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Inventor(s)

Hinkle; Gregory

KETOHEXOKINASE (KHK) iRNA COMPOSITIONS AND METHODS OF USE THEREOF

Abstract

The present invention relates to RNAi agents, e.g., dsRNA agents, targeting the ketohexokinase (KHK) gene. The invention also relates to methods of using such RNAi agents to inhibit expression of a KHK gene and to methods of treating or preventing a KHK-associated disease in a subject.

Inventors: Hinkle; Gregory (Plymouth, MA)

Applicant: Alnylam Pharmaceuticals, Inc. (Cambridge, MA)

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Background/Summary

RELATED APPLICATIONS [0001] This application is a continuation of U.S. patent application Ser. No. 18/215,848, filed Jun. 29, 2023, which is a continuation of U.S. patent application Ser. No. 17/254,421, filed on Dec. 21, 2020 which in turn is a 35 U.S.C. § 371 national stage filing of International Application No.: PCT/US2019/051429, filed on Sep. 17, 2019, which in turn claims the benefit of priority to U.S. Provisional Patent Application No. 62/732,600, filed on Sep. 18, 2018. The entire contents of each of the foregoing applications are hereby incorporated herein by reference.

REFERENCE TO ELECTRONIC SEQUENCE LISTING

[0002] The application contains a Sequence Listing which has been submitted electronically in .XML format and is hereby incorporated by reference in its entirety. Said .XML copy, created on Dec. 18, 2024, is named “121301_06005_SL.xml” and is 4,933,365 bytes in size. The sequence listing contained in this .XML file is part of the specification and is hereby incorporated by reference herein in its entirety.

BACKGROUND OF THE INVENTION

[0003] Epidemiological studies have shown that a western diet is one of the leading causes of the modern obesity pandemic. Increase in fructose uptake, associated with the use of enriched soft drinks and processed food, is proposed to be a major contributing factor to the epidemic. High fructose corn sweeteners started gaining widespread use in the food industry by 1967. Although glucose and fructose have the same caloric value per molecule, the two sugars are metabolized differently and utilize different GLUT transporters. Fructose is almost exclusively metabolized in the liver, and unlike the glucose metabolism pathway, the fructose metabolism pathway is not regulated by feedback inhibition by the product (Khaitan Z et al., (2013) *J. Nutr. Metab.* 2013, Article ID 682673, 1-12). While hexokinase and phosphofructokinase (PFK) regulate the production of glyceraldehyde-3-P from glucose, fructokinase or ketohexokinase (KHK), which is responsible for phosphorylation of fructose to fructose-1-phosphate in the liver, it is not down regulated by increasing concentrations of fructose-1-phosphate. As a result, all fructose entering the cell is rapidly phosphorylated. (Cirillo P. et al., (2009) *J. Am. Soc. Nephrol.* 20: 545-553). Continued utilization of ATP to phosphorylate the fructose to fructose-1-phosphate results in intracellular phosphate depletion, ATP depletion, activation of AMP deaminase and formation of uric acid (Khaitan Z. et al., (2013) *J. Nutr. Metab.* Article ID 682673, 1-12). Increased uric acid further stimulates the up-regulation of KHK (Lanaspa M. A. et al., (2012) *PLOS ONE* 7(10): 1-11) and causes endothelial cell and adipocyte dysfunction. Fructose-1-phosphate is subsequently converted to glyceraldehyde by the action of aldolase B and is phosphorylated to glyceraldehyde-3-phosphate. The latter proceeds downstream to the glycolysis pathway to form pyruvate, which enters the citric acid cycle, wherefrom, under well-fed conditions, citrate is exported to the cytosol from the mitochondria, providing Acetyl Coenzyme A for lipogenesis (FIG. 1).

[0004] The phosphorylation of fructose by KHK, and subsequent activation of lipogenesis leads to, for example, fatty liver, hypertriglyceridemia, dyslipidemia, and insulin resistance.

Proinflammatory changes in renal proximal tubular cells have also been shown to be induced by KHK activity (Cirillo P. et al., (2009) *J. Am. Soc. Nephrol.* 20: 545-553). The phosphorylation of fructose by KHK is associated with diseases, disorders or conditions such as liver disease (e.g., fatty liver, steatohepatitis), dyslipidemia (e.g., hyperlipidemia, high LDL cholesterol, low HDL cholesterol, hypertriglyceridemia, postprandial hypertriglyceridemia), disorders of glycemic control (e.g., insulin resistance, type 2 diabetes), cardiovascular disease (e.g., hypertension, endothelial cell dysfunction), kidney disease (e.g., acute kidney disorder, tubular dysfunction, proinflammatory changes to the proximal tubules, chronic kidney disease), metabolic syndrome, adipocyte

dysfunction, visceral adipose deposition, obesity, hyperuricemia, gout, eating disorders, and excessive sugar craving. Accordingly, there is a need in the art for compositions and methods for treating diseases, disorders, and conditions associated with KHK activity.

SUMMARY OF THE INVENTION

[0005] The present invention provides compositions comprising RNAi agents, e.g., double stranded RNAi agents, targeting ketohexokinase (KHK). The present invention also provides methods of using the compositions of the invention for inhibiting KHK expression or for treating a subject having a disorder that would benefit from reducing the expression of a KHK gene, e.g., a KHK-associated disease, such as liver disease (e.g., fatty liver, steatohepatitis, especially non-alcoholic steatohepatitis (NASH)), dyslipidemia (e.g., hyperlipidemia, high LDL cholesterol, low HDL cholesterol, hypertriglyceridemia, postprandial hypertriglyceridemia), disorders of glycemic control (e.g., insulin resistance, type 2 diabetes), cardiovascular disease (e.g., hypertension, endothelial cell dysfunction), kidney disease (e.g., acute kidney disorder, tubular dysfunction, proinflammatory changes to the proximal tubules, chronic kidney disease), metabolic syndrome, adipocyte dysfunction, visceral adipose deposition, obesity, hyperuricemia, gout, eating disorders, and excessive sugar craving.

[0006] In an aspect, the invention provides a double stranded ribonucleic acid (dsRNA) agent for inhibiting expression of ketohexokinase (KHK), wherein the dsRNA comprises a sense strand and an antisense strand, wherein the sense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO:1 and the antisense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO:2.

[0007] In certain embodiments, the sense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of any one of nucleotides 89-107, 176-194, 264-282, 474-492, 508-526, 529-547, 562-580, 616-646, 682-700, 705-723, 705-757, 705-799, 739-757, 739-799, 760-799, 804-822, 837-855, 892-910, 959-977, 992-1010, 922-1041, 1013-1041, 1069-1108, 1169-1140, 1111-1140, 1155-1196, 1221-1261, 1267-1294, or 1320-1350 of SEQ ID NO:1. In certain embodiments, the sense strand comprises at least 15 contiguous nucleotides of the nucleotide sequence of SEQ ID NO: 1, or one of the foregoing portions of the nucleotide sequence of SEQ ID NO: 1.

[0008] In certain embodiments, the sense strands and antisense strands comprise a nucleotide sequence selected from the group consisting of any one of the nucleotide sequences in Table 3 or 5.

[0009] In certain embodiments, the sense strand or the antisense strand comprise any one of the nucleotide sequences in any one of the duplexes selected from the group consisting of AD-72506, AD-72319, AD-72502, AD-72513, AD-72499, AD-72303, AD-72500, AD-72522, AD-72512, AD-72304, AD-72514, AD-72257, AD-72295, AD-72332, AD-72507, AD-72311, AD-72501, AD-72508, AD-72293, AD-72322, AD-72264, AD-72290, AD-72338, AD-72315, AD-72272, AD-72337, AD-72298, AD-72503, AD-72327, AD-72521, AD-72309, AD-72313, AD-72517, AD-72316, AD-72335, AD-72317, provided in Table 3 or 5. In certain embodiments, the sense strand and the antisense strand comprise the nucleotide sequence of any one of the duplexes selected from the group consisting of AD-72506, AD-72319, AD-72502, AD-72513, AD-72499, AD-72303, AD-72500, AD-72522, AD-72512, AD-72304, AD-72514, AD-72257, AD-72295, AD-72332, AD-72507, AD-72311, AD-72501, AD-72508, AD-72293, AD-72322, AD-72264, AD-72290, AD-72338, AD-72315, AD-72272, AD-72337, AD-72298, AD-72503, AD-72327, AD-72521, AD-72309, AD-72313, AD-72517, AD-72316, AD-72335, of AD-72317, provided in Table 3 or 5.

[0010] In an aspect, the invention provides a double stranded ribonucleic acid (dsRNA) agent for inhibiting expression of a ketohexokinase (KHK) gene, wherein the dsRNA comprises a sense strand and an antisense strand, the antisense strand comprising a region of complementarity which comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from any one of the antisense sequences listed in Table 3 or 5. In certain embodiments, the dsRNA comprises a

sense strand and an antisense strand, the antisense strand comprising a region of complementarity which comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from any one of the antisense sequences in any one of the duplexes selected from the group consisting of AD-72506, AD-72319, AD-72502, AD-72513, AD-72499, AD-72303, AD-72500, AD-72522, AD-72512, AD-72304, AD-72514, AD-72257, AD-72295, AD-72332, AD-72507, AD-72311, AD-72501, AD-72508, AD-72293, AD-72322, AD-72264, AD-72290, AD-72338, AD-72315, AD-72272, AD-72337, AD-72298, AD-72503, AD-72327, AD-72521, AD-72309, AD-72313, AD-72517, AD-72316, AD-72335, or AD-72317.

[0011] In certain embodiments, the dsRNA comprises at least one modified nucleotide. In some embodiments, all of the nucleotides of the sense strand and all of the nucleotides of the antisense strand comprise a modification.

[0012] In an aspect, the invention provides a double stranded RNAi agent for inhibiting expression of a ketohexokinase (KHK) gene, wherein the dsRNA agent comprises a sense strand and an antisense strand forming a double stranded region, wherein the sense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO:1 and the antisense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO:2, wherein substantially all of the nucleotides of the sense strand and substantially all of the nucleotides of the antisense strand are modified nucleotides, and wherein the sense strand is conjugated to a ligand attached at the 3'-terminus. In certain embodiments, the sense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of any one of nucleotides 89-107, 176-194, 264-282, 474-492, 508-526, 529-547, 562-580, 616-646, 682-700, 705-723, 705-757, 705-799, 739-757, 739-799, 760-799, 804-822, 837-855, 892-910, 959-977, 992-1010, 922-1041, 1013-1041, 1069-1108, 1169-1140, 1111-1140, 1155-1196, 1221-1261, 1267-1294, or 1320-1350 of SEQ ID NO:1 and the antisense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO:2, wherein substantially all of the nucleotides of the sense strand and substantially all of the nucleotides of the antisense strand are modified nucleotides, and wherein the sense strand is conjugated to a ligand attached at the 3'-terminus. In certain embodiments, the sense strand comprises at least 15 contiguous nucleotides of SEQ ID NO: 1, or one of the foregoing portions of the nucleotide sequence of SEQ ID NO: 1, and at least 15 contiguous nucleotides of the corresponding portion of SEQ ID NO: 2, such that the sense and antisense strands are complementary to each other.

[0013] In one aspect, the present invention provides double stranded RNAi agents for inhibiting expression of KHK, which comprise a sense strand and an antisense strand forming a double stranded region, wherein the sense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of any one of nucleotides 89-107, 176-194, 264-282, 474-492, 508-526, 529-547, 562-580, 616-646, 682-700, 705-723, 705-757, 705-799, 739-757, 739-799, 760-799, 804-822, 837-855, 892-910, 959-977, 992-1010, 922-1041, 1013-1041, 1069-1108, 1169-1140, 1111-1140, 1155-1196, 1221-1261, 1267-1294, or 1320-1350 of SEQ ID NO:1 and the antisense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the corresponding position of the nucleotide sequence of SEQ ID NO:2, such that the antisense strand is complementary to the at least 15 contiguous nucleotides differing by no more than 3 nucleotides in the sense strand. In certain embodiments, substantially all of the nucleotides of the sense strand or substantially all of the nucleotides of the antisense strand are modified nucleotides, or substantially all of the nucleotides of both strands are modified; and wherein the sense strand is conjugated to a ligand attached at the 3'-terminus.

[0014] In certain embodiments, the sense strand comprises at least 15 contiguous nucleotides of any one of nucleotides 89-107, 176-194, 264-282, 474-492, 508-526, 529-547, 562-580, 616-646, 682-700, 705-723, 705-757, 705-799, 739-757, 739-799, 760-799, 804-822, 837-855, 892-910, 959-977, 992-1010, 922-1041, 1013-1041, 1069-1108, 1169-1140, 1111-1140, 1155-1196, 1221-

1261, 1267-1294, or 1320-1350 of SEQ ID NO:1, and the antisense strand comprises at least 15 contiguous nucleotides from the corresponding portion of nucleotide sequence of SEQ ID NO:2 such that the antisense strand is complementary to the at least 15 contiguous nucleotides differing by no more than 3 nucleotides in the sense strand. In certain embodiments, substantially all of the nucleotides of the sense strand or substantially all of the nucleotides of the antisense strand are modified nucleotides, or substantially all of the nucleotides of both strands are modified; and wherein the sense strand is conjugated to a ligand attached at the 3'-terminus.

[0015] In an aspect, the present invention also provides double stranded RNAi agents for inhibiting expression of KHK, which comprise a sense strand and an antisense strand forming a double stranded region, wherein the sense strand comprises at least 15 contiguous nucleotides of any one of nucleotides 89-107, 176-194, 264-282, 474-492, 508-526, 529-547, 562-580, 616-646, 682-700, 705-723, 705-757, 705-799, 739-757, 739-799, 760-799, 804-822, 837-855, 892-910, 959-977, 992-1010, 922-1041, 1013-1041, 1069-1108, 1169-1140, 1111-1140, 1155-1196, 1221-1261, 1267-1294, or 1320-1350 of SEQ ID NO:1 and the antisense strand comprises at least 15 contiguous nucleotides from the corresponding position of the nucleotide sequence of SEQ ID NO:2 such that the antisense strand is complementary to the at least 15 contiguous nucleotides in the sense strand. In certain embodiments, substantially all of the nucleotides of the sense strand are modified nucleotides. In certain embodiments, substantially all of the nucleotides of the antisense strand are modified nucleotides. In certain embodiments, substantially all of the nucleotides of both strands are modified. In preferred embodiments, the sense strand is conjugated to a ligand attached at the 3'-terminus.

[0016] In certain embodiments, the antisense strand comprises a region of complementarity which comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from any one of the antisense sequences listed in any one of Tables 3 and 5. For example, in a certain embodiments, the antisense strand may comprise a region of complementarity which comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from any one of the antisense sequences of any one of the duplexes selected from the group consisting of AD-72506, AD-72319, AD-72502, AD-72513, AD-72499, AD-72303, AD-72500, AD-72522, AD-72512, AD-72304, AD-72514, AD-72257, AD-72295, AD-72332, AD-72507, AD-72311, AD-72501, AD-72508, AD-72293, AD-72322, AD-72264, AD-72290, AD-72338, AD-72315, AD-72272, AD-72337, AD-72298, AD-72503, AD-72327, AD-72521, AD-72309, AD-72313, AD-72517, AD-72316, AD-72335, and AD-72317. In certain embodiments, the antisense strand comprises a region of complementarity to SEQ ID NO: 1 which comprises at least 15 contiguous nucleotides of any one of the antisense sequences of the foregoing duplexes.

[0017] In some embodiments, all of the nucleotides of the sense strand and all of the nucleotides of the antisense strand comprise a modification.

[0018] In one embodiment, at least one of the modified nucleotides is selected from the group consisting of a deoxy-nucleotide, a 3'-terminal deoxy-thymine (dT) nucleotide, a 2'-O-methyl modified nucleotide, a 2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an unlocked nucleotide, a conformationally restricted nucleotide, a constrained ethyl nucleotide, an abasic nucleotide, a 2'-amino-modified nucleotide, a 2'-O-allyl-modified nucleotide, 2'-C-alkyl-modified nucleotide, 2'-hydroxyl-modified nucleotide, a 2'-methoxyethyl modified nucleotide, a 2'-O-alkyl-modified nucleotide, a morpholino nucleotide, a phosphoramidate, a non-natural base comprising nucleotide, a tetrahydropyran modified nucleotide, a 1,5-anhydrohexitol modified nucleotide, a cyclohexenyl modified nucleotide, a nucleotide comprising a phosphorothioate group, a nucleotide comprising a methylphosphonate group, a nucleotide comprising a 5'-phosphate, and a nucleotide comprising a 5'-phosphate mimic. In another embodiment, the modified nucleotides comprise a short sequence of 3'-terminal deoxy-thymine nucleotides (dT).

[0019] In certain embodiments, substantially all of the nucleotides of the sense strand are modified.

In certain embodiments, substantially all of the nucleotides of the antisense strand are modified. In certain embodiments, substantially all of the nucleotides of both the sense strand and the antisense strand are modified.

[0020] In certain embodiments, the duplex comprises a modified antisense strand nucleotide sequence provided in Table 5. In certain embodiments, the duplex comprises a modified sense strand nucleotide sequence provided in Table 5. In certain embodiments, the duplex comprises a modified duplex provided in Table 5.

[0021] In certain embodiments, the region of complementarity between the antisense strand and the target mRNA nucleotide sequence is at least 17 nucleotides in length. For example, the region of complementarity between the antisense strand and the target is 19 to 21 nucleotides in length, for example, the region of complementarity is 21 nucleotides in length. In preferred embodiments, each strand is no more than 30 nucleotides in length.

[0022] In other embodiments, one or both of the strands of the double stranded RNAi agents of the invention is up to 66 nucleotides in length, e.g., 36-66, 26-36, 25-36, 31-60, 22-43, 27-53 nucleotides in length, with a region of at least 19 contiguous nucleotides that is substantially complementary to at least a part of an mRNA transcript of an KHK gene. In some embodiments, the sense and antisense strands form a duplex of 18-30 contiguous nucleotides.

[0023] In one embodiment, at least one strand of the dsRNA agent comprises a 3' overhang of at least 1 nucleotide. In certain embodiments, at least one strand comprises a 3' overhang of at least 2 nucleotides, e.g., 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, or 15 nucleotides. In other embodiments, at least one strand of the RNAi agent comprises a 5' overhang of at least 1 nucleotide. In certain embodiments, at least one strand comprises a 5' overhang of at least 2 nucleotides, e.g., 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, or 15 nucleotides. In still other embodiments, both the 3' and the 5' end of one strand of the RNAi agent comprise an overhang of at least 1 nucleotide.

[0024] In certain embodiments, the double stranded RNAi agent further comprises a ligand. In certain embodiments, the ligand is an N-acetylgalactosamine (GalNAc). The ligand may be one or more GalNAc attached to the RNAi agent through a monovalent, a bivalent, or a trivalent branched linker. The ligand may be conjugated to the 3' end of the sense strand of the double stranded RNAi agent, the 5' end of the sense strand of the double stranded RNAi agent, the 3' end of the antisense strand of the double stranded RNAi agent, or the 5' end of the antisense strand of the double stranded RNAi agent.

[0025] In some embodiments, the double stranded RNAi agents of the invention comprise a plurality, e.g., 2, 3, 4, 5, or 6, of GalNAc, each independently attached to a plurality of nucleotides of the double stranded RNAi agent through a plurality of monovalent linkers.

[0026] In certain embodiments, the ligand is

##STR00001##

[0027] In certain embodiments, the double stranded RNAi agent is conjugated to the ligand as shown in the following schematic

##STR00002##

and, wherein X is O or S. In one embodiment, the X is O.

[0028] In certain embodiments, the region of complementarity comprises any one of the antisense nucleotide sequences of Table 3 or Table 5. In another embodiment, the region of complementarity consists of one of the antisense nucleotide sequences of Table 3 or Table 5.

[0029] In another aspect, the invention provides a double stranded RNAi agent for inhibiting the expression of a KHK gene, wherein the double stranded RNAi agent comprises a sense strand, wherein the sense strand comprises at least 15 contiguous nucleotides of the nucleotide sequence of any one of nucleotides 89-107, 176-194, 264-282, 474-492, 508-526, 529-547, 562-580, 616-646, 682-700, 705-723, 705-757, 705-799, 739-757, 739-799, 760-799, 804-822, 837-855, 892-910, 959-977, 992-1010, 922-1041, 1013-1041, 1069-1108, 1169-1140, 1111-1140, 1155-1196, 1221-1261, 1267-1294, or 1320-1350 of SEQ ID NO: 1, and the sense strand is complementary to the

antisense strand, wherein the antisense strand comprises a region complementary to part of an mRNA encoding KHK, wherein each strand is about 14 to about 30 nucleotides in length, wherein the dsRNA agent is represented by formula (III):

sense: 5' n.sub.p-N.sub.a-(XXX).sub.i—N.sub.b—YYY—N.sub.b—(ZZZ).sub.j—N.sub.a-n.sub.q
3'

antisense: 3' n.sub.p'—N.sub.a'—(X'X'X').sub.k—N.sub.b'—Y'Y'Y'—N.sub.b'—(Z'Z'Z').sub.l—
N.sub.a'-n.sub.q' 5' (III) [0030] wherein: i, j, k, and l are each independently 0 or 1; p, p', q,
and q' are each independently 0-6; each N.sub.a and N.sub.a' independently represents an
oligonucleotide sequence comprising 0-25 nucleotides which are either modified or unmodified or
combinations thereof, each sequence comprising at least two differently modified nucleotides; each
N.sub.b and N.sub.b' independently represents an oligonucleotide sequence comprising 0-10
nucleotides which are either modified or unmodified or combinations thereof; each n.sub.p,
n.sub.p', n.sub.q, and n.sub.q', each of which may or may not be present, independently represents
an overhang nucleotide; XXX, YYY, ZZZ, X'X'X', Y'Y'Y', and Z'Z'Z' each independently
represent one motif of three identical modifications on three consecutive nucleotides; modifications
on N.sub.b differ from the modification on Y and modifications on N.sub.b' differ from the
modification on Y'; and wherein the sense strand is conjugated to at least one ligand.

[0031] In certain embodiments, i is 0; j is 0; i is 1; j is 1; both i and j are 0; or both i and j are 1. In
another embodiment, k is 0; l is 0; k is 1; l is 1; both k and l are 0; or both k and l are 1. In another
embodiment, XXX is complementary to X'X'X', YYY is complementary to Y'Y'Y', and ZZZ is
complementary to Z'Z'Z'. In another embodiment, the YYY motif occurs at or near the cleavage
site of the sense strand. In another embodiment, the Y'Y'Y' motif occurs at the 11, 12 and 13
positions of the antisense strand from the 5'-end. In one embodiment, the Y' is 2'-O-methyl.

[0032] For example, formula (III) can be represented by formula (IIIa):

sense: 5' n.sub.p-N.sub.a—YYY—N.sub.a-n.sub.q 3'

antisense: 3' n.sub.p'-N.sub.a'—Y'Y'Y'—N.sub.a'-n.sub.q'-5' (IIIa).

[0033] In another embodiment, formula (III) is represented by formula (IIIb):

sense: 5' n.sub.p-N.sub.a—YYY—N.sub.b—ZZZ-n.sub.a-n.sub.q 3'

antisense: 3' n.sub.p'-N.sub.a'—Y'Y'Y'—N.sub.b'—Z'Z'Z'-n.sub.a'-n.sub.q' 5' (IIIb) [0034]
wherein each N.sub.b and N.sub.b' independently represents an oligonucleotide sequence
comprising 1-5 modified nucleotides.

[0035] Alternatively, formula (III) can be represented by formula (IIIc):

sense: 5' n.sub.p-N.sub.a—XXX—N.sub.b—YYY—N.sub.a-n.sub.q 3'

antisense: 3' n.sub.p'-N.sub.a'—X'X'X'—N.sub.b'—Y'Y'Y'—N.sub.a'-n.sub.q' 5' (IIIc)
wherein each N.sub.b and N.sub.b' independently represents an oligonucleotide sequence
comprising 1-5 modified nucleotides.

[0036] Further, formula (III) can be represented by formula (IIId):

sense: 5' n.sub.p-N.sub.a—XXX—N.sub.b—YYY—N.sub.b—ZZZ—N.sub.a-n.sub.q 3'

antisense: 3' n.sub.p'—N.sub.a'—X'X'X'—N.sub.b'—Y'Y'Y'—N.sub.b'—Z'Z'Z'—N.sub.a'-
n.sub.q' 5' (IIId) [0037] wherein each N.sub.b and N.sub.b' independently represents an

oligonucleotide sequence comprising 1-5 modified nucleotides and each N.sub.a and N.sub.a' independently represents an oligonucleotide sequence comprising 2-10 modified nucleotides. [0038] In certain embodiments, the double stranded region is 15-30 nucleotide pairs in length. For example, the double stranded region can be 17-23 nucleotide pairs in length. The double stranded region can be 17-25 nucleotide pairs in length. The double stranded region can be 23-27 nucleotide pairs in length. The double stranded region can be 19-21 nucleotide pairs in length. The double stranded region can be 21-23 nucleotide pairs in length.

[0039] In certain embodiments, each strand has 15-30 nucleotides. In other embodiments, each strand has 19-30 nucleotides.

[0040] Modifications on the nucleotides may be selected from the group including, but not limited to, LNA, HNA, CeNA, 2'-methoxyethyl, 2'-O-alkyl, 2'-O-allyl, 2'-C-allyl, 2'-fluoro, 2'-deoxy, 2'-hydroxyl, and combinations thereof. In one embodiment, the modifications on the nucleotides are 2'-O-methyl or 2'-fluoro modifications.

[0041] In certain embodiments, the ligand is an N-acetylgalactosamine (GalNAc). The ligand may be one or more GalNAc attached to the RNAi agent through a monovalent, a bivalent, or a trivalent branched linker. The ligand may be conjugated to the 3' end of the sense strand of the double stranded RNAi agent, the 5' end of the sense strand of the double stranded RNAi agent, the 3' end of the antisense strand of the double stranded RNAi agent, or the 5' end of the antisense strand of the double stranded RNAi agent.

[0042] In some embodiments, the double stranded RNAi agents of the invention comprise a plurality, e.g., 2, 3, 4, 5, or 6, of GalNAc, each independently attached to a plurality of nucleotides of the double stranded RNAi agent through a plurality of monovalent linkers. In one embodiment, the

##STR00003##

[0043] The ligand can be attached to the 3' end of the sense strand.

[0044] An exemplary structure of a dsRNAi agent conjugated to the ligand is shown in the following schematic

##STR00004##

[0045] In certain embodiments, the RNAi agent further comprises at least one phosphorothioate or methylphosphonate internucleotide linkage. For example the phosphorothioate or methylphosphonate internucleotide linkage can be at the 3'-terminus of one strand, i.e., the sense strand or the antisense strand; or at the ends of both strands, the sense strand and the antisense strand.

[0046] In certain embodiments, the phosphorothioate or methylphosphonate internucleotide linkage is at the 5'-terminus of one strand, i.e., the sense strand or the antisense strand; or at the ends of both strands, the sense strand and the antisense strand.

[0047] In certain embodiments, the phosphorothioate or methylphosphonate internucleotide linkage is at both the 5'- and 3'-terminus of one strand, i.e., the sense strand or the antisense strand; or at the ends of both strands, the sense strand and the antisense strand.

[0048] In certain embodiments, the base pair at the 1 position of the 5'-end of the antisense strand of the duplex is an AU base pair.

[0049] In certain embodiments, the Y nucleotides contain a 2'-fluoro modification. In another embodiment, the Y' nucleotides contain a 2'-O-methyl modification. In another embodiment, $p' > 0$.

[0050] In some embodiments, $p' = 2$. In some embodiments, $q' = 0$, $p = 0$, $q = 0$, and p' overhang nucleotides are complementary to the target mRNA. In some embodiments, $q' = 0$, $p = 0$, $q = 0$, and p' overhang nucleotides are non-complementary to the target mRNA.

[0051] In certain embodiments, the sense strand has a total of 21 nucleotides and the antisense strand has a total of 23 nucleotides.

[0052] In certain embodiments, at least one n.sub.p' is linked to a neighboring nucleotide via a phosphorothioate linkage. In other embodiments, all n.sub.p' are linked to neighboring nucleotides

via phosphorothioate linkages.

[0053] In certain embodiments, the dsRNAi agent is selected from the group of dsRNAi agents listed in Tables 3 and 5. In certain embodiments, all of the nucleotides of the sense strand and all of the nucleotides of the antisense strand comprise a modification.

[0054] In an aspect, the invention provides a double stranded RNAi agent capable of inhibiting the expression of KHK in a cell, wherein the dsRNA agent comprises a sense strand, wherein the sense strand comprises an at least 15 contiguous nucleotide portion of any one of nucleotides 89-107, 176-194, 264-282, 474-492, 508-526, 529-547, 562-580, 616-646, 682-700, 705-723, 705-757, 705-799, 739-757, 739-799, 760-799, 804-822, 837-855, 892-910, 959-977, 992-1010, 922-1041, 1013-1041, 1069-1108, 1169-1140, 1111-1140, 1155-1196, 1221-1261, 1267-1294, or 1320-1350 of SEQ ID NO: 1, and the sense strand is complementary to an antisense strand, wherein the antisense strand comprises a region complementary to part of an mRNA encoding KHK, wherein each strand is about 14 to about 30 nucleotides in length, wherein the dsRNA agent is represented by formula (III):

sense: 5' n.sub.p-N.sub.a—(XXX).sub.i—N.sub.b—YYY—N.sub.b—(ZZZ).sub.j—N.sub.a-n.sub.q 3'

antisense: 3' n.sub.p'—N.sub.a'—(X'X'X').sub.k—N.sub.b'—Y'Y'Y'—N.sub.b'—(Z'Z'Z').sub.l—N.sub.a'-n.sub.q' 5' (III) [0055] wherein i, j, k, and l are each independently 0 or 1; p, p', q, and q' are each independently 0-6; each N.sub.a and N.sub.a' independently represents an oligonucleotide sequence comprising 0-25 nucleotides which are either modified or unmodified or combinations thereof, each sequence comprising at least two differently modified nucleotides; each N.sub.b and N.sub.b' independently represents an oligonucleotide sequence comprising 0-10 nucleotides which are either modified or unmodified or combinations thereof; each n.sub.p, n.sub.p', n.sub.q, and n.sub.q', each of which may or may not be present independently represents an overhang nucleotide; XXX, YYY, ZZZ, X'X'X', Y'Y'Y', and Z'Z'Z' each independently represent one motif of three identical modifications on three consecutive nucleotides, and wherein the modifications are 2'-O-methyl or 2'-fluoro modifications; modifications on N.sub.b differ from the modification on Y and modifications on N.sub.b' differ from the modification on Y'; and wherein the sense strand is conjugated to at least one ligand.

[0056] In an aspect, the invention provides a double stranded RNAi agent capable of inhibiting the expression of KHK in a cell, wherein the dsRNA agent comprises a sense strand, wherein the sense strand preferably comprises an at least 15 contiguous nucleotide portion of any one of nucleotides 89-107, 176-194, 264-282, 474-492, 508-526, 529-547, 562-580, 616-646, 682-700, 705-723, 705-757, 705-799, 739-757, 739-799, 760-799, 804-822, 837-855, 892-910, 959-977, 992-1010, 922-1041, 1013-1041, 1069-1108, 1169-1140, 1111-1140, 1155-1196, 1221-1261, 1267-1294, or 1320-1350 of SEQ ID NO: 1, and the sense strand is complementary to an antisense strand, wherein the antisense strand comprises a region complementary to part of an mRNA encoding KHK, wherein each strand is about 14 to about 30 nucleotides in length, wherein the dsRNA agent is represented by formula (III):

sense: 5' n.sub.p-N.sub.a—(XXX).sub.i—N.sub.b—YYY—N.sub.b—(ZZZ).sub.j—N.sub.a-n.sub.q 3'

antisense: 3' n.sub.p'—N.sub.a'—(X'X'X').sub.k—N.sub.b'—Y'Y'Y'—N.sub.b'—(Z'Z'Z').sub.l—N.sub.a'-n.sub.q' 5' (III) [0057] wherein: i, j, k, and l are each independently 0 or 1; each n.sub.p, n.sub.q, and n.sub.q', each of which may or may not be present, independently represents an overhang nucleotide; [0058] p, q, and q' are each independently 0-6; n.sub.p>0 and at least one n.sub.p' is linked to a neighboring nucleotide via a phosphorothioate linkage; each N.sub.a and

N.sub.a' independently represents an oligonucleotide sequence comprising 0-25 nucleotides which are either modified or unmodified or combinations thereof, each sequence comprising at least two differently modified nucleotides; each N.sub.b and N.sub.b' independently represents an oligonucleotide sequence comprising 0-10 nucleotides which are either modified or unmodified or combinations thereof; XXX, YYY, ZZZ, X'X'X', Y'Y'Y', and Z'Z'Z' each independently represent one motif of three identical modifications on three consecutive nucleotides, and wherein the modifications are 2'-O-methyl or 2'-fluoro modifications; modifications on N.sub.b differ from the modification on Y and modifications on N.sub.b' differ from the modification on Y'; and wherein the sense strand is conjugated to at least one ligand.

[0059] In certain embodiments, the invention provides a double stranded RNAi agent capable of inhibiting the expression of KHK in a cell, wherein the double stranded RNAi agent comprises a sense strand, wherein the sense strand comprises an at least 15 contiguous nucleotide portion of any one of nucleotides 89-107, 176-194, 264-282, 474-492, 508-526, 529-547, 562-580, 616-646, 682-700, 705-723, 705-757, 705-799, 739-757, 739-799, 760-799, 804-822, 837-855, 892-910, 959-977, 992-1010, 922-1041, 1013-1041, 1069-1108, 1169-1140, 1111-1140, 1155-1196, 1221-1261, 1267-1294, or 1320-1350 of SEQ ID NO: 1, and the sense strand is complementary to an antisense strand, wherein the antisense strand comprises a region complementary to part of an mRNA encoding KHK, wherein each strand is about 14 to about 30 nucleotides in length, wherein the dsRNA agent is represented by formula (III):

sense: 5' n.sub.p-N.sub.a—(XXX).sub.i—N.sub.b—YYY—N.sub.b—(ZZZ).sub.j—N.sub.a-n.sub.q 3'

antisense: 3' n.sub.p'—N.sub.a'—(X'X'X').sub.k—N.sub.b'—Y'Y'Y'—N.sub.b'—(Z'Z'Z').sub.l—N.sub.a'-n.sub.q' 5' (III) [0060] wherein i, j, k, and l are each independently 0 or 1; each n.sub.p, n.sub.q, and n.sub.q', each of which may or may not be present, independently represents an overhang nucleotide; p, q, and q' are each independently 0-6; n.sub.p' > 0 and at least one n.sub.p' is linked to a neighboring nucleotide via a phosphorothioate linkage; each N.sub.a and N.sub.a' independently represents an oligonucleotide sequence comprising 0-25 nucleotides which are either modified or unmodified or combinations thereof, each sequence comprising at least two differently modified nucleotides; each N.sub.b and N.sub.b' independently represents an oligonucleotide sequence comprising 0-10 nucleotides which are either modified or unmodified or combinations thereof; XXX, YYY, ZZZ, X'X'X', Y'Y'Y', and Z'Z'Z' each independently represent one motif of three identical modifications on three consecutive nucleotides, and wherein the modifications are 2'-O-methyl or 2'-fluoro modifications; modifications on N.sub.b differ from the modification on Y and modifications on N.sub.b' differ from the modification on Y'; and wherein the sense strand is conjugated to at least one ligand, wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker.

[0061] In an aspect, the invention provides a double stranded RNAi agent capable of inhibiting the expression of KHK in a cell, wherein the dsRNA agent comprises a sense strand, wherein the sense strand comprises an at least 15 contiguous nucleotide portion of any one of nucleotides 89-107, 176-194, 264-282, 474-492, 508-526, 529-547, 562-580, 616-646, 682-700, 705-723, 705-757, 705-799, 739-757, 739-799, 760-799, 804-822, 837-855, 892-910, 959-977, 992-1010, 922-1041, 1013-1041, 1069-1108, 1169-1140, 1111-1140, 1155-1196, 1221-1261, 1267-1294, or 1320-1350 of SEQ ID NO: 1, and the sense strand is complementary to an antisense strand, wherein the antisense strand comprises a region complementary to part of an mRNA encoding KHK, wherein each strand is about 14 to about 30 nucleotides in length, wherein the dsRNA agent is represented by formula (III):

sense: 5' n.sub.p-N.sub.a—(XXX).sub.i—N.sub.b—YYY—N.sub.b—(ZZZ).sub.j—N.sub.a-

n.sub.q 3'

antisense: 3' n.sub.p'—N.sub.a'—(X'X'X').sub.k—N.sub.b'—Y'Y'Y'—N.sub.b'—(Z'Z'Z').sub.l—N.sub.a'-n.sub.q' 5' (III) [0062] wherein i, j, k, and l are each independently 0 or 1; each n.sub.p, n.sub.q, and n.sub.q', each of which may or may not be present, independently represents an overhang nucleotide; p, q, and q' are each independently 0-6; n.sub.p'>0 and at least one n.sub.p' is linked to a neighboring nucleotide via a phosphorothioate linkage; each N.sub.a and N.sub.a' independently represents an oligonucleotide sequence comprising 0-25 nucleotides which are either modified or unmodified or combinations thereof, each sequence comprising at least two differently modified nucleotides; each N.sub.b and N.sub.b' independently represents an oligonucleotide sequence comprising 0-10 nucleotides which are either modified or unmodified or combinations thereof; XXX, YYY, ZZZ, X'X'X', Y'Y'Y', and Z'Z'Z' each independently represent one motif of three identical modifications on three consecutive nucleotides, and wherein the modifications are 2'-O-methyl or 2'-fluoro modifications; modifications on N.sub.b differ from the modification on Y and modifications on N.sub.b' differ from the modification on Y'; wherein the sense strand comprises at least one phosphorothioate linkage; and wherein the sense strand is conjugated to at least one ligand, wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker.

[0063] In an aspect, the invention provides a double stranded RNAi agent capable of inhibiting the expression of KHK in a cell, wherein the dsRNA agent comprises a sense strand, wherein the sense strand comprises an at least 15 contiguous nucleotide portion of any one of nucleotides 89-107, 176-194, 264-282, 474-492, 508-526, 529-547, 562-580, 616-646, 682-700, 705-723, 705-757, 705-799, 739-757, 739-799, 760-799, 804-822, 837-855, 892-910, 959-977, 992-1010, 922-1041, 1013-1041, 1069-1108, 1169-1140, 1111-1140, 1155-1196, 1221-1261, 1267-1294, or 1320-1350 of SEQ ID NO: 1, and the sense strand is complementary to an antisense strand, wherein the antisense strand comprises a region complementary to part of an mRNA encoding KHK, wherein each strand is about 14 to about 30 nucleotides in length, wherein the dsRNA agent is represented by formula (III):

sense: 5' n.sub.p-N.sub.a—YYY—N.sub.a-n.sub.q 3'

antisense: 3' n.sub.p'—N.sub.a'—Y'Y'Y'—N.sub.a'-n.sub.q' 5' (IIIa) [0064] wherein each n.sub.p, n.sub.q, and n.sub.q', each of which may or may not be present, independently represents an overhang nucleotide; p, q, and q' are each independently 0-6; n.sub.p'>0 and at least one n.sub.p' is linked to a neighboring nucleotide via a phosphorothioate linkage; each N.sub.a and N.sub.a' independently represents an oligonucleotide sequence comprising 0-25 nucleotides which are either modified or unmodified or combinations thereof, each sequence comprising at least two differently modified nucleotides; YYY and Y'Y'Y' each independently represent one motif of three identical modifications on three consecutive nucleotides, and wherein the modifications are 2'-O-methyl or 2'-fluoro modifications; wherein the sense strand comprises at least one phosphorothioate linkage; and wherein the sense strand is conjugated to at least one ligand, wherein the ligand is one or more GalNAc derivatives attached through a bivalent or trivalent branched linker.

[0065] In an aspect, the invention provides a double stranded RNAi agent for inhibiting expression of KHK, wherein the double stranded RNAi agent comprises a sense strand and an antisense strand forming a double stranded region, wherein the sense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO:1, for example, 15 contiguous nucleotides differing by no more than 3 nucleotides selected from the nucleotide sequence of any one of nucleotides 89-107, 176-194, 264-282, 474-492, 508-526, 529-547, 562-580, 616-646, 682-700, 705-723, 705-757, 705-799, 739-757, 739-799, 760-799, 804-822, 837-855, 892-910, 959-977, 992-1010, 922-1041, 1013-1041, 1069-1108, 1169-1140, 1111-

1140, 1155-1196, 1221-1261, 1267-1294, or 1320-1350 or SEQ ID NO: 1, and the antisense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO:2, wherein substantially all of the nucleotides of the sense strand comprise a modification selected from a 2'-O-methyl modification and a 2'-fluoro modification, wherein the sense strand comprises two phosphorothioate internucleotide linkages at the 5'-terminus, wherein substantially all of the nucleotides of the antisense strand comprise a modification selected from a 2'-O-methyl modification and a 2'-fluoro modification, wherein the antisense strand comprises two phosphorothioate internucleotide linkages at the 5'-terminus and two phosphorothioate internucleotide linkages at the 3'-terminus, and wherein the sense strand is conjugated to one or more GalNAc derivatives attached through a monovalent or branched bivalent or trivalent linker at the 3'-terminus. In certain embodiments, the sense strand comprises at least 15 contiguous nucleotides of SEQ ID NO: 1, or any one of the foregoing indicated portions of SEQ ID NO: 1, and at least 15 contiguous nucleotides of the corresponding portion of SEQ ID NO: 2 such that the antisense strand is complementary to the at least 15 contiguous nucleotides differing by no more than 3 nucleotides in the sense strand. In certain embodiments, the sense strand and the antisense strand comprise at least 15 consecutive nucleotides of SEQ ID NO: 1 and SEQ ID NO: 2, or any one of the indicated portions of SEQ ID NO: 1 and the corresponding portion of SEQ ID NO: 2.

