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(54) **Title:** METHODS AND COMPOSITIONS FOR INHALATION DELIVERY OF CONJUGATED OLIGONUCLEOTIDE

(57) **Abstract:** The present invention provides an inhalable formulation comprising a ligand conjugated oligonucleotide and particles of a physiologically acceptable pharmacologically-inert carrier.

## Methods and Compositions for Inhalation Delivery of Conjugated Oligonucleotide

### FIELD OF INVENTION

The present invention relates to the field of therapeutic agent inhalation delivery using ligand conjugated oligonucleotides. In particular, the present invention provides inhalation delivery of carbohydrate conjugates iRNA agents. Additionally, the present invention provides methods of making these compositions, as well as methods of introducing these oligonucleotides into subjects using these compositions, *e.g.*, for the treatment of various disease conditions.

### BACKGROUND

Oligonucleotide compounds have important therapeutic applications in medicine. Oligonucleotides can be used to silence genes that are responsible for a particular disease. Gene-silencing prevents formation of a protein by inhibiting translation. Importantly, gene-silencing agents are a promising alternative to traditional small, organic compounds that inhibit the function of the protein linked to the disease. siRNA, antisense RNA, and micro-RNA are oligonucleotides that prevent the formation of proteins by gene-silencing.

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 (Fire *et al.* (1998) *Nature* **391**, 806-811; Elbashir *et al.* (2001) *Genes Dev.* **15**, 188-200). Short dsRNA directs gene-specific, post-transcriptional silencing in many organisms, including vertebrates, and has provided a new tool for studying gene function. RNAi is mediated by RNA-induced silencing complex (RISC), a sequence-specific, multi-component nuclease that destroys messenger RNAs homologous to the silencing trigger. RISC is known to contain short RNAs (approximately 22 nucleotides) derived from the double-stranded RNA trigger, but the protein components of this activity remained unknown.

siRNA compounds are promising agents for a variety of diagnostic and therapeutic purposes. siRNA compounds can be used to identify the function of a gene. In addition, siRNA compounds offer enormous potential as a new type of pharmaceutical agent which acts by silencing disease-causing genes. Research is currently underway to develop interference RNA therapeutic agents for the treatment of many diseases including central-nervous-system diseases, inflammatory diseases, metabolic disorders, oncology, infectious diseases, and ocular disease.

siRNA has been shown to be extremely effective as a potential anti-viral therapeutic with numerous published examples appearing recently. siRNA molecules directed against targets in the viral genome dramatically reduce viral titers by orders of magnitude in animal models of influenza (Ge *et al.*, (2004) *Proc. Natl. Acad. Sci. USA*, **101**, 8676-8681; Tompkins *et al.* (2004) *Proc. Natl. Acad. Sci. USA*, **101**, 8682-8686; Thomas *et al.* (2005) *Expert Opin. Biol. Ther.* **5**, 495-505), respiratory syncytial virus (RSV) (Bitko *et al.* (2005) *Nat. Med.* **11**, 50-55), hepatitis B virus (HBV) (Morrissey *et al.* (2005) *Nat. Biotechnol.* **23**, 1002-1007), hepatitis C virus (Kapadia *et al.* (2003) *Proc. Natl. Acad. Sci. USA*, **100**, 2014-2018; Wilson *et al.* (2003) *Proc. Natl. Acad. Sci. USA*, **100**, 2783-2788) and SARS coronavirus (Li *et al.* (2005) *Nat. Med.* **11**, 944-951).

Efficient delivery to cells *in vivo* requires specific targeting and substantial protection from the extracellular environment, particularly serum proteins. One method of achieving specific targeting is to conjugate a targeting moiety to the iRNA agent. The targeting moiety helps in targeting the iRNA agent to the required target site. One way a targeting moiety can improve delivery is by receptor mediated endocytotic activity. This mechanism of uptake involves the movement of iRNA agent bound to membrane receptors into the interior of an area that is enveloped by the membrane via invagination of the membrane structure or by fusion of the delivery system with the cell membrane. This process is initiated via activation of a cell-surface or membrane receptor following binding of a specific ligand to the receptor. Many receptor-mediated endocytotic systems are known and have been studied, including those that recognize sugars such as galactose, mannose, mannose-6-phosphate, peptides and proteins such as transferrin, asialoglycoprotein, vitamin B12, insulin and epidermal growth factor (EGF). The Asialoglycoprotein receptor (ASGP-R) is a high capacity receptor, which is highly abundant on hepatocytes. The ASGP-R shows a 50-fold higher affinity for N-Acetyl-D-Galactosylamine (GalNAc) than D-Gal. Previous work has shown that multivalency is required to achieve nM affinity, while spacing among sugars is also crucial.

The Mannose receptor, with its high affinity to D-mannose represents another important carbohydrate-based ligand-receptor pair. The mannose receptor is highly expressed on specific cell types such as macrophages and possibly dendritic cells. Mannose conjugates as well as mannosylated drug carriers have been successfully used to target drug molecules to those cells. For examples, see Biessen *et al.* (1996) *J. Biol. Chem.* **271**, 28024-28030; Kinzel *et al.* (2003) *J. Peptide Sci.* **9**, 375-385; Barratt *et al.*

(1986) *Biochim. Biophys. Acta* **862**, 153-64; Diebold *et al.* (2002) *Somat. Cell Mol. Genetics* **27**, 65-74.

Lipophilic moieties, such as cholesterol or fatty acids, when attached to highly hydrophilic molecules such as nucleic acids can substantially enhance plasma protein binding and consequently circulation half life. In addition, binding to certain plasma proteins, such as lipoproteins, has been shown to increase uptake in specific tissues expressing the corresponding lipoprotein receptors (e.g., LDL-receptor HDL-receptor or the scavenger receptor SR-B1). For examples, see Bijsterbosch, M. K., Rump, E. T. *et al.* (2000) *Nucleic Acids Res.* **28**, 2717-25; Wolfrum, C., Shi, S. *et al.* (2007) **25**, 1149-57.

Lipophilic conjugates can also be used in combination with the targeting ligands in order to improve the intracellular trafficking of the targeted delivery approach.

Pulmozyme® is provided as a liquid protein formulation ready for use in nebulizer systems. In addition to nebulizer systems, pulmonary administration of drugs and other pharmaceuticals can be accomplished by provision of an inhalable solution formulated for inhalation by means of suitable liquid-based inhalers known as metered dosage inhalers or a dry powder formulation for inhalation by means of suitable inhalers known as dry powder inhalers (DPIs).

There is a clear need for efficient *in vivo* delivery of ligand conjugated iRNA agents and methods for their preparation. The present invention is directed to this very important end.

## SUMMARY

The present invention there is provided an inhalable formulation comprising a ligand conjugated oligonucleotide and particles of a physiologically acceptable pharmacologically-inert carrier.

## BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1.** Microsprayer Dosing of GalNAc-FVII or GalNAc-TTR results in Dose Dependent Reduction of Target. (A) Serum FVII levels determined by FVII Activity Assay analyzed 7 days after each dose (n=4 per group) (B) Serum mTTR levels measured by ELISA analyzed 7 days after each dose (n=4 per group);

**Figure 2.** FVII Activity or Serum TTR Levels Reveal Dose Dependent and Durable Knockdown Following Conjugate Delivery by Microsprayer or Subcutaneous Delivery. Efficacy profile in wild type C57BL/6 mice following a single Microsprayer or

SC dose of 3, 1, or 0.3mg/kg FVII-GalNAc or TTR-GalNAc (N=4 per group). Serum collected pre-dose, 7, 14 and 21 days post dose for analysis. A) FVII levels normalized to the individual animal pre-dose. Reduction of FVII activity reaches maximum suppression at approximately 7 days post-dose. Duration of FVII silencing is observed out to Day 21.

5 B) TTR levels normalized to the individual animal pre-dose. Reduction of TTR reaches maximum suppression at approximately 7 days post-dose. Microsprayer dosing leads to comparable silencing observed with SC administration at the dose levels examined.

**Figure 3.** Plasma siRNA Levels are Comparable Following Conjugate Delivery by Microsprayer or SC Dosing. siRNA levels assessed by stem-loop PCR method in  
10 tissues from wild type C57BL/6 mice following a single Microsprayer or SC dose of 3, 1, or 0.3mg/kg mTTR-GalNAc (N=4 per group). Plasma collected at 1, 6 and 24 hours post dose for analysis.

### DETAILED DESCRIPTION

15 This invention is based on the finding that conjugation of a carbohydrate moiety to an iRNA agent can be delivered effectively into the airways of a subject by inhalation. Inhalation delivery would provide a needle-free injection of oligonucleotide conjugates in clinic as an alternative strategy to achieve systemic exposure to the liver.

According to an aspect of some embodiments of the present invention there is  
20 provided an inhalable formulation comprising a ligand conjugated oligonucleotide and particles of a physiologically acceptable pharmacologically-inert carrier.

According to an aspect of some embodiments of the present invention there is provided an inhalable formulation comprising a ligand conjugated oligonucleotide and particles of a physiologically acceptable pharmacologically-inert carrier, wherein said  
25 ligand conjugated oligonucleotide is a multivalent N-Acetylgalactosamine conjugated oligonucleotide.

According to an aspect of some embodiments of the present invention there is provided an inhalable formulation comprising a ligand conjugated oligonucleotide and particles of a physiologically acceptable pharmacologically-inert carrier, wherein said  
30 physiologically acceptable pharmacologically-inert carrier is a dry powder.

According to an aspect of some embodiments of the present invention there is provided an inhalable formulation comprising a ligand conjugated oligonucleotide and particles of a physiologically acceptable pharmacologically-inert carrier, wherein said physiologically acceptable pharmacologically-inert carrier is a dry powder, wherein said

dry powder carrier is selected from the group consisting of (a) at least one crystalline sugar selected from the group consisting of glucose, arabinose, maltose, saccharose, dextrose, and lactose; and (b) at least one polyalcohol selected from the group consisting of mannitol, maltitol, lactitol, and sorbitol.

5           According to an aspect of some embodiments of the present invention there is provided an inhalable formulation comprising a ligand conjugated oligonucleotide and particles of a physiologically acceptable pharmacologically-inert carrier, wherein said physiologically acceptable pharmacologically-inert carrier is a dry powder, wherein said dry powder carrier is in a form of finely divided particles having a mass median diameter  
10 (MMD) in the range of 0.5 to 10 microns.

          According to an aspect of some embodiments of the present invention there is provided an inhalable formulation comprising a ligand conjugated oligonucleotide and particles of a physiologically acceptable pharmacologically-inert carrier, wherein said physiologically acceptable pharmacologically-inert carrier is a dry powder, wherein said  
15 dry powder carrier is in a form of finely divided particles having a mass median diameter (MMD) in the range of 1.0 to 6.0 microns.

          According to an aspect of some embodiments of the present invention there is provided an inhalable formulation comprising a ligand conjugated oligonucleotide and particles of a physiologically acceptable pharmacologically-inert carrier, wherein said  
20 physiologically acceptable pharmacologically-inert carrier is a dry powder, wherein said dry powder wherein said carrier is in a form of coarse particles having a mass diameter of 50- 500 microns.

          According to an aspect of some embodiments of the present invention there is provided an inhalable formulation comprising a ligand conjugated oligonucleotide and  
25 particles of a physiologically acceptable pharmacologically-inert carrier, wherein said physiologically acceptable pharmacologically-inert carrier is a dry powder, wherein said dry powder wherein said coarse particles have a mass diameter of 150 microns to 400 microns.

          According to an aspect of some embodiments of the present invention there is  
30 provided an inhalable formulation comprising a ligand conjugated oligonucleotide and particles of a physiologically acceptable pharmacologically-inert carrier further comprising, as an active ingredient, a magnesium salt.

          According to an aspect of some embodiments of the present invention there is provided an inhalable formulation comprising a ligand conjugated oligonucleotide and

particles of a physiologically acceptable pharmacologically-inert carrier further comprising one or more additive materials selected from the group consisting of an amino acid, a water soluble surface active agent, a lubricant, and a glidant.

5 According to an aspect of some embodiments of the present invention there is provided a dry powder inhaler device, comprising the inhalable dry powder formulation comprising a ligand conjugated oligonucleotide and particles of a physiologically acceptable pharmacologically-inert solid carrier a means for introducing the inhalable dry powder formulation into the airways of a subject by inhalation.

10 According to an aspect of some embodiments of the present invention there is provided a dry powder inhaler device, comprising the inhalable dry powder formulation comprising a ligand conjugated oligonucleotide and particles of a physiologically acceptable pharmacologically-inert solid carrier a means for introducing the inhalable dry powder formulation into the airways of a subject by inhalation, wherein the dry powder inhaler device is a single dose or a multidose inhaler.

15 According to an aspect of some embodiments of the present invention there is provided a dry powder inhaler device, comprising the inhalable dry powder formulation comprising a ligand conjugated oligonucleotide and particles of a physiologically acceptable pharmacologically-inert solid carrier a means for introducing the inhalable dry powder formulation into the airways of a subject by inhalation, wherein said device is  
20 pre- metered or device-metered.

According to an aspect of some embodiments of the present invention there is provided an inhalable formulation comprising a ligand conjugated oligonucleotide and particles of a physiologically acceptable pharmacologically-inert carrier for use in reducing or inhibiting the expression of an aberrant protein in a subject in need thereof,  
25 the method comprising administering to the subject in need thereof an effective amount of the inhalable formulation comprising a ligand conjugated oligonucleotide.

According to an aspect of some embodiments of the present invention there is provided an inhalable formulation comprising a ligand conjugated oligonucleotide and particles of a physiologically acceptable pharmacologically-inert carrier for use in  
30 reducing or inhibiting the expression of an aberrant protein in a subject in need thereof, the method comprising administering to the subject in need thereof, wherein said subject is suffering from a disease or condition selected from the group consisting of male infertility, metastatic cancer, a viral, bacterial, fungal or protozoan infection, sepsis, atherosclerosis, diabetes, delayed type hypersensitivity and a uterine disorder.

According to an aspect of some embodiments of the present invention there is provided an inhalable formulation comprising a ligand conjugated oligonucleotide and particles of a physiologically acceptable pharmacologically-inert carrier, wherein said physiologically acceptable pharmacologically-inert carrier, wherein said physiologically acceptable pharmacologically-inert carrier, wherein said physiologically acceptable pharmacologically-inert carrier is an inert liquid carrier.

According to an aspect of some embodiments of the present invention there is provided an inhalable formulation comprising a ligand conjugated oligonucleotide and particles of a physiologically acceptable pharmacologically-inert carrier, wherein said physiologically acceptable pharmacologically-inert carrier, wherein said physiologically acceptable pharmacologically-inert carrier is an inert liquid carrier, wherein said liquid carrier is selected from the group consisting of water, an aqueous alcoholic solution, perfluorocarbon and saline.

According to an aspect of some embodiments of the present invention there is provided an inhalable formulation comprising a ligand conjugated oligonucleotide and particles of a physiologically acceptable pharmacologically-inert carrier, wherein said physiologically acceptable pharmacologically-inert carrier and further comprising a magnesium salt.

According to an aspect of some embodiments of the present invention there is provided an inhalable formulation comprising a ligand conjugated oligonucleotide and particles of a physiologically acceptable pharmacologically-inert carrier, wherein said physiologically acceptable pharmacologically-inert carrier and further comprising one or more additive materials selected from the group consisting of a surfactant, a mucolytic agent, an adsorption enhancer and a lubricant.

According to an aspect of some embodiments of the present invention there is provided an inhalable formulation comprising a ligand conjugated oligonucleotide and particles of a physiologically acceptable pharmacologically-inert carrier, wherein said physiologically acceptable pharmacologically-inert carrier and further, wherein said ligand conjugated oligonucleotide is formulated in liposomes.

According to an aspect of some embodiments of the present invention there is provided a liquid inhaler device, comprising the inhalable pharmaceutical composition comprising a ligand conjugated oligonucleotide and a physiologically acceptable pharmacologically-inert liquid carrier, and a means for introducing the pharmaceutical composition into the airways of a subject by inhalation.



According to an aspect of some embodiments of the present invention there is provided a liquid inhaler device, comprising the inhalable pharmaceutical composition comprising a ligand conjugated oligonucleotide and a physiologically acceptable pharmacologically-inert liquid carrier, wherein said device is a single dose or a multidose inhaler.

According to an aspect of some embodiments of the present invention there is provided a liquid inhaler device, comprising the inhalable pharmaceutical composition comprising a ligand conjugated oligonucleotide and a physiologically acceptable pharmacologically-inert liquid carrier, wherein said device is pre-metered or device-metered.

According to an aspect of some embodiments of the present invention there is provided a liquid inhaler device, comprising the inhalable pharmaceutical composition comprising a ligand conjugated oligonucleotide and a physiologically acceptable pharmacologically-inert liquid carrier, wherein said device is a metered dose inhaler or a nebulizer.

According to an aspect of some embodiments of the present invention there is provided a liquid inhaler device, comprising the inhalable pharmaceutical composition comprising a ligand conjugated oligonucleotide and a physiologically acceptable pharmacologically-inert liquid carrier, wherein said formulation is provided for inhalation in particles ranging from about 1 to 10 microns in size.

According to an aspect of some embodiments of the present invention there is provided a liquid inhaler device, comprising the inhalable pharmaceutical composition comprising a ligand conjugated oligonucleotide and a physiologically acceptable pharmacologically-inert liquid carrier, wherein said formulation is provided for inhalation in particles ranging from about 2 to 5 microns in size.

According to an aspect of some embodiments of the present invention there is provided an inhalable formulation comprising a ligand conjugated oligonucleotide and particles of a physiologically acceptable pharmacologically-inert carrier, wherein said oligonucleotide is selected from a siRNA, a shRNA an antisense or a miRNA.

According to an aspect of some embodiments of the present invention there is provided an inhalable formulation comprising an iRNA agent that is conjugated with at least one carbohydrate ligand, e.g., monosaccharide, disaccharide, trisaccharide, tetrasaccharide, oligosaccharide, polysaccharide. These carbohydrate-conjugated iRNA agents target, in particular, the parenchymal cells of the liver. In one embodiment, the

iRNA agent includes more than one carbohydrate ligand, preferably two or three. In one embodiment, the iRNA agent comprises one or more galactose moiety. In another embodiment, the iRNA agent includes at least one (e.g., two or three or more) lactose molecules (lactose is a glucose coupled to a galactose). In another embodiment, the iRNA agent includes at least one (e.g., two or three or more) N-Acetyl-Galactosamine (GalNAc), N-Ac-Glucosamine (GluNAc), or mannose (e.g., mannose-6-phosphate). In one embodiment, iRNA agent comprises at least one mannose ligand, and the iRNA agent targets macrophages.

According to an aspect of some embodiments of the present invention there is provided an inhalable formulation comprising an iRNA agent comprising a carbohydrate ligand, and the presence of the carbohydrate ligand can increase delivery of the iRNA agent to the liver. Thus an iRNA agent comprising a carbohydrate ligand can be useful for targeting a gene for which expression is undesired in the liver. For example, an iRNA agent comprising a carbohydrate ligand can target a nucleic acid expresses by a hepatitis virus (e.g., hepatitis C, hepatitis B, hepatitis A, hepatitis D, hepatitis E, hepatitis F, hepatitis G, or hepatitis H).

According to an aspect of some embodiments of the present invention there is provided an inhalable formulation comprising a carbohydrate-conjugated iRNA agent that targets a gene of the hepatitis C virus. In another embodiment, the iRNA agent that targets a gene of the hepatitis C virus can be administered to a human having or at risk for developing hepatitis, e.g., acute or chronic hepatitis, or inflammation of the liver. A human who is a candidate for treatment with a carbohydrate-conjugated iRNA agent, e.g., an iRNA agent that targets a gene of HCV, can present symptoms indicative of HCV infection, such as jaundice, abdominal pain, liver enlargement and fatigue.

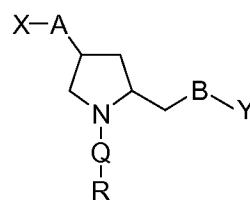
According to an aspect of some embodiments of the present invention there is provided an inhalable formulation comprising a carbohydrate-conjugated iRNA agent targets the 5' core region of HCV. This region lies just downstream of the ribosomal toe-print straddling the initiator methionine. In another embodiment, an iRNA agent targets any one of the nonstructural proteins of HCV, such as NS3, NS4A, NS4B, NS5A, or NS5B. In another embodiment, an iRNA agent targets the E1, E2, or C gene of HCV.

According to an aspect of some embodiments of the present invention there is provided an inhalable formulation comprising, the carbohydrate-conjugated iRNA agent targets a hepatitis B virus (HBV), and the iRNA agent has a sequence that is substantially similar to a sequence of a gene of HBV, e.g., the protein X (HBx) gene of HBV.

In one embodiment, the inhalable formulation comprising a carbohydrate-conjugated iRNA agent can also be used to treat other liver disorders, including disorders characterized by unwanted cell proliferation, hematological disorders, metabolic disorders, and disorders characterized by inflammation. A proliferation disorder of the liver can be, for example, a benign or malignant disorder, e.g., a cancer, e.g., a hepatocellular carcinoma (HCC), hepatic metastasis, or hepatoblastoma. A hepatic hematology or inflammation disorder can be a disorder involving clotting factors, a complement-mediated inflammation or a fibrosis, for example. Metabolic diseases of the liver include dyslipidemias and irregularities in glucose regulation. In one embodiment, a liver disorder is treated by administering one or more iRNA agents that have a sequence that is substantially identical to a sequence in a gene involved in the liver disorder.

In one embodiment, the inhalable formulation comprising a carbohydrate-conjugated iRNA agent targets a nucleic acid expressed in the liver, such as an ApoB RNA, c-jun RNA, beta-catenin RNA, or glucose-6-phosphatase mRNA.

According to an aspect of some embodiments of the present invention there is provided an inhalable formulation comprising a ligand conjugated oligonucleotide and particles of a physiologically acceptable pharmacologically-inert carrier, wherein said ligand conjugated oligonucleotide having the structure shown in formula (I'):



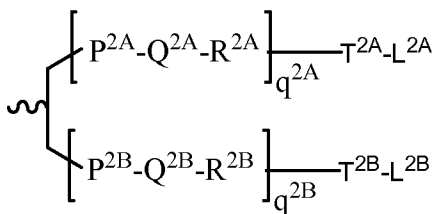
Formula (I')

wherein:

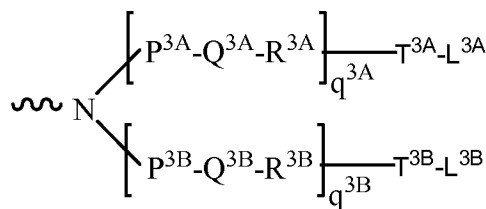
A and B are each independently for each occurrence O, N(R<sup>N</sup>) or S;

X and Y are each independently for each occurrence H, a protecting group, a phosphate group, a phosphodiester group, an activated phosphate group, an activated phosphite group, a phosphoramidite, a solid support, -P(Z')(Z'')O-nucleoside, -P(Z')(Z'')O-oligonucleotide, a lipid, a PEG, a steroid, a polymer, a nucleotide, a nucleoside, -P(Z')(Z'')O-R<sup>1</sup>-Q'-R<sup>2</sup>-OP(Z''')(Z''''')O-oligonucleotide, or an oligonucleotide, -P(Z')(Z'')-formula(I), -P(Z')(Z'')- or -Q-R;

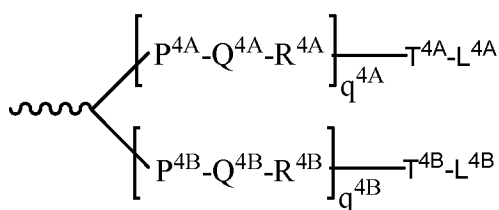
R is L<sup>1</sup> or has the structure shown in formula (II) – (V):



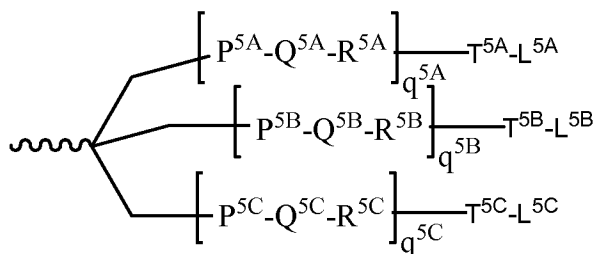
Formula (II)



Formula (III)



Formula (IV)



Formula (V)

$q^{2A}$ ,  $q^{2B}$ ,  $q^{3A}$ ,  $q^{3B}$ ,  $q^{4A}$ ,  $q^{4B}$ ,  $q^{5A}$ ,  $q^{5B}$  and  $q^{5C}$  represent independently for each

occurrence 0-20 and wherein the repeating unit can be the same or different;

5 Q and Q' are independently for each occurrence is absent,  $-(P^7-Q^7-R^7)_p-T^7-$  or  $-T^7-Q^7-T^7-B-T^{8'}-Q^8-T^8$ ;

$P^{2A}$ ,  $P^{2B}$ ,  $P^{3A}$ ,  $P^{3B}$ ,  $P^{4A}$ ,  $P^{4B}$ ,  $P^{5A}$ ,  $P^{5B}$ ,  $P^{5C}$ ,  $P^7$ ,  $T^{2A}$ ,  $T^{2B}$ ,  $T^{3A}$ ,  $T^{3B}$ ,  $T^{4A}$ ,  $T^{4B}$ ,  $T^{4A}$ ,  $T^{5B}$ ,  $T^{5C}$ ,  $T^7$ ,  $T^7$ ,  $T^8$  and  $T^{8'}$  are each independently for each occurrence absent, CO, NH, O, S, OC(O), NHC(O), CH<sub>2</sub>, CH<sub>2</sub>NH or CH<sub>2</sub>O;

10 B is  $-\text{CH}_2-\text{N}(\text{B}^L)-\text{CH}_2-$ ;

$\text{B}^L$  is  $-\text{T}^B-\text{Q}^B-\text{T}^{B'}-\text{R}^x$ ;

$Q^{2A}$ ,  $Q^{2B}$ ,  $Q^{3A}$ ,  $Q^{3B}$ ,  $Q^{4A}$ ,  $Q^{4B}$ ,  $Q^{5A}$ ,  $Q^{5B}$ ,  $Q^{5C}$ ,  $Q^7$ ,  $Q^8$  and  $Q^B$  are independently for each occurrence absent, alkylene, substituted alkylene and wherein one or more methylenes can be interrupted or terminated by one or more of O, S, S(O), SO<sub>2</sub>, N(R<sup>N</sup>),

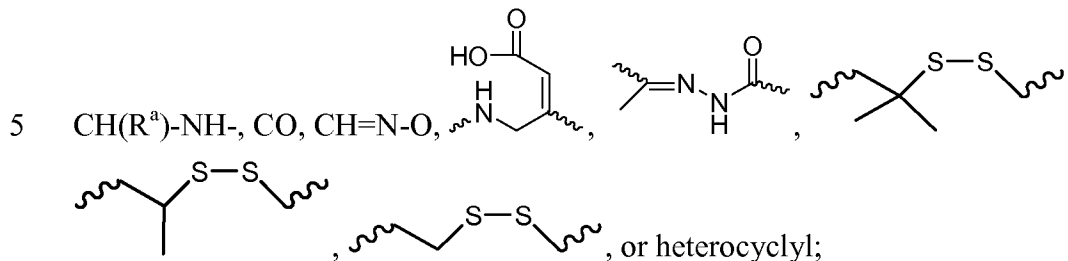
15  $\text{C}(\text{R}')=\text{C}(\text{R}')$ ,  $\text{C}\equiv\text{C}$  or  $\text{C}(\text{O})$ ;

$\text{T}^B$  and  $\text{T}^{B'}$  are each independently for each occurrence absent, CO, NH, O, S, OC(O), OC(O)O, NHC(O), NHC(O)NH, NHC(O)O, CH<sub>2</sub>, CH<sub>2</sub>NH or CH<sub>2</sub>O;

$\text{R}^x$  is a lipophile (e.g., cholesterol, cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-Bis-O(hexadecyl)glycerol, geranyloxyhexyl group, 20 hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, myristic acid, O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine), a vitamin (e.g., folate, vitamin A, vitamin E, biotin, pyridoxal), a peptide, a carbohydrate (e.g., monosaccharide, disaccharide, trisaccharide, tetrasaccharide, oligosaccharide, polysaccharide), an endosomolytic component, a steroid (e.g., uvaol,

hecigenin, diosgenin), a terpene (e.g., triterpene, e.g., sarsasapogenin, Friedelin, epifriedelanol derivatized lithocholic acid), or a cationic lipid;

$R^1, R^2, R^{2A}, R^{2B}, R^{3A}, R^{3B}, R^{4A}, R^{4B}, R^{5A}, R^{5B}, R^{5C}, R^7$  are each independently for each occurrence absent, NH, O, S,  $CH_2$ ,  $C(O)O$ ,  $C(O)NH$ ,  $NHCH(R^a)C(O)$ ,  $-C(O)-$



$L^1, L^{2A}, L^{2B}, L^{3A}, L^{3B}, L^{4A}, L^{4B}, L^{5A}, L^{5B}$  and  $L^{5C}$  are each independently for each occurrence a carbohydrate, e.g., monosaccharide, disaccharide, trisaccharide, tetrasaccharide, oligosaccharide and polysaccharide;

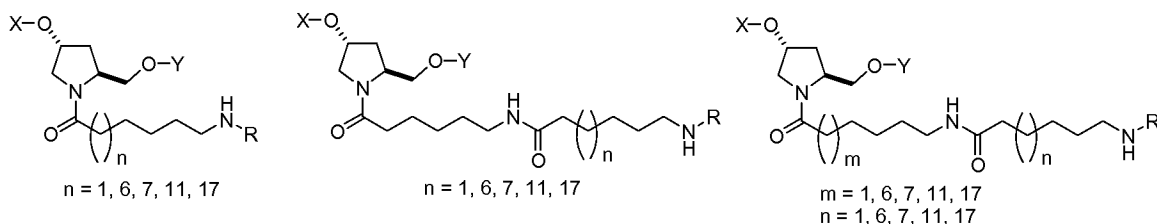
10  $R'$  and  $R''$  are each independently H,  $C^1-C_6$  alkyl, OH, SH, or  $N(R^N)_2$ ;  
 $R^N$  is independently for each occurrence H, methyl, ethyl, propyl, isopropyl, butyl or benzyl;

$R^a$  is H or amino acid side chain;

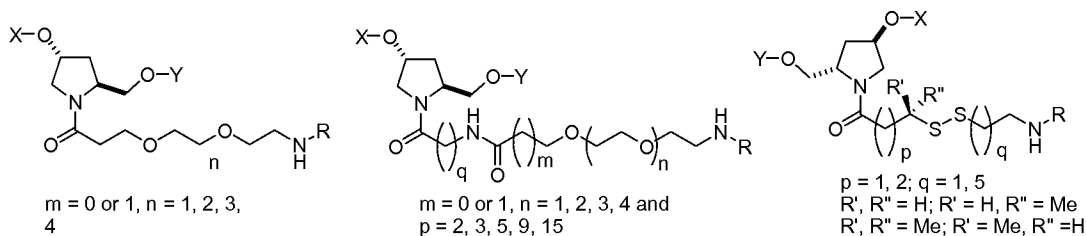
$Z', Z'', Z'''$  and  $Z''''$  are each independently for each occurrence O or S;

15 p represent independently for each occurrence 0-20.

