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(71) Applicant *(for all designated States except US)*: **ALNYLAM PHARMACEUTICALS, INC.** [US/US]; 300 Third Street, Third Floor, Cambridge, MA 02142 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **BUMCROT, David** [US/US]; c/o Alnylam Pharmaceuticals, Inc., 300 Third Street, Third Floor, Cambridge, MA 02142 (US). **AKINC, Akin** [US/US]; c/o Alnylam Pharmaceuticals, Inc., 300 Third Street, Third Floor, Cambridge, MA 02142 (US). **SAH, Dinah** [US/US]; c/o Alnylam Pharmaceuticals, Inc., 300 Third Street, Third Floor, Cambridge, MA 02142 (US). **NOVOBRANTSEVA, Tatiana** [RU/US]; c/o Alnylam Pharmaceuticals, Inc., 300 Third Street, Third Floor, Cambridge, MA 02142 (US).

(74) Agents: **HUBL, Susan, T.** et al.; Fenwick & West LLP, Silicon Valley Center, 801 California Street, Mountain View, CA 94041 (US).

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(54) **Title**: LIPID FORMULATED COMPOSITIONS AND METHODS FOR INHIBITING EXPRESSION OF Eg5 AND VEGF GENES

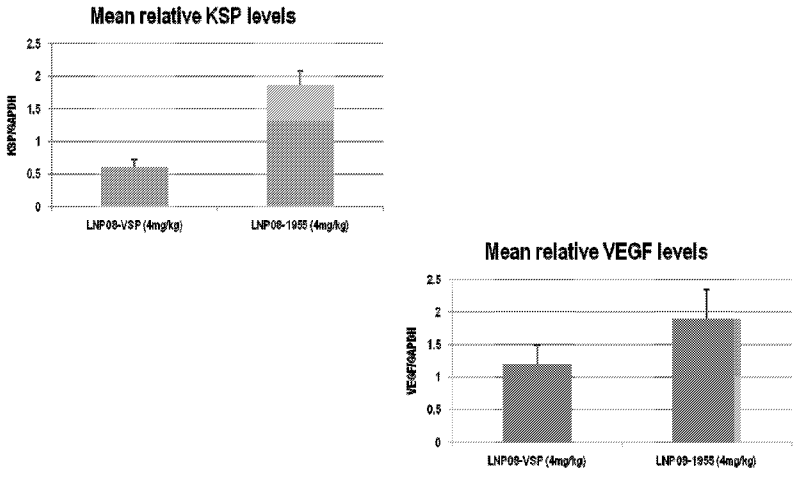

FIG. 18

(57) **Abstract**: This invention relates to compositions containing double-stranded ribonucleic acid (dsRNA) in a lipid formulation, and methods of using the compositions to inhibit the expression of the Human kinesin family member 11 (Eg5) and Vascular Endothelial Growth Factor (VEGF), and methods of using the compositions to treat pathological processes mediated by Eg5 and VEGF expression, such as cancer.

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LIPID FORMULATED COMPOSITIONS AND METHODS FOR INHIBITING EXPRESSION OF Eg5 AND VEGF GENES

Field of the Invention

This invention relates to lipid formulated compositions containing double-stranded ribonucleic acid (dsRNA), and their use in mediating RNA interference to inhibit the expression of a combination of genes, *e.g.*, the Eg5 and Vascular Endothelial Growth Factor (VEGF) genes. The dsRNA are formulated in a lipid formulation and can include a lipoprotein, e.g., apolipoprotein E. Also included in the invention is the use of the compositions to treat pathological processes mediated by Eg5 and VEGF expression, such as cancer.

Cross Reference to Related Applications

This application claims the benefit of U.S. Provisional Application Serial No. 61/159,788, filed March 12, 2009; U.S. Provisional Application Serial No. 61/231,579, filed August 5, 2009, and U. S. Provisional Application Serial No. 61/285,947, filed December 11, 2009, all of which are incorporated herein by reference, in their entirety, for all purposes.

Reference to a Sequence Listing

This application includes a Sequence Listing submitted electronically as a text file named 16564US_sequencelisting.txt, created on Month, XX, 2010, with a size of XXX,XXX bytes. The sequence listing is incorporated by reference.

Background of the Invention

The maintenance of cell populations within an organism is governed by the cellular processes of cell division and programmed cell death. Within normal cells, the cellular events associated with the initiation and completion of each process is highly regulated. In proliferative disease such as cancer, one or both of these processes may be perturbed. For example, a cancer cell may have lost its regulation (checkpoint control) of the cell division cycle through either the overexpression of a positive regulator or the loss of a negative regulator, perhaps by mutation.

Alternatively, a cancer cell may have lost the ability to undergo programmed cell death through the overexpression of a negative regulator. Hence, there is a need to develop new chemotherapeutic drugs that will restore the processes of checkpoint control and programmed cell death to cancerous cells.

One approach to the treatment of human cancers is to target a protein that is essential for cell cycle progression. In order for the cell cycle to proceed from one phase to the next, certain prerequisite events must be completed. There are checkpoints within the cell cycle that enforce the proper order of events and phases. One such checkpoint is the spindle checkpoint that occurs during the metaphase stage of mitosis. Small molecules that target proteins with essential

functions in mitosis may initiate the spindle checkpoint to arrest cells in mitosis. Of the small molecules that arrest cells in mitosis, those which display anti-tumor activity in the clinic also induce apoptosis, the morphological changes associated with programmed cell death. An effective chemotherapeutic for the treatment of cancer may thus be one which induces checkpoint control and programmed cell death. Unfortunately, there are few compounds available for controlling these processes within the cell. Most compounds known to cause mitotic arrest and apoptosis act as tubulin binding agents. These compounds alter the dynamic instability of microtubules and indirectly alter the function/structure of the mitotic spindle thereby causing mitotic arrest. Because most of these compounds specifically target the tubulin protein which is a component of all microtubules, they may also affect one or more of the numerous normal cellular processes in which microtubules have a role. Hence, there is also a need for agents that more specifically target proteins associated with proliferating cells.

Eg5 is one of several kinesin-like motor proteins that are localized to the mitotic spindle and known to be required for formation and/or function of the bipolar mitotic spindle. Recently, there was a report of a small molecule that disturbs bipolarity of the mitotic spindle (Mayer, T. U. *et al.* 1999. Science 286(5441) 971-4, herein incorporated by reference). More specifically, the small molecule induced the formation of an aberrant mitotic spindle wherein a monoastral array of microtubules emanated from a central pair of centrosomes, with chromosomes attached to the distal ends of the microtubules. The small molecule was dubbed "monastrol" after the monoastral array. This monoastral array phenotype had been previously observed in mitotic cells that were immunodepleted of the *Eg5* motor protein. This distinctive monoastral array phenotype facilitated identification of monastrol as a potential inhibitor of *Eg5*. Indeed, monastrol was further shown to inhibit the *Eg5* motor-driven motility of microtubules in an *in vitro* assay. The *Eg5* inhibitor monastrol had no apparent effect upon the related kinesin motor or upon the motor(s) responsible for golgi apparatus movement within the cell. Cells that display the monoastral array phenotype either through immunodepletion of *Eg5* or monastrol inhibition of *Eg5* arrest in M-phase of the cell cycle. However, the mitotic arrest induced by either immunodepletion or inhibition of *Eg5* is transient (Kapoor, T. M., 2000. J Cell Biol 150(5) 975-80). Both the monoastral array phenotype and the cell cycle arrest in mitosis induced by monastrol are reversible. Cells recover to form a normal bipolar mitotic spindle, to complete mitosis and to proceed through the cell cycle and normal cell proliferation. These data suggest that an inhibitor of *Eg5* which induced a transient mitotic arrest may not be effective for the treatment of cancer cell proliferation. Nonetheless, the discovery that monastrol causes mitotic arrest is intriguing and hence there is a need to further study and identify compounds which can

be used to modulate the *Eg5* motor protein in a manner that would be effective in the treatment of human cancers. There is also a need to explore the use of these compounds in combination with other antineoplastic agents.

VEGF (vascular endothelial growth factor, also known as vascular permeability factor, VPF) is a multifunctional cytokine that stimulates angiogenesis, epithelial cell proliferation, and endothelial cell survival. VEGF can be produced by a wide variety of tissues, and its overexpression or aberrant expression can result in a variety disorders, including cancers and retinal disorders, such as age-related macular degeneration and other angiogenic disorders.

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

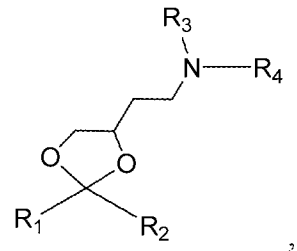
Summary of the Invention

The invention provides compositions and methods for inhibiting the expression of human Eg5/KSP and VEGF genes in a cell using lipid formulated compositions containing dsRNA.

Compositions of the invention include a nucleic acid lipid particle having a first double-stranded ribonucleic acid (dsRNA) for inhibiting the expression of a human kinesin family member 11 (Eg5/KSP) gene in a cell and a second dsRNA for inhibiting expression of a human VEGF in a cell. The nucleic acid lipid particle has a lipid formulation having 45-65 mol % of a cationic lipid, 5 mol % to about 10 mol %, of a non-cationic lipid, 25-40 mol % of a sterol, and 0.5-5 mol % of a PEG or PEG-modified lipid. The first dsRNA targeting Eg5/KSP includes a first sense strand and a first antisense strand, and the first sense strand having a first sequence and the first antisense strand has a second sequence complementary to at least 15 contiguous nucleotides of SEQ ID NO:1311 (5'-UCGAGAAUCUAAACUAACU-3'), wherein the first sequence is complementary to the second sequence and wherein the first dsRNA is between 15 and 30 base pairs in length. The second dsRNA includes a second sense strand and a second antisense strand, the second sense strand having a third sequence and the second antisense strand having a fourth sequence complementary to at least 15 contiguous nucleotides of SEQ ID

NO:1538 (5'-GCACAUAGGAGAGAUGAGCUU-3'), wherein the third sequence is complementary to the fourth sequence and wherein the second dsRNA is between 15 and 30 base pairs in length.

In one embodiment, the cationic lipid of the composition has formula A, wherein formula A is



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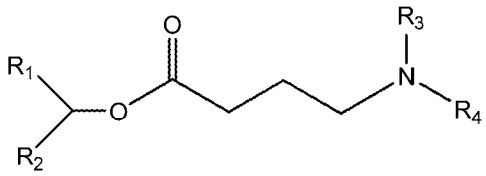

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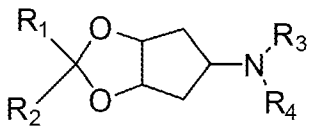


where R1 and R2 are independently alkyl, alkenyl or alkynyl, each can be optionally substituted, and R3 and R4 are independently lower alkyl or R3 and R4 can be taken together to form an optionally substituted heterocyclic ring.

In other embodiments, the cationic lipid is XTC (2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane). In a related embodiment, the cationic lipid is XTC, the non-cationic lipid is DSPC, the sterol is cholesterol and the PEG lipid has PEG-DMG. In a yet related embodiment, the cationic lipid is XTC and the formulation is selected from the group consisting of:

LNP05	XTC/DSPC/Cholesterol/PEG-DMG 57.5/7.5/31.5/3.5 lipid:siRNA ~ 6:1
LNP06	XTC/DSPC/Cholesterol/PEG-DMG 57.5/7.5/31.5/3.5 lipid:siRNA ~ 11:1
LNP07	XTC/DSPC/Cholesterol/PEG-DMG 60/7.5/31/1.5, lipid:siRNA ~ 6:1
LNP08	XTC/DSPC/Cholesterol/PEG-DMG 60/7.5/31/1.5, lipid:siRNA ~ 11:1

LNP09	XTC/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 lipid:siRNA ~ 10:1
LNP13	XTC/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 lipid:siRNA ~ 33:1
LNP22	XTC/DSPC/Cholesterol/PEG-DSG 50/10/38.5/1.5 lipid:siRNA ~10

In another embodiment, the cationic lipid of the composition is ALNY-100 ((3aR,5s,6aS)-N,N-dimethyl-2,2-di((9Z,12Z)-octadeca-9,12-dienyl)tetrahydro-3aH-cyclopenta[d][1,3]dioxol-5-amine)). In other embodiments, the cationic lipid is ALNY-100 and the formulation includes:

LNP10	ALNY-100/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 lipid:siRNA ~ 10:1

In other embodiments, the cationic lipid is MC3 (((6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate). In a related embodiment, the cationic lipid 9s MC3 and the lipid formulation is selected from the group consisting of:

LNP11	MC3/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 lipid:siRNA ~ 10:1
LNP14	MC3/DSPC/Cholesterol/PEG-DMG 40/15/40/5 lipid:siRNA ~11
LNP15	MC3/DSPC/Cholesterol/PEG-DSG/GalNAc-PEG-DSG 50/10/35/4.5/0.5 lipid:siRNA ~11
LNP16	MC3/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 lipid:siRNA ~7
LNP17	MC3/DSPC/Cholesterol/PEG-DSG 50/10/38.5/1.5 lipid:siRNA ~10
LNP18	MC3/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 lipid:siRNA ~12
LNP19	MC3/DSPC/Cholesterol/PEG-DMG 50/10/35/5 lipid:siRNA ~8
LNP20	MC3/DSPC/Cholesterol/PEG-DPG 50/10/38.5/1.5 lipid:siRNA ~10

In another embodiment, the first dsRNA includes a sense strand consisting of SEQ ID NO:1534 (5'-UCGAGAAUCUAAACUAACUTT-3') and an antisense strand consisting of SEQ ID NO:1535 (5'-AGUUAGUUUAGAUUCCUGATT-3') and the second dsRNA includes a sense strand consisting of SEQ ID NO:1536 (5'-GCACAUAGGAGAGAUGAGCUU-3'), and an antisense strand consisting of SEQ ID NO:1537 (5'-AAGCUCAUCUCUCCUAUGUGCUG-3'). In yet another embodiment, each strand is modified as follows to include a 2'-O-methyl ribonucleotide as indicated by a lower case letter "c" or "u" and a phosphorothioate as indicated by a lower case letter "s": the first dsRNA includes a sense strand consisting of SEQ ID NO:1240 (5'-ucGAGAAucuAAAcuAAcuTsT-3') and an antisense strand consisting of SEQ ID NO:1241 (5'-AGUuAGUUuAGAUUCUCGATsT); the second dsRNA includes a sense strand consisting of SEQ ID NO:1242 (5'-GcAcAuAGGAGAGAuGAGCUsU-3') and an antisense strand consisting of SEQ ID NO:1243 (5'-AAGCUcAUCUCUCCuAuGuGCusG-3').

In other embodiments, the first and second dsRNA includes at least one modified nucleotide. In some embodiments, the modified nucleotide is chosen from the group of: a 2'-O-methyl modified nucleotide, a nucleotide having a 5'-phosphorothioate group, and a terminal nucleotide linked to a cholesteryl derivative or dodecanoic acid bisdecylamide group. In another embodiment, the modified nucleotide is chosen from the group of: a 2'-deoxy-2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an abasic nucleotide, 2'-amino-modified nucleotide, 2'-alkyl-modified nucleotide, morpholino nucleotide, a phosphoramidate, and a non-natural base having nucleotide. In yet another embodiment, the first and second dsRNA each comprise at least one 2'-O-methyl modified ribonucleotide and at least one nucleotide having a 5'-phosphorothioate group.

In some embodiments, each dsRNA is 19-23 bases in length. In another embodiment, each strand of each dsRNA is 21-23 bases in length. In yet another embodiment, each strand of the first dsRNA is 21 bases in length, the sense strand of the second dsRNA is 21 bases in length and the antisense strand of the second dsRNA is 23 bases in length. In other embodiments, the first and second dsRNA are present in an equimolar ratio. In one embodiment, the composition further has Sorafenib. In another embodiment, the composition further has a lipoprotein. In another embodiment, the composition further has apolipoprotein E (ApoE).

In another embodiment, the composition, upon contact with a cell expressing Eg5, inhibits expression of Eg5 by at least 40%. In yet another embodiment, the composition, upon contact with a cell expressing VEGF, inhibits expression of VEGF by at least 40%. In other embodiments, the administration of the composition to a cell decreases expression of Eg5 and

VEGF in the cell. In a related embodiment, the composition is administered in a nM concentration. In a yet related embodiment, the administration of the composition to a cell increases monoaster formation in the cell.

In other embodiments, the administration of the composition to a mammal results in at least one effect selected from the group consisting of prevention of tumor growth, reduction in tumor growth, or prolonged survival in the mammal. In some embodiments, the effect is measured using at least one assay selected from the group consisting of determination of body weight, determination of organ weight, visual inspection, mRNA analysis, serum AFP analysis and survival monitoring.

The invention also provides methods for inhibiting the expression of Eg5/KSP and VEGF in a cell. The methods includes the steps ofadministering the composition of the invention to a cell. The invention also provides methods for preventing tumor growth, reducing tumor growth, or prolonging survival in a mammal in need of treatment for cancer. The methods include the step of administering the composition of the inventionto the mammal. In one embodiment, the mammal has liver cancer. In another embodiment, the mammal is a human with liver cancer. In some embodiments, a dose containing between 0.25 mg/kg and 4 mg/kg dsRNA is administered to the mammal. In other embodiments, the dsRNA is administered to a human at about 0.01, 0.1, 0.5, 1.0, 2.5, or 5.0 mg/kg.

In yet another embodiment, the invention provides methods for reducing tumor growth in a mammal in need of treatment for cancer. The methods include administering the composition of the invention to the mammal, the method reducing tumor growth by at least 20%. In another embodiment, the method reduces KSP expression by at least 60%.

Brief Description of the Figures

FIG. 1 is a graph showing liver weights as a percentage of body weight following administration of SNALP-siRNAs in a Hep3B mouse model.

FIG. 2A is a graph showing the effect of PBS on body weight in a Hep3B mouse model.

FIG. 2B is a graph showing the effect of a SNALP-siRNA (VEGF/KSP) on body weight in a Hep3B mouse model.

FIG. 2C is a graph showing the effect of a SNALP-siRNA (KSP/Luciferase) on body weight in a Hep3B mouse model.

FIG. 2D is a graph showing the effect of SNALP-siRNA (VEGF/Luciferase) on body weight in a Hep3B mouse model.

FIG. 3 is a graph showing the effects of SNALP-siRNAs on body weight in a Hep3B mouse model.

FIG. 4 is a graph showing the body weight in untreated control animals.

FIG. 5 is a graph showing the effects of control luciferase-SNALP siRNAs on body weight in a Hep3B mouse model.

FIG. 6 is a graph showing the effects of VSP-SNALP siRNAs on body weight in a Hep3B mouse model.

FIG. 7A is a graph showing the effects of SNALP-siRNAs on human GAPDH levels normalized to mouse GAPDH levels in a Hep3B mouse model.

FIG. 7B is a graph showing the effects of SNALP-siRNAs on serum AFP levels as measured by serum ELISA in a Hep3B mouse model.

FIG. 8 is a graph showing the effects of SNALP-siRNAs on human GAPDH levels normalized to mouse GAPDH levels in a Hep3B mouse model.

FIG. 9 is a graph showing the effects of SNALP-siRNAs on human KSP levels normalized to human GAPDH levels in a Hep3B mouse model.

FIG. 10 is a graph showing the effects of SNALP-siRNAs on human VEGF levels normalized to human GAPDH levels in a Hep3B mouse model.

FIG. 11A is a graph showing the effects of SNALP-siRNAs on mouse VEGF levels normalized to human GAPDH levels in a Hep3B mouse model.

FIG. 11B is a set of graphs showing the effects of SNALP-siRNAs on human GAPDH levels and serum AFP levels in a Hep3B mouse model.

FIG. 12A is a graph showing the effect of PBS, Luciferase, and ALN-VSP on tumor KSP measured by percentage of relative hKSP mRNA in a Hep3B mouse model.

FIG. 12B is a graph showing the effect of PBS, Luciferase, and SNALP-VSP on tumor VEGF measured by percentage of relative hVEGF mRNA in a Hep3B mouse model.

FIG. 12C is a graph showing the effect of PBS, Luciferase, and SNALP-VSP on GAPDH levels measured by percentage of relative hGAPDH mRNA in a Hep3B mouse model.

FIG. 13A is a graph showing the effect of SNALP si-RNAs on survival in mice with hepatic tumors. Treatment was started at 18 days after tumor cell seeding.

FIG. 13B is a graph showing the effect of SNALP-siRNAs on survival in mice with hepatic tumors. Treatment was started at 26 days after tumor cell seeding.

FIG. 14 is a graph showing the effects of SNALP-siRNAs on serum alpha fetoprotein (AFP) levels.

FIG. 15A is an image of H&E stained sections in tumor bearing animals (three weeks after Hep3B cell implantation) that were administered 2 mg/kg SNALP-VSP. Twenty four hours

later, tumor bearing liver lobes were processed for histological analysis. Arrows indicate mono asters.

FIG. 15B is an image of H&E stained sections in tumor bearing animals (three weeks after Hep3B cell implantation) that were administered 2 mg/kg SNALP-Luc. Twenty four hours later, tumor bearing liver lobes were processed for histological analysis.

FIG. 16 is a graph illustrating the effects on survival of administration SNALP formulated siRNA and Sorafenib.

FIG. 17 is a flow chart of the in-line mixing method.

FIG. 18 are graphs illustrating the effects on KSP and VEGF expression in intrahepatic Hep3B tumors in mice following treatment with LNP-08 formulated VSP.

FIG. 19 illustrates the chemical structures of PEG-DSG and PEG-C-DSA.

FIG. 20 illustrates the structures of cationic lipids ALNY-100, MC3, and XTC.

FIG. 21 are graphs illustrating the effects on KSP and VEGF expression in intrahepatic Hep3B tumors in mice treated with SNALP-1955 (Luc), ALN-VSP02, and SNALP-T-VSP LNP11 and LNP-12 formulated VSP.

FIG. 22 is a set of graphs comparing the effects on KSP and VEGF expression in intrahepatic Hep3B tumors in mice treated with LNP08-Luc, ALN-VSP02, and LNP-08 and LNP08-C18 formulated VSP.

Detailed Description of the Invention

The invention provides compositions and methods for inhibiting the expression of the Eg5 gene and VEGF gene in a cell or mammal using the dsRNAs. The dsRNAs are packaged in a lipid nucleic acid particle. The invention also provides compositions and methods for treating pathological conditions and diseases, such as liver cancer, in a mammal caused by the expression of the Eg5 gene and VEGF genes. The dsRNA directs the sequence-specific degradation of mRNA through a process known as RNA interference (RNAi).

The following detailed description discloses how to make and use the compositions containing dsRNAs to inhibit the expression of the Eg5 gene and VEGF genes, respectively, as well as compositions and methods for treating diseases and disorders caused by the expression of these genes, such as cancer. The pharmaceutical compositions featured in the invention include a dsRNA having an antisense strand comprising a region of complementarity which is less than 30 nucleotides in length, generally 19-24 nucleotides in length, and is substantially complementary to at least part of an RNA transcript of the Eg5 gene, together with a pharmaceutically acceptable carrier. The compositions featured in the invention also include a dsRNA having an antisense strand having a region of complementarity which is less than 30

nucleotides in length, generally 19-24 nucleotides in length, and is substantially complementary to at least part of an RNA transcript of the VEGF gene.

Accordingly, certain aspects of the invention provide pharmaceutical compositions containing the Eg5 and VEGF dsRNAs and a pharmaceutically acceptable carrier, methods of using the compositions to inhibit expression of the Eg5 gene and the VEGF gene respectively, and methods of using the pharmaceutical compositions to treat diseases caused by expression of the Eg5 and VEGF genes.

I. Definitions

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

"G," "C," "A" and "U" each generally stand for a nucleotide that contains guanine, cytosine, adenine, and uracil as a base, respectively. "T" and "dT" are used interchangeably herein and refer to a deoxyribonucleotide wherein the nucleobase is thymine, e.g., deoxyribothymine. However, it will be understood that the term "ribonucleotide" or "nucleotide" can also refer to a modified nucleotide, as further detailed below, or a surrogate replacement moiety. The skilled person is well aware that guanine, cytosine, adenine, and uracil may be replaced by other moieties without substantially altering the base pairing properties of an oligonucleotide comprising a nucleotide bearing such replacement moiety. For example, without limitation, a nucleotide comprising inosine as its base may base pair with nucleotides containing adenine, cytosine, or uracil. Hence, nucleotides containing uracil, guanine, or adenine may be replaced in the nucleotide sequences of the invention by a nucleotide containing, for example, inosine. In another example, adenine and cytosine anywhere in the oligonucleotide can be replaced with guanine and uracil, respectively to form G-U Wobble base pairing with the target mRNA. Sequences comprising such replacement moieties are embodiments of the invention.

As used herein, "Eg5" refers to the human kinesin family member 11, which is also known as KIF11, Eg5, HKSP, KSP, KNSL1 or TRIP5. Eg5 sequence can be found as NCBI GeneID:3832, HGNC ID: HGNC:6388 and RefSeq ID number:NM_004523. The terms "Eg5" and "KSP" and "Eg5/KSP" are used interchangeably

As used herein, "VEGF," also known as vascular permeability factor, is an angiogenic growth factor. VEGF is a homodimeric 45 kDa glycoprotein that exists in at least three different isoforms. VEGF isoforms are expressed in endothelial cells. The VEGF gene contains 8 exons that express a 189-amino acid protein isoform. A 165-amino acid isoform lacks the residues

encoded by exon 6, whereas a 121-amino acid isoform lacks the residues encoded by exons 6 and 7. VEGF145 is an isoform predicted to contain 145 amino acids and to lack exon 7. VEGF can act on endothelial cells by binding to an endothelial tyrosine kinase receptor, such as Flt-1 (VEGFR-1) or KDR/flk-1 (VEGFR-2). VEGFR-2 is expressed in endothelial cells and is involved in endothelial cell differentiation and vasculogenesis. A third receptor, VEGFR-3, has been implicated in lymphogenesis.

The various isoforms have different biologic activities and clinical implications. For example, VEGF145 induces angiogenesis and like VEGF189 (but unlike VEGF165), VEGF145 binds efficiently to the extracellular matrix by a mechanism that is not dependent on extracellular matrix-associated heparin sulfates. VEGF displays activity as an endothelial cell mitogen and chemoattractant *in vitro* and induces vascular permeability and angiogenesis *in vivo*. VEGF is secreted by a wide variety of cancer cell types and promotes the growth of tumors by inducing the development of tumor-associated vasculature. Inhibition of VEGF function has been shown to limit both the growth of primary experimental tumors as well as the incidence of metastases in immunocompromised mice. Various dsRNAs directed to VEGF are described in co-pending US Ser. No. 11/078,073 and 11/340,080, which are hereby incorporated by reference in their entirety.

As used herein, "target sequence" refers to a contiguous portion of the nucleotide sequence of an mRNA molecule formed during the transcription of the Eg5/KSP and/or VEGF gene, including mRNA that is a product of RNA processing of a primary transcription product.

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

As used herein, and unless otherwise indicated, the term "complementary," when used to describe a first nucleotide sequence in relation to a second nucleotide sequence, refers to the ability of an oligonucleotide or polynucleotide comprising the first nucleotide sequence to hybridize and form a duplex structure under certain conditions with an oligonucleotide or polynucleotide comprising the second nucleotide sequence, as will be understood by the skilled person. Such conditions can, for example, be stringent conditions, where stringent conditions may include: 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50°C or 70°C for 12-16 hours followed by washing. Other conditions, such as physiologically relevant conditions as may be encountered inside an organism, can apply. The skilled person will be able to determine the set of conditions most appropriate for a test of complementarity of two sequences in accordance with the ultimate application of the hybridized nucleotides.

The term "complementary" includes base-pairing of the oligonucleotide or polynucleotide comprising the first nucleotide sequence to the oligonucleotide or polynucleotide comprising the second nucleotide sequence over the entire length of the first and second nucleotide sequence. Such sequences can be referred to as "fully complementary" with respect to each other herein. However, where a first sequence is referred to as "substantially complementary" with respect to a second sequence herein, the two sequences can be fully complementary, or they may form one or more, but generally not more than 4, 3 or 2 mismatched base pairs upon hybridization, while retaining the ability to hybridize under the conditions most relevant to their ultimate application. However, where two oligonucleotides are designed to form, upon hybridization, one or more single stranded overhangs, such overhangs shall not be regarded as mismatches with regard to the determination of complementarity. For example, a dsRNA comprising one oligonucleotide 21 nucleotides in length and another oligonucleotide 23 nucleotides in length, wherein the longer oligonucleotide comprises a sequence of 21 nucleotides that is fully complementary to the shorter oligonucleotide, may yet be referred to as "fully complementary" for the purposes of the invention.

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

The terms "complementary," "fully complementary" and "substantially complementary" herein may be used with respect to the base matching between the sense strand and the antisense strand of a dsRNA, or between the antisense strand of a dsRNA and a target sequence, as will be understood from the context of their use.

As used herein, a polynucleotide which is "substantially complementary to at least part of" a messenger RNA (mRNA) refers to a polynucleotide which is substantially complementary to a contiguous portion of the mRNA of interest (*e.g.*, encoding Eg5/KSP and/or VEGF) including a 5' untranslated region (UTR), an open reading frame (ORF), or a 3' UTR. For example, a polynucleotide is complementary to at least a part of a Eg5 mRNA if the sequence is substantially complementary to a non-interrupted portion of a mRNA encoding Eg5.

The term "double-stranded RNA" or "dsRNA", as used herein, refers to a duplex structure comprising two anti-parallel and substantially complementary, as defined above, nucleic acid strands. In general, the majority of nucleotides of each strand are ribonucleotides, but as described in detail herein, each or both strands can also include at least one non-

ribonucleotide, e.g., a deoxyribonucleotide and/or a modified nucleotide. In addition, as used in this specification, "dsRNA" may include chemical modifications to ribonucleotides, including substantial modifications at multiple nucleotides and including all types of modifications disclosed herein or known in the art. Any such modifications, as used in an siRNA type molecule, are encompassed by "dsRNA" for the purposes of this specification and claims.

The two strands forming the duplex structure may be different portions of one larger RNA molecule, or they may be separate RNA molecules. Where the two strands are part of one larger molecule, and therefore are connected by an uninterrupted chain of nucleotides between the 3' end of one strand and the 5' end of the respective other strand forming the duplex structure, the connecting RNA chain is referred to as a "hairpin loop". Where the two strands are connected covalently by means other than an uninterrupted chain of nucleotides between the 3' end of one strand and the 5'end of the respective other strand forming the duplex structure, the connecting structure is referred to as a "linker." The RNA strands may have the same or a different number of nucleotides. The maximum number of base pairs is the number of nucleotides in the shortest strand of the dsRNA minus any overhangs that are present in the duplex. In addition to the duplex structure, a dsRNA may comprise one or more nucleotide overhangs. In general, the majority of nucleotides of each strand are ribonucleotides, but as described in detail herein, each or both strands can also include at least one non-ribonucleotide, e.g., a deoxyribonucleotide and/or a modified nucleotide. In addition, as used in this specification, "dsRNA" may include chemical modifications to ribonucleotides, including substantial modifications at multiple nucleotides and including all types of modifications disclosed herein or known in the art. Any such modifications, as used in an siRNA type molecule, are encompassed by "dsRNA" for the purposes of this specification and claims.

As used herein, a "nucleotide overhang" refers to the unpaired nucleotide or nucleotides that protrude from the duplex structure of a dsRNA when a 3' end of one strand of the dsRNA extends beyond the 5' end of the other strand, or vice versa. "Blunt" or "blunt end" means that there are no unpaired nucleotides at that end of the dsRNA, *i.e.*, no nucleotide overhang. A "blunt ended" dsRNA is a dsRNA that is double-stranded over its entire length, *i.e.*, no nucleotide overhang at either end of the molecule. In some embodiments the dsRNA can have a nucleotide overhang at one end of the duplex and a blunt end at the other end.

The term "antisense strand" refers to the strand of a dsRNA which includes a region that is substantially complementary to a target sequence. As used herein, the term "region of complementarity" refers to the region on the antisense strand that is substantially complementary to a sequence, for example a target sequence, as defined herein. Where the region of

complementarity is not fully complementary to the target sequence, the mismatches may be in the internal or terminal regions of the molecule. Generally, the most tolerated mismatches are in the terminal regions, e.g., within 6, 5, 4, 3, or 2 nucleotides of the 5' and/or 3' terminus.

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

"Introducing into a cell," when referring to a dsRNA, means facilitating uptake or absorption into the cell, as is understood by those skilled in the art. Absorption or uptake of dsRNA can occur through unaided diffusive or active cellular processes, or by auxiliary agents or devices. The meaning of this term is not limited to cells *in vitro*. A dsRNA may also be "introduced into a cell", wherein the cell is part of a living organism. In such instance, introduction into the cell will include the delivery to the organism. For example, for *in vivo* delivery, dsRNA can be injected into a tissue site or administered systemically. *In vitro* introduction into a cell includes methods known in the art such as electroporation and lipofection.

The terms "silence" and "inhibit the expression of" "down-regulate the expression of," "suppress the expression of" and the like, in as far as they refer to the Eg5 and/or VEGF gene, herein refer to the at least partial suppression of the expression of the Eg5 gene, as manifested by a reduction of the amount of Eg5 mRNA and/or VEGF mRNA which may be isolated from a first cell or group of cells in which the Eg5 and/or VEGF gene is transcribed and which has or have been treated such that the expression of the Eg5 and/or VEGF gene is inhibited, as compared to a second cell or group of cells substantially identical to the first cell or group of cells but which has or have not been so treated (control cells). The degree of inhibition is usually expressed in terms of

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

Alternatively, the degree of inhibition may be given in terms of a reduction of a parameter that is functionally linked to Eg5 and/or VEGF gene expression, *e.g.* the amount of protein encoded by the Eg5 and/or VEGF gene which is produced by a cell, or the number of cells displaying a certain phenotype, *e.g.* apoptosis. In principle, target gene silencing can be determined in any cell expressing the target, either constitutively or by genomic engineering, and by any appropriate assay. However, when a reference is needed in order to determine whether a given dsRNA inhibits the expression of the Eg5 gene by a certain degree and therefore is encompassed by the instant invention, the assay provided in the Examples below shall serve as such reference.

For example, in certain instances, expression of the Eg5 gene (or VEGF gene) is suppressed by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50% by administration of the double-stranded oligonucleotide of the invention. In some embodiments, the Eg5 and/or VEGF gene is suppressed by at least about 60%, 70%, or 80% by administration of the double-stranded oligonucleotide of the invention. In other embodiments, the Eg5 and/or VEGF gene is suppressed by at least about 85%, 90%, or 95% by administration of the double-stranded oligonucleotide of the invention. The Tables and Example below provides values for inhibition of expression using various Eg5 and/or VEGF dsRNA molecules at various concentrations.

As used herein in the context of Eg5 expression (or VEGF expression), the terms "treat," "treatment," and the like, refer to relief from or alleviation of pathological processes mediated by Eg5 and/or VEGF expression. In the context of the present invention, insofar as it relates to any of the other conditions recited herein below (other than pathological processes mediated by Eg5 and/or VEGF expression), the terms "treat," "treatment," and the like mean to relieve or alleviate at least one symptom associated with such condition, or to slow or reverse the progression of such condition, such as the slowing and progression of hepatic carcinoma.

As used herein, the phrases "therapeutically effective amount" and "prophylactically effective amount" refer to an amount that provides a therapeutic benefit in the treatment, prevention, or management of pathological processes mediated by Eg5 and/or VEGF expression or an overt symptom of pathological processes mediated by Eg5 and/or VEGF expression. The specific amount that is therapeutically effective can be readily determined by ordinary medical practitioner, and may vary depending on factors known in the art, such as, *e.g.*, the type of pathological processes mediated by Eg5 and/or VEGF expression, the patient's history and age, the stage of pathological processes mediated by Eg5 and/or VEGF expression, and the administration of other anti-pathological processes mediated by Eg5 and/or VEGF expression agents.

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

The term "pharmaceutically acceptable carrier" refers to a carrier for administration of a therapeutic agent. As described in more detail below, such carriers include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The term specifically excludes cell culture medium. For drugs administered orally, pharmaceutically acceptable carriers include, but are not limited to, pharmaceutically acceptable excipients, such as inert diluents, disintegrating agents, binding agents, lubricating agents, sweetening agents, flavoring agents, coloring agents and preservatives. Suitable inert diluents include sodium and calcium carbonate, sodium and calcium phosphate, and lactose, while corn starch and alginic acid are suitable disintegrating agents. Binding agents may include starch and gelatin, while the lubricating agent, if present, will generally be magnesium stearate, stearic acid or talc. If desired, the tablets may be coated with a material such as glyceryl monostearate or glyceryl distearate, to delay absorption in the gastrointestinal tract.

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

II. Double-stranded ribonucleic acid (dsRNA)

As described in more detail herein, the invention provides double-stranded ribonucleic acid (dsRNA) molecules for inhibiting the expression of the Eg5 and/or VEGF gene in a cell or mammal, wherein the dsRNA comprises an antisense strand comprising a region of complementarity which is complementary to at least a part of an mRNA formed in the expression of the Eg5 and/or VEGF gene, and wherein the region of complementarity is less than 30 nucleotides in length, generally 19-24 nucleotides in length, and wherein said dsRNA, upon contact with a cell expressing said Eg5 and/or VEGF gene, inhibits the expression of said Eg5 and/or VEGF gene. The dsRNA of the invention can further include one or more single-stranded nucleotide overhangs.

The dsRNA can be synthesized by standard methods known in the art as further discussed below, *e.g.*, by use of an automated DNA synthesizer, such as are commercially available from, for example, Biosearch, Applied Biosystems, Inc. The dsRNA comprises two strands that are sufficiently complementary to hybridize to form a duplex structure. One strand of the dsRNA (the antisense strand) comprises a region of complementarity that is substantially complementary, and generally fully complementary, to a target sequence, derived from the sequence of an mRNA formed during the expression of the Eg5 and/or VEGF gene, the other strand (the sense strand) comprises a region which is complementary to the antisense strand, such that the two strands hybridize and form a duplex structure when combined under suitable conditions. Generally, the duplex structure is between 15 and 30, or between 25 and 30, or

between 18 and 25, or between 19 and 24, or between 19 and 21, or 19, 20, or 21 base pairs in length. In one embodiment the duplex is 19 base pairs in length. In another embodiment the duplex is 21 base pairs in length. When two different siRNAs are used in combination, the duplex lengths can be identical or can differ.

Each strand of the dsRNA of invention is generally between 15 and 30, or between 18 and 25, or 18, 19, 20, 21, 22, 23, or 24 nucleotides in length. In other embodiments, each is strand is 25-30 base pairs in length. Each strand of the duplex can be the same length or of different lengths. When two different siRNAs are used in combination, the lengths of each strand of each siRNA can be identical or can differ. For example, a composition can include a dsRNA targeted to Eg5 with a sense strand of 21 nucleotides and an antisense strand of 21 nucleotides, and a second dsRNA targeted to VEGF with a sense strand of 21 nucleotides and an antisense strand of 23 nucleotides.

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

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

As described in more detail herein, the composition of the invention includes a first dsRNA targeting Eg5 and a second dsRNA targeting VEGF. The first and second dsRNA can have the same overhang architecture, e.g., number of nucleotide overhangs on each strand, or each dsRNA can have a different architecture. In one embodiment, the first dsRNA targeting

Eg5 includes a 2 nucleotide overhang at the 3' end of each strand and the second dsRNA targeting VEGF includes a 2 nucleotide overhang on the 3' end of the antisense strand and a blunt end at the 5' end of the antisense strand (e.g., the 3' end of the sense strand).

In one embodiment, the Eg5 gene targeted by the dsRNA of the invention is the human Eg5 gene. In one embodiment, the antisense strand of the dsRNA targeting Eg5 comprises at least 15 contiguous nucleotides of one of the antisense sequences of Tables 1-3. In specific embodiments, the first sequence of the dsRNA is selected from one of the sense strands of Tables 1-3, and the second sequence is selected from the group consisting of the antisense sequences of Tables 1-3. Alternative antisense agents that target elsewhere in the target sequence provided in Tables 1-3 can readily be determined using the target sequence and the flanking Eg5 sequence. In some embodiments, the dsRNA targeted to Eg5 will comprise at least two nucleotide sequence selected from the groups of sequences provided in Tables 1-3. One of the two sequences is complementary to the other of the two sequences, with one of the sequences being substantially complementary to a sequence of an mRNA generated in the expression of the Eg5 gene. As such, the dsRNA will comprises two oligonucleotides, wherein one oligonucleotide is described as the sense strand in Tables 1-3, and the second oligonucleotide is described as the antisense strand in Tables 1-3.

In embodiments using a second dsRNA targeting VEGF, such agents are exemplified in the Examples, Tables 4a and 4b, and in co-pending US Serial Nos: 11/078,073 and 11/340,080, herein incorporated by reference. In one embodiment the dsRNA targeting VEGF has an antisense strand complementary to at least 15 contiguous nucleotides of the VEGF target sequences described in Table 4a. In other embodiments, the dsRNA targeting VEGF comprises one of the antisense sequences of Table 4b, or one of the sense sequences of Table 4b, or comprises one of the duplexes (sense and antisense strands) of Table 4b.

The skilled person is well aware that dsRNAs comprising a duplex structure of between 20 and 23, but specifically 21, base pairs have been hailed as particularly effective in inducing RNA interference (Elbashir *et al.*, EMBO 2001, 20:6877-6888). However, others have found that shorter or longer dsRNAs can be effective as well. In the embodiments described above, by virtue of the nature of the oligonucleotide sequences provided in Tables 1-3, the dsRNAs of the invention can comprise at least one strand of a length of minimally 21 nt. It can be reasonably expected that shorter dsRNAs comprising one of the sequences of Tables 1-3 minus only a few nucleotides on one or both ends may be similarly effective as compared to the dsRNAs described above. Hence, dsRNAs comprising a partial sequence of at least 15, 16, 17, 18, 19, 20, or more contiguous nucleotides from one of the sequences of Tables 1-3, and differing in their ability to

inhibit the expression of the Eg5 gene in a FACS assay as described herein below by not more than 5, 10, 15, 20, 25, or 30 % inhibition from a dsRNA comprising the full sequence, are contemplated by the invention. Further dsRNAs that cleave within the target sequence provided in Tables 1-3 can readily be made using the Eg5 sequence and the target sequence provided. Additional dsRNA targeting VEGF can be designed in a similar matter using the sequences disclosed in Tables 4a and 4b, the Examples and co-pending US Serial Nos: 11/078,073 and 11/340,080, herein incorporated by reference.

In addition, the RNAi agents provided in Tables 1-3 identify a site in the Eg5 mRNA that is susceptible to RNAi based cleavage. As such the present invention further includes RNAi agents, e.g., dsRNA, that target within the sequence targeted by one of the agents of the present invention. As used herein a second RNAi agent is said to target within the sequence of a first RNAi agent if the second RNAi agent cleaves the message anywhere within the mRNA that is complementary to the antisense strand of the first RNAi agent. Such a second agent will generally consist of at least 15 contiguous nucleotides from one of the sequences provided in Tables 1-3 coupled to additional nucleotide sequences taken from the region contiguous to the selected sequence in the Eg5 gene. For example, the last 15 nucleotides of SEQ ID NO:1 combined with the next 6 nucleotides from the target Eg5 gene produces a single strand agent of 21 nucleotides that is based on one of the sequences provided in Tables 1-3. Additional RNAi agents, e.g., dsRNA, targeting VEGF can be designed in a similar matter using the sequences disclosed in Tables 4a and 4b, the Examples and co-pending US Serial Nos: 11/078,073 and 11/340,080, herein incorporated by reference.

The dsRNA of the invention can contain one or more mismatches to the target sequence. In a preferred embodiment, the dsRNA of the invention contains no more than 3 mismatches. If the antisense strand of the dsRNA contains mismatches to a target sequence, it is preferable that the area of mismatch not be located in the center of the region of complementarity. If the antisense strand of the dsRNA contains mismatches to the target sequence, it is preferable that the mismatch be restricted to 5 nucleotides from either end, for example 5, 4, 3, 2, or 1 nucleotide from either the 5' or 3' end of the region of complementarity. For example, for a 23 nucleotide dsRNA strand which is complementary to a region of the Eg5 gene, the dsRNA generally does not contain any mismatch within the central 13 nucleotides. The methods described within the invention can be used to determine whether a dsRNA containing a mismatch to a target sequence is effective in inhibiting the expression of the Eg5 gene. Consideration of the efficacy of dsRNAs with mismatches in inhibiting expression of the Eg5

gene is important, especially if the particular region of complementarity in the Eg5 gene is known to have polymorphic sequence variation within the population.

Modifications

In yet another embodiment, the dsRNA is chemically modified to enhance stability. The nucleic acids of the invention may be synthesized and/or modified by methods well established in the art, such as those described in "Current protocols in nucleic acid chemistry," Beaucage, S.L. *et al.* (Edrs.), John Wiley & Sons, Inc., New York, NY, USA, which is hereby incorporated herein by reference. Specific examples of preferred dsRNA compounds useful in this invention include dsRNAs containing modified backbones or no natural internucleoside linkages. As defined in this specification, dsRNAs having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified dsRNAs that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified dsRNA backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

Representative U.S. patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S. Pat. Nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,195; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,316; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050, each of which is herein incorporated by reference

Preferred modified dsRNA backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatoms and alkyl or cycloalkyl internucleoside linkages, or ore or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide,

sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH2 component parts.

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

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

Most preferred embodiments of the invention are dsRNAs with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular --CH_2--NH--CH_2--, --CH_2--N(CH_3)--O--CH_2--[known as a methylene (methylimino) or MMI backbone], --CH_2--O--N(CH_3)--CH_2--, --CH_2--N(CH_3)--N(CH_3)--CH_2-- and --N(CH_3)--CH_2--CH_2--[wherein the native phosphodiester backbone is represented as --O--P--O--CH_2--] of the above-referenced U.S. Pat. No. 5,489,677, and the amide backbones of the above-referenced U.S. Pat. No. 5,602,240. Also preferred are dsRNAs having morpholino backbone structures of the above-referenced U.S. Pat. No. 5,034,506.

Modified dsRNAs may also contain one or more substituted sugar moieties. Preferred dsRNAs comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Particularly preferred are O[$(CH_2)_nO]_mCH_3$, O$(CH_2)_nOCH_3$, O$(CH_2)_nNH_2$, O$(CH_2)_nCH_3$, O$(CH_2)_nONH_2$, and

$O(CH_2)_nON[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about 10. Other preferred dsRNAs comprise one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an dsRNA, or a group for improving the pharmacodynamic properties of an dsRNA, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O--$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin *et al.*, Helv. Chim. Acta, 1995, 78, 486-504) *i.e.*, an alkoxy-alkoxy group. A further preferred modification includes 2'-dimethylaminooxyethoxy, *i.e.*, a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in examples herein below, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), *i.e.*, 2'-O--CH_2--O--CH_2--$N(CH_2)_2$, also described in examples herein below.

Other preferred modifications include 2'-methoxy (2'-OCH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the dsRNA, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked dsRNAs and the 5' position of 5' terminal nucleotide. dsRNAs may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative U.S. patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Pat. Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

dsRNAs may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl anal other 8-

substituted adenines and guanines, 5-halo, particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosine's, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-daazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in The Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859, Kroschwitz, J. L, ed. John Wiley & Sons, 1990, those disclosed by Englisch *et al.*, Angewandte Chemie, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y S., Chapter 15, DsRNA Research and Applications, pages 289-302, Crooke, S. T. and Lebleu, B., Ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and 0-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2 degrees Celcius. (Sanghvi, Y. S., Crooke, S. T. and Lebleu, B., Eds., DsRNA Research and Applications, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative U.S. patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. Pat. No. 3,687,808, as well as U.S. Pat. Nos. 4,845,205; 5,130,30; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; and 5,681,941, each of which is herein incorporated by reference, and U.S. Pat. No. 5,750,692, also herein incorporated by reference.

Conjugates

Another modification of the dsRNAs of the invention involves chemically linking to the dsRNA one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the dsRNA. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger *et al.*, Proc. Natl. Acid. Sci. USA, 199, 86, 6553-6556), cholic acid (Manoharan *et al.*, Biorg. Med. Chem. Let., 1994 4 1053-1060), a thioether, *e.g.*, beryl-S-tritylthiol (Manoharan *et al.*, Ann. N.Y. Acad. Sci., 1992, 660, 306-309; Manoharan *et al.*, Biorg. Med. Chem. Let., 1993, 3, 2765-2770), a thiocholesterol (Oberhauser *et al.*, Nucl. Acids Res., 1992, 20, 533-538), an aliphatic chain, *e.g.*, dodecandiol or undecyl residues (Saison-Behmoaras *et al.*, EMBO J, 1991, 10, 1111-1118; Kabanov *et al.*, FEBS Lett., 1990, 259, 327-330; Svinarchuk *et al.*, Biochimie, 1993, 75, 49-54), a phospholipid, *e.g.*, di-hexadecyl-rac-glycerol or triethyl-ammonium 1,2-di-O-hexadecyl-rac-glycero-3-Hphosphonate (Manoharan *et al.*,

Tetrahedron Lett., 1995, 36, 3651-3654; Shea *et al.*, Nucl. Acids Res., 1990, 18, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan *et al.*, Nucleosides & Nucleotides, 1995, 14, 969-973), or adamantane acetic acid (Manoharan *et al.*, Tetrahedron Lett., 1995, 36, 3651-3654), a palmityl moiety (Mishra *et al.*, Biochim. Biophys. Acta, 1995, 1264, 229-237), or an octadecylamine or hexylamino-carbonyloxycholesterol moiety (Crooke *et al.*, J. Pharmacol. Exp. Ther., 1996, 277, 923-937).

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

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within a dsRNA. The present invention also includes dsRNA compounds which are chimeric compounds. "Chimeric" dsRNA compounds or "chimeras," in the context of this invention, are dsRNA compounds, particularly dsRNAs, which contain two or more chemically distinct regions, each made up of at least one monomer unit, *i.e.*, a nucleotide in the case of an dsRNA compound. These dsRNAs typically contain at least one region wherein the dsRNA is modified so as to confer upon the dsRNA increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the dsRNA may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of dsRNA inhibition of gene expression. Consequently, comparable results can often be obtained with shorter dsRNAs when chimeric dsRNAs are used, compared to phosphorothioate deoxy dsRNAs hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

In certain instances, the dsRNA may be modified by a non-ligand group. A number of non-ligand molecules have been conjugated to dsRNAs in order to enhance the activity, cellular distribution or cellular uptake of the dsRNA, and procedures for performing such conjugations are available in the scientific literature. Such non-ligand moieties have included lipid moieties, such as cholesterol (Letsinger *et al.*, Proc. Natl. Acad. Sci. USA, 1989, 86:6553), cholic acid (Manoharan *et al.*, Bioorg. Med. Chem. Lett., 1994, 4:1053), a thioether, *e.g.*, hexyl-S-tritylthiol (Manoharan *et al.*, Ann. N.Y. Acad. Sci., 1992, 660:306; Manoharan *et al.*, Bioorg. Med. Chem. Let., 1993, 3:2765), a thiocholesterol (Oberhauser *et al.*, Nucl. Acids Res., 1992, 20:533), an aliphatic chain, *e.g.*, dodecandiol or undecyl residues (Saison-Behmoaras *et al.*, EMBO J., 1991, 10:111; Kabanov *et al.*, FEBS Lett., 1990, 259:327; Svinarchuk *et al.*, Biochimie, 1993, 75:49), a phospholipid, *e.g.*, di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan *et al.*, Tetrahedron Lett., 1995, 36:3651; Shea *et al.*, Nucl. Acids Res., 1990, 18:3777), a polyamine or a polyethylene glycol chain (Manoharan *et al.*, Nucleosides & Nucleotides, 1995, 14:969), or adamantane acetic acid (Manoharan *et al.*, Tetrahedron Lett., 1995, 36:3651), a palmityl moiety (Mishra *et al.*, Biochim. Biophys. Acta, 1995, 1264:229), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke *et al.*, J. Pharmacol. Exp. Ther., 1996, 277:923). Representative United States patents that teach the preparation of such dsRNA conjugates have been listed above. Typical conjugation protocols involve the synthesis of dsRNAs bearing an aminolinker at one or more positions of the sequence. The amino group is then reacted with the molecule being conjugated using appropriate coupling or activating reagents. The conjugation reaction may be performed either with the dsRNA still bound to the solid support or following cleavage of the dsRNA in solution phase. Purification of the dsRNA conjugate by HPLC typically affords the pure conjugate.

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

Vector encoded siRNA agents

In another aspect of the invention, Eg5 and VEGF specific dsRNA molecules that are expressed from transcription units inserted into DNA or RNA vectors (see, *e.g.*, Couture, A, *et al.*, *TIG.* (1996), **12**:5-10; Skillern, A., *et al.*, International PCT Publication No. WO 00/22113, Conrad, International PCT Publication No. WO 00/22114, and Conrad, US Pat. No. 6,054,299). These transgenes can be introduced as a linear construct, a circular plasmid, or a viral vector, which can be incorporated and inherited as a transgene integrated into the host genome. The

transgene can also be constructed to permit it to be inherited as an extrachromosomal plasmid (Gassmann, *et al.*, Proc. Natl. Acad. Sci. USA (1995) 92:1292).

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

The recombinant dsRNA expression vectors are generally DNA plasmids or viral vectors. dsRNA expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus (for a review, see Muzyczka, *et al.*, Curr. Topics Micro. Immunol. (1992) 158:97-129)); adenovirus (see, for example, Berkner, *et al.*, BioTechniques (1998) 6:616), Rosenfeld *et al.* (1991, Science 252:431-434), and Rosenfeld *et al.* (1992), Cell 68:143-155)); or alphavirus as well as others known in the art. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells, *in vitro* and/or *in vivo* (see, *e.g.*, Eglitis, *et al.*, Science (1985) 230:1395-1398; Danos and Mulligan, Proc. Natl. Acad. Sci. USA (1998) 85:6460-6464; Wilson *et al.*, 1988, Proc. Natl. Acad. Sci. USA 85:3014-3018; Armentano *et al.*, 1990, Proc. Natl. Acad. Sci. USA 87:61416145; Huber *et al.*, 1991, Proc. Natl. Acad. Sci. USA 88:8039-8043; Ferry *et al.*, 1991, Proc. Natl. Acad. Sci. USA 88:8377-8381; Chowdhury *et al.*, 1991, Science 254:1802-1805; van Beusechem. *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89:7640-19 ; Kay *et al.*, 1992, Human Gene Therapy 3:641-647; Dai *et al.*, 1992, Proc. Natl.Acad. Sci. USA 89:10892-10895; Hwu *et al.*, 1993, J. Immunol. 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573). Recombinant retroviral vectors capable of transducing and expressing genes inserted into the genome of a cell can be produced by transfecting the recombinant retroviral genome into suitable packaging cell lines such as PA317 and Psi-CRIP (Comette *et al.*, 1991, Human Gene Therapy 2:5-10; Cone *et al.*, 1984, Proc. Natl. Acad. Sci. USA 81:6349). Recombinant adenoviral vectors can be used to infect a wide variety of cells and tissues in susceptible hosts (*e.g.*, rat, hamster, dog, and chimpanzee) (Hsu *et al.*, 1992, J. Infectious Disease, 166:769), and also have the advantage of not requiring mitotically active cells for infection.

Any viral vector capable of accepting the coding sequences for the dsRNA molecule(s) to be expressed can be used, for example vectors derived from adenovirus (AV); adeno-associated virus (AAV); retroviruses (*e.g.*, lentiviruses (LV), Rhabdoviruses, murine leukemia virus); herpes virus, and the like. The tropism of viral vectors can be modified by pseudotyping the

vectors with envelope proteins or other surface antigens from other viruses, or by substituting different viral capsid proteins, as appropriate.

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

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

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

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

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

The promoter driving dsRNA expression in either a DNA plasmid or viral vector of the invention may be a eukaryotic RNA polymerase I (*e.g.* ribosomal RNA promoter), RNA

polymerase II (*e.g.* CMV early promoter or actin promoter or U1 snRNA promoter) or generally RNA polymerase III promoter (*e.g.* U6 snRNA or 7SK RNA promoter) or a prokaryotic promoter, for example the T7 promoter, provided the expression plasmid also encodes T7 RNA polymerase required for transcription from a T7 promoter. The promoter can also direct transgene expression to the pancreas (see, *e.g.*, the insulin regulatory sequence for pancreas (Bucchini *et al.*, 1986, Proc. Natl. Acad. Sci. USA 83:2511-2515)).

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

Generally, recombinant vectors capable of expressing dsRNA molecules are delivered as described below, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of dsRNA molecules. Such vectors can be repeatedly administered as necessary. Once expressed, the dsRNAs bind to target RNA and modulate its function or expression. Delivery of dsRNA expressing vectors can be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from the patient followed by reintroduction into the patient, or by any other means that allows for introduction into a desired target cell.

dsRNA expression DNA plasmids are typically transfected into target cells as a complex with cationic lipid carriers (*e.g.* Oligofectamine) or non-cationic lipid-based carriers (*e.g.* Transit-TKO^{TM}). Multiple lipid transfections for dsRNA-mediated knockdowns targeting different regions of a single EG5 gene (or VEGF gene) or multiple Eg5 genes (or VEGF genes) over a period of a week or more are also contemplated by the invention. Successful introduction of the vectors of the invention into host cells can be monitored using various known methods. For example, transient transfection can be signaled with a reporter, such as a fluorescent marker, such as Green Fluorescent Protein (GFP). Stable transfection of ex vivo cells can be ensured using markers that provide the transfected cell with resistance to specific environmental factors (*e.g.*, antibiotics and drugs), such as hygromycin B resistance.

The Eg5 specific dsRNA molecules and VEGF specific dsRNA molecules can also be inserted into vectors and used as gene therapy vectors for human patients. Gene therapy vectors

can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Patent 5,328,470) or by stereotactic injection (see *e.g.*, Chen *et al.* (1994) Proc. Natl. Acad. Sci. USA 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can include a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

Pharmaceutical compositions containing dsRNA

In one embodiment, the invention provides pharmaceutical compositions containing a dsRNA, as described herein, and a pharmaceutically acceptable carrier and methods of administering the same. The pharmaceutical composition containing the dsRNA is useful for treating a disease or disorder associated with the expression or activity of a Eg5/KSP and/or VEGF gene, such as pathological processes mediated by Eg5/KSP and/or VEGF expression, e.g., liver cancer. Such pharmaceutical compositions are formulated based on the mode of delivery.

Dosage

The pharmaceutical compositions featured herein are administered in dosages sufficient to inhibit expression of EG5/KSP and/or VEGF genes. In general, a suitable dose of dsRNA will be in the range of 0.01 to 200.0 milligrams (mg) per kilogram (kg) body weight of the recipient per day, generally in the range of 1 to 50 mg per kilogram body weight per day. For example, the dsRNA can be administered at 0.01 mg/kg, 0.05 mg/kg, 0.5 mg/kg, 1 mg/kg, 1.5 mg/kg, 2 mg/kg, 3 mg/kg, 5.0 mg/kg, 10 mg/kg, 20 mg/kg, 30 mg/kg, 40 mg/kg, or 50 mg/kg per single dose.

The pharmaceutical composition can be administered once daily, or the dsRNA may be administered as two, three, or more sub-doses at appropriate intervals throughout the day. The effect of a single dose on EG5/KSP and/or VEGF levels is long lasting, such that subsequent doses are administered at not more than 7 day intervals, or at not more than 1, 2, 3, or 4 week intervals.

In some embodiments the dsRNA is administered using continuous infusion or delivery through a controlled release formulation. In that case, the dsRNA contained in each sub-dose must be correspondingly smaller in order to achieve the total daily dosage. The dosage unit can also be compounded for delivery over several days, *e.g.*, using a conventional sustained release formulation which provides sustained release of the dsRNA over a several day period. Sustained release formulations are well known in the art and are particularly useful for delivery of agents at

a particular site, such as could be used with the agents of the present invention. In this embodiment, the dosage unit contains a corresponding multiple of the daily dose.

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

Advances in mouse genetics have generated a number of mouse models for the study of various human diseases, such as pathological processes mediated by EG5/KSP AND/OR VEGF expression. Such models are used for *in vivo* testing of dsRNA, as well as for determining a therapeutically effective dose. A suitable mouse model is, for example, a mouse containing a plasmid expressing human EG5/KSP AND/OR VEGF. Another suitable mouse model is a transgenic mouse carrying a transgene that expresses human EG5/KSP AND/OR VEGF.

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

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

doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

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

Administration

The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical, pulmonary, *e.g.*, by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal, and subdermal, oral or parenteral, e.g., subcutaneous.

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

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

Formulations

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active

ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

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

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

In addition, dsRNA that target the EG5/KSP and/or VEGF gene can be formulated into compositions containing the dsRNA admixed, encapsulated, conjugated, or otherwise associated with other molecules, molecular structures, or mixtures of nucleic acids. For example, a composition containing one or more dsRNA agents that target the Eg5/KSP and/or VEGF gene can contain other therapeutic agents, such as other cancer therapeutics or one or more dsRNA compounds that target non-EG5/KSP AND/OR VEGF genes.

Oral, parenteral, topical, and biologic formulations

Compositions and formulations for oral administration include powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable. In some embodiments, oral formulations are those in which dsRNAs featured in the invention are administered in conjunction with one or more penetration enhancers surfactants and chelators. Suitable surfactants include fatty acids and/or esters or salts thereof, bile acids and/or salts thereof. Suitable bile acids/salts include chenodeoxycholic acid (CDCA) and ursodeoxychenodeoxycholic acid (UDCA), cholic acid, dehydrocholic acid, deoxycholic acid, glucholic acid, glycholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, sodium tauro-24,25-dihydro-fusidate and sodium glycodihydrofusidate. Suitable fatty acids include arachidonic acid, undecanoic acid, oleic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine,

an acylcholine, or a monoglyceride, a diglyceride or a pharmaceutically acceptable salt thereof (*e.g.,* sodium). In some embodiments, combinations of penetration enhancers are used, for example, fatty acids/salts in combination with bile acids/salts. One exemplary combination is the sodium salt of lauric acid, capric acid and UDCA. Further penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether. dsRNAs featured in the invention may be delivered orally, in granular form including sprayed dried particles, or complexed to form micro or nanoparticles. dsRNA complexing agents include poly-amino acids; polyimines; polyacrylates; polyalkylacrylates, polyoxethanes, polyalkylcyanoacrylates; cationized gelatins, albumins, starches, acrylates, polyethyleneglycols (PEG) and starches; polyalkylcyanoacrylates; DEAE-derivatized polyimines, pollulans, celluloses and starches. Suitable complexing agents include chitosan, N-trimethylchitosan, poly-L-lysine, polyhistidine, polyornithine, polyspermines, protamine, polyvinylpyridine, polythiodiethylaminomethylethylene P(TDAE), polyaminostyrene (*e.g.,* p-amino), poly(methylcyanoacrylate), poly(ethylcyanoacrylate), poly(butylcyanoacrylate), poly(isobutylcyanoacrylate), poly(isohexylcynaoacrylate), DEAE-methacrylate, DEAE-hexylacrylate, DEAE-acrylamide, DEAE-albumin and DEAE-dextran, polymethylacrylate, polyhexylacrylate, poly(D,L-lactic acid), poly(DL-lactic-co-glycolic acid (PLGA), alginate, and polyethyleneglycol (PEG). Oral formulations for dsRNAs and their preparation are described in detail in U.S. Patent 6,887,906, U.S. Patent Publication. No. 20030027780, and U.S. Patent No. 6,747,014, each of which is incorporated herein by reference.

