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[54] COMPOSITIONS FOR ENHANCING DELIVERY OF DOUBLE-STRANDED RNA TO REGULATE GENE EXPRESSION IN MAMMALIAN CELLS 增強對哺乳動物細胞基因表達的調節用雙鏈 RNA 輸送的組合物

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(54) **Compositions for enhancing delivery of double-stranded RNA to regulate gene expression in mammalian cells**

Zusammensetzungen zur Erhöhung der Abgabe von doppelsträngiger DNA zur Regulierung der Genexpression in Säugetierzellen

Compositions pour améliorer la fourniture d'ARN à double brin pour réguler l'expression des gènes dans des cellules de mammifères

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(56) References cited:
WO-A-2004/011647 WO-A-2004/048545
WO-A-2004/061081 WO-A-2004/065601

- **MORRIS M.C. ET AL.:** "A peptide carrier for the delivery of biologically active proteins into mammalian cells" **NATURE BIOTECHNOLOGY**, vol. 19, 2001, pages 1173-1176, XP002559236
- **TUSCHL T. AND BORKHARDT A.:** "Small interfering RNAs: a revolutionary tool for the analysis of gene function and gene therapy" **MOLECULARINTERVENTIONS**, vol. 2, no. 3, June 2002 (2002-06), pages 158-165, XP002294002
- **LAMBERTON J.S. AND CHRISTIAN A.T.:** "Varying the nucleic acid composition of siRNA molecules dramatically varies the duration and degree of gene silencing" **MOLECULAR BIOTECHNOLOGY**, vol. 24, no. 2, June 2003 (2003-06), pages 111-119, XP008062635
- **DUGAN L. ET AL.:** "Tracking of dual-labeled SiHybrid gene-silencing molecules in individual human cells using confocal laser scanning microscopy" **BIOPHYSICAL JOURNAL**, vol. 8, no. 1, January 2004 (2004-01), page 598A, XP008062636

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Description

[0001] RNA interference is the process of sequence-specific post transcriptional gene silencing in cells initiated by double-stranded RNA (dsRNA) that is homologous in sequence to a portion of a targeted mRNA. Introduction of dsRNA into cells leads to the destruction of the endogenous RNAs that share the same sequence as the dsRNA. The dsRNA molecules are cleaved by an RNase III family nuclease called Dicer into short-interfering RNAs (siRNA), which are 19-23 nucleotides (nt) in length. The siRNAs are incorporated into a multicomponent nuclease complex (RISC, RNA-induced silencing complex), which identifies mRNA substrates through their homology to the siRNA, binds to and destroys the targeted mRNA. In mammalian cells, dsRNAs longer than 30 base pairs can activate the dsRNA-dependent kinase PKR and 2'-5'-oligoadenylate synthetase, normally induced by interferon. By virtue of its small size, synthetic siRNA avoids activation of the interferon response. The activated PKR inhibits general translation by phosphorylation of the translation factor eukaryotic initiation factor 2a (eIF2a), while 2'-5'-oligoadenylate synthetase causes nonspecific mRNA degradation via activation of RNase L.

[0002] In contrast to the nonspecific effect of long dsRNA, siRNA can mediate selective gene silencing in the mammalian system. Hairpin RNA with a short loop and 19 to 27 base pairs in the stem also selectively silences expression of genes that are homologous to the sequence in the double-stranded stem. Mammalian cells can convert short hairpin RNA into siRNA to mediate selective gene silencing.

[0003] RISC mediates cleavage of single stranded RNA having sequence complementary to the antisense strand of the siRNA duplex. Cleavage of the target RNA takes place in the middle of the region complementary to the antisense strand of the siRNA duplex.

[0004] Studies have shown that 21 nucleotide siRNA duplexes are most active when containing two nucleotide 3'-overhangs. Furthermore, complete substitution of one or both siRNA strands with 2'-deoxy (2'-H) or 2'-O-methyl nucleotides abolishes RNAi activity, whereas substitution of the 3'-terminal siRNA overhang nucleotides with deoxy nucleotides (2'-H) was shown to be tolerated.

[0005] Studies have shown that replacing the 3'-overhanging segments of a 21-mer siRNA duplex having 2 nucleotide 3' overhangs with deoxyribonucleotides does not have an adverse effect on RNAi activity. Replacing up to 4 nucleotides on each end of the siRNA with deoxyribonucleotides has been reported to be well tolerated whereas complete substitution with deoxyribonucleotides results in no RNAi activity.

[0006] WO 2004/048545 describes siRNA delivery methods including conjugation with delivery peptides and mixing with dendrimers. Morris et al. (Nature Biotechnology, vol. 19, 2001, p.1173-1176) describe a peptide carrier for the delivery of biologically active proteins into mammalian cells. WO 2004/011647 describes double-stranded RNA molecules that mediate RNA interference in target cells such as hepatic cells.

[0007] RNA interference is emerging as a promising means for reducing the expression of specific gene products, and thus may be useful for developing therapeutic drugs to treat viral infections, cancers, autoimmune diseases, and other diseases and conditions amenable to treatment by down-regulation of mRNA expression. However, there remains an important need in the art for additional tools and methods to design, produce, formulate, deliver, and use siRNAs as therapeutic tools, including for therapies targeted to specific tissues and cells.

Brief Description of the Drawings**[0008]**

Figure 1 illustrates serum effects on cellular uptake of a cholesterol-conjugated siRNA in complex with a delivery enhancing agent (comprising a permeabilizing peptide, PN73), and on an unconjugated siRNA in complex with PN73—expressed as percentage uptake.

Figure 2 illustrates serum effects on cellular uptake of a cholesterol-conjugated siRNA in complex with PN73, and on an unconjugated siRNA in complex with PN73—expressed as mean fluorescence intensity (MFI).

Figure 3 illustrates the effects of increasing concentrations of serum on cellular uptake of a cholesterol-conjugated siRNA in the presence or absence of a second delivery enhancing agent, lipofectamine—expressed as percentage uptake.

Figure 4 illustrates the effects of increasing concentrations of serum on cellular uptake of a cholesterol-conjugated siRNA in the presence or absence of a second delivery enhancing agent, lipofectamine—expressed as MFI.

Description

[0009] The present invention provides delivery-enhancing peptides and compositions, as described in the claims.

[0010] The present specification provides double-stranded nucleic acids conjugated to a cholesterol moiety to facilitate delivery of the nucleic acids into a selected target cell or tissue. In particular the present specification is directed towards methods and compositions to administer double-stranded ribonucleic acid to a mammal so as to effectuate transfection of the double-stranded RNA into a desired tissue of the mammal. In certain embodiments the double-stranded RNA has 30 or fewer nucleotides, and is a short interfering RNA (siRNA).

[0011] It has been surprisingly discovered that selectively conjugating a cholesterol moiety to a siRNA at selective ends of the siRNA sense and/or antisense strands increases the silencing of the targeted mRNA. For example, the following siRNA/cholesterol moiety constructs increase the silencing effect of the targeted mRNA in comparison to siRNA having no cholesterol conjugated to it:

1. A siRNA construct having a cholesterol moiety linked to the 5' end of the sense strand and the 5' end of the antisense, and no cholesterol moiety at the other ends;

2. A siRNA construct having a cholesterol moiety linked to the 3' end of the antisense strand, and no cholesterol moiety linked to the other ends of the siRNA strands;

3. A siRNA construct having cholesterol moiety linked to the 5' end of the sense strand, and no cholesterol moiety linked to the other ends of the siRNA strands;

4. A siRNA construct having a cholesterol moiety linked to the 3' end of the sense strand and no cholesterol moiety linked to the other ends of the siRNA strands;

5. A siRNA construct having a cholesterol moiety linked to the 3' end of the sense strand, a cholesterol moiety linked to the 3' end of the antisense strand and no cholesterol moiety linked to the other ends of the siRNA strands; and

6. A siRNA construct having a cholesterol moiety linked to the 5' end of the antisense strand and no cholesterol moiety linked to the other ends of the siRNA strands.

[0012] Thus, the constructs listed above are embodiments described in the present specification, as well as those constructs in which the ds nucleic acid is a siHybrid in which the sense strand is a DNA molecule.

[0013] The following constructs showed a progressively decreased silencing of the targeted mRNA in comparison to a siRNA having no cholesterol moieties conjugated to any of its ends:

1. A siRNA construct having a cholesterol moiety linked to the 3' end of the sense strand, a cholesterol moiety linked 5' end of the antisense strand and no cholesterol moiety linked to the other ends of the siRNA strands;

2. A siRNA construct having a cholesterol moiety linked to the 3' end of the antisense strand, a cholesterol moiety linked to the 5' end of the antisense strand and no cholesterol moiety linked to the other ends of the siRNA strands;

3. A siRNA construct having a cholesterol moiety linked to the 5' end of the sense strand a cholesterol moiety linked to the 3' end of the antisense strand and no cholesterol moiety linked to the other ends of the siRNA strands;

4. A siRNA construct having a cholesterol moiety linked to 5' end of the sense strand, a cholesterol moiety linked to the 3' end of the sense strand, and no cholesterol moiety linked to the other ends of the siRNA strands;

5. A siRNA construct having a cholesterol moiety linked to 5' end of the sense strand, a cholesterol moiety linked to the 3' end of the antisense strand, a cholesterol moiety linked to the 5' end of the antisense strand and no cholesterol moiety linked to the 3', end of the sense strand;

6. A siRNA construct having a cholesterol moiety linked to 5' end of the sense strand, a cholesterol moiety linked to the 3' end of the sense strand, a cholesterol moiety linked to the 3' end of the antisense strand and no cholesterol moiety linked to the 5' end of the antisense strand;

7. A siRNA construct having a cholesterol moiety linked to 5' end of the sense strand, a cholesterol moiety linked to the 3' end of the sense strand, a cholesterol moiety linked to the 5' end of the antisense strand and no cholesterol

moiety linked to the 3' end of the antisense strand;

8. A siRNA construct having a cholesterol moiety linked to 3' end of the sense strand, a cholesterol moiety linked to the 3' end of the sense strand, a cholesterol moiety linked to the 3' end of the antisense strand, a cholesterol moiety linked to the 5' end of the antisense strand, and no cholesterol moiety linked to the 5' end of the sense strand;

9. A siRNA construct having a cholesterol moiety on the 5' end of the sense strand, a cholesterol moiety on the 3' end of the sense strand, a cholesterol moiety on the 3' end of the antisense strand and a cholesterol moiety on the 5' end of the antisense strand.

Definitions

[0014] As used herein, the term "inverted repeat" refers to a nucleic acid sequence comprising a sense and an antisense element positioned so that they are able to form a double stranded siRNA when the repeat is transcribed. The inverted repeat may optionally include a linker or a heterologous sequence such as a self-cleaving ribozyme between the two elements of the repeat. The elements of the inverted repeat have a length sufficient to form a double stranded RNA. Typically, each element of the inverted repeat is about 15 to about 100 nucleotides in length, preferably about 20-30 base nucleotides, preferably about 20-25 nucleotides in length, e.g., 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length.

[0015] "Silencing" refers to partial or complete loss-of-function through targeted inhibition of gene expression in a cell and may also be referred to as "knock down". Depending on the circumstances and the biological problem to be addressed, it may be preferable to partially reduce gene expression. Alternatively, it might be desirable to reduce gene expression as much as possible. The extent of silencing may be determined by any method known in the art, some of which are summarized in International Publication No. WO 99/32619. Depending on the assay, quantitation of gene expression permits detection of various amounts of inhibition for example, greater than 10%, 33%, 50%, 90%, 95% or 99%.

[0016] The phrase "inhibiting expression of a target gene" refers to the ability of a siRNA of the specification to initiate gene silencing of the target gene. To examine the extent of gene silencing, samples or assays of the organism of interest or cells in culture expressing a particular construct are compared to control samples lacking expression of the construct. Control samples (lacking construct expression) are assigned a relative value of 100%. Inhibition of expression of a target gene is achieved when the test value relative to the control is about 90%, preferably 50%, more preferably 25-0%. Suitable assays include, e.g., examination of protein or mRNA levels using techniques known to those of skill in the art such as dot blots, northern blots, in situ hybridization, ELISA, immunoprecipitation, enzyme function, as well as phenotypic assays known to those of skill in the art.

[0017] "Nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

[0018] "Large double-stranded RNA" refers to any double-stranded RNA having a size greater than about 40 base pairs (bp) for example, larger than 100 bp or more particularly larger than 300 bp. The sequence of a large dsRNA may represent a segment of a mRNA or the entire mRNA. The maximum size of the large dsRNA is not limited herein. The double-stranded RNA may include modified bases where the modification may be to the phosphate sugar backbone or to the nucleoside. Such modifications may include a nitrogen or sulfur heteroatom or any other modification known in the art.

[0019] The double-stranded structure may be formed by self-complementary RNA strand such as occurs for a hairpin or a micro RNA or by annealing of two distinct complementary RNA strands.

[0020] "Overlapping" refers to when two RNA fragments have sequences which overlap by a plurality of nucleotides on one strand, for example, where the plurality of nucleotides (nt) numbers as few as 2-5 nucleotides or by 5-10 nucleotides or more.

[0021] "One or more dsRNAs" refers to dsRNAs that differ from each other on the basis of sequence.

[0022] "Target gene or mRNA" refers to any gene or mRNA of interest. Any of the genes previously identified by genetics or by sequencing can be implemented as a target. Target genes or mRNA can include developmental genes and regulatory genes, as well as metabolic or structural genes or genes encoding enzymes. The target gene may be expressed in cells in which a phenotype is being investigated, or in an organism in a manner that directly or indirectly impacts a phenotypic characteristic. The target gene may be endogenous or exogenous. Such cells include any cell in the body of an adult or embryonic animal or plant including gamete or any isolated cell such as occurs in an immortal cell line or primary cell culture.

[0023] In this specification and the appended claims, the singular forms of "a", "an" and "the" include plural reference unless the context clearly dictates otherwise.

[0024] "siRNA" means a small interfering RNA that is a short-length double-stranded RNA that are not toxic in mammalian cells. The length is not limited to 21 to 23 bp long. There is no particular limitation in the length of siRNA as long as it does not show toxicity. "siRNAs" can be, for example, 15 to 49 bp, preferably 15 to 35 bp, and more preferably 21 to 30 bp long. Alternatively, the double-stranded RNA portion of a final transcription product of siRNA to be expressed can be, for example, 15 to 49 bp, preferably 15 to 35 bp, and more preferably 21 to 30 bp long. The double-stranded RNA portions of siRNAs in which two RNA strands pair up are not limited to the completely paired ones, and may contain nonpairing portions due to mismatch (the corresponding nucleotides are not complementary), bulge (lacking in the corresponding complementary nucleotide on one strand), and the like. Nonpairing portions can be contained to the extent that they do not interfere with siRNA formation. The "bulge" used herein preferably comprise 1 to 2 nonpairing nucleotides, and the double-stranded RNA region of siRNAs in which two RNA strands pair up contains preferably 1 to 7, more preferably 1 to 5 bulges. In addition, the "mismatch" used herein is contained in the double-stranded RNA region of siRNAs in which two RNA strands pair up, preferably 1 to 7, more preferably 1 to 5, in number. In a preferable mismatch, one of the nucleotides is guanine, and the other is uracil. Such a mismatch is due to a mutation from C to T, G to A, or mixtures thereof in DNA coding for sense RNA, but not particularly limited to them. Furthermore, in the present specification the double-stranded RNA region of siRNAs in which two RNA strands pair up may contain both bulge and mismatched, which sum up to, preferably 1 to 7, more preferably 1 to 5 in number.

[0025] The terminal structure of siRNA may be either blunt or cohesive (overhanging) as long as siRNA enables to silence the target gene expression due to its RNAi effect. The cohesive (overhanging) end structure is not limited only to the 3' overhang as reported by Tuschl et al. (ibid.), and the 5' overhanging structure may be included as long as it is capable of inducing the RNAi effect. In addition, the number of overhanging nucleotides is not limited to the reported 2 or 3, but can be any numbers as long as the overhang is capable of inducing the RNAi effect. For example, the overhang may be 1 to 8, or 2 to 4 nucleotides. Herein, the total length of siRNA having cohesive end structure is expressed as the sum of the length of the paired double-stranded portion and that of a pair comprising overhanging single-strands at both ends. For example, in the case of 19 bp double-stranded RNA portion with 4 nucleotide overhangs at both ends, the total length is expressed as 23 bp. Furthermore, since this overhanging sequence has low specificity to a target gene, it is not necessarily complementary (antisense) or identical (sense) to the target gene sequence. Furthermore, as long as the siRNA is able to maintain its gene silencing effect on the target gene, it may comprise a low molecular weight RNA (which may be a natural RNA molecule such as tRNA, rRNA or viral RNA, or an artificial RNA molecule), for example, in the overhanging portion at its one end.

[0026] In addition, the terminal structure of the "siRNA" is necessarily the cut off structure at both ends as described above, and may have a stem-loop structure in which ends of one side of double-stranded RNA are connected by a linker RNA. The length of the double-stranded RNA region (stem-loop portion) can be, for example, 15 to 49 bp, preferably 15 to 35 bp, and more preferably 21 to 30 bp long. Alternatively, the length of the double-stranded RNA region that is a final transcription product of siRNAs to be expressed is, for example, 15 to 49 bp, preferably 15 to 35 bp, and more preferably 21 to 30 bp long. Furthermore, there is no particular limitation in the length of the linker as long as it has a length so as not to hinder the pairing of the stem portion. For example, for stable pairing of the stem portion and suppression of the recombination between DNAs coding for the portion, the linker portion may have a clover-leaf tRNA structure. Even though the linker has a length that hinders pairing of the stem portion, it is possible, for example, to construct the linker portion to include introns so that the introns are excised during processing of precursor RNA into mature RNA, thereby allowing pairing of the stem portion. In the case of a stem-loop siRNA, either end (head or tail) of RNA with no loop structure may have a low molecular weight RNA. As described above, this low molecular weight RNA may be a natural RNA molecule such as tRNA, rRNA or viral RNA, or an artificial RNA molecule.

[0027] "Antisense RNA" is an RNA strand having a sequence complementary to a target gene mRNA, and thought to induce RNAi by binding to the target gene mRNA. "Sense RNA" has a sequence complementary to the antisense RNA, and annealed to its complementary antisense RNA to form siRNA. These antisense and sense RNAs have been conventionally synthesized with an RNA synthesizer.

[0028] As used herein, the term "RNAi construct" is a generic term used throughout the specification to include small interfering RNAs (siRNAs), hairpin RNAs, and other RNA species which can be cleaved *in vivo* to form siRNAs. RNAi constructs herein also include expression vectors (also referred to as RNAi expression vectors) capable of giving rise to transcripts which form dsRNAs or hairpin RNAs in cells, and/or transcripts which can produce siRNAs *in vivo*. Optionally, the siRNA include single strands or double strands of siRNA.

[0029] An siHybrid molecule is a double-stranded nucleic acid that has a similar function to siRNA. Instead of a double-stranded RNA molecule, a siHybrid is comprised of an RNA strand and a DNA strand. Preferably, the RNA strand is the antisense strand as that is the strand that binds to the target mRNA. The siHybrid created by the hybridization of the DNA and RNA strands have a hybridized complementary portion and preferably at least one 3' overhanging end.

[0030] A cholesterol moiety is a cholesterol molecule, sterol or any compound derived from cholesterol including

cholestanol, ergosterol, stimastanol, stigmasterol, methyl-lithocholic acid, cortisol, corticosterone, Δ^5 -pregnenolone, progesterone, deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, 11-dioxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, aldosterone, 18-hydroxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, cortisone, prednisone, 6 α -methylprednisone, 9 α -fluoro-16 α -hydroxyprednisolone, 9 α -fluoro-16 α -methylprednisolone, 9 α -fluorocortisol, testosterone, dihydrotestosterone, androstenediol, androstenedione, androstenedione, 3 α ,5 α -androstenediol, estrone, estradiol, estrogen, spermidine cholesterol carbamate, N⁴-spermidine cholesteryl carbamate, N⁴-spermidine cholesteryl carbamate di HCl salt, N⁴-spermidine-7 dehydro cholesteryl carbamate, N⁴-spermine cholesteryl carbamate, N,N bis(3-aminopropyl) cholesteryl carbamate, N,N bis(6-aminohexyl) cholesteryl carbamate, N⁴-spermidine dihydrocholesteryl carbamate, N⁴-spermidine lithocholic carbamate methyl ester, N¹,N⁸-bis (3-aminopropyl-N⁴-spermidine cholesteryl carbamate, N(N⁴-3aminopropylspermidine) cholesteryl carbamate, N,N-bis(4-aminobutyl) cholesteryl carbamate, N⁴-spermidine cholesteryl urea, N⁴-spermine cholesteryl urea, N⁴-spermidine dihydro cholesteryl urea, N⁴-spermine dihydro cholesteryl urea, N,N-bis(N¹-3-aminopropyl-N⁴-aminobutyl) cholesteryl carbamate, N⁴spermidine cholesteryl carboxamide, and N-[N¹,N⁴,N⁸-tris (3-aminopropyl)spermidine] cholesteryl carbamate, lumisterol, cholic acid, desoxycholic acid, chenodesoxycholic acid and lithocholic acid and derivatives thereof (see, e.g., U.S. Patent No. 6,331,524).

[0031] The following exemplary cholesterol-RNA constructs are illustrative of various embodiments of the specification:

1. A siRNA or siHybrid construct having a cholesterol moiety linked to the 5' end of the sense strand and the 5' end of the antisense and no cholesterol moiety at the other ends;

2. A siRNA or siHybrid construct having a cholesterol moiety linked to the 3' end of the antisense strand and no cholesterol moiety linked to the other ends of the siRNA or siHybrid strands;

3. A siRNA or siHybrid construct having cholesterol moiety linked to the 5' end of the sense strand and no cholesterol moiety linked to the other ends of the siRNA or siHybrid strands;

4. A siRNA or siHybrid construct having a cholesterol moiety linked to the 3' end of the sense strand and no cholesterol moiety linked to the other ends of the siRNA or siHybrid strands;

5. A siRNA or siHybrid construct having a cholesterol moiety linked to the 3' end of the sense strand, a cholesterol moiety linked to the 3' end of the antisense strand and no cholesterol moiety linked to the other ends of the siRNA or siHybrid strands; and 6. A siRNA or siHybrid construct having a cholesterol moiety linked to the 5' end of the antisense strand and no cholesterol moiety linked to the other ends of the siRNA or siHybrid strands.