In some embodiments, the formula (I') has the structure

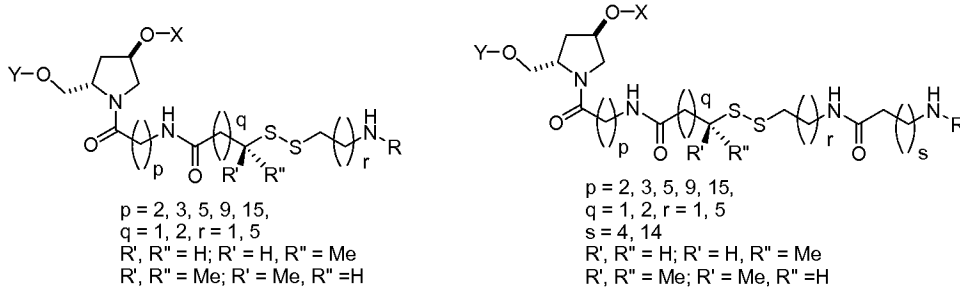


In some embodiments, the formula (I') has the structure

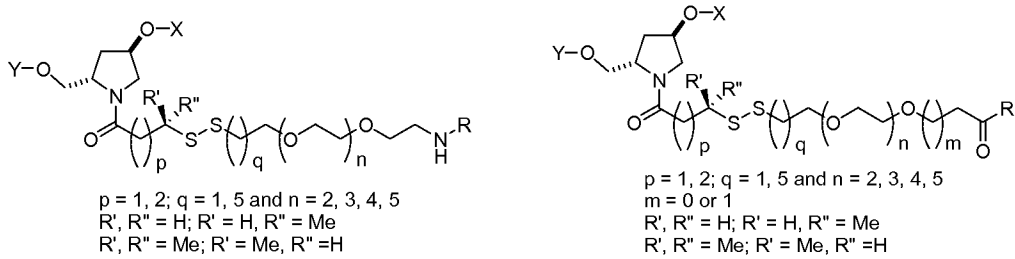


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In some embodiments, the formula (I') has the structure

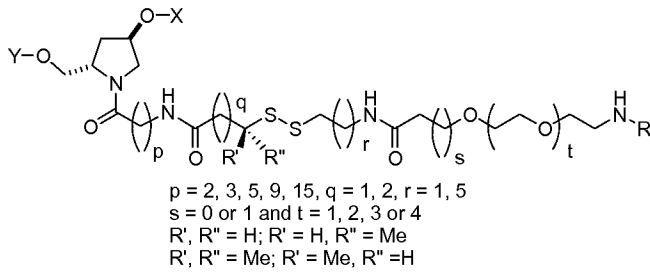


In some embodiments, the formula (I') has the structure

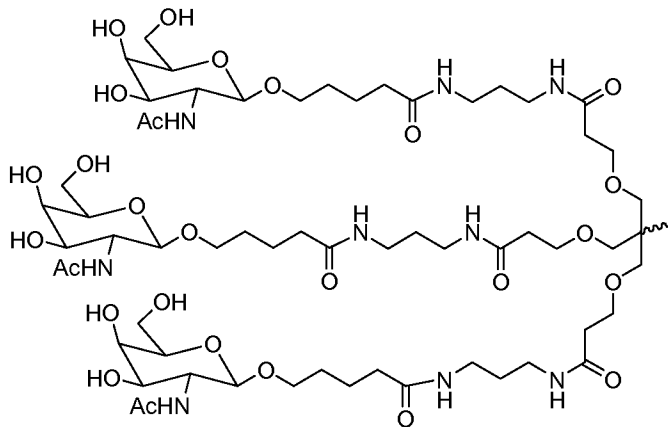


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In some embodiments, the formula (I') has the structure

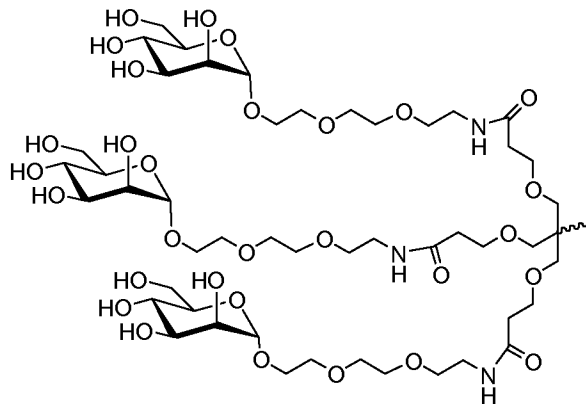


In some embodiments, R is

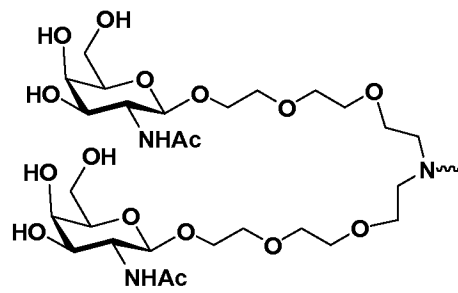


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In some embodiments, R is

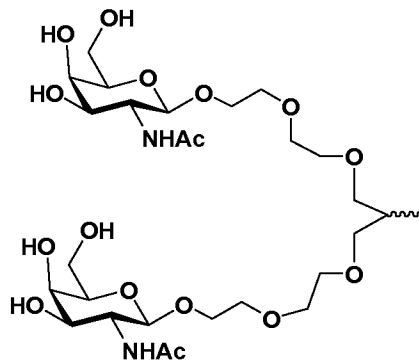


In some embodiments, R is



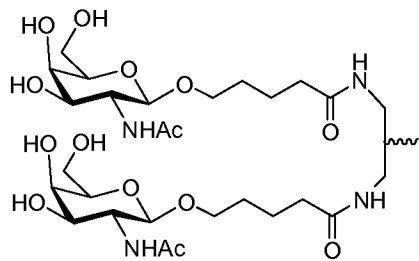
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In some embodiments, R is

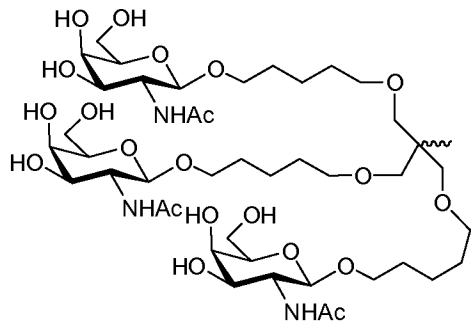


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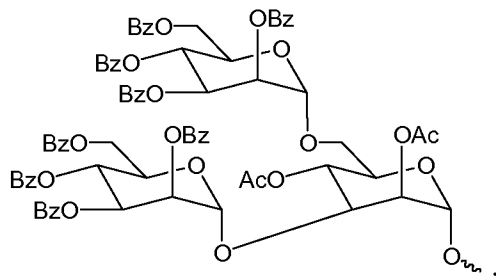
In some embodiments, R is



In some embodiments, R is

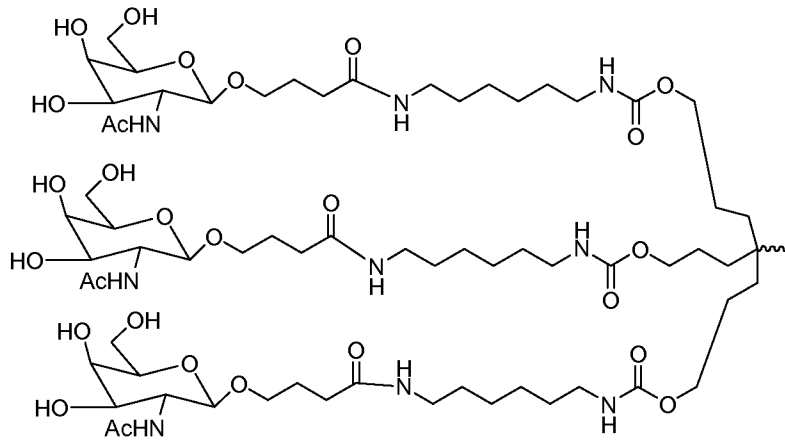


In some embodiments, R is

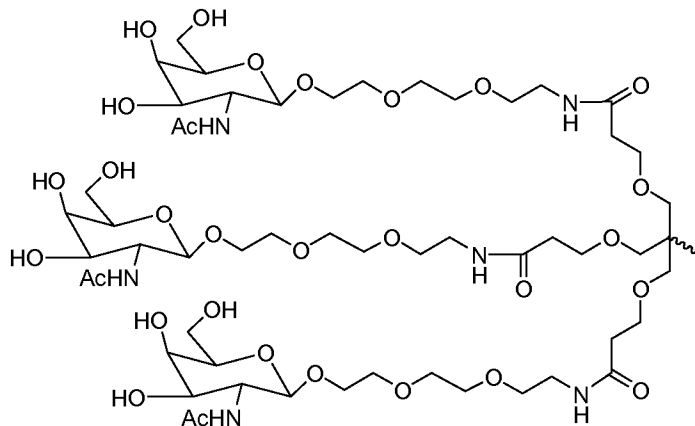


5

In some embodiments, R is



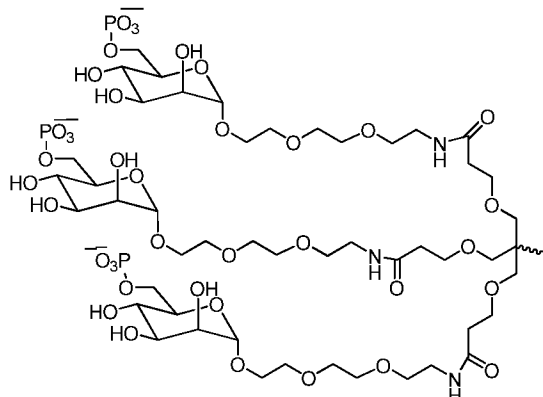
In some embodiments, R is



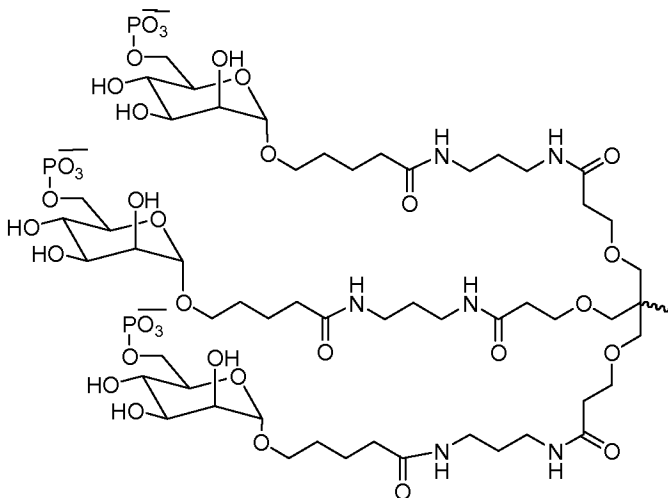
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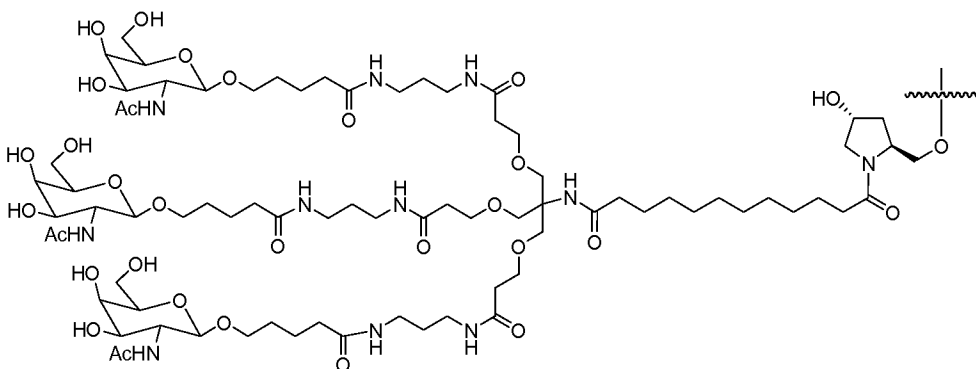
In some preferred embodiments, R is



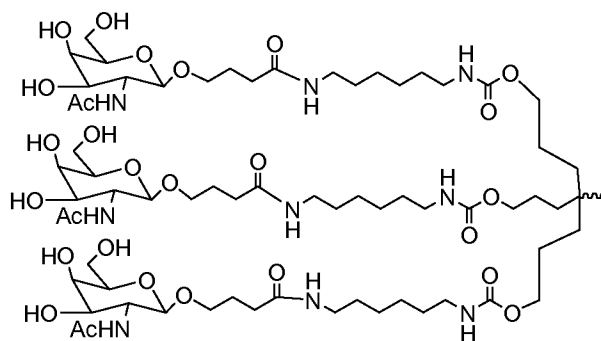
5 In some preferred embodiments, R is



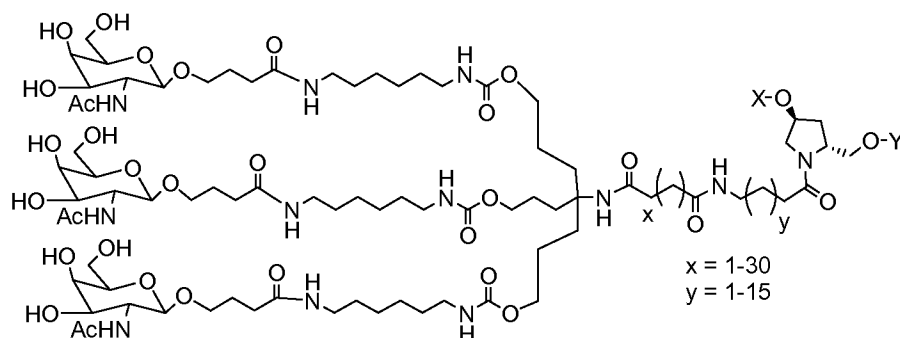
In some preferred embodiments, formula (I) has the structure



In some embodiments R is

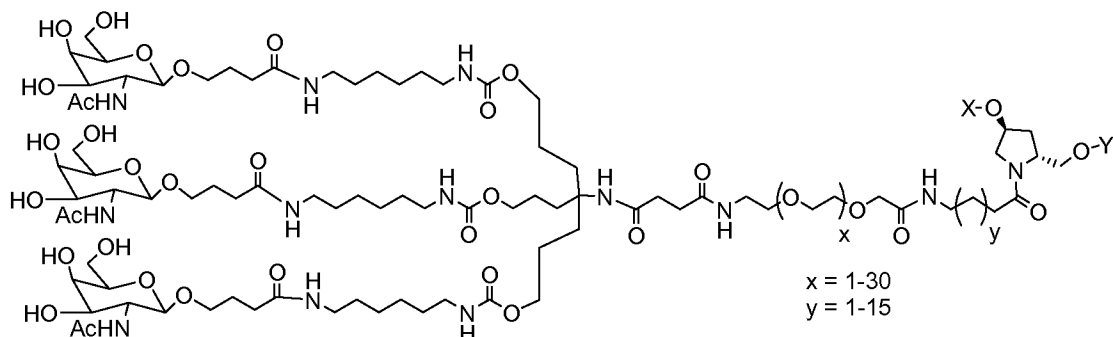


In some embodiments monomer of formula (I) has the structure



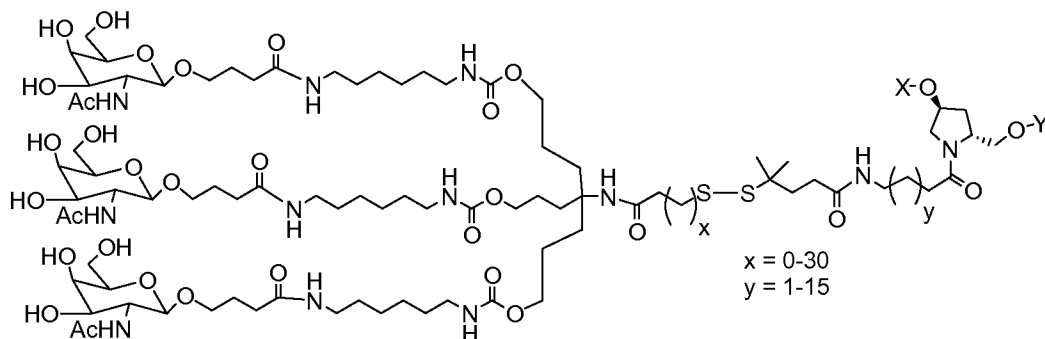
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In some embodiments monomer of formula (I) has the structure

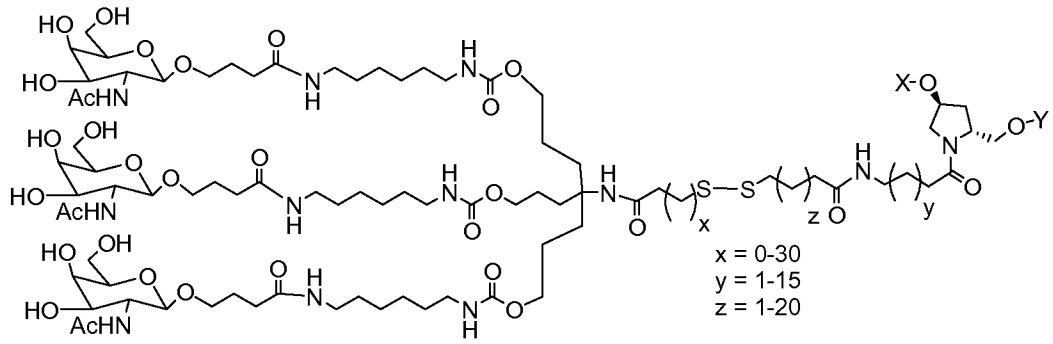


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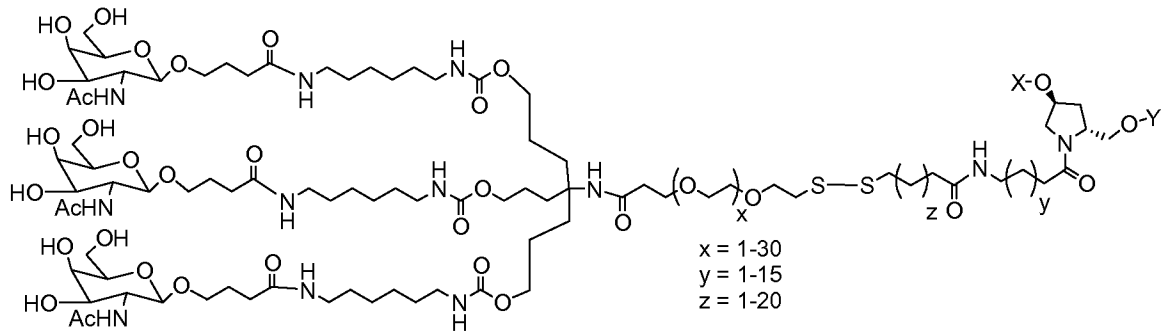
In some embodiments monomer of formula (I) has the structure



In some embodiments monomer of formula (I) has the structure

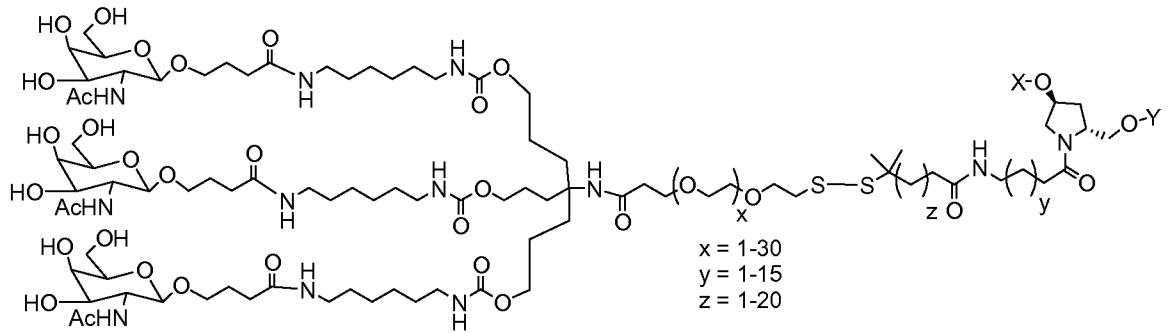


In some embodiments monomer of formula (I) has the structure



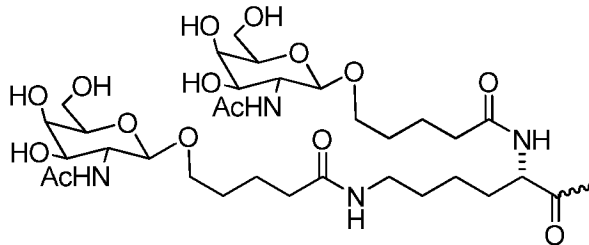
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In some embodiments monomer of formula (I) has the structure

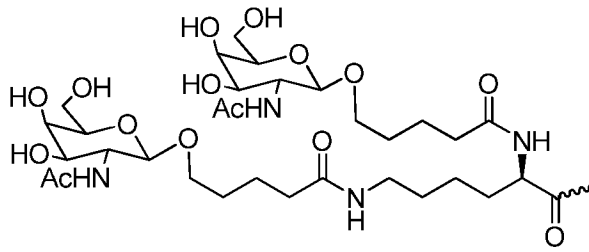


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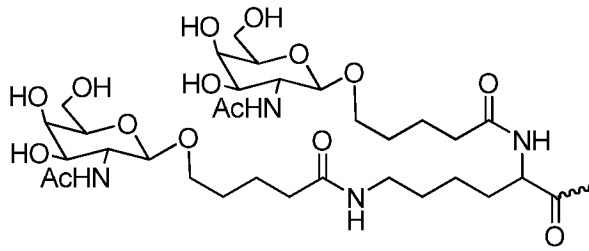
In some embodiments, R is



In some embodiments, R is

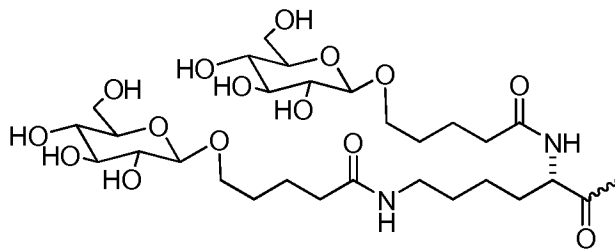


In some embodiments, R is

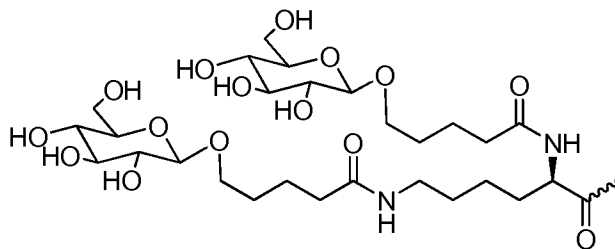


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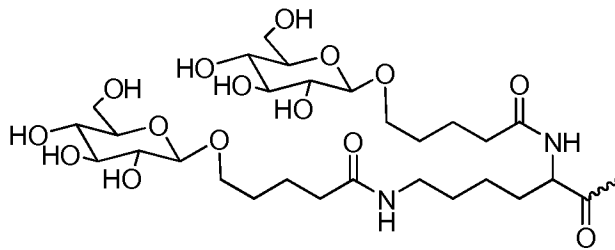
In some embodiments, R is



