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(71) Applicant: **ALNYLAM PHARMACEUTICALS, INC.** [US/US]; 675 West Kendall Street, Henri A. Termeer Square, Cambridge, MA 02142 (US).

(72) Inventors: **PENG, Haiyan**; 675 West Kendall Street, Henri A. Termeer Square, Cambridge, MA 02142 (US). **BOSTWICK, Bret, Lee**; 675 West Kendall Street, Henri A. Termeer Square, Cambridge, MA 02142 (US). **MCININCH, James, D.**; 675 West Kendall Street, Henri A. Termeer Square, Cambridge, MA 02142 (US). **SCHLEGEL, Mark, K.**; 675 West Kendall Street, Henri A. Termeer Square, Cambridge, MA 02142 (US). **CASTORENO, Adam**; 675 West Kendall Street, Henri A. Termeer Square, Cambridge, MA 02142 (US). **NGUYEN, Tuyen, M.**; 675 West Kendall Street, Henri A. Termeer Square, Cambridge, MA 02142 (US).

(74) Agent: **ZACHARAKIS, Maria, Laccotripe** et al.; McCarter & English, LLP, 265 Franklin Street, Boston, MA 02110 (US).

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(54) Title: PRION PROTEIN (PRNP) IRNA COMPOSITIONS AND METHODS AND METHODS OF USE THEREOF

(57) Abstract: The disclosure relates to double stranded ribonucleic acid (dsRNAi) agents and compositions targeting a prion protein (PRNP) gene, as well as methods of inhibiting expression of a PRNP gene and methods of treating subjects having a PRNP-associated disease or disorder, e.g., Prion diseases, using such dsRNAi agents and compositions.

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PRION PROTEIN (PRNP) IRNA COMPOSITIONS AND METHODS OF USE THEREOF

RELATED APPLICATIONS

This application claims the benefit of priority to U.S. Provisional Application No. 63/153,411 filed on February 25, 2021, the entire contents of which is incorporated herein by reference.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on February 17, 2022, is named 121301_13920_SL.txt and is 185,376 bytes in size.

BACKGROUND OF THE INVENTION

Prion diseases, or transmissible spongiform encephalopathies (TSEs), are a group of neurodegenerative disorders that affect both humans and animals. Prion disease typically progresses quickly with rapid cognitive decline, and is fatal within a few months of symptoms onset. Prion disease is caused by misfolding of the cellular prion protein highly expressed in neuronal and glial cells, PrP^{C}, into an autocatalytically self-propagating conformer called the scrapie prion protein, or PrP^{Sc}, which is insoluble and relatively resistant to degradation by proteases. The accumulation of PrP^{Sc} lead to neurodegeration and/or neuronal injury and loss.

There are three different subtypes of prion disease categorized by how the disease is contracted: sporadic, genetic and acquired. Inherent mutations in the prion protein gene (*PRNP*) cause the genetic subtype of prion disease, such as, Creutzfeldt-Jakob Disease (CJD), Gerstmann-Straussler-Scheinker Disease (GSS), and Fatal Familial Insomnia (FFI). More than 60 variants of PRNP have been identified. In pateints with genetic prion diseases, the mutations in the *PRNP* gene cause the cells to produce PrP^{Sc}. The acquired subtype of prion disease is casued by prion proteins and included Mad Cow Disease and Kuru. The sporadic subtype of prion disease is caused by unknown factors and accounts for approximately 85% of all prion disease.

Previous studies have shown that homozygous deletion of PrP prevents prion infection in mice and goats (Büeler H., Aguzzi A., *et al. Cell.* 1993; 73:1339–1347; Salvesen Ø., *et al. Vet. Res.* 2020; 51:1), while heterozygous PrP knockout delays development of disease following prion infection (Salvesen Ø., *et al. Vet. Res.* 2020; 51:1; Büeler H., *et al. Mol. Med.* 1994; 1:19–30; Prusiner S.B., *et al. Proc. Natl. Acad. Sci. U.S.A.* 1993; 90:10608–10612; Sakaguchi S., *et al. J. Virol.* 1995; 69:7586–7592) and transgenic PrP overexpression accelerates it (Fischer M., *et al. EMBO J.* 1996; 15:1255–1264.), providing genetic evidence of a dose-response relationship between PrP dosage and disease susceptibility. Conditional knockout systems have confirmed that post-natal depletion provides a significant survival benefit (Mallucci G., *et al. Science*. 2003; 302:871–874; Safar J.G., *et al. J. Gen. Virol.* 2005; 86:2913–2923.). Knockout animals are healthy (Büeler H., *et al. Nature*. 1992; 356:577–582; Richt J.A., *et al. Nat. Biotechnol.* 2007; 25:132–138; Benestad S.L., *et al. Vet. Res.* 2012; 43:87.). Furthermore,

heterozygous inactivating mutations also appear to be tolerated in humans (Minikel E.V., *et al. Sci. Transl. Med.* 2016; 8:322ra9; Minikel E.V., *et al. Nature*. 2020; 581:459–464), minimizing any concern about on-target toxicity of pharmacologic PrP lowering.

Effective treatments for PRNP-associated diseases, such as prion diseases, are currently not available and any treatments that are available are palliative. Thus, there remains a need for agents that can selectively and efficiently silence the PRNP gene using the cell's own RNAi machinery that have both high biological activity and *in vivo* stability, and that can effectively inhibit expression of a target PRNP gene.

BRIEF SUMMARY OF THE INVENTION

The present disclosure provides RNAi agent compositions which effect the RNA-induced silencing complex (RISC)-mediated cleavage of RNA transcripts of a prion protein (PRNP) gene. The PRNP gene may be within a cell, *e.g.,* a cell within a subject, such as a human. The present disclosure also provides methods of using the RNAi agent compositions of the disclosure for inhibiting the expression of a PRNP gene or for treating a subject who would benefit from inhibiting or reducing the expression of a PRNP gene, *e.g.,* a subject suffering or prone to suffering from a PRNP-associated disease, *e.g.,* a prion disease, such as a genetic prion disease, *e.g.,* familial Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI); a sporadic prion disease, *e.g.,* sporadic Creutzfeldt-Jakob disease, Sporadic Fatal Insomnia (sFI), and Variably Proteinase-Sensitive Prionopathy (VPSPr), or an acquired prion disease, *e.g.,* Iatrogenic CJD (iCJD), Kuru, and Variant CJD (vCJD).

Accordingly, in one aspect, the instant disclosure provides a double stranded ribonucleic acid (RNAi) agent for inhibiting expression of a PRNP 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, *e.g.,* 15, 16, 17, 18, 19, 20, or 21, contiguous nucleotides differing by no more than 0, 1, 2, or 3 nucleotides from the nucleotide sequence of SEQ ID NO:1 and the antisense strand comprises at least 15, *e.g.,* 15, 16, 17, 18, 19, 20, or 21, contiguous nucleotides differing by no more than 1, 2, or 3 nucleotides from the nucleotide sequence of SEQ ID NO:5, and wherein the sense strand, the antisense strand, or both the sense strand and the antisense strand is conjugated to one or more lipophilic moieties.

In certain embodiments, the sense strand comprises at least 15 contiguous nucleotides of the nucleotide sequence of SEQ ID NO:1 and the antisense strand comprises at least 15 contiguous nucleotides of the nucleotide sequence of SEQ ID NO:5. In certain embodiments, the sense strand comprises at least 17 contiguous nucleotides of the nucleotide sequence of SEQ ID NO:1 and the antisense strand comprises at least 17 contiguous nucleotides of the nucleotide sequence of SEQ ID NO:5. In certain embodiments, the sense strand comprises at least 19 contiguous nucleotides of the nucleotide sequence of SEQ ID NO:1 and the antisense strand comprises at least 19 contiguous nucleotides of the nucleotide sequence of SEQ ID NO:5.

In some embodiments, the nucleotide sequence of the sense strand comprises any one of the sense strand nucleotide sequences in any one of Tables 2-3.

In another aspect, the instant disclosure provides a double stranded ribonucleic acid (RNAi) agent for inhibiting expression of a PRNP gene, where the RNAi agent includes a sense strand and an antisense strand forming a double stranded region, wherein the antisense strand comprises a region of complementarity to an mRNA encoding PRNP, and wherein the region of complementarity comprises at least 15, *e.g.,* 15, 16, 17, 18, 19, 20, or 21, contiguous nucleotides differing by no more than 3 nucleotides (*i.e.,* differing by 3, 2, 1, or 0 nucleotides) from any one of the antisense sequences listed in any one of Table 2-3, and wherein the sense strand, the antisense strand, or both the sense strand and the antisense strand is conjugated to one or more lipophilic moieties.

In certain embodiments, the antisense strand includes a region of complementarity which includes at least 15 contiguous nucleotides of any one of the antisense sequences listed in any one of Table 2-3. In certain embodiments, the antisense strand includes a region of complementarity which includes at least 17 contiguous nucleotides of any one of the antisense sequences listed in any one of Table 2-3. In certain embodiments, the antisense strand includes a region of complementarity which includes at least 19 contiguous nucleotides of any one of the antisense sequences listed in any one of Tables 2-3. In certain embodiments, the antisense strand includes a region of complementarity which includes at least 20 contiguous nucleotides of any one of the antisense sequences listed in any one of Table 2-3. In certain embodiments, the antisense strand includes a region of complementarity which includes at least 21 contiguous nucleotides of any one of the antisense sequences listed in any one of Table 2-3. In certain embodiments, thymine-to-uracil or uracil-to-thymine differences between aligned (compared) sequences are not counted as nucleotides that differ between the aligned (compared) sequences.

In one aspect, the present invention provides a double stranded ribonucleic acid (dsRNA) agent for inhibiting expression of prion protein (PRNP) 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, *e.g.,* 15, 16, 17, 18, 19, 20, or 21, contiguous nucleotides differing by no more than three nucleotides from any one of the nucleotide sequence of nucleotides 502-524, 507-529, 502-529, 540-562, 566-588, 575-597, 576-598, 589-611, 575-611, 593-615, 594-616, 600-622, 593-622, 650-672, 858-880, 976-998, 1100-1122, 1126-1148, 1220-1242, 1221-1243, 1220-1243, 1304-1326, 1328-1350, 1410-1432, 1445-1467, 1481-1503, 1532-1554, 1610-1632, 1615-1637, 1617-1639, 1621-1643, 1610-1643, 1610-1639, 1688-1710, 1694-1714, , 1830-1852, 1831-1853, 1854-1876, 1830-1853, 1872-1894, 1873-1895, 1938-1960, 2011-2033, 2015-2037, 2031-2053, 2034-2056, 2069-2091, 2076-2098, 2077-2099, 1872-1895, 2011-2037, 2069-2099, 2079-2101, 2031-2056, 2069-2098, 2138-2160, 2143-2165, 2158-2180, 2167-2189, 2168-2190, 2170-2192, 2174-2196, 2175-2197, 2177-2199, 2185-2207, 2189-2211, 2196-2218, 2200-2222, 2202-2224, 2221-2243, 2222-2244, 2223-2245, 2238-2260, 2241-2263, 2242-2264, 2138-2264, 2257-2358, 2174-2245, 2174-2224, 2138-2196, 2177-2224, 2223-2245, 2138-2189, 2177-2211, 2196-2224, 2257-2279, 2258-2280, 2335-2537, 2336-2358, 2257-2358, 2342-2364, 2397-2419, 2398-2420, 2399-2421, 2400-2422, 2401-2423, 2404-2426, 2397-2426, 2394-2423, 2397-2421, 2399-

2426, and 2398-2421 of SEQ ID NO: 1, and the antisense strand comprises at least 15, *e.g.,* 15, 16, 17, 18, 19, 20, or 21, contiguous nucleotides from the corresponding nucleotide sequence of SEQ ID NO:5, and wherein the sense strand, the antisense strand, or both the sense strand and the antisense strand is conjugated to one or more lipophilic moieties.

In one aspect, the present invention provides a double stranded ribonucleic acid (dsRNA) agent for inhibiting expression of prion protein (PRNP) 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, *e.g.,* 15, 16, 17, 18, 19, 20, or 21, contiguous nucleotides differing by no more than three nucleotides from any one of the nucleotide sequence of nucleotides 530-570, 535-565, 539-561, 540-562, 555-605, 560-605, 560-600, 566-588, 567-589, 568-590, 569-591, 570-592, 575-597, 580-620-585-620, 589-611, 590-612, 591-613, 592-614, 593-615, 594-616, 600-650, 610-650, 613-635, 614-636, 615-637, 616-638, 617-639, 618-640, 640-680, 649-671, 650-672, 651-673, 670-700, 674-696, 675-697, 795-830, 804-826, 2325-2375, 2325-2370, 2330-2370, 2335-2357, 2336-2358, 2337-2359, 2338-2360, 2339-2361, or 2340-2362 of SEQ ID NO: 1, and the antisense strand comprises at least 15, *e.g.,* 15, 16, 17, 18, 19, 20, or 21, contiguous nucleotides from the corresponding nucleotide sequence of SEQ ID NO:5, and wherein the sense strand, the antisense strand, or both the sense strand and the antisense strand is conjugated to one or more lipophilic moieties .

In one aspect, the present invention provides a double stranded ribonucleic acid (dsRNA) agent for inhibiting expression of prion protein (PRNP) 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, *e.g.,* 15, 16, 17, 18, 19, 20, or 21, contiguous nucleotides differing by no more than three nucleotides from any one of the nucleotide sequence of nucleotides 502-524, 540-562, 589-611, 594-616, 650-672, 1445-1467, 1688-1710, 2011-2033, 2031-2053, 2185-2207, 2222-2244, 2336-2358, 2339-2361, 2399-2421 or 2258-2280 of SEQ ID NO: 1, and the antisense strand comprises at least 15 contiguous nucleotides from the corresponding nucleotide sequence of SEQ ID NO:5, and wherein the sense strand, the antisense strand, or both the sense strand and the antisense strand is conjugated to one or more lipophilic moieties.

In some embodiments, the antisense strand comprises at least 15, *e.g.,* 15, 16, 17, 18, 19, 20, or 21, contiguous nucleotides differing by no more than three nucleotides from any one of the antisense strand nucleotide sequences of a duplex selected from the group consisting of AD-1070511, AD-1070462, AD-1070553, AD-1072048, AD-1070516 and AD-1072050, AD-1071769, AD-1071949, AD-1070444, AD-1071505, AD-1071912, AD-1071789, AD-1071265, AD-1072084, and AD-1071985.

In some embodiments, the lipophilic moiety is conjugated *via* a linker or a carrier.

The sense strand, the antisense strand, or both the sense strand and the antisense strand may be conjugated to one or more lipophilic moieties. In some embodiments, the lipophilic moiety is conjugated to one or more internal positions in the double stranded region of the dsRNA agent, *e.g.,* the one or more lipophilic moieties may be conjugated to one or more internal positions on the antisense strand. In some

embodiments, the one or more lipophilic moieties are conjugated to one or more internal positions on at least one strand *via* a linker or carrier.

In some embodiments, lipophilicity of the lipophilic moiety, measured by logKow, exceeds 0.

In some embodiments, the hydrophobicity of the dsRNA agent, measured by the unbound fraction in a plasma protein binding assay of the dsRNA agent, exceeds 0.2. In some embodiments, the plasma protein binding assay is an electrophoretic mobility shift assay using human serum albumin protein.

In some embodiments, the internal positions include all positions except the terminal two positions from each end of the sense strand or the antisense strand. In other embodiments, the internal positions include all positions except the terminal three positions from each end of the sense strand or the antisense strand.

In some embodiments, the internal positions exclude a cleavage site region of the sense strand, *e.g.*, the internal positions include all positions except positions 9-12, counting from the 5'-end of the sense strand or the internal positions include all positions except positions 11-13, counting from the 3'-end of the sense strand.

In some embodiments, the internal positions exclude a cleavage site region of the antisense strand. In other embodiments, the internal positions include all positions except positions 12-14, counting from the 5'-end of the antisense strand. In some embodiments, the internal positions include all positions except positions 11-13 on the sense strand, counting from the 3'-end, and positions 12-14 on the antisense strand, counting from the 5'-end.

In some embodiments, the one or more lipophilic moieties are conjugated to one or more of the internal positions selected from the group consisting of positions 4-8 and 13-18 on the sense strand, and positions 6-10 and 15-18 on the antisense strand, counting from the 5'end of each strand.

In some embodiments, the one or more lipophilic moieties are conjugated to one or more of the internal positions selected from the group consisting of positions 5, 6, 7, 15, and 17 on the sense strand, and positions 15 and 17 on the antisense strand, counting from the 5'-end of each strand.

In some embodiments, the positions in the double stranded region exclude a cleavage site region of the sense strand.

In some embodiments, the sense strand is 21 nucleotides in length, the antisense strand is 23 nucleotides in length, and the lipophilic moiety is conjugated to position 20, position 15, position 1, position 7, position 6, or position 2 of the sense strand or position 16 of the antisense strand.

In other embodiments, the sense strand is 21 nucleotides in length, the antisense strand is 23 nucleotides in length, and the lipophilic moiety is conjugated to position 21, position 20, position 15, position 1, position 7, position 6, or position 2 of the sense strand or position 16 of the antisense strand.

In some embodiments, the lipophilic moiety is an aliphatic, alicyclic, or polyalicyclic compound.

In some embodiments, the lipophilic moiety is selected from the group consisting of lipid, cholesterol, retinoic acid, cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-bis-O(hexadecyl)glycerol, geranyloxyhexyanol, hexadecylglycerol, borneol, menthol, 1,3-

propanediol, heptadecyl group, palmitic acid, myristic acid, O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine.

In some embodiments, the lipophilic moiety is selected from the group consisting of cholesterol, lithocholic acid (LCA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosanoic acid (DCA), choline, vitamin A, vitamin E, retinoic acid, and alpha-tocopheryl succinate. In some embodiments, the lipophilic moiety is selected from the group consisting of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and docosanoic acid (DCA). In some embodiments, the lipophilic moiety is not cholesterol.

In some embodiments, the lipophilic moiety contains a saturated or unsaturated C4-C30 hydrocarbon chain, and an optional functional group selected from the group consisting of hydroxyl, amine, carboxylic acid, sulfonate, phosphate, thiol, azide, and alkyne.

In some embodiments, the lipophilic moiety contains a saturated or unsaturated C6-C18 hydrocarbon chain.

In some embodiments, the lipophilic moiety contains a saturated or unsaturated C16 hydrocarbon chain. In some embodiments, the saturated or unsaturated C16 hydrocarbon chain is conjugated to position 6, counting from the 5'-end of the strand.

In some embodiments, the lipophilic moiety is conjugated *via* a carrier that replaces one or more nucleotide(s) in the internal position(s) or the double stranded region. In some embodiments, the carrier is a cyclic group selected from the group consisting of pyrrolidinyl, pyrazolinyl, pyrazolidinyl, imidazolinyl, imidazolidinyl, piperidinyl, piperazinyl, [1,3]dioxolanyl, oxazolidinyl, isoxazolidinyl, morpholinyl, thiazolidinyl, isothiazolidinyl, quinoxalinyl, pyridazinonyl, tetrahydrofuranyl, and decalinyl; or is an acyclic moiety based on a serinol backbone or a diethanolamine backbone.

In some embodiments, the lipophilic moiety is conjugated to the dsRNA agent *via* a linker containing an ether, thioether, urea, carbonate, amine, amide, maleimide-thioether, disulfide, phosphodiester, sulfonamide linkage, a product of a click reaction, or carbamate.

In some embodiments, the lipophilic moiety is conjugated to a nucleobase, sugar moiety, or internucleosidic linkage.

In some embodiments, the dsRNA agent comprises at least one modified nucleotide. In some embodiments, no more than five of the sense strand nucleotides and no more than five of the nucleotides of the antisense strand are unmodified nucleotides. In other embodiments, all of the nucleotides of the sense strand and all of the nucleotides of the antisense strand comprise a modification.

In some embodiments, at least one of the modified nucleotides is selected from the group a deoxy-nucleotide, a 3'-terminal deoxythimidine (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 5'-phosphorothioate group, a nucleotide comprising a 5'-methylphosphonate group, a nucleotide comprising a 5' phosphate or 5' phosphate mimic, a nucleotide comprising vinyl phosphonate, a glycol nucleic acid (GNA) or a glycol nucleic acid S-isomer (S-GNA) (e.g., a nucleotide comprising adenosine-glycol nucleic acid (GNA) ora nucleotide comprising thymidine-glycol nucleic acid (GNA) S-Isomer), a nucleotide comprising 2-hydroxymethyl-tetrahydrofurane-5-phosphate, a nucleotide comprising 2'-deoxythymidine-3'phosphate, a nucleotide comprising 2'-deoxyguanosine-3'-phosphate, and a terminal nucleotide linked to a cholesteryl derivative and a dodecanoic acid bisdecylamide group; and combinations thereof.

In other embodiments, the modified nucleotide is selected from the group consisting of a 2'-deoxy-2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, 3'-terminal deoxythimidine nucleotides (dT), a locked nucleotide, an abasic nucleotide, a 2'-amino-modified nucleotide, a 2'-alkyl-modified nucleotide, a morpholino nucleotide, a phosphoramidate, and a non-natural base comprising nucleotide.

In some embodiments, at least one of the modified nucleotides is selected from the group consisting of a deoxy-nucleotide, a 2'-O-methyl modified nucleotide, a 2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a glycol modified nucleotide (GNA), and a vinyl-phosphonate nucleotide; and combinations thereof.

In some embodiments, at least one of the modifications on the nucleotides is a thermally destabilizing nucleotide modification. In some embodiments, the thermally destabilizing nucleotide modification is selected from the group consisting of an abasic modification; a mismatch with the opposing nucleotide in the duplex; and destabilizing sugar modification, a 2'-deoxy modification, an acyclic nucleotide, an unlocked nucleic acids (UNA), and a glycerol nucleic acid (GNA)

In some embodiments, the modified nucleotide comprises a short sequence of 3'-terminal deoxythimidine nucleotides (dT).

In some embodiments, the modifications on the nucleotides are 2'-O-methyl, GNA and 2'fluoro modifications.

In some embodiments, the dsRNA agent further comprises at least one phosphorothioate internucleotide linkage. In some embodiments, the dsRNA agent comprises 6-8 phosphorothioate internucleotide linkages. In one embodiment, the phosphorothioate or methylphosphonate internucleotide linkage is at the 3'-terminus of one strand. Optionally, the strand is the antisense strand. In another embodiment, the strand is the sense strand. In a related embodiment, the phosphorothioate or methylphosphonate internucleotide linkage is at the 5'-terminus of one strand. Optionally, the strand is the antisense strand. In another embodiment, the strand is the sense strand. In another embodiment, the phosphorothioate or methylphosphonate internucleotide linkage is at the both the 5'- and 3'-terminus of one strand. Optionally, the strand is the antisense strand. In another embodiment, the strand is the sense strand.

In some embodiments, each strand is no more than 30 nucleotides in length.

In some embodiments, at least one strand comprises a 3' overhang of at least 1 nucleotide or a 3' overhang of at least 2 nucleotides.

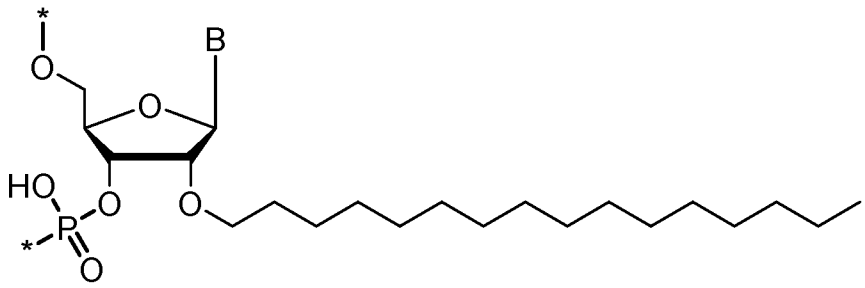
The double stranded region may be 15-30 nucleotide pairs in length; 17-23 nucleotide pairs in length; 17-25 nucleotide pairs in length; 23-27 nucleotide pairs in length; 19-21 nucleotide pairs in length; or 21-23 nucleotide pairs in length.

Each strand may be 19-30 nucleotides; 19-23 nucleotides; or 21-23 nucleotides.

In some embodiments, the dsRNA agent further comprises a targeting ligand that targets a liver tissue. In some embodiments, the targeting ligand is a GalNAc conjugate.

In certain embodiments, the double-stranded RNAi agent further includes a targeting ligand that targets a receptor which mediates delivery to a CNS tissue, *e.g.*, a hydrophilic ligand.

In certain embodiments, the targeting ligand is a C16 ligand. In one embodiment, the ligand is conjugated at the 2'-position of a nucleotide or modified nucleotide within the sense or antisense strand. For example, a C16 ligand may be conjugated as shown in the following structure:



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where * denotes a bond to an adjacent nucleotide, and B is a nucleobase or a nucleobase analog, optionally where B is adenine, guanine, cytosine, thymine or uracil.

In some embodiments, the lipophilic moeity or targeting ligand is conjugated *via* a bio-clevable linker selected from the group consisting of DNA, RNA, disulfide, amide, funtionalized monosaccharides or oligosaccharides of galactosamine, glucosamine, glucose, galactose, mannose, and combinations thereof.

In some embodiments, the 3' end of the sense strand is protected *via* an end cap which is a cyclic group having an amine, said cyclic group being selected from the group consisting of pyrrolidinyl, pyrazolinyl, pyrazolidinyl, imidazolinyl, imidazolidinyl, piperidinyl, piperazinyl, [1,3]dioxolanyl, oxazolidinyl, isoxazolidinyl, morpholinyl, thiazolidinyl, isothiazolidinyl, quinoxalinyl, pyridazinonyl, tetrahydrofuranyl, and decalinyl.

In some embodiments, the dsRNA agent further comprises a terminal, chiral modification occurring at the first internucleotide linkage at the 3' end of the antisense strand, having the linkage phosphorus atom in Sp configuration, a terminal, chiral modification occurring at the first internucleotide linkage at the 5' end of the antisense strand, having the linkage phosphorus atom in Rp configuration, and a terminal, chiral modification occurring at the first internucleotide linkage at the 5' end of the sense strand, having the linkage phosphorus atom in either Rp configuration or Sp configuration.

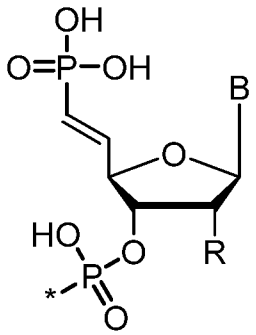
In some embodiments, the dsRNA agent further comprises a terminal, chiral modification occurring at the first and second internucleotide linkages at the 3' end of the antisense strand, having the linkage phosphorus atom in Sp configuration, a terminal, chiral modification occurring at the first internucleotide linkage at the 5' end of the antisense strand, having the linkage phosphorus atom in Rp configuration, and a terminal, chiral modification occurring at the first internucleotide linkage at the 5' end of the sense strand, having the linkage phosphorus atom in either Rp or Sp configuration.

In some embodiments, the dsRNA agent further comprises a terminal, chiral modification occurring at the first, second and third internucleotide linkages at the 3' end of the antisense strand, having the linkage phosphorus atom in Sp configuration, a terminal, chiral modification occurring at the first internucleotide linkage at the 5' end of the antisense strand, having the linkage phosphorus atom in Rp configuration, and a terminal, chiral modification occurring at the first internucleotide linkage at the 5' end of the sense strand, having the linkage phosphorus atom in either Rp or Sp configuration.

In some embodiments, the dsRNA agent further comprises a terminal, chiral modification occurring at the first, and second internucleotide linkages at the 3' end of the antisense strand, having the linkage phosphorus atom in Sp configuration, a terminal, chiral modification occurring at the third internucleotide linkages at the 3' end of the antisense strand, having the linkage phosphorus atom in Rp configuration, a terminal, chiral modification occurring at the first internucleotide linkage at the 5' end of the antisense strand, having the linkage phosphorus atom in Rp configuration, and a terminal, chiral modification occurring at the first internucleotide linkage at the 5' end of the sense strand, having the linkage phosphorus atom in either Rp or Sp configuration.

In some embodiments, the dsRNA agent further comprises a terminal, chiral modification occurring at the first, and second internucleotide linkages at the 3' end of the antisense strand, having the linkage phosphorus atom in Sp configuration, a terminal, chiral modification occurring at the first, and second internucleotide linkages at the 5' end of the antisense strand, having the linkage phosphorus atom in Rp configuration, and a terminal, chiral modification occurring at the first internucleotide linkage at the 5' end of the sense strand, having the linkage phosphorus atom in either Rp or Sp configuration.

In some embodiments, the dsRNA agent further comprises a phosphate or phosphate mimic at the 5'-end of the antisense strand. In some embodiments, the phosphate mimic is a 5'-vinyl phosphonate (VP). When the phosphate mimic is a 5'-vinyl phosphonate (VP), the 5'-terminal nucleotide can have the following structure,



wherein * indicates the location of the bond to 5'-position of the adjacent nucleotide;

R is hydrogen, hydroxy, methoxy, fluoro, or another 2'-modification described herein (e.g., hydroxy or methoxy); and

B is a nucleobase or a modified nucleobase, optionally where B is adenine, guanine, cytosine, thymine or uracil.

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

In some embodiments, the sense strand has a total of 21 nucleotides and the antisense strand has a total of 23 nucleotides.

In one embodiment, the dsRNA agent comprises at least one modified nucleotide.

In one embodiment, substantially all of the nucleotides of the sense strand; substantially all of the nucleotides of the antisense strand comprise a modification; or substantially all of the nucleotides of the sense strand and substantially all of the nucleotides of the antisense strand comprise a modification.

In one embodiment, all of the nucleotides of the sense strand comprise a modification; all of the nucleotides of the antisense strand comprise a modification; or all of the nucleotides of the sense strand and all of the nucleotides of the antisense strand comprise a modification.

In one embodiment, at least one of the modified nucleotides is selected from the group consisting of a deoxy-nucleotide, a 3'-terminal deoxythimidine (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, 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, a nucleotide comprising a 5'-phosphate mimic, a thermally destabilizing nucleotide, a glycol modified nucleotide (GNA), and a 2-O-(N-methylacetamide) modified nucleotide; and combinations thereof.

In one embodiment, the modifications on the nucleotides are selected from the group consisting of LNA, glycol nucleic acid (GNA), hexitol nucleic acid (HNA), 2′-methoxyethyl, 2′-O-alkyl, 2′-O-allyl, 2′-C- allyl, 2′-fluoro, 2′-deoxy, 2'-hydroxyl, and glycol; and combinations thereof.

In one embodiment, at least one of the modified nucleotides is selected from the group consisting of a deoxy-nucleotide, a 2'-O-methyl modified nucleotide, a 2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a glycol modified nucleotide (GNA), *e.g.,* Ggn, Cgn, Tgn, or Agn, and, a vinyl-phosphonate nucleotide; and combinations thereof.

In another embodiment, at least one of the modifications on the nucleotides is a thermally destabilizing nucleotide modification.

In one embodiment, the thermally destabilizing nucleotide modification is selected from the group consisting of an abasic modification; a mismatch with the opposing nucleotide in the duplex; and

destabilizing sugar modification, a 2'-deoxy modification, an acyclic nucleotide, an unlocked nucleic acid (UNA), and a glycerol nucleic acid (GNA).

The double stranded region may be 19-30 nucleotide pairs in length; 19-25 nucleotide pairs in length; 19-23 nucleotide pairs in length; 23-27 nucleotide pairs in length; or 21-23 nucleotide pairs in length.

In one embodiment, each strand is independently no more than 30 nucleotides in length.

In one embodiment, the sense strand is 21 nucleotides in length and the antisense strand is 23 nucleotides in length.

The region of complementarity may be at least 17 nucleotides in length; 19-23 nucleotides in length; or 19 nucleotides in length.

In one embodiment, at least one strand comprises a 3' overhang of at least 1 nucleotide. In another embodiment, at least one strand comprises a 3' overhang of at least 2 nucleotides.

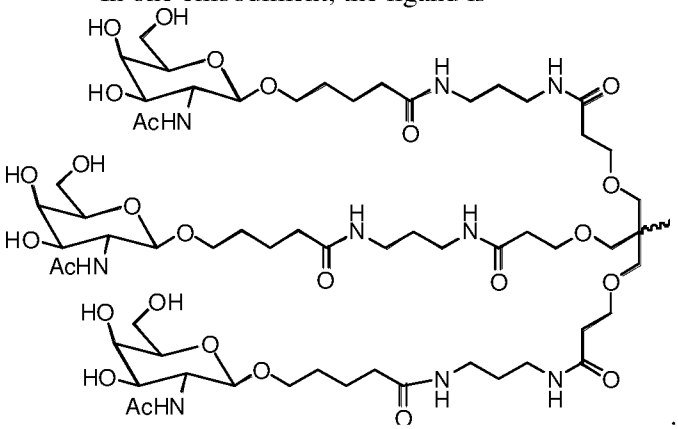
In one embodiment, the dsRNA agent further comprises a ligand.

In one embodiment, the ligand is conjugated to the 3' end of the sense strand of the dsRNA agent.

In one embodiment, the ligand is an N-acetylgalactosamine (GalNAc) derivative.

In one embodiment, the ligand is one or more GalNAc derivatives attached through a monovalent, bivalent, or trivalent branched linker.

In one embodiment, the ligand is



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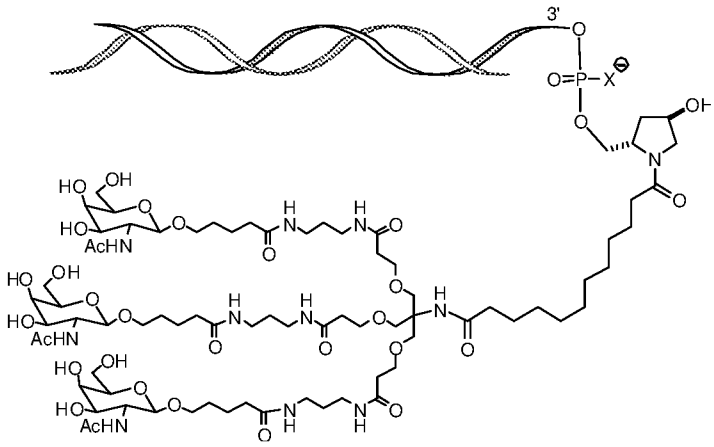
In one embodiment, the dsRNA agent is conjugated to the ligand as shown in the following schematic



and, wherein X is O or S.

In one embodiment, the X is O.

In one embodiment, the dsRNA agent further comprises at least one phosphorothioate or methylphosphonate internucleotide linkage.

In one embodiment, the phosphorothioate or methylphosphonate internucleotide linkage is at the 3'-terminus of one strand, *e.g.,* the antisense strand or the sense strand.

In another embodiment, the phosphorothioate or methylphosphonate internucleotide linkage is at the 5'-terminus of one strand, *e.g.,* the antisense strand or the sense strand.

In one embodiment, the phosphorothioate or methylphosphonate internucleotide linkage is at the both the 5'- and 3'-terminus of one strand. In one embodiment, the strand is the antisense strand.

In one embodiment, the base pair at the 1 position of the 5′-end of the antisense strand of the duplex is an AU base pair.

The present invention also provides cells containing any of the dsRNA agents of the invention and pharmaceutical compositions comprising any of the dsRNA agents of the invention.

The pharmaceutical composition of the invention may include dsRNA agent in an unbuffered solution, *e.g.,* saline or water, or the pharmaceutical composition of the invention may include the dsRNA agent is in a buffer solution, *e.g.,* a buffer solution comprising acetate, citrate, prolamine, carbonate, or phosphate or any combination thereof; or phosphate buffered saline (PBS).

In one aspect, the present invention provides a method of inhibiting expression of a PRNP gene in a cell. The method includes contacting the cell with any of the dsRNAs of the invention or any of the pharmaceutical compositions of the invention, thereby inhibiting expression of the PRNP gene in the cell.

In one embodiment, the cell is within a subject, *e.g.*, a human subject, *e.g.*, a subject having a PRNP-associated disease.

In certain embodiments, the PRNP-associated disease is a prion disease. In certain embodiments, the prion disease is a genetic prion diseass selected from the group consisting of familial Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI). In some embodiments, the prion disease is a sporadic prion disease selected from the group consisting of

sporadic Creutzfeldt-Jakob disease, Sporadic Fatal Insomnia (sFI), and Variably Proteinase-Sensitive Prionopathy (VPSPr). In another embodiment, the prion disease is an acquired prion disease selected from the group consisting of Iatrogenic CJD (iCJD), Kuru, and Variant CJD (vCJD).

In some embodiments, contacting the cell with the dsRNA agent inhibits the expression of PRNP by at least 50%, 60%, 70%, 80%, 90%, or 95%. In some embodiments, inhibiting expression of PRNP decreases PRNP protein levels in serum of the subject by at least 50%, 60%, 70%, 80%, 90%, or 95%.

In one aspect, the present invention provide a method of treating a subject having a disorder that would benefit from reduction in PRNP expression, the method comprising administering to the subject a therapeutically effective amount of any one of the dsRNA agents of the invention, or any one of the pharmaceutical compositions of the invention, thereby treating the subject having the disorder that would benefit from reduction in PRNP expression.

In another aspect, the present invention provides a method of preventing at least one symptom in a subject having a disorder that would benefit from reduction in PRNP expression, the method comprising administering to the subject a prophylactically effective amount of any one of the dsRNA agents of the invention, or any one of the pharmaceutical compositions of the invention, thereby preventing at least one symptom in the subject having the disorder that would benefit from reduction in PRNP expression.

In some embodiments, the disorder is a PRNP-associated disease. In some embodiments, the PRNP-associated disease is a prion disease. In some embodiments, the prion disease is a genetic prion disease selected from the group consisting of familial Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI). In some embodiments, the prion disease is a sporadic prion disease selected from the group consisting of sporadic Creutzfeldt-Jakob disease, Sporadic Fatal Insomnia (sFI), and Variably Proteinase-Sensitive Prionopathy (VPSPr). In another embodiment, the prion disease is an acquired prion disease selected from the group consisting of Iatrogenic CJD (iCJD), Kuru, and Variant CJD (vCJD).

Another aspect of the instant disclosure provides a method of inhibiting the expression of PRNP in a subject, the method comprising administering to the subject a therapeutically effective amount of any one of the dsRNA agents of the invention, or any one of the pharmaceutical compositions of the invention, thereby inhibiting the expression of PRNP in the subject.

An additional aspect of the disclosure provides a method for treating or preventing a PRNP-associated disorder in a subject, the method comprising administering to the subject a therapeutically effective amount of any one of the dsRNA agents of the invention, or any one of the pharmaceutical compositions of the invention, thereby treating or preventing a PRNP-associated disorder in the subject.

In certain embodiments, the PRNP-associated disorder is selected from the group consisting of a prion disease, such as a genetic prion disease, *e.g.,* familial Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI). In some embodiments, the prion disease is a sporadic prion disease selected from the group consisting of sporadic Creutzfeldt-Jakob disease, Sporadic Fatal Insomnia (sFI), and Variably Proteinase-Sensitive Prionopathy (VPSPr). In

another embodiment, the prion disease is an acquired prion disease selected from the group consisting of Iatrogenic CJD (iCJD), Kuru, and Variant CJD (vCJD).

In some embodiments, the subject is human.

In some embodiments, the dsRNA agent is administered to the subject at a dose of about 0.01 mg/kg to 50 mg/kg or at a dose of about 5 mg to 1000 mg.

In some embodiments, the dsRNA agent is administered to the subject intrathecally.

In some embodiments, the dsRNA agent is administered to the subject subcutaneously.

In one embodiment, the method reduces the expression of a PRNP gene in a brain or spine tissue, *e.g.*, striatum, cortex, cerebellum, cervical spine, lumbar spine, or thoracic spine.

In one embodiment, the method reduces the expression of a PRNP gene in the brain.

In some embodiments, the methods of the invention further comprise administering to the subject an additional agent for treatment or prevention of a PRNP-associated disorder. Exemplary additional therapeutics and treatments include, for example, sedatives, antidepressants, clonazepam, sodium valproate, opiates, antiepileptic drugs, cholinesterase inhibitors, memantine, benzodiazepines, levodopa, COMT inhibitors (*e.g.*, tolcapone and entacapone), dopamine agonists (*e.g.*, bromocriptine, pergolide, pramipexole, ropinirole, piribedil, cabergoline, apomorphine and lisuride), MAO-B inhibitors (*e.g.*, safinamide, selegiline and rasagiline), amantadine, an anticholinergic, modafinil, pimavanserin, doxepin, rasagline, an antipsychotic, an atypical antipsychotic (*e.g.*, amisulpride, olanzapine, risperidone, and clozapine), riluzole, edaravone , deep brain stimulation, non-invasive ventilation (NIV), invasive ventilation physical therapy, occupational therapy, speech therapy, dietary changes and swallowing technique a feeding tube, a PEG tube, probiotics, and psychological therapy.

The present invention also provides kits comprising any of the dsRNAs of the invention or any of the pharmaceutical compositions of the invention, and optionally, instructions for use.

In another embodiment, the RNAi agent is a pharmaceutically acceptable salt thereof. "Pharmaceutically acceptable salts" of each of RNAi agents herein include, but are not limited to, a sodium salt, a calcium salt, a lithium salt, a potassium salt, an ammonium salt, a magnesium salt, an mixtures thereof. One skilled in the art will appreciate that the RNAi agent, when provided as a polycationic salt having one cation per free acid group of the optionally modified phosophodiester backbone and/or any other acidic modifications (*e.g.*, 5'-terminal phosphonate groups). For example, an oligonucleotide of "n" nucleotides in length contains n-1 optionally modified phosophodiesters, so that an oligonucleotide of 21 nt in length may be provided as a salt having up to 20 cations (*e.g.*, 20 sodium cations). Similarly, an RNAi agentshaving a sense strand of 21 nt in length and an antisense strand of 23 nt in length may be provided as a salt having up to 42 cations (*e.g.*, 42 sodium cations). In the preceding example, where the RNAi agent also includes a 5'-terminal phosphate or a 5'-terminal vinylphosphonate group, the RNAi agent may be provided as a salt having up to 44 cations (*e.g.*, 44 sodium cations).

The present invention is further illustrated by the following detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph depicting the level of PRNP mRNA in the right-hemipsheres of wild-type mice at Day 28 following a single intracerebroventricular (ICV) injection of 50 μg, 150 μg, or 300 μg dose of an exemplary dsRNA agent targeting PRNP, AD-1070516, or control, artificial CSF (aCSF) on Day 0.

DETAILED DESCRIPTION OF THE INVENTION

The present disclosure provides RNAi compositions, which effect the RNA-induced silencing complex (RISC)-mediated cleavage of RNA transcripts of a PRNP gene. The PRNP 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 (PRNP gene) in mammals.

The iRNAs of the invention have been designed to target a PRNP gene, including portions of the gene that are conserved in the PRNP 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.

Accordingly, the present disclosure also provides methods of using the RNAi compositions of the disclosure for inhibiting the expression of a PRNP gene or for treating a subject having a disorder that would benefit from inhibiting or reducing the expression of a PRNP gene, *e.g.,* a PRNP-associated disesase, *e.g.,* a prion disease, such as a genetic prion disease, such as, Creutzfeldt-Jakob Disease (CJD), Gerstmann-Straussler-Scheinker Disease (GSS), and Fatal Familial Insomnia (FFI), an acquired prion disease, such as Mad Cow Disease, Iatrogenic CJD (iCJD), Kuru, and Variant CJD (vCJD), or a sporadic prion diseasesuch as sporadic Creutzfeldt-Jakob disease, Sporadic Fatal Insomnia (sFI), and Variably Proteinase-Sensitive Prionopathy (VPSPr).

The RNAi agents of the disclosure 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 PRNP gene. In certain embodiments, the RNAi agents of the disclosure include an RNA strand (the antisense strand) having a region which is about 21-23 nucleotides in length, which region is substantially complementary to at least part of an mRNA transcript of a PRNP gene.

In certain embodiments, the RNAi agents of the disclosure 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 PRNP gene. These RNAi agents with the longer length antisense strands can, for example, 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.

The use of these RNAi agents enables the targeted degradation of mRNAs of a PRNP gene in mammals. Thus, methods and compositions including these RNAi agents are useful for treating a subject who would benefit by a reduction in the levels or activity of a PRNP protein, such as a subject having a PRNP-associated disease, *e.g.,* a prion disease.

The following detailed description discloses how to make and use compositions containing RNAi agents to inhibit the expression of a PRNP gene, as well as compositions and methods for treating subjects having diseases and disorders that would benefit from inhibition or reduction of the expression of the PRNP gene.

I. Definitions

In order that the present disclosure 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 disclosure.

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.

The term "including" is used herein to mean, and is used interchangeably with, the phrase "including but not limited to".

The term "or" is used herein to mean, and is used interchangeably with, the term "and/or," unless context clearly indicates otherwise.

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 ±10%. In certain embodiments, about means ±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.

The term “at least” , “no less than”, or “or more” 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.

As used herein, “no more than” or “or less” 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. As used herein, ranges include both the upper and lower limit.

As used herein, methods of detection can include determination that the amount of analyte present is below the level of detection of the method.

In the event of a conflict between an indicated target site and the nucleotide sequence for a sense or antisense strand, the indicated sequence takes precedence.

In the event of a conflict between a chemical structure and a chemical name, the chemical structure takes precedence.

As used herein, the term "prion protein," used interchangeably with the term "PRNP," refers to the well-known gene and polypeptide, also known in the art as AltPrP, ASCR, CD230 antigen, CJD, GSS, Kuru, prion protein (p27-30), PRIP, PrP, PrP27-30, PrP33-35C and PrPc.

Prion protein genes are highly conserved in mammals, and have been implicated in neurogenesis and differentiation of neural stem cells, neuritogenesis, involvement and interaction with signal transduction pathways, synaptogenesis, neuronal survival via anti- or pro-apoptotic functions, copper binding, redox homeostasis, long-term renewal of hemopoietic stem cells, activation and development of T cells, differentiation and modulation of phagocytosis of leukocytes, and altering leukocyte recruitment to sites of inflammation (Caughey B, Baron GS. *Nature*. 2006;443:803–810).

Multiple isoforms of the prion protein exist, two of which are most relevant to prion disease, cellular prion protein (PrP^{C}) and scrapie prion protein (PrP^{Sc}). Both PrP^{C} and PrP^{Sc} isoforms have the same amino acid sequence, however, PrP^{c} consists mainly of α-helices while PrP^{Sc} has undergone a three-dimentsonal structure change to have an increased β-sheet structure. This conformational difference makes PrP^{Sc} insoluble in detergents and relatively resistant to degradation by proteases. PrP^{Sc} has been shown to be infectious and to self-propagate by acting as a template that induces the conversion of PrP^{C} to PrP^{Sc}. PrP^{Sc} propagates as oligomers, which polymerize to form amyloid fibrils. The abnormal PrP^{Sc} protein builds up in the brain, forming clumps that damage or destroy neurons. The loss of these cells creates microscopic sponge-like holes (vacuoles) in the brain, which leads to the signs and symptoms of prion disease.

The term "PRNP" includes human PRNP, the amino acid and nucleotide sequence of which may be found in, for example, GenBank Accession No. NM_000311.5 (GI: 1653962152; SEQ ID NO:1); mouse PRNP, the amino acid and nucleotide sequence of which may be found in, for example, GenBank Accession No. NM_001278256.1 (GI: 506326230; SEQ ID NO:2); and rat PRNP, the amino acid and nucleotide sequence of which may be found in, for example, GenBank Accession No. XM_006235062.3 (GI:1046878307; SEQ ID NO:3). The term "PRNP" also includes Macaca fascicularis PRNP, the amino acid and nucleotide sequence of which may be found in, for example, GenBank Accession No. XM_005568413.2 (GI:982273619; SEQ ID NO:4).

Additional examples of PRNP mRNA sequences are readily available using, *e.g.*, GenBank, UniProt, OMIM, and the Macaca genome project web site.

Exemplary PRNP nucleotide sequences may also be found in SEQ ID NOs:1-4. SEQ ID NOs:5-8 are the reverse complement sequences of SEQ ID NOs:1-4, respectively.

Further information on PRNP is provided, for example in the NCBI Gene database at www.ncbi.nlm.nih.gov/gene/5621.

The entire contents of each of the foregoing GenBank Accession numbers and the Gene database numbers are incorporated herein by reference as of the date of filing this application.

The terms "prion protein" and "PRNP," as used herein, also refers to naturally occurring DNA sequence variations of the PRNP gene. Numerous sequence variations within the PRNP gene have been identified and may be found at, for example, NCBI dbSNP and UniProt (see, *e.g.*, www.ncbi.nlm.nih.gov/snp?LinkName=gene_snp&from_uid=5621, the entire contents of which is incorporated herein by reference as of the date of filing this application.

As used herein, "target sequence" refers to a contiguous portion of the nucleotide sequence of an mRNA molecule formed during the transcription of a PRNP gene, including mRNA that is a product of RNA processing of a primary transcription product. In one embodment, the target portion of the sequence will be at least long enough to serve as a substrate for RNAi-directed cleavage at or near that portion of the nucleotide sequence of an mRNA molecule formed during the transcription of a PRNP gene. In one embodiment, the target sequence is within the protein coding region of the PRNP gene. In another embodiment, the target sequence is within the 3' UTR of the PRNP gene.

