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

(72) Inventors; and

(75) Inventors/Applicants (for US only): **DE
FOUGEROLLES, Antonin** [CA/US]; 66
Summit Avenue, Brookline, MA 02446 (US).
FRANK-KAMENETSKY, Maria [US/US]; 8 Fairbanks

Street, Apartment #3, Brookline, MA 02446 (US).
MANOHARAN, Muthiah [US/US]; 25 Circle Dr.,
Weston, MA 02493 (US). **RAJEEV, Kallanthottahil, G.**
[IN/US]; 30 Cambridgepark Drive, Apt. 4101, Cambridge,
MA 02140 (US). **HADWIGER, Phillipp** [AT/DE];
Schlesierstr. 6, D-96264 Altenkunstadt (DE).

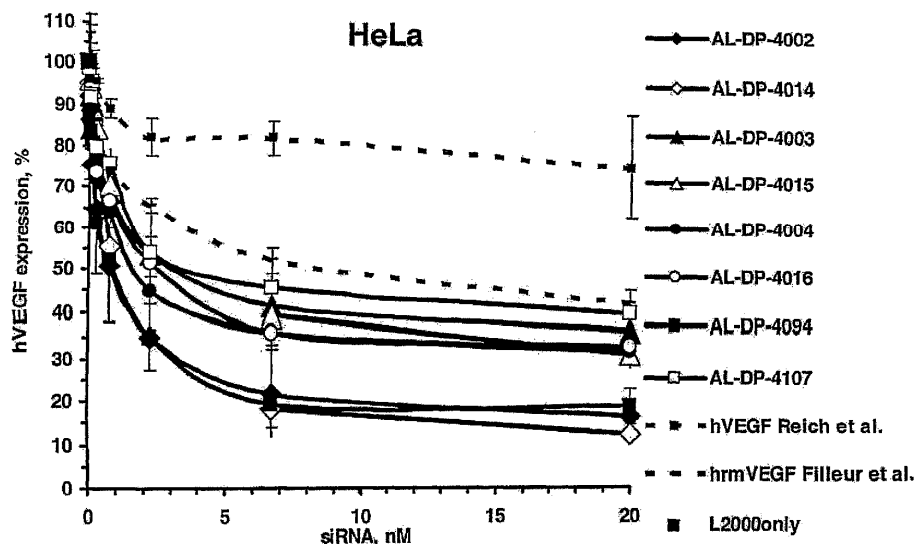
(74) Agents: **MYERS, Louis** et al.; Fish & Richardson P.C.,
225 Franklin Street, Boston, MA 02110-2804 (US).

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(54) Title: **iRNA AGENTS TARGETING VEGF**



(57) Abstract: The features of the present invention relate to compounds, compositions and methods useful for modulating the expression of vascular endothelial growth factor (VEGF), such as by the mechanism of RNA interference (RNAi). The compounds and compositions include iRNA agents that can be unmodified or chemically-modified.

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iRNA AGENTS TARGETING VEGF

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/552,620, filed March 12, 2004, U.S. Provisional Application No. 60/559,824, filed April 5, 2004, and U.S. Provisional Application No. 60/647,191, filed January 25, 2005. All three provisional applications are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

The present invention is in the field of iRNA agents that can inhibit expression of vascular endothelial growth factor (VEGF). The invention also relates to the use of siRNA targeting VEGF sequences to treat conditions or disorders related to unwanted expression of VEGF, *e.g.*, age-related macular degeneration or diabetic retinopathy.

BACKGROUND

VEGF (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 of disorders, including retinal disorders such as age-related macular degeneration and diabetic retinopathy, cancer, asthma, and other angiogenic disorders.

Macular degeneration is a major cause of blindness in the United States and the frequency of this disorder increases with age. Macular degeneration refers to the group of diseases in which sight-sensing cells in the macular zone of the retina malfunction or lose function and which can result in debilitating loss of vital central or detail vision. Adult macular degeneration (AMD), which is the most common form of macular degeneration, occurs in two main forms. Ninety percent of people with AMD have the form described as “dry” macular degeneration. An area of the retina is affected, which leads to slow breakdown of cells in the macula, and a gradual loss of central vision. The other form of AMD is “wet” macular degeneration. Although only 10% of people with AMD have this type, it accounts for 90% of blindness from the disease. As dry AMD progresses, new blood vessels may begin to grow and

cause “wet” AMD. These new blood vessels often leak blood and fluid under the macula. This causes rapid damage to the macula that can lead to loss of central vision in a short time. iRNA agents targeting VEGF can be useful for the treatment of wet and dry macular degeneration.

RNA interference or “RNAi” is a term initially coined by Fire and co-workers to describe the observation that double-stranded RNA (dsRNA) can block gene expression when it is introduced into worms (Fire *et al.*, *Nature* 391:806-811, 1998). Short dsRNA directs gene-specific, post-transcriptional silencing in many organisms, including vertebrates, and has provided a new tool for studying gene function. RNAi has been suggested as a method of developing a new class of therapeutic agents. However, to date, these have remained mostly as suggestions with no demonstrate proof that RNAi can be used therapeutically.

The present invention advances the art by providing a detailed gene walk across the VEGF gene and a detailed structural analysis of modifications that can be employed to stabilize the molecule against degradation and increase cellular uptake and targeting.

SUMMARY OF THE INVENTION

The invention provides compounds, compositions and methods useful for modulating the expression of VEGF. The invention provides compounds, compositions and methods useful for modulating the expression of VEGF activity by RNA interference (RNAi) using small nucleic acid molecules, such as short interfering RNA (siRNA), double-stranded RNA (dsRNA), microRNA (miRNA) and short hairpin RNA (shRNA) molecules, which collectively fall under the general term of iRNA agents. The iRNA agents can be unmodified or chemically-modified nucleic acid molecules. The iRNA agents can be chemically synthesized or expressed from a vector or enzymatically synthesized. The invention provides various chemically-modified synthetic iRNA agents capable of modulating VEGF gene expression or activity in cells and in a mammal by RNAi. The use of a chemically-modified iRNA agent can improve one or more properties of an iRNA agent through increased resistance to degradation, increased specificity to target moieties, improved cellular uptake, and the like.

In one aspect, the invention provides an iRNA agent that down-regulates expression of a VEGF gene. The VEGF gene can include a VEGF encoding sequence and/or VEGF regulatory sequences such as may exist 5’ or 3’ of a VEGF open reading frame (ORF).

In one embodiment, the invention provides an isolated iRNA agent including a sense and antisense sequence, where the sense and antisense sequences can form an RNA duplex. The sense sequence can include a nucleotide sequence that is identical or substantially identical to a target sequence of about 19 to 23 nucleotides of a VEGF sequence. In one embodiment, the VEGF sequence that is targeted includes the sequence of any one of SEQ ID NOs:2-401 (see Table 1).

In one embodiment, the sense sequence of the iRNA agent includes a sequence identical or substantially identical to any of the VEGF target sequences, *e.g.*, substantially identical to any of sense sequences provided in Table 1, SEQ ID NOs:2-401. In another embodiment, the antisense sequence of the iRNA agent can include a sequence complementary to or substantially complementary to, any of the target sequences, *e.g.*, complementary to any of SEQ ID NOs:2-401. By “substantially identical” is meant that the mismatch between the nucleotide sequences is less than 50%, 40%, 30%, 20%, 10%, 5%, or 1%. Preferably, no more than 1, 2, 3, 4, or 5 nucleotides differ between the target sequence and sense sequence. Furthermore, sequences that are “complementary” to each other (*e.g.*, sense and antisense sequences) can be fully complementary, or can have no more than 1, 2, 3, 4, or 5 nucleotides that lack full complementarity.

In one embodiment, the sense and antisense pairs of sequences of an iRNA agent includes any one of the agents provided in Table 2, or a sequence which differs in the sense strand from the recited sequence by no more than 1, 2, 3, 4, or 5 nucleotides, or in the antisense strand by no more than 1, 2, 3, 4, or 5 nucleotides, or in both strands by no more than 1, 2, 3, 4, or 5 nucleotides.

In one preferred embodiment, the sense sequence of an iRNA agent includes a sequence that is selected from the group consisting of SEQ ID NO:456, SEQ ID NO:550, SEQ ID NO:608, and SEQ ID NO:634, or a sequence that differs from the recited sequence by no more than 1, 2, 3, 4, or 5 nucleotides.

In another embodiment, the antisense sequence of the iRNA agent includes a sequence fully complementary or substantially complementary to any of the VEGF target sequences, *e.g.*, complementary or substantially complementary to any of SEQ ID NOs:2-401.

In another embodiment, the antisense sequence of an iRNA agent includes a sequence selected from the group consisting any of the antisense sequences provided in Table 2, or a sequence which differs from the recited sequence by no more than 1, 2, 3, 4, or 5 nucleotides. In a preferred embodiment, this antisense sequence is fully complementary to a sense sequence or has no more than 1, 2, 3, 4, or 5 nucleotide mismatches with the sense sequence.

In a preferred embodiment, the antisense sequence of an iRNA agent includes a sequence selected from the group consisting of SEQ ID NO:457, SEQ ID NO:551, SEQ ID NO:609, and SEQ ID NO:635, or a sequence that differs from the recited sequence by no more than 1, 2, 3, 4, or 5 nucleotides.

In another embodiment, the iRNA agent is chemically modified. For example, the iRNA agent can include a non-nucleotide moiety. A chemical modification or other non-nucleotide moiety can stabilize the sense and antisense sequences against nucleolytic degradation. Additionally, conjugates can be used to increase uptake and target uptake of the iRNA agent to particular cell types. Preferred modifications include those specifically provided in the Examples, Tables 6-19.

In another embodiment, the iRNA agent includes a 3'-overhang that ranges from 1 to about 6 nucleotides. As used herein, a "3' overhang" refers to at least one unpaired nucleotide extending from the 3' end of an iRNA sequence. The 3' overhang can include ribonucleotides or deoxyribonucleotides or modified ribonucleotides or modified deoxyribonucleotides. The 3' overhang is preferably from 1 to about 5 nucleotides in length, more preferably from 1 to about 4 nucleotides in length and most preferably from about 2 to about 4 nucleotides in length. The 3' overhang can occur on the sense or antisense sequence, or on both sequences of an iRNA agent.

In one preferred embodiment, the iRNA agent of the invention includes an antisense sequence having 23 nucleotides complementary to the target VEGF sequence and a sense sequence having at least 21 nucleotides. Each sequence can include at least 21 nucleotides that are complementary to each other, and at least the antisense sequence can have a 3' overhang of two nucleotides.

In one embodiment, both the sense and antisense sequences of the iRNA agent include a 3' overhang, the length of which can be the same or different for each sequence. In one embodiment, the 3' overhang on each sequence ranges from 1 to about 6 (*e.g.*, from 1 to about 3)

nucleotides in length. In a preferred embodiment, the 3' overhang is on both sequences of the iRNA agent and is two nucleotides in length. In another preferred embodiment, the 3' overhang is on both sequences of the iRNA agent and the 3' overhangs include two thymidylic acid residues ("TT").

5 In one embodiment, an iRNA agent includes an antisense sequence having about 19 to 25 (e.g., about 19, 20, 21, 22, 23, 24, or 25) nucleotides with complementarity to an RNA sequence encoding a VEGF protein. The iRNA agent can further include a sense sequence having about 19 to 25 (e.g., about 19, 20, 21, 22, 23, 24, or 25) nucleotides, and the antisense and sense sequences can have distinct nucleotide sequences with at least about 19, 20, or 21

10 complementary nucleotides.

In one embodiment, an iRNA agent of the invention includes an antisense region having about 19 to about 25 (e.g., about 19 to about 23) nucleotides with complementarity to an RNA sequence encoding VEGF, and a sense region having about 19 to 25 (e.g., about 19 to about 23) nucleotides. The sense and antisense regions can be included in a linear molecule with at least
15 about 19 complementary nucleotides. The sense sequence can include a nucleotide sequence that is substantially identical to a nucleotide sequence of VEGF.

In one embodiment, the iRNA agent includes an antisense sequence of about 21 nucleotides complementary to the VEGF target sequence and a sense sequence of about 21 nucleotides complementary to the antisense sequence. The iRNA agent can include a non-
20 nucleotide moiety. In one embodiment, the sense or antisense sequence of the iRNA agent can include a 2'-O-methyl (2'-OMe) pyrimidine nucleotide, 2'-deoxy nucleotide (e.g., deoxycytidine), 2'-deoxy-2'-fluoro (2'-F) pyrimidine nucleotide, 2'-O-methoxyethyl (2'-O-MOE), 2'-O-aminopropyl (2'-O-AP), 2'-O-N-methylacetamido (2'-O-NMA), 2'-O-dimethylaminoethoxyethyl (2'-DMAEOE), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-
25 dimethylaminopropyl (2'-O-AP), 2'-hydroxy nucleotide, or a 2'-ara-fluoro nucleotide, or a locked nucleic acid (LNA), extended nucleic acid (ENA), hexose nucleic acid (HNA), cyclohexene nucleic acid (CeNA), ribo-difluorotoluy, 5-allylamino-pyrimidines, or 5-Me-2'-modified pyrimidines. A 2' modification is preferably a 2'-OMe modification, and more preferably, a 2'-fluoro modification. In a preferred embodiment, one or more 2' modified nucleotides are on the
30 sense strand of the iRNA agent.

In one embodiment, an iRNA agent includes a nucleobase modification, such as a cationic modification, such as a 3'-abasic cationic modification. The cationic modification can be, *e.g.*, an alkylamino-dT (*e.g.*, a C6 amino-dT), an allylamino conjugate, a pyrrolidine conjugate, a pthalamido a hydroxyprolinol conjugate or an aminooxy conjugate, on one or more of the
5 terminal nucleotides of the iRNA agent. An alkylamino-dT conjugate is preferably attached to the 3' end of the sense or antisense strand of an iRNA agent. A pyrrolidine linker is preferably attached to the 3' or 5' end of the sense strand, or the 3' end of the antisense strand. An allyl amine uridine is preferably on the 3' or 5' end of the sense strand, and not on the 5' end of the antisense strand. An aminooxy conjugate can be attached to a hydroxyl prolinol and at the 3' or
10 5' end of either the sense or antisense strands.

In another embodiment, an iRNA agent that targets VEGF includes a conjugate, *e.g.*, to facilitate entry into a cell or to inhibit exo- or endonucleolytic cleavage. The conjugate can be, for example, a lipophile, a terpene, a protein binding agent, a vitamin, a carbohydrate, a retinoid or a peptide. For example, the conjugate can be naproxen, nitroindole (or another conjugate that
15 contributes to stacking interactions), folate, ibuprofen, retinol or a C5 pyrimidine linker. In other embodiments, the conjugates are glyceride lipid conjugates (*e.g.* a dialkyl glyceride derivatives), vitamin E conjugates, or thio-cholesterols. Preferably, conjugates are on the 3' end of the antisense strand, or on the 5' or 3' end of the sense strand, and preferably the conjugates are not on the 3' end of the antisense strand and on the 3' end of the sense strand.

In one embodiment, the conjugate is naproxen, and the conjugate is preferably on the 5' or 3' end of the sense or antisense strands. In one embodiment, the conjugate is cholesterol or thiocholesterol, and the conjugate is preferably on the 5' or 3' end of the sense strand and preferably not present on the antisense strand. In some embodiments, the cholesterol is conjugated to the iRNA agent by a pyrrolidine linker, or serinol linker, or hydroxyprolinol linker.
20 In another embodiment, the conjugate is cholanolic acid, and the cholanolic acid is attached to the 5' or 3' end of the sense strand, or the 3' end of the antisense strand. In one embodiment, the cholanolic acid is attached to the 3' end of the sense strand and the 3' end of the antisense strand. In another embodiment, the conjugate is retinol acid, and the retinol acid is attached to the 5' or 3' end of the sense strand, or the 3' end of the antisense strand. In one embodiment, the retinol
25 acid is attached to the 3' end of the sense strand and the 3' end of the antisense strand.
30

In one aspect, an iRNA agent of the invention has RNAi activity that modulates expression of RNA encoded by a VEGF gene. VEGF genes can share some degree of sequence identity with each other, and thus, iRNA agents can target a class of VEGF genes, or alternatively, specific VEGF genes, by targeting sequences that are either shared amongst
5 different VEGF targets or that are unique for a specific VEGF target. Therefore, in one embodiment, an iRNA agent can target a conserved region of a VEGF nucleotide sequence (*e.g.*, RNA sequence). The conserved region can have sequence identity with several different VEGF-related sequences (*e.g.*, different VEGF isoforms, splice variants, mutant genes, *etc.*). Thus, one iRNA agent can target several different VEGF-related sequences.

10 In one embodiment, an iRNA agent is chemically modified. In another embodiment the iRNA agent includes a duplex molecule wherein one or more sequences of the duplex molecule is chemically modified. Non-limiting examples of such chemical modifications include phosphorothioate internucleotide linkages, 2'-deoxyribonucleotides, 2'-O-methyl
15 ribonucleotides, 2'-deoxy-2'-fluoro ribonucleotides, "universal base" nucleotides, "acyclic" nucleotides, 5'-C-methyl nucleotides, and terminal glyceryl and/or inverted deoxy abasic residue incorporation. These chemical modifications, when used in iRNA agents, can help to preserve RNAi activity of the agents in cells and can increase the serum stability of the iRNA agents.

In one embodiment, an iRNA agent includes one or more chemical modifications and the sense and antisense sequences of the double-stranded RNA is about 21 nucleotides long.

20 In a preferred embodiment, the first and preferably the first two internucleotide linkages at the 5' end of the antisense and/or sense sequences are modified, preferably by a phosphorothioate. In a preferred embodiment, the first, and preferably the first two, three, or four internucleotide linkages at the 3' end of a sense and/or antisense sequence are modified, preferably by a phosphorothioate. More preferably, the 5' end of both the sense and antisense
25 sequences, and the 3' end of both the sense and antisense sequences are modified as described.

In another aspect, an iRNA agent that mediates the down-regulation of VEGF expression includes one or more chemical modifications that modulate the binding affinity between the sense and the antisense sequences of the iRNA construct.

30 In one embodiment, the invention features an iRNA agent that includes one or more chemical modifications that can modulate the cellular uptake of the iRNA agent.

In another embodiment, the invention features an iRNA agent that includes one or more chemical modifications that improve the pharmacokinetics of the iRNA agent. Such chemical modifications include but are not limited to conjugates, such as ligands for cellular receptors, *e.g.*, peptides derived from naturally occurring protein ligands; protein localization sequences; antibodies; nucleic acid aptamers; vitamins and other co-factors, such as folate, retinoids and N-acetylgalactosamine; polymers, such as polyethyleneglycol (PEG, *e.g.* PEG 5 and PEG20); phospholipids; polyamines, such as spermine or spermidine; and others.

In one embodiment, the iRNA agent includes a duplex molecule selected from the group consisting of AL-DP-4003, AL-DP-4116, AL-DP-4015, AL-DP-4120, AL-DP-4002, AL-DP-4115, AL-DP-4014, AL-DP-4119, AL-DP-4094, AL-DP-4118, AL-DP-4107, AL-DP-4122, AL-DP-4004, AL-DP-4117, AL-DP-4016, AL-DP-4121, AL-DP-4127, AL-DP-4128, AL-DP-4129, and AL-DP-4055 (see Tables 2 and 3).

In one preferred embodiment, the iRNA agent includes a duplex described as AL-DP-4094, which includes the antisense sequence 5' AAGCUCAUCUCUCCUAUGUGCUG 3' (SEQ ID NO:609) and the sense sequence 5' GCACAUAGGAGAGAUGAGCUU 3' (SEQ ID NO:608).

In another preferred embodiment, the iRNA agent includes a duplex described as AL-DP-4004, which includes the antisense sequence 5' CUUUCUUUGGUCUGCAUUCACAU 3' (SEQ ID NO:635) and the sense sequence 5' GUGAAUGCAGACCAAAGAAAG 3' (SEQ ID NO:634).

In another preferred embodiment, the iRNA agent includes a duplex described as AL-DP-4015, which includes the antisense sequence 5' GUACUCCUGGAAGAUGUCCTT 3' (SEQ ID NO:551) and the sense sequence 5' GGACAUUCUCCAGGAGUACTT 3' (SEQ ID NO:550).

In another preferred embodiment, the iRNA agent includes a duplex described as AL-DP-4055, which includes the antisense sequence 5' UGCAGCCUGGGACCACUUGTT 3' (SEQ ID NO:457) and the sense sequence 5' CAAGUGGUCCCAGGCUGCATT 3' (SEQ ID NO:456).

In one embodiment, the antisense sequence of an iRNA agent described herein does not hybridize to an off-target sequence. For example, the antisense sequence can have less than 5, 4, 3, 2, or 1 nucleotides complementary to an off-target sequence. By "off-target" is meant a sequence other than a VEGF nucleotide sequence.

In another embodiment, the sense strand is modified to inhibit off-target silencing. The sense strand can include a cholesterol moiety, such as cholesterol attached to the sense strand by a pyrrolidine linker.

5 In another embodiment, the antisense sequence of an iRNA agent described herein can hybridize to a VEGF sequence in a human and a VEGF sequence in a non-human mammal, *e.g.*, a mouse, rat, or monkey.

In another aspect, the invention provides a method of delivering an iRNA agent, *e.g.*, an iRNA agent described herein, to the eye of a subject, *e.g.*, a mammalian subject, such as a mouse, a rat, a monkey or a human.

10 In another aspect, the invention provides a method of delivering an iRNA agent to the eye of a subject, *e.g.*, a mammalian subject, such as a mouse, a rat, a monkey or a human.

In one embodiment, the iRNA agent can be delivered to a cell or cells in a choroid region of the eye. In one preferred embodiment, the iRNA agent down-regulates expression of the VEGF gene at a target site within the eye. An iRNA agent delivered to the eye, *e.g.*, choroid
15 cells of the eye, can be an unmodified iRNA agent.

In one embodiment, the iRNA agent can be stabilized with phosphorothioate linkages. In another embodiment, the 3' end of the sense or antisense sequences, or both, of the iRNA agent can be modified with a cationic group, such as a 3'-abasic cationic modification. The cationic modification can be, *e.g.*, an alkylamino-dT (*e.g.*, a C6 amino-dT), an allylamine, a pyrrolidine, a
20 pthalamido, a hydroxyprolinol, a polyamine, a cationic peptide, or a cationic amino acid on one or more of the terminal nucleotides of the iRNA agent. The modification can be an external or terminal cationic residue. In preferred embodiments, a pyrrolidine cap is attached to the 3' or 5' end of the sense strand, or the 3' end of the antisense strand.

In one embodiment, the sense or antisense sequence, or both, of the iRNA agent can be
25 modified with a sugar, *e.g.*, a glycoconjugate or alkylglycoside component, *e.g.*, glucose, mannose, 2-deoxy-glucose, or an analog thereof. In another embodiment, the iRNA agent can be conjugated to an enzyme substrate, *e.g.*, a substrate for which the relative enzyme is present in a higher amount, as compared to the enzyme level in other tissues of the body, *e.g.*, in tissues other than the eye.

In one embodiment, at least about 30%, 40%, 50%, 60%, 70%, 80%, 90% or more of the iRNA agent administered to the subject reaches the eye. In a preferred embodiment, between about 30-90%, 40-80% or 50-70% of the iRNA agent administered to the subject reaches the eye.

In another aspect, the invention features a composition, *e.g.*, a pharmaceutical
5 composition that includes an iRNA agent of the present invention in a pharmaceutically acceptable carrier or diluent. The iRNA agent can be any agent described herein. In one embodiment, the iRNA agent is chemically modified, such as with any chemical modification described herein. Preferred modified iRNA agents includes those provided in Tables 2-19.

In another aspect, the invention features a method for treating or preventing a disease or
10 condition in a subject. The method can include administering to the subject a composition of the invention under conditions suitable for the treatment or prevention of the disease or condition in the subject, alone or in conjunction with one or more other therapeutic compounds.

In one embodiment, the iRNA agent is administered at or near the site of unwanted VEGF expression, *e.g.*, by a catheter or other placement device (*e.g.*, a retinal pellet or an implant
15 including a porous, non-porous, or gelatinous material). In one embodiment the iRNA agent is administered via an intraocular implant, which can be inserted, for example, into an anterior or posterior chamber of the eye; or into the sclera, transchoroidal space, or an avascularized region exterior to the vitreous. In another embodiment, the implant is positioned over an avascular region, such as on the sclera, so as to allow for transcleral diffusion of the drug to the desired site
20 of treatment, *e.g.*, to the intraocular space and macula of the eye. Furthermore, the site of transcleral diffusion is preferably in proximity to the macula.

In another embodiment, an iRNA agent is administered to the eye by injection, *e.g.*, by intraocular, retinal, or subretinal injection.

In another embodiment, an iRNA agent is administered topically to the eye, such as by a
25 patch or liquid eye drops, or by iontophoresis. Ointments or droppable liquids can be delivered by ocular delivery systems known in the art such as applicators or eye droppers.

In one embodiment, an iRNA is delivered at or near a site of neovascularization.

In one embodiment, an iRNA agent is administered repeatedly. Administration of an iRNA agent can be carried out over a range of time periods. It can be administered hourly, daily,
30 once every few days, weekly, or monthly. The timing of administration can vary from patient to

patient, depending upon such factors as the severity of a patient's symptoms. For example, an effective dose of an iRNA agent can be administered to a patient once a month for an indefinite period of time, or until the patient no longer requires therapy. In addition, sustained release compositions containing an iRNA agent can be used to maintain a relatively constant dosage in the area of the target VEGF nucleotide sequences.

In another embodiment, an iRNA agent is delivered to the eye at a dosage on the order of about 0.00001 mg to about 3 mg per eye, or preferably about 0.0001-0.001 mg per eye, about 0.03- 3.0 mg per eye, about 0.1-3.0 mg per eye or about 0.3-3.0 mg per eye.

In another embodiment, an iRNA agent is administered prophylactically such as to prevent or slow the onset of a disorder or condition that affects the eye. For example, an iRNA can be administered to a patient who is susceptible to or otherwise at risk for a neovascular disorder.

In one embodiment one eye of a human is treated with an iRNA agent described herein, and in another embodiment, both eyes of a human are treated.

In another aspect, a method of inhibiting VEGF expression is provided. One such method includes administering an effective amount of an iRNA agent of the present invention.

In another aspect, a method of treating adult onset macular degeneration is provided. The method includes administering a therapeutically effective amount of an iRNA agent of the present invention.

In one embodiment, a human has been diagnosed with dry adult macular degeneration (AMD), and in another embodiment the human has been diagnosed with wet AMD.

In one embodiment, a human treated with an iRNA agent described herein is over the age of 50, *e.g.*, between the ages of 75 and 80, and the human has been diagnosed with adult onset macular degeneration. In another embodiment, a human treated with an iRNA agent described herein is between the ages of 30-50, and the human has been diagnosed with late onset macular degeneration. In another embodiment, a human treated with an iRNA agent described herein is between the ages of 5-20, and the human has been diagnosed with middle onset macular degeneration. In another embodiment, a human treated with an iRNA agent described herein is 7 years old or younger, and the human has been diagnosed with early onset macular degeneration.

In one aspect, methods of treating any disease or disorder characterized by unwanted VEGF expression are provided. Particularly preferred embodiments include the treatment of disorders of the eye or retina, which are characterized by unwanted VEGF expression. The disease or disorder can be a diabetic retinopathy, neovascular glaucoma, a tumor or metastatic cancer (*e.g.*, colon or breast cancer), a pulmonary disease (*e.g.*, asthma or bronchitis), rheumatoid arthritis, or psoriasis. Other angiogenic disorders can be treated by the methods featured in the invention.

In another aspect, the invention features a kit containing an iRNA agent of the invention. The iRNA agent of the kit can be chemically modified and can be useful for modulating the expression of a VEGF target gene in a cell, tissue or organism. In one embodiment, the kit contains more than one iRNA agent of the invention.

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 pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, useful methods and materials are described below. The materials, methods, and examples are illustrative only and not intended to be limiting. Other features and advantages of the invention will be apparent from the accompanying drawings and description, and from the claims. The contents of all references, pending patent applications and published patents, cited throughout this application are hereby expressly incorporated by reference. In case of conflict, the present specification, including definitions, will control.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 is the nucleotide sequence of the mRNA of the 121 amino acid form of vascular endothelial growth factor, VEGF₁₂₁. The first nucleotide of the initiator codon is nucleotide 1. The signal peptide is from nucleotide 1 through 78.

FIGURE 2 is a graphical representation of a comparative analysis of the activities of single- and double-overhang siRNAs in *in vitro* assays in HeLa cells. Solid lines with filled symbols represent the single-overhang siRNA, solid lines with open symbols represent the double-overhang siRNAs; dashed lines represent the control siRNAs. The control siRNA

hVEGF is described in Reich *et al.* (*Mol. Vis.* 9:210, 2003); the control siRNA hrmVEGF is described in Filleur *et al.* (*Cancer Res.* 63:3919, 2003). “L2000” refers to Lipofectamine 2000 reagent. hVEGF expression (y-axis) refers to endogenous VEGF expression.

FIGURE 3 is a graphical representation of a comparative analysis of the activities of single- and double-overhang siRNAs in ARPE-19 cells. Solid lines with filled symbols represent the single-overhang siRNA; solid lines with open symbols represent the double-overhang siRNAs; dashed lines represent the control siRNAs. The control siRNA hVEGF is described in Reich *et al.* (*Mol. Vis.* 9:210, 2003); the control siRNA hrmVEGF is described in Filleur *et al.* (*supra*). “L2000” refers to Lipofectamine 2000 reagent. hVEGF expression (y-axis) refers to endogenous VEGF expression.

FIGURE 4 is a graphical representation of a comparative analysis of the siRNAs activities in HeLa cells of single-overhang siRNAs with their analogous blunt siRNAs in which the number of base-paired nucleotides is 21. The control siRNA hVEGF is described in Reich *et al.* (*Mol. Vis.* 9:210, 2003); the control siRNA hrmVEGF is described in Filleur *et al.* (*supra*). “L2000” refers to Lipofectamine 2000 reagent. hVEGF expression (y-axis) refers to endogenous VEGF expression.

FIGURE 5 is a graphical representation of a comparative analysis of the siRNAs activities in HeLa cells of double-overhang siRNAs with their analogous blunt siRNAs in which the number of base-paired nucleotides is 19. The control siRNA hVEGF is described in Reich *et al.* (*supra*); the control siRNA hrmVEGF is described in Filleur *et al.* (*supra*). “L2000” refers to Lipofectamine 2000 reagent. hVEGF expression (y-axis) refers to endogenous VEGF expression.

FIGURE 6A is a graphical representation of the activities of single-overhang and double overhang siRNAs targeting ORF 319 (SEQ ID NO:320) (AL-DP-4002 and AL-DP-4014, respectively) and ORF 343 (SEQ ID NO:344) (AL-DP-4094 and AL-DP-4107, respectively) in cells under normal oxygen (normoxia, 20% oxygen).

FIGURE 6B is a graphical representation of the activities of single-overhang and double overhang siRNAs targeting ORF 319 (SEQ ID NO:320) (AL-DP-4002 and AL-DP-4014, respectively) and ORF 343 (SEQ ID NO:344) (AL-DP-4094 and AL-DP-4107, respectively) in cells under hypoxic conditions (1% oxygen).

FIGURE 6C is a graphical representation of the activities of single-overhang and double overhang siRNAs targeting ORF 319 (SEQ ID NO:320) (AL-DP-4002 and AL-DP-4014, respectively) and ORF 343 (SEQ ID NO:344) (AL-DP-4094 and AL-DP-4107, respectively) in cells under hypoxic conditions (130 μ M defoxamine).

5 FIGURE 7 is a graphical representation of the comparative activities of double-overhang (AL-DP-4014) unmodified siRNA and phosphorothioate-modified (AL-DP-4127, AL-DP-4128, AL-DP-4129) siRNAs targeting ORF 319 (SEQ ID NO:320) in HeLa cells. The control siRNA hVEGF is described in Reich *et al. (supra)*; the control siRNA hrmVEGF is described in Filleur *et al. (supra)*. “L2000” refers to Lipofectamine 2000 reagent. hVEGF expression (y-axis) refers
10 to endogenous VEGF expression.

FIGURE 8A is a graphical representation of the activities of siRNAs targeting ORF 319 (SEQ ID NO:320) (AL-DP-4014 and AL-DP-4127) and a mutated version AL-DP-4140 (Table 5) in cells under normal oxygen conditions (normoxia, 20% oxygen). The control siRNA Cand5 is identical to the hVEGF control of FIGURE. 7 and is described in Reich *et al. (supra)*.

15 “L2000” refers to Lipofectamine 2000 reagent. VEGF expression (y-axis) refers to endogenous VEGF expression.

FIGURE 8B is a graphical representation of the activities of siRNAs targeting ORF 319 (SEQ ID NO:320) (AL-DP-4014 and AL-DP-4127) and a mutated version AL-DP-4140 (Table 5) in cells under normal or hypoxic conditions (hypoxia, 1% Oxygen). The control siRNAs are
20 as described for FIGURE. 8A.

FIGURES 9A-9E are graphical representations of the activities of siRNAs having the sequence of AL-DP-4094 but differing in the inclusion of nucleotide modifications (see Table 4). The control siRNA “Acuity” is identical to the Cand5 control of FIGURE. 8A and the hVEGF control of FIGURE. 7. The “Filleur” control siRNA is the equivalent of the hrmVEGF control
25 siRNA of FIGURE. 7.

FIGURE 10 is a graphical representation of siRNA silencing activity *in vitro* in HeLa cells.

FIGURE 11 is an RP-HPLC scan of AL-DP-4094 siRNA following incubation in human serum.

FIGURE 12 is a summary of AL-DP-4094 fragment mapping as determined by LC/MS. The analysis was performed following incubation of the siRNA in human serum.

FIGURES 13-29 are graphs of silencing activity of 2'-O-methyl and/or 2'-fluoro modified siRNAs *in vitro* in HeLa cells (Table 6).

5 FIGURE 30 are graphs of silencing activity of alternating 2'-O-methyl and 2'-fluoro modified siRNAs *in vitro* in HeLa cells (Table 7).

FIGURES 31-33 are graphs of silencing activity of cholesterol and colonic conjugated siRNAs *in vitro* in HeLa cells (Table 8).

10 FIGURE 34 is a graph of silencing activity of naproxen conjugated siRNAs *in vitro* in HeLa cells (Table 9).

FIGURE 35 is a graph of silencing activity of biotin conjugated siRNAs *in vitro* in HeLa cells (Table 10).

FIGURE 36 is a graph of silencing activity of 5'-retinal conjugated siRNAs *in vitro* in HeLa cells (Table 11).

15 FIGURE 37 is a graph of silencing activity of ribo-difluorotoluidyl modified siRNAs *in vitro* in HeLa cells (Table 13).

FIGURE 38 is a graph of silencing activity of 2'-arafluoro-2'-deoxy-nucleoside modified siRNAs *in vitro* in HeLa cells (Table 14).

20 FIGURE 39 5'-O-DMTr-2'-deoxy-2'-fluoro A, C, G and U CPG supports for oligonucleotide synthesis. These supports were used for syntheses of selected sequences listed Tables 6 and 7.

FIGURE 40 Cholesterol and 5 β -cholanic (or cholanic) acid conjugate building blocks for conjugation to oligonucleotides. These building blocks were used for syntheses of selected sequences listed in Table 8.

FIGURE 41 $^{5\text{Me}}\text{C}$ and $^{5\text{Me}}\text{U}$ RNA building blocks for oligonucleotide synthesis. These building blocks were used for syntheses of selected sequences listed in Table 8.

FIGURE 42. Naproxen – *trans*-4- hydroxy-L-prolinol and naproxen-serinol building blocks for conjugation to oligonucleotides. These building blocks were used for syntheses of selected sequences listed in Table 9.

FIGURE 43 Biotin – *trans*-4- hydroxy-L-prolinol and biotin-serinol building blocks for conjugation to oligonucleotides. These building blocks were used for syntheses of selected sequences listed in Table 10.

FIGURE 44 Building blocks for post-synthetic conjugation – Oxime approach. These building blocks were/are used for syntheses of selected sequences listed in Table 11.

FIGURE 45 Building blocks for post-synthetic conjugation – Active ester approach. These building blocks were used for syntheses of selected sequences listed in Table 12.

FIGURE 46 DFT amidite and CPG for oligonucleotide synthesis. These building blocks were used for syntheses of selected sequences listed in Table 13.

FIGURE 47 2'-Deoxy-2'-araF amidite for oligonucleotide synthesis. These building blocks were used for syntheses of selected sequences listed in Table 14.

FIGURE 48 *P*-methylphosphonamidite of ribo $^{5\text{Me}}\text{U}$ and ribo $\text{C}(\text{N}^{\text{Ac}})$. These building blocks were used for syntheses of selected sequences listed in Table 15.

FIGURE 49 C5-aminoallyl U amidite. These building blocks were used for syntheses of selected sequences listed in Table 16.

FIGURE 50 Thiocholesterol conjugate building blocks.

BRIEF DESCRIPTION OF THE TABLES

Table 1 provides the sequences in the VEGF gene that are targeted by the agents of the present invention. These sequence can also be the sense strand of some of the iRNA agents of the present invention.

5 Table 2 provides 123 iRNA duplexes that target the VEGF gene, the target sequence in the VEGF gene and activity data that is described in the Examples.

Table 3 provides iRNA duplexes that are modified to contain phosphorothioate stabilizations and activity data that is described in the Examples.

10 Table 4 provides iRNA duplexes based on the AL-DP-4094 duplex that are modified for stabilization and activity data that is described in the Examples.

Table 5 provides iRNA duplexes activity data in HeLa cells for several iRNA agents of the present invention.

Table 6 provides iRNA agents with activity data in HeLa cells for agents containing one or more phosphorothioate, 2'-O-methyl and 2'-fluoro modifications.

15 Table 7 provides iRNA agents with activity data in HeLa cells for agents containing alternating 2'-O-methyl and 2'-fluoro modifications.

Table 8 A and B provides iRNA agents with activity data in HeLa cells for agents containing cholesterol or cholanolic acid conjugates.

20 Table 9 provides iRNA agents with activity data in HeLa cells for agents containing naproxen conjugates.

Table 10 provides iRNA agents with activity data in HeLa cells for agents containing biotin conjugates.

Table 11 provides iRNA agents containing aldehydes, retinal and other retinoid conjugates.

25 Table 12 provides iRNA agents containing polyethylene glycol conjugates.

Table 13 provides iRNA agents with activity data in HeLa cells for agents containing ribo-difluorotoluyll modifications.

Table 14 provides iRNA agents with activity data in HeLa cells for agents containing 2'-arafluoro-2'-deoxy-nucleoside modifications.

30 Table 15 provides iRNA agents containing methylphosphonate modifications.

Table 16 provides iRNA agents containing C-5 allyamino modifications.

Table 17 provides iRNA agents containing a variety and combinations of the modifications as noted in the Table.

Table 18 provides physical characterization of iRNA agents containing a variety and
5 combinations of the modifications as noted in the Table.

DETAILED DESCRIPTION

Double-stranded (dsRNA) directs the sequence-specific silencing of mRNA through a
10 process known as RNA interference (RNAi). The process occurs in a wide variety of organisms, including mammals and other vertebrates.

It has been demonstrated that 21-23 nt fragments of dsRNA are sequence-specific mediators of RNA silencing, *e.g.*, by causing RNA degradation. While not wishing to be bound by theory, it may be that a molecular signal, which may be merely the specific length of the
15 fragments, present in these 21-23 nt fragments recruits cellular factors that mediate RNAi. Described herein are methods for preparing and administering these 21-23 nt fragments, and other iRNAs agents, and their use for specifically inactivating gene function. The use of iRNA agents (or recombinantly produced or chemically synthesized oligonucleotides of the same or similar nature) enables the targeting of specific mRNAs for silencing in mammalian cells. In
20 addition, longer dsRNA agent fragments can also be used, *e.g.*, as described below.

Although, in mammalian cells, long dsRNAs can induce the interferon response, which is frequently deleterious, siRNAs do not trigger the interferon response, at least not to an extent that is deleterious to the cell and host. In particular, the length of the sense and antisense sequences in an iRNA agent can be less than 31, 30, 28, 25, or 23 nt, *e.g.*, sufficiently short to
25 avoid inducing a deleterious interferon response. Thus, the administration of a composition of iRNA agents (*e.g.*, formulated as described herein) to a mammalian cell can be used to silence expression of a target gene while circumventing the interferon response. Further, use of a discrete species of iRNA agent can be used to selectively target one allele of a target gene, *e.g.*, in a subject heterozygous for the allele.

30 The target-complementary sequence (the antisense sequence) of an iRNA agent, such as an iRNA duplex, can have a 5' phosphate and ATP may be utilized to maintain the 5'-phosphate

moiety on the siRNA (Nykanen *et al.*, *Cell* 107:309, 2001); however, iRNA agents lacking a 5'-phosphate have been shown to be active when introduced exogenously, suggesting that 5'-phosphorylation of siRNA constructs may occur *in vivo*.

Vascular endothelial growth factor (VEGF) 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. The mRNA corresponding to the coding sequence of human VEGF121 (Genbank Accession Number AF214570, SEQ ID NO:1) is shown in FIG. 1. 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. VEGF is also expressed at abnormally high levels in inflammatory diseases such as rheumatoid arthritis and psoriasis, and is involved in the inflammation, airway and vascular remodeling that occurs during asthmatic episodes. Elevated VEGF expression is also correlated with several forms of ocular neovascularization that often lead to severe vision loss, including diabetic retinopathy, retinopathy of prematurity, and macular degeneration.

iRNA Agents An "RNA agent," as used herein, is an unmodified RNA, modified RNA, or nucleoside surrogate. Preferred examples include those which have greater resistance to nuclease degradation than do unmodified RNAs. Preferred examples include those which have a 2' sugar modification, a modification in a single strand overhang, preferably a 3' single strand overhang, or, particularly if single stranded, a 5' modification which includes one or more phosphate groups or one or more analogs of a phosphate group.

An "iRNA agent," as used herein, is an RNA agent which can, or which can be cleaved into an RNA agent which can, down regulate the expression of a target gene, preferably an endogenous or pathogen target RNA. While not wishing to be bound by theory, an iRNA agent may act by one or more of a number of mechanisms, including post-transcriptional cleavage of a target mRNA sometimes referred to in the art as RNAi, or pre-transcriptional or pre-translational mechanisms. An iRNA agent can include a single strand or can include more than one strands, *e.g.*, it can be a double stranded iRNA agent. If the iRNA agent is a single strand it is particularly preferred that it include a 5' modification which includes one or more phosphate groups or one or more analogs of a phosphate group.

The iRNA agent should include a region of sufficient homology to the target gene, and be of sufficient length in terms of nucleotides, such that the iRNA agent, or a fragment thereof, can mediate down regulation of the target gene. (For ease of exposition the term nucleotide or ribonucleotide is sometimes used herein in reference to one or more monomeric subunits of an RNA agent. It will be understood herein that the usage of the term "ribonucleotide" or "nucleotide," herein can, in the case of a modified RNA or nucleotide surrogate, also refer to a modified nucleotide, or surrogate replacement moiety at one or more positions.) Thus, the iRNA agent is or includes a region which is at least partially, and in some embodiments fully, complementary to the target RNA. It is not necessary that there be perfect complementarity between the iRNA agent and the target, but the correspondence must be sufficient to enable the iRNA agent, or a cleavage product thereof, to direct sequence specific silencing, *e.g.*, by RNAi cleavage of the target RNA, *e.g.*, mRNA.

Complementarity, or degree of homology with the target strand, is most critical in the antisense strand. While perfect complementarity, particularly in the antisense strand, is often desired some embodiments can include, particularly in the antisense strand, one or more but

preferably 6, 5, 4, 3, 2, or fewer mismatches (with respect to the target RNA). The mismatches, particularly in the antisense strand, are most tolerated in the terminal regions and if present are preferably in a terminal region or regions, *e.g.*, within 6, 5, 4, or 3 nucleotides of the 5' and/or 3' terminus. The sense strand need only be sufficiently complementary with the antisense strand to maintain the overall double strand character of the molecule.

Single stranded regions of an iRNA agent will often be modified or include nucleoside surrogates, *e.g.*, the unpaired region or regions of a hairpin structure, *e.g.*, a region which links two complementary regions, can have modifications or nucleoside surrogates. Modification to stabilize one or more 3'- or 5'-terminus of an iRNA agent, *e.g.*, against exonucleases, or to favor the antisense sRNA agent to enter into RISC are also favored. Modifications can include C3 (or C6, C7, C12) amino linkers, thiol linkers, carboxyl linkers, non-nucleotidic spacers (C3, C6, C9, C12, abasic, triethylene glycol, hexaethylene glycol), special biotin or fluorescein reagents that come as phosphoramidites and that have another DMT-protected hydroxyl group, allowing multiple couplings during RNA synthesis.

iRNA agents include: molecules that are long enough to trigger the interferon response (which can be cleaved by Dicer (Bernstein *et al.*, *Nature* 409:363-366, 2001)) and enter a RISC (RNAi-induced silencing complex); and molecules that are sufficiently short that they do not trigger the interferon response (which molecules can also be cleaved by Dicer and/or enter a RISC), *e.g.*, molecules which are of a size which allows entry into a RISC, *e.g.*, molecules which resemble Dicer-cleavage products. Molecules that are short enough that they do not trigger an interferon response are termed sRNA agents or shorter iRNA agents herein. "sRNA agent or shorter iRNA agent" as used herein, refers to an iRNA agent, *e.g.*, a double stranded RNA agent or single strand agent, that is sufficiently short that it does not induce a deleterious interferon response in a human cell, *e.g.*, it has a duplexed region of less than 60 but preferably less than 50, 40, or 30 nucleotide pairs. The sRNA agent, or a cleavage product thereof, can down regulate a target gene, *e.g.*, by inducing RNAi with respect to a target RNA, preferably an endogenous or pathogen target RNA.

Each strand of a sRNA agent can be equal to or less than 30, 25, 24, 23, 22, 21, or 20 nucleotides in length. The strand is preferably at least 19 nucleotides in length. For example, each strand can be between 21 and 25 nucleotides in length. Preferred sRNA agents have a

duplex region of 17, 18, 19, 29, 21, 22, 23, 24, or 25 nucleotide pairs, and one or more overhangs, preferably one or two 3' overhangs, of 2- 3 nucleotides.

A "single strand iRNA agent" as used herein, is an iRNA agent which is made up of a single molecule. It may include a duplexed region, formed by intra-strand pairing, *e.g.*, it may be, or include, a hairpin or pan-handle structure. Single strand iRNA agents are preferably antisense with regard to the target molecule. In preferred embodiments single strand iRNA agents are 5' phosphorylated or include a phosphoryl analog at the 5' prime terminus. 5'-phosphate modifications include those which are compatible with RISC mediated gene silencing. Suitable modifications include: 5'-monophosphate ((HO)₂(O)P-O-5'); 5'-diphosphate ((HO)₂(O)P-O-P(HO)(O)-O-5'); 5'-triphosphate ((HO)₂(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-guanosine cap (7-methylated or non-methylated) (7m-G-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-adenosine cap (A_{ppp}), and any modified or unmodified nucleotide cap structure (N-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-monothiophosphate (phosphorothioate; (HO)₂(S)P-O-5'); 5'-monodithiophosphate (phosphorodithioate; (HO)(HS)(S)P-O-5'), 5'-phosphorothiolate ((HO)₂(O)P-S-5'); any additional combination of oxygen/sulfur replaced monophosphate, diphosphate and triphosphates (*e.g.* 5'-alpha-thiotriphosphate, 5'-gamma-thiotriphosphate, etc.), 5'-phosphoramidates ((HO)₂(O)P-NH-5', (HO)(NH₂)(O)P-O-5'), 5'-alkylphosphonates (R=alkyl=methyl, ethyl, isopropyl, propyl, etc., *e.g.* RP(OH)(O)-O-5', (OH)₂(O)P-5'-CH₂-), 5'-alkyletherphosphonates (R=alkylether=methoxymethyl (MeOCH₂-), ethoxymethyl, etc., *e.g.* RP(OH)(O)-O-5'-). (These modifications can also be used with the antisense strand of a double stranded iRNA.)

A single strand iRNA agent should be sufficiently long that it can enter the RISC and participate in RISC mediated cleavage of a target mRNA. A single strand iRNA agent is at least 14, and more preferably at least 15, 20, 25, 29, 35, 40, or 50 nucleotides in length. It is preferably less than 200, 100, or 60 nucleotides in length.

Hairpin iRNA agents will have a duplex region equal to or at least 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotide pairs. The duplex region will preferably be equal to or less than 200, 100, or 50, in length. Preferred ranges for the duplex region are 15-30, 17 to 23, 19 to 23, and 19 to 21 nucleotides pairs in length. The hairpin will preferably have a single strand overhang or

terminal unpaired region, preferably the 3', and preferably of the antisense side of the hairpin. Preferred overhangs are 2-3 nucleotides in length.

A "double stranded (ds) iRNA agent" as used herein, is an iRNA agent which includes more than one, and preferably two, strands in which interchain hybridization can form a region of duplex structure.

Other suitable modifications to a sugar, base, or backbone of an iRNA agent are described in co-owned PCT Application No. PCT/US2004/01193, filed January 16, 2004. An iRNA agent can include a non-naturally occurring base, such as the bases described in co-owned PCT Application No. PCT/US2004/011822, filed April 16, 2004. An iRNA agent can include a non-naturally occurring sugar, such as a non-carbohydrate cyclic carrier molecule. Exemplary features of non-naturally occurring sugars for use in iRNA agents are described in co-owned PCT Application No. PCT/US2004/11829 filed April 16, 2003.

An iRNA agent can include an internucleotide linkage (*e.g.*, the chiral phosphorothioate linkage) useful for increasing nuclease resistance. In addition, or in the alternative, an iRNA agent can include a ribose mimic for increased nuclease resistance. Exemplary internucleotide linkages and ribose mimics for increased nuclease resistance are described in co-owned PCT Application No. PCT/US2004/07070 filed on March 8, 2004.

An iRNA agent can have a ZXY structure, such as is described in co-owned PCT Application No. PCT/US2004/07070 filed on March 8, 2004.

An iRNA agent can be complexed with an amphipathic moiety. Exemplary amphipathic moieties for use with iRNA agents are described in co-owned PCT Application No. PCT/US2004/07070 filed on March 8, 2004.

In another embodiment, the iRNA agent can be complexed to a delivery agent that features a modular complex. The complex can include a carrier agent linked to one or more of (preferably two or more, more preferably all three of): (a) a condensing agent (*e.g.*, an agent capable of attracting, *e.g.*, binding, a nucleic acid, *e.g.*, through ionic or electrostatic interactions); (b) a fusogenic agent (*e.g.*, an agent capable of fusing and/or being transported through a cell membrane); and (c) a targeting group, *e.g.*, a cell or tissue targeting agent, *e.g.*, a lectin, glycoprotein, lipid or protein, *e.g.*, an antibody, that binds to a specified cell type. iRNA

agents complexed to a delivery agent are described in co-owned PCT Application No. PCT/US2004/07070 filed on March 8, 2004.

An iRNA agent can have non-canonical pairings, such as between the sense and antisense sequences of the iRNA duplex. Exemplary features of non-canonical iRNA agents are described in co-owned PCT Application No. PCT/US2004/07070 filed on March 8, 2004.

Many of these types of modifications are provided in the Examples and are described in Tables 3-18.

Design of iRNA

The present invention is based on a gene walk of the VEGF gene to identify active iRNA agents that can be used to reduce the level of VEGF mRNA in a cell. Not all potential iRNA agent sequences in the VEGF gene are active, many of which also having significant off-target effects. The present invention advances the art by selecting those sequences which are active and do not have significant off-target effects. Further, the sequence chosen for the iRNA agents of the present invention are conserved amongst multiple species allowing one to use a single agent for animal and toxicological studies as well as using it for therapeutic purposes in humans.

Based on these results, the invention specifically provides an iRNA agent that can be used in treating VEGF mediated disorders, particularly in the eye such as AMD, in isolated form and as a pharmaceutical composition described below. Such agents will include a sense strand having at least 15 or more contiguous nucleotides that are complementary to the VEGF gene and an antisense strand having at least 15 or more contiguous nucleotides that are complementary to the sense strand sequence. Particularly useful are iRNA agents that have a sense strand that comprises, consist essentially of or consists of a nucleotide sequence provided in Table 1, such as those agents proved in Table 2, or any of the modifications provided in Tables 3-18.

Candidate iRNA agents can be designed by performing, as done herein, a gene walk analysis of the VEGF gene that will serve as the iRNA target. Overlapping, adjacent, or closely spaced candidate agents corresponding to all or some of the transcribed region can be generated and tested. Each of the iRNA agents can be tested and evaluated for the ability to down regulate the target gene expression (see below, "Evaluation of Candidate iRNA agents").

Preferably, the iRNA agents of the present invention are based on and comprise at least 15 or more contiguous nucleotides from one of the iRNA agents shown to be active in Table 2, or the modified sequences provided in Tables 3-18. In such agents, the agent can comprise, consist of or consist essentially of the entire sequence provided in the Table or can comprise 15 or more
5 contiguous residues along with additional nucleotides from contiguous regions of the target gene.

An iRNA agent can be rationally designed based on sequence information and desired characteristics and the information of the target sequence provided in Table 1. For example, an iRNA agent can be designed according to the relative melting temperature of the candidate duplex. Generally, the duplex should have a lower melting temperature at the 5' end of the
10 antisense strand than at the 3' end of the antisense strand.

Accordingly, the present invention provides iRNA agents comprising a sense strand and antisense strand each comprising a sequence of at least 15, 16, 17, 18, 19, 20, 21 or 23 nucleotides which is essentially identical to one of the agents provided in Table 1 or 2.

The antisense strand of an iRNA agent should be equal to or at least, 15, 16 17, 18, 19,
15 25, 29, 40, or 50 nucleotides in length. It should be equal to or less than 50, 40, or 30, nucleotides in length. Preferred ranges are 15-30, 17 to 25, 19 to 23, and 19 to 21 nucleotides in length. Exemplified iRNA agents include those that comprise 15 or more nucleotides from one of the agents in Table 2 (or are complementary to the target sequence provided in Table 1) but are not longer than 25 nucleotides in length.

The sense strand of an iRNA agent should be equal to or at least 15, 16 17, 18, 19, 25, 29,
20 40, or 50 nucleotides in length. It should be equal to or less than 50, 40, or 30 nucleotides in length. Preferred ranges are 15-30, 17 to 25, 19 to 23, and 19 to 21 nucleotides in length. Exemplified iRNA agents include those that comprise 15 or more nucleotides from one of the agents in Table 2 (or the target sequence in Table 2) but are not longer than 25 nucleotides in
25 length.

The double stranded portion of an iRNA agent should be equal to or at least, 15, 16 17, 18, 19, 20, 21, 22, 23, 24, 25, 29, 40, or 50 nucleotide pairs in length. It should be equal to or less than 50, 40, or 30 nucleotides pairs in length. Preferred ranges are 15-30, 17 to 25, 19 to 23, and 19 to 21 nucleotides pairs in length.

The agents provided in Table 2 are 23 nucleotides in length for each strand. The iRNA agents contain a 21 nucleotide double stranded region with a 2 nucleotide overhang on each of the 3' ends of the agent. These agents can be modified as described herein to obtain equivalent agents comprising at least a portion of these sequences (15 or more contiguous nucleotides) and
5 or modifications to the oligonucleotide bases and linkages. Particularly preferred are the modification and agents provided in Tables 3-18.

Generally, the iRNA agents of the instant invention include a region of sufficient complementarity to the VEGF gene and are of sufficient length in terms of nucleotides that the iRNA agent, or a fragment thereof, can mediate down regulation of the VEGF gene. The
10 antisense strands of the iRNA agents of the present invention are preferably fully complementary to the mRNA sequences of VEGF gene. However, it is not necessary that there be perfect complementarity between the iRNA agent and the target, but the correspondence must be sufficient to enable the iRNA agent, or a cleavage product thereof, to direct sequence specific silencing, *e.g.*, by RNAi cleavage of a VEGF mRNA.

Therefore, the iRNA agents of the instant invention include agents comprising a sense strand and antisense strand each comprising a sequence of at least 16, 17 or 18 nucleotides which is essentially identical, as defined below, to one of the sequences of the VEGF gene, such as those agent provided in Table 2, except that not more than 1, 2 or 3 nucleotides per strand, respectively, have been substituted by other nucleotides (*e.g.* adenosine replaced by uracil), while
20 essentially retaining the ability to inhibit VEGF expression. These agents will therefore possess at least 15 or more nucleotides identical to the VEGF gene but 1, 2 or 3 base mismatches with respect to either the VEGF mRNA sequence or between the sense and antisense strand are introduced. Mismatches to the target VEGF mRNA sequence, particularly in the antisense strand, are most tolerated in the terminal regions and if present are preferably in a terminal
25 region or regions, *e.g.*, within 6, 5, 4, or 3 nucleotides of a 5' and/or 3' terminus, most preferably within 6, 5, 4, or 3 nucleotides of the 5'-terminus of the sense strand or the 3'-terminus of the antisense strand. The sense strand need only be sufficiently complementary with the antisense strand to maintain the overall double stranded character of the molecule.

It is preferred that the sense and antisense strands be chosen such that the iRNA agent
30 includes a single strand or unpaired region at one or both ends of the molecule, such as those

exemplified in Table 2 (as well as Tables 3-18). Thus, an iRNA agent contains sense and antisense strands, preferably paired to contain an overhang, *e.g.*, one or two 5' or 3' overhangs but preferably a 3' overhang of 2-3 nucleotides. Most embodiments will have a 3' overhang. Preferred siRNA agents will have single-stranded overhangs, preferably 3' overhangs, of 1 to 4, or preferably 2 or 3 nucleotides, in length, on one or both ends of the iRNA agent. The overhangs can be the result of one strand being longer than the other, or the result of two strands of the same length being staggered. 5'-ends are preferably phosphorylated.

Preferred lengths for the duplexed region is between 15 and 30, most preferably 18, 19, 20, 21, 22, and 23 nucleotides in length, *e.g.*, in the siRNA agent range discussed above.

Embodiments in which the two strands of the siRNA agent are linked, *e.g.*, covalently linked are also included. Hairpin, or other single strand structures which provide the required double stranded region, and preferably a 3' overhang are also within the invention.

Synthesis of iRNA Agents Oligonucleotides (*e.g.*, certain modified oligonucleotides or portions of oligonucleotides lacking ribonucleotides) can be synthesized using protocols known in the art, for example as described in Caruthers *et al.*, *Methods in Enzymology* 211:3, 1992; Thompson *et al.*, International PCT Publication No. WO 99/54459; Wincott *et al.*, *Nucleic Acids Res.* 23:2677, 1995; Wincott *et al.*, *Methods Mol. Bio.* 74:59, 1997; Brennan *et al.*, *Biotechnol. Bioeng.* 61:33, 1998; and Brennan, U. S. Pat. No. 6,001,311. All of these references are incorporated herein by reference. The synthesis of oligonucleotides makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end.

The method of synthesis used for RNA including certain iRNA agents of the invention follows the procedure as described in Usman *et al.*, *J. Chem. Soc.* 109:7845, 1987; Scaringe *et al.*, *Nucleic Acids Res.* 18:5433, 1990; Wincott *et al.*, *Nucleic Acids Res.* 23:2677, 1995; and Wincott *et al.*, *Methods Mol. Bio.* 74:59, 1997; and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. Detailed descriptions of a variety of synthetic methods to produce modified iRNA agents are provided in the Examples.

Alternatively, the nucleic acid molecules of the present invention can be synthesized separately and joined together post-synthetically, for example, by ligation (Moore *et al.*, *Science* 256:9923, 1992; Draper *et al.*, International PCT publication No. WO 93/23569; Shabarova *et al.*, *Nucleic Acids Res.* 19:4247, 1991; Bellon *et al.*, *Nucleosides & Nucleotides* 16:951, 1997; 5 Bellon *et al.*, *Bioconjugate* 8:204, 1997), or by hybridization following synthesis and/or deprotection.

An iRNA agent can also be assembled from two distinct nucleic acid sequences or fragments wherein one fragment includes the sense region and the second fragment includes the antisense region of the iRNA agent.

10 iRNA agents can be modified extensively to enhance stability by modification with nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-fluoro, difluorotoluy, 5-allylamino-pyrimidines, 2'-O-methyl, 2'-H (for a review see Usman and Cedergren, *Trends in Biochem. Sci.* 17:34, 1992). iRNA constructs can be purified by gel electrophoresis using general methods or can be purified by high pressure liquid chromatography (HPLC; see Wincott *et al.*, 15 *supra*, the totality of which is hereby incorporated herein by reference) and re-suspended in water.