In some embodiments, R is

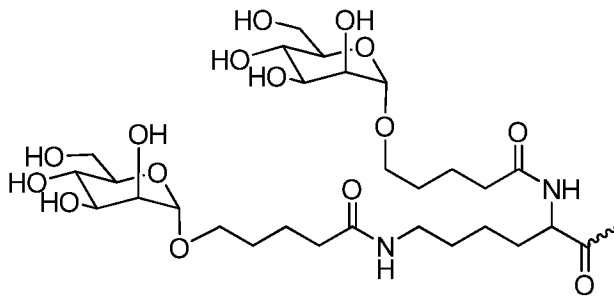


In some embodiments, R is

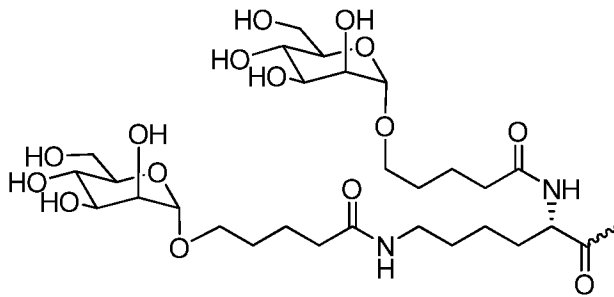


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In some embodiments, R is

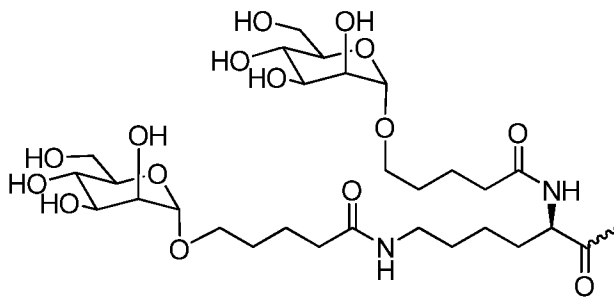


In some embodiments, R is

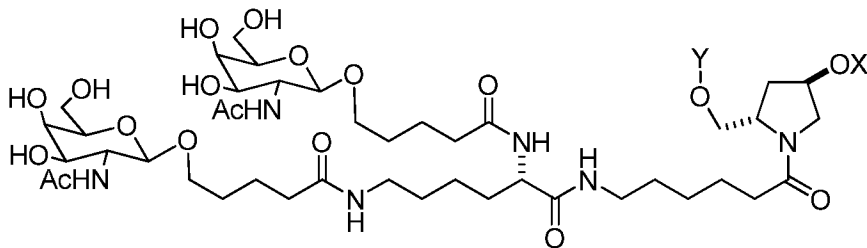


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In some embodiments, R is

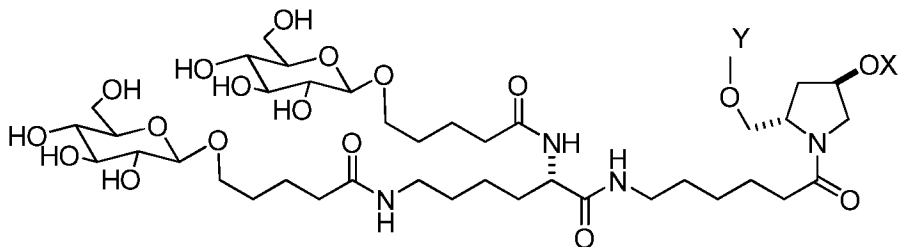


In some preferred embodiments, formula (I) has the structure

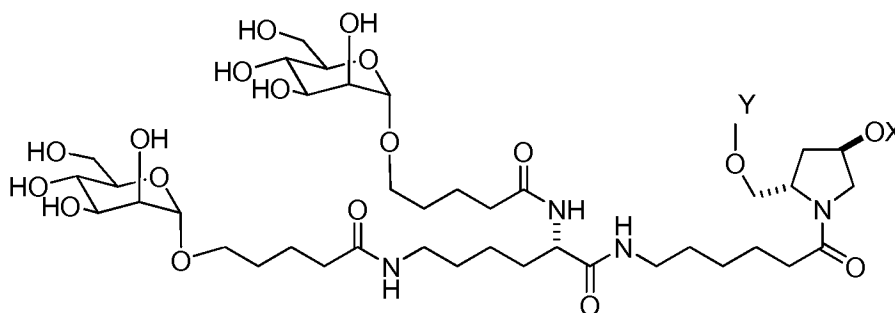


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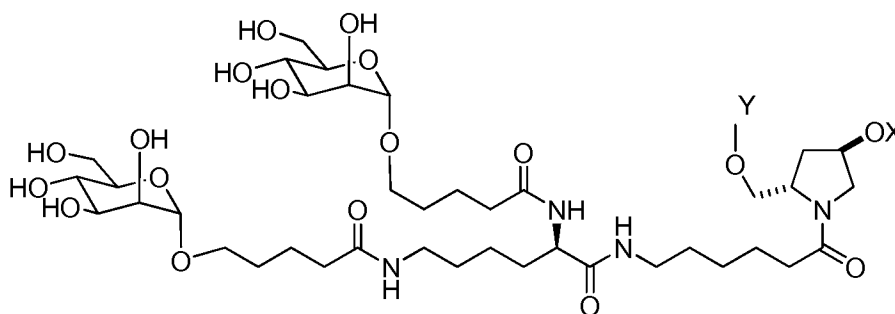
In some preferred embodiments, formula (I) has the structure



In some preferred embodiments, formula (I) has the structure

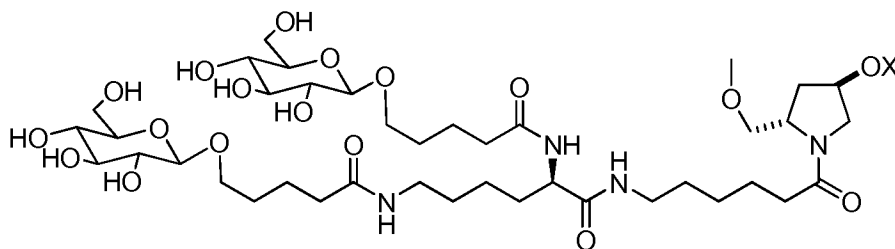


In some preferred embodiments, formula (I) has the structure

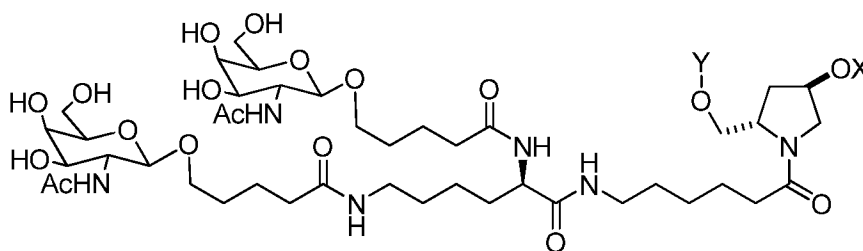


5

In some preferred embodiments, formula (I) has the structure



In some preferred embodiments, formula (I) has the structure



In some preferred embodiments both L<sup>2A</sup> and L<sup>2B</sup> are the same.

10

In some embodiments both L<sup>2A</sup> and L<sup>2B</sup> are different.

In some preferred embodiments both L<sup>3A</sup> and L<sup>3B</sup> are the same.

In some embodiments both L<sup>3A</sup> and L<sup>3B</sup> are different.

In some preferred embodiments both L<sup>4A</sup> and L<sup>4B</sup> are the same.

In some embodiments both L<sup>4A</sup> and L<sup>4B</sup> are different.

15

In some preferred embodiments all of L<sup>5A</sup>, L<sup>5B</sup> and L<sup>5C</sup> are the same.

In some embodiments two of L<sup>5A</sup>, L<sup>5B</sup> and L<sup>5C</sup> are the same.

In some embodiments  $L^{5A}$  and  $L^{5B}$  are the same.

In some embodiments  $L^{5A}$  and  $L^{5C}$  are the same.

In some embodiments  $L^{5B}$  and  $L^{5C}$  are the same.

5 In another aspect, the invention features, an iRNA agent comprising at least one monomer of formula (I).

In some embodiments, the iRNA agent will comprise 1, 2, 3, 4 or 5 monomers of formula (I), more preferably 1, 2 or 3 monomers of formula (I), more preferably 1 or 2 monomers of formula (I), even more preferably only one monomer of formula (I).

10 In some embodiments, all the monomers of formula (I) are on the same strand of a double stranded iRNA agent.

In some embodiments, the monomers of formula (I) are on the separate strands of a double strand of an iRNA agent.

15 In some embodiments, all monomers of formula (I) in an iRNA agent are the same.

In some embodiments, the monomers of formula (I) in an iRNA agent are all different.

In some embodiments, only some monomers of formula (I) in an iRNA agent are the same.

20 In some embodiments, the monomers of formula (I) will be next to each other in the iRNA agent.

In some embodiments, the monomers of formula (I) will not be next to each other in the iRNA agent.

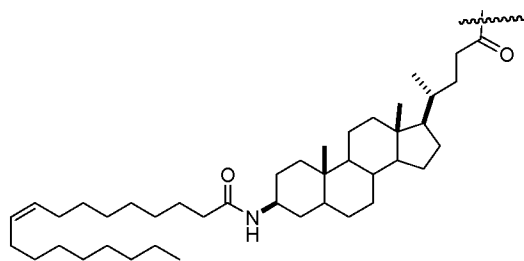
25 In some embodiments, the monomer of formula (I) will be on the 5'-end, 3'-end, at an internal position, both the 3'- and the 5'-end, both 5'-end and an internal position, both 3'-end and internal position, and at all three positions (5'-end, 3'-end and an internal position) of the iRNA agent.

In some preferred embodiments,  $R^x$  is cholesterol.

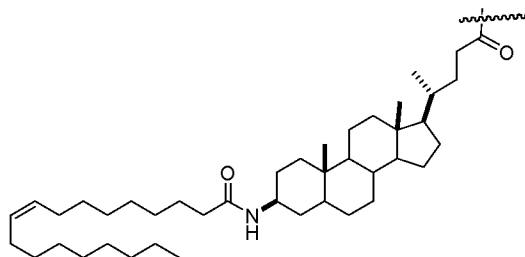
In some preferred embodiments,  $R^x$  is lithocholic.

30 In some preferred embodiments,  $R^x$  is oleyl lithocholic.

In some preferred embodiments, R<sup>x</sup> has the structure

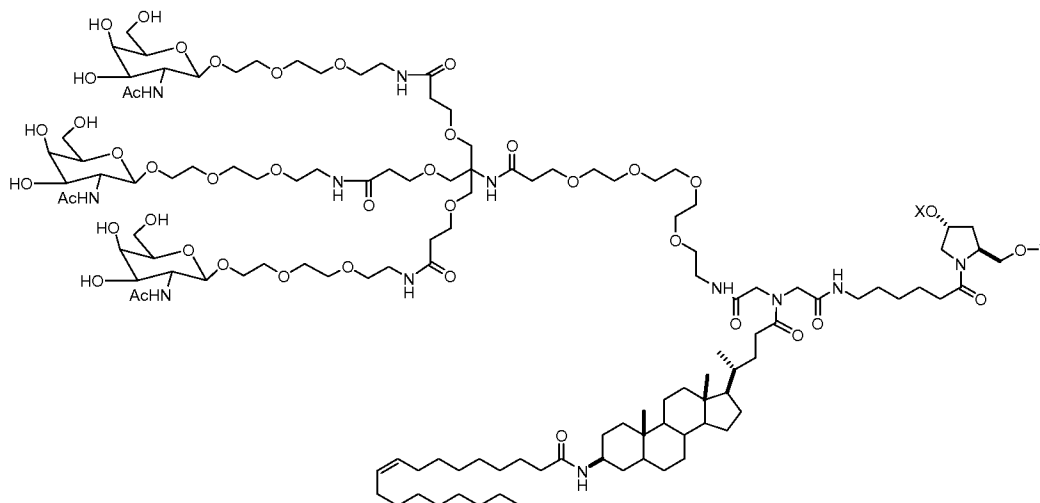


In some preferred embodiments, B<sup>L</sup> has the structure

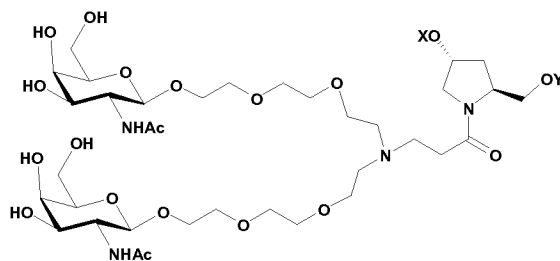


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In some preferred embodiments, formula (I) has the structure



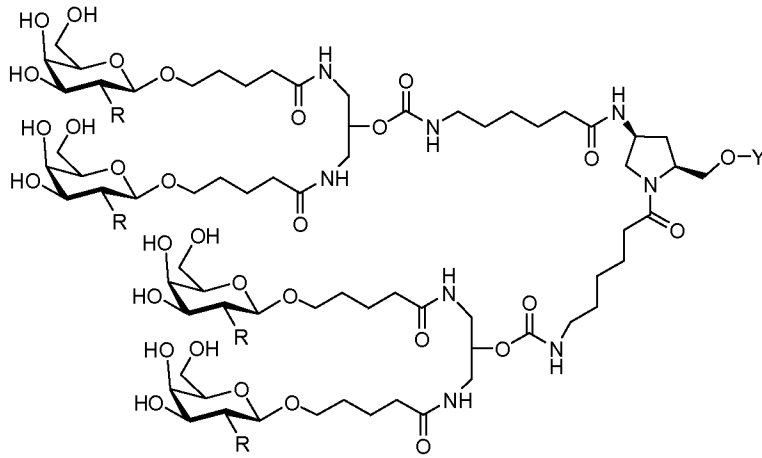
In some preferred embodiments, formula (I) has the structure



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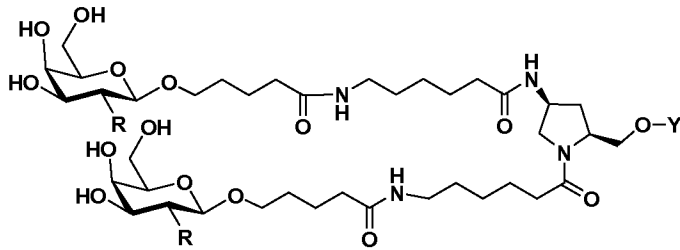
In some preferred embodiments, formula (I) has the structure



, wherein R is OH or

NHCOOH.

In some preferred embodiments, formula (I) has the structure

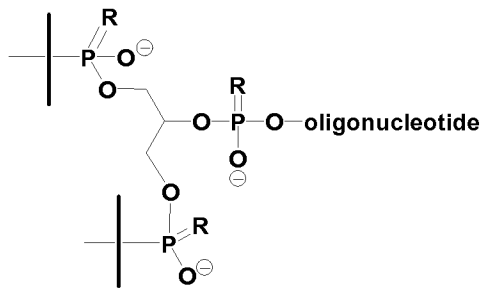


5

, wherein R is OH or

NHCOOH.

In some preferred embodiments, monomer of formula (I) is linked to the iRNA agent through a linker of formula (VII)

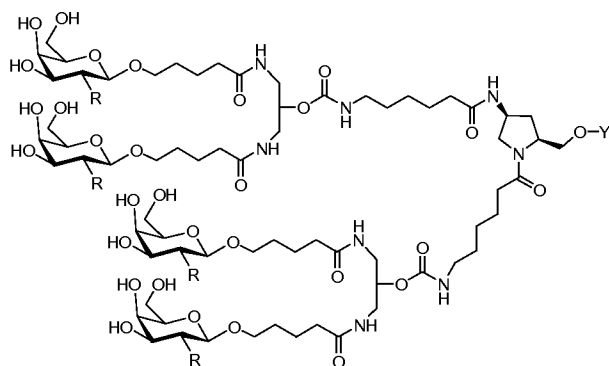


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Formula (VII)

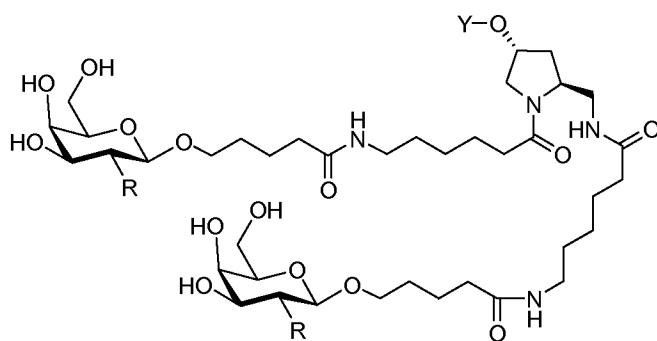
, wherein R is O or S.

In some preferred embodiments, formula (I) has the structure



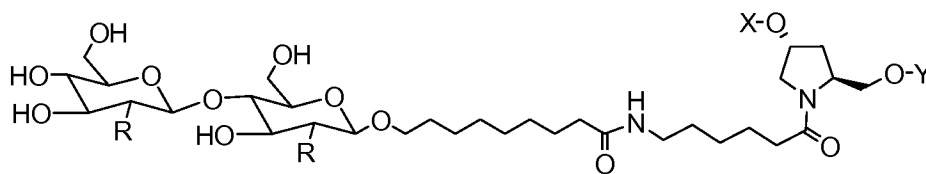
, wherein R is OH or NHCOOH.

In some preferred embodiments, formula (I) has the structure



5

In some preferred embodiments, formula (I) has the structure

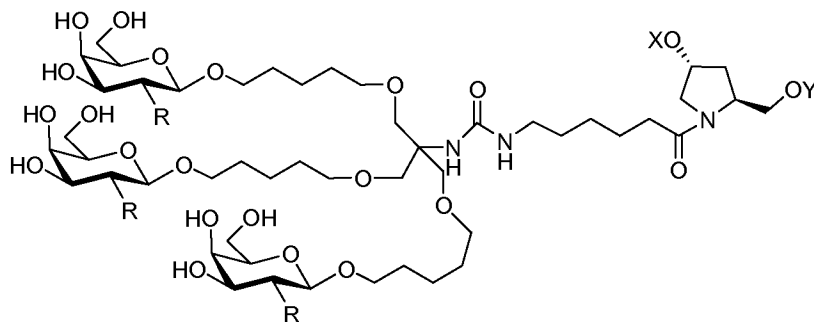


, where in

R is OH or NHCOOH.

10

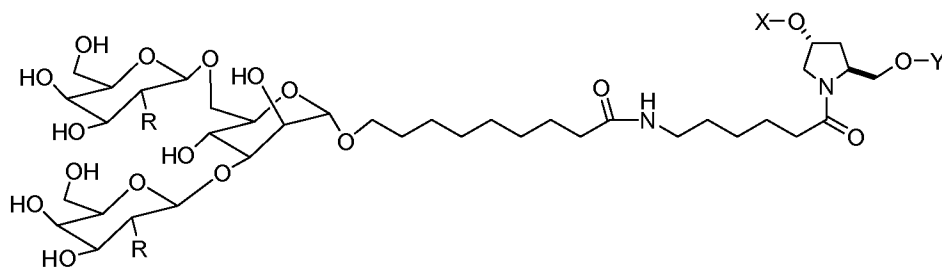
In some preferred embodiments, formula (I) has the structure



, wherein R

is OH or NHCOOH.

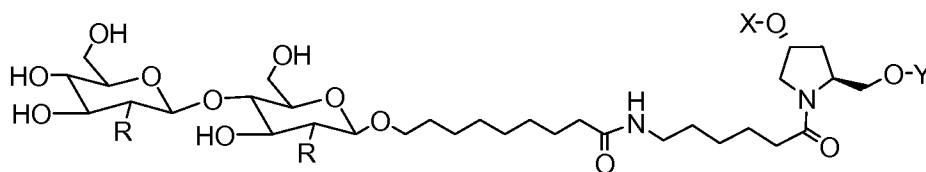
In some preferred embodiments, formula (I) has the structure



, wherein R is

OH or NHCOOH.

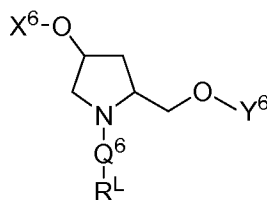
5 In some preferred embodiments, formula (I) has the structure



, wherein R is OH

or NHCOOH.

10 In some embodiments, the iRNA agent will have a monomer with the structure shown in formula (VI) in addition to monomer of formula (I)



Formula (VI)

wherein X<sup>6</sup> and Y<sup>6</sup> are each independently H, OH, a hydroxyl protecting group, a phosphate group, a phosphodiester group, an activated phosphate group, an activated phosphite group, a phosphoramidite, a solid support, -P(Z')(Z'')O-nucleoside, -P(Z')(Z'')O-oligonucleotide, a lipid, a PEG, a steroid, a polymer, -P(Z')(Z'')O-R<sup>1</sup>-Q'-R<sup>2</sup>-OP(Z''')(Z''''O)-oligonucleotide, a nucleotide, or an oligonucleotide, -P(Z')(Z'')-

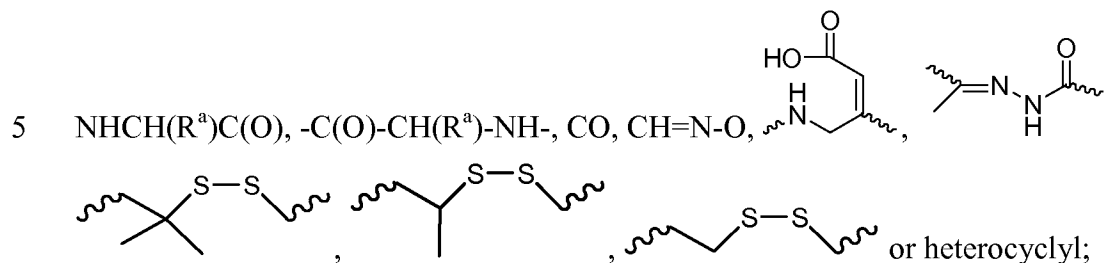
15 formula(I) or -P(Z')(Z'')-;

Q<sup>6</sup> is absent or -(P<sup>6</sup>-Q<sup>6</sup>-R<sup>6</sup>)<sub>v</sub>-T<sup>6</sup>-;

20 P<sup>6</sup> and T<sup>6</sup> are each independently for each occurrence absent, CO, NH, O, S, OC(O), NHC(O), CH<sub>2</sub>, CH<sub>2</sub>NH or CH<sub>2</sub>O;

$Q^6$  is independently for each occurrence absent, substituted alkylene wherein one or more methylenes can be intercepted or terminated by one or more of O, S, S(O), SO<sub>2</sub>, N(R<sup>N</sup>), C(R')=C(R'), C≡C or C(O);

R<sup>6</sup> is independently for each occurrence absent, NH, O, S, CH<sub>2</sub>, C(O)O, C(O)NH,



R' and R'' are each independently H, C<sub>1</sub>-C<sub>6</sub> alkyl OH, SH, N(R<sup>N</sup>)<sub>2</sub>;

R<sup>N</sup> is independently for each occurrence methyl, ethyl, propyl, isopropyl, butyl or  
10 benzyl;

R<sup>a</sup> is H or amino acid side chain;

Z', Z'', Z''' and Z'''' are each independently for each occurrence O or S;

v represent independently for each occurrence 0-20;

R<sup>L</sup> is a lipophile (e.g., cholesterol, cholic acid, adamantane acetic acid, 1-pyrene  
15 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), a vitamin (e.g., folate, vitamin A, biotin, pyridoxal), a peptide, a carbohydrate (e.g., monosaccharide, disaccharide, trisaccharide, tetrasaccharide,  
20 oligosaccharide, polysaccharide), an endosomolytic component, a steroid (e.g., uvaol, hecigenin, diosgenin), a terpene (e.g., triterpene, e.g., sarsasapogenin, Friedelin, epifriedelanol derivatized lithocholic acid), or a cationic lipid.

In some embodiments, one or more, e.g., 1, 2, 3, 4 or 5, monomers of formula (VI) in addition to one or more, e.g. 1, 2, 3, 4, or 5, monomers of formula (I) are present  
25 in the iRNA agent.

In some preferred embodiments only 1 monomer of formula (I) and 1 monomer of formula (VI) are present in the iRNA agent.

In some embodiments, R<sup>L</sup> is cholesterol.

In some embodiments, R<sup>L</sup> is lithocholic.

30 In some embodiments, R<sup>L</sup> is oleyl lithocholic.

In some embodiments, monomer of formula (I) is covalently linked with the monomer of formula (VI).

In some preferred embodiments, monomer of formula (I) is linked with the monomer of formula (VI) through a phosphate linkage, e.g. a phosphodiester linkage, a phosphorothioate linkage, a phosphorodithioate linkage.

In some preferred embodiments, monomer of formula (I) is linked to the iRNA agent through the monomer of formula (VI).

In some embodiments, monomer of formula (I) intervenes between the iRNA agent and the monomer of formula (VI).

In some embodiments, monomer of formula (I) and monomer of formula (II) are directly linked to each other.

In some embodiments, monomer of formula (I) and monomer of formula (II) are not directly linked to each other.

In some embodiments, monomer of formula (I) and monomer of formula (VI) are on separate strands of a double stranded iRNA agent.

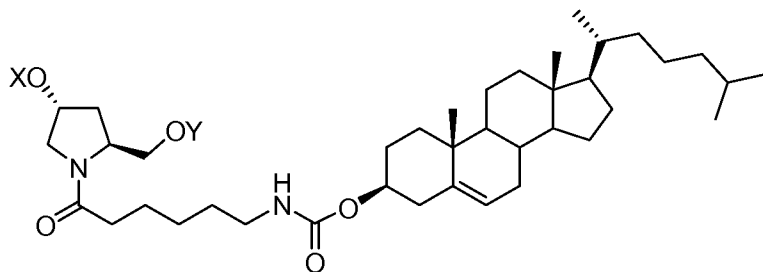
In some embodiments, monomer of formula (I) and monomer of formula (VI) are on opposite terminal ends of the iRNA agent.

In some embodiments, monomer of formula (I) and monomer of formula (VI) are on the same terminal end of the iRNA agent.

In some embodiments, one of monomer of formula (I) or monomer of formula (VI) is at an internal position while the other is at a terminal position of an iRNA agent.

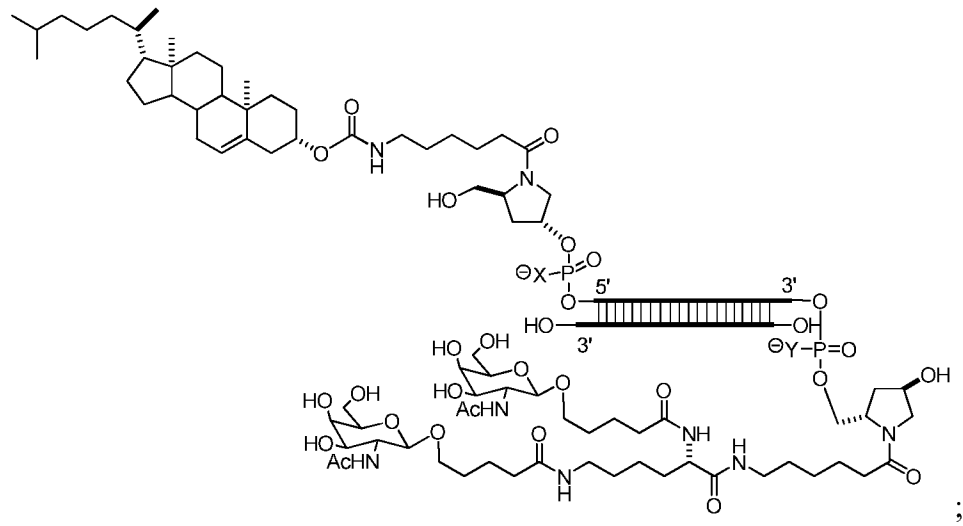
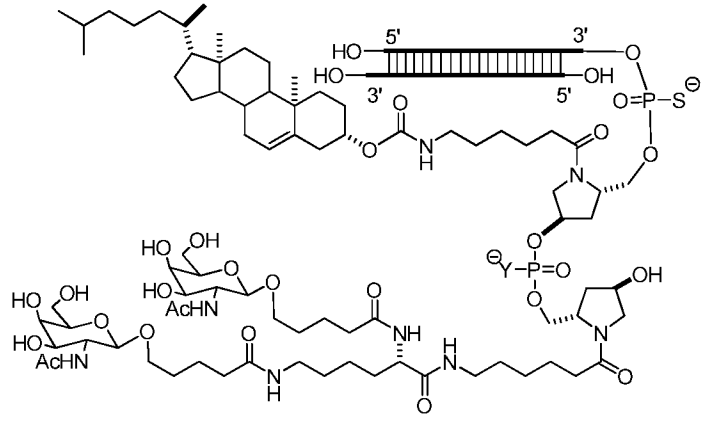
In some embodiments, monomer of formula (I) and monomer of formula (VI) are both at an internal position of the iRNA agent.