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

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Suitable topical formulations include those in which the dsRNAs featured in the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Suitable lipids and liposomes include neutral (*e.g.,* dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearolyphosphatidyl choline) negative (*e.g.,* dimyristoylphosphatidyl glycerol DMPG) and cationic (*e.g.,* dioleoyltetramethylaminopropyl

DOTAP and dioleoylphosphatidyl ethanolamine DOTMA). dsRNAs featured in the invention may be encapsulated within liposomes or may form complexes thereto, in particular to cationic liposomes. Alternatively, dsRNAs may be complexed to lipids, in particular to cationic lipids. Suitable fatty acids and esters include but are not limited to arachidonic acid, oleic acid, eicosanoic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C_{1-10} alkyl ester (*e.g.*, isopropylmyristate IPM), monoglyceride, diglyceride or pharmaceutically acceptable salt thereof. Topical formulations are described in detail in U.S. Patent No. 6,747,014, which is incorporated herein by reference. In addition, dsRNA molecules can be administered to a mammal as biologic or abiologic means as described in, for example, U.S. Pat. No. 6,271,359. Abiologic delivery can be accomplished by a variety of methods including, without limitation, (1) loading liposomes with a dsRNA acid molecule provided herein and (2) complexing a dsRNA molecule with lipids or liposomes to form nucleic acid-lipid or nucleic acid-liposome complexes. The liposome can be composed of cationic and neutral lipids commonly used to transfect cells *in vitro*. Cationic lipids can complex (*e.g.*, charge-associate) with negatively charged nucleic acids to form liposomes. Examples of cationic liposomes include, without limitation, lipofectin, lipofectamine, lipofectace, and DOTAP. Procedures for forming liposomes are well known in the art. Liposome compositions can be formed, for example, from phosphatidylcholine, dimyristoyl phosphatidylcholine, dipalmitoyl phosphatidylcholine, dimyristoyl phosphatidylglycerol, or dioleoyl phosphatidylethanolamine. Numerous lipophilic agents are commercially available, including Lipofectin™ (Invitrogen/Life Technologies, Carlsbad, Calif.) and Effectene™ (Qiagen, Valencia, Calif.). In addition, systemic delivery methods can be optimized using commercially available cationic lipids such as DDAB or DOTAP, each of which can be mixed with a neutral lipid such as DOPE or cholesterol. In some cases, liposomes such as those described by Templeton *et al.* (Nature Biotechnology, 15: 647-652 (1997)) can be used. In other embodiments, polycations such as polyethyleneimine can be used to achieve delivery *in vivo* and ex vivo (Boletta *et al.*, J. Am Soc. Nephrol. 7: 1728 (1996)). Additional information regarding the use of liposomes to deliver nucleic acids can be found in U.S. Pat. No. 6,271,359, PCT Publication WO 96/40964 and Morrissey, D. *et al.* 2005. Nat Biotechnol. 23(8):1002-7.

Biologic delivery can be accomplished by a variety of methods including, without limitation, the use of viral vectors. For example, viral vectors (*e.g.*, adenovirus and herpes virus vectors) can be used to deliver dsRNA molecules to liver cells. Standard molecular biology

techniques can be used to introduce one or more of the dsRNAs provided herein into one of the many different viral vectors previously developed to deliver nucleic acid to cells. These resulting viral vectors can be used to deliver the one or more dsRNAs to cells by, for example, infection.

Liposomal formulations

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

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

In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable to use a liposome which is highly deformable and able to pass through such fine pores.

Further advantages of liposomes include: liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; and liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomes start to merge with the cellular membranes and as the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the active agent may act.

Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many drugs. There is growing evidence that for topical administration, liposomes

present several advantages over other formulations. Such advantages include reduced side-effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and hydrophobic, into the skin.

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

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

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

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

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

liposomal formulation was superior to aqueous administration (du Plessis *et al.*, Antiviral Research, 1992, 18, 259-265).

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome™ I (glyceryl dilaurate/cholesterol/po- lyoxyethylene-10-stearyl ether) and Novasome™ II (glyceryl distearate/cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver cyclosporin-A into the dermis of mouse skin. Results indicated that such non-ionic liposomal systems were effective in facilitating the deposition of cyclosporin-A into different layers of the skin (Hu *et al.*, S.T.P. Pharma. Sci., 1994, 4, 6, 466).

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

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

Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto *et al.* (Bull. Chem. Soc. Jpn., 1980, 53, 2778) described liposomes comprising a nonionic detergent, $2C_{1215G}$, that contains a PEG moiety. Illum *et al.* (FEBS Lett., 1984, 167, 79) noted that hydrophilic coating

of polystyrene particles with polymeric glycols results in significantly enhanced blood half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups of polyalkylene glycols (*e.g.*, PEG) are described by Sears (U.S. Pat. Nos. 4,426,330 and 4,534,899). Klibanov *et al.* (FEBS Lett., 1990, 268, 235) described experiments demonstrating that liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant increases in blood circulation half-lives. Blume *et al.* (Biochimica et Biophysica Acta, 1990, 1029, 91) extended such observations to other PEG-derivatized phospholipids, *e.g.*, DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG. Liposomes having covalently bound PEG moieties on their external surface are described in European Patent No. EP 0 445 131 B1 and WO 90/04384 to Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by Woodle *et al.* (U.S. Pat. Nos. 5,013,556 and 5,356,633) and Martin *et al.* (U.S. Pat. No. 5,213,804 and European Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Pat. No. 5,225,212 (both to Martin *et al.*) and in WO 94/20073 (Zalipsky *et al.*) Liposomes comprising PEG-modified ceramide lipids are described in WO 96/10391 (Choi *et al*). U.S. Pat. No. 5,540,935 (Miyazaki *et al.*) and U.S. Pat. No. 5,556,948 (Tagawa *et al.*) describe PEG-containing liposomes that can be further derivatized with functional moieties on their surfaces.

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

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

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

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

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

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

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

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

Nucleic acid lipid particles

In one embodiment, a dsRNA featured in the invention is fully encapsulated in the lipid formulation, e.g., to form a nucleic acid-lipid particle, e.g., . Nucleic acid-lipid particles

typically contain a cationic lipid, a non-cationic lipid, a sterol, and a lipid that prevents aggregation of the particle (*e.g.*, a PEG-lipid conjugate). Nucleic acid-lipid particles are extremely useful for systemic applications, as they exhibit extended circulation lifetimes following intravenous (i.v.) injection and accumulate at distal sites (*e.g.*, sites physically separated from the administration site). In addition, the nucleic acids when present in the nucleic acid-lipid particles of the present invention are resistant in aqueous solution to degradation with a nuclease. Nucleic acid-lipid particles and their method of preparation are disclosed in, *e.g.*, U.S. Patent Nos. 5,976,567; 5,981,501; 6,534,484; 6,586,410; 6,815,432; and PCT Publication No. WO 96/40964.

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

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

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

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

The particles of the present invention typically have a mean diameter of about 50 nm to about 150 nm, more typically about 60 nm to about 130 nm, more typically about 70 nm to about 110 nm, most typically about 70 nm to about 90 nm, and are substantially nontoxic

In one embodiment, the lipid to drug ratio (mass/mass ratio) (*e.g.*, lipid to dsRNA ratio) will be in the range of from about 1:1 to about 50:1, from about 1:1 to about 25:1, from about 3:1

to about 15:1, from about 4:1 to about 10:1, from about 5:1 to about 9:1, or about 6:1 to about 9:1, or about 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, or 33:1.

Cationic lipids

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

Other cationic lipids, which carry a net positive charge at about physiological pH, in addition to those specifically described above, may also be included in lipid particles of the invention. Such cationic lipids include, but are not limited to, N,N-dioleyl-N,N-dimethylammonium chloride ("DODAC"); N-(2,3-dioleyloxy)propyl-N,N-N-triethylammonium chloride ("DOTMA"); N,N-distearyl-N,N-dimethylammonium bromide ("DDAB"); N-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride ("DOTAP"); 1,2-Dioleyloxy-3-trimethylaminopropane chloride salt ("DOTAP.Cl"); 3β-(N-(N',N'-dimethylaminoethane)-carbamoyl)cholesterol ("DC-Chol"), N-(1-(2,3-dioleyloxy)propyl)-N-2-(sperminecarboxamido)ethyl)-N,N-dimethylammonium trifluoracetate ("DOSPA"), dioctadecylamidoglycyl carboxyspermine ("DOGS"), 1,2-dileoyl-sn-3-phosphoethanolamine ("DOPE"), 1,2-dioleoyl-3-dimethylammonium propane ("DODAP"), N, N-dimethyl-2,3-

dioleyloxy)propylamine ("DODMA"), and N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide ("DMRIE"). Additionally, a number of commercial preparations of cationic lipids can be used, such as, *e.g.*, LIPOFECTIN (including DOTMA and DOPE, available from GIBCO/BRL), and LIPOFECTAMINE (comprising DOSPA and DOPE, available from GIBCO/BRL). In particular embodiments, a cationic lipid is an amino lipid.

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

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

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

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

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

In one embodiment, the cationic lipid XTC (2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane) is used to prepare nucleic acid-lipid particles . Synthesis of XTC is described in United States provisional patent application number 61/107,998 filed on October 23, 2008, which is herein incorporated by reference.

In another embodiment, the cationic lipid MC3 ((6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate), (e.g., DLin-M-C3-DMA) is used to prepare nucleic acid-lipid particles . Synthesis of MC3 and MC3 comprising formulations are described, *e.g.*, in U.S. Provisional Serial No. 61/244,834, filed September 22, 2009, and U.S. Provisional Serial No. 61/185,800, filed June 10, 2009, which are hereby incorporated by reference.

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

FIG. 20 illustrates the structures of ALNY-100, MC3, and XTC.

The cationic lipid may comprise from about 20 mol % to about 70 mol % or about 45-65 mol % or about 40 mol % of the total lipid present in the particle.

Non-cationic lipids

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

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

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

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

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

Conjugated lipids

Conjugated lipids can be used in nucleic acid-lipid particle to prevent aggregation, including polyethylene glycol (PEG)-modified lipids, monosialoganglioside Gm1, and polyamide oligomers ("PAO") such as (described in US Pat. No. 6,320,017). Other compounds with uncharged, hydrophilic, steric-barrier moieties, which prevent aggregation during formulation, like PEG, Gm1 or ATTA, can also be coupled to lipids for use as in the methods and compositions of the invention. ATTA-lipids are described, *e.g.*, in U.S. Patent No.

6,320,017, and PEG-lipid conjugates are described, *e.g.*, in U.S. Patent Nos. 5,820,873, 5,534,499 and 5,885,613. Typically, the concentration of the lipid component selected to reduce aggregation is about 1 to 15% (by mole percent of lipids).

Specific examples of PEG-modified lipids (or lipid-polyoxyethylene conjugates) that are useful in the invention can have a variety of "anchoring" lipid portions to secure the PEG portion to the surface of the lipid vesicle. Examples of suitable PEG-modified lipids include PEG-modified phosphatidylethanolamine and phosphatidic acid, PEG-ceramide conjugates (*e.g.*, PEG-CerC14 or PEG-CerC20) which are described in co-pending USSN 08/486,214, incorporated herein by reference, PEG-modified dialkylamines and PEG-modified 1,2-diacyloxypropan-3-amines. Particularly preferred are PEG-modified diacylglycerols and dialkylglycerols.

In embodiments where a sterically-large moiety such as PEG or ATTA are conjugated to a lipid anchor, the selection of the lipid anchor depends on what type of association the conjugate is to have with the lipid particle. It is well known that mePEG (mw2000)-diastearoylphosphatidylethanolamine (PEG-DSPE) will remain associated with a liposome until the particle is cleared from the circulation, possibly a matter of days. Other conjugates, such as PEG-CerC20 have similar staying capacity. PEG-CerC14, however, rapidly exchanges out of the formulation upon exposure to serum, with a $T_{1/2}$ less than 60 mins. in some assays. As illustrated in US Pat. Application SN 08/486,214, at least three characteristics influence the rate of exchange: length of acyl chain, saturation of acyl chain, and size of the steric-barrier head group. Compounds having suitable variations of these features may be useful for the invention. For some therapeutic applications, it may be preferable for the PEG-modified lipid to be rapidly lost from the nucleic acid-lipid particle *in vivo* and hence the PEG-modified lipid will possess relatively short lipid anchors. In other therapeutic applications, it may be preferable for the nucleic acid-lipid particle to exhibit a longer plasma circulation lifetime and hence the PEG-modified lipid will possess relatively longer lipid anchors. Exemplary lipid anchors include those having lengths of from about C_{14} to about C_{22}, preferably from about C_{14} to about C_{16}. In some embodiments, a PEG moiety, for example an mPEG-NH_2, has a size of about 1000, 2000, 5000, 10,000, 15,000 or 20,000 daltons.

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

The conjugated lipid that inhibits aggregation of particles may be, for example, a polyethyleneglycol (PEG)-lipid including, without limitation, a PEG-diacylglycerol (DAG), a PEG-dialkyloxypropyl (DAA), a PEG-phospholipid, a PEG-ceramide (Cer), or a mixture thereof. The PEG-DAA conjugate may be, for example, a PEG-dilauryloxypropyl (C_{12}), a PEG-dimyristyloxypropyl (C_{14}), a PEG-dipalmityloxypropyl (C_{16}), or a PEG- distearyloxypropyl (C_{18}). Additional conjugated lipids include polyethylene glycol - didimyristoyl glycerol (C14-PEG or PEG-C14, where PEG has an average molecular weight of 2000 Da) (PEG-DMG); (R)-2,3-bis(octadecyloxy)propyl1-(methoxy poly(ethylene glycol)2000)propylcarbamate) (PEG-DSG); PEG-carbamoyl-1,2-dimyristyloxypropylamine, in which PEG has an average molecular weight of 2000 Da (PEG-cDMA); N-Acetylgalactosamine-((R)-2,3-bis(octadecyloxy)propyl1-(methoxy poly(ethylene glycol)2000)propylcarbamate)) (GalNAc-PEG-DSG); and polyethylene glycol –dipalmitoylglycerol (PEG-DPG).

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

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

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

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

Lipoproteins

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

Suitable apolipoproteins include, but are not limited to, ApoA-I, ApoA-II, ApoA-IV, ApoA-V and ApoE, and active polymorphic forms, isoforms, variants and mutants as well as fragments or truncated forms thereof. In certain embodiments, the apolipoprotein is a thiol containing apolipoprotein. "Thiol containing apolipoprotein" refers to an apolipoprotein, variant, fragment or isoform that contains at least one cysteine residue. The most common thiol

containing apolipoproteins are ApoA-I Milano (ApoA-I_M) and ApoA-I Paris (ApoA-I_P) which contain one cysteine residue (Jia *et al.,* 2002, Biochem. Biophys. Res. Comm. 297: 206-13; Bielicki and Oda, 2002, Biochemistry 41: 2089-96). ApoA-II, ApoE2 and ApoE3 are also thiol containing apolipoproteins. Isolated ApoE and/or active fragments and polypeptide analogues thereof, including recombinantly produced forms thereof, are described in U.S. Pat. Nos. 5,672,685; 5,525,472; 5,473,039; 5,182,364; 5,177,189; 5,168,045; 5,116,739; the disclosures of which are herein incorporated by reference. ApoE3 is disclosed in Weisgraber, *et al.*, "Human E apoprotein heterogeneity: cysteine-arginine interchanges in the amino acid sequence of the apo-E isoforms," J. Biol. Chem. (1981) 256: 9077-9083; and Rall, *et al.,* "Structural basis for receptor binding heterogeneity of apolipoprotein E from type III hyperlipoproteinemic subjects," Proc. Nat. Acad. Sci. (1982) 79: 4696-4700. (See also GenBank accession number K00396.)

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

In certain embodiments, the apolipoprotein can be a fragment, variant or isoform of the apolipoprotein. The term "fragment" refers to any apolipoprotein having an amino acid sequence shorter than that of a native apolipoprotein and which fragment retains the activity of native apolipoprotein, including lipid binding properties. By "variant" is meant substitutions or alterations in the amino acid sequences of the apolipoprotein, which substitutions or alterations, e.g., additions and deletions of amino acid residues, do not abolish the activity of native apolipoprotein, including lipid binding properties. Thus, a variant can comprise a protein or peptide having a substantially identical amino acid sequence to a native apolipoprotein provided herein in which one or more amino acid residues have been conservatively substituted with chemically similar amino acids. Examples of conservative substitutions include the substitution of at least one hydrophobic residue such as isoleucine, valine, leucine or methionine for another. Likewise, the present invention contemplates, for example, the substitution of at least one hydrophilic residue such as, for example, between arginine and lysine, between glutamine and asparagine, and between glycine and serine (see U.S. Pat. Nos. 6,004,925, 6,037,323 and

6,046,166). The term "isoform" refers to a protein having the same, greater or partial function and similar, identical or partial sequence, and may or may not be the product of the same gene and usually tissue specific (see Weisgraber 1990, J. Lipid Res. 31(8):1503-11; Hixson and Powers 1991, J. Lipid Res. 32(9):1529-35; Lackner *et al.*, 1985, J. Biol. Chem. 260(2):703-6; Hoeg *et al.*, 1986, J. Biol. Chem. 261(9):3911-4; Gordon *et al.*, 1984, J. Biol. Chem. 259(1):468-74; Powell et al., 1987, Cell 50(6):831-40; Aviram *et al.*, 1998, Arterioscler. Thromb. Vase. Biol. 18(10):1617-24; Aviram et al., 1998, J. Clin. Invest. 101(8):1581-90; Billecke et al., 2000, Drug Metab. Dispos. 28(11):1335-42; Draganov et al., 2000, J. Biol. Chem. 275(43):33435-42; Steinmetz and Utermann 1985, J. Biol. Chem. 260(4):2258-64; Widler *et al.*, 1980, J. Biol. Chem. 255(21):10464-71; Dyer *et al.*, 1995, J. Lipid Res. 36(1):80-8; Sacre *et al.*, 2003, FEBS Lett. 540(1-3):181-7; Weers, et al., 2003, Biophys. Chem. 100(1-3):481-92; Gong *et al.*, 2002, J. Biol. Chem. 277(33):29919-26; Ohta et al., 1984, J. Biol. Chem. 259(23):14888-93 and U.S. Pat. No. 6,372,886).

In certain embodiments, the methods and compositions of the present invention include the use of a chimeric construction of an apolipoprotein. For example, a chimeric construction of an apolipoprotein can be comprised of an apolipoprotein domain with high lipid binding capacity associated with an apolipoprotein domain containing ischemia reperfusion protective properties. A chimeric construction of an apolipoprotein can be a construction that includes separate regions within an apolipoprotein (*i.e.,* homologous construction) or a chimeric construction can be a construction that includes separate regions between different apolipoproteins (*i.e.*, heterologous constructions). Compositions comprising a chimeric construction can also include segments that are apolipoprotein variants or segments designed to have a specific character (*e.g.*, lipid binding, receptor binding, enzymatic, enzyme activating, antioxidant or reduction-oxidation property) (see Weisgraber 1990, J. Lipid Res. 31(8):1503-11; Hixson and Powers 1991, J. Lipid Res. 32(9):1529-35; Lackner *et al.*, 1985, J. Biol. Chem. 260(2):703-6; Hoeg *et al.*, 1986, J. Biol. Chem. 261(9):3911-4; Gordon *et al.*, 1984, J. Biol. Chem. 259(1):468-74; Powell *et al.*, 1987, Cell 50(6):831-40; Aviram *et al.*, 1998, Arterioscler. Thromb. Vasc. Biol. 18(10):1617-24; Aviram *et al.*, 1998, J. Clin. Invest. 101(8):1581-90; Billecke *et al.*, 2000, Drug Metab. Dispos. 28(11):1335-42; Draganov *et al.*, 2000, J. Biol. Chem. 275(43):33435-42; Steinmetz and Utermann 1985, J. Biol. Chem. 260(4):2258-64; Widler *et al.*, 1980, J. Biol. Chem. 255(21):10464-71; Dyer *et al.*, 1995, J. Lipid Res. 36(1):80-8; Sorenson *et al.*, 1999, Arterioscler. Thromb. Vasc. Biol. 19(9):2214-25; Palgunachari 1996, Arterioscler. Throb. Vasc. Biol. 16(2):328-38: Thurberg *et al.*, J. Biol. Chem. 271(11):6062-70; Dyer 1991, J. Biol. Chem. 266(23):150009-15; Hill 1998, J. Biol. Chem. 273(47):30979-84).

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

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

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

In certain embodiments, the apolipoprotein can be a mixture of apolipoproteins. In one embodiment, the apolipoprotein can be a homogeneous mixture, that is, a single type of apolipoprotein. In another embodiment, the apolipoprotein can be a heterogeneous mixture of apolipoproteins, that is, a mixture of two or more different apolipoproteins. Embodiments of heterogenous mixtures of apolipoproteins can comprise, for example, a mixture of an

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

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

Other components

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

Also suitable for inclusion in the lipid particles of the invention are programmable fusion lipids. Such lipid particles have little tendency to fuse with cell membranes and deliver their payload until a given signal event occurs. This allows the lipid particle to distribute more evenly after injection into an organism or disease site before it starts fusing with cells. The signal event can be, for example, a change in pH, temperature, ionic environment, or time. In the latter case, a fusion delaying or "cloaking" component, such as an ATTA-lipid conjugate or a PEG-lipid conjugate, can simply exchange out of the lipid particle membrane over time. Exemplary lipid anchors include those having lengths of from about C_{14} to about C_{22}, preferably from about C_{14} to about C_{16}. In some embodiments, a PEG moiety, for example an mPEG-NH_2, has a size of about 1000, 2000, 5000, 10,000, 15,000 or 20,000 daltons.

A lipid particle conjugated to a nucleic acid agent can also include a targeting moiety, e.g., a targeting moiety that is specific to a cell type or tissue. Targeting of lipid particles using a variety of targeting moieties, such as ligands, cell surface receptors, glycoproteins, vitamins (*e.g.,* riboflavin) and monoclonal antibodies, has been previously described (*see, e.g.*, U.S. Patent Nos. 4,957,773 and 4,603,044). The targeting moieties can include the entire protein or fragments thereof. Targeting mechanisms generally require that the targeting agents be positioned on the surface of the lipid particle in such a manner that the targeting moiety is available for interaction with the target, for example, a cell surface receptor. A variety of different targeting agents and methods are known and available in the art, including those described, *e.g.*, in Sapra, P. and Allen, TM, *Prog. Lipid Res.* 42(5):439-62 (2003); and Abra, RM *et al., J. Liposome Res.* 12:1-3, (2002).

The use of lipid particles, *i.e.*, liposomes, with a surface coating of hydrophilic polymer chains, such as polyethylene glycol (PEG) chains, for targeting has been proposed (Allen, *et al.*, *Biochimica et Biophysica Acta* 1237: 99-108 (1995); DeFrees, *et al., Journal of the American Chemistry Society* 118: 6101-6104 (1996); Blume, *et al., Biochimica et Biophysica Acta* 1149: 180-184 (1993); Klibanov, *et al., Journal of Liposome Research* 2: 321-334 (1992); U.S. Patent No. 5,013556; Zalipsky, *Bioconjugate Chemistry* 4: 296-299 (1993); Zalipsky, *FEBS Letters* 353: 71-74 (1994); Zalipsky, in Stealth Liposomes Chapter 9 (Lasic and Martin, Eds) CRC Press, Boca Raton Fl (1995). In one approach, a ligand, such as an antibody, for targeting the lipid particle is linked to the polar head group of lipids forming the lipid particle. In another approach, the targeting ligand is attached to the distal ends of the PEG chains forming the hydrophilic polymer coating (Klibanov, *et al., Journal of Liposome Research* 2: 321-334 (1992); Kirpotin *et al., FEBS Letters* 388: 115-118 (1996)).

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

Liposomes, 149 Methods in Enzymology 111-119 (Academic Press, Inc. 1987)). Other targeting methods include the biotin-avidin system.

Production of nucleic acid-lipid particles

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

The extrusion method (also refer to as preformed method or batch process) is a method where the empty liposomes (i.e. no nucleic acid) are prepared first, followed by the addition of nucleic acid to the empty liposome. Extrusion of liposome compositions through a small-pore polycarbonate membrane or an asymmetric ceramic membrane results in a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired liposome complex size distribution is achieved. The liposomes may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in liposome size. In some instances, the lipid-nucleic acid compositions which are formed can be used without any sizing. These methods are disclosed in the US 5,008,050; US 4,927,637; US 4,737,323; *Biochim Biophys Acta.* 1979 Oct 19;557(1):9-23; *Biochim Biophys Acta.* 1980 Oct 2;601(3):559-7; *Biochim Biophys Acta.* 1986 Jun 13;858(1):161-8; and *Biochim. Biophys. Acta* 1985 812, 55-65, which are hereby incorporated by reference in their entirety.

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

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

Characterization of nucleic acid-lipid particles

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

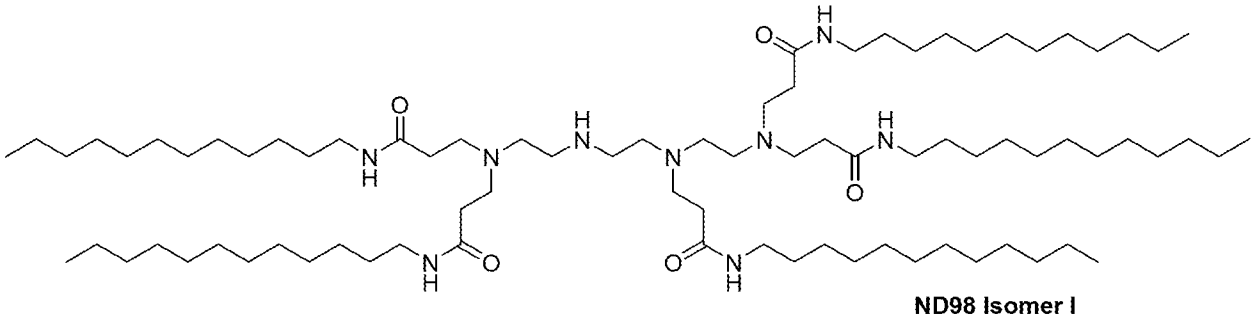
an RNA-binding dye, such as Ribogreen (Molecular Probes) in the presence or absence of a formulation disrupting surfactant, *e.g.*, 0.5% Triton-X100. The total siRNA in the formulation can be determined by the signal from the sample containing the surfactant, relative to a standard curve. The entrapped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

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

Formulations of nucleic acid-lipid particles

LNP01

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



Formula 1

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

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

Name	**cationic lipid/non-cationic lipid/cholesterol/PEG-lipid conjugate mol % ratio Lipid:siRNA ratio**
SNALP	DLinDMA/DPPC/Cholesterol/PEG-cDMA (57.1/7.1/34.4/1.4) lipid:siRNA ~ 7:1
LNP-S-X	XTC/DPPC/Cholesterol/PEG-cDMA 57.1/7.1/34.4/1.4 lipid:siRNA ~ 7:1
LNP05	XTC/DSPC/Cholesterol/PEG-DMG 57.5/7.5/31.5/3.5 lipid:siRNA ~ 6:1
LNP06	XTC/DSPC/Cholesterol/PEG-DMG 57.5/7.5/31.5/3.5 lipid:siRNA ~ 11:1
LNP07	XTC/DSPC/Cholesterol/PEG-DMG 60/7.5/31/1.5, lipid:siRNA ~ 6:1
LNP08	XTC/DSPC/Cholesterol/PEG-DMG 60/7.5/31/1.5, lipid:siRNA ~ 11:1
LNP09	XTC/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 lipid:siRNA ~ 10:1
LNP10	ALNY-100/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 lipid:siRNA ~ 10:1
LNP11	MC3/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 lipid:siRNA ~ 10:1
LNP13	XTC/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 lipid:siRNA ~ 33:1
LNP14	MC3/DSPC/Cholesterol/PEG-DMG 40/15/40/5 lipid:siRNA ~11:1

LNP15	MC3/DSPC/Cholesterol/PEG-DSG/GalNAc-PEG-DSG 50/10/35/4.5/0.5 lipid:siRNA ~11:1
LNP16	MC3/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 lipid:siRNA ~7:1
LNP17	MC3/DSPC/Cholesterol/PEG-DSG 50/10/38.5/1.5 lipid:siRNA ~10:1
LNP18	MC3/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 lipid:siRNA ~12:1
LNP19	MC3/DSPC/Cholesterol/PEG-DMG 50/10/35/5 lipid:siRNA ~8:1
LNP20	MC3/DSPC/Cholesterol/PEG-DPG 50/10/38.5/1.5 lipid:siRNA ~10:1
LNP22	XTC/DSPC/Cholesterol/PEG-DSG 50/10/38.5/1.5 lipid:siRNA ~10:1

XTC comprising formulations are described, e.g., in U.S. Provisional Serial No. 61/239,686, filed September 3, 2009, which is hereby incorporated by reference.

MC3 comprising formulations are described, e.g., in U.S. Provisional Serial No. 61/244,834, filed September 22, 2009, and U.S. Provisional Serial No. 61/185,800, filed June 10, 2009, which are hereby incorporated by reference.

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

Additional representative formulations delineated in Tables 25 and 26. Lipid refers to a cationic lipid.

Table 25: Composition of exemplary nucleic acid-lipid particle (mole %) prepared via extrusion methods.

Lipid (mol %)	DSPC (mol %)	Chol (mol %)	PEG (mol %)	Lipid/ siRNA
20	30	40	10	2.13
20	30	40	10	2.35
20	30	40	10	2.37
20	30	40	10	3.23
20	30	40	10	3.91
30	20	40	10	2.89
30	20	40	10	3.34
30	20	40	10	3.34
30	20	40	10	4.10

Lipid (mol %)	DSPC (mol %)	Chol (mol %)	PEG (mol %)	Lipid/ siRNA
30	20	40	10	5.64
40	10	40	10	3.02
40	10	40	10	3.35
40	10	40	10	3.74
40	10	40	10	5.80
40	10	40	10	8.00
45	5	40	10	3.27
45	5	40	10	3.30
45	5	40	10	4.45
45	5	40	10	7.00
45	5	40	10	9.80
50	0	40	10	27.03
20	35	40	5	3.00
20	35	40	5	3.32
20	35	40	5	3.05
20	35	40	5	3.67
20	35	40	5	4.71
30	25	40	5	2.47
30	25	40	5	2.98
30	25	40	5	3.29
30	25	40	5	4.99
30	25	40	5	7.15
40	15	40	5	2.79
40	15	40	5	3.29
40	15	40	5	4.33
40	15	40	5	7.05
40	15	40	5	9.63
45	10	40	5	2.44
45	10	40	5	3.21
45	10	40	5	4.29
45	10	40	5	6.50
45	10	40	5	8.67
20	35	40	5	4.10
20	35	40	5	4.83
30	25	40	5	3.86
30	25	40	5	5.38
30	25	40	5	7.07

Lipid (mol %)	DSPC (mol %)	Chol (mol %)	PEG (mol %)	Lipid/ siRNA
40	15	40	5	3.85
40	15	40	5	4.88
40	15	40	5	7.22
40	15	40	5	9.75
45	10	40	5	2.83
45	10	40	5	3.85
45	10	40	5	4.88
45	10	40	5	7.05
45	10	40	5	9.29
45	20	30	5	4.01
45	20	30	5	3.70
50	15	30	5	4.75
50	15	30	5	3.80
55	10	30	5	3.85
55	10	30	5	4.13
60	5	30	5	5.09
60	5	30	5	4.67
65	0	30	5	4.75
65	0	30	5	6.06
56.5	10	30	3.5	3.70
56.5	10	30	3.5	3.56
57.5	10	30	2.5	3.48
57.5	10	30	2.5	3.20
58.5	10	30	1.5	3.24
58.5	10	30	1.5	3.13
59.5	10	30	0.5	3.24
59.5	10	30	0.5	3.03
45	10	40	5	7.57
45	10	40	5	7.24
45	10	40	5	7.48
45	10	40	5	7.84
65	0	30	5	4.01
60	5	30	5	3.70
55	10	30	5	3.65
50	10	35	5	3.43
50	15	30	5	3.80
45	15	35	5	3.70

Lipid (mol %)	DSPC (mol %)	Chol (mol %)	PEG (mol %)	Lipid/ siRNA
45	20	30	5	3.75
45	25	25	5	3.85
55	10	32.5	2.5	3.61
60	10	27.5	2.5	3.65
60	10	25	5	4.07
55	5	38.5	1.5	3.75
60	10	28.5	1.5	3.43
55	10	33.5	1.5	3.48
60	5	33.5	1.5	3.43
55	5	37.5	2.5	3.75
60	5	32.5	2.5	4.52
60	5	32.5	2.5	3.52
45	15 (DMPC)	35	5	3.20
45	15 (DPPC)	35	5	3.43
45	15 (DOPC)	35	5	4.52
45	15 (POPC)	35	5	3.85
55	5	37.5	2.5	3.96
55	10	32.5	2.5	3.56
60	5	32.5	2.5	3.80
60	10	27.5	2.5	3.75
60	5	30	5	4.19
60	5	33.5	1.5	3.48
60	5	33.5	1.5	6.64
60	5	30	5	3.90
60	5	30	5	4.65
60	5	30	5	5.88
60	5	30	5	7.51
60	5	30	5	9.51
60	5	30	5	11.06
62.5	2.5	50	5	6.63
45	15	35	5	3.31
45	15	35	5	6.80
60	5	25	10	6.48
60	5	32.5	2.5	3.43
60	5	30	5	3.90
60	5	30	5	7.61
45	15	35	5	3.13
45	15	35	5	6.42

Lipid (mol %)	DSPC (mol %)	Chol (mol %)	PEG (mol %)	Lipid/ siRNA
60	5	25	10	6.48
60	5	32.5	2.5	3.03
60	5	30	5	3.43
60	5	30	5	6.72
60	5	30	5	4.13
70	5	20	5	5.48
80	5	10	5	5.94
90	5	0	5	9.50
60	5	30	5 C12PEG	3.85
60	5	30	5	3.70
60	5	30	5 C16PEG	3.80
60	5	30	5	4.19
60	5	29	5	4.07
60	5	30	5	3.56
60	5	30	5	3.39
60	5	30	5	3.96
60	5	30	5	4.01
60	5	30	5	4.07
60	5	30	5	4.25
60	5	30	5	3.80
60	5	30	5	3.31
60	5	30	5	4.83
60	5	30	5	4.67
60	5	30	5	3.96
57.5	7.5	33.5	1.5	3.39
57.5	7.5	32.5	2.5	3.39
57.5	7.5	31.5	3.5	3.52
57.5	7.5	30	5	4.19
60	5	30	5	3.96
60	5	30	5	3.96
60	5	30	5	3.56
60	5	33.5	1.5	3.52
60	5	25	10	5.18
60	5 (DPPC)	30	5	4.25
60	5	32.5	2.5	3.70
57.5	7.5	31.5	3.5	3.06
57.5	7.5	31.5	3.5	3.65
57.5	7.5	31.5	3.5	4.70

Lipid (mol %)	DSPC (mol %)	Chol (mol %)	PEG (mol %)	Lipid/ siRNA
57.5	7.5	31.5	3.5	6.56

Table 26: Composition of exemplary nucleic acid-lipid particles prepared via in-line mixing

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

Lipid (mol %)	DSPC (mol %)	Chol (mol %)	PEG (mol %)	Lipid A/ siRNA
70	10	19	1	3.85
60	5	34	1	3.52
60	5	34	1	3.70
60	5	34	1	3.52
60	7.5	27.5	5	5.18
60	7.5	29	3.5	4.45
60	5	31.5	3.5	4.83
60	7.5	31	1.5	3.48
57.5	7.5	30	5	4.75
57.5	7.5	31.5	3.5	4.83
57.5	5	34	3.5	4.67
57.5	7.5	33.5	1.5	3.43
55	7.5	32.5	5	4.38
55	7.5	34	3.5	4.13
55	5	36.5	3.5	4.38
55	7.5	36	1.5	3.35

Synthesis of cationic lipids.

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

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

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

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

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

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

The terms "optionally substituted alkyl", "optionally substituted alkenyl", "optionally substituted alkynyl", "optionally substituted acyl", and "optionally substituted heterocycle" means that, when substituted, at least one hydrogen atom is replaced with a substituent. In the case of an oxo substituent (=O) two hydrogen atoms are replaced. In this regard, substituents include oxo, halogen, heterocycle, -CN, $-OR^x$, $-NR^xR^y$, $-NR^xC(=O)R^y$, $-NR^xSO_2R^y$, $-C(=O)R^x$, $-C(=O)OR^x$, $-C(=O)NR^xR^y$, $-SO_nR^x$ and $-SO_nNR^xR^y$, wherein n is 0, 1 or 2, R^x and R^y are the same or different and independently hydrogen, alkyl or heterocycle, and each of said alkyl and heterocycle substituents may be further substituted with one or more of oxo, halogen, -OH, -CN, alkyl, $-OR^x$, heterocycle, $-NR^xR^y$, $-NR^xC(=O)R^y$, $-NR^xSO_2R^y$, $-C(=O)R^x$, $-C(=O)OR^x$, $-C(=O)NR^xR^y$, $-SO_nR^x$ and $-SO_nNR^xR^y$.

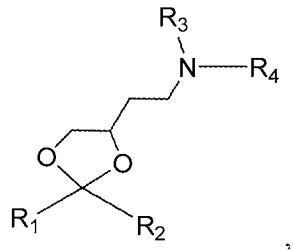
"Halogen" means fluoro, chloro, bromo and iodo.

In some embodiments, the methods of the invention may require the use of protecting groups. Protecting group methodology is well known to those skilled in the art (*see, for example*, PROTECTIVE GROUPS IN ORGANIC SYNTHESIS, Green, T.W. *et al.*, Wiley-Interscience, New York City, 1999). Briefly, protecting groups within the context of this invention are any

group that reduces or eliminates unwanted reactivity of a functional group. A protecting group can be added to a functional group to mask its reactivity during certain reactions and then removed to reveal the original functional group. In some embodiments an "alcohol protecting group" is used. An "alcohol protecting group" is any group which decreases or eliminates unwanted reactivity of an alcohol functional group. Protecting groups can be added and removed using techniques well known in the art.

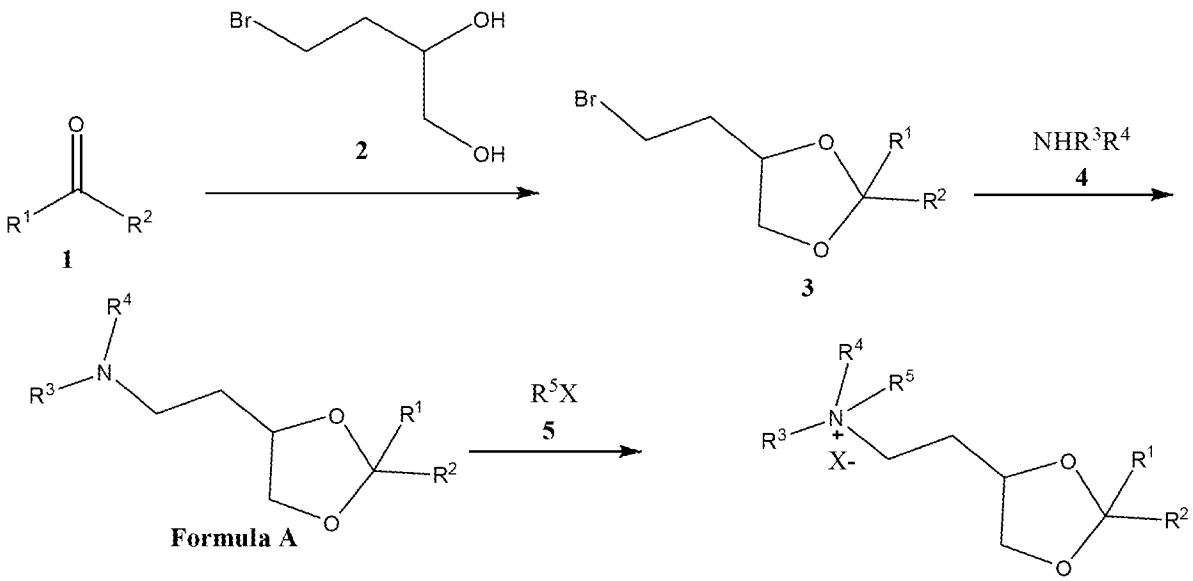
Synthesis of Formula A

In one embodiments, nucleic acid-lipid particles of the invention are formulated using a cationic lipid of formula A:



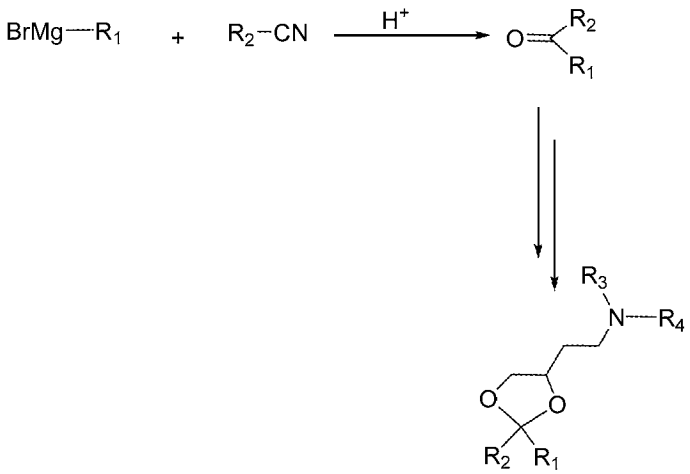
where R1 and R2 are independently alkyl, alkenyl or alkynyl, each can be optionally substituted, and R3 and R4 are independently lower alkyl or R3 and R4 can be taken together to form an optionally substituted heterocyclic ring. In some embodiments, the cationic lipid is XTC (2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane).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

Scheme 1



Lipid A, where R_1 and R_2 are independently alkyl, alkenyl or alkynyl, each can be optionally substituted, and R_3 and R_4 are independently lower alkyl or R_3 and R_4 can be taken together to form an optionally substituted heterocyclic ring, can be prepared according to Scheme 1. Ketone 1 and bromide 2 can be purchased or prepared according to methods known to those of ordinary skill in the art. Reaction of 1 and 2 yields ketal 3. Treatment of ketal 3 with amine 4 yields lipids of formula A. The lipids of formula A can be converted to the corresponding ammonium salt with an organic salt of formula 5, where X is anion counter ion selected from halogen, hydroxide, phosphate, sulfate, or the like.

Scheme 2



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

Synthesis of MC3

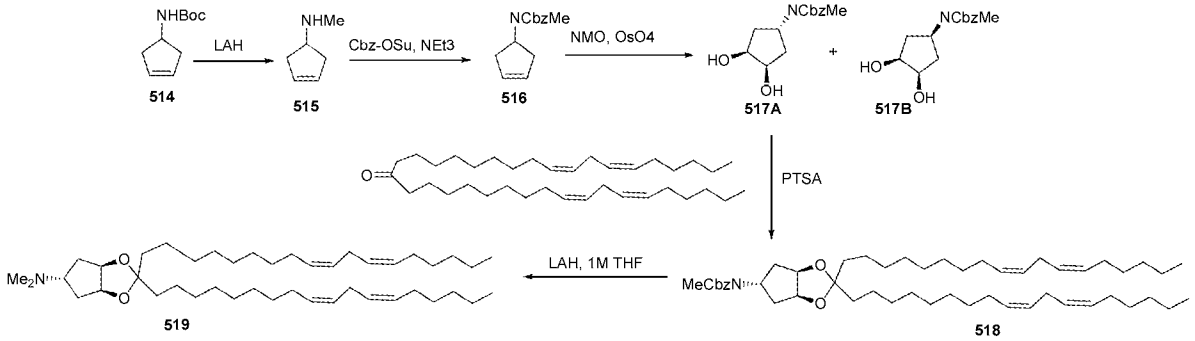
Preparation of DLin-M-C3-DMA (i.e., (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate) was as follows. A solution of (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-ol (0.53 g), 4-N,N-dimethylaminobutyric acid hydrochloride (0.51 g), 4-N,N-dimethylaminopyridine (0.61g) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.53 g) in dichloromethane (5 mL) was stirred at room temperature overnight. The solution was washed with dilute hydrochloric acid followed by dilute aqueous sodium bicarbonate. The organic fractions were dried over anhydrous magnesium sulphate, filtered and the solvent removed on a rotovap. The residue was passed down a silica gel column (20 g) using a 1-5% methanol/dichloromethane elution gradient.

Fractions containing the purified product were combined and the solvent removed, yielding a colorless oil (0.54 g).

Synthesis of ALNY-100

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

Scheme 3



Synthesis of 515:

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

Synthesis of 516:

To a stirred solution of compound 515 in 100 mL dry DCM in a 250 mL two neck RBF, was added NEt3 (37.2 mL, 0.2669 mol) and cooled to 0 0C under nitrogen atmosphere. After a slow addition of N-(benzyloxy-carbonyloxy)-succinimide (20 g, 0.08007 mol) in 50 mL dry DCM, reaction mixture was allowed to warm to room temperature. After completion of the reaction (2-3 h by TLC) mixture was washed successively with 1N HCl solution (1 x 100 mL) and saturated NaHCO3 solution (1 x 50 mL). The organic layer was then dried over anhyd. Na2SO4 and the solvent was evaporated to give crude material which was purified by silica gel column chromatography to get 516 as sticky mass. Yield: 11g (89%). 1H-NMR (CDCl3,

400MHz): δ = 7.36-7.27(m, 5H), 5.69 (s, 2H), 5.12 (s, 2H), 4.96 (br., 1H) 2.74 (s, 3H), 2.60(m, 2H), 2.30-2.25(m, 2H). LC-MS [M+H] -232.3 (96.94%).

Synthesis of 517A and 517B:

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

517A - Peak-1 (white solid), 5.13 g (96%). 1H-NMR (DMSO, 400MHz): δ= 7.39-7.31(m, 5H), 5.04(s, 2H), 4.78-4.73 (m, 1H), 4.48-4.47(d, 2H), 3.94-3.93(m, 2H), 2.71(s, 3H), 1.72- 1.67(m, 4H). LC-MS - [M+H]-266.3, [M+NH4 +]-283.5 present, HPLC-97.86%. Stereochemistry confirmed by X-ray.

Synthesis of 518:

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

General Procedure for the Synthesis of Compound 519:

A solution of compound 518 (1 eq) in hexane (15 mL) was added in a drop-wise fashion to an ice-cold solution of LAH in THF (1 M, 2 eq). After complete addition, the mixture was heated at 40□C over 0.5 h then cooled again on an ice bath. The mixture was carefully hydrolyzed with saturated aqueous Na2SO4 then filtered through celite and reduced to an oil. Column chromatography provided the pure 519 (1.3 g, 68%) which was obtained as a colorless oil. 13C NMR □ = 130.2, 130.1 (x2), 127.9 (x3), 112.3, 79.3, 64.4, 44.7, 38.3, 35.4, 31.5, 29.9 (x2), 29.7, 29.6 (x2), 29.5 (x3), 29.3 (x2), 27.2 (x3), 25.6, 24.5, 23.3, 226, 14.1; Electrospray MS (+ve): Molecular weight for C44H80NO2 (M + H)+ Calc. 654.6, Found 654.6.

Therapeutic Agent-Lipid Particle Compositions and Formulations

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

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

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

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

In various embodiments, therapeutic agents include any therapeutically effective agent or drug, such as anti-inflammatory compounds, anti-depressants, stimulants, analgesics, antibiotics, birth control medication, antipyretics, vasodilators, anti-angiogenics, cytovascular agents, signal

transduction inhibitors, cardiovascular drugs, *e.g.*, anti-arrhythmic agents, vasoconstrictors, hormones, and steroids.

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

Additional formulations

Emulsions

The compositions of the present invention may be prepared and formulated as emulsions. Emulsions are typically heterogeneous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 μm in diameter (Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi *et al.*, in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa., 1985, p. 301). Emulsions are often biphasic systems comprising two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be of either the water-in-oil (w/o) or the oil-in-water (o/w) variety. When an aqueous phase is finely

divided into and dispersed as minute droplets into a bulk oily phase, the resulting composition is called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase, the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions may contain additional components in addition to the dispersed phases, and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in emulsions as needed. Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous phase provides an o/w/o emulsion.

Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion may be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that may be incorporated into either phase of the emulsion. Emulsifiers may broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (Rieger, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc., New York, N.Y., 1988, volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants may be classified into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (Rieger,

in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

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

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

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

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used may be free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture have been reviewed in the literature (Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of ease of formulation, as well as efficacy from an absorption and bioavailability standpoint (Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

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

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Block, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335). Compared to conventional emulsions, microemulsions offer the

advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions may, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (Constantinides *et al.*, Pharmaceutical Research, 1994, 11, 1385-1390; Ritschel, Meth. Find. Exp. Clin. Pharmacol., 1993, 13, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (Constantinides *et al.*, Pharmaceutical Research, 1994, 11, 1385; Ho *et al.*, J. Pharm. Sci., 1996, 85, 138-143). Often microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be particularly advantageous when formulating thermolabile drugs, peptides or dsRNAs. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will

facilitate the increased systemic absorption of dsRNAs and nucleic acids from the gastrointestinal tract, as well as improve the local cellular uptake of dsRNAs and nucleic acids.

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

Penetration Enhancers

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

Penetration enhancers may be classified as belonging to one of five broad categories, *i.e.*, surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p.92). Each of the above mentioned classes of penetration enhancers are described below in greater detail.

Surfactants: In connection with the present invention, surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of dsRNAs through the mucosa is enhanced. In addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p.92); and perfluorochemical emulsions, such as FC-43. Takahashi *et al.*, J. Pharm. Pharmacol., 1988, 40, 252).

Fatty acids: Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C_{1-10} alkyl esters thereof (*e.g.*,

methyl, isopropyl and t-butyl), and mono- and di-glycerides thereof (*i.e.*, oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, *etc.*) (Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p.92; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; El Hariri *et al.*, J. Pharm. Pharmacol., 1992, 44, 651-654).

Bile salts: The physiological role of bile includes the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 in: Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th Ed., Hardman *et al.* Eds., McGraw-Hill, New York, 1996, pp. 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus the term "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. Suitable bile salts include, for example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucholic acid (sodium glucholate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92; Swinyard, Chapter 39 In: Remington's Pharmaceutical Sciences, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, Pa., 1990, pages 782-783; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; Yamamoto *et al.*, J. Pharm. Exp. Ther., 1992, 263, 25; Yamashita *et al.*, J. Pharm. Sci., 1990, 79, 579-583).

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

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

Agents that enhance uptake of dsRNAs at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi *et al*, U.S. Pat. No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo *et al.*, PCT Application WO 97/30731), are also known to enhance the cellular uptake of dsRNAs.

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

Carriers

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

Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (*i.e.*, does not possess biological activity per se) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting

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

Excipients

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

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

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

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

Other Components

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

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

Combination therapy

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

In one aspect of the invention, the subject compounds may be administered in combination with one or more separate agents that modulate protein kinases involved in various disease states. Examples of such kinases may include, but are not limited to: serine/threonine

specific kinases, receptor tyrosine specific kinases and non-receptor tyrosine specific kinases. Serine/threonine kinases include mitogen activated protein kinases (MAPK), meiosis specific kinase (MEK), RAF and aurora kinase. Examples of receptor kinase families include epidermal growth factor receptor (EGFR) (*e.g.*, HER2/neu, HER3, HER4, ErbB, ErbB2, ErbB3, ErbB4, Xmrk, DER, Let23); fibroblast growth factor (FGF) receptor (*e.g.* FGF-Rl, GFF-R2/BEK/CEK3, FGF-R3/CEK2, FGF-R4/TKF, KGF-R); hepatocyte growth/scatter factor receptor (HGFR) (*e.g.*, MET, RON, SEA, SEX); insulin receptor (*e.g.* IGFI-R); Eph (*e.g.* CEK5, CEK8, EBK, ECK, EEK, EHK-I, EHK-2, ELK, EPH, ERK, HEK, MDK2, MDK5, SEK); AxI (*e.g.* Mer/Nyk, Rse); RET; and platelet- derived growth factor receptor (PDGFR) (*e.g.* PDGFα-R, PDGβ-R, CSFl - R/FMS, SCF- R/C-KIT, VEGF-R/FLT, NEK/FLK1, FLT3/FLK2/STK-1). Non-receptor tyrosine kinase families include, but are not limited to, BCR-ABL (*e.g.* $p43^{abl}$, ARG); BTK (*e.g.* ITK/EMT, TEC); CSK, FAK, FPS, JAK, SRC, BMX, FER, CDK and SYK.

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

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

In certain preferred embodiments, the compounds of the invention are administered in combination with a chemotherapeutic agent. Chemotherapeutic agents encompass a wide range of therapeutic treatments in the field of oncology. These agents are administered at various stages of the disease for the purposes of shrinking tumors, destroying remaining cancer cells left over after surgery, inducing remission, maintaining remission and/or alleviating symptoms relating to the cancer or its treatment. Examples of such agents include, but are not limited to, alkylating agents such as mustard gas derivatives (Mechlorethamine, cylophosphamide, chlorambucil, melphalan, ifosfamide), ethylenimines (thiotepa, hexamethylmelanine), Alkylsulfonates (Busulfan), Hydrazines and Triazines (Altretamine, Procarbazine, Dacarbazine and Temozolomide), Nitrosoureas (Carmustine, Lomustine and Streptozocin), Ifosfamide and metal salts (Carboplatin, Cisplatin, and Oxaliplatin); plant alkaloids such as Podophyllotoxins

(Etoposide and Tenisopide), Taxanes (Paclitaxel and Docetaxel), Vinca alkaloids (Vincristine, Vinblastine, Vindesine and Vinorelbine), and Camptothecan analogs (Irinotecan and Topotecan); anti-tumor antibiotics such as Chromomycins (Dactinomycin and Plicamycin), Anthracyclines (Doxorubicin, Daunorubicin, Epirubicin, Mitoxantrone, Valrubicin and Idarubicin), and miscellaneous antibiotics such as Mitomycin, Actinomycin and Bleomycin; anti-metabolites such as folic acid antagonists (Methotrexate, Pemetrexed, Raltitrexed, Aminopterin), pyrimidine antagonists (5-Fluorouracil, Floxuridine, Cytarabine, Capecitabine, and Gemcitabine), purine antagonists (6-Mercaptopurine and 6-Thioguanine) and adenosine deaminase inhibitors (Cladribine, Fludarabine, Mercaptopurine, Clofarabine, Thioguanine, Nelarabine and Pentostatin); topoisomerase inhibitors such as topoisomerase I inhibitors (Ironotecan, topotecan) and topoisomerase II inhibitors (Amsacrine, etoposide, etoposide phosphate, teniposide); monoclonal antibodies (Alemtuzumab, Gemtuzumab ozogamicin, Rituximab, Trastuzumab, Ibritumomab Tioxetan, Cetuximab, Panitumumab, Tositumomab, Bevacizumab); and miscellaneous anti-neoplasties such as ribonucleotide reductase inhibitors (Hydroxyurea); adrenocortical steroid inhibitor (Mitotane); enzymes (Asparaginase and Pegaspargase); anti-microtubule agents (Estramustine); and retinoids (Bexarotene, Isotretinoin, Tretinoin (ATRA). In certain preferred embodiments, the compounds of the invention are administered in combination with a chemoprotective agent. Chemoprotective agents act to protect the body or minimize the side effects of chemotherapy. Examples of such agents include, but are not limited to, amfostine, mesna, and dexrazoxane.

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

It will be appreciated that compounds of the invention can be used in combination with an immunotherapeutic agent. One form of immunotherapy is the generation of an active systemic tumor-specific immune response of host origin by administering a vaccine composition at a site distant from the tumor. Various types of vaccines have been proposed, including isolated tumor-antigen vaccines and anti-idiotype vaccines. Another approach is to use tumor cells from the

subject to be treated, or a derivative of such cells (reviewed by Schirrmacher *et al.* (1995) J. Cancer Res. Clin. Oncol. 121 :487). In U.S. Pat. No. 5,484,596, Hanna Jr. *et al.* claim a method for treating a resectable carcinoma to prevent recurrence or metastases, comprising surgically removing the tumor, dispersing the cells with collagenase, irradiating the cells, and vaccinating the patient with at least three consecutive doses of about 10^7 cells.

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

The compounds of the invention can be co-administered with siRNA that target other genes. For example, a compound of the invention can be co-administered with an siRNA targeted to a c-Myc gene. In one example, AD-12115 can be co-administered with a c-Myc

siRNA. Examples of c-Myc targeted siRNAs are disclosed in United States patent application number 12/373,039 which is herein incorporated by reference.

Methods for treating diseases caused by expression of the Eg5 and VEGF genes

The invention relates in particular to the use of a composition containing at least two dsRNAs, one targeting an Eg5 gene, and one targeting a VEGF gene, for the treatment of a cancer, such as liver cancer, *e.g.*, for inhibiting tumor growth and tumor metastasis. For example, a composition, such as pharmaceutical composition, may be used for the treatment of solid tumors, like intrahepatic tumors such as may occur in cancers of the liver. A composition containing a dsRNA targeting Eg5 and a dsRNA targeting VEGF may also be used to treat other tumors and cancers, such as breast cancer, lung cancer, head and neck cancer, brain cancer, abdominal cancer, colon cancer, colorectal cancer, esophagus cancer, gastrointestinal cancer, glioma, tongue cancer, neuroblastoma, osteosarcoma, ovarian cancer, pancreatic cancer, prostate cancer, retinoblastoma, Wilm's tumor, multiple myeloma and for the treatment of skin cancer, like melanoma, for the treatment of lymphomas and blood cancer. The invention further relates to the use of a composition containing an Eg5 dsRNA and a VEGF dsRNA for inhibiting accumulation of ascites fluid and pleural effusion in different types of cancer, *e.g.*, liver cancer, breast cancer, lung cancer, head cancer, neck cancer, brain cancer, abdominal cancer, colon cancer, colorectal cancer, esophagus cancer, gastrointestinal cancer, glioma, tongue cancer, neuroblastoma, osteosarcoma, ovarian cancer, pancreatic cancer, prostate cancer, retinoblastoma, Wilm's tumor, multiple myeloma, skin cancer, melanoma, lymphomas and blood cancer. Owing to the inhibitory effects on Eg5 and VEGF expression, a composition according to the invention or a pharmaceutical composition prepared therefrom can enhance the quality of life.

In one embodiment, a patient having a tumor associated with AFP expression, or a tumor secreting AFP, *e.g.*, a hepatoma or teratoma, is treated. In certain embodiments, the patient has a malignant teratoma, an endodermal sinus tumor (yolk sac carcinoma), a neuroblastoma, a hepatoblastoma, a heptocellular carcinoma, testicular cancer or ovarian cancer.

The invention furthermore relates to the use of a dsRNA or a pharmaceutical composition thereof, *e.g.*, for treating cancer or for preventing tumor metastasis, in combination with other pharmaceuticals and/or other therapeutic methods, *e.g.*, with known pharmaceuticals and/or known therapeutic methods, such as, for example, those which are currently employed for treating cancer and/or for preventing tumor metastasis. Preference is given to a combination with radiation therapy and chemotherapeutic agents, such as cisplatin, cyclophosphamide, 5-fluorouracil, adriamycin, daunorubicin or tamoxifen.

The invention can also be practiced by including with a specific RNAi agent, in combination with another anti-cancer chemotherapeutic agent, such as any conventional chemotherapeutic agent. The combination of a specific binding agent with such other agents can potentiate the chemotherapeutic protocol. Numerous chemotherapeutic protocols will present themselves in the mind of the skilled practitioner as being capable of incorporation into the method of the invention. Any chemotherapeutic agent can be used, including alkylating agents, antimetabolites, hormones and antagonists, radioisotopes, as well as natural products. For example, the compound of the invention can be administered with antibiotics such as doxorubicin and other anthracycline analogs, nitrogen mustards such as cyclophosphamide, pyrimidine analogs such as 5-fluorouracil, cisplatin, hydroxyurea, taxol and its natural and synthetic derivatives, and the like. As another example, in the case of mixed tumors, such as adenocarcinoma of the breast, where the tumors include gonadotropin-dependent and gonadotropin-independent cells, the compound can be administered in conjunction with leuprolide or goserelin (synthetic peptide analogs of LH-RH). Other antineoplastic protocols include the use of a tetracycline compound with another treatment modality, *e.g.*, surgery, radiation, *etc.*, also referred to herein as "adjunct antineoplastic modalities." Thus, the method of the invention can be employed with such conventional regimens with the benefit of reducing side effects and enhancing efficacy.

Methods for inhibiting expression of the Eg5 gene and the VEGF gene

In yet another aspect, the invention provides a method for inhibiting the expression of the Eg5 gene and the VEGF gene in a mammal. The method includes administering a composition featured in the invention to the mammal such that expression of the target Eg5 gene and the target VEGF gene is silenced.

In one embodiment, a method for inhibiting Eg5 gene expression and VEGF gene expression includes administering a composition containing two different dsRNA molecules, one having a nucleotide sequence that is complementary to at least a part of an RNA transcript of the Eg5 gene and the other having a nucleotide sequence that is complementary to at least a part of an RNA transcript of the VEGF gene of the mammal to be treated. When the organism to be treated is a mammal such as a human, the composition may be administered by any means known in the art including, but not limited to oral or parenteral routes, including intravenous, intramuscular, subcutaneous, transdermal, airway (aerosol), nasal, rectal, and topical (including buccal and sublingual) administration. In preferred embodiments, the compositions are administered by intravenous infusion or injection.

Methods of preparing lipid particles

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

As described above, several of these cationic lipids are amino lipids that are charged at a pH below the pK_a of the amino group and substantially neutral at a pH above the pK_a. These cationic lipids are termed titratable cationic lipids and can be used in the formulations of the invention using a two-step process. First, lipid vesicles can be formed at the lower pH with titratable cationic lipids and other vesicle components in the presence of nucleic acids. In this manner, the vesicles will encapsulate and entrap the nucleic acids. Second, the surface charge of the newly formed vesicles can be neutralized by increasing the pH of the medium to a level above the pK_a of the titratable cationic lipids present, *i.e.*, to physiological pH or higher. Particularly advantageous aspects of this process include both the facile removal of any surface adsorbed nucleic acid and a resultant nucleic acid delivery vehicle which has a neutral surface. Liposomes or lipid particles having a neutral surface are expected to avoid rapid clearance from circulation and to avoid certain toxicities which are associated with cationic liposome preparations. Additional details concerning these uses of such titratable cationic lipids in the formulation of nucleic acid-lipid particles are provided in US Patent 6,287,591 and US Patent 6,858,225, incorporated herein by reference.

It is further noted that the vesicles formed in this manner provide formulations of uniform vesicle size with high content of nucleic acids. Additionally, the vesicles have a size range of from about 30 to about 150 nm, more preferably about 30 to about 90 nm.

Without intending to be bound by any particular theory, it is believed that the very high efficiency of nucleic acid encapsulation is a result of electrostatic interaction at low pH. At

acidic pH (e.g. pH 4.0) the vesicle surface is charged and binds a portion of the nucleic acids through electrostatic interactions. When the external acidic buffer is exchanged for a more neutral buffer (*e.g.*. pH 7.5) the surface of the lipid particle or liposome is neutralized, allowing any external nucleic acid to be removed. More detailed information on the formulation process is provided in various publications (*e.g.*, US Patent 6,287,591 and US Patent 6,858,225).

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

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

In preparing the nucleic acid-lipid particles of the invention, the mixture of lipids is typically a solution of lipids in an organic solvent. This mixture of lipids can then be dried to form a thin film or lyophilized to form a powder before being hydrated with an aqueous buffer to form liposomes. Alternatively, in a preferred method, the lipid mixture can be solubilized in a water miscible alcohol, such as ethanol, and this ethanolic solution added to an aqueous buffer resulting in spontaneous liposome formation. In most embodiments, the alcohol is used in the form in which it is commercially available. For example, ethanol can be used as absolute ethanol (100%), or as 95% ethanol, the remainder being water. This method is described in more detail in US Patent 5,976,567).

In accordance with the invention, the lipid mixture is combined with a buffered aqueous solution that may contain the nucleic acids. The buffered aqueous solution of is typically a solution in which the buffer has a pH of less than the pK_a of the protonatable lipid in the lipid mixture. Examples of suitable buffers include citrate, phosphate, acetate, and MES. A

particularly preferred buffer is citrate buffer. Preferred buffers will be in the range of 1-1000 mM of the anion, depending on the chemistry of the nucleic acid being encapsulated, and optimization of buffer concentration may be significant to achieving high loading levels (*see, e.g.,* US Patent 6,287,591 and US Patent 6,858,225). Alternatively, pure water acidified to pH 5-6 with chloride, sulfate or the like may be useful. In this case, it may be suitable to add 5% glucose, or another non-ionic solute which will balance the osmotic potential across the particle membrane when the particles are dialyzed to remove ethanol, increase the pH, or mixed with a pharmaceutically acceptable carrier such as normal saline. The amount of nucleic acid in buffer can vary, but will typically be from about 0.01 mg/mL to about 200 mg/mL, more preferably from about 0.5 mg/mL to about 50 mg/mL.

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

Optionally, the lipid-encapsulated therapeutic agent (*e.g.*, nucleic acid) complexes which are produced by combining the lipid mixture and the buffered aqueous solution of therapeutic agents (nucleic acids) can be sized to achieve a desired size range and relatively narrow distribution of lipid particle sizes. Preferably, the compositions provided herein will be sized to a mean diameter of from about 70 to about 200 nm, more preferably about 90 to about 130 nm. Several techniques are available for sizing liposomes to a desired size. One sizing method is described in U.S. Pat. No. 4,737,323, incorporated herein by reference. Sonicating a liposome suspension either by bath or probe sonication produces a progressive size reduction down to small unilamellar vesicles (SUVs) less than about 0.05 microns in size. Homogenization is

another method which relies on shearing energy to fragment large liposomes into smaller ones. In a typical homogenization procedure, multilamellar vesicles are recirculated through a standard emulsion homogenizer until selected liposome sizes, typically between about 0.1 and 0.5 microns, are observed. In both methods, the particle size distribution can be monitored by conventional laser-beam particle size determination. For certain methods herein, extrusion is used to obtain a uniform vesicle size.