[0066] In certain embodiments, all of the nucleotides of the sense strand and all of the nucleotides of the antisense strand are modified nucleotides. In certain embodiments, each strand has 19-30 nucleotides.

[0067] In certain embodiments, substantially all of the nucleotides of the sense strand are modified. In certain embodiments, substantially all of the nucleotides of the antisense strand are modified. In certain embodiments, substantially all of the nucleotides of both the sense strand and the antisense strand are modified.

[0068] In an aspect, the invention provides a cell containing a dsRNA agent as described herein.

[0069] In an aspect, the invention provides a vector encoding at least one strand of a dsRNA agent, wherein the antisense strand comprises a region of complementarity to at least a part of an mRNA encoding KHK, wherein the dsRNA is 30 base pairs or less in length, and wherein the dsRNA agent targets the mRNA for cleavage. In certain embodiments, the region of complementarity is at least 15 nucleotides in length. In certain embodiments, the region of complementarity is 19 to 23 nucleotides in length.

[0070] In an aspect, the invention provides a cell comprising a vector as described herein.

[0071] In an aspect, the invention provides a pharmaceutical composition for inhibiting expression of a KHK gene comprising the dsRNA agent of the invention. In one embodiment, the dsRNAi agent is administered in an unbuffered solution. In certain embodiments, the unbuffered solution is saline or water. In other embodiments, the dsRNAi agent is administered with a buffer solution. In such embodiments, the buffer solution can comprise acetate, citrate, prolamine, carbonate, or phosphate, or any combination thereof. For example, the buffer solution can be phosphate buffered saline (PBS).

[0072] In an aspect, the invention provides a pharmaceutical composition comprising the dsRNA agent of the invention and a lipid formulation. In certain embodiments, the lipid formulation comprises a LNP. In certain embodiments, the lipid formulation comprises a MC3.

[0073] In an aspect, the invention provides a method of inhibiting KHK expression in a cell, the method comprising (a) contacting the cell with the dsRNA agent of the invention or a pharmaceutical composition of the invention; and (b) maintaining the cell produced in step (a) for a time sufficient to obtain degradation of the mRNA transcript of a KHK gene, thereby inhibiting expression of the KHK gene in the cell. In certain embodiments, the cell is within a subject, for example, a human subject, for example a female human or a male human. In certain embodiments, the subject has or is susceptible to reduced kidney function. In preferred embodiments, KHK

expression is inhibited by at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% as compared to an appropriate control, or lowered to below the threshold of detection. In certain embodiments, the sense strand comprises at least 15 contiguous nucleotides with no more than three mismatches from SEQ ID NO: 1, or comprises any one of nucleotides 89-107, 176-194, 264-282, 474492, 508-526, 529-547, 562-580, 616-646, 682-700, 705-723, 705-757, 705-799, 739-757, 739-799, 760-799, 804-822, 837-855, 892-910, 959-977, 992-1010, 922-1041, 1013-1041, 1069-1108, 1169-1140, 1111-1140, 1155-1196, 1221-1261, 1267-1294, or 1320-1350 of SEQ ID NO: 1 and at least 15 contiguous nucleotides of the corresponding portion of SEQ ID NO: 2 such that the antisense strand is complementary to the at least 15 contiguous nucleotides differing by no more than 3 nucleotides in the sense strand. In certain embodiments, the sense strand and the antisense strand comprise at least 15 consecutive nucleotides of SEQ ID NO: 1 and SEQ ID NO: 2 or one of the indicated portions of SEQ ID NO: 1 and the corresponding portion of SEQ ID NO: 2 such that the antisense strand is complementary to the at least 15 contiguous nucleotides differing by no more than 3 nucleotides in the sense strand.

[0074] In an aspect, the invention provides a method of treating a subject having a disease or disorder that would benefit from reduction in KHK expression, the method comprising administering to the subject a therapeutically effective amount of the dsRNA agent of the invention or a pharmaceutical composition of the invention, thereby treating the subject.

[0075] In an aspect, the invention provides a method of preventing at least one symptom in a subject having a disease or disorder that would benefit from reduction in KHK expression, the method comprising administering to the subject a prophylactically effective amount of the dsRNA agent of the invention or a pharmaceutical composition of the invention, thereby preventing at least one symptom in the subject having a disorder that would benefit from reduction in KHK expression.

[0076] In certain embodiments, the administration of the dsRNA to the subject causes a decrease in fructose metabolism. In certain embodiments, the administration of the dsRNA causes a decrease in the level of KHK in the subject, especially hepatic KHK, especially KHK-C in a subject with elevated KHK. In certain embodiments, the administration of the dsRNA causes a decrease in fructose metabolism in the subject. In certain embodiments, the administration of the dsRNA causes a decrease in the level of uric acid, e.g., serum uric acid, in a subject with elevated serum uric acid, e.g., elevated serum uric acid associated with gout. In certain embodiments, the administration of the dsRNA causes a normalization of serum lipids, e.g., triglycerides including postprandial triglycerides, LDL, HDL, or cholesterol, in a subject with at least one abnormal serum lipid level. In certain embodiments, the administration of the dsRNA causes a normalization of lipid deposition, e.g., a decrease of lipid deposition in the liver (e.g., decrease of NAFLD or NASH), a decrease of visceral fat deposition, a decrease in body weight. In certain embodiments, the administration of the dsRNA causes a normalization of insulin or glucose response in a subject with abnormal insulin response not related to an immune response to insulin, or abnormal glucose response. In certain embodiments, the administration of the dsRNA results in an improvement of kidney function, or a stoppage or reduction of the rate of loss of kidney function. In certain embodiments, the dsRNA causes a reduction of hypertension, i.e., elevated blood pressure.

[0077] In certain embodiments, the KHK-associated disease is a liver disease, e.g., fatty liver disease such as NAFLD or NASH. In certain embodiments, the KHK-associated disease is dyslipidemia, e.g., elevated serum triglycerides, elevated serum LDL, elevated serum cholesterol, lowered serum HDL, postprandial hypertriglyceridemia. In another embodiment, the KHK-associated disease is a disorder of glycemic control, e.g., insulin resistance not resulting from an immune response against insulin, glucose resistance, type 2 diabetes. In certain embodiments, the KHK-associated disease is a cardiovascular disease, e.g., hypertension, endothelial cell dysfunction. In certain embodiments, the KHK-associated disease is a kidney disease, e.g., acute kidney disorder, tubular dysfunction, proinflammatory changes to the proximal tubules, chronic

kidney disease. In certain embodiments, the disease is metabolic syndrome. In certain embodiments, the KHK-associated disease is a disease of lipid deposition or dysfunction, e.g., visceral adipose deposition, fatty liver, obesity. In certain embodiments, the KHK-associated disease is a disease of elevated uric acid, e.g., gout, hyperuricemia.

[0078] In certain embodiments the KHK-associated disease is an eating disorder such as excessive sugar craving.

[0079] In certain embodiments, the invention further comprises administering an additional agent to a subject with a KHK-associated disease.

[0080] In certain embodiments, treatments known in the art for the various KHK-associated diseases are used in combination with the RNAi agents of the invention. Such treatments are discussed below.

[0081] In various embodiments, the dsRNAi agent is administered to a subject at a dose of about 0.01 mg/kg to about 10 mg/kg or about 0.5 mg/kg to about 50 mg/kg. In some embodiments, the dsRNA agent is administered to a subject at a dose of about 10 mg/kg to about 30 mg/kg. In certain embodiments, the dsRNA agent is administered to a subject at a dose selected from 0.5 mg/kg 1 mg/kg, 1.5 mg/kg, 3 mg/kg, 5 mg/kg, 10 mg/kg, and 30 mg/kg. In certain embodiments, the RNAi agent is administered about once per week, once per month, once every other two months, or once a quarter (i.e., once every three months) at a dose of about 0.1 mg/kg to about 5.0 mg/kg.

[0082] In certain embodiments, the dsRNAi agent is administered to the subject once a week. In certain embodiments, the dsRNAi agent is administered to the subject once a month. In certain embodiments, the dsRNAi agent is administered to a subject once per quarter (i.e., every three months).

[0083] In some embodiments, the dsRNAi agent is administered to the subject subcutaneously.

[0084] In some embodiments, the dsRNAi agent is administered to the subject intramuscularly.

[0085] In various embodiments, the methods of the invention further comprise measuring the uric acid level, especially serum uric acid level, in the subject. In various embodiments, the methods of the invention further comprise measuring the urine fructose level in the subject. In various embodiments, the methods of the invention further comprise measuring a serum lipid level in a subject. In certain embodiments, the methods of the invention further include measuring insulin or glucose sensitivity in a subject. In certain embodiments, a decrease in the levels of expression or activity of fructose metabolism indicates that the KHK-associated disease is being treated or prevented.

Description

BRIEF DESCRIPTION OF THE DRAWINGS

[0086] FIG. 1 depicts the classic and alternative lipogenic pathways of fructose. In the classical pathway, triglycerides (TG) are a direct product of fructose metabolism by the action of multiple enzymes including aldolase B (Aldo B) and fatty acid synthase (FAS). In an alternative pathway, uric acid produced from the nucleotide turnover that occurs during the phosphorylation of fructose to fructose-1-phosphate (F-1-P) results in the generation of mitochondrial oxidative stress (mtROS), which causes a decrease in the activity of aconitase (ACO2) in the Krebs cycle. As a consequence, the ACO2 substrate, citrate, accumulates and is released to the cytosol where it acts as substrate for TG synthesis through the activation of ATP citrate lyase (ACL) and fatty acid synthase. AMPD2, AMP deaminase 2; IMP, inosine monophosphate; PO.sub.4, phosphate (from Johnson et al. (2013) *Diabetes*. 62:3307-3315).

[0087] FIG. 2 depicts the exon arrangement on the human KHK gene for the transcript products of ketohexokinase A (NM_000221.2, SEQ ID NO: 3), ketohexokinase C (NM_006488.2, SEQ ID NO: 1) and transcript variant X5 (XM_005264298.1, SEQ ID NO: 5).

DETAILED DESCRIPTION OF THE INVENTION

[0088] The present invention provides compositions comprising RNAi agents, e.g., double stranded iRNA agents, targeting KHK. The present invention also provides methods of using the compositions of the invention for inhibiting KHK expression and for treating KHK-associated disease, disorders, or conditions, e.g., liver disease (e.g., fatty liver, steatohepatitis, NAFLD, NASH), dyslipidemia (e.g., hyperlipidemia, high LDL cholesterol, low HDL cholesterol, hypertriglyceridemia, postprandial hypertriglyceridemia), disorders of glycemic control (e.g., insulin resistance not due to an immune response to insulin, type 2 diabetes), cardiovascular disease (e.g., hypertension, endothelial cell dysfunction), kidney disease (e.g., acute kidney disorder, tubular dysfunction, proinflammatory changes to the proximal tubules, chronic kidney disease), metabolic syndrome, adipocyte dysfunction, visceral adipose deposition, obesity, hyperuricemia, gout, eating disorders, and excessive sugar craving (Khaitan Z. et al., (2013) *J. Nutr. Metab.*, Article ID 682673, 1-12; Diggle C. P. et al., (2009) *J. Hisotchem. Cytochem.*, 57(8): 763-774; Cirillo P. et al., (2009) *J. Am. Soc. Nephrol.*, 20: 545-553; Lanaspá M. A. et al., (2012) *PLOS ONE* 7(10): 1-11).

[0089] The KHK (Ketohehexokinase) gene is located on chromosome 2p23 and encodes ketohehexokinase, also known as fructokinase. KHK is a phosphotransferase enzyme with an alcohol as the phosphate acceptor. KHK belongs to the ribokinase family of carbohydrate kinases (Trinh et al., *ACTA Cryst.*, D65: 201-211). Two isoforms of ketohehexokinase have been identified, KHK-A and KHK-C, that result from alternative splicing of the full length mRNA. These isoforms differ by inclusion of either exon 3a or 3c, and differ by 32 amino acids between positions 72 and 115 (see, e.g., FIG. 2). KHK-C mRNA is expressed at high levels, predominantly in the liver, kidney and small intestine. KHK-C has a much lower $K_{sub.m}$ for fructose binding than KHK-A, and as a result, is highly effective in phosphorylating dietary fructose. The sequence of a human KHK-C mRNA transcript may be found at, for example, GenBank Accession No. GI: 153218447 (NM_006488.2; SEQ ID NO:1). The sequence of a human KHK-A mRNA transcript may be found at, for example GenBank Accession No. GI: 153218446 (NM_000221.2; SEQ ID NO:3). The sequence of full-length human KHK mRNA is provided in GenBank Accession No. GI: 530367552 (XM_005264298.1; SEQ ID NO:5) was used (FIG. 2).

[0090] The present invention provides iRNA agents, compositions and methods for modulating the expression of a KHK gene. In certain embodiments, expression of KHK is reduced using a KHK-specific iRNA agent, thereby leading to a decrease in the phosphorylation of fructose to fructose-1-phosphate and thereby preventing an increase in uric acid levels and an increase in lipogenesis resulting from metabolism through the fructose metabolic pathway. Thus, inhibition of KHK gene expression or activity using the iRNA compositions of the invention is useful as a therapy to reduce the lipogenic effects of dietary fructose and prevent the concomitant accumulation of uric acid in a subject. Such inhibition is useful for treating diseases, disorders, or conditions such as liver disease (e.g., fatty liver, steatohepatitis, NAFLD, NASH), dyslipidemia (e.g., hyperlipidemia, high LDL cholesterol, low HDL cholesterol, hypertriglyceridemia, postprandial hypertriglyceridemia), disorders of glycemic control (e.g., insulin resistance, diabetes), cardiovascular disease (e.g., hypertension, endothelial cell dysfunction), kidney disease (e.g., acute kidney disorder, tubular dysfunction, proinflammatory changes to the proximal tubules, chronic kidney disease), metabolic syndrome, adipocyte dysfunction, visceral adipose deposition, obesity, hyperuricemia, gout, eating disorders, and excessive sugar craving.

[0091] The present invention provides iRNA compositions which affect the RNA-induced silencing complex (RISC)-mediated cleavage of RNA transcripts of a ketohehexokinase (KHK) gene. The gene may be within a cell, e.g., a cell within a subject, such as a human. The use of these iRNAs enables the targeted degradation of mRNAs of the corresponding gene (KHK gene) in mammals.

[0092] The iRNAs of the invention have been designed to target the human KHK gene, including portions of the gene that are conserved in the KHK orthologs of other mammalian species. Without

intending to be limited by theory, it is believed that a combination or sub-combination of the foregoing properties and the specific target sites or the specific modifications in these iRNAs confer to the iRNAs of the invention improved efficacy, stability, potency, durability, and safety. [0093] Accordingly, the present invention also provides methods for treating a subject having a disorder that would benefit from inhibiting or reducing the expression of a KHK gene, e.g., a KHK-associated disease, using iRNA compositions which effect the RNA-induced silencing complex (RISC)-mediated cleavage of RNA transcripts of a KHK gene.

[0094] Very low dosages of the iRNAs of the invention, in particular, can specifically and efficiently mediate RNA interference (RNAi), resulting in significant inhibition of expression of the corresponding gene (KHK gene).

[0095] The iRNAs of the invention may include an RNA strand (the antisense strand) having a region which is about 30 nucleotides or less in length, e.g., 15-30, 15-29, 15-28, 15-27, 15-26, 15-25, 15-24, 15-23, 15-22, 15-21, 15-20, 15-19, 15-18, 15-17, 18-30, 18-29, 18-28, 18-27, 18-26, 18-25, 18-24, 18-23, 18-22, 18-21, 18-20, 19-30, 19-29, 19-28, 19-27, 19-26, 19-25, 19-24, 19-23, 19-22, 19-21, 19-20, 20-30, 20-29, 20-28, 20-27, 20-26, 20-25, 20-24, 20-23, 20-22, 20-21, 21-30, 21-29, 21-28, 21-27, 21-26, 21-25, 21-24, 21-23, or 21-22 nucleotides in length, which region is substantially complementary to at least part of an mRNA transcript of a KHK gene.

[0096] In certain embodiments, the iRNAs of the invention include an RNA strand (the antisense strand) which can include longer lengths, for example up to 66 nucleotides, e.g., 36-66, 26-36, 25-36, 31-60, 22-43, 27-53 nucleotides in length with a region of at least 19 contiguous nucleotides that is substantially complementary to at least a part of an mRNA transcript of a KHK gene. These iRNAs with the longer length antisense strands preferably include a second RNA strand (the sense strand) of 20-60 nucleotides in length wherein the sense and antisense strands form a duplex of 18-30 contiguous nucleotides.

[0097] The use of the iRNAs of the invention enables the targeted degradation of mRNAs of the corresponding gene (KHK gene) in mammals. Very low dosages of the iRNAs of the invention, in particular, can specifically and efficiently mediate RNA interference (RNAi), resulting in significant inhibition of expression of the corresponding gene (KHK gene). Using in vitro and in vivo assays, the present inventors have demonstrated that iRNAs targeting a KHK gene can mediate RNAi, resulting in significant inhibition of expression of KHK, as well as reducing fructose metabolism which will decrease one or more of the symptoms associated with a KHK-associated disease. Thus, methods and compositions including these iRNAs are useful for treating a subject having a KHK-associated disease. The methods and compositions herein are useful for reducing the level of KHK in a subject, preferably KHK-C in a subject e.g., hepatic KHK-C in a subject.

[0098] The following detailed description discloses how to make and use compositions containing iRNAs to inhibit the expression of a KHK gene as well as compositions, uses, and methods for treating subjects having diseases and disorders that would benefit from reduction of the expression of a KHK gene.

I. Definitions

[0099] In order that the present invention may be more readily understood, certain terms are first defined. In addition, it should be noted that whenever a value or range of values of a parameter are recited, it is intended that values and ranges intermediate to the recited values are also intended to be part of this invention.

[0100] The articles “a” and “an” are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element, e.g., a plurality of elements.

[0101] The term “including” is used herein to mean, and is used interchangeably with, the phrase “including but not limited to”.

[0102] The term “or” is used herein to mean, and is used interchangeably with, the term “and/or,”

unless context clearly indicates otherwise. For example, “sense strand or antisense strand” is understood as “sense strand or antisense strand or sense strand and antisense strand.”

[0103] The term “about” is used herein to mean within the typical ranges of tolerances in the art. For example, “about” can be understood as about 2 standard deviations from the mean. In certain embodiments, about means \pm 10%. In certain embodiments, about means \pm 5%. When about is present before a series of numbers or a range, it is understood that “about” can modify each of the numbers in the series or range.

[0104] The term “at least” prior to a number or series of numbers is understood to include the number adjacent to the term “at least”, and all subsequent numbers or integers that could logically be included, as clear from context. For example, the number of nucleotides in a nucleic acid molecule must be an integer. For example, “at least 18 nucleotides of a 21 nucleotide nucleic acid molecule” means that 18, 19, 20, or 21 nucleotides have the indicated property. When at least is present before a series of numbers or a range, it is understood that “at least” can modify each of the numbers in the series or range.

[0105] As used herein, “no more than” or “less than” is understood as the value adjacent to the phrase and logical lower values or intergers, as logical from context, to zero. For example, a duplex with an overhang of “no more than 2 nucleotides” has a 2, 1, or 0 nucleotide overhang. When “no more than” is present before a series of numbers or a range, it is understood that “no more than” can modify each of the numbers in the series or range.

[0106] As used herein, “ketohexokinase” or “KHK” is an enzyme that catalyzes conversion of fructose to fructose-1-phosphate. The product of this gene is the first enzyme in the pathway that catabolizes dietary fructose. Alternatively spliced transcript variants encoding different isoforms have been identified. The gene is also known as fructokinase. Further information on KHK is provided, for example, in the NCBI Gene database at www.ncbi.nlm.nih.gov/gene/3975 (which is incorporated herein by reference as of the date of filing this application).

[0107] As used herein, “ketohexokinase,” used interchangeably with the term “KHK,” refers to the naturally occurring gene that encodes a KHK protein. The amino acid and complete coding sequences of the reference sequence of the human KHK gene may be found in, for example, GenBank Accession No. GI: 153218447 (RefSeq Accession No. NM_006488; SEQ ID NO:1; SEQ ID NO:2), GenBank Accession No. GI: 153218446 (RefSeq Accession No. NM_000221.2; SEQ ID NO: 3 and 4), and GenBank Accession No. 767914480 (RefSeq Accession No. XM_005264298.2; SEQ ID NO: 5 and 6). Mammalian orthologs of the human KHK gene may be found in, for example, GI: 887209819 (RefSeq Accession No. NM_008439.4, mouse; SEQ ID NO:7 and SEQ ID NO:8); GI: 126432547 (RefSeq Accession No. NM_031855.3, rat; SEQ ID NO:9 and SEQ ID NO:10); GenBank Accession Nos. GI: 982291245 (RefSeq Accession No. XM_005576321, cynomolgus monkey; SEQ ID NO:11 and SEQ ID NO:12).

[0108] There are two KHK isoforms produced by alternative splicing of the KHK pre-mRNA. KHK-C is abundant in fructose metabolizing organs, e.g., liver, kidney, and intestines. It is highly active and responsible for most fructose metabolism. KHK-A has a lower affinity to fructose and is widely expressed in most tissues. The iRNA agents provided herein can be capable of silencing one or both KHK isoforms. In preferred embodiments, the iRNA agent is capable of silencing at least KHK-C and expression of at least the KHK-C isoform is inhibited.

[0109] A number of naturally occurring SNPs are known and can be found, for example, in the SNP database at the NCBI at www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=3795 (which is incorporated herein by reference as of the date of filing this application) which provides SNPs in human KHK. In preferred embodiments, such naturally occurring variants are included within the scope of the KHK gene sequence.

[0110] Additional examples of KHK mRNA sequences are readily available using publicly available databases, e.g., GenBank, UniProt, and OMIM.

[0111] As used herein, “target sequence” refers to a contiguous portion of the nucleotide sequence

of an mRNA molecule formed during the transcription of a KHK gene, including mRNA that is a product of RNA processing of a primary transcription product. The target portion of the sequence will be at least long enough to serve as a substrate for iRNA-directed cleavage at or near that portion of the nucleotide sequence of an mRNA molecule formed during the transcription of a KHK gene. In one embodiment, the target sequence is within the protein coding region of KHK. [0112] The target sequence may be from about 9-36 nucleotides in length, e.g., about 15-30 nucleotides in length. For example, the target sequence can be from about 15-30 nucleotides, 15-29, 15-28, 15-27, 15-26, 15-25, 15-24, 15-23, 15-22, 15-21, 15-20, 15-19, 15-18, 15-17, 18-30, 18-29, 18-28, 18-27, 18-26, 18-25, 18-24, 18-23, 18-22, 18-21, 18-20, 19-30, 19-29, 19-28, 19-27, 19-26, 19-25, 19-24, 19-23, 19-22, 19-21, 19-20, 20-30, 20-29, 20-28, 20-27, 20-26, 20-25, 20-24, 20-23, 20-22, 20-21, 21-30, 21-29, 21-28, 21-27, 21-26, 21-25, 21-24, 21-23, or 21-22 nucleotides in length. Ranges and lengths intermediate to the above recited ranges and lengths are also contemplated to be part of the invention.

[0113] As used herein, the term “strand comprising a sequence” refers to an oligonucleotide comprising a chain of nucleotides that is described by the sequence referred to using the standard nucleotide nomenclature.

[0114] “G,” “C,” “A,” “T,” and “U” each generally stand for a nucleotide that contains guanine, cytosine, adenine, thymidine, and uracil as a base, respectively. However, it will be understood that the term “ribonucleotide” or “nucleotide” can also refer to a modified nucleotide, as further detailed below, or a surrogate replacement moiety (see, e.g., Table 2). The skilled person is well aware that guanine, cytosine, adenine, and uracil can be replaced by other moieties without substantially altering the base pairing properties of an oligonucleotide comprising a nucleotide bearing such replacement moiety. For example, without limitation, a nucleotide comprising inosine as its base can base pair with nucleotides containing adenine, cytosine, or uracil. Hence, nucleotides containing uracil, guanine, or adenine can be replaced in the nucleotide sequences of dsRNA featured in the invention by a nucleotide containing, for example, inosine. In another example, adenine and cytosine anywhere in the oligonucleotide can be replaced with guanine and uracil, respectively to form G-U Wobble base pairing with the target mRNA. Sequences containing such replacement moieties are suitable for the compositions and methods featured in the invention. [0115] The terms “iRNA”, “RNAi agent,” “iRNA agent,” “RNA interference agent” as used interchangeably herein, refer to an agent that contains RNA as that term is defined herein, and which mediates the targeted cleavage of an RNA transcript via an RNA-induced silencing complex (RISC) pathway. iRNA directs the sequence-specific degradation of mRNA through a process known as RNA interference (RNAi). The iRNA modulates, e.g., inhibits, the expression of a KHK gene in a cell, e.g., a cell within a subject, such as a mammalian subject.

[0116] In one embodiment, an RNAi agent of the invention includes a single stranded RNA that interacts with a target RNA sequence, e.g., a KHK target mRNA sequence, to direct the cleavage of the target RNA. Without wishing to be bound by theory it is believed that long double stranded RNA introduced into cells is broken down into siRNA by a Type III endonuclease known as Dicer (Sharp et al. (2001) *Genes Dev.* 15:485). Dicer, a ribonuclease-III-like enzyme, processes the dsRNA into 19-23 base pair short interfering RNAs with characteristic two base 3' overhangs (Bernstein, et al., (2001) *Nature* 409:363). The siRNAs are then incorporated into an RNA-induced silencing complex (RISC) where one or more helicases unwind the siRNA duplex, enabling the complementary antisense strand to guide target recognition (Nykanen, et al., (2001) *Cell* 107:309). Upon binding to the appropriate target mRNA, one or more endonucleases within the RISC cleave the target to induce silencing (Elbashir, et al., (2001) *Genes Dev.* 15:188). Thus, in one aspect the invention relates to a single stranded RNA (siRNA) generated within a cell and which promotes the formation of a RISC complex to effect silencing of the target gene, i.e., a KHK gene. Accordingly, the term “siRNA” is also used herein to refer to an iRNA as described above.

[0117] In certain embodiments, the RNAi agent may be a single-stranded siRNA (ssRNAi) that is

introduced into a cell or organism to inhibit a target mRNA. Single-stranded RNAi agents bind to the RISC endonuclease, Argonaute 2, which then cleaves the target mRNA. The single-stranded siRNAs are generally 15-30 nucleotides and are chemically modified. The design and testing of single-stranded siRNAs are described in U.S. Pat. No. 8,101,348 and in Lima et al., (2012) *Cell* 150:883-894, the entire contents of each of which are hereby incorporated herein by reference. Any of the antisense nucleotide sequences described herein may be used as a single-stranded siRNA as described herein or as chemically modified by the methods described in Lima et al., (2012) *Cell* 150:883-894.

[0118] In certain embodiments, an “iRNA” for use in the compositions, uses, and methods of the invention is a double stranded RNA and is referred to herein as a “double stranded RNAi agent,” “double stranded RNA (dsRNA) molecule,” “dsRNA agent,” or “dsRNA”. The term “dsRNA”, refers to a complex of ribonucleic acid molecules, having a duplex structure comprising two anti-parallel and substantially complementary nucleic acid strands, referred to as having “sense” and “antisense” orientations with respect to a target RNA, i.e., a KHK gene. In some embodiments of the invention, a double stranded RNA (dsRNA) triggers the degradation of a target RNA, e.g., an mRNA, through a post-transcriptional gene-silencing mechanism referred to herein as RNA interference or RNAi.

[0119] In general, the majority of nucleotides of each strand of a dsRNA molecule are ribonucleotides, but as described in detail herein, each or both strands can also include one or more non-ribonucleotides, e.g., a deoxyribonucleotide or a modified nucleotide. In addition, as used in this specification, an “iRNA” may include ribonucleotides with chemical modifications; an iRNA may include substantial modifications at multiple nucleotides. As used herein, the term “modified nucleotide” refers to a nucleotide having, independently, a modified sugar moiety, a modified internucleotide linkage, or modified nucleobase, or any combination thereof. Thus, the term modified nucleotide encompasses substitutions, additions or removal of, e.g., a functional group or atom, to internucleoside linkages, sugar moieties, or nucleobases. The modifications suitable for use in the agents of the invention include all types of modifications disclosed herein or known in the art. Any such modifications, as used in a siRNA type molecule, are encompassed by “iRNA” or “RNAi agent” for the purposes of this specification and claims.

[0120] The majority of nucleotides of each strand of a dsRNA molecule may be ribonucleotides, but as described in detail herein, each or both strands can also include one or more non-ribonucleotides, e.g., a deoxyribonucleotide or a modified nucleotide. In addition, as used in this specification, an “iRNA” may include ribonucleotides with chemical modifications; an iRNA agent may include substantial modifications at multiple nucleotides. As used herein, the term “modified nucleotide” refers to a nucleotide having, independently, a modified sugar moiety, a modified internucleotide linkage, or modified nucleobase. Thus, the term modified nucleotide encompasses substitutions, additions or removal of, e.g., a functional group or atom, to internucleoside linkages, sugar moieties, or nucleobases. The modifications suitable for use in the agents of the invention include all types of modifications disclosed herein or known in the art. Any such modifications, as used in a siRNA type molecule, are encompassed by “iRNA” or “RNAi agent” for the purposes of this specification and claims.

[0121] The duplex region may be of any length that permits specific degradation of a desired target RNA through a RISC pathway, and may range from about 9 to 36 base pairs in length, e.g., about 15-30 base pairs in length, for example, about 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, or 36 base pairs in length, such as about 15-30, 15-29, 15-28, 15-27, 15-26, 15-25, 15-24, 15-23, 15-22, 15-21, 15-20, 15-19, 15-18, 15-17, 18-30, 18-29, 18-28, 18-27, 18-26, 18-25, 18-24, 18-23, 18-22, 18-21, 18-20, 19-30, 19-29, 19-28, 19-27, 19-26, 19-25, 19-24, 19-23, 19-22, 19-21, 19-20, 20-30, 20-29, 20-28, 20-27, 20-26, 20-25, 20-24, 20-23, 20-22, 20-21, 21-30, 21-29, 21-28, 21-27, 21-26, 21-25, 21-24, 21-23, or 21-22 base pairs in length. Ranges and lengths intermediate to the above recited ranges and lengths are also

contemplated to be part of the invention.

[0122] The two strands forming the duplex structure may be different portions of one larger RNA molecule, or they may be separate RNA molecules. Where the two strands are part of one larger molecule, and therefore are connected by an uninterrupted chain of nucleotides between the 3'-end of one strand and the 5'-end of the respective other strand forming the duplex structure, the connecting RNA chain is referred to as a "hairpin loop." A hairpin loop can comprise at least one unpaired nucleotide. In some embodiments, the hairpin loop can comprise at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 23 or more unpaired nucleotides. In some embodiments, the hairpin loop can be 10 or fewer nucleotides. In some embodiments, the hairpin loop can be 8 or fewer unpaired nucleotides. In some embodiments, the hairpin loop can be 4-10 unpaired nucleotides. In some embodiments, the hairpin loop can be 4-8 nucleotides.

[0123] Where the two substantially complementary strands of a dsRNA are comprised by separate RNA molecules, those molecules need not, but can be covalently connected. Where the two strands are connected covalently by means other than an uninterrupted chain of nucleotides between the 3'-end of one strand and the 5'-end of the respective other strand forming the duplex structure, the connecting structure is referred to as a "linker." The RNA strands may have the same or a different number of nucleotides. The maximum number of base pairs is the number of nucleotides in the shortest strand of the dsRNA minus any overhangs that are present in the duplex. In addition to the duplex structure, an RNAi may comprise one or more nucleotide overhangs.

[0124] In certain embodiments, an iRNA agent of the invention is a dsRNA, each strand of which comprises 19-23 nucleotides, that interacts with a target RNA sequence, e.g., a KHK gene, without wishing to be bound by theory, long double stranded RNA introduced into cells is broken down into siRNA by a Type III endonuclease known as Dicer (Sharp et al. (2001) *Genes Dev.* 15:485). Dicer, a ribonuclease-III-like enzyme, processes the dsRNA into 19-23 base pair short interfering RNAs with characteristic two base 3' overhangs (Bernstein, et al., (2001) *Nature* 409:363). The siRNAs are then incorporated into an RNA-induced silencing complex (RISC) where one or more helicases unwind the siRNA duplex, enabling the complementary antisense strand to guide target recognition (Nykanen, et al., (2001) *Cell* 107:309). Upon binding to the appropriate target mRNA, one or more endonucleases within the RISC cleave the target to induce silencing (Elbashir, et al., (2001) *Genes Dev.* 15:188).

[0125] In some embodiments, an iRNA of the invention is a dsRNA of 24-30 nucleotides that interacts with a target RNA sequence, e.g., a KHK target mRNA sequence, to direct the cleavage of the target RNA. Without wishing to be bound by theory, long double stranded RNA introduced into cells is broken down into siRNA by a Type III endonuclease known as Dicer (Sharp et al. (2001) *Genes Dev.* 15:485). Dicer, a ribonuclease-III-like enzyme, processes the dsRNA into 19-23 base pair short interfering RNAs with characteristic two base 3' overhangs (Bernstein, et al., (2001) *Nature* 409:363). The siRNAs are then incorporated into an RNA-induced silencing complex (RISC) where one or more helicases unwind the siRNA duplex, enabling the complementary antisense strand to guide target recognition (Nykanen, et al., (2001) *Cell* 107:309). Upon binding to the appropriate target mRNA, one or more endonucleases within the RISC cleave the target to induce silencing (Elbashir, et al., (2001) *Genes Dev.* 15:188).

[0126] As used herein, the term "nucleotide overhang" refers to at least one unpaired nucleotide that protrudes from the duplex structure of a double stranded iRNA. For example, when a 3'-end of one strand of a dsRNA extends beyond the 5'-end of the other strand, or vice versa, there is a nucleotide overhang. A dsRNA can comprise an overhang of at least one nucleotide; alternatively the overhang can comprise at least two nucleotides, at least three nucleotides, at least four nucleotides, at least five nucleotides or more. A nucleotide overhang can comprise or consist of a nucleotide/nucleoside analog, including a deoxynucleotide/nucleoside. The overhang(s) can be on the sense strand, the antisense strand, or any combination thereof. Furthermore, the nucleotide(s) of an overhang can be present on the 5'-end, 3'-end, or both ends of either an antisense or sense strand

of a dsRNA.

[0127] In certain embodiments, the antisense strand of a dsRNA has a 1-10 nucleotide, e.g., a 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotide, overhang at the 3'-end or the 5'-end. In certain embodiments, the overhang on the sense strand or the antisense strand, or both, can include extended lengths longer than 10 nucleotides, e.g., 1-30 nucleotides, 2-30 nucleotides, 10-30 nucleotides, or 10-15 nucleotides in length. In certain embodiments, the extended overhang can include a self complementary portion, i.e., the overhang is capable of forming a stable hairpin structure, e.g., a duplex of at least 3 nucleotides, or a duplex of at least four nucleotides. In certain embodiments, an extended overhang is on the sense strand of the duplex. In certain embodiments, an extended overhang is present on the 3'-end of the sense strand of the duplex. In certain embodiments, an extended overhang is present on the 5' end of the sense strand of the duplex. In certain embodiments, an extended overhang is on the antisense strand of the duplex. In certain embodiments, an extended overhang is present on the 3' end of the antisense strand of the duplex. In certain embodiments, an extended overhang is present on the 5' end of the antisense strand of the duplex. In certain embodiments, one or more of the nucleotides in the overhang is replaced with a nucleoside thiophosphate. In certain embodiments, the overhang includes a self-complementary portion such that the overhang is capable of forming a hairpin structure that is stable under physiological conditions.

[0128] "Blunt" or "blunt end" means that there are no unpaired nucleotides at that end of the double stranded RNAi agent, i.e., no nucleotide overhang. A "blunt ended" double stranded RNAi agent is double stranded over its entire length, i.e., no nucleotide overhang at either end of the molecule. The RNAi agents of the invention include RNAi agents with no nucleotide overhang at one end (i.e., agents with one overhang and one blunt end) or with no nucleotide overhangs at either end.

[0129] The term "antisense strand" or "guide strand" refers to the strand of an iRNA, e.g., a dsRNA, which includes a region that is substantially complementary to a target sequence, e.g., a KHK mRNA. As used herein, the term "region of complementarity" refers to the region on the antisense strand that is substantially complementary to a sequence, for example a target sequence, e.g., a KHK nucleotide sequence, as defined herein. Where the region of complementarity is not fully complementary to the target sequence, the mismatches can be in the internal or terminal regions of the molecule. Generally, the most tolerated mismatches are in the terminal regions, e.g., within 5, 4, 3, 2, or 1 nucleotides of the 5'- or 3'-end of the iRNA. In some embodiments, a double stranded RNAi agent of the invention includes a nucleotide mismatch in the antisense strand. In some embodiments, a double stranded RNAi agent of the invention includes a nucleotide mismatch in the sense strand. In some embodiments, the nucleotide mismatch is, for example, within 5, 4, 3, 2, or 1 nucleotides from the 3'-end of the iRNA. In another embodiment, the nucleotide mismatch is, for example, in the 3'-terminal nucleotide of the iRNA.

[0130] The term "sense strand" or "passenger strand" as used herein, refers to the strand of an iRNA that includes a region that is substantially complementary to a region of the antisense strand as that term is defined herein.

[0131] As used herein, "substantially all of the nucleotides are modified" are largely but not wholly modified and can include not more than 5, 4, 3, 2, or 1 unmodified nucleotides.

[0132] As used herein, the term "cleavage region" refers to a region that is located immediately adjacent to the cleavage site. The cleavage site is the site on the target at which cleavage occurs. In some embodiments, the cleavage region comprises three bases on either end of, and immediately adjacent to, the cleavage site. In some embodiments, the cleavage region comprises two bases on either end of, and immediately adjacent to, the cleavage site. In some embodiments, the cleavage site specifically occurs at the site bound by nucleotides 10 and 11 of the antisense strand, and the cleavage region comprises nucleotides 11, 12 and 13.

[0133] As used herein, and unless otherwise indicated, the term "complementary," when used to

describe a first nucleotide sequence in relation to a second nucleotide sequence, refers to the ability of an oligonucleotide or polynucleotide comprising the first nucleotide sequence to hybridize and form a duplex structure under certain conditions with an oligonucleotide or polynucleotide comprising the second nucleotide sequence, as will be understood by the skilled person. Such conditions can, for example, be stringent conditions, where stringent conditions can include: 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50° C. or 70° C. for 12-16 hours followed by washing (see, e.g., “Molecular Cloning: A Laboratory Manual, Sambrook, et al. (1989) Cold Spring Harbor Laboratory Press). Other conditions, such as physiologically relevant conditions as can be encountered inside an organism, can apply. The skilled person will be able to determine the set of conditions most appropriate for a test of complementarity of two sequences in accordance with the ultimate application of the hybridized nucleotides.

[0134] Complementary sequences within an iRNA, e.g., within a dsRNA as described herein, include base-pairing of the oligonucleotide or polynucleotide comprising a first nucleotide sequence to an oligonucleotide or polynucleotide comprising a second nucleotide sequence over the entire length of one or both nucleotide sequences. Such sequences can be referred to as “fully complementary” with respect to each other herein. However, where a first sequence is referred to as “substantially complementary” with respect to a second sequence herein, the two sequences can be fully complementary, or they can form one or more, but generally not more than 5, 4, 3, or 2 mismatched base pairs upon hybridization for a duplex up to 30 base pairs, while retaining the ability to hybridize under the conditions most relevant to their ultimate application, e.g., inhibition of gene expression via a RISC pathway. However, where two oligonucleotides are designed to form, upon hybridization, one or more single stranded overhangs, such overhangs shall not be regarded as mismatches with regard to the determination of complementarity. For example, a dsRNA comprising one oligonucleotide 21 nucleotides in length and another oligonucleotide 23 nucleotides in length, wherein the longer oligonucleotide comprises a sequence of 21 nucleotides that is fully complementary to the shorter oligonucleotide, can yet be referred to as “fully complementary” for the purposes described herein.

[0135] “Complementary” sequences, as used herein, can also include, or be formed entirely from, non-Watson-Crick base pairs or base pairs formed from non-natural and modified nucleotides, in so far as the above requirements with respect to their ability to hybridize are fulfilled. Such non-Watson-Crick base pairs include, but are not limited to, G:U Wobble or Hoogsteen base pairing.

[0136] The terms “complementary,” “fully complementary,” and “substantially complementary” herein can be used with respect to the base matching between the sense strand and the antisense strand of a dsRNA, or between the antisense strand of a double stranded RNAi agent and a target sequence, as will be understood from the context of their use.

[0137] As used herein, a polynucleotide that is “substantially complementary to at least part of” a messenger RNA (mRNA) refers to a polynucleotide that is substantially complementary to a contiguous portion of the mRNA of interest (e.g., an mRNA encoding a KHK gene). For example, a polynucleotide is complementary to at least a part of a KHK mRNA if the sequence is substantially complementary to a non-interrupted portion of an mRNA encoding a KHK gene.

[0138] Accordingly, in some embodiments, the antisense polynucleotides disclosed herein are fully complementary to the target KHK sequence. In other embodiments, the antisense polynucleotides disclosed herein are substantially complementary to the target KHK sequence and comprise a contiguous nucleotide sequence which is at least about 80% complementary over its entire length to the equivalent region of the nucleotide sequence of any one of SEQ ID NOs:1, 3, 5, 7, 9, and 11, preferably of SEQ ID NOs: 1, 3, and 5, or a fragment of any one of SEQ ID NOs:1, 3, 5, 7, 9, and 11, preferably of SEQ ID NOs: 1, 3, and 5, such as at least 85%, 86%, 87%, 88%, 89%, about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% complementary or 100% complementary.