[0032] In more detailed embodiments of the specification, a cholesterol-conjugated siRNA or siHybrid is formulated with, or delivered in a coordinate administration method with, one or more secondary delivery-enhancing agent(s) that is/are further effective to enhance delivery of the cholesterol-conjugated siRNA or siHybrid into mammalian cells. Typically the second delivery-enhancing agent(s) is/are effective to facilitate delivery of the cholesterol-conjugated siRNA or siHybrid across the plasma membrane and into the cytoplasm of a targeted mammalian cell. The targeted cell may be any cell for which delivery of a cholesterol-conjugated siRNA or siHybrid into the cell for regulation of gene expression is desired. Exemplary target cells in this context include pulmonary alveolar or other airway cells, skin cells, hepatic cells, renal cells, pancreatic cells, endothelial cells, nucleated blood cells (e.g., lymphocytes, monocytes, macrophages, or dendritic cells), muscle cells (e.g., cardiac or smooth muscle cells), mammary cells, peripheral or central nervous system (CNS) cells, cells of the stomach or intestinal tract, tumor cells, and other cells that are amenable to gene regulation for therapeutic purposes according to the methods and compositions of the specification.

[0033] In one exemplary embodiment, the cholesterol-conjugated siRNA or siHybrid are targeted for delivery to mucosal epithelial cells, for example nasal mucosal epithelial cells.

[0034] Within these and related aspects of the specification, the secondary delivery-enhancing agent(s) may be selected from one or any combination of the following:

- (a) an aggregation inhibitory agent;
- (b) a charge modifying agent;
- (c) a pH control agent;
- (d) a degradative enzyme inhibitory agent;
- (e) a mucolytic or mucus clearing agent;
- (f) a ciliostatic agent;
- (g) a membrane penetration-enhancing agent selected from (i) a surfactant, (ii) a bile salt, (iii) a phospholipid additive, mixed micelle, liposome, or carrier, (iv) an alcohol, (v) an enamine, (vi) an NO donor compound, (vii) a long-chain

amphipathic molecule (viii) a small hydrophobic penetration enhancer; (ix) sodium or a salicylic acid derivative; (x) a glycerol ester of acetoacetic acid (xi) a cyclodextrin or beta-cyclodextrin derivative, (xii) a medium-chain fatty acid, (xiii) a chelating agent, (xiv) an amino acid or salt thereof, (xv) an N-acetylamino acid or salt thereof, (xvi) an enzyme degradative to a selected membrane component, (xvii) an inhibitor of fatty acid synthesis, or (xviii) an inhibitor of cholesterol synthesis; or (xix) any combination of the membrane penetration enhancing agents recited in (g)(i)-(xix); (h) a delivery-enhancing peptide; (i) a vasodilator agent; (j) a selective transport-enhancing agent; and (k) a stabilizing delivery vehicle, carrier, support or complex-forming species with which the cholesterol-conjugated siRNA or siHybrid is effectively combined, associated, contained, encapsulated or bound resulting in stabilization of the siRNA or siHybrid for enhanced delivery.

[0035] In additional aspects of the specification, the delivery-enhancing agent(s) comprise(s) any one or any combination of two or more of the foregoing delivery-enhancing agents recited in (a)-(k), and the formulation of the cholesterol-conjugated siRNA or siHybrid with the delivery-enhancing agents provides for increased delivery of the cholesterol-conjugated siRNA or siHybrid into the cytoplasm of target cells for gene regulation by the cholesterol-conjugated siRNA or siHybrid.

[0036] Any one or combination of the foregoing secondary delivery-enhancing agents may be added to a pharmaceutical composition comprising a cholesterol-conjugated siRNA or siHybrid as described herein, to yield a combinatorial formulation providing greater delivery enhancement in comparison to intracellular delivery of the cholesterol-conjugated siRNA or siHybrid without the secondary delivery-enhancing agent(s).

[0037] Within coordinate administration methods of the specification, the cholesterol-conjugated siRNA or siHybrid is administered to a target cell, tissue, or individual in combination with one or more secondary delivery-enhancing agents in a coordinate administration protocol. Within these coordinate administration methods, the cholesterol-conjugated siRNA or siHybrid is administered to the same cell, tissue, or individual as the secondary delivery-enhancing agent(s), prior to, simultaneous with, or after administration of the secondary delivery-enhancing agent(s), which similarly may be selected from any one or combination of the following:

- (a) an aggregation inhibitory agent;
- (b) a charge modifying agent;
- (c) a pH control agent;
- (d) a degradative enzyme inhibitory agent;
- (e) a mucolytic or mucus clearing agent;
- (f) a ciliostatic agent;
- (g) a membrane penetration-enhancing agent selected from (i) a surfactant, (ii) a bile salt, (iii) a phospholipid additive, mixed micelle, liposome, or carrier, (iv) an alcohol, (v) an enamine, (vi) an NO donor compound, (vii) a long-chain amphipathic molecule (viii) a small hydrophobic penetration enhancer; (ix) sodium or a salicylic acid derivative; (x) a glycerol ester of acetoacetic acid (xi) a cyclodextrin or beta-cyclodextrin derivative, (xii) a medium-chain fatty acid, (xiii) a chelating agent, (xiv) an amino acid or salt thereof, (xv) an N-acetylamino acid or salt thereof, (xvi) an enzyme degradative to a selected membrane component, (xvii) an inhibitor of fatty acid synthesis, or (xviii) an inhibitor of cholesterol synthesis; or (xix) any combination of the membrane penetration enhancing agents recited in (g)(i)-(xix); (h) a delivery-enhancing peptide; (i) a vasodilator agent; (j) a selective transport-enhancing agent; and (k) a stabilizing delivery vehicle, carrier, support or complex-forming species with which the cholesterol-conjugated siRNA or siHybrid is effectively combined, associated, contained, encapsulated or bound resulting in stabilization of the siRNA or siHybrid for enhanced intracellular delivery. The coordinate administration of the cholesterol-conjugated siRNA or siHybrid and secondary delivery-enhancing agent(s) provides for increased uptake of the cholesterol-conjugated siRNA or siHybrid into the cytoplasm of targeted cells, typically enhancing gene regulation (e.g., increasing knockdown of mRNA translation to thereby reduce expression of one or more selected protein(s), such as TNF- α , in the target cell.

[0038] Additional detailed description pertaining to secondary delivery-enhancing agents, is provided, for example, in United States Provisional Patent Applications No.s: 60/612,121, filed September 21, 2004; 60/667,835, filed April 1, 2005; 60/612,285, filed September 21, 2004; 60/667,871, filed April 1, 2005; 60/613,416, filed September 27, 2004; and 60/667,833, filed April 1, 2005.

[0039] Within exemplary embodiments of the specification, a delivery-enhancing peptide is employed as the secondary delivery-enhancing agent. The delivery-enhancing peptide may be conjugated to, combinatorially formulated with, or

coordinately administered with, the cholesterol-conjugated siRNA or siHybrid to enhance intracellular uptake of the cholesterol-conjugated siRNA or siHybrid and improve gene regulation results achieved thereby. Delivery-enhancing peptides in this context may include natural or synthetic, therapeutically or prophylactically active, peptides (comprised of two or more covalently linked amino acids), proteins, peptide or protein fragments, peptide or protein analogs, peptide or protein mimetics, and chemically modified derivatives or salts of active peptides or proteins. Thus, as used herein, the term "delivery-enhancing peptide" will often be intended to embrace all of these active species, i.e., peptides and proteins, peptide and protein fragments, peptide and protein analogs, peptide and protein mimetics, and chemically modified derivatives and salts of active peptides or proteins. Often, the delivery-enhancing peptide comprises a mutein that is readily obtainable by partial substitution, addition, or deletion of amino acids within a naturally occurring or native (e.g., wild-type, naturally occurring mutant, or allelic variant) peptide or protein sequence (e.g., a sequence of a naturally occurring "cell penetrating peptide" or peptide fragment of a native protein, such as a tight junction protein). Additionally, biologically active fragments of native peptides or proteins are included. Such mutant derivatives and fragments substantially retain the desired cell penetrating or other delivery-enhancing activity of the corresponding native peptide or proteins. In the case of peptides or proteins having carbohydrate chains, biologically active variants marked by alterations in these carbohydrate species are also included within the specification.

[0040] The delivery-enhancing peptides, proteins, analogs and mimetics for use within the methods and compositions of the specification are may be conjugated to, or formulated with, the cholesterol-conjugated siRNA or siHybrid to yield a pharmaceutical composition that includes a delivery-enhancing effective amount of the delivery-enhancing peptide, protein, analog or mimetic (i.e., an amount of the peptide sufficient to detectably enhance intracellular delivery of the cholesterol-conjugated siRNA or siHybrid).

[0041] Exemplary delivery-enhancing peptides for use within the methods and compositions of the specification include any one or combination of the following peptides, or active fragments, muteins, conjugates, or complexes thereof:

RKKRRQRRRPPQCAAVALLPAVLLALLAP (SEQ ID NO: 1);
 RQIKIWFQNRRMKWKK (SEQ ID NO: 2);
 GWTLNSAGYLLGKINLKALAALAKKIL (SEQ ID NO: 3);
 KLALKLALKALKAAALKLA (SEQ ID NO: 4);
 KLWSAWPSLWSSLWKP (SEQ ID NO: 7);
 AAVALLPAVLLALLAPRKKRRQRRRPPQ (SEQ ID NO: 8);
 LLETLLKPFQCRICMRNFSTRQARRNHRRRHRR (SEQ ID NO: 9);
 RRRQRRKRGGDIMGEWGNEIFGAIAGFLG (SEQ ID NO: 10);
 KETWWETWWTEWSQPGRKKRRQRRRPPQ (SEQ ID NO: 11);
 GLGSLLKAGKKLKQPKSKRKV (SEQ ID NO: 12); and
 KGSKKAHTKAQKKDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 13)

[0042] Delivery-enhancing peptides of the specification may further include various modifications known in the art, e.g., for modifying the charge, membrane permeability, half-life, degradative potential, reactivity (e.g., to form conjugates), immunogenicity, or other desired properties of the subject peptide. Exemplary modified delivery-enhancing peptides in this context may include, for example, peptides modified by incorporation of one or more selected amino- or carboxy-terminal chemical modifications. For example, amino- and/or carboxy-terminal amide, BrAc, or maleimide groups may be included, as exemplified by the modified delivery-enhancing peptides shown in Table 1.

Table 1

Peptide	Sequences	Effects
PN0028	RKKRRQRRRPPQCAAVALLPAVLLALLAP-amide (SEQ ID NO:1)	+
PN0058	RQIKIWFQNRRMKWKK-amide (SEQ ID NO: 2)	+
PN0064	BrAc-GWTLNSAGYLLGKINLKALAALAKKILamide (SEQ ID NO: 3)	+
PN0068	BrAc-KLALKLALKALKAAALKLA-amide (SEQ ID NO: 4)	+
PN0069	GRKKRRQRRRPQ-amide (SEQ ID NO: 5)	-
PN0071	RRRRRRR (SEQ ID NO: 6)	-
PN0228	NH2-KLWSAWPSLWSSLWKP-amide (SEQ ID NO: 7)	+/-
PN027	NH2-AAVALLPAVLLALLAPRKKRRQRRRPPQ-amide (SEQ ID NO: 8)	+
PN202	NH2-LLETLLKPFQCRICMRNFSTRQARRNHRRRHRR-amide (SEQ ID NO: 9)	+
PN250	NH2-RRRQRRKRGGDIMGEWGNEIFGAIAGFLG-amide (SEQ ID NO: 10)	+
PN183	NH2-KETWWETWWTEWSQPGRKKRRQRRRPPQ-amide (SEQ ID NO: 11)	+

(continued)

Peptide	Sequences	Effects
PN283	Maleimide-GLGSLLKKAGKKLKQPKSKRKV-amide (SEQ ID NO: 12)	+
PN073	KGSKKA/TKAQQDKKKRKRSRKESYSVYVYKVLKQ-amide (SEQ ID NO: 13)	+
Assay medium only		-

[0043] The + and - notations indicated in Table 1 for the listed peptides relate to activity of the peptides to enhance permeation of across epithelial monolayers-as determined by measurement of peptide-mediated changes in trans-epithelial electrical resistance (TEER). A + notation indicates that the subject peptide enhances epithelial permeation of macromolecules. The peptides that exhibit permeation-enhancing activity can be tested and selected according to the methods herein to determine their utility for enhancing delivery of cholesterol-conjugated siRNA or siHybrid into the cytoplasm of targeted cells to enhance gene regulation

[0044] The above disclosure generally describes the present specification, which is further exemplified by the following examples. These examples are described solely for purposes of illustration. Although specific terms and values have been employed herein, such terms and values will likewise be understood as exemplary.

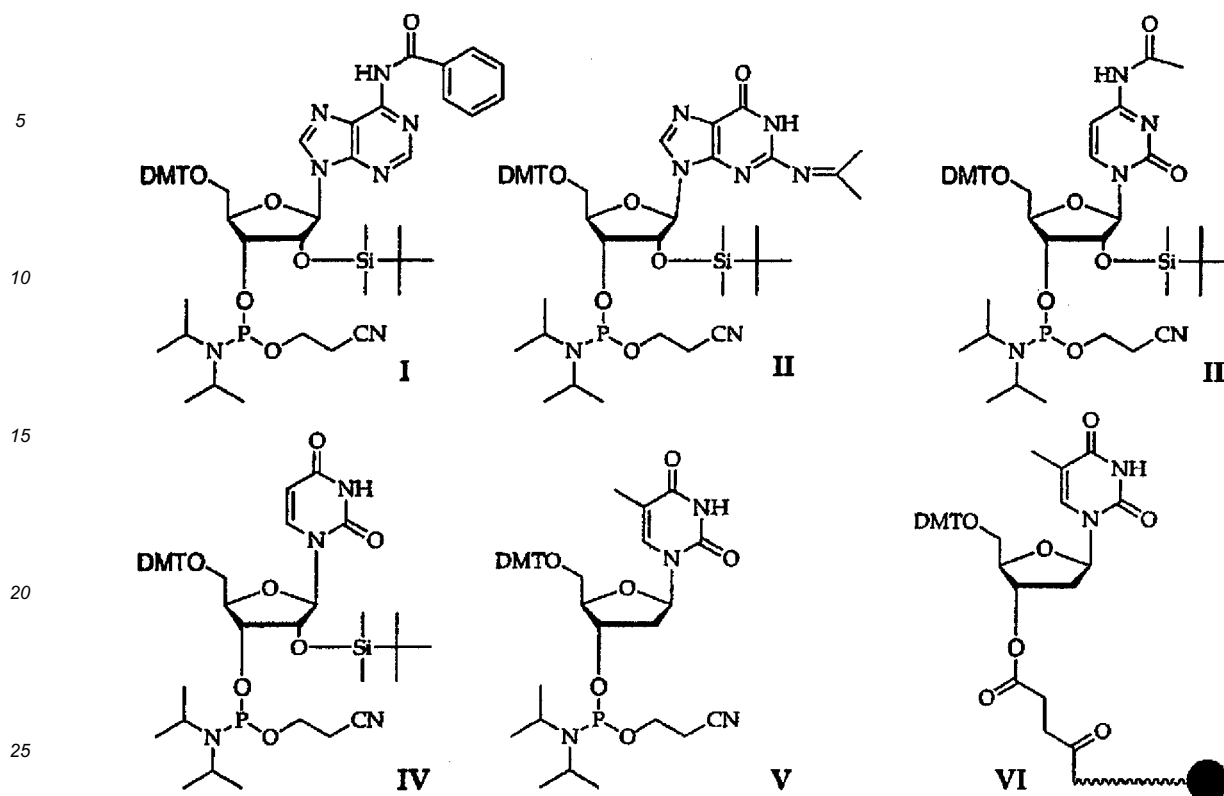
Example 1

Synthesis and Purification of Cholesteryl-Labeled siRNA

Synthesis of unmodified siRNAs:

[0045] Unmodified siRNAs were synthesized according to the general strategy for solid-phase oligonucleotide synthesis. The syntheses proceeded from the 3'- to 5'-direction [current protocols in nucleic acid chemistry, chapter 3]. The first step involved attachment of a mononucleoside/tide to the surface of an insoluble solid support through a covalent bond. All unmodified siRNAs described here were synthesized starting with a CPG-bound deoxythymidine (purchased from Glen Research, Sterling VA). The thymidine nucleoside is covalently attached to the solid support through 3'-hydroxyl group using a base labile linker. Before chain elongation can proceed, the terminal-protecting group (dimethoxytrityl, DMT) on the nucleoside is removed. This exposes a free 5'-OH group where the next nucleotide unit can be added. An excess of reagents is used to force the coupling reaction to occur on as many of the immobilized nucleotides as possible. After the coupling reaction, excess reagents are washed away. The reaction is followed by a c capping step, to block off non-extended sites, and an oxidation step. The process of terminal-protecting group removal and chain extension is then repeated using different bases until the desired sequence has been assembled. Some or all of the protecting groups may optionally be removed, and then the covalent attachment to the support is hydrolyzed to release the product. Removal of the protecting groups were carried out with 3:1 mixture of concentrated ammonia: ethanol. After removal of any remaining protecting groups, the oligonucleotide is ready for purification and use.

[0046] RNA syntheses were carried out by Applied Biosystems 3400 using standard phosphoramidite chemistry. The corresponding building blocks, 5'-dimethoxytrityl-*N*-benzoyladenine-2'-*O*-(*t*-butyldimethylsilyl)-3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (Bz-A-CE phosphoramidite) (I), 5'-dimethoxytrityl-*N*-dimethylformamide-guanosine, 2'-*O*-(*t*-butyldimethylsilyl)-3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (dmf-G-CE phosphoramidite) (II), 5'-dimethoxytrityl-*N*-acetylcytidine-2'-*O*-(*t*-butyldimethylsilyl)-3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (Ac-C-CE phosphoramidite) (III), 5'-dimethoxytrityluridine-2'-*O*-(*t*-butyldimethylsilyl)-3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (U-CE phosphoramidite) (IV) and 5'-dimethoxytrityl-2'-deoxythymidine-3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (V) were purchased from Glen Research Inc. (Sterling VA). For un-modified sequences, the syntheses started on Controlled Pore glass (CPG) bound deoxythymidine (VI) (Applied Biosystems, Foster City, CA) in 0.2 or 1.0 μ mol scale. Other reagents and solvents were purchased from Glen Research (Sterling, VA) and/or Applied Biosystems (Foster City, CA).



DMT = 4,4'-dimethoxytrytyl

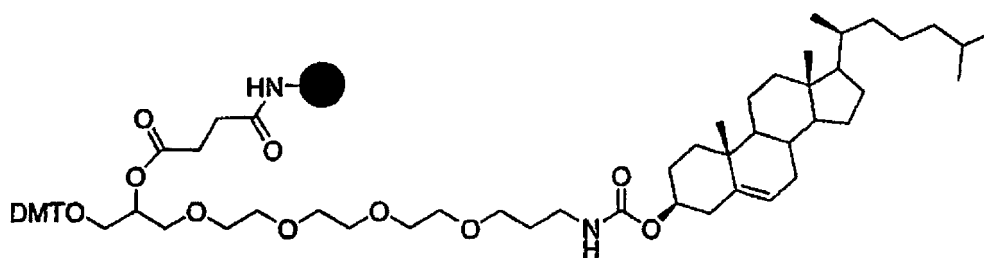
Commonly used protected phosphoramidites for the synthesis of RNA

Synthesis of 3'-cholesteryl-labeled siRNA

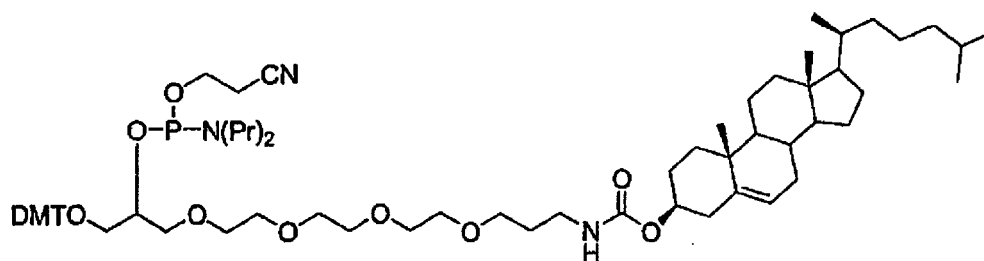
[0047] The synthesis of 3'-cholesteryl-labelled siRNAs was carried out using the modified support strategy. In this method a new modified solid phase synthesis support must be prepared for each 3'-reporter group or conjugate. The solid phase support for attaching cholesteryl group to the 3'-termini of oligonucleotides is commercially available. The synthesis of the 3'-cholesteryl-labelled oligonucleotides were accomplished using 1-dimethoxytrytyloxy-3-O-(*N*-cholesteryl-3-aminopropyl)-triethyleneglycol-glyceryl-2-O-succinoyl-long-chain-alkylamino-CPG (**VII**, Glen Research, Sterling VA). The designed 21 nucleotide sequence was then assembled on this modified solid support using standard phosphoramidite protocols for RNA synthesis as described herein above.