Extrusion of liposome compositions through a small-pore polycarbonate membrane or an asymmetric ceramic membrane results in a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired liposome complex size distribution is achieved. The liposomes may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in liposome size. In some instances, the lipid-nucleic acid compositions which are formed can be used without any sizing.

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

Optionally the lipid vesicles (*i.e.*, lipid particles) can be formed by hydration in an aqueous buffer and sized using any of the methods described above prior to addition of the nucleic acid. As described above, the aqueous buffer should be of a pH below the pKa of the amino lipid. A solution of the nucleic acids can then be added to these sized, preformed vesicles. To allow encapsulation of nucleic acids into such "pre-formed" vesicles the mixture should contain an alcohol, such as ethanol. In the case of ethanol, it should be present at a concentration of about 20% (w/w) to about 45% (w/w). In addition, it may be necessary to warm the mixture of pre-formed vesicles and nucleic acid in the aqueous buffer-ethanol mixture to a temperature of about 25° C to about 50° C depending on the composition of the lipid vesicles and the nature of the nucleic acid. It will be apparent to one of ordinary skill in the art that optimization of the encapsulation process to achieve a desired level of nucleic acid in the lipid vesicles will require

manipulation of variable such as ethanol concentration and temperature. Examples of suitable conditions for nucleic acid encapsulation are provided in the Examples. Once the nucleic acids are encapsulated within the preformed vesicles, the external pH can be increased to at least partially neutralize the surface charge. Unencapsulated and surface adsorbed nucleic acids can then be removed as described above.

Method of Use

The lipid particles of the invention may be used to deliver a therapeutic agent to a cell, *in vitro* or *in vivo*. In particular embodiments, the therapeutic agent is a nucleic acid, which is delivered to a cell using a nucleic acid-lipid particles of the invention. While the following description of various methods of using the lipid particles and related pharmaceutical compositions of the invention are exemplified by description related to nucleic acid-lipid particles, it is understood that these methods and compositions may be readily adapted for the delivery of any therapeutic agent for the treatment of any disease or disorder that would benefit from such treatment.

In certain embodiments, the invention provides methods for introducing a nucleic acid into a cell. Preferred nucleic acids for introduction into cells are siRNA, immune-stimulating oligonucleotides, plasmids, antisense and ribozymes. These methods may be carried out by contacting the particles or compositions of the invention with the cells for a period of time sufficient for intracellular delivery to occur.

The compositions of the invention can be adsorbed to almost any cell type. Once adsorbed, the nucleic acid-lipid particles can either be endocytosed by a portion of the cells, exchange lipids with cell membranes, or fuse with the cells. Transfer or incorporation of the nucleic acid portion of the complex can take place via any one of these pathways. Without intending to be limited with respect to the scope of the invention, it is believed that in the case of particles taken up into the cell by endocytosis the particles then interact with the endosomal membrane, resulting in destabilization of the endosomal membrane, possibly by the formation of non-bilayer phases, resulting in introduction of the encapsulated nucleic acid into the cell cytoplasm. Similarly in the case of direct fusion of the particles with the cell plasma membrane, when fusion takes place, the liposome membrane is integrated into the cell membrane and the contents of the liposome combine with the intracellular fluid. Contact between the cells and the lipid-nucleic acid compositions, when carried out *in vitro*, will take place in a biologically compatible medium. The concentration of compositions can vary widely depending on the particular application, but is generally between about 1 μmol and about 10 mmol. In certain embodiments, treatment of the cells with the lipid-nucleic acid compositions will generally be

carried out at physiological temperatures (about 37°C) for periods of time from about 1 to 24 hours, preferably from about 2 to 8 hours. For *in vitro* applications, the delivery of nucleic acids can be to any cell grown in culture, whether of plant or animal origin, vertebrate or invertebrate, and of any tissue or type. In preferred embodiments, the cells will be animal cells, more preferably mammalian cells, and most preferably human cells.

In one group of embodiments, a lipid-nucleic acid particle suspension is added to 60-80% confluent plated cells having a cell density of from about 10^3 to about 10^5 cells/mL, more preferably about 2×10^4 cells/mL. The concentration of the suspension added to the cells is preferably of from about 0.01 to 20 μg/mL, more preferably about 1 μg/mL.

Typical applications include using well known procedures to provide intracellular delivery of siRNA to knock down or silence specific cellular targets. Alternatively applications include delivery of DNA or mRNA sequences that code for therapeutically useful polypeptides. In this manner, therapy is provided for genetic diseases by supplying deficient or absent gene products (*i.e.,* for Duchenne's dystrophy, see Kunkel, *et al., Brit. Med. Bull.* 45(3):630-643 (1989), and for cystic fibrosis, see Goodfellow, *Nature* 341:102-103 (1989)). Other uses for the compositions of the invention include introduction of antisense oligonucleotides in cells (see, Bennett, *et al., Mol. Pharm.* 41:1023-1033 (1992)).

Alternatively, the compositions of the invention can also be used for deliver of nucleic acids to cells *in vivo*, using methods which are known to those of skill in the art. With respect to application of the invention for delivery of DNA or mRNA sequences, Zhu, *et al., Science* 261:209-211 (1993), incorporated herein by reference, describes the intravenous delivery of cytomegalovirus (CMV)-chloramphenicol acetyltransferase (CAT) expression plasmid using DOTMA-DOPE complexes. Hyde, *et al., Nature* 362:250-256 (1993), incorporated herein by reference, describes the delivery of the cystic fibrosis transmembrane conductance regulator (CFTR) gene to epithelia of the airway and to alveoli in the lung of mice, using liposomes. Brigham, *et al., Am. J. Med. Sci.* 298:278-281 (1989), incorporated herein by reference, describes the *in vivo* transfection of lungs of mice with a functioning prokaryotic gene encoding the intracellular enzyme, chloramphenicol acetyltransferase (CAT). Thus, the compositions of the invention can be used in the treatment of infectious diseases.

For *in vivo* administration, the pharmaceutical compositions are preferably administered parenterally, *i.e.*, intraarticularly, intravenously, intraperitoneally, subcutaneously, or intramuscularly. In particular embodiments, the pharmaceutical compositions are administered intravenously or intraperitoneally by a bolus injection. For one example, see Stadler, *et al.*, U.S. Patent No. 5,286,634, which is incorporated herein by reference. Intracellular nucleic acid

delivery has also been discussed in Straubringer, *et al.,* METHODS IN ENZYMOLOGY, Academic Press, New York. 101:512-527 (1983); Mannino, *et al., Biotechniques* 6:682-690 (1988); Nicolau, *et al., Crit. Rev. Ther. Drug Carrier Syst.* 6:239-271 (1989), and Behr, *Acc. Chem. Res.* 26:274-278 (1993). Still other methods of administering lipid-based therapeutics are described in, for example, Rahman *et al.,* U.S. Patent No. 3,993,754; Sears, U.S. Patent No. 4,145,410; Papahadjopoulos *et al.,* U.S. Patent No. 4,235,871; Schneider, U.S. Patent No. 4,224,179; Lenk *et al.,* U.S. Patent No. 4,522,803; and Fountain *et al.,* U.S. Patent No. 4,588,578.

In other methods, the pharmaceutical preparations may be contacted with the target tissue by direct application of the preparation to the tissue. The application may be made by topical, "open" or "closed" procedures. By "topical," it is meant the direct application of the pharmaceutical preparation to a tissue exposed to the environment, such as the skin, oropharynx, external auditory canal, and the like. "Open" procedures are those procedures which include incising the skin of a patient and directly visualizing the underlying tissue to which the pharmaceutical preparations are applied. This is generally accomplished by a surgical procedure, such as a thoracotomy to access the lungs, abdominal laparotomy to access abdominal viscera, or other direct surgical approach to the target tissue. "Closed" procedures are invasive procedures in which the internal target tissues are not directly visualized, but accessed via inserting instruments through small wounds in the skin. For example, the preparations may be administered to the peritoneum by needle lavage. Likewise, the pharmaceutical preparations may be administered to the meninges or spinal cord by infusion during a lumbar puncture followed by appropriate positioning of the patient as commonly practiced for spinal anesthesia or metrazamide imaging of the spinal cord. Alternatively, the preparations may be administered through endoscopic devices.

The lipid-nucleic acid compositions can also be administered in an aerosol inhaled into the lungs (*see,* Brigham, *et al., Am. J. Sci.* 298(4):278-281 (1989)) or by direct injection at the site of disease (Culver, Human Gene Therapy, MaryAnn Liebert, Inc., Publishers, New York. pp.70-71 (1994)).

The methods of the invention may be practiced in a variety of hosts. Preferred hosts include mammalian species, such as humans, non-human primates, dogs, cats, cattle, horses, sheep, and the like.

Dosages for the lipid-therapeutic agent particles of the invention will depend on the ratio of therapeutic agent to lipid and the administrating physician's opinion based on age, weight, and condition of the patient.

In one embodiment, the invention provides a method of modulating the expression of a target polynucleotide or polypeptide. These methods generally comprise contacting a cell with a lipid particle of the invention that is associated with a nucleic acid capable of modulating the expression of a target polynucleotide or polypeptide. As used herein, the term "modulating" refers to altering the expression of a target polynucleotide or polypeptide. In different embodiments, modulating can mean increasing or enhancing, or it can mean decreasing or reducing. Methods of measuring the level of expression of a target polynucleotide or polypeptide are known and available in the arts and include, e.g., methods employing reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemical techniques. In particular embodiments, the level of expression of a target polynucleotide or polypeptide is increased or reduced by at least 10%, 20%, 30%, 40%, 50%, or greater than 50% as compared to an appropriate control value. For example, if increased expression of a polypeptide desired, the nucleic acid may be an expression vector that includes a polynucleotide that encodes the desired polypeptide. On the other hand, if reduced expression of a polynucleotide or polypeptide is desired, then the nucleic acid may be, *e.g.*, an antisense oligonucleotide, siRNA, or microRNA that comprises a polynucleotide sequence that specifically hybridizes to a polynucleotide that encodes the target polypeptide, thereby disrupting expression of the target polynucleotide or polypeptide. Alternatively, the nucleic acid may be a plasmid that expresses such an antisense oligonucleotide, siRNA, or microRNA.

In one particular embodiment, the invention provides a method of modulating the expression of a polypeptide by a cell, comprising providing to a cell a lipid particle that consists of or consists essentially of a cationic lipid of formula A, a neutral lipid, a sterol, a PEG of PEG-modified lipid, e.g., in a molar ratio of about 35-65% of cationic lipid of formula A, 3-12% of the neutral lipid, 15-45% of the sterol, and 0.5-10% of the PEG or PEG-modified lipid, wherein the lipid particle is associated with a nucleic acid capable of modulating the expression of the polypeptide. In particular embodiments, the molar lipid ratio is approximately 60/7.5/31/1.5 or 57.5/7.5/31.5/3.5 (mol% LIPID A/DSPC/Chol/PEG-DMG). In another group of embodiments, the neutral lipid in these compositions is replaced with DPPC (dipalmitoylphosphatidylcholine), POPC, DOPE or SM.

In particular embodiments, the therapeutic agent is selected from an siRNA, a microRNA, an antisense oligonucleotide, and a plasmid capable of expressing an siRNA, a microRNA, or an antisense oligonucleotide, and wherein the siRNA, microRNA, or antisense RNA comprises a polynucleotide that specifically binds to a polynucleotide that encodes the polypeptide, or a complement thereof, such that the expression of the polypeptide is reduced.

In other embodiments, the nucleic acid is a plasmid that encodes the polypeptide or a functional variant or fragment thereof, such that expression of the polypeptide or the functional variant or fragment thereof is increased.

In related embodiments, the invention provides a method of treating a disease or disorder characterized by overexpression of a polypeptide in a subject, comprising providing to the subject a pharmaceutical composition of the invention, wherein the therapeutic agent is selected from an siRNA, a microRNA, an antisense oligonucleotide, and a plasmid capable of expressing an siRNA, a microRNA, or an antisense oligonucleotide, and wherein the siRNA, microRNA, or antisense RNA comprises a polynucleotide that specifically binds to a polynucleotide that encodes the polypeptide, or a complement thereof.

In one embodiment, the pharmaceutical composition comprises a lipid particle that consists of or consists essentially of Lipid A, DSPC, Chol and PEG-DMG, PEG-C-DOMG or PEG-DMA, *e.g.*, in a molar ratio of about 35-65% of cationic lipid of formula A, 3-12% of the neutral lipid, 15-45% of the sterol, and 0.5-10% of the PEG or PEG-modified lipid PEG-DMG, PEG-C-DOMG or PEG-DMA, wherein the lipid particle is associated with the therapeutic nucleic acid. In particular embodiments, the molar lipid ratio is approximately 60/7.5/31/1.5 or 57.5/7.5/31.5/3.5 (mol% LIPID A/DSPC/Chol/PEG-DMG). In another group of embodiments, the neutral lipid in these compositions is replaced with DPPC, POPC, DOPE or SM.

In another related embodiment, the invention includes a method of treating a disease or disorder characterized by underexpression of a polypeptide in a subject, comprising providing to the subject a pharmaceutical composition of the invention, wherein the therapeutic agent is a plasmid that encodes the polypeptide or a functional variant or fragment thereof.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

EXAMPLES

Example 1. dsRNA synthesis

Source of reagents

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

siRNA synthesis

For screening of dsRNA, single-stranded RNAs were produced by solid phase synthesis on a scale of 1 µmole using an Expedite 8909 synthesizer (Applied Biosystems, Applera Deutschland GmbH, Darmstadt, Germany) and controlled pore glass (CPG, 500Å, Proligo Biochemie GmbH, Hamburg, Germany) as solid support. RNA and RNA containing *2'-O*-methyl nucleotides were generated by solid phase synthesis employing the corresponding phosphoramidites and *2'-O*-methyl phosphoramidites, respectively (Proligo Biochemie GmbH, Hamburg, Germany). These building blocks were incorporated at selected sites within the sequence of the oligoribonucleotide chain using standard nucleoside phosphoramidite chemistry such as described in Current protocols in nucleic acid chemistry, Beaucage, S.L. *et al.* (Edrs.), John Wiley & Sons, Inc., New York, NY, USA. Phosphorothioate linkages were introduced by replacement of the iodine oxidizer solution with a solution of the Beaucage reagent (Chruachem Ltd, Glasgow, UK) in acetonitrile (1%). Further ancillary reagents were obtained from Mallinckrodt Baker (Griesheim, Germany).

Deprotection and purification of the crude oligoribonucleotides by anion exchange HPLC were carried out according to established procedures. Yields and concentrations were determined by UV absorption of a solution of the respective RNA at a wavelength of 260 nm using a spectral photometer (DU 640B, Beckman Coulter GmbH, Unterschleißheim, Germany). Double stranded RNA was generated by mixing an equimolar solution of complementary strands in annealing buffer (20 mM sodium phosphate, pH 6.8; 100 mM sodium chloride), heated in a water bath at 85 - 90°C for 3 minutes and cooled to room temperature over a period of 3 - 4 hours. The annealed RNA solution was stored at –20 °C until use.

dsRNA targeting the Eg5 gene

Initial Screening set

siRNA design was carried out to identify siRNAs targeting Eg5 (also known as KIF11, HSKP, KNSL1 and TRIP5). Human mRNA sequences to Eg5, RefSeq ID number:NM_004523, was used.

siRNA duplexes cross-reactive to human and mouse Eg5 were designed. Twenty-four duplexes were synthesized for screening. (Table 1a). A second screening set was defined with 266 siRNAs targeting human Eg5, as well as its rhesus monkey ortholog (Table 2a). An expanded screening set was selected with 328 siRNA targeting human Eg5, with no necessity to hit any Eg5 mRNA of other species (Table 3a).

The sequences for human and a partial rhesus Eg5 mRNAs were downloaded from NCBI Nucleotide database and the human sequence was further on used as reference sequence (Human EG5:NM_004523.2, 4908 bp, and Rhesus EG5: XM_001087644.1, 878 bp (only 5' part of human EG5).

For the Tables: Key: A,G,C,U-ribonucleotides: T-deoxythymidine: u,c-2'-O-methyl nucleotides: s-phosphorothioate linkage.

Table 1a. Sequences of Eg5/ KSP dsRNA duplexes

position in human Eg5/KSP sequence	SEQ ID NO:	sequence of 23mer target site	SEQ ID NO:	sense sequence (5'-3')	SEQ ID No:	antisense sequence (5'-3')	duplex name
385-407	1244	ACCGAAGUGUUGUUUGUCCAAUU	1	cGAAGuGuuGuuuGuccAATsT	2	UUGGAcAAAcAAcACUUCGTsT	AL-DP-6226
347-369	1245	UAUGGUGUUUGGAGCAUCUACUA	3	uGGuGuuuGGAGcAucuAcTsT	4	GuAGAUGCUCcAAAcACcATsT	AL-DP-6227
1078-1100	1246	AAUCUAAACUAACUAGAAUCCUC	5	ucuAAAcuAAcuAGAAuccTsT	6	GGAUUCuAGUuAGUUuAGATsT	AL-DP-6228
1067-1089	1247	UCCUUAUCGAGAAUCUAAACUAA	7	cuuAucGAGAAucuAAAcuTsT	8	AGUUuAGAUUCUCGAuAAGTsT	AL-DP-6229
374-396	1248	GAUUGAUGUUUACCGAAGUGUUG	9	uuGAuGuuuAccGAAGuGuTsT	10	AcACUUCGGuAAAcAUcAATsT	AL-DP-6230
205-227	1249	UGGUGAGAUGCAGACCAUUUAAU	11	GuGAGAuGcAGAccAuuuATsT	12	uAAAUGGUCUGcAUCUcACTsT	AL-DP-6231
1176-1198	1250	ACUCUGAGUACAUUGGAAUAUGC	13	ucuGAGuAcAuuGGAAuAuTsT	14	AuAUUCcAAUGuACUcAGATsT	AL-DP-6232
386-408	1251	CCGAAGUGUUGUUUGUCCAAUUC	15	GAAGuGuuGuuuGuccAAuTsT	16	AUUGGAcAAAcAAcACUUCTsT	AL-DP-6233
416-438	1252	AGUUAUUAUGGGCUAUAAUUGCA	17	uuAuuAuGGGcuAuAAuuGTsT	18	cAAUuAuAGCCcAuAAuAATsT	AL-DP-6234
485-507	1253	GGAAGGUGAAAGGUCACCUAAUG	19	AAGGuGAAAGGucAccuAATsT	20	UuAGGUGACCUUUcACCUUTsT	AL-DP-6235
476-498	1254	UUUUACAAUGGAAGGUGAAAGGU	21	uuAcAAuGGAAGGuGAAAGTsT	22	CUUUcACCUUCcAUUGuAATsT	AL-DP-6236
486-508	1255	GAAGGUGAAAGGUCACCUAAUGA	23	AGGuGAAAGGucAccuAAuTsT	24	AUuAGGUGACCUUUcACCUTsT	AL-DP-6237
487-509	1256	AAGGUGAAAGGUCACCUAAUGAA	25	GGuGAAAGGucAccuAAuGTsT	26	cAUuAGGUGACCUUUcACCTsT	AL-DP-6238
1066-1088	1257	UUCCUUAUCGAGAAUCUAAACUA	27	ccuuAucGAGAAucuAAAcTsT	28	GUUuAGAUUCUCGAuAAGGTsT	AL-DP-6239
1256-1278	1258	AGCUCUUAUUAAGGAGUAUACGG	29	cucuuAuuAAGGAGuAuAcTsT	30	GuAuACUCCUuAAuAAGAGTsT	AL-DP-6240
2329-2351	1259	CAGAGAGAUUCUGUGCUUUGGAG	31	GAGAGAuucuGuGcuuuGGTsT	32	CcAAAGcAcAGAAUCUCUCTsT	AL-DP-6241
1077-1099	1260	GAAUCUAAACUAACUAGAAUCCU	33	AucuAAAcuAAcuAGAAucTsT	34	GAUUCuAGUuAGUUuAGAUTsT	AL-DP-6242
1244-1266	1261	ACUCACCAAAAAAGCUCUUAUUA	35	ucAccAAAAAAGcucuuATsT	36	AuAAGAGCUUUUUUGGUGATsT	AL-DP-6243
637-659	1262	AAGAGCUUUUUGAUCUUCUUAAU	37	GAGcuuuuuGAucuucuuATsT	38	uAAGAAGAUcAAAAAGCUCTsT	AL-DP-6244
1117-1139	1263	GGCGUACAAGAACAUCUAUAAUU	39	cGuAcAAGAAcAucuAuATsT	40	UuAuAGAUGUUCUUGuACGTsT	AL-DP-6245
373-395	1264	AGAUUGAUGUUUACCGAAGUGUU	41	AuuGAuGuuuAccGAAGuGTsT	42	cACUUCGGuAAAcAUcAAUTsT	AL-DP-6246

1079-1101	1265	AUCUAAACUAACUAGAAUCCUCC	43	cuAAAcuAAcuAGAAuccuTsT	44	AGGAUUCuAGUuAGUUuAGTsT	AL-DP-6247
383-405	1266	UUACCGAAGUGUUGUUUGUCCAA	45	AccGAAGuGuuGuuuGuccTsT	46	GGAcAAAcAAcACUUCGGUTsT	AL-DP-6248
200-222	1267	GGUGGUGGUGAGAUGCAGACCAU	47	uGGuGGuGAGAuGcAGAccTsT	48	GGUCUGcAUCUcACcACcATsT	AL-DP-6249

Table 1b. Analysis of Eg5/KSP ds duplexes

duplex name	single dose screen @ 25 nM [% residual mRNA]	SDs 2nd screen (among quadruplicates)
AL-DP-6226	23%	3%
AL-DP-6227	69%	10%
AL-DP-6228	33%	2%
AL-DP-6229	2%	2%
AL-DP-6230	66%	11%
AL-DP-6231	17%	1%
AL-DP-6232	9%	3%
AL-DP-6233	24%	6%
AL-DP-6234	91%	2%
AL-DP-6235	112%	4%
AL-DP-6236	69%	4%
AL-DP-6237	42%	2%
AL-DP-6238	45%	2%
AL-DP-6239	2%	1%
AL-DP-6240	48%	2%
AL-DP-6241	41%	2%
AL-DP-6242	8%	2%
AL-DP-6243	7%	1%
AL-DP-6244	6%	2%
AL-DP-6245	12%	2%
AL-DP-6246	28%	3%
AL-DP-6247	71%	4%
AL-DP-6248	5%	2%
AL-DP-6249	28%	3%

Table 2a. Sequences of Eg5/ KSP dsRNA duplexes

SEQ ID NO:	sequence of 19-mer target site	SEQ ID NO.	sense sequence (5´-3´)	SEQ ID NO.	antisense sequence (5´-3´)	duplex name
1268	CAUACUCUAGUCGUUCCCA	49	cAuAcucuAGucGuucccATsT	50	UGGGAACGACuAGAGuAUGTsT	AD-12072
1269	AGCGCCCAUUCAAUAGUAG	51	AGcGcccAuucAAuAGuAGTsT	52	CuACuAUUGAAUGGGCGCUTsT	AD-12073
1270	GGAAAGCUAGCGCCCAUUC	53	GGAAAGcuAGcGcccAuucTsT	54	GAAUGGGCGCuAGCUUUCCTsT	AD-12074
1271	GAAAGCUAGCGCCCAUUCA	55	GAAAGcuAGcGcccAuucATsT	56	UGAAUGGGCGCuAGCUUUCTsT	AD-12075
1272	AGAAACUACGAUUGAUGGA	57	AGAAAcuAcGAuuGAuGGATsT	58	UCcAUcAAUCGuAGUUUCUTsT	AD-12076
1273	UGUUCCUUAUCGAGAAUCU	59	uGuuccuuAucGAGAAucuTsT	60	AGAUUCUCGAuAAGGAAcATsT	AD-12077
1274	CAGAUUACCUCUGCGAGCC	61	cAGAuuAccucuGcGAGccTsT	62	GGCUCGcAGAGGuAAUCUGTsT	AD-12078
1275	GCGCCCAUUCAAUAGUAGA	63	GcGcccAuucAAuAGuAGATsT	64	UCuACuAUUGAAUGGGCGCTsT	AD-12079
1276	UUGCACUAUCUUUGCGUAU	65	uuGcAcuAucuuuGcGuAuTsT	66	AuACGcAAAGAuAGUGcAATsT	AD-12080
1277	CAGAGCGGAAAGCUAGCGC	67	cAGAGcGGAAAGcuAGcGcTsT	68	GCGCuAGCUUUCCGCUCUGTsT	AD-12081
1278	AGACCUUAUUUGGUAAUCU	69	AGAccuuAuuuGGuAAucuTsT	70	AGAUuACcAAAuAAGGUCUTsT	AD-12082
1279	AUUCUCUUGGAGGGCGUAC	71	AuucucuuGGAGGGcGuAcTsT	72	GuACGCCCUCcAAGAGAAUTsT	AD-12083
1280	GGCUGGUAUAAUUCCACGU	73	GGcuGGuAuAAuuccAcGuTsT	74	ACGUGGAAUuAuACcAGCCTsT	AD-12084
1281	GCGGAAAGCUAGCGCCCAU	75	GcGGAAAGcuAGcGcccAuTsT	76	AUGGGCGCuAGCUUUCCGCTsT	AD-12085
1282	UGCACUAUCUUUGCGUAUG	77	uGcAcuAucuuuGcGuAuGTsT	78	cAuACGcAAAGAuAGUGcATsT	AD-12086
1283	GUAUAAUUCCACGUACCCU	79	GuAuAAuuccAcGuAcccuTsT	80	AGGGuACGUGGAAUuAuACTsT	AD-12087
1284	AGAAUCUAAACUAACUAGA	81	AGAAucuAAAcuAAcuAGATsT	82	UCuAGUuAGUUuAGAUUCUTsT	AD-12088

SEQ ID NO:	sequence of 19-mer target site	SEQ ID NO.	sense sequence (5′-3′)	SEQ ID NO.	antisense sequence (5′-3′)	duplex name
1285	AGGAGCUGAAUAGGGUUAC	83	AGGAGcuGAAuAGGGuuAcTsT	84	GuAACCCuAUUcAGCUCCUTsT	AD-12089
1286	GAAGUACAUAAGACCUUAU	85	GAAGuAcAuAAGAccuuAuTsT	86	AuAAGGUCUuAUGuACUUCTsT	AD-12090
1287	GACAGUGGCCGAUAAGAUA	87	GAcAGuGGccGAuAAGAuATsT	88	uAUCUuAUCGGCcACUGUCTsT	AD-12091
1288	AAACCACUUAGUAGUGUCC	89	AAAccAcuuAGuAGuGuccTsT	90	GGAcACuACuAAGUGGUUUTsT	AD-12092
1289	UCCCUAGACUUCCCUAUUU	91	ucccuAGAcuucccuAuuuTsT	92	AAAuAGGGAAGUCuAGGGATsT	AD-12093
1290	UAGACUUCCCUAUUUCGCU	93	uAGAcuucccuAuuucGcuTsT	94	AGCGAAAuAGGGAAGUCuATsT	AD-12094
1291	GCGUCGCAGCCAAAUUCGU	95	GcGucGcAGccAAAuucGuTsT	96	ACGAAUUUGGCUGCGACGCTsT	AD-12095
1292	AGCUAGCGCCCAUUCAAUA	97	AGcuAGcGcccAuucAAuATsT	98	uAUUGAAUGGGCGCuAGCUTsT	AD-12096
1293	GAAACUACGAUUGAUGGAG	99	GAAAcuAcGAuuGAuGGAGTsT	100	CUCcAUcAAUCGuAGUUUCTsT	AD-12097
1294	CCGAUAAGAUAGAAGAUCA	101	ccGAuAAGAuAGAAGAucATsT	102	UGAUCUUCuAUCUuAUCGGTsT	AD-12098
1295	UAGCGCCCAUUCAAUAGUA	103	uAGcGcccAuucAAuAGuATsT	104	uACuAUUGAAUGGGCGCuATsT	AD-12099
1296	UUUGCGUAUGGCCAAACUG	105	uuuGcGuAuGGccAAAcuGTsT	106	cAGUUUGGCcAuACGcAAATsT	AD-12100
1297	CACGUACCCUUCAUCAAAU	107	cACGuAcccuucAucAAAuTsT	108	AUUUGAUGAAGGGuACGUGTsT	AD-12101
1298	UCUUUGCGUAUGGCCAAAC	109	ucuuuGcGuAuGGccAAAcTsT	110	GUUUGGCcAuACGcAAAGATsT	AD-12102
1299	CCGAAGUGUUGUUUGUCCA	111	ccGAAGuGuuGuuuGuccATsT	112	UGGAcAAAcAAcACUUCGGTsT	AD-12103
1300	AGAGCGGAAAGCUAGCGCC	113	AGAGcGGAAAGcuAGcGccTsT	114	GGCGCuAGCUUUCCGCUCUTsT	AD-12104
1301	GCUAGCGCCCAUUCAAUAG	115	GcuAGcGcccAuucAAuAGTsT	116	CuAUUGAAUGGGCGCuAGCTsT	AD-12105
1302	AAGUUAGUGUACGAACUGG	117	AAGuuAGuGuAcGAAcuGGTsT	118	CcAGUUCGuAcACuAACUUTsT	AD-12106
1303	GUACGAACUGGAGGAUUGG	119	GuAcGAAcuGGAGGAuuGGTsT	120	CcAAUCCUCcAGUUCGuACTsT	AD-12107
1304	ACGAACUGGAGGAUUGGCU	121	AcGAAcuGGAGGAuuGGcuTsT	122	AGCcAAUCCUCcAGUUCGUTsT	AD-12108
1305	AGAUUGAUGUUUACCGAAG	123	AGAuuGAuGuuuAccGAAGTsT	124	CUUCGGuAAAcAUcAAUCUTsT	AD-12109
1306	UAUGGGCUAUAAUUGCACU	125	uAuGGGcuAuAAuuGcAcuTsT	126	AGUGcAAUuAuAGCCcAuATsT	AD-12110
1307	AUCUUUGCGUAUGGCCAAA	127	AucuuuGcGuAuGGccAAATsT	128	UUUGGCcAuACGcAAAGAUTsT	AD-12111
1308	ACUCUAGUCGUUCCCACUC	129	AcucuAGucGuucccAcucTsT	130	GAGUGGGAACGACuAGAGUTsT	AD-12112
1309	AACUACGAUUGAUGGAGAA	131	AAcuAcGAuuGAuGGAGAATsT	132	UUCUCcAUcAAUCGuAGUUTsT	AD-12113
1310	GAUAAGAGAGCUCGGGAAG	133	GAuAAGAGAGcucGGGAAGTsT	134	CUUCCCGAGCUCUCUuAUCTsT	AD-12114
1311	UCGAGAAUCUAAACUAACU	135	ucGAGAAucuAAAcuAAcuTsT	136	AGUuAGUUuAGAUUCUCGATsT	AD-12115
1312	AACUAACUAGAAUCCUCCA	137	AAcuAAcuAGAAuccuccATsT	138	UGGAGGAUUCuAGUuAGUUTsT	AD-12116
1313	GGAUCGUAAGAAGGCAGUU	139	GGAucGuAAGAAGGcAGuuTsT	140	AACUGCCUUCUuACGAUCCTsT	AD-12117
1314	AUCGUAAGAAGGCAGUUGA	141	AucGuAAGAAGGcAGuuGATsT	142	UcAACUGCCUUCUuACGAUTsT	AD-12118
1315	AGGCAGUUGACCAACACAA	143	AGGcAGuuGAccAAcAcAATsT	144	UUGUGUUGGUcAACUGCCUTsT	AD-12119
1316	UGGCCGAUAAGAUAGAAGA	145	uGGccGAuAAGAuAGAAGATsT	146	UCUUCuAUCUuAUCGGCcATsT	AD-12120
1317	UCUAAGGAUAUAGUCAACA	147	ucuAAGGAuAuAGucAAcATsT	148	UGUUGACuAuAUCCUuAGATsT	AD-12121
1318	ACUAAGCUUAAUUGCUUUC	149	AcuAAGcuuAAuuGcuuucTsT	150	GAAAGcAAUuAAGCUuAGUTsT	AD-12122
1319	GCCCAGAUCAACCUUUAAU	151	GcccAGAucAAccuuuAAuTsT	152	AUuAAAGGUUGAUCUGGGCTsT	AD-12123
1320	UUAAUUUGGCAGAGCGGAA	153	uuAAuuuGGcAGAGcGGAATsT	154	UUCCGCUCUGCcAAAUuAATsT	AD-12124
1321	UUAUCGAGAAUCUAAACUA	155	uuAucGAGAAucuAAAcuATsT	156	uAGUUuAGAUUCUCGAuAATsT	AD-12125
1322	CUAGCGCCCAUUCAAUAGU	157	cuAGcGcccAuucAAuAGuTsT	158	ACuAUUGAAUGGGCGCuAGTsT	AD-12126
1323	AAUAGUAGAAUGUGAUCCU	159	AAuAGuAGAAuGuGAuccuTsT	160	AGGAUcAcAUUCuACuAUUTsT	AD-12127
1324	UACGAAAAGAAGUUAGUGU	161	uAcGAAAAGAAGuuAGuGuTsT	162	AcACuAACUUCUUUUCGuATsT	AD-12128
1325	AGAAGUUAGUGUACGAACU	163	AGAAGuuAGuGuAcGAAcuTsT	164	AGUUCGuAcACuAACUUCUTsT	AD-12129
1326	ACUAAACAGAUUGAUGUUU	165	AcuAAAcAGAuuGAuGuuuTsT	166	AAAcAUcAAUCUGUUuAGUTsT	AD-12130
1327	CUUUGCGUAUGGCCAAACU	167	cuuuGcGuAuGGccAAAcuTsT	168	AGUUUGGCcAuACGcAAAGTsT	AD-12131
1328	AAUGAAGAGUAUACCUGGG	169	AAuGAAGAGuAuAccuGGGTsT	170	CCcAGGuAuACUCUUcAUUTsT	AD-12132
1329	AUAAUUCCACGUACCCUUC	171	AuAAuucCAcGuAcccuucTsT	172	GAAGGGuACGUGGAAUuAUTsT	AD-12133
1330	ACGUACCCUUCAUCAAAUU	173	AcGuAcccuucAucAAAuuTsT	174	AAUUUGAUGAAGGGuACGUTsT	AD-12134
1331	CGUACCCUUCAUCAAAUUU	175	cGuAcccuucAucAAAuuuTsT	176	AAAUUUGAUGAAGGGuACGTsT	AD-12135
1332	GUACCCUUCAUCAAAUUUU	177	GuAcccuucAucAAAuuuuTsT	178	AAAAUUUGAUGAAGGGuACTsT	AD-12136
1333	AACUUACUGAUAAUGGUAC	179	AAcuuAcuGAuAAuGGuAcTsT	180	GuACcAUuAUcAGuAAGUUTsT	AD-12137
1334	UUCAGUCAAAGUGUCUCUG	181	uucAGucAAAGuGucucuGTsT	182	cAGAGAcACUUUGACUGAATsT	AD-12138
1335	UUCUUAAUCCAUCAUCUGA	183	uucuuAAuccAucAucuGATsT	184	UcAGAUGAUGGAUuAAGAATsT	AD-12139
1336	ACAGUACACAACAAGGAUG	185	AcAGuAcAcAAcAAGGAuGTsT	186	cAUCCUUGUUGUGuACUGUTsT	AD-12140
1337	AAGAAACUACGAUUGAUGG	187	AAGAAAcuAcGAuuGAuGGTsT	188	CcAUcAAUCGuAGUUUCUUTsT	AD-12141
1338	AAACUACGAUUGAUGGAGA	189	AAAcuAcGAuuGAuGGAGATsT	190	UCUCcAUcAAUCGuAGUUUTsT	AD-12142
1339	UGGAGCUGUUGAUAAGAGA	191	uGGAGcuGuuGAuAAGAGATsT	192	UCUCUuAUcAAcAGCUCcATsT	AD-12143
1340	CUAACUAGAAUCCUCCAGG	193	cuAAcuAGAAuccuccAGGTsT	194	CCUGGAGGAUUCuAGUuAGTsT	AD-12144
1341	GAAUAUGCUCAUAGAGCAA	195	GAAuAuGcucAuAGAGcAATsT	196	UUGCUCuAUGAGcAuAUUCTsT	AD-12145
1342	AUGCUCAUAGAGCAAAGAA	197	AuGcucAuAGAGcAAAGAATsT	198	UUCUUUGCUCuAUGAGcAUTsT	AD-12146
1343	AAAAAUUGGUGCUGUUGAG	199	AAAAAuuGGuGcuGuuGAGTsT	200	CUcAAcAGcACcAAUUUUUTsT	AD-12147
1344	GAGGAGCUGAAUAGGGUUA	201	GAGGAGcuGAAuAGGGuuATsT	202	uAACCCuAUUcAGCUCCUCTsT	AD-12148
1345	GGAGCUGAAUAGGGUUACA	203	GGAGcuGAAuAGGGuuAcATsT	204	UGuAACCCuAUUcAGCUCCTsT	AD-12149
1346	GAGCUGAAUAGGGUUACAG	205	GAGcuGAAuAGGGuuAcAGTsT	206	CUGuAACCCuAUUcAGCUCTsT	AD-12150
1347	AGCUGAAUAGGGUUACAGA	207	AGcuGAAuAGGGuuAcAGATsT	208	UCUGuAACCCuAUUcAGCUTsT	AD-12151
1348	GCUGAAUAGGGUUACAGAG	209	GcuGAAuAGGGuuAcAGAGTsT	210	CUCUGuAACCCuAUUcAGCTsT	AD-12152
1349	CCAAACUGGAUCGUAAGAA	211	ccAAAcuGGAucGuAAGAATsT	212	UUCUuACGAUCcAGUUUGGTsT	AD-12153
1350	GAUCGUAAGAAGGCAGUUG	213	GAucGuAAGAAGGcAGuuGTsT	214	cAACUGCCUUCUuACGAUCTsT	AD-12154
1351	ACCUUAUUUGGUAAUCUGC	215	AccuuAuuuGGuAAucuGcTsT	216	GcAGAUuACcAAAuAAGGUTsT	AD-12155
1352	UUAGAUACCAUUACUACAG	217	uuAGAuAccAuuAcuAcAGTsT	218	CUGuAGuAAUGGuAUCuAATsT	AD-12156
1353	AUACCAUUACUACAGUAGC	219	AuAccAuuAcuAcAGuAGcTsT	220	GCuACUGuAGuAAUGGuAUTsT	AD-12157
1354	UACUACAGUAGCACUUGGA	221	uAcuAcAGuAGcAcuuGGATsT	222	UCcAAGUGCuACUGuAGuATsT	AD-12158
1355	AAAGUAAAACUGUACUACA	223	AAAGuAAAAcuGuAcuAcATsT	224	UGuAGuAcAGUUUuACUUUTsT	AD-12159
1356	CUCAAGACUGAUCUUCUAA	225	cucAAGAcuGAucuucuAATsT	226	UuAGAAGAUcAGUCUUGAGTsT	AD-12160
1357	UUGACAGUGGCCGAUAAGA	227	uuGAcAGuGGccGAuAAGATsT	228	UCUuAUCGGCcACUGUcAATsT	AD-12161
1358	UGACAGUGGCCGAUAAGAU	229	uGAcAGuGGccGAuAAGAuTsT	230	AUCUuAUCGGCcACUGUcATsT	AD-12162

SEQ ID NO:	sequence of 19-mer target site	SEQ ID NO.	sense sequence (5′-3′)	SEQ ID NO.	antisense sequence (5′-3′)	duplex name
1359	GCAAUGUGGAAACCUAACU	231	GcAAuGuGGAAAccuAAcuTsT	232	AGUuAGGUUUCcAcAUUGCTsT	AD-12163
1360	CCACUUAGUAGUGUCCAGG	233	ccAcuuAGuAGuGuccAGGTsT	234	CCUGGAcACuACuAAGUGGTsT	AD-12164
1361	AGAAGGUACAAAAUUGGUU	235	AGAAGGuAcAAAAuuGGuuTsT	236	AACcAAUUUUGuACCUUCUTsT	AD-12165
1362	UGGUUUGACUAAGCUUAAU	237	uGGuuuGAcuAAGcuuAAuTsT	238	AUuAAGCUuAGUcAAACcATsT	AD-12166
1363	GGUUUGACUAAGCUUAAUU	239	GGuuuGAcuAAGcuuAAuuTsT	240	AAUuAAGCUuAGUcAAACCTsT	AD-12167
1364	UCUAAGUCAAGAGCCAUCU	241	ucuAAGucAAGAGccAucuTsT	242	AGAUGGCUCUUGACUuAGATsT	AD-12168
1365	UCAUCCCUAUAGUUCACUU	243	ucAucccuAuAGuucAcuuTsT	244	AAGUGAACuAuAGGGAUGATsT	AD-12169
1366	CAUCCCUAUAGUUCACUUU	245	cAucccuAuAGuucAcuuuTsT	246	AAAGUGAACuAuAGGGAUGTsT	AD-12170
1367	CCCUAGACUUCCCUAUUUC	247	cccuAGAcuucccuAuuucTsT	248	GAAAuAGGGAAGUCuAGGGTsT	AD-12171
1368	AGACUUCCCUAUUUCGCUU	249	AGAcuucccuAuuucGcuuTsT	250	AAGCGAAAuAGGGAAGUCUTsT	AD-12172
1369	UCACCAAACCAUUUGUAGA	251	ucAccAAAccAuuuGuAGATsT	252	UCuAcAAAUGGUUUGGUGATsT	AD-12173
1370	UCCUUUAAGAGGCCUAACU	253	uccuuuAAGAGGccuAAcuTsT	254	AGUuAGGCCUCUuAAAGGATsT	AD-12174
1371	UUUAAGAGGCCUAACUCAU	255	uuuAAGAGGccuAAcucAuTsT	256	AUGAGUuAGGCCUCUuAAATsT	AD-12175
1372	UUAAGAGGCCUAACUCAUU	257	uuAAGAGGccuAAcucAuuTsT	258	AAUGAGUuAGGCCUCUuAATsT	AD-12176
1373	GGCCUAACUCAUUCACCCU	259	GGccuAAcucAuucAcccuTsT	260	AGGGUGAAUGAGUuAGGCCTsT	AD-12177
1374	UGGUAUUUUUGAUCUGGCA	261	uGGuAuuuuuGAucuGGcATsT	262	UGCcAGAUcAAAAAuACcATsT	AD-12178
1375	AGUUUAGUGUGUAAAGUUU	263	AGuuuAGuGuGuAAAGuuuTsT	264	AAACUUuAcAcACuAAACUTsT	AD-12179
1376	GCCAAAUUCGUCUGCGAAG	265	GccAAAuucGucuGcGAAGTsT	266	CUUCGcAGACGAAUUUGGCTsT	AD-12180
1377	AAUUCGUCUGCGAAGAAGA	267	AAuucGucuGcGAAGAAGATsT	268	UCUUCUUCGcAGACGAAUUTsT	AD-12181
1378	UGAAAGGUCACCUAAUGAA	269	uGAAAGGucAccuAAuGAATsT	270	UUcAUuAGGUGACCUUUcATsT	AD-12182
1379	CAGACCAUUUAAUUUGGCA	271	cAGAccAuuuAAuuuGGcATsT	272	UGCcAAAUuAAAUGGUCUGTsT	AD-12183
1380	AGACCAUUUAAUUUGGCAG	273	AGAccAuuuAAuuuGGcAGTsT	274	CUGCcAAAUuAAAUGGUCUTsT	AD-12184
1381	AGUUAUUAUGGGCUAUAAU	275	AGuuAuuAuGGGcuAuAAuTsT	276	AUuAuAGCCcAuAAuAACUTsT	AD-12185
1382	GCUGGUAUAAUUCCACGUA	277	GcuGGuAuAAuuccAcGuATsT	278	uACGUGGAAUuAuACcAGCTsT	AD-12186
1383	AUUUAAUUUGGCAGAGCGG	279	AuuuAAuuuGGcAGAGcGGTsT	280	CCGCUCUGCcAAAUuAAAUTsT	AD-12187
1384	UUUAAUUUGGCAGAGCGGA	281	uuuAAuuuGGcAGAGcGGATsT	282	UCCGCUCUGCcAAAUuAAATsT	AD-12188
1385	UUUGGCAGAGCGGAAAGCU	283	uuuGGcAGAGcGGAAAGcuTsT	284	AGCUUUCCGCUCUGCcAAATsT	AD-12189
1386	UUUUACAAUGGAAGGUGAA	285	uuuuAcAAuGGAAGGuGAATsT	286	UUcACCUUCcAUUGuAAAATsT	AD-12190
1387	AAUGGAAGGUGAAAGGUCA	287	AAuGGAAGGuGAAAGGucATsT	288	UGACCUUUcACCUUCcAUUTsT	AD-12191
1388	UGAGAUGCAGACCAUUUAA	289	uGAGAuGcAGAccAuuuAATsT	290	UuAAAUGGUCUGcAUCUcATsT	AD-12192
1389	UCGCAGCCAAAUUCGUCUG	291	ucGcAGccAAAuucGucuGTsT	292	cAGACGAAUUUGGCUGCGATsT	AD-12193
1390	GGCUAUAAUUGCACUAUCU	293	GGcuAuAAuuGcAcuAucuTsT	294	AGAuAGUGcAAUuAuAGCCTsT	AD-12194
1391	AUUGACAGUGGCCGAUAAG	295	AuuGAcAGuGGccGAuAAGTsT	296	CUuAUCGGCcACUGUcAAUTsT	AD-12195
1392	CUAGACUUCCCUAUUUCGC	297	cuAGAcuucccuAuuucGcTsT	298	GCGAAAuAGGGAAGUCuAGTsT	AD-12196
1393	ACUAUCUUUGCGUAUGGCC	299	AcuAucuuuGcGuAuGGccTsT	300	GGCcAuACGcAAAGAuAGUTsT	AD-12197
1394	AUACUCUAGUCGUUCCCAC	301	AuAcucuAGucGuucccAcTsT	302	GUGGGAACGACuAGAGuAUTsT	AD-12198
1395	AAAGAAACUACGAUUGAUG	303	AAAGAAAcuAcGAuuGAuGTsT	304	cAUcAAUCGuAGUUUCUUUTsT	AD-12199
1396	GCCUUGAUUUUUUGGCGGG	305	GccuuGAuuuuuuGGcGGGTsT	306	CCCGCcAAAAAAUcAAGGCTsT	AD-12200
1397	CGCCCAUUCAAUAGUAGAA	307	cGcccAuucAAuAGuAGAATsT	308	UUCuACuAUUGAAUGGGCGTsT	AD-12201
1398	CCUUAUUUGGUAAUCUGCU	309	ccuuAuuuGGuAAucuGcuTsT	310	AGcAGAUuACcAAAuAAGGTsT	AD-12202
1399	AGAGACAAUUCCGGAUGUG	311	AGAGAcAAuuccGGAuGuGTsT	312	cAcAUCCGGAAUUGUCUCUTsT	AD-12203
1400	UGACUUUGAUAGCUAAAUU	313	uGAcuuuGAuAGcuAAAuuTsT	314	AAUUuAGCuAUcAAAGUcATsT	AD-12204
1401	UGGCAGAGCGGAAAGCUAG	315	uGGcAGAGcGGAAAGcuAGTsT	316	CuAGCUUUCCGCUCUGCcATsT	AD-12205
1402	GAGCGGAAAGCUAGCGCCC	317	GAGcGGAAAGcuAGcGcccTsT	318	GGGCGCuAGCUUUCCGCUCTsT	AD-12206
1403	AAAGAAGUUAGUGUACGAA	319	AAAGAAGuuAGuGuAcGAATsT	320	UUCGuAcACuAACUUCUUUTsT	AD-12207
1404	AUUGCACUAUCUUUGCGUA	321	AuuGcAcuAucuuuGcGuATsT	322	uACGcAAAGAuAGUGcAAUTsT	AD-12208
1405	GGUAUAAUUCCACGUACCC	323	GGuAuAAuuccAcGuAcccTsT	324	GGGuACGUGGAAUuAuACCTsT	AD-12209
1406	UACUCUAGUCGUUCCCACU	325	uAcucuAGucGuucccAcuTsT	326	AGUGGGAACGACuAGAGuATsT	AD-12210
1407	UAUGAAAGAAACUACGAUU	327	uAuGAAAGAAAcuAcGAuuTsT	328	AAUCGuAGUUUCUUUcAuATsT	AD-12211
1408	AUGCUAGAAGUACAUAAGA	329	AuGcuAGAAGuAcAuAAGATsT	330	UCUuAUGuACUUCuAGcAUTsT	AD-12212
1409	AAGUACAUAAGACCUUAUU	331	AAGuAcAuAAGAccuuAuuTsT	332	AAuAAGGUCUuAUGuACUUTsT	AD-12213
1410	ACAGCCUGAGCUGUUAAUG	333	AcAGccuGAGcuGuuAAuGTsT	334	cAUuAAcAGCUcAGGCUGUTsT	AD-12214
1411	AAAGAAGAGACAAUUCCGG	335	AAAGAAGAGAcAAuuccGGTsT	336	CCGGAAUUGUCUCUUCUUUTsT	AD-12215
1412	CACACUGGAGAGGUCUAAA	337	cAcAcuGGAGAGGucuAAATsT	338	UUuAGACCUCUCcAGUGUGTsT	AD-12216
1413	CACUGGAGAGGUCUAAAGU	339	cAcuGGAGAGGucuAAAGuTsT	340	ACUUuAGACCUCUCcAGUGTsT	AD-12217
1414	ACUGGAGAGGUCUAAAGUG	341	AcuGGAGAGGucuAAAGuGTsT	342	cACUUuAGACCUCUCcAGUTsT	AD-12218
1415	CGUCGCAGCCAAAUUCGUC	343	cGucGcAGccAAAuucGucTsT	344	GACGAAUUUGGCUGCGACGTsT	AD-12219
1416	GAAGGCAGUUGACCAACAC	345	GAAGGcAGuuGAccAAcAcTsT	346	GUGUUGGUcAACUGCCUUCTsT	AD-12220
1417	CAUUCACCCUGACAGAGUU	347	cAuucAcccuGAcAGAGuuTsT	348	AACUCUGUcAGGGUGAAUGTsT	AD-12221
1418	AAGAGGCCUAACUCAUUCA	349	AAGAGGccuAAcucAuucATsT	350	UGAAUGAGUuAGGCCUCUUTsT	AD-12222
1419	GAGACAAUUCCGGAUGUGG	351	GAGAcAAuuccGGAuGuGGTsT	352	CcAcAUCCGGAAUUGUCUCTsT	AD-12223
1420	UUCCGGAUGUGGAUGUAGA	353	uuccGGAuGuGGAuGuAGATsT	354	UCuAcAUCcAcAUCCGGAATsT	AD-12224
1421	AAGCUAGCGCCCAUUCAAU	355	AAGcuAGcGcccAuucAAuTsT	356	AUUGAAUGGGCGCuAGCUUTsT	AD-12225
1422	GAAGUUAGUGUACGAACUG	357	GAAGuuAGuGuAcGAAcuGTsT	358	cAGUUCGuAcACuAACUUCTsT	AD-12226
1423	UAUAAUUCCACGUACCCUU	359	uAuAAuuccAcGuAcccuuTsT	360	AAGGGuACGUGGAAUuAuATsT	AD-12227
1424	ACAGUGGCCGAUAAGAUAG	361	AcAGuGGccGAuAAGAuAGTsT	362	CuAUCUuAUCGGCcACUGUTsT	AD-12228
1425	UCUGUCAUCCCUAUAGUUC	363	ucuGucAucccuAuAGuucTsT	364	GAACuAuAGGGAUGAcAGATsT	AD-12229
1426	UUCUUGCUAUGACUUGUGU	365	uucuuGcuAuGAcuuGuGuTsT	366	AcAcAAGUcAuAGcAAGAATsT	AD-12230
1427	GUAAGAAGGCAGUUGACCA	367	GuAAGAAGGcAGuuGAccATsT	368	UGGUcAACUGCCUUCUuACTsT	AD-12231
1428	CAUUGACAGUGGCCGAUAA	369	cAuuGAcAGuGGccGAuAATsT	370	UuAUCGGCcACUGUcAAUGTsT	AD-12232
1429	AGAAACCACUUAGUAGUGU	371	AGAAAccAcuuAGuAGuGuTsT	372	AcACuACuAAGUGGUUUCUTsT	AD-12233
1430	GGAUUGUUCAUCAAUUGGC	373	GGAuuGuucAucAAuuGGcTsT	374	GCcAAUUGAUGAAcAAUCCTsT	AD-12234
1431	UAAGAGGCCUAACUCAUUC	375	uAAGAGGccuAAcucAuucTsT	376	GAAUGAGUuAGGCCUCUuATsT	AD-12235
1432	AGUUAGUGUACGAACUGGA	377	AGuuAGuGuAcGAAcuGGATsT	378	UCcAGUUCGuAcACuAACUTsT	AD-12236

SEQ ID NO:	sequence of 19-mer target site	SEQ ID NO.	sense sequence (5′-3′)	SEQ ID NO.	antisense sequence (5′-3′)	duplex name
1433	AGUACAUAAGACCUUAUUU	379	AGuAcAuAAGAccuuAuuuTsT	380	AAAuAAGGUCUuAUGuACUTsT	AD-12237
1434	UGAGCCUUGUGUAUAGAUU	381	uGAGccuuGuGuAuAGAuuTsT	382	AAUCuAuAcAcAAGGCUcATsT	AD-12238
1435	CCUUUAAGAGGCCUAACUC	383	ccuuuAAGAGGccuAAcucTsT	384	GAGUuAGGCCUCUuAAAGGTsT	AD-12239
1436	ACCACUUAGUAGUGUCCAG	385	AccAcuuAGuAGuGuccAGTsT	386	CUGGAcACuACuAAGUGGUTsT	AD-12240
1437	GAAACUUCCAAUUAUGUCU	387	GAAAcuuccAAuuAuGucuTsT	388	AGAcAuAAUUGGAAGUUUCTsT	AD-12241
1438	UGCAUACUCUAGUCGUUCC	389	uGcAuAcucuAGucGuuccTsT	390	GGAACGACuAGAGuAUGcATsT	AD-12242
1439	AGAAGGCAGUUGACCAACA	391	AGAAGGcAGuuGAccAAcATsT	392	UGUUGGUcAACUGCCUUCUTsT	AD-12243
1440	GUACAUAAGACCUUAUUUG	393	GuAcAuAAGAccuuAuuuGTsT	394	cAAAuAAGGUCUuAUGuACTsT	AD-12244
1441	UAUAAUUGCACUAUCUUUG	395	uAuAAuuGcAcuAucuuuGTsT	396	cAAAGAuAGUGcAAUuAuATsT	AD-12245
1442	UCUCUGUUACAAUACAUAU	397	ucucuGuuAcAAuAcAuAuTsT	398	AuAUGuAUUGuAAcAGAGATsT	AD-12246
1443	UAUGCUCAUAGAGCAAAGA	399	uAuGcucAuAGAGcAAAGATsT	400	UCUUUGCUCuAUGAGcAuATsT	AD-12247
1444	UGUUGUUUGUCCAAUUCUG	401	uGuuGuuuGuccAAuucuGTsT	402	cAGAAUUGGAcAAAcAAcATsT	AD-12248
1445	ACUAACUAGAAUCCUCCAG	403	AcuAAcuAGAAuccuccAGTsT	404	CUGGAGGAUUCuAGUuAGUTsT	AD-12249
1446	UGUGGUGUCUAUACUGAAA	405	uGuGGuGucuAuAcuGAAATsT	406	UUUcAGuAuAGAcACcAcATsT	AD-12250
1447	UAUUAUGGGAGACCACCCA	407	uAuuAuGGGAGAccAcccATsT	408	UGGGUGGUCUCCcAuAAuATsT	AD-12251
1448	AAGGAUGAAGUCUAUCAAA	409	AAGGAuGAAGucuAucAAATsT	410	UUUGAuAGACUUcAUCCUUTsT	AD-12252
1449	UUGAUAAGAGAGCUCGGGA	411	uuGAuAAGAGAGcucGGGATsT	412	UCCCGAGCUCUCUuAUcAATsT	AD-12253
1450	AUGUUCCUUAUCGAGAAUC	413	AuGuuccuuAucGAGAAucTsT	414	GAUUCUCGAuAAGGAAcAUTsT	AD-12254
1451	GGAAUAUGCUCAUAGAGCA	415	GGAAuAuGcucAuAGAGcATsT	416	UGCUCuAUGAGcAuAUUCCTsT	AD-12255
1452	CCAUUCCAAACUGGAUCGU	417	ccAuuccAAAcuGGAucGuTsT	418	ACGAUCcAGUUUGGAAUGGTsT	AD-12256
1453	GGCAGUUGACCAACACAAU	419	GGcAGuuGAccAAcAcAAuTsT	420	AUUGUGUUGGUcAACUGCCTsT	AD-12257
1454	CAUGCUAGAAGUACAUAAG	421	cAuGcuAGAAGuAcAuAAGTsT	422	CUuAUGuACUUCuAGcAUGTsT	AD-12258
1455	CUAGAAGUACAUAAGACCU	423	cuAGAAGuAcAuAAGAccuTsT	424	AGGUCUuAUGuACUUCuAGTsT	AD-12259
1456	UUGGAUCUCUCACAUCUAU	425	uuGGAucucucAcAucuAuTsT	426	AuAGAUGUGAGAGAUCcAATsT	AD-12260
1457	AACUGUGGUGUCUAUACUG	427	AAcuGuGGuGucuAuAcuGTsT	428	cAGuAuAGAcACcAcAGUUTsT	AD-12261
1458	UCAUUGACAGUGGCCGAUA	429	ucAuuGAcAGuGGccGAuATsT	430	uAUCGGCcACUGUcAAUGATsT	AD-12262
1459	AUAAAGCAGACCCAUUCCC	431	AuAAAGcAGAcccAuucccTsT	432	GGGAAUGGGUCUGCUUuAUTsT	AD-12263
1460	ACAGAAACCACUUAGUAGU	433	AcAGAAAccAcuuAGuAGuTsT	434	ACuACuAAGUGGUUUCUGUTsT	AD-12264
1461	GAAACCACUUAGUAGUGUC	435	GAAAccAcuuAGuAGuGucTsT	436	GAcACuACuAAGUGGUUUCTsT	AD-12265
1462	AAAUCUAAGGAUAUAGUCA	437	AAAucuAAGGAuAuAGucATsT	438	UGACuAuAUCCUuAGAUUUTsT	AD-12266
1463	UUAUUUAUACCCAUCAACA	439	uuAuuuAuAcccAucAAcATsT	440	UGUUGAUGGGuAuAAAuAATsT	AD-12267
1464	ACAGAGGCAUUAACACACU	441	AcAGAGGcAuuAAcAcAcuTsT	442	AGUGUGUuAAUGCCUCUGUTsT	AD-12268
1465	ACACACUGGAGAGGUCUAA	443	AcAcAcuGGAGAGGucuAATsT	444	UuAGACCUCUCcAGUGUGUTsT	AD-12269
1466	ACACUGGAGAGGUCUAAAG	445	AcAcuGGAGAGGucuAAAGTsT	446	CUUuAGACCUCUCcAGUGUTsT	AD-12270
1467	CGAGCCCAGAUCAACCUUU	447	cGAGcccAGAucAAccuuuTsT	448	AAAGGUUGAUCUGGGCUCGTsT	AD-12271
1468	UCCCUAUUUCGCUUUCUCC	449	ucccuAuuucGcuuucuccTsT	450	GGAGAAAGCGAAAuAGGGATsT	AD-12272
1469	UCUAAAAUCACUGUCAACA	451	ucuAAAAucAcuGucAAcATsT	452	UGUUGAcAGUGAUUUuAGATsT	AD-12273
1470	AGCCAAAUUCGUCUGCGAA	453	AGccAAAuucGucuGcGAATsT	454	UUCGcAGACGAAUUUGGCUTsT	AD-12274
1471	CCCAUUCAAUAGUAGAAUG	455	cccAuucAAuAGuAGAAuGTsT	456	cAUUCuACuAUUGAAUGGGTsT	AD-12275
1472	GAUGAAUGCAUACUCUAGU	457	GAuGAAuGcAuAcucuAGuTsT	458	ACuAGAGuAUGcAUUcAUCTsT	AD-12276
1473	CUCAUGUUCCUUAUCGAGA	459	cucAuGuuccuuAucGAGATsT	460	UCUCGAuAAGGAAcAUGAGTsT	AD-12277
1474	GAGAAUCUAAACUAACUAG	461	GAGAAucuAAAcuAAcuAGTsT	462	CuAGUuAGUUuAGAUUCUCTsT	AD-12278
1475	UAGAAGUACAUAAGACCUU	463	uAGAAGuAcAuAAGAccuuTsT	464	AAGGUCUuAUGuACUUCuATsT	AD-12279
1476	CAGCCUGAGCUGUUAAUGA	465	cAGccuGAGcuGuuAAuGATsT	466	UcAUuAAcAGCUcAGGCUGTsT	AD-12280
1477	AAGAAGAGACAAUUCCGGA	467	AAGAAGAGAcAAuuccGGATsT	468	UCCGGAAUUGUCUCUUCUUTsT	AD-12281
1478	UGCUGGUGUGGAUUGUUCA	469	uGcuGGuGuGGAuuGuucATsT	470	UGAAcAAUCcAcACcAGcATsT	AD-12282
1479	AAAUUCGUCUGCGAAGAAG	471	AAAuucGucuGcGAAGAAGTsT	472	CUUCUUCGcAGACGAAUUUTsT	AD-12283
1480	UUUCUGGAAGUUGAGAUGU	473	uuucuGGAAGuuGAGAuGuTsT	474	AcAUCUcAACUUCcAGAAATsT	AD-12284
1481	UACUAAACAGAUUGAUGUU	475	uAcuAAAcAGAuuGAuGuuTsT	476	AAcAUcAAUCUGUUuAGuATsT	AD-12285
1482	GAUUGAUGUUUACCGAAGU	477	GAuuGAuGuuuAccGAAGuTsT	478	ACUUCGGuAAAcAUcAAUCTsT	AD-12286
1483	GCACUAUCUUUGCGUAUGG	479	GcAcuAucuuuGcGuAuGGTsT	480	CcAuACGcAAAGAuAGUGCTsT	AD-12287
1484	UGGUAUAAUUCCACGUACC	481	uGGuAuAAuuccAcGuAccTsT	482	GGuACGUGGAAUuAuACcATsT	AD-12288
1485	AGCAAGCUGCUUAAACACAG	483	AGcAAGcuGcuuAAcAcAGTsT	484	CUGUGUuAAGcAGCUUGCUTsT	AD-12289
1486	CAGAAACCACUUAGUAGUG	485	cAGAAAccAcuuAGuAGuGTsT	486	cACuACuAAGUGGUUUCUGTsT	AD-12290
1487	AACUUAUUGGAGGUUGUAA	487	AAcuuAuuGGAGGuuGuAATsT	488	UuAcAACCUCcAAuAAGUUTsT	AD-12291
1488	CUGGAGAGGUCUAAAGUGG	489	cuGGAGAGGucuAAAGuGGTsT	490	CcACUUuAGACCUCUCcAGTsT	AD-12292
1489	AAAAAAGAUAUAAGGCAGU	491	AAAAAAGAuAuAAGGcAGuTsT	492	ACUGCCUuAuAUCUUUUUUTsT	AD-12293
1490	GAAUUUUGAUAUCUACCCA	493	GAAuuuuGAuAucuAcccATsT	494	UGGGuAGAuAUcAAAAUUCTsT	AD-12294
1491	GUAUUUUUGAUCUGGCAAC	495	GuAuuuuuGAucuGGcAAcTsT	496	GUUGCcAGAUcAAAAAuACTsT	AD-12295
1492	AGGAUCCCUUGGCUGGUAU	497	AGGAucccuuGGcuGGuAuTsT	498	AuACcAGCcAAGGGAUCCUTsT	AD-12296
1493	GGAUCCCUUGGCUGGUAUA	499	GGAucccuuGGcuGGuAuATsT	500	uAuACcAGCcAAGGGAUCCTsT	AD-12297
1494	CAAUAGUAGAAUGUGAUCC	501	cAAuAGuAGAAuGuGAuccTsT	502	GGAUcAcAUUCuACuAUUGTsT	AD-12298
1495	GCUAUAAUUGCACUAUCUU	503	GcuAuAAuuGcAcuAucuuTsT	504	AAGAuAGUGcAAUuAuAGCTsT	AD-12299
1496	UACCCUUCAUCAAAUUUUU	505	uAcccuucAucAAAuuuuuTsT	506	AAAAAUUUGAUGAAGGGuATsT	AD-12300
1497	AGAACAUAUUGAAUAAGCC	507	AGAAcAuAuuGAAuAAGccTsT	508	GGCUuAUUcAAuAUGUUCUTsT	AD-12301
1498	AAAUUGGUGCUGUUGAGGA	509	AAAuuGGuGcuGuuGAGGATsT	510	UCCUcAAcAGcACcAAUUUTsT	AD-12302
1499	UGAAUAGGGUUACAGAGUU	511	uGAAuAGGGuuAcAGAGuuTsT	512	AACUCUGuAACCCuAUUcATsT	AD-12303
1500	AAGAACUUGAAACCACUCA	513	AAGAAcuuGAAAccAcucATsT	514	UGAGUGGUUUcAAGUUCUUTsT	AD-12304
1501	AAUAAAGCAGACCCAUUCC	515	AAuAAAGcAGAcccAuucCTsT	516	GGAAUGGGUCUGCUUuAUUTsT	AD-12305
1502	AUACCCAUCAACACUGGUA	517	AuAcccAucAAcAcuGGuATsT	518	uACcAGUGUUGAUGGGuAUTsT	AD-12306
1503	UGGAUUGUUCAUCAAUUGG	519	uGGAuuGuucAucAAuuGGTsT	520	CcAAUUGAUGAAcAAUCcATsT	AD-12307
1504	UGGAGAGGUCUAAAGUGGA	521	uGGAGAGGucuAAAGuGGATsT	522	UCcACUUuAGACCUCUCcATsT	AD-12308
1505	GUCAUCCCUAUAGUUCACU	523	GucAucccuAuAGuucAcuTsT	524	AGUGAACuAuAGGGAUGACTsT	AD-12309
1506	AUAAUGGCUAUAAUUUCUC	525	AuAAuGGcuAuAAuuucuCTsT	526	GAGAAAUuAuAGCcAUuAUTsT	AD-12310