[0139] In one embodiment, an RNAi agent of the invention includes a sense strand that is substantially complementary to an antisense polynucleotide which, in turn, is complementary to a

target KHK sequence and comprises a contiguous nucleotide sequence which is at least about 80% complementary over its entire length to any one of the sense strand nucleotide sequences in any one of Tables Table 3 or Table 5, or a fragment of any one of the sense strands in Table 3 or Table 5, such as about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% complementary, or 100% complementary.

[0140] In some embodiments, an iRNA of the invention includes an antisense strand that is substantially complementary to the target KHK sequence and comprises a contiguous nucleotide sequence which is at least about 80% complementary over its entire length to the equivalent region of the nucleotide sequence of any one of the antisense strands in Table 3 or 5, or a fragment of any one of the antisense strands in Table 3 or 5, such as about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% complementary, or 100% complementary.

[0141] In general, the majority of nucleotides of each strand are ribonucleotides, but as described in detail herein, each or both strands can also include one or more non-ribonucleotides, e.g., a deoxyribonucleotide or a modified nucleotide. In addition, an “iRNA” may include ribonucleotides with chemical modifications. Such modifications may include all types of modifications disclosed herein or known in the art. Any such modifications, as used in an dsRNA molecule, are encompassed by “iRNA” for the purposes of this specification and claims.

[0142] In an aspect of the invention, an agent for use in the methods and compositions of the invention is a single-stranded antisense oligonucleotide molecule that inhibits a target mRNA via an antisense inhibition mechanism. The single-stranded antisense oligonucleotide molecule is complementary to a sequence within the target mRNA. The single-stranded antisense oligonucleotides can inhibit translation in a stoichiometric manner by base pairing to the mRNA and physically obstructing the translation machinery, see Dias, N. et al., (2002) *Mol Cancer Ther* 1:347-355. The single-stranded antisense oligonucleotide molecule may be about 14 to about 30 nucleotides in length and have a sequence that is complementary to a target sequence. For example, the single-stranded antisense oligonucleotide molecule may comprise a sequence that is at least about 14, 15, 16, 17, 18, 19, 20, or more contiguous nucleotides from any one of the antisense sequences described herein.

[0143] The phrase “contacting a cell with an iRNA,” such as a dsRNA, as used herein, includes contacting a cell by any possible means. Contacting a cell with an iRNA includes contacting a cell in vitro with the iRNA or contacting a cell in vivo with the iRNA. The contacting may be done directly or indirectly. Thus, for example, the iRNA may be put into physical contact with the cell by the individual performing the method, or alternatively, the iRNA may be put into a situation that will permit or cause it to subsequently come into contact with the cell.

[0144] Contacting a cell in vitro may be done, for example, by incubating the cell with the iRNA. Contacting a cell in vivo may be done, for example, by injecting the iRNA into or near the tissue where the cell is located, or by injecting the iRNA into another area, e.g., the bloodstream or the subcutaneous space, such that the agent will subsequently reach the tissue where the cell to be contacted is located. For example, the iRNA may contain or be coupled to a ligand, e.g., a GalNAc, e.g., GalNAc3, that directs the iRNA to a site of interest, e.g., the liver. Combinations of in vitro and in vivo methods of contacting are also possible. For example, a cell may also be contacted in vitro with an iRNA and subsequently transplanted into a subject.

[0145] In certain embodiments, contacting a cell with an iRNA includes “introducing” or “delivering the iRNA into the cell” by facilitating or effecting uptake or absorption into the cell. Absorption or uptake of an iRNA can occur through unaided diffusion or active cellular processes, or by auxiliary agents or devices. Introducing an iRNA into a cell may be in vitro or in vivo. For example, for in vivo introduction, iRNA can be injected into a tissue site or administered systemically. In vivo delivery can also be done by a beta-glucan delivery system, such as those described in U.S. Pat. Nos. 5,032,401 and 5,607,677, and US Publication No. 2005/0281781, the entire contents of which are hereby incorporated herein by reference. In vitro introduction into a

cell includes methods known in the art such as electroporation and lipofection. Further approaches are described herein below or are known in the art.

[0146] The term “lipid nanoparticle” or “LNP” is a vesicle comprising a lipid layer encapsulating a pharmaceutically active molecule, such as a nucleic acid molecule, e.g., an iRNA or a plasmid from which an iRNA is transcribed. LNPs are described in, for example, U.S. Pat. Nos. 6,858,225, 6,815,432, 8,158,601, and 8,058,069, the entire contents of which are hereby incorporated herein by reference.

[0147] As used herein, a “subject” is an animal, such as a mammal, including a primate (such as a human, a non-human primate, e.g., a monkey, and a chimpanzee), a non-primate (such as a cow, a pig, a camel, a llama, a horse, a goat, a rabbit, a sheep, a hamster, a guinea pig, a cat, a dog, a rat, a mouse, a horse, and a whale), or a bird (e.g., a duck or a goose) that expresses the target gene, either endogenously or heterologously. In certain embodiments, the subject is a human, such as a human being treated or assessed for a disease, disorder, or condition that would benefit from reduction in KHK gene expression or replication; a human at risk for a disease, disorder, or condition that would benefit from reduction in KHK gene expression; a human having a disease, disorder, or condition that would benefit from reduction in KHK gene expression; or human being treated for a disease, disorder or condition that would benefit from reduction in KHK gene expression, as described herein. In some embodiments, the subject is a female human. In other embodiments, the subject is a male human.

[0148] As used herein, the terms “treating” or “treatment” refer to a beneficial or desired result including, but not limited to, alleviation or amelioration of one or more signs or symptoms associated with KHK gene expression or KHK protein production, especially elevated KHK gene expression or elevated KHK protein production. “Treatment” can also mean prolonging survival as compared to expected survival in the absence of treatment.

[0149] The term “lower” in the context of the level of KHK gene expression or KHK protein production in a subject, or a disease marker or symptom refers to a statistically significant decrease in such level. The decrease can be, for example, at least 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%, or below the level of detection for the detection method. In certain embodiments, the expression of the target is normalized, i.e., decreased towards or to a level accepted as within the range of normal for an individual without such disorder, e.g., normalization of body weight, blood pressure, or a serum lipid level. As used here, “lower” in a subject can refer to lowering of gene expression or protein production in a cell in a subject does not require lowering of expression in all cells or tissues of a subject. For example, as used herein, lowering in a subject can include lowering of gene expression or protein production in the liver of a subject.

[0150] The term “lower” can also be used in association with normalizing a symptom of a disease or condition, i.e. decreasing the difference between a level in a subject suffering from a KHK-associated disease towards or to a level in a normal subject not suffering from a KHK-associated disease. For example, if a subject with a normal weight of 70 kg weighs 90 kg prior to treatment (20 kg overweight) and 80 kg after treatment (10 kg overweight), the subject's weight is lowered towards a normal weight by 50% ($10/20 \times 100\%$). Similarly, if the HDL level of a woman is increased from 50 mg/dL (poor) to 57 mg/dL, with a normal level being 60 mg/dL, the difference between the prior level of the subject and the normal level is decreased by 70% (difference of 10 mg/dL between subject level and normal is decreased by 7 mg/dL, $7/10 \times 100\%$). As used herein, if a disease is associated with an elevated value for a symptom. “normal” is considered to be the upper limit of normal. If a disease is associated with a decreased value for a symptom. “normal” is considered to be the lower limit of normal.

[0151] As used herein, “prevention” or “preventing,” when used in reference to a disease, disorder or condition thereof, that would benefit from a reduction in expression of a KHK gene or production of KHK protein, refers to a reduction in the likelihood that a subject will develop a

symptom associated with such a disease, disorder, or condition, e.g., a sign or symptom of KHK gene expression or KHK activity and increased fructose metabolism. Without being bound by mechanism, it is known that fructose phosphorylation catalyzed by KHK to form fructose-1-phosphate is not regulated by feedback inhibition which can result in depletion of ATP and intracellular phosphate, an increase AMP levels, which results in the production of uric acid. Further, the fructose-1-phosphate is metabolized to glyceraldehyde which feeds into the citric acid cycle increasing the production of acetyl Co-A stimulating fatty acid synthesis. Diseases and conditions associated with elevated uric acid and fatty acid synthesis include, e.g., liver disease (e.g., fatty liver, steatohepatitis including non-alcoholic steatohepatitis (NASH)), dyslipidemia (e.g., hyperlipidemia, high LDL cholesterol, low HDL cholesterol, hypertriglyceridemia, postprandial hypertriglyceridemia), disorders of glycemic control (e.g., insulin resistance not related to immune response to insulin, type 2 diabetes), cardiovascular disease (e.g., hypertension, endothelial cell dysfunction), kidney disease (e.g., acute kidney disorder, tubular dysfunction, proinflammatory changes to the proximal tubules, chronic kidney disease), metabolic syndrome, disease of lipid deposition or dysfunction (e.g., adipocyte dysfunction, visceral adipose deposition, obesity), disease of elevated uric acid (e.g., hyperuricemia, gout), and eating disorders such as excessive sugar craving. The failure to develop a disease, disorder or condition, or the reduction in the development of a symptom or comorbidity associated with such a disease, disorder or condition (e.g., by at least about 10% on a clinically accepted scale for that disease or disorder), or the exhibition of delayed signs or symptoms or disease progression by days, weeks, months or years is considered effective prevention.

[0152] As used herein, the term “ketohexokinase disease” or “KHK-associated disease,” is a disease or disorder that is caused by, or associated with KHK gene expression or KHK protein production. The term “KHK-associated disease” includes a disease, disorder or condition that would benefit from a decrease in KHK gene expression, replication, or protein activity. Non-limiting examples of KHK-associated diseases include, for example, liver disease (e.g., fatty liver, steatohepatitis including non-alcoholic steatohepatitis (NASH)), dyslipidemia (e.g., hyperlipidemia, high LDL cholesterol, low HDL cholesterol, hypertriglyceridemia, postprandial hypertriglyceridemia), disorders of glycemic control (e.g., insulin resistance not related to immune response to insulin, type 2 diabetes), cardiovascular disease (e.g., hypertension, endothelial cell dysfunction), kidney disease (e.g., acute kidney disorder, tubular dysfunction, proinflammatory changes to the proximal tubules, chronic kidney disease), metabolic syndrome, disease of lipid deposition or dysfunction (e.g., adipocyte dysfunction, visceral adipose deposition, obesity), disease of elevated uric acid (e.g., hyperuricemia, gout), and eating disorders such as excessive sugar craving. Further details regarding signs and symptoms of the various diseases or conditions are provided herein and are well known in the art.

[0153] In certain embodiments, a KHK-associated disease is associated with elevated uric acid (e.g. hyperuricemia, gout).

[0154] In certain embodiments, a KHK-associated disease is associated with elevated lipid levels (e.g., fatty liver, steatohepatitis including non-alcoholic steatohepatitis (NASH), dyslipidemia).

[0155] “Therapeutically effective amount,” as used herein, is intended to include the amount of an iRNA that, when administered to a patient for treating a subject having a KHK-associated disease, is sufficient to effect treatment of the disease (e.g., by diminishing, ameliorating, or maintaining the existing disease or one or more symptoms of disease or its related comorbidities). The “therapeutically effective amount” may vary depending on the iRNA, how it is administered, the disease and its severity and the history, age, weight, family history, genetic makeup, stage of pathological processes mediated by KHK gene expression, the types of preceding or concomitant treatments, if any, and other individual characteristics of the patient to be treated.

[0156] “Prophylactically effective amount,” as used herein, is intended to include the amount of an iRNA that, when administered to a subject who does not yet experience or display symptoms of a

KHK-associated diseases, but who may be predisposed to a KHK-associated disease, is sufficient to prevent or delay the development or progression of the disease or one or more symptoms of the disease for a clinically significant period of time. The “prophylactically effective amount” may vary depending on the iRNA, how it is administered, the degree of risk of disease, and the history, age, weight, family history, genetic makeup, the types of preceding or concomitant treatments, if any, and other individual characteristics of the patient to be treated.

[0157] A “therapeutically-effective amount” or “prophylactically effective amount” also includes an amount of an iRNA that produces some desired local or systemic effect at a reasonable benefit/risk ratio applicable to any treatment. iRNAs employed in the methods of the present invention may be administered in a sufficient amount to produce a reasonable benefit/risk ratio applicable to such treatment.

[0158] The phrase “pharmaceutically acceptable” is employed herein to refer to those compounds, materials, compositions, or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human subjects and animal subjects without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[0159] The phrase “pharmaceutically-acceptable carrier” as used herein means a pharmaceutically-acceptable material, composition, or vehicle, such as a liquid or solid filler, diluent, excipient, manufacturing aid (e.g., lubricant, talc magnesium, calcium or zinc stearate, or steric acid), or solvent encapsulating material, involved in carrying or transporting the subject compound from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be “acceptable” in the sense of being compatible with the other ingredients of the formulation and not injurious to the subject being treated. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) lubricating agents, such as magnesium stearate, sodium lauryl sulfate and talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) pH buffered solutions; (21) polyesters, polycarbonates or polyanhydrides; (22) bulking agents, such as polypeptides and amino acids (23) serum component, such as serum albumin, HDL and LDL; and (22) other non-toxic compatible substances employed in pharmaceutical formulations.

[0160] The term “sample,” as used herein, includes a collection of similar fluids, cells, or tissues isolated from a subject, as well as fluids, cells, or tissues present within a subject. Examples of biological fluids include blood, serum and serosal fluids, plasma, cerebrospinal fluid, ocular fluids, lymph, urine, saliva, and the like. Tissue samples may include samples from tissues, organs, or localized regions. For example, samples may be derived from particular organs, parts of organs, or fluids or cells within those organs. In certain embodiments, samples may be derived from the liver (e.g., whole liver or certain segments of liver or certain types of cells in the liver, such as, e.g., hepatocytes). In some embodiments, a “sample derived from a subject” refers to urine obtained from the subject. A “sample derived from a subject” can refer to blood (which can be readily converted to plasma or serum) drawn from the subject.

I. iRNAs of the Invention

[0161] The present invention provides iRNAs which inhibit the expression of a KHK gene. In preferred embodiments, the iRNA includes double stranded ribonucleic acid (dsRNA) molecules for inhibiting the expression of a KHK gene in a cell, such as a cell within a subject, e.g., a

mammal, such as a human having or susceptible to a KHK-associated disease. The dsRNAi agent includes an antisense strand having a region of complementarity which is complementary to at least a part of an mRNA formed in the expression of a KHK gene. The region of complementarity is about 30 nucleotides or less in length (e.g., about 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, or 18 nucleotides or less in length). Upon contact with a cell expressing the KHK gene, the iRNA inhibits the expression of the KHK gene (e.g., a human, a primate, a non-primate, or a bird KHK gene) by at least about 20% as assayed by, for example, a PCR or branched DNA (bDNA)-based method, or by a protein-based method, such as by immunofluorescence analysis, using, for example, western blotting or flowcytometric techniques. In preferred embodiments, inhibition of expression is determined by the qPCR method provided in the Example 2, preferably at a 10 nM concentration of the iRNA in the appropriate species matched cell line and delivered to the cell line in the manner provided therein.

[0162] A dsRNA includes two RNA strands that are complementary and hybridize to form a duplex structure under conditions in which the dsRNA will be used. One strand of a dsRNA (the antisense strand) includes a region of complementarity that is substantially complementary, and generally fully complementary, to a target sequence. The target sequence can be derived from the sequence of an mRNA formed during the expression of a KHK gene. The other strand (the sense strand) includes a region that is complementary to the antisense strand, such that the two strands hybridize and form a duplex structure when combined under suitable conditions. As described elsewhere herein and as known in the art, the complementary sequences of a dsRNA can also be contained as self-complementary regions of a single nucleic acid molecule, as opposed to being on separate oligonucleotides.

[0163] Generally, the duplex structure is 15 to 30 base pairs in length, e.g., 15-29, 15-28, 15-27, 15-26, 15-25, 15-24, 15-23, 15-22, 15-21, 15-20, 15-19, 15-18, 15-17, 18-30, 18-29, 18-28, 18-27, 18-26, 18-25, 18-24, 18-23, 18-22, 18-21, 18-20, 19-30, 19-29, 19-28, 19-27, 19-26, 19-25, 19-24, 19-23, 19-22, 19-21, 19-20, 20-30, 20-29, 20-28, 20-27, 20-26, 20-25, 20-24, 20-23, 20-22, 20-21, 21-30, 21-29, 21-28, 21-27, 21-26, 21-25, 21-24, 21-23, or 21-22 base pairs in length. Ranges and lengths intermediate to the above recited ranges and lengths are also contemplated to be part of the invention.

[0164] Similarly, the region of complementarity to the target sequence is 15 to 30 nucleotides in length, e.g., 15-29, 15-28, 15-27, 15-26, 15-25, 15-24, 15-23, 15-22, 15-21, 15-20, 15-19, 15-18, 15-17, 18-30, 18-29, 18-28, 18-27, 18-26, 18-25, 18-24, 18-23, 18-22, 18-21, 18-20, 19-30, 19-29, 19-28, 19-27, 19-26, 19-25, 19-24, 19-23, 19-22, 19-21, 19-20, 20-30, 20-29, 20-28, 20-27, 20-26, 20-25, 20-24, 20-23, 20-22, 20-21, 21-30, 21-29, 21-28, 21-27, 21-26, 21-25, 21-24, 21-23, or 21-22 nucleotides in length. Ranges and lengths intermediate to the above recited ranges and lengths are also contemplated to be part of the invention.

[0165] In some embodiments, the dsRNA is about 15 to about 23 nucleotides in length, or about 25 to about 30 nucleotides in length. In general, the dsRNA is long enough to serve as a substrate for the Dicer enzyme. For example, it is well-known in the art that dsRNAs longer than about 21-23 nucleotides in length may serve as substrates for Dicer. As the ordinarily skilled person will also recognize, the region of an RNA targeted for cleavage will most often be part of a larger RNA molecule, often an mRNA molecule. Where relevant, a “part” of an mRNA target is a contiguous sequence of an mRNA target of sufficient length to allow it to be a substrate for RNAi-directed cleavage (i.e., cleavage through a RISC pathway).

[0166] One of skill in the art will also recognize that the duplex region is a primary functional portion of a dsRNA, e.g., a duplex region of about 9 to about 36 base pairs, e.g., about 10-36, 11-36, 12-36, 13-36, 14-36, 15-36, 9-35, 10-35, 11-35, 12-35, 13-35, 14-35, 15-35, 9-34, 10-34, 11-34, 12-34, 13-34, 14-34, 15-34, 9-33, 10-33, 11-33, 12-33, 13-33, 14-33, 15-33, 9-32, 10-32, 11-32, 12-32, 13-32, 14-32, 15-32, 9-31, 10-31, 11-31, 12-31, 13-32, 14-31, 15-31, 15-30, 15-29, 15-28, 15-27, 15-26, 15-25, 15-24, 15-23, 15-22, 15-21, 15-20, 15-19, 15-18, 15-17, 18-30, 18-29, 18-28, 18-

27, 18-26, 18-25, 18-24, 18-23, 18-22, 18-21, 18-20, 19-30, 19-29, 19-28, 19-27, 19-26, 19-25, 19-24, 19-23, 19-22, 19-21, 19-20, 20-30, 20-29, 20-28, 20-27, 20-26, 20-25, 20-24, 20-23, 20-22, 20-21, 21-30, 21-29, 21-28, 21-27, 21-26, 21-25, 21-24, 21-23, or 21-22 base pairs. Thus, in one embodiment, to the extent that it becomes processed to a functional duplex, of e.g., 15-30 base pairs, that targets a desired RNA for cleavage, an RNA molecule or complex of RNA molecules having a duplex region greater than 30 base pairs is a dsRNA. Thus, an ordinarily skilled artisan will recognize that in one embodiment, a miRNA is a dsRNA. In another embodiment, a dsRNA is not a naturally occurring miRNA. In another embodiment, an iRNA agent useful to target KHK gene expression is not generated in the target cell by cleavage of a larger dsRNA.

[0167] A dsRNA as described herein can further include one or more single-stranded nucleotide overhangs e.g., 1-4, 24, 1-3, 2-3, 1, 2, 3, or 4 nucleotides. dsRNAs having at least one nucleotide overhang can have superior inhibitory properties relative to their blunt-ended counterparts. A nucleotide overhang can comprise or consist of a nucleotide/nucleoside analog, including a deoxynucleotide/nucleoside. The overhang(s) can be on the sense strand, the antisense strand, or any combination thereof. Furthermore, the nucleotide(s) of an overhang can be present on the 5'-end, 3'-end, or both ends of an antisense or sense strand of a dsRNA.

[0168] In certain embodiments, the overhang on the sense strand or the antisense strand, or both, can include extended lengths longer than 10 nucleotides, e.g., 10-30 nucleotides, 10-25 nucleotides, 10-20 nucleotides or 10-15 nucleotides in length. In certain embodiments, an extended overhang is on the sense strand of the duplex. In certain embodiments, an extended overhang is present on the 3'-end of the sense strand of the duplex. In certain embodiments, an extended overhang is present on the 5'-end of the sense strand of the duplex. In certain embodiments, an extended overhang is on the antisense strand of the duplex. In certain embodiments, an extended overhang is present on the 3'-end of the antisense strand of the duplex. In certain embodiments, an extended overhang is present on the 5'-end of the antisense strand of the duplex. In certain embodiments, one or more of the nucleotides in the extended overhang is replaced with a nucleoside thiophosphate.

[0169] A dsRNA can be synthesized by standard methods known in the art as further discussed below, e.g., by use of an automated DNA synthesizer, such as are commercially available from, for example, Biosearch, Applied Biosystems, Inc.

[0170] Double stranded RNAi compounds of the invention may be prepared using a two-step procedure. First, the individual strands of the double stranded RNA molecule are prepared separately. Then, the component strands are annealed. The individual strands of the siRNA compound can be prepared using solution-phase or solid-phase organic synthesis or both. Organic synthesis offers the advantage that the oligonucleotide strands comprising unnatural or modified nucleotides can be easily prepared. Similarly, single-stranded oligonucleotides of the invention can be prepared using solution-phase or solid-phase organic synthesis or both.

[0171] In an aspect, a dsRNA of the invention includes at least two nucleotide sequences, a sense sequence and an anti-sense sequence. The sense strand is selected from the group of sequences provided in Tables 3 and 5, and the corresponding antisense strand of the sense strand is selected from the group of sequences of Tables 3 and 5. In this aspect, one of the two sequences is complementary to the other of the two sequences, with one of the sequences being substantially complementary to a sequence of an mRNA generated in the expression of a KHK gene. As such, in this aspect, a dsRNA will include two oligonucleotides, where one oligonucleotide is described as the sense strand in Table 3 or 5, and the second oligonucleotide is described as the corresponding antisense strand of the sense strand in Table 3 or 5. In certain embodiments, the substantially complementary sequences of the dsRNA are contained on separate oligonucleotides. In other embodiments, the substantially complementary sequences of the dsRNA are contained on a single oligonucleotide.

[0172] It will be understood that, although the sequences in Table 3 are not described as modified or conjugated sequences, the RNA of the iRNA of the invention e.g., a dsRNA of the invention,

may comprise any one of the sequences set forth in Table 3 that are modified and/or conjugated, or the sequences of Table 5 that are unmodified and/or unconjugated. In other words, the invention encompasses dsRNA of Tables 3 and 5 which are un-modified, un-conjugated, modified, and/or conjugated, as described herein.

[0173] The skilled person is well aware that dsRNAs having a duplex structure of between about 20 and 23 base pairs, e.g., 21, base pairs have been hailed as particularly effective in inducing RNA interference (Elbashir et al., *EMBO* 2001, 20:6877-6888). However, others have found that shorter or longer RNA duplex structures can also be effective (Chu and Rana (2007) *RNA* 14:1714-1719; Kim et al. (2005) *Nat Biotech* 23:222-226). In the embodiments described above, by virtue of the nature of the oligonucleotide sequences provided in any one of Tables 3 and 5, dsRNAs described herein can include at least one strand of a length of minimally 21 nucleotides. It can be reasonably expected that shorter duplexes having one of the sequences of Tables 3 and 5 minus only a few nucleotides on one or both ends can be similarly effective as compared to the dsRNAs described above. Hence, dsRNAs having a sequence of at least 15, 16, 17, 18, 19, 20, or more contiguous nucleotides derived from one of the sequences of Tables 3 and 5, and differing in their ability to inhibit the expression of a KHK gene by not more than about 5, 10, 15, 20, 25, or 30% inhibition from a dsRNA comprising the full sequence, are contemplated to be within the scope of the present invention.

[0174] In addition, the RNAs provided in Tables 3 and 5 identify a site(s) in a KHK transcript that is susceptible to RISC-mediated cleavage. As such, the present invention further features iRNAs that target within one of these sites. As used herein, an iRNA is said to target within a particular site of an RNA transcript if the iRNA promotes cleavage of the transcript anywhere within that particular site. Such an iRNA will generally include at least about 15 contiguous nucleotides from one of the sequences provided in Tables 3 and 5 coupled to additional nucleotide sequences taken from the region contiguous to the selected sequence in a KHK gene.

[0175] While a target sequence is generally about 15-30 nucleotides in length, there is wide variation in the suitability of particular sequences in this range for directing cleavage of any given target RNA. Various software packages and the guidelines set out herein provide guidance for the identification of optimal target sequences for any given gene target, but an empirical approach can also be taken in which a “window” or “mask” of a given size (as a non-limiting example, 21 nucleotides) is literally or figuratively (including, e.g., in silico) placed on the target RNA sequence to identify sequences in the size range that can serve as target sequences. By moving the sequence “window” progressively one nucleotide upstream or downstream of an initial target sequence location, the next potential target sequence can be identified, until the complete set of possible sequences is identified for any given target size selected. This process, coupled with systematic synthesis and testing of the identified sequences (using assays as described herein or as known in the art or provided herein) to identify those sequences that perform optimally can identify those RNA sequences that, when targeted with an iRNA agent, mediate the best inhibition of target gene expression. Thus, while the sequences identified, for example, in Tables 3 and 5 represent effective target sequences, it is contemplated that further optimization of inhibition efficiency can be achieved by progressively “walking the window” one nucleotide upstream or downstream of the given sequences to identify sequences with equal or better inhibition characteristics.

[0176] Further, it is contemplated that for any sequence identified, e.g., in Tables 3 and 5, further optimization could be achieved by systematically either adding or removing nucleotides to generate longer or shorter sequences and testing those sequences generated by walking a window of the longer or shorter size up or down the target RNA from that point. Again, coupling this approach to generating new candidate targets with testing for effectiveness of iRNAs based on those target sequences in an inhibition assay as known in the art or as described herein can lead to further improvements in the efficiency of inhibition. Further still, such optimized sequences can be adjusted by, e.g., the introduction of modified nucleotides as described herein or as known in the

art, addition or changes in overhang, or other modifications as known in the art or discussed herein to further optimize the molecule (e.g., increasing serum stability or circulating half-life, increasing thermal stability, enhancing transmembrane delivery, targeting to a particular location or cell type, increasing interaction with silencing pathway enzymes, increasing release from endosomes) as an expression inhibitor.

[0177] An iRNA as described herein can contain one or more mismatches to the target sequence. In one embodiment, an iRNA as described herein contains no more than 3 mismatches. If the antisense strand of the iRNA contains mismatches to a target sequence, it is preferable that the area of mismatch is not located in the center of the region of complementarity. If the antisense strand of the iRNA contains mismatches to the target sequence, it is preferable that the mismatch be restricted to be within the last 5 nucleotides from either the 5'- or 3'-end of the region of complementarity. For example, for a 23 nucleotide iRNA agent the strand which is complementary to a region of a KHK gene, generally does not contain any mismatch within the central 13 nucleotides. The methods described herein or methods known in the art can be used to determine whether an iRNA containing a mismatch to a target sequence is effective in inhibiting the expression of a KHK gene. Consideration of the efficacy of iRNAs with mismatches in inhibiting expression of a KHK gene is important, especially if the particular region of complementarity in a KHK gene is known to have polymorphic sequence variation within the population.

II. Modified iRNAs of the Invention

[0178] In certain embodiments, the RNA of the iRNA of the invention e.g., a dsRNA, is unmodified, and does not comprise, e.g., chemical modifications or conjugations known in the art and described herein. In other embodiments, the RNA of an iRNA of the invention, e.g., a dsRNA, is chemically modified to enhance stability or other beneficial characteristics. In certain embodiments of the invention, substantially all of the nucleotides of an iRNA of the invention are modified. In other embodiments of the invention, all of the nucleotides of an iRNA or substantially all of the nucleotides of an iRNA are modified, i.e., not more than 5, 4, 3, 2, or 1 unmodified nucleotides are present in a strand of the iRNA.

[0179] The nucleic acids featured in the invention can be synthesized or modified by methods well established in the art, such as those described in "Current protocols in nucleic acid chemistry," Beaucage, S. L. et al. (Edrs.), John Wiley & Sons, Inc., New York, NY, USA, which is hereby incorporated herein by reference. Modifications include, for example, end modifications, e.g., 5'-end modifications (phosphorylation, conjugation, inverted linkages) or 3'-end modifications (conjugation, DNA nucleotides, inverted linkages, etc.); base modifications, e.g., replacement with stabilizing bases, destabilizing bases, or bases that base pair with an expanded repertoire of partners, removal of bases (abasic nucleotides), or conjugated bases; sugar modifications (e.g., at the 2'-position or 4'-position) or replacement of the sugar; or backbone modifications, including modification or replacement of the phosphodiester linkages. Specific examples of iRNA compounds useful in the embodiments described herein include, but are not limited to RNAs containing modified backbones or no natural internucleoside linkages. RNAs having modified backbones include, among others, those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified RNAs that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides. In some embodiments, a modified iRNA will have a phosphorus atom in its internucleoside backbone.

[0180] Modified RNA backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5'-linked

analog of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

[0181] Representative US Patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S. Pat. Nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,195; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,316; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,625,050; 6,028,188; 6,124,445; 6,160,109; 6,169,170; 6,172,209; 6,239,265; 6,277,603; 6,326,199; 6,346,614; 6,444,423; 6,531,590; 6,534,639; 6,608,035; 6,683,167; 6,858,715; 6,867,294; 6,878,805; 7,015,315; 7,041,816; 7,273,933; 7,321,029; and U.S. Pat. RE39464, the entire contents of each of which are hereby incorporated herein by reference.

[0182] Modified RNA backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatoms and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S, and CH₂ component parts.

[0183] Representative US patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S. Pat. Nos. 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,64,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439, the entire contents of each of which are hereby incorporated herein by reference.

[0184] Suitable RNA mimetics are contemplated for use in iRNAs provided herein, in which both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound in which an RNA mimetic that has been shown to have excellent hybridization properties is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar backbone of an RNA is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative US patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Pat. Nos. 5,539,082; 5,714,331; and 5,719,262, the entire contents of each of which are hereby incorporated herein by reference. Additional PNA compounds suitable for use in the iRNAs of the invention are described in, for example, in Nielsen et al., *Science*, 1991, 254, 1497-1500.

[0185] Some embodiments featured in the invention include RNAs with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular —CH₂—NH—CH₂—, —CH₂—N(CH₃)—O—CH₂—[known as a methylene (methylimino) or MMI backbone], —CH₂—O—N(CH₃)—CH₂—, —CH₂—N(CH₃)—N(CH₃)—CH₂— and —N(CH₃)—CH₂—CH₂—[wherein the native phosphodiester backbone is represented as —O—P—O—CH₂—] of the above-referenced U.S. Pat. No. 5,489,677, and the amide backbones of the above-referenced U.S. Pat. No. 5,602,240. In some embodiments, the RNAs featured herein have morpholino backbone structures of the above-referenced U.S. Pat. No. 5,034,506.

[0186] Modified RNAs can also contain one or more substituted sugar moieties. The iRNAs, e.g., dsRNAs, featured herein can include one of the following at the 2'-position: OH; F; O—, S—, or

N-alkyl; O—, S—, or N-alkenyl; O—, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl can be substituted or unsubstituted C.sub.1 to C.sub.10 alkyl or C.sub.2 to C.sub.10 alkenyl and alkynyl. Exemplary suitable modifications include O[(CH.sub.2).sub.nO].sub.mCH.sub.3, O(CH.sub.2).sub.-nOCH.sub.3, O(CH.sub.2).sub.nNH.sub.2, O(CH.sub.2).sub.nCH.sub.3, O(CH.sub.2).sub.nONH.sub.2, and O(CH.sub.2).sub.nON[(CH.sub.2).sub.-nCH.sub.3].sub.2, where n and m are from 1 to about 10. In other embodiments, dsRNAs include one of the following at the 2' position: C.sub.1 to C.sub.10 lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH.sub.3, OCN, Cl, Br, CN, CF.sub.3, OCF.sub.3, SOCH.sub.3, SO.sub.2CH.sub.3, ONO.sub.2, NO.sub.2, N.sub.3, NH.sub.2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an iRNA, or a group for improving the pharmacodynamic properties of an iRNA, and other substituents having similar properties. In some embodiments, the modification includes a 2'-methoxyethoxy (2'-O—CH.sub.2CH.sub.2OCH.sub.3, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta*, 1995, 78:486-504) i.e., an alkoxy-alkoxy group. Another exemplary modification is 2'-dimethylaminoethoxy, i.e., a O(CH.sub.2).sub.2ON(CH.sub.3).sub.2 group, also known as 2'-DMAOE, as described in examples herein below, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), i.e., 2'-O—CH.sub.2—O—CH.sub.2—N(CH.sub.2).sub.2. Further exemplary modifications include: 5'-Me-2'-F nucleotides, 5'-Me-2'-OMe nucleotides, 5'-Me-2'-deoxynucleotides, (both R and S isomers in these three families); 2'-alkoxyalkyl; and 2'-NMA (N-methylacetamide).

[0187] Other modifications include 2'-methoxy (2'-OCH.sub.3), 2-aminopropoxy (2'-OCH.sub.2CH.sub.2CH.sub.2NH.sub.2) and 2'-fluoro (2'-F). Similar modifications can also be made at other positions on the RNA of an iRNA, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked dsRNAs and the 5' position of 5' terminal nucleotide. iRNAs can also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative US patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Pat. Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, certain of which are commonly owned with the instant application. The entire contents of each of the foregoing are hereby incorporated herein by reference.

[0188] An iRNA can also include nucleobase (often referred to in the art simply as “base”) modifications or substitutions. As used herein, “unmodified” or “natural” nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C), and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as deoxy-thymine (dT), 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo, particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-daazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in *Modified Nucleosides in Biochemistry, Biotechnology and Medicine*, Herdewijn, P. ed. Wiley-VCH, 2008; those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J. L., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., *Angewandte Chemie, International Edition*, 1991, 30, 613, and those disclosed by Sanghvi, Y S.,

Chapter 15, dsRNA Research and Applications, pages 289-302, Crooke, S. T. and Lebleu, B., Ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds featured in the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2° C. (Sanghvi, Y. S., Crooke, S. T. and Lebleu, B., Eds., dsRNA Research and Applications, CRC Press, Boca Raton, 1993, pp. 276-278) and are exemplary base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

[0189] Representative US patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. Pat. Nos. 3,687,808, 4,845,205; 5,130,30; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,681,941; 5,750,692; 6,015,886; 6,147,200; 6,166,197; 6,222,025; 6,235,887; 6,380,368; 6,528,640; 6,639,062; 6,617,438; 7,045,610; 7,427,672; and 7,495,088, the entire contents of each of which are hereby incorporated herein by reference.

[0190] The RNA of an iRNA can also be modified to include one or more locked nucleic acids (LNA). A locked nucleic acid is a nucleotide having a modified ribose moiety in which the ribose moiety comprises an extra bridge connecting the 2' and 4' carbons. This structure effectively "locks" the ribose in the 3-endo structural conformation. The addition of locked nucleic acids to siRNAs has been shown to increase siRNA stability in serum, and to reduce off-target effects (Elmen, J. et al., (2005) *Nucleic Acids Research* 33(1):439-447; Mook, OR. et al., (2007) *Mol Canc Ther* 6(3):833-843; Grunweller, A. et al., (2003) *Nucleic Acids Research* 31(12):3185-3193).

[0191] In some embodiments, the iRNA of the invention comprises one or more monomers that are UNA (unlocked nucleic acid) nucleotides. UNA is unlocked acyclic nucleic acid, wherein any of the bonds of the sugar has been removed, forming an unlocked "sugar" residue. In one example, UNA also encompasses monomer with bonds between C1'-C4' have been removed (i.e. the covalent carbon-oxygen-carbon bond between the C1' and C4' carbons). In another example, the C2'-C3' bond (i.e. the covalent carbon-carbon bond between the C2' and C3' carbons) of the sugar has been removed (see *Nuc. Acids Symp. Series*, 52, 133-134 (2008) and Fluiter et al., *Mol. Biosyst.*, 2009, 10, 1039 hereby incorporated by reference).

[0192] Representative US publications that teach the preparation of UNA include, but are not limited to, U.S. Pat. No. 8,314,227; and US Patent Publication Nos. 2013/0096289; 2013/0011922; and 2011/0313020, the entire contents of each of which are hereby incorporated herein by reference.

[0193] The RNA of an iRNA can also be modified to include one or more bicyclic sugar moieties. A "bicyclic sugar" is a furanosyl ring modified by the bridging of two atoms. A "bicyclic nucleoside" ("BNA") is a nucleoside having a sugar moiety comprising a bridge connecting two carbon atoms of the sugar ring, thereby forming a bicyclic ring system. In certain embodiments, the bridge connects the 4'-carbon and the 2'-carbon of the sugar ring. Thus, in some embodiments an agent of the invention may include one or more locked nucleic acids (LNA). A locked nucleic acid is a nucleotide having a modified ribose moiety in which the ribose moiety comprises an extra bridge connecting the 2' and 4' carbons. In other words, an LNA is a nucleotide comprising a bicyclic sugar moiety comprising a 4'-CH₂-O-2' bridge. This structure effectively "locks" the ribose in the 3'-endo structural conformation. The addition of locked nucleic acids to siRNAs has been shown to increase siRNA stability in serum, and to reduce off-target effects (Elmen, J. et al., (2005) *Nucleic Acids Research* 33(1):439-447; Mook, OR. et al., (2007) *Mol Canc Ther* 6(3):833-843; Grunweller, A. et al., (2003) *Nucleic Acids Research* 31(12):3185-3193). Examples of bicyclic nucleosides for use in the polynucleotides of the invention include without limitation nucleosides comprising a bridge between the 4' and the 2' ribosyl ring atoms. In certain embodiments, the

antisense polynucleotide agents of the invention include one or more bicyclic nucleosides comprising a 4' to 2' bridge. Examples of such 4' to 2' bridged bicyclic nucleosides, include but are not limited to 4'-(CH₂)—O-2' (LNA); 4'-(CH₂)—S-2'; 4'-(CH₂)₂-O-2' (ENA); 4'-CH(CH₃)—O-2' (also referred to as “constrained ethyl” or “cEt”) and 4'-CH(CH₂CH₃)—O-2' (and analogs thereof; see, e.g., U.S. Pat. No. 7,399,845); 4'-C(CH₃)CH₂—O-2' (and analogs thereof; see e.g., U.S. Pat. No. 8,278,283); 4'-CH₂—N(OCH₃)-2' (and analogs thereof; see e.g., U.S. Pat. No. 8,278,425); 4'-CH₂—O—N(CH₃)-2' (see, e.g., US Patent Publication No. 2004/0171570); 4'-CH₂—N(R)—O-2', wherein R is H, C₁-C₁₂ alkyl, or a protecting group (see, e.g., U.S. Pat. No. 7,427,672); 4'-CH₂—C(HXCH₃)-2' (see, e.g., Chattopadhyaya et al., J. Org. Chem., 2009, 74, 118-134); and 4'-CH₂—C(=CH₂)-2' (and analogs thereof; see, e.g., U.S. Pat. No. 8,278,426). The entire contents of each of the foregoing are hereby incorporated herein by reference.

[0194] Additional representative US patents and US Patent Publications that teach the preparation of locked nucleic acid nucleotides include, but are not limited to, the following: U.S. Pat. Nos. 6,268,490; 6,525,191; 6,670,461; 6,770,748; 6,794,499; 6,998,484; 7,053,207; 7,034,133; 7,084,125; 7,399,845; 7,427,672; 7,569,686; 7,741,457; 8,022,193; 8,030,467; 8,278,425; 8,278,426; 8,278,283; US 2008/0039618; and US 2009/0012281, the entire contents of each of which are hereby incorporated herein by reference.

[0195] Any of the foregoing bicyclic nucleosides can be prepared having one or more stereochemical sugar configurations including for example α -L-ribofuranose and β -D-ribofuranose (see WO 99/14226).

[0196] The RNA of an iRNA can also be modified to include one or more constrained ethyl nucleotides. As used herein, a “constrained ethyl nucleotide” or “cEt” is a locked nucleic acid comprising a bicyclic sugar moiety comprising a 4'-CH(CH₃)—O-2' bridge. In one embodiment, a constrained ethyl nucleotide is in the S conformation referred to herein as “S-cEt.”

[0197] An iRNA of the invention may also include one or more “conformationally restricted nucleotides” (“CRN”). CRN are nucleotide analogs with a linker connecting the C2' and C4' carbons of ribose or the C3 and —C5' carbons of ribose. CRN lock the ribose ring into a stable conformation and increase the hybridization affinity to mRNA. The linker is of sufficient length to place the oxygen in an optimal position for stability and affinity resulting in less ribose ring puckering.