In some preferred embodiments, monomer of formula (VI) has the structure

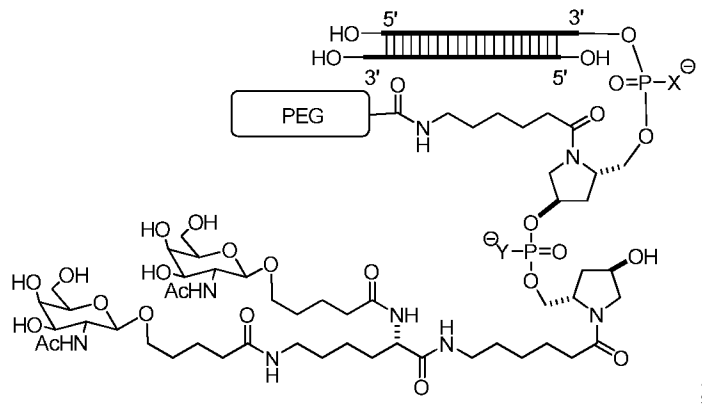


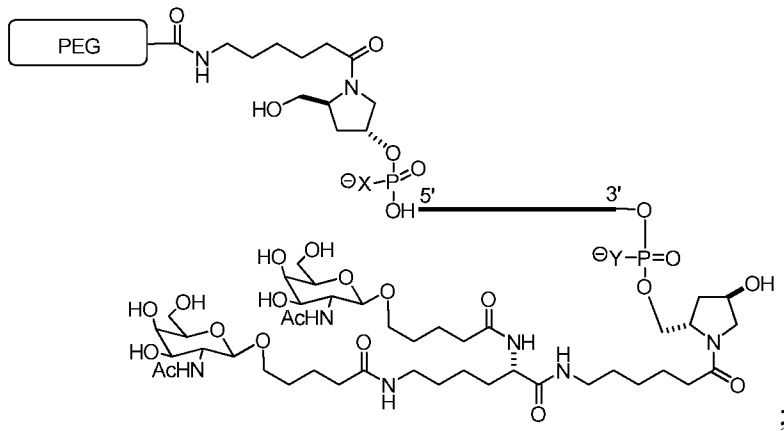
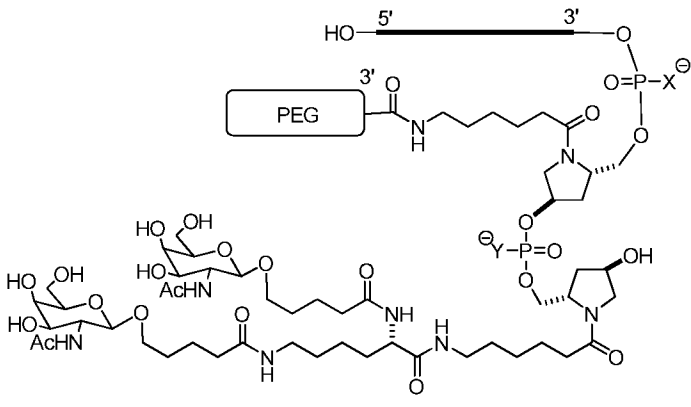
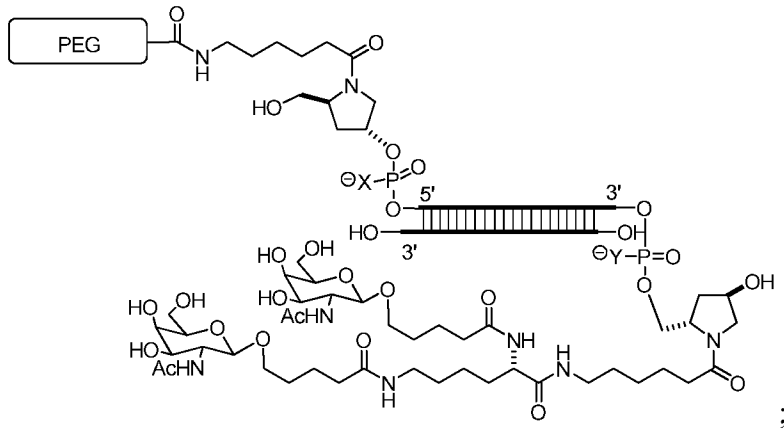
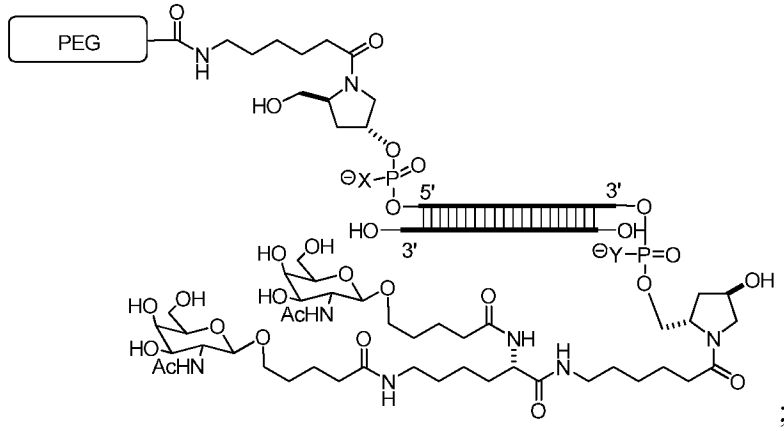
According to an aspect of some embodiments of the present invention there is provided an inhalable formulation comprising a ligand conjugated oligonucleotide and

particles of a physiologically acceptable pharmacologically-inert carrier, wherein said ligand conjugated oligonucleotide is selected from the group consisting of:

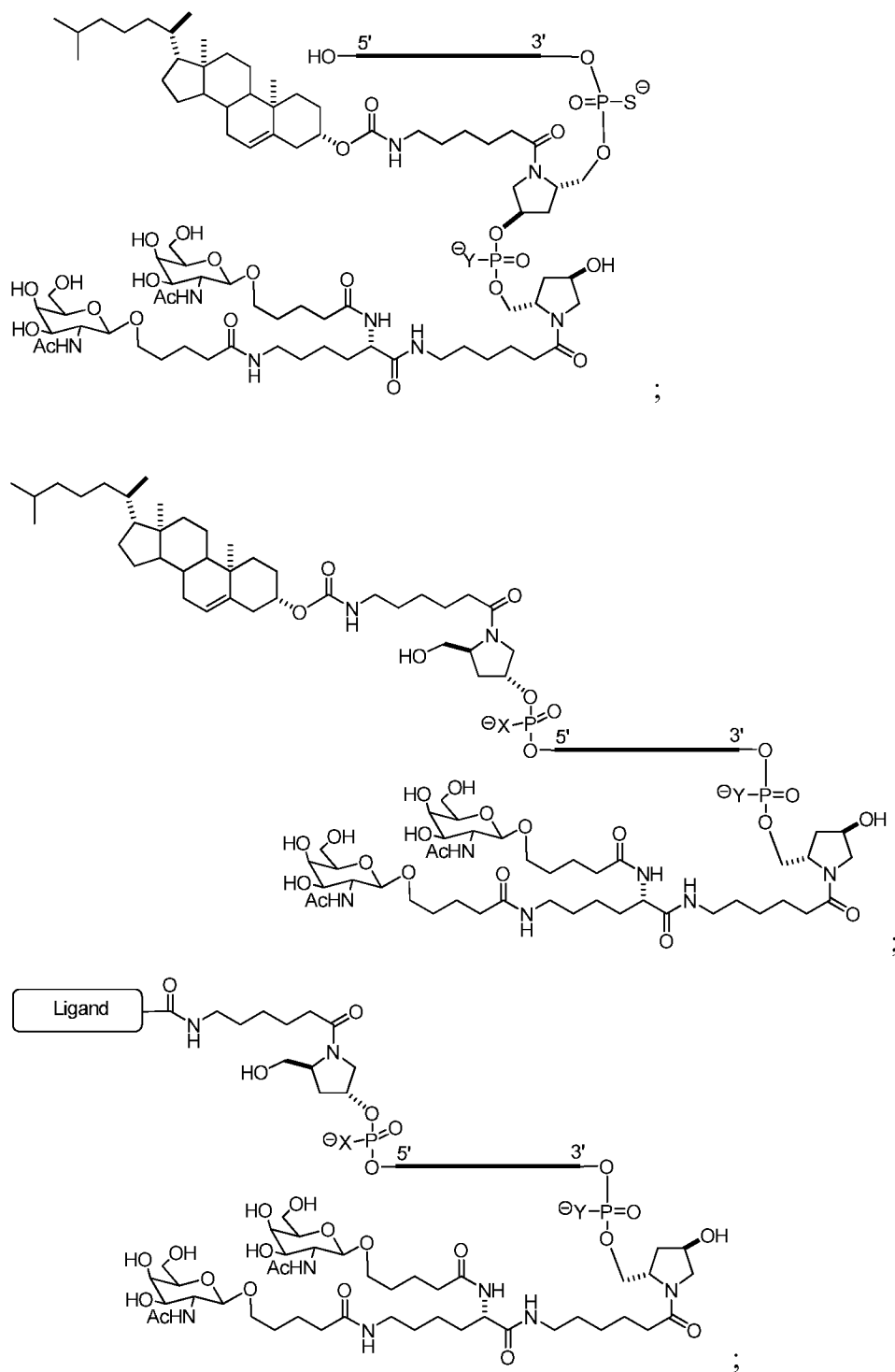


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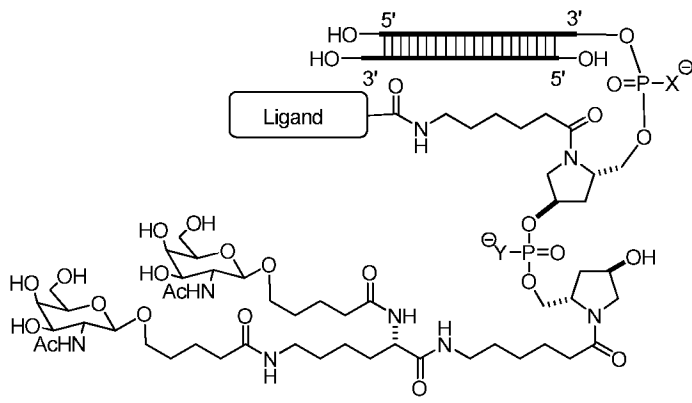
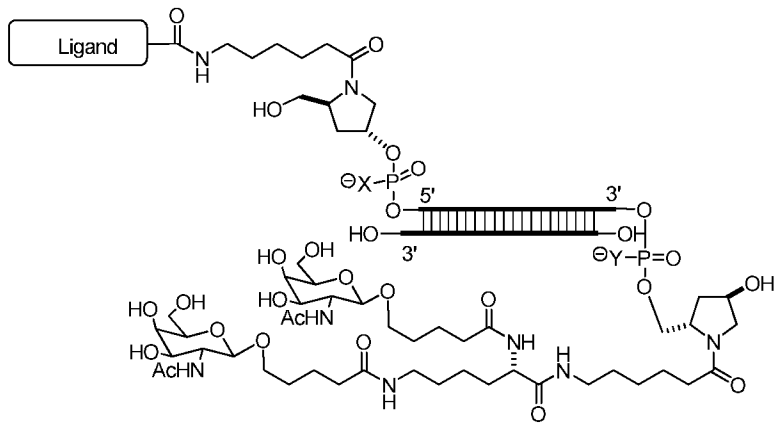
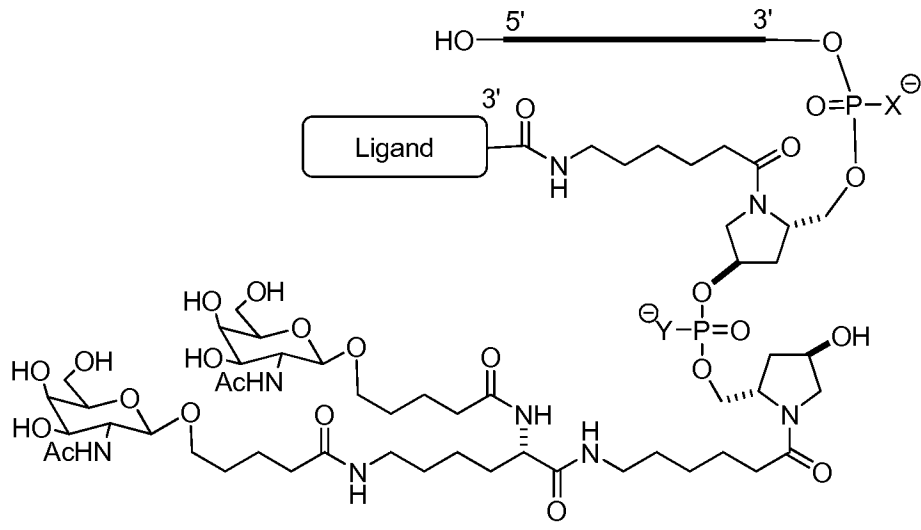


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wherein the ligand is a PK modulator: X = O or S; Y = O or S; PEG stands for ω-OH, ω-amino, ω-methoxy, ω-SH, ω-propargyl, ω-azido and ω-ligand PEGS with MW between 200 and 100,000.

10

### Endosomolytic Components

For macromolecular drugs and hydrophilic drug molecules, which cannot easily cross bilayer membranes, entrapment in endosomal/lysosomal compartments of the cell is thought to be the biggest hurdle for effective delivery to their site of action. In recent years, a number of approaches and strategies have been devised to address this problem. For liposomal formulations, the use of fusogenic lipids in the formulation has been the most common approach (Singh, R. S., Goncalves, C. et al. (2004). On the Gene Delivery Efficacies of pH-Sensitive Cationic Lipids via Endosomal Protonation. A Chemical Biology Investigation. *Chem. Biol.* **11**, 713-723.). Other components, which exhibit pH-sensitive endosomolytic activity through protonation and/or pH-induced conformational changes, include charged polymers and peptides. Examples may be found in Hoffman, A. S., Stayton, P. S. et al. (2002). Design of "smart" polymers that can direct intracellular drug delivery. *Polymers Adv. Technol.* **13**, 992-999; Kakudo, Chaki, T., S. et al. (2004). Transferrin-Modified Liposomes Equipped with a pH-Sensitive Fusogenic Peptide: An Artificial Viral-like Delivery System. *Biochemistry* **436**, 5618-5628; Yessine, M. A. and Leroux, J. C. (2004). Membrane-destabilizing polyanions: interaction with lipid bilayers and endosomal escape of biomacromolecules. *Adv. Drug Deliv. Rev.* **56**, 999-1021; Oliveira, S., van Rooy, I. et al. (2007). Fusogenic peptides enhance endosomal escape improving siRNA-induced silencing of oncogenes. *Int. J. Pharm.* **331**, 211-4. They have generally been used in the context of drug delivery systems, such as liposomes or lipoplexes. For folate receptor-mediated delivery using liposomal formulations, for instance, a pH-sensitive fusogenic peptide has been incorporated into the liposomes and shown to enhance the activity through improving the unloading of drug during the uptake process (Turk, M. J., Reddy, J. A. et al. (2002). Characterization of a novel pH-sensitive peptide that enhances drug release from folate-targeted liposomes at endosomal pHs. *Biochim. Biophys. Acta* **1559**, 56-68).

In certain embodiments, the endosomolytic components of the present invention may be polyanionic peptides or peptidomimetics which show pH-dependent membrane activity and/or fusogenicity. A peptidomimetic may be a small protein-like chain designed to mimic a peptide. A peptidomimetic may arise from modification of an existing peptide in order to alter the molecule's properties, or the synthesis of a peptide-like molecule using unnatural amino acids or their analogs. In certain embodiments, they have improved stability and/or biological activity when compared to a peptide. In certain embodiments, the endosomolytic component assumes its active conformation at

endosomal pH (e.g., pH 5-6). The “active” conformation is that conformation in which the endosomolytic component promotes lysis of the endosome and/or transport of the modular composition of the invention, or its any of its components (e.g., a nucleic acid), from the endosome to the cytoplasm of the cell.

5 Libraries of compounds may be screened for their differential membrane activity at endosomal pH versus neutral pH using a hemolysis assay. Promising candidates isolated by this method may be used as components of the modular compositions of the invention. A method for identifying an endosomolytic component for use in the compositions and methods of the present invention may comprise: providing a library of  
10 compounds; contacting blood cells with the members of the library, wherein the pH of the medium in which the contact occurs is controlled; determining whether the compounds induce differential lysis of blood cells at a low pH (e.g., about pH 5-6) versus neutral pH (e.g., about pH 7-8).

Exemplary endosomolytic components include the GALA peptide (Subbarao et al., Biochemistry, 1987, 26: 2964-2972), the EALA peptide (Vogel et al., J. Am. Chem. Soc., 1996, 118: 1581-1586), and their derivatives (Turk et al., Biochem. Biophys. Acta, 2002, 1559: 56-68). In certain embodiments, the endosomolytic component may contain a chemical group (e.g., an amino acid) which will undergo a change in charge or protonation in response to a change in pH. The endosomolytic component may be linear  
20 or branched. Exemplary primary sequences of endosomolytic components include H<sub>2</sub>N-(AALEALAEALEALAEALAEALAEAAAAGGC)-CO<sub>2</sub>H; H<sub>2</sub>N-(AALAEALAEALAEALAEALAEALAAAAGGC)-CO<sub>2</sub>H; and H<sub>2</sub>N-(ALEALAEALEALAEA)-CONH<sub>2</sub>.

In certain embodiments, more than one endosomolytic component may be  
25 incorporated into ligand conjugated oligonucleotide of the invention. In some embodiments, this will entail incorporating more than one of the same endosomolytic component into the iRNA agent in addition to the monomers of formula (I). In other embodiments, this will entail incorporating two or more different endosomolytic components into iRNA agent in addition to the monomers of formula (I).

30 These endosomolytic components may mediate endosomal escape by, for example, changing conformation at endosomal pH. In certain embodiments, the endosomolytic components may exist in a random coil conformation at neutral pH and rearrange to an amphipathic helix at endosomal pH. As a consequence of this conformational transition, these peptides may insert into the lipid membrane of the

endosome, causing leakage of the endosomal contents into the cytoplasm. Because the conformational transition is pH-dependent, the endosomolytic components can display little or no fusogenic activity while circulating in the blood (pH ~7.4). Fusogenic activity is defined as that activity which results in disruption of a lipid membrane by the

5 endosomolytic component. One example of fusogenic activity is the disruption of the endosomal membrane by the endosomolytic component, leading to endosomal lysis or leakage and transport of one or more components of the modular composition of the invention (e.g., the nucleic acid) from the endosome into the cytoplasm.

10 In addition to the hemolysis assay described herein, suitable endosomolytic components can be tested and identified by a skilled artisan using other methods. For example, the ability of a compound to respond to, e.g., change charge depending on, the pH environment can be tested by routine methods, e.g., in a cellular assay. In certain embodiments, a test compound is combined with or contacted with a cell, and the cell is allowed to internalize the test compound, e.g., by endocytosis. An endosome preparation

15 can then be made from the contacted cells and the endosome preparation compared to an endosome preparation from control cells. A change, e.g., a decrease, in the endosome fraction from the contacted cell vs. the control cell indicates that the test compound can function as a fusogenic agent. Alternatively, the contacted cell and control cell can be evaluated, e.g., by microscopy, e.g., by light or electron microscopy, to determine a

20 difference in the endosome population in the cells. The test compound and/or the endosomes can be labeled, e.g., to quantify endosomal leakage.

In another type of assay, an iRNA agent described herein is constructed using one or more test or putative fusogenic agents. The iRNA agent can be labeled for easy visualization. The ability of the endosomolytic component to promote endosomal escape,

25 once the iRNA agent is taken up by the cell, can be evaluated, e.g., by preparation of an endosome preparation, or by microscopy techniques, which enable visualization of the labeled iRNA agent in the cytoplasm of the cell. In certain other embodiments, the inhibition of gene expression, or any other physiological parameter, may be used as a surrogate marker for endosomal escape.

30 In other embodiments, circular dichroism spectroscopy can be used to identify compounds that exhibit a pH-dependent structural transition.

A two-step assay can also be performed, wherein a first assay evaluates the ability of a test compound alone to respond to changes in pH, and a second assay evaluates the

ability of a modular composition that includes the test compound to respond to changes in pH.

### Peptides

5           Peptides suitable for use with the present invention can be a natural peptide, .e.g. tat or antennopedia peptide, a synthetic peptide or a peptidomimetic. Furthermore, the peptide can be a modified peptide, for example peptide can comprise non-peptide or pseudo-peptide linkages, and D-amino acids. A peptidomimetic (also referred to herein as an oligopeptidomimetic) is a molecule capable of folding into a defined three-  
10           dimensional structure similar to a natural peptide. The attachment of peptide and peptidomimetics to the oligonucleotide can affect pharmacokinetic distribution of the oligonucleotide, such as by enhancing cellular recognition and absorption. The peptide or peptidomimetic moiety can be about 5-50 amino acids long, *e.g.*, about 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids long (see Table 1, for example).

**Table 1.** Exemplary Cell Permeation Peptides

Cell Permeation Peptide	Amino acid Sequence	Reference
Penetratin	RQIKIWFQNRRMKWKK	Derossi <i>et al.</i> , J. Biol. Chem. 269:10444, 1994
Tat fragment (48-60)	GRKKRRQRRRPPQC	Vives <i>et al.</i> , J. Biol. Chem., 272:16010, 1997
Signal Sequence-based peptide	GALFLGWLGAAGSTMGAWSQPKKK RKV	Chaloin <i>et al.</i> , Biochem. Biophys. Res. Commun., 243:601, 1998
PVEC	LLIILRRRIRKQAHHSK	Elmquist <i>et al.</i> , Exp. Cell Res., 269:237, 2001
Transportan	GWTLNSAGYLLKINLKALAALAKKIL	Pooga <i>et al.</i> , FASEB J., 12:67, 1998
Amphiphilic model peptide	KLALKLALKALKAAKLA	Oehlke <i>et al.</i> , Mol. Ther., 2:339, 2000
Arg <sub>9</sub>	RRRRRRRRR	Mitchell <i>et al.</i> , J. Pept. Res., 56:318, 2000
Bacterial cell wall permeating	KFFKFFKFFK	
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDF LRNLVPRTES	
Cecropin P1	SWLSKTAKKLENSAKKRISGIAIAIQ GGPR	
$\alpha$ -defensin	ACYCRIPACIAGERRYGTCTIYQGRLW AFCC	
b-defensin	DHYNCVSSGGQCLYSACPIFTKIQTGTC YRGKAKCCK	
Bactenecin	RKCRIVVIRVCR	
PR-39	RRRPRPPYLPRPRPPFFPPRLPPRIPPG FPPRFPPRFPGKR-NH <sub>2</sub>	
Indolicidin	ILPWKWPWWPWRR-NH <sub>2</sub>	

A peptide or peptidomimetic can be, for example, a cell permeation peptide, cationic peptide, amphipathic peptide, or hydrophobic peptide (*e.g.*, consisting primarily of Tyr, Trp or Phe). The peptide moiety can be a dendrimer peptide, constrained peptide or crosslinked peptide. In another alternative, the peptide moiety can include a hydrophobic membrane translocation sequence (MTS). An exemplary hydrophobic MTS-containing peptide is RFGF having the amino acid sequence AAVALLPAVLLALLAP. A RFGF analogue (*e.g.*, amino acid sequence AALLPVLLAAP) containing a hydrophobic MTS can also be a targeting moiety. The peptide moiety can be a “delivery” peptide, which can carry large polar molecules including peptides, oligonucleotides, and protein across cell membranes. For example, sequences from the HIV Tat protein (GRKKRRQRRRPPQ) and the *Drosophila* Antennapedia protein (RQIKIWFQNRRMKWKK) have been found to be capable of functioning as delivery peptides. A peptide or peptidomimetic can be encoded by a random sequence of DNA, such as a peptide identified from a phage-display library, or one-bead-one-compound (OBOC) combinatorial library (Lam *et al.*, *Nature*, 354:82-84, 1991). Preferably the peptide or peptidomimetic tethered to the lipid is a cell targeting peptide such as an arginine-glycine-aspartic acid (RGD)-peptide, or RGD mimic. A peptide moiety can range in length from about 5 amino acids to about 40 amino acids. The peptide moieties can have a structural modification, such as to increase stability or direct conformational properties. Any of the structural modifications described below can be utilized.

An RGD peptide moiety can be used to target a tumor cell, such as an endothelial tumor cell or a breast cancer tumor cell (Zitzmann *et al.*, *Cancer Res.*, 62:5139-43, 2002). An RGD peptide can facilitate targeting to tumors of a variety of other tissues, including the lung, kidney, spleen, or liver (Aoki *et al.*, *Cancer Gene Therapy* 8:783-787, 2001). Preferably, the RGD peptide will facilitate targeting of the lipid particle to the kidney. The RGD peptide can be linear or cyclic, and can be modified, *e.g.*, glycosylated or methylated to facilitate targeting to specific tissues. For example, a glycosylated RGD peptide can target a tumor cell expressing  $\alpha_v\beta_3$  (Haubner *et al.*, *Jour. Nucl. Med.*, 42:326-336, 2001).

Peptides that target markers enriched in proliferating cells can be used. *E.g.*, RGD containing peptides and peptidomimetics can target cancer cells, in particular cells that exhibit an  $I_V\beta_3$  integrin. Thus, one could use RGD peptides, cyclic peptides containing

RGD, RGD peptides that include D-amino acids, as well as synthetic RGD mimics. In addition to RGD, one can use other moieties that target the  $I_V\text{-}\alpha_3$  integrin ligand. Generally, such ligands can be used to control proliferating cells and angiogenesis.

A “cell permeation peptide” is capable of permeating a cell, *e.g.*, a microbial cell, such as a bacterial or fungal cell, or a mammalian cell, such as a human cell. A microbial cell-permeating peptide can be, for example, an  $\alpha$ -helical linear peptide (*e.g.*, LL-37 or Ceropin P1), a disulfide bond-containing peptide (*e.g.*,  $\alpha$ -defensin,  $\beta$ -defensin or bactenecin), or a peptide containing only one or two dominating amino acids (*e.g.*, PR-39 or indolicidin). A cell permeation peptide can also include a nuclear localization signal (NLS). For example, a cell permeation peptide can be a bipartite amphipathic peptide, such as MPG, which is derived from the fusion peptide domain of HIV-1 gp41 and the NLS of SV40 large T antigen (Simeoni *et al.*, Nucl. Acids Res. 31:2717-2724, 2003).

The term “oligonucleotide” refers to a nucleic acid molecule (RNA or DNA) for example of length less than 100, 200, 300, 400, 500, 600, 700, 800, 900 or 1000 nucleotides, and include terms such as iRNA agent, antisense, ribozyme, aptamer, mRNA, dsiRNA, decoy, microRNA, tRNA, shRNA, RNA agent, and the like.

#### iRNA Agents

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 downregulation 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, or for example, 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 may be in a terminal region or regions, *e.g.*, within 6, 5, 4, or 3 nucleotides of the 5' and/or 3' termini. The sense strand need  
5 only be sufficiently complementary with the antisense strand to maintain the overall double stranded character of the molecule.

As discussed elsewhere herein, and in the material incorporated by reference in its entirety, an iRNA agent will often be modified or include nucleoside surrogates. Single stranded regions of an iRNA agent will often be modified or include nucleoside  
10 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'-termini of an iRNA agent, *e.g.*, against exonucleases, or to favor the antisense siRNA agent to enter into RISC are also envisioned. Modifications can include C3 (or C6, C7, C12) amino linkers, thiol linkers,  
15 carboxyl linkers, non-nucleotide 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  
20 response (which can be cleaved by Dicer (Bernstein *et al.* 2001. Nature, 409:363-366) and enter a RISC (RNAi-induced silencing complex)); and, molecules which 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.  
25 Molecules that are short enough that they do not trigger an interferon response are termed siRNA agents or shorter iRNA agents herein. "siRNA 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, 50, 40, or 30 nucleotide pairs.  
30 The siRNA agent, or a cleavage product thereof, can down regulate a target gene, *e.g.*, by inducing RNAi with respect to a target RNA, wherein the target may comprise an endogenous or pathogen target RNA.

Each strand of an siRNA agent can be equal to or less than 30, 25, 24, 23, 22, 21, or 20 nucleotides in length. The strand may be at least 19 nucleotides in length. For

example, each strand can be between 21 and 25 nucleotides in length. siRNA agents may have a duplex region of 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotide pairs, and one or more overhangs, or one or two 3' overhangs, of 2- 3 nucleotides.