SEQ ID NO:	sequence of 19-mer target site	SEQ ID NO.	sense sequence (5′-3′)	SEQ ID NO.	antisense sequence (5′-3′)	duplex name
1507	AUCCCUUGGCUGGUAUAAU	527	AucccuuGGcuGGuAuAAuTsT	528	AUuAuACcAGCcAAGGGAUTsT	AD-12311
1508	GGGCUAUAAUUGCACUAUC	529	GGGcuAuAAuuGcAcuAucTsT	530	GAuAGUGcAAUuAuAGCCCTsT	AD-12312
1509	GAUUCUCUUGGAGGGCGUA	531	GAuucucuuGGAGGGcGuATsT	532	uACGCCCUCcAAGAGAAUCTsT	AD-12313
1510	GCAUCUCUCAAUCUUGAGG	533	GcAucucucAAucuuGAGGTsT	534	CCUcAAGAUUGAGAGAUGCTsT	AD-12314
1511	CAGCAGAAAUCUAAGGAUA	535	cAGcAGAAAucuAAGGAuATsT	536	uAUCCUuAGAUUUCUGCUGTsT	AD-12315
1512	GUCAAGAGCCAUCUGUAGA	537	GucAAGAGccAucuGuAGATsT	538	UCuAcAGAUGGCUCUUGACTsT	AD-12316
1513	AAACAGAGGCAUUAACACA	539	AAAcAGAGGcAuuAAcAcATsT	540	UGUGUuAAUGCCUCUGUUUTsT	AD-12317
1514	AGCCCAGAUCAACCUUUAA	541	AGcccAGAucAAccuuuAATsT	542	UuAAAGGUUGAUCUGGGCUTsT	AD-12318
1515	UAUUUUUGAUCUGGCAACC	543	uAuuuuuGAucuGGcAAccTsT	544	GGUUGCcAGAUcAAAAAuATsT	AD-12319
1516	UGUUUGGAGCAUCUACUAA	545	uGuuuGGAGcAucuAcuAATsT	546	UuAGuAGAUGCUCcAAAcATsT	AD-12320
1517	GAAAUUACAGUACACAACA	547	GAAAuuAcAGuAcAcAAcATsT	548	UGUUGUGuACUGuAAUUUCTsT	AD-12321
1518	ACUUGACCAGUGUAAAUCU	549	AcuuGAccAGuGuAAAucuTsT	550	AGAUUuAcACUGGUcAAGUTsT	AD-12322
1519	ACCAGUGUAAAUCUGACCU	551	AccAGuGuAAAucuGAccuTsT	552	AGGUcAGAUUuAcACUGGUTsT	AD-12323
1520	AGAACAAUCAUUAGCAGCA	553	AGAAcAAucAuuAGcAGcATsT	554	UGCUGCuAAUGAUUGUUCUTsT	AD-12324
1521	CAAUGUGGAAACCUAACUG	555	cAAuGuGGAAAccuAAcuGTsT	556	cAGUuAGGUUUCcAcAUUGTsT	AD-12325
1522	ACCAAGAAGGUACAAAAUU	557	AccAAGAAGGuAcAAAAuuTsT	558	AAUUUUGuACCUUCUUGGUTsT	AD-12326
1523	GGUACAAAAUUGGUUGAAG	559	GGuAcAAAAuuGGuuGAAGTsT	560	CUUcAACcAAUUUUGuACCTsT	AD-12327
1524	GGUGUGGAUUGUUCAUCAA	561	GGuGuGGAuuGuucAucAATsT	562	UUGAUGAAcAAUCcAcACCTsT	AD-12328
1525	AGAGUUCACAAAAAGCCCA	563	AGAGuucAcAAAAAGcccATsT	564	UGGGCUUUUUGUGAACUCUTsT	AD-12329
1526	UGAUAGCUAAAUUAAACCA	565	uGAuAGcuAAAuuAAAccATsT	566	UGGUUuAAUUuAGCuAUcATsT	AD-12330
1527	AAUAAGCCUGAAGUGAAUC	567	AAuAAGccuGAAGuGAAucTsT	568	GAUUcACUUcAGGCUuAUUTsT	AD-12331
1528	CAGUUGACCAACACAAUGC	569	cAGuuGAccAAcAcAAuGcTsT	570	GcAUUGUGUUGGUcAACUGTsT	AD-12332
1529	UGGUGUGGAUUGUUCAUCA	571	uGGuGuGGAuuGuucAucATsT	572	UGAUGAAcAAUCcAcACcATsT	AD-12333
1530	AUUCACCCUGACAGAGUUC	573	AuucAcccuGAcAGAGuucTsT	574	GAACUCUGUcAGGGUGAAUTsT	AD-12334
1531	UAAGACCUUAUUUGGUAAU	575	uAAGAccuuAuuuGGuAAuTsT	576	AUuACcAAAuAAGGUCUuATsT	AD-12335
1532	AAGCAAUGUGGAAACCUAA	577	AAGcAAuGuGGAAAccuAATsT	578	UuAGGUUUCcAcAUUGCUUTsT	AD-12336
1533	UCUGAAACUGGAUAUCCCA	579	ucuGAAAcuGGAuAucccATsT	580	UGGGAuAUCcAGUUUcAGATsT	AD-12337

Table 2b. Analysis of Eg5/KSP dsRNA duplexes

Eg5/ KSP duplex Name	1st single dose screen @ 50 nM [% resudual mRNA]	SDs 1st screen (among quadruplicates)	2nd single dose screen @ 25 nM [% resudual mRNA]	SDs 2nd screen (among quadruplicates)	3rd single dose screen @ 25 nM	SDs 3rd screen (among quadruplicates)
AD-12072	65%	2%	82%	5%		
AD-12073	84%	1%	61%	6%		
AD-12074	51%	3%	36%	9%		
AD-12075	56%	4%	36%	4%		
AD-12076	21%	4%	13%	3%		
AD-12077	11%	2%	6%	1%		
AD-12078	22%	3%	9%	2%		
AD-12079	22%	10%	15%	7%		
AD-12080	68%	4%	52%	13%		
AD-12081	34%	8%	35%	24%		
AD-12082	20%	2%	92%	5%		
AD-12083	85%	6%	63%	10%		
AD-12084	18%	6%	17%	4%		
AD-12085	13%	4%	12%	4%		
AD-12086	26%	5%	17%	3%		
AD-12087	95%	4%	80%	4%		
AD-12088	29%	6%	29%	2%		
AD-12089	69%	5%	64%	7%		
AD-12090	46%	15%	34%	5%		
AD-12091	16%	6%	17%	3%		
AD-12092	82%	26%	63%	5%		
AD-12093	84%	4%	70%	4%		
AD-12094	46%	3%	34%	1%		
AD-12095	14%	2%	13%	1%		
AD-12096	26%	11%	17%	1%		
AD-12097	23%	2%	21%	1%		
AD-12098	41%	14%	17%	3%		
AD-12099	57%	2%	48%	6%		
AD-12100	101%	11%	98%	8%		
AD-12101	46%	7%	32%	2%		
AD-12102	96%	17%	88%	18%		
AD-12103	19%	5%	20%	2%		
AD-12104	40%	8%	24%	2%		
AD-12105	39%	2%	36%	10%		
AD-12106	87%	6%	79%	19%		
AD-12107	29%	2%	32%	16%		
AD-12108	38%	4%	39%	8%		
AD-12109	49%	3%	44%	10%		
AD-12110	85%	5%	80%	14%		
AD-12111	64%	6%	71%	18%		
AD-12112	48%	4%	41%	5%		
AD-12113	13%	0%	14%	3%		
AD-12114	32%	6%	16%	4%		
AD-12115	8%	4%	7%	5%		
AD-12116	74%	5%	61%	7%		
AD-12117	21%	4%	20%	2%		
AD-12118	44%	4%	42%	6%		
AD-12119	37%	4%	24%	3%		
AD-12120	22%	2%	15%	4%		
AD-12121	32%	1%	22%	2%		
AD-12122	36%	16%	19%	5%		
AD-12123	28%	1%	16%			
AD-12124	28%	2%	16%			
AD-12125	15%	1%	14%			
AD-12126	51%	22%	27%			
AD-12127	54%	4%	42%	9%		
AD-12128	29%	1%	20%	2%		
AD-12129	22%	3%	19%	3%		
AD-12130	53%	6%	42%	7%		
AD-12131	28%	5%	22%	3%		
AD-12132	88%	2%	90%	18%		
AD-12133	34%	2%	26%	6%		
AD-12134	18%	3%	14%	2%		
AD-12135	50%	6%	37%	4%		
AD-12136	42%	19%	22%	2%		

Eg5/ KSP duplex Name	1st single dose screen @ 50 nM [% resuduai mRNA]	SDs 1st screen (among quadruplicates)	2nd single dose screen @ 25 nM [% resuduai mRNA]	SDs 2nd screen (among quadruplicates)	3rd single dose screen @ 25 nM	SDs 3rd screen (among quadruplicates)
AD-12137	85%	12%	92%	4%		
AD-12138	47%	6%	49%	1%		
AD-12139	80%	5%	72%	4%		
AD-12140	97%	22%	67%	9%		
AD-12141	120%	4%	107%	10%		
AD-12142	55%	8%	33%	4%		
AD-12143	64%	34%	19%	2%		
AD-12144	58%	29%	17%	2%		
AD-12145	27%	8%	18%	2%		
AD-12146	19%	20%	15%	1%		
AD-12147	29%	9%	35%	3%		
AD-12148	30%	3%	56%	5%		
AD-12149	8%	2%	12%	3%		
AD-12150	31%	2%	31%	7%		
AD-12151	9%	5%	14%	2%		
AD-12152	3%	3%	23%	3%		
AD-12153	20%	6%	34%	4%		
AD-12154	24%	7%	44%	3%		
AD-12155	33%	6%	53%	11%		
AD-12156	35%	5%	40%	5%		
AD-12157	8%	3%	23%	4%		
AD-12158	13%	2%	22%	5%		
AD-12159	34%	6%	46%	5%		
AD-12160	19%	3%	31%	4%		
AD-12161	88%	4%	83%	7%		
AD-12162	26%	7%	32%	7%		
AD-12163	55%	9%	40%	3%		
AD-12164			21%	3%		
AD-12165	30%	3%	41%	4%		
AD-12166	9%	10%	22%	9%		
AD-12167	26%	3%	30%	2%		
AD-12168	54%	4%	59%	20%		
AD-12169	41%	4%	51%	16%		
AD-12170	43%	4%	52%	20%		
AD-12171	67%	3%	73%	25%		
AD-12172	53%	15%	37%	2%		
AD-12173	39%	0%	39%	0%		
AD-12174	41%	5%	27%	0%		
AD-12175	29%	0%	38%	14%		
AD-12176	43%	2%	56%	25%		
AD-12177	68%	6%	74%	30%		
AD-12178	41%	4%	41%	6%		
AD-12179	53%	5%	44%	5%		
AD-12180	16%	2%	13%	4%		
AD-12181	19%	3%	14%	2%		
AD-12182	16%	4%	18%	8%		
AD-12183	26%	3%	19%	4%		
AD-12184	54%	2%	77%	8%		
AD-12185	8%	1%	9%	1%		
AD-12186	36%	3%	41%	6%		
AD-12187	34%	17%	27%	1%		
AD-12188	30%	3%	27%	4%		
AD-12189	51%	4%	48%	5%		
AD-12190	33%	2%	26%	4%		
AD-12191	20%	2%	13%	0%		
AD-12192	21%	1%	23%	10%		
AD-12193	64%	8%	98%	6%		
AD-12194	8%	2%	15%	4%		
AD-12195	34%	2%	48%	3%		
AD-12196	34%	2%	51%	3%		
AD-12197	75%	4%	93%	6%		
AD-12198	55%	5%	48%	2%		
AD-12199	102%	6%	118%	9%		
AD-12200	75%	6%	60%	12%		
AD-12201	42%	3%	16%	4%		
AD-12202	29%	4%	9%	3%		
AD-12203	114%	14%	89%	20%		

Eg5/ KSP duplex Name	1st single dose screen @ 50 nM [% resuduai mRNA]	SDs 1st screen (among quadruplicates)	2nd single dose screen @ 25 nM [% resuduai mRNA]	SDs 2nd screen (among quadruplicates)	3rd single dose screen @ 25 nM	SDs 3rd screen (among quadruplicates)
AD-12204	64%	7%	26%	5%		
AD-12205	66%	12%	35%	4%		
AD-12206	46%	3%	32%	12%		
AD-12207	57%	5%	40%	6%		
AD-12208	30%	8%	10%	5%		
AD-12209	101%	6%	102%	23%		
AD-12210	38%	11%	27%	14%		
AD-12211	16%	6%	10%	5%		
AD-12212	59%	8%	65%	5%		
AD-12213	24%	9%	12%	2%		
AD-12214	67%	14%	70%	12%		
AD-12215	29%	13%	13%	4%		
AD-12216	36%	4%	13%	1%		
AD-12217	36%	9%	11%	2%		
AD-12218	35%	5%	17%	3%		
AD-12219	41%	9%	14%	1%		
AD-12220	37%	5%	23%	3%		
AD-12221	58%	7%	39%	6%		
AD-12222	74%	9%	53%	3%		
AD-12223	74%	10%	67%	7%		
AD-12224	24%	2%	11%	2%		
AD-12225	75%	5%	76%	14%		
AD-12226	45%	8%	40%	3%		
AD-12227	61%	6%	47%	5%		
AD-12228	28%	3%	25%	5%		
AD-12229	54%	13%	37%	6%		
AD-12230	70%	17%	65%	4%		
AD-12231	32%	12%	22%	6%		
AD-12232	30%	3%	17%	2%		
AD-12233	38%	2%	32%	3%		
AD-12234	90%	5%	95%	7%		
AD-12235	57%	7%	46%	3%		
AD-12236	34%	8%	16%	2%		
AD-12237	42%	9%	32%	8%		
AD-12238	42%	6%	34%	6%		
AD-12239	42%	3%	40%	4%		
AD-12240	47%	6%	36%	5%		
AD-12241	69%	5%	70%	8%		
AD-12242	61%	2%	47%	3%		
AD-12243	26%	7%	15%	1%		
AD-12244	25%	6%	15%	1%		
AD-12245	65%	6%	83%	13%		
AD-12246	29%	7%	31%	6%		
AD-12247	57%	13%	50%	3%		
AD-12248	36%	8%	20%	3%	15%	7%
AD-12249	44%	3%	70%	11%	103%	34%
AD-12250	47%	5%	18%	5%	17%	4%
AD-12251	121%	28%	35%	8%	60%	42%
AD-12252	94%	19%	8%	3%	5%	3%
AD-12253	94%	33%	42%	8%	49%	27%
AD-12254	101%	58%	70%	5%	80%	32%
AD-12255	163%	27%	28%	6%	36%	10%
AD-12256	112%	62%	18%	3%	9%	4%
AD-12257	10%	4%	9%	2%	6%	2%
AD-12258	27%	9%	18%	3%	20%	6%
AD-12259	20%	5%	12%	2%	13%	5%
AD-12260	22%	7%	81%	7%	65%	13%
AD-12261	122%	11%	66%	7%	80%	22%
AD-12262	97%	30%	33%	6%	44%	18%
AD-12263	177%	57%	85%	11%	84%	15%
AD-12264	37%	6%	10%	1%	10%	4%
AD-12265	40%	8%	17%	1%	20%	10%
AD-12266	33%	9%	9%	1%	8%	4%
AD-12267	34%	13%	11%	1%	6%	2%
AD-12268	34%	6%	11%	1%	9%	2%
AD-12269	54%	6%	33%	4%	29%	7%
AD-12270	52%	5%	29%	4%	27%	6%

Eg5/ KSP duplex Name	1st single dose screen @ 50 nM [% resuduai mRNA]	SDs 1st screen (among quadruplicates)	2nd single dose screen @ 25 nM [% resuduai mRNA]	SDs 2nd screen (among quadruplicates)	3rd single dose screen @ 25 nM	SDs 3rd screen (among quadruplicates)
AD-12271	53%	7%	27%	3%	19%	6%
AD-12272	85%	15%	57%	7%	51%	16%
AD-12273	36%	6%	26%	2%	30%	5%
AD-12274	75%	21%	40%	2%	50%	19%
AD-12275	29%	9%	8%	1%	8%	4%
AD-12276	45%	19%	15%	2%	16%	12%
AD-12277	58%	17%	32%	2%	55%	14%
AD-12278	120%	35%	96%	10%	124%	38%
AD-12279	47%	29%	17%	1%	12%	4%
AD-12280	2%	0%	3%	1%		
AD-12281	2%	0%	5%	2%		
AD-12282	3%	0%	25%	5%		
AD-12283	3%	1%	35%	4%		
AD-12284	5%	2%	49%	8%		
AD-12285	7%	7%	21%	26%		
AD-12286	28%	34%	12%	7%		
AD-12287	40%	21%	51%	23%		
AD-12288	26%	7%	155%	146%		
AD-12289	43%	21%	220%	131%		
AD-12290	2%	1%	81%	23%		
AD-12291	4%	1%	70%	3%		
AD-12292	2%	1%	6%	2%		
AD-12293	4%	2%	36%	3%		
AD-12294	10%	6%	38%	3%		
AD-12295	29%	31%	37%	3%		
AD-12296	82%	4%	89%	2%		
AD-12297	75%	3%	65%	2%		
AD-12298	73%	4%	60%	3%		
AD-12299	76%	4%	66%	4%		
AD-12300	36%	4%	15%	1%		
AD-12301	33%	4%	18%	2%		
AD-12302	66%	5%	65%	3%		
AD-12303	35%	6%	17%	2%		
AD-12304	70%	8%	70%	6%		
AD-12305	63%	8%	80%	7%		
AD-12306	23%	6%	20%	3%		
AD-12307	78%	10%	58%	5%		
AD-12308	27%	8%	15%	2%		
AD-12309	58%	11%	42%	3%		
AD-12310	106%	23%	80%	2%		
AD-12311	73%	12%	60%	2%		
AD-12312	39%	3%	36%	3%		
AD-12313	64%	9%	49%	6%		
AD-12314	28%	7%	14%	6%		
AD-12315	31%	7%	13%	2%		
AD-12316	42%	5%	14%	2%		
AD-12317	34%	9%	15%	5%		
AD-12318	46%	4%	28%	4%		
AD-12319	77%	3%	56%	4%		
AD-12320	55%	7%	41%	3%		
AD-12321	21%	3%	10%	2%		
AD-12322	27%	8%	30%	12%		
AD-12323	26%	7%	35%	18%		
AD-12324	27%	8%	27%	14%		
AD-12325	32%	12%	32%	22%		
AD-12326	42%	22%	45%	41%		
AD-12327	36%	14%	37%	32%		
AD-12328	45%	2%	31%	3%		
AD-12329	61%	4%	34%	3%		
AD-12330	63%	5%	38%	4%		
AD-12331	50%	2%	26%	5%		
AD-12332	80%	4%	51%	7%		
AD-12333	34%	6%	12%	2%		
AD-12334	27%	2%	18%	3%		
AD-12335	84%	6%	60%	7%		
AD-12336	45%	4%	36%	4%		
AD-12337	30%	7%	19%	2%		

Table 3. Sequences and analysis of Eg5/KSP dsRNA duplexes

Sense sequence (5′-3′)	SEQ ID NO.	Antisense sequence (5′-3′)	SEQ ID NO.	duplex name	single dose screen @ 25 nM [% residual mRNA]	SDs 2nd screen (among quadruplicates)
ccAuuAcuAcAGuAGcAcuTsT	582	AGUGCuACUGuAGuAAUGGTsT	583	AD-14085	19%	1%
AucuGGcAAccAuAuuucuTsT	584	AGAAAuAUGGUUGCcAGAUTsT	585	AD-14086	38%	1%
GAuAGcuAAAuuAAAccAATsT	586	UUGGUUuAAUUuAGCuAUCTsT	587	AD-14087	75%	10%
AGAuAccAuuAcuAcAGuATsT	588	uACUGuAGuAAUGGuAUCUTsT	589	AD-14088	22%	8%
GAuuGuucAucAAuuGGcGTsT	590	CGCcAAUUGAUGAAcAAUCTsT	591	AD-14089	70%	12%
GcuuucuccucGGcucAcuTsT	592	AG**uG**AGCCGAGGAGAAAGCTsT	593	AD-14090	79%	11%
GGAGGAuuGGcuGAcAAGATsT	594	UCUUGUcAGCcAAUCCUCCTsT	595	AD-14091	29%	3%
uAAuGAAGAGuAuAccuGGTsT	596	CcAGGuAuACUCUUcAUuATsT	597	AD-14092	23%	2%
uuucAccAAAccAuuuGuATsT	598	uAcAAAUGGUUUGGUGAAATsT	599	AD-14093	60%	2%
cuuAuuAAGGAGuAuAcGGTsT	600	CCGuAuACUCCUuAAuAAGTsT	601	AD-14094	11%	3%
GAAAucAGAuGGAcGuAAGTsT	602	CUuACGUCcAUCUGAUUUCTsT	603	AD-14095	10%	2%
cAGAuGucAGcAuAAGcGATsT	604	UCGCUuAUGCUGAcAUCUGTsT	605	AD-14096	27%	2%
AucuAAcccuAGuuGuAucTsT	606	GAuAcAACuAGGGUuAGAUTsT	607	AD-14097	45%	6%
AAGAGcuuGuuAAAAucGGTsT	608	CCGAUUUuAAcAAGCUCUUTsT	609	AD-14098	50%	10%
uuAAGGAGuAuAcGGAGGATsT	610	UCCUCCGuAuACUCCUuAATsT	611	AD-14099	12%	4%
uuGcAAuGuAAAuAcGuAuTsT	612	AuACGuAUUuAcAUUGcAATsT	613	AD-14100	49%	7%
ucuAAcccuAGuuGuAuccTsT	614	GGAuAcAACuAGGGUuAGATsT	615	AD-14101	36%	1%
cAuGuAucuuuuuucucGAuTsT	616	AUCGAGAAAAAGAuAcAUGTsT	617	AD-14102	49%	3%
GAuGucAGcAuAAGcGAuGTsT	618	cAUCGCUuAUGCUGAcAUCTsT	619	AD-14103	74%	5%
ucccAAcAGGuAcGAcAccTsT	620	GGUGUCGuACCUGUUGGGATsT	621	AD-14104	27%	3%
uGcucAcGAuGAGuuuAGuTsT	622	ACuAAACUcAUCGUGAGcATsT	623	AD-14105	34%	4%
AGAGcuuGuuAAAAucGGATsT	624	UCCGAUUUuAAcAAGCUCUTsT	625	AD-14106	9%	2%
GcGuAcAAGAAcAucuAuATsT	626	uAuAGAUGUUCUUGuACGCTsT	627	AD-14107	5%	1%
GAGGuuGuAAGccAAuGuuTsT	628	AAcAUUGGCUuAcAACCUCTsT	629	AD-14108	15%	1%
AAcAGGuAcGAcAccAcAGTsT	630	CUGUGGUGUCGuACCUGUUTsT	631	AD-14109	91%	2%
AAcccuAGuuGuAucccucTsT	632	GAGGGAuAcAACuAGGGUUTsT	633	AD-14110	66%	5%
GcAuAAGcGAuGGAuAAuATsT	634	uAUuAUCcAUCGCUuAUGCTsT	635	AD-14111	33%	3%
AAGcGAuGGAuAAuAccuATsT	636	uAGGuAUuAUCcAUCGCUUTsT	637	AD-14112	51%	3%
uGAuccuGuAcGAAAAGAATsT	638	UUCUUUUCGuAcAGGAUcATsT	639	AD-14113	22%	3%
AAAAcAuuGGccGuucuGGTsT	640	CcAGAACGGCcAAUGUUUUTsT	641	AD-14114	117%	8%
cuuGGAGGcGuAcAAGAATsT	642	UUCUUGuACGCCCUCcAAGTsT	643	AD-14115	50%	8%
GGcGuAcAAGAAcAucuAuTsT	644	AuAGAUGUUCUUGuACGCCTsT	645	AD-14116	14%	3%
AcucuGAGuAcAuuGGAAuTsT	646	AUUCcAAUGuACUcAGAGUTsT	647	AD-14117	12%	4%
uuAuuAAGGAGuAuAcGGATsT	648	UCCGuAuACUCCUuAAuAATsT	649	AD-14118	26%	4%
uAAGGAGuAuAcGGAGGAGTsT	650	CUCCUCCGuAuACUCCUuATsT	651	AD-14119	24%	5%
AAAucAAuAGucAAcuAAATsT	652	UUuAGUUGACuAUUGAUUUTsT	653	AD-14120	8%	1%
AAucAAuAGucAAcuAAAGTsT	654	CUUuAGUUGACuAUUGAUUTsT	655	AD-14121	24%	2%
uucucAGuAuAcuGuGuAATsT	656	UuAcAcAGuAuACUGAGAATsT	657	AD-14122	10%	1%
uGuGAAAcAcucuGAuAAATsT	658	UUuAUcAGAGUGUUUcAcATsT	659	AD-14123	8%	1%
AGAuGuGAAucucuGAAcATsT	660	UGUUcAGAGAUUcAcAUCUTsT	661	AD-14124	9%	2%
AGGuuGuAAGccAAuGuuGTsT	662	cAAcAUUGGCUuAcAACCUTsT	663	AD-14125	114%	6%
uGAGAAAucAGAuGGAcGuTsT	664	ACGUCcAUCUGAUUUCUcATsT	665	AD-14126	9%	1%
AGAAAucAGAuGGAcGuAATsT	666	UuACGUCcAUCUGAUUUCUTsT	667	AD-14127	57%	6%
AuAucccAAcAGGuAcGAcTsT	668	GUCGuACCUGUUGGGAuAUTsT	669	AD-14128	104%	6%
cccAAcAGGuAcGAcAccATsT	670	UGGUGUCGuACCUGUUGGGTsT	671	AD-14129	21%	2%
AGuAuAcuGAAGAAccucuTsT	672	AGAGGUUCUUcAGuAuACUTsT	673	AD-14130	57%	6%
AuAuAuAucAGccGGcGcTsT	674	GCGCCCGGCUGAuAuAuAUTsT	675	AD-14131	93%	6%
AAucuAAcccuAGuuGuAuTsT	676	AuAcAACuAGGGUuAGAUUTsT	677	AD-14132	75%	8%
cuAAcccuAGuuGuAucccTsT	678	GGGAuAcAACuAGGGUuAGTsT	679	AD-14133	66%	4%
cuAGuuGuAucccucccuuuTsT	680	AAAGGAGGGAuAcAACuAGTsT	681	AD-14134	44%	6%
AGAcAucuGAcuAAuGGcuTsT	682	AGCcAUuAGUcAGAUGUCUTsT	683	AD-14135	55%	6%
GAAGcucAcAAuGAuuuAATsT	684	UuAAAUcAUUGUGAGCUUCTsT	685	AD-14136	29%	3%
AcAuGuAucuuuuuucucGATsT	686	UCGAGAAAAAGAuAcAUGUTsT	687	AD-14137	40%	3%
ucGAuucAAAucuuAAcccTsT	688	GGGUuAAGAUUUGAAUCGATsT	689	AD-14138	39%	5%
ucuuAAcccuuAGGAcucuTsT	690	AGAGUCCuAAGGGUuAAGATsT	691	AD-14139	71%	11%
GcucAcGAuGAGuuuAGuGTsT	692	cACuAAACUcAUCGUGAGCTsT	693	AD-14140	43%	15%
cAuAAGcGAuGGAuAAuAcTsT	694	GuAUuAUCcAUCGCUuAUGTsT	695	AD-14141	33%	6%
AuAAGcGAuGGAuAAuAccTsT	696	GGuAUuAUCcAUCGCUuAUTsT	697	AD-14142	51%	14%
ccuAAuAAAcuGcccucAGTsT	698	CUGAGGGcAGUUuAUuAGGTsT	699	AD-14143	42%	1%
ucGGAAAGuuGAAcuuGGuTsT	700	ACcAAGUUcAACUUUCCGATsT	701	AD-14144	4%	4%
GAAAAcAuuGGccGuucuGTsT	702	cAGAACGGCcAAUGUUUUCTsT	703	AD-14145	92%	5%
AAGAcuGAucuucuAAGuuTsT	704	AACUuAGAAGAUcAGUCUUTsT	705	AD-14146	13%	2%
GAGcuuGuuAAAAucGGAATsT	706	UUCCGAUUUuAAcAAGCUCTsT	707	AD-14147	8%	1%
AcAuuGGccGuucuGGAGcTsT	708	GCUCcAGAACGGCcAAUGUTsT	709	AD-14148	80%	7%
AAGAAcAucuAuAAuuGcATsT	710	UGcAAUuAuAGAUGUUCUUTsT	711	AD-14149	44%	7%

Sense sequence (5′-3′)	SEQ ID NO.	Antisense sequence (5′-3′)	SEQ ID NO.	duplex name	single dose screen @ 25 nM [% residual mRNA]	SDs 2nd screen (among quadruplicates)
AAAuGuGucuAcucAuGuuTsT	712	AAcAUGAGuAGAcAcAUUUTsT	713	AD-14150	32%	29%
uGucuAcucAuGuuucucATsT	714	UGAGAAAcAUGAGuAGAcATsT	715	AD-14151	75%	11%
GuAuAcuGuGuAAcAAucuTsT	716	AGAUUGUuAcAcAGuAuACTsT	717	AD-14152	8%	5%
uAuAcuGuGuAAcAAucuATsT	718	uAGAUUGUuAcAcAGuAuATsT	719	AD-14153	17%	11%
cuuAGuAGuGuccAGGAAATsT	720	UUUCCUGGAcACuACuAAGTsT	721	AD-14154	16%	4%
ucAGAuGGAcGuAAGGcAGTsT	722	CUGCCUuACGUCcAUCUGATsT	723	AD-14155	11%	1%
AGAuAAAuuGAuAGcAcAATsT	724	UUGUGCuAUcAAUUuAUCUTsT	725	AD-14156	10%	1%
cAAcAGGuAcGAcAccAcATsT	726	UGUGGUGUCGuACCUGUUGTsT	727	AD-14157	29%	3%
uGcAAuGuAAAuAcGuAuuTsT	728	AAuACGuAUUuAcAUUGcATsT	729	AD-14158	51%	3%
AGucAGAAuuuuAucuAGATsT	730	UCuAGAuAAAAUUCUGACUTsT	731	AD-14159	53%	5%
cuAGAAAucuuuuAAcAccTsT	732	GGUGUuAAAAGAUUUCuAGTsT	733	AD-14160	40%	3%
AAuAAAucuAAcccuAGuuTsT	734	AACuAGGGUuAGAUUuAUUTsT	735	AD-14161	83%	7%
AAuuuucuGcucAcGAuGATsT	736	UcAUCGUGAGcAGAAAAUUTsT	737	AD-14162	44%	6%
GcccucAGuAAAuccAuGGTsT	738	CcAUGGAUUuACUGAGGGCTsT	739	AD-14163	57%	3%
AcGuuuAAAAcGAGAucuuTsT	740	AAGAUCUCGUUUuAAACGUTsT	741	AD-14164	4%	1%
AGGAGAuAGAAcGuuuAAATsT	742	UUuAAACGUUCuAUCUCCUTsT	743	AD-14165	11%	1%
GAccGucAuGGcGucGcAGTsT	744	CUGCGACGCcAUGACGGUCTsT	745	AD-14166	90%	5%
AccGucAuGGcGucGcAGcTsT	746	GCUGCGACGCcAUGACGGUTsT	747	AD-14167	49%	1%
GAAcGuuuAAAAcGAGAucTsT	748	GAUCUCGUUUuAAACGUUCTsT	749	AD-14168	12%	2%
uuGAGcuuAAcAuAGGuAATsT	750	UuACCuAUGUuAAGCUcAATsT	751	AD-14169	66%	4%
AcuAAAuuGAucucGuAGATsT	752	UCuACGAGAUcAAUUuAGUTsT	753	AD-14170	52%	6%
ucGuAGAAuuAucuuAAuATsT	754	uAUuAAGAuAAUUCuACGATsT	755	AD-14171	42%	4%
GGAGAuAGAAcGuuuAAAATsT	756	UUUuAAACGUUCuAUCUCCTsT	757	AD-14172	3%	1%
AcAAcuuAuuGGAGGuuGuTsT	758	AcAACCUCcAAuAAGUUGUTsT	759	AD-14173	29%	2%
uGAGcuuAAcAuAGGuAAATsT	760	UUuACCuAUGUuAAGCUcATsT	761	AD-14174	69%	2%
AucucGuAGAAuuAucuuATsT	762	uAAGAuAAUUCuACGAGAUTsT	763	AD-14175	53%	3%
cuGcGuGcAGucGGuccucTsT	764	GAGGACCGACUGcACGcAGTsT	765	AD-14176	111%	4%
cAcGcAGcGcccGAGAGuATsT	766	uACUCUCGGGCGCUGCGUGTsT	767	AD-14177	87%	6%
AGuAccAGGGAGAcuccGGTsT	768	CCGGAGUCUCCCUGGuACUTsT	769	AD-14178	59%	2%
AcGGAGGAGAuAGAAcGuuTsT	770	AACGUUCuAUCUCCUCCGUTsT	771	AD-14179	9%	2%
AGAAcGuuuAAAAcGAGAuTsT	772	AUCUCGUUUuAAACGUUCUTsT	773	AD-14180	43%	2%
AAcGuuuAAAAcGAGAucuTsT	774	AGAUCUCGUUUuAAACGUUTsT	775	AD-14181	70%	10%
AGcuuGAGcuuAAcAuAGGTsT	776	CCuAUGUuAAGCUcAAGCUTsT	777	AD-14182	100%	7%
AGcuuAAcAuAGGuAAAuATsT	778	uAUUuACCuAUGUuAAGCUTsT	779	AD-14183	60%	5%
uAGAGcuAcAAAAccuAucTsT	780	GAuAGGUUUUGuAGCUCuATsT	781	AD-14184	129%	6%
uAGuuGuAucccuccuuuATsT	782	uAAAGGAGGGAuAcAACuATsT	783	AD-14185	62%	4%
AccAcccAGAcAucuGAcuTsT	784	AGUcAGAUGUCUGGGUGGUTsT	785	AD-14186	42%	3%
AGAAAcuAAAuuGAucucGTsT	786	CGAGAUcAAUUuAGUUUCUTsT	787	AD-14187	123%	12%
ucucGuAGAAuuAucuuAATsT	788	UuAAGAuAAUUCuACGAGATsT	789	AD-14188	38%	2%
cAAcuuAuuGGAGGuuGuATsT	790	uAcAACCUCcAAuAAGUUGTsT	791	AD-14189	13%	1%
uuGuAucccuccuuuAAGuTsT	792	ACUuAAAGGAGGGAuAcAATsT	793	AD-14190	59%	3%
ucAcAAcuuAuuGGAGGuuTsT	794	AACCUCcAAuAAGUUGUGATsT	795	AD-14191	93%	3%
AGAAcuGuAcucuucucAGTsT	796	CUGAGAAGAGuAcAGUUCUTsT	797	AD-14192	45%	5%
GAGcuuAAcAuAGGuAAAuTsT	798	AUUuACCuAUGUuAAGCUCTsT	799	AD-14193	57%	3%
cAccAAcAucuGuccuuAGTsT	800	CuAAGGAcAGAUGUUGGUGTsT	801	AD-14194	51%	4%
AAAGcccAcuuuAGAGuAuTsT	802	AuACUCuAAAGUGGGCUUUTsT	803	AD-14195	77%	5%
AAGcccAcuuuAGAGuAuATsT	804	uAuACUCuAAAGUGGGCUUTsT	805	AD-14196	42%	6%
GAccuuAuuuGGuAAucuGTsT	806	cAGAUuACcAAAuAAGGUCTsT	807	AD-14197	15%	2%
GAuuAAuGuAcucAAGAcuTsT	808	AGUCUUGAGuAcAUuAAUCTsT	809	AD-14198	12%	2%
cuuuAAGAGGccuAAcucATsT	810	UGAGUuAGGCCUCUuAAAGTsT	811	AD-14199	18%	2%
uuAAAccAAAcccuAuuGATsT	812	UcAAuAGGGUUUGGUUuAATsT	813	AD-14200	72%	9%
ucuGuuGGAGAucuAuAAuTsT	814	AUuAuAGAUCUCcAAcAGATsT	815	AD-14201	9%	3%
cuGAuGuuucuGAGAGAcuTsT	816	AGUCUCUcAGAAAcAUcAGTsT	817	AD-14202	25%	3%
GcAuAcucuAGucGuuccccTsT	818	GGGAACGACuAGAGuAUGCTsT	819	AD-14203	21%	1%
GuuccuuAucGAGAAucuATsT	820	uAGAUUCUCGAuAAGGAACTsT	821	AD-14204	4%	2%
GcAcuuGGAucucucAcAuTsT	822	AUGUGAGAGAUCcAAGUGCTsT	823	AD-14205	5%	1%
AAAAAAGGAAcuAGAuGGcTsT	824	GCcAUCuAGUUCCUUUUUUTsT	825	AD-14206	79%	6%
AGAGcAGAuuAccucuGcGTsT	826	CGcAGAGGuAAUCUGCUCUTsT	827	AD-14207	55%	2%
AGcAGAuuAccucuGcGAGTsT	828	CUCGcAGAGGuAAUCUGCUTsT	829	AD-14208	100%	4%
cccuGAcAGAGuucAcAAATsT	830	UUUGUGAACUCUGUcAGGGTsT	831	AD-14209	34%	3%
GuuuAccGAAGuGuuGuuuTsT	832	AAAcAAcACUUCGGuAAACTsT	833	AD-14210	13%	2%
uuAcAGuAcAcAAcAAGGATsT	834	UCCUUGUUGUGuACUGuAATsT	835	AD-14211	9%	1%
AcuGGAucGuAAGAAGGcATsT	836	UGCCUUCUuACGAUCcAGUTsT	837	AD-14212	20%	3%
GAGcAGAuuAccucuGcGATsT	838	UCGcAGAGGuAAUCUGCUCTsT	839	AD-14213	48%	5%
AAAAGAAGuuAGuGuAcGATsT	840	UCGuAcACuAACUUCUUUUUTsT	841	AD-14214	28%	18%
GAccAuuuAAuuuGGcAGATsT	842	UCUGCcAAAUuAAAUGGUCTsT	843	AD-14215	132%	0%
GAGAGGAGuGAuAAuuAAATsT	844	UUuAAUuAUcACUCCUCUCTsT	845	AD-14216	3%	0%
cuGGAGGAuuGGcuGAcAATsT	846	UUGUcAGCcAAUCCUCcAGTsT	847	AD-14217	19%	1%
cucuAGucGuucccAcucATsT	848	UGAGUGGGAACGACuAGAGTsT	849	AD-14218	67%	8%
GAuAccAuuAcuAcAGuAGTsT	850	CuACUGuAGuAAUGGuAUCTsT	851	AD-14219	76%	4%

Sense sequence (5′-3′)	SEQ ID NO.	Antisense sequence (5′-3′)	SEQ ID NO.	duplex name	single dose screen @ 25 nM [% residual mRNA]	SDs 2nd screen (among quadruplicates)
uucGucuGcGAAGAAGAAATsT	852	UUUCUUCUUCGcAGACGAATsT	853	AD-14220	33%	8%
GAAAAGAAGuuAGuGuAcGTsT	854	CGuAcACuAACUUCUUUUCTsT	855	AD-14221	25%	2%
uGAuGuuuAccGAAGuGuuTsT	856	AAcACUUCGGuAAAcAUcATsT	857	AD-14222	7%	2%
uGuuuGuccAAuucuGGAuTsT	858	AUCcAGAAUUGGAcAAAcATsT	859	AD-14223	19%	2%
AuGAAGAGuAuAccuGGGATsT	860	UCCcAGGuAuACUCUUcAUTsT	861	AD-14224	13%	1%
GcuAcucuGAuGAAuGcAuTsT	862	AUGcAUUcAUcAGAGuAGCTsT	863	AD-14225	15%	2%
GcccuuGuAGAAAGAAcAcTsT	864	GUGUUCUUUCuAcAAGGGCTsT	865	AD-14226	11%	0%
ucAuGuuccuuAucGAGAATsT	866	UUCUCGAuAAGGAAcAUGATsT	867	AD-14227	5%	1%
GAAuAGGGuuAcAGAGuuGTsT	868	cAACUCUGuAACCCuAUUCTsT	869	AD-14228	34%	3%
cAAAcuGGAucGuAAGAAGTsT	870	CUUCUuACGAUCcAGUUUGTsT	871	AD-14229	15%	2%
cuuAuuuGGuAAucuGcuGTsT	872	cAGcAGAUuACcAAAuAAGTsT	873	AD-14230	20%	1%
AGcAAuGuGGAAAccuAAcTsT	874	GUuAGGUUUCcAcAUUGCUTsT	875	AD-14231	18%	1%
AcAAuAAAGcAGAcccAuuTsT	876	AAUGGGUCUGCUUuAUUGUTsT	877	AD-14232	21%	1%
AAccAcuuAGuAGuGuccATsT	878	UGGAcACuACuAAGUGGUUTsT	879	AD-14233	106%	12%
AGucAAGAGccAucuGuAGTsT	880	CuAcAGAUGGCUCUUGACUTsT	881	AD-14234	35%	3%
cucccuAGAcuucccuAuuTsT	882	AAuAGGGAAGUCuAGGGAGTsT	883	AD-14235	48%	4%
AuAGcuAAAuuAAAccAAATsT	884	UUUGGUUuAAUUuAGCuAUTsT	885	AD-14236	23%	3%
uGGcuGGuAuAAuuccAcGTsT	886	CGUGGAAUuAuACcAGCcATsT	887	AD-14237	79%	9%
uuAuuuGGuAAucuGcuGuTsT	888	AcAGcAGAUuACcAAAuAATsT	889	AD-14238	92%	7%
AAcuAGAuGGcuuucucAGTsT	890	CUGAGAAAGCcAUCuAGUUTsT	891	AD-14239	20%	2%
ucAuGGcGucGcAGccAAATsT	892	UUUGGCUGCGACGCcAUGATsT	893	AD-14240	71%	6%
AcuGGAGGAuuGGcuGAcATsT	894	UGUcAGCcAAUCCUCcAGUTsT	895	AD-14241	14%	1%
cuAuAAuuGcAcuAucuuuTsT	896	AAAGAuAGUGcAAUuAuAGTsT	897	AD-14242	11%	2%
AAAGGucAccuAAuGAAGATsT	898	UCUUcAUuAGGUGACCUUUTsT	899	AD-14243	11%	1%
AuGAAuGcAuAcucuAGucTsT	900	GACuAGAGuAUGcAUUcAUTsT	901	AD-14244	15%	2%
AAcAuAuuGAAuAAGccuGTsT	902	cAGGCUuAUUcAAuAUGUUTsT	903	AD-14245	80%	7%
AAGAAGGcAGuuGAccAAcTsT	904	GUUGGUcAACUGCCUUCUUTsT	905	AD-14246	57%	5%
GAuAcuAAAAGAAcAAucATsT	906	UGAUUGUUCUUUuAGuAUCTsT	907	AD-14247	9%	3%
AuAcuGAAAAucAAuAGucTsT	908	GACuAUUGAUUUUcAGuAUTsT	909	AD-14248	39%	4%
AAAAAGGAAcuAGAuGGcuTsT	910	AGCcAUCuAGUUCCUUUUUTsT	911	AD-14249	64%	2%
GAAcuAGAuGGcuuucucATsT	912	UGAGAAAGCcAUCuAGUUCTsT	913	AD-14250	18%	2%
GAAAccuAAcuGAAGAccuTsT	914	AGGUCUUcAGUuAGGUUUCTsT	915	AD-14251	56%	6%
uAcccAucAAcAcuGGuAATsT	916	UuACcAGUGUUGAUGGGuATsT	917	AD-14252	48%	6%
AuuuuGAuAucuAcccAuuTsT	918	AAUGGGuAGAuAUcAAAAUTsT	919	AD-14253	39%	5%
AucccuAuAGuucAcuuuGTsT	920	cAAAGUGAACuAuAGGGAUTsT	921	AD-14254	44%	8%
AuGGGcuAuAAuuGcAcuATsT	922	uAGUGcAAUuAuAGCCcAUTsT	923	AD-14255	108%	8%
AGAuuAccucuGcGAGcccTsT	924	GGGCUCGcAGAGGuAAUCUTsT	925	AD-14256	108%	6%
uAAuuccAcGuAcccuucATsT	926	UGAAGGGuACGUGGAAUuATsT	927	AD-14257	23%	2%
GucGuucccAcucAGuuuuTsT	928	AAAAC**uG**AG**uG**GGAACGACTsT	929	AD-14258	21%	3%
AAAucAAucccuGuuGAcuTsT	930	AGUcAAcAGGGAUUGAUUUTsT	931	AD-14259	19%	2%
ucAuGAGAcAAAGAAcAuATsT	932	uAUGUUCUUUGCUCuAUGATsT	933	AD-14260	10%	1%
uuAcuAcAGuAGcAcuuGGTsT	934	CcAAGUGCuACUGuAGuAATsT	935	AD-14261	76%	3%
AuGuGGAAAccuAAcuGAATsT	936	UUcAGUuAGGUUUCcAcAUTsT	937	AD-14262	13%	2%
uGuGGAAAccuAAcuGAAGTsT	938	CUUcAGUuAGGUUUCcAcATsT	939	AD-14263	14%	2%
ucuuccuuAAAuGAAAGGGTsT	940	CCCUUUcAUUuAAGGAAGATsT	941	AD-14264	65%	3%
uGAAGAAccucuAAGucAATsT	942	UUGACUuAGAGGUUCUUcATsT	943	AD-14265	13%	1%
AGAGGucuAAAGuGGAAGATsT	944	UCUUCcACUUuAGACCUCUTsT	945	AD-14266	18%	3%
AuAucuAcccAuuuuucuGTsT	946	cAGAAAAAUGGGuAGAuAUTsT	947	AD-14267	50%	9%
uAAGccuGAAGuGAAucAGTsT	948	CUGAUUcACUUcAGGCUuATsT	949	AD-14268	13%	3%
AGAuGcAGAccAuuuAAuuTsT	950	AAUuAAAUGGUCUGcAUCUTsT	951	AD-14269	19%	4%
AGuGuuGuuuGuccAAuucTsT	952	GAAUUGGAcAAAcAAcACUTsT	953	AD-14270	11%	2%
cuAuAAuGAAGAGcuuuuuTsT	954	AAAAAGCUCUUcAUuAuAGTsT	955	AD-14271	11%	1%
AGAGGAGuGAuAAuuAAAGTsT	956	CUUuAAUuAUcACUCCUCUTsT	957	AD-14272	7%	1%
uuucucuGuuAcAAuAcAuTsT	958	AUGuAUUGuAAcAGAGAAATsT	959	AD-14273	14%	2%
AAcAucuAuAAuuGcAAcATsT	960	UGUUGcAAUuAuAGAUGUUTsT	961	AD-14274	73%	4%
uGcuAGAAGuAcAuAAGAcTsT	962	GUCUuAUGuACUUCuAGcATsT	963	AD-14275	10%	1%
AAuGuAcucAAGAcuGAucTsT	964	GAUcAGUCUUGAGuAcAUUTsT	965	AD-14276	89%	2%
GuAcucAAGAcuGAucuucTsT	966	GAAGAUcAGUCUUGAGuACTsT	967	AD-14277	7%	1%
cAcucuGAuAAAcucAAuGTsT	968	cAUUGAGUUuAUcAGAGUGTsT	969	AD-14278	12%	1%
AAGAGcAGAuuAccucuGcTsT	970	GcAGAGGuAAUCUGCUCUUTsT	971	AD-14279	104%	3%
ucuGcGAGcccAGAucAAcTsT	972	GUUGAUCUGGGCUCGcAGATsT	973	AD-14280	21%	2%
AAcuuGAGccuuGuGuAuATsT	974	uAuAcAcAAGGCUcAAGUUTsT	975	AD-14281	43%	3%
GAAuAuAuAuAucAGccGGTsT	976	CCGGCUGAuAuAuAuAUUCTsT	977	AD-14282	45%	6%
uGucAuccccuAuAGuucAcTsT	978	GUGAACuAuAGGGAUGAcATsT	979	AD-14283	35%	5%
GAucuGGcAAccAuAuuucTsT	980	GAAAuAUGGUUGCcAGAUCTsT	981	AD-14284	58%	3%
uGGcAAccAuAuuucuGGATsT	982	UCcAGAAAuAUGGUUGCcATsT	983	AD-14285	48%	3%
GAuGuuuAccGAAGuGuuGTsT	984	cAAcACUUCGGuAAAcAUCTsT	985	AD-14286	49%	3%
uuccuuAucGAGAAucuAATsT	986	UuAGAUUCUCGAuAAGGAATsT	987	AD-14287	6%	1%
AGcuuAAuuGcuuucuGGATsT	988	UCcAGAAAGcAAUuAAGCUTsT	989	AD-14288	50%	2%
uuGcuAuuAuGGGAGAccATsT	990	UGGUCUCCcAuAAuAGcAATsT	991	AD-14289	48%	1%

Sense sequence (5′-3′)	SEQ ID NO.	Antisense sequence (5′-3′)	SEQ ID NO.	duplex name	single dose screen @ 25 nM [% residual mRNA]	SDs 2nd screen (among quadruplicates)
GucAuGGcGucGcAGccAATsT	992	UUGGCUGCGACGCcAUGACTsT	993	AD-14290	112%	7%
uAAuuGcAcuAucuuuGcGTsT	994	CGcAAAGAuAGUGcAAUuATsT	995	AD-14291	77%	2%
cuAucuuuGcGuAuGGccATsT	996	UGGCcAuACGcAAAGAuAGTsT	997	AD-14292	80%	6%
ucccuAuAGuucAcuuuGuTsT	998	AcAAAGUGAACuAuAGGGATsT	999	AD-14293	58%	2%
ucAAccuuuAAuucAcuuGTsT	1000	cAAGUGAAUuAAAGGUUGATsT	1001	AD-14294	77%	2%
GGcAAccAuAuuucuGGAATsT	1002	UUCcAGAAAuAUGGUUGCCTsT	1003	AD-14295	62%	2%
AuGuAcucAAGAcuGAucuTsT	1004	AGAUcAGUCUUGAGuAcAUTsT	1005	AD-14296	59%	4%
GcAGAccAuuuAAuuuGGcTsT	1006	GCcAAAUuAAAUGGUCUGCTsT	1007	AD-14297	37%	1%
ucuGAGAGAcuAcAGAuGuTsT	1008	AcAUCUGuAGUCUCUcAGATsT	1009	AD-14298	21%	1%
uGcucAuAGAGcAAAGAAcTsT	1010	GUUCUUUGCUCuAUGAGcATsT	1011	AD-14299	6%	1%
AcAuAAGAccuuAuuuGGuTsT	1012	ACcAAAuAAGGUCUuAUGUTsT	1013	AD-14300	17%	2%
uuuGuGcuGAuucuGAuGGTsT	1014	CcAUcAGAAUcAGcAcAAATsT	1015	AD-14301	97%	6%
ccAucAAcAcuGGuAAGAATsT	1016	UUCUuACcAGUGUUGAUGGTsT	1017	AD-14302	13%	1%
AGAcAAuuccGGAuGuGGATsT	1018	UCcAcAUCCGGAAUUGUCUTsT	1019	AD-14303	13%	3%
GAAcuuGAGccuuGuGuAuTsT	1020	AuAcAcAAGGCUcAAGUUCTsT	1021	AD-14304	38%	2%
uAAuuuGGcAGAGcGGAAATsT	1022	UUUCCGCUCUGCcAAAUuATsT	1023	AD-14305	14%	2%
uGGAuGAAGuuAuuAuGGGTsT	1024	CCcAuAAuAACUUcAUCcATsT	1025	AD-14306	22%	4%
AucuAcAuGAAcuAcAAGATsT	1026	UCUUGuAGUUcAUGuAGAUTsT	1027	AD-14307	26%	6%
GGuAuuuuuGAucuGGcAATsT	1028	UUGCcAGAUcAAAAAuACCTsT	1029	AD-14308	62%	8%
cuAAuGAAGAGuAuAccuGTsT	1030	cAGGuAuACUCUUcAUuAGTsT	1031	AD-14309	52%	5%
uuuGAGAAAcuuAcuGAuATsT	1032	uAUcAGuAAGUUUCUcAAATsT	1033	AD-14310	32%	3%
cGAuAAGAuAGAAGAucAATsT	1034	UUGAUCUUCuAUCUuAUCGTsT	1035	AD-14311	23%	2%
cuGGcAAccAuAuuucuGGTsT	1036	CcAGAAAuAUGGUUGCcAGTsT	1037	AD-14312	49%	6%
uAGAuAccAuuAcuAcAGuTsT	1038	ACUGuAGuAAUGGuAUCuATsT	1039	AD-14313	69%	4%
GuAuuAAAuuGGGuuucAuTsT	1040	AUGAAACCcAAUUuAAuACTsT	1041	AD-14314	52%	3%
AAGAccuuAuuuGGuAAucTsT	1042	GAUuACcAAAuAAGGUCUUTsT	1043	AD-14315	66%	4%
GcuGuuGAuAAGAGAGcucTsT	1044	GAGCUCUCUuAUcAAcAGCTsT	1045	AD-14316	19%	4%
uAcucAuGuuucucAGAuuTsT	1046	AAUCUGAGAAAcAUGAGuATsT	1047	AD-14317	16%	5%
cAGAuGGAcGuAAGGcAGcTsT	1048	GCUGCCUuACGUCcAUCUGTsT	1049	AD-14318	52%	11%
uAucccAAcAGGuAcGAcATsT	1050	UGUCGuACCUGUUGGGAuATsT	1051	AD-14319	28%	11%
cAuuGcuAuuAuGGGAGAcTsT	1052	GUCUCCcAuAAuAGcAAUGTsT	1053	AD-14320	52%	10%
cccucAGuAAAuccAuGGuTsT	1054	ACcAUGGAUUuACUGAGGGTsT	1055	AD-14321	53%	6%
GGucAuuAcuGcccuuGuATsT	1056	uAcAAGGGcAGuAAUGACCTsT	1057	AD-14322	20%	2%
AAccAcucAAAAAcAuuuGTsT	1058	cAAAUGUUUUUGAGUGGUUTsT	1059	AD-14323	116%	6%
uuuGcAAGuuAAuGAAucuTsT	1060	AGAUUcAUuAACUUGcAAATsT	1061	AD-14324	14%	2%
uuAuuuucAGuAGucAGAATsT	1062	UUCUGACuACUGAAAAuAATsT	1063	AD-14325	50%	2%
uuuucucGAuucAAAucuuTsT	1064	AAGAUU**uG**AAUCGAGAAAATsT	1065	AD-14326	47%	3%
GuAcGAAAAGAAGuuAGuGTsT	1066	cACuAACUUCUUUUCGuACTsT	1067	AD-14327	18%	2%
uuuAAAAcGAGAucuuGcuTsT	1068	AGcAAGAUCUCGUUUuAAATsT	1069	AD-14328	19%	1%
GAAuuGAuuAAuGuAcucATsT	1070	UGAGuAcAUuAAUcAAUUCTsT	1071	AD-14329	94%	10%
GAuGGAcGuAAGGcAGcucTsT	1072	GAGCUGCCUuACGUCcAUCTsT	1073	AD-14330	60%	4%
cAucuGAcuAAuGGcucuGTsT	1074	cAGAGCcAUuAGUcAGAUGTsT	1075	AD-14331	54%	7%
GuGAuccuGuAcGAAAAGATsT	1076	UCUUUUCGuAcAGGAUcACTsT	1077	AD-14332	22%	4%
AGcucuuAuuAAGGAGuAuTsT	1078	AuACUCCUuAAuAAGAGCUTsT	1079	AD-14333	70%	10%
GcucuuAuuAAGGAGuAuATsT	1080	uAuACUCCUuAAuAAGAGCTsT	1081	AD-14334	18%	3%
ucuuAuuAAGGAGuAuAcGTsT	1082	CGuAuACUCCUuAAuAAGATsT	1083	AD-14335	38%	6%
uAuuAAGGAGuAuAcGGAGTsT	1084	CUCCGuAuACUCCUuAAuATsT	1085	AD-14336	16%	3%
cuGcAGcccGuGAGAAAAATsT	1086	UUUUUCUcACGGGCUGcAGTsT	1087	AD-14337	65%	4%
ucAAGAcuGAucuucuAAGTsT	1088	CUuAGAAGAUcAGUCUUGATsT	1089	AD-14338	18%	0%
cuucuAAGuucAcuGGAAATsT	1090	UUUCcAGUGAACUuAGAAGTsT	1091	AD-14339	20%	4%
uGcAAGuuAAuGAAucuuuTsT	1092	AAAGAUUcAUuAACUUGcATsT	1093	AD-14340	24%	1%
AAucuAAGGAuAuAGucAATsT	1094	UUGACuAuAUCCUuAGAUUTsT	1095	AD-14341	27%	3%
AucucuGAAcAcAAGAAcATsT	1096	UGUUCUUGUGUUcAGAGAUTsT	1097	AD-14342	13%	1%
uucuGAAcAGuGGGuAucuTsT	1098	AGAuACCcACUGUUcAGAATsT	1099	AD-14343	19%	1%
AGuuAuuuAuAcccAucAATsT	1100	UUGAUGGGuAuAAAuAACUTsT	1101	AD-14344	23%	2%
AuGcuAAAcuGuucAGAAATsT	1102	UUUCUGAAcAGUUuAGcAUTsT	1103	AD-14345	21%	4%
cuAcAGAGcAcuuGGuuAcTsT	1104	GuAACcAAGUGCUCUGuAGTsT	1105	AD-14346	18%	2%
uAuAuAucAGccGGGcGcGTsT	1106	CGCGCCCGGCUGAuAuAuATsT	1107	AD-14347	67%	2%
AuGuAAAuAcGuAuuucuATsT	1108	uAGAAAuACGuAUUuAcAUTsT	1109	AD-14348	39%	3%
uuuuucucGAuucAAAucuTsT	1110	AGAUU**uG**AAUCGAGAAAAATsT	1111	AD-14349	83%	6%
AAucuuAAcccuuAGGAcuTsT	1112	AGUCCuAAGGGUuAAGAUUTsT	1113	AD-14350	54%	2%
ccuuAGGAcucuGGuAuuuTsT	1114	AAAuACcAGAGUCCuAAGGTsT	1115	AD-14351	57%	8%
AAuAAAcuGcccucAGuAATsT	1116	UuACUGAGGGcAGUUuAUUTsT	1117	AD-14352	82%	3%
GAuccuGuAcGAAAAGAAGTsT	1118	CUUCUUUUCGuAcAGGAUCTsT	1119	AD-14353	2%	1%
AAuGuGAuccuGuAcGAAATsT	1120	UUUCGuAcAGGAUcAcAUUTsT	1121	AD-14354	18%	11%
GuGAAAAcAuuGGccGuucTsT	1122	GAACGGCcAAUGUUUUcACTsT	1123	AD-14355	2%	1%
cuuGAGGAAAcucuGAGuATsT	1124	uACUcAGAGUUUCCUcAAGTsT	1125	AD-14356	8%	2%
cGuuuAAAAcGAGAucuuGTsT	1126	cAAGAUCUCGUUUuAAACGTsT	1127	AD-14357	6%	3%
uuAAAAcGAGAucuuGcuGTsT	1128	cAGcAAGAUCUCGUUUuAATsT	1129	AD-14358	98%	17%
AAAGAuGuAucuGGucuccTsT	1130	GGAGACcAGAuAcAUCUUUTsT	1131	AD-14359	10%	1%

Sense sequence (5′-3′)	SEQ ID NO.	Antisense sequence (5′-3′)	SEQ ID NO.	duplex name	single dose screen @ 25 nM [% residual mRNA]	SDs 2nd screen (among quadruplicates)
cAGAAAAuGuGucuAcucATsT	1132	UGAGuAGAcAcAUUUUCUGTsT	1133	AD-14360	6%	4%
cAGGAAuuGAuuAAuGuAcTsT	1134	GuAcAUuAAUcAAUUCCUGTsT	1135	AD-14361	30%	5%
AGucAAcuAAAGcAuAuuuTsT	1136	AAAuAUGCUUuAGUUGACUTsT	1137	AD-14362	28%	2%
uGuGuAAcAAucuAcAuGATsT	1138	UcAUGuAGAUUGUuAcAcATsT	1139	AD-14363	60%	6%
AuAccAuuuGuuccuuGGuTsT	1140	ACcAAGGAAcAAAUGGuAUTsT	1141	AD-14364	12%	9%
GcAGAAAucuAAGGAuAuATsT	1142	uAuAUCCUuAGAUUUCUGCTsT	1143	AD-14365	5%	2%
uGGcuucucAcAGGAAcucTsT	1144	GAGUUCCUGUGAGAAGCcATsT	1145	AD-14366	28%	5%
GAGAuGuGAAucucuGAAcTsT	1146	GUUcAGAGAUUcAcAUCUCTsT	1147	AD-14367	42%	4%
uGuAAGccAAuGuuGuGAGTsT	1148	CUcAcAAcAUUGGCUuAcATsT	1149	AD-14368	93%	12%
AGccAAuGuuGuGAGGcuuTsT	1150	AAGCCUcAcAAcAUUGGCUTsT	1151	AD-14369	65%	4%
uuGuGAGGcuucAAGuucATsT	1152	UGAACUUGAAGCCUcAcAATsT	1153	AD-14370	5%	2%
AGGcAGcucAuGAGAAAcATsT	1154	UGUUUCUcAUGAGCUGCCUTsT	1155	AD-14371	54%	5%
AuAAAuuGAuAGcAcAAAATsT	1156	UUUUGUGCuAUcAAUUuAUTsT	1157	AD-14372	4%	1%
AcAAAAucuAGAAcuuAAuTsT	1158	AUuAAGUUCuAGAUUUUGUTsT	1159	AD-14373	5%	1%
GAuAucccAAcAGGuAcGATsT	1160	UCGuACCUGUUGGGAuAUCTsT	1161	AD-14374	92%	6%
AAGuuAuuuAuAcccAucATsT	1162	UGAUGGGuAuAAAuAACUUTsT	1163	AD-14375	76%	4%
uGuAAAAuAcGuAuuuucuAGTsT	1164	CuAGAAAAuACGuAUUuAcATsT	1165	AD-14376	70%	5%
ucuAGuuuucAuAuAAAGuTsT	1166	ACUUuAuAUGAAAACuAGATsT	1167	AD-14377	48%	4%
AuAAAGuAGuucuuuuAuATsT	1168	uAuAAAAGAACuACUUuAUTsT	1169	AD-14378	48%	3%
ccAuuuGuAGAGcuAcAAATsT	1170	UUUGuAGCUCuAcAAAUGGTsT	1171	AD-14379	44%	5%
uAuuuucAGuAGucAGAAuTsT	1172	AUUCUGACuACUGAAAAuATsT	1173	AD-14380	35%	16%
AAAucuAAcccuAGuuGuATsT	1174	uAcAACuAGGGUuAGAUUUTsT	1175	AD-14381	44%	5%
cuuuAGAGuAuAcAuuGcuTsT	1176	AGcAAUGuAuACUCuAAAGTsT	1177	AD-14382	28%	1%
AucuGAcuAAuGGcucuGuTsT	1178	AcAGAGCcAUuAGUcAGAUTsT	1179	AD-14383	55%	11%
cAcAAuGAuuuAAGGAcuGTsT	1180	cAGUCCUuAAAUcAUUGUGTsT	1181	AD-14384	48%	9%
ucuuuuucucGAuucAAAuTsT	1182	AUU**uG**AAUCGAGAAAAAGATsT	1183	AD-14385	36%	2%
cuuuuucucGAuucAAAucTsT	1184	GAUU**uG**AAUCGAGAAAAAGTsT	1185	AD-14386	41%	7%
AuuuucuGcucAcGAuGAGTsT	1186	CUcAUCGUGAGcAGAAAAUTsT	1187	AD-14387	38%	3%
uuucuGcucAcGAuGAGuuTsT	1188	AACUcAUCGUGAGcAGAAATsT	1189	AD-14388	50%	4%
AGAGcuAcAAAAccuAuccTsT	1190	GGAuAGGUUUUGuAGCUCUTsT	1191	AD-14389	98%	6%
GAGccAAAGGuAcAccAcuTsT	1192	AGUGGUGuACCUUUGGCUCTsT	1193	AD-14390	43%	8%
GccAAAGGuAcAccAcuAcTsT	1194	GuAGUGGUGuACCUUUGGCTsT	1195	AD-14391	48%	4%
GAAcuGuAcucuucucAGcTsT	1196	GCUGAGAAGAGuAcAGUUCTsT	1197	AD-14392	44%	3%
AGGuAAAuAucAccAAcAuTsT	1198	AUGUUGGUGAuAUUuACCUTsT	1199	AD-14393	37%	2%
AGcuAcAAAAccuAuccuuTsT	1200	AAGGAuAGGUUUUGuAGCUTsT	1201	AD-14394	114%	7%
uGuGAAAGcAuuuAAuuccTsT	1202	GGAAUuAAAUGCUUUcAcATsT	1203	AD-14395	55%	4%
GcccAcuuuAGAGuAuAcATsT	1204	UGuAuACUCuAAAGUGGGCTsT	1205	AD-14396	49%	5%
uGuGccAcAcuccAAGAccTsT	1206	GGUCUUGGAGUGUGGcAcATsT	1207	AD-14397	71%	6%
AAAcuAAAuuGAucucGuATsT	1208	uACGAGAUcAAUUuAGUUUTsT	1209	AD-14398	81%	7%
uGAucucGuAGAAuuAucuTsT	1210	AGAuAAUUCuACGAGAUcATsT	1211	AD-14399	38%	4%
GcGuGcAGucGGucccuccATsT	1212	UGGAGGACCGACUGcACGCTsT	1213	AD-14400	106%	8%
AAAGuuuAGAGAcAucuGATsT	1214	UcAGAUGUCUCuAAACUUUTsT	1215	AD-14401	47%	3%
cAGAAGGAAuAuGuAcAAATsT	1216	UUUGuAcAuAUUCCUUCUGTsT	1217	AD-14402	31%	1%
cGcccGAGAGuAccAGGGATsT	1218	UCCCUGGuACUCUCGGGCGTsT	1219	AD-14403	105%	4%
cGGAGGAGAuAGAAcGuuuTsT	1220	AAACGUUCuAUCUCCUCCGTsT	1221	AD-14404	3%	1%
AGAuAGAAcGuuuAAAAcGTsT	1222	CGUUUuAAACGUUCuAUCUTsT	1223	AD-14405	15%	1%
GGAAcAGGAAcuucAcAAcTsT	1224	GU**uGuG**AAGUUCC**uG**UUCCTsT	1225	AD-14406	44%	5%
GuGAGccAAAGGuAcAccATsT	1226	UGGUGuACCUUUGGCUcACTsT	1227	AD-14407	41%	4%
AucccucccuAGAcuuccccuTsT	1228	AGGGAAGUCuAGGGAGGAUTsT	1229	AD-14408	104%	3%
cAcAcuccAAGAccuGuGcTsT	1230	GcAcAGGUCUUGGAGUGUGTsT	1231	AD-14409	67%	4%
AcAGAAGGAAuAuGuAcAATsT	1232	UUGuAcAuAUUCCUUCUGUTsT	1233	AD-14410	22%	1%
uuAGAGAcAucuGAcuuuGTsT	1234	cAAAGUcAGAUGUCUCuAATsT	1235	AD-14411	29%	3%
AAuuGAucucGuAGAAuuATsT	1236	uAAUUCuACGAGAUcAAUUTsT	1237	AD-14412	31%	4%

dsRNA targeting the VEGF gene

Four hundred target sequences were identified within exons 1-5 of the VEGF-A121 mRNA sequence. Reference transcript is : NM_003376.

```
1   augaacuuuc ugcugucuug ggugcauugg agccuugccu ugcugcucua ccuccaccau

61  gccaaguggu cccaggcugc acccauggca gaaggaggag ggcagaauca ucacgaagug

121 gugaaguuca uggaugucua ucagcgcagc uacugccauc caaucgagac ccugguggac
```

181 aucuuccagg aguacccuga ugagaucgag uacaucuuca agccauccug ugugccccug

241 augcgaugcg ggggcugcug caaugacgag ggccuggagu gugugcccac ugaggagucc

301 aacaucacca ugcagauuau gcggaucaaa ccucaccaag gccagcacau aggagagaug

361 agcuuccuac agcacaacaa augugaaugc agaccaaaga aagauagagc aagacaagaa

421 aaaugugaca agccgaggcg guga (SEQ ID NO:1539)

Table 4a includes the identified target sequences. Corresponding siRNAs targeting these sequences were subjected to a bioinformatics screen.