[0198] Representative publications that teach the preparation of certain of the above noted CRN include, but are not limited to, US Patent Publication No. 2013/0190383; and PCT publication WO 2013/036868, the entire contents of each of which are hereby incorporated herein by reference.

[0199] Potentially stabilizing modifications to the ends of RNA molecules can include N-(acetylaminocaproyl)-4-hydroxyprolinol (Hyp-C6-NHAc), N-(caproyl)-4-hydroxyprolinol (Hyp-C6), N-(acetyl-4-hydroxyprolinol (Hyp-NHAc), thymidine-2'-O-deoxythymidine (ether), N-(aminocaproyl)-4-hydroxyprolinol (Hyp-C6-amino), 2-docosanoyl-uridine-3'-phosphate, inverted base dT(idT) and others. Disclosure of this modification can be found in PCT Publication No. WO 2011/005861.

[0200] Other modifications of the nucleotides of an iRNA of the invention include a 5' phosphate or 5' phosphate mimic, e.g., a 5'-terminal phosphate or phosphate mimic on the antisense strand of an iRNA. Suitable phosphate mimics are disclosed in, for example US Patent Publication No. 2012/0157511, the entire contents of which are incorporated herein by reference.

A. Modified iRNAs Comprising Motifs of the Invention

[0201] In certain aspects of the invention, the double stranded RNAi agents of the invention include agents with chemical modifications as disclosed, for example, in WO2013/075035, the entire contents of each of which are incorporated herein by reference. WO2013/075035 provides motifs of three identical modifications on three consecutive nucleotides into a sense strand or antisense strand of a dsRNAi agent, particularly at or near the cleavage site. In some embodiments,

the sense strand and antisense strand of the dsRNAi agent may otherwise be completely modified. The introduction of these motifs interrupts the modification pattern, if present, of the sense or antisense strand. The dsRNAi agent may be optionally conjugated with a GalNAc derivative ligand, for instance on the sense strand.

[0202] More specifically, when the sense strand and antisense strand of the double stranded RNAi agent are completely modified to have one or more motifs of three identical modifications on three consecutive nucleotides at or near the cleavage site of at least one strand of a dsRNAi agent, the gene silencing activity of the dsRNAi agent was observed.

[0203] Accordingly, the invention provides double stranded RNAi agents capable of inhibiting the expression of a target gene (i.e., KHK gene) in vivo. The RNAi agent comprises a sense strand and an antisense strand. Each strand of the RNAi agent may be, independently, 12-30 nucleotides in length. For example, each strand may independently be 14-30 nucleotides in length, 17-30 nucleotides in length, 25-30 nucleotides in length, 27-30 nucleotides in length, 17-23 nucleotides in length, 17-21 nucleotides in length, 17-19 nucleotides in length, 19-25 nucleotides in length, 19-23 nucleotides in length, 19-21 nucleotides in length, 21-25 nucleotides in length, or 21-23 nucleotides in length.

[0204] The sense strand and antisense strand typically form a duplex double stranded RNA (“dsRNA”), also referred to herein as “dsRNAi agent.” The duplex region of an dsRNAi agent may be 12-30 nucleotide pairs in length. For example, the duplex region can be 14-30 nucleotide pairs in length, 17-30 nucleotide pairs in length, 27-30 nucleotide pairs in length, 17-23 nucleotide pairs in length, 17-21 nucleotide pairs in length, 17-19 nucleotide pairs in length, 19-25 nucleotide pairs in length, 19-23 nucleotide pairs in length, 19-21 nucleotide pairs in length, 21-25 nucleotide pairs in length, or 21-23 nucleotide pairs in length. In another example, the duplex region is selected from 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, and 27 nucleotides in length.

[0205] In certain embodiments, the dsRNAi agent may contain one or more overhang regions or capping groups at the 3'-end, 5'-end, or both ends of one or both strands. The overhang can be, independently, 1-6 nucleotides in length, for instance 2-6 nucleotides in length, 1-5 nucleotides in length, 2-5 nucleotides in length, 1-4 nucleotides in length, 2-4 nucleotides in length, 1-3 nucleotides in length, 2-3 nucleotides in length, or 1-2 nucleotides in length. In certain embodiments, the overhang regions can include extended overhang regions as provided above. 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. The overhang can form a mismatch with the target mRNA or it can be complementary to the gene sequences being targeted or can be another sequence. The first and second strands can also be joined, e.g., by additional bases to form a hairpin, or by other non-base linkers.

[0206] In certain embodiments, the nucleotides in the overhang region of the dsRNAi agent can each independently be a modified or unmodified nucleotide including, but not limited to 2'-sugar modified, such as, 2'-F, 2'-O-methyl, thymidine (T), 2'-O-methoxyethyl-5-methyluridine (Teo), 2'-O-methoxyethyladenosine (Aeo), 2'-O-methoxyethyl-5-methylcytidine (m5Ceo), and any combinations thereof. For example, TT can be an overhang sequence for either end on either strand. The overhang can form a mismatch with the target mRNA or it can be complementary to the gene sequences being targeted or can be another sequence.

[0207] The 5'- or 3'-overhangs at the sense strand, antisense strand, or both strands of the dsRNAi agent may be phosphorylated. In some embodiments, the overhang region(s) contains two nucleotides having a phosphorothioate between the two nucleotides, where the two nucleotides can be the same or different. In some embodiments, the overhang is present at the 3'-end of the sense strand, antisense strand, or both strands. In some embodiments, this 3'-overhang is present in the antisense strand. In some embodiments, this 3'-overhang is present in the sense strand.

[0208] The dsRNAi agent may contain only a single overhang, which can strengthen the interference activity of the RNAi, without affecting its overall stability. For example, the single-

stranded overhang may be located at the 3'-end of the sense strand or, alternatively, at the 3'-end of the antisense strand. The RNAi may also have a blunt end, located at the 5'-end of the antisense strand (or the 3'-end of the sense strand) or vice versa. Generally, the antisense strand of the dsRNAi agent has a nucleotide overhang at the 3'-end, and the 5'-end is blunt. While not wishing to be bound by theory, the asymmetric blunt end at the 5'-end of the antisense strand and 3'-end overhang of the antisense strand favor the guide strand loading into RISC process.

[0209] In certain embodiments, the dsRNAi agent is a double ended bluntmer of 19 nucleotides in length, wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides at positions 7, 8, 9 from the 5'-end. The antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at positions 11, 12, 13 from the 5'-end.

[0210] In other embodiments, the dsRNAi agent is a double ended bluntmer of 20 nucleotides in length, wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides at positions 8, 9, 10 from the 5'-end. The antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at positions 11, 12, 13 from the 5'-end.

[0211] In yet other embodiments, the dsRNAi agent is a double ended bluntmer of 21 nucleotides in length, wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides at positions 9, 10, 11 from the 5'-end. The antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at positions 11, 12, 13 from the 5'-end.

[0212] In certain embodiments, the dsRNAi agent comprises a 21 nucleotide sense strand and a 23 nucleotide antisense strand, wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides at positions 9, 10, 11 from the 5'-end; the antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at positions 11, 12, 13 from the 5'-end, wherein one end of the RNAi agent is blunt, while the other end comprises a 2 nucleotide overhang. Preferably, the 2 nucleotide overhang is at the 3'-end of the antisense strand.

[0213] When the 2 nucleotide overhang is at the 3'-end of the antisense strand, there may be two phosphorothioate internucleotide linkages between the terminal three nucleotides, wherein two of the three nucleotides are the overhang nucleotides, and the third nucleotide is a paired nucleotide next to the overhang nucleotide. In one embodiment, the RNAi agent additionally has two phosphorothioate internucleotide linkages between the terminal three nucleotides at both the 5'-end of the sense strand and at the 5'-end of the antisense strand. In certain embodiments, every nucleotide in the sense strand and the antisense strand of the dsRNAi agent, including the nucleotides that are part of the motifs are modified nucleotides. In certain embodiments each residue is independently modified with a 2'-O-methyl or 3'-fluoro, e.g., in an alternating motif. Optionally, the dsRNAi agent further comprises a ligand (preferably GalNAc.sub.3).

[0214] In certain embodiments, the dsRNAi agent comprises a sense and an antisense strand, wherein the sense strand is 25-30 nucleotide residues in length, wherein starting from the 5' terminal nucleotide (position 1) positions 1 to 23 of the first strand comprise at least 8 ribonucleotides; the antisense strand is 36-66 nucleotide residues in length and, starting from the 3' terminal nucleotide, comprises at least 8 ribonucleotides in the positions paired with positions 1-23 of sense strand to form a duplex; wherein at least the 3' terminal nucleotide of antisense strand is unpaired with sense strand, and up to 6 consecutive 3' terminal nucleotides are unpaired with sense strand, thereby forming a 3' single stranded overhang of 1-6 nucleotides; wherein the 5' terminus of antisense strand comprises from 10-30 consecutive nucleotides which are unpaired with sense strand, thereby forming a 10-30 nucleotide single stranded 5' overhang; wherein at least the sense strand 5' terminal and 3' terminal nucleotides are base paired with nucleotides of antisense strand when sense and antisense strands are aligned for maximum complementarity, thereby forming a

substantially duplexed region between sense and antisense strands; and antisense strand is sufficiently complementary to a target RNA along at least 19 ribonucleotides of antisense strand length to reduce target gene expression when the double stranded nucleic acid is introduced into a mammalian cell; and wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides, where at least one of the motifs occurs at or near the cleavage site. The antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at or near the cleavage site.

[0215] In certain embodiments, the dsRNAi agent comprises sense and antisense strands, wherein the dsRNAi agent comprises a first strand having a length which is at least 25 and at most 29 nucleotides and a second strand having a length which is at most 30 nucleotides with at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at position 11, 12, 13 from the 5' end; wherein the 3' end of the first strand and the 5' end of the second strand form a blunt end and the second strand is 1-4 nucleotides longer at its 3' end than the first strand, wherein the duplex region which is at least 25 nucleotides in length, and the second strand is sufficiently complementary to a target mRNA along at least 19 nucleotide of the second strand length to reduce target gene expression when the RNAi agent is introduced into a mammalian cell, and wherein Dicer cleavage of the dsRNAi agent preferentially results in an siRNA comprising the 3'-end of the second strand, thereby reducing expression of the target gene in the mammal.

Optionally, the dsRNAi agent further comprises a ligand.

[0216] In certain embodiments, the sense strand of the dsRNAi agent contains at least one motif of three identical modifications on three consecutive nucleotides, where one of the motifs occurs at the cleavage site in the sense strand.

[0217] In certain embodiments, the antisense strand of the dsRNAi agent can also contain at least one motif of three identical modifications on three consecutive nucleotides, where one of the motifs occurs at or near the cleavage site in the antisense strand.

[0218] For a dsRNAi agent having a duplex region of 17-23 nucleotide in length, the cleavage site of the antisense strand is typically around the 10, 11, and 12 positions from the 5'-end. Thus the motifs of three identical modifications may occur at the 9, 10, 11 positions; the 10, 11, 12 positions; the 11, 12, 13 positions; the 12, 13, 14 positions; or the 13, 14, 15 positions of the antisense strand, the count starting from the first nucleotide from the 5'-end of the antisense strand, or, the count starting from the first paired nucleotide within the duplex region from the 5'-end of the antisense strand. The cleavage site in the antisense strand may also change according to the length of the duplex region of the dsRNAi agent from the 5'-end.

[0219] The sense strand of the dsRNAi agent may contain at least one motif of three identical modifications on three consecutive nucleotides at the cleavage site of the strand; and the antisense strand may have at least one motif of three identical modifications on three consecutive nucleotides at or near the cleavage site of the strand. When the sense strand and the antisense strand form a dsRNA duplex, the sense strand and the antisense strand can be so aligned that one motif of the three nucleotides on the sense strand and one motif of the three nucleotides on the antisense strand have at least one nucleotide overlap, i.e., at least one of the three nucleotides of the motif in the sense strand forms a base pair with at least one of the three nucleotides of the motif in the antisense strand. Alternatively, at least two nucleotides may overlap, or all three nucleotides may overlap.

[0220] In some embodiments, the sense strand of the dsRNAi agent may contain more than one motif of three identical modifications on three consecutive nucleotides. The first motif may occur at or near the cleavage site of the strand and the other motifs may be a wing modification. The term "wing modification" herein refers to a motif occurring at another portion of the strand that is separated from the motif at or near the cleavage site of the same strand. The wing modification is either adjacent to the first motif or is separated by at least one or more nucleotides. When the motifs are immediately adjacent to each other then the chemistries of the motifs are distinct from each other, and when the motifs are separated by one or more nucleotide than the chemistries can

be the same or different. Two or more wing modifications may be present. For instance, when two wing modifications are present, each wing modification may occur at one end relative to the first motif which is at or near cleavage site or on either side of the lead motif.

[0221] Like the sense strand, the antisense strand of the dsRNAi agent may contain more than one motifs of three identical modifications on three consecutive nucleotides, with at least one of the motifs occurring at or near the cleavage site of the strand. This antisense strand may also contain one or more wing modifications in an alignment similar to the wing modifications that may be present on the sense strand.

[0222] In some embodiments, the wing modification on the sense strand or antisense strand of the dsRNAi agent typically does not include the first one or two terminal nucleotides at the 3'-end, 5'-end, or both ends of the strand.

[0223] In other embodiments, the wing modification on the sense strand or antisense strand of the dsRNAi agent typically does not include the first one or two paired nucleotides within the duplex region at the 3'-end, 5'-end, or both ends of the strand.

[0224] When the sense strand and the antisense strand of the dsRNAi agent each contain at least one wing modification, the wing modifications may fall on the same end of the duplex region, and have an overlap of one, two, or three nucleotides.

[0225] When the sense strand and the antisense strand of the dsRNAi agent each contain at least two wing modifications, the sense strand and the antisense strand can be so aligned that two modifications each from one strand fall on one end of the duplex region, having an overlap of one, two, or three nucleotides; two modifications each from one strand fall on the other end of the duplex region, having an overlap of one, two or three nucleotides; two modifications one strand fall on each side of the lead motif, having an overlap of one, two or three nucleotides in the duplex region.

[0226] In some embodiments, every nucleotide in the sense strand and antisense strand of the dsRNAi agent, including the nucleotides that are part of the motifs, may be modified. Each nucleotide may be modified with the same or different modification which can include one or more alteration of one or both of the non-linking phosphate oxygens or of one or more of the linking phosphate oxygens; alteration of a constituent of the ribose sugar, e.g., of the 2'-hydroxyl on the ribose sugar; wholesale replacement of the phosphate moiety with "dephospho" linkers; modification or replacement of a naturally occurring base; and replacement or modification of the ribose-phosphate backbone.

[0227] As nucleic acids are polymers of subunits, many of the modifications occur at a position which is repeated within a nucleic acid, e.g., a modification of a base, or a phosphate moiety, or 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 region, 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. A modification may occur only in the double strand region of a dsRNAi agent or may only occur in a single strand region of a dsRNAi agent. For example, a phosphorothioate modification at a non-linking O position may only occur at one or both ends, may only occur in a terminal region, 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 the ends. The 5'-end or ends can be phosphorylated.

[0228] It may be 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. For example, 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 may be modified, e.g., with a modification described herein. Modifications can include, e.g., the use of modifications at the 2' position of the ribose sugar with modifications that are known in the art, e.g., the use of

deoxyribonucleotides, 2'-deoxy-2'-fluoro (2'-F) or 2'-O-methyl modified instead of the ribosugar of the nucleobase, and modifications in the phosphate group, e.g., phosphorothioate modifications. Overhangs need not be homologous with the target sequence.

[0229] In some embodiments, each residue of the sense strand and antisense strand is independently modified with LNA, CRN, cET, UNA, HNA, CeNA, 2'-methoxyethyl, 2'-O-methyl, 2'-O-allyl, 2'-C-allyl, 2'-deoxy, 2'-hydroxyl, or 2'-fluoro. The strands can contain more than one modification. In one embodiment, each residue of the sense strand and antisense strand is independently modified with 2'-O-methyl or 2'-fluoro.

[0230] At least two different modifications are typically present on the sense strand and antisense strand. Those two modifications may be the 2'-O-methyl or 2'-fluoro modifications, or others.

[0231] In certain embodiments, the N.sub.a or N.sub.b comprise modifications of an alternating pattern. The term "alternating motif" as used herein refers to a motif having one or more modifications, each modification occurring on alternating nucleotides of one strand. The alternating nucleotide may refer to one per every other nucleotide or one per every three nucleotides, or a similar pattern. For example, if A, B and C each represent one type of modification to the nucleotide, the alternating motif can be "ABABABABABAB . . .," "AABBAABBAABB . . .," "AABAABAABAAB . . .," "AAABAAABAAAB . . .," "AAABBBAAABBB . . .," or "ABCABCABCABC . . .," etc.

[0232] The type of modifications contained in the alternating motif may be the same or different. For example, if A, B, C, D each represent one type of modification on the nucleotide, the alternating pattern, i.e., modifications on every other nucleotide, may be the same, but each of the sense strand or antisense strand can be selected from several possibilities of modifications within the alternating motif such as "ABABAB . . .," "ACACAC . . ." "BDBDBD . . ." or "CDCDCD . . .," etc.

[0233] In some embodiments, the dsRNAi agent of the invention comprises the modification pattern for the alternating motif on the sense strand relative to the modification pattern for the alternating motif on the antisense strand is shifted. The shift may be such that the modified group of nucleotides of the sense strand corresponds to a differently modified group of nucleotides of the antisense strand and vice versa. For example, the sense strand when paired with the antisense strand in the dsRNA duplex, the alternating motif in the sense strand may start with "ABABAB" from 5' to 3' of the strand and the alternating motif in the antisense strand may start with "BABABA" from 5' to 3' of the strand within the duplex region. As another example, the alternating motif in the sense strand may start with "AABBAABB" from 5' to 3' of the strand and the alternating motif in the antisense strand may start with "BBAABBAA" from 5' to 3' of the strand within the duplex region, so that there is a complete or partial shift of the modification patterns between the sense strand and the antisense strand.

[0234] In some embodiments, the dsRNAi agent comprises the pattern of the alternating motif of 2'-O-methyl modification and 2'-F modification on the sense strand initially has a shift relative to the pattern of the alternating motif of 2'-O-methyl modification and 2'-F modification on the antisense strand initially, i.e., the 2'-O-methyl modified nucleotide on the sense strand base pairs with a 2'-F modified nucleotide on the antisense strand and vice versa. The 1 position of the sense strand may start with the 2'-F modification, and the 1 position of the antisense strand may start with the 2'-O-methyl modification.

[0235] The introduction of one or more motifs of three identical modifications on three consecutive nucleotides to the sense strand or antisense strand interrupts the initial modification pattern present in the sense strand or antisense strand. This interruption of the modification pattern of the sense or antisense strand by introducing one or more motifs of three identical modifications on three consecutive nucleotides to the sense or antisense strand may enhance the gene silencing activity against the target gene.

[0236] In some embodiments, when the motif of three identical modifications on three consecutive

nucleotides is introduced to any of the strands, the modification of the nucleotide next to the motif is a different modification than the modification of the motif. For example, the portion of the sequence containing the motif is “. . . N.sub.aYYYN.sub.b . . . ,” where “Y” represents the modification of the motif of three identical modifications on three consecutive nucleotide, and “N.sub.a” and “N.sub.b” represent a modification to the nucleotide next to the motif “YYY” that is different than the modification of Y, and where N.sub.a and N.sub.b can be the same or different modifications. Alternatively, N.sub.a or N.sub.b may be present or absent when there is a wing modification present.

[0237] The iRNA may further comprise at least one phosphorothioate or methylphosphonate internucleotide linkage. The phosphorothioate or methylphosphonate internucleotide linkage modification may occur on any nucleotide of the sense strand, antisense strand, or both strands in any position of the strand. For instance, the internucleotide linkage modification may occur on every nucleotide on the sense strand or antisense strand; each internucleotide linkage modification may occur in an alternating pattern on the sense strand or antisense strand; or the sense strand or antisense strand may contain both internucleotide linkage modifications in an alternating pattern. The alternating pattern of the internucleotide linkage modification on the sense strand may be the same or different from the antisense strand, and the alternating pattern of the internucleotide linkage modification on the sense strand may have a shift relative to the alternating pattern of the internucleotide linkage modification on the antisense strand. In one embodiment, a double-stranded RNAi agent comprises 6-8phosphorothioate internucleotide linkages. In some embodiments, the antisense strand comprises two phosphorothioate internucleotide linkages at the 5'-end and two phosphorothioate internucleotide linkages at the 3'-end, and the sense strand comprises at least two phosphorothioate internucleotide linkages at either the 5'-end or the 3'-end.

[0238] In some embodiments, the dsRNAi agent comprises a phosphorothioate or methylphosphonate internucleotide linkage modification in the overhang region. For example, the overhang region may contain two nucleotides having a phosphorothioate or methylphosphonate internucleotide linkage between the two nucleotides. Internucleotide linkage modifications also may be made to link the overhang nucleotides with the terminal paired nucleotides within the duplex region. For example, at least 2, 3, 4, or all the overhang nucleotides may be linked through phosphorothioate or methylphosphonate internucleotide linkage, and optionally, there may be additional phosphorothioate or methylphosphonate internucleotide linkages linking the overhang nucleotide with a paired nucleotide that is next to the overhang nucleotide. For instance, there may be at least two phosphorothioate internucleotide linkages between the terminal three nucleotides, in which two of the three nucleotides are overhang nucleotides, and the third is a paired nucleotide next to the overhang nucleotide. These terminal three nucleotides may be at the 3'-end of the antisense strand, the 3'-end of the sense strand, the 5'-end of the antisense strand, or the 5'-end of the antisense strand.

[0239] In some embodiments, the 2-nucleotide overhang is at the 3'-end of the antisense strand, and there are two phosphorothioate internucleotide linkages between the terminal three nucleotides, wherein two of the three nucleotides are the overhang nucleotides, and the third nucleotide is a paired nucleotide next to the overhang nucleotide. Optionally, the dsRNAi agent may additionally have two phosphorothioate internucleotide linkages between the terminal three nucleotides at both the 5'-end of the sense strand and at the 5'-end of the antisense strand.

[0240] In one embodiment, the dsRNAi agent comprises mismatch(es) with the target, within the duplex, or combinations thereof. The mismatch may occur in the overhang region or the duplex region. The base pair may be ranked on the basis of their propensity to promote dissociation or melting (e.g., on the free energy of association or dissociation of a particular pairing, the simplest approach is to examine the pairs on an individual pair basis, though next neighbor or similar analysis can also be used). In terms of promoting dissociation: A:U is preferred over G:C; G:U is preferred over G:C; and I:C is preferred over G:C (I=inosine). Mismatches, e.g., non-canonical or

other than canonical pairings (as described elsewhere herein) are preferred over canonical (A:T, A:U, G:C) pairings; and pairings which include a universal base are preferred over canonical pairings.

[0241] In certain embodiments, the dsRNAi agent comprises at least one of the first 1, 2, 3, 4, or 5 base pairs within the duplex regions from the 5'-end of the antisense strand independently selected from the group of: A:U, G:U, I:C, and mismatched pairs, e.g., non-canonical or other than canonical pairings or pairings which include a universal base, to promote the dissociation of the antisense strand at the 5'-end of the duplex.

[0242] In certain embodiments, the nucleotide at the 1 position within the duplex region from the 5'-end in the antisense strand is selected from A, dA, dU, U, and dT. Alternatively, at least one of the first 1, 2, or 3 base pair within the duplex region from the 5'-end of the antisense strand is an AU base pair. For example, the first base pair within the duplex region from the 5'-end of the antisense strand is an AU base pair.

[0243] In other embodiments, the nucleotide at the 3'-end of the sense strand is deoxy-thymine (dT) or the nucleotide at the 3'-end of the antisense strand is deoxy-thymine (dT). For example, there is a short sequence of deoxy-thymine nucleotides, for example, two dT nucleotides on the 3'-end of the sense, antisense strand, or both strands.

[0244] In certain embodiments, the sense strand sequence may be represented by formula (I):

5' n.sub.p-N.sub.a—(XXX).sub.i—N.sub.b—YYY—N.sub.b—(ZZZ).sub.j—N.sub.a-n.sub.q
3' (I) [0245] wherein: [0246] i and j are each independently 0 or 1; [0247] p and q are each independently 0-6; [0248] each N.sub.a independently represents an oligonucleotide sequence comprising 0-25 modified nucleotides, each sequence comprising at least two differently modified nucleotides; [0249] each N.sub.b independently represents an oligonucleotide sequence comprising 0-10 modified nucleotides; [0250] each n.sub.p and n.sub.q independently represent an overhang nucleotide; [0251] wherein Nb and Y do not have the same modification; and [0252] XXX, YYY, and ZZZ each independently represent one motif of three identical modifications on three consecutive nucleotides. Preferably YYY is all 2'-F modified nucleotides.

[0253] In some embodiments, the N.sub.a or N.sub.b comprises modifications of alternating pattern.

[0254] In some embodiments, the YYY motif occurs at or near the cleavage site of the sense strand. For example, when the dsRNAi agent has a duplex region of 17-23 nucleotides in length, the YYY motif can occur at or the vicinity of the cleavage site (e.g.: can occur at positions 6, 7, 8; 7, 8, 9; 8, 9, 10; 9, 10, 11; 10, 11,12; or 11, 12, 13) of the sense strand, the count starting from the first nucleotide, from the 5'-end; or optionally, the count starting at the first paired nucleotide within the duplex region, from the 5'-end.

[0255] In one embodiment, i is 1 and j is 0, or i is 0 and j is 1, or both i and j are 1. The sense strand can therefore be represented by the following formulas:

5' n.sub.p-N.sub.a—YYY—N.sub.b—ZZZ—N.sub.a-n.sub.q 3' (Ib);

5' n.sub.p-N.sub.a—XXX—N.sub.b—YYY—N.sub.a-n.sub.q 3' (Ic); or

5' n.sub.p-N.sub.a—XXX—N.sub.b—YYY—N.sub.b—ZZZ—N.sub.a-n.sub.q 3' (Id).

[0256] When the sense strand is represented by formula (Ib), N.sub.b represents an oligonucleotide sequence comprising 0-10, 0-7, 0-5, 04, 0-2, or 0 modified nucleotides. Each N.sub.a independently can represent an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

[0257] When the sense strand is represented as formula (Ic), N.sub.b represents an oligonucleotide sequence comprising 0-10, 0-7, 0-10, 0-7, 0-5, 04, 0-2, or 0 modified nucleotides. Each N.sub.a can

independently represent an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

[0258] When the sense strand is represented as formula (Id), each N.sub.b independently represents an oligonucleotide sequence comprising 0-10, 0-7, 0-5, 0-4, 0-2, or 0 modified nucleotides.

Preferably, N.sub.b is 0, 1, 2, 3, 4, 5, or 6 Each N.sub.a can independently represent an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

[0259] Each of X, Y and Z may be the same or different from each other.

[0260] In other embodiments, i is 0 and j is 0, and the sense strand may be represented by the formula:

5' n.sub.p-N.sub.a—YYY—N.sub.a-n.sub.q 3' (Ia).

[0261] When the sense strand is represented by formula (Ia), each N.sub.a independently can represent an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

[0262] In one embodiment, the antisense strand sequence of the RNAi may be represented by formula (II):

5' n.sub.q'-N.sub.a'—(Z'Z'Z').sub.k—N.sub.b'—Y'Y'Y'—N.sub.b'(X'X'X').sub.l—N'-n.sub.p' 3' (II) [0263] wherein: [0264] k and l are each independently 0 or 1; [0265] p' and q' are each

independently 0-6; [0266] each N.sub.a' independently represents an oligonucleotide sequence comprising 0-25 modified nucleotides, each sequence comprising at least two differently modified nucleotides; [0267] each N.sub.b' independently represents an oligonucleotide sequence comprising 0-10 modified nucleotides; [0268] each n.sub.p' and n.sub.q' independently represent an overhang nucleotide; [0269] wherein N.sub.b' and Y' do not have the same modification; and [0270] X'X'X', Y'Y'Y', and Z'Z'Z' each independently represent one motif of three identical modifications on three consecutive nucleotides.

[0271] In some embodiments, the N.sub.a' or N.sub.b' comprises modifications of alternating pattern.

[0272] The Y'Y'Y' motif occurs at or near the cleavage site of the antisense strand. For example, when the dsRNAi agent has a duplex region of 17-23 nucleotides in length, the Y'Y'Y' motif can occur at positions 9, 10, 11; 10, 11, 12; 11, 12, 13; 12, 13, 14; or 13, 14, 15 of the antisense strand, with the count starting from the first nucleotide, from the 5'-end; or optionally, the count starting at the first paired nucleotide within the duplex region, from the 5'-end. Preferably, the Y'Y'Y' motif occurs at positions 11, 12, 13.

[0273] In certain embodiments, Y'Y'Y' motif is all 2'-OMe modified nucleotides.

[0274] In certain embodiments, k is 1 and l is 0, or k is 0 and l is 1, or both k and l are 1.

[0275] The antisense strand can therefore be represented by the following formulas:

5' n.sub.q'-N.sub.a'—Z'Z'Z'—N.sub.b'—Y'Y'Y'—N.sub.a'-n.sub.p' 3' (IIb);

5' n.sub.q'-N.sub.a'—Y'Y'Y'—N.sub.b'—X'X'X'-n.sub.p' 3' (IIc); or

5' n.sub.q'-N.sub.a'—Z'Z'Z'—N.sub.b'—Y'Y'Y'—N.sub.b'—X'X'X'—N.sub.a'-n.sub.p' 3' (IId).

[0276] When the antisense strand is represented by formula (IIb), N.sub.b' represents an oligonucleotide sequence comprising 0-10, 0-7, 0-10, 0-7, 0-5, 04, 0-2, or 0 modified nucleotides. Each N.sub.a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

[0277] When the antisense strand is represented as formula (IIc), N.sub.b' represents an oligonucleotide sequence comprising 0-10, 0-7, 0-10, 0-7, 0-5, 04, 0-2, or 0 modified nucleotides. Each N.sub.a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10

modified nucleotides.

[0278] When the antisense strand is represented as formula (IIId), each N.sub.b' independently represents an oligonucleotide sequence comprising 0-10, 0-7, 0-10, 0-7, 0-5, 0-4, 0-2, or 0 modified nucleotides. Each N.sub.a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides. Preferably, N.sub.b is 0, 1, 2, 3, 4, 5, or 6.

[0279] In other embodiments, k is 0 and l is 0 and the antisense strand may be represented by the formula:

5' n.sub.p'-N.sub.a'—Y'Y'Y'—N.sub.a'-n.sub.q' 3' (Ia).

[0280] When the antisense strand is represented as formula (IIa), each N.sub.a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides. Each of X', Y' and Z' may be the same or different from each other.

[0281] Each nucleotide of the sense strand and antisense strand may be independently modified with LNA, CRN, UNA, cEt, HNA, CeNA, 2'-methoxyethyl, 2'-O-methyl, 2'-O-allyl, 2'-C-allyl, 2'-hydroxyl, or 2'-fluoro. For example, each nucleotide of the sense strand and antisense strand is independently modified with 2'-O-methyl or 2'-fluoro. Each X, Y, Z, X', Y', and Z', in particular, may represent a 2'-O-methyl modification or a 2'-fluoro modification.

[0282] In some embodiments, the sense strand of the dsRNAi agent may contain YYY motif occurring at 9, 10, and 11 positions of the strand when the duplex region is 21 nt, the count starting from the first nucleotide from the 5'-end, or optionally, the count starting at the first paired nucleotide within the duplex region, from the 5'-end; and Y represents 2'-F modification. The sense strand may additionally contain XXX motif or ZZZ motifs as wing modifications at the opposite end of the duplex region; and XXX and ZZZ each independently represents a 2'-OMe modification or 2'-F modification.

[0283] In some embodiments the antisense strand may contain Y'Y'Y' motif occurring at positions 11, 12, 13 of the strand, the count starting from the first nucleotide from the 5'-end, or optionally, the count starting at the first paired nucleotide within the duplex region, from the 5'-end; and Y' represents 2'-O-methyl modification. The antisense strand may additionally contain X'X'X' motif or Z'Z'Z' motifs as wing modifications at the opposite end of the duplex region; and X'X'X' and Z'Z'Z' each independently represents a 2'-OMe modification or 2'-F modification.

[0284] The sense strand represented by any one of the above formulas (Ia), (Ib), (Ic), and (Id) forms a duplex with a antisense strand being represented by any one of formulas (IIa), (IIb), (IIc), and (IIId), respectively.

[0285] Accordingly, the dsRNAi agents for use in the methods of the invention may comprise a sense strand and an antisense strand, each strand having 14 to 30 nucleotides, the iRNA duplex represented by formula (III):

sense: 5' n.sub.p-N.sub.a—(XXX).sub.i—N.sub.b—YYY—N.sub.b—(ZZZ).sub.j—N.sub.a-n.sub.q 3'

antisense: 3' n.sub.p'—N.sub.a'—(X'X'X').sub.k—N.sub.b'—Y'Y'Y'—N.sub.b'—(Z'Z'Z').sub.l—N.sub.a'-n.sub.q' 5' (III) [0286] wherein: [0287] i, j, k, and l are each independently 0 or 1;

[0288] p, p', q, and q' are each independently 0-6; [0289] each N.sub.a and N.sub.a' independently represents an oligonucleotide sequence comprising 0-25 modified nucleotides, each sequence comprising at least two differently modified nucleotides; [0290] each N.sub.b and N.sub.b' independently represents an oligonucleotide sequence comprising 0-10 modified nucleotides; [0291] wherein each n.sub.p', n.sub.p, n.sub.q', and n.sub.q, each of which may or may not be present, independently represents an overhang nucleotide; and [0292] XXX, YYY, ZZZ, X'X'X', Y'Y'Y', and Z'Z'Z' each independently represent one motif of three identical modifications on three consecutive nucleotides.

[0293] In one embodiment, i is 0 and j is 0; or i is 1 and j is 0; or i is 0 and j is 1; or both i and j are 0; or both i and j are 1. In another embodiment, k is 0 and l is 0; or k is 1 and l is 0; k is 0 and l is 1; or both k and l are 0; or both k and l are 1.

[0294] Exemplary combinations of the sense strand and antisense strand forming an iRNA duplex include the formulas below:

5' n.sub.P-N.sub.a—YYY—N.sub.a-n.sub.q 3'

3' n.sub.p'—N.sub.a'—Y'Y'Y'—N.sub.a'n.sub.q' 5' (IIIa)

5' n.sub.P-N.sub.a—YYY—N.sub.b—ZZZ—N.sub.an.sub.q 3'

3' n.sub.p'—N.sub.a'—Y'Y'Y'—N.sub.b'—Z'Z'Z'—N.sub.a'n.sub.q' 5' (IIIb)

5' n.sub.p-N.sub.a—XXX—N.sub.b—YYY—N.sub.a-n.sub.q 3'

3' n.sub.p'—N.sub.a'—X'X'X'—N.sub.b'—Y'Y'Y'—N'-n.sub.q' 5' (IIIc)

5' n.sub.p-N.sub.a—XXX—N.sub.b—YYY—N.sub.b—ZZZ—N.sub.a-n.sub.q 3'

3' n.sub.p'—N.sub.a'—X'X'X'—N.sub.b'—Y'Y'Y'—N.sub.b'—Z'Z'Z'—N.sub.a-n.sub.q' 5' (IIId)

[0295] When the dsRNAi agent is represented by formula (IIIa), each N.sub.a independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

[0296] When the dsRNAi agent is represented by formula (IIIb), each N.sub.b independently represents an oligonucleotide sequence comprising 1-10, 1-7, 1-5, or 1-4 modified nucleotides. Each N.sub.a independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

[0297] When the dsRNAi agent is represented as formula (IIIc), each N.sub.b, N.sub.b' independently represents an oligonucleotide sequence comprising 0-10, 0-7, 0-10, 0-7, 0-5, 04, 0-2, or 0 modified nucleotides. Each N.sub.a independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

[0298] When the dsRNAi agent is represented as formula (IIId), each N.sub.b, N.sub.b' independently represents an oligonucleotide sequence comprising 0-10, 0-7, 0-10, 0-7, 0-5, 04, 0-2, or 0 modified nucleotides. Each N.sub.a, N.sub.a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides. Each of N.sub.a, N.sub.a', N.sub.b, and N.sub.b' independently comprises modifications of alternating pattern.

[0299] Each of X, Y, and Z in formulas (III), (IIIa), (IIIb), (IIIc), and (IIId) may be the same or different from each other.

[0300] When the dsRNAi agent is represented by formula (III), (IIIa), (IIIb), (IIIc), and (IIId), at least one of the Y nucleotides may form a base pair with one of the Y' nucleotides. Alternatively, at least two of the Y nucleotides form base pairs with the corresponding Y' nucleotides; or all three of the Y nucleotides all form base pairs with the corresponding Y' nucleotides.

[0301] When the dsRNAi agent is represented by formula (IIIb) or (IIId), at least one of the Z nucleotides may form a base pair with one of the Z' nucleotides. Alternatively, at least two of the Z nucleotides form base pairs with the corresponding Z' nucleotides; or all three of the Z nucleotides all form base pairs with the corresponding Z' nucleotides.

[0302] When the dsRNAi agent is represented as formula (IIIc) or (IIId), at least one of the X nucleotides may form a base pair with one of the X' nucleotides. Alternatively, at least two of the X nucleotides form base pairs with the corresponding X' nucleotides; or all three of the X nucleotides

all form base pairs with the corresponding X' nucleotides.

[0303] In certain embodiments, the modification on the Y nucleotide is different than the modification on the Y' nucleotide, the modification on the Z nucleotide is different than the modification on the Z' nucleotide, or the modification on the X nucleotide is different than the modification on the X' nucleotide.

[0304] In certain embodiments, when the dsRNAi agent is represented by formula (IIId), the N.sub.a modifications are 2'-O-methyl or 2'-fluoro modifications. In other embodiments, when the RNAi agent is represented by formula (IIId), the N.sub.a modifications are 2'-O-methyl or 2'-fluoro modifications and n.sub.p' > 0 and at least one n.sub.p' is linked to a neighboring nucleotide a via phosphorothioate linkage. In yet other embodiments, when the RNAi agent is represented by formula (IIId), the N.sub.a modifications are 2'-O-methyl or 2'-fluoro modifications, n.sub.p' > 0 and at least one n.sub.p' is linked to a neighboring nucleotide via phosphorothioate linkage, and the sense strand is conjugated to one or more GalNAc derivatives attached through a bivalent or trivalent branched linker (described below). In other embodiments, when the RNAi agent is represented by formula (IIId), the N.sub.a modifications are 2'-O-methyl or 2'-fluoro modifications, n.sub.p' > 0 and at least one n.sub.p' is linked to a neighboring nucleotide via phosphorothioate linkage, the sense strand comprises at least one phosphorothioate linkage, and the sense strand is conjugated to one or more GalNAc derivatives attached through a monovalent, bivalent or trivalent branched linker.

[0305] In some embodiments, when the dsRNAi agent is represented by formula (IIIa), the N.sub.a modifications are 2'-O-methyl or 2'-fluoro modifications, n.sub.p' > 0 and at least one n.sub.p' is linked to a neighboring nucleotide via phosphorothioate linkage, the sense strand comprises at least one phosphorothioate linkage, and the sense strand is conjugated to one or more GalNAc derivatives attached through a bivalent or trivalent branched linker.

[0306] In some embodiments, the dsRNAi agent is a multimer containing at least two duplexes represented by formula (III), (IIIa), (IIIb), (IIIc), and (IIId), wherein the duplexes are connected by a linker. The linker can be cleavable or non-cleavable. Optionally, the multimer further comprises a ligand. Each of the duplexes can target the same gene or two different genes; or each of the duplexes can target same gene at two different target sites.

[0307] In some embodiments, the dsRNAi agent is a multimer containing three, four, five, six, or more duplexes represented by formula (III), (IIIa), (IIIb), (IIIc), and (IIId), wherein the duplexes are connected by a linker. The linker can be cleavable or non-cleavable. Optionally, the multimer further comprises a ligand. Each of the duplexes can target the same gene or two different genes; or each of the duplexes can target same gene at two different target sites.

[0308] In one embodiment, two dsRNAi agents represented by at least one of formulas (III), (IIIa), (IIIb), (IIIc), and (IIId) are linked to each other at the 5' end, and one or both of the 3' ends, and are optionally conjugated to a ligand. Each of the agents can target the same gene or two different genes; or each of the agents can target same gene at two different target sites.

[0309] Various publications describe multimeric iRNAs that can be used in the methods of the invention. Such publications include WO2007/091269, U.S. Pat. No. 7,858,769, WO2010/141511, WO2007/117686, WO2009/014887, and WO2011/031520 the entire contents of each of which are hereby incorporated herein by reference.

[0310] As described in more detail below, the iRNA that contains conjugations of one or more carbohydrate moieties to an iRNA can optimize one or more properties of the iRNA. In many cases, the carbohydrate moiety will be attached to a modified subunit of the iRNA. For example, the ribose sugar of one or more ribonucleotide subunits of a iRNA can be replaced with another moiety, e.g., a non-carbohydrate (preferably cyclic) carrier to which is attached a carbohydrate ligand. A ribonucleotide subunit in which the ribose sugar of the subunit has been so replaced is referred to herein as a ribose replacement modification subunit (RRMS). A cyclic carrier may be a carbocyclic ring system. i.e., all ring atoms are carbon atoms, or a heterocyclic ring system. i.e.,

one or more ring atoms may be a heteroatom, e.g., nitrogen, oxygen, sulfur. The cyclic carrier may be a monocyclic ring system. or may contain two or more rings, e.g. fused rings. The cyclic carrier may be a fully saturated ring system. or it may contain one or more double bonds.