In another aspect of the invention, iRNA agents can be expressed from transcription units inserted into DNA or RNA vectors. The recombinant vectors can be DNA plasmids or viral vectors. iRNA agent-expressing viral vectors can be constructed based on, but not limited to, 20 adeno-associated virus, retrovirus, adenovirus, or alphavirus. The recombinant vectors capable of expressing the iRNA agents can be delivered as described herein, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of iRNA agents.

Evaluating iRNA agents Any of the iRNA agents described herein can be evaluated and 25 modified as follows.

An iRNA agent may be susceptible to cleavage by an endonuclease or exonuclease, such as when the iRNA agent is introduced into the body of a subject. Methods can be used to determine sites of cleavage, *e.g.*, endo- and exonucleolytic cleavage on an iRNA agent and to determine the mechanism of cleavage. An iRNA agent can be modified to inhibit such cleavage.

A dsRNA, *e.g.*, an iRNA agent, can be evaluated to identify sites that are susceptible to modification, particularly cleavage, *e.g.*, cleavage by a component found in the body of a subject. The component can be specific for a particular area of the body, such as a particular tissue, organ, or bodily fluid (*e.g.*, blood, plasma, or serum). Sites in an iRNA agent that are susceptible to cleavage, either by endonucleolytic or exonucleolytic cleavage, in certain areas of the body, may be resistant to cleavage in other areas of the body.

A method for evaluating an iRNA agent can include: (1) determining the point or points at which a substance present in the body of a subject, and preferably a component present in a compartment of the body into which a therapeutic dsRNA is to be introduced (this includes compartments into which the therapeutic is directly introduced, *e.g.*, the circulation, as well as in compartments to which the therapeutic is eventually targeted, *e.g.*, the liver or kidney; in some cases, *e.g.*, the eye, the two are the same), cleaves a dsRNA, *e.g.*, an iRNA agent; and (2) identifying one or more points of cleavage, *e.g.*, endonucleolytic, exonucleolytic, or both, in the dsRNA. Optionally, the method further includes providing an RNA (*e.g.*, an iRNA agent) modified to inhibit cleavage at such sites.

The steps described above can be accomplished by using one or more of the following assays:

(i) (a) contacting a candidate dsRNA, *e.g.*, an iRNA agent, with a test agent (*e.g.*, a biological agent),

(b) using a size-based assay, *e.g.*, gel electrophoresis to determine if the iRNA agent is cleaved. In a preferred embodiment a time course is taken and a number of samples incubated for different times are applied to the size-based assay. In preferred embodiments, the candidate dsRNA is not labeled. The method can be a "stains all" method.

(ii) (a) supplying a candidate dsRNA, *e.g.*, an iRNA agent, which is radiolabeled;

(b) contacting the candidate dsRNA with a test agent,

(c) using a size-based assay, *e.g.*, gel electrophoresis to determine if the iRNA agent is cleaved. In a preferred embodiment, a time course is taken where a number of samples are incubated for different times and applied to the size-based assay. In preferred embodiments the determination is made under conditions that allow determination of the number of

nucleotides present in a fragment. For example, an incubated sample is run on a gel having

markers that allow assignment of the length of cleavage products. The gel can include a standard that is a "ladder" digestion. Either the sense or antisense strand can be labeled. Preferably only one strand is labeled in a particular experiment. The label can be incorporated at the 5' end, 3' end, or at an internal position. Length of a fragment (and thus the point of cleavage) can be determined from the size of the fragment based on the ladder and mapping using a site-specific endonuclease such as RNase T1.

(iii) Fragments produced by any method, *e.g.*, one described herein, *e.g.*, one of those above, can be analyzed by mass spectrometry. Following contacting the iRNA with the test agent, the iRNA can be purified (*e.g.*, partially purified), such as by phenol-chloroform extraction followed by precipitation. Liquid chromatography can then be used to separate the fragments and mass spectrometry can be used to determine the mass of each fragment. This allows determination of the mechanism of cleavage, *e.g.*, if by direct phosphate cleavage, such as by 5' or 3' exonuclease cleavage, or mediated by the 2'OH via formation of a cyclic phosphate.

In another embodiment, the information relating to a site of cleavage is used to select a backbone atom, a sugar or a base, for modification, *e.g.*, a modification to decrease cleavage.

Exemplary modifications include modifications that inhibit endonucleolytic degradation, including the modifications described herein. Particularly favored modifications include: 2' modification, *e.g.*, a 2'-O-methylated nucleotide or 2'-deoxy nucleotide (*e.g.*, 2'-deoxycytidine), or a 2'-fluoro, difluorotoluidyl, 5-Me-2'-pyrimidines, 5-allylamino-pyrimidines, 2'-O-methoxyethyl, 2'-hydroxy, or 2'-ara-fluoro nucleotide, or a locked nucleic acid (LNA), extended nucleic acid (ENA), hexose nucleic acid (HNA), or cyclohexene nucleic acid (CeNA). In one embodiment, the 2' modification is on the uridine of at least one 5'-uridine-adenine-3' (5'-UA-3') dinucleotide, at least one 5'-uridine-guanine-3' (5'-UG-3') dinucleotide, at least one 5'-uridine-uridine-3' (5'-UU-3') dinucleotide, or at least one 5'-uridine-cytidine-3' (5'-UC-3') dinucleotide, or on the cytidine of at least one 5'-cytidine-adenine-3' (5'-CA-3') dinucleotide, at least one 5'-cytidine-cytidine-3' (5'-CC-3') dinucleotide, or at least one 5'-cytidine-uridine-3' (5'-CU-3') dinucleotide. The 2' modification can also be applied to all the pyrimidines in an iRNA agent. In one preferred embodiment, the 2' modification is a 2'OMe modification on the sense strand of an iRNA agent. In a more preferred embodiment the 2' modification is a 2' fluoro modification, and the 2' fluoro is on the sense or antisense strand or on both strands.

Modification of the backbone, *e.g.*, with the replacement of an O with an S, in the phosphate backbone, *e.g.*, the provision of a phosphorothioate modification can be used to inhibit endonuclease activity. In some embodiments, an iRNA agent has been modified by replacing one or more ribonucleotides with deoxyribonucleotides. Preferably, adjacent

5 deoxyribonucleotides are joined by phosphorothioate linkages, and the iRNA agent does not include more than four consecutive deoxyribonucleotides on the sense or the antisense strands. Replacement of the U with a C5 amino linker; replacement of an A with a G (sequence changes are preferred to be located on the sense strand and not the antisense strand); or modification of the sugar at the 2', 6', 7', or 8' position can also inhibit endonuclease cleavage of the iRNA

10 agent. Preferred embodiments are those in which one or more of these modifications are present on the sense but not the antisense strand, or embodiments where the antisense strand has fewer of such modifications.

Exemplary modifications also include those that inhibit degradation by exonucleases. Examples of modifications that inhibit exonucleolytic degradation can be found herein. In one

15 embodiment, an iRNA agent includes a phosphorothioate linkage or P-alkyl modification in the linkages between one or more of the terminal nucleotides of an iRNA agent. In another embodiment, one or more terminal nucleotides of an iRNA agent include a sugar modification, *e.g.*, a 2' or 3' sugar modification. Exemplary sugar modifications include, for example, a 2'-O-methylated nucleotide, 2'-deoxy nucleotide (*e.g.*, deoxy-cytidine), 2'-deoxy-2'-fluoro (2'-F)

20 nucleotide, 2'-O-methoxyethyl (2'-O-MOE), 2'-O-aminopropyl (2'-O-AP), 2'-O-N-methylacetamido (2'-O-NMA), 2'-O-dimethylaminoethoxyethyl (2'-DMAEOE), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-AP), 2'-hydroxy nucleotide, or a 2'-ara-fluoro nucleotide, or a locked nucleic acid (LNA), extended nucleic acid (ENA), hexose nucleic acid (HNA), or cyclohexene nucleic acid (CeNA). A 2' modification is

25 preferably 2'OMe, more preferably, 2'fluoro.

The modifications described to inhibit exonucleolytic cleavage can be combined onto a single iRNA agent. For example, in one embodiment, at least one terminal nucleotide of an iRNA agent has a phosphorothioate linkage and a 2' sugar modification, *e.g.*, a 2'F or 2'OMe modification. In another embodiment, at least one terminal nucleotide of an iRNA agent has a 5'

30 Me-pyrimidine and a 2' sugar modification, *e.g.*, a 2'F or 2'OMe modification.

To inhibit exonuclease cleavage, an iRNA agent can include a nucleobase modification, such as a cationic modification, such as a 3'-abasic cationic modification. The cationic modification can be, *e.g.*, an alkylamino-dT (*e.g.*, a C6 amino-dT), an allylamino conjugate, a pyrrolidine conjugate, a pthalamido or a hydroxyprolinol conjugate, on one or more of the terminal nucleotides of the iRNA agent. An alkylamino-dT conjugate is preferably attached to the 3' end of the sense or antisense strand of an iRNA agent. A pyrrolidine linker is preferably attached to the 3' or 5' end of the sense strand, or the 3' end of the antisense strand. An allyl amine uridine is preferably on the 3' or 5' end of the sense strand, and not on the 5' end of the antisense strand.

In another embodiment, the iRNA agent includes a conjugate on one or more of the terminal nucleotides of the iRNA agent. The conjugate can be, for example, a lipophile, a terpene, a protein binding agent, a vitamin, a carbohydrate, a retinoid, or a peptide. For example, the conjugate can be naproxen, nitroindole (or another conjugate that contributes to stacking interactions), folate, ibuprofen, cholesterol, retinoids, PEG, or a C5 pyrimidine linker. In other embodiments, the conjugates are glyceride lipid conjugates (*e.g.* a dialkyl glyceride derivatives), vitamin E conjugates, or thio-cholesterols. Preferably, conjugates are on the 3' end of the antisense strand, or on the 5' or 3' end of the sense strand, and preferably the conjugates are not on the 3' end of the antisense strand and on the 3' end of the sense strand.

In one embodiment, the conjugate is naproxen, and the conjugate is preferably on the 5' or 3' end of the sense or antisense strands. In one embodiment, the conjugate is cholesterol, and the conjugate is preferably on the 5' or 3' end of the sense strand and preferably not present on the antisense strand. In some embodiments, the cholesterol is conjugated to the iRNA agent by a pyrrolidine linker, or serinol linker, aminooxy, or hydroxyprolinol linker. In other embodiments, the conjugate is a dU-cholesterol, or cholesterol is conjugated to the iRNA agent by a disulfide linkage. In another embodiment, the conjugate is cholanic acid, and the cholanic acid is attached to the 5' or 3' end of the sense strand, or the 3' end of the antisense strand. In one embodiment, the cholanic acid is attached to the 3' end of the sense strand and the 3' end of the antisense strand. In another embodiment, the conjugate is PEG5, PEG20, naproxen or retinal.

In another embodiment, one or more terminal nucleotides have a 2'-5' linkage. Preferably, a 2'-5' linkage occurs on the sense strand, *e.g.*, the 5' end of the sense strand.

In one embodiment, the iRNA agent includes an L-sugar, preferably at the 5' or 3' end of the sense strand.

In one embodiment, the iRNA agent includes a methylphosphonate at one or more terminal nucleotides to enhance exonuclease resistance, *e.g.*, at the 3' end of the sense or
5 antisense strands of the iRNA agent.

In one embodiment, an iRNA agent has been modified by replacing one or more ribonucleotides with deoxyribonucleotides. Preferably, adjacent deoxyribonucleotides are joined by phosphorothioate linkages, and the iRNA agent does not include more than four consecutive deoxyribonucleotides on the sense or the antisense strands.

10 In some embodiments, an iRNA agent having increased stability in cells and biological samples includes a difluorotoluy (DFT) modification, *e.g.*, 2,4-difluorotoluy uracil, or a guanidine to inosine substitution.

The methods described can be used to select and/or optimize a therapeutic dsRNA, *e.g.*, iRNA agent. dsRNAs, *e.g.*, iRNA agents, made by a method described herein are within the
15 invention.

The methods can be used to evaluate a candidate dsRNA, *e.g.*, a candidate iRNA agent, which is unmodified or which includes a modification, *e.g.*, a modification that inhibits degradation, targets the dsRNA molecule, or modulates hybridization. Such modifications are described herein. A cleavage assay can be combined with an assay to determine the ability of a
20 modified or non-modified candidate to silence the target. For example, one might (optionally) test a candidate to evaluate its ability to silence a target (or off-target sequence), evaluate its susceptibility to cleavage, modify it (*e.g.*, as described herein, *e.g.*, to inhibit degradation) to produce a modified candidate, and test the modified candidate for one or both of the ability to silence and the ability to resist degradation. The procedure can be repeated. Modifications can
25 be introduced one at a time or in groups. It will often be convenient to use a cell-based method to monitor the ability to silence a target RNA. This can be followed by a different method, *e.g.*, a whole animal method, to confirm activity.

The invention includes using information on cleavage sites obtained by a method described herein to modify a dsRNA, *e.g.*, an iRNA agent.

Optimizing the activity of the nucleic acid molecules of the invention Chemically synthesizing nucleic acid molecules with modifications (base, sugar and/or phosphate) can prevent their degradation by serum ribonucleases, which can increase their potency (see *e.g.*, Eckstein *et al.*, International Publication No. WO 92/07065; Perrault *et al.*, *Nature* 344:565, 5 1990; Phieken *et al.*, *Science* 253:314, 1991; Usman and Cedergren, *Trends in Biochem. Sci.* 17:334, 1992; Usman *et al.*, International Publication No. WO 93/15187; and Rossi *et al.*, International Publication No. WO 91/03162; Sproat, U.S. Pat. No. 5,334,711; Gold *et al.*, U.S. Pat. No. 6,300,074; and Burgin *et al.*, *supra* ; all of which are incorporated by reference herein). All of the above references describe various chemical modifications that can be made to the base, 10 phosphate and/or sugar moieties of the nucleic acid molecules described herein. Modifications that enhance their efficacy in cells, and removal of bases from nucleic acid molecules to shorten oligonucleotide synthesis times and reduce chemical requirements are desired.

Other suitable modifications to a sugar, base, or backbone of an iRNA agent are described elsewhere herein.

15 There are several examples in the art describing sugar, base and phosphate modifications that can be introduced into nucleic acid molecules with significant enhancement in their nuclease stability and efficacy. For example, oligonucleotides are modified to enhance stability and/or enhance biological activity by modification with nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-fluoro, 2'-O-methyl, 2'-O-allyl, 2'-H, nucleotide base modifications (for a 20 review see Usman and Cedergren, *Trends in Biochem. Sci.* 17:34, 1992; Usman *et al.*, *Nucleic Acids Symp. Ser.* 31:163, 1994; Burgin *et al.*, *Biochemistry* 35:14090, 1996). Sugar modification of nucleic acid molecules have been extensively described in the art (see Eckstein *et al.*, International Publication PCT No. WO 92/07065; Perrault *et al.*, *Nature* 344:565, 1990; Phieken *et al.*, *Science* 253:314, 1991; Usman and Cedergren, *Trends in Biochem. Sci.* 17:334, 1992; 25 Usman *et al.*, International Publication PCT No. WO93/15187; Sproat, U.S. Pat. No. 5,334,711, and Beigelman *et al.*, *J. Biol. Chem.* 270:25702, 1995; Beigelman *et al.*, International PCT publication No. WO 97/26270; Beigelman *et al.*, U.S. Pat. No. 5,716,824; Usman *et al.*, U.S. Pat. No. 5,627,053; Woolf *et al.*, International PCT Publication No. WO 98/13526; Karpeisky *et al.*, *Tetrahedron Lett.* 39:1131, 1998; Earnshaw and Gait, *Biopolymers (Nucleic Acid Sciences)* 30 48:39, 1998; Verma and Eckstein, *Annu. Rev. Biochem.* 67:99, 1998; and Burlina *et al.*, *Bioorg.*

Med. Chem. 5:1999, 1997; all of the references are hereby incorporated in their totality by reference herein). Such publications describe general methods and strategies to determine the location of incorporation of sugar, base and/or phosphate modifications and the like into nucleic acid molecules without modulating catalysis, and are incorporated by reference herein. In view of such teachings, similar modifications can be used as described herein to modify the iRNA nucleic acid molecules of the instant invention so long as the ability of iRNA agents to promote RNAi in cells is not significantly inhibited.

While chemical modification of oligonucleotide internucleotide linkages with phosphorothioate, phosphorodithioate, and/or 5'-methylphosphonate linkages improves stability, excessive modifications can cause some toxicity or decreased activity. Therefore, when designing nucleic acid molecules, the amount of these internucleotide linkages should be minimized. The reduction in the concentration of these linkages should lower toxicity, resulting in increased efficacy and higher specificity of these molecules.

The 3' and 5' ends of an iRNA agent can be modified. Such modifications can be at the 3' end, 5' end or both ends of the molecule. They can include modification or replacement of an entire terminal phosphate or of one or more of the atoms of the phosphate group. For example, the 3' and 5' ends of an oligonucleotide can be conjugated to other functional molecular entities such as labeling moieties, *e.g.*, fluorophores (*e.g.*, pyrene, TAMRA, fluorescein, Cy3 or Cy5 dyes) or protecting groups (based *e.g.*, on sulfur, silicon, boron or ester). The functional molecular entities can be attached to the sugar through a phosphate group and/or a spacer. The terminal atom of the spacer can connect to or replace the linking atom of the phosphate group or the C-3' or C-5' O, N, S or C group of the sugar. Alternatively, the spacer can connect to or replace the terminal atom of a nucleotide surrogate (*e.g.*, PNAs). These spacers or linkers can include *e.g.*, $-(CH_2)_n-$, $-(CH_2)_nN-$, $-(CH_2)_nO-$, $-(CH_2)_nS-$, $O(CH_2CH_2O)_nCH_2CH_2OH$ (*e.g.*, $n = 3$ or 6), abasic sugars, amide, carboxy, amine, oxyamine, oxyimine, thioether, disulfide, thiourea, sulfonamide, or morpholino, or biotin and fluorescein reagents. When a spacer/phosphate-functional molecular entity-spacer/phosphate array is interposed between two sequences of an iRNA agent, the array can substitute for a hairpin RNA loop in a hairpin-type RNA agent. The 3' end can be an $-OH$ group. While not wishing to be bound by theory, it is believed that conjugation of certain moieties can improve transport, hybridization, and

specificity properties. Again, while not wishing to be bound by theory, it may be desirable to introduce terminal alterations that improve nuclease resistance. Other examples of terminal modifications include dyes, intercalating agents (*e.g.*, acridines), cross-linkers (*e.g.*, psoralene, mitomycin C), porphyrins (TPPC4, texaphyrin, Sapphyrin), polycyclic aromatic hydrocarbons (*e.g.*, phenazine, dihydrophenazine), artificial endonucleases (*e.g.*, EDTA), lipophilic carriers (*e.g.*, cholesterol, cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-Bis-O(hexadecyl)glycerol, geranyloxyhexyl group, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, myristic acid, O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine) and peptide conjugates (*e.g.*, antennapedia peptide, Tat peptide), alkylating agents, phosphate, amino, mercapto, PEG (*e.g.*, PEG-40K), MPEG, [MPEG]₂, polyamino, alkyl, substituted alkyl, radiolabeled markers, enzymes, haptens (*e.g.*, biotin), transport/absorption facilitators (*e.g.*, aspirin, vitamin E, folic acid), and synthetic ribonucleases (*e.g.*, imidazole, bisimidazole, histamine, imidazole clusters, acridine-imidazole conjugates, Eu³⁺ complexes of tetraazamacrocycles). In some embodiments, conjugates such as retinol or retinoic acid can be attached to the 5' or 3' end, or both ends, of an iRNA agent. Use of such conjugates may improve specific uptake and delivery of iRNA agents to cells that express retinol receptors, such as retinal pigment epithelial cells.

Terminal modifications can be added for a number of reasons, such as to modulate activity or to modulate resistance to degradation. Terminal modifications useful for modulating activity include modification of the 5' end with phosphate or phosphate analogs. For example, in preferred embodiments iRNA agents, especially antisense sequences, are 5' phosphorylated or include a phosphoryl analog at the 5' prime terminus. 5'-phosphate modifications include those which are compatible with RISC mediated gene silencing. Suitable modifications include: 5'-monophosphate ((HO)₂(O)P-O-5'); 5'-diphosphate ((HO)₂(O)P-O-P(HO)(O)-O-5'); 5'-triphosphate ((HO)₂(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-guanosine cap (7-methylated or non-methylated) (7m-G-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-adenosine cap (Appp), and any modified or unmodified nucleotide cap structure (N-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-monothio phosphate (phosphorothioate; (HO)₂(S)P-O-5'); 5'-monodithio phosphate (phosphorodithioate; (HO)(HS)(S)P-O-5'), 5'-phosphorothiolate ((HO)₂(O)P-S-5'); any additional combination of oxygen/sulfur replaced monophosphate,

diphosphate and triphosphates (*e.g.*, 5'-alpha-thiotriphosphate, 5'-gamma-thiotriphosphate, *etc.*), 5'-phosphoramidates ((HO)2(O)P-NH-5', (HO)(NH2)(O)P-O-5'), 5'-alkylphosphonates (R=alkyl=methyl, ethyl, isopropyl, propyl, *etc.*, *e.g.*, RP(OH)(O)-O-5', (OH)2(O)P-5'-CH2-), 5'-alkyletherphosphonates (R=alkylether=methoxymethyl (MeOCH2-), ethoxymethyl, *etc.*, *e.g.*, RP(OH)(O)-O-5'-).

In another embodiment, the invention features conjugates and/or complexes of iRNA agents of the invention. Such conjugates and/or complexes can be used to facilitate delivery of iRNA agents into a biological system, such as a cell. The conjugates and complexes provided by the instant invention can impart therapeutic activity by transferring therapeutic compounds across cellular membranes, altering the pharmacokinetics, and/or modulating the localization of nucleic acid molecules of the invention. The present invention encompasses the design and synthesis of novel conjugates and complexes for the delivery of molecules, including, but not limited to, small molecules, lipids, phospholipids, nucleosides, nucleotides, nucleic acids, antibodies, toxins, negatively charged polymers and other polymers, for example, proteins, peptides, hormones, carbohydrates, polyethylene glycols, or polyamines, across cellular membranes. In general, the transporters described are designed to be used either individually or as part of a multi-component system, with or without degradable linkers. These compounds are expected to improve delivery and/or localization of nucleic acid molecules of the invention into a number of cell types originating from different tissues, in the presence or absence of serum (see Sullenger and Cech, U.S. Pat. No. 5,854,038). Conjugates of the molecules described herein can be attached to biologically active molecules via linkers that are biodegradable, such as biodegradable nucleic acid linker molecules.

Administration of the iRNA Agents A patient who has been diagnosed with a disorder characterized by unwanted VEGF expression can be treated by administration of an iRNA agent described herein to block the negative effects of VEGF, thereby alleviating the symptoms associated with unwanted VEGF gene expression. For example, the iRNA agent can alleviate symptoms associated with a disease of the eye, such as a neovascular disorder. In other examples, the iRNA agent can be administered to treat a patient who has a tumor or metastatic cancer, such as colon or breast cancer; a pulmonary disease, such as asthma or bronchitis; or an

autoimmune disease such as rheumatoid arthritis or psoriasis. The anti-VEGF iRNA agents can be administered systemically, *e.g.*, orally or by intramuscular injection or by intravenous injection, in admixture with a pharmaceutically acceptable carrier adapted for the route of administration. An iRNA agent can comprise a delivery vehicle, including liposomes, for administration to a subject, carriers and diluents and their salts, and/or can be present in pharmaceutically acceptable formulations. Methods for the delivery of nucleic acid molecules are described in Akhtar *et al.*, *Trends in Cell Bio.* 2:139, 1992; Delivery Strategies for Antisense Oligonucleotide Therapeutics, ed. Akhtar, 1995; Maurer *et al.*, *Mol. Membr. Biol.*, 16:129, 1999; Hofland and Huang, *Handb. Exp. Pharmacol.* 137:165, 1999; and Lee *et al.*, *ACS Symp. Ser.* 752:184, 2000, all of which are incorporated herein by reference. Beigelman *et al.*, U.S. Pat. No. 6,395,713 and Sullivan *et al.*, PCT WO 94/02595 further describe the general methods for delivery of nucleic acid molecules. Nucleic acid molecules can be administered to cells by a variety of methods known to those of skill in the art, including, but not restricted to, encapsulation in liposomes, by ionophoresis, or by incorporation into other vehicles, such as hydrogels, cyclodextrins (see for example Gonzalez *et al.*, *Bioconjugate Chem.* 10:1068, 1999), biodegradable nanocapsules, and bioadhesive microspheres, or by proteinaceous vectors (O'Hare and Normand, International PCT Publication No. WO 00/53722).

In the present methods, the iRNA agent can be administered to the subject either as naked iRNA agent, in conjunction with a delivery reagent, or as a recombinant plasmid or viral vector which expresses the iRNA agent. Preferably, the iRNA agent is administered as naked iRNA.

The iRNA agent of the invention can be administered to the subject by any means suitable for delivering the iRNA agent to the cells of the tissue at or near the area of unwanted VEGF expression, such as at or near an area of neovascularization. For example, the iRNA agent can be administered by gene gun, electroporation, or by other suitable parenteral administration routes.

Suitable enteral administration routes include oral delivery.

Suitable parenteral administration routes include intravascular administration (*e.g.*, intravenous bolus injection, intravenous infusion, intra-arterial bolus injection, intra-arterial infusion and catheter instillation into the vasculature); peri- and intra-tissue injection (*e.g.*, intraocular injection, intra-retinal injection, or sub-retinal injection); subcutaneous injection or

deposition including subcutaneous infusion (such as by osmotic pumps); direct application to the area at or near the site of neovascularization, for example by a catheter or other placement device (e.g., a retinal pellet or an implant comprising a porous, non-porous, or gelatinous material). It is preferred that injections or infusions of the iRNA agent be given at or near the site of
5 neovascularization.

The iRNA agent of the invention can be delivered using an intraocular implant. Such implants can be biodegradable and/or biocompatible implants, or may be non-biodegradable implants. The implants may be permeable or impermeable to the active agent, and may be inserted into a chamber of the eye, such as the anterior or posterior chambers, or may be
10 implanted in the sclera, transchoroidal space, or an avascularized region exterior to the vitreous. In a preferred embodiment, the implant may be positioned over an avascular region, such as on the sclera, so as to allow for transcleral diffusion of the drug to the desired site of treatment, e.g., the intraocular space and macula of the eye. Furthermore, the site of transcleral diffusion is preferably in proximity to the macula.

The iRNA agent of the invention can also be administered topically, for example, by patch or by direct application to the eye, or by iontophoresis. Ointments, sprays, or droppable liquids can be delivered by ocular delivery systems known in the art such as applicators or eyedroppers. The compositions can be administered directly to the surface of the eye or to the interior of the eyelid. Such compositions can include mucomimetics such as hyaluronic acid,
20 chondroitin sulfate, hydroxypropyl methylcellulose or poly(vinyl alcohol), preservatives such as sorbic acid, EDTA or benzylchromium chloride, and the usual quantities of diluents and/or carriers.

The iRNA agent of the invention may be provided in sustained release compositions, such as those described in, for example, U.S. Patent Nos. 5,672,659 and 5,595,760. The use of
25 immediate or sustained release compositions depends on the nature of the condition being treated. If the condition consists of an acute or over-acute disorder, treatment with an immediate release form will be preferred over a prolonged release composition. Alternatively, for certain preventative or long-term treatments, a sustained release composition may be appropriate.

An iRNA agent can be injected into the interior of the eye, such as with a needle or other
30 delivery device.

The iRNA agent of the invention can be administered in a single dose or in multiple doses. Where the administration of the iRNA agent of the invention is by infusion, the infusion can be a single sustained dose or can be delivered by multiple infusions. Injection of the agent directly into the tissue is at or near the site of neovascularization is preferred. Multiple injections
5 of the agent into the tissue at or near the site of neovascularization are also preferred.

Dosage levels on the order of about 1 µg/kg to 100 mg/kg of body weight per administration are useful in the treatment of the neovascular diseases. When administered directly to the eye, the preferred dosage range is about 0.00001 mg to about 3 mg per eye, or preferably about 0.0001-0.001 mg per eye, about 0.03- 3.0 mg per eye, about 0.1-3.0 mg per eye
10 or about 0.3-3.0 mg per eye. One skilled in the art can also readily determine an appropriate dosage regimen for administering the iRNA agent of the invention to a given subject. For example, the iRNA agent can be administered to the subject once, *e.g.*, as a single injection or deposition at or near the neovascularization site. Alternatively, the iRNA agent can be administered once or twice daily to a subject for a period of from about three to about twenty-
15 eight days, more preferably from about seven to about ten days. In a preferred dosage regimen, the iRNA agent is injected at or near a site of unwanted VEGF expression (such as near a site of neovascularization) once a day for seven days. Where a dosage regimen comprises multiple administrations, it is understood that the effective amount of iRNA agent administered to the subject can comprise the total amount of iRNA agent administered over the entire dosage
20 regimen. One skilled in the art will appreciate that the exact individual dosages may be adjusted somewhat depending on a variety of factors, including the specific iRNA agent being administered, the time of administration, the route of administration, the nature of the formulation, the rate of excretion, the particular disorder being treated, the severity of the disorder, the pharmacodynamics of the iRNA agent, and the age, sex, weight, and general health
25 of the patient. Wide variations in the necessary dosage level are to be expected in view of the differing efficiencies of the various routes of administration. For instance, oral administration generally would be expected to require higher dosage levels than administration by intravenous or intravitreal injection. Variations in these dosage levels can be adjusted using standard empirical routines of optimization, which are well-known in the art. The precise therapeutically

effective dosage levels and patterns are preferably determined by the attending physician in consideration of the above-identified factors.

In addition to treating pre-existing neovascular diseases, iRNA agents of the invention can be administered prophylactically in order to prevent or slow the onset of these and related disorders. In prophylactic applications, an iRNA of the invention is administered to a patient
5 susceptible to or otherwise at risk of a particular neovascular disorder.

The iRNA agents featured by the invention are preferably formulated as pharmaceutical compositions prior to administering to a subject, according to techniques known in the art. Pharmaceutical compositions of the present invention are characterized as being at least sterile
10 and pyrogen-free. As used herein, "pharmaceutical formulations" include formulations for human and veterinary use. Methods for preparing pharmaceutical compositions of the invention are within the skill in the art, for example as described in Remington's Pharmaceutical Science, 18th ed., Mack Publishing Company, Easton, Pa. (1990), and The Science and Practice of Pharmacy, 2003, Gennaro *et al.*, the entire disclosures of which are herein incorporated by
15 reference.

The present pharmaceutical formulations comprise an iRNA agent of the invention (*e.g.*, 0.1 to 90% by weight), or a physiologically acceptable salt thereof, mixed with a physiologically acceptable carrier medium. Preferred physiologically acceptable carrier media are water, buffered water, normal saline, 0.4% saline, 0.3% glycine, hyaluronic acid and the like.

20 Pharmaceutical compositions of the invention can also comprise conventional pharmaceutical excipients and/or additives. Suitable pharmaceutical excipients include stabilizers, antioxidants, osmolality adjusting agents, buffers, and pH adjusting agents. Suitable additives include physiologically biocompatible buffers (*e.g.*, tromethamine hydrochloride), additions of chelants (such as, for example, DTPA or DTPA-bisamide) or calcium chelate
25 complexes (as for example calcium DTPA, CaNaDTPA-bisamide), or, optionally, additions of calcium or sodium salts (for example, calcium chloride, calcium ascorbate, calcium gluconate or calcium lactate). Pharmaceutical compositions of the invention can be packaged for use in liquid form, or can be lyophilized.

For solid compositions, conventional non-toxic solid carriers can be used; for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like.

For example, a solid pharmaceutical composition for oral administration can comprise any of the carriers and excipients listed above and 10-95%, preferably 25%-75%, of one or more iRNA agents of the invention.

By "pharmaceutically acceptable formulation" is meant a composition or formulation that allows for the effective distribution of the nucleic acid molecules of the instant invention in the physical location most suitable for their desired activity. Non-limiting examples of agents suitable for formulation with the nucleic acid molecules of the instant invention include: P-glycoprotein inhibitors (such as PluronicP85), which can enhance entry of drugs into the CNS (Joliet-Riant and Tillement, *Fundam. Clin. Pharmacol.* 13:16, 1999); biodegradable polymers, such as poly (DL-lactide-coglycolide) microspheres for sustained release delivery. Other non-limiting examples of delivery strategies for the nucleic acid molecules of the instant invention include material described in Boado *et al.*, *J. Pharm. Sci.* 87:1308, 1998; Tyler *et al.*, *FEBS Lett.* 421:280, 1999; Pardridge *et al.*, *PNAS USA* 92:5592, 1995; Boado, *Adv. Drug Delivery Rev.* 15:73, 1995; Aldrian-Herrada *et al.*, *Nucleic Acids Res.* 26:4910, 1998; and Tyler *et al.*, *PNAS USA* 96:7053, 1999.

The invention also features the use of the composition comprising surface-modified liposomes containing poly (ethylene glycol) lipids (PEG-modified, or long-circulating liposomes or stealth liposomes). These formulations offer a method for increasing the accumulation of drugs in target tissues. This class of drug carriers resists opsonization and elimination by the mononuclear phagocytic system (MPS or RES), thereby enabling longer blood circulation times and enhanced tissue exposure for the encapsulated drug (Lasic *et al.*, *Chem. Rev.* 95:2601, 1995; Ishiwata *et al.*, *Chem.Phare. Bull.* 43:1005, 1995).

Such liposomes have been shown to accumulate selectively in tumors, presumably by extravasation and capture in the neovascularized target tissues (Lasic *et al.*, *Science* 267:1275, 1995; Oku *et al.*, *Biochim. Biophys. Acta* 1238:86, 1995). The long-circulating liposomes enhance the pharmacokinetics and pharmacodynamics of DNA and RNA, particularly compared to conventional cationic liposomes which are known to accumulate in tissues of the MPS (Liu *et*

al., *J. Biol. Chem.* 42:24864, 1995; Choi *et al.*, International PCT Publication No. WO 96/10391; Ansell *et al.*, International PCT Publication No. WO 96/10390 ; Holland *et al.*, International PCT Publication No. WO 96/10392). Long-circulating liposomes are also likely to protect drugs from nuclease degradation to a greater extent compared to cationic liposomes, based on their ability to avoid accumulation in metabolically aggressive MPS tissues such as the liver and spleen.

The present invention also includes compositions prepared for storage or administration that include a pharmaceutically effective amount of the desired compounds in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro edit. 1985), hereby incorporated by reference herein. For example, preservatives, stabilizers, dyes and flavoring agents can be provided. These include sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid. In addition, antioxidants and suspending agents can be used.

The nucleic acid molecules of the present invention can also be administered to a subject in combination with other therapeutic compounds to increase the overall therapeutic effect. The use of multiple compounds to treat an indication can increase the beneficial effects while reducing the presence of side effects.

Alternatively, certain iRNA agents of the instant invention can be expressed within cells from eukaryotic promoters (*e.g.*, Izant and Weintraub, *Science* 229:345, 1985; McGarry and Lindquist, *Proc. Natl. Acad. Sci. USA* 83:399, 1986; Scanlon *et al.*, *Proc. Natl. Acad. Sci. USA* 88:10591, 1991; Kashani-Sabet *et al.*, *Antisense Res. Dev.* 2:3, 1992; Dropulic *et al.*, *J. Virol.* 66:1432, 1992; Weerasinghe *et al.*, *J. Virol.* 65:5531, 1991; Ojwanget *et al.*, *Proc. Natl. Acad. Sci. USA* 89:10802, 1992; Chen *et al.*, *Nucleic Acids Res.* 20:4581, 1992; Sarver *et al.*, *Science* 247:1222, 1990; Thompson *et al.*, *Nucleic Acids Res.* 23:2259, 1995; Good *et al.*, *Gene Therapy* 4:45, 1997). Those skilled in the art realize that any nucleic acid can be expressed in eukaryotic cells from the appropriate DNA/RNA vector. The activity of such nucleic acids can be augmented by their release from the primary transcript by a enzymatic nucleic acid (Draper *et al.*, PCT WO 93/23569, and Sullivan *et al.*, PCT WO 94/02595; Ohkawa *et al.*, *Nucleic Acids Symp. Ser.* 27:156, 1992; Taira *et al.*, *Nucleic Acids Res.* 19:5125, 1991; Ventura *et al.*, *Nucleic Acids Res.* 21:3249, 1993; Chowrira *et al.*, *J. Biol. Chem.* 269:25856, 1994).

In another aspect of the invention, RNA molecules of the present invention can be expressed from transcription units (see for example Couture *et al.*, *Trends in Genetics* 12:510, 1996) inserted into DNA or RNA vectors. The recombinant vectors can be DNA plasmids or viral vectors. iRNA agent-expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. In another embodiment, pol III based constructs are used to express nucleic acid molecules of the invention (see for example Thompson, U. S. Pats. Nos. 5,902,880 and 6,146,886). The recombinant vectors capable of expressing the iRNA agents can be delivered as described above, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of nucleic acid molecules. Such vectors can be repeatedly administered as necessary. Once expressed, the iRNA agent interacts with the target mRNA and generates an RNAi response. Delivery of iRNA agent-expressing vectors can be systemic, such as by intravenous or intra-muscular administration, by administration to target cells ex-planted from a subject followed by reintroduction into the subject, or by any other means that would allow for introduction into the desired target cell (for a review see Couture *et al.*, *Trends in Genetics* 12:510, 1996).

Additional ophthalmic indications for the iRNA agents of the invention include proliferative diabetic retinopathy (the most severe stage of diabetic retinopathy), uveitis (an inflammatory condition of the eye that often leads to macular edema), cystoid macular edema following cataract surgery, myopic degeneration (a condition in which a patient with a high degree of nearsightedness develops choroidal neovascularization), inflammatory macular degeneration (a condition in which a patient with inflammation in the macular area due to infections or other causes, develops choroidal neovascularization), and iris neovascularization (a serious complication of diabetic retinopathy or retinal vein occlusion involving new blood vessel growth on the surface of the iris).

Additional non-ophthalmic indications for the iRNA agents of the invention include cancer, including but not limited to renal and colon cancer, and psoriasis. Solid tumors and their metastases rely on new blood vessel growth for their survival.

Psoriasis is a chronic inflammatory skin disease that causes skin cells to grow too quickly, resulting in thick white or red patches of skin. Preclinical and clinical data suggest that

VEGF-induced blood vessel growth and blood vessel leakage play a role in the development of this condition.

The invention is further illustrated by the following examples, which should not be construed as further limiting.

5

EXAMPLES

Example 1: siRNA Design

Four hundred target sequences were identified within exons 1-5 of the VEGF-A121 mRNA sequence (See Table 1, SEQ ID NOs 2-401) and corresponding siRNAs targeting these 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 2).

Example 2: Synthesis of the siRNA oligonucleotides

RNA was synthesized on Expedite 8909, ABI 392 and ABI394 Synthesizers (Applied Biosystems, Applera Deutschland GmbH, Frankfurter Str. 129b, 64293 Darmstadt, Germany) at 1 μ mole scale employing CPG solid support and Expedite RNA phosphoramidites (both from Prologo Biochemie GmbH, Georg-Hyken-Str.14, Hamburg, Germany). Ancillary reagents were obtained from Mallinckrodt Baker (Im Leuschnerpark 4:64347 Griesheim, Germany). Phosphorothioate linkages were introduced by replacement of the iodine oxidizer solution with a solution of the Beaucage reagent in acetonitrile (5% weight per volume).

30

Cleavage of the oligoribonucleotides from the solid support and base deprotection was accomplished with a 3:1 (v/v) mixture of methylamine (41%) in water and methylamine (33%) in ethanol. 2'-Desilylation was carried out according to established procedures (Wincott *et al.*, *Nucleic Acids Res.* 23:2677-2684, 1995). Crude oligoribonucleotides were purified by anion exchange HPLC using a 22x250 mm DNAPac PA 100 column with buffer A containing 10 mM NaClO₄, 20 mM Tris, pH 6.8, 6 M urea and buffer B containing 400 mM NaClO₄, 20 mM Tris, pH 6.8, 6 M Urea. Flow rate was 4.5 mL/min starting with 15% Buffer B which was increased to 55% over 45 minutes.

The purified compounds were characterized by LC/ESI-MS (LC: Ettan Micro, Amersham Biosciences Europe GmbH, Munzinger Strasse 9, 79111 Freiburg, Germany, ESI-MS: LCQ, Deca XP, Thermo Finnigan, Im Steingrund 4-6, 63303 Dreieich, Germany) and capillary electrophoresis (P/ACE MDQ Capillary Electrophoresis System, Beckman Coulter GmbH, 85702 Unterschleißheim, Germany). Purity of the isolated oligoribonucleotides was at least 85%.

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. 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), heating in a water bath at 85 - 90 °C for 3 minutes and cooling to room temperature over a period of 3 - 4 hours. The RNA was kept at -20 °C until use.

Example 3: Efficacy Screen of siRNAs

Using two efficacy screens, the VEGF siRNA were screened for their ability to become a lead candidate. Table 2 shows the relative efficiencies of some of the siRNAs in their ability to inhibit expression of an endogenous VEGF gene. In this process the number of candidate siRNAs was winnowed. Human HeLa or ARPE-19 (human retinal pigment epithelial cell line with differentiated properties (Dunn *et al.*, *Exp. Eye Res.* 62:155, 1996) were plated in 96-well plates (17,000 cells/well) in 100 µl 10% fetal bovine serum in Dulbecco's Modified Eagle Medium (DMEM). When the cells reached approximately 90% confluence (approximately 24 hours later) they were transfected with serial three-fold dilutions of siRNA starting at 20 nM 0.4 µl of transfection reagent Lipofectamine™ 2000 (Invitrogen Corporation, Carlsbad, CA) was

used per well and transfections were performed according to the manufacturer's protocol. Namely, the siRNA: Lipofectamine™ 2000 complexes were prepared as follows. The appropriate amount of siRNA was diluted in Opti-MEM I Reduced Serum Medium without serum and mixed gently. The Lipofectamine™ 2000 was mixed gently before use, then for each well of a 96 well plate, 0.4 µl was diluted in 25 µl of Opti-MEM I Reduced Serum Medium without serum and mixed gently and incubated for 5 minutes at room temperature. After the 5 minute incubation, 1 µl of the diluted siRNA was combined with the diluted Lipofectamine™ 2000 (total volume is 26.4 µl). The complex was mixed gently and incubated for 20 minutes at room temperature to allow the siRNA: Lipofectamine™ 2000 complexes to form. Then 100 µl of 10% fetal bovine serum in DMEM was added to each of the siRNA:Lipofectamine™ 2000 complexes and mixed gently by rocking the plate back and forth. 100 µl of the above mixture was added to each well containing the cells and the plates were incubated at 37°C in a CO₂ incubator for 24 hours, then the culture medium was removed and 100 µl 10% fetal bovine serum in DMEM was added. Following the medium change, conditioned medium was collected at 24 hours (HeLa cells) or 72 hours (ARPE-19 cells) and a human VEGF ELISA was performed using the DuoSet human VEGF ELISA Development kit (R&D Systems, Inc. Minneapolis, MN 55413). This kit contains the basic component required for the development of sandwich ELISAs to measure natural and recombinant human VEGF in cell culture supernatants and serum.

The materials used included:

Capture Antibody – 576 µg/ml of goat anti-human VEGF when reconstituted with 0.25 ml PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.2-7.4, 0.2 µm filtered). After reconstitution, stored at 2-8°C for up to 60 days or aliquoted and stored at -20°C to -70°C in a manual defrost freezer for up to 6 months. Diluted to a working concentration of 0.8 µg/ml in PBS without carrier protein.

Detection antibody – 4.5 µg/ml of biotinylated goat anti-human VEGF when reconstituted with 1.0 ml of Reagent Diluent (1% bovine serum albumin in PBS, pH 7.2-7.4, 0.2 µm filtered). After reconstitution, stored at 2-8°C for up to 60 days or aliquoted and stored at -20°C to -70°C in a manual defrost freezer for up to 6 months. Diluted to a working concentration of 25 ng/ml in Reagent Diluent.

Standard: 110 ng/ml of recombinant when reconstituted with 0.5 ml of Reagent Diluent. Allowed the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. The reconstituted Standard can be stored at 2-8°C for up to 60 days or aliquoted and stored at -20°C to -70°C in a manual defrost freezer for up to 6 months. A seven point standard
 5 curve using 2-fold serial dilutions in Reagent Diluent, and a high standard of 4000 pg/ml is recommended.

Streptavidin-HRP: 1.0 ml of streptavidin conjugated to horseradish-peroxidase. Stored at 2-8°C for up to 6 months. Diluted to the working concentration specified on the vial label.

General ELISA protocol was followed (R&D Systems, Inc., Minneapolis, MN).

10 Controls included no siRNA, human VEGF siRNA (Cand5, (a.k.a., hVEGF5) Reich *et al.*, *Mol Vis.* 9:210, 2003) and an siRNA matching a 21-nt sequence conserved between the human, rat and mouse VEGF (hrmVEGF, Filleur *et al.*, *Cancer Res.* 63:3919-3922, 2003).

The activities of the siRNAs were compared to the activity of the control human VEGF siRNA of Reich *et al.* (*supra*) with “+” representing a lower activity, “++” representing similar
 15 activity and “+++” representing a higher activity than the control human VEGF siRNA (Table 2). FIG. 2 shows the activities of single- and double-overhang siRNAs in HeLa cells. Solid lines with filled symbols represent the single-overhang siRNA, solid lines with open symbols represent the double-overhang siRNAs; dashed lines represent the control siRNAs. All of the siRNAs are more active than the control siRNAs and may inhibit expression of VEGF by
 20 approximately 80%. In contrast, the siRNA from Reich *et al.* (*supra*) reduced the level of endogenous hVEGF by approximately 20% under the same experimental conditions. Similarly, under the same experimental conditions, the siRNA based on consensus sequence hrmVEGF (Filleur *et al.*, *supra*) reduced the expression level by approximately 45%.

FIG. 3 shows the activities of single- and double-overhang siRNAs in ARPE-19 cells.
 25 Solid lines with filled symbols represent the single-overhang siRNA, solid lines with open symbols represent the double-overhang siRNAs; dashed lines represent the control siRNAs. All of the siRNAs are more active than the control siRNAs and may inhibit expression of VEGF by approximately 90%. In contrast, the siRNA from Reich *et al.* (*Mol. Vis.* 9:210, 2003) reduced the level of hVEGF by approximately 35% under the same experimental conditions. Similarly, under

the same experimental conditions, the siRNA based on consensus sequence hrmVEGF (Filleur *et al.*, *supra*) reduced the expression level by approximately 70%.

FIGs. 4 and 5 show the results of a comparison of single- and double-overhang siRNAs with their analogous blunt-ended siRNAs, respectively in HeLa cells. The results are in agreement with the data of Elbashir *et al.* (*Genes & Development* 15:188, 2001) in that the presence of an overhang in an siRNA confers greater efficiency in inhibition of gene silencing. However, it is important to note that the activity of the blunt ended siRNAs are comparable to the results achieved using the control siRNAs.

Example 4: *In vitro* assay for the silencing of VEGF synthesis under hypoxic conditions

Human HeLa cells were plated in 96 well plates at 10,000 cells/well in 100 μ l of growth medium (10% FBS in DMEM). 24 hours post cell seeding when the cells had reached approximately 50% confluence they were transfected with serial three fold dilutions of siRNA starting at 30 nM. 0.2 μ l of Lipofectamine™ 2000 transfection reagent (Invitrogen Corporation, Carlsbad CA) was used per well and transfections were carried out as described in the Invitrogen product insert. Controls included no siRNA, human VEGF siRNA (Reich *et al.*, *Mol. Vis.* 9:210, 2003) and an siRNA matching a 21-nt sequence conserved between the human, rat and mouse VEGF (hrmVEGF, Filleur *et al.*, *Cancer Res.* 63:3919-3922, 2003). Transfections were done in duplicate on each plate. Additionally, duplicate plates were transfected so that 24 hours post transfection the growth media could be changed and one plate could be kept in normal oxygen growth conditions (37°C, 5% CO₂, 20% oxygen) and the duplicate plate could be kept in hypoxic conditions (37°C, 1% oxygen, balance nitrogen). Hypoxic conditions were maintained by using a Pro-ox Oxygen Controller (BioSpherix, Ltd., Redfield, NY) attached to a Pro-ox *in vitro* culture chamber. Cells were maintained in either normoxic or hypoxic conditions for 24 hours post media change. Conditioned culture media was then collected from both plates and tested for secreted VEGF levels in a DuoSet VEGF ELISA (R&D Systems, Minneapolis, MN). The assays were performed according to the manufacturer's protocol and as described in Example 2.

For deferoxamine chemically induced hypoxia, 130 μ M deferoxamine (Sigma D9533), was used. Deferoxamine was added to the fresh growth media 24 hours post-transfection. Cells

treated with deferoxamine were then grown under normal growth conditions (37°C, 5% CO₂, 20% oxygen).

FIG. 6 shows the results obtained with siRNAs (both single overhang siRNAs and double overhangs siRNAs) directed against ORF regions having the first nucleotides corresponding to 319 and 343 respectively, together with the control siRNAs. Under hypoxic conditions, either 1% oxygen (FIG. 6B) or 130 µM deferoxamine (FIG. 6C), three of the experimental siRNAs achieved almost 95% inhibition of expression of VEGF, namely AL-DP-4094 (single-overhang) directed at ORF 343, and both of the siRNAs (single and double-overhangs) directed at ORF 319. The control siRNAs of Reich *et al* (*supra*) and Filleur *et al*. (*supra*) demonstrate an ability to inhibit VEGF expression by 45% and 85% respectively.

FIGs. 8A and 8B show the results obtained with the siRNAs AL-DP-4014, a phosphorothioate modified version of AL-DP-4014 (AL-DP-4127, see Table 3) and a mutated version of AL-DP-4014 (AL-DP-4140, see Table 5). Under both normal and hypoxic conditions, the unmodified (AL-DP-4014) and the phosphorothioate-modified siRNA (AL-DP-4127) reduced endogenous VEGF expression to less than 20% of its original expression level. Under hypoxic conditions, the phosphorothioate-modified siRNA essentially abolished VEGF expression.

Example 5: Modified VEGF siRNA molecules retain full activity and show enhanced stability

Phosphorothioate derivatives were made for the AL-DP-4014, targeting ORF 319 of VEGF, and are presented in Table 3. These siRNAs were tested in the HeLa cell assay described in Example 3, and FIG. 7 shows that these derivatives are as active in the HeLa assay as the unmodified siRNA.

A panel of siRNAs were synthesized that retained the sequence of the AL-DP-4094 siRNA (Table 1) but included different modifications including phosphorothioate linkages, O-methyl-modified nucleotides, and 2'-fluoro-modified nucleotides (Table 4). The panel of siRNAs was tested in HeLa cells, and FIGs. 9A-9E demonstrate that all modified versions of the AL-DP-4094 siRNA effectively reduced VEGF expression by greater than 90%, exhibiting greater efficacy than either of the two previously identified VEGF siRNAs ("Acuity" in Reich *et*

al. (supra), and “Filleur” in Filleur *et al. (supra)*). FIG. 10 also shows data from *in vitro* assays in HeLa cells. The graph in FIG. 10 shows that the unmodified AL-DP-4094 siRNA and a phosphorothioate-modified AL-DP-4004 siRNA (AL-DP-4219) reduced VEGF expression by more than 70% (FIG. 10). Scrambled versions of the compound AL-DP-4094 (*e.g.*, AL-DP-4216 and AL-DP-4218 (sequences shown below; underlined nucleotides represent mismatched nucleotides as compared to AL-DP-4094)) did not inhibit VEGF expression. An siRNA targeting the firefly luciferase gene (AL-DP-3015; see below) also did not inhibit VEGF expression.

AL-DP-4216	AL4094 MI s	5'- GCACA <u>UUGGACAGUUGGUU</u> -3'
	AL4094 MI	
	as	'3-GUCGUGUA <u>ACCUGUCAACACCAA</u> -5'
	AL4094 M5	
AL-DP-4218 s		5'- GCACA <u>UAGAAGUGACGCGCUU</u> -3'
	AL4094 M5	
	as	'3-GUCGUGUA <u>UCUUCACUGCGCGAA</u> -5'
AL-DP-3015		5'- GAACUGUGUGUGAGAGGUCCU-3'
		'3-CGCUUGACACACACUCUCCAGGA-5'

10

The Stains-All technique (Sigma, St. Louis, MO) was performed to examine the stability of the modified siRNAs. To perform the assay, an siRNA duplex was incubated in 90% human serum at 37°C. Samples of the reaction mix were quenched at various time points (at 0, 0.25, 1, 2, 4, and 24 hours) and subjected to polyacrylamide gel electrophoresis. Cleavage of the RNA over the time course provided information regarding the susceptibility of the siRNA duplex to serum nuclease degradation.

O-methyl and 2'-fluoro modifications used in combination with phosphorothioate modifications were found to enhance stability to a greater extent than when phosphorothioate modifications were used alone. For example, modified versions of the AL-DP-4094 siRNA included a phosphorothioate-modified siRNA (AL-DP-4198), a phosphorothioate plus O-methyl modified siRNAs (*e.g.*, AL-DP-4180, AL-DP-4175, and AL-DP-4220), and phosphorothioate plus O-methyl plus 2'-fluoro modified siRNAs (*e.g.*, AL-DP-4197 and AL-DP-4221) (Table 4).

The AL-DP-4180, AL-DP-4175, and AL-DP-4197 siRNAs were found to be more stable in human serum than the AL-DP-4198 siRNA. It was determined that the phosphorothioate modification stabilized the siRNAs against exonucleolytic degradation, and the O-methyl and 2'-fluoro modifications stabilized the siRNAs against endonucleolytic degradation.

5

Example 6: *In vitro* Stability Assay of VEGF siRNAs in Different Rat Serum and Ocular tissues

1. Preparation of Tissue Homogenates

Tissues from pooled whole eyes, retinas, vitreous humors from at least three rats were excised and frozen immediately in liquid nitrogen. The frozen tissue was pulverized over dry ice, using instruments that were pre-chilled on dry ice. 1 ml of RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1mM Na2EDTA, 0.5% Na-deoxycholate deoxycholic acid, 1% IGEPAL CA-630, 0.05% SDS) was added to the frozen tissue powder and the mixture was mixed thoroughly and vigorously. The homogenate was centrifuged at 10,000 x g for 5 min at 4°C and the pellet was discarded. 100 µl aliquots of the supernatant were transferred to pre-chilled microcentrifuge tubes and stored at -70°C or used immediately in the stability assay.

2. 5'-end labeling of single stranded sense or antisense siRNA using T4 polynucleotide kinase and $\gamma^{32}\text{P}$ -ATP

The following reagents were used:

- 20 T4 Polynucleotide Kinase (PNK) 10 units/µl (New England Biolabs, Beverly, MA)
- 10X T4 PNK buffer (700 mM Tris-HCl, 100 mM MgCl₂, 50 mM Dithiothreitol (DTT), pH7.6)
- Gamma-³²P-ATP (PerkinElmer, Shelton, CT) 250 µCi, 3000 Ci/mmol (3.3 µM)
- 10 µM stocks of synthetic RNA oligo diluted in H₂O
- 25 Microspin Sephadex™ G-25 columns (Amersham Biosciences)
- RNAse-free Water and 0.65 ml microcentrifuge (1.5 ml) tubes

A 25 µl kinase reaction contained:

- 2.5µl from 10 µM stock sense or antisense (1µM final conc.)
- 2.5µl 10X PNK Buffer (1X)
- 30 1.5µl $\gamma^{32}\text{P}$ -ATP (approximately 0.2 nM)

1.0μl 10 unit/μl T4 PNK (10 units)

17.5μl dH₂O

The reaction mix was incubated at 37°C for 1 hour (water bath) prior to fractionating the labeled siRNA through Sephadex™ G-25 spin columns (Amersham). 0.5 μL was used to determine the number of counts per minute (cpm)/ml of the radiolabeled sample.

3. Partial alkaline hydrolysis ladder of radiolabeled single-stranded siRNA

To generate a sample of size markers a portion of the 5' γ³²P-end- labeled siRNA was subjected to alkaline hydrolysis as follows:

30 μl hydrolysis reaction containing 2.5 μl 5' end –labeled siRNA (sense or antisense), 6.0 μl 0.5M Na₂CO₃/NaHCO₃ (pH 9.5), 1.5μl 10mg/ml tRNA, and 20.0μl dH₂O was incubated at 90°C for 7.5 min, then chilled on ice or at 4°C. 30μl of 90% formamide, 50mM Na₂EDTA, 10mM DTT, and XC&BB (xylene cyanol and bromophenol blue), of which 1μl+4μl formamide dye was used for the gel electrophoretic analysis.

4. Annealing of radiolabeled 1μM stock siRNA duplexes

30μl 1μM stock of different siRNA duplexes were prepared in which either the sense strand or the antisense strand was radiolabeled.

The samples were heated at 90°C for 2 min and then incubated at 37°C for 1 hour and then stored at -20°C until used.

5. Quality control of siRNA duplex:

Samples of the siRNA duplex were analyzed by electrophoresis through 15% polyacrylamide in Tris-Borate, EDTA (TBE) Gel. Electrophoresis was performed at 150V for 1 hour prior to running the samples through. Samples were prepared by mixing 0.5-1 μl siRNA duplex or single stranded, 3-3.5 μl 0.5X TBE, 1 μl 5X native loading dye (total volume = 5 μl).

6. Stability reactions

2 μl siRNA duplex was added to 18 μl serum or tissue lysate or buffer control in PCR tube (0.2ml). A zero time point sample was removed immediately following the addition of the siRNA duplex by removing 2 μl and adding it to 18 μl 90% formamide, 50 mM EDTA, 10 mM DTT and xylene cyanol and bromophenol blue (XC & BB). Other samples were

removed after 15 min, 30 min, 1 hour, 2 hours, and 4 hours and treated similarly. These samples were stored in a 96-well plate. In some experiments the time points were extended to 8, 24 and 48 hours. Time point samples for the buffer (phosphate buffered saline, PBS, 1X working PBS contains 0.14 M Sodium Chloride, 0.003 M Potassium Chloride, 0.002 M Potassium phosphate, 0.01 M Sodium phosphate) were taken at zero and the last time point of the experiment. Samples were analyzed by electrophoresis through 20% polyacrylamide gels (pre-run at 75 W for 1 hour) in 1 X TBE (10 X = 890 mM Tris, 890 mM Boric acid, 20 mM EDTA, pH 8.0). The gel was transferred to a phosphorimager cassette, covered with an enhancer screen and scanned after overnight exposure.

Polyacrylamide gel analysis indicated that the ocular environment contains fewer nucleases than human serum. Testing the unmodified form of the VEGF siRNA AL-DP-4014 for stability in rat eye extract revealed only the presence of exonuclease activity. In human serum, experiments with AL-DP-4127 and -4140 (Tables 4 and 5) indicated that terminally modified phosphorothioate modifications protected against exonucleolytic degradation but not against endonucleolytic activity. These results were consistent with experiments performed in rat whole eye extracts. The terminally modified phosphorothioate derivatives AL-DP-4127 and -4140 were stabilized against exonuclease activity as compared to the unmodified AL-DP-4014 siRNA and the unmodified Cand5 siRNA (Reich *et al. (supra)*). However, the -4127 and -4140 siRNAs were still subject to endonucleolytic degradation.

Modifications to the lead compound AL-DP-4094 stabilized the siRNA against exonucleolytic and endonucleolytic degradation. The phosphorothioate-modified siRNA AL-DP-4198 was degraded to a similar extent as the unmodified 4094 compound, but the addition of O-methyl modifications, as in AL-DP-4180 and AL-DP-4220, stabilized the siRNAs in rat whole eye extracts.

Notably, the siRNAs were generally more stable in rat retina lysates than in the rat whole eye extracts described above. Neither the unmodified AL-DP-4094, nor the modified AL-DP-4198, -4180, or -4220 siRNAs were degraded in the retina lysates.

Example 7. Endonuclease-sensitive sites were mapped on AL-DP-4094 siRNA.

The stability of the AL-DP-4094 siRNA was examined by the Stains-All and radiolabeled techniques following incubation in human serum (see above). These assays revealed susceptibility to exo- and endonucleases. RP-HPLC was used to examine the fragment profile of the siRNA following incubation in serum FIG. 11.

Following incubation of the -4094 siRNA in human serum, the fragments were phenol-chloroform extracted and precipitated, and then subjected to LC/MS analysis. FIG. 12 describes the identified fragments and associated characteristics.

10 **Example 8: Detailed study of modifications to siRNAs targeting VEGF (Table 6)**

Eight major different patterns of chemical modification of siRNA duplexes that target the VEGF mRNA were synthesized and evaluated (Table 6). The ribose sugar modifications used were either 2'-O-methyl (2'OMe) or 2'-fluoro (2'F). Both pyrimidines (Py) and purines (Pu) could be modified as provided in Table 6.