Synthesis of 5'-cholesteryl-labeled siRNA

[0048] A protected oligonucleotide with a free hydroxyl group at the 5'-end, immobilized on the solid support, may easily be obtained by solid phase synthesis using either methodologies described herein above. The 5'-terminal hydroxyl can then be reacted with phosphoramidites. Phosphoramidites often obtained from a molecule having a hydroxyl functionality allow the direct introduction of a functional group or ligand to the chain after oxidation and deprotection. To incorporate a cholesteryl group to the 5'-end of siRNA molecules, dimethoxytrytyloxy-3-O-(*N*-cholesteryl-3-aminopropyl)-triethyleneglycol-glyceryl-2-O-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite (**VIII**) was purchased from Glen Research (Sterling, VA). During the solid support synthesis of siRNA, after the incorporation of the last nucleoside/tide, the 5'-dimethoxytrytyl protecting group was cleaved and **VIII** was coupled to the grown chain



1-dimethoxytrityloxy-3-O-(*N*-cholesteryl-3-aminopropyl)-triethyleneglycol-glycerol-2-O-succinoyl-long-chain-alkylamino-CPG (VII)



dimethoxytrityloxy-3-O-(*N*-cholesteryl-3-aminopropyl)-triethyleneglycol-glycerol-2-O-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite (VII)

Syntheses of 3' and 5'-dicholesteryl-labeled siRNAs

[0049] Syntheses of 3',5'-dicholesteryl-labeled siRNAs were accomplished using a combination of the methods described above. The synthesis of such a molecule started with using **VII** as the "modified solid support", and elongation and incorporation of the 5'-cholesteryl moiety were carried out as described above.

Example 2

Cholesterol-Enhanced Uptake of siRNA and Silencing of Beta-Galactosidase mRNA Expression

Transfection of 9L/LacZ cells:

[0050]

Day 0:

a) Take saturated 9L/LacZ culture from T75 flask, detach cell and dilute into 10ml with complete medium (DMEM, 1xPS, 1xNa Pyruvate, 1x NEAA).

b) Further dilute the cell to 1:15, and seed 100 μ l into each 96 well, which should give 50% confluence cell the next day for transfection. Remember to leave the edge well empty and fill with 250 μ l water, do not stack up plates in the incubator.

c) Incubate overnight at 37°C, 5% CO₂ incubator.

Day 1:

a) Prepare the transfection complex in Opti-MEM, 50 μ l each well.

b) Dump the medium in plates, wash each well once with 200 μ l PBS or Opti-MEM.

c) Blot the plates dry completely with tissue by inversion.

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d) Add the transfection mixture (50 µl/well) into each well, add 250 µl water into wells on the edge to prevent wells from drying.

e) Incubate for at least 3 hours at 37°C, 5% CO₂ incubator.

f) Dump the transfection mixture, replace with 100 µl of complete medium (DMEM, 1xPS, 1xNa Pyruvate, 1x NEAA).

β-Gal/BCA assay in 96 well format

Cell Lysis

[0051]

a) Dump the medium, wash once with 200 µl PBS, blot the plate dry with inversion.

b) Add 30 µl lysis buffer from β-Gal Kit into each well.

c) Freeze-Thaw the cells twice to generate lysate.

β-Gal assay

[0052]

a) Prepare assay mix (50 µl 1xbuffer, 17 µl ONPG each well)

b) Take new plate and add 65ul assay mix into each well.

c) Add 10 µl of cell lysate into each well. There should be blank wells for subtraction of the background activities.

d) Incubate at 37°C for about 20 minutes, prevent long incubation which will use up all ONPG and biased the high expression.

e) Add 100 µl of the Stop solution.

f) Measure the OD at 420nm.

BCA assay

[0053]

a) Prepare BSA standard (150 ul per well), every points should be duplicated oneach plate.

b) Put 145 µl of water into each well, add 5 ul of cell lysate into each well.

c) Prepare Assay Reagent (A:B:C: 25:24:1), mix right before use.

d) Add 150 µl of Assay Reagent into each well.

e) Incubate at 37°C for about 20 minutes.

f) Measure the OD at 562nm.

Flow Cytometry Measurement of FITC/FAM conjugated siRNA

[0054]

a) After transfection, incubate cell for at least 3 hours.

b) Wash with 200 μ l PBS.

c) Detach cell with 15 μ l TE, incubate at 37°C.

d) Re-suspend five wells with 30 μ l FACS solution (PBS with 0.5% BSA, and 0.1% sodium Azide)

e) Combine all five wells into a tube.

f) Add PI 5 μ l into each tube.

g) Analyze the cells with fluorescence activated cell sorting (FCAS) with BD FACscan instrument according to manufacture's instruction.

Results

Cholesterol conjugation of siRNA

[0055] The transfection was performed with either regular siRNA or cholesterol-conjugated siRNA with lipofectamine (Invitrogen) on 9L/beta-gal cells. The siRNA was designed to specifically knock down beta-galactosidase mRNA and activities are expressed as percentage of beta-gal activities from control (transfected cells by lipofectamine alone).

1. siRNA sequence and structure information of cholesterol-conjugated siRNA

[0056]

C.U.A.C.A.C.A.A.U.C.A.G.C.G.A.U.U.U.dT.dT (Sense) (SEQ ID NO: 14)

A.A.A.U.C.G.C.U.G.A.U.U.U.G.U.G.U.A.G.dT.dT (Antisense) (SEQ ID NO: 15)

Designation of cholesterol conjugated siRNA

[0057]

A. regular sense or antisense strand

B. 5' end labeled sense strand

C. 3' end labeled sense strand

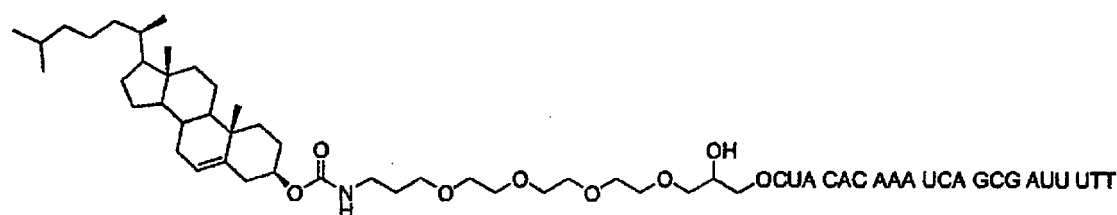
D. both ends labeled sense strand

E. 5' end labeled antisense strand

F. 3' end labeled antisense strand

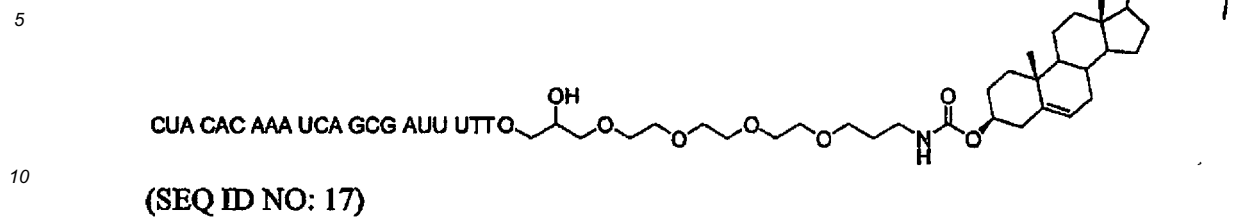
G. both end labeled antisense strand

Sense (B)

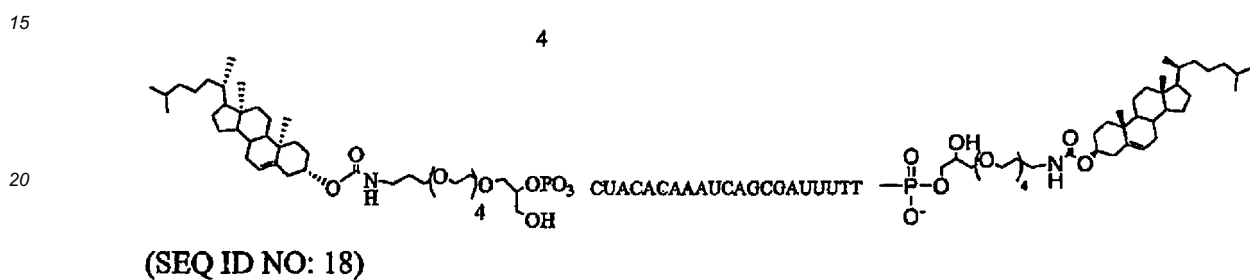


(SEQ ID NO: 16)

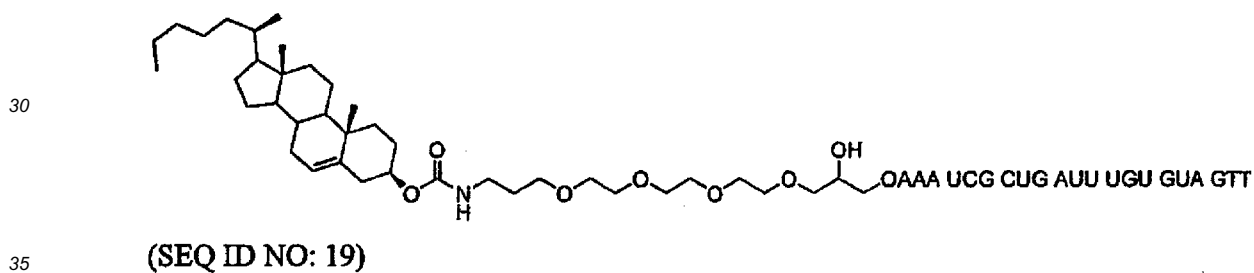
Sense (C)



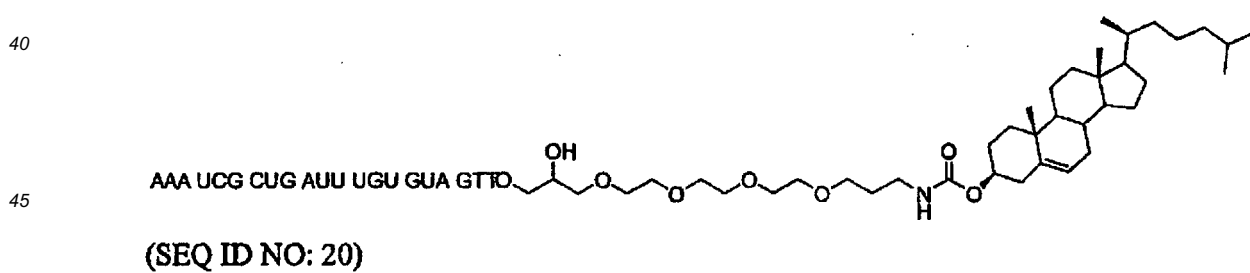
Sense (D)



Antisense (E)



Antisense (F)



Antisense (G)

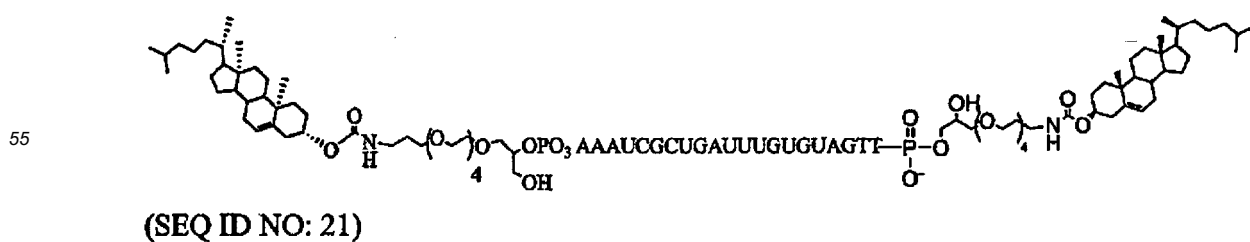


Table 1 Cholesterol siRNA Activities Post-Transfection

Duplexes	Activity (% of control)		Duplexes	Activity (% of control)
AA	23.12		AG	29.87
BE	10.27		BF	32.02
AF	11.99		DA	33.99
BA	12.09		BG	46.39
EA	16.18		DF	65.4
CE	16.76		DE	77.12
AE	19.02		CG	77.80
CE	27.62		DG	98.84

[0058] Table 1 above provides results of transfection and mRNA silencing experiments using the siRNA constructs made using sense and antisense strands designated above. The transfection and silencing assay results show cholesterol-enhanced delivery of exemplary siRNAs of the invention, and demonstrate silencing of the beta-galactosidase mRNA by the cholesterol-conjugated siRNAs. The "Activity (% of control)" indicates the beta-galactosidase activity remaining after the transfection. The lower the percentage, the greater was the efficacy of the siRNA construct. The double letters represent a double-stranded siRNA. Thus, the exemplary constructs, BE, AF, BA, CA, CF, and AE are representative of the nature and activity of cholesterol conjugated dsRNAs of the present invention. These constructs show greater silencing efficacy than the corresponding unconjugated siRNAs. siRNA constructs CE, AG, BF, DA, BG, DF, DE, CG and DG showed lower efficacy than the unconjugated siRNA construct AA.

[0059] AA is a siRNA construct with no cholesterol conjugated to any of the ends of the sense or antisense RNA strands. This construct was transfected into the cells resulting in silencing of the beta-galactosidase mRNA so that 23.12% of the activity of the beta-galactosidase mRNA remained.

[0060] BE is a siRNA construct having a cholesterol moiety linked to the 5' end of the sense strand and a cholesterol moiety linked to the 5' end of the antisense strand, and no cholesterol moiety linked to the other ends of the siRNA. This construct was transfected into the cells resulting in silencing the beta-galactosidase mRNA so that only 10.27% of the activity of the beta-galactosidase mRNA remained. This is unexpectedly superior to the unconjugated siRNA.

[0061] AF is a siRNA construct having a cholesterol moiety linked to the 3' end of the antisense strand and no cholesterol moiety linked to the other ends of the siRNA strands. This construct was transfected into the cells resulting in silencing the beta-galactosidase mRNA so that only 11.99% of the activity of the beta-galactosidase mRNA remained. This is unexpectedly superior to the unconjugated siRNA.

[0062] BA is a siRNA construct having a cholesterol moiety linked to the 5' end of the sense strand and no cholesterol moiety linked to the other ends of the siRNA strands. This construct was transfected into the cells resulting in silencing the beta-galactosidase mRNA so that only 12.09% of the activity of the beta-galactosidase mRNA remained. This is unexpectedly superior to the unconjugated siRNA.

[0063] CA is a siRNA construct having a cholesterol moiety linked to the 3' end of the sense strand and no cholesterol moiety linked to the other ends of the siRNA strands. This construct was transfected into the cells resulting in silencing the beta-galactosidase mRNA so that only 16.18% of the activity of the beta-galactosidase mRNA remained. This is unexpectedly superior to the unconjugated siRNA.

[0064] CF is a siRNA construct having a cholesterol moiety linked to the 3' end of the sense strand, a cholesterol moiety linked to the 3' end of the antisense strand, and no cholesterol moiety linked to the other ends of the siRNA strands. This construct was transfected into the cells resulting in silencing the beta-galactosidase mRNA so that only 16.76% of the activity of the beta-galactosidase mRNA remained. This is unexpectedly superior to the unconjugated siRNA.

[0065] AE is a siRNA construct having a cholesterol moiety linked to the 5' end of the antisense strand and no cholesterol moiety linked to the other ends of the siRNA strands. This construct was transfected into the cells resulting in silencing the beta-galactosidase mRNA so that only 19.02% of the activity of the beta-galactosidase mRNA remained. This is unexpectedly superior to the unconjugated siRNA.

[0066] The constructs listed below showed lower ability to silence the beta-galactosidase reporter than was determined for the corresponding, unconjugated siRNA.

[0067] CE is a siRNA construct having a cholesterol moiety linked to the 3' end of the sense strand, a cholesterol moiety linked 5' end of the antisense strand, and no cholesterol moiety linked to the other ends of the siRNA strands. This construct was transfected into the cells resulting in silencing the beta-galactosidase mRNA so that 27.62% of the

activity of the beta-galactosidase mRNA remained. This silencing effect was lower than that observed for the corresponding, unconjugated siRNA.

[0068] AG is a siRNA construct having a cholesterol moiety linked to the 3' end of the antisense strand, a cholesterol moiety linked to the 5' end of the antisense strand, and no cholesterol moiety linked to the other ends of the siRNA strands. This construct was transfected into the cells resulting in silencing the beta-galactosidase mRNA so that 29.87% of the activity of the beta-galactosidase mRNA remained. This silencing effect was lower than that observed for the corresponding, unconjugated siRNA.

[0069] BF is a siRNA construct having a cholesterol moiety linked to the 5' end of the sense strand, a cholesterol moiety linked to the 3' end of the antisense strand, and no cholesterol moiety linked to the other ends of the siRNA strands. This construct was transfected into the cells resulting in silencing the beta-galactosidase mRNA so that 32.02% of the activity of the beta-galactosidase mRNA remained. This silencing effect was lower than that observed for the corresponding, unconjugated siRNA.

[0070] DA is a siRNA construct having a cholesterol moiety linked to 5' end of the sense strand, a cholesterol moiety linked to the 3' end of the sense strand, and no cholesterol moiety linked to the other ends of the siRNA strands. This construct was transfected into the cells resulting in silencing the beta-galactosidase mRNA so that 33.99% of the activity of the beta-galactosidase mRNA remained. This silencing effect was lower than that observed for the corresponding, unconjugated siRNA.

[0071] BG is a siRNA construct having a cholesterol moiety linked to 5' end of the sense strand, a cholesterol moiety linked to the 3' end of the antisense strand, a cholesterol moiety linked to the 5' end of the antisense strand, and no cholesterol moiety linked to the 3' end of the sense strand. This construct was transfected into the cells resulting in silencing the beta-galactosidase mRNA so that 46.39% of the activity of the beta-galactosidase mRNA remained. This silencing effect was lower than that observed for the corresponding, unconjugated siRNA.

[0072] DF is a siRNA construct having a cholesterol moiety linked to 5' end of the sense strand, a cholesterol moiety linked to the 3' end of the sense strand, a cholesterol moiety linked to the 3' end of the antisense strand, and no cholesterol moiety linked to the 5' end of the antisense strand. This construct was transfected into the cells resulting in silencing the beta-galactosidase mRNA so that 65.40% of the activity of the beta-galactosidase mRNA remained. This silencing effect was lower than that observed for the corresponding, unconjugated siRNA.

[0073] DE is a siRNA construct having a cholesterol moiety linked to 5' end of the sense strand, a cholesterol moiety linked to the 3' end of the sense strand, a cholesterol moiety linked to the 5' end of the antisense strand and no cholesterol moiety linked to the 3' end of the antisense strand. This construct was transfected into the cells resulting in silencing the beta-galactosidase mRNA so that 77.12% of the activity of the beta-galactosidase mRNA remained. This silencing effect was lower than that observed for the corresponding, unconjugated siRNA.

[0074] BG is a siRNA construct having a cholesterol moiety linked to 3' end of the sense strand, a cholesterol moiety linked to the 3' end of the sense strand, a cholesterol moiety linked to the 3' end of the antisense strand, a cholesterol moiety linked to the 5' end of the antisense strand, and no cholesterol moiety linked to the 5' end of the sense strand. This construct was transfected into the cells resulting in silencing the beta-galactosidase mRNA so that 77.80% of the activity of the beta-galactosidase mRNA remained. This silencing effect was lower than that observed for the corresponding, unconjugated siRNA.

[0075] DG is a siRNA construct having a cholesterol moiety on the 5' end of the sense strand, a cholesterol moiety on the 3' end of the sense strand, a cholesterol moiety on the 3' end of the antisense strand, and a cholesterol moiety on the 5' end of the antisense strand. This construct was transfected into the cells resulting in silencing the beta-galactosidase mRNA so that 98.84% of the activity of the beta-galactosidase mRNA remained. This silencing effect was lower than that observed for the corresponding, unconjugated siRNA.

Example 3

Serum Inhibition of Cholesterol-Enhanced siRNA Uptake, and Rescue of Cholesterol Enhancement of Uptake by Additional Delivery-Enhancing Agents

Human Monocyte Isolation and purity

[0076] Fresh human blood samples from healthy donors were purchased from Golden West Biologicals (Temecula, CA). For isolation of monocytes, blood samples were diluted with PBS at 1:1 ratio immediately after receiving. Peripheral blood mononuclear cells (PBMC) were first isolated by Ficoll (Amersham, CA, USA) gradient from whole blood. Then monocytes were further purified from PBMCs using Miltenyi CD14 positive selection kit (MILTENYI BIOTEC GmbH, Germany) by following the manufacturer's instructions. The purity of the monocytes was greater than 95%, judged by flow cytometry stained with anti-CD14 antibody (BD Biosciences, CA). Purified human monocytes were maintained overnight in complete media before induction and knockdown assay.

Flow Cytometry

[0077] Fluorescence activated cell sorting (FACS) analysis were performed using Beckman Coulter FC500 cell analyzer (Fullerton, CA). The instrument was adjusted according to the fluorescence probes used (FAM or Cy5 for siRNA and FITC and PE for CD14). Propidium iodide (Fluka, St Louis, MO) and AnnexinV (R&D systems, Minneapolis, MN) were used as indicators for cell viability and cytotoxicity.

[0078] For siRNA uptake analysis, cells were washed with PBS, treated with trypsin (attached cells only), and then analyzed by flow cytometry. Uptake of the siRNA designated BA, described above, was also measured by intensity of Cy5 or FAM fluorescence in the cells and cellular viability assessed by addition of propidium iodide or AnnexinV-PE. In order to differentiate the cellular uptake from the membrane insertion of fluorescence labeled siRNA, trypan blue was used to quench the fluorescence on the cell membrane surface.

Table 2 Higher MFI with PN73 compared with cholesterol siRNA alone

Serum	Cholesterol siRNA alone	Unconjugated siRNA with 20 μ M PN73
0	24.8	32.9
5%	1.55	11.5
10%	1.34	5.39
20%	1.19	5.85

[0079] The data in Table 2 show that the presence of serum significantly reduces cellular uptake of the siRNA conjugated to a cholesterol moiety according to the invention. Serum also inhibits unconjugated siRNA uptake in the presence of an exemplary delivery-enhancing peptide, PN73 (KGSKKA VTKAQKKDGKKRKR SRKESYSVYVYKVLKQ-amide; SEQ ID NO: 13), but to a lesser extent than the inhibition noted for the cholesterol-conjugated siRNA.