[0311] The ligand may be attached to the polynucleotide via a carrier. The carriers include (i) at least one “backbone attachment point,” preferably two “backbone attachment points” and (ii) at least one “tethering attachment point.” A “backbone attachment point” as used herein refers to a functional group, e.g. a hydroxyl group, or generally, a bond available for, and that is suitable for incorporation of the carrier into the backbone, e.g., the phosphate, or modified phosphate, e.g., sulfur containing, backbone, of a ribonucleic acid. A “tethering attachment point” (TAP) in some embodiments refers to a constituent ring atom of the cyclic carrier, e.g., a carbon atom or a heteroatom (distinct from an atom which provides a backbone attachment point), that connects a selected moiety. The moiety can be, e.g., a carbohydrate, e.g. monosaccharide, disaccharide, trisaccharide, tetrasaccharide, oligosaccharide, or polysaccharide. Optionally, the selected moiety is connected by an intervening tether to the cyclic carrier. Thus, the cyclic carrier will often include a functional group, e.g., an amino group, or generally, provide a bond, that is suitable for incorporation or tethering of another chemical entity, e.g., a ligand to the constituent ring.

[0312] The iRNA may be conjugated to a ligand via a carrier, wherein the carrier can be cyclic group or acyclic group; preferably, the cyclic group is selected from pyrrolidinyl, pyrazolinyl, pyrazolidinyl, imidazolinyl, imidazolidinyl, piperidinyl, piperazinyl, [1,3]dioxolane, oxazolidinyl, isoxazolidinyl, morpholinyl, thiazolidinyl, isothiazolidinyl, quinoxalinyl, pyridazinonyl, tetrahydrofuryl, and decalin; preferably, the acyclic group is a serinol backbone or diethanolamine backbone.

[0313] In certain embodiments, the iRNA for use in the methods of the invention is an agent selected from agents listed in Table 3 or Table 5. These agents may further comprise a ligand.

III. iRNAs Conjugated to Ligands

[0314] Another modification of the RNA of an iRNA of the invention involves chemically linking to the iRNA one or more ligands, moieties or conjugates that enhance the activity, cellular distribution, or cellular uptake of the iRNA e.g., into a cell. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, 1989, 86: 6553-6556), cholic acid (Manoharan et al., *Biorg. Med. Chem. Let.*, 1994, 4:1053-1060), a thioether, e.g., beryl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, 1992, 660:306-309; Manoharan et al., *Biorg. Med. Chem. Let.*, 1993, 3:2765-2770), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, 1992, 20:533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J*, 1991, 10:1111-1118; Kabanov et al., *FEBS Lett.*, 1990, 259:327-330; Svinarchuk et al., *Biochimie*, 1993, 75:49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-phosphonate (Manoharan et al., *Tetrahedron Lett.*, 1995, 36:3651-3654; Shea et al., *Nucl. Acids Res.*, 1990, 18:3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, 1995, 14:969-973), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, 1995, 36:3651-3654), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, 1995, 1264:229-237), or an octadecylamine or hexylamino-carboxyloxycholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, 1996, 277:923-937).

[0315] In certain embodiments, a ligand alters the distribution, targeting or lifetime of an iRNA agent into which it is incorporated. In preferred embodiments a ligand provides an enhanced affinity for a selected target, e.g., molecule, cell or cell type, compartment, e.g., a cellular or organ compartment, tissue, organ or region of the body, as, e.g., compared to a species absent such a ligand. Preferred ligands do not take part in duplex pairing in a duplexed nucleic acid.

[0316] Ligands can include a naturally occurring substance, such as a protein (e.g., human serum albumin (HSA), low-density lipoprotein (LDL), or globulin); carbohydrate (e.g., a dextran, pullulan, chitin, chitosan, inulin, cyclodextrin, N-acetylgalactosamine, or hyaluronic acid); or a

lipid. The ligand can also be a recombinant or synthetic molecule, such as a synthetic polymer, e.g., a synthetic polyamino acid. Examples of polyamino acids include polyamino acid is a polylysine (PLL), poly L-aspartic acid, poly L-glutamic acid, styrene-maleic acid anhydride copolymer, poly(L-lactide-co-glycolide) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-hydroxypropyl)methacrylamide copolymer (HMPA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacrylic acid), N-isopropylacrylamide polymers, or polyphosphazine. Example of polyamines include: polyethylenimine, polylysine (PLL), spermine, spermidine, polyamine, pseudopeptide-polyamine, peptidomimetic polyamine, dendrimer polyamine, arginine, amidine, protamine, cationic lipid, cationic porphyrin, quaternary salt of a polyamine, or an alpha helical peptide.

[0317] Ligands can also include targeting groups, e.g., a cell or tissue targeting agent, e.g., a lectin, glycoprotein, lipid or protein, e.g., an antibody, that binds to a specified cell type such as a kidney cell. A targeting group can be a thyrotropin, melanotropin, lectin, glycoprotein, surfactant protein A, Mucin carbohydrate, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucoseamine multivalent mannose, multivalent fucose, glycosylated polyaminoacids, multivalent galactose, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipid, cholesterol, a steroid, bile acid, folate, vitamin B12, vitamin A, biotin, or an RGD peptide or RGD peptide mimetic.

[0318] Other examples of ligands 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 molecules, 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 phenoxazine) and peptide conjugates (e.g., antennapedia peptide, Tat peptide), alkylating agents, phosphate, amino, mercapto, PEG (e.g., PEG-40K), MPEG, [MPEG].sub.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, Eu³⁺ complexes of tetraazamacrocycles), dinitrophenyl, HRP, or AP.

[0319] Ligands can be proteins, e.g., glycoproteins, or peptides, e.g., molecules having a specific affinity for a co-ligand, or antibodies e.g., an antibody, that binds to a specified cell type such as a hepatic cell. Ligands can also include hormones and hormone receptors. They can also include non-peptidic species, such as lipids, lectins, carbohydrates, vitamins, cofactors, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucoseamine multivalent mannose, or multivalent fucose. The ligand can be, for example, a lipopolysaccharide, an activator of p38 MAP kinase, or an activator of NF- κ B.

[0320] The ligand can be a substance, e.g., a drug, which can increase the uptake of the iRNA agent into the cell, for example, by disrupting the cell's cytoskeleton, e.g., by disrupting the cell's microtubules, microfilaments, or intermediate filaments. The drug can be, for example, taxon, vincristine, vinblastine, cytochalasin, nocodazole, japlakinolide, latrunculin A, phalloidin, swinholide A, indanocine, or myoservin.

[0321] In some embodiments, a ligand attached to an iRNA as described herein acts as a pharmacokinetic modulator (PK modulator). PK modulators include lipophiles, bile acids, steroids, phospholipid analogues, peptides, protein binding agents, PEG, vitamins etc. Exemplary PK modulators include, but are not limited to, cholesterol, fatty acids, cholic acid, lithocholic acid, dialkylglycerides, diacylglyceride, phospholipids, sphingolipids, naproxen, ibuprofen, vitamin E, biotin etc. Oligonucleotides that comprise a number of phosphorothioate linkages are also known to bind to serum protein, thus short oligonucleotides, e.g., oligonucleotides of about 5 bases, 10

bases, 15 bases, or 20 bases, comprising multiple of phosphorothioate linkages in the backbone are also amenable to the present invention as ligands (e.g. as PK modulating ligands). In addition, aptamers that bind serum components (e.g. serum proteins) are also suitable for use as PK modulating ligands in the embodiments described herein.

[0322] Ligand-conjugated iRNAs of the invention may be synthesized by the use of an oligonucleotide that bears a pendant reactive functionality, such as that derived from the attachment of a linking molecule onto the oligonucleotide (described below). This reactive oligonucleotide may be reacted directly with commercially-available ligands, ligands that are synthesized bearing any of a variety of protecting groups, or ligands that have a linking moiety attached thereto.

[0323] The oligonucleotides used in the conjugates of the present invention may be conveniently and routinely made through the well-known technique of solid-phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, Calif.). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is also known to use similar techniques to prepare other oligonucleotides, such as the phosphorothioates and alkylated derivatives.

[0324] In the ligand-conjugated iRNAs and ligand-molecule bearing sequence-specific linked nucleosides of the present invention, the oligonucleotides and oligonucleosides may be assembled on a suitable DNA synthesizer utilizing standard nucleotide or nucleoside precursors, or nucleotide or nucleoside conjugate precursors that already bear the linking moiety, ligand-nucleotide or nucleoside-conjugate precursors that already bear the ligand molecule, or non-nucleoside ligand-bearing building blocks.

[0325] When using nucleotide-conjugate precursors that already bear a linking moiety, the synthesis of the sequence-specific linked nucleosides is typically completed, and the ligand molecule is then reacted with the linking moiety to form the ligand-conjugated oligonucleotide. In some embodiments, the oligonucleotides or linked nucleosides of the present invention are synthesized by an automated synthesizer using phosphoramidites derived from ligand-nucleoside conjugates in addition to the standard phosphoramidites and non-standard phosphoramidites that are commercially available and routinely used in oligonucleotide synthesis.

A. Lipid Conjugates

[0326] In certain embodiments, the ligand or conjugate is a lipid or lipid-based molecule. Such a lipid or lipid-based molecule preferably binds a serum protein, e.g., human serum albumin (HSA). An HSA binding ligand allows for distribution of the conjugate to a target tissue, e.g., a non-kidney target tissue of the body. For example, the target tissue can be the liver, including parenchymal cells of the liver. Other molecules that can bind HSA can also be used as ligands. For example, naproxen or aspirin can be used. A lipid or lipid-based ligand can (a) increase resistance to degradation of the conjugate, (b) increase targeting or transport into a target cell or cell membrane, or (c) can be used to adjust binding to a serum protein, e.g., HSA.

[0327] A lipid based ligand can be used to inhibit, e.g., control the binding of the conjugate to a target tissue. For example, a lipid or lipid-based ligand that binds to HSA more strongly will be less likely to be targeted to the kidney and therefore less likely to be cleared from the body. A lipid or lipid-based ligand that binds to HSA less strongly can be used to target the conjugate to the kidney.

[0328] In certain embodiments, the lipid based ligand binds HSA. Preferably, it binds HSA with a sufficient affinity such that the conjugate will be preferably distributed to a non-kidney tissue. However, it is preferred that the affinity not be so strong that the HSA-ligand binding cannot be reversed.

[0329] In other embodiments, the lipid based ligand binds HSA weakly or not at all, such that the conjugate will be preferably distributed to the kidney. Other moieties that target to kidney cells can also be used in place of, or in addition to, the lipid based ligand.

[0330] In another aspect, the ligand is a moiety, e.g., a vitamin, which is taken up by a target cell, e.g., a proliferating cell. These are particularly useful for treating disorders characterized by

unwanted cell proliferation, e.g., of the malignant or non-malignant type, e.g., cancer cells. Exemplary vitamins include vitamin A, E, and K. Other exemplary vitamins include are B vitamin, e.g., folic acid, B12, riboflavin, biotin, pyridoxal or other vitamins or nutrients taken up by target cells such as liver cells. Also included are HSA and low density lipoprotein (LDL).

B. Cell Permeation Agents

[0331] In another aspect, the ligand is a cell-permeation agent, preferably a helical cell-permeation agent. Preferably, the agent is amphipathic. An exemplary agent is a peptide such as tat or antennopodia. If the agent is a peptide, it can be modified, including a peptidylmimetic, invertomers, non-peptide or pseudo-peptide linkages, and use of D-amino acids. The helical agent is preferably an alpha-helical agent, which preferably has a lipophilic and a lipophobic phase.

[0332] The ligand can be a peptide or peptidomimetic. A peptidomimetic (also referred to herein as an oligopeptidomimetic) is a molecule capable of folding into a defined three-dimensional structure similar to a natural peptide. The attachment of peptide and peptidomimetics to iRNA agents can affect pharmacokinetic distribution of the iRNA, such as by enhancing cellular recognition and absorption. The peptide or peptidomimetic moiety can be about 5-50 amino acids long, e.g., about 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids long.

[0333] A peptide or peptidomimetic can be, for example, a cell permeation peptide, cationic peptide, amphipathic peptide, or hydrophobic peptide (e.g., consisting primarily of Tyr, Trp, or Phe). The peptide moiety can be a dendrimer peptide, constrained peptide or crosslinked peptide. In another alternative, the peptide moiety can include a hydrophobic membrane translocation sequence (MTS). An exemplary hydrophobic MTS-containing peptide is RFGF having the amino acid sequence AAVALLPAVLLALLAP (SEQ ID NO: 13). An RFGF analogue (e.g., amino acid sequence AALLPVLLAAP (SEQ ID NO:14) containing a hydrophobic MTS can also be a targeting moiety. The peptide moiety can be a "delivery" peptide, which can carry large polar molecules including peptides, oligonucleotides, and protein across cell membranes. For example, sequences from the HIV Tat protein (GRKKRRQRRRPPQ (SEQ ID NO:15) and the *Drosophila* Antennapedia protein (RQIKIWFQNRRMKWKK (SEQ ID NO:16) have been found to be capable of functioning as delivery peptides. A peptide or peptidomimetic can be encoded by a random sequence of DNA, such as a peptide identified from a phage-display library, or one-bead-one-compound (OBOC) combinatorial library (Lam et al., Nature, 354:82-84, 1991). Examples of a peptide or peptidomimetic tethered to a dsRNA agent via an incorporated monomer unit for cell targeting purposes is an arginine-glycine-aspartic acid (RGD)-peptide, or RGD mimic. A peptide moiety can range in length from about 5 amino acids to about 40 amino acids. The peptide moieties can have a structural modification, such as to increase stability or direct conformational properties. Any of the structural modifications described below can be utilized.

[0334] An RGD peptide for use in the compositions and methods of the invention may be linear or cyclic, and may be modified, e.g., glycosylated or methylated, to facilitate targeting to a specific tissue(s). RGD-containing peptides and peptidomimetics may include D-amino acids, as well as synthetic RGD mimics. In addition to RGD, one can use other moieties that target the integrin ligand. Preferred conjugates of this ligand target PECAM-1 or VEGF.

[0335] A "cell permeation peptide" is capable of permeating a cell, e.g., a microbial cell, such as a bacterial or fungal cell, or a mammalian cell, such as a human cell. A microbial cell-permeating peptide can be, for example, an α -helical linear peptide (e.g., LL-37 or Ceropin P1), a disulfide bond-containing peptide (e.g., α -defensin, β -defensin or bactenecin), or a peptide containing only one or two dominating amino acids (e.g., PR-39 or indolicidin). A cell permeation peptide can also include a nuclear localization signal (NLS). For example, a cell permeation peptide can be a bipartite amphipathic peptide, such as MPG, which is derived from the fusion peptide domain of HIV-1 gp41 and the NLS of SV40 large T antigen (Simeoni et al., *Nucl. Acids Res.* 31:2717-2724, 2003).

C. Carbohydrate Conjugates

[0336] In some embodiments of the compositions and methods of the invention, an iRNA further comprises a carbohydrate. The carbohydrate conjugated iRNA is advantageous for the in vivo delivery of nucleic acids, as well as compositions suitable for in vivo therapeutic use, as described herein. As used herein, “carbohydrate” refers to a compound which is either a carbohydrate per se made up of one or more monosaccharide units having at least 6 carbon atoms (which can be linear, branched or cyclic) with an oxygen, nitrogen or sulfur atom bonded to each carbon atom; or a compound having as a part thereof a carbohydrate moiety made up of one or more monosaccharide units each having at least six carbon atoms (which can be linear, branched or cyclic), with an oxygen, nitrogen or sulfur atom bonded to each carbon atom. Representative carbohydrates include the sugars (mono-, di-, tri-, and oligosaccharides containing from about 4, 5, 6, 7, 8, or 9 monosaccharide units), and polysaccharides such as starches, glycogen, cellulose and polysaccharide gums. Specific monosaccharides include HBV and above (e.g., HBV, C6, C7, or C8) sugars; di- and trisaccharides include sugars having two or three monosaccharide units (e.g., HBV, C6, C7, or C8).

[0337] In certain embodiments, a carbohydrate conjugate for use in the compositions and methods of the invention is a monosaccharide. In one embodiment, the monosaccharide is an N-acetylgalactosamine, such as

##STR00005##

[0338] In other embodiments, a carbohydrate conjugate for use in the compositions and methods of the invention is selected from the group:

##STR00006## ##STR00007## ##STR00008## ##STR00009##

[0339] Another representative carbohydrate conjugate for use in the embodiments described herein includes, but is not limited to,

##STR00010##

when one of X or Y is an oligonucleotide, the other is a hydrogen.

[0340] In certain embodiments of the invention, the GalNAc or GalNAc derivative is attached to an iRNA agent of the invention via a monovalent linker. In some embodiments, the GalNAc or GalNAc derivative is attached to an iRNA agent of the invention via a bivalent linker. In yet other embodiments of the invention, the GalNAc or GalNAc derivative is attached to an iRNA agent of the invention via a trivalent linker.

[0341] In one embodiment, the double stranded RNAi agents of the invention comprise one GalNAc or GalNAc derivative attached to the iRNA agent. In another embodiment, the double stranded RNAi agents of the invention comprise a plurality (e.g., 2, 3, 4, 5, or 6) GalNAc or GalNAc derivatives, each independently attached to a plurality of nucleotides of the double stranded RNAi agent through a plurality of monovalent linkers.

[0342] In some embodiments, for example, when the two strands of an iRNA agent of the invention are part of one larger molecule connected by an uninterrupted chain of nucleotides between the 3'-end of one strand and the 5'-end of the respective other strand forming a hairpin loop comprising, a plurality of unpaired nucleotides, each unpaired nucleotide within the hairpin loop may independently comprise a GalNAc or GalNAc derivative attached via a monovalent linker.

[0343] In some embodiments, the carbohydrate conjugate further comprises one or more additional ligands as described above, such as, but not limited to, a PK modulator or a cell permeation peptide.

[0344] Additional carbohydrate conjugates suitable for use in the present invention include those described in PCT Publication Nos. WO 2014/179620 and WO 2014/179627, the entire contents of each of which are incorporated herein by reference.

D. Linkers

[0345] In some embodiments, the conjugate or ligand described herein can be attached to an iRNA oligonucleotide with various linkers that can be cleavable or non-cleavable.

[0346] The term “linker” or “linking group” means an organic moiety that connects two parts of a

compound, e.g., covalently attaches two parts of a compound. Linkers typically comprise a direct bond or an atom such as oxygen or sulfur, a unit such as NR₈, C(O), C(O)NH, SO, SO.sub.2, SO.sub.2NH or a chain of atoms, such as, but not limited to, substituted or unsubstituted alkyl, substituted or unsubstituted alkenyl, substituted or unsubstituted alkynyl, arylalkyl, arylalkenyl, arylalkynyl, heteroarylalkyl, heteroarylalkenyl, heteroarylalkynyl, heterocyclalkyl, heterocyclalkenyl, heterocyclalkynyl, aryl, heteroaryl, heterocycl, cycloalkyl, cycloalkenyl, alkylarylalkyl, alkylarylalkenyl, alkylarylalkynyl, alkenylarylalkyl, alkenylarylalkenyl, alkenylarylalkynyl, alkynylarylalkyl, alkynylarylalkenyl, alkynylarylalkynyl, alkylheteroarylalkyl, alkylheteroarylalkenyl, alkylheteroarylalkynyl, alkenylheteroarylalkyl, alkenylheteroarylalkenyl, alkenylheteroarylalkynyl, alkynylheteroarylalkyl, alkynylheteroarylalkenyl, alkynylheteroarylalkynyl, alkylheterocyclalkyl, alkylheterocyclalkenyl, alkylheterocyclalkynyl, alkenylheterocyclalkyl, alkenylheterocyclalkenyl, alkenylheterocyclalkynyl, alkynylheterocyclalkyl, alkynylheterocyclalkenyl, alkynylheterocyclalkynyl, alkylaryl, alkenylaryl, alkynylaryl, alkylheteroaryl, alkenylheteroaryl, alkynylheteroaryl, which one or more methylenes can be interrupted or terminated by O, S, S(O), SO.sub.2, N(R₈), C(O), substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, or substituted or unsubstituted heterocyclic; where R₈ is hydrogen, acyl, aliphatic, or substituted aliphatic. In one embodiment, the linker is between about 1-24 atoms, 2-24, 3-24, 4-24, 5-24, 6-24, 6-18, 7-18, 8-18, 7-17, 8-17, 6-16, 7-16, or 8-16 atoms.

[0347] A cleavable linking group is one which is sufficiently stable outside the cell, but which upon entry into a target cell is cleaved to release the two parts the linker is holding together. In a preferred embodiment, the cleavable linking group is cleaved at least about 10 times, 20, times, 30 times, 40 times, 50 times, 60 times, 70 times, 80 times, 90 times, or 100 times faster in a target cell or under a first reference condition (which can, e.g., be selected to mimic or represent intracellular conditions) than in the blood of a subject, or under a second reference condition (which can, e.g., be selected to mimic or represent conditions found in the blood or serum).

[0348] Cleavable linking groups are susceptible to cleavage agents, e.g., pH, redox potential, or the presence of degradative molecules. Generally, cleavage agents are more prevalent or found at higher levels or activities inside cells than in serum or blood. Examples of such degradative agents include: redox agents which are selected for particular substrates or which have no substrate specificity, including, e.g., oxidative or reductive enzymes or reductive agents such as mercaptans, present in cells, that can degrade a redox cleavable linking group by reduction; esterases; endosomes or agents that can create an acidic environment, e.g., those that result in a pH of five or lower; enzymes that can hydrolyze or degrade an acid cleavable linking group by acting as a general acid, peptidases (which can be substrate specific), and phosphatases.

[0349] A cleavable linkage group, such as a disulfide bond can be susceptible to pH. The pH of human serum is 7.4, while the average intracellular pH is slightly lower, ranging from about 7.1-7.3. Endosomes have a more acidic pH, in the range of 5.5-6.0, and lysosomes have an even more acidic pH at around 5.0. Some linkers will have a cleavable linking group that is cleaved at a preferred pH, thereby releasing a cationic lipid from the ligand inside the cell, or into the desired compartment of the cell.

[0350] A linker can include a cleavable linking group that is cleavable by a particular enzyme. The type of cleavable linking group incorporated into a linker can depend on the cell to be targeted. For example, a liver-targeting ligand can be linked to a cationic lipid through a linker that includes an ester group. Liver cells are rich in esterases, and therefore the linker will be cleaved more efficiently in liver cells than in cell types that are not esterase-rich. Other cell-types rich in esterases include cells of the lung, renal cortex, and testis.

[0351] Linkers that contain peptide bonds can be used when targeting cell types rich in peptidases, such as liver cells and synoviocytes.

[0352] In general, the suitability of a candidate cleavable linking group can be evaluated by testing

the ability of a degradative agent (or condition) to cleave the candidate linking group. It will also be desirable to also test the candidate cleavable linking group for the ability to resist cleavage in the blood or when in contact with other non-target tissue. Thus, one can determine the relative susceptibility to cleavage between a first and a second condition, where the first is selected to be indicative of cleavage in a target cell and the second is selected to be indicative of cleavage in other tissues or biological fluids, e.g., blood or serum. The evaluations can be carried out in cell free systems, in cells, in cell culture, in organ or tissue culture, or in whole animals. It can be useful to make initial evaluations in cell-free or culture conditions and to confirm by further evaluations in whole animals. In preferred embodiments, useful candidate compounds are cleaved at least about 2, 4, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 times faster in the cell (or under in vitro conditions selected to mimic intracellular conditions) as compared to blood or serum (or under in vitro conditions selected to mimic extracellular conditions).

i. Redox Cleavable Linking Groups

[0353] In certain embodiments, a cleavable linking group is a redox cleavable linking group that is cleaved upon reduction or oxidation. An example of reductively cleavable linking group is a disulphide linking group (—S—S—). To determine if a candidate cleavable linking group is a suitable “reductively cleavable linking group,” or for example is suitable for use with a particular iRNA moiety and particular targeting agent one can look to methods described herein. For example, a candidate can be evaluated by incubation with dithiothreitol (DTT), or other reducing agent using reagents known in the art, which mimic the rate of cleavage which would be observed in a cell, e.g., a target cell. The candidates can also be evaluated under conditions which are selected to mimic blood or serum conditions. In one, candidate compounds are cleaved by at most about 10% in the blood. In other embodiments, useful candidate compounds are degraded at least about 2, 4, 10, 20, 30, 40, 50, 60, 70, 80, 90, or about 100 times faster in the cell (or under in vitro conditions selected to mimic intracellular conditions) as compared to blood (or under in vitro conditions selected to mimic extracellular conditions). The rate of cleavage of candidate compounds can be determined using standard enzyme kinetics assays under conditions chosen to mimic intracellular media and compared to conditions chosen to mimic extracellular media.

ii. Phosphate-Based Cleavable Linking Groups

[0354] In other embodiments, a cleavable linker comprises a phosphate-based cleavable linking group. A phosphate-based cleavable linking group is cleaved by agents that degrade or hydrolyze the phosphate group. An example of an agent that cleaves phosphate groups in cells are enzymes such as phosphatases in cells. Examples of phosphate-based linking groups are —O—P(O)(ORk)—O— , —O—P(S)(ORk)—O— , —O—P(S)(SRk)—O— , —S—P(O)(ORk)—O— , —O—P(O)(ORk)—S— , —S—P(O)(ORk)—S— , —O—P(S)(ORk)—S— , —S—P(S)(ORk)—O— , —O—P(O)(Rk)—O— , —O—P(S)(Rk)—O— , —S—P(O)(Rk)—O— , —S—P(S)(Rk)—O— , —S—P(O)(Rk)—S— , —O—P(S)(Rk)—S— . Preferred embodiments are —O—P(O)(OH)—O— , —O—P(S)(OH)—O— , —O—P(S)(SH)—O— , —S—P(O)(OH)—O— , —O—P(O)(OH)—S— , —S—P(O)(OH)—S— , —O—P(S)(OH)—S— , —S—P(S)(OH)—O— , —O—P(O)(H)—O— , —O—P(S)(H)—O— , —S—P(O)(H)—O— , —S—P(S)(H)—O— , —S—P(O)(H)—S— , and —O—P(S)(H)—S— . A preferred embodiment is —O—P(O)(OH)—O— . These candidates can be evaluated using methods analogous to those described above.

iii. Acid cleavable linking groups

[0355] In other embodiments, a cleavable linker comprises an acid cleavable linking group. An acid cleavable linking group is a linking group that is cleaved under acidic conditions. In preferred embodiments acid cleavable linking groups are cleaved in an acidic environment with a pH of about 6.5 or lower (e.g., about 6.0, 5.5, 5.0, or lower), or by agents such as enzymes that can act as a general acid. In a cell, specific low pH organelles, such as endosomes and lysosomes can provide a cleaving environment for acid cleavable linking groups. Examples of acid cleavable linking groups include but are not limited to hydrazones, esters, and esters of amino acids. Acid cleavable

groups can have the general formula —C=NN— , C(O)O , or —OC(O) . A preferred embodiment is when the carbon attached to the oxygen of the ester (the alkoxy group) is an aryl group, substituted alkyl group, or tertiary alkyl group such as dimethyl pentyl or t-butyl. These candidates can be evaluated using methods analogous to those described above.

iv. Ester-Based Linking Groups

[0356] In other embodiments, a cleavable linker comprises an ester-based cleavable linking group. An ester-based cleavable linking group is cleaved by enzymes such as esterases and amidases in cells. Examples of ester-based cleavable linking groups include, but are not limited to, esters of alkylene, alkenylene and alkynylene groups. Ester cleavable linking groups have the general formula —C(O)O— , or —OC(O)— . These candidates can be evaluated using methods analogous to those described above.

v. Peptide-Based Cleaving Groups

[0357] In yet other embodiments, a cleavable linker comprises a peptide-based cleavable linking group. A peptide-based cleavable linking group is cleaved by enzymes such as peptidases and proteases in cells. Peptide-based cleavable linking groups are peptide bonds formed between amino acids to yield oligopeptides (e.g., dipeptides, tripeptides etc.) and polypeptides. Peptide-based cleavable groups do not include the amide group (—C(O)NH—). The amide group can be formed between any alkylene, alkenylene or alkynylene. A peptide bond is a special type of amide bond formed between amino acids to yield peptides and proteins. The peptide based cleavage group is generally limited to the peptide bond (i.e., the amide bond) formed between amino acids yielding peptides and proteins and does not include the entire amide functional group. Peptide-based cleavable linking groups have the general formula $\text{—NHCHRAC(O)NHCHRBC(O)—}$, where RA and RB are the R groups of the two adjacent amino acids. These candidates can be evaluated using methods analogous to those described above.

[0358] In some embodiments, an iRNA of the invention is conjugated to a carbohydrate through a linker. Non-limiting examples of iRNA carbohydrate conjugates with linkers of the compositions and methods of the invention include, but are not limited to,

##STR00011## ##STR00012## [0359] when one of X or Y is an oligonucleotide, the other is a hydrogen.

[0360] In certain embodiments of the compositions and methods of the invention, a ligand is one or more “GalNAc” (N-acetylgalactosamine) derivatives attached through a bivalent or trivalent branched linker.

[0361] In certain embodiments, a dsRNA of the invention is conjugated to a bivalent or trivalent branched linker selected from the group of structures shown in any of formula (XXXII)—(XXXV):
##STR00013##

wherein: [0362] q2A, q2B, q3A, q3B, q4A, q4B, q5A, q5B and q5C represent independently for each occurrence 0-20 and wherein the repeating unit can be the same or different; [0363] p.sup.2A, p.sup.2B, p.sup.3A, p.sup.3B, p.sup.4A, p.sup.4B, p.sup.5A, p.sup.5B, p.sup.5C, T.sup.2A, T.sup.2B, V.sup.3A, T.sup.3B, T.sup.4A, T.sup.4B, T.sup.4A, T.sup.5B, T.sup.5C are each independently for each occurrence absent, CO, NH, O, S, OC(O), NHC(O), CH.sub.2, CH.sub.2NH, or CH.sub.2O; [0364] Q.sup.2A, Q.sup.2B, Q.sup.3A, Q.sup.3B, Q.sup.4A, Q.sup.4B, Q.sup.5A, Q.sup.5B, Q.sup.5C are independently for each occurrence absent, alkylene, substituted alkylene wherein one or more methylenes can be interrupted or terminated by one or more of O, S, S(O), SO.sub.2, N(R.sup.N), C(R')=C(R''), C≡C, or C(O); [0365] R.sup.2A, R.sup.2B, R.sup.3A, R.sup.3B, R.sup.4A, R.sup.4B, R.sup.5A, R.sup.5B, R.sup.5C are each independently for each occurrence absent, NH, O, S, CH.sub.2, C(O)O, C(O)NH, NHCH(R.sup.a)C(O), $\text{—C(O)—CH(R.sup.a)—NH—}$, CO, CH=N—O,
##STR00014##

heterocyclyl; [0366] L.sup.2A, L.sup.2B, L.sup.3A, L.sup.3B, L.sup.4A, L.sup.4B, L.sup.5A, L.sup.5B, and L.sup.5C represent the ligand; i.e. each independently for each occurrence a

monosaccharide (such as GalNAc), disaccharide, trisaccharide, tetrasaccharide, oligosaccharide, or polysaccharide; and R.sup.a is H or amino acid side chain. Trivalent conjugating GalNAc derivatives are particularly useful for use with RNAi agents for inhibiting the expression of a target gene, such as those of formula (XXXV):

##STR00015## [0367] wherein L.sup.5A, L.sup.5B and L.sup.5C represent a monosaccharide, such as GalNAc derivative.

[0368] Examples of suitable bivalent and trivalent branched linker groups conjugating GalNAc derivatives include, but are not limited to, the structures recited above as formulas II, VII, XI, X, and XIII.

[0369] Representative US patents that teach the preparation of RNA conjugates include, but are not limited to, U.S. Pat. Nos. 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241; 5,391,723; 5,416,203; 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928; 5,688,941; 6,294,664; 6,320,017; 6,576,752; 6,783,931; 6,900,297; 7,037,646; and 8,106,022, the entire contents of each of which are hereby incorporated herein by reference.

[0370] It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications can be incorporated in a single compound or even at a single nucleoside within an iRNA. The present invention also includes iRNA compounds that are chimeric compounds.

[0371] "Chimeric" iRNA compounds or "chimeras," in the context of this invention, are iRNA compounds, preferably dsRNAi agents, that contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of a dsRNA compound. These iRNAs typically contain at least one region wherein the RNA is modified so as to confer upon the iRNA increased resistance to nuclease degradation, increased cellular uptake, or increased binding affinity for the target nucleic acid. An additional region of the iRNA can serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of iRNA inhibition of gene expression. Consequently, comparable results can often be obtained with shorter iRNAs when chimeric dsRNAs are used, compared to phosphorothioate deoxy dsRNAs hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

[0372] In certain instances, the RNA of an iRNA can be modified by a non-ligand group. A number of non-ligand molecules have been conjugated to iRNAs in order to enhance the activity, cellular distribution or cellular uptake of the iRNA, and procedures for performing such conjugations are available in the scientific literature. Such non-ligand moieties have included lipid moieties, such as cholesterol (Kubo, T. et al., *Biochem. Biophys. Res. Comm.*, 2007, 365(1):54-61; Letsinger et al., *Proc. Natl. Acad. Sci. USA*, 1989, 86:6553), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1994, 4:1053), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, 1992, 660:306; Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1993, 3:2765), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, 1992, 20:533), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, 1991, 10:111; Kabanov et al., *FEBS Lett.*, 1990, 259:327; Svinarchuk et al., *Biochimie*, 1993, 75:49), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, 1995, 36:3651; Shea et al., *Nucl. Acids Res.*, 1990, 18:3777), a polyamine or a

polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, 1995, 14:969), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, 1995, 36:3651), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, 1995, 1264:229), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, 1996, 277:923).

Representative United States patents that teach the preparation of such RNA conjugates have been listed above. Typical conjugation protocols involve the synthesis of RNAs bearing an aminolinker at one or more positions of the sequence. The amino group is then reacted with the molecule being conjugated using appropriate coupling or activating reagents. The conjugation reaction can be performed either with the RNA still bound to the solid support or following cleavage of the RNA, in solution phase. Purification of the RNA conjugate by HPLC typically affords the pure conjugate.

IV. Delivery of an iRNA of the Invention

[0373] The delivery of an iRNA of the invention to a cell e.g., a cell within a subject, such as a human subject (e.g., a subject in need thereof, such as a subject having a disease, disorder, or condition associated with KHK gene expression) can be achieved in a number of different ways. For example, delivery may be performed by contacting a cell with an iRNA of the invention either in vitro or in vivo. In vivo delivery may also be performed directly by administering a composition comprising an iRNA, e.g., a dsRNA, to a subject. Alternatively, in vivo delivery may be performed indirectly by administering one or more vectors that encode and direct the expression of the iRNA.

[0374] These alternatives are discussed further below.

[0375] In general, any method of delivering a nucleic acid molecule (in vitro or in vivo) can be adapted for use with an iRNA of the invention (see e.g., Akhtar S. and Julian R L. (1992) *Trends Cell. Biol.* 2(5):139-144 and WO94/02595, which are incorporated herein by reference in their entireties).

[0376] For in vivo delivery, factors to consider in order to deliver an iRNA molecule include, for example, biological stability of the delivered molecule, prevention of non-specific effects, and accumulation of the delivered molecule in the target tissue. The non-specific effects of an iRNA can be minimized by local administration, for example, by direct injection or implantation into a tissue or topically administering the preparation. Local administration to a treatment site maximizes local concentration of the agent, limits the exposure of the agent to systemic tissues that can otherwise be harmed by the agent or that can degrade the agent, and permits a lower total dose of the iRNA molecule to be administered. Several studies have shown successful knockdown of gene products when a dsRNAi agent is administered locally. For example, intraocular delivery of a VEGF dsRNA by intravitreal injection in cynomolgus monkeys (Tolentino, M J, et al (2004) *Retina* 24:132-138) and subretinal injections in mice (Reich, S J., et al (2003) *Mol. Vis.* 9:210-216) were both shown to prevent neovascularization in an experimental model of age-related macular degeneration. In addition, direct intratumoral injection of a dsRNA in mice reduces tumor volume (Pille, J., et al (2005) *Mol. Ther.* 11:267-274) and can prolong survival of tumor-bearing mice (Kim, W J., et al (2006) *Mol. Ther.* 14:343-350; Li, S., et al (2007) *Mol. Ther.* 15:515-523). RNA interference has also shown success with local delivery to the CNS by direct injection (Dorn, G., et al. (2004) *Nucleic Acids* 32:e49; Tan, P H., et al (2005) *Gene Ther.* 12:59-66; Makimura, H., et al (2002) *BMC Neurosci.* 3:18; Shishkina, G T., et al (2004) *Neuroscience* 129:521-528; Thakker, E R., et al (2004) *Proc. Natl. Acad. Sci. U.S.A.* 101:17270-17275; Akaneya, Y., et al (2005) *J. Neurophysiol.* 93:594-602) and to the lungs by intranasal administration (Howard, K A., et al (2006) *Mol. Ther.* 14:476-484; Zhang, X., et al (2004) *J. Biol. Chem.* 279:10677-10684; Bitko, V., et al (2005) *Nat. Med.* 11:50-55). For administering an iRNA systemically for the treatment of a disease, the RNA can be modified or alternatively delivered using a drug delivery system; both methods act to prevent the rapid degradation of the dsRNA by endo- and exo-nucleases in vivo. Modification of the RNA or the pharmaceutical carrier can also permit targeting of the iRNA to the target tissue and avoid undesirable off-target effects. iRNA molecules can be modified by chemical conjugation to lipophilic groups such as cholesterol to enhance cellular uptake and prevent

degradation. For example, an iRNA directed against ApoB conjugated to a lipophilic cholesterol moiety was injected systemically into mice and resulted in knockdown of apoB mRNA in both the liver and jejunum (Soutschek, J., et al (2004) *Nature* 432:173-178). Conjugation of an iRNA to an aptamer has been shown to inhibit tumor growth and mediate tumor regression in a mouse model of prostate cancer (McNamara, J O, et al (2006) *Nat. Biotechnol.* 24:1005-1015). In an alternative embodiment, the iRNA can be delivered using drug delivery systems such as a nanoparticle, a dendrimer, a polymer, liposomes, or a cationic delivery system. Positively charged cationic delivery systems facilitate binding of an iRNA molecule (negatively charged) and also enhance interactions at the negatively charged cell membrane to permit efficient uptake of an iRNA by the cell. Cationic lipids, dendrimers, or polymers can either be bound to an iRNA, or induced to form a vesicle or micelle (see e.g., Kim S H, et al (2008) *Journal of Controlled Release* 129(2):107-116) that encases an iRNA. The formation of vesicles or micelles further prevents degradation of the iRNA when administered systemically. Methods for making and administering cationic-iRNA complexes are well within the abilities of one skilled in the art (see e.g., Sorensen, D R, et al (2003) *J. Mol. Biol* 327:761-766; Verma, U N, et al (2003) *Clin. Cancer Res.* 9:1291-1300; Arnold, A S et al (2007) *J. Hypertens.* 25:197-205, which are incorporated herein by reference in their entirety). Some non-limiting examples of drug delivery systems useful for systemic delivery of iRNAs include DOTAP (Sorensen, D R., et al (2003), supra; Verma, U N, et al (2003), supra), Oligofectamine, "solid nucleic acid lipid particles" (Zimmermann, T S, et al (2006) *Nature* 441:111-114), cardiolipin (Chien, P Y, et al (2005) *Cancer Gene Ther.* 12:321-328; Pal, A, et al (2005) *Int J. Oncol.* 26:1087-1091), polyethyleneimine (Bonnet M E, et al (2008) *Pharm. Res.* August 16 Epub ahead of print; Aigner, A. (2006) *J. Biomed. Biotechnol.* 71659), Arg-Gly-Asp (RGD) peptides (Liu, S. (2006) *Mol. Pharm.* 3:472-487), and polyamidoamines (Tomalia, D A, et al (2007) *Biochem. Soc. Trans.* 35:61-67; Yoo, H., et al (1999) *Pharm. Res.* 16:1799-1804). In some embodiments, an iRNA forms a complex with cyclodextrin for systemic administration. Methods for administration and pharmaceutical compositions of iRNAs and cyclodextrins can be found in U.S. Pat. No. 7,427,605, which is herein incorporated by reference in its entirety.

A. Vector Encoded iRNAs of the Invention

[0377] iRNA targeting the KHK gene can be expressed from transcription units inserted into DNA or RNA vectors (see, e.g., Couture, A, et al., *TIG.* (1996), 12:5-10; Skillern, A, et al., International PCT Publication No. WO 00/22113, Conrad, International PCT Publication No. WO 00/22114, and Conrad, U.S. Pat. No. 6,054,299). Expression can be transient (on the order of hours to weeks) or sustained (weeks to months or longer), depending upon the specific construct used and the target tissue or cell type. These transgenes can be introduced as a linear construct, a circular plasmid, or a viral vector, which can be an integrating or non-integrating vector. The transgene can also be constructed to permit it to be inherited as an extrachromosomal plasmid (Gassmann, et al., *Proc. Natl. Acad. Sci. USA* (1995) 92:1292).

[0378] The individual strand or strands of an iRNA can be transcribed from a promoter on an expression vector. Where two separate strands are to be expressed to generate, for example, a dsRNA, two separate expression vectors can be co-introduced (e.g., by transfection or infection) into a target cell. Alternatively each individual strand of a dsRNA can be transcribed by promoters both of which are located on the same expression plasmid. In one embodiment, a dsRNA is expressed as inverted repeat polynucleotides joined by a linker polynucleotide sequence such that the dsRNA has a stem and loop structure.