15 The first four patterns(A-D) incorporated 2'OMe on both strands at every other position. Four configurations were synthesized: 1) at each even position on the sense strand and at each odd position of the antisense strand, 2) at each odd position on the sense strand and at each even position of the antisense strand, 3) at even positions on both strands, and 4) at odd positions on both strands.;

20 The fifth pattern (E) incorporated the 2'OMe modification at all pyrimidine nucleotides on both the sense and antisense strands of the duplex.

Pattern F included duplexes with 2'OMe modifications only on pyrimidines in 5'-PyPu-3' dinucleotides, especially at only at UA, CA, UG sites (both strands).

Pattern G duplexes had the 2'F modification on pyrimidines of the antisense strand and 25 2'OMe modifications on pyrimidines in the sense strand.

Pattern (H) had antisense strands with 2'F-modified pyrimidines in 5'-PyPu-3' dinucleotides, only at UA, CA, UG sites (both strands) and sense strands with 2'OMe modifications only on pyrimidines in 5'-PyPu-3' dinucleotides, only at UA, CA, UG sites (both strands).

30 **A-D:** Full Alternating 2'-OMe (both strands)

Four configurations: Even/Odd; Odd/Even; Even/Even; Odd/Odd

E: 2'-OMe Py (both strands)

F: 2'-OMe Py only at UA, CA, UG sites (both strands)

G: 2'-OMe All Py (sense)

5 2'-F All Py (anti-sense)

H: 2'-OMe Py only at UA, CA, UG sites (sense)

2'-F Py only at UA, CA, UG sites (anti-sense)

17 different parent VEGF duplexes from Table 2 tested

10 1. Evaluation of serum stability of siRNA duplexes

2 μ M siRNA duplexes (final concentration) were incubated in 90% pooled human serum at 37°C. Samples were quenched on dry ice after 30 minutes, 4 hours, and 24 hours. For each siRNA sequence, a sample at the same concentration was incubated in the absence of serum (in PBS) at 37°C for 24 hours. After all samples were quenched, RNA was extracted using
15 phenol:chloroform and concentrated by ethanol precipitation. Samples were air dried and resuspended in a denaturing loading buffer. One third of each time point was analyzed on a 20% acrylamide (19:1), 7 M urea, 1XTBE gel run at 60°C. RNA was visualized by staining with stains-all solution. A qualitative assessment of the stability of each modified siRNA was made by comparison to the parent unmodified siRNA for each duplex set. PBS controls served as
20 markers for the quality of the input siRNA.

2. Stability of VEGF modular chemistries

Four modular chemistries were screened 1) all pyrimidines substituted with 2'-O-methyl (2'OMe) in both sense and antisense strands, 2) pyrimidines in UA, UG, CA pairs substituted with 2'OMe in both sense and antisense strands, 3) all pyrimidines substituted with 2'OMe in the
25 sense strand and 2'-fluoro (2'F) in the antisense strand, 4) pyrimidines in UA, UG, CA pairs substituted with 2'OMe in the sense strand and 2'F in the antisense strand. In total, 85 siRNAs were screened including the unmodified parent duplexes plus the four modular chemistries.

Of the 85 siRNAs screened, 35 were stable for at least 24hours as assessed by visual comparison with the parent unmodified duplexes. These 35 duplexes had 2'OMe pyrimidines in
30 both strands or 2'OMe pyrimidines in the sense strand and 2'F in the antisense strand

(chemistries 1 and 3 above). Of the duplexes with fewer modified residues, only five had at least ~50% full length material remaining at the 4 hour time point as compared to their unmodified parent.

Substitution of all pyrimidines with either 2'OMe or 2'F protects siRNAs from serum nuclease degradation for ~24hr in 90% human serum at 37°C. The protected duplexes had roughly 85%-100% full length material remaining at 24 hours as compared to duplex incubated in the absence of serum. Minimal modification of pyrimidines in UA, UG, and CA dinucleotide pairs only stabilized several siRNAs relative to their unmodified parent but did not stabilize sufficiently for long-term nuclease resistance. Some potential RNase A sites were not protected by methylation (YpN, *e.g.* UC, UU) and this is likely the reason for the lower resistance to serum endonucleases.

3. Analysis of Duplex activity

Duplexes were tested for activity in the HeLa cell assay described above. Table 6 and Figures 13-29 provides summary and graphs of duplex activities in HeLa cells for each of the modifications described above.

Synthesis of the iRNA agents

RNA Synthesis using "fast" deprotection monomers

1. RNA synthesis

Oligoribonucleotides were synthesized using phosphoramidite technology on solid phase employing an AKTA 10 synthesizer (Amersham Biosciences) at scales ranging from 35 to 60 μ mol. Synthesis was performed on solid supports made of controlled pore glass (CPG, 520Å, with a loading of 70 μ mol/g) or polystyrene (with a loading of 71 μ mol/g). All amidites were dissolved in anhydrous acetonitrile (70 mM) and molecular sieves (3Å) were added. 5-Ethyl thiotetrazole (ETT, 600 mM in acetonitrile) was used as the activator solution. Coupling times were 8 minutes. Oxidation was carried out either with a mixture of iodine/water/pyridine (50 mM/10%/90% (v/v)) or by employing a 100 mM solution of 3-ethoxy-1,2,4-dithiazoline-5-one (EDITH) in anhydrous acetonitrile in order to introduce phosphorothioate linkages. Standard capping reagents were used. Cholesterol was conjugated to RNA *via* the either the 5' or the 3'-end of the sense strand by starting from a CPG modified with cholesterol (described below)

using a hydroxyprolinol linker. The DMT protecting group was removed from cholesterol-conjugated RNA, but the DMT was left on unconjugated RNA to facilitate purification.

2. Cleavage and deprotection of support bound oligonucleotide.

After solid-phase synthesis, the RNA was cleaved from the support by passing 14 mL of a 3:1 (v/v) mixture of 40% methylamine in water and methylamine in ethanol through the synthesis column over a 30 min time period. For the cholesterol-conjugated RNA, the ratio of methylamine in water to methylamine in ethanol was 1:13. The eluent was divided into four 15 mL screw cap vials and heated to 65°C for additional 30 min. This solution was subsequently dried down under reduced pressure in a speedvac. The residue in each vial was dissolved in 250 µL *N*-methylpyrrolidin-2-one (NMP), and 120 µL triethylamine (TEA) and 160 µL TEA·3HF were added. This mixture was brought to 65°C for 2h. After cooling to ambient temperature, 1.5 mL NMP and 1 mL of ethoxytrimethylsilane were added. After 10 min, the oligoribonucleotide was precipitated by adding 3 mL of ether. The pellets were collected by centrifugation, the supernatants were discarded, and the solids were reconstituted in 1 mL buffer 10 mM sodium phosphate.

3. Purification of oligoribonucleotides

Crude oligonucleotides were purified by reversed phase HPLC on an AKTA Explorer system (Amersham Biosciences) using a 16/10 HR column (Amersham Biosciences) packed to a bed height of 10 cm with Source RPC 15. Buffer A was 10 mM sodium phosphate and buffer B contained 65% acetonitrile in buffer A. A flow rate of 6.5 mL/min was employed. UV traces at 260, 280, and 290 nm were recorded. For DMT-on oligoribonucleotides a gradient of 7% B to 45% B within 10 column volumes (CV) was used and for cholesterol-conjugated RNA a gradient of 5% B to 100% B within 14 CV was employed. Appropriate fractions were pooled and concentrated under reduced pressure to roughly 10 mL. DMT-on oligonucleotides were treated with one-third volume 1M NaOAc, pH 4.25 for several hours at ambient temp.

Finally, the purified oligonucleotides were desalted by size exclusion chromatography on a column containing Sephadex G-25. The oligonucleotide solutions were concentrated to a volume <15 mL. The concentrations of the solutions were determined by measurement of the absorbance at 260 nm in a UV spectrophotometer. Until annealing the individual strands were stored as frozen solutions at -20°C.

4. Analysis of oligoribonucleotides

Cholesterol conjugated RNA was analyzed by CGE and LC/MS. Unconjugated RNA was also analyzed by IEX-HPLC. CGE analysis was performed on a BeckmanCoulter PACE MDQ CE instrument, equipped with a fixed wavelength detector at 254 nm. An eCap DNA capillary (BeckmanCoulter) with an effective length of 20 cm was used. All single stranded RNA samples were analyzed under denaturing conditions containing 6 M urea (eCap ssDNA100 Gel Buffer Kit, BeckmanCoulter) at 40°C. Samples were injected electrokinetically with 10 kV for 5-8 sec. The run voltage was 15 kV.

IEX HPLC analysis was performed on a Dionex BioLC system equipped with a fixed wavelength detector (260 and 280 nm), column oven, autosampler, and internal degasser. A Dionex DNAPac P100 column (4*250mm) was used as at a flow rate of 1.0 mL/min and 30°C. Unconjugated RNA (20 μ L, 1 OD/mL concentration) was injected. Eluent A contained 20 mM Na₂HPO₄, 10 mM NaBr, 10% acetonitrile, pH 11 and Eluent B was 1 M NaBr in Eluent A. The elution started with 20% B for 1 min and then a linear gradient with a target concentration of 80% B over 20 min was employed.

LC-MS analysis was performed on an Ettan μ LC-system (Amersham Bioscience) equipped with a Jetstream column heater and a fixed wavelength detector (254nm). A ThermoFinnigan LCQ DecaXP ESI-MS system with micro-spray source and ion trap detector was coupled online to the HPLC. Oligonucleotide samples (25 μ L sample, 1 OD/mL concentration in water for unconjugated RNA and 40 μ L for cholesterol-conjugated RNA) were injected onto a Waters Xterra C8 MS column (2.1 x 50 mm; 2.5 μ m particle size) with a flow rate of 200 μ L/min at 60°C. Composition of eluent A was 400 mM hexafluoroisopropanol (HFIP), 16.3 mM TEA in H₂O, pH 7.9 and eluent B was methanol. For unconjugated RNA elution started with 7% B for 3 min and then a gradient from 7% B to 25% B in 13 min was used. For cholesterol-conjugated material the starting conditions were 35% B for 3 min and then the concentration of eluent B was increased to 75% B in 30 min. Analysis figures are provided in Table 6.

5. Annealing of oligoribonucleotides

Complementary strands were annealed by combining equimolar RNA solutions. The mixture was lyophilized and reconstituted with an appropriate volume of annealing buffer (100

mM NaCl, 20 mM sodium phosphate, pH 6.8) to achieve the desired concentration. This solution was placed into a water bath at 95°C and then cooled to ambient temp. within 3h. Extent of duplex formation was monitored by native 10% polyacrylamide gel electrophoresis (PAGE) and bands were visualized by staining with the "stains all" reagent (Sigma).

5

RNA Synthesis using "standard" deprotection monomers including ribo and 2'-O-methyl phosphoramidites.

A. RNA/2'OMe (Thioate ends)

The chimeric RNA molecules with 2'-OMe nucleotides were synthesized on a 394 ABI machine using the standard cycle written by the manufacturer with modifications to a few wait steps. The solid support was CPG (500A). The monomers were either RNA phosphoramidites or 2' OMe RNA phosphoramidites with standard protecting groups and used at concentrations of 0.15 M in acetonitrile (CH₃CN) unless otherwise stated. Specifically the RNA phosphoramidites were 5'-O-Dimethoxytrityl-N⁶-benzoyl-2'-O-*t*butyldimethylsilyl-adenosine-3'-O-(β-cyanoethyl-N,N'-diisopropyl) phosphoramidite, 5'-O-Dimethoxytrityl-N²-isobutyryl-2'-O-*t*butyldimethylsilyl-guanosine-3'-O-(β-cyanoethyl-N,N'-diisopropyl)phosphoramidite, 5'-O-Dimethoxytrityl-N⁴-acetyl-2'-O-*t*butyldimethylsilyl-cytidine-3'-O-(β-cyanoethyl-N,N'-diisopropyl)phosphoramidite and 5'-O-Dimethoxytrityl-2'-O-*t*butyldimethylsilyl-uridine-3'-O-(β-cyanoethyl-N,N'-diisopropyl)phosphoramidite; the 2'OMe RNA phosphoramidites were 5'-O-Dimethoxytrityl-N⁶-benzoyl-2'-O-methyl-adenosine-3'-O-(β-cyanoethyl-N,N'-diisopropyl) phosphoramidite, 5'-O-Dimethoxytrityl-N²-isobutyryl-2'-O-methyl-guanosine-3'-O-(β-cyanoethyl-N,N'-diisopropyl)phosphoramidite, 5'-O-Dimethoxytrityl-N⁴-acetyl-2'-O-methyl-cytidine-3'-O-(β-cyanoethyl-N,N'-diisopropyl)phosphoramidite and 5'-O-Dimethoxytrityl-2'-O-methyl-uridine-3'-O-(β-cyanoethyl-N,N'-diisopropyl)phosphoramidite. The coupling times were 10 min for all monomers. Details of the other reagents are as follows: Activator: 5-(ethylthio)-1H-tetrazole (0.25M); Cap A: 5% acetic anhydride/THF/pyridine; Cap B: 10% N-methylimidazole/THF. Phosphate oxidation involved THBP (10% in ACN) for 10 min while

phosphorothioate oxidation utilized 0.05 M EDITH reagent /acetonitrile. Detritylation was achieved with 3% TCA/dichloromethane. The DMT protecting group was removed after the last step of the cycle.

After completion of synthesis the controlled pore glass (CPG) was transferred to a screw cap, sterile microfuge tube. The oligonucleotide was cleaved and simultaneously the base and phosphate groups deprotected with 1.0 mL of a mixture of ethanolic methylamine:ammonia (8 M methylamine in ethanol/ 30% aq ammonia) (1:1) for 5 hours at 55°C. The tube was cooled briefly on ice and then the solution was transferred to a 5 mL centrifuge tube; this was followed by washing three times with 0.25 mL of 50% acetonitrile. The tubes were cooled at -80°C for 15 min, before drying in a lyophilizer.

The white residue obtained was resuspended in 200 uL of NMP/Et₃N/Et₃N-HF and heated at 65°C for 1.5h to remove the TBDMS groups at the 2'-position. The oligonucleotides were then precipitated in dry diethyl ether (400 uL) containing Et₃N (1%). The liquid was removed carefully to yield a pellet at the bottom of the tube. Residual ether was removed in the speed vacuum to give the "crude" RNA as a white fluffy material. Samples were dissolved in 1mL RNase free water and quantitated by measuring absorbance at 260 nm. This crude material was stored at -20°C.

The crude oligonucleotides were analyzed and purified by HPLC. The crude oligonucleotides were analyzed and purified by Reverse Phase IonPair (RP IP) HPLC. The RP HPLC analysis was performed on a Gilson LC system, equipped with a fixed wavelength detector (260 and 280 nm), column oven, autosampler and internal degasser. An XTerra C18 column (4.6*250mm) was used at a flow rate of 1.0 mL/min at 65°C. RNA (20 µL for analytical run, 1 mL for a preparative run at 1 OD/mL concentration) was injected. Eluent A contained 0.1 M TEAAc, HPLC water, pH 7.0 and Eluent B was 0.1 M TEAAc in HPLC water, 70% acetonitrile, pH 7.0. The elution started with 10% B for 2 min, followed by 25% B in 4 min and then a linear gradient with a target concentration of 50% B over another 30 min was employed. The purified dry oligonucleotides were then desalted using Sephadex G25M.

B. Synthesis of oligonucleotides with 2'-Fluoro modifications

The RNA molecules were synthesized on a 394 ABI machine using the standard cycle written by the manufacturer with modifications to a few wait steps. The solid support was CPG

(500A, TsT AG 001 from AM Chemicals LLC and the rC and rU were from Prime Synthesis).

The monomers were either RNA phosphoramidites or 2' F phosphoramidites with standard protecting groups and used at concentrations of 0.15 M in acetonitrile (CH₃CN) unless otherwise stated. Specifically the RNA phosphoramidites were 5'-O-Dimethoxytrityl-N⁶-benzoyl-2'-O-*t*-butyldimethylsilyl-adenosine-3'-O-(β-cyanoethyl-N,N'-diisopropyl) phosphoramidite, 5'-O-Dimethoxytrityl-N²-isobutyryl-2'-O-*t*-butyldimethylsilyl-guanosine-3'-O-(β-cyanoethyl-N,N'-diisopropyl)phosphoramidite, 5'-O-Dimethoxytrityl-N⁴-acetyl-2'-O-*t*-butyldimethylsilyl-cytidine-3'-O-(β-cyanoethyl-N,N'-diisopropyl)phosphoramidite, and 5'-O-Dimethoxytrityl-2'-O-*t*-butyldimethylsilyl-uridine-3'-O-(β-cyanoethyl-N,N'-diisopropyl)phosphoramidite; the 2'F RNA phosphoramidites were 5'-O-Dimethoxytrityl-N⁴-acetyl-2'-*fluoro*-2'-deoxy-cytidine-3'-O-(β-cyanoethyl-N,N'-diisopropyl)phosphoramidite and 5'-O-Dimethoxytrityl-2'-*fluoro*-2'-deoxy-uridine-3'-O-(β-cyanoethyl-N,N'-diisopropyl)phosphoramidite. The coupling times were 10 min for all monomers. Details of the other reagents are as follows: Activator: 5-ethyl thiotetrazole (0.25 M); Cap A: 5% acetic anhydride/THF/pyridine; Cap B: 10% N-methylimidazole/THF; phosphate oxidation involved THBP (10% in ACN) for 10 min while phosphorothioate oxidation utilized 0.05 M EDITH reagent/acetonitrile. Detritylation was achieved with 3%

TCA/dichloromethane. The DMT protecting group was removed after the last step of the cycle.

After completion of synthesis, CPG was transferred to a screw cap, sterile microfuge tube. The oligonucleotide was cleaved and the base and phosphate groups were simultaneously deprotected with 1.0 mL of a mixture of ethanolic ammonia (1:3) for 7 hours at 55°C. The tube was cooled briefly on ice and then the solution was transferred to a 5 mL centrifuge tube; this was followed by washing three times with 0.25 mL of 50% acetonitrile. The tubes were cooled at -80°C for 15 min, before drying in a lyophilizer.

The white residue obtained was resuspended in 200 uL of NMP/Et₃N/Et₃N-HF and heated at 50°C for 16 h to remove the TBDMS groups at the 2' position. The oligonucleotides were then precipitated in dry diethyl ether (400 uL) containing Et₃N (1 %). The liquid was removed carefully to yield a pellet at the bottom of the tube. Residual ether was removed in the speed vacuum to give the "crude" RNA as a white fluffy material. Samples were dissolved in 1 mL RNase free water and quantitated by measuring the absorbance at 260 nm. This crude material was stored at -20°C.

The crude oligonucleotides were analyzed and purified by HPLC. The purified dry oligonucleotides were then desalted using Sephadex G25M.

C. Synthesis of phosphorothioate RNA oligoribonucleotides

5 The oligonucleotides were synthesized on a 394 ABI machine (ALN 0208) using the standard 93 step cycle written by the manufacturer with modifications to a few steps as described below. The solid support was controlled pore glass (CPG, 2 μ mole rA CPG, 520A, or rU CPG, 500A). The monomers were RNA phosphoramidites with standard protecting groups used at concentrations of 0.15 M in acetonitrile (CH_3CN) unless otherwise stated. Specifically the RNA
10 phosphoramidites were 5'-O-Dimethoxytrityl- N⁶-benzoyl-2'-O-*t*-butyldimethylsilyl- adenosine-3'-O-(β -cyanoethyl-N,N'-diisopropyl) phosphoramidite, 5'-O-Dimethoxytrityl- N²-isobutyryl-2'-O-*t*-butyldimethylsilyl- guanosine-3'-O-(β -cyanoethyl-N,N'-diisopropyl)phosphoramidite, 5'-O-Dimethoxytrityl- N⁴-acetyl-2'-O-*t*-butyldimethylsilyl- cytidine-3'-O-(β -cyanoethyl-N,N'-diisopropyl)phosphoramidite and 5'-O-Dimethoxytrityl-2'-O-*t*-butyldimethylsilyl- uridine-3'-O-
15 (β -cyanoethyl-N,N'-diisopropyl)phosphoramidite. The coupling times were 10 min. Details of the other reagents are as follows: activator: 5-ethyl thiotetrazole (0.25M); Cap A: 5% acetic anhydride/THF/pyridine; Cap B: 10% N-methylimidazole/THF; PS-oxidation, 0.05M EDITH reagent /acetonitrile. Detritylation was achieved with 3% TCA/dichloromethane.

After completion of synthesis the CPG was transferred to a screw cap sterile microfuge
20 tube. The oligonucleotide was cleaved and simultaneously the base and phosphate groups deprotected with 1.0 mL of a mixture of ethanolic methylamine:ammonia (1:1) for 5 hours at 55°C. The tube was cooled briefly on ice and then the solution was transferred to a 5 mL centrifuge tube; this was followed by washing with 3 x 0.25 mL of 50% acetonitrile. The tubes were cooled at -80°C for 15 min, before drying in a lyophilizer.

25 The white residue obtained was resuspended in 200 μ L of TEA:3HF and heated at 65°C for 1.5 h to remove the TBDMS groups at the 2'-position. The oligonucleotides were then precipitated by addition of 400 μ L dry MeOH. The liquid was removed after spinning in a microcentrifuge for 5 minutes on the highest speed available. Residual methanol was removed in speed vacuum. Samples were dissolved in 1 mL RNase free water and quantitated by

measuring the absorbance at 260 nm. The crude material was stored at -20°C. The oligonucleotides were analyzed and purified by HPLC and then desalted using Sephadex G25M.

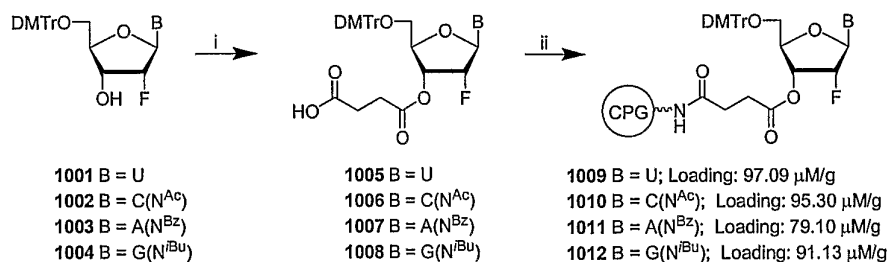
Example 9. Synthesis of oligonucleotides with alternating 2'-F RNA and 2'-O-Me RNA

(Table 7)

A. Synthesis of CPGs for 2'-F.

CPGs of 5'-O-DMTr-2'-deoxy-2'-fluororibonucleosides with appropriate base protection were synthesized as shown in Scheme A. 5'-O-DMTr-2'-Deoxy-2'-fluoro-N^{Bz}-A and 5'-O-DMTr-2'-Deoxy-2'-fluoro-N^{iBu}-G were synthesized as reported (Kawasaki *et al.*, *J. Med. Chem.*, **1993**, 36, 831). Reaction of compounds **1001** with succinic anhydride in the presence of DMAP in ethylenedichloride yielded compound **1005**. Compound **1005** was treated with 2,2'-dithiobis(5-nitropyridine) (DTNP) and triphenylphosphine in the presence of DMAP in acetonitrile-ethylenedichloride and subsequently with lcaa CPG as reported by Kumar *et al.* (*Nucleosides & Nucleotides*, **1996**, 15, 879) yielded the desired CPG **1009**. Loading of the CPG was determined as reported in the literature (Prakash *et al.*, *J. Org. Chem.*, **2002**, 67, 357). CPGs of suitably protected 2'-deoxy-2'-fluoro A, C and G were obtained as described above (Scheme A).

Scheme A^a: lcaa CPG of 2'-deoxy-2'-F A(N^{Bz}), C(N^{Ac}), G(N^{iBu}) and U



^a (i) Succinic anhydride, DMAP/EDC (ii) DTNP, Ph₃P, DMAP and lcaa CPG.

The chimeric RNA molecules with alternating 2'-F RNA and 2'-O-Me RNA were synthesized on a 394 ABI machine using the standard cycle written by the manufacturer with modifications to a few wait steps. The solid support were CPG (500A). The monomers were either 2'-F RNA phosphoramidites or 2' OMe RNA phosphoramidites with standard protecting

groups and used at concentrations of 0.15 M in acetonitrile (CH_3CN) unless otherwise stated. Specifically the 2'OMe RNA phosphoramidites were 5'-O-Dimethoxytrityl-N⁶-benzoyl-2'-O-methyl-adenosine-3'-O-(β -cyanoethyl-N,N'-diisopropyl) phosphoramidite, 5'-O-Dimethoxytrityl-N²-isobutyryl-2'-O-methyl-guanosine-3'-O-(β -cyanoethyl-N,N'-diisopropyl)phosphoramidite, 5'-O-Dimethoxytrityl-N⁴-acetyl-2'-O-methyl-cytidine-3'-O-(β -cyanoethyl-N,N'-diisopropyl)phosphoramidite and 5'-O-Dimethoxytrityl-2'-O-methyl-uridine-3'-O-(β -cyanoethyl-N,N'-diisopropyl)phosphoramidite. The 2'F RNA phosphoramidites 5'-O-Dimethoxytrityl-N⁴-acetyl-2'-fluoro-2'-deoxy-cytidine-3'-O-(β -cyanoethyl-N,N'-diisopropyl)phosphoramidite, 5'-O-Dimethoxytrityl-2'-fluoro-2'-deoxy-uridine-3'-O-(β -cyanoethyl-N,N'-diisopropyl)phosphoramidite, 5'-O-Dimethoxytrityl-2'-fluoro-N²-isobutyryl-2'-deoxy-guanosine-3'-O-(β -cyanoethyl-N,N'-diisopropyl)phosphoramidite and 5'-O-Dimethoxytrityl-2'-fluoro-N²-isobutyryl-2'-deoxy-guanosine-3'-O-(β -cyanoethyl-N,N'-diisopropyl)phosphoramidite. The coupling times were 10 min for all monomers. Details of the other reagents are as follows: Activator: 5-ethyl thiotetrazole (0.25M); Cap A: 5% acetic anhydride/THF/pyridine; Cap B: 10% N-methylimidazole/THF; phosphate oxidation involved 0.02M I_2 /THF/ H_2O , while PS-oxidation was carried out using EDITH reagent as described above. Detritylation was achieved with 3% TCA/dichloromethane. The final DMT protecting group was removed in the synthesizer.

After completion of synthesis the CPG was transferred to a screw cap, sterile microfuge tube. The oligonucleotide was cleaved and the base and phosphate groups were simultaneously deprotected with 1.0 mL of a mixture of ethanolic:ammonia (1:3) for 7 hours at 55°C. The tube was cooled briefly on ice and then the solution was transferred to a 5 mL centrifuge tube; this was followed by washing three times with 0.25 mL of 50% acetonitrile. The tubes were cooled at -80°C for 15 min before drying in a lyophilizer to give the "crude" RNA as a white fluffy material. Samples were dissolved in 1mL RNase free water and quantitated by measuring the absorbance at 260 nm. This crude material was stored at -20°C.

The crude oligonucleotides were analyzed and purified by 20% polyacrylimide denaturing gels. The purified dry oligonucleotides were then desalted using Sephadex G25M (Amersham Biosciences).

B. Analysis of Duplex activity

Duplexes were tested for activity in the HeLa cell assay described above. Table 7 and Figure 30 provides graphs of the activities in HeLa cells for each of the modifications described above.

5 Example 10 Conjugated VEGF molecules (Tables 8, 9, 10 and 18)

1. Synthesis:

The RNA molecules were synthesized on an ABI-394 machine (Applied Biosystems) using the standard 93 step cycle written by the manufacturer with modifications to a few wait steps as described below. The solid support was controlled pore glass (CPG, 1umole, 500 A) and the monomers were RNA phosphoramidites with standard protecting groups (5'-O-dimethoxytrityl-N6-benzoyl-2'-O-t-butyl dimethylsilyl-adenosine-3'-O-N,N'-diisopropyl-2-cyanoethylphosphoramidite, 5'-O-dimethoxytrityl-N4-acetyl-2'-O-t-butyl dimethylsilyl-cytidine-3'-O-N,N'-diisopropyl-2-cyanoethylphosphoramidite, 5'-O-dimethoxytrityl-N2-isobutryl-2'-O-t-butyl dimethylsilyl-guanosine-3'-O-N,N'-diisopropyl-2-cyanoethylphosphoramidite, and 5'-O-dimethoxytrityl-2'-O-t-butyl dimethylsilyl-uridine-3'-O-N,N'-diisopropyl-2-cyanoethylphosphoramidite. All amidites were used at a concentration of 0.15M in acetonitrile (CH₃CN) and a coupling time of 6 min for unmodified and 2'-O-Me modified monomers and 12 min for modified and conjugated monomers. 5-ethyl thiotetrazole (0.25M) was used as an activator. For the PO-oxidation Iodine/Water/Pyridine and for PS-oxidation Beaucage reagent (20 %) in anhy. acetonitrile was used. The sulfurization time was about 6 min. All syntheses was performed on a 1 umole scale.

Reagents	Concentration		Wait or Coupling step
Activator:	0.25M	5-Ethylthio-1H-tetrazole	720 sec
PO-oxidation	0.02M	Iodine in THF/Water/Pyridine	20 sec
PO-oxidation	0.02M	t-Butyl-hydrogen peroxide	600 sec
PS-oxidation	2 %	Beaucage reagent /anhy. Acetonitrile	360 Sec (200 sec, wait +30 sec pulse+130 sec wait
Cap A	5%	5%Phenoxyacetic anhydride/THF/pyridine	20 sec

Cap B	10%	10% N-methylimidazole/THF	20 sec
Detritylation	3% TCA	Trichloro Acetic Acid /dichloromethane	70 sec

The following types of modifications were used to perform the synthesis using these protocols:

1. Unmodified phosphodiester backbone (PO) only
2. Phosphorothioate (PS) only
3. 2'-O-Me, PS
4. 3'-Naproxen, 2'F- 5Me-U, PS
5. 5'-Cholesterol, PS
6. 3'-Cholesterol, PS
7. 2'F- 5Me-U, PS
8. 3'-Biotin, 2'F- 5Me-U, PS
9. 3'-cholanic acid, 2'F- 5Me-U, PS
10. Methylphosphonate
11. C-5 allylamino rU

2. Deprotection- I (Nucleobase Deprotection)

After completion of the synthesis, the controlled pore glass (CPG) was transferred to a screw cap vial or screw cap RNase free microfuge tube. The oligonucleotide was cleaved from the support and the base and phosphate protecting groups were simultaneously removed by using a mixture of ethanolic ammonia (ammonia (28-30 % : ethanol (3:1))- (1.0 mL) for 15h at 55°C. The vial was cooled briefly on ice and then the ethanolic ammonia mixture was transferred to a new microfuge tube. The CPG was washed with portions of deionized water (2 x 0.1 mL). The supernatant was combined, cooled in dry ice for 10 min and then dried in a speed vac.

3. Deprotection-II (Removal of 2'-O- TBDMS group)

The white residue obtained was resuspended in a mixture of triethylamine, triethylamine trihydrofluoride (TEA.3HF ca. 24% HF)) and 1-Methyl-2-Pyrrolidinone (NMP) (4:3:7) (400 ul) and heated at 65°C for 90 min to remove the tert-butyldimethylsilyl (TBDMS) groups at the 2'-

position. The reaction was then quenched with isopropoxytrimethylsilane (iPrOMe₃Si, 400 ul) and further incubated on the heating block leaving the caps open for 10min; This causes the volatile isopropoxytrimethylsilyl fluoride adduct to vaporize. The residual quenching reagent was removed by drying in a speed vac. 3% Triethylamine in diethyl ether (1.5 ml) was added.

5 The mixture was subjected to centrifugation. A pellet of RNA formed. The supernatant was pipetted out without disturbing the pellet. The pellet was dried in a speed vac. The crude RNA was obtained as a white fluffy material in the microfuge tube.

10

4. Quantitation of Crude Oligomer or Raw Analysis

Samples were dissolved in deionized water (1.0mL) and quantitated as follows: Blanking was first performed with water alone (1mL). A sample of the RNA solution (20ul) was diluted with water (980 uL) and mixed well in a microfuge tube, then transferred to a cuvette and the

15 absorbance reading was obtained at 260 nm. The crude material was dried down and stored at -20 °C

5. MS analysis:

The crude samples (0.1 OD) analyzed using LC-MS.

20

6. Purification of Oligomers

(a) Polyacrylamide Gel Electrophoresis (PAGE) Purification

The oligonucleotides were purified by vertical slab polyacrylamide gel electrophoresis (PAGE) using an Owl's Separation Systems (Portsmouth, NH). Electrophoresis grade

25 acrylamide (40%), N,N'-methylene-bis(acrylamide) (BIS), ammonium persulfate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED), bromophenol blue (BPB), xylene cyanol (XC) 10 x TBE (0.89 M tris-hydroxy-methylaminomethane, borate pH 8.3, 20mM disodium ethylenediaminetetraacetate) were from National Diagnostics (Atlanta, GA). The 12 % denaturing gel was prepared for purification of unmodified and modified oligoribonucleotides.

The thickness of the preparative gels was 1.5 mm. Loading buffer was 80% formamide in 10x TBE. After removal of the glass plates, the gels were covered with Saran Wrap® and placed over a fluorescent TLC plate illuminated by a hand-held UV lamp for visualization. The desired bands were excised and shaken overnight in 2mL of water or 0.03 M Sodium Acetate. The eluent was removed by drying in a speed vac.

(b) High Performance Liquid chromatography (HPLC) Purification:

Condition A: Purification of unmodified, 2'-O-Me/PS Oligoribonucleotides:

Amount of injected sample is about ~100 OD.

Column: Dionex PA-100 Semiprep.

Buffer A: Water

Buffer B: 0.25 M Tris.Cl pH 8.0

Buffer C: 0.375 M Sod.Perchlorate

Heating: 65 °C

Time	Flow	Buffer A	Buffer B	Buffer C	TotalYield	Purity
0	5.00	88 %	10 %	2.0 %	40-60%	85-98 %
3.0	5.00	88 %	10 %	2.0 %		
30.0	5.00	57.0	10 %	33.0		
35.0	5.00	88 %	10 %	2.0 %		
40.0	5.00	88 %	10 %	2.0 %		

Condition B: Protocols for Purification of 2'-O-Me/PS Oligoribonucleotides:

Column: Dionex PA-100 Semiprep.

Buffer A: Water

Buffer B 0.25 M Tris.Cl pH 8.0

Buffer C 0.8 M Sod.Perchlorate

Heating: 65 °C

Time	Flow	Buffer A	Buffer B	Buffer C	Total Yield	Purity
0	5.00	88 %	10 %	2.0 %	~40-60%	85-98 %
3.0	5.00	88 %	10 %	2.0 %		
30.0	5.00	57.0	10 %	33.0		

35.0	5.00	88 %	10 %	2.0 %		
40.0	5.00	88 %	10 %	2.0 %		

7. Desalting of Purified Oligomer

The purified dry oligomer was then desalted using Sephadex G-25 M. The cartridge was conditioned with 10 mL of deionised water thrice. Finally the purified oligomer dissolved thoroughly in 2.5mL RNAse free water was applied to the cartridge with a very slow drop-wise elution. The salt free oligomer was eluted with 3.5 ml deionized water directly into a screw cap vial. The purified RNA material was dried down in speed vac and stored at -20°C.

Biotin conjugated siRNAs (Table 10)

1. Synthesis:

The RNA molecules were synthesized on an ABI-394 machine (Applied Biosystems) using the standard 93 step cycle written by the manufacturer with modifications to a few wait steps as described below. The solid support was controlled pore glass (CPG, 1umole, 500 A) and the monomers were RNA phosphoramidites with standard protecting groups (5'-O-dimethoxytrityl N6-Benzoyl-2'-O-t-butyldimethylsilyl- adenosine-3'-O-N,N'-diisopropyl-2-cyanoethylphosphoramidite, 5'-O-dimethoxytrityl-N4-acetyl-2'-O-t-butyldimethylsilyl-cytidine-3'-O-N,N'-diisopropyl-2-cyanoethylphosphoramidite, 5'-O-dimethoxytrityl-N2-isobutryl-2'-O-t-butyldimethylsilyl-guanosine-3'-O-N,N'-diisopropyl-2-cyanoethylphosphoramidite, and 5'-O-dimethoxytrityl-2'-O-t-butyldimethylsilyl-uridine-3'-O-N,N'-diisopropyl-2-cyanoethylphosphoramidite. The modified CPG and amidites were synthesized using known methods and as described herein. . All amidites were used at a concentration of 0.15M in acetonitrile (CH₃CN) and a coupling time of 6 min for unmodified and 2'-O-Me monomers and 12 min for modified and conjugated monomers. 5-Ethylthio-1H-tetrazole (0.25M) was used as an activator. For the PO-oxidation Iodine/Water/Pyridine and for PS-oxidation Beaucage reagent (2 %) in anhy. acetonitrile was used. The sulfurization time is about 6 min. For synthesis of 3'-biotin conjugated siRNAs, t-butyl-hydrogen peroxide was used as oxidizing agent (oxidation time 10 min).

Reagent	Concentration		Wait or Coupling step
Activator:	0.25M	5-Ethylthio-tetrazole	300 sec for unmodified and 720 sec for modified oligos.
PO-oxidation	0.02M	Iodine in THF/water/pyridine	20sec
PO-oxidation	0.02M	t-Butyl-hydrogen peroxide	600 sec
PS-oxidation	2 %	Beaucage reagent /anhy. Acetonitrile	360 Sec (200 sec, wait +30 sec pulse+130 sec wait
Cap A	5%	5% Phenoxyacetic anhydride/THF/pyridine	20sec
Cap B	10%	10% N-Methylimidazole/THF	20sec
Detritylation	3% TCA	Trichloro Acetic Acid/dichloromethane	70sec

2. Deprotection- I (Nucleobase Deprotection)

After completion of synthesis the controlled pore glass (CPG) was transferred to a screw cap vial or a screw cap RNase free microfuge tube. The oligonucleotide was cleaved from the support with the simultaneous removal of base and phosphate protecting groups with a mixture of ethanolic ammonia [ammonia (28-30%): ethanol (3:1) 1.0 mL] for 15h at 55°C. The vial was cooled briefly on ice and then the ethanolic ammonia mixture was transferred to a new microfuge tube. The CPG was washed with portions of deionized water (2 x 0.1 mL). The combined filtrate was then put in dry ice for 10 min dried in a speed vac.

3. Deprotection-II (Removal of 2'-O- TBDMS group)

The white residue obtained was resuspended in a mixture of triethylamine, triethylamine trihydrofluoride (TEA.3HF ca, 24% HF) and 1-Methyl-2-Pyrrolidinone (NMP) (4:3:7) (400 ul) and heated at 65°C for 90 min to remove the tert-butyldimethylsilyl (TBDMS) groups at the 2' position. The reaction was then quenched with isopropoxytrimethylsilane (iPrOMe₃Si, 400 ul) and further incubated on the heating block leaving the caps open for 10min; (This causes the volatile isopropoxytrimethylsilylfluoride adduct to vaporize). The residual quenching reagent was

removed by drying in a speed vac. 3% Triethylamine in diethyl ether (1.5 ml) was added and the mixture was subjected to centrifugation to afford a pellet of RNA. The supernatant was pipetted out without disturbing the pellet. The pellet was dried in a speed vac. The crude RNA was obtained as a white fluffy material in the microfuge tube.

5

4. Quantitation of Crude Oligomer or Raw Analysis

Samples were dissolved in deionized water (1.0mL) and quantitated as follows: Blanking was first performed with water alone (1mL). A sample of the RNA solution (20ul) was diluted with water (980 uL) and mixed well in a microfuge tube, then transferred to a cuvette and the absorbance reading was obtained at 260 nm. The crude material was dried down and stored at -20°C.

10

5. MS analysis:

Samples of the RNA (0.1 OD) were analyzed using MS.

15

6. Purification of Oligomers

Polyacrylamide Gel Electrophoresis (PAGE) Purification

The oligonucleotides were purified by vertical slab polyacrylamide gel electrophoresis (PAGE) using an Owl's Separation Systems (Portsmouth, NH). Electrophoresis grade acrylamide (40%), N,N'-methylene-bis(acrylamide) (BIS), ammonium persulfate (APS, 20 acrylamide (40%), N,N'-methylene-bis(acrylamide) (BIS), ammonium persulfate (APS, N,N,N',N'-tetramethylethylenediamine (TEMED), bromophenol blue (BPB), xylene cyanol (XC) 10 x TBE (0.89 M). Trishydroxy-methylaminomethane, borate (pH 8.3), 20mM disodium ethylenediaminetetraacetate) were from National Diagnostics (Atlanta, GA). The 12 % Denaturing gel was prepared for purification of oligoribonucleotides. The thickness of the preparative gel was 1.5 mm. Loading buffer was 80% formamide in 10x TBE. After removal of the PAGE glass plates, the gels were covered with Saran Wrap® and placed over a fluorescent TLC plate illuminated by a hand-held UV lamp (Upland, CA) for visualization. The desired bands were excised and shaken overnight in water (2mL) or 0.03 M sodium acetate. The eluent was removed and dried in a speed vac. All biotin conjugated sequences were purified by PAGE.

25

7. Desalting of Purified Oligomer

The purified dry oligomer was then desalted using Sephadex G-25 M (Amersham Biosciences). The cartridge was conditioned with deionized water thrice (10 mL each).
5 Finally the purified oligomer dissolved thoroughly in 2.5mL RNase free water was applied to the cartridge with very slow drop- wise elution. The salt free oligomer was eluted with deionized water (3.5 ml) directly into a screw cap vial. The purified RNA material was dried down on speed vac and stored at -20°C

10

8. Quality Control

(a) Capillary Gel Electrophoresis (CGE)

(b) Electrospray LC/MS

15 A sample of the oligomer (approx. 0.10 OD) was dissolved in water (50 ul & 100 ml in separate tubes) and then pipetted into special vials for CGE and LC/MS analysis.

9. Analysis of Duplex activity

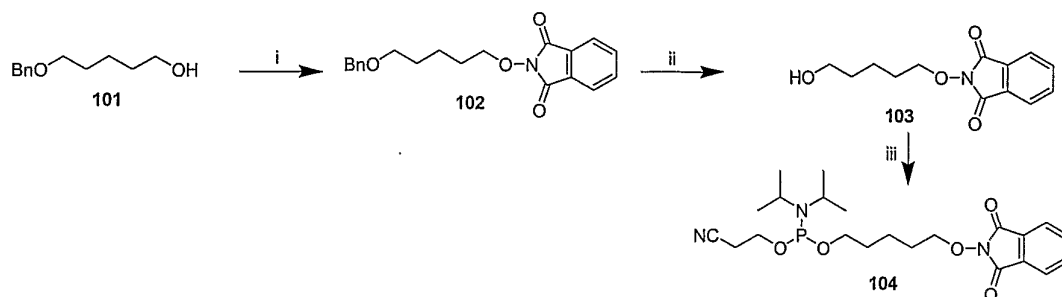
Duplexes were tested for activity in the HeLa cell assay described above. Tables 8, 9, 10 and 18 and Figures 31-35 provides data and graphs of the activities in HeLa cells for each of the
20 modifications described above.

Example 11 Conjugation of retinoids to RNA (Table 14)

Conjugation of all-*trans*-retinal to Oligonucleotides (RNA):

Phosphoramidite **104** was synthesized as shown in Scheme B for retinal conjugation to
25 oligonucleotides.

Scheme B: Synthesis of Post-synthetic conjugation building blocks for retinal conjugation – oxime approach 1 for 5'-conjugation.



^a (i) Ph_3P , DIAD, *N*-hydroxyphthalimide / MeCN; (ii) H_2 , Pd-C (10 %), 1 atm/EtOAc; (iii) Phosphitylation

5 Step 1: Compound 102: Monobenzylpentan-1,5-diol (15.70 g, 80.82 mmol), Ph_3P (25.43 g, 96.84 mmol) and *N*-hydroxyphthalimide (116.0g, 98.08 mmol) were taken in anhydrous CH_3CN (100 ml) under argon atm. Neat DIAD (20.0 mL, 103.25 mmol) was added dropwise into the stirring solution over a period of 20 minutes and the stirring was continued for 24h. The reaction was monitored by TLC. Solvents were removed *in vacuo*; and the residue was triturated with diethyl ether and filtered. Residue was washed with ether, filtered and combined the filtrate. Hexane was added dropwise into the filtrate until it gave turbidity and subsequently the solution was made homogeneous by adding ether into it. The homogeneous solution was stored at 5 °C for 24 h. Precipitated Ph_3PO was filtered off, washed with ether-hexane mixture (1:1). Combined filtrate was evaporated to dryness and the residue was purified by flash silica gel column chromatography (10-15 % EtOAc in Hexane) to obtain 24.5 g (89.3 %) of compound 102 as a viscous pale yellow oil. ^1H NMR (400 MHz, CDCl_3 , 25 °C): 7.84-7.82 (m, 2H); 7.75-7.73 (m, 2H); 7.34-7.33 (m, 4H); 7.29-7.26 (m, 1H); 4.51 (s, 2H); 4.22-4.18 (t, $J(\text{H,H}) = 6.71$ Hz, 2H); 3.52-3.48 (t, $J(\text{H,H}) = 6.4$ Hz, 2H); 2.04-1.78 (m, 2H); 1.73-1.56 (m, 4H). ^{13}C NMR (100 MHz, CDCl_3 , 25 °C): 163.9, 138.8, 134.6, 129.2, 128.6, 127.8, 127.7, 123.7, 78.6, 73.1, 70.3, 29.6, 28.2, 22.5.

20 Step 2: Compound 103: Compound 102 (23.5 g, 69.29 mmol) was taken in 100 ml of EtOAc/methanol (1:1). The mixture was degassed and purged with argon, to this 2.4 g of Pd-C (10%- wet Degusa type) was added. The mixture was then hydrogenated overnight, filtered through a celite bed over a sintered funnel. The residue was subsequently passed through a

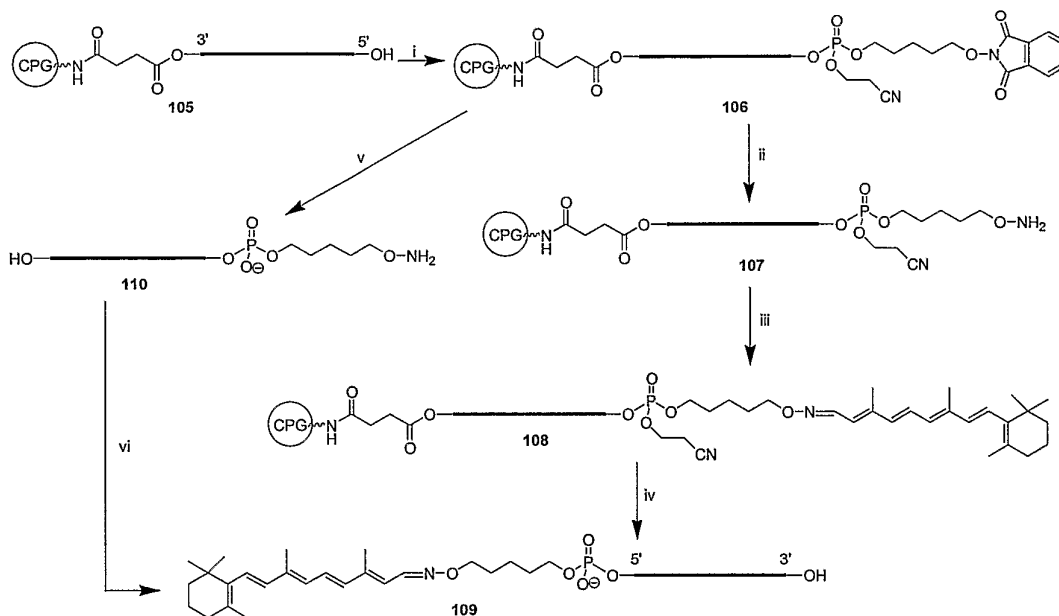
column of silica gel and eluted out using 40 % EtOAc in hexane to obtain compound **103** (15.70 g, 90.9 %) as a white solid. ^1H NMR (400 MHz, CDCl_3 , 25 °C) 7.83-7.81 (bm, 2H); 7.75-7.73 (bm, 2H); 4.23-4.19 (t, $J(\text{H,H}) = 6.4$ Hz, 2H); 3.70-3.66 (t, $J(\text{H,H}) = 5.80$ Hz, 2H); 1.83-1.79 (m, 2H); 1.67-1.60 (m, 4H). ^{13}C NMR (100 MHz, CDCl_3 , 25 °C) δ 163.9, 134.7, 129.1, 123.7, 78.6, 62.7, 32.4, 28.0, 22.0.

Step 3: Compound **104**: Compound **103** (5.4 g, 21.67 mmol) and triethylamine (4 ml, 28.69 mmol) were taken in anhydrous EtOAc(30 ml) under argon. 2-Cyanoethyl diisopropylchlorophosphoramidite (5.00ml, 21.97 mmol) was added to the reaction mixture dropwise. A white precipitate of $\text{Et}_3\text{N.HCl}$ was formed immediately after the addition of the reagent and the reaction was complete in 10 min (monitored by TLC). The precipitate was filtered through a sintered funnel and solvent was removed under reduced pressure. The residue was directly loaded on a silica gel column for purification. Eluted with hexane/EtOAc 9:1 to afford compound **104** as a yellow oil, 8.68g (89.13%). ^1H NMR (400 MHz, CDCl_3 , 25 °C) δ 7.85-7.81 (m, 2H); δ 7.77-7.72 (m, 2H); 4.22-4.19 (t, $J(\text{H,H}) = 6.80$ Hz, 2H); 3.91-3.76 (m, 2H); 3.72-3.53 (m, 4H); 2.67-2.63 (t, $J(\text{H,H}) = 6.71$ Hz, 2H); 1.86-1.78 (m, 2H); 1.73-1.66 (m, 2H); 1.62-1.56 (m, 2H); 1.19-1.16 (m, 12H). ^{31}P NMR (162 MHz, CDCl_3 , 25 °C) δ 145.09. ^{13}C NMR (100 MHz, CDCl_3 , 25 °C) δ 163.9, 134.7, 129.2, 123.7, 117.9, 78.6, 64.0, 63.4, 58.7, 58.5, 43.2, 43.1, 31.1, 31.0, 28.1, 24.9, 24.8, 24.7, 22.3, 20.6, 20.5.

Step 4: Conjugation of all-*trans*-retinal to Oligonucleotide: All-*trans*-retinal was conjugated to oligonucleotide as shown in the Scheme C. Compound **104** was coupled to solid bound oligonucleotide **105** under standard solid phase oligonucleotide synthesis conditions to obtain compound **106**. Phthalimido protecting group on compound **106** was selectively removed by treating with hydrazinium hydrate as reported by Salo *et al.* (*Bioconjugate Chem.* **1999**, *10*, 815) to obtain compound **107**. Treatment of compound **107** with all-*trans*-retinal under dark condition gave compound **108** as reported in the literature (*Bioconjugate Chem.* **1999**, *10*, 815). Standard RNA oligonucleotide deprotection and purification under dark yielded the desired oligonucleotide-retinal conjugate **109**. Compound **109** was also obtained from compound **110** as shown in Scheme C. Complete deprotection and purification of compound **106** yielded an

unbound free oligonucleotide **110** which was subsequently reacted with all-*trans*-retinal to afford the desired compound **109**.

Scheme C: Conjugation of all-*trans*-retinal to oligonucleotides



5

- ^a (i) Phosphoramidite **104**, (standard oligonucleotide synthesis cycle); (ii) Hydrazinium hydrate/Py/AcOH (0.124/4/7); (iii) all-*trans*-retinal in DMF or MeCN; (iv) Oligonucleotide (RNA) deprotection (MeNH₂, TEA.3HF) and purification; (v) Oligonucleotide (RNA) deprotection (MeNH₂, TEA.3HF) and purification; (vi) all-*trans*-retinal in DMSO-H₂O
- 10

Step 4.1. Oligonucleotide Synthesis:

All oligonucleotides except AL-3166 were synthesized on an ABI 490 DNA synthesizer. Commercially available controlled pore glass solid supports (dT-CPG and U-CPG, 500Å) 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-

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guanosine-3'-O-N,N'-diisopropyl-2-cyanoethylphosphoramidite, and 5'-O-dimethoxytrityl-2'-*t*-butyldimethylsilyl-uridine-3'-O-N,N'-diisopropyl-2-cyanoethylphosphoramidite were used for the oligonucleotide synthesis. All phosphoramidites were used at a concentration of 0.15M in acetonitrile (CH₃CN). Coupling time of 10 minutes was used. The activator was 5-ethyl thiotetrazole (0.25M), for the PO-oxidation Iodine/Water/Pyridine was used.

Sequence AL-3166 was synthesized on the AKTAoligopilot synthesizer. All phosphoramidites were used at a concentration of 0.2M in acetonitrile (CH₃CN) except for guanosine which was used at 0.2M concentration in 10% THF/acetonitrile (v/v). Coupling/recycling time of 16 minutes was used. The activator was 5-ethyl thiotetrazole (0.75M), for the PO-oxidation Iodine/Water/Pyridine was used and for the PS-oxidation PADS (2 %) in 2,6-lutidine/ACN (1:1 v/v) was used.

The aminoxy-linker phosphoramidite was synthesized as described above and used at a concentration of 0.15M in acetonitrile. Coupling time for the aminoxy-linker phosphoramidite was 15 minutes. For all sequences, coupling of the aminoxy-linker phosphoramidite was carried out on the ABI 390 DNA synthesizer.

Step 4.2. Cleavage of the phthalimido-protecting group from the aminoxy-linker oligonucleotides

After coupling of the aminoxy-linker, the CPG was treated with 2.5 ml of 0.5M hydrazinium acetate in pyridine (0.16/4/2 hydrazine anhydrous, pyridine, acetic acid) using the dual syringe method. Every 5 minutes the syringes were pushed back and forth to get new solution on the CPG. After the hydrazinium acetate treatment, the CPG was washed with 2x5 ml of pyridine followed by 3x5ml of acetonitrile. Flushing with dry argon for 30 seconds then dried CPG.

Step 4.3. On support conjugation with the aldehydes

The 1-pyrene-carboxaldehyde and the all-*trans*-retinal were from Aldrich and used at concentrations of 0.5M in DMF. The 4-keto-retinol was used at a concentration of 0.13M in DMF. The CPG from above was added to the aldehyde solutions. Conjugation was carried out

overnight (~16 hrs) at room temperature. After the reaction was complete, the CPG was rinsed with DMF followed by acetonitrile and air dried for 10-15 minutes. For sequence AL-3213, the conjugation with both all-*trans*-retinal and 1-pyrene-carboxaldehyde was also carried out in acetonitrile. In the case of 1-pyrene-carboxaldehyde, the aldehyde did not fully dissolved at 0.5M and the solution was used as is without filtration to get rid of the undissolved aldehyde.

Step 4.4. Deprotection- I (Nucleobase Deprotection) of on support conjugated oligonucleotides

For on support retinal conjugated oligonucleotides, the support was transferred to a 5 ml tube (VWR). The oligonucleotide was cleaved from the support with simultaneous deprotection of base and phosphate groups with 1 mL of 40% aq. methylamine 15 mins at 65°C. The tube was cooled briefly on ice and then the methylamine was filtered into a new 15 ml tube. The CPG was washed with 3 x 1 mL portions of DMSO.

Step 4.5. Deprotection-II (Removal of 2' TBDMS group) of on support conjugated oligonucleotides

To the above mixture was added 1.5 ml of triethylamine trihydrofluoride (TREAT-HF) and heated at 60°C for 15 minutes to remove the *tert*-butyldimethylsilyl (TBDMS) groups at the 2' position. The reaction was then quenched with 5.5 ml of 50mM sodium acetate (pH 5.5) and stored in freezer until purification.

Step 4.6. After deprotection conjugation with aldehydes

Conjugation with the aldehydes (1-pyrene-carboxaldehyde and all-*trans*-retinal) after deprotection of the aminoxy-linker oligonucleotides was also carried out as an alternative conjugation strategy.

Step 4.7. Deprotection- I (Nucleobase Deprotection) for after deprotection conjugation

The support was transferred to a 2 ml screw cap tube. The oligonucleotide was cleaved from the support with simultaneous deprotection of base and phosphate groups with 0.5 mL of 40% aq. methylamine 15 mins at 65°C. The tube was cooled briefly on ice and then the

methylamine was filtered into a new 15 ml tube. The CPG was washed with 2 x 0.5 mL portions of 50% acetonitrile/water. The mixture was then frozen on dry ice and dried under vacuum on a speed vac.

5 Step 4.8. Deprotection-II (Removal of 2' TBDMS group) for after deprotection conjugation

The dried residue was resuspended in 0.5 ml of triethylamine trihydrofluoride (TEA.3HF) and heated at 60°C for 15 minutes to remove the *tert*-butyldimethylsilyl (TBDMS) groups at the 2' position. The reaction mixture was then cooled to room temperature and RNA
10 precipitated with 2 ml of dry methanol and dried under vacuum on a speed vac. The sample was then dissolved in 2 ml of water and kept frozen in freezer till further analysis.

Step 4.9. Quantitation of Crude Oligomer or Raw Analysis

For all samples, a 1µl, a 10µl or 30µl aliquote was diluted with 999µl, 990µl or 970µl of
15 deionised nuclease free water (1.0 mL) and absorbance reading obtained at 260 nm.

Step 4.10. Purification of conjugated Oligomers

(a) Cude LC/MS analysis

The crude oligomers were first analyzed by LC/MS, to look at the presence and
20 abundance of the expected final product.

(b) Reverse-phase purification

The conjugated samples were purified by reverse-phase HPLC on an RPC-Source15 column (21.5 x 1 cm). The buffer system was: A = 20 mM sodium acetate in 10% ACN, pH 8.5
25 and B = 20 mM sodium acetate in 70% ACN, pH 8.5, with a flow rate of 5.0 mL/min, and wavelengths 260 and 375. The fractions containing the full-length oligonucleotides were then individually desalted.

Step 4.11. Desalting of purified oligonucleotides

The purified oligonucleotide fractions were desalted using the PD-10 Sephadex G-25 columns. First the columns were equilibrated with 25-30 ml of water. The samples were then applied in a volume of 2.5 ml. The samples were then eluted in salt-free fraction of 3.5 ml. The desalted fractions were combined together and kept frozen till needed.

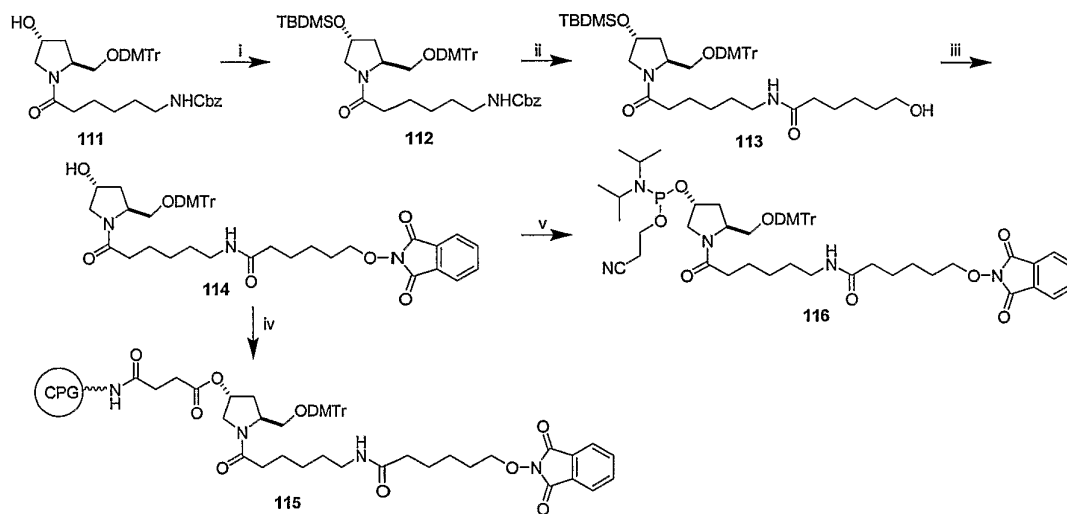
5 Step 4.12. Capillary Gel Electrophoresis (CGE), Ion-Exchange HPLC (IEX) and Electrospray LC/MS

Approximately 0.3 OD of desalted oligonucleotides were diluted in water to 300 μ l and then pipetted in special vials for CGE, IEX and LC/MS analysis.

10 Step 5 Conjugation of all-*trans*-retinal to 3'-end of Oligonucleotides (RNA):

Phosphoramidite **116** for 5'-conjugation and CPG support **115** for 3'-conjugation of retinoids were synthesized as shown in the Scheme D. The CPG support **115** is used for 3' conjugation of retinoids to oligonucleotides

Scheme D^a. Synthesis of Post-synthetic conjugation building blocks for retinal conjugation – oxime approach 2 for 3' and 5' conjugation.