To ensure that the sequences were specific to VEGF sequence and not to sequences from any other genes, the target sequences were checked against the sequences in Genbank using the BLAST search engine provided by NCBI. The use of the BLAST algorithm is described in Altschul *et al.*, J. Mol. Biol. 215:403, 1990; and Altschul and Gish, Meth. Enzymol. 266:460, 1996.

siRNAs were also prioritized for their ability to cross react with monkey, rat and human VEGF sequences.

Of these 400 potential target sequences 80 were selected for analysis by experimental screening in order to identify a small number of lead candidates. A total of 114 siRNA molecules were designed for these 80 target sequences 114 (Table 4b).

Table 4a. Target sequences in VEGF-121

SEQ ID NO:	position in VEGF-121 ORF	TARGET SEQUENCE IN VEGF121 mRNA 5′ to 3′	SEQ ID NO:	position in VEGF-121 ORF	TARGET SEQUENCE IN VEGF121 mRNA 5′ to 3′
1540	1	AUGAACUUUCUGCUGUCUUGGGU	1584	45	GCUCUACCUCCACCAUGCCAAGU
1541	2	UGAACUUUCUGCUGUCUUGGGUG	1585	46	CUCUACCUCCACCAUGCCAAGUG
1542	3	GAACUUUCUGCUGUCUUGGGUGC	1586	47	UCUACCUCCACCAUGCCAAGUGG
1543	4	AACUUUCUGCUGUCUUGGGUGCA	1587	48	CUACCUCCACCAUGCCAAGUGGU
1544	5	ACUUUCUGCUGUCUUGGGUGCAU	1588	49	UACCUCCACCAUGCCAAGUGGUC
1545	6	CUUUCUGCUGUCUUGGGUGCAUU	1589	50	ACCUCCACCAUGCCAAGUGGUCC
1546	7	UUUCUGCUGUCUUGGGUGCAUUG	1590	51	CCUCCACCAUGCCAAGUGGUCCC
1547	8	UUCUGCUGUCUUGGGUGCAUUGG	1591	52	CUCCACCAUGCCAAGUGGUCCCA
1548	9	UCUGCUGUCUUGGGUGCAUUGGA	1592	53	UCCACCAUGCCAAGUGGUCCCAG
1549	10	CUGCUGUCUUGGGUGCAUUGGAG	1593	54	CCACCAUGCCAAGUGGUCCCAGG
1550	11	UGCUGUCUUGGGUGCAUUGGAGC	1594	55	CACCAUGCCAAGUGGUCCCAGGC
1551	12	GCUGUCUUGGGUGCAUUGGAGCC	1595	56	ACCAUGCCAAGUGGUCCCAGGCU
1552	13	CUGUCUUGGGUGCAUUGGAGCCU	1596	57	CCAUGCCAAGUGGUCCCAGGCUG
1553	14	UGUCUUGGGUGCAUUGGAGCCUU	1597	58	CAUGCCAAGUGGUCCCAGGCUGC
1554	15	GUCUUGGGUGCAUUGGAGCCUUG	1598	59	AUGCCAAGUGGUCCCAGGCUGCA
1555	16	UCUUGGGUGCAUUGGAGCCUUGC	1599	60	UGCCAAGUGGUCCCAGGCUGCAC
1556	17	CUUGGGUGCAUUGGAGCCUUGCC	1600	61	GCCAAGUGGUCCCAGGCUGCACC
1557	18	UUGGGUGCAUUGGAGCCUUGCCU	1601	62	CCAAGUGGUCCCAGGCUGCACCC

SEQ ID NO:	position in VEGF-121 ORF	TARGET SEQUENCE IN VEGF121 mRNA 5′ to 3′	SEQ ID NO:	position in VEGF-121 ORF	TARGET SEQUENCE IN VEGF121 mRNA 5′ to 3′
1558	19	UGGGUGCAUUGGAGCCUUGCCUU	1602	63	CAAGUGGUCCCAGGCUGCACCCA
1559	20	GGGUGCAUUGGAGCCUUGCCUUG	1603	64	AAGUGGUCCCAGGCUGCACCCAU
1560	21	GGUGCAUUGGAGCCUUGCCUUGC	1604	65	AGUGGUCCCAGGCUGCACCCAUG
1561	22	GUGCAUUGGAGCCUUGCCUUGCU	1605	66	GUGGUCCCAGGCUGCACCCAUGG
1562	23	UGCAUUGGAGCCUUGCCUUGCUG	1606	67	UGGUCCCAGGCUGCACCCAUGGC
1563	24	GCAUUGGAGCCUUGCCUUGCUGC	1607	68	GGUCCCAGGCUGCACCCAUGGCA
1564	25	CAUUGGAGCCUUGCCUUGCUGCU	1608	69	GUCCCAGGCUGCACCCAUGGCAG
1565	26	AUUGGAGCCUUGCCUUGCUGCUC	1609	70	UCCCAGGCUGCACCCAUGGCAGA
1566	27	UUGGAGCCUUGCCUUGCUGCUCU	1610	71	CCCAGGCUGCACCCAUGGCAGAA
1567	28	UGGAGCCUUGCCUUGCUGCUCUA	1611	72	CCAGGCUGCACCCAUGGCAGAAG
1568	29	GGAGCCUUGCCUUGCUGCUCUAC	1612	73	CAGGCUGCACCCAUGGCAGAAGG
1569	30	GAGCCUUGCCUUGCUGCUCUACC	1613	74	AGGCUGCACCCAUGGCAGAAGGA
1570	31	AGCCUUGCCUUGCUGCUCUACCU	1614	75	GGCUGCACCCAUGGCAGAAGGAG
1571	32	GCCUUGCCUUGCUGCUCUACCUC	1615	76	GCUGCACCCAUGGCAGAAGGAGG
1572	33	CCUUGCCUUGCUGCUCUACCUCC	1616	77	CUGCACCCAUGGCAGAAGGAGGA
1573	34	CUUGCCUUGCUGCUCUACCUCCA	1617	78	UGCACCCAUGGCAGAAGGAGGAG
1574	35	UUGCCUUGCUGCUCUACCUCCAC	1618	79	GCACCCAUGGCAGAAGGAGGAGG
1575	36	UGCCUUGCUGCUCUACCUCCACC	1619	80	CACCCAUGGCAGAAGGAGGAGGG
1576	37	GCCUUGCUGCUCUACCUCCACCA	1620	81	ACCCAUGGCAGAAGGAGGAGGGC
1577	38	CCUUGCUGCUCUACCUCCACCAU	1621	82	CCCAUGGCAGAAGGAGGAGGGCA
1578	39	CUUGCUGCUCUACCUCCACCAUG	1622	83	CCAUGGCAGAAGGAGGAGGGCAG
1579	40	UUGCUGCUCUACCUCCACCAUGC	1623	84	CAUGGCAGAAGGAGGAGGGCAGA
1580	41	UGCUGCUCUACCUCCACCAUGCC	1624	85	AUGGCAGAAGGAGGAGGGCAGAA
1581	42	GCUGCUCUACCUCCACCAUGCCA	1625	86	UGGCAGAAGGAGGAGGGCAGAAU
1582	43	CUGCUCUACCUCCACCAUGCCAA	1626	87	GGCAGAAGGAGGAGGGCAGAAUC
1583	44	UGCUCUACCUCCACCAUGCCAAG	1627	88	GCAGAAGGAGGAGGGCAGAAUCA
1628	89	CAGAAGGAGGAGGGCAGAAUCAU	1674	135	UGUCUAUCAGCGCAGCUACUGCC
1629	90	AGAAGGAGGAGGGCAGAAUCAUC	1675	136	GUCUAUCAGCGCAGCUACUGCCA
1630	91	GAAGGAGGAGGGCAGAAUCAUCA	1676	137	UCUAUCAGCGCAGCUACUGCCAU
1631	92	AAGGAGGAGGGCAGAAUCAUCAC	1677	138	CUAUCAGCGCAGCUACUGCCAUC
1632	93	AGGAGGAGGGCAGAAUCAUCACG	1678	139	UAUCAGCGCAGCUACUGCCAUCC
1633	94	GGAGGAGGGCAGAAUCAUCACGA	1679	140	AUCAGCGCAGCUACUGCCAUCCA
1634	95	GAGGAGGGCAGAAUCAUCACGAA	1680	141	UCAGCGCAGCUACUGCCAUCCAA
1635	96	AGGAGGGCAGAAUCAUCACGAAG	1681	142	CAGCGCAGCUACUGCCAUCCAAU
1636	97	GGAGGGCAGAAUCAUCACGAAGU	1682	143	AGCGCAGCUACUGCCAUCCAAUC
1637	98	GAGGGCAGAAUCAUCACGAAGUG	1683	144	GCGCAGCUACUGCCAUCCAAUCG
1638	99	AGGGCAGAAUCAUCACGAAGUGG	1684	145	CGCAGCUACUGCCAUCCAAUCGA
1639	100	GGGCAGAAUCAUCACGAAGUGGU	1685	146	GCAGCUACUGCCAUCCAAUCGAG
1640	101	GGCAGAAUCAUCACGAAGUGGUG	1686	147	CAGCUACUGCCAUCCAAUCGAGA
1641	102	GCAGAAUCAUCACGAAGUGGUGA	1687	148	AGCUACUGCCAUCCAAUCGAGAC
1642	103	CAGAAUCAUCACGAAGUGGUGAA	1688	149	GCUACUGCCAUCCAAUCGAGACC
1643	104	AGAAUCAUCACGAAGUGGUGAAG	1689	150	CUACUGCCAUCCAAUCGAGACCC
1644	105	GAAUCAUCACGAAGUGGUGAAGU	1690	151	UACUGCCAUCCAAUCGAGACCCU
1645	106	AAUCAUCACGAAGUGGUGAAGUU	1691	152	ACUGCCAUCCAAUCGAGACCCUG
1646	107	AUCAUCACGAAGUGGUGAAGUUC	1692	153	CUGCCAUCCAAUCGAGACCCUGG
1647	108	UCAUCACGAAGUGGUGAAGUUCA	1693	154	UGCCAUCCAAUCGAGACCCUGGU
1648	109	CAUCACGAAGUGGUGAAGUUCAU	1694	155	GCCAUCCAAUCGAGACCCUGGUG

SEQ ID NO:	position in VEGF-121 ORF	TARGET SEQUENCE IN VEGF121 mRNA 5′ to 3′	SEQ ID NO:	position in VEGF-121 ORF	TARGET SEQUENCE IN VEGF121 mRNA 5′ to 3′
1649	110	AUCACGAAGUGGUGAAGUUCAUG	1695	156	CCAUCCAAUCGAGACCCUGGUGG
1650	111	UCACGAAGUGGUGAAGUUCAUGG	1696	157	CAUCCAAUCGAGACCCUGGUGGA
1651	112	CACGAAGUGGUGAAGUUCAUGGA	1697	158	AUCCAAUCGAGACCCUGGUGGAC
1652	113	ACGAAGUGGUGAAGUUCAUGGAU	1698	159	UCCAAUCGAGACCCUGGUGGACA
1653	114	CGAAGUGGUGAAGUUCAUGGAUG	1699	160	CCAAUCGAGACCCUGGUGGACAU
1654	115	GAAGUGGUGAAGUUCAUGGAUGU	1700	161	CAAUCGAGACCCUGGUGGACAUC
1655	116	AAGUGGUGAAGUUCAUGGAUGUC	1701	162	AAUCGAGACCCUGGUGGACAUCU
1656	117	AGUGGUGAAGUUCAUGGAUGUCU	1702	163	AUCGAGACCCUGGUGGACAUCUU
1657	118	GUGGUGAAGUUCAUGGAUGUCUA	1703	164	UCGAGACCCUGGUGGACAUCUUC
1658	119	UGGUGAAGUUCAUGGAUGUCUAU	1704	165	CGAGACCCUGGUGGACAUCUUCC
1659	120	GGUGAAGUUCAUGGAUGUCUAUC	1705	166	GAGACCCUGGUGGACAUCUUCCA
1660	121	GUGAAGUUCAUGGAUGUCUAUCA	1706	167	AGACCCUGGUGGACAUCUUCCAG
1661	122	UGAAGUUCAUGGAUGUCUAUCAG	1707	168	GACCCUGGUGGACAUCUUCCAGG
1662	123	GAAGUUCAUGGAUGUCUAUCAGC	1708	169	ACCCUGGUGGACAUCUUCCAGGA
1663	124	AAGUUCAUGGAUGUCUAUCAGCG	1709	170	CCCUGGUGGACAUCUUCCAGGAG
1664	125	AGUUCAUGGAUGUCUAUCAGCGC	1710	171	CCUGGUGGACAUCUUCCAGGAGU
1665	126	GUUCAUGGAUGUCUAUCAGCGCA	1711	172	CUGGUGGACAUCUUCCAGGAGUA
1666	127	UUCAUGGAUGUCUAUCAGCGCAG	1712	173	UGGUGGACAUCUUCCAGGAGUAC
1667	128	UCAUGGAUGUCUAUCAGCGCAGC	1713	174	GGUGGACAUCUUCCAGGAGUACC
1668	129	CAUGGAUGUCUAUCAGCGCAGCU	1714	175	GUGGACAUCUUCCAGGAGUACCC
1669	130	AUGGAUGUCUAUCAGCGCAGCUA	1715	176	UGGACAUCUUCCAGGAGUACCCU
1670	131	UGGAUGUCUAUCAGCGCAGCUAC	1716	177	GGACAUCUUCCAGGAGUACCCUG
1671	132	GGAUGUCUAUCAGCGCAGCUACU	1717	178	GACAUCUUCCAGGAGUACCCUGA
1672	133	GAUGUCUAUCAGCGCAGCUACUG	1718	179	ACAUCUUCCAGGAGUACCCUGAU
1673	134	AUGUCUAUCAGCGCAGCUACUGC	1719	180	CAUCUUCCAGGAGUACCCUGAUG
1720	181	AUCUUCCAGGAGUACCCUGAUGA	1766	227	CCUGUGUGCCCCUGAUGCGAUGC
1721	182	UCUUCCAGGAGUACCCUGAUGAG	1767	228	CUGUGUGCCCCUGAUGCGAUGCG
1722	183	CUUCCAGGAGUACCCUGAUGAGA	1768	229	UGUGUGCCCCUGAUGCGAUGCGG
1723	184	UUCCAGGAGUACCCUGAUGAGAU	1769	230	GUGUGCCCCUGAUGCGAUGCGGG
1724	185	UCCAGGAGUACCCUGAUGAGAUC	1770	231	UGUGCCCCUGAUGCGAUGCGGGG
1725	186	CCAGGAGUACCCUGAUGAGAUCG	1771	232	GUGCCCCUGAUGCGAUGCGGGGG
1726	187	CAGGAGUACCCUGAUGAGAUCGA	1772	233	UGCCCCUGAUGCGAUGCGGGGGC
1727	188	AGGAGUACCCUGAUGAGAUCGAG	1773	234	GCCCCUGAUGCGAUGCGGGGGCU
1728	189	GGAGUACCCUGAUGAGAUCGAGU	1774	235	CCCCUGAUGCGAUGCGGGGGCUG
1729	190	GAGUACCCUGAUGAGAUCGAGUA	1775	236	CCCUGAUGCGAUGCGGGGGCUGC
1730	191	AGUACCCUGAUGAGAUCGAGUAC	1776	237	CCUGAUGCGAUGCGGGGGCUGCU
1731	192	GUACCCUGAUGAGAUCGAGUACA	1777	238	CUGAUGCGAUGCGGGGGCUGCUG
1732	193	UACCCUGAUGAGAUCGAGUACAU	1778	239	UGAUGCGAUGCGGGGGCUGCUGC
1733	194	ACCCUGAUGAGAUCGAGUACAUC	1779	240	GAUGCGAUGCGGGGGCUGCUGCA
1734	195	CCCUGAUGAGAUCGAGUACAUCU	1780	241	AUGCGAUGCGGGGGCUGCUGCAA
1735	196	CCUGAUGAGAUCGAGUACAUCUU	1781	242	UGCGAUGCGGGGGCUGCUGCAAU
1736	197	CUGAUGAGAUCGAGUACAUCUUC	1782	243	GCGAUGCGGGGGCUGCUGCAAUG
1737	198	UGAUGAGAUCGAGUACAUCUUCA	1783	244	CGAUGCGGGGGCUGCUGCAAUGA
1738	199	GAUGAGAUCGAGUACAUCUUCAA	1784	245	GAUGCGGGGGCUGCUGCAAUGAC
1739	200	AUGAGAUCGAGUACAUCUUCAAG	1785	246	AUGCGGGGGCUGCUGCAAUGACG
1740	201	UGAGAUCGAGUACAUCUUCAAGC	1786	247	UGCGGGGGCUGCUGCAAUGACGA
1741	202	GAGAUCGAGUACAUCUUCAAGCC	1787	248	GCGGGGGCUGCUGCAAUGACGAG

SEQ ID NO:	position in VEGF-121 ORF	TARGET SEQUENCE IN VEGF121 mRNA 5′ to 3′	SEQ ID NO:	position in VEGF-121 ORF	TARGET SEQUENCE IN VEGF121 mRNA 5′ to 3′
1742	203	AGAUCGAGUACAUCUUCAAGCCA	1788	249	CGGGGGCUGCUGCAAUGACGAGG
1743	204	GAUCGAGUACAUCUUCAAGCCAU	1789	250	GGGGGCUGCUGCAAUGACGAGGG
1744	205	AUCGAGUACAUCUUCAAGCCAUC	1790	251	GGGGCUGCUGCAAUGACGAGGGC
1745	206	UCGAGUACAUCUUCAAGCCAUCC	1791	252	GGGCUGCUGCAAUGACGAGGGCC
1746	207	CGAGUACAUCUUCAAGCCAUCCU	1792	253	GGCUGCUGCAAUGACGAGGGCCU
1747	208	GAGUACAUCUUCAAGCCAUCCUG	1793	254	GCUGCUGCAAUGACGAGGGCCUG
1748	209	AGUACAUCUUCAAGCCAUCCUGU	1794	255	CUGCUGCAAUGACGAGGGCCUGG
1749	210	GUACAUCUUCAAGCCAUCCUGUG	1795	256	UGCUGCAAUGACGAGGGCCUGGA
1750	211	UACAUCUUCAAGCCAUCCUGUGU	1796	257	GCUGCAAUGACGAGGGCCUGGAG
1751	212	ACAUCUUCAAGCCAUCCUGUGUG	1797	258	CUGCAAUGACGAGGGCCUGGAGU
1752	213	CAUCUUCAAGCCAUCCUGUGUGC	1798	259	UGCAAUGACGAGGGCCUGGAGUG
1753	214	AUCUUCAAGCCAUCCUGUGUGCC	1799	260	GCAAUGACGAGGGCCUGGAGUGU
1754	215	UCUUCAAGCCAUCCUGUGUGCCC	1800	261	CAAUGACGAGGGCCUGGAGUGUG
1755	216	CUUCAAGCCAUCCUGUGUGCCCC	1801	262	AAUGACGAGGGCCUGGAGUGUGU
1756	217	UUCAAGCCAUCCUGUGUGCCCCU	1802	263	AUGACGAGGGCCUGGAGUGUGUG
1757	218	UCAAGCCAUCCUGUGUGCCCCUG	1803	264	UGACGAGGGCCUGGAGUGUGUGC
1758	219	CAAGCCAUCCUGUGUGCCCCUGA	1804	265	GACGAGGGCCUGGAGUGUGUGCC
1759	220	AAGCCAUCCUGUGUGCCCCUGAU	1805	266	ACGAGGGCCUGGAGUGUGUGCCC
1760	221	AGCCAUCCUGUGUGCCCCUGAUG	1806	267	CGAGGGCCUGGAGUGUGUGCCCA
1761	222	GCCAUCCUGUGUGCCCCUGAUGC	1807	268	GAGGGCCUGGAGUGUGUGCCCAC
1762	223	CCAUCCUGUGUGCCCCUGAUGCG	1808	269	AGGGCCUGGAGUGUGUGCCCACU
1763	224	CAUCCUGUGUGCCCCUGAUGCGA	1809	270	GGGCCUGGAGUGUGUGCCCACUG
1764	225	AUCCUGUGUGCCCCUGAUGCGAU	1810	271	GGCCUGGAGUGUGUGCCCACUGA
1765	226	UCCUGUGUGCCCCUGAUGCGAUG	1811	272	GCCUGGAGUGUGUGCCCACUGAG
1812	273	CCUGGAGUGUGUGCCCACUGAGG	1858	319	AUGCGGAUCAAACCUCACCAAGG
1813	274	CUGGAGUGUGUGCCCACUGAGGA	1859	320	UGCGGAUCAAACCUCACCAAGGC
1814	275	UGGAGUGUGUGCCCACUGAGGAG	1860	321	GCGGAUCAAACCUCACCAAGGCC
1815	276	GGAGUGUGUGCCCACUGAGGAGU	1861	322	CGGAUCAAACCUCACCAAGGCCA
1816	277	GAGUGUGUGCCCACUGAGGAGUC	1862	323	GGAUCAAACCUCACCAAGGCCAG
1817	278	AGUGUGUGCCCACUGAGGAGUCC	1863	324	GAUCAAACCUCACCAAGGCCAGC
1818	279	GUGUGUGCCCACUGAGGAGUCCA	1864	325	AUCAAACCUCACCAAGGCCAGCA
1819	280	UGUGUGCCCACUGAGGAGUCCAA	1865	326	UCAAACCUCACCAAGGCCAGCAC
1820	281	GUGUGCCCACUGAGGAGUCCAAC	1866	327	CAAACCUCACCAAGGCCAGCACA
1821	282	UGUGCCCACUGAGGAGUCCAACA	1867	328	AAACCUCACCAAGGCCAGCACAU
1822	283	GUGCCCACUGAGGAGUCCAACAU	1868	329	AACCUCACCAAGGCCAGCACAUA
1823	284	UGCCCACUGAGGAGUCCAACAUC	1869	330	ACCUCACCAAGGCCAGCACAUAG
1824	285	GCCCACUGAGGAGUCCAACAUCA	1870	331	CCUCACCAAGGCCAGCACAUAGG
1825	286	CCCACUGAGGAGUCCAACAUCAC	1871	332	CUCACCAAGGCCAGCACAUAGGA
1826	287	CCACUGAGGAGUCCAACAUCACC	1872	333	UCACCAAGGCCAGCACAUAGGAG
1827	288	CACUGAGGAGUCCAACAUCACCA	1873	334	CACCAAGGCCAGCACAUAGGAGA
1828	289	ACUGAGGAGUCCAACAUCACCAU	1874	335	ACCAAGGCCAGCACAUAGGAGAG
1829	290	CUGAGGAGUCCAACAUCACCAUG	1875	336	CCAAGGCCAGCACAUAGGAGAGA
1830	291	UGAGGAGUCCAACAUCACCAUGC	1876	337	CAAGGCCAGCACAUAGGAGAGAU
1831	292	GAGGAGUCCAACAUCACCAUGCA	1877	338	AAGGCCAGCACAUAGGAGAGAUG
1832	293	AGGAGUCCAACAUCACCAUGCAG	1878	339	AGGCCAGCACAUAGGAGAGAUGA
1833	294	GGAGUCCAACAUCACCAUGCAGA	1879	340	GGCCAGCACAUAGGAGAGAUGAG
1834	295	GAGUCCAACAUCACCAUGCAGAU	1880	341	GCCAGCACAUAGGAGAGAUGAGC

SEQ ID NO:	position in VEGF-121 ORF	TARGET SEQUENCE IN VEGF121 mRNA 5′ to 3′	SEQ ID NO:	position in VEGF-121 ORF	TARGET SEQUENCE IN VEGF121 mRNA 5′ to 3′
1835	296	AGUCCAACAUCACCAUGCAGAUU	1881	342	CCAGCACAUAGGAGAGAUGAGCU
1836	297	GUCCAACAUCACCAUGCAGAUUA	1882	343	CAGCACAUAGGAGAGAUGAGCUU
1837	298	UCCAACAUCACCAUGCAGAUUAU	1883	344	AGCACAUAGGAGAGAUGAGCUUC
1838	299	CCAACAUCACCAUGCAGAUUAUG	1884	345	GCACAUAGGAGAGAUGAGCUUCC
1839	300	CAACAUCACCAUGCAGAUUAUGC	1885	346	CACAUAGGAGAGAUGAGCUUCCU
1840	301	AACAUCACCAUGCAGAUUAUGCG	1886	347	ACAUAGGAGAGAUGAGCUUCCUA
1841	302	ACAUCACCAUGCAGAUUAUGCGG	1887	348	CAUAGGAGAGAUGAGCUUCCUAC
1842	303	CAUCACCAUGCAGAUUAUGCGGA	1888	349	AUAGGAGAGAUGAGCUUCCUACA
1843	304	AUCACCAUGCAGAUUAUGCGGAU	1889	350	UAGGAGAGAUGAGCUUCCUACAG
1844	305	UCACCAUGCAGAUUAUGCGGAUC	1890	351	AGGAGAGAUGAGCUUCCUACAGC
1845	306	CACCAUGCAGAUUAUGCGGAUCA	1891	352	GGAGAGAUGAGCUUCCUACAGCA
1846	307	ACCAUGCAGAUUAUGCGGAUCAA	1892	353	GAGAGAUGAGCUUCCUACAGCAC
1847	308	CCAUGCAGAUUAUGCGGAUCAAA	1893	354	AGAGAUGAGCUUCCUACAGCACA
1848	309	CAUGCAGAUUAUGCGGAUCAAAC	1894	355	GAGAUGAGCUUCCUACAGCACAA
1849	310	AUGCAGAUUAUGCGGAUCAAACC	1895	356	AGAUGAGCUUCCUACAGCACAAC
1850	311	UGCAGAUUAUGCGGAUCAAACCU	1896	357	GAUGAGCUUCCUACAGCACAACA
1851	312	GCAGAUUAUGCGGAUCAAACCUC	1897	358	AUGAGCUUCCUACAGCACAACAA
1852	313	CAGAUUAUGCGGAUCAAACCUCA	1898	359	UGAGCUUCCUACAGCACAACAAA
1853	314	AGAUUAUGCGGAUCAAACCUCAC	1899	360	GAGCUUCCUACAGCACAACAAAU
1854	315	GAUUAUGCGGAUCAAACCUCACC	1900	361	AGCUUCCUACAGCACAACAAAUG
1855	316	AUUAUGCGGAUCAAACCUCACCA	1901	362	GCUUCCUACAGCACAACAAAUGU
1856	317	UUAUGCGGAUCAAACCUCACCAA	1902	363	CUUCCUACAGCACAACAAAUGUG
1857	318	UAUGCGGAUCAAACCUCACCAAG	1903	364	UUCCUACAGCACAACAAAUGUGA
1904	365	UCCUACAGCACAACAAAUGUGAA			
1905	366	CCUACAGCACAACAAAUGUGAAU			
1906	367	CUACAGCACAACAAAUGUGAAUG			
1907	368	UACAGCACAACAAAUGUGAAUGC			
1908	369	ACAGCACAACAAAUGUGAAUGCA			
1909	370	CAGCACAACAAAUGUGAAUGCAG			
1910	371	AGCACAACAAAUGUGAAUGCAGA			
1911	372	GCACAACAAAUGUGAAUGCAGAC			
1912	373	CACAACAAAUGUGAAUGCAGACC			
1913	374	ACAACAAAUGUGAAUGCAGACCA			
1914	375	CAACAAAUGUGAAUGCAGACCAA			
1915	376	AACAAAUGUGAAUGCAGACCAAA			
1916	377	ACAAAUGUGAAUGCAGACCAAAG			
1917	378	CAAAUGUGAAUGCAGACCAAAGA			
1918	379	AAAUGUGAAUGCAGACCAAAGAA			
1919	380	AAUGUGAAUGCAGACCAAAGAAA			
1920	381	AUGUGAAUGCAGACCAAAGAAAG			
1921	382	UGUGAAUGCAGACCAAAGAAAGA			
1922	383	GUGAAUGCAGACCAAAGAAAGAU			
1923	384	UGAAUGCAGACCAAAGAAAGAUA			
1924	385	GAAUGCAGACCAAAGAAAGAUAG			
1925	386	AAUGCAGACCAAAGAAAGAUAGA			
1926	387	AUGCAGACCAAAGAAAGAUAGAG			
1927	388	UGCAGACCAAAGAAAGAUAGAGC			

SEQ ID NO:	position in VEGF-121 ORF	TARGET SEQUENCE IN VEGF121 mRNA 5′ to 3′	SEQ ID NO:	position in VEGF-121 ORF	TARGET SEQUENCE IN VEGF121 mRNA 5′ to 3′
1928	389	GCAGACCAAAGAAAGAUAGAGCA			
1929	390	CAGACCAAAGAAAGAUAGAGCAA			
1930	391	AGACCAAAGAAAGAUAGAGCAAG			
1931	392	GACCAAAGAAAGAUAGAGCAAGA			
1932	393	ACCAAAGAAAGAUAGAGCAAGAC			
1933	394	CCAAAGAAAGAUAGAGCAAGACA			
1934	395	CAAAGAAAGAUAGAGCAAGACAA			
1935	396	AAAGAAAGAUAGAGCAAGACAAG			
1936	397	AAGAAAGAUAGAGCAAGACAAGA			
1937	398	AGAAAGAUAGAGCAAGACAAGAA			
1938	399	GAAAGAUAGAGCAAGACAAGAAA			
1939	400	AAAGAUAGAGCAAGACAAGAAAA			

Table 4b: VEGF targeted duplexes

Strand: S= sense, AS=Antisense

position in ORF	SEQ ID NO:	Target sequence (5′-3′)	Duplex ID	Strand	SEQ ID NO:	Strand Sequences
1	2184	AUGAACUUUCUGCUGUCUUGGGU	**AL-DP-4043**	S	1940	5 GAACUUUCUGCUGUCUUGGGU 3
				AS	1941	3 UACUUGAAAGACGACAGAACCCA 5
22	2185	GUGCAUUGGAGCCUUGCCUUGCU	**AL-DP-4077**	S	1942	5 GCAUUGGAGCCUUGCCUUGCU 3
				AS	1943	3 CACGUAACCUCGGAACGGAACGA 5
47	2186	UCUACCUCCACCAUGCCAAGUGG	**AL-DP-4021**	S	1944	5 UACCUCCACCAUGCCAAGUTT 3
				AS	1945	3 TTAUGGAGGUGGUACGGUUCA 5
48	2187	CUACCUCCACCAUGCCAAGUGGU	**AL-DP-4109**	S	1946	5 ACCUCCACCAUGCCAAGUGTT 3
				AS	1947	3 TTUGGAGGUGGUACGGUUCAC 5
50	2188	ACCUCCACCAUGCCAAGUGGUCC	**AL-DP-4006**	S	1948	5 CUCCACCAUGCCAAGUGGUCC 3
				AS	1949	3 UGGAGGUGGUACGGUUCACCAGG 5
			AL-DP-4083	S	1950	5 CUCCACCAUGCCAAGUGGUTT 3
				AS	1951	3 TTGAGGUGGUACGGUUCACCA 5
51	2189	CCUCCACCAUGCCAAGUGGUCCC	**AL-DP-4047**	S	1952	5 UCCACCAUGCCAAGUGGUCCC 3
				AS	1953	3 GGAGGUGGUACGGUUCACCAGGG 5
			AL-DP-4017	S	1954	5 UCCACCAUGCCAAGUGGUCTT 3
				AS	1955	3 TTAGGUGGUACGGUUCACCAG 5
52	2190	CUCCACCAUGCCAAGUGGUCCCA	**AL-DP-4048**	S	1956	5 CCACCAUGCCAAGUGGUCCCA 3
				AS	1957	3 GAGGUGGUACGGUUCACCAGGGU 5
			AL-DP-4103	S	1958	5 CCACCAUGCCAAGUGGUCCTT 3
				AS	1959	3 TTGGUGGUACGGUUCACCAGG 5

position in ORF	SEQ ID NO:	Target sequence (5′-3′)	Duplex ID	Strand	SEQ ID NO:	Strand Sequences
53	2191	UCCACCAUGCCAAGUGGUCCCAG	AL-DP-4035	S	1960	5 CACCAUGCCAAGUGGUCCCAG 3
				AS	1961	3 AGGUGGUACGGUUCACCAGGGUC 5
			AL-DP-4018	S	1962	5 CACCAUGCCAAGUGGUCCCTT 3
				AS	1963	3 TTGUGGUACGGUUCACCAGGG 5
54	2192	CCACCAUGCCAAGUGGUCCCAGG	AL-DP-4036	S	1964	5 ACCAUGCCAAGUGGUCCCAGG 3
				AS	1965	3 GGUGGUACGGUUCACCAGGGUCC 5
			AL-DP-4084	S	1966	5 ACCAUGCCAAGUGGUCCCATT 3
				AS	1967	3 TTUGGUACGGUUCACCAGGGU 5
55	2193	CACCAUGCCAAGUGGUCCCAGGC	AL-DP-4093	S	1968	5 CCAUGCCAAGUGGUCCCAGGC 3
				AS	1969	3 GUGGUACGGUUCACCAGGGUCCG 5
			AL-DP-4085	S	1970	5 CCAUGCCAAGUGGUCCCAGTT 3
				AS	1971	3 TTGGUACGGUUCACCAGGGUC 5
56	2194	ACCAUGCCAAGUGGUCCCAGGCU	AL-DP-4037	S	1972	5 CAUGCCAAGUGGUCCCAGGCU 3
				AS	1973	3 UGGUACGGUUCACCAGGGUCCGA 5
			AL-DP-4054	S	1974	5 CAUGCCAAGUGGUCCCAGGTT 3
				AS	1975	3 TTGUACGGUUCACCAGGGUCC 5
57	2195	CCAUGCCAAGUGGUCCCAGGCUG	AL-DP-4038	S	1976	5 AUGCCAAGUGGUCCCAGGCUG 3
				AS	1977	3 GGUACGGUUCACCAGGGUCCGAC 5
			AL-DP-4086	S	1978	5 AUGCCAAGUGGUCCCAGGCTT 3
				AS	1979	3 TTUACGGUUCACCAGGGUCCG 5
58	2196	CAUGCCAAGUGGUCCCAGGCUGC	AL-DP-4049	S	1980	5 UGCCAAGUGGUCCCAGGCUGC 3
				AS	1981	3 GUACGGUUCACCAGGGUCCGACG 5
			AL-DP-4087	S	1982	5 UGCCAAGUGGUCCCAGGCUTT 3
				AS	1983	3 TTACGGUUCACCAGGGUCCGA 5
59	2197	AUGCCAAGUGGUCCCAGGCUGCA	AL-DP-4001	S	1984	5 GCCAAGUGGUCCCAGGCUGCA 3
				AS	1985	3 UACGGUUCACCAGGGUCCGACGU 5
			AL-DP-4052	A	1986	5 GCCAAGUGGUCCCAGGCUGTT 3
				AS	1987	3 TTCGGUUCACCAGGGUCCGAC 5
60	2198	UGCCAAGUGGUCCCAGGCUGCAC	AL-DP-4007	S	1988	5 CCAAGUGGUCCCAGGCUGCAC 3
				AS	1989	3 ACGGUUCACCAGGGUCCGACGUG 5
			AL-DP-4088	S	1990	5 CCAAGUGGUCCCAGGCUGCTT 3
				AS	1991	3 TTGGUUCACCAGGGUCCGACG 5
61	2199	GCCAAGUGGUCCCAGGCUGCACC	AL-DP-4070	S	1992	5 CAAGUGGUCCCAGGCUGCACC 3
				AS	1993	3 CGGUUCACCAGGGUCCGACGUGG 5

position in ORF	SEQ ID NO:	Target sequence (5′-3′)	Duplex ID	Strand	SEQ ID NO:	Strand Sequences
			AL-DP-4055	S	1994	5 CAAGUGGUCCCAGGCUGCATT 3
				AS	1995	3 TTGUUCACCAGGGUCCGACGU 5
62	2200	CCAAGUGGUCCCAGGCUGCACCC	AL-DP-4071	S	1996	5 AAGUGGUCCCAGGCUGCACCC 3
				AS	1997	3 GGUUCACCAGGGUCCGACGUGGG 5
			AL-DP-4056	S	1998	5 AAGUGGUCCCAGGCUGCACTT 3
				AS	1999	3 TTUUCACCAGGGUCCGACGUG 5
63	2201	CAAGUGGUCCCAGGCUGCACCCA	AL-DP-4072	S	2000	5 AGUGGUCCCAGGCUGCACCCA 3
				AS	2001	3 GUUCACCAGGGUCCGACGUGGGU 5
			AL-DP-4057	S	2002	5 AGUGGUCCCAGGCUGCACCTT 3
				AS	2003	3 TTUCACCAGGGUCCGACGUGG 5
64	2202	AAGUGGUCCCAGGCUGCACCCAU	AL-DP-4066	S	2004	5 GUGGUCCCAGGCUGCACCCTT 3
				AS	2005	3 TTCACCAGGGUCCGACGUGGG 5
99	2203	AGGGCAGAAUCAUCACGAAGUGG	AL-DP-4022	S	2006	5 GGCAGAAUCAUCACGAAGUTT 3
				AS	2007	3 TTCCGUCUUAGUAGUGCUUCA 5
100	2204	GGGCAGAAUCAUCACGAAGUGGU	AL-DP-4023	S	2008	5 GCAGAAUCAUCACGAAGUGTT 3
				AS	2009	3 TTCGUCUUAGUAGUGCUUCAC 5
101	2205	GGCAGAAUCAUCACGAAGUGGUG	AL-DP-4024	S	2010	5 CAGAAUCAUCACGAAGUGGTT 3
				AS	2011	3 TTGUCUUAGUAGUGCUUCACC 5
102	2206	GCAGAAUCAUCACGAAGUGGUGA	AL-DP-4076	S	2012	5 AGAAUCAUCACGAAGUGGUGA 3
				AS	2013	3 CGUCUUAGUAGUGCUUCACCACU 5
			AL-DP-4019	S	2014	5 AGAAUCAUCACGAAGUGGUTT 3
				AS	2015	3 TTUCUUAGUAGUGCUUCACCA 5
103	2207	CAGAAUCAUCACGAAGUGGUGAA	AL-DP-4025	S	2016	5 GAAUCAUCACGAAGUGGUGTT 3
				AS	2017	3 TTCUUAGUAGUGCUUCACCAC 5
104	2208	AGAAUCAUCACGAAGUGGUGAAG	AL-DP-4110	S	2018	5 AAUCAUCACGAAGUGGUGATT 3
				AS	2019	3 TTUUAGUAGUGCUUCACCACU 5
105	2209	GAAUCAUCACGAAGUGGUGAAGU	AL-DP-4068	S	2020	5 AUCAUCACGAAGUGGUGAATT 3
				AS	2021	3 TTUAGUAGUGCUUCACCACUU 5
113	2210	ACGAAGUGGUGAAGUUCAUGGAU	AL-DP-4078	S	2022	5 GAAGUGGUGAAGUUCAUGGAU 3
				AS	2023	3 UGCUUCACCACUUCAAGUACCUA 5
121	2211	GUGAAGUUCAUGGAUGUCUAUCA	AL-DP-4080	S	2024	5 GAAGUUCAUGGAUGUCUAUCA 3
				AS	2025	3 CACUUCAAGUACCUACAGAUAGU 5
129	2212	CAUGGAUGUCUAUCAGCGCAGCU	AL-DP-4111	S	2026	5 UGGAUGUCUAUCAGCGCAGTT 3
				AS	2027	3 TTACCUACAGAUAGUCGCGUC 5

position in ORF	SEQ ID NO:	Target sequence (5′-3′)	Duplex ID	Strand	SEQ ID NO:	Strand Sequences
130	2213	AUGGAUGUCUAUCAGCGCAGCUA	AL-DP-4041	S	2028	5 GGAUGUCUAUCAGCGCAGCUA 3
				AS	2029	3 UACCUACAGAUAGUCGCGUCGAU 5
			AL-DP-4062	S	2030	5 GGAUGUCUAUCAGCGCAGCTT 3
				AS	2031	3 TTCCUACAGAUAGUCGCGUCG 5
131	2214	UGGAUGUCUAUCAGCGCAGCUAC	AL-DP-4069	S	2032	5 GAUGUCUAUCAGCGCAGCUTT 3
				AS	2033	3 TTCUACAGAUAGUCGCGUCGA 5
132	2215	GGAUGUCUAUCAGCGCAGCUACU	AL-DP-4112	S	2034	5 AUGUCUAUCAGCGCAGCUATT 3
				AS	2035	3 TTUACAGAUAGUCGCGUCGAU 5
133	2216	GAUGUCUAUCAGCGCAGCUACUG	AL-DP-4026	S	2036	5 UGUCUAUCAGCGCAGCUACTT 3
				AS	2037	3 TTACAGAUAGUCGCGUCGAUG 5
134	2217	AUGUCUAUCAGCGCAGCUACUGC	AL-DP-4095	S	2038	5 GUCUAUCAGCGCAGCUACUGC 3
				AS	2039	3 UACAGAUAGUCGCGUCGAUGACG 5
			AL-DP-4020	S	2040	5 GUCUAUCAGCGCAGCUACUTT 3
				AS	2041	3 TTCAGAUAGUCGCGUCGAUGA 5
135	2218	UGUCUAUCAGCGCAGCUACUGCC	AL-DP-4027	S	2042	5 UCUAUCAGCGCAGCUACUGTT 3
				AS	2043	3 TTAGAUAGUCGCGUCGAUGAC 5
144	2219	GCGCAGCUACUGCCAUCCAAUCG	AL-DP-4081	S	2044	5 GCAGCUACUGCCAUCCAAUCG 3
				AS	2045	3 CGCGUCGAUGACGGUAGGUUAGC 5
146	2220	GCAGCUACUGCCAUCCAAUCGAG	AL-DP-4098	S	2046	5 AGCUACUGCCAUCCAAUCGAG 3
				AS	2047	3 CGUCGAUGACGGUAGGUUAGCUC 5
149	2221	GCUACUGCCAUCCAAUCGAGACC	AL-DP-4028	S	2048	5 UACUGCCAUCCAAUCGAGATT 3
				AS	2049	3 TTAUGACGGUAGGUUAGCUCU 5
150	2222	CUACUGCCAUCCAAUCGAGACCC	AL-DP-4029	S	2050	5 ACUGCCAUCCAAUCGAGACTT 3
				AS	2051	3 TTUGACGGUAGGUUAGCUCUG 5
151	2223	UACUGCCAUCCAAUCGAGACCCU	AL-DP-4030	S	2052	5 CUGCCAUCCAAUCGAGACCTT 3
				AS	2053	3 TTGACGGUAGGUUAGCUCUGG 5
152	2224	ACUGCCAUCCAAUCGAGACCCUG	AL-DP-4031	S	2054	5 UGCCAUCCAAUCGAGACCCTT 3
				AS	2055	3 TTACGGUAGGUUAGCUCUGGG 5
166	2225	GAGACCCUGGUGGACAUCUUCCA	AL-DP-4008	S	2056	5 GACCCUGGUGGACAUCUUCCA 3
				AS	2057	3 CUCUGGGACCACCUGUAGAAGGU 5
			AL-DP-4058	S	2058	5 GACCCUGGUGGACAUCUUCTT 3
				AS	2059	3 TTCUGGGACCACCUGUAGAAG 5
167	2226	AGACCCUGGUGGACAUCUUCCAG	AL-DP-4009	S	2060	5 ACCCUGGUGGACAUCUUCCAG 3
				AS	2061	3 UCUGGGACCACCUGUAGAAGGUC 5

position in ORF	SEQ ID NO:	Target sequence (5′-3′)	Duplex ID	Strand	SEQ ID NO:	Strand Sequences
			AL-DP-4059	S	2062	5 ACCCUGGUGGACAUCUUCCTT 3
				AS	2063	3 TTUGGGACCACCUGUAGAAGG 5
168	2227	GACCCUGGUGGACAUCUUCCAGG	AL-DP-4010	S	2064	5 CCCUGGUGGACAUCUUCCAGG 3
				AS	2065	3 CUGGGACCACCUGUAGAAGGUCC 5
			AL-DP-4060	S	2066	5 CCCUGGUGGACAUCUUCCATT 3
				AS	2067	3 TTGGGACCACCUGUAGAAGGU 5
169	2228	ACCCUGGUGGACAUCUUCCAGGA	AL-DP-4073	S	2068	5 CCUGGUGGACAUCUUCCAGGA 3
				AS	2069	3 UGGGACCACCUGUAGAAGGUCCU 5
			AL-DP-4104	S	2070	5 CCUGGUGGACAUCUUCCAGTT 3
				AS	2071	3 TTGGACCACCUGUAGAAGGUC 5
170	2229	CCCUGGUGGACAUCUUCCAGGAG	AL-DP-4011	S	2072	5 CUGGUGGACAUCUUCCAGGAG 3
				AS	2073	3 GGGACCACCUGUAGAAGGUCCUC 5
			AL-DP-4089	S	2074	5 CUGGUGGACAUCUUCCAGGTT 3
				AS	2075	3 TTGACCACCUGUAGAAGGUCC 5
171	2230	CCUGGUGGACAUCUUCCAGGAGU	AL-DP-4074	S	2076	5 UGGUGGACAUCUUCCAGGAGU 3
				AS	2077	3 GGACCACCUGUAGAAGGUCCUCA 5
			AL-DP-4090	S	2078	5 UGGUGGACAUCUUCCAGGATT 3
				AS	2079	3 TTACCACCUGUAGAAGGUCCU 5
172	2231	CUGGUGGACAUCUUCCAGGAGUA	AL-DP-4039	S	2080	5 GGUGGACAUCUUCCAGGAGUA 3
				AS	2081	3 GACCACCUGUAGAAGGUCCUCAU 5
			AL-DP-4091	S	2082	5 GGUGGACAUCUUCCAGGAGTT 3
				AS	2083	3 TTCCACCUGUAGAAGGUCCUC 5
175	2232	GUGGACAUCUUCCAGGAGUACCC	AL-DP-4003	S	2084	5 GGACAUCUUCCAGGAGUACCC 3
				AS	2085	3 CCUGUAGAAGGUCCUCAUGGG 5
			AL-DP-4116	S	2086	5 GGACAUCUUCCAGGAGUACCC 3
				AS	2087	3 CCUGUAGAAGGUCCUCAUGGG 5
			AL-DP-4015	S	2088	5 GGACAUCUUCCAGGAGUACTT 3
				AS	2089	3 TTCCUGUAGAAGGUCCUCAUG 5
			AL-DP-4120	S	2090	5 GGACAUCUUCCAGGAGUAC 3
				AS	2091	3 CCUGUAGAAGGUCCUCAUG 5
179	2233	ACAUCUUCCAGGAGUACCCUGAU	AL-DP-4099	S	2092	5 AUCUUCCAGGAGUACCCUGAU 3
				AS	2093	3 UGUAGAAGGUCCUCAUGGGACUA 5
191	2234	AGUACCCUGAUGAGAUCGAGUAC	AL-DP-4032	S	2094	5 UACCCUGAUGAGAUCGAGUTT 3
				AS	2095	3 TTAUGGGACUACUCUAGCUCA 5

position in ORF	SEQ ID NO:	Target sequence (5′-3′)	Duplex ID	Strand	SEQ ID NO:	Strand Sequences
192	2235	GUACCCUGAUGAGAUCGAGUACA	AL-DP-4042	S	2096	5 ACCCUGAUGAGAUCGAGUACA 3
				AS	2097	3 CAUGGGACUACUCUAGCUCAUGU 5
			AL-DP-4063	S	2098	5 ACCCUGAUGAGAUCGAGUATT 3
				AS	2099	3 TTUGGGACUACUCUAGCUCAU 5
209	2236	AGUACAUCUUCAAGCCAUCCUGU	AL-DP-4064	S	2100	5 UACAUCUUCAAGCCAUCCUTT 3
				AS	2101	3 TTAUGUAGAAGUUCGGUAGGA 5
260	2237	GCAAUGACGAGGGCCUGGAGUGU	AL-DP-4044	S	2102	5 AAUGACGAGGGCCUGGAGUGU 3
				AS	2103	3 CGUUACUGCUCCCGGACCUCACA 5
263	2238	AUGACGAGGGCCUGGAGUGUGUG	AL-DP-4045	S	2104	5 GACGAGGGCCUGGAGUGUGUG 3
				AS	2105	3 UACUGCUCCCGGACCUCACACAC 5
279	2239	GUGUGUGCCCACUGAGGAGUCCA	AL-DP-4046	S	2106	5 GUGUGCCCACUGAGGAGUCCA 3
				AS	2107	3 CACACACGGGUGACUCCUCAGGU 5
281	2240	GUGUGCCCACUGAGGAGUCCAAC	AL-DP-4096	S	2108	5 GUGCCCACUGAGGAGUCCAAC 3
				AS	2109	3 CACACGGGUGACUCCUCAGGUUG 5
283	2241	GUGCCCACUGAGGAGUCCAACAU	AL-DP-4040	S	2110	5 GCCCACUGAGGAGUCCAACAU 3
				AS	2111	3 CACGGGUGACUCCUCAGGUUGUA 5
289	2242	ACUGAGGAGUCCAACAUCACCAU	AL-DP-4065	S	2112	5 UGAGGAGUCCAACAUCACCTT 3
				AS	2113	3 TTACUCCUCAGGUUGUAGUGG 5
302	2243	ACAUCACCAUGCAGAUUAUGCGG	AL-DP-4100	S	2114	5 AUCACCAUGCAGAUUAUGCGG 3
				AS	2115	3 UGUAGUGGUACGUCUAAUACGCC 5
305	2244	UCACCAUGCAGAUUAUGCGGAUC	AL-DP-4033	S	2116	5 ACCAUGCAGAUUAUGCGGATT 3
				AS	2117	3 TTUGGUACGUCUAAUACGCCU 5
310	2245	AUGCAGAUUAUGCGGAUCAAACC	AL-DP-4101	S	2118	5 GCAGAUUAUGCGGAUCAAACC 3
				AS	2119	3 UACGUCUAAUACGCCUAGUUUGG 5
312	2246	GCAGAUUAUGCGGAUCAAACCUC	AL-DP-4102	S	2120	5 AGAUUAUGCGGAUCAAACCUC 3
				AS	2121	3 CGUCUAAUACGCCUAGUUUGGAG 5
315	2247	GAUUAUGCGGAUCAAACCUCACC	AL-DP-4034	S	2122	5 UUAUGCGGAUCAAACCUCATT 3
				AS	2123	3 TTAAUACGCCUAGUUUGGAGU 5
316	2248	AUUAUGCGGAUCAAACCUCACCA	AL-DP-4113	S	2124	5 UAUGCGGAUCAAACCUCACTT 3
				AS	2125	3 TTAUACGCCUAGUUUGGAGUG 5
317	2249	UUAUGCGGAUCAAACCUCACCAA	AL-DP-4114	S	2126	5 AUGCGGAUCAAACCUCACCTT 3
				AS	2127	3 TTUACGCCUAGUUUGGAGUGG 5
319	2250	AUGCGGAUCAAACCUCACCAAGG	AL-DP-4002	S	2128	5 GCGGAUCAAACCUCACCAAGG 3
				AS	2129	3 UACGCCUAGUUUGGAGUGGUUCC 5

position in ORF	SEQ ID NO:	Target sequence (5′-3′)	Duplex ID	Strand	SEQ ID NO:	Strand Sequences
			AL-DP-4115	S	2130	5 GCGGAUCAAACCUCACCAA 3
				AS	2131	3 CGCCUAGUUUGGAGUGGUU 5
			AL-DP-4014	S	2132	5 GCGGAUCAAACCUCACCAATT 3
				AS	2133	3 TTCGCCUAGUUUGGAGUGGUU 5
			AL-DP-4119	S	2134	5 GCGGAUCAAACCUCACCAA 3
				AS	2135	3 CGCCUAGUUUGGAGUGGUU 5
321	2251	GCGGAUCAAACCUCACCAAGGCC	**AL-DP-4013**	S	2136	5 GGAUCAAACCUCACCAAGGCC 3
				AS	2137	3 CGCCUAGUUUGGAGUGGUUCCGG 5
341	2252	GCCAGCACAUAGGAGAGAUGAGC	**AL-DP-4075**	S	2138	5 CAGCACAUAGGAGAGAUGAGC 3
				AS	2139	3 CGGUCGUGUAUCCUCUCUACUCG 5
			AL-DP-4105	S	2140	5 CAGCACAUAGGAGAGAUGATT 3
				AS	2141	3 TTGUCGUGUAUCCUCUCUACU 5
342	2253	CCAGCACAUAGGAGAGAUGAGCU	**AL-DP-4050**	S	2142	5 AGCACAUAGGAGAGAUGAGCU 3
				AS	2143	3 GGUCGUGUAUCCUCUCUACUCGA 5
			AL-DP-4106	S	2144	5 AGCACAUAGGAGAGAUGAGTT 3
				AS	2145	3 TTUCGUGUAUCCUCUCUACUC 5
343	2254	CAGCACAUAGGAGAGAUGAGCUU	**AL-DP-4094**	S	2146	5 GCACAUAGGAGAGAUGAGCUU 3
				AS	2147	3 GUCGUGUAUCCUCUCUACUCGAA 5
			AL-DP-4118	S	2148	5 GCACAUAGGAGAGAUGAGCUU 3
				AS	2149	3 CGUGUAUCCUCUCUACUCGAA 5
			AL-DP-4107	S	2150	5 GCACAUAGGAGAGAUGAGCTT 3
				AS	2151	3 TTCGUGUAUCCUCUCUACUCG 5
			AL-DP-4122	S	2152	5 GCACAUAGGAGAGAUGAGC 3
				AS	2153	3 CGUGUAUCCUCUCUACUCG 5
344	2255	AGCACAUAGGAGAGAUGAGCUUC	**AL-DP-4012**	S	2154	5 CACAUAGGAGAGAUGAGCUUC 3
				AS	2155	3 UCGUGUAUCCUCUCUACUCGAAG 5
			AL-DP-4108	S	2156	5 CACAUAGGAGAGAUGAGCUTT 3
				AS	2157	3 TTGUGUAUCCUCUCUACUCGA 5
346	2256	CACAUAGGAGAGAUGAGCUUCCU	**AL-DP-4051**	S	2158	5 CAUAGGAGAGAUGAGCUUCCU 3
				AS	2159	3 GUGUAUCCUCUCUACUCGAAGGA 5
			AL-DP-4061	S	2160	5 CAUAGGAGAGAUGAGCUUCTT 3
				AS	2161	3 TTGUAUCCUCUCUACUCGAAG 5
349	2257	AUAGGAGAGAUGAGCUUCCUACA	**AL-DP-4082**	S	2162	5 AGGAGAGAUGAGCUUCCUACA 3
				AS	2163	3 UAUCCUCUCUACUCGAAGGAUGU 5

position in ORF	SEQ ID NO:	Target sequence (5′-3′)	Duplex ID	Strand	SEQ ID NO:	Strand Sequences
369	2258	ACAGCACAACAAAUGUGAAUGCA	AL-DP-4079	S	2164	5 AGCACAACAAAUGUGAAUGCA 3
				AS	2165	3 UGUCGUGUUGUUUACACUUACGU 5
372	2259	GCACAACAAAUGUGAAUGCAGAC	AL-DP-4097	S	2166	5 ACAACAAAUGUGAAUGCAGAC 3
				AS	2167	3 CGUGUUGUUUACACUUACGUCUG 5
379	2260	AAAUGUGAAUGCAGACCAAAGAA	AL-DP-4067	S	2168	5 AUGUGAAUGCAGACCAAAGTT 3
				AS	2169	3 TTUACACUUACGUCUGGUUUC 5
380	2261	AAUGUGAAUGCAGACCAAAGAAA	AL-DP-4092	S	2170	5 UGUGAAUGCAGACCAAAGATT 3
				AS	2171	3 TTACACUUACGUCUGGUUUCU 5
381	2262	AUGUGAAUGCAGACCAAAGAAAG	AL-DP-4004	S	2172	5 GUGAAUGCAGACCAAAGAAAG 3
				AS	2173	3 UACACUUACGUCUGGUUUCUUUC 5
			AL-DP-4117	S	2174	5 GUGAAUGCAGACCAAAGAAAG 3
				AS	2175	3 CACUUACGUCUGGUUUCUUUC 5
			AL-DP-4016	S	2176	5 GUGAAUGCAGACCAAAGAATT 3
				AS	2177	3 TTCACUUACGUCUGGUUUCUU 5
			AL-DP-4121	S	2178	5 GUGAAUGCAGACCAAAGAA 3
				AS	2179	3 CACUUACGUCUGGUUUCUU 5
383	2263	GUGAAUGCAGACCAAAGAAAGAU	AL-DP-4005	S	2180	5 GAAUGCAGACCAAAGAAAGAU 3
				AS	2181	3 CACUUACGUCUGGUUUCUUUCUA 5
			AL-DP-4053	S	2182	5 GAAUGCAGACCAAAGAAAGTT 3
				AS	2183	3 TTCUUACGUCUGGUUUCUUUC 5

Example 2. Eg5 siRNA *in vitro* screening via cell proliferation

As silencing of Eg5 has been shown to cause mitotic arrest (Weil, D, *et al* [2002] Biotechniques 33: 1244-8), a cell viability assay was used for siRNA activity screening. HeLa cells (14000 per well [Screens 1 and 3] or 10000 per well [Screen2])) were seeded in 96-well plates and simultaneously transfected with Lipofectamine 2000 (Invitrogen) at a final siRNA concentration in the well of 30 nM and at final concentrations of 50 nM (1st screen) and 25 nM (2nd screen). A subset of duplexes was tested at 25 nM in a third screen (Table 5).

Seventy-two hours post-transfection, cell proliferation was assayed the addition of WST-1 reagent (Roche) to the culture medium, and subsequent absorbance measurement at 450 nm. The absorbance value for control (non-transfected) cells was considered 100 percent, and absorbances for the siRNA transfected wells were compared to the control value. Assays were performed in sextuplicate for each of three screens. A subset of the siRNAs was further tested

at a range of siRNA concentrations. Assays were performed in HeLa cells (14000 per well; method same as above, Table 5).

Table 5: Effects of Eg5 targeted duplexes on cell viability at 25nM.

	Relative absorbance at 450 nm					
	Screen I		Screen II		Screen III	
Duplex	mean	sd	Mean	sd	mean	Sd
AL-DP-6226	20	10	28	11	43	9
AL-DP-6227	66	27	96	41	108	33
AL-DP-6228	56	28	76	22	78	18
AL-DP-6229	17	3	31	9	48	13
AL-DP-6230	48	8	75	11	73	7
AL-DP-6231	8	1	21	4	41	10
AL-DP-6232	16	2	37	7	52	14
AL-DP-6233	31	9	37	6	49	12
AL-DP-6234	103	40	141	29	164	45
AL-DP-6235	107	34	140	27	195	75
AL-DP-6236	48	12	54	12	56	12
AL-DP-6237	73	14	108	18	154	37
AL-DP-6238	64	9	103	10	105	24
AL-DP-6239	9	1	20	4	31	11
AL-DP-6240	99	7	139	16	194	43
AL-DP-6241	43	9	54	12	66	19
AL-DP-6242	6	1	15	7	36	8
AL-DP-6243	7	2	19	5	33	13
AL-DP-6244	7	2	19	3	37	13
AL-DP-6245	25	4	45	10	58	9
AL-DP-6246	34	8	65	10	66	13
AL-DP-6247	53	6	78	14	105	20
AL-DP-6248	7	0	22	7	39	12
AL-DP-6249	36	8	48	13	61	7

The nine siRNA duplexes that showed the greatest growth inhibition in Table 5 were re-tested at a range of siRNA concentrations in HeLa cells. The siRNA concentrations tested were 100 nM, 33.3 nM, 11.1 nM, 3.70 nM, 1.23 nM, 0.41 nM, 0.14 nM and 0.046 nM. Assays were performed in sextuplicate, and the concentration of each siRNA resulting in fifty percent inhibition of cell proliferation (IC_{50}) was calculated. This dose-response analysis was performed between two and four times for each duplex. Mean IC_{50} values (nM) are given in Table 6.