In addition to homology to target RNA and the ability to down regulate a target gene, an iRNA agent may have one or more of the following properties:

(1) it may be of the Formula VII, VIII, IX or X set out in the RNA Agent section below;

(2) if single stranded it may have a 5' modification which includes one or more phosphate groups or one or more analogs of a phosphate group;

(3) it may, despite modifications, even to a very large number, or all of the nucleosides, have an antisense strand that can present bases (or modified bases) in the proper three dimensional framework so as to be able to form correct base pairing and form a duplex structure with a homologous target RNA which is sufficient to allow down regulation of the target, *e.g.*, by cleavage of the target RNA;

(4) it may, despite modifications, even to a very large number, or all of the nucleosides, still have "RNA-like" properties, *i.e.*, it may possess the overall structural, chemical and physical properties of an RNA molecule, even though not exclusively, or even partly, of ribonucleotide-based content. For example, an iRNA agent can contain, *e.g.*, a sense and/or an antisense strand in which all of the nucleotide sugars contain *e.g.*, 2' fluoro in place of 2' hydroxyl. This deoxyribonucleotide-containing agent can still be expected to exhibit RNA-like properties. While not wishing to be bound by theory, the electronegative fluorine prefers an axial orientation when attached to the C2' position of ribose. This spatial preference of fluorine can, in turn, force the sugars to adopt a C<sub>3'</sub>-*endo* pucker. This is the same puckering mode as observed in RNA molecules and gives rise to the RNA-characteristic A-family-type helix. Further, since fluorine is a good hydrogen bond acceptor, it can participate in the same hydrogen bonding interactions with water molecules that are known to stabilize RNA structures. A modified moiety at the 2' sugar position may be able to enter into H bonding which is more characteristic of the OH moiety of a ribonucleotide than the H moiety of a deoxyribonucleotide. Certain iRNA agents will: exhibit a C<sub>3'</sub>-*endo* pucker in all, or at least 50, 75, 80, 85, 90, or 95 % of its sugars; exhibit a C<sub>3'</sub>-*endo* pucker in a sufficient amount of its sugars that it can give rise to a the RNA-characteristic A-family-type helix; will have no more than 20, 10, 5, 4, 3, 2, or 1 sugar which is not a C<sub>3'</sub>-*endo* pucker structure. Regardless of the nature of the modification, and even though the RNA agent can contain deoxynucleotides or modified

deoxynucleotides, particularly in overhang or other single strand regions, it is certain DNA molecules, or any molecule in which more than 50, 60, or 70 % of the nucleotides in the molecule, or more than 50, 60, or 70 % of the nucleotides in a duplexed region are deoxyribonucleotides, or modified deoxyribonucleotides which are deoxy at the 2'

5 position, are excluded from the definition of RNA agent.

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 may be antisense with regard to the target molecule. In certain 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)<sub>2</sub>(O)P-O-5'); 5'-diphosphate ((HO)<sub>2</sub>(O)P-O-P(HO)(O)-O-5'); 5'-triphosphate ((HO)<sub>2</sub>(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<sub>ppp</sub>), 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)<sub>2</sub>(S)P-O-5'); 5'-monodithiophosphate (phosphorodithioate; (HO)(HS)(S)P-O-5'), 5'-phosphorothiolate ((HO)<sub>2</sub>(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)<sub>2</sub>(O)P-NH-5', (HO)(NH<sub>2</sub>)(O)P-O-5'), 5'-alkylphosphonates (R=alkyl=methyl, ethyl, isopropyl, propyl, etc., *e.g.*, RP(OH)(O)-O-5', (OH)<sub>2</sub>(O)P-5'-CH<sub>2</sub>-), 5'-alkyletherphosphonates (R=alkylether=methoxymethyl (MeOCH<sub>2</sub>-), 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 may 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 in other embodiments at least 15, 20, 25, 29, 35, 40, or 50 nucleotides in length. In certain embodiments, it is 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, 29, 21, 22, 23, 24, or 25 nucleotide pairs. The duplex region will may be equal to or less than 200, 100, or 50, in length. In certain embodiments, ranges for the duplex region are 15-

30, 17 to 23, 19 to 23, and 19 to 21 nucleotides pairs in length. The hairpin may have a single strand overhang or terminal unpaired region, in some embodiments at the 3', and in certain embodiments on the antisense side of the hairpin. In some embodiments, the overhangs are 2-3 nucleotides in length.

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

The antisense strand of a double stranded iRNA agent may be equal to or at least, 14, 15, 16 17, 18, 19, 25, 29, 40, or 60 nucleotides in length. It may be equal to or less  
10 than 200, 100, or 50, nucleotides in length. Ranges may be 17 to 25, 19 to 23, and 19 to 21 nucleotides in length.

The sense strand of a double stranded iRNA agent may be equal to or at least 14, 15, 16 17, 18, 19, 25, 29, 40, or 60 nucleotides in length. It may be equal to or less than 200, 100, or 50, nucleotides in length. Ranges may be 17 to 25, 19 to 23, and 19 to 21  
15 nucleotides in length.

The double strand portion of a double stranded iRNA agent may be equal to or at least, 14, 15, 16 17, 18, 19, 20, 21, 22, 23, 24, 25, 29, 40, or 60 nucleotide pairs in length. It may be equal to or less than 200, 100, or 50, nucleotides pairs in length. Ranges may be 15-30, 17 to 23, 19 to 23, and 19 to 21 nucleotides pairs in length.

20 In many embodiments, the ds iRNA agent is sufficiently large that it can be cleaved by an endogenous molecule, *e.g.*, by Dicer, to produce smaller ds iRNA agents, *e.g.*, siRNAs agents

It may be desirable to modify one or both of the antisense and sense strands of a double strand iRNA agent. In some cases they will have the same modification or the  
25 same class of modification but in other cases the sense and antisense strand will have different modifications, *e.g.*, in some cases it is desirable to modify only the sense strand. It may be desirable to modify only the sense strand, *e.g.*, to inactivate it, *e.g.*, the sense strand can be modified in order to inactivate the sense strand and prevent formation of an active siRNA/protein or RISC. This can be accomplished by a modification which  
30 prevents 5'-phosphorylation of the sense strand, *e.g.*, by modification with a 5'-O-methyl ribonucleotide (see Nykänen *et al.*, (2001) ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell 107, 309-321.) Other modifications which prevent phosphorylation can also be used, *e.g.*, simply substituting the 5'-OH by H rather than O-Me. Alternatively, a large bulky group may be added to the 5'-phosphate

turning it into a phosphodiester linkage, though this may be less desirable as phosphodiesterases can cleave such a linkage and release a functional siRNA 5'-end. Antisense strand modifications include 5' phosphorylation as well as any of the other 5' modifications discussed herein, particularly the 5' modifications discussed above in the section on single stranded iRNA molecules.

The sense and antisense strands may be chosen such that the ds iRNA agent includes a single strand or unpaired region at one or both ends of the molecule. Thus, a ds iRNA agent may contain sense and antisense strands, paired to contain an overhang, *e.g.*, one or two 5' or 3' overhangs, or a 3' overhang of 2-3 nucleotides. Many embodiments will have a 3' overhang. Certain siRNA agents will have single-stranded overhangs, in some embodiments, 3' overhangs, of 1 or 2 or 3 nucleotides in length at each end. 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 may be phosphorylated.

In some embodiments, the length for the duplexed region is between 15 and 30, or 18, 19, 20, 21, 22, and 23 nucleotides in length, *e.g.*, in the siRNA agent range discussed above. siRNA agents can resemble in length and structure the natural Dicer processed products from long dsRNAs. 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 a 3' overhang are also within the invention.

The isolated iRNA agents described herein, including ds iRNA agents and siRNA agents can mediate silencing of a target RNA, *e.g.*, mRNA, *e.g.*, a transcript of a gene that encodes a protein. For convenience, such mRNA is also referred to herein as mRNA to be silenced. Such a gene is also referred to as a target gene. In general, the RNA to be silenced is an endogenous gene or a pathogen gene. In addition, RNAs other than mRNA, *e.g.*, tRNAs, and viral RNAs, can also be targeted.

As used herein, the phrase "mediates RNAi" refers to the ability to silence, in a sequence specific manner, a target RNA. While not wishing to be bound by theory, it is believed that silencing uses the RNAi machinery or process and a guide RNA, *e.g.*, an siRNA agent of 21 to 23 nucleotides.

As used herein, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity such that stable and specific binding occurs between a compound of the invention and a target RNA molecule.

Specific binding requires a sufficient degree of complementarity to avoid non-specific binding of the oligomeric compound to non-target sequences under conditions in which specific binding is desired, *i.e.*, under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or in the case of *in vitro* assays, under conditions in which the assays are performed. The non-target sequences typically differ by at least 5 nucleotides.

In one embodiment, an iRNA agent is “sufficiently complementary” to a target RNA, *e.g.*, a target mRNA, such that the iRNA agent silences production of protein encoded by the target mRNA. In another embodiment, the iRNA agent is “exactly complementary” to a target RNA, *e.g.*, the target RNA and the iRNA agent anneal, for example to form a hybrid made exclusively of Watson-Crick base pairs in the region of exact complementarity. A “sufficiently complementary” target RNA can include an internal region (*e.g.*, of at least 10 nucleotides) that is exactly complementary to a target RNA. Moreover, in some embodiments, the iRNA agent specifically discriminates a single-nucleotide difference. In this case, the iRNA agent only mediates RNAi if exact complementary is found in the region (*e.g.*, within 7 nucleotides of) the single-nucleotide difference.

RNA agents discussed herein include unmodified RNA as well as RNA which have been modified, *e.g.*, to improve efficacy, and polymers of nucleoside surrogates. Unmodified RNA refers to a molecule in which the components of the nucleic acid, namely sugars, bases, and phosphate moieties, are the same or essentially the same as that which occur in nature, for example as occur naturally in the human body. The art has often referred to rare or unusual, but naturally occurring, RNAs as modified RNAs, see, *e.g.*, Limbach *et al.*, (1994) Summary: the modified nucleosides of RNA, *Nucleic Acids Res.* 22: 2183-2196. Such rare or unusual RNAs, often termed modified RNAs (apparently because they are typically the result of a post transcriptionally modification) are within the term unmodified RNA, as used herein. Modified RNA refers to a molecule in which one or more of the components of the nucleic acid, namely sugars, bases, and phosphate moieties, are different from that which occurs in nature, for example, different from that which occurs in the human body. While they are referred to as modified “RNAs,” they will of course, because of the modification, include molecules which are not RNAs. Nucleoside surrogates are molecules in which the ribophosphate backbone is replaced with a non-ribophosphate construct that allows the bases to be presented in the correct spatial relationship such that hybridization is substantially similar

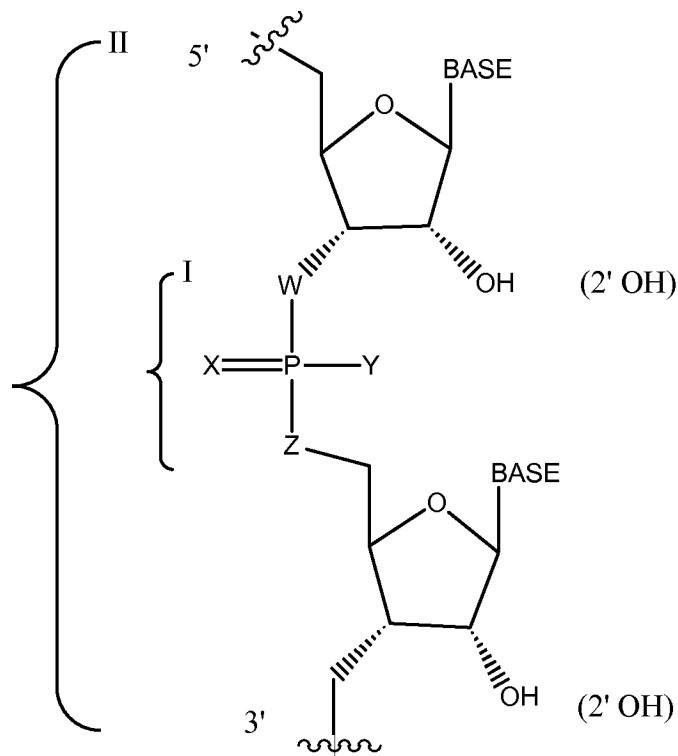
to what is seen with a ribophosphate backbone, *e.g.*, non-charged mimics of the ribophosphate backbone. Examples of all of the above are discussed herein.

Much of the discussion below refers to single strand molecules. In many embodiments of the invention a double stranded iRNA agent, *e.g.*, a partially double stranded iRNA agent, is envisioned. Thus, it is understood that that double stranded structures (*e.g.*, where two separate molecules are contacted to form the double stranded region or where the double stranded region is formed by intramolecular pairing (*e.g.*, a hairpin structure)) made of the single stranded structures described below are within the invention. Lengths are described elsewhere herein.

As nucleic acids are polymers of subunits, many of the modifications described below occur at a position which is repeated within a nucleic acid, *e.g.*, a modification of a base, or a phosphate moiety, or the a non-linking O of a phosphate moiety. In some cases the modification will occur at all of the subject positions in the nucleic acid but in many cases it will not. By way of example, a modification may only occur at a 3' or 5' terminal position, may only occur in a terminal regions, *e.g.*, at a position on a terminal nucleotide or in the last 2, 3, 4, 5, or 10 nucleotides of a strand. A modification may occur in a double strand region, a single strand region, or in both. A modification may occur only in the double strand region of an RNA or may only occur in a single strand region of an RNA. *E.g.*, a phosphorothioate modification at a non-linking O position may only occur at one or both termini, may only occur in a terminal regions, *e.g.*, at a position on a terminal nucleotide or in the last 2, 3, 4, 5, or 10 nucleotides of a strand, or may occur in double strand and single strand regions, particularly at termini. The 5' end or ends can be phosphorylated.

In some embodiments it is possible, *e.g.*, to enhance stability, to include particular bases in overhangs, or to include modified nucleotides or nucleotide surrogates, in single strand overhangs, *e.g.*, in a 5' or 3' overhang, or in both. *E.g.*, it can be desirable to include purine nucleotides in overhangs. In some embodiments all or some of the bases in a 3' or 5' overhang will be modified, *e.g.*, with a modification described herein. Modifications can include, *e.g.*, the use of modifications at the 2' OH group of the ribose sugar, *e.g.*, the use of deoxyribonucleotides, *e.g.*, deoxythymidine, instead of ribonucleotides, and modifications in the phosphate group, *e.g.*, phosphothioate modifications. Overhangs need not be homologous with the target sequence.

Modifications and nucleotide surrogates are discussed below.



FORMULA (VII)

The scaffold presented above in Formula VII represents a portion of a ribonucleic acid. The basic components are the ribose sugar, the base, the terminal phosphates, and phosphate internucleotide linkers. Where the bases are naturally occurring bases, *e.g.*, adenine, uracil, guanine or cytosine, the sugars are the unmodified 2' hydroxyl ribose sugar (as depicted) and W, X, Y, and Z are all O, Formula VII represents a naturally occurring unmodified oligoribonucleotide.

Unmodified oligoribonucleotides may be less than optimal in some applications, *e.g.*, unmodified oligoribonucleotides can be prone to degradation by *e.g.*, cellular nucleases. Nucleases can hydrolyze nucleic acid phosphodiester bonds. However, chemical modifications to one or more of the above RNA components can confer improved properties, and, *e.g.*, can render oligoribonucleotides more stable to nucleases.

Modified nucleic acids and nucleotide surrogates can include one or more of:

- (i) alteration, *e.g.*, replacement, of one or both of the non-linking (X and Y) phosphate oxygens and/or of one or more of the linking (W and Z) phosphate oxygens (When the phosphate is in the terminal position, one of the positions W or Z will not link the phosphate to an additional element in a naturally occurring ribonucleic acid. However, for simplicity of terminology, except where otherwise noted, the W position at the 5' end of a nucleic acid and the terminal Z position at



the 3' end of a nucleic acid, are within the term "linking phosphate oxygens" as used herein);

(ii) alteration, *e.g.*, replacement, of a constituent of the ribose sugar, *e.g.*, of the 2' hydroxyl on the ribose sugar;

5 (iii) wholesale replacement of the phosphate moiety (bracket I) with "dephospho" linkers;

(iv) modification or replacement of a naturally occurring base;

(v) replacement or modification of the ribose-phosphate backbone (bracket II);

10 (vi) modification of the 3' end or 5' end of the RNA, *e.g.*, removal, modification or replacement of a terminal phosphate group or conjugation of a moiety, *e.g.*, a fluorescently labeled moiety, to either the 3' or 5' end of RNA.

The terms replacement, modification, alteration, and the like, as used in this context, do not imply any process limitation, *e.g.*, modification does not mean that one must start with a reference or naturally occurring ribonucleic acid and modify it to produce a modified ribonucleic acid but rather modified simply indicates a difference from a naturally occurring molecule.

It is understood that the actual electronic structure of some chemical entities cannot be adequately represented by only one canonical form (*i.e.*, Lewis structure).

20 While not wishing to be bound by theory, the actual structure can instead be some hybrid or weighted average of two or more canonical forms, known collectively as resonance forms or structures. Resonance structures are not discrete chemical entities and exist only on paper. They differ from one another only in the placement or "localization" of the bonding and nonbonding electrons for a particular chemical entity. It can be possible for one resonance structure to contribute to a greater extent to the hybrid than the others.

25 Thus, the written and graphical descriptions of the embodiments of the present invention are made in terms of what the art recognizes as the predominant resonance form for a particular species. For example, any phosphoroamidate (replacement of a nonlinking oxygen with nitrogen) would be represented by  $X = O$  and  $Y = N$  in the above figure.

30 Specific modifications are discussed in more detail below.

### The Phosphate Group

The phosphate group is a negatively charged species. The charge is distributed equally over the two non-linking oxygen atoms (*i.e.*, X and Y in Formula 1 above).

5 However, the phosphate group can be modified by replacing one of the oxygens with a different substituent. One result of this modification to RNA phosphate backbones can be increased resistance of the oligoribonucleotide to nucleolytic breakdown. Thus while not wishing to be bound by theory, it can be desirable in some embodiments to introduce alterations which result in either an uncharged linker or a charged linker with  
10 unsymmetrical charge distribution.

Examples of modified phosphate groups include phosphorothioate, phosphoroselenates, borano phosphates, borano phosphate esters, hydrogen phosphonates, phosphoramidates, alkyl or aryl phosphonates and phosphotriesters. Phosphorodithioates have both non-linking oxygens replaced by sulfur. Unlike the  
15 situation where only one of X or Y is altered, the phosphorus center in the phosphorodithioates is achiral which precludes the formation of oligoribonucleotides diastereomers. Diastereomer formation can result in a preparation in which the individual diastereomers exhibit varying resistance to nucleases. Further, the hybridization affinity of RNA containing chiral phosphate groups can be lower relative to the corresponding  
20 unmodified RNA species. Thus, while not wishing to be bound by theory, modifications to both X and Y which eliminate the chiral center, *e.g.*, phosphorodithioate formation, may be desirable in that they cannot produce diastereomer mixtures. Thus, X can be any one of S, Se, B, C, H, N, or OR (R is alkyl or aryl). Thus Y can be any one of S, Se, B, C, H, N, or OR (R is alkyl or aryl). Replacement of X and/or Y with sulfur is possible.

25 The phosphate linker can also be modified by replacement of a linking oxygen (*i.e.*, W or Z in Formula 1) with nitrogen (bridged phosphoramidates), sulfur (bridged phosphorothioates) and carbon (bridged methylenephosphonates). The replacement can occur at a terminal oxygen (position W (3') or position Z (5')). Replacement of W with carbon or Z with nitrogen is possible.

30 Candidate agents can be evaluated for suitability as described below.

### The Sugar Group

A modified RNA can include modification of all or some of the sugar groups of the ribonucleic acid. *E.g.*, the 2' hydroxyl group (OH) can be modified or replaced with a number of different “oxy” or “deoxy” substituents. While not being bound by theory, enhanced stability is expected since the hydroxyl can no longer be deprotonated to form a 2' alkoxide ion. The 2' alkoxide can catalyze degradation by intramolecular nucleophilic attack on the linker phosphorus atom. Again, while not wishing to be bound by theory, it can be desirable to some embodiments to introduce alterations in which alkoxide formation at the 2' position is not possible.

Examples of “oxy”-2' hydroxyl group modifications include alkoxy or aryloxy (OR, *e.g.*, R = H, alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar); polyethyleneglycols (PEG),  $O(CH_2CH_2O)_nCH_2CH_2OR$ ; “locked” nucleic acids (LNA) in which the 2' hydroxyl is connected, *e.g.*, by a methylene bridge, to the 4' carbon of the same ribose sugar; O-AMINE (AMINE =  $NH_2$ ; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino) and aminoalkoxy,  $O(CH_2)_nAMINE$ , (*e.g.*, AMINE =  $NH_2$ ; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino). It is noteworthy that oligonucleotides containing only the methoxyethyl group (MOE),  $(OCH_2CH_2OCH_3)$ , a PEG derivative, exhibit nuclease stabilities comparable to those modified with the robust phosphorothioate modification.

“Deoxy” modifications include hydrogen (*i.e.*, deoxyribose sugars, which are of particular relevance to the overhang portions of partially ds RNA); halo (*e.g.*, fluoro); amino (*e.g.*,  $NH_2$ ; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, or amino acid);  $NH(CH_2CH_2NH)_nCH_2CH_2-$ AMINE (AMINE =  $NH_2$ ; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino),  $-NHC(O)R$  (R = alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar), cyano; mercapto; alkyl-thio-alkyl; thioalkoxy; and alkyl, cycloalkyl, aryl, alkenyl and alkynyl, which may be optionally substituted with *e.g.*, an amino functionality. Other substituents of certain embodiments include 2'-methoxyethyl, 2'-OCH<sub>3</sub>, 2'-O-allyl, 2'-C-allyl, and 2'-fluoro.

The sugar group can also contain one or more carbons that possess the opposite stereochemical configuration than that of the corresponding carbon in ribose. Thus, a modified RNA can include nucleotides containing *e.g.*, arabinose, as the sugar.

Modified RNA's can also include "abasic" sugars, which lack a nucleobase at C-  
5 1'. These abasic sugars can also be further contain modifications at one or more of the constituent sugar atoms.

To maximize nuclease resistance, the 2' modifications can be used in combination with one or more phosphate linker modifications (*e.g.*, phosphorothioate). The so-called "chimeric" oligonucleotides are those that contain two or more different modifications.

10 Candidate modifications can be evaluated as described below.

#### Replacement of the Phosphate Group

The phosphate group can be replaced by non-phosphorus containing connectors (*cf.* Bracket I in Formula 1 above). While not wishing to be bound by theory, it is  
15 believed that since the charged phosphodiester group is the reaction center in nucleolytic degradation, its replacement with neutral structural mimics should impart enhanced nuclease stability. Again, while not wishing to be bound by theory, it can be desirable, in some embodiment, to introduce alterations in which the charged phosphate group is replaced by a neutral moiety.

20 Examples of moieties which can replace the phosphate group include siloxane, carbonate, carboxymethyl, carbamate, amide, thioether, ethylene oxide linker, sulfonate, sulfonamide, thioformacetal, formacetal, oxime, methyleneimino, methylenemethylimino, methylenehydrazo, methylenedimethylhydrazo and methyleneoxymethylimino. In certain  
25 embodiments, replacements may include the methylenecarbonylamino and methylenemethylimino groups.

Candidate modifications can be evaluated as described below.

#### Replacement of Ribophosphate Backbone

Oligonucleotide- mimicking scaffolds can also be constructed wherein the  
30 phosphate linker and ribose sugar are replaced by nuclease resistant nucleoside or nucleotide surrogates (see Bracket II of Formula 1 above). While not wishing to be bound by theory, it is believed that the absence of a repetitively charged backbone diminishes binding to proteins that recognize polyanions (*e.g.*, nucleases). Again, while

not wishing to be bound by theory, it can be desirable in some embodiment, to introduce alterations in which the bases are tethered by a neutral surrogate backbone.

Examples include the morpholino, cyclobutyl, pyrrolidine and peptide nucleic acid (PNA) nucleoside surrogates. In certain embodiments, PNA surrogates may be used.

5 Candidate modifications can be evaluated as described below.

### Terminal Modifications

The 3' and 5' ends of an oligonucleotide can be modified. Such modifications can be at the 3' end, 5' end or both ends of the molecule. They can include modification or  
10 replacement of an entire terminal phosphate or of one or more of the atoms of the phosphate group. *E.g.*, 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  
15 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  
20 sugars, amide, carboxy, amine, oxyamine, oximine, 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 strands of iRNA agents, this 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  
25 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),  
30 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, O<sub>3</sub>-(oleoyl)lithocholic acid, O<sub>3</sub>-(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]<sub>2</sub>, polyamino, alkyl, substituted alkyl, radiolabeled  
 5 markers, enzymes, haptens (*e.g.*, biotin), transport/absorption facilitators (*e.g.*, aspirin, vitamin E, folic acid), synthetic ribonucleases (*e.g.*, imidazole, bisimidazole, histamine, imidazole clusters, acridine-imidazole conjugates, Eu<sup>3+</sup> complexes of tetraazamacrocycles).

Terminal modifications can be added for a number of reasons, including as  
 10 discussed elsewhere herein 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. *E.g.*, in certain embodiments, iRNA agents, especially antisense strands, are 5' phosphorylated or include a phosphoryl analog at the 5' prime terminus. 5'-phosphate modifications include those which are compatible with  
 15 RISC mediated gene silencing. Suitable modifications include: 5'-monophosphate ((HO)<sub>2</sub>(O)P-O-5'); 5'-diphosphate ((HO)<sub>2</sub>(O)P-O-P(HO)(O)-O-5'); 5'-triphosphate ((HO)<sub>2</sub>(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<sub>ppp</sub>), and any modified or unmodified nucleotide cap structure (N-O-5'-(HO)(O)P-O-  
 20 (HO)(O)P-O-P(HO)(O)-O-5'); 5'-monothiophosphate (phosphorothioate; (HO)<sub>2</sub>(S)P-O-5'); 5'-monodithiophosphate (phosphorodithioate; (HO)(HS)(S)P-O-5'), 5'-phosphorothiolate ((HO)<sub>2</sub>(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)<sub>2</sub>(O)P-NH-5',  
 25 (HO)(NH<sub>2</sub>)(O)P-O-5'), 5'-alkylphosphonates (R=alkyl=methyl, ethyl, isopropyl, propyl, etc., *e.g.*, RP(OH)(O)-O-5'-, (OH)<sub>2</sub>(O)P-5'-CH<sub>2</sub>-), 5'-alkyletherphosphonates (R=alkylether=methoxymethyl (MeOCH<sub>2</sub>-), ethoxymethyl, etc., *e.g.*, RP(OH)(O)-O-5'-).

Terminal modifications can also be useful for increasing resistance to degradation.

Terminal modifications can also be useful for monitoring distribution, and in such  
 30 cases the groups to be added may include fluorophores, *e.g.*, fluorescein or an Alexa dye, *e.g.*, Alexa 488. . Terminal modifications can also be useful for enhancing uptake, useful modifications for this include cholesterol. Terminal modifications can also be useful for cross-linking an RNA agent to another moiety; modifications useful for this include mitomycin C.

Candidate modifications can be evaluated as described below.

### The Bases

Adenine, guanine, cytosine and uracil are the most common bases found in RNA.

- 5 These bases can be modified or replaced to provide RNA's having improved properties. *E.g.*, nuclease resistant oligoribonucleotides can be prepared with these bases or with synthetic and natural nucleobases (*e.g.*, inosine, thymine, xanthine, hypoxanthine, nubularine, isoguanisine, or tubercidine) and any one of the above modifications. Alternatively, substituted or modified analogs of any of the above bases and “universal
- 10 bases” can be employed. Examples include 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 5-halouracil, 5-(2-aminopropyl)uracil, 5-amino allyl uracil, 8-halo, amino, thiol, thioalkyl, hydroxyl and other 8-substituted
- 15 adenines and guanines, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine, 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine, dihydrouracil, 3-deaza-5-azacytosine, 2-aminopurine, 5-alkyluracil, 7-alkylguanine, 5-alkyl cytosine, 7-deazaadenine, N6, N6-dimethyladenine, 2,6-
- 20 diaminopurine, 5-amino-allyl-uracil, N3-methyluracil, substituted 1,2,4-triazoles, 2-pyridinone, 5-nitroindole, 3-nitropyrrole, 5-methoxyuracil, uracil-5-oxyacetic acid, 5-methoxycarbonylmethyluracil, 5-methyl-2-thiouracil, 5-methoxycarbonylmethyl-2-thiouracil, 5-methylaminomethyl-2-thiouracil, 3-(3-amino-3carboxypropyl)uracil, 3-methylcytosine, 5-methylcytosine, N<sup>4</sup>-acetyl cytosine, 2-thiocytosine, N6-methyladenine,
- 25 N6-isopentyladenine, 2-methylthio-N6-isopentenyladenine, N-methylguanines, or O-alkylated bases. Further purines and pyrimidines include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in the Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859, Kroschwitz, J. I., ed. John Wiley & Sons, 1990, and those disclosed by Englisch *et al.*, *Angewandte Chemie*, International Edition, 1991, 30, 613.
- 30 Generally, base changes are not used for promoting stability, but they can be useful for other reasons, *e.g.*, some, *e.g.*, 2,6-diaminopurine and 2 amino purine, are fluorescent. Modified bases can reduce target specificity. This may be taken into consideration in the design of iRNA agents.