[0080] Figures 1 and 2 illustrate the effects of 5% serum on cellular uptake of a cholesterol-conjugated siRNA according to the invention in complex with a permeabilizing peptide delivery enhancing agent, PN73 (cholesterol siRNA+PN73), and on an unconjugated siRNA in complex with PN73 (siRNA+PN73). For these and related uptake assays, cholesterol-conjugated siRNA and siRNA/PN73 complex were transfected into human monocytes in Opti-MEM® media (Invitrogen) as described above, with serum added in fixed or varied concentration(s). The final concentration of siRNA for both cholesterol and complex were 0.2 μ M. The uptake efficiency and Mean fluorescence intensity were assessed by flow cytometry. The cellular uptake values shown in Figures 1 and 2 were determined with variation of PN73 concentrations in the presence of a fixed, 5% concentration of serum.

[0081] Figures 3 and 4 illustrate the effects of varying concentrations of serum on cellular uptake of a cholesterol-conjugated siRNA in the presence or absence of a second delivery enhancing agent, lipofectamine, as determined by flow cytometry.

[0082] The foregoing studies demonstrate that cholesterol-conjugation of siRNAs can significantly enhance their cellular uptake. However, uptake of cholesterol-conjugated siRNAs can be substantially diminished or even eliminated by the presence of serum. This is likely due to binding of the cholesterol moiety with serum proteins--inhibiting the ability of the cholesterol-bound siRNAs to enter target cells. In the presence of a selected delivery enhancing agent, Lipofectamine, this inhibitory effect of serum on cholesterol-siRNA uptake can be effectively diminished. In addition, the presence of a different kind of delivery enhancing agent, exemplified by the permeabilizing peptide PN73, can also mediate rescue of siRNA delivery blocked by serum. More specifically, the addition of a permeabilizing peptide to a delivery formulation comprising a siRNA conjugated to a cholesterol moiety reduces the inhibitory effects of serum on cholesterol-siRNA uptake in a dose dependent manner. This discovery indicates that, although cholesterol conjugation to siRNA alone may not optimize siRNA delivery, additional delivery-enhancing agents including, but not limited to, Lipofectamine and PN73, can further enhance siRNA delivery to mammalian cells and tissues *in vitro* and *in vivo*.

[0083] Further embodiments of the specification are described below and are referred to as Clauses 1-96.

[0084] Clause 1. A double-stranded (ds) RNA or a dsHybrid having a sense and an anti-sense strand, each strand having a 5' and a 3' end, wherein a cholesterol moiety is linked to the 5' end of the sense strand and a cholesterol moiety is linked to the 5' end of the antisense and wherein no cholesterol moiety is linked to the other ends of the strands.

[0085] Clause 2. The dsRNA or dsHybrid of Clause 1 wherein the dsRNA or dsHybrid has a length of 19 to 21 base pairs.

[0086] Clause 3. A dsHybrid of Clause 1 wherein the sense strand is a DNA strand.

[0087] Clause 4. The dsRNA or dsHybrid of Clause 1 wherein the cholesterol moiety is selected from the group consisting of cholesterol, sterol, any compound derived from cholesterol, cholestanol, ergosterol, stigmasterol, methyl-lithocholic acid, cortisol, corticosterone, Δ^5 -pregnenolone, progesterone, deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, 11-dioxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androsten-

edione, aldosterone, 18-hydroxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, cortisone, prednisone, 6 α -methylprednisone, 9 α -fluoro-16 α -hydroxyprednisolone, 9 α -fluoro-16 α -methylprednisolone, 9 α -fluorocortisol, testosterone, dihydrotestosterone, androstenediol, androstenedione, androstenedione, 3 α ,5 α -androstenediol, estrone, estradiol, estrogen, spermidine cholesterol carbamate, N⁴-spermidine cholesteryl carbamate, N⁴-spermidine cholesteryl carbamate di HCl salt, N⁴-spermidine-7 dehydro cholesteryl carbamate, N⁴-spermine cholesteryl carbamate, N,N bis(3-aminopropyl) cholesteryl carbamate, N,N bis(6-aminoethyl) cholesteryl carbamate, N⁴-spermidine dihydrocholesteryl carbamate, N⁴-spermidine lithocholic carbamate methyl ester, N¹,N⁸-bis (3-aminopropyl-N⁴-spermidine cholesteryl carbamate, N (N⁴-3aminopropylspermidine) cholesteryl carbamate, N,N-bis(4-aminobutyl) cholesteryl carbamate, N⁴-spermidine cholesteryl urea, N⁴-spermine cholesteryl urea, N⁴-spermidine dihydro cholesteryl urea, N⁴-spermine dihydro cholesteryl urea, N,N-bis(N⁴-3-aminopropyl-N⁴-aminobutyl) cholesteryl carbamate, N⁴spermidine cholesteryl carboxamide, and N-[N¹,N⁴,N⁸-tris (3-aminopropyl)spermidine] cholesteryl carbamate, lumisterol, cholic acid, desoxycholic acid, chenodesoxycholic acid and lithocholic acid and derivative thereof.

[0088] Clause 5. A double-stranded (ds) RNA or a dsHybrid having a sense and an antisense strand, each strand having a 5' and a 3' end, wherein a cholesterol moiety is linked to the 3' end of the antisense strand and wherein no cholesterol moiety is linked to the other ends of the strands.

[0089] Clause 6. The dsRNA or dsHybrid of Clause 5 wherein the dsRNA or dsHybrid has a length of 19 to 21 base pairs.

[0090] Clause 7. A dsHybrid of Clause 5 wherein the sense strand is a DNA strand.

[0091] Clause 8. The dsRNA or dsHybrid of Clause 5 wherein the cholesterol moiety is selected from the group consisting of cholesterol, sterol, any compound derived from cholesterol, cholestanol, ergosterol, stigmasterol, methyl-lithocholic acid, cortisol, corticosterone, Δ^5 -pregnenolone, progesterone, deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, 11-dioxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, aldosterone, 18-hydroxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, cortisone, prednisone, 6 α -methylprednisone, 9 α -fluoro-16 α -hydroxyprednisolone, 9 α -fluoro-16 α -methylprednisolone, 9 α -fluorocortisol, testosterone, dihydrotestosterone, androstenediol, androstenedione, androstenedione, 3 α ,5 α -androstenediol, estrone, estradiol, estrogen, spermidine cholesterol carbamate, N⁴-spermidine cholesteryl carbamate, N⁴-spermidine cholesteryl carbamate di HCl salt, N⁴-spermidine-7 dehydro cholesteryl carbamate, N⁴-spermine cholesteryl carbamate, N,N bis(3-aminopropyl) cholesteryl carbamate, N,N bis(6-aminoethyl) cholesteryl carbamate, N⁴-spermidine dihydrocholesteryl carbamate, N⁴-spermidine lithocholic carbamate methyl ester, N¹,N⁸-bis (3-aminopropyl-N⁴-spermidine cholesteryl carbamate, N (N⁴-3aminopropylspermidine) cholesteryl carbamate, N,N-bis(4-aminobutyl) cholesteryl carbamate, N⁴-spermidine cholesteryl urea, N⁴-spermine cholesteryl urea, N⁴-spermidine dihydro cholesteryl urea, N⁴-spermine dihydro cholesteryl urea, N,N-bis(N⁴-3-aminopropyl-N⁴-aminobutyl) cholesteryl carbamate, N⁴spermidine cholesteryl carboxamide, and N-[N¹,N⁴,N⁸-tris (3-aminopropyl)spermidine] cholesteryl carbamate, lumisterol, cholic acid, desoxycholic acid, chenodesoxycholic acid and lithocholic acid and derivative thereof.

[0092] Clause 9. A double-stranded (ds) RNA or a dsHybrid having a sense and an anti-sense strand, each strand having a 5' and a 3' end, wherein a cholesterol moiety is linked to the 5' end of the sense strand and no cholesterol moiety is linked to the other ends of the strands.

[0093] Clause 10. The dsRNA or dsHybrid of Clause 9 wherein the dsRNA or dsHybrid has a length of 19 to 21 base pairs.

[0094] Clause 11. A dsHybrid of Clause 9 wherein the sense strand is a DNA strand.

[0095] Clause 12. The dsRNA or dsHybrid of Clause 9 wherein the cholesterol moiety is selected from the group consisting of cholesterol, sterol, any compound derived from cholesterol, cholestanol, ergosterol, stigmasterol, methyl-lithocholic acid, cortisol, corticosterone, Δ^5 -pregnenolone, progesterone, deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, 11-dioxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, aldosterone, 18-hydroxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, cortisone, prednisone, 6 α -methylprednisone, 9 α -fluoro-16 α -hydroxyprednisolone, 9 α -fluoro-16 α -methylprednisolone, 9 α -fluorocortisol, testosterone, dihydrotestosterone, androstenediol, androstenedione, androstenedione, 3 α ,5 α -androstenediol, estrone, estradiol, estrogen, spermidine cholesterol carbamate, N⁴-spermidine cholesteryl carbamate, N⁴-spermidine cholesteryl carbamate di HCl salt, N⁴-spermidine-7 dehydro cholesteryl carbamate, N⁴-spermine cholesteryl carbamate, N,N bis(3-aminopropyl) cholesteryl carbamate, N,N bis(6-aminoethyl) cholesteryl carbamate, N⁴-spermidine dihydrocholesteryl carbamate, N⁴-spermidine lithocholic carbamate methyl ester, N¹,N⁸-bis (3-aminopropyl-N⁴-spermidine cholesteryl carbamate, N (N⁴-3aminopropylspermidine) cholesteryl carbamate, N,N-bis(4-aminobutyl) cholesteryl carbamate, N⁴-spermidine cholesteryl urea, N⁴-spermine cholesteryl urea, N⁴-spermidine dihydro cholesteryl urea, N⁴-spermine dihydro cholesteryl urea, N,N-bis(N⁴-3-aminopropyl-N⁴-aminobutyl) cholesteryl carbamate, N⁴spermidine cholesteryl carboxamide, and N-[N¹,N⁴,N⁸-tris (3-aminopropyl)spermidine] cholesteryl carbamate, lumisterol, cholic acid, desoxycholic acid, chenodesoxycholic acid and lithocholic acid and derivative thereof.

[0096] Clause 13. A double-stranded (ds) RNA or a dsHybrid having a sense and an anti-sense strand, each strand having a 5' and a 3' end, wherein a cholesterol moiety is linked to the 3' end of the sense strand and no cholesterol moiety is linked to the other ends of the strands.

[0097] Clause 14. The dsRNA or dsHybrid of Clause 13 wherein the dsRNA or dsHybrid has a length of 19 to 21 base pairs.

[0098] Clause 15. A dsHybrid of Clause 13 wherein the sense strand is a DNA strand.

[0099] Clause 16. The dsRNA or dsHybrid of Clause 13 wherein the cholesterol moiety is selected from the group consisting of cholesterol, sterol, any compound derived from cholesterol, cholestanol, ergosterol, stimastanol, stigmasterol, methyl-lithocholic acid, cortisol, corticosterone, Δ^5 -pregnenolone, progesterone, deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, 11-dioxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, aldosterone, 18-hydroxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, cortisone, prednisone, 6 α -methylprednisone, 9 α -fluoro-16 α -hydroxyprednisolone, 9 α -fluoro-16 α -methylprednisolone, 9 α -fluorocortisol, testosterone, dihydrotestosterone, androstenediol, androstenedione, androstenedione, 3 α ,5 α -androstenediol, estrone, estradiol, estrogen, spermidine cholesterol carbamate, N⁴-spermidine cholesteryl carbamate, N⁴-spermidine cholesteryl carbamate di HCl salt, N⁴-spermidine-7 dehydro cholesteryl carbamate, N⁴-spermine cholesteryl carbamate, N,N bis(3-aminopropyl) cholesteryl carbamate, N,N bis(6-aminoheptyl) cholesteryl carbamate, N⁴-spermidine dihydrocholesteryl carbamate, N⁴-spermidine lithocholic carbamate methyl ester, N¹,N⁸-bis (3-aminopropyl-N⁴-spermidine cholesteryl carbamate, N (N⁴-3aminopropylspermidine) cholesteryl carbamate, N,N-bis(4-aminobutyl) cholesteryl carbamate, N⁴-spermidine cholesteryl urea, N⁴-spermine cholesteryl urea, N⁴-spermidine dihydro cholesteryl urea, N⁴-spermine dihydro cholesteryl urea, N,N-bis(N¹-3-aminopropyl-N⁴-aminobutyl) cholesteryl carbamate, N⁴spermidine cholesteryl carboxamide, and N-[N¹,N⁴,N⁸-tris (3-aminopropyl)spermidine] cholesteryl carbamate, lumisterol, cholic acid, desoxycholic acid, chenodesoxycholic acid and lithocholic acid and derivative thereof.

[0100] Clause 17. A double-stranded (ds) RNA or a dsHybrid having a sense and an anti-sense strand, each strand having a 5' and a 3' end, wherein a cholesterol moiety is linked to the 3' end of the sense strand, a cholesterol moiety is linked to the 3' end of the antisense strand and no cholesterol moiety is linked to the other ends of the strands.

[0101] Clause 18. The dsRNA or dsHybrid of Clause 17 wherein the dsRNA or dsHybrid has a length of 19 to 21 base pairs.

[0102] Clause 19. A dsHybrid of Clause 17 wherein the sense strand is a DNA strand.

[0103] Clause 20. The dsRNA or dsHybrid of Clause 17 wherein the cholesterol moiety is selected from the group consisting of cholesterol, sterol, any compound derived from cholesterol, cholestanol, ergosterol, stimastanol, stigmasterol, methyl-lithocholic acid, cortisol, corticosterone, Δ^5 -pregnenolone, progesterone, deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, 11-dioxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, aldosterone, 18-hydroxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, cortisone, prednisone, 6 α -methylprednisone, 9 α -fluoro-16 α -hydroxyprednisolone, 9 α -fluoro-16 α -methylprednisolone, 9 α -fluorocortisol, testosterone, dihydrotestosterone, androstenediol, androstenedione, androstenedione, 3 α ,5 α -androstenediol, estrone, estradiol, estrogen, spermidine cholesterol carbamate, N⁴-spermidine cholesteryl carbamate, N⁴-spermidine cholesteryl carbamate di HCl salt, N⁴-spermidine-7 dehydro cholesteryl carbamate, N⁴-spermine cholesteryl carbamate, N,N bis(3-aminopropyl) cholesteryl carbamate, N,N bis(6-aminoheptyl) cholesteryl carbamate, N⁴-spermidine dihydrocholesteryl carbamate, N⁴-spermidine lithocholic carbamate methyl ester, N¹,N⁸-bis (3-aminopropyl-N⁴-spermidine cholesteryl carbamate, N (N⁴-3aminopropylspermidine) cholesteryl carbamate, N,N-bis(4-aminobutyl) cholesteryl carbamate, N⁴-spermidine cholesteryl urea, N⁴-spermine cholesteryl urea, N⁴-spermidine dihydro cholesteryl urea, N⁴-spermine dihydro cholesteryl urea, N,N-bis(N¹-3-aminopropyl-N⁴-aminobutyl) cholesteryl carbamate, N⁴spermidine cholesteryl carboxamide, and N-[N¹,N⁴,N⁸-tris (3-aminopropyl)spermidine] cholesteryl carbamate, lumisterol, cholic acid, desoxycholic acid, chenodesoxycholic acid and lithocholic acid and derivative thereof.

[0104] Clause 21. A double-stranded (ds) RNA or a dsHybrid having a sense and an antisense strand, each strand having a 5' and a 3' end, wherein a cholesterol moiety is linked to the 5' end of the antisense strand and no cholesterol moiety is linked to the other ends of the strands.

[0105] Clause 22. The dsRNA or dsHybrid of Clause 21 wherein the dsRNA or dsHybrid has a length of 19 to 21 base pairs.

[0106] Clause 23. A dsHybrid of Clause 21 wherein the sense strand is a DNA.

[0107] Clause 24. The dsRNA or dsHybrid of Clause 21 wherein the cholesterol moiety is selected from the group consisting of cholesterol, sterol, any compound derived from cholesterol, cholestanol, ergosterol, stimastanol, stigmasterol, methyl-lithocholic acid, cortisol, corticosterone, Δ^5 -pregnenolone, progesterone, deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, 11-dioxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, aldosterone, 18-hydroxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, cortisone, prednisone, 6 α -methylprednisone, 9 α -fluoro-16 α -hydroxyprednisolone, 9 α -fluoro-16 α -methylprednisolone, 9 α -fluorocortisol, testosterone, dihydrotestosterone, androstenediol, androstenedione, androstenedione, 3 α ,5 α -androstenediol, estrone, estradiol, estrogen, spermidine cholesterol carbamate, N⁴-spermidine cholesteryl carbamate, N⁴-spermidine cholesteryl carbamate di HCl salt, N⁴-spermidine-7 dehydro cholesteryl carbamate, N⁴-spermine cholesteryl carbamate, N,N bis(3-aminopropyl) cholesteryl carbamate, N,N bis(6-aminoheptyl) cholesteryl carbamate, N⁴-spermidine dihydrocholesteryl carbamate, N⁴-spermidine lithocholic carbamate methyl ester, N¹,N⁸-bis (3-aminopropyl-N⁴-spermidine cholesteryl carbamate, N

(N⁴-3aminopropylspermidine) cholesteryl carbamate, N,N-bis(4-aminobutyl) cholesteryl carbamate, N⁴-spermidine cholesteryl urea, N⁴-spermine cholesteryl urea, N⁴-spermidine dihydro cholesteryl urea, N⁴-spermine dihydro cholesteryl urea, N,N-bis(N¹-3-aminopropyl-N⁴-aminobutyl) cholesteryl carbamate, N⁴spermidine cholesteryl carboxamide, and N-[N¹,N⁴,N⁸-tris (3-aminopropyl)spermidine] cholesteryl carbamate, lumisterol, cholic acid, desoxycholic acid, cheno-desoxycholic acid and lithocholic acid and derivative thereof.

[0108] Clause 25. A double-stranded (ds) RNA or a dsHybrid having a sense and an antisense strand, each strand having a 5' and a 3' end, wherein the 3' end of the sense strand is connected to the 5' end of the antisense strand by means of a loop, and wherein a cholesterol moiety is linked to the 3' end of the antisense strand and wherein no cholesterol moiety is linked to the other ends of the strands.

[0109] Clause 26. The dsRNA or dsHybrid of Clause 25 wherein the dsRNA or dsHybrid has a length of 19 to 21 base pairs.

[0110] Clause 27. A dsHybrid of Clause 25 wherein the sense strand is a DNA strand.

[0111] Clause 28. The dsRNA or dsHybrid of Clause 25 wherein the cholesterol moiety is selected from the group consisting of cholesterol, sterol, any compound derived from cholesterol, cholestanol, ergosterol, stimastanol, stigmasterol, methyl-lithocholic acid, cortisol, corticosterone, Δ^5 -pregnenolone, progesterone, deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, 11-dioxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, aldosterone, 18-hydroxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, cortisone, prednisone, 6 α -methylprednisone, 9 α -fluoro-16 α -hydroxyprednisolone, 9 α -fluoro-16 α -methylprednisolone, 9 α -fluorocortisol, testosterone, dihydrotestosterone, androstenediol, androstenedione, androstenedione, 3 α ,5 α -androstenediol, estrone, estradiol, estrogen, spermidine cholesterol carbamate, N⁴-spermidine cholesteryl carbamate, N⁴-spermidine cholesteryl carbamate di HCl salt, N⁴-spermidine-7 dehydro cholesteryl carbamate, N⁴-spermine cholesteryl carbamate, N,N bis(3-aminopropyl) cholesteryl carbamate, N,N bis(6-aminoheptyl) cholesteryl carbamate, N⁴-spermidine dihydrocholesteryl carbamate, N⁴-spermidine lithocholic carbamate methyl ester, N¹,N⁸-bis (3-aminopropyl-N⁴-spermidine cholesteryl carbamate, N (N⁴-3aminopropylspermidine) cholesteryl carbamate, N,N-bis(4-aminobutyl) cholesteryl carbamate, N⁴-spermidine cholesteryl urea, N⁴-spermine cholesteryl urea, N⁴-spermidine dihydro cholesteryl urea, N⁴-spermine dihydro cholesteryl urea, N,N-bis(N¹-3-aminopropyl-N⁴-aminobutyl) cholesteryl carbamate, N⁴spermidine cholesteryl carboxamide, and N-[N¹,N⁴,N⁸-tris (3-aminopropyl)spermidine] cholesteryl carbamate, lumisterol, cholic acid, desoxycholic acid, cheno-desoxycholic acid and lithocholic acid and derivative thereof.

[0112] Clause 29. A double-stranded (ds) RNA or a dsHybrid having a sense and an antisense strand, each strand having a 5' and a 3' end, wherein the 3' end of the sense strand is connected to the 5' end of the antisense strand by means of a loop, wherein a cholesterol moiety is linked to the 5' end of the sense strand and no cholesterol moiety is linked to the other ends of the strands.

[0113] Clause 30. The dsRNA or dsHybrid of Clause 29 wherein the dsRNA or dsHybrid has a length of 19 to 21 base pairs.

[0114] Clause 31. A dsHybrid of Clause 29 wherein the sense strand is a DNA strand.