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. In some embodiments, the target sequence is about 19 to about 30 nucleotides in length. In other embodiments, the target sequence is about 19 to about 25 nucleotides in length. In still other embodiments, the target sequence is about 19 to about 23 nucleotides in length. In some embodiments, the target sequence is about 21 to about 23 nucleotides in length. Ranges and lengths intermediate to the above recited ranges and lengths are also contemplated to be part of the invention.

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.

"G," "C," "A," "T", and "U" each generally stand for a nucleotide that contains guanine, cytosine, adenine, thymidine, and uracil as a base, respectively in the context of a modified or unmodified nucleotide. 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 1). The skilled person is well aware that guanine, cytosine, adenine, thymidine, 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 disclosure 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 disclosure.

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. RNA interference (RNAi) is a process that directs the sequence-specific degradation of mRNA. RNAi modulates, *e.g.,* inhibits, the expression of PRNP in a cell, *e.g.,* a cell within a subject, such as a mammalian subject.

In one embodiment, an RNAi agent of the disclosure includes a single stranded RNAi that interacts with a target RNA sequence, *e.g.,* a PRNP 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 double-stranded short interfering RNAs (siRNAs) comprising a sense strand and an antisense strand by a Type III endonuclease known as Dicer (Sharp *et al.* (2001) *Genes Dev*. 15:485). Dicer, a ribonuclease-III-like enzyme, processes these dsRNA into 19-23 base pair short interfering RNAs with characteristic two base 3' overhangs (Bernstein, *et al.*, (2001) *Nature* 409:363). These 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 disclosure relates to a single stranded RNA (ssRNA) (the antisense strand of a siRNA duplex) generated within a cell and which promotes the formation of a RISC complex to effect silencing of the target gene, *i.e.,* a PRNP gene. Accordingly, the term "siRNA" is also used herein to refer to an RNAi as described above.

In another embodiment, the RNAi agent may be a single-stranded RNA 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 RNAs are described in U.S. Patent 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.

In another embodiment, a "RNAi agent" for use in the compositions and methods of the disclosure 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 PRNP gene. In some embodiments of the disclosure, 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.

In general, a dsRNA molecule can include ribonucleotides, but as described in detail herein, each or both strands can also include one or more non-ribonucleotides, *e.g.*, a deoxyribonucleotide, a modified nucleotide. In addition, as used in this specification, an "RNAi agent" may include ribonucleotides with chemical modifications; an RNAi agent may include substantial modifications at multiple nucleotides; an RNAi 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 a 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 disclosure 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 "RNAi agent" for the purposes of this specification and claims.

In certain embodiments of the instant disclosure, inclusion of a deoxy-nucleotide – which is acknowledged as a naturally occurring form of nucleotide – if present within a RNAi agent can be considered to constitute a modified nucleotide.

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.

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 at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 20, at least 23 or more unpaired nucleotides or nucleotides not directed to the target site of the dsRNA. 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.

In certain embodiments, the two strands of double-stranded oligomeric compound can be linked together. The two strands can be linked to each other at both ends, or at one end only. By linking at one end is meant that 5'-end of first strand is linked to the 3'-end of the second strand or 3'-end of first strand is linked to 5'-end of the second strand. When the two strands are linked to each other at both ends, 5'-end of first strand is linked to 3'-end of second strand and 3'-end of first strand is linked to 5'-end of second strand. The two strands can be linked together by an oligonucleotide linker including, but not limited to, (N)n; wherein N is independently a modified or unmodified nucleotide and n is 3-23. In some embodiments, n is 3-10, *e.g.*, 3, 4, 5, 6, 7, 8, 9, or 10. In some embodiments, the oligonucleotide linker is selected from the group consisting of GNRA, (G)4, (U)4, and (dT)4, wherein N is a modified or unmodified nucleotide and R is a modified or unmodified purine nucleotide. Some of the nucleotides in the linker can be involved in base-pair interactions with other nucleotides in the linker. The two strands can also be linked together by a non-nucleosidic linker, *e.g.* a linker described herein. It will be appreciated by one of skill in the art that any oligonucleotide chemical modifications or variations describe herein can be used in the oligonucleotide linker.

Hairpin and dumbbell type oligomeric compounds will have a duplex region equal to or at least 14, 15, 15, 16, 17, 18, 19, 29, 21, 22, 23, 24, or 25 nucleotide pairs. The duplex region can be equal to or less than 200, 100, or 50, in length. In some embodiments, ranges for the duplex region are 15-30, 17 to 23, 19 to 23, and 19 to 21 nucleotides pairs in length.

The hairpin oligomeric compounds can have a single strand overhang or terminal unpaired region, in some embodiments at the 3', and in some embodiments on the antisense side of the hairpin. In some embodiments, the overhangs are 1-4, more generally 2-3 nucleotides in length. The hairpin oligomeric compounds that can induce RNA interference are also referred to as "shRNA" herein.

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.

In one embodiment, an RNAi agent of the invention is a dsRNA, each strand of which is 24-30 nucleotides in length, that interacts with a target RNA sequence, *e.g.*, a PRNP 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).

In one embodiment, an RNAi agent of the invention is a dsRNA agent, each strand of which comprises 19-23 nucleotides that interacts with a PRNP RNA 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). In one embodiment, an RNAi agent of the invention is a dsRNA of 24-30 nucleotides that interacts with a PRNP RNA sequence to direct the cleavage of the target RNA.

As used herein, the term "nucleotide overhang" refers to at least one unpaired nucleotide that protrudes from the duplex structure of a RNAi agent, *e.g.*, a dsRNA. 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.

In one embodiment, 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 one embodiment, the sense 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 another embodiment, one or more of the nucleotides in the overhang is replaced with a nucleoside thiophosphate.

In certain embodiments, the antisense strand of a dsRNA has a 1-10 nucleotide, *e.g.*, 0-3, 1-3, 2-4, 2-5, 4-10, 5-10, *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 one embodiment, the sense 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 another embodiment, one or more of the nucleotides in the overhang is replaced with a nucleoside thiophosphate.

In certain embodiments, the overhang on the sense strand or the antisense strand, can include extended lengths longer than 10 nucleotides, *e.g.*, 1-30 nucleotides, 2-30 nucleotides, 10-30 nucleotides,

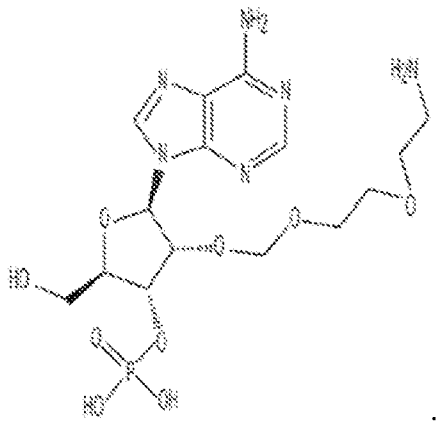
or 10-15 nucleotides in length. In certain embodiments, an extended overhang is on the sense strand of the duplex. In certain embodiments, an extended overhang is present on the 3'end of the sense strand of the duplex. In certain embodiments, an extended overhang is present on the 5'end of the sense 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.

In certain embodiments, at least one end of at least one strand is extended beyond a duplex targeting region, including structures where one of the strands includes a thermodynamically -stabilizing tetraloop structure (see, *e.g.*, U.S. Patent Nos. 8,513,207 and 8,927,705, as well as W02010033225, the entire contents of each of which are incorporated by reference herein). Such structures may include single-stranded extensions (on one or both sides of the molecule)as well as double-stranded extensions.

In certain embodiments, the 3' end of the sense strand and the 5' end of the antisense strand are joined by a polynucleotide sequence comprising ribonucleotides, deoxyribonucleotides or both, optionally wherein the polynucleotide sequence comprises a tetraloop sequence. In certain embodiments, the sense strand is 25-35 nucleotides in length.

A tetraloop may contain ribonucleotides, deoxyribonucleotides, modified nucleotides, and combinations thereof. Typically, a tetraloop has 4 to 5 nucleotides. In some embodiments, the loop comprises a sequence set forth as GAAA. In some embodiments, at least one of the nucleotide of the loop (GAAA) comprises a nucleotide modification. In some embodiments, the modified nucleotide comprises a 2'-modification. In some embodiments, the 2 '-modification is a modification selected from the group consisting of 2'-aminoethyl, 2'-fluoro, 2'-O-methyl, 2'-O-methoxyethyl, 2'-aminodiethoxymethanol, 2'- adem, and 2'-deoxy-2'-fhioro- -d-arabinonucleic acid. In some embodiments, all of the nucleotides of the loop are modified. In some embodiments, the G in the GAAA sequence comprises a 2'-OH. In some embodiments, each of the nucleotides in the GAAA sequence comprises a 2'-O-methyl modification. In some embodiments, each of the A in the GAAA sequence comprises a 2'-OH and the G in the GAAA sequence comprises a 2'-O-methyl modification. In some embodiments, each of the A in the GAAA sequence comprises a 2'-O-methoxyethyl (MOE) modification and the G in the GAAA sequence comprises a 2'-O-methyl modification; or each of the A in the GAAA sequence comprises a 2'- adem modification and the G in the GAAA sequence comprises a 2'-O-methyl modification. See, *e.g.,* PCT Publication No. WO 2020/206350, the entire contents of which are incorporated herein by reference.

An exemplary 2'adem modified nucleotide is shown below:



In one embodiment of the dsRNA, at least one strand comprises a 3' overhang of at least 1 nucleotide. In another embodiment, 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.

In one embodiment, the antisense strand of a dsRNA has a 1-10 nucleotide, *e.g.*, 0-3, 1-3, 2-4, 2-5, 4-10, 5-10, *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 one embodiment, the sense 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 another embodiment, one or more of the nucleotides in the overhang is replaced with a nucleoside thiophosphate.

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, 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.

The terms "blunt" or "blunt ended" as used herein in reference to a dsRNA mean that there are no unpaired nucleotides or nucleotide analogs at a given terminal end of a dsRNA, *i.e.*, no nucleotide overhang. One or both ends of a dsRNA can be blunt. Where both ends of a dsRNA are blunt, the dsRNA

is said to be blunt ended. To be clear, a "blunt ended" dsRNA is a dsRNA that is blunt at both ends, *i.e.*, no nucleotide overhang at either end of the molecule. Most often such a molecule will be double stranded over its entire length.

The term "antisense strand" or "guide strand" refers to the strand of a RNAi agent, *e.g.*, a dsRNA, which includes a region that is substantially complementary to a target sequence, *e.g.,* a PRNP 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 PRNP 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, or 2 nucleotides of the 5'- or 3'-terminus of the RNAi agent.

In some embodiments, a double stranded RNA agent of the invention includes a nucleotide mismatch in the antisense strand. In some embodiments, the antisense strand of the double stranded RNA agent of the invention includes no more than 4 mismatches with the target mRNA, *e.g.*, the antisense strand includes 4, 3, 2, 1, or 0 mismatches with the target mRNA. In some embodiments, the antisense strand double stranded RNA agent of the invention includes no more than 4 mismatches with the sense strand, *e.g.*, the antisense strand includes 4, 3, 2, 1, or 0 mismatches with the sense strand. In some embodiments, a double stranded RNA agent of the invention includes a nucleotide mismatch in the sense strand. In some embodiments, the sense strand of the double stranded RNA agent of the invention includes no more than 4 mismatches with the antisense strand, *e.g.*, the sense strand includes 4, 3, 2, 1, or 0 mismatches with the antisense strand. In some embodiments, the nucleotide mismatch is, for example, within 5, 4, 3 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 agent. In some embodiments, the mismatch(s) is not in the seed region.

Thus, an RNAi agent as described herein can contain one or more mismatches to the target sequence. In one embodiment, a RNAi agent as described herein contains no more than 3 mismatches (*i.e.,* 3, 2, 1, or 0 mismatches). In one embodiment, an RNAi agent as described herein contains no more than 2 mismatches. In one embodiment, an RNAi agent as described herein contains no more than 1 mismatch. In one embodiment, an RNAi agent as described herein contains 0 mismatches. In certain embodiments, if the antisense strand of the RNAi agent contains mismatches to the target sequence, the mismatch can optionally be restricted to be within the last 5 nucleotides from either the 5'- or 3'-end of the region of complementarity. For example, in such embodiments, for a 23 nucleotide RNAi agent, the strand which is complementary to a region of a PRNP 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 RNAi agent containing a mismatch to a target sequence is effective in inhibiting the expression of a PRNP gene. Consideration of the efficacy of RNAi agents with mismatches in inhibiting expression of a PRNP gene is important, especially if the particular region of complementarity in a PRNP gene is known to have polymorphic sequence variation within the population.

The term “sense strand” or "passenger strand" as used herein, refers to the strand of a RNAi agent that includes a region that is substantially complementary to a region of the antisense strand as that term is defined herein.

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.

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.

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 be, for example, “stringent conditions”, where stringent conditions can include: 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50 ºC or 70 ºC 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.

Complementary sequences within a RNAi agent, *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, in vitro or in vivo.. 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.

"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.

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 two oligonucleotides or polynucleotides, such as the antisense strand of a RNAi agent and a target sequence, as will be understood from the context of their use.

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 PRNP). For example, a polynucleotide is complementary to at least a part of a PRNP mRNA if the sequence is substantially complementary to a non-interrupted portion of an mRNA encoding PRNP.

Accordingly, in some embodiments, the antisense strand polynucleotides disclosed herein are fully complementary to the target PRNP sequence.

In other embodiments, the antisense strand polynucleotides disclosed herein are substantially complementary to the target PRNP 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 SEQ ID NOs: 1-4 for PRNP, or a fragment of SEQ ID NOs: 1-4, such as about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% complementary

In other embodiments, the antisense polynucleotides disclosed herein are substantially complementary to the target PRNP sequence and comprise 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 2-3, or a fragment of any one of the sense strand nucleotide sequences in any one of Tables 2-3, such as about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% complementary.

In one embodiment, an RNAi agent of the disclosure includes a sense strand that is substantially complementary to an antisense polynucleotide which, in turn, is the same as a target PRNP sequence, and wherein the sense strand polynucleotide 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 SEQ ID NOs: 5-8, or a fragment of any one of SEQ ID NOs: 5-8, such as about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% complementary.

In some embodiments, the antisense polynucleotides disclosed herein are substantially complementary to a fragment of a target PRNP sequence and comprise a contiguous nucleotide sequence

which is at least 80% complementary over its entire length to a fragment of SEQ ID NO: 1 selected from the group of nucleotides 502-524, 507-529, 502-529, 540-562, 566-588, 575-597, 576-598, 589-611, 575-611, 593-615, 594-616, 600-622, 593-622, 650-672, 858-880, 976-998, 1100-1122, 1126-1148, 1220-1242, 1221-1243, 1220-1243, 1304-1326, 1328-1350, 1410-1432, 1445-1467, 1481-1503, 1532-1554, 1610-1632, 1615-1637, 1617-1639, 1621-1643, 1610-1643, 1610-1639, 1688-1710, 1694-1714, , 1830-1852, 1831-1853, 1854-1876, 1830-1853, 1872-1894, 1873-1895, 1938-1960, 2011-2033, 2015-2037, 2031-2053, 2034-2056, 2069-2091, 2076-2098, 2077-2099, 1872-1895, 2011-2037, 2069-2099, 2079-2101, 2031-2056, 2069-2098, 2138-2160, 2143-2165, 2158-2180, 2167-2189, 2168-2190, 2170-2192, 2174-2196, 2175-2197, 2177-2199, 2185-2207, 2189-2211, 2196-2218, 2200-2222, 2202-2224, 2221-2243, 2222-2244, 2223-2245, 2238-2260, 2241-2263, 2242-2264, 2138-2264, 2257-2358, 2174-2245, 2174-2224, 2138-2196, 2177-2224, 2223-2245, 2138-2189, 2177-2211, 2196-2224, 2257-2279, 2258-2280, 2335-2537, 2336-2358, 2257-2358, 2342-2364, 2397-2419, 2398-2420, 2399-2421, 2400-2422, 2401-2423, 2404-2426, 2397-2426, 2394-2423, 2397-2421, 2399-2426, and 2398-2421 of SEQ ID NO: 1, such as about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% complementary.

In some embodiments, the antisense polynucleotides disclosed herein are substantially complementary to a fragment of a target PRNP sequence and comprise a contiguous nucleotide sequence which is at least 80% complementary over its entire length to a fragment of SEQ ID NO: 1 selected from the group of nucleotides 530-570, 535-565, 539-561, 540-562, 555-605, 560-605, 560-600, 566-588, 567-589, 568-590, 569-591, 570-592, 575-597, 580-620-585-620, 589-611, 590-612, 591-613, 592-614, 593-615, 594-616, 600-650, 610-650, 613-635, 614-636, 615-637, 616-638, 617-639, 618-640, 640-680, 649-671, 650-672, 651-673, 670-700, 674-696, 675-697, 795-830, 804-826, 2325-2375, 2325-2370, 2330-2370, 2335-2357, 2336-2358, 2337-2359, 2338-2360, 2339-2361, or 2340-2362 of SEQ ID NO: 1, such as about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% complementary.

In other embodiments, the antisense polynucleotides disclosed herein are substantially complementary to a fragment of a target PRNP sequence and comprise a contiguous nucleotide sequence which is at least 80% complementary over its entire length to a fragment of SEQ ID NO: 1 selected from the group of nucleotides 502-524, 540-562, 589-611, 594-616, 650-672, 1445-1467, 1688-1710, 2011-2033, 2031-2053, 2185-2207, 2222-2244, 2336-2358, 2339-2361, 2399-2421 or 2258-2280 of SEQ ID NO: 1, such as about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% complementary.

In some embodiments, an iRNA of the invention includes a sense strand that is substantially complementary to an antisense polynucleotide which, in turn, is complementary to a target PRNP sequence, and wherein the sense strand polynucleotide comprises a contiguous nucleotide sequence which is at least about 80% complementary over its entire length to any one of the antisense strand nucleotide sequences in any one of any one of Tables 2-3, or a fragment of any one of the antisense strand nucleotide

sequences in any one of Tables 2-3, such as about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% complementary.

In certain embodiments, the sense and antisense strands are selected from any one of duplexes AD-1070511, AD-1070462, AD-1070553, AD-1072048, AD-1070516 and AD-1072050, AD-1071769, AD-1071949, AD-1070444, AD-1071505, AD-1071912, AD-1071789, AD-1071265, AD-1072084, and AD-1071985.

In some embodiments, the double-stranded region of a double-stranded iRNA agent is equal to or at least, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 23, 24, 25, 26, 27, 28, 29, 30 or more nucleotide pairs in length.

In some embodiments, the antisense strand of a double-stranded iRNA agent is equal to or at least 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length.

In some embodiments, the sense strand of a double-stranded iRNA agent is equal to or at least 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length.

In one embodiment, the sense and antisense strands of the double-stranded iRNA agent are each independently 15 to 30 nucleotides in length.

In one embodiment, the sense and antisense strands of the double-stranded iRNA agent are each independently 19 to 25 nucleotides in length.

In one embodiment, the sense and antisense strands of the double-stranded iRNA agent are each independently 21 to 23 nucleotides in length.

In one embodiment, the sense strand of the iRNA agent is 21-nucleotides in length, and the antisense strand is 23-nucleotides in length, wherein the strands form a double-stranded region of 21 consecutive base pairs having a 2-nucleotide long single stranded overhangs at the 3'-end.

In one aspect of the invention, an agent for use in the methods and compositions of the invention is a single-stranded antisense nucleic acid molecule that inhibits a target mRNA *via* an antisense inhibition mechanism. The single-stranded antisense RNA 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 RNA molecule may be about 15 to about 30 nucleotides in length and have a sequence that is complementary to a target sequence. For example, the single-stranded antisense RNA molecule may comprise a sequence that is at least about 15, 16, 17, 18, 19, 20, or more contiguous nucleotides from any one of the antisense sequences described herein.

In one embodiment, at least partial suppression of the expression of a PRNP gene, is assessed by a reduction of the amount of PRNP mRNA which can be isolated from or detected in a first cell or group of cells in which a PRNP gene is transcribed and which has or have been treated such that the expression of a PRNP gene is inhibited, as compared to a second cell or group of cells substantially identical to the first cell or group of cells but which has or have not been so treated (control cells). The degree of inhibition may be expressed in terms of:

$$\frac{(\text{mRNA in control cells}) - (\text{mRNA in treated cells})}{(\text{mRNA in control cells})} \bullet 100\%$$

In one embodiment, inhibition of expression is determined by the dual luciferase method in Example 1 wherein the RNAi agent is present at 10 nM.

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

Contacting a cell *in vitro* may be done, for example, by incubating the cell with the RNAi agent. Contacting a cell *in vivo* may be done, for example, by injecting the RNAi agent into or near the tissue where the cell is located, or by injecting the RNAi agent into another area, *e.g.*, the central nervous system (CNS), optionally *via* intrathecal or other injection, or to 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 RNAi agent may contain or be coupled to a ligand, *e.g.*, a lipophilic moiety or moieties as described below and further detailed, *e.g.*, in PCT Publication No. WO 2019/217459, which is incorporated herein by reference, that directs or otherwise stabilizes the RNAi agent at a site of interest, *e.g.*, the CNS. In some embodiments, the RNAi agent may contain or be coupled to a ligand, *e.g.*, one or more GalNAc derivatives as described below, that directs or otherwise stabilizes the RNAi agent at a site of interest, *e.g.*, the liver. In other embodiments, the RNAi agent may contain or be coupled to a lipophilic moiety or moieties and one or more GalNAc derivatives. 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 RNAi agent and subsequently transplanted into a subject.

In one embodiment, contacting a cell with an RNAi agent includes "introducing" or "delivering the RNAi agent into the cell" by facilitating or effecting uptake or absorption into the cell. Absorption or uptake of a RNAi agent can occur through unaided diffusive or active cellular processes, or by auxiliary agents or devices. Introducing a RNAi agent into a cell may be *in vitro* or *in vivo*. For example, for *in vivo* introduction, a RNAi agent can be injected into a tissue site or administered systemically. *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.

The term "lipophile" or "lipophilic moiety" broadly refers to any compound or chemical moiety having an affinity for lipids. One way to characterize the lipophilicity of the lipophilic moiety is by the octanol-water partition coefficient, $\log K_{ow}$, where K_{ow} is the ratio of a chemical's concentration in the octanol-phase to its concentration in the aqueous phase of a two-phase system at equilibrium. The octanol-water partition coefficient is a laboratory-measured property of a substance. However, it may also be predicted by using coefficients attributed to the structural components of a chemical which are calculated using first-principle or empirical methods (see, for example, Tetko *et al.*, *J. Chem. Inf. Comput.*

Sci. 41:1407-21 (2001), which is incorporated herein by reference in its entirety). It provides a thermodynamic measure of the tendency of the substance to prefer a non-aqueous or oily milieu rather than water (*i.e.* its hydrophilic/lipophilic balance). In principle, a chemical substance is lipophilic in character when its $\log K_{ow}$ exceeds 0. Typically, the lipophilic moiety possesses a $\log K_{ow}$ exceeding 1, exceeding 1.5, exceeding 2, exceeding 3, exceeding 4, exceeding 5, or exceeding 10. For instance, the $\log K_{ow}$ of 6-amino hexanol, for instance, is predicted to be approximately 0.7. Using the same method, the $\log K_{ow}$ of cholesteryl N-(hexan-6-ol) carbamate is predicted to be 10.7.

The lipophilicity of a molecule can change with respect to the functional group it carries. For instance, adding a hydroxyl group or amine group to the end of a lipophilic moiety can increase or decrease the partition coefficient (*e.g.*, $\log K_{ow}$) value of the lipophilic moiety.

Alternatively, the hydrophobicity of the double-stranded RNAi agent, conjugated to one or more lipophilic moieties, can be measured by its protein binding characteristics. For instance, in certain embodiments, the unbound fraction in the plasma protein binding assay of the double-stranded RNAi agent could be determined to positively correlate to the relative hydrophobicity of the double-stranded RNAi agent, which could then positively correlate to the silencing activity of the double-stranded RNAi agent.

In one embodiment, the plasma protein binding assay determined is an electrophoretic mobility shift assay (EMSA) using human serum albumin protein. An exemplary protocol of this binding assay is illustrated in detail in, *e.g.*, PCT Publication No. WO 2019/217459. The hydrophobicity of the double-stranded RNAi agent, measured by fraction of unbound siRNA in the binding assay, exceeds 0.15, exceeds 0.2, exceeds 0.25, exceeds 0.3, exceeds 0.35, exceeds 0.4, exceeds 0.45, or exceeds 0.5 for an enhanced *in vivo* delivery of siRNA.

Accordingly, conjugating the lipophilic moieties to the internal position(s) of the double-stranded RNAi agent provides optimal hydrophobicity for the enhanced *in vivo* delivery of siRNA.

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.,* a rNAi agent or a plasmid from which a RNAi agent is transcribed. LNPs are described in, for example, U.S. Patent 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.

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), or a non-primate (such as a cow, sheep, rat, or a mouse). In one embodiment, 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 PRNP expression; a human at risk for a disease, disorder, or condition that would benefit from reduction in PRNP expression; a human having a disease, disorder, or condition that would benefit from reduction in PRNP expression; or human being treated for a disease, disorder, or condition that would benefit from reduction in PRNP expression as described herein.

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 PRNP gene expression or PRNP protein production, *e.g.,* a PRNP-associated disease, *e.g.*, a prion disease, such as a genetic prion disease, a sporadic prion disease, or an acquired prion disease, in subjects having such diseases. "Treatment" can also mean prolonging survival as compared to expected survival in the absence of treatment.

The term "lower" in the context of the level of PRNP 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 10%, 15%, 20%, 25%, 30%, %, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or more. In certain embodiments, a decrease is at least 20%. In certain embodiments, the decrease is at least 50% in a disease marker, *e.g.*, protein or gene expression level. "Lower" in the context of the level of PRNP in a subject is a decrease to a level accepted as within the range of normal for an individual without such disorder. In certain embodiments, "lower" is the decrease in the difference between the level of a marker or symptom for a subject suffering from a disease and a level accepted within the range of normal for an individual, *e.g.*, the level of decrease in bodyweight between an obese individual and an individual having a weight accepted within the range of normal.

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 PRNP gene or production of a PRNP 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 symptom of a PRNP-associated disease. The failure to develop a disease, disorder, or condition, or the reduction in the development of a symptom 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 symptoms delayed (*e.g.*, by days, weeks, months or years) is considered effective prevention.

As used herein, the term "PRNP-associated disease" or "PRNP-associated disorder" is understood as any disease or disorder that would benefit from reduction in the expression or activity of PRNP. Such PRNP-associated diseases are characterized by increasing deposition of the abnormally folded scrapie form of PrP protein (PrP^{sc}) in areas of the brain associated with neuronal cell death in such diseases, *e.g.,* prion diseases, such as a genetic prion disease, *e.g.*, Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI), a sporadic prion disease, *e.g.,* sporadic Creutzfeldt-Jakob disease, Sporadic Fatal Insomnia (sFI), and Variably Proteinase-Sensitive Prionopathy (VPSPr), or an acquired prion disease, *e.g.*, Iatrogenic CJD (iCJD), Kuru, and Variant CJD (vCJD).

In one embodiment, a PRNP-associated disease is prion disease. Prion disease is a chronic neurodegenerative disease and is caused by abnormal prions, microscopic infectious agents made of protein. Prions cause a number of diseases in a variety of mammals, including bovine spongiform encephalopathy (BSE or mad cow disease) in cattle and scrapie in sheep. Spongiform refers to the characteristic appearance of infected brains, which become filled with holes until they resemble sponges

when examined under a microscope. There are three different subtypes of prion disease categorized by how the disease is contracted. All differ slightly with regards to typical signs, symptoms and duration of illness. The subtypes are sporadic, genetic and acquired. In some embodiments, the PRNP-associated disease is a genetic prion disease, *e.g.,* familial Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI). In some embodiments, the PRNP-associated disease is a sporatic prion disease, *e.g.,* sporadic Creutzfeldt-Jakob disease, sporadic fatal insomnia. In other embodiments, the PRNP-associated disease is an acquired prion disease, *e.g.,* iatogenic Creutzfeldt-Jakob disease, variant Creutzfeldt-Jakob disease or Kuru.

Prion disease usually starts slowly and gradually worsens over time. The common symptoms of prion disease include difficulties with thinking, memory andjudgment, personality changes such as apathy, agitation and depression, confusion or disorientation (including easily getting lost),involuntary muscle spasms (myoclonus), loss of coordination (ataxia), trouble sleeping (insomnia), difficult or slurred speech and impaired vision or blindness. As a person's condition declines, they often withdraw from family and society. Gradually, bodily functions are lost, ultimately leading to death.

Creutzfeldt-Jakob disease (CJD) is the most common of the known human prion disease. CJD is a rare, degenerative, fatal brain disorder. It affects about one person in every one million per year worldwide; in the United States there are about 350 cases per year. CJD usually appears in later life and runs a rapid course. Typical onset of symptoms occurs at about age 60, and about 70 percent of individuals die within one year. In the early stages of the disease, people may have failing memory, behavioral changes, lack of coordination, and visual disturbances. As the illness progresses, mental deterioration becomes pronounced and involuntary movements, blindness, weakness of extremities, and coma may occur.

There are three major categories of CJD. In sporadic CJD, the disease appears even though the person has no known risk factors for the disease. This is by far the most common type of CJD and accounts for at least 85 percent of cases. In genetic CJD, the person may have a family history of the disease and test positive for a genetic mutation associated with CJD. About 10 to 15 percent of cases of CJD in the United States are hereditary. In acquired CJD, the disease is transmitted by exposure to brain or nervous system tissue, usually through certain medical procedures. There is no evidence that CJD is contagious through casual contact with someone who has CJD. Since CJD was first described in 1920, fewer than one percent of cases have been acquired CJD. A type of CJD called variant CJD (or vCJD) can be acquired by eating meat from cattle affected by a disease similar to CJD called bovine spongiform encephalopathy (BSE) or, commonly, “mad cow” disease.

CJD is characterized by rapidly progressive dementia. Initially, individuals experience problems with muscle coordination, personality changes (including impaired memory, judgment, and thinking), and impaired vision. People with the disease, especially with FFI, also may experience insomnia, depression, or unusual sensations. As the illness progresses, peoples’ mental impairment becomes severe. They often develop involuntary muscle jerks called myoclonus, and they may go blind. They eventually lose the ability to move and speak, and enter a coma. Pneumonia and other infections often occur in these

individuals and can lead to death. Variant CJD begins primarily with psychiatric symptoms, affects younger individuals than other types of CJD, and has a longer than usual duration from onset of symptoms to death.

Some symptoms of CJD can be similar to symptoms of other progressive neurological disorders, such as Alzheimer's and Huntington's disease. However, CJD causes unique changes in brain tissue which can be seen at autopsy. It also tends to cause more rapid deterioration of a person's abilities than Alzheimer's disease or most other types of dementia.

"Therapeutically effective amount," as used herein, is intended to include the amount of an RNAi agent that, when administered to a subject having a PRNP-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). The "therapeutically effective amount" may vary depending on the RNAi agent, how the agent is administered, the disease and its severity and the history, age, weight, family history, genetic makeup, the types of preceding or concomitant treatments, if any, and other individual characteristics of the subject to be treated.

"Prophylactically effective amount," as used herein, is intended to include the amount of a RNAi agent that, when administered to a subject having a PRNP-associated disorder, is sufficient to prevent or ameliorate the disease or one or more symptoms of the disease. Ameliorating the disease includes slowing the course of the disease or reducing the severity of later-developing disease. The "prophylactically effective amount" may vary depending on the RNAi agent, how the agent 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.

A "therapeutically-effective amount" or "prophylacticaly effective amount" also includes an amount of a RNAi agent that produces some desired local or systemic effect at a reasonable benefit/risk ratio applicable to any treatment. A RNAi agent employed in the methods of the present disclosure may be administered in a sufficient amount to produce a reasonable benefit/risk ratio applicable to such treatment.

The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials (including salts), 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.

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 state, 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.

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 brain (*e.g.*, whole brain or certain segments of brain, *e.g.,* striatum, or certain types of cells in the brain, such as, *e.g.*, neurons and glial cells (astrocytes, oligodendrocytes, microglial cells)). In other embodiments, a "sample derived from a subject" refers to liver tissue (or subcomponents thereof) derived from the subject. In some embodiments, a "sample derived from a subject" refers to blood drawn from the subject or plasma or serum derived therefrom. In further embodiments, a "sample derived from a subject" refers to brain tissue (or subcomponents thereof) or retinal tissue (or subcomponents thereof) derived from the subject.

II. RNAi Agents of the Disclosure

Described herein are RNAi agents which inhibit the expression of a PRNP gene. In one embodiment, the RNAi agent includes double stranded ribonucleic acid (dsRNA) molecules for inhibiting the expression of a PRNP gene in a cell, such as a cell within a subject, *e.g.,* a mammal, such as a human having a PRNP-associated disease, *e.g.*, a prion disease, such as a genetic prion disease, *e.g.,* familial Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI); a sporadic prion disease, *e.g.,* sporadic Creutzfeldt-Jakob disease, Sporadic Fatal Insomnia (sFI), and Variably Proteinase-Sensitive Prionopathy (VPSPr); or an acquired prion disease, *e.g.,* Iatrogenic CJD (iCJD), Kuru, and Variant CJD (vCJD).

The dsRNA 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 PRNP gene. The region of complementarity is about 15-30 nucleotides or less in length. Upon contact with a cell expressing the PRNP gene, the RNAi agent inhibits the expression of the PRNP gene (*e.g.,* a human gene, a primate

gene, a non-primate gene) by at least 50% 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 certain embodiments, inhibition of expression is by at least 50% as assayed by the Dual-Glo lucifierase assay in Example 1where the siRNA is at a 10 nM concentration.

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 PRNP 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.

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. In certain embodiments, the duplex structure is 18 to 25 base pairs in length, *e.g.*, 18-25, 18-24, 18-23, 18-22, 18-21, 18-20, 19-25, 19-24, 19-23, 19-22, 19-21, 19-20, 20-25, 20-24,20-23, 20-22, 20-21, 21-25, 21-24, 21-23, 21-22, 22-25, 22-24, 22-23, 23-25, 23-24 or 24-25 base pairs in length, for example, 19-21 basepairs in length. Ranges and lengths intermediate to the above recited ranges and lengths are also contemplated to be part of the disclosure.

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, for example 19-23 nucleotides in length or 21-23 nucleotides in length. Ranges and lengths intermediate to the above recited ranges and lengths are also contemplated to be part of the disclosure.

In some embodiments, the duplex structure is 19 to 30 base pairs in length. Similarly, the region of complementarity to the target sequence is 19 to 30 nucleotides in length.

In some embodiments, the dsRNA is 15 to 23 nucleotides in length, or 25 to 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 can 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).

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 15 to 36 base pairs, *e.g.*, 15-36, 15-35, 15-34, 15-33, 15-32, 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, for example, 19-21 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, a RNAi agent useful to target PRNP expression is not generated in the target cell by cleavage of a larger dsRNA.

A dsRNA as described herein can further include one or more single-stranded nucleotide overhangs *e.g.*, 1, 2, 3, or 4 nucleotides. 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. In certain embodiments, longer, extended overhangs are possible.

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.

iRNA 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. Single-stranded oligonucleotides of the invention can be prepared using solution-phase or solid-phase organic synthesis or both.

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

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

A large bioreactor, *e.g.*, the OligoPilot II from Pharmacia Biotec AB (Uppsala Sweden), can be used to produce a large amount of a particular RNA strand for a given siRNA. The OligoPilotII reactor can efficiently couple a nucleotide using only a 1.5 molar excess of a phosphoramidite nucleotide. To

make an RNA strand, ribonucleotides amidites are used. Standard cycles of monomer addition can be used to synthesize the 21 to 23 nucleotide strand for the siRNA. Typically, the two complementary strands are produced separately and then annealed, *e.g.*, after release from the solid support and deprotection.

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

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

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

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

In one aspect, a dsRNA of the disclosure includes at least two nucleotide sequences, a sense sequence and an antisense sequence. The sense strand sequence for PRNP may be selected from the group of sequences provided in any one of Tables 2-3, and the corresponding nucleotide sequence of the antisense strand of the sense strand may be selected from the group of sequences of any one of Tables 2-3. 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 PRNP gene. As such, in this aspect, a dsRNA will include two oligonucleotides, where one oligonucleotide is described as the sense strand (passenger strand) in any one of Tables 2-3, and the second oligonucleotide is described as the corresponding antisense strand (guide strand) of the sense strand in any one of Tables 2-3 for PRNP.

In one embodiment, the substantially complementary sequences of the dsRNA are contained on separate oligonucleotides. In another embodiment, the substantially complementary sequences of the dsRNA are contained on a single oligonucleotide.

It will be understood that, although the sequences provided herein are described as modified or conjugated sequences, the RNA of the RNAi agent of the disclosure *e.g.*, a dsRNA of the disclosure, may comprise any one of the sequences set forth in any one of Tables 2-3 that is un-modified, un-conjugated, or modified or conjugated differently than described therein. One or more lipophilic ligands or one or

more GalNAc ligands can be included in any of the positions of the RNAi agents provided in the instant application.

The skilled person is well aware that dsRNAs having a duplex structure of about 20 to 23 base pairs, *e.g.,* 21, base pairs have been hailed as particularly effective in inducing RNA interference (Elbashir *et al.*, (2001) *EMBO J.*, 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 herein, dsRNAs described herein can include at least one strand of a length of minimally 21 nucleotides. It can be reasonably expected that shorter duplexes 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 provided herein, and differing in their ability to inhibit the expression of a PRNP gene by not more than 10, 15, 20, 25, or 30 % inhibition from a dsRNA comprising the full sequence using the *in vitro* assay with Cos7 and a 10 nM concentration of the RNA agent and the PCR assay as provided in the examples herein, are contemplated to be within the scope of the present disclosure.

In addition, the RNAs described herein identify a site(s) in a PRNP transcript that is susceptible to RISC-mediated cleavage. As such, the present disclosure further features RNAi agents that target within this site(s). As used herein, a RNAi agent is said to target within a particular site of an RNA transcript if the RNAi agent promotes cleavage of the transcript anywhere within that particular site. Such a RNAi agent will generally include at least about 15 contiguous nucleotides, such as at least 19 nucleotides, from one of the sequences provided herein coupled to additional nucleotide sequences taken from the region contiguous to the selected sequence in a PRNP gene.

An RNAi agent as described herein can contain one or more mismatches to the target sequence. In one embodiment, an RNAi agent as described herein contains no more than 3 mismatches (*i.e.,* 3, 2, 1, or 0 mismatches). In one embodiment, an RNAi agent as described herein contains no more than 2 mismatches. In one embodiment, an RNAi agent as described herein contains no more than 1 mismatch. In one embodiment, an RNAi agent as described herein contains 0 mismatches. In certain embodiments, if the antisense strand of the RNAi agent contains mismatches to the target sequence, the mismatch can optionally be restricted to be within the last 5 nucleotides from either the 5'- or 3'-end of the region of complementarity. For example, in such embodiments, for a 23 nucleotide RNAi agent, the strand which is complementary to a region of a PRNP 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 RNAi agent containing a mismatch to a target sequence is effective in inhibiting the expression of a PRNP gene. Consideration of the efficacy of RNAi agents with mismatches in inhibiting expression of a PRNP gene is important, especially if the particular region of complementarity in a PRNP gene is known to have polymorphic sequence variation within the population.

III. Modified RNAi Agents of the Disclosure

In one embodiment, the RNA of the RNAi agent of the disclosure *e.g.*, a dsRNA, is un-modified, and does not comprise, *e.g.,* chemical modifications or conjugations known in the art and described herein. In certain embodiments, the RNA of an RNAi agent of the disclosure, *e.g.*, a dsRNA, is chemically modified to enhance stability or other beneficial characteristics. In certain embodiments of the disclosure, substantially all of the nucleotides of an RNAi agent of the disclosure are modified. In other embodiments of the disclosure, all of the nucleotides of an RNAi agent of the disclosure are modified. RNAi agents of the disclosure in which "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. In still other embodiments of the disclosure, RNAi agents of the disclosure can include not more than 5, 4, 3, 2 or 1 modified nucleotides.

The nucleic acids featured in the disclosure 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 RNAi agents 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 RNAi agent will have a phosphorus atom in its internucleoside backbone.

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 analogs 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, *e.g.,* sodium salts, mixed salts and free acid forms are also included.

Representative U.S. patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S. Patent 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 US Pat RE39464, the entire contents of each of which are hereby incorporated herein by reference.

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 CH_2 component parts.

Representative U.S. patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S. Patent 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.

In other embodiments, suitable RNA mimetics are contemplated for use in RNAi agents, 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, 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 U.S. patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Patent 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 RNAi agents of the disclosure are described in, for example, in Nielsen *et al.*, *Science*, 1991, 254, 1497-1500.

Some embodiments featured in the disclosure include RNAs with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular --CH_2--NH--CH_2-, --CH_2--N(CH_3)--O--CH_2--[known as a methylene (methylimino) or MMI backbone], --CH_2--O--N(CH_3)--CH_2--, --CH_2--N(CH_3)--N(CH_3)--CH_2-- and --N(CH_3)--CH_2--CH_2--of the above-referenced U.S. Patent No. 5,489,677, and the amide backbones of the above-referenced U.S. Patent No. 5,602,240. In some embodiments, the RNAs featured herein have morpholino backbone structures of the above-referenced US5,034,506. The native phosphodiester backbone can be represented as -O-P(O)(OH)-OCH_2-.

Modified RNAs can also contain one or more substituted sugar moieties. The RNAi agents, *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_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Exemplary suitable modifications include $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about 10. In other embodiments, dsRNAs include one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of a RNAi agent, or a group for improving the pharmacodynamic properties of a RNAi agent, and other substituents having similar properties. In some embodiments, the modification includes a 2'-methoxyethoxy (2'-O--$CH_2CH_2OCH_3$, 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'-dimethylaminooxyethoxy, *i.e.*, a $O(CH_2)_2ON(CH_3)_2$ 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_2--O--CH_2--$N(CH_3)_2$. 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).

Other modifications include 2'-methoxy (2'-OCH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$), 2'-*O*-hexadecyl, and 2'-fluoro (2'-F). Similar modifications can also be made at other positions on the RNA of a RNAi agent, 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. RNAi agents can also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative U.S. 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.

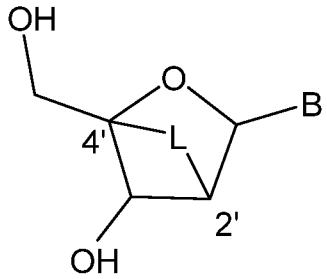
An RNAi agent of the disclosure can also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 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 anal 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, these disclosed by Englisch *et al.*, (1991) *Angewandte Chemie, International Edition*, 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 disclosure. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and 0-6 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.

Representative U.S. 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. Patent 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.

An RNAi agent of the disclosure can also be modified to include one or more bicyclic sugar moities. A “bicyclic sugar” is a furanosyl ring modified by a ring formed by the bridging of two carbons, whether adjacent or non-adjacent. A “bicyclic nucleoside” (“BNA”) is a nucleoside having a sugar moiety comprising a ring formed by bridging two carbons, whether adjacent or non-adjacent, 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, optionally, via the 2’-acyclic oxygen atom. Thus, in some embodiments an agent of the disclosure 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 disclosure 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 disclosure include one or more bicyclic nucleosides comprising a 4′ to 2′ bridge.

A locked nucleoside can be represented by the structure (omitting stereochemistry),



wherein B is a nucleobase or modified nucleobase and L is the linking group that joins the 2'-carbon to the 4'-carbon of the ribose ring. Examples of such 4′ to 2′ bridged bicyclic nucleosides, include but are not limited to 4′-(CH2)—O-2′ (LNA); 4′-(CH_2)—S-2′; 4′-(CH_2)$_2$—O-2′ (ENA); 4′-CH(CH3)—O-2′ (also referred to as "constrained ethyl" or "cEt") and 4′-CH(CH_2OCH_3)—O-2′ (and analogs thereof; see, *e.g.*, U.S. Pat. No. 7,399,845); 4′-C(CH_3)(CH_3)—O-2′ (and analogs thereof; see *e.g.*, US Patent No. 8,278,283); 4′-CH_2—N(OCH_3)-2′ (and analogs thereof; see *e.g.*, US Patent No. 8,278,425); 4′-CH_2—O—N(CH_3)-2′ (see, *e.g.*,U.S. Patent Publication No. 2004/0171570); 4′-CH_2—N(R)—O-2′, wherein R is H, C_1-C_{12} alkyl, or a nitrogen protecting group (see, *e.g.*, U.S. Pat. No. 7,427,672); 4′-CH_2—C(H)(CH_3)-2′ (see, *e.g.*, Chattopadhyaya *et al., J. Org. Chem.*, 2009, 74, 118-134); and 4′-CH_2—C(=CH_2)-2′ (and analogs thereof; see, *e.g.*, US Patent No. 8,278,426). The entire contents of each of the foregoing are hereby incorporated herein by reference.

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: US Patent 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.

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).

An RNAi agent of the disclosure 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(CH_3)-O-2' bridge (i.e., L in the preceding structure). In one embodiment, a constrained ethyl nucleotide is in the S conformation referred to herein as "S-cEt."

An RNAi agent of the disclosure 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 PRNP 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.

Representative publications that teach the preparation of certain of the above noted CRN include, but are not limited to, US 2013/0190383; and WO 2013/036868, the entire contents of each of which are hereby incorporated herein by reference.

In some embodiments, a RNAi agent of the disclosure 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'-PRNP' bond (*i.e.* the covalent carbon-carbon bond between the C2' and PRNP' 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).

Representative U.S. publications that teach the preparation of UNA include, but are not limited to, US8,314,227; 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.

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 2'-deoxy-modified ribonucleotide, such as inverted dT(idT), inverted dA (idA), and inverted abasic 2'-deoxyribonucleotide (iAb) and others. Disclosure of this modification can be found in WO 2011/005861.

In one example, the 3' or 5' terminal end of a oligonucleotide is linked to an inverted 2'-deoxy-modified ribonucleotide, such as inverted dT(idT), inverted dA (idA), or a inverted abasic 2'-deoxyribonucleotide (iAb). In one particular example, the inverted 2'-deoxy-modified ribonucleotide is linked to the 3'end of an oligonucleotide, such as the 3'-end of a sense strand described herein, where the linking is via a 3'-3' phosphodiester linkage or a 3'-3'-phosphorothioate linkage.

In another example, the 3'-end of a sense strand is linked via a 3'-3'-phosphorothioate linkage to an inverted abasic ribonucleotide (iAb). In another example, the 3'-end of a sense strand is linked via a 3'-3'-phosphorothioate linkage to an inverted dA (idA).

In one particular example, the inverted 2'-deoxy-modified ribonucleotide is linked to the 3'end of an oligonucleotide, such as the 3'-end of a sense strand described herein, where the linking is via a 3'-3' phosphodiester linkage or a 3'-3'-phosphorothioate linkage.

In another example, the 3'-terminal nucleotides of a sense strand is an inverted dA (idA) and is linked to the preceding nucleotide via a 3'-3'- linkage (*e.g.*, 3'-3'-phosphorothioate linkage).

Other modifications of a RNAi agent of the disclosure include a 5' phosphate or 5' phosphate mimic, *e.g.,* a 5'-terminal phosphate or phosphate mimic on the antisense strand of a RNAi agent. Suitable phosphate mimics are disclosed in, for example US 2012/0157511, the entire contents of which are incorporated herein by reference.

A. Modified RNAi agents Comprising Motifs of the Disclosure

In certain aspects of the disclosure, the double-stranded RNAi agents of the disclosure include agents with chemical modifications as disclosed, for example, in WO 2013/075035, the entire contents of

which are incorporated herein by reference. As shown herein and in WO 2013/075035, one or more motifs of three identical modifications on three consecutive nucleotides may be introduced into a sense strand or antisense strand of an RNAi agent, particularly at or near the cleavage site. In some embodiments, the sense strand and antisense strand of the RNAi agent may otherwise be completely modified. The introduction of these motifs interrupts the modification pattern, if present, of the sense or antisense strand. The RNAi agent may be optionally conjugated with a lipophilic ligand, *e.g.*, a C16 ligand, for instance on the sense strand. The RNAi agent may be optionally modified with a (*S*)-glycol nucleic acid (GNA) modification, for instance on one or more residues of the antisense strand.

Accordingly, the disclosure provides double stranded RNAi agents capable of inhibiting the expression of a target gene (*i.e.*, a PRNP gene) *in vivo*. The RNAi agent comprises a sense strand and an antisense strand. Each strand of the RNAi agent may be 15-30 nucleotides in length. For example, each strand may be 16-30 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. In certain embodiments, each strand is 19-23 nucleotides in length.

The sense strand and antisense strand typically form a duplex double stranded RNA ("dsRNA"), also referred to herein as an "RNAi agent." The duplex region of an RNAi agent may be 15-30 nucleotide pairs in length. For example, the duplex region can be 16-30 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. In certain embodiments, the duplex region is 19-21 nucleotide pairs in length.