^a (i) TBDMS-Cl, Imidazole/Py, rt; (ii) (a) H₂, Pd-C (10 %)/EtOAc-MeOH, 4 h and (b) \square -Caprolactone, TEA, 55 °C, 24 h; (iii) TEA.3HF/THF; (iv) (a) Succinic anhydride, DMAP/EDC, 24 h and (b) DTNP, Ph₃P, DMAP followed by addition of lcaa CPG; (v) Phosphitylation

Step 5.1: Compound 112: Compound **111** (120.0 g, 30.01 mmol) was stirred with TBDMS-Cl (5.43 g, 36.02 mmol) in the presence of imidazole (7.5 g, 110.16 mmol) in anhydrous pyridine (100 mL) overnight. After removing pyridine, the product was extracted into ethyl acetate (300 mL), washed with aqueous sodium bicarbonate, followed by standard workup. Residue obtained was subjected to flash silica gel column chromatography using 1 % methanol in dichloromethane as eluent to afford compound **112a** as a pale white solid (24.4 g, quant. ¹H NMR (500 MHz, [D₆]DMSO, 25 °C): δ 7.33-7.13 (bm, 15H, accounted for 14H after D₂O exchange); 6.87-6.82 (bm, 4H); 5.01 (s, 0.2H, rotamer minor); 4.99 (s, 1.8H, rotamer major), 4.68-4.64 (m, 0.72 H, major rotamer); 4.14-4.07 (bm, 1H), 3.72 (s, 7H), 3.38-3.36 (m, 0.6H, rotamer minor); 3.26-3.21 (m, 1.4H, rotamer major); 3.08-3.07 (m, 0.3H, rotamer, minor); 2.99-2.89 (m, 2.7H, rotamer, major); 2.22-2.12 (m, 2H), 2.04-1.78 (m, 2H); 1.48-1.23 (m, 6H), 0.84, 0.82 (s, 9H, rotamers major and minor); 0.05 (d, $J(\text{H,H}) = 1.5 \text{ Hz}$, 4.3H, rotamer major); 0.03-0.02 (d, $J(\text{H,H}) = 5.5 \text{ Hz}$, 1.7H).

Step 5.2: Compound 113: Compound **112** (9.4 g, 14.54 mmol) was suspended in 15 mL of β -caprolactone and 10 mL of TEA was added into the suspension. The reaction mixture was stirred under argon at 55 °C bath temperature for 24 h. Completion of the reaction was monitored by TLC analysis. TEA was removed from the reaction mixture *in vacuo* and 150 mL of dichloromethane-hexane (2:1 mixture) was added into the residue. The homogeneous solution thus obtained was directly loaded on a column of silica gel and eluted with dichloromethane-hexane (2:1) followed by neat dichloromethane. Elution of the silica column with 4 % methanol in dichloromethane afforded the desired compound **113** as a white solid (8.73 g, 78.9 %). ¹H NMR (400 MHz, [D₆]DMSO, 25 °C) δ 7.72-7.68 (bm, 1H, exchangeable with D₂O); 7.33-7.16 (m, 9H); 6.88-6.84 (m, 4H); 4.68-4.62 (m, 0.8H); 4.57-4.52 (m, 0.2H); 4.34-4.31 (t, $J(\text{H,H}) = 5.18 \text{ Hz}$, 1H, exchangeable with D₂O); 4.14-4.08 (bm, 1H); 3.74-3.67 (m, 7H); 3.39-3.32 (m, 3.3H); 3.25-3.21 (m, 1.7H); 3.09-2.88 (m, 4H)

6. Analysis of Duplex activity

Duplexes were tested for activity in the HeLa cell assay described above. Table 14 and Figure 38 provides data and a graph of the activities in HeLa cells for each of the modifications described above.

Example 12 Conjugation of Polyethylene glycol to siRNA (Table 12)**Amino linker oligonucleotides for PEG Conjugation**

General. Ion exchange preparative chromatography was performed on TSKgel-SuperQ-5PW (Tosoh). Ion exchange analytical chromatography was performed on a DNAPac Pa100 (Dionex). Electron spray ionization mass spectra were recorded with an Agilent 1100 MSD-SL.

HPLC Techniques. The RNA was analyzed by ion-exchange chromatography (column, DNAPac Pa100, 4x250mm, analytical), heated to 30°C, flow rate 1.5 mL min⁻¹, buffer A = 0.020M Na₂HPO₄ in 10% CH₃CN, pH 11; buffer B = buffer A + 1 M NaBr in 10% CH₃CN, pH 11, linear gradient from 0 to 75% in 53 min. The LC/ESI-MS conditions were as follows: column XTerra C8 (2.1x30 mm, 2.5µm), linear gradient from 5 to 35% in 2 min and from 35 to 70% in 30.5 min, flow rate 0.200 mL min⁻¹, buffer A = 400mM HFIP/16.3mM TEA in H₂O, buffer B = 100% methanol. The RNA was purified by ion-exchange chromatography (5cm in-house packed column, TSKgel-SuperQ-5PW, 20µm), heated to 75°C, flow rate 50 mL min⁻¹, buffer A = 0.020M Na₂HPO₄ in 10% CH₃CN, pH 8.5; buffer B = buffer A + 1 M NaBr in 10% CH₃CN, pH 8.5, linear gradient from 20 to 55% in 120 min.

RNA synthesis. The protected RNA was assembled on an AKTA Oligo Pilot 100 on a 100-150 µmol scale using custom in-house support and phosphoramidite chemistry. Phosphoramidites were used as 0.2 mol L⁻¹ solutions in dry CH₃CN, with a 900s coupling time and the manufacturer's recommended synthesis protocols were used. After synthesis, the support-bound RNA was treated with aqueous CH₃NH₂ (40%) for 90 minutes at 45°C, cooled, filtered and washed with DMSO (3x40mL). The filtrate was then treated with TEA.3HF (60mL) for 60 minutes at 40°C, and quenched with aq. NaOAc (0.05M, pH 5.5, 200mL). The synthesis was followed by analytical ion-exchange HPLC, preparative HPLC, then desalting on Sephadex G-25.

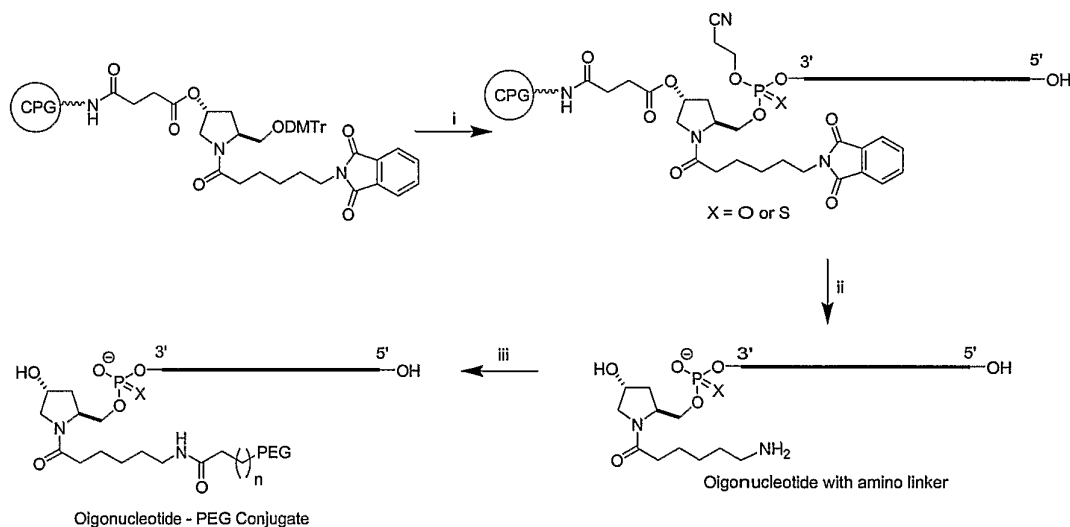
Step 1. Oligonucleotide Synthesis:

A general conjugation approach is shown in the Scheme E.

All oligonucleotides were synthesized on an AKTA oligopilot synthesizer. Commercially available controlled pore glass solid support (dT-CPG, 500Å) or the phthalimido-hydroxy-

prolinol solid support 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 were used for the oligonucleotide synthesis. All phosphoramidites were used at a concentration of 0.2M in acetonitrile (CH₃CN) except for guanosine which was used at 0.2M concentration in 10% THF/acetonitrile (v/v). Coupling/recycling time of 16 minutes was used. The activator was 5-ethyl thiotetrazole (0.75M), for the PO-oxidation Iodine/Water/Pyridine was used and for the PS-oxidation PADS (2 %) in 2,6-lutidine/ACN (1:1 v/v) was used. The amino-linker phosphoramidite was synthesized and used at a concentration of 0.2M in acetonitrile. Coupling/recycling time for the amino-linker phosphoramidite was 16 minutes.

Scheme E^a: Pegylation of RNA Oligonucleotides



^a (i) Solid phase Oligonucleotide synthesis; (ii) Deprotection and purification; (iii) PEG-NHS ester, NaHCO₃, pH 8.1, 1 h.

Step 2. Deprotection- I (Nucleobase Deprotection)

After completion of synthesis, the support was transferred to a 100 ml glass bottle. The oligonucleotide was cleaved from the support with simultaneous deprotection of base and phosphate groups with 40 mL of a 40% aq. methyl amine 90 mins at 45°C. The bottle was cooled briefly on ice and then the methylamine was filtered into a new 500 ml bottle. The CPG was washed with 3 x 40 mL portions of DMSO. The mixture was then cooled on dry ice.

Step 3. Deprotection-II (Removal of 2' TBDMS group)

To the above mixture was added 60 ml triethylamine trihydrofluoride (TREAT-HF) and heated at 40°C for 60 minutes to remove the *tert*-butyldimethylsilyl (TBDMS) groups at the 2' position. The reaction was then quenched with 220 ml of 50mM sodium acetate (pH 5.5) and stored in freezer until purification.

Step 4. Quantitation of Crude Oligomer or Raw Analysis

For all samples, a 10µl aliquote was diluted with 990 µl of deionised nuclease free water (1.0 mL) and absorbance reading obtained at 260 nm.

Step 5. Purification of Oligomers

(a) HPLC Purification

The crude oligomers were first analyzed by HPLC (Dionex PA 100). The buffer system was: A = 20 mM phosphate pH 11, B = 20 mM phosphate, 1.8 M NaBr, pH 11, flow rate 1.0 mL/min, and wavelength 260-280 nm. Injections of 5-15 µl were done for each sample. The samples were purified by HPLC on an TSK-Gel SuperQ-5PW (20) column (17.3 x 5 cm). The buffer system was: A = 20 mM phosphate in 10% ACN, pH 8.5 and B = 20 mM phosphate, 1.0 M NaBr in 10% ACN, pH 8.5, with a flow rate of 50.0 mL/min, and wavelength 260 and 294. The fractions containing the fulllength oligonucleotides were then pooled together, evaporated and reconstituted to ~100 ml with deionised water.

Step 6. Desalting of Purified Oligomer

The purified oligonucleotides were desalted on an AKTA Explorer (Amersham Biosciences) using Sephadex G-25 column. First column was washed with water at a flow rate of 25 ml/min for 20-30 min. The sample was then applied in 25 ml fractions. The eluted salt-free fractions were combined together, dried down and reconstituted in 50 ml of RNase free water.

Step 7. Capillary Gel Electrophoresis (CGE) and Electrospray LC/MS

Approximately 0.15 OD of desalted oligonucleotides were diluted in water to 150 μ l and then pipetted in special vials for CGE and LC/MS analysis.

Step 8. PEG conjugation.

A) Initial reaction conditions. The purified and desalted RNA was lyophilized. RNA (1mg) was dissolved in aq. NaHCO₃ (0.1M, 200 μ L, pH 8.1) and DMF (200 μ L each). 5 K (13 equivalents, 10mg) or 20KPEG (3.4 equivalents, 10mg) was added directly to reaction vial and vortexed thoroughly. The reaction continued overnight at 4°C, and was followed by analytical ion-exchange HPLC. When the reaction reached >85% completion, it was quenched with aq. NaOAc (0.05M, pH 5.5) until the pH was ~7.

B) Borate buffer conjugation. The purified and desalted RNA was lyophilized. A sample of RNA (1mg) was dissolved in sodium borate buffer (200 μ L, 0.05M, pH 10). 5KPEG (3mg, 4.5 equivalents Sunbright ME-50HS, NOF Corp.) was dissolved in CH₃CN (200 μ L). The RNA solution was added to the PEG solution and vortexed thoroughly. The reaction continued for one hour at room temperature, and was followed by analytical ion-exchange HPLC. When reaction reached >85% completion, it was quenched with aq. NaOAc (0.05M, pH 5.5) until the pH was ~7.

C) PEG linker (AS and HS) comparison. A sample of RNA (1mg) was dissolved in aq. NaHCO₃ (0.1M, 200 μ L, pH 8.1) and DMF (200 μ L). 5KPEG (13.5 eq, 10mg, Sunbright ME-50HS or Sunbright ME-50AS, NOF Corp.) was added directly to the reaction vial and vortexed thoroughly. The reaction continued overnight at 4°C, and was followed by analytical ion-

exchange HPLC. When the reaction reached >85% completion, it was quenched with aq. NaOAc (0.05M, pH 5.5) until the pH was ~7.

D) Final optimized PEG conjugation. The purified and desalted RNA was lyophilized. A sample of RNA (50mg) was dissolved in aq. NaHCO₃ (0.1M, 2mL pH 8.1) and DMF (1mL).
 5 20KPEG (approximately 2.7 eq, 400-520mg Sunbright ME-200HS, different amounts for different sequences within this range) was dissolved in CH₃CN (2mL). The RNA solution was added to the PEG solution and vortexed thoroughly. H₂O (250mL) was added to the reaction to decrease turbidity. The reaction continued for one hour at room temperature, and was followed by analytical ion-exchange HPLC. When the reaction reached >85% completion, it was
 10 quenched with aq. NaOAc (0.05M, pH 5.5) until the pH was ~7.

Step 9. Analysis of Duplex activity

Duplexes were tested for activity in the HeLa cell assay described above. Table 12 and Figure 45 provide data and graphs of the activities in HeLa cells for each of the modifications described above.

Example 13 Synthesis of oligonucleotides containing the ribo-difluorotoluy (DFT) nucleoside (Table 13)

The RNA molecules were synthesized on a 394 ABI machine using the standard cycle written by the manufacturer with modifications to a few wait steps. The solid support was 500 Å dT CPG (2 umole). The monomers were either RNA phosphoramidites or the ribo-difluorotoluy
 20 amidite. All had standard protecting groups and were used at concentrations of 0.15 M in acetonitrile (CH₃CN) unless otherwise stated. Specifically the phosphoramidites were 5'-O-Dimethoxytrityl-N⁶-benzoyl-2'-O-*t*-butyldimethylsilyl-adenosine-3'-O-(β-cyanoethyl-N,N'-diisopropyl) phosphoramidite, 5'-O-Dimethoxytrityl-N²-isobutyryl-2'-O-*t*-butyldimethylsilyl-
 25 guanosine-3'-O-(β-cyanoethyl-N,N'-diisopropyl)phosphoramidite, 5'-O-Dimethoxytrityl-N⁴-acetyl-2'-O-*t*-butyldimethylsilyl-cytidine-3'-O-(β-cyanoethyl-N,N'-diisopropyl)phosphoramidite, and 5'-O-Dimethoxytrityl-2'-O-*t*-butyldimethylsilyl-uridine-3'-O-(β-cyanoethyl-N,N'-
 diisopropyl)phosphoramidite, 5'-O-Dimethoxytrityl-difluorotoluy O-*t*-butyldimethylsilyl-3'-O-(β-cyanoethyl-N,N'-diisopropyl)phosphoramidite (0.12 M). The coupling times were 7 min for
 30 all RNA monomers and 10 min for the DFT monomer. Details of the other reagents are as

follows: Activator: 5-ethylthio-1H-tetrazole (0.25M), Cap A: 5% acetic anhydride/THF/pyridine, Cap B: 10% N-methylimidazole/THF; phosphate oxidation involved 0.02M I₂/THF/H₂O.

Detritylation was achieved with 3% TCA/dichloromethane. The DMT protecting group was removed after the last step of the cycle.

5 After completion of synthesis the CPG was transferred to a screw cap, sterile microfuge tube. The oligonucleotide was cleaved and the base and phosphate groups were simultaneously deprotected with 1.0 mL of a mixture of ethanolic ammonia (1:3) for 16 hours at 55°C. The tube was cooled briefly on ice and then the solution was transferred to a 5 mL centrifuge tube; this was followed by washing three times with 0.25 mL of 50% acetonitrile. The tubes were cooled
10 at -80°C for 15 min, before drying in a lyophilizer.

 The white residue obtained was resuspended in 200 uL of triethylamine trihydrofluoride and heated at 65°C for 1.5 h to remove the TBDMS groups at the 2'-position. The oligonucleotides were then precipitated in dry methanol (400 uL). The liquid was removed carefully to yield a pellet at the bottom of the tube. Residual methanol was removed in the speed
15 vacuum to give a white fluffy material. Samples were dissolved in 1 mL RNase free water and quantitated by measuring the absorbance at 260 nm. This crude material was stored at -20°C.

 The crude oligonucleotides were analyzed and purified by 20% polyacrylamide denaturing gels. The purified dry oligonucleotides were then desalted using Sephadex G25M.

 Duplexes were tested for activity in the HeLa cell assay described above. Table 13 and
20 Figure 46 provide data and graphs of the activities in HeLa cells for each of the modifications described above.

Example 14 Synthesis of RNA modified with 2'-ara-fluoro-2'-deoxy-nucleosides (Table 14)

 The chimeric RNA molecules were synthesized on a 394 ABI machine using the standard
25 cycle written by the manufacturer with modifications to a few wait steps. The solid support was 500 Å dT CPG (2 μmole). The monomers were either RNA phosphoramidites, or 2'-ara-fluoro-2'-deoxy (2' ara F) phosphoramidites. All monomers had standard protecting groups and were used at concentrations of 0.15 M in acetonitrile (CH₃CN) unless otherwise stated. Specifically the RNA phosphoramidites were 5'-O-Dimethoxytrityl-N⁶-benzoyl-2'-O-*t*-butyldimethylsilyl-
30 adenosine-3'-O-(α-cyanoethyl-N,N'-diisopropyl) phosphoramidite, 5'-O-Dimethoxytrityl-N²-

isobutyryl-2'-O-*t*-butyldimethylsilyl-guanosine-3'-O-(β -cyanoethyl-N,N'-diisopropyl)phosphoramidite, 5'-O-Dimethoxytrityl-N⁴-acetyl-2'-O-*t*-butyldimethylsilyl-cytidine-3'-O-(β -cyanoethyl-N,N'-diisopropyl)phosphoramidite, and 5'-O-Dimethoxytrityl-2'-O-*t*-butyldimethylsilyl-uridine-3'-O-(β -cyanoethyl-N,N'-diisopropyl)phosphoramidite; the 2'ara F phosphoramidites were 5'-O-Dimethoxytrityl-N⁴-benzoyl-2'-*ara*fluoro-2'-deoxy-cytidine-3'-O-(β -cyanoethyl-N,N'-diisopropyl)phosphoramidite, and 5'-O-Dimethoxytrityl-2'-*ara*fluoro-2'-deoxy-uridine-3'-O-(β -cyanoethyl-N,N'-diisopropyl)phosphoramidite, and 5'-O-Dimethoxytrityl-2'-*ara*fluoro-thymidine-3'-O-(β -cyanoethyl-N,N'-diisopropyl)phosphoramidite.

The coupling times were 10 min for all monomers. Details of the other reagents are as follows:

Activator: 5-ethylthio-1H-tetrazole (0.25M), Cap A: 5% acetic anhydride/THF/pyridine, Cap B: 10% N-methylimidazole/THF; phosphate oxidation involved 0.02 M I₂/THF/H₂O. Detritylation was achieved with 3% TCA/dichloromethane. The final DMT protecting group was removed after the last cycle.

After completion of synthesis the CPG was transferred to a screw cap, sterile microfuge tube. The oligonucleotide was cleaved and the base and phosphate groups were simultaneously deprotected with 1.0 mL of a mixture of ethanolic ammonia conc (1:3) for 5 hours at 55°C. The tube was cooled briefly on ice and then the solution was transferred to a 5 mL centrifuge tube; this was followed by washing three times with 0.25 mL of 50% acetonitrile. The tubes were cooled at -80°C for 15 min, before drying in a lyophilizer.

The white residue obtained was resuspended in 200 μ L of triethylamine trihydrofluoride and heated at 65°C for 1.5h to remove the TBDMS groups at the 2'-OH position. The oligonucleotides were then precipitated in dry methanol (400 μ L). The liquid was removed carefully to yield a pellet at the bottom of the tube. Residual methanol was removed in the speed vacuum to give a white fluffy material. Samples were dissolved in 1 mL RNase free water and quantitated by measuring the absorbance at 260 nm. This crude material was stored at -20°C.

The crude oligonucleotides were analyzed and purified by 20% polyacrylamide denaturing gels. The purified dry oligonucleotides were then desalted using Sephadex G25M (Amersham Biosciences).

Duplexes were tested for activity in the HeLa cell assay described above. Table 14 and Figure 47 provide data and graphs of the activities in HeLa cells for each of the modifications described above.

5 **Example 15 Deprotection of Methylphosphonate Modified siRNAs (Table 15)**

Deprotection step 1:

After completion of the synthesis, the controlled pore glass (CPG) was transferred to a screw cap vial. A solution (0.5 ml) consisting of Acetonitrile/Ethanol/NH₄OH (45:45:10) was added to the support. The vial was sealed and left at room temperature for 30 min.

10 Ethylenediamine (0.5 mL) was added to the vial and left at room temperature for an additional 6 hours. The supernatant was decanted and the support was washed twice with 1:1 acetonitrile/water (0.5 mL). The combined supernatant was diluted with water (15 mL). The pH was adjusted to 7.0 with 6 M HCl in AcCN/H₂O (1:9). The sample was desalted using a Sep-pak C₁₈ cartridge and then dried in a speed vac.

15

Deprotection step 2 (Removal of 2'-O- TBDMS group)

The white residue obtained was resuspended in a mixture of triethylamine, triethylamine trihydrofluoride (TEA.3HF ca, 24% HF) and 1-Methyl-2-Pyrrolidinone (NMP) (4:3:7) (400 ul) and heated at 65°C for 90 min to remove the tert-butyldimethylsilyl (TBDMS) groups at the 2'-
20 position. The reaction was then quenched with isopropoxytrimethylsilane (iPrOMe₃Si, 400 ul) and further incubated on the heating block leaving the caps open for 10min; (This causes the volatile isopropoxytrimethylsilyl fluoride adduct to vaporize). The residual quenching reagent was removed by drying in a speed vac. 3% Triethylamine in diethyl ether (1.5 ml) was added and the mixture was subjected to centrifugation to afford a pellet of RNA. The supernatant was pipetted
25 out without disturbing the pellet. The pellet was dried in a speed vac. The crude RNA was obtained as a white fluffy material in the microfuge tube.

Purification:

All methylphosphonate modified sequences were purified by PAGE

30

Analysis of Duplex activity

Duplexes were tested for activity in the HeLa cell assay described above. Table 15 and Figure 48 provide data and graphs of the activities in HeLa cells for each of the modifications described above.

TABLE 1. Target sequences in VEGF 121

SEQ ID NO:	ORF	TARGET SEQUENCE IN VEGF121 mRNA 5' to 3'
2	1	AUGAACUUUCUGCUGUCUUGGGU
3	2	UGAACUUUCUGCUGUCUUGGGUG
4	3	GAACUUUCUGCUGUCUUGGGUGC
5	4	AACUUUCUGCUGUCUUGGGUGCA
6	5	ACUUUCUGCUGUCUUGGGUGCAU
7	6	CUUUCUGCUGUCUUGGGUGCAUU
8	7	UUUCUGCUGUCUUGGGUGCAUUG
9	8	UUCUGCUGUCUUGGGUGCAUUGG
10	9	UCUGCUGUCUUGGGUGCAUUGGA
11	10	CUGCUGUCUUGGGUGCAUUGGAG
12	11	UGCUGUCUUGGGUGCAUUGGAGC
13	12	GCUGUCUUGGGUGCAUUGGAGCC
14	13	CUGUCUUGGGUGCAUUGGAGCCU
15	14	UGUCUUGGGUGCAUUGGAGCCUU
16	15	GUCUUGGGUGCAUUGGAGCCUUG
17	16	UCUUGGGUGCAUUGGAGCCUUGC
18	17	CUUGGGUGCAUUGGAGCCUUGCC
19	18	UUGGGUGCAUUGGAGCCUUGCCU
20	19	UGGGUGCAUUGGAGCCUUGCCUU
21	20	GGGUGCAUUGGAGCCUUGCCUUG
22	21	GGUGCAUUGGAGCCUUGCCUUGC
23	22	GUGCAUUGGAGCCUUGCCUUGC U
24	23	UGCAUUGGAGCCUUGCCUUGCUG
25	24	GCAUUGGAGCCUUGCCUUGCUGC
26	25	CAUUGGAGCCUUGCCUUGCUGCU
27	26	AUUGGAGCCUUGCCUUGCUGCUC
28	27	UUGGAGCCUUGCCUUGCUGCUCU
29	28	UGGAGCCUUGCCUUGCUGCUCUA
30	29	GGAGCCUUGCCUUGCUGCUCUAC
31	30	GAGCCUUGCCUUGCUGCUCUACC
32	31	AGCCUUGCCUUGCUGCUCUACCU
33	32	GCCUUGCCUUGCUGCUCUACCUC
34	33	CCUUGCCUUGCUGCUCUACCUC C
35	34	CUUGCCUUGCUGCUCUACCUC C A
36	35	UUGCCUUGCUGCUCUACCUC C A C
37	36	UGCCUUGCUGCUCUACCUC C A C C
38	37	GCCUUGCUGCUCUACCUC C A C C A
39	38	CCUUGCUGCUCUACCUC C A C C A U
40	39	CUUGCUGCUCUACCUC C A C C A U G

41	40	UUGCUGCUCUACCUCACCAUGC
42	41	UGCUGCUCUACCUCACCAUGCC
43	42	GCUGCUCUACCUCACCAUGCCA
44	43	CUGCUCUACCUCACCAUGCCAA
45	44	UGCUCUACCUCACCAUGCCAAG
46	45	GCUCUACCUCACCAUGCCAAGU
47	46	CUCUACCUCACCAUGCCAAGUG
48	47	UCUACCUCACCAUGCCAAGUGG
49	48	CUACCUCACCAUGCCAAGUGGU
50	49	UACCUCACCAUGCCAAGUGGUC
51	50	ACCUCACCAUGCCAAGUGGUCC
52	51	CCUCCACCAUGCCAAGUGGUCCC
53	52	CUCCACCAUGCCAAGUGGUCCCA
54	53	UCCACCAUGCCAAGUGGUCCCAG
55	54	CCACCAUGCCAAGUGGUCCCAGG
56	55	CACCAUGCCAAGUGGUCCCAGGC
57	56	ACCAUGCCAAGUGGUCCCAGGCU
58	57	CCAUGCCAAGUGGUCCCAGGCUG
59	58	CAUGCCAAGUGGUCCCAGGCUGC
60	59	AUGCCAAGUGGUCCCAGGCUGCA
61	60	UGCCAAGUGGUCCCAGGCUGCAC
62	61	GCCAAGUGGUCCCAGGCUGCACC
63	62	CCAAGUGGUCCCAGGCUGCACCC
64	63	CAAGUGGUCCCAGGCUGCACCCA
65	64	AAGUGGUCCCAGGCUGCACCCAU
66	65	AGUGGUCCCAGGCUGCACCCAUG
67	66	GUGGUCCCAGGCUGCACCCAUGG
68	67	UGGUCCCAGGCUGCACCCAUGGC
69	68	GGUCCCAGGCUGCACCCAUGGCA
70	69	GUCCCAGGCUGCACCCAUGGCAG
71	70	UCCCAGGCUGCACCCAUGGCAGA
72	71	CCCAGGCUGCACCCAUGGCAGAA
73	72	CCAGGCUGCACCCAUGGCAGAAG
74	73	CAGGCUGCACCCAUGGCAGAAGG
75	74	AGGCUGCACCCAUGGCAGAAGGA
76	75	GGCUGCACCCAUGGCAGAAGGAG
77	76	GCUGCACCCAUGGCAGAAGGAGG
78	77	CUGCACCCAUGGCAGAAGGAGGA
79	78	UGCACCCAUGGCAGAAGGAGGAG
80	79	GCACCCAUGGCAGAAGGAGGAGG
81	80	CACCCAUGGCAGAAGGAGGAGGG
82	81	ACCCAUGGCAGAAGGAGGAGGGC
83	82	CCCAUGGCAGAAGGAGGAGGGCA
84	83	CCAUGGCAGAAGGAGGAGGGCAG

85	84	CAUGGCAGAAGGAGGAGGGCAGA
86	85	AUGGCAGAAGGAGGAGGGCAGAA
87	86	UGGCAGAAGGAGGAGGGCAGAAU
88	87	GGCAGAAGGAGGAGGGCAGAAUC
89	88	GCAGAAGGAGGAGGGCAGAAUCA
90	89	CAGAAGGAGGAGGGCAGAAUCAU
91	90	AGAAGGAGGAGGGCAGAAUCAUC
92	91	GAAGGAGGAGGGCAGAAUCAUCA
93	92	AAGGAGGAGGGCAGAAUCAUCAC
94	93	AGGAGGAGGGCAGAAUCAUCACG
95	94	GGAGGAGGGCAGAAUCAUCACGA
96	95	GAGGAGGGCAGAAUCAUCACGAA
97	96	AGGAGGGCAGAAUCAUCACGAAG
98	97	GGAGGGCAGAAUCAUCACGAAGU
99	98	GAGGGCAGAAUCAUCACGAAGUG
100	99	AGGGCAGAAUCAUCACGAAGUGG
101	100	GGGCAGAAUCAUCACGAAGUGGU
102	101	GGCAGAAUCAUCACGAAGUGGUG
103	102	GCAGAAUCAUCACGAAGUGGUGA
104	103	CAGAAUCAUCACGAAGUGGUGAA
105	104	AGAAUCAUCACGAAGUGGUGAAG
106	105	GAAUCAUCACGAAGUGGUGAAGU
107	106	AAUCAUCACGAAGUGGUGAAGUU
108	107	AUCAUCACGAAGUGGUGAAGUUC
109	108	UCAUCACGAAGUGGUGAAGUUCA
110	109	CAUCACGAAGUGGUGAAGUUCAU
111	110	AUCACGAAGUGGUGAAGUUCAUG
112	111	UCACGAAGUGGUGAAGUUCAUGG
113	112	CACGAAGUGGUGAAGUUCAUGGA
114	113	ACGAAGUGGUGAAGUUCAUGGAU
115	114	CGAAGUGGUGAAGUUCAUGGAUG
116	115	GAAGUGGUGAAGUUCAUGGAUGU
117	116	AAGUGGUGAAGUUCAUGGAUGUC
118	117	AGUGGUGAAGUUCAUGGAUGUCU
119	118	GUGGUGAAGUUCAUGGAUGUCUA
120	119	UGGUGAAGUUCAUGGAUGUCUAU
121	120	GGUGAAGUUCAUGGAUGUCUAUC
122	121	GUGAAGUUCAUGGAUGUCUAUCA
123	122	UGAAGUUCAUGGAUGUCUAUCAG
124	123	GAAGUUCAUGGAUGUCUAUCAGC
125	124	AAGUUCAUGGAUGUCUAUCAGCG
126	125	AGUUCAUGGAUGUCUAUCAGCGC
127	126	GUUCAUGGAUGUCUAUCAGCGCA
128	127	UUCAUGGAUGUCUAUCAGCGCAG

129	128	UCAUGGAUGUCUAUCAGCGCAGC
130	129	CAUGGAUGUCUAUCAGCGCAGCU
131	130	AUGGAUGUCUAUCAGCGCAGCUA
132	131	UGGAUGUCUAUCAGCGCAGCUAC
133	132	GGAUGUCUAUCAGCGCAGCUACU
134	133	GAUGUCUAUCAGCGCAGCUACUG
135	134	AUGUCUAUCAGCGCAGCUACUGC
136	135	UGUCUAUCAGCGCAGCUACUGCC
137	136	GUCUAUCAGCGCAGCUACUGCCA
138	137	UCUAUCAGCGCAGCUACUGCCAU
139	138	CUAUCAGCGCAGCUACUGCCAUC
140	139	UAUCAGCGCAGCUACUGCCAUCC
141	140	AUCAGCGCAGCUACUGCCAUCCA
142	141	UCAGCGCAGCUACUGCCAUCCAA
143	142	CAGCGCAGCUACUGCCAUCCAAU
144	143	AGCGCAGCUACUGCCAUCCAAUC
145	144	GCGCAGCUACUGCCAUCCAAUCG
146	145	CGCAGCUACUGCCAUCCAAUCGA
147	146	GCAGCUACUGCCAUCCAAUCGAG
148	147	CAGCUACUGCCAUCCAAUCGAGA
149	148	AGCUACUGCCAUCCAAUCGAGAC
150	149	GCUACUGCCAUCCAAUCGAGACC
151	150	CUACUGCCAUCCAAUCGAGACCC
152	151	UACUGCCAUCCAAUCGAGACCCU
153	152	ACUGCCAUCCAAUCGAGACCCUG
154	153	CUGCCAUCCAAUCGAGACCCUGG
155	154	UGCCAUCCAAUCGAGACCCUGGU
156	155	GCCAUCCAAUCGAGACCCUGGUG
157	156	CCAUCCAAUCGAGACCCUGGUGG
158	157	CAUCCAAUCGAGACCCUGGUGGA
159	158	AUCCAAUCGAGACCCUGGUGGAC
160	159	UCCAAUCGAGACCCUGGUGGACA
161	160	CCAAUCGAGACCCUGGUGGACAU
162	161	CAAUCGAGACCCUGGUGGACAUC
163	162	AAUCGAGACCCUGGUGGACAUCU
164	163	AUCGAGACCCUGGUGGACAUCUU
165	164	UCGAGACCCUGGUGGACAUCUUC
166	165	CGAGACCCUGGUGGACAUCUUCC
167	166	GAGACCCUGGUGGACAUCUUCCA
168	167	AGACCCUGGUGGACAUCUUCCAG
169	168	GACCCUGGUGGACAUCUUCCAGG
170	169	ACCCUGGUGGACAUCUUCCAGGA
171	170	CCCUGGUGGACAUCUUCCAGGAG
172	171	CCUGGUGGACAUCUUCCAGGAGU

173	172	CUGGUGGACAUCUCCAGGAGUA
174	173	UGGUGGACAUCUCCAGGAGUAC
175	174	GGUGGACAUCUCCAGGAGUACC
176	175	GUGGACAUCUCCAGGAGUACCC
177	176	UGGACAUCUCCAGGAGUACCCU
178	177	GGACAUCUCCAGGAGUACCCUG
179	178	GACAUCUCCAGGAGUACCCUGA
180	179	ACAUCUCCAGGAGUACCCUGAU
181	180	CAUCUCCAGGAGUACCCUGAUG
182	181	AUCUCCAGGAGUACCCUGAUGA
183	182	UCUCCAGGAGUACCCUGAUGAG
184	183	CUCCAGGAGUACCCUGAUGAGA
185	184	UCCAGGAGUACCCUGAUGAGAU
186	185	UCCAGGAGUACCCUGAUGAGAU
187	186	CCAGGAGUACCCUGAUGAGAU
188	187	CAGGAGUACCCUGAUGAGAU
189	188	AGGAGUACCCUGAUGAGAU
190	189	GGAGUACCCUGAUGAGAU
191	190	GAGUACCCUGAUGAGAU
192	191	AGUACCCUGAUGAGAU
193	192	GUACCCUGAUGAGAU
194	193	UACCCUGAUGAGAU
195	194	ACCCUGAUGAGAU
196	195	CCCUGAUGAGAU
197	196	CCUGAUGAGAU
198	197	CUGAUGAGAU
199	198	UGAUGAGAU
200	199	GAUGAGAU
201	200	AUGAGAU
202	201	UGAGAU
203	202	GAGAU
204	203	AGAUGAU
205	204	GAUCGAGUA
206	205	AUCGAGUA
207	206	UCGAGUA
208	207	CGAGUA
209	208	GAGUA
210	209	AGUA
211	210	GUACAUCU
212	211	UACAUCU
213	212	ACAUCU
214	213	CAUCU
215	214	AUCU
216	215	UCU

217	216	CUUCAAGCCAUCCUGUGUGCCCC
218	217	UUCAAGCCAUCCUGUGUGCCCCU
219	218	UCAAGCCAUCCUGUGUGCCCCUG
220	219	CAAGCCAUCCUGUGUGCCCCUGA
221	220	AAGCCAUCCUGUGUGCCCCUGAU
222	221	AGCCAUCCUGUGUGCCCCUGAUG
223	222	GCCAUCCUGUGUGCCCCUGAUGC
224	223	CCAUCCUGUGUGCCCCUGAUGCG
225	224	CAUCCUGUGUGCCCCUGAUGCGA
226	225	AUCCUGUGUGCCCCUGAUGCGAU
227	226	UCCUGUGUGCCCCUGAUGCGAUG
228	227	CCUGUGUGCCCCUGAUGCGAUGC
229	228	CUGUGUGCCCCUGAUGCGAUGCG
230	229	UGUGUGCCCCUGAUGCGAUGCGG
231	230	GUGUGCCCCUGAUGCGAUGCGGG
232	231	UGUGCCCCUGAUGCGAUGCGGGG
233	232	GUGCCCCUGAUGCGAUGCGGGGG
234	233	UGCCCCUGAUGCGAUGCGGGGGC
235	234	GCCCCUGAUGCGAUGCGGGGGCU
236	235	CCCCUGAUGCGAUGCGGGGGCUG
237	236	CCUGAUGCGAUGCGGGGGCUGC
238	237	CCUGAUGCGAUGCGGGGGCUGCU
239	238	CUGAUGCGAUGCGGGGGCUGCUG
240	239	UGAUGCGAUGCGGGGGCUGCUGC
241	240	GAUGCGAUGCGGGGGCUGCUGCA
242	241	AUGCGAUGCGGGGGCUGCUGCAA
243	242	UGCAGUGCGGGGGCUGCUGCAAU
244	243	GCGAUGCGGGGGCUGCUGCAAUG
245	244	CGAUGCGGGGGCUGCUGCAAUGA
246	245	GAUGCGGGGGCUGCUGCAAUGAC
247	246	AUGCGGGGGCUGCUGCAAUGACG
248	247	UGCAGGGGGCUGCUGCAAUGACGA
249	248	GCGGGGGCUGCUGCAAUGACGAG
250	249	CGGGGGCUGCUGCAAUGACGAGG
251	250	GGGGGCUGCUGCAAUGACGAGGG
252	251	GGGGCUGCUGCAAUGACGAGGGC
253	252	GGGCUGCUGCAAUGACGAGGGCC
254	253	GGCUGCUGCAAUGACGAGGGCCU
255	254	GCUGCUGCAAUGACGAGGGCCUG
256	255	CUGCUGCAAUGACGAGGGCCUGG
257	256	UGCUGCAAUGACGAGGGCCUGGA
258	257	GCUGCAAUGACGAGGGCCUGGAG
259	258	CUGCAAUGACGAGGGCCUGGAGU
260	259	UGCAAUGACGAGGGCCUGGAGUG

261	260	GCAAUGACGAGGGCCUGGAGUGU
262	261	CAAUGACGAGGGCCUGGAGUGUG
263	262	AAUGACGAGGGCCUGGAGUGUGU
264	263	AUGACGAGGGCCUGGAGUGUGUG
265	264	UGACGAGGGCCUGGAGUGUGUGC
266	265	GACGAGGGCCUGGAGUGUGUGCC
267	266	ACGAGGGCCUGGAGUGUGUGCCC
268	267	CGAGGGCCUGGAGUGUGUGCCCA
269	268	GAGGGCCUGGAGUGUGUGCCCAC
270	269	AGGGCCUGGAGUGUGUGCCCACU
271	270	GGGCCUGGAGUGUGUGCCCACUG
272	271	GGCCUGGAGUGUGUGCCCACUGA
273	272	GCCUGGAGUGUGUGCCCACUGAG
274	273	CCUGGAGUGUGUGCCCACUGAGG
275	274	CUGGAGUGUGUGCCCACUGAGGA
276	275	UGGAGUGUGUGCCCACUGAGGAG
277	276	GGAGUGUGUGCCCACUGAGGAGU
278	277	GAGUGUGUGCCCACUGAGGAGUC
279	278	AGUGUGUGCCCACUGAGGAGUCC
280	279	GUGUGUGCCCACUGAGGAGUCCA
281	280	UGUGUGCCCACUGAGGAGUCCAA
282	281	GUGUGCCCACUGAGGAGUCCAAC
283	282	UGUGCCCACUGAGGAGUCCAACA
284	283	GUGCCCACUGAGGAGUCCAACAU
285	284	UGCCCACUGAGGAGUCCAACAUC
286	285	GCCCACUGAGGAGUCCAACAUCA
287	286	CCCACUGAGGAGUCCAACAUCAC
288	287	CCACUGAGGAGUCCAACAUCACC
289	288	CACUGAGGAGUCCAACAUCACCA
290	289	ACUGAGGAGUCCAACAUCACCAU
291	290	CUGAGGAGUCCAACAUCACCAUG
292	291	UGAGGAGUCCAACAUCACCAUGC
293	292	GAGGAGUCCAACAUCACCAUGCA
294	293	AGGAGUCCAACAUCACCAUGCAG
295	294	GGAGUCCAACAUCACCAUGCAGA
296	295	GAGUCCAACAUCACCAUGCAGAU
297	296	AGUCCAACAUCACCAUGCAGAUU
298	297	GUCCAACAUCACCAUGCAGAUUA
299	298	UCCAACAUCACCAUGCAGAUUAU
300	299	CCAACAUCACCAUGCAGAUUAUG
301	300	CAACAUCACCAUGCAGAUUAUGC
302	301	AACAUCACCAUGCAGAUUAUGCG
303	302	ACAUCACCAUGCAGAUUAUGCGG
304	303	CAUCACCAUGCAGAUUAUGCGGA

305	304	AUCACCAUGCAGAUUAUGCGGAU
306	305	UCACCAUGCAGAUUAUGCGGAUC
307	306	CACCAUGCAGAUUAUGCGGAUCA
308	307	ACCAUGCAGAUUAUGCGGAUCAA
309	308	CCAUGCAGAUUAUGCGGAUCAA
310	309	CAUGCAGAUUAUGCGGAUCAAAC
311	310	AUGCAGAUUAUGCGGAUCAAACC
312	311	UGCAGAUUAUGCGGAUCAAACCU
313	312	GCAGAUUAUGCGGAUCAAACCUC
314	313	CAGAUUAUGCGGAUCAAACCUCA
315	314	AGAUUAUGCGGAUCAAACCUCAC
316	315	GAUUAUGCGGAUCAAACCUCACC
317	316	AUUAUGCGGAUCAAACCUCACCA
318	317	UUAUGCGGAUCAAACCUCACCAA
319	318	UAUGCGGAUCAAACCUCACCAAG
320	319	AUGCGGAUCAAACCUCACCAAGG
321	320	UGCGGAUCAAACCUCACCAAGGC
322	321	GCGGAUCAAACCUCACCAAGGCC
323	322	CGGAUCAAACCUCACCAAGGCCA
324	323	GGAUCAAACCUCACCAAGGCCAG
325	324	GAUCAAACCUCACCAAGGCCAGC
326	325	AUCAAAACCUCACCAAGGCCAGCA
327	326	UCAAAACCUCACCAAGGCCAGCAC
328	327	CAAACCUCACCAAGGCCAGCACA
329	328	AAACCUCACCAAGGCCAGCACA
330	329	AACCUCACCAAGGCCAGCACAUA
331	330	ACCUCACCAAGGCCAGCACAUA
332	331	CCUCACCAAGGCCAGCACAUA
333	332	CUCACCAAGGCCAGCACAUA
334	333	UCACCAAGGCCAGCACAUA
335	334	CACCAAGGCCAGCACAUA
336	335	ACCAAGGCCAGCACAUA
337	336	CCAAGGCCAGCACAUA
338	337	CAAGGCCAGCACAUA
339	338	AAGGCCAGCACAUA
340	339	AGGCCAGCACAUA
341	340	GGCCAGCACAUA
342	341	GCCAGCACAUA
343	342	CCAGCACAUA
344	343	CAGCACAUA
345	344	AGCACAUA
346	345	GCACAUA
347	346	CACAUA
348	347	ACAUA

349	348	CAUAGGAGAGAUGAGCUUCCUAC
350	349	AUAGGAGAGAUGAGCUUCCUACA
351	350	UAGGAGAGAUGAGCUUCCUACAG
352	351	AGGAGAGAUGAGCUUCCUACAGC
353	352	GGAGAGAUGAGCUUCCUACAGCA
354	353	GAGAGAUGAGCUUCCUACAGCAC
355	354	AGAGAUGAGCUUCCUACAGCACA
356	355	GAGAUGAGCUUCCUACAGCACAA
357	356	AGAUGAGCUUCCUACAGCACAAC
358	357	GAUGAGCUUCCUACAGCACAACA
359	358	AUGAGCUUCCUACAGCACAACAA
360	359	UGAGCUUCCUACAGCACAACAAA
361	360	GAGCUUCCUACAGCACAACAAAU
362	361	AGCUUCCUACAGCACAACAAUUG
363	362	GCUUCCUACAGCACAACAAUUGU
364	363	CUUCCUACAGCACAACAAUUGUG
365	364	UCCUACAGCACAACAAUUGUGA
366	365	UCCUACAGCACAACAAUUGUGAA
367	366	CCUACAGCACAACAAUUGUGAAU
368	367	CUACAGCACAACAAUUGUGAAUG
369	368	UACAGCACAACAAUUGUGAAUGC
370	369	ACAGCACAACAAUUGUGAAUGCA
371	370	CAGCACAACAAUUGUGAAUGCAG
372	371	AGCACAACAAUUGUGAAUGCAGA
373	372	GCACAACAAUUGUGAAUGCAGAC
374	373	CACAACAAUUGUGAAUGCAGACC
375	374	ACAACAAUUGUGAAUGCAGACCA
376	375	CAACAAUUGUGAAUGCAGACCAA
377	376	AACAAUUGUGAAUGCAGACCAAA
378	377	ACAAUUGUGAAUGCAGACCAAAG
379	378	CAAUUGUGAAUGCAGACCAAAGA
380	379	AAAUUGUGAAUGCAGACCAAAGAA
381	380	AAUGUGAAUGCAGACCAAAGAAA
382	381	AUGUGAAUGCAGACCAAAGAAAG
383	382	UGUGAAUGCAGACCAAAGAAAGA
384	383	GUGAAUGCAGACCAAAGAAAGAU
385	384	UGAAUGCAGACCAAAGAAAGAU
386	385	GAAUGCAGACCAAAGAAAGAUAG
387	386	AAUGCAGACCAAAGAAAGAUAGA
388	387	AUGCAGACCAAAGAAAGAUAGAG
389	388	UGCAGACCAAAGAAAGAUAGAGC
390	389	GCAGACCAAAGAAAGAUAGAGCA
391	390	CAGACCAAAGAAAGAUAGAGCAA
392	391	AGACCAAAGAAAGAUAGAGCAAG

393	392	GACCAAAGAAAGAUAGAGCAAGA
394	393	ACCAAAGAAAGAUAGAGCAAGAC
395	394	CCAAAGAAAGAUAGAGCAAGACA
396	395	CAAAGAAAGAUAGAGCAAGACAA
397	396	AAAGAAAGAUAGAGCAAGACAAG
398	397	AAGAAAGAUAGAGCAAGACAAGA
399	398	AGAAAGAUAGAGCAAGACAAGAA
400	399	GAAAGAUAGAGCAAGACAAGAAA
401	400	AAAGAUAGAGCAAGACAAGAAAA

TABLE 2

Position in ORF	SEQ ID NO:	Target sequence (5'-3')	Alnylam DUP ID	Strand	SEQ ID NO:	Sequences	Efficacy HeLa	Efficacy hrPE
1	2	AUGAACUUUCUGUCUGUCUUGGGU	AL-DP-4043	S AS	402 403	5 GAACUUUCUGUCUGUCUUGGGU 3 3 UACUUGAAGAGACGACAGAACCCA 5	+++	NA
22	23	GUGCAUUGGAGCCUUGCCUUGCU	AL-DP-4077	S AS	404 405	5 GCAUUGGAGCCUUGCCUUGCU 3 3 CACGUAACCCUGGAAACGGAACGA 5	+++	NA
47	48	UCUACCUCCACCAUGCCCAAGUGG	AL-DP-4021	S AS	406 407	5 UACCUCCACCAUGCCCAAGUTT 3 3 TTAUGGAGGUGUACGCUCA 5	+	NA
48	49	CUACCUCCACCAUGCCCAAGUGGU	AL-DP-4109	S AS	408 409	5 ACCUCCACCAUGCCCAAGUGTT 3 3 TTUGGAGGUGUACGCUUCAC 5	+	NA
50	51	ACCUCCACCAUGCCCAAGUGGUCC	AL-DP-4006	S AS	410 411	5 CUCCACCAUGCCCAAGUGUCC 3 3 UGGAGGUGGUAACGCUUACACGAG 5	++	+
			AL-DP-4083	S AS	412 413	5 CUCCACCAUGCCCAAGUGGUTT 3 3 TTGAGGUGGUACGCUUACCA 5	++	++
51	52	CCUCCACCAUGCCCAAGUGGUCCC	AL-DP-4047	S AS	414 415	5 UCCACCAUGCCCAAGUGUCCC 3 3 GGAGGUGGUACGCUUACACGAGG 5	+	NA
			AL-DP-4017	S AS	416 417	5 UCCACCAUGCCCAAGUGGUCTT 3 3 TTAGGUGGUACGCUUACACGAG 5	+	NA
52	53	CUCCACCAUGCCCAAGUGGUCCCA	AL-DP-4048	S AS	418 419	5 CCACCAUGCCCAAGUGGUCCCA 3 3 GAGGUGGUACGCUUACACGAGG 5	++	++
			AL-DP-4103	S AS	420 421	5 CCACCAUGCCCAAGUGGUCCCTT 3 3 TTGGUGGUACGCUUACACGAG 5	++/+	++
53	54	UCCACCAUGCCCAAGUGGUCCAG	AL-DP-4035	S AS	422 423	5 CACCAUGCCCAAGUGGUCCAG 3 3 AGGUGGUACGCUUACACGAGG 5	++	+
			AL-DP-4018	S AS	424 425	5 CACCAUGCCCAAGUGGUCCCTT 3 3 TTGUGGUACGCUUACACGAGG 5	++/+	+
54	55	CCACCAUGCCCAAGUGGUCCAGG	AL-DP-4036	S AS	426 427	5 ACCAUGCCCAAGUGGUCCAGG 3 3 GGUGGUACGCUUACACGAGG 5	+++	++

			AL-DP-4084	S	428	5 ACCAUGCCAAAGUGGUCCATT 3 3 TTUGGUACGGUUCACCAAGGU 5	++	+
55	56	CACCAUGCCAAAGUGGUCCAGGC	AL-DP-4093	AS	429			
				S	430	5 CCAUGCCAAAGUGGUCCAGGC 3	++	+
				AS	431	3 GUGGUACGGUUCACCAAGGUCCG 5		
			AL-DP-4085	S	432	5 CCAUGCCAAAGUGGUCCAGTT 3	+	+
				AS	433	3 TTGGUACGGUUCACCAAGGU 5		
56	57	ACCAUGCCAAAGUGGUCCAGGCU	AL-DP-4037	S	434	5 CAUGCCAAAGUGGUCCAGGCU 3	+	+
				AS	435	3 UGGUACGGUUCACCAAGGUCCGA 5		
			AL-DP-4054	S	436	5 CAUGCCAAAGUGGUCCAGTT 3	++	+
				AS	437	3 TTGUACGGUUCACCAAGGUCC 5		
57	58	CCAUGCCAAAGUGGUCCAGGCU	AL-DP-4038	S	438	5 AUGCCAAAGUGGUCCAGGCU 3	++	++
				AS	439	3 GGUACGGUUCACCAAGGUCCGAC 5		
			AL-DP-4086	S	440	5 AUGCCAAAGUGGUCCAGGCTT 3	+	+
				AS	441	3 TTUACGGUUCACCAAGGUCCG 5		
58	59	CAUGCCAAAGUGGUCCAGGCU	AL-DP-4049	S	442	5 UGCCAAAGUGGUCCAGGCU 3	++	++
				AS	443	3 GUACGGUUCACCAAGGUCCGACG 5		
			AL-DP-4087	S	444	5 UGCCAAAGUGGUCCAGGCU 3	+	+
				AS	445	3 TTACGGUUCACCAAGGUCCGA 5		
59	60	AUGCCAAAGUGGUCCAGGCU	AL-DP-4001	S	446	5 GCCAAAGUGGUCCAGGCU 3	++	++
				AS	447	3 UACGGUUCACCAAGGUCCGACGU 5		
			AL-DP-4052	A	448	5 GCCAAAGUGGUCCAGGCU 3	+++	++
				AS	449	3 TTGGUUCACCAAGGUCCGAC 5		
60	61	UGCCAAAGUGGUCCAGGCU	AL-DP-4007	S	450	5 CCAAGUGGUCCAGGCU 3	+++	++
				AS	451	3 ACGGUUCACCAAGGUCCGACGU 5		
			AL-DP-4088	S	452	5 CCAAGUGGUCCAGGCU 3	+++	++
				AS	453	3 TTGGUUCACCAAGGUCCGACG 5		
61	62	GCCAAAGUGGUCCAGGCU	AL-DP-4070	S	454	5 CAAAGUGGUCCAGGCU 3	++	++
				AS	455	3 CGGUUCACCAAGGUCCGACGU 5		
			AL-DP-4055	S	456	5 CAAAGUGGUCCAGGCU 3	+++	+
				AS	457	3 TTGUUCACCAAGGUCCGACGU 5		
62	63	CCAAGUGGUCCAGGCU	AL-DP-4071	S	458	5 AAGUGGUCCAGGCU 3	+	NA
				AS	459	3 GGUUCACCAAGGUCCGACGU 5		

63	64	AL-DP-4056	S AS	460 461	5 AAGUGUCCAGGUGCATT 3 3 TTUACACAGGUGCCAGUG 5	++	NA
		AL-DP-4072	S AS	462 463	5 AGUGUCCAGGUGCACCAC 3 3 GUUCACACAGGUGCCAGUGGU 5	++	+
		AL-DP-4057	S AS	464 465	5 AGUGUCCAGGUGCACCATT 3 3 TTUACACAGGUGCCAGUGGU 5	++/+	++
64	65	AL-DP-4066	S AS	466 467	5 GUGUCCAGGUGCACCCTT 3 3 TTACACAGGUGCCAGUGGU 5	+	NA
99	100	AL-DP-4022	S AS	468 469	5 GGCGAUAUACACGAAGUTT 3 3 TTCCGUCUAGUAGUCUACA 5	+++	NA
100	101	AL-DP-4023	S AS	470 471	5 GCAGAAUACACGAAGUGTT 3 3 TTCCGUCUAGUAGUCUACA 5	++	NA
101	102	AL-DP-4024	S AS	472 473	5 CAGAAUACACGAAGUGTT 3 3 TTGUCUAGUAGUCUACCAC 5	+	NA
102	103	AL-DP-4076	S AS	474 475	5 AGAAUACACGAAGUGUGA 3 3 CGUCUAGUAGUCUACCACU 5	++	NA
103	104	AL-DP-4019	S AS	476 477	5 AGAAUACACGAAGUGUGTT 3 3 TTUCUAGUAGUCUACCAC 5	++	NA
104	105	AL-DP-4025	S AS	478 479	5 GAAUACACGAAGUGUGTT 3 3 TTUAGUAGUCUACCAC 5	++	NA
105	106	AL-DP-4110	S AS	480 481	5 AAUACACGAAGUGUGATT 3 3 TTUAGUAGUCUACCACU 5	+	NA
113	114	AL-DP-4078	S AS	482 483	5 AUCAUACGAAGUGUGAATT 3 3 TTUAGUAGUCUACCACU 5	+	NA
121	122	AL-DP-4080	S AS	484 485	5 GAAGUGGUGAAGUUAUGGAU 3 3 UGCUACACCAUUAAGUACCUA 5	+++	NA
129	130	AL-DP-4111	S AS	486 487	5 GAAGUUAUGGUGUUAUCA 3 3 CACUACAGUACCUACAGUAGU 5	+++	NA
130	131	AL-DP-4041	S AS	488 489	5 UGGAUGUCUAUACAGCGCAGTT 3 3 TTACCUACAGUAGUCGCGUC 5	+++	NA
		AL-DP-4041	S AS	490 491	5 GGAUGUCUAUACAGCGCAGCUA 3 3 UACCUACAGUAGUCGCGUAGU 5	+++	NA

			AL-DP-4062	S	492	5 GGAUGUCUAUCAGCGCAGCTT 3 3 TTCCUACAGAUAGUCGCGUCG 5	+++	NA
131	132	UGGAUGUCUAUCAGCGCAGCUAC	AL-DP-4069	S	494	5 GAUGUCUAUCAGCGCAGCTT 3 3 TTTCUACAGAUAGUCGCGUGA 5	+++	NA
132	133	GGAUGUCUAUCAGCGCAGCUACU	AL-DP-4112	S	496	5 AUGUCUAUCAGCGCAGCUATT 3 3 TTUACAGAUAGUCGCGUGAU 5	+	NA
133	134	GAUGUCUAUCAGCGCAGCUACUG	AL-DP-4026	S	498	5 UGUCUAUCAGCGCAGCUACTT 3 3 TTACAGAUAGUCGCGUGCAUG 5	++	NA
134	135	AUGUCUAUCAGCGCAGCUACUGC	AL-DP-4095	S	500	5 GUCUAUCAGCGCAGCUACUGC 3 3 UACAGAUAGUCGCGUGCAGCG 5	+++	NA
135	136	UGUCUAUCAGCGCAGCUACUGCC	AL-DP-4027	S	504	5 UCUAUCAGCGCAGCUACUGTT 3 3 TTAGAUAGUCGCGUGCAGGAC 5	+	NA
144	145	GCGCAGCUACUGCCAUCCAAUUG	AL-DP-4081	S	506	5 GCAGCUACUGCCAUCCAAUUG 3 3 CGCGUCGAGUACGGUAGGUAGC 5	+++	NA
146	147	GCAGCUACUGCCAUCCAAUUGAG	AL-DP-4098	S	508	5 AGCUACUGCCAUCCAAUUGAG 3 3 CGUCGAGUACGGUAGGUAGCUC 5	+++	NA
149	150	GCUACUGCCAUCCAAUUGGAGCC	AL-DP-4028	S	510	5 UACUGCCAUCCAAUUGGAGATT 3 3 TTAUGACGGUAGGUAGCUCU 5	++	NA
150	151	CUACUGCCAUCCAUUGGAGCCC	AL-DP-4029	S	512	5 ACUGCCAUCCAUUGGAGACTT 3 3 TTUGACGGUAGGUAGCUCUG 5	+	NA
151	152	UACUGCCAUCCAAUUGGAGCCCU	AL-DP-4030	S	514	5 CUGCCAUCCAAUUGGAGACTT 3 3 TTGACGGUAGGUAGCUCUGG 5	+++	NA
152	153	ACUGCCAUCCAAUUGGAGCCUG	AL-DP-4031	S	516	5 UGCCAUCCAUUGGAGCCCTT 3 3 TTACGGUAGGUAGCUCUGGG 5	+	NA
166	167	GAGACCGUGGAGCAUCUUCCA	AL-DP-4008	S	518	5 GACCGUGGAGCAUCUUCCA 3 3 CUUCGGGACCAUUGAGAGGU 5	++	+
167	168	AGACCGUGGAGCAUCUUCAG	AL-DP-4009	S	522	5 ACCCGUGGAGCAUCUUCAG 3 3 UCVGGGACCAUUGAGAGGU 5	++	NA