[0115] Clause 32. The dsRNA or dsHybrid of Clause 29 wherein the cholesterol moiety is selected from the group consisting of cholesterol, sterol, any compound derived from cholesterol, cholestanol, ergosterol, stimastanol, stigmasterol, methyl-lithocholic acid, cortisol, corticosterone, Δ^5 -pregnenolone, progesterone, deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, 11-dioxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, aldosterone, 18-hydroxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, cortisone, prednisone, 6 α -methylprednisone, 9 α -fluoro-16 α -hydroxyprednisolone, 9 α -fluoro-16 α -methylprednisolone, 9 α -fluorocortisol, testosterone, dihydrotestosterone, androstenediol, androstenedione, androstenedione, 3 α ,5 α -androstenediol, estrone, estradiol, estrogen, spermidine cholesterol carbamate, N⁴-spermidine cholesteryl carbamate, N⁴-spermidine cholesteryl carbamate di HCl salt, N⁴-spermidine-7 dehydro cholesteryl carbamate, N⁴-spermine cholesteryl carbamate, N,N bis(3-aminopropyl) cholesteryl carbamate, N,N bis(6-aminoheptyl) cholesteryl carbamate, N⁴-spermidine dihydrocholesteryl carbamate, N⁴-spermidine lithocholic carbamate methyl ester, N¹,N⁸-bis (3-aminopropyl-N⁴-spermidine cholesteryl carbamate, N (N⁴-3aminopropylspermidine) cholesteryl carbamate, N,N-bis(4-aminobutyl) cholesteryl carbamate, N⁴-spermidine cholesteryl urea, N⁴-spermine cholesteryl urea, N⁴-spermidine dihydro cholesteryl urea, N⁴-spermine dihydro cholesteryl urea, N,N-bis(N¹-3-aminopropyl-N⁴-aminobutyl) cholesteryl carbamate, N⁴spermidine cholesteryl carboxamide, and N-[N¹,N⁴,N⁸-tris (3-aminopropyl)spermidine] cholesteryl carbamate, lumisterol, cholic acid, desoxycholic acid, cheno-desoxycholic acid and lithocholic acid and derivative thereof.

[0116] Clause 33. A method for producing double-stranded (ds) RNA comprising hybridizing an RNA sense and an RNA antisense strand together, each strand having a 5' and a 3' end, wherein a cholesterol moiety is linked to the 5' end of the sense strand and a cholesterol moiety is linked to the 5' end of the antisense and wherein no cholesterol moiety is linked to the other ends of the strands.

[0117] Clause 34. The method of Clause 33 wherein the dsRNA has a length of 19 to 21 base pairs.

[0118] Clause 35. The method of Clause 33 wherein the cholesterol moiety is selected from the group consisting of cholesterol, sterol, any compound derived from cholesterol, cholestanol, ergosterol, stimastanol, stigmasterol, methyl-

lithocholic acid, cortisol, corticosterone, Δ^5 -pregnenolone, progesterone, deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, 11-dioxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, aldosterone, 18-hydroxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, cortisone, prednisone, 6 α -methylprednisone, 9 α -fluoro-16 α -hydroxyprednisolone, 9 α -fluoro-16 α -methylprednisolone, 9 α -fluorocortisol, testosterone, dihydrotestosterone, androstenediol, androstenedione, androstenedione, 3 α ,5 α -androstenediol, estrone, estradiol, estrogen, spermidine cholesterol carbamate, N⁴-spermidine cholesteryl carbamate, N⁴-spermidine cholesteryl carbamate di HCl salt, N⁴-spermidine-7 dehydro cholesteryl carbamate, N⁴-spermine cholesteryl carbamate, N,N bis(3-aminopropyl) cholesteryl carbamate, N,N bis(6-aminoethyl) cholesteryl carbamate, N⁴-spermidine dihydrocholesteryl carbamate, N⁴-spermidine lithocholic carbamate methyl ester, N¹,N⁸-bis (3-aminopropyl-N⁴-spermidine cholesteryl carbamate, N (N⁴-3aminopropylspermidine) cholesteryl carbamate, N,N-bis(4-aminobutyl) cholesteryl carbamate, N⁴-spermidine cholesteryl urea, N⁴-spermine cholesteryl urea, N⁴-spermidine dihydro cholesteryl urea, N⁴-spermine dihydro cholesteryl urea, N,N-bis(N¹-3-aminopropyl-N⁴-aminobutyl) cholesteryl carbamate, N⁴spermidine cholesteryl carboxamide, and N-[N¹,N⁴,N⁸-tris (3-aminopropyl)spermidine] cholesteryl carbamate, lumisterol, cholic acid, desoxycholic acid, chenodesoxycholic acid and lithocholic acid and derivative thereof.

[0119] Clause 36. A method for producing double-stranded (ds) RNA comprising hybridizing an RNA sense and an RNA antisense strand together, each strand having a 5' and a 3' end, wherein a cholesterol moiety is linked to the 3' end of the antisense strand and wherein no cholesterol moiety is linked to the other ends of the strands.

[0120] Clause 37. The method of Clause 36 wherein the dsRNA has a length of 19 to 21 base pairs.

[0121] Clause 38. The method of Clause 36 wherein the cholesterol moiety is selected from the group consisting of cholesterol, sterol, any compound derived from cholesterol, chlolestanol, ergosterol, stimastanol, stigmastanol, methyl-lithocholic acid, cortisol, corticosterone, Δ^5 -pregnenolone, progesterone, deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, 11-dioxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, aldosterone, 18-hydroxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, cortisone, prednisone, 6 α -methylprednisone, 9 α -fluoro-16 α -hydroxyprednisolone, 9 α -fluoro-16 α -methylprednisolone, 9 α -fluorocortisol, testosterone, dihydrotestosterone, androstenediol, androstenedione, androstenedione, 3 α ,5 α -androstenediol, estrone, estradiol, estrogen, spermidine cholesterol carbamate, N⁴-spermidine cholesteryl carbamate, N⁴-spermidine cholesteryl carbamate di HCl salt, N⁴-spermidine-7 dehydro cholesteryl carbamate, N⁴-spermine cholesteryl carbamate, N,N bis(3-aminopropyl) cholesteryl carbamate, N,N bis(6-aminoethyl) cholesteryl carbamate, N⁴-spermidine dihydrocholesteryl carbamate, N⁴-spermidine lithocholic carbamate methyl ester, N¹,N⁸-bis (3-aminopropyl-N⁴-spermidine cholesteryl carbamate, N (N⁴-3aminopropylspermidine) cholesteryl carbamate, N,N-bis(4-aminobutyl) cholesteryl carbamate, N⁴-spermidine cholesteryl urea, N⁴-spermine cholesteryl urea, N⁴-spermidine dihydro cholesteryl urea, N⁴-spermine dihydro cholesteryl urea, N,N-bis(N¹-3-aminopropyl-N⁴-aminobutyl) cholesteryl carbamate, N⁴spermidine cholesteryl carboxamide, and N-[N¹,N⁴,N⁸-tris (3-aminopropyl)spermidine] cholesteryl carbamate, lumisterol, cholic acid, desoxycholic acid, chenodesoxycholic acid and lithocholic acid and derivative thereof.

[0122] Clause 39. A method for producing double-stranded (ds) RNA comprising hybridizing an RNA sense and an RNA antisense strand together, each strand having a 5' and a 3' end, wherein a cholesterol moiety is linked to the 5' end of the sense strand and no cholesterol moiety is linked to the other ends of the siRNA strands.

[0123] Clause 40. The method of Clause 39 wherein the dsRNA has a length of 19 to 21 base pairs.

[0124] Clause 41. The method of Clause 39 wherein the cholesterol moiety is selected from the group consisting of cholesterol, sterol, any compound derived from cholesterol, chlolestanol, ergosterol, stimastanol, stigmastanol, methyl-lithocholic acid, cortisol, corticosterone, Δ^5 -pregnenolone, progesterone, deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, 11-dioxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, aldosterone, 18-hydroxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, cortisone, prednisone, 6 α -methylprednisone, 9 α -fluoro-16 α -hydroxyprednisolone, 9 α -fluoro-16 α -methylprednisolone, 9 α -fluorocortisol, testosterone, dihydrotestosterone, androstenediol, androstenedione, androstenedione, 3 α ,5 α -androstenediol, estrone, estradiol, estrogen, spermidine cholesterol carbamate, N⁴-spermidine cholesteryl carbamate, N⁴-spermidine cholesteryl carbamate di HCl salt, N⁴-spermidine-7 dehydro cholesteryl carbamate, N⁴-spermine cholesteryl carbamate, N,N bis(3-aminopropyl) cholesteryl carbamate, N,N bis(6-aminoethyl) cholesteryl carbamate, N⁴-spermidine dihydrocholesteryl carbamate, N⁴-spermidine lithocholic carbamate methyl ester, N¹,N⁸-bis (3-aminopropyl-N⁴-spermidine cholesteryl carbamate, N (N⁴-3aminopropylspermidine) cholesteryl carbamate, N,N-bis(4-aminobutyl) cholesteryl carbamate, N⁴-spermidine cholesteryl urea, N⁴-spermine cholesteryl urea, N⁴-spermidine dihydro cholesteryl urea, N⁴-spermine dihydro cholesteryl urea, N,N-bis(N¹-3-aminopropyl-N⁴-aminobutyl) cholesteryl carbamate, N⁴spermidine cholesteryl carboxamide, and N-[N¹,N⁴,N⁸-tris (3-aminopropyl)spermidine] cholesteryl carbamate, lumisterol, cholic acid, desoxycholic acid, chenodesoxycholic acid and lithocholic acid and derivative thereof.

[0125] Clause 42. A method for producing double-stranded (ds) RNA comprising hybridizing an RNA sense and an RNA antisense strand together, each strand having a 5' and a 3' end, wherein a cholesterol moiety is linked to the 3' end of the sense strand and no cholesterol moiety is linked to the other ends of the siRNA strands.

[0126] Clause 43. The method of Clause 42 wherein the dsRNA has a length of 19 to 21 base pairs.

[0127] Clause 44. The dsRNA or dsHybrid of Clause 42 wherein the cholesterol moiety is selected from the group consisting of cholesterol, sterol, any compound derived from cholesterol, cholestanol, ergosterol, stimastanol, stigmasterol, methyl-lithocholic acid, cortisol, corticosterone, Δ^5 -pregnenolone, progesterone, deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, 11-dioxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, aldosterone, 18-hydroxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, cortisone, prednisone, 6 α -methylprednisone, 9 α -fluoro-16 α -hydroxyprednisolone, 9 α -fluoro-16 α -methylprednisolone, 9 α -fluorocortisol, testosterone, dihydrotestosterone, androstenediol, androstenedione, androstenedione, 3 α ,5 α -androstenediol, estrone, estradiol, estrogen, spermidine cholesterol carbamate, N⁴-spermidine cholesteryl carbamate, N⁴-spermidine cholesteryl carbamate di HCl salt, N⁴-spermidine-7 dehydro cholesteryl carbamate, N⁴-spermine cholesteryl carbamate, N,N bis(3-aminopropyl) cholesteryl carbamate, N,N bis(6-aminoethyl) cholesteryl carbamate, N⁴-spermidine dihydrocholesteryl carbamate, N⁴-spermidine lithocholic carbamate methyl ester, N¹,N⁸-bis (3-aminopropyl-N⁴-spermidine cholesteryl carbamate, N (N⁴-3aminopropylspermidine) cholesteryl carbamate, N,N-bis(4-aminobutyl) cholesteryl carbamate, N⁴-spermidine cholesteryl urea, N⁴-spermine cholesteryl urea, N⁴-spermidine dihydro cholesteryl urea, N⁴-spermine dihydro cholesteryl urea, N,N-bis(N'-3-aminopropyl-N''4-aminobutyl) cholesteryl carbamate, N⁴spermidine cholesteryl carboxamide, and N-[N¹,N⁴,N⁸-tris (3-aminopropyl)spermidine] cholesteryl carbamate, lumisterol, cholic acid, desoxycholic acid, chenodesoxycholic acid and lithocholic acid and derivative thereof.

[0128] Clause 45. A method for producing double-stranded (ds) RNA comprising hybridizing an RNA sense and an RNA anti-sense strand together, each strand having a 5' and a 3' end, wherein a cholesterol moiety is linked to the 3' end of the sense strand, a cholesterol moiety is linked to the 3' end of the antisense strand and no cholesterol moiety linked to the other ends of the siRNA or strands.

[0129] Clause 46. The method of Clause 45 wherein the dsRNA has a length of 19 to 21 base pairs.

[0130] Clause 47. The method of Clause 45 wherein the cholesterol moiety is selected from the group consisting of cholesterol, sterol, any compound derived from cholesterol, cholestanol, ergosterol, stimastanol, stigmasterol, methyl-lithocholic acid, cortisol, corticosterone, Δ^5 -pregnenolone, progesterone, deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, 11-dioxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, aldosterone, 18-hydroxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, cortisone, prednisone, 6 α -methylprednisone, 9 α -fluoro-16 α -hydroxyprednisolone, 9 α -fluoro-16 α -methylprednisolone, 9 α -fluorocortisol, testosterone, dihydrotestosterone, androstenediol, androstenedione, androstenedione, 3 α ,5 α -androstenediol, estrone, estradiol, estrogen, spermidine cholesterol carbamate, N⁴-spermidine cholesteryl carbamate, N⁴-spermidine cholesteryl carbamate di HCl salt, N⁴-spermidine-7 dehydro cholesteryl carbamate, N⁴-spermine cholesteryl carbamate, N,N bis(3-aminopropyl) cholesteryl carbamate, N,N bis(6-aminoethyl) cholesteryl carbamate, N⁴-spermidine dihydrocholesteryl carbamate, N⁴-spermidine lithocholic carbamate methyl ester, N¹,N⁸-bis (3-aminopropyl-N⁴-spermidine cholesteryl carbamate, N (N⁴-3aminopropylspermidine) cholesteryl carbamate, N,N-bis(4-aminobutyl) cholesteryl carbamate, N⁴-spermidine cholesteryl urea, N⁴-spermine cholesteryl urea, N⁴-spermidine dihydro cholesteryl urea, N⁴-spermine dihydro cholesteryl urea, N,N-bis(N'-3-aminopropyl-N''4-aminobutyl) cholesteryl carbamate, N⁴spermidine cholesteryl carboxamide, and N-[N¹,N⁴,N⁸-tris (3-aminopropyl)spermidine] cholesteryl carbamate, lumisterol, cholic acid, desoxycholic acid, chenodesoxycholic acid and lithocholic acid and derivative thereof.

[0131] Clause 48. A method for producing double-stranded (ds) RNA comprising hybridizing an RNA sense and an RNA antisense strand together, each strand having a 5' and a 3' end, wherein a cholesterol moiety is linked to the 5' end of the antisense strand and no cholesterol moiety is linked to the other ends of the dsRNA strands.

[0132] Clause 49. The method of Clause 48 wherein the dsRNA or has a length of 19 to 21 base pairs.

[0133] Clause 50. The method of Clause 48 wherein the cholesterol moiety is selected from the group consisting of cholesterol, sterol, any compound derived from cholesterol, cholestanol, ergosterol, stimastanol, stigmasterol, methyl-lithocholic acid, cortisol, corticosterone, Δ^5 -pregnenolone, progesterone, deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, 11-dioxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, aldosterone, 18-hydroxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, cortisone, prednisone, 6 α -methylprednisone, 9 α -fluoro-16 α -hydroxyprednisolone, 9 α -fluoro-16 α -methylprednisolone, 9 α -fluorocortisol, testosterone, dihydrotestosterone, androstenediol, androstenedione, androstenedione, 3 α ,5 α -androstenediol, estrone, estradiol, estrogen, spermidine cholesterol carbamate, N⁴-spermidine cholesteryl carbamate, N⁴-spermidine cholesteryl carbamate di HCl salt, N⁴-spermidine-7 dehydro cholesteryl carbamate, N⁴-spermine cholesteryl carbamate, N,N bis(3-aminopropyl) cholesteryl carbamate, N,N bis(6-aminoethyl) cholesteryl carbamate, N⁴-spermidine dihydrocholesteryl carbamate, N⁴-spermidine lithocholic carbamate methyl ester, N¹,N⁸-bis (3-aminopropyl-N⁴-spermidine cholesteryl carbamate, N (N⁴-3aminopropylspermidine) cholesteryl carbamate, N,N-bis(4-aminobutyl) cholesteryl carbamate, N⁴-spermidine cholesteryl urea, N⁴-spermine cholesteryl urea, N⁴-spermidine dihydro cholesteryl urea, N⁴-spermine dihydro cholesteryl urea, N,N-bis(N'-3-aminopropyl-N''4-aminobutyl) cholesteryl carbamate, N⁴spermidine cholesteryl carboxamide, and N-[N¹,N⁴,N⁸-tris (3-aminopropyl)spermidine] cholesteryl carbamate, lumisterol, cholic acid, desoxycholic acid, chenodesoxycholic acid and lithocholic acid and derivative thereof.

[0134] Clause 51. A method for producing double-stranded (ds) RNA comprising hybridizing an RNA sense and an

RNA antisense strand together, each strand having a 5' and a 3' end, wherein the 3' end of the sense strand is connected to the 5' end of the antisense strand by means of a loop, and wherein a cholesterol moiety is linked to the 3' end of the antisense strand and wherein no cholesterol moiety is linked to the other ends of the strands of the dsRNA.

[0135] Clause 52. The method of Clause 51 wherein the dsRNA has a length of 19 to 21 base pairs.

[0136] Clause 53. The method of Clause 51 wherein the cholesterol moiety is selected from the group consisting of cholesterol, sterol, any compound derived from cholesterol, chlolestanol, ergosterol, stimastanol, stigmaterol, methyl-lithocholic acid, cortisol, corticosterone, Δ^5 -pregnenolone, progesterone, deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, 11-dioxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, aldosterone, 18-hydroxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, cortisone, prednisone, 6 α -methylprednisone, 9 α -fluoro-16 α -hydroxyprednisolone, 9 α -fluoro-16 α -methylprednisolone, 9 α -fluorocortisol, testosterone, dihydrotestosterone, androstenediol, androstenedione, androstenedione, 3 α ,5 α -androstenediol, estrone, estradiol, estrogen, spermidine cholesterol carbamate, N⁴-spermidine cholesteryl carbamate, N⁴-spermidine cholesteryl carbamate di HCl salt, N⁴-spermidine-7 dehydro cholesteryl carbamate, N⁴-spermine cholesteryl carbamate, N,N bis(3-aminopropyl) cholesteryl carbamate, N,N bis(6-aminoheptyl) cholesteryl carbamate, N⁴-spermidine dihydrocholesteryl carbamate, N⁴-spermidine lithocholic carbamate methyl ester, N¹,N⁸-bis (3-aminopropyl-N⁴-spermidine cholesteryl carbamate, N (N⁴-3aminopropylspermidine) cholesteryl carbamate, N,N-bis(4-aminobutyl) cholesteryl carbamate, N⁴-spermidine cholesteryl urea, N⁴-spermine cholesteryl urea, N⁴-spermidine dihydro cholesteryl urea, N⁴-spermine dihydro cholesteryl urea, N,N-bis(N⁴-3-aminopropyl-N⁴-aminobutyl) cholesteryl carbamate, N⁴spermidine cholesteryl carboxamide, and N-[N¹,N⁴,N⁸-tris (3-aminopropyl)spermidine] cholesteryl carbamate, lumisterol, cholic acid, desoxycholic acid, chenodesoxycholic acid and lithocholic acid and derivative thereof.

[0137] Clause 54. A method for producing double-stranded (ds) RNA comprising hybridizing an RNA sense and an RNA antisense strand together, wherein the 3' end of the sense strand is connected to the 5' end of the antisense strand by means of a loop, wherein a cholesterol moiety is linked to the 5' end of the sense strand and no cholesterol moiety is linked to the other ends of the dsRNA strands.

[0138] Clause 55. The method of Clause 54 wherein the dsRNA has a length of 19 to 21 base pairs.

[0139] Clause 56. The method of Clause 54 wherein the cholesterol moiety is selected from the group consisting of cholesterol, sterol, any compound derived from cholesterol, chlolestanol, ergosterol, stimastanol, stigmaterol, methyl-lithocholic acid, cortisol, corticosterone, Δ^5 -pregnenolone, progesterone, deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, 11-dioxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, aldosterone, 18-hydroxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, cortisone, prednisone, 6 α -methylprednisone, 9 α -fluoro-16 α -hydroxyprednisolone, 9 α -fluoro-16 α -methylprednisolone, 9 α -fluorocortisol, testosterone, dihydrotestosterone, androstenediol, androstenedione, androstenedione, 3 α ,5 α -androstenediol, estrone, estradiol, estrogen, spermidine cholesterol carbamate, N⁴-spermidine cholesteryl carbamate, N⁴-spermidine cholesteryl carbamate di HCl salt, N⁴-spermidine-7 dehydro cholesteryl carbamate, N⁴-spermine cholesteryl carbamate, N,N bis(3-aminopropyl) cholesteryl carbamate, N,N bis(6-aminoheptyl) cholesteryl carbamate, N⁴-spermidine dihydrocholesteryl carbamate, N⁴-spermidine lithocholic carbamate methyl ester, N¹,N⁸-bis (3-aminopropyl-N⁴-spermidine cholesteryl carbamate, N (N⁴-3aminopropylspermidine) cholesteryl carbamate, N,N-bis(4-aminobutyl) cholesteryl carbamate, N⁴-spermidine cholesteryl urea, N⁴-spermine cholesteryl urea, N⁴-spermidine dihydro cholesteryl urea, N⁴-spermine dihydro cholesteryl urea, N,N-bis(N⁴-3-aminopropyl-N⁴-aminobutyl) cholesteryl carbamate, N⁴spermidine cholesteryl carboxamide, and N-[N¹,N⁴,N⁸-tris (3-aminopropyl)spermidine] cholesteryl carbamate, lumisterol, cholic acid, desoxycholic acid, chenodesoxycholic acid and lithocholic acid and derivative thereof.

[0140] Clause 57. A method for producing a double-stranded (ds) Hybrid comprising hybridizing a DNA sense and an RNA antisense strand together, each strand having a 5' and a 3' end, wherein a cholesterol moiety is linked to the 5' end of the sense strand and a cholesterol moiety is linked to the 5' end of the antisense strand and wherein no cholesterol moiety is linked to the other ends of the strands.

[0141] Clause 58. The method of Clause 57 wherein the dsHybrid has a length of 19 to 21 base pairs.