In one embodiment, the RNAi 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 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 nucleotide overhang region is 2 nucleotides in length. 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.

In one embodiment, the nucleotides in the overhang region of the RNAi agent can each independently be a modified or unmodified nucleotide including, but no limited to 2'-sugar modified, such as, 2-F, 2'-O-methyl, thymidine (T), 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.

The 5'- or 3'- overhangs at the sense strand, antisense strand or both strands of the RNAi 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 one embodiment, the overhang is present at the 3'-end of the sense strand, antisense strand, or both strands. In one embodiment, this 3'-overhang is present in the antisense strand. In one embodiment, this 3'-overhang is present in the sense strand.

The RNAi 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'-terminal end of the sense strand or, alternatively, at the 3'-terminal end of the antisense strand. The RNAi may also have a blunt end, located at the 5'-end of the antisense strand (i.e., the 3'-end of the sense strand) or *vice versa*. Generally, the antisense strand of the RNAi 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.

In one embodiment, the RNAi agent is a double blunt-ended 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, and 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, and 13 from the 5'end.

In another embodiment, the RNAi agent is a double blunt-ended 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, and 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, and 13 from the 5'end.

In yet another embodiment, the RNAi agent is a double blunt-ended 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, and 13 from the 5'end.

In one embodiment, the RNAi 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, and 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, and 13 from the 5'end, wherein one end of the RNAi agent is blunt, while the other end comprises a 2 nucleotide overhang. In one example, the two nucleotide overhang is at the 3'-end of the antisense strand. When the two 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 one embodiment, every nucleotide in the sense strand and the antisense strand of the RNAi agent, including the nucleotides that are part of the motifs are modified nucleotides. In one embodiment each residue is independently modified with a 2'-O-methyl or 2'-fluoro, *e.g.,* in an alternating motif. Optionally, the RNAi agent further comprises a ligand (*e.g.*, a lipophilic ligand, optionally a C16 ligand).

In one embodiment, the RNAi 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.

In one embodiment, the RNAi agent comprises sense and antisense strands, wherein the RNAi 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, and 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 region which is at least 25 nucleotides in length, and the second strand is sufficiently complemenatary 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 RNAi agent results in an siRNA comprising the 3' end of the second strand, thereby reducing expression of the target gene in the mammal. Optionally, the RNAi agent further comprises a ligand.

In one embodiment, the sense strand of the RNAi 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.

In one embodiment, the antisense strand of the RNAi 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.

For an RNAi 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, and 11 positions; 10, 11, and 12 positions; 11, 12, and 13 positions; 12, 13, and 14 positions; or 13, 14, and 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 RNAi from the 5'-end.

The sense strand of the RNAi 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.

In one embodiment, the sense strand of the RNAi 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 adajacent 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 chemistry 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.

Like the sense strand, the antisense strand of the RNAi agent may contain more than one motif 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.

In one embodiment, the wing modification on the sense strand or antisense strand of the RNAi agent typically does not include the first one or two terminal nucleotides at the 3'-end, 5'-end or both ends of the strand.

In another embodiment, the wing modification on the sense strand or antisense strand of the RNAi 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.

When the sense strand and the antisense strand of the RNAi 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.

When the sense strand and the antisense strand of the RNAi 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.

In one embodiment, the RNAi agent comprises mismatch(es) with the target, within the duplex, or combinations thereof. The mistmatch 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.

In one embodiment, the RNAi 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.

In one embodiment, the nucleotide at the 1 position within the duplex region from the 5'-end in the antisense strand is selected from the group consisting of 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.

In another embodiment, the nucleotide at the 3'-end of the sense strand is deoxythimidine (dT). In another embodiment, the nucleotide at the 3'-end of the antisense strand is deoxythimidine (dT). In one embodiment, there is a short sequence of deoxythimidine nucleotides, for example, two dT nucleotides on the 3'-end of the sense or antisense strand.

In one embodiment, the sense strand sequence may be represented by formula (I):

5' n_p-N_a-(X X X)$_i$-N_b-Y Y Y -N_b-(Z Z Z)$_j$-N_a-n_q 3' (I)

wherein:

i and j are each independently 0 or 1;

p and q are each independently 0-6;

each N_a independently represents an oligonucleotide sequence comprising 0-25 modified nucleotides, each sequence comprising at least two differently modified nucleotides;

each N_b independently represents an oligonucleotide sequence comprising 0-10 modified nucleotides;

each n_p and n_q independently represent an overhang nucleotide;

wherein Nb and Y do not have the same modification; and

XXX, YYY and ZZZ each independently represent one motif of three identical modifications on three consecutive nucleotides. In one embodiment, YYY is all 2'-F modified nucleotides.

In one embodiment, the N_a or N_b comprise modifications of alternating pattern.

In one embodiment, the YYY motif occurs at or near the cleavage site of the sense strand. For example, when the RNAi 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 1st nucleotide, from the 5'-end; or optionally, the count starting at the 1st paired nucleotide within the duplex region, from the 5'- end.

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_p-N_a-YYY-N_b-ZZZ-N_a-n_q 3' (Ib);

5' n_p-N_a-XXX-N_b-YYY-N_a-n_q 3' (Ic); or

5' n_p-N_a-XXX-N_b-YYY-N_b-ZZZ-N_a-n_q 3' (Id).

When the sense strand is represented by formula (Ib), N_b represents an oligonucleotide sequence comprising 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides.

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

When the sense strand is represented as formula (Ic), N_b 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_a can independently represent an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the sense strand is represented as formula (Id), each N_b independently represents an oligonucleotide sequence comprising 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides. In one embodiment, N_b is 0, 1, 2, 3, 4, 5 or 6. Each N_a can independently represent 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.

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

5' n_p-N_a-YYY- N_a-n_q 3' (Ia).

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

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

$$5' \; n_{q'}\text{-}N_a'\text{-}(Z'Z'Z')_k\text{-}N_b'\text{-}Y'Y'Y'\text{-}N_b'\text{-}(X'X'X')_l\text{-}N'_a\text{-}n_p' \; 3' \quad \text{(II)}$$

wherein:

k and l are each independently 0 or 1;

p' and q' are each independently 0-6;

each N_a' independently represents an oligonucleotide sequence comprising 0-25 modified nucleotides, each sequence comprising at least two differently modified nucleotides;

each N_b' independently represents an oligonucleotide sequence comprising 0-10 modified nucleotides;

each n_p' and n_q' independently represent an overhang nucleotide;

wherein N_b' and Y' do not have the same modification; and

X′X′X′, Y′Y′Y′ and Z′Z′Z′ each independently represent one motif of three identical modifications on three consecutive nucleotides.

In one embodiment, the N_a' or N_b' comprise modifications of alternating pattern.

The Y′Y′Y′ motif occurs at or near the cleavage site of the antisense strand. For example, when the RNAi agent has a duplex region of 17-23nucleotidein 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 1st nucleotide, from the 5'-end; or optionally, the count starting at the 1st paired nucleotide within the duplex region, from the 5'- end. In another emobodiment, the Y′Y′Y′ motif occurs at positions 11, 12, 13.

In one embodiment, Y′Y′Y′ motif is all 2'-OMe modified nucleotides.

In one embodiment, k is 1 and l is 0, or k is 0 and l is 1, or both k and l are 1.

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

$$5' \; n_{q'}\text{-}N_a'\text{-}Z'Z'Z'\text{-}N_b'\text{-}Y'Y'Y'\text{-}N_a'\text{-}n_{p'} \; 3' \quad \text{(IIb)};$$

$$5' \; n_{q'}\text{-}N_a'\text{-}Y'Y'Y'\text{-}N_b'\text{-}X'X'X'\text{-}n_{p'} \; 3' \; \text{(IIc)}; \text{ or}$$

$$5' \; n_{q'}\text{-}N_a'\text{-}Z'Z'Z'\text{-}N_b'\text{-}Y'Y'Y'\text{-}N_b'\text{-}X'X'X'\text{-}N_a'\text{-}n_{p'} \; 3' \; \text{(IId)}.$$

When the antisense strand is represented by formula (IIb), N_b' 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_a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the antisense strand is represented as formula (IIc), N_b' 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_a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the antisense strand is represented as formula (IId), each N_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_a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides. In another emobodiment, N_b is 0, 1, 2, 3, 4, 5 or 6.

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

5' $n_{p'}$-$N_{a'}$-Y'Y'Y'- $N_{a'}$-$n_{q'}$ 3' (Ia).

When the antisense strand is represented as formula (IIa), each N_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.

Each nucleotide of the sense strand and antisense strand may be independently modified with LNA, 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.

In one embodiment, the sense strand of the RNAi 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 1st nucleotide from the 5'-end, or optionally, the count starting at the 1st 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.

In one embodiment the antisense strand may contain Y′Y′Y′ motif occurring at positions 11, 12, 13 of the strand, the count starting from the 1st nucleotide from the 5'-end, or optionally, the count starting at the 1st 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.

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 (IId), respectively.

Accordingly, the RNAi agents for use in the methods of the disclosure may comprise a sense strand and an antisense strand, each strand having 14 to 30 nucleotides, the RNAi duplex represented by formula (III):

sense: 5' n_p -N_a-(X X X)$_i$ -N_b- Y Y Y -N_b -(Z Z Z)$_j$-N_a-n_q 3'

antisense: 3' n_p'-N_a'-(X'X'X')$_k$-N_b'-Y'Y'Y'-N_b'-(Z'Z'Z')$_l$-N_a'-n_q' 5'

(III)

wherein:

i, j, k, and l are each independently 0 or 1;

p, p′, q, and q′ are each independently 0-6;

each N_a and N_a' independently represents an oligonucleotide sequence comprising 0-25 modified nucleotides, each sequence comprising at least two differently modified nucleotides;

each N_b and N_b' independently represents an oligonucleotide sequence comprising 0-10 modified nucleotides;

wherein

each n_p', n_p, n_q', and n_q, each of which may or may not be present, independently represents an overhang nucleotide; and

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.

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.

Exemplary combinations of the sense strand and antisense strand forming a RNAi duplex include the formulas below:

5' n_p - N_a -Y Y Y -N_a-n_q 3'

3' n_p'-N_a'-Y′Y′Y′ -$N_a'$$n_q'$ 5'

(IIIa)

5' n_p -N_a -Y Y Y -N_b -Z Z Z -N_a-n_q 3'

3' n_p'-N_a'-Y′Y′Y′-N_b'-Z′Z′Z′-$N_a'$$n_q'$ 5'

(IIIb)

5' n_p-N_a- X X X -N_b -Y Y Y - N_a-n_q 3'

3' n_p'-N_a'-X′X′X′-N_b'-Y′Y′Y′-N_a'-n_q' 5'

(IIIc)

5' n_p -N_a -X X X -N_b-Y Y Y -N_b- Z Z Z -N_a-n_q 3'

3' n_p'-N_a'-X′X′X′-N_b'-Y′Y′Y′-N_b'-Z′Z′Z′-N_a-n_q' 5'

(IIId)

When the RNAi agent is represented by formula (IIIa), each N_a independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the RNAi agent is represented by formula (IIIb), each N_b independently represents an oligonucleotide sequence comprising 1-10, 1-7, 1-5 or 1-4 modified nucleotides. Each N_a independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the RNAi agent is represented as formula (IIIc), each N_b, N_b' independently represents an oligonucleotide sequence comprising 0-10, 0-7, 0-10, 0-7, 0-5, 0-4, 0-2 or 0modified nucleotides. Each N_a independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the RNAi agent is represented as formula (IIId), each N_b, N_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_a, N_a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides. Each of N_a, N_a', N_b and N_b' independently comprises modifications of alternating pattern.

In one embodiment, when the RNAi agent is represented by formula (IIId), the N_a modifications are 2′-O-methyl or 2′-fluoro modifications. In another embodiment, when the RNAi agent is represented by formula (IIId), the N_a modifications are 2′-O-methyl or 2′-fluoro modifications and $n_p' > 0$ and at least one n_p' is linked to a neighboring nucleotide a via phosphorothioate linkage. In yet another embodiment, when the RNAi agent is represented by formula (IIId), the N_a modifications are 2′-O-methyl or 2′-fluoro modifications , $n_p' > 0$ and at least one n_p' is linked to a neighboring nucleotide via phosphorothioate linkage, and the sense strand is conjugated to one or more C16 (or related) moieties attached through a bivalent or trivalent branched linker (described below). In another embodiment, when the RNAi agent is represented by formula (IIId), the N_a modifications are 2′-O-methyl or 2′-fluoro modifications , $n_p' > 0$ and at least one n_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 lipophilic, *e.g.*, C16 (or related) moieties, optionally attached through a bivalent or trivalent branched linker.

In one embodiment, when the RNAi agent is represented by formula (IIIa), the N_a modifications are 2′-O-methyl or 2′-fluoro modifications , $n_p' > 0$ and at least one n_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 lipophilic, *e.g.*, C16 (or related) moieties attached through a bivalent or trivalent branched linker.

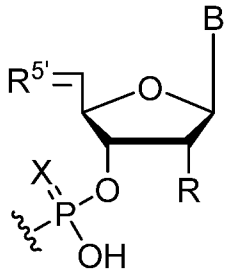
In one embodiment, the RNAi agent is a multimer containing at least two duplexes represented by formula (III), (IIIa), (IIIb), (IIIc), and (IIId), wherein the duplexes are connected by a linker. The linker can be cleavable or non-cleavable. Optionally, the multimer further comprises a ligand. Each of 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.

In one embodiment, the RNAi 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.

In one embodiment, two RNAi agents represented by formula (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 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.

Various publications describe multimeric RNAi agents that can be used in the methods of the disclosure. Such publications include WO2007/091269, WO2010/141511, WO2007/117686, WO2009/014887, and WO2011/031520; and US 7858769, the entire contents of each of which are hereby incorporated herein by reference.

In certain embodiments, the compositions and methods of the disclosure include a vinyl phosphonate (VP) modification of an RNAi agent as described herein. In exemplary embodiments, a 5'-vinyl phosphonate modified nucleotide of the disclosure has the structure:



wherein X is O or S;

R is hydrogen, hydroxy, fluoro, or C_{1-20}alkoxy (e.g., methoxy or n-hexadecyloxy);

$R^{5'}$ is $=C(H)-P(O)(OH)_2$ and the double bond between the C5' carbon and $R^{5'}$ is in the *E* or *Z* orientation (e.g., *E* orientation); and

B is a nucleobase or a modified nucleobase, optionally where B is adenine, guanine, cytosine, thymine, or uracil.

In one embodiment, $R^{5'}$ is $=C(H)-P(O)(OH)_2$ and the double bond between the C5' carbon and R5' is in the E orientation. In another embodiment, R is methoxy and $R^{5'}$ is $=C(H)-P(O)(OH)_2$ and the double bond between the C5' carbon and R5' is in the E orientation. In another embodiment, X is S, R is methoxy, and $R^{5'}$ is $=C(H)-P(O)(OH)_2$ and the double bond between the C5' carbon and R5' is in the E orientation.

A vinyl phosphonate of the instant disclosure may be attached to either the antisense or the sense strand of a dsRNA of the disclosure. In certain embodiments, a vinyl phosphonate of the instant disclosure is attached to the antisense strand of a dsRNA, optionally at the 5' end of the antisense strand of the dsRNA.

Vinyl phosphate modifications are also contemplated for the compositions and methods of the instant disclosure. An exemplary vinyl phosphate structure includes the preceding structure, where $R^{5'}$ is $=C(H)-OP(O)(OH)_2$ and the double bond between the C5' carbon and $R^{5'}$ is in the *E* or *Z* orientation (e.g., *E* orientation).

A. *Thermally Destabilizing Modifications*

In certain embodiments, a dsRNA molecule can be optimized for RNA interference by incorporating thermally destabilizing modifications in the seed region of the antisense strand. As used herein "seed region" means at positions 2-9 of the 5'-end of the referenced strand. For example, thermally destabilizing modifications can be incorporated in the seed region of the antisense strand to reduce or inhibit off-target gene silencing.

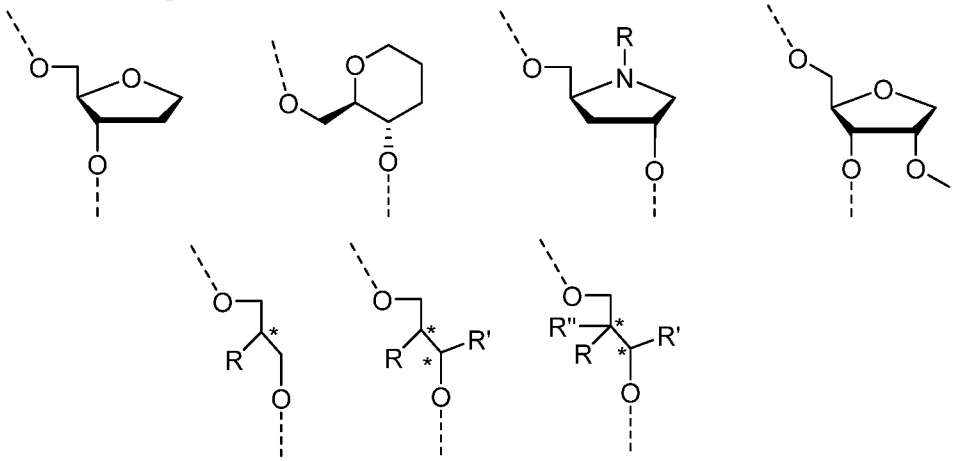
The term "thermally destabilizing modification(s)" includes modification(s) that would result with a dsRNA with a lower overall melting temperature (T_m) than the T_m of the dsRNA without having such modification(s). For example, the thermally destabilizing modification(s) can decrease the T_m of the

dsRNA by 1 – 4 °C, such as one, two, three or four degrees Celcius. And, the term "thermally destabilizing nucleotide" refers to a nucleotide containing one or more thermally destabilizing modifications.

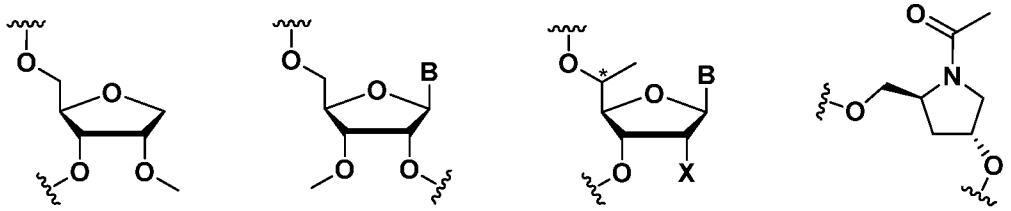
It has been discovered that dsRNAs with an antisense strand comprising at least one thermally destabilizing modification of the duplex within the first 9 nucleotide positions, counting from the 5' end, of the antisense strand have reduced off-target gene silencing activity. Accordingly, in some embodiments, the antisense strand comprises at least one (*e.g.*, one, two, three, four, five or more) thermally destabilizing modification of the duplex within the first 9 nucleotide positions of the 5' region of the antisense strand. In some embodiments, one or more thermally destabilizing modification(s) of the duplex is/are located in positions 2-9, such as positions 4-8, from the 5'-end of the antisense strand. In some further embodiments, the thermally destabilizing modification(s) of the duplex is/are located at position 6, 7 or 8 from the 5'-end of the antisense strand. In still some further embodiments, the thermally destabilizing modification of the duplex is located at position 7 from the 5'-end of the antisense strand. In some embodiments, the thermally destabilizing modification of the duplex is located at position 2, 3, 4, 5 or 9 from the 5'-end of the antisense strand.

The thermally destabilizing modifications can include, but are not limited to, abasic modification; mismatch with the opposing nucleotide in the opposing strand; and sugar modification such as 2'-deoxy modification, acyclic nucleotide, *e.g.*, unlocked nucleic acids (UNA) or glycol nucleic acid (GNA); and 2'-5'-linked ribonucleotides ("3'-RNA").

Exemplified abasic modifications include, but are not limited to the following:



Wherein R = H, Me, Et or OMe; R' = H, Me, Et or OMe; R" = H, Me, Et or OMe



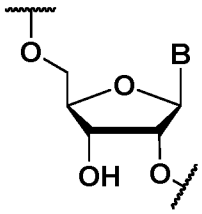
Mod2 (2'-OMe Abasic Spacer)

Mod3 (3'-OMe)

Mod4 (5'-Me)

X = OMe, F

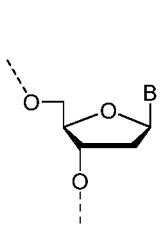
Mod5 (Hyp-spacer)



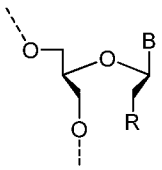
(3'-RNA)

wherein B is a modified or unmodified nucleobase.

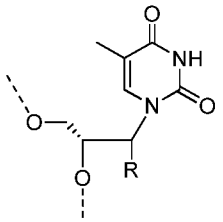
Exemplified sugar modifications include, but are not limited to the following:



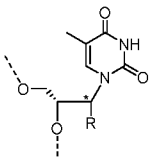
2'-deoxy



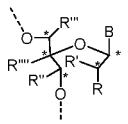
unlocked nucleic acid
R= H, OH, O-alkyl



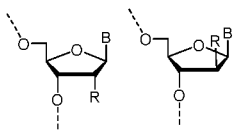
glycol nucleic acid
R= H, OH, O-alkyl



glycol nucleic acid
R= H, OH, O-alkyl



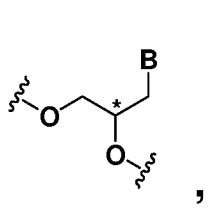
unlocked nucleic acid
R= H, OH, CH_3, CH_2CH_3, O-alkyl, NH_2, NHMe, NMe_2
R' = H, OH, CH_3, CH_2CH_3, O-alkyl, NH_2, NHMe, NMe_2
R'' = H, OH, CH_3, CH_2CH_3, O-alkyl, NH_2, NHMe, NMe_2
R''' = H, OH, CH_3, CH_2CH_3, O-alkyl, NH_2, NHMe, NMe_2
R'''' = H, OH, CH_3, CH_2CH_3, O-alkyl, NH_2, NHMe, NMe_2



R = H, methyl, ethyl

wherein B is a modified or unmodified nucleobase.

In some embodiments the thermally destabilizing modification of the duplex is selected from the group consisting of: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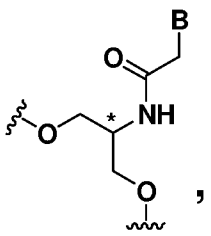
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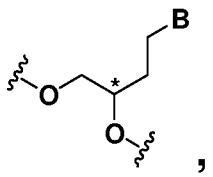
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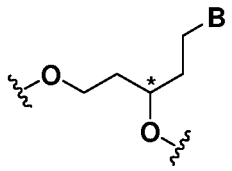
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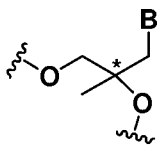
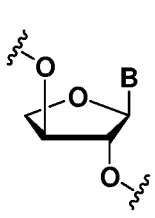
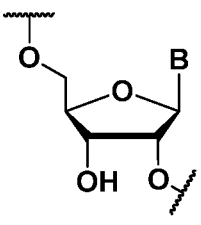
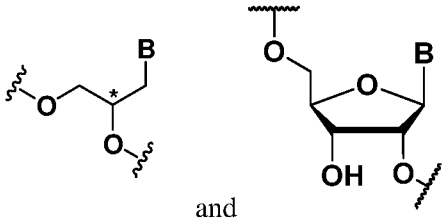

, and

and


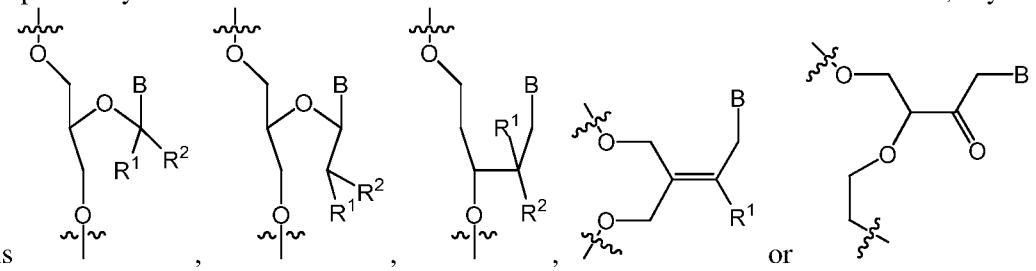
wherein B is a modified or unmodified nucleobase and the asterisk on each structure represents either *R*, *S* or *racemic*.

In some embodiments the thermally destabilizing modification of the duplex is selected from the group consisting of:

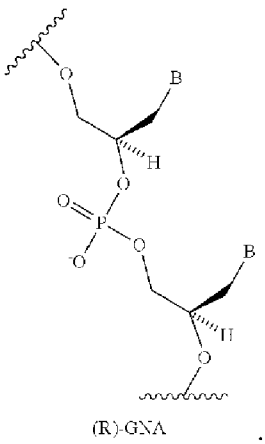


and

wherein B is a modified or unmodified nucleobase and the asterisk represents either *R*, *S* or *racemic* (e.g. S).

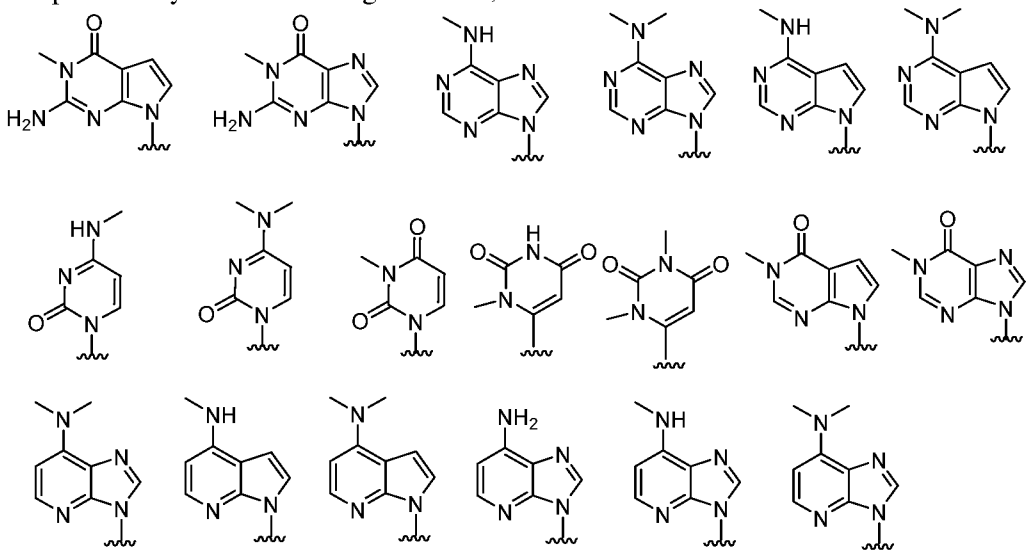
The term "acyclic nucleotide" refers to any nucleotide having an acyclic ribose sugar, for example, where any of bonds between the ribose carbons (*e.g.*, C1'-C2', C2'-PRNP', PRNP'-C4', C4'-O4', or C1'-O4') is absent or at least one of ribose carbons or oxygen (*e.g.*, C1', C2', PRNP', C4', or O4') are independently or in combination absent from the nucleotide. In some embodiments, acyclic nucleotide is  wherein B is a modified or unmodified nucleobase, R^1 and R^2 independently are H, halogen, OR_3, or alkyl; and R_3 is H, alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar). The term "UNA" refers to 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 monomers with bonds between C1'-C4' being removed (*i.e.* the covalent carbon-oxygen-carbon bond between the C1' and C4' carbons). In another example, the C2'-PRNP' bond (*i.e.* the covalent carbon-carbon bond between the C2' and PRNP' carbons) of the sugar is removed (see Mikhailov et. al., Tetrahedron Letters, 26 (17): 2059 (1985); and Fluiter *et al.*, Mol. Biosyst., 10: 1039 (2009), which are hereby incorporated by reference in their entirety). The acyclic derivative provides greater backbone flexibility without affecting the Watson-Crick pairings. The acyclic nucleotide can be linked via 2'-5' or 3'-5' linkage.

The term 'GNA' refers to glycol nucleic acid which is a polymer similar to DNA or RNA but differing in the composition of its "backbone" in that is composed of repeating glycerol units linked by phosphodiester bonds:



The thermally destabilizing modification of the duplex can be mismatches (*i.e.*, noncomplementary base pairs) between the thermally destabilizing nucleotide and the opposing nucleotide in the opposite strand within the dsRNA duplex. Exemplary mismatch base pairs include G:G, G:A, G:U, G:T, A:A, A:C, C:C, C:U, C:T, U:U, T:T, U:T, or a combination thereof. Other mismatch base pairings known in the art are also amenable to the present invention. A mismatch can occur between nucleotides that are either naturally occurring nucleotides or modified nucleotides, *i.e.*, the mismatch base pairing can occur between the nucleobases from respective nucleotides independent of the modifications on the ribose sugars of the nucleotides. In certain embodiments, the dsRNA molecule contains at least one nucleobase in the mismatch pairing that is a 2'-deoxy nucleobase; *e.g.*, the 2'-deoxy nucleobase is in the sense strand.

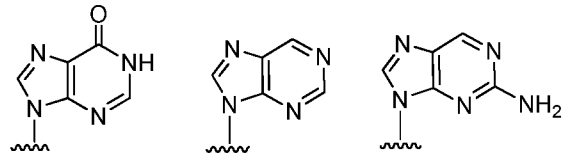
In some embodiments, the thermally destabilizing modification of the duplex in the seed region of the antisense strand includes nucleotides with impaired Watson-Crick hydrogen-bonding to the complementary base on the target mRNA, such as modified nucleobases:



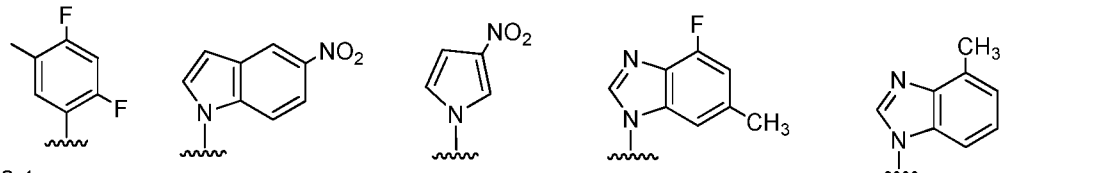
More examples of abasic nucleotide, acyclic nucleotide modifications (including UNA and GNA), and mismatch modifications have been described in detail in WO 2011/133876, which is herein incorporated by reference in its entirety.

The thermally destabilizing modifications may also include universal base with reduced or abolished capability to form hydrogen bonds with the opposing bases, and phosphate modifications.

In some embodiments, the thermally destabilizing modification of the duplex includes nucleotides with non-canonical bases such as, but not limited to, nucleobase modifications with impaired or completely abolished capability to form hydrogen bonds with bases in the opposite strand. These nucleobase modifications have been evaluated for destabilization of the central region of the dsRNA duplex as described in WO 2010/0011895, which is herein incorporated by reference in its entirety. Exemplary nucleobase modifications are:

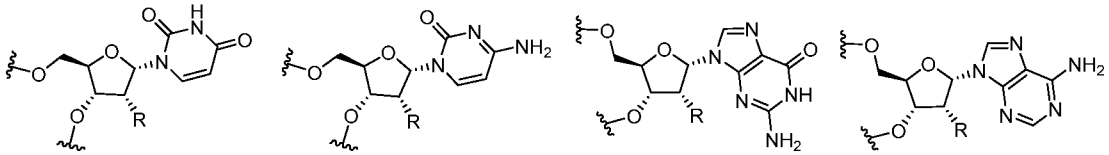


inosine nebularine 2-aminopurine



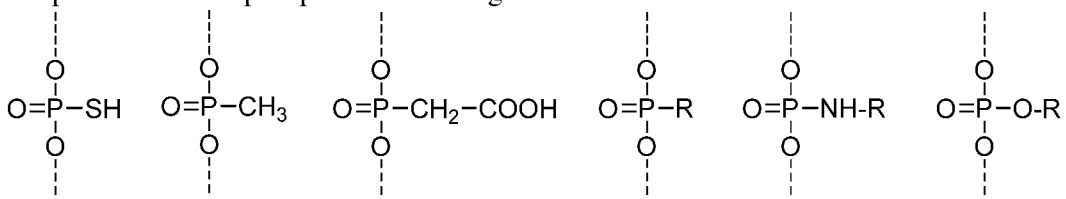
2,4-difluorotoluene 5-nitroindole 3-nitropyrrole 4-Fluoro-6-methylbenzimidazole 4-Methylbenzimidazole

In some embodiments, the thermally destabilizing modification of the duplex in the seed region of the antisense strand includes one or more α-nucleotide complementary to the base on the target mRNA, such as:



wherein R is H, OH, OCH_3, F, NH_2, NHMe, NMe_2 or O-alkyl.

Exemplary phosphate modifications known to decrease the thermal stability of dsRNA duplexes compared to natural phosphodiester linkages are:



R = alkyl

The alkyl for the R group can be a C_1-C_6alkyl. Specific alkyls for the R group include, but are not limited to methyl, ethyl, propyl, isopropyl, butyl, pentyl and hexyl.

As the skilled artisan will recognize, in view of the functional role of nucleobases is defining specificity of a RNAi agent of the disclosure, while nucleobase modifications can be performed in the various manners as described herein, *e.g.*, to introduce destabilizing modifications into a RNAi agent of the disclosure, *e.g.*, for purpose of enhancing on-target effect relative to off-target effect, the range of modifications available and, in general, present upon RNAi agents of the disclosure tends to be much greater for non-nucleobase modifications, *e.g.*, modifications to sugar groups or phosphate backbones of

polyribonucleotides. Such modifications are described in greater detail in other sections of the instant disclosure and are expressly contemplated for RNAi agents of the disclosure, either possessing native nucleobases or modified nucleobases as described above or elsewhere herein.

In addition to the antisense strand comprising a thermally destabilizing modification, the dsRNA can also comprise one or more stabilizing modifications. For example, the dsRNA can comprise at least two (*e.g.*, two, three, four, five, six, seven, eight, nine, ten or more) stabilizing modifications. Without limitations, the stabilizing modifications all can be present in one strand. In some embodiments, both the sense and the antisense strands comprise at least two stabilizing modifications. The stabilizing modification can occur on any nucleotide of the sense strand or antisense strand. For instance, the stabilizing modification can occur on every nucleotide on the sense strand or antisense strand; each stabilizing modification can occur in an alternating pattern on the sense strand or antisense strand; or the sense strand or antisense strand comprises both stabilizing modification in an alternating pattern. The alternating pattern of the stabilizing modifications on the sense strand may be the same or different from the antisense strand, and the alternating pattern of the stabilizing modifications on the sense strand can have a shift relative to the alternating pattern of the stabilizing modifications on the antisense strand.

In some embodiments, the antisense strand comprises at least two (*e.g.*, two, three, four, five, six, seven, eight, nine, ten or more) stabilizing modifications. Without limitations, a stabilizing modification in the antisense strand can be present at any positions. In some embodiments, the antisense comprises stabilizing modifications at positions 2, 6, 8, 9, 14, and 16 from the 5'-end. In some other embodiments, the antisense comprises stabilizing modifications at positions 2, 6, 14, and 16 from the 5'-end. In still some other embodiments, the antisense comprises stabilizing modifications at positions 2, 14, and 16 from the 5'-end.

In some embodiments, the antisense strand comprises at least one stabilizing modification adjacent to the destabilizing modification. For example, the stabilizing modification can be the nucleotide at the 5'-end or the 3'-end of the destabilizing modification, *i.e.*, at position -1 or +1 from the position of the destabilizing modification. In some embodiments, the antisense strand comprises a stabilizing modification at each of the 5'-end and the 3'-end of the destabilizing modification, *i.e.*, positions -1 and +1 from the position of the destabilizing modification.

In some embodiments, the antisense strand comprises at least two stabilizing modifications at the 3'-end of the destabilizing modification, *i.e.*, at positions +1 and +2 from the position of the destabilizing modification.

In some embodiments, the sense strand comprises at least two (*e.g.*, two, three, four, five, six, seven, eight, nine, ten or more) stabilizing modifications. Without limitations, a stabilizing modification in the sense strand can be present at any positions. In some embodiments, the sense strand comprises stabilizing modifications at positions 7, 10, and 11 from the 5'-end. In some other embodiments, the sense strand comprises stabilizing modifications at positions 7, 9, 10, and 11 from the 5'-end. In some embodiments, the sense strand comprises stabilizing modifications at positions opposite or complimentary to positions 11, 12, and 15 of the antisense strand, counting from the 5'-end of the

antisense strand. In some other embodiments, the sense strand comprises stabilizing modifications at positions opposite or complimentary to positions 11, 12, 13, and 15 of the antisense strand, counting from the 5'-end of the antisense strand. In some embodiments, the sense strand comprises a block of two, three, or four stabilizing modifications.

In some embodiments, the sense strand does not comprise a stabilizing modification in position opposite or complimentary to the thermally destabilizing modification of the duplex in the antisense strand.

Exemplary thermally stabilizing modifications include, but are not limited to, 2'-fluoro modifications. Other thermally stabilizing modifications include, but are not limited to, LNA.

In some embodiments, the dsRNA of the disclosure comprises at least four (*e.g.*, four, five, six, seven, eight, nine, ten, or more) 2'-fluoro nucleotides. Without limitations, the 2'-fluoro nucleotides all can be present in one strand. In some embodiments, both the sense and the antisense strands comprise at least two 2'-fluoro nucleotides. The 2'-fluoro modification can occur on any nucleotide of the sense strand or antisense strand. For instance, the 2'-fluoro modification can occur on every nucleotide on the sense strand or antisense strand; each 2'-fluoro modification can occur in an alternating pattern on the sense strand or antisense strand; or the sense strand or antisense strand comprises both 2'-fluoro modifications in an alternating pattern. The alternating pattern of the 2'-fluoro modifications on the sense strand may be the same or different from the antisense strand, and the alternating pattern of the 2'-fluoro modifications on the sense strand can have a shift relative to the alternating pattern of the 2'-fluoro modifications on the antisense strand.

In some embodiments, the antisense strand comprises at least two (*e.g.*, two, three, four, five, six, seven, eight, nine, ten, or more) 2'-fluoro nucleotides. Without limitations, a 2'-fluoro modification in the antisense strand can be present at any positions. In some embodiments, the antisense comprises 2'-fluoro nucleotides at positions 2, 6, 8, 9, 14, and 16 from the 5'-end. In some other embodiments, the antisense comprises 2'-fluoro nucleotides at positions 2, 6, 14, and 16 from the 5'-end. In still some other embodiments, the antisense comprises 2'-fluoro nucleotides at positions 2, 14, and 16 from the 5'-end.

In some embodiments, the antisense strand comprises at least one 2'-fluoro nucleotide adjacent to the destabilizing modification. For example, the 2'-fluoro nucleotide can be the nucleotide at the 5'-end or the 3'-end of the destabilizing modification, *i.e.*, at position -1 or +1 from the position of the destabilizing modification. In some embodiments, the antisense strand comprises a 2'-fluoro nucleotide at each of the 5'-end and the 3'-end of the destabilizing modification, *i.e.*, positions -1 and +1 from the position of the destabilizing modification.

In some embodiments, the antisense strand comprises at least two 2'-fluoro nucleotides at the 3'-end of the destabilizing modification, *i.e.*, at positions +1 and +2 from the position of the destabilizing modification.

In some embodiments, the sense strand comprises at least two (*e.g.*, two, three, four, five, six, seven, eight, nine, ten, or more) 2'-fluoro nucleotides. Without limitations, a 2'-fluoro modification in the sense strand can be present at any positions. In some embodiments, the antisense comprises 2'-fluoro

nucleotides at positions 7, 10, and 11 from the 5'-end. In some other embodiments, the sense strand comprises 2'-fluoro nucleotides at positions 7, 9, 10, and 11 from the 5'-end. In some embodiments, the sense strand comprises 2'-fluoro nucleotides at positions opposite or complimentary to positions 11, 12, and 15 of the antisense strand, counting from the 5'-end of the antisense strand. In some other embodiments, the sense strand comprises 2'-fluoro nucleotides at positions opposite or complimentary to positions 11, 12, 13, and 15 of the antisense strand, counting from the 5'-end of the antisense strand. In some embodiments, the sense strand comprises a block of two, three or four 2'-fluoro nucleotides.

In some embodiments, the sense strand does not comprise a 2'-fluoro nucleotide in position opposite or complimentary to the thermally destabilizing modification of the duplex in the antisense strand.

In some embodiments, the dsRNA molecule of the disclosure comprises a 21 nucleotides (nt) sense strand and a 23 nucleotides (nt) antisense, wherein the antisense strand contains at least one thermally destabilizing nucleotide, where the at least one thermally destabilizing nucleotide occurs in the seed region of the antisense strand (*i.e.*, at position 2-9 of the 5'-end of the antisense strand), wherein one end of the dsRNA is blunt, while the other end is comprises a 2 nt overhang, and wherein the dsRNA optionally further has at least one (*e.g.*, one, two, three, four, five, six or all seven) of the following characteristics: (i) the antisense comprises 2, 3, 4, 5, or 6 2'-fluoro modifications; (ii) the antisense comprises 1, 2, 3, 4, or 5 phosphorothioate internucleotide linkages; (iii) the sense strand is conjugated with a ligand; (iv) the sense strand comprises 2, 3, 4, or 5 2'-fluoro modifications; (v) the sense strand comprises 1, 2, 3, 4, or 5 phosphorothioate internucleotide linkages; (vi) the dsRNA comprises at least four 2'-fluoro modifications; and (vii) the dsRNA comprises a blunt end at 5'-end of the antisense strand. In another emobodiment, the two nucleotide overhang is at the 3'-end of the antisense.

In some embodiments, the dsRNA molecule of the disclosure comprising a sense and antisense strands, 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 said sense strand comprise at least 8 ribonucleotides; antisense strand is 36-66 nucleotide residues in length and, starting from the 3' terminal nucleotide, 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 said double stranded nucleic acid is introduced into a mammalian cell; and wherein the antisense strand contains at least one thermally destabilizing nucleotide, where at least one thermally destabilizing nucleotide is in the seed region of the

antisense strand (*i.e.* at position 2-9 of the 5'-end of the antisense strand). For example, the thermally destabilizing nucleotide occurs between positions opposite or complimentary to positions 14-17 of the 5'-end of the sense strand, and wherein the dsRNA optionally further has at least one (*e.g.*, one, two, three, four, five, six or all seven) of the following characteristics: (i) the antisense comprises 2, 3, 4, 5, or 6 2'-fluoro modifications; (ii) the antisense comprises 1, 2, 3, 4, or 5 phosphorothioate internucleotide linkages; (iii) the sense strand is conjugated with a ligand; (iv) the sense strand comprises 2, 3, 4, or 5 2'-fluoro modifications; (v) the sense strand comprises 1, 2, 3, 4, or 5 phosphorothioate internucleotide linkages; and (vi) the dsRNA comprises at least four 2'-fluoro modifications; and (vii) the dsRNA comprises a duplex region of 12-30 nucleotide pairs in length.

In some embodiments, the dsRNA molecule of the disclosure comprises a sense and antisense strands, wherein said dsRNA molecule comprises a sense strand having a length which is at least 25 and at most 29 nucleotides and an antisense strand having a length which is at most 30 nucleotides with the sense strand comprises a modified nucleotide that is susceptible to enzymatic degradation at position 11 from the 5'end, wherein the 3' end of said sense strand and the 5' end of said antisense strand form a blunt end and said antisense strand is 1-4 nucleotides longer at its 3' end than the sense strand, wherein the duplex region which is at least 25 nucleotides in length, and said antisense strand is sufficiently complementary to a target mRNA along at least 19 nucleotides of said antisense strand length to reduce target gene expression when said dsRNA molecule is introduced into a mammalian cell, and wherein dicer cleavage of said dsRNA results in an siRNA comprising said 3' end of said antisense strand, thereby reducing expression of the target gene in the mammal, wherein the antisense strand contains at least one thermally destabilizing nucleotide, where the at least one thermally destabilizing nucleotide is in the seed region of the antisense strand (*i.e.* at position 2-9 of the 5'-end of the antisense strand), and wherein the dsRNA optionally further has at least one (*e.g.*, one, two, three, four, five, six or all seven) of the following characteristics: (i) the antisense comprises 2, 3, 4, 5, or 6 2'-fluoro modifications; (ii) the antisense comprises 1, 2, 3, 4, or 5 phosphorothioate internucleotide linkages; (iii) the sense strand is conjugated with a ligand; (iv) the sense strand comprises 2, 3, 4, or 5 2'-fluoro modifications; (v) the sense strand comprises 1, 2, 3, 4, or 5 phosphorothioate internucleotide linkages; and (vi) the dsRNA comprises at least four 2'-fluoro modifications; and (vii) the dsRNA has a duplex region of 12-29 nucleotide pairs in length.

In some embodiments, every nucleotide in the sense strand and antisense strand of the dsRNA molecule 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.

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 an RNA or may only occur in a single strand region of an RNA. *E.g.*, a phosphorothioate modification at a non-linking O position may only occur at one or both termini, may only occur in a terminal 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 termini. The 5' end or ends can be phosphorylated.

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. *E.g.*, it can be desirable to include purine nucleotides in overhangs. In some embodiments all or some of the bases in a 3' or 5' overhang 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.

In some embodiments, each residue of the sense strand and antisense strand is independently modified with LNA, HNA, CeNA, 2'-methoxyethyl, 2'- O-methyl, 2'-O-allyl, 2'-C- allyl, 2'-deoxy, or 2'-fluoro. The strands can contain more than one modification. In some embodiments, each residue of the sense strand and antisense strand is independently modified with 2'-O-methyl or 2'-fluoro. It is to be understood that these modifications are in addition to the at least one thermally destabilizing modification of the duplex present in the antisense strand.

At least two different modifications are typically present on the sense strand and antisense strand. Those two modifications may be the 2'-deoxy, 2'- O-methyl or 2'-fluoro modifications, acyclic nucleotides or others. In some embodiments, the sense strand and antisense strand each comprises two differently modified nucleotides selected from 2'-O-methyl or 2'-deoxy. In some embodiments, each residue of the sense strand and antisense strand is independently modified with 2'-O-methyl nucleotide, 2'-deoxy nucleotide, 2´-deoxy-2'-fluoro nucleotide, 2'-O-N-methylacetamido (2'-O-NMA , 2'O-$CH_2C(O)N(Me)H$) nucleotide, a 2'-O-dimethylaminoethoxyethyl (2'-O-DMAEOE) nucleotide, 2'-O-aminopropyl (2'-O-AP) nucleotide, or 2'-ara-F nucleotide. Again, it is to be understood that these modifications are in addition to the at least one thermally destabilizing modification of the duplex present in the antisense strand.

In some embodiments, the dsRNA molecule of the disclosure comprises modifications of an alternating pattern. The term "alternating motif" or "alternative pattern" 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.

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.

In some embodiments, the dsRNA molecule of the disclosure 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'-3' of the strand and the alternating motif in the antisense strand may start with "BABABA" from 3'-5'of the strand within the duplex region. As another example, the alternating motif in the sense strand may start with "AABBAABB" from 5'-3' of the strand and the alternating motif in the antisense strand may start with "BBAABBAA" from 3'-5'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.

In one particular example, the alternating motif in the sense strand is "ABABAB" sfrom 5'-3' of the strand, where each A is an unmodified ribonucleotide and each B is a 2'-Omethyl modified nucleotide.

In one particular example, the alternating motif in the sense strand is "ABABAB" sfrom 5'-3' of the strand, where each A is an 2'-deoxy-2'-fluoro modified nucleotide and each B is a 2'-Omethyl modified nucleotide.

In another particular example, the alternating motif in the antisense strand is "BABABA" from 3'-5'of the strand, where each A is a 2'-deoxy-2'-fluoro modified nucleotide and each B is a 2'-Omethyl modified nucleotide.

In one particular example, the alternating motif in the sense strand is "ABABAB" sfrom 5'-3' of the strand and the alternating motif in the antisense strand is "BABABA" from 3'-5'of the strand, where each A is an unmodified ribonucleotide and each B is a 2'-Omethyl modified nucleotide.

In one particular example, the alternating motif in the sense strand is "ABABAB" sfrom 5'-3' of the strand and the alternating motif in the antisense strand is "BABABA" from 3'-5'of the strand, where each A is a 2'-deoxy-2'-fluoro modified nucleotide and each B is a 2'-Omethyl modified nucleotide.

The dsRNA molecule of the disclosure 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 or antisense strand or both 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 comprises 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 some embodiments, the dsRNA molecule comprises the phosphorothioate or methylphosphonate internucleotide linkage modification in the overhang region. For example, the overhang region comprises 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 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. In one emobodiment, these terminal three nucleotides may be at the 3'-end of the antisense strand.

In some embodiments, the sense strand of the dsRNA molecule comprises 1-10 blocks of two to ten phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and the said sense strand is paired with an antisense strand comprising any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphosphonate or phosphate linkage.

In some embodiments, the antisense strand of the dsRNA molecule comprises two blocks of two phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and the said antisense strand is paired with a sense strand comprising any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphosphonate or phosphate linkage.