168	169		AL-DP-4059	S	524	5 ACCUGGUGGACAUUUCCTT 3 3 TTUGGACCAACUGUAGAGG 5	+	NA
			AL-DP-4010	S	525	5 CCUGUGGACAUUUCAGG 3 3 CUGGACCAACUGUAGAGUCC 5	+	+
			AL-DP-4060	S	526	5 CCCUGGUGGACAUUCCATT 3 3 TTGGACCAACUGUAGAGGU 5	+++	++
			AL-DP-4073	S	527	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	++	+
			AL-DP-4104	S	528	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	+++ / +	++
			AL-DP-4011	S	529	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	+	NA
			AL-DP-4089	S	530	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	+	NA
			AL-DP-4074	S	531	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	++	+
			AL-DP-4090	S	532	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	++	++
			AL-DP-4039	S	533	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	+	+
			AL-DP-4091	S	534	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	++	++
			AL-DP-4003	S	535	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	+	+
			AL-DP-4116	S	536	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	+	+
			AL-DP-4015	S	537	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	++	++
			AL-DP-4120	S	538	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	+	+
			AL-DP-4099	S	539	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	+++	NA
179	170		AL-DP-4059	S	540	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	+	NA
			AL-DP-4010	S	541	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	++	+
			AL-DP-4060	S	542	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	+	+
			AL-DP-4073	S	543	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	++	++
			AL-DP-4104	S	544	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	+	+
			AL-DP-4011	S	545	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	++	++
			AL-DP-4089	S	546	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	+	+
			AL-DP-4074	S	547	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	++	++
			AL-DP-4090	S	548	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	+	+
			AL-DP-4039	S	549	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	++	++
			AL-DP-4091	S	550	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	+	+
			AL-DP-4003	S	551	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	++	++
			AL-DP-4116	S	552	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	+	+
			AL-DP-4015	S	553	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	++	++
			AL-DP-4120	S	554	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	+	+
			AL-DP-4099	S	555	5 CCUGUGGACAUUCCAGTT 3 3 TTGGACCAACUGUAGAGGU 5	+++	NA

191	192	AGUACCCUGAUGAGAUCCGAGUAC	AL-DP-4032	S	556	5 UACCCUGAUGAGAUCCGAGUATT 3	+++	NA
192	193	GUACCCUGAUGAGAUCCGAGUACA	AL-DP-4042	AS	557	3 TTAUGGGGACUACUCUAGCUCA 5	+++	NA
			AL-DP-4063	S	558	5 ACCCUGAUGAGAUCCGAGUACA 3	+++	NA
				AS	559	3 CAUGGGACUACUCUAGCUCAUGU 5	+++	NA
209	210	AGUACAUUCUACAAGCCAUCUGU	AL-DP-4064	S	560	5 ACCCUGAUGAGAUCCGAGUATT 3	+	NA
260	261	GCAUAACGAGGGCCUGGAGUGU	AL-DP-4044	AS	561	3 TTUGGGACUACUCUAGCUCAU 5	+	NA
263	264	AUGACGAGGGCCUGGAGUGUG	AL-DP-4045	S	562	5 UACAUCUACAAGCCAUCUATT 3	+	NA
279	280	GUGUGUGCCACUGAGGAGUCCA	AL-DP-4046	AS	563	3 TTAUGUAGAAGUUCGUAGA 5	+	NA
281	282	GUGUGCCACUGAGGAGUCCAAC	AL-DP-4096	S	564	5 AAUACGAGGGCCUGGAGUGU 3	+	NA
283	284	GUGCCACUGAGGAGUCCAACAU	AL-DP-4040	AS	565	3 CGUUAUGUGUCCCGGACCUCA 5	+	NA
289	290	ACUGAGGAGUCCAACAUCAACAU	AL-DP-4065	S	566	5 GACGAGGGCCUGGAGUGUG 3	+	NA
302	303	ACAUCAACUAGCAGAUUAUGCGG	AL-DP-4100	AS	567	3 UACUGUCUCCCGGACCUCAACAC 5	+++	NA
305	306	UCACCAUGCAGAUUAUGCGGAGU	AL-DP-4033	S	568	5 GUGUGCCACUGAGGAGUCCA 3	+++	NA
310	311	AUGCAGAUUAUGCGGAGUCAAACC	AL-DP-4101	AS	569	3 CACACACGGGUGACUCCUAGGU 5	+++	NA
312	313	GCAGAUUAUGCGGAGUCAAACCUC	AL-DP-4102	S	570	5 GUGCCACUGAGGAGUCCAAC 3	+	NA
315	316	GAUUUUGCGGAGUCAAACCUCACC	AL-DP-4034	AS	571	3 CACACGGGUGACUCCUAGGU 5	+++	NA
316	317	AUUUUGCGGAGUCAAACCUCACCA	AL-DP-4113	S	572	5 GCCCAGUGAGGAGUCCAACAU 3	+++	NA
				AS	573	3 CACGGGUGACUCCUAGGU 5	+	NA
				S	574	5 UGAGGAGUCCAACAUCACTT 3	++	NA
				AS	575	3 TTAUCUCCUAGGUUUGAGUGG 5	++	NA
				S	576	5 AUCACCAUGCAGAUUAUGCGG 3	+++	NA
				AS	577	3 UGUAGUGGAGUCCUAAUAGCGC 5	+++	NA
				S	578	5 ACCAUGCAGAUUAUGCGGATT 3	+++	NA
				AS	579	3 TTUGGUAGGUCUAAUACGCCU 5	+++	NA
				S	580	5 GCAGAUUAUGCGGAGUCAAACC 3	+++	NA
				AS	581	3 UACGUCUAAUACGCCUAGUUUGG 5	+++	NA
				S	582	5 AGAUUAUGCGGAGUCAAACCUC 3	+++	NA
				AS	583	3 CGUCUAAUACGCCUAGUUUGGAG 5	++	NA
				S	584	5 UUAUGCGGAGUCAAACCUCATT 3	++	NA
				AS	585	3 TTAUAUACGCCUAGUUUGGAGU 5	++	NA
				S	586	5 UUAUGCGGAGUCAAACCUCATT 3	++	NA
				AS	587	3 TTAUACGCCUAGUUUGGAGUG 5	++	NA

317	318	UUUUGGGAUCAAACCUACACCAA	AL-DP-4114	S AS	588 589	5 AUGCGAUCAAACCUACACCTT 3 3 TTUACGCCUAGUUUGAGUGG 5	+	NA
319	320	AUGCGAUCAAACCUACCAAGG	AL-DP-4002	S AS	590 591	5 GCAGAUCAAACCUACCAAGG 3 3 UACGCCUAGUUUGAGUGGUCC 5	+++	+++
			AL-DP-4115	S AS	592 593	5 GCAGAUCAAACCUACACCAA 3 3 CGCCUAGUUUGAGUGGUU 5	+++	NA
			AL-DP-4014	S AS	594 595	5 GCAGAUCAAACCUACCAATT 3 3 TTGCGCUAGUUUGAGUGGUU 5	+++	+++
			AL-DP-4119	S AS	596 597	5 GCAGAUCAAACCUACACCAA 3 3 CGCCUAGUUUGAGUGGUU 5	+++	NA
321	322	GCAGAUCAAACCUACCAAGGCC	AL-DP-4013	S AS	598 599	5 GGAUCAAAACCUACCAAGGCC 3 3 CGCCUAGUUUGAGUGGUUCCG 5	++	NA
341	342	GCCAGCAUAUAGGAGAGAGC	AL-DP-4075	S AS	600 601	5 CAGCAUAUAGGAGAGAGC 3 3 CGGUGUUAUCCUCUACUCG 5	+++	++
			AL-DP-4105	S AS	602 603	5 CAGCAUAUAGGAGAGAGATT 3 3 TTGUGUGUAUCCUCUACUCU 5	++	++
342	343	CCAGCAUAUAGGAGAGAGCU	AL-DP-4050	S AS	604 605	5 AGCAUAUAGGAGAGAGAGCU 3 3 GGUUGUAUCCUCUACUCGA 5	+++	+++
			AL-DP-4106	S AS	606 607	5 AGCAUAUAGGAGAGAGATT 3 3 TTGUGUAUCCUCUACUC 5	++	+++
343	344	CAGCAUAUAGGAGAGAGCUU	AL-DP-4094	S AS	608 609	5 GCACUAUAGGAGAGAGAGCUU 3 3 GUUGUAUCCUCUACUCGAA 5	+++	+++
			AL-DP-4118	S AS	610 611	5 GCACUAUAGGAGAGAGAGCUU 3 3 CGUGUAUCCUCUACUCGAA 5	*	NA
			AL-DP-4107	S AS	612 613	5 GCACUAUAGGAGAGAGAGCTT 3 3 TTGUGUAUCCUCUACUCG 5	+++	+++
			AL-DP-4122	S AS	614 615	5 GCACUAUAGGAGAGAGAGC 3 3 CGUGUAUCCUCUACUCG 5	++	NA
344	345	AGCACAUAUAGGAGAGAGCUUC	AL-DP-4012	S AS	616 617	5 CACAUAUAGGAGAGAGCUUC 3 3 UCGUGUAUCCUCUACUCGAG 5	+++	+++
			AL-DP-4108	S AS	618 619	5 CACAUAUAGGAGAGAGCTT 3 3 TTGUGUAUCCUCUACUCGA 5	+++	+++

346	347	CACAUAGGAGAGAGAGCUUCU	AL-DP-4051	S	620	5 CAUAGGAGAGAGAGCUUCU 3	+++	+++
			AL-DP-4061	AS	621	3 GUGUAUCCUCUACUCGAGGA 5		
				S	622	5 CAUAGGAGAGAGAGCUUCTT 3	+++	+++
				AS	623	3 TTGUUACUCCUCUACUCGAAG 5		
349	350	AUAGGAGAGAGAGCUUCUACA	AL-DP-4082	S	624	5 AGGAGAGAGAGAGCUUCUACA 3	+++	NA
				AS	625	3 UAUCCUCUACUCGAGAGAGU 5		
369	370	ACAGCACACACAAAUUGUAAUGCA	AL-DP-4079	S	626	5 AGCACACACAAAUUGUAAUGCA 3	++	NA
				AS	627	3 UGUCGUGUGUUUACACUUAAGU 5		
372	373	GCACACACAAUUGUAAUGCAGAC	AL-DP-4097	S	628	5 ACACACACAAUUGUAAUGCAGAC 3	++	NA
				AS	629	3 CGUGUUGUUUACACUUAAGU 5		
379	380	AAUUGUAAUGCAGACCAAGAA	AL-DP-4067	S	630	5 AUGUAAUGCAGACCAAGATT 3	++	NA
				AS	631	3 TTUACACUUAAGUUGUUGU 5		
380	381	AAUUGUAAUGCAGACCAAGAAA	AL-DP-4092	S	632	5 UGUGAAUGCAGACCAAGATT 3	+++	NA
				AS	633	3 TTACACUUAAGUUGUUGU 5		
381	382	AUGUAAUGCAGACCAAGAAAG	AL-DP-4004	S	634	5 GUGAAUGCAGACCAAGAAAG 3	+++	++
				AS	635	3 UACACUUAAGUUGUUGUUGU 5		
			AL-DP-4117	S	636	5 GUGAAUGCAGACCAAGAAAG 3	+++	NA
				AS	637	3 CACUUAAGUUGUUGUUGU 5		
			AL-DP-4016	S	638	5 GUGAAUGCAGACCAAGAAATT 3	+++	+++
				AS	639	3 TTACUUAAGUUGUUGUUGU 5		
			AL-DP-4121	S	640	5 GUGAAUGCAGACCAAGAA 3	++	NA
				AS	641	3 CACUUAAGUUGUUGUUGU 5		
383	384	GUGAAUGCAGACCAAGAAAGAU	AL-DP-4005	S	642	5 GAUAGCAGACCAAGAAAGAU 3	+++	++
				AS	643	3 CACUUAAGUUGUUGUUGUUGU 5		
			AL-DP-4053	S	644	5 GAAUGCAGACCAAGAAAGATT 3	+++	++
				AS	645	3 TTUUAAGUUGUUGUUGUUGU 5		

TABLE 3- Phosphorothioate stabilized siRNA Molecules are modified versions of AL-DP-4014.

ORF Position	Aln Duplex #	Duplex Sequence	SEQ ID NO:	Efficacy
319	ALN-DP-4127	5' -G*C*GGAUCAAAACCUCACCA*A*dT*dT-3' dT*dT*C*GCCUAGUUUGGAGUGG*U*U-5'	3'- 646 647	+++
319	ALN-DP-4128	5' -G*C*GGAUCAAAACCUC*ACC*A*A*dT*dT-3' 3'-dT*dT*CGCCUAGUUUGGAGUGGU*U-5'	648 649	+++
319	ALN-DP-4129	5' -G*C*GGAUCAAAACCUC*ACC*A*A*dT*dT-3' 3'-dT*dT*C*GCCUAGUUUGGAGUGG*U*U-5'	650 651	+++

* indicates the position of a phosphorothioate group

Table 4. *In vitro* efficacy of Modified AL-DP-4094 series

SiRNA			Efficacy	5'-sense strand-3' 3'-antisense strand-5'
AL-DP-4198	AL4554	+++		5'- GsCACAUAAGGAGAGAUGAGCU _s U-3'
	AL4557			3'-GsUCGUGUAUCCUCUCUACUCGAsA-5'
AL-DP-4165	AL4554	+++		5'- GsCACAUAAGGAGAGAUGAGCU _s U-3'
	AL4558			3'-GsU _{OMe} C _{OMe} GUGUAUCCUCUCUACUGAsA-5'
AL-DP-4166	AL4554	+++		5'- GsCACAUAAGGAGAGAUGAGCU _s U-3'
	AL4559			3'-GsU _{OMe} C _{OMe} GUGUAU _{OMe} CCUCUCUACUCGAsA-5'
AL-DP-4167	AL4554	+++		5'- GsCACAUAAGGAGAGAUGAGCU _s U-3'
	AL4560			3'-GsU _{OMe} CGU _{OMe} GU _{OMe} AU _{OMe} CCUCUCUAC _{OMe} UCGAA-5'
AL-DP-4168	AL4554	+++		5'- GsCACAUAAGGAGAGAUGAGCU _s U-3'
	AL4561			3'-GsU _{OMe} C _{OMe} GU _{OMe} GU _{OMe} AU _{OMe} CCUCUCUAC _{OMe} UCGAA-5'
AL-DP-4169	AL4554	+++		5'- GsCACAUAAGGAGAGAUGAGCU _s U-3'
	AL4562			3'-GsU _{OMe} dCGU _{OMe} GU _{OMe} AU _{OMe} CCUCUCUAdCUCGAA-5'
AL-DP-4170	AL4555	+++		5'- GsCACAU _{2'} _{OMe} AGGAGAGAUGAGCU _s U-3'
	AL4557			3'-GsUCGUGUAUCCUCUCUACUCGAsA-5'
AL-DP-4171	AL4555	+++		5'- GsCACAU _{2'} _{OMe} AGGAGAGAUGAGCU _s U-3'
	AL4558			3'-GsU _{OMe} C _{OMe} GUGUAUCCUCUCUACUGAsA-5'
AL-DP-4172	AL4555	+++		5'- GsCACAU _{2'} _{OMe} AGGAGAGAUGAGCU _s U-3'
	AL4559			3'-GsU _{OMe} C _{OMe} GUGUAU _{OMe} CCUCUCUACUGAsA-5'
AL-DP-4173	AL4555	+++		5'- GsCACAU _{2'} _{OMe} AGGAGAGAUGAGCU _s U-3'
	AL4560			3'-GsU _{OMe} CGU _{OMe} GU _{OMe} AU _{OMe} CCUCUCUAC _{OMe} UCGAA-5'
AL-DP-4174	AL4555	+++		5'- GsCACAU _{2'} _{OMe} AGGAGAGAUGAGCU _s U-3'
	AL4561			3'-GsU _{OMe} C _{OMe} GU _{OMe} GU _{OMe} AU _{OMe} CCUCUCUAC _{OMe} UCGAA-5'
AL-DP-4175	AL4555	+++		5'- GsCACAU _{2'} _{OMe} AGGAGAGAUGAGCU _s U-3'
	AL4562			3'-GsU _{OMe} dCGU _{OMe} GU _{OMe} AU _{OMe} CCUCUCUAdCUCGAA-5'
AL-DP-4176	AL4556	+++		5'-GC _{OMe} AC _{OMe} AU _{OMe} AGGAGAGAU _{OMe} GAGCU _{OMe} SU-3'
	AL4557			3'-GsUCGUGUAUCCUCUCUACUCGAsA-5'
AL-DP-4177	AL4556	+++		5'-GC _{OMe} AC _{OMe} AU _{OMe} AGGAGAGAU _{OMe} GAGCU _{OMe} SU-3'
	AL4558			3'-GsU _{OMe} C _{OMe} GUGUAUCCUCUCUACUGAsA-5'
AL-DP-4178	AL4556	+++		5'-GC _{OMe} AC _{OMe} AU _{OMe} AGGAGAGAU _{OMe} GAGCU _{OMe} SU-3'
	AL4559			3'-GsU _{OMe} C _{OMe} GUGUAU _{OMe} CCUCUCUACUGAsA-5'
AL-DP-4179	AL4556	+++		5'-GC _{OMe} AC _{OMe} AU _{OMe} AGGAGAGAU _{OMe} GAGCU _{OMe} SU-3'
	AL4560			3'-GsU _{OMe} CGU _{OMe} GU _{OMe} AU _{OMe} CCUCUCUAC _{OMe} UCGAA-5'
AL-DP-4180	AL4556	+++		5'-GC _{OMe} AC _{OMe} AU _{OMe} AGGAGAGAU _{OMe} GAGCU _{OMe} SU-3'
	AL4561			3'-GsU _{OMe} C _{OMe} GU _{OMe} GU _{OMe} AU _{OMe} CCUCUCUAC _{OMe} UCGAA-5'

AL-DP-4181	AL4556	+++	5'-G _{OMe} AC _{OMe} AU _{OMe} AGGAGAGAU _{OMe} GAGCU _{OMe} SU-3'
	AL4562		3'-GsU _{OMe} dCGU _{OMe} GU _{OMe} AU _{OMe} CCUCUCUAdCUCGAA-5'
AL-DP-4220	AL2780	+++	5'-GsC _{OMe} AC _{OMe} AU _{OMe} AGGAGAGAU _{OMe} GAGCU _{OMe} SU-3'
	AL2781		3'-GsU _{OMe} C _{OMe} GU _{OMe} GU _{OMe} AU _{OMe} CCUCUCUAC _{OMe} UCGAsA-5'
AL-DP-4182	AL4563	+++	5'-G dC A dC AU _{OMe} AGGAGAGAU _{OMe} GAGCU _{OMe} SU-3'
	AL4557		3'-GsUCGUGUAUCCUCUCUACUCGAsA-5'
AL-DP-4183	AL4563	+++	5'-G dC A dC AU _{OMe} AGGAGAGAU _{OMe} GAGCU _{OMe} SU-3'
	AL4558		3'-GsU _{OMe} C _{OMe} GUGUAUCCUCUCUACUGAsA-5'
AL-DP-4184	AL4563	+++	5'-G dC A dC AU _{OMe} AGGAGAGAU _{OMe} GAGCU _{OMe} SU-3'
	AL4559		3'-GsU _{OMe} C _{OMe} GUGUAU _{OMe} CCUCUCUACUGAsA-5'
AL-DP-4185	AL4563	+++	5'-G dC A dC AU _{OMe} AGGAGAGAU _{OMe} GAGCU _{OMe} SU-3'
	AL4560		3'-GsU _{OMe} CGU _{OMe} GU _{OMe} AU _{OMe} CCUCUCUAC _{OMe} UCGAA-5'
AL-DP-4186	AL4563	+++	5'-G dC A dC AU _{OMe} AGGAGAGAU _{OMe} GAGCU _{OMe} SU-3'
	AL4561		3'-GsU _{OMe} C _{OMe} GU _{OMe} GU _{OMe} AU _{OMe} CCUCUCUAC _{OMe} UCGAA-5'
AL-DP-4187	AL4563	+++	5'-G dC A dC AU _{OMe} AGGAGAGAU _{OMe} GAGCU _{OMe} SU-3'
	AL4562		3'-GsU _{OMe} dCGU _{OMe} GU _{OMe} AU _{OMe} CCUCUCUAdCUCGAA-5'
AL-DP-4188	AL4564	+++	5'-GsCACAU _F AGGAGAGAUGAGCU _S U-3'
	AL4557		3'-GsUCGUGUAUCCUCUCUACUCGAsA-5'
AL-DP-4189	AL4565	+++	5'-GC _F AC _F AU _F AGGAGAGAU _F GAGCU _F SU-3'
	AL4557		3'-GsUCGUGUAUCCUCUCUACUCGAsA-5'
AL-DP-4190	AL4566	+++	5'-GC _F AC _F AU _{OMe} AGGAGAGAU _{OMe} GAGCU _{OMe} SU-3'
	AL4557		3'-GsUCGUGUAUCCUCUCUACUCGAsA-5'
AL-DP-4191	AL4567	+++	5'-GC _{OMe} AC _{OMe} AU _F AGGAGAGAU _F GAGCU _F SU-3'
	AL4557		3'-GsUCGUGUAUCCUCUCUACUCGAsA-5'
AL-DP-4192	AL4554	+++	5'-GsCACAUAGGAGAGAUGAGCU _S U-3'
	AL4568		3'-GsU _F CGU _F GU _F AU _F CCUCUCUAC _F UCGAA-5'
AL-DP-4193	AL4554	+++	5'-GsCACAUAGGAGAGAUGAGCU _S U-3'
	AL4569		3'-GsU _F CGU _F GU _F AU _F CCUCUCUAC _{OMe} UCGAA-5'
AL-DP-4194	AL4554	+++	5'-GsCACAUAGGAGAGAUGAGCU _S U-3'
	AL4570		3'-GsU _{OMe} CGU _{OMe} GU _{OMe} AU _{OMe} CCUCUCUAC _F UCGAA-5'
AL-DP-4197	AL4556	ND	5'-GC _{OMe} AC _{OMe} AU _{OMe} AGGAGAGAU _{OMe} GAGCU _{OMe} SU-3'
	AL4568		3'-GsU _F CGU _F GU _F AU _F CCUCUCUAC _F UCGAA-5'
AL-DP-4221	AL2780	+++	5'-GsC _{OMe} AC _{OMe} AU _{OMe} AGGAGAGAU _{OMe} GAGCU _{OMe} SU-3'
	AL2782		3'-GsU _F CGU _F GU _F AU _F CCUCUCUAC _F UCGAsA-5'
"Atugen Design" based on single overhang			
AL-DP-4195	AL4571	+	5'-GcAcAuAgGaGaUgAgCusU-3'

	AL4572	ˆ3-gsUcGuGuAuCcUcUaCuCgAa-ˆ5
d	deoxynucleotide	
OMe	2'O-Methyl	
F	2'Flouro	
s	phosphorothioate linkage	
5	<u>N</u>	Mismatches in scrambled controls

Table 5. *In vitro* efficacy of siRNAs in HeLa cells

siRNA	Unmodified parent	Strand #	Efficacy	5'-sense strand- 3' 3'- antisense strand-5'
AL-DP-4374	AL-DP-4055	AL2732 AL2740	+++	5' CsAAGUGGUCCCAGGCUGCATsT 3' 3' TsTGUUCACCAGGGUCCGACGsU 5'
AL-DP-4375	AL-DP-4015	AL2728 AL2730	+++	5' GsGACAUCUUCAGGAGUACTsT 3' 3' TsTCCUGUAGAAGGUCCUCAUsG 5'
AL-DP-4379	AL-DP-4088	AL2963 AL2964	+++	5' C _{OMe} C _{OMe} AAGU _{OMe} GGU _{OMe} C _{OMe} C _{OMe} C _{OMe} AGGC _{OMe} U _{OMe} GC _{OMe} TsT 3' 3' TsTGGU _F U _F C _F AC _F C _F AGGGU _F C _F C _F GAC _F G 5'
AL-DP-4380	AL-DP-4014	AL2966 AL2967	+++	5' GC _{OMe} GGAU _{OMe} C _{OMe} AAAC _{OMe} C _{OMe} U _{OMe} C _{OMe} AC _{OMe} C _{OMe} AATsT 3' 3' 'TsTC _F GC _F C _F U _F AGU _F U _F GGAGU _F GGU _F U _F 5'
AL-DP-4219	AL-DP-4004	AL2712 AL2720	+++	5' GsUGAAUGCAGACCAAAGAAAsG 3' 3' UsACACUUACGUCUGGUUUCUUUsC 5'
AL-DP-4140	AL-DP-4014	AL2281 AL2282	-	5' GsCsGGAACAAUCCUGACCAsAsTsT 3' 3' TsTCGCCUUGUAGGACUGGsUsU 3'

OMe 2'O-Methyl
F 2'Flouro
s phosphorothioate linkage
N Mismatches in scrambled controls

5

Table 6. Oligonucleotides with phosphorothioate, 2'-O-methyl, and 2'-fluoro modifications and *in vitro* efficacy against VEGF.

Parent AL-DP-# and ORF	AL-DP-#	AL-SQ #	Duplex Sequence and Modifications	<i>in vitro</i> Efficacy	Mass	Extinction Coefficient
4103 ORF 52		4034	CCACCAUGCCAAGUGGUCCdTdT	++		
		4132	dTdTGGUGGUACGGUUCACCCAGG			
	4222	2510	CsC _{OMe} sAC _{OMe} CA _{OMe} UG _{OMe} CC _{OMe} AA _{OMe} GU _{OMe} GG _{OMe} UC _{OMe} sCsdTsdT	-	6810.3	189.72
		2511	dTsdTsGsG _{OMe} UG _{OMe} GU _{OMe} AC _{OMe} GG _{OMe} UU _{OMe} CA _{OMe} CC _{OMe} AsG _{OMe} sG		6947.4	199.62
	4223	2540	C _{OMe} sCsA _{OMe} CC _{OMe} AU _{OMe} GC _{OMe} CA _{OMe} AG _{OMe} UG _{OMe} GU _{OMe} CsC _{OMe} sdTsdT	-	6824.3	189.72
		2541	dTsdTsG _{OMe} sGU _{OMe} GG _{OMe} UA _{OMe} CG _{OMe} GU _{OMe} UC _{OMe} AC _{OMe} CA _{OMe} sGsG _{OMe}		6961.4	199.62
	4224	2510	CsC _{OMe} sAC _{OMe} CA _{OMe} UG _{OMe} CC _{OMe} AA _{OMe} GU _{OMe} GG _{OMe} UC _{OMe} sCsdTsdT	+/-	6810.3	189.72
		2541	dTsdTsG _{OMe} sGU _{OMe} GG _{OMe} UA _{OMe} CG _{OMe} GU _{OMe} UC _{OMe} AC _{OMe} CA _{OMe} sGsG _{OMe}		6961.4	199.62
	4225	2540	C _{OMe} sCsA _{OMe} CC _{OMe} AU _{OMe} GC _{OMe} CA _{OMe} AG _{OMe} UG _{OMe} GU _{OMe} CsC _{OMe} sdTsdT	-	6824.3	189.72
		2511	dTsdTsGsG _{OMe} UG _{OMe} GU _{OMe} AC _{OMe} GG _{OMe} UU _{OMe} CA _{OMe} CC _{OMe} AsG _{OMe} sG		6947.4	199.62
	4226	2570	C _{OMe} sC _{OMe} AC _{OMe} CA _{OMe} AU _{OMe} GC _{OMe} CA _{OMe} AA _{OMe} GU _{OMe} GG _{OMe} UC _{OMe} dTsdT	-	6790.4	189.72
		2571	dTsdTGGU _{OMe} GGU _{OMe} AC _{OMe} GGU _{OMe} U _{OMe} CA _{OMe} AC _{OMe} AGsG		6885.4	199.62
	4227	2600	CsC _{OMe} ACC _{OMe} AU _{OMe} GCC _{OMe} AA _{OMe} GU _{OMe} GGUCCdTsdT	-	6706.2	189.72
		2601	dTsdTGGU _{OMe} GGU _{OMe} AC _{OMe} GGU _{OMe} UCAC _{OMe} CAGsG		6843.3	199.62
	4228	2570	C _{OMe} sC _{OMe} AC _{OMe} CA _{OMe} AU _{OMe} GC _{OMe} CA _{OMe} AA _{OMe} GU _{OMe} GGU _{OMe} C _{OMe} dTsdT	-	6790.4	189.72
		2631	dTsdTGGU _{OMe} GGU _{OMe} AC _{OMe} GGU _{OMe} U _{OMe} CA _{OMe} C _{OMe} AGsG		6789.1	199.62
	4229	2600	CsC _{OMe} ACC _{OMe} AU _{OMe} GCC _{OMe} AA _{OMe} GU _{OMe} GGUCCdTsdT	+	6706.2	189.72
		2661	dTsdTGGU _{OMe} GGU _{OMe} AC _{OMe} GGU _{OMe} UCAC _{OMe} CAGsG		6783.1	199.62
4088 ORF 60		4042	CCAAUGGUCCCGGCGUCdTdT	+++		
		4140	dTdTGGUUCACAGGGUCCGACG			

4230	2512	CsCom ₆ AAOm ₆ GUOm ₆ GGOm ₆ UCOm ₆ CCOm ₆ AGOm ₆ GCm ₆ UGOm ₆ SCsdTsdT	-	6866.3	190.35
	2513	cTsdTsGsGom ₆ UUm ₆ CAOm ₆ CCOm ₆ AGOm ₆ GGOm ₆ UCOm ₆ CGOm ₆ AsCom ₆ SG		6906.4	194.31
4231	2542	Com ₆ SCsAAOm ₆ AGOm ₆ UGOm ₆ GUOm ₆ CCOm ₆ CAOm ₆ GGOm ₆ CUOm ₆ GsCom ₆ sdTsdT	-	6880.4	190.35
	2543	cTsdTsGom ₆ GUOm ₆ UCOm ₆ ACOm ₆ CAOm ₆ GGOm ₆ GUOm ₆ CCOm ₆ GAOm ₆ SCsGom ₆		6920.4	194.31
4232	2512	CsCom ₆ SAOm ₆ GUOm ₆ GGOm ₆ UCOm ₆ CCOm ₆ AGOm ₆ GCm ₆ UGOm ₆ SCsdTsdT	-	6866.3	190.35
	2543	cTsdTsGom ₆ GUOm ₆ UCOm ₆ ACOm ₆ CAOm ₆ GGOm ₆ GUOm ₆ CCOm ₆ GAOm ₆ SCsGom ₆		6920.4	194.31
4233	2542	Com ₆ SCsAAOm ₆ AGOm ₆ UGOm ₆ GUOm ₆ CCOm ₆ CAOm ₆ GGOm ₆ CUOm ₆ GsCom ₆ sdTsdT	-	6880.4	190.35
	2513	cTsdTsGsGom ₆ UUm ₆ CAOm ₆ CCOm ₆ AGOm ₆ GGOm ₆ UCOm ₆ CGOm ₆ AsCom ₆ SG		6906.4	194.31
4234	2572	Com ₆ SCom ₆ AAAGUOm ₆ GGUOm ₆ Com ₆ Com ₆ AGGGCom ₆ UOm ₆ GCm ₆ dTsdT	-	6832.4	190.35
	2573	cTsdTGGUOm ₆ UOm ₆ Com ₆ ACOm ₆ Com ₆ AGGGUOm ₆ Com ₆ Com ₆ GACOm ₆ SG		6858.4	194.31
4235	2602	CsCom ₆ AAAGUOm ₆ GGUCCCm ₆ AGGCUOm ₆ GCdTsdt	+	6748.2	190.35
	2603	cTsdTGGUUCACOm ₆ CAGGGUOm ₆ CCGACOm ₆ SG		6788.2	194.31
4236	2572	Com ₆ SCom ₆ AAAGUOm ₆ GGUOm ₆ Com ₆ Com ₆ AGGGCom ₆ UOm ₆ GCm ₆ dTsdT	+++	6832.4	190.35
	2633	cTsdTGGUUF-CF-AC-CF-AGGGUUF-CF-GACOm ₆ SG		6750.1	194.31
4237	2602	CsCom ₆ AAAGUOm ₆ GGUCCCm ₆ AGGCUOm ₆ GCdTsdt	+++	6748.2	190.35
	2663	cTsdTGGUUFUCACFCAGGGUUFCCGACFSG		6740.1	194.31
4055 ORF 61	4043	CAAGUGGUCCCCAGGUCGAdTdT	+++		
	4141	cTdTGUUACACAGGGUCCCGACGU			
4358	2736	CAOm ₆ AGOm ₆ UGOm ₆ GUOm ₆ CCOm ₆ CAOm ₆ GGOm ₆ CUOm ₆ GCm ₆ AdTsdt	-		
	2744	cTsdTGUOm ₆ UCOm ₆ ACOm ₆ CAOm ₆ GGOm ₆ GUOm ₆ CCOm ₆ GAOm ₆ CGOm ₆ U			
4359	2737	Com ₆ AAOm ₆ GUOm ₆ GGOm ₆ UCOm ₆ CCOm ₆ AGOm ₆ GCm ₆ UGOm ₆ CAOm ₆ dTsdt	-		
	2745	cTsdTGom ₆ UUm ₆ CAOm ₆ CCOm ₆ AGOm ₆ GGOm ₆ UCOm ₆ CGOm ₆ ACOm ₆ GUOm ₆			
4360	2736	CAOm ₆ AGOm ₆ UGOm ₆ GUOm ₆ CCOm ₆ CAOm ₆ GGOm ₆ CUOm ₆ GCm ₆ AdTsdt	-		
	2745	cTsdTGom ₆ UUm ₆ CAOm ₆ CCOm ₆ AGOm ₆ GGOm ₆ UCOm ₆ CGOm ₆ ACOm ₆ GUOm ₆			

	4361	2737	CoMe ₂ AAoMeGU _{OMe} GG _{OMe} UC _{OMe} CC _{OMe} AG _{OMe} GC _{OMe} UG _{OMe} CA _{OMe} dTsdt	-		
		2744	dTsdtGU _{OMe} UC _{OMe} AC _{OMe} CA _{OMe} GG _{OMe} GU _{OMe} CC _{OMe} GA _{OMe} CG _{OMe} U			
	4362	2735	CoMe ₂ AAAGU _{OMe} GGU _{OMe} CoMe ₂ CoMe ₂ AGGCoMe ₂ U _{OMe} GC _{OMe} AdTsdt	-		
		2743	dTsdtGU _{OMe} U _{OMe} CoMe ₂ AC _{OMe} CoMe ₂ AGGGU _{OMe} CoMe ₂ CoMe ₂ GAC _{OMe} GU _{OMe}			
	4363	2734	CoMe ₂ AAGU _{OMe} GGUCCCoMe ₂ AGGCU _{OMe} GC _{OMe} AdTsdt	-		
		2742	dTsdtGU _{OMe} UCAC _{OMe} CAGGGU _{OMe} CCGAC _{OMe} GU _{OMe}			
	4364	2735	CoMe ₂ AAGU _{OMe} GGU _{OMe} CoMe ₂ CoMe ₂ AGGCoMe ₂ U _{OMe} GC _{OMe} AdTsdt	-?		
		2747	dTsdtGU _{OMe} U _{OMe} AC _{OMe} AC _{OMe} AGGGU _{OMe} CF _{OMe} GAC _{OMe} GU _{OMe}			
	4365	2734	CoMe ₂ AAGU _{OMe} GGUCCCoMe ₂ AGGCU _{OMe} GC _{OMe} AdTsdt	+++		
		2746	dTsdtGU _{OMe} UCAC _{OMe} CF _{OMe} CAGGGU _{OMe} CCGAC _{OMe} GU _{OMe}			
4019 ORF 102		4003	AGAAUCAUCACGAAGUGGUdTdt	++		
		4070	dTdTTUCUUAAGUAGUCUUCACCA			
	4238	2514	AsG _{OMe} SA _{OMe} AA _{OMe} UC _{OMe} AUCA _{OMe} CG _{OMe} AA _{OMe} GU _{OMe} GG _{OMe} sUsdtTsdt	-	6923.4	216.9
		2515	dTsdtTsUsCoMe ₂ UU _{OMe} AG _{OMe} UA _{OMe} GU _{OMe} GC _{OMe} UU _{OMe} CA _{OMe} CsCoMe ₂ SA		6774.2	191.16
	4239	2544	Ac _{OMe} sGsAc _{OMe} AA _{OMe} UC _{OMe} CA _{OMe} UC _{OMe} AC _{OMe} GA _{OMe} AG _{OMe} UG _{OMe} GsU _{OMe} sdtTsdt	-	6937.4	216.9
		2545	dTsdtTsUsCoMe ₂ CU _{OMe} UA _{OMe} GU _{OMe} AG _{OMe} UG _{OMe} CU _{OMe} UC _{OMe} AC _{OMe} CsAc _{OMe}		6788.3	191.16
	4240	2514	AsG _{OMe} SA _{OMe} AA _{OMe} UC _{OMe} AUCA _{OMe} CG _{OMe} AA _{OMe} GU _{OMe} GG _{OMe} sUsdtTsdt	-	6923.4	216.9
		2545	dTsdtTsUsCoMe ₂ CU _{OMe} UA _{OMe} GU _{OMe} AG _{OMe} UG _{OMe} CU _{OMe} UC _{OMe} AC _{OMe} CsAc _{OMe}		6788.3	191.16
	4241	2544	Ac _{OMe} sGsAc _{OMe} AA _{OMe} UC _{OMe} CA _{OMe} UC _{OMe} AC _{OMe} GA _{OMe} AG _{OMe} UG _{OMe} GsU _{OMe} sdtTsdt	-	6937.4	216.9
		2515	dTsdtTsUsCoMe ₂ CU _{OMe} UA _{OMe} GU _{OMe} AG _{OMe} UG _{OMe} CU _{OMe} UC _{OMe} AC _{OMe} CsAc _{OMe} SA		6774.2	191.16
	4242	2574	Ac _{OMe} sGAAU _{OMe} CoMe ₂ CoMe ₂ AC _{OMe} CoMe ₂ GAAGU _{OMe} GGU _{OMe} dTsdt	-	6847.4	216.9
		2575	dTsdtTU _{OMe} CoMe ₂ CoMe ₂ U _{OMe} AGU _{OMe} AGU _{OMe} GC _{OMe} U _{OMe} CoMe ₂ CoMe ₂ SA		6788.3	191.16
	4243	2604	AsGAAU _{OMe} CoMe ₂ CoMe ₂ ACGAAGU _{OMe} GGUdTdt	-	6791.2	216.9
		2605	dTsdtTUCUUAAGU _{OMe} AGU _{OMe} GCUUCAC _{OMe} CsA		6842.1	191.16

	4244	2574 2635	A _{OMe} sGAAU _{OMe} C _{OMe} AU _{OMe} C _{OMe} AC _{OMe} GAAGU _{OMe} GGU _{OMe} dTsdT cTsdTU _F Cr _F U _F AGU _F AGU _F GC _{OMe} U _F U _F Cr _F AC _F Cr _F SA	+	6847.4 6624.0	216.9 191.16
	4245	2604 2665	AsGAAU _{OMe} AUC _{OMe} ACGAAGU _{OMe} GGUdTsdT cTsdTUCUUAAGU _F AGU _F GCUCACAC _F CsA	++	6791.2 6606.0	216.9 191.16
4111 ORF 129		4007 4074	UGGAUGUCUAUCAGCGCAGdTdT cTdTACCUCAGAUAGUCGCGUC	+++		
	4246	2516 2517	UsG _{OMe} sGA _{OMe} UG _{OMe} UC _{OMe} UA _{OMe} UC _{OMe} AG _{OMe} CG _{OMe} CA _{OMe} sGsdTsdT cTsdTsAsC _{OMe} CU _{OMe} AC _{OMe} AG _{OMe} AU _{OMe} AG _{OMe} UC _{OMe} GC _{OMe} GsU _{OMe} sC	-	6892.3 6835.3	200.34 198.36
	4247	2546 2547	U _{OMe} sGsG _{OMe} AU _{OMe} GU _{OMe} CU _{OMe} AU _{OMe} CA _{OMe} GC _{OMe} AsG _{OMe} sdtTsdT dTsdTsA _{OMe} sCC _{OMe} UA _{OMe} CA _{OMe} GA _{OMe} UA _{OMe} GU _{OMe} CG _{OMe} sUsC _{OMe}	-	6906.4 6849.4	200.34 198.36
	4248	2516 2547	UsG _{OMe} sGA _{OMe} UG _{OMe} UC _{OMe} UA _{OMe} UC _{OMe} AG _{OMe} CG _{OMe} CA _{OMe} sGsdTsdT dTsdTsA _{OMe} sCC _{OMe} UA _{OMe} CA _{OMe} GA _{OMe} UA _{OMe} GU _{OMe} CG _{OMe} sUsC _{OMe}	-	6892.3 6849.4	200.34 198.36
	4249	2546 2517	U _{OMe} sGsG _{OMe} AU _{OMe} GU _{OMe} CU _{OMe} AU _{OMe} CA _{OMe} GC _{OMe} AsG _{OMe} sdtTsdT dTsdTsAsC _{OMe} CU _{OMe} AC _{OMe} AG _{OMe} AU _{OMe} AG _{OMe} UC _{OMe} GC _{OMe} GsU _{OMe} sC	-	6906.4 6835.3	200.34 198.36
	4250	2576 2577	U _{OMe} sGGAU _{OMe} GUC _{OMe} U _{OMe} AU _{OMe} CA _{OMe} AGC _{OMe} GC _{OMe} A _{OMe} GdTsdT dTsdTAC _{OMe} C _{OMe} U _{OMe} AC _{OMe} AGAU _{OMe} AGU _{OMe} C _{OMe} GC _{OMe} sUsC _{OMe}	-	6844.3 6801.4	200.34 198.36
	4251	2606 2607	UsGGAU _{OMe} GUCU _{OMe} AU _{OMe} AGC _{OMe} AGC _{OMe} AGdTsdT dTsdTAC _{OMe} CUAC _{OMe} AGAU _{OMe} AGU _{OMe} CGCGU _{OMe} sC	-	6788.2 6731.2	200.34 198.36
	4252	2576 2637	U _{OMe} sGGAU _{OMe} GUC _{OMe} U _{OMe} AU _{OMe} CA _{OMe} AGC _{OMe} GC _{OMe} A _{OMe} GdTsdT dTsdTAC _F Cr _F U _F AC _F AGAU _{OMe} AGU _F Cr _F Cr _F GU _F sC _F	+	6844.3 6681.1	200.34 198.36
	4253	2606 2667	UsGGAU _{OMe} GUCU _{OMe} AU _{OMe} AGC _{OMe} AGC _{OMe} AGdTsdT dTsdTAC _F CUAC _F AGAU _F AGU _F CGCGU _F sC	+++	6788.2 6671.1	200.34 198.36
4028 ORF 149	++	4014 4081	UACUGCCAUCCAAUCGAGdTdT dTdT AUGACGGUAGGUUAGCUCU			

	4254	2518	UsA _{OMe} sCU _{OMe} GC _{OMe} CA _{OMe} UC _{OMe} CA _{OMe} AU _{OMe} CG _{OMe} AG _{OMe} sAsdTsdT	No data	6819.3	201.69
		2519	dTsdTsAsU _{OMe} GA _{OMe} CG _{OMe} GU _{OMe} AG _{OMe} GU _{OMe} UA _{OMe} GC _{OMe} sUsC _{OMe} sU		6893.3	201.69
	4255	2548	U _{OMe} sAsC _{OMe} UG _{OMe} CC _{OMe} AU _{OMe} CC _{OMe} AA _{OMe} UC _{OMe} GA _{OMe} sAsdTsdT	No data	6833.4	201.69
		2549	dTsdTsA _{OMe} sU _{OMe} GC _{OMe} AC _{OMe} GG _{OMe} UA _{OMe} GG _{OMe} UU _{OMe} AG _{OMe} CU _{OMe} sCsU _{OMe}		6907.4	201.69
	4256	2518	UsA _{OMe} sCU _{OMe} GC _{OMe} CA _{OMe} UC _{OMe} CA _{OMe} AU _{OMe} CG _{OMe} AG _{OMe} sAsdTsdT	No data	6819.3	201.69
		2549	dTsdTsA _{OMe} sU _{OMe} GC _{OMe} AC _{OMe} GG _{OMe} UA _{OMe} GG _{OMe} UU _{OMe} AG _{OMe} CU _{OMe} sCsU _{OMe}		6907.4	201.69
	4257	2548	U _{OMe} sAsC _{OMe} UG _{OMe} CC _{OMe} AU _{OMe} CC _{OMe} AA _{OMe} UC _{OMe} GA _{OMe} sAsdTsdT	No data	6833.4	201.69
		2519	dTsdTsAsU _{OMe} GA _{OMe} CG _{OMe} GU _{OMe} AG _{OMe} GU _{OMe} UA _{OMe} GC _{OMe} sUsC _{OMe} sU		6893.3	201.69
	4258	2578	U _{OMe} sAC _{OMe} U _{OMe} GC _{OMe} C _{OMe} AU _{OMe} C _{OMe} AAU _{OMe} C _{OMe} GA _{OMe} sAsdTsdT	-	6785.4	201.69
		2579	dTsdTAU _{OMe} GA _{OMe} CG _{OMe} GU _{OMe} AG _{OMe} GU _{OMe} UA _{OMe} GC _{OMe} sUsC _{OMe} sU		6845.3	201.69
	4259	2608	UsACU _{OMe} GCC _{OMe} AUCC _{OMe} AAUCGAGAdTsdT	++	6701.2	201.69
		2609	dTsdTAU _{OMe} GA _{OMe} CG _{OMe} GU _{OMe} AG _{OMe} GU _{OMe} UA _{OMe} GC _{OMe} sUsC _{OMe} sU		6775.2	201.69
	4260	2578	U _{OMe} sAC _{OMe} U _{OMe} GC _{OMe} C _{OMe} AU _{OMe} C _{OMe} AAU _{OMe} C _{OMe} GA _{OMe} sAsdTsdT	+	6785.4	201.69
		2639	dTsdTAU _{OMe} GA _{OMe} CG _{OMe} GU _{OMe} AG _{OMe} GU _{OMe} UA _{OMe} GC _{OMe} sUsC _{OMe} sU		6721.1	201.69
	4261	2608	UsACU _{OMe} GCC _{OMe} AUCC _{OMe} AAUCGAGAdTsdT	+	6701.2	201.69
		2669	dTsdTAU _{OMe} GA _{OMe} CG _{OMe} GU _{OMe} AG _{OMe} GU _{OMe} UA _{OMe} GC _{OMe} sUsC _{OMe} sU		6727.1	201.69
4060		4061	CCCUGGUGGACAUCCUCCAdTdT	+++		
ORF 168		4159	dTdTGGGACCACCUAGAGAGG			
	4262	2520	CsC _{OMe} sCU _{OMe} GG _{OMe} UG _{OMe} GA _{OMe} CA _{OMe} UC _{OMe} CC _{OMe} sAsdTsdT	-	6788.3	185.13
		2521	dTsdTsGsG _{OMe} GA _{OMe} CC _{OMe} AC _{OMe} CU _{OMe} GU _{OMe} AG _{OMe} AA _{OMe} GA _{OMe} sUsC _{OMe} sU		6954.4	208.89
	4263	2550	C _{OMe} sCsC _{OMe} UG _{OMe} GU _{OMe} GG _{OMe} AC _{OMe} AU _{OMe} CU _{OMe} UC _{OMe} CsA _{OMe} sAsdTsdT	-	6802.3	185.13
		2551	dTsdTsG _{OMe} sGG _{OMe} AC _{OMe} CA _{OMe} CC _{OMe} UG _{OMe} UA _{OMe} GA _{OMe} AG _{OMe} sGsU _{OMe}		6968.5	208.89
	4264	2520	CsC _{OMe} sCU _{OMe} GG _{OMe} UG _{OMe} GA _{OMe} CA _{OMe} UC _{OMe} CC _{OMe} sAsdTsdT	-	6788.3	185.13
		2551	dTsdTsG _{OMe} sGG _{OMe} AC _{OMe} CA _{OMe} CC _{OMe} UG _{OMe} UA _{OMe} GA _{OMe} AG _{OMe} sGsU _{OMe}		6968.5	208.89

	4265	2550	C _{OMe} sCsC _{OMe} UG _{OMe} GU _{OMe} GG _{OMe} AC _{OMe} AU _{OMe} CU _{OMe} UC _{OMe} CsA _{OMe} sdtTsdT	-	6802.3	185.13
		2521	dTsdTsGsG _{OMe} GA _{OMe} CC _{OMe} AC _{OMe} CU _{OMe} GU _{OMe} AG _{OMe} AA _{OMe} GsG _{OMe} sU		6954.4	208.89
	4266	2580	C _{OMe} sC _{OMe} C _{OMe} U _{OMe} GGU _{OMe} GGAC _{OMe} AU _{OMe} C _{OMe} U _{OMe} U _{OMe} C _{OMe} CoMeAdTsdT	-	6782.3	185.13
		2581	dTsdTGGGAC _{OMe} C _{OMe} AC _{OMe} C _{OMe} U _{OMe} GU _{OMe} AGAAAGGsU _{OMe}		6878.4	208.89
	4267	2610	CsCCU _{OMe} GGU _{OMe} GGAC _{OMe} AUCU _{OMe} UCC _{OMe} AdTsdT	+	6670.1	185.13
		2611	dTsdTGGGAC _{OMe} CAC _{OMe} CUGU _{OMe} AGAAAGGsU _{OMe}		6836.3	208.89
	4268	2580	C _{OMe} sC _{OMe} C _{OMe} U _{OMe} GGU _{OMe} GGAC _{OMe} AU _{OMe} C _{OMe} U _{OMe} U _{OMe} C _{OMe} CoMeAdTsdT	++	6782.3	185.13
		2641	dTsdTGGGAC _{OMe} C _{OMe} AC _{OMe} C _{OMe} U _{OMe} GU _{OMe} AGAAAGGsU _{OMe}		6778.2	208.89
	4269	2610	CsCCU _{OMe} GGU _{OMe} GGAC _{OMe} AUCU _{OMe} UCC _{OMe} AdTsdT	+	6670.1	185.13
		2671	dTsdTGGGAC _{OMe} CAC _{OMe} CUGU _{OMe} AGAAAGGsU _{OMe}		6772.2	208.89
4015		4066	GGACAUCU _{OMe} UCCAGGAGUACdTdT	+++		
ORF 175		4164	dTdTCCUGUAGAGGUCCUCAUG			
	4270	2522	GsG _{OMe} sAC _{OMe} AU _{OMe} CU _{OMe} UC _{OMe} CA _{OMe} GG _{OMe} AG _{OMe} UA _{OMe} sCsdTsdT	-	6875.4	202.32
		2523	dTsdTsCsC _{OMe} UG _{OMe} UA _{OMe} GA _{OMe} AG _{OMe} GU _{OMe} CC _{OMe} UC _{OMe} AsU _{OMe} sG		6852.3	196.38
	4271	2552	G _{OMe} sGsA _{OMe} CA _{OMe} UC _{OMe} UU _{OMe} CC _{OMe} AG _{OMe} GA _{OMe} GU _{OMe} AsC _{OMe} sdtTsdT	-	6889.4	202.32
		2553	dTsdTsC _{OMe} sC _{OMe} CU _{OMe} GU _{OMe} AG _{OMe} AA _{OMe} GG _{OMe} UC _{OMe} CU _{OMe} CA _{OMe} sUsG _{OMe}		6866.3	196.38
	4272	2522	GsG _{OMe} sAC _{OMe} AU _{OMe} CU _{OMe} UC _{OMe} CA _{OMe} GG _{OMe} AG _{OMe} UA _{OMe} sCsdTsdT	-	6875.4	202.32
		2553	dTsdTsC _{OMe} sC _{OMe} CU _{OMe} GU _{OMe} AG _{OMe} AA _{OMe} GG _{OMe} UC _{OMe} CU _{OMe} CA _{OMe} sUsG _{OMe}		6866.3	196.38
	4273	2552	G _{OMe} sGsA _{OMe} CA _{OMe} UC _{OMe} UU _{OMe} CC _{OMe} AG _{OMe} GA _{OMe} GU _{OMe} AsC _{OMe} sdtTsdT	-	6889.4	202.32
		2523	dTsdTsCsC _{OMe} UG _{OMe} UA _{OMe} GA _{OMe} AG _{OMe} GU _{OMe} CC _{OMe} UC _{OMe} AsU _{OMe} sG		6852.3	196.38
	4274	2582	G _{OMe} sGAC _{OMe} AU _{OMe} C _{OMe} U _{OMe} C _{OMe} CoMeAGGAGU _{OMe} AC _{OMe} dTsdT	-	6827.4	202.32
		2583	dTsdTC _{OMe} C _{OMe} U _{OMe} GU _{OMe} AGAAAGGU _{OMe} C _{OMe} CoMeU _{OMe} C _{OMe} AU _{OMe} sG		6818.3	196.38
	4275	2612	GsGAC _{OMe} AUCU _{OMe} UCC _{OMe} AGGAGU _{OMe} ACdTsdT	-	6743.2	202.32
		2613	dTsdTCCUGU _{OMe} AGAAAGGU _{OMe} CCUCAU _{OMe} sG		6720.1	196.38

	4276	2582	G _{OMe} S _{GAC} _{OMe} AU _{OMe} C _{OMe} U _{OMe} U _{OMe} C _{OMe} C _{OMe} AGGAGU _{OMe} AG _{OMe} dTsdtT	-	6827.4	202.32
		2643	dTsdtTC _F C _F U _F GU _F AGAAGGU _F C _F C _F U _F C _F AU _F sG		6698.0	196.38
	4277	2612	GsGAC _{OMe} AUCUUC _{OMe} AGGAGU _{OMe} ACdTsdtT	+++	6743.2	202.32
		2673	dTsdtTCCUGU _F AGAAGGU _F CCUCAU _F sG		6684.0	196.38
4032 ORF 191		4025	UACCCUGAUGAGAU _{OMe} CGAGU _{OMe} dTdT	+++		
		4092	cTdTAUGGGACUACUCUAGCUCU			
	4278	2524	UsA _{OMe} sCC _{OMe} CU _{OMe} GA _{OMe} U _{OMe} AG _{OMe} AU _{OMe} CG _{OMe} AG _{OMe} sUsdTsdT	+	6876.3	203.67
		2525	dTsdtTsAsU _{OMe} GG _{OMe} GA _{OMe} CU _{OMe} AC _{OMe} UC _{OMe} UA _{OMe} GC _{OMe} UsC _{OMe} sA		6836.3	199.71
	4279	2554	U _{OMe} sAsC _{OMe} CC _{OMe} UG _{OMe} AU _{OMe} GA _{OMe} GA _{OMe} UC _{OMe} GA _{OMe} GsU _{OMe} sdTsdT	+	6890.4	203.67
		2555	dTsdtTsA _{OMe} sUG _{OMe} GG _{OMe} AC _{OMe} UA _{OMe} CU _{OMe} AG _{OMe} CU _{OMe} sCsA _{OMe}		6850.3	199.71
	4280	2524	UsA _{OMe} sCC _{OMe} CU _{OMe} GA _{OMe} U _{OMe} AG _{OMe} AU _{OMe} CG _{OMe} AG _{OMe} sUsdTsdT	++	6876.3	203.67
		2555	dTsdtTsA _{OMe} sUG _{OMe} GG _{OMe} AC _{OMe} UA _{OMe} CU _{OMe} AG _{OMe} CU _{OMe} sCsA _{OMe}		6850.3	199.71
	4281	2554	U _{OMe} sAsC _{OMe} CC _{OMe} UG _{OMe} AU _{OMe} GA _{OMe} GA _{OMe} UC _{OMe} GA _{OMe} GsU _{OMe} sdTsdT	-	6890.4	203.67
		2525	dTsdtTsAsU _{OMe} GG _{OMe} GA _{OMe} CU _{OMe} AC _{OMe} UC _{OMe} UA _{OMe} GC _{OMe} UsC _{OMe} sA		6836.3	199.71
	4282	2584	U _{OMe} sAC _{OMe} C _{OMe} C _{OMe} U _{OMe} GAU _{OMe} GAU _{OMe} C _{OMe} GAGU _{OMe} dTsdtT	-	6828.3	203.67
		2585	dTsdtTAU _{OMe} GGGACU _{OMe} ACUCU _{OMe} AGCUC _{OMe} sA		6802.3	199.71
	4283	2614	UsACCCU _{OMe} GAU _{OMe} GAGAU _{OMe} CGAGU _{OMe} dTsdtT	+++	6744.2	203.67
		2615	dTsdtTAU _{OMe} GGGAC _{OMe} UAC _{OMe} UCUAGCUCsA		6704.1	199.71
	4284	2584	U _{OMe} sAC _{OMe} C _{OMe} C _{OMe} U _{OMe} GAU _{OMe} GAGAU _{OMe} C _{OMe} GAGU _{OMe} dTsdtT	+++	6828.3	203.67
		2645	dTsdtTAU _F GGGAC _F U _F AC _F U _F C _F U _F AGC _F U _F C _F sA		6682.0	199.71
	4285	2614	UsACCCU _{OMe} GAU _{OMe} GAGAU _{OMe} CGAGU _{OMe} dTsdtT	++	6744.2	203.67
		2675	dTsdtTAU _F GGGAC _F UAC _F UCUAGCUCsA		6668.0	199.71
4033 ORF 305		4026	ACCAUGCAGAUUAUGCGGAdTdT	++		
		4093	cTdTUGGUAGCUCUAAUACGCCU			

	4286	2526	As _{COMe} sCA _{OMe} UG _{OMe} CA _{OMe} GA _{OMe} JU _{OMe} AU _{OMe} GU _{OMe} GC _{OMe} GG _{OMe} sAsdTsdT	++	6899.4	209.61
		2527	dTsdTsUsG _{OMe} GU _{OMe} AC _{OMe} GU _{OMe} CU _{OMe} AA _{OMe} UA _{OMe} CG _{OMe} CsC _{OMe} sU		6813.3	193.77
	4287	2556	AO _{Me} sCsC _{OMe} AU _{OMe} GC _{OMe} AG _{OMe} AU _{OMe} UA _{OMe} UG _{OMe} CG _{OMe} GsA _{OMe} sdTsdT	+	6913.4	209.61
		2557	dTsdTsU _{OMe} sGG _{OMe} UA _{OMe} CG _{OMe} UC _{OMe} UA _{OMe} AU _{OMe} AC _{OMe} GC _{OMe} sCsU _{OMe}		6827.3	193.77
	4288	2526	As _{COMe} sCA _{OMe} UG _{OMe} CA _{OMe} GA _{OMe} JU _{OMe} AU _{OMe} GU _{OMe} GC _{OMe} GG _{OMe} sAsdTsdT	-	6899.4	209.61
		2557	dTsdTsU _{OMe} sGG _{OMe} UA _{OMe} CG _{OMe} UC _{OMe} UA _{OMe} AU _{OMe} AC _{OMe} GC _{OMe} sCsU _{OMe}		6827.3	193.77
	4289	2556	AO _{Me} sCsC _{OMe} AU _{OMe} GC _{OMe} AG _{OMe} AU _{OMe} UA _{OMe} UG _{OMe} CG _{OMe} GsA _{OMe} sdTsdT	-	6913.4	209.61
		2527	dTsdTsUsG _{OMe} GU _{OMe} AC _{OMe} GU _{OMe} CU _{OMe} AA _{OMe} UA _{OMe} CG _{OMe} CsC _{OMe} sU		6813.3	193.77
	4290	2586	AO _{Me} sCC _{OMe} AU _{OMe} GC _{OMe} AGAU _{OMe} U _{OMe} AU _{OMe} GU _{OMe} GGAdTsdT	-	6837.4	209.61
		2587	dTsdTU _{OMe} GGU _{OMe} AC _{OMe} GU _{OMe} CU _{OMe} AA _{OMe} UA _{OMe} CG _{OMe} CsC _{OMe} sU _{OMe}		6793.3	193.77
	4291	2616	AsCC _{OMe} AU _{OMe} GC _{OMe} AGAU _{OMe} U _{OMe} GU _{OMe} GGGAdTsdT	-	6795.3	209.61
		2617	dTsdTU _{OMe} GGU _{OMe} AC _{OMe} GU _{OMe} CU _{OMe} AA _{OMe} UA _{OMe} CG _{OMe} CsC _{OMe} sU		6709.2	193.77
	4292	2586	AO _{Me} sCC _{OMe} AU _{OMe} GC _{OMe} AGAU _{OMe} U _{OMe} AU _{OMe} GU _{OMe} GGAdTsdT	+++	6837.4	209.61
		2647	dTsdTU _{OMe} GGU _{OMe} AC _{OMe} GU _{OMe} CU _{OMe} AA _{OMe} UA _{OMe} CG _{OMe} CsC _{OMe} sU		6645	193.77
	4293	2616	AsCC _{OMe} AU _{OMe} GC _{OMe} AGAU _{OMe} U _{OMe} GU _{OMe} GGGAdTsdT	+++	6795.3	209.61
		2677	dTsdTU _{OMe} GGU _{OMe} AC _{OMe} GU _{OMe} CU _{OMe} AA _{OMe} UA _{OMe} CG _{OMe} CsC _{OMe} sU		6649.0	193.77
4014		4112	GCGGAUCAAACCCUACCAAdTdt	+++		
ORF 319		4180	dTdtCGCCUAGUUUGGAGUGGUU			
	4294	2528	GsC _{OMe} sGG _{OMe} AU _{OMe} CA _{OMe} AA _{OMe} CC _{OMe} UC _{OMe} CA _{OMe} sAsdTsdT	+	6841.4	206.28
		2529	dTsdTsCsC _{OMe} CC _{OMe} UA _{OMe} GU _{OMe} UU _{OMe} GG _{OMe} AG _{OMe} UG _{OMe} GsU _{OMe} sU		6886.3	192.42
	4295	2558	GO _{Me} sCsG _{OMe} GA _{OMe} UC _{OMe} AA _{OMe} CC _{OMe} CU _{OMe} CA _{OMe} CC _{OMe} AsA _{OMe} sdTsdT	-	6855.4	206.28
		2559	dTsdTsC _{OMe} sGG _{OMe} CU _{OMe} AG _{OMe} JU _{OMe} UG _{OMe} GA _{OMe} GU _{OMe} GG _{OMe} sUsU _{OMe}		6900.3	192.42
	4296	2528	GsC _{OMe} sGG _{OMe} AU _{OMe} CA _{OMe} AA _{OMe} CC _{OMe} UC _{OMe} CA _{OMe} sAsdTsdT	-	6841.4	206.28
		2559	dTsdTsC _{OMe} sGG _{OMe} CU _{OMe} AG _{OMe} JU _{OMe} UG _{OMe} GA _{OMe} GU _{OMe} GG _{OMe} sUsU _{OMe}		6900.3	192.42