[0142] Clause 59. The method of Clause 57 wherein the cholesterol moiety is selected from the group consisting of cholesterol, sterol, any compound derived from cholesterol, chlolestanol, ergosterol, stimastanol, stigmaterol, methyl-lithocholic acid, cortisol, corticosterone, Δ^5 -pregnenolone, progesterone, deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, 11-dioxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, aldosterone, 18-hydroxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, cortisone, prednisone, 6 α -methylprednisone, 9 α -fluoro-16 α -hydroxyprednisolone, 9 α -fluoro-16 α -methylprednisolone, 9 α -fluorocortisol, testosterone, dihydrotestosterone, androstenediol, androstenedione, androstenedione, 3 α ,5 α -androstenediol, estrone, estradiol, estrogen, spermidine cholesterol carbamate, N⁴-spermidine cholesteryl carbamate, N⁴-spermidine cholesteryl carbamate di HCl salt, N⁴-spermidine-7 dehydro cholesteryl carbamate, N⁴-spermine cholesteryl carbamate, N,N bis(3-aminopropyl) cholesteryl carbamate, N,N bis(6-aminoheptyl) cholesteryl carbamate, N⁴-spermidine dihydrocholesteryl carbamate, N⁴-spermidine lithocholic carbamate methyl ester, N¹,N⁸-bis (3-aminopropyl-N⁴-spermidine cholesteryl carbamate, N (N⁴-3aminopropylspermidine) cholesteryl carbamate, N,N-bis(4-aminobutyl) cholesteryl carbamate, N⁴-spermidine

cholesteryl urea, N⁴-spermine cholesteryl urea, N⁴-spermidine dihydro cholesteryl urea, N⁴-spermine dihydro cholesteryl urea, N,N-bis(N'-3-aminopropyl-N"4-aminobutyl) cholesteryl carbamate, N⁴spermidine cholesteryl carboxamide, and N-[N¹,N⁴,N⁸-tris (3-aminopropyl)spermidine] cholesteryl carbamate, lumisterol, cholic acid, desoxycholic acid, cheno-desoxycholic acid and lithocholic acid and derivative thereof.

[0143] Clause 60. A method for producing double-stranded (ds) Hybrid comprising hybridizing a DNA sense and an RNA antisense strand together, each strand having a 5' and a 3' end, wherein a cholesterol moiety is linked to the 3' end of the antisense strand and wherein no cholesterol moiety is linked to the other ends of the strands.

[0144] Clause 61. The method of Clause 60 wherein the dsHybrid has a length of 19 to 21 base pairs.

[0145] Clause 62. The method of Clause 60 wherein the cholesterol moiety is selected from the group consisting of cholesterol, sterol, any compound derived from cholesterol, cholestanol, ergosterol, stimastanol, stigmaterol, methyl-lithocholic acid, cortisol, corticosterone, Δ^5 -pregnenolone, progesterone, deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, 11-dioxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, aldosterone, 18-hydroxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, cortisone, prednisone, 6 α -methylpredisone, 9 α -fluoro-16 α -hydroxyprednisolone, 9 α -fluoro-16 α -methylprednisolone, 9 α -fluorocortisol, testosterone, dihydrotestosterone, androstenediol, androstenedione, androstenedione, 3 α ,5 α -androstenediol, estrone, estradiol, estrogen, spermidine cholesterol carbamate, N⁴-spermidine cholesteryl carbamate, N⁴-spermidine cholesteryl carbamate di HCl salt, N⁴-spermidine-7 dehydro cholesteryl carbamate, N⁴-spermine cholesteryl carbamate, N,N bis(3-aminopropyl) cholesteryl carbamate, N,N bis(6-aminoheptyl) cholesteryl carbamate, N⁴-spermidine dihydrocholesteryl carbamate, N⁴-spermidine lithocholic carbamate methyl ester, N¹,N⁸-bis (3-aminopropyl-N⁴-spermidine cholesteryl carbamate, N (N⁴-3aminopropyl)spermidine) cholesteryl carbamate, N,N-bis(4-aminobutyl) cholesteryl carbamate, N⁴-spermidine cholesteryl urea, N⁴-spermine cholesteryl urea, N⁴-spermidine dihydro cholesteryl urea, N⁴-spermine dihydro cholesteryl urea, N,N-bis(N'-3-aminopropyl-N"4-aminobutyl) cholesteryl carbamate, N⁴spermidine cholesteryl carboxamide, and N-[N¹,N⁴,N⁸-tris (3-aminopropyl)spermidine] cholesteryl carbamate, lumisterol, cholic acid, desoxycholic acid, cheno-desoxycholic acid and lithocholic acid and derivative thereof.

[0146] Clause 63. A method for producing double-stranded (ds) Hybrid comprising hybridizing a DNA sense and an RNA antisense strand together, each strand having a 5' and a 3' end, wherein a cholesterol moiety is linked to the 5' end of the sense strand and no cholesterol moiety is linked to the other ends of the strands.

[0147] Clause 64. The method of Clause 63 wherein the dsHybrid has a length of 19 to 21 base pairs.

[0148] Clause 65. The method of Clause 63 wherein the cholesterol moiety is selected from the group consisting of cholesterol, sterol, any compound derived from cholesterol, cholestanol, ergosterol, stimastanol, stigmaterol, methyl-lithocholic acid, cortisol, corticosterone, Δ^5 -pregnenolone, progesterone, deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, 11-dioxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, aldosterone, 18-hydroxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, cortisone, prednisone, 6 α -methylpredisone, 9 α -fluoro-16 α -hydroxyprednisolone, 9 α -fluoro-16 α -methylprednisolone, 9 α -fluorocortisol, testosterone, dihydrotestosterone, androstenediol, androstenedione, androstenedione, 3 α ,5 α -androstenediol, estrone, estradiol, estrogen, spermidine cholesterol carbamate, N⁴-spermidine cholesteryl carbamate, N⁴-spermidine cholesteryl carbamate di HCl salt, N⁴-spermidine-7 dehydro cholesteryl carbamate, N⁴-spermine cholesteryl carbamate, N,N bis(3-aminopropyl) cholesteryl carbamate, N,N bis(6-aminoheptyl) cholesteryl carbamate, N⁴-spermidine dihydrocholesteryl carbamate, N⁴-spermidine lithocholic carbamate methyl ester, N¹,N⁸-bis (3-aminopropyl-N⁴-spermidine cholesteryl carbamate, N (N⁴-3aminopropyl)spermidine) cholesteryl carbamate, N,N-bis(4-aminobutyl) cholesteryl carbamate, N⁴-spermidine cholesteryl urea, N⁴-spermine cholesteryl urea, N⁴-spermidine dihydro cholesteryl urea, N⁴-spermine dihydro cholesteryl urea, N,N-bis(N'-3-aminopropyl-N"4-aminobutyl) cholesteryl carbamate, N⁴spermidine cholesteryl carboxamide, and N-[N¹,N⁴,N⁸-tris (3-aminopropyl)spermidine] cholesteryl carbamate, lumisterol, cholic acid, desoxycholic acid, cheno-desoxycholic acid and lithocholic acid and derivative thereof.

[0149] Clause 66. A method for producing double-stranded (ds) Hybrid comprising hybridizing a DNA sense and an RNA antisense strand together, each strand having a 5' and a 3' end, wherein a cholesterol moiety is linked to the 3' end of the sense strand and no cholesterol moiety is linked to the other ends of the strands.

[0150] Clause 67. The method of Clause 66 wherein the dsHybrid has a length of 19 to 21 base pairs.

[0151] Clause 68. The method of Clause 66 wherein the cholesterol moiety is selected from the group consisting of cholesterol, sterol, any compound derived from cholesterol, cholestanol, ergosterol, stimastanol, stigmaterol, methyl-lithocholic acid, cortisol, corticosterone, Δ^5 -pregnenolone, progesterone, deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, 11-dioxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, aldosterone, 18-hydroxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, cortisone, prednisone, 6 α -methylpredisone, 9 α -fluoro-16 α -hydroxyprednisolone, 9 α -fluoro-16 α -methylprednisolone, 9 α -fluorocortisol, testosterone, dihydrotestosterone, androstenediol, androstenedione, androstenedione, 3 α ,5 α -androstenediol, estrone, estradiol, estrogen, spermidine cholesterol carbamate, N⁴-spermidine cholesteryl carbamate, N⁴-spermidine cholesteryl carbamate di HCl salt, N⁴-spermidine-7 dehydro cholesteryl carbamate, N⁴-spermine cholesteryl carbamate, N,N bis(3-aminopropyl) cholesteryl carbamate, N,N bis(6-aminoheptyl) cholesteryl carbamate, N⁴-spermidine dihydrocholesteryl carbamate,

N⁴-spermidine lithocholic carbamate methyl ester, N¹,N⁸-bis (3-aminopropyl-N⁴-spermidine cholesteryl carbamate, N (N⁴-3aminopropylspermidine) cholesteryl carbamate, N,N-bis(4-aminobutyl) cholesteryl carbamate, N⁴-spermidine cholesteryl urea, N⁴-spermine cholesteryl urea, N⁴-spermidine dihydro cholesteryl urea, N⁴-spermine dihydro cholesteryl urea, N,N-bis(N¹-3-aminopropyl-N⁴-aminobutyl) cholesteryl carbamate, N⁴spermidine cholesteryl carboxamide, and N-[N¹,N⁴,N⁸-tris (3-aminopropyl)spermidine] cholesteryl carbamate, lumisterol, cholic acid, desoxycholic acid, cheno-desoxycholic acid and lithocholic acid and derivative thereof.

[0152] Clause 69. A method for producing double-stranded (ds) Hybrid comprising hybridizing a DNA sense and an RNA antisense strand together, each strand having a 5' and a 3' end, wherein a cholesterol moiety is linked to the 3' end of the sense strand, a cholesterol moiety is linked to the 3' end of the antisense strand and no cholesterol moiety is linked to the other ends of the strands.

[0153] Clause 70. The method of Clause 69 wherein the dsHybrid has a length of 19 to 21 base pairs.

[0154] Clause 71. The method of Clause 69 wherein the cholesterol moiety is selected from the group consisting of cholesterol, sterol, any compound derived from cholesterol, chlolestanol, ergosterol, stimastanol, stigmaterol, methyl-lithocholic acid, cortisol, corticosterone, Δ^5 -pregnenolone, progesterone, deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, 11-dioxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, aldosterone, 18-hydroxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, cortisone, prednisone, 6 α -methylpredisone, 9 α -fluoro-16 α -hydroxyprednisolone, 9 α -fluoro-16 α -methylprednisolone, 9 α -fluorocortisol, testosterone, dihydrotestosterone, androstenediol, androstenedione, androstenedione, 3 α ,5 α -androstanediol, estrone, estradiol, estrogen, spermidine cholesterol carbamate, N⁴-spermidine cholesteryl carbamate, N⁴-spermidine cholesteryl carbamate di HCl salt, N⁴-spermidine-7 dehydro cholesteryl carbamate, N⁴-spermine cholesteryl carbamate, N,N bis(3-aminopropyl) cholesteryl carbamate, N,N bis(6-aminohexyl) cholesteryl carbamate, N⁴-spermidine dihydrocholesteryl carbamate, N⁴-spermidine lithocholic carbamate methyl ester, N¹,N⁸-bis (3-aminopropyl-N⁴-spermidine cholesteryl carbamate, N (N⁴-3aminopropylspermidine) cholesteryl carbamate, N,N-bis(4-aminobutyl) cholesteryl carbamate, N⁴-spermidine cholesteryl urea, N⁴-spermine cholesteryl urea, N⁴-spermidine dihydro cholesteryl urea, N⁴-spermine dihydro cholesteryl urea, N,N-bis(N¹-3-aminopropyl-N⁴-aminobutyl) cholesteryl carbamate, N⁴spermidine cholesteryl carboxamide, and N-[N¹,N⁴,N⁸-tris (3-aminopropyl)spermidine] cholesteryl carbamate, lumisterol, cholic acid, desoxycholic acid, cheno-desoxycholic acid and lithocholic acid and derivative thereof.

[0155] Clause 72. A method for producing double-stranded (ds)Hybrid comprising hybridizing a DNA sense and an RNA antisense strand together, each strand having a 5' and a 3' end, wherein a cholesterol moiety is linked to the 5' end of the antisense strand and no cholesterol moiety is linked to the other ends of the dsRNA strands.

[0156] Clause 73. The method of Clause 72 wherein the dsHybrid has a length of 19 to 21 base pairs.

[0157] Clause 74. The method of Clause 72 wherein the cholesterol moiety is selected from the group consisting of cholesterol, sterol, any compound derived from cholesterol, chlolestanol, ergosterol, stimastanol, stigmaterol, methyl-lithocholic acid, cortisol, corticosterone, Δ^5 -pregnenolone, progesterone, deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, 11-dioxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, aldosterone, 18-hydroxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, cortisone, prednisone, 6 α -methylpredisone, 9 α -fluoro-16 α -hydroxyprednisolone, 9 α -fluoro-16 α -methylprednisolone, 9 α -fluorocortisol, testosterone, dihydrotestosterone, androstenediol, androstenedione, androstenedione, 3 α ,5 α -androstanediol, estrone, estradiol, estrogen, spermidine cholesterol carbamate, N⁴-spermidine cholesteryl carbamate, N⁴-spermidine cholesteryl carbamate di HCl salt, N⁴-spermidine-7 dehydro cholesteryl carbamate, N⁴-spermine cholesteryl carbamate, N,N bis(3-aminopropyl) cholesteryl carbamate, N,N bis(6-aminohexyl) cholesteryl carbamate, N⁴-spermidine dihydrocholesteryl carbamate, N⁴-spermidine lithocholic carbamate methyl ester, N¹,N⁸-bis (3-aminopropyl-N⁴-spermidine cholesteryl carbamate, N (N⁴-3aminopropylspermidine) cholesteryl carbamate, N,N-bis(4-aminobutyl) cholesteryl carbamate, N⁴-spermidine cholesteryl urea, N⁴-spermine cholesteryl urea, N⁴-spermidine dihydro cholesteryl urea, N⁴-spermine dihydro cholesteryl urea, N,N-bis(N¹-3-aminopropyl-N⁴-aminobutyl) cholesteryl carbamate, N⁴spermidine cholesteryl carboxamide, and N-[N¹,N⁴,N⁸-tris (3-aminopropyl)spermidine] cholesteryl carbamate, lumisterol, cholic acid, desoxycholic acid, cheno-desoxycholic acid and lithocholic acid and derivative thereof.

[0158] Clause 75. A method for producing a double-stranded (ds) Hybrid comprising hybridizing a DNA sense and an RNA antisense strand together, each strand having a 5' and a 3' end, wherein the 3' end of the sense strand is connected to the 5' end of the antisense strand by means of a loop, and wherein a cholesterol moiety is linked to the 3' end of the antisense strand and wherein no cholesterol moiety is linked to the other ends of the strands of the dsHybrid.

[0159] Clause 76. The method of Clause 75 wherein the dsHybrid has a length of 19 to 21 base pairs.

[0160] Clause 77. The method of Clause 75 wherein the cholesterol moiety is selected from the group consisting of cholesterol, sterol, any compound derived from cholesterol, chlolestanol, ergosterol, stimastanol, stigmaterol, methyl-lithocholic acid, cortisol, corticosterone, Δ^5 -pregnenolone, progesterone, deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, 11-dioxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, aldosterone, 18-hydroxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, cortisone, prednisone, 6 α -methylpredisone, 9 α -fluoro-16 α -hydroxyprednisolone, 9 α -fluoro-16 α -methylprednisolone, 9 α -fluorocortisol, testosterone, dihy-

drotestosterone, androstenediol, androstenedione, androstenedione, $3\alpha,5\alpha$ -androstenediol, estrone, estradiol, estrogen, spermidine cholesterol carbamate, N^4 -spermidine cholesteryl carbamate, N^4 -spermidine cholesteryl carbamate di HCl salt, N^4 -spermidine-7 dehydro cholesteryl carbamate, N^4 -spermine cholesteryl carbamate, N,N bis(3-aminopropyl) cholesteryl carbamate, N,N bis(6-aminoethyl) cholesteryl carbamate, N^4 -spermidine dihydrocholesteryl carbamate, N^4 -spermidine lithocholic carbamate methyl ester, N^1,N^8 -bis (3-aminopropyl- N^4 -spermidine cholesteryl carbamate, N (N^4 -3aminopropylspermidine) cholesteryl carbamate, N,N-bis(4-aminobutyl) cholesteryl carbamate, N^4 -spermidine cholesteryl urea, N^4 -spermine cholesteryl urea, N^4 -spermidine dihydro cholesteryl urea, N^4 -spermine dihydro cholesteryl urea, N,N-bis(N^1 -3-aminopropyl- N^4 -aminobutyl) cholesteryl carbamate, N^4 spermidine cholesteryl carboxamide, and N-[N^1,N^4,N^8 -tris(3-aminopropyl)spermidine] cholesteryl carbamate, lumisterol, cholic acid, desoxycholic acid, chenodesoxycholic acid and lithocholic acid and derivative thereof.

[0161] Clause 78. A method for producing double-stranded (ds) Hybrid comprising hybridizing a DNA sense and an RNA antisense strand together, wherein the 3' end of the sense strand is connected to the 5' end of the antisense strand by means of a loop, wherein a cholesterol moiety is linked to the 5' end of the sense strand and no cholesterol moiety linked to the other ends of the dsHybrid strands.

[0162] Clause 79. The method of Clause 78 wherein the dsHybrid has a length of 19 to 21 base pairs.

[0163] Clause 80. The method of Clause 78 wherein the cholesterol moiety is selected from the group consisting of cholesterol, sterol, any compound derived from cholesterol, chlolestanol, ergosterol, stimastanol, stigmastanol, methyl-lithocholic acid, cortisol, corticosterone, Δ^5 -pregnenolone, progesterone, deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, 11-dioxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, aldosterone, 18-hydroxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, cortisone, prednisone, 6 α -methylprednisone, 9 α -fluoro-16 α -hydroxyprednisolone, 9 α -fluoro-16 α -methylprednisolone, 9 α -fluorocortisol, testosterone, dihydrotestosterone, androstenediol, androstenedione, androstenedione, $3\alpha,5\alpha$ -androstenediol, estrone, estradiol, estrogen, spermidine cholesterol carbamate, N^4 -spermidine cholesteryl carbamate, N^4 -spermidine cholesteryl carbamate di HCl salt, N^4 -spermidine-7 dehydro cholesteryl carbamate, N^4 -spermine cholesteryl carbamate, N,N bis(3-aminopropyl) cholesteryl carbamate, N,N bis(6-aminoethyl) cholesteryl carbamate, N^4 -spermidine dihydrocholesteryl carbamate, N^4 -spermidine lithocholic carbamate methyl ester, N^1,N^8 -bis (3-aminopropyl- N^4 -spermidine cholesteryl carbamate, N (N^4 -3aminopropylspermidine) cholesteryl carbamate, N,N-bis(4-aminobutyl) cholesteryl carbamate, N^4 -spermidine cholesteryl urea, N^4 -spermine cholesteryl urea, N^4 -spermidine dihydro cholesteryl urea, N^4 -spermine dihydro cholesteryl urea, N,N-bis(N^1 -3-aminopropyl- N^4 -aminobutyl) cholesteryl carbamate, N^4 spermidine cholesteryl carboxamide, and N-[N^1,N^4,N^8 -tris (3-aminopropyl)spermidine] cholesteryl carbamate, lumisterol, cholic acid, desoxycholic acid, chenodesoxycholic acid and lithocholic acid and derivative thereof.

[0164] Clause 81. A pharmaceutical composition for enhancing delivery of a double-stranded (ds) RNA or a dsHybrid into a cytoplasm of a mammalian target cell comprising:

a double-stranded (ds) RNA or a dsHybrid having a sense and an anti-sense strand, each strand having a 5' and a 3' end, wherein a cholesterol moiety is linked to the 5' end of the sense strand and a cholesterol moiety is linked to the 5' end of the antisense and wherein no cholesterol moiety is linked to the other ends of the strands; and

one or more secondary delivery-enhancing agent(s) in an amount effective to enhance delivery of the double-stranded (ds) RNA or dsHybrid linked to the cholesterol moiety into the cytoplasm of the target cell selected from:

- (a) an aggregation inhibitory agent;
- (b) a charge modifying agent;
- (c) a pH control agent;
- (d) a degradative enzyme inhibitory agent;
- (e) a mucolytic or mucus clearing agent;
- (f) a ciliostatic agent;
- (g) a membrane penetration-enhancing agent selected from (i) a surfactant, (ii) a bile salt, (iii) a phospholipid additive, mixed micelle, liposome, or carrier, (iv) an alcohol, (v) an enamine, (vi) an NO donor compound, (vii) a long-chain amphipathic molecule (viii) a small hydrophobic penetration enhancer; (ix) sodium or a salicylic acid derivative; (x) a glycerol ester of acetoacetic acid (xi) a cyclodextrin or beta-cyclodextrin derivative, (xii) a medium-chain fatty acid, (xiii) a chelating agent, (xiv) an amino acid or salt thereof, (xv) an N-acetyl amino acid or salt thereof, (xvi) an enzyme degradative to a selected membrane component, (xvii) an inhibitor of fatty acid synthesis, or (xviii) an inhibitor of cholesterol synthesis; or (xix) any combination of the membrane penetration enhancing agents recited in (g)(i)-(xix);
- (h) a delivery-enhancing peptide;
- (i) a vasodilator agent;
- (j) a selective transport-enhancing agent; and

(k) a stabilizing delivery vehicle, carrier, support or complex-forming species with which the cholesterol-conjugated siRNA or siHybrid is effectively combined, associated, contained, encapsulated or bound resulting in stabilization of the siRNA.

[0165] Clause 82. The pharmaceutical composition of Clause 81 wherein the dsRNA or dsHybrid has a length of 19 to 21 base pairs.

[0166] Clause 83. The pharmaceutical composition of Clause 81 wherein the sense strand is a DNA strand.