[0379] iRNA expression vectors are generally DNA plasmids or viral vectors. Expression vectors compatible with eukaryotic cells, preferably those compatible with vertebrate cells, can be used to produce recombinant constructs for the expression of an iRNA as described herein. Eukaryotic cell expression vectors are well known in the art and are available from a number of commercial sources. Typically, such vectors are provided containing convenient restriction sites for insertion of the desired nucleic acid segment. Delivery of iRNA expressing vectors can be systemic, such as by

intravenous or intramuscular administration, by administration to target cells ex-planted from the patient followed by reintroduction into the patient, or by any other means that allows for introduction into a desired target cell.

[0380] Viral vector systems which can be utilized with the methods and compositions described herein include, but are not limited to, (a) adenovirus vectors; (b) retrovirus vectors, including but not limited to lentiviral vectors, moloney murine leukemia virus, etc.; (c) adeno-associated virus vectors; (d) herpes simplex virus vectors; (e) SV 40 vectors; (f) polyoma virus vectors; (g) papilloma virus vectors; (h) picornavirus vectors; (i) pox virus vectors such as an orthopox, e.g., vaccinia virus vectors or avipox, e.g. canary pox or fowl pox; and (j) a helper-dependent or gutless adenovirus. Replication-defective viruses can also be advantageous. Different vectors will or will not become incorporated into the cells' genome. The constructs can include viral sequences for transfection, if desired. Alternatively, the construct can be incorporated into vectors capable of episomal replication, e.g. EPV and EBV vectors. Constructs for the recombinant expression of an iRNA will generally require regulatory elements, e.g., promoters, enhancers, etc., to ensure the expression of the iRNA in target cells. Other aspects to consider for vectors and constructs are known in the art.

V. Pharmaceutical Compositions of the Invention

[0381] The present invention also includes pharmaceutical compositions and formulations which include the iRNAs of the invention. In one embodiment, provided herein are pharmaceutical compositions containing an iRNA, as described herein, and a pharmaceutically acceptable carrier.

[0382] The pharmaceutical compositions containing the iRNA are useful for treating a disease or disorder associated with the expression or activity of a KHK gene. Such pharmaceutical compositions are formulated based on the mode of delivery. One example is compositions that are formulated for systemic administration via parenteral delivery, e.g., by subcutaneous (SC) or intravenous (IV) delivery. The pharmaceutical compositions of the invention may be administered in dosages sufficient to inhibit expression of a KHK gene.

[0383] The pharmaceutical compositions of the invention may be administered in dosages sufficient to inhibit expression of a KHK gene. In general, a suitable dose of an iRNA of the invention will be in the range of about 0.001 to about 200.0 milligrams per kilogram body weight of the recipient per day, generally in the range of about 1 to 50 mg per kilogram body weight per day. Typically, a suitable dose of an iRNA of the invention will be in the range of about 0.1 mg/kg to about 5.0 mg/kg, preferably about 0.3 mg/kg and about 3.0 mg/kg. A repeat-dose regimen may include administration of a therapeutic amount of iRNA on a regular basis, such as every other day or once a year. In certain embodiments, the iRNA is administered about once per month to about once per quarter (i.e., about once every three months).

[0384] After an initial treatment regimen, the treatments can be administered on a less frequent basis. For example, after administration weekly or biweekly for three months, administration can be repeated once per month, for six months, or a year; or longer.

[0385] The pharmaceutical composition can be administered once daily, or the iRNA can be administered as two, three, or more sub-doses at appropriate intervals throughout the day or even using continuous infusion or delivery through a controlled release formulation. In that case, the iRNA contained in each sub-dose must be correspondingly smaller in order to achieve the total daily dosage. The dosage unit can also be compounded for delivery over several days, e.g., using a conventional sustained release formulation which provides sustained release of the iRNA over a several day period. Sustained release formulations are well known in the art and are particularly useful for delivery of agents at a particular site, such as could be used with the agents of the present invention. In this embodiment, the dosage unit contains a corresponding multiple of the daily dose.

[0386] In other embodiments, a single dose of the pharmaceutical compositions can be long lasting, such that subsequent doses are administered at not more than 3, 4, or 5 day intervals, or at not more than 1, 2, 3, or 4 week intervals. In some embodiments of the invention, a single dose of the

pharmaceutical compositions of the invention is administered once per week. In other embodiments of the invention, a single dose of the pharmaceutical compositions of the invention is administered bi-monthly.

[0387] The skilled artisan will appreciate that certain factors can influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of a composition can include a single treatment or a series of treatments. Estimates of effective dosages and in vivo half-lives for the individual iRNAs encompassed by the invention can be made using conventional methodologies or on the basis of in vivo testing using an appropriate animal model, as known in the art. Appropriate animal models for various diseases and conditions are provided herein.

[0388] The pharmaceutical compositions of the present invention can be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration can be topical (e.g., by a transdermal patch), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal, oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal, or intramuscular injection or infusion; subdermal, e.g., via an implanted device; or intracranial, e.g., by intraparenchymal, intrathecal or intraventricular administration.

[0389] The iRNA can be delivered in a manner to target a particular tissue (e.g., vascular endothelial cells).

[0390] Pharmaceutical compositions and formulations for topical or transdermal administration can 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 can be necessary or desirable. Coated condoms, gloves and the like can also be useful. Suitable topical formulations include those in which the iRNAs featured in the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Suitable lipids and liposomes include neutral (e.g., dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearoylphosphatidyl choline) negative (e.g., dimyristoylphosphatidyl glycerol DMPG) and cationic (e.g., dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA). iRNAs featured in the invention can be encapsulated within liposomes or can form complexes thereto, in particular to cationic liposomes. Alternatively, iRNAs can be complexed to lipids, in particular to cationic lipids. Suitable fatty acids and esters include but are not limited to arachidonic acid, oleic acid, eicosanoic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprinate, tricaprinate, monoolein, dilaurin, glyceryl 1-monocaprinate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C.sub.1-20 alkyl ester (e.g., isopropylmyristate IPM), monoglyceride, diglyceride or pharmaceutically acceptable salt thereof). Topical formulations are described in detail in U.S. Pat. No. 6,747,014, which is incorporated herein by reference.

A. iRNA Formulations Comprising Membranous Molecular Assemblies

[0391] An iRNA for use in the compositions and methods of the invention 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 iRNA. The lipophilic material isolates the aqueous interior from an aqueous exterior, which typically does not include the iRNA 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 iRNA are delivered into the cell where the iRNA can specifically bind to a target RNA and can mediate RNA interference. In some cases the liposomes are also specifically targeted, e.g., to direct the iRNA to particular cell types.

[0392] A liposome containing an iRNA agent 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 iRNA agent preparation is then added to the micelles that include the lipid component. The cationic groups on the lipid interact with the iRNA agent and condense around the iRNA agent to form a liposome. After condensation, the detergent is removed, e.g., by dialysis, to yield a liposomal preparation of iRNA agent.

[0393] 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 adjusted to favor condensation.

[0394] Methods for producing stable polynucleotide delivery vehicles, which incorporate a polynucleotide/cationic lipid complex as structural components of the delivery vehicle, are further described in, e.g., WO 96/37194, the entire contents of which are incorporated herein by reference. 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. Nos. 4,897,355; 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 iRNA agent preparations into liposomes.

[0395] Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged nucleic acid molecules to form a stable complex. The positively charged nucleic acid/liposome complex binds to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang et al., *Biochem. Biophys. Res. Commun.*, 1987, 147, 980-985).

[0396] Liposomes which are pH-sensitive or negatively-charged, entrap nucleic acids rather than complex with it. Since both the nucleic acid and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some nucleic acid is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver nucleic acids 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*, 1992, 19, 269-274).

[0397] 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 two or more of phospholipid, phosphatidylcholine, and cholesterol.

[0398] Examples of other methods to introduce liposomes into cells in vitro and in vivo include U.S. Pat. Nos. 5,283,185 and 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.

[0399] 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 Novasomer™ II (glyceryl distearate/cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver cyclosporin-A into the dermis of mouse skin. Results indicated that such non-ionic liposomal systems were effective in facilitating the deposition of cyclosporine A into different layers of the skin (Hu et al. *S.T.P. Pharma. Sci.*, 1994, 4(6) 466).

[0400] Liposomes also include “sterically stabilized” liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside G.sub.M1, or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of the reticuloendothelial system (RES) (Allen et al., *FEBS Letters*, 1987, 223, 42; Wu et al., *Cancer Research*, 1993, 53, 3765).

[0401] Various liposomes comprising one or more glycolipids are known in the art. Papahadjopoulos et al. (*Ann. N.Y. Acad. Sci.*, 1987, 507, 64) reported the ability of monosialoganglioside G.sub.M1, galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were expounded upon by Gabizon et al. (*Proc. Natl. Acad. Sci. U.S.A.*, 1988, 85, 6949). U.S. Pat. No. 4,837,028 and WO 88/04924, both to Allen et al., disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside GmI or a galactocerebroside sulfate ester. U.S. Pat. No. 5,543,152 (Webb et al.) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-sn-dimyristoylphosphatidylcholine are disclosed in WO 97/13499 (Lim et al.).

[0402] In some embodiments, 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 iRNA agents to macrophages.

[0403] 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 iRNAs 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.

[0404] A positively charged synthetic cationic lipid, N-[1-(2,3-dioleoyloxy)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 iRNA agent (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).

[0405] 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.

[0406] 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 dioctaoleoylamide ("DOGS") (Transfectam™, Promega, Madison, Wisconsin) and dipalmitoylphosphatidylethanolamine 5-carboxyspermyl-amide ("DPPES") (see, e.g., U.S. Pat. No. 5,171,678).

[0407] 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.

[0408] 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 iRNA agent into the skin. In some implementations, liposomes are used for delivering iRNA agent to epidermal cells and also to enhance the penetration of iRNA agent 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).

[0409] 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 iRNA agent are useful for treating a dermatological disorder.

[0410] Liposomes that include iRNA 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 iRNAs can be delivered, for example, subcutaneously by infection in order to deliver iRNAs 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.

[0411] Other formulations amenable to the present invention are described in WO/2008/042973.

[0412] Transfersomes are yet another type of liposomes, and are highly deformable lipid aggregates which are attractive candidates for drug delivery vehicles. Transfersomes can be described as lipid droplets which are so highly deformable that they are easily able to penetrate through pores which are smaller than the droplet. Transfersomes are adaptable to the environment in which they are used, e.g., they are self-optimizing (adaptive to the shape of pores in the skin), self-repairing, frequently reach their targets without fragmenting, and often self-loading. To make transfersomes it is possible to add surface edge-activators, usually surfactants, to a standard liposomal composition. Transfersomes have been used to deliver serum albumin to the skin. The transfersome-mediated delivery of serum albumin has been shown to be as effective as subcutaneous injection of a solution containing serum albumin.

[0413] Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes. 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 (also known as the “head”) provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in “Pharmaceutical Dosage Forms”, Marcel Dekker, Inc., New York, N.Y., 1988, p. 285).

[0414] If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic 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.

[0415] 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.

[0416] 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.

[0417] 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.

[0418] The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in “Pharmaceutical Dosage Forms”, Marcel Dekker, Inc., New York, N.Y., 1988, p. 285).

[0419] The iRNA for use in the methods of the invention can also be provided as micellar

formulations. "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. [0420] A mixed micellar formulation suitable for delivery through transdermal membranes may be prepared by mixing an aqueous solution of iRNA, an alkali metal C₈ to C_n 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.

[0421] In one method a first micellar composition is prepared which contains the RNAi 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 RNAi, 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.

[0422] Phenol or m-cresol may be added to the mixed micellar composition to stabilize the formulation and protect against bacterial growth. Alternatively, phenol 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.

[0423] 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.

[0424] 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.

[0425] 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.

B. Lipid Particles

[0426] iRNAs, e.g., dsRNAi agents of in the invention may be fully encapsulated in a lipid formulation, e.g., a LNP, or other nucleic acid-lipid particle.

[0427] As used herein, the term "LNP" refers to a stable nucleic acid-lipid particle. LNPs typically contain a cationic lipid, a non-cationic lipid, and a lipid that prevents aggregation of the particle (e.g., a PEG-lipid conjugate). LNPs are extremely useful for systemic applications, as they exhibit extended circulation lifetimes following intravenous (i.v.) injection and accumulate at distal sites (e.g., sites physically separated from the administration site). LNPs include "pSPLP," which include an encapsulated condensing agent-nucleic acid complex as set forth in PCT Publication No. WO 00/03683. The particles of the present invention typically have a mean diameter of about 50

nm to about 150 nm, more typically about 60 nm to about 130 nm, more typically about 70 nm to about 110 nm, most typically about 70 nm to about 90 nm, and are substantially nontoxic. In addition, the nucleic acids when present in the nucleic acid-lipid particles of the present invention are resistant in aqueous solution to degradation with a nuclease. Nucleic acid-lipid particles and their method of preparation are disclosed in, e.g., U.S. Pat. Nos. 5,976,567; 5,981,501; 6,534,484; 6,586,410; 6,815,432; US Publication No. 2010/0324120 and PCT Publication No. WO 96/40964. [0428] In one embodiment, the lipid to drug ratio (mass/mass ratio) (e.g., lipid to dsRNA ratio) will be in the range of from about 1:1 to about 50:1, from about 1:1 to about 25:1, from about 3:1 to about 15:1, from about 4:1 to about 10:1, from about 5:1 to about 9:1, or about 6:1 to about 9:1. Ranges intermediate to the above recited ranges are also contemplated to be part of the invention. [0429] The cationic lipid can be, for example, N,N-dioleoyl-N,N-dimethylammonium chloride (DODAC), N,N-distearoyl-N,N-dimethylammonium bromide (DDAB), N—(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), N—(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-dimethyl-2,3-dioleoyloxypropylamine (DODMA), 1,2-DiLinoleoyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), 1,2-Dilinoleylcarbamoxyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-Dilinoleoyloxy-3-(dimethylamino)acetoxyp propane (DLin-DAC), 1,2-Dilinoleoyloxy-3-morpholinopropane (DLin-MA), 1,2-Dilinoleoyl-3-dimethylaminopropane (DLinDAP), 1,2-Dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), 1-Linoleoyl-2-linoleoyloxy-3-dimethylaminopropane (DLin-2-DMAP), 1,2-Dilinoleoyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-Dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), 1,2-Dilinoleoyloxy-3-(N-methylpiperazino)propane (DLin-MPZ), or 3-(N,N-Dilinoleylamino)-1,2-propanediol (DLinAP), 3-(N,N-Dioleylamino)-1,2-propanedio (DOAP), 1,2-Dilinoleoyloxy-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), 1,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLinDMA), 2,2-Dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA) or analogs thereof, (3aR,5s,6aS)—N,N-dimethyl-2,2-di((9Z,12Z)-octadeca-9,12-dienyl)tetrahydro-3aH-cyclopenta[d][1,3]dioxol-5-amine (ALN100), (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (MC3), 1,1'-(2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl)(2-hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethylazanediy)l)didodecan-2-ol (Tech G1), or a mixture thereof. The cationic lipid can comprise from about 20 mol % to about 50 mol % or about 40 mol % of the total lipid present in the particle. [0430] In some embodiments, the compound 2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane can be used to prepare lipid-siRNA nanoparticles.

[0431] In some embodiments, the lipid-siRNA particle includes 40% 2, 2-Dilinoleyl 4-dimethylaminoethyl-[1,3]-dioxolane: 10% DSPC: 40% Cholesterol: 10% PEG-C-DOMG (mole percent) with a particle size of 63.0 ± 20 nm and a 0.027 siRNA/Lipid Ratio.

[0432] The ionizable/non-cationic lipid can be an anionic lipid or a neutral lipid including, but not limited to, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoyl-phosphatidylethanolamine (DOPE), palmitoyl-oleoylphosphatidylcholine (POPC), palmitoyl-oleoylphosphatidylethanolamine (POPE), dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidyl-ethanolamine (DSPE), 16-O-monomethyl PE, 16-O-dimethyl PE, 18-1-trans PE, 1-stearoyl-2-oleoyl-phosphatidylethanolamine (SOPE), cholesterol, or a mixture thereof. The non-cationic lipid can be from about 5 mol % to about 90 mol %, about 10 mol %, or about 58 mol % if cholesterol is included, of the total lipid present in the particle.

[0433] The conjugated lipid that inhibits aggregation of particles can be, for example, a polyethyleneglycol (PEG)-lipid including, without limitation, a PEG-diacylglycerol (DAG), a PEG-dialkyloxypropyl (DAA), a PEG-phospholipid, a PEG-ceramide (Cer), or a mixture thereof.

The PEG-DAA conjugate can be, for example, a PEG-dilauryloxypropyl (Ci.sub.2), a PEG-dimyristyloxypropyl (Ci.sub.4), a PEG-dipalmitoyloxypropyl (Ci.sub.6), or a PEG-distearoyloxypropyl (C)s). The conjugated lipid that prevents aggregation of particles can be from 0 mol % to about 20 mol % or about 2 mol % of the total lipid present in the particle.

[0434] In some embodiments, the nucleic acid-lipid particle further includes cholesterol at, e.g., about 10 mol % to about 60 mol % or about 48 mol % of the total lipid present in the particle.

[0435] In one embodiment, the lipidoid ND98.Math.4HCl (MW 1487) (see U.S. patent application Ser. No. 12/056,230, filed Mar. 26, 2008, which is incorporated herein by reference), Cholesterol (Sigma-Aldrich), and PEG-Ceramide C16 (Avanti Polar Lipids) can be used to prepare lipid-dsRNA nanoparticles (i.e., LNP01 particles). Stock solutions of each in ethanol can be prepared as follows: ND98, 133 mg/ml; Cholesterol, 25 mg/ml, PEG-Ceramide C16, 100 mg/ml. The ND98, Cholesterol, and PEG-Ceramide C16 stock solutions can then be combined in a, e.g., 42:48:10 molar ratio. The combined lipid solution can be mixed with aqueous dsRNA (e.g., in sodium acetate pH 5) such that the final ethanol concentration is about 35-45% and the final sodium acetate concentration is about 100-300 mM. Lipid-dsRNA nanoparticles typically form spontaneously upon mixing. Depending on the desired particle size distribution, the resultant nanoparticle mixture can be extruded through a polycarbonate membrane (e.g., 100 nm cut-off) using, for example, a thermobarrel extruder, such as Lipex Extruder (Northern Lipids, Inc). In some cases, the extrusion step can be omitted. Ethanol removal and simultaneous buffer exchange can be accomplished by, for example, dialysis or tangential flow filtration. Buffer can be exchanged with, for example, phosphate buffered saline (PBS) at about pH 7, e.g., about pH 6.9, about pH 7.0, about pH 7.1, about pH 7.2, about pH 7.3, or about pH 7.4.

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[0436] LNP01 formulations are described, e.g., in International Application Publication No. WO 20081042973, which is hereby incorporated by reference.

[0437] Additional exemplary lipid-dsRNA formulations are described in Table 1.

TABLE-US-00001 TABLE 1 cationic lipid/non-cationic lipid/ cholesterol/PEG-lipid conjugate

Ionizable/Cationic Lipid	Lipid:siRNA ratio	SNALP-1	1,2-Dilinolenyloxy-N,N-DLinDMA/DPPC/Cholesterol/PEG-cDMA	dimethylaminopropane (DLinDMA)
(57.1/7.1/34.4/1.4)	lipid:siRNA~7:1	2-XTC	2,2-Dilinoleyl-4-dimethylaminoethyl-XTC/DPPC/Cholesterol/PEG-cDMA	[1,3]-dioxolane (XTC) 57.1/7.1/34.4/1.4 lipid:siRNA~7:1
LNP05	2,2-Dilinoleyl-4-dimethylaminoethyl-XTC/DSPC/Cholesterol/PEG-DMG	[1,3]-dioxolane (XTC) 57.5/7.5/31.5/3.5	lipid:siRNA~6:1	LNP06 2,2-Dilinoleyl-4-dimethylaminoethyl-XTC/DSPC/Cholesterol/PEG-DMG [1,3]-dioxolane (XTC) 57.5/7.5/31.5/3.5 lipid:siRNA~11:1
LNP07	2,2-Dilinoleyl-4-dimethylaminoethyl-XTC/DSPC/Cholesterol/PEG-DMG	[1,3]-dioxolane (XTC) 60/7.5/31/1.5,	lipid:siRNA~6:1	LNP08 2,2-Dilinoleyl-4-dimethylaminoethyl-XTC/DSPC/Cholesterol/PEG-DMG [1,3]-dioxolane (XTC) 60/7.5/31/1.5, lipid:siRNA~11:1
LNP09	2,2-Dilinoleyl-4-dimethylaminoethyl-XTC/DSPC/Cholesterol/PEG-DMG	[1,3]-dioxolane (XTC) 50/10/38.5/1.5	Lipid:siRNA 10:1	LNP10 (3aR,5s,6aS)-N,N-dimethyl-2,2-ALN100/DSPC/Cholesterol/PEG-DMG di((9Z,12Z)-octadeca-9,12-
	dienyl)tetrahydro-3aH- Lipid:siRNA 10:1	cyclopenta[d][1,3]dioxol-5-amine (ALN100)	LNP11 (6Z,9Z,28Z,31Z)-heptatriaconta-	MC-3/DSPC/Cholesterol/PEG-DMG 6,9,28,31-tetraen-19-yl 4-
	50/10/38.5/1.5 (dimethylamino)butanoate (MC3)	Lipid:siRNA 10:1	LNP12 1,1'-(2-(4-(2-((2-(bis(2- Tech G1/DSPC/Cholesterol/PEG-DMG hydroxydodecyl)amino)ethyl)(2-	50/10/38.5/1.5 hydroxydodecyl)amino)ethyl)piperazin- Lipid:siRNA 10:1
	1-yl)ethylazanediyldidodecan-2-ol (Tech G1)	LNP13 XTC XTC/DSPC/Chol/PEG-DMG 50/10/38.5/1.5	Lipid:siRNA: 33:1	LNP14 MC3 MC3/DSPC/Chol/PEG-DMG 40/15/40/5 Lipid:siRNA: 11:1
	LNP15 MC3 MC3/DSPC/Chol/PEG-DSG/GalNAc-PEG-DSG 50/10/35/4.5/0.5	Lipid:siRNA: 11:1	LNP16 MC3 MC3/DSPC/Chol/PEG-DMG 50/10/38.5/1.5	Lipid:siRNA: 7:1
	LNP17 MC3 MC3/DSPC7Chol/PEG-DSG 50/10/38.5/1.5	Lipid:siRNA: 10:1	LNP18 MC3	

MC3/DSPC/Chol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA: 12:1 LNP19 MC3 MC3/DSPC7Chol/PEG-DPG 50/10/38.5/1.5 Lipid:siRNA: 10:1 LNP21 C12-200 C12-200/DSPC/Chol/PEG-DSG 50/10/38.5/1.5 Lipid:siRNA: 7:1 LNP22 XTC XTC/DSPC/Chol/PEG-DSG 50/10/38.5/1.5 Lipid:siRNA: 10:1 DSPC: distearoylphosphatidylcholine DPPC: dipalmitoylphosphatidylcholine PEG-DMG: PEG-didimyristoyl glycerol (C14-PEG, or PEG-C14) (PEG with avg mol wt of 2000) PEG-DSG: PEG-distyryl glycerol (C18-PEG, or PEG-C18) (PEG with avg mol wt of 2000) PEG-cDMA: PEG-carbamoyl-1,2-dimyristyloxypropylamine (PEG with avg mol wt of 2000) SNALP (1,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLinDMA)) comprising formulations are described in International Publication No. WO2009/127060, filed Apr. 15, 2009, which is hereby incorporated by reference. XTC comprising formulations are described, e.g., in International Application No. PCT/US2010/022614, filed Jan. 29, 2010, which is hereby incorporated by reference. MC3 comprising formulations are described, e.g., in US Patent Publication No. 2010/0324120, filed Jun. 10, 2010, the entire contents of which are hereby incorporated by reference. ALNY-100 comprising formulations are described, e.g., International patent application number PCT/US09/63933, filed on Nov. 10, 2009, which is hereby incorporated by reference. C12-200 comprising formulations are described in WO2010/129709, which is hereby incorporated by reference.

[0438] Compositions and formulations for oral administration include powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitables. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids, or binders can be desirable. In some embodiments, oral formulations are those in which dsRNAs featured in the invention are administered in conjunction with one or more penetration enhancer surfactants and chelators. Suitable surfactants include fatty acids or esters or salts thereof, bile acids or salts thereof. Suitable bile acids/salts include chenodeoxycholic acid (CDCA) and ursodeoxychenodeoxycholic acid (UDCA), cholic acid, dehydrocholic acid, deoxycholic acid, glucolic acid, glycholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, sodium tauro-24,25-dihydro-fusidate and sodium glycodihydrofusidate. Suitable fatty acids include arachidonic acid, undecanoic acid, oleic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a monoglyceride, a diglyceride or a pharmaceutically acceptable salt thereof (e.g., sodium). In some embodiments, combinations of penetration enhancers are used, for example, fatty acids/salts in combination with bile acids/salts. One exemplary combination is the sodium salt of lauric acid, capric acid and UDCA. Further penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether. DsRNAs featured in the invention can be delivered orally, in granular form including sprayed dried particles, or complexed to form micro or nanoparticles. DsRNA complexing agents include poly-amino acids; polyimines; polyacrylates; polyalkylacrylates, polyoxethanes, polyalkylcyanoacrylates; cationized gelatins, albumins, starches, acrylates, polyethyleneglycols (PEG), and starches; polyalkylcyanoacrylates; DEAE-derivatized polyimines, pollulans, celluloses, and starches. Suitable complexing agents include chitosan, N-trimethylchitosan, poly-L-lysine, polyhistidine, polyornithine, polyspermines, protamine, polyvinylpyridine, polythiodiethylaminomethylethylene P(TDAE), polyaminostyrene (e.g., p-amino), poly(methylcyanoacrylate), poly(ethylcyanoacrylate), poly(butylcyanoacrylate), poly(isobutylcyanoacrylate), poly(isohexylcynaoacrylate), DEAE-methacrylate, DEAE-hexylacrylate, DEAE-acrylamide, DEAE-albumin and DEAE-dextran, polymethylacrylate, polyhexylacrylate, poly(D,L-lactic acid), poly(DL-lactic-co-glycolic acid (PLGA), alginate, and polyethyleneglycol (PEG). Oral formulations for dsRNAs and their preparation are described in detail in U.S. Pat. No. 6,887,906, US Publn. No. 20030027780, and U.S. Pat. No. 6,747,014, each of which is incorporated herein by reference.

[0439] Compositions and formulations for parenteral, intraparenchymal (into the brain), intrathecal, intraventricular or intrahepatic administration can include sterile aqueous solutions which can also contain buffers, diluents, and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds, and other pharmaceutically acceptable carriers or excipients.

[0440] Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions can be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids, and self-emulsifying semisolids. Formulations include those that target the liver when treating hepatic disorders such as hepatic carcinoma.

[0441] The pharmaceutical formulations of the present invention, which can conveniently be presented in unit dosage form, can be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

[0442] The compositions of the present invention can be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention can also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions can further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol or dextran. The suspension can also contain stabilizers.

C. Additional Formulations

i. Emulsions

[0443] The iRNAs of the present invention can be prepared and formulated as emulsions.

Emulsions are typically heterogeneous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 μm in diameter (see e.g., Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi et al., in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa., 1985, p. 301). Emulsions are often biphasic systems comprising two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions can be of either the water-in-oil (w/o) or the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase, the resulting composition is called a water-in-oil (w/o) emulsion.

Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase, the resulting composition is called an oil-in-water (ow) emulsion. Emulsions can contain additional components in addition to the dispersed phases, and the active drug which can be present as a solution either in the aqueous phase, oily phase or itself as a separate phase.

Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants can also be present in emulsions as needed. Pharmaceutical emulsions can also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous phase provides an o/w/o emulsion.

[0444] Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or

discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion can be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that can be incorporated into either phase of the emulsion. Emulsifiers can broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (see e.g., Ansel's *Pharmaceutical Dosage Forms and Drug Delivery Systems*, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

[0445] Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (see e.g., Ansel's *Pharmaceutical Dosage Forms and Drug Delivery Systems*, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc., New York, N.Y., 1988, volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants can be classified into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic, and amphoteric (see e.g., Ansel's *Pharmaceutical Dosage Forms and Drug Delivery Systems*, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

[0446] Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate, and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

[0447] A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives, and antioxidants (Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

[0448] Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

[0449] Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that can readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-

hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used can be free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

[0450] The application of emulsion formulations via dermatological, oral, and parenteral routes, and methods for their manufacture have been reviewed in the literature (see e.g., Ansel's *Pharmaceutical Dosage Forms and Drug Delivery Systems*, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of ease of formulation, as well as efficacy from an absorption and bioavailability standpoint (see e.g., Ansel's *Pharmaceutical Dosage Forms and Drug Delivery Systems*, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins, and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

ii. Microemulsions

[0451] In one embodiment of the present invention, the iRNAs are formulated as microemulsions. A microemulsion can be defined as a system of water, oil, and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (see e.g., Ansel's *Pharmaceutical Dosage Forms and Drug Delivery Systems*, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, in: *Controlled Release of Drugs: Polymers and Aggregate Systems*, Rosoff, M., Ed., 1989, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, Pa., 1985, p. 271).

[0452] The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (see e.g., Ansel's *Pharmaceutical Dosage Forms and Drug Delivery Systems*, Allen, L V., Popovich N G., and Ansel H C., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335). Compared to conventional emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

[0453] Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij® 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid

esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (M0310), hexaglycerol monooleate (P0310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (M0750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions can, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase can typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase can include, but is not limited to, materials such as Captex® 300, Captex® 355, Capmul® MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils, and silicone oil.

[0454] Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (see e.g., U.S. Pat. Nos. 6,191,105; 7,063,860; 7,070,802; 7,157,099; Constantinides et al., *Pharmaceutical Research*, 1994, 11, 1385-1390; Ritschel, *Meth. Find. Exp. Clin. Pharmacol.*, 1993, 13, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (see e.g., U.S. Pat. Nos. 6,191,105; 7,063,860; 7,070,802; 7,157,099; Constantinides et al., *Pharmaceutical Research*, 1994, 11, 1385; Ho et al., *J. Pharm. Sci.*, 1996, 85, 138-143). Often microemulsions can form spontaneously when their components are brought together at ambient temperature. This can be particularly advantageous when formulating thermolabile drugs, peptides or iRNAs. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of iRNAs and nucleic acids from the gastrointestinal tract, as well as improve the local cellular uptake of iRNAs and nucleic acids.

[0455] Microemulsions of the present invention can also contain additional components and additives such as sorbitan monostearate (Grill® 3), Labrasol®, and penetration enhancers to improve the properties of the formulation and to enhance the absorption of the iRNAs and nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present invention can be classified as belonging to one of five broad categories—surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p. 92). Each of these classes has been discussed above.

iii. Microparticles

[0456] An iRNA of the invention 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.

iv. Penetration Enhancers

[0457] In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly iRNAs, to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic drugs can cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration

enhancers also enhance the permeability of lipophilic drugs.

[0458] Penetration enhancers can be classified as belonging to one of five broad categories, i.e., surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (see e.g., Malmsten, M. *Surfactants and polymers in drug delivery*, Informa Health Care, New York, NY, 2002; Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p. 92). Such compounds are well known in the art.

v. Carriers

[0459] Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (i.e., does not possess biological activity per se) but is recognized as a nucleic acid by in vivo processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioate dsRNA in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid (Miyao et al., *DsRNA Res. Dev.*, 1995, 5, 115-121; Takakura et al., *DsRNA & Nucl. Acid Drug Dev.*, 1996, 6, 177-183).

vi. Excipients

[0460] In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent, or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient can be liquid or solid and is selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, etc., when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to, binding agents (e.g., pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, etc.); fillers (e.g., lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, etc.); lubricants (e.g., magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, etc.); disintegrants (e.g., starch, sodium starch glycolate, etc.); and wetting agents (e.g., sodium lauryl sulphate, etc.).

[0461] Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone, and the like.

[0462] Formulations for topical administration of nucleic acids can include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions can also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with nucleic acids can be used.

[0463] Suitable pharmaceutically acceptable excipients include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone, and the like.

vii. Other Components

[0464] The compositions of the present invention can additionally contain other adjunct

components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions can contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or can contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

[0465] Aqueous suspensions can contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol, or dextran. The suspension can also contain stabilizers.

[0466] In some embodiments, pharmaceutical compositions featured in the invention include (a) one or more iRNA and (b) one or more agents which function by a non-iRNA mechanism and which are useful in treating a KHK-associated disorder.

[0467] Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds that exhibit high therapeutic indices are preferred.

[0468] The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of compositions featured herein in the invention lies generally within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage can vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the methods featured in the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose can be formulated in animal models to achieve a circulating plasma concentration range of the compound or, when appropriate, of the polypeptide product of a target sequence (e.g., achieving a decreased concentration of the polypeptide) that includes the IC50 (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma can be measured, for example, by high performance liquid chromatography.

[0469] In addition to their administration, as discussed above, the iRNAs featured in the invention can be administered in combination with other known agents effective in treatment of pathological processes mediated by KHK expression. In any event, the administering physician can adjust the amount and timing of iRNA administration on the basis of results observed using standard measures of efficacy known in the art or described herein.

VI. Methods for Inhibiting KHK Expression

[0470] The present invention also provides methods of inhibiting expression of a KHK gene in a cell. The methods include contacting a cell with an RNAi agent, e.g., double stranded RNAi agent, in an amount effective to inhibit expression of KHK in the cell, thereby inhibiting expression of KHK in the cell.

[0471] Contacting of a cell with an iRNA, e.g., a double stranded RNAi agent, may be done in vitro or in vivo. Contacting a cell in vivo with the iRNA includes contacting a cell or group of cells within a subject, e.g., a human subject, with the iRNA. Combinations of in vitro and in vivo methods of contacting a cell are also possible. Contacting a cell may be direct or indirect, as discussed above. Furthermore, contacting a cell may be accomplished via a targeting ligand,

including any ligand described herein or known in the art. In preferred embodiments, the targeting ligand is a carbohydrate moiety, e.g., a GalNAc.sub.3 ligand, or any other ligand that directs the RNAi agent to a site of interest.

[0472] The term “inhibiting,” as used herein, is used interchangeably with “reducing,” “silencing,” “downregulating”, “suppressing”, and other similar terms, and includes any level of inhibition.

[0473] The phrase “inhibiting expression of a KHK” is intended to refer to inhibition of expression of any KHK gene (such as, e.g., a mouse KHK gene, a rat KHK gene, a monkey KHK gene, or a human KHK gene) as well as variants or mutants of a KHK gene. Thus, the KHK gene may be a wild-type KHK gene, a mutant KHK gene, or a transgenic KHK gene in the context of a genetically manipulated cell, group of cells, or organism.

[0474] “Inhibiting expression of a KHK gene” includes any level of inhibition of a KHK gene, e.g., at least partial suppression of the expression of a KHK gene. The expression of the KHK gene may be assessed based on the level, or the change in the level, of any variable associated with KHK gene expression, e.g., KHK mRNA level or KHK protein level. This level may be assessed in an individual cell or in a group of cells, including, for example, a sample derived from a subject. In certain embodiments, expression inhibition may be assessed in liver cells in a subject.

[0475] Inhibition may be assessed by a decrease in an absolute or relative level of one or more variables that are associated with KHK expression compared with a control level. The control level may be any type of control level that is utilized in the art, e.g., a pre-dose baseline level, or a level determined from a similar subject, cell, or sample that is untreated or treated with a control (such as, e.g., buffer only control or inactive agent control).

[0476] It is understood that the degree and duration of elevation of a sign of a KHK-associated disease will vary depending upon the sign. For example, lipid signs, e.g., fasting lipid levels, NAFLD, NASH, obesity; signs of liver and kidney function, and glucose or insulin response, are durable signs that will not vary in a clinically significant manner within a day or even within a week. Other markers, e.g., serum uric acid and glucose levels, and urine fructose levels, will vary within and likely between days. Blood pressure can be elevated transiently and durably in response to fructose. As fructose likely results in weight gain at least in part by reducing satiety, fructose consumption in conjunction with caloric limitation may not result in weight gain.

[0477] Further, depending on the disease state in the subject, as many as one third of adults and two thirds of children malabsorb fructose (Johnson et al. (2013) Diabetes. 62:3307-3315), e.g., due to variations in expression of the GLUT5 transporter in the gut. However, repeated exposure to fructose can increase fructose absorption. Fructose metabolism has demonstrated to be different depending on the source of fructose, e.g., in high fructose corn syrup vs. in natural fruit, and at high concentrations, such as those provided by soft drinks, glucose can be converted to fructose by the polyol pathway. However, fructose will have more metabolic effects than glucose. Body composition, e.g., lean body mass, has also been demonstrated to affect fructose metabolism. Therefore, both the timing of testing and controls must be carefully selected.

[0478] In some embodiments of the methods of the invention, expression of a KHK gene, preferably expression of the KHK gene in the liver, is inhibited by at least 20%, a 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%, or to below the level of detection of the assay as compared to an appropriate control. Further, it is understood that obtaining liver samples for monitoring expression levels is not routine in the art. Therefore, in certain embodiments, the level of KHK expression is inhibited sufficiently to provide a clinical benefit to the subject, e.g., by treating or preventing at least one sign or symptom of a KHK associated disease.

[0479] Inhibition of the expression of a KHK gene may be manifested by a reduction of the amount of mRNA expressed by a first cell or group of cells (such cells may be present, for example, in a sample derived from a subject) in which a KHK gene is transcribed and which has or have been treated (e.g., by contacting the cell or cells with an iRNA of the invention, or by administering an iRNA of the invention to a subject in which the cells are or were present) such that the expression

of a KHK 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 not or have not been so treated (control cell(s) not treated with an iRNA or not treated with an iRNA targeted to the gene of interest). In preferred embodiments, the inhibition is assessed by the method provided in Example 2 in the cell type listed wherein the RNAi agent is delivered at a 10 nM concentration using the method provided therein and expressing the level of mRNA in treated cells as a percentage of the level of mRNA in control cells assessed using the PCR method provided therein and calculated using the following formula:

$$[00001] \frac{(mRNA_{incontrolcells}) - (mRNA_{intreatedcells})}{(mRNA_{incontrolcells})} \cdot \text{Math. } 100\%$$

[0480] In other embodiments, inhibition of the expression of a KHK gene may be assessed in terms of a reduction of a parameter that is functionally linked to KHK gene expression, e.g., KHK protein expression or fructose metabolism. KHK gene silencing may be determined in any cell expressing KHK, either endogenous or heterologous from an expression construct, and by any assay known in the art.

[0481] Inhibition of the expression of a KHK protein may be manifested by a reduction in the level of the KHK protein that is expressed by a cell or group of cells (e.g., the level of protein expressed in a sample derived from a subject). As explained above, for the assessment of mRNA suppression, the inhibition of protein expression levels in a treated cell or group of cells may similarly be expressed as a percentage of the level of protein in a control cell or group of cells.

[0482] A control cell or group of cells that may be used to assess the inhibition of the expression of a KHK gene includes a cell or group of cells that has not yet been contacted with an RNAi agent of the invention. For example, the control cell or group of cells may be derived from an individual subject (e.g., a human or animal subject) prior to treatment of the subject with an RNAi agent.

[0483] In certain embodiments, the level of expression of KHK in a sample is determined by detecting a transcribed polynucleotide, or portion thereof, e.g., mRNA of the KHK gene. RNA may be extracted from cells using RNA extraction techniques including, for example, using acid phenol/guanidine isothiocyanate extraction (RNAzol B; Biogenesis), RNeasy™ RNA preparation kits (Qiagen®) or PAXgene® (PreAnalytix, Switzerland). Typical assay formats utilizing ribonucleic acid hybridization include nuclear run-on assays, RT-PCR, RNase protection assays, northern blotting, in situ hybridization, and microarray analysis.

[0484] In some embodiments, the level of expression of KHK is determined using a nucleic acid probe. The term “probe”, as used herein, refers to any molecule that is capable of selectively binding to a specific KHK. Probes can be synthesized by one of skill in the art, or derived from appropriate biological preparations. Probes may be specifically designed to be labeled. Examples of molecules that can be utilized as probes include, but are not limited to, RNA, DNA, proteins, antibodies, and organic molecules.

[0485] Isolated mRNA can be used in hybridization or amplification assays that include, but are not limited to, Southern or northern analyses, polymerase chain reaction (PCR) analyses and probe arrays. One method for the determination of mRNA levels involves contacting the isolated mRNA with a nucleic acid molecule (probe) that can hybridize to KHK mRNA. In one embodiment, the mRNA is immobilized on a solid surface and contacted with a probe, for example by running the isolated mRNA on an agarose gel and transferring the mRNA from the gel to a membrane, such as nitrocellulose. In an alternative embodiment, the probe(s) are immobilized on a solid surface and the mRNA is contacted with the probe(s), for example, in an Affymetrix® gene chip array. A skilled artisan can readily adapt known mRNA detection methods for use in determining the level of KHK mRNA.

[0486] An alternative method for determining the level of expression of KHK in a sample involves the process of nucleic acid amplification or reverse transcriptase (to prepare cDNA) of for example mRNA in the sample, e.g., by RT-PCR (the experimental embodiment set forth in Mullis, 1987, U.S. Pat. No. 4,683,202), ligase chain reaction (Barany (1991) *Proc. Natl. Acad. Sci. USA* 88:189-193), self sustained sequence replication (Guatelli et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:1874-

1878), transcriptional amplification system (Kwoh et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:1173-1177), Q-Beta Replicase (Lizardi et al. (1988) *Bio/Technology* 6:1197), rolling circle replication (Lizardi et al., U.S. Pat. No. 5,854,033) or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers. In particular aspects of the invention, the level of expression of KHK is determined by quantitative fluorogenic RT-PCR (i.e., the TaqMan™ System).