	4297	2558	G _{OMe} S _{Cs} G _{OMe} GA _{OMe} UC _{OMe} AA _{OMe} AC _{OMe} CU _{OMe} CA _{OMe} CC _{OMe} As _{OMe} sdtTsdT	-	6855.4	206.28
		2529	dTsdTsCsG _{OMe} CC _{OMe} UA _{OMe} GU _{OMe} UU _{OMe} GG _{OMe} AG _{OMe} UG _{OMe} GsU _{OMe} sU		6886.3	192.42
	4298	2588	G _{OMe} S _{Cs} G _{OMe} GAU _{OMe} C _{OMe} AAAC _{OMe} C _{OMe} U _{OMe} CoMeAC _{OMe} CoMeAAAdTsdT	-	6793.4	206.28
		2589	dTsdTC _{OMe} GC _{OMe} C _{OMe} U _{OMe} AGU _{OMe} U _{OMe} U _{OMe} GGAGU _{OMe} GGU _{OMe} sU _{OMe}		6852.3	192.42
	4299	2618	GsCGGAUC _{OMe} AAACCUC _{OMe} ACC _{OMe} AAAdTsdT	-	6709.2	206.28
		2619	dTsdTCGCCUAGU _{OMe} UU _{OMe} GGAGU _{OMe} GGU _{OMe} sU		6754.1	192.42
	4300	2588	G _{OMe} S _{Cs} G _{OMe} GAU _{OMe} C _{OMe} AAAC _{OMe} C _{OMe} U _{OMe} CoMeAC _{OMe} CoMeAAAdTsdT	+	6793.4	206.28
		2649	dTsdTC _{OMe} GC _{OMe} C _{OMe} U _{OMe} AGU _{OMe} U _{OMe} U _{OMe} GGAGU _{OMe} GGU _{OMe} sU _{OMe}		6716.0	192.42
	4301	2618	GsCGGAUC _{OMe} AAACCUC _{OMe} ACC _{OMe} AAAdTsdT	+++	6709.2	206.28
		2679	dTsdTCGCCUAGU _{OMe} UU _{OMe} GGAGU _{OMe} GGU _{OMe} sU		6718.0	192.42
4123		4362	ACCUCACCAAGGCCAGCACdTdT	++		
ORF 330		4363	dTdTUGGAGUGGUUCCGGUCGUG			
	4302	2530	As _{OMe} sCU _{OMe} CA _{OMe} CC _{OMe} AA _{OMe} GG _{OMe} CC _{OMe} AG _{OMe} CA _{OMe} sCsdTsdT	+	6816.4	197.64
		2531	dTsdTsUsG _{OMe} GA _{OMe} GU _{OMe} GG _{OMe} UU _{OMe} CC _{OMe} GG _{OMe} UC _{OMe} GsU _{OMe} sG		6941.3	191.7
	4303	2560	A _{OMe} sCsC _{OMe} UC _{OMe} AC _{OMe} CA _{OMe} AG _{OMe} GC _{OMe} CA _{OMe} sCsdTsdT	-	6830.4	197.64
		2561	dTsdTsU _{OMe} sGG _{OMe} AG _{OMe} UG _{OMe} GU _{OMe} UC _{OMe} CG _{OMe} sUsG _{OMe}		6955.4	191.7
	4304	2530	As _{OMe} sCU _{OMe} CA _{OMe} CC _{OMe} AA _{OMe} GG _{OMe} CC _{OMe} AG _{OMe} CA _{OMe} sCsdTsdT	-	6816.4	197.64
		2561	dTsdTsU _{OMe} sGG _{OMe} AG _{OMe} UG _{OMe} GU _{OMe} UC _{OMe} CG _{OMe} sUsG _{OMe}		6955.4	191.7
	4305	2560	A _{OMe} sCsC _{OMe} UC _{OMe} AC _{OMe} CA _{OMe} AG _{OMe} GC _{OMe} CA _{OMe} sCsdTsdT	-	6830.4	197.64
		2531	dTsdTsUsG _{OMe} GA _{OMe} GU _{OMe} GG _{OMe} UU _{OMe} CC _{OMe} GG _{OMe} UC _{OMe} GsU _{OMe} sG		6941.3	191.7
	4306	2590	A _{OMe} sC _{OMe} C _{OMe} U _{OMe} CoMeAC _{OMe} CoMeAAGGCoMeCoMeAGC _{OMe} dTsdT	-	6782.4	197.64
		2591	dTsdTU _{OMe} GGAGU _{OMe} GGU _{OMe} U _{OMe} CoMeCoMeGGU _{OMe} CoMeGU _{OMe} sG _{OMe}		6893.3	191.7
	4307	2620	AsCCUC _{OMe} ACC _{OMe} AAGGCC _{OMe} AGC _{OMe} ACdTsdt	-	6698.2	197.64
		2621	dTsdTUGGAGU _{OMe} GGU _{OMe} UCCGGU _{OMe} CGU _{OMe} sG		6823.2	191.7

	4308	2590 2651	A _{OMe} S _{COMe} C _{OMe} U _{OMe} C _{OMe} AC _{OMe} C _{OMe} AAGGC _{OMe} C _{OMe} AGC _{OMe} AC _{OMe} dTsdt cTsdTU _F GGAGU _F GGU _F U _F C _F C _F GGU _F C _F GU _F S _{COMe}	-	6782.4 6785.1	197.64 191.7
	4309	2620 2681	AsCCU _{COMe} ACC _{COMe} AAGGCC _{COMe} AGC _{COMe} AcdTsdt cTsdTUGGAGU _F GGU _F UCCGGU _F CGU _F SG	+	6698.2 6775.1	197.64 191.7
4094so ORF 343		4326 4327	GCACAUAGGAGAGAUAGGCUU GUCCGUUAUCCUCUCUACUCGAA	+++		
	4310	2532 2533	GsC _{OMe} S _{COMe} AC _{COMe} AU _{OMe} AG _{OMe} GA _{OMe} GA _{OMe} U _{OMe} AG _{OMe} AG _{OMe} sCsU _{OMe} sU GsU _{OMe} sCsG _{OMe} UG _{OMe} UA _{OMe} UC _{OMe} CU _{OMe} CU _{OMe} AC _{OMe} UC _{OMe} GsA _{OMe} sA	-	7019.5 7487.6	222.12 206.91
	4311	2562 2563	G _{OMe} sCsA _{OMe} CA _{OMe} UA _{OMe} GG _{OMe} AG _{OMe} AG _{OMe} AU _{OMe} GA _{OMe} GsC _{OMe} sUsU _{OMe} C _{OMe} sGsU _{OMe} sGU _{OMe} AU _{OMe} CC _{OMe} UC _{OMe} UA _{OMe} CU _{OMe} CG _{OMe} sAsA _{OMe}	-	7033.5 7501.7	222.12 206.91
	4312	2532 2563	GsC _{OMe} S _{COMe} AC _{COMe} AU _{OMe} AG _{OMe} GA _{OMe} GA _{OMe} U _{OMe} AG _{OMe} AG _{OMe} sCsU _{OMe} sU C _{OMe} sGsU _{OMe} sGU _{OMe} AU _{OMe} CC _{OMe} UC _{OMe} UA _{OMe} CU _{OMe} CG _{OMe} sAsA _{OMe}	-	7019.5 7501.7	222.12 206.91
	4313	2562 2533	G _{OMe} sCsA _{OMe} CA _{OMe} UA _{OMe} GG _{OMe} AG _{OMe} AG _{OMe} AU _{OMe} GA _{OMe} GsC _{OMe} sUsU _{OMe} GsU _{OMe} sCsG _{OMe} UG _{OMe} UA _{OMe} UC _{OMe} CU _{OMe} CU _{OMe} AC _{OMe} UC _{OMe} GsA _{OMe} sA	-	7033.5 7487.6	222.12 206.91
	4314	2592 2593	GsC _{OMe} AC _{COMe} AU _{OMe} AGGAGAGAU _{OMe} GAGC _{OMe} U _{OMe} sU _{OMe} GsU _{OMe} C _{OMe} GU _{OMe} GU _{OMe} AU _{OMe} C _{OMe} C _{OMe} U _{OMe} C _{OMe} U _{OMe} AC _{OMe} U _{OMe} C _{OMe} GASA	+	6929.4 7495.8	222.12 206.91
	4315	2622 2623	GsC _{OMe} AC _{COMe} AU _{OMe} AGGAGAGAU _{OMe} GAGC _{OMe} U _{OMe} GsU _{OMe} CGU _{OMe} GU _{OMe} AU _{OMe} CCUCUCUAC _{COMe} UCGASA	+++	6887.3 7355.5	222.12 206.91
	4316	2592 2653	GsC _{OMe} AC _{COMe} AU _{OMe} AGGAGAGAU _{OMe} GAGC _{OMe} U _{OMe} sU _{OMe} GsU _{OMe} CGU _{OMe} GU _{OMe} AU _{OMe} C _{OMe} C _{OMe} U _{OMe} C _{OMe} U _{OMe} AC _{OMe} U _{OMe} C _{OMe} GASA	+++	6929.4 7283.3	222.12 206.91
	4317	2622 2683	GsC _{OMe} AC _{COMe} AU _{OMe} AGGAGAGAU _{OMe} GAGC _{OMe} U _{OMe} GsU _{OMe} CGU _{OMe} GU _{OMe} AU _{OMe} CCUCUCUAC _{COMe} UCGASA	+	6887.3 7291.3	222.12 206.91
4107do ORF 343		4117 4185	GCACAUAGGAGAGAUAGGdtdT dTTCGUUAUCCUCUCUACUCG	+++		
	4318	2534	GsC _{OMe} S _{COMe} AC _{COMe} AU _{OMe} AG _{OMe} GA _{OMe} GA _{OMe} U _{OMe} AG _{OMe} sCsTsdt	+	7001.5	222.12

		2535	dTsdTtCsG _{OMe} UG _{OMe} UA _{OMe} UC _{OMe} CU _{OMe} CU _{OMe} AC _{OMe} UsC _{OMe} sG		6726.2	176.58
	4319	2564	G _{OMe} sCsA _{OMe} CA _{OMe} UA _{OMe} GG _{OMe} AG _{OMe} AU _{OMe} GA _{OMe} GsC _{OMe} sdtTsdT	-	7015.5	222.12
		2565	dTsdTtCsC _{OMe} sSGU _{OMe} GU _{OMe} AU _{OMe} CC _{OMe} UC _{OMe} UA _{OMe} CU _{OMe} sCsG _{OMe}		6740.2	176.58
	4320	2534	GsC _{OMe} sAC _{OMe} AU _{OMe} AG _{OMe} GA _{OMe} GA _{OMe} UG _{OMe} AG _{OMe} sCsdTsdT	-	7001.5	222.12
		2565	dTsdTtCsC _{OMe} sSGU _{OMe} GU _{OMe} AU _{OMe} CC _{OMe} UC _{OMe} UA _{OMe} CU _{OMe} sCsG _{OMe}		6740.2	176.58
	4321	2564	G _{OMe} sCsA _{OMe} CA _{OMe} UA _{OMe} GG _{OMe} AG _{OMe} AU _{OMe} GA _{OMe} GsC _{OMe} sdtTsdT	-	7015.5	222.12
		2535	dTsdTtCsGsG _{OMe} UG _{OMe} UA _{OMe} UC _{OMe} CU _{OMe} AC _{OMe} UsC _{OMe} sG		6726.2	176.58
	4322	2594	G _{OMe} sC _{OMe} AC _{OMe} AU _{OMe} AGGAGAGAU _{OMe} GAGC _{OMe} dtTsdT	-	6897.4	222.12
		2595	dTsdTtC _{OMe} GU _{OMe} GU _{OMe} AU _{OMe} C _{OMe} U _{OMe} C _{OMe} U _{OMe} AC _{OMe} U _{OMe} C _{OMe} sG		6748.3	176.58
	4323	2624	GsC _{OMe} AC _{OMe} AU _{OMe} AGGAGAGAU _{OMe} GAGCdTsdT	+++	6883.3	222.12
		2625	dTsdTtCGU _{OMe} GU _{OMe} AU _{OMe} CCUCUCUAC _{OMe} UCsG		6608.0	176.58
	4324	2594	G _{OMe} sC _{OMe} AC _{OMe} AU _{OMe} AGGAGAGAU _{OMe} GAGC _{OMe} dtTsdT	+++	6897.4	222.12
		2655	dTsdTtC _{OMe} GU _{OMe} GU _{OMe} AC _{OMe} U _{OMe} AC _{OMe} U _{OMe} AC _{OMe} U _{OMe} AC _{OMe} sG		6579.9	176.58
	4325	2624	GsC _{OMe} AC _{OMe} AU _{OMe} AGGAGAGAU _{OMe} GAGCdTsdT	+++	6883.3	222.12
		2685	dTsdTtCGU _{OMe} GU _{OMe} AC _{OMe} U _{OMe} CCUCUCUAC _{OMe} UCsG		6559.9	176.58
4061		4119	CAUAGGAGAGAGAGCUUCdtT	+++		
ORF 346		4187	dtTtGUAUCCUCUCUACUCGAAG			
	4326	2536	CsA _{OMe} sUA _{OMe} GG _{OMe} AG _{OMe} AG _{OMe} GA _{OMe} GC _{OMe} UU _{OMe} sCsdTsdT	+	6939.4	213.57
		2537	dTsdTtGsU _{OMe} AU _{OMe} CC _{OMe} UC _{OMe} UA _{OMe} CU _{OMe} CG _{OMe} AsA _{OMe} sG		6773.2	189.81
	4327	2566	C _{OMe} sAsU _{OMe} AG _{OMe} GA _{OMe} GA _{OMe} GA _{OMe} UG _{OMe} AG _{OMe} CU _{OMe} UsC _{OMe} sdtTsdT	-	6953.4	213.57
		2567	dTsdTtGs _{OMe} sUA _{OMe} UC _{OMe} CU _{OMe} AC _{OMe} GA _{OMe} sAsG _{OMe}		6787.3	189.81
	4328	2536	CsA _{OMe} sUA _{OMe} GG _{OMe} AG _{OMe} AG _{OMe} AU _{OMe} GA _{OMe} GC _{OMe} UU _{OMe} sCsdTsdT	-	6939.4	213.57
		2567	dTsdTtGs _{OMe} sUA _{OMe} UC _{OMe} CU _{OMe} AC _{OMe} UC _{OMe} sAsG _{OMe}		6787.3	189.81
	4329	2566	C _{OMe} sAsU _{OMe} AG _{OMe} GA _{OMe} GA _{OMe} UG _{OMe} AG _{OMe} CU _{OMe} UsC _{OMe} sdtTsdT	-	6953.4	213.57

		2537	dTsdTsGsU _{OMe} AAU _{OMe} CC _{OMe} UC _{OMe} UA _{OMe} CU _{OMe} CG _{OMe} AsA _{OMe} SG		6773.2	189.81
4330		2596	C _{OMe} sAU _{OMe} AGGAGAGAU _{OMe} GAGC _{OMe} U _{OMe} U _{OMe} ComedTsdT	-	6863.4	213.57
		2597	dTsdTTGU _{OMe} AAU _{OMe} CC _{OMe} U _{OMe} Com _{OMe} U _{OMe} Com _{OMe} GAAsG		6767.3	189.81
4331		2626	CsAU _{OMe} AGGAGAGAU _{OMe} GAGCUUCdTsdT	++	6807.2	213.57
		2627	dTsdTGU _{OMe} AAU _{OMe} CCUCUCUAC _{OMe} UCGAAsG		6841.1	189.81
4332		2596	C _{OMe} sAU _{OMe} AGGAGAGAU _{OMe} GAGC _{OMe} U _{OMe} U _{OMe} ComedTsdT	++	6863.4	213.57
		2657	dTsdTTGU _{OMe} AAU _{OMe} CCUCUCUAC _{OMe} UCGAAsG		6623.0	189.81
4333		2626	CsAU _{OMe} AGGAGAGAU _{OMe} GAGCUUCdTsdT	+++	6807.2	213.57
		2687	dTsdTTGU _{OMe} AAU _{OMe} CCUCUCUAC _{OMe} UCGAAsG		6605.0	189.81
4092		4123	UGUGAAUUCAGACCAAGAdTdT	+++		
ORF 380		4191	dTdTACACUUACGUCUGGUUCU			
4334		2538	UsG _{OMe} sUG _{OMe} AA _{OMe} UG _{OMe} CA _{OMe} GA _{OMe} CC _{OMe} AA _{OMe} AG _{OMe} sAsdTsdT	+	6946.5	222.84
		2539	dTsdTsAsC _{OMe} AAU _{OMe} AA _{OMe} UG _{OMe} CA _{OMe} GA _{OMe} CC _{OMe} AA _{OMe} AG _{OMe} sAsdTsdT		6751.2	185.22
4335		2568	U _{OMe} sGsU _{OMe} GA _{OMe} AAU _{OMe} AA _{OMe} UG _{OMe} CA _{OMe} GA _{OMe} CC _{OMe} AA _{OMe} AG _{OMe} sAsdTsdT	-	6960.5	222.84
		2569	dTsdTsA _{OMe} sCA _{OMe} AAU _{OMe} AA _{OMe} UG _{OMe} CA _{OMe} GA _{OMe} CC _{OMe} AA _{OMe} AG _{OMe} sAsdTsdT		6765.2	185.22
4336		2538	UsG _{OMe} sUG _{OMe} AA _{OMe} UG _{OMe} CA _{OMe} GA _{OMe} CC _{OMe} AA _{OMe} AG _{OMe} sAsdTsdT	-	6946.5	222.84
		2569	dTsdTsA _{OMe} sCA _{OMe} AAU _{OMe} AA _{OMe} UG _{OMe} CA _{OMe} GA _{OMe} CC _{OMe} AA _{OMe} AG _{OMe} sAsdTsdT		6765.2	185.22
4337		2568	U _{OMe} sGsU _{OMe} GA _{OMe} AAU _{OMe} AA _{OMe} UG _{OMe} CA _{OMe} GA _{OMe} CC _{OMe} AA _{OMe} AG _{OMe} sAsdTsdT	+	6960.5	222.84
		2539	dTsdTsAsC _{OMe} AAU _{OMe} AA _{OMe} UG _{OMe} CA _{OMe} GA _{OMe} CC _{OMe} AA _{OMe} AG _{OMe} sAsdTsdT		6751.2	185.22
4338		2598	U _{OMe} sGU _{OMe} GAU _{OMe} GC _{OMe} AGAC _{OMe} Com _{OMe} AAAAGAdTsdT	-	6856.4	222.84
		2599	dTsdTACAC _{OMe} U _{OMe} U _{OMe} AA _{OMe} UG _{OMe} CA _{OMe} GA _{OMe} CC _{OMe} AA _{OMe} AG _{OMe} sAsdTsdT		6759.3	185.22
4339		2628	UsGU _{OMe} GAU _{OMe} GC _{OMe} AGAC _{OMe} Com _{OMe} AAAAGAdTsdT	-	6842.3	222.84
		2629	dTsdTAC _{OMe} U _{OMe} U _{OMe} AA _{OMe} UG _{OMe} CA _{OMe} GA _{OMe} CC _{OMe} AA _{OMe} AG _{OMe} sAsdTsdT		6647.1	185.22
4340		2598	U _{OMe} sGU _{OMe} GAU _{OMe} GC _{OMe} AGAC _{OMe} Com _{OMe} AAAAGAdTsdT	+++	6856.4	222.84

			2659	dTsdTAC _F AC _F U _F U _F AC _F GU _F C _F U _F GGU _F U _F U _F C _F SU _F			185.22
	4341		2628	UsGU _{OMe} GAU _{OMe} GC _{OMe} AGACC _{OMe} AAAAGAdTsdT	++	6586.9	222.84
			2689	dTsdTAC _F AC _F UUAC _F GU _F CUGGU _F UUCSU		6586.9	185.22
4004 so			4338	GUGAAUGCAGACCAAAAGAAAG	+++		
ORF 381			4339	UACACUUACGUCUGGUUUUUUC			
	4366		2716	GsU _{OMe} GA _{OMe} AU _{OMe} GC _{OMe} AG _{OMe} AC _{OMe} CA _{OMe} AA _{OMe} GA _{OMe} AA _{OMe} SG	+		
			2724	UsA _{OMe} CA _{OMe} CU _{OMe} UA _{OMe} CG _{OMe} UC _{OMe} UG _{OMe} GU _{OMe} UU _{OMe} CU _{OMe} UU _{OMe} SC			
	4367		2717	G _{OMe} sUG _{OMe} AA _{OMe} UG _{OMe} CA _{OMe} GA _{OMe} CC _{OMe} AA _{OMe} AG _{OMe} AA _{OMe} AsG _{OMe}	-		
			2725	U _{OMe} sAC _{OMe} AC _{OMe} UU _{OMe} AC _{OMe} GU _{OMe} CU _{OMe} GG _{OMe} UU _{OMe} UC _{OMe} UU _{OMe} USC _{OMe}	+		
	4368		2716	GsU _{OMe} GA _{OMe} AU _{OMe} GC _{OMe} AG _{OMe} AC _{OMe} CA _{OMe} AA _{OMe} GA _{OMe} AA _{OMe} SG			
			2725	U _{OMe} sAC _{OMe} AC _{OMe} UU _{OMe} AC _{OMe} GU _{OMe} CU _{OMe} GG _{OMe} UU _{OMe} UC _{OMe} UU _{OMe} USC _{OMe}	+		
	4369		2717	G _{OMe} sUG _{OMe} AA _{OMe} UG _{OMe} CA _{OMe} GA _{OMe} CC _{OMe} AA _{OMe} AG _{OMe} AA _{OMe} AsG _{OMe}	+		
			2724	UsA _{OMe} CA _{OMe} CU _{OMe} UA _{OMe} CG _{OMe} UC _{OMe} UG _{OMe} GU _{OMe} UU _{OMe} CU _{OMe} UU _{OMe} SC			
	4370		2715	GsU _{OMe} GAU _{OMe} GC _{OMe} AGAC _{OMe} CA _{OMe} AAAAGAAAsG	-		
			2723	U _{OMe} sAC _{OMe} AC _{OMe} UU _{OMe} AC _{OMe} GU _{OMe} CU _{OMe} GGU _{OMe} UU _{OMe} U _{OMe} U _{OMe} USC _{OMe}			
	4371		2714	GsU _{OMe} GAU _{OMe} GC _{OMe} AGACC _{OMe} AAAAGAAAsG	+++		
			2722	UsACAC _{OMe} UUAC _{OMe} GU _{OMe} CUGGU _{OMe} UUUCUUUsC			
	4372		2715	GsU _{OMe} GAU _{OMe} GC _{OMe} AGAC _{OMe} CA _{OMe} AAAAGAAAsG	+++		
			2727	UsAC _F AC _F U _F U _F AC _F GU _F C _F U _F GGU _F U _F C _F U _F U _F S _F C _F			
	4373		2714	GsU _{OMe} GAU _{OMe} GC _{OMe} AGACC _{OMe} AAAAGAAAsG	++		
			2726	UsACAC _F UUAC _F GU _F CUGGU _F UUUCUUUsC			

Duplexes are shown with the sense strand written 5' to 3'. The complementary antisense strand is written below the sense strand in the 3' to 5' direction. Lower case "d" indicates a deoxy nucleotide; all other positions are ribo. Lower case "s" indicates a phosphorothioate linkage. Subscript "OMe" indicates a 2'-O-methyl sugar and subscript "F" indicates a 2'-fluoro modified sugar. The extinction coefficient is the value at 260 nm (*10⁻³).

Table 7 Oligonucleotides with alternating 2'-O-methyl and 2'-fluoro modifications targeting VEGF.

Parent AL-DP- #	AL- DP-#	AL- SQ-#	Duplex Sequence and Modifications	Efficacy	Observed Mass	OD/mg	Extinction Coefficient
4060	4399	3082 3091	ComesCfComesUfGomesGfUomesGfGomesAfComesAfUomesCfUomesUfComesCfSAomes GfSGomesGfAomesCfComesAfComesCfUomesGfUomesAfGomesAfAomesGfComesSUf	-	6151.47	27.5	169
4015	4400	3083 3092	GomesGfAomesCfAomesUfComesUfComesCfComesAfGomesGfAomesGfUomesAfSAomes CfSAomesUfGomesUfAomesGfAomesAfGomesCfComesUfComesGf	+	6238.49	29	186
4032	4401	3084 3093	UomesSAfComesCfComesUfGomesAfUomesGfAomesGfAomesUfComesGfSAomes AfSUomesGfComesGfAomesCfUomesAfComesUfComesUfAomesGfComesSAf	+++	6239.47	31.8	188
4033	4402	3085 3094	AomesCfComesAfUomesGfComesAfGomesAfUomesUfAomesUfGomesCfGomesGfSAomes UfSGomesGfUomesAfComesGfUomesCfUomesAfAomesUfAomesCfComesSUf	+	6262.54	30.7	194
4014	4403	3086 3095	GomesCfComesGfAomesUfComesAfComesAfComesCfUomesCfAomesCfComesAfSAomes CfSGomesCfComesUfAomesGfUomesUfUomesGfComesAfGomesUfGomesGfUSUf	++	6204.65	26.4	190
4094so	4404	3087 3096	GomesCfAomesCfAomesUfAomesGfGomesAfGomesAfGomesAfUomesGfAomesGfSAomes CfSGomesUfGomesUfAomesUfComesCfUomesCfUomesCfUomesAfComesUfComesGf	+	6364.57	31.3	206
4061	4405	3088 3097	ComesSAfUomesAfGomesGfAomesGfAomesUfGomesAfGomesCfUomesUfSAomes GfSUomesAfUomesCfComesUfComesUfComesCfUomesCfGomesAfAomesGf	+++	6302.59	32.8	198
4092	4406	3089 3098	UomesGfUomesGfAomesAfUomesGfComesAfGomesAfComesCfAomesGfSAomes AfSAomesAfComesUfUomesAfComesGfUomesCfUomesGfComesUfComesUfComesSUf	++	6309.63	33.6	207
4004 so	4407	3090 3099	GomesSUfGomesAfAomesUfGomesCfAomesGfAomesCfComesAfAomesAfGomesAfSAomes CfSAomesCfUomesUfAomesCfComesUfComesUfGomesUfGomesGfUomesCfUomesUf	+++	6332.67	30.5	213

Duplexes are shown with the sense strand written 5' to 3'. The complementary antisense strand is written below the sense strand in the 3' to 5' direction. Lower case "s" indicates a phosphorothioate linkage. Subscript "OMe" indicates a 2'-O-methyl sugar and subscript "F" indicates a 2'-fluoro modified sugar. The parent duplexes had unpaired nucleotides at one or both ends of the duplex. These duplexes have blunt ends. The extinction coefficient is the value at 260 nm ($\times 10^{-3}$).

Table 8A-B. Cholesterol and cholanolic acid conjugates of active VEGF sequences (single strands).

Parent AL-DP-#	AL-SQ #	Strand	Sequence and Modifications	Calculated Mass	Found Mass	Purity	OD
4014	2363	sense	GsCsGGGAUCAAACCCUC _{OMe} ACC _{OMe} AsAdTsdTs- Chol	7466.5	7463.8	98.2	
4014	2697	sense	Chol -sGsCGGAUC _{OMe} AA ACCUC _{OMe} ACC _{OMe} AadTsdT	7232.3	7430.3	98.0	
4014	2698	sense	Chol -sGsCGGAUC _{OMe} AAACCCUC _{OMe} AC _{OMe} CoMeAAdTsdT	7446.3	7444.3	91.0	
4014	2699	sense	GsCGGAUC _{OMe} AAACCCUC _{OMe} ACC _{OMe} AadTs- Chol	7265.7	7265.7	98.0	
4060	4940	sense	Chol -C _{OMe} CoMeCoMeCoMeU _{OMe} GGU _{OMe} GGAC _{OMe} AU _{OMe} CoMeU _{OMe} CoMeCoMeAdTsdT			100	550
4060	2641	sense	Chol -sC _{OMe} CoMeCoMeCoMeU _{OMe} GGU _{OMe} GGAC _{OMe} AU _{OMe} CoMeU _{OMe} CoMeCoMeAdTsdT			100	583
4033	4935	sense	Chol -A _{OMe} CC _{OMe} AU _{OMe} GC _{OMe} AGAU _{OMe} U _{OMe} GC _{OMe} GGAdTsdT			100	562
4033	4941	sense	Chol -sA _{OMe} CC _{OMe} AU _{OMe} GC _{OMe} AGAU _{OMe} U _{OMe} GC _{OMe} GGAdTsdT			100	480
4061	4936	sense	Chol -C _{OMe} AU _{OMe} AGGAGAGAU _{OMe} GAGC _{OMe} U _{OMe} CoMeAdTsdT			100	532
4061	4942	sense	Chol -sC _{OMe} AU _{OMe} AGGAGAGAU _{OMe} GAGC _{OMe} U _{OMe} CoMeAdTsdT			98.2	514
4094	2965	sense	Chol -GC _{OMe} AC _{OMe} AU _{OMe} AGGAGAGAU _{OMe} GAGC _{OMe} U _{OMe} CoMeS _{OMe}	7205.7	7205.4	89.0	
4014	2701	sense	GsCGGAUC _{OMe} AAACCCUC _{OMe} ACC _{OMe} AadTs- Cholanolic	7219.8	7219.4	88.2	
4014	2702	sense	GsCGGAUC _{OMe} AAACCCUC _{OMe} AC _{OMe} CoMeAAdTs- Cholanolic	7276.3	7274.9	71.3	
4014	2696	antisense	U _S ^{5Me} U _F GG ^{5Me} U _F GAGGU ^{5Me} U _F GAUCCGCCdTs- Cholanolic				

The strands are shown written 5' to 3'. Lower case "s" indicates a phosphorothioate linkage. The lower case "d" indicates a deoxy residue. Subscript "OMe" indicates a 2'-O-methyl sugar. Subscript "F" indicates a 2'-fluoro. "**Chol**-" indicates a hydroxyprolinol cholesterol conjugate. "**Cholanolic**" indicates a cholanolic acid conjugate. "^{5Me}U" indicates a 5-methyl-uridine.

Table 8B.

Parent AL-DP- #		AL-DP-#	AL-SQ #	Strand	Sequence and Modifications	Efficacy	Calculated Mass	Found Mass	Purity
4014	4206		2363	sense	GsCsGGAUCAAAACCUCOMeACCOMeAsAdTsdTs-Chol	+	7466.5	7463.8	98.2
			2381	as	UsUGGUGAGGUUUGAUCCGCdTsdT				
4014	4351		2697	sense	Chol-sGsCGGAUCOMeAA ACCUCOMeAACCOMeAadTsdT	-	7232.3	7430.3	98.0
			4180	as	UUUGUGAGGUUUGAUCCGCTT				
4014	4352		2698	sense	Chol-sGsCGGAUCOMeAAACCUCOMeACOMeAAAdTsdT	-	7446.3	7444.3	91.0
			4180	as	UUUGUGAGGUUUGAUCCGCTT				
4014	4353		2699	sense	GsCGGAUCOMeAAAACCUCOMeACCOMeAadTs-Chol	++	7265.7	7265.7	98.0
			4180		UUUGUGAGGUUUGAUCCGCTT				
4094	4381		2965	sense	Chol-GCOMeACOMeAUOMeAGGAGAGAUOMeGAGCOMeUOMesUOMe	++	7205.7	7205.4	89.0
			2945	as	AAGtCfUcAfUcFtUcFcUcFuUgUgUgUgUgcUsg				
4014	4209		2701	sense	GsCGGAUCOMeAAACCUCOMeACCOMeAAAdTs-Cholanic	++	7219.8	7219.4	88.2
			2381	as	UsUGGUGAGGUUUGAUCCGCdTsdT				
4014	4210		2702	sense	GsCGGAUCOMeAAACCUCOMeACCOMeAAAdTs-Cholanic	++	7276.3	7274.9	71.3
			2381	as	UsUGGUGAGGUUUGAUCCGCdTsdT				
4014	4357		4112	sense	GCGGAUCAAAACCUCACCAATT	+++			
			2696	antisense	US ^{5Me} UFGG ^{5Me} UF ^{5Me} GAGGU ^{5Me} UF ^{5Me} UFGAUCCGCCdTs-Cholanic				
4094	4390		2949	ss	Chol-GCOMeCACOMeCACOMeUAAGGAGAGAComUGAGCOMeUsU	+++			
			2945	as	AAGtCfUcAfUcFtUcFcUcFuUgUgUgUgUgcUsg				
4094	4391		2950	ss	GSOMeCACOMeCACOMeUAAGGAGAGACOMeUGAGCOMeUU-Chol	+++			

			as	AAGfCfUfCAfUfCfUfCfUfCfUfUfUGfUGfCfUsG				
4094	4392	2945	SS	Thio-Chol-G _{OMe} CA _{OMe} CA _{OMe} UAGGAGAGA _{OMe} UGAGCC _{OMe} UsU	+++			
		2945	as	AAGfCfUfCAfUfCfUfCfUfCfUfUfUGfUGfCfUsG				
4094	4393	2948	SS	Chol-G _{OMe} CA _{OMe} CA _{OMe} UAGGAGAGA _{OMe} UGAGCC _{OMe} UU-NH2	+++			
		2945	as	AAGfCfUfCAfUfCfUfCfUfCfUfUfUGfUGfCfUsG				
4094	4394	2949	SS	Chol-G _{OMe} CA _{OMe} CA _{OMe} UAGGAGAGA _{OMe} UGAGCC _{OMe} UsU	+++			
		4327	as	AAGCUCAUCUCUCUUAUGUGCUG				
4094	4395	2950	SS	G _{OMe} CA _{OMe} CA _{OMe} UAGGAGAGA _{OMe} UGAGCC _{OMe} UU-Chol	+++			
		4327	as	AAGCUCAUCUCUCUUAUGUGCUG				
4094	4396	2951	SS	Thio-Chol-G _{OMe} CA _{OMe} CA _{OMe} UAGGAGAGA _{OMe} UGAGCC _{OMe} UsU	+++			
		4327	as	AAGCUCAUCUCUCUUAUGUGCUG				

The strands are shown written 5' to 3'. Lower case "s" indicates a phosphorothioate linkage. The lower case "d" indicates a deoxy residue. Subscript "OMe" indicates a 2'-O-methyl sugar. Subscript "F" indicates a 2'-fluoro. "Chol" indicates a hydroxyprolinol cholesterol conjugate. "Cholanic" indicates a cholanolic acid conjugate. "⁵MeU" indicates a 5-methyl-uridine.

Table 9. Naproxen conjugates of active VEGF sequence.

Parent AL-DP-#	AL-DP-#	AL-SQ #	Sequence and Modifications	Efficacy	Calculated Mass	Found Mass	Purity
4014	4355	2694 as	U ^{5Me} _S U ^{5Me} _F GGU ^{5Me} _F GAGGU ^{5Me} _F U ^{5Me} _F GAUCCGCdTsdTs-Naproxen	+++	7269.4	7270.7	80.1
		4112 ss	GCGGAUCAAACCCUACCAATT				

5 The antisense strand of the duplex is shown written 5' to 3'. Lower case "s" indicates a phosphorothioate linkage. Lower case "d" indicates a deoxy. Subscript "F" indicates a 2'-fluor sugar. "^{5Me}U" indicates a 5-methyl-uridine. "Naproxen" indicates a naproxen conjugated to the oligonucleotide through a serinol linker.

Table 10. Biotin conjugates of active oligonucleotides targeting VEGF.

Parent AL-DP-#	AL-DP-#	AL-SQ-#	Strand	Sequence and Modifications	Efficacy	Calc. Mass	Exp. Mass	Purity
4014	4356	4112	sense	5 GCGGAUCAAAACCCACCAATT 3	+++			
		2695	antisense	Us ^{5Me} U _F GG ^{5Me} U _F GAGGU ^{5Me} U _F ^{5Me} U _F GAUCCGCdTsdTs-Biotin		7285.4	7284.3	70.2
4220		3071	sense	AsAGCUC _{OMe} AUCUCUCUCCU _{OMe} AU _{OMe} GU _{OMe} GC _{OMe} U _{OMe} sGs-Biotin	Used for ELISA	7872.1	7871.89	82.02

The oligonucleotides are written 5' to 3'. Lower case "s" indicates a phosphorothioate linkage. Lower case "d" indicates a deoxy. Subscript "OMe" indicates a 2'-O-methyl sugar and subscript "F" indicates a 2'-fluoro modified sugar. "^{5Me}U" indicates a 5-methyl uridine.

Table 11a-b. Conjugation of aldehydes, Retinal and other Retinoids to VEGF siRNAs and model oligonucleotides.

Sequence ID	Sequence*	Cal Mass	Found Mass	CGE (%)
AL-3174	Q25-dTdTdTdTdTdTdT dTdTdTdTdTdTdT	3767.22	3769.09	A
AL-3175	Q26-dTdTdTdTdTdTdTdT dTdTdTdTdTdTdT	3980.07	3981.37	A
AL-3176	Q27-dTdTdTdTdTdTdTdTdT dTdTdTdTdTdTdT	4034.24	4035.56	A
AL-4326	GCACAUAGGAGAGAGAGCUU	6799.22	6798.88	A
AL-3177	Q25-GCACAUAGGAGAGAGAGCUU		B	A
AL-3178	Q27-GCACAUAGGAGAGAGAGCUU	7246.66	7246.53	97% ^c
AL-3166	GCACAUAGGAGAGAGAGCUUsU	6815.16	6815.10	A
AL-3184	Q25-GCACAUAGGAGAGAGAGCUUsU	6995.16	B	A
AL-3185	Q27-GCACAUAGGAGAGAGAGCUUsU	7261.6	7262.47	97.8 ^c
AL-3193	Q28- GCACAUAGGAGAGAGAGCUUsU	7277.61	E	F
AL-3211	GAACUGUGUGAGAGAGGUCCsU	6785.10	B	A
AL-3212	Q25-GAACUGUGUGAGAGAGGUCCsU	6965.10	G	G
AL-3213	Q27-GAACUGUGUGAGAGAGGUCCsU	7231.54	G	G
AL-3214	Q26-GAACUGUGUGAGAGAGGUCCsU	7177.37	G	G

Table 11. b

AL-DP-#	AL-SQ-#	5'-3' Sequence	Comments
AL-DP-4410	AL3178	Q27-GCACAUAGGAGAGAGAGCUU	5'Retinal4094
	AL4327	AAGCUCAUCUCUCCUAUGUGCUG	
AL-DP-4413	AL3185	Q27-GCACAUAGGAGAGAGAGCUsU	5'Retinal, 3'PS 4094
	AL3167	AAGCUCAUCUCUCCUAUGUGCUsG	

Q25 = aminoxy-linker
Q26 = 1-pyrene-carboxaldehyde-aminoxy
Q27 = all-*trans*-retinal-aminoxy
Q28 = 4-keto-retinol

- (A) These samples were not purified and thus no CGE analysis.
- (B) These samples were not analyzed as they were used in the conjugation reaction in the next step.
- (C) There are two isomers (E and Z) and while two peaks were seen in the CGE, only one peak was seen in the LC/MS with one mass only.
The CGE % therefore is the areas of the two peaks in the CGE added together.
- (D) Only a little bit of the desired product was present in the crude mixture.
- (E) Two peaks in the LC/MS were seen with masses of 7276.42 and 7277.72. The masses can be explained by the easy oxidation of retinal to retinal.
- (F) The two main products are 33% and 67% by CGE.
- (G) To be determined.

Table 12. Polyethylene glycol conjugates of active VEGF sequences and control conjugates.

Parent AL-DP #	AL-SQ #	Strand ¹	Sequence and Modifications	MW Expected	MW Observed ²	HPLC retention time	Starting amount	% Yield
4094	3194	VEGF sense	GCACAUAGGAGAGAGACGUUs-HP-NH2	7107.46	7107.2	37.497	466.67mg	25.9
4094	3195	VEGF sense	GCACAUAGGAGAGAGACGUUs-HP-NH2-20KPEG	27213.19	28333.51- 29614.44	31.283	50mg	33.8
5167	3164	control	GsUCAUCACACUGAAUACCAAU-HP-NH2	6932.33	6932.15	19.733	491.4 mg	34.7
5167	3170	control	GsUCAUCACACUGAAUACCAAU-HP-NH2-5KPEG	11746.19	11000- 13000	16.822	50mg	38.4
5167	3171	control	GsUCAUCACACUGAAUACCAAU-HP-NH2-20KPEG	26746.19	27456- 29524	16.164	50mg	39.2
1000	2936	control	NH2-HP-CUUACGCUGAGUACUUCGAdTsdT	6915.3	6915.01	20.506		
1000	3187	control	5KPEG-NH2-HP-CUUACGCUGAGUACUUCGAdTsdT	12021.46	11847- 13256	17.829	50mg	39.2
1000	3188	control	20KPEG-NH2-HP-CUUACGCUGAGUACUUCGAdTsdT	27021.46	27440- 29289	16.921	50mg	33.6
1000	2937	control	CsUUACGCUGAGUACUUCGAdTdT-HP-NH2	6915.3	6915.06	20.537		
1000	3172	control	CsUUACGCUGAGUACUUCGAdTdT-HP-NH2-5KPEG	12021.46	12300- 13034	17.578	50mg	48.0
1000	3173	control	CsUUACGCUGAGUACUUCGAdTdT-HP-NH2-20KPEG	27021.46	27000- 29000	17.087	50mg	52.0

The strands are shown written 5' to 3'. Lower case "s" indicates a phosphorothioate linkage. The lower case "d" indicates a deoxy residue. "HP-NH2" or "NH2-HP" indicates a hydroxyprolinol amine conjugate used as a control. "HP-NH2-20KPEG" or "20KPEG-NH2-HP" indicates

5 conjugation to polyethylene glycol (20K) through the hydroxyprolinol linker. "HP-NH2-5KPEG" or "5KPEG-NH2-HP" indicates conjugation to polyethylene glycol (20K) through the hydroxyprolinol linker.

¹ The control in this column indicates that the oligonucleotide is not complementary to VEGF. Oligonucleotides 3164, 3170, and 3171 target ApoB and oligonucleotides 2936, 3187, 3188, 2937, 3172, and 3173 target luciferase.

² The range in observed molecular weight is due to the polydispersity of PEG starting material.

Table 13. Oligonucleotides targeting VEGF with the ribo-difluorotoluy/ modification.

Parent AL-DP-#	AL-DP-#	AL-SQ-#	Duplex Sequence and Modifications	Type	<i>In vitro</i> efficacy	T _m (°C)
4014	4014	4112 4180	<u>CGGGAUCAAACCUACCAAdTdT</u> dTdTTCGCCUAGUUUGGAGUGGUU	Control	+++	80
4014		4112 2957	CGGGAUCAAACCUACCAAdTdT dTdTTCGCCUAGUUUGGAGUGGUU	Mismatch antisense	+	75
4014		4112 2958	CGGGAUCAAACCUACCAAdTdT dTdTTCGCCUAGUUUGGAGUGGUU	Mismatch antisense	+	75
4014		4112 2959	CGGGAUCAAACCUACCAAdTdT dTdTTCGCCUAGUUUGGAGUGGUU	Mismatch antisense	++	75
4014	4347	4112 2472	CGGGAUCAAACCUACCAAdTdT dTdTTCGCCUAGUUUGGAGUGGUU	Difluorotoluy/	++	76
4014	4348	4112 2473	CGGGAUCAAACCUACCAAdTdT dTdTTCGCCUAGUUUGGAGUGGUU	Difluorotoluy/	++	
4014	4349	4112 2474	CGGGAUCAAACCUACCAAdTdT dTdTTCGCCUAGUUUGGAGUGGUU	Difluorotoluy/	++	
4014	4350	4112 2475	CGGGAUCAAACCUACCAAdTdT dTdTTCGCCUAGUUUGGAGUGGUU	Difluorotoluy/	+	70
4014		2953	CGGGAUCAAAGCCUCACCAAdTdT	Mismatch sense		77
4014		4180 2954	dTdTTCGCCUAGUUUGGAGUGGUU CGGGAUCAAACCUACCAAdTdT	Mismatch sense		73
4014		4180 2955 4180	dTdTTCGCCUAGUUUGGAGUGGUU CGGGAUCAAACCUACCAAdTdT dTdTTCGCCUAGUUUGGAGUGGUU	Mismatch sense		73

Duplexes are shown with the sense strand written 5' to 3'. The complementary antisense strand is written 3' to 5'. Lower case "d" indicates a deoxy nucleotide; all other positions are ribo. Lower case "s" indicates a phosphorothioate linkage. "F" indicates a ribo-difluorotoluy/ modification. Positions altered relative to the control duplex are indicated in bold face type.

Table 14. Oligonucleotides with 2'-araffluoro-2'-deoxy-nucleosides targeting VEGF.

Parent AL-DP- #	AL-DP- #	AL- SQ-#	Strand	Sequence and Modifications	Efficacy	Expected Mass	Observed Mass	HPLC Purity
4014	4342	2478 4112	antisense sense	UT _{araF} GGT _{araF} GAGGUUT _{araF} GAUCCGCdTdT GCGGAUCAAAACCUCACCAATT	++	6728.02	6727.25	92.82
4014	4343	2479 4112	antisense sense	UT _{araF} GGT _{araF} GAGGUUT _{araF} T _{araF} GAUCCGCdTdT GCGGAUCAAAACCUCACCAATT	+++	6744.04	6743.22	91.97
4014	4344	2480 4112	antisense sense	UU _{araF} GGU _{araF} GAGGUUU _{araF} GAUCCGCdTdT GCGGAUCAAAACCUCACCAATT	++	6685.94	6685.13	94.83
4014	4345	2481 4112	antisense sense	UU _{araF} GGU _{araF} GAGGUUU _{araF} U _{araF} GAUCCGCdTdT GCGGAUCAAAACCUCACCAATT	+++	6687.93	6687.11	91.97
4014	4346	2814 4180	sense antisense	GCGGAUC _{araF} AA ACCUC _{araF} AC _{araF} C _{araF} AAAdTdT UUGGUGAGGUUUUGAUCCGCTT	+++	6699.14	6698.42	97.60

Sequences are shown written 5' to 3'. Lower case "d" indicates a deoxy nucleotide. "U_{araF}" indicates a 2'-araffluoro-2'-deoxy-uridine, "T_{araF}" indicates a 2'-araffluoro-thymidine, and "C_{araF}" indicates a 2'-araffluoro-2'-deoxy-cytidine.

Table 15. Methylphosphonate-modified VEGF RNAs.

Parent AL-DP-#	AL-SQ #	Strand	Sequence and Modifications	Calculated Mass	Found Mass	Purity
4014	2501	sense	GsCsGGAUC _{mp} AA ACCUC _{mp} A CcmpAsAsdTsdT	6712.50		
4014	2502	antisense	UsU _{mp} sGGUGAGGUU _{mp} UGAUCCGsCsdTsdT	6758.97	6766.1	
4014	2503	antisense	UsU _{mp} sGGU _{mp} GAGGUU _{mp} U _{mp} GAUCCGsCsdTsdT	6756.44	6743.99	

5 The oligonucleotides are shown written 5' to 3'. Lower case "s" indicates a phosphorothioate linkage. Subscript "mp" indicates a methyl phosphonate linkage. Lower case "d" indicates a deoxy nucleotide.

Table 16. C-5 Allylamino -modified VEGF RNAs.

Parent AL-DP-#	AL-SQ #	Strand	Sequence and Modifications	Calculated Mass	Found Mass	Purity
4014	2504	antisense	UsU _{aa} sG GU _{aa} GAGGUUU _{aa} GAUCCG _s CsdTsdT	6925.38	6924.9	92.4
4014	2505	antisense	UsU _{aa} sGGU _{aa} GAGGUU _{aa} U _{aa} GAUCCG _s CsdTsdT	6980.40	6979.8	90.0

The oligonucleotides are shown written 5' to 3'. Lower case "s" indicates a phosphorothioate linkage. Subscript "aa" indicates an allylamino modification. Lower case "d" indicates a deoxy nucleotide.

Table 17. Miscellaneous Modifications to VEGF RNA (single strands).

Parent AL-DP-#	AL-SQ #	Strand	Sequence and Modifications	Calculated Mass	Found Mass	Purity
4107	2192	sense	GsCACAUAGGAGAGAGAGCsdTsdT	6843.36	6842.6	84.0
4107	2193	antisense	GsCUCAUCUCUCC*UAUGUGCsdTsdT	6584.3	6584.1	80.0
4107	2194	sense	GsCsACAUAGGAGAGAGAGCsdTsdT	6875.0	6874.2	88.7
4107	2196	antisense	GsCACAUAGGAGAGAGAGCsdTsdT	6875.5	6874.0	88.7
4014	2281	sense mismatch	GsCsGGAAACAUAUCCUGACCAsAsdTsdT	6755.4	6753.9	82.9
4014	2282	antisense mismatch	UsUsGGUCAGGAUUGUCCGsCsdTsdT	6720.0	6719.9	96.7
4014	2299	sense mismatch	GCGGAACAUAUCCUGACCAATT	6675.0	6673.8	85.9
4014	2300	antisense mismatch	UUGGUCAGGAUUGUCCGCTT	6639.9	6638.5	86.5
4014	2200	sense	GsCsGGAUCAAAACCUCACCAsAsdTsdT	6715.4	6714.3	86.0
4014	2201	antisense	UsUsGGUGAGGUUUUGAUCCGsCsdTsdT	6760.3	6759.6	91.2
4014	2202	sense	GsCGGAUCAAAACCUCACCAAsdTsdT	6683.2	6682.3	95.7
4014	2203	antisense	UsUGGUGAGGUUUUGAUCCGsCsdTsdT	6728.1	6727.3	87.6
4351	2206	sense	UUCUUUGGUCUGCAU UCAC	5913.4	5912.3	98.0
4359	2207	sense	UsUGGUGAGGUUUUGAUCCGsCsdTsdT	6760.3	6759.05	92.0
4014	2210	sense	GsCsGGAUCAAAACCUCsACCsAsAsdTsdT	6747.5	6746.6	82.7
4014	2212	sense	GsCsUCAUCUCUCCUUsAUGUGsCsdTsdT	6616.3	6614.8	78.9
4014	2323	sense	GsCsGGAUCAAAACCUC _{OMe} ACC _{OMe} AsAsdTsdT	6743.4	6742.3	90.0
4014	2324	sense	GsCsGGAUCAAAACCUC _{OMe} AC _{OMe} AsAsdTsdT	6771.5	6770.4	86.8
4014	2325	sense	GsCsGGAUCAAAACCUC _{OMe} sACC _{OMe} sAsAsdTsdT	6775.5	6774.6	87.6
4014	2499	sense	GsCsGGAUCA _{OMe} AAACCUC _{OMe} AC _{OMe} AsAsdTsdT	6771	6771.1	84.8

4014	2500	sense	GsCsGGAUdCAAACCUdCdCdCsAsdTsdT	6651.4	6650.6	82.6
4014	2506	antisense	Us ^{5Me} U _F sGGG ^{5Me} U _F GAGGU ^{5Me} U _F GUAUCGsCsdTsdT	6808.4	6808	82.0
4014	2507	antisense	UsU _F sGG ^{5Me} U _F GAGGU ^{5Me} U _F ^{5Me} U _F GUAUCCGsCsdTsdT	6824.3	6823.3	80.2
4014	2508	antisense	Us ^{5Me} U _F sGGG ^{5Me} U _F GAGG ^{5Me} U _F ^{5Me} U _F UGAUUCCGsCsdTsdT	6824.3	6823.4	84.3
4014	2509	antisense	UsU _{OMe} sGGU _{OMe} GAGGU ^{5Me} U _F ^{5Me} U _F GUAUCCGsCsdTsdT	6820.3	6822.0	85.0
4220	2780	antisense	GsCo _{Me} ACo _{Me} AU _{OMe} AGGAGAGAU _{OMe} GAGCU _{OMe} sU	6901.38	6900.77	89.29
4060 ¹	2808	sense	AsGsCsUsUsAsAsCsCsUsGsUsCsCsUsUsCsAsA	6230.57		
4060 ¹	2809	antisense	UsUsGsAsAsGsGsAsCsAsGsGsUsUsAsAsGsCsU	6413.73		

The oligonucleotides are shown written 5' to 3'. Lower case "s" indicates a phosphorothioate linkage. Lower case "d" indicates a deoxy. Subscript "OMe" indicates a 2'-O-methyl sugar. Subscript "F" indicates a 2'-fluoro. "^{5Me}U" indicates a 5-methyl uridine. ¹ The parent duplex has dT overhangs. The phosphorothioate-modified duplex has blunt ends.

Table 18. Physical characteristics of VEGF compounds derived from duplexes 4094, 4060, 4033, 4061, 4004, 4014, 4107 and 4003

Parent duplex	AL-SQ-#	Sense strands Antisense strands	Modif Seq	Calc. mass	Obs. mass
AL-DP-4094	4326	5'-GCACAUAGGAGAGAGAGCUU-3'		6670,1	6670,0
	4327	3'-GUCCGUAUCCUCUCUACUCGAA-5'		7220,3	7220,0
			Modif Seq		
	4554	5'-G*CACAUAGGAGAGAGAGCU*U-3'		6830,3	6830,0
	4557	5'-A*AGCUCAUCUCUCCUAGUGCU*G-3'	2PS	7252,4	7252,0
	4555	5'-G*CACAUAGGAGAGAGAGCU*U-3'		6844,3	6844,0
	4558	5'-A*AGCUCAUCUCUCCUAGUGCU*G-3'	2PS, 1xOMe	7280,4	7280,0
	4556	5'-GcAcAuAGGAGAGAGAGCU*U-3'	1xPS; 5xOMe	6884,3	6884,0
	4559	5'-A*AGCUCAUCUCUCCUAGUGCU*G-3'	2xPS, 3xOMe	7294,4	7293,0
	4563	5'-G(dC)A(dC)AuAGGAGAGAGAGCU*U-3'	1xPS, 3xOMe, 2xdC	6824,3	6824,0
	4560	5'-AAGCUcAUCUCUCCUAGUGCU*G-3'	1xPS, 5xOMe	7306,4	7306,0
	4564	5'-G*CACAU _{2F} AGGAGAGAGAGCU*U-3'	2xPS; 1x2'F	6832,2	6831,0
	4561	5'-AAGCUcAUCUCUCCUAGUGCU*G-3'	1xPS, 6xOMe	7320,4	7320,0
	4565	5'-GC _{2F} AC _{2F} AU _{2F} AGGAGAGAU _{2F} GAGCU _{2F} *U-3'	1xPS; 5x2'F	6824,3	6823,0
	4562	5'-AAGCU(dC)AUCUCUCCUAGUGCU*G-3'	1xPS, 4xOMe, 2xdC	7260,4	7260,0
	4566	5'-GC _{2F} AC _{2F} AUAGGAGAGAGAGCU*U-3'	1xPS, 3xOMe, 2x2'F	6860,3	6859,0
	4568	5'-AAGCUc _{2F} AUCUCUCCU _{2F} AU _{2F} GU _{2F} GCU _{2F} *G-3'	1xPS, 5x2'F	7246,4	7244,0
	4567	5'-GcAcAU _{2F} AGGAGAGAU _{2F} GAGCU _{2F} *U-3'	1xPS, 2xOMe, 3x2'F	6848,3	6847,0
	4569	5'-AAGCUcAUCUCUCCU _{2F} AU _{2F} GU _{2F} GCU _{2F} *G-3'	1xPS, 1xOMe, 4x2'F	7258,4	tbd
	4567	5'-GcAcAU _{2F} AGGAGAGAU _{2F} GAGCU _{2F} *U-3'	1xPS, 2xOMe, 3x2'F	6848,3	6847,0
	4570	5'-AAGCUc _{2F} AUCUCUCCU _{2F} AU _{2F} GU _{2F} GCU*G-3'	1xPS, 4xOMe, 1x2'F	7294,4	7292,0
	4571	5'-GcAcAuAgGaGaUgAgCU*U-3'	1xPS, altern. 2'OMe	6954,3	6953,0
	4572	5'-aAgCUcAUcUcCUcAUcGUcGU*G-3'	1xPS, altern. 2'OMe	7404,4	7403,0
	4352	5'-GCACAUAGGAGAGAGAGC-3'	blunt	6185,8	6186,0
	4353	5'-GCUCACUCUCCUAGUGC-3'	blunt	5910,5	5910,8
AL-DP-4060	4061	5'-CCCUGGUGGACAUCCUCCATT-3'		6581,0	Tbd
	4159	3'-TTGGGACCAUCCUAGAGGU-5'		6747,2	tbd
			Modif Seq		
	2580	5'-cccuGGUGGACAUccuCCAT*T	Modifications		
	2641	3'-T*GGGAC _{2F} C _{2F} AC _{2F} C _{2F} U _{2F} GU _{2F} AGAAGGU _{2F} -5'	1xPS, 2'OMe@Py, 1xPS, 2'F@Py	6765,1 6777,3	6764,0 6777,9
	4934	5'-(Chol)cccuGGUGGACAUccuCCAT*T	1xPS, 2'OMe@Py, 5'Chol	7470,0	7468,0

	2641	3'-T*TGGAAC _{2F} C _{2F} AC _{2F} C _{2F} U _{2F} GU _{2F} AGAAGGU _{2F} -5'	1xPS, 2'F@Py	6777,3	6777,9
	4940	5'-(Chol)*ccuGGuGGACuuccAT*T	2xPS, 2'OMe@Py, 5'Chol	7486,0	7485,0
	2641	3'-T*TGGAAC _{2F} C _{2F} AC _{2F} C _{2F} U _{2F} GU _{2F} AGAAGGU _{2F} -5'	1xPS, 2'F@Py	6777,3	6777,9
AL-DP-4033	4026	5'-ACCAUGCAGAUUAUGCGGATT		6692,1	Tbd
	4093	3'-TTUGGUACGUCUAAUACGCCU-5'		6606,0	tbd
	Modif Seq		Modifications		
	2586	5'-aCcAuGcAGAUuGcGGAT*T	1xPS, 8x 2'OMe	6820,2	6819,0
	2647	3'-T*TU _{2F} GGU _{2F} AC _{2F} GU _{2F} C _{2F} U _{2F} AAU _{2F} AC _{2F} GC _{2F} C _{2F} U _{2F}	1xPS, 2'F@Py	6644,0	6644,0
	4935	5'-(Chol)aCcAuGcAGAUuGcGGAT*T	1xPS, 8x 2'OMe; 5'Chol	7525,1	Tbd
	2647	3'-T*TU _{2F} GGU _{2F} AC _{2F} GU _{2F} C _{2F} U _{2F} AAU _{2F} AC _{2F} GC _{2F} C _{2F} U _{2F}	1xPS, 2'F@Py	6644,0	6644,0
	4941	5'-(Chol)*aCcAuGcAGAUuGcGGAT*T	2xPS, 8x 2'OMe; 5'Chol	7541,1	7539,0
	2647	3'-T*TU _{2F} GGU _{2F} AC _{2F} GU _{2F} C _{2F} U _{2F} AAU _{2F} AC _{2F} GC _{2F} C _{2F} U _{2F}	1xPS, 2'F@Py	6644,0	6644,0
AL-DP-4061	4119	5'-CAUAGGAGAGAGAGCUUCTT		6732,2	Tbd
	4187	3'-TTGUAUCCUCUCUACUCGAAG-5'		6566,0	tbd
	Modif Seq		Modifications		
	2596	5'-CAUAGGAGAGAGAGCUUCTT	1xPS, 2'OMe @allPy	6846,3	6845,0
	2657	3'-TTGuAuuccucucUcGGAAG-5'	1xPS, 2'F@Py	6604,1	6605,0
	4936	5'-(Chol)CAUAGGAGAGAGAGCUUCTT	1xPS, 2'OMe @Py,	7551,2	Tbd
	2657	3'-TTGuAuuccucucUcGGAAG-5'	5'Chol	6604,1	6605,0
			1xPS, 2'F@Py		
	4937	5'-(Chol)*CAUAGGAGAGAGAGCUUCTT	2xPS, 2'OMe@Py, 5'Chol	7567,2	7565,0
	2657	3'-TTGuAuuccucucUcGGAAG-5'	1xPS, 2'F@Py	6604,1	6605,0
AL-DP-4331	2626	5'-cAUAGGAGAGAGAGCUUCTT-3'	1xPS, 3x 2'OMe	6790,3	6789,0
	2627	3'-T*TGUAUCCUCUCUAcUCGAAG-5'	1xPS, 3x 2'OMe	6624,1	6624,0
AL-DP-4004	4338	5'-GUGAAUGCAGACCAAGAAAG-3'		6828,3	tbd
	4339	3'-UACACUACGUCUGGUUUCUUUC-5'			
	Modif Seq		Modifications		
	4350	5'-GUGAAUGCAGACCAAGAA-3'	blunt	6153,8	6154,0
	4351	5'-UUCUUUGGUCUGCAUUCAC-3'	blunt	5912,5	5911,8
	4338	5'-GUGAAUGCAGACCAAGAAAG-3'	blunt	6829,3	6523,5
	4344	5'-CUUUUUUGGUCUGCAUUCAC-3'	blunt	6523,9	6523,5
AL-DP-4371	2714	5'-GuGAUuGcAGACCAAGAAAG-3'	1xPS, 4x 2'OMe	6900,4	6900,0
	2722	3'-U*ACAcUUAUGUGGUUUUCUUUC-5'	1xPS, 4x 2'OMe	7231,3	7230,0
AL-DP-4014	4112	5'-GCGGAUCAAACCCUCACCAATT-3'		6634,1	6634,5
	4180	3'-TTCGCCUAGUUUGGAGUGGUU-5'		6679,1	6680,3

	Modif Seq		Modifications		
	4318	5'-GCGGAUCAAACCCUACCAAGG-3'	blunt	6717,2	tbd
	4342	5'-CCUUGGUGAGGUUUGAUCCGC-3'	blunt	6681,0	6683,3
	4346	5'-GCGGAUCAAACCCUACCA-3'	blunt	6025,7	6026,5
	4347	5'-UUGGUGAGGUUUGAUCCGC-3'	blunt	6070,6	6071,3
AL-DP- 4127	4358	5'-G*C*GGAUCAAACCCUACCA*AT*T-3'	(2+3)PS	6714,4	6714,8
	2201	3'-T*T*C*GCCUAGUUGGAGUGG*U*U-5'	(2+3)PS	6759,3	tbd
AL-DP- 4107	4117	5'-GCACAUAGGAGAGAGAGCCTT-3'		6794,2	6794,0
	4185	3'-TTCGUGAUCCUCUCUACUCG-5'		6518,9	6519,0
	Modif Seq		Modifications		
	4326	5'-GCACAUAGGAGAGAGAGCUU-3'		6799,2	tbd
	4345	5'-AAGCUCAUCUCCUAGUGC-3'	blunt	6569,0	6568,5
	4354	5'-G*CACAUAGGAGAGAGAGC*T*T-3'	(1+2)PS	6842,4	6842,5
	4356	5'-G*C*ACAUAGGAGAGAGAG*C*T*T-3'	(2+3)PS	6874,5	tbd
AL-DP- 4003	4286	5'-GGACAUUCCAGGAGUACCC-3'		6670,1	6669,5
	4287	5'-GGGUACUCCUGGAAGAUUCCAC-3'		7361,5	7362,0
	Modif Seq		Modifications		
	4348	5'-GGACAUUCCAGGAGUAC-3'	Blunt	6059,7	6059,5
	4349	5'-GUACUCCUGGAAGAUUCC-3'	blunt	6036,7	6036,8
	4286	5'-GGACAUUCCAGGAGUACCC-3'	blunt	6671,1	tbd
	4343	5'-GGGUACUCCUGGAAGAUUCC-3'	blunt	6727,1	6727,5

Abbreviations used :

Lower case letters : 2'OMe ribonucleotides

Upper case letters followed by subscript 2'F: 2'F ribonucleotides

Upper case letters: regular ribonucleotides

T: Deoxythymidine

(dC): Deoxycytidine

*: Phosphorothioate linkage

(Chol): Cholesterol

Tbd: to be determined

Altern.: alternating

OTHER EMBODIMENTS

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

WE CLAIM:

1. An isolated iRNA agent, comprising a sense sequence and an antisense sequence, wherein the sense and the antisense sequences form an RNA duplex, and wherein the antisense sequence comprises a nucleotide sequence sufficiently complementary to a target sequence of about 19 to 23 nucleotides of a VEGF nucleotide sequence and wherein said target sequence differs by no more than 1, 2, or 3 nucleotides from a sequence selected from the group consisting of SEQ ID NOs:1-401.
2. The iRNA agent of claim 1, wherein the sense sequence comprises a sequence that differs by no more than 1, 2, or 3 nucleotides from a sequence selected from the group consisting of SEQ ID NOs:2-401.
3. The iRNA agent of claim 1, wherein the sense sequence comprises a sequence that differs by no more than 1, 2, or 3 nucleotides from a sequence selected from the group consisting of SEQ ID NO:456, SEQ ID NO:546, SEQ ID NO:548, SEQ ID NO:550, SEQ ID NO:552, SEQ ID NO:590, SEQ ID NO:592, SEQ ID NO:594, SEQ ID NO:596, SEQ ID NO:608, SEQ ID NO:610, SEQ ID NO:612, SEQ ID NO:614, SEQ ID NO:634, SEQ ID NO:636, SEQ ID NO:638, SEQ ID NO:640, SEQ ID NO:646, SEQ ID NO: 648, and SEQ ID NO:650.
4. The sense sequence of claim 3, wherein the sequence differs by no more than 1, 2, or 3 nucleotides from SEQ ID NO:608.
5. The iRNA agent of claim 1, wherein the antisense sequence comprises a sequence that differs by no more than 1, 2, or 3 nucleotides from a sequence selected from the group consisting of SEQ ID NO:457, SEQ ID NO:547, SEQ ID NO:549, SEQ ID NO:551, SEQ ID NO:553, SEQ ID NO:591, SEQ ID NO:593, SEQ ID NO:595, SEQ ID NO:597, SEQ ID NO:609, SEQ ID NO:611, SEQ ID NO:613, SEQ ID NO:615, SEQ ID NO:635, SEQ ID NO:637, SEQ ID NO:639, SEQ ID NO:641, SEQ ID NO:647, SEQ ID NO: 649, and SEQ ID NO:651.