[0167] Clause 84. The pharmaceutical composition of Clause 81 wherein the cholesterol moiety is selected from the group consisting of cholesterol, sterol, any compound derived from cholesterol, cholestanol, ergosterol, stimastanol, stigmaterol, methyl-lithocholic acid, cortisol, corticosterone, Δ^5 -pregnenolone, progesterone, deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, 11-dioxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, aldosterone, 18-hydroxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, cortisone, prednisone, 6 α -methylpredisone, 9 α -fluoro-16 α -hydroxyprednisolone, 9 α -fluoro-16 α -methylprednisolone, 9 α -fluorocortisol, testosterone, dihydrotestosterone, androstenediol, androstenedione, androstenedione, 3 α ,5 α -androstenediol, estrone, estradiol, estrogen, spermidine cholesterol carbamate, N⁴-spermidine cholesteryl carbamate, N⁴-spermidine cholesteryl carbamate di HCl salt, N⁴-spermidine-7 dehydro cholesteryl carbamate, N⁴-spermine cholesteryl carbamate, N,N bis (3-aminopropyl) cholesteryl carbamate, N,N bis(6-aminohexyl) cholesteryl carbamate, N⁴-spermidine dihydrocholesteryl carbamate, N⁴-spermidine lithocholic carbamate methyl ester, N¹,N⁸-bis (3-aminopropyl-N⁴-spermidine cholesteryl carbamate, N(N⁴-3aminopropylspermidine) cholesteryl carbamate, N,N-bis(4-aminobutyl) cholesteryl carbamate, N⁴-spermidine cholesteryl urea, N⁴-spermine cholesteryl urea, N⁴-spermidine dihydro cholesteryl urea, N⁴-spermine dihydro cholesteryl urea, N,N-bis(N'-3-aminopropyl-N"4-aminobutyl) cholesteryl carbamate, N⁴spermidine cholesteryl carboxamide, and N-[N¹,N⁴,N⁸-tris (3-aminopropyl)spermidine] cholesteryl carbamate, lumisterol, cholic acid, desoxycholic acid, chenodesoxycholic acid and lithocholic acid and derivative thereof.

[0168] Clause 85. The pharmaceutical composition of Clause 81 wherein the secondary delivery-enhancing agent(s) include(s) a delivery-enhancing peptide.

[0169] Clause 86. The pharmaceutical composition of Clause 85 wherein the delivery-enhancing peptide comprises an amino acid sequence selected from:

RKKRRQRRRPPQCAAVALLPAVLLALLAP (SEQ ID NO: 1);
 RQIKIWFAQNRRMKWKK (SEQ ID NO: 2);
 GWTLNSAGYLLGKINLKALAALAKKIL (SEQ ID NO: 3);
 KLALKLALKALKAAALKLA (SEQ ID NO: 4);
 KLWSAWPSLWSSLWKP (SEQ ID NO: 7);
 AAVALLPAVLLALLAPRKKRRQRRRPPQ (SEQ ID NO: 8);
 LLETLLKPFQCRICMRNFSTRQARRNHRRRHRR (SEQ ID NO: 9);
 RRRQRRKRGGDIMGEWGNIEFGAIGFLG (SEQ ID NO: 10);
 KETWWETWTEWSQPGRKKRRQRRRPPQ (SEQ ID NO: 11);
 GLGSLLKKAGKKLKQPKSKRKV (SEQ ID NO: 12); and
 KGSKKAVTKAQKKGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 13)

[0170] Clause 87. The pharmaceutical composition of Clause 85 wherein the delivery-enhancing peptide is combined with or conjugated to the double-stranded (ds) RNA or dsHybrid linked to the cholesterol moiety.

[0171] Clause 88. The pharmaceutical composition of Clause 81 which is effective to deliver the double-stranded (ds) RNA or dsHybrid linked to the cholesterol moiety to a target mammalian cell selected from pulmonary alveolar cells, skin cells, hepatic cells, renal cells, pancreatic cells, endothelial cells, nucleated blood cells, muscle cells, mammary cells, peripheral or central nervous system (CNS) cells, gastrointestinal cells, or tumor cells.

[0172] Clause 89. A pharmaceutical composition for enhancing delivery of a double-stranded (ds) RNA or a dsHybrid into a cytoplasm of a mammalian target cell comprising:

a double-stranded (ds) RNA or a dsHybrid having a sense and an antisense strand, each strand having a 5' and a 3' end, wherein a cholesterol moiety is linked to the 3' end of the antisense strand and wherein no cholesterol moiety is linked to the other ends of the strands; and

one or more secondary delivery-enhancing agent(s) in an amount effective to enhance delivery of the double-stranded (ds) RNA or dsHybrid linked to the cholesterol moiety into the cytoplasm of the target cell selected from:

- (a) an aggregation inhibitory agent;
- (b) a charge modifying agent;

- (c) a pH control agent;
- (d) a degradative enzyme inhibitory agent;
- (e) a mucolytic or mucus clearing agent;
- (f) a ciliostatic agent;

(g) a membrane penetration-enhancing agent selected from (i) a surfactant, (ii) a bile salt, (iii) a phospholipid additive, mixed micelle, liposome, or carrier, (iv) an alcohol, (v) an enamine, (vi) an NO donor compound, (vii) a long-chain amphipathic molecule (viii) a small hydrophobic penetration enhancer; (ix) sodium or a salicylic acid derivative; (x) a glycerol ester of acetoacetic acid (xi) a cyclodextrin or beta-cyclodextrin derivative, (xii) a medium-chain fatty acid, (xiii) a chelating agent, (xiv) an amino acid or salt thereof, (xv) an N-acetylamino acid or salt thereof, (xvi) an enzyme degradative to a selected membrane component, (xvii) an inhibitor of fatty acid synthesis, or (xviii) an inhibitor of cholesterol synthesis; or (xix) any combination of the membrane penetration enhancing agents recited in (g)(i)-(xix);

(h) a delivery-enhancing peptide;

(i) a vasodilator agent;

(j) a selective transport-enhancing agent; and

(k) a stabilizing delivery vehicle, carrier, support or complex-forming species with which the cholesterol-conjugated siRNA or siHybrid is effectively combined, associated, contained, encapsulated or bound resulting in stabilization of the siRNA.

[0173] Clause 90. The pharmaceutical composition of Clause 89 wherein the dsRNA or dsHybrid has a length of 19 to 21 base pairs.

[0174] Clause 91. The pharmaceutical composition of Clause 89 wherein the sense strand is a DNA strand.

[0175] Clause 92. The pharmaceutical composition of Clause 89 wherein the cholesterol moiety is selected from the group consisting of cholesterol, sterol, any compound derived from cholesterol, chlolestanol, ergosterol, stigmasterol, methyl-lithocholic acid, cortisol, corticosterone, Δ^5 -pregnenolone, progesterone, deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, 11-dioxycortisol, dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, aldosterone, 18-hydroxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, cortisone, prednisone, 6 α -methylprednisone, 9 α -fluoro-16 α -hydroxyprednisolone, 9 α -fluoro-16 α -methylprednisolone, 9 α -fluorocortisol, testosterone, dihydrotestosterone, androstenediol, androstenedione, androstenedione, 3 α ,5 α -androstenediol, estrone, estradiol, estrogen, spermidine cholesterol carbamate, N⁴-spermidine cholesteryl carbamate, N⁴-spermidine cholesteryl carbamate di HCl salt, N⁴-spermidine-7 dehydro cholesteryl carbamate, N⁴-spermine cholesteryl carbamate, N,N bis (3-aminopropyl) cholesteryl carbamate, N,N bis(6-aminohexyl) cholesteryl carbamate, N⁴-spermidine dihydrocholesteryl carbamate, N⁴-spermidine lithocholic carbamate methyl ester, N¹,N⁸-bis (3-aminopropyl-N⁴-spermidine cholesteryl carbamate, N(N⁴-3aminopropyl)spermidine) cholesteryl carbamate, N,N-bis(4-aminobutyl) cholesteryl carbamate, N⁴-spermidine cholesteryl urea, N⁴-spermine cholesteryl urea, N⁴-spermidine dihydro cholesteryl urea, N⁴-spermine dihydro cholesteryl urea, N,N-bis(N¹-3-aminopropyl-N⁴-aminobutyl) cholesteryl carbamate, N⁴spermidine cholesteryl carboxamide, and N-[N¹,N⁴,N⁸-tris (3-aminopropyl)spermidine] cholesteryl carbamate, lumisterol, cholic acid, desoxycholic acid, chenodesoxycholic acid and lithocholic acid and derivative thereof.

[0176] Clause 93. The pharmaceutical composition of Clause 89 wherein the secondary delivery-enhancing agent(s) include(s) a delivery-enhancing peptide.

[0177] Clause 94. The pharmaceutical composition of Clause 93 wherein the delivery-enhancing peptide comprises an amino acid sequence selected from:

RKKRRQRRRPPQCAAVALLPAVLLALLAP (SEQ ID NO: 1);

RQIKIWFQNRRMKWKK (SEQ ID NO: 2);

GWTLNSAGYLLGKINLKALAALAKKIL (SEQ ID NO: 3);

KLALKLALKALKAAALKLA (SEQ ID NO: 4);

KLWSAWPSLWSSLWKP (SEQ ID NO: 7);

AAVALLPAVLLALLAPRKKRRQRRRPPQ (SEQ ID NO: 8);

LLETLLKPFQCRICMRNFSTRQARRNHRHRHRR (SEQ ID NO: 9);

RRRQRRKRGDIMGEGWNEIFGAIAGFLG (SEQ ID NO: 10);

KETWWETWWTEWSQPGRKKRRQRRRPPQ (SEQ ID NO: 11);

GLGSLLKKAGKKLKQPKSKRKV (SEQ ID NO: 12); and

KGSKKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 13)

[0178] Clause 95. The pharmaceutical composition of Clause 93 wherein the delivery-enhancing peptide is combined with or conjugated to the double-stranded (ds) RNA or dsHybrid linked to the cholesterol moiety.

[0179] Clause 96. The pharmaceutical composition of Clause 89 which is effective to deliver the double-stranded (ds)

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RNA or dsHybrid linked to the cholesterol moiety to a target mammalian cell selected from pulmonary alveolar cells, skin cells, hepatic cells, renal cells, pancreatic cells, endothelial cells, nucleated blood cells, muscle cells, mammary cells, peripheral or central nervous system (CNS) cells, gastrointestinal cells, or tumor cells.

5 SEQUENCE LISTING

[0180]

10 <110> MDRNA, INC.

<120> COMPOSITIONS FOR ENHANCING DELIVERY OF DOUBLE-STRANDED RNA TO REGULATE GENE
EXPRESSION IN MAMMALIAN CELLS

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<140> Not yet assigned
<141> 2005-04-15

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1 5 10 15

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<213> Human immunodeficiency virus

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EP 2 145 957 B1

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Arg

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EP 2 145 957 B1

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EP 2 145 957 B1

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<223> Description of Combined DNA/RNA Molecule: Synthetic siRNA oligonucleotide construct

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<213> Artificial Sequence

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<223> Description of Artificial Sequence: Synthetic siRNA oligonucleotide construct

55 <220>

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EP 2 145 957 B1

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<223> Description of Artificial Sequence: Synthetic siRNA oligonucleotide construct

<220>

<223> both ends labeled antisense strand

<400> 21

aaaucgcuga uuuguguagt t 21

Claims

1. A delivery-enhancing peptide consisting of the amino acid sequence of SEQ ID NO: 11 or salt thereof.

2. A delivery-enhancing peptide according to Claim 1, wherein the amino acid sequence of SEQ ID NO: 11 is modified by incorporation of one or more amino- and/or carboxy-terminal chemical modifications, preferably by inclusion of one or more amino- and/or carboxy-terminal amide, BrAc, or maleimide groups.

3. A composition comprising a peptide according to Claim 1 or 2 conjugated to or in a complex with a cholesterol-conjugated siRNA.

4. A composition according to Claim 3, wherein the siRNA has a sense and an anti-sense strand, each strand having a 5' and a 3' end, wherein a cholesterol moiety is linked to the 5' end of the sense strand and a cholesterol moiety is linked to the 5' end of the antisense strand and wherein no cholesterol moiety is linked to the other ends of the strands.

5. A composition according to Claim 3, wherein the siRNA has a sense and an anti-sense strand, each strand having a 5' and a 3' end, wherein a cholesterol moiety is linked to the 3' end of the antisense strand and wherein no cholesterol moiety is linked to the other ends of the strands.

6. A composition according to Claim 3, wherein the siRNA has a sense and an anti-sense strand, each strand having a 5' and a 3' end, wherein a cholesterol moiety is linked to the 5' end of the sense strand and no cholesterol moiety is linked to the other ends of the strands.

7. A composition according to Claim 3, wherein the siRNA has a sense and an anti-sense strand, each strand having a 5' and a 3' end, wherein a cholesterol moiety is linked to the 3' end of the sense strand and no cholesterol moiety is linked to the other ends of the strands.

8. A composition according to Claim 3, wherein the siRNA has a sense and an anti-sense strand, each strand having a 5' and a 3' end, wherein a cholesterol moiety is linked to the 3' end of the sense strand, a cholesterol moiety is linked to the 3' end of the antisense strand and no cholesterol moiety is linked to the other ends of the strands.

9. A composition according to Claim 3, wherein the siRNA has a sense and an anti-sense strand, each strand having a 5' and a 3' end, wherein a cholesterol moiety is linked to the 5' end of the antisense strand and no cholesterol moiety is linked to the other ends of the strands.

10. A composition according to Claim 3, wherein the siRNA has a sense and an anti-sense strand, each strand having a 5' and a 3' end, wherein the 3' end of the sense strand is connected to the 5' end of the antisense strand by means of a loop, and wherein a cholesterol moiety is linked to the 3' end of the antisense strand and wherein no cholesterol moiety is linked to the other ends of the strands.

11. A composition according to Claim 3, wherein the siRNA has a sense and an anti-sense strand, each strand having a 5' and a 3' end, wherein the 3' end of the sense strand is connected to the 5' end of the antisense strand by means of a loop, wherein a cholesterol moiety is linked to the 5' end of the sense strand and no cholesterol moiety is linked to the other ends of the strands.

12. A pharmaceutical composition comprising a composition according to any of Claims 3 to 11, preferably wherein the pharmaceutical composition further comprises one or more secondary delivery-enhancing agent(s) selected from the group consisting of:

(a) an aggregation inhibitory agent;

(b) a charge modifying agent;

(c) a pH control agent;

(d) a degradative enzyme inhibitory agent;

(e) a mucolytic or mucus clearing agent;

(f) a ciliostatic agent;

(g) a membrane penetration-enhancing agent selected from (i) a surfactant, (ii) a bile salt, (iii) a phospholipid additive, mixed micelle, liposome, or carrier, (iv) an alcohol, (v) an enamine, (vi) an NO donor compound, (vii) a long-chain amphipathic molecule (viii) a small hydrophobic penetration enhancer; (ix) sodium or a salicylic acid derivative; (x) a glycerol ester of acetoacetic acid (xi) a cyclodextrin or beta-cyclodextrin derivative, (xii) a medium-chain fatty acid, (xiii) a chelating agent, (xiv) an amino acid or salt thereof, (xv) an N-acetyl amino acid or salt thereof, (xvi) an enzyme degradative to a selected membrane component, (xvii) an inhibitor of fatty acid

synthesis, or (xviii) an inhibitor of cholesterol synthesis; or (xix) any combination of the membrane penetration enhancing agents recited in (g)(i)-(xix);
 (h) a delivery-enhancing peptide;
 (i) a vasodilator agent;
 (j) a selective transport-enhancing agent; and
 (k) a stabilizing delivery vehicle, carrier, support or complex-forming species with which the siRNA is effectively combined, associated, contained, encapsulated or bound resulting in stabilization of the siRNA.

13. A delivery-enhancing peptide according to Claim 1 or 2, or a composition according to any of Claims 3 to 12, for delivering a siRNA to a target cell.

14. A delivery-enhancing peptide according to Claim 13, or a composition according to Claim 14, wherein the target cell is a mammalian cell selected from pulmonary alveolar cells, skin cells, hepatic cells, renal cells, pancreatic cells, endothelial cells, nucleated blood cells, muscle cells, mammary cells, peripheral or central nervous system (CNS) cells, gastrointestinal cells, or tumor cells.

Patentansprüche

1. Abgabe erhöhendes Peptid bestehend aus der Aminosäuresequenz von SEQ ID NR: 11 oder einem Salz davon.
2. Abgabe erhöhendes Peptid nach Anspruch 1, wobei die Aminosäuresequenz von SEQ ID NR: 11 durch Einbauen von ein oder mehreren Amino- und/oder Carboxy-terminalen chemischen Modifikationen, vorzugsweise durch Einschließen von ein oder mehreren Amino- und/oder Carboxy-terminalen Amid-, BrAc- oder Maleimidgruppen modifiziert wird.
3. Zusammensetzung, die ein Peptid nach Anspruch 1 oder 2 im Konjugat mit oder in einem Komplex mit einer Cholesterin-konjugierten siRNA beinhaltet.
4. Zusammensetzung nach Anspruch 3, wobei die siRNA einen Sense- und einen Anti-Sense-Strang hat, wobei jeder Strang ein 5'- und ein 3'-Ende hat, wobei ein Cholesterinanteil mit dem 5'-Ende des Sense-Strangs verknüpft ist und ein Cholesterinanteil mit dem 5'-Ende des Anti-Sense-Strangs verknüpft ist und kein Cholesterinanteil mit den anderen Enden der Stränge verknüpft ist.
5. Zusammensetzung nach Anspruch 3, wobei die siRNA einen Sense- und einen Anti-Sense-Strang hat, wobei jeder Strang ein 5'- und ein 3'-Ende hat, wobei ein Cholesterinanteil mit dem 3'-Ende des Anti-Sense-Strangs verknüpft ist und kein Cholesterinanteil mit den anderen Enden der Stränge verknüpft ist.
6. Zusammensetzung nach Anspruch 3, wobei die siRNA einen Sense- und einen Anti-Sense-Strang hat, wobei jeder Strang ein 5'- und ein 3'-Ende hat, wobei ein Cholesterinanteil mit dem 5'-Ende des Sense-Strangs verknüpft ist und kein Cholesterinanteil mit den anderen Enden der Stränge verknüpft ist.
7. Zusammensetzung nach Anspruch 3, wobei die siRNA einen Sense- und einen Anti-Sense-Strang hat, wobei jeder Strang ein 5'- und ein 3'-Ende hat, wobei ein Cholesterinanteil mit dem 3'-Ende des Sense-Strangs verknüpft ist und kein Cholesterinanteil mit den anderen Enden der Stränge verknüpft ist.
8. Zusammensetzung nach Anspruch 3, wobei die siRNA einen Sense- und einen Anti-Sense-Strang hat, wobei jeder Strang ein 5'- und ein 3'-Ende hat, wobei ein Cholesterinanteil mit dem 3'-Ende des Sense-Strangs verknüpft ist, ein Cholesterinanteil mit dem 3'-Ende des Anti-Sense-Strangs verknüpft ist und kein Cholesterinanteil mit den anderen Enden der Stränge verknüpft ist.
9. Zusammensetzung nach Anspruch 3, wobei die siRNA einen Sense- und einen Anti-Sense-Strang hat, wobei jeder Strang ein 5'- und ein 3'-Ende hat, wobei ein Cholesterinanteil mit dem 5'-Ende des Anti-Sense-Strangs verknüpft ist und kein Cholesterinanteil mit den anderen Enden der Stränge verknüpft ist.
10. Zusammensetzung nach Anspruch 3, wobei die siRNA einen Sense- und einen Anti-Sense-Strang hat, wobei jeder Strang ein 5'- und ein 3'-Ende hat, wobei das 3'-Ende des Sense-Strangs mit dem 5'-Ende des Anti-Sense-Strangs durch eine Schleife verbunden ist, und wobei ein Cholesterinanteil mit dem 3'-Ende des Anti-Sense-Strangs verknüpft

ist und kein Cholesterinanteil mit den anderen Enden der Stränge verknüpft ist.

11. Zusammensetzung nach Anspruch 3, wobei die siRNA einen Sense- und einen Anti-Sense-Strang hat, wobei jeder Strang ein 5'- und ein 3'-Ende hat, wobei das 3'-Ende des Sense-Strangs mit dem 5'-Ende des Anti-Sense-Strangs durch eine Schleife verbunden ist, wobei ein Cholesterinanteil mit dem 5'-Ende des Sense-Strangs verknüpft ist und kein Cholesterinanteil mit den anderen Enden der Stränge verknüpft ist.

12. Pharmazeutische Zusammensetzung, die eine Zusammensetzung nach einem der Ansprüche 3 bis 11 beinhaltet, wobei die pharmazeutische Zusammensetzung vorzugsweise ferner ein oder mehrere sekundäre die Abgabe erhöhende Agenzien umfasst, die ausgewählt sind aus der Gruppe bestehend aus:

- (a) einem aggregationshemmenden Agens;
- (b) einem ladungsmodifizierenden Agens;
- (c) einem pH-Regulierungsagens;
- (d) einem Abbauenzym hemmenden Agens;
- (e) einem mukolytischen oder schleimbeseitigenden Agens;
- (f) einem ciliostatischen Agens;
- (g) einem Membranpenetration verbessernden Agens, ausgewählt aus (i) einem Tensid, (ii) einem Gallensalz, (iii) einem/r Phospholipidadditiv, Mischmicelle, Liposom oder Träger, (iv) einem Alkohol, (v) einem Enamin, (vi) einer NO-Spenderverbindung, (vii) einem langkettigen amphipathischen Molekül, (viii) einem kleinen hydrophoben Penetrationsverbesserer; (ix) Natrium oder einem Salicylsäurederivat; (x) einem Glycerolester von Acetessigsäure, (xi) einem Cyclodextrin oder Beta-Cyclodextrin-Derivat, (xii) einer mittelkettigen Fettsäure, (xiii) einem Gelatbildner, (xiv) einer Aminosäure oder einem Salz davon, (xv) einer N-Acetylamino-säure oder einem Salz davon, (xvi) einem Enzymabbau-mittel zu einer gewählten Membrankomponente, (xvii) einem Fettsäure-synthese-Inhibitor, oder (xviii) einem Cholesterinsynthese-Inhibitor; oder (xix) einer beliebigen Kombination der in (g)(i)-(xix) aufgeführten die Membranpenetration verbessernden Agenzien;
- (h) einem die Abgabe verbessernden Peptid;
- (i) einem Vasodilatatoragens;
- (j) einem selektiven transportverbessernden Agens; und
- (k) einem/r stabilisierenden Abgabevehikel, Träger, Substrat oder komplexbildenden Spezies, mit dem/der die siRNA effektiv kombiniert, assoziiert, aufgenommen, verkapselt oder gebunden ist, was zur Stabilisierung der siRNA führt.

