(19) **United States**

(12) **Patent Application Publication** (10) Pub. No.: **US 2017/0000815 A1**
Fitzgerald et al. (43) **Pub. Date:** **Jan. 5, 2017**

(54) **LIPID FORMULATED DSRNA TARGETING THE PCSK9 GENE**

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(21) Appl. No.: **15/060,492**

(22) Filed: **Mar. 3, 2016**

Related U.S. Application Data

(63) Continuation of application No. 14/058,052, filed on Oct. 18, 2013, now abandoned, which is a continuation of application No. 13/568,898, filed on Aug. 7, 2012, now Pat. No. 8,598,139, which is a continuation of application No. 12/816,207, filed on Jun. 15, 2010, now Pat. No. 8,273,869.

(60) Provisional application No. 61/187,169, filed on Jun. 15, 2009, provisional application No. 61/218,350, filed on Jun. 18, 2009, provisional application No. 61/244,790, filed on Sep. 22, 2009, provisional application No. 61/285,598, filed on Dec. 11, 2009, provisional application No. 61/293,474, filed on Jan. 8, 2010, provisional application No. 61/313,578, filed on Mar. 12, 2010.

Publication Classification

(51) **Int. Cl.**
A61K 31/713	(2006.01)
A61K 47/48	(2006.01)
A61K 38/17	(2006.01)
C12N 15/113	(2006.01)

(52) **U.S. Cl.**
CPC *A61K 31/713* (2013.01); *C12N 15/1137* (2013.01); *A61K 47/48815* (2013.01); *A61K 47/48061* (2013.01); *A61K 47/481* (2013.01); *A61K 38/1709* (2013.01); *C12N 2310/3515* (2013.01); *C12N 2310/321* (2013.01); *C12N 2310/322* (2013.01); *C12N 2310/315* (2013.01)

(57) **ABSTRACT**

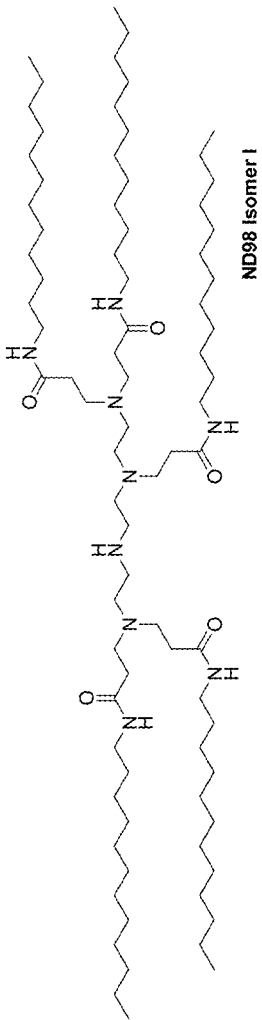This invention relates to composition and methods using lipid formulated siRNA targeted to a PCSK9 gene.

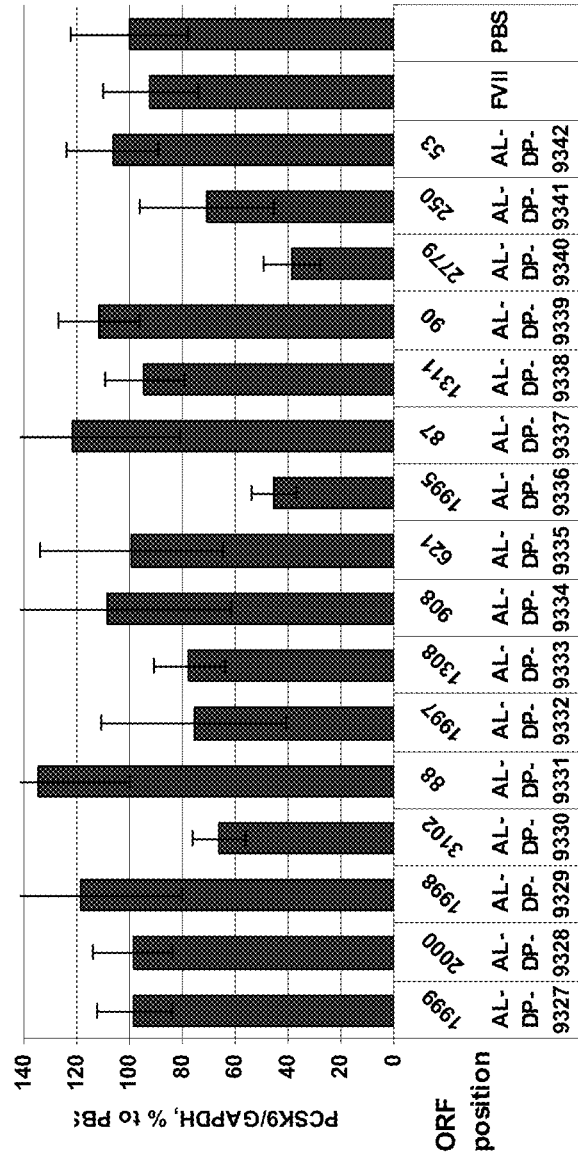ND98 Isomer I

FIG. 1

FIG. 2

FIG. 3

FIG. 4

FIG. 5

FIG. 6A

FIG. 6B
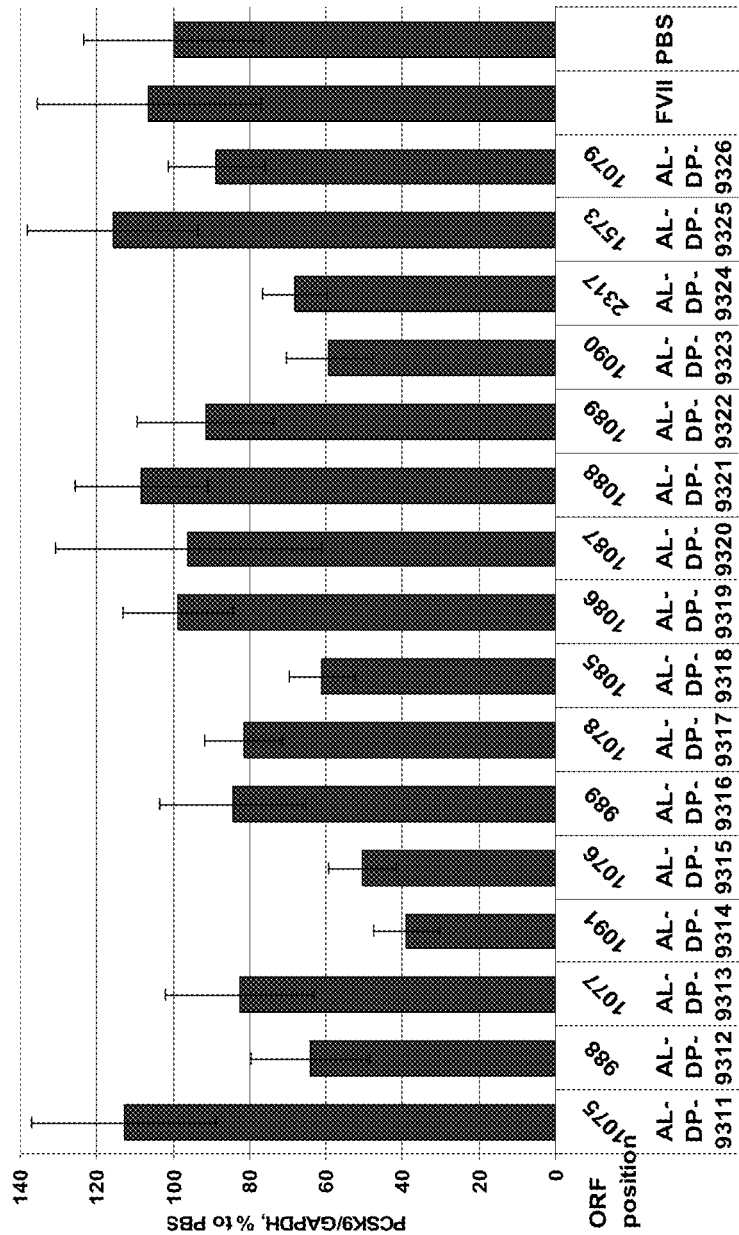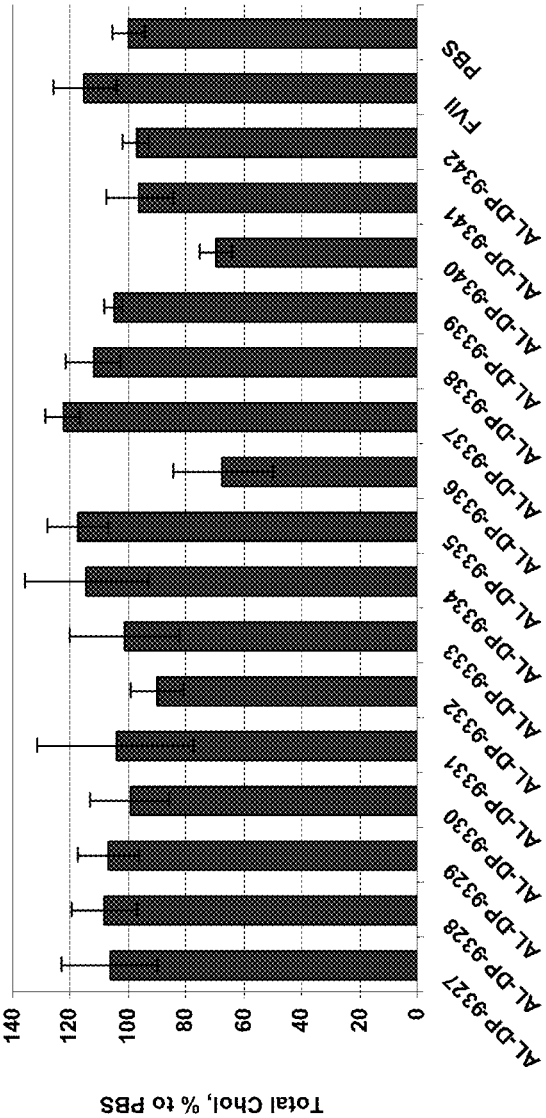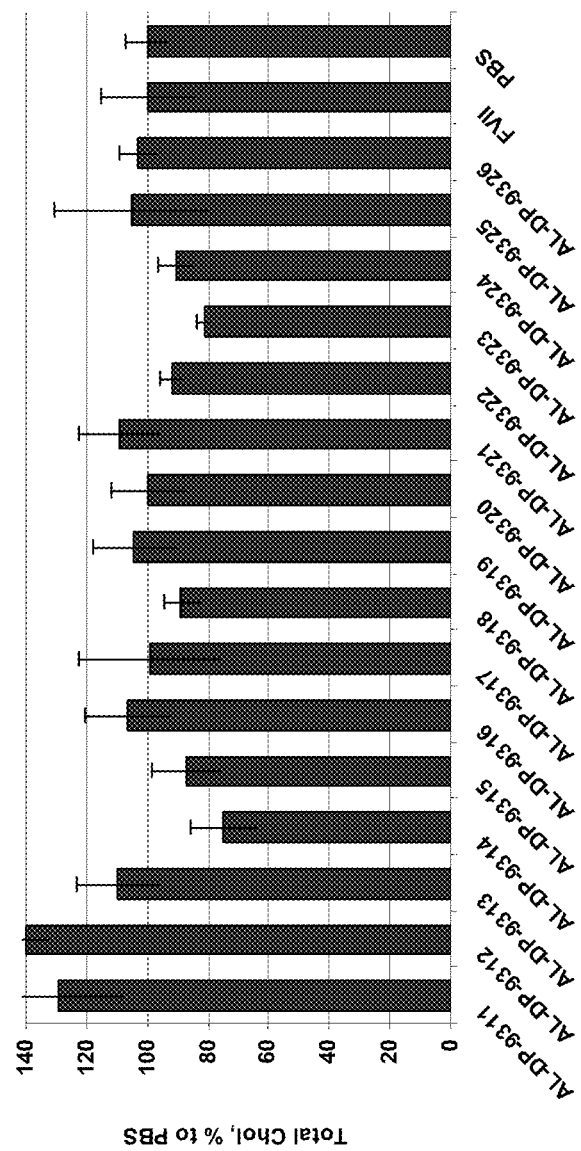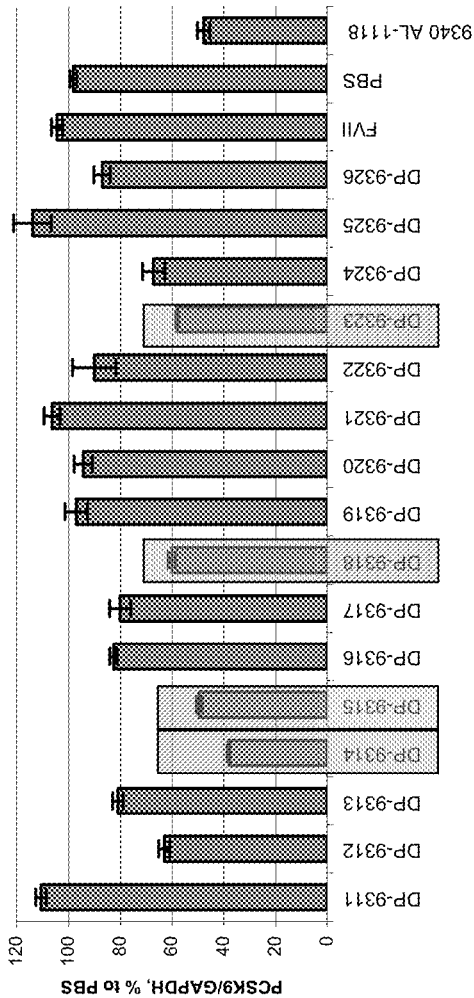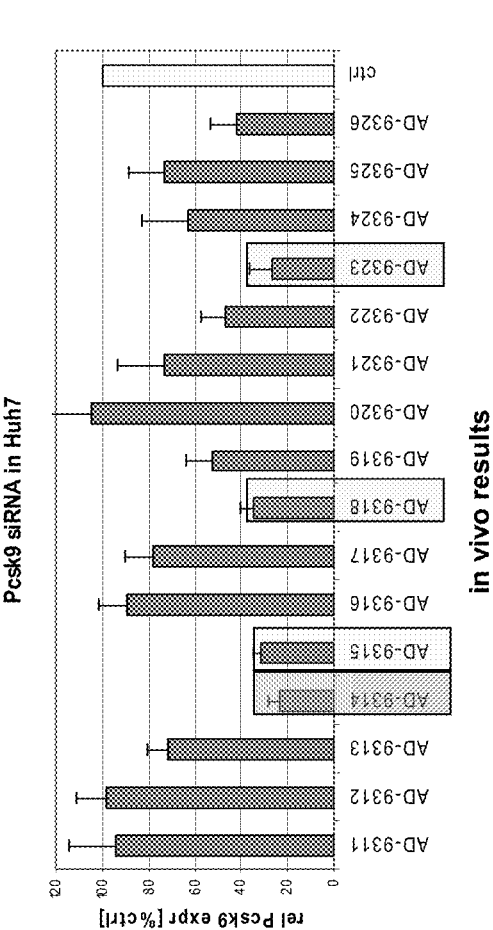
FIG. 7A
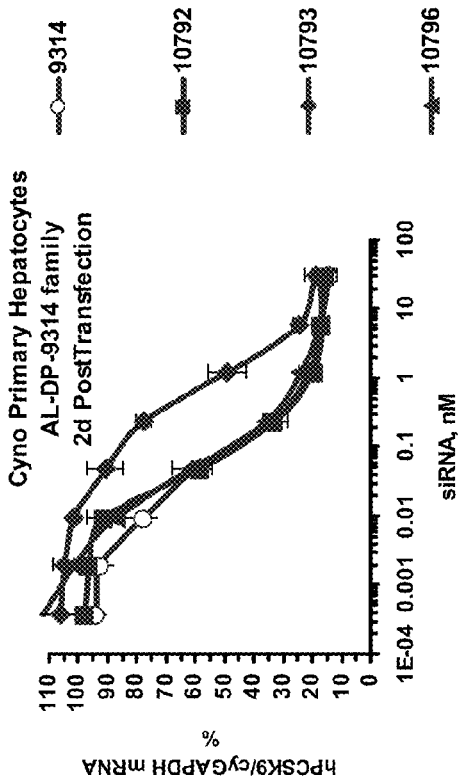
FIG. 7B
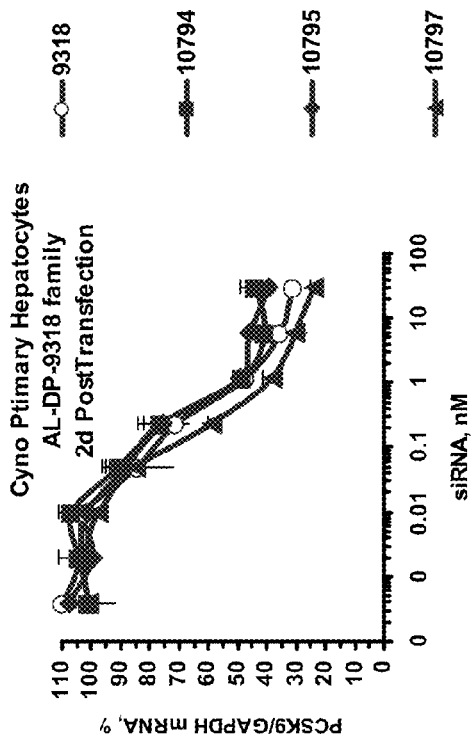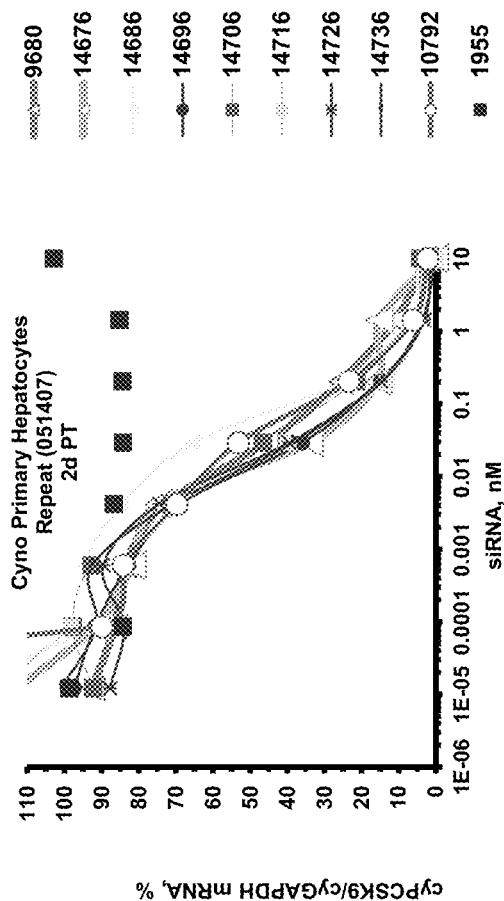
FIG. 7C
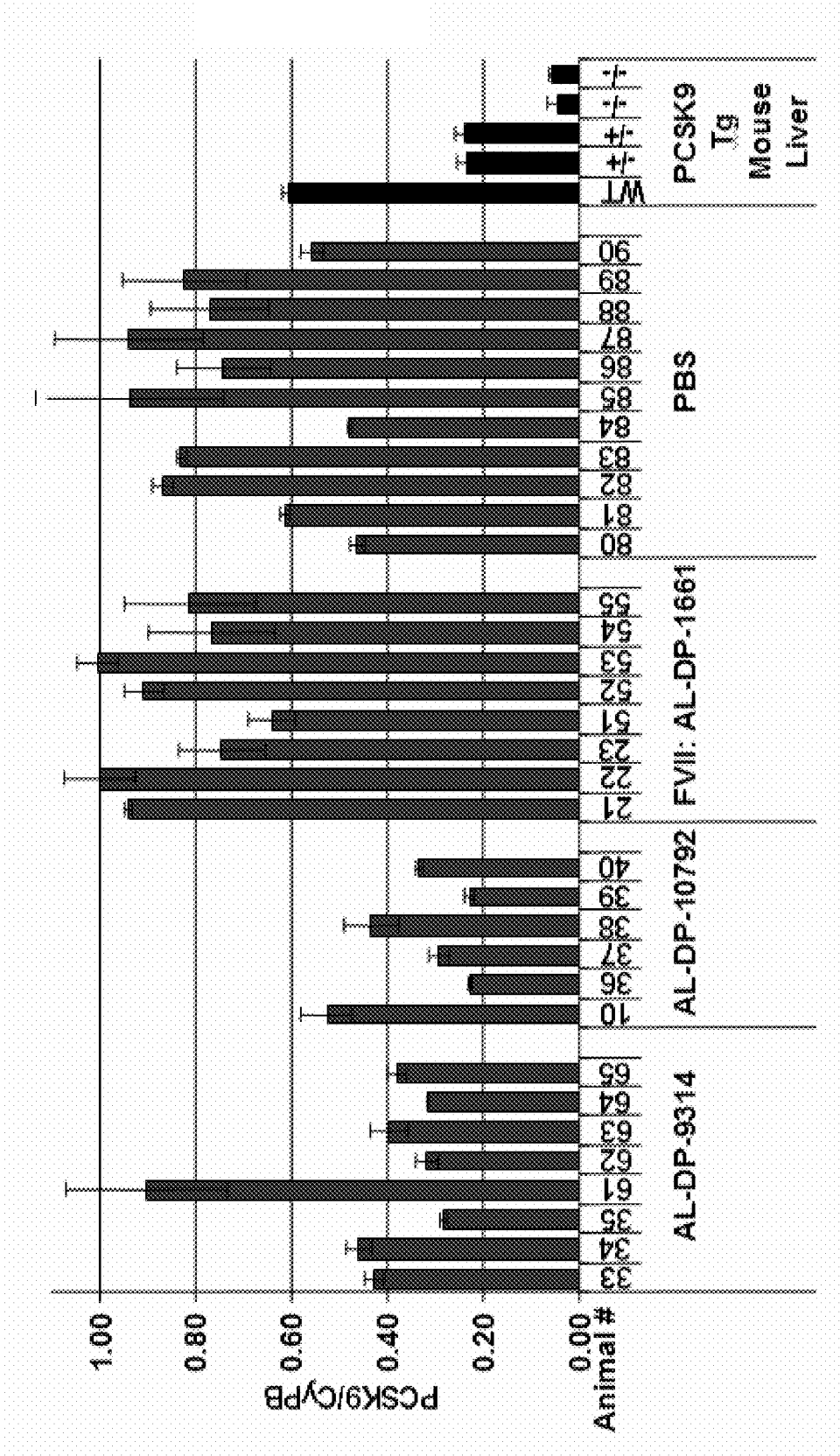
FIG. 8

FIG. 9A
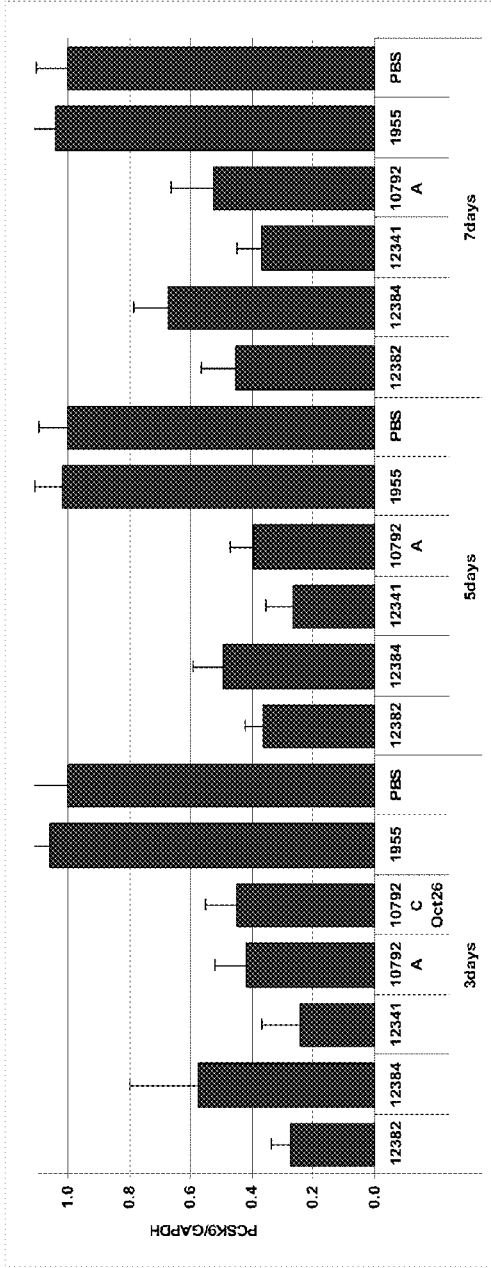
FIG. 9B
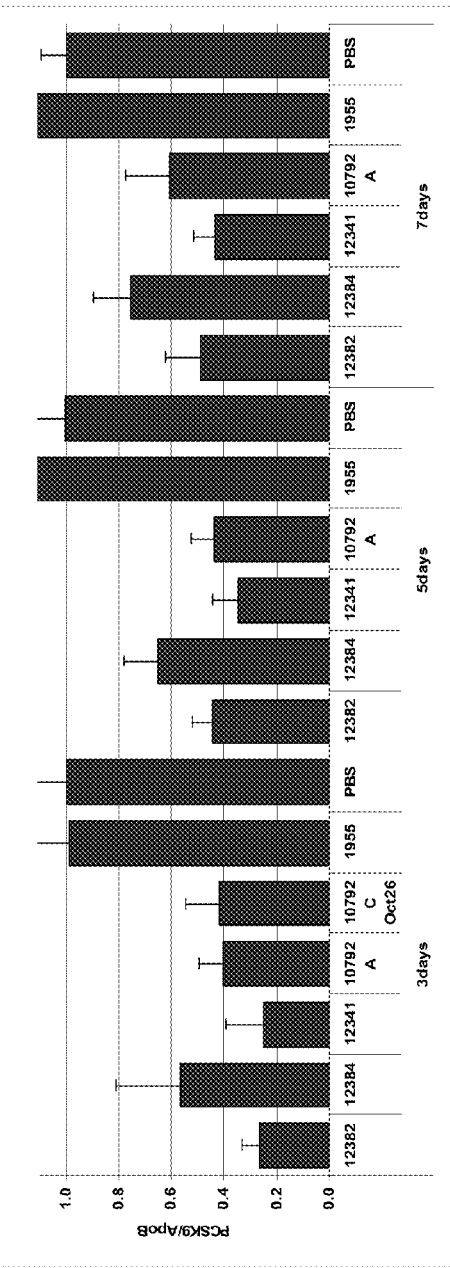
FIG. 10A
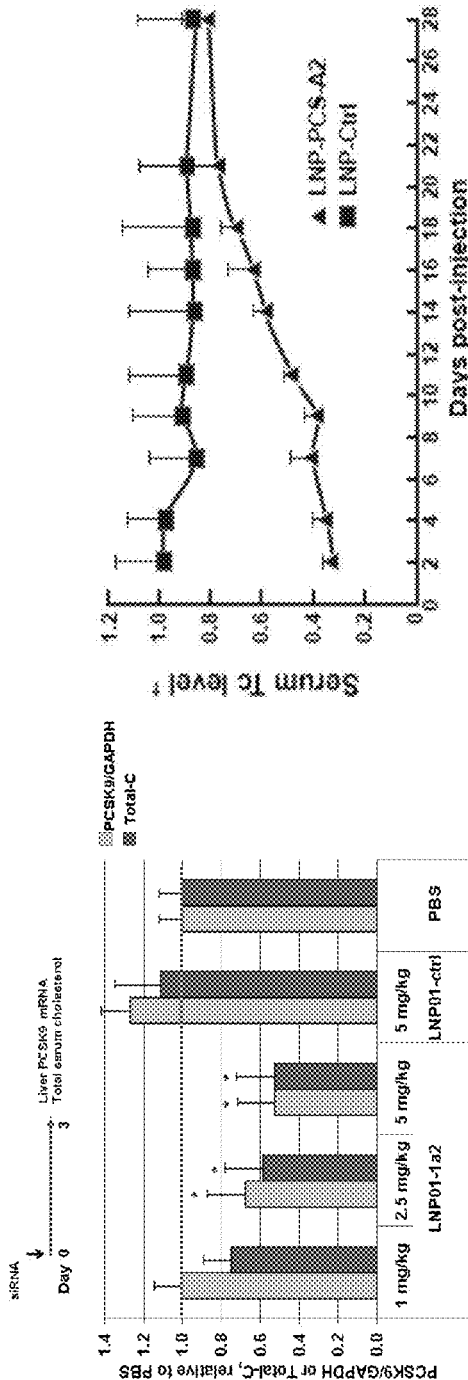
FIG. 10B
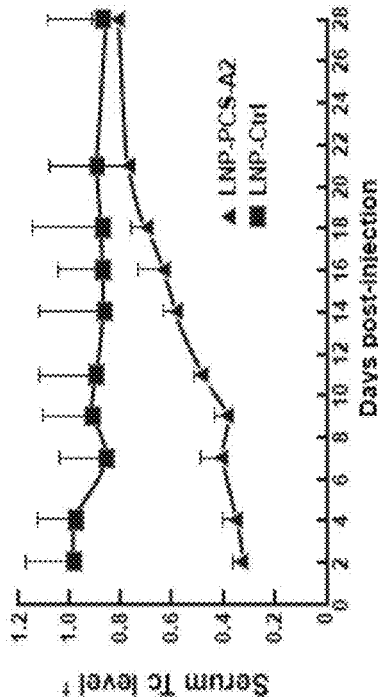
FIG. 10C
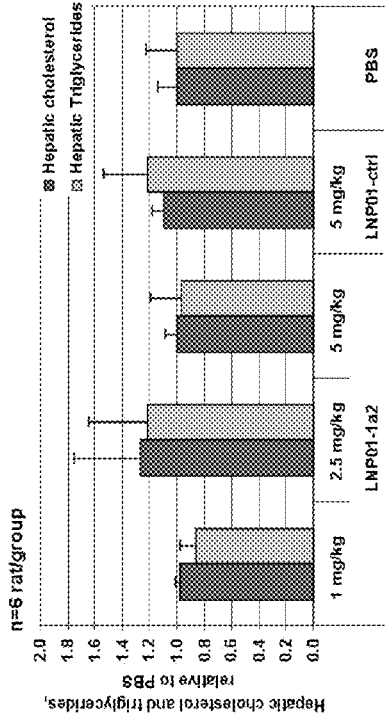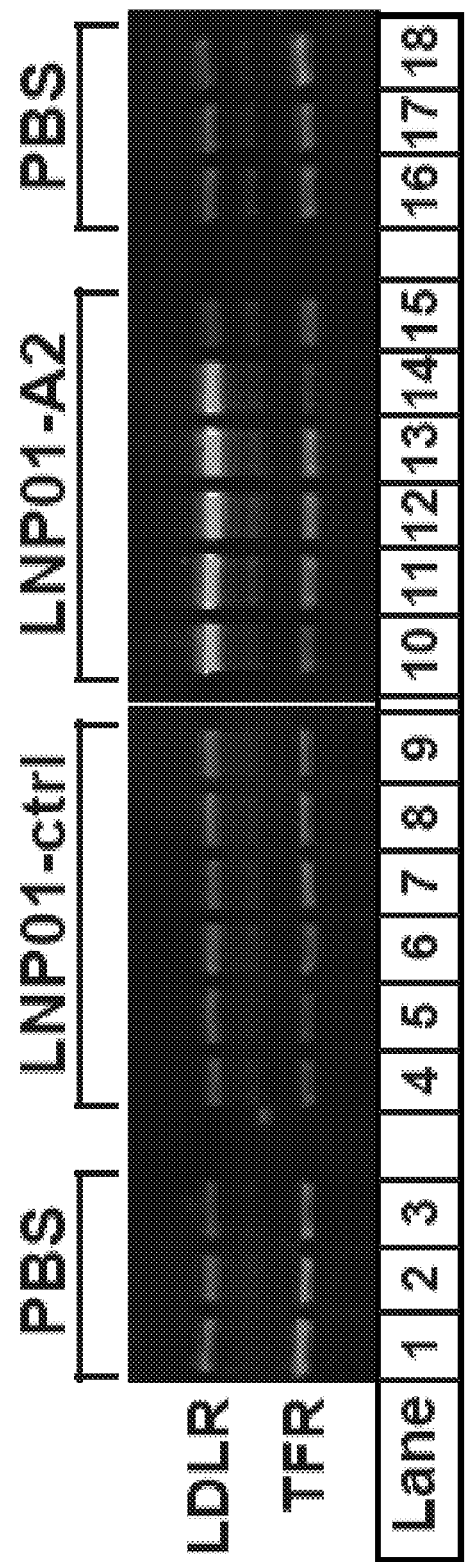
FIG. 11

FIG. 12B
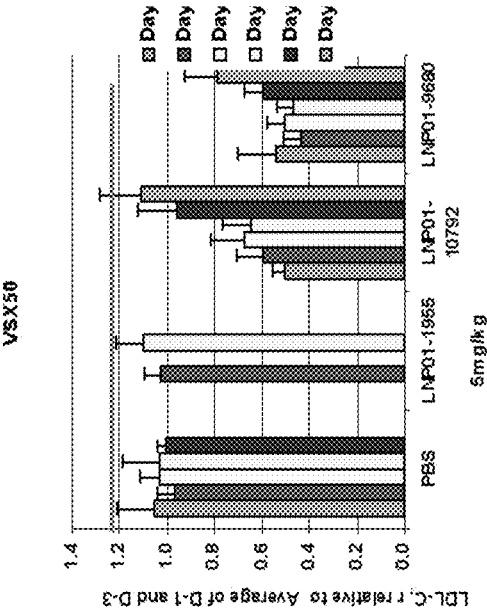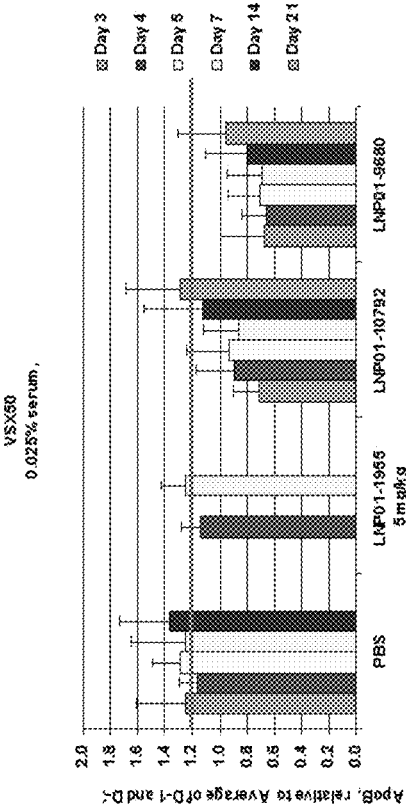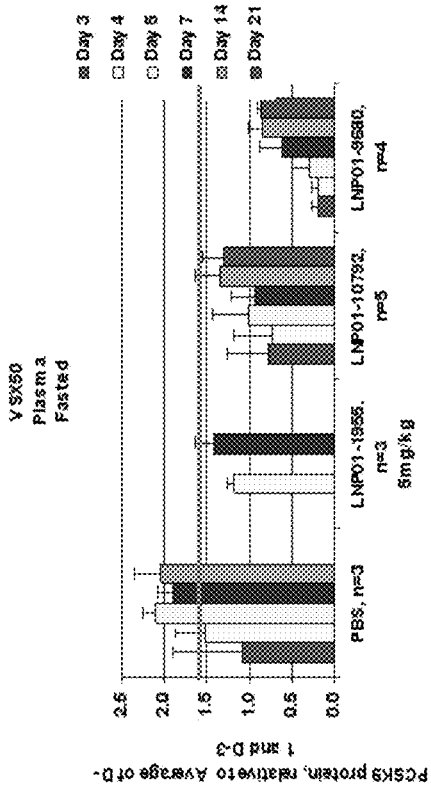
FIG. 12A

FIG. 12D

FIG. 12C
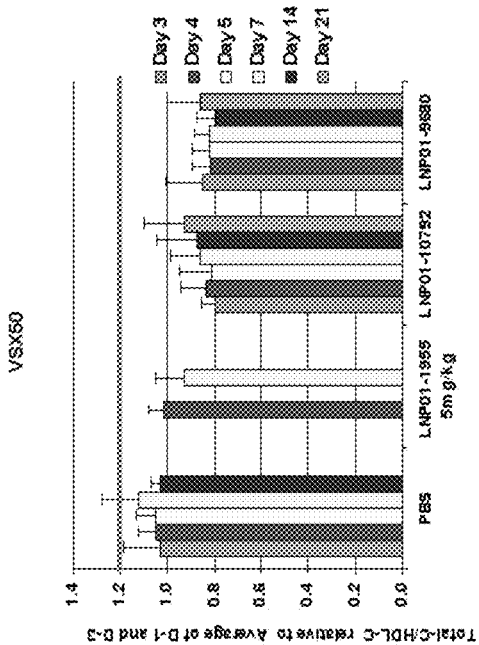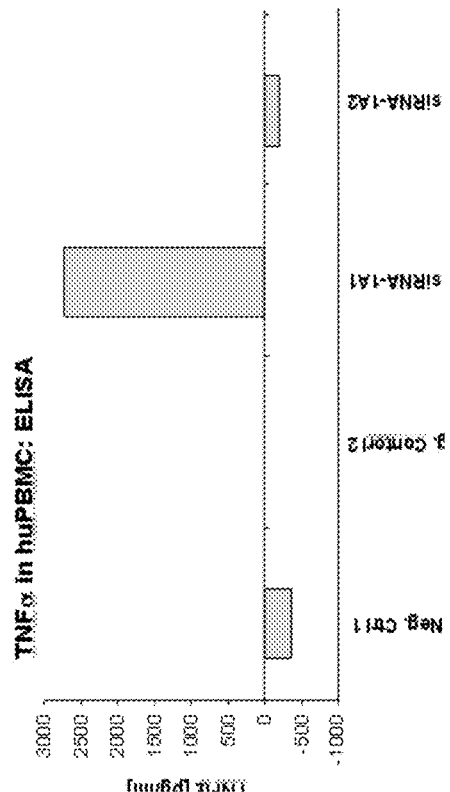
FIG. 13B
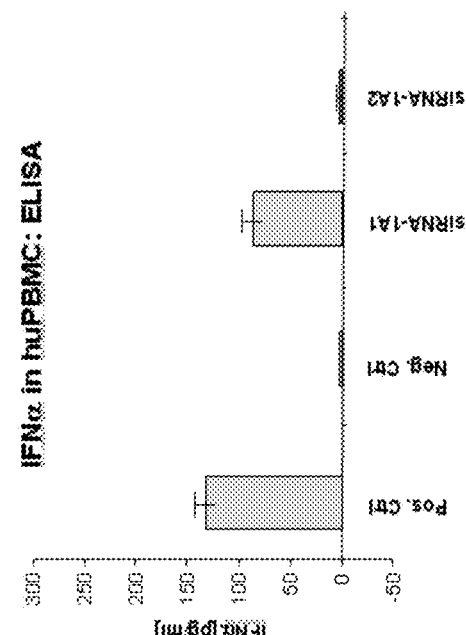
FIG. 13A
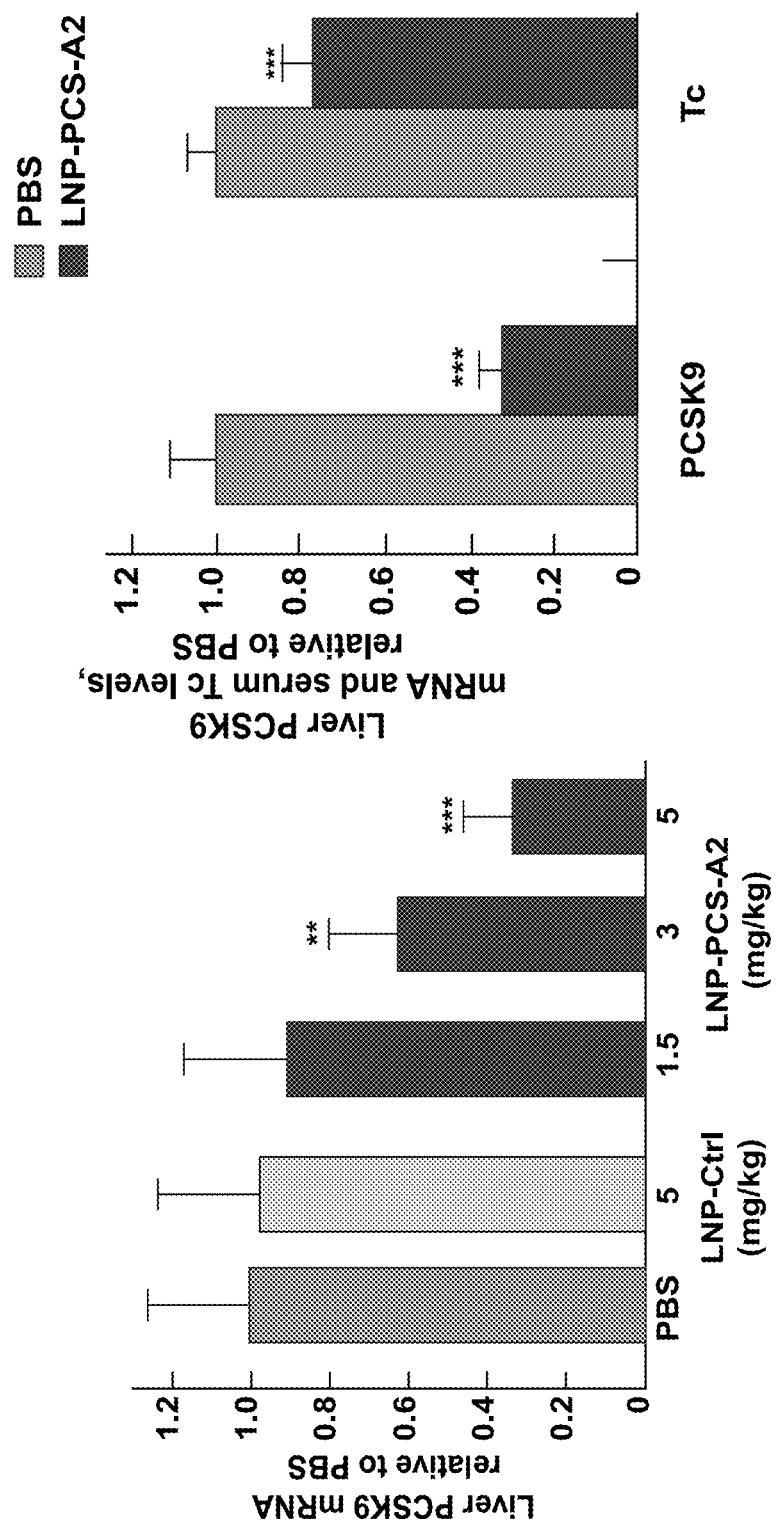
FIG. 14A