6. The antisense sequence of claim 5, wherein the sequence differs by no more than 1, 2, or 3 nucleotides from SEQ ID NO:609.
7. The iRNA agent of claim 1, wherein the iRNA agent further comprises a non-nucleotide moiety.
8. The iRNA of claim 7, wherein the sense and antisense sequences are stabilized against nucleolytic degradation.
9. The iRNA agent of claim 1, further comprising one 3'-overhang wherein said 3'-overhang comprises from 1 to 6 nucleotides.
10. The iRNA of claim 9, further comprising a second 3'-overhang wherein said second 3'-overhang comprises from 1 to 6 nucleotides.
11. The iRNA agent of claim 1, further comprising a phosphorothioate at the first internucleotide linkage at the 5' end of the antisense and sense sequences.
12. The iRNA agent of claim 1, further comprising a phosphorothioate at the first internucleotide linkage at the 3' end of the antisense and sense sequences.
13. The iRNA agent of claim 1, further comprising a phosphorothioate at the first internucleotide linkage at the 5' end of the antisense and sense sequences, and a phosphorothioate at the first internucleotide linkage at the 3' end of the antisense and sense sequences.
14. The iRNA agent of claim 1, further comprising a 2'-modified nucleotide.
15. The iRNA agent of claim 14, wherein the 2'-modified nucleotide comprises a modification selected from the group consisting of: 2'-deoxy, 2'-deoxy-2'-fluoro, 2'-O-methyl, 2'-O-methoxyethyl (2'-O-MOE), 2'-O-aminopropyl (2'-O-AP), 2'-O-

dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), 2'-O-dimethylaminoethoxyethyl (2'-O-DMAEOE), and 2'-O-N-methylacetamido (2'-O-NMA).

16. A method of reducing the amount of VEGF RNA in a cell of a subject, comprising contacting the cell with an iRNA agent of any one of the claims 1-15.

17. A method of making an iRNA agent of any of claims 1-15, the method comprising the synthesis of the iRNA agent, wherein the sense and antisense strands comprise at least one modification that stabilizes the iRNA agent against nucleolytic degradation.

18. A pharmaceutical composition comprising an iRNA agent of claim 1 and a pharmaceutically acceptable carrier.

19. A method of inhibiting VEGF expression comprising administering an effective amount of an iRNA agent of claim 1.

20. A method of treating a human diagnosed as having or at risk for having adult macular degeneration (AMD), comprising administering to a subject in need of such treatment a therapeutically effective amount of an iRNA agent of claim 1.

Figure 1

```

1   augaacuunc ugcugucucug ggugcauugg agccumgccu ugcugucucua ccuccaccan
61   gccaaaguggu cccaggcugc acccauggca gaaggaggag gccagaaauca uacacgaagug
121  gugaaguuca uggaugucua ucaggcgcagc uacugccauc caaucgagac ccuggguggac
181  aucuuccagg aguaccucga ugagaucgag uacauucuca agccauccug ugnugcccccug
241  augcgaugcg ggggcugcug caaugacgag gccuuggagu gugugccac ugaaggagucc
301  aacauaccca ugcagauuuu gccggaucaaa ccuacccaag gccagcaca aggagagaug
361  agcuuuccuac agcacaacaa augugaaugc agaccaaaga aagauagagc aagacaagaa
421  aaauugaca agccgaggcg guga (SEQ ID NO:1)

```

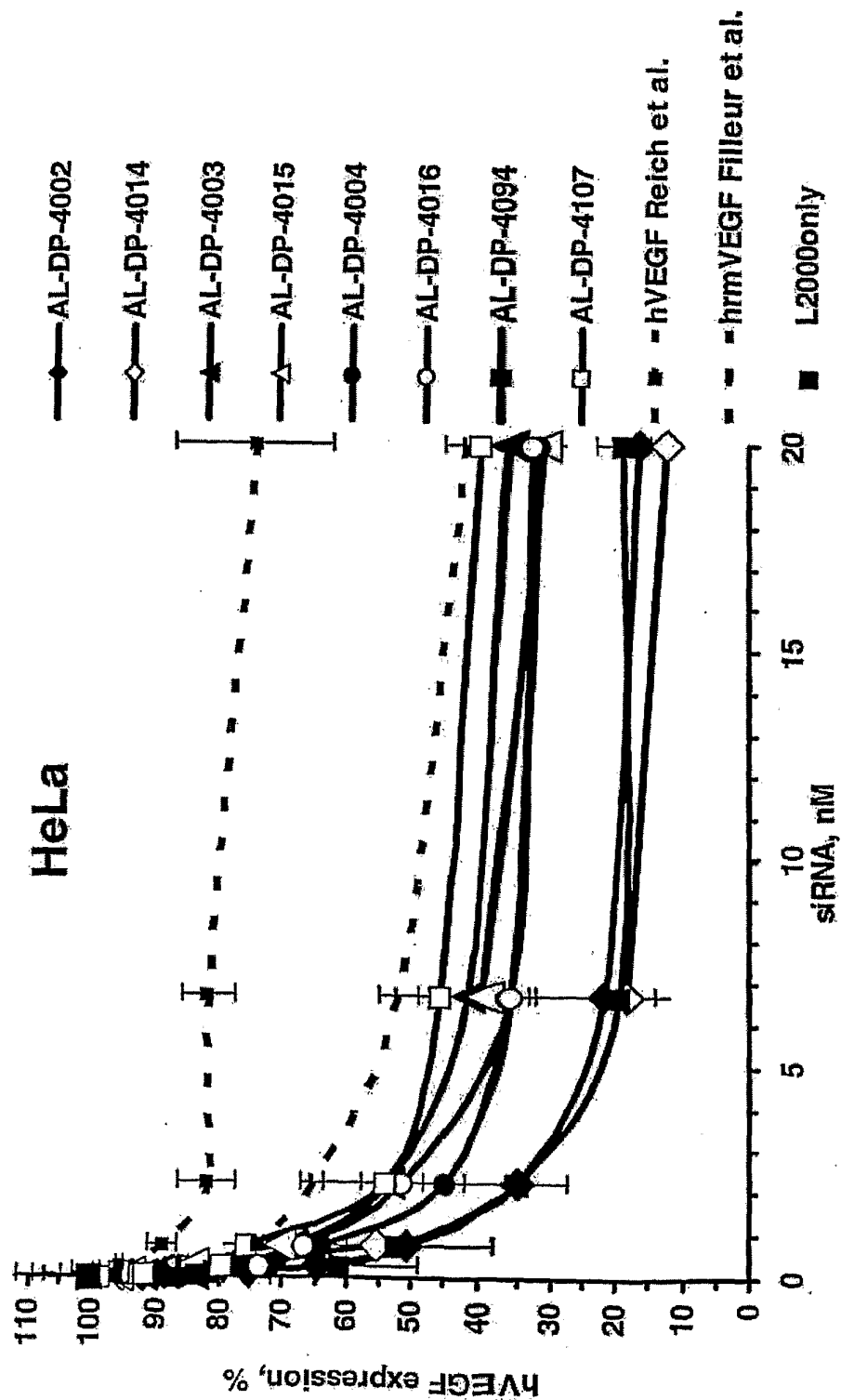


FIGURE 2

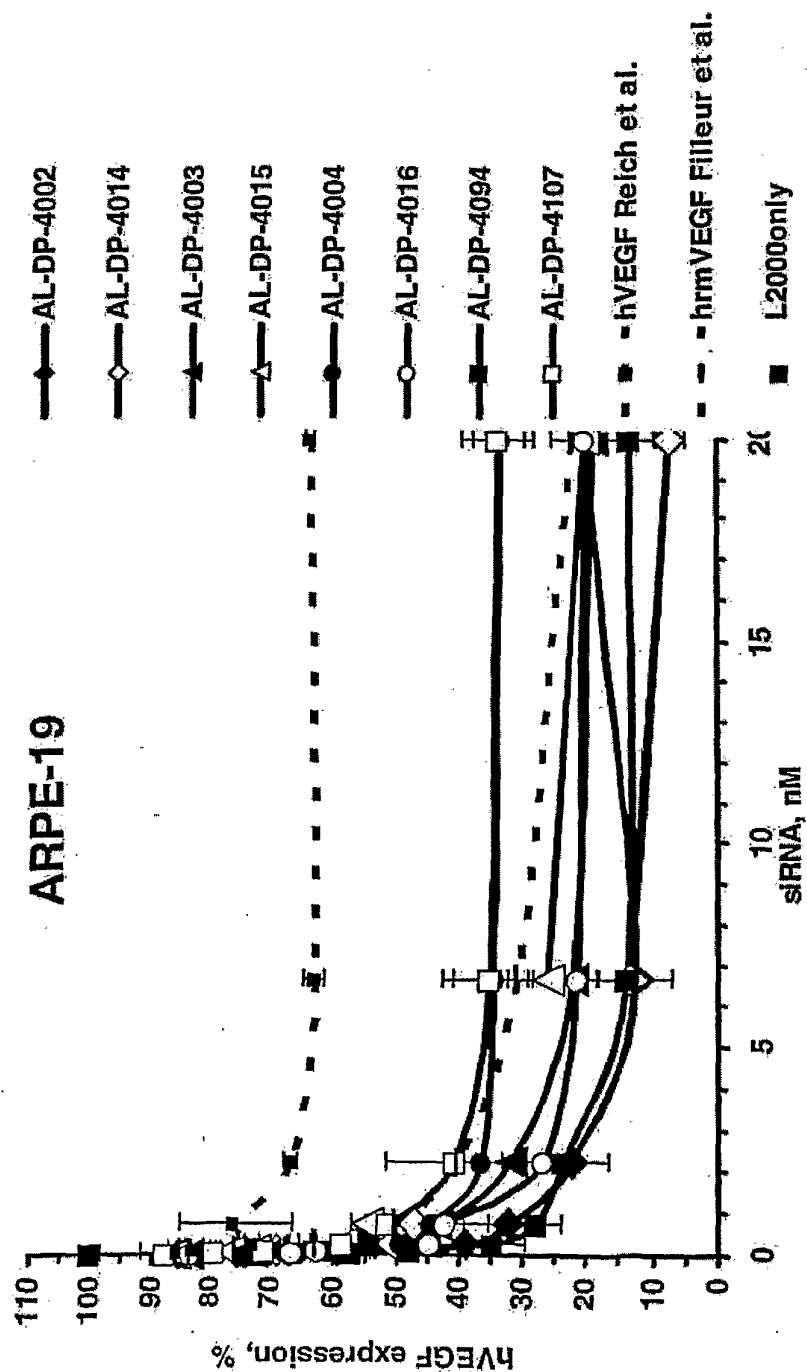


FIGURE 3

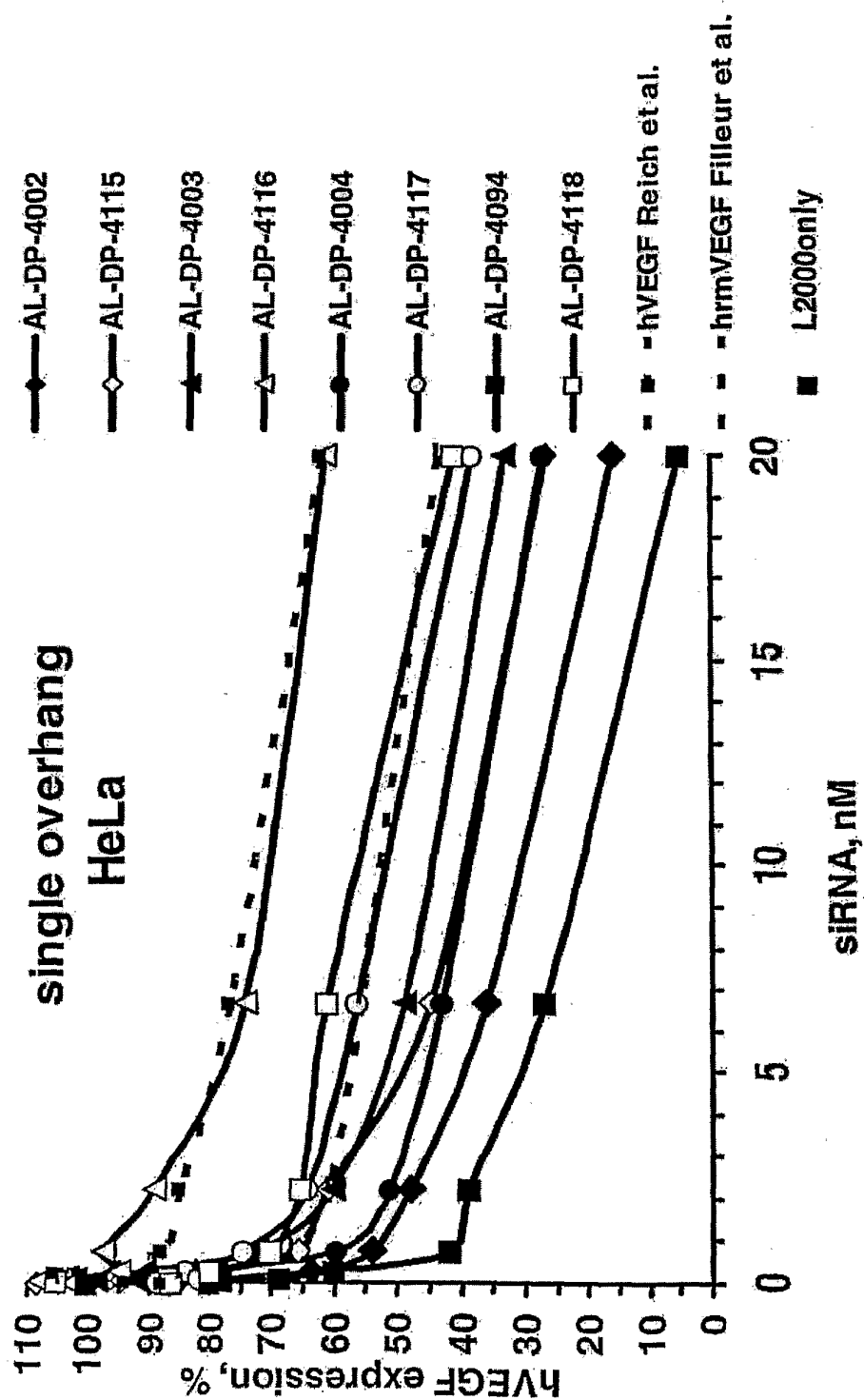


FIGURE 4

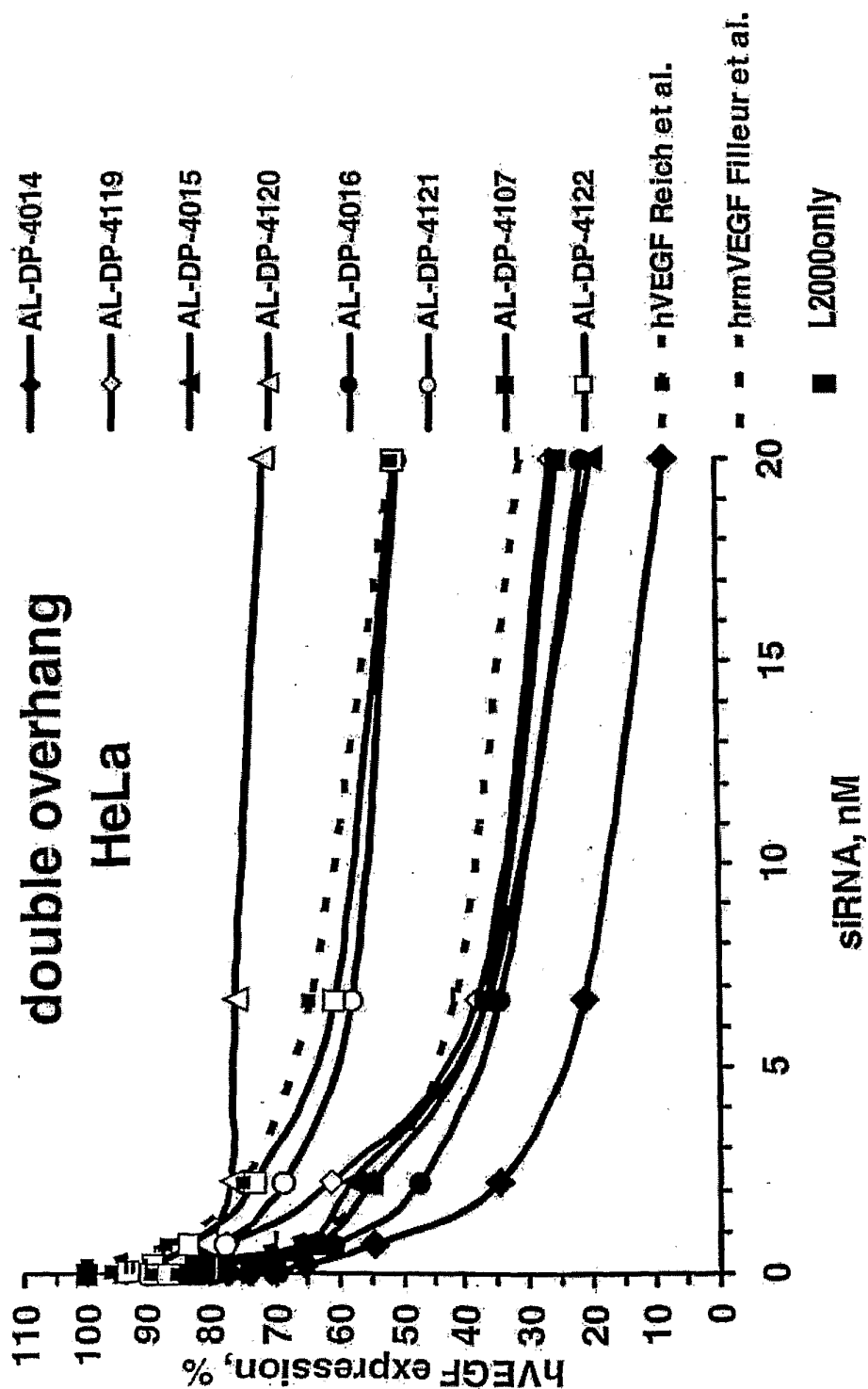


FIGURE 5

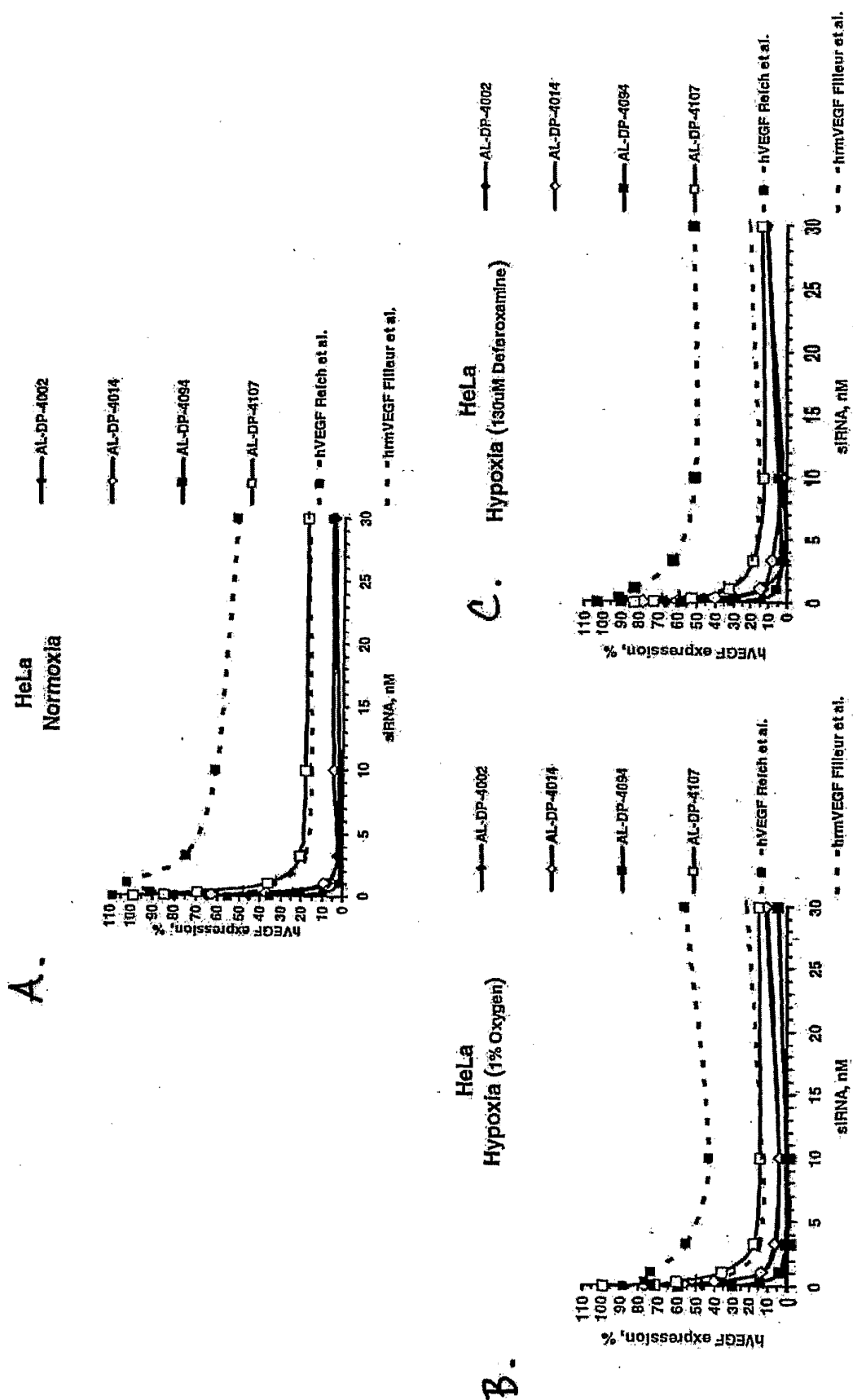


FIGURE 6

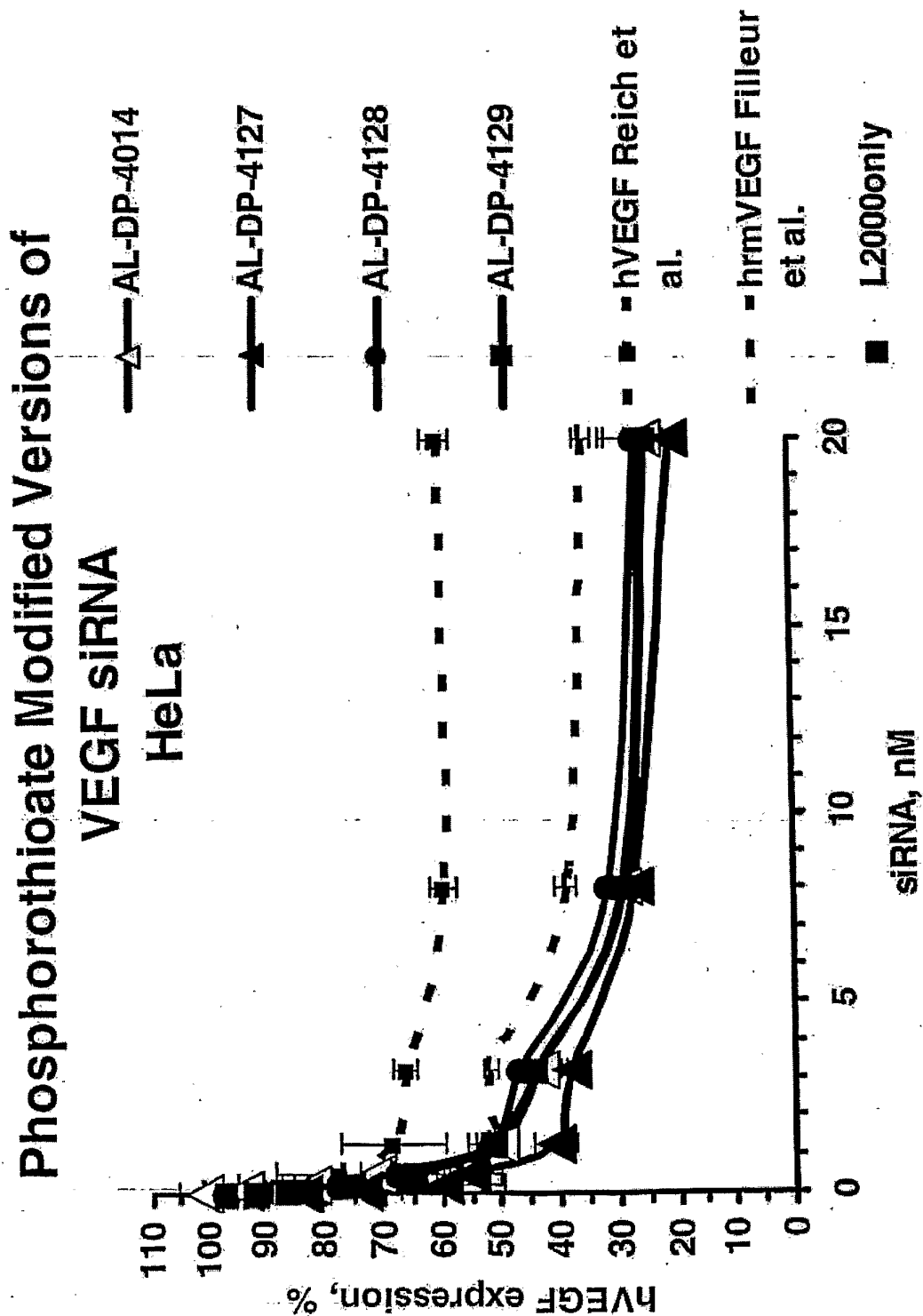


FIGURE 7

FIG. 8A

HeLa - Normoxia

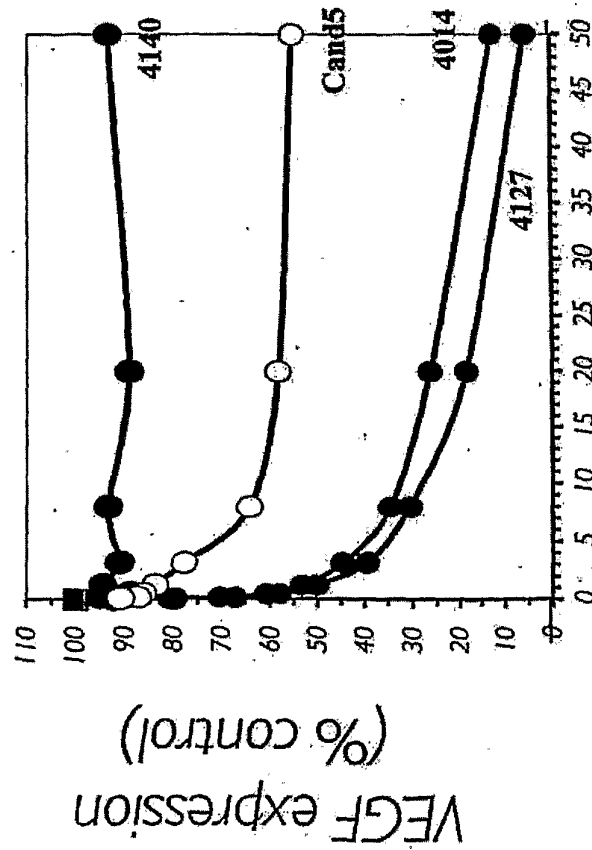
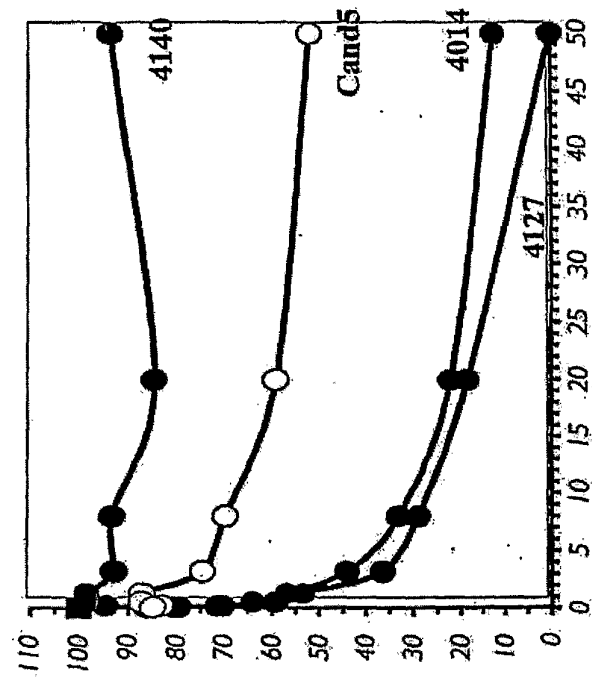


FIG. 8B

HeLa - Hypoxia



siRNA (nM)

—●— AL-DP-4014 (Unmodified) —○— Cand5 Reich et al.

—●— AL-DP-4127 (PS Modified)

■ L2000 only

—●— AL-DP-4140 (Mutated Modified)

A.

VEGF expression, %

h

AL-OP-4198
AL-OP-4185
AL-OP-4168
AL-OP-4167
AL-OP-4168
AL-OP-4188
AL-OP-4084
Acuity
Filler
L2000 only

B.

VEGF expression, %

h

AL-OP-4170
AL-OP-4171
AL-OP-4172
AL-OP-4173
AL-OP-4174
AL-OP-4175
AL-OP-4084
AL-OP-4188
Acuity
Filler
L2000 only

C.

VEGF expression, %

h

AL-OP-4188
AL-OP-4185
AL-OP-4184
AL-OP-4183
AL-OP-4182
AL-OP-4181
AL-OP-4180
AL-OP-4179
AL-OP-4178
AL-OP-4177
AL-OP-4176
AL-OP-4175
AL-OP-4174
AL-OP-4173
AL-OP-4172
AL-OP-4171
AL-OP-4170
Acuity
Filler
L2000 only

FIG. 10

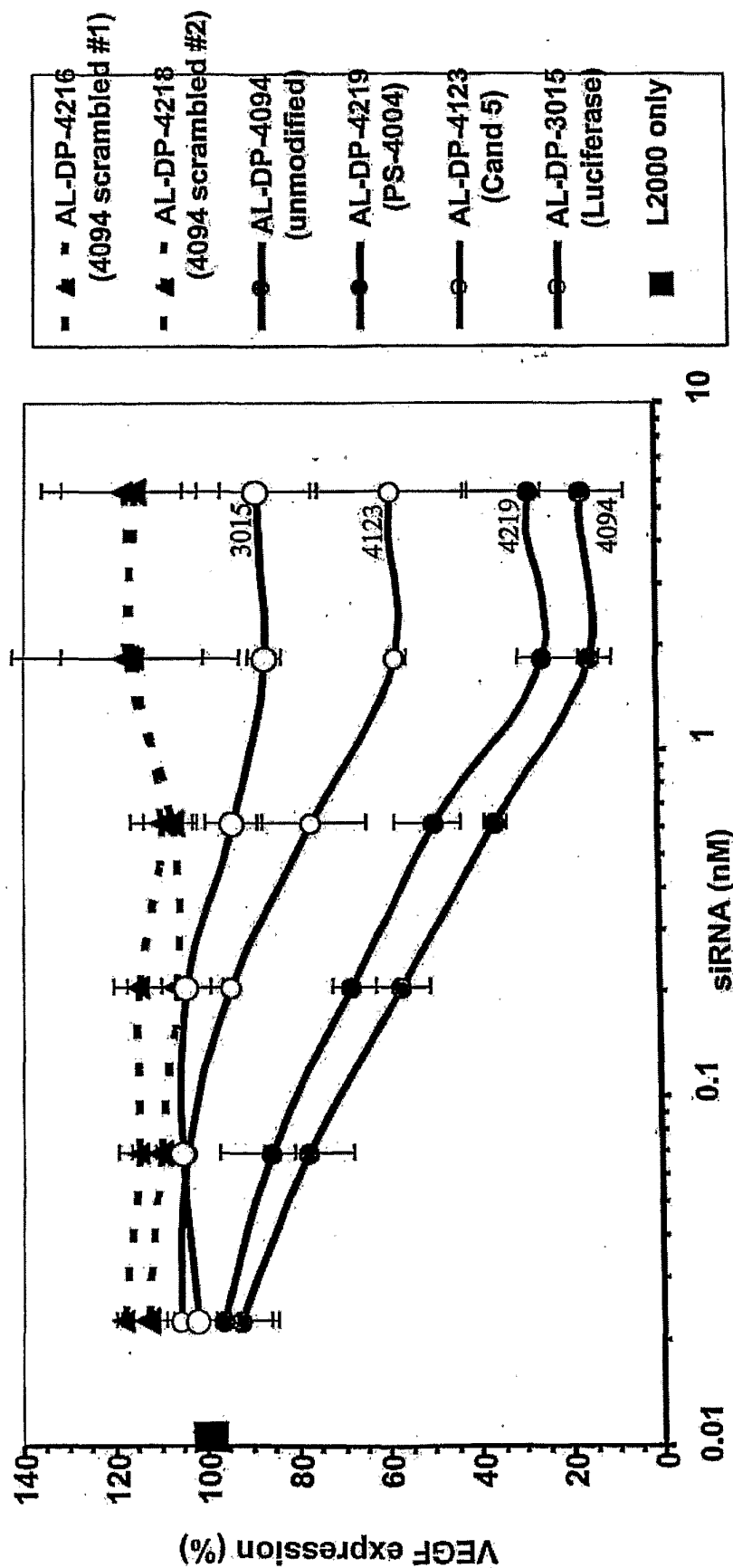


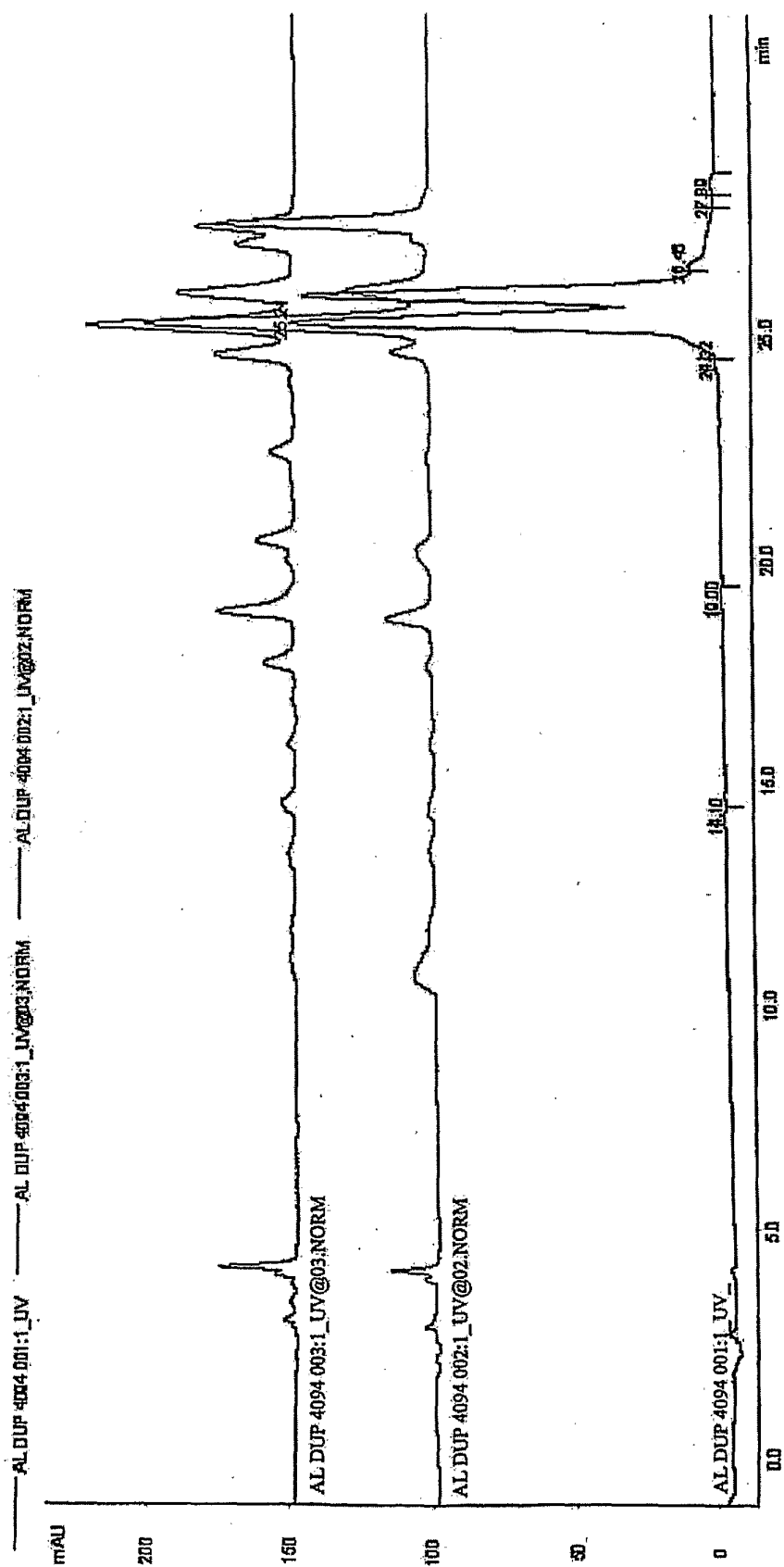
FIG. 11

FIG. 12

AL-DUP-4094_120min				5' - GCACAUAGGAGAGAUAGAGCUU-3'					
				3' - GUUGUGUAUCCUCUCUAGUCGAA-5'					
RT [min]	Strand	3-Exo/Endo	cleavage site	Fragment	Phosphate	cal. Mass	exp. Mass	Remarks	
1,17	sense	endo	U ₆ -A ₇	5' - GCACAU	3-Phosphate	1920,2	1920,0	+ some unidentified masses	
10,34	antisense	endo	U ₆ -C ₂	UACUCGAA-5'	cyclic Phosphate	2554,5	2554,0	small after 30min - slow kinetics	
14,61	sense	3-exo of next fragment		AGGAGAGAUAGAGCU	-	4571,8	4572,0		
15,85	sense	endo	U ₆ -A ₇	AGGAGAGAUAGAGCUU-3'	-	4878,0	4878,0		
17,32	antisense	endo	U ₁₅ -A ₁₆	UCCUCUCUACUCGAA-5'	cyclic Phosphate	4693,8	4693,0		
19,37	antisense	endo	U ₁₅ -A ₁₆	UCCUCUCUACUCGAA-5'	3-Phosphate	4711,8	4711,0	not detected after 30min	
21,45	sense	3-exo	n-1	5' - GCACAUAGGAGAGAUAGAGCU	-	6492,0	6492,5	small after 30min	
22,16	sense	FLP		5' - GCACAUAGGAGAGAUAGAGCUU	-	6798,2	6797,5	only small amounts of as-FLP detected	
22,86	antisense	3-exo	n-2	CGUGUAUCCUCUCUACUCGAA-5'	cyclic Phosphate	6630,9	6631,0		
23,91	antisense	3-exo	n-2	CGUGUAUCCUCUCUACUCGAA-5'	3-Phosphate	6648,9	6648,0	not detected after 30min	
24,32	antisense	3-exo	n-1	UCGUGUAUCCUCUCUACUCGAA-5'	3-Phosphate	6955,1	6954,8		