13. Abgabe erhöhendes Peptid nach Anspruch 1 oder 2 oder Zusammensetzung nach einem der Ansprüche 3 bis 12 zum Abgeben einer siRNA an eine Zielzelle.

14. Abgabe erhöhendes Peptid nach Anspruch 13 oder Zusammensetzung nach Anspruch 14, wobei die Zielzelle eine Säugetierzelle ist, ausgewählt aus pulmonalen Alveolarzellen, Hautzellen, Leberzellen, Nierenzellen, Bauspeicheldrüsenzellen, Endothelzellen, nukleierten Blutzellen, Muskelzellen, Brustzellen, Zellen des peripheren oder zentralen Nervensystems (CNS), Magendarmzellen oder Tumorzellen.

Revendications

1. Peptide améliorant un apport constitué de la séquence d'acides aminés SEQ. n° 11 ou d'un sel de celle-ci.
2. Peptide améliorant un apport selon la revendication 1, dans lequel la séquence d'acides aminés SEQ. n° 11 est modifiée par incorporation d'un ou plusieurs modificateurs chimiques à terminaison amino et/ou carboxy, préféra-blement par inclusion d'un ou plusieurs groupes amide, BrAc ou maléimide à terminaison amino et/ou carboxy.
3. Composition comprenant un peptide selon la revendication 1 ou 2, conjugué à ou dans un complexe comprenant un ARNsi conjugué à un groupe cholestérol.
4. Composition selon la revendication 3, dans laquelle l'ARNsi comporte un brin sens et un brin antisens, chaque brin comportant une extrémité 5' et une extrémité 3', dans laquelle une fraction cholestérol est liée à l'extrémité 5' du brin sens et une fraction cholestérol est liée à l'extrémité 5' du brin antisens et dans laquelle aucune fraction cholestérol n'est liée aux autres extrémités des brins.

5. Composition selon la revendication 3, dans laquelle l'ARNsi comporte un brin sens et un brin antisens, chaque brin comportant une extrémité 5' et une extrémité 3', dans laquelle une fraction cholestérol est liée à l'extrémité 3' du brin antisens et dans laquelle aucune fraction cholestérol n'est liée aux autres extrémités des brins.
- 5 6. Composition selon la revendication 3, dans laquelle l'ARNsi comporte un brin sens et un brin antisens, chaque brin comportant une extrémité 5' et une extrémité 3', dans laquelle une fraction cholestérol est liée à l'extrémité 5' du brin sens et aucune fraction cholestérol n'est liée aux autres extrémités des brins.
- 10 7. Composition selon la revendication 3, dans laquelle l'ARNsi comporte un brin sens et un brin antisens, chaque brin comportant une extrémité 5' et une extrémité 3', dans laquelle une fraction cholestérol est liée à l'extrémité 3' du brin sens et aucune fraction cholestérol n'est liée aux autres extrémités des brins.
- 15 8. Composition selon la revendication 3, dans laquelle l'ARNsi comporte un brin sens et un brin antisens, chaque brin comportant une extrémité 5' et une extrémité 3', dans laquelle une fraction cholestérol est liée à l'extrémité 3' du brin sens, une fraction cholestérol est liée à l'extrémité 3' du brin antisens et aucune fraction cholestérol n'est liée aux autres extrémités des brins.
- 20 9. Composition selon la revendication 3, dans laquelle l'ARNsi comporte un brin sens et un brin antisens, chaque brin comportant une extrémité 5' et une extrémité 3', dans laquelle une fraction cholestérol est liée à l'extrémité 5' du brin antisens et aucune fraction cholestérol n'est liée aux autres extrémités des brins.
- 25 10. Composition selon la revendication 3, dans laquelle l'ARNsi comporte un brin sens et un brin antisens, chaque brin comportant une extrémité 5' et une extrémité 3', dans laquelle l'extrémité 3' du brin sens est liée à l'extrémité 5' du brin antisens au moyen d'une boucle, dans laquelle une fraction cholestérol est liée à l'extrémité 3' du brin antisens et dans laquelle aucune fraction cholestérol n'est liée aux autres extrémités des brins.
- 30 11. Composition selon la revendication 3, dans laquelle l'ARNsi comporte un brin sens et un brin antisens, chaque brin comportant une extrémité 5' et une extrémité 3', dans laquelle l'extrémité 3' du brin sens est liée à l'extrémité 5' du brin antisens au moyen d'une boucle, et dans laquelle une fraction cholestérol est liée à l'extrémité 5' du brin sens et aucune fraction cholestérol n'est liée aux autres extrémités des brins.
- 35 12. Composition pharmaceutique comprenant une composition selon l'une quelconque des revendications 3 à 11, ladite composition pharmaceutique comprenant préférentiellement en outre un ou plusieurs agents secondaires améliorant un apport sélectionnés dans le groupe constitué de :
 - (a) un agent anti-agrégation ;
 - (b) un agent modificateur de charge ;
 - (c) un agent de contrôle de pH ;
 - (d) un agent inhibiteur d'enzyme de dégradation ;
 - 40 (e) un groupe mucolytique ou d'élimination du mucus ;
 - (f) un agent ciliostatique ;
 - (g) un agent améliorant la pénétration membranaire sélectionné parmi (i) un tensioactif, (ii) un sel biliaire, (iii) un additif, une micelle mixte, un liposome ou un vecteur phospholipidique, (iv) un alcool, (v), une énamine, (vi) un composé donneur d'oxyde nitrique, (vii) une molécule amphipathique à chaîne longue, (viii) un activateur de pénétration hydrophobe de petite taille, (ix) un dérivé de sodium ou d'acide salicylique, (x) un ester glycérolique d'acide acétoacétique, (xi) un dérivé de cyclodextrine ou de bêta-cyclodextrine, (xii) un acide gras à chaîne
 - 45 moyenne, (xiii) un chélateur, (xiv) un acide aminé ou un sel de celui-ci, (xv) un acide N-acétylaminé ou un sel de celui-ci, (xvi) une enzyme de dégradation d'un constituant membranaire sélectionné, (xvii) un inhibiteur de la synthèse des acides gras, (xviii) un inhibiteur de la synthèse du cholestérol, ou (xix) toute combinaison des agents améliorant la pénétration membranaire cités dans (g) (i) - (xix) ;
 - 50 (h) un peptide améliorant un apport ;
 - (i) un agent vasodilatateur ;
 - (j) un agent améliorant sélectivement un transport ; et
 - (k) un véhicule, un vecteur, un support ou une espèce formant un complexe, ayant un effet stabilisateur et utilisé
 - 55 (e) pour un apport, avec lequel/laquelle l'ARNsi est efficacement combiné, associé, contenu, encapsulé ou lié, l'ARNsi étant ainsi stabilisé.
13. Peptide améliorant un apport selon la revendication 1 ou 2, ou composition selon l'une quelconque des revendications

3 à 12, pour l'apport d'un ARNsi dans une cellule cible.

- 5 **14.** Peptide améliorant un apport selon la revendication 13, ou composition selon la revendication 14, la cellule cible étant une cellule mammifère sélectionnée parmi des cellules alvéolaires pulmonaires, des cellules cutanées, des cellules hépatiques, des cellules rénales, des cellules pancréatiques, des cellules endothéliales, des cellules sanguines nucléées, des cellules musculaires, des cellules mammaires, des cellules du système nerveux périphérique ou du système nerveux central (SNC), des cellules gastro-intestinales, ou des cellules tumorales.
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Figure 1

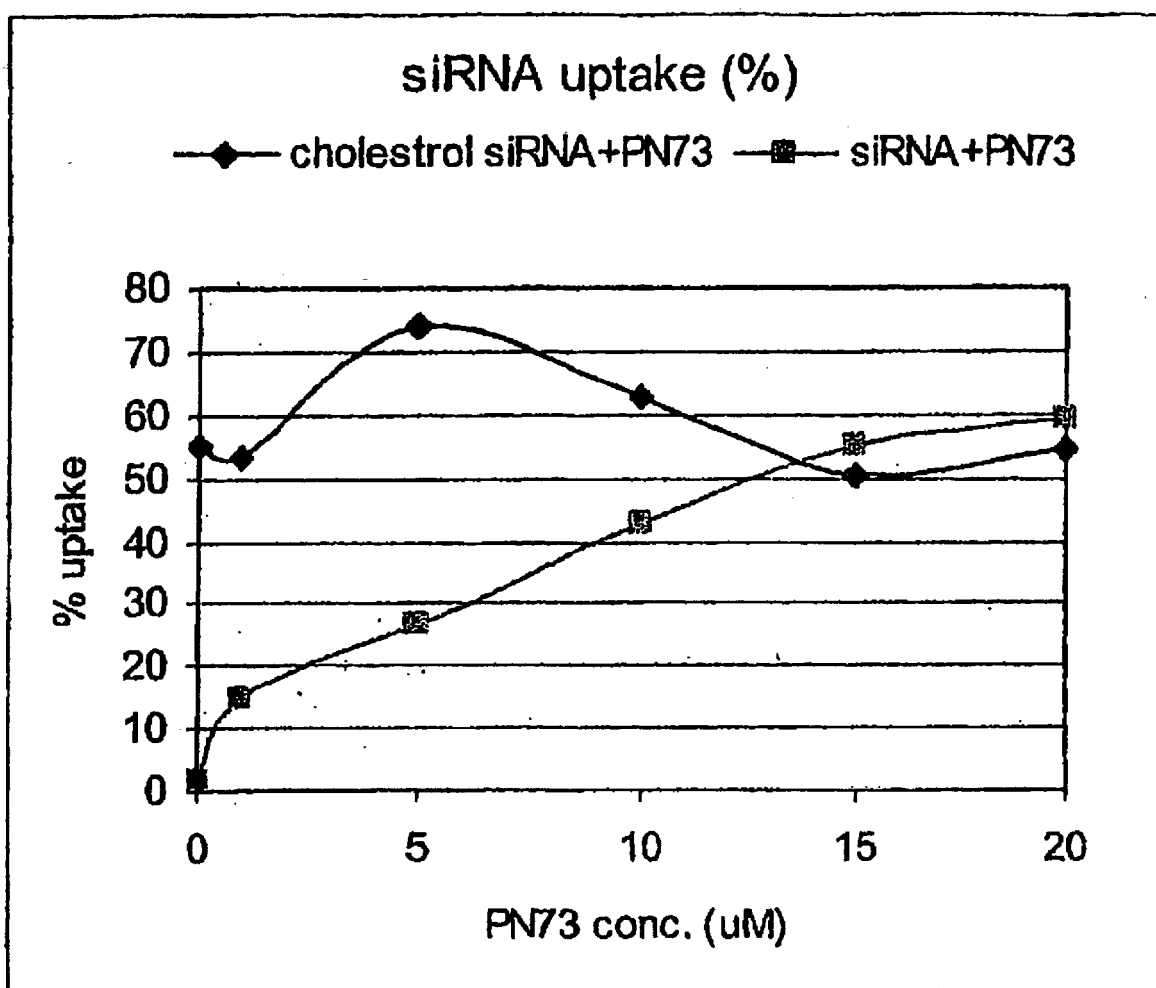


Figure 2

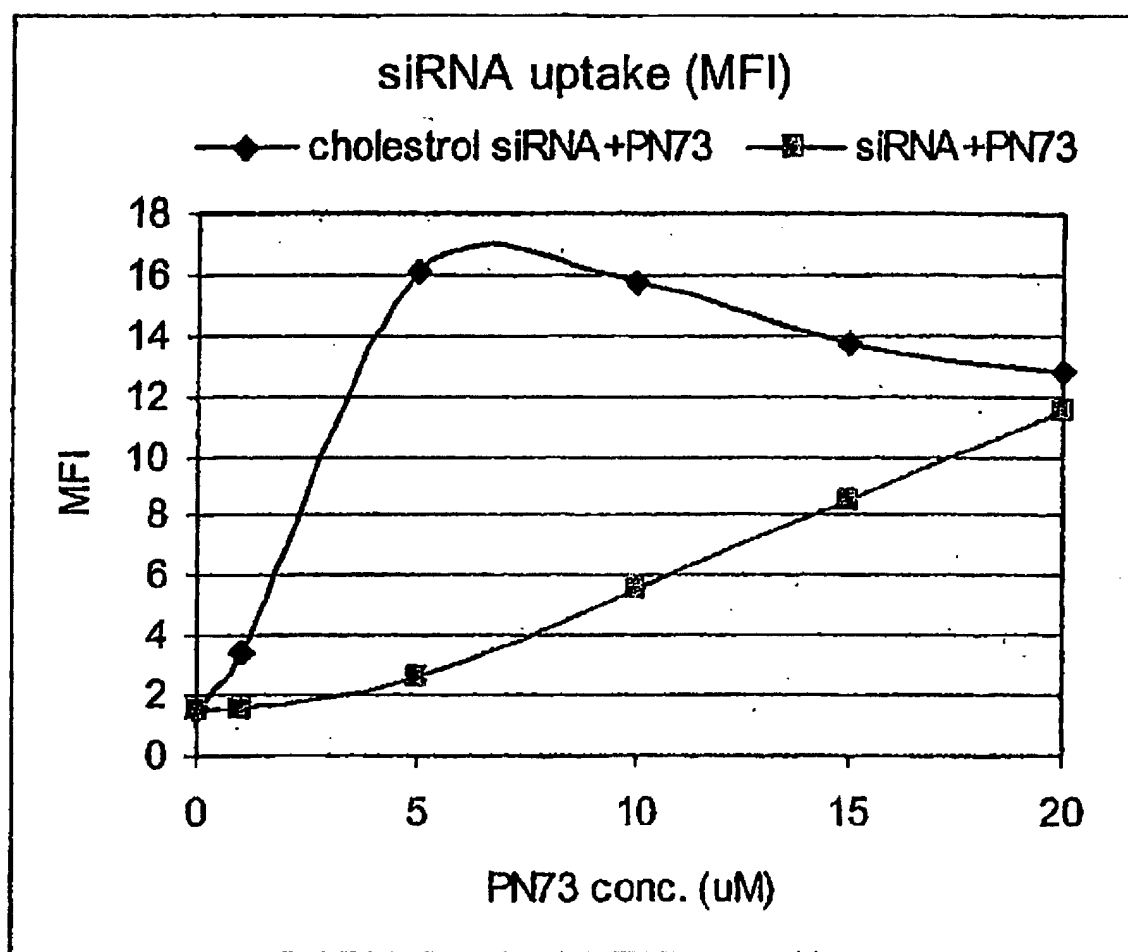


Figure 3

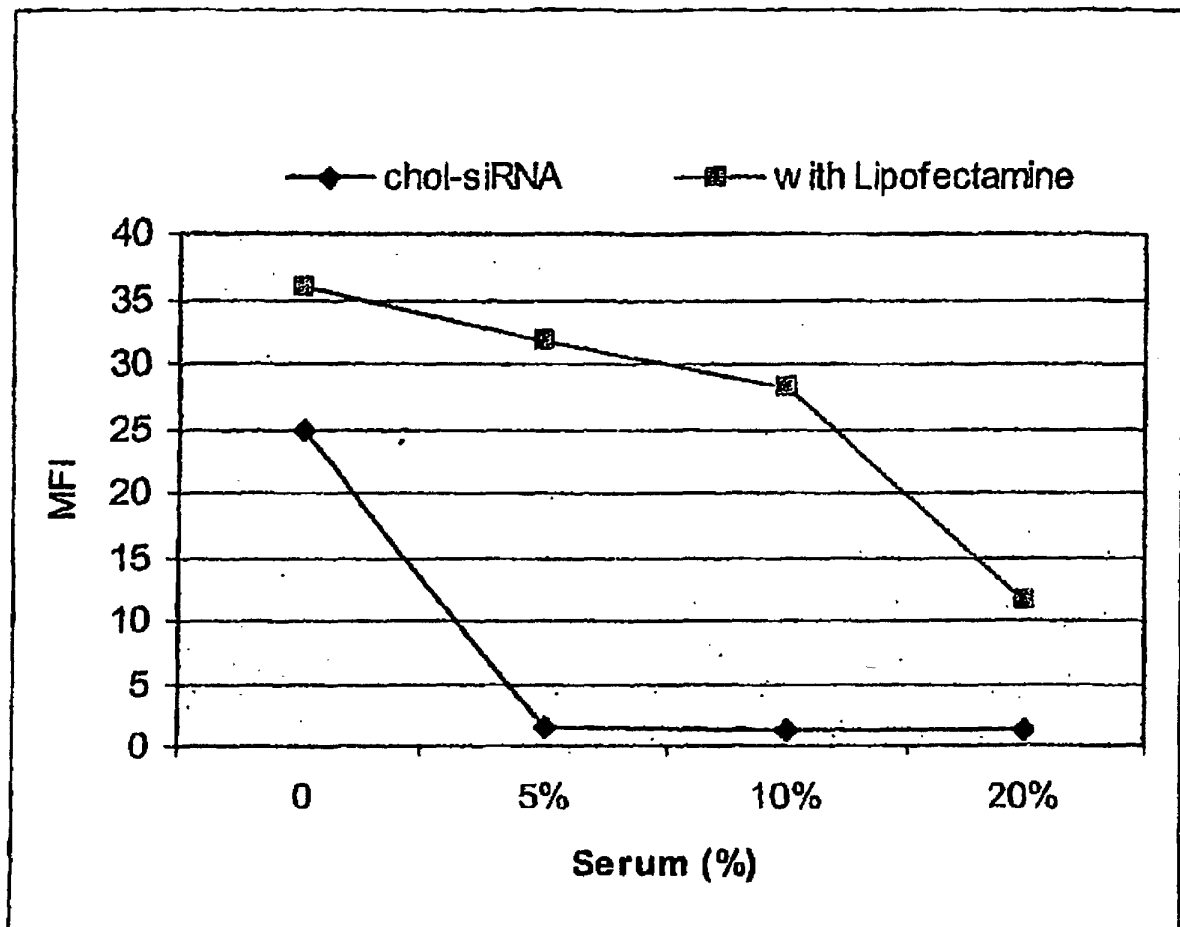
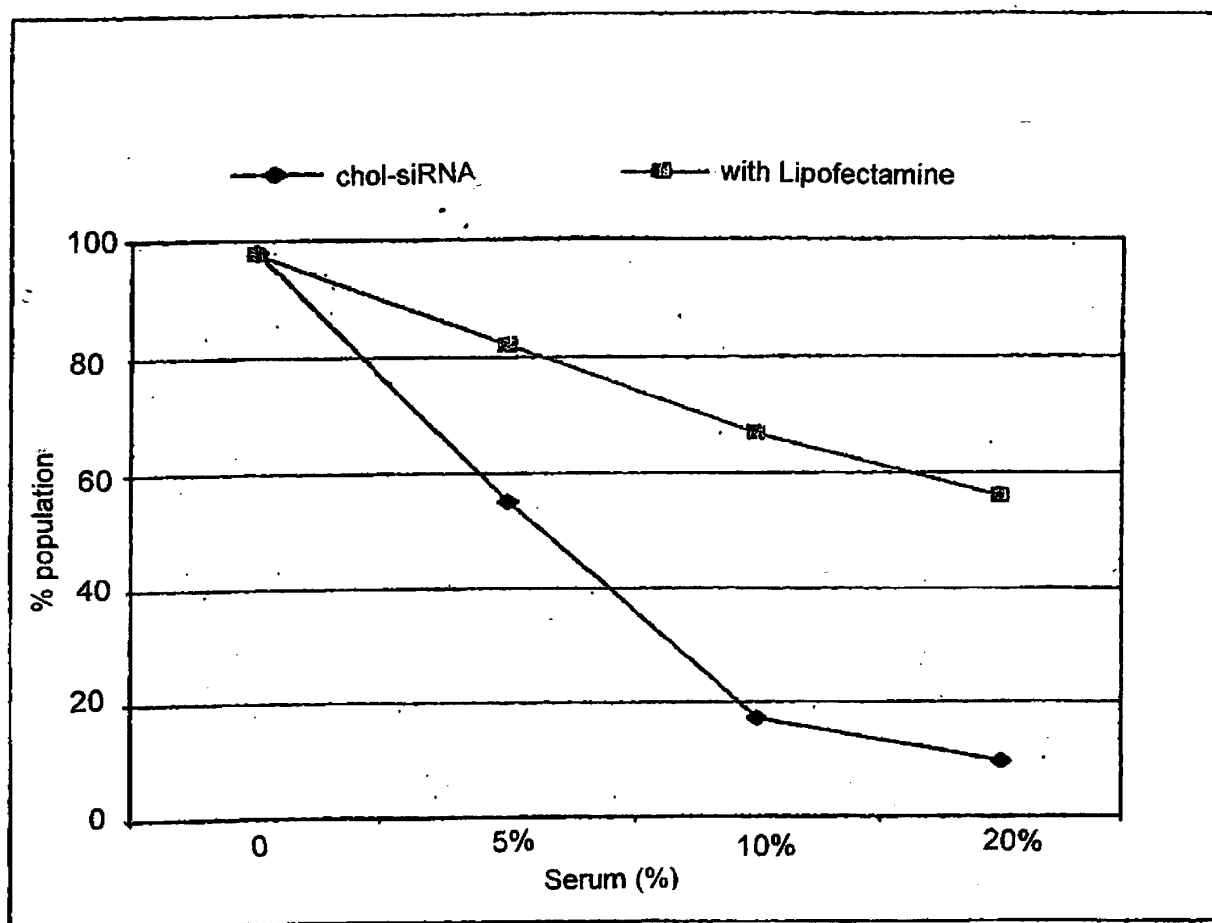


Figure 4



REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- WO 2004048545 A [0006]
- WO 2004011647 A [0006]
- WO 9932619 A [0015]
- US 6331524 B [0030]
- US 60612121 B [0038]
- US 60667835 B [0038]
- US 60612285 B [0038]
- US 60667871 B [0038]
- US 60613416 B [0038]
- US 60667833 B [0038]

Non-patent literature cited in the description

- **MORRIS et al.** *Nature Biotechnology*, 2001, vol. 19, 1173-1176 [0006]