In some embodiments, the antisense strand of the dsRNA molecule comprises two blocks of three phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and

the said antisense strand is paired with a sense strand comprising any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphosphonate or phosphate linkage.

In some embodiments, the antisense strand of the dsRNA molecule comprises two blocks of four phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and the said antisense strand is paired with a sense strand comprising any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphosphonate or phosphate linkage.

In some embodiments, the antisense strand of the dsRNA molecule comprises two blocks of five phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and the said antisense strand is paired with a sense strand comprising any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphosphonate or phosphate linkage.

In some embodiments, the antisense strand of the dsRNA molecule comprises two blocks of six phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and the said antisense strand is paired with a sense strand comprising any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphosphonate or phosphate linkage.

In some embodiments, the antisense strand of the dsRNA molecule comprises two blocks of seven phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, 4, 5, 6, 7, or 8 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and the said antisense strand is paired with a sense strand comprising any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphosphonate or phosphate linkage.

In some embodiments, the antisense strand of the dsRNA molecule comprises two blocks of eight phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, 4, 5, or 6 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and the said antisense strand is paired with a sense strand comprising any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphosphonate or phosphate linkage.

In some embodiments, the antisense strand of the dsRNA molecule comprises two blocks of nine phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, or 4 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and the said antisense strand is paired with a sense strand comprising any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphosphonate or phosphate linkage.

In some embodiments, the dsRNA molecule of the disclosure further comprises one or more phosphorothioate or methylphosphonate internucleotide linkage modification within 1-10 nucleotides of the termini position(s) of the sense or antisense strand. For example, at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides may be linked through phosphorothioate or methylphosphonate internucleotide linkage at one end or both ends of the sense or antisense strand.

In some embodiments, the dsRNA molecule of the disclosure further comprises one or more phosphorothioate or methylphosphonate internucleotide linkage modification within 1-10 nucleotides of the internal region of the duplex of each of the sense or antisense strand. For example, at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides may be linked through phosphorothioate or methylphosphonate internucleotide linkage at position 8-16 of the duplex region counting from the 5'-end of the sense strand; the dsRNA molecule can optionally further comprise one or more phosphorothioate or methylphosphonate internucleotide linkage modification within 1-10 of the termini position(s).

In some embodiments, the dsRNA molecule of the disclosure further comprises one to five phosphorothioate or methylphosphonate internucleotide linkage modification(s) within position 1-5 and one to five phosphorothioate or methylphosphonate internucleotide linkage modification(s) within position 18-23 of the sense strand (counting from the 5'-end), and one to two phosphorothioate or methylphosphonate internucleotide linkage modification at positions 1 and 2 and one to five within positions 18-23 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises one phosphorothioate internucleotide linkage modification within position 1-5 and one phosphorothioate or methylphosphonate internucleotide linkage modification within position 18-23 of the sense strand (counting from the 5'-end), and one phosphorothioate internucleotide linkage modification at positions 1 or 2 and two phosphorothioate or methylphosphonate internucleotide linkage modifications within positions 18-23 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises two phosphorothioate internucleotide linkage modifications within position 1-5 and one phosphorothioate internucleotide linkage modification within position 18-23 of the sense strand (counting from the 5'-end), and one phosphorothioate internucleotide linkage modification at positions 1 and 2 and two phosphorothioate internucleotide linkage modifications within positions 18-23 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises two phosphorothioate internucleotide linkage modifications within position 1-5 and two phosphorothioate internucleotide linkage modifications within position 18-23 of the sense strand (counting from the 5'-end), and one phosphorothioate internucleotide linkage modification at positions 1 and 2 and two phosphorothioate internucleotide linkage modifications within positions 18-23 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises two phosphorothioate internucleotide linkage modifications within position 1-5 and two phosphorothioate internucleotide linkage modifications within position 18-23 of the sense strand (counting from the 5'-end), and one phosphorothioate internucleotide linkage modification at positions 1 and 2 and one phosphorothioate internucleotide linkage modification within positions 18-23 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises one phosphorothioate internucleotide linkage modification within position 1-5 and one phosphorothioate internucleotide linkage modification within position 18-23 of the sense strand (counting from the 5'-end), and two phosphorothioate internucleotide linkage modifications at positions 1 and 2 and two phosphorothioate internucleotide linkage modifications within positions 18-23 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises one phosphorothioate internucleotide linkage modification within position 1-5 and one within position 18-23 of the sense strand (counting from the 5'-end), and two phosphorothioate internucleotide linkage modification at positions 1 and 2 and one phosphorothioate internucleotide linkage modification within positions 18-23 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises one phosphorothioate internucleotide linkage modification within position 1-5 (counting from the 5'-end) of the sense strand, and two phosphorothioate internucleotide linkage modifications at positions 1 and 2 and one phosphorothioate internucleotide linkage modification within positions 18-23 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises two phosphorothioate internucleotide linkage modifications within position 1-5 (counting from the 5'-end) of the sense strand, and one phosphorothioate internucleotide linkage modification at positions 1 and 2 and two phosphorothioate internucleotide linkage modifications within positions 18-23 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises two phosphorothioate internucleotide linkage modifications within position 1-5 and one within position 18-23 of the sense strand (counting from the 5'-end), and two phosphorothioate internucleotide linkage modifications at positions 1 and 2 and one phosphorothioate internucleotide linkage modification within positions 18-23 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises two phosphorothioate internucleotide linkage modifications within position 1-5 and one phosphorothioate internucleotide linkage modification within position 18-23 of the sense strand (counting from the 5'-end), and two phosphorothioate internucleotide linkage modifications at positions 1 and 2 and two phosphorothioate internucleotide linkage modifications within positions 18-23 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises two phosphorothioate internucleotide linkage modifications within position 1-5 and one phosphorothioate internucleotide linkage modification within position 18-23 of the sense strand (counting from the 5'-end), and one phosphorothioate internucleotide linkage modification at positions 1 and 2 and two phosphorothioate internucleotide linkage modifications within positions 18-23 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises two phosphorothioate internucleotide linkage modifications at position 1 and 2, and two phosphorothioate internucleotide linkage modifications at position 20 and 21 of the sense strand (counting from the 5'-end), and one phosphorothioate internucleotide linkage modification at positions 1 and one at position 21 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises one phosphorothioate internucleotide linkage modification at position 1, and one phosphorothioate internucleotide linkage modification at position 21 of the sense strand (counting from the 5'-end), and two phosphorothioate internucleotide linkage modifications at positions 1 and 2 and two phosphorothioate internucleotide linkage modifications at positions 20 and 21 the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises two phosphorothioate internucleotide linkage modifications at position 1 and 2, and two phosphorothioate internucleotide linkage modifications at position 21 and 22 of the sense strand (counting from the 5'-end), and one phosphorothioate internucleotide linkage modification at positions 1 and one phosphorothioate internucleotide linkage modification at position 21 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises one phosphorothioate internucleotide linkage modification at position 1, and one phosphorothioate internucleotide linkage modification at position 21 of the sense strand (counting from the 5'-end), and two phosphorothioate internucleotide linkage modifications at positions 1 and 2 and two phosphorothioate internucleotide linkage modifications at positions 21 and 22 the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises two phosphorothioate internucleotide linkage modifications at position 1 and 2, and two phosphorothioate internucleotide linkage modifications at position 22 and 23 of the sense strand (counting from the 5'-end),

and one phosphorothioate internucleotide linkage modification at positions 1 and one phosphorothioate internucleotide linkage modification at position 21 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises one phosphorothioate internucleotide linkage modification at position 1, and one phosphorothioate internucleotide linkage modification at position 21 of the sense strand (counting from the 5'-end), and two phosphorothioate internucleotide linkage modifications at positions 1 and 2 and two phosphorothioate internucleotide linkage modifications at positions 23 and 23 the antisense strand (counting from the 5'-end).

In some embodiments, compound of the disclosure comprises a pattern of backbone chiral centers. In some embodiments, a common pattern of backbone chiral centers comprises at least 5 internucleotidic linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 6 internucleotidic linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 7 internucleotide linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 8 internucleotide linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 9 internucleotide linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 10 internucleotide linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 11 internucleotide linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 12 internucleotide linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 13 internucleotide linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 14 internucleotide linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 15 internucleotide linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 16 internucleotide linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 17 internucleotide linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 18 internucleotide linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 19 internucleotide linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises no more than 8 internucleotide linkages in the Rp configuration. In some embodiments, a common pattern of backbone chiral centers comprises no more than 7 internucleotide linkages in the Rp configuration. In some embodiments, a common pattern of backbone chiral centers comprises no more than 6 internucleotide linkages in the Rp configuration. In some embodiments, a common pattern of backbone chiral centers comprises no more than 5 internucleotide linkages in the Rp configuration. In some embodiments, a common pattern of backbone chiral centers comprises no more than 4 internucleotide linkages in the Rp configuration. In some embodiments, a common pattern of backbone chiral centers comprises no more than 3 internucleotide linkages in the Rp configuration. In some embodiments, a

common pattern of backbone chiral centers comprises no more than 2 internucleotide linkages in the Rp configuration. In some embodiments, a common pattern of backbone chiral centers comprises no more than 1 internucleotide linkages in the Rp configuration. In some embodiments, a common pattern of backbone chiral centers comprises no more than 8 internucleotide linkages which are not chiral (as a non-limiting example, a phosphodiester). In some embodiments, a common pattern of backbone chiral centers comprises no more than 7 internucleotide linkages which are not chiral. In some embodiments, a common pattern of backbone chiral centers comprises no more than 6 internucleotide linkages which are not chiral. In some embodiments, a common pattern of backbone chiral centers comprises no more than 5 internucleotide linkages which are not chiral. In some embodiments, a common pattern of backbone chiral centers comprises no more than 4 internucleotide linkages which are not chiral. In some embodiments, a common pattern of backbone chiral centers comprises no more than 3 internucleotide linkages which are not chiral. In some embodiments, a common pattern of backbone chiral centers comprises no more than 2 internucleotide linkages which are not chiral. In some embodiments, a common pattern of backbone chiral centers comprises no more than 1 internucleotide linkage which is not chiral. In some embodiments, a common pattern of backbone chiral centers comprises at least 10 internucleotide linkages in the Sp configuration, and no more than 8 internucleotide linkages which are not chiral. In some embodiments, a common pattern of backbone chiral centers comprises at least 11 internucleotide linkages in the Sp configuration, and no more than 7 internucleotide linkages which are not chiral. In some embodiments, a common pattern of backbone chiral centers comprises at least 12 internucleotide linkages in the Sp configuration, and no more than 6 internucleotide linkages which are not chiral. In some embodiments, a common pattern of backbone chiral centers comprises at least 13 internucleotide linkages in the Sp configuration, and no more than 6 internucleotide linkages which are not chiral. In some embodiments, a common pattern of backbone chiral centers comprises at least 14 internucleotide linkages in the Sp configuration, and no more than 5 internucleotide linkages which are not chiral. In some embodiments, a common pattern of backbone chiral centers comprises at least 15 internucleotide linkages in the Sp configuration, and no more than 4 internucleotide linkages which are not chiral. In some embodiments, the internucleotide linkages in the Sp configuration are optionally contiguous or not contiguous. In some embodiments, the internucleotide linkages in the Rp configuration are optionally contiguous or not contiguous. In some embodiments, the internucleotide linkages which are not chiral are optionally contiguous or not contiguous.

In some embodiments, compound of the disclosure comprises a block is a stereochemistry block. In some embodiments, a block is an Rp block in that each internucleotidic linkage of the block is Rp. In some embodiments, a 5'-block is an Rp block. In some embodiments, a 3'-block is an Rp block. In some embodiments, a block is an Sp block in that each internucleotidic linkage of the block is Sp. In some embodiments, a 5'-block is an Sp block. In some embodiments, a 3'-block is an Sp block. In some embodiments, provided oligonucleotides comprise both Rp and Sp blocks. In some embodiments, provided oligonucleotides comprise one or more Rp but no Sp blocks. In some embodiments, provided oligonucleotides comprise one or more Sp but no Rp blocks. In some embodiments, provided

oligonucleotides comprise one or more natural phosphate linkage (PO) blocks wherein each internucleotide linkage in a natural phosphate linkage.

In some embodiments, compound of the disclosure comprises a 5'-block is an Sp block wherein each sugar moiety comprises a 2'-F modification. In some embodiments, a 5'-block is an Sp block wherein each of internucleotide linkages is a modified internucleotide linkage and each sugar moiety comprises a 2'-F modification. In some embodiments, a 5'-block is an Sp block wherein each of internucleotide linkage is a phosphorothioate linkage and each sugar moiety comprises a 2'-F modification. In some embodiments, a 5'-block comprises 4 or more nucleoside units. In some embodiments, a 5'-block comprises 5 or more nucleoside units. In some embodiments, a 5'-block comprises 6 or more nucleoside units. In some embodiments, a 5'-block comprises 7 or more nucleoside units. In some embodiments, a 3'-block is an Sp block wherein each sugar moiety comprises a 2'-F modification. In some embodiments, a 3'-block is an Sp block wherein each of internucleotide linkage is a modified internucleotide linkage and each sugar moiety comprises a 2'-F modification. In some embodiments, a 3'-block is an Sp block wherein each of internucleotide linkage is a phosphorothioate linkage and each sugar moiety comprises a 2'-F modification. In some embodiments, a 3'-block comprises 4 or more nucleoside units. In some embodiments, a 3'-block comprises 5 or more nucleoside units. In some embodiments, a 3'-block comprises 6 or more nucleoside units. In some embodiments, a 3'-block comprises 7 or more nucleoside units.

In some embodiments, compound of the disclosure comprises a type of nucleoside in a region or an oligonucleotide is followed by a specific type of internucleotide linkage, *e.g.*, natural phosphate linkage, modified internucleotide linkage, Rp chiral internucleotide linkage, Sp chiral internucleotide linkage, etc. In some embodiments, A is followed by Sp. In some embodiments, A is followed by Rp. In some embodiments, A is followed by natural phosphate linkage (PO). In some embodiments, U is followed by Sp. In some embodiments, U is followed by Rp. In some embodiments, U is followed by natural phosphate linkage (PO). In some embodiments, C is followed by Sp. In some embodiments, C is followed by Rp. In some embodiments, C is followed by natural phosphate linkage (PO). In some embodiments, G is followed by Sp. In some embodiments, G is followed by Rp. In some embodiments, G is followed by natural phosphate linkage (PO). In some embodiments, C and U are followed by Sp. In some embodiments, C and U are followed by Rp. In some embodiments, C and U are followed by natural phosphate linkage (PO). In some embodiments, A and G are followed by Sp. In some embodiments, A and G are followed by Rp.

In some embodiments, the antisense strand comprises phosphorothioate internucleotide linkages between nucleotide positions 21 and 22, and between nucleotide positions 22 and 23, wherein the antisense strand contains at least one thermally destabilizing modification of the duplex located in the seed region of the antisense strand (*i.e.*, at position 2-9 of the 5'-end of the antisense strand), and wherein the dsRNA optionally further has at least one (*e.g.*, one, two, three, four, five, six, seven or all eight) of the following characteristics: (i) the antisense comprises 2, 3, 4, 5, or 6 2'-fluoro modifications; (ii) the antisense comprises 3, 4, or 5 phosphorothioate internucleotide linkages; (iii) the sense strand is

conjugated with a ligand; (iv) the sense strand comprises 2, 3, 4 or 5 2'-fluoro modifications; (v) the sense strand comprises 1, 2, 3, 4, or 5 phosphorothioate internucleotide linkages; (vi) the dsRNA comprises at least four 2'-fluoro modifications; (vii) the dsRNA comprises a duplex region of 12-40 nucleotide pairs in length; and (viii) the dsRNA has a blunt end at 5'-end of the antisense strand.

In some embodiments, the antisense strand comprises phosphorothioate internucleotide linkages between nucleotide positions 1 and 2, between nucleotide positions 2 and 3, between nucleotide positions 21 and 22, and between nucleotide positions 22 and 23, wherein the antisense strand contains at least one thermally destabilizing modification of the duplex located in the seed region of the antisense strand (*i.e.*, at position 2-9 of the 5'-end of the antisense strand), and wherein the dsRNA optionally further has at least one (*e.g.*, one, two, three, four, five, six, seven or all eight) of the following characteristics: (i) the antisense comprises 2, 3, 4, 5, or 6 2'-fluoro modifications; (ii) the sense strand is conjugated with a ligand; (iii) the sense strand comprises 2, 3, 4 or 5 2'-fluoro modifications; (iv) the sense strand comprises 1, 2, 3, 4, or 5 phosphorothioate internucleotide linkages; (v) the dsRNA comprises at least four 2'-fluoro modifications; (vi) the dsRNA comprises a duplex region of 12-40 nucleotide pairs in length; (vii) the dsRNA comprises a duplex region of 12-40 nucleotide pairs in length; and (viii) the dsRNA has a blunt end at 5'-end of the antisense strand.

In some embodiments, the sense strand comprises phosphorothioate internucleotide linkages between nucleotide positions 1 and 2, and between nucleotide positions 2 and 3, wherein the antisense strand contains at least one thermally destabilizing modification of the duplex located in the seed region of the antisense strand (*i.e.*, at position 2-9 of the 5'-end of the antisense strand), and wherein the dsRNA optionally further has at least one (*e.g.*, one, two, three, four, five, six, seven or all eight) of the following characteristics: (i) the antisense comprises 2, 3, 4, 5, or 6 2'-fluoro modifications; (ii) the antisense comprises 1, 2, 3, 4, or 5 phosphorothioate internucleotide linkages; (iii) the sense strand is conjugated with a ligand; (iv) the sense strand comprises 2, 3, 4 or 5 2'-fluoro modifications; (v) the sense strand comprises 3, 4 or 5 phosphorothioate internucleotide linkages; (vi) the dsRNA comprises at least four 2'-fluoro modifications; (vii) the dsRNA comprises a duplex region of 12-40 nucleotide pairs in length; and (viii) the dsRNA has a blunt end at 5'-end of the antisense strand.

In some embodiments, the sense strand comprises phosphorothioate internucleotide linkages between nucleotide positions 1 and 2, and between nucleotide positions 2 and 3, the antisense strand comprises phosphorothioate internucleotide linkages between nucleotide positions 1 and 2, between nucleotide positions 2 and 3, between nucleotide positions 21 and 22, and between nucleotide positions 22 and 23, wherein the antisense strand contains at least one thermally destabilizing modification of the duplex located in the seed region of the antisense strand (*i.e.*, at position 2-9 of the 5'-end of the antisense strand), and wherein the dsRNA optionally further has at least one (*e.g.*, one, two, three, four, five, six or all seven) of the following characteristics: (i) the antisense comprises 2, 3, 4, 5 or 6 2'-fluoro modifications; (ii) the sense strand is conjugated with a ligand; (iii) the sense strand comprises 2, 3, 4 or 5 2'-fluoro modifications; (iv) the sense strand comprises 3, 4 or 5 phosphorothioate internucleotide linkages; (v) the dsRNA comprises at least four 2'-fluoro modifications; (vi) the dsRNA comprises a

duplex region of 12-40 nucleotide pairs in length; and (vii) the dsRNA has a blunt end at 5'-end of the antisense strand.

In some embodiments, the dsRNA molecule of the disclosure comprises mismatch(es) with the target, within the duplex, or combinations thereof. The mismatch can occur in the overhang region or the duplex region. The base pair can 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.

In some embodiments, the dsRNA molecule of the disclosure 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 can be chosen independently 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.

In some embodiments, the nucleotide at the 1 position within the duplex region from the 5'-end in the antisense strand is selected from the group consisting of 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.

It was found that introducing 4'-modified or 5'-modified nucleotide to the 3'-end of a phosphodiester (PO), phosphorothioate (PS), or phosphorodithioate (PS2) linkage of a nucleotide at any position of single stranded or double stranded oligonucleotide can exert steric effect to the internucleotide linkage and, hence, protecting or stabilizing it against nucleases.

In some embodiments, 5'-modified nucleotide is introduced at the 3'-end of a dinucleotide at any position of single stranded or double stranded siRNA. For instance, a 5'-alkylated nucleotide may be introduced at the 3'-end of a dinucleotide at any position of single stranded or double stranded siRNA. The alkyl group at the 5' position of the ribose sugar can be racemic or chirally pure *R* or *S* isomer. An exemplary 5'-alkylated nucleoside is 5'-methyl nucleotide. The 5'-methyl can be either racemic or chirally pure *R* or *S* isomer.

In some embodiments, 4'-modified nucleotide is introduced at the 3'-end of a dinucleotide at any position of single stranded or double stranded siRNA. For instance, a 4'-alkylated nucleotide may be introduced at the 3'-end of a dinucleotide at any position of single stranded or double stranded siRNA. The alkyl group at the 4' position of the ribose sugar can be racemic or chirally pure *R* or *S* isomer. An exemplary 4'-alkylated nucleotide is 4'-methyl nucleoside. The 4'-methyl can be either racemic or chirally pure *R* or *S* isomer. Alternatively, a 4'-*O*-alkylated nucleotide may be introduced at the 3'-end of a dinucleotide at any position of single stranded or double stranded siRNA. The 4'-*O*-alkyl of the ribose

sugar can be racemic or chirally pure *R* or *S* isomer. An exemplary 4'-*O*-alkylated nucleotide is 4'-*O*-methyl nucleoside. The 4'-*O*-methyl can be either racemic or chirally pure *R* or *S* isomer.

In some embodiments, 5'-alkylated nucleotide is introduced at any position on the sense strand or antisense strand of a dsRNA, and such modification maintains or improves potency of the dsRNA. The 5'-alkyl can be either racemic or chirally pure *R* or *S* isomer. An exemplary 5'-alkylated nucleotide is 5'-methyl nucleotide. The 5'-methyl can be either racemic or chirally pure *R* or *S* isomer.

In some embodiments, 4'-alkylated nucleotide is introduced at any position on the sense strand or antisense strand of a dsRNA, and such modification maintains or improves potency of the dsRNA. The 4'-alkyl can be either racemic or chirally pure *R* or *S* isomer. An exemplary 4'-alkylated nucleotide is 4'-methyl nucleoside. The 4'-methyl can be either racemic or chirally pure *R* or *S* isomer.

In some embodiments, 4'-*O*-alkylated nucleotide is introduced at any position on the sense strand or antisense strand of a dsRNA, and such modification maintains or improves potency of the dsRNA. The 5'-alkyl can be either racemic or chirally pure *R* or *S* isomer. An exemplary 4'-*O*-alkylated nucleotide is 4'-*O*-methyl nucleotide. The 4'-*O*-methyl can be either racemic or chirally pure *R* or *S* isomer.

In some embodiments, the dsRNA molecule of the disclosure can comprise 2'-5' linkages (with 2'-H, 2'-OH and 2'-OMe and with P=O or P=S). For example, the 2'-5' linkages modifications can be used to promote nuclease resistance or to inhibit binding of the sense to the antisense strand, or can be used at the 5' end of the sense strand to avoid sense strand activation by RISC.

In another embodiment, the dsRNA molecule of the disclosure can comprise L sugars (*e.g.*, L ribose, L-arabinose with 2'-H, 2'-OH and 2'-OMe). For example, these L sugars modifications can be used to promote nuclease resistance or to inhibit binding of the sense to the antisense strand, or can be used at the 5' end of the sense strand to avoid sense strand activation by RISC.

Various publications describe multimeric siRNA which can all be used with the dsRNA of the disclosure. Such publications include WO2007/091269, US 7858769, WO2010/141511, WO2007/117686, WO2009/014887, and WO2011/031520 which are hereby incorporated by their entirely.

As described in more detail below, the RNAi agent that contains conjugations of one or more carbohydrate moieties to an RNAi agent can optimize one or more properties of the RNAi agent. In many cases, the carbohydrate moiety will be attached to a modified subunit of the RNAi agent. For example, the ribose sugar of one or more ribonucleotide subunits of a dsRNA agent can be replaced with another moiety, *e.g.*, a non-carbohydrate (such as, 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.

The ligand may be attached to the polynucleotide via a carrier. The carriers include (i) at least one "backbone attachment point," such as 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 and 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.

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

In certain specific embodiments, the RNAi agent for use in the methods of the disclosure is an agent selected from the group of agents listed in any one of Tables 2-3. These agents may further comprise a ligand, such as one or more lipophilic moieties, one or more GalNAc derivatives, or both of one of more lipophilic moieties and one or more GalNAc derivatives.

IV. iRNAs Conjugated to Ligands

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. Acid. 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 triethyl-ammonium 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-carbonyloxycholesterol moiety (Crooke *et al.*, *J. Pharmacol. Exp. Ther.*, 1996, 277:923-937).

In certain embodiments, a ligand alters the distribution, targeting or lifetime of an iRNA agent into which it is incorporated. In some 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. Typical ligands will not take part in duplex pairing in a duplexed nucleic acid.

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 or hyaluronic acid); or a lipid. The ligand may 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-glycolied) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-hydroxypropyl)methacrylamide copolymer (HMPA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacryllic 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 α helical peptide.

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-glucosamine multivalent mannose, multivalent fucose, glycosylated polyaminoacids, multivalent galactose, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipid, cholesterol, a steroid, bile acid, folate, vitamin B12, biotin, or an RGD peptide or RGD peptide mimetic. In certain embodiments, the ligand is a multivalent galactose, *e.g.*, an N-acetyl-galactosamine.

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, $[MPEG]_2$, 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.

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 cancer cell, endothelial cell, or bone cell. Ligands may 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-glucosamine multivalent mannose, or multivalent fucose. The ligand can be, for example, a lipopolysaccharide, an activator of p38 MAP kinase, or an activator of NF-κB.

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.

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.

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.

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.

In the ligand-conjugated oligonucleotides 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.

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

In certain embodiments, the ligand or conjugate is a lipid or lipid-based molecule. Such a lipid or lipid-based molecule can typically bind a serum protein, such as 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.

A lipid-based ligand can be used to modulate, *e.g.*, control (*e.g.*, inhibit) 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.

In certain embodiments, the lipid-based ligand binds HSA. For example, the ligand can bind HSA with a sufficient affinity such that distribution of the conjugate to a non-kidney tissue is enhanced. However, the affinity is typically not so strong that the HSA-ligand binding cannot be reversed.

In certain embodiments, the lipid-based ligand binds HSA weakly or not at all, such that distribution of the conjugate to the kidney is enhanced. Other moieties that target to kidney cells can also be used in place of or in addition to the lipid-based ligand.

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 cancer cells. Also included are HSA and low density lipoprotein (LDL).

B. Cell Permeation Agents

In another aspect, the ligand is a cell-permeation agent, such as a helical cell-permeation agent. In certain embodiments, the agent is amphipathic. An exemplary agent is a peptide such as tat or antennopedia. 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 typically an α-helical agent and can have a lipophilic and a lipophobic phase.

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.

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: 9). An RFGF analogue (*e.g.*, amino acid sequence AALLPVLLAAP (SEQ ID NO: 10)) 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: 11)) and the *Drosophila Antennapedia* protein (RQIKIWFQNRRMKWKK (SEQ ID NO: 12)) 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). Typically, the peptide or peptidomimetic tethered to a dsRNA agent via an incorporated monomer unit is a cell targeting peptide such as 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.

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 peptidiomimemtics 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, such as PECAM-1 or VEGF.

An RGD peptide moiety can be used to target a particular cell type, *e.g.*, a tumor cell, such as an endothelial tumor cell or a breast cancer tumor cell (Zitzmann *et al.*, *Cancer Res.*, 62:5139-43, 2002). An RGD peptide can facilitate targeting of an dsRNA agent to tumors of a variety of other tissues, including

the lung, kidney, spleen, or liver (Aoki *et al.*, *Cancer Gene Therapy* 8:783-787, 2001). Typically, the RGD peptide will facilitate targeting of an iRNA agent to the kidney. The RGD peptide can be linear or cyclic, and can be modified, *e.g.,* glycosylated or methylated to facilitate targeting to specific tissues. For example, a glycosylated RGD peptide can deliver an iRNA agent to a tumor cell expressing $\alpha_V\beta_3$ (Haubner *et al.*, *Jour. Nucl. Med.*, 42:326-336, 2001).

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

In some embodiments of the compositions and methods of the invention, an iRNA further comprises a carbohydrate. The carbohydrate conjugated iRNA are 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 C5 and above (*e.g.,* C5, C6, C7, or C8) sugars; di- and tri-saccharides include sugars having two or three monosaccharide units (*e.g.,* C5, C6, C7, or C8).

In certain embodiments, a carbohydrate conjugate comprises a monosaccharide.

In certain embodiments, the monosaccharide is an N-acetylgalactosamine (GalNAc). GalNAc conjugates, which comprise one or more N-acetylgalactosamine (GalNAc) derivatives, are described, for example, in US 8,106,022, the entire content of which is hereby incorporated herein by reference. In some embodiments, the GalNAc conjugate serves as a ligand that targets the iRNA to particular cells. In some embodiments, the GalNAc conjugate targets the iRNA to liver cells, *e.g.*, by serving as a ligand for the asialoglycoprotein receptor of liver cells (*e.g.*, hepatocytes).

In some embodiments, the carbohydrate conjugate comprises one or more GalNAc derivatives. The GalNAc derivatives may be attached via a linker, *e.g.*, a bivalent or trivalent branched linker. In some embodiments the GalNAc conjugate is conjugated to the 3' end of the sense strand. In some

embodiments, the GalNAc conjugate is conjugated to the iRNA agent (*e.g.*, to the 3' end of the sense strand) via a linker, *e.g.*, a linker as described herein. In some embodiments the GalNAc conjugate is conjugated to the 5' end of the sense strand. In some embodiments, the GalNAc conjugate is conjugated to the iRNA agent (*e.g.*, to the 5' end of the sense strand) via a linker, *e.g.*, a linker as described herein.

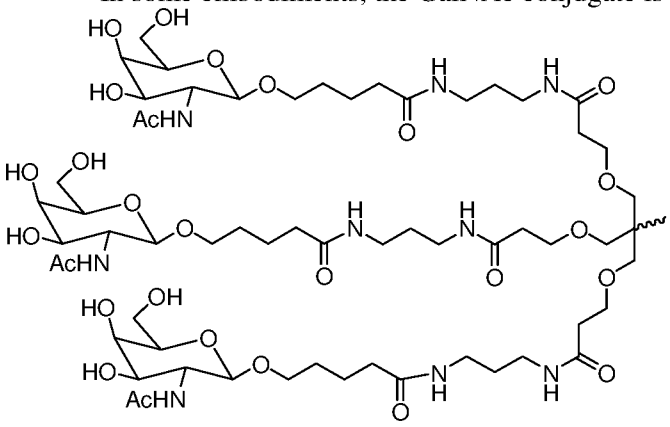
In certain embodiments of the invention, the GalNAc or GalNAc derivative is attached to an iRNA agent of the invention via a monovalent linker. In some embodiments, the GalNAc or GalNAc derivative is attached to an iRNA agent of the invention via a bivalent linker. In yet other embodiments of the invention,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

In certain embodiments, the double stranded RNAi agents of the invention comprise one GalNAc or GalNAc derivative attached to the iRNA agent. In certain embodiments, 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.

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. The hairpin loop may also be formed by an extended overhang in one strand of the duplex.

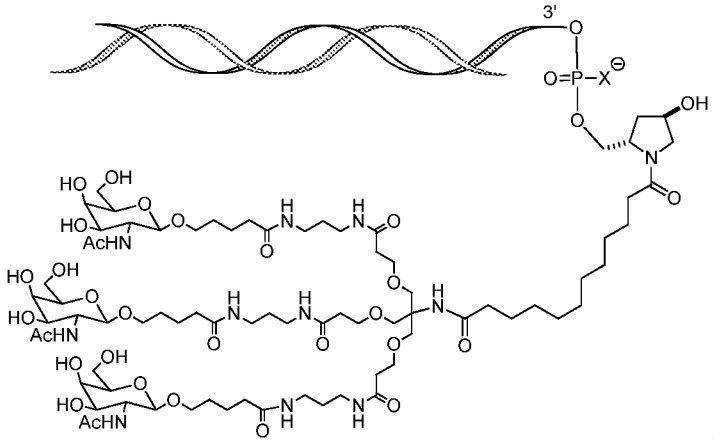
In some embodiments, for example, when the two strands of an iRNA agent of the invention are part of one larger molecule connected by an uninterrupted chain of nucleotides between the 3'-end of one strand and the 5'-end of the respective other strand forming a hairpin loop comprising, a plurality of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

In some embodiments, the GalNAc conjugate is

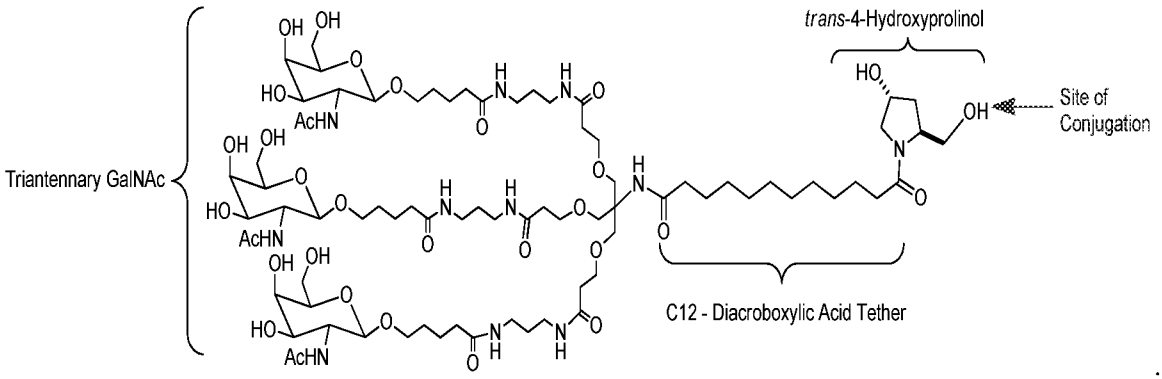


Formula II.

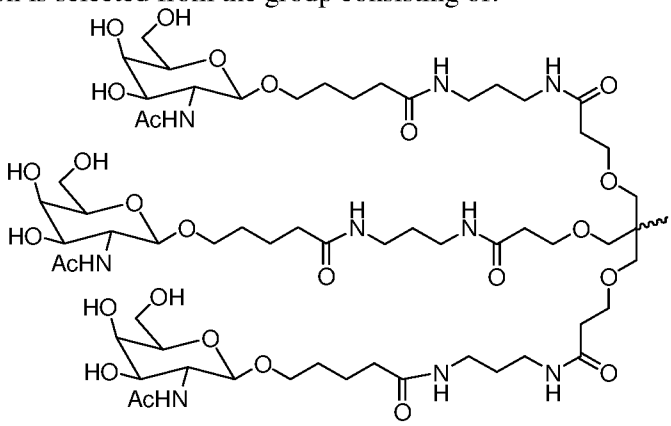
In some embodiments, the RNAi agent is attached to the carbohydrate conjugate via a linker as shown in the following schematic, wherein X is O or S



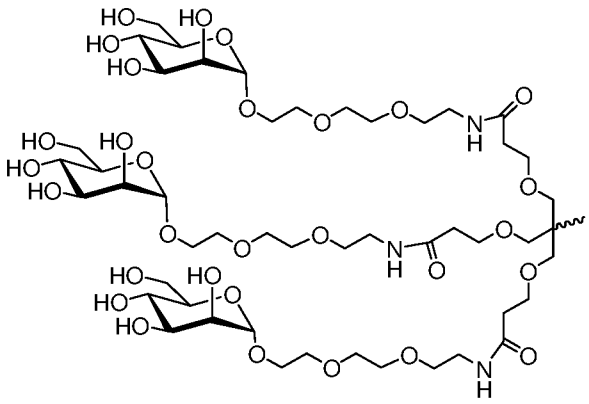
In some embodiments, the RNAi agent is conjugated to L96 as defined in Table 1 and shown below:



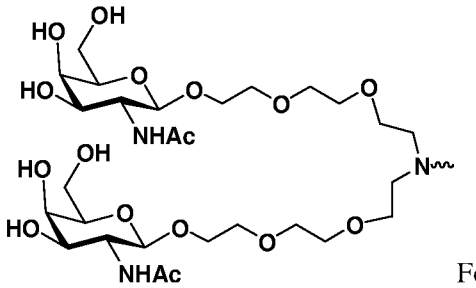
In certain embodiments, a carbohydrate conjugate for use in the compositions and methods of the invention is selected from the group consisting of:



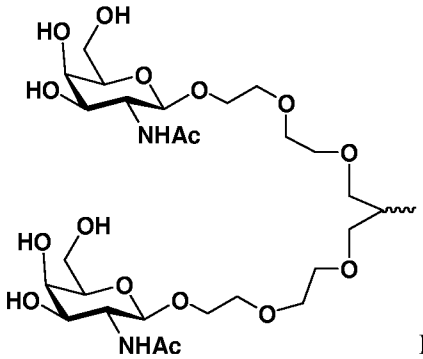
Formula II,



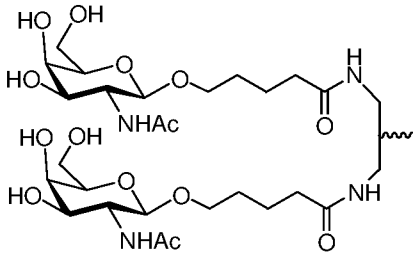
Formula III,



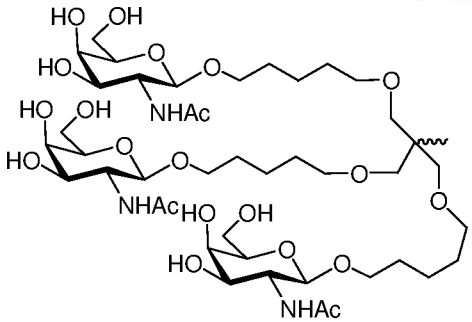
Formula IV,



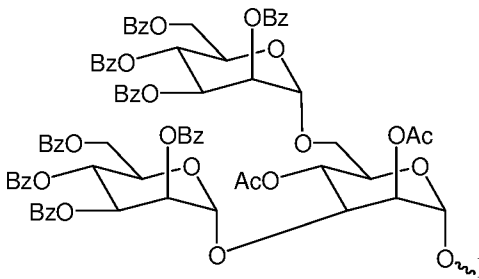
Formula V,



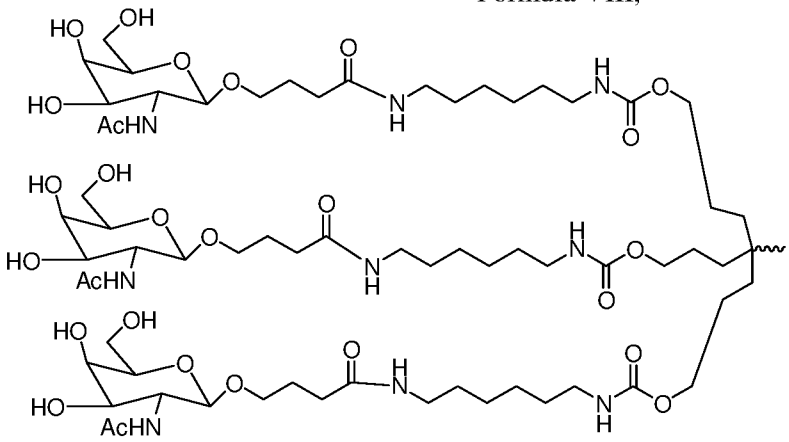
Formula VI,



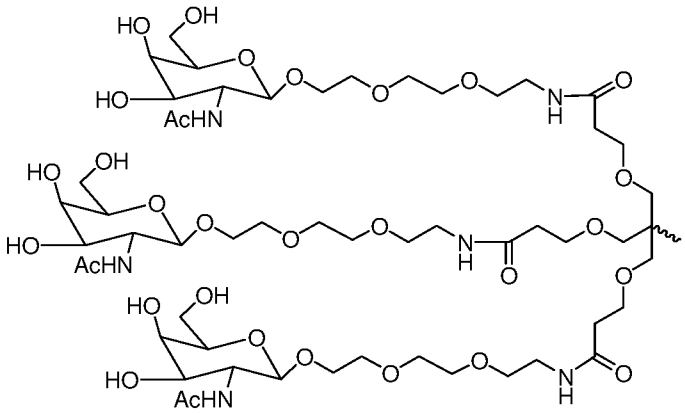
Formula VII,



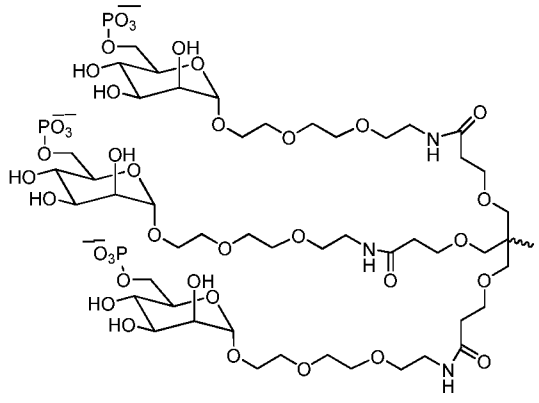
Formula VIII,



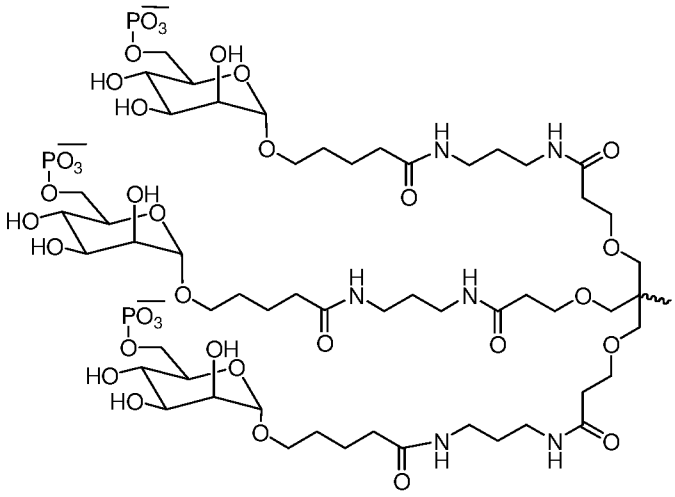
Formula IX,



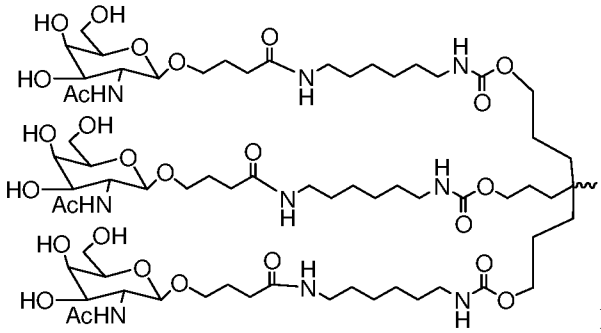
Formula X,



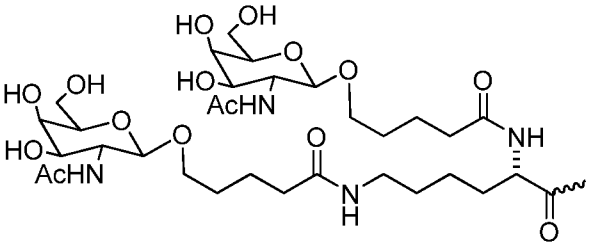
Formula XI,



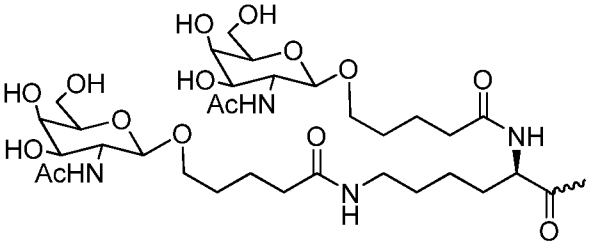
Formula XII,



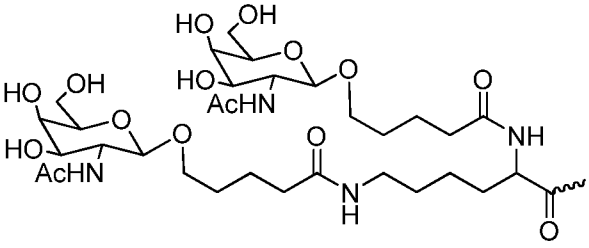
Formula XIII,



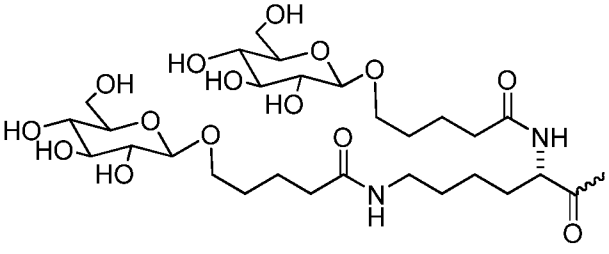
Formula XIV,



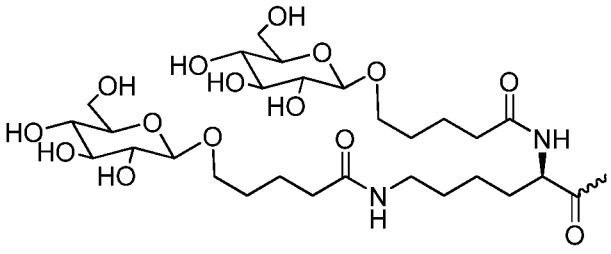
Formula XV,



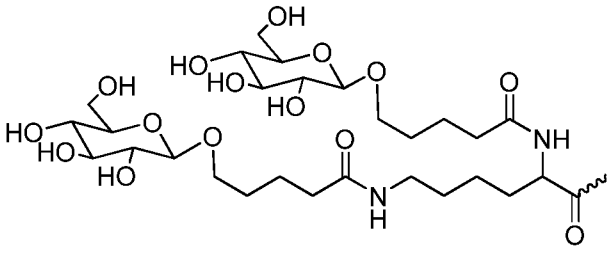
Formula XVI,



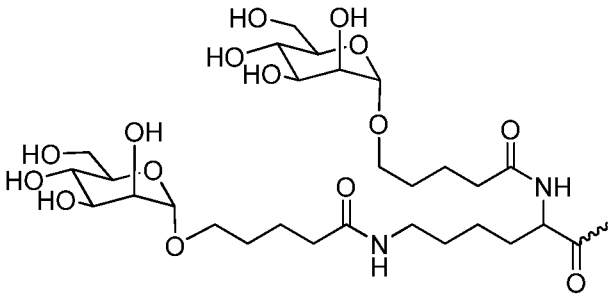
Formula XVII,



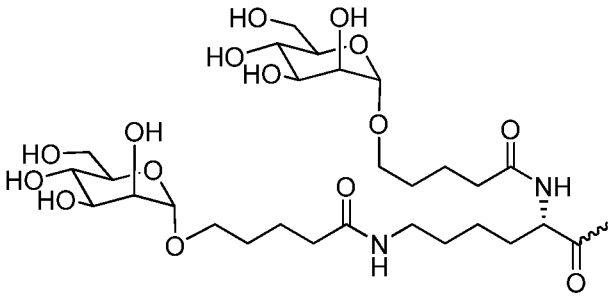
Formula XVIII,



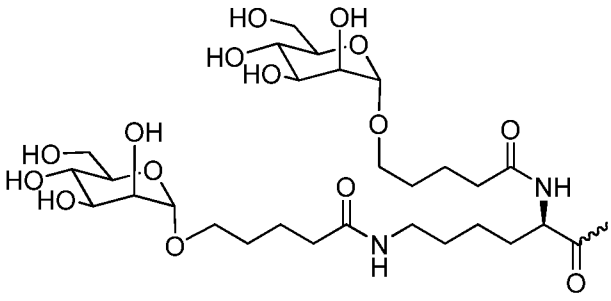
Formula XIX,



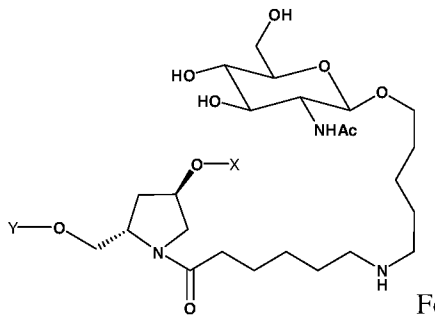
Formula XX,



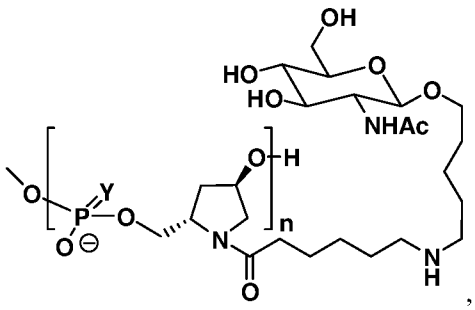
Formula XXI,



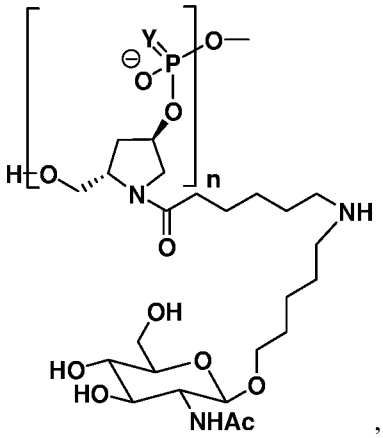
Formula XXII,



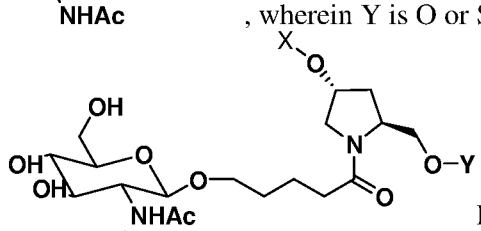
Formula XXIII;