FIG. 14B

** = P<.01; *** = P<.001 (ANOVA)

FIG. 15B
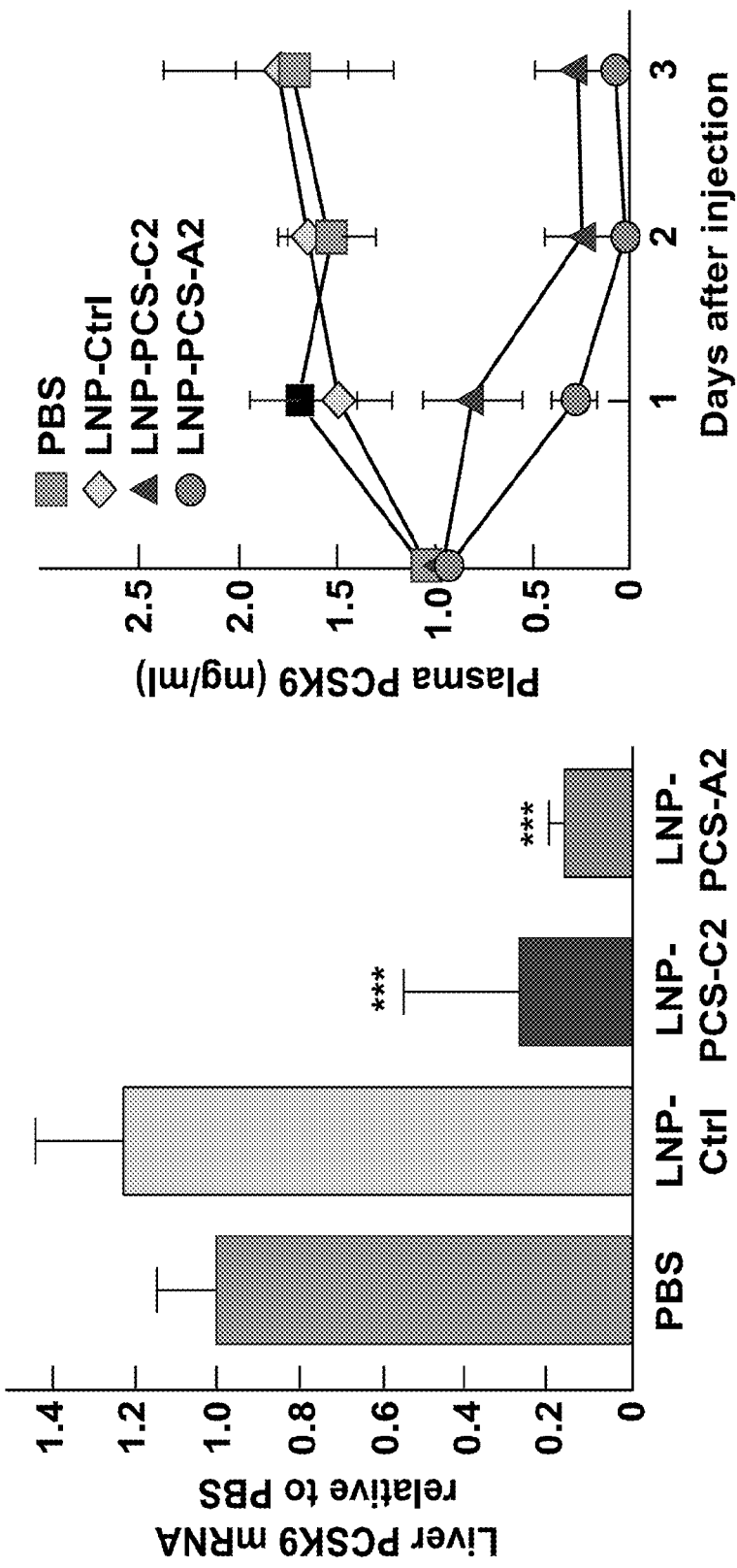
FIG. 15A

*** = P < .001 (ANOVA)

FIG. 16

X= O or S
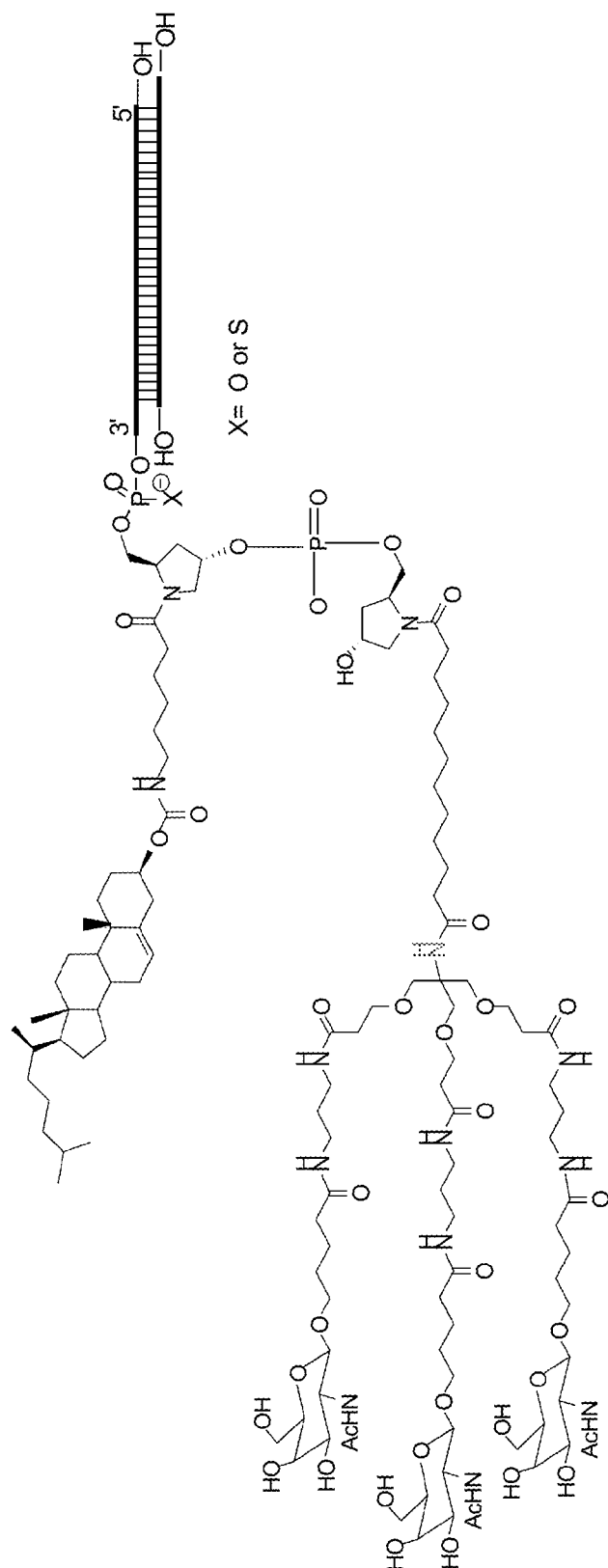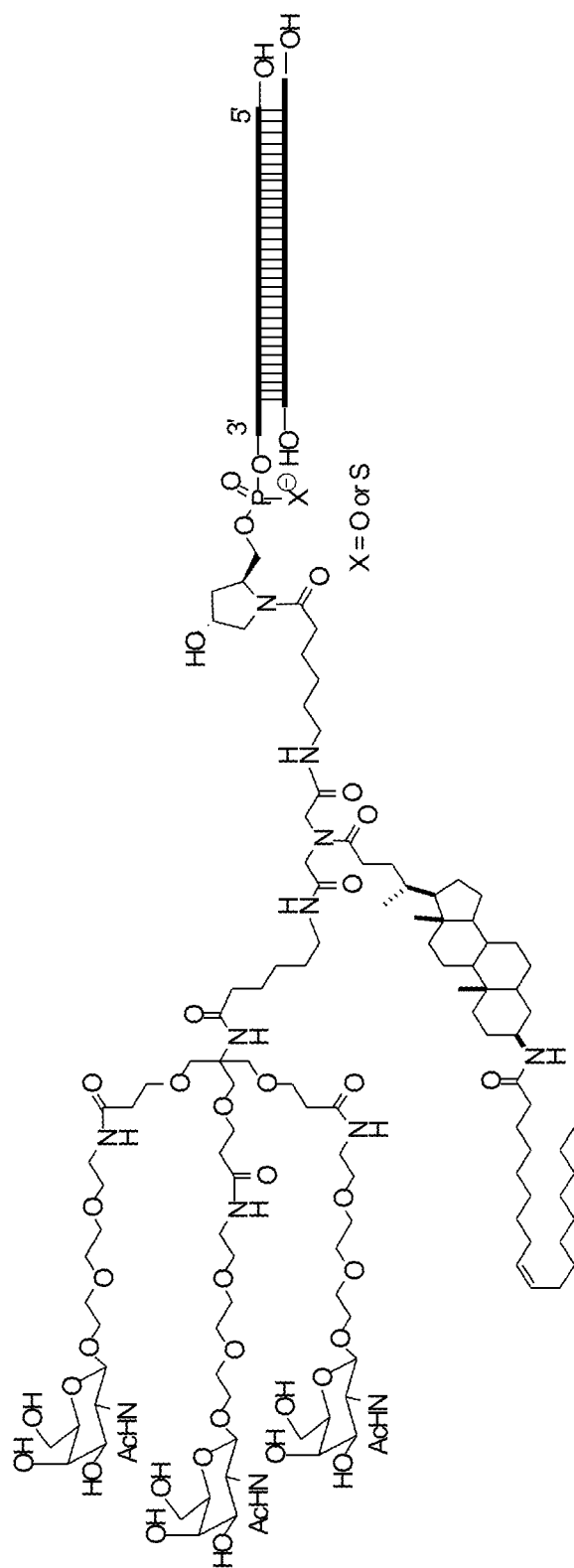
FIG. 17
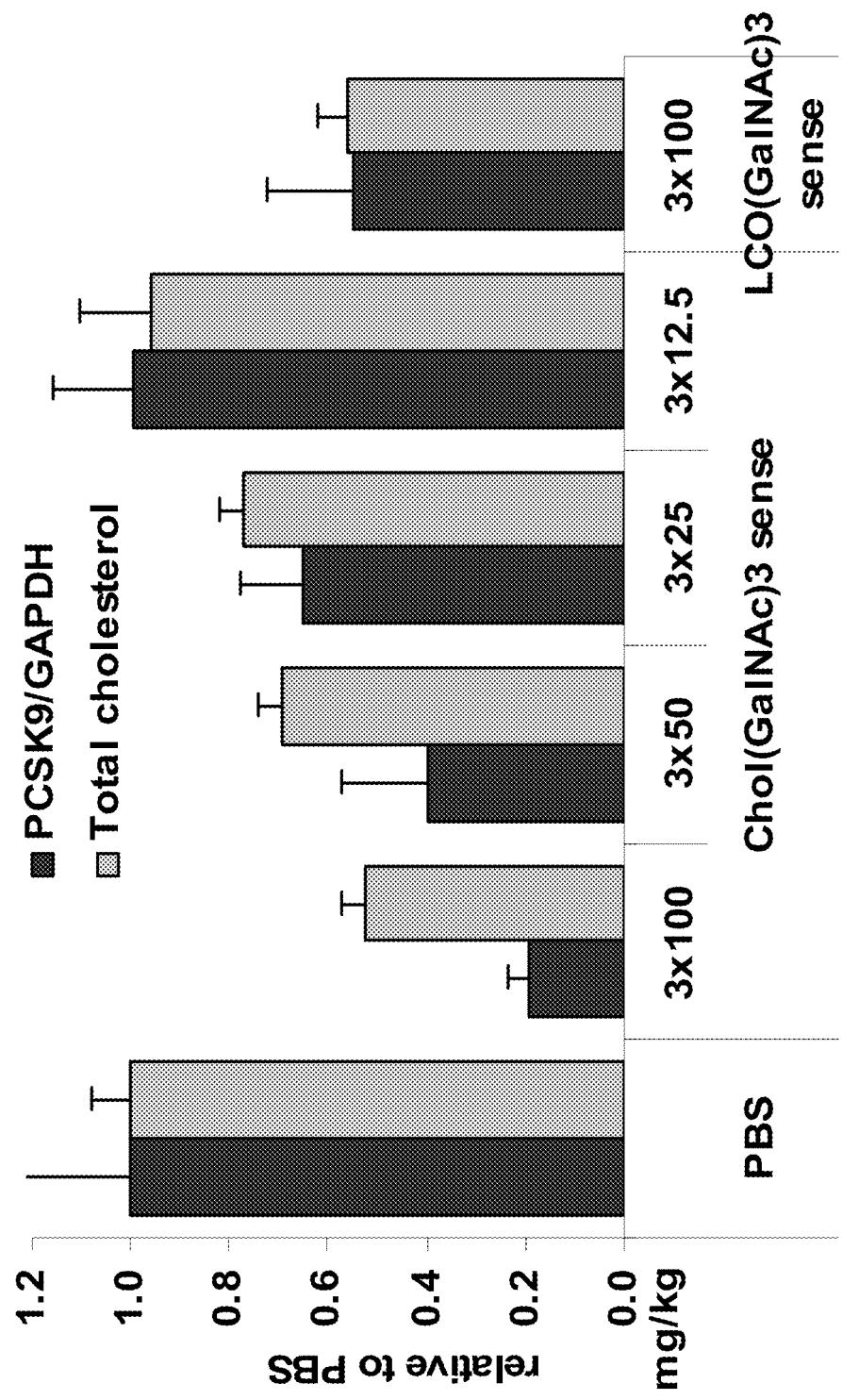
FIG. 18
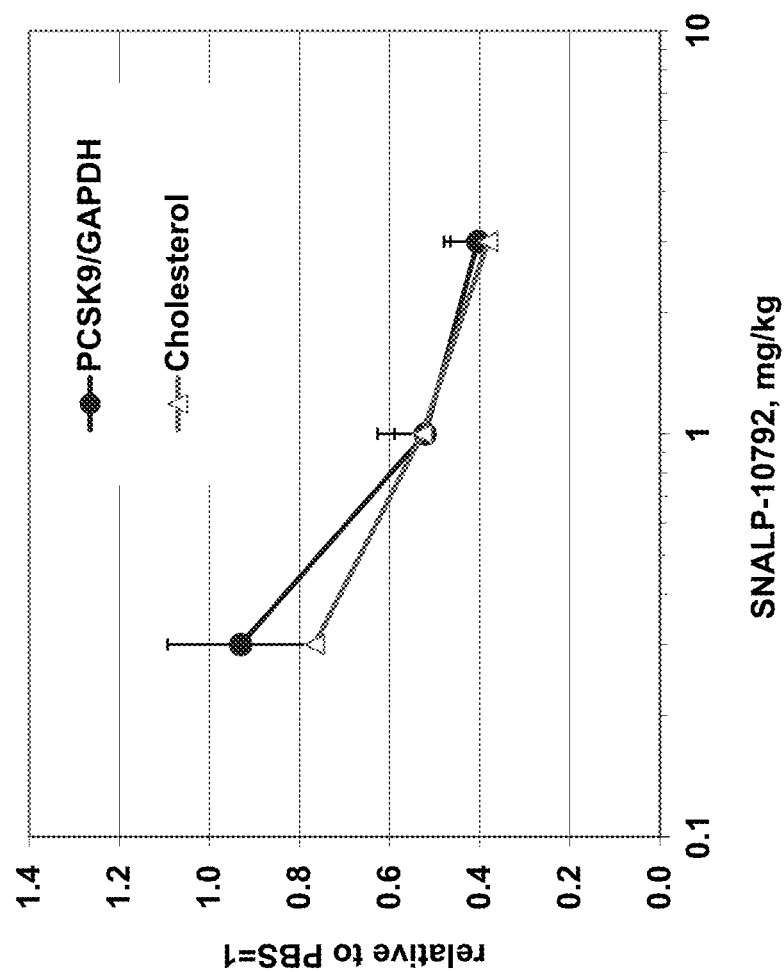
FIG. 19
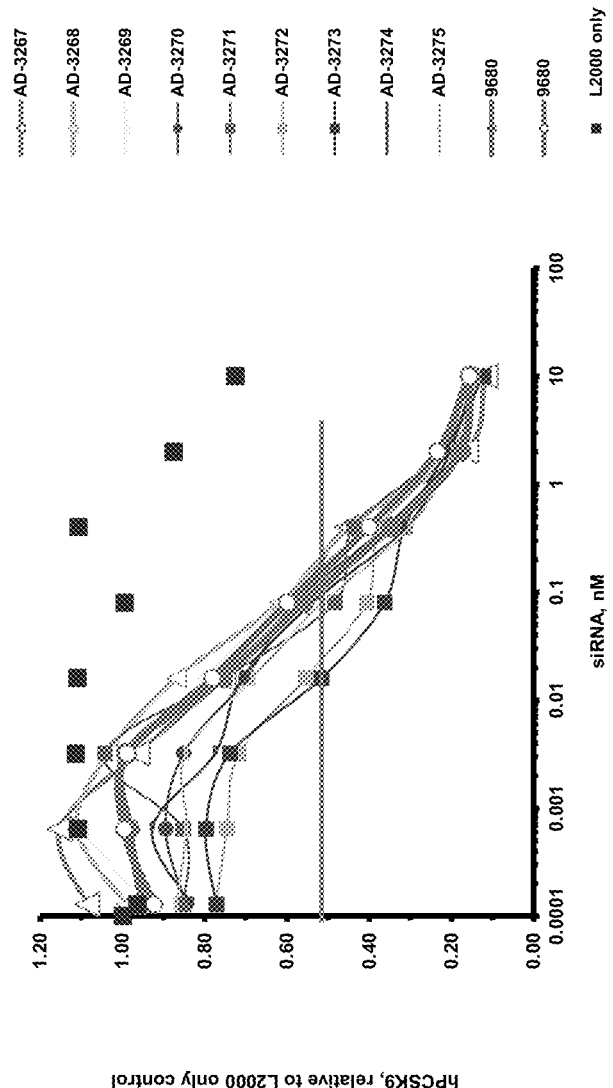
FIG. 20
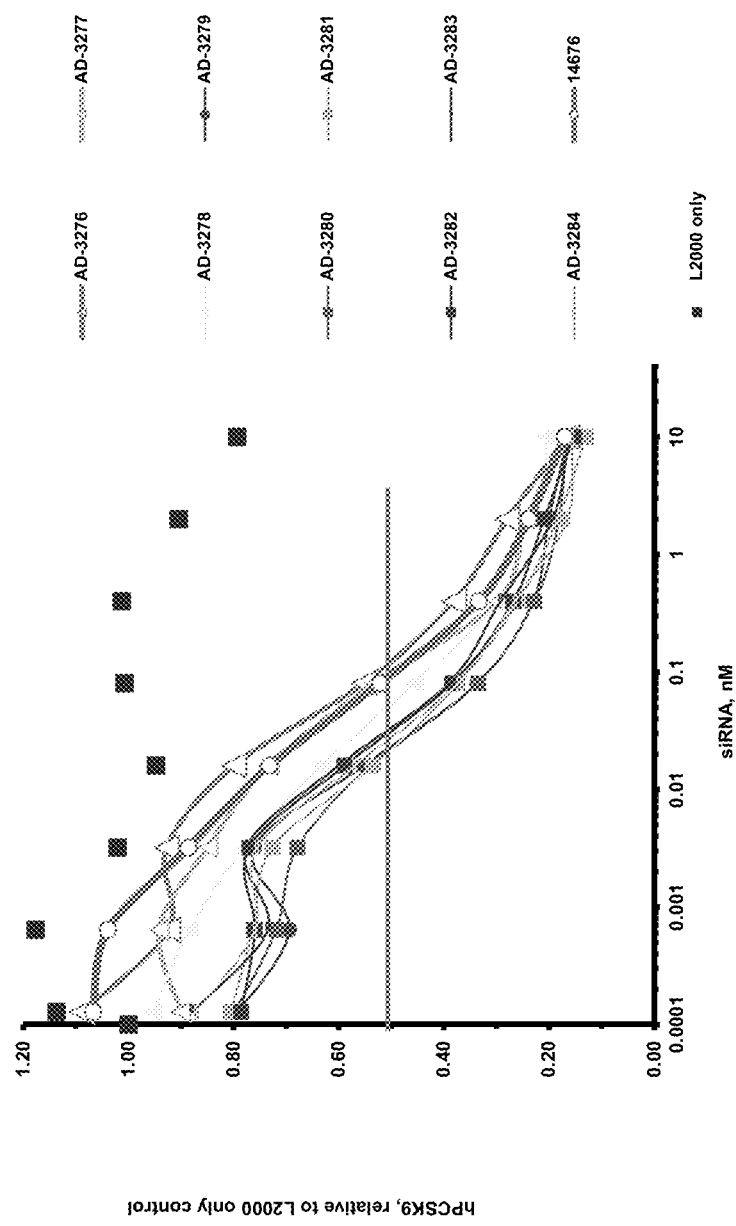
FIG. 21
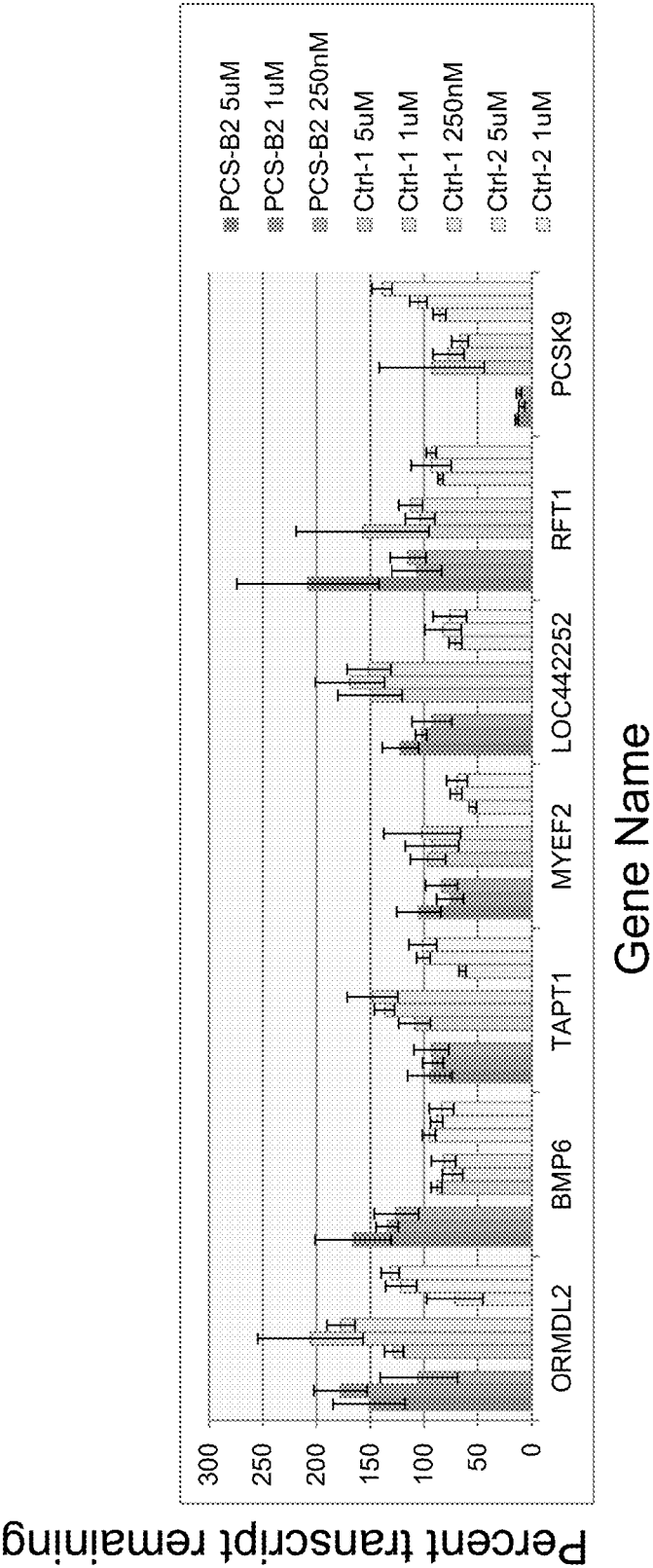
FIG. 22
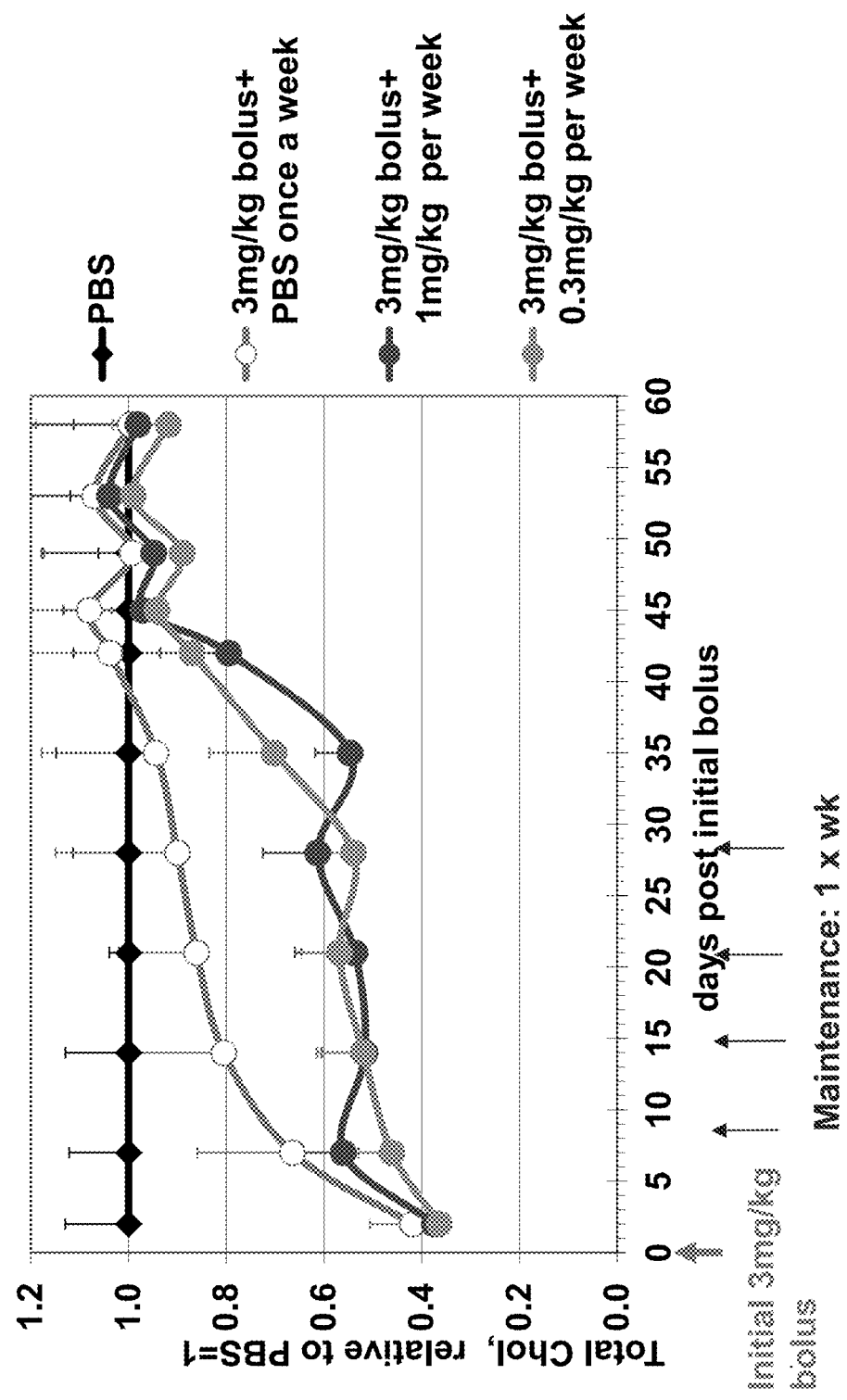
FIG. 23
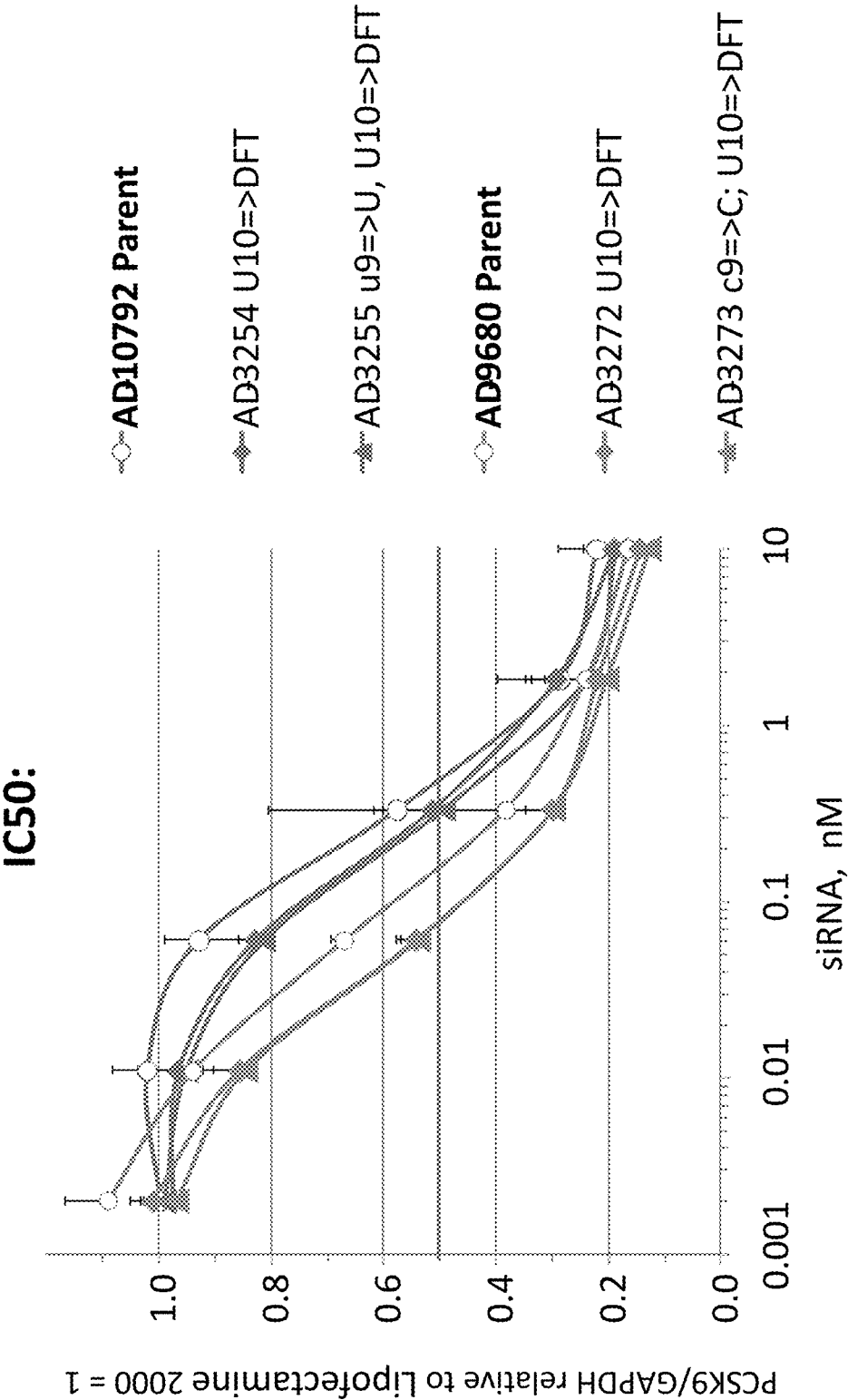
FIG. 24
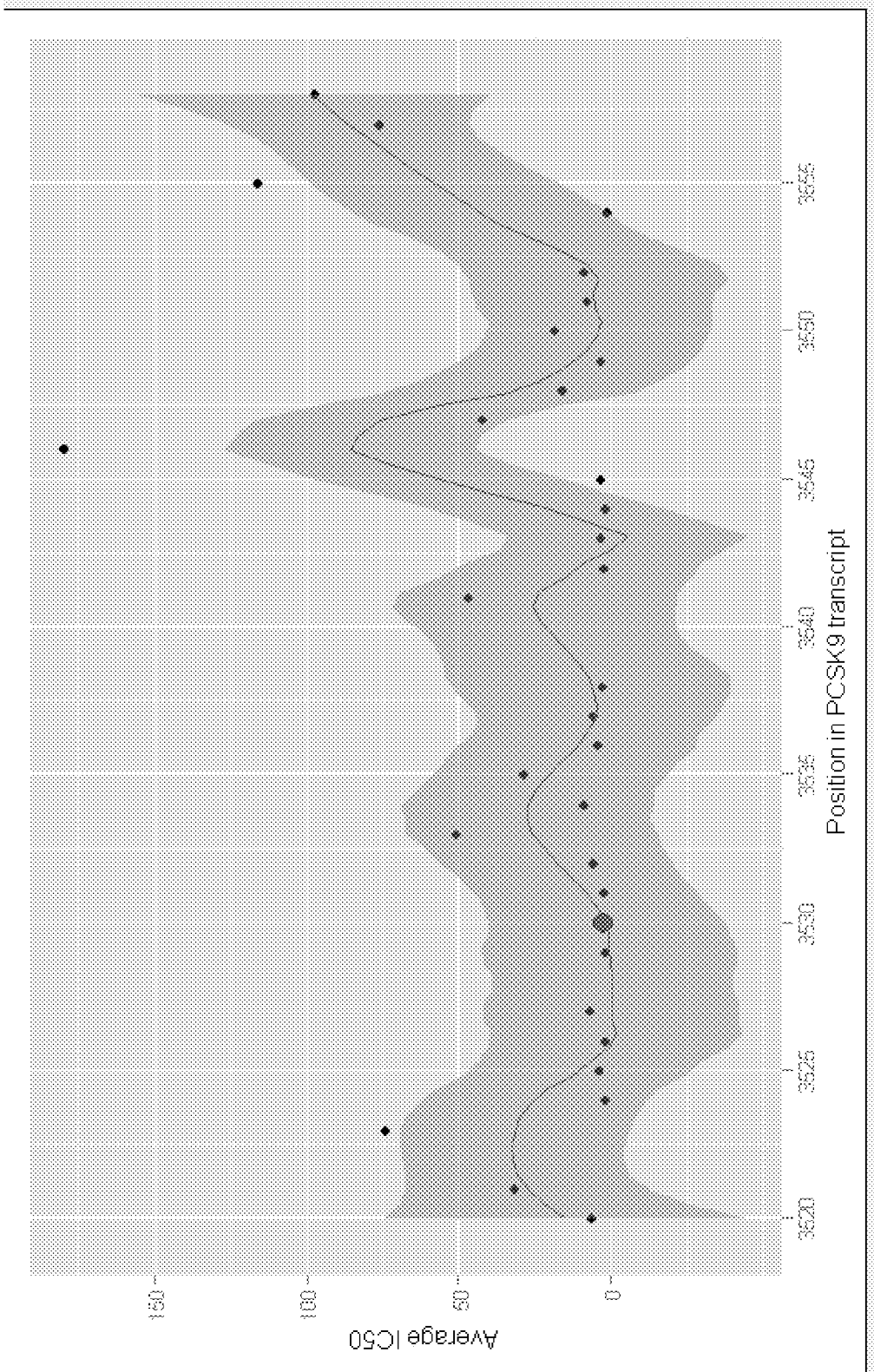
FIG. 25
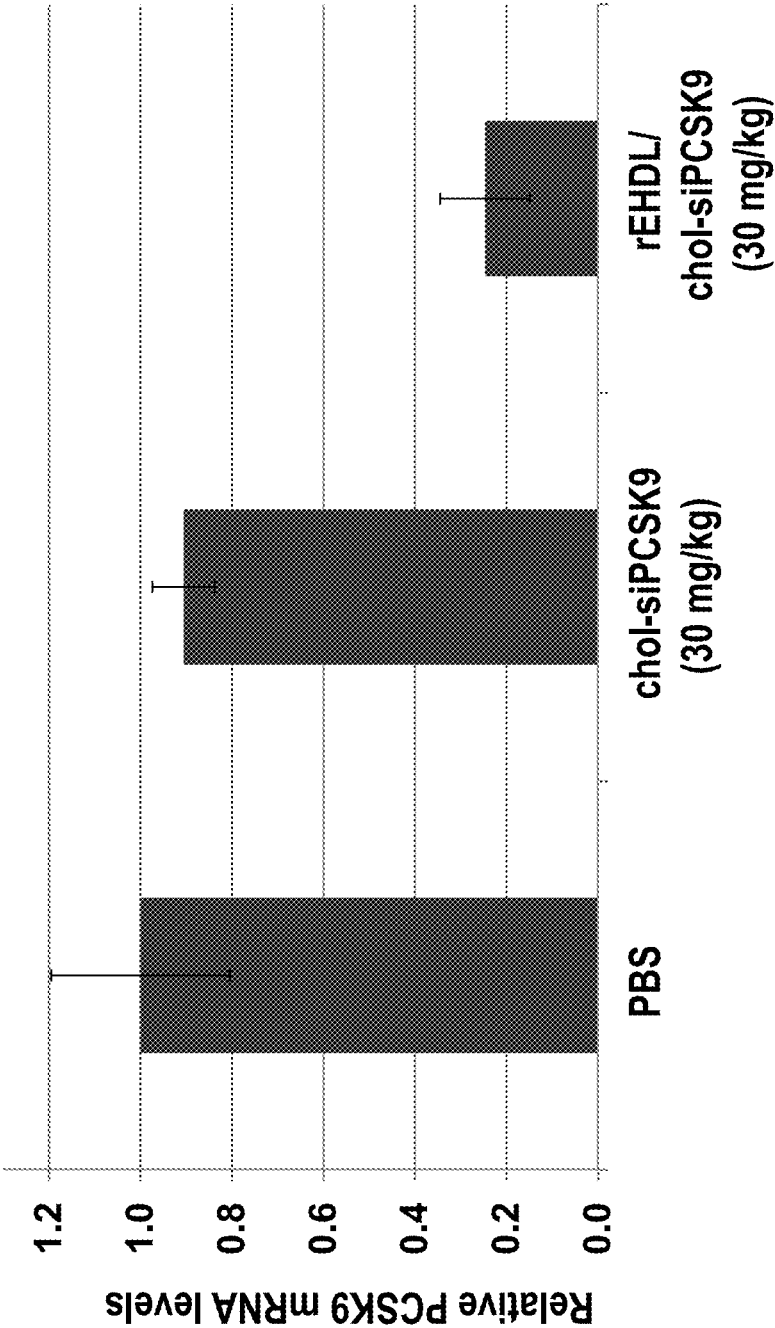
FIG. 26
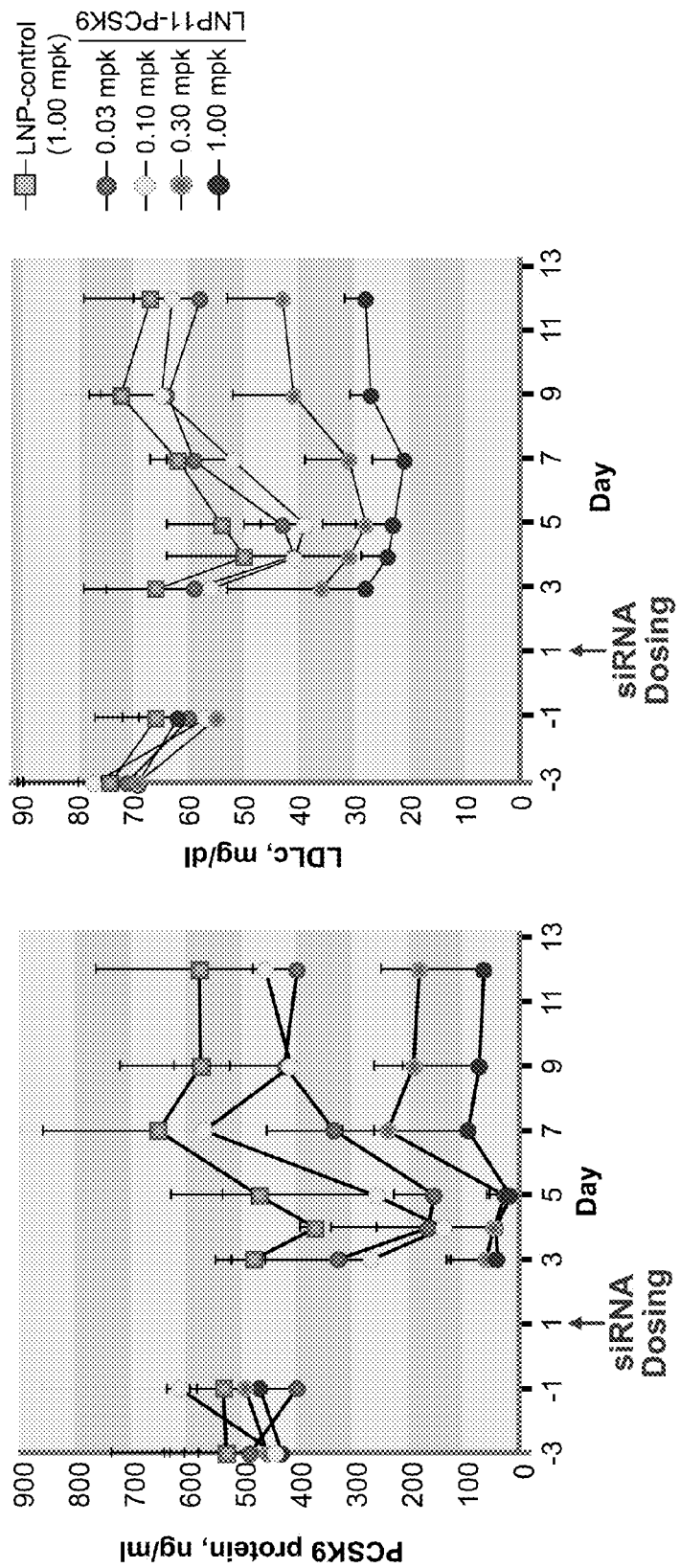
FIG. 27A
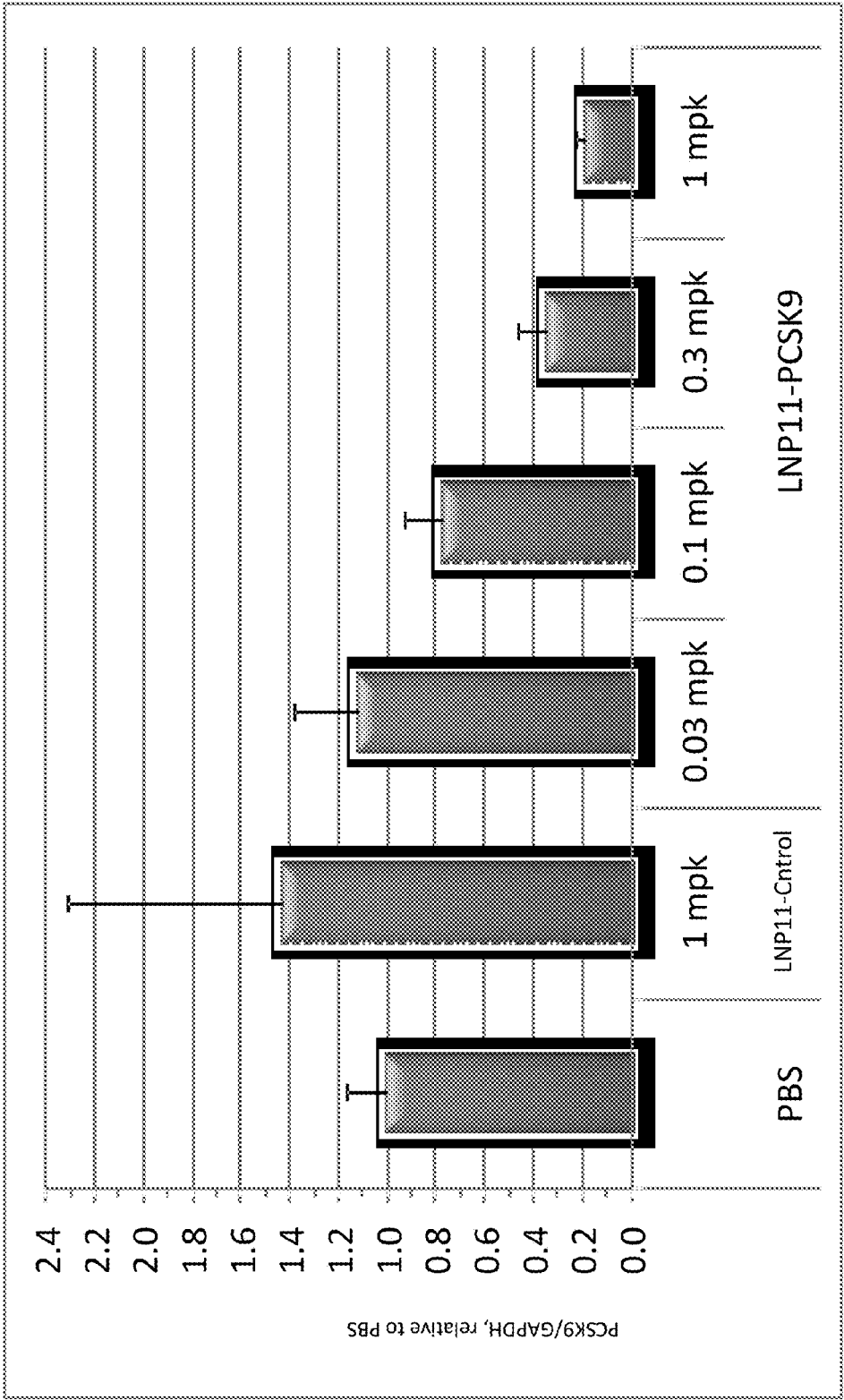
FIG. 27B
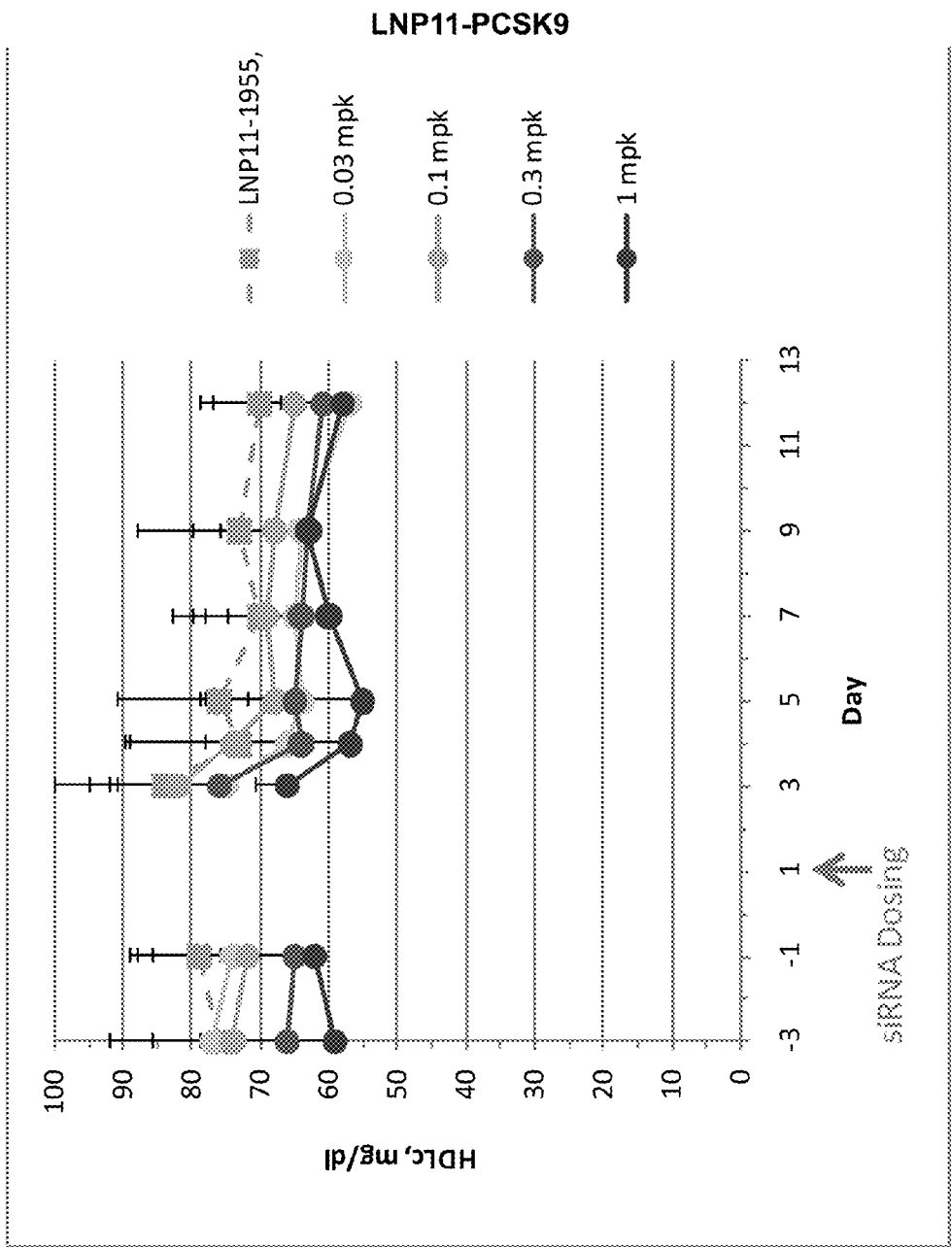
FIG. 27C
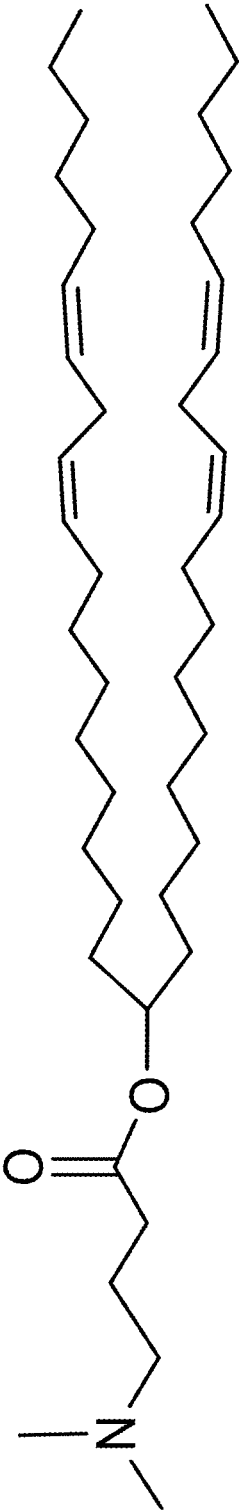
MC3
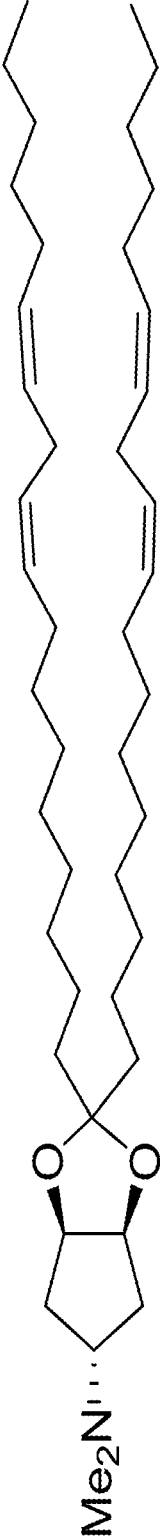
ALNY-100

FIG. 28
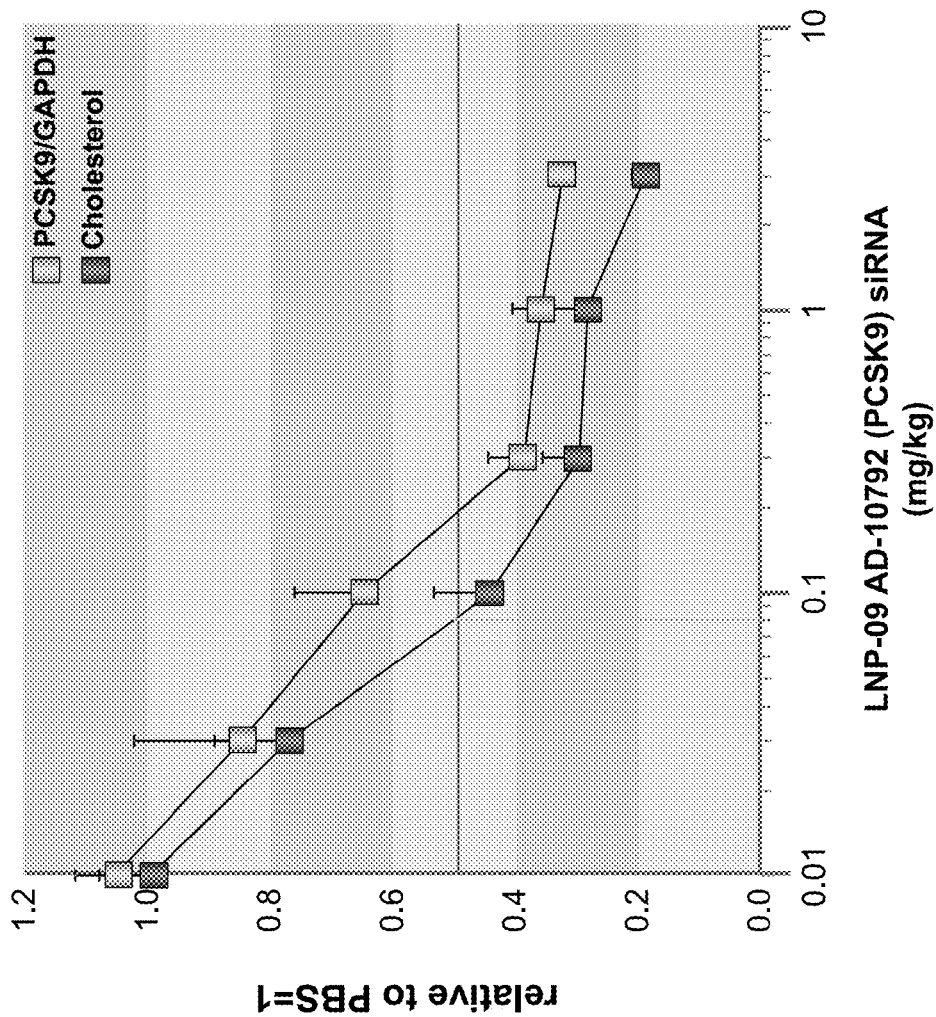
FIG. 29
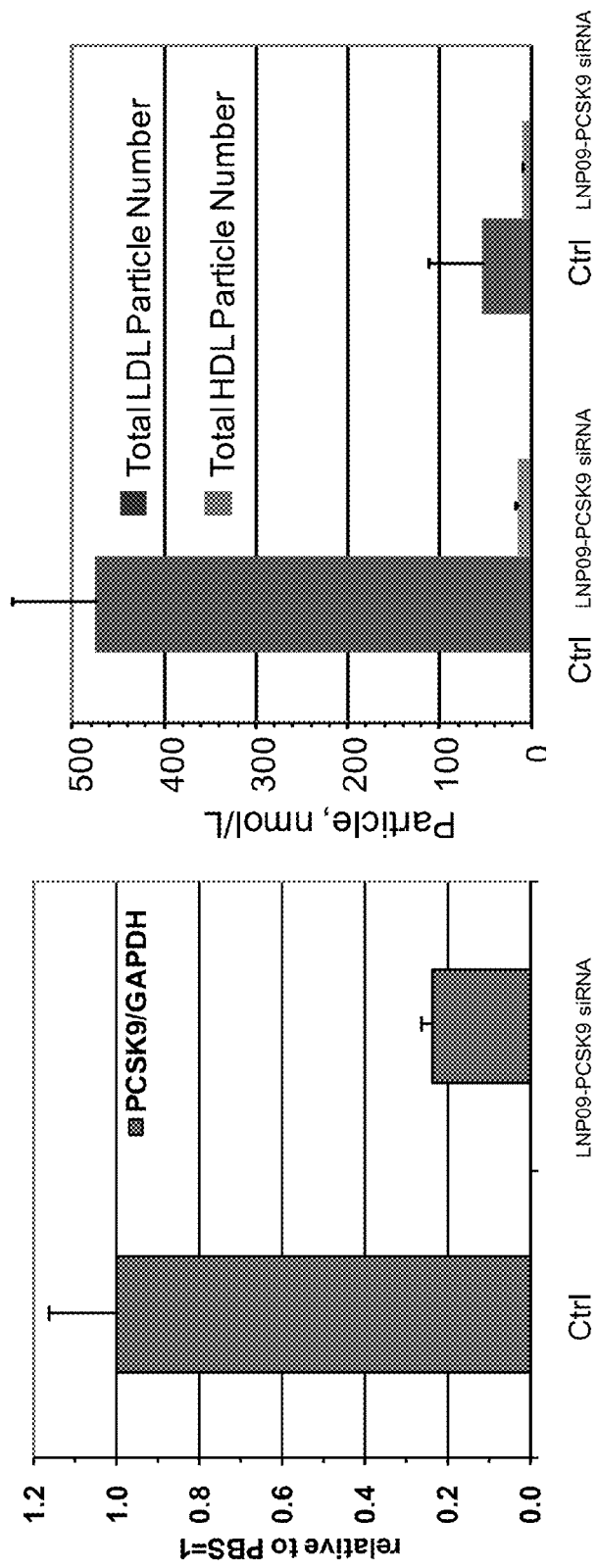
FIG. 30

LIPID FORMULATED DSRNA TARGETING THE PCSK9 GENE

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation of pending U.S. patent application Ser. No. 14/058,052 filed Oct. 18, 2013, which is a continuation of U.S. patent application Ser. No. 13/568,898, filed Aug. 7, 2012, now U.S. Pat. No. 8,598, 139, issued Dec. 3, 2013, which is a continuation of U.S. patent application Ser. No. 12/816,207, filed Jun. 15, 2010, now U.S. Pat. No. 8,273,869, issued Sep. 25, 2012, which claims the benefit of U.S. Provisional Application Ser. No. 61/187,169, filed Jun. 15, 2009; and U.S. Provisional Application Ser. No. 61/218,350, filed Jun. 18, 2009; and U.S. Provisional Application Ser. No. 61/244,790, filed Sep. 22, 2009; and U.S. Provisional Application Ser. No. 61/285,598, filed Dec. 11, 2009; and U.S. Provisional Application Ser. No. 61/293,474, filed Jan. 8, 2010; and U.S. Provisional Application Ser. No. 61/313,578, filed Mar. 12, 2010, all of which are incorporated herein by reference, in their entirety, for all purposes.

FIELD OF THE INVENTION

[0002] This invention relates to compositions comprising lipid formulated dsRNA targeting a PCSK9 gene and methods for treating diseases caused by PCSK9 gene expression.

REFERENCE TO A SEQUENCE LISTING

[0003] This application includes a Sequence Listing submitted electronically as a text file named 33334_US_sequencelisting.txt, created on Feb. 29, 2016, with a size of 565,248 bytes, with 1730 sequences. The sequence listing is incorporated by reference.

BACKGROUND OF THE INVENTION

[0004] Proprotein convertase subtilisin kexin 9 (PCSK9) is a member of the subtilisin serine protease family. The other eight mammalian subtilisin proteases, PCSK1-PCSK8 (also called PC1/3, PC2, furin, PC4, PC5/6, PACE4, PC7, and S1P/SKI-1) are proprotein convertases that process a wide variety of proteins in the secretory pathway and play roles in diverse biological processes (Bergeron, F. (2000) *J. Mol. Endocrinol.* 24, 1-22, Gensberg, K., (1998) *Semin. Cell Dev. Biol.* 9, 11-17, Seidah, N. G. (1999) *Brain Res.* 848, 45-62, Taylor, N. A., (2003) *FASEB J.* 17, 1215-1227, and Zhou, A., (1999) *J. Biol. Chem.* 274, 20745-20748). PCSK9 has been proposed to play a role in cholesterol metabolism. PCSK9 mRNA expression is down-regulated by dietary cholesterol feeding in mice (Maxwell, K. N., (2003) *J. Lipid Res.* 44, 2109-2119), up-regulated by statins in HepG2 cells (Dubuc, G., (2004) *Arterioscler. Thromb. Vasc. Biol.* 24, 1454-1459), and up-regulated in sterol regulatory element binding protein (SREBP) transgenic mice (Horton, J. D., (2003) *Proc. Natl. Acad. Sci. USA* 100, 12027-12032), similar to the cholesterol biosynthetic enzymes and the low-density lipoprotein receptor (LDLR). Furthermore, PCSK9 missense mutations have been found to be associated with a form of autosomal dominant hypercholesterolemia (Hchola3) (Abifadel, M., et al. (2003) *Nat. Genet.* 34, 154-156, Timms, K. M., (2004) *Hum. Genet.* 114, 349-353, Leren, T. P. (2004) *Clin. Genet.* 65, 419-422). PCSK9 may also play a role in determining LDL cholesterol levels in the general population, because single-nucleotide polymorphisms (SNPs) have been associated with cholesterol levels in a Japanese population (Shioji, K., (2004) *J. Hum. Genet.* 49, 109-114).

[0005] Autosomal dominant hypercholesterolemias (ADHs) are monogenic diseases in which patients exhibit elevated total and LDL cholesterol levels, tendon xanthomas, and premature atherosclerosis (Rader, D. J., (2003) 1 *Clin. Invest.* 111, 1795-1803). The pathogenesis of ADHs and a recessive form, autosomal recessive hypercholesterolemia (ARH) (Cohen, J. C., (2003) *Curr. Opin. Lipidol.* 14, 121-127), is due to defects in LDL uptake by the liver. ADH may be caused by LDLR mutations, which prevent LDL uptake, or by mutations in the protein on LDL, apolipoprotein B, which binds to the LDLR. ARH is caused by mutations in the ARH protein that are necessary for endocytosis of the LDLR-LDL complex via its interaction with clathrin. Therefore, if PCSK9 mutations are causative in Hchola3 families, it seems likely that PCSK9 plays a role in receptor-mediated LDL uptake.

[0006] Overexpression studies point to a role for PCSK9 in controlling LDLR levels and, hence, LDL uptake by the liver (Maxwell, K. N. (2004) *Proc. Natl. Acad. Sci. USA* 101, 7100-7105, Benjannet, S., et al. (2004) *J. Biol. Chem.* 279, 48865-48875, Park, S. W., (2004) *J. Biol. Chem.* 279, 50630-50638). Adenoviral-mediated overexpression of mouse or human PCSK9 for 3 or 4 days in mice results in elevated total and LDL cholesterol levels; this effect is not seen in LDLR knockout animals (Maxwell, K. N. (2004) *Proc. Natl. Acad. Sci. USA* 101, 7100-7105, Benjannet, S., et al. (2004) *J. Biol. Chem.* 279, 48865-48875, Park, S. W., (2004) *J. Biol. Chem.* 279, 50630-50638). In addition, PCSK9 overexpression results in a severe reduction in hepatic LDLR protein, without affecting LDLR mRNA levels, SREBP protein levels, or SREBP protein nuclear to cytoplasmic ratio.

[0007] Loss of function mutations in PCSK9 have been designed in mouse models (Rashid et al., (2005) *PNAS,* 102, 5374-5379), and identified in human individuals (Cohen et al. (2005) *Nature Genetics* 37:161-165). In both cases loss of PCSK9 function lead to lowering of total and LDLc cholesterol. In a retrospective outcome study over 15 years, loss of one copy of PCSK9 was shown to shift LDLc levels lower and to lead to an increased risk-benefit protection from developing cardiovascular heart disease (Cohen et al., (2006)*N Engl. J. Med.,* 354:1264-1272).

[0008] Recently, double-stranded RNA molecules (dsRNA) have been shown to block gene expression in a highly conserved regulatory mechanism known as RNA interference (RNAi). WO 99/32619 (Fire et al.) discloses the use of a dsRNA of at least 25 nucleotides in length to inhibit the expression of genes in *C. elegans*. dsRNA has also been shown to degrade target RNA in other organisms, including plants (see, e.g., WO 99/53050, Waterhouse et al.; and WO 99/61631, Heifetz et al.), *Drosophila* (see, e.g., Yang, D., et al., *Curr. Biol.* (2000) 10:1191-1200), and mammals (see WO 00/44895, Limmer; and DE 101 00 586.5, Kreutzer et al.). This natural mechanism has now become the focus for the development of a new class of pharmaceutical agents for treating disorders that are caused by the aberrant or unwanted regulation of a gene.

[0009] A description of siRNA targeting PCSK9 can be found in U.S. Pat. No. 7,605,251 and WO 2007/134161.

Additional disclosure can be found in U.S. Patent Publication No. 2010/0010066 and WO 2009/134487

SUMMARY OF THE INVENTION

[0010] As described in more detail below, disclosed herein are compositions comprising lipid formulated siRNA targeting PCSK9, e.g., MC3 formulated siRNA targeting PCSK9. Also disclosed are methods of using the compositions for inhibition of PCSK9 expression and for treatment of pathologies related to PCSK9 expression, e.g., hyperlipidemia

[0011] Accordingly, one aspect of the invention is a compositing comprising a nucleic acid lipid particle comprising a double-stranded ribonucleic acid (dsRNA) for inhibiting the expression of a human PCSK9 gene in a cell, wherein the nucleic acid lipid particle comprises a lipid formulation comprising 45-65 mol % of a cationic lipid, 5 mol % to about 10 mol %, of a non-cationic lipid, 25-40 mol % of a sterol, and 0.5-5 mol % of a PEG or PEG-modified lipid, the dsRNA consists of a sense strand and an antisense strand, and the sense strand comprises a first sequence and the antisense strand comprises a second sequence complementary to at least 15 contiguous nucleotides of SEQ ID NO:1523 (5'-UUCUAGACCUGUUUUGCUU-3'), wherein the first sequence is complementary to the second sequence and wherein the dsRNA is between 15 and 30 base pairs in length.

[0012] As described herein the composition includes a cationic lipid. In one embodiment, the cationic lipid comprises MC3 (((6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate. For example, the lipid formulation can be selected from the following:

LNP11	MC3/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5
LNP14	MC3/DSPC/Cholesterol/PEG-DMG 40/15/40/5
LNP15	MC3/DSPC/Cholesterol/PEG-DSG/GalNAc-PEG-DSG 50/10/35/4.5/0.5
LNP16	MC3/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5
LNP17	MC3/DSPC/Cholesterol/PEG-DSG 50/10/38.5/1.5
LNP18	MC3/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5
LNP19	MC3/DSPC/Cholesterol/PEG-DMG 50/10/35/5
LNP20	MC3/DSPC/Cholesterol/PEG-DPG 50/10/38.5/1.5

[0013] In other embodiments, the cationic lipid comprises formula A wherein formula A is
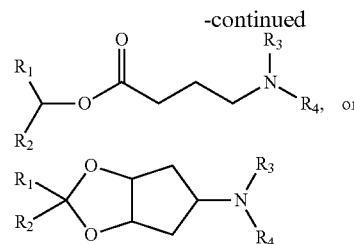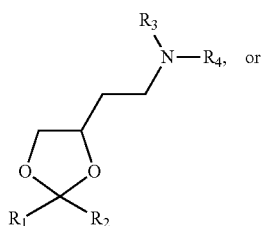
[0014] where R1 and R2 are independently alkyl, alkenyl or alkynyl, each can be optionally substituted, and R3 and R4 are independently lower alkyl or R3 and R4 can be taken together to form an optionally substituted heterocyclic ring. In some embodiments the cationic lipid comprises formula A and is XTC (2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane). The lipid formulation can include the cationic lipid XTC, the non-cationic lipid DSPC, the sterol cholesterol and the PEG lipid PEG-DMG. In other embodiments the cationic lipid comprises XTC and the formulation is selected from the group consisting of:

LNP05	XTC/DSPC/Cholesterol/PEG-DMG 57.5/7.5/31.5/3.5
LNP06	XTC/DSPC/Cholesterol/PEG-DMG 57.5/7.5/31.5/3.5
LNP07	XTC/DSPC/Cholesterol/PEG-DMG 60/7.5/31/1.5,
LNP08	XTC/DSPC/Cholesterol/PEG-DMG 60/7.5/31/1.5
LNP09	XTC/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5
LNP13	XTC/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5
LNP22	XTC/DSPC/Cholesterol/PEG-DSG 50/10/38.5/1.5

[0015] In still further embodiments, the cationic lipid comprises ALNY-100 ((3aR,5s,6aS)—N,N-dimethyl-2,2-di((9Z,12Z)-octadeca-9,12-dienyl)tetrahydro-3aH-cyclopenta[d][1,3]dioxol-5-amine)). For example, the cationic lipid comprises ALNY-100 and the formulation consists of ALNY-100/DSPC/Cholesterol/PEG-DMG in a ratio of 50/10/38.5/1.5

[0016] The composition includes a dsRNA targeting PCSK9. In some embodiments, the sense strand comprises SEQ ID NO:1227 and the antisense strand comprises SEQ ID NO:1228. In other embodiments, the sense strand consists of SEQ ID NO:1227 and the antisense strand consists of SEQ ID NO:1228. One or both strands can be modified, e.g., each strand is modified as follows to include a 2'-O-methyl ribonucleotide as indicated by a lower case letter "c" or "u" and a phosphorothioate as indicated by a lower case letter "s": the dsRNA consists of a sense strand consisting of

SEQ ID NO: 1229
(5'-uucuAGAccuGuuuuGcuuTsT-3')

[0017] and an antisense strand consisting of

SEQ ID NO: 1230
(5'-AAGcAAAAcAGGUCuAGAATsT-3').

[0018] In other embodiments, the dsRNA comprises at least one modified nucleotide, e.g., a modified nucleotide chosen from the group of: a 2'-O-methyl modified nucleotide, a nucleotide comprising a 5'-phosphorothioate group, and a terminal nucleotide linked to a cholesteryl derivative or dodecanoic acid bisdecylamide group, and/or, e.g., the modified nucleotide is chosen from the group of: a 2'-deoxy-2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an abasic nucleotide, 2'-amino-modified nucleotide, 2'-alkyl-modified nucleotide, morpholino nucleotide, a phosphoramidate, and a non-natural base comprising nucleotide. In one embodiment, dsRNA comprises at least one 2'-O-methyl modified ribonucleotide and at least one nucleotide comprising a 5'-phosphorothioate group.

[0019] The compositions include a dsRNA between 15 and 30 base pairs in length. In one embodiment, each strand of the dsRNA is 19-23 bases in length, or, e.g., 21-23 bases in length, or, e.g. 21 bases in length.

[0020] In one aspect, the compositions include a lipoprotein, e.g., apolipoprotein E (ApoE). In some embodiments, the compositions include a lipoprotein and the dsRNA is conjugated to a lipophile, e.g., a cholesterol. The ApoE can be reconstituted with at least one amphiphilic agent, e.g., a phospholipid, e.g., a phospholipid selected from the group consisting of dimyristoyl phosphatidyl choline (DMPC), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), egg phosphatidylcholine (EPC), distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), -phosphatidylethanolamine (POPE), dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), and combinations thereof. In some embodiments, the ApoE is reconstituted high density lipoprotein (rHDL).

[0021] The compositions, e.g., lipid formulated dsRNA targeting PCSK9, can be administered to a cell or subject, e.g., a primate, e.g., a human. In one aspect, administration of the compositions inhibits expression of PCSK9 by at least 40% compared to administration of a control and/or reduces PCSK9 protein levels in the mammal compared to administration of a control, and/or reduces LDLc levels in a mammal compared to administration of a control and/or reduces both PCSK9 hepatic mRNA and total serum cholesterol at a dosage of less than 0.1 mg/kg compared to administration of a control and/or reduces PCSK9 hepatic mRNA at an ED50 of about 0.2 mg/kg and reduces total serum cholesterol with an ED50 of about 0.08 mg/kg compared to administration of a control and/or reduces serum LDL particle numbers by more than 90% or reduces serum HDL particle numbers by more than 75% compared to administration of a control.

[0022] The invention also provides methods comprising administering the lipid formulated PCSK9 targeted dsRNA compositions described herein. In one embodiment, the invention includes a method for inhibiting the expression of PCSK9 in a cell comprising administering the compositions to the cell. In another embodiment, the invention includes a method for reducing LDLc levels in a mammal in need of treatment comprising administering the compositions to the mammal.

[0023] As described in more detail below, the methods can include any appropriate dosage, e.g., between 0.25 mg/kg and 4 mg/kg dsRNA, or e.g., at about 0.01, 0.1, 0.5, 1.0, 2.5, or 5.0 mg/kg dsRNA.

[0024] Also described herein are methods for inhibiting expression of a PCSK9 gene in a subject, comprising administering to the subject the lipid formulated PCSK9 targeted dsRNA compositions described herein at a first dose of about 3 mg/kg followed by administering at least one subsequent dose once a week, wherein the subsequent dose is lower than the first dose. The subject can be, e.g., a rat or a non-human primate or a human. The subsequent dose can be about 1.0 mg/kg or about 0.3 mg/kg. In some embodiments, the subsequent dose is administered once a week for four weeks. In some embodiments, administration of the first dose decreases total cholesterol levels by about 15-60%.

[0025] The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0026] The prefixes "AD-" "DP-" and "AL-DP-" are used interchangeably e.g., AL-DP-9327 and AD-9237.

[0027] FIG. 1 shows the structure of the ND-98 lipid.

[0028] FIG. 2 shows the results of the in vivo screen of 16 mouse specific (AL-DP-9327 through AL-DP-9342) PCSK9 siRNAs directed against different ORF regions of PCSK9 mRNA (having the first nucleotide corresponding to the ORF position indicated on the graph) in C57/BL6 mice (5 animals/group). The ratio of PCSK9 mRNA to GAPDH mRNA in liver lysates was averaged over each treatment group and compared to a control group treated with PBS or a control group treated with an unrelated siRNA (blood coagulation factor VII).

[0029] FIG. 3 shows the results of the in vivo screen of 16 human/mouse/rat cross-reactive (AL-DP-9311 through AL-DP-9326) PCSK9 siRNAs directed against different ORF regions of PCSK9 mRNA (having the first nucleotide corresponding to the ORF position indicated on the graph) in C57/BL6 mice (5 animals/group). The ratio of PCSK9 mRNA to GAPDH mRNA in liver lysates was averaged over each treatment group and compared to a control group treated with PBS or a control group treated with an unrelated siRNA (blood coagulation factor VII).

[0030] FIG. 4 shows the results of the in vivo screen of 16 mouse specific (AL-DP-9327 through AL-DP-9342) PCSK9 siRNAs in C57/BL6 mice (5 animals/group). Total serum cholesterol levels were averaged over each treatment group and compared to a control group treated with PBS or a control group treated with an unrelated siRNA (blood coagulation factor VII).

[0031] FIG. 5 shows the results of the in vivo screen of 16 human/mouse/rat cross-reactive (AL-DP-9311 through AL-DP-9326) PCSK9 siRNAs in C57/BL6 mice (5 animals/group). Total serum cholesterol levels were averaged over each treatment group and compared to a control group treated with PBS or a control group treated with an unrelated siRNA (blood coagulation factor VII).

[0032] FIG. 6A and FIG. 6B compare in vitro and in vivo results, respectively, for silencing PCSK9.

[0033] FIG. 7A and FIG. 7B are examples of in vitro results for silencing PCSK9 using monkey primary hepatocytes.

[0034] FIG. 7C shows results for silencing of PCSK9 in monkey primary hepatocytes using AL-DP-9680 and chemically modified version of AL-DP-9680.

[0035] FIG. 8 shows in vivo activity of LNP-01 formulated siRNAs to PCSK-9.

[0036] FIG. 9A and FIG. 9B show in vivo activity of LNP-01 Formulated chemically modified 9314 and derivatives with chemical modifications such as AD-10792, AD-12382, AD-12384, AD-12341 at different times post a single dose in mice.

[0037] FIG. 10A shows the effect of PCSK9 siRNAs on PCSK9 transcript levels and total serum cholesterol levels in rats after a single dose of formulated AD-10792. FIG. 10B shows the effect of PCSK9 siRNAs on serum total cholesterol levels in the experiment as FIG. 10A. A single dose of formulated AD-10792 results in an ~60% lowering of total cholesterol in the rats that returns to baseline by ~3-4 weeks. FIG. 10C shows the effect of PCSK9 siRNAs on hepatic cholesterol and triglyceride levels in the same experiment as FIG. 10A.

[0038] FIG. 11 is a Western blot showing that liver LDL receptor levels were upregulated following administration of PCSK9 siRNAs in rat.

[0039] FIG. 12A, FIG. 12B, FIG. 12C, and FIG. 12D show the effects of PCSK9 siRNAs on LDLc and ApoB protein levels, total cholesterol/HDLc ratios, and PCSK9 protein levels, respectively, in nonhuman primates following a single dose of formulated AD-10792 or AD-9680.

[0040] FIG. 13A is a graph showing that unmodified siRNA-AD-A1A (AD-9314), but not 2'OMe modified siRNA-AD-1A2 (AD-10792), induced IFN-alpha in human primary blood monocytes. FIG. 13B is a graph showing that unmodified siRNA-AD-A1A (AD-9314), but not 2'OMe modified siRNA-AD-1A2 (AD-10792), also induced TNF-alpha in human primary blood monocytes.

[0041] FIG. 14A is a graph showing that the PCSK9 siRNA siRNA-AD-1A2 (a.k.a. LNP-PCS-A2 or a.k.a. "formulated AD-10792") decreased PCSK9 mRNA levels in mice liver in a dose-dependent manner. FIG. 14B is a graph showing that single administration of 5 mg/kg siRNA-AD-1A2 decreased serum total cholesterol levels in mice within 48 hours.

[0042] FIG. 15A is a graph showing that PCSK9 siRNAs targeting human and monkey PCSK9 (LNP-PCS-C2) (a.k.a. "formulated AD-9736"), and PCSK9 siRNAs targeting mouse PCSK9 (LNP-PCS-A2) (a.k.a. "formulated AD-10792"), reduced liver PCSK9 levels in transgenic mice expressing human PCSK9. FIG. 15B is a graph showing that LNP-PCS-C2 and LNP-PCS-A2 reduced plasma PCSK9 levels in the same transgenic mice.

[0043] FIG. 16 shows the structure of an siRNA conjugated to Chol-p-(GalNAc)3 via phosphate linkage at the 3' end.

[0044] FIG. 17 shows the structure of an siRNA conjugated to LCO(GalNAc)3 (a (GalNAc)3-3'-Lithocholicoleoyl siRNA Conjugate).

[0045] FIG. 18 is a graph showing the results of conjugated siRNAs on PCSK9 transcript levels and total serum cholesterol in mice.

[0046] FIG. 19 is a graph showing the results of lipid formulated siRNAs on PCSK9 transcript levels and total serum cholesterol in rats.

[0047] FIG. 20 is a graph showing the results of siRNA transfection on PCSK9 transcript levels in HeLa cells using AD-9680 and variations of AD-9680 as described in Table 6.

[0048] FIG. 21 is a graph showing the results of siRNA transfection on PCSK9 transcript levels in HeLa cells using AD-14676 and variations of AD-14676 as described in Table 6.

[0049] FIG. 22 is a graph with the results of PCSK9 targeted siRNA transfection of Hep3B cells and the effects on PCSK9 and off-target gene levels.

[0050] FIG. 23 shows the results of treatment in rats with a maintenance dose of PCSK9 targeted siRNA.

[0051] FIG. 24 is a dose response curve of treatment of HeLa cells with modified siRNAs.

[0052] FIG. 25 is a graph of average IC50 of siRNA vs. target position in human PCSK9 transcript. The large blue dot indicates the IC50 and location of AD-9680.

[0053] FIG. 26 is a graph with the results of administration of rEHDL formulated cholesterol conjugated siRNA.

[0054] FIG. 27A is a graph with results of administration of second generation LNP formulated PCSK9 targeted siRNA (AD-9680 in LNP11) to non-human primates, demonstrating a reduction in both PCSK9 protein and LDLc levels. LDLc: low density lipoprotein cholesterol; mpk: mg per kg.

[0055] FIG. 27B is a bar graph showing dose dependent PCSK mRNA silencing in non-human primates after treatment with LNP formulated siRNA targeting PCSK9.

[0056] FIG. 27C is a graph with the results of administration of second generation LNP formulated PCSK9 targeted siRNA (AD-9680) to non-human primates, demonstrating a no change in HDLc levels.

[0057] FIG. 28 illustrates the chemical structures of the cationic lipids MC3 and ALNY-100.

[0058] FIG. 29 is a graph of effects on PCSK9 mRNA and serum cholesterol levels in rats after administration of LNP-09 formulated AD-10792, an siRNA targeting rodent PCSK9.

[0059] FIG. 30 are graphs of the effects on PCSK9 mRNA and LDL/HDL particle numbers in CETP/ApoB tg mice after administration of LNP-09 formulated AD-10792, an siRNA targeting rodent PCSK9.

DETAILED DESCRIPTION OF THE INVENTION

[0060] The invention provides a solution to the problem of treating diseases that can be modulated by the down regulation of the PCSK9 gene, such as hyperlipidemia, by using double-stranded ribonucleic acid (dsRNA) to silence the PCSK9 gene.

[0061] The invention provides compositions and methods for inhibiting the expression of the PCSK9 gene in a subject using a dsRNA. The invention also provides compositions and methods for treating pathological conditions and diseases, such as hyperlipidemia, that can be modulated by down regulating the expression of the PCSK9 gene. dsRNA directs the sequence-specific degradation of mRNA through a process known as RNA interference (RNAi).

[0062] The dsRNA useful for the compositions and methods of an invention include an RNA strand (the antisense

strand) having a region that is less than 30 nucleotides in length, generally 19-24 nucleotides in length, and is substantially complementary to at least part of an mRNA transcript of the PCSK9 gene. The use of these dsRNAs enables the targeted degradation of an mRNA that is involved in the regulation of the LDL Receptor and circulating cholesterol levels. Using cell-based and animal assays, the present inventors have demonstrated that very low dosages of these dsRNAs can specifically and efficiently mediate RNAi, resulting in significant inhibition of expression of the PCSK9 gene. Thus, methods and compositions including these dsRNAs are useful for treating pathological processes that can be mediated by down regulating PCSK9, such as in the treatment of hyperlipidemia.

[0063] The following detailed description discloses how to make and use the dsRNA and compositions containing dsRNA to inhibit the expression of the target PCSK9 gene, as well as compositions and methods for treating diseases that can be modulated by down regulating the expression of PCSK9, such as hyperlipidemia. The pharmaceutical compositions of the invention include a dsRNA having an antisense strand having a region of complementarity that is less than 30 nucleotides in length, generally 19-24 nucleotides in length, and that is substantially complementary to at least part of an RNA transcript of the PCSK9 gene, together with a pharmaceutically acceptable carrier.

[0064] Accordingly, certain aspects of the invention provide pharmaceutical compositions including the dsRNA that targets PCSK9 together with a pharmaceutically acceptable carrier, methods of using the compositions to inhibit expression of the PCSK9 gene, and methods of using the pharmaceutical compositions to treat diseases by down regulating the expression of PCSK9.

DEFINITIONS

[0065] For convenience, the meaning of certain terms and phrases used in the specification, examples, and appended claims, are provided below. If there is an apparent discrepancy between the usage of a term in other parts of this specification and its definition provided in this section, the definition in this section shall prevail.

[0066] "G," "C," "A" and "U" each generally stand for a nucleotide that contains guanine, cytosine, adenine, and uracil as a base, respectively. "T" and "dT" are used interchangeably herein and refer to a deoxyribonucleotide wherein the nucleobase is thymine, e.g., deoxyribothymine. However, it will be understood that the term "ribonucleotide" or "nucleotide" or "deoxyribonucleotide" can also refer to a modified nucleotide, as further detailed below, or a surrogate replacement moiety. The skilled person is well aware that guanine, cytosine, adenine, and uracil may 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 may base pair with nucleotides containing adenine, cytosine, or uracil. Hence, nucleotides containing uracil, guanine, or adenine may be replaced in the nucleotide sequences of the invention by a nucleotide containing, for example, inosine. Sequences comprising such replacement moieties are embodiments of the invention.

[0067] As used herein, "PCSK9" refers to the proprotein convertase subtilisin kexin 9 gene or protein (also known as FH3, HCHOLA3, NARC-1, NARC1). Examples of mRNA

sequences to PCSK9 include but are not limited to the following: human: NM_174936; mouse: NM_153565, and rat: NM_199253. Additional examples of PCSK9 mRNA sequences are readily available using, e.g., GenBank.

[0068] As used herein, "target sequence" refers to a contiguous portion of the nucleotide sequence of an mRNA molecule formed during the transcription of the PCSK9 gene, including mRNA that is a product of RNA processing of a primary transcription product.

[0069] 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.

[0070] As used herein, and unless otherwise indicated, the term "complementary," when used to describe a first nucleotide sequence in relation to a second nucleotide sequence, refers to the ability of an oligonucleotide or polynucleotide comprising the first nucleotide sequence to hybridize and form a duplex structure under certain conditions with an oligonucleotide or polynucleotide comprising the second nucleotide sequence, as will be understood by the skilled person. Such conditions can, for example, be stringent conditions, where stringent conditions may include: 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50° C. or 70° C. for 12-16 hours followed by washing. Other conditions, such as physiologically relevant conditions as may 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.

[0071] This includes base-pairing of the oligonucleotide or polynucleotide having the first nucleotide sequence to the oligonucleotide or polynucleotide having the second nucleotide sequence over the entire length of the first and second nucleotide sequences. Such sequences can be referred to as "fully complementary" with respect to each other. 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 may form one or more, but generally not more than 4, 3 or 2 mismatched base pairs upon hybridization, while retaining the ability to hybridize under the conditions most relevant to their ultimate application. 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 having one oligonucleotide 21 nucleotides in length and another oligonucleotide 23 nucleotides in length, wherein the longer oligonucleotide has a sequence of 21 nucleotides that is fully complementary to the shorter oligonucleotide, may yet be referred to as "fully complementary."

[0072] "Complementary" sequences, as used herein, may also include, or be formed entirely from, non-Watson-Crick base pairs and/or base pairs formed from non-natural and modified nucleotides, in as far as the above requirements with respect to their ability to hybridize are fulfilled. Such non-Watson-Crick base pairs includes, but not limited to, G:U Wobble or Hoogstein base pairing.

[0073] The terms "complementary", "fully complementary" and "substantially complementary" herein may be used with respect to the base matching between the sense strand and the antisense strand of a dsRNA, or between the

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antisense strand of a dsRNA and a target sequence, as will be understood from the context of their use.

[0074] As used herein, a polynucleotide which 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., encoding PCSK9) including a 5" UTR, an open reading frame (ORF), or a 3' UTR. For example, a polynucleotide is complementary to at least a part of a PCSK9 mRNA if the sequence is substantially complementary to a non-interrupted portion of an mRNA encoding PCSK9.

[0075] The term "double-stranded RNA" or "dsRNA", as used herein, refers a duplex structure comprising two anti-parallel and substantially complementary, as defined above, nucleic acid strands. In general, the majority of nucleotides of each strand are ribonucleotides, but as described in detail herein, each or both strands can also include at least one non-ribonucleotide, e.g., a deoxyribonucleotide and/or a modified nucleotide. In addition, as used in this specification, "dsRNA" may include chemical modifications to ribonucleotides, including substantial modifications at multiple nucleotides and including all types of modifications disclosed herein or known in the art. Any such modifications, as used in an siRNA type molecule, are encompassed by "dsRNA" for the purposes of this specification and claims.

[0076] The two strands forming the duplex structure may be different portions of one larger RNA molecule, or they may be separate RNA molecules. Where separate RNA molecules, such dsRNA are often referred to in the literature as siRNA ("short interfering RNA"). 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", "short hairpin RNA" or "shRNA". 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, a dsRNA may comprise one or more nucleotide overhangs. In general, the majority of nucleotides of each strand are ribonucleotides, but as described in detail herein, each or both strands can also include at least one non-ribonucleotide, e.g., a deoxyribonucleotide and/or a modified nucleotide. In addition, as used in this specification, "dsRNA" may include chemical modifications to ribonucleotides, including substantial modifications at multiple nucleotides and including all types of modifications disclosed herein or known in the art. Any such modifications, as used in an siRNA type molecule, are encompassed by "dsRNA" for the purposes of this specification and claims.

[0077] As used herein, a "nucleotide overhang" refers to the unpaired nucleotide or nucleotides that protrude from the duplex structure of a dsRNA when a 3'-end of one strand of the dsRNA extends beyond the 5'-end of the other strand, or vice versa. "Blunt" or "blunt end" means that there are no unpaired nucleotides at that end of the dsRNA, i.e., no nucleotide overhang. A "blunt ended" dsRNA is a dsRNA that is double-stranded over its entire length, i.e., no nucleotide overhang at either end of the molecule. For clarity, chemical caps or non-nucleotide chemical moieties conjugated to the 3' end or 5' end of an siRNA are not considered in determining whether an siRNA has an overhang or is blunt ended.

[0078] The term "antisense strand" refers to the strand of a dsRNA which includes a region that is substantially complementary to a target sequence. 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, as defined herein. Where the region of complementarity is not fully complementary to the target sequence, the mismatches may be in the internal or terminal regions of the molecule. Generally the most tolerated mismatches are in the terminal regions, e.g., within 6, 5, 4, 3, or 2 nucleotides of the 5' and/or 3' terminus.

[0079] The term "sense strand," as used herein, refers to the strand of a dsRNA that includes a region that is substantially complementary to a region of the antisense strand.

[0080] "Introducing into a cell", when referring to a dsRNA, means facilitating uptake or absorption into the cell, as is understood by those skilled in the art. Absorption or uptake of dsRNA can occur through unaided diffusive or active cellular processes, or by auxiliary agents or devices. The meaning of this term is not limited to cells in vitro; a dsRNA may also be "introduced into a cell", wherein the cell is part of a living organism. In such instance, introduction into the cell will include the delivery to the organism. For example, for in vivo delivery, dsRNA 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.

[0081] The terms "silence," "inhibit the expression of," "down-regulate the expression of," "suppress the expression of," and the like, in as far as they refer to the PCSK9 gene, herein refer to the at least partial suppression of the expression of the PCSK9 gene, as manifested by a reduction of the amount of PCSK9 mRNA which may be isolated from a first cell or group of cells in which the PCSK9 gene is transcribed and which has or have been treated such that the expression of the PCSK9 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 is usually expressed in terms of

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

[0082] Alternatively, the degree of inhibition may be given in terms of a reduction of a parameter that is functionally linked to PCSK9 gene expression, e.g. the amount of protein encoded by the PCSK9 gene which is produced by a cell, or the number of cells displaying a certain phenotype. In principle, target gene silencing can be determined in any cell expressing the target, either constitutively or by genomic engineering, and by any appropriate assay. However, when a reference is needed in order to determine whether a given dsRNA inhibits the expression of the PCSK9 gene by a certain degree and therefore is encom-

passed by the instant invention, the assays provided in the Examples below shall serve as such reference.

[0083] As used herein in the context of PCSK9 expression, the terms "treat", "treatment", and the like, refer to relief from or alleviation of pathological processes which can be mediated by down regulating the PCSK9 gene. In the context of the present invention insofar as it relates to any of the other conditions recited herein below (other than pathological processes which can be mediated by down regulating the PCSK9 gene), the terms "treat", "treatment", and the like mean to relieve or alleviate at least one symptom associated with such condition, or to slow or reverse the progression of such condition. For example, in the context of hyperlipidemia, treatment will involve a decrease in serum lipid levels.

[0084] As used herein, the phrases "therapeutically effective amount" and "prophylactically effective amount" refer to an amount that provides a therapeutic benefit in the treatment, prevention, or management of pathological processes that can be mediated by down regulating the PCSK9 gene or an overt symptom of pathological processes which can be mediated by down regulating the PCSK9 gene. The specific amount that is therapeutically effective can be readily determined by an ordinary medical practitioner, and may vary depending on factors known in the art, such as, e.g., the type of pathological processes that can be mediated by down regulating the PCSK9 gene, the patient's history and age, the stage of pathological processes that can be mediated by down regulating PCSK9 gene expression, and the administration of other anti-pathological processes that can be mediated by down regulating PCSK9 gene expression.

[0085] As used herein, a "pharmaceutical composition" includes a pharmacologically effective amount of a dsRNA and a pharmaceutically acceptable carrier. As used herein, "pharmacologically effective amount," "therapeutically effective amount" or simply "effective amount" refers to that amount of an RNA effective to produce the intended pharmacological, therapeutic or preventive result. For example, if a given clinical treatment is considered effective when there is at least a 25% reduction in a measurable parameter associated with a disease or disorder, a therapeutically effective amount of a drug for the treatment of that disease or disorder is the amount necessary to effect at least a 25% reduction in that parameter.

[0086] The term "pharmaceutically acceptable carrier" refers to a carrier for administration of a therapeutic agent. Such carriers include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof and are described in more detail below. The term specifically excludes cell culture medium.

[0087] As used herein, a "transformed cell" is a cell into which a vector has been introduced from which a dsRNA molecule may be expressed.

[0088] Double-Stranded Ribonucleic Acid (dsRNA)

[0089] As described in more detail below, the invention provides methods and composition having double-stranded ribonucleic acid (dsRNA) molecules for inhibiting the expression of the PCSK9 gene in a cell or mammal, wherein the dsRNA includes an antisense strand having a region of complementarity that is complementary to at least a part of an mRNA formed in the expression of the PCSK9 gene, and wherein the region of complementarity is less than 30 nucleotides in length, generally 19-24 nucleotides in length.

In some embodiments, the dsRNA, upon contact with a cell expressing the PCSK9 gene, inhibits the expression of said PCSK9 gene, e.g., as measured such as by an assay described herein. For example, expression of a PCSK9 gene in cell culture, such as in HepB3 cells, can be assayed by measuring PCSK9 mRNA levels, such as by bDNA or TaqMan assay, or by measuring protein levels, such as by ELISA assay. The dsRNA of the invention can further include one or more single-stranded nucleotide overhangs.

[0090] The 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. The dsRNA includes two nucleic acid strands that are sufficiently complementary to hybridize to form a duplex structure. One strand of the dsRNA (the antisense strand) can have a region of complementarity that is substantially complementary, and generally fully complementary, to a target sequence, derived from the sequence of an mRNA formed during the expression of the PCSK9 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. Generally, the duplex structure is between 15 and 30, or between 25 and 30, or between 18 and 25, or between 19 and 24, or between 19 and 21, or 19, 20, or 21 base pairs in length. In one embodiment the duplex is 19 base pairs in length. In another embodiment the duplex is 21 base pairs in length. When two different siRNAs are used in combination, the duplex lengths can be identical or can differ.

[0091] Each strand of the dsRNA of invention is generally between 15 and 30, or between 18 and 25, or 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length. In other embodiments, each is strand is 25-30 nucleotides in length. Each strand of the duplex can be the same length or of different lengths. When two different siRNAs are used in combination, the lengths of each strand of each siRNA can be identical or can differ.