[0487] The expression levels of KHK mRNA may be monitored using a membrane blot (such as used in hybridization analysis such as northern, Southern, dot, and the like), or microwells, sample tubes, gels, beads or fibers (or any solid support comprising bound nucleic acids). See U.S. Pat. Nos. 5,770,722, 5,874,219, 5,744,305, 5,677,195 and 5,445,934, which are incorporated herein by reference. The determination of KHK expression level may also comprise using nucleic acid probes in solution.

[0488] In preferred embodiments, the level of mRNA expression is assessed using branched DNA (bDNA) assays or real time PCR (qPCR). The use of these methods and conditions described and exemplified in the Examples presented herein are preferred.

[0489] The level of KHK protein expression may be determined using any method known in the art for the measurement of protein levels. Such methods include, for example, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, fluid or gel precipitin reactions, absorption spectroscopy, a colorimetric assays, spectrophotometric assays, flow cytometry, immunodiffusion (single or double), immunoelectrophoresis, western blotting, radioimmunoassay (RIA), enzyme-linked immunosorbent assays (ELISAs), immunofluorescent assays, electrochemiluminescence assays, and the like.

[0490] In some embodiments, the efficacy of the methods of the invention in the treatment of a KHK-related disease is assessed by a decrease in KHK mRNA level (by liver biopsy) or KHK.

[0491] In some embodiments, the efficacy of the methods of the invention in the treatment of KHK-associated diseases can be monitored by evaluating a subject for normalization of at least one sign or symptom of the disease previously displayed in the subject including, normalization of serum uric acid level, normalization of serum lipids, normalization of body weight, normalization of lipid deposition, e.g., in the liver, in the viscera; normalization of glucose or insulin responsiveness; normalization of blood sugar, normalization of kidney function, normalization of liver function, normalization of blood pressure. These symptoms may be assessed in vitro or in vivo using any method known in the art and as compared to an appropriate control. In some embodiments of the methods of the invention, the iRNA is administered to a subject such that the iRNA is delivered to a specific site within the subject. There are two KHK isoforms produced by alternative splicing of the KHK pre-mRNA. KHK-C is abundant in fructose metabolizing organs, e.g., liver, kidney, and intestines. It is highly active and responsible for most fructose metabolism. KHK-A has a lower affinity to fructose and is widely expressed in most tissues. The iRNA agents provided herein can be capable of silencing one or both KHK isoforms. In preferred embodiments, the iRNA agent is capable of silencing at least KHK-C and expression of at least the KHK-C isoform is inhibited. Studies using knockout mice have demonstrated that inhibiting expression of KHK-C is both necessary and sufficient to reduce the adverse effects observed resulting from consumption of excess fructose (see, e.g., Marek et al. (2015) *Diabetes*. 64:508-518). The inhibition of expression of KHK may be assessed using measurements of the level or change in the level of KHK mRNA or KHK protein in a sample derived from fluid or tissue from the specific site within the subject.

[0492] As used herein, the terms detecting or determining a level of an analyte are understood to mean performing the steps to determine if a material, e.g., protein, RNA, is present. As used herein,

methods of detecting or determining include detection or determination of an analyte level that is below the level of detection for the method used.

VII. Methods of Treating or Preventing KHK-Associated Diseases

[0493] The present invention also provides methods of using an iRNA of the invention or a composition containing an iRNA of the invention to reduce or inhibit KHK expression in a cell. The methods include contacting the cell with a dsRNA of the invention and maintaining the cell for a time sufficient to obtain degradation of the mRNA transcript of a KHK gene, thereby inhibiting expression of the KHK gene in the cell. Reduction in gene expression can be assessed by any methods known in the art. For example, a reduction in the expression of KHK may be determined by determining the mRNA expression level of KHK, e.g., in a liver sample, using methods routine to one of ordinary skill in the art, e.g., northern blotting, qRT-PCR; by determining the protein level of KHK using methods routine to one of ordinary skill in the art, such as western blotting, immunological techniques. A reduction in the expression of KHK may also be assessed indirectly by measuring a decrease in fructose metabolism by detecting one or more indicators of fructose metabolism. e.g., the presence of fructose in the urine indicating lack of fructose metabolism. The steps of fructose metabolism are also discussed herein.

[0494] In the methods of the invention the cell may be contacted in vitro or in vivo, i.e., the cell may be within a subject.

[0495] A cell suitable for treatment using the methods of the invention may be any cell that expresses a KHK gene, preferably a KHK-C gene, typically a liver cell. A cell suitable for use in the methods of the invention may be a mammalian cell, e.g., a primate cell (such as a human cell or a non-human primate cell, e.g., a monkey cell or a chimpanzee cell), a non-primate cell (such as a cow cell, a pig cell, a camel cell, a llama cell, a horse cell, a goat cell, a rabbit cell, a sheep cell, a hamster, a guinea pig cell, a cat cell, a dog cell, a rat cell, a mouse cell, a lion cell, a tiger cell, a bear cell, or a buffalo cell), or a bird cell (e.g., a duck cell or a goose cell). In one embodiment, the cell is a human cell, e.g., a human liver cell.

[0496] KHK expression is inhibited in the cell by at least 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%, or to a level below the level of detection of the assay.

[0497] The in vivo methods of the invention may include administering to a subject a composition containing an iRNA, where the iRNA includes a nucleotide sequence that is complementary to at least a part of an RNA transcript of the KHK gene of the mammal to be treated. When the organism to be treated is a mammal such as a human, the composition can be administered by any means known in the art including, but not limited to oral, intraperitoneal, or parenteral routes, including intracranial (e.g., intraventricular, intraparenchymal, and intrathecal), intravenous, intramuscular, subcutaneous, transdermal, airway (aerosol), nasal, rectal, and topical (including buccal and sublingual) administration. In certain embodiments, the compositions are administered by intravenous infusion or injection. In certain embodiments, the compositions are administered by subcutaneous injection.

[0498] In some embodiments, the administration is via a depot injection. A depot injection may release the iRNA in a consistent way over a prolonged time period. Thus, a depot injection may reduce the frequency of dosing needed to obtain a desired effect, e.g., a desired inhibition of KHK, or a therapeutic or prophylactic effect. A depot injection may also provide more consistent serum concentrations. Depot injections may include subcutaneous injections or intramuscular injections. In preferred embodiments, the depot injection is a subcutaneous injection.

[0499] In some embodiments, the administration is via a pump. The pump may be an external pump or a surgically implanted pump. In certain embodiments, the pump is a subcutaneously implanted osmotic pump. In other embodiments, the pump is an infusion pump. An infusion pump may be used for intravenous, subcutaneous, arterial, or epidural infusions. In preferred embodiments, the infusion pump is a subcutaneous infusion pump. In other embodiments, the

pump is a surgically implanted pump that delivers the iRNA to the liver.

[0500] The mode of administration may be chosen based upon whether local or systemic treatment is desired and based upon the area to be treated. The route and site of administration may be chosen to enhance targeting.

[0501] In one aspect, the present invention also provides methods for inhibiting the expression of a KHK gene in a mammal. The methods include administering to the mammal a composition comprising a dsRNA that targets a KHK gene in a cell of the mammal and maintaining the mammal for a time sufficient to obtain degradation of the mRNA transcript of the KHK gene, thereby inhibiting expression of the KHK gene in the cell. Reduction in gene expression can be assessed by any methods known in the art and by methods, e.g. qRT-PCR, described herein. Reduction in protein production can be assessed by any methods known in the art and by methods, e.g. ELISA, described herein. In one embodiment, a puncture liver biopsy sample serves as the tissue material for monitoring the reduction in the KHK gene or protein expression.

[0502] The present invention further provides methods of treatment of a subject in need thereof. The treatment methods of the invention include administering an iRNA of the invention to a subject, e.g., a subject that would benefit from a reduction or inhibition of KHK expression, in a therapeutically effective amount of an iRNA targeting a KHK gene or a pharmaceutical composition comprising an iRNA targeting a KHK gene.

[0503] An iRNA of the invention may be administered as a “free iRNA.” A free iRNA is administered in the absence of a pharmaceutical composition. The naked iRNA may be in a suitable buffer solution. The buffer solution may comprise acetate, citrate, prolamine, carbonate, or phosphate, or any combination thereof. In one embodiment, the buffer solution is phosphate buffered saline (PBS). The pH and osmolarity of the buffer solution containing the iRNA can be adjusted such that it is suitable for administering to a subject.

[0504] Alternatively, an iRNA of the invention may be administered as a pharmaceutical composition, such as a dsRNA liposomal formulation.

[0505] Subjects that would benefit from a reduction or inhibition of KHK gene expression are those having a disorder of elevated KHK expression such as those discussed herein.

[0506] The invention further provides methods for the use of an iRNA or a pharmaceutical composition thereof, e.g., for treating a subject that would benefit from reduction or inhibition of KHK expression, e.g., a subject having a KHK-associated disease, in combination with other pharmaceuticals or other therapeutic methods, e.g., with known pharmaceuticals or known therapeutic methods, such as, for example, those which are currently employed for treating these disorders. For example, in certain embodiments, an iRNA targeting KHK is administered in combination with an agent useful in treating a KHK-associated disorder as described elsewhere herein. The agent to be administered will depend, for example, on the specific KHK-associated disease that the subject is suffering from.

[0507] The iRNA and additional therapeutic agents may be administered at the same time or in the same combination, e.g., parenterally, or the additional therapeutic agent can be administered as part of a separate composition or at separate times or by another method known in the art or described herein.

[0508] In one embodiment, the method includes administering a composition featured herein such that expression of the target KHK gene is decreased, such as for about 1, 2, 3, 4, 5, 6, 7, 8, 12, 16, 18, 24 hours, 28, 32, or about 36 hours. In one embodiment, expression of the target KHK gene is decreased for an extended duration, e.g., at least about two, three, four days or more, e.g., about one week, two weeks, three weeks, or four weeks or longer.

[0509] Preferably, the iRNAs useful for the methods and compositions featured herein specifically target RNAs (primary or processed) of the target KHK gene. Compositions and methods for inhibiting the expression of these genes using iRNAs can be prepared and performed as described herein.

[0510] Administration of the iRNA according to the methods of the invention may result in a reduction of the severity, signs, symptoms, or markers of such diseases or disorders in a patient with a disorder of elevated KHK. By “reduction” in this context is meant a statistically significant decrease in such level. The reduction (absolute reduction or reduction of the difference between the elevated level in the subject and a normal level) can be, for example, at least about 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%, or to below the level of detection of the assay used.

[0511] Efficacy of treatment or prevention of disease can be assessed, for example by measuring disease progression, disease remission, symptom severity, reduction in pain, quality of life, dose of a medication required to sustain a treatment effect, level of a disease marker, or any other measurable parameter appropriate for a given disease being treated or targeted for prevention. It is well within the ability of one skilled in the art to monitor efficacy of treatment or prevention by measuring any one of such parameters, or any combination of parameters. As discussed herein, the specific parameters to be measured depend on the KHK-associated disease that the subject is suffering from.

[0512] Comparisons of the later readings with the initial readings provide a physician an indication of whether the treatment is effective. It is well within the ability of one skilled in the art to monitor efficacy of treatment or prevention by measuring any one of such parameters, or any combination of parameters. In connection with the administration of an iRNA targeting KHK or pharmaceutical composition thereof, “effective against” a KHK related disorder indicates that administration in a clinically appropriate manner results in a beneficial effect for at least a statistically significant fraction of patients, such as a improvement of symptoms, a cure, a reduction in disease, extension of life, improvement in quality of life, or other effect generally recognized as positive by medical doctors familiar with treating KHK-related disorders.

[0513] A treatment or preventive effect is evident when there is a statistically significant improvement in one or more parameters of disease status, or by a failure to worsen or to develop symptoms where they would otherwise be anticipated. As an example, a favorable change of at least 10% in a measurable parameter of disease, and preferably at least 20%, 30%, 40%, 50% or more can be indicative of effective treatment. Efficacy for a given iRNA drug or formulation of that drug can also be judged using an experimental animal model for the given disease as known in the art. When using an experimental animal model, efficacy of treatment is evidenced when a statistically significant reduction in a marker or symptom is observed.

[0514] Alternatively, the efficacy can be measured by a reduction in the severity of disease as determined by one skilled in the art of diagnosis based on a clinically accepted disease severity grading scale. Any positive change resulting in e.g., lessening of severity of disease measured using the appropriate scale, represents adequate treatment using an iRNA or iRNA formulation as described herein.

[0515] Subjects can be administered a therapeutic amount of iRNA, such as about 0.01 mg/kg to about 200 mg/kg.

[0516] The iRNA can be administered by intravenous infusion over a period of time, on a regular basis. In certain embodiments, after an initial treatment regimen, the treatments can be administered on a less frequent basis. Administration of the iRNA can reduce KHK levels, e.g., in a cell, tissue, blood, urine, or other compartment of the patient by at least about 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%, or below the level of detection of the assay method used. As KHK-C is expressed in the liver, it is unlikely that treatment will be monitored by measuring KHK-C expression in the liver. In certain embodiments, the efficacy of treatment is assessed by measuring on or more signs or symptoms of the KHK-associated disease from which the subject is suffering.

[0517] Before administration of a full dose of the iRNA, patients can be administered a smaller dose, such as a 5% infusion reaction, and monitored for adverse effects, such as an allergic

reaction. In another example, the patient can be monitored for unwanted immunostimulatory effects, such as increased cytokine (e.g., TNF-alpha or INF-alpha) levels.

[0518] Alternatively, the iRNA can be administered subcutaneously, i.e., by subcutaneous injection. One or more injections may be used to deliver the desired daily dose of iRNA to a subject. The injections may be repeated over a period of time. The administration may be repeated on a regular basis. In certain embodiments, after an initial treatment regimen, the treatments can be administered on a less frequent basis. A repeat-dose regimen may include administration of a therapeutic amount of iRNA on a regular basis, such as every other day or to once a year. In certain embodiments, the iRNA is administered about once per month to about once per quarter (i.e., about once every three months).

IX. Diagnostic Criteria and Treatment for KHK-Associated Diseases

[0519] Diagnostic criteria, therapeutic agents, and considerations for treatment for various KHK-associated diseases are provided below.

A. Hyperuricemia

[0520] Serum uric acid levels are not routinely obtained as clinical lab values. However, hyperuricemia (elevated uric acid) is associated with a number of diseases and conditions including gout, NAFLD, NASH, metabolic disorder, insulin resistance (not resulting from an immune response to insulin), cardiovascular disease, hypertension, and type 2 diabetes. It is expected that decreasing KHK expression can be useful in the prevention or treatment of one or more conditions associated with elevated serum uric acid levels. Further, it is expected that a subject would derive clinical benefit from normalization of serum uric acid levels towards or to a normal serum uric acid level, e.g., no more than 6.8 mg/dl, preferably no more than 6 mg/dl, even in the absence of overt signs or symptoms of one or more conditions associated with elevated uric acid.

[0521] Animal models of hyperuricemia include, for example, high fructose diet, e.g., in rats and mice, which can induce one or more of fat accumulation including fatty liver, insulin resistance, type 2 diabetes, obesity including visceral obesity, metabolic syndrome, decreased adiponectin secretion, reduced renal function, and inflammation (see, e.g., Johnson et al. (2013) Diabetes. 62:3307-3315). Administration of oxonic acid, a uricase inhibitor, can also be used to induce hyperuricemia (see, e.g., Mazalli et al. (2001) Hypertens. 38:1101-1106). Genetic models of hyperuricemia include the B6; 129S7-Uox^{sup.tmlBay/J} mouse available from Jackson Laboratory ([/jaxmice.jax.org/strain/002223.html](http://jaxmice.jax.org/strain/002223.html)) which develops hyperuricemia, with 10-fold higher levels of serum uric acid levels.

[0522] Various treatments for hyperuricemia are known in the art. However, some of the agents can only be used in limited populations. For example, allopurinol is a xanthine oxidase inhibitor that is used to reduce serum uric acid levels for the treatment of a number of conditions, e.g., gout, cardiovascular disease including ischemia-reperfusion injury, hypertension, atherosclerosis, and stroke, and inflammatory diseases (Pacher et al., (2006) Pharma. Rev. 58:87-114). However, the use of allopurinol is contraindicated in subjects with impaired renal function, e.g., chronic kidney disease, hypothyroidism, hyperinsulinemia, or insulin resistance; or in subjects predisposed to kidney disease or impaired renal function, e.g., subjects with hypertension, metabolic disorder, diabetes, and the elderly. Further, allopurinol should not be taken by subjects taking oral coagulants or probenecid as well as subjects taking diuretics, especially thiazide diuretics or other drugs that can reduce kidney function or have potential kidney toxicity.

[0523] In certain embodiments, the compositions and methods of the invention are used in combination with other compositions and methods to treat hyperuricemia, e.g., allopurinol, oxypurinol, febuxostat. In certain embodiments, the compositions and methods of the invention are used for treatment of subjects with reduced kidney function or susceptible to reduced kidney function, e.g., due to age, comorbidities, or drug interactions.

B. Gout

[0524] Gout affects approximately 1 in 40 adults, most commonly men between 30-60 years of

age. Gout less commonly affects women. Gout is one of a few types of arthritis where future damage to joints can be avoided by treatment. Gout is characterized by recurrent attacks of acute inflammatory arthritis caused by an inflammatory reaction to uric acid crystals in the joint due to hyperuricemia resulting from insufficient renal clearance of uric acid or excessive uric acid production. Fructose associated gout is sometimes associated with variants of transporters expressed in the kidney, intestine, and liver. Gout is characterized by the formation and deposition of tophi, monosodium urate (MSU) crystals, in the joints and subcutaneously. Pain associated with gout is not related to the size of the tophi, but is a result of an immune response against the MSU crystals. There is a linear inverse relation between serum uric acid and the rate of decrease in tophus size. For example, in one study of 18 patients with non-tophaceous gout, serum uric acid declined to 2.7-5.4 mg/dL (0.16-0.32 mM) in all subjects within 3 months of starting urate lowering therapy (Pascual and Sivera (2007) *Ann. Rheum. Dis.* 66:1056-1058). However, it took 12 months with normalized serum uric acid for MSU crystals to disappear from asymptomatic knee or first MTP joints in patients who had gout for less than 10 years, vs. 18 months in those with gout for more than 10 years. Therefore, effective treatment of gout does not require complete clearance of tophi or resolution of all symptoms, e.g., joint pain and swelling, inflammation, but simply a reduction in at least one sign or symptom of gout, e.g., reduction in severity or frequency of gout attacks, in conjunction with a reduction in serum urate levels.

[0525] Animal models of gout include oxonic acid-induced hyperuricemia (see, e.g., Jang et al. (2014) *Mycobiology*. 42:296-300).

[0526] Currently available treatments for gout are contraindicated or ineffective in a number of subjects. Allopurinol, a common first line treatment to reduce uric acid levels in subjects with gout, is contraindicated in a number of populations, especially those with compromised renal function, as discussed above. Further, a number of subjects fail treatment with allopurinol, e.g., subjects who suffer gout flares despite treatment, or subjects who suffer from rashes or hypersensitivity reactions associated with allopurinol.

[0527] In certain embodiments, the compositions and methods of the invention are used in combination with other agents to reduce serum uric acid. In certain embodiments, the compositions and methods of the invention are used in combination with agents for treatment of symptoms of gout, e.g., analgesic or anti-inflammatory agents, e.g., NSAIDS. In certain embodiments, the compositions and methods of the invention are used for treatment of subjects with reduced kidney function or susceptible to reduced kidney function, e.g., due to age, comorbidities, or drug interactions.

C. Liver Disease

[0528] NAFLD is associated with hyperuricemia (Xu et al. (2015) *J. Hepatol.* 62:1412-1419) which, in turn, is associated with elevated fructose metabolism. The definition of nonalcoholic fatty liver disease (NAFLD) requires that (a) there is evidence of hepatic steatosis, either by imaging or by histology and (b) there are no causes for secondary hepatic fat accumulation such as significant alcohol consumption, use of steatogenic medication or hereditary disorders. In the majority of patients, NAFLD is associated with metabolic risk factors such as obesity, diabetes mellitus, and dyslipidemia. NAFLD is histologically further categorized into nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH). NAFL is defined as the presence of hepatic steatosis with no evidence of hepatocellular injury in the form of ballooning of the hepatocytes. NASH is defined as the presence of hepatic steatosis and inflammation with hepatocyte injury (ballooning) with or without fibrosis (Chalasani et al. (2012) *Hepatol.* 55:2005-2023). It is generally agreed that patients with simple steatosis have very slow, if any, histological progression, while patients with NASH can exhibit histological progression to cirrhotic-stage disease. The long term outcomes of patients with NAFLD and NASH have been reported in several studies. Their findings can be summarized as follows; (a) patients with NAFLD have increased overall mortality compared to matched control populations, (b) the most common cause of death in patients with NAFLD, NAFL, and NASH is

cardiovascular disease, and (c) patients with NASH (but not NAFL) have an increased liver-related mortality rate.

[0529] Animal models of NAFLD include various high fat- or high fructose-fed animal models. Genetic models of NAFLD include the B6.129S7-Ldlr.sup.tmlHer/J and the B6.129S4-Pten.sup.tmlHwu/J mice available from The Jackson Laboratory.

[0530] Treatment of NAFLD is typically to manage the conditions that resulted in development of NAFLD. For example, patients with dyslipidemia are treated with agents to normalize cholesterol or triglycerides, as needed, to treat or prevent further progression of NAFLD. Patients with type 2 diabetes are treated with agents to normalize glucose or insulin sensitivity. Lifestyle changes, e.g., changes in diet and exercise, are also used to treat NAFLD. In a mouse model of NAFLD, treatment with allopurinol both prevented the development of hepatic steatosis, but also significantly ameliorated established hepatic steatosis in mice (Xu et al., *J. Hepatol.* 62:1412-1419, 2015).

[0531] In certain embodiments, the compositions and methods of the invention are used in combination with other agents to reduce serum uric acid. In certain embodiments, the compositions and methods of the invention are used in combination with agents for treatment of symptoms of NAFLD. In certain embodiments, the compositions and methods of the invention are used for treatment of subjects with reduced kidney function or susceptible to reduced kidney function, e.g., due to age, comorbidities, or drug interactions.

D. Dyslipidemia, Disorders of Glycemic Control, Metabolic Syndrome, and Obesity

[0532] Dyslipidemia (e.g., hyperlipidemia, high LDL cholesterol, low HDL cholesterol, hypertriglyceridemia, postprandial hypertriglyceridemia), disorders of glycemic control (e.g., insulin resistance, type 2 diabetes), metabolic syndrome, adipocyte dysfunction, visceral adipose deposition, obesity, and excessive sugar craving are associated with elevated fructose metabolism. Characteristics or diagnostic criteria for the conditions are provided below. Animal models of metabolic disorder and the component features include various high fat- or high fructose-fed animal models. Genetic models include leptin deficient B6.Cg-Lep.sup.ob/J, commonly known as ob or ob/ob mice, which are available from The Jackson Laboratory.

[0533] Normal and abnormal fasting levels of the lipids are provided in the table below.

TABLE-US-00002 Lipid Value Interpretation
Total Cholesterol
Below 200 mg/dL Desirable
200-239 mg/dL Borderline high
240 mg/dL and above High
LDL Cholesterol
Below 70 mg/dL Best for people who have cholesterol heart disease or diabetes.
70-100 mg/dL Optimal for people at risk of heart disease.
100-129 mg/dL Near optimal if there is no heart disease. High if there is heart disease.
130-159 mg/dL Borderline high if there is no heart disease. High if there is heart disease.
160-189 mg/dL High if there is no heart disease. Very high if there is heart disease.
190 mg/dL and above Very high
HDL Cholesterol
Below 40 mg/dL (men) Poor
40-59 mg/dL (men) Moderate
60 mg/dL and above Normal
Triglycerides
Below 150 mg/dL Desirable
150-199 mg/dL Borderline high
200-499 mg/dL High
500 mg/dL and above Very High

[0534] Postprandial hypertriglyceridemia is principally initiated by overproduction or decreased catabolism of triglyceride-rich lipoproteins (TRLs) and is a consequence of predisposing genetic variations and medical conditions such as obesity and insulin resistance.

[0535] Insulin resistance is characterized by the presence of at least one of: [0536] 1. A fasting blood glucose level of 100-125 mg/dL taken at two different times; or [0537] 2. An oral glucose tolerance test with a result of a glucose level of 140-199 mg/dL at 2 hours after glucose consumption.

[0538] As used herein, insulin resistance does not include a lack of response to insulin as a result of an immune response to administered insulin as often occurs in late stages of insulin dependent diabetes, especially type 1 diabetes.

[0539] Type 2 diabetes is characterized by at least one of: [0540] 1. A fasting blood glucose level ≥ 126 mg/dL taken at two different times; [0541] 2. A hemoglobin A1c (A1C) test with a result of

≥6.5% or higher; or 3. An oral glucose tolerance test with a result of a glucose level ≥200 mg/dL at 2 hours after glucose consumption.

[0542] Pharmacological treatments for type 2 diabetes and insulin resistance include treatment with agents to normalize blood sugar such as metformin (e.g., glucophage, glumetza), sulfonylureas (e.g., glyburide, glipizide, glimepiride), meglitinides (e.g., repaglinide, nateglinide), thiazolidinediones (rosiglitazone, pioglitazone), DPP4 inhibitors (sitagliptin, saxagliptin, linagliptin), GLP-1 receptor antagonists (exenatide, liraglutide), and SGLT2 inhibitors (e.g., canagliflozin, dapagliflozin).

[0543] Obesity is characterized as disease of excess body fat. Body mass index (BMI), which is calculated by dividing body weight in kilograms (kg) by height in meters (m) squared, provides a reasonable estimate of body fat for most, but not all, people. Generally, a BMI below 18.5 is characterized as underweight, 18.5 to 24.9 is normal, 25.0-29.9 is overweight, 30.0-34.9 is obese (class I), 35-39.9 is obese (class II), and 40.0 and higher is extremely obese (class III).

[0544] Methods for assessment of subcutaneous vs. visceral fat are provided, for example, in Wajchenberg (2000) Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome, *Endocr Rev.* 21:697-738, which is incorporated herein by reference.

[0545] Metabolic syndrome is characterized by a cluster of conditions defined as at least three of the five following metabolic risk factors: [0546] 1. Large waistline (≥35 inches for women or ≥40 inches for men); [0547] 2. High triglyceride level (≥150 mg/dl); [0548] 3. Low HDL cholesterol (≤50 mg/dl for women or ≤40 mg/dl for men); [0549] 4. Elevated blood pressure (≥130/85) or on medicine to treat high blood pressure; and [0550] 5. High fasting blood sugar (≥100 mg/dl) or being in medicine to treat high blood sugar.

[0551] As with NAFLD, the agents for treatment of metabolic syndrome depend on the specific risk factors present, e.g., normalize lipids when lipids are abnormal, normalize glucose or insulin sensitivity when they are abnormal.

[0552] Metabolic syndrome, insulin resistance, and type 2 diabetes are often associated with decreased renal function or the potential for decreased renal function.

[0553] In certain embodiments, the compositions and methods of the invention are for use in treatment of subjects with dyslipidemia, disorders of glycemic control, metabolic syndrome, and obesity. For example, in certain embodiments, the compositions and methods of the invention are for use in subjects with metabolic syndrome, insulin resistance, or type 2 diabetes and chronic kidney disease. In certain embodiments, the compositions and methods are for use in subjects with metabolic syndrome, insulin resistance, or type 2 diabetes who are suffering from one or more of cardiovascular disease, hypothyroidism, or inflammatory disease; or elderly subjects (e.g., over 65). In certain embodiments, the compositions and methods are for use in subjects with metabolic syndrome, insulin resistance, or type 2 diabetes who are also taking a drug that can reduce kidney function as demonstrated by the drug label. For example, in certain embodiments the compositions and methods of the invention are for use in subjects with metabolic syndrome, insulin resistance, or type 2 diabetes who are being treated with oral coagulants or probenecid. For example, in certain embodiments the compositions and methods of the invention are for use in subjects with metabolic syndrome, insulin resistance, or type 2 diabetes who are being treated with diuretics, especially thiazide diuretics.

[0554] In certain embodiments, the compositions and methods of the invention are used in combination with other agents to reduce serum uric acid. In certain embodiments, the compositions and methods of the invention are used in combination with agents for treatment of symptoms of metabolic syndrome, insulin resistance, or type 2 diabetes. In certain embodiments, subjects are treated with e.g., agents to decrease blood pressure, e.g., diuretics, beta-blockers, ACE inhibitors, angiotensin II receptor blockers, calcium channel blockers, alpha blockers, alpha-2 receptor antagonists, combined alpha- and beta-blockers, central agonists, peripheral adrenergic inhibitors, and blood vessel dilators; agents to decrease cholesterol, e.g., statins, selective cholesterol

absorption inhibitors, resins, or lipid lowering therapies; or agents to normalize blood sugar, e.g., metformin, sulfonylureas, meglitinides, thiazolidinediones, DPP-4 inhibitors, GLP-1 receptor antagonists, and SGLT2 inhibitors.

[0555] In certain embodiments, the compositions and methods of the invention are used for treatment of subjects with reduced kidney function or susceptible to reduced kidney function, e.g., due to age, comorbidities, or drug interactions.

[0556] The iRNA and additional therapeutic agents may be administered at the same time or in the same combination, e.g., parenterally, or the additional therapeutic agent can be administered as part of a separate composition or at separate times or by another method known in the art or described herein.

E. Cardiovascular Disease

[0557] In certain embodiments, the compositions and methods of the invention are for use in treatment of subjects with cardiovascular disease. For example, in certain embodiments, the compositions and methods of the invention are for use in subjects with cardiovascular disease and chronic kidney disease. In certain embodiments, the compositions and methods are for use in subjects with cardiovascular disease who are suffering from one or more of metabolic disorder, insulin resistance, hyperinsulinemia, diabetes, hypothyroidism, or inflammatory disease. In certain embodiments, the compositions and methods are for use in subjects with cardiovascular disease who are also taking a drug that can reduce kidney function as demonstrated by the drug label. For example, in certain embodiments the compositions and methods of the invention are for use in subjects with cardiovascular disease who are being treated with oral coagulants or probenecid. For example, in certain embodiments the compositions and methods of the invention are for use in subjects with cardiovascular disease who are being treated with diuretics, especially thiazide diuretics. For example, in certain embodiments the compositions and methods of the invention are for use in subjects with cardiovascular disease who have failed treatment with allopurinol.

[0558] In certain embodiments, the compositions and methods of the invention are used in combination with other agents to reduce serum uric acid. In certain embodiments, the compositions and methods of the invention are used in combination with agents for treatment of symptoms of cardiovascular disease, e.g., agents to decrease blood pressure, e.g., diuretics, beta-blockers, ACE inhibitors, angiotensin II receptor blockers, calcium channel blockers, alpha blockers, alpha-2 receptor antagonists, combined alpha- and beta-blockers, central agonists, peripheral adrenergic inhibitors, and blood vessel dilators; or agents to decrease cholesterol, e.g., statins, selective cholesterol absorption inhibitors, resins, or lipid lowering therapies.

F. Kidney Disease

[0559] Kidney disease includes, for example, acute kidney disorder, tubular dysfunction, proinflammatory changes to the proximal tubules, and chronic kidney disease.

[0560] Acute kidney (renal) failure occurs when the kidneys suddenly become unable to filter waste products from the blood resulting in accumulation of dangerous levels of wastes in serum and systemic chemical imbalance. Acute kidney failure can develop rapidly over a few hours or a few days, and is most common in individuals who are already hospitalized, particularly in critically ill individuals who need intensive care. Acute kidney failure can be fatal and requires intensive treatment. However, acute kidney failure may be reversible. If you're otherwise in good health, you may recover normal or nearly normal kidney function.

[0561] Chronic kidney disease, also called chronic kidney failure, describes the gradual loss of kidney function. When chronic kidney disease reaches an advanced stage, dangerous levels of fluid, electrolytes and wastes can accumulate in the body. Signs and symptoms of kidney disease may include nausea, vomiting, loss of appetite, fatigue and weakness, sleep problems, changes in urine output, decreased mental sharpness, muscle twitches and cramps, hiccups, swelling of feet and ankles, persistent itching, chest pain, if fluid builds up around the lining of the heart, shortness of breath, if fluid builds up in the lungs, high blood pressure (hypertension) that's difficult to

control. Signs and symptoms of chronic kidney disease are often nonspecific and can develop slowly, and may not appear until irreversible damage has occurred.

[0562] Kidney disease is treated by removing the damaging agent or condition that is causing kidney damage, e.g. normalize blood pressure to improve kidney function, end treatment with agents that can induce kidney damage, reduce inflammation that is causing kidney damage, or by providing renal support (e.g., renal dialysis) to assist kidney function.

[0563] Renal function is typically determined using one or more routine laboratory tests, BUN (blood urea nitrogen), creatinine (blood), creatinine (urine), or creatinine clearance (see, e.g., www.nlm.nih.gov/medlineplus/ency/article/003435.htm). The tests may also be diagnostic of conditions in other organs.

[0564] Generally, a BUN level of 6 to 20 mg/dL is considered normal, although normal values may vary among different laboratories. Elevated BUN level can be indicative of kidney disease, including glomerulonephritis, pyelonephritis, and acute tubular necrosis, or kidney failure.

[0565] A normal result for blood creatinine is 0.7 to 1.3 mg/dL for men and 0.6 to 1.1 mg/dL for women. Elevated blood creatinine can be indicative of compromised kidney function due to kidney damage or failure, infection, or reduced blood flow.

[0566] Urine creatinine (24-hour sample) values can range from 500 to 2000 mg/day. Results depend on age and amount of lean body mass. Normal results are 14 to 26 mg per kg of body mass per day for men

[0567] And 11 to 20 mg per kg of body mass per day for women. Abnormal results can be indicative of kidney damage, such as damage to the tubule cells, kidney failure, decreased blood flow to the kidneys, or kidney infection (pyelonephritis).

[0568] The creatinine clearance test helps provide information regarding kidney function by comparing the creatinine level in urine with the creatinine level in blood. Clearance is often measured as milliliters per minute (ml/min). Normal values are 97 to 137 ml/min. for men and 88 to 128 ml/min. for women. Lower than normal creatinine clearance can be indicative of kidney damage, such as damage to the tubule cells, kidney failure, decreased blood flow to the kidneys, or reduced glomerular filtration in the kidneys.

[0569] In certain embodiments, the compositions and methods of the invention can be used for the treatment of kidney disease. It is expected that such agents would not cause damage to the kidney.

[0570] This invention is further illustrated by the following examples which should not be construed as limiting. The entire contents of all references, patents and published patent applications cited throughout this application, as well as the Sequence Listing, are hereby incorporated herein by reference.

EXAMPLES

Example 1. iRNA Synthesis

Source of Reagents

[0571] Where the source of a reagent is not specifically given herein, such reagent can be obtained from any supplier of reagents for molecular biology at a quality/purity standard for application in molecular biology.

Transcripts and siRNA Design

[0572] A set of dsRNA agents targeting human KHK (human NCBI refseqID: XM_005264298; NCBI GeneID: 3795) were designed using custom R and Python scripts. The human KHK REFSEQ mRNA has a length of 2144 bases. The rationale and method for the set of dsRNA agent designs is as follows: the predicted efficacy for every potential 19mer RNAi agent from position 10 through position 2144 was determined with a linear model derived the direct measure of mRNA knockdown from more than 20,000 distinct dsRNA agents designs targeting a large number of vertebrate genes. The custom Python script built the set of dsRNAs by systematically selecting an RNAi agent every 11 bases along the target mRNA starting at position 10. At each of the positions, the neighboring RNA agent (one position to the 5' end of the mRNA, one position to the 3' end of

the mRNA) was swapped into the design set if the predicted efficacy was better than the efficacy at the exact every-11.sup.th RNAi agent. Low complexity RNAi agents, i.e., those with Shannon Entropy measures below 1.35 were excluded from the set.

[0573] A detailed list of the unmodified KHK sense and antisense strand sequences is shown in Table 3. A detailed list of the umodified KHK sense and antisense strand sequences is shown in Table 5.

[0574] RNAi agents were synthesized and annealed using routine methods known in the art.

Example 2—In Vitro Screening

Cell culture and transfections:

[0575] Hep3B (ATCC) cells were transfected by adding 4.9 μ l of Opti-MEM plus 0.1 μ l of Lipofectamine RNAiMax per well (Invitrogen®, Carlsbad CA. cat #13778-150) to 5 μ l of dsRNA duplexes per well into a 384-well plate and incubated at room temperature for 15 minutes. Forty μ l of EMEM containing $\sim 5 \times 10^5$ cells were then added to the siRNA mixture. Cells were incubated for twenty-four hours prior to RNA purification. Single dose experiments were performed at 10 nM final duplex concentration.

Total RNA Isolation Using DYNABEADS mRNA Isolation Kit:

[0576] RNA was isolated using an automated protocol on a BioTek-EL406 platform using DYNABEADS (Invitrogen, cat #61012). Briefly, 50 μ l of Lysis/Binding Buffer and 25 μ l of lysis buffer containing 3 μ l of magnetic beads were added to the plate with cells. Plates were incubated on an electromagnetic shaker for 10 minutes at room temperature and then magnetic beads were captured and the supernatant was removed. Bead-bound RNA was then washed 2 times with 150 μ l Wash Buffer A and once with Wash Buffer B. Beads were then washed with 150 μ l Elution Buffer, re-captured and supernatant removed.

cDNA Synthesis Using ABI High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, Cat #4368813):

[0577] Ten μ l of a master mix containing 1 μ l 10 \times Buffer, 0.4 μ l 25 \times dNTPs, 1 μ l 10 \times Random primers, 0.5 μ l Reverse Transcriptase, 0.5 μ l RNase inhibitor and 6.6 μ l of H.sub.2O per reaction was added to RNA isolated above. Plates were sealed, mixed, and incubated on an electromagnetic shaker for 10 minutes at room temperature, followed by two hours 37° C.

Real Time PCR:

[0578] Two μ l of cDNA were added to a master mix containing 0.5 μ l of GAPDH TaqMan Probe (Hs99999905 m1), 0.5 μ l KHK probe (Hs00240827_m1) and 5 μ l Lightcycler 480 probe master mix (Roche Cat #04887301001) per well in a 384 well plates (Roche cat #04887301001). Real time PCR was done in a LightCycler480 Real Time PCR system (Roche). Each duplex was tested at least two times and data were normalized to naïve cells or cells transfected with a non-targeting control siRNA.

[0579] To calculate relative fold change, real time data were analyzed using the $\Delta\Delta C_t$ method and normalized to assays performed with cells transfected with 10 nM AD-1955, or mock transfected cells.