Candidate modifications can be evaluated as described below.

### Evaluation of Candidate RNAs

One can evaluate a candidate RNA agent, *e.g.*, a modified RNA, for a selected property by exposing the agent or modified molecule and a control molecule to the appropriate conditions and evaluating for the presence of the selected property. For example, resistance to a degradant can be evaluated as follows. A candidate modified RNA (and a control molecule, usually the unmodified form) can be exposed to degradative conditions, *e.g.*, exposed to a milieu, which includes a degradative agent, *e.g.*, a nuclease. *E.g.*, one can use a biological sample, *e.g.*, one that is similar to a milieu, which might be encountered, in therapeutic use, *e.g.*, blood or a cellular fraction, *e.g.*, a cell-free homogenate or disrupted cells. The candidate and control could then be evaluated for resistance to degradation by any of a number of approaches. For example, the candidate and control could be labeled prior to exposure, with, *e.g.*, a radioactive or enzymatic label, or a fluorescent label, such as Cy3 or Cy5. Control and modified RNA's can be incubated with the degradative agent, and optionally a control, *e.g.*, an inactivated, *e.g.*, heat inactivated, degradative agent. A physical parameter, *e.g.*, size, of the modified and control molecules are then determined. They can be determined by a physical method, *e.g.*, by polyacrylamide gel electrophoresis or a sizing column, to assess whether the molecule has maintained its original length, or assessed functionally. Alternatively, Northern blot analysis can be used to assay the length of an unlabeled modified molecule.

A functional assay can also be used to evaluate the candidate agent. A functional assay can be applied initially or after an earlier non-functional assay, (*e.g.*, assay for resistance to degradation) to determine if the modification alters the ability of the molecule to silence gene expression. For example, a cell, *e.g.*, a mammalian cell, such as a mouse or human cell, can be co-transfected with a plasmid expressing a fluorescent protein, *e.g.*, GFP, and a candidate RNA agent homologous to the transcript encoding the fluorescent protein (see, *e.g.*, WO 00/44914). For example, a modified dsRNA homologous to the GFP mRNA can be assayed for the ability to inhibit GFP expression by monitoring for a decrease in cell fluorescence, as compared to a control cell, in which the transfection did not include the candidate dsRNA, *e.g.*, controls with no agent added and/or controls with a non-modified RNA added. Efficacy of the candidate agent on gene expression can be assessed by comparing cell fluorescence in the presence of the modified and unmodified dsRNA agents.



In an alternative functional assay, a candidate dsiRNA agent homologous to an endogenous mouse gene, for example, a maternally expressed gene, such as *c-mos*, can be injected into an immature mouse oocyte to assess the ability of the agent to inhibit gene expression *in vivo* (see, *e.g.*, WO 01/36646). A phenotype of the oocyte, *e.g.*, the ability to maintain arrest in metaphase II, can be monitored as an indicator that the agent is inhibiting expression. For example, cleavage of *c-mos* mRNA by a dsiRNA agent would cause the oocyte to exit metaphase arrest and initiate parthenogenetic development (Colledge *et al.* Nature 370: 65-68, 1994; Hashimoto *et al.* Nature, 370:68-71, 1994). The effect of the modified agent on target RNA levels can be verified by Northern blot to assay for a decrease in the level of target mRNA, or by Western blot to assay for a decrease in the level of target protein, as compared to a negative control. Controls can include cells in which with no agent is added and/or cells in which a non-modified RNA is added.

#### 15 RNA Structure References

The disclosure of all publications, patents, and published patent applications listed herein are hereby incorporated by reference.

#### General References

20 The oligoribonucleotides and oligoribonucleosides used in accordance with this invention may be with solid phase synthesis, see for example "Oligonucleotide synthesis, a practical approach", Ed. M. J. Gait, IRL Press, 1984; "Oligonucleotides and Analogues, A Practical Approach", Ed. F. Eckstein, IRL Press, 1991 (especially Chapter 1, Modern machine-aided methods of oligodeoxyribonucleotide synthesis, Chapter 2, Oligoribonucleotide synthesis, Chapter 3, 2'-O--Methyloligoribonucleotide- s: synthesis and applications, Chapter 4, Phosphorothioate oligonucleotides, Chapter 5, Synthesis of oligonucleotide phosphorodithioates, Chapter 6, Synthesis of oligo-2'-deoxyribonucleoside methylphosphonates, and. Chapter 7, Oligodeoxynucleotides containing modified bases. Other particularly useful synthetic procedures, reagents, 30 blocking groups and reaction conditions are described in Martin, P., *Helv. Chim. Acta*, **1995**, 78, 486-504; Beaucage, S. L. and Iyer, R. P., *Tetrahedron*, **1992**, 48, 2223-2311 and Beaucage, S. L. and Iyer, R. P., *Tetrahedron*, **1993**, 49, 6123-6194, or references referred to therein. Modification described in WO 00/44895, WO01/75164, or WO02/44321 can be used herein.

### Phosphate Group References

The preparation of phosphinate oligoribonucleotides is described in U.S. Pat. No. 5,508,270. The preparation of alkyl phosphonate oligoribonucleotides is described in  
5 U.S. Pat. No. 4,469,863. The preparation of phosphoramidite oligoribonucleotides is described in U.S. Pat. No. 5,256,775 or U.S. Pat. No. 5,366,878. The preparation of phosphotriester oligoribonucleotides is described in U.S. Pat. No. 5,023,243. The preparation of borano phosphate oligoribonucleotide is described in U.S. Pat. Nos. 5,130,302 and 5,177,198. The preparation of 3'-Deoxy-3'-amino phosphoramidate  
10 oligoribonucleotides is described in U.S. Pat. No. 5,476,925. 3'-Deoxy-3'-methylene phosphonate oligoribonucleotides is described in An, H, *et al. J. Org. Chem.* **2001**, *66*, 2789-2801. Preparation of sulfur bridged nucleotides is described in Sproat *et al. Nucleosides Nucleotides* **1988**, *7*,651 and Crosstick *et al. Tetrahedron Lett.* **1989**, *30*, 4693.

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### Sugar Group References

Modifications to the 2' modifications can be found in Verma, S. *et al. Annu. Rev. Biochem.* **1998**, *67*, 99-134 and all references therein. Specific modifications to the ribose can be found in the following references: 2'-fluoro (Kawasaki *et. al., J. Med. Chem.*, **1993**, *36*, 831-841), 2'-MOE (Martin, P. *Helv. Chim. Acta* **1996**, *79*, 1930-1938), "LNA" (Wengel, J. *Acc. Chem. Res.* **1999**, *32*, 301-310).

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### Replacement of the Phosphate Group References

Methylenemethylimino linked oligoribonucleosides, also identified herein as MMI  
25 linked oligoribonucleosides, methylenedimethylhydrazo linked oligoribonucleosides, also identified herein as MDH linked oligoribonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified herein as amide-3 linked oligoribonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified herein as amide-4 linked oligoribonucleosides as well as mixed backbone compounds having, as for  
30 instance, alternating MMI and PO or PS linkages can be prepared as is described in U.S. Pat. Nos. 5,378,825, 5,386,023, 5,489,677 and in published PCT applications PCT/US92/04294 and PCT/US92/04305 (published as WO 92/20822 WO and 92/20823, respectively). Formacetal and thioformacetal linked oligoribonucleosides can be prepared as is described in U.S. Pat. Nos. 5,264,562 and 5,264,564. Ethylene oxide linked

oligoribonucleosides can be prepared as is described in U.S. Pat. No. 5,223,618. Siloxane replacements are described in Cormier, J.F. *et al. Nucleic Acids Res.* **1988**, *16*, 4583. Carbonate replacements are described in Tittensor, J.R. *J. Chem. Soc. C* **1971**, 1933. Carboxymethyl replacements are described in Edge, M.D. *et al. J. Chem. Soc. Perkin Trans. 1* **1972**, 1991. Carbamate replacements are described in Stirchak, E.P. *Nucleic Acids Res.* 1989, *17*, 6129.

#### Replacement of the Phosphate-Ribose Backbone References

Cyclobutyl sugar surrogate compounds can be prepared as is described in U.S. Pat. No. 5,359,044. Pyrrolidine sugar surrogate can be prepared as is described in U.S. Pat. No. 5,519,134. Morpholino sugar surrogates can be prepared as is described in U.S. Pat. Nos. 5,142,047 and 5,235,033, and other related patent disclosures. Peptide Nucleic Acids (PNAs) are known per se and can be prepared in accordance with any of the various procedures referred to in *Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications*, Bioorganic & Medicinal Chemistry, 1996, *4*, 5-23. They may also be prepared in accordance with U.S. Pat. No. 5,539,083.

#### Terminal Modification References

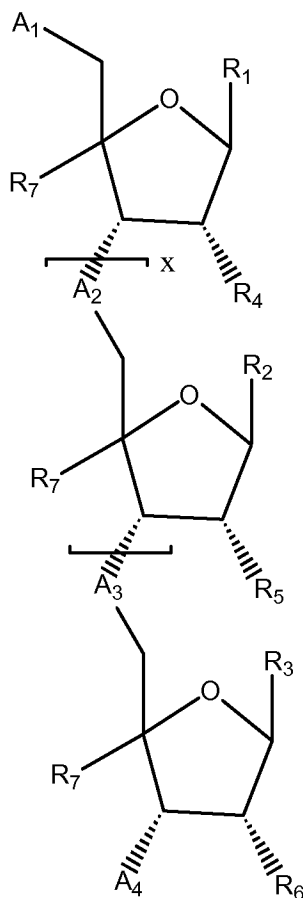
Terminal modifications are described in Manoharan, M. *et al. Antisense and Nucleic Acid Drug Development* *12*, 103-128 (2002) and references therein.

#### Base References

N-2 substituted purine nucleoside amidites can be prepared as is described in U.S. Pat. No. 5,459,255. 3-Deaza purine nucleoside amidites can be prepared as is described in U.S. Pat. No. 5,457,191. 5,6-Substituted pyrimidine nucleoside amidites can be prepared as is described in U.S. Pat. No. 5,614,617. 5-Propynyl pyrimidine nucleoside amidites can be prepared as is described in U.S. Pat. No. 5,484,908. Additional references can be disclosed in the above section on base modifications.

Additional RNA Agents

Certain RNA agents have the following structure (Formula VIII):



FORMULA VIII

5

wherein:

$R^1$ ,  $R^2$ , and  $R^3$  are independently H, (*i.e.*, abasic nucleotides), adenine, guanine, cytosine and uracil, inosine, thymine, xanthine, hypoxanthine, nubularine, tubercidine, isoguanisine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 5-halouracil, 5-(2-aminopropyl)uracil, 5-amino allyl uracil, 8-halo, amino, thiol, thioalkyl, hydroxyl and other 8-substituted adenines and guanines, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine, 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine, dihydrouracil, 3-deaza-5-azacytosine, 2-aminopurine, 5-alkyluracil, 7-alkylguanine, 5-alkyl cytosine, 7-deazaadenine, 7-deazaguanine, N6, N6-dimethyladenine, 2,6-

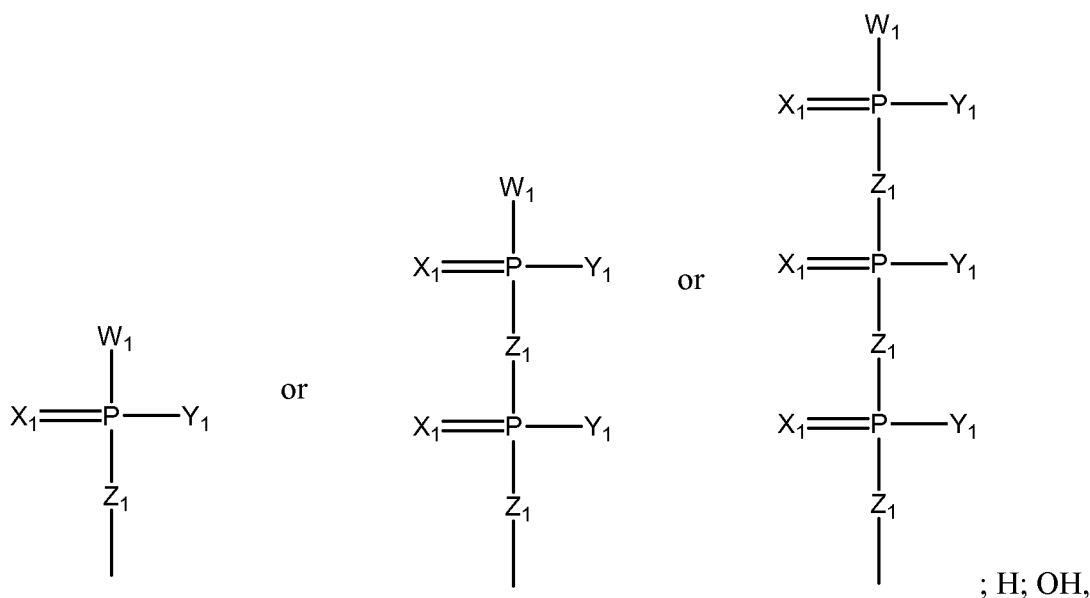
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diaminopurine, 5-amino-allyl-uracil, N3-methyluracil, substituted 1,2,4-triazoles, 2-pyridinone, 5-nitroindole, 3-nitropyrrole, 5-methoxyuracil, uracil-5-oxyacetic acid, 5-methoxycarbonylmethyluracil, 5-methyl-2-thiouracil, 5-methoxycarbonylmethyl-2-thiouracil, 5-methylaminomethyl-2-thiouracil, 3-(3-amino-3carboxypropyl)uracil, 3-methylcytosine, 5-methylcytosine, N<sup>4</sup>-acetyl cytosine, 2-thiocytosine, N6-methyladenine, N6-isopentyladenine, 2-methylthio-N6-isopentenyladenine, N-methylguanines, or O-alkylated bases;

R<sup>4</sup>, R<sup>5</sup>, and R<sup>6</sup> are independently OR<sup>8</sup>, O(CH<sub>2</sub>CH<sub>2</sub>O)<sub>m</sub>CH<sub>2</sub>CH<sub>2</sub>OR<sup>8</sup>; O(CH<sub>2</sub>)<sub>n</sub>R<sup>9</sup>; O(CH<sub>2</sub>)<sub>n</sub>OR<sup>9</sup>, H; halo; NH<sub>2</sub>; NHR<sup>8</sup>; N(R<sup>8</sup>)<sub>2</sub>; NH(CH<sub>2</sub>CH<sub>2</sub>NH)<sub>m</sub>CH<sub>2</sub>CH<sub>2</sub>NHR<sup>9</sup>; NHC(O)R<sup>8</sup>; cyano; mercapto, SR<sup>8</sup>; alkyl-thio-alkyl; alkyl, aralkyl, cycloalkyl, aryl, heteroaryl, alkenyl, alkynyl, each of which may be optionally substituted with halo, hydroxy, oxo, nitro, haloalkyl, alkyl, alkaryl, aryl, aralkyl, alkoxy, aryloxy, amino, alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, acylamino, alkylcarbamoyl, arylcarbamoyl, aminoalkyl, alkoxy carbonyl, carboxy, hydroxyalkyl, alkanesulfonyl, alkanesulfonamido, arenesulfonamido, aralkylsulfonamido, alkylcarbonyl, acyloxy, cyano, or ureido; or R<sup>4</sup>, R<sup>5</sup>, or R<sup>6</sup> together combine with R<sup>7</sup> to form an [-O-CH<sub>2</sub>-] covalently bound bridge between the sugar 2' and 4' carbons;

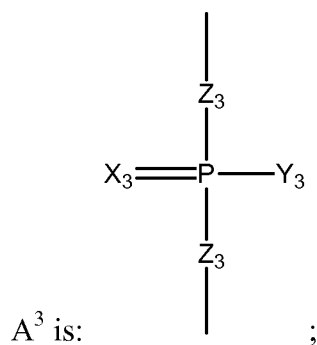
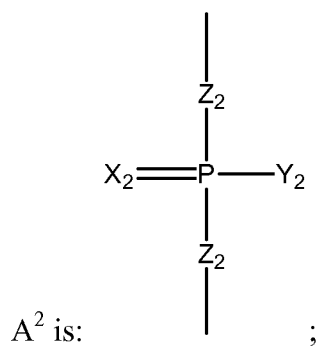
A<sup>1</sup> is:



OCH<sub>3</sub>, W<sup>1</sup>; an abasic nucleotide; or absent;

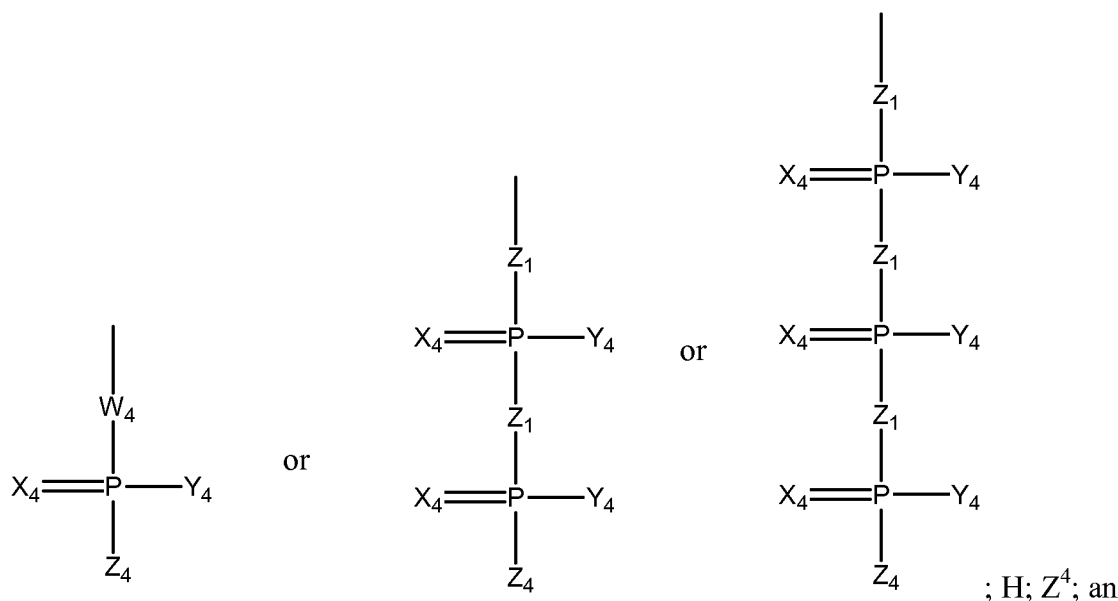
(in some embodiments, A<sup>1</sup>, especially with regard to anti-sense strands, is chosen from 5'-monophosphate ((HO)<sub>2</sub>(O)P-O-5'), 5'-diphosphate ((HO)<sub>2</sub>(O)P-O-P(HO)(O)-O-5'), 5'-triphosphate ((HO)<sub>2</sub>(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'), 5'-guanosine cap (7-

- 5 methylated or non-methylated) (7m-G-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'), 5'-adenosine cap (App), 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)<sub>2</sub>(S)P-O-5'), 5'-monodithiophosphate (phosphorodithioate; (HO)(HS)(S)P-O-5'), 5'-phosphorothiolate ((HO)<sub>2</sub>(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)<sub>2</sub>(O)P-NH-5', (HO)(NH<sub>2</sub>)(O)P-O-5'), 5'-alkylphosphonates (R=alkyl=methyl, ethyl, isopropyl, propyl, etc., *e.g.*, RP(OH)(O)-O-5', (OH)<sub>2</sub>(O)P-5'-CH<sub>2</sub>-), 5'-alkyletherphosphonates (R=alkylether=methoxymethyl (MeOCH<sub>2</sub>-), ethoxymethyl, etc., *e.g.*, RP(OH)(O)-O-5'-);



15

A<sup>4</sup> is:



inverted nucleotide; an abasic nucleotide; or absent;

- 5            W<sup>1</sup> is OH, (CH<sub>2</sub>)<sub>n</sub>R<sup>10</sup>, (CH<sub>2</sub>)<sub>n</sub>NHR<sup>10</sup>, (CH<sub>2</sub>)<sub>n</sub> OR<sup>10</sup>, (CH<sub>2</sub>)<sub>n</sub> SR<sup>10</sup>; O(CH<sub>2</sub>)<sub>n</sub>R<sup>10</sup>;  
 O(CH<sub>2</sub>)<sub>n</sub>OR<sup>10</sup>, O(CH<sub>2</sub>)<sub>n</sub>NR<sup>10</sup>, O(CH<sub>2</sub>)<sub>n</sub>SR<sup>10</sup>; O(CH<sub>2</sub>)<sub>n</sub>SS(CH<sub>2</sub>)<sub>n</sub>OR<sup>10</sup>, O(CH<sub>2</sub>)<sub>n</sub>C(O)OR<sup>10</sup>,  
 NH(CH<sub>2</sub>)<sub>n</sub>R<sup>10</sup>; NH(CH<sub>2</sub>)<sub>n</sub>NR<sup>10</sup>; NH(CH<sub>2</sub>)<sub>n</sub>OR<sup>10</sup>, NH(CH<sub>2</sub>)<sub>n</sub>SR<sup>10</sup>; S(CH<sub>2</sub>)<sub>n</sub>R<sup>10</sup>,  
 S(CH<sub>2</sub>)<sub>n</sub>NR<sup>10</sup>, S(CH<sub>2</sub>)<sub>n</sub>OR<sup>10</sup>, S(CH<sub>2</sub>)<sub>n</sub>SR<sup>10</sup> O(CH<sub>2</sub>CH<sub>2</sub>O)<sub>m</sub>CH<sub>2</sub>CH<sub>2</sub>OR<sup>10</sup>;  
 O(CH<sub>2</sub>CH<sub>2</sub>O)<sub>m</sub>CH<sub>2</sub>CH<sub>2</sub>NHR<sup>10</sup>, NH(CH<sub>2</sub>CH<sub>2</sub>NH)<sub>m</sub>CH<sub>2</sub>CH<sub>2</sub>NHR<sup>10</sup>; Q-R<sup>10</sup>, O-Q-R<sup>10</sup> N-Q-  
 10 R<sup>10</sup>, S-Q-R<sup>10</sup> or -O-;

W<sup>4</sup> is O, CH<sub>2</sub>, NH, or S;

X<sup>1</sup>, X<sup>2</sup>, X<sup>3</sup>, and X<sup>4</sup> are each independently O or S;

15

Y<sup>1</sup>, Y<sup>2</sup>, Y<sup>3</sup>, and Y<sup>4</sup> are each independently OH, O<sup>-</sup>, OR<sup>8</sup>, S, Se, BH<sub>3</sub><sup>-</sup>, H, NHR<sup>9</sup>, N(R<sup>9</sup>)<sub>2</sub>  
 alkyl, cycloalkyl, aralkyl, aryl, or heteroaryl, each of which may be optionally substituted;

Z<sup>1</sup>, Z<sup>2</sup>, and Z<sup>3</sup> are each independently O, CH<sub>2</sub>, NH, or S;

20

Z<sup>4</sup> is OH, (CH<sub>2</sub>)<sub>n</sub>R<sup>10</sup>, (CH<sub>2</sub>)<sub>n</sub>NHR<sup>10</sup>, (CH<sub>2</sub>)<sub>n</sub> OR<sup>10</sup>, (CH<sub>2</sub>)<sub>n</sub> SR<sup>10</sup>; O(CH<sub>2</sub>)<sub>n</sub>R<sup>10</sup>;  
 O(CH<sub>2</sub>)<sub>n</sub>OR<sup>10</sup>, O(CH<sub>2</sub>)<sub>n</sub>NR<sup>10</sup>, O(CH<sub>2</sub>)<sub>n</sub>SR<sup>10</sup>, O(CH<sub>2</sub>)<sub>n</sub>SS(CH<sub>2</sub>)<sub>n</sub>OR<sup>10</sup>, O(CH<sub>2</sub>)<sub>n</sub>C(O)OR<sup>10</sup>;  
 NH(CH<sub>2</sub>)<sub>n</sub>R<sup>10</sup>; NH(CH<sub>2</sub>)<sub>n</sub>NR<sup>10</sup>; NH(CH<sub>2</sub>)<sub>n</sub>OR<sup>10</sup>, NH(CH<sub>2</sub>)<sub>n</sub>SR<sup>10</sup>; S(CH<sub>2</sub>)<sub>n</sub>R<sup>10</sup>,

$S(CH_2)_nNR^{10}$ ,  $S(CH_2)_nOR^{10}$ ,  $S(CH_2)_nSR^{10}O(CH_2CH_2O)_mCH_2CH_2OR^{10}$ ,  
 $O(CH_2CH_2O)_mCH_2CH_2NHR^{10}$ ,  $NH(CH_2CH_2NH)_mCH_2CH_2NHR^{10}$ ;  $Q-R^{10}$ ,  $O-Q-R^{10}$   $N-Q-R^{10}$ ,  $S-Q-R^{10}$ ;

5 x is 5-100, chosen to comply with a length for an RNA agent described herein;

$R^7$  is H; or is together combined with  $R^4$ ,  $R^5$ , or  $R^6$  to form an [-O-CH<sub>2</sub>-] covalently bound bridge between the sugar 2' and 4' carbons;

10  $R^8$  is alkyl, cycloalkyl, aryl, aralkyl, heterocyclyl, heteroaryl, amino acid, or sugar;

$R^9$  is NH<sub>2</sub>, alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, or amino acid;

15  $R^{10}$  is H; fluorophore (pyrene, TAMRA, fluorescein, Cy3 or Cy5 dyes); sulfur, silicon, boron or ester protecting group; 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 (cholesterol, cholic acid, adamantane acetic acid, 1-pyrene butyric acid,  
 20 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),  
 25 alkylating agents, phosphate, amino, mercapto, PEG (*e.g.*, PEG-40K), MPEG, [MPEG]<sub>2</sub>, polyamino; alkyl, cycloalkyl, aryl, aralkyl, heteroaryl; radiolabelled markers, enzymes, haptens (*e.g.*, biotin), transport/absorption facilitators (*e.g.*, aspirin, vitamin E, folic acid), synthetic ribonucleases (*e.g.*, imidazole, bisimidazole, histamine, imidazole clusters, acridine-imidazole conjugates, Eu<sup>3+</sup> complexes of tetraazamacrocycles); or an RNA agent;;

30

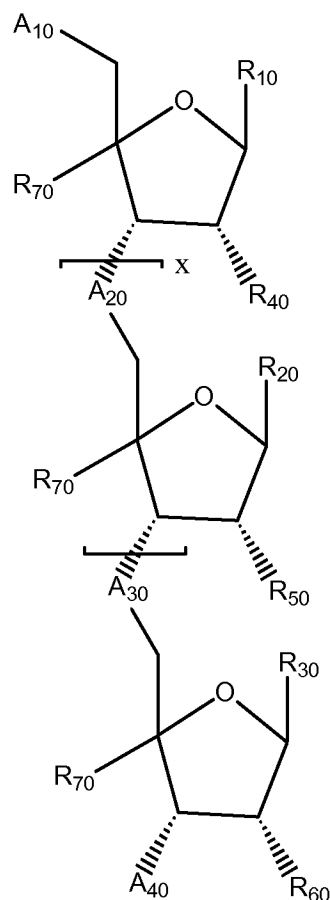
m is 0-1,000,000;

n is 0-20.



Q is a spacer selected from the group consisting of abasic sugar, amide, carboxy, oxyamine, oxyimine, thioether, disulfide, thiourea, sulfonamide, or morpholino, biotin or fluorescein reagents.