Table 6: IC50 of siRNA: cell proliferation in HeLa cells

Duplex	**Mean IC_{50}**
AL-DP-6226	**15.5**
AL-DP-6229	**3.4**
AL-DP-6231	**4.2**
AL-DP-6232	**17.5**

AL-DP-6239	**4.4**
AL-DP-6242	**5.2**
AL-DP-6243	**2.6**
AL-DP-6244	**8.3**
AL-DP-6248	**1.9**

Example 3. Eg5 siRNA in vitro screening via mRNA inhibition

Directly before transfection, HeLa S3 (ATCC-Number: CCL-2.2, LCG Promochem GmbH, Wesel, Germany) cells were seeded at 1.5 x 10^4 cells / well on 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) in 75 µl of growth medium (Ham's F12, 10% fetal calf serum, 100u penicillin / 100 µg/ml streptomycin, all from Bookroom AG, Berlin, Germany). Transfections were performed in quadruplicates. For each well 0.5 µl Lipofectamine2000 (Invitrogen GmbH, Karlsruhe, Germany) were mixed with 12 µl Opti-MEM (Invitrogen) and incubated for 15 min at room temperature. For the siRNA concentration being 50 nM in the 100 µl transfection volume, 1 µl of a 5 µM siRNA were mixed with 11.5 µl Opti-MEM per well, combined with the Lipofectamine2000-Opti-MEM mixture and again incubated for 15 minutes at room temperature. siRNA-Lipofectamine2000-complexes were applied completely (25 µl each per well) to the cells and cells were incubated for 24 h at 37°C and 5 % CO_2 in a humidified incubator (Heroes GmbH, Hanau). The single dose screen was done once at 50 nM and at 25 nM, respectively.

Cells were harvested by applying 50 µl of lysis mixture (content of the QuantiGene bDNA-kit from Genospectra, Fremont, USA) to each well containing 100 µl of growth medium and were lysed at 53°C for 30 min. Afterwards, 50 µl of the lists were incubated with probe sets specific to human Eg5 and human GAPDH and proceeded according to the manufacturer's protocol for QuantiGene. In the end chemoluminescence was measured in a Victor2-Light (Perkin Elmer, Wiesbaden, Germany) as RLUs (relative light units) and values obtained with the hEg5 probe set were normalized to the respective GAPDH values for each well. Values obtained with siRNAs directed against Eg5 were related to the value obtained with an unspecific siRNA (directed against HCV) which was set to 100% (Tables 1b, 2b and 3b).

Effective siRNAs from the screen were further characterized by dose response curves. Transfections of dose response curves were performed at the following concentrations: 100 nM, 16.7 nM, 2.8 nM, 0.46 nM, 77 picoM, 12.8 picoM, 2.1 picoM, 0.35 picoM, 59.5 fM, 9.9 fM and mock (no siRNA) and diluted with Opti-MEM to a final concentration of 12.5 µl according to the above protocol. Data analysis was performed by using the Microsoft Excel add-in software XL-fit 4.2 (IDBS, Guildford, Surrey, UK) and applying the dose response model number 205 (Tables 1b, 2b and 3b).

The lead siRNA AD12115 was additionally analyzed by applying the WST-proliferation assay from Roche (as previously described).

A subset of 34 duplexes from Table 2 that showed greatest activity was assayed by transfection in HeLa cells at final concentrations ranging from 100nM to 10fM. Transfections were performed in quadruplicate. Two dose-response assays were performed for each duplex. The concentration giving 20% (IC20), 50% (IC50) and 80% (IC80) reduction of KSP mRNA was calculated for each duplex (Table 7).

Table 7: Dose response mRNA inhibition of Eg5/KSP duplexes in HeLa cells

Concentrations given in pM

	IC20s		IC50s		IC80s	
Duplex name	1st screen	2nd screen	1st screen	2nd screen	1st screen	2nd screen
AD12077	1.19	0.80	6.14	10.16	38.63	76.16
AD12078	25.43	25.43	156.18	156.18	ND	ND
AD12085	9.08	1.24	40.57	8.52	257.68	81.26
AD12095	1.03	0.97	9.84	4.94	90.31	60.47
AD12113	4.00	5.94	17.18	28.14	490.83	441.30
AD12115	0.60	0.41	3.79	3.39	23.45	23.45
AD12125	31.21	22.02	184.28	166.15	896.85	1008.11
AD12134	2.59	5.51	17.87	22.00	116.36	107.03
AD12149	0.72	0.50	4.51	3.91	30.29	40.89
AD12151	0.53	6.84	4.27	10.72	22.88	43.01
AD12152	155.45	7.56	867.36	66.69	13165.27	ND
AD12157	0.30	26.23	14.60	92.08	14399.22	693.31
AD12166	0.20	0.93	3.71	3.86	46.28	20.59
AD12180	28.85	28.85	101.06	101.06	847.21	847.21
AD12185	2.60	0.42	15.55	13.91	109.80	120.63
AD12194	2.08	1.11	5.37	5.09	53.03	30.92
AD12211	5.27	4.52	11.73	18.93	26.74	191.07
AD12257	4.56	5.20	21.68	22.75	124.69	135.82
AD12280	2.37	4.53	6.89	20.23	64.80	104.82
AD12281	8.81	8.65	19.68	42.89	119.01	356.08
AD12282	7.71	456.42	20.09	558.00	ND	ND
AD12285	ND	1.28	57.30	7.31	261.79	42.53
AD12292	40.23	12.00	929.11	109.10	ND	ND
AD12252	0.02	18.63	6.35	68.24	138.09	404.91
AD12275	25.76	25.04	123.89	133.10	1054.54	776.25
AD12266	4.85	7.80	10.00	32.94	41.67	162.65
AD12267	1.39	1.21	12.00	4.67	283.03	51.12
AD12264	0.92	2.07	8.56	15.12	56.36	196.78
AD12268	2.29	3.67	22.16	25.64	258.27	150.84
AD12279	1.11	28.54	23.19	96.87	327.28	607.27

AD12256	**7.20**	**33.52**	**46.49**	**138.04**	**775.54**	**1076.76**
AD12259	**2.16**	**8.31**	**8.96**	**40.12**	**50.05**	**219.42**
AD12276	**19.49**	**6.14**	**89.60**	**59.60**	**672.51**	**736.72**
AD12321	**4.67**	**4.91**	**24.88**	**19.43**	**139.50**	**89.49**

(ND-not determined)

Example 4. Silencing of liver Eg5/KSP in juvenile rats following single-bolus administration of LNP01 formulated siRNA

From birth until approximately 23 days of age, Eg5/KSP expression can be detected in the growing rat liver. Target silencing with a formulated Eg5/KSP siRNA was evaluated in juvenile rats using duplex AD-6248.

KSP Duplex Tested

Duplex ID	Target	Sense	Antisense
AD6248	KSP	AccGAAGuGuuGuuuGuccTsT (SEQ ID NO:1238)	GGAcAAAcAAcACUUCGGUTsT (SEQ ID NO:1239)

Methods

Dosing of animals. Male, juvenile Sprague-Dawley rats (19 days old) were administered single doses of lipidoid ("LNP01") formulated siRNA via tail vein injection. Groups of ten animals received doses of 10 milligrams per kilogram (mg/kg) bodyweight of either AD6248 or an unspecific siRNA. Dose level refers to the amount of siRNA duplex administered in the formulation. A third group received phosphate-buffered saline. Animals were sacrificed two days after siRNA administration. Livers were dissected, flash frozen in liquid Nitrogen and pulverized into powders.

mRNA measurements. Levels of Eg5/KSP mRNA were measured in livers from all treatment groups. Samples of each liver powder (approximately ten milligrams) were homogenized in tissue lysis buffer containing proteinase K. Levels of Eg5/KSP and GAPDH mRNA were measured in triplicate for each sample using the Quantigene branched DNA assay (GenoSpectra). Mean values for Eg5/KSP were normalized to mean GAPDH values for each sample. Group means were determined and normalized to the PBS group for each experiment.

Statistical analysis. Significance was determined by ANOVA followed by the Tukey post-hoc test.

Results

Data Summary

Mean values (±standard deviation) for Eg5/KSP mRNA are given. Statistical significance (p value) versus the PBS group is shown (ns, not significant [p>0.05]).

Table 8. Experiment 1

	KSP/GAPDH	p value

PBS		1.0±0.47	
AD6248	10 mg/kg	0.47±0.12	<0.001
unspec	10 mg/kg	1.0±0.26	ns

A statistically significant reduction in liver Eg5/KSP mRNA was obtained following treatment with formulated AD6248 at a dose of 10 mg/kg.

Example 5. Silencing of rat liver VEGF following intravenous infusion of LNP01 formulated VSP

A "lipidoid" formulation comprising an equimolar mixture of two siRNAs was administered to rats. As used herein, VSP refers to a composition having two siRNAs, one directed to Eg5/KSP and one directed to VEGF. For this experiment the duplex AD3133 directed towards VEGF and AD12115 directed towards Eg5/KSP were used. Since Eg5/KSP expression is nearly undetectable in the adult rat liver, only VEGF levels were measured following siRNA treatment.

siRNA duplexes administered (VSP)

Duplex ID	Target	Sense	Antisense
AD12115	Eg5/KSP	ucGAGAAucuAAAcuAAcuTsT (SEQ ID NO:1240)	AGUuAGUUuAGAUUCUCGATsT (SEQ ID NO:1241)
AD3133	VEGF	GcAcAuAGGAGAGAuGAGCUsU (SEQ ID NO:1242)	AAGCUcAUCUCUCCuAuGuGCusG (SEQ ID NO:1243)

Key: A,G,C,U-ribonucleotides; c,u-2'-O-Me ribonucleotides; s-phosphorothioate.

Unmodified versions of each strand and the targets for each siRNA are as follows

Eg5/KSP	unmod sense	5′ UCGAGAAUCUAAACUAACUTT 3′	SEQ ID NO:1534
	unmod antisense	3′ TTAGUCCUUAGAUUUGAUUGA 5′	SEQ ID NO:1535
	target	5′ UCGAGAAUCUAAACUAACU 3′	SEQ ID NO:1311
VEGF	unmod sense	5′ GCACAUAGGAGAGAUGAGCUU 3′	SEQ ID NO:1536
	unmod antisense	3′ GUCGUGUAUCCUCUCUACUCGAA 5′	SEQ ID NO:1537
	target	5′ GCACAUAGGAGAGAUGAGCUU 3′	SEQ ID NO:1538

Methods

Dosing of animals. Adult, female Sprague-Dawley rats were administered lipidoid ("LNP01") formulated siRNA by a two-hour infusion into the femoral vein. Groups of four animals received doses of 5, 10 and 15 milligrams per kilogram (mg/kg) bodyweight of formulated siRNA. Dose level refers to the total amount of siRNA duplex administered in the formulation. A fourth group received phosphate-buffered saline. Animals were sacrificed 72 hours after the end of the siRNA infusion. Livers were dissected, flash frozen in liquid Nitrogen and pulverized into powders.

Formulation Procedure

The lipidoid ND98·4HCl (MW 1487) (Formula 1, above), Cholesterol (Sigma-Aldrich), and PEG-Ceramide C16 (Avanti Polar Lipids) were used to prepare lipid-siRNA nanoparticles.

Stock solutions of each in ethanol were prepared: ND98, 133 mg/mL; Cholesterol, 25 mg/mL, PEG-Ceramide C16, 100 mg/mL. ND98, Cholesterol, and PEG-Ceramide C16 stock solutions were then combined in a 42:48:10 molar ratio. Combined lipid solution was mixed rapidly with aqueous siRNA (in sodium acetate pH 5) such that the final ethanol concentration was 35-45% and the final sodium acetate concentration was 100-300 mM. Lipid-siRNA nanoparticles formed spontaneously upon mixing. Depending on the desired particle size distribution, the resultant nanoparticle mixture was in some cases extruded through a polycarbonate membrane (100 nm cut-off) using a thermobarrel extruder (Lipex Extruder, Northern Lipids, Inc). In other cases, the extrusion step was omitted. Ethanol removal and simultaneous buffer exchange was accomplished by either dialysis or tangential flow filtration. Buffer was exchanged to phosphate buffered saline (PBS) pH 7.2.

Characterization of formulations

Formulations prepared by either the standard or extrusion-free method are characterized in a similar manner. Formulations are first characterized by visual inspection. They should be whitish translucent solutions free from aggregates or sediment. Particle size and particle size distribution of lipid-nanoparticles are measured by dynamic light scattering using a Malvern Zetasizer Nano ZS (Malvern, USA). Particles should be 20-300 nm, and ideally, 40-100 nm in size. The particle size distribution should be unimodal. The total siRNA concentration in the formulation, as well as the entrapped fraction, is estimated using a dye exclusion assay. A sample of the formulated siRNA is incubated with the RNA-binding dye Ribogreen (Molecular Probes) in the presence or absence of a formulation disrupting surfactant, 0.5% Triton-X100. The total siRNA in the formulation is determined by the signal from the sample containing the surfactant, relative to a standard curve. The entrapped fraction is determined by subtracting the "free" siRNA content (as measured by the signal in the absence of surfactant) from the total siRNA content. Percent entrapped siRNA is typically >85%. For SNALP formulation, the particle size is at least 30 nm, at least 40 nm, at least 50 nm, at least 60 nm, at least 70 nm, at least 80 nm, at least 90 nm, at least 100 nm, at least 110 nm, and at least 120 nm. The preferred range is about at least 50 nm to about at least 110 nm, preferably about at least 60 nm to about at least 100 nm, most preferably about at least 80 nm to about at least 90 nm. In one example, each of the particle size comprises at least about 1:1 ratio of Eg5 dsRNA to VEGF dsRNA.

mRNA measurements. Samples of each liver powder (approximately ten milligrams) were homogenized in tissue lysis buffer containing proteinase K. Levels of VEGF and GAPDH mRNA were measured in triplicate for each sample using the Quantigene branched DNA assay

(GenoSpectra). Mean values for VEGF were normalized to mean GAPDH values for each sample. Group means were determined and normalized to the PBS group for each experiment.

Protein measurements. Samples of each liver powder (approximately 60 milligrams) were homogenized in 1 ml RIPA buffer. Total protein concentrations were determined using the Micro BCA protein assay kit (Pierce). Samples of total protein from each animal were used to determine VEGF protein levels using a VEGF ELISA assay (R&D systems). Group means were determined and normalized to the PBS group for each experiment.

Statistical analysis. Significance was determined by ANOVA followed by the Tukey post-hoc test

Results

Data Summary

Mean values (±standard deviation) for mRNA (VEGF/GAPDH) and protein (rel. VEGF) are shown for each treatment group. Statistical significance (p value) versus the PBS group for each experiment is shown.

Table 9.

	VEGF/GAPDH	p value	rel VEGF	p value
PBS	1.0±0.17		1.0±0.17	
5 mg/kg	0.74±0.12	<0.05	0.23±0.03	<0.001
10 mg/kg	0.65±0.12	<0.005	0.22±0.03	<0.001
15 mg/kg	0.49±0.17	<0.001	0.20±0.04	<0.001

Statistically significant reductions in liver VEGF mRNA and protein were measured at all three siRNA dose levels.

Example 6. Assessment of VSP SNALP in mouse models of human hepatic tumors.

These studies utilized a VSP siRNA cocktail containing dsRNAs targeting KSP/Eg5 and dsRNAs targeting VEGF. As used herein, VSP refers to a composition having two siRNAs, one directed to Eg5/KSP and one directed to VEGF. For this experiment the duplexes AD3133 (directed towards VEGF) and AD12115 (directed towards Eg5/KSP) were used. The siRNA cocktail was formulated in SNALP as described below.

The maximum study size utilized 20-25 mice. To test the efficacy of the siRNA SNALP cocktail to treat liver cancer, 1x10^6 tumor cells were injected directly into the left lateral lobe of test mice. The incisions were closed by sutures, and the mice allowed to recover for 2-5 hours. The mice were fully recovered within 48-72 hours. The SNALP siRNA treatment was initiated 8-11 days after tumor seeding.

The SNALP formulations utilized were (i) VSP (KSP + VEGF siRNA cocktail (1:1 molar ratio)); (ii) KSP (KSP + Luc siRNA cocktail); and (iii) VEGF (VEGF + Luc siRNA

cocktail). All formulations contained equal amounts (mg) of each active siRNA. All mice received a total siRNA/lipid dose, and each cocktail was formulated into 1:57 cDMA SNALP (1.4% PEG-cDMA; 57.1% DLinDMA; 7.1% DPPC; and 34.3% cholesterol), 6:1 lipid:drug using original citrate buffer conditions.

Human Hep3B Study A: anti-tumor activity of VSP-SNALP

Human Hepatoma Hep3B tumors were established in scid/beige mice by intrahepatic seeding. Group A (n=6) animals were administered PBS; Group B (n=6) animals were administered VSP SNALP; Group C (n=5) animals were administered KSP/Luc SNALP; Group D (n=5) animals were administered VEGF/Luc SNALP.

SNALP treatment was initiated eight days after tumor seeding. The SNALP was dosed at 3 mg/kg total siRNA, twice weekly (Monday and Thursday), for a total of six doses (cumulative 18 mg/kg siRNA). The final dose was administered at day 25, and the terminal endpoint was at day 27.

Tumor burden was assayed by (a) body weight; (b) liver weight; (c) visual inspection + photography at day 27; (d) human-specific mRNA analysis; and (e) blood alpha-fetoprotein levels measured at day 27.

Table 10 below illustrates the results of visual scoring of tumor burden measured in the seeded (left lateral) liver lobe. Score: "-" = no visible tumor; "+"= evidence of tumor tissue at injection site; "++" = Discrete tumor nodule protruding from liver lobe; "+++" = large tumor protruding on both sides of liver lobe; "++++" = large tumor, multiple nodules throughout liver lobe.

Table 10.

	Mouse	Tumor Burden
Group A: PBS, day 27	1	++++
	2	++++
	3	++
	4	+++
	5	++++
	6	++++
Group B: VSP (VEGF + KSP/Eg5, d. 27	1	+
	2	-
	3	-
	4	-
	5	++
	6	-
Group C: KSP (Luc + KSP), d. 27	1	+
	2	++
	3	-

	4	+
	5	++
Group D: VEGF (Luc + VEGF), d. 27	1	++++
	2	-
	3	++++
	4	+++
	5	++++

Liver weights, as percentage of body weight, are shown in FIG. 1. FIG.. 2A, FIG. 2B, FIG. 2C and FIG. 2D show the effects of PBS, VSP, KSP and VEGF on body weight on Human Hepatoma Hep3B tumors in mice.

From this study, the following conclusions were made. (1) VSP SNALP demonstrated potent anti-tumor effects in Hep3B 1H model; (2) the anti-tumor activity of the VSP cocktail appeared largely associated with the KSP component; (3) anti-KSP activity was confirmed by single dose histological analysis; and (4) VEGF siRNA showed no measurable effect on inhibition of tumor growth in this model.

Human Hep3B Study B: prolonged survival with VSP treatment

In a second Hep3B study, human hepatoma Hep3B tumors were established by intrahepatic seeding into scid/beige mice. These mice were deficient for lymphocytes and natural killer (NK) cells, which is the minimal scope for immune-mediated anti-tumor effects. Group A (n=6) mice were untreated; Group B (n=6) mice were administered luciferase (luc) 1955 SNALP (Lot No. AP10-02); and Group C (n=7) mice were administered VSP SNALP (Lot No. AP10-01). SNALP was 1:57 cDMA SNALP, and 6:1 lipid:drug.

SNALP treatment was initiated eight days after tumor seeding. SNALP was dosed at 3 mg/kg siRNA, twice weekly (Mondays and Thursdays), for a total of six doses (cumulative 18 mg/kg siRNA). The final dose was delivered at day 25, and the terminal endpoint of the study was at day 27.

Tumor burden was assayed by (1) body weight; (2) visual inspection + photography at day 27; (3) human-specific mRNA analysis; and (4) blood alpha-fetoprotein measured at day 27.

FIG. 3 shows body weights were measured at each day of dosing (days 8, 11, 14, 18, 21, and 25) and on the day of sacrifice.

Table 11.

	Mouse	Tumor Burden by macroscopic observation
Group A: untreated, day 27	A1R	++
	A1G	++++
	A1W	-

	A2R	++++
	A2G	+++
	A2W	++++
Group B: 1955 Luc SNALP, day 27	B1R	++++
	B1G	++++
	B1W	+++
	B2R	++
	B2G	+++
	B2W	++++
Group C: VSP SNALP, day 27	C1R	-
	C1G	-
	C1B	-
	C1W	+
	C2R	+
	C2G	+
	C2W	-

Score: "-" = no visible tumor; "+"= evidence of tumor tissue at injection site; "++" = Discrete tumor nodule protruding from liver lobe; "+++" = large tumor protruding on both sides of liver lobe; "++++" = large tumor, multiple nodules throughout liver lobe.

The correlation between body weights and tumor burden are shown in FIGs. 4, 5 and 6. FIG. 4 shows percentage body weight over 27 days in untreated mice. FIG. 5 shows percentage body weight over 27 days in 1955 Luc SNALP treated mice. FIG. 6 shows percentage body weight over 27 days in VSP SNALP treated mice.

A single dose of VSP SNALP (2 mg/kg) to Hep3B mice also resulted in the formation of mitotic spindles in liver tissue samples examined by histological staining.

Tumor burden was quantified by quantitative RT-PCR (pRT-PCR) (Taqman). Human GAPDH was normalized to mouse GAPDH via species-specific Taqman assays. FIG. 7A shows tumor scores as shown by macroscopic observation in the table above correlated with GADPH levels.

Serum ELISA was performed to measure alpha-fetoprotein (AFP) secreted by the tumor. As described below, if levels of AFP go down after treatment, the tumor is not growing. FIG. 7B shows that the treatment with VSP lowered AFP levels in some animals compared to treatment with controls.

Human HepB3 Study C:

In a third study, human HCC cells (HepB3) were injected directly into the liver of SCID/beige mice, and treatment was initiated 20 days later. Group A animals were administered PBS; Group B animals were administered 4 mg/kg Luc-1955 SNALP; Group C animals were administered 4 mg/kg SNALP-VSP; Group D animals were administered 2 mg/kg SNALP-VSP;

and Group E animals were administered 1 mg/kg SNALP-VSP. Treatment was with a single intravenous (iv) dose, and mice were sacrificed 24 hr. later.

Tumor burden and target silencing was assayed by qRT-PCR (Taqman). Tumor score was also measured visually as described above, and the results are shown in the following table. hGAPDH levels, as shown in FIG. 8, correlates with macroscopic tumor score as shown in the table below.

Table 12.

	Mouse	Tumor Burden by macroscopic observation
Group A: PBS	A2	+++
	A3	+++
	A4	+++
Group B: 4 mg/kg Luc-1955 SNALP	B1	+
	B2	+++
	B3	+++
	B4	+++
Group C: 4 mg/kg SNALP-VSP	C1	++
	C2	++
	C3	++
	C4	+++
Group D: 2 mg/kg SNALP-VSP	D1	++
	D2	+
	D3	+
	D4	++
Group E: 1 mg/kg SNALP-VSP	E1	+++
	E2	+
	E3	++
	E4	+

Score: "+"= variable tumor take/ some small tumors; "++" = Discrete tumor nodule protruding from liver lobe; "+++" = large tumor protruding on both sides of liver lobe

Human (tumor-derived) KSP silencing was assayed by Taqman analysis and the results are shown in FIG. 9. hKSP expression was normalized to hGAPDH. About 80% tumor KSP silencing was observed at 4 mg/kg SNALP-VSP, and efficacy was evident at 1 mg/kg. The clear bars in FIG. 9 represent the results from small (low GAPDH) tumors.

Human (tumor-derived) VEGF silencing was assayed by Taqman analysis and the results are shown in FIG. 10. hVEGF expression was normalized to hGAPDH. About 60% tumor VEGF silencing was observed at 4 mg/kg SNALP-VSP, and efficacy was evident at 1 mg/kg. The clear bars in FIG. 10 represent the results from small (low GAPDH) tumors.

Mouse (liver-derived) VEGF silencing was assayed by Taqman analysis and the results are shown in FIG. 11A. mVEGF expression was normalized to hGAPDH. About 50% liver VEGF silencing was observed at 4 mg/kg SNALP-VSP, and efficacy was evident at 1 mg/kg.

Human HepB3 Study D: contribution of each dsRNA to tumor growth

In a fourth study, human HCC cells (HepB3) were injected directly into the liver of SCID/beige mice, and treatment was initiated 8 days later. Treatment was with intravenous (iv) bolus injections, twice weekly, for a total of six does. The final dose was administered at day 25, and the terminal endpoint was at day 27.

Tumor burden was assayed by gross histology, human-specific mRNA analysis (hGAPDH qPCR), and blood alpha-fetoprotein levels (serum AFP via ELISA).

In Study 1, Group A was treated with PBS, Group B was treated with SNALP-KSP+Luc (3 mg/kg), Group C was treated with SNALP-VEGF+Luc (3 mg/kg), and Group D was treated with SNALP-VSP (3 mg/kg).

In Study 2, Group A was treated with PBS; Group B was treated with SNALP-KSP+Luc (1 mg/kg), Group C was treated with ALN-VSP02 (1 mg/kg).

Both GAPDH mRNA levels and serum AFP levels were shown to decrease after treatment with SNALP-VSP (as shown in FIG. 11B).

Histology Studies:

Human hepatoma Hep3B tumors were established by intrahepatic seeding in mice. SNALP treatment was initiated 20 days after tumor seeding. Tumor-bearing mice (three per group) were treated with a single intravenous (IV) dose of (i) VSP SNALP or (ii) control (Luc) SNALP at 2 mg/kg total siRNA.

Liver/tumor samples were collected for conventional H&E histology 24 hours after single SNALP administration.

Large macroscopic tumor nodules (5-10 mm) were evident at necroscopy.

Effect of SNALP-VSP in Hep3B mice:

SNALP-VSP (a cocktail of KSP dsRNA and VEGF dsRNA) treatment reduced tumor burden and expression of tumor-derived KSP and VEGF. GAPDH mRNA levels, a measure of tumor burden, were also observed to decline following administration of SNALP-VSP dsRNA (shown in FIG. 12A, FIG. 12B and FIG. 12C). A decrease in tumor burden by visual macroscopic observation was also evident following administration of SNALP-VSP.

A single IV bolus injection of SNALP-VSP also resulted in mitotic spindle formation that was clearly detected in liver tissue samples from Hep3B mice. This observation indicated cell cycle arrest.

Example 7. Survival of SNALP-VSP animals versus SNALP-Luc treated animals

To test the effect of siRNA SNALP on survival rates of cancer subjects, tumors were established by intrahepatic seeding in mice and the mice were treated with SNALP-siRNA.

These studies utilized a VSP siRNA cocktail containing dsRNAs targeting KSP/Eg5 and VEGF. Control was dsRNA targeting Luc. The siRNA cocktail was formulated in SNALPs.

Tumor cells (Human Hepatoma Hep3B, 1x10^6) were injected directly into the left lateral lobe of scid/beige mice. These mice were deficient for lymphocytes and natural killer (NK) cells, which is the minimal scope for immune-mediated anti-tumor effects. The incisions were closed by sutures, and the mice allowed to recover for 2-5 hours. The mice were fully recovered within 48-72 hours.

All mice received a total siRNA/lipid intravenous (iv) dose, and each cocktail was formulated into 1:57 cDMA SNALP (1.4% PEG-cDMA; 57.1% DLinDMA; 7.1% DPPC; and 34.3% cholesterol), 6:1 lipid:drug using original citrate buffer conditions.

siRNA-SNALP treatment was initiated on the day indicated below (18 or 26 days) after tumor seeding. siRNA-SNALP were administered twice a week for three weeks after 18 or 26 days at a dose of 4 mg/kg. Survival was monitored and animals were euthanized based on humane surrogate endpoints (*e.g.*, animal body weight, abdominal distension/discoloration, and overall health).

The survival data for treatment initiated 18 days after tumor seeing is summarized in Table 13, Table 14, and FIG. 13A.

Table 13. Kaplan-Meier (survival) data (% Surviving)

Day	SNALP-Luc	SNALP-VSP
18	100%	100%
22	100%	100%
25	100%	100%
27	100%	100%
28	100%	100%
28	86%	100%
29	86%	100%
32	86%	100%
33	86%	100%
33	43%	100%
35	43%	100%
36	43%	100%
36	29%	100%
38	29%	100%
38	14%	100%
38	14%	88%
40	14%	88%
43	14%	88%
45	14%	88%
49	14%	88%

51	14%	88%
51	14%	50%
53	14%	50%
53	14%	25%
55	14%	25%
57	14%	25%
57	0%	0%

Table 14. Survival in days, for each animal.

Animal	Treatment group	Survival	
1	SNALP-Luc	28	days
2	SNALP-Luc	33	days
3	SNALP-Luc	33	days
4	SNALP-Luc	33	days
5	SNALP-Luc	36	days
6	SNALP-Luc	38	days
7	SNALP-Luc	57	days
8	SNALP-VSP	38	days
9	SNALP-VSP	51	days
10	SNALP-VSP	51	days
11	SNALP-VSP	51	days
12	SNALP-VSP	53	days
13	SNALP-VSP	53	days
14	SNALP-VSP	57	days
15	SNALP-VSP	57	days

FIG. 13A shows the mean survival of SNALP-VSP animals and SNALP-Luc treated animals versus days after tumor seeding. The mean survival of SNALP-VSP animals was extended by approximately 15 days versus SNALP-Luc treated animals.

Table 15. Serum alpha fetoprotein (AFP) concentration, for each animal, at a time pre-treatment and at end of treatment (concentration in μg/ml)

		pre-Rx	End of Rx
1	SNALP-Luc	30.858	454.454
2	SNALP-Luc	10.088	202.082
3	SNALP-Luc	23.736	648.952
4	SNALP-Luc	1.696	13.308
5	SNALP-Luc	4.778	338.688
6	SNALP-Luc	15.004	826.972
7	SNALP-Luc	11.036	245.01
8	SNALP-VSP	37.514	182.35
9	SNALP-VSP	91.516	248.06
10	SNALP-VSP	25.448	243.13
11	SNALP-VSP	24.862	45.514
12	SNALP-VSP	57.774	149.352
13	SNALP-VSP	12.446	78.724
14	SNALP-VSP	2.912	9.61
15	SNALP-VSP	4.516	11.524

Tumor burden was monitored using serum AFP levels during the course of the experiment. Alpha-fetoprotein (AFP) is a major plasma protein produced by the yolk sac and the liver during fetal life. The protein is thought to be the fetal counterpart of serum albumin, and human AFP and albumin gene are present in tandem in the same transcriptional orientation on chromosome 4. AFP is found in monomeric as well as dimeric and trimeric forms, and binds copper, nickel, fatty acids and bilirubin. AFP levels decrease gradually after birth, reaching adult levels by 8-12 months. Normal adult AFP levels are low, but detectable. AFP has no known function in normal adults and AFP expression in adults is often associated with a subset of tumors such as hepatoma and teratoma. AFP is a tumor marker used to monitor testicular cancer, ovarian cancer, and malignant teratoma. Principle tumors that secrete AFP include endodermal sinus tumor (yolk sac carcinoma), neuroblastoma, hepatoblastoma, and heptocellular carcinoma. In patients with AFP-secreting tumors, serum levels of AFP often correlate with tumor size. Serum levels are useful in assessing response to treatment. Typically, if levels of AFP go down after treatment, the tumor is not growing. A temporary increase in AFP immediately following chemotherapy may indicate not that the tumor is growing but rather that it is shrinking (and releasing AFP as the tumor cells die). Resection is usually associated with a fall in serum levels. As shown in FIG. 14, tumor burden in SNALP-VSP treated animals was significantly reduced.

The experiment was repeated with SNALP-siRNA treatment at 26, 29, 32 35, 39, and 42 days after implantation. The data is shown in FIG. 13B. The mean survival of SNALP-VSP animals was extended by approximately 15 days versus SNALP-Luc treated animals by approximately 19 days, or 38%.

Example 8. Induction of Mono-asters in Established Tumors

Inhibition of KSP in dividing cells leads to the formation of mono asters that are readily observable in histological sections. To determine whether mono aster formation occurred in SNALP-VSP treated tumors, tumor bearing animals (three weeks after Hep3B cell implantation) were administered 2 mg/kg SNALP-VSP via tail vein injection. Control animals received 2 mg/kg SNALP-Luc. Each cocktail was formulated into 1:57 cDMA SNALP (1.4% PEG-cDMA; 57.1% DLinDMA; 7.1% DPPC; and 34.3% cholesterol), 6:1 lipid:drug using original citrate buffer conditions.

Twenty four hours later, animals were sacrificed, and tumor bearing liver lobes were processed for histological analysis. Representative images of H&E stained tissue sections are shown in FIG. 15. Extensive mono aster formation was evident in SNALP-VSP treated (A), but

not SNALP-Luc treated (B), tumors. In the latter, normal mitotic figures were evident. The generation of mono asters is a characteristic feature of KSP inhibition and provides further evidence that SNALP-VSP has significant activity in established liver tumors.

Example 9. Manufacturing Process and Product specification of ALN-VSP02 (SNALP-VSP)

ALN-VSP02 product contains 2 mg/mL of drug substance ALN-VSPDS01 formulated in a sterile lipid particle formulation (referred to as SNALP) for IV administration via infusion. Drug substance ALN-VSPDS01 consists of two siRNAs (ALN-12115 targeting KSP and ALN-3133 targeting VEGF) in an equimolar ratio. The drug product is packaged in 10 mL glass vials with a fill volume of 5 mL.

The drug substance can be formulated in other nucleic acid-lipid particle formulations as described herein, e.g., with cationic lipids XTC, ALNY-100, and MC3.

The following terminology is used herein:

Drug Substance	siRNA Duplexes	Single Strand Intermediates
ALN-VSPDS01	ALN-12115*	Sense: A-19562
		Antisense: A-19563
	ALN-3133**	Sense: A-3981
		Antisense: A-3982

*Alternate names = AD-12115, AD12115; ** Alternate names = AD-3133, AD3133

9.1 Preparation of drug substance ALN-VSPDS01

The two siRNA components of drug substance ALN-VSPDS01, ALN-12115 and ALN-3133, are chemically synthesized using commercially available synthesizers and raw materials. The manufacturing process consists of synthesizing the two single strand oligonucleotides of each duplex (A 19562 sense and A 19563 antisense of ALN 12115 and A 3981 sense and A 3982 antisense of ALN 3133) by conventional solid phase oligonucleotide synthesis using phosphoramidite chemistry and 5' O dimethoxytriphenylmethyl (DMT) protecting group with the 2' hydroxyl protected with tert butyldimethylsilyl (TBDMS) or the 2' hydroxyl replaced with a 2' methoxy group (2' OMe). Assembly of an oligonucleotide chain by the phosphoramidite method on a solid support such as controlled pore glass or polystyrene. The cycle consists of 5' deprotection, coupling, oxidation, and capping. Each coupling reaction is carried out by activation of the appropriately protected ribo, 2' OMe , or deoxyribonucleoside amidite using 5 (ethylthio) 1H tetrazole reagent followed by the coupling of the free 5' hydroxyl group of a support immobilized protected nucleoside or oligonucleotide. After the appropriate number of cycles, the final 5' protecting group is removed by acid treatment. The crude oligonucleotide is cleaved from the solid support by aqueous methylamine treatment with

concomitant removal of the cyanoethyl protecting group as well as nucleobase protecting groups. The 2' O TBDMS group is then cleaved using a hydrogen fluoride containing reagent to yield the crude oligoribonucleotide, which is purified using strong anion exchange high performance liquid chromatography (HPLC) followed by desalting using ultrafiltration. The purified single strands are analyzed to confirm the correct molecular weight, the molecular sequence, impurity profile and oligonucleotide content, prior to annealing into the duplexes. The annealed duplex intermediates ALN 12115 and ALN 3133 are either lyophilized and stored at 20°C or mixed in 1:1 molar ratio and the solution is lyophilized to yield drug substance ALN VSPDS01. If the duplex intermediates were stored as dry powder, they are re-dissolved in water before mixing. The equimolar ratio is achieved by monitoring the mixing process by an HPLC method.

Example specifications are shown in Table 16a.

Table 16a. Example specifications for ALN-VSPDS01

Test	Method	Acceptance Criteria
Appearance	Visual	White to off-white powder
Identity, ALN-VSPDS01 ALN-3133 ALN-12115	Duplex AX-HPLC	Duplex retention times are consistent with those of reference standards
Identity, ALN-VSPDS01	MS	Molecular weight of single strands are within the following ranges: A-3981: 6869-6873 Da A-3982: 7305-7309 Da A-19562: 6762-6766 Da A-19563: 6675-6679 Da
Sodium counter ion (%w/w on anhydrous basis)	Flame AAS or ICP-OES	Report data
ALN-VSPDS01 assay	Denaturing AX-HPLC	90 – 110%
Purity of ALN-VSPDS01	SEC	≥ 90.0 area %
Single strand purity, ALN-VSPDS01	Denaturing AX-HPLC	Report data Report area % for total impurities
siRNA molar ratio	Duplex AX-HPLC	1.0 ± 0.1
Moisture content	Karl Fischer titration	≤ 15%
Residual solvents Acetonitrile Ethanol Isopropanol	HS-Capillary GC	 ≤ 410 ppm ≤ 5000 ppm ≤ 5000 ppm
pH of 1% solution	USP <791>	Report data
Heavy metals As, Cd, Cu, Cr, Fe, Ni, Pb, Sn	ICP-MS	Report data
Bacterial endotoxins	USP <85>	≤ 0.5 EU/mg
Bioburden	Modified USP <61>	< 100 CFU/g

The results of up to 12 month stability testing for ALN-VSPDS01 drug substance are shown in Tables 16b. The assay methods were chosen to assess physical property (appearance, pH, moisture), purity (by SEC and denaturing anion exchange chromatography) and potency (by denaturing anion exchange chromatography [AX-HPLC]).

Table 16b: Stability of drug substance

Lot No.: A05M07001N			Study Storage Conditions: -20°C (Storage Condition)				
Test	Method	Acceptance Criteria	Results				
			Initial	1 Month	3 Months	6 Months	12 Months
Appearance	Visual	White to off-white powder	Pass	Pass	Pass	Pass	Pass
pH	USP <791>	Report data	6.7	6.4	6.6	6.4	6.8
Moisture content (%w/w)	Karl Fischer titration	≤ 15%	3.6*	6.7	6.2	5.6	5.0
Purity (area %)	SEC	≥ 90.0 area%	95	95	94	92	95
A-3981 (sense) (area %)	Denaturing AX-HPLC	Report data	24	23	23	23	23
A-3982 (antisense) (area %)	Denaturing AX-HPLC	Report data	23	23	23	23	24
A-19562 (sense) (area %)	Denaturing AX-HPLC	Report data	22	21	21	21	21
A-19563 (antisense) (area %)	Denaturing AX-HPLC	Report data	23	22	22	22	22

9.2 Preparation of drug product ALN-VSP02

ALN VSP02, is a sterile formulation of the two siRNAs (in a 1:1 molar ratio) with lipid excipients in isotonic buffer. The lipid excipients associate with the two siRNAs, protect them from degradation in the circulatory system, and aid in their delivery to the target tissue. The specific lipid excipients and the quantitative proportion of each (shown in Table 17) have been selected through an iterative series of experiments comparing the physicochemical properties, stability, pharmacodynamics, pharmacokinetics, toxicity and product manufacturability of numerous different formulations. The excipient DLinDMA is a titratable aminolipid that is positively charged at low pH, such as that found in the endosome of mammalian cells, but relatively uncharged at the more neutral pH of whole blood. This feature facilitates the efficient encapsulation of the negatively charged siRNAs at low pH, preventing formation of empty particles, yet allows for adjustment (reduction) of the particle charge by replacing the formulation buffer with a more neutral storage buffer prior to use. Cholesterol and the neutral lipid DPPC are incorporated in order to provide physicochemical stability to the particles. The polyethyleneglycol lipid conjugate PEG2000 C DMA aids drug product stability, and provides optimum circulation time for the proposed use. ALN VSP02 lipid particles have a mean diameter of approximately 80-90 nm with low polydispersity values. At neutral pH, the particles are essentially uncharged, with Zeta Potential values of less than 6 mV. There is no evidence of empty (non loaded) particles based on the manufacturing process.

Table 17: Quantitative Composition of ALN-VSP02

Component, grade	Proportion (mg/mL)
ALN-VSPDS01, cGMP	2.0*
DLinDMA (1,2-Dilinoleyloxy-N,N-dimethyl-3-aminopropane), cGMP	7.3
DPPC (R-1,2-Dipalmitoyl-sn-glycero-3-phosphocholine), cGMP	1.1
Cholesterol, Synthetic, cGMP	2.8
PEG2000-C-DMA (3-N-[(ω-Methoxy poly(ethylene glycol) 2000) carbamoyl]-1,2-dimyristyloxy-propylamine), cGMP	0.8
Phosphate Buffered Saline, cGMP	q.s.

* The 1:1 molar ratio of the two siRNAs in the drug product is maintained throughout the size distribution of the drug product particles.

Solutions of lipid (in ethanol) and ALN VSPDS01 drug substance (in aqueous buffer) are mixed and diluted to form a colloidal dispersion of siRNA lipid particles with an average particle size of approximately 80-90 nm. This dispersion is then filtered through 0.45/0.2 µm filters, concentrated, and diafiltered by Tangential Flow Filtration. After in process testing and concentration adjustment to 2.0 mg/mL, the product is sterile filtered, aseptically filled into glass vials, stoppered, capped and placed at 5 ± 3°C. The ethanol and all aqueous buffer components are USP grade; all water used is USP Sterile Water For Injection grade.ALN-VSP02.

A similar method is used to formulate ALN-VSPDS01 in other lipid formulations, e.g., those with cationic lipids XTC, ALNY-100, and MC3.

Example 10. In Vitro Efficacy of ALN-VSP02 in Human Cancer Cell Lines

The efficacy of ALN-VSP02 treatment in human cancer cell lines was determined via measurement of KSP mRNA, VEGF mRNA, and cell viability after treatment. IC50 (nM) values determined for KSP and VEGF in each cell line.

Table 19: cell lines

Cell line tested	ATCC cat number
HELA	ATCC Cat N: CCL-2
KB	ATCC Cat N: CCL-17
HEP3B	ATCC Cat N: HB-8064
SKOV-3	ATCC Cat N: HTB-77
HCT-116	ATCC Cat N: CCL-247
HT-29	ATCC Cat N: HTB-38
PC-3	ATCC Cat N: CRL-1435
A549	ATCC Cat N: CCL-185
MDA-MB-231	ATCC Cat N: HTB-26

Cells were plated in 96 well plates in complete media at day 1 to reach a density of 70% on day 2. On day 2 media was replaced with Opti-MEM reduced serum media (Invitrogen Cat N: 11058-021) and cells were transfected with either ALN-VSP02 or control SNALP-Luc with concentration range starting at 1.8 μM down to 10 pM. After 6 hours the media was changed to complete media. Three replicate plates for each cell line for each experiment was done.

ALN-VSP02 was formulated as described in Table 17.

Cells were harvested 24 hours after transfection. KSP levels were measured using bDNA; VEGF mRNA levels were measured using human TaqMan assay.

Viability was measured using Cell Titer Blue reagent (Promega Cat N: G8080) at 48 and/or 72h following manufacturer's recommendations.

As shown in Table 20, nM concentrations of VSP02 are effective in reducing expression of both KSP and VEGF in multiple human cell lines. Viability of treated cells was not

Table 20: Results

Cell line	**IC50 (nM) KSP**	**IC50 (nM) VEGF**
HeLa	8.79	672
SKOV-3	142	1347
HCT116	31.6	27.5
Hep3B	1.3	14.5
HT-29	262	ND
PC3	127	ND
KB	50.6	ND
A549	201	ND
MB231	187	ND

Example 11. Anti-tumor efficacy of VSP SNALP vs. Sorafenib in established Hep3B intrahepatic tumors

The anti-tumor effects of multi-dosing VSP SNALP verses Sorafenib in scid/beige mice bearing established Hep3B intrahepatic tumors was studied. Sorafenib is a small molecule inhibitor of protein kinases approved for treatment of hepatic cellular carcinoma (HCC).

Tumors were established by intrahepatic seeding in scid/beige mice as described herein. Treatment was initiated 11 days post-seeding. Mice were treated with Sorafenib and a control siRNA-SNALP, Sorafenib and VSP siRNA-SNALP, or VSP siRNA-SNALP only. Control mice were treated with buffers only (DMSO for Sorafenib and PBS for siRNA-SNALP). Sorafenib was administered intraparenterally from Mon to Fri for three weeks, at 15 mg/kg according to body weight for a total of 15 injections. Sorafenib was administered a minimum of 1 hour after

SNALP injections. The siRNA-SNALPS were administered intravenously via the lateral tail vein according at 3 mg/kg based on the most recently recorded body weight (10 ml/kg) for 3 weeks (total of 6 doses) on days 1, 4, 7, 10, 14, and 17.

Each siRNA-SNALP was formulated into 1:57 cDMA SNALP (1.4% PEG-cDMA; 57.1% DLinDMA; 7.1% DPPC; and 34.3% cholesterol), 6:1 lipid:drug using original citrate buffer conditions.

Mice were euthanized based on an assessment of tumor burden including progressive weight loss and clinical signs including condition, abdominal distension/discoloration and mobility.

The percent survival data are shown in FIG. 16. Co-administration of VSP siRNA-SNALP with Sorafenib increased survival proportion compared to administration of Sorafenib or VSP siRNA-SNALP alone. VSP siRNA-SNALP increased survival proportion compared to Sorafenib.

Example 12. In vitro efficacy of VSP using variants of AD-12115 and AD-3133

Two sets of duplexes targeted to Eg5/KSP and VEGF were designed and synthesized. Each set included duplexes tiling 10 nucleotides in each direction of the target sites for either AD-12115 and AD-3133.

Sequences of the target, sense strand, and antisense strand for each duplex are shown in the Table below.

Each duplex is assayed for inhibition of expression using the assays described herein. The duplexes are administered alone and/or in combination, *e.g.*, an Eg5/KSP dsRNA in combination with a VEGF dsRNA. In some embodiments, the dsRNA are administered in a nucleic-acid lipid particle, e.g., SNALP, formulation as described herein.

Table 21: Sequences of dsRNA targeted to VEGF and Eg5/KSP (tiling)

Duplex ID	target gene	target sequence 5′ to 3′	SEQ ID NO:	Sense Strand Antisense strand 5′ to 3′	SEQ ID NO:
AD-20447.1	VEGFA	ACCAAGGCCAGCACAUAGG	2264	AccAAGGccAGcAcAuAGGTsT	2304
				CCuAUGUGCUGGCCUUGGUTsT	2305
AD-20448.1	VEGFA	CCAAGGCCAGCACAUAGGA	2265	ccAAGGccAGcAcAuAGGATsT	2306
				UCCuAUGUGCUGGCCUUGGTsT	2307
AD-20449.1	VEGFA	CCAAGGCCAGCACAUAGGA	2266	ccAAGGccAGcAcAuAGGATsT	2308
				CUCCuAUGUGCUGGCCUUGTsT	2309
AD-20450.1	VEGFA	AAGGCCAGCACAUAGGAGA	2267	AAGGccAGcAcAuAGGAGATsT	2310
				UCUCCuAUGUGCUGGCCUUTsT	2311
AD-20451.1	VEGFA	AGGCCAGCACAUAGGAGAG	2268	AGGccAGcAcAuAGGAGAGTsT	2312
				CUCUCCuAUGUGCUGGCCUTsT	2313
AD-20452.1	VEGFA	GGCCAGCACAUAGGAGAGA	2269	GGccAGcAcAuAGGAGAGATsT	2314
				UCUCUCCuAUGUGCUGGCCTsT	2315
AD-20453.1	VEGFA	GCCAGCACAUAGGAGAGAU	2270	GccAGcAcAuAGGAGAGAuTsT	2316
				AUCUCUCCuAUGUGCUGGCTsT	2317
AD-20454.1	VEGFA	CCAGCACAUAGGAGAGAUG	2271	ccAGcAcAuAGGAGAGAuGTsT	2318
				cAUCUCUCCuAUGUGCUGGTsT	2319
AD-20455.1	VEGFA	CAGCACAUAGGAGAGAUGA	2272	cAGcAcAuAGGAGAGAuGATsT	2320
				UcAUCUCUCCuAUGUGCUGTsT	2321
AD-20456.1	VEGFA	AGCACAUAGGAGAGAUGAG	2273	AGcAcAuAGGAGAGAuGAGTsT	2322
				CUcAUCUCUCCuAUGUGCUTsT	2323
AD-20457.1	VEGFA	CACAUAGGAGAGAUGAGCU	2274	cAcAuAGGAGAGAuGAGcuTsT	2324
				AGCUcAUCUCUCCuAUGUGTsT	2325
AD-20458.1	VEGFA	ACAUAGGAGAGAUGAGCUU	2275	AcAuAGGAGAGAuGAGcuuTsT	2326
				AAGCUcAUCUCUCCuAUGUTsT	2327
AD-20459.1	VEGFA	CAUAGGAGAGAUGAGCUUC	2276	cAuAGGAGAGAuGAGcuucTsT	2328
				GAAGCUcAUCUCUCCuAUGTsT	2329
AD-20460.1	VEGFA	AUAGGAGAGAUGAGCUUCC	2277	AuAGGAGAGAuGAGcuuccTsT	2330
				GGAAGCUcAUCUCUCCuAUTsT	2331
AD-20461.1	VEGFA	UAGGAGAGAUGAGCUUCCU	2278	uAGGAGAGAuGAGcuuccuTsT	2332
				AGGAAGCUcAUCUCUCCuATsT	2333

Duplex ID	target gene	target sequence 5′ to 3′	SEQ ID NO:	Sense Strand Antisense strand 5′ to 3′	SEQ ID NO:
AD-20462.1	VEGFA	AGGAGAGAUGAGCUUCCUA	2279	AGGAGAGAuGAGcuuccuATsT	2334
				uAGGAAGCUcAUCUCUCCUTsT	2335
AD-20463.1	VEGFA	GGAGAGAUGAGCUUCCUAC	2280	GGAGAGAuGAGcuuccuAcTsT	2336
				GuAGGAAGCUcAUCUCUCCTsT	2337
AD-20464.1	VEGFA	GAGAGAUGAGCUUCCUACA	2281	GAGAGAuGAGcuuccuAcATsT	2338
				UGuAGGAAGCUcAUCUCUCTsT	2339
AD-20465.1	VEGFA	AGAGAUGAGCUUCCUACAG	2282	AGAGAuGAGcuuccuAcAGTsT	2340
				CUGuAGGAAGCUcAUCUCUTsT	2341
AD-20466.1	VEGFA	GAGAUGAGCUUCCUACAGC	2283	GAGAuGAGcuuccuAcAGcTsT	2342
				GCUGuAGGAAGCUcAUCUCTsT	2343
AD-20467.1	KSP	AUGUUCCUUAUCGAGAAUC	2284	AuGuuccuuAucGAGAAucTsT	2344
				GAUUCUCGAuAAGGAAcAUTsT	2345
AD-20468.1	KSP	UGUUCCUUAUCGAGAAUCU	2285	uGuuccuuAucGAGAAucuTsT	2346
				AGAUUCUCGAuAAGGAAcATsT	2347
AD-20469.1	KSP	GUUCCUUAUCGAGAAUCUA	2286	GuuccuuAucGAGAAucuATsT	2348
				uAGAUUCUCGAuAAGGAACTsT	2349
AD-20470.1	KSP	UUCCUUAUCGAGAAUCUAA	2287	uuccuuAucGAGAAucuAATsT	2350
				UuAGAUUCUCGAuAAGGAATsT	2351
AD-20471.1	KSP	UCCUUAUCGAGAAUCUAAA	2288	uccuuAucGAGAAucuAAATsT	2352
				UUuAGAUUCUCGAuAAGGATsT	2353
AD-20472.1	KSP	CCUUAUCGAGAAUCUAAAC	2289	ccuuAucGAGAAucuAAAcTsT	2354
				GUUuAGAUUCUCGAuAAGGTsT	2355
AD-20473.1	KSP	CUUAUCGAGAAUCUAAACU	2290	cuuAucGAGAAucuAAAcuTsT	2356
				AGUUuAGAUUCUCGAuAAGTsT	2357
AD-20474.1	KSP	UUAUCGAGAAUCUAAACUA	2291	uuAucGAGAAucuAAAcuATsT	2358
				uAGUUuAGAUUCUCGAuAATsT	2359
AD-20475.1	KSP	UAUCGAGAAUCUAAACUAA	2292	uAucGAGAAucuAAAcuAATsT	2360
				UuAGUUuAGAUUCUCGAuATsT	2361
AD-20476.1	KSP	AUCGAGAAUCUAAACUAAC	2293	AucGAGAAucuAAAcuAAcTsT	2362
				GUuAGUUuAGAUUCUCGAUTsT	2363
AD-20477.1	KSP	CGAGAAUCUAAACUAACUA	2294	cGAGAAucuAAAcuAAcuATsT	2364

Duplex ID	target gene	target sequence 5′ to 3′	SEQ ID NO:	Sense Strand Antisense strand 5′ to 3′	SEQ ID NO:
					2365
AD-20478.1	KSP	GAGAAUCUAAACUAACUAG	2295	GAGAAucuAAAcuAAcuAGTsT	2366
				CuAGUuAGUUuAGAUUCUCTsT	2367
AD-20479.1	KSP	AGAAUCUAAACUAACUAGA	2296	AGAAucuAAAcuAAcuAGATsT	2368
				UCuAGUuAGUUuAGAUUCUTsT	2369
AD-20480.1	KSP	GAAUCUAAACUAACUAGAA	2297	GAAucuAAAcuAAcuAGAATsT	2370
				UUCuAGUuAGUUuAGAUUCTsT	2371
AD-20481.1	KSP	AAUCUAAACUAACUAGAAU	2298	AAucuAAAcuAAcuAGAAuTsT	2372
				AUUCuAGUuAGUUuAGAUUTsT	2373
AD-20482.1	KSP	AUCUAAACUAACUAGAAUC	2299	AucuAAAcuAAcuAGAAucTsT	2374
				GAUUCuAGUuAGUUuAGAUTsT	2375
AD-20483.1	KSP	UCUAAACUAACUAGAAUCC	2300	ucuAAAcuAAcuAGAAuccTsT	2376
				GGAUUCuAGUuAGUUuAGATsT	2377
AD-20484.1	KSP	CUAAACUAACUAGAAUCCU	2301	cuAAAcuAAcuAGAAuccuTsT	2378
				AGGAUUCuAGUuAGUUuAGTsT	2379
AD-20485.1	KSP	UAAACUAACUAGAAUCCUC	2302	uAAAcuAAcuAGAAuccucTsT	2380
				GAGGAUUCuAGUuAGUUuATsT	2381
AD-20486.1	KSP	AAACUAACUAGAAUCCUCC	2303	AAAcuAAcuAGAAuccucccTsT	2382
				GGAGGAUUCuAGUuAGUUUTsT	2383

Example 13. VEGF targeted dsRNA with a single blunt end

A set of dsRNA duplexes targeted to VEGF were designed and synthesized. The set included duplexes tiling 10 nucleotides in each direction of the target sites for AD-3133. Each duplex includes a 2 base overhang at the end corresponding to the 3' end of the antisense strand and no overhang, *e.g.*, a blunt end, at the end corresponding to the 5' end of the antisense strand.

The sequences of each strand of these duplexes are shown in the following table.

Each duplex is assayed for inhibition of expression using the assays described herein. The VEGF duplexes are administered alone and/or in combination with an Eg5/KSP dsRNA (e.g., AD-12115). In some embodiments, the dsRNA are administered in a nucleic-acid lipid particle, e.g., SNALP, formulation as described herein.

Table 22: Target sequences of blunt ended dsRNA targeted to VEGF

duplex ID	SEQ	VEGF target sequence	position on

	ID NO:	5' to 3'	VEGF gene
AD-20447.1	2384	ACCAAGGCCAGCACAUAGG	1365
AD-20448.1	2385	CCAAGGCCAGCACAUAGGA	1366
AD-20449.1	2386	CAAGGCCAGCACAUAGGAG	1367
AD-20450.1	2387	AAGGCCAGCACAUAGGAGA	1368
AD-20451.1	2388	AGGCCAGCACAUAGGAGAG	1369
AD-20452.1	2389	GGCCAGCACAUAGGAGAGA	1370
AD-20453.1	2390	GCCAGCACAUAGGAGAGAU	1371
AD-20454.1	2391	CCAGCACAUAGGAGAGAUG	1372
AD-20455.1	2392	CAGCACAUAGGAGAGAUGA	1373
AD-20456.1	2393	AGCACAUAGGAGAGAUGAG	1374
AD-20457.1	2394	CACAUAGGAGAGAUGAGCU	1376
AD-20458.1	2395	ACAUAGGAGAGAUGAGCUU	1377
AD-20459.1	2396	CAUAGGAGAGAUGAGCUUC	1378
AD-20460.1	2397	AUAGGAGAGAUGAGCUUCC	1379
AD-20461.1	2398	UAGGAGAGAUGAGCUUCCU	1380
AD-20462.1	2399	AGGAGAGAUGAGCUUCCUA	1381
AD-20463.1	2400	GGAGAGAUGAGCUUCCUAC	1382
AD-20464.1	2401	GAGAGAUGAGCUUCCUACA	1383
AD-20465.1	2402	AGAGAUGAGCUUCCUACAG	1384
AD-20466.1	2403	GAGAUGAGCUUCCUACAGC	1385

Table 23: Strand sequences of blunt ended dsRNA targeted to VEGF

duplex ID	Sense strand (5' to 3')	SEQ ID NO:	Antisense strand (5' to 3')	SEQ ID NO:
AD-20447.1	ACCAAGGCCAGCACAUAGGAG	2404	CUCCUAUGUGCUGGCCUUGGUGA	2424
AD-20448.1	CCAAGGCCAGCACAUAGGAGA	2405	UCUCCUAUGUGCUGGCCUUGGUG	2425
AD-20449.1	CAAGGCCAGCACAUAGGAGAG	2406	CUCUCCUAUGUGCUGGCCUUGGU	2426
AD-20450.1	AAGGCCAGCACAUAGGAGAGA	2407	UCUCUCCUAUGUGCUGGCCUUGG	2427
AD-20451.1	AGGCCAGCACAUAGGAGAGAU	2408	AUCUCUCCUAUGUGCUGGCCUUG	2428
AD-20452.1	GGCCAGCACAUAGGAGAGAUG	2409	CAUCUCUCCUAUGUGCUGGCCUU	2429
AD-20453.1	GCCAGCACAUAGGAGAGAUGA	2410	UCAUCUCUCCUAUGUGCUGGCCU	2430
AD-20454.1	CCAGCACAUAGGAGAGAUGAG	2411	CUCAUCUCUCCUAUGUGCUGGCC	2431
AD-20455.1	CAGCACAUAGGAGAGAUGAGC	2412	GCUCAUCUCUCCUAUGUGCUGGC	2432
AD-20456.1	AGCACAUAGGAGAGAUGAGCU	2413	AGCUCAUCUCUCCUAUGUGCUGG	2433
AD-20457.1	CACAUAGGAGAGAUGAGCUUC	2414	GAAGCUCAUCUCUCCUAUGUGCU	2434
AD-20458.1	ACAUAGGAGAGAUGAGCUUCC	2415	GGAAGCUCAUCUCUCCUAUGUGC	2435
AD-20459.1	CAUAGGAGAGAUGAGCUUCCU	2416	AGGAAGCUCAUCUCUCCUAUGUG	2436
AD-20460.1	AUAGGAGAGAUGAGCUUCCUA	2417	UAGGAAGCUCAUCUCUCCUAUGU	2437
AD-20461.1	UAGGAGAGAUGAGCUUCCUAC	2418	GUAGGAAGCUCAUCUCUCCUAUG	2438
AD-20462.1	AGGAGAGAUGAGCUUCCUACA	2419	UGUAGGAAGCUCAUCUCUCCUAU	2439
AD-20463.1	GGAGAGAUGAGCUUCCUACAG	2420	CUGUAGGAAGCUCAUCUCUCCUA	2440
AD-20464.1	GAGAGAUGAGCUUCCUACAGC	2421	GCUGUAGGAAGCUCAUCUCUCCU	2441
AD-20465.1	AGAGAUGAGCUUCCUACAGCA	2422	UGCUGUAGGAAGCUCAUCUCUCC	2442

AD-20466.1	GAGAUGAGCUUCCUACAGCAC	2423	GUGCUGUAGGAAGCUCAUCUCUC	2443

Example 14: dsRNA Oligonucleotide Synthesis

Synthesis

All oligonucleotides are synthesized on an AKTAoligopilot synthesizer. Commercially available controlled pore glass solid support (dT-CPG, 500Å, Prime Synthesis) and RNA phosphoramidites with standard protecting groups, 5'-*O*-dimethoxytrityl N6-benzoyl-2'-*t*-butyldimethylsilyl-adenosine-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite, 5'-*O*-dimethoxytrityl-N4-acetyl-2'-*t*-butyldimethylsilyl-cytidine-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite, 5'-*O*-dimethoxytrityl-N2--isobutryl-2'-*t*-butyldimethylsilyl-guanosine-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite, and 5'-*O*-dimethoxytrityl-2'-*t*-butyldimethylsilyl-uridine-3'-O-N,N'-diisopropyl-2-cyanoethylphosphoramidite (Pierce Nucleic Acids Technologies) were used for the oligonucleotide synthesis. The 2'-F phosphoramidites, 5'-*O*-dimethoxytrityl-N4-acetyl-2'-fluro-cytidine-3'-*O*-N,N'-diisopropyl-2-cyanoethyl-phosphoramidite and 5'-*O*-dimethoxytrityl-2'-fluro-uridine-3'-O-N,N'-diisopropyl-2-cyanoethyl-phosphoramidite are purchased from (Promega). All phosphoramidites are used at a concentration of 0.2M in acetonitrile (CH_3CN) except for guanosine which is used at 0.2M concentration in 10% THF/ANC (v/v). Coupling/recycling time of 16 minutes is used. The activator is 5-ethyl thiotetrazole (0.75M, American International Chemicals); for the PO-oxidation iodine/water/pyridine is used and for the PS-oxidation PADS (2%) in 2,6-lutidine/ACN (1:1 v/v) is used.

3'-ligand conjugated strands are synthesized using solid support containing the corresponding ligand. For example, the introduction of cholesterol unit in the sequence is performed from a hydroxyprolinol-cholesterol phosphoramidite. Cholesterol is tethered to trans-4-hydroxyprolinol via a 6-aminohexanoate linkage to obtain a hydroxyprolinol-cholesterol moiety. 5'-end Cy-3 and Cy-5.5 (fluorophore) labeled siRNAs are synthesized from the corresponding Quasar-570 (Cy-3) phosphoramidite are purchased from Biosearch Technologies. Conjugation of ligands to 5'-end and or internal position is achieved by using appropriately protected ligand-phosphoramidite building block. An extended 15 min coupling of 0.1 M solution of phosphoramidite in anhydrous CH3CN in the presence of 5-(ethylthio)-1H-tetrazole activator to a solid-support-bound oligonucleotide. Oxidation of the internucleotide phosphite to the phosphate is carried out using standard iodine-water as reported (1) or by treatment with tert-butyl hydroperoxide/acetonitrile/water (10: 87: 3) with 10 min oxidation wait time conjugated oligonucleotide. Phosphorothioate is introduced by the oxidation of phosphite to

phosphorothioate by using a sulfur transfer reagent such as DDTT (purchased from AM Chemicals), PADS and or Beaucage reagent. The cholesterol phosphoramidite is synthesized in house and used at a concentration of 0.1 M in dichloromethane. Coupling time for the cholesterol phosphoramidite is 16 minutes.

Deprotection I (Nucleobase Deprotection)

After completion of synthesis, the support is transferred to a 100 mL glass bottle (VWR). The oligonucleotide is cleaved from the support with simultaneous deprotection of base and phosphate groups with 80 mL of a mixture of ethanolic ammonia [ammonia: ethanol (3:1)] for 6.5 h at 55°C. The bottle is cooled briefly on ice and then the ethanolic ammonia mixture is filtered into a new 250-mL bottle. The CPG is washed with 2 x 40 mL portions of ethanol/water (1:1 v/v). The volume of the mixture is then reduced to ~ 30 mL by roto-vap. The mixture is then frozen on dry ice and dried under vacuum on a speed vac.