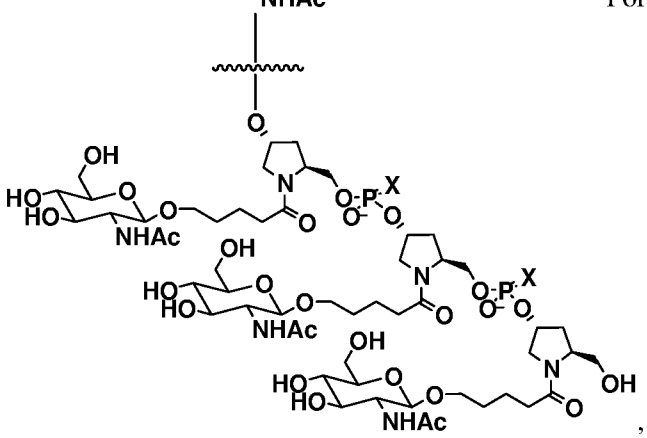
, wherein Y is O or S and n is 3 -6 (Formula XXIV);



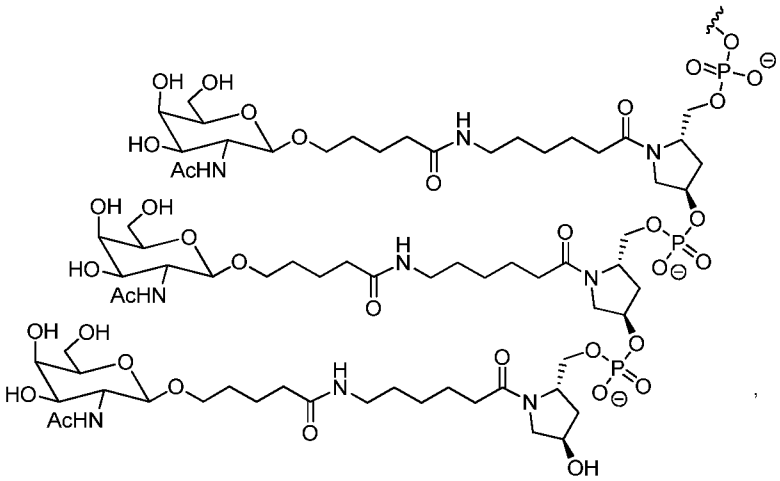
, wherein Y is O or S and n is 3-6 (Formula XXV);

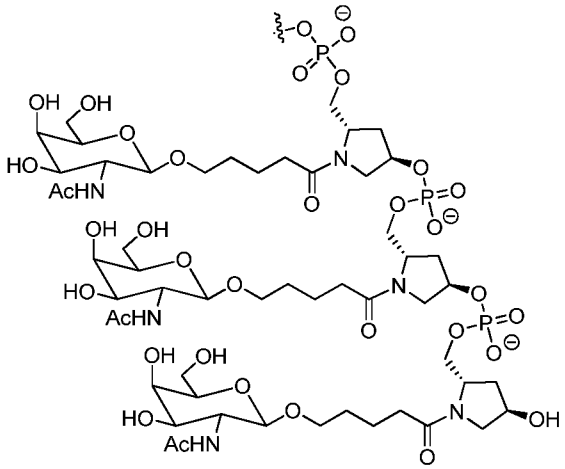


Formula XXVI;

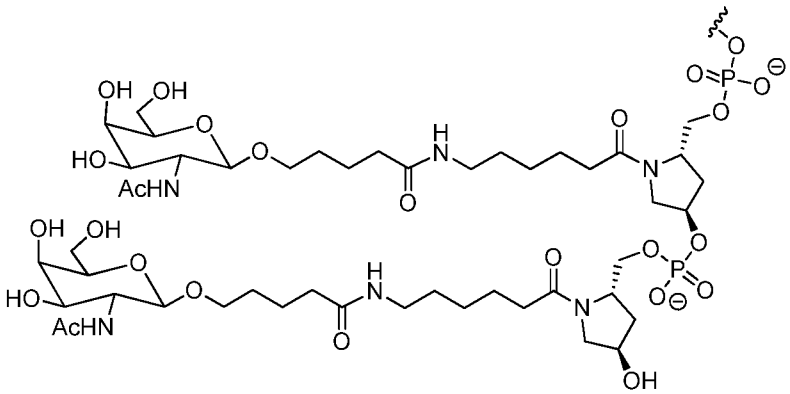


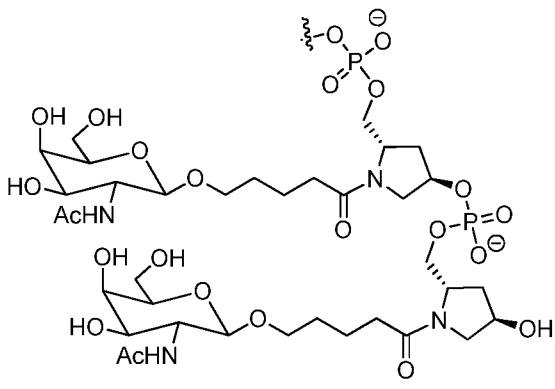
, wherein X is O or S (Formula XXVII);





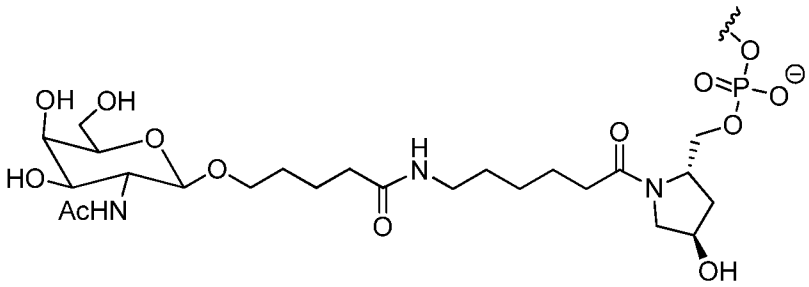
Formula XXVII; Formula XXIX;



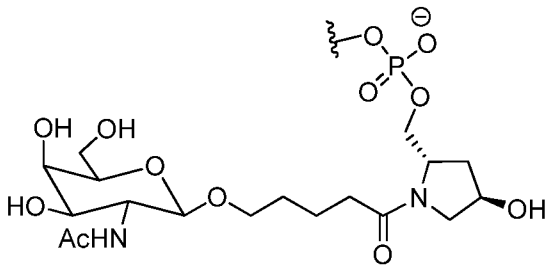


Formula XXX;

Formula XXXI;

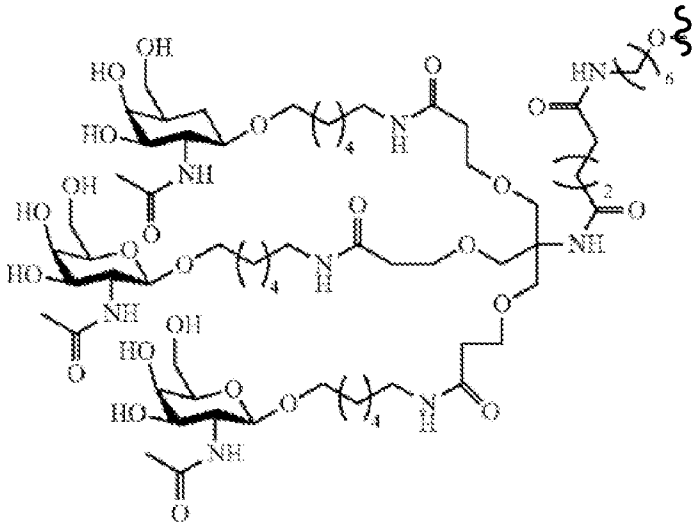


, and



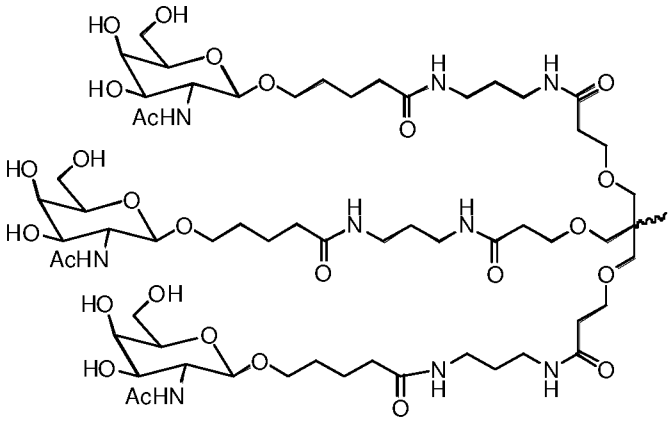
Formula XXXII;

Formula XXXIII.



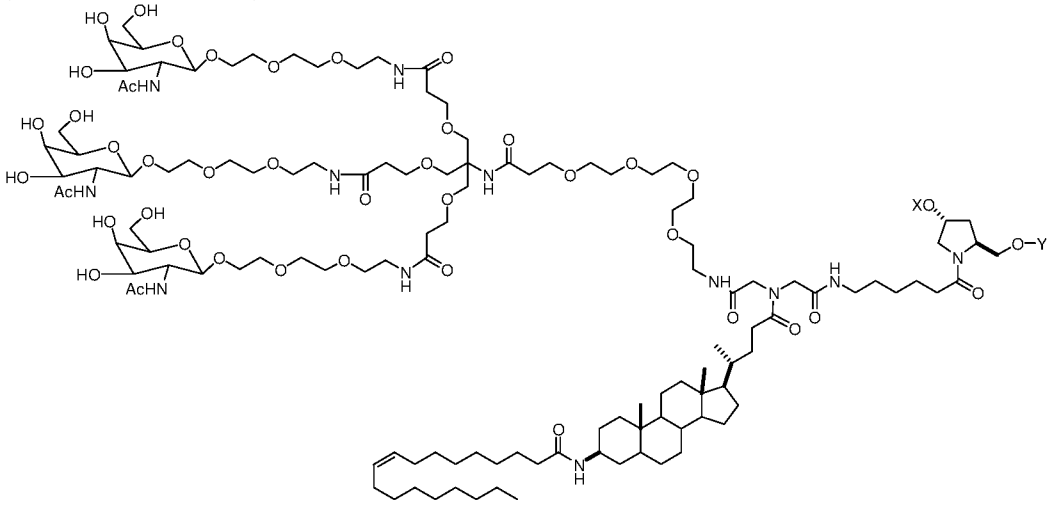
Formula XXXIV.

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



Formula II.

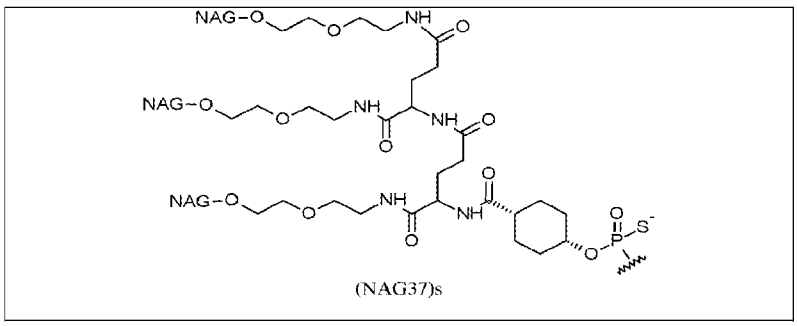
Another representative carbohydrate conjugate for use in the embodiments described herein includes, but is not limited to,



(Formula XXXVI),

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

In some embodiments, a suitable ligand is a ligand disclosed in WO 2019/055633, the entire contents of which are incorporated herein by reference. In one embodiment the ligand comprises the structure below:



In certain embodiments, the RNAi agents of the disclosure may include GalNAc ligands, even if such GalNAc ligands are currently projected to be of limited value for the intrathecal/CNS delivery route(s) of the instant disclosure.

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. In other embodiments of the invention, the GalNAc or GalNAc derivative is attached to an iRNA agent of the invention *via* a tetravalent linker.

In certain embodiments, the double stranded RNAi agents of the invention comprise one GalNAc or GalNAc derivative attached to the iRNA agent, *e.g.,* the 5'end of the sense strand of a dsRNA agent, or the 5' end of one or both sense strands of a dual targeting RNAi agent as described herein. In certain embodiments, 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.

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.

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.

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

D. Linkers

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.

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_2, SO_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, heterocyclylalkyl, heterocyclylalkenyl, heterocyclylalkynyl, aryl, heteroaryl, heterocyclyl, cycloalkyl, cycloalkenyl, alkylarylalkyl, alkylarylalkenyl, alkylarylalkynyl, alkenylarylalkyl, alkenylarylalkenyl, alkenylarylalkynyl, alkynylarylalkyl, alkynylarylalkenyl, alkynylarylalkynyl, alkylheteroarylalkyl, alkylheteroarylalkenyl, alkylheteroarylalkynyl, alkenylheteroarylalkyl, alkenylheteroarylalkenyl, alkenylheteroarylalkynyl, alkynylheteroarylalkyl, alkynylheteroarylalkenyl, alkynylheteroarylalkynyl, alkylheterocyclylalkyl, alkylheterocyclylalkenyl, alkylhererocyclylalkynyl, alkenylheterocyclylalkyl, alkenylheterocyclylalkenyl,

alkenylheterocyclylalkynyl, alkynylheterocyclylalkyl, alkynylheterocyclylalkenyl, alkynylheterocyclylalkynyl, alkylaryl, alkenylaryl, alkynylaryl, alkylheteroaryl, alkenylheteroaryl, alkynylhereroaryl, which one or more methylenes can be interrupted or terminated by O, S, S(O), SO_2, N(R8), C(O), substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocyclic; where R8 is hydrogen, acyl, aliphatic or substituted aliphatic. In certain embodiments, the linker is between about 1-24 atoms, 2-24, 3-24, 4-24, 5-24, 6-24, 6-18, 7-18, 8-18 atoms, 7-17, 8-17, 6-16, 7-16, or 8-16 atoms.

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 another 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 more, or at least about 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).

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.

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 selected pH, thereby releasing a cationic lipid from the ligand inside the cell, or into the desired compartment of the cell.

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.

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

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 certain embodiments, useful candidate compounds are cleaved 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 serum (or under *in vitro* conditions selected to mimic extracellular conditions).

i. Redox cleavable linking groups

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 know 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

In certain 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-, wherein Rk at each occurrence can be, independently, C1-C20 alkyl, C1-C20 haloalkyl, C6-C10 aryl, or C7-C12 aralkyl. . Exemplary embodiments include -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-. In certain embodiments a phosphate-based linking group is -O-P(O)(OH)-O-. These candidates can be evaluated using methods analogous to those described above.

iii. Acid cleavable linking groups

In certain embodiments, a cleavable linker comprises an acid cleavable linking group. An *a*cid cleavable linking group is a linking group that is cleaved under acidic conditions. In other 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, 5.75, 5.5, 5.25, 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). An exemplary 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.

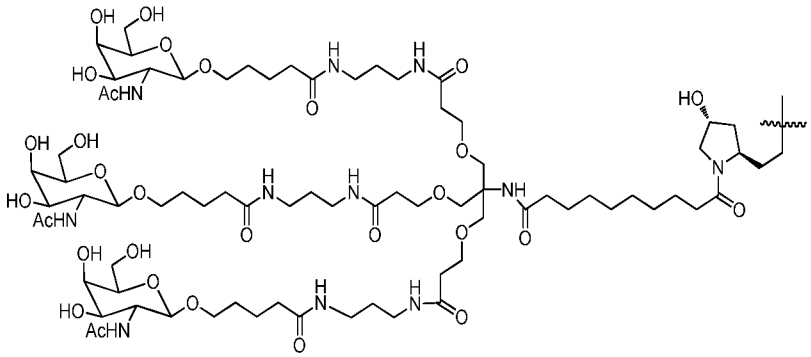
iv. Ester-based cleavable linking groups

In certain 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.

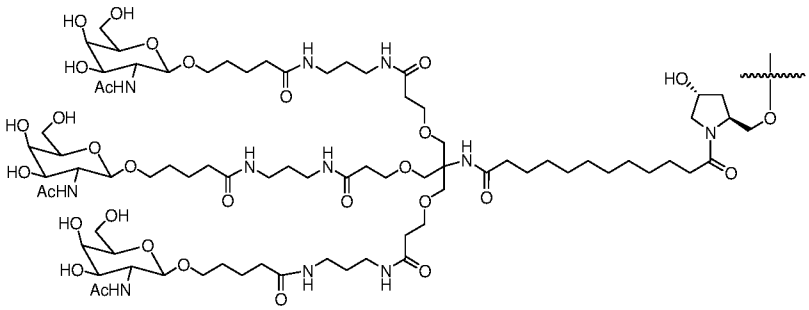
v. Peptide-based cleavable linking groups

In yet another embodiment, 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 alkynelene. 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 – 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.

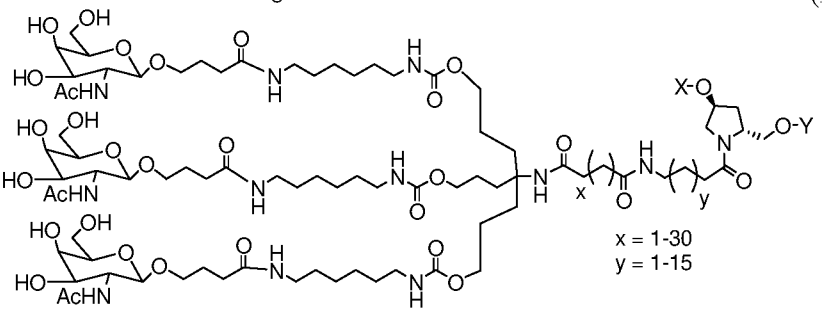
In some embodiments, an iRNA of the invention is conjugated to a carbohydrate through a linker. Non-limiting examples of iRNA carbohydrate conjugates with linkers of the compositions and methods of the invention include, but are not limited to,



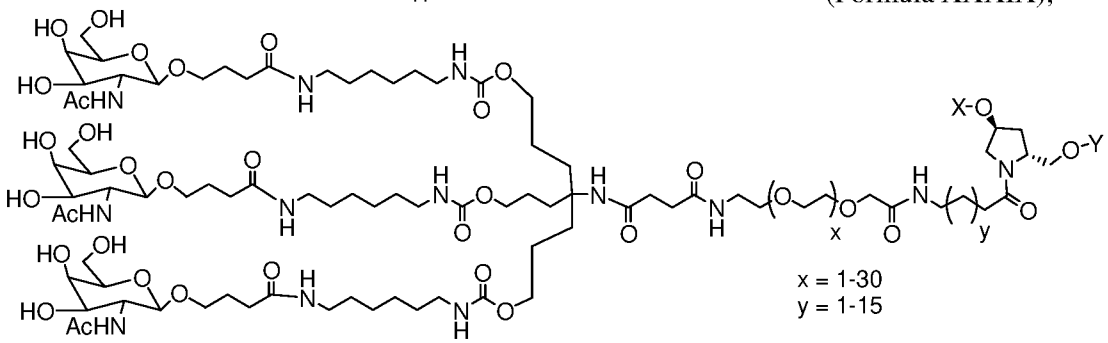
(Formula XXXVII),



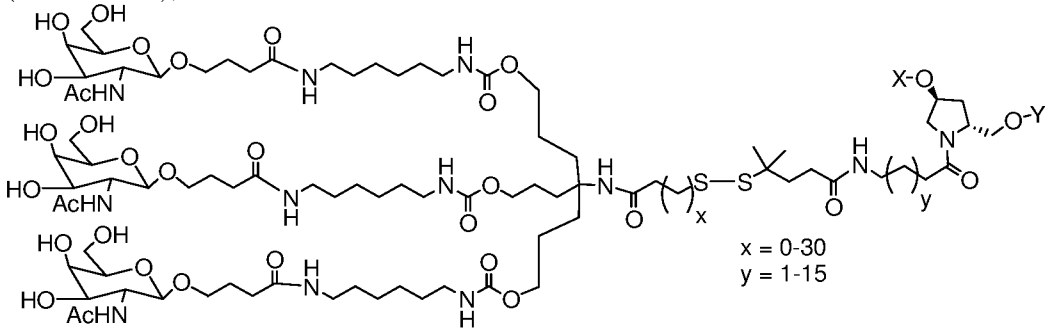
(Formula XXXVIII),



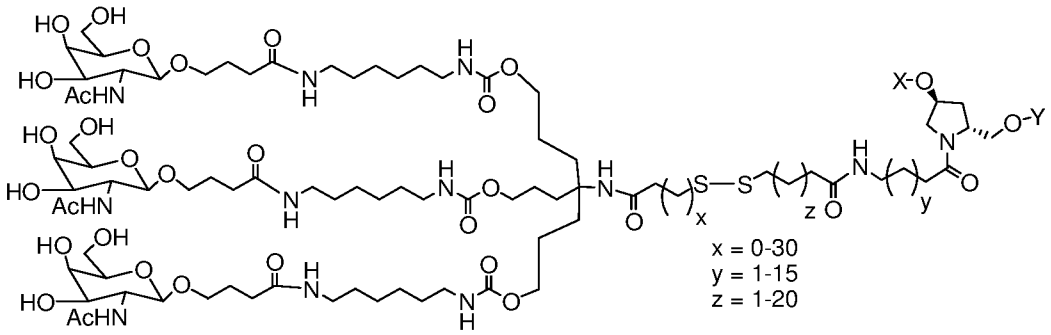
(Formula XXXIX),



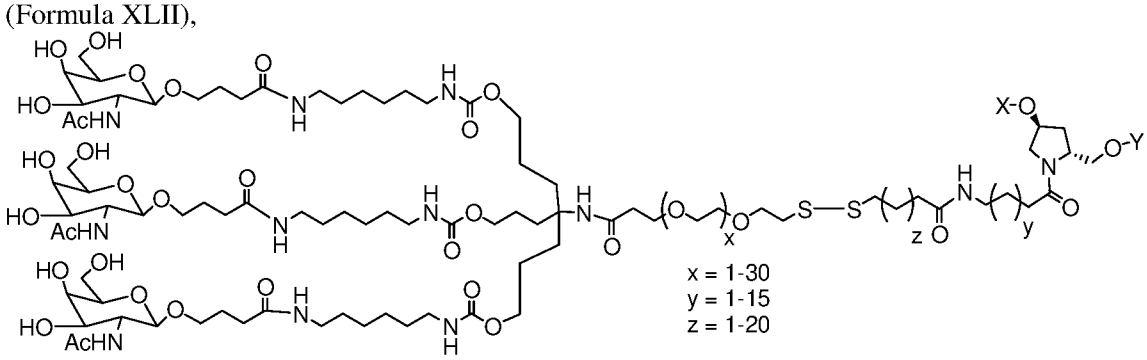
(Formula XL),



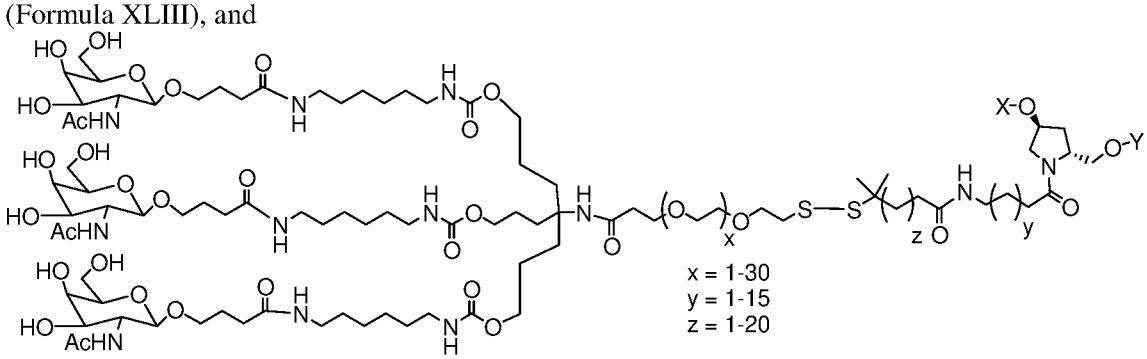
(Formula XLI),



(Formula XLII),



(Formula XLIII), and

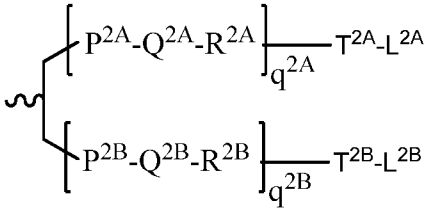


(Formula XLIV), when one of X or Y is an oligonucleotide, the other is a hydrogen.

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.

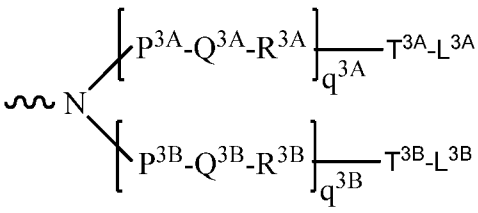
In certain embodiments, a dsRNA of the invention is conjugated to a bivalent or trivalent branched linker selected from the group of structures shown in any of formula (XLV) – (XLVI):

Formula XXXXV

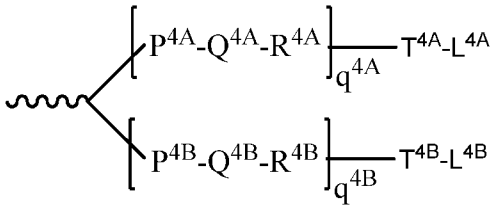


(IV)

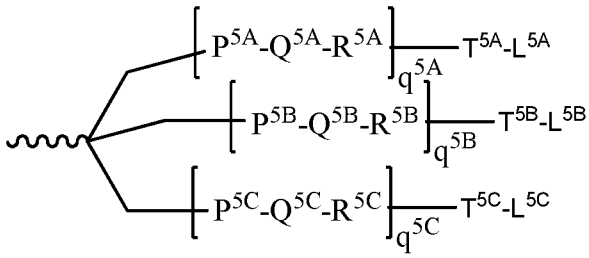
Formula XLVI



V)



Formula XLVII



Formula XLVIII

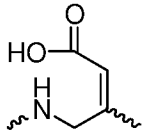
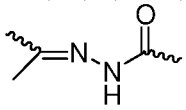
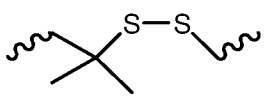
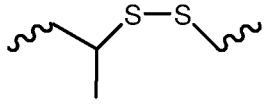
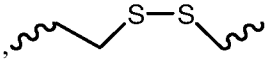
;

wherein:

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;

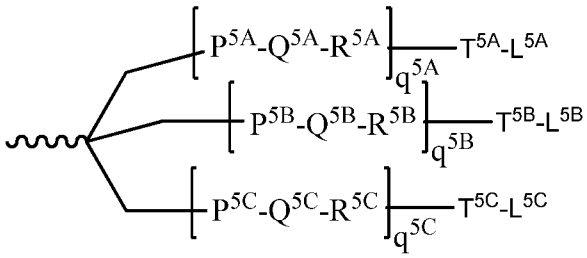
P^{2A}, P^{2B}, P^{3A}, P^{3B}, P^{4A}, P^{4B}, P^{5A}, P^{5B}, P^{5C}, T^{2A}, T^{2B}, T^{3A}, T^{3B}, T^{4A}, T^{4B}, T^{4A}, T^{5B}, T^{5C} are each independently for each occurrence absent, CO, NH, O, S, OC(O), NHC(O), CH_2, CH_2NH or CH_2O;

Q^{2A}, Q^{2B}, Q^{3A}, Q^{3B}, Q^{4A}, Q^{4B}, Q^{5A}, Q^{5B}, Q^{5C} are independently for each occurrence absent, alkylene, substituted alkylene wherin one or more methylenes can be interrupted or terminated by one or more of O, S, S(O), SO_2, $N(R^N)$, C(R')=C(R''), C≡C or C(O);

R^{2A}, R^{2B}, R^{3A}, R^{3B}, R^{4A}, R^{4B}, R^{5A}, R^{5B}, R^{5C} are each independently for each occurrence absent, NH, O, S, CH_2, C(O)O, C(O)NH, $NHCH(R^a)C(O)$, -C(O)-$CH(R^a)$-NH-, CO, CH=N-O,  ,  ,  ,  ,  or heterocyclyl;

L^{2A}, L^{2B}, L^{3A}, L^{3B}, L^{4A}, L^{4B}, L^{5A}, L^{5B} and L^{5C} represent the ligand; *i.e.* each independently for each occurrence a monosaccharide (such as GalNAc), disaccharide, trisaccharide, tetrasaccharide, oligosaccharide, or polysaccharide; andR^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 (XLIX):

Formula XLIX



Form ,

wherein L^{5A}, L^{5B} and L^{5C} represent a monosaccharide, such as GalNAc derivative.

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.

Representative U.S. Patents that teach the preparation of RNA conjugates include, but are not limited to, U.S. Patent 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.

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.

"Chimeric" iRNA compounds or "chimeras," in the context of this invention, are iRNA compounds, such as dsRNA 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.

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. Let.*, 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.

V. Delivery of an RNAi Agent of the Disclosure

The delivery of a RNAi agent of the disclosure 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 PRNP-associated disorder, *e.g.,* a prion disease, such as a genetic prion disease, *e.g.,* familial Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI), can be achieved in a number of different ways. For example, delivery may be performed by contacting a cell with an RNAi agent of the disclosure either *in vitro* or *in vivo*. *In vivo* delivery may also be performed directly by administering a composition comprising an RNAi agent, *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 RNAi agent. These alternatives are discussed further below.

In general, any method of delivering a nucleic acid molecule (*in vitro* or *in vivo*) can be adapted for use with a RNAi agent of the disclosure (see *e.g.*, Akhtar S. and Julian RL., (1992) *Trends Cell. Biol.* 2(5):139-144 and WO94/02595, which are incorporated herein by reference in their entireties). For *in vivo* delivery, factors to consider in order to deliver an RNAi agent include, for example, biological stability of the delivered agent, prevention of non-specific effects, and accumulation of the delivered agent in the

target tissue. The non-specific effects of an RNAi agent 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 RNAi agent to be administered. Several studies have shown successful knockdown of gene products when an RNAi agent is administered locally. For example, intraocular delivery of a VEGF dsRNA by intravitreal injection in cynomolgus monkeys (Tolentino, MJ. *et al.*, (2004) *Retina* 24:132-138) and subretinal injections in mice (Reich, SJ. *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, WJ. *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, PH. *et al.* (2005) *Gene Ther.* 12:59-66; Makimura, H. *et a.l* (2002) *BMC Neurosci.* 3:18; Shishkina, GT., *et al.* (2004) *Neuroscience* 129:521-528; Thakker, ER., *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, KA. *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 a RNAi agent 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 RNAi agent to the target tissue and avoid undesirable off-target effects (*e.g.*, without wishing to be bound by theory, use of GNAs as described herein has been identified to destabilize the seed region of a dsRNA, resulting in enhanced preference of such dsRNAs for on-target effectiveness, relative to off-target effects, as such off-target effects are significantly weakened by such seed region destabilization). RNAi agents can be modified by chemical conjugation to lipophilic groups such as cholesterol to enhance cellular uptake and prevent degradation. For example, a RNAi agent 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 RNAi agent to an aptamer has been shown to inhibit tumor growth and mediate tumor regression in a mouse model of prostate cancer (McNamara, JO. *et al.*, (2006) *Nat. Biotechnol.* 24:1005-1015). In an alternative embodiment, the RNAi agent 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 molecule RNAi agent (negatively charged) and also enhance interactions at the negatively charged cell membrane to permit efficient uptake of an RNAi agent by the cell. Cationic lipids, dendrimers, or polymers can either be bound to an RNAi agent, or induced to form a vesicle or micelle (see *e.g.*, Kim SH. *et al.*, (2008) *Journal of Controlled Release* 129(2):107-116) that encases an RNAi agent. The formation of vesicles or micelles further

prevents degradation of the RNAi agent when administered systemically. Methods for making and administering cationic- RNAi agent complexes are well within the abilities of one skilled in the art (see *e.g.*, Sorensen, DR., *et al.* (2003) *J. Mol. Biol* 327:761-766; Verma, UN. *et al.*, (2003) *Clin. Cancer Res.* 9:1291-1300; Arnold, AS *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 RNAi agents include DOTAP (Sorensen, DR., *et al* (2003), supra; Verma, UN. *et al.*, (2003), supra), Oligofectamine, "solid nucleic acid lipid particles" (Zimmermann, TS. *et al.,* (2006) *Nature* 441:111-114), cardiolipin (Chien, PY. *et al.*, (2005) *Cancer Gene Ther.* 12:321-328; Pal, A. *et al.*, (2005) *Int J. Oncol.* 26:1087-1091), polyethyleneimine (Bonnet ME. *et al.*, (2008) *Pharm. Res.* Aug 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, DA. *et al.*, (2007) *Biochem. Soc. Trans.* 35:61-67; Yoo, H. *et al.*, (1999) *Pharm. Res.* 16:1799-1804). In some embodiments, a RNAi agent forms a complex with cyclodextrin for systemic administration. Methods for administration and pharmaceutical compositions of RNAi agents and cyclodextrins can be found in U.S. Patent No. 7, 427, 605, which is herein incorporated by reference in its entirety.

Certain aspects of the instant disclosure relate to a method of reducing the expression of a PRNP target gene in a cell, comprising contacting said cell with the double-stranded RNAi agent of the disclosure. In one embodiment, the cell is a hepatic cell, optionally a hepatocyte. In one embodiment, the cell is an extrahepatic cell, optionally a CNS cell, *e.g.,* a neuron or a glial cell.

Another aspect of the disclosure relates to a method of reducing the expression of a PRNP target gene in a subject, comprising administering to the subject the double-stranded RNAi agent of the disclosure.

Another aspect of the disclosure relates to a method of treating a subject having a PRNP-associated disorder, comprising administering to the subject a therapeutically effective amount of the double-stranded RNAi agent of the disclosure, thereby treating the subject. Exemplary PRNP-associated disorders that can be treated by the method of the disclosure include prion diseases, such as a genetic prion disease, *e.g.,* familial Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI); a sporadic prion disease, *e.g.,* sporadic Creutzfeldt-Jakob disease, Sporadic Fatal Insomnia (sFI), and Variably Proteinase-Sensitive Prionopathy (VPSPr), or an acquired prion disease, *e.g.,* Iatrogenic CJD (iCJD), Kuru, and Variant CJD (vCJD).

In one embodiment, the double-stranded RNAi agent is administered subcutaneously.

In one embodiment, the double-stranded RNAi agent is administered by intraventricular administration.

In one embodiment, the double-stranded RNAi agent is administered intrathecally. By intrathecal administration of the double-stranded RNAi agent, the method can reduce the expression of a PRNP target gene in a brain or spine tissue, for instance, cortex, cerebellum, striatum, cervical spine, lumbar spine, and thoracic spine.

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

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

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

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

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

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

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

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

In one embodiment, the administration of the siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, composition is parenteral, *e.g.,* intravenous (*e.g.*, as a bolus or as a diffusible infusion), intradermal, intraperitoneal, intramuscular, intrathecal, intraventricular, intracranial, subcutaneous, transmucosal, buccal, sublingual, endoscopic, rectal, oral, vaginal, topical, pulmonary, intranasal, urethral, or ocular. Administration can be provided by the subject or by another person, *e.g.*, a health care provider. The medication can be provided in measured doses or in a dispenser which delivers a metered dose. Selected modes of delivery are discussed in more detail below.

Intrathecal Administration.

In one embodiment, the double-stranded RNAi agent is delivered by intrathecal injection (*i.e.*, injection into the spinal fluid which bathes the brain and spinal cord tissue). Intrathecal injection of RNAi agents into the spinal fluid can be performed as a bolus injection or via minipumps which can be implanted beneath the skin, providing a regular and constant delivery of siRNA into the spinal fluid. The circulation of the spinal fluid from the choroid plexus, where it is produced, down around the spinal cord and dorsal root ganglia and subsequently up past the cerebellum and over the cortex to the arachnoid granulations, where the fluid can exit the CNS, that, depending upon size, stability, and solubility of the compounds injected, molecules delivered intrathecally could hit targets throughout the entire CNS.

In some embodiments, the intrathecal administration is via a pump. The pump may be a surgically implanted osmotic pump. In one embodiment, the osmotic pump is implanted into the subarachnoid space of the spinal canal to facilitate intrathecal administration.

In some embodiments, the intrathecal administration is via an intrathecal delivery system for a pharmaceutical including a reservoir containing a volume of the pharmaceutical agent, and a pump configured to deliver a portion of the pharmaceutical agent contained in the reservoir. More details about this intrathecal delivery system may be found in WO 2015/116658, which is incorporated by reference in its entirety.

The amount of intrathecally injected RNAi agents may vary from one target gene to another target gene and the appropriate amount that has to be applied may have to be determined individually for each target gene. Typically, this amount ranges from 10 μg to 2 mg, or 50 μg to 1500 μg, or 100 μg to 1000 μg.

Vector encoded RNAi agents of the Disclosure

RNAi agents targeting the PRNP 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; WO 00/22113, WO 00/22114, and US 6,054,299). Expression can be sustained (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.*, (1995) *Proc. Natl. Acad. Sci. USA* 92:1292).

The individual strand or strands of a RNAi agent 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.

RNAi agent expression vectors are generally DNA plasmids or viral vectors. Expression vectors compatible with eukaryotic cells, such as those compatible with vertebrate cells, can be used to produce recombinant constructs for the expression of a RNAi agent as described herein. Delivery of RNAi agent 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.

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 a RNAi agent will generally require regulatory elements, *e.g.*, promoters, enhancers, *etc.*, to ensure the expression of the RNAi agent in target cells. Other aspects to consider for vectors and constructs are known in the art.

VI. Pharmaceutical Compositions of the Invention

The present disclosure also includes pharmaceutical compositions and formulations which include the RNAi agents of the disclosure. In one embodiment, provided herein are pharmaceutical compositions containing an RNAi agent, as described herein, and a pharmaceutically acceptable carrier. The pharmaceutical compositions containing the RNAi agent are useful for treating a disease or disorder associated with the expression or activity of PRNP, *e.g.*, a PRNP-associated disease, such as prion diseases, *e.g.*, a genetic prion disease, *e.g.,* familial Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI); a sporadic prion disease, *e.g.,* sporadic Creutzfeldt-Jakob disease, Sporadic Fatal Insomnia (sFI), and Variably Proteinase-Sensitive

Prionopathy (VPSPr), or an acquired prion disease, *e.g.,* Iatrogenic CJD (iCJD), Kuru, and Variant CJD (vCJD).

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 intravenous (IV), intramuscular (IM), or for subcutaneous (subQ) delivery. Another example is compositions that are formulated for direct delivery into the central nervous system, *e.g.*, by intrathecal or intraventricular routes of injection, optionally by infusion into the brain (*e.g.,* striatum), such as by continuous pump infusion.

In some embodiments, the pharmaceutical compositions of the invention are pyrogen free or non-pyrogenic.

The pharmaceutical compositions of the disclosure may be administered in dosages sufficient to inhibit expression of a PRNP gene. In general, a suitable dose of an RNAi agent of the disclosure will be a flat dose in the range of about 0.001 to about 200.0 mgabout once per month to about once per year, typically about once per quarter (*i.e.*, about once every three months) to about once per year, generally a flat dose in the range of about 1 to 50 mg about once per month to about once per year, typically about once per quarter to about once per year.

After an initial treatment regimen (*e.g.*, loading dose), the treatments can be administered on a less frequent basis.

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.

Advances in mouse genetics have generated a number of mouse models for the study of various PRNP-associated diseases that would benefit from reduction in the expression of PRNP. Such models can be used for *in vivo* testing of RNAi agents, as well as for determining a therapeutically effective dose. Suitable mouse models are known in the art and include, for example, the mouse models described elsewhere herein.

The pharmaceutical compositions of the present disclosure 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.

The RNAi agents can be delivered in a manner to target a particular tissue, such as the liver, the CNS (*e.g.*, neuronal, glial or vascular tissue of the brain), or both the liver and CNS.

Pharmaceutical compositions and formulations for topical 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 RNAi agents featured in the disclosure 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, distearolyphosphatidyl choline) negative (*e.g.,* dimyristoylphosphatidyl glycerol DMPG) and cationic (*e.g.,* dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA). RNAi agents featured in the disclosure can be encapsulated within liposomes or can form complexes thereto, in particular to cationic liposomes. Alternatively, RNAi agents 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, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C_{1-20} alkyl ester (*e.g.,* isopropylmyristate IPM), monoglyceride, diglyceride or pharmaceutically acceptable salt thereof. Topical formulations are described in detail in US 6,747,014, which is incorporated herein by reference.

A. RNAi Agent Formulations Comprising Membranous Molecular Assemblies

A RNAi agent for use in the compositions and methods of the disclosure 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 RNAi agent composition. The lipophilic material isolates the aqueous interior from an aqueous exterior, which typically does not include the RNAi agent 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 RNAi agent are delivered into the cell where the RNAi agent can specifically bind to a target RNA and can mediate RNAi. In some cases the liposomes are also specifically targeted, *e.g.*, to direct the RNAi agent to particular cell types.

A liposome containing an RNAi 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 RNAi agent preparation is then added to the micelles that include the lipid component. The cationic groups on the

lipid interact with the RNAi agent and condense around the RNAi agent to form a liposome. After condensation, the detergent is removed, *e.g.*, by dialysis, to yield a liposomal preparation of RNAi agent.

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.

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.*, (1987) *Proc. Natl. Acad. Sci. USA* 8:7413-7417; United States Patent No. 4,897,355; United States Patent No. 5,171,678; Bangham *et al.*, (1965) *M. Mol. Biol.* 23:238; Olson *et al.*, (1979) *Biochim. Biophys. Acta* 557:9; Szoka *et al.*, (1978) *Proc. Natl. Acad. Sci.* 75: 4194; Mayhew *et al.*, (1984) *Biochim. Biophys. Acta* 775:169; Kim *et al.*, (1983) *Biochim. Biophys. Acta* 728:339; and Fukunaga *et al.*, (1984) *Endocrinol.* 115:757. 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.*, (1986) *Biochim. Biophys. Acta* 858:161. Microfluidization can be used when consistently small (50 to 200 nm) and relatively uniform aggregates are desired (Mayhew *et al.*, (1984) *Biochim. Biophys. Acta* 775:169. These methods are readily adapted to packaging RNAi agent preparations into liposomes.

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.* (1987) *Biochem. Biophys. Res. Commun.*, 147:980-985).

Liposomes, which are pH-sensitive or negatively charged, entrap nucleic acids rather than complex with them. 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.* (1992) *Journal of Controlled Release*, 19:269-274).

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

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

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 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.*, (1994) *S.T.P.Pharma. Sci.*, 4(6):466).

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_{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.*, (1987) *FEBS Letters,* 223:42; Wu *et al.*, (1993) *Cancer Research*, 53:3765).

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_{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). United States Patent No. 4,837,028 and WO 88/04924, both to Allen *et al.*, disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside G_{M1} or a galactocerebroside sulfate ester. United States Patent 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*).

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

Further advantages of liposomes include: liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect encapsulated RNAi agents in their internal compartments from metabolism and degradation (Rosoff, in "Pharmaceutical Dosage Forms," Lieberman, Rieger and Banker (Eds.), 1988,

volume 1, p. 245). Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

A positively charged synthetic cationic lipid, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) can be used to form small liposomes that interact spontaneously with nucleic acid to form lipid-nucleic acid complexes which are capable of fusing with the negatively charged lipids of the cell membranes of tissue culture cells, resulting in delivery of RNAi agent (see, *e.g.*, Felgner, P. L. *et al.*, (1987) *Proc. Natl. Acad. Sci. USA* 8:7413-7417, and United States Patent No.4,897,355 for a description of DOTMA and its use with DNA).

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

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

Another cationic lipid conjugate includes derivatization of the lipid with cholesterol ("DC-Chol") which has been formulated into liposomes in combination with DOPE (See, Gao, X. and Huang, L., (1991) *Biochim. Biophys. Res. Commun.* 179:280). Lipopolylysine, made by conjugating polylysine to DOPE, has been reported to be effective for transfection in the presence of serum (Zhou, X. *et al.*, (1991) *Biochim. Biophys. Acta* 1065:8). For certain cell lines, these liposomes containing conjugated cationic lipids, are said to exhibit lower toxicity and provide more efficient transfection than the DOTMA-containing compositions. Other commercially available cationic lipid products include DMRIE and DMRIE-HP (Vical, La Jolla, California) and Lipofectamine (DOSPA) (Life Technology, Inc., Gaithersburg, Maryland). Other cationic lipids suitable for the delivery of oligonucleotides are described in WO 98/39359 and WO 96/37194.

Liposomal formulations are particularly suited for topical administration, liposomes present several advantages over other formulations. Such advantages include reduced side effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer RNAi agent into the skin. In some implementations,

liposomes are used for delivering RNAi agent to epidermal cells and also to enhance the penetration of RNAi 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.*, (1992) *Journal of Drug Targeting*, vol. 2,405-410 and du Plessis *et al.*, (1992) *Antiviral Research*, 18:259-265; Mannino, R. J. and Fould-Fogerite, S., (1998) *Biotechniques* 6:682-690; Itani, T. *et al.*, (1987) *Gene* 56:267-276; Nicolau, C. *et al.* (1987) *Meth. Enzymol.* 149:157-176; Straubinger, R. M. and Papahadjopoulos, D. (1983) *Meth. Enzymol.* 101:512-527; Wang, C. Y. and Huang, L., (1987) *Proc. Natl. Acad. Sci. USA* 84:7851-7855).

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 RNAi agent are useful for treating a dermatological disorder.

Liposomes that include RNAi agents 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. Transferosomes can be made by adding surface edge activators, usually surfactants, to a standard liposomal composition. Transfersomes that include RNAi agent can be delivered, for example, subcutaneously by infection in order to deliver RNAi agent 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 transferosomes can be self-optimizing (adaptive to the shape of pores, *e.g.*, in the skin), self-repairing, and can frequently reach their targets without fragmenting, and often self-loading.

Other formulations amenable to the present disclosure are described in PCT publication No. WO 2008/042973.

Transfersomes, yet another type of liposomes, 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.

Surfactants find wide application in formulations such as those described herein, particularlay in 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).

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.

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

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

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

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

The RNAi agent for use in the methods of the disclosure 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.

A mixed micellar formulation suitable for delivery through transdermal membranes may be prepared by mixing an aqueous solution of the siRNA composition, an alkali metal C_8 to C_{22} alkyl sulphate, and a micelle forming compounds. Exemplary micelle forming compounds include lecithin, hyaluronic acid, pharmaceutically acceptable salts of hyaluronic acid, glycolic acid, lactic acid, chamomile extract, cucumber extract, oleic acid, linoleic acid, linolenic acid, monoolein, monooleates, monolaurates, borage oil, evening of primrose oil, menthol, trihydroxy oxo cholanyl glycine and pharmaceutically acceptable salts thereof, glycerin, polyglycerin, lysine, polylysine, triolein, polyoxyethylene ethers and analogues thereof, polidocanol alkyl ethers and analogues thereof,

chenodeoxycholate, deoxycholate, and mixtures thereof. The micelle forming compounds may be added at the same time or after addition of the alkali metal alkyl sulphate. Mixed micelles will form with substantially any kind of mixing of the ingredients but vigorous mixing in order to provide smaller size micelles.

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

Phenol 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.

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

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

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

Lipid particles

RNAi agents, *e.g.,* dsRNAs of in the disclosure may be fully encapsulated in a lipid formulation, *e.g.,* a LNP, or other nucleic acid-lipid particle.

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 WO 00/03683. The particles of the present disclosure 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 disclosure 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. Patent Nos. 5,976,567; 5,981,501; 6,534,484; 6,586,410; 6,815,432; United States Patent publication No. 2010/0324120 and WO 96/40964.

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 disclosure.

Certain specific LNP formulations for delivery of RNAi agents have been described in the art, including, *e.g.*, "LNP01" formulations as described in, *e.g.*, WO 2008/042973, which is hereby incorporated by reference.

Additional exemplary lipid-dsRNA formulations are identified in the table below.