Certain RNA agents in which the entire phosphate group has been replaced have the following structure (Formula IX):



FORMULA IX

wherein:

10  $A^{10}$ - $A^{40}$  is L-G-L;  $A^{10}$  and/or  $A^{40}$  may be absent, wherein

L is a linker, wherein one or both L may be present or absent and is selected from the group consisting of  $\text{CH}_2(\text{CH}_2)_g$ ;  $\text{N}(\text{CH}_2)_g$ ;  $\text{O}(\text{CH}_2)_g$ ;  $\text{S}(\text{CH}_2)_g$ ;

G is a functional group selected from the group consisting of siloxane, carbonate, 15 carboxymethyl, carbamate, amide, thioether, ethylene oxide linker, sulfonate, sulfonamide, thioformacetal, formacetal, oxime, methyleneimino, methylenemethylimino, methylenehydrazo, methylenedimethylhydrazo and methyleneoxymethylimino;

$R^{10}$ ,  $R^{20}$ , and  $R^{30}$  are independently H, (*i.e.*, abasic nucleotides), adenine, guanine, cytosine and uracil, inosine, thymine, xanthine, hypoxanthine, nubularine, tubercidine, isoguanisine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 5-halouracil, 5-(2-aminopropyl)uracil, 5-amino allyl uracil, 8-halo, amino, thiol, thioalkyl, hydroxyl and other 8-substituted adenines and guanines, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine, 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine, dihydrouracil, 3-deaza-5-azacytosine, 2-aminopurine, 5-alkyluracil, 7-alkylguanine, 5-alkyl cytosine, 7-deazaadenine, 7-deazaguanine, N6, N6-dimethyladenine, 2,6-diaminopurine, 5-amino-allyl-uracil, N3-methyluracil substituted 1,2,4-triazoles, 2-pyridinone, 5-nitroindole, 3-nitropyrrole, 5-methoxyuracil, uracil-5-oxyacetic acid, 5-methoxycarbonylmethyluracil, 5-methyl-2-thiouracil, 5-methoxycarbonylmethyl-2-thiouracil, 5-methylaminomethyl-2-thiouracil, 3-(3-amino-3-carboxypropyl)uracil, 3-methylcytosine, 5-methylcytosine, N<sup>4</sup>-acetyl cytosine, 2-thiocytosine, N6-methyladenine, N6-isopentyladenine, 2-methylthio-N6-isopentenyladenine, N-methylguanines, or O-alkylated bases;

20

$R^{40}$ ,  $R^{50}$ , and  $R^{60}$  are independently  $OR^8$ ,  $O(CH_2CH_2O)_mCH_2CH_2OR^8$ ;  $O(CH_2)_nR^9$ ;  $O(CH_2)_nOR^9$ , H; halo;  $NH_2$ ;  $NHR^8$ ;  $N(R^8)_2$ ;  $NH(CH_2CH_2NH)_mCH_2CH_2R^9$ ;  $NHC(O)R^8$ ; cyano; mercapto,  $SR^7$ ; alkyl-thio-alkyl; alkyl, aralkyl, cycloalkyl, aryl, heteroaryl, alkenyl, alkynyl, each of which may be optionally substituted with halo, hydroxy, oxo, nitro, haloalkyl, alkyl, alkaryl, aryl, aralkyl, alkoxy, aryloxy, amino, alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, acylamino, alkylcarbamoyl, arylcarbamoyl, aminoalkyl, alkoxy-carbonyl, carboxy, hydroxyalkyl, alkanesulfonyl, alkanesulfonamido, arenesulfonamido, aralkylsulfonamido, alkylcarbonyl, acyloxy, cyano, and ureido groups; or  $R^{40}$ ,  $R^{50}$ , or  $R^{60}$  together combine with  $R^{70}$  to form an [-O-CH<sub>2</sub>-] covalently bound bridge between the sugar 2' and 4' carbons;

30

x is 5-100 or chosen to comply with a length for an RNA agent described herein;

R<sup>70</sup> is H; or is together combined with R<sup>40</sup>, R<sup>50</sup>, or R<sup>60</sup> to form an [-O-CH<sub>2</sub>-] covalently bound bridge between the sugar 2' and 4' carbons;

R<sup>8</sup> is alkyl, cycloalkyl, aryl, aralkyl, heterocyclyl, heteroaryl, amino acid, or sugar;

5

R<sup>9</sup> is NH<sub>2</sub>, alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, or amino acid;

m is 0-1,000,000;

10

n is 0-20;

g is 0-2.

15 Certain nucleoside surrogates have the following structure (Formula X):



FORMULA X

20 wherein:

S is a nucleoside surrogate selected from the group consisting of morpholino, cyclobutyl, pyrrolidine and peptide nucleic acid;

L is a linker and is selected from the group consisting of CH<sub>2</sub>(CH<sub>2</sub>)<sub>g</sub>; N(CH<sub>2</sub>)<sub>g</sub>; O(CH<sub>2</sub>)<sub>g</sub>; S(CH<sub>2</sub>)<sub>g</sub>; -C(O)(CH<sub>2</sub>)<sub>n</sub>-or may be absent;

25

M is an amide bond; sulfonamide; sulfinate; phosphate group; modified phosphate group as described herein; or may be absent;

30 R<sup>100</sup>, R<sup>200</sup>, and R<sup>300</sup> are independently H (*i.e.*, abasic nucleotides), adenine, guanine, cytosine and uracil, inosine, thymine, xanthine, hypoxanthine, nubularine, tubercidine, isoguanisine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 5-halouracil, 5-(2-aminopropyl)uracil, 5-

amino allyl uracil, 8-halo, amino, thiol, thioalkyl, hydroxyl and other 8-substituted adenines and guanines, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine, 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-  
 5 propynylcytosine, dihydrouracil, 3-deaza-5-azacytosine, 2-aminopurine, 5-alkyluracil, 7-alkylguanine, 5-alkyl cytosine, 7-deazaadenine, 7-deazaguanine, N6, N6-dimethyladenine, 2,6-diaminopurine, 5-amino-allyl-uracil, N3-methyluracil substituted 1, 2, 4,-triazoles, 2-pyridinones, 5-nitroindole, 3-nitropyrrole, 5-methoxyuracil, uracil-5-oxyacetic acid, 5-methoxycarbonylmethyluracil, 5-methyl-2-thiouracil, 5-methoxycarbonylmethyl-2-  
 10 thiouracil, 5-methylaminomethyl-2-thiouracil, 3-(3-amino-3carboxypropyl)uracil, 3-methylcytosine, 5-methylcytosine, N<sup>4</sup>-acetyl cytosine, 2-thiocytosine, N6-methyladenine, N6-isopentyladenine, 2-methylthio-N6-isopentenyladenine, N-methylguanines, or O-alkylated bases;

x is 5-100, or chosen to comply with a length for an RNA agent described herein;  
 15 g is 0-2.

### Definitions

The term “halo” refers to any radical of fluorine, chlorine, bromine or iodine. The term “alkyl” refers to saturated and unsaturated non-aromatic hydrocarbon chains that  
 20 may be a straight chain or branched chain, containing the indicated number of carbon atoms (these include without limitation propyl, allyl, or propargyl), which may be optionally inserted with N, O, or S. For example, C<sub>1</sub>-C<sub>10</sub> indicates that the group may have from 1 to 10 (inclusive) carbon atoms in it. The term “alkoxy” refers to an -O-alkyl radical. The term “alkylene” refers to a divalent alkyl (*i.e.*, -R-). The term  
 25 “alkylenedioxo” refers to a divalent species of the structure -O-R-O-, in which R represents an alkylene. The term “aminoalkyl” refers to an alkyl substituted with an amino. The term “mercapto” refers to an -SH radical. The term “thioalkoxy” refers to an -S-alkyl radical.

The term “aryl” refers to a 6-carbon monocyclic or 10-carbon bicyclic aromatic  
 30 ring system wherein 0, 1, 2, 3, or 4 atoms of each ring may be substituted by a substituent. Examples of aryl groups include phenyl, naphthyl and the like. The term “arylalkyl” or the term “aralkyl” refers to alkyl substituted with an aryl. The term “arylalkoxy” refers to an alkoxy substituted with aryl.

The term "cycloalkyl" as employed herein includes saturated and partially unsaturated cyclic hydrocarbon groups having 3 to 12 carbons, for example, 3 to 8 carbons, and, for example, 3 to 6 carbons, wherein the cycloalkyl group additionally may be optionally substituted. Cycloalkyl groups include, without limitation, cyclopropyl, cyclobutyl, cyclopentyl, cyclopentenyl, cyclohexyl, cyclohexenyl, cycloheptyl, and cyclooctyl.

The term "heteroaryl" refers to an aromatic 5-8 membered monocyclic, 8-12 membered bicyclic, or 11-14 membered tricyclic ring system having 1-3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, said heteroatoms selected from O, N, or S (*e.g.*, carbon atoms and 1-3, 1-6, or 1-9 heteroatoms of N, O, or S if monocyclic, bicyclic, or tricyclic, respectively), wherein 0, 1, 2, 3, or 4 atoms of each ring may be substituted by a substituent. Examples of heteroaryl groups include pyridyl, furyl or furanyl, imidazolyl, benzimidazolyl, pyrimidinyl, thiophenyl or thienyl, quinolinyl, indolyl, thiazolyl, and the like. The term "heteroarylalkyl" or the term "heteroaralkyl" refers to an alkyl substituted with a heteroaryl. The term "heteroarylalkoxy" refers to an alkoxy substituted with heteroaryl.

The term "heterocyclyl" refers to a nonaromatic 5-8 membered monocyclic, 8-12 membered bicyclic, or 11-14 membered tricyclic ring system having 1-3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, said heteroatoms selected from O, N, or S (*e.g.*, carbon atoms and 1-3, 1-6, or 1-9 heteroatoms of N, O, or S if monocyclic, bicyclic, or tricyclic, respectively), wherein 0, 1, 2 or 3 atoms of each ring may be substituted by a substituent. Examples of heterocyclyl groups include trizolyl, tetrazolyl, piperazinyl, pyrrolidinyl, dioxanyl, morpholinyl, tetrahydrofuranlyl, and the like.

The term "oxo" refers to an oxygen atom, which forms a carbonyl when attached to carbon, an N-oxide when attached to nitrogen, and a sulfoxide or sulfone when attached to sulfur.

The term "acyl" refers to an alkylcarbonyl, cycloalkylcarbonyl, arylcarbonyl, heterocyclylcarbonyl, or heteroarylcarbonyl substituent, any of which may be further substituted by substituents.

The term "substituted" refers to the replacement of one or more hydrogen radicals in a given structure with the radical of a specified substituent including, but not limited to:

halo, alkyl, alkenyl, alkynyl, aryl, heterocyclyl, thiol, alkylthio, arylthio, alkylthioalkyl, arylthioalkyl, alkylsulfonyl, alkylsulfonylalkyl, arylsulfonylalkyl, alkoxy,

aryloxy, aralkoxy, aminocarbonyl, alkylaminocarbonyl, arylaminocarbonyl, alkoxy carbonyl, aryloxy carbonyl, haloalkyl, amino, trifluoromethyl, cyano, nitro, alkylamino, arylamino, alkylaminoalkyl, arylaminoalkyl, aminoalkylamino, hydroxy, alkoxyalkyl, carboxyalkyl, alkoxy carbonylalkyl, aminocarbonylalkyl, acyl, 5 aralkoxy carbonyl, carboxylic acid, sulfonic acid, sulfonyl, phosphonic acid, aryl, heteroaryl, heterocyclic, and aliphatic. It is understood that the substituent can be further substituted.

### Palindromes

10 The iRNA agents of the invention can target more than one RNA region. For example, an iRNA agent can include a first and second sequence that are sufficiently complementary to each other to hybridize. The first sequence can be complementary to a first target RNA region and the second sequence can be complementary to a second target RNA region. The first and second sequences of the iRNA agent can be on different RNA 15 strands, and the mismatch between the first and second sequences can be less than 50%, 40%, 30%, 20%, 10%, 5%, or 1%. The first and second sequences of the iRNA agent are on the same RNA strand, and in a related embodiment more than 50%, 60%, 70%, 80%, 90%, 95%, or 1% of the iRNA agent can be in bimolecular form. The first and second sequences of the iRNA agent can be fully complementary to each other.

20 The first target RNA region can be encoded by a first gene and the second target RNA region can be encoded by a second gene, or the first and second target RNA regions can be different regions of an RNA from a single gene. The first and second sequences can differ by at least 1 nucleotide.

The first and second target RNA regions can be on transcripts encoded by first and 25 second sequence variants, e.g., first and second alleles, of a gene. The sequence variants can be mutations, or polymorphisms, for example. The first target RNA region can include a nucleotide substitution, insertion, or deletion relative to the second target RNA region, or the second target RNA region can be a mutant or variant of the first target region.

The first and second target RNA regions can comprise viral or human RNA 30 regions. The first and second target RNA regions can also be on variant transcripts of an oncogene or include different mutations of a tumor suppressor gene transcript. In addition, the first and second target RNA regions can correspond to hot-spots for genetic variation.

The compositions of the invention can include mixtures of iRNA agent molecules. For example, one iRNA agent can contain a first sequence and a second sequence sufficiently complementary to each other to hybridize, and in addition the first sequence is complementary to a first target RNA region and the second sequence is complementary to a second target RNA region. The mixture can also include at least one additional iRNA agent variety that includes a third sequence and a fourth sequence sufficiently complementary to each other to hybridize, and where the third sequence is complementary to a third target RNA region and the fourth sequence is complementary to a fourth target RNA region. In addition, the first or second sequence can be sufficiently complementary to the third or fourth sequence to be capable of hybridizing to each other. The first and second sequences can be on the same or different RNA strands, and the third and fourth sequences can be on the same or different RNA strands.

The target RNA regions can be variant sequences of a viral or human RNA, and in certain embodiments, at least two of the target RNA regions can be on variant transcripts of an oncogene or tumor suppressor gene. The target RNA regions can correspond to genetic hot-spots.

Methods of making an iRNA agent composition can include obtaining or providing information about a region of an RNA of a target gene (*e.g.*, a viral or human gene, or an oncogene or tumor suppressor, *e.g.*, p53), where the region has high variability or mutational frequency (*e.g.*, in humans). In addition, information about a plurality of RNA targets within the region can be obtained or provided, where each RNA target corresponds to a different variant or mutant of the gene (*e.g.*, a region including the codon encoding p53 248Q and/or p53 249S). The iRNA agent can be constructed such that a first sequence is complementary to a first of the plurality of variant RNA targets (*e.g.*, encoding 249Q) and a second sequence is complementary to a second of the plurality of variant RNA targets (*e.g.*, encoding 249S), and the first and second sequences can be sufficiently complementary to hybridize.

Sequence analysis, *e.g.*, to identify common mutants in the target gene, can be used to identify a region of the target gene that has high variability or mutational frequency. A region of the target gene having high variability or mutational frequency can be identified by obtaining or providing genotype information about the target gene from a population.

Expression of a target gene can be modulated, *e.g.*, downregulated or silenced, by providing an iRNA agent that has a first sequence and a second sequence sufficiently

complementary to each other to hybridize. In addition, the first sequence can be complementary to a first target RNA region and the second sequence can be complementary to a second target RNA region.

An iRNA agent can include a first sequence complementary to a first variant RNA target region and a second sequence complementary to a second variant RNA target region. The first and second variant RNA target regions can correspond to first and second variants or mutants of a target gene, *e.g.*, viral gene, tumor suppressor or oncogene. The first and second variant target RNA regions can include allelic variants, mutations (*e.g.*, point mutations), or polymorphisms of the target gene. The first and second variant RNA target regions can correspond to genetic hot-spots.

A plurality of iRNA agents (*e.g.*, a panel or bank) can be provided.

#### Other Embodiments

In yet another embodiment, iRNAs agents are produced in a cell *in vivo*, *e.g.*, from exogenous DNA templates that are delivered into the cell. For example, the DNA templates can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (U.S. Pat. No. 5,328,470), or by stereotactic injection (see, *e.g.*, Chen *et al.* (1994) Proc. Natl. Acad. Sci. USA 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. The DNA templates, for example, can include two transcription units, one that produces a transcript that includes the top strand of a iRNA agent and one that produces a transcript that includes the bottom strand of a iRNA agent. When the templates are transcribed, the iRNA agent is produced, and processed into siRNA agent fragments that mediate gene silencing.

#### Antagomirs

Antagomirs are RNA-like oligonucleotides that harbor various modifications for RNase protection and pharmacologic properties, such as enhanced tissue and cellular uptake. They differ from normal RNA by, for example, complete 2'-*O*-methylation of sugar, phosphorothioate backbone and, for example, a cholesterol-moiety at 3'-end. Antagomirs may be used to efficiently silence endogenous miRNAs thereby preventing miRNA-induced gene silencing. An example of antagomir-mediated miRNA silencing is



the silencing of miR-122, described in Krutzfeldt et al, Nature, 2005, 438: 685-689, which is expressly incorporated by reference herein, in its entirety.

#### Decoy Oligonucleotides

5           Because transcription factors can recognize their relatively short binding sequences, even in the absence of surrounding genomic DNA, short oligonucleotides bearing the consensus binding sequence of a specific transcription factor can be used as tools for manipulating gene expression in living cells. This strategy involves the intracellular delivery of such “decoy oligonucleotides”, which are then recognized and  
10 bound by the target factor. Occupation of the transcription factor’s DNA-binding site by the decoy renders the transcription factor incapable of subsequently binding to the promoter regions of target genes. Decoys can be used as therapeutic agents, either to inhibit the expression of genes that are activated by a transcription factor, or to upregulate genes that are suppressed by the binding of a transcription factor. Examples of the  
15 utilization of decoy oligonucleotides may be found in Mann et al., J. Clin. Invest., 2000, 106: 1071-1075, which is expressly incorporated by reference herein, in its entirety.

#### Antisense Oligonucleotides

          Antisense oligonucleotides are single strands of DNA or RNA that are at least  
20 partially complementary to a chosen sequence. In the case of antisense RNA, they prevent translation of complementary RNA strands by binding to it. Antisense DNA can also be used to target a specific, complementary (coding or non-coding) RNA. If binding takes place, the DNA/RNA hybrid can be degraded by the enzyme RNase H. Examples of the utilization of antisense oligonucleotides may be found in Dias et al., Mol. Cancer  
25 Ther., 2002, 1: 347-355, which is expressly incorporated by reference herein, in its entirety.

#### Aptamers

          Aptamers are nucleic acid molecules that bind a specific target molecule or  
30 molecules. Aptamers may be RNA or DNA based, and may include a riboswitch. A riboswitch is a part of an mRNA molecule that can directly bind a small target molecule, and whose binding of the target affects the gene's activity. Thus, an mRNA that contains a riboswitch is directly involved in regulating its own activity, depending on the presence or absence of its target molecule.

### Physiological Effects

The iRNA agents described herein can be designed such that determining therapeutic toxicity is made easier by the complementarity of the iRNA agent with both a human and a non-human animal sequence. By these methods, an iRNA agent can consist of a sequence that is fully complementary to a nucleic acid sequence from a human and a nucleic acid sequence from at least one non-human animal, e.g., a non-human mammal, such as a rodent, ruminant or primate. For example, the non-human mammal can be a mouse, rat, dog, pig, goat, sheep, cow, monkey, Pan paniscus, Pan troglodytes, Macaca mulatto, or Cynomolgus monkey. The sequence of the iRNA agent could be complementary to sequences within homologous genes, e.g., oncogenes or tumor suppressor genes, of the non-human mammal and the human. By determining the toxicity of the iRNA agent in the non-human mammal, one can extrapolate the toxicity of the iRNA agent in a human. For a more strenuous toxicity test, the iRNA agent can be complementary to a human and more than one, e.g., two or three or more, non-human animals.

The methods described herein can be used to correlate any physiological effect of an iRNA agent on a human, e.g., any unwanted effect, such as a toxic effect, or any positive, or desired effect.

20

### Increasing cellular uptake of dsRNAs

A method of the invention that includes administering an iRNA agent and a drug that affects the uptake of the iRNA agent into the cell. The drug can be administered before, after, or at the same time that the iRNA agent is administered. The drug can be covalently linked to the iRNA agent. The drug can be, for example, a lipopolysaccharid, an activator of p38 MAP kinase, or an activator of NF- $\kappa$ B. The drug can have a transient effect on the cell.

The drug can increase the uptake of the iRNA agent into the cell, for example, by disrupting the cell's cytoskeleton, e.g., by disrupting the cell's microtubules, microfilaments, and/or intermediate filaments. The drug can be, for example, taxon, vincristine, vinblastine, cytochalasin, nocodazole, japlakinolide, latrunculin A, phalloidin, swinholidide A, indanocine, or myoservin.

30

The drug can also increase the uptake of the iRNA agent into the cell by activating an inflammatory response, for example. Exemplary drug's that would have such an effect include tumor necrosis factor alpha (TNFalpha), interleukin-1 beta, or gamma interferon.

#### 5 iRNA Conjugates

An iRNA agent can be coupled, *e.g.*, covalently coupled, to a second agent. For example, an iRNA agent used to treat a particular disorder can be coupled to a second therapeutic agent, *e.g.*, an agent other than the iRNA agent. The second therapeutic agent can be one which is directed to the treatment of the same disorder. For example, in the  
10 case of an iRNA used to treat a disorder characterized by unwanted cell proliferation, *e.g.*, cancer, the iRNA agent can be coupled to a second agent which has an anti-cancer effect. For example, it can be coupled to an agent which stimulates the immune system, *e.g.*, a CpG motif, or more generally an agent that activates a toll-like receptor and/or increases the production of gamma interferon.

15

#### iRNA Production

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

#### 20 Organic Synthesis

An iRNA can be made by separately synthesizing each respective strand of a double-stranded RNA molecule. The component strands can then be annealed.

A large bioreactor, *e.g.*, the OligoPilot II from Pharmacia Biotec AB (Uppsala Sweden), can be used to produce a large amount of a particular RNA strand for a given  
25 iRNA. The OligoPilotII reactor can efficiently couple a nucleotide using only a 1.5 molar excess of a phosphoramidite nucleotide. To make an RNA strand, ribonucleotides amidites are used. Standard cycles of monomer addition can be used to synthesize the 21 to 23 nucleotide strand for the iRNA. Typically, the two complementary strands are produced separately and then annealed, *e.g.*, after release from the solid support and  
30 deprotection.

Organic synthesis can be used to produce a discrete iRNA species. The complementary of the species to a particular target gene can be precisely specified. For example, the species may be complementary to a region that includes a polymorphism, *e.g.*, a single nucleotide polymorphism. Further the location of the polymorphism can be

precisely defined. In some embodiments, the polymorphism is located in an internal region, *e.g.*, at least 4, 5, 7, or 9 nucleotides from one or both of the termini.

#### dsiRNA Cleavage

5 iRNAs can also be made by cleaving a larger ds iRNA. The cleavage can be mediated *in vitro* or *in vivo*. For example, to produce iRNAs by cleavage *in vitro*, the following method can be used:

*In vitro* transcription. dsiRNA is produced by transcribing a nucleic acid (DNA) segment in both directions. For example, the HiScribe™ RNAi transcription kit (New  
10 England Biolabs) provides a vector and a method for producing a dsiRNA for a nucleic acid segment that is cloned into the vector at a position flanked on either side by a T7 promoter. Separate templates are generated for T7 transcription of the two complementary strands for the dsiRNA. The templates are transcribed *in vitro* by addition of T7 RNA polymerase and dsiRNA is produced. Similar methods using PCR  
15 and/or other RNA polymerases (*e.g.*, T3 or SP6 polymerase) can also be used. In one embodiment, RNA generated by this method is carefully purified to remove endotoxins that may contaminate preparations of the recombinant enzymes.

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

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

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

### Formulation

The iRNA agents described herein can be formulated for administration to a subject.

5 For ease of exposition the formulations, compositions and methods in this section are discussed largely with regard to unmodified iRNA agents. It may be understood, however, that these formulations, compositions and methods can be practiced with other iRNA agents, *e.g.*, modified iRNA agents, and such practice is within the invention.

10 A formulated iRNA composition can assume a variety of states. In some examples, the composition is at least partially crystalline, uniformly crystalline, and/or anhydrous (*e.g.*, less than 80, 50, 30, 20, or 10% water). In another example, the iRNA is in an aqueous phase, *e.g.*, in a solution that includes water.

15 The aqueous phase or the crystalline compositions can, *e.g.*, be incorporated into a delivery vehicle, *e.g.*, a liposome (particularly for the aqueous phase) or a particle (*e.g.*, a microparticle as can be appropriate for a crystalline composition). Generally, the iRNA composition is formulated in a manner that is compatible with the intended method of administration (see, below).

20 In particular embodiments, the composition is prepared by at least one of the following methods: spray drying, lyophilization, vacuum drying, evaporation, fluid bed drying, or a combination of these techniques; or sonication with a lipid, freeze-drying, condensation and other self-assembly.

25 A iRNA preparation can be formulated in combination with another agent, *e.g.*, another therapeutic agent or an agent that stabilizes a iRNA, *e.g.*, a protein that complexes with iRNA to form an iRNP. Still other agents include chelators, *e.g.*, EDTA (*e.g.*, to remove divalent cations such as  $Mg^{2+}$ ), salts, RNase inhibitors (*e.g.*, a broad specificity RNase inhibitor such as RNAsin) and so forth.

30 In one embodiment, the iRNA preparation includes another iRNA agent, *e.g.*, a second iRNA that can mediated RNAi with respect to a second gene, or with respect to the same gene. Still other preparation can include at least 3, 5, ten, twenty, fifty, or a hundred or more different iRNA species. Such iRNAs can mediate RNAi with respect to a similar number of different genes.

In one embodiment, the iRNA preparation includes at least a second therapeutic agent (*e.g.*, an agent other than an RNA or a DNA). For example, a iRNA composition for the treatment of a viral disease, *e.g.*, HIV, might include a known antiviral agent (*e.g.*,

a protease inhibitor or reverse transcriptase inhibitor). In another example, a iRNA composition for the treatment of a cancer might further comprise a chemotherapeutic agent.

Exemplary formulations are discussed below:

5

### Liposomes

For ease of exposition the formulations, compositions and methods in this section are discussed largely with regard to unmodified iRNA agents. It may be understood, however, that these formulations, compositions and methods can be practiced with other iRNA agents, *e.g.*, modified iRNA agents, and such practice is within the invention. An iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be processed into a siRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, or precursor thereof) preparation can be formulated for delivery in a membranous molecular assembly, *e.g.*, a liposome or a micelle. As used herein, the term “liposome” refers to a vesicle composed of amphiphilic lipids arranged in at least one bilayer, *e.g.*, one bilayer or a plurality of bilayers. Liposomes include unilamellar and multilamellar vesicles that have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the iRNA composition. The lipophilic material isolates the aqueous interior from an aqueous exterior, which typically does not include the iRNA composition, although in some examples, it may. Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomal bilayer fuses with bilayer of the cellular membranes. As the merging of the liposome and cell progresses, the internal aqueous contents that include the iRNA are delivered into the cell where the iRNA can specifically bind to a target RNA and can mediate RNAi. In some cases the liposomes are also specifically targeted, *e.g.*, to direct the iRNA to particular cell types.

A liposome containing a iRNA can be prepared by a variety of methods.

30 In one example, the lipid component of a liposome is dissolved in a detergent so that micelles are formed with the lipid component. For example, the lipid component can be an amphipathic cationic lipid or lipid conjugate. The detergent can have a high critical micelle concentration and may be nonionic. Exemplary detergents include cholate, CHAPS, octylglucoside, deoxycholate, and lauroyl sarcosine. The iRNA

preparation is then added to the micelles that include the lipid component. The cationic groups on the lipid interact with the iRNA and condense around the iRNA to form a liposome. After condensation, the detergent is removed, *e.g.*, by dialysis, to yield a liposomal preparation of iRNA.

5 If necessary a carrier compound that assists in condensation can be added during the condensation reaction, *e.g.*, by controlled addition. For example, the carrier compound can be a polymer other than a nucleic acid (*e.g.*, spermine or spermidine). pH can also be adjusted to favor condensation.

Further description of methods for producing stable polynucleotide delivery  
10 vehicles, which incorporate a polynucleotide/cationic lipid complex as structural components of the delivery vehicle, are described in, *e.g.*, WO 96/37194. Liposome formation can also include one or more aspects of exemplary methods described in Felgner, P. L. *et al.*, *Proc. Natl. Acad. Sci.*, USA 8:7413-7417, 1987; U.S. Pat. No. 4,897,355; U.S. Pat. No. 5,171,678; Bangham, *et al. M. Mol. Biol.* 23:238, 1965; Olson,  
15 *et al. Biochim. Biophys. Acta* 557:9, 1979; Szoka, *et al. Proc. Natl. Acad. Sci.* 75: 4194, 1978; Mayhew, *et al. Biochim. Biophys. Acta* 775:169, 1984; Kim, *et al. Biochim. Biophys. Acta* 728:339, 1983; and Fukunaga, *et al. Endocrinol.* 115:757, 1984. Commonly used techniques for preparing lipid aggregates of appropriate size for use as delivery vehicles include sonication and freeze-thaw plus extrusion (see, *e.g.*, Mayer, *et*  
20 *al. Biochim. Biophys. Acta* 858:161, 1986). Microfluidization can be used when consistently small (50 to 200 nm) and relatively uniform aggregates are desired (Mayhew, *et al. Biochim. Biophys. Acta* 775:169, 1984). These methods are readily adapted to packaging iRNA preparations into liposomes.