TABLE-US-00003 TABLE 2 Abbreviations of nucleotide monomers used in nucleic acid sequence representation. It will be understood that these monomers, when present in an oligonucleotide, are mutually linked by 5'-3'-phosphodiester bonds. Abbreviation Nucleotide(s) A Adenosine-3'-phosphate Af 2'-fluoroadenosine-3'-phosphate Afs 2'-fluoroadenosine-3'-phosphorothioate As adenosine-3'-phosphorothioate C cytidine-3'-phosphate Cf 2'-fluorocytidine-3'-phosphate Cfs 2'-fluorocytidine-3'-phosphorothioate Cs cytidine-3'-phosphorothioate G guanosine-3'-phosphate Gf 2'-fluoroguanosine-3'-phosphate Gfs 2'-fluoroguanosine-3'-phosphorothioate Gs guanosine-3'-phosphorothioate T 5'-methyluridine-3'-phosphate Tf 2'-fluoro-5-methyluridine-3'-phosphate Tfs 2'-fluoro-5-methyluridine-3'-phosphorothioate Ts 5-methyluridine-3'-phosphorothioate U Uridine-3'-phosphate Uf 2'-fluorouridine-3'-phosphate Ufs 2'-fluorouridine-3'-phosphorothioate Us uridine-3'-phosphorothioate N any nucleotide (G, A, C, T or U) a 2'-O-methyladenosine-3'-phosphate as 2'-

O-methyladenosine-3'-phosphorothioate c 2'-O-methylcytidine-3'-phosphate cs 2'-O-methylcytidine-3'-phosphorothioate g 2'-O-methylguanosine-3'-phosphate gs 2'-O-methylguanosine-3'-phosphorothioate t 2'-O-methyl-5-methyluridine-3'-phosphate ts 2'-O-methyl-5-methyluridine-3'-phosphorothioate u 2'-O-methyluridine-3'-phosphate us 2'-O-methyluridine-3'-phosphorothioate s phosphorothioate linkage L96 N-[tris(GalNAc-alkyl)-amidodecanoyl]- 4-hydroxyprolinol Hyp-(GalNAc-alkyl)3 dT 2'-deoxythymidinc-3'-phosphate dC 2'-deoxycytidinc-3'-phosphate Y44 inverted abasic DNA (2-hydroxymethyl- tetrahydrofurane-5-phosphate) (Tgn) Thymidine-glycol nucleic acid (GNA) S-Isomer P Phosphate VP Vinyl-phosphate (Aam) 2'-O-(N-methylacetamide)adenosine-3'-phosphate

CGCGAGGAAGGCCCU 43 310-328 A-144866 AGCAGGCCUCCUCCG 225
310-328 AD-72277 A-144867 GGGCCCUGCUCCUUUCGUU 44 319-337 A-144868
AACGAAAGGAGCAGGGCCC 226 319-337 AD-72278 A-144871
UGCACCCCUGGCCGUGCA 45 341-359 A-144872 UGCAGCGGCCAGGGGUGCA 227
341-359 AD-72279 A-144873 CCGCUGCAGGUGGCUCCU 46 352-370 A-144874
AGGGAGCCACCUGCAGCGG 228 352-370 AD-72280 A-144875
GCUCCCUGGAGGAGGAGCU 47 364-382 A-144876 AGCUCCUCCUCCAGGGAGC 229
364-382 AD-72281 A-144877 AGGAGCUCCACGCGGAGG 48 376-394 A-144878
CCUCCGCGUGGGAGCUCCU 230 376-394 AD-72282 A-144879
ACGCGGAGGAGGAGCCAGG 49 386-404 A-144880 CCUGGCUCCUCCUCCGCGU 231
386-404 AD-72283 A-144881 AGCCAGGGCAGCUGGGAGC 50 398-416 A-144882
GCUCCCAGCUGCCCUGGCU 232 398-416 AD-72284 A-144883
CUGGGAGCGGGGACACCAU 51 409-427 A-144884 AUGGUGUCCCCGCUCCAG 233
409-427 AD-72285 A-144885 GGACACCAUCCUCCUGGAU 52 419-437 A-144886
AUCCAGGAGGAUGGUGUCC 234 419-437 AD-72286 A-144887
CCUGGAUAAGAGGCAGAGG 53 431-449 A-144888 CCUCUGCCUCUUAUCCAGG 235
431-449 AD-72287 A-144889 AGGCAGAGGCCGGGAGGAA 54 441-459 A-144890
UCCUCCCCGGCCUCUGCCU 236 441-459 AD-72288 A-144891
GGAGGAACCCCGUCAGCCG 55 453-471 A-144892 CGGCUGACGGGGUCCUCC 237
453-471 AD-72289 A-144893 CGUCAGCCGGGCGGGCAGG 56 463-481 A-144894
CCUGCCCGCCCGGCUGACG 238 463-481 AD-72290 A-144895
CGGGCAGGAAGCUCUGGGA 57 474-492 A-144896 UCCAGAGCUUCCUGCCCG 239
474-492 AD-72291 A-144897 UCUGGGAGUAGCCUCAUGG 58 486-504 A-144898
CCAUGAGGCUACUCCCAGA 240 486-504 AD-72292 A-144899
AGCCUCAUGGAAGAGAAGC 59 495-513 A-144900 GCUUCUCUCCAUGAGGCU 241
495-513 AD-72293 A-144901 AGAAGCAGAUCCUGUGCGU 60 508-526 A-144902
ACGCACAGGAUCUGCUUCU 242 508-526 AD-72294 A-144903
CUGUGCGUGGGGCUAGUGG 61 519-537 A-144904 CCACUAGCCCCACGCACAG 243
519-537 AD-72295 A-144905 GGCUAGUGGUGCUGGACGU 62 529-547 A-144906
ACGUCCAGCACCACUAGCC 244 529-547 AD-72296 A-144907
UGGACGUCAUCAGCCUGGU 63 541-559 A-144908 ACCAGGCUGAUGACGUCCA 245
541-559 AD-72297 A-144909 AGCCUGGUGGACAAGUACC 64 552-570 A-144910
GGUACUUGUCCACCAGGCU 246 552-570 AD-72298 A-144911
ACAAGUACCCUAAGGAGGA 65 562-580 A-144912 UCCUCCUUAGGGUACUUGU 247
562-580 AD-72299 A-144913 AAGGAGGACUCGGAGAUAA 66 573-591 A-144914
UUAUCUCCGAGUCCUCCUU 248 573-591 AD-72300 A-144915
CGGAGAUAAAGGAGCCUGCC 67 583-601 A-144916 GGCAGGCUCCUUAUCUCCG 249
583-601 AD-72301 A-144917 AGCCUGCCAGAUGUGUCUG 68 594-612 A-144918
CAGACACAUCUGGCAGGCU 250 594-612 AD-72302 A-144919
UGUGUCUGCUACAGACUUU 69 605-623 A-144920 AAAGUCUGUAGCAGACACA 251
605-623 AD-72303 A-144921 CAGACUUUGAGAAGGUUGA 70 616-634 A-144922
UCAACCUUCUCAAGUCUG 252 616-634 AD-72304 A-144923
AGGUUGAUCUGACCCAGUU 71 628-646 A-144924 AACUGGGUCAGAUAACCU 253
628-646 AD-72305 A-144925 ACCCAGUUCAAGUGGAUCC 72 639-657 A-144926
GGAUCCACUUGAACUGGGU 254 639-657 AD-72306 A-144927
AGUGGAUCCACAUUGAGGG 73 649-667 A-144928 CCCUCAUUGUGGAUCCACU 255
649-667 AD-72307 A-144929 UUGAGGGCCGGAACGCAUC 74 661-679 A-144930
GAUGCGUUCCGGCCCUCAA 256 661-679 AD-72308 A-144931
AACGCAUCGGAGCAGGUGA 75 672-690 A-144932 UCACCUGCUCCGAUGCGUU 257
672-690 AD-72309 A-144933 AGCAGGUGAAGAUGCUGCA 76 682-700 A-144934

UGAGCAUUCACCUUACCUUACCUU 258 682-700 AD-72310 A-144935
 UGCUGCAGCGGAUAGACGC 77 694-712 A-144936 GCGUCUAUCCGCUGCAGCA 259
 694-712 AD-72311 A-144937 AUAGACGCACACAACACCA 78 705-723 A-144938
 UGGUGUUGUGUGCGUCUAU 260 705-723 AD-72312 A-144941
 CAGCCUCCAGAGCAGAAGA 79 726-744 A-144942 UCUUCUGCUCUGGAGGCUG 261
 726-744 AD-72313 A-144943 AGAAGAUCCGGGUGUCCGU 80 739-757 A-144944
 ACGGACACCCGGAUCUUCU 262 739-757 AD-72314 A-144945
 GUGUCCGUGGAGGUGGAGA 81 750-768 A-144946 UCUCCACCUCACGGACAC 263
 750-768 AD-72315 A-144947 AGGUGGAGAAGCCACGAGA 82 760-778 A-144948
 UCUCGUGGCUUCUCCACCU 264 760-778 AD-72316 A-144949
 CCACGAGAGGAGCUCUCC 83 771-789 A-144950 GGAAGAGCUCUCUCUGUGG 265
 771-789 AD-72317 A-144951 AGCUCUCCAGCUGUUUGG 84 781-799 A-144952
 CCAAACAGCUGGAAGAGCU 266 781-799 AD-72318 A-144953
 UGUUUGGCUACGGAGACGU 85 793-811 A-144954 ACGUCUCCGUAGCCAAACA 267
 793-811 AD-72319 A-144955 GGAGACGUGGUGUUUGUCA 86 804-822 A-144956
 UGACAAACACCACGUCUCC 268 804-822 AD-72320 A-144957
 UUUGUCAGCAAAGAUGUGG 87 816-834 A-144958 CCACAUCUUUGCUGACAAA 269
 816-834 AD-72321 A-144959 AAAGAUGUGGCCAAGCACU 88 825-843 A-144960
 AGUGCUUGGCCACAUCUUU 270 825-843 AD-72322 A-144961
 AAGCACUUGGGGUUCCAGU 89 837-855 A-144962 ACUGGAACCCCAAGUGCUU 271
 837-855 AD-72323 A-144963 UCCAGUCAGCAGAGGAAG 90 849-867 A-144964
 CUUCCUCUGCUGACUGGAA 272 849-867 AD-72324 A-144965
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 860-878 AD-72325 A-144967 UUGAGGGGCUUGUAUGGUC 92 870-888 A-144968
 GACCAUACAAGCCCCUCA 274 870-888 AD-72326 A-144969
 UAUGGUCGUGUGAGGAAAG 93 882-900 A-144970 CUUCCUCACACGACCAUA 275
 882-900 AD-72327 A-144971 UGAGGAAAGGGGCUGUGCU 94 892-910 A-144972
 AGCACAGCCCCUUUCCUCA 276 892-910 AD-72328 A-144975
 CUGUGCCUGGGCUGAGGAG 95 914-932 A-144976 CUCCUCAGCCCAGGCACAG 277
 914-932 AD-72329 A-144977 UGAGGAGGGCGCCGACGCC 96 926-944 A-144978
 GGCGUCGGCGCCCUCCUCA 278 926-944 AD-72330 A-144979
 CCGACGCCCUUGGGCCCUCA 97 937-955 A-144980 UCAGGGCCCAGGGCGUCGG 279
 937-955 AD-72331 A-144981 UGGGCCCUGAUGGCAAU 98 946-964 A-144982
 AAUUUGCCAUCAGGGCCCA 280 946-964 AD-72332 A-144983
 CAAAUUGCUCACUCGGAU 99 959-977 A-144984 AUCCGAGUGGAGCAAUUUG 281
 959-977 AD-72333 A-144985 ACUCGGAUGC UUUCCCGCC 100 970-988 A-144986
 GGCGGGAAAGCAUCCGAGU 282 970-988 AD-72334 A-144987
 UUUCCCGCCACCCCGCGUG 101 980-998 A-144988 CACGCGGGGUGGCGGGAAA 283
 980-998 AD-72335 A-144989 CCGCGUGGUGGAUACACUG 102 992-1010 A-144990
 CAGUGUAUCCACCACGCGG 284 992-1010 AD-72336 A-144991
 AUACACUGGGAGCUGGAGA 103 1003-1021 A-144992 UCUCCAGCUCUCCAGUGUAU 285
 1003-1021 AD-72337 A-144993 AGCUGGAGACACCUUCAU 104 1013-1031 A-144994
 AUUGAAGGUGUCUCCAGCU 286 1013-1031 AD-72338 A-144995
 ACCUUCAAUGCCUCCGUCA 105 1023-1041 A-144996 UGACGGAGGCAUUGAAGGU 287
 1023-1041 AD-72339 A-144997 UCCGUCAUCUUCAGCCUCU 106 1035-1053 A-144998
 AGAGGCUGAAGAUGACGGA 288 1035-1053 AD-72498 A-144999
 AGCCUCUCCAGGGGAGGA 107 1047-1065 A-145000 UCCUCCCCUGGGAGAGGCU 289
 1047-1065 AD-72499 A-145003 UGCAGGAAGCACUGAGAU 108 1069-1087 A-145004
 AAUCUCAGUGCUUCCUGCA 290 1069-1087 AD-72500 A-145005
 CUGAGAUUCGGGUGCCAGG 109 1080-1098 A-145006 CCUGGCACCCGAAUCUCAG 291

1080-1098 AD-72501 A-145007 GGUGCCAGGUGGCCGCAA 110 1090-1108 A-145008
UUGCCGGCCACCUGGCACC 292 1090-1108 AD-72502 A-145011
AGUGUGGCCUGCAGGGCUU 111 1111-1129 A-145012 AAGCCCUGCAGGCCACACU 293
1111-1129 AD-72503 A-145013 CAGGGCUUUGAUGGCAUCG 112 1122-1140 A-145014
CGAUGCCAUCAAGCCCUG 294 1122-1140 AD-72504 A-145015
GCAUCGUGUGAGAGCAGGU 113 1135-1153 A-145016 ACCUGCUCUCACACGAUGC 295
1135-1153 AD-72505 A-145017 AGAGCAGGUGCCGGCUCCU 114 1145-1163 A-145018
AGGAGCCGGCACCUGCUCU 296 1145-1163 AD-72506 A-145019
CCGGCUCCUCACACACCAU 115 1155-1173 A-145020 AUGGUGUGUGAGGAGCCGG 297
1155-1173 AD-72507 A-145021 CACACCAUGGAGACUACCA 116 1166-1184 A-145022
UGGUAGUCUCCAUGGUGUG 298 1166-1184 AD-72508 A-145023
ACUACCAUUGCGGCUGCAU 117 1178-1196 A-145024 AUGCAGCCGCAAUGGUAGU 299
1178-1196 AD-72509 A-145025 CGGCUGCAUCGCCUUCUCC 118 1188-1206 A-145026
GGAGAAGGCGAUGCAGCCG 300 1188-1206 AD-72510 A-145027
UUCUCCCCUCCAUCCAGCC 119 1201-1219 A-145028 GGCUGGAUGGAGGGGAGAA 301
1201-1219 AD-72511 A-145029 AUCCAGCCUGGCGUCCAGG 120 1212-1230 A-145030
CCUGGACGCCAGGCUGGAU 302 1212-1230 AD-72512 A-145031
GGCGUCCAGGUUGCCCUGU 121 1221-1239 A-145032 ACAGGGCAACCUGGACGCC 303
1221-1239 AD-72513 A-145033 CCCUGUUCAGGGGACAGAU 122 1234-1252 A-145034
AUCUGUCCCCUGAACAGGG 304 1234-1252 AD-72514 A-145035
GGGGACAGAUGCAAGCUGU 123 1243-1261 A-145036 ACAGCUUGCAUCUGUCCCC 305
1243-1261 AD-72515 A-145037 CAAGCUGUGGGGAGGACUC 124 1254-1272 A-145038
GAGUCCUCCCCACAGCUUG 306 1254-1272 AD-72516 A-145039
AGGACUCUGCCUGUGUCCU 125 1266-1284 A-145040 AGGACACAGGCAGAGUCCU 307
1266-1284 AD-72517 A-145041 CUGUGUCCUGUGUUCCCCA 126 1276-1294 A-145042
UGGGGAACACAGGACACAG 308 1276-1294 AD-72518 A-145043
UUCCCCACAGGGAGAGGCU 127 1288-1306 A-145044 AGCCUCUCCCUGUGGGGAA 309
1288-1306 AD-72519 A-145045 AGAGGCUCUGGGGGGAUGG 128 1300-1318 A-145046
CCAUCCCCCAGAGCCUCU 310 1300-1318 AD-72520 A-145047
GGGGGGAUGGCUGGGGGAU 129 1309-1327 A-145048 AUCCCCCAGCCAUCCCCC 311
1309-1327 AD-72521 A-145049 UGGGGGAUGCAGAGCCUCA 130 1320-1338 A-145050
UGAGGCUCUGCAUCCCCCA 312 1320-1338 AD-72522 A-145051
AGCCUCAGAGCAAAUAAAU 131 1332-1350 A-145052 AUUUAUUUGCUCUGAGGCU 313
1332-1350 AD-72523 A-145053 AAUAAAUUCUUCCUCAGAG 132 1343-1361 A-145054
CUCUGAGGAAGAUUUAUUU 314 1343-1361 AD-72524 A-145055
CCUCAGAGCCAGCUUCUCC 133 1354-1372 A-145056 GGAGAAGCUGGCUCUGAGG 315
1354-1372 AD-72525 A-145057 AGCUUCUCCUCUCAUGUC 134 1364-1382 A-145058
GACAUUGAGAGGAGAAGCU 316 1364-1382 AD-72526 A-145059
UCAAUGUCUGAACUGCUCU 135 1375-1393 A-145060 AGAGCAGUUCAGACAUUGA 317
1375-1393 AD-72527 A-145061 UGCUCUGGCUGGGCAUUC 136 1388-1406 A-145062
GGAAUGCCCAGCCAGAGCA 318 1388-1406 AD-72528 A-145063
UGGGCAUUCCUGAGGCUCU 137 1397-1415 A-145064 AGAGCCUCAGGAAUGCCCA 319
1397-1415 AD-72529 A-145065 GAGGCUCUGACUCUUCGAU 138 1408-1426 A-145066
AUCGAAGAGUCAGAGCCUC 320 1408-1426 AD-72530 A-145071
CCAUUCCCCAAAUUAACCU 139 1442-1460 A-145072 AGGUUAAUUUGGGGAAUGG 321
1442-1460 AD-72531 A-145073 UUAACCUCUCCGCCCAGGC 140 1454-1472 A-145074
GCCUGGGCGGAGAGGUUAA 322 1454-1472 AD-72532 A-145075
GCCCAGGCCCAGAGGAGGG 141 1465-1483 A-145076 CCCUCCUCUGGGCCUGGGC 323
1465-1483 AD-72533 A-145077 CAGAGGAGGGGCUGCCUGG 142 1474-1492 A-145078
CCAGGCAGCCCCUCCUCUG 324 1474-1492 AD-72534 A-145079

UGCCUGGGCAGCAGCAGCG 143 1486-1504 A-14500 CGCUGUCUAGCCCCAGGCA 325
1486-1504 AD-72535 A-145081 AGAGCAGCGAGAAGUGCCC 144 1496-1514 A-145082
GGGCACUUCUCGCGUCUCU 326 1496-1514 AD-72536 A-145083
AAGUGCCCUGGGCUUGCCA 145 1507-1525 A-145084 UGGCAAGCCCAGGGGCACUU 327
1507-1525 AD-72537 A-145085 UUGCCACCAGCUCUGCCCU 146 1520-1538 A-145086
AGGGCAGAGCUGGUGGCAA 328 1520-1538 AD-72538 A-145087
CUCUGCCCUGGCUGGGGAG 147 1530-1548 A-145088 CUCCCCAGCCAGGGCAGAG 329
1530-1548 AD-72539 A-145089 GCUGGGGAGGACACUCGGU 148 1540-1558 A-145090
ACCGAGUGUCCUCCCCAGC 330 1540-1558 AD-72540 A-145093
ACACCCAGUGAACCUGCCA 149 1564-1582 A-145094 UGGCAGGUUCACUGGGUGU 331
1564-1582 AD-72541 A-145095 AACCUGCCAAAGAAACCGU 150 1574-1592 A-145096
ACGGUUUCUUUGGCAGGUU 332 1574-1592 AD-72542 A-145097
AGAAACCGUGAGAGCUCUU 151 1584-1602 A-145098 AAGAGCUCUCACGGUUUCU 333
1584-1602 AD-72543 A-145099 GCUCUUCGGGGCCUGCGU 152 1597-1615 A-145100
ACGCAGGGCCCCGAAGAGC 334 1597-1615 AD-72544 A-145101
CCCUGCGUUGUGCAGACUC 153 1608-1626 A-145102 GAGUCUGCACAACGCAGGG 335
1608-1626 AD-72545 A-145103 UGCAGACUCUAUUCCCACA 154 1618-1636 A-145104
UGUGGGAAUAGAGUCUGCA 336 1618-1636 AD-72546 A-145105
UUCCCACAGCUCAGAAGCU 155 1629-1647 A-145106 AGCUUCUGAGCUGUGGGAA 337
1629-1647 AD-72547 A-145107 CAGAAGCUGGGAGUCCACA 156 1640-1658 A-145108
UGUGGACUCCCAGCUUCUG 338 1640-1658 AD-72548 A-145109
GAGUCCACACCGCUGAGCU 157 1650-1668 A-145110 AGCUCAGCGGUGUGGACUC 339
1650-1668 AD-72549 A-145111 UGAGCUGAACUGACAGGCC 158 1663-1681 A-145112
GGCCUGUCAGUUCAGCUCA 340 1663-1681 AD-72550 A-145113
UGACAGGCCAGUGGGGGGC 159 1673-1691 A-145114 GCCCCCACUGGCCUGUCA 341
1673-1691 AD-72551 A-145115 UGGGGGGCAGGGGUGCGCC 160 1684-1702 A-145116
GGCGCACCCCUGCCCCCCA 342 1684-1702 AD-72552 A-145117
GGUGCGCCUCCUCUGCCCU 161 1695-1713 A-145118 AGGGCAGAGGAGGCGCACC 343
1695-1713 AD-72553 A-145119 UCUGCCCUGCCCACCAGCC 162 1706-1724 A-145120
GGCUGGUGGGCAGGGCAGA 344 1706-1724 AD-72554 A-145121
ACCAGCCUGUGAUUUGAUG 163 1718-1736 A-145122 CAUCAAUACACAGGCUGGU 345
1718-1736 AD-72555 A-145123 UGAUUUGAUGGGGUCUUCA 164 1727-1745 A-145124
UGAAGACCCCAUCAAUUA 346 1727-1745 AD-72556 A-145125
GUCUUCAUUGUCCAGAAAU 165 1739-1757 A-145126 AUUUCUGGACAAUGAAGAC 347
1739-1757 AD-72557 A-145127 UCCAGAAAUACCUCUCCC 166 1749-1767 A-145128
GGGAGGAGGUUUUCUGGA 348 1749-1767 AD-72558 A-145129
UCCUCCCGCUGACUGCCCC 167 1761-1779 A-145130 GGGGCAGUCAGCGGGAGGA 349
1761-1779 AD-72559 A-145131 ACUGCCCCAGAGCCUGAAA 168 1772-1790 A-145132
UUUCAGGCUCUGGGGCAGU 350 1772-1790 AD-72560 A-145133
AGCCUGAAAGUCUCACCCU 169 1782-1800 A-145134 AGGGUGAGACUUUCAGGCU 351
1782-1800 AD-72561 A-145135 UCACCCUUGGAGCCCACCU 170 1794-1812 A-145136
AGGUGGGCUCCAAGGGUGA 352 1794-1812 AD-72562 A-145137
CCCACCUUGGAAUUAAGGG 171 1806-1824 A-145138 CCCUAAUUCCAAGGUGGG 353
1806-1824 AD-72563 A-145139 GAAUUAAGGGCGUGCCUCA 172 1815-1833 A-145140
UGAGGCACGCCCUUAAUUC 354 1815-1833 AD-72564 A-145141
UGCCUCAGCCACAAUGUG 173 1827-1845 A-145142 CACAUUUGUGGCUGAGGCA 355
1827-1845 AD-72565 A-145143 ACAAUGUGACCCAGGAUA 174 1837-1855 A-145144
UAUCCUGGGUCACAUUUGU 356 1837-1855 AD-72566 A-145145
CAGGAUACAGAGUGUUGCU 175 1849-1867 A-145146 AGCAACACUCUGUAUCCUG 357
1849-1867 AD-72567 A-145147 AGUGUUGCUGUCCUCAGGG 176 1859-1877 A-145148

CCGAGCAGCAGCA 358 1859-1877 AD-72568 A-145149
 CCUCAGGGAGGUCCGAUCU 177 1870-1888 A-145150 AGAUCGGACCUCUCCUGAGG 359
 1870-1888 AD-72569 A-145151 UCCGAUCUGGAACACAUAU 178 1881-1899 A-145152
 AUAUGUGUUCCAGAUCGGA 360 1881-1899 AD-72570 A-145153
 ACACAUAUUGGAAUUGGGG 179 1892-1910 A-145154 CCCCAAUUCCAAUAUGUGU 361
 1892-1910 AD-72571 A-145155 UUGGGGCCAACUCCAAUAU 180 1905-1923 A-145156
 AUUAUUGGAGUUGGCCCCAA 362 1905-1923 AD-72572 A-145157
 ACUCCAAUAUAGGGUGGGU 181 1914-1932 A-145158 ACCCACCCUAUAUUGGAGU 363
 1914-1932 AD-72573 A-145159 GUGGGUAAGGCCUUAUAAU 182 1927-1945 A-145160
 AUUAUAAGGCCUUAACCCAC 364 1927-1945 AD-72574 A-145161
 CCUUAUAAUGUAAAGAGCA 183 1937-1955 A-145162 UGCUCUUUACAUAUAAGG 365
 1937-1955 AD-72575 A-145163 AAGAGCAUAUAAUGUAAAG 184 1949-1967 A-145164
 CUUUACAUAUAUAGCUCUU 366 1949-1967 AD-72576 A-145165
 UAAUGUAAAGGGCUUUAGA 185 1958-1976 A-145166 UCUAAGCCCUUACAUAUA 367
 1958-1976 AD-72577 A-145167 UUUAGAGUGAGACAGACCU 186 1971-1989 A-145168
 AGGUCUGUCUCACUCUAAA 368 1971-1989 AD-72578 A-145169
 ACAGACCUGGAUUA AAAAUC 187 1982-2000 A-145170 GAUUUUA AUCCAGGUCUGU 369
 1982-2000 AD-72579 A-145171 UAAAAUCUGCCAUAUAAU 188 1993-2011 A-145172
 AUUAAAUGGCAGAUUUUAA 370 1993-2011 AD-72580 A-145173
 CAUUUAAUUAAGCUGCAUAU 189 2004-2022 A-145174 AUAUGCAGCUAAUUA AAAUG 371
 2004-2022 AD-72581 A-145177 CUUAGGGUACAGCACUUA 190 2026-2044 A-145178
 UUAAGUGCUGUACCCUAAG 372 2026-2044 AD-72582 A-145179
 CAGCACUUAACGCAAUCUG 191 2035-2053 A-145180 CAGAUUGCGUUAAGUGCUG 373
 2035-2053 AD-72583 A-145181 GCAAUCUGCCUCAAUUUCU 192 2046-2064 A-145182
 AGAAAUUGAGGCAGAUUGC 374 2046-2064 AD-72584 A-145183
 AAUUUCUUCAUCUGUCAAA 193 2058-2076 A-145184 UUUGACAGAUGAAGAAAUU 375
 2058-2076 AD-72590 A-145187 GAACCAAUUCUGCUUGGCU 194 2079-2097 A-145188
 AGCCAAGCAGAAUUGGUUC 376 2079-2097 AD-72591 A-145189
 UUGGCUACAGAAUUAUUGU 195 2092-2110 A-145190 ACAAUAAUUCUGUAGCCAA 377
 2092-2110 AD-72592 A-145191 AUUAUUGUGAGGAUAAAAU 196 2103-2121 A-145192
 AUUUUAUCCUCACAAUAUAU 378 2103-2121 AD-72593 A-145193
 AGGAUAAAAUCAUAUAUAU 197 2112-2130 A-145194 UUAUAUAUGAUUUUAUCCU 379
 2112-2130 AD-72594 A-145195 UAUUA AAAAUGCCCAGCAU 198 2124-2142 A-145196
 AUGCUGGGCAUUUUAUAUA 380 2124-2142

TABLE-US-00005 TABLE 4 KHK Single Dose Screen in Hep3B Data are expressed as percent
 message remaining relative to AD-1955 non-targeting control Duplex ID 10 nM AVG 10 nM SD
 Range AD-72250 94.30 16.80 13-31 AD-72251 75.82 25.36 23-41 AD-72252 68.85 8.30 34-52
 AD-72253 62.42 20.81 44-62 AD-72254 55.80 19.86 56-74 AD-72255 52.78 18.60 68-86 AD-
 72256 66.12 24.48 77-95 AD-72257 33.16 13.92 89-107 AD-72258 90.29 26.86 99-117 AD-
 72259 117.30 21.51 111-129 AD-72260 125.66 49.57 122-140 AD-72261 91.29 39.54 145-163
 AD-72262 61.75 15.29 156-174 AD-72263 58.14 22.74 166-184 AD-72264 40.11 11.92 176-194
 AD-72265 83.05 19.86 189-207 AD-72266 91.87 34.68 199-217 AD-72267 106.26 16.23 211-229
 AD-72268 96.90 20.89 222-240 AD-72269 78.58 16.73 233-251 AD-72270 130.44 33.41 244-262
 AD-72271 80.59 19.99 255-273 AD-72272 44.06 22.56 264-282 AD-72273 109.26 37.17 275-293
 AD-72274 117.78 17.41 286-304 AD-72275 100.90 18.81 299-317 AD-72276 132.55 40.22 310-
 328 AD-72277 76.26 23.91 319-337 AD-72278 120.43 29.66 341-359 AD-72279 55.06 26.33 352-
 370 AD-72280 105.22 15.92 364-382 AD-72281 103.75 11.62 376-394 AD-72282 99.25 17.09
 386-404 AD-72283 88.63 21.23 398-416 AD-72284 102.01 15.30 409-427 AD-72285 90.62 21.65
 419-437 AD-72286 100.16 14.03 431-449 AD-72287 56.78 3.45 441-459 AD-72288 87.30 18.82
 453-471 AD-72289 110.55 17.40 463-481 AD-72290 40.35 9.04 474-492 AD-72291 77.68 8.34

486-504 AD-72292 22.36 495-513 AD-72293 39.06 7.41 508-526 AD-72294 85.87 7.80
519-537 AD-72295 33.96 7.27 529-547 AD-72296 96.53 21.78 541-559 AD-72297 100.84 27.67
552-570 AD-72298 44.37 8.01 562-580 AD-72299 59.40 15.10 573-591 AD-72300 81.29 11.12
583-601 AD-72301 104.74 25.20 594-612 AD-72302 60.08 2.58 605-623 AD-72303 29.22 5.19
616-634 AD-72304 32.08 8.14 628-646 AD-72305 65.71 21.88 639-657 AD-72306 77.49 18.16
649-667 AD-72307 109.94 17.59 661-679 AD-72308 55.01 7.88 672-690 AD-72309 47.80 8.17
682-700 AD-72310 66.01 13.17 694-712 AD-72311 35.44 5.89 705-723 AD-72312 56.48 8.52
726-744 AD-72313 48.74 14.39 739-757 AD-72314 60.81 15.77 750-768 AD-72315 41.93 6.20
760-778 AD-72316 49.25 12.18 771-789 AD-72317 49.74 13.54 781-799 AD-72318 83.39 7.50
793-811 AD-72319 25.46 2.59 804-822 AD-72320 84.90 20.01 816-834 AD-72321 63.72 20.06
825-843 AD-72322 40.03 7.16 837-855 AD-72323 65.00 4.11 849-867 AD-72324 77.03 14.56
860-878 AD-72325 58.38 8.54 870-888 AD-72326 60.72 7.58 882-900 AD-72327 46.52 16.36
892-910 AD-72328 69.89 6.20 914-932 AD-72329 87.57 17.58 926-944 AD-72330 52.91 7.57
937-955 AD-72331 87.15 8.65 946-964 AD-72332 34.22 11.41 959-977 AD-72333 56.06 15.55
970-988 AD-72334 92.79 19.23 980-998 AD-72335 49.64 12.66 992-1010 AD-72336 72.76 24.19
1003-1021 AD-72337 44.07 9.09 1013-1031 AD-72338 40.67 9.91 1023-1041 AD-72339 68.84
13.54 1035-1053 AD-72498 89.12 19.36 1047-1065 AD-72499 29.04 8.75 1069-1087 AD-72500
29.75 16.22 1080-1098 AD-72501 36.97 13.21 1090-1108 AD-72502 27.57 11.60 1111-1129 AD-
72503 45.60 7.81 1122-1140 AD-72504 77.11 17.10 1135-1153 AD-72505 78.01 2.91 1145-1163
AD-72506 24.58 4.99 1155-1173 AD-72507 35.39 4.85 1166-1184 AD-72508 37.27 2.49 1178-
1196 AD-72509 51.05 15.67 1188-1206 AD-72510 91.60 32.85 1201-1219 AD-72511 69.19 17.33
1212-1230 AD-72512 31.86 7.21 1221-1239 AD-72513 28.70 6.98 1234-1252 AD-72514 32.78
9.58 1243-1261 AD-72515 50.05 11.73 1254-1272 AD-72516 51.22 10.85 1266-1284 AD-72517
49.12 6.15 1276-1294 AD-72518 67.90 12.78 1288-1306 AD-72519 59.04 17.32 1300-1318 AD-
72520 72.96 32.80 1309-1327 AD-72521 46.81 8.84 1320-1338 AD-72522 31.78 14.81 1332-1350
AD-72523 72.17 5.70 1343-1361 AD-72524 76.18 23.61 1354-1372 AD-72525 91.94 29.44 1364-
1382 AD-72526 79.86 21.67 1375-1393 AD-72527 59.88 21.52 1388-1406 AD-72528 91.91 14.45
1397-1415 AD-72529 66.07 8.24 1408-1426 AD-72530 78.63 10.38 1442-1460 AD-72531 102.17
14.26 1454-1472 AD-72532 102.14 14.06 1465-1483 AD-72533 75.21 22.35 1474-1492 AD-72534
93.37 36.21 1486-1504 AD-72535 107.76 15.58 1496-1514 AD-72536 87.72 14.74 1507-1525 AD-
72537 104.07 22.46 1520-1538 AD-72538 102.86 18.44 1530-1548 AD-72539 85.81 8.99 1540-
1558 AD-72540 81.34 18.04 1564-1582 AD-72541 97.44 19.13 1574-1592 AD-72542 95.88 10.07
1584-1602 AD-72543 117.02 26.69 1597-1615 AD-72544 81.45 56.89 1608-1626 AD-72545 77.35
7.88 1618-1636 AD-72546 107.55 11.98 1629-1647 AD-72547 77.98 5.37 1640-1658 AD-72548
63.79 21.20 1650-1668 AD-72549 101.19 4.19 1663-1681 AD-72550 106.89 12.57 1673-1691 AD-
72551 88.46 5.34 1684-1702 AD-72552 111.87 11.75 1695-1713 AD-72553 136.07 28.97 1706-
1724 AD-72554 122.41 4.39 1718-1736 AD-72555 77.55 21.53 1727-1745 AD-72556 73.87 38.83
1739-1757 AD-72557 93.39 9.13 1749-1767 AD-72558 107.67 18.87 1761-1779 AD-72559 88.92
7.98 1772-1790 AD-72560 95.18 6.86 1782-1800 AD-72561 87.30 18.93 1794-1812 AD-72562
95.70 7.13 1806-1824 AD-72563 93.46 9.82 1815-1833 AD-72564 117.28 27.22 1827-1845 AD-
72565 63.45 22.28 1837-1855 AD-72566 102.19 40.02 1849-1867 AD-72567 66.73 28.77 1859-
1877 AD-72568 105.83 32.44 1870-1888 AD-72569 99.51 17.09 1881-1899 AD-72570 110.18
25.33 1892-1910 AD-72571 96.82 17.18 1905-1923 AD-72572 71.24 22.70 1914-1932 AD-72573
102.94 14.30 1927-1945 AD-72574 91.16 39.27 1937-1955 AD-72575 90.54 24.48 1949-1967 AD-
72576 99.62 16.22 1958-1976 AD-72577 97.43 21.17 1971-1989 AD-72578 96.51 28.91 1982-
2000 AD-72579 113.62 31.85 1993-2011 AD-72580 74.56 9.76 2004-2022 AD-72581 94.40 14.63
2026-2044 AD-72582 71.79 18.70 2035-2053 AD-72583 85.71 17.88 2046-2064 AD-72584 113.53
44.49 2058-2076 AD-72590 86.23 8.29 2079-2097 AD-72591 101.14 18.17 2092-2110 AD-72592
94.39 16.72 2103-2121 AD-72593 87.27 14.27 2112-2130 AD-72594 84.50 30.12 2124-2142
TABLE-US-00006 TABLE 5 KHK Modified Sequences Sense SEQ Antisense SEQ SEQ

Duplex Oligo ID Oligo ID Name Sense sequence NO
mRNA target sequence NO AD-72250 A-144811 AGGCAGGGCUGCAGAUGCgdTdT 381
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144815 GCCCAGCUGUACCUCGCGuTdT 383 A-144816
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144819 CCGGGUCGGGAGUCGGAGAdTdT 385 A-144820
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144825 AGUGCGGGGCAAGUAGCGCdTdT 388 A-144826
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 2220 agagctgggc ctgcatctgc agccctgcct gaagac 2256

EQUIVALENTS

[0580] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments and methods described herein. Such equivalents are intended to be encompassed by the scope of the following claims.

Claims

1. A double stranded ribonucleic acid (dsRNA) agent for inhibiting expression of a ketohexokinase (KHK) gene, wherein the dsRNA agent comprises a sense strand and an antisense strand forming a double stranded region, wherein the sense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of any one of nucleotides 89-107, 176-194, 264-282, 474-492, 508-526, 529-547, 562-580, 616-646, 682-700, 705-723, 705-757, 705-799, 739-757, 739-799, 760-799, 804-822, 837-855, 892-910, 959-977, 992-1010, 922-1041, 1013-1041, 1069-1108, 1169-1140, 1111-1140, 1155-1196, 1221-1261, 1267-1294, or 1320-1350 of SEQ ID NO:1, and the antisense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO:2.

2.-4. (canceled)

5. The dsRNA agent of claim 1, wherein the dsRNA comprises at least one modified nucleotide.

6. (canceled)

7. A double stranded ribonucleic acid (dsRNA) agent for inhibiting expression of a ketohexokinase (KHK) gene, wherein the dsRNA agent comprises a sense strand and an antisense strand forming a double stranded region, wherein the sense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of any one of nucleotides 89-107, 176-194, 264-282, 474-492, 508-526, 529-547, 562-580, 616-646, 682-700, 705-723, 705-757, 705-799, 739-757, 739-799, 760-799, 804-822, 837-855, 892-910, 959-977, 992-1010, 922-1041, 1013-1041, 1069-1108, 1169-1140, 1111-1140, 1155-1196, 1221-1261, 1267-1294, or 1320-1350 of SEQ ID NO:1 and the antisense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of SEQ ID NO:2, wherein all of the nucleotides of the sense strand and all of the nucleotides of the antisense strand are modified nucleotides, and wherein the sense strand is conjugated to a ligand attached at the 3'-terminus.

8. (canceled)

9. The dsRNA agent of claim 7, wherein at least one of the modified nucleotides is selected from the group consisting of a deoxy-nucleotide, a 3'-terminal deoxy-thymine (dT) nucleotide, a 2'-O-methyl modified nucleotide, a 2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an unlocked nucleotide, a conformationally restricted nucleotide, a constrained ethyl nucleotide, an abasic nucleotide, a 2'-amino-modified nucleotide, a 2'-O-allyl-modified nucleotide, 2'-C-alkyl-modified nucleotide, 2'-hydroxly-modified nucleotide, a 2'-methoxyethyl modified nucleotide, a 2'-O-alkyl-modified nucleotide, a morpholino nucleotide, a phosphoramidate, a non-natural base comprising nucleotide, a tetrahydropyran modified

nucleotide, a 1,5-anhydrohexitol modified nucleotide, a cyclohexenyl modified nucleotide, a nucleotide comprising a phosphorothioate group, a nucleotide comprising a methylphosphonate group, a nucleotide comprising a 5'-phosphate, and a nucleotide comprising a 5'-phosphate mimic.

10.-13. (canceled)

14. The dsRNA agent of claim 1, wherein each strand is no more than 30 nucleotides in length.

15. The dsRNA agent of claim 1, wherein at least one strand comprises a 3' overhang of at least 1 nucleotide.

16. (canceled)

17. The dsRNA agent of claim 1, further comprising a ligand.

18. The dsRNA agent of claim 17, wherein the ligand is conjugated to the 3' end of the sense strand of the dsRNA agent.

19. The dsRNA agent of claim 17, wherein the ligand is an N-acetylgalactosamine (GalNAc) derivative.

20. The dsRNA agent of claim 19, wherein the ligand is ##STR00017##

21. The dsRNA agent of claim 19, wherein the dsRNA agent is conjugated to the ligand as shown in the following schematic ##STR00018## and, wherein X is O or S.

22. The dsRNA agent of claim 21, wherein the X is O.

23.-35. (canceled)

36. The dsRNA agent of claim 1, wherein the double stranded region is 15-30 nucleotide pairs in length.

37.-41. (canceled)

42.-45. (canceled)

46. The dsRNA agent of claim 17, wherein the ligand is one or more GalNAc derivatives attached through a monovalent, a bivalent or a trivalent branched linker.

47.-49. (canceled)

50. The dsRNA agent of claim 1, wherein the agent further comprises at least one phosphorothioate or methylphosphonate internucleotide linkage.

51.-75. (canceled)

76. A double stranded ribonucleic acid (dsRNA) agent for inhibiting the expression of a ketohexokinase (KHK) gene in a cell, wherein the dsRNA agent comprises a sense strand and an antisense strand forming a double stranded region, wherein the sense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of any one of nucleotides 89-107, 176-194, 264-282, 474-492, 508-526, 529-547, 562-580, 616-646, 682-700, 705-723, 705-757, 705-799, 739-757, 739-799, 760-799, 804-822, 837-855, 892-910, 959-977, 992-1010, 922-1041, 1013-1041, 1069-1108, 1169-1140, 1111-1140, 1155-1196, 1221-1261, 1267-1294, or 1320-1350 of the nucleotide sequence of SEQ ID NO:1 and the antisense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the corresponding nucleotide sequence of SEQ ID NO:2, wherein substantially all of the nucleotides of the sense strand comprise a modification selected from the group consisting of a 2'-O-methyl modification and a 2'-fluoro modification, wherein the sense strand comprises two phosphorothioate internucleotide linkages at the 5'-terminus, wherein substantially all of the nucleotides of the antisense strand comprise a modification selected from the group consisting of a 2'-O-methyl modification and a 2'-fluoro modification, wherein the antisense strand comprises two phosphorothioate internucleotide linkages at the 5'-terminus and two phosphorothioate internucleotide linkages at the 3'-terminus, and wherein the sense strand is conjugated to one or more GalNAc derivatives attached through a monovalent, a bivalent or a trivalent branched linker at the 3'-terminus.

77. (canceled)

78. (canceled)

79. An isolated cell containing the dsRNA agent of claim 1.

- 80.** A pharmaceutical composition for inhibiting expression of a ketohexokinase (KHK) gene comprising the dsRNA agent of claim 1.
- 81.** (canceled)
- 82.** (canceled)
- 83.** A method of inhibiting expression of a ketohexokinase (KHK) gene in a cell, the method comprising: (a) contacting the cell with the double stranded RNAi agent of claim 1; and (b) maintaining the cell produced in step (a) for a time sufficient to obtain degradation of the mRNA transcript of the KHK gene, thereby inhibiting expression of the KHK gene in the cell.
- 84.-86.** (canceled)
- 87.** A method of treating a subject having a disease or disorder that would benefit from reduction in expression of ketohexokinase (KHK), the method comprising administering to the subject a therapeutically effective amount of the dsRNA agent of claim 1, thereby treating the subject.
- 88.** (canceled)
- 89.** (canceled)
- 90.** The method of claim 87, wherein the disorder is a KHK-associated disease.
- 91.-102.** (canceled)
- 103.** The method of claim 87, wherein the subject is human.
- 104.** (canceled)
- 105.** The method of claim 87, further comprising administering an agent for the treatment of a KHK-associated disease.
- 106.-111.** (canceled)
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