Deprotection II (Removal of 2'-TBDMS group)

The dried residue is resuspended in 26 mL of triethylamine, triethylamine trihydrofluoride (TEA•3HF) or pyridine-HF and DMSO (3:4:6) and heated at 60oC for 90 minutes to remove the tert-butyldimethylsilyl (TBDMS) groups at the 2' position. The reaction is then quenched with 50 mL of 20 mM sodium acetate and the pH is adjusted to 6.5. Oligonucleotide is stored in a freezer until purification.

Analysis

The oligonucleotides are analyzed by high-performance liquid chromatography (HPLC) prior to purification and selection of buffer and column depends on nature of the sequence and or conjugated ligand.

HPLC Purification

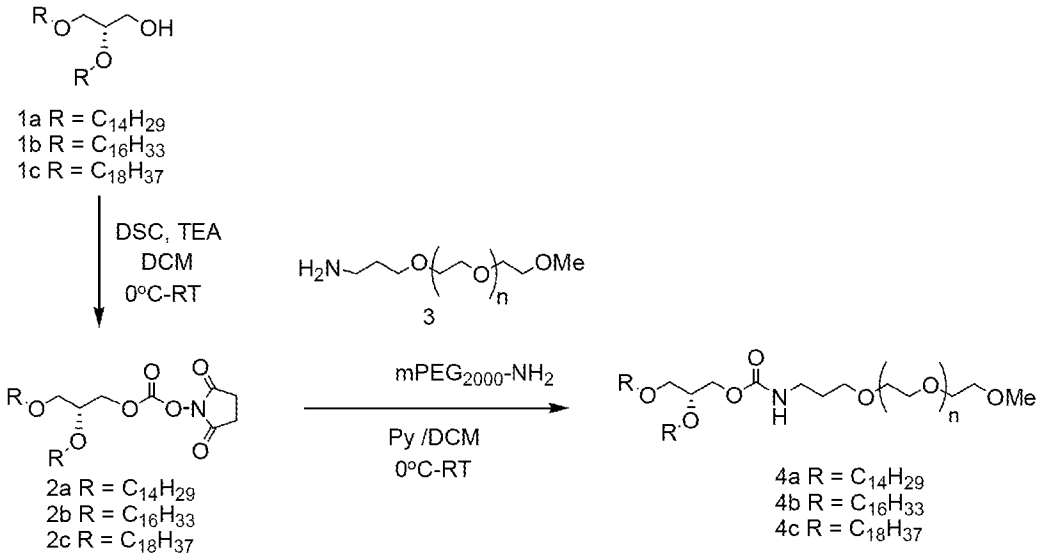
The ligand-conjugated oligonucleotides are purified by reverse-phase preparative HPLC. The unconjugated oligonucleotides are purified by anion-exchange HPLC on a TSK gel column packed in house. The buffers are 20 mM sodium phosphate (pH 8.5) in 10% CH_3CN (buffer A) and 20 mM sodium phosphate (pH 8.5) in 10% CH_3CN, 1M NaBr (buffer B). Fractions containing full-length oligonucleotides are pooled, desalted, and lyophilized. Approximately 0.15 OD of desalted oligonucleotides are diluted in water to 150 µL and then pipetted into special vials for CGE and LC/MS analysis. Compounds are then analyzed by LC-ESMS and CGE.

siRNA preparation

For the preparation of siRNA, equimolar amounts of sense and antisense strand are heated in 1xPBS at 95°C for 5 min and slowly cooled to room temperature. Integrity of the duplex is confirmed by HPLC analysis. AD-3133 and AD-AD-12115, described herein are synthesized.

Example 15: Synthesis of conjugated lipids:

The PEG-lipids, such as mPEG2000-1,2-Di-*O*-alkyl-*sn*3-carbomoylglyceride (PEG-DMG) were synthesized using the following procedures:



mPEG2000-1,2-Di-*O*-alkyl-*sn*3-carbomoylglyceride

Preparation of compound 4a: 1,2-Di-*O*-tetradecyl-*sn*-glyceride **1a** (30 g, 61.80 mmol) and *N,N'*-succinimidylcarboante (DSC, 23.76 g, 1.5eq) were taken in dichloromethane (DCM, 500 mL) and stirred over an ice water mixture. Triethylamine (25.30 mL, 3eq) was added to stirring solution and subsequently the reaction mixture was allowed to stir overnight at ambient temperature. Progress of the reaction was monitored by TLC. The reaction mixture was diluted with DCM (400 mL) and the organic layer was washed with water (2X500 mL), aqueous $NaHCO_3$ solution (500 mL) followed by standard work-up. Residue obtained was dried at ambient temperature under high vacuum overnight. After drying the crude carbonate 2a thus obtained was dissolved in dichloromethane (500 mL) and stirred over an ice bath. To the stirring solution $mPEG_{2000}$-NH_2 (**3,** 103.00 g, 47.20 mmol, purchased from NOF Corporation, Japan) and anhydrous pyridine (80 mL, excess) were added under argon. In some embodiments, the methoxy-(PEG)x-amine has an x= from 45-49, preferably 47-49, and more preferably 49. The reaction mixture was then allowed stir at ambient temperature overnight. Solvents and volatiles were removed under vacuum and the residue was dissolved in DCM (200 mL) and charged on a column of silica gel packed in ethyl acetate. The column was initially eluted with ethyl acetate

and subsequently with gradient of 5-10 % methanol in dichloromethane to afford the desired PEG-Lipid 4a as a white solid (105.30g, 83%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 5.20-5.12(m, 1H), 4.18-4.01(m, 2H), 3.80-3.70(m, 2H), 3.70-3.20(m, -O-CH_2-CH_2-O-, PEG-CH_2), 2.10-2.01(m, 2H), 1.70-1.60 (m, 2H), 1.56-1.45(m, 4H), 1.31-1.15(m, 48H), 0.84(t, J= 6.5Hz, 6H). MS range found: 2660-2836.

Preparation of 4b: 1,2-Di-*O*-hexadecyl-*sn*-glyceride **1b** (1.00 g, 1.848 mmol) and DSC (0.710 g, 1.5eq) were taken together in dichloromethane (20 mL) and cooled down to 0°C in an ice water mixture. Triethylamine (1.00 mL, 3eq) was added to that and stirred overnight. The reaction was followed by TLC, diluted with DCM, washed with water (2 times), $NaHCO_3$ solution and dried over sodium sulfate. Solvents were removed under reduced pressure and the residue **2b** under high vacuum overnight. This compound was directly used for the next reaction without further purification. $MPEG_{2000}$-NH_2 **3** (1.50g, 0.687 mmol, purchased from NOF Corporation, Japan) and compound from previous step **2b** (0.702g, 1.5eq) were dissolved in dichloromethane (20 mL) under argon. The reaction was cooled to 0°C. Pyridine (1 mL, excess) was added to that and stirred overnight. The reaction was monitored by TLC. Solvents and volatiles were removed under vacuum and the residue was purified by chromatography (first Ethyl acetate then 5-10% MeOH/DCM as a gradient elution) to get the required compound **4b** as white solid (1.46 g, 76 %). ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 5.17(t, J= 5.5Hz, 1H), 4.13(dd, J= 4.00Hz, 11.00 Hz, 1H), 4.05(dd, J= 5.00Hz, 11.00 Hz, 1H), 3.82-3.75(m, 2H), 3.70-3.20(m, -O-CH_2-CH_2-O-, PEG-CH_2), 2.05-1.90(m, 2H), 1.80-1.70 (m, 2H), 1.61-1.45(m, 6H), 1.35-1.17(m, 56H), 0.85(t, J= 6.5Hz, 6H). MS range found: 2716-2892.

Preparation of 4c: 1,2-Di-*O*-octadecyl-*sn*-glyceride **1c** (4.00 g, 6.70 mmol) and DSC (2.58 g, 1.5eq) were taken together in dichloromethane (60 mL) and cooled down to 0°C in an ice water mixture. Triethylamine (2.75 mL, 3eq) was added to that and stirred overnight. The reaction was followed by TLC, diluted with DCM, washed with water (2 times), $NaHCO_3$ solution and dried over sodium sulfate. Solvents were removed under reduced pressure and the residue under high vacuum overnight. This compound was directly used for the next reaction with further purification. $MPEG_{2000}$-NH_2 **3** (1.50g, 0.687 mmol, purchased from NOF Corporation, Japan) and compound from previous step **2c** (0.760g, 1.5eq) were dissolved in dichloromethane (20 mL) under argon. The reaction was cooled to 0°C. Pyridine (1 mL, excess) was added to that and stirred overnight. The reaction was monitored by TLC. Solvents and volatiles were removed under vacuum and the residue was purified by chromatography (first Ethyl acetate then 5-10% MeOH/DCM as a gradient elution) to get the required compound **4c** as white solid (0.92 g, 48 %). ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 5.22-5.15(m, 1H), 4.16(dd, J=

4.00Hz, 11.00 Hz, 1H), 4.06 (dd, J= 5.00Hz, 11.00 Hz, 1H), 3.81-3.75(m, 2H), 3.70-3.20(m, -O-CH_2-CH_2-O-, PEG-CH_2), 1.80-1.70 (m, 2H), 1.60-1.48(m, 4H), 1.31-1.15(m, 64H), 0.85(t, J= 6.5Hz, 6H). MS range found: 2774-2948.

Example 16: General protocol for the extrusion method

Lipids (e.g., Lipid A, DSPC, cholesterol, DMG-PEG) are solubilized and mixed in ethanol according to the desired molar ratio. Liposomes are formed by an ethanol injection method where mixed lipids are added to sodium acetate buffer at pH 5.2. This results in the spontaneous formation of liposomes in 35 % ethanol. The liposomes are extruded through a 0.08 μm polycarbonate membrane at least 2 times. A stock siRNA solution is prepared in sodium acetate and 35% ethanol and is added to the liposome to load. The siRNA-liposome solution is incubated at 37°C for 30 min and, subsequently, diluted. Ethanol is removed and exchanged to PBS buffer by dialysis or tangential flow filtration.

Example 17: General protocol for the in-line mixing method

Individual and separate stock solutions are prepared – one containing lipid and the other siRNA. Lipid stock containing, e.g., lipid A, DSPC, cholesterol and PEG lipid is prepared by solubilized in 90% ethanol. The remaining 10% is low pH citrate buffer. The concentration of the lipid stock is 4 mg/mL. The pH of this citrate buffer can range between pH 3-5, depending on the type of fusogenic lipid employed. The siRNA is also solubilized in citrate buffer at a concentration of 4 mg/mL. For small scale, 5 mL of each stock solution is prepared.

Stock solutions are completely clear and lipids must be completely solubilized before combining with siRNA. Therefore stock solutions may be heated to completely solubilize the lipids. The siRNAs used in the process may be unmodified oligonucleotides or modified and may be conjugated with lipophilic moieties such as cholesterol.

The individual stocks are combined by pumping each solution to a T-junction. A dual-head Watson-Marlow pump is used to simultaneously control the start and stop of the two streams. A 1.6 mm polypropylene tubing is further downsized to a 0.8 mm tubing in order to increase the linear flow rate. The polypropylene line (ID = 0.8 mm) are attached to either side of a T-junction. The polypropylene T has a linear edge of 1.6 mm for a resultant volume of 4.1 mm^3. Each of the large ends (1.6 mm) of polypropylene line is placed into test tubes containing either solubilized lipid stock or solubilized siRNA. After the T-junction a single tubing is placed where the combined stream will emit. The tubing is then extending into a container with 2× volume of PBS. The PBS is rapidly stirring. The flow rate for the pump is at a setting of 300 rpm or 110 mL/min. Ethanol is removed and exchanged for PBS by dialysis. The lipid

formulations are then concentrated using centrifugation or diafiltration to an appropriate working concentration.

FIG. 17 shows a schematic of the in-line mixing method.

Example 18: siRNA silencing by LNP-08 formulated VSP in intrahepatic Hep3B tumors in mice.

Silencing of VSP (VEGF and KSP) was performed in orthotopic (intrahepatic) Hep3B tumors following intravenous administration of siRNAs formulated in XTC containing nucleic acid-lipid particles, e.g., LNP-08.

Tumors were established by implantation of $1X10^6$ Hep3B cells into the right flank of 8 week-old female Fox scid/beige mice. The cells were engineered to stably express firefly Luciferase. Tumor burden was monitored weekly by in vivo biophotonic imaging using the IVIS system (Caliper, Inc.). Approximately 4 weeks after tumor implantation, cohorts of tumor-bearing animals received intravenous (tail vein) injections of test article as follows:

Group	Test article	Dose (siRNA)	n
1	LNP08-1955	4 mg/kg	5
2	LNP08-VSP	4 mg/kg	5

LNP08-1955 is siRNA AD-1955 (targeting firefly Luciferase) formulated in lipid nanoparticles comprising XTC (60 mol%), DSPC (7.5 mol%), Cholesterol (31 mol%) and PEG-cDMG (1.5 mol%) at an N:P ratio of approximately 3.0.

LNP08-VSP is siRNAs AD-12115 (targeting KSP) and AD-3133 (targeting VEGF) in a 1:1 molar ratio formulated in lipid nanoparticles comprising XTC (60 mol%), DSPC (7.5 mol%), Cholesterol (31 mol%) and PEG-cDMG (1.5 mol%) at an N:P ratio of approximately 3.0.

One day following treatment, animals were sacrificed and tumor-bearing liver lobes collected for analysis. Total RNA was extracted followed by cDNA synthesis by random priming. Levels of human KSP and human VEGF, normalized to human GAPDH, were measured using human-specific custom Taqman® assays (Applied Biosystems, Inc.). Group averages were calculated and normalized to the LNP08-1955 treatment group.

As shown in FIG. 18, treatment with LNP08-VSP (Group 2) resulted in a greater than 60%, e.g., 68% reduction in tumor KSP mRNA ($p<0.001$) and at least 40% reduction in VEGF mRNA ($p<0.05$) relative to the LNP08-1955 treatment (Group 1).

Example 19: Evaluation of LNP-011 and LNP-012 lipid formulations in the mouse Hep3b tumor model

The effects of various VSP formulations on KSP and VEGF expression in intrahepatic Hep3B tumors in mice were compared. Thirty five female Fox Scid beige mice were injected

with 1X10^6 Hep3B-Luc cells suspeneded in 0.025 cc PBS via direct intrahepatic surgery. Tumor growth was monitered via Luc readings by Xenogen.

Mice received a single bolus dose (4 mg/kg) of one of the following: SNALP-1955 (luciferase control); ALN-VSP02; SNALP-T-VSP (with C-18 PEG)-VSP; LNP-11-VSP, and LNP-12 VSP. Animal were euthanized at 24 hours post does, and the TaqMan protocol was used for detection of tumor specific KSP and VEGF knockdown.

The results are shown in FIG. 21. SNAPL-T-VSP; LNP-11-VSP, and LNP-12 VSP demonstrated increased knockdown of KSP expression compared to ALN-VSP02.

Example 20: Evaluation of LNP-08 +/- C18 lipid formulations in the mouse Hep3b tumor model

The effects of the following VSP formulations were tested in a HEP3B tumor model. Tumor-bearing (intrahepatic) mice were injected with one of the following formulations, prepared and administered as a single bolus IV dose according to protocols described above:

Group	Test article	Dose (siRNA)	n
1	ALN-VSP02	4 mg/kg	6
2	LNP08-Luc	4 mg/kg	4
3	LNP08-VSP	4 mg/kg	7
4	LNP08-VSP	1 mg/kg	7
5	LNP08-VSP	0.25 mg/kg	7
6	LNP08-C18-VSP	4 mg/kg	7
7	LNP08-C18-VSP	1 mg/kg	7
8	LNP08-C18-VSP	0.25 mg/kg	7

Formulation of ALN-VSP02 was as described in Example 9.

LNP08-Luc is siRNA AD-1955 (targeting firefly Luciferase) formulated in lipid nanoparticles comprising XTC (60 mol%), DSPC (7.5 mol%), Cholesterol (31 mol%) and PEG-cDMG (1.5 mol%) at an N:P ratio of approximately 3.0.

LNP08-VSP is siRNA AD-12115 (targeting KSP) and AD-3133 (targeting VEGF) in a 1:1 molar ratio formulated in lipid nanoparticles comprising XTC (60 mol%), DSPC (7.5 mol%), Cholesterol (31 mol%) and PEG-cDMG (1.5 mol%) at an N:P ratio of approximately 3.0.

LNP08-C18-VSP is siRNA AD-12115 (targeting KSP) and AD-3133 (targeting VEGF) in a 1:1 molar ratio formulated in lipid nanoparticles comprising XTC (60 mol%), DSPC (7.5 mol%), Cholesterol (31 mol%) and PEG-cDSG (1.5 mol%) at an N:P ratio of approximately 3.0.

FIG. 19 illustrates the chemical structures of PEG-DSG and PEG-C-DSA. PEG-DSG is polyethylene glycol distyryl glycerol, in which PEG is either C18-PEG or PEG-C18 and the PEG has an average molecular weight of 2000 Da.

Twenty-four hours following treatment, animals were sacrificed and tumors collected for analysis. Total RNA was extracted from tumors, followed by cDNA synthesis by random priming. Levels of human KSP and human VEGF, normalized to human GAPDH, were measured using human-specific custom Taqman® assays (Applied Biosystems, Inc.).

The results are shown the graphs in FIG. 22 and show KSP and VEGF silencing comparable to silencing by ALN-VSP02.

Example 21: Role of ApoE in the Cellular Uptake of Liposomes in HeLa Cells

LNP formulated dsRNAs are prepared with the addition of recombinant human ApoE. The resulting LNP-ApoE formulated dsRNA are tested in HeLa cells for the effect on uptake of the dsRNA by the cells. Compositions and methods utilizing ApoE in conjunction with ionizable lipids is described in International patent application No., PCT/US10/22614, which is herein incorporated by reference in its entirety.

Experimental protocol:

HeLa cells are seeded in 96 well plates (Grenier) at 6000 cells per well overnight. Three different liposome formulations of Alexa-fluor 647 labeled GFP siRNA: 1) LNP01, 2) SNALP, 3) LNP05 are diluted in one of 3 media conditions to a 50nM final concentration. Media conditions examined are OptiMem, DMEM with 10% FBS or DMEM with 10% FBS plus 10ug/mL of human recombinant ApoE (Fitzgerald Industries). The indicated liposomes either in media or in media-precomplexed with ApoE for 10 minutes are added to cells for either 4, 6, or 24 hours. Three replicated are performed for each experimental condition. After addition to HeLa cells in plates for indicated time points cells are fixed in 4% paraformaldehyde for 15 minutes then nuclei and cytoplasm stained with DAPI and Syto dye. Images are acquired using an Opera spinning disc automated confocal system from Perkin Elmer. Quantitation of Alexa Fluor 647 siRNA uptake is performed using Acapella software. Four different parameters are quantified: 1) Cell number, 2) the number of siRNA positive spots per field, 3) the number of siRNA positive spots per cell and 4) the integrated spot signal or the average number of siRNA spots per cell times the average spot intensity. The average spot signal therefore is a rough estimate of the total amount of siRNA content per cell.

In addition, the 4 different LNP-ApoE formulated dsRNA are tested (SNALP (DLinDMa), XTC, MC3, ALNY-100) in the following cell lines and the effect on uptake of the dsRNA by the cells is determined:

A375 (melanoma), B16F10 (melanoma), BT-474 (breast), GTL-16 (gastric carcinoma), Hct116 (colon), Hep3b (Hepatic), HepG2 (liver), HeLa (cervical), HUH 7 (liver), MCF7 (breast) , Mel-285 (uveal melanoma), NCI-H1975 (lung), OMM-1.3 (uveal melanoma), PC3 (prostate), SKOV-3 (ovarian), U87 (glioblastoma).

Example 22: K_d of KSP siRNA in the presence of ApoE.

The effect of ApoE on the Kd (affinity) of LNP-08 formulated siRNA targeting KSP was evaluated in multiple cell lines. Both LNP08 and LNP08 with C18PEG formulated siRNA were used. The KSP targeted siRNA duplex was AL-DP-6248.

position in human Eg5/KSP sequence	SEQ ID NO:	sense sequence (5′-3′)	SEQ ID No:	antisense sequence (5′-3′)	duplex name
383-405	45	AccGAAGuGuuGuuuGuccTsT	46	GGAcAAAcAAcACUUCGGUTsT	AL-DP-6248

The following cell lines were used.

Cell Line	Cell Type	Species
HeLa	**Cervical Adenocarcinoma**	**Human**
HCT116	**Colorectal carcinoma**	**Human**
A375	**Melanoma**	**Human**
MCF7	**Breast adenocarcinoma**	**Human**
B16F10	**Melanoma**	**Mouse**
Hep3b	**Hepatic**	**Human**
HUH 7	**Hepatic**	**Human**
HepG2	**Hepatic**	**Human**
Skov 3	**Ovarian**	**Human**
U87	**Glioblastoma**	**Human**
PC3	**Prostate**	**Human**

On day 1, cells were plated in 96 well plates at 20,000 cells/well. On day 2, formulated siRNA were incubated with serum-containing media +/- ApoE at 37°C for 15-30 minutes. Media was removed from cells and pre-warmed complexes were layered on the cells at 100uL/well at an siRNA concentration of 20nM. ApoE concentration was titrated at 1.0, 3.0, 9.0, and 20.0 µg/ml. Cells were incubated with formulated duplexes for 24 hours. At day 3, cells lysed and prepared for bDNA analysis and kD calculations.

The presence of Apo E improved kD in a number of cell lines including HCT-116, HeLa, A375, and B16F10 (data not shown).

Example 23: IC_{50} of KSP siRNA in the presence of ApoE.

The effect of ApoE on the IC_{50} (efficacy) of LNP-08 formulated siRNA targeting KSP was evaluated in multiple cell lines. Both LNP08 and LNP08 with C18PEG formulated siRNA were used. The KSP targeted siRNA duplex was AL-DP-6248.

At day 0, cells were plated at 15,000-20,000 per well in 96 well plates. At day 1, serum-containing media, formulated duplex, and +/- 3ug/ml ApoE were incubated at 37°C for 15-30 minutes. Serial dilutions of siRNA were used in the 0.01 nM to 1.0 μM range. Media was removed from cells and pre-warmed complexes were layered on cells at 100uL/well. Cells were incubated with siRNA for 24 hours. At day 2, cells were lysed and prepared for bDNA analysis as described herein. KSP mRNA levels were determined using a Quantigene 1.0 to determine KSP levels in comparison to GAPDH. Negative control was luciferase targeted siRNA, AD-1955.

The results are shown in the table below. LNP-08 formulated siRNA was active in all cell lines. In some cell lines the addition of ApoE improved efficacy of siRNA treatment as demonstrated by a lower IC_{50}.

			IC_{50}			
Cell Line	Cell Type	Species	LNP08 C18	LNP08 C18 + 3ug/mL ApoE	LNP08	LNP08 + 3ug/mL ApoE
HeLa	Cervical Adenocarcinoma	Human	7.02	3.51	2.75	2.02
HCT116	Colorectal carcinoma	Human	4.71	3.89	0.4	0.44
A375	Melanoma	Human	>500	24.82	7.08	0.94
MCF7	Breast adenocarcinoma	Human	>500	>500	19.98	10.26
B16F10	Melanoma	Mouse	13.92	>500	18.52	2.37
Hep3b	Hepatic	Human	60.47*/NA	22.13 */>600	1.4	8.98
HUH 7	Hepatic	Human	NA	>600	14.26	1.8
HepG2	Hepatic	Human	433nM	67.3(1ug/ml) /0.45(3ug/ml)	1.27	0.38
Skov 3	Ovarian	Human	NA	NA	3.95	7.26
U87	Glioblastoma	Human	NA	NA	464.74	283.68
PC3	Prostate	Human	NA	>600	96.62	59

Example 24. Inhibition of Eg5/KSP and VEGF expression in humans

A human subject is treated with a pharmaceutical composition, e.g., a nucleic acid-lipid particle having both a dsRNA targeted to a Eg5/KSP gene and a dsRNA targeted to a VEGF gene to inhibit expression of the Eg5/KSP and VEGF genes in a nucleic acid-lipid particle. The nucleic acid-lipid particle comprises, e.g., XTC, MC3, or ALNY-100.

A subject in need of treatment is selected or identified. The subject can be in need of cancer treatment, *e.g.*, liver cancer.

At time zero, a suitable first dose of the composition is subcutaneously administered to the subject. The composition is formulated as described herein. After a period of time, the subject's condition is evaluated, *e.g.*, by measurement of tumor growth, measuring serum AFP levels, and the like. This measurement can be accompanied by a measurement of Eg5/KSP and/or VEGF expression in said subject, and/or the products of the successful siRNA-targeting of Eg5/KSP and/or VEGF mRNA. Other relevant criteria can also be measured. The number and strength of doses are adjusted according to the subject's needs.

After treatment, the subject's condition is compared to the condition existing prior to the treatment, or relative to the condition of a similarly afflicted but untreated subject.

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

CLAIMS

We claim:

1. A composition comprising a nucleic acid lipid particle comprising a first double-stranded ribonucleic acid (dsRNA) for inhibiting the expression of a human kinesin family member 11 (Eg5/KSP) gene in a cell and a second dsRNA for inhibiting expression of a human VEGF in a cell, wherein:

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

the first dsRNA consists of a first sense strand and a first antisense strand, and the first sense strand comprises a first sequence and the first antisense strand comprises a second sequence complementary to at least 15 contiguous nucleotides of

SEQ ID NO:1311 (5'-UCGAGAAUCUAAACUAACU-3'),

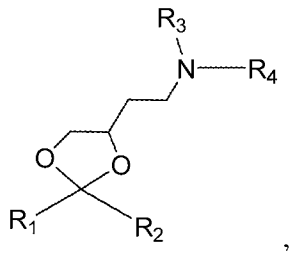
wherein the first sequence is complementary to the second sequence and wherein the first dsRNA is between 15 and 30 base pairs in length; and

the second dsRNA consists of a second sense strand and a second antisense strand, the second sense strand comprising a third sequence and the second antisense strand comprising a fourth sequence complementary to at least 15 contiguous nucleotides of

SEQ ID NO:1538 (5'-GCACAUAGGAGAGAUGAGCUU-3'),

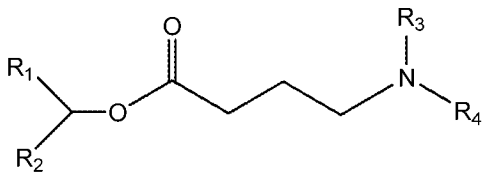
wherein the third sequence is complementary to the fourth sequence and wherein the second dsRNA is between 15 and 30 base pairs in length.

2. The composition of claim 1, wherein the cationic lipid comprises formula A wherein formula A is

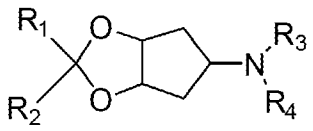


,

or



, or



where R1 and R2 are independently alkyl, alkenyl or alkynyl, each can be optionally substituted, and R3 and R4 are independently lower alkyl or R3 and R4 can be taken together to form an optionally substituted heterocyclic ring.

3. The composition of claim 2, wherein the cationic lipid comprises XTC (2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane).

4. The composition of claim 2, wherein the cationic lipid comprises XTC, the non-cationic lipid comprises DSPC, the sterol comprises cholesterol and the PEG lipid comprises PEG-DMG.

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

LNP05	XTC/DSPC/Cholesterol/PEG-DMG 57.5/7.5/31.5/3.5 lipid:siRNA ~ 6:1
LNP06	XTC/DSPC/Cholesterol/PEG-DMG 57.5/7.5/31.5/3.5 lipid:siRNA ~ 11:1
LNP07	XTC/DSPC/Cholesterol/PEG-DMG 60/7.5/31/1.5, lipid:siRNA ~ 6:1
LNP08	XTC/DSPC/Cholesterol/PEG-DMG 60/7.5/31/1.5, lipid:siRNA ~ 11:1
LNP09	XTC/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 lipid:siRNA ~ 10:1
LNP13	XTC/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 lipid:siRNA ~ 33:1
LNP22	XTC/DSPC/Cholesterol/PEG-DSG 50/10/38.5/1.5 lipid:siRNA ~10

6. The composition of claim 1, wherein the cationic lipid comprises ALNY-100 ((3aR,5s,6aS)-N,N-dimethyl-2,2-di((9Z,12Z)-octadeca-9,12-dienyl)tetrahydro-3aH-cyclopenta[d][1,3]dioxol-5-amine)).

7. The composition of claim 6, wherein the cationic lipid comprises ALNY-100 and the formulation consists of:

LNP10	ALNY-100/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 lipid:siRNA ~ 10:1

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

9. The composition of claim 8, wherein the cationic lipid comprises MC3 and the lipid formulation is selected from the group consisting of:

LNP11	MC3/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 lipid:siRNA ~ 10:1
LNP14	MC3/DSPC/Cholesterol/PEG-DMG 40/15/40/5 lipid:siRNA ~11
LNP15	MC3/DSPC/Cholesterol/PEG-DSG/GalNAc-PEG-DSG 50/10/35/4.5/0.5 lipid:siRNA ~11
LNP16	MC3/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 lipid:siRNA ~7
LNP17	MC3/DSPC/Cholesterol/PEG-DSG 50/10/38.5/1.5 lipid:siRNA ~10
LNP18	MC3/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 lipid:siRNA ~12
LNP19	MC3/DSPC/Cholesterol/PEG-DMG 50/10/35/5 lipid:siRNA ~8
LNP20	MC3/DSPC/Cholesterol/PEG-DPG 50/10/38.5/1.5 lipid:siRNA ~10

10. The composition of claim 1, wherein the first dsRNA consists of a sense strand consisting of SEQ ID NO:1534 (5'-UCGAGAAUCUAAACUAACUTT-3') and an antisense strand consisting of SEQ ID NO:1535 (5'-AGUUAGUUUAGAUUCCUGATT-3') and the second dsRNA consists of a sense strand consisting of SEQ ID NO:1536 (5'-GCACAUAGGAGAGAUGAGCUU-3'), and an antisense strand consisting of SEQ ID NO:1537 (5'-AAGCUCAUCUCUCCUAUGUGCUG-3').

11. The composition of claim 10, wherein each strand is modified as follows to include a 2'-O-methyl ribonucleotide as indicated by a lower case letter "c" or "u" and a phosphorothioate as indicated by a lower case letter "s":
the first dsRNA consists of a sense strand consisting of
SEQ ID NO:1240 (5'-ucGAGAAucuAAAcuAAcuTsT-3')
and an antisense strand consisting of
SEQ ID NO:1241 (5'-AGUuAGUUuAGAUUCUCGATsT);
the second dsRNA consists of a sense strand consisting of
SEQ ID NO:1242 (5'-GcAcAuAGGAGAGAuGAGCUsU-3')
and an antisense strand consisting of
SEQ ID NO:1243 (5'-AAGCUcAUCUCUCCuAuGuGCusG-3').

12. The composition of claim 1, wherein the first and second dsRNA comprises at least one modified nucleotide.

13. The composition of claim 12, wherein the modified nucleotide is chosen from the group of: a 2'-O-methyl modified nucleotide, a nucleotide comprising a 5'-phosphorothioate group, and a terminal nucleotide linked to a cholesteryl derivative or dodecanoic acid bisdecylamide group.

14. The composition of claim 12, wherein the modified nucleotide is chosen from the group of: a 2'-deoxy-2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an abasic nucleotide, 2'-amino-modified nucleotide, 2'-alkyl-modified nucleotide, morpholino nucleotide, a phosphoramidate, and a non-natural base comprising nucleotide.

15. The composition of claim 1, wherein the first and second dsRNA each comprise at least one 2'-O-methyl modified ribonucleotide and at least one nucleotide comprising a 5'-phosphorothioate group.

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

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

18. The composition of claim 1, wherein each strand of the first dsRNA is 21 bases in length and the sense strand of the second dsRNA is 21 bases in length and the antisense strand of the second dsRNA is 23 bases in length.

19. The composition of claim 1, wherein the first and second dsRNA are present in an equimolar ratio.

20. The composition of claim 1, further comprising Sorafenib.

21. The composition of claim 1, further comprising a lipoprotein.

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

23. The composition of claim 1, wherein the composition, upon contact with a cell expressing Eg5, inhibits expression of Eg5 by at least 40%.

24. The composition of claim 1, wherein the composition, upon contact with a cell expressing VEGF, inhibits expression of VEGF by at least 40%.

25. The composition of claim 1 wherein administration of the composition to a cell decreases expression of Eg5 and VEGF in the cell.

26. The composition of claim 25, wherein the composition is administered in a nM concentration.

27. The composition of claim 1, wherein administration of the composition to a cell increases monoaster formation in the cell.

28. The composition of claim 1, wherein administration of the composition to a mammal results in at least one effect selected from the group consisting of prevention of tumor growth, reduction in tumor growth, or prolonged survival in the mammal.

29. The composition of claim 28, wherein the effect is measured using at least one assay selected from the group consisting of determination of body weight, determination of organ weight, visual inspection, mRNA analysis, serum AFP analysis and survival monitoring.

30. A method for inhibiting the expression of Eg5/KSP and VEGF in a cell comprising administering the composition of claim 1 to the cell.

31. A method for preventing tumor growth, reducing tumor growth, or prolonging survival in a mammal in need of treatment for cancer comprising administering the composition of claim 1 to the mammal.

32. The method of claim 31, wherein the mammal has liver cancer.

33. The method of claim 31, wherein the mammal is a human with liver cancer.

34. The method of claim 31, wherein a dose containing between 0.25 mg/kg and 4 mg/kg dsRNA is administered to the mammal.

35. The method of claim 31, wherein the dsRNA is administered to a human at about 0.01, 0.1, 0.5, 1.0, 2.5, or 5.0 mg/kg.

36. A method for reducing tumor growth in a mammal in need of treatment for cancer comprising administering the composition of claim 1 to the mammal, the method reducing tumor growth by at least 20%.

37. The method of claim 36, wherein the method reduces KSP expression by at least 60%.

5

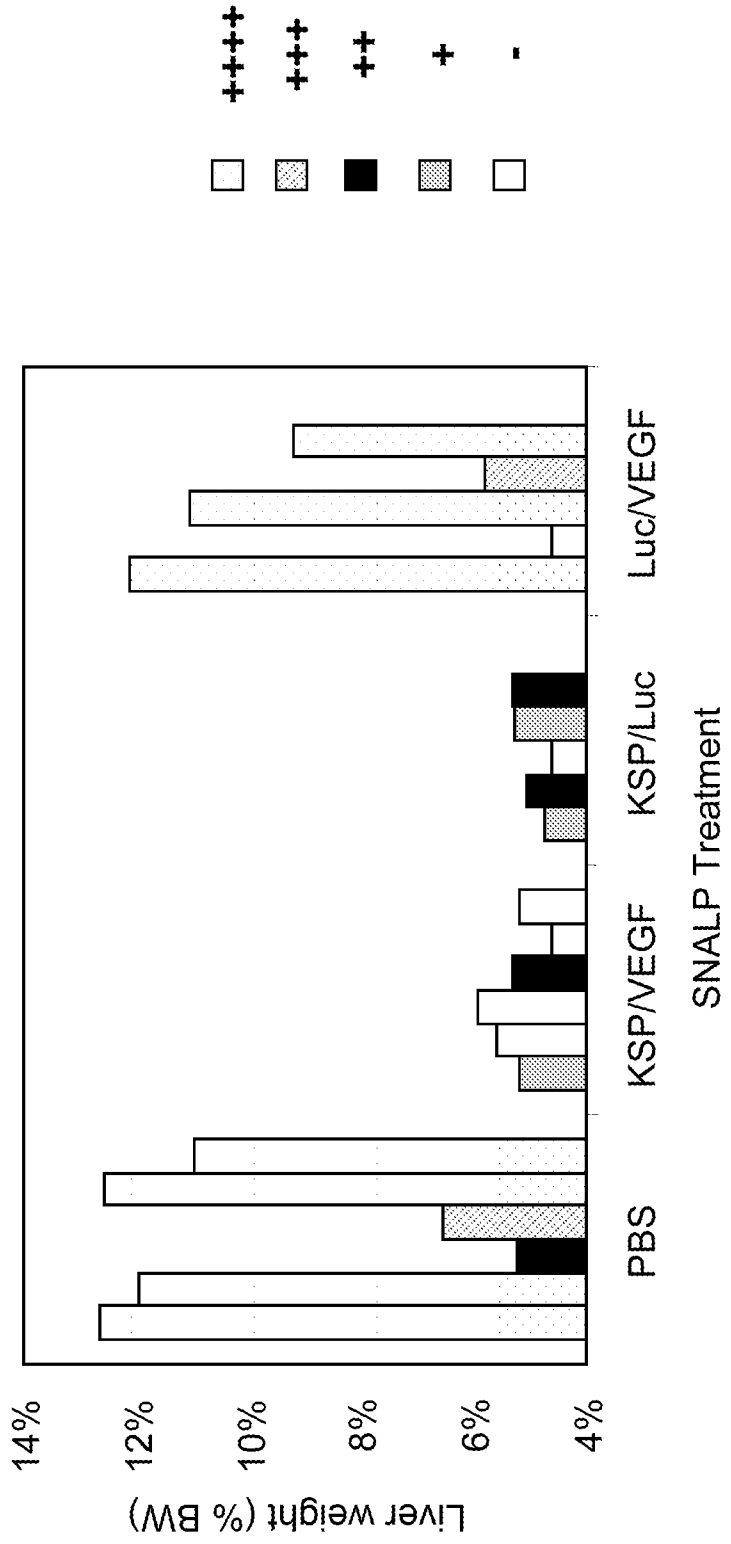

++++
+++
++
+
-
Liver weight (% BW)
14%
12%
10%
8%
6%
4%
PBS
KSP/VEGF
KSP/Luc
Luc/VEGF
SNALP Treatment

FIG. 1

FIG. 2A

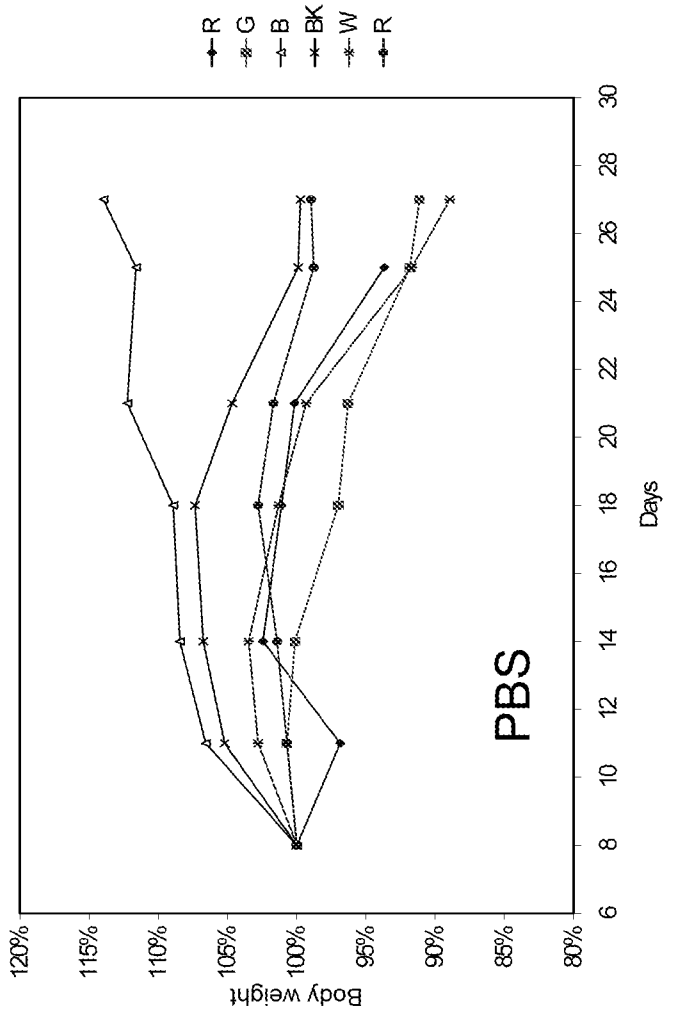
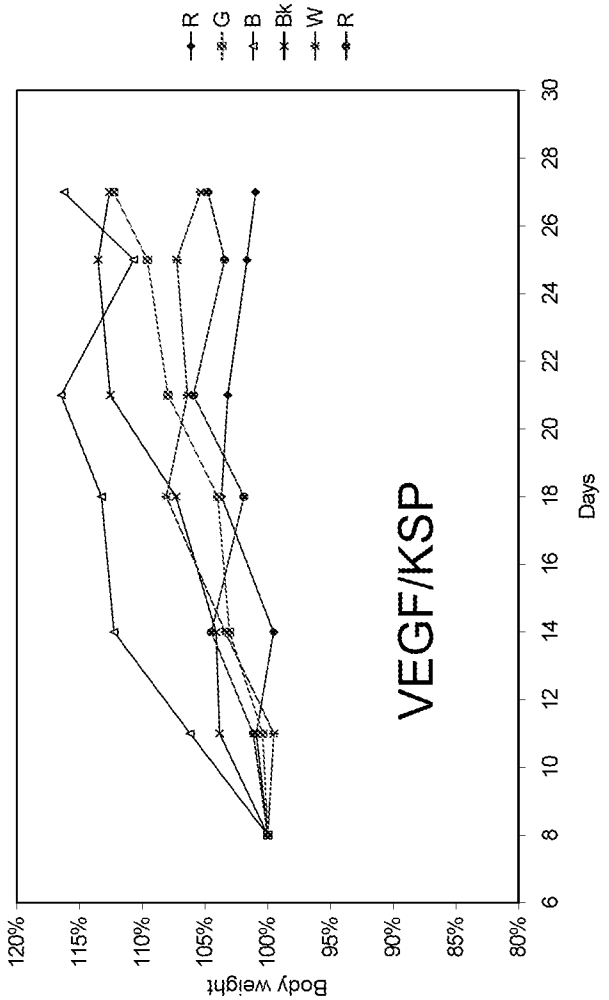

R
G
B
BK
W
R
PBS
Body weight
120%
115%
110%
105%
100%
95%
90%
85%
80%
6
8
10
12
14
16
18
20
22
24
26
28
30
Days

FIG. 2B


R
G
B
Bk
W
R
VEGF/KSP
Body weight
120%
115%
110%
105%
100%
95%
90%
85%
80%
6
8
10
12
14
16
18
20
22
24
26
28
30
Days

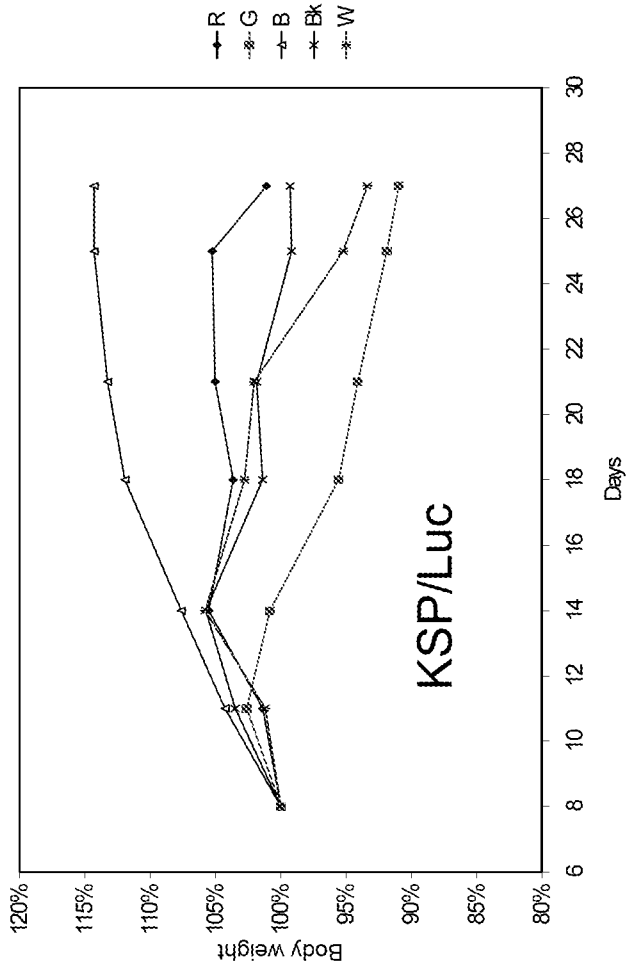

KSP/Luc
Body weight
Days
R
G
B
Bk
W
120%
115%
110%
105%
100%
95%
90%
85%
80%
6
8
10
12
14
16
18
20
22
24
26
28
30

FIG. 2C

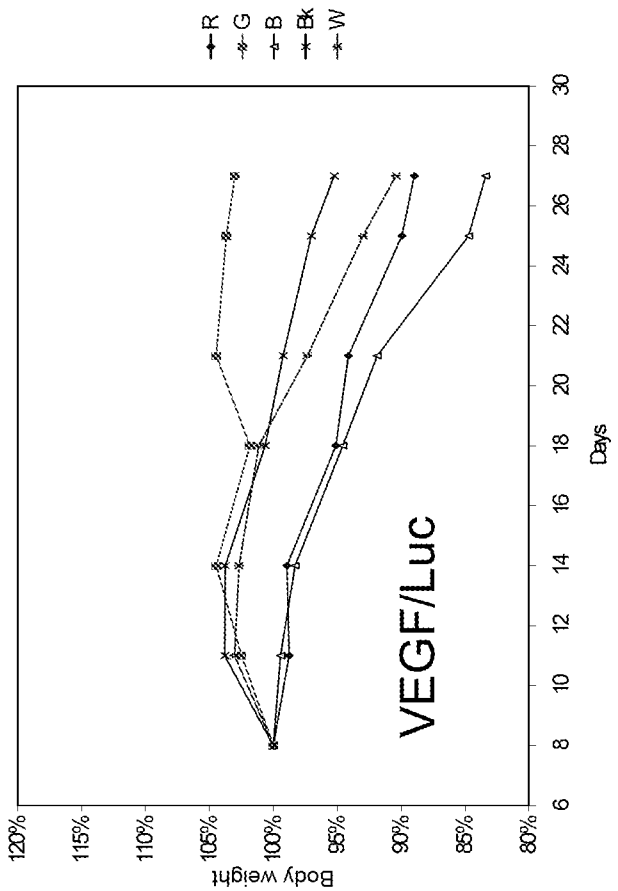

VEGF/Luc
Body weight
Days
R
G
B
Bk
W
120%
115%
110%
105%
100%
95%
90%
85%
80%
6
8
10
12
14
16
18
20
22
24
26
28
30

FIG. 2D

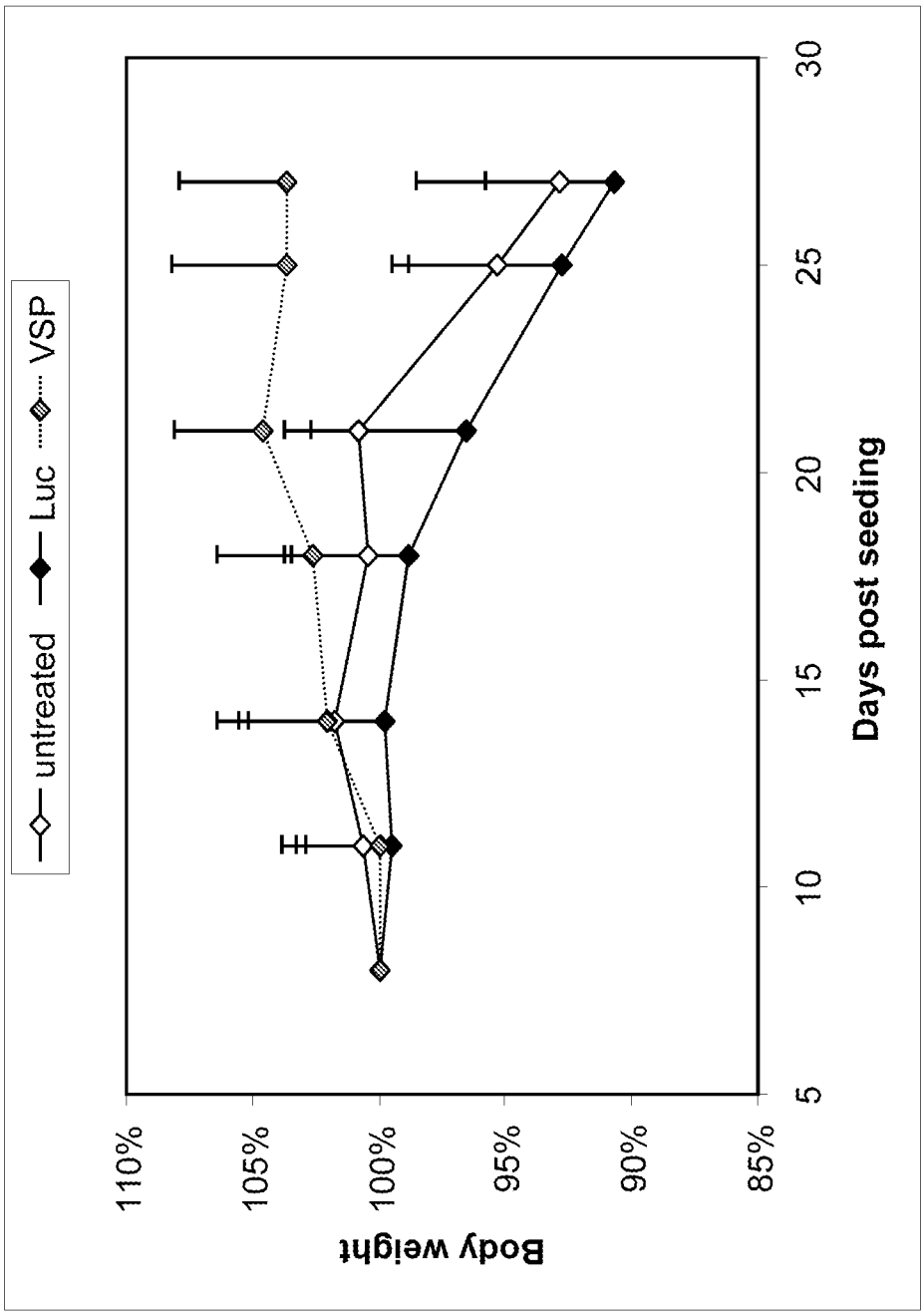

untreated
Luc
VSP
Body weight
110%
105%
100%
95%
90%
85%
5
10
15
20
25
30
Days post seeding

FIG. 3

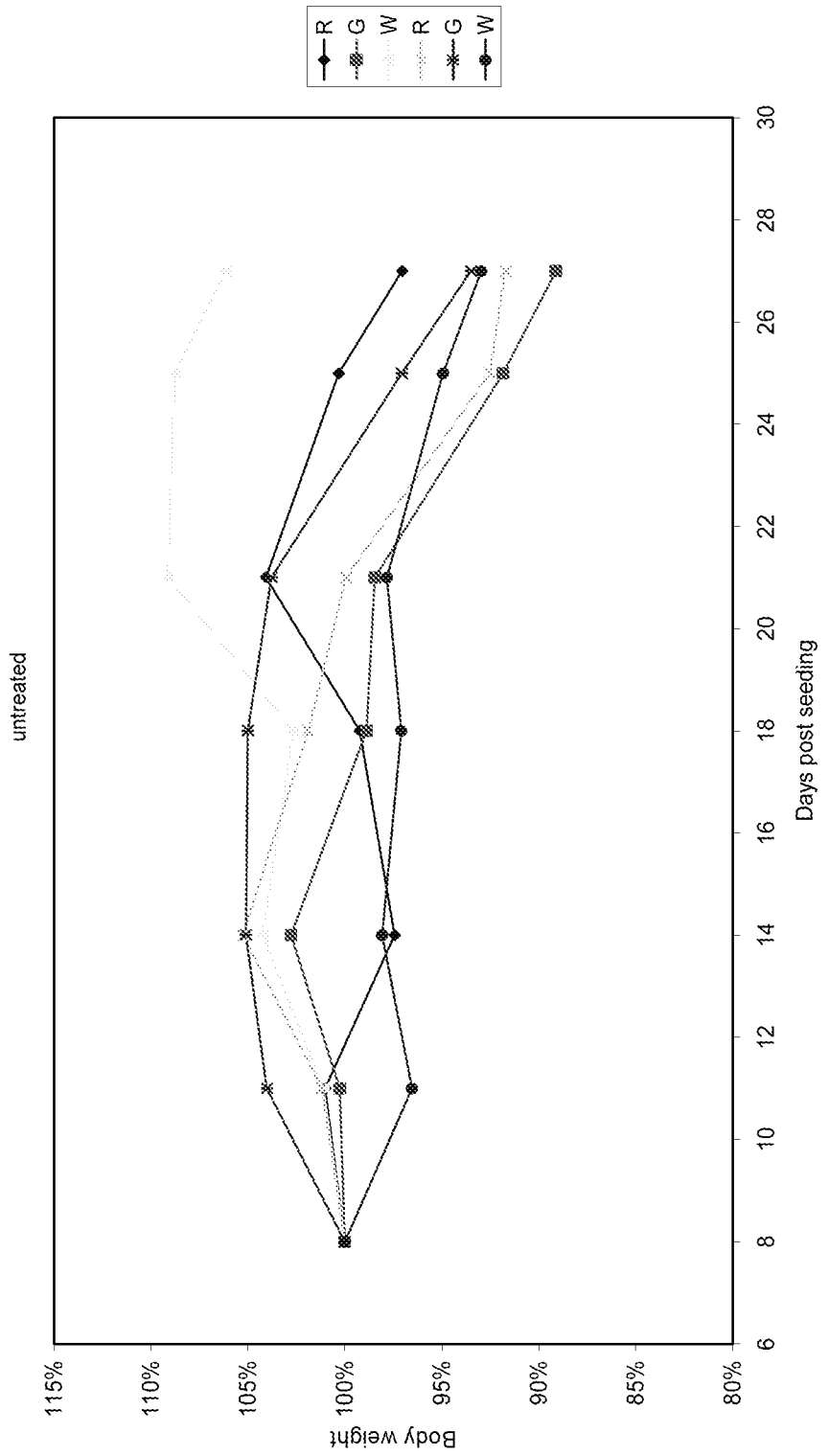

R
G
W
R
G
W
untreated
Body weight
115%
110%
105%
100%
95%
90%
85%
80%
6
8
10
12
14
16
18
20
22
24
26
28
30
Days post seeding

FIG. 4

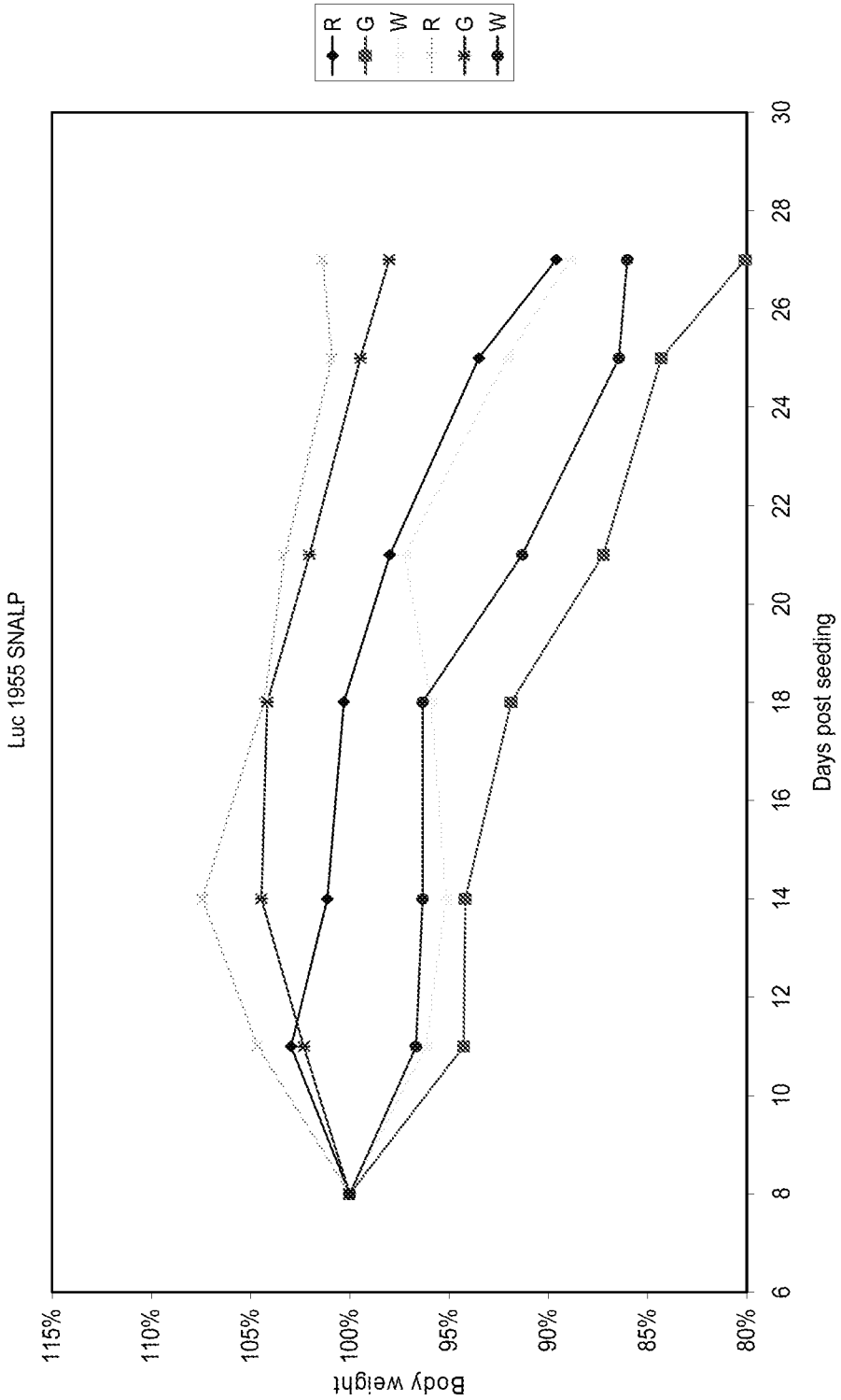

R
G
W
R
G
W
Luc 1955 SNALP
Body weight
115%
110%
105%
100%
95%
90%
85%
80%
6
8
10
12
14
16
18
20
22
24
26
28
30
Days post seeding

FIG. 5

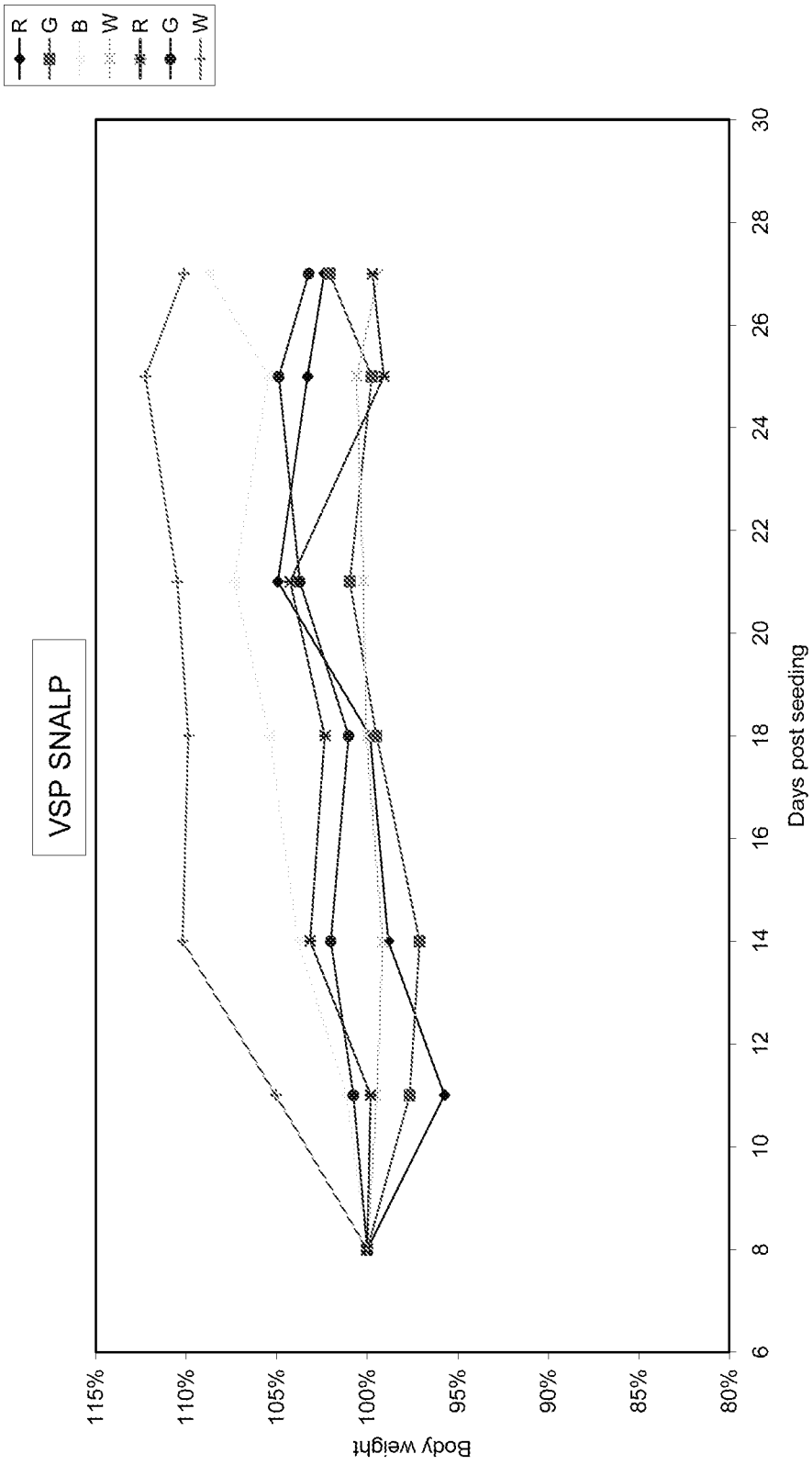

VSP SNALP
R
G
B
W
R
G
W
Body weight
115%
110%
105%
100%
95%
90%
85%
80%
6
8
10
12
14
16
18
20
22
24
26
28
30
Days post seeding

FIG. 6

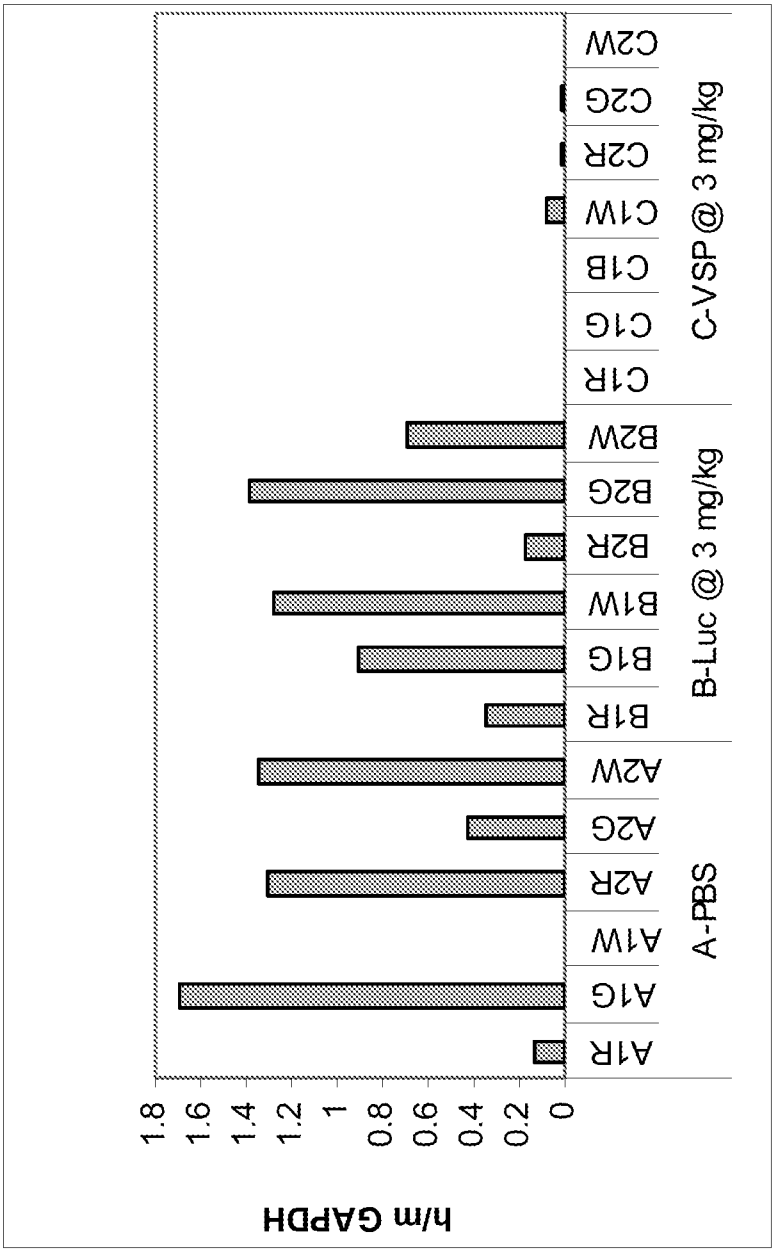

h/m GAPDH
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
A1R
A1G
A1W
A2R
A2G
A2W
B1R
B1G
B1W
B2R
B2G
B2W
C1R
C1G
C1B
C1W
C2R
C2G
C2W
A-PBS
B-Luc @ 3 mg/kg
C-VSP @ 3 mg/kg
Treatment in Hep3B mice