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

One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl

phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

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

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

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

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

A DOTMA analogue, 1,2-bis(oleoyloxy)-3-(trimethylammonia)propane (DOTAP) can be used in combination with a phospholipid to form DNA-complexing vesicles. Lipofectin™ (Bethesda Research Laboratories, Gaithersburg, Md.) is an effective agent for the delivery of highly anionic nucleic acids into living tissue culture cells that comprise positively charged DOTMA liposomes which interact spontaneously with



negatively charged polynucleotides to form complexes. When enough positively charged liposomes are used, the net charge on the resulting complexes is also positive. Positively charged complexes prepared in this way spontaneously attach to negatively charged cell surfaces, fuse with the plasma membrane, and efficiently deliver functional nucleic acids into, for example, tissue culture cells. Another commercially available cationic lipid, 1,2-bis(oleoyloxy)-3,3-(trimethylammonia)propane (“DOTAP”) (Boehringer Mannheim, Indianapolis, Indiana) differs from DOTMA in that the oleoyl moieties are linked by ester, rather than ether linkages.

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

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

Liposomal formulations are particularly suited for topical administration, liposomes present several advantages over other formulations. Such advantages include reduced side effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer iRNA, into the skin. In some implementations, liposomes are used for delivering iRNA to epidermal cells and also to enhance the penetration of iRNA into dermal tissues, *e.g.*, into skin. For example, the liposomes can be applied topically. Topical delivery of drugs formulated as liposomes to the skin has been documented (see, *e.g.*, Weiner *et al.*, *Journal of Drug Targeting*, 1992, vol. 2,405-410 and du Plessis *et al.*,

*Antiviral Research*, 18, 1992, 259-265; Mannino, R. J. and Fould-Fogerite, S.,  
Biotechniques 6:682-690, 1988; Itani, T. *et al.* Gene 56:267-276. 1987; Nicolau, C. *et al.*  
Meth. Enz. 149:157-176, 1987; Straubinger, R. M. and Papahadjopoulos, D. Meth. Enz.  
101:512-527, 1983; Wang, C. Y. and Huang, L., Proc. Natl. Acad. Sci. USA 84:7851-  
5 7855, 1987).

Non-ionic liposomal systems have also been examined to determine their utility in  
the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant  
and cholesterol. Non-ionic liposomal formulations comprising Novasome I (glyceryl  
dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome II (glyceryl  
10 distearate/ cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver a drug into  
the dermis of mouse skin. Such formulations with iRNA are useful for treating a  
dermatological disorder.

Liposomes that include iRNA can be made highly deformable. Such  
deformability can enable the liposomes to penetrate through pore that are smaller than the  
15 average radius of the liposome. For example, transfersomes are a type of deformable  
liposomes. Transfersomes can be made by adding surface edge activators, usually  
surfactants, to a standard liposomal composition. Transfersomes that include iRNA can  
be delivered, for example, subcutaneously by infection in order to deliver iRNA to  
keratinocytes in the skin. In order to cross intact mammalian skin, lipid vesicles must  
20 pass through a series of fine pores, each with a diameter less than 50 nm, under the  
influence of a suitable transdermal gradient. In addition, due to the lipid properties, these  
transfersomes can be self-optimizing (adaptive to the shape of pores, *e.g.*, in the skin),  
self-repairing, and can frequently reach their targets without fragmenting, and often self-  
loading.

25

### Surfactants

For ease of exposition the formulations, compositions and methods in this section  
are discussed largely with regard to unmodified iRNA agents. It may be understood,  
however, that these formulations, compositions and methods can be practiced with other  
30 iRNA agents, *e.g.*, modified iRNA agents, and such practice is within the invention.  
Surfactants find wide application in formulations such as emulsions (including  
microemulsions) and liposomes (see above). iRNA (or a precursor, *e.g.*, a larger dsRNA  
which can be processed into a iRNA, or a DNA which encodes a iRNA or precursor)  
compositions can include a surfactant. In one embodiment, the iRNA is formulated as an

emulsion that includes a surfactant. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in "Pharmaceutical Dosage Forms," Marcel Dekker, Inc., New York, NY, 1988, p. 285).

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

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

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

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

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

#### Micelles and other Membranous Formulations

For ease of exposition the micelles and other formulations, compositions and methods in this section are discussed largely with regard to unmodified iRNA agents. It

may be understood, however, that these micelles and other formulations, compositions and methods can be practiced with other iRNA agents, *e.g.*, modified iRNA agents, and such practice is within the invention. The iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be  
5 processed into a siRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, or precursor thereof)) composition can be provided as a micellar formulation. "Micelles" are defined herein as a particular type of molecular assembly in which amphipathic molecules are arranged in a spherical structure such that all the hydrophobic portions of the molecules are directed inward, leaving the hydrophilic  
10 portions in contact with the surrounding aqueous phase. The converse arrangement exists if the environment is hydrophobic.

A mixed micellar formulation suitable for delivery through transdermal membranes may be prepared by mixing an aqueous solution of the iRNA composition, an alkali metal C<sub>8</sub> to C<sub>22</sub> alkyl sulphate, and a micelle forming compounds. Exemplary  
15 micelle forming compounds include lecithin, hyaluronic acid, pharmaceutically acceptable salts of hyaluronic acid, glycolic acid, lactic acid, chamomile extract, cucumber extract, oleic acid, linoleic acid, linolenic acid, monoolein, monooleates, monolaurates, borage oil, evening of primrose oil, menthol, trihydroxy oxo cholanyl glycine and pharmaceutically acceptable salts thereof, glycerin, polyglycerin, lysine,  
20 polylysine, triolein, polyoxyethylene ethers and analogues thereof, polidocanol alkyl ethers and analogues thereof, chenodeoxycholate, deoxycholate, and mixtures thereof. The micelle forming compounds may be added at the same time or after addition of the alkali metal alkyl sulphate. Mixed micelles will form with substantially any kind of mixing of the ingredients but vigorous mixing in order to provide smaller size micelles.

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

Phenol and/or m-cresol may be added to the mixed micellar composition to stabilize the formulation and protect against bacterial growth. Alternatively, phenol

and/or m-cresol may be added with the micelle forming ingredients. An isotonic agent such as glycerin may also be added after formation of the mixed micellar composition.

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

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

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

### Particles

For ease of exposition the particles, formulations, compositions and methods in this section are discussed largely with regard to unmodified iRNA agents. It may be understood, however, that these particles, formulations, compositions and methods can be practiced with other iRNA agents, *e.g.*, modified iRNA agents, and such practice is within the invention. In another embodiment, an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be processed into a siRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, or precursor thereof) preparations may be incorporated into a particle, *e.g.*, a microparticle. Microparticles can be produced by spray-drying, but may also be produced by other methods including lyophilization, evaporation, fluid bed drying, vacuum drying, or a combination of these techniques. See below for further description.

**Sustained -Release Formulations.** An iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be processed into a siRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, or precursor thereof) described herein can be

formulated for controlled, *e.g.*, slow release. Controlled release can be achieved by disposing the iRNA within a structure or substance which impedes its release. *E.g.*, iRNA can be disposed within a porous matrix or in an erodable matrix, either of which allow release of the iRNA over a period of time.

5 Polymeric particles, *e.g.*, polymeric in microparticles can be used as a sustained-release reservoir of iRNA that is taken up by cells only released from the microparticle through biodegradation. The polymeric particles in this embodiment should therefore be large enough to preclude phagocytosis (*e.g.*, larger than 10  $\mu\text{m}$  or larger than 20  $\mu\text{m}$ ). Such particles can be produced by the same methods to make smaller particles, but with  
10 less vigorous mixing of the first and second emulsions. That is to say, a lower homogenization speed, vortex mixing speed, or sonication setting can be used to obtain particles having a diameter around 100  $\mu\text{m}$  rather than 10  $\mu\text{m}$ . The time of mixing also can be altered.

Larger microparticles can be formulated as a suspension, a powder, or an  
15 implantable solid, to be delivered by intramuscular, subcutaneous, intradermal, intravenous, or intraperitoneal injection; via inhalation (intranasal or intrapulmonary); orally; or by implantation. These particles are useful for delivery of any iRNA when slow release over a relatively long term is desired. The rate of degradation, and consequently of release, varies with the polymeric formulation.

20 Microparticles may include pores, voids, hollows, defects or other interstitial spaces that allow the fluid suspension medium to freely permeate or perfuse the particulate boundary. For example, the perforated microstructures can be used to form hollow, porous spray dried microspheres.

Polymeric particles containing iRNA (*e.g.*, a siRNA) can be made using a double  
25 emulsion technique, for instance. First, the polymer is dissolved in an organic solvent. A polymer may be polylactic-co-glycolic acid (PLGA), with a lactic/glycolic acid weight ratio of 65:35, 50:50, or 75:25. Next, a sample of nucleic acid suspended in aqueous solution is added to the polymer solution and the two solutions are mixed to form a first emulsion. The solutions can be mixed by vortexing or shaking, and in the mixture can be  
30 sonicated. Any method by which the nucleic acid receives the least amount of damage in the form of nicking, shearing, or degradation, while still allowing the formation of an appropriate emulsion is possible. For example, acceptable results can be obtained with a Vibra-cell model VC-250 sonicator with a 1/8" microtip probe, at setting #3.

### Spray Drying

An iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be processed into a siRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, or precursor thereof)) can be prepared by spray drying. Spray dried iRNA can be administered to a subject or be subjected to further formulation. A pharmaceutical composition of iRNA can be prepared by spray drying a homogeneous aqueous mixture that includes a iRNA under conditions sufficient to provide a dispersible powdered composition, *e.g.*, a pharmaceutical composition. The material for spray drying can also include one or more of: a pharmaceutically acceptable excipient, or a dispersibility-enhancing amount of a physiologically acceptable, water-soluble protein. The spray-dried product can be a dispersible powder that includes the iRNA.

Spray drying is a process that converts a liquid or slurry material to a dried particulate form. Spray drying can be used to provide powdered material for various administrative routes including inhalation. See, for example, M. Sacchetti and M. M. Van Oort in: *Inhalation Aerosols: Physical and Biological Basis for Therapy*, A. J. Hickey, ed. Marcel Dekkar, New York, 1996.

Spray drying can include atomizing a solution, emulsion, or suspension to form a fine mist of droplets and drying the droplets. The mist can be projected into a drying chamber (*e.g.*, a vessel, tank, tubing, or coil) where it contacts a drying gas. The mist can include solid or liquid pore forming agents. The solvent and pore forming agents evaporate from the droplets into the drying gas to solidify the droplets, simultaneously forming pores throughout the solid. The solid (typically in a powder, particulate form) then is separated from the drying gas and collected.

Spray drying includes bringing together a highly dispersed liquid, and a sufficient volume of air (*e.g.*, hot air) to produce evaporation and drying of the liquid droplets. The preparation to be spray dried can be any solution, course suspension, slurry, colloidal dispersion, or paste that may be atomized using the selected spray drying apparatus. Typically, the feed is sprayed into a current of warm filtered air that evaporates the solvent and conveys the dried product to a collector. The spent air is then exhausted with the solvent. Several different types of apparatus may be used to provide the desired product. For example, commercial spray dryers manufactured by Buchi Ltd. or Niro Corp. can effectively produce particles of desired size.

Spray-dried powdered particles can be approximately spherical in shape, nearly uniform in size and frequently hollow. There may be some degree of irregularity in shape depending upon the incorporated medicament and the spray drying conditions. In many instances the dispersion stability of spray-dried microspheres appears to be more effective  
5 if an inflating agent (or blowing agent) is used in their production. Certain embodiments may comprise an emulsion with an inflating agent as the disperse or continuous phase (the other phase being aqueous in nature). An inflating agent may be dispersed with a surfactant solution, using, for instance, a commercially available microfluidizer at a pressure of about 5000 to 15,000 psi. This process forms an emulsion, which may be  
10 stabilized by an incorporated surfactant, typically comprising submicron droplets of water immiscible blowing agent dispersed in an aqueous continuous phase. The formation of such dispersions using this and other techniques are common and well known to those in the art. The blowing agent may be a fluorinated compound (*e.g.*, perfluorohexane, perfluorooctyl bromide, perfluorodecalin, perfluorobutyl ethane) which vaporizes during  
15 the spray-drying process, leaving behind generally hollow, porous aerodynamically light microspheres. As will be discussed in more detail below, other suitable blowing agents include chloroform, freons, and hydrocarbons. Nitrogen gas and carbon dioxide are also contemplated as a suitable blowing agent.

Although the perforated microstructures may be formed using a blowing agent as  
20 described above, it will be appreciated that, in some instances, no blowing agent is required and an aqueous dispersion of the medicament and surfactant(s) are spray dried directly. In such cases, the formulation may be amenable to process conditions (*e.g.*, elevated temperatures) that generally lead to the formation of hollow, relatively porous microparticles. Moreover, the medicament may possess special physicochemical  
25 properties (*e.g.*, high crystallinity, elevated melting temperature, surface activity, etc.) that make it particularly suitable for use in such techniques.

The perforated microstructures may optionally be associated with, or comprise, one or more surfactants. Moreover, miscible surfactants may optionally be combined with the suspension medium liquid phase. It will be appreciated by those skilled in the art  
30 that the use of surfactants may further increase dispersion stability, simplify formulation procedures or increase bioavailability upon administration. Of course combinations of surfactants, including the use of one or more in the liquid phase and one or more associated with the perforated microstructures are contemplated as being within the scope of the invention. By “associated with or comprise” it is meant that the structural matrix or



perforated microstructure may incorporate, adsorb, absorb, be coated with or be formed by the surfactant.

Surfactants suitable for use include any compound or composition that aids in the formation and maintenance of the stabilized respiratory dispersions by forming a layer at the interface between the structural matrix and the suspension medium. The surfactant may comprise a single compound or any combination of compounds, such as in the case of co-surfactants. Particularly certain surfactants are substantially insoluble in the propellant, nonfluorinated, and selected from the group consisting of saturated and unsaturated lipids, nonionic detergents, nonionic block copolymers, ionic surfactants, and combinations of such agents. It may be emphasized that, in addition to the aforementioned surfactants, suitable (*i.e.*, biocompatible) fluorinated surfactants are compatible with the teachings herein and may be used to provide the desired stabilized preparations.

Lipids, including phospholipids, from both natural and synthetic sources may be used in varying concentrations to form a structural matrix. Generally, compatible lipids comprise those that have a gel to liquid crystal phase transition greater than about 40° C. In certain embodiments, the incorporated lipids are relatively long chain (*i.e.*, C<sub>6</sub>-C<sub>22</sub>) saturated lipids and may comprise phospholipids. Exemplary phospholipids useful in the disclosed stabilized preparations comprise egg phosphatidylcholine, dilauroylphosphatidylcholine, dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine, distearylphosphatidylcholine, short-chain phosphatidylcholines, phosphatidylethanolamine, dioleoylphosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, glycolipids, ganglioside GM1, sphingomyelin, phosphatidic acid, cardiolipin; lipids bearing polymer chains such as, polyethylene glycol, chitin, hyaluronic acid, or polyvinylpyrrolidone; lipids bearing sulfonated mono-, di-, and polysaccharides; fatty acids such as palmitic acid, stearic acid, and oleic acid; cholesterol, cholesterol esters, and cholesterol hemisuccinate. Due to their excellent biocompatibility characteristics, phospholipids and combinations of phospholipids and poloxamers are particularly suitable for use in the stabilized dispersions disclosed herein.

Compatible nonionic detergents comprise: sorbitan esters including sorbitan trioleate (Spans™ 85), sorbitan sesquioleate, sorbitan monooleate, sorbitan monolaurate, polyoxyethylene (20) sorbitan monolaurate, and polyoxyethylene (20) sorbitan monooleate, oleyl polyoxyethylene (2) ether, stearyl polyoxyethylene (2) ether, lauryl

polyoxyethylene (4) ether, glycerol esters, and sucrose esters. Other suitable nonionic detergents can be easily identified using McCutcheon's Emulsifiers and Detergents (McPublishing Co., Glen Rock, N.J.). Certain block copolymers include diblock and triblock copolymers of polyoxyethylene and polyoxypropylene, including poloxamer 188 (Pluronic.RTM. F68), poloxamer 407 (Pluronic.RTM. F-127), and poloxamer 338. Ionic surfactants such as sodium sulfosuccinate, and fatty acid soaps may also be utilized. In certain embodiments, the microstructures may comprise oleic acid or its alkali salt.

In addition to the aforementioned surfactants, cationic surfactants or lipids may be used, especially in the case of delivery of an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be processed into a siRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, or precursor thereof). Examples of suitable cationic lipids include: DOTMA, N-[-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium-chloride; DOTAP, 1,2-dioleyloxy-3-(trimethylammonio)propane; and DOTB, 1,2-dioleyl-3-(4'-trimethylammonio)butanoyl-sn-glycerol. Polycationic amino acids such as polylysine, and polyarginine are also contemplated.

For the spraying process, such spraying methods as rotary atomization, pressure atomization and two-fluid atomization can be used. Examples of the devices used in these processes include "Parubisu [phonetic rendering] Mini-Spray GA-32" and "Parubisu Spray Drier DL-41", manufactured by Yamato Chemical Co., or "Spray Drier CL-8," "Spray Drier L-8," "Spray Drier FL-12," "Spray Drier FL-16" or "Spray Drier FL-20," manufactured by Okawara Kakoki Co., can be used for the method of spraying using rotary-disk atomizer.

While no particular restrictions are placed on the gas used to dry the sprayed material, it is recommended to use air, nitrogen gas or an inert gas. The temperature of the inlet of the gas used to dry the sprayed materials such that it does not cause heat deactivation of the sprayed material. The range of temperatures may vary between about 50°C to about 200°C, for example, between about 50°C and 100°C. The temperature of the outlet gas used to dry the sprayed material, may vary between about 0°C and about 150°C, for example, between 0°C and 90°C, and for example between 0°C and 60°C.

The spray drying is done under conditions that result in substantially amorphous powder of homogeneous constitution having a particle size that is respirable, a low moisture content and flow characteristics that allow for ready aerosolization. In some cases, the particle size of the resulting powder is such that more than about 98% of the

mass is in particles having a diameter of about 10  $\mu\text{m}$  or less with about 90% of the mass being in particles having a diameter less than 5  $\mu\text{m}$ . Alternatively, about 95% of the mass will have particles with a diameter of less than 10  $\mu\text{m}$  with about 80% of the mass of the particles having a diameter of less than 5  $\mu\text{m}$ .

5           The dispersible pharmaceutical-based dry powders that include the iRNA preparation may optionally be combined with pharmaceutical carriers or excipients which are suitable for respiratory and pulmonary administration. Such carriers may serve simply as bulking agents when it is desired to reduce the iRNA concentration in the powder which is being delivered to a patient, but may also serve to enhance the stability of the  
10 iRNA compositions and to improve the dispersibility of the powder within a powder dispersion device in order to provide more efficient and reproducible delivery of the iRNA and to improve handling characteristics of the iRNA such as flowability and consistency to facilitate manufacturing and powder filling.

          Such carrier materials may be combined with the drug prior to spray drying, *i.e.*,  
15 by adding the carrier material to the purified bulk solution. In that way, the carrier particles will be formed simultaneously with the drug particles to produce a homogeneous powder. Alternatively, the carriers may be separately prepared in a dry powder form and combined with the dry powder drug by blending. The powder carriers will usually be crystalline (to avoid water absorption), but might in some cases be amorphous or mixtures  
20 of crystalline and amorphous. The size of the carrier particles may be selected to improve the flowability of the drug powder, typically being in the range from 25  $\mu\text{m}$  to 100  $\mu\text{m}$ . A carrier material may be crystalline lactose having a size in the above-stated range.

          Powders prepared by any of the above methods will be collected from the spray dryer in a conventional manner for subsequent use. For use as pharmaceuticals and other  
25 purposes, it will frequently be desirable to disrupt any agglomerates which may have formed by screening or other conventional techniques. For pharmaceutical uses, the dry powder formulations will usually be measured into a single dose, and the single dose sealed into a package. Such packages are particularly useful for dispersion in dry powder inhalers, as described in detail below. Alternatively, the powders may be packaged in  
30 multiple-dose containers.

          Methods for spray drying hydrophobic and other drugs and components are described in U.S. Pat. Nos. 5,000,888; 5,026,550; 4,670,419, 4,540,602; and 4,486,435. Bloch and Speison (1983) *Pharm. Acta Helv* 58:14-22 teaches spray drying of hydrochlorothiazide and chlorthalidone (lipophilic drugs) and a hydrophilic adjuvant

(pentaerythritol) in azeotropic solvents of dioxane-water and 2-ethoxyethanol-water. A number of Japanese Patent application Abstracts relate to spray drying of hydrophilic-hydrophobic product combinations, including JP 806766; JP 7242568; JP 7101884; JP 7101883; JP 71018982; JP 7101881; and JP 4036233. Other foreign patent publications  
5 relevant to spray drying hydrophilic-hydrophobic product combinations include FR 2594693; DE 2209477; and WO 88/07870.

### Lyophilization

An iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be processed into a siRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, or precursor thereof) preparation can be made by lyophilization. Lyophilization is a freeze-drying process in which water is sublimed from the composition after it is frozen. The particular advantage associated with the lyophilization process is that biologicals and  
10 pharmaceuticals that are relatively unstable in an aqueous solution can be dried without elevated temperatures (thereby eliminating the adverse thermal effects), and then stored in a dry state where there are few stability problems. With respect to the instant invention such techniques are particularly compatible with the incorporation of nucleic acids in perforated microstructures without compromising physiological activity. Methods for  
15 providing lyophilized particulates are known to those of skill in the art and it would clearly not require undue experimentation to provide dispersion compatible microstructures in accordance with the teachings herein. Accordingly, to the extent that lyophilization processes may be used to provide microstructures having the desired porosity and size, they are conformance with the teachings herein and are expressly  
20 contemplated as being within the scope of the instant invention.

### Genes

In one aspect, the invention features, a method of treating a subject at risk for or afflicted with a disease that may benefit from the administration of the iRNA agent of the  
30 invention. The method comprises administering the iRNA agent of the invention to a subject in need thereof, thereby treating the subject. The iRNA agent that is administered will depend on the disease being treated.

In certain embodiments, the iRNA agent silences a growth factor or growth factor receptor gene, a kinase, *e.g.*, a protein tyrosine, serine or threonine kinase gene, an

adaptor protein gene, a gene encoding a G protein superfamily molecule, or a gene encoding a transcription factor.

### Dosage

5           In one aspect, the invention features a method of administering an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, to a subject (*e.g.*, a human subject). The method includes administering a unit dose of the iRNA agent, *e.g.*, a siRNA agent, *e.g.*, double stranded siRNA agent that (a) the double-stranded part is 19-25 nucleotides (nt) long, for example, 21-23 nt, (b) is complementary to a target RNA (*e.g.*, an  
10    endogenous or pathogen target RNA), and, optionally, (c) includes at least one 3' overhang 1-5 nucleotide long. In one embodiment, the unit dose is less than 1.4 mg per kg of bodyweight, or less than 10, 5, 2, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005 or 0.00001 mg per kg of bodyweight, and less than 200 nmole of RNA agent (*e.g.*, about  $4.4 \times 10^{16}$  copies) per kg of bodyweight, or less than 1500, 750, 300,  
15    150, 75, 15, 7.5, 1.5, 0.75, 0.15, 0.075, 0.015, 0.0075, 0.0015, 0.00075, 0.00015 nmole of RNA agent per kg of bodyweight.

          The defined amount can be an amount effective to treat or prevent a disease or disorder, *e.g.*, a disease or disorder associated with the target RNA. The unit dose, for  
20    example, can be administered by injection (*e.g.*, intravenous or intramuscular), an inhaled dose, or a topical application. In some embodiments dosages may be less than 2, 1, or 0.1 mg/kg of body weight.

          In some embodiments, the unit dose is administered less frequently than once a day, *e.g.*, less than every 2, 4, 8 or 30 days. In another embodiment, the unit dose is not administered with a frequency (*e.g.*, not a regular frequency). For example, the unit dose  
25    may be administered a single time.

          In one embodiment, the effective dose is administered with other traditional therapeutic modalities. In one embodiment, the subject has a viral infection and the modality is an antiviral agent other than an iRNA agent, *e.g.*, other than a double-stranded iRNA agent, or siRNA agent,. In another embodiment, the subject has atherosclerosis  
30    and the effective dose of an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, is administered in combination with, *e.g.*, after surgical intervention, *e.g.*, angioplasty.

          In one embodiment, a subject is administered an initial dose and one or more maintenance doses of an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA

agent, (e.g., a precursor, e.g., a larger iRNA agent which can be processed into a siRNA agent, or a DNA which encodes an iRNA agent, e.g., a double-stranded iRNA agent, or siRNA agent, or precursor thereof). The maintenance dose or doses are generally lower than the initial dose, e.g., one-half less of the initial dose. A maintenance regimen can include treating the subject with a dose or doses ranging from 0.01  $\mu\text{g}$  to 1.4 mg/kg of body weight per day, e.g., 10, 1, 0.1, 0.01, 0.001, or 0.00001 mg per kg of bodyweight per day. The maintenance doses are, for example, administered no more than once every 5, 10, or 30 days. Further, the treatment regimen may last for a period of time which will vary depending upon the nature of the particular disease, its severity and the overall condition of the patient. In certain embodiments the dosage may be delivered no more than once per day, e.g., no more than once per 24, 36, 48, or more hours, e.g., no more than once for every 5 or 8 days. Following treatment, the patient can be monitored for changes in his condition and for alleviation of the symptoms of the disease state. The dosage of the compound may either be increased in the event the patient does not respond significantly to current dosage levels, or the dose may be decreased if an alleviation of the symptoms of the disease state is observed, if the disease state has been ablated, or if undesired side-effects are observed.

The effective dose can be administered in a single dose or in two or more doses, as desired or considered appropriate under the specific circumstances. If desired to facilitate repeated or frequent infusions, implantation of a delivery device, e.g., a pump, semi-permanent stent (e.g., intravenous, intraperitoneal, intracisternal or intracapsular), or reservoir may be advisable.

In one embodiment, the iRNA agent pharmaceutical composition includes a plurality of iRNA agent species. In another embodiment, the iRNA agent species has sequences that are non-overlapping and non-adjacent to another species with respect to a naturally occurring target sequence. In another embodiment, the plurality of iRNA agent species is specific for different naturally occurring target genes. In another embodiment, the iRNA agent is allele specific.

In some cases, a patient is treated with a iRNA agent in conjunction with other therapeutic modalities. For example, a patient being treated for a viral disease, e.g., an HIV associated disease (e.g., AIDS), may be administered a iRNA agent specific for a target gene essential to the virus in conjunction with a known antiviral agent (e.g., a protease inhibitor or reverse transcriptase inhibitor). In another example, a patient being

treated for cancer may be administered a iRNA agent specific for a target essential for tumor cell proliferation in conjunction with a chemotherapy.

Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the compound of the invention is administered in maintenance doses, ranging from 0.01  $\mu$ g to 100 g per kg of body weight (see US 6,107,094).

The concentration of the iRNA agent composition is an amount sufficient to be effective in treating or preventing a disorder or to regulate a physiological condition in humans. The concentration or amount of iRNA agent administered will depend on the parameters determined for the agent and the method of administration, e.g., nasal, buccal, pulmonary. For example, nasal formulations tend to require much lower concentrations of some ingredients in order to avoid irritation or burning of the nasal passages. It is sometimes desirable to dilute an oral formulation up to 10-100 times in order to provide a suitable nasal formulation.

Certain factors may influence the dosage required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of an iRNA agent, e.g., a double-stranded iRNA agent, or siRNA agent, (e.g., a precursor, e.g., a larger iRNA agent which can be processed into a siRNA agent, or a DNA which encodes an iRNA agent, e.g., a double-stranded