	Ionizable/Cationic Lipid	**cationic lipid/non-cationic lipid/cholesterol/PEG-lipid conjugate Lipid:siRNA ratio**
SNALP-1	l,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLinDMA)	DLinDMA/DPPC/Cholesterol/PEG-cDM (57.1/7.1/34.4/1.4) lipid:siRNA ~ 7:1
2-XTC	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DPPC/Cholesterol/PEG-cDMA 57.1/7.1/34.4/1.4 lipid:siRNA ~ 7:1
LNP05	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 57.5/7.5/31.5/3.5 lipid:siRNA ~ 6:1
LNP06	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 57.5/7.5/31.5/3.5 lipid:siRNA ~ 11:1
LNP07	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 60/7.5/31/1.5, lipid:siRNA ~ 6:1
LNP08	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 60/7.5/31/1.5, lipid:siRNA ~ 11:1
LNP09	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA 10:1

LNP10	(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)	ALN100/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA 10:1
LNP11	(6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (MPRNP)	MC-3/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA 10:1
LNP12	1,1'-(2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl)(2-hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethylazanediyl)didodecan-2-ol (Tech G1)	Tech G1/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA 10:1
LNP13	XTC	XTC/DSPC/Chol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA: 33:1
LNP14	MPRNP	MPRNP/DSPC/Chol/PEG-DMG 40/15/40/5 Lipid:siRNA: 11:1
LNP15	MPRNP	MPRNP/DSPC/Chol/PEG-DSG/GalNAc PEG-DSG 50/10/35/4.5/0.5 Lipid:siRNA: 11:1
LNP16	MPRNP	MPRNP/DSPC/Chol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA: 7:1
LNP17	MPRNP	MPRNP/DSPC/Chol/PEG-DSG 50/10/38.5/1.5 Lipid:siRNA: 10:1
LNP18	MPRNP	MPRNP/DSPC/Chol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA: 12:1
LNP19	MPRNP	MPRNP/DSPC/Chol/PEG-DMG 50/10/35/5 Lipid:siRNA: 8:1
LNP20	MPRNP	MPRNP/DSPC/Chol/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 (l,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLinDMA)) comprising formulations are described in WO 2009/127060, which is hereby incorporated by reference.

XTC comprising formulations are described in WO 2010/088537, the entire contents of which are hereby incorporated herein by reference.

MPRNP comprising formulations are described, *e.g.*, in United States Patent Publication No. 2010/0324120, the entire contents of which are hereby incorporated by reference.

ALNY-100 comprising formulations are described in WO 2010/054406, the entire contents of which are hereby incorporated herein by reference.

C12-200 comprising formulations are described in WO 2010/129709, the entire contents of which are hereby incorporated herein by reference.

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 minitablets. 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 disclosure 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, glucholic 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 disclosure 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. Patent 6,887,906, U.S. 2003/0027780, and U.S. Patent No. 6,747,014, each of which is incorporated herein by reference.

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.

Pharmaceutical compositions of the present disclosure 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. Particularly useful formulations include those that target the brain when treating PRNP-associated diseases or disorders.

The pharmaceutical formulations of the present disclosure, 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.

The compositions of the present disclosure 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 disclosure 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.

Additional Formulations

i. Emulsions

The compositions of the present disclosure 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 0.1μm 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 (o/w) emulsion. Emulsions can contain additional components in addition to the dispersed phases, and the active drug which can be present as a solution in either 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.

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).

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).

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.

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).

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.

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.

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

In one embodiment of the present disclosure, the compositions of RNAi agents and nucleic acids 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).

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, 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; 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.

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 (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), 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.

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. Patent 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. Patent 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 RNAi agents. 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 disclosure will facilitate the increased systemic absorption of RNAi agents and nucleic acids from the gastrointestinal tract, as well as improve the local cellular uptake of RNAi agents and nucleic acids.

Microemulsions of the present disclosure 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 RNAi agents and nucleic acids of the present disclosure. Penetration enhancers used in the microemulsions of the present disclosure 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

An RNAi agent of the disclosure 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

In one embodiment, the present disclosure employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly RNAi agents, 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.

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). Each of the above mentioned classes of penetration enhancers are described below in greater detail.

Surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of RNAi agents through the mucosa is enhanced. In addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (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); and perfluorochemical emulsions, such as FC-43. Takahashi *et al.*, J. Pharm. Pharmacol., 1988, 40, 252).

Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C_{1-20} alkyl esters thereof (*e.g.*, methyl, isopropyl and t-butyl), and mono- and di-glycerides thereof (*i.e.*, oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, *etc.*) (see *e.g.*, Touitou, E., *et al.* Enhancement in Drug Delivery, CRC Press, Danvers, MA, 2006; Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p.92; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; El Hariri *et al.*, J. Pharm. Pharmacol., 1992, 44, 651-654).

The physiological role of bile includes the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (see *e.g.*, Malmsten, M. Surfactants and polymers in drug delivery, Informa Health

Care, New York, NY, 2002; Brunton, Chapter 38 in: Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th Ed., Hardman *et al.* Eds., McGraw-Hill, New York, 1996, pp. 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus the term "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. Suitable bile salts include, for example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucholic acid (sodium glucholate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (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, page 92; Swinyard, Chapter 39 In: Remington's Pharmaceutical Sciences, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, Pa., 1990, pages 782-783; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; Yamamoto *et al.*, J. Pharm. Exp. Ther., 1992, 263, 25; Yamashita *et al.*, J. Pharm. Sci., 1990, 79, 579-583).

Chelating agents, as used in connection with the present disclosure, can be defined as compounds that remove metallic ions from solution by forming complexes therewith, with the result that absorption of RNAi agents through the mucosa is enhanced. With regards to their use as penetration enhancers in the present disclosure, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, J. Chromatogr., 1993, 618, 315-339). Suitable chelating agents include but are not limited to disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (*e.g.*, sodium salicylate, 5-methoxysalicylate and homovanilate), N-acyl derivatives of collagen, laureth-9 and N-amino acyl derivatives of beta-diketones (enamines)(see *e.g.*, Katdare, A. *et al.*, Excipient development for pharmaceutical, biotechnology, and drug delivery, CRC Press, Danvers, MA, 2006; Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; Buur *et al.*, J. Control Rel., 1990, 14, 43-51).

As used herein, non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of RNAi agents through the alimentary mucosa (see *e.g.*, Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33). This class of penetration enhancers includes, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita *et al.*, J. Pharm. Pharmacol., 1987, 39, 621-626).

Agents that enhance uptake of RNAi agents at the cellular level can also be added to the pharmaceutical and other compositions of the present disclosure. For example, cationic lipids, such as

lipofectin (Junichi *et al*, U.S. Pat. No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (WO 97/30731), are also known to enhance the cellular uptake of dsRNAs.

Other agents can be utilized to enhance the penetration of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

vi. Excipients

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*).

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 disclosure. 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.

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.

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

The compositions of the present disclosure 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 disclosure, 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 disclosure. 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.

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.

In some embodiments, pharmaceutical compositions featured in the disclosure include (a) one or more RNAi agents and (b) one or more agents which function by a non-RNAi mechanism and which are useful in treating a PRNP-associated disorder. Examples of such agents include, but are not lmited to SSRIs, venlafaxine, bupropion, and atypical antipsychotics.

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 LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (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 LD_{50}/ED_{50}. Compounds that exhibit high therapeutic indices are preferred.

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 disclosure lies generally within a range of circulating concentrations that include the ED_{50} 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 disclosure, 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 IC_{50} (*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.

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

VII. Methods for Inhibiting PRNP Expression

The present disclosure also provides methods of inhibiting expression of a PRNP 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 PRNP in the cell, thereby inhibiting expression of PRNP in the cell. In certain embodiments of the disclosure, PRNP is inhibited preferentially in CNS (*e.g.*, brain) cells. In some embodiments of the disclosure, PRNP is inhibited in liver cells (*e.g.*, hepatocytes). In certain embodiments of the disclosure, PRNP is inhibited in CNS (*e.g.*, brain) cells and in liver (*e.g.*, hepatocytes) cells.

Contacting of a cell with a RNAi agent, *e.g.*, a double stranded RNAi agent, may be done *in vitro* or *in vivo*. Contacting a cell *in vivo* with the RNAi agent includes contacting a cell or group of cells within a subject, *e.g.*, a human subject, with the RNAi agent. 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 some embodiments, the targeting ligand is a carbohydrate moiety, *e.g.*, a GalNAc ligand, or any other ligand that directs the RNAi agent to a site of interest.

The term "inhibiting," as used herein, is used interchangeably with "reducing," "silencing," "downregulating," "suppressing" and other similar terms, and includes any level of inhibition. In certain embodiments, a level of inhibition, *e.g.*, for an RNAi agent of the instant disclosure, can be assessed in cell culture conditions, *e.g.*, wherein cells in cell culture are transfected *via* Lipofectamine™-mediated transfection at a concentration in the vicinity of a cell of 10 nM or less, 1 nM or less, *etc*. Knockdown of a given RNAi agent can be determined *via* comparison of pre-treated levels in cell culture versus post-treated levels in cell culture, optionally also comparing against cells treated in parallel with a scrambled or other form of control RNAi agent. Knockdown in cell culture of, *e.g.*, preferably 50% or more, can thereby be identified as indicative of "inhibiting" or "reducing", "downregulating" or "suppressing", *etc*. having occurred. It is expressly contemplated that assessment of targeted mRNA or encoded protein levels (and therefore an extent of "inhibiting", *etc*. caused by a RNAi agent of the disclosure) can also be assessed in *in vivo* systems for the RNAi agents of the instant disclosure, under properly controlled conditions as described in the art.

The phrase "inhibiting expression of a PRNP gene" or "inhibiting expression of PRNP," as used herein, includes inhibition of expression of any PRNP gene (such as, *e.g.*, a mouse PRNP gene, a rat PRNP gene, a monkey PRNP gene, or a human PRNP gene) as well as variants or mutants of a PRNP gene that encode a PRNP protein. Thus, the PRNP gene may be a wild-type PRNP gene, a mutant PRNP gene, or a transgenic PRNP gene in the context of a genetically manipulated cell, group of cells, or organism.

"Inhibiting expression of a PRNP gene" includes any level of inhibition of a PRNP gene, *e.g.*, at least partial suppression of the expression of a PRNP gene, such as an inhibition by at least 20%. In certain embodiments, inhibition is by at least 30%, at least 40%, preferably at least 50%, at least about 60%, at least 70%, at least about 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%; or to below the level of detection of the assay method. In a preferred

method, inhibition is measured at a 10 nM concentration of the siRNA using the luciferase assay provided in Example 1.

The expression of a PRNP gene may be assessed based on the level of any variable associated with PRNP gene expression, *e.g.*, PRNP mRNA level or PRNP protein level, or, for example, the level of PRNP deposition in areas of the brain associated with neuronal cell death.

Inhibition may be assessed by a decrease in an absolute or relative level of one or more of these variables 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).

In some embodiments of the methods of the disclosure, expression of a PRNP gene is inhibited by at least 20%, 30%, 40%, preferably at least 50%, 60%, 70%, 80%, 85%, 90%, or 95%, or to below the level of detection of the assay. In certain embodiments, the methods include a clinically relevant inhibition of expression of PRNP, *e.g.* as demonstrated by a clinically relevant outcome after treatment of a subject with an agent to reduce the expression of PRNP.

Inhibition of the expression of a PRNP 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 PRNP gene is transcribed and which has or have been treated (*e.g.*, by contacting the cell or cells with a RNAi agent of the disclosure, or by administering a RNAi agent of the disclosure to a subject in which the cells are or were present) such that the expression of a PRNP 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 a RNAi agent or not treated with a RNAi agent targeted to the gene of interest). The degree of inhibition may be expressed in terms of:

$$\frac{(\text{mRNA in control cells}) - (\text{mRNA in treated cells})}{(\text{mRNA in control cells})} \bullet 100\%$$

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

Inhibition of the expression of a PRNP protein may be manifested by a reduction in the level of the PRNP 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 inhibiton 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.

A control cell or group of cells that may be used to assess the inhibition of the expression of a PRNP gene includes a cell or group of cells that has not yet been contacted with an RNAi agent of the disclosure. 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.

The level of PRNP mRNA that is expressed by a cell or group of cells may be determined using any method known in the art for assessing mRNA expression. In one embodiment, the level of expression of PRNP in a sample is determined by detecting a transcribed polynucleotide, or portion thereof, *e.g.*, mRNA of the PRNP 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. Circulating PRNP mRNA may be detected using methods the described in WO2012/177906, the entire contents of which are hereby incorporated herein by reference.

In some embodiments, the level of expression of PRNP 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 PRNP nucleic acid or protein, or fragment thereof. 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.

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 PRNP 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 PRNP mRNA.

An alternative method for determining the level of expression of PRNP 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, US Patent 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.*, US Patent 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 disclosure, the level of expression of PRNP is determined by quantitative fluorogenic RT-PCR (*i.e.*, the TaqMan™ System), by a Dual-Glo® Luciferase assay, or by other art-recognized method for measurement of PRNP expression or mRNA level.

The expression level of PRNP 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 US Patent 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 PRNP expression level may also comprise using nucleic acid probes in solution.

In some embodiments, the level of mRNA expression is assessed using branched DNA (bDNA) assays or real time PCR (qPCR). The use of this PCR method is described and exemplified in the Examples presented herein. Such methods can also be used for the detection of PRNP nucleic acids.

The level of PRNP 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. Such assays can also be used for the detection of proteins indicative of the presence or replication of PRNP proteins.

In some embodiments, the efficacy of the methods of the disclosure in the treatment of a PRNP-related disease is assessed by a decrease in PRNP mRNA level (*e.g*, by assessment of a CSF sample for PRNP level, by brain biopsy, or otherwise).

In some embodiments, the efficacy of the methods of the disclosure in the treatment of a PRNP-related disease is assessed by a decrease in PRNP mRNA level (*e.g*, by assessment of a liver sample for PRNP level, by biopsy, or otherwise).

In some embodiments of the methods of the disclosure, the RNAi agent is administered to a subject such that the RNAi agent is delivered to a specific site within the subject. The inhibition of expression of PRNP may be assessed using measurements of the level or change in the level of PRNP mRNA or PRNP protein in a sample derived from a specific site within the subject, *e.g.*, CNS cells. In certain embodiments, the methods include a clinically relevant inhibition of expression of PRNP, *e.g.* as demonstrated by a clinically relevant outcome after treatment of a subject with an agent to reduce the expression of PRNP.

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.

VIII. Methods of Treating or Preventing PRNP-Associated Diseases

The present disclosure also provides methods of using a RNAi agent of the disclosure or a composition containing a RNAi agent of the disclosure to reduce or inhibit PRNP expression in a cell. The methods include contacting the cell with a dsRNA of the disclosure and maintaining the cell for a

time sufficient to obtain degradation of the mRNA transcript of a PRNP gene, thereby inhibiting expression of the PRNP 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 PRNP may be determined by determining the mRNA expression level of PRNP using methods routine to one of ordinary skill in the art, *e.g.,* northern blotting, qRT-PCR; by determining the protein level of PRNP using methods routine to one of ordinary skill in the art, such as western blotting, immunological techniques.

In the methods of the disclosure the cell may be contacted *in vitro* or *in vivo, i.e.,* the cell may be within a subject.

A cell suitable for treatment using the methods of the disclosure may be any cell that expresses a PRNP gene. A cell suitable for use in the methods of the disclosure 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 rat cell, or a mouse cell. In one embodiment, the cell is a human cell, *e.g.,* a human CNS cell. In one embodiment, the cell is a human cell, *e.g.,* a human liver cell. In one embodiment, the cell is a human cell, *e.g.,* a human CNS cell or a human liver cell.

PRNP expression is inhibited in the cell by at least about 30, 40, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, or about 100%, *i.e.*, to below the level of detection. In preferred embodiments, PRNP expression is inhibited by at least 50 %.

The *in vivo* methods of the disclosure may include administering to a subject a composition containing a RNAi agent, where the RNAi agent includes a nucleotide sequence that is complementary to at least a part of an RNA transcript of the PRNP 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. In certain embodiments, the compositions are administered by intrathecal injection.

In some embodiments, the administration is *via* a depot injection. A depot injection may release the RNAi agent 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 PRNP, 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.

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 intracranial, 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 RNAi agent to the CNS.

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.

In one aspect, the present disclosure also provides methods for inhibiting the expression of a PRNP gene in a mammal. The methods include administering to the mammal a composition comprising a dsRNA that targets a PRNP gene in a cell of the mammal and maintaining the mammal for a time sufficient to obtain degradation of the mRNA transcript of the PRNP gene, thereby inhibiting expression of the PRNP gene in the cell. Reduction in gene expression can be assessed by any methods known it the art and by methods, *e.g.* qRT-PCR, described herein. Reduction in protein production can be assessed by any methods known it the art and by methods, *e.g.* ELISA, described herein. In one embodiment, a CNS biopsy sample or a cerebrospinal fluid (CSF) sample serves as the tissue material for monitoring the reduction in PRNP gene or protein expression (or of a proxy therefore).

The present disclosure further provides methods of treatment of a subject in need thereof. The treatment methods of the disclosure include administering an RNAi agent of the disclosure to a subject, *e.g.*, a subject that would benefit from inhibition of PRNP expression, in a therapeutically effective amount of a RNAi agent targeting a PRNP gene or a pharmaceutical composition comprising a RNAi agent targeting a PRNP gene.

In addition, the present disclosure provides methods of preventing, treating or inhibiting the progression of a PRNP-associated disease or disorder, such as prion diseases, *e.g.*, a genetic prion disease, *e.g.,* familial Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI); a sporadic prion disease, *e.g.,* sporadic Creutzfeldt-Jakob disease, Sporadic Fatal Insomnia (sFI), and Variably Proteinase-Sensitive Prionopathy (VPSPr), or an acquired prion disease, *e.g.,* Iatrogenic CJD (iCJD), Kuru, and Variant CJD (vCJD). The methods include administering to the subject a therapeutically effective amount of any of the RNAi agent, *e.g.,* dsRNA agents, or the pharmaceutical composition provided herein, thereby preventing, treating, or inhibiting the progression of the PRNP-associated disease or disorder in the subject.

An RNAi agent of the disclosure may be administered as a "free RNAi agent." A free RNAi agent is administered in the absence of a pharmaceutical composition. The naked RNAi agent 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 RNAi agent can be adjusted such that it is suitable for administering to a subject.

Alternatively, an RNAi agent of the disclosure may be administered as a pharmaceutical composition, such as a dsRNA liposomal formulation.

Subjects that would benefit from a reduction or inhibition of PRNP gene expression are those having a PRNP-associated disease.

The disclosure further provides methods for the use of a RNAi agent or a pharmaceutical composition thereof, *e.g.*, for treating a subject that would benefit from reduction or inhibition of PRNP expression, *e.g.,* a subject having a PRNP-associated disorder, 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 RNAi agent targeting PRNP is administered in combination with, *e.g.*, an agent useful in treating a PRNP-associated compldisorder as described elsewhere herein or as otherwise known in the art. For example, additional agents and treatments suitable for treating a subject that would benefit from reducton in PRNP expression, *e.g.,* a subject having a PRNP-associated disorder, may include agents currently used to treat symptoms of PRNP. The RNAi agent and additional therapeutic agents may be administered at the same time or in the same combination, *e.g.*, intrathecally, 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.

Exemplary additional therapeutics and treatments include, for example, sedatives, antidepressants, clonazepam, sodium valproate, opiates, antiepileptic drugs, cholinesterase inhibitors, memantine, benzodiazepines, levodopa, COMT inhibitors (*e.g.,* tolcapone and entacapone), dopamine agonists (*e.g.,* bromocriptine, pergolide, pramipexole, ropinirole, piribedil, cabergoline, apomorphine and lisuride), MAO-B inhibitors (*e.g.,* safinamide, selegiline and rasagiline), amantadine, an anticholinergic, modafinil, pimavanserin, doxepin, rasagline, an antipsychotic, an atypical antipsychotic (*e.g.,* amisulpride, olanzapine, risperidone, and clozapine), riluzole, edaravone , deep brain stimulation, non-invasive ventilation (NIV), invasive ventilation physical therapy, occupational therapy, speech therapy, dietary changes and swallowing technique a feeding tube, a PEG tube, probiotics, and psychological therapy.

In one embodiment, the method includes administering a composition featured herein such that expression of the target PRNP gene is decreased, for at least one month. In some embodiments, expression is decreased for at least 2 months, or 6 months.

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

Administration of the dsRNA according to the methods of the disclosure may result in a reduction of the severity, signs, symptoms, or markers of such diseases or disorders in a patient with a PRNP-associated disorder. By "reduction" in this context is meant a statistically significant or clinically significant decrease in such level. The reduction can be, for example, at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or about 100%.

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. For example, efficacy of treatment of a PRNP-associated disorder may be assessed, for example, by periodic monitoring of a subject's cognition, learning, or memory. 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 a RNAi agent targeting PRNP or pharmaceutical composition thereof, "effective against" a PRNP-associated 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 an 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 PRNP-associated disorders and the related causes.

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 RNAi agent 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.

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 a RNAi agent or RNAi agent formulation as described herein.

Subjects can be administered a therapeutic amount of dsRNA, such as about 0.01 mg/kg to about 200 mg/kg.

The RNAi agent can be administered intrathecally, intraventricularly, or 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 RNAi agent can reduce PRNP levels, *e.g.*, in a cell, tissue, blood, CSF sample or other compartment of the patient by at least 20%, 30%, 40%, 50%, 55%, 60%, 65%, 70,% 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or at least about 99% or more. In a preferred embodiment, administration of the RNAi agent can reduce PRNP levels, *e.g.*, in a cell, tissue, blood, CSF sample or other compartment of the patient by at least 50%.

Before administration of a full dose of the RNAi agent, 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.

Alternatively, the RNAi agent can be administered subcutaneously, *i.e.*, by subcutaneous injection. One or more injections may be used to deliver the desired, *e.g.*, monthly dose of RNAi agent 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 regimine may include administration of a therapeutic amount of RNAi agent on a regular basis, such as monthly or extending to once a quarter, twice per year, once per year. In certain embodiments, the RNAi agent is administered about once per month to about once per quarter (*i.e.*, about once every three months).

IX. Kits

In certain aspects, the instant disclosure provides kits that include a suitable container containing a pharmaceutical formulation of a siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a ssiRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, or precursor thereof).

Such kits include one or more dsRNA agent(s) and instructions for use, *e.g.*, instructions for administering a prophylactically or therapeutically effective amount of a dsRNA agent(s). The dsRNA agent may be in a vial or a pre-filled syringe. The kits may optionally further comprise means for administering the dsRNA agent (*e.g.*, an injection device, such as a pre-filled syringe), or means for measuring the inhibition of PRNP (*e.g.*, means for measuring the inhibition of PRNP mRNA, PRNP protein, and/or PRNP activity). Such means for measuring the inhibition of PRNP may comprise a means for obtaining a sample from a subject, such as, *e.g.*, a CSF and/or plasma sample. The kits of the invention may optionally further comprise means for determining the therapeutically effective or prophylactically effective amount.

In certain embodiments the individual components of the pharmaceutical formulation may be provided in one container, *e.g.*, a vial or a pre-filled syringe. Alternatively, it may be desirable to provide the components of the pharmaceutical formulation separately in two or more containers, *e.g.*, one container for a siRNA compound preparation, and at least another for a carrier compound. The kit may be packaged in a number of different configurations such as one or more containers in a single box. The different components can be combined, *e.g.*, according to instructions provided with the kit. The components can be combined according to a method described herein, *e.g.*, to prepare and administer a pharmaceutical composition. The kit can also include a delivery device.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the RNAi agents and methods featured in the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are

incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

An inforrmal Sequence Listing is filed herewith and forms part of the specification as filed.

EXAMPLES

Example 1. RNAi Agent Design, Synthesis, Selection, and *In Vitro* Evaluation

This Example describes methods for the design, synthesis, selection, and *in vitro* evaluation of PRNP RNAi agents.

Source of reagents

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.

Bioinformatics

siRNAs targeting the human prion protein (PRNP) gene (human: NCBI refseqID NM_000311.5; NCBI GeneID: 5261) were designed using custom R and Python scripts. The human NM_000311.5 REFSEQ mRNA, has a length of 2435 bases.

Detailed lists of the unmodified PRNP sense and antisense strand nucleotide sequences are shown in Table 2. Detailed lists of the modified PRNP sense and antisense strand nucleotide sequences are shown in Table 3.

It is to be understood that, throughout the application, a duplex name without a decimal is equivalent to a duplex name with a decimal which merely references the batch number of the duplex. For example, AD-564727 is equivalent to AD-564727.1.

siRNA Synthesis

siRNAs were synthesized and annealed using routine methods known in the art. Briefly, siRNA sequences were synthesized on a 1 μmol scale using a Mermade 192 synthesizer (BioAutomation) with phosphoramidite chemistry on solid supports. The solid support was controlled pore glass (500-1000 Å) loaded with a custom GalNAc ligand (3'-GalNAc conjugates), universal solid support (AM Chemicals), or the first nucleotide of interest. Ancillary synthesis reagents and standard 2-cyanoethyl phosphoramidite monomers (2'-deoxy-2'-fluoro, 2'-O-methyl, RNA, DNA) were obtained from Thermo-Fisher (Milwaukee, WI), Hongene (China), or Chemgenes (Wilmington, MA, USA). Additional phosphoramidite monomers were procured from commercial suppliers, prepared in-house, or procured using custom synthesis from various CMOs. Phosphoramidites were prepared at a concentration of 100

mM in either acetonitrile or 9:1 acetonitrile:DMF and were coupled using 5-Ethylthio-1H-tetrazole (ETT, 0.25 M in acetonitrile) with a reaction time of 400 s. Phosphorothioate linkages were generated using a 100 mM solution of 3-((Dimethylamino-methylidene) amino)-3H-1,2,4-dithiazole-3-thione (DDTT, obtained from Chemgenes (Wilmington, MA, USA)) in anhydrous acetonitrile/pyridine (9:1 v/v). Oxidation time was 5 minutes. All sequences were synthesized with final removal of the DMT group ("DMT-Off").

Upon completion of the solid phase synthesis, solid-supported oligoribonucleotides were treated with 300 µL of Methylamine (40% aqueous) at room temperature in 96 well plates for approximately 2 hours to afford cleavage from the solid support and subsequent removal of all additional base-labile protecting groups. For sequences containing any natural ribonucleotide linkages (2'-OH) protected with a tert-butyl dimethyl silyl (TBDMS) group, a second deprotection step was performed using TEA.3HF (triethylamine trihydrofluoride). To each oligonucleotide solution in aqueous methylamine was added 200 µL of dimethyl sulfoxide (DMSO) and 300 µL TEA.3HF and the solution was incubated for approximately 30 mins at 60 °C. After incubation, the plate was allowed to come to room temperature and crude oligonucleotides were precipitated by the addition of 1 mL of 9:1 acetontrile:ethanol or 1:1 ethanol:isopropanol. The plates were then centrifuged at 4 °C for 45 mins and the supernatant carefully decanted with the aid of a multichannel pipette. The oligonucleotide pellet was resuspended in 20 mM NaOAc and subsequently desalted using a HiTrap size exclusion column (5 mL, GE Healthcare) on an Agilent LC system equipped with an autosampler, UV detector, conductivity meter, and fraction collector. Desalted samples were collected in 96 well plates and then analyzed by LC-MS and UV spectrometry to confirm identity and quantify the amount of material, respectively.

Duplexing of single strands was performed on a Tecan liquid handling robot. Sense and antisense single strands were combined in an equimolar ratio to a final concentration of 10 µM in 1x PBS in 96 well plates, the plate sealed, incubated at 100 °C for 10 minutes, and subsequently allowed to return slowly to room temperature over a period of 2-3 hours. The concentration and identity of each duplex was confirmed and then subsequently utilized for in vitro screening assays.

In vitro Screening Assays

Cell culture and transfections

Transfection experiments are performed in human neuroblastoma BE(2)C cells (ATCC CRL-2268) with EMEM:F12 media (Gibco catalog no. 11765054) and mouse neuroblastoma Neuro2A cells (ATCC CCL-131) with EMEM media. Cells are transfected by adding 4.9 µL of Opti-MEM plus 0.1 µL of RNAiMAX per well (Invitrogen, Carlsbad CA. cat # 13778-150) to 5 µL of siRNA duplexes per well, with 4 replicates of each siRNA duplex, into a 384-well plate, and incubated at room temperature for 15 minutes. Forty µL of MEDIA containing ~5 $x10^3$ cells are then added to the siRNA mixture. Cells are incubated for 24 hours prior to RNA purification. Experiments are performed at 50nM, 10nM, 1nM and 0.1nM.

Total RNA isolation using DYNABEADS mRNA Isolation Kit

RNA is isolated using an automated protocol on a BioTek-EL406 platform using DYNABEADs (Invitrogen, cat#61012). Briefly, 70 µL of Lysis/Binding Buffer and 10 µL of lysis buffer containing 3 µL of magnetic beads are added to the plate with cells. Plates are incubated on an electromagnetic shaker for 10 minutes at room temperature and then magnetic beads are captured and the supernatant is removed. Bead-bound RNA are then washed 2 times with 150 µL Wash Buffer A and once with Wash Buffer B. Beads are then washed with 150 µL Elution Buffer, re-captured and supernatant removed.

cDNA synthesis using ABI High capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, Cat #4368813)

Ten µL of a master mix containing 1 µL 10X Buffer, 0.4 µL 25X dNTPs, 1 µL 10x Random primers, 0.5 µL Reverse Transcriptase, 0.5 µL RNase inhibitor and 6.6 µL of H_2O per reaction is added to RNA isolated above. Plates are sealed, mixed, and incubated on an electromagnetic shaker for 10 minutes at room temperature, followed by 2 hour incubation at 37°C.

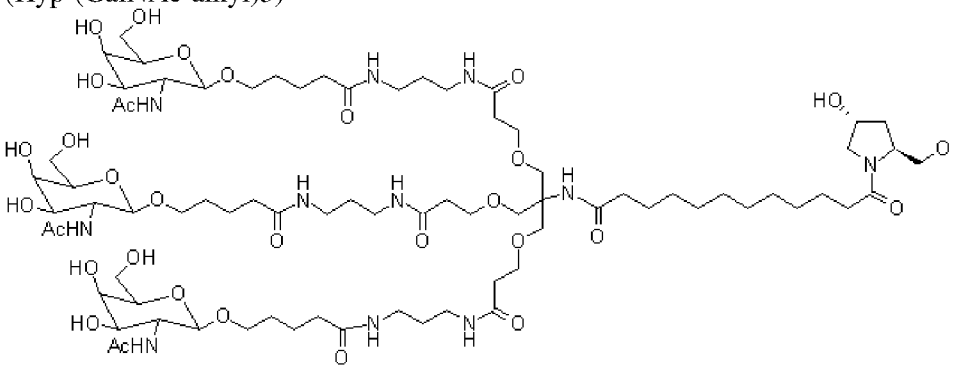
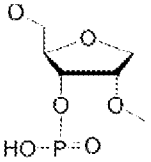
Real time PCR

Two µL of cDNA are added to a master mix containing 0.5 µL of human or mouse GAPDH TaqMan Probe (ThermoFisher cat 4352934E or 4351309) and 0.5 µL of appropriate PRNP probe (commercially available, *e.g.*, from Thermo Fisher) and 5 µL Lightcycler 480 probe master mix (Roche Cat # 04887301001) per well in a 384 well plates (Roche cat # 04887301001). Real time PCR is done in a LightCycler480 Real Time PCR system (Roche). Each duplex is tested with N=4 and data were normalized to cells transfected with a non-targeting control siRNA. To calculate relative fold change, real time data are analyzed using the ΔΔCt method and normalized to assays performed with cells transfected with a non-targeting control siRNA.

The results of the transfection experiments of the dsRNA agents listed in Tables 2 and 3 in BE2C cells are shown in Table 4, and the results of the transfection experiments of the dsRNA agents listed in Tables 2 and 3 in Neuro2a are shown in Table 5.

Table 1. 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; and it is understood that when the nucleotide contains a 2'-fluoro modification, then the fluoro replaces the hydroxy at that position in the parent nucleotide (i.e., it is a 2'-deoxy-2'-fluoronucleotide).

Abbreviation	**Nucleotide(s)**
A	Adenosine-3'-phosphate
Ab	beta-L-adenosine-3`-phosphate
Abs	beta-L-adenosine-3`-phosphorothioate
Af	2'-fluoroadenosine-3'-phosphate
Afs	2'-fluoroadenosine-3'-phosphorothioate
As	adenosine-3'-phosphorothioate

Abbreviation	Nucleotide(s)
C	cytidine-3’-phosphate
Cb	beta-L-cytidine-3`-phosphate
Cbs	beta-L-cytidine-3'-phosphorothioate
Cf	2’-fluorocytidine-3’-phosphate
Cfs	2’-fluorocytidine-3’-phosphorothioate
Cs	cytidine-3’-phosphorothioate
G	guanosine-3’-phosphate
Gb	beta-L-guanosine-3`-phosphate
Gbs	beta-L-guanosine-3`-phosphorothioate
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, modified or unmodified
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) 
Y34	2-hydroxymethyl-tetrahydrofurane-4-methoxy-3-phosphate (abasic 2'-OMe furanose) 

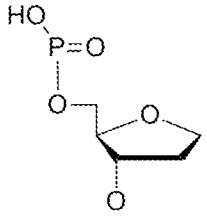
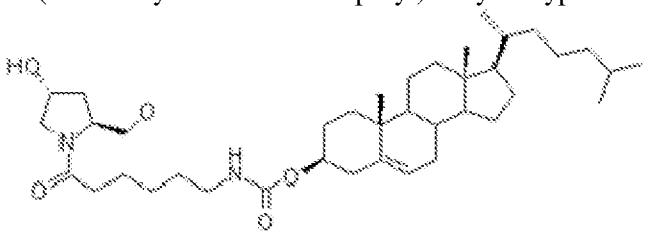
Abbreviation	Nucleotide(s)
Y44	inverted abasic DNA (2-hydroxymethyl-tetrahydrofurane-5-phosphate) 
L10	N-(cholesterylcarboxamidocaproyl)-4-hydroxyprolinol (Hyp-C6-Chol) 
(Agn)	Adenosine-glycol nucleic acid (GNA) S-Isomer
(Cgn)	Cytidine-glycol nucleic acid (GNA) S-Isomer
(Ggn)	Guanosine-glycol nucleic acid (GNA) S-Isomer
(Tgn)	Thymidine-glycol nucleic acid (GNA) S-Isomer
P	Phosphate
VP	Vinyl-phosphonate
dA	2`-deoxyadenosine-3`-phosphate
dAs	2`-deoxyadenosine-3`-phosphorothioate
dC	2`-deoxycytidine-3`-phosphate
dCs	2`-deoxycytidine-3`-phosphorothioate
dG	2`-deoxyguanosine-3`-phosphate
dGs	2`-deoxyguanosine-3`-phosphorothioate
dT	2`-deoxythymidine-3`-phosphate
dTs	2`-deoxythymidine-3`-phosphorothioate
dU	2`-deoxyuridine
dUs	2`-deoxyuridine-3`-phosphorothioate
(C2p)	cytidine-2`-phosphate
(G2p)	guanosine-2`-phosphate
(U2p)	uridine-2`-phosphate
(A2p)	adenosine-2`-phosphate
(Ahd)	2'-O-hexadecyl-adenosine-3'-phosphate
(Ahds)	2'-O-hexadecyl-adenosine-3'-phosphorothioate
(Chd)	2'-O-hexadecyl-cytidine-3'-phosphate
(Chds)	2'-O-hexadecyl-cytidine-3'-phosphorothioate
(Ghd)	2'-O-hexadecyl-guanosine-3'-phosphate
(Ghds)	2'-O-hexadecyl-guanosine-3'-phosphorothioate
(Uhd)	2'-O-hexadecyl-uridine-3'-phosphate
(Uhds)	2'-O-hexadecyl-uridine-3'-phosphorothioate
s	phosphorothioate

Table 2. Unmodified Sense and Antisense Strand Sequences of PRNP dsRNA Agents

Duplex Name	Sense Sequence 5' to 3'	SEQ ID NO:	Range in NM__000311.5	Antisense Sequence 5' to 3'	SEQ ID NO:	Range in NM_000311.5
AD-1071792.1	CCUAGCAGAUAUGUAUUACUA	13	2036-2056	UAGUAAUACAUAUCUGCUAGGUG	14	2034-2056
AD-1071769.1	CCCUGAAUUGUUUGAUAUUGA	15	2013-2033	UCAAUAUCAAACAAUUCAGGGAA	16	2011-2033
AD-1071773.1	GAAUUGUUUGAUAUUGUCACA	17	2017-2037	UGUGACAAUAUCAAACAAUUCAG	18	2015-2037
AD-1071718.1	GGAGAUGACAGAAAUAUGAUA	19	1940-1960	UAUCAUAUUUCUGUCAUCUCCAA	20	1938-1960
AD-1071870.1	UUAGGAGUUUUGUUUAGAGCA	21	2145-2165	UGCUCUAAACAAAACUCCUAAGU	22	2143-2165
AD-1071904.1	GUGUCUAAUGCAUUAACUUUA	23	2179-2199	UAAAGUUAAUGCAUUAGACACUU	24	2177-2199
AD-1071789.1	UCACCUAGCAGAUAUGUAUUA	25	2033-2053	UAAUACAUAUCUGCUAGGUGACA	26	2031-2053
AD-1071671.1	CAUAGUUUCUGUAAUUGGCUA	27	1875-1895	UAGCCAAUUACAGAAACUAUGAA	28	1873-1895
AD-1071628.1	AUUUCUGUCUCAUAAUUGUCA	29	1832-1852	UGACAAUUAUGAGACAGAAAUAA	30	1830-1852
AD-1071865.1	CAGACUUAGGAGUUUUGUUUA	31	2140-2160	UAAACAAAACUCCUAAGUCUGUC	32	2138-2160
AD-1071670.1	UCAUAGUUUCUGUAAUUGGCA	33	1874-1894	UGCCAAUUACAGAAACUAUGAAC	34	1872-1894
AD-1071901.1	GAAGUGUCUAAUGCAUUAACA	35	2176-2196	UGUUAAUGCAUUAGACACUUCAG	36	2174-2196
AD-1071837.1	CACUUUGUGAGUAUUCUAUGA	37	2081-2101	UCAUAGAAUACUCACAAAGUGCA	38	2079-2101
AD-1071929.1	GGUACUGAAUACUUAAUAUGA	39	2204-2224	UCAUAUUAAGUAUUCAGUACCUU	40	2202-2224
AD-1071827.1	UAUUGGCUUGCACUUUGUGAA	41	2071-2091	UUCACAAAGUGCAAGCCAAUAAU	42	2069-2091
AD-1071923.1	UUGUAAGGUACUGAAUACUUA	43	2198-2218	UAAGUAUUCAGUACCUUACAAAA	44	2196-2218
AD-1071834.1	UUGCACUUUGUGAGUAUUCUA	45	2078-2098	UAGAAUACUCACAAAGUGCAAGC	46	2076-2098
AD-1071984.1	UGUGCACUGAAUCGUUUCAUA	47	2259-2279	UAUGAAACGAUUCAGUGCACAUU	48	2257-2279
AD-1071629.1	UUUCUGUCUCAUAAUUGUCAA	49	1833-1853	UUGACAAUUAUGAGACAGAAAUA	50	1831-1853
AD-1071668.1	GUUCAUAGUUUCUGUAAUUGA	51	1872-1892	UCAAUUACAGAAACUAUGAACUU	52	1870-1892
AD-1070449.1	CGUUACUAUCGUGAAAACAUA	53	509-529	UAUGUUUUCACGAUAGUAACGGU	54	507-529
AD-1071836.1	GCACUUUGUGAGUAUUCUAUA	55	2080-2100	UAUAGAAUACUCACAAAGUGCAA	56	2078-2100
AD-1071897.1	AUCUGAAGUGUCUAAUGCAUA	57	2172-2192	UAUGCAUUAGACACUUCAGAUGU	58	2170-2192
AD-1071864.1	ACAGACUUAGGAGUUUUGUUA	59	2139-2159	UAACAAAACUCCUAAGUCUGUCC	60	2137-2159
AD-1071927.1	AAGGUACUGAAUACUUAAUAA	61	2202-2222	UUAUUAAGUAUUCAGUACCUUAC	62	2200-2222
AD-1070444.1	AGGACCGUUACUAUCGUGAAA	63	504-524	UUUCACGAUAGUAACGGUCCUCA	64	502-524

AD-1071981.1	CAAUGUGCACUGAAUCGUUUA	65	2256-2276	UAAACGAUUCAGUGCACAUUGUA	66	2254-2276
AD-1071509.1	UCCUUUGUCCAUUUACCUGGA	67	1694-1714	UCCAGGUAAAUGGACAAAGGAGA	68	1692-1714
AD-1071902.1	AAGUGUCUAAUGCAUUAACUA	69	2177-2197	UAGUUAAUGCAUUAGACACUUCA	70	2175-2197
AD-1071084.1	AUCUUAGUAGAGAUUUCAUAA	71	1222-1242	UUAUGAAAUCUCUACUAAGAUAG	72	1220-1242
AD-1071948.1	GUGGGAAACCCUUUUGCGUGA	73	2223-2243	UCACGCAAAAGGGUUUCCCACAU	74	2221-2243
AD-1071437.1	GCAUUCCUUUCUUUAAACUAA	75	1619-1639	UUAGUUUAAAGAAAGGAAUGCCA	76	1617-1639
AD-1071508.1	CUCCUUUGUCCAUUUACCUGA	77	1693-1713	UCAGGUAAAUGGACAAAGGAGAU	78	1691-1713
AD-1071265.1	UUCAACAAGAGUAAAUAUUUGA	79	1447-1467	UCAAUAUUUACUCUUGUUGAACA	80	1445-1467
AD-1070450.1	GUUACUAUCGUGAAAACAUGA	81	510-530	UCAUGUUUUCACGAUAGUAACGG	82	508-530
AD-1070522.1	UGCGUCAAUAUCACAAUCAAA	83	602-622	UUUGAUUGUGAUAUUGACGCAGU	84	600-622
AD-1071969.1	UCCUUAGGCUUACAAUGUGCA	85	2244-2264	UGCACAUUGUAAGCCUAAGGACC	86	2242-2264
AD-1071352.1	CUUCUGGGACUUGAAAUCAAA	87	1534-1554	UUUGAUUUCAAGUCCCAGAAGCC	88	1532-1554
AD-1071912.1	UGCAUUAACUUUUGUAAGGUA	89	2187-2207	UACCUUACAAAAGUUAAUGCAUU	90	2185-2207
AD-1071885.1	AGAGCAGUUAACACUCUGAAGA	91	2160-2180	UCUUCAGAUGUUAACUGCUCUAA	92	2158-2180
AD-1071949.1	UGGGAAACCCUUUUGCGUGGA	93	2224-2244	UCCACGCAAAAGGGUUUCCCACA	94	2222-2244
AD-1071085.1	UCUUAGUAGAGAUUUCAUAGA	95	1223-1243	UCUAUGAAAUCUCUACUAAGAUA	96	1221-1243
AD-1070516.1	CACGACUGCGUCAAUAUCACA	97	596-616	UGUGAUAUUGACGCAGUCGUGCA	98	594-616
AD-1071895.1	ACAUCUGAAGUGUCUAAUGCA	99	2170-2190	UGCAUUAGACACUUCAGAUGUUA	100	2168-2190
AD-1071430.1	AUAUGUGGCAUUCCUUUCUUA	101	1612-1632	UAAGAAAGGAAUGCCACACAUAUAG	102	1610-1632
AD-1070990.1	UUGAGGCUAAAAACAAAUCUCA	103	1128-1148	UGAGAUUUGUUUUAGCCUCAACC	104	1126-1148
AD-1071506.1	AUCUCCUUUGUCCAUUUACCA	105	1691-1711	UGGUAAAUGGACAAAGGAGAUUUU	106	1689-1711
AD-1071985.1	GUGCACUGAAUCGUUUCAUGA	107	2260-2280	UCAUGAAACGAUUCAGUGCACAU	108	2258-2280
AD-1070511.1	UUGUGCACGACUGCGUCAAUA	109	591-611	UAUUGACGCAGUCGUGCACAAAG	110	589-611
AD-1071301.1	CUGGCUAGAGGACAUAUUCAA	111	1483-1503	UUGAAUAUGUCCUCUAGCCAGAG	112	1481-1503
AD-1071835.1	UGCACUUUGUGAGUAUUCUAA	113	2079-2099	UUAGAAUACUCACAAAGUGCAAG	114	2077-2099
AD-1072054.1	AUUUGUAACUUUGCAUGUUCA	115	2344-2364	UGAACAUGCAAAGUUACAAAUAU	116	2342-2364
AD-1070861.1	CACUGGAAAACAUAGAGUAGA	117	978-998	UCUACUCUAUGUUUUCCAGUGCC	118	976-998
AD-1071230.1	UUUUACUUUUCACAGUAUGGA	119	1412-1432	UCCAUACUGUGAAAAGUAAAACA	120	1410-1432
AD-1071435.1	UGGCAUUCCUUUCUUUAAACA	121	1617-1637	UGUUUAAAGAAAGGAAUGCCACA	122	1615-1637

AD-1070553.1	GGAGAACUUCACCGAGACCGA	123	652-672	UCGGUCUCGGUGAAGUUCUCCCC	124	650-672
AD-1071894.1	AACAUCUGAAGUGUCUAAUGA	125	2169-2189	UCAUUAGACACUUCAGAUGUUAA	126	2167-2189
AD-1071652.1	ACCAGAAUUAGGUCAAGUUCA	127	1856-1876	UGAACUUGACCUAAUUCUGGUUU	128	1854-1876
AD-1072050.1	CUAUAUUUGUAACUUUGCAUA	129	2340-2360	UAUGCAAAGUUACAAAUAUAGAA	130	2338-2360
AD-1072052.1	AUAUUUGUAACUUUGCAUGUA	131	2342-2362	UACAUGCAAAGUUACAAAUAUAG	132	2340-2362
AD-1071968.1	GUCCUUAGGCUUACAAUGUGA	133	2243-2263	UCACAUUGUAAGCCUAAGGACCA	134	2241-2263
AD-1071441.1	UCCUUUCUUUAAACUAUAGGA	135	1623-1643	UCCUAUAGUUUAAAGAAAGGAAU	136	1621-1643
AD-1071168.1	AUCCUAGAGAUUCUUAGCUCA	137	1330-1350	UGAGCUAAGAAUCUCUAGGAUUU	138	1328-1350
AD-1070332.1	GUGGAACAAGCCGAGUAAGCA	139	361-381	UGCUUACUCGGCUUGUUCCACUG	140	359-381
AD-1070964.1	CUUAUUUUUGGACUUAGUGCA	141	1102-1122	UGCACUAAGUCCAAAAAUAAGUC	142	1100-1122
AD-1070974.1	GACUUAGUGCAACAGGUUGAA	143	1112-1132	UUCAACCUGUUGCACUAAGUCCA	144	1110-1132
AD-1070935.1	AACAGCAAAUAACCAUUGGUA	145	1073-1093	UACCAAUGGUUAUUUGCUGUUAU	146	1071-1093
AD-1071016.1	GUCUGAAAUACCUUUGCCUGA	147	1154-1174	UCAGGCAAAGGUAUUUCAGACUG	148	1152-1174
AD-1071163.1	GCCACAUGAUACUUAUUCAAA	149	1306-1326	UUUGAAUAAGUAUCAUGUGGCCU	150	1304-1326
AD-1071500.1	AGGCAAAUCUCCUUUGUCCAA	151	1685-1705	UUGGACAAAGGAGAUUUGCCUUC	152	1683-1705
AD-1071664.1	UCAAGUUCAUAGUUUCUGUAA	153	1868-1888	UUACAGAAACUAUGAACUUGACC	154	1866-1888
AD-1071983.1	AUGUGCACUGAAUCGUUUCAA	155	2258-2278	UUGAAACGAUUCAGUGCACAUUG	156	2256-2278
AD-1072083.1	AUCUGACUGAAAUUAAACGAA	157	2400-2420	UUCGUUUAAUUUCAGUCAGAUAU	158	2398-2420
AD-1071505.1	AAUCUCCUUUGUCCAUUUACA	159	1690-1710	UGUAAAUGGACAAAGGAGAUUUG	160	1688-1710
AD-1071916.1	UUAACUUUUGUAAGGUACUGA	161	2191-2211	UCAGUACCUUACAAAAGUUAAUG	162	2189-2211
AD-1071662.1	GGUCAAGUUCAUAGUUUCUGA	163	1866-1886	UCAGAAACUAUGAACUUGACCUA	164	1864-1886
AD-1071074.1	CUAAUGCCCUAUCUUAGUAGA	165	1212-1232	UCUACUAAGAUAGGGCAUUAGUA	166	1210-1232
AD-1070975.1	ACUUAGUGCAACAGGUUGAGA	167	1113-1133	UCUCAACCUGUUGCACUAAGUCC	168	1111-1133
AD-1072048.1	UUCUAUAUUUGUAACUUUGCA	169	2338-2358	UGCAAAGUUACAAAUAUAGAAAA	170	2336-2358
AD-1070752.1	UAAUCUUUUUCCAGCUUGAGA	171	859-879	UCUCAAGCUGGAAAAAGAUUAGA	172	857-879
AD-1071491.1	AGACACUGAAGGCAAAUCUCA	173	1676-1696	UGAGAUUUGCCUUCAGUGUCUAG	174	1674-1696
AD-1071965.1	GUGGUCCUUAGGCUUACAAUA	175	2240-2260	UAUUGUAAGCCUAAGGACCACGC	176	2238-2260
AD-1070963.1	ACUUAUUUUUGGACUUAGUGA	177	1101-1121	UCACUAAGUCCAAAAAUAAGUCC	178	1099-1121
AD-1070515.1	GCACGACUGCGUCAAUAUCAA	179	595-615	UUGAUAUUGACGCAGUCGUGCAC	180	593-615