[0092] The dsRNA of the invention can include one or more single-stranded overhang(s) of one or more nucleotides. In one embodiment, at least one end of the dsRNA has a single-stranded nucleotide overhang of 1 to 4, generally 1 or 2 nucleotides. In another embodiment, the antisense strand of the dsRNA has 1-10 nucleotides overhangs each at the 3' end and the 5' end over the sense strand. In further embodiments, the sense strand of the dsRNA has 1-10 nucleotides overhangs each at the 3' end and the 5' end over the antisense strand.

[0093] A dsRNAs having at least one nucleotide overhang can have unexpectedly superior inhibitory properties than the blunt-ended counterpart. In some embodiments the presence of only one nucleotide overhang strengthens the interference activity of the dsRNA, without affecting its overall stability. A dsRNA having only one overhang has proven particularly stable and effective in vivo, as well as in a variety of cells, cell culture mediums, blood, and serum. Generally, the single-stranded overhang is located at the 3'-terminal end of the antisense strand or, alternatively, at the 3'-terminal end of the sense strand. The dsRNA can also have a blunt end, generally located at the 5'-end of the antisense strand. Such dsRNAs can have improved stability and inhibitory activity, thus allowing administration at low dosages, i.e., less than 5 mg/kg body weight of the recipient

per day. Generally, the antisense strand of the dsRNA has a nucleotide overhang at the 3'-end, and the 5'-end is blunt. In another embodiment, one or more of the nucleotides in the overhang is replaced with a nucleoside thiophosphate.

[0094] The 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. In one embodiment, the PCSK9 gene is a human PCSK9 gene. In other embodiments, the antisense strand of the dsRNA includes a first strand selected from the sense sequences of Table 1a, Table 2a, and Table 5a, and a second strand selected from the antisense sequences of Table 1a, Table 2a, and Table 5a. Alternative antisense agents that target elsewhere in the target sequence provided in Table 1a, Table 2a, and Table 5a, can readily be determined using the target sequence and the flanking PCSK9 sequence.

[0095] For example, the dsRNA AD-9680 (from Table 1a) targets the PCSK 9 gene at 3530-3548; therefore the target sequence is as follows: 5' UUCUAGACCUGUUUUGCUU 3' (SEQ ID NO:1523). The dsRNA AD-10792 (from Table 1a) targets the PCSK9 gene at 1091-1109; therefore the target sequence is as follows: 5' GCCUG-GAGUUUAUUCGGAA 3' (SEQ ID NO:1524). Included in the invention are dsRNAs that have regions of complementarity to SEQ ID NO:1523 and SEQ ID NO:1524.

[0096] In further embodiments, the dsRNA includes at least one nucleotide sequence selected from the groups of sequences provided in Table 1a, Table 2a, and Table 5a. In other embodiments, the dsRNA includes at least two sequences selected from this group, where one of the at least two sequences is complementary to another of the at least two sequences, and one of the at least two sequences is substantially complementary to a sequence of an mRNA generated in the expression of the PCSK9 gene. Generally, the dsRNA includes two oligonucleotides, where one oligonucleotide is described as the sense strand in Table 1a, Table 2a, and Table 5a and the second oligonucleotide is described as the antisense strand in Table 1a, Table 2a, and Table 5a

[0097] The skilled person is well aware that dsRNAs having a duplex structure of between 20 and 23, but specifically 21, base pairs have been hailed as particularly effective in inducing RNA interference (Elbashir et al., EMBO 2001, 20:6877-6888). However, others have found that shorter or longer dsRNAs can be effective as well. In the embodiments described above, by virtue of the nature of the oligonucleotide sequences provided in Table 1a, Table 2a, and Table 5a, the dsRNAs of the invention can include at least one strand of a length of minimally 21 nt. It can be reasonably expected that shorter dsRNAs having one of the sequences of Table 1a, Table 2a, and Table 5a minus only a few nucleotides on one or both ends may be similarly effective as compared to the dsRNAs described above. Hence, dsRNAs having a partial sequence of at least 15, 16, 17, 18, 19, 20, or more contiguous nucleotides from one of the sequences of Table 1a, Table 2a, and Table 5a, and differing in their ability to inhibit the expression of the PCSK9 gene in a FACS assay as described herein below by not more than 5, 10, 15, 20, 25, or 30% inhibition from a dsRNA comprising the full sequence, are contemplated by the invention. Further dsRNAs that cleave within the target sequence provided in Table 1a, Table 2a, and Table 5a can readily be made using the PCSK9 sequence and the target sequence provided.

[0098] In addition, the RNAi agents provided in Table 1a, Table 2a, and Table 5a identify a site in the PCSK9 mRNA that is susceptible to RNAi based cleavage. As such the present invention further includes RNAi agents that target within the sequence targeted by one of the agents of the present invention. As used herein a second RNAi agent is said to target within the sequence of a first RNAi agent if the second RNAi agent cleaves the message anywhere within the mRNA that is complementary to the antisense strand of the first RNAi agent. Such a second agent will generally consist of at least 15 contiguous nucleotides from one of the sequences provided in Table 1a, Table 2a, and Table 5a coupled to additional nucleotide sequences taken from the region contiguous to the selected sequence in the PCSK9 gene. For example, the last 15 nucleotides of SEQ ID NO:1 (minus the added AA sequences) combined with the next 6 nucleotides from the target PCSK9 gene produces a single strand agent of 21 nucleotides that is based on one of the sequences provided in Table 1a, Table 2a, and Table 5a.

[0099] The dsRNA of the invention can contain one or more mismatches to the target sequence. In one embodiment, the dsRNA of the invention contains no more than 1, no more than 2, or no more than 3 mismatches. In one embodiment, the antisense strand of the dsRNA contains mismatches to the target sequence, and the area of mismatch is not located in the center of the region of complementarity. In another embodiment, the antisense strand of the dsRNA contains mismatches to the target sequence and the mismatch is restricted to 5 nucleotides from either end, for example 5, 4, 3, 2, or 1 nucleotide from either the 5' or 3' end of the region of complementarity. For example, for a 23 nucleotide dsRNA strand which is complementary to a region of the PCSK9 gene, the dsRNA does not contain any mismatch within the central 13 nucleotides. The methods described within the invention can be used to determine whether a dsRNA containing a mismatch to a target sequence is effective in inhibiting the expression of the PCSK9 gene. Consideration of the efficacy of dsRNAs with mismatches in inhibiting expression of the PCSK9 gene is important, especially if the particular region of complementarity in the PCSK9 gene is known to have polymorphic sequence variation within the population.

[0100] In one embodiment, at least one end of the dsRNA has a single-stranded nucleotide overhang of 1 to 4, generally 1 or 2 nucleotides. dsRNAs having at least one nucleotide overhang have unexpectedly superior inhibitory properties than their blunt-ended counterparts. Moreover, the present inventors have discovered that the presence of only one nucleotide overhang strengthens the interference activity of the dsRNA, without affecting its overall stability. dsRNA having only one overhang has proven particularly stable and effective in vivo, as well as in a variety of cells, cell culture mediums, blood, and serum. Generally, the single-stranded overhang is located at the 3'-terminal end of the antisense strand or, alternatively, at the 3'-terminal end of the sense strand. The dsRNA may also have a blunt end, generally located at the 5'-end of the antisense strand. Such dsRNAs have improved stability and inhibitory activity, thus allowing administration at low dosages, i.e., less than 5 mg/kg body weight of the recipient per day. Generally, the antisense strand of the dsRNA has a nucleotide overhang at the 3'-end, and the 5'-end is blunt. In another embodiment, one or more of the nucleotides in the overhang is replaced with a nucleoside thiophosphate.

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[0101] Chemical Modifications and Conjugates

[0102] In yet another embodiment, the dsRNA is chemically modified to enhance stability. The nucleic acids of the invention may be synthesized and/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, N.Y., USA, which is hereby incorporated herein by reference. Chemical modifications may include, but are not limited to 2' modifications, modifications at other sites of the sugar or base of an oligonucleotide, introduction of non-natural bases into the oligonucleotide chain, covalent attachment to a ligand or chemical moiety, and replacement of internucleotide phosphate linkages with alternate linkages such as thiophosphates. More than one such modification may be employed.

[0103] Chemical linking of the two separate dsRNA strands may be achieved by any of a variety of well-known techniques, for example by introducing covalent, ionic or hydrogen bonds; hydrophobic interactions, van der Waals or stacking interactions; by means of metal-ion coordination, or through use of purine analogues. Generally, the chemical groups that can be used to modify the dsRNA include, without limitation, methylene blue; bifunctional groups, generally bis-(2-chloroethyl)amine; N-acetyl-N'-(p-glyoxylbenzoyl)cystamine; 4-thiouracil; and psoralen. In one embodiment, the linker is a hexa-ethylene glycol linker. In this case, the dsRNA are produced by solid phase synthesis and the hexa-ethylene glycol linker is incorporated according to standard methods (e.g., Williams, D. J., and K. B. Hall, *Biochem*. (1996) 35:14665-14670). In a particular embodiment, the 5'-end of the antisense strand and the 3'-end of the sense strand are chemically linked via a hexaethylene glycol linker. In another embodiment, at least one nucleotide of the dsRNA comprises a phosphorothioate or phosphorodithioate groups. The chemical bond at the ends of the dsRNA is generally formed by triple-helix bonds. Table 1a, Table 2a, and Table 5a provides examples of modified RNAi agents of the invention.

[0104] In yet another embodiment, the nucleotides at one or both of the two single strands may be modified to prevent or inhibit the degradation activities of cellular enzymes, such as, for example, without limitation, certain nucleases. Techniques for inhibiting the degradation activity of cellular enzymes against nucleic acids are known in the art including, but not limited to, 2'-amino modifications, 2'-amino sugar modifications, 2'-F sugar modifications, 2'-F modifications, 2'-alkyl sugar modifications, uncharged backbone modifications, morpholino modifications, 2'-O-methyl modifications, and phosphoramidate (see, e.g., Wagner, *Nat. Med*. (1995) 1:1116-8). Thus, at least one 2'-hydroxyl group of the nucleotides on a dsRNA is replaced by a chemical group, generally by a 2'-amino or a 2'-methyl group. Also, at least one nucleotide may be modified to form a locked nucleotide. Such locked nucleotide contains a methylene bridge that connects the 2'-oxygen of ribose with the 4'-carbon of ribose. Oligonucleotides containing the locked nucleotide are described in Koshkin, A. A., et al., *Tetrahedron* (1998), 54: 3607-3630) and Obika, S. et al., *Tetrahedron Lett*. (1998), 39: 5401-5404). Introduction of a locked nucleotide into an oligonucleotide improves the affinity for complementary sequences and increases the melting temperature by several degrees (Braasch, D. A. and D. R. Corey, *Chem. Biol*. (2001), 8:1-7).

[0105] Conjugating a ligand to a dsRNA can enhance its cellular absorption as well as targeting to a particular tissue or uptake by specific types of cells such as liver cells. In certain instances, a hydrophobic ligand is conjugated to the dsRNA to facilitate direct permeation of the cellular membrane and or uptake across the liver cells. Alternatively, the ligand conjugated to the dsRNA is a substrate for receptor-mediated endocytosis. These approaches have been used to facilitate cell permeation of antisense oligonucleotides as well as dsRNA agents. For example, cholesterol has been conjugated to various antisense oligonucleotides resulting in compounds that are substantially more active compared to their non-conjugated analogs. See M. Manoharan *Antisense & Nucleic Acid Drug Development* 2002, 12, 103. Other lipophilic compounds that have been conjugated to oligonucleotides include 1-pyrene butyric acid, 1,3-bis-O-(hexadecyl)glycerol, and menthol. One example of a ligand for receptor-mediated endocytosis is folic acid. Folic acid enters the cell by folate-receptor-mediated endocytosis. dsRNA compounds bearing folic acid would be efficiently transported into the cell via the folate-receptor-mediated endocytosis. Li and coworkers report that attachment of folic acid to the 3'-terminus of an oligonucleotide resulted in an 8-fold increase in cellular uptake of the oligonucleotide. Li, S.; Deshmukh, H. M.; Huang, L. *Pharm. Res*. 1998, 15, 1540. Other ligands that have been conjugated to oligonucleotides include polyethylene glycols, carbohydrate clusters, cross-linking agents, porphyrin conjugates, delivery peptides and lipids such as cholesterol and cholesterylamine. Examples of carbohydrate clusters include Chol-p-(GalNAc)$_3$ (N-acetyl galactosamine cholesterol) and LCO(GalNAc)$_3$ (N-acetyl galactosamine-3'-Lithocholic-oleoyl.

[0106] In certain instances, conjugation of a cationic ligand to oligonucleotides results in improved resistance to nucleases. Representative examples of cationic ligands are propylammonium and dimethylpropylammonium. Interestingly, antisense oligonucleotides were reported to retain their high binding affinity to mRNA when the cationic ligand was dispersed throughout the oligonucleotide. See M. Manoharan *Antisense & Nucleic Acid Drug Development* 2002, 12, 103 and references therein.

[0107] In some cases, a ligand can be multifunctional and/or a dsRNA can be conjugated to more than one ligand. For example, the dsRNA can be conjugated to one ligand for improved uptake and to a second ligand for improved release.

[0108] The ligand-conjugated dsRNA of the invention may be synthesized by the use of a dsRNA that bears a pendant reactive functionality, such as that derived from the attachment of a linking molecule onto the dsRNA. 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 methods of the invention facilitate the synthesis of ligand-conjugated dsRNA by the use of, in some embodiments, nucleoside monomers that have been appropriately conjugated with ligands and that may further be attached to a solid-support material. Such ligand-nucleoside conjugates, optionally attached to a solid-support material, are prepared according to certain embodiments of the methods described herein via reaction of a selected serum-binding ligand with a linking moiety located on the 5' position of a nucleoside or oligonucleotide. In certain instances, a dsRNA bearing an aralkyl

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ligand attached to the 3'-terminus of the dsRNA is prepared by first covalently attaching a monomer building block to a controlled-pore-glass support via a long-chain aminoalkyl group. Then, nucleotides are bonded via standard solid-phase synthesis techniques to the monomer building-block bound to the solid support. The monomer building block may be a nucleoside or other organic compound that is compatible with solid-phase synthesis.

[0109] The dsRNA used in the conjugates of the 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.

[0110] Synthesis

[0111] Teachings regarding the synthesis of particular modified oligonucleotides may be found in the following U.S. patents: U.S. Pat. Nos. 5,138,045 and 5,218,105, drawn to polyamine conjugated oligonucleotides; U.S. Pat. No. 5,212,295, drawn to monomers for the preparation of oligonucleotides having chiral phosphorus linkages; U.S. Pat. Nos. 5,378,825 and 5,541,307, drawn to oligonucleotides having modified backbones; U.S. Pat. No. 5,386,023, drawn to backbone-modified oligonucleotides and the preparation thereof through reductive coupling; U.S. Pat. No. 5,457,191, drawn to modified nucleobases based on the 3-deazapurine ring system and methods of synthesis thereof; U.S. Pat. No. 5,459,255, drawn to modified nucleobases based on N-2 substituted purines; U.S. Pat. No. 5,521,302, drawn to processes for preparing oligonucleotides having chiral phosphorus linkages; U.S. Pat. No. 5,539,082, drawn to peptide nucleic acids; U.S. Pat. No. 5,554,746, drawn to oligonucleotides having β-lactam backbones; U.S. Pat. No. 5,571,902, drawn to methods and materials for the synthesis of oligonucleotides; U.S. Pat. No. 5,578,718, drawn to nucleosides having alkylthio groups, wherein such groups may be used as linkers to other moieties attached at any of a variety of positions of the nucleoside; U.S. Pat. Nos. 5,587,361 and 5,599,797, drawn to oligonucleotides having phosphorothioate linkages of high chiral purity; U.S. Pat. No. 5,506,351, drawn to processes for the preparation of 2'-O-alkyl guanosine and related compounds, including 2,6-diaminopurine compounds; U.S. Pat. No. 5,587,469, drawn to oligonucleotides having N-2 substituted purines; U.S. Pat. No. 5,587,470, drawn to oligonucleotides having 3-deazapurines; U.S. Pat. No. 5,223,168, and U.S. Pat. No. 5,608,046, both drawn to conjugated 4'-desmethyl nucleoside analogs; U.S. Pat. Nos. 5,602,240, and 5,610,289, drawn to backbone-modified oligonucleotide analogs; U.S. Pat. Nos. 6,262,241, and 5,459,255, drawn to, inter alia, methods of synthesizing 2'-fluoro-oligonucleotides.

[0112] In the ligand-conjugated dsRNA and ligand-molecule bearing sequence-specific linked nucleosides of the 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.

[0113] 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. Oligonucleotide conjugates bearing a variety of molecules such as steroids, vitamins, lipids and reporter molecules, has previously been described (see Manoharan et al., PCT Application WO 93/07883). In one embodiment, the oligonucleotides or linked nucleosides featured in the 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.

[0114] The incorporation of a 2'-O-methyl, 2'-O-ethyl, 2'-O-propyl, 2'-O-allyl, 2'-O-aminoalkyl or 2'-deoxy-2'-fluoro group in nucleosides of an oligonucleotide confers enhanced hybridization properties to the oligonucleotide. Further, oligonucleotides containing phosphorothioate backbones have enhanced nuclease stability. Thus, functionalized, linked nucleosides of the invention can be augmented to include either or both a phosphorothioate backbone or a 2'-O-methyl, 2'-O-ethyl, 2'-O-propyl, 2'-O-aminoalkyl, 2'-O-allyl or 2'-deoxy-2'-fluoro group. A summary listing of some of the oligonucleotide modifications known in the art is found at, for example, PCT Publication WO 200370918.

[0115] In some embodiments, functionalized nucleoside sequences of the invention possessing an amino group at the 5'-terminus are prepared using a DNA synthesizer, and then reacted with an active ester derivative of a selected ligand. Active ester derivatives are well known to those skilled in the art. Representative active esters include N-hydrosuccinimide esters, tetrafluorophenolic esters, pentafluorophenolic esters and pentachlorophenolic esters. The reaction of the amino group and the active ester produces an oligonucleotide in which the selected ligand is attached to the 5'-position through a linking group. The amino group at the 5'-terminus can be prepared utilizing a 5'-Amino-Modifier C6 reagent. In one embodiment, ligand molecules may be conjugated to oligonucleotides at the 5'-position by the use of a ligand-nucleoside phosphoramidite wherein the ligand is linked to the 5'-hydroxy group directly or indirectly via a linker. Such ligand-nucleoside phosphoramidites are typically used at the end of an automated synthesis procedure to provide a ligand-conjugated oligonucleotide bearing the ligand at the 5'-terminus.

[0116] Examples of modified internucleoside linkages or 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, mixed salts and free-acid forms are also included.

[0117] Representative United States patents relating to the preparation of the above phosphorus-atom-containing linkages include, but are not limited to, U.S. Pat. Nos. 3,687,

808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 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; 5,541,306; 5,550, 111; 5,563,253; 5,571,799; 5,587,361; 5,625,050; and 5,697, 248, each of which is herein incorporated by reference.

[0118] Examples of modified internucleoside linkages or backbones that do not include a phosphorus atom therein (i.e., oligonucleosides) have backbones that are formed by short chain alkyl or cycloalkyl intersugar linkages, mixed heteroatom and alkyl or cycloalkyl intersugar linkages, or one or more short chain heteroatomic or heterocyclic inter-sugar linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleo-side); siloxane backbones; sulfide, sulfoxide and sulfone backbones;

[0119] formacetyl and thioformacetyl backbones; methyl-ene formacetyl and thioformacetyl backbones; alkene con-taining backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfona-mide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

[0120] Representative United States patents relating to the preparation of the above oligonucleosides include, but are not limited to, U.S. Pat. Nos. 5,034,506; 5,166,315; 5,185, 444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 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; 5,610, 289; 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, each of which is herein incorporated by reference.

[0121] In certain instances, the oligonucleotide may be modified by a non-ligand group. A number of non-ligand molecules have been conjugated to oligonucleotides in order to enhance the activity, cellular distribution or cellular uptake of the oligonucleotide, and procedures for perform-ing such conjugations are available in the scientific litera-ture. Such non-ligand moieties have included lipid moieties, such as cholesterol (Letsinger et al., Proc. Natl. Acad. Sci. USA, 1989, 86:6553), cholic acid (Manoharan et al., Bioorg. Med. Chem. Left., 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; Svinar-chuk 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., Tet-rahedron Lett., 1995, 36:3651), a palmityl moiety (Mishra et al., Biochim. Biophys. Acta, 1995, 1264:229), or an octa-decylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., J. Pharmacol. Exp. Ther., 1996, 277:923). Representative United States patents that teach the prepara-tion of such oligonucleotide conjugates have been listed above. Typical conjugation protocols involve the synthesis of oligonucleotides bearing an aminolinker at one or more positions of the sequence. The amino group is then reacted with the molecule being conjugated using appropriate cou-

pling or activating reagents. The conjugation reaction may be performed either with the oligonucleotide still bound to the solid support or following cleavage of the oligonucle-otide in solution phase. Purification of the oligonucleotide conjugate by HPLC typically affords the pure conjugate. The use of a cholesterol conjugate is particularly preferred since such a moiety can increase targeting liver cells, a site of PCSK9 expression.

[0122] Vector Encoded RNAi Agents

[0123] In another aspect of the invention, PCSK9 specific dsRNA molecules that modulate PCSK9 gene expression activity are expressed from transcription units inserted into DNA or RNA vectors (see, e.g., Couture, A, et al., TIG. (1996), 12:5-10; Skillern, A., et al., International PCT Pub-lication No. WO 00/22113, Conrad, International PCT Pub-lication No. WO 00/22114, and Conrad, U.S. Pat. No. 6,054,299). These transgenes can be introduced as a linear construct, a circular plasmid, or a viral vector, which can be incorporated and inherited as a transgene integrated into the host genome. The transgene can also be constructed to permit it to be inherited as an extrachromosomal plasmid (Gassmann, et al., Proc. Natl. Acad. Sci. USA (1995) 92:1292).

[0124] The individual strands of a dsRNA can be tran-scribed by promoters on two separate expression vectors and co-transfected into a target cell. Alternatively each indi-vidual strand of the dsRNA can be transcribed by promoters both of which are located on the same expression plasmid. In one embodiment, a dsRNA is expressed as an inverted repeat joined by a linker polynucleotide sequence such that the dsRNA has a stem and loop structure.

[0125] The recombinant dsRNA expression vectors are generally DNA plasmids or viral vectors. dsRNA expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus (for a review, see Muzyczka, et al., Curr. Topics Micro. Immunol. (1992) 158:97-129)); adeno-virus (see, for example, Berkner, et al., BioTechniques (1998) 6:616), Rosenfeld et al. (1991, Science 252:431-434), and Rosenfeld et al. (1992), Cell 68:143-155)); or alphavirus as well as others known in the art. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells, in vitro and/or in vivo (see, e.g., Eglitis, et al., Science (1985) 230:1395-1398; Danos and Mulligan, Proc. Natl. Acad. Sci. USA (1998) 85:6460-6464; Wilson et al., 1988, Proc. Natl. Acad. Sci. USA 85:3014-3018; Armentano et al., 1990, Proc. Natl. Acad. Sci. USA 87:61416145; Huber et al., 1991, Proc. Natl. Acad. Sci. USA 88:8039-8043; Ferry et al., 1991, Proc. Natl. Acad. Sci. USA 88:8377-8381; Chowdhury et al., 1991, Science 254:1802-1805; van Beusechem. et al., 1992, Proc. Nad. Acad. Sci. USA 89:7640-19; Kay et al., 1992, Human Gene Therapy 3:641-647; Dai et al., 1992, Proc. Natl. Acad. Sci. USA 89:10892-10895; Hwu et al., 1993, J. Immunol. 150:4104-4115; U.S. Pat. No. 4,868,116; U.S. Pat. No. 4,980,286; PCT Application WO 89/07136; PCT Applica-tion WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573). Recombinant retroviral vectors capable of transducing and expressing genes inserted into the genome of a cell can be produced by transfecting the recombinant retroviral genome into suitable packaging cell lines such as PA317 and Psi-CRIP (Comette et al., 1991, Human Gene Therapy 2:5-10; Cone et al., 1984, Proc. Natl. Acad. Sci. USA 81:6349). Recombinant adenoviral vectors can be used to infect a wide variety of cells and tissues in

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susceptible hosts (e.g., rat, hamster, dog, and chimpanzee) (Hsu et al., 1992, *J. Infectious Disease*, 166:769), and also have the advantage of not requiring mitotically active cells for infection.

[0126] Any viral vector capable of accepting the coding sequences for the dsRNA molecule(s) to be expressed can be used, for example vectors derived from adenovirus (AV); adeno-associated virus (AAV); retroviruses (e.g., lentiviruses (LV), Rhabdoviruses, murine leukemia virus); herpes virus, and the like. The tropism of viral vectors can be modified by pseudotyping the vectors with envelope proteins or other surface antigens from other viruses, or by substituting different viral capsid proteins, as appropriate.

[0127] For example, lentiviral vectors of the invention can be pseudotyped with surface proteins from vesicular stomatitis virus (VSV), rabies, Ebola, Mokola, and the like. AAV vectors of the invention can be made to target different cells by engineering the vectors to express different capsid protein serotypes. For example, an AAV vector expressing a serotype 2 capsid on a serotype 2 genome is called AAV 2/2. This serotype 2 capsid gene in the AAV 2/2 vector can be replaced by a serotype 5 capsid gene to produce an AAV 2/5 vector. Techniques for constructing AAV vectors which express different capsid protein serotypes are within the skill in the art; see, e.g., Rabinowitz J E et al. (2002), *J. Virol* 76:791-801, the entire disclosure of which is herein incorporated by reference.

[0128] Selection of recombinant viral vectors suitable for use in the invention, methods for inserting nucleic acid sequences for expressing the dsRNA into the vector, and methods of delivering the viral vector to the cells of interest are within the skill in the art. See, for example, Dornburg R (1995), *Gene Therap.* 2: 301-310; Eglitis M A (1988), *Biotechniques* 6: 608-614; Miller A D (1990), *Hum Gene Therap.* 1: 5-14; Anderson W F (1998), *Nature* 392: 25-30; and Rubinson D A et al., *Nat. Genet.* 33: 401-406, the entire disclosures of which are herein incorporated by reference.

[0129] Preferred viral vectors are those derived from AV and AAV. In a particularly preferred embodiment, the dsRNA of the invention is expressed as two separate, complementary single-stranded RNA molecules from a recombinant AAV vector having, for example, either the U6 or H1 RNA promoters, or the cytomegalovirus (CMV) promoter.

[0130] A suitable AV vector for expressing the dsRNA of the invention, a method for constructing the recombinant AV vector, and a method for delivering the vector into target cells, are described in Xia H et al. (2002), Nat. Biotech. 20: 1006-1010.

[0131] Suitable AAV vectors for expressing the dsRNA of the invention, methods for constructing the recombinant AV vector, and methods for delivering the vectors into target cells are described in Samulski R et al. (1987), J. Virol. 61: 3096-3101; Fisher K J et al. (1996), J. Virol, 70: 520-532; Samulski R et al. (1989), J. Virol. 63: 3822-3826; U.S. Pat. No. 5,252,479; U.S. Pat. No. 5,139,941; International Patent Application No. WO 94/13788; and International Patent Application No. WO 93/24641, the entire disclosures of which are herein incorporated by reference.

[0132] The promoter driving dsRNA expression in either a DNA plasmid or viral vector of the invention may be a eukaryotic RNA polymerase I (e.g. ribosomal RNA promoter), RNA polymerase II (e.g. CMV early promoter or actin promoter or U1 snRNA promoter) or generally RNA polymerase III promoter (e.g. U6 snRNA or 7SK RNA promoter) or a prokaryotic promoter, for example the T7 promoter, provided the expression plasmid also encodes T7 RNA polymerase required for transcription from a T7 promoter. The promoter can also direct transgene expression to the pancreas (see, e.g., the insulin regulatory sequence for pancreas (Bucchini et al., 1986, Proc. Natl. Acad. Sci. USA 83:2511-2515)).

[0133] In addition, expression of the transgene can be precisely regulated, for example, by using an inducible regulatory sequence and expression systems such as a regulatory sequence that is sensitive to certain physiological regulators, e.g., circulating glucose levels, or hormones (Docherty et al., 1994, FASEB J. 8:20-24). Such inducible expression systems, suitable for the control of transgene expression in cells or in mammals include regulation by ecdysone, by estrogen, progesterone, tetracycline, chemical inducers of dimerization, and isopropyl-beta-D1-thiogalactopyranoside (EPTG). A person skilled in the art would be able to choose the appropriate regulatory/promoter sequence based on the intended use of the dsRNA transgene.

[0134] Generally, recombinant vectors capable of expressing dsRNA molecules are delivered as described below, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of dsRNA molecules. Such vectors can be repeatedly administered as necessary. Once expressed, the dsRNAs bind to target RNA and modulate its function or expression. Delivery of dsRNA 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.

[0135] dsRNA expression DNA plasmids are typically transfected into target cells as a complex with cationic lipid carriers (e.g. Oligofectamine) or non-cationic lipid-based carriers (e.g. Transit-TKO™). Multiple lipid transfections for dsRNA-mediated knockdowns targeting different regions of a single PCSK9 gene or multiple PCSK9 genes over a period of a week or more are also contemplated by the invention. Successful introduction of the vectors of the invention into host cells can be monitored using various known methods. For example, transient transfection. can be signaled with a reporter, such as a fluorescent marker, such as Green Fluorescent Protein (GFP). Stable transfection of ex vivo cells can be ensured using markers that provide the transfected cell with resistance to specific environmental factors (e.g., antibiotics and drugs), such as hygromycin B resistance.

[0136] The PCSK9 specific dsRNA molecules can also be inserted into vectors and used as gene therapy vectors for human patients. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Pat. No. 5,328,470) or by stereotactic injection (see e.g., Chen et al. (1994) Proc. Natl. Acad. Sci. USA 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can include a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, e.g., retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

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[0137] Pharmaceutical Compositions Containing dsRNA

[0138] In one embodiment, the invention provides pharmaceutical compositions containing a dsRNA, as described herein, and a pharmaceutically acceptable carrier and methods of administering the same. The pharmaceutical composition containing the dsRNA is useful for treating a disease or disorder associated with the expression or activity of a PCSK9 gene, such as pathological processes mediated by PCSK9 expression, e.g., hyperlipidemia. Such pharmaceutical compositions are formulated based on the mode of delivery.

[0139] Dosage

[0140] The pharmaceutical compositions featured herein are administered in dosages sufficient to inhibit expression of PCSK9 genes. In general, a suitable dose of dsRNA will be in the range of 0.01 to 200.0 milligrams per kilogram body weight of the recipient per day, generally in the range of 1 to 50 mg per kilogram body weight per day. For example, the dsRNA can be administered at 0.01 mg/kg, 0.05 mg/kg, 0.5 mg/kg, 1 mg/kg, 1.5 mg/kg, 2.0 mg/kg, 3.0 mg/kg, 5.0 mg/kg, 10 mg/kg, 20 mg/kg, 30 mg/kg, 40 mg/kg, or 50 mg/kg per single dose.

[0141] In another embodiment, the dosage is between 0.01 and 0.2 mg/kg. For example, the dsRNA can be administered at a dose of 0.01 mg/kg, 0.02 mg/kg, 0.03 mg/kg, 0.04 mg/kg, 0.05 mg/kg, 0.06 mg/kg, 0.07 mg/kg 0.08 mg/kg 0.09 mg/kg, 0.10 mg/kg, 0.11 mg/kg, 0.12 mg/kg, 0.13 mg/kg, 0.14 mg/kg, 0.15 mg/kg, 0.16 mg/kg, 0.17 mg/kg, 0.18 mg/kg, 0.19 mg/kg, or 0.20 mg/kg.

[0142] In one embodiment, the dosage is between 0.2 mg/kg and 1.5 mg/kg. For example, the dsRNA can be administered at a dose of 0.2 mg/kg, 0.3 mg/kg, 0.4 mg/kg, 0.5 mg/kg, 0.6 mg/kg, 0.7 mg/kg, 0.8 mg/kg, 0.9 mg/kg, 1 mg/kg, 1.1 mg/kg, 1.2 mg/kg, 1.3 mg/kg, 1.4 mg/kg, or 1.5 mg/kg.

[0143] The dsRNA can be administered at a dose of 0.03, 0.1, 0.3, or 1.3, or 3.0 mg/kg.

[0144] The pharmaceutical composition can be administered once daily, or the dsRNA may be administered as two, three, or more sub-doses at appropriate intervals throughout the day. The effect of a single dose on PCSK9 levels is long lasting, such that subsequent doses are administered at not more than 7 day intervals, or at not more than 1, 2, 3, or 4 week intervals.

[0145] In one embodiment the lipid formulated PCSK9 targeted dsRNA is administered at a first dose of about 3 mg/kg followed by administering at least one subsequent dose once a week, wherein the subsequent dose is lower than the first dose, e.g., the subsequent dose is about 1.0 mg/kg or about 0.3 mg/kg. The subsequent dose can be administered, e.g., once a week for four weeks.

[0146] In some embodiments the dsRNA is administered using continuous infusion or delivery through a controlled release formulation. In that case, the dsRNA contained in each sub-dose must be correspondingly smaller in order to achieve the total daily dosage. The dosage unit can also be compounded for delivery over several days, e.g., using a conventional sustained release formulation which provides sustained release of the dsRNA over a several day period. Sustained release formulations are well known in the art and are particularly useful for delivery of agents at a particular site, such as could be used with the agents of the present invention. In this embodiment, the dosage unit contains a corresponding multiple of the daily dose.

[0147] The skilled artisan will appreciate that certain factors may 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 and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of a composition can include a single treatment or a series of treatments. Estimates of effective dosages and in vivo half-lives for the individual dsRNAs encompassed by the invention can be made using conventional methodologies or on the basis of in vivo testing using an appropriate animal model, as described elsewhere herein.

[0148] Advances in mouse genetics have generated a number of mouse models for the study of various human diseases, such as pathological processes mediated by PCSK9 expression. Such models are used for in vivo testing of dsRNA, as well as for determining a therapeutically effective dose. A suitable mouse model is, for example, a mouse containing a plasmid expressing human PCSK9. Another suitable mouse model is a transgenic mouse carrying a transgene that expresses human PCSK9.

[0149] Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds that exhibit high therapeutic indices are preferred.

[0150] 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 in the invention lies generally within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the methods featured in the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range of the compound or, when appropriate, of the polypeptide product of a target sequence (e.g., achieving a decreased concentration of the polypeptide) that includes the IC50 (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

[0151] In addition to their administration, as discussed above, the dsRNAs featured in the invention can be administered in combination with other known agents effective in treatment of pathological processes mediated by target gene expression. In any event, the administering physician can adjust the amount and timing of dsRNA administration on the basis of results observed using standard measures of efficacy known in the art or described herein.

[0152] Administration

[0153] The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical,

pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal, and subdermal, oral or parenteral, e.g., subcutaneous.

[0154] Typically, when treating a mammal with hyperlipidemia, the dsRNA molecules are administered systemically via parental means. Parenteral administration includes intravenous, intra-arterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intraparenchymal, intrathecal or intraventricular, administration. For example, dsRNAs, conjugated or unconjugate or formulated with or without liposomes, can be administered intravenously to a patient. For such, a dsRNA molecule can be formulated into compositions such as sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or solutions in liquid or solid oil bases. Such solutions also can contain buffers, diluents, and other suitable additives. For parenteral, intrathecal, or intraventricular administration, a dsRNA molecule can be formulated into compositions such as sterile aqueous solutions, which also can contain buffers, diluents, and other suitable additives (e.g., penetration enhancers, carrier compounds, and other pharmaceutically acceptable carriers). Formulations are described in more detail herein.

[0155] The dsRNA can be delivered in a manner to target a particular tissue, such as the liver (e.g., the hepatocytes of the liver).

[0156] Formulations

[0157] The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may 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.

[0158] The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

[0159] Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids. In one aspect are formulations that target the liver when treating hepatic disorders such as hyperlipidemia.

[0160] In addition, dsRNA that target the PCSK9 gene can be formulated into compositions containing the dsRNA admixed, encapsulated, conjugated, or otherwise associated with other molecules, molecular structures, or mixtures of nucleic acids. For example, a composition containing one or more dsRNA agents that target the PCSK9 gene can contain other therapeutic agents, such as other cancer therapeutics or one or more dsRNA compounds that target non-PCSK9 genes.

[0161] Oral, Parenteral, Topical, and Biologic Formulations

[0162] 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 may be desirable. In some embodiments, oral formulations are those in which dsRNAs featured in the invention are administered in conjunction with one or more penetration enhancers surfactants and chelators. Suitable surfactants include fatty acids and/or esters or salts thereof, bile acids and/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 invention may 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(isohexylcyanoacrylate), DEAE-methacrylate, DEAE-hexylacrylate, DEAE-acrylamide, DEAE-albumin and DEAE-dextran, polymethylacrylate, polyhexylacrylate, poly(D,L-lactic acid), poly(DL-lactic-co-glycolic acid (PLGA), alginate, and polyethyleneglycol (PEG). Oral formulations for dsRNAs and their preparation are described in detail in U.S. Pat. No. 6,887,906, U.S. Patent Publication. No. 20030027780, and U.S. Pat. No. 6,747,014, each of which is incorporated herein by reference.

[0163] Compositions and formulations for parenteral, intraparenchymal (into the brain), intrathecal, intraventricular or intrahepatic administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetra-

tion enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

[0164] Pharmaceutical compositions and 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. Suitable topical formulations include those in which the dsRNAs featured in the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Suitable lipids and liposomes include neutral (e.g., dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearolyphosphatidyl choline) negative (e.g., dimyristoylphosphatidyl glycerol DMPG) and cationic (e.g., dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA). dsRNAs featured in the invention may be encapsulated within liposomes or may form complexes thereto, in particular to cationic liposomes. Alternatively, dsRNAs may 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_{1-10} alkyl ester (e.g., isopropylmyristate IPM), monoglyceride, diglyceride or pharmaceutically acceptable salt thereof. Topical formulations are described in detail in U.S. Pat. No. 6,747,014, which is incorporated herein by reference. In addition, dsRNA molecules can be administered to a mammal as biologic or abiologic means as described in, for example, U.S. Pat. No. 6,271,359. Abiologic delivery can be accomplished by a variety of methods including, without limitation, (1) loading liposomes with a dsRNA acid molecule provided herein and (2) complexing a dsRNA molecule with lipids or liposomes to form nucleic acid-lipid or nucleic acid-liposome complexes. The liposome can be composed of cationic and neutral lipids commonly used to transfect cells in vitro. Cationic lipids can complex (e.g., charge-associate) with negatively charged nucleic acids to form liposomes. Examples of cationic liposomes include, without limitation, lipofectin, lipofectamine, lipofectace, and DOTAP. Procedures for forming liposomes are well known in the art. Liposome compositions can be formed, for example, from phosphatidylcholine, dimyristoyl phosphatidylcholine, dipalmitoyl phosphatidylcholine, dimyristoyl phosphatidylglycerol, or dioleoyl phosphatidylethanolamine. Numerous lipophilic agents are commercially available, including Lipofectin™ (Invitrogen/Life Technologies, Carlsbad, Calif.) and Effectene (Qiagen, Valencia, Calif.). In addition, systemic delivery methods can be optimized using commercially available cationic lipids such as DDAB or DOTAP, each of which can be mixed with a neutral lipid such as DOPE or cholesterol. In some cases, liposomes such as those described by Templeton et al. (Nature Biotechnology, 15: 647-652 (1997)) can be used. In other embodiments, polycations such as polyethyleneimine can be used to achieve delivery in vivo and ex vivo (Boletta et al., J. Am Soc. Nephrol. 7: 1728 (1996)). Additional information regarding the use of liposomes to deliver nucleic acids can be found in U.S. Pat. No. 6,271,359, PCT Publication WO 96/40964 and Morrissey, D. et al. 2005. Nat Biotechnol. 23(8):1002-7.

[0165] Biologic delivery can be accomplished by a variety of methods including, without limitation, the use of viral vectors. For example, viral vectors (e.g., adenovirus and herpes virus vectors) can be used to deliver dsRNA molecules to liver cells. Standard molecular biology techniques can be used to introduce one or more of the dsRNAs provided herein into one of the many different viral vectors previously developed to deliver nucleic acid to cells. These resulting viral vectors can be used to deliver the one or more dsRNAs to cells by, for example, infection.

[0166] Liposomal Formulations

[0167] There are many organized surfactant structures besides microemulsions that have been studied and used for the formulation of drugs. These include monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, have attracted great interest because of their specificity and the duration of action they offer from the standpoint of drug delivery. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers.

[0168] Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the composition to be delivered. Cationic liposomes possess the advantage of being able to fuse to the cell wall. Non-cationic liposomes, although not able to fuse as efficiently with the cell wall, are taken up by macrophages in vivo.

[0169] 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. Therefore, it is desirable to use a liposome which is highly deformable and able to pass through such fine pores.

[0170] 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; and liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., 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.

[0171] 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 liposomes start to merge with the cellular membranes and as the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the active agent may act.

[0172] Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many drugs. There is growing evidence that 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 a wide variety of drugs, both hydrophilic and hydrophobic, into the skin.

[0173] Several reports have detailed the ability of liposomes to deliver agents including high-molecular weight DNA into the skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have been administered to the skin. The majority of applications resulted in the targeting of the upper epidermis

[0174] Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged DNA molecules to form a stable complex. The positively charged DNA/liposome complex binds to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang et al., Biochem. Biophys. Res. Commun., 1987, 147, 980-985).

[0175] Liposomes which are pH-sensitive or negatively-charged, entrap DNA rather than complex with it. Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some DNA is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou et al., Journal of Controlled Release, 1992, 19, 269-274).

[0176] One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

[0177] Several studies have assessed the topical delivery of liposomal drug formulations to the skin. Application of liposomes containing interferon to guinea pig skin resulted in a reduction of skin herpes sores while delivery of interferon via other means (e.g., as a solution or as an emulsion) were ineffective (Weiner et al., Journal of Drug Targeting, 1992, 2, 405-410). Further, an additional study tested the efficacy of interferon administered as part of a liposomal formulation to the administration of interferon using an aqueous system, and concluded that the liposomal formulation was superior to aqueous administration (du Plessis et al., Antiviral Research, 1992, 18, 259-265).

[0178] 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/po-lyoxyethylene-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 cyclosporin-A into different layers of the skin (Hu et al., S. T. P. Pharma. Sci., 1994, 4, 6, 466).

[0179] 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_{mi}, or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of the reticuloendothelial system (RES) (Allen et al., FEBS Letters, 1987, 223, 42; Wu et al., Cancer Research, 1993, 53, 3765).

[0180] 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). U.S. Pat. No. 4,837,028 and WO 88/04924, both to Allen et al., disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside G_{M1} or a galactocerebroside sulfate ester. U.S. Pat. No. 5,543,152 (Webb et al.) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-sn-dimyristoylphosphat-idylcholine are disclosed in WO 97/13499 (Lim et al.).

[0181] Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto et al. (Bull. Chem. Soc. Jpn., 1980, 53, 2778) described liposomes comprising a nonionic detergent, $2C_{1215G}$, that contains a PEG moiety. Illum et al. (FEBS Lett., 1984, 167, 79) noted that hydrophilic coating of polystyrene particles with polymeric glycols results in significantly enhanced blood half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups of polyalkylene glycols (e.g., PEG) are described by Sears (U.S. Pat. Nos. 4,426,330 and 4,534, 899). Klibanov et al. (FEBS Lett., 1990, 268, 235) described experiments demonstrating that liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant increases in blood circulation half-lives. Blume et al. (Biochimica et Biophysica Acta, 1990, 1029, 91) extended such observations to other PEG-derivatized phospholipids, e.g., DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG. Liposomes having covalently bound PEG moieties on their external surface are described in European Patent No. EP 0 445 131 B1 and WO 90/04384 to Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by Woodle et al. (U.S. Pat. Nos. 5,013,556 and 5,356,633) and Martin et al. (U.S. Pat. No. 5,213,804 and European Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Pat. No. 5,225,212 (both to Martin et al.) and in WO 94/20073 (Zalipsky et al.) Liposomes comprising PEG-modified ceramide lipids are described in WO 96/10391 (Choi et al). U.S. Pat. No.

5,540,935 (Miyazaki et al.) and U.S. Pat. No. 5,556,948 (Tagawa et al.) describe PEG-containing liposomes that can be further derivatized with functional moieties on their surfaces.

[0182] A number of liposomes comprising nucleic acids are known in the art. WO 96/40062 to Thierry et al. discloses methods for encapsulating high molecular weight nucleic acids in liposomes. U.S. Pat. No. 5,264,221 to Tagawa et al. discloses protein-bonded liposomes and asserts that the contents of such liposomes may include a dsRNA. U.S. Pat. No. 5,665,710 to Rahman et al. describes certain methods of encapsulating oligodeoxynucleotides in liposomes. WO 97/04787 to Love et al. discloses liposomes comprising dsRNAs targeted to the raf gene.

[0183] Transfersomes are yet another type of liposomes and are highly deformable lipid aggregates which are attractive candidates for drug delivery vehicles. Transfersomes may 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.

[0184] Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in Pharmaceutical Dosage Forms, Marcel Dekker, Inc., New York, N.Y., 1988, p. 285).

[0185] 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.

[0186] 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.

[0187] 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.

[0188] 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.

[0189] 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).

[0190] Nucleic Acid Lipid Particles

[0191] In one embodiment, a dsRNA featured in the invention is fully encapsulated in the lipid formulation, e.g., to form a nucleic acid-lipid particle, e.g., Nucleic acid-lipid particles typically contain a cationic lipid, a non-cationic lipid, a sterol, and a lipid that prevents aggregation of the particle (e.g., a PEG-lipid conjugate). Nucleic acid-lipid particles 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). In addition, the nucleic acids when present in the nucleic acid-lipid particles of the present invention are resistant in aqueous solution to degradation with a nuclease. Nucleic acid-lipid particles and their method of preparation are disclosed in, e.g., U.S. Pat. Nos. 5,976,567; 5,981,501; 6,534,484; 6,586,410; 6,815,432; and PCT Publication No. WO 96/40964.

[0192] Nucleic acid-lipid particles can further include one or more additional lipids and/or other components such as cholesterol. Other lipids may be included in the liposome compositions for a variety of purposes, such as to prevent lipid oxidation or to attach ligands onto the liposome surface. Any of a number of lipids may be present, including amphipathic, neutral, cationic, and anionic lipids. Such lipids can be used alone or in combination. Specific examples of additional lipid components that may be present are described herein.

[0193] Additional components that may be present in a nucleic acid-lipid particle include bilayer stabilizing components such as polyamide oligomers (see, e.g., U.S. Pat. No. 6,320,017), peptides, proteins, detergents, lipid-derivatives, such as PEG coupled to phosphatidylethanolamine and PEG conjugated to ceramides (see, U.S. Pat. No. 5,885, 613).

[0194] A nucleic acid-lipid particle can include one or more of a second amino lipid or cationic lipid, a neutral lipid, a sterol, and a lipid selected to reduce aggregation of lipid particles during formation, which may result from steric stabilization of particles which prevents charge-induced aggregation during formation.

[0195] Nucleic acid-lipid particles include, e.g., a SPLP, pSPLP, and SNALP. The term "SNALP" refers to a stable nucleic acid-lipid particle, including SPLP. The term "SPLP" refers to a nucleic acid-lipid particle comprising plasmid DNA encapsulated within a lipid vesicle. SPLPs include "pSPLP," which include an encapsulated condensing agent-nucleic acid complex as set forth in PCT Publication No. WO 00/03683.

[0196] The particles of the present invention typically have a mean diameter of about 50 nm to about 150 nm, more

typically about 60 nm to about 130 nm, more typically about 70 nm to about 110 nm, most typically about 70 nm to about 90 nm, and are substantially nontoxic

[0197]   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, or about 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, or 33:1.

[0198]   Cationic Lipids

[0199]   The nucleic acid-lipid particles of the invention typically include a cationic lipid. The cationic lipid may be, for example, N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N—(I-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), N—(I-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-dimethyl-2,3-dioleyloxy)propylamine (DODMA), 1,2-DiLinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), 1,2-Dilinoleylcarbamoyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-Dilinoleyoxy-3-(dimethylamino)acetoxypropane (DLin-DAC), 1,2-Dilinoleyoxy-3-morpholinopropane (DLin-MA), 1,2-Dilinoleoyl-3-dimethylaminopropane (DLinDAP), 1,2-Dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), 1-Linoleoyl-2-linoleyloxy-3-dimethylaminopropane (DLin-2-DMAP), 1,2-Dilinoleyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-Dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), 1,2-Dilinoleyloxy-3-(N-methylpiperazino)propane (DLin-MPZ), or 3-(N,N-Dilinoleylamino)-1,2-propanediol (DLinAP), 3-(N,N-Dioleylamino)-1,2-propanedio (DOAP), 1,2-Dilinoleyloxo-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), 1,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLinDMA), 2,2-Dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA) or analogs thereof, (3aR,5s,6aS)—N,N-dimethyl-2,2-di((9Z,12Z)-octadeca-9,12-dienyl)tetrahydro-3aH-cyclopenta[d][1,3]dioxol-5-amine (ALNY-100), (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (MC3), or a mixture thereof.

[0200]   Other cationic lipids, which carry a net positive charge at about physiological pH, in addition to those specifically described above, may also be included in lipid particles of the invention. Such cationic lipids include, but are not limited to, N,N-dioleyl-N,N-dimethylammonium chloride ("DODAC"); N-(2,3-dioleyloxy)propyl-N,N—N-triethylammonium chloride ("DOTMA"); N,N-distearyl-N,N-dimethylammonium bromide ("DDAB"); N-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride ("DOTAP"); 1,2-Dioleyloxy-3-trimethylaminopropane chloride salt ("DOTAP.Cl"); 3β-(N—(N',N'-dimethylaminoethane)-carbamoyl)cholesterol ("DC-Chol"), N-(1-(2,3-dioleyloxy)propyl)-N-2-(sperminecarboxamido)ethyl)-N,N-dimethylammonium trifluoracetate ("DOSPA"), dioctadecylamidoglycyl carboxyspermine ("DOGS"), 1,2-dileoyl-sn-3-phosphoethanolamine ("DOPE"), 1,2-dioleoyl-3-dimethylammonium propane ("DODAP"), N, N-dimethyl-2,3-dioleyloxy)propylamine ("DODMA"), and N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide ("DMRIE"). Additionally, a number of commercial preparations of cationic lipids can be used, such as, e.g., LIPOFECTIN (including DOTMA and DOPE,

available from GIBCO/BRL), and LIPOFECTAMINE (comprising DOSPA and DOPE, available from GIBCO/BRL). In particular embodiments, a cationic lipid is an amino lipid.

[0201]   As used herein, the term "amino lipid" is meant to include those lipids having one or two fatty acid or fatty alkyl chains and an amino head group (including an alkylamino or dialkylamino group) that may be protonated to form a cationic lipid at physiological pH.

[0202]   Other amino lipids would include those having alternative fatty acid groups and other dialkylamino groups, including those in which the alkyl substituents are different (e.g., N-ethyl-N-methylamino-, N-propyl-N-ethylamino- and the like). For those embodiments in which R^{11} and R^{12} are both long chain alkyl or acyl groups, they can be the same or different. In general, amino lipids having less saturated acyl chains are more easily sized, particularly when the complexes must be sized below about 0.3 microns, for purposes of filter sterilization. Amino lipids containing unsaturated fatty acids with carbon chain lengths in the range of C_{14} to C_{22} are preferred. Other scaffolds can also be used to separate the amino group and the fatty acid or fatty alkyl portion of the amino lipid. Suitable scaffolds are known to those of skill in the art.

[0203]   In certain embodiments, amino or cationic lipids of the invention have at least one protonatable or deprotonatable group, such that the lipid is positively charged at a pH at or below physiological pH (e.g. pH 7.4), and neutral at a second pH, preferably at or above physiological pH. It will, of course, be understood that the addition or removal of protons as a function of pH is an equilibrium process, and that the reference to a charged or a neutral lipid refers to the nature of the predominant species and does not require that all of the lipid be present in the charged or neutral form. Lipids that have more than one protonatable or deprotonatable group, or which are zwiterrionic, are not excluded from use in the invention.

[0204]   In certain embodiments, protonatable lipids according to the invention have a pKa of the protonatable group in the range of about 4 to about 11. Most preferred is pKa of about 4 to about 7, because these lipids will be cationic at a lower pH formulation stage, while particles will be largely (though not completely) surface neutralized at physiological pH around pH 7.4. One of the benefits of this pKa is that at least some nucleic acid associated with the outside surface of the particle will lose its electrostatic interaction at physiological pH and be removed by simple dialysis; thus greatly reducing the particle's susceptibility to clearance.

[0205]   One example of a cationic lipid is 1,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLinDMA). Synthesis and preparation of nucleic acid-lipid particles including DLinDMA is described in International application number PCT/CA2009/00496, filed Apr. 15, 2009.

[0206]   In one embodiment, the cationic lipid XTC (2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane) is used to prepare nucleic acid-lipid particles. Synthesis of XTC is described in U.S. provisional patent application No. 61/107,998 filed on Oct. 23, 2008, which is herein incorporated by reference.

[0207] In one aspect, the cationic lipids have the structure

$$R_3 - E - \overset{R_1}{\underset{R_2}{<}}$$
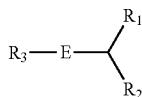
[0208] and salts or isomers thereof, wherein R_1 and R_2 are each independently for each occurrence optionally substituted C_{10}-C_{30} alkyl, optionally substituted C_{10}-C_{30} alkenyl, optionally substituted C_{10}-C_{30} alkynyl, optionally substituted C_{10}-C_{30} acyl, or -linker-ligand; R_3 is H, optionally substituted C_1-C_{10} alkyl, optionally substituted C_2-C_{10} alkenyl, optionally substituted C_2-C_{10} alkynyl, alkylhetrocycle, alkylphosphate, alkylphosphorothioate, alkylphosphorodithioate, alkylphosphonates, alkylamines, hydroxyalkyls, ω-aminoalkyls, ω-(substituted)aminoalkyls, ω-phosphoalkyls, ω-thiophosphoalkyls, optionally substituted polyethylene glycol (PEG, mw 100-40K), optionally substituted mPEG (mw 120-40K), heteroaryl, heterocycle, or linker-ligand; and E is C(O)O or OC(O). Synthesis and use of this lipid family is described in WO 2010/054401 (PCTUS2009/063927 filed on Nov. 10, 2009. The cationic lipid MC3 is one embodiment of this family of cationic lipids.

[0209] In another embodiment, the cationic lipid MC3 ((6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate), (e.g., DLin-M-C3-DMA) is used to prepare nucleic acid-lipid particles. Synthesis of MC3 and MC3 comprising formulations are described, e.g., in U.S. Provisional Ser. No. 61/244,834, filed Sep. 22, 2009, and U.S. Provisional Ser. No. 61/185,800, filed Jun. 10, 2009, and U.S. patent application Ser. No. 12/813,448 filed on Jun. 10, 2010, which are hereby incorporated by reference.

[0210] In another embodiment, the cationic lipid ALNY-100 ((3aR,5s,6aS)—N,N-dimethyl-2,2-di((9Z,12Z)-octadeca-9,12-dienyl)tetrahydro-3aH-cyclopenta[d][1,3]dioxol-5-amine) is used to prepare nucleic acid-lipid particles. Synthesis of ALNY-100 is described in International patent application number PCT/US09/63933 filed on Nov. 10, 2009, which is herein incorporated by reference.

[0211] FIG. 28 illustrates the structures of ALNY-100 and MC3.

[0212] The cationic lipid may comprise from about 20 mol % to about 70 mol % or about 45-65 mol % or about 10, 20, 30, 40, 50, 60, or 70 mol % of the total lipid present in the particle.

[0213] Non-Cationic Lipids

[0214] The nucleic acid-lipid particles of the invention can include a non-cationic lipid. The non-cationic lipid may be an anionic lipid or a neutral lipid. Examples include but not limited to, distearoylphosphatidylcholine (DSPC), dioleoyl-phosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoyl-phosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoylphosphatidylethanolamine (POPE), dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidyl-ethanolamine (DSPE), 16-O-monom-ethyl PE, 16-O-dimethyl PE, 18-1-trans PE, 1-stearoyl-2-oleoyl-phosphatidyethanolamine (SOPE), cholesterol, or a mixture thereof.

[0215] Anionic lipids suitable for use in lipid particles of the invention include, but are not limited to, phosphatidylglycerol, cardiolipin, diacylphosphatidylserine, diacylphosphatidic acid, N-dodecanoyl phosphatidylethanoloamine, N-succinyl phosphatidylethanolamine, N-glutaryl phosphatidylethanolamine, lysylphosphatidylglycerol, and other anionic modifying groups joined to neutral lipids.

[0216] Neutral lipids, when present in the lipid particle, can be any of a number of lipid species which exist either in an uncharged or neutral zwitterionic form at physiological pH. Such lipids include, for example diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, dihydrosphingomyelin, cephalin, and cerebrosides. The selection of neutral lipids for use in the particles described herein is generally guided by consideration of, e.g., liposome size and stability of the liposomes in the bloodstream. Preferably, the neutral lipid component is a lipid having two acyl groups, (i.e., diacylphosphatidylcholine and diacylphosphatidylethanolamine). Lipids having a variety of acyl chain groups of varying chain length and degree of saturation are available or may be isolated or synthesized by well-known techniques. In one group of embodiments, lipids containing saturated fatty acids with carbon chain lengths in the range of C_{14} to C_{22} are preferred. In another group of embodiments, lipids with mono- or di-unsaturated fatty acids with carbon chain lengths in the range of C_{14} to C_{22} are used. Additionally, lipids having mixtures of saturated and unsaturated fatty acid chains can be used. Preferably, the neutral lipids used in the invention are DOPE, DSPC, POPC, or any related phosphatidylcholine. The neutral lipids useful in the invention may also be composed of sphingomyelin, dihydrosphingomyeline, or phospholipids with other head groups, such as serine and inositol.

[0217] In one embodiment the non-cationic lipid is distearoylphosphatidylcholine (DSPC). In another embodiment the non-cationic lipid is dipalmitoylphosphatidylcholine (DPPC).

[0218] The non-cationic lipid may be from about 5 mol % to about 90 mol %, about 5 mol % to about 10 mol %, about 10 mol %, or about 58 mol % if cholesterol is included, of the total lipid present in the particle.

[0219] Conjugated Lipids

[0220] Conjugated lipids can be used in nucleic acid-lipid particle to prevent aggregation, including polyethylene glycol (PEG)-modified lipids, monosialoganglioside Gml, and polyamide oligomers ("PAO") such as (described in U.S. Pat. No. 6,320,017). Other compounds with uncharged, hydrophilic, steric-barrier moieties, which prevent aggregation during formulation, like PEG, Gml or ATTA, can also be coupled to lipids for use as in the methods and compositions of the invention. ATTA-lipids are described, e.g., in U.S. Pat. No. 6,320,017, and PEG-lipid conjugates are described, e.g., in U.S. Pat. Nos. 5,820,873, 5,534,499 and 5,885,613. Typically, the concentration of the lipid component selected to reduce aggregation is about 1 to 15% (by mole percent of lipids).

[0221] Specific examples of PEG-modified lipids (or lipid-polyoxyethylene conjugates) that are useful in the invention can have a variety of "anchoring" lipid portions to secure the PEG portion to the surface of the lipid vesicle.

Examples of suitable PEG-modified lipids include PEG-modified phosphatidylethanolamine and phosphatidic acid, PEG-ceramide conjugates (e.g., PEG-CerC14 or PEG-CerC20) which are described in co-pending U.S. Ser. No. 08/486,214, incorporated herein by reference, PEG-modified dialkylamines and PEG-modified 1,2-diacyloxypropan-3-amines. Particularly preferred are PEG-modified diacylglycerols and dialkylglycerols.

[0222] In embodiments where a sterically-large moiety such as PEG or ATTA are conjugated to a lipid anchor, the selection of the lipid anchor depends on what type of association the conjugate is to have with the lipid particle. It is well known that mePEG (mw2000)-diastearoylphosphatidylethanolamine (PEG-DSPE) will remain associated with a liposome until the particle is cleared from the circulation, possibly a matter of days. Other conjugates, such as PEG-CerC20 have similar staying capacity. PEG-CerC14, however, rapidly exchanges out of the formulation upon exposure to serum, with a $T_{1/2}$ less than 60 minutes in some assays. As illustrated in U.S. patent application Ser. No. 08/486,214, at least three characteristics influence the rate of exchange: length of acyl chain, saturation of acyl chain, and size of the steric-barrier head group. Compounds having suitable variations of these features may be useful for the invention. For some therapeutic applications, it may be preferable for the PEG-modified lipid to be rapidly lost from the nucleic acid-lipid particle in vivo and hence the PEG-modified lipid will possess relatively short lipid anchors. In other therapeutic applications, it may be preferable for the nucleic acid-lipid particle to exhibit a longer plasma circulation lifetime and hence the PEG-modified lipid will possess relatively longer lipid anchors. Exemplary lipid anchors include those having lengths of from about C_{14} to about C_{22}, preferably from about C_{14} to about C_{16}. In some embodiments, a PEG moiety, for example an mPEG-NH$_2$, has a size of about 1000, 2000, 5000, 10,000, 15,000 or 20,000 Daltons.

[0223] It should be noted that aggregation preventing compounds do not necessarily require lipid conjugation to function properly. Free PEG or free ATTA in solution may be sufficient to prevent aggregation. If the particles are stable after formulation, the PEG or ATTA can be dialyzed away before administration to a subject.

[0224] The conjugated lipid that inhibits aggregation of particles may be, for example, a polyethyleneglycol (PEG)-lipid including, without limitation, a PEG-diacylglycerol (DAG), a PEG-dialkyloxypropyl (DAA), a PEG-phospholipid, a PEG-ceramide (Cer), or a mixture thereof. The PEG-DAA conjugate may be, for example, a PEG-dilauryloxypropyl (Ci$_2$), a PEG-dimyristyloxypropyl (Ci$_4$), a PEG-dipalmityloxypropyl (Ci$_6$), or a PEG-distearyloxypropyl (Ci$_8$). Additional conjugated lipids include polyethylene glycol-didimyristoyl glycerol (C14-PEG or PEG-C14, where PEG has an average molecular weight of 2000 Da) (PEG-DMG); (R)-2,3-bis(octadecyloxy)propyl1-(methoxy poly(ethylene glycol)2000)propylcarbamate) (PEG-DSG); PEG-carbamoyl-1,2-dimyristyloxypropylamine, in which PEG has an average molecular weight of 2000 Da (PEG-cDMA); N-Acetylgalactosamine-((R)-2,3-bis(octadecyloxy)propyl1-(methoxy poly(ethylene glycol)2000)propylcarbamate)) (GalNAc-PEG-DSG); and polyethylene glycol-dipalmitoylglycerol (PEG-DPG).

[0225] In one embodiment the conjugated lipid is PEG-DMG. In another embodiment the conjugated lipid is PEG-cDMA. In still another embodiment the conjugated lipid is PEG-DPG. Alternatively the conjugated lipid is GalNAc-PEG-DSG.

[0226] The conjugated lipid that prevents aggregation of particles may be from 0 mol % to about 20 mol % or about 0.5 to about 5.0 mol % or about 2 mol % of the total lipid present in the particle.

[0227] The sterol component of the lipid mixture, when present, can be any of those sterols conventionally used in the field of liposome, lipid vesicle or lipid particle preparation. A preferred sterol is cholesterol.

[0228] In some embodiments, the nucleic acid-lipid particle further includes a sterol, e.g., a cholesterol at, e.g., about 10 mol % to about 60 mol % or about 25 to about 40 mol % or about 48 mol % of the total lipid present in the particle.

[0229] Lipoproteins

[0230] In one embodiment, the formulations of the invention further comprise an apolipoprotein. As used herein, the term "apolipoprotein" or "lipoprotein" refers to apolipoproteins known to those of skill in the art and variants and fragments thereof and to apolipoprotein agonists, analogues or fragments thereof described below.

[0231] Suitable apolipoproteins include, but are not limited to, ApoA-I, ApoA-II, ApoA-IV, ApoA-V and ApoE, and active polymorphic forms, isoforms, variants and mutants as well as fragments or truncated forms thereof. In certain embodiments, the apolipoprotein is a thiol containing apolipoprotein. "Thiol containing apolipoprotein" refers to an apolipoprotein, variant, fragment or isoform that contains at least one cysteine residue. The most common thiol containing apolipoproteins are ApoA-I Milano (ApoA-I$_M$) and ApoA-I Paris (ApoA-Ip) which contain one cysteine residue (Jia et al., 2002, Biochem. Biophys. Res. Comm. 297: 206-13; Bielicki and Oda, 2002, Biochemistry 41: 2089-96). ApoA-II, ApoE2 and ApoE3 are also thiol containing apolipoproteins. Isolated ApoE and/or active fragments and polypeptide analogues thereof, including recombinantly produced forms thereof, are described in U.S. Pat. Nos. 5,672,685; 5,525,472; 5,473,039; 5,182,364; 5,177,189; 5,168,045; 5,116,739; the disclosures of which are herein incorporated by reference. ApoE3 is disclosed in Weisgraber, et al., "Human E apoprotein heterogeneity: cysteine-arginine interchanges in the amino acid sequence of the apo-E isoforms," J. Biol. Chem. (1981) 256: 9077-9083; and Rall, et al., "Structural basis for receptor binding heterogeneity of apolipoprotein E from type III hyperlipoproteinemic subjects," Proc. Nat. Acad. Sci. (1982) 79: 4696-4700. (See also GenBank accession number K00396.)

[0232] In certain embodiments, the apolipoprotein can be in its mature form, in its preproapolipoprotein form or in its proapolipoprotein form. Homo- and heterodimers (where feasible) of pro- and mature ApoA-I (Duverger et al., 1996, Arterioscler. Thromb. Vasc. Biol. 16(12):1424-29), ApoA-I Milano (Klon et al., 2000, Biophys. J. 79:(3)1679-87; Franceschini et al., 1985, J. Biol. Chem. 260: 1632-35), ApoA-I Paris (Daum et al., 1999, J. Mol. Med. 77:614-22), ApoA-II (Shelness et al., 1985, J. Biol. Chem. 260(14):8637-46; Shelness et al., 1984, J. Biol. Chem. 259(15):9929-35), ApoA-IV (Duverger et al., 1991, Euro. J. Biochem. 201(2): 373-83), and ApoE (McLean et al., 1983, J. Biol. Chem. 258(14):8993-9000) can also be utilized within the scope of the invention.

[0233] In certain embodiments, the apolipoprotein can be a fragment, variant or isoform of the apolipoprotein. The term "fragment" refers to any apolipoprotein having an amino acid sequence shorter than that of a native apolipoprotein and which fragment retains the activity of native apolipoprotein, including lipid binding properties. By "variant" is meant substitutions or alterations in the amino acid sequences of the apolipoprotein, which substitutions or alterations, e.g., additions and deletions of amino acid residues, do not abolish the activity of native apolipoprotein, including lipid binding properties. Thus, a variant can comprise a protein or peptide having a substantially identical amino acid sequence to a native apolipoprotein provided herein in which one or more amino acid residues have been conservatively substituted with chemically similar amino acids. Examples of conservative substitutions include the substitution of at least one hydrophobic residue such as isoleucine, valine, leucine or methionine for another. Likewise, the present invention contemplates, for example, the substitution of at least one hydrophilic residue such as, for example, between arginine and lysine, between glutamine and asparagine, and between glycine and serine (see U.S. Pat. Nos. 6,004,925, 6,037,323 and 6,046,166). The term "isoform" refers to a protein having the same, greater or partial function and similar, identical or partial sequence, and may or may not be the product of the same gene and usually tissue specific (see Weisgraber 1990, J. Lipid Res. 31(8):1503-11; Hixson and Powers 1991, J. Lipid Res. 32(9):1529-35; Lackner et al., 1985, J. Biol. Chem. 260(2): 703-6; Hoeg et al., 1986, J. Biol. Chem. 261(9):3911-4; Gordon et al., 1984, J. Biol. Chem. 259(1):468-74; Powell et al., 1987, Cell 50(6):831-40; Aviram et al., 1998, Arterioscler. Thromb. Vasc. Biol. 18(10):1617-24; Aviram et al., 1998, J. Clin. Invest. 101(8):1581-90; Billecke et al., 2000, Drug Metab. Dispos. 28(11):1335-42; Draganov et al., 2000, J. Biol. Chem. 275(43):33435-42; Steinmetz and Utermann 1985, J. Biol. Chem. 260(4):2258-64; Widler et al., 1980, J. Biol. Chem. 255(21):10464-71; Dyer et al., 1995, J. Lipid Res. 36(1):80-8; Sacre et al., 2003, FEBS Lett. 540(1-3): 181-7; Weers, et al., 2003, Biophys. Chem. 100(1-3):481-92; Gong et al., 2002, J. Biol. Chem. 277(33):29919-26; Ohta et al., 1984, J. Biol. Chem. 259(23):14888-93 and U.S. Pat. No. 6,372,886).