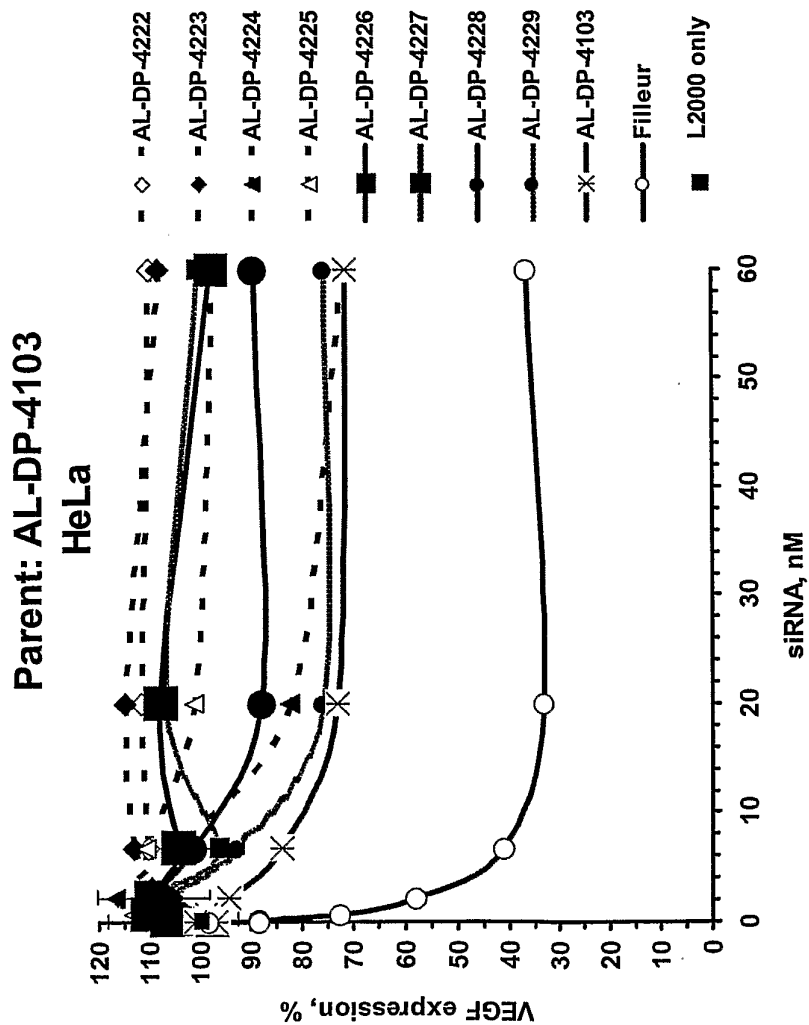


FIGURE 13

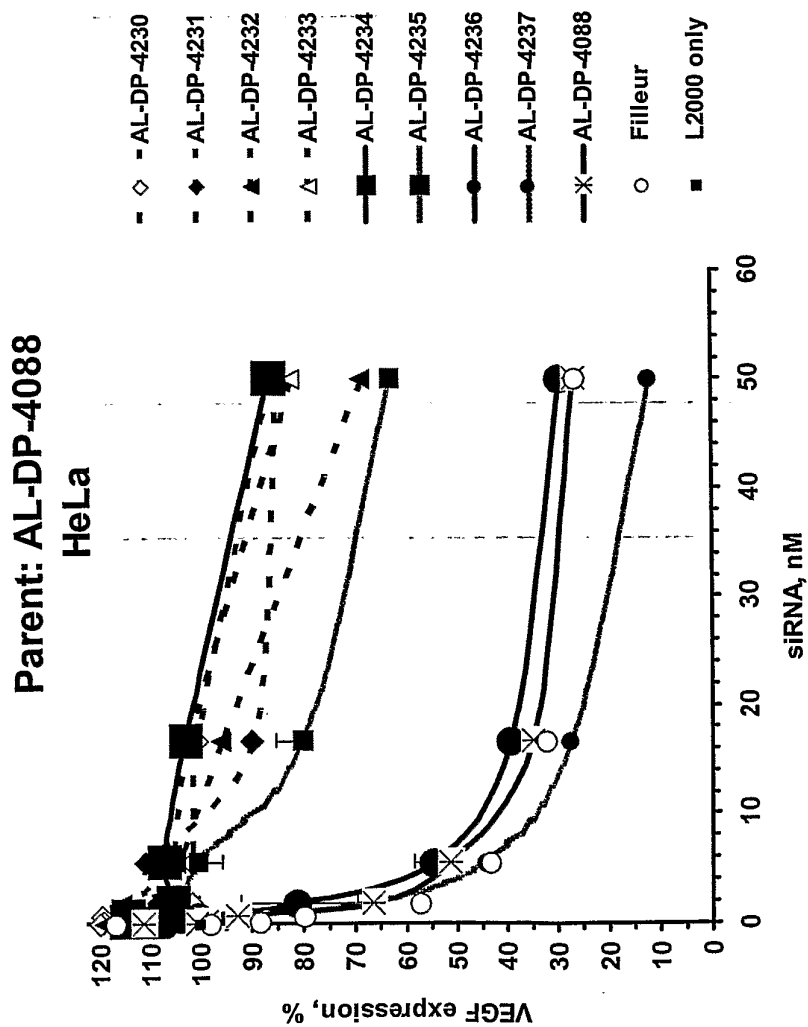


FIGURE 14

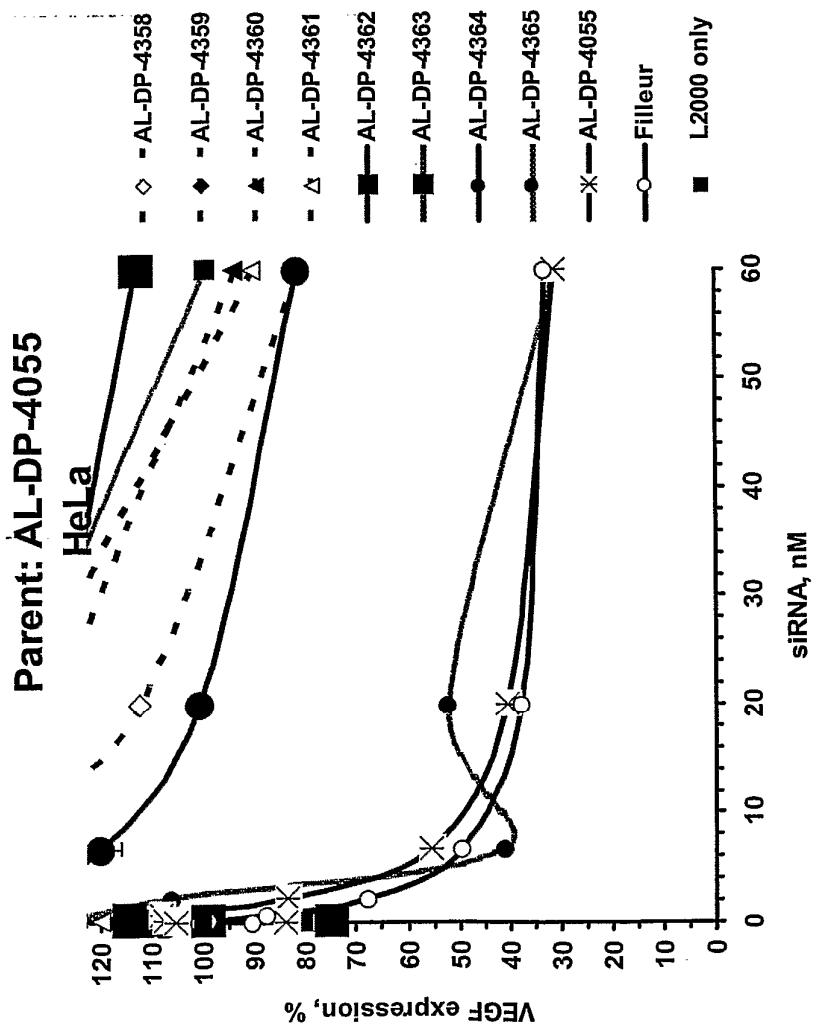


FIGURE 15

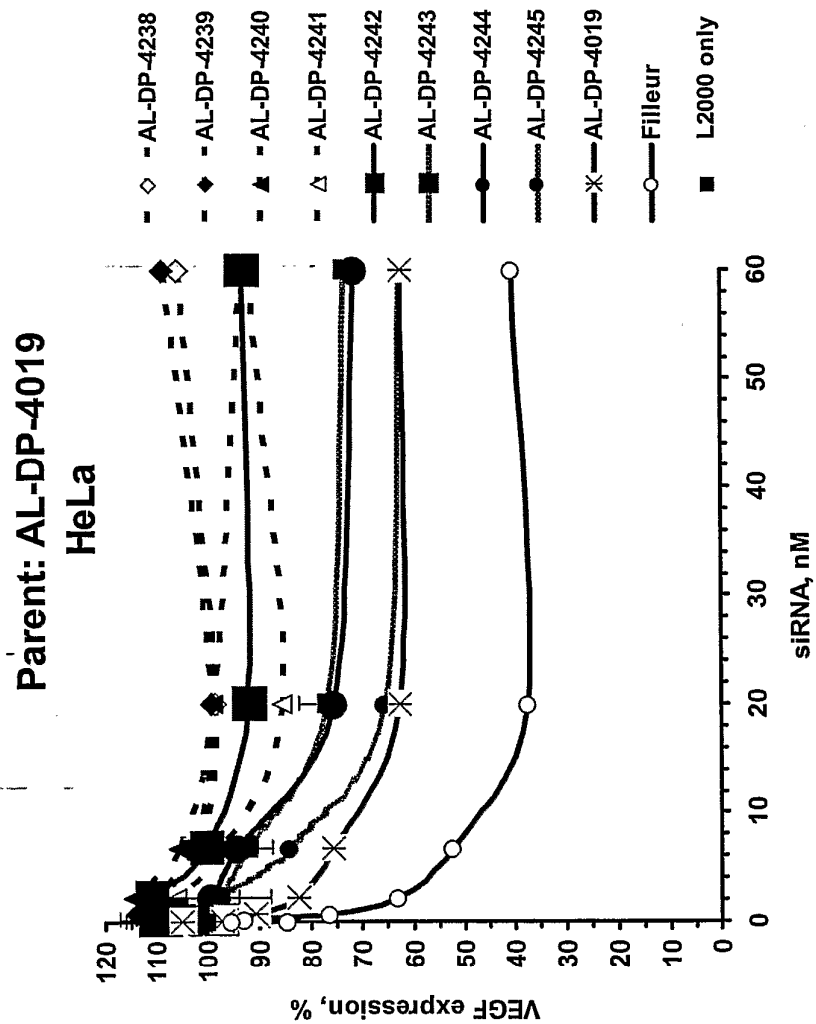


FIGURE 16

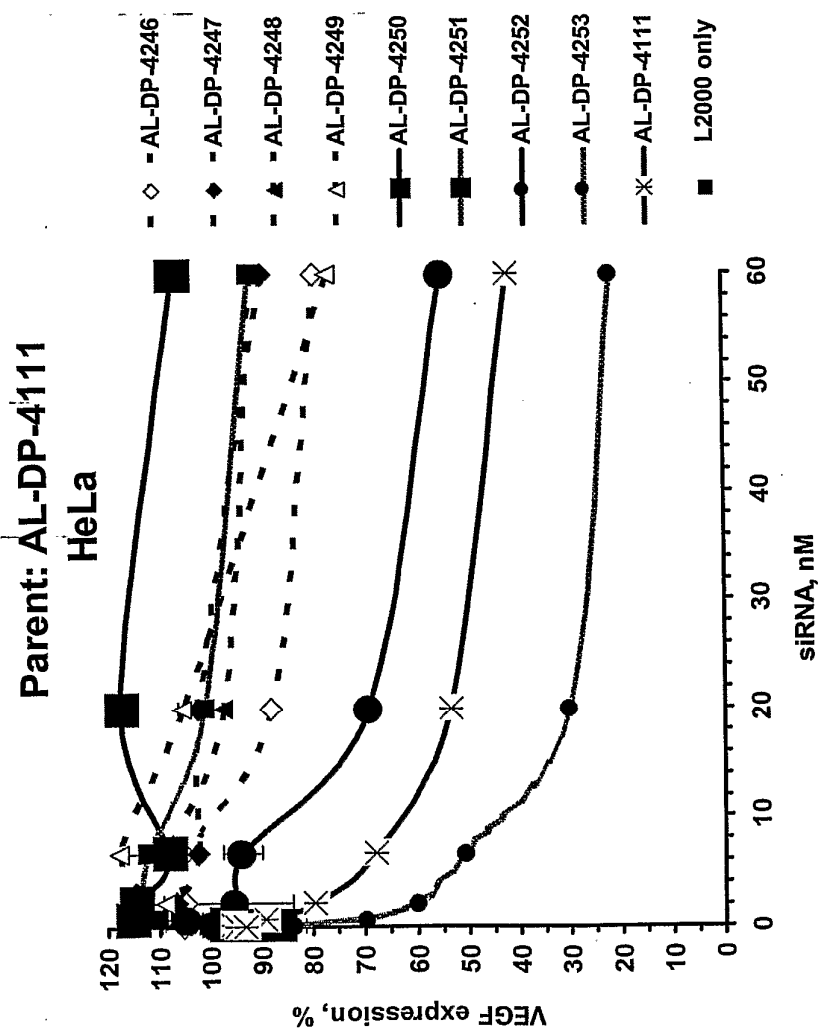


FIGURE 17

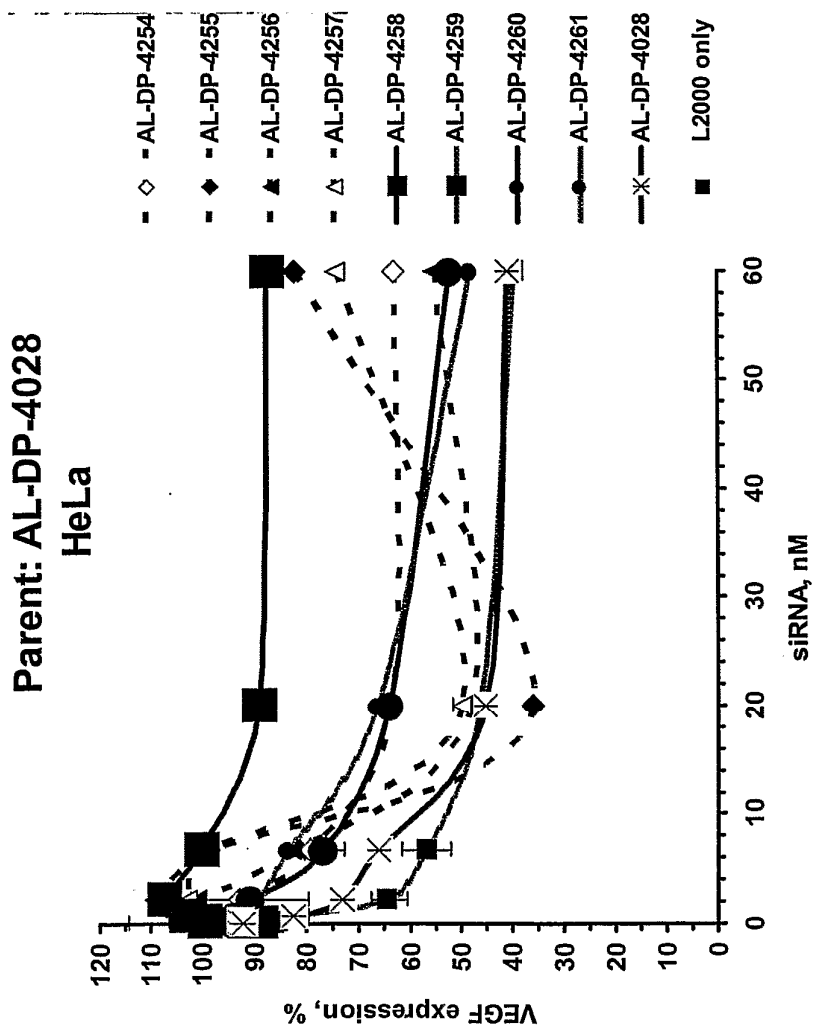


FIGURE 18

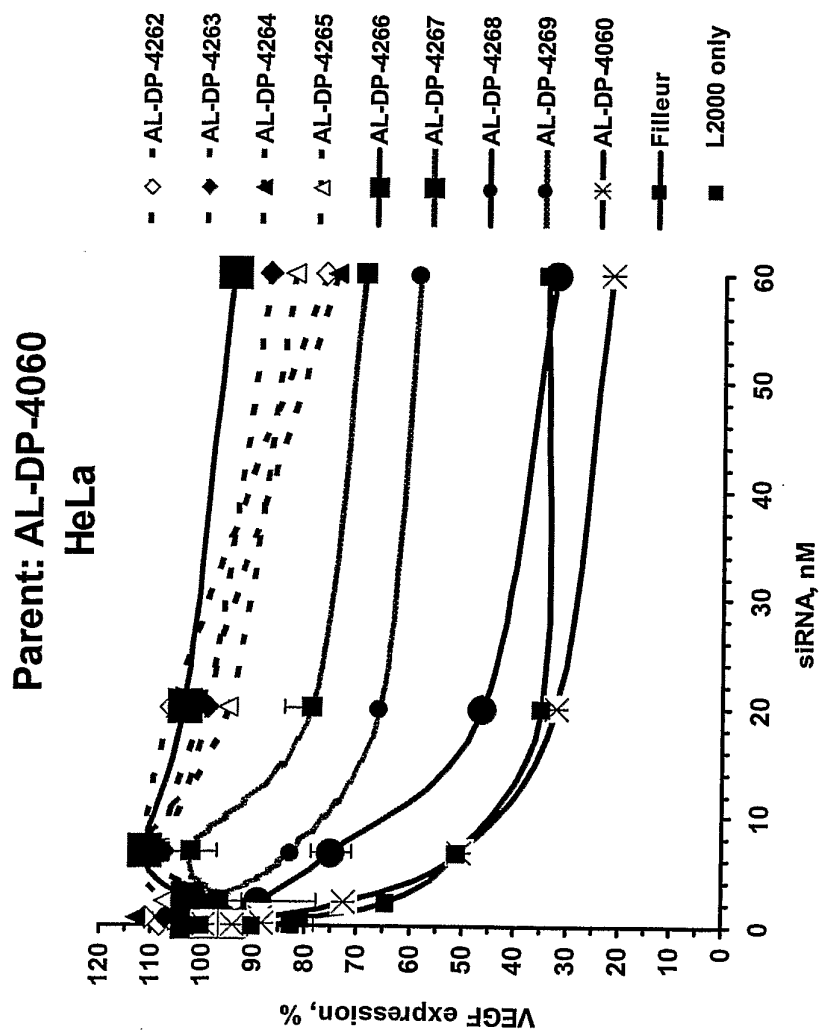


FIGURE 19

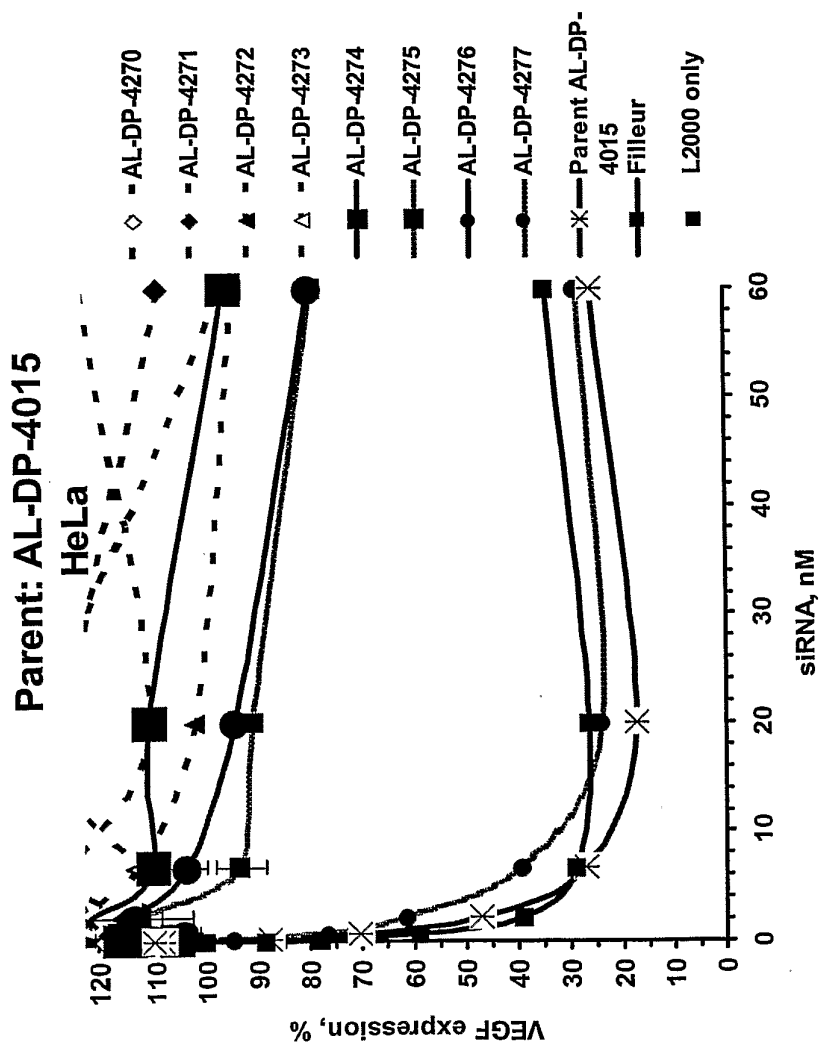


FIGURE 20

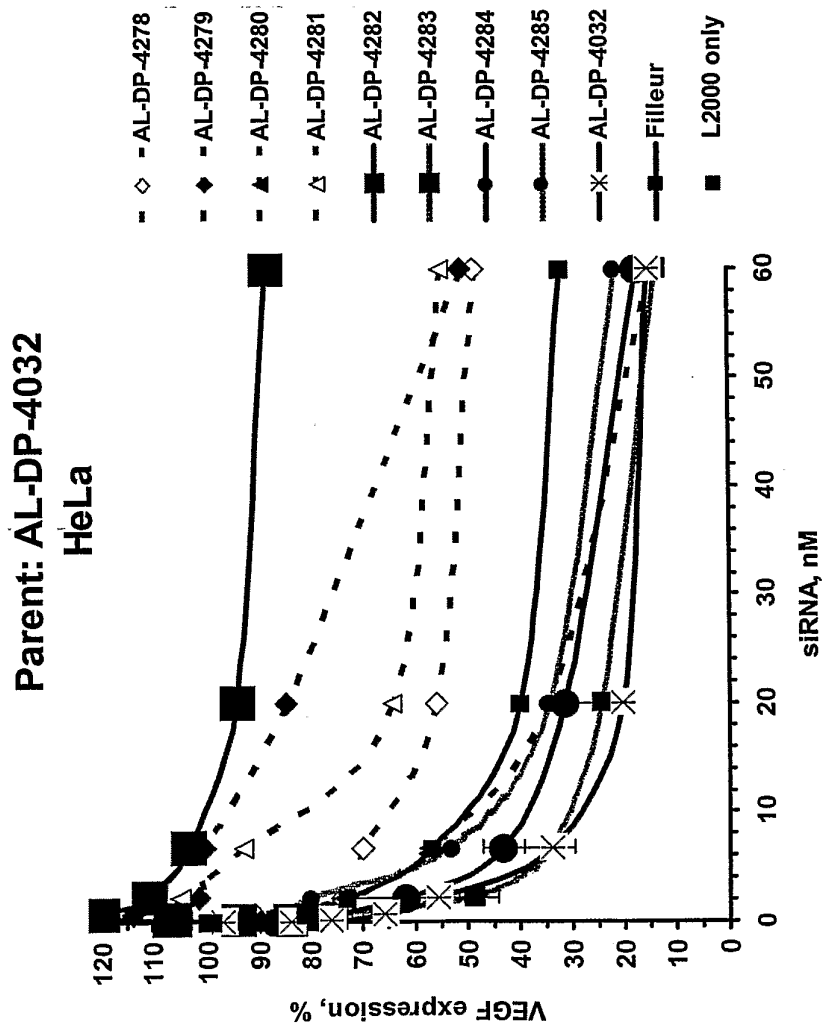


FIGURE 21

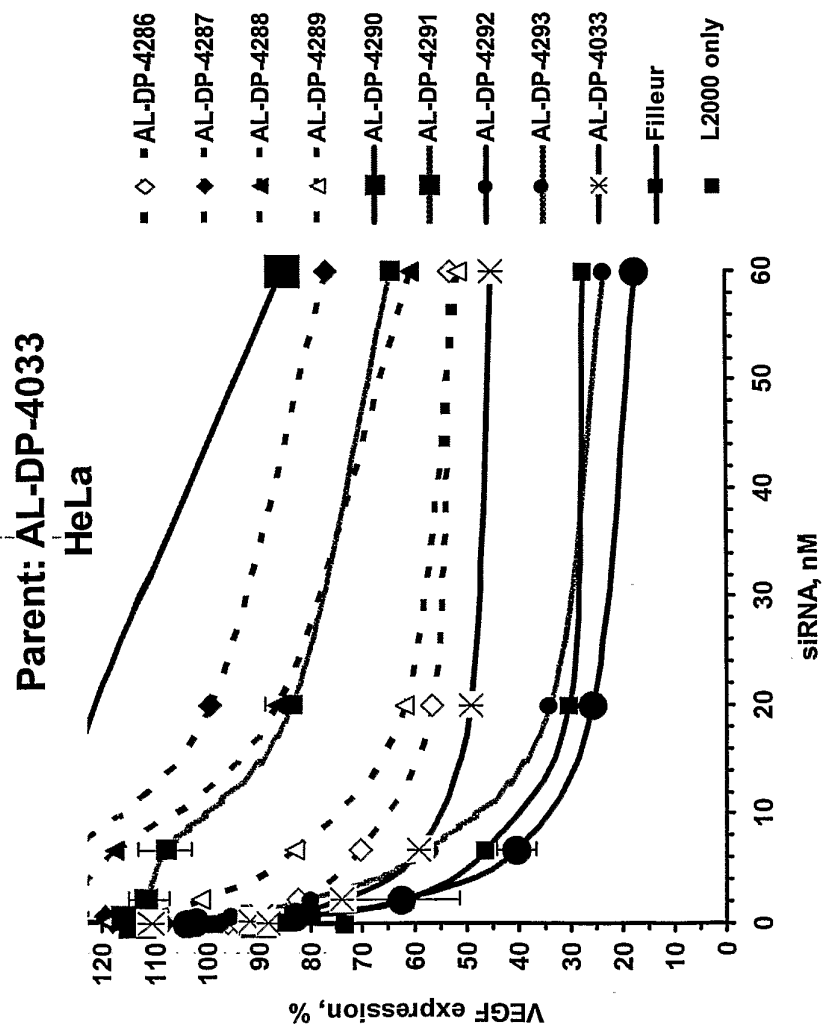


FIGURE 22

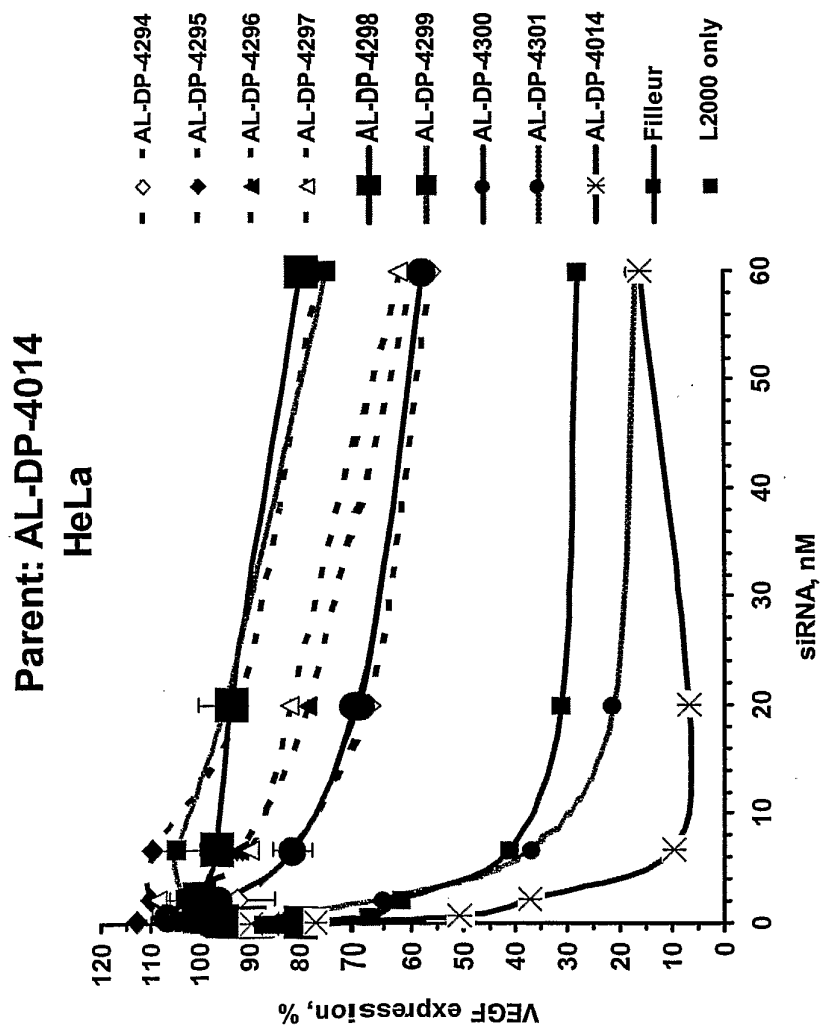


FIGURE 23

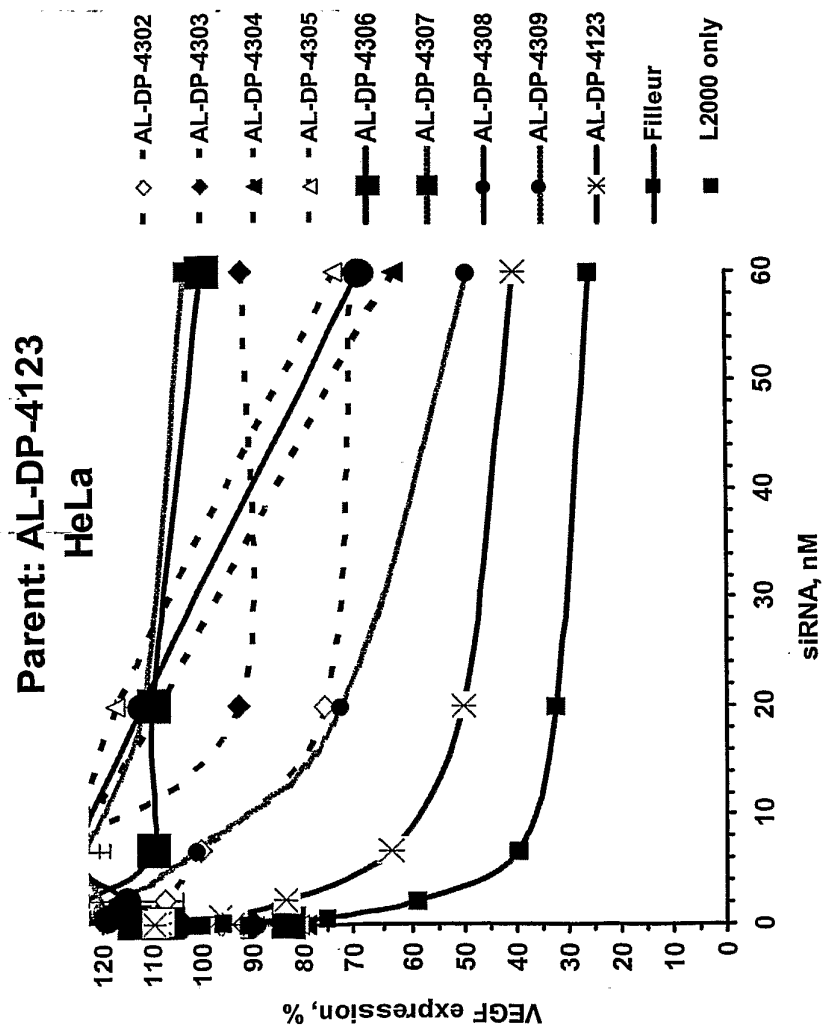


FIGURE 24

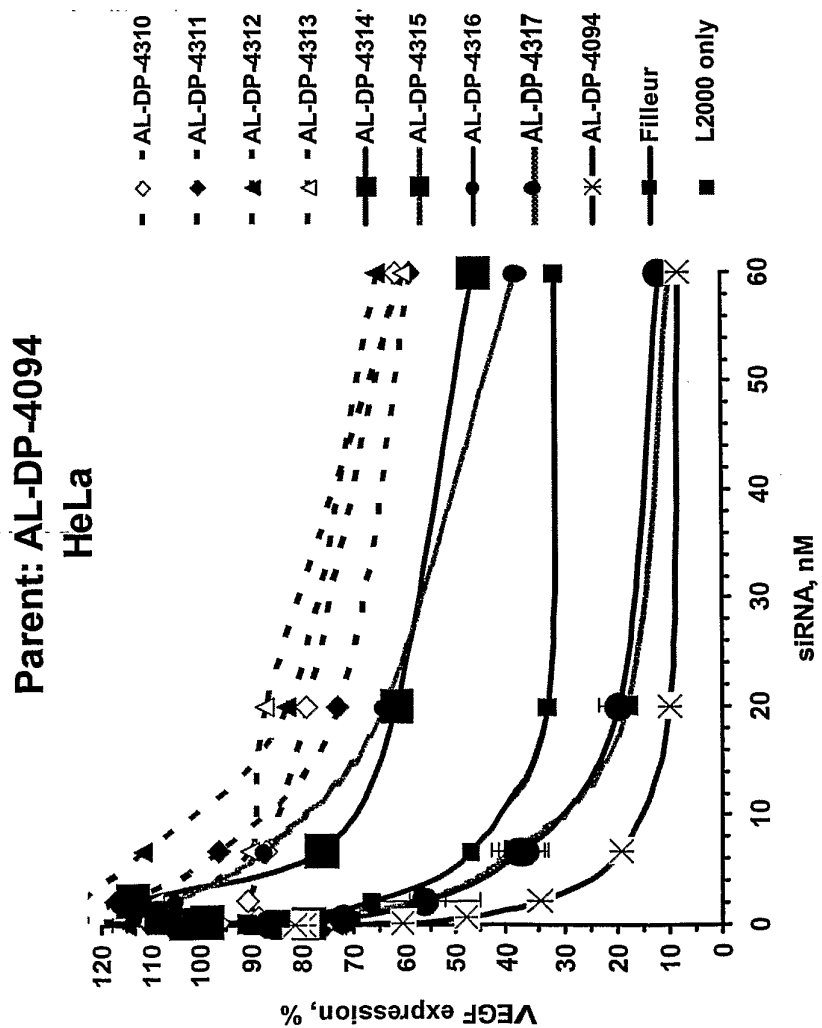


FIGURE 25

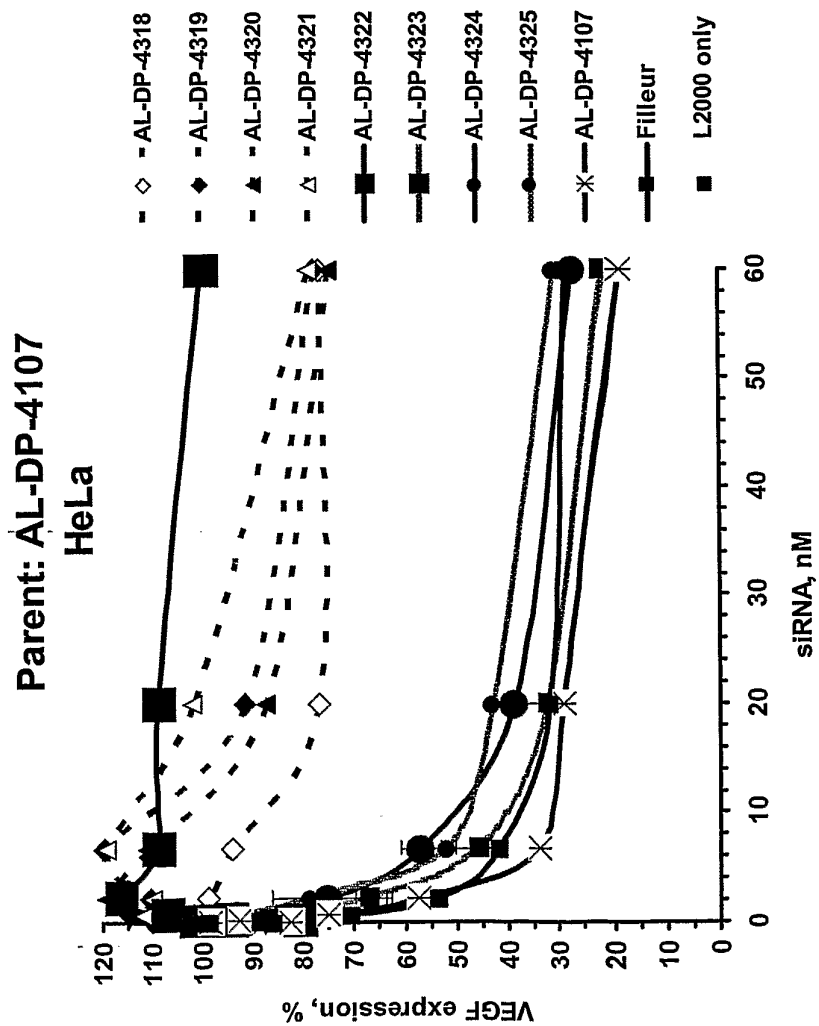


FIGURE 26

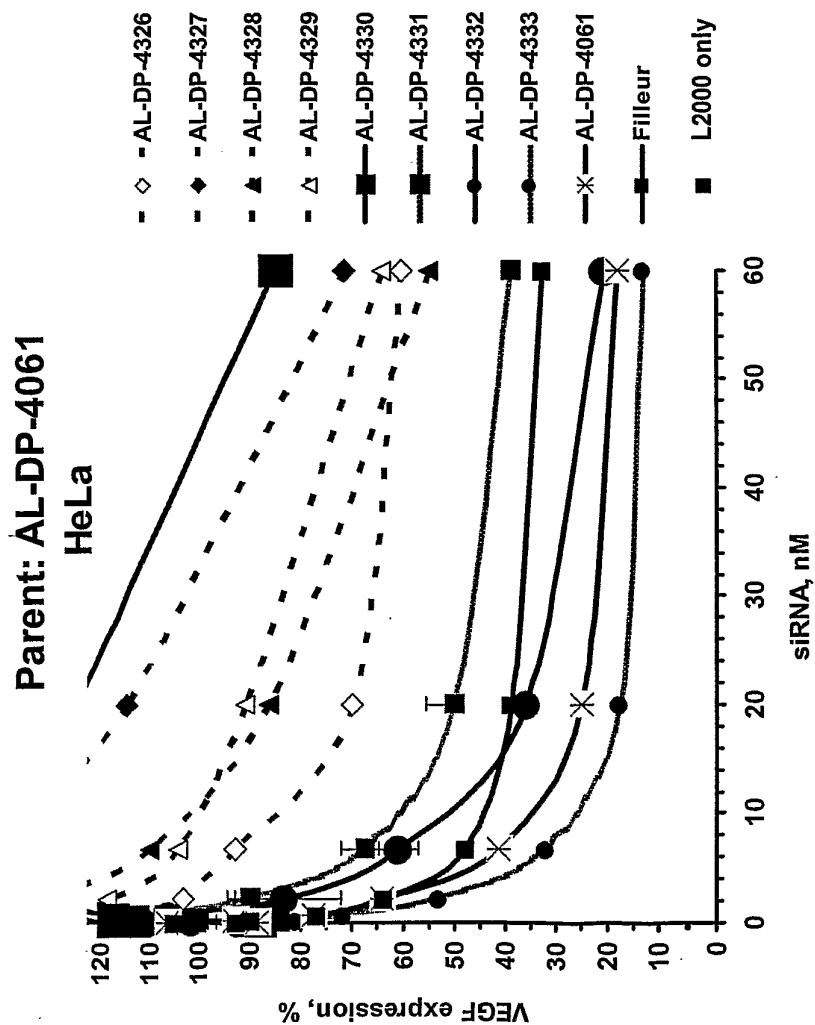


FIGURE 27

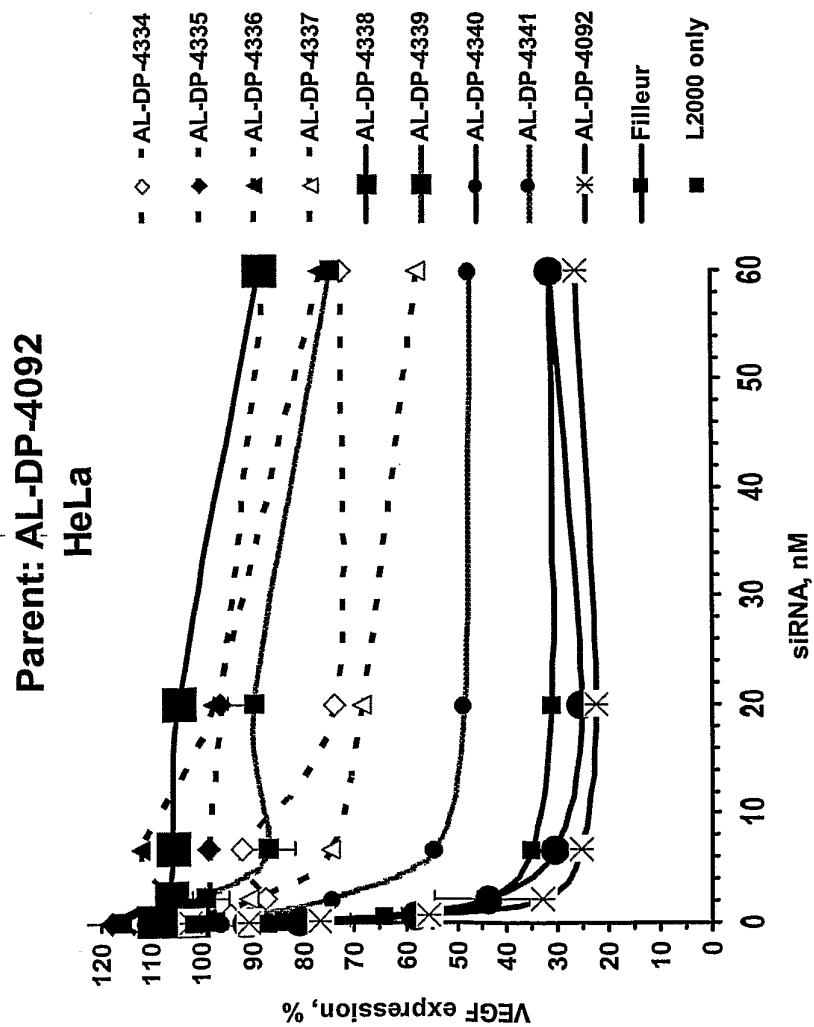


FIGURE 28

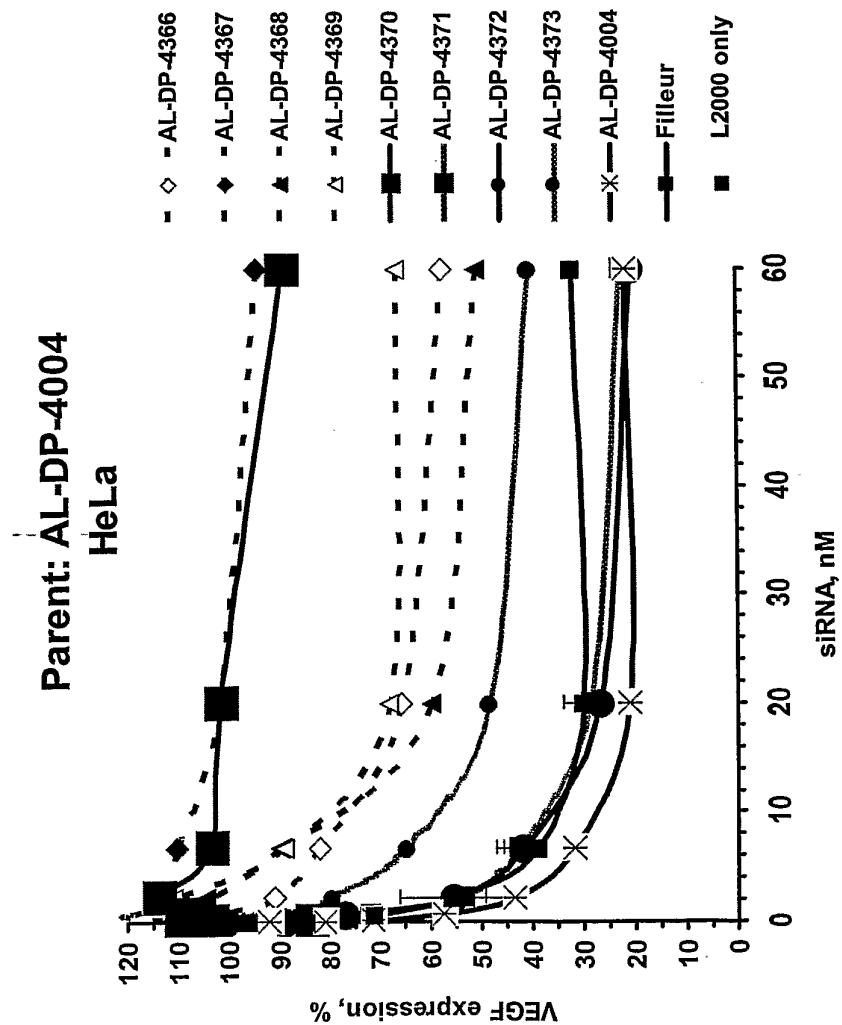
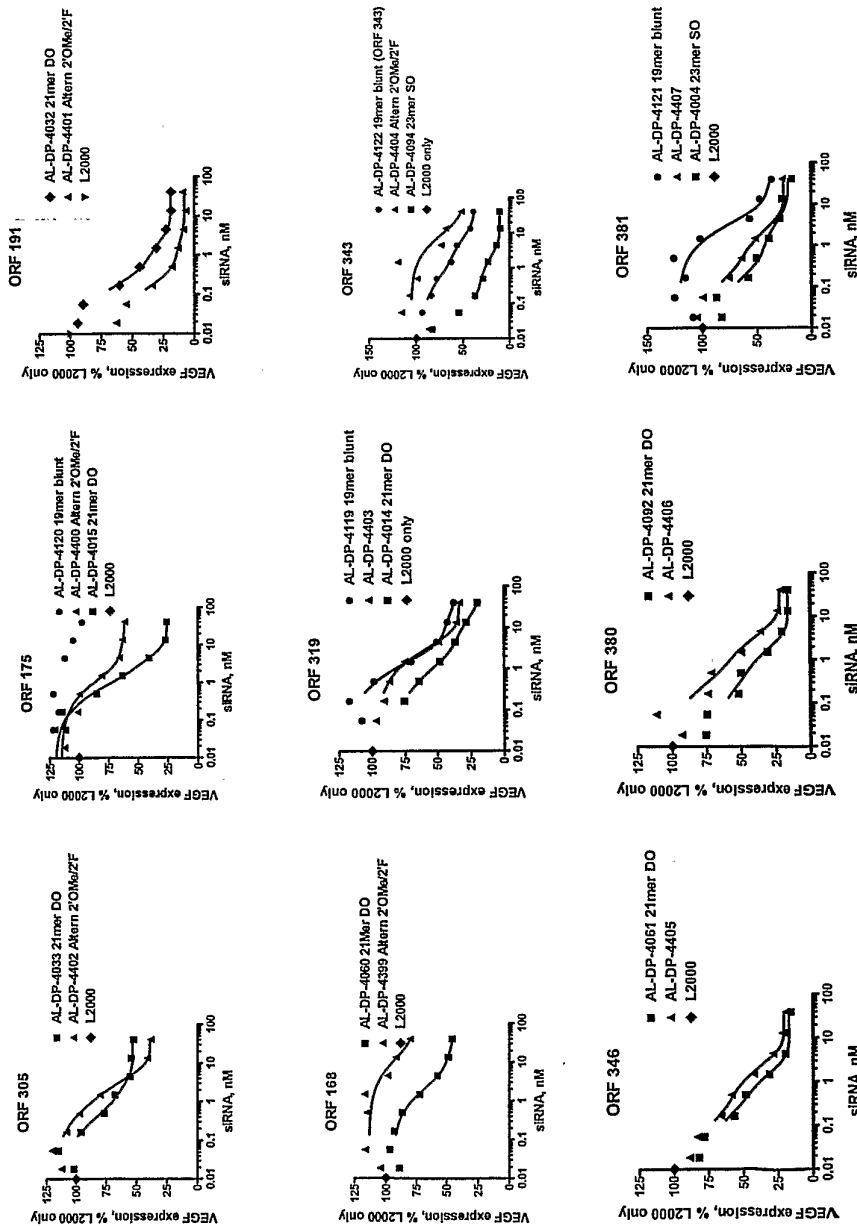


FIGURE 29

ALTERNATING 2'OMe and 2'F



Graph for Table 7

FIGURE 30

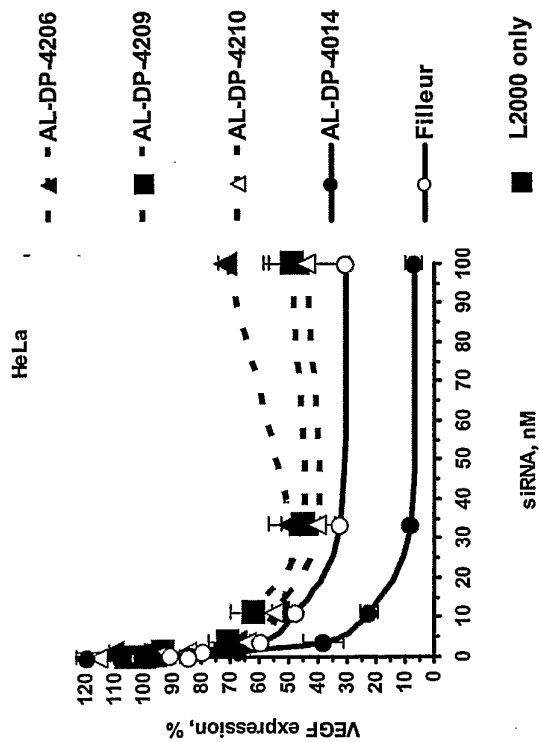


FIGURE 31

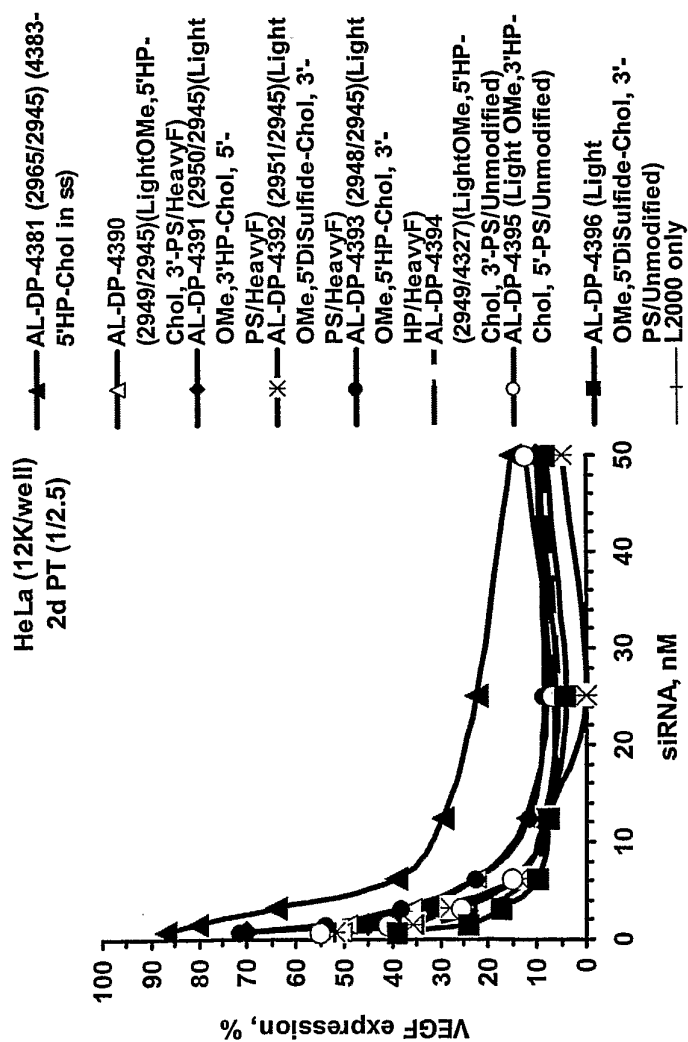


FIGURE 32

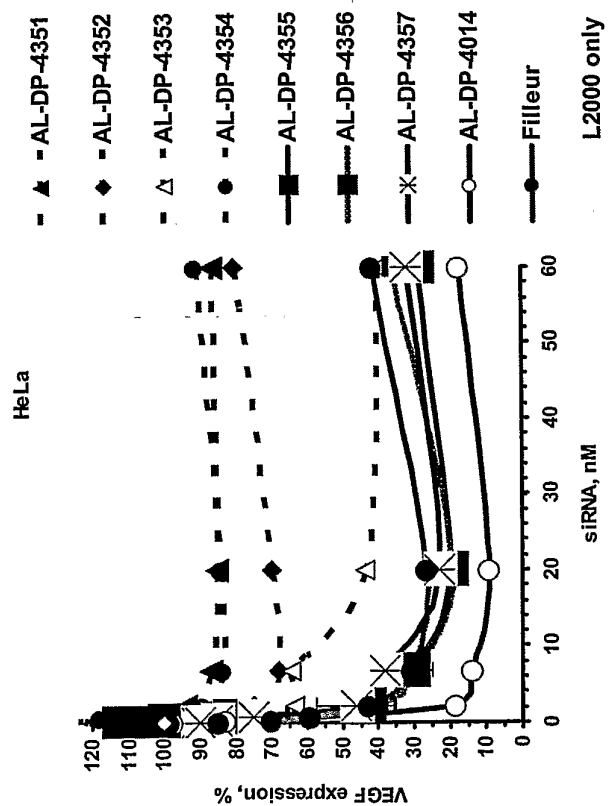


FIGURE 33

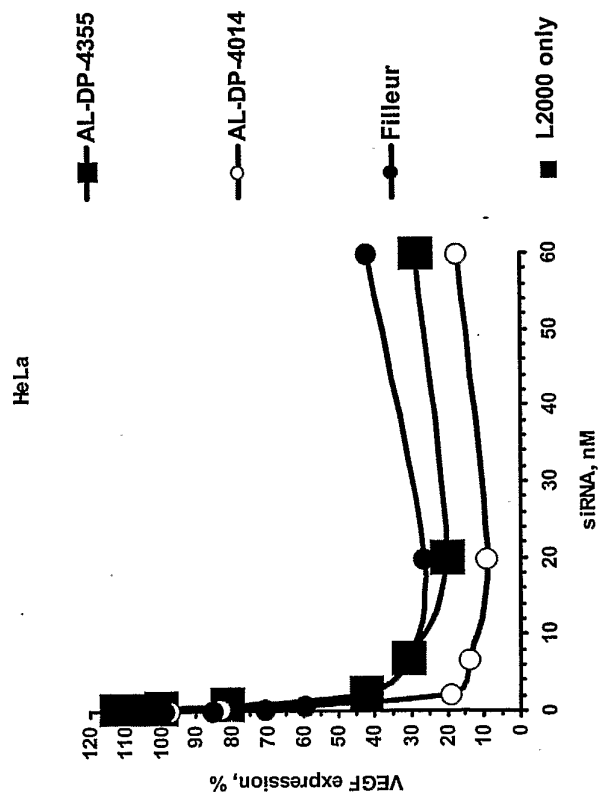


FIGURE 34

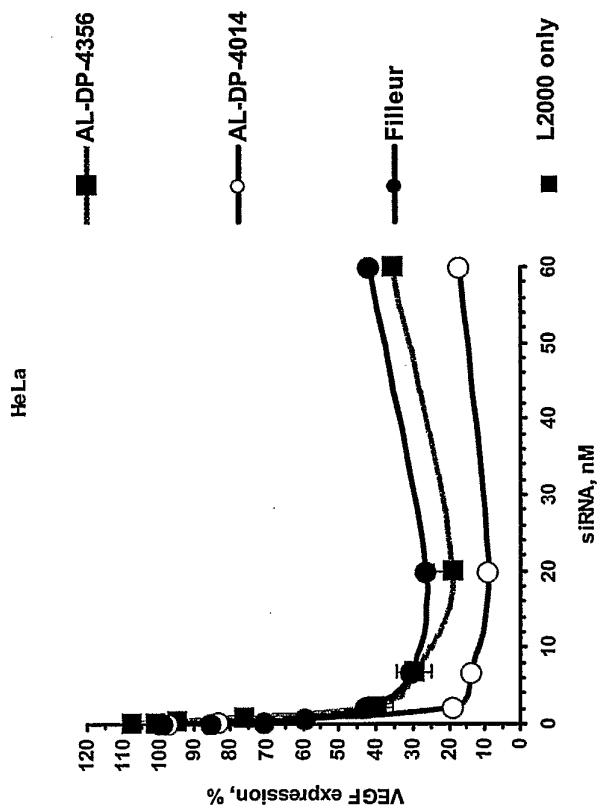


FIGURE 35

(A) 5'Retinal (in ss) Conjugates of 4094 unmodified and 4094 3'PS

ARPE-19

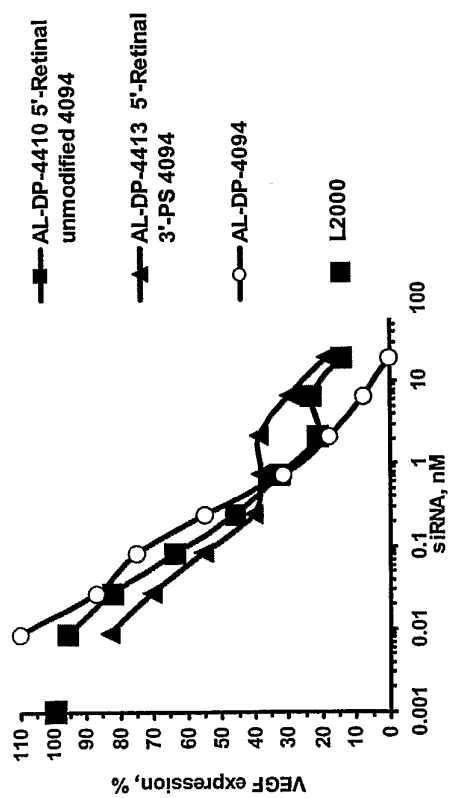


FIGURE 36

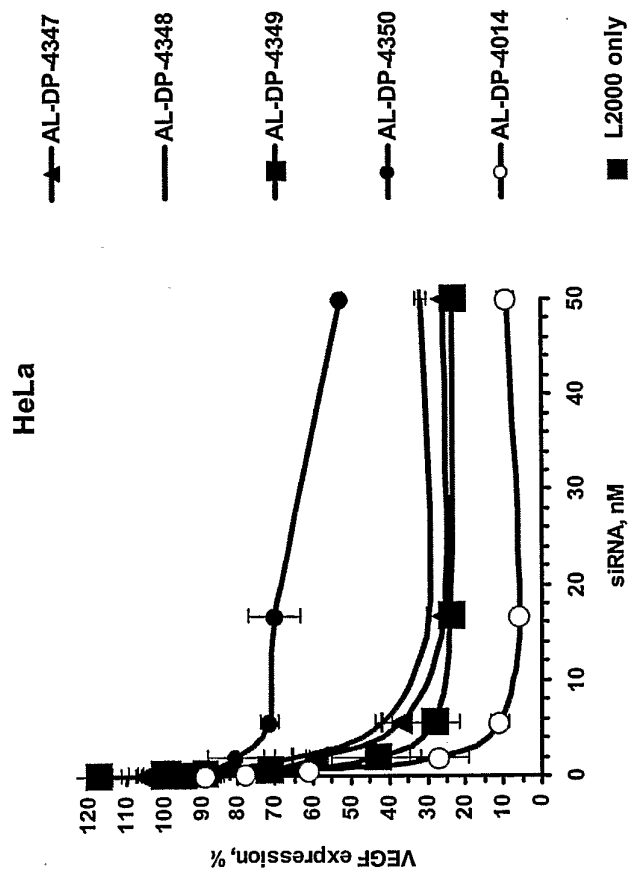


FIGURE 37

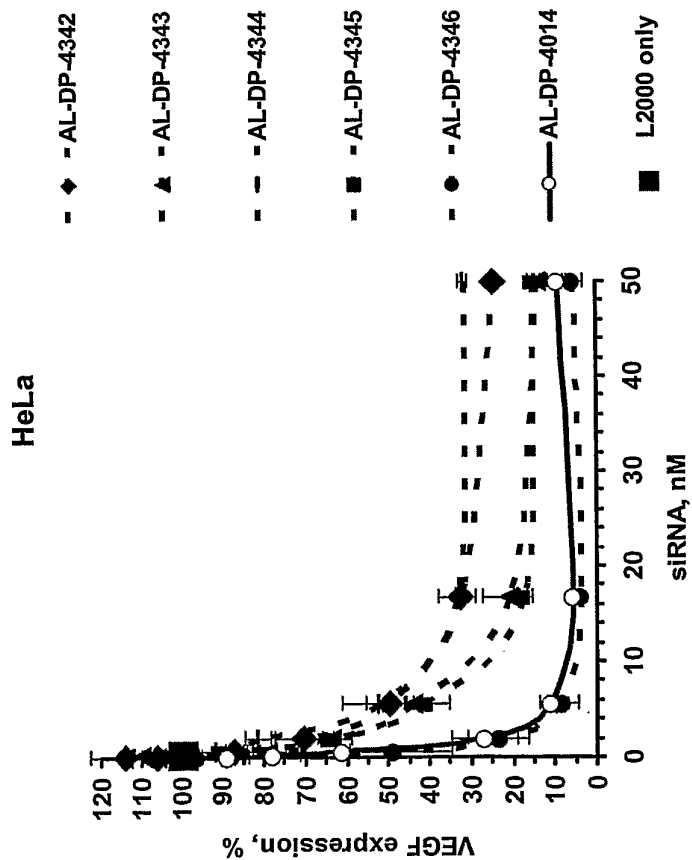
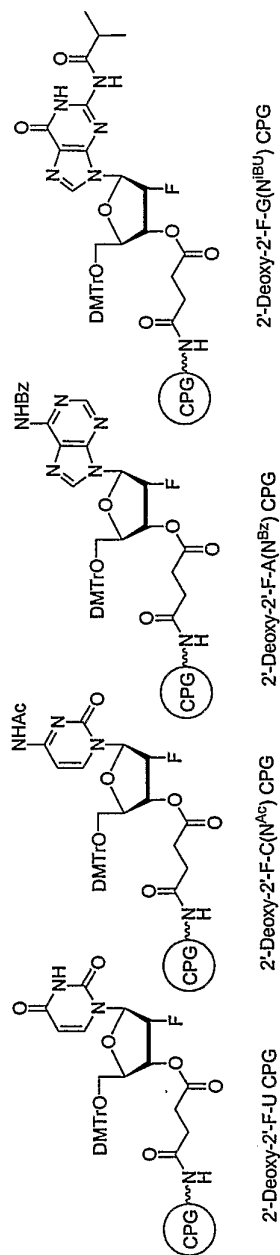
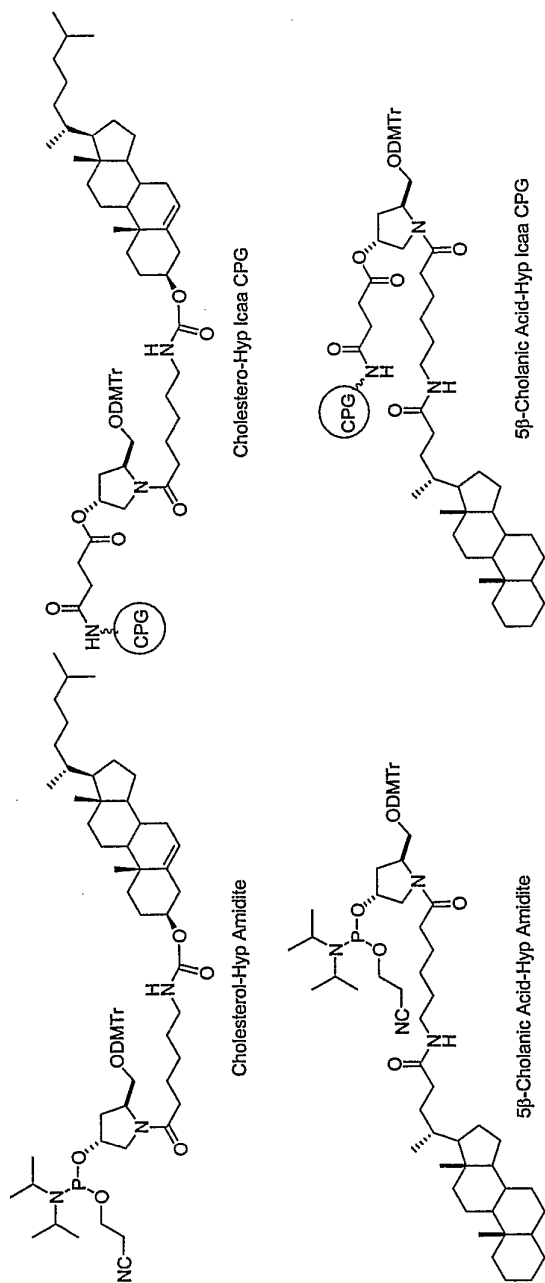
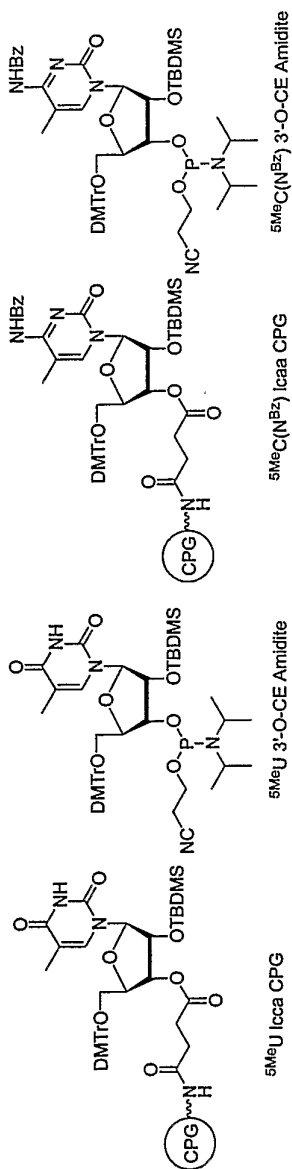
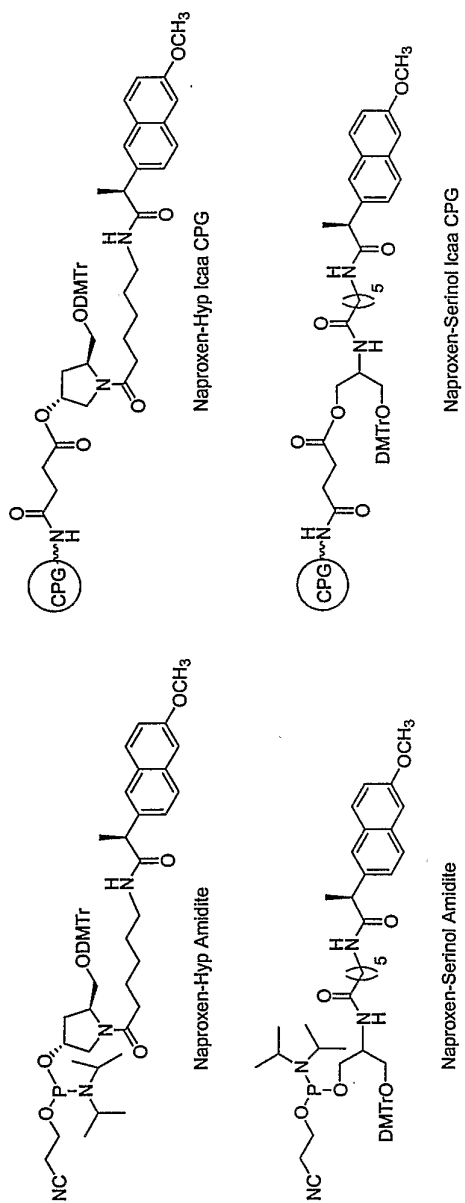


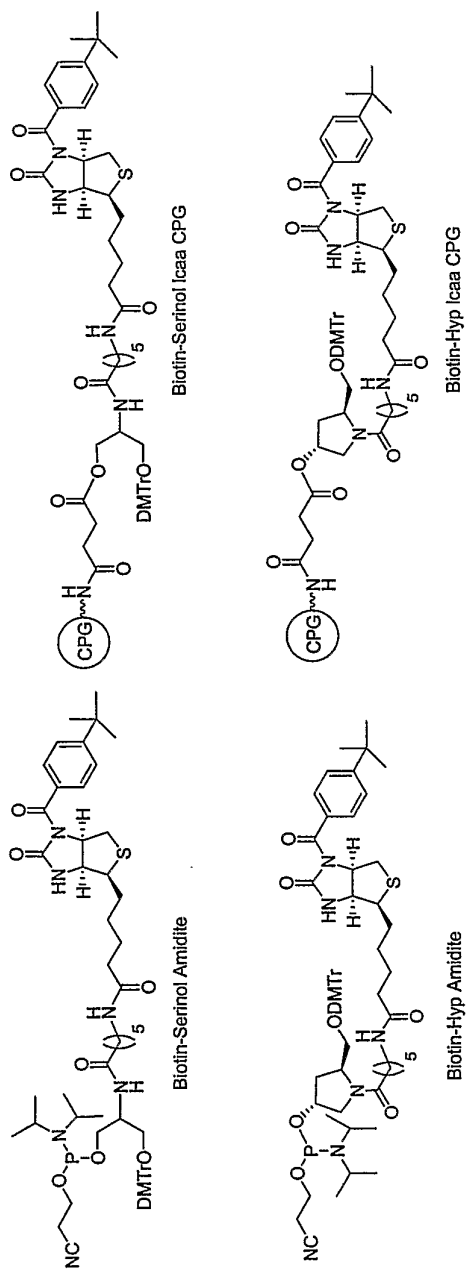
FIGURE 38

**FIGURE 39**

**FIGURE 40**

**FIGURE 41**

**FIGURE 42**

**FIGURE 43**

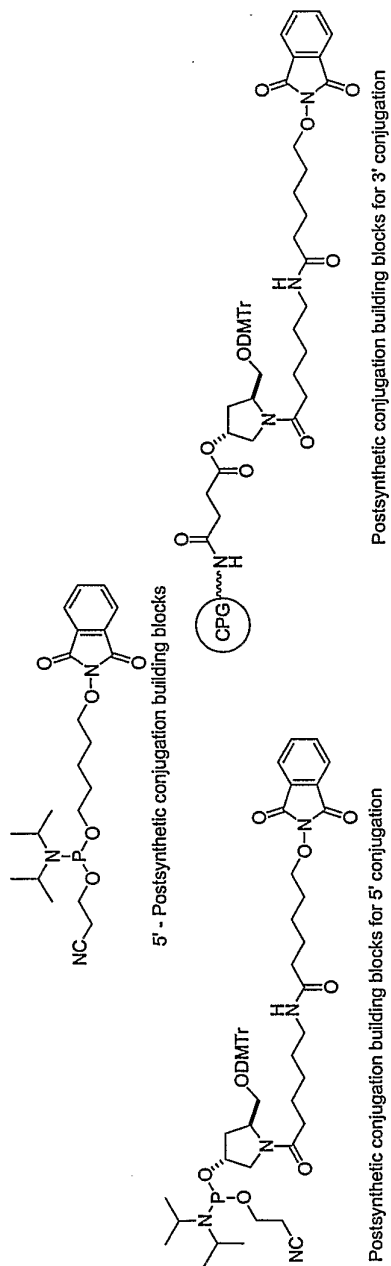
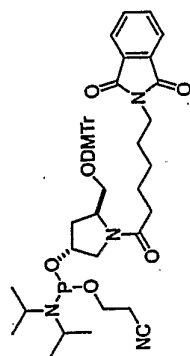
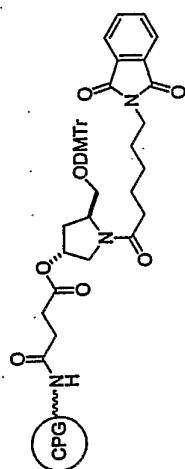


FIGURE 44

**FIGURE 45**

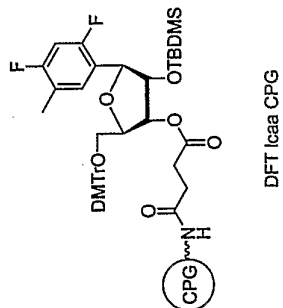
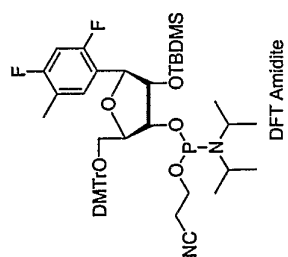


FIGURE 46

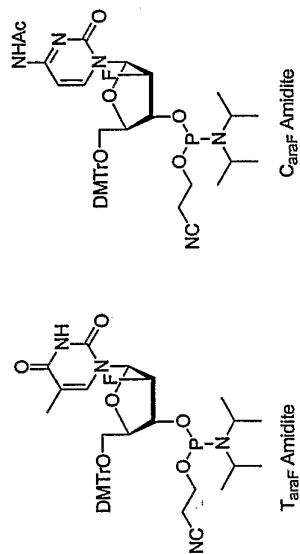
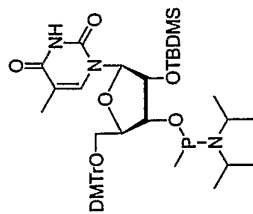
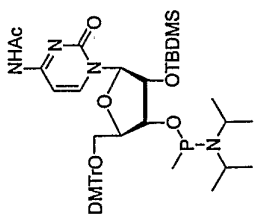


FIGURE 47

**FIGURE 48**

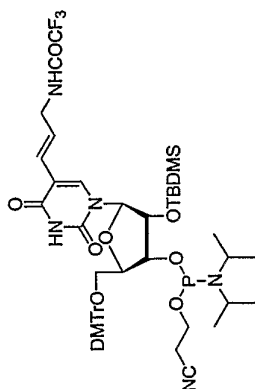
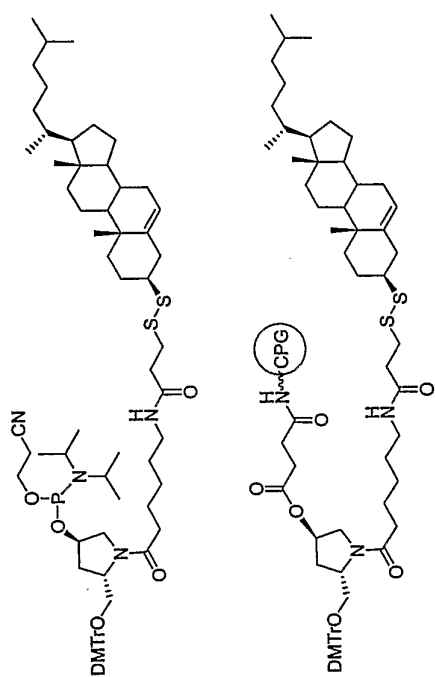


FIGURE 49

**FIGURE 50**