AD-1070488.1	UGAGUACAGCAACCAGAACAA	181	568-588	UUGUUCUGGUUGCUGUACUCAUC	182	566-588
AD-1070462.1	AACCAAGUGUACUACAGGCCA	183	542-562	UGGCCUGUAGUACACUUGGUUGG	184	540-562
AD-1072085.1	CUGACUGAAAUUAAACGAGCA	185	2402-2422	UGCUCGUUUAAUUUCAGUCAGAU	186	2400-2422
AD-1071396.1	GGCAACCUCCCAUUUUAGAUA	187	1578-1598	UAUCUAAAAUGGGAGGUUGCCUC	188	1576-1598
AD-1070491.1	GUACAGCAACCAGAACAACUA	189	571-591	UAGUUGUUCUGGUUGCUGUACUC	190	569-591
AD-1072084.1	UCUGACUGAAAUUAAACGAGA	191	2401-2421	UCUCGUUUAAUUUCAGUCAGAUA	192	2399-2421
AD-1071474.1	AAAGUAAAUUGCCUUCUAGAA	193	1659-1679	UUCUAGAAGGCAAUUUACUUUUC	194	1657-1679
AD-1071093.1	GAGAUUUCAUAGCUAUUUAGA	195	1231-1251	UCUAAAUAGCUAUGAAAUCUCUA	196	1229-1251
AD-1070498.1	AACCAGAACAACUUUGUGCAA	197	578-598	UUGCACAAAGUUGUUCUGGUUGC	198	576-598
AD-1072086.1	UGACUGAAAUUAAACGAGCGA	199	2403-2423	UCGCUCGUUUAAUUUCAGUCAGA	200	2401-2423
AD-1071213.1	CAUGAGCUCUGUGUGUACCGA	201	1377-1397	UCGGUACACACAGAGCUCAUGCU	202	1375-1397
AD-1070492.1	UACAGCAACCAGAACAACUUA	203	572-592	UAAGUUGUUCUGGUUGCUGUACU	204	570-592
AD-1070753.1	AAUCUUUUUCCAGCUUGAGGA	205	860-880	UCCUCAAGCUGGAAAAAGAUUAG	206	858-880
AD-1071144.1	CUGCCAGGUUUGUUAGGAGGA	207	1287-1307	UCCUCCUAACAAACCUGGCAGAA	208	1285-1307
AD-1072089.1	CUGAAAUUAAACGAGCGAAGA	209	2406-2426	UCUUCGCUCGUUUAAUUUCAGUC	210	2404-2426
AD-1071950.1	GGGAAACCCUUUUGCGUGGUA	211	2225-2245	UACCACGCAAAAGGGUUUCCCAC	212	2223-2245
AD-1072051.1	UAUAUUUGUAACUUUGCAUGA	213	2341-2361	UCAUGCAAAGUUACAAAUAUAGA	214	2339-2361
AD-1070514.1	UGCACGACUGCGUCAAUAUCA	215	594-614	UGAUAUUGACGCAGUCGUGCACA	216	592-614
AD-1070554.1	GAGAACUUCACCGAGACCGAA	217	653-673	UUCGGUCUCGGUGAAGUUCUCCC	218	651-673
AD-1070340.1	AGCCGAGUAAGCCAAAAACCA	219	369-389	UGGUUUUUGGCUUACUCGGCUUG	220	367-389
AD-1070539.1	CAAGCAGCACACGGUCACCAA	221	619-639	UUGGUGACCGUGUGCUGCUUGAU	222	617-639
AD-1072049.1	UCUAUAUUUGUAACUUUGCAA	223	2339-2359	UUGCAAAGUUACAAAUAUAGAAA	224	2337-2359
AD-1071507.1	UCUCCUUUGUCCAUUUACCUA	225	1692-1712	UAGGUAAAUGGACAAAGGAGAUU	226	1690-1712
AD-1072047.1	UUUCUAUAUUUGUAACUUUGA	227	2337-2357	UCAAAGUUACAAAUAUAGAAAAU	228	2335-2357
AD-1070497.1	CAACCAGAACAACUUUGUGCA	229	577-597	UGCACAAAGUUGUUCUGGUUGCU	230	575-597
AD-1071669.1	UUCAUAGUUUCUGUAAUUGGA	231	1873-1893	UCCAAUUACAGAAACUAUGAACU	232	1871-1893
AD-1070461.1	CAACCAAGUGUACUACAGGCA	233	541-561	UGCCUGUAGUACACUUGGUUGGG	234	539-561
AD-1071499.1	AAGGCAAAUCUCCUUUGUCCA	235	1684-1704	UGGACAAAGGAGAUUUGCCUUCA	236	1682-1704
AD-1070578.1	AAGAUGAUGGAGCGCGUGGUA	237	677-697	UACCACGCGCUCCAUCAUCUUAA	238	675-697

AD-1070537.1	AUCAAGCAGCACACGGUCACA	239	617-637	UGUGACCGUGUGCUGCUUGAUUG	240	615-637
AD-1070538.1	UCAAGCAGCACACGGUCACCA	241	618-638	UGGUGACCGUGUGCUGCUUGAUU	242	616-638
AD-1070489.1	GAGUACAGCAACCAGAACAAA	243	569-589	UUUGUUCUGGUUGCUGUACUCAU	244	567-589
AD-1070941.1	AAAUAACCAUUGGUUAAUCUA	245	1079-1099	UAGAUUAACCAAUGGUUAUUUGC	246	1077-1099
AD-1070490.1	AGUACAGCAACCAGAACAACA	247	570-590	UGUUGUUCUGGUUGCUGUACUCA	248	568-590
AD-1070552.1	GGGAGAACUUCACCGAGACCA	249	651-671	UGGUCUCGGUGAAGUUCUCCCCC	250	649-671
AD-1071220.1	UCUGUGUGUACCGAGAACUGA	251	1384-1404	UCAGUUCUCGGUACACACAGAGC	252	1382-1404
AD-1070577.1	UAAGAUGAUGGAGCGCGUGGA	253	676-696	UCCACGCGCUCCAUCAUCUUAAC	254	674-696
AD-1070540.1	AAGCAGCACACGGUCACCACA	255	620-640	UGUGGUGACCGUGUGCUGCUUGA	256	618-640
AD-1070331.1	AGUGGAACAAGCCGAGUAAGA	257	360-380	UCUUACUCGGCUUGUUCCACUGA	258	358-380
AD-1072082.1	UAUCUGACUGAAAUUAAACGA	259	2399-2419	UCGUUUAAUUUCAGUCAGAUAUU	260	2397-2419
AD-1070536.1	AAUCAAGCAGCACACGGUCAA	261	616-636	UUGACCGUGUGCUGCUUGAUUGU	262	614-636
AD-1070512.1	UGUGCACGACUGCGUCAAUAA	263	592-612	UUAUUGACGCAGUCGUGCACAAA	264	590-612
AD-1070535.1	CAAUCAAGCAGCACACGGUCA	265	615-635	UGACCGUGUGCUGCUUGAUUGUG	266	613-635
AD-1070513.1	GUGCACGACUGCGUCAAUAUA	267	593-613	UAUAUUGACGCAGUCGUGCACAA	268	591-613
AD-1070702.1	CUCAUCUUCCUGAUAGUGGGA	269	806-826	UCCCACUAUCAGGAAGAUGAGGA	270	804-826
AD-1070576.1	UUAAGAUGAUGGAGCGCGUGA	271	675-695	UCACGCGCUCCAUCAUCUUAACG	272	673-695

Table 3. Modified Sense and Antisense Strand Sequences of PRNP dsRNA Agents

Duplex Name	Sense Sequence 5' to 3'	SEQ ID NO:	Antisense Sequence 5' to 3'	SEQ ID NO:	mRNA target sequence	SEQ ID NO:
AD-1071792.1	cscsuag(Chd)AfgAfUfAfuguauuacsusa	273	VPusAfsguaAfuAfCfauauCfuGfcuaggsusg	274	CACCUAGCAGAUAUGUAUUACUU	275
AD-1071769.1	cscscug(Ahd)AfuUfGfUfuugauauusgsa	276	VPusCfsaauAfuCfAfaacaAfuUfcagggsasa	277	UUCCCUGAAUUGUUUGAUAUUGU	278
AD-1071773.1	gsasauu(Ghd)UfuUfGfAfuauugucascsa	279	VPusGfsugaCfaAfUfaucaAfaCfaauucsasg	280	CUGAAUUGUUUGAUAUUGUCACC	281

Duplex Name	Sense Sequence 5' to 3'	SEQ ID NO:	Antisense Sequence 5' to 3'	SEQ ID NO:	mRNA target sequence	SEQ ID NO:
AD-1071718.1	gsgsaga(Uhd)GfaCfAfGfaaauaugas usa	282	VPusAfsucaUfaUfUfucugUfcAfucucc sasa	283	UUGGAGAUGACAGAAAUAUG AUU	284
AD-1071870.1	ususagg(Ahd)GfuUfUfUfguuuagags csa	285	VPusGfscucUfaAfAfcaaaAfcUfccuaas gsu	286	ACUUAGGAGUUUUGUUUAGA GCA	287
AD-1071904.1	gsusguc(Uhd)AfaUfGfCfauuaacuus usa	288	VPusAfsaagUfuAfAfugcaUfuAfgacac susu	289	AAGUGUCUAAUGCAUUAACU UUU	290
AD-1071789.1	uscsacc(Uhd)AfgCfAfGfauauguaus usa	291	VPusAfsauaCfaUfAfucugCfuAfgguga scsa	292	UGUCACCUAGCAGAUAUGUA UUA	293
AD-1071671.1	csasuag(Uhd)UfuCfUfGfuaauuggcs usa	294	VPusAfsgccAfaUfUfacagAfaAfcuaug sasa	295	UUCAUAGUUUCUGUAAUUGG CUU	296
AD-1071628.1	asusuuc(Uhd)GfuCfUfCfauaauugus csa	297	VPusGfsacaAfuUfAfugagAfcAfgaaau sasa	298	UUAUUUCUGUCUCAUAAUUG UCA	299
AD-1071865.1	csasgac(Uhd)UfaGfGfAfguuuuguus usa	300	VPusAfsaacAfaAfAfcuccUfaAfgucug susc	301	GACAGACUUAGGAGUUUUGU UUA	302
AD-1071670.1	uscsaua(Ghd)UfuUfCfUfguaauuggs csa	303	VPusGfsccaAfuUfAfcagaAfaCfuauga sasc	304	GUUCAUAGUUUCUGUAAUUG GCU	305
AD-1071901.1	gsasagu(Ghd)UfcUfAfAfugcauuaas csa	306	VPusGfsuuaAfuGfCfauuaGfaCfacuuc sasg	307	CUGAAGUGUCUAAUGCAUUA ACU	308
AD-1071837.1	csascuu(Uhd)GfuGfAfGfuauucuaus gsa	309	VPusCfsauaGfaAfUfacucAfcAfaagug scsa	310	UGCACUUUGUGAGUAUUCUA UGU	311
AD-1071929.1	gsgsuac(Uhd)GfaAfUfAfcuuaauaus gsa	312	VPusCfsauaUfuAfAfguauUfcAfguacc susu	313	AAGGUACUGAAUACUUAAUA UGU	314
AD-1071827.1	usasuug(Ghd)CfuUfGfCfacuuugugs asa	315	VPusUfscacAfaAfGfugcaAfgCfcaaua sasu	316	AUUAUUGGCUUGCACUUUGU GAG	317
AD-1071923.1	ususgua(Ahd)GfgUfAfCfugaauacus usa	318	VPusAfsaguAfuUfCfaguaCfcUfuacaa sasa	319	UUUUGUAAGGUACUGAAUAC UUA	320
AD-1071834.1	ususgca(Chd)UfuUfGfUfgaguauucs usa	321	VPusAfsgaaUfaCfUfcacaAfaGfugcaas gsc	322	GCUUGCACUUUGUGAGUAUU CUA	323
AD-1071984.1	usgsugc(Ahd)CfuGfAfAfucguuucas usa	324	VPusAfsugaAfaCfGfauucAfgUfgcaca susu	325	AAUGUGCACUGAAUCGUUUC AUG	326
AD-1071629.1	ususucu(Ghd)UfcUfCfAfuaauugucs asa	327	VPusUfsgacAfaUfUfaugaGfaCfagaaa susa	328	UAUUUCUGUCUCAUAAUUGU CAA	329
AD-	gsusuca(Uhd)AfgUfUfUfcuguaauus	330	VPusCfsaauUfaCfAfgaaaCfuAfugaacs	331	AAGUUCAUAGUUUCUGUAAU	332

Duplex Name	Sense Sequence 5' to 3'	SEQ ID NO:	Antisense Sequence 5' to 3'	SEQ ID NO:	mRNA target sequence	SEQ ID NO:
1071668.1	gsa		usu		UGG	
AD-1070449.1	csgsuua(Chd)UfaUfCfGfugaaaacasusa	333	VPusAfsuguUfuUfCfacgaUfaGfuaacgsgsu	334	ACCGUUACUAUCGUGAAAACAUG	335
AD-1071836.1	gscsacu(Uhd)UfgUfGfAfguauucuasusa	336	VPusAfsuagAfaUfAfcucaCfaAfagugcsasa	337	UUGCACUUUGUGAGUAUUCUAUG	338
AD-1071897.1	asuscug(Ahd)AfgUfGfUfcuaaugcasusa	339	VPusAfsugcAfuUfAfgacaCfuUfcagausgsu	340	ACAUCUGAAGUGUCUAAUGCAUU	341
AD-1071864.1	ascsaga(Chd)UfuAfGfGfaguuuugususa	342	VPusAfsacaAfaAfCfuccuAfaGfucugucscsc	343	GGACAGACUUAGGAGUUUUGUUU	344
AD-1071927.1	asasggu(Ahd)CfuGfAfAfuacuuaausasa	345	VPusUfsauuAfaGfUfauucAfgUfaccuusasc	346	GUAAGGUACUGAAUACUUAAUAU	347
AD-1070444.1	asgsgac(Chd)GfuUfAfCfuaucgugasasa	348	VPusUfsucaCfgAfUfaguaAfcGfguccuscsa	349	UGAGGACCGUUACUAUCGUGAAA	350
AD-1071981.1	csasaug(Uhd)GfcAfCfUfgaaucguususa	351	VPusAfsaacGfaUfUfcaguGfcAfcauugsusa	352	UACAAUGUGCACUGAAUCGUUUC	353
AD-1071509.1	uscscuu(Uhd)GfuCfCfAfuuuaccugsgsa	354	VPusCfscagGfuAfAfauggAfcAfaaggasgsa	355	UCUCCUUUGUCCAUUUACCUGGA	356
AD-1071902.1	asasgug(Uhd)CfuAfAfUfgcauuaacsusa	357	VPusAfsguuAfaUfGfcauuAfgAfcacuuscsa	358	UGAAGUGUCUAAUGCAUUAACUU	359
AD-1071084.1	asuscuu(Ahd)GfuAfGfAfgauuucausasa	360	VPusUfsaugAfaAfUfcucuAfcUfaagausasg	361	CUAUCUUAGUAGAGAUUUCAUAG	362
AD-1071948.1	gsusggg(Ahd)AfaCfCfCfuuuugcgusgsa	363	VPusCfsacgCfaAfAfagggUfuUfcccacsasu	364	AUGUGGGAAACCCUUUUGCGUGG	365
AD-1071437.1	gscsauu(Chd)CfuUfUfCfuuuaaacusasa	366	VPusUfsaguUfuAfAfagaaAfgGfaaugcscsa	367	UGGCAUUCCUUUCUUUAAACUAU	368
AD-1071508.1	csusccu(Uhd)UfgUfCfCfauuuaccusgsa	369	VPusCfsaggUfaAfAfuggaCfaAfaggagsasu	370	AUCUCCUUUGUCCAUUUACCUGG	371
AD-1071265.1	ususcaa(Chd)AfaGfAfGfuaaauauusgsa	372	VPusCfsaauAfuUfUfacucUfuGfuugaascsa	373	UGUUCAACAAGAGUAAAUAUUGU	374
AD-1070450.1	gsusuac(Uhd)AfuCfGfUfgaaaacausgsa	375	VPusCfsaugUfuUfUfcacgAfuAfguaacsgsg	376	CCGUUACUAUCGUGAAAACAUGC	377
AD-1070522.1	usgscgu(Chd)AfaUfAfUfcacaaucasasa	378	VPusUfsugaUfuGfUfgauaUfuGfacgcasgsu	379	ACUGCGUCAAUAUCACAAUCAAG	380

Duplex Name	Sense Sequence 5' to 3'	SEQ ID NO:	Antisense Sequence 5' to 3'	SEQ ID NO:	mRNA target sequence	SEQ ID NO:
AD-1071969.1	uscscuu(Ahd)GfgCfUfUfacaaugugscsa	381	VPusGfscacAfuUfGfuaagCfcUfaaggascsc	382	GGUCCUUAGGCUUACAAUGUGCA	383
AD-1071352.1	csusucu(Ghd)GfgAfCfUfugaaaucasasa	384	VPusUfsugaUfuUfCfaaguCfcCfagaagscsc	385	GGCUUCUGGGACUUGAAAUCAAA	386
AD-1071912.1	usgscau(Uhd)AfaCfUfUfuguaaggsusa	387	VPusAfsccuUfaCfAfaaagUfuAfaugcasusu	388	AAUGCAUUAACUUUUGUAAGGUA	389
AD-1071885.1	asgsagc(Ahd)GfuUfAfAfcaucugaasgsa	390	VPusCfsuucAfgAfUfguuaAfcUfgcucusasa	391	UUAGAGCAGUUAACAUCUGAAGU	392
AD-1071949.1	usgsgga(Ahd)AfcCfCfUfuuugcgugsgsa	393	VPusCfscacGfcAfAfaaggGfuUfucccascsa	394	UGUGGGAAACCCUUUUGCGUGGU	395
AD-1071085.1	uscsuua(Ghd)UfaGfAfGfauuucauasgsa	396	VPusCfsuauGfaAfAfucucUfaCfuaagasusa	397	UAUCUUAGUAGAGAUUUCAUAGC	398
AD-1070516.1	csascga(Chd)UfgCfGfUfcaauaucascsa	399	VPusGfsugaUfaUfUfgacgCfaGfucgugscsa	400	UGCACGACUGCGUCAAUAUCACA	401
AD-1071895.1	ascsauc(Uhd)GfaAfGfUfgucuaaugscsa	402	VPusGfscauUfaGfAfcacuUfcAfgaugususa	403	UAACAUCUGAAGUGUCUAAUGCA	404
AD-1071430.1	asusaug(Uhd)GfgCfAfUfuccuuucususa	405	VPusAfsagaAfaGfGfaaugCfcAfcauausasg	406	CUAUAUGUGGCAUUCCUUUCUUU	407
AD-1070990.1	ususgag(Ghd)CfuAfAfAfacaaaucuscsa	408	VPusGfsagaUfuUfGfuuuuAfgCfcucaascsc	409	GGUUGAGGCUAAAACAAAUCUCA	410
AD-1071506.1	asuscuc(Chd)UfuUfGfUfccauuuacscsa	411	VPusGfsguaAfaUfGfgacaAfaGfgagaususu	412	AAAUCUCCUUUGUCCAUUUACCU	413
AD-1071985.1	gsusgca(Chd)UfgAfAfUfcguuucausgsa	414	VPusCfsaugAfaAfCfgauuCfaGfugcacsasu	415	AUGUGCACUGAAUCGUUUCAUGU	416
AD-1070511.1	ususgug(Chd)AfcGfAfCfugcgucaasusa	417	VPusAfsuugAfcGfCfagucGfuGfcacaasasg	418	CUUUGUGCACGACUGCGUCAAUA	419
AD-1071301.1	csusggc(Uhd)AfgAfGfGfacauauucsasa	420	VPusUfsgaaUfaUfGfuccuCfuAfgccagsasg	421	CUCUGGCUAGAGGACAUAUUCAC	422
AD-1071835.1	usgscac(Uhd)UfuGfUfGfaguauucusasa	423	VPusUfsagaAfuAfCfucacAfaAfgugcasasg	424	CUUGCACUUUGUGAGUAUUCUAU	425
AD-1072054.1	asusuug(Uhd)AfaCfUfUfugcauguuscsa	426	VPusGfsaacAfuGfCfaaagUfuAfcaaausasu	427	AUAUUUGUAACUUUGCAUGUUCU	428
AD-	csascug(Ghd)AfaAfAfCfauagaguas	429	VPusCfsuacUfcUfAfuguuUfuCfcagugsusu	430	GGCACUGGAAAACAUAGAGU	431

Duplex Name	Sense Sequence 5' to 3'	SEQ ID NO:	Antisense Sequence 5' to 3'	SEQ ID NO:	mRNA target sequence	SEQ ID NO:
1070861.1	gsa		scsc		AGA	
AD-1071230.1	ususuua(Chd)UfuUfUfCfacaguaugsgsa	432	VPusCfscauAfcUfGfugaaAfaGfuaaaascsa	433	UGUUUUACUUUUCACAGUAUGGG	434
AD-1071435.1	usgsgca(Uhd)UfcCfUfUfucuuuaaascsa	435	VPusGfsuuuAfaAfGfaaagGfaAfugccascsa	436	UGUGGCAUUCCUUUCUUUAAACU	437
AD-1070553.1	gsgsaga(Ahd)CfuUfCfAfccgagaccsgsa	438	VPusCfsgguCfuCfGfgugaAfgUfucuccscsc	439	GGGGAGAACUUCACCGAGACCGA	440
AD-1071894.1	asascau(Chd)UfgAfAfGfugucuaausgsa	441	VPusCfsauuAfgAfCfacuuCfaGfauguusasa	442	UUAACAUCUGAAGUGUCUAAUGC	443
AD-1071652.1	ascscag(Ahd)AfuUfAfGfgucaaguuscsa	444	VPusGfsaacUfuGfAfccuaAfuUfcuggususu	445	AAACCAGAAUUAGGUCAAGUUCA	446
AD-1072050.1	csusaua(Uhd)UfuGfUfAfacuuugcasusa	447	VPusAfsugcAfaAfGfuuacAfaAfuauagsasa	448	UUCUAUAUUUGUAACUUUGCAUG	449
AD-1072052.1	asusauu(Uhd)GfuAfAfCfuuugcaugsusa	450	VPusAfscauGfcAfAfaguuAfcAfaauausasg	451	CUAUAUUUGUAACUUUGCAUGUU	452
AD-1071968.1	gsusccu(Uhd)AfgGfCfUfuacaaugusgsa	453	VPusCfsacaUfuGfUfaagcCfuAfaggacscsa	454	UGGUCCUUAGGCUUACAAUGUGC	455
AD-1071441.1	uscscuu(Uhd)CfuUfUfAfaacuauagsgsa	456	VPusCfscuaUfaGfUfuuaaAfgAfaaggasasu	457	AUUCCUUUCUUUAAACUAUAGGU	458
AD-1071168.1	asusccu(Ahd)GfaGfAfUfucuuagcuscsa	459	VPusGfsagcUfaAfGfaaucUfcUfaggaususu	460	AAAUCCUAGAGAUUCUUAGCUCU	461
AD-1070332.1	gsusgga(Ahd)CfaAfGfCfcgaguaagscsa	462	VPusGfscuuAfcUfCfggcuUfgUfuccacsusg	463	CAGUGGAACAAGCCGAGUAAGCC	464
AD-1070964.1	csusuau(Uhd)UfuUfGfGfacuuagugscsa	465	VPusGfscacUfaAfGfuccaAfaAfauaagsusc	466	GACUUAUUUUUGGACUUAGUGCA	467
AD-1070974.1	gsascuu(Ahd)GfuGfCfAfacagguugsasa	468	VPusUfscaaCfcUfGfuugcAfcUfaagucscsa	469	UGGACUUAGUGCAACAGGUUGAG	470
AD-1070935.1	asascag(Chd)AfaAfUfAfaccauuggsusa	471	VPusAfsccaAfuGfGfuuauUfuGfcuguusasu	472	AUAACAGCAAAUAACCAUUGGUU	473
AD-1071016.1	gsuscug(Ahd)AfaUfAfCfcuuugccusgsa	474	VPusCfsaggCfaAfAfgguaUfuUfcagacsusg	475	CAGUCUGAAAUACCUUUGCCUGG	476
AD-1071163.1	gscscac(Ahd)UfgAfUfAfcuuauucasasa	477	VPusUfsugaAfuAfAfguauCfaUfguggcscsu	478	AGGCCACAUGAUACUUAUUCAAA	479

Duplex Name	Sense Sequence 5' to 3'	SEQ ID NO:	Antisense Sequence 5' to 3'	SEQ ID NO:	mRNA target sequence	SEQ ID NO:
AD-1071500.1	asgsgca(Ahd)AfuCfUfCfcuuuguccsasa	480	VPusUfsggaCfaAfAfggagAfuUfugccususc	481	GAAGGCAAAUCUCCUUUGUCCAU	482
AD-1071664.1	uscsaag(Uhd)UfcAfUfAfguuucugusasa	483	VPusUfsacaGfaAfAfcuauGfaAfcuugascsc	484	GGUCAAGUUCAUAGUUUCUGUAA	485
AD-1071983.1	asusgug(Chd)AfcUfGfAfaucguuucsasa	486	VPusUfsgaaAfcGfAfuucaGfuGfcacaususg	487	CAAUGUGCACUGAAUCGUUUCAU	488
AD-1072083.1	asuscug(Ahd)CfuGfAfAfauuaaacgsasa	489	VPusUfscguUfuAfAfuuucAfgUfcagausasu	490	AUAUCUGACUGAAAUUAAACGAG	491
AD-1071505.1	asasucu(Chd)CfuUfUfGfuccauuuascsa	492	VPusGfsuaaAfuGfGfacaaAfgGfagauususg	493	CAAAUCUCCUUUGUCCAUUUACC	494
AD-1071916.1	ususaac(Uhd)UfuUfGfUfaagguacusgsa	495	VPusCfsaguAfcCfUfuacaAfaAfguuaasusg	496	CAUUAACUUUUGUAAGGUACUGA	497
AD-1071662.1	gsgsuca(Ahd)GfuUfCfAfuaguuucusgsa	498	VPusCfsagaAfaCfUfaugaAfcUfugaccsusa	499	UAGGUCAAGUUCAUAGUUUCUGU	500
AD-1071074.1	csusaau(Ghd)CfcCfUfAfucuuaguasgsa	501	VPusCfsuacUfaAfGfauagGfgCfauuagsusa	502	UACUAAUGCCCUAUCUUAGUAGA	503
AD-1070975.1	ascsuua(Ghd)UfgCfAfAfcagguugasgsa	504	VPusCfsucaAfcCfUfguugCfaCfuaaguscsc	505	GGACUUAGUGCAACAGGUUGAGG	506
AD-1072048.1	ususcua(Uhd)AfuUfUfGfuaacuuugscsa	507	VPusGfscaaAfgUfUfacaaAfuAfuagaasasa	508	UUUUCUAUAUUUGUAACUUUGCA	509
AD-1070752.1	usasauc(Uhd)UfuUfUfCfcagcuugasgsa	510	VPusCfsucaAfgCfUfggaaAfaAfgauuasgsa	511	UCUAAUCUUUUUCCAGCUUGAGG	512
AD-1071491.1	asgsaca(Chd)UfgAfAfGfgcaaaucuscsa	513	VPusGfsagaUfuUfGfccuuCfaGfugucusasg	514	CUAGACACUGAAGGCAAAUCUCC	515
AD-1071965.1	gsusggu(Chd)CfuUfAfGfgcuuacaasusa	516	VPusAfsuugUfaAfGfccuaAfgGfaccacsgsc	517	GCGUGGUCCUUAGGCUUACAAUG	518
AD-1070963.1	ascsuua(Uhd)UfuUfUfGfgacuuagusgsa	519	VPusCfsacuAfaGfUfccaaAfaAfuaaguscsc	520	GGACUUAUUUUUGGACUUAGUGC	521
AD-1070515.1	gscsacg(Ahd)CfuGfCfGfucaauaucsasa	522	VPusUfsgauAfuUfGfacgcAfgUfcgugcsasc	523	GUGCACGACUGCGUCAAUAUCAC	524
AD-1070488.1	usgsagu(Ahd)CfaGfCfAfaccagaacsasa	525	VPusUfsguuCfuGfGfuugcUfgUfacucasusc	526	GAUGAGUACAGCAACCAGAACAA	527
AD-	asascca(Ahd)GfuGfUfAfcuacaggcsc	528	VPusGfsgccUfgUfAfguacAfcUfugguu	529	CCAACCAAGUGUACUACAGG	530

Duplex Name	Sense Sequence 5' to 3'	SEQ ID NO:	Antisense Sequence 5' to 3'	SEQ ID NO:	mRNA target sequence	SEQ ID NO:
1070462.1	sa		sgsg		CCC	
AD-1072085.1	csusgac(Uhd)GfaAfAfUfuaaacgagscsa	531	VPusGfscucGfuUfUfaauuUfcAfgucagsasu	532	AUCUGACUGAAAUUAAACGAGCG	533
AD-1071396.1	gsgscaa(Chd)CfuCfCfCfauuuuagasusa	534	VPusAfsucuAfaAfAfugggAfgGfuugccsusc	535	GAGGCAACCUCCCAUUUUAGAUG	536
AD-1070491.1	gsusaca(Ghd)CfaAfCfCfagaacaacsusa	537	VPusAfsguuGfuUfCfugguUfgCfuguacsusc	538	GAGUACAGCAACCAGAACAACUU	539
AD-1072084.1	uscsuga(Chd)UfgAfAfAfuuaaacgasgsa	540	VPusCfsucgUfuUfAfauuuCfaGfucagasusa	541	UAUCUGACUGAAAUUAAACGAGC	542
AD-1071474.1	asasagu(Ahd)AfaUfUfGfccuucuagsasa	543	VPusUfscuaGfaAfGfgcaaUfuUfacuuususc	544	GAAAAGUAAAUUGCCUUCUAGAC	545
AD-1071093.1	gsasgau(Uhd)UfcAfUfAfgcuauuuasgsa	546	VPusCfsuaaAfuAfGfcuauGfaAfaucucsusa	547	UAGAGAUUUCAUAGCUAUUUAGA	548
AD-1070498.1	asascca(Ghd)AfaCfAfAfcuuugugcsasa	549	VPusUfsgcaCfaAfAfguugUfuCfugguusgsc	550	GCAACCAGAACAACUUUGUGCAC	551
AD-1072086.1	usgsacu(Ghd)AfaAfUfUfaaacgagcsgsa	552	VPusCfsgcuCfgUfUfuaauUfuCfagucasgsa	553	UCUGACUGAAAUUAAACGAGCGA	554
AD-1071213.1	csasuga(Ghd)CfuCfUfGfuguguaccsgsa	555	VPusCfsgguAfcAfCfacagAfgCfucaugscsu	556	AGCAUGAGCUCUGUGUGUACCGA	557
AD-1070492.1	usascag(Chd)AfaCfCfAfgaacaacususa	558	VPusAfsaguUfgUfUfcuggUfuGfcuguascsu	559	AGUACAGCAACCAGAACAACUUU	560
AD-1070753.1	asasucu(Uhd)UfuUfCfCfagcuugagsgsa	561	VPusCfscucAfaGfCfuggaAfaAfagauusasg	562	CUAAUCUUUUUCCAGCUUGAGGG	563
AD-1071144.1	csusgcc(Ahd)GfgUfUfUfguuaggagsgsa	564	VPusCfscucCfuAfAfcaaaCfcUfggcagsasa	565	UUCUGCCAGGUUUGUUAGGAGGC	566
AD-1072089.1	csusgaa(Ahd)UfuAfAfAfcgagcgaasgsa	567	VPusCfsuucGfcUfCfguuuAfaUfuucagsusc	568	GACUGAAAUUAAACGAGCGAAGA	569
AD-1071950.1	gsgsgaa(Ahd)CfcCfUfUfuugcguggsusa	570	VPusAfsccaCfgCfAfaaagGfgUfuucccsasc	571	GUGGGAAACCCUUUUGCGUGGUC	572
AD-1072051.1	usasuau(Uhd)UfgUfAfAfcuuugcausgsa	573	VPusCfsaugCfaAfAfguuaCfaAfauauasgsa	574	UCUAUAUUUGUAACUUUGCAUGU	575
AD-1070514.1	usgscac(Ghd)AfcUfGfCfgucaauauscsa	576	VPusGfsauaUfuGfAfcgcaGfuCfgugcascsa	577	UGUGCACGACUGCGUCAAUAUCA	578

Duplex Name	Sense Sequence 5' to 3'	SEQ ID NO:	Antisense Sequence 5' to 3'	SEQ ID NO:	mRNA target sequence	SEQ ID NO:
AD-1070554.1	gsasgaa(Chd)UfuCfAfCfcgagaccgsasa	579	VPusUfscggUfcUfCfggugAfaGfuucucscsc	580	GGGAGAACUUCACCGAGACCGAC	581
AD-1070340.1	asgsccg(Ahd)GfuAfAfGfccaaaaacscsa	582	VPusGfsguuUfuUfGfgcuuAfcUfcggcususg	583	CAAGCCGAGUAAGCCAAAAACCA	584
AD-1070539.1	csasagc(Ahd)GfcAfCfAfcggucaccsasa	585	VPusUfsgguGfaCfCfguguGfcUfgcuugsasu	586	AUCAAGCAGCACACGGUCACCAC	587
AD-1072049.1	uscsuau(Ahd)UfuUfGfUfuaacuuugcsasa	588	VPusUfsgcaAfaGfUfuacaAfaUfauagasasa	589	UUUCUAUAUUUGUAACUUUGCAU	590
AD-1071507.1	uscsucc(Uhd)UfuGfUfCfcauuuaccsusa	591	VPusAfsgguAfaAfUfggacAfaAfggagasusu	592	AAUCUCCUUUGUCCAUUUACCUG	593
AD-1072047.1	ususucu(Ahd)UfaUfUfUfUfguaacuuusgsa	594	VPusCfsaaaGfuUfAfcaaaUfaUfagaaasasu	595	AUUUUCUAUAUUUGUAACUUUGC	596
AD-1070497.1	csasacc(Ahd)GfaAfCfAfacuuugugscsa	597	VPusGfscacAfaAfGfuuguUfcUfgguugscsu	598	AGCAACCAGAACAACUUUGUGCA	599
AD-1071669.1	ususcau(Ahd)GfuUfUfCfuguaauugsgsa	600	VPusCfscaaUfuAfCfagaaAfcUfaugaascsu	601	AGUUCAUAGUUUCUGUAAUUGGC	602
AD-1070461.1	csasacc(Ahd)AfgUfGfUfacuacaggscsa	603	VPusGfsccuGfuAfGfuacaCfuUfgguugsgsg	604	CCCAACCAAGUGUACUACAGGCC	605
AD-1071499.1	asasggc(Ahd)AfaUfCfUfccuuugucscsa	606	VPusGfsgacAfaAfGfgagaUfuUfgccuuscsa	607	UGAAGGCAAAUCUCCUUUGUCCA	608
AD-1070578.1	asasgau(Ghd)AfuGfGfAfgcgcguggssasa	609	VPusAfsccaCfgCfGfcuccAfuCfaucuusasa	610	UUAAGAUGAUGGAGCGCGUGGUU	611
AD-1070537.1	asuscaa(Ghd)CfaGfCfAfcacggucascsusg	612	VPusGfsugaCfcGfUfgugcUfgCfuugaususg	613	CAAUCAAGCAGCACACGGUCACC	614
AD-1070538.1	uscsaag(Chd)AfgCfAfCfacggucacscsusu	615	VPusGfsgugAfcCfGfugugCfuGfcuugasusu	616	AAUCAAGCAGCACACGGUCACCA	617
AD-1070489.1	gsasgua(Chd)AfgCfAfAfccagaacasasasu	618	VPusUfsuguUfcUfGfguugCfuGfuacucsasu	619	AUGAGUACAGCAACCAGAACAAC	620
AD-1070941.1	asasaua(Ahd)CfcAfUfUfgguuaaucsusa	621	VPusAfsgauUfaAfCfcaauGfgUfuauuusgsc	622	GCAAAUAACCAUUGGUUAAUCUG	623
AD-1070490.1	asgsuac(Ahd)GfcAfAfCfcagaacaaascsa	624	VPusGfsuugUfuCfUfgguuGfcUfguacuscsa	625	UGAGUACAGCAACCAGAACAACU	626
AD-	gsgsgag(Ahd)AfcUfUfCfaccgagacsc	627	VPusGfsgucUfcGfGfugaaGfuUfcuccc	628	GGGGGAGAACUUCACCGAGA	629

Duplex Name	Sense Sequence 5' to 3'	SEQ ID NO:	Antisense Sequence 5' to 3'	SEQ ID NO:	mRNA target sequence	SEQ ID NO:
1070552.1	sa		scsc		CCG	
AD-1071220.1	uscsugu(Ghd)UfgUfAfCfcgagaacus gsa	630	VPusCfsaguUfcUfCfgguaCfaCfacagas gsc	631	GCUCUGUGUGUACCGAGAAC UGG	632
AD-1070577.1	usasaga(Uhd)GfaUfGfGfagcgcgugs gsa	633	VPusCfscacGfcGfCfuccaUfcAfucuuas asc	634	GUUAAGAUGAUGGAGCGCGU GGU	635
AD-1070540.1	asasgca(Ghd)CfaCfAfCfggucaccasc sa	636	VPusGfsuggUfgAfCfcgugUfgCfugcu usgsa	637	UCAAGCAGCACACGGUCACCA CA	638
AD-1070331.1	asgsugg(Ahd)AfcAfAfGfccgaguaas gsa	639	VPusCfsuuaCfuCfGfgcuuGfuUfccacu sgsa	640	UCAGUGGAACAAGCCGAGUA AGC	641
AD-1072082.1	usasucu(Ghd)AfcUfGfAfaauuaaacs gsa	642	VPusCfsguuUfaAfUfuucaGfuCfagaua susu	643	AAUAUCUGACUGAAAUUAAA CGA	644
AD-1070536.1	asasuca(Ahd)GfcAfGfCfacacggucsa sa	645	VPusUfsgacCfgUfGfugcuGfcUfugauu sgsu	646	ACAAUCAAGCAGCACACGGU CAC	647
AD-1070512.1	usgsugc(Ahd)CfgAfCfUfgcgucaaus asa	648	VPusUfsauuGfaCfCfGfcaguCfgUfgcaca sasa	649	UUUGUGCACGACUGCGUCAA UAU	650
AD-1070535.1	csasauc(Ahd)AfgCfAfGfcacacggusc sa	651	VPusGfsaccGfuGfUfgcugCfuUfgauug susg	652	CACAAUCAAGCAGCACACGG UCA	653
AD-1070513.1	gsusgca(Chd)GfaCfUfGfcgucaauasu sa	654	VPusAfsuauUfgAfCfgcagUfcGfugcac sasa	655	UUGUGCACGACUGCGUCAAU AUC	656
AD-1070702.1	csuscau(Chd)UfuCfCfUfgauaguggs gsa	657	VPusCfsccaCfuAfUfcaggAfaGfaugag sgsa	658	UCCUCAUCUUCCUGAUAGUG GGA	659
AD-1070576.1	ususaag(Ahd)UfgAfUfGfgagcgcgus gsa	660	VPusCfsacgCfgCfUfccauCfaUfcuuaas csg	661	CGUUAAGAUGAUGGAGCGCG UGG	662

Table 4. ***In Vitro*** **Screen in BE2C Cells**

Duplex Name	50 nM		10 nM		1 nM		0.1 nM	
	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD
AD-1071792.1	17.47	8.28	16.15	10.06	10.06	3.24	37.48	19.74
AD-1071769.1	11.27	2.39	11.93	2.20	11.22	4.10	21.08	3.34
AD-1071773.1	22.46	15.85	10.22	2.73	12.20	4.36	21.58	5.45
AD-1071718.1	15.03	9.00	17.61	12.55	12.47	4.35	33.70	3.59
AD-1071870.1	11.69	5.41	16.47	2.36	13.28	2.33	40.70	6.94
AD-1071904.1	9.51	3.39	14.86	3.76	13.71	1.39	34.61	15.59
AD-1071789.1	17.18	8.61	14.47	4.92	13.82	1.75	30.99	20.42
AD-1071671.1	14.05	3.51	16.77	4.90	14.80	2.99	41.95	5.57
AD-1071628.1	16.00	3.78	17.77	7.31	15.97	5.42	43.15	13.81
AD-1071865.1	10.00	2.37	21.96	10.32	16.27	2.91	40.54	7.10
AD-1071670.1	9.11	0.89	11.69	1.14	16.77	5.40	34.60	1.81
AD-1071901.1	12.51	3.05	14.84	2.61	16.78	1.94	37.54	2.01
AD-1071837.1	9.12	1.88	16.85	2.48	16.94	4.38	44.41	6.44
AD-1071929.1	9.32	2.11	17.08	4.51	17.05	1.29	34.83	7.02
AD-1071827.1	11.05	6.16	17.80	10.37	17.23	0.49	37.22	11.67
AD-1071923.1	17.18	6.06	15.54	1.53	18.49	5.79	32.98	4.96
AD-1071834.1	12.28	5.19	18.10	1.38	18.51	3.90	38.85	5.39
AD-1071984.1	9.00	3.95	12.34	1.56	18.82	6.52	49.61	33.26

Duplex Name	50 nM		10 nM		1 nM		0.1 nM	
	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD
AD-1071629.1	18.88	8.46	18.61	13.02	18.85	5.56	31.10	2.15
AD-1071668.1	43.39	24.72	25.49	17.25	19.25	9.19	132.66	48.78
AD-1070449.1	8.63	1.56	12.57	2.05	19.29	2.33	36.71	16.16
AD-1071836.1	10.40	4.50	15.13	2.16	19.65	3.55	56.15	8.86
AD-1071897.1	19.77	12.34	16.13	0.93	20.08	3.99	43.47	1.36
AD-1071864.1	13.56	4.01	30.25	10.94	20.31	4.09	60.81	10.43
AD-1071927.1	14.34	6.84	14.95	2.74	20.46	3.96	36.32	6.99
AD-1070444.1	11.11	3.88	11.80	1.80	20.51	4.24	36.92	5.85
AD-1071981.1	17.27	9.46	19.34	8.52	20.51	4.88	104.28	14.93
AD-1071509.1	9.48	1.67	15.96	7.29	20.52	3.78	24.08	7.09
AD-1071902.1	10.79	2.51	17.11	2.36	20.56	1.95	41.58	5.37
AD-1071084.1	17.34	1.45	15.60	1.22	20.70	4.43	36.63	1.77
AD-1071948.1	11.47	5.92	17.10	4.37	21.47	4.72	46.75	13.97
AD-1071437.1	16.29	5.45	14.94	1.13	21.62	1.35	29.34	1.20
AD-1071508.1	21.65	14.42	19.80	7.17	22.14	4.13	66.88	38.71
AD-1071265.1	15.44	2.13	15.53	1.45	22.15	1.41	35.37	3.69
AD-1070450.1	12.22	4.33	12.87	1.53	22.52	4.85	57.46	34.12
AD-1070522.1	30.78	25.39	20.05	11.00	23.00	4.53	43.74	22.48
AD-	22.44	10.98	13.73	2.03	23.45	3.44	40.87	4.51

Duplex Name	50 nM		10 nM		1 nM		0.1 nM	
	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD
1071969.1								
AD-1071352.1	16.53	1.53	20.86	11.93	23.57	2.72	43.35	23.87
AD-1071912.1	16.84	6.88	14.11	5.90	23.63	3.42	29.95	19.98
AD-1071885.1	14.16	1.81	17.43	7.96	24.11	4.52	40.04	11.20
AD-1071949.1	14.12	11.28	15.16	2.90	24.38	10.38	41.91	17.02
AD-1071085.1	17.83	1.85	16.38	2.14	24.69	1.12	35.46	3.03
AD-1070516.1	16.80	5.01	14.12	2.37	24.69	2.63	37.01	4.77
AD-1071895.1	16.62	6.56	17.65	3.00	25.00	2.95	46.41	7.80
AD-1071430.1	14.71	8.00	15.75	11.23	25.25	9.56	34.47	12.14
AD-1070990.1	20.86	2.43	22.25	12.33	25.25	2.78	38.04	6.47
AD-1071506.1	22.00	8.69	18.76	6.18	25.37	5.22	62.07	21.73
AD-1071985.1	15.99	7.42	13.43	9.06	25.49	7.38	20.34	2.40
AD-1070511.1	17.85	4.67	16.92	3.17	26.08	5.59	46.62	17.19
AD-1071301.1	21.07	2.50	17.79	0.54	26.11	2.97	37.92	5.47
AD-1071835.1	16.79	5.65	16.93	1.83	26.14	6.68	46.38	5.31
AD-1072054.1	17.36	6.48	9.72	2.10	26.28	8.39	14.55	2.08
AD-1070861.1	18.15	6.69	25.62	10.65	26.68	6.60	37.64	4.79
AD-1071230.1	17.46	1.82	18.05	0.85	26.70	2.83	45.85	3.14
AD-1071435.	20.04	8.16	21.28	9.02	27.04	11.48	45.74	15.23

Duplex Name	50 nM		10 nM		1 nM		0.1 nM	
	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD
1								
AD-1070553.1	34.84	26.85	26.45	15.99	27.09	5.03	43.38	6.85
AD-1071894.1	15.96	3.91	23.71	8.49	27.17	2.63	41.76	0.99
AD-1071652.1	36.23	17.71	25.61	16.27	27.53	10.93	49.67	4.80
AD-1072050.1	19.12	6.28	21.30	10.37	28.19	6.62	137.16	77.57
AD-1072052.1	14.58	0.52	15.80	1.98	28.97	7.89	73.13	41.68
AD-1071968.1	15.41	2.70	16.54	3.35	29.13	0.87	48.53	17.78
AD-1071441.1	16.47	2.01	16.45	1.96	29.53	4.89	45.17	7.41
AD-1071168.1	18.27	9.81	23.36	13.98	29.63	6.27	46.19	7.80
AD-1070332.1	14.29	4.28	17.99	2.02	30.06	8.58	50.79	9.18
AD-1070964.1	20.85	2.82	19.99	2.13	30.50	2.58	40.97	0.46
AD-1070974.1	22.87	4.98	15.73	1.56	30.65	2.38	55.25	14.22
AD-1070935.1	23.79	10.91	26.11	15.47	30.85	6.09	47.77	6.33
AD-1071016.1	24.86	16.45	21.15	9.75	31.49	11.08	53.30	15.23
AD-1071163.1	21.69	11.58	23.86	16.73	31.64	16.18	45.37	21.95
AD-1071500.1	42.34	6.25	20.52	2.55	31.68	4.69	53.90	1.99
AD-1071664.1	54.16	24.09	30.13	17.28	31.75	14.85	112.13	41.96
AD-1071983.1	13.13	4.38	17.97	7.85	32.01	17.93	81.02	42.25
AD-1072083.1	16.88	1.96	17.22	6.51	32.19	4.36	18.37	1.43

Duplex Name	50 nM		10 nM		1 nM		0.1 nM	
	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD
AD-1071505.1	18.23	10.45	13.06	3.36	32.52	18.96	30.63	5.89
AD-1071916.1	22.27	14.90	19.60	8.12	33.61	5.74	35.64	4.65
AD-1071662.1	26.98	13.00	19.00	12.89	33.81	19.58	83.91	24.35
AD-1071074.1	30.21	17.95	26.37	14.41	34.03	4.54	59.20	10.49
AD-1070975.1	26.19	2.80	18.36	0.64	34.12	1.10	52.79	2.12
AD-1072048.1	18.07	2.52	21.39	9.96	34.22	3.37	35.28	0.71
AD-1070752.1	29.62	1.13	23.45	1.97	34.30	2.62	61.82	7.91
AD-1071491.1	51.03	4.63	28.84	6.29	34.55	9.06	62.54	1.56
AD-1071965.1	23.00	10.09	32.99	11.56	34.63	9.57	48.15	3.44
AD-1070963.1	25.65	3.23	19.48	3.70	34.94	3.30	52.08	4.23
AD-1070515.1	13.47	1.43	14.96	1.83	35.20	19.23	44.92	5.34
AD-1070488.1	28.54	6.88	19.56	2.24	36.01	7.20	48.85	7.05
AD-1070462.1	19.16	5.46	24.26	5.58	36.65	12.00	42.39	6.83
AD-1072085.1	22.24	10.46	21.57	12.75	37.04	4.96	26.52	9.44
AD-1071396.1	21.70	3.55	31.26	22.06	37.55	12.62	53.79	3.90
AD-1070491.1	20.67	10.15	26.75	15.47	37.82	2.32	56.74	8.77
AD-1072084.1	19.72	2.52	15.77	4.18	37.96	3.72	22.77	4.39
AD-1071474.1	49.90	12.53	23.15	3.16	38.07	3.24	61.66	10.09
AD-	28.55	2.06	21.24	1.09	38.15	4.93	57.90	3.25