[0234] In certain embodiments, the methods and compositions of the present invention include the use of a chimeric construction of an apolipoprotein. For example, a chimeric construction of an apolipoprotein can be comprised of an apolipoprotein domain with high lipid binding capacity associated with an apolipoprotein domain containing ischemia reperfusion protective properties. A chimeric construction of an apolipoprotein can be a construction that includes separate regions within an apolipoprotein (i.e., homologous construction) or a chimeric construction can be a construction that includes separate regions between different apolipoproteins (i.e., heterologous constructions). Compositions comprising a chimeric construction can also include segments that are apolipoprotein variants or segments designed to have a specific character (e.g., lipid binding, receptor binding, enzymatic, enzyme activating, antioxidant or reduction-oxidation property) (see Weisgraber 1990, J. Lipid Res. 31(8):1503-11; Hixson and Powers 1991, J. Lipid Res. 32(9):1529-35; Lackner et al., 1985, J. Biol. Chem. 260(2): 703-6; Hoeg et al., 1986, J. Biol. Chem. 261(9):3911-4; Gordon et al., 1984, J. Biol. Chem. 259(1):468-74; Powell et

al., 1987, Cell 50(6):831-40; Aviram et al., 1998, Arterioscler. Thromb. Vasc. Biol. 18(10):1617-24; Aviram et al., 1998, J. Clin. Invest. 101(8):1581-90; Billecke et al., 2000, Drug Metab. Dispos. 28(11):1335-42; Draganov et al., 2000, J. Biol. Chem. 275(43):33435-42; Steinmetz and Utermann 1985, J. Biol. Chem. 260(4):2258-64; Widler et al., 1980, J. Biol. Chem. 255(21):10464-71; Dyer et al., 1995, J. Lipid Res. 36(1):80-8; Sorenson et al., 1999, Arterioscler. Thromb. Vasc. Biol. 19(9):2214-25; Palgunachari 1996, Arterioscler. Throb. Vasc. Biol. 16(2):328-38: Thurberg et al., J. Biol. Chem. 271(11):6062-70; Dyer 1991, J. Biol. Chem. 266(23): 150009-15; Hill 1998, J. Biol. Chem. 273(47):30979-84).

[0235] Apolipoproteins utilized in the invention also include recombinant, synthetic, semi-synthetic or purified apolipoproteins. Methods for obtaining apolipoproteins or equivalents thereof, utilized by the invention are well-known in the art. For example, apolipoproteins can be separated from plasma or natural products by, for example, density gradient centrifugation or immunoaffinity chromatography, or produced synthetically, semi-synthetically or using recombinant DNA techniques known to those of the art (see, e.g., Mulugeta et al., 1998, J. Chromatogr. 798(1-2): 83-90; Chung et al., 1980, J. Lipid Res. 21(3):284-91; Cheung et al., 1987, J. Lipid Res. 28(8):913-29; Persson, et al., 1998, J. Chromatogr. 711:97-109; U.S. Pat. Nos. 5,059, 528, 5,834,596, 5,876,968 and 5,721,114; and PCT Publications WO 86/04920 and WO 87/02062).

[0236] Apolipoproteins utilized in the invention further include apolipoprotein agonists such as peptides and peptide analogues that mimic the activity of ApoA-I, ApoA-I Milano (ApoA-I$_M$), ApoA-I Paris (ApoA-I$_P$), ApoA-II, ApoA-IV, and ApoE. For example, the apolipoprotein can be any of those described in U.S. Pat. Nos. 6,004,925, 6,037,323, 6,046,166, and 5,840,688, the contents of which are incorporated herein by reference in their entireties.

[0237] Apolipoprotein agonist peptides or peptide analogues can be synthesized or manufactured using any technique for peptide synthesis known in the art including, e.g., the techniques described in U.S. Pat. Nos. 6,004,925, 6,037, 323 and 6,046,166. For example, the peptides may be prepared using the solid-phase synthetic technique initially described by Merrifield (1963, J. Am. Chem. Soc. 85:2149-2154). Other peptide synthesis techniques may be found in Bodanszky et al., Peptide Synthesis, John Wiley & Sons, 2d Ed., (1976) and other references readily available to those skilled in the art. A summary of polypeptide synthesis techniques can be found in Stuart and Young, Solid Phase Peptide. Synthesis, Pierce Chemical Company, Rockford, Ill., (1984). Peptides may also be synthesized by solution methods as described in The Proteins, Vol. II, 3d Ed., Neurath et al., Eds., p. 105-237, Academic Press, New York, N.Y. (1976). Appropriate protective groups for use in different peptide syntheses are described in the above-mentioned texts as well as in McOmie, Protective Groups in Organic Chemistry, Plenum Press, New York, N.Y. (1973). The peptides of the present invention might also be prepared by chemical or enzymatic cleavage from larger portions of, for example, apolipoprotein A-I.

[0238] In certain embodiments, the apolipoprotein can be a mixture of apolipoproteins. In one embodiment, the apolipoprotein can be a homogeneous mixture, that is, a single type of apolipoprotein. In another embodiment, the apolipoprotein can be a heterogeneous mixture of apolipoproteins, that is, a mixture of two or more different apolipo-

proteins. Embodiments of heterogeneous mixtures of apolipoproteins can comprise, for example, a mixture of an apolipoprotein from an animal source and an apolipoprotein from a semi-synthetic source. In certain embodiments, a heterogeneous mixture can comprise, for example, a mixture of ApoA-I and ApoA-I Milano. In certain embodiments, a heterogeneous mixture can comprise, for example, a mixture of ApoA-I Milano and ApoA-I Paris. Suitable mixtures for use in the methods and compositions of the invention will be apparent to one of skill in the art.

[0239] If the apolipoprotein is obtained from natural sources, it can be obtained from a plant or animal source. If the apolipoprotein is obtained from an animal source, the apolipoprotein can be from any species. In certain embodiments, the apolipoprotien can be obtained from an animal source. In certain embodiments, the apolipoprotein can be obtained from a human source. In preferred embodiments of the invention, the apolipoprotein is derived from the same species as the individual to which the apolipoprotein is administered.

[0240] Other Components

[0241] In numerous embodiments, amphipathic lipids are included in lipid particles of the invention. "Amphipathic lipids" refer to any suitable material, wherein the hydrophobic portion of the lipid material orients into a hydrophobic phase, while the hydrophilic portion orients toward the aqueous phase. Such compounds include, but are not limited to, phospholipids, aminolipids, and sphingolipids. Representative phospholipids include sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoyloleoyl phosphatdylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine, or dilinoleylphosphatidylcholine. Other phosphorus-lacking compounds, such as sphingolipids, glycosphingolipid families, diacylglycerols, and β-acyloxyacids, can also be used. Additionally, such amphipathic lipids can be readily mixed with other lipids, such as triglycerides and sterols.

[0242] Also suitable for inclusion in the lipid particles of the invention are programmable fusion lipids. Such lipid particles have little tendency to fuse with cell membranes and deliver their payload until a given signal event occurs. This allows the lipid particle to distribute more evenly after injection into an organism or disease site before it starts fusing with cells. The signal event can be, for example, a change in pH, temperature, ionic environment, or time. In the latter case, a fusion delaying or "cloaking" component, such as an ATTA-lipid conjugate or a PEG-lipid conjugate, can simply exchange out of the lipid particle membrane over time. Exemplary lipid anchors include those having lengths of from about C_{14} to about C_{22}, preferably from about C_{14} to about C_{16}. In some embodiments, a PEG moiety, for example an mPEG-NH$_2$, has a size of about 1000, 2000, 5000, 10,000, 15,000 or 20,000 Daltons.

[0243] A lipid particle conjugated to a nucleic acid agent can also include a targeting moiety, e.g., a targeting moiety that is specific to a cell type or tissue. Targeting of lipid particles using a variety of targeting moieties, such as ligands, cell surface receptors, glycoproteins, vitamins (e.g., riboflavin) and monoclonal antibodies, has been previously described (see, e.g., U.S. Pat. Nos. 4,957,773 and 4,603,044). The targeting moieties can include the entire protein or fragments thereof. Targeting mechanisms generally require

that the targeting agents be positioned on the surface of the lipid particle in such a manner that the targeting moiety is available for interaction with the target, for example, a cell surface receptor. A variety of different targeting agents and methods are known and available in the art, including those described, e.g., in Sapra, P. and Allen, T M, *Prog. Lipid Res.* 42(5):439-62 (2003); and Abra, R M et al., *J. Liposome Res.* 12:1-3, (2002).

[0244] The use of lipid particles, i.e., liposomes, with a surface coating of hydrophilic polymer chains, such as polyethylene glycol (PEG) chains, for targeting has been proposed (Allen, et al., *Biochimica et Biophysica Acta* 1237: 99-108 (1995); DeFrees, et al., *Journal of the American Chemistry Society* 118: 6101-6104 (1996); Blume, et al., *Biochimica et Biophysica Acta* 1149: 180-184 (1993); Klibanov, et al., *Journal of Liposome Research* 2: 321-334 (1992); U.S. Pat. No. 5,013,556; Zalipsky, Bioconjugate Chemistry 4: 296-299 (1993); *Zalipsky, FEBS Letters* 353: 71-74 (1994); Zalipsky, in Stealth Liposomes Chapter 9 (Lasic and Martin, Eds) CRC Press, Boca Raton Fla. (1995). In one approach, a ligand, such as an antibody, for targeting the lipid particle is linked to the polar head group of lipids forming the lipid particle. In another approach, the targeting ligand is attached to the distal ends of the PEG chains forming the hydrophilic polymer coating (Klibanov, et al., *Journal of Liposome Research* 2: 321-334 (1992); Kirpotin et al., *FEBS Letters* 388: 115-118 (1996)).

[0245] Standard methods for coupling the target agents can be used. For example, phosphatidylethanolamine, which can be activated for attachment of target agents, or derivatized lipophilic compounds, such as lipid-derivatized bleomycin, can be used. Antibody-targeted liposomes can be constructed using, for instance, liposomes that incorporate protein A (see, Renneisen, et al., *J. Bio. Chem.*, 265:16337-16342 (1990) and Leonetti, et al., *Proc. Natl. Acad. Sci.* (*USA*), 87:2448-2451 (1990). Other examples of antibody conjugation are disclosed in U.S. Pat. No. 6,027,726, the teachings of which are incorporated herein by reference. Examples of targeting moieties can also include other proteins, specific to cellular components, including antigens associated with neoplasms or tumors. Proteins used as targeting moieties can be attached to the liposomes via covalent bonds (see, Heath, *Covalent Attachment of Proteins to Liposomes*, 149 *Methods in Enzymology* 111-119 (Academic Press, Inc. 1987)). Other targeting methods include the biotin-avidin system.

[0246] Production of Nucleic Acid-Lipid Particles

[0247] In one embodiment, the nucleic acid-lipid particle formulations of the invention are produced via an extrusion method or an in-line mixing method.

[0248] The extrusion method (also referred to as preformed method or batch process) is a method where the empty liposomes (i.e. no nucleic acid) are prepared first, followed by the addition of nucleic acid to the empty liposome. Extrusion of liposome compositions through a small-pore polycarbonate membrane or an asymmetric ceramic membrane results in a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired liposome complex size distribution is achieved. The liposomes may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in liposome size. In some instances, the lipid-nucleic acid compositions which are formed can be used without any sizing. These methods are

disclosed in the U.S. Pat. No. 5,008,050; U.S. Pat. No. 4,927,637; U.S. Pat. No. 4,737,323; *Biochim Biophys Acta.* 1979 Oct. 19; 557(1):9-23; *Biochim Biophys Acta.* 1980 Oct. 2; 601(3):559-7; *Biochim Biophys Acta.* 1986 Jun. 13; 858(1):161-8; and *Biochim. Biophys. Acta* 1985 812, 55-65, which are hereby incorporated by reference in their entirety.

[0249] The in-line mixing method is a method wherein both the lipids and the nucleic acid are added in parallel into a mixing chamber. The mixing chamber can be a simple T-connector or any other mixing chamber that is known to one skill in the art. These methods are disclosed in U.S. Pat. No. 6,534,018 and U.S. Pat. No. 6,855,277; US publication 2007/0042031 and Pharmaceuticals Research, Vol. 22, No. 3, March 2005, p. 362-372, which are hereby incorporated by reference in their entirety.

[0250] It is further understood that the formulations of the invention can be prepared by any methods known to one of ordinary skill in the art.

[0251] Characterization of Nucleic Acid-Lipid Particles

[0252] Formulations prepared by either the standard or extrusion-free method can be characterized in similar manners. For example, formulations are typically characterized by visual inspection. They should be whitish translucent solutions free from aggregates or sediment. Particle size and particle size distribution of lipid-nanoparticles can be measured by light scattering using, for example, a Malvern Zetasizer Nano ZS (Malvern, USA). Particles should be about 20-300 nm, such as 40-100 nm in size. The particle size distribution should be unimodal. The total siRNA concentration in the formulation, as well as the entrapped fraction, is estimated using a dye exclusion assay. A sample

least 110 nm, about at least 60 nm to about at least 100 nm, or about at least 80 nm to about at least 90 nm.

[0254] Formulations of Nucleic Acid-Lipid Particles

[0255] LNP01

[0256] One example of synthesis of a nucleic acid-lipid particle is as follows. Nucleic acid-lipid particles are synthesized using the lipidoid ND98.4HCl (MW 1487) (Formula 1), Cholesterol (Sigma-Aldrich), and PEG-Ceramide C16 (Avanti Polar Lipids). This nucleic acid-lipid particle is sometimes referred to as a LNP01 particle. Stock solutions of each in ethanol can be prepared as follows: ND98, 133 mg/ml; Cholesterol, 25 mg/ml, PEG-Ceramide C16, 100 mg/ml. The ND98, Cholesterol, and PEG-Ceramide C16 stock solutions can then be combined in a, e.g., 42:48:10 molar ratio. The combined lipid solution can be mixed with aqueous siRNA (e.g., in sodium acetate pH 5) such that the final ethanol concentration is about 35-45% and the final sodium acetate concentration is about 100-300 mM. Lipid-siRNA nanoparticles typically form spontaneously upon mixing. Depending on the desired particle size distribution, the resultant nanoparticle mixture can be extruded through a polycarbonate membrane (e.g., 100 nm cut-off) using, for example, a thermobarrel extruder, such as Lipex Extruder (Northern Lipids, Inc). In some cases, the extrusion step can be omitted. Ethanol removal and simultaneous buffer exchange can be accomplished by, for example, dialysis or tangential flow filtration. Buffer can be exchanged with, for example, phosphate buffered saline (PBS) at about pH 7, e.g., about pH 6.9, about pH 7.0, about pH 7.1, about pH 7.2, about pH 7.3, or about pH 7.4.

Formula 1

ND98 Isomer I
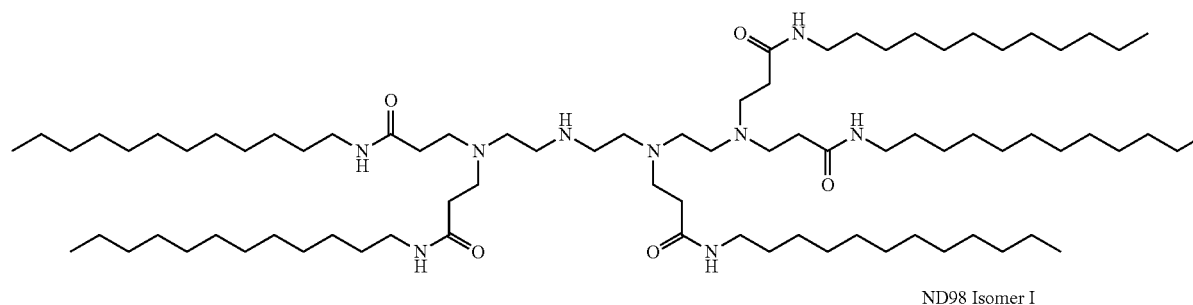
of the formulated siRNA can be incubated with an RNA-binding dye, such as Ribogreen (Molecular Probes) in the presence or absence of a formulation disrupting surfactant, e.g., 0.5% Triton-X100. The total siRNA in the formulation can be determined by the signal from the sample containing the surfactant, relative to a standard curve. The entrapped fraction is determined by subtracting the "free" siRNA content (as measured by the signal in the absence of surfactant) from the total siRNA content. Percent entrapped siRNA is typically >85%. In one embodiment, the formulations of the invention are entrapped by at least 75%, at least 80% or at least 90%.

[0253] For nucleic acid-lipid particle formulations, the particle size is at least 30 nm, at least 40 nm, at least 50 nm, at least 60 nm, at least 70 nm, at least 80 nm, at least 90 nm, at least 100 nm, at least 110 nm, and at least 120 nm. The suitable range is typically about at least 50 nm to about at

[0257] LNP01 formulations are described, e.g., in International Application Publication No. WO 2008/042973, which is hereby incorporated by reference.

[0258] Additional exemplary nucleic acid-lipid particle formulations are described in the following table. It is to be understood that the name of the nucleic acid-lipid particle in the table is not meant to be limiting. For example, as used herein, the term SNALP refers to formulations that include the cationic lipid DLinDMA.

Name	cationic lipid/non-cationic lipid/cholesterol/PEG-lipid conjugate mol % ratio Lipid:siRNA ratio
SNALP	DLinDMA/DPPC/Cholesterol/PEG-cDMA (57.1/7.1/34.4/1.4) lipid:siRNA ~7:1

-continued

Name	cationic lipid/non-cationic lipid/cholesterol/PEG-lipid conjugate mol % ratio Lipid:siRNA ratio
LNP-S-X	XTC/DPPC/Cholesterol/PEG-cDMA 57.1/7.1/34.4/1.4 lipid:siRNA ~7:1
LNP05	XTC/DSPC/Cholesterol/PEG-DMG 57.5/7.5/31.5/3.5 lipid:siRNA ~6:1
LNP06	XTC/DSPC/Cholesterol/PEG-DMG 57.5/7.5/31.5/3.5 lipid:siRNA ~11:1
LNP07	XTC/DSPC/Cholesterol/PEG-DMG 60/7.5/31/1.5, lipid:siRNA ~6:1
LNP08	XTC/DSPC/Cholesterol/PEG-DMG 60/7.5/31/1.5, lipid:siRNA ~11:1
LNP09	XTC/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 lipid:siRNA ~10:1
LNP10	ALNY-100/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 lipid:siRNA ~10:1
LNP11	MC3/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 lipid:siRNA ~10:1
LNP13	XTC/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 lipid:siRNA ~33:1
LNP14	MC3/DSPC/Cholesterol/PEG-DMG 40/15/40/5 lipid:siRNA ~11:1
LNP15	MC3/DSPC/Cholesterol/PEG-DSG/GalNAc-PEG-DSG 50/10/35/4.5/0.5 lipid:siRNA ~11:1
LNP16	MC3/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 lipid:siRNA ~7:1
LNP17	MC3/DSPC/Cholesterol/PEG-DSG 50/10/38.5/1.5 lipid:siRNA ~10:1
LNP18	MC3/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 lipid:siRNA ~12:1
LNP19	MC3/DSPC/Cholesterol/PEG-DMG 50/10/35/5 lipid:siRNA ~8:1
LNP20	MC3/DSPC/Cholesterol/PEG-DPG 50/10/38.5/1.5 lipid:siRNA ~10:1
LNP22	XTC/DSPC/Cholesterol/PEG-DSG 50/10/38.5/1.5 lipid:siRNA ~10:1

TABLE 11

Composition of exemplary nucleic acid-lipid particle (mole %) prepared via extrusion methods.				
Lipid (mol %)	DSPC (mol %)	Chol (mol %)	PEG (mol %)	Lipid/siRNA
20	30	40	10	2.13
20	30	40	10	2.35
20	30	40	10	2.37
20	30	40	10	3.23
20	30	40	10	3.91
30	20	40	10	2.89
30	20	40	10	3.34
30	20	40	10	3.34
30	20	40	10	4.10
30	20	40	10	5.64
40	10	40	10	3.02
40	10	40	10	3.35
40	10	40	10	3.74
40	10	40	10	5.80
40	10	40	10	8.00
45	5	40	10	3.27
45	5	40	10	3.30
45	5	40	10	4.45
45	5	40	10	7.00
45	5	40	10	9.80
50	0	40	10	27.03
20	35	40	5	3.00
20	35	40	5	3.32
20	35	40	5	3.05
20	35	40	5	3.67
20	35	40	5	4.71
30	25	40	5	2.47
30	25	40	5	2.98
30	25	40	5	3.29
30	25	40	5	4.99
30	25	40	5	7.15
40	15	40	5	2.79
40	15	40	5	3.29
40	15	40	5	4.33
40	15	40	5	7.05
40	15	40	5	9.63
45	10	40	5	2.44
45	10	40	5	3.21
45	10	40	5	4.29
45	10	40	5	6.50
45	10	40	5	8.67
20	35	40	5	4.10
20	35	40	5	4.83
30	25	40	5	3.86
30	25	40	5	5.38
30	25	40	5	7.07
40	15	40	5	3.85
40	15	40	5	4.88
40	15	40	5	7.22
40	15	40	5	9.75
45	10	40	5	2.83
45	10	40	5	3.85
45	10	40	5	4.88
45	10	40	5	7.05
45	10	40	5	9.29
45	20	30	5	4.01
45	20	30	5	3.70
50	15	30	5	4.75
50	15	30	5	3.80
55	10	30	5	3.85
55	10	30	5	4.13
60	5	30	5	5.09
60	5	30	5	4.67
65	0	30	5	4.75
65	0	30	5	6.06
56.5	10	30	3.5	3.70
56.5	10	30	3.5	3.56
57.5	10	30	2.5	3.48
57.5	10	30	2.5	3.20
58.5	10	30	1.5	3.24
58.5	10	30	1.5	3.13

[0259] XTC comprising formulations are described, e.g., in U.S. Provisional Ser. No. 61/239,686, filed Sep. 3, 2009, which is hereby incorporated by reference.

[0260] MC3 comprising formulations are described, e.g., in U.S. Provisional Ser. No. 61/244,834, filed Sep. 22, 2009, and U.S. Provisional Ser. No. 61/185,800, filed Jun. 10, 2009, which are hereby incorporated by reference.

[0261] ALNY-100 comprising formulations are described, e.g., International patent application number PCT/US09/63933, filed on Nov. 10, 2009, which is hereby incorporated by reference.

[0262] Additional representative formulations delineated in Tables 11 and 12. Lipid refers to a cationic lipid.

TABLE 11-continued

Composition of exemplary nucleic acid-lipid particle (mole %) prepared via extrusion methods.

Lipid (mol %)	DSPC (mol %)	Chol (mol %)	PEG (mol %)	Lipid/siRNA
59.5	10	30	0.5	3.24
59.5	10	30	0.5	3.03
45	10	40	5	7.57
45	10	40	5	7.24
45	10	40	5	7.48
45	10	40	5	7.84
65	0	30	5	4.01
60	5	30	5	3.70
55	10	30	5	3.65
50	10	35	5	3.43
50	15	30	5	3.80
45	15	35	5	3.70
45	20	30	5	3.75
45	25	25	5	3.85
55	10	32.5	2.5	3.61
60	10	27.5	2.5	3.65
60	10	25	5	4.07
55	5	38.5	1.5	3.75
60	10	28.5	1.5	3.43
55	10	33.5	1.5	3.48
60	5	33.5	1.5	3.43
55	5	37.5	2.5	3.75
60	5	32.5	2.5	4.52
60	5	32.5	2.5	3.52
45	15 (DMPC)	35	5	3.20
45	15 (DPPC)	35	5	3.43
45	15 (DOPC)	35	5	4.52
45	15 (POPC)	35	5	3.85
55	5	37.5	2.5	3.96
55	10	32.5	2.5	3.56
60	5	32.5	2.5	3.80
60	10	27.5	2.5	3.75
60	5	30	5	4.19
60	5	33.5	1.5	3.48
60	5	33.5	1.5	6.64
60	5	30	5	3.90
60	5	30	5	4.65
60	5	30	5	5.88
60	5	30	5	7.51
60	5	30	5	9.51
60	5	30	5	11.06
62.5	2.5	50	5	6.63
45	15	35	5	3.31
45	15	35	5	6.80
60	5	25	10	6.48
60	5	32.5	2.5	3.43
60	5	30	5	3.90
60	5	30	5	7.61
45	15	35	5	3.13
45	15	35	5	6.42
60	5	25	10	6.48
60	5	32.5	2.5	3.03
60	5	30	5	3.43
60	5	30	5	6.72
60	5	30	5	4.13
70	5	20	5	5.48
80	5	10	5	5.94
90	5	0	5	9.50
60	5	30	5 C12PEG	3.85
60	5	30	5	3.70
60	5	30	5 C16PEG	3.80
60	5	30	5	4.19
60	5	29	5	4.07
60	5	30	5	3.56
60	5	30	5	3.39
60	5	30	5	3.96
60	5	30	5	4.01
60	5	30	5	4.07
60	5	30	5	4.25
60	5	30	5	3.80
60	5	30	5	3.31

TABLE 11-continued

Composition of exemplary nucleic acid-lipid particle (mole %) prepared via extrusion methods.

Lipid (mol %)	DSPC (mol %)	Chol (mol %)	PEG (mol %)	Lipid/siRNA
60	5	30	5	4.83
60	5	30	5	4.67
60	5	30	5	3.96
57.5	7.5	33.5	1.5	3.39
57.5	7.5	32.5	2.5	3.39
57.5	7.5	31.5	3.5	3.52
57.5	7.5	30	5	4.19
60	5	30	5	3.96
60	5	30	5	3.96
60	5	30	5	3.56
60	5	33.5	1.5	3.52
60	5	25	10	5.18
60	5 (DPPC)	30	5	4.25
60	5	32.5	2.5	3.70
57.5	7.5	31.5	3.5	3.06
57.5	7.5	31.5	3.5	3.65
57.5	7.5	31.5	3.5	4.70
57.5	7.5	31.5	3.5	6.56

TABLE 12

Composition of exemplary nucleic acid-lipid particles prepared via in-line mixing

Lipid (mol %)	DSPC (mol %)	Chol (mol %)	PEG (mol %)	Lipid A/siRNA
55	5	37.5	2.5	3.96
55	10	32.5	2.5	3.56
60	5	32.5	2.5	3.80
60	10	27.5	2.5	3.75
60	5	30	5	4.19
60	5	33.5	1.5	3.48
60	5	33.5	1.5	6.64
60	5	25	10	6.79
60	5	32.5	2.5	3.96
60	5	34	1	3.75
60	5	34.5	0.5	3.28
50	5	40	5	3.96
60	5	30	5	4.75
70	5	20	5	5.00
80	5	10	5	5.18
60	5	30	5	13.60
60	5	30	5	14.51
60	5	30	5	6.20
60	5	30	5	4.60
60	5	30	5	6.20
60	5	30	5	5.82
40	5	54	1	3.39
40	7.5	51.5	1	3.39
40	10	49	1	3.39
50	5	44	1	3.39
50	7.5	41.5	1	3.43
50	10	39	1	3.35
60	5	34	1	3.52
60	7.5	31.5	1	3.56
60	10	29	1	3.80
70	5	24	1	3.70
70	7.5	21.5	1	4.13
70	10	19	1	3.85
60	5	34	1	3.52
60	5	34	1	3.70
60	5	34	1	3.52
60	7.5	27.5	5	5.18
60	7.5	29	3.5	4.45
60	5	31.5	3.5	4.83
60	7.5	31	1.5	3.48
57.5	7.5	30	5	4.75

TABLE 12-continued

Composition of exemplary nucleic acid-lipid particles prepared via in-line mixing

Lipid (mol %)	DSPC (mol %)	Chol (mol %)	PEG (mol %)	Lipid A/siRNA
57.5	7.5	31.5	3.5	4.83
57.5	5	34	3.5	4.67
57.5	7.5	33.5	1.5	3.43
55	7.5	32.5	5	4.38
55	7.5	34	3.5	4.13
55	5	36.5	3.5	4.38
55	7.5	36	1.5	3.35

[0263] Synthesis of Cationic Lipids.

[0264] Any of the compounds, e.g., cationic lipids and the like, used in the nucleic acid-lipid particles of the invention may be prepared by known organic synthesis techniques, including the methods described in more detail in the Examples. All substituents are as defined below unless indicated otherwise.

[0265] "Alkyl" means a straight chain or branched, non-cyclic or cyclic, saturated aliphatic hydrocarbon containing from 1 to 24 carbon atoms. Representative saturated straight chain alkyls include methyl, ethyl, n-propyl, n-butyl, n-pentyl, n-hexyl, and the like; while saturated branched alkyls include isopropyl, sec-butyl, isobutyl, tert-butyl, isopentyl, and the like. Representative saturated cyclic alkyls include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, and the like; while unsaturated cyclic alkyls include cyclopentenyl and cyclohexenyl, and the like.

[0266] "Alkenyl" means an alkyl, as defined above, containing at least one double bond between adjacent carbon atoms. Alkenyls include both cis and trans isomers. Representative straight chain and branched alkenyls include ethylenyl, propylenyl, 1-butenyl, 2-butenyl, isobutylenyl, 1-pentenyl, 2-pentenyl, 3-methyl-1-butenyl, 2-methyl-2-butenyl, 2,3-dimethyl-2-butenyl, and the like.

[0267] "Alkynyl" means any alkyl or alkenyl, as defined above, which additionally contains at least one triple bond between adjacent carbons. Representative straight chain and branched alkynyls include acetylenyl, propynyl, 1-butynyl, 2-butynyl, 1-pentynyl, 2-pentynyl, 3-methyl-1 butynyl, and the like.

[0268] "Acyl" means any alkyl, alkenyl, or alkynyl wherein the carbon at the point of attachment is substituted with an oxo group, as defined below. For example, —C(=O)alkyl, —C(=O)alkenyl, and —C(=O)alkynyl are acyl groups.

[0269] "Heterocycle" means a 5- to 7-membered mono-cyclic, or 7- to 10-membered bicyclic, heterocyclic ring which is either saturated, unsaturated, or aromatic, and which contains from 1 or 2 heteroatoms independently selected from nitrogen, oxygen and sulfur, and wherein the nitrogen and sulfur heteroatoms may be optionally oxidized, and the nitrogen heteroatom may be optionally quaternized, including bicyclic rings in which any of the above heterocycles are fused to a benzene ring. The heterocycle may be attached via any heteroatom or carbon atom. Heterocycles include heteroaryls as defined below. Heterocycles include morpholinyl, pyrrolidinonyl, pyrrolidinyl, piperidinyl, piperizynyl, hydantoinyl, valerolactamyl, oxiranyl, oxetanyl, tetrahydrofuranyl, tetrahydropyranyl, tetrahydropyridinyl, tetrahydroprimidinyl, tetrahydrothiophenyl, tetrahydrothiopyranyl, tetrahydropyrimidinyl, tetrahydrothiophenyl, tetrahydrothiopyranyl, and the like.

[0270] The terms "optionally substituted alkyl", "optionally substituted alkenyl", "optionally substituted alkynyl", "optionally substituted acyl", and "optionally substituted heterocycle" means that, when substituted, at least one hydrogen atom is replaced with a substituent. In the case of an oxo substituent (=O) two hydrogen atoms are replaced. In this regard, substituents include oxo, halogen, heterocycle, —CN, —OR^x, —NR^{x}R^y, —NR^{x}C(=O)R^y, —NR^{x}SO$_2$R^y, —C(=O)R^x, —C(=O)OR^x, —C(=O) NR^{x}R^y, —SO$_n$R^x and —SO$_n$NR^{x}R^y, wherein n is 0, 1 or 2, R^x and R^y are the same or different and independently hydrogen, alkyl or heterocycle, and each of said alkyl and heterocycle substituents may be further substituted with one or more of oxo, halogen, —OH, —CN, alkyl, —OR', heterocycle, —NR^{x}R^y, —NR^{x}C(=O)R^y, —NR^{x}SO$_2$R^y, —C(=O)R^x, —C(=O)OR^x, —C(=O)NR^{x}R^y, —SO—R^x and —SO$_n$NR^{x}R^y.

[0271] "Halogen" means fluoro, chloro, bromo and iodo.

[0272] In some embodiments, the methods of the invention may require the use of protecting groups. Protecting group methodology is well known to those skilled in the art (see, for example, PROTECTIVE GROUPS IN ORGANIC SYNTHESIS, Green, T. W. et al., Wiley-Interscience, New York City, 1999). Briefly, protecting groups within the context of this invention are any group that reduces or eliminates unwanted reactivity of a functional group. A protecting group can be added to a functional group to mask its reactivity during certain reactions and then removed to reveal the original functional group. In some embodiments an "alcohol protecting group" is used. An "alcohol protecting group" is any group which decreases or eliminates unwanted reactivity of an alcohol functional group. Protecting groups can be added and removed using techniques well known in the art.

[0273] Synthesis of MC3

[0274] Preparation of DLin-M-C3-DMA (i.e., (6Z,9Z, 28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate) was as follows. A solution of (6Z,9Z, 28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-ol (0.53 g), 4-N,N-dimethylaminobutyric acid hydrochloride (0.51 g), 4-N,N-dimethylaminopyridine (0.61 g) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.53 g) in dichloromethane (5 mL) was stirred at room temperature overnight. The solution was washed with dilute hydrochloric acid followed by dilute aqueous sodium bicarbonate. The organic fractions were dried over anhydrous magnesium sulphate, filtered and the solvent removed on a rotovap. The residue was passed down a silica gel column (20 g) using a 1-5% methanol/dichloromethane elution gradient. Fractions containing the purified product were combined and the solvent removed, yielding a colorless oil (0.54 g). Further description is provided in WO 2010/054401 (PCTUS2009/063927 filed on Nov. 10, 2009 and U.S. patent application Ser. No. 12/813,448 filed on Jun. 10, 2010.

[0275] Synthesis of Formula A

[0276] In one embodiment, nucleic acid-lipid particles of the invention are formulated using a cationic lipid of formula A:
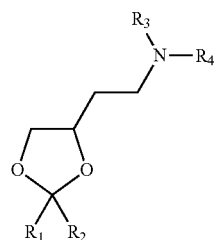
[0277] where R1 and R2 are independently alkyl, alkenyl or alkynyl, each can be optionally substituted, and R3 and R4 are independently lower alkyl or R3 and R4 can be taken together to form an optionally substituted heterocyclic ring. In some embodiments, the cationic lipid is XTC (2,2-Dilinoleyl-4-dimethylaminoethyl[1,3]-dioxolane). In general, the lipid of formula A above may be made by the following Reaction Schemes 1 or 2, wherein all substituents are as defined above unless indicated otherwise.

R_3 and R_4 are independently lower alkyl or R_3 and R_4 can be taken together to form an optionally substituted heterocyclic ring, can be prepared according to Scheme 1. Ketone 1 and bromide 2 can be purchased or prepared according to methods known to those of ordinary skill in the art. Reaction of 1 and 2 yields ketal 3. Treatment of ketal 3 with amine 4 yields lipids of formula A. The lipids of formula A can be converted to the corresponding ammonium salt with an organic salt of formula 5, where Xis anion counter ion selected from halogen, hydroxide, phosphate, sulfate, or the like.

Scheme 1

Formula A

[0278] Lipid A, where R_1 and R_2 are independently alkyl, alkenyl or alkynyl, each be optionally substituted, and

Scheme 2

[0279] Alternatively, the ketone 1 starting material can be prepared according to Scheme 2. Grignard reagent 6 and cyanide 7 can be purchased or prepared according to methods known to those of ordinary skill in the art. Reaction of 6 and 7 yields ketone 1. Conversion of ketone 1 to the corresponding lipids of formula A is as described in Scheme 1.

[0280] Synthesis of ALNY-100

[0281] Synthesis of ketal 519 [ALNY-100] was performed using the following scheme 3:

Scheme 3

-continued
LAH,
1M THF

519 ← 518
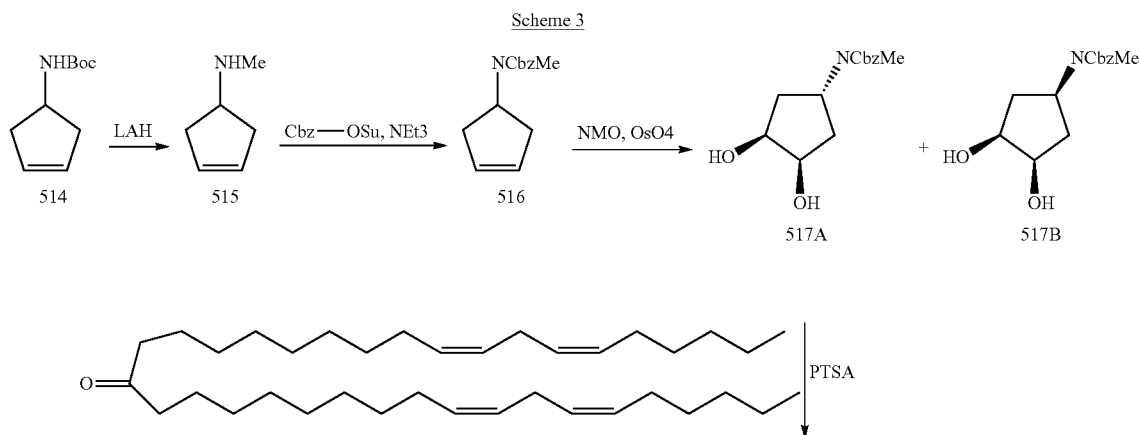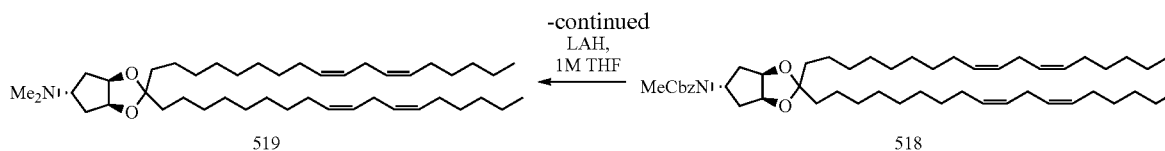
[0282] Synthesis of 515:

[0283] To a stirred suspension of LiAlH4 (3.74 g, 0.09852 mol) in 200 ml anhydrous THF in a two neck RBF (1 L), was added a solution of 514 (10 g, 0.04926 mol) in 70 mL of THF slowly at 0° C. under nitrogen atmosphere. After complete addition, reaction mixture was warmed to room temperature and then heated to reflux for 4 h. Progress of the reaction was monitored by TLC. After completion of reaction (by TLC) the mixture was cooled to 0° C. and quenched with careful addition of saturated Na2SO4 solution. Reaction mixture was stirred for 4 h at room temperature and filtered off. Residue was washed well with THF. The filtrate and washings were mixed and diluted with 400 mL dioxane and 26 mL conc. HCl and stirred for 20 minutes at room temperature. The volatilities were stripped off under vacuum to furnish the hydrochloride salt of 515 as a white solid. Yield: 7.12 g 1H-NMR (DMSO, 400 MHz): δ=9.34 (broad, 2H), 5.68 (s, 2H), 3.74 (m, 1H), 2.66-2.60 (m, 2H), 2.50-2.45 (m, 5H).

[0284] Synthesis of 516:

[0285] To a stirred solution of compound 515 in 100 mL dry DCM in a 250 mL two neck RBF, was added NEt3 (37.2 mL, 0.2669 mol) and cooled to 0° C. under nitrogen atmosphere. After a slow addition of N-(benzyloxy-carbonyloxy)-succinimide (20 g, 0.08007 mol) in 50 mL dry DCM, reaction mixture was allowed to warm to room temperature. After completion of the reaction (2-3 h by TLC) mixture was washed successively with 1N HCl solution (1×100 mL) and saturated NaHCO3 solution (1×50 mL). The organic layer was then dried over anhyd. Na2SO4 and the solvent was evaporated to give crude material which was purified by silica gel column chromatography to get 516 as sticky mass. Yield: 11 g (89%). 1H-NMR (CDCl3, 400 MHz): δ=7.36-7.27 (m, 5H), 5.69 (s, 2H), 5.12 (s, 2H), 4.96 (br., 1H) 2.74 (s, 3H), 2.60 (m, 2H), 2.30-2.25 (m, 2H). LC-MS [M+H]-232.3 (96.94%).

[0286] Synthesis of 517A and 517B:

[0287] The cyclopentene 516 (5 g, 0.02164 mol) was dissolved in a solution of 220 mL acetone and water (10:1) in a single neck 500 mL RBF and to it was added N-methyl morpholine-N-oxide (7.6 g, 0.06492 mol) followed by 4.2 mL of 7.6% solution of OsO4 (0.275 g, 0.00108 mol) in tert-butanol at room temperature. After completion of the reaction (~3 h), the mixture was quenched with addition of solid Na2SO3 and resulting mixture was stirred for 1.5 h at room temperature. Reaction mixture was diluted with DCM (300 mL) and washed with water (2×100 mL) followed by saturated NaHCO3 (1×50 mL) solution, water (1×30 mL) and finally with brine (lx 50 mL). Organic phase was dried over an.Na2SO4 and solvent was removed in vacuum. Silica gel column chromatographic purification of the crude material was afforded a mixture of diastereomers, which were separated by prep HPLC. Yield:—6 g crude

[0288] 517A—Peak-1 (white solid), 5.13 g (96%). 1H-NMR (DMSO, 400 MHz): δ=7.39-7.31 (m, 5H), 5.04 (s, 2H), 4.78-4.73 (m, 1H), 4.48-4.47 (d, 2H), 3.94-3.93 (m,

2H), 2.71 (s, 3H), 1.72-1.67 (m, 4H). LC-MS—[M+H]-266. 3, [M+NH4+]-283.5 present, HPLC-97.86%. Stereochemistry confirmed by X-ray.

[0289] Synthesis of 518:

[0290] Using a procedure analogous to that described for the synthesis of compound 505, compound 518 (1.2 g, 41%) was obtained as a colorless oil. 1H-NMR (CDCl3, 400 MHz): δ=7.35-7.33 (m, 4H), 7.30-7.27 (m, 1H), 5.37-5.27 (m, 8H), 5.12 (s, 2H), 4.75 (m, 1H), 4.58-4.57 (m, 2H), 2.78-2.74 (m, 7H), 2.06-2.00 (m, 8H), 1.96-1.91 (m, 2H), 1.62 (m, 4H), 1.48 (m, 2H), 1.37-1.25 (br m, 36H), 0.87 (m, 6H). HPLC-98.65%.

[0291] General Procedure for the Synthesis of Compound 519:

[0292] A solution of compound 518 (1 eq) in hexane (15 mL) was added in a drop-wise fashion to an ice-cold solution of LAH in THF (1 M, 2 eq). After complete addition, the mixture was heated at 40□C over 0.5 h then cooled again on an ice bath. The mixture was carefully hydrolyzed with saturated aqueous Na2SO4 then filtered through celite and reduced to an oil. Column chromatography provided the pure 519 (1.3 g, 68%) which was obtained as a colorless oil. 13C NMR □=130.2, 130.1 (×2), 127.9 (×3), 112.3, 79.3, 64.4, 44.7, 38.3, 35.4, 31.5, 29.9 (×2), 29.7, 29.6 (×2), 29.5 (×3), 29.3 (×2), 27.2 (×3), 25.6, 24.5, 23.3, 226, 14.1; Electrospray MS (+ve): Molecular weight for C44H80NO2 (M+H)+ Calc. 654.6. Found 654.6.

[0293] Therapeutic Agent-Lipid Particle Compositions and Formulations

[0294] The invention includes compositions comprising a lipid particle of the invention and an active agent, wherein the active agent is associated with the lipid particle. In particular embodiments, the active agent is a therapeutic agent. In particular embodiments, the active agent is encapsulated within an aqueous interior of the lipid particle. In other embodiments, the active agent is present within one or more lipid layers of the lipid particle. In other embodiments, the active agent is bound to the exterior or interior lipid surface of a lipid particle.

[0295] "Fully encapsulated" as used herein indicates that the nucleic acid in the particles is not significantly degraded after exposure to serum or a nuclease assay that would significantly degrade free DNA. In a fully encapsulated system, preferably less than 25% of particle nucleic acid is degraded in a treatment that would normally degrade 100% of free nucleic acid, more preferably less than 10% and most preferably less than 5% of the particle nucleic acid is degraded. Alternatively, full encapsulation may be determined by an Oligreen® assay. Oligreen® is an ultra-sensitive fluorescent nucleic acid stain for quantitating oligonucleotides and single-stranded DNA in solution (available from Invitrogen Corporation, Carlsbad, Calif.). Fully encapsulated also suggests that the particles are serum stable, that is, that they do not rapidly decompose into their component parts upon in vivo administration.

[0296] Active agents, as used herein, include any molecule or compound capable of exerting a desired effect on a cell, tissue, organ, or subject. Such effects may be biological, physiological, or cosmetic, for example. Active agents may be any type of molecule or compound, including e.g., nucleic acids, peptides and polypeptides, including, e.g., antibodies, such as, e.g., polyclonal antibodies, monoclonal antibodies, antibody fragments; humanized antibodies, recombinant antibodies, recombinant human antibodies, and Primatized™ antibodies, cytokines, growth factors, apoptotic factors, differentiation-inducing factors, cell surface receptors and their ligands; hormones; and small molecules, including small organic molecules or compounds.

[0297] In one embodiment, the active agent is a therapeutic agent, or a salt or derivative thereof. Therapeutic agent derivatives may be therapeutically active themselves or they may be prodrugs, which become active upon further modification. Thus, in one embodiment, a therapeutic agent derivative retains some or all of the therapeutic activity as compared to the unmodified agent, while in another embodiment, a therapeutic agent derivative lacks therapeutic activity.

[0298] In various embodiments, therapeutic agents include any therapeutically effective agent or drug, such as anti-inflammatory compounds, anti-depressants, stimulants, analgesics, antibiotics, birth control medication, antipyretics, vasodilators, anti-angiogenics, cytovascular agents, signal transduction inhibitors, cardiovascular drugs, e.g., anti-arrhythmic agents, vasoconstrictors, hormones, and steroids.

[0299] In certain embodiments, the therapeutic agent is an oncology drug, which may also be referred to as an anti-tumor drug, an anti-cancer drug, a tumor drug, an antineoplastic agent, or the like. Examples of oncology drugs that may be used according to the invention include, but are not limited to, adriamycin, alkeran, allopurinol, altretamine, amifostine, anastrozole, araC, arsenic trioxide, azathioprine, bexarotene, biCNU, bleomycin, busulfan intravenous, busulfan oral, capecitabine (Xeloda), carboplatin, carmustine, CCNU, celecoxib, chlorambucil, cisplatin, cladribine, cyclosporin A, cytarabine, cytosine arabinoside, daunorubicin, cytoxan, daunorubicin, dexamethasone, dexrazoxane, dodetaxel, doxorubicin, doxorubicin, DTIC, epirubicin, estramustine, etoposide phosphate, etoposide and VP-16, exemestane, FK506, fludarabine, fluorouracil, 5-FU, gemcitabine (Gemzar), gemtuzumab-ozogamicin, goserelin acetate, hydrea, hydroxyurea, idarubicin, ifosfamide, imatinib mesylate, interferon, irinotecan (Camptostar, CPT-111), letrozole, leucovorin, leustatin, leuprolide, levamisole, litretinoin, megastrol, melphalan, L-PAM, mesna, methotrexate, methoxsalen, mithramycin, mitomycin, mitoxantrone, nitrogen mustard, paclitaxel, pamidronate, Pegademase, pentostatin, porfimer sodium, prednisone, rituxan, streptozocin, STI-571, tamoxifen, taxotere, temozolamide, teniposide, VM-26, topotecan (Hycamtin), toremifene, tretinoin, ATRA, valrubicin, velban, vinblastine, vincristine, VP16, and vinorelbine. Other examples of oncology drugs that may be used according to the invention are ellipticin and ellipticin analogs or derivatives, epothilones, intracellular kinase inhibitors and camptothecins.

[0300] Additional Formulations

[0301] Emulsions

[0302] The compositions of the present invention may be prepared and formulated as emulsions. Emulsions are typically heterogeneous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 μm in diameter (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 may 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 may contain additional components in addition to the dispersed phases, and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in emulsions as needed. Pharmaceutical emulsions may 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.

[0303] 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 may 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 may be incorporated into either phase of the emulsion. Emulsifiers may broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

[0304] Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (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 may be classified into different classes based on the nature of the hydrophilic group: non-ionic, anionic, cationic and amphoteric (Rieger, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

[0305] 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, non-swelling 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.

[0306] Large varieties 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).

[0307] 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.

[0308] Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may 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 may 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.

[0309] The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture has been reviewed in the literature (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 (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.

[0310] In one embodiment of the present invention, the compositions of dsRNAs and nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (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).

[0311] 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 (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.

[0312] 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 may, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying

microemulsion systems are known in the art. The aqueous phase may 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 may 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.

[0313] 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 (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 (Constantinides et al., Pharmaceutical Research, 1994, 11, 1385; Ho et al., J. Pharm. Sci., 1996, 85, 138-143). Often microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be particularly advantageous when formulating thermolabile drugs, peptides or dsRNAs. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of dsRNAs and nucleic acids from the gastrointestinal tract, as well as improve the local cellular uptake of dsRNAs and nucleic acids.

[0314] Microemulsions of the present invention may 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 dsRNAs and nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present invention may 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.

[0315] Penetration Enhancers

[0316] In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly dsRNAs, 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 may 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.

[0317] Penetration enhancers may be classified as belonging to one of five broad categories, i.e., 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 the above mentioned classes of penetration enhancers are described below in greater detail.

[0318] Surfactants: In connection with the present invention, 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 dsRNAs 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) (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).

[0319] Fatty acids: 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_{1-10} 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.) (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).

[0320] Bile salts: The physiological role of bile includes the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (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) (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).

[0321] Chelating Agents: Chelating agents, as used in connection with the present invention, can be defined as compounds that remove metallic ions from solution by forming complexes therewith, with the result that absorption of dsRNAs through the mucosa is enhanced. With regards to

their use as penetration enhancers in the present invention, 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-methox-ysalicylate and homovanilate), N-acyl derivatives of collagen, laureth-9 and N-amino acyl derivatives of beta-dike-tones (enamines)(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).

[0322] Non-chelating non-surfactants: 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 dsRNAs through the alimentary mucosa (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, indometha-cin and phenylbutazone (Yamashita et al., J. Pharm. Phar-macol., 1987, 39, 621-626).

[0323] Agents that enhance uptake of dsRNAs at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. 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 (Lollo et al., PCT Application WO 97/30731), are also known to enhance the cellular uptake of dsRNAs.

[0324] Other agents may 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.

[0325] Carriers

[0326] dsRNAs of the present invention can be formulated in a pharmaceutically acceptable carrier or diluent. A "pharmaceutically acceptable carrier" (also referred to herein as an "excipient") is a pharmaceutically acceptable solvent, suspending agent, or any other pharmacologically inert vehicle. Pharmaceutically acceptable carriers can be liquid or solid, and can be selected with the planned manner of administration in mind so as to provide for the desired bulk, consistency, and other pertinent transport and chemical properties. Typical pharmaceutically acceptable carriers include, by way of example and not limitation: water; saline solution; binding agents (e.g., polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose and other sugars, gelatin, or calcium sulfate); lubricants (e.g., starch, polyethylene glycol, or sodium acetate); disintegrates (e.g., starch or sodium starch glycolate); and wetting agents (e.g., sodium lauryl sulfate).

[0327] Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (i.e., does not possess biological activity per se) but is recognized as a nucleic acid

by in vivo processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The co-administration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extra-circulatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioate dsRNA in hepatic tissue can be reduced when it is co-administered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-ac-etamido-4'isothiocyano-stilbene-2,2'-disulfonic acid (Miyao et al., DsRNA Res. Dev., 1995, 5, 115-121; Takakura et al., DsRNA & Nucl. Acid Drug Dev., 1996, 6, 177-183.

[0328] Excipients

[0329] 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 may 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).

[0330] Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

[0331] Formulations for topical administration of nucleic acids may 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 may 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.

[0332] 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, hydroxymethyl-cellulose, polyvinylpyrrolidone and the like.

[0333] Other Components

[0334] The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-

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

[0335] Aqueous suspensions may contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

[0336] Combination Therapy

[0337] In one aspect, a composition of the invention can be used in combination therapy. The term "combination therapy" includes the administration of the subject compounds in further combination with other biologically active ingredients (such as, but not limited to, a second and different antineoplastic agent) and non-drug therapies (such as, but not limited to, surgery or radiation treatment). For instance, the compounds of the invention can be used in combination with other pharmaceutically active compounds, preferably compounds that are able to enhance the effect of the compounds of the invention. The compounds of the invention can be administered simultaneously (as a single preparation or separate preparation) or sequentially to the other drug therapy. In general, a combination therapy envisions administration of two or more drugs during a single cycle or course of therapy.

[0338] In one aspect of the invention, the subject compounds may be administered in combination with one or more separate agents that modulate protein kinases involved in various disease states. Examples of such kinases may include, but are not limited to: serine/threonine specific kinases, receptor tyrosine specific kinases and non-receptor tyrosine specific kinases. Serine/threonine kinases include mitogen activated protein kinases (MAPK), meiosis specific kinase (MEK), RAF and aurora kinase. Examples of receptor kinase families include epidermal growth factor receptor (EGFR) (e.g., HER2/neu, HER3, HERO, ErbB, ErbB2, ErbB3, ErbB4, Xmrk, DER, Let23); fibroblast growth factor (FGF) receptor (e.g. FGF-R1, GFF-R2/BEK/CEK3, FGF-R3/CEK2, FGF-R4/TKF, KGF-R); hepatocyte growth/scatter factor receptor (HGFR) (e.g., MET, RON, SEA, SEX); insulin receptor (e.g. IGFI-R); Eph (e.g. CEKS, CEK8, EBK, ECK, EEK, EHK-I, EHK-2, ELK, EPH, ERK, HEK, MDK2, MDKS, SEK); AxI (e.g. Mer/Nyk, Rse); RET; and platelet-derived growth factor receptor (PDGFR) (e.g. PDGFα-R, PDGβ-R, CSF1-R/FMS, SCF-R/C-KIT, VEGF-R/FLT, NEK/FLK1, FLT3/FLK2/STK-1). Non-receptor tyrosine kinase families include, but are not limited to, BCR-ABL (e.g. p43^{abl} ARG); BTK (e.g. ITK/EMT, TEC); CSK, FAK, FPS, JAK, SRC, BMX, FER, CDK and SYK.

[0339] In another aspect of the invention, the subject compounds may be administered in combination with one or more agents that modulate non-kinase biological targets or processes. Such targets include histone deacetylases (HDAC), DNA methyltransferase (DNMT), heat shock proteins (e.g., HSP90), and proteosomes.

[0340] In one embodiment, subject compounds may be combined with antineoplastic agents (e.g. small molecules, monoclonal antibodies, antisense RNA, and fusion proteins) that inhibit one or more biological targets such as Zolinza, Tarceva, Iressa, Tykerb, Gleevec, Sutent, Sprycel, Nexavar, Sorafenib, CNF2024, RG108, BMS387032, Affmitak, Avastin, Herceptin, Erbitux, AG24322, PD325901, ZD6474, PD 184322, Obatodax, ABT737 and AEE788. Such combinations may enhance therapeutic efficacy over efficacy achieved by any of the agents alone and may prevent or delay the appearance of resistant mutational variants.

[0341] In certain preferred embodiments, the compounds of the invention are administered in combination with a chemotherapeutic agent. Chemotherapeutic agents encompass a wide range of therapeutic treatments in the field of oncology. These agents are administered at various stages of the disease for the purposes of shrinking tumors, destroying remaining cancer cells left over after surgery, inducing remission, maintaining remission and/or alleviating symptoms relating to the cancer or its treatment. Examples of such agents include, but are not limited to, alkylating agents such as mustard gas derivatives (Mechlorethamine, cylophosphamide, chlorambucil, melphalan, ifosfamide), ethylenimines (thiotepa, hexamethylmelanine), Alkylsulfonates (Busulfan), Hydrazines and Triazines (Altretamine, Procarbazine, Dacarbazine and Temozolomide), Nitrosoureas (Carmustine, Lomustine and Streptozocin), Ifosfamide and metal salts (Carboplatin, Cisplatin, and Oxaliplatin); plant alkaloids such as Podophyllotoxins (Etoposide and Tenisopide), Taxanes (Paclitaxel and Docetaxel), Vinca alkaloids (Vincristine, Vinblastine, Vindesine and Vinorelbine), and Camptothecan analogs (Irinotecan and Topotecan); antitumor antibiotics such as Chromomycins (Dactinomycin and Plicamycin), Anthracyclines (Doxorubicin, Daunorubicin, Epirubicin, Mitoxantrone, Valrubicin and Idarubicin), and miscellaneous antibiotics such as Mitomycin, Actinomycin and Bleomycin; anti-metabolites such as folic acid antagonists (Methotrexate, Pemetrexed, Raltitrexed, Aminopterin), pyrimidine antagonists (5-Fluorouracil, Floxuridine, Cytarabine, Capecitabine, and Gemcitabine), purine antagonists (6-Mercaptopurine and 6-Thioguanine) and adenosine deaminase inhibitors (Cladribine, Fludarabine, Mercaptopurine, Clofarabine, Thioguanine, Nelarabine and Pentostatin); topoisomerase inhibitors such as topoisomerase I inhibitors (Ironotecan, topotecan) and topoisomerase II inhibitors (Amsacrine, etoposide, etoposide phosphate, teniposide); monoclonal antibodies (Alemtuzumab, Gemtuzumab ozogamicin, Rituximab, Trastuzumab, Ibritumomab Tioxetan, Cetuximab, Panitumumab, Tositumomab, Bevacizumab); and miscellaneous anti-neoplasties such as ribonucleotide reductase inhibitors (Hydroxyurea); adrenocortical steroid inhibitor (Mitotane); enzymes (Asparaginase and Pegaspargase); anti-microtubule agents (Estramustine); and retinoids (Bexarotene, Isotretinoin, Tretinoin (ATRA). In certain preferred embodiments, the compounds of the invention are administered in combination with a chemoprotective agent. Chemoprotective agents act to protect the body or minimize the side effects of chemotherapy. Examples of such agents include, but are not limited to, amfostine, mesna, and dexrazoxane.

[0342] In one aspect of the invention, the subject compounds are administered in combination with radiation therapy. Radiation is commonly delivered internally (implantation of radioactive material near cancer site) or externally from a machine that employs photon (x-ray or gamma-ray) or particle radiation. Where the combination therapy further comprises radiation treatment, the radiation treatment may be conducted at any suitable time so long as a beneficial effect from the co-action of the combination of the therapeutic agents and radiation treatment is achieved. For example, in appropriate cases, the beneficial effect is still achieved when the radiation treatment is temporally removed from the administration of the therapeutic agents, perhaps by days or even weeks.

[0343] It will be appreciated that compounds of the invention can be used in combination with an immunotherapeutic agent. One form of immunotherapy is the generation of an active systemic tumor-specific immune response of host origin by administering a vaccine composition at a site distant from the tumor. Various types of vaccines have been proposed, including isolated tumor-antigen vaccines and anti-idiotype vaccines. Another approach is to use tumor cells from the subject to be treated, or a derivative of such cells (reviewed by Schirrmacher et al. (1995) J. Cancer Res. Clin. Oncol. 121:487). In U.S. Pat. No. 5,484,596, Hanna Jr. et al. claim a method for treating a resectable carcinoma to prevent recurrence or metastases, comprising surgically removing the tumor, dispersing the cells with collagenase, irradiating the cells, and vaccinating the patient with at least three consecutive doses of about 10^7 cells.

[0344] It will be appreciated that the compounds of the invention may advantageously be used in conjunction with one or more adjunctive therapeutic agents. Examples of suitable agents for adjunctive therapy include steroids, such as corticosteroids (amcinonide, betamethasone, betamethasone dipropionate, betamethasone valerate, budesonide, clobetasol, clobetasol acetate, clobetasol butyrate, clobetasol 17-propionate, cortisone, deflazacort, desoximetasone, diflucortolone valerate, dexamethasone, dexamethasone sodium phosphate, desonide, furoate, fluocinonide, fluocinolone acetonide, halcinonide, hydrocortisone, hydrocortisone butyrate, hydrocortisone sodium succinate, hydrocortisone valerate, methyl prednisolone, mometasone, prednicarbate, prednisolone, triamcinolone, triamcinolone acetonide, and halobetasol proprionate); a 5HTi agonist, such as a triptan (e.g. sumatriptan or naratriptan); an adenosine A1 agonist; an EP ligand; an NMDA modulator, such as a glycine antagonist; a sodium channel blocker (e.g. lamotrigine); a substance P antagonist (e.g. an NKi antagonist); a cannabinoid; acetaminophen or phenacetin; a 5-lipoxygenase inhibitor; a leukotriene receptor antagonist; a DMARD (e.g. methotrexate); gabapentin and related compounds; a tricyclic antidepressant (e.g. amitryptilline); a neurone stabilizing antiepileptic drug; a mono-aminergic uptake inhibitor (e.g. venlafaxine); a matrix metalloproteinase inhibitor; a nitric oxide synthase (NOS) inhibitor, such as an iNOS or an nNOS inhibitor; an inhibitor of the release, or action, of tumour necrosis factor α; an antibody therapy, such as a monoclonal antibody therapy; an antiviral agent, such as a nucleoside inhibitor (e.g. lamivudine) or an immune system modulator (e.g. interferon); an opioid analgesic; a local anaesthetic; a stimulant, including caffeine; an H2-antagonist (e.g. ranitidine); a proton pump inhibitor (e.g. omeprazole); an antacid (e.g. aluminium or magnesium hydroxide; an antiflatulent (e.g. simethicone); a decongestant (e.g. phenylephrine, phenylpropanolamine, pseudoephedrine, oxymetazoline, epinephrine, naphazoline, xylometazoline, propylhexedrine, or levo-desoxyephedrine); an antitussive (e.g. codeine, hydrocodone, carmiphen, carbetapentane, or dextramethorphan); a diuretic; or a sedating or non-sedating antihistamine.

[0345] The compounds of the invention can be co-administered with siRNA that target other genes. For example, a compound of the invention can be co-administered with an siRNA targeted to a c-Myc gene. In one example, AD-12115 can be co-administered with a c-Myc siRNA. Examples of c-Myc targeted siRNAs are disclosed in U.S. patent application Ser. No. 12/373,039 which is herein incorporated by reference.

[0346] Methods of Preparing Lipid Particles

[0347] The methods and compositions of the invention make use of certain cationic lipids, the synthesis, preparation and characterization of which is described below and in the accompanying Examples. In addition, the present invention provides methods of preparing lipid particles, including those associated with a therapeutic agent, e.g., a nucleic acid. In the methods described herein, a mixture of lipids is combined with a buffered aqueous solution of nucleic acid to produce an intermediate mixture containing nucleic acid encapsulated in lipid particles wherein the encapsulated nucleic acids are present in a nucleic acid/lipid ratio of about 3 wt % to about 25 wt %, preferably 5 to 15 wt %. The intermediate mixture may optionally be sized to obtain lipid-encapsulated nucleic acid particles wherein the lipid portions are unilamellar vesicles, preferably having a diameter of 30 to 150 nm, more preferably about 40 to 90 nm. The pH is then raised to neutralize at least a portion of the surface charges on the lipid-nucleic acid particles, thus providing an at least partially surface-neutralized lipid-encapsulated nucleic acid composition.

[0348] As described above, several of these cationic lipids are amino lipids that are charged at a pH below the pK_a of the amino group and substantially neutral at a pH above the pK_a. These cationic lipids are termed titratable cationic lipids and can be used in the formulations of the invention using a two-step process. First, lipid vesicles can be formed at the lower pH with titratable cationic lipids and other vesicle components in the presence of nucleic acids. In this manner, the vesicles will encapsulate and entrap the nucleic acids. Second, the surface charge of the newly formed vesicles can be neutralized by increasing the pH of the medium to a level above the pK_a of the titratable cationic lipids present, i.e., to physiological pH or higher. Particularly advantageous aspects of this process include both the facile removal of any surface adsorbed nucleic acid and a resultant nucleic acid delivery vehicle which has a neutral surface. Liposomes or lipid particles having a neutral surface are expected to avoid rapid clearance from circulation and to avoid certain toxicities which are associated with cationic liposome preparations. Additional details concerning these uses of such titratable cationic lipids in the formulation of nucleic acid-lipid particles are provided in U.S. Pat. No. 6,287,591 and U.S. Pat. No. 6,858,225, incorporated herein by reference.

[0349] It is further noted that the vesicles formed in this manner provide formulations of uniform vesicle size with high content of nucleic acids. Additionally, the vesicles have

a size range of from about 30 to about 150 nm, more preferably about 30 to about 90 nm.

[0350] Without intending to be bound by any particular theory, it is believed that the very high efficiency of nucleic acid encapsulation is a result of electrostatic interaction at low pH. At acidic pH (e.g. pH 4.0) the vesicle surface is charged and binds a portion of the nucleic acids through electrostatic interactions. When the external acidic buffer is exchanged for a more neutral buffer (e.g., pH 7.5) the surface of the lipid particle or liposome is neutralized, allowing any external nucleic acid to be removed. More detailed information on the formulation process is provided in various publications (e.g., U.S. Pat. No. 6,287,591 and U.S. Pat. No. 6,858,225).

[0351] In view of the above, the present invention provides methods of preparing lipid/nucleic acid formulations. In the methods described herein, a mixture of lipids is combined with a buffered aqueous solution of nucleic acid to produce an intermediate mixture containing nucleic acid encapsulated in lipid particles, e.g., wherein the encapsulated nucleic acids are present in a nucleic acid/lipid ratio of about 10 wt % to about 20 wt %. The intermediate mixture may optionally be sized to obtain lipid-encapsulated nucleic acid particles wherein the lipid portions are unilamellar vesicles, preferably having a diameter of 30 to 150 nm, more preferably about 40 to 90 nm. The pH is then raised to neutralize at least a portion of the surface charges on the lipid-nucleic acid particles, thus providing an at least partially surface-neutralized lipid-encapsulated nucleic acid composition.

[0352] In certain embodiments, the mixture of lipids includes at least two lipid components: a first amino lipid component of the present invention that is selected from among lipids which have a pKa such that the lipid is cationic at pH below the pKa and neutral at pH above the pKa, and a second lipid component that is selected from among lipids that prevent particle aggregation during lipid-nucleic acid particle formation. In particular embodiments, the amino lipid is a novel cationic lipid of the present invention.

[0353] In preparing the nucleic acid-lipid particles of the invention, the mixture of lipids is typically a solution of lipids in an organic solvent. This mixture of lipids can then be dried to form a thin film or lyophilized to form a powder before being hydrated with an aqueous buffer to form liposomes. Alternatively, in a preferred method, the lipid mixture can be solubilized in a water miscible alcohol, such as ethanol, and this ethanolic solution added to an aqueous buffer resulting in spontaneous liposome formation. In most embodiments, the alcohol is used in the form in which it is commercially available. For example, ethanol can be used as absolute ethanol (100%), or as 95% ethanol, the remainder being water. This method is described in more detail in U.S. Pat. No. 5,976,567).

[0354] In accordance with the invention, the lipid mixture is combined with a buffered aqueous solution that may contain the nucleic acids. The buffered aqueous solution of is typically a solution in which the buffer has a pH of less than the pK_a of the protonatable lipid in the lipid mixture. Examples of suitable buffers include citrate, phosphate, acetate, and MES. A particularly preferred buffer is citrate buffer. Preferred buffers will be in the range of 1-1000 mM of the anion, depending on the chemistry of the nucleic acid being encapsulated, and optimization of buffer concentration may be significant to achieving high loading levels (see, e.g.,

U.S. Pat. No. 6,287,591 and U.S. Pat. No. 6,858,225). Alternatively, pure water acidified to pH 5-6 with chloride, sulfate or the like may be useful. In this case, it may be suitable to add 5% glucose, or another non-ionic solute which will balance the osmotic potential across the particle membrane when the particles are dialyzed to remove ethanol, increase the pH, or mixed with a pharmaceutically acceptable carrier such as normal saline. The amount of nucleic acid in buffer can vary, but will typically be from about 0.01 mg/mL to about 200 mg/mL, more preferably from about 0.5 mg/mL to about 50 mg/mL.

[0355] The mixture of lipids and the buffered aqueous solution of therapeutic nucleic acids is combined to provide an intermediate mixture. The intermediate mixture is typically a mixture of lipid particles having encapsulated nucleic acids. Additionally, the intermediate mixture may also contain some portion of nucleic acids which are attached to the surface of the lipid particles (liposomes or lipid vesicles) due to the ionic attraction of the negatively-charged nucleic acids and positively-charged lipids on the lipid particle surface (the amino lipids or other lipid making up the protonatable first lipid component are positively charged in a buffer having a pH of less than the pK_a of the protonatable group on the lipid). In one group of preferred embodiments, the mixture of lipids is an alcohol solution of lipids and the volumes of each of the solutions are adjusted so that upon combination, the resulting alcohol content is from about 20% by volume to about 45% by volume. The method of combining the mixtures can include any of a variety of processes, often depending upon the scale of formulation produced. For example, when the total volume is about 10-20 mL or less, the solutions can be combined in a test tube and stirred together using a vortex mixer. Large-scale processes can be carried out in suitable production scale glassware.