FIG. 7A

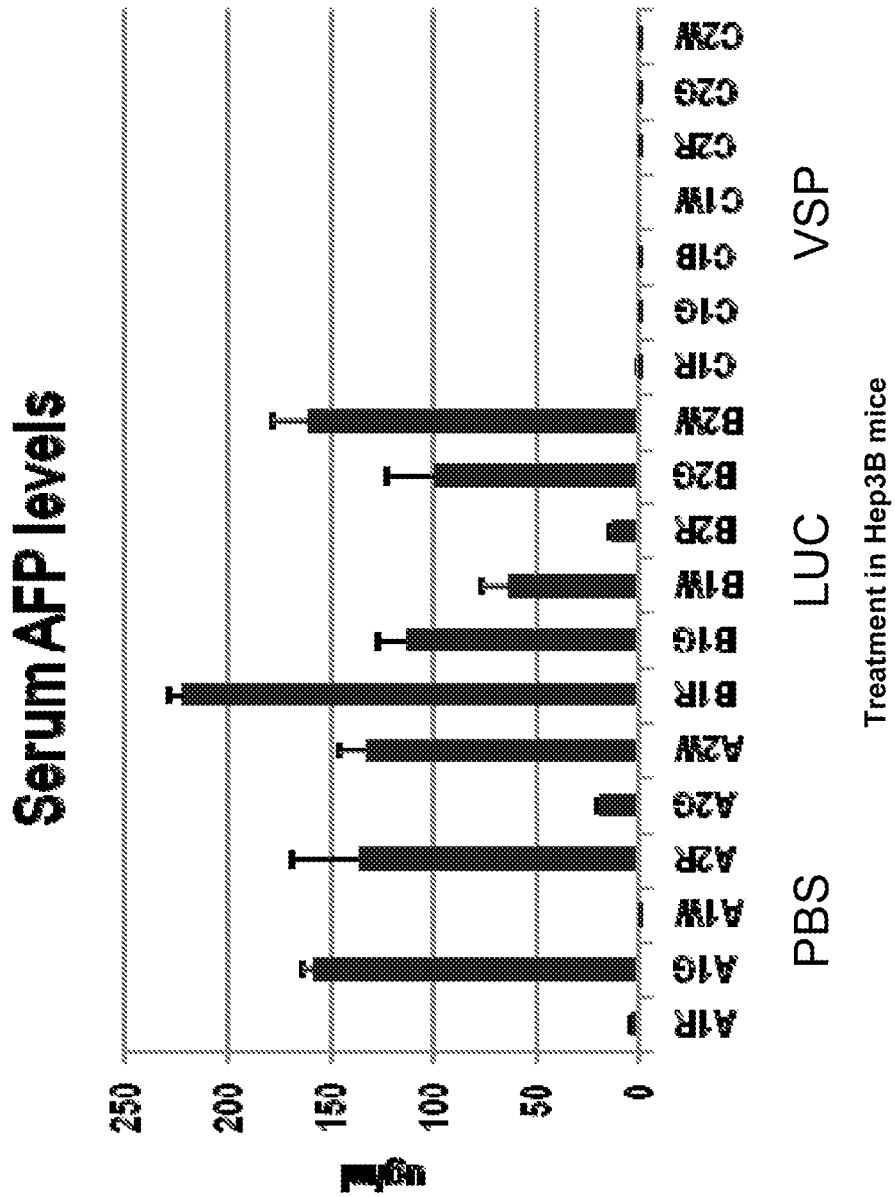

Serum AFP levels
250
200
150
100
50
0
A1R
A1G
A1W
A2R
A2G
A2W
B1R
B1G
B1W
B2R
B2G
B2W
C1R
C1G
C1B
C1W
C2R
C2G
C2W
PBS
LUC
VSP
Treatment in Hep3B mice

FIG. 7B

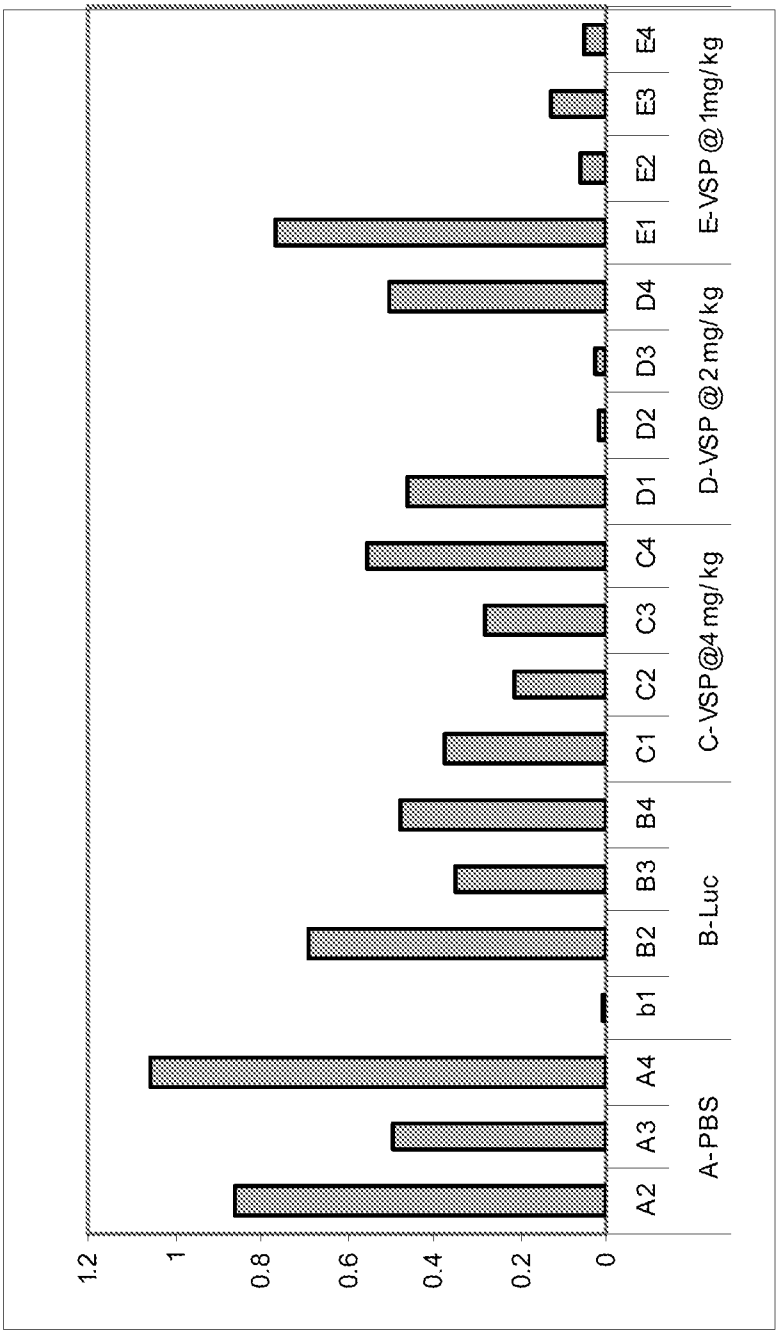

h/m GAPDH
1.2
1
0.8
0.6
0.4
0.2
0
A2
A3
A4
b1
B2
B3
B4
C1
C2
C3
C4
D1
D2
D3
D4
E1
E2
E3
E4
A-PBS
B-Luc
C-VSP@4 mg/kg
D-VSP @2 mg/kg
E-VSP @1mg/kg
Treatment in Hep3B mice

FIG. 8

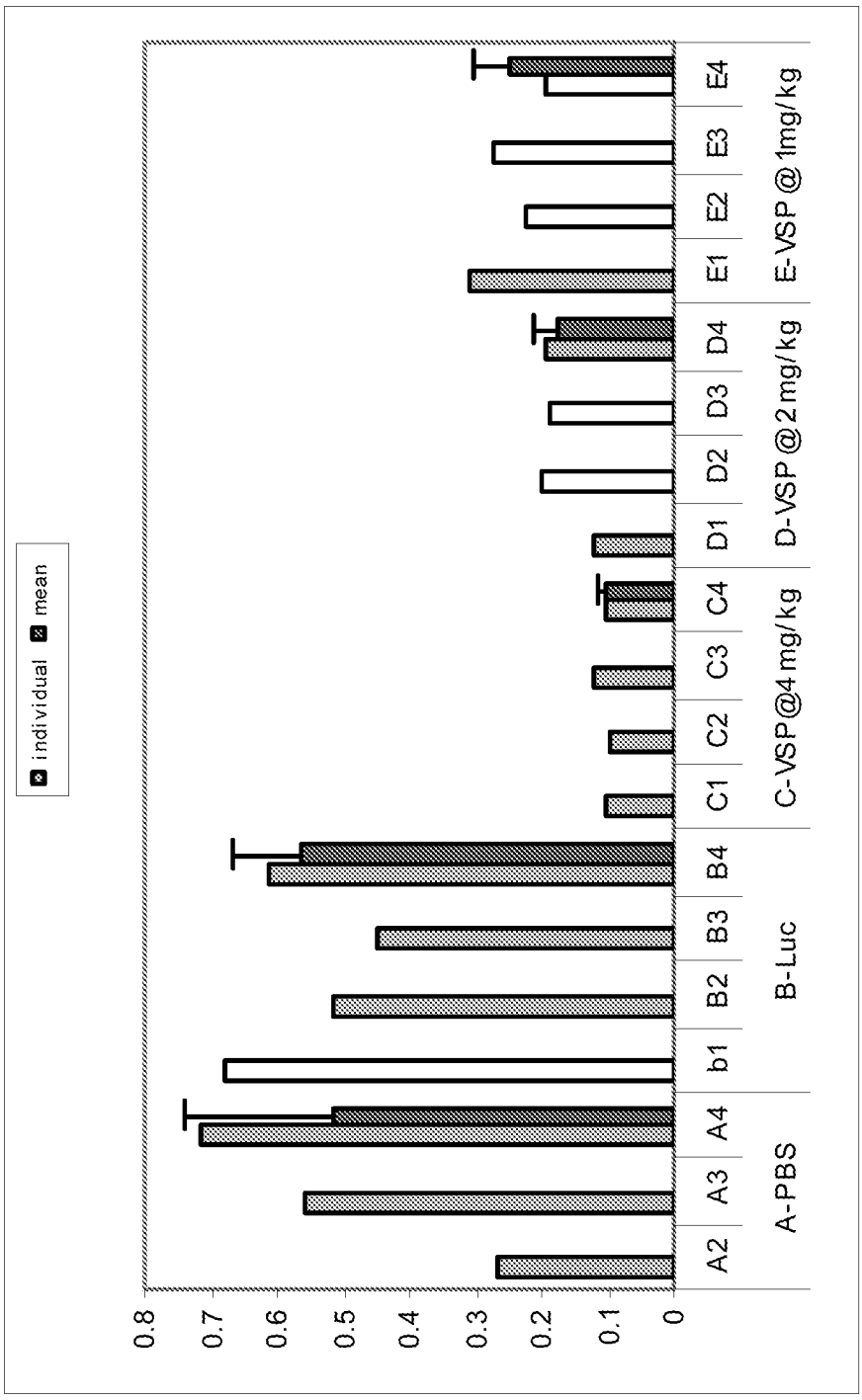

hKSP/h GAPDH
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
individual
mean
A2
A3
A4
b1
B2
B3
B4
C1
C2
C3
C4
D1
D2
D3
D4
E1
E2
E3
E4
A-PBS
B-Luc
C-VSP@4 mg/kg
D-VSP @2 mg/kg
E-VSP @1mg/kg
Treatment in Hep3B mice

FIG. 9

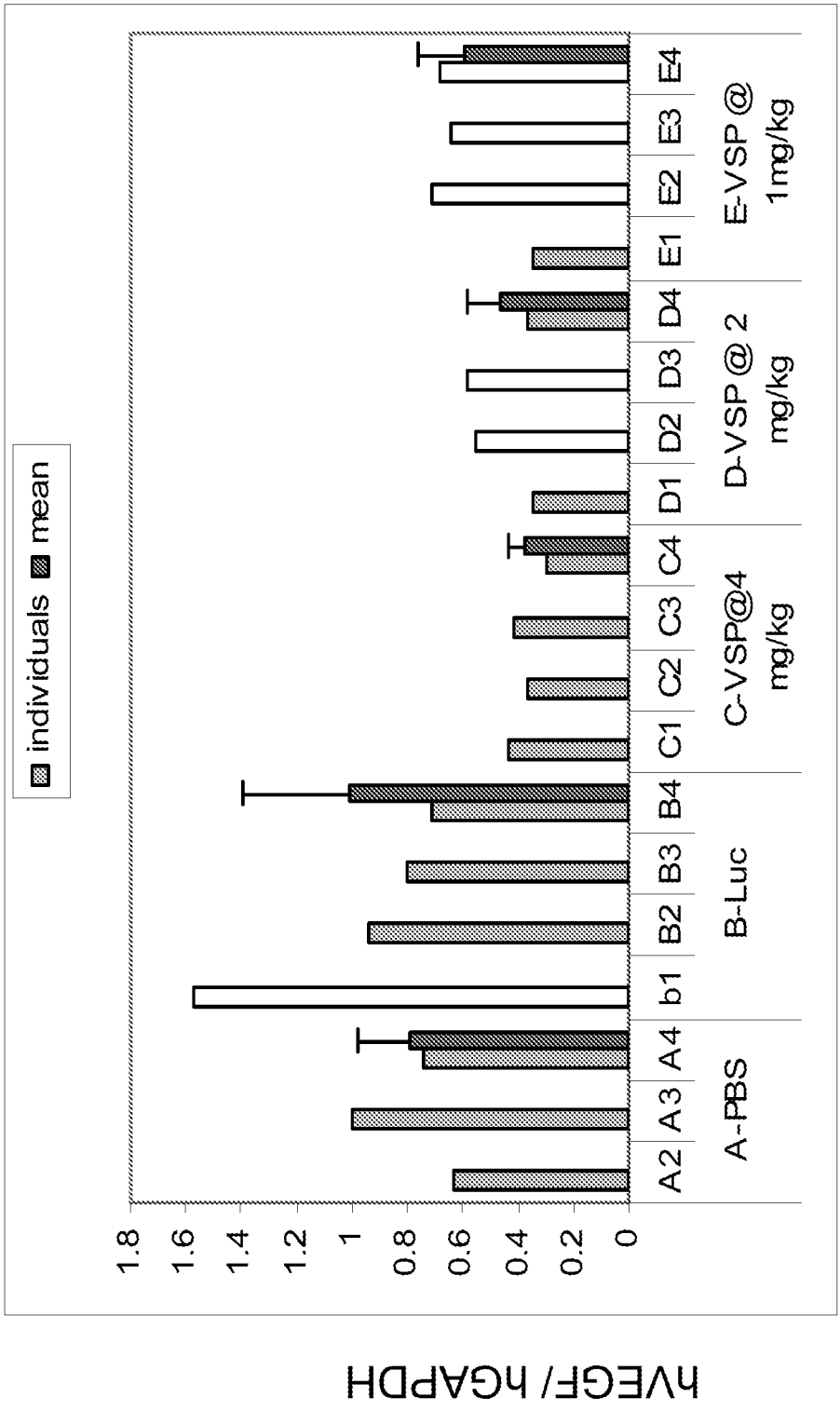

individuals
mean
hVEGF/ hGAPDH
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
A2
A3
A4
b1
B2
B3
B4
C1
C2
C3
C4
D1
D2
D3
D4
E1
E2
E3
E4
A-PBS
B-Luc
C-VSP@4 mg/kg
D-VSP @ 2 mg/kg
E-VSP @ 1mg/kg
Treatment in Hep3B mice

FIG. 10

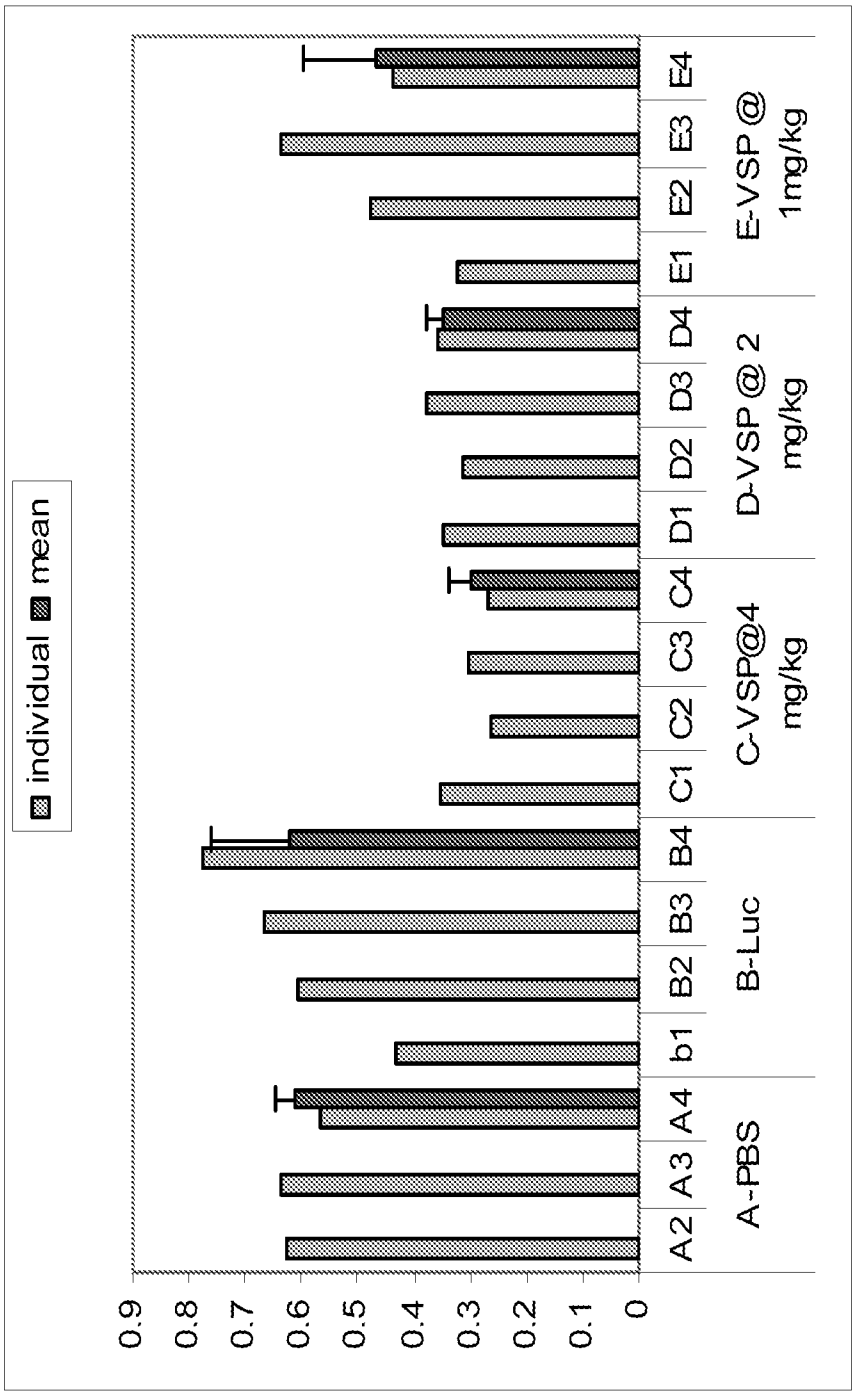

individual
mean
mVEGF/ hGAPDH
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
A2
A3
A4
b1
B2
B3
B4
C1
C2
C3
C4
D1
D2
D3
D4
E1
E2
E3
E4
A-PBS
B-Luc
C-VSP@4 mg/kg
D-VSP @ 2 mg/kg
E-VSP @ 1mg/kg
Treatment in Hep3B mice

FIG. 11A

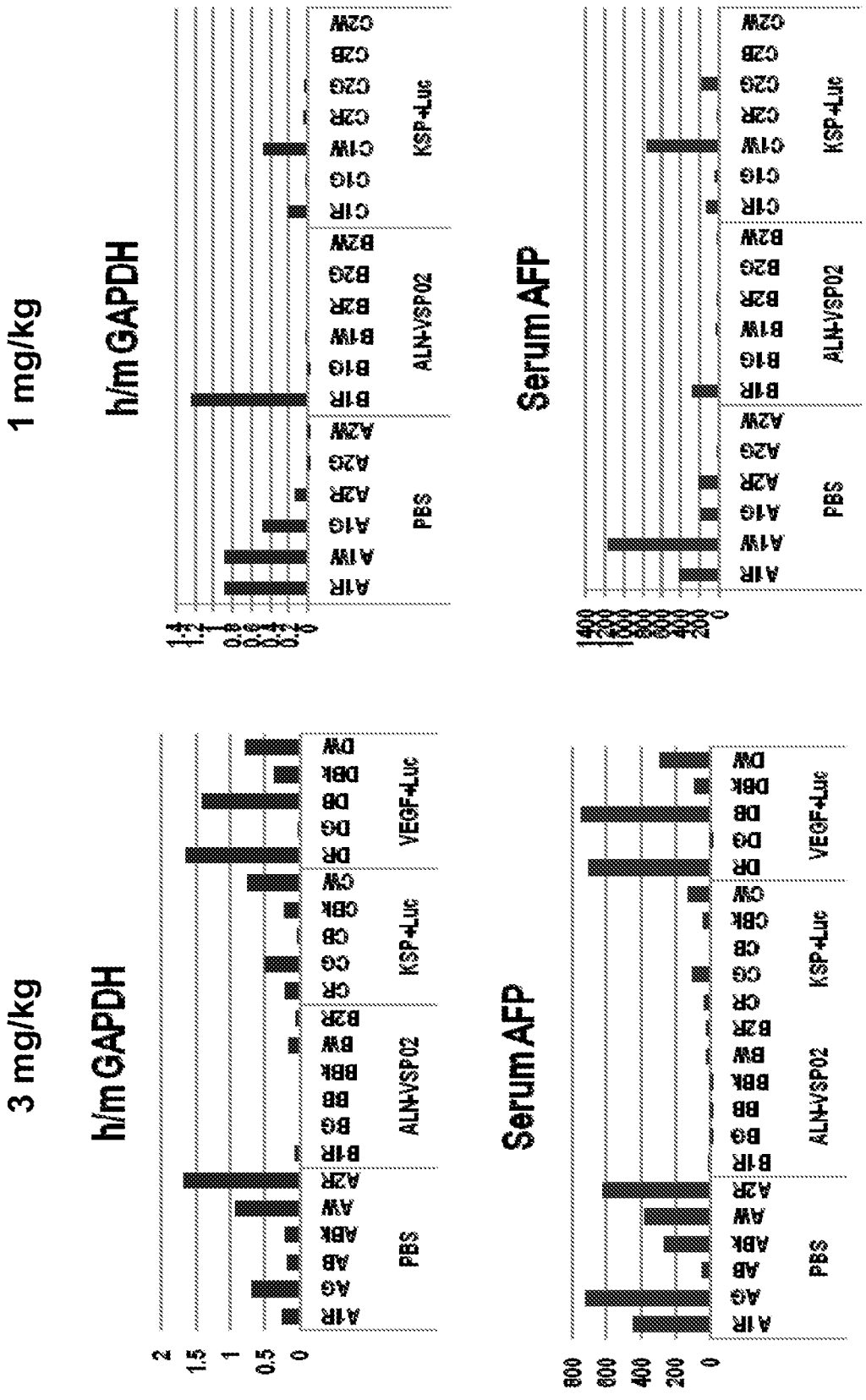

1 mg/kg
h/m GAPDH
Serum AFP
3 mg/kg
h/m GAPDH
Serum AFP
PBS
ALN-VSP02
KSP-Luc
VEGF-Luc

FIG. 11B

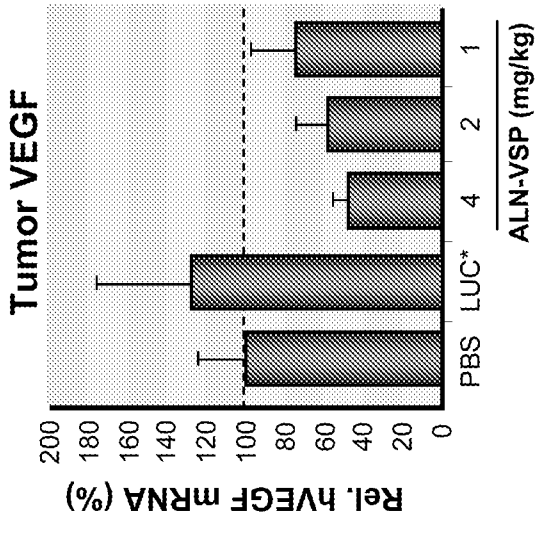


FIG. 12B

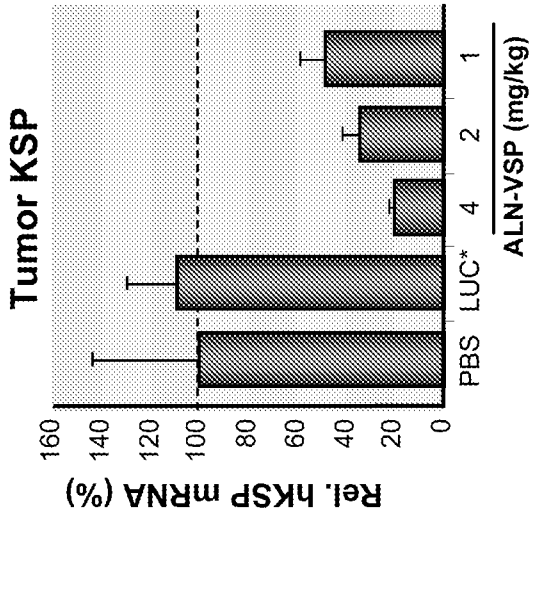


FIG. 12A

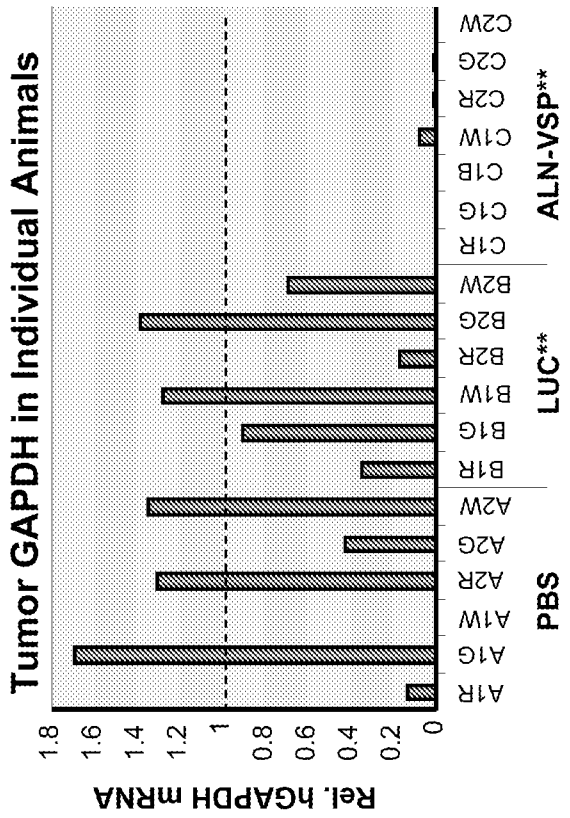

Tumor GAPDH in Individual Animals
Rel. hGAPDH mRNA
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
A1R
A1G
A1W
A2R
A2G
A2W
B1R
B1G
B1W
B2R
B2G
B2W
C1R
C1G
C1B
C1W
C2R
C2G
C2W
PBS
LUC**
ALN-VSP**
Treatment in Hep3B mice
* 4 mg/kg dose
** 3 mg/kg dose

FIG. 12C

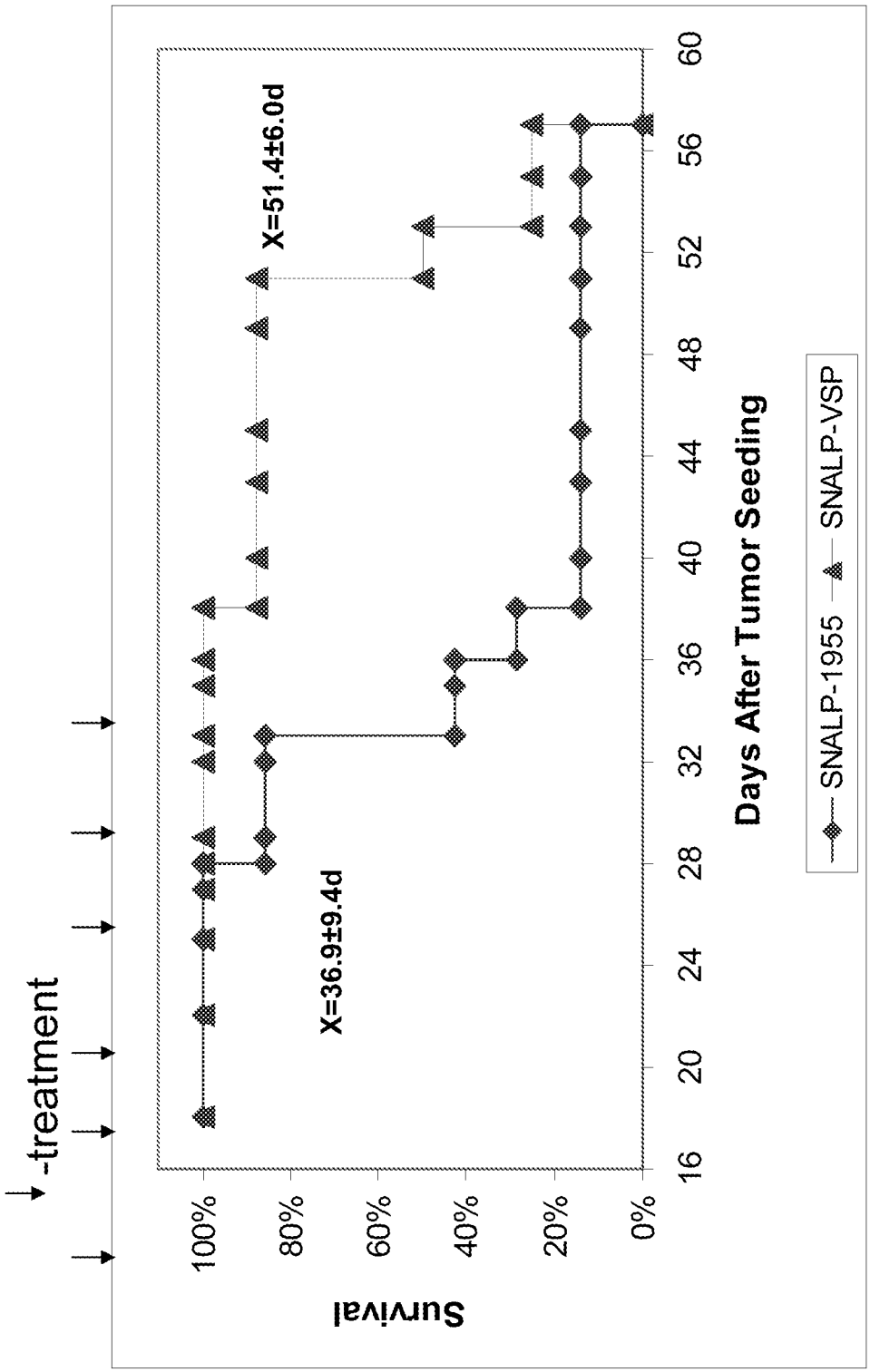

-treatment
100%
80%
60%
40%
20%
0%
Survival
16
20
24
28
32
36
40
44
48
52
56
60
Days After Tumor Seeding
X=36.9±9.4d
X=51.4±6.0d
SNALP-1955
SNALP-VSP

FIG. 13A

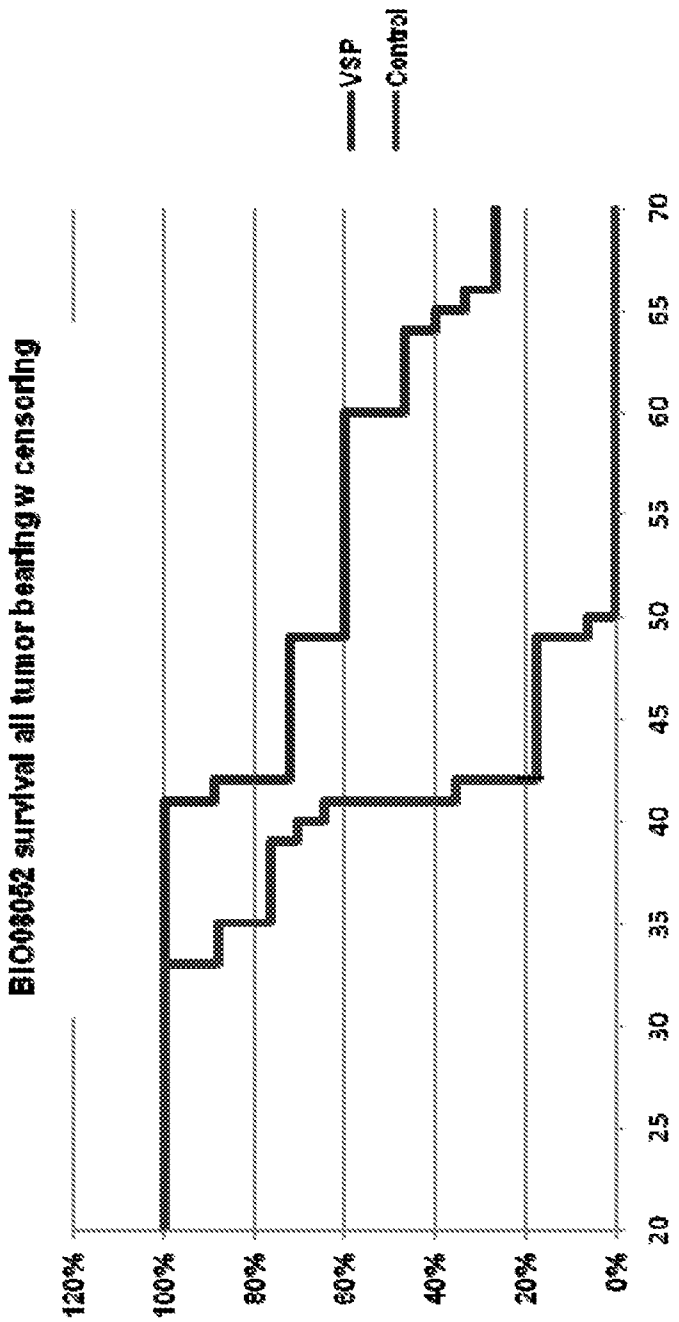
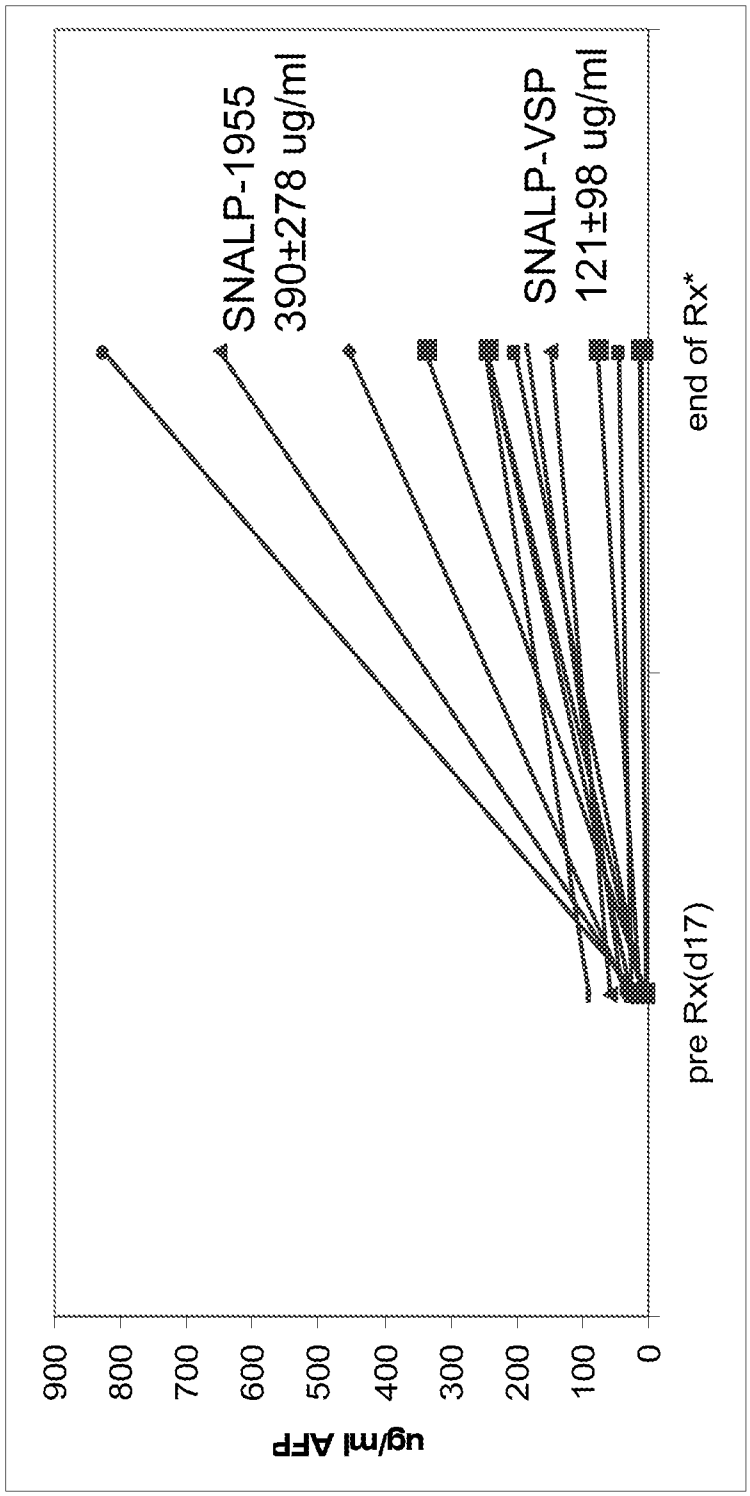

BIO08052 survival all tumor bearing w censoring
VSP
Control
120%
100%
80%
60%
40%
20%
0%
20
25
30
35
40
45
50
55
60
65
70

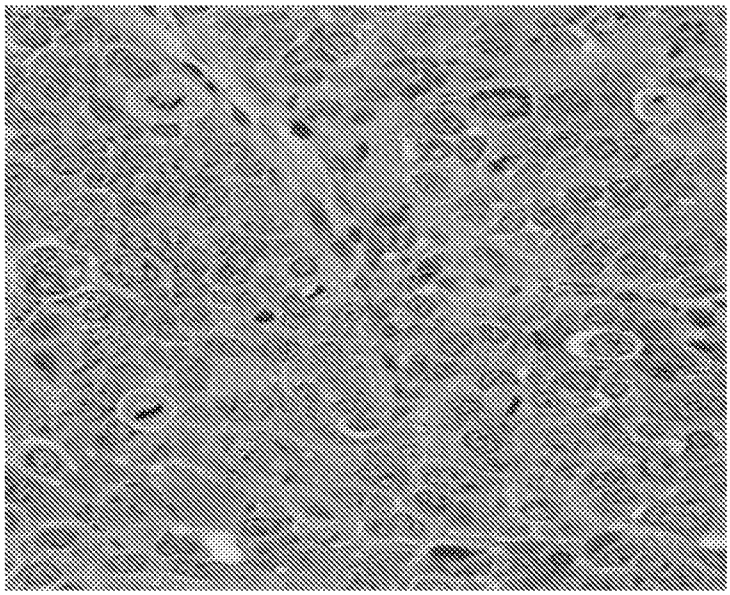
FIG. 13B



*ALN-VSP02: d32(n=8)
SNALP-1955: d28 (n=1), d32 (n=3), d33 (n=3)

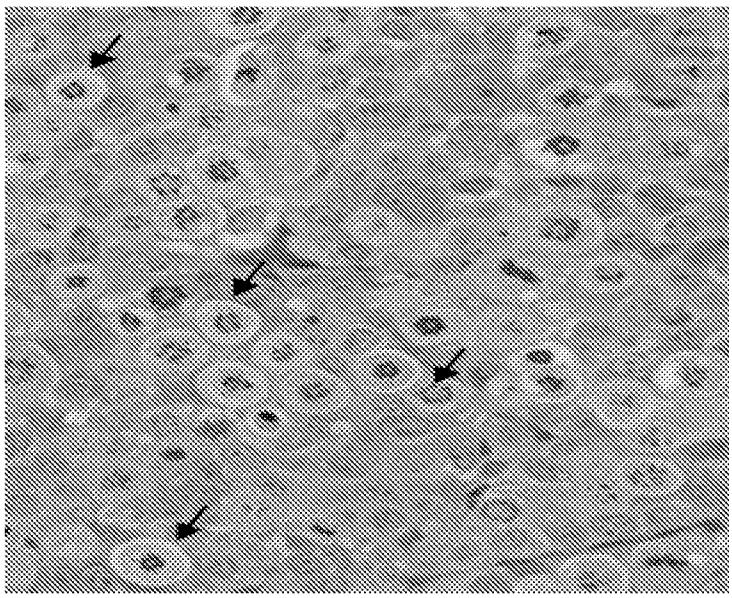
Treatment of SNALP-siRNA in Hep3B mice

FIG. 14



SNALP-Luc treated

FIG. 15B



SNALP-VSP treated

FIG. 15A

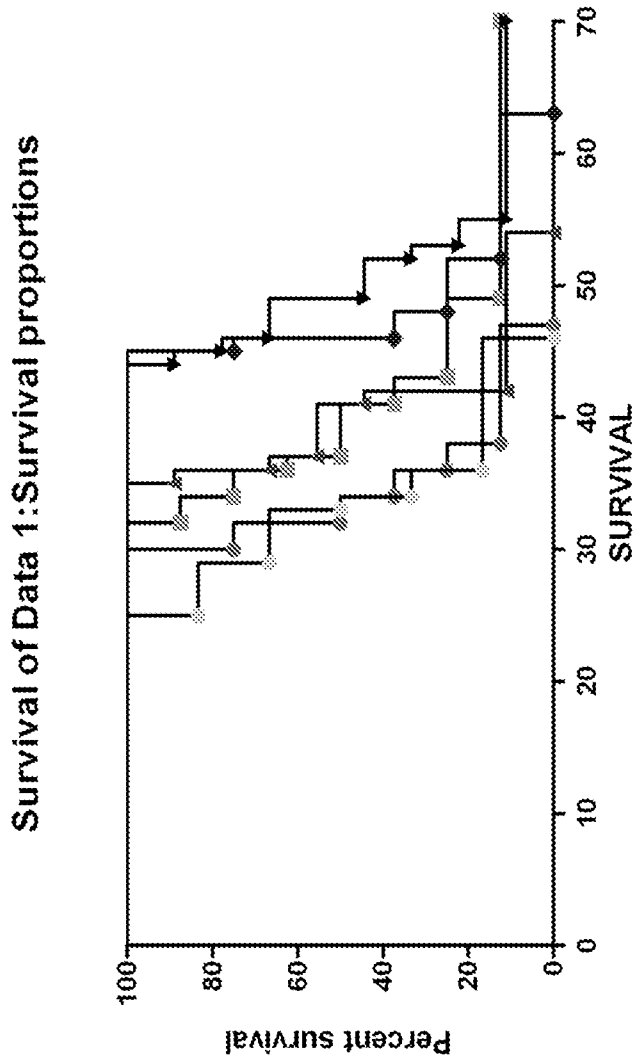

Survival of Data 1:Survival proportions
Percent survival
100
80
60
40
20
0
0
10
20
30
40
50
60
70
SURVIVAL
Soraf + PBS
Soraf + Luc SNALP
Soraf + VSP SNALP
DMSO + VSP SNALP
DMSO + PBS
NAIVE

FIG. 16

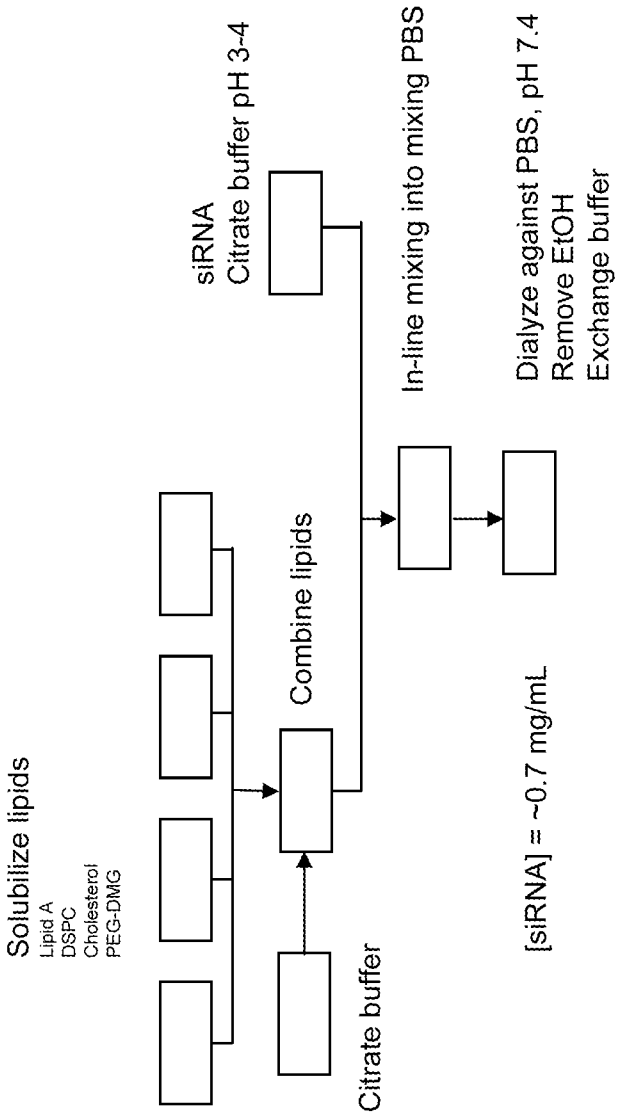

Solubilize lipids
Lipid A
DSPC
Cholesterol
PEG-DMG
Citrate buffer
Combine lipids
siRNA
Citrate buffer pH 3-4
In-line mixing into mixing PBS
Dialyze against PBS, pH 7.4
Remove EtOH
Exchange buffer
[siRNA] = ~0.7 mg/mL

FIG. 17

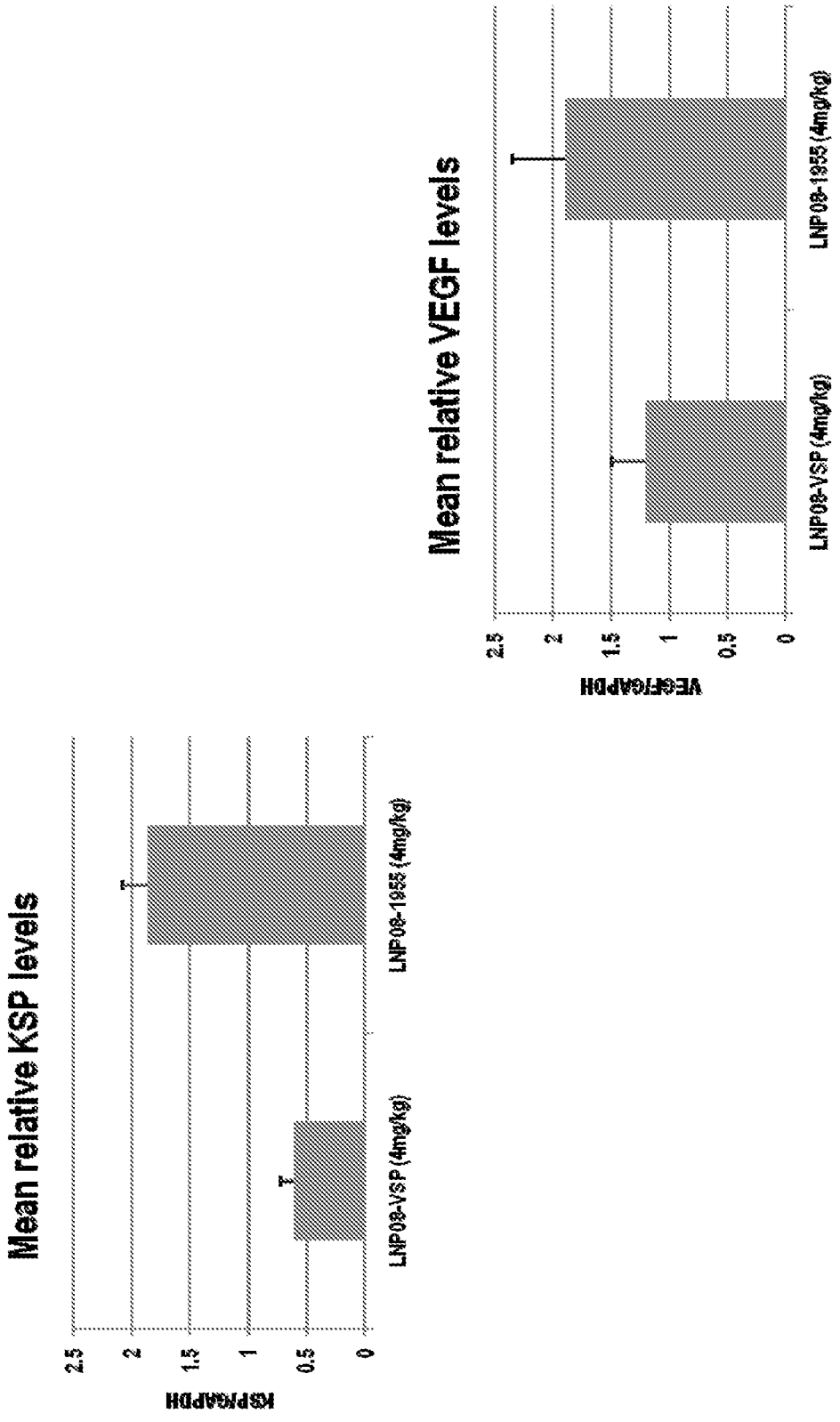
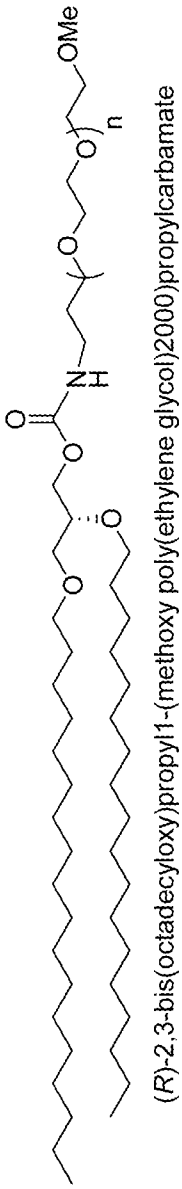
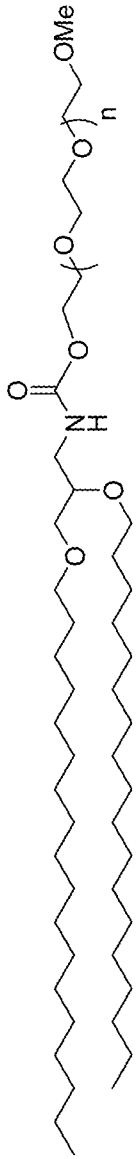

Mean relative KSP levels
KSP/GAPDH
2.5
2
1.5
1
0.5
0
LNP08-VSP (4mg/kg)
LNP08-1955 (4mg/kg)
Mean relative VEGF levels
VEGF/GAPDH
2.5
2
1.5
1
0.5
0
LNP08-VSP (4mg/kg)
LNP08-1955 (4mg/kg)

FIG. 18



(*R*)-2,3-bis(octadecyloxy)propyl1-(methoxy poly(ethylene glycol)2000)propylcarbamate

PEG-DSG



3-N-(methoxy poly(ethylene glycol)2000)carbamoyl-1,2-distearyloxy-propylamine

PEG-C-DSA

FIG. 19

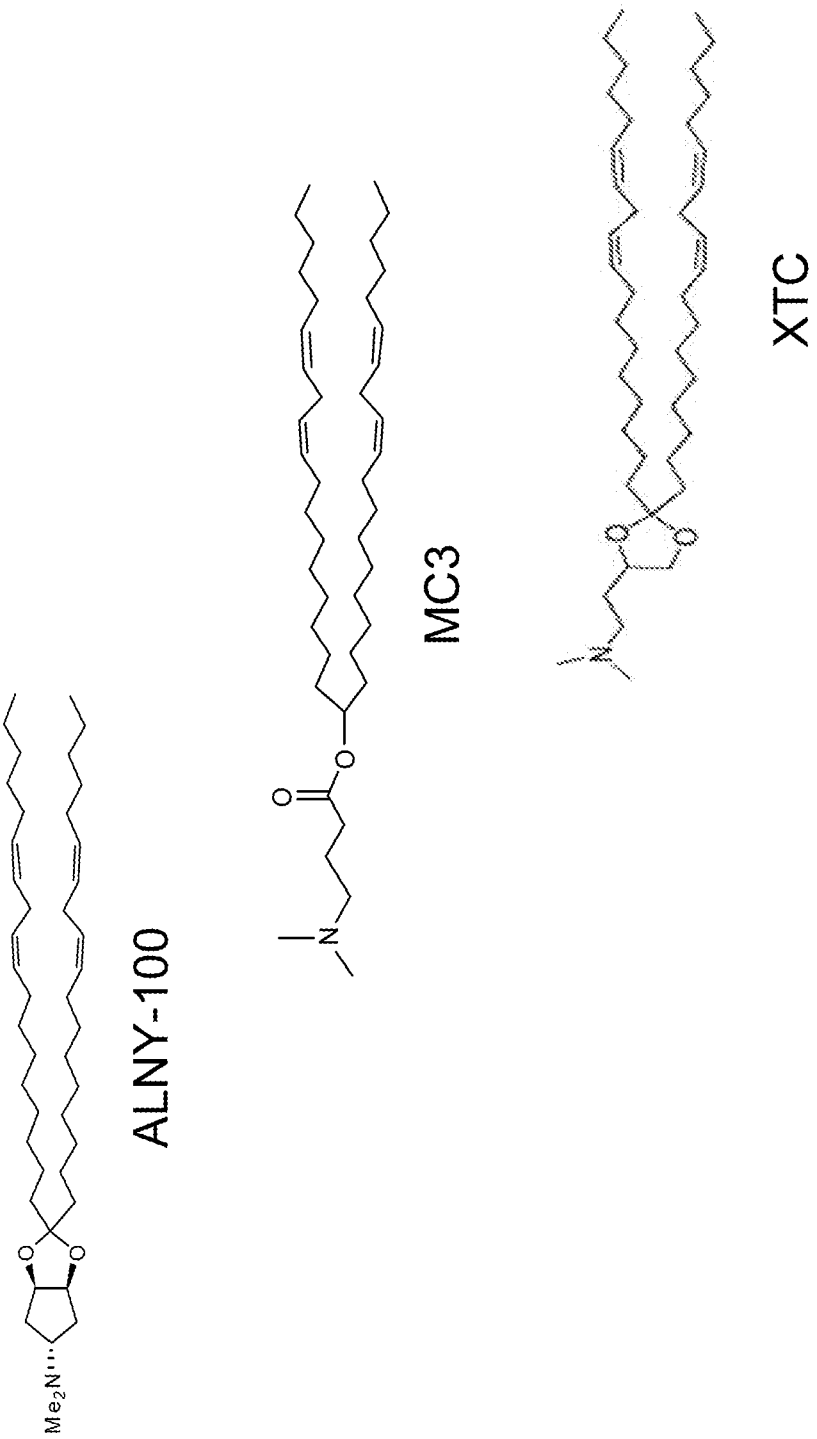


FIG. 20

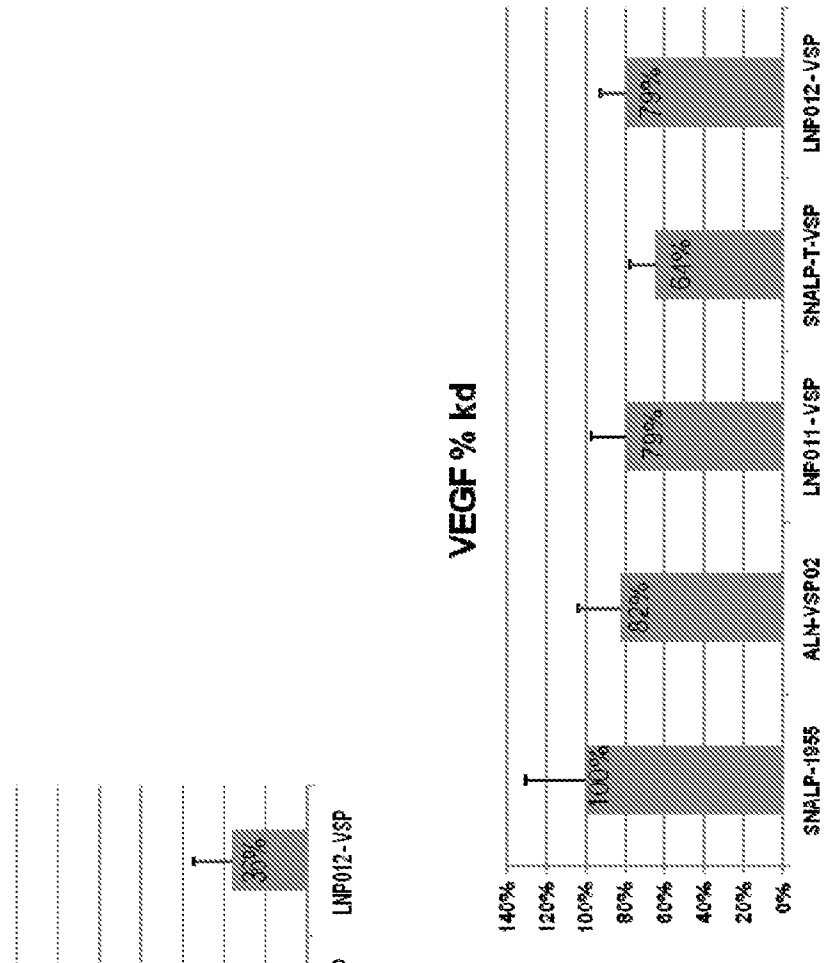

KSP % KD
140%
120%
100%
80%
60%
40%
20%
0%
100%
75%
29%
40%
33%
SNALP-1855
ALN-VSP02
LNP011-VSP
SNALP-T-VSP
LNP012-VSP
VEGF % kd
140%
120%
100%
80%
60%
40%
20%
0%
100%
82%
70%
63%
70%
SNALP-1855
ALN-VSP02
LNP011-VSP
SNALP-T-VSP
LNP012-VSP

FIG. 21

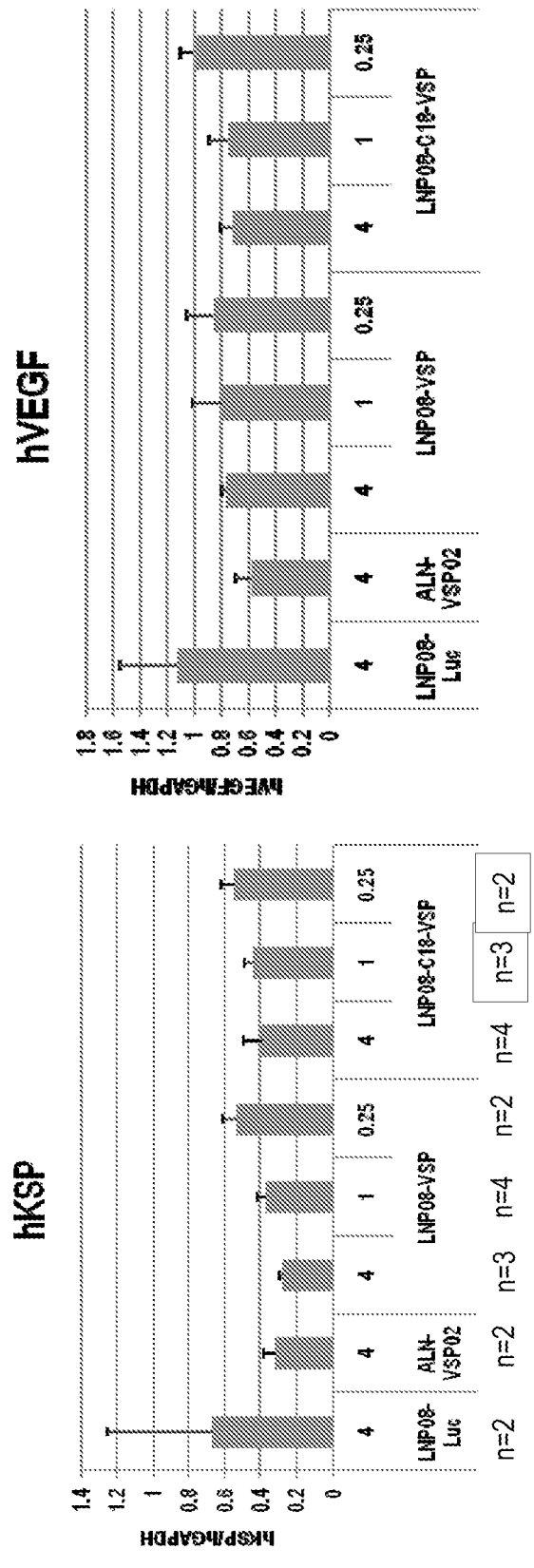

hVEGF
hVEGF/hGAPDH
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
LNP08-Luc
ALN-VSP02
LNP08-VSP
LNP08-C18-VSP
4
4
4
1
0.25
hKSP
hKSP/hGAPDH
1.4
1.2
1
0.8
0.6
0.4
0.2
0
n=2
n=2
n=3
n=4
n=2
n=4
n=3
n=2

FIG. 22

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2010/027210

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/113 C12N15/88
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, Sequence Search, BIOSIS, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/115168 A2 (ALNYLAM PHARMACEUTICALS INC [US]; BUMCROT DAVID [US]; TAN PAMELA [DE];) 11 October 2007 (2007-10-11)	1-19, 21-37
Y	page 8 - last paragraph pages 41-47 page 84 - page 88	20
X,P	WO 2009/111658 A2 (ALNYLAM PHARMACEUTICALS INC [US]; BUMCROT DAVID [US]) 11 September 2009 (2009-09-11) page 98 - page 99	1-37

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 June 2010	11/06/2010

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Cupido, Marinus

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2010/027210

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TAKIMOTO CHRIS H ET AL: "Safety and anti-tumor activity of sorafenib (Nexavar®) in combination with other anti-cancer agents: a review of clinical trials" CANCER CHEMOTHERAPY AND PHARMACOLOGY, SPRINGER VERLAG, BERLIN LNKD- DOI:10.1007/S00280-007-0639-9, vol. 61, no. 4, 1 April 2008 (2008-04-01), pages 535-548, XP002543476 ISSN: 0344-5704 the whole document	20
A	ZIMMERMANN TRACY S ET AL: "RNAi-mediated gene silencing in non-human primates" NATURE, NATURE PUBLISHING GROUP, LONDON, GB LNKD- DOI:10.1038/NATURE04688, vol. 441, no. 7089, 4 May 2006 (2006-05-04), pages 111-114, XP002412249 ISSN: 0028-0836 the whole document	1-37
X,P	LI LEIMING ET AL: "Overcoming obstacles to develop effective and safe siRNA therapeutics." EXPERT OPINION ON BIOLOGICAL THERAPY MAY 2009 LNKD- PUBMED:19392577, vol. 9, no. 5, May 2009 (2009-05), pages 609-619, XP008122949 ISSN: 1744-7682 page 615, line 23 - line 29	1-37

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2010/027210

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007115168 A2	11-10-2007	AU 2007233109 A1	11-10-2007
		CA 2647728 A1	11-10-2007
		EA 200870401 A1	28-08-2009
		EP 2008274 A2	31-12-2008
		JP 2009532036 T	10-09-2009
		KR 20090010174 A	29-01-2009
		US 2007281899 A1	06-12-2007
WO 2009111658 A2	11-09-2009	US 2010087508 A1	08-04-2010