Duplex Name	50 nM		10 nM		1 nM		0.1 nM	
	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD
1071093.1								
AD-1070498.1	23.83	11.12	19.17	7.33	38.24	23.94	45.59	14.06
AD-1072086.1	24.22	0.89	17.86	3.97	38.58	3.09	29.83	8.60
AD-1071213.1	29.38	11.30	32.31	16.24	38.62	5.23	73.09	4.46
AD-1070492.1	26.44	15.25	24.90	12.34	39.25	7.95	57.33	3.12
AD-1070753.1	20.64	1.92	19.37	1.56	39.57	25.07	49.93	3.30
AD-1071144.1	29.83	5.39	23.05	1.22	39.66	0.55	70.63	11.07
AD-1072089.1	24.61	8.76	14.76	2.58	39.95	20.78	33.58	8.06
AD-1071950.1	18.68	9.46	19.82	9.85	40.56	18.97	37.85	21.56
AD-1072051.1	28.96	14.70	20.24	6.78	40.77	24.94	133.71	21.10
AD-1070514.1	37.51	21.18	23.12	1.91	41.27	20.56	56.70	9.42
AD-1070554.1	33.95	22.98	26.13	15.48	42.00	9.82	73.40	28.36
AD-1070340.1	38.09	9.13	25.30	7.35	42.12	13.13	59.19	13.17
AD-1070539.1	40.43	5.47	27.37	3.65	42.75	6.07	76.09	2.72
AD-1072049.1	19.89	1.03	26.59	13.84	42.85	19.07	88.46	43.34
AD-1071507.1	28.69	13.06	23.25	12.73	43.46	16.20	73.34	29.19
AD-1072047.1	32.91	3.31	31.45	9.38	44.81	6.75	44.83	5.00
AD-1070497.1	20.50	11.12	30.33	18.25	45.39	22.49	45.24	6.67
AD-1071669.	27.16	21.57	22.08	4.52	45.41	24.19	82.08	40.75

Duplex Name	50 nM		10 nM		1 nM		0.1 nM	
	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD
1								
AD-1070461.1	23.85	6.02	27.50	7.24	46.32	7.41	51.70	11.06
AD-1071499.1	23.48	8.07	30.42	15.92	49.08	19.84	68.85	4.77
AD-1070578.1	56.69	5.51	35.64	5.34	50.30	8.67	77.05	4.01
AD-1070537.1	43.58	16.98	33.83	18.17	51.57	14.89	57.20	5.70
AD-1070538.1	49.19	0.85	39.65	14.45	52.47	5.67	80.55	8.10
AD-1070489.1	34.37	8.36	29.52	10.36	52.55	8.31	82.03	22.09
AD-1070941.1	56.55	28.66	34.60	2.97	53.73	14.84	85.09	12.61
AD-1070490.1	82.85	9.46	74.05	26.29	53.97	8.14	83.24	18.67
AD-1070552.1	57.15	32.27	59.94	46.24	54.95	2.00	71.29	5.28
AD-1071220.1	62.91	4.26	49.89	5.27	55.41	17.90	88.53	5.03
AD-1070577.1	38.75	1.69	33.00	9.30	59.74	5.02	83.43	12.28
AD-1070540.1	87.17	12.58	71.93	3.00	63.54	6.18	82.46	4.78
AD-1070331.1	67.10	13.65	69.48	5.53	63.62	22.88	70.53	5.96
AD-1072082.1	30.61	9.66	25.36	6.85	64.38	46.29	22.40	5.98
AD-1070536.1	56.35	12.92	35.57	7.90	67.27	4.78	86.71	13.81
AD-1070512.1	41.77	8.33	39.06	12.83	67.73	11.53	77.92	11.50
AD-1070535.1	57.79	0.69	70.08	14.12	69.35	15.26	72.22	3.84
AD-1070513.1	48.22	3.35	36.95	2.11	70.81	21.72	81.56	0.86

Duplex Name	50 nM		10 nM		1 nM		0.1 nM	
	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD
AD-1070702.1	130.85	16.96	82.78	8.78	82.89	8.68	99.63	6.28
AD-1070576.1	127.24	8.36	73.19	14.90	103.56	26.59	98.24	6.13

Table 5. ***In Vitro*** **Screen in Neuro2a Cells**

Duplex Name	50 nM		10 nM		1 nM		0.1 nM	
	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD
AD-1071792.1	34.38	9.77	12.82	3.01	45.40	13.73	47.23	9.08
AD-1071769.1	29.61	4.81	13.54	2.08	27.44	5.65	24.12	7.99
AD-1071773.1	31.38	3.83	13.93	1.77	25.11	5.50	51.29	17.72
AD-1071718.1	42.22	6.35	24.08	19.71	31.14	7.13	40.39	7.07
AD-1071870.1	37.85	9.69	24.13	4.28	44.08	10.20	46.12	4.49
AD-1071904.1	50.07	7.77	25.11	4.11	41.24	4.74	67.32	2.90
AD-1071789.1	54.49	11.50	25.53	5.72	42.35	4.14	46.28	4.63
AD-1071671.1	46.19	17.09	26.77	9.50	52.09	14.65	30.46	11.70
AD-1071628.1	19.22	4.46	29.86	3.48	41.39	22.54	48.33	24.33
AD-1071865.1	87.81	30.20	32.26	7.58	111.93	10.04	58.00	35.11
AD-1071670.1	47.61	8.96	32.62	10.07	61.37	20.89	67.38	12.60
AD-1071901.1	90.86	11.90	32.62	13.27	73.57	6.97	67.21	10.67
AD-1071837.1	50.04	17.07	33.40	5.05	75.41	30.69	67.93	39.92
AD-1071929.	90.08	13.95	34.31	8.05	103.06	29.12	78.33	22.52

Duplex Name	50 nM		10 nM		1 nM		0.1 nM	
	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD
1								
AD-1071827.1	63.32	26.41	35.00	22.57	38.97	3.90	52.13	7.57
AD-1071923.1	34.35	2.24	35.65	11.55	69.61	36.37	73.64	29.50
AD-1071834.1	76.33	28.41	36.92	10.07	58.93	15.73	47.05	4.21
AD-1071984.1	55.04	12.30	38.47	13.10	46.13	4.79	53.61	7.20
AD-1071629.1	64.52	8.78	39.19	7.18	69.39	10.23	78.22	7.51
AD-1071668.1	45.79	10.17	39.30	3.89	41.27	7.20	58.51	19.53
AD-1070449.1	62.88	12.61	40.70	12.34	112.27	6.56	73.56	33.45
AD-1071836.1	65.02	5.39	43.26	8.79	67.21	3.88	74.79	7.47
AD-1071897.1	99.71	16.56	46.16	13.74	50.53	6.00	64.45	4.95
AD-1071864.1	67.98	8.26	48.34	6.27	63.18	4.33	57.79	11.27
AD-1071927.1	100.13	5.94	49.52	22.56	72.59	1.76	86.05	9.89
AD-1070444.1	54.43	21.40	49.98	15.55	48.02	14.09	39.57	13.53
AD-1071981.1	88.01	19.40	50.45	3.73	72.49	5.65	65.67	6.40
AD-1071509.1	80.40	7.94	53.40	6.15	85.03	9.25	80.53	8.93
AD-1071902.1	59.23	5.23	54.05	24.96	55.60	8.83	78.72	20.75
AD-1071084.1	48.04	7.38	55.99	15.71	49.79	4.20	74.79	22.35
AD-1071948.1	129.42	23.41	56.05	40.63	66.66	9.28	82.30	14.08
AD-1071437.1	92.84	13.95	56.71	7.28	64.96	7.84	81.99	11.01

Duplex Name	50 nM		10 nM		1 nM		0.1 nM	
	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD
AD-1071508.1	63.19	6.25	57.55	17.37	77.51	20.38	83.72	19.72
AD-1071265.1	74.44	10.96	57.81	12.01	71.11	2.26	52.04	5.93
AD-1070450.1	84.37	11.62	58.85	16.08	76.86	2.04	74.82	11.68
AD-1070522.1	74.59	9.94	60.15	13.28	85.81	9.39	70.93	10.95
AD-1071969.1	72.57	24.69	65.13	22.96	88.15	2.71	77.02	14.55
AD-1071352.1	80.83	7.94	65.17	8.49	98.66	18.82	74.26	10.42
AD-1071912.1	60.57	5.20	65.24	13.45	79.56	18.72	78.51	15.60
AD-1071885.1	91.84	8.30	65.33	15.87	111.85	36.46	94.34	19.71
AD-1071949.1	94.15	6.94	68.19	9.24	121.01	21.98	86.93	13.55
AD-1071085.1	81.43	10.07	68.99	23.76	70.75	2.75	80.42	9.33
AD-1070516.1	99.84	9.66	71.40	11.83	91.42	6.98	85.74	17.99
AD-1071895.1	50.58	4.85	72.17	5.64	112.73	9.95	137.55	23.38
AD-1071430.1	97.90	8.83	72.25	12.12	91.48	12.84	88.25	37.15
AD-1070990.1	111.59	4.01	75.10	6.83	124.18	18.32	91.73	8.62
AD-1071506.1	109.84	3.10	76.05	11.51	90.66	16.11	113.19	20.89
AD-1071985.1	115.76	19.02	77.08	20.31	108.11	7.18	106.89	10.74
AD-1070511.1	85.82	14.10	77.15	32.69	76.18	5.31	69.73	10.27
AD-1071301.1	81.47	16.32	77.18	18.11	92.80	23.07	95.27	12.82
AD-	105.75	3.70	78.72	9.04	95.79	11.58	127.56	9.53

Duplex Name	50 nM		10 nM		1 nM		0.1 nM	
	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD
1071835.1								
AD-1072054.1	105.86	12.13	79.76	13.27	93.40	14.38	126.85	45.73
AD-1070861.1	91.25	9.17	81.24	1.84	94.30	18.65	99.09	26.52
AD-1071230.1	121.89	3.95	81.34	10.29	95.25	13.61	98.27	5.67
AD-1071435.1	114.13	14.06	81.41	13.52	124.42	8.82	81.45	16.69
AD-1070553.1	87.29	25.97	81.94	8.12	63.31	14.88	69.98	23.52
AD-1071894.1	102.82	10.02	81.95	12.88	83.52	18.17	151.91	31.14
AD-1071652.1	85.35	12.47	83.55	19.17	95.16	20.85	103.29	21.11
AD-1072050.1	92.69	17.25	83.84	21.31	84.74	5.70	103.08	23.42
AD-1072052.1	83.77	37.72	84.30	16.14	88.31	28.47	95.71	13.03
AD-1071968.1	125.96	30.92	85.39	35.18	137.38	24.64	89.12	6.18
AD-1071441.1	102.71	4.69	86.57	16.86	98.29	7.01	96.48	22.41
AD-1071168.1	142.25	9.05	88.06	18.37	126.73	18.27	93.48	4.76
AD-1070332.1	96.86	4.90	88.15	14.57	94.80	11.70	106.48	23.83
AD-1070964.1	122.62	6.79	90.88	11.93	118.13	7.70	129.78	3.87
AD-1070974.1	95.24	14.87	91.26	9.29	101.14	24.33	124.98	34.39
AD-1070935.1	86.41	15.15	91.87	30.52	78.96	11.28	135.28	51.14
AD-1071016.1	116.74	10.64	93.07	6.51	103.56	9.02	124.33	19.10
AD-1071163.	122.96	12.35	93.79	10.92	97.44	11.62	116.77	16.31

Duplex Name	50 nM		10 nM		1 nM		0.1 nM	
	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD
1								
AD-1071500.1	109.41	6.89	93.94	28.57	98.22	10.78	115.10	18.64
AD-1071664.1	95.74	6.19	95.09	9.07	90.81	9.59	103.19	20.73
AD-1071983.1	107.75	2.14	95.97	17.57	87.62	16.91	122.64	28.85
AD-1072083.1	117.49	16.88	96.13	15.38	112.89	21.43	97.41	12.53
AD-1071505.1	114.58	9.62	96.28	2.09	97.86	12.64	125.20	16.30
AD-1071916.1	114.74	15.20	97.63	19.37	113.46	23.96	98.20	20.90
AD-1071662.1	117.08	9.61	99.34	15.09	101.57	12.56	95.41	19.81
AD-1071074.1	119.35	2.18	99.59	16.26	102.18	4.57	102.16	14.17
AD-1070975.1	98.62	13.33	100.91	34.91	103.73	7.46	127.03	19.01
AD-1072048.1	120.38	25.28	101.59	23.74	91.98	13.31	118.03	36.71
AD-1070752.1	116.16	18.74	101.61	3.18	112.35	3.45	111.09	15.27
AD-1071491.1	101.74	14.83	101.77	17.15	81.61	13.70	106.75	25.59
AD-1071965.1	128.49	15.51	101.89	8.10	186.60	83.49	109.00	14.64
AD-1070963.1	116.03	4.30	103.79	9.94	91.63	6.48	113.09	24.80
AD-1070515.1	151.97	10.28	104.16	15.21	144.50	9.60	103.67	10.60
AD-1070488.1	91.93	10.44	104.22	24.68	118.10	15.51	115.55	7.51
AD-1070462.1	104.43	7.33	104.94	11.72	104.45	7.57	139.73	23.99
AD-1072085.1	94.67	7.70	108.46	24.98	97.93	21.14	153.63	56.04

Duplex Name	50 nM		10 nM		1 nM		0.1 nM	
	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD
AD-1071396.1	99.92	1.54	110.16	11.59	120.27	32.11	133.61	7.49
AD-1070491.1	102.35	4.34	110.59	35.66	107.80	32.46	111.62	17.60
AD-1072084.1	89.07	20.17	111.77	6.42	116.62	18.82	126.35	16.31
AD-1071474.1	122.36	11.57	113.05	11.96	163.66	14.39	132.01	26.76
AD-1071093.1	96.13	13.52	113.42	14.09	147.35	18.16	118.48	9.33
AD-1070498.1	91.67	13.45	113.88	9.19	79.30	8.81	108.63	14.96
AD-1072086.1	123.20	15.92	115.49	29.56	108.55	4.25	138.48	25.53
AD-1071213.1	116.69	26.05	116.37	17.21	103.04	14.69	117.48	13.13
AD-1070492.1	109.34	29.24	116.40	21.85	122.34	32.17	143.29	11.39
AD-1070753.1	84.35	7.70	118.18	41.84	82.38	19.87	106.33	20.40
AD-1071144.1	83.13	12.45	119.74	19.55	130.42	12.03	150.23	12.13
AD-1072089.1	108.05	10.98	121.02	10.65	113.55	10.44	108.01	12.96
AD-1071950.1	108.92	4.02	121.58	28.99	92.61	5.28	131.06	30.82
AD-1072051.1	112.57	16.35	121.99	50.78	119.33	21.82	152.50	44.08
AD-1070514.1	164.35	21.93	122.06	5.95	162.77	23.04	97.49	24.93
AD-1070554.1	115.57	8.06	122.17	20.42	147.90	15.76	124.16	9.95
AD-1070340.1	100.00	4.72	122.41	21.00	132.46	17.07	110.72	8.55
AD-1070539.1	111.19	25.82	123.37	14.99	114.30	13.27	136.79	9.18
AD-	136.76	27.06	125.13	16.79	150.71	14.99	121.57	23.32

Duplex Name	50 nM		10 nM		1 nM		0.1 nM	
	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD
1072049.1								
AD-1071507.1	115.01	13.93	126.11	20.25	159.30	23.79	143.74	23.89
AD-1072047.1	127.69	18.48	127.04	34.27	93.99	2.90	195.73	65.72
AD-1070497.1	124.13	13.87	127.08	33.92	109.48	11.43	150.21	33.50
AD-1071669.1	111.13	20.07	128.56	5.92	112.14	18.56	131.53	26.85
AD-1070461.1	120.03	14.13	128.77	18.29	143.32	17.52	117.40	17.84
AD-1071499.1	83.75	23.18	132.22	64.67	91.81	24.10	117.40	31.24
AD-1070578.1	85.97	8.52	133.03	48.79	82.80	16.31	115.63	4.31
AD-1070537.1	128.99	13.40	133.78	27.81	181.83	15.77	117.64	8.85
AD-1070538.1	95.05	9.61	133.78	26.83	130.27	9.25	120.34	12.46
AD-1070489.1	110.30	11.95	135.81	28.93	99.69	13.89	133.95	31.84
AD-1070941.1	101.03	9.64	136.63	35.20	133.57	16.32	139.77	16.05
AD-1070490.1	90.56	13.57	137.27	31.55	106.33	16.44	140.73	31.60
AD-1070552.1	126.07	30.96	139.83	30.54	153.89	39.72	138.49	17.84
AD-1071220.1	108.36	11.18	141.55	51.36	123.19	12.88	124.52	25.74
AD-1070577.1	145.82	23.47	143.05	34.14	129.74	17.34	104.41	13.32
AD-1070540.1	106.04	11.03	147.21	12.57	125.13	16.85	140.54	13.23
AD-1070331.1	93.65	16.00	148.85	48.97	99.22	15.81	178.66	42.91
AD-1072082.	194.79	6.73	148.93	6.06	186.41	47.20	107.32	18.83

Duplex Name	50 nM		10 nM		1 nM		0.1 nM	
	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD	% Message Remaining	SD
1								
AD-1070536.1	99.40	8.26	154.48	53.42	78.37	6.15	128.99	34.79
AD-1070512.1	111.85	16.96	161.58	36.38	143.82	10.92	170.38	23.08
AD-1070535.1	149.99	45.62	169.10	90.63	137.56	31.17	150.26	43.80
AD-1070513.1	124.05	38.23	169.88	25.75	99.67	21.72	163.95	64.45
AD-1070702.1	160.56	14.16	186.42	28.42	204.87	8.68	157.17	42.65
AD-1070576.1	134.96	9.17	214.43	54.48	123.38	12.51	132.97	6.87

Example 2. Dose dependent knockdown of PRNP *in vivo*

To determine the appropriate dose of a dsRNA agent targeting PRNP to administer intrathecally, wild-type mice (n=4) were administered a single 50 µg, 150 µg, or 300 µg dose of an exemplary dsRNA agent targeting PRNP, AD-1070516, or control, artificial CSF (aCSF) *via* intracerebroventricular (ICV) injection on Day 0. The dose of dsRNA agent was administered in a volume of 10 µl and the dose of aCSF was administered in a volume of 5 µL. On Day 28 post-dose, animals were sacrificed and tissue samples were collected. The level of PRNP mRNA was determined by qPCR in right hemisphere samples and the level of PRNP protein was determined by ELISA in left hemisphere samples.

As depicted in Figure 1, the data demonstrate a dose dependent knockdown of PRNP mRNA with the 50 µg group having about a 15% knockdown of PRNP mRNA, the 150 µg group having about a 40% knockdown of PRNP mRNA, and the 300 µg having about a 60% knockdown of PRNP mRNA

We claim:

1. A double stranded ribonucleic acid (dsRNA) agent for inhibiting expression of prion protein (PRNP) in a cell,

wherein the dsRNA agent comprises a sense strand and an antisense strand forming a double stranded region,

wherein the sense starnd comprises a nucleotide sequence comprising at least 15 contiguous nucleotides differing by no more than 3 nucleotides from a portion of the nucleotide sequence of SEQ ID NO: 1, and the antisense strand comprises a nucleotide sequence comprising at least 15 contiguous nucleotides differing by no more than 3 nucleotides of the corresponding portion of the nucleotide sequence of SEQ ID NO:5, and

wherein the sense strand, the antisense strand, or both the sense strand and the antisense strand is conjugated to one or more lipophilic moieties.

2. The dsRNA agent of claim 1, wherein the nucleotide sequence of the sense strand comprises any one of the sense strand nucleotide sequences in any one of Tables 2-3.

3. A double stranded ribonucleic acid (dsRNA) agent for inhibiting expression of prion protein (PRNP) in a cell,

wherein the dsRNA agent comprises a sense strand and an antisense strand forming a double stranded region,

wherein the antisense strand comprises a region of complementarity to an mRNA encoding PRNP, and wherein the region of complementarity comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from any one of the antisense nucleotide sequences in any one of Tables 2-3, and

wherein the sense strand, the antisense strand, or both the sense strand and the antisense strand is conjugated to one or more lipophilic moieties.

4. A double stranded ribonucleic acid (dsRNA) agent for inhibiting expression of prion protein (PRNP) 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 three nucleotides from any one of the nucleotide sequence of nucleotides 502-524, 507-529, 502-529, 540-562, 566-588, 575-597, 576-598, 589-611, 575-611, 593-615, 594-616, 600-622, 593-622, 650-672, 858-880, 976-998, 1100-1122, 1126-1148, 1220-1242, 1221-1243, 1220-1243, 1304-1326, 1328-1350, 1410-1432, 1445-1467, 1481-1503, 1532-1554, 1610-1632, 1615-1637, 1617-1639, 1621-1643, 1610-1643, 1610-1639, 1688-1710, 1694-1714, 1830-1852, 1831-1853, 1854-1876, 1830-1853, 1872-1894, 1873-1895,

1938-1960, 2011-2033, 2015-2037, 2031-2053, 2034-2056, 2069-2091, 2076-2098, 2077-2099, 1872-1895, 2011-2037, 2069-2099, 2079-2101, 2031-2056, 2069-2098, 2138-2160, 2143-2165, 2158-2180, 2167-2189, 2168-2190, 2170-2192, 2174-2196, 2175-2197, 2177-2199, 2185-2207, 2189-2211, 2196-2218, 2200-2222, 2202-2224, 2221-2243, 2222-2244, 2223-2245, 2238-2260, 2241-2263, 2242-2264, 2138-2264, 2257-2358, 2174-2245, 2174-2224, 2138-2196, 2177-2224, 2223-2245, 2138-2189, 2177-2211, 2196-2224, 2257-2279, 2258-2280, 2335-2537, 2336-2358, 2257-2358, 2342-2364, 2397-2419, 2398-2420, 2399-2421, 2400-2422, 2401-2423, 2404-2426, 2397-2426, 2394-2423, 2397-2421, 2399-2426, and 2398-2421 of SEQ ID NO: 1, and the antisense strand comprises at least 15 contiguous nucleotides from the corresponding nucleotide sequence of SEQ ID NO:5, and

wherein the sense strand, the antisense strand, or both the sense strand and the antisense strand is conjugated to one or more lipophilic moieties.

5. A double stranded ribonucleic acid (dsRNA) agent for inhibiting expression of prion protein (PRNP) 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 three nucleotides from any one of the nucleotide sequence of nucleotides 530-570, 535-565, 539-561, 540-562, 555-605, 560-605, 560-600, 566-588, 567-589, 568-590, 569-591, 570-592, 575-597, 580-620-585-620, 589-611, 590-612, 591-613, 592-614, 593-615, 594-616, 600-650, 610-650, 613-635, 614-636, 615-637, 616-638, 617-639, 618-640, 640-680, 649-671, 650-672, 651-673, 670-700, 674-696, 675-697, 795-830, 804-826, 2325-2375, 2325-2370, 2330-2370, 2335-2357, 2336-2358, 2337-2359, 2338-2360, 2339-2361, or 2340-2362 of SEQ ID NO: 1, and the antisense strand comprises at least 15 contiguous nucleotides from the corresponding nucleotide sequence of SEQ ID NO:5, and. wherein the sense strand, the antisense strand, or both the sense strand and the antisense strand is conjugated to one or more lipophilic moieties.

6. The dsRNA agent of any one of claims 1-5, wherein the antisense strand comprises at least 15 contiguous nucleotides differing by no more than three nucleotides from any one of the antisense strand nucleotide sequences of a duplex selected from the group consisting of AD-1070511, AD-1070462, AD-1070553, AD-1072048, AD-1070516, AD-1072050; AD-1071769, AD-1071949, AD-1070444, AD-1071505, AD-1071912, AD-1071789, AD-1071265, AD-1072084 and AD-1071985.

7. The dsRNA agent of any one of claims 1-6, wherein the one or more lipophilic moieties are conjugated to one or more internal positions in the double stranded region of the dsRNA agent.

8. The dsRNA agent of any one of claims 1-7, wherein the one or more lipophilic moieties are conjugated to one or more internal positions on the antisense strand.

9. The dsRNA agent of any one of claims 1-8, wherein the one or more lipophilic moieties are conjugated to one or more internal positions on at least one strand *via* a linker or carrier.

10. The dsRNA agent of any one of claims 1-9, wherein lipophilicity of the lipophilic moiety, measured by $\log K_{ow}$, exceeds 0.

11. The dsRNA agent of any one of claims 1-10, wherein the hydrophobicity of the dsRNA agent, measured by the unbound fraction in a plasma protein binding assay of the dsRNA agent, exceeds 0.2.

12. The dsRNA agent of claim 11, wherein the plasma protein binding assay is an electrophoretic mobility shift assay using human serum albumin protein.

13. The dsRNA agent of any one of claims 1-12, wherein the internal positions include all positions except the terminal two positions from each end of the sense strand or the antisense strand.

14. The dsRNA agent of claim 13, wherein the internal positions include all positions except the terminal three positions from each end of the sense strand or the antisense strand.

15. The dsRNA agent of any one of claims 1-14, wherein the internal positions exclude a cleavage site region of the sense strand.

16. The dsRNA agent of claim 15, wherein the internal positions include all positions except positions 9-12, counting from the 5'-end of the sense strand.

17. The dsRNA agent of claim 15, wherein the internal positions include all positions except positions 11-13, counting from the 3'-end of the sense strand.

18. The dsRNA agent of any one of claims 1-14, wherein the internal positions exclude a cleavage site region of the antisense strand.

19. The dsRNA agent of claim 18, wherein the internal positions include all positions except positions 12-14, counting from the 5'-end of the antisense strand.

20. The dsRNA agent of any one of claims 1-19, wherein the internal positions include all positions except positions 11-13 on the sense strand, counting from the 3'-end, and positions 12-14 on the antisense strand, counting from the 5'-end.

21. The dsRNA agent of any one of claims 1-20, wherein the one or more lipophilic moieties are conjugated to one or more of the internal positions selected from the group consisting of positions 4-8 and 13-18 on the sense strand, and positions 6-10 and 15-18 on the antisense strand, counting from the 5'end of each strand.

22. The dsRNA agent of claim 21, wherein the one or more lipophilic moieties are conjugated to one or more of the internal positions selected from the group consisting of positions 5, 6, 7, 15, and 17 on the sense strand, and positions 15 and 17 on the antisense strand, counting from the 5'-end of each strand.

23. The dsRNA agent of claim 7, wherein the positions in the double stranded region exclude a cleavage site region of the sense strand.

24. The dsRNA agent of any one of claims 1-23, wherein the sense strand is 21 nucleotides in length, the antisense strand is 23 nucleotides in length, and the lipophilic moiety is conjugated to position 20, position 15, position 1, position 7, position 6, or position 2 of the sense strand or position 16 of the antisense strand.

25. The dsRNA agent of any one of claims 1-23, wherein the sense strand is 21 nucleotides in length, the antisense strand is 23 nucleotides in length, and the lipophilic moiety is conjugated to position 21, position 20, position 15, position 1, position 7, position 6, or position 2 of the sense strand or position 16 of the antisense strand.

26. The dsRNA agent of any one of claims 1-25, wherein the lipophilic moiety is an aliphatic, alicyclic, or polyalicyclic compound.

27. The dsRNA agent of claim 26, wherein the lipophilic moiety is selected from the group consisting of lipid, cholesterol, retinoic acid, cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-bis-O(hexadecyl)glycerol, geranyloxyhexyanol, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, myristic acid, O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine.

28. The dsRNA agent of claim 27, wherein the lipophilic moiety contains a saturated or unsaturated C4-C30 hydrocarbon chain, and an optional functional group selected from the group consisting of hydroxyl, amine, carboxylic acid, sulfonate, phosphate, thiol, azide, and alkyne.

29. The dsRNA agent of claim 28, wherein the lipophilic moiety contains a saturated or unsaturated C6-C18 hydrocarbon chain.

30. The dsRNA agent of claim 29, wherein the lipophilic moiety contains a saturated or unsaturated C16 hydrocarbon chain.

31. The dsRNA agent of claim 30, wherein the saturated or unsaturated C16 hydrocarbon chain is conjugated to position 6, counting from the 5'-end of the strand.

32. The dsRNA agent of any one of claims 1-31, wherein the lipophilic moiety is conjugated *via* a carrier that replaces one or more nucleotide(s) in the internal position(s) or the double stranded region.

33. The dsRNA agent of claim 32, wherein the carrier is a cyclic group selected from the group consisting of pyrrolidinyl, pyrazolinyl, pyrazolidinyl, imidazolinyl, imidazolidinyl, piperidinyl, piperazinyl, [1,3]dioxolanyl, oxazolidinyl, isoxazolidinyl, morpholinyl, thiazolidinyl, isothiazolidinyl, quinoxalinyl, pyridazinonyl, tetrahydrofuranyl, and decalinyl; or is an acyclic moiety based on a serinol backbone or a diethanolamine backbone.

34. The dsRNA agent of any one of claims 1-33, wherein the lipophilic moiety is conjugated to the dsRNA agent *via* a linker containing an ether, thioether, urea, carbonate, amine, amide, maleimide-thioether, disulfide, phosphodiester, sulfonamide linkage, a product of a click reaction, or carbamate.

35. The double-stranded iRNA agent of any one of claims 1-34, wherein the lipophilic moiety is conjugated to a nucleobase, sugar moiety, or internucleosidic linkage.

36. The dsRNA agent of any one of claims 1-35, wherein the dsRNA agent comprises at least one modified nucleotide.

37. The dsRNA agent of claim 36, wherein no more than five of the sense strand nucleotides and no more than five of the nucleotides of the antisense strand are unmodified nucleotides

38. The dsRNA agent of claim 36, wherein all of the nucleotides of the sense strand and all of the nucleotides of the antisense strand are modified nucleotides.

39. The dsRNA agent of any one of claims 36-38, wherein at least one of the modified nucleotides is selected from the group a deoxy-nucleotide, a 3'-terminal deoxythimidine (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 5'-phosphorothioate group, a nucleotide comprising a 5'-methylphosphonate group, a nucleotide comprising a 5' phosphate or 5' phosphate mimic, a nucleotide comprising vinyl phosphonate, a nucleotide comprising adenosine-glycol nucleic acid (GNA), a nucleotide comprising thymidine-glycol nucleic acid (GNA) S-Isomer, a nucleotide comprising 2-hydroxymethyl-tetrahydrofurane-5-phosphate, a nucleotide comprising 2'-deoxythymidine-3'phosphate, a nucleotide comprising 2'-deoxyguanosine-3'-phosphate, and a terminal nucleotide linked to a cholesteryl derivative, a dodecanoic acid bisdecylamide group; acytidine-2`-phosphate, a guanosine-2`-phosphate, a uridine-2`-phosphate, a adenosine-2`-phosphate, a 2'-O-hexadecyl-adenosine-3'-phosphate, a 2'-O-hexadecyl-cytidine-3'-phosphate, a 2'-O-hexadecyl-guanosine-3'-phosphate, and a 2'-O-hexadecyl-uridine-3'-phosphate, and combinations thereof.

40. The dsRNA agent of claim 39, wherein the modified nucleotide is selected from the group consisting of a 2'-deoxy-2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, 3'-terminal deoxythimidine nucleotides (dT), a locked nucleotide, an abasic nucleotide, a 2'-amino-modified nucleotide, a 2'-alkyl-modified nucleotide, a morpholino nucleotide, a phosphoramidate, and a non-natural base comprising nucleotide.

41. The dsRNA of claim 39, wherein at least one of the modified nucleotides is selected from the group consisting of a deoxy-nucleotide, a 2'-O-methyl modified nucleotide, a 2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a glycol modified nucleotide (GNA), and, a vinyl-phosphonate nucleotide; and combinations thereof.

42. The dsRNA of claim 39, wherein at least one of the modifications on the nucleotides is a thermally destabilizing nucleotide modification.

43. The dsRNA of claim 42, wherein the thermally destabilizing nucleotide modification is selected from the group consisting of an abasic modification; a mismatch with the opposing nucleotide in the duplex; and destabilizing sugar modification, a 2'-deoxy modification, an acyclic nucleotide, an unlocked nucleic acids (UNA), and a glycerol nucleic acid (GNA).

44. The dsRNA agent of claim 39, wherein the modified nucleotide comprises a short sequence of 3'-terminal deoxythimidine nucleotides (dT).

45. The dsRNA agent of claim 39, wherein the modifications on the nucleotides are 2'-O-methyl, GNA and 2'fluoro modifications.

46. The dsRNA agent of any one of claims 1-45, further comprising at least one phosphorothioate internucleotide linkage.

47. The dsRNA agent of claim 46, wherein the dsRNA agent comprises 6-8 phosphorothioate internucleotide linkages.

48. The dsRNA agent of any one of claims 1-46, wherein each strand is no more than 30 nucleotides in length.

49. The dsRNA agent of any one of claims 1-48, wherein at least one strand comprises a 3' overhang of at least 1 nucleotide.

50. The dsRNA agent of any one of claims 1-48, wherein at least one strand comprises a 3' overhang of at least 2 nucleotides.

51. The dsRNA agent of any one of claims 1-50, wherein the double stranded region is 15-30 nucleotide pairs in length.

52. The dsRNA agent of claim 51, wherein the double stranded region is 17-23 nucleotide pairs in length.

53. The dsRNA agent of claim 51, wherein the double stranded region is 17-25 nucleotide pairs in length.

54. The dsRNA agent of claim 51, wherein the double stranded region is 23-27 nucleotide pairs in length.

55. The dsRNA agent of claim 51, wherein the double stranded region is 19-21 nucleotide pairs in length.

56. The dsRNA agent of claim 51, wherein the double stranded region is 21-23 nucleotide pairs in length.

57. The dsRNA agent of any one of claims 1-56, wherein each strand has 19-30 nucleotides.

58. The dsRNA agent of any one of claims 1-56, wherein each strand has 19-23 nucleotides.

59. The dsRNA agent of any one of claims 1-56, wherein each strand has 21-23 nucleotides.

60. The dsRNA agent of any one of claims 1-59, further comprising a targeting ligand that targets a liver tissue.

61. The dsRNA agent of claim 60, wherein the targeting ligand is a GalNAc conjugate.

62. The dsRNA agent of any one of claims 1-61, wherein the lipophilic moeity or targeting ligand is conjugated *via* a bio-clevable linker selected from the group consisting of DNA, RNA, disulfide, amide, funtionalized monosaccharides or oligosaccharides of galactosamine, glucosamine, glucose, galactose, mannose, and combinations thereof.

63. The dsRNA agent of any one of claims 1-62, wherein the 3' end of the sense strand is protected *via* an end cap which is a cyclic group having an amine, said cyclic group being selected from the group consisting of pyrrolidinyl, pyrazolinyl, pyrazolidinyl, imidazolinyl, imidazolidinyl, piperidinyl, piperazinyl, [1,3]dioxolanyl, oxazolidinyl, isoxazolidinyl, morpholinyl, thiazolidinyl, isothiazolidinyl, quinoxalinyl, pyridazinonyl, tetrahydrofuranyl, and decalinyl.

64. The dsRNA agent of any one of claims 1-63, further comprising

a terminal, chiral modification occurring at the first internucleotide linkage at the 3' end of the antisense strand, having the linkage phosphorus atom in Sp configuration,

a terminal, chiral modification occurring at the first internucleotide linkage at the 5' end of the antisense strand, having the linkage phosphorus atom in Rp configuration, and

a terminal, chiral modification occurring at the first internucleotide linkage at the 5' end of the sense strand, having the linkage phosphorus atom in either Rp configuration or Sp configuration.

65. The dsRNA agent of any one of claims 1-63, further comprising

a terminal, chiral modification occurring at the first and second internucleotide linkages at the 3' end of the antisense strand, having the linkage phosphorus atom in Sp configuration,

a terminal, chiral modification occurring at the first internucleotide linkage at the 5' end of the antisense strand, having the linkage phosphorus atom in Rp configuration, and

a terminal, chiral modification occurring at the first internucleotide linkage at the 5' end of the sense strand, having the linkage phosphorus atom in either Rp or Sp configuration.

66. The dsRNA agent of any one of claims 1-63, further comprising

a terminal, chiral modification occurring at the first, second and third internucleotide linkages at the 3' end of the antisense strand, having the linkage phosphorus atom in Sp configuration,

a terminal, chiral modification occurring at the first internucleotide linkage at the 5' end of the antisense strand, having the linkage phosphorus atom in Rp configuration, and

a terminal, chiral modification occurring at the first internucleotide linkage at the 5' end of the sense strand, having the linkage phosphorus atom in either Rp or Sp configuration.

67. The dsRNA agent of any one of claims 1-63, further comprising

a terminal, chiral modification occurring at the first, and second internucleotide linkages at the 3' end of the antisense strand, having the linkage phosphorus atom in Sp configuration,

a terminal, chiral modification occurring at the third internucleotide linkages at the 3' end of the antisense strand, having the linkage phosphorus atom in Rp configuration,

a terminal, chiral modification occurring at the first internucleotide linkage at the 5' end of the antisense strand, having the linkage phosphorus atom in Rp configuration, and

a terminal, chiral modification occurring at the first internucleotide linkage at the 5' end of the sense strand, having the linkage phosphorus atom in either Rp or Sp configuration.

68. The dsRNA agent of any one of claims 1-63, further comprising

a terminal, chiral modification occurring at the first, and second internucleotide linkages at the 3' end of the antisense strand, having the linkage phosphorus atom in Sp configuration,

a terminal, chiral modification occurring at the first, and second internucleotide linkages at the 5' end of the antisense strand, having the linkage phosphorus atom in Rp configuration, and

a terminal, chiral modification occurring at the first internucleotide linkage at the 5' end of the sense strand, having the linkage phosphorus atom in either Rp or Sp configuration.

69. The dsRNA agent of any one of claims 1-68, further comprising a phosphate or phosphate mimic at the 5'-end of the antisense strand.

70. The dsRNA agent of claim 69, wherein the phosphate mimic is a 5'-vinyl phosphonate (VP).

71. The dsRNA agent of any one of claims 1-70, wherein the base pair at the 1 position of the 5′-end of the antisense strand of the duplex is an AU base pair.

72. The dsRNA agent of any one of claims 1-71, wherein the sense strand has a total of 21 nucleotides and the antisense strand has a total of 23 nucleotides.

73. A cell containing the dsRNA agent of any one of claims 1-72.

74. A pharmaceutical composition for inhibiting expression of a gene encoding prion protein (PRNP) comprising the dsRNA agent of any one of claims 1-72.

75. The pharmaceutical composition of claim 74, wherein dsRNA agent is in an unbuffered solution.

76. The pharmaceutical composition of claim 75, wherein the unbuffered solution is saline or water.

77. The pharmaceutical composition of claim 74, wherein said dsRNA agent is in a buffer solution.

78. The pharmaceutical composition of claim 77, wherein the buffer solution comprises acetate, citrate, prolamine, carbonate, or phosphate or any combination thereof.

79. The pharmaceutical composition of claim 78, wherein the buffer solution is phosphate buffered saline (PBS).

80. A method of inhibiting expression of a prion protein (PRNP) gene in a cell, the method comprising contacting the cell with the dsRNA agent of any one of claims 1-72, or the pharmaceutical composition of any one of claims 74-79, thereby inhibiting expression of the PRNP gene in the cell.

81. The method of claim 80, wherein the cell is within a subject.

82. The method of claim 81, wherein the subject is a human.

83. The method of claim 82, wherein the subject has a PRNP-associated disorder.

84. The method of claim 83, wherein the PRNP-associated disease is a prion disease.

85. The method of claim 84, wherein the prion disease is a genetic prion disease selected from the group consisting of familial Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI).

86. The method of claim 84, wherein the prion disease is a sporadic prion disease selected from the group consisting of sporadic Creutzfeldt-Jakob disease, Sporadic Fatal Insomnia (sFI), and Variably Proteinase-Sensitive Prionopathy (VPSPr).

87. The method of claim 84, wherein the prion disease is an acquired prion disease selected from the group consisting of Iatrogenic CJD (iCJD), Kuru, and Variant CJD (vCJD).

88. The method of any one of claims 80-87, wherein contacting the cell with the dsRNA agent inhibits the expression of PRNP by at least 50%, 60%, 70%, 80%, 90%, or 95%.

89. The method of any one of claims 80-88, wherein inhibiting expression of PRNP decreases PRNP protein levels in serum of the subject by at least 50%, 60%, 70%, 80%, 90%, or 95%.

90. A method of treating a subject having a disorder that would benefit from reduction in PRNP expression, the method comprising administering to the subject a therapeutically effective amount of the dsRNA agent of any one of claims 1-72, or the pharmaceutical composition of any one of claims 74-79, thereby treating the subject having the disorder that would benefit from reduction in PRNP expression.

91. A method of preventing at least one symptom in a subject having a disorder that would benefit from reduction in PRNP expression, the method comprising administering to the subject a prophylactically effective amount of the dsRNA agent of any one of claims 1-72, or the pharmaceutical composition of any one of claims 74-79, thereby preventing at least one symptom in the subject having the disorder that would benefit from reduction in PRNP expression.

92. The method of claim 90 or 91, wherein the disorder is a PRNP-associated disease.

93. The method of claim 92, wherein the PRNP-associated disease is a prion disease.

94. The method of claim 93, wherein the prion disease is a genetic prion disease selected from the group consisting of familial Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI).

95. The method of claim 93, wherein the prion disease is a sporadic prion disease selected from the group consisting of sporadic Creutzfeldt-Jakob disease, Sporadic Fatal Insomnia (sFI), and Variably Proteinase-Sensitive Prionopathy (VPSPr).

96. The method of claim 93, wherein the prion disease is an acquired prion disease selected from the group consisting of Iatrogenic CJD (iCJD), Kuru, and Variant CJD (vCJD).

97. A method of inhibiting the expression of PRNP in a subject, the method comprising administering to the subject a therapeutically effective amount of the dsRNA agent of any one of claims 1-72, or the pharmaceutical composition of any one of claims 74-79, thereby inhibiting the expression of PRNP in the subject.

98. A method for treating or preventing a PRNP-associated disorder in a subject, the method comprising administering to the subject a therapeutically effective amount of the dsRNA agent of any one of claims 1-72, or the pharmaceutical composition of any one of claims 74-79, thereby treating or preventing a PRNP-associated disorder in the subject.

99. The method of claim 97 or 98, wherein the PRNP-associated disease is a prion disease.

100. The method of claim 99, wherein the prion disease is a genetic prion disease selected from the group consisting of familial Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI).

101. The method of claim 99, wherein the prion disease is a sporadic prion disease selected from the group consisting of sporadic Creutzfeldt-Jakob disease, Sporadic Fatal Insomnia (sFI), and Variably Proteinase-Sensitive Prionopathy (VPSPr).

102. The method of claim 99, wherein the prion disease is an acquired prion disease selected from the group consisting of Iatrogenic CJD (iCJD), Kuru, and Variant CJD (vCJD).

103. The method of any one of claims 90-102, wherein the subject is human.

104. The method of any one of claims 90-103, wherein the dsRNA agent is administered to the subject at a dose of about 0.01 mg/kg to 50 mg/kg or at a dose of about 5 mg to 1000 mg.

105. The method of any one of claims 90-104, wherein the dsRNA agent is administered to the subject intrathecally.

106. The method of any one of claims 90-104, wherein the dsRNA agent is administered to the subject subcutaneously.

107. The method of any one of claims 90-106, wherein administration of the dsRNA agent reduced the expression of PRNP in a brain or spine tissue.

108. The method of any one of claims 90-107, further comprising administering to the subject an additional agent for treatment or prevention of a PRNP-associated disorder.

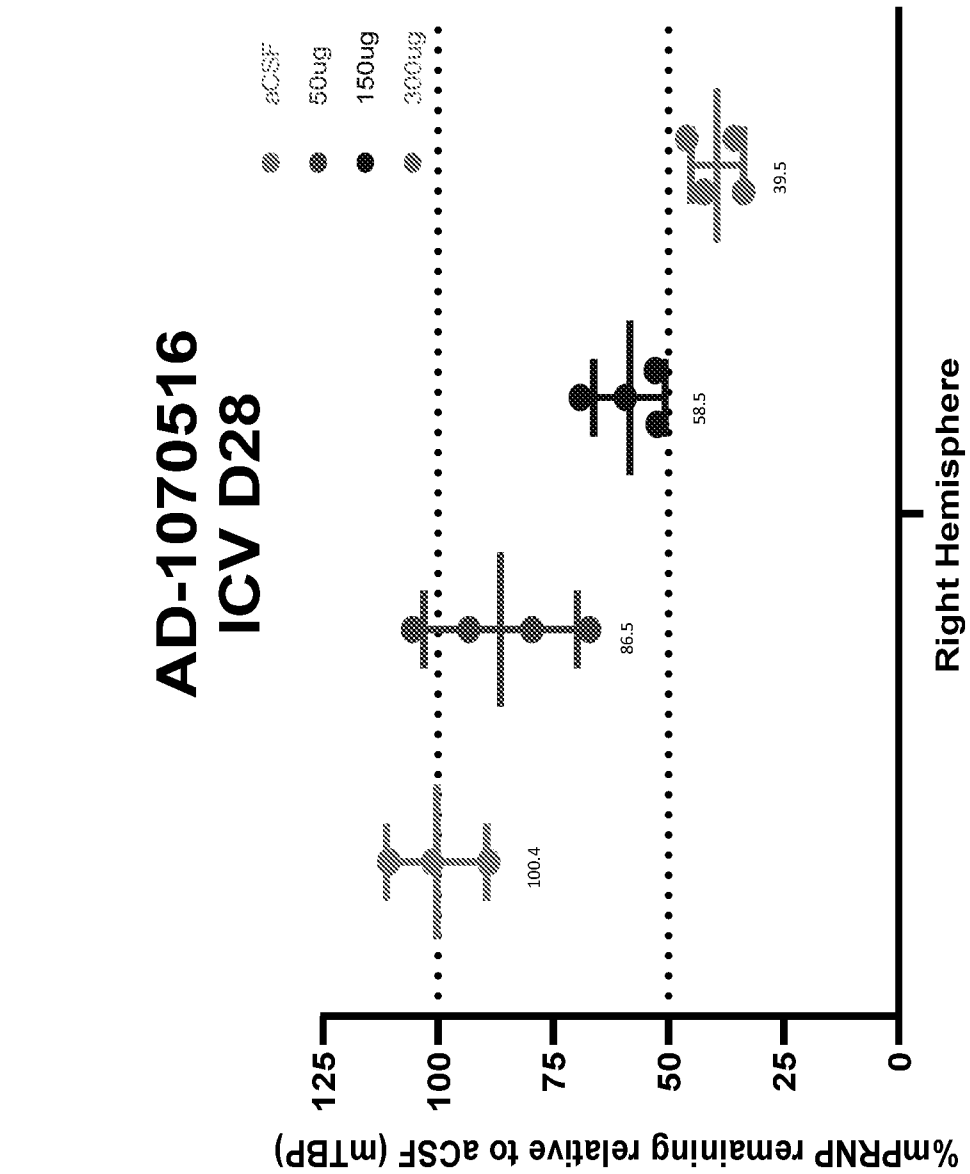

AD-1070516
ICV D28
aCSF
50ug
150ug
300ug
100.4
86.5
58.5
39.5
125
100
75
50
25
0
%mPRNP remaining relative to aCSF (mTBP)
Right Hemisphere

Figure 1

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2022/017690

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/113
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 2020/106996 A1 (IONIS PHARMACEUTICALS INC [US]) 28 May 2020 (2020-05-28) claims 1, 64, 66, 70-81; tables 19-22; sequences 1360, 1437, 1514, 1591, 1668, 1744 the whole document	1-4, 6-108 1-4, 6-108
Y	WO 2019/217459 A1 (ALNYLAM PHARMACEUTICALS INC [US]) 14 November 2019 (2019-11-14) cited in the application claims 1-56; figures 1-3, 13-20 the whole document	1-4, 6-108
A	WO 2016/188729 A1 (RIESNER DETLEV [DE]; HEINRICH-HEINE-UNIVERSITÄT DÜSSELDORF [DE] ET AL.) 1 December 2016 (2016-12-01) claims 1, 7-9, 23; examples 8-10; sequence 115 the whole document	1-4, 6-108

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 May 2022	01/08/2022

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Spindler, Mark-Peter

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2022/017690

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).

      [ ] on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2022/017690

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims;; it is covered by claims Nos.:

1-4, 6-108(all partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-4, 6-108(all partially)

dsRNA for inhibiting expression of PRNP comprising a sense strand comprising at least 15 contiguous nucleotides differing by no more than 3 nucleotides from a portion of SEQ ID NO: 1 and an antisense strand comprising at least 15 contiguous nucleotides differing by no more than 3 nucleotides from a portion of SEQ ID NO: 5, and being conjugated to at least one lipophilic moiety, wherein the dsRNA targets the region 2031-2056 of NM_000311.5 (strands defined by SEQ ID NO: 13/14, 25/26, 273/274, 291/292); cell comprising said dsRNA; pharmaceutical composition comprising said dsRNA; methods employing said dsRNA

2-130. claims: 1-108(partially)

as for invention 1) but wherein the dsRNA targets one of the regions listed in table 2 or 3

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2022/017690

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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