[0356] Optionally, the lipid-encapsulated therapeutic agent (e.g., nucleic acid) complexes which are produced by combining the lipid mixture and the buffered aqueous solution of therapeutic agents (nucleic acids) can be sized to achieve a desired size range and relatively narrow distribution of lipid particle sizes. Preferably, the compositions provided herein will be sized to a mean diameter of from about 70 to about 200 nm, more preferably about 90 to about 130 nm. Several techniques are available for sizing liposomes to a desired size. One sizing method is described in U.S. Pat. No. 4,737,323, incorporated herein by reference. Sonicating a liposome suspension either by bath or probe sonication produces a progressive size reduction down to small unilamellar vesicles (SUVs) less than about 0.05 microns in size. Homogenization is another method which relies on shearing energy to fragment large liposomes into smaller ones. In a typical homogenization procedure, multilamellar vesicles are recirculated through a standard emulsion homogenizer until selected liposome sizes, typically between about 0.1 and 0.5 microns, are observed. In both methods, the particle size distribution can be monitored by conventional laser-beam particle size determination. For certain methods herein, extrusion is used to obtain a uniform vesicle size.

[0357] Extrusion of liposome compositions through a small-pore polycarbonate membrane or an asymmetric ceramic membrane results in a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired liposome

complex size distribution is achieved. The liposomes may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in liposome size. In some instances, the lipid-nucleic acid compositions which are formed can be used without any sizing.

[0358] In particular embodiments, methods of the present invention further comprise a step of neutralizing at least some of the surface charges on the lipid portions of the lipid-nucleic acid compositions. By at least partially neutralizing the surface charges, unencapsulated nucleic acid is freed from the lipid particle surface and can be removed from the composition using conventional techniques. Preferably, unencapsulated and surface adsorbed nucleic acids are removed from the resulting compositions through exchange of buffer solutions. For example, replacement of a citrate buffer (pH about 4.0, used for forming the compositions) with a HEPES-buffered saline (HBS pH about 7.5) solution, results in the neutralization of liposome surface and nucleic acid release from the surface. The released nucleic acid can then be removed via chromatography using standard methods, and then switched into a buffer with a pH above the pKa of the lipid used.

[0359] Optionally the lipid vesicles (i.e., lipid particles) can be formed by hydration in an aqueous buffer and sized using any of the methods described above prior to addition of the nucleic acid. As described above, the aqueous buffer should be of a pH below the pKa of the amino lipid. A solution of the nucleic acids can then be added to these sized, preformed vesicles. To allow encapsulation of nucleic acids into such "pre-formed" vesicles the mixture should contain an alcohol, such as ethanol. In the case of ethanol, it should be present at a concentration of about 20% (w/w) to about 45% (w/w). In addition, it may be necessary to warm the mixture of pre-formed vesicles and nucleic acid in the aqueous buffer-ethanol mixture to a temperature of about 25° C. to about 50° C. depending on the composition of the lipid vesicles and the nature of the nucleic acid. It will be apparent to one of ordinary skill in the art that optimization of the encapsulation process to achieve a desired level of nucleic acid in the lipid vesicles will require manipulation of variable such as ethanol concentration and temperature. Examples of suitable conditions for nucleic acid encapsulation are provided in the Examples. Once the nucleic acids are encapsulated within the preformed vesicles, the external pH can be increased to at least partially neutralize the surface charge. Unencapsulated and surface adsorbed nucleic acids can then be removed as described above.

[0360] Methods for Inhibiting Expression of the PCSK9 Gene

[0361] In yet another aspect, the invention provides a method for inhibiting the expression of the PCSK9 gene in a mammal. The method includes administering a composition of the invention to the mammal such that expression of the target PCSK9 gene is decreased for an extended duration, e.g., at least one week, two weeks, three weeks, or four weeks or longer.

[0362] For example, in certain instances, expression of the PCSK9 gene is suppressed by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50% by administration of a double-stranded oligonucleotide described herein. In some embodiments, the PCSK9 gene is suppressed by at least about 60%, 70%, or 80% by administration of the double-stranded oligonucleotide. In some embodiments, the PCSK9 gene is suppressed by at least about 85%, 90%, or

95% by administration of the double-stranded oligonucleotide. Table 1b, Table 2b, and Table 5b provide a wide range of values for inhibition of expression obtained in an in vitro assay using various PCSK9 dsRNA molecules at various concentrations.

[0363] The effect of the decreased target PCSK9 gene preferably results in a decrease in LDLc (low density lipoprotein cholesterol) levels in the blood, and more particularly in the serum, of the mammal. In some embodiments, LDLc levels are decreased by at least 10%, 15%, 20%, 25%, 30%, 40%, 50%, or 60%, or more, as compared to pretreatment levels.

[0364] The method includes administering a composition containing a dsRNA, where the dsRNA has a nucleotide sequence that is complementary to at least a part of an RNA transcript of the PCSK9 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 or parenteral routes, including intravenous, intramuscular, subcutaneous, transdermal, and airway (aerosol) administration. In some embodiments, the compositions are administered by intravenous infusion or injection.

[0365] The methods and compositions described herein can be used to treat diseases and conditions that can be modulated by down regulating PCSK9 gene expression. For example, the compositions described herein can be used to treat hyperlipidemia and other forms of lipid imbalance such as hypercholesterolemia, hypertriglyceridemia and the pathological conditions associated with these disorders such as heart and circulatory diseases. In some embodiments, a patient treated with a PCSK9 dsRNA is also administered a non-dsRNA therapeutic agent, such as an agent known to treat lipid disorders.

[0366] In one aspect, the invention provides a method of inhibiting the expression of the PCSK9 gene in a subject, e.g., a human. The method includes administering a first single dose of dsRNA, e.g., a dose sufficient to depress levels of PCSK9 mRNA for at least 5, more preferably 7, 10, 14, 21, 25, 30 or 40 days; and optionally, administering a second single dose of dsRNA, wherein the second single dose is administered at least 5, more preferably 7, 10, 14, 21, 25, 30 or 40 days after the first single dose is administered, thereby inhibiting the expression of the PCSK9 gene in a subject.

[0367] In one embodiment, doses of dsRNA are administered not more than once every four weeks, not more than once every three weeks, not more than once every two weeks, or not more than once every week. In another embodiment, the administrations can be maintained for one, two, three, or six months, or one year or longer.

[0368] In another embodiment, administration can be provided when Low Density Lipoprotein cholesterol (LDLc) levels reach or surpass a predetermined minimal level, such as greater than 70 mg/dL, 130 mg/dL, 150 mg/dL, 200 mg/dL, 300 mg/dL, or 400 mg/dL.

[0369] In one embodiment, the subject is selected, at least in part, on the basis of needing (as opposed to merely selecting a patient on the grounds of who happens to be in need of) LDL lowering, LDL lowering without lowering of HDL, ApoB lowering, or total cholesterol lowering without HDL lowering.

[0370] In one embodiment, the dsRNA does not activate the immune system, e.g., it does not increase cytokine levels,

such as TNF-alpha or IFN-alpha levels. For example, when measured by an assay, such as an in vitro PBMC assay, such as described herein, the increase in levels of TNF-alpha or IFN-alpha, is less than 30%, 20%, or 10% of control cells treated with a control dsRNA, such as a dsRNA that does not target PCSK9.

[0371] In one aspect, the invention provides a method for treating, preventing or managing a disorder, pathological process or symptom, which, for example, can be mediated by down regulating PCSK9 gene expression in a subject, such as a human subject. In one embodiment, the disorder is hyperlipidemia. The method includes administering a first single dose of dsRNA, e.g., a dose sufficient to depress levels of PCSK9 mRNA for at least 5, more preferably 7, 10, 14, 21, 25, 30 or 40 days; and optionally, administering a second single dose of dsRNA, wherein the second single dose is administered at least 5, more preferably 7, 10, 14, 21, 25, 30 or 40 days after the first single dose is administered, thereby inhibiting the expression of the PCSK9 gene in a subject.

[0372] In another embodiment, a composition containing a dsRNA featured in the invention, i.e., a dsRNA targeting PCSK9, is administered with a non-dsRNA therapeutic agent, such as an agent known to treat a lipid disorders, such as hypercholesterolemia, atherosclerosis or dyslipidemia. For example, a dsRNA featured in the invention can be administered with, e.g., an HMG-CoA reductase inhibitor (e.g., a statin), a fibrate, a bile acid sequestrant, niacin, an antiplatelet agent, an angiotensin converting enzyme inhibitor, an angiotensin II receptor antagonist (e.g., losartan potassium, such as Merck & Co.'s Cozaar®), an acylCoA cholesterol acetyltransferase (ACAT) inhibitor, a cholesterol absorption inhibitor, a cholesterol ester transfer protein (CETP) inhibitor, a microsomal triglyceride transfer protein (MTTP) inhibitor, a cholesterol modulator, a bile acid modulator, a peroxisome proliferation activated receptor (PPAR) agonist, a gene-based therapy, a composite vascular protectant (e.g., AGI-1067, from Atherogenics), a glycoprotein IIb/IIIa inhibitor, aspirin or an aspirin-like compound, an IBAT inhibitor (e.g., S-8921, from Shionogi), a squalene synthase inhibitor, or a monocyte chemoattractant protein (MCP)-I inhibitor. Exemplary HMG-CoA reductase inhibitors include atorvastatin (Pfizer's Lipitor®/Tahor/Sortis/Torvast/Cardyl), pravastatin (Bristol-Myers Squibb's Pravachol, Sankyo's Mevalotin/Sanaprav), simvastatin (Merck's Zocor®/Sinvacor, Boehringer Ingelheim's Denan, Banyu's Lipovas), lovastatin (Merck's Mevacor/Mevinacor, Bexal's Lovastatina, Cepa; Schwarz Pharma's Liposcler), fluvastatin (Novartis' Lescol®/Locol/Lochol, Fujisawa's Cranoc, Solvay's Digaril), cerivastatin (Bayer's Lipobay/GlaxoSmithKline's Baycol), rosuvastatin (AstraZeneca's Crestor®), and pitivastatin (itavastatin/risivastatin) (Nissan Chemical, Kowa Kogyo, Sankyo, and Novartis). Exemplary fibrates include, e.g., bezafibrate (e.g., Roche's Befizal®/Cedur®/Bezalip®, Kissei's Bezatol), clofibrate (e.g., Wyeth's Atromid-S®), fenofibrate (e.g., Fournier's Lipidil/Lipantil, Abbott's Tricor®, Takeda's Lipantil, generics), gemfibrozil (e.g., Pfizer's Lopid/Lipur) and ciprofibrate (Sanofi-Synthelabo's Modalim®). Exemplary bile acid sequestrants include, e.g., cholestyramine (Bristol-Myers Squibb's Questran® and Questran Light™), colestipol (e.g., Pharmacia's Colestid), and colesevelam (Genzyme/Sankyo's WelChol™). Exemplary niacin therapies include, e.g., immediate release formulations, such as Aventis' Nicobid,

Upsher-Smith's Niacor, Aventis' Nicolar, and Sanwakagaku's Perycit. Niacin extended release formulations include, e.g., Kos Pharmaceuticals' Niaspan and Upsher-Smith's Slo-Niacin. Exemplary antiplatelet agents include, e.g., aspirin (e.g., Bayer's aspirin), clopidogrel (Sanofi-Synthelabo/Bristol-Myers Squibb's Plavix), and ticlopidine (e.g., Sanofi-Synthelabo's Ticlid and Daiichi's Panaldine). Other aspirin-like compounds useful in combination with a dsRNA targeting PCSK9 include, e.g., Asacard (slow-release aspirin, by Pharmacia) and Pamicogrel (Kanebo/Angelini Ricerche/CEPA). Exemplary angiotensin-converting enzyme inhibitors include, e.g., ramipril (e.g., Aventis' Altace) and enalapril (e.g., Merck & Co.'s Vasotec). Exemplary acyl CoA cholesterol acetyltransferase (ACAT) inhibitors include, e.g., avasimibe (Pfizer), eflucimibe (BioMérieux Pierre Fabre/Eli Lilly), CS-505 (Sankyo and Kyoto), and SMP-797 (Sumito). Exemplary cholesterol absorption inhibitors include, e.g., ezetimibe (Merck/Schering-Plough Pharmaceuticals Zetia®) and Pamaqueside (Pfizer). Exemplary CETP inhibitors include, e.g., Torcetrapib (also called CP-529414, Pfizer), JTT-705 (Japan Tobacco), and CETi-I (Avant Immunotherapeutics). Exemplary microsomal triglyceride transfer protein (MTTP) inhibitors include, e.g., implitapide (Bayer), R-103757 (Janssen), and CP-346086 (Pfizer). Other exemplary cholesterol modulators include, e.g., NO-1886 (Otsuka/TAP Pharmaceutical), CI-1027 (Pfizer), and WAY-135433 (Wyeth-Ayerst). Exemplary bile acid modulators include, e.g., HBS-107 (Hisamitsu/Banyu), Btg-511 (British Technology Group), BARI-1453 (Aventis), S-8921 (Shionogi), SD-5613 (Pfizer), and AZD-7806 (AstraZeneca). Exemplary peroxisome proliferation activated receptor (PPAR) agonists include, e.g., tesaglitazar (AZ-242) (AstraZeneca), Netoglitazone (MCC-555) (Mitsubishi/Johnson & Johnson), GW-409544 (Ligand Pharniaceuticals/GlaxoSmithKline), GW-501516 (Ligand Pharmaceuticals/GlaxoSmithKline), LY-929 (Ligand Pharmaceuticals and Eli Lilly), LY-465608 (Ligand Pharmaceuticals and Eli Lilly), LY-518674 (Ligand Pharmaceuticals and Eli Lilly), and MK-767 (Merck and Kyorin). Exemplary gene-based therapies include, e.g., AdGWEGF121.10 (GenVec), ApoAl (UCB Pharma/Groupe Fournier), EG-004 (Trinam) (Ark Therapeutics), and ATP-binding cassette transporter-A1 (ABCA1) (CV Therapeutics/Incyte, Aventis, Xenon). Exemplary Glycoprotein IIb/IIIa inhibitors include, e.g., roxifiban (also called DMP754, Bristol-Myers Squibb), Gantofiban (Merck KGaA/Yamanouchi), and Cromafiban (Millennium Pharmaceuticals). Exemplary squalene synthase inhibitors include, e.g., BMS-1884941 (Bristol-Myers Squibb), CP-210172 (Pfizer), CP-295697 (Pfizer), CP-294838 (Pfizer), and TAK-475 (Takeda). An exemplary MCP-I inhibitor is, e.g., RS-504393 (Roche Bioscience). The anti-atherosclerotic agent BO-653 (Chugai Pharmaceuticals), and the nicotinic acid derivative Nyclin (Yamanouchi Pharmacuticals) are also appropriate for administering in combination with a dsRNA featured in the invention. Exemplary combination therapies suitable for administration with a dsRNA targeting PCSK9 include, e.g., advicor (Niacin/lovastatin from Kos Pharmaceuticals), amlodipine/atorvastatin (Pfizer), and ezetimibe/simvastatin (e.g., Vytorin® 10/10, 10/20, 10/40, and 10/80 tablets by Merck/Schering-Plough Pharmaceuticals). Agents for treating hypercholesterolemia, and suitable for administration in combination with a dsRNA targeting PCSK9 include, e.g., lovastatin, niacin Altoprev® Extended-Release Tablets (An-

drx Labs), lovastatin Caduet® Tablets (Pfizer), amlodipine besylate, atorvastatin calcium Crestor® Tablets (AstraZeneca), rosuvastatin calcium Lescol® Capsules (Novartis), fluvastatin sodium Lescol® (Reliant, Novartis), fluvastatin sodium Lipitor® Tablets (Parke-Davis), atorvastatin calcium Lofibra® Capsules (Gate), Niaspan Extended-Release Tablets (Kos), niacin Pravachol Tablets (Bristol-Myers Squibb), pravastatin sodium TriCor® Tablets (Abbott), fenofibrate Vytorin® 10/10 Tablets (Merck/Schering-Plough Pharmaceuticals), ezetimibe, simvastatin WelChol™ Tablets (Sankyo), colesevelam hydrochloride Zetia® Tablets (Schering), ezetimibe Zetia® Tablets (Merck/Schering-Plough Pharmaceuticals), and ezetimibe Zocor® Tablets (Merck).

[0373] In one embodiment, a dsRNA targeting PCSK9 is administered in combination with an ezetimibe/simvastatin combination (e.g., Vytorin® (Merck/Schering-Plough Pharmaceuticals)).

[0374] In one embodiment, the PCSK9 dsRNA is administered to the patient, and then the non-dsRNA agent is administered to the patient (or vice versa). In another embodiment, the PCSK9 dsRNA and the non-dsRNA therapeutic agent are administered at the same time.

[0375] In another aspect, the invention features, a method of instructing an end user, e.g., a caregiver or a subject, on how to administer a dsRNA described herein. The method includes, optionally, providing the end user with one or more doses of the dsRNA, and instructing the end user to administer the dsRNA on a regimen described herein, thereby instructing the end user.

[0376] In yet another aspect, the invention provides a method of treating a patient by selecting a patient on the basis that the patient is in need of LDL lowering, LDL lowering without lowering of HDL, ApoB lowering, or total cholesterol lowering. The method includes administering to the patient a dsRNA targeting PCSK9 in an amount sufficient to lower the patient's LDL levels or ApoB levels, e.g., without substantially lowering HDL levels.

[0377] In another aspect, the invention provides a method of treating a patient by selecting a patient on the basis that the patient is in need of lowered ApoB levels, and administering to the patient a dsRNA targeting PCSK9 in an amount sufficient to lower the patient's ApoB levels. In one embodiment, the amount of PCSK9 is sufficient to lower LDL levels as well as ApoB levels. In another embodiment, administration of the PCSK9 dsRNA does not affect the level of HDL cholesterol in the patient.

[0378] 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 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.

EXAMPLES

Example 1

Gene Walking of the PCSK9 Gene

[0379] siRNA Design was Carried Out to Identify in Two Separate Selections

[0380] a) siRNAs targeting PCSK9 human and either mouse or rat mRNA and

[0381] b) all human reactive siRNAs with predicted specificity to the target gene PCSK9.

[0382] mRNA sequences to human, mouse and rat PCSK9 were used: Human sequence NM_174936.2 was used as reference sequence during the complete siRNA selection procedure.

[0383] 19 mer stretches conserved in human and mouse, and human and rat PCSK9 mRNA sequences were identified in the first step, resulting in the selection of siRNAs cross-reactive to human and mouse, and siRNAs cross-reactive to human and rat targets

[0384] SiRNAs specifically targeting human PCSK9 were identified in a second selection. All potential 19mer sequences of human PCSK9 were extracted and defined as candidate target sequences. Sequences cross-reactive to human, monkey, and those cross-reactive to mouse, rat, human and monkey are all listed in Tables 1a and 2a. Chemically modified versions of those sequences and their activity in both in vitro and in vivo assays are also listed in Tables 1a and 2a. The data is described in the examples and in FIGS. 2-8.

[0385] In order to rank candidate target sequences and their corresponding siRNAs and select appropriate ones, their predicted potential for interacting with irrelevant targets (off-target potential) was taken as a ranking parameter. siRNAs with low off-target potential were defined as preferable and assumed to be more specific in vivo.

[0386] For predicting siRNA-specific off-target potential, the following assumptions were made:

[0387] 1) positions 2 to 9 (counting 5' to 3') of a strand (seed region) may contribute more to off-target potential than rest of sequence (non-seed and cleavage site region)

[0388] 2) positions 10 and 11 (counting 5' to 3') of a strand (cleavage site region) may contribute more to off-target potential than non-seed region

[0389] 3) positions 1 and 19 of each strand are not relevant for off-target interactions

[0390] 4) an off-target score can be calculated for each gene and each strand, based on complementarity of siRNA strand sequence to the gene's sequence and position of mismatches

[0391] 5) number of predicted off-targets as well as highest off-target score must be considered for off-target potential

[0392] 6) off-target scores are to be considered more relevant for off-target potential than numbers of off-targets

[0393] 7) assuming potential abortion of sense strand activity by internal modifications introduced, only off-target potential of antisense strand will be relevant

[0394] To identify potential off-target genes, 19mer candidate sequences were subjected to a homology search against publically available human mRNA sequences.

[0395] The following off-target properties for each 19mer input sequence were extracted for each off-target gene to calculate the off-target score:

[0396] Number of mismatches in non-seed region

[0397] Number of mismatches in seed region

[0398] Number of mismatches in cleavage site region

[0399] The off-target score was calculated for considering assumption 1 to 3 as follows:

Off-target score=number of seed mismatches*10+ number of cleavage site mismatches*1.2+number of non-seed mismatches*1

[0400] The most relevant off-target gene for each siRNA corresponding to the input 19mer sequence was defined as the gene with the lowest off-target score. Accordingly, the lowest off-target score was defined as the relevant off-target score for each siRNA.

Example 2

dsRNA Synthesis

[0401] Source of Reagents
[0402] Where the source of a reagent is not specifically given herein, such reagent may be obtained from any supplier of reagents for molecular biology at a quality/purity standard for application in molecular biology.
[0403] siRNA Synthesis
[0404] Single-stranded RNAs were produced by solid phase synthesis on a scale of 1 μmole using an Expedite 8909 synthesizer (Applied Biosystems, Applera Deutschland GmbH, Darmstadt, Germany) and controlled pore glass (CPG, 500 Å, Proligo Biochemie GmbH, Hamburg, Germany) as solid support. RNA and RNA containing 2'-O-methyl nucleotides were generated by solid phase synthesis employing the corresponding phosphoramidites and 2'-O-methyl phosphoramidites, respectively (Proligo Biochemie GmbH, Hamburg, Germany). These building blocks were incorporated at selected sites within the sequence of the oligoribonucleotide chain using standard nucleoside phosphoramidite chemistry such as described in Current protocols in nucleic acid chemistry, Beaucage, S. L. et al. (Edrs.), John Wiley & Sons, Inc., New York, N.Y., USA. Phosphorothioate linkages were introduced by replacement of the iodine oxidizer solution with a solution of the Beaucage reagent (Chruachem Ltd, Glasgow, UK) in acetonitrile (1%). Further ancillary reagents were obtained from Mallinckrodt Baker (Griesheim, Germany).
[0405] Deprotection and purification of the crude oligoribonucleotides by anion exchange HPLC were carried out according to established procedures. Yields and concentrations were determined by UV absorption of a solution of the respective RNA at a wavelength of 260 nm using a spectral photometer (DU 640B, Beckman Coulter GmbH, Unterschleißheim, Germany). Double stranded RNA was generated by mixing an equimolar solution of complementary strands in annealing buffer (20 mM sodium phosphate, pH 6.8; 100 mM sodium chloride), heated in a water bath at 85-90° C. for 3 minutes and cooled to room temperature over a period of 3-4 hours. The annealed RNA solution was stored at −20° C. until use.
[0406] Conjugated siRNAs
[0407] For the synthesis of 3'-cholesterol-conjugated siRNAs (herein referred to as -Chol-3'), an appropriately modified solid support was used for RNA synthesis. The modified solid support was prepared as follows:

Diethyl-2-azabutane-1,4-dicarboxylate AA

[0408]
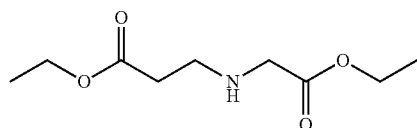
AA

[0409] A 4.7 M aqueous solution of sodium hydroxide (50 ml) was added into a stirred, ice-cooled solution of ethyl glycinate hydrochloride (32.19 g, 0.23 mole) in water (50 ml). Then, ethyl acrylate (23.1 g, 0.23 mole) was added and the mixture was stirred at room temperature until completion of the reaction was ascertained by TLC. After 19 h the solution was partitioned with dichloromethane (3×100 ml). The organic layer was dried with anhydrous sodium sulfate, filtered and evaporated. The residue was distilled to afford AA (28.8 g, 61%).

3-{Ethoxycarbonylmethyl-[6-(9H-fluoren-9-yl-methoxycarbonyl-amino)-hexanoyl]-amino}-propionic acid ethyl ester AB

[0410]
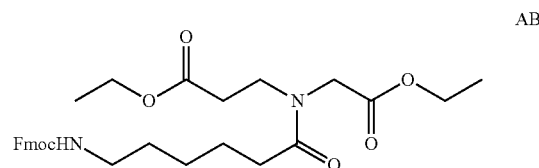
AB

[0411] Fmoc-6-amino-hexanoic acid (9.12 g, 25.83 mmol) was dissolved in dichloromethane (50 ml) and cooled with ice. Diisopropylcarbodiimde (3.25 g, 3.99 ml, 25.83 mmol) was added to the solution at 0° C. It was then followed by the addition of Diethyl-azabutane-1,4-dicarboxylate (5 g, 24.6 mmol) and dimethylamino pyridine (0.305 g, 2.5 mmol). The solution was brought to room temperature and stirred further for 6 h. Completion of the reaction was ascertained by TLC. The reaction mixture was concentrated under vacuum and ethyl acetate was added to precipitate diisopropyl urea. The suspension was filtered. The filtrate was washed with 5% aqueous hydrochloric acid, 5% sodium bicarbonate and water. The combined organic layer was dried over sodium sulfate and concentrated to give the crude product which was purified by column chromatography (50% EtOAC/Hexanes) to yield 11.87 g (88%) of AB.

3-[(6-Amino-hexanoyl)-ethoxycarbonylmethyl-amino]-propionic acid ethyl ester AC

[0412]
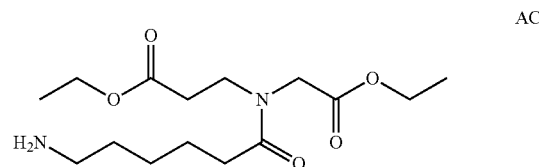
AC

[0413] 3-{Ethoxycarbonylmethyl-[6-(9H-fluoren-9-yl-methoxycarbonylamino)-hexanoyl]-amino}-propionic acid ethyl ester AB (11.5 g, 21.3 mmol) was dissolved in 20% piperidine in dimethylformamide at 0° C. The solution was continued stirring for 1 h. The reaction mixture was concentrated under vacuum, water was added to the residue, and the product was extracted with ethyl acetate. The crude product was purified by conversion into its hydrochloride salt.

3-({6-[17-(1,5-Dimethyl-hexyl)-10,13-dimethyl-2,3, 4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H- cyclopenta[a]phenanthren-3-yloxycarbonylamino]- hexanoyl}ethoxycarbonylmethyl-amino)-propionic acid ethyl ester AD

[0414]

AD
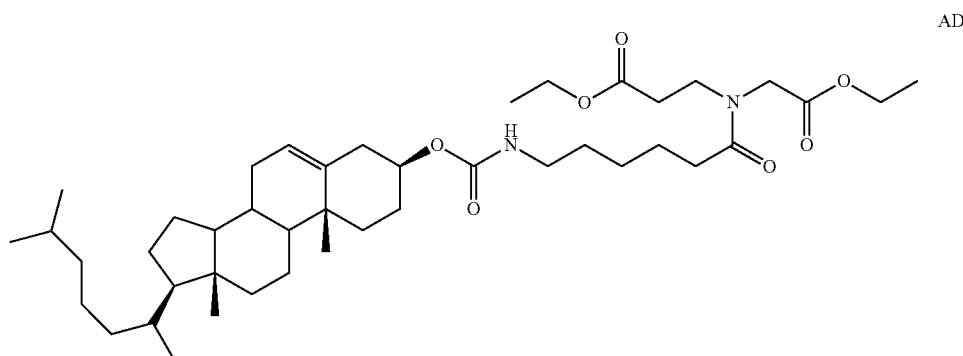
[0415] The hydrochloride salt of 3-[(6-Amino-hexanoyl)- ethoxycarbonylmethyl-amino]-propionic acid ethyl ester AC (4.7 g, 14.8 mmol) was taken up in dichloromethane. The suspension was cooled to 0° C. on ice. To the suspension diisopropylethylamine (3.87 g, 5.2 ml, 30 mmol) was added. To the resulting solution cholesteryl chloroformate (6.675 g, 14.8 mmol) was added. The reaction mixture was stirred overnight. The reaction mixture was diluted with dichloromethane and washed with 10% hydrochloric acid. The product was purified by flash chromatography (10.3 g, 92%).

1-{6-[17-(1,5-Dimethyl-hexyl)-10,13-dimethyl-2,3, 4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H- cyclopenta[a] phenanthren-3-yloxycarbonylamino]- hexanoyl}-4-oxo-pyrrolidine-3-carboxylic acid ethyl ester AE

[0416]

[0417] Potassium t-butoxide (1.1 g, 9.8 mmol) was slur- ried in 30 ml of dry toluene. The mixture was cooled to 0° C. on ice and 5 g (6.6 mmol) of diester AD was added slowly with stirring within 20 mins. The temperature was kept below 5° C. during the addition. The stirring was continued for 30 mins at 0° C. and 1 ml of glacial acetic acid was added, immediately followed by 4 g of $NaH_2PO_4.H_2O$ in 40 ml of water The resultant mixture was extracted twice with 100 ml of dichloromethane each and the combined organic extracts were washed twice with 10 ml of phosphate buffer each, dried, and evaporated to dryness. The residue was dissolved in 60 ml of toluene, cooled to 0° C. and extracted with three 50 ml portions of cold pH 9.5 carbonate buffer. The aqueous extracts were adjusted to pH 3 with phosphoric acid, and extracted with five 40 ml portions of chloroform which were combined, dried and evaporated to dryness. The residue was purified by column chromatography using 25% ethylacetate/hexane to afford 1.9 g of b-ketoester (39%).

AE
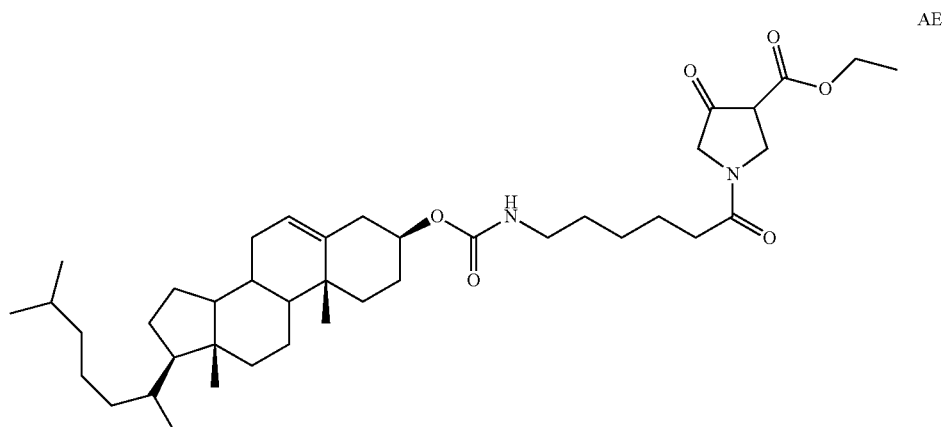
[6-(3-Hydroxy-4-hydroxymethyl-pyrrolidin-1-yl)-6-
oxo-hexyl]-carbamic acid 17-(1,5-dimethyl-hexyl)-
10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-
tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl
ester AF

[0418]
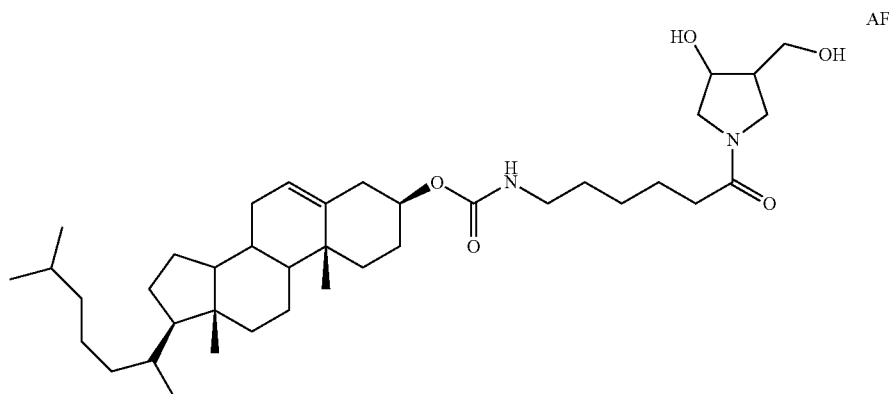
[0419] Methanol (2 ml) was added dropwise over a period of 1 h to a refluxing mixture of b-ketoester AE (1.5 g, 2.2 mmol) and sodium borohydride (0.226 g, 6 mmol) in tetrahydrofuran (10 ml). Stirring was continued at reflux temperature for 1 h. After cooling to room temperature, 1 N HCl (12.5 ml) was added, the mixture was extracted with ethylacetate (3×40 ml). The combined ethylacetate layer was dried over anhydrous sodium sulfate and concentrated under vacuum to yield the product which was purified by column chromatography (10% MeOH/CHCl$_3$) (89%).

(6-{3-[Bis-(4-methoxy-phenyl)-phenyl-methoxym-
ethyl]-4-hydroxy-pyrrolidin-1-yl}-6-oxo-hexyl)-
carbamic acid 17-(1,5-dimethyl-hexyl)-10,13-dim-
ethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-
tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl
ester AG

[0420]
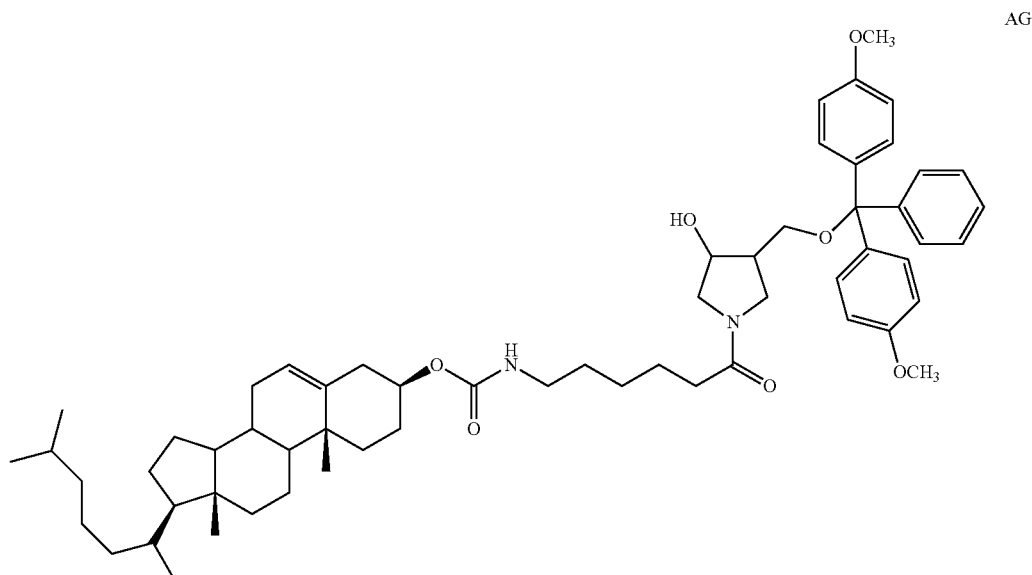
[0421] Diol AF (1.25 gm 1.994 mmol) was dried by evaporating with pyridine (2×5 ml) in vacuo. Anhydrous pyridine (10 ml) and 4,4'-dimethoxytritylchloride (0.724 g, 2.13 mmol) were added with stirring. The reaction was carried out at room temperature overnight. The reaction was quenched by the addition of methanol. The reaction mixture was concentrated under vacuum and to the residue dichloromethane (50 ml) was added. The organic layer was washed with 1M aqueous sodium bicarbonate. The organic layer was dried over anhydrous sodium sulfate, filtered and concentrated. The residual pyridine was removed by evaporating with toluene. The crude product was purified by column chromatography (2% MeOH/Chloroform, Rf=0.5 in 5% MeOH/CHCl₃) (1.75 g, 95%).

Succinic acid mono-(4-[bis-(4-methoxy-phenyl)-phenyl-methoxymethyl]-1-{6-[17-(1,5-dimethyl-hexyl)-10,13-dimethyl 2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H cyclopenta[a]phenanthren-3-yloxycarbonylamino]-hexanoyl}-pyrrolidin-3-yl) ester AH

[0422]
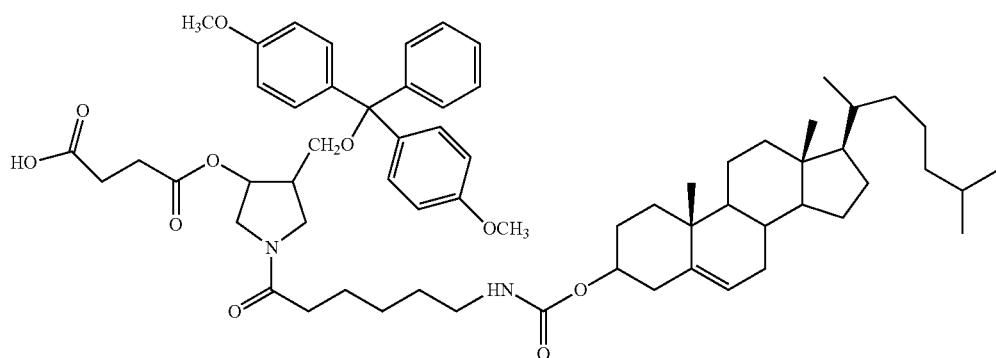
AH

[0423] Compound AG (1.0 g, 1.05 mmol) was mixed with succinic anhydride (0.150 g, 1.5 mmol) and DMAP (0.073 g, 0.6 mmol) and dried in a vacuum at 40° C. overnight. The mixture was dissolved in anhydrous dichloroethane (3 ml), triethylamine (0.318 g, 0.440 ml, 3.15 mmol) was added and the solution was stirred at room temperature under argon atmosphere for 16 h. It was then diluted with dichloromethane (40 ml) and washed with ice cold aqueous citric acid (5 wt %, 30 ml) and water (2×20 ml). The organic phase was dried over anhydrous sodium sulfate and concentrated to dryness. The residue was used as such for the next step.
[0424] Cholesterol Derivatised CPG AI
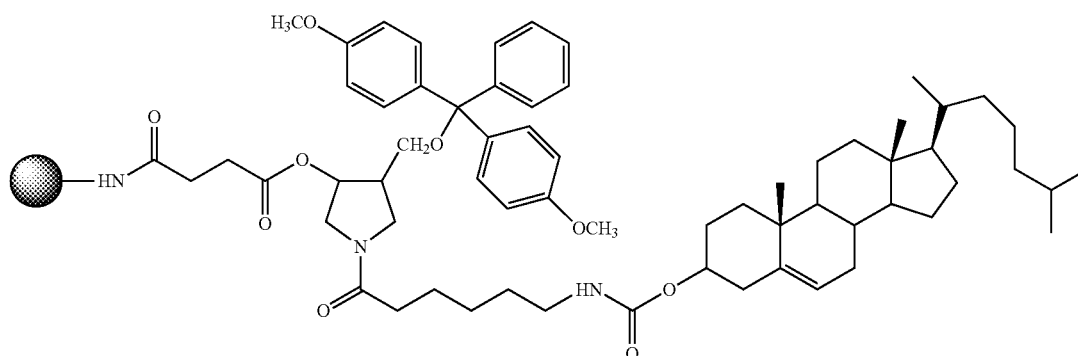
AI

[0425] Succinate AH (0.254 g, 0.242 mmol) was dissolved in a mixture of dichloromethane/acetonitrile (3:2, 3 ml). To that solution DMAP (0.0296 g, 0.242 mmol) in acetonitrile (1.25 ml), 2,2'-Dithio-bis(5-nitropyridine) (0.075 g, 0.242 mmol) in acetonitrile/dichloroethane (3:1, 1.25 ml) were added successively. To the resulting solution triphenylphosphine (0.064 g, 0.242 mmol) in acetonitrile (0.6 ml) was added. The reaction mixture turned bright orange in color. The solution was agitated briefly using a wrist-action shaker (5 mins). Long chain alkyl amine-CPG (LCAA-CPG) (1.5 g, 61 mM) was added. The suspension was agitated for 2 h. The CPG was filtered through a sintered funnel and washed with acetonitrile, dichloromethane and ether successively. Unreacted amino groups were masked using acetic anhydride/pyridine. The achieved loading of the CPG was measured by taking UV measurement (37 mM/g).

[0426] The synthesis of siRNAs bearing a 5'-12-dodecanoic acid bisdecylamide group (herein referred to as "5'-C32-") or a 5'-cholesteryl derivative group (herein referred to as "5'-Chol-") was performed as described in WO 2004/065601, except that, for the cholesteryl derivative, the oxidation step was performed using the Beaucage reagent in order to introduce a phosphorothioate linkage at the 5'-end of the nucleic acid oligomer.

[0427] Synthesis of dsRNAs conjugated to Chol-p-(Gal-NAc)₃ (N-acetyl galactosamine-cholesterol) (FIG. **16**) and LCO(GalNAc)₃ (N-acetyl galactosamine-3'-Lithocholic-oleoyl) (FIG. **17**) is described in U.S. patent application Ser. No. 12/328,528, filed on Dec. 4, 2008, which is hereby incorporated by reference.

Example 3

PCSK9 siRNA Screening in HuH7, HepG2, HeLa and Primary Monkey Hepatocytes Discovers Highly Active Sequences

[0428] HuH-7cells were obtained from JCRB Cell Bank (Japanese Collection of Research Bioresources) (Shinjuku, Japan, cat. No.: JCRB0403) Cells were cultured in Dulbecco's MEM (Biochrom AG, Berlin, Germany, cat. No. F0435) supplemented to contain 10% fetal calf serum (FCS) (Biochrom AG, Berlin, Germany, cat. No. S0115), Penicillin 100 U/ml, Streptomycin 100 μg/ml (Biochrom AG, Berlin, Germany, cat. No. A2213) and 2 mM L-Glutamin (Biochrom AG, Berlin, Germany, cat. No K0282) at 37° C. in an atmosphere with 5% CO_2 in a humidified incubator (Heraeus HERAcell, Kendro Laboratory Products, Langenselbold, Germany). HepG2 and HeLa cells were obtained from American Type Culture Collection (Rockville, Md., cat. No. HB-8065) and cultured in MEM (Gibco Invitrogen, Karlsruhe, Germany, cat. No. 21090-022) supplemented to contain 10% fetal calf serum (FCS) (Biochrom AG, Berlin, Germany, cat. No. S0115), Penicillin 100 U/ml, Streptomycin 100 μg/ml (Biochrom AG, Berlin, Germany, cat. No. A2213), lx Non Essential Amino Acids (Biochrom AG, Berlin, Germany, cat. No. K-0293), and 1 mM Sodium Pyruvate (Biochrom AG, Berlin, Germany, cat. No. L-0473) at 37° C. in an atmosphere with 5% CO_2 in a humidified incubator (Heraeus HERAcell, Kendro Laboratory Products, Langenselbold, Germany).

[0429] For transfection with siRNA, HuH7, HepG2, or HeLa cells were seeded at a density of 2.0×10^4 cells/well in 96-well plates and transfected directly. Transfection of siRNA (30 nM for single dose screen) was carried out with lipofectamine 2000 (Invitrogen GmbH, Karlsruhe, Germany, cat. No. 11668-019) as described by the manufacturer.

[0430] 24 hours after transfection HuH7 and HepG2 cells were lysed and PCSK9 mRNA levels were quantified with the Quantigene Explore Kit (Genosprectra, Dumbarton Circle Fremont, USA, cat. No. QG-000-02) according to the protocol. PCSK9 mRNA levels were normalized to GAP-DH mRNA. For each siRNA eight individual datapoints were collected. siRNA duplexes unrelated to PCSK9 gene were used as control. The activity of a given PCSK9 specific siRNA duplex was expressed as percent PCSK9 mRNA concentration in treated cells relative to PCSK9 mRNA concentration in cells treated with the control siRNA duplex.

[0431] Primary cynomolgus monkey hepatocytes (cryo-preserved) were obtained from In vitro Technologies, Inc. (Baltimore, Md., USA, cat No MO0305) and cultured in InVitroGRO CP Medium (cat No Z99029) at 37° C. in an atmosphere with 5% CO_2 in a humidified incubator.

[0432] For transfection with siRNA, primary cynomolgus monkey cells were seeded on Collagen coated plates (Fisher Scientific, cat. No. 08-774-5) at a density of 3.5×104 cells/well in 96-well plates and transfected directly. Transfection of siRNA (eight 2-fold dilution series starting from 30 nM) in duplicates was carried out with lipofectamine 2000 (Invitrogen GmbH, Karlsruhe, Germany, cat. No. 11668-019) as described by the manufacturer.

[0433] 16 hours after transfection medium was changed to fresh InVitroGRO CP Medium with Torpedo Antibiotic Mix (In vitro Technologies, Inc, cat. No Z99000) added.

[0434] 24 hours after medium change primary cynomolgus monkey cells were lysed and PCSK9 mRNA levels were quantified with the Quantigene Explore Kit (Genosprectra, Dumbarton Circle Fremont, USA, cat. No. QG-000-02) according to the protocol. PCSK9 mRNA levels were normalized to GAPDH mRNA. Normalized PCSK9/GAPDH ratios were then compared to PCSK9/GAPDH ratio of lipofectamine 2000 only control.

[0435] Tables 1b and 2b (and FIG. **6A**) summarize the results and provide examples of in vitro screens in different cell lines at different doses. Silencing of PCSK9 transcript was expressed as percentage of remaining transcript at a given dose.

[0436] Highly active sequences are those with less than 70% transcript remaining post treatment with a given siRNA at a dose less than or equal to 100 nM. Very active sequences are those that have less than 60% of transcript remaining after treatment with a dose less than or equal to 100 nM. Active sequences are those that have less than 90% transcript remaining after treatment with a high dose (100 nM).

[0437] Examples of active siRNAs were also screened in vivo in mouse in lipidoid formulations as described below. Active sequences in vitro were also generally active in vivo (See FIGS. **6A** and **6B** and example 4).

Example 4

In Vivo Efficacy Screen of PCSK9 siRNAs in Mice

[0438] 32 PCSK9 siRNAs formulated in LNP-01 liposomes were tested in vivo in a mouse model. LNP01 is a lipidoid formulation formed from cholesterol, mPEG2000-C14 Glyceride, and dsRNA. The LNP01 formulation is useful for delivering dsRNAs to the liver.

[0439] Formulation Procedure

[0440] The lipidoid LNP-01.4HCl (MW 1487) (FIG. 1), Cholesterol (Sigma-Aldrich), and PEG-Ceramide C16 (Avanti Polar Lipids) were used to prepare lipid-siRNA nanoparticles. Stock solutions of each in ethanol were prepared: LNP-01, 133 mg/ml; Cholesterol, 25 mg/ml, PEG-Ceramide C16, 100 mg/ml. LNP-01, Cholesterol, and PEG-Ceramide C16 stock solutions were then combined in a 42:48:10 molar ratio. Combined lipid solution was mixed rapidly with aqueous siRNA (in sodium acetate pH 5) such that the final ethanol concentration was 35-45% and the final sodium acetate concentration was 100-300 mM. Lipid-siRNA nanoparticles formed spontaneously upon mixing. Depending on the desired particle size distribution, the resultant nanoparticle mixture was in some cases extruded through a polycarbonate membrane (100 nm cut-off) using a thermobarrel extruder (Lipex Extruder, Northern Lipids, Inc). In other cases, the extrusion step was omitted. Ethanol removal and simultaneous buffer exchange was accomplished by either dialysis or tangential flow filtration. Buffer was exchanged to phosphate buffered saline (PBS) pH 7.2.

[0441] Characterization of Formulations

[0442] Formulations prepared by either the standard or extrusion-free method are characterized in a similar manner. Formulations are first characterized by visual inspection. They should be whitish translucent solutions free from aggregates or sediment. Particle size and particle size distribution of lipid-nanoparticles are measured by dynamic light scattering using a Malvern Zetasizer Nano ZS (Malvern, USA). Particles should be 20-300 nm, and ideally, 40-100 nm in size. The particle size distribution should be unimodal. The total siRNA concentration in the formulation, as well as the entrapped fraction, is estimated using a dye exclusion assay. A sample of the formulated siRNA is incubated with the RNA-binding dye Ribogreen (Molecular Probes) in the presence or absence of a formulation disrupting surfactant, 0.5% Triton-X100. The total siRNA in the formulation is determined by the signal from the sample containing the surfactant, relative to a standard curve. The entrapped fraction is determined by subtracting the "free" siRNA content (as measured by the signal in the absence of surfactant) from the total siRNA content. Percent entrapped siRNA is typically >85%.

[0443] Bolus Dosing

[0444] Bolus dosing of formulated siRNAs in C57/BL6 mice (5/group, 8-10 weeks old, Charles River Laboratories, MA) was performed by tail vein injection using a 27 G needle. SiRNAs were formulated in LNP-01 (and then dialyzed against PBS) at 0.5 mg/ml concentration allowing the delivery of the 5 mg/kg dose in 10 µl/g body weight. Mice were kept under an infrared lamp for approximately 3 min prior to dosing to ease injection.

[0445] 48 hour post dosing mice were sacrificed by CO_2-asphyxiation. 0.2 ml blood was collected by retro-orbital bleeding and the liver was harvested and frozen in liquid nitrogen. Serum and livers were stored at −80° C. µl

[0446] Frozen livers were grinded using 6850 Freezer/Mill Cryogenic Grinder (SPEX CentriPrep, Inc) and powders stored at −80° C. until analysis.

[0447] PCSK9 mRNA levels were detected using the branched-DNA technology based kit from QuantiGene Reagent System (Genospectra) according to the protocol. 10-20 mg of frozen liver powders was lysed in 600 µl of 0.16 µg/ml Proteinase K (Epicentre, #MPRK092) in Tissue and Cell Lysis Solution (Epicentre, #MTC096H) at 65° C. for 3 hours. Then 10 µl of the lysates were added to 90 µl of Lysis Working Reagent (1 volume of stock Lysis Mixture in two volumes of water) and incubated at 52° C. overnight on Genospectra capture plates with probe sets specific to mouse PCSK9 and mouse GAPDH or cyclophilin B. Nucleic acid sequences for Capture Extender (CE), Label Extender (LE) and blocking (BL) probes were selected from the nucleic acid sequences of PCSK9, GAPDH and cyclophilin B with the help of the QuantiGene ProbeDesigner Software 2.0 (Genospectra, Fremont, Calif., USA, cat. No. QG-002-02). Chemo luminescence was read on a Victor2-Light (Perkin Elmer) as Relative light units. The ratio of PCSK9 mRNA to GAPDH or cyclophilin B mRNA in liver lysates was averaged over each treatment group and compared to a control group treated with PBS or a control group treated with an unrelated siRNA (blood coagulation factor VII).

[0448] Total serum cholesterol in mouse serum was measured using the StanBio Cholesterol LiquiColor kit (StanBio Laboratory, Boerne, Tex., USA) according to manufacturer's instructions. Measurements were taken on a Victor2 1420 Multilabel Counter (Perkin Elmer) at 495 nm.

[0449] Results

[0450] At least 10 PCSK9 siRNAs showed more than 40% PCSK9 mRNA knock down compared to a control group treated with PBS, while control group treated with an unrelated siRNA (blood coagulation factor VII) had no effect (FIGS. 2-3). Silencing of PCSK9 transcript also correlated with a lowering of total serum cholesterol in these animals (FIGS. 4-5). The most efficacious siRNAs with respect to knocking down PCSK9 mRNAs also showed the most pronounced cholesterol lowering effects (compare FIGS. 2-3 and FIGS. 4-5). In addition there was a strong correlation between those molecules that were active in vitro and those active in vivo (compare FIGS. 6A and 6B).

[0451] Sequences containing different chemical modifications were also screened in vitro (Tables 1 and 2) and in vivo. As an example, less modified sequences AD-9314 and AD-9318, and a more modified versions of that sequence AD-9314 (AD-10792, AD-10793, and AD-10796); AD-9318-(AD-10794, AD-10795, AD-10797) were tested both in vitro (in primary monkey hepatocytes) or in vivo (AD-9314 and AD-10792) formulated in LNP-01. FIG. 7 (also see Tables 1 and 2) shows that the parent molecules AD-9314 and AD-9318 and the modified versions were all active in vitro. FIG. 8 as an example shows that both the parent AD-9314 and the more highly modified AD-10792 sequences were active in vivo displaying 50-60% silencing of endogenous PCSK9 in mice. FIG. 9 further exemplifies that activity of other chemically modified versions of AD-9314 and AD-0792.

[0452] AD-3511, a derivative of AD-10792, was as efficacious as 10792 (IC50 of ~0.07-0.2 nM) (data not shown). The sequences of the sense and antisense strands of AD-3511 are as follows:

```
Sense strand:
                           SEQ ID NO: 1521
5'-GccuGGAGuuuAuucGGAAdTsdT

Antisense strand:
                           SEQ ID NO: 1522
5'-puUCCGAAuAAACUCcAGGCdTsdT
```

Example 5

PCSK9 Duration of Action Experiments in Rats and NHP

[0453] Rats
[0454] Rats were treated via tail vein injection with 5 mg/kg of LNP01-10792 (Formulated ALDP-10792). Blood was drawn at the indicated time points (see Table 3) and the amount of total cholesterol compared to PBS treated animals was measured by standard means. Total cholesterol levels decreased at day two ~60% and returned to baseline by day 28. These data show that formulated versions of PCSK9 siRNAs lower cholesterol levels for extended periods of time.
[0455] Monkeys
[0456] Cynomolgus monkeys were treated with LNP01 formulated dsRNA and LDL-C levels were evaluated. A total of 19 cynomolgus monkeys were assigned to dose groups. Beginning on Day –11, animals were limit-fed twice-a-day according to the following schedule: feeding at 9 a.m., feed removal at 10 a.m., feeding at 4 p.m., feed removal at 5 p.m. On the first day of dosing all animals were dosed once via 30-minute intravenous infusion. The animals were evaluated for changes in clinical signs, body weight, and clinical pathology indices, including direct LDL and HDL cholesterol.
[0457] Venipuncture through the femoral vein was used to collect blood samples. Samples were collected prior to the morning feeding (i.e., before 9 a.m.) and at approximately 4 hours (beginning at 1 p.m.) after the morning feeding on Days –3, –1, 3, 4, 5, and 7 for Groups 1-7; on Day 14 for Groups 1, 4, and 6; on Days 18 and 21 for Group 1; and on Day 21 for Groups 4 and 6. At least two 1.0 ml samples were collected at each time point.
[0458] No anticoagulant was added to the 1.0 ml serum samples, and the dry anticoagulant Ethylenediaminetet-raacetic acid (K2) was added to each 1.0 ml plasma sample. Serum samples were allowed to stand at room temperature for at least 20 minutes to facilitate coagulation and then the samples were placed on ice. Plasma samples were placed on ice as soon as possible following sample collection. Samples were transported to the clinical pathology lab within 30 minutes for further processing.
[0459] Blood samples were processed to serum or plasma as soon as possible using a refrigerated centrifuge, per Testing Facility Standard operating procedure. Each sample was split into 3 approximately equal volumes, quickly frozen in liquid nitrogen, and placed at –70° C. Each aliquot should have had a minimum of approximately 50 µL. If the total sample volume collected was under 150 µL, the residual sample volume went into the last tube. Each sample was labeled with the animal number, dose group, day of collection, date, nominal collection time, and study number (s). Serum LDL cholesterol was measured directly per standard procedures on a Beckman analyzer according to manufactures instructions.
[0460] The results are shown in Table 4. LNP01-10792 and LNP01-9680 administered at 5 mg/kg decreased serum LDL cholesterol within 3 to 7 days following dose administration. Serum LDL cholesterol returned to baseline levels by Day 14 in most animals receiving LNP01-10792 and by Day 21 in animals receiving LNP01-9680. This data demonstrated a greater than 21 day duration of action for cholesterol lowering of LNP01 formulated ALDP-9680.

Example 6

PCSK9 siRNAs Cause Decreased PCSK mRNA in Liver Extracts, and Lower Serum Cholesterol Levels in Mice and Rats

[0461] To test if acute silencing of the PCSK9 transcript by a PCSK9 siRNA (and subsequent PCSK9 protein down-regulation), would result in acutely lower total cholesterol levels, siRNA molecule AD-1a2 (AD-10792) was formulated in an LNP01 lipidoid formulation. Sequences and modifications of these dsRNAs are shown in Table 5a. Liposomal formulated siRNA duplex AD-1a2 (LNP01-1a2) was injected via tail vein in low volumes (~0.2 ml for mouse and ~1.0 ml for rats) at different doses into C57/BL6 mice or Sprague Dawley rats.
[0462] In mice, livers were harvested 48 hours post-injection, and levels of PCSK9 transcript were determined. In addition to liver, blood was harvested and subjected to a total cholesterol analysis. LNP01-1a2 displayed a clear dose response with maximal PCSK9 message suppression (~60-70%) as compared to a control siRNA targeting luciferase (LNP01-ctrl) or PBS treated animals (FIG. 14A). The decrease of PCSK9 transcript at the highest dose translated into a ~30% lowering of total cholesterol in mice (FIG. 14B). This level of cholesterol reduction is between that reported for heterozygous and homozygous PCSK9 knock-out mice (Rashid et al., Proc. Natl. Acad. Sci. USA 102: 5374-9, 2005, epub Apr. 1, 2005). Thus, lowering of PCSK9 transcript through an RNAi mechanism is capable of acutely decreasing total cholesterol in mice. Moreover the effect on the PCSK9 transcript persisted between 20-30 days, with higher doses displaying greater initial transcript level reduction, and subsequently more persistent effects.
[0463] Down-modulation of total cholesterol in rats has been historically difficult as cholesterol levels remain unchanged even at high doses of HMG-CoA reductase inhibitors. Interestingly, as compared to mice, rats appear to have a much higher level of PCSK9 basal transcript levels as measured by bDNA assays. Rats were dosed with a single injection of LNP01-a2 via tail vein at 1, 2.5 and 5 mg/kg. Liver tissue and blood were harvested 72 hours post-injection. LNP01-1a2 exhibited a clear dose response effect with maximal 50-60% silencing of the PCSK9 transcript at the highest dose, as compared to a control luciferase siRNA and PBS (FIG. 10A). The mRNA silencing was associate with an acute ~50-60% decrease of serum total cholesterol (FIGS. 10A and 10B) lasting 10 days, with a gradual return to pre-dose levels by ~3 weeks (FIG. 10B). This result demonstrated that lowering of PCSK9 via siRNA targeting had acute, potent and lasting effects on total cholesterol in the rat model system. To confirm that the transcript reduction observed was due to a siRNA mechanism, liver extracts from treated or control animals were subjected to 5' RACE, a method previously utilized to demonstrate that the predicted siRNA cleavage event occurs (Zimmermann et al., Nature. 441:111-4, 2006, Epub 2006 Mar. 26). PCR amplification and detection of the predicted site specific mRNA cleavage event was observed in animals treated with LNP01-1a2, but not PBS or LNP01-ctrl control animals. (Frank-Kamanetsky et al. (2008) PNAS 105:119715-11920) This result demonstrated that the effects of LNP01-1a2 observed were due to cleavage of the PCSK9 transcript via an siRNA specific mechanism.

[0464] The mechanism by which PCSK9 impacts cholesterol levels has been linked to the number of LDLRs on the cell surface. Rats (as opposed to mice, NHP, and humans) control their cholesterol levels through tight regulation of cholesterol synthesis and to a lesser degree through the control of LDLR levels. To investigate whether modulation of LDLR was occurring upon RNAi therapeutic targeting of PCSK9, we quantified the liver LDLR levels (via western blotting) in rats treated with 5 mg/kg LNP01-1a2. As shown in FIG. **11**, LNP01-1a2 treated animals had a significant (~3-5 fold average) induction of LDLR levels 48 hours post a single dose of LNP01-1a2 compared to PBS or LNP01-ctrl control siRNA treated animals.

[0465] Assays were also performed to test whether reduction of PCSK9 changes the levels of triglycerides and cholesterol in the liver itself. Acute lowering of genes involved in VLDL assembly and secretion such as microsomal triglyceride transfer protein (MTP) or ApoB by genetic deletion, compounds, or siRNA inhibitors results in increased liver triglycerides (see, e.g., Akdim et al., *Curr. Opin. Lipidol.* 18:397-400, 2007). Increased clearance of plasma cholesterol induced by PCSK9 silencing in the liver (and a subsequent increase in liver LDLR levels) was not predicted to result in accumulation of liver triglycerides. However, to address this possibility, liver cholesterol and triglyceride concentrations in livers of the treated or control animals were quantified. As shown in FIG. **10**C, there was no statistical difference in liver TG levels or cholesterol levels of rats administered PCSK9 siRNAs compared to the controls. These results indicated that PCSK9 silencing and subsequent cholesterol lowering is unlikely to result in excess hepatic lipid accumulation.

Example 7

Additional Modifications to siRNAs do not Affect Silencing and Duration of Cholesterol Reduction in Rats

[0466] Phosphorothioate modifications at the 3' ends of both sense and antisense strands of a dsRNA can protect against exonucleases. 2'OMe and 2'F modifications in both the sense and antisense strands of a dsRNA can protect against endonucleases. AD-1a2 (see Table 5b) contains 2'OMe modifications on both the sense and antisense strands. Experiments were performed to determine if the inherent stability (as measured by siRNA stability in human serum) or the degree or type of chemical modification (2'OMe versus 2'F or a mixture) was related to either the observed rat efficacy or the duration of silencing effects. Stability of siRNAs with the same AD-1a2 core sequence, but containing different chemical modifications were created and tested for activity in vitro in primary Cyno monkey hepatocytes. A series of these molecules that maintained similar activity as measured by in vitro IC50 values for PCSK9 silencing (Table 5b), were then tested for their stability against exo and endonuclease cleavage in human serum. Each duplex was incubated in human serum at 37° C. (a time course), and subjected to HPLC analysis. The parent sequence AD-1a2 had a T½ of ~7 hours in pooled human serum. Sequences AD-1a3, AD-1a5, and AD-1a4, which were more heavily modified (see chemical modifications in Table 5) all had T½'s greater than 24 hours. To test whether the differences in chemical modification or stability resulted in changes in efficacy, AD-1a2, AD-1a3, AD-1a5, AD-1a4,

and an AD-control sequence were formulated and injected into rats. Blood was collected from animals at various days post-dose, and total cholesterol concentrations were measured. Previous experiments had shown a very tight correlation between the lowering of PCSK9 transcript levels and total cholesterol values in rats treated with LNP01-1a2 (FIG. **10**A). All four molecules were observed to decrease total cholesterol by ~60% day 2 post-dose (versus PBS or control siRNA), and all of the molecules had equal effects on total cholesterol levels displaying similar magnitude and duration profiles. There was no statistical difference in the magnitude of cholesterol lowering and the duration of effect demonstrated by these molecules, regardless of their different chemistries or stabilities in human serum.

Example 8

LNP01-1a2 and LNP01-3a1 Silence Human PCSK9 and Circulating Human PCSK9 Protein in Transgenic Mice

[0467] The efficacy of LNP01-1a2 (i.e., PCS-A2 or AD-10792) and another molecule, AD-3a1 (i.e., PCS-C2 or AD-9736) (which targets only human and monkey PCSK9 message), to silence the human PCSK9 gene was tested in vivo. A line of transgenic mice expressing human PCSK9 under the ApoE promoter was used (Lagace et al., *J. Clin Invest.* 116:2995-3005, 2006). Specific PCR reagents and antibodies were designed that detected the human but not the mouse transcripts and protein respectively. Cohorts of the humanized mice were injected with a single dose of LNP01-1a2 (a.k.a. LNP-PCS-A2) or LNP01-3a1 (a.k.a. LNP-PCS-C2), and 48 hours later both livers and blood were collected. A single dose of LNP01-1a2 or LNP01-3a1 was able to decrease the human PCSK9 transcript levels by >70% (FIG. **15**A), and this transcript down-regulation resulted in significantly lower levels of circulating human PCSK9 protein as measured by ELISA (FIG. **15**B). These results demonstrated that both siRNAs were capable of silencing the human transcript and subsequently reducing the amount of circulating plasma human PCSK9 protein.

Example 9

Secreted PCSK9 Levels are Regulated by Diet in NHP

[0468] In mice, PCSK9 mRNA levels are regulated by the transcription factor sterol regulatory element binding protein-2 and are reduced by fasting. In clinical practice, and standard NHP studies, blood collection and cholesterol levels are measured after an overnight fasting period. This is due in part to the potential for changes in circulating TGs to interfere with the calculation of LDLc values. Given the regulation of PCSK9 levels by fasting and feeding behavior in mice, experiments were performed to understand the effect of fasting and feeding in NHP.

[0469] Cyno monkeys were acclimated to a twice daily feeding schedule during which food was removed after a one hour period. Animals were fed from 9-10 am in the morning, after which food was removed. The animals were next fed once again for an hour between 5 pm-6 pm with subsequent food removal. Blood was drawn after an overnight fast (6 pm until 9 am the next morning), and again, 2 and 4 hours following the 9 am feeding. PCSK9 levels in blood plasma or serum were determined by ELISA assay (see Methods).

Interestingly, circulating PCSK9 levels were found to be higher after the overnight fasting and decreased 2 and 4 hours after feeding. This data was consistent with rodent models where PCSK9 levels were highly regulated by food intake. However, unexpectedly, the levels of PCSK9 went down the first few hours post-feeding. This result enabled a more carefully designed NHP experiment to probe the efficacy of formulated AD-1a2 and another PCSK9 siRNA (AD-2a1) that was highly active in primary Cyno hepatocytes.

Example 10

PCSK9 siRNAs Reduce Circulating LDLc, ApoB, and PCSK9, but not HDLc in Non-Human Primates (NHPs)

[0470] siRNAs targeting PCSK9 acutely lowered both PCSK9 and total cholesterol levels by 72 hours post-dose and lasted ~21-30 days after a single dose in mice and rats. To extend these findings to a species whose lipoprotein profiles most closely mimic that of humans, further experiments were performed in the Cynomologous (Cyno) monkey model.

[0471] siRNA 1 (LNP01-10792) and siRNA 2 (LNP-01-9680), both targeting PCSK9 were administered to cynomologous monkeys. As shown in FIG. 12, both siRNAs caused significant lipid lowering for up to 7 days post administration. siRNA 2 caused ~50% lipid lowering for at least 7 days post-administration, and ~60% lipid lowering at day 14 post-administration, and siRNA 1 caused ~60% LDLc lowering for at least 7 days.

[0472] Male Cynos were first pre-screened for those that had LDLc of 40 mg/dl or higher. Chosen animals were then put on a fasted/fed diet regime and acclimated for 11 days. At day −3 and −1 pre-dose, serum was drawn at both fasted and 4 hours post-fed time points and analyzed for total cholesterol (Tc), LDL (LDLc), HDL cholesterol (HDLc) as well as triglycerides (TG), and PCSK9 plasma levels. Animals were randomized based on their day −3 LDLc levels. On the day of dosing (designated day 1), either 1 mg/kg or 5 mg/kg of LNP01-1a2 and 5 mg/kg LNP01-2a1 were injected, along with PBS and 1 mg/kg LNP01-ctrl as controls. All doses were well tolerated with no in-life findings. As the experiment progressed it became apparent (based on LDLc lowering) that the lower dose was not efficacious. We therefore dosed the PBS group animals on day 14 with 5 mg/kg LNP01-ctrl control siRNA, which could then serve as an additional control for the high dose groups of 5 mg/kg LNP01-1a2 and 5 mg/kg LNP01-2a1. Initially blood was drawn from animals on days 3, 4, 5, and 7 post-dose and Tc, HDLc, LDLc, and TGs concentrations were measured. Additional blood draws from the LNP01-1a2, LNP01-2a1 high dose groups were carried out at day 14 and day 21 post-dose (as the LDLc levels had not returned to baseline by day 7).

[0473] As shown in FIG. 12A, a single dose of LNP01-1a2 or LNP01-2a1 resulted in a statistically significant reduction of LDLc beginning at day 3 post-dose that returned to baseline over ~14 days (for LNP01-1a2) and ~21 days (LNP01-2a1). This effect was not seen in either the PBS, the control siRNA groups, or the 1 mg/kg treatment groups. LNP01-2a1 resulted in an average lowering of LDLc of 56% 72 hours post-dose, with 1 of 4 animals achieving nearly 70% LDLc, and all others achieving >50% LDLc decrease,

as compared to pre-dose levels, (see FIG. 12A. As expected, the lowering of LDLc in the treated animals also correlated with a reduction of circulating ApoB levels as measured by serum ELISA (FIG. 12B). Interestingly, the degree of LDLc lowering observed in this study of Cyno monkey was greater than those that have been reported for high dose statins, as well as, for other current standard of care compounds used for hypercholesterolemia. The onset of action is also much more acute than that of statins with effects being seen as early as 48 hours post-dose.

[0474] Neither LNP01-1a2 nor LNP01-2a1 treatments resulted in a lowering of HDLc. In fact, both molecules resulted (on average) in a trend towards a decreased Tc/HDL ratio (FIG. 12C). In addition, circulating triglyceride levels, and with the exception of one animal, ALT and AST levels were not significantly impacted.

[0475] PCSK9 protein levels were also measured in treated and control animals. As shown in FIG. 11, LNP01-1a2 and LNP01-2a1 treatment each resulted in trends toward decreased circulating PCSK9 protein levels versus pre-dose. Specifically, the more active siRNA LNP01-2a1 demonstrated significant reduction of circulating PCSK9 protein versus both PBS (day 3-21) and LNP01-ctrl siRNA control (day 4, day 7).

Example 11

Modified siRNA and Activation of Immune Responses in hPBMCs

[0476] siRNAs were tested for activation of the immune system in primary human blood monocytes (hPBMC). Two control inducing sequences and the unmodified parental compound AD-1a1 was found to induce both IFN-alpha and TNF-alpha. However, chemically modified versions of this sequence (AD-1a2, AD-1a3, AD-1a5, and AD-1a4) as well as AD-2a1 were negative for both IFN-alpha and TNF-alpha induction in these same assays (see Table 5, and FIGS. 13A and 13B). Thus chemical modifications are capable of dampening both IFN-alpha and TNF-alpha responses to siRNA molecules. In addition, neither AD-1a2, nor AD-2a1 activated IFN-alpha when formulated into liposomes and tested in mice.

Example 12

Evaluation of siRNA Conjugates in Mice

[0477] AD-10792 was conjugated to GalNAc)3/Cholesterol (FIG. 16) or GalNAc)3/LCO (FIG. 17). The sense strand was synthesized with the conjugate on the 3' end. The conjugated siRNAs were assayed for effects on PCSK9 transcript levels and total serum cholesterol in mice using the methods described below.

[0478] Briefly, mice were dosed via tail injection with one of the 2 conjugated siRNAs or PBS on three consecutive days: day 0, day 1 and day 2 with a dosage of about 100, 50, 25 or 12.5 mg/kg. Each dosage group included 6 mice. 24 hour post last dosing mice were sacrificed and blood and liver samples were obtained, stored, and processed to determine PCSK9 mRNA levels and total serum cholesterol.

[0479] The results are shown in FIG. **18**. Compared to control PBS, both siRNA conjugates demonstrated activity with an ED50 of 3×50 mg/kg for GalNAc)3/Cholesterol conjugated AD-10792 and 3×100 mg/kg for GalNAc)3/LCO conjugated AD-10792. The results indicate that Cholesterol conjugated siRNA with GalNAc are active and capable of silencing PCSK9 in the liver resulting in cholesterol lowering.

[0480] Bolus Dosing

[0481] Bolus dosing of formulated siRNAs in C57/BL6 mice (6/group, 8-10 weeks old, Charles River Laboratories, MA) was performed by tail vein injection using a 27 G needle. SiRNAs were formulated in LNP-01 (and then dialyzed against PBS) and diluted with PBS to concentrations 1.0, 0.5, 0.25 and 0.125 mg/ml allowing the delivery of 100; 50; 25 and 12.5 mg/kg doses in 10 µl/g body weight. Mice were kept under an infrared lamp for approximately 3 min prior to dosing to ease injection.

[0482] 24 hour post last dose mice were sacrificed by CO_2-asphyxiation. 0.2 ml blood was collected by retro-orbital bleeding and the liver was harvested and frozen in liquid nitrogen. Serum and livers were stored at −80° C. Frozen livers were grinded using 6850 Freezer/Mill Cryogenic Grinder (SPEX CentriPrep, Inc) and powders stored at −80° C. until analysis.

[0483] PCSK9 mRNA levels were detected using the branched-DNA technology based kit from QuantiGene Reagent System (Panomics, USA) according to the protocol. 10-20 mg of frozen liver powders was lysed in 600 µl of 0.16 µg/ml Proteinase K (Epicentre, #MPRK092) in Tissue and Cell Lysis Solution (Epicentre, #MTC096H) at 65° C. for 3 hours. Then 10 µl of the lysates were added to 90 µl of Lysis Working Reagent (1 volume of stock Lysis Mixture in two volumes of water) and incubated at 52° C. overnight on Genospectra capture plates with probe sets specific to mouse PCSK9 and mouse GAPDH. Probes sets for mouse PCSK9 and mouse GAPDH were purchased from Panomics, USA. Chemo luminescence was read on a Victor2-Light (Perkin Elmer) as Relative light units. The ratio of PCSK9 mRNA to mGAPDH mRNA in liver lysates was averaged over each treatment group and compared to a control group treated with PBS or a control group treated with an unrelated siRNA (blood coagulation factor VII).

[0484] Total serum cholesterol in mouse serum was measured using the Total Cholesterol Assay (Wako, USA) according to manufacturer's instructions. Measurements were taken on a Victor2 1420 Multilabel Counter (Perkin Elmer) at 600 nm.

Example 13

Evaluation of Lipid Formulated siRNAs in Rats

[0485] Briefly, rats were dosed via tail injection with SNALP formulated siRNAs or PBS with a single dosage of about 0.3, 1.0, and 3.0 mg/kg of SNALP formulated AD-10792. Each dosage group included 5 rats. 72 hour post dosing rats were sacrificed and blood and liver samples were obtained, stored, and processed to determine PCSK9 mRNA and total serum cholesterol levels. The results are shown in FIG. **19**. Compared to control PBS, SNALP formulated AD-10792 (FIG. **19**A) had an ED50 of about 1.0 mg/kg for both lowering of PCSK9 transcript levels and total serum cholesterol levels. These results show that administration of

SNALP formulated siRNA results in effective and efficient silencing of PCSK9 and subsequent lowering of total cholesterol in vivo.

[0486] Bolus Dosing

[0487] Bolus dosing of formulated siRNAs in Sprague-Dawley rats (5/group, 170-190 g body weight, Charles River Laboratories, MA) was performed by tail vein injection using a 27 G needle. SiRNAs were formulated in SNALP (and then dialyzed against PBS) and diluted with PBS to concentrations 0.066; 0.2 and 0.6 mg/ml allowing the delivery of 0.3; 1.0 and 3.0 mg/kg of SNALP formulated AD-10792 in 5 µl/g body weight. Rats were kept under an infrared lamp for approximately 3 min prior to dosing to ease injection.

[0488] 72 hour post last dose rats were sacrificed by CO_2-asphyxiation. 0.2 ml blood was collected by retro-orbital bleeding and the liver was harvested and frozen in liquid nitrogen. Serum and livers were stored at −80° C. Frozen livers were grinded using 6850 Freezer/Mill Cryogenic Grinder (SPEX CentriPrep, Inc) and powders stored at −80° C. until analysis.

[0489] PCSK9 mRNA levels were detected using the branched-DNA technology based kit from QuantiGene Reagent System (Panomics, USA) according to the protocol. 10-20 mg of frozen liver powders was lysed in 600 µl of 0.16 µg/ml Proteinase K (Epicentre, #MPRK092) in Tissue and Cell Lysis Solution (Epicentre, #MTC096H) at 65° C. for 3 hours. Then 10 µl of the lysates were added to 90 µl of Lysis Working Reagent (1 volume of stock Lysis Mixture in two volumes of water) and incubated at 52° C. overnight on Genospectra capture plates with probe sets specific to rat PCSK9 and rat GAPDH. Probes sets for rat PCSK9 and rat GAPDH were purchased from Panomics, USA. Chemo luminescence was read on a Victor2-Light (Perkin Elmer) as Relative light units. The ratio of rat PCSK9 mRNA to rat GAPDH mRNA in liver lysates was averaged over each treatment group and compared to a control group treated with PBS or a control group treated with an unrelated siRNA (blood coagulation factor VII).

[0490] Total serum cholesterol in rat serum was measured using the Total Cholesterol Assay (Wako, USA) according to manufacturer's instructions. Measurements were taken on a Victor2 1420 Multilabel Counter (Perkin Elmer) at 600 nm.

Example 14

In Vitro Efficacy Screen in HeLa Cells of Mismatch Walk of AD-9680 and AD-14676

[0491] The effects of variations in sequence or modification on the effectiveness of AD-9680, AD-14676, and AD-10792 were assayed in HeLa cells. A number of variants were synthesized as shown in Table 6 and include adding DFT (2,4-Difluorotoluyl, a thymidine triphosphate shape analog lacking Watson-Crick pairing); adding single or combination mismatches; and testing two different backbone chemistries: leading with a 2'-O methyl, or alternating with 2'F.

[0492] Sequences of the 3 parent duplexes can be found in Table 1a and are duplicated as follows:

target region	Sense strand (5' to 3')	SEQ ID NO:	Antisense strand (5' to 3')	SEQ ID NO:	Duplex
3530-3548	uucuAGAccuGuuuuGcuuTsT	1229	AAGcAAAAcAGGUCuAGAATsT	1230	AD-9680
3530-3548	UfuCfuAfgAfcCfuGfuUfuUfg CfuUfTsT	1231	P-aAfgCfaAfaAfcAfgGfuCfuAfgA faTsT	1232	AD-14676
1091-1109	GccuGGAGuuuAuucGGAATsT	459	UUCCGAAuAAACUCcAGGCTsT	460	AD-10792

[0493] HeLa were plated in 96-well plates (8,000-10,000 cells/well) in 100 μl 10% fetal bovine serum in Dulbecco's Modified Eagle Medium (DMEM). When the cells reached approximately 50% confluence (~24 hours later) they were transfected with serial four-fold dilutions of siRNA starting at 10 nM. 0.4 μl of transfection reagent Lipofectamine™ 2000 (Invitrogen Corporation, Carlsbad, Calif.) was used per well and transfections were performed according to the manufacturer's protocol. Namely, the siRNA: Lipofectamine™ 2000 complexes were prepared as follows. The appropriate amount of siRNA was diluted in Opti-MEM I Reduced Serum Medium without serum and mixed gently. The Lipofectamine™ 2000 was mixed gently before use, then for each well of a 96 well plate 0.4 μl was diluted in 25 μl of Opti-MEM I Reduced Serum Medium without serum and mixed gently and incubated for 5 minutes at room temperature. After the 5 minute incubation, 1 μl of the diluted siRNA was combined with the diluted Lipofectamine™ 2000 (total volume is 26.4 μl). The complex was mixed gently and incubated for 20 minutes at room temperature to allow the siRNA: Lipofectamine™ 2000 complexes to form. Then 100 μl of 10% fetal bovine serum in DMEM was added to each of the siRNA:Lipofectamine™ 2000 complexes and mixed gently by rocking the plate back and forth. 100 μl of the above mixture was added to each well containing the cells and the plates were incubated at 37° C. in a CO2 incubator for 24 hours, then the culture medium was removed and 100 μl 10% fetal bovine serum in DMEM was added.

[0494] 24 hours post medium change medium was removed, cells were lysed and cell lysates assayed for PCSK9 mRNA silencing by bDNA assay (Panomics, USA) following the manufacturer's protocol. Chemo luminescence was read on a Victor2-Light (Perkin Elmer) as Relative light units. The ratio of human PCSK9 mRNA to human GAPDH mRNA in cell lysates was compared to that of cells treated with Lipofectamine™ 2000 only control.

[0495] FIG. 20 is dose response curves of a series of compounds related to AD-9680. FIG. 21 is a dose response curve of a series of compounds related to AD-14676 The results show that DFTs or mismatches in certain positions are able increase the activity (as evidenced by lower IC50 values) of both parent compounds. FIG. 24 is a dose response curve comparing the efficiency of parent duplexes AD-9680 and AD-10792 with modified duplexes wherein a DFT is inserted at position 10 of the sense strand. This modification improves the efficiency by about 2 fold in HeLa cells.

[0496] Without being bound by theory, it is hypothesized that destabilization of the sense strand through the introduction of mismatches, or DFT might result in quicker removal of the sense strand.

Example 15

Lack of Off Target Effects in Hep3B Cells at High Concentrations

[0497] A lipid formulated PCSK9 targeted siRNA (AD-9680) was transfected into Hep3B cells at concentrations of 250 nM, 1 uM and 5 uM in triplicates using the reagent RNAiMAX (Invitrogen) according to the manufacture's instruction: 1ul of transfection reagent; reverse transfection protocol. Samples were collected 48 hrs post transfection. Total RNA was purified using MagMAX™-96 Total RNA Isolation Kit (Ambion); cDNA was synthesized with High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (ABI) from 13.5 μl of RNA prep; ABI Gene Expression Taqman assays were used; q-PCR reactions were set up according to manufacturer's instruction using Taq-Man® Gene Expression Master Mix (ABI) and run on ABI 7900 machine. Delta delta Ct method was used to calculate values. Samples were normalized to hGAPDH and calibrated to mock transfection.

[0498] Transcript levels were measured for the following genes having the closest homology to the target sequence: ORMDL2, BMP6, TAPT1, MYEF2, LOC442252, RFT1, and PCSK9.

[0499] The results are shown in FIG. 22. No off target effects were observed at high concentrations of dsRNA (PCS-B2=AD-9680).

AD-9680	S	1531	uucuAGAccuGuuuuGcuudTsdT
	AS	1532	AAGcAAAAcAGGUCuAGAAdTsdT

Example 16

Maintenance of Decrease in Total Cholesterol Levels in Rats by Lower Dosage of AD-10792

[0500] Rats were treated with 3 mg/kg bolus dose of SNALP-DlinDMA formulated AD-10792. At day 2, total serum cholesterol levels were determined. This was followed by once a week dosing at 1.0 and 0.3 mg/kg for four

weeks. Rats were bled one day prior to repeat dosing and total serum cholesterol levels were determined. The negative control was PBS.

[0501] The results are shown in the graph of FIG. **23**. After 3 mg/kg bolus dose, total cholesterol levels decreased by 60% and were maintained at about 50% by repeated once a week 1.0 and 0.3 mg/kg dosing and come back to pre dose levels after repeated dosing is stopped.

[0502] A 10 fold lower (than EC50), once a week, maintenance dose effectively maintains silencing with cholesterol levels returning to baseline by 15 days post last injection. The initial does of PCSK9 increased LDLR levels as reflected by the decrease in total serum cholesterol. This increase in LDLR levels increased the efficacy of the PCSK9 targeted siRNA as reflected by the lower dosage of subsequent administration AD-10792.

Example 17

Assay of Effects on Cholesterol Levels in Rats after Administration of Various Lipid Formulations of AD-10792

[0503] Rats were treated with four different lipid formulations of AD-10792 including SNALP and LNP08, described herein. At day 3, total serum cholesterol levels were determined. The experiment was performed using the methods described herein. Administration of LNP-08 formulated AD-10792 results in the lowest EC50 of 0.08 mg/kg compared to LNP01 formulated (EC50 of 2.0 mg/kg) and SNALP formulated (EC50 of 1.0 mg/kg). (data not shown).

Example 18

PCSK9 siRNA Filing Experiment

[0504] Bioinformatic Selection of PCSK9 Tiling Set

[0505] Sense and antisense oligomers were designed to target the human PCSK9 transcript in the flanking regions immediately upstream and downstream of the 19 base target region of ALN-PCSK9 (AD-9680). We used the NCBI Refseq NM_174936.2 as the reference human transcript for the PCSK9 gene. The antisense oligo of AD-9680 contains 19 contiguous bases complementary to the bases in the region of NM_174936 from positions 3530 through 3548 relative to the start of the mRNA. A set of siRNA molecules was designed to each unique 19mer of the subset of the transcript sequence defined by 10 bases upstream from the 5' end to 10 bases downstream from the 3' end of the target region of AD-9680. With respect to the NM_174936.2 transcript, the first base at the 5' position of the sense oligo 19mer extends from positions 3520 to positions 3558 (Tables 7 and 8).

[0506] Synthesis of PCSK9 Tiling Sequences:

[0507] PCSK9 sequences were synthesized on MerMade 192 synthesizer. Two sets of sequences were made. The first set contained no chemical modifications (unmodified) and a second set was made with endolight chemical modifications. In sequences containing endolight chemical modification, all pyrimidines (cytosine and uridine) in the sense strand were replaced with corresponding 2'-O-Methyl bases (2' 0-Methyl C and 2'-O-Methyl U). In the antisense strand, pyrimidines adjacent to (towards 5' position) ribo A nucleoside were replaced with their corresponding 2-O-Methyl nucleosides. A two base dTsdT extension at the 3' end of both sense and anti sense sequences was introduced. The sequence file was

converted to a text file to make it compatible for loading in the MerMade 192 synthesis software.

[0508] The synthesis of PCSK9 sequences used solid supported oligonucleotide synthesis using phosphoramidite chemistry. The synthesis of the above sequences was performed at 1 μm scale in 96 well plates. The amidite solutions were prepared at 0.1 M concentration and ethyl thio tetrazole (0.6M in Acetonitrile) was used as activator. The synthesized sequences were cleaved and deprotected in 96 well plates, using methylamine in the first step and Fluoride ion in the second step. The crude sequences thus obtained were precipitated using acetone: ethanol mix and the pellet were re-suspended in 0.2M sodium acetate buffer. Samples from each sequence were analyzed by LC-MS and the resulting mass data confirmed the identity of the sequences. A selected set of samples were also analyzed by IEX chromatography. All sequences were purified on AKTA explorer purification system using Source 15Q column. A single peak corresponding to the full length sequence was collected in the eluent and was subsequently analyzed for purity by ion exchange chromatography. The purified sequences were desalted on a Sephadex G25 column using AKTA purifier. The desalted PCSK9 sequences were analyzed for concentration and purity. The single strands were then submitted for annealing.

[0509] In Vitro Screening of PCSK9 Tiling siRNAs:

[0510] Cell Culture and Transfection:

[0511] Hela cells (ATCC, Manassas, Va.) were grown to near confluence at 37° C. in an atmosphere of 5% CO_2 in Eagle's Minimum Essential Medium (EMEM, ATCC) supplemented with 10% FBS, streptomycin, and glutamine (ATCC) before being released from the plate by trypsinization. Reverse transfection was carried out by adding 5 μl of Opti-MEM to 5 μl of siRNA duplexes per well into a 96-well plate along with 10 μl of Opti-MEM plus 0.2 μl of Lipofectamine RNAiMax per well (Invitrogen, Carlsbad Calif. cat #13778-150) and incubated at room temperature for 15 minutes. 80 μl of complete growth media without antibiotic containing 2.0×10^4 Hela cells were then added. Cells were incubated for 24 hours prior to RNA purification. Experiments were performed at 0.1 or 10 nM final duplex concentration. For dose response screens, HeLa cells were transfected with siRNAs over seven, ten-fold serial dilutions from 1 nM to 1 fM.

[0512] Total RNA was isolated using MagMAX-96 Total RNA Isolation Kit (Applied Biosystem, Forer City Calif., part #: AM1830). Cells were harvested and lysed in 140 μl of Lysis/Binding Solution then mixed for 1 minute at 850 rpm using and Eppendorf Thermomixer (the mixing speed was the same throughout the process). Twenty micro liters of magnetic beads and Lysis/Binding Enhancer mixture were added into cell-lysate and mixed for 5 minutes. Magnetic beads were captured using magnetic stand and the supernatant was removed without disturbing the beads. After removing supernatant, magnetic beads were washed with Wash Solution 1 (isopropanol added) and mixed for 1 minute. Beads were capture again and supernatant removed. Beads were then washed with 150 μl Wash Solution 2 (Ethanol added), captured and supernatant was removed. 50 μl of DNase mixture (MagMax turbo DNase Buffer and Turbo DNase) was then added to the beads and they were mixed for 10 to 15 minutes. After mixing, 100 μl of RNA Rebinding Solution was added and mixed for 3 minutes. Supernatant was removed and magnetic beads were washed again with 150 μl Wash Solution 2 and mixed for 1 minute and

supernatant was removed completely. The magnetic beads were mixed for 2 minutes to dry before RNA was eluted with 50 μl of water.

[0513] cDNA was synthesized using ABI High capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, Calif., Cat #4368813). A master mix of 2 μl 10× Buffer, 0.8 μl 25×dNTPs, 2 μl Random primers, 1 μl Reverse Transcriptase, 1 μl RNase inhibitor and 3.2 μl of H_2O per reaction were added into 10 μl total RNA. cDNA was generated using a Bio-Rad C-1000 or S-1000 thermal cycler (Hercules, Calif.) through the following steps: 25° C. 10 min, 37° C. 120 min, 85° C. 5 sec, 4° C. hold.

[0514] Real time PCR was performed as follows. 2 μl of cDNA were added to a master mix containing 1 μl GAPDH TaqMan Probe (Applied Biosystems Cat #4326317E), 1 μl PCSK9 TaqMan probe (Applied Biosystems cat # HS03037355_M1) and 10 μl Roche Probes Master Mix

Example 19

ApoE3-Based Reconstituted HDL Complexed with dsRNAs Targeting PCSK9

[0520] C57BL6 mice were administered 30 mg/kg rEHDL/chol-siPCSK9 by intravenous administration (tail vein injection) in a single bolus dose.

[0521] Chol-siPCSK9 (dsRNA Duplex AD-20583) has the following sequence:

```
Sense:
                              (SEQ ID NO: 1729)
GccuGGAGuuuAuucGGAAdTsdTL10

Antisense:
                              (SEQ ID NO:1730)
PuUfcCfgAfaUfaAfaCfuCfcAfgGfcdTsdT
```

[0522] The structure of L10 is:

$C_{39}H_{65}N_2O_5$
Exact Mass: 641.4893
Mol. Wt.: 641.9438
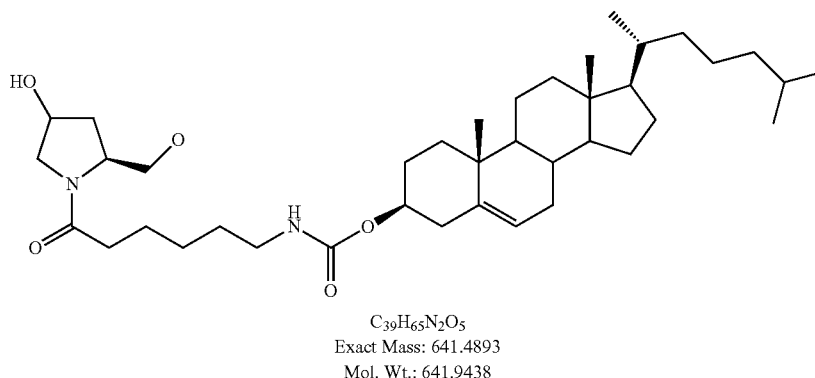
(Roche Cat #04887301001) per well in a LightCycler 480 384 well plate (Roche cat #0472974001). Real time PCR was done in a LightCycler 480 Real Time PCR machine (Roche). Each duplex was tested in two independent transfections and each transfections was assayed in duplicate.

[0515] Real time data were analyzed using the ΔΔ Ct method. Each sample was normalized to GAPDH expression and knockdown was assessed relative to cells transfected with the non-targeting duplex AD-1955. IC50s were defined using a 4 parameter fit model in XLfit.

[0516] The data for the single dose experiments are shown in Table 9. Data are expressed as the percent of message remaining relative to cells targeted with control AD-1955.

[0517] The data for the dose response screen is shown in Table 10. Data are expressed as dose in pM that results in 50% inhibition relative to AD-1955. Each dose response was repeated twice (Rep1 and Rep2). Average of the IC50s generated in the two dose response screens is shown.

[0518] The average IC50 for siRNA flanking AD-9680 was plotted vs. the starting position of the target region in the human PCSK9 transcript FIG. 25.

[0519] Thus, targeting nucleotide region 3520-3555 of PCSK9 with an RNAi agent is highly effective at inhibiting PCSK9.

[0523] After injection, mice were fasted overnight (~14 hours), and then sacrificed at 48 h post-injection. mRNA levels from liver were determined by bDNA assay, and normalized to GAPDH mRNA levels.

[0524] Results

[0525] The results of the bDNA assays are shown in FIG. 26, which indicate that there was a significant reduction in PCSK9 following administration of rEHDL/chol-siPCSK9, but not following administration of uncomplexed siRNAs (chol-siPCSK9). rEHDL/chol-siPCSK9 decreased PCSK9 mRNA levels by about 80%.

Example 20

LNP-11 Formulated siRNA in Non-Human Primates (NHPs)

[0526] An siRNA targeting PCSK9 (AD-9680) was formulated in a LNP-11 formulation (described herein) and administered to cynomologous monkeys. Control was AD-1955. The lipid formulated siRNAs were administered via a 30 minute infusion on day 1 at dosages of 0.03, 0.1, 0.3, and 1.0 mg/kg. Control was administered at 1.0 mg/kg. On day 3, liver biopsies were performed for measurement of PCSK9 transcript. Blood samples were collected on days −3, −1, 3, 4, 5, 7, 9, 11, 12, 15, 22, 30, and 37 and PCSK9 protein levels and LDLc numbers and HDLc numbers were determined.

[0527] The results are shown in FIG. 27A, FIG. 27B, and FIG. 27C.

[0528] As shown in FIG. 27A and FIG. 27B, administration resulted in a rapid and durable dose dependent reduction in PCSK9 protein levels and resulted in >50% reduction in LDLc (LDL cholesterol) levels. These effects were very potent with ED50 dose levels between 30 and 100 micrograms per kilogram. As shown in FIG. 27C, administration resulted in no change in HDLc levels.

Example 21

Dose Response in Rats with LNP-09 Formulated PCSK9 dsRNA

[0529] The dsRNA AD-10792 (targeting rate PCSK9) was encapsulated in a XTC containing formulation, e.g., a LNP09 formulation. LNP09 formulation was XTC/DSPC/Cholesterol/PEG-DMG at a % mol ratio of 50/10/38.5/1.5 and a lipid:siRNA ratio of 10:1.

[0530] Formulations were injected via tail vein, single dose (DRC) into rats. Livers and plasma were harvested 72 hours post-injection (5 animals per group). PCSK9 transcript levels were measured via bDNA in livers prepared as manufacturer's protocol. GAPDH transcript levels were also measured and the PCSK9 to GAPDH ratios were normalized to those of PBS control and graphed. Total cholesterol was measured in serum using cholesterol kit from WAKO TX.

[0531] The results are shown in FIG. 29. With this formulation PCSK9 silencing and total cholesterol lowering in rats was achieved at doses <0.1 mg/kg. The ED_{50} for was 0.2 mg/kg for lowering PCSK9 mRNA and 0.2 mg/kg and 0.08 for lowering serum cholesterol.

Example 22

Treatment of Transgenic Mice with LNP-09 Formulated PCSK9 dsRNA

[0532] Transgenic mice that overexpress human CETP and ApoB100 (CETP/ApoB double humanized transgenic mice, Taconic Labs) more closely mimic the LDL/HDL ratios found in man.

[0533] CETP/ApoB double humanized transgenic mice were purchased from Taconic labs. Animals were injected through tail vein (single injection) of 5 mg/kg of LNP09 formulated AD-10792 (standard formulation procedure), or AD-1955 Luciferase control (4 animals per group). Livers and plasma were harvested 72 hours post-injection (5 animals per group) and liver PCSK9 mRNA, LDL particle, and HDL particle number were determined.

[0534] PCSK9 transcript levels were measured via bDNA in livers prepared according to manufacturer's protocol. GAPDH transcript levels were also measured and the PCSK9 to GAPDH ratios were graphed, normalized to those of PBS control. LDL and HDL particle numbers/concentration were measured by NMR (Liposciences Inc.) based on their SOP.

[0535] The results are shown in FIG. 30. Silencing of PCSK9 lowered LDL particle concentrations ~90%, while HDL levels were modestly lower (as compared to those treated animals treated with PBS controls). This demonstrates significant lowering of PCSK9 levels with subsequent LDLc lowering in these animals.

Example 23

Inhibition of PCSK9 Expression in Humans

[0536] A human subject is treated with a lipid formulated dsRNA targeted to a PCSK9 gene, described herein, to inhibit expression of the PCSK9 gene and lower cholesterol levels for an extended period of time following a single dose. In one embodiment, the lipid formulated dsRNA includes the lipid MC3.

[0537] A subject in need of treatment is selected or identified. The subject can be in need of LDL lowering, LDL lowering without lowering of HDL, ApoB lowering, or total cholesterol lowering. The identification of the subject can occur in a clinical setting, or elsewhere, e.g., in the subject's home through the subject's own use of a self-testing kit.

[0538] At time zero, a suitable first dose of an anti-PCSK9 siRNA is subcutaneously administered to the subject. The dsRNA is formulated as described herein. After a period of time following the first dose, e.g., 7 days, 14 days, and 21 days, the subject's condition is evaluated, e.g., by measuring LDL, ApoB, and/or total cholesterol levels. This measurement can be accompanied by a measurement of PCSK9 expression in said subject, and/or the products of the successful siRNA-targeting of PCSK9 mRNA. Other relevant criteria can also be measured. The number and strength of doses are adjusted according to the subject's needs.

[0539] After treatment, the subject's LDL, ApoB, or total cholesterol levels are lowered relative to the levels existing prior to the treatment, or relative to the levels measured in a similarly afflicted but untreated subject.

[0540] Those skilled in the art are familiar with methods and compositions in addition to those specifically set out in the present disclosure which will allow them to practice this invention to the full scope of the claims hereinafter appended.

TABLE 1a

dsRNA sequences targeted to PCSK9					
position in human access. # NM_174936	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense-strand sequence (5'-3')[1]	SEQ ID NO:	Duplex name
2-20	AGCGACGUCGAGGCGCUCATT	1	UGAGCGCCUCGACGUCGCUTT	2	AD-15220
15-33	CGCUCAUGGUUGCAGGCGGTT	3	CCGCCUGCAACCAUGAGCGTT	4	AD-15275

TABLE 1a-continued

dsRNA sequences targeted to PCSK9					
position in human access. # NM_174936	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense-strand sequence (5'-3')[1]	SEQ ID NO:	Duplex name
16-34	GCUCAUGGUUGCAGGCGGGTT	5	CCCGCCUGCAACCAUGAGCTT	6	AD-15301
30-48	GCGGGCGCCGCCGUUCAGUTT	7	ACUGAACGGCGGCGCCCGCTT	a	AD-15276
31-49	CGGGCGCCGCCGUUCAGUUTT	9	AACUGAACGGCGGCGCCCGTT	10	AD-15302
32-50	GGGCGCCGCCGUUCAGUUCTT	11	GAACUGAACGGCGGCGCCCTT	12	AD-15303
40-58	CCGUUCAGUUCAGGGUCUGTT	13	CAGACCCUGAACUGAACGGTT	14	AD-15221
43-61	UUCAGUUCAGGGUCUGAGCTT	15	GCUCAGACCCUGAACUGAATT	16	AD-15413
82-100	GUGAGACUGGCUCGGGCGGTT	17	CCGCCCGAGCCAGUCUCACTT	18	AD-15304
100-118	GGCCGGGACGCGUCGUUGCTT	19	GCAACGACGCGUCCCGGCCTT	20	AD-15305
101-119	GCCGGGACGCGUCGUUGCATT	21	UGCAACGACGCGUCCCGGCTT	22	AD-15306
102-120	CCGGGACGCGUCGUUGCAGTT	23	CUGCAACGACGCGUCCCGGTT	24	AD-15307
105-123	GGACGCGUCGUUGCAGCAGTT	25	CUGCUGCAACGACGCGUCCTT	26	AD-15277
135-153	UCCCAGCCAGGAUUCCGCGTsT	27	CGCGGAAUCCUGGCUGGGATsT	28	AD-9526
135-153	ucccAGccAGGAuuccGcGTsT	29	CGCGGAAUCCUGGCUGGGATsT	30	AD-9652
136-154	CCCAGCCAGGAUUCCGCGCTsT	31	GCGCGGAAUCCUGGCUGGGTsT	32	AD-9519
136-154	cccAGccAGGAuuccGcGcTsT	33	GCGCGGAAUCCUGGCUGGGTsT	34	AD-9645
138-156	CAGCCAGGAUUCCGCGCGCTsT	35	GCGCGCGGAAUCCUGGCUGTsT	36	AD-9523
138-156	cAGccAGGAuuccGcGcGcTsT	37	GCGCGCGGAAUCCUGGCUGTsT	38	AD-9649
185-203	AGCUCCUGCACAGUCCUCCTsT	39	GGAGGACUGUGCAGGAGCUTsT	40	AD-9569
185-203	AGcuccuGcAcAGuccuccTsT	41	GGAGGACUGUGcAGGAGCUTsT	42	AD-9695
205-223	CACCGCAAGGCUCAAGGCGTT	43	CGCCUUGAGCCUUGCGGUGTT	44	AD-15222
208-226	CGCAAGGCUCAAGGCGCCGTT	45	CGGCGCCUUGAGCCUUGCGTT	46	AD-15278
210-228	CAAGGCUCAAGGCGCCGCCTT	47	GGCGGCGCCUUGAGCCUUGTT	48	AD-15178
232-250	GUGGACCGCGCACGGCCUCTT	49	GAGGCCGUGCGCGGUCCACTT	50	AD-15308

TABLE 1a-continued

dsRNA sequences targeted to PCSK9

position in human access. # NM_174936	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense-strand sequence (5'-3')[1]	SEQ ID NO:	Duplex name
233-251	UGGACCGCGCACGGCCUCUTT	51	AGAGGCCGUGCGCGGUCCATT	52	AD-15223
234-252	GGACCGCGCACGGCCUCUATT	53	UAGAGGCCGUGCGCGGUCCTT	54	AD-15309
235-253	GACCGCGCACGGCCUCUAGTT	55	CUAGAGGCCGUGCGCGGUCTT	56	AD-15279
236-254	ACCGCGCACGGCCUCUAGGTT	57	CCUAGAGGCCGUGCGCGGUTT	58	AD-15194
237-255	CCGCGCACGGCCUCUAGGUTT	59	ACCUAGAGGCCGUGCGCGGTT	60	AD-15310
238-256	CGCGCACGGCCUCUAGGUCTT	61	GACCUAGAGGCCGUGCGCGTT	62	AD-15311
239-257	GCGCACGGCCUCUAGGUCUTT	63	AGACCUAGAGGCCGUGCGCTT	64	AD-15392
240-258	CGCACGGCCUCUAGGUCUCTT	65	GAGACCUAGAGGCCGUGCGTT	66	AD-15312
248-266	CUCUAGGUCUCCUCGCCAGTT	67	CUGGCGAGGAGACCUAGAGTT	68	AD-15313
249-267	UCUAGGUCUCCUCGCCAGGTT	69	CCUGGCGAGGAGACCUAGATT	70	AD-15280
250-268	CUAGGUCUCCUCGCCAGGATT	71	UCCUGGCGAGGAGACCUAGTT	72	AD-15267
252-270	AGGUCUCCUCGCCAGGACATT	73	UGUCCUGGCGAGGAGACCUTT	74	AD-15314
258-276	CCUCGCCAGGACAGCAACCTT	75	GGUUGCUGUCCUGGCGAGGTT	76	AD-15315
300-318	CGUCAGCUCCAGGCGGUCCTsT	77	GGACCGCCUGGAGCUGACGTsT	78	AD-9624
300-318	cGucAGcuccAGGcGGuccTsT	79	GGACCGCCUGGAGCUGACGTsT	80	AD-9750
301-319	GUCAGCUCCAGGCGGUCCUTsT	81	AGGACCGCCUGGAGCUGACTsT	82	AD-9623
301-319	GucAGcuccAGGcGGuccuTsT	83	AGGACCGCCUGGAGCUGACTsT	84	AD-9749
370-388	GGCGCCCGUGCGCAGGAGGTT	85	CCUCCUGCGCACGGGCGCCTT	86	AD-15384
408-426	GGAGCUGGUGCUAGCCUUGTsT	87	CAAGGCUAGCACCAGCUCCTsT	88	AD-9607
408-426	GGAGcuGGuGcuAGccuuGTsT	89	cAAGGCuAGcACcAGCUCCTsT	90	AD-9733
411-429	GCUGGUGCUAGCCUUGCGUTsT	91	ACGCAAGGCUAGCACCAGCTsT	92	AD-9524
411-429	GcuGGuGcuAGccuuGcGuTsT	93	ACGcAAGGCuAGcACcAGCTsT	94	AD-9650
412-430	CUGGUGCUAGCCUUGCGUUTsT	95	AACGCAAGGCUAGCACCAGTsT	96	AD-9520

TABLE 1a-continued

dsRNA sequences targeted to PCSK9

position in human access. # NM_174936	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense-strand sequence (5'-3')[1]	SEQ ID NO:	Duplex name
412-430	CUGGUGCUAGCCUUGCGUUTsT	97	AACGCAAGGCUAGCACCAGTsT	98	AD-9520
412-430	cuGGuGcuAGccuuGcGuuTsT	99	AACGcAAGGCuAGcACcAGTsT	100	AD-9646
416-434	UGCUAGCCUUGCGUUCCGATsT	101	UCGGAACGCAAGGCUAGCATsT	102	AD-9608
416-434	uGcuAGccuuGcGuuccGATsT	103	UCGGAACGcAAGGCuAGcATsT	104	AD-9734
419-437	UAGCCUUGCGUUCCGAGGATsT	105	UCCUCGGAACGCAAGGCUATsT	106	AD-9546
419-437	uAGc cuuGcGuuccGAGGATsT	107	UCCUCGGAACGcAAGGCuATsT	108	AD-9672
439-457	GACGGCCUGGCCGAAGCACTT	109	GUGCUUCGGCCAGGCCGUCTT	110	AD-15385
447-465	GGCCGAAGCACCCGAGCACTT	111	GUGCUCGGGUGCUUCGGCCTT	112	AD-15393
448-466	GCCGAAGCACCCGAGCACGTT	113	CGUGCUCGGGUGCUUCGGCTT	114	AD-15316
449-467	CCGAAGCACCCGAGCACGGTT	115	CCGUGCUCGGGUGCUUCGGTT	116	AD-15317
458-476	CCGAGCACGGAACCACAGCTT	117	GCUGUGGUUCCGUGCUCGGTT	118	AD-15318
484-502	CACCGCUGCGCCAAGGAUCTT	119	GAUCCUUGGCGCAGCGGUGTT	120	AD-15195
486-504	CCGCUGCGCCAAGGAUCCGTT	121	CGGAUCCUUGGCGCAGCGGTT	122	AD-15224
487-505	CGCUGCGCCAAGGAUCCGUTT	123	ACGGAUCCUUGGCGCAGCGTT	124	AD-15188
489-507	CUGCGCCAAGGAUCCGUGGTT	125	CCACGGAUCCUUGGCGCAGTT	126	AD-15225
500-518	AUCCGUGGAGGUUGCCUGGTT	127	CCAGGCAACCUCCACGGAUTT	128	AD-15281
509-527	GGUUGCCUGGCACCUACGUTT	129	ACGUAGGUGCCAGGCAACCTT	130	AD-15282
542-560	AGGAGACCCACCUCUCGCATT	131	UGCGAGAGGUGGGUCUCCUTT	132	AD-15319
543-561	GGAGACCCACCUCUCGCAGTT	133	CUGCGAGAGGUGGGUCUCCTT	134	AD-15226
544-562	GAGACCCACCUCUCGCAGUTT	135	ACUGCGAGAGGUGGGUCUCTT	136	AD-15271
549-567	CCACCUCUCGCAGUCAGAGTT	137	CUCUGACUGCGAGAGGUGGTT	138	AD-15283
552-570	CCUCUCGCAGUCAGAGCGCTT	139	GCGCUCUGACUGCGAGAGGTT	140	AD-15284
553-571	CUCUCGCAGUCAGAGCGCATT	141	UGCGCUCUGACUGCGAGAGTT	142	AD-15189

TABLE 1a-continued

dsRNA sequences targeted to PCSK9

position in human access. # NM_174936	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense-strand sequence (5'-3')[1]	SEQ ID NO:	Duplex name
554-572	UCUCGCAGUCAGAGCGCACUT	143	GUGCGCUCUGACUGCGAGATT	144	AD-15227
555-573	CUCGCAGUCAGAGCGCACUTsT	145	AGUGCGCUCUGACUGCGAGTsT	146	AD-9547
555-573	cucGcAGucAGAGcGcAcuTsT	147	AGUGCGCUCUGACUGCGAGTsT	148	AD-9673
558-576	GCAGUCAGAGCGCACUGCCTsT	149	GGCAGUGCGCUCUGACUGCTsT	150	AD-9548
558-576	GcAGucAGAGcGcAcuGccTsT	151	GGcAGUGCGCUCUGACUGCTsT	152	AD-9674
606-624	GGGAUACCUCACCAAGAUCTsT	153	GAUCUUGGUGAGGUAUCCCTsT	154	AD-9529
606-624	GGGAuAccucAccAAGAucTsT	155	GAUCUUGGUGAGGuAUCCCTsT	156	AD-9655
659-677	UGGUGAAGAUGAGUGGCGATsT	157	UCGCCACUCAUCUUCACCATsT	158	AD-9605
659-677	uGGuGAAGAuGAGuGGcGATsT	159	UCGCcACUcAUCUUcACcATsT	160	AD-9731
663-681	GAAGAUGAGUGGCGACCUGTsT	161	CAGGUCGCCACUCAUCUUCTsT	162	AD-9596
663-681	GAAGAuGAGuGGcGAccuGTsT	163	cAGGUCGCcACUcAUCUUCTsT	164	AD-9722
704-722	CCCAUGUCGACUACAUCGATsT	165	UCGAUGUAGUCGACAUGGGTsT	166	AD-9583
704-722	cccAuGucGAcuAcAucGATsT	167	UCGAUGuAGUCGAcAUGGGTsT	168	AD-9709
718-736	AUCGAGGAGGACUCCUCUGTsT	169	CAGAGGAGUCCUCCUCGAUTsT	170	AD-9579
718-736	AucGAGGAGGAcuccucuGTsT	171	cAGAGGAGUCCUCCUCGAUTsT	172	AD-9705
758-776	GGAACCUGGAGCGGAUUACTT	173	GUAAUCCGCUCCAGGUUCCTT	174	AD-15394
759-777	GAACCUGGAGCGGAUUACCTT	175	GGUAAUCCGCUCCAGGUUCTT	176	AD-15196
760-778	AACCUGGAGCGGAUUACCCTT	177	GGGUAAUCCGCUCCAGGUUTT	178	AD-15197
777-795	CCCUCCACGGUACCGGGCGTT	179	CGCCCGGUACCGUGGAGGGTT	180	AD-15198
782-800	CACGGUACCGGGCGGAUGATsT	181	UCAUCCGCCCGGUACCGUGTsT	182	AD-9609
782-800	cAcGGuAccGGGcGGAuGATsT	183	UcAUCCGCCCGGuACCGUGTsT	184	AD-9735
783-801	ACGGUACCGGGCGGAUGAATsT	185	UUCAUCCGCCCGGUACCGUTsT	186	AD-9537
783-801	AcGGuAccGGGcGGAuGAATsT	187	UUcAUCCGCCCGGuACCGUTsT	188	AD-9663

TABLE 1a-continued

		dsRNA sequences targeted to PCSK9			

position in human access. # NM_174936	Sense strand sequence (5'-3')¹	SEQ ID NO:	Antisense- strand sequence (5'-3')¹	SEQ ID NO:	Duplex name
784-802	CGGUACCGGGCGGAUGAAUTsT	189	AUUCAUCCGCCCGGUACCGTsT	190	AD-9528
784-802	cGGuAccGGGcGGAuGAAuTsT	191	AUUcAUCCGCCCGGuACCGTsT	192	AD-9654
785-803	GGUACCGGGCGGAUGAAUATsT	193	UAUUCAUCCGCCCGGUACCTsT	194	AD-9515
785-803	GGuAccGGGcGGAuGAAuATsT	195	uAUUcAUCCGCCCGGuACCTsT	196	AD-9641
786-804	GUACCGGGCGGAUGAAUACTsT	197	GUAUUCAUCCGCCCGGUACTsT	198	AD-9514
786-804	GuAccGGGcGGAuGAAuAcTsT	199	GuAUUcAUCCGCCCGGuACTsT	200	AD-9640
788-806	ACCGGGCGGAUGAAUACCATsT	201	UGGUAUUCAUCCGCCCGGUTsT	202	AD-9530
788-806	AccGGGcGGAuGAAuAccATsT	203	UGGuAUUcAUCCGCCCGGUTsT	204	AD-9656
789-807	CCGGGCGGAUGAAUACCAGTsT	205	CUGGUAUUCAUCCGCCCGGTsT	206	AD-9538
789-807	ccGGGcGGAuGAAuAccAGTsT	207	CUGGuAUUcAUCCGCCCGGTsT	208	AD-9664
825-843	CCUGGUGGAGGUGUAUCUCTsT	209	GAGAUACACCUCCACCAGGTsT	210	AD-9598
825-843	ccuGGuGGAGGuGuAucucTsT	211	GAGAuAcACCUCcAccAGGTsT	212	AD-9724
826-844	CUGGUGGAGGUGUAUCUCCTsT	213	GGAGAUACACCUCCACCAGTsT	214	AD-9625
826-844	cuGGuGGAGGuGuAucuccTsT	215	GGAGAuAcACCUCcAccAGTsT	216	AD-9751
827-845	UGGUGGAGGUGUAUCUCCUTsT	217	AGGAGAUACACCUCCACCATsT	218	AD-9556
827-845	uGGuGGAGGuGuAucuccuTsT	219	AGGAGAuAcACCUCcAccATsT	220	AD-9682
828-846	GGUGGAGGUGUAUCUCCUATsT	221	UAGGAGAUACACCUCCACCTsT	222	AD-9539
828-846	GGuGGAGGuGuAucuccuATsT	223	uAGGAGAuAcACCUCcACCTsT	224	AD-9665
831-849	GGAGGUGUAUCUCCUAGACTsT	225	GUCUAGGAGAUACACCUCCTsT	226	AD-9517
831-849	GGAGGuGuAucuccuAGAcTsT	227	GUCuAGGAGAuAcACCUCCTsT	228	AD-9643
833-851	AGGUGUAUCUCCUAGACACTsT	229	GUGUCUAGGAGAUACACCUTsT	230	AD-9610
833-851	AGGuGuAucuccuAGAcAcTsT	231	GUGUCuAGGAGAuAcACCUTsT	232	AD-9736
833-851	AfgGfuGfuAfuCfuCfcUfaGfaCfaC fTsT	233	p- gUfgUfcUfaGfgAfgAfuAfcAfcCfuTsT	234	AD-14681

TABLE 1a-continued

dsRNA sequences targeted to PCSK9

position in human access. # NM_174936	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense-strand sequence (5'-3')[1]	SEQ ID NO:	Duplex name
833-851	AGGUfGUfAUfCfUfCfCfUfAGACfAC fTsT	235	GUfGUfCfUfAGGAGAUfACfACfCfUfTsT	236	AD-14691
833-851	AgGuGuAuCuCcUaGaCaCTsT	237	p-gUfgUfcUfaGfgAfgAfuAfcAfcCfuTsT	238	AD-14701
833-851	AgGuGuAuCuCcUaGaCaCTsT	239	GUfGUfCfUfAGGAGAUfACfACfCfUfTsT	240	AD-14711
833-851	AfgGfuGfuAfuCfuCfcUfaGfaCfaC fTsT	241	GUGUCuaGGagAUACAccuTsT	242	AD-14721
833-851	AGGUfGUfAUfCfUfCfCfUfAGACfAC fTsT	243	GUGUCuaGGagAUACAccuTsT	244	AD-14731
833-851	AgGuGuAuCuCcUaGaCaCTsT	245	GUGUCuaGGagAUACAccuTsT	246	AD-14741
833-851	GfcAfcCfcUfcAfuAfgGfcCfuGfgA fTsT	247	p-uCfcAfgGfccCfuAfuGfaGfgGfuGfcTsT	248	AD-15087
833-851	GCfACfCfCfUfCfAUfAGGCfCfUfGG ATsT	249	UfCfCfAGGCfCfUfAUfGAGGGUfGCfTsT	250	AD-15097
833-851	GcAcCcUcAuAgGcCuGgATsT	251	p-uCfcAfgGfccCfuAfuGfaGfgGfuGfcTsT	252	AD-15107
833-851	GcAcCcUcAuAgGcCuGgATsT	253	UfCfCfAGGCfCfUfAUfGAGGGUfGCfTsT	254	AD-15117
833-851	GfcAfcCfcUfcAfuAfgGfcCfuGfgA fTsT	255	UCCAGgcCUauGAGGGugcTsT	256	AD-15127
833-851	GCfACfCfCfUfCfAUfAGGCfCfUfGG ATsT	257	UCCAGgcCUauGAGGGugcTsT	258	AD-15137
833-851	GcAcCcUcAuAgGcCuGgATsT	259	UCCAGgcCUauGAGGGugcTsT	260	AD-15147
836-854	UGUAUCUCCUAGACACCAGTsT	261	CUGGUGUCUAGGAGAUACATsT	262	AD-9516
836-854	uGuAucuccuAGAcAccAGTsT	263	CUGGUGUCUaGGAGAuAcATsT	264	AD-9642
840-858	UCUCCUAGACACCAGCAUATsT	265	UAUGCUGGUGUCUAGGAGATsT	266	AD-9562
840-858	ucuccuAGAcAccAGcAuATsT	267	uAUGCUGGUGUCUaGGAGATsT	268	AD-9688
840-858	UfcUfcCfuAfgAfcAfcCfaGfcAfuA fTsT	269	p-uAfuGfcUfgGfuGfuCfuAfgGfaGfaTsT	270	AD-14677
840-858	UfCfUfCfCfUfAGACfACfCfAGCfAU fATsT	271	UfAUfGCfUfGGUfGUfCfUfAGGAGATsT	272	AD-14687
840-858	UcUcCuAgAcAcCaGcAuATsT	273	p-uAfuGfcUfgGfuGfuCfuAfgGfaGfaTsT	274	AD-14697
840-858	UcUcCuAgAcAcCaGcAuATsT	275	UfAUfGCfUfGGUfGUfCfUfAGGAGATsT	276	AD-14707
840-858	UfcUfcCfuAafAfcAfcCfaGfcAfuA fTsT	277	UAUGCugGUguCUAGGagaTsT	278	AD-14717
840-858	UfCfUfCfCfUfAGACfACfCfAGCfAU fATsT	279	UAUGCugGUguCUAGGagaTsT	280	AD-14727

TABLE 1a-continued

dsRNA sequences targeted to PCSK9

position in human access. # NM_174936	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense-strand sequence (5'-3')[1]	SEQ ID NO:	Duplex name
840-858	UcUcCuAgAcAcCaGcAuATsT	281	UAUGCugGUguCUAGGagaTsT	282	AD-14737
840-858	AfgGfcCfuGfgAfgUfuUfaUfuCfgGfTsT	283	p-cCfgAfaUfaAfaCfuCfcAfgGfcCfuTsT	284	AD-15083
840-858	AGGCfCfUfGGAGUfUfUfAUfUfCfGGTsT	285	CfCfGAAUfAAACfUfCfCfAGGCfCfUfTsT	286	AD-15093
840-858	AgGcCuGgAgUuUaUuCgGTsT	287	p-cCfgAfaUfaAfaCfuCfcAfgGfcCfuTsT	288	AD-15103
840-858	AgGcCuGgAgUuUaUuCgGTsT	289	CfCfGAAUfAAACfUfCfCfAGGCfCfUfTsT	290	AD-15113
840-858	AfgGfcCfuGfgAfgUfuUfaUfuCfgGfTsT	291	CCGAAuaAAcuCCAGGccuTsT	292	AD-15123
840-858	AGGCfCfUfGGAGUfUfUfAUfUfCfGGTsT	293	CCGAAuaAAcuCCAGGccuTsT	294	AD-15133
840-858	AgGcCuGgAgUuUaUuCgGTsT	295	CCGAAuaAAcuCCAGGccuTsT	296	AD-15143
841-859	CUCCUAGACACCAGCAUACTsT	297	GUAUGCUGGUGUCUAGGAGTsT	298	AD-9521
841-859	cuccuAGAcAccAGcAuAcTsT	299	GuAUGCUGGUGUCuAGGAGTsT	300	AD-9647
842-860	UCCUAGACACCAGCAUACATsT	301	UGUAUGCUGGUGUCUAGGATsT	302	AD-9611
842-860	uccuAGAcAccAGcAuAcATsT	303	UGuAUGCUGGUGUCuAGGATsT	304	AD-9737
843-861	CCUAGACACCAGCAUACAGTsT	305	CUGUAUGCUGGUGUCUAGGTsT	306	AD-9592
843-861	ccuAGAcAccAGcAuAcAGTsT	307	CUGuAUGCUGGUGUCuAGGTsT	308	AD-9718
847-865	GACACCAGCAUACAGAGUGTsT	309	CACUCUGUAUGCUGGUGUCTsT	310	AD-9561
847-865	GAcAccAGcAuAcAGAGuGTsT	311	cACUCUGuAUGCUGGUGUCTsT	312	AD-9687
855-873	CAUACAGAGUGACCACCGGTsT	313	CCGGUGGUCACUCUGUAUGTsT	314	AD-9636
855-873	cAuAcAGAGuGAccAccGGTsT	315	CCGGUGGUcACUCUGuAUGTsT	316	AD-9762
860-878	AGAGUGACCACCGGGAAAUTsT	317	AUUUCCCGGUGGUCACUCUTsT	318	AD-9540
860-878	AGAGuGAccAccGGGAAAuTsT	319	AUUUCCCGGUGGUcACUCUTsT	320	AD-9666
861-879	GAGUGACCACCGGGAAAUCTsT	321	GAUUUCCCGGUGGUCACUCTsT	322	AD-9535
861-879	GAGuGAccAccGGGAAAucTsT	323	GAUUUCCCGGUGGUcACUCTsT	324	AD-9661
863-881	GUGACCACCGGGAAAUCGATsT	325	UCGAUUUCCCGGUGGUCACTsT	326	AD-9559

TABLE 1a-continued

	dsRNA sequences targeted to PCSK9			
position in human access. # NM_174936	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense- strand sequence (5'-3')[1]	SEQ ID NO:	Duplex name
863-881	GuGAccAccGGGAAAucGATsT	327	UCGAUUUCCCGGUGGUcACTsT	328	AD-9685
865-883	GACCACCGGGAAAUCGAGGTsT	329	CCUCGAUUUCCCGGUGGUCTsT	330	AD-9533
865-883	GAccAccGGGAAAucGAGGTsT	331	CCUCGAUUUCCCGGUGGUCTsT	332	AD-9659
866-884	ACCACCGGGAAAUCGAGGGTsT	333	CCCUCGAUUUCCCGGUGGUTsT	334	AD-9612
866-884	AccAccGGGAAAucGAGGGTsT	335	CCCUCGAUUUCCCGGUGGUTsT	336	AD-9738
867-885	CCACCGGGAAAUCGAGGGCTsT	337	GCCCUCGAUUUCCCGGUGGTsT	338	AD-9557
867-885	ccAccGGGAAAucGAGGGcTsT	339	GCCCUCGAUUUCCCGGUGGTsT	340	AD-9683
875-893	AAAUCGAGGGCAGGGUCAUTsT	341	AUGACCCUGCCCUCGAUUUTsT	342	AD-9531
875-893	AAAucGAGGGcAGGGucAuTsT	343	AUGACCCUGCCCUCGAUUUTsT	344	AD-9657
875-893	AfaAfuCfgAfgGfgCfaGfgGfuCfaUfTsT	345	p-aUfgAfcCfcUfgCfcCfuCfgAfuUfuTsT	346	AD-14673
875-893	AAAUfCfGAGGGCfAGGGUfCfAUfTsT	347	AUfGACfCfCfUfGCfCfCfUfCfGAUfUfUfTsT	348	AD-14683
875-893	AaAuCgAgGgCaGgGuCaUTsT	349	p-aUfgAfcCfcUfgCfcCfuCfgAfuUfuTsT	350	AD-14693
875-893	AaAuCgAgGgCaGgGuCaUfTsT	351	AUfGACfCfCfUfGCfCfCfUfCfGAUfUfUfU	352	AD-14703
875-893	AfaAfuCfgAfgGfgCfaGfgGfuCfaUfTsT	353	AUGACccUGccCUCGAuuuTsT	354	AD-14713
875-893	AAAUfCfGAGGGCfAGGGUfCfAUfTsT	355	AUGACccUGccCUCGAuuuTsT	356	AD-14723
875-893	AaAuCgAgGgCaGgGuCaUTsT	357	AUGACccUGccCUCGAuuuTsT	358	AD-14733
875-893	CfgGfcAfcCfcUfcAfuAfgGfcCfuGfTsT	359	p-cAfgGfcCfuAfuGfaGfgGfuGfcCfgTsT	360	AD-15079
875-893	CfGGCfACfCfCfUfCfAUfAGGCfCfUfGTsT	361	CfAGGCfCfUfAUfGAGGGUfGCfCfGTsT	362	AD-15089
875-893	CgGcAcCcUcAuAgGcCuGTsT	363	p-cAfgGfcCfuAfuGfaGfgGfuGfcCfgTsT	364	AD-15099
875-893	CgGcAcCcUcAuAgGcCuGTsT	365	CfAGGCfCfUfAUfGAGGGUfGCfCfGTsT	366	AD-15109
875-893	CfgGfcAfcCfcUfcAfuAfgGfcCfuGfTsT	367	CAGGCcuAUgaGGGUGccgTsT	368	AD-15119
875-893	CfGGCfACfCfCfUfCfAUfAGGCfCfUfGTsT	369	CAGGCcuAUgaGGGUGccgTsT	370	AD-15129
875-893	CgGcAcCcUcAuAgGcCuGTsT	371	CAGGCcuAUgaGGGUGccgTsT	372	AD-15139

TABLE 1a-continued

dsRNA sequences targeted to PCSK9

position in human access. # NM_174936	Sense strand sequence (5'-3')¹	SEQ ID NO:	Antisense-strand sequence (5'-3')¹	SEQ ID NO:	Duplex name
877-895	AUCGAGGGCAGGGUCAUGGTsT	373	CCAUGACCCUGCCCUCGAUTsT	374	AD-9542
877-895	AucGAGGGcAGGGucAuGGTsT	375	CcAUGACCCUGCCCUCGAUTsT	376	AD-9668
878-896	cGAGGGcAGGGucAuGGucTsT	377	GACcAUGACCCUGCCCUCGTsT	378	AD-9739
880-898	GAGGGCAGGGUCAUGGUCATsT	379	UGACCAUGACCCUGCCCUCTsT	380	AD-9637
880-898	GAGGGcAGGGucAuGGucATsT	381	UGACcAUGACCCUGCCCUCTsT	382	AD-9763
882-900	GGGCAGGGUCAUGGUCACCTsT	383	GGUGACCAUGACCCUGCCCTsT	384	AD-9630
882-900	GGGcAGGGucAuGGucAccTsT	385	GGUGACcAUGACCCUGCCCTsT	386	AD-9756
885-903	CAGGGUCAUGGUCACCGACTsT	387	GUCGGUGACCAUGACCCUGTsT	388	AD-9593
885-903	cAGGGucAuGGucAccGAcTsT	389	GUCGGUGACcAUGACCCUGTsT	390	AD-9719
886-904	AGGGUCAUGGUCACCGACUTsT	391	AGUCGGUGACCAUGACCCUTsT	392	AD-9601
886-904	AGGGucAuGGucAccGAcuTsT	393	AGUCGGUGACcAUGACCCUTsT	394	AD-9727
892-910	AUGGUCACCGACUUCGAGATsT	395	UCUCGAAGUCGGUGACCAUTsT	396	AD-9573
892-910	AuGGucAccGAcuucGAGATsT	397	UCUCGAAGUCGGUGACcAUTsT	398	AD-9699
899-917	CCGACUUCGAGAAUGUGCCTT	399	GGCACAUUCUCGAAGUCGGTT	400	AD-15228
921-939	GGAGGACGGGACCCGCUUCTT	401	GAAGCGGGUCCCGUCCUCCTT	402	AD-15395
993-1011	CAGCGGCCGGGAUGCCGGCTsT	403	GCCGGCAUCCCGGCCGCUGTsT	404	AD-9602
993-1011	cAGcGGccGGGAuGccGGcTsT	405	GCCGGcAUCCCGGCCGCUGTsT	406	AD-9728
1020-1038	GGGUGCCAGCAUGCGCAGCTT	407	GCUGCGCAUGCUGGCACCCTT	408	AD-15386
1038-1056	CCUGCGCGUGCUCAACUGCTsT	409	GCAGUUGAGCACGCGCAGGTsT	410	AD-9580
1038-1056	ccuGcGcGuGcucAAcuGcTsT	411	GcAGUUGAGcACGCGcAGGTsT	412	AD-9706
1040-1058	UGCGCGUGCUCAACUGCCATsT	413	UGGCAGUUGAGCACGCGCATsT	414	AD-9581
1040-1058	uGcGcGuGcucAAcuGccATsT	415	UGGCAGUUGAGcACGCGcATsT	416	AD-9707
1042-1060	CGCGUGCUCAACUGCCAAGTsT	417	CUUGGCAGUUGAGCACGCGTsT	418	AD-9543

TABLE 1a-continued

dsRNA sequences targeted to PCSK9

position in human access. # NM_174936	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense-strand sequence (5'-3')[1]	SEQ ID NO:	Duplex name
1042-1060	cGcGuGcucAAcuGccAAGTsT	419	CUUGGcAGUUGAGcACGCGTsT	420	AD-9669
1053-1071	CUGCCAAGGGAAGGGCACGTsT	421	CGUGCCCUUCCCUUGGCAGTsT	422	AD-9574
1053-1071	cuGccAAGGGAAGGGcAcGTsT	423	CGUGCCCUUCCCUUGGcAGTsT	424	AD-9700
1057-1075	CAAGGGAAGGGCACGGUUATT	425	UAACCGUGCCCUUCCCUUGTT	426	AD-15320
1058-1076	AAGGGAAGGGCACGGUUAGTT	427	CUAACCGUGCCCUUCCCUUTT	428	AD-15321
1059-1077	AGGGAAGGGCACGGUUAGCTT	429	GCUAACCGUGCCCUUCCCUTT	430	AD-15199
1060-1078	GGGAAGGGCACGGUUAGCGTT	431	CGCUAACCGUGCCCUUCCCTT	432	AD-15167
1061-1079	GGAAGGGCACGGUUAGCGGTT	433	CCGCUAACCGUGCCCUUCCTT	434	AD-15164
1062-1080	GAAGGGCACGGUUAGCGGCTT	435	GCCGCUAACCGUGCCCUUCTT	436	AD-15166
1063-1081	AAGGGCACGGUUAGCGGCATT	437	UGCCGCUAACCGUGCCCUUTT	438	AD-15322
1064-1082	AGGGCACGGUUAGCGGCACTT	439	GUGCCGCUAACCGUGCCCUTT	440	AD-15200
1068-1086	CACGGUUAGCGGCACCCUCTT	441	GAGGGUGCCGCUAACCGUGTT	442	AD-15213
1069-1087	ACGGUUAGCGGCACCCUCATT	443	UGAGGGUGCCGCUAACCGUTT	444	AD-15229
1072-1090	GUUAGCGGCACCCUCAUAGTT	445	CUAUGAGGGUGCCGCUAACTT	446	AD-15215
1073-1091	UUAGCGGCACCCUCAUAGGTT	447	CCUAUGAGGGUGCCGCUAATT	448	AD-15214
1076-1094	GCGGCACCCUCAUAGGCCUTsT	449	AGGCCUAUGAGGGUGCCGCTsT	450	AD-9315
1079-1097	GCACCCUCAUAGGCCUGGATsT	451	UCCAGGCCUAUGAGGGUGCTsT	452	AD-9326
1085-1103	UCAUAGGCCUGGAGUUUAUTsT	453	AUAAACUCCAGGCCUAUGATsT	454	AD-9318
1090-1108	GGCCUGGAGUUUAUUCGGATsT	455	UCCGAAUAAACUCCAGGCCTsT	456	AD-9323
1091-1109	GCCUGGAGUUUAUUCGGAATsT	457	UUCCGAAUAAACUCCAGGCTsT	458	AD-9314
1091-1109	GccuGGAGuuuAuucGGAATsT	459	UUCCGAAuAAACUCcAGGCTsT	460	AD-10792
1091-1109	GccuGGAGuuuAuucGGAATsT	461	UUCCGAAUAACUCCAGGCTsT	462	AD-10796
1093-1111	CUGGAGUUUAUUCGGAAAATsT	463	UUUUCCGAAUAAACUCCAGTsT	464	AD-9638

TABLE 1a-continued

dsRNA sequences targeted to PCSK9

position in human access. # NM_174936	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense-strand sequence (5'-3')[1]	SEQ ID NO:	Duplex name
1093-1111	cuGGAGuuuAuucGGAAAATsT	465	UUUUCCGAAuAAACUCcAGTsT	466	AD-9764
1095-1113	GGAGUUUAUUCGGAAAAGCTsT	467	GCUUUUCCGAAUAAACUCCTsT	468	AD-9525
1095-1113	GGAGuuuAuucGGAAAAGcTsT	469	GCUUUUCCGAAuAAACUCCTsT	470	AD-9651
1096-1114	GAGUUUAUUCGGAAAAGCCTsT	471	GGCUUUUCCGAAUAAACUCTsT	472	AD-9560
1096-1114	GAGuuuAuucGGAAAAGccTsT	473	GGCUUUUCCGAAuAAACUCTsT	474	AD-9686
1100-1118	UUAUUCGGAAAAGCCAGCUTsT	475	AGCUGGCUUUUCCGAAUAATsT	476	AD-9536
1100-1118	uuAuucGGAAAAGccAGcuTsT	477	AGCUGGCUUUUCCGAAuAATsT	478	AD-9662
1154-1172	CCCUGGCGGGUGGGUACAGTsT	479	CUGUACCCACCCGCCAGGGTsT	480	AD-9584
1154-1172	cccuGGcGGGuGGGuAcAGTsT	481	CUGuACCcACCCGCcAGGGTsT	482	AD-9710
1155-1173	CCUGGCGGGUGGGUACAGCTT	483	GCUGUACCCACCCGCCAGGTT	484	AD-15323
1157-1175	UGGCGGGUGGGUACAGCCGTsT	485	CGGCUGUACCCACCCGCCATsT	486	AD-9551
1157-1175	uGGcGGGuGGGuAcAGccGTsT	487	CGGCUGuACCcACCCGCcATsT	488	AD-9677
1158-1176	GGCGGGUGGGUACAGCCGCTT	489	GCGGCUGUACCCACCCGCCTT	490	AD-15230
1162-1180	GGUGGGUACAGCCGCGUCCTT	491	GGACGCGGCUGUACCCACCTT	492	AD-15231
1164-1182	UGGGUACAGCCGCGUCCUCTT	493	GAGGACGCGGCUGUACCCATT	494	AD-15285
1172-1190	GCCGCGUCCUCAACGCCGCTT	495	GCGGCGUUGAGGACGCGGCTT	496	AD-15396
1173-1191	CCGCGUCCUCAACGCCGCCTT	497	GGCGGCGUUGAGGACGCGGTT	498	AD-15397
1216-1234	GUCGUGCUGGUCACCGCUGTsT	499	CAGCGGUGACCAGCACGACTsT	500	AD-9600
1216-1234	GucGuGcuGGucAccGcuGTsT	501	cAGCGGUGACcAGcACGACTsT	502	AD-9726
1217-1235	UCGUGCUGGUCACCGCUGCTsT	503	GCAGCGGUGACCAGCACGATsT	504	AD-9606
1217-1235	ucGuGcuGGucAccGcuGcTsT	505	GcAGCGGUGACcAGcACGATsT	506	AD-9732
1223-1241	UGGUCACCGCUGCCGGCAATsT	507	UUGCCGGCAGCGGUGACCATsT	508	AD-9633
1223-1241	uGGucAccGcuGccGGcAATsT	509	UUGCCGGcAGCGGUGACcATsT	510	AD-9759

TABLE 1a-continued

dsRNA sequences targeted to PCSK9

position in human access. # NM_174936	Sense strand sequence (5'-3')¹	SEQ ID NO:	Antisense-strand sequence (5'-3')¹	SEQ ID NO:	Duplex name
1224-1242	GGUCACCGCUGCCGGCAACTsT	511	GUUGCCGGCAGCGGUGACCTsT	512	AD-9588
1224-1242	GGucAccGcuGccGGcAAcTsT	513	GUUGCCGGcAGCGGUGACCTsT	514	AD-9714
1227-1245	CACCGCUGCCGGCAACUUCTsT	515	GAAGUUGCCGGCAGCGGUGTsT	516	AD-9589
1227-1245	cAccGcuGccGGcAAcuucTsT	517	GAAGUUGCCGGcAGCGGUGTsT	518	AD-9715
1229-1247	CCGCUGCCGGCAACUUCCGTsT	519	CGGAAGUUGCCGGCAGCGGTsT	520	AD-9575
1229-1247	ccGcuGccGGcAAcuuccGTsT	521	CGGAAGUUGCCGGcAGCGGTsT	522	AD-9701
1230-1248	CGCUGCCGGCAACUUCCGGTsT	523	CCGGAAGUUGCCGGCAGCGTsT	524	AD-9563
1230-1248	cGcuGccGGcAAcuuccGGTsT	525	CCGGAAGUUGCCGGcAGCGTsT	526	AD-9689
1231-1249	GCUGCCGGCAACUUCCGGGTsT	527	CCCGGAAGUUGCCGGCAGCTsT	528	AD-9594
1231-1249	GcuGccGGcAAcuuccGGGTsT	529	CCCGGAAGUUGCCGGcAGCTsT	530	AD-9720
1236-1254	CGGCAACUUCCGGGACGAUTsT	531	AUCGUCCCGGAAGUUGCCGTsT	532	AD-9585
1236-1254	cGGcAAcuuccGGGAcGAuTsT	533	AUCGUCCCGGAAGUUGCCGTsT	534	AD-9711
1237-1255	GGCAACUUCCGGGACGAUGTsT	535	CAUCGUCCCGGAAGUUGCCTsT	536	AD-9614
1237-1255	GGcAAcuuccGGGAcGAuGTsT	537	cAUCGUCCCGGAAGUUGCCTsT	538	AD-9740
1243-1261	UUCCGGGACGAUGCCUGCCTsT	539	GGCAGGCAUCGUCCCGGAATsT	540	AD-9615
1243-1261	uuccGGGAcGAuGccuGccTsT	541	GGcAGGcAUCGUCCCGGAATsT	542	AD-9741
1248-1266	GGACGAUGCCUGCCUCUACTsT	543	GUAGAGGCAGGCAUCGUCCTsT	544	AD-9534
1248-1266	GGACGAUGCCUGCCUCUACTsT	545	GUAGAGGCAGGCAUCGUCCTsT	546	AD-9534
1248-1266	GGAcGAuGccuGccucuAcTsT	547	GuAGAGGcAGGcAUCGUCCTsT	548	AD-9660
1279-1297	GCUCCCGAGGUCAUCACAGTT	549	CUGUGAUGACCUCGGGAGCTT	550	AD-15324
1280-1298	CUCCCGAGGUCAUCACAGUTT	551	ACUGUGAUGACCUCGGGAGTT	552	AD-15232
1281-1299	UCCCGAGGUCAUCACAGUUTT	553	AACUGUGAUGACCUCGGGATT	554	AD-15233
1314-1332	CCAAGACCAGCCGGUGACCTT	555	GGUCACCGGCUGGUCUUGGTT	556	AD-15234

TABLE 1a-continued

dsRNA sequences targeted to PCSK9

position in human access. # NM_174936	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense- strand sequence (5'-3')[1]	SEQ ID NO:	Duplex name
1315-1333	CAAGACCAGCCGGUGACCCTT	557	GGGUCACCGGCUGGUCUUGTT	558	AD- 15286
1348-1366	ACCAACUUUGGCCGCUGUGTsT	559	CACAGCGGCCAAAGUUGGUTsT	560	AD- 9590
1348-1366	AccAAcuuuGGccGcuGuGTsT	561	cAcAGCGGCcAAAGUUGGUTsT	562	AD- 9716
1350-1368	CAACUUUGGCCGCUGUGUGTsT	563	CACACAGCGGCCAAAGUUGTsT	564	AD- 9632
1350-1368	cAAcuuuGGccGcuGuGuGTsT	565	cAcAcAGCGGCcAAAGUUGTsT	566	AD- 9758
1360-1378	CGCUGUGUGGACCUCUUUGTsT	567	CAAAGAGGUCCACACAGCGTsT	568	AD- 9567
1360-1378	cGcuGuGuGGAccucuuuGTsT	569	cAAAGAGGUCcAcAcAGCGTsT	570	AD- 9693
1390-1408	GACAUCAUUGGUGCCUCCATsT	571	UGGAGGCACCAAUGAUGUCTsT	572	AD- 9586
1390-1408	GAcAucAuuGGuGccuccATsT	573	UGGAGGcACcAAUGAUGUCTsT	574	AD- 9712
1394-1412	UCAUUGGUGCCUCCAGCGATsT	575	UCGCUGGAGGCACCAAUGATsT	576	AD- 9564
1394-1412	ucAuuGGuGccuccAGcGATsT	577	UCGCUGGAGGcAccAAUGATsT	578	AD- 9690
1417-1435	AGCACCUGCUUUGUGUCACTsT	579	GUGACACAAAGCAGGUGCUTsT	580	AD- 9616
1417-1435	AGcAccuGcuuuGuGucAcTsT	581	GUGAcAcAAAGcAGGUGCUTsT	582	AD- 9742
1433-1451	CACAGAGUGGGACAUCACATT	583	UGUGAUGUCCCACUCUGUGTT	584	AD- 15398
1486-1504	AUGCUGUCUGCCGAGCCGGTsT	585	CCGGCUCGGCAGACAGCAUTsT	586	AD- 9617
1486-1504	AuGcuGucuGccGAGccGGTsT	587	CCGGCUCGGcAGAcAGcAUTsT	588	AD- 9743
1491-1509	GUCUGCCGAGCCGGAGCUCTsT	589	GAGCUCCGGCUCGGCAGACTsT	590	AD- 9635
1491-1509	GucuGccGAGccGGAGcucTsT	591	GAGCUCCGGCUCGGcAGACTsT	592	AD- 9761
1521-1539	GUUGAGGCAGAGACUGAUCTsT	593	GAUCAGUCUCUGCCUCAACTsT	594	AD- 9568
1521-1539	GuuGAGGcAGAGAcuGAucTsT	595	GAUcAGUCUCUGCCUcAACTsT	596	AD- 9694
1527-1545	GCAGAGACUGAUCCACUUCTsT	597	GAAGUGGAUCAGUCUCUGCTsT	598	AD- 9576
1527-1545	GcAGAGAcuGAuccAcuucTsT	599	GAAGUGGAUcAGUCUCUGCTsT	600	AD- 9702
1529-1547	AGAGACUGAUCCACUUCUCTsT	601	GAGAAGUGGAUCAGUCUCUTsT	602	AD- 9627

TABLE 1a-continued

dsRNA sequences targeted to PCSK9

position in human access. # NM_174936	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense-strand sequence (5'-3')[1]	SEQ ID NO:	Duplex name
1529-1547	AGAGAcuGAuccAcuucucTsT	603	GAGAAGUGGAUcAGUCUCUTsT	604	AD-9753
1543-1561	UUCUCUGCCAAAGAUGUCATsT	605	UGACAUCUUUGGCAGAGAATsT	606	AD-9628
1543-1561	uucucuGccAAAGAuGucATsT	607	UGAcAUCUUUGGcAGAGAATsT	608	AD-9754
1545-1563	CUCUGCCAAAGAUGUCAUCTsT	609	GAUGACAUCUUUGGCAGAGTsT	610	AD-9631
1545-1563	cucuGccAAAGAuGucAucTsT	611	GAUGAcAUCUUUGGcAGAGTsT	612	AD-9757
1580-1598	CUGAGGACCAGCGGGUACUTsT	613	AGUACCCGCUGGUCCUCAGTsT	614	AD-9595
1580-1598	cuGAGGAccAGcGGGuAcuTsT	615	AGuACCCGCUGGUCCUcAGTsT	616	AD-9721
1581-1599	UGAGGACCAGCGGGUACUGTsT	617	CAGUACCCGCUGGUCCUCATsT	618	AD-9544
1581-1599	uGAGGAccAGcGGGuAcuGTsT	619	cAGuACCCGCUGGUCCUcATsT	620	AD-9670
1666-1684	ACUGUAUGGUCAGCACACUTT	621	AGUGUGCUGACCAUACAGUTT	622	AD-15235
1668-1686	UGUAUGGUCAGCACACUCGTT	623	CGAGUGUGCUGACCAUACATT	624	AD-15236
1669-1687	GUAUGGUCAGCACACUCGGTT	625	CCGAGUGUGCUGACCAUACTT	626	AD-15168
1697-1715	GGAUGGCCACAGCCGUCGCTT	627	GCGACGGCUGUGGCCAUCCTT	628	AD-15174
1698-1716	GAUGGCCACAGCCGUCGCCTT	629	GGCGACGGCUGUGGCCAUCTT	630	AD-15325
1806-1824	CAAGCUGGUCUGCCGGGCCTT	631	GGCCCGGCAGACCAGCUUGTT	632	AD-15326
1815-1833	CUGCCGGGCCCACAACGCUTsT	633	AGCGUUGUGGGCCCGGCAGTsT	634	AD-9570
1815-1833	cuGccGGGcccAcAAcGcuTsT	635	AGCGUUGUGGGCCCGGcAGTsT	636	AD-9696
1816-1834	UGCCGGGCCCACAACGCUUTsT	637	AAGCGUUGUGGGCCCGGCATsT	638	AD-9566
1816-1834	uGccGGGcccAcAAcGcuuTsT	639	AAGCGUUGUGGGCCCGGcATsT	640	AD-9692
1818-1836	CCGGGCCCACAACGCUUUUTsT	641	AAAAGCGUUGUGGGCCCGGTsT	642	AD-9532
1818-1836	ccGGGcccAcAAcGcuuuuTsT	643	AAAAGCGUUGUGGGCCCGGTsT	644	AD-9658
1820-1838	GGGCCCACAACGCUUUUGGTsT	645	CCAAAAGCGUUGUGGGCCCTsT	646	AD-9549
1820-1838	GGGcccAcAAcGcuuuuGGTsT	647	CcAAAAGCGUUGUGGGCCCTsT	648	AD-9675

TABLE 1a-continued

dsRNA sequences targeted to PCSK9					
position in human access. # NM_174936	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense-strand sequence (5'-3')[1]	SEQ ID NO:	Duplex name
1840-1858	GGUGAGGGUGUCUACGCCATsT	649	UGGCGUAGACACCCUCACCTsT	650	AD-9541
1840-1858	GGuGAGGGuGucuAcGccATsT	651	UGGCGuAGAcACCCUcACCTsT	652	AD-9667
1843-1861	GAGGGUGUCUACGCCAUUGTsT	653	CAAUGGCGUAGACACCCUCTsT	654	AD-9550
1843-1861	GAGGGuGucuAcGccAuuGTsT	655	cAAUGGCGuAGAcACCCUCTsT	656	AD-9676
1861-1879	GCCAGGUGCUGCCUGCUACTsT	657	GUAGCAGGCAGCACCUGGCTsT	658	AD-9571
1861-1879G	ccAGGuGcuGccuGcuAcTsT	659	GuAGcAGGcAGcACCUGGCTsT	660	AD-9697
1862-1880	CCAGGUGCUGCCUGCUACCTsT	661	GGUAGCAGGCAGCACCUGGTsT	662	AD-9572
1862-1880	ccAGGuGcuGccuGcuAccTsT	663	GGuAGcAGGcAGcACCUGGTsT	664	AD-9698
2008-2026	ACCCACAAGCCGCCUGUGCTT	665	GCACAGGCGGCUUGUGGGUTT	666	AD-15327
2023-2041	GUGCUGAGGCCACGAGGUCTsT	667	GACCUCGUGGCCUCAGCACTsT	668	AD-9639
2023-2041	GuGcuGAGGccAcGAGGucTsT	669	GACCUCGUGGCCUcAGcACTsT	670	AD-9765
2024-2042	UGCUGAGGCCACGAGGUCATsT	671	UGACCUCGUGGCCUCAGCATsT	672	AD-9518
2024-2042	UGCUGAGGCCACGAGGUCATsT	673	UGACCUCGUGGCCUCAGCATsT	674	AD-9518
2024-2042	uGcuGAGGccAcGAGGucATsT	675	UGACCUCGUGGCCUcAGcATsT	676	AD-9644
2024-2042	UfgCfuGfaGfgCfcAfcGfaGfgUfCAfTsT	677	p-uGfaCfcUfcGfuGfgCfcUfcAfgCfaTsT	678	AD-14672
2024-2042	UfGCfUfGAGGCfCfACfGAGGUfCfATST	679	UfGACfCfUfCfGUfGGCfCfUfCfAGCfATST	680	AD-14682
2024-2042	UgCuGaGgCcAcGaGgUcATsT	681	p-uGfaCfcUfcGfuGfgCfcUfcAfgCfaTsT	682	AD-14692
2024-2042	UgCuGaGgCcAcGaGgUcATsT	683	UfGACfCfUfCfGUfGGCfCfUfCfAGCfATsT	684	AD-14702
2024-2042	UfgCfuGfaGfgCfcAfcGfaGfgUfCAfTsT	685	UGACCUcGUggCCUCAgcaTsT	686	AD-14712
2024-2042	UfGCfUfGAGGCfCfACfGAGGUfCfATST	687	UGACCUcGUggCCUCAgcaTsT	688	AD-14722
2024-2042	UgCuGaGgCcAcGaGgUcATsT	689	UGACCUcGUggCCUCAgcaTsT	690	AD-14732
2024-2042	GfuGfgUfcAfgCfgGfcCfgGfgAfuGfTsT	691	p-cAfuCfcCfgGfcCfgCfuGfaCfcAfcTsT	692	AD-15078
2024-2042	GUfGGUfcfAGCffGGCfCffGGGAUfGTsT	693	CfAUfCfCfCfGGCfCfGCfUfGACfCfACfTsT	694	AD-15088

TABLE 1a-continued

dsRNA sequences targeted to PCSK9

position in human access. # NM_174936	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense-strand sequence (5'-3')[1]	SEQ ID NO:	Duplex name
2024-2042	GuGgUcAgCgGcCgGgAuGTsT	695	p-cAfuCfcCfgGfcCfgCfuGfaCfcAfcTsT	696	AD-15098
2024-2042	GuGgUcAgCgGcCgGgAuGTsT	697	CfAUfCfCfCfGGCfCfGCfUfGACfCfACfTsT	698	AD-15108
2024-2042	GfuGfgUfcAfgCfgGfcCfgGfgAfuGfTsT	699	CAUCCcgGCcgCUGACcacTsT	700	AD-15118
2024-2042	GUfGGUfCfAGCfGGCfCfGGGAUfGTsT	701	CAUCCcgGCcgCUGACcacTsT	702	AD-15128
2024-2042	GuGgUcAgCgGcCgGgAuGTsT	703	CAUCCcgGCcgCUGACcacTsT	704	AD-15138
2030-2048	GGCCACGAGGUCAGCCCAATT	705	UUGGGCUGACCUCGUGGCCTT	706	AD-15237
2035-2053	CGAGGUCAGCCCAACCAGUTT	707	ACUGGUUGGGCUGACCUCGTT	708	AD-15287
2039-2057	GUCAGCCCAACCAGUGCGUTT	709	ACGCACUGGUUGGGCUGACTT	710	AD-15238
2041-2059	CAGCCCAACCAGUGCGUGGTT	711	CCACGCACUGGUUGGGCUGTT	712	AD-15328
2062-2080	CACAGGGAGGCCAGCAUCCTT	713	GGAUGCUGGCCUCCCUGUGTT	714	AD-15399
2072-2090	CCAGCAUCCACGCUUCCUGTsT	715	CAGGAAGCGUGGAUGCUGGTsT	716	AD-9582
2072-2090	ccAGcAuccAcGcuuccuGTsT	717	cAGGAAGCGUGGAUGCUGGTsT	718	AD-9708
2118-2136	AGUCAAGGAGCAUGGAAUCTsT	719	GAUUCCAUGCUCCUUGACUTsT	720	AD-9545
2118-2136	AGucAAGGAGcAuGGAAucTsT	721	GAUUCcAUGCUCCUUGACUTsT	722	AD-9671
2118-2136	AfgUfcAfaGfgAfgCfaUfgGfaAfuCfTsT	723	p-gAfuUfcCfaUfgCfuCfcUfuGfaCfuTsT	724	AD-14674
2118-2136	AGUfCfAAGGAGCfAUfGGAAUfCfTsT	725	GAUfUfCfCfAUfGCfUfCfCfUfUfGACfUfTsT	726	AD-14684
2118-2136	AgUcAaGgAgCaUgGaAuCTsT	727	p-gAfuUfcCfaUfgCfuCfcUfuGfaCfuTsT	728	AD-14694
2118-2136	AgUcAaGgAgCaUgGaAuCTsT	729	GAUfUfCfCfAUfGCfUfCfCfUfUfGACfUfTsT	730	AD-14704
2118-2136	AfgUfcAfaGfgAfgCfaUfgGfaAfuCfTsT	731	GAUUCcaUGcuCCUUGacuTsT	732	AD-14714
2118-2136	AGUfCfAAGGAGCfAUfGGAAUfCfTsT	733	GAUUCcaUGcuCCUUGacuTsT	734	AD-14724
2118-2136	AgUcAaGgAgCaUgGaAuCTsT	735	GAUUCcaUGcuCCUUGacuTsT	736	AD-14734
2118-2136	GfcGfgCfaCfcCfuCfaUfaGfgCfcUfTsT	737	p-aGfgCfcUfaUfgAfgGfgUfgCfcGfcTsT	738	AD-15080
2118-2136	GCfGGCfACfCfCfUfCfAUfAGGCfCfUfTsT	739	AGGCfCfUfAUfGAGGGUfGCfCfGCfTsT	740	AD-15090

TABLE 1a-continued

dsRNA sequences targeted to PCSK9

position in human access. # NM_174936	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense-strand sequence (5'-3')[1]	SEQ ID NO:	Duplex name
2118-2136	GcGgCaCcCuCaUaGgCcUTsT	741	p-aGfgCfcUfaUfgAfgGfgUfgCfcGfcTsT	742	AD-15100
2118-2136	GcGgCaCcCuCaUaGgCcUTsT	743	AGGCfCfUfAUfGAGGGUfGCfCfGCfTsT	744	AD-15110
2118-2136	GfcGfgCfaCfcCfuCfaUfaGfgCfcUfTsT	745	AGGCCuaUGagGGUGCcgcTsT	746	AD-15120
2118-2136	GCfGGCfACfCfCfUfCfAUfAGGCfCfUfTsT	747	AGGCCuaUGagGGUGCcgcTsT	748	AD-15130
2118-2136	GcGgCaCcCuCaUaGgCcUTsT	749	AGGCCuaUGagGGUGCcgcTsT	750	AD-15140
2122-2140	AAGGAGCAUGGAAUCCCGGTsT	751	CCGGGAUUCCAUGCUCCUUTsT	752	AD-9522
2122-2140	AAGGAGcAuGGAAucccGGTsT	753	CCGGGAUUCcAUGCUCCUUTsT	754	AD-9648
2123-2141	AGGAGCAUGGAAUCCCGGCTsT	755	GCCGGGAUUCCAUGCUCCUTsT	756	AD-9552
2123-2141	AGGAGcAuGGAAucccGGcTsT	757	GCCGGGAUUCcAUGCUCCUTsT	758	AD-9678
2125-2143	GAGCAUGGAAUCCCGGCCCTsT	759	GGGCCGGGAUUCCAUGCUCTsT	760	AD-9618
2125-2143	GAGcAuGGAAucccGGcccTsT	761	GGGCCGGGAUUCcAUGCUCTsT	762	AD-9744
2230-2248	GCCUACGCCGUAGACAACATT	763	UGUUGUCUACGGCGUAGGCTT	764	AD-15239
2231-2249	CCUACGCCGUAGACAACACTT	765	GUGUUGUCUACGGCGUAGGTT	766	AD-15212
2232-2250	CUACGCCGUAGACAACACGTT	767	CGUGUUGUCUACGGCGUAGTT	768	AD-15240
2233-2251	UACGCCGUAGACAACACGUTT	769	ACGUGUUGUCUACGGCGUATT	770	AD-15177
2235-2253	CGCCGUAGACAACACGUGUTT	771	ACACGUGUUGUCUACGGCGTT	772	AD-15179
2236-2254	GCCGUAGACAACACGUGUGTT	773	CACACGUGUUGUCUACGGCTT	774	AD-15180
2237-2255	CCGUAGACAACACGUGUGUTT	775	ACACACGUGUUGUCUACGGTT	776	AD-15241
2238-2256	CGUAGACAACACGUGUGUATT	777	UACACACGUGUUGUCUACGTT	778	AD-15268
2240-2258	UAGACAACACGUGUGUAGUTT	779	ACUACACACGUGUUGUCUATT	780	AD-15242
2241-2259	AGACAACACGUGUGUAGUCTT	781	GACUACACACGUGUUGUCUTT	782	AD-15216
2242-2260	GACAACACGUGUGUAGUCATT	783	UGACUACACACGUGUUGUCTT	784	AD-15176
2243-2261	ACAACACGUGUGUAGUCAGTT	785	CUGACUACACACGUGUUGUTT	786	AD-15181

TABLE 1a-continued

dsRNA sequences targeted to PCSK9

position in human access. # NM_174936	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense-strand sequence (5'-3')[1]	SEQ ID NO:	Duplex name
2244-2262	CAACACGUGUGUAGUCAGGTT	787	CCUGACUACACACGUGUUGTT	788	AD-15243
2247-2265	CACGUGUGUAGUCAGGAGCTT	789	GCUCCUGACUACACACGUGTT	790	AD-15182
2248-2266	ACGUGUGUAGUCAGGAGCCTT	791	GGCUCCUGACUACACACGUTT	792	AD-15244
2249-2267	CGUGUGUAGUCAGGAGCCGTT	793	CGGCUCCUGACUACACACGTT	794	AD-15387
2251-2269	UGUGUAGUCAGGAGCCGGGTT	795	CCCGGCUCCUGACUACACATT	796	AD-15245
2257-2275	GUCAGGAGCCGGGACGUCATsT	797	UGACGUCCCGGCUCCUGACTsT	798	AD-9555
2257-2275	GucAGGAGccGGGAcGucATsT	799	UGACGUCCCGGCUCCUGACTsT	800	AD-9681
2258-2276	UCAGGAGCCGGGACGUCAGTsT	801	CUGACGUCCCGGCUCCUGATsT	802	AD-9619
2258-2276	ucAGGAGccGGGAcGucAGTsT	803	CUGACGUCCCGGCUCCUGATsT	804	AD-9745
2259-2277	CAGGAGCCGGGACGUCAGCTsT	805	GCUGACGUCCCGGCUCCUGTsT	806	AD-9620
2259-2277	cAGGAGccGGGAcGucAGcTsT	807	GCUGACGUCCCGGCUCCUGTsT	808	AD-9746
2263-2281	AGCCGGGACGUCAGCACUATT	809	UAGUGCUGACGUCCCGGCUTT	810	AD-15288
2265-2283	CCGGGACGUCAGCACUACATT	811	UGUAGUGCUGACGUCCCGGTT	812	AD-15246
2303-2321	CCGUGACAGCCGUUGCCAUTT	813	AUGGCAACGGCUGUCACGGTT	814	AD-15289
2317-2335	GCCAUCUGCUGCCGGAGCCTsT	815	GGCUCCGGCAGCAGAUGGCTsT	816	AD-9324
2375-2393	CCCAUCCCAGGAUGGGUGUTT	817	ACACCCAUCCUGGGAUGGGTT	818	AD-15329
2377-2395	CAUCCCAGGAUGGGUGUCUTT	819	AGACACCCAUCCUGGGAUGTT	820	AD-15330
2420-2438	AGCUUUAAAAUGGUUCCGATT	821	UCGGAACCAUUUUAAAGCUTT	822	AD-15169
2421-2439	GCUUUAAAAUGGUUCCGACTT	823	GUCGGAACCAUUUUAAAGCTT	824	AD-15201
2422-2440	CUUUAAAAUGGUUCCGACUTT	825	AGUCGGAACCAUUUUAAAGTT	826	AD-15331
2423-2441	UUUAAAAUGGUUCCGACUUTT	827	AAGUCGGAACCAUUUUAAATT	828	AD-15190
2424-2442	UUAAAAUGGUUCCGACUUGTT	829	CAAGUCGGAACCAUUUUAATT	830	AD-15247
2425-2443	UAAAAUGGUUCCGACUUGUTT	831	ACAAGUCGGAACCAUUUUATT	832	AD-15248

TABLE 1a-continued

dsRNA sequences targeted to PCSK9

position in human access. # NM_174936	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense-strand sequence (5'-3')[1]	SEQ ID NO:	Duplex name
2426-2444	AAAAUGGUUCCGACUUGUCUTT	833	GACAAGUCGGAACCAUUUUTT	834	AD-15175
2427-2445	AAAUGGUUCCGACUUGUCCTT	835	GGACAAGUCGGAACCAUUUTT	836	AD-15249
2428-2446	AAUGGUUCCGACUUGUCCCTT	837	GGGACAAGUCGGAACCAUUTT	838	AD-15250
2431-2449	GGUUCCGACUUGUCCCUCUTT	839	AGAGGGACAAGUCGGAACCTT	840	AD-15400
2457-2475	CUCCAUGGCCUGGCACGAGTT	841	CUCGUGCCAGGCCAUGGAGTT	842	AD-15332
2459-2477	CCAUGGCCUGGCACGAGGGTT	843	CCCUCGUGCCAGGCCAUGGTT	844	AD-15388
2545-2563	GAACUCACUCACUCUGGGUTT	845	ACCCAGAGUGAGUGAGUUCTT	846	AD-15333
2549-2567	UCACUCACUCUGGGUGCCUTT	847	AGGCACCCAGAGUGAGUGATT	848	AD-15334
2616-2634	UUUCACCAUUCAAACAGGUTT	849	ACCUGUUUGAAUGGUGAAATT	850	AD-15335
2622-2640	CAUUCAAACAGGUCGAGCUTT	851	AGCUCGACCUGUUUGAAUGTT	852	AD-15183
2623-2641	AUUCAAACAGGUCGAGCUGTT	853	CAGCUCGACCUGUUUGAAUTT	854	AD-15202
2624-2642	UUCAAACAGGUCGAGCUGUTT	855	ACAGCUCGACCUGUUUGAATT	856	AD-15203
2625-2643	UCAAACAGGUCGAGCUGUGTT	857	CACAGCUCGACCUGUUUGATT	858	AD-15272
2626-2644	CAAACAGGUCGAGCUGUGCTT	859	GCACAGCUCGACCUGUUUGTT	860	AD-15217
2627-2645	AAACAGGUCGAGCUGUGCUTT	861	AGCACAGCUCGACCUGUUUTT	862	AD-15290
2628-2646	AACAGGUCGAGCUGUGCUCTT	863	GAGCACAGCUCGACCUGUUTT	864	AD-15218
2630-2648	CAGGUCGAGCUGUGCUCGGTT	865	CCGAGCACAGCUCGACCUGTT	866	AD-15389
2631-2649	AGGUCGAGCUGUGCUCGGGTT	867	CCCGAGCACAGCUCGACCUTT	868	AD-15336
2633-2651	GUCGAGCUGUGCUCGGGUGTT	869	CACCCGAGCACAGCUCGACTT	870	AD-15337
2634-2652	UCGAGCUGUGCUCGGGUGCTT	871	GCACCCGAGCACAGCUCGATT	872	AD-15191
2657-2675	UCGAGCUGUGCUCGGGUGCTT	873	CGGCACAUUGGGAGCAGCUTT	874	AD-15390
2658-2676	GCUGCUCCCAAUGUGCCGATT	875	UCGGCACAUUGGGAGCAGCTT	876	AD-15338
2660-2678	UGCUCCCAAUGUGCCGAUGTT	877	CAUCGGCACAUUGGGAGCATT	878	AD-15204

TABLE 1a-continued

dsRNA sequences targeted to PCSK9

position in human access. # NM_174936	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense-strand sequence (5'-3')[1]	SEQ ID NO:	Duplex name
2663-2681	UCCCAAUGUGCCGAUGUCCTT	879	GGACAUCGGCACAUUGGGATT	880	AD-15251
2665-2683	CCAAUGUGCCGAUGUCCGUTT	881	ACGGACAUCGGCACAUUGGTT	882	AD-15205
2666-2684	CAAUGUGCCGAUGUCCGUGTT	883	CACGGACAUCGGCACAUUGTT	884	AD-15171
2667-2685	AAUGUGCCGAUGUCCGUGGTT	885	CCACGGACAUCGGCACAUUTT	886	AD-15252
2673-2691	CCGAUGUCCGUGGGCAGAATT	887	UUCUGCCCACGGACAUCGGTT	888	AD-15339
2675-2693	GAUGUCCGUGGGCAGAAUGTT	889	CAUUCUGCCCACGGACAUCTT	890	AD-15253
2678-2696	GUCCGUGGGCAGAAUGACUTT	891	AGUCAUUCUGCCCACGGACTT	892	AD-15340
2679-2697	UCCGUGGGCAGAAUGACUUTT	893	AAGUCAUUCUGCCCACGGATT	894	AD-15291
2683-2701	UGGGCAGAAUGACUUUUAUTT	895	AUAAAAGUCAUUCUGCCCATT	896	AD-15341
2694-2712	ACUUUUAUUGAGCUCUUGUTT	897	ACAAGAGCUCAAUAAAAGUTT	898	AD-15401
2700-2718	AUUGAGCUCUUGUUCCGUGTT	899	CACGGAACAAGAGCUCAAUTT	900	AD-15342
2704-2722	AGCUCUUGUUCCGUGCCAGTT	901	CUGGCACGGAACAAGAGCUTT	902	AD-15343
2705-2723	GCUCUUGUUCCGUGCCAGGTT	903	CCUGGCACGGAACAAGAGCTT	904	AD-15292
2710-2728	UGUUCCGUGCCAGGCAUUCTT	905	GAAUGCCUGGCACGGAACATT	906	AD-15344
2711-2729	GUUCCGUGCCAGGCAUUCATT	907	UGAAUGCCUGGCACGGAACTT	908	AD-15254
2712-2730	UUCCGUGCCAGGCAUUCAATT	909	UUGAAUGCCUGGCACGGAATT	910	AD-15345
2715-2733	CGUGCCAGGCAUUCAAUCCTT	911	GGAUUGAAUGCCUGGCACGTT	912	AD-15206
2716-2734	GUGCCAGGCAUUCAAUCCUTT	913	AGGAUUGAAUGCCUGGCACTT	914	AD-15346
2728-2746	CAAUCCUCAGGUCUCCACCTT	915	GGUGGAGACCUGAGGAUUGTT	916	AD-15347
2743-2761	CACCAAGGAGGCAGGAUUCTsT	917	GAAUCCUGCCUCCUUGGUGTsT	918	AD-9577
2743-2761	cAccAAGGAGGcAGGAuucTsT	919	GAAUCCUGCCUCCUUGGUGTsT	920	AD-9703
2743-2761	CfaCfcAfaGfgAfgGfcAfgGfaUfuCffTsT	921	p-gAfaUfcCfuGfcCfuCfcUfuGfgUfgTsT	922	AD-14678
2743-2761	CfACfCfAAGGAGGCfAGGAUfUfCfTsT	923	GAAUfCfCfUfGCfCfUfCfCfUfUfGGUfGTsT	924	AD-14688

TABLE 1a-continued

			dsRNA sequences targeted to PCSK9		
position in human access. # NM_174936	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense- strand sequence (5'-3')[1]	SEQ ID NO:	Duplex name
2743-2761	CaCcAaGgAgGcAgGaUuCTsT	925	p- gAfaUfcCfuGfcCfuCfcUfuGfgUfgTsT	926	AD- 14698
2743-2761	CaCcAaGgAgGcAgGaUuCTsT	927	GAAUfCfCfUfGCfCfUfCfCfUfUfGGUfG TsT	928	AD- 14708
2743-2761	CfaCfcAfaGfgAfgGfcAfgGfaUfuC fTsT	929	GAAUCcuGCcuCCUUGgugTsT	930	AD- 14718
2743-2761	CfACfCfAAGGAGGCfAGGAUfUfCfTs T	931	GAAUCcuGCcuCCUUGgugTsT	932	AD- 14728
2743-2761	CaCcAaGgAgGcAgGaUuCTsT	933	GAAUCcuGCcuCCUUGgugTsT	934	AD- 14738
2743-2761	GfgCfcUfgGfaGfuUfuAfuUfcGfgA fTsT	935	p- uCfcGfaAfuAfaAfcUfcCfaGfgCfcTsT	936	AD- 15084
2743-2761	GGCfCfUfGGAGUfUfUfAUfUfCfGGA TsT	937	UfCfCfGAAUfAAACfUfCfCfAGGCfCfTs T	938	AD- 15094
2743-2761	GgCcUgGaGuUuAuUcGgATsT	939	p- uCfcGfaAfuAfaAfcUfcCfaGfgCfcTsT	940	AD- 15104
2743-2761	GgCcUgGaGuUuAuUcGgATsT	941	UfCfCfGAAUfAAACfUfCfCfAGGCfCfTs T	942	AD- 15114
2743-2761	GfgCfcUfgGfaGfuUfuAfuUfcGfgA fTsT	943	UCCGAauAAacUCCAGgccTsT	944	AD- 15124
2743-2761	GGCfCfUfGGAGUfUfUfAUfUfCfGGA TsT	945	UCCGAauAAacUCCAGgccTsT	946	AD- 15134
2743-2761	GgCcUgGaGuUuAuUcGgATsT	947	UCCGAauAAacUCCAGgccTsT	948	AD- 15144
2753-2771	GCAGGAUUCUUCCCAUGGATT	949	UCCAUGGGAAGAAUCCUGCTT	950	AD- 15391
2794-2812	UGCAGGGACAAACAUCGUUTT	951	AACGAUGUUUGUCCCUGCATT	952	AD- 15348
2795-2813	GCAGGGACAAACAUCGUUGTT	953	CAACGAUGUUUGUCCCUGCTT	954	AD- 15349
2797-2815	AGGGACAAACAUCGUUGGGTT	955	CCCAACGAUGUUUGUCCCUTT	956	AD- 15170
2841-2859	CCCUCAUCUCCAGCUAACUTT	957	AGUUAGCUGGAGAUGAGGGTT	958	AD- 15350
2845-2863	CAUCUCCAGCUAACUGUGGTT	959	CCACAGUUAGCUGGAGAUGTT	960	AD- 15402
2878-2896	GCUCCCUGAUUAAUGGAGGTT	961	CCUCCAUUAAUCAGGGAGCTT	962	AD- 15293
2881-2899	CCCUGAUUAAUGGAGGCUUTT	963	AAGCCUCCAUUAAUCAGGGTT	964	AD- 15351
2882-2900	CCUGAUUAAUGGAGGCUUATT	965	UAAGCCUCCAUUAAUCAGGTT	966	AD- 15403
2884-2902	UGAUUAAUGGAGGCUUAGCTT	967	GCUAAGCCUCCAUUAAUCATT	968	AD- 15404
2885-2903	GAUUAAUGGAGGCUUAGCUTT	969	AGCUAAGCCUCCAUUAAUCTT	970	AD- 15207

TABLE 1a-continued

dsRNA sequences targeted to PCSK9					
position in human access. # NM_174936	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense-strand sequence (5'-3')[1]	SEQ ID NO:	Duplex name
2886-2904	AUUAAUGGAGGCUUAGCUUTT	971	AAGCUAAGCCUCCAUUAAUTT	972	AD-15352
2887-2905	UUAAUGGAGGCUUAGCUUUTT	973	AAAGCUAAGCCUCCAUUAATT	974	AD-15255
2903-2921	UUUCUGGAUGGCAUCUAGCTsT	975	GCUAGAUGCCAUCCAGAAATsT	976	AD-9603
2903-2921	uuucuGGAuGGcAucuAGcTsT	977	GCuAGAUGCcAUCcAGAAATsT	978	AD-9729
2904-2922	UUCUGGAUGGCAUCUAGCCTsT	979	GGCUAGAUGCCAUCCAGAATsT	980	AD-9599
2904-2922	uucuGGAuGGcAucuAGccTsT	981	GGCuAGAUGCcAUCcAGAATsT	982	AD-9725
2905-2923	UCUGGAUGGCAUCUAGCCATsT	983	UGGCUAGAUGCCAUCCAGATsT	984	AD-9621
2905-2923	ucuGGAuGGcAucuAGccATsT	985	UGGCuAGAUGCcAUCcAGATsT	986	AD-9747
2925-2943	AGGCUGGAGACAGGUGCGCTT	987	GCGCACCUGUCUCCAGCCUTT	988	AD-15405
2926-2944	GGCUGGAGACAGGUGCGCCTT	989	GGCGCACCUGUCUCCAGCCTT	990	AD-15353
2927-2945	GCUGGAGACAGGUGCGCCCTT	991	GGGCGCACCUGUCUCCAGCTT	992	AD-15354
2972-2990	UUCCUGAGCCACCUUUACUTT	993	AGUAAAGGUGGCUCAGGAATT	994	AD-15406
2973-2991	UCCUGAGCCACCUUUACUCTT	995	GAGUAAAGGUGGCUCAGGATT	996	AD-15407
2974-2992	CCUGAGCCACCUUUACUCUTT	997	AGAGUAAAGGUGGCUCAGGTT	998	AD-15355
2976-2994	UGAGCCACCUUUACUCUGCTT	999	GCAGAGUAAAGGUGGCUCATT	1000	AD-15356
2978-2996	AGCCACCUUUACUCUGCUCTT	1001	GAGCAGAGUAAAGGUGGCUTT	1002	AD-15357
2981-2999	CACCUUUACUCUGCUCUAUTT	1003	AUAGAGCAGAGUAAAGGUGTT	1004	AD-15269
2987-3005	UACUCUGCUCUAUGCCAGGTsT	1005	CCUGGCAUAGAGCAGAGUATsT	1006	AD-9565
2987-3005	uAcucuGcucuAuGccAGGTsT	1007	CCUGGcAuAGAGcAGAGuATsT	1008	AD-9691
2998-3016	AUGCCAGGCUGUGCUAGCATT	1009	UGCUAGCACAGCCUGGCAUTT	1010	AD-15358
3003-3021	AGGCUGUGCUAGCAACACCTT	1011	GGUGUUGCUAGCACAGCCUTT	1012	AD-15359
3006-3024	CUGUGCUAGCAACACCCAATT	1013	UUGGGUGUUGCUAGCACAGTT	1014	AD-15360
3010-3028	GCUAGCAACACCCAAAGGUTT	1015	ACCUUUGGGUGUUGCUAGCTT	1016	AD-15219

TABLE 1a-continued

dsRNA sequences targeted to PCSK9

position in human access. # NM_174936	Sense strand sequence (5'-3')¹	SEQ ID NO:	Antisense-strand sequence (5'-3')¹	SEQ ID NO:	Duplex name
3038-3056	GGAGCCAUCACCUAGGACUTT	1017	AGUCCUAGGUGAUGGCUCCTT	1018	AD-15361
3046-3064	CACCUAGGACUGACUCGGCTT	1019	GCCGAGUCAGUCCUAGGUGTT	1020	AD-15273
3051-3069	AGGACUGACUCGGCAGUGUTT	1021	ACACUGCCGAGUCAGUCCUTT	1022	AD-15362
3052-3070	GGACUGACUCGGCAGUGUGTT	1023	CACACUGCCGAGUCAGUCCTT	1024	AD-15192
3074-3092	UGGUGCAUGCACUGUCUCATT	1025	UGAGACAGUGCAUGCACCATT	1026	AD-15256
3080-3098	AUGCACUGUCUCAGCCAACTT	1027	GUUGGCUGAGACAGUGCAUTT	1028	AD-15363
3085-3103	CUGUCUCAGCCAACCCGCUTT	1029	AGCGGGUUGGCUGAGACAGTT	1030	AD-15364
3089-3107	CUCAGCCAACCCGCUCCACTsT	1031	GUGGAGCGGGUUGGCUGAGTsT	1032	AD-9604
3089-3107	cucAGccAAcccGcuccAcTsT	1033	GUGGAGCGGGUUGGCUGAGTsT	1034	AD-9730
3093-3111	GCCAACCCGCUCCACUACCTsT	1035	GGUAGUGGAGCGGGUUGGCTsT	1036	AD-9527
3093-3111	GccAAcccGcuccAcuAccTsT	1037	GGuAGUGGAGCGGGUUGGCTsT	1038	AD-9653
3096-3114	AACCCGCUCCACUACCCGGTT	1039	CCGGGUAGUGGAGCGGGUUTT	1040	AD-15365
3099-3117	CCGCUCCACUACCCGGCAGTT	1041	CUGCCGGGUAGUGGAGCGGTT	1042	AD-15294
3107-3125	CUACCCGGCAGGGUACACATT	1043	UGUGUACCCUGCCGGGUAGTT	1044	AD-15173
3108-3126	UACCCGGCAGGGUACACAUTT	1045	AUGUGUACCCUGCCGGGUATT	1046	AD-15366
3109-3127	ACCCGGCAGGGUACACAUUTT	1047	AAUGUGUACCCUGCCGGGUTT	1048	AD-15367
3110-3128	CCCGGCAGGGUACACAUUCTT	1049	GAAUGUGUACCCUGCCGGGTT	1050	AD-15257
3112-3130	CGGCAGGGUACACAUUCGCTT	1051	GCGAAUGUGUACCCUGCCGTT	1052	AD-15184
3114-3132	GCAGGGUACACAUUCGCACTT	1053	GUGCGAAUGUGUACCCUGCTT	1054	AD-15185
3115-3133	CAGGGUACACAUUCGCACCTT	1055	GGUGCGAAUGUGUACCCUGTT	1056	AD-15258
3116-3134	AGGGUACACAUUCGCACCCTT	1057	GGGUGCGAAUGUGUACCCUTT	1058	AD-15186
3196-3214	GGAACUGAGCCAGAAACGCTT	1059	GCGUUUCUGGCUCAGUUCCTT	1060	AD-15274
3197-3215	GAACUGAGCCAGAAACGCATT	1061	UGCGUUUCUGGCUCAGUUCTT	1062	AD-15368

TABLE 1a-continued

dsRNA sequences targeted to PCSK9

position in human access. # NM_174936	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense- strand sequence (5'-3')[1]	SEQ ID NO:	Duplex name
3198-3216	AACUGAGCCAGAAACGCAGTT	1063	CUGCGUUUCUGGCUCAGUUTT	1064	AD-15369
3201-3219	UGAGCCAGAAACGCAGAUUTT	1065	AAUCUGCGUUUCUGGCUCATT	1066	AD-15370
3207-3225	AGAAACGCAGAUUGGGCUGTT	1067	CAGCCCAAUCUGCGUUUCUTT	1068	AD-15259
3210-3228	AACGCAGAUUGGGCUGGCUTT	1069	AGCCAGCCCAAUCUGCGUUTT	1070	AD-15408
3233-3251	AGCCAAGCCUCUUCUUACUTsT	1071	AGUAAGAAGAGGCUUGGCUTsT	1072	AD-9597
3233-3251	AGccAAGccucuucuuAcuTsT	1073	AGuAAGAAGAGGCUUGGCUTsT	1074	AD-9723
3233-3251	AfgCfcAfaGfcCfuCfuUfcUfuAfcUfTsT	1075	p-aGfuAfaGfaAfgGfgGfcUfuGfgCfuTsT	1076	AD-14680
3233-3251	AGCfCfAAGCfCfUfCfUfUfCfUfUfACfUfTsT	1077	AGUfAAGAAGAGGCfUfUfGGCfUfTsT	1078	AD-14690
3233-3251	AgCcAaGcCuCuUcUuAcUTsT	1079	p-aGfuAfaGfaAfgGfgGfcUfuGfgCfuTsT	1080	AD-14700
3233-3251	AgCcAaGcCuCuUcUuAcUTsT	1081	AGUfAAGAAGAGGCfUfUfGGCfUfTsT	1082	AD-14710
3233-3251	AfgCfcAfaGfcCfuCfuUfcUfuAfcUfTsT	1083	AGUAAgaAGagGCUUGgcuTsT	1084	AD-14720
3233-3251	AGCfCfAAGCfCfUfCfUfUfCfUfUfACfUfTsT	1085	AGUAAgaAGagGCUUGgcuTsT	1086	AD-14730
3233-3251	AgCcAaGcCuCuUcUuAcUTsT	1087	AGUAAgaAGagGCUUGgcuTsT	1088	AD-14740
3233-3251	UfgGfuUfcCfcUfgAfgGfaCfcAfgCfTsT	1089	p-gCfuGfgUfcCfuCfaGfgGfaAfcCfaTsT	1090	AD-15086
3233-3251	UfGGUfUfCfCfCfUfGAGGACfCfAGCfTsT	1091	GCfUfGGUfCfCfUfCfAGGGAACfCfATsT	1092	AD-15096
3233-3251	UgGuUcCcUgAgGaCcAgCTsT	1093	p-gCfuGfgUfcCfuCfaGfgGfaAfcCfaTsT	1094	AD-15106
3233-3251	UgGuUcCcUgAgGaCcAgCTsT	1095	GCfUfGGUfCfCfUfCfAGGGAACfCfATsT	1096	AD-15116
32333251-	UfgGfuUfcCfcUfgAfgGfaCfcAfgCfTsT	1097	GCUGGucCUcaGGGAAccaTsT	1098	AD-15126
3233-3251	UfGGUfUfCfCfCfUfGAGGACfCfAGCfTsT	1099	GCUGGucCUcaGGGAAccaTsT	1100	AD-15136
3233-3251	UgGuUcCcUgAgGaCcAgCTsT	1101	GCUGGucCUcaGGGAAccaTsT	1102	AD-15146
3242-3260	UCUUCUUACUUCACCCGGCTT	1103	GCCGGGUGAAGUAAGAAGATT	1104	AD-15260
3243-3261	CUUCUUACUUCACCCGGCUTT	1105	AGCCGGGUGAAGUAAGAAGTT	1106	AD-15371
3244-3262	UUCUUACUUCACCCGGCUGTT	1107	CAGCCGGGUGAAGUAAGAATT	1108	AD-15372

TABLE 1a-continued

dsRNA sequences targeted to PCSK9					
position in human access. # NM_174936	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense- strand sequence (5'-3')[1]	SEQ ID NO:	Duplex name
3262-3280	GGGCUCCUCAUUUUUACGGUU	1109	CCGUAAAAAUGAGGAGCCCUU	1110	AD-15172
3263-3281	GGCUCCUCAUUUUUACGGGUU	1111	CCCGUAAAAAUGAGGAGCCUU	1112	AD-15295
3264-3282	GCUCCUCAUUUUUACGGGUUU	1113	ACCCGUAAAAAUGAGGAGCUU	1114	AD-15373
3265-3283	CUCCUCAUUUUUACGGGUAUU	1115	UACCCGUAAAAAUGAGGAGUU	1116	AD-15163
3266-3284	UCCUCAUUUUUACGGGUAAUU	1117	UUACCCGUAAAAAUGAGGAUU	1118	AD-15165
3267-3285	CCUCAUUUUUACGGGUAACUU	1119	GUUACCCGUAAAAAUGAGGUU	1120	AD-15374
3268-3286	CUCAUUUUUACGGGUAACAUU	1121	UGUUACCCGUAAAAAUGAGUU	1122	AD-15296
3270-3288	CAUUUUUACGGGUAACAGUU	1123	ACUGUUACCCGUAAAAAUGUU	1124	AD-15261
3271-3289	AUUUUUACGGGUAACAGUGUU	1125	CACUGUUACCCGUAAAAAUUU	1126	AD-15375
3274-3292	UUUACGGGUAACAGUGAGGUU	1127	CCUCACUGUUACCCGUAAAUU	1128	AD-15262
3308-3326	CAGACCAGGAAGCUCGGUGUU	1129	CACCGAGCUUCCUGGUCUGUU	1130	AD-15376
3310-3328	GACCAGGAAGCUCGGUGAGUU	1131	CUCACCGAGCUUCCUGGUCUU	1132	AD-15377
3312-3330	CCAGGAAGCUCGGUGAGUGUU	1133	CACUCACCGAGCUUCCUGGUU	1134	AD-15409
3315-3333	GGAAGCUCGGUGAGUGAUGUU	1135	CAUCACUCACCGAGCUUCCUU	1136	AD-15378
3324-3342	GUGAGUGAUGGCAGAACGAUU	1137	UCGUUCUGCCAUCACUCACUU	1138	AD-15410
3326-3344	GAGUGAUGGCAGAACGAUGUU	1139	CAUCGUUCUGCCAUCACUCUU	1140	AD-15379
3330-3348	GAUGGCAGAACGAUGCCUGUU	1141	CAGGCAUCGUUCUGCCAUCUU	1142	AD-15187
3336-3354	AGAACGAUGCCUGCAGGCAUU	1143	UGCCUGCAGGCAUCGUUCUUU	1144	AD-15263
3339-3357	ACGAUGCCUGCAGGCAUGGUU	1145	CCAUGCCUGCAGGCAUCGUUU	1146	AD-15264
3348-3366	GCAGGCAUGGAACUUUUUCUU	1147	GAAAAAGUUCCAUGCCUGCUU	1148	AD-15297
3356-3374	GGAACUUUUUCCGUUAUCAUU	1149	UGAUAACGGAAAAAGUUCCUU	1150	AD-15208
3357-3375	GAACUUUUUCCGUUAUCACUU	1151	GUGAUAACGGAAAAAGUUCUU	1152	AD-15209
3358-3376	AACUUUUUCCGUUAUCACCUU	1153	GGUGAUAACGGAAAAAGUUUU	1154	AD-15193

TABLE 1a-continued

dsRNA sequences targeted to PCSK9

position in human access. # NM_174936	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense- strand sequence (5'-3')[1]	SEQ ID NO:	Duplex name
3370-3388	UAUCACCCAGGCCUGAUUCTT	1155	GAAUCAGGCCUGGGUGAUATT	1156	AD-15380
3378-3396	AGGCCUGAUUCACUGGCCUTT	1157	AGGCCAGUGAAUCAGGCCUTT	1158	AD-15298
3383-3401	UGAUUCACUGGCCUGGCGGTT	1159	CCGCCAGGCCAGUGAAUCATT	1160	AD-15299
3385-3403	AUUCACUGGCCUGGCGGAGTT	1161	CUCCGCCAGGCCAGUGAAUTT	1162	AD-15265
3406-3424	GCUUCUAAGGCAUGGUCGGTT	1163	CCGACCAUGCCUUAGAAGCTT	1164	AD-15381
3407-3425	CUUCUAAGGCAUGGUCGGGTT	1165	CCCGACCAUGCCUUAGAAGTT	1166	AD-15210
3429-3447	GAGGGCCAACAACUGUCCCTT	1167	GGGACAGUUGUUGGCCCUCTT	1168	AD-15270
3440-3458	ACUGUCCCUCCUUGAGCACTsT	1169	GUGCUCAAGGAGGGACAGUTsT	1170	AD-9591
3440-3458	AcuGucccuccuuGAGcAcTsT	1171	GUGCUcAAGGAGGGAcAGUTsT	1172	AD-9717
3441-3459	CUGUCCCUCCUUGAGCACCTsT	1173	GGUGCUCAAGGAGGGACAGTsT	1174	AD-9622
3441-3459	cuGucccuccuuGAGcAccTsT	1175	GGUGCUcAAGGAGGGAcAGTsT	1176	AD-9748
3480-3498	ACAUUUAUCUUUUGGGUCUTsT	1177	AGACCCAAAAGAUAAAUGUTsT	1178	AD-9587
3480-3498	AcAuudAucuuuuGGGucuTsT	1179	AGACCcAAAAGAuAAAUGUTsT	1180	AD-9713
3480-3498	AfcAfuUfuAfuCfuUfuUfgGfgUfCUfTsT	1181	p-aGfaCfcCfaAfaAfgAfuAfaAfuGfuTsT	1182	AD-14679
3480-3498	ACfAUfUfUfAUfCfUfUfUfUfGGGUfCfUfTsT	1183	AGACfCfCfAAAAGAUfAAAUfGUfTsT	1184	AD-14689
3480-3498	AcAuUuAuCuUuUgGgUcUTsT	1185	P-aGfaCfcCfaAfaAfgAfuAfaAfuGfuTsT	1186	AD-14699
3480-3498	AcAuUuAuCuUuUgGgUcUTsT	1187	AGACfCfCfAAAAGAUfAAAUfGUfTsT	1188	AD-14709
3480-3498	AfcAfuUfuAfuCfuUfuUfgGfgUfCUfTsT	1189	AGACCcaAAagAUAAAuguTsT	1190	AD-14719
3480-3498	ACfAUfUfUfAUfCfUfUfUfUfGGGUfCfUfTsT	1191	AGACCcaAAagAUAAAuguTsT	1192	AD-14729
3480-3498	AcAuUuAuCuUuUgGgUcUTsT	1193	AGACCcaAAagAUAAAuguTsT	1194	AD-14739
3480-3498	GfcCfaUfcUfgCfuGfcCfgGfaGfcCfTsT	1195	p-gGfcUfcCfgGfcAfgCfaGfaUfgGfcTsT	1196	AD-15085
3480-3498	GCfCfAUfCfUfGCfUfGCfCfGGAGCfCfTsT	1197	GGCfUfCfCfGGCfAGCfAGAUfGGCfTsT	1198	AD-15095
3480-3498	GcCaUcUgCuGcCgGaGcCTsT	1199	P-gGfcUfcCfgGfcAfgCfaGfaUfgGfcTsT	1200	AD-15105

TABLE 1a-continued

	dsRNA sequences targeted to PCSK9				
position in human access. # NM_174936	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense- strand sequence (5'-3')[1]	SEQ ID NO:	Duplex name
3480-3498	GcCaUcUgCuGcCgGaGcCTsT	1201	GGCfUfCfCfGGCfAGCfAGAUfGGCfTsT	1202	AD-15115
3480-3498	GfcCfaUfcUfgCfuGfcCfgGfaGfcC fTsT	1203	GGCUCauGCagCAGUggcTsT	1204	AD-15125
3480-3498	GCfCfAUfCfUfGCfUfGCfCfGGAGCf CfTsT	1205	GGCUCauGCagCAGAUggcTsT	1206	AD-15135
3480-3498	GcCaUcUgCuGcCgGaGcCTsT	1207	GGCUCauGCagCAGAUggcTsT	1208	AD-15145
3481-3499	CAUUUAUCUUUUGGGUCUGTsT	1209	CAGACCCAAAAGAUAAAUGTsT	1210	AD-9578
3481-3499	cAuuuAucuuuuGGGucuGTsT	1211	cAGACCcAAAAGAuAAAUGTsT	1212	AD-9704
3485-3503	UAUCUUUUGGGUCUGUCCUTsT	1213	AGGACAGACCCAAAAGAUATsT	1214	AD-9558
3485-3503	uAucuuuuGGGucuGuccuTsT	1215	AGGAcAGACCcAAAAGAuATsT	1216	AD-9684
3504-3522	CUCUGUUGCCUUUUUACAGTsT	1217	CUGUAAAAAGGCAACAGAGTsT	1218	AD-9634
3504-3522	cucuGuuGccuuuuuAcAGTsT	1219	CUGuAAAAAGGcAAcAGAGTsT	1220	AD-9760
3512-3530	CCUUUUUACAGCCAACUUUTT	1221	AAAGUUGGCUGUAAAAAGGTT	1222	AD-15411
3521-3539	AGCCAACUUUUCUAGACCUTT	1223	AGGUCUAGAAAAGUUGGCUTT	1224	AD-15266
3526-3544	ACUUUUCUAGACCUGUUUUTT	1225	AAAACAGGUCUAGAAAAGUTT	1226	AD-15382
3530-3548	UUCUAGACUGUUUUGCUUTsT	1227	AAGCAAAACAGGUCUAGAATsT	1228	AD-9554
3530-3548	uucuAGAccuGuuuuGcuuTsT	1229	AAGcAAAAcAGGUCuAGAATsT	1230	AD-9680
3530-3548	UfuCfuAfgAfcCfuGfuUfuUfgCfuU fTsT	1231	p-aAfgCfaAfaAfcAfgGfuCfuAfgAfaTsT	1232	AD-14676
3530-3548	UfUfCfUfAGACfCfUfGUfUfUfUfGC fUfUfTsT	1233	AAGCfAAAACfAGGUfCfUfAGAATsT	1234	AD-14686
3530-3548	UuCuAgAcCuGuUuUgCuUTsT	1235	p-aAfgCfaAfaAfcAfgGfuCfuAfgAfaTsT	1236	AD-14696
3530-3548	UuCuAgAcCuGuUuUgCuUTsT	1237	AAGCfAAAACfAGGUfCfUfAGAATsT	1238	AD-14706
3530-3548	UfuCfuAfgAfcCfuGfuUfuUffCfuU fTsT	1239	AAGcAaaACagGUCUAgaaTsT	1240	AD-14716
3530-3548	UfUfCfUfAGACfCfUfGUfUfUfUfGC fUfUfTsT	1241	AAGcAaaACagGUCUAgaaTsT	1242	AD-14726
3530-3548	UuCuAgAcCuGuUuUgCuUTsT	1243	AAGcAaaACagGUCUAgaaTsT	1244	AD-14736
3530-3548	CfaUfaGfgCfcUfgGfaGfuUfuAfuU fTsT	1245	p-aAfuAfaAfcUfcCfaGfgCfcUfaUfgTsT	1246	AD-15082

TABLE 1a-continued

dsRNA sequences targeted to PCSK9

position in human access. # NM_174936	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense-strand sequence (5'-3')[1]	SEQ ID NO:	Duplex name
3530-3548	CfAUfAGGCfCfUfGGAGUfUfUfAUfUfTsT	1247	AAUfAAACfUfCfCfAGGCfCfUfAUfGTsT	1248	AD-15092
3530-3548	CaUaGgCcUgGaGuUuAuUTsT	1249	P-aAfuAfaAfcUfcCfaGfgCfcUfaUfgTsT	1250	AD-15102
3530-3548	CaUaGgCcUgGaGuUuAuUTsT	1251	AAUfAAACfUfCfCfAGGCfCfUfAUfGTsT	1252	AD-15112
3530-3548	CfaUfaGfgCfcUfgGfaGfuUfuAfuUfTsT	1253	AAUAAacUCcaGGCCUaugTsT	1254	AD-15122
3530-3548	CfAUfAGGCfCfUfGGAGUfUfUfAUfUfTsT	1255	AAUAAacUCcaGGCCUaugTsT	1256	AD-15132
3530-3548	CaUaGgCcUgGaGuUuAuUTsT	1257	AAUAAacUCcaGGCCUaugTsT	1258	AD-15142
3531-3549	UCUAGACCUGUUUUGCUUUTsT	1259	AAAGCAAAACAGGUCUAGATsT	1260	AD-9553
3531-3549	ucuAGAccuGuuuuGcuuuTsT	1261	AAAGcAAAAcAGGUCuAGATsT	1262	AD-9679
3531-3549	UfcUfaGfaCfcUfgUfuUfuGfcUfuUfTsT	1263	p-aAfaGfcAfaAfaCfaGfgUfcUfaGfaTsT	1264	AD-14675
3531-3549	UfCfUfAGACfCfUfGUfUfUfUfGCfUfUfUfTsT	1265	AAAGCfAAAACfAGGUfCfUfAGATsT	1266	AD-14685
3531-3549	UcUaGaCcUgUuUuGcUuUTsT	1267	P-aAfaGfcAfaAfaCfaGfgUfcUfaGfaTsT	1268	AD-14695
3531-3549	UcUaGaCcUgUuUuGcUuUTsT	1269	AAAGCfAAAACfAGGUfCfUfAGATsT	1270	AD-14705
3531-3549	UfcUfaGfaCfcUfgUfuUfuGfcUfuUfTsT	1271	AAAGCaaAAcaGGUCUagaTsT	1272	AD-14715
3531-3549	UfCfUfAGACfCfUfGUfUfUfUfGCfUfUfUfTsT	1273	AAAGCaaAAcaGGUCUagaTsT	1274	AD-14725
3531-3549	UcUaGaCcUgUuUuGcUuUTsT	1275	AAAGCaaAAcaGGUCUagaTsT	1276	AD-14735
3531-3549	UfcAfuAfgGfcCfuGfgAfgUfuUfaUfTsT	1277	p-aUfaAfaCfuCfcAfgGfcCfuAfuGfaTsT	1279	AD-15081
3531-3549	UfCfAUfAGGCfCfUfGGAGUfUfUfAUfTsT	1279	AUfAAACfUfCfCfAGGCfCfUfAUfGATsT	1280	AD-15091
3531-3549	UcAuAgGcCuGgAgUuUaUTsT	1281	P-aUfaAfaCfuCfcAfgGfcCfuAfuGfaTsT	1282	AD-15101
3531-3549	UcAuAgGcCuGgAgUuUaUTsT	1283	AUfAAACfUfCfCfAGGCfCfUfAUfGATsT	1284	AD-15111
3531-3549	UfcAfuAfgGfcCfuGfgAfgUfuUfaUfTsT	1285	AUAAAcuCCagGCCUaugaTsT	1287	AD-15121
3531-3549	UfCfAUfAGGCfCfUfGGAGUfUfUfAUfTsT	1287	AUAAAcuCCagGCCUaugaTsT	1288	AD-15131
3531-3549	UcAuAgGcCuGgAgUuUaUTsT	1289	AUAAAcuCCagGCCUaugaTsT	1290	AD-15141
3557-3575	UGAAGAUAUUUAUUCUGGGTsT	1291	CCCAGAAUAAAUAUCUUCATsT	1292	AD-9626

TABLE 1a-continued

dsRNA sequences targeted to PCSK9					
position in human access. # NM_174936	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense-strand sequence (5'-3')[1]	SEQ ID NO:	Duplex name
3557-3575	uGAAGAuAuuuAuucuGGGTsT	1293	CCcAGAAuAAAuAUCUUcATsT	1294	AD-9752
3570-3588	CUGGGUUUUGUAGCAUUUTsT	1295	AAAUGCUACAAAACCCAGATsT	1296	AD-9629
3570-3588	ucuGGGuuuuGuAGcAuuuTsT	1297	AAAUGCuAcAAAACCcAGATsT	1298	AD-9755
3613-3631	AUAAAAACAAACAAACGUUTT	1299	AACGUUUGUUUGUUUUUAUTT	1300	AD-15412
3617-3635	AAACAAACAAACGUUGUCCTT	1301	GGACAACGUUUGUUUGUUUTT	1302	AD-15211
3618-3636	AACAAACAAACGUUGUCCUTT	1303	AGGACAACGUUUGUUUGUUTT	1304	AD-15300

U, C, A, G: corresponding ribonucleotide; T: deoxythymidine; u, c, a, g: corresponding 2'O-methyl ribonucleotide; Uf, Cf, Af, Gf: corresponding 2'-deoxy-2'-fluoro ribonucleotide; where nucleotides are written in sequence, they are connected by 3'-5' phosphodiester groups; nucleotides with interjected "s" are connected by 3'-O-5'-O phosphorothiodiester groups; unless denoted by prefix "p-", oligonucleotides are devoid of a 5'-phosphate group on the 5'-most nucleotide; all oligonucleotides bear 3'-OH on the 3'-most nucleotide

TABLE 1b

Screening of siRNAs targeted to PCSK9					
	Mean percent remaining mRNA transcript at siRNA concentration/in cell type				
Duplex name	100 nM/ HepG2	30 nM/ HepG2	3 nM/ HepG2	30 nM/ HeLa	IC50 in HepG2 [nM]	IC50 in Cynomolgous monkey Hepatocyte [nM]s
AD-15220				35		
AD-15275				56		
AD-15301				70		
AD-15276				42		
AD-15302				32		
AD-15303				37		
AD-15221				30		
AD-15413				61		
AD-15304				70		
AD-15305				36		
AD-15306				20		
AD-15307				38		
AD-15277				50		
AD-9526	74	89				
AD-9652		97				
AD-9519		78				
AD-9645		66				
AD-9523		55				
AD-9649		60				
AD-9569		112				
AD-9695		102				
AD-15222				75		
AD-15278				78		
AD-15178				83		
AD-15308				84		
AD-15223				67		
AD-15309				34		
AD-15279				44		
AD-15194				63		
AD-15310				42		
AD-15311				30		
AD-15392				18		
AD-15312				21		
AD-15313				19		
AD-15280				81		

Note: the header row with the four numeric columns (100 nM/HepG2, 30 nM/HepG2, 3 nM/HepG2, 30 nM/HeLa) falls under the spanning header "Mean percent remaining mRNA transcript at siRNA concentration/in cell type"; IC50 in HepG2 [nM] and IC50 in Cynomolgous monkey Hepatocyte [nM]s are separate columns.

TABLE 1b-continued

	Screening of siRNAs targeted to PCSK9					
	Mean percent remaining mRNA transcript at siRNA concentration/in cell type				IC50 in	IC50 in Cynomolgous
Duplex name	100 nM/ HepG2	30 nM/ HepG2	3 nM/ HepG2	30 nM/ HeLa	HepG2 [nM]	monkey Hepatocyte [nM]s
AD-15267				82		
AD-15314				32		
AD-15315				74		
AD-9624		94				
AD-9750		96				
AD-9623	43	66				
AD-9749		105				
AD-15384				48		
AD-9607		32	28		0.20	
AD-9733		78	73			
AD-9524		23	28		0.07	
AD-9650		91	90			
AD-9520		23	32			
AD-9520		23				
AD-9646		97	108			
AD-9608		37				
AD-9734		91				
AD-9546		32				
AD-9672		57				
AD-15385				54		
AD-15393				31		
AD-15316				37		
AD-15317				37		
AD-15318				63		
AD-15195				45		
AD-15224				57		
AD-15188				42		
AD-15225				51		
AD-15281				89		
AD-15282				75		
AD-15319				61		
AD-15226				56		
AD-15271				25		
AD-15283				25		
AD-15284				64		
AD-15189				17		
AD-15227				62		
AD-9547		31	29		0.20	
AD-9673		56	57			
AD-9548		54	60			
AD-9674		36	57			
AD-9529		60				
AD-9655		140				
AD-9605		27	31		0.27	
AD-9731		31	31		0.32	
AD-9596		37				
AD-9722		76				
AD-9583		42				
AD-9709		104				
AD-9579		113				
AD-9705		81				
AD-15394				32		
AD-15196				72		
AD-15197				85		
AD-15198				71		
AD-9609	66	71				
AD-9735		115				
AD-9537		145				
AD-9663		102				
AD-9528		113				
AD-9654		107				
AD-9515		49				
AD-9641		92				
AD-9514		57				
AD-9640		89				
AD-9530		75				
AD-9656		77				
AD-9538	79	80				
AD-9664		53				

TABLE 1b-continued

	Screening of siRNAs targeted to PCSK9					
	Mean percent remaining mRNA transcript at siRNA concentration/in cell type				IC50 in	IC50 in Cynomolgous
Duplex name	100 nM/ HepG2	30 nM/ HepG2	3 nM/ HepG2	30 nM/ HeLa	HepG2 [nM]	monkey Hepatocyte [nM]s
AD-9598	69	83				
AD-9724		127				
AD-9625	58	88				
AD-9751		60				
AD-9556		46				
AD-9682		38				
AD-9539	56	63				
AD-9665		83				
AD-9517		36				
AD-9643		40				
AD-9610		36	34		0.04	
AD-9736		22	29		0.04	0.5
AD-14681				33		
AD-14691				27		
AD-14701				32		
AD-14711				33		
AD-14721				22		
AD-14731				21		
AD-14741				22		
AD-15087				37		
AD-15097				51		
AD-15107				26		
AD-15117				28		
AD-15127				33		
AD-15137				54		
AD-15147				52		
AD-9516		94				
AD-9642		105				
AD-9562		46	51			
AD-9688		26	34		4.20	
AD-14677				38		
AD-14687				52		
AD-14697				35		
AD-14707				58		
AD-14717				42		
AD-14727				50		
AD-14737				32		
AD-15083				16		
AD-15093				24		
AD-15103				11		
AD-15113				34		
AD-15123				19		
AD-15133				15		
AD-15143				16		
AD-9521		50				
AD-9647		62				
AD-9611		48				
AD-9737		68				
AD-9592	46	55				
AD-9718		78				
AD-9561		64				
AD-9687		84				
AD-9636		42	41		2.10	
AD-9762		9	28		0.40	0.5
AD-9540		45				
AD-9666		81				
AD-9535	48	73				
AD-9661		83				
AD-9559		35				
AD-9685		77				
AD-9533		100				
AD-9659		88				
AD-9612		122				
AD-9738		83				
AD-9557	75	96				
AD-9683		48				
AD-9531		31	32		0.53	
AD-9657		23	29		0.66	0.5
AD-14673				81		

TABLE 1b-continued

	Screening of siRNAs targeted to PCSK9					
	Mean percent remaining mRNA transcript at siRNA concentration/in cell type				IC50 in	IC50 in Cynomolgous
Duplex name	100 nM/ HepG2	30 nM/ HepG2	3 nM/ HepG2	30 nM/ HeLa	HepG2 [nM]	monkey Hepatocyte [nM]s
AD-14683				56		
AD-14693				56		
AD-14703				68		
AD-14713				55		
AD-14723				24		
AD-14733				34		
AD-15079				85		
AD-15089				54		
AD-15099				70		
AD-15109				67		
AD-15119				67		
AD-15129				57		
AD-15139				69		
AD-9542		160				
AD-9668		92				
AD-9739		109				
AD-9637	56	83				
AD-9763		79				
AD-9630		82				
AD-9756		63				
AD-9593		55				
AD-9719		115				
AD-9601		111				
AD-9727		118				
AD-9573		36	42		1.60	
AD-9699		32	36		2.50	
AD-15228				26		
AD-15395				53		
AD-9602		126				
AD-9728		94				
AD-15386				45		
AD-9580		112				
AD-9706		86				
AD-9581		35				
AD-9707		81				
AD-9543		51				
AD-9669		97				
AD-9574		74				
AD-9700						
AD-15320				26		
AD-15321				34		
AD-15199				64		
AD-15167				86		
AD-15164				41		
AD-15166				43		
AD-15322				64		
AD-15200				46		
AD-15213				27		
AD-15229				44		
AD-15215				49		
AD-15214				101		
AD-9315		15	32		0.98	
AD-9326		35	51			
AD-9318		14	37		0.40	
AD-9323		14	33			
AD-9314		11	22		0.04	
AD-10792					0.10	0.10
AD-10796					0.1	0.1
AD-9638		101				
AD-9764		112				
AD-9525		53				
AD-9651		58				
AD-9560		97				
AD-9686		111				
AD-9536		157				
AD-9662		81				
AD-9584	52	68				
AD-9710		111				
AD-15323				62		

TABLE 1b-continued

Screening of siRNAs targeted to PCSK9

Duplex name	Mean percent remaining mRNA transcript at siRNA concentration/in cell type				IC50 in HepG2 [nM]	IC50 in Cynomolgous monkey Hepatocyte [nM]s
	100 nM/ HepG2	30 nM/ HepG2	3 nM/ HepG2	30 nM/ HeLa		
AD-9551		91				
AD-9677		62				
AD-15230				52		
AD-15231				25		
AD-15285				36		
AD-15396				27		
AD-15397				56		
AD-9600		112				
AD-9726		95				
AD-9606		107				
AD-9732		105				
AD-9633	56	75				
AD-9759		111				
AD-9588		66				
AD-9714		106				
AD-9589	67	85				
AD-9715		113				
AD-9575		120				
AD-9701		100				
AD-9563		103				
AD-9689		81				
AD-9594	80	95				
AD-9720		92				
AD-9585		83				
AD-9711		122				
AD-9614		100				
AD-9740		198				
AD-9615		116				
AD-9741		130				
AD-9534		32	30			
AD-9534		32				
AD-9660		89	79			
AD-15324				46		
AD-15232				19		
AD-15233				25		
AD-15234				59		
AD-15286				109		
AD-9590		122				
AD-9716		114				
AD-9632		34				
AD-9758		96				
AD-9567		41				
AD-9693		50				
AD-9586	81	104				
AD-9712		107				
AD-9564		120				
AD-9690		92				
AD-9616	74	84				
AD-9742		127				
AD-15398				24		
AD-9617		111				
AD-9743		104				
AD-9635	73	90				
AD-9761		15	33			0.5
AD-9568		76				
AD-9694		52				
AD-9576		47				
AD-9702		79				
AD-9627		69				
AD-9753		127				
AD-9628		141				
AD-9754		89				
AD-9631		80				
AD-9757		78				
AD-9595		31	32			
AD-9721		87	70			
AD-9544		68				
AD-9670		67				
AD-15235				25		

TABLE 1b-continued

	Screening of siRNAs targeted to PCSK9					
	Mean percent remaining mRNA transcript at siRNA concentration/in cell type				IC50 in	IC50 in Cynomolgous
Duplex name	100 nM/ HepG2	30 nM/ HepG2	3 nM/ HepG2	30 nM/ HeLa	HepG2 [nM]	monkey Hepatocyte [nM]s
AD-15236				73		
AD-15168				100		
AD-15174				92		
AD-15325				81		
AD-15326				65		
AD-9570	35	42				
AD-9696		77				
AD-9566		38				
AD-9692		78				
AD-9532		100				
AD-9658		102				
AD-9549		50				
AD-9675		78				
AD-9541		43				
AD-9667		73				
AD-9550		36				
AD-9676		100				
AD-9571		27	32			
AD-9697		74	89			
AD-9572	47	53				
AD-9698		73				
AD-15327				82		
AD-9639		30	35			
AD-9765		82	74			
AD-9518		31	35		0.60	
AD-9518		31				
AD-9644		35	37		2.60	0.5
AD-14672				26		
AD-14682				27		
AD-14692				22		
AD-14702				19		
AD-14712				25		
AD-14722				18		
AD-14732				32		
AD-15078				86		
AD-15088				97		
AD-15098				74		
AD-15108				67		
AD-15118				76		
AD-15128				86		
AD-15138				74		
AD-15237				30		
AD-15287				30		
AD-15238				36		
AD-15328				35		
AD-15399				47		
AD-9582		37				
AD-9708		81				
AD-9545		31	43			
AD-9671		15	33		2.50	
AD-14674				16		
AD-14684				26		
AD-14694				18		
AD-14704				27		
AD-14714				20		
AD-14724				18		
AD-14734				18		
AD-15080				29		
AD-15090				23		
AD-15100				26		
AD-15110				23		
AD-15120				20		
AD-15130				20		
AD-15140				19		
AD-9522		59				
AD-9648		78				
AD-9552		80				
AD-9678		76				
AD-9618		90				

TABLE 1b-continued

	Screening of siRNAs targeted to PCSK9					
	Mean percent remaining mRNA transcript at siRNA concentration/in cell type				IC50 in	IC50 in Cynomolgous
Duplex name	100 nM/ HepG2	30 nM/ HepG2	3 nM/ HepG2	30 nM/ HeLa	HepG2 [nM]	monkey Hepatocyte [nM]s
AD-9744		91				
AD-15239				38		
AD-15212				19		
AD-15240				43		
AD-15177				59		
AD-15179				13		
AD-15180				15		
AD-15241				14		
AD-15268				42		
AD-15242				21		
AD-15216				28		
AD-15176				35		
AD-15181				35		
AD-15243				22		
AD-15182				42		
AD-15244				31		
AD-15387				23		
AD-15245				18		
AD-9555		34				
AD-9681		55				
AD-9619	42	61				
AD-9745		56				
AD-9620	44	77				
AD-9746		89				
AD-15288				19		
AD-15246				16		
AD-15289				37		
AD-9324		59	67			
AD-15329				103		
AD-15330				62		
AD-15169				22		
AD-15201				6		
AD-15331				14		
AD-15190				47		
AD-15247				61		
AD-15248				22		
AD-15175				45		
AD-15249				51		
AD-15250				96		
AD-15400				12		
AD-15332				22		
AD-15388				30		
AD-15333				20		
AD-15334				96		
AD-15335				75		
AD-15183				16		
AD-15202				41		
AD-15203				39		
AD-15272				49		
AD-15217				16		
AD-15290				15		
AD-15218				13		
AD-15389				13		
AD-15336				40		
AD-15337				19		
AD-15191				33		
AD-15390				25		
AD-15338				9		
AD-15204				33		
AD-15251				76		
AD-15205				14		
AD-15171				16		
AD-15252				58		
AD-15339				20		
AD-15253				15		
AD-15340				18		
AD-15291				17		
AD-15341				11		
AD-15401				13		

TABLE 1b-continued

	Screening of siRNAs targeted to PCSK9					
	Mean percent remaining mRNA transcript at siRNA concentration/in cell type				IC50 in	IC50 in Cynomolgous
Duplex name	100 nM/ HepG2	30 nM/ HepG2	3 nM/ HepG2	30 nM/ HeLa	HepG2 [nM]	monkey Hepatocyte [nM]s
AD-15342				30		
AD-15343				21		
AD-15292				16		
AD-15344				20		
AD-15254				18		
AD-15345				18		
AD-15206				15		
AD-15346				16		
AD-15347				62		
AD-9577		33	31			
AD-9703		17	26			1
AD-14678				22		
AD-14688				23		
AD-14698				23		
AD-14708				14		
AD-14718				31		
AD-14728				25		
AD-14738				31		
AD-15084				19		
AD-15094				11		
AD-15104				16		
AD-15114				15		
AD-15124				11		
AD-15134				12		
AD-15144				9		
AD-15391				7		
AD-15348				13		
AD-15349				8		
AD-15170				40		
AD-15350				14		
AD-15402				27		
AD-15293				27		
AD-15351				14		
AD-15403				11		
AD-15404				38		
AD-15207				15		
AD-15352				23		
AD-15255				31		
AD-9603		123				
AD-9729		56				
AD-9599		139				
AD-9725		38				
AD-9621		77				
AD-9747		63				
AD-15405				32		
AD-15353				39		
AD-15354				49		
AD-15406				35		
AD-15407				39		
AD-15355				18		
AD-15356				50		
AD-15357				54		
AD-15269				23		
AD-9565		74				
AD-9691		49				
AD-15358				12		
AD-15359				24		
AD-15360				13		
AD-15219				19		
AD-15361				24		
AD-15273				36		
AD-15362				31		
AD-15192				20		
AD-15256				19		
AD-15363				33		
AD-15364				24		
AD-9604	35	49				
AD-9730		85				
AD-9527		45				

TABLE 1b-continued

Screening of siRNAs targeted to PCSK9

Duplex name	Mean percent remaining mRNA transcript at siRNA concentration/in cell type				IC50 in HepG2 [nM]	IC50 in Cynomolgous monkey Hepatocyte [nM]s
	100 nM/ HepG2	30 nM/ HepG2	3 nM/ HepG2	30 nM/ HeLa		
AD-9653		86				
AD-15365				62		
AD-15294				30		
AD-15173				12		
AD-15366				21		
AD-15367				11		
AD-15257				18		
AD-15184				50		
AD-15185				12		
AD-15258				73		
AD-15186				36		
AD-15274				19		
AD-15368				7		
AD-15369				17		
AD-15370				19		
AD-15259				38		
AD-15408				52		
AD-9597		23	21		0.04	
AD-9723		12	26			0.5
AD-14680				15		
AD-14690				18		
AD-14700				15		
AD-14710				15		
AD-14720				18		
AD-14730				18		
AD-14740				17		
AD-15086				85		
AD-15096				70		
AD-15106				71		
AD-15116				73		
AD-15126				71		
AD-15136				56		
AD-15146				72		
AD-15260				79		
AD-15371				24		
AD-15372				52		
AD-15172				27		
AD-15295				22		
AD-15373				11		
AD-15163				18		
AD-15165				13		
AD-15374				23		
AD-15296				13		
AD-15261				20		
AD-15375				90		
AD-15262				72		
AD-15376				14		
AD-15377				19		
AD-15409				17		
AD-15378				18		
AD-15410				8		
AD-15379				11		
AD-15187				36		
AD-15263				18		
AD-15264				75		
AD-15297				21		
AD-15208				6		
AD-15209				28		
AD-15193				131		
AD-15380				88		
AD-15298				43		
AD-15299				99		
AD-15265				95		
AD-15381				18		
AD-15210				40		
AD-15270				83		
AD-9591	75	95				
AD-9717		105				
AD-9622		94				

TABLE 1b-continued

	Screening of siRNAs targeted to PCSK9					
	Mean percent remaining mRNA transcript at siRNA concentration/in cell type				IC50 in	IC50 in Cynomolgous
Duplex name	100 nM/ HepG2	30 nM/ HepG2	3 nM/ HepG2	30 nM/ HeLa	HepG2 [nM]	monkey Hepatocyte [nM]s
AD-9748		103				
AD-9587		63	49			
AD-9713		22	25			0.5
AD-14679				19		
AD-14689				24		
AD-14699				19		
AD-14709				21		
AD-14719				24		
AD-14729				23		
AD-14739				24		
AD-15085				74		
AD-15095				60		
AD-15105				33		
AD-15115				30		
AD-15125				54		
AD-15135				51		
AD-15145				49		
AD-9578	49	61				
AD-9704		111				
AD-9558		66				
AD-9684		63				
AD-9634		29	30			
AD-9760		14	27			
AD-15411				5		
AD-15266				23		
AD-15382				12		
AD-9554		23	24			
AD-9680		12	22		0.1	0.1
AD-14676				12		
AD-14686				13		
AD-14696				12		.1
AD-14706				18		.1
AD-14716				17		.1
AD-14726				16		.1
AD-14736				9		.1
AD-15082				27		
AD-15092				28		
AD-15102				19		
AD-15112				17		
AD-15122				56		
AD-15132				39		
AD-15142				46		
AD-9553		27	22		0.02	
AD-9679		17	21			0.1
AD-14675				11		
AD-14685				19		
AD-14695				12		
AD-14705				16		
AD-14715				19		
AD-14725				19		
AD-14735				19		
AD-15081				30		
AD-15091				16		
AD-15101				16		
AD-15111				11		
AD-15121				19		
AD-15131				17		
AD-15141				18		
AD-9626		97	68			
AD-9752		28	33			
AD-9629		23	24			
AD-9755		28	29			0.5
AD-15412				21		
AD-15211				73		
AD-15300				41		

TABLE 2a

Sequences of modified dsRNA targeted to PCSK9

Duplex number	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense-strand sequence (5'-3')[1]	SEQ ID NO:
AD-10792	GccuGGAGuuuAuucGGAATsT	1305	UUCCGAAuAAACUCcAGGCTsT	1306
AD-10793	GccuGGAGuuuAuucGGAATsT	1307	uUcCGAAuAAACUccAGGCTsT	1308
AD-10796	GccuGGAGuuuAuucGGAATsT	1309	UUCCGAAUAAACUCCAGGCTsT	1310
AD-12038	GccuGGAGuuuAuucGGAATsT	1311	uUCCGAAUAAACUCCAGGCTsT	1312
AD-12039	GccuGGAGuuuAuucGGAATsT	1313	UuCCGAAUAAACUCCAGGCTsT	1314
AD-12040	GccuGGAGuuuAuucGGAATsT	1315	UUcCGAAUAAACUCCAGGCTsT	1316
AD-12041	GccuGGAGuuuAuucGGAATsT	1317	UUCcGAAUAAACUCCAGGCTsT	1318
AD-12042	GCCUGGAGUUUAUUCGGAATsT	1319	uUCCGAAUAAACUCCAGGCTsT	1320
AD-12043	GCCUGGAGUUUAUUCGGAATsT	1321	UuCCGAAUAAACUCCAGGCTsT	1322
AD-12044	GCCUGGAGUUUAUUCGGAATsT	1323	UUcCGAAUAAACUCCAGGCTsT	1324
AD-12045	GCCUGGAGUUUAUUCGGAATsT	1325	UUCcGAAUAAACUCCAGGCTsT	1326
AD-12046	GccuGGAGuuuAuucGGAA	1327	UUCCGAAUAAACUCCAGGCscsu	1328
AD-12047	GccuGGAGuuuAuucGGAAA	1329	UUUCCGAAUAAACUCCAGGCscsu	1330
AD-12048	GccuGGAGuuuAuucGGAAAA	1331	UUUUCCGAAUAAACUCCAGGCscsu	1332
AD-12049	GccuGGAGuuuAuucGGAAAAG	1333	CUUUUCCGAAUAAACUCCAGGCscsu	1334
AD-12050	GccuGGAGuuuAuucGGAATTab	1335	UUCCGAAUAAACUCCAGGCTTab	1336
AD-12051	GccuGGAGuuuAuucGGAAATTab	1337	UUUCCGAAuAAACUCCAGGCTTab	1338
AD-12052	GccuGGAGuuuAuucGGAAAATTab	1339	UUUUCCGAAUAAACUCCAGGCTTab	1340
AD-12053	GccuGGAGuuuAuucGGAAAAGTTab	1341	CUUUUCCGAAUAAACUCCAGGCTTab	1342
AD-12054	GCCUGGAGUUUAUUCGGAATsT	1343	UUCCGAAUAAACUCCAGGCscsu	1344
AD-12055	GccuGGAGuuuAuucGGAATsT	1345	UUCCGAAUAAACUCCAGGCscsu	1346
AD-12056	GcCuGgAgUuUaUuCgGaA	1347	UUCCGAAUAAACUCCAGGCTTab	1348
AD-12057	GcCuGgAgUuUaUuCgGaA	1349	UUCCGAAUAAACUCCAGGCTsT	1350
AD-12058	GcCuGgAgUuUaUuCgGaA	1351	UUCCGAAuAAACUCcAGGCTsT	1352
AD-12059	GcCuGgAgUuUaUuCgGaA	1353	uUcCGAAuAAACUccAGGCTsT	1354
AD-12060	GcCuGgAgUuUaUuCgGaA	1355	UUCCGaaUAaaCUCCAggc	1356
AD-12061	GcCuGgnAgUuUaUuCgGaATsT	1357	UUCCGaaUAaaCUCCAggcTsT	1358
AD-12062	GcCuGgAgUuUaUuCgGaATTab	1359	UUCCGaaUAaaCUCCAggcTTab	1360
AD-12063	GcCuGgAgUuUaUuCgGaA	1361	UUCCGaaUAaaCUCCAggcscsu	1362
AD-12064	GcCuGgnAgUuUaUuCgGaATsT	1363	UUCCGAAUAAACUCcAGGCTsT	1364
AD-12065	GcCuGgAgUuUaUuCgGaATTab	1365	UUCCGAAuAAACUCcAGGCTTab	1366
AD-12066	GcCuGgAgUuUaUuCgGaA	1367	UUCCGAAUAAACUCcAGGCscsu	1368
AD-12067	GcCuGgnAgUuUaUuCgGaATsT	1369	UUCCGAAUAAACUCCAGGCTsT	1370
AD-12068	GcCuGgAgUuUaUuCgGaATTab	1371	UUCCGAAUAAACUCCAGGCTTab	1372
AD-12069	GcCuGgAgUuUaUuCgGaA	1373	UUCCGAAUAAACUCCAGGCscsu	1374
AD-12338	GfcCfuGfgAfgUfuUfaUfuCfgGfaAf	1375	P-uUfcCfgAfaUfaAfaCfuCfcAfgGfc	1376

TABLE 2a-continued

Sequences of modified dsRNA targeted to PCSK9

Duplex number	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense-strand sequence (5'-3')[1]	SEQ ID NO:
AD-12339	GcCuGgAgUuUaUuCgGaA	1377	P-uUfcCfgAfaUfaAfaCfuCfcAfgGfc	1378
AD-12340	GccuGGAGuuuAuucGGAA	1379	P-uUfcCfgAfaUfaAfaCfuCfcAfgGfc	1380
AD-12341	GfcCfuGfgAfgUfuUfaUfuCfgGfaAfTsT	1381	P-uUfcCfgAfaUfaAfaCfuCfcAfgGfcTsT	1382
AD-12342	GfcCfuGfgAfgUfuUfaUfuCfgGfaAfTsT	1383	UUCCGAAuAAACUCcAGGCTsT	1384
AD-12343	GfcCfuGfgAfgUfuUfaUfuCfgGfaAfTsT	1385	uUcCGAAuAAACUccAGGCTsT	1386
AD-12344	GfcCfuGfgAfgUfuUfaUfuCfgGfaAfTsT	1387	UUCCGAAUAAACUCCAGGCTsT	1388
AD-12345	GfcCfuGfgAfgUfuUfaUfuCfgGfaAfTsT	1389	UUCCGAAUAAACUCCAGGCscsu	1390
AD-12346	GfcCfuGfgAfgUfuUfaUfuCfgGfaAfTsT	1391	UUCCGaaUAaaCUCCAggcscsu	1392
AD-12347	GCCUGGAGUUUAUUCGGAATsT	1393	P-uUfcCfgAfaUfaAfaCfuCfcAfgGfcTsT	1394
AD-12348	GccuGGAGuuuAuucGGAATsT	1395	P-uUfcCfgAfaUfaAfaCfuCfcAfgGfcTsT	1396
AD-12349	GcCuGgnAgUuUaUuCgGaATsT	1397	P-uUfcCfgAfaUfaAfaCfuCfcAfgGfcTsT	1398
AD-12350	GfcCfuGfgAfgUfuUfaUfuCfgGfaAfTTab	1399	P-uUfcCfgAfaUfaAfaCfuCfcAfgGfcTTab	1400
AD-12351	GfcCfuGfgAfgUfuUfaUfuCfgGfaAf	1401	P-uUfcCfgAfaUfaAfaCfuCfcAfgGfcsCfsu	1402
AD-12352	GfcCfuGfgAfgUfuUfaUfuCfgGfaAf	1403	UUCCGaaUAaaCUCCAggcscsu	1404
AD-12354	GfcCfuGfgAfgUfuUfaUfuCfgGfaAf	1405	UUCCGAAUAAACUCCAGGCscsu	1406
AD-12355	GfcCfuGfgAfgUfuUfaUfuCfgGfaAf	1407	UUCCGAAUAAACUCcAGGCTsT	1408
AD-12356	GfcCfuGfgAfgUfuUfaUfuCfgGfaAf	1409	uUcCGAAUAAACUccAGGCTsT	1410
AD-12357	GmocCmouGmogAm02gUmouUmoaUmouCmogGmoaA	1411	UUCCGaaUAaaCUCCAggc	1412
AD-12358	GmocCmouGmogAm02gUmouUmoaUmouCmogGmoaA	1413	P-uUfcCfgAfaUfaAfaCfuCfcAfgGfc	1414
AD-12359	GmocCmouGmogAm02gUmouUmoaUmouCmogGmoaA	1415	P-uUfcCfgAfaUfaAfaCfuCfcAfgGfcsCfsu	1416
AD-12360	GmocCmouGmogAm02gUmouUmoaUmouCmogGmoaA	1417	UUCCGAAUAAACUCCAGGCscsu	1418
AD-12361	GmocCmouGmogAm02gUmouUmoaUmouCmogGmoaA	1419	UUCCGAAUAAACUCcAGGCTsT	1420
AD-12362	GmocCmouGmogAm02gUmouUmoaUmouCmogGmoaA	1421	uUcCGAAUAAACUccAGGCTsT	1422
AD-12363	GmocCmouGmogAm02gUmouUmoaUmouCmogGmoaA	1423	UUCCGaaUAaaCUCCAggcscsu	1424
AD-12364	GmocCmouGmogAmogUmouUmoaUmouCmogGmoaATsT	1425	UUCCGaaUAaaCUCCAggcTsT	1426
AD-12365	GmocCmouGmogAmogUmouUmoaUmouCmogGmoaATsT	1427	UUCCGAAUAAACUCcAGGCTsT	1428
AD-12366	GmocCmouGmogAmogUmouUmoaUmouCmogGmoaATsT	1429	UUCCGAAUAAACUCCAGGCTsT	1430
AD-12367	GmocmocmouGGAGmoumoumouAmoumoumoucGGAATsT	1431	UUCCGaaUAaaCUCCAggcTsT	1432
AD-12368	GmocmocmouGGAGmoumoumouAmoumoumoucGGAATsT	1433	UUCCGAAUAAACUCcAGGCTsT	1434

TABLE 2a-continued

	Sequences of modified dsRNA targeted to PCSK9			
Duplex number	Sense strand sequence (5'-3')[1]	SEQ ID NO:	Antisense-strand sequence (5'-3')[1]	SEQ ID NO:
AD-12369	GmocmocmouGGAGmoumoumouAmoumoum ocGGAATsT	1435	UUCCGAAUAAACUCCAGGCTsT	1436
AD-12370	GmocmocmouGGAGmoumoumouAmoumoum ocGGAATsT	1437	P-UfaCfCfGAAUfAAACMCfCfAGGCfTsT	1438
AD-12371	GmocmocmouGGAGmoumoumouAmoumoum ocGGAATsT	1439	P-UfUfCfCfGAAUfAAACfUfCfCfAGGCfsCfsUf	1440
AD -12372	GmocmocmouGGAGmoumoumouAmoumoum ocGGAATsT	1441	P-uUfcCfgAfaUfaAfaCfuCfcAfgGfcsCfsu	1442
AD -12373	GmocmocmouGGAGmoumoumouAmoumoum ocGGAATsT	1443	UUCCGAAUAAACUCCAGGCTsT	1444
AD-12374	GCfCfUfGGAGUfUfUfAUfUfCfGGAATsT	1445	UfUfCfCfGAAUfAAACfUfCfCfAGGCfTsT	1446
AD-12375	GCfCfUfGGAGUfUfUfAUfUfCfGGAATsT	1447	UUCCGAAUAAACUCCAGGCTsT	1448
AD-12377	GCfCfUfGGAGUfUfUfAUfUfCfGGAATsT	1449	uUcCGAAuAAACUccAGGCTsT	1450
AD-12378	GCfCfUfGGAGUfUfUfAUfUfCfGGAATsT	1451	UUCCGaaUAaaCUCCAggcscsu	1452
AD-12379	GCfCfUfGGAGUfUfUfAUfUfCfGGAATsT	1453	UUCCGAAUAAACUCCAGGCscsu	1454
AD-12380	GCfCfUfGGAGUfUfUfAUfUfCfGGAATsT	1455	P-uUfcCfgAfaUfaAfaCfuCfcAfgGfcsCfsu	1456
AD-12381	GCfCfUfGGAGUfUfUfAUfUfCfGGAATsT	1457	P-uUfcCfgAfaUfaAfaCfuCfcAfgGfcTsT	1458
AD-12382	GCfCfUfGGAGUfUfUfAUfUfCfGGAATsT	1459	P-UfUfCfCfGAAUfAAACfUfCfCfAGGCfTsT	1460
AD-12383	GCCUGGAGUUUAUUCGGAATsT	1461	P-UfUfCfCfGAAUfAAACfUfCfCfAGGCfTsT	1462
AD-12384	GccuGGAGuuuAuucGGAATsT	1463	P-UfUfCfCfGAAUfAAACfUfCfCfAGGCfTsT	1464
AD-12385	GcCuGgnAgUuUaUuCgGaATsT	1465	P-UfUfCfCfGAAUfAAACfUfCfCfAGGCfTsT	1466
AD-12386	GfcCfuGfgAfgUfuUfaUfuCfgGfaAf	1467	P-UfUfCfCfGAAUfAAACfUfCfCfAGGCfTsT	1468
AD-12387	GCfCfUfGGAGGUfUfUfAUfUfCfGGAA	1469	UfUfCfCfGAAUfAAACfUfCfCfAGGCfsCfsUf	1470
AD-12388	GCfCfUfGGAGGUfUfUfAUfUfCfGGAA	1471	P-uUfcCfgAfaUfaAfaCfuCfcAfgGfc	1472
AD-12389	GCfCfUfGGAGGUfUfUfAUfUfCfGGAA	1473	P-uUfcCfgAfaUfaAfaCfuCfcAfgGfcsCfsu	1474
AD-12390	GCfCfUfGGAGGUfUfUfAUfUfCfGGAA	1475	UUCCGAAUAAACUCCAGGCscsu	1476
AD-12391	GCfCfUfGGAGGUfUfUfAUfUfCfGGAA	1477	UUCCGaaUAaaCUCCAggc	1478
AD-12392	GCfCfUfGGAGGUfUfUfAUfUfCfGGAA	1479	UUCCGAAUAAACUCCAGGCTsT	1480
AD-12393	GCfCfUfGGAGGUfUfUfAUfUfCfGGAA	1481	UUCCGAAuAAACUCcAGGCTsT	1482
AD-12394	GCfCfUfGGAGGUfUfUfAUfUfCfGGAA	1483	uUcCGAAuAAACUccAGGCTsT	1484
AD-12395	GmocCmouGmogAmogUmouUmoaUmouCmo gGmoaATsT	1485	P-UfUfCfCfGAAUfAAACfUfCfCfAGGCfsCfsUf	1486
AD-12396	GmocCmouGmogAm02gUmouUmoaUmouCm ogGmoaA	1487	P-UfUfCfCfGAAUfAAACfUfCfCfAGGCfsCfsUf	1488
AD-12397	GfccCfuGfgAfgUfuUfaUfuCfgGfaAf	1489	P-UfUfCfCfGAAUfAAACfUfCfCfAGGCfsCfsUf	1490
AD-12398	GfccCfuGfgAfgUfuUfaUfuCfgGfaAfTsT	1491	P-UfUfCfCfGAAUfAAACfUfCfCfAGGCfsCfsUf	1492
AD-12399	GcCuGgnAgUuUaUuCgGaATsT	1493	P-UfUfCfCfGAAUfAAACfUfCfCfAGGCfsCfsUf	1494
AD-12400	GCCUGGAGUUUAUUCGGAATsT	1495	P-UfUfCfCfGAAUfAAACfUfCfCfAGGCfsCfsUf	1496
AD-12401	GccuGGAGuuuAuucGGAATsT	1497	P-UfUfCfCfGAAUfAAACfUfCfCfAGGCfsCfsUf	1498

TABLE 2a-continued

Sequences of modified dsRNA targeted to PCSK9

Duplex number	Sense strand sequence (5'-3')¹	SEQ ID NO:	Antisense-strand sequence (5'-3')¹	SEQ ID NO:
AD-12402	GccuGGAGuuuAuucGGAA	1499	P-UfUfCfCfGAAUfAAACfUfCfCfAGGCfsCfsUf	1500
AD-12403	GCfCfUfGGAGGUfUfUfAUfUfCfGGAA	1501	P-UfUfCfCfGAAUfAAACfUfCfCfAGGCfsCfsUf	1502
AD-9314	GCCUGGAGUUUAUUCGGAATsT	1503	UUCCGAAUAAACUCCAGGCTsT	1504
AD-10794	ucAuAGGccuGGAGuuuAudTsdT	1525	AuAAACUCcAGGCCuAUGAdTsdT	1526
AD-10795	ucAuAGGccuGGAGuuuAudTsdT	1527	AuAAACUccAGGcCuAuGAdTsdT	1528
AD-10797	ucAuAGGccuGGAGuuuAudTsdT	1529	AUAAACUCCAGGCCUAUGAdTsdT	1530

[0541] U, C, A, G: corresponding ribonucleotide; T: deoxythymidine; u, c, a, g: corresponding 2'-O-methyl ribonucleotide; Uf, Cf, Af, Gf: corresponding 2'-deoxy-2'-fluoro ribonucleotide; moc, mou, mog, moa: corresponding 2'-MOE nucleotide; where nucleotides are written in sequence, they are connected by 3'-5' phosphodiester groups; ab: 3'-terminal abasic nucleotide; nucleotides with interjected "s" are connected by 3'-O-5'-O phosphorothiodiester groups; unless denoted by prefix "p-", oligonucleotides are devoid of a 5'-phosphate group on the 5'-most nucleotide; all oligonucleotides bear 3'-OH on the 3'-most nucleotide

TABLE 2b

Screening of dsRNAs targeted to PCSK9

Duplex number	Remaining mRNA in % of controls at siRNA conc. of 30 nM
AD-10792	15
AD-10793	32
AD-10796	13
AD-12038	13
AD-12039	29
AD-12040	10
AD-12041	11
AD-12042	12
AD-12043	13
AD-12044	7
AD-12045	8
AD-12046	13
AD-12047	17
AD-12048	43
AD-12049	34
AD-12050	16
AD-12051	31
AD-12052	81
AD-12053	46
AD-12054	8
AD-12055	13
AD-12056	11
AD-12057	8
AD-12058	9
AD-12059	23
AD-12060	10
AD-12061	7
AD-12062	10
AD-12063	19
AD-12064	15
AD-12065	16
AD-12066	20
AD-12067	17

TABLE 2b-continued

Screening of dsRNAs targeted to PCSK9

Duplex number	Remaining mRNA in % of controls at siRNA conc. of 30 nM
AD-12068	18
AD-12069	13
AD-12338	15
AD-12339	14
AD-12340	19
AD-12341	12
AD-12342	13
AD-12343	24
AD-12344	9
AD-12345	12
AD-12346	13
AD-12347	11
AD-12348	8
AD-12349	11
AD-12350	17
AD-12351	11
AD-12352	11
AD-12354	11
AD-12355	9
AD-12356	25
AD-12357	56
AD-12358	29
AD-12359	30
AD-12360	15
AD-12361	20
AD-12362	51
AD-12363	11
AD-12364	25
AD-12365	18
AD-12366	23
AD-12367	42
AD-12368	40
AD-12369	26
AD-12370	68
AD-12371	60
AD-12372	60
AD-12373	55
AD-12374	9
AD-12375	16
AD-12377	88
AD-12378	6
AD-12379	6
AD-12380	8
AD-12381	10
AD-12382	7
AD-12383	7
AD-12384	8
AD-12385	8
AD-12386	11
AD-12387	13

TABLE 2b-continued

Screening of dsRNAs targeted to PCSK9

Duplex number	Remaining mRNA in % of controls at siRNA conc. of 30 nM
AD-12388	19
AD-12389	16
AD-12390	17
AD-12391	21
AD-12392	28
AD-12393	17
AD-12394	75
AD-12395	55
AD-12396	59
AD-12397	20
AD-12398	11
AD-12399	13
AD-12400	12
AD-12401	13
AD-12402	14
AD-12403	4
AD-9314	9

TABLE 3

Cholesterol levels of rats treated with LNP01-10792

Dosage of 5 mg/kg, n = 6 rats per group

Day	Total serum cholesterol (relative to PBS control)
2	0.329 ± 0.035
4	0.350 ± 0.055
7	0.402 ± 0.09
9	0.381 ± 0.061
11	0.487 ± 0.028
14	0.587 ± 0.049
16	0.635 ± 0.107
18	0.704 ± 0.060
21	0.775 ± 0.102
28	0.815 ± 0.103

TABLE 4

Serum LDL-C levels of cynomolgus monkeys treated with LNP formulated dsRNAs

	Serum LDL-C (relative to pre-dose)					
	Day 3	Day 4	Day 5	Day 7	Day 14	Day 21
PBS n = 3	1.053 ± 0.158	0.965 ± 0.074	1.033 ± 0.085	1.033 ± 0.157	1.009 ± 0.034	
LNP01-1955 n = 3		1.027 ± 0.068		1.104 ± 0.114		
LNP01-10792 n = 5	0.503 ± 0.055	0.596 ± 0.111	0.674 ± 0.139	0.644 ± 0.121	0.958 ± 0.165	1.111 ± 0.172
LNP01-9680 n = 4	0.542 ± 0.155	0.437 ± 0.076	0.505 ± 0.071	0.469 ± 0.066	0.596 ± 0.080	0.787 ± 0.138

TABLE 5a

Modified dsRNA targeted to PCSK9

Name	Position in human access.#	Sense	Antisense	Sequence 5'-3'	SEQ ID NO:
AD-1a1	1091	unmodified	unmodified	GCCUGGAGUUUAUUCGGAAdTdT UUCCGAAUAAACUCCAGGCdTsdT	1505 1506
AD-1a2	1091	2'OMe	2'OMe	GccuGGAGuuuAuucGGAAdTsdT UUCCGAAuAAACUCcAGGCdTsdT	1507 1508
AD-1a3	1091	Alt 2'F, 2'OMe	Alt 2'F, 2'OMe	GfcCfuGfgAfgUfuUfaUfuCfgGfaAfdTdT puUfcCfgAfaUfaAfaCfuCfcAfgGfcdTsdT	1509 1510
AD-1a4	1091	2'OMe	2'F all Py, 5'Phosphate	GccuGGAGuuuAuucGGAAdTsdT PUfUfCfCfgAAUfAAACfUfCfCfAGGCfdTsdT	1511 1512
AD-1a5	1091	2'F	2'F all Py, 5'Phosphate	GCfCfUfGGAGUfUfUfAUfUfCfGGAAdTsdT PUfUfCfCfgAAUfAAACfUfCfCfAGGCfdTsdT	1513 1514
AD-2a1	3530 (3'UTR)	2'OMe	2'OMe	uucuAGAccuGuuuuGcuudTsdT AAGcAAAAcAGGUCuAGAAdTsdT	1515 1516
AD-3a1	833	2'OMe	2'OMe	AGGuGuAucuccuAGAcAcdTsdT GUGUCuAGGAGAuAcACCUdTsdT	1517 1518

TABLE 5a-continued

					SEQ
	Position in human				ID
Name	access.#	Sense	Antisense	Sequence 5'-3'	NO:
AD-ctrl (Luc.)	N/A	2'OMe	2'OMe	cuuAcGcuGAGuAcuucGAdTsdT	1519
				UCGAAGuACUcAGCGuAAGdTsdT	1520

U, C, A, G: corresponding ribonucleotide; T: deoxythymidine; u, c, a, g: corresponding 2'-O-methyl ribonucleotide; Uf, Cf, Af, Gf: corresponding 2'-deoxy-2'-fluoro ribonucleotide; where nucleotides are written in sequence, they are connected by 3'-5' phosphodiester groups; nucleotides with interjected "s" are connected by 3'-O-5'-O phosphorothiodiester groups; unless denoted by prefix "p-", oligonucleotides are devoid of a 5'-phosphate group on the 5'-most nucleotide; all oligonucleotides bear 3'-OH on the 3'-most nucleotide.

TABLE 5b

Silencing activity of modified dsRNA in monkey hepatocytes

Name	Position in human access.#	IFN-α/ TNF-α Induction	Sense	Antisense	Primary Cynomolgus Monkey Hepatocytes ~IC50, nM
AD-1a1	1091	Yes/Yes	unmodified	unmodified	0.07-0.2
AD-1a2	1091	No/No	2'OMe	2'OMe	0.07-0.2
AD-1a3	1091	No/No	Alt 2'F, 2'OMe	Alt 2'F, 2'OMe	0.07-0.2
AD-1a4	1091	No/No	2'OMe	2'F all Py, 5'Phosphate	0.07-0.2
AD-1a5	1091	No/No	2'F	2'F all Py, 5'Phosphate	0.07-0.2
AD-2a1	3530 (3'UTR)	No/No	2'OMe	2'OMe	0.07-0.2
AD-3a1	833	No/No	2'OMe	2'OMe	0.1-0.3
AD-ctrl (Luc.)	N/A	No/No	2'OMe	2'OMe	N/A

TABLE 6

dsRNA targeted to PCSK9: mismatches and modifications

Duplex #	Strand	SEQ ID NO:	Sequence 5' to 3'
AD-9680	S	1531	uucuAGAccuGuuuuGcuudTsdT
	AS	1532	AAGcAAAAcAGGUCuAGAAdTsdT
AD-3267	S	1535	uucuAGAcCuGuuuuGcuuTsT
	AS	1536	AAGcAAAAcAGGUCuAGAATsT
AD-3268	S	1537	uucuAGAccUGuuuuGcuuTsT
	AS	1538	AAGcAAAAcAGGUCuAGAATsT
AD-3269	S	1539	uucuAGAcCUGuuuuGcuuTsT
	AS	1540	AAGcAAAAcAGGUCuAGAATsT
AD-3270	S	1541	uucuAGAcY1uGuuuuGcuuTsT
	AS	1542	AAGcAAAAcAGGUCuAGAATsT
AD-3271	S	1543	uucuAGAcY1UGuuuuGcuuTsT
	AS	1544	AAGcAAAAcAGGUCuAGAATsT
AD-3272	S	1545	uucuAGAccY1GuuuuGcuuTsT
	AS	1546	AAGcAAAAcAGGUCuAGAATsT
AD-3273	S	1547	uucuAGAcCY1GuuuuGcuuTsT
	AS	1548	AAGcAAAAcAGGUCuAGAATsT
AD-3274	S	1549	uucuAGAccuY1uuuuGcuuTsT
	AS	1550	AAGcAAAAcAGGUCuAGAATsT

TABLE 6-continued

		SEQ ID	
Duplex #	Strand	NO:	Sequence 5' to 3'

dsRNA targeted to PCSK9: mismatches and modifications

Duplex #	Strand	SEQ ID NO:	Sequence 5' to 3'
AD-3275	S	1551	uucuAGAcCUY1uuuuGcuuTsT
	AS	1552	AAGcAAAAcAGGUCuAGAATsT
AD-14676	S	1553	UfuCfuAfgAfcCfuGfuUfuUfgCfuUfTsT
	AS	1554	p-aAfgCfaAfaAfcAfgGfuCfuAfgAfaTsT
AD-3276	S	1555	UfuCfuAfgAfcCuGfuUfuUfgCfuUfTsT
	AS	1556	p-aAfgCfaAfaAfcAfgGfuCfuAfgAfaTsT
AD-3277	S	1557	UfuCfuAfgAfcCfUGfuUfuUfgCfuUfTsT
	AS	1558	p-aAfgCfaAfaAfcAfgGfuCfuAfgAfaTsT
AD-3278	S	1559	UfuCfuAfgAfcCUGfuUfuUfgCfuUfTsT
	AS	1560	p-aAfgCfaAfaAfcAfgGfuCfuAfgAfaTsT
AD-3279	S	1561	UfuCfuAfgAfcYluGfuUfuUfgCfuUfTsT
	AS	1562	p-aAfgCfaAfaAfcAfgGfuCfuAfgAfaTsT
AD-3280	S	1563	UfuCfuAfgAfcY1UGfuUfuUfgCfuUfTsT
	AS	1564	p-aAfgCfaAfaAfcAfgGfuCfuAfgAfaTsT
AD-3281	S	1565	UfuCfuAfgAfcCfY1GfuUfuUfgCfuUfTsT
	AS	1566	p-aAfgCfaAfaAfcAfgGfuCfuAfgAfaTsT
AD-3282	S	1567	UfuCfuAfgAfcCY1GfuUfuUfgCfuUfTsT
	AS	1568	p-aAfgCfaAfaAfcAfgGfuCfuAfgAfaTsT
AD-3283	S	1569	UfuCfuAfgAfcCfuY1uUfuUfgCfuUfTsT
	AS	1570	p-aAfgCfaAfaAfcAfgGfuCfuAfgAfaTsT
AD-3284	S	1571	UfuCfuAfgAfcCUY1uUfuUfgCfuUfTsT
	AS	1572	p-aAfgCfaAfaAfcAfgGfuCfuAfgAfaTsT
AD-10792	S	459	GccuGGAGuuuAuucGGAATsT
	AS	460	UUCCGAAuAAACUCcAGGCTsT
AD-3254	S	1573	GccuGGAGuY1uAuucGGAATsT
	AS	1574	UUCCGAAuAAACUCcAGGCTsT
AD-3255	S	1575	GccuGGAGUY1uAuucGGAATsT
	AS	1576	UUCCGAAuAAACUCcAGGCTsT

Strand: S/Sense; AS/Antisense
U, C, A, G: corresponding ribonucleotide; T: deoxythymidine; u, c, a, g: corresponding 2'-O-methyl ribonucleotide; Uf, Cf, Af, Gf: corresponding 2'-deoxy-2'-fluoro ribonucleotide; Y1 corresponds to DFT difluorotoluyl ribo (or deoxyribo)nucleotide; where nucleotides are written in sequence, they are connected by 3'-5' phosphodiester groups; nucleotides with interjected "s" are connected by 3'-O-5'-O phosphorothiodiester groups; unless denoted by prefix "p-", oligonucleotides are devoid of a 5'-phosphate group on the 5'-most nucleotide; all oligonucleotides bear 3'-OH on the 3'-most nucleotide

TABLE 7

Sequences of unmodified siRNA flanking AD-9680

Duplex	Type	Sequence (5' to 3')	Target site	SEQ ID NO:
AD-22169-b1	sense	CAGCCAACUUUUCUAGACCdTsdT	3520	1577
	antis	GGUCUAGAAAAGUUGGCUGdTsdT	3520	1578
AD-22170-b1	sense	AGCCAACUUUUCUAGACCUdTsdT	3521	1579
	antis	AGGUCUAGAAAAGUUGGCUdTsdT	3521	1580
AD-22171-b1	sense	GCCAACUUUUCUAGACCUGdTsdT	3522	1581
	antis	CAGGUCUAGAAAAGUUGGCdTsdT	3522	1582
AD-22172-b1	sense	CCAACUUUUCUAGACCUGUdTsdT	3523	1583
	antis	ACAGGUCUAGAAAAGUUGGdTsdT	3523	1584
AD-22173-b1	sense	CAACUUUUCUAGACCUGUUdTsdT	3524	1585
	antis	AACAGGUCUAGAAAAGUUGdTsdT	3524	1586

TABLE 7-continued

Sequences of unmodified siRNA flanking AD-9680

Duplex	Type	Sequence (5' to 3')	Target site	SEQ ID NO:
AD-22174-b1	sense	AACUUUUCUAGACCUGUUUdTsdT	3525	1587
	antis	AAACAGGUCUAGAAAAGUUdTsdT	3525	1588
AD-22175-b1	sense	ACUUUUCUAGACCUGUUUUdTsdT	3526	1589
	antis	AAAACAGGUCUAGAAAAGUdTsdT	3526	1590
AD-22176-b1	sense	CUUUUCUAGACCUGUUUUGdTsdT	3527	1591
	antis	CAAAACAGGUCUAGAAAAGdTsdT	3527	1592
AD-22177-b1	sense	UUUUCUAGACCUGUUUUGCdTsdT	3528	1593
	antis	GCAAAACAGGUCUAGAAAAdTsdT	3528	1594
AD-22178-b1	sense	UUUCUAGACCUGUUUUGCUdTsdT	3529	1595
	antis	AGCAAAACAGGUCUAGAAAdTsdT	3529	1596
AD-22179-b1	sense	UCUAGACCUGUUUUGCUUUdTsdT	3531	1597
	antis	AAAGCAAAACAGGUCUAGAdTsdT	3531	1598
AD-22180-b1	sense	CUAGACCUGUUUUGCUUUUdTsdT	3532	1599
	antis	AAAAGCAAAACAGGUCUAGdTsdT	3532	1600
AD-22181-b1	sense	UAGACCUGUUUUGCUUUUGdTsdT	3533	1601
	antis	CAAAAGCAAAACAGGUCUAdTsdT	3533	1602
AD-22182-b1	sense	AGACCUGUUUUGCUUUUGUdTsdT	3534	1603
	antis	ACAAAAGCAAAACAGGUCUdTsdT	3534	1604
AD-22183-b1	sense	GACCUGUUUUGCUUUUGUAdTsdT	3535	1605
	antis	UACAAAAGCAAAACAGGUCdTsdT	3535	1606
AD-22184-b1	sense	ACCUGUUUUGCUUUUGUAAdTsdT	3536	1607
	antis	UUACAAAAGCAAAACAGGUdTsdT	3536	1608
AD-22185-b1	sense	CCUGUUUUGCUUUUGUAACdTsdT	3537	1609
	antis	GUUACAAAAGCAAAACAGGdTsdT	3537	1610
AD-22186-b1	sense	CUGUUUUGCUUUUGUAACUdTsdT	3538	1611
	antis	AGUUACAAAAGCAAAACAGdTsdT	3538	1612
AD-22187-b1	sense	UGUUUUGCUUUUGUAACUUdTsdT	3539	1613
	antis	AAGUUACAAAAGCAAAACAdTsdT	3539	1614
AD-22188-b1	sense	GUUUUGCUUUUGUAACUUGdTsdT	3540	1615
	antis	CAAGUUACAAAAGCAAAACdTsdT	3540	1616
AD-22189-b1	sense	UUUUGCUUUUGUAACUUGAdTsdT	3541	1617
	antis	UCAAGUUACAAAAGCAAAAdTsdT	3541	1618
AD-22190-b1	sense	UUUGCUUUUGUAACUUGAAdTsdT	3542	1619
	antis	UUCAAGUUACAAAAGCAAAdTsdT	3542	1620
AD-22191-b1	sense	UUGCUUUUGUAACUUGAAGdTsdT	3543	1621
	antis	CUUCAAGUUACAAAAGCAAdTsdT	3543	1622
AD-22192-b1	sense	UGCUUUUGUAACUUGAAGAdTsdT	3544	1623
	antis	UCUUCAAGUUACAAAAGCAdTsdT	3544	1624
AD-22193-b1	sense	GCUUUUGUAACUUGAAGAUdTsdT	3545	1625
	antis	AUCUUCAAGUUACAAAAGCdTsdT	3545	1626
AD-22194-b1	sense	CUUUUGUAACUUGAAGAUAdTsdT	3546	1627
	antis	UAUCUUCAAGUUACAAAAGdTsdT	3546	1628
AD-22195-b1	sense	UUUUGUAACUUGAAGAUAUdTsdT	3547	1629
	antis	AUAUCUUCAAGUUACAAAAdTsdT	3547	1630
AD-22196-b1	sense	UUUGUAACUUGAAGAUAUUdTsdT	3548	1631
	antis	AAUAUCUUCAAGUUACAAAdTsdT	3548	1632
AD-22197-b1	sense	UUGUAACUUGAAGAUAUUUdTsdT	3549	1633
	antis	AAAUAUCUUCAAGUUACAAdTsdT	3549	1634

TABLE 7-continued

Sequences of unmodified siRNA flanking AD-9680

Duplex	Type	Sequence (5' to 3')	Target site	SEQ ID NO:
AD-22198-b1	sense	UGUAACUUGAAGAUAUUUAdTsdT	3550	1635
	antis	UAAAUAUCUUCAAGUUACAdTsdT	3550	1636
AD-22199-b1	sense	GUAACUUGAAGAUAUUUAUdTsdT	3551	1637
	antis	AUAAAUAUCUUCAAGUUACdTsdT	3551	1638
AD-22200-b1	sense	UAACUUGAAGAUAUUUAUUdTsdT	3552	1639
	antis	AAUAAAUAUCUUCAAGUUAdTsdT	3552	1640
AD-22201-b1	sense	AACUUGAAGAUAUUUAUUCdTsdT	3553	1641
	antis	GAAUAAAUAUCUUCAAGUUdTsdT	3553	1642
AD-22202-b1	sense	ACUUGAAGAUAUUUAUUCUdTsdT	3554	1643
	antis	AGAAUAAAUAUCUUCAAGUdTsdT	3554	1644
AD-22203-b1	sense	CUUGAAGAUAUUUAUUCUGdTsdT	3555	1645
	antis	CAGAAUAAAUAUCUUCAAGdTsdT	3555	1646
AD-22204-b1	sense	UUGAAGAUAUUUAUUCUGGdTsdT	3556	1647
	antis	CCAGAAUAAAUAUCUUCAAdTsdT	3556	1648
AD-22205-b1	sense	UGAAGAUAUUUAUUCUGGGdTsdT	3557	1649
	antis	CCCAGAAUAAAUAUCUUCAdTsdT	3557	1650
AD-22206-b1	sense	GAAGAUAUUUAUUCUGGGUdTsdT	3558	1651
	antis	ACCCAGAAUAAAUAUCUUCdTsdT	3558	1652

TABLE 8

Sequences of modified siRNA flanking AD-9680

Duplex	Type	Sequence (5' to 3')	Target	SEQ ID NO:
AD-22098-b1	sense	cAGccAAcuuuucuAGAccdTsdT	3520	1653
	antis	GGUCuAGAAAAGUUGGCUGdTsdT	3520	1654
AD-22099-b1	sense	AGccAAcuuuucuAGAccudTsdT	3521	1655
	antis	AGGUCuAGAAAAGUUGGCUdTsdT	3521	1656
AD-22100-b1	sense	GccAAcuuuucuAGAccuGdTsdT	3522	1657
	antis	cAGGUCuAGAAAAGUUGGCdTsdT	3522	1658
AD-22101-b1	sense	ccAAcuuuucuAGAccuGudTsdT	3523	1659
	antis	AcAGGUCuAGAAAAGUUGGdTsdT	3523	1660
AD-22102-b1	sense	cAAcuuuucuAGAccuGuudTsdT	3524	1661
	antis	AAcAGGUCuAGAAAAGUUGdTsdT	3524	1662
AD-22103-b1	sense	AAcuuuucuAGAccuGuuudTsdT	3525	1663
	antis	AAAcAGGUCuAGAAAAGUUdTsdT	3525	1664
AD-22104-b1	sense	AcuuuucuAGAccuGuuuudTsdT	3526	1665
	antis	AAAAcAGGUCuAGAAAAGUdTsdT	3526	1666
AD-22105-b1	sense	cuuuucuAGAccuGuuuuGdTsdT	3527	1667
	antis	cAAAAcAGGUCuAGAAAAGdTsdT	3527	1668
AD-22106-b1	sense	uuuucuAGAccuGuuuuGcdTsdT	3528	1669
	antis	GcAAAAcAGGUCuAGAAAAdTsdT	3528	1670
AD-22107-b1	sense	uuucuAGAccuGuuuuGcudTsdT	3529	1671
	antis	AGcAAAAcAGGUCuAGAAAdTsdT	3529	1672
AD-22108-b1	sense	ucuAGAccuGuuuuGcuuudTsdT	3531	1673
	antis	AAAGcAAAAcAGGUCuAGAdTsdT	3531	1674
AD-22109-b1	sense	cuAGAccuGuuuuGcuuuudTsdT	3532	1675
	antis	AAAAGcAAAAcAGGUCuAGdTsdT	3532	1676

TABLE 8-continued

Sequences of modified siRNA flanking AD-9680				
Duplex	Type	Sequence (5' to 3')	Target	SEQ ID NO:
AD-22110-b1	sense	uAGAccuGuuuuGcuuuuGudTsdT	3533	1677
	antis	cAAAAGcAAAAcAGGUCuAdTsdT	3533	1678
AD-22111-b1	sense	AGAccuGuuuuGcuuuuGudTsdT	3534	1679
	antis	AcAAAAGcAAAAcAGGUCUdTsdT	3534	1680
AD-22112-b1	sense	GAccuGuuuuGcuuuuGuAdTsdT	3535	1681
	antis	uAcAAAAGcAAAAcAGGUCdTsdT	3535	1682
AD-22113-b1	sense	AccuGuuuuGcuuuuGuAAdTsdT	3536	1683
	antis	UuAcAAAAGcAAAAcAGGUdTsdT	3536	1684
AD-22114-b1	sense	ccuGuuuuGcuuuuGuAAcdTsdT	3537	1685
	antis	GUuAcAAAAGcAAAAcAGGdTsdT	3537	1686
AD-22115-b1	sense	cuGuuuuGcuuuuGuAAcudTsdT	3538	1687
	antis	AGUuAcAAAAGcAAAAcAGdTsdT	3538	1688
	sense	uGuuuuGcuuuuGuAAcuudTsdT	3539	1689
	antis	AAGUuAcAAAAGcAAAAcAdTsdT	3539	1690
AD-22116-b1	sense	GuuuuGcuuuuGuAAcuuGdTsdT	3540	1691
	antis	cAAGUuAcAAAAGcAAAACdTsdT	3540	1692
AD-22117-b1	sense	uuuuGcuuuuGuAAcuuGAdTsdT	3541	1693
	antis	UcAAGUuAcAAAAGcAAAAdTsdT	3541	1694
AD-22118-b1	sense	uuuGcuuuuGuAAcuuGAAdTsdT	3542	1695
	antis	UUcAAGUuAcAAAAGcAAAdTsdT	3542	1696
AD-22119-b1	sense	uuGcuuuuGuAAcuuGAAGdTsdT	3543	1697
	antis	CUUcAAGUuAcAAAAGcAAdTsdT	3543	1698
AD-22120-b1	sense	uGcuuuuGuAAcuuGAAGAdTsdT	3544	1699
	antis	UCUUcAAGUuAcAAAAGcAdTsdT	3544	1700
AD-22121-b1	sense	GcuuuuGuAAcuuGAAGAudTsdT	3545	1701
	antis	AUCUUcAAGUuAcAAAAGCdTsdT	3545	1702
AD-22122-b1	sense	cuuuuGuAAcuuGAAGAuAdTsdT	3546	1703
	antis	uAUCUUcAAGUuAcAAAAGdTsdT	3546	1704
AD-22123-b1	sense	uuuuGuAAcuuGAAGAuAudTsdT	3547	1705
	antis	AuAUCUUcAAGUuAcAAAAdTsdT	3547	1706
AD-22124-b1	sense	uuuGuAAcuuGAAGAuAuudTsdT	3548	1707
	antis	AAuAUCUUcAAGUuAcAAAdTsdT	3548	1708
AD-22125-b1	sense	uuGuAAcuuGAAGAuAuuudTsdT	3549	1709
	antis	AAAuAUCUUcAAGUuAcAAdTsdT	3549	1710
AD-22126-b1	sense	uGuAAcuuGAAGAuAuuuAdTsdT	3550	1711
	antis	uAAAuAUCUUcAAGUuAcAdTsdT	3550	1712
AD-22127-b1	sense	GuAAcuuGAAGAuAuuuAudTsdT	3551	1713
	antis	AuAAAuAUCUUcAAGUuACdTsdT	3551	1714
AD-22128-b1	sense	uAAcuuGAAGAuAuuuAuudTsdT	3552	1715
	antis	AAuAAAuAUCUUcAAGUuAdTsdT	3552	1716
AD-22129-b1	sense	AAcuuGAAGAuAuuuAuucdTsdT	3553	1717
	antis	GAAuAAAuAUCUUcAAGUUdTsdT	3553	1718
AD-22130-b1	sense	AcuuGAAGAuAuuuAuucudTsdT	3554	1719
	antis	AGAAuAAAuAUCUUcAAGUdTsdT	3554	1720
AD-22131-b1	sense	cuuGAAGAuAuuuAuucuGdTsdT	3555	1721
	antis	cAGAAuAAAuAUCUUcAAGdTsdT	3555	1722
AD-22132-b1	sense	uuGAAGAuAuuuAuucuGGdTsdT	3556	1723
	antis	CcAGAAuAAAuAUCUUcAAdTsdT	3556	1724

TABLE 8-continued

Sequences of modified siRNA flanking AD-9680

Duplex	Type	Sequence (5' to 3')	Target	SEQ ID NO:
AD-22133-b1	sense	uGAAGAuAuuuAuucuGGGdTsdT	3557	1725
	antis	CCcAGAAuAAAuAUCUUcAdTsdT	3557	1726
AD-22134-b1	sense	GAAGAuAuuuAuucuGGGudTsdT	3558	1727
	antis	ACCcAGAAuAAAuAUCUUCdTsdT	3558	1728

TABLE 9

Single dose treatment of HeLa cells with siRNA flanking AD-9680

Duplex ID	% message remaining 0.1 nM	SD 0.1 nM	% message remaining 10 nM	SD 10 nM
AD-22098-b1	10.6	1.9	9.2	3.7
AD-22098-b1	7.7	1.7	7.9	0.7
AD-22099-b1	21.3	4.5	27.4	7.2
AD-22099-b1	25.9	2.4	29.6	9.1
AD-22100-b1	58.6	9.6	35.8	11.1
AD-22100-b1	62.5	0.3	27.4	3.5
AD-22101-b1	21.9	3.8	12.9	1.4
AD-22101-b1	19.3	0.3	9.7	1.3
AD-22102-b1	6.6	0.1	7.7	3.3
AD-22103-b1	8.7	0.0	8.2	1.3
AD-22104-b1	7.6	0.2	8.5	2.8
AD-22105-b1	13.4	1.0	8.1	2.3
AD-22106-b1	59.1	0.4	35.4	4.6
AD-22107-b1	9.1	0.8	8.4	3.7
AD-22108-b1	8.8	0.9	6.2	1.7
AD-22109-b1	9.8	0.9	8.2	1.7
AD-22110-b1	24.8	1.7	15.3	5.9
AD-22111-b1	8.3	0.7	6.2	1.7
AD-22112-b1	15.1	0.0	10.3	2.9
AD-22113-b1	10.9	0.6	10.0	2.0
AD-22114-b1	8.9	1.1	7.3	1.3
AD-22115-b1	5.3	0.8	3.7	0.7
AD-22116-b1	58.1	0.4	34.5	7.3
AD-22117-b1	19.9	0.9	12.2	2.9
AD-22118-b1	5.3	0.0	4.4	1.0
AD-22119-b1	8.6	1.9	5.8	2.3
AD-22120-b1	7.2	0.8	5.8	2.4
AD-22121-b1	7.3	0.9	6.4	2.1
AD-22122-b1	32.5	2.5	18.1	6.3
AD-22123-b1	14.7	0.8	16.7	7.0
AD-22124-b1	12.8	1.9	10.5	5.3
AD-22125-b1	7.4	0.6	9.0	4.6
AD-22126-b1	12.8	0.4	16.4	7.3
AD-22127-b1	8.8	0.5	9.6	5.0
AD-22128-b1	9.9	0.2	12.4	5.9
AD-22129-b1	85.9	10.3	94.9	49.8
AD-22130-b1	5.6	1.0	6.2	4.1
AD-22131-b1	26.9	8.4	12.9	7.3
AD-22132-b1	78.5	18.5	67.5	34.1
AD-22133-b1	26.4	7.1	15.0	6.7
AD-22134-b1	26.9	0.1	22.4	6.5
AD-22169-b1	7.3	0.6	6.0	1.5
AD-22169-b1	7.0	1.1	6.1	1.3
AD-22170-b1	9.3	1.6	7.2	1.8
AD-22170-b1	9.7	1.1	11.2	1.0
AD-22171-b1	7.1	2.3	4.5	0.2
AD-22171-b1	6.5	1.9	4.4	2.8
AD-22172-b1	7.2	1.1	7.6	3.7
AD-22172-b1	7.0	0.4	7.0	2.4
AD-22173-b1	15.7	12.5	5.9	0.1
AD-22174-b1	8.9	2.7	6.4	0.9
AD-22175-b1	10.7	4.3	7.9	2.4
AD-22176-b1	9.6	0.8	8.4	3.1
AD-22177-b1	38.9	5.9	21.4	1.2
AD-22178-b1	6.5	0.5	5.6	0.9
AD-22179-b1	7.0	0.8	5.9	0.1

TABLE 9-continued

Single dose treatment of HeLa cells with siRNA flanking AD-9680

Duplex ID	% message remaining 0.1 nM	SD 0.1 nM	% message remaining 10 nM	SD 10 nM
AD-22180-b1	7.3	3.7	7.2	1.6
AD-22181-b1	11.1	0.9	10.0	1.0
AD-22182-b1	5.4	1.4	4.0	1.5
AD-22183-b1	3.8	0.4	2.9	0.4
AD-22184-b1	5.1	0.2	3.7	0.7
AD-22185-b1	5.7	0.6	5.0	1.5
AD-22186-b1	5.3	0.3	5.7	1.0
AD-22187-b1	5.3	1.2	5.3	1.4
AD-22188-b1	12.6	2.6	11.6	0.2
AD-22189-b1	5.2	0.5	4.5	1.8
AD-22190-b1	4.7	1.3	3.4	1.1
AD-22191-b1	10.5	0.6	7.9	0.9
AD-22192-b1	6.9	2.2	5.8	3.5
AD-22193-b1	7.5	1.5	5.2	0.6
AD-22194-b1	8.0	1.4	6.5	1.9
AD-22195-b1	7.0	1.9	4.9	2.3
AD-22196-b1	5.4	0.0	3.8	0.9
AD-22197-b1	6.6	0.4	5.2	1.2
AD-22198-b1	7.3	0.8	8.5	2.4
AD-22199-b1	5.5	0.7	4.2	1.2
AD-22200-b1	11.0	0.5	12.5	3.1
AD-22201-b1	44.0	3.1	47.3	8.3
AD-22202-b1	9.0	1.2	7.2	0.9
AD-22203-b1	12.5	0.0	12.7	2.2
AD-22204-b1	57.1	5.2	50.2	10.2
AD-22205-b1	27.0	0.4	24.5	0.0
AD-22206-b1	13.9	1.1	11.4	1.3
AD-9680	7.1	ND	9.3	ND

TABLE 10

IC50 in HeLa cells using siRNA flanking AD-9680

Duplex Name	Rep1 IC50 (pM)	Rep2 IC50 (pM)	Average IC50 (pM)
AD-22098	6.0	6.7	6.4
AD-22099	25.0	37.8	31.4
AD-22101	66.5	81.9	74.2
AD-22102	2.3	1.5	1.9
AD-22103	6.3	1.2	3.8
AD-22104	2.2	1.4	1.8
AD-22105	13.3	0.1	6.7
AD-22107	2.2	0.9	1.6
AD-22108	2.3	2.0	2.1
AD-22109	5.5	6.3	5.9
AD-22110	59.1	42.2	50.7
AD-22111	9.1	8.2	8.7
AD-22112	25.8	31.0	28.4
AD-22113	4.2	4.4	4.3
AD-22114	6.9	4.0	5.5
AD-22115	3.0	2.2	2.6
AD-22117	56.0	37.6	46.8

TABLE 10-continued

IC50 in HeLa cells using siRNA flanking AD-9680			
Duplex Name	Rep1 IC50 (pM)	Rep2 IC50 (pM)	Average IC50 (pM)
AD-22118	2.9	1.7	2.3
AD-22119	6.7	0.0	3.4
AD-22120	2.0	1.2	1.6
AD-22121	2.1	4.1	3.1
AD-22122	203.3	156.3	179.8
AD-22123	33.1	50.7	41.9
AD-22124	18.8	13.1	15.9
AD-22125	3.3	2.6	3.0
AD-22126	17.9	18.5	18.2

TABLE 10-continued

IC50 in HeLa cells using siRNA flanking AD-9680			
Duplex Name	Rep1 IC50 (pM)	Rep2 IC50 (pM)	Average IC50 (pM)
AD-22127	11.1	4.3	7.7
AD-22128	14.6	3.3	8.9
AD-22130	1.7	0.3	1.0
AD-22131	172.5	59.6	116.0
AD-22133	94.6	57.2	75.9
AD-22134	113.0	81.3	97.2
AD-9680	3.8	2.4	3.1

SEQUENCE LISTING

The patent application contains a lengthy "Sequence Listing" section. A copy of the "Sequence Listing" is available in electronic form from the USPTO web site (http://seqdata.uspto.gov/?pageRequest=docDetail&DocID=US20170000815A1). An electronic copy of the "Sequence Listing" will also be available from the USPTO upon request and payment of the fee set forth in 37 CFR 1.19(b)(3).

1. A composition comprising a nucleic acid lipid particle comprising a double-stranded ribonucleic acid (dsRNA) for inhibiting the expression of a human PCSK9 gene in a cell, wherein:

  the nucleic acid lipid particle comprises a lipid formulation comprising 45-65 mol % of a cationic lipid, 5 mol % to about 10 mol %, of a non-cationic lipid, 25-40 mol % of a sterol, and 0.5-5 mol % of a PEG or PEG-modified lipid,

  the dsRNA consists of a sense strand and an antisense strand, and the sense strand comprises a first sequence and the antisense strand comprises a second sequence complementary to at least 15 contiguous nucleotides of a nucleotide sequence of a target sequence of a dsRNA found in Table 1a, Table 2a, Table 5a, Table 6, Table 7, Table 8,

  wherein the first sequence is complementary to the second sequence and wherein the dsRNA is between 15 and 30 base pairs in length.

2. The composition of claim **1**, wherein the cationic lipid comprises MC3 (((6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate).

3. The composition of claim **2**, wherein the cationic lipid comprises MC3 and the lipid formulation is selected from the group consisting of:

LNP11	MC3/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5
LNP14	MC3/DSPC/Cholesterol/PEG-DMG 40/15/40/5
LNP15	MC3/DSPC/Cholesterol/PEG-DSG/GalNAc-PEG-DSG 50/10/35/4.5/0.5
LNP16	MC3/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5

-continued

LNP17	MC3/DSPC/Cholesterol/PEG-DSG 50/10/38.5/1.5
LNP18	MC3/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5
LNP19	MC3/DSPC/Cholesterol/PEG-DMG 50/10/35/5
LNP20	MC3/DSPC/Cholesterol/PEG-DPG 50/10/38.5/1.5

4.-**9**. (canceled)

10. The composition of claim **1**, wherein the sense strand comprises SEQ ID NO:1227 and the antisense strand comprises SEQ ID NO:1228.

11. (canceled)

12. (canceled)

13. The composition of claim **1**, wherein the dsRNA comprises at least one modified nucleotide.

14. The composition of claim **13**, wherein the modified nucleotide is chosen from the group of: a 2'-O-methyl modified nucleotide, a nucleotide comprising a 5'-phosphorothioate group, and a terminal nucleotide linked to a cholesteryl derivative or dodecanoic acid bisdecylamide group.

15. The composition of claim **13**, wherein the modified nucleotide is chosen from the group of: a 2'-deoxy-2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an abasic nucleotide, 2'-amino-modified nucleotide, 2'-alkyl-modified nucleotide, morpholino nucleotide, a phosphoramidate, and a non-natural base comprising nucleotide.

16. The composition of claim **1**, wherein the dsRNA comprises at least one 2'-O-methyl modified ribonucleotide and at least one nucleotide comprising a 5'-phosphorothioate group.

17. The composition of claim **1**, wherein each strand of the dsRNA is 19-23 bases in length.

18. The composition of claim **1**, wherein each strand of the dsRNA is 21-23 bases in length.

19. The composition of claim **1**, wherein each strand of the dsRNA is 21 bases in length.

20. The composition of claim **1**, further comprising a lipoprotein.

21. The composition of claim **1**, further comprising apolipoprotein E (ApoE).

22. The composition of claim **21**, wherein the dsRNA is conjugated to a lipophile.

23. The composition of claim **22**, wherein the lipophile is cholesterol.

24. The composition of claim **21**, wherein the ApoE is reconstituted with at least one amphiphilic agent.

25. The composition of claim **24**, wherein the amphiphilic agent is a phospholipid.

26. The composition of claim **24**, wherein the amphilic agent is a phospholipid selected from the group consisting of dimyristoyl phosphatidyl choline (DMPC), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), egg phosphatidylcholine (EPC), di stearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), -phosphatidylethanolamine (POPE), dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), and combinations thereof.

27. The composition of claim **25**, wherein the ApoE is reconstituted high density lipoprotein (rHDL).

28.-33. (canceled)

34. A method for inhibiting the expression of PCSK9 in a cell comprising administering the composition of claim **1** to the cell.

35.-43. (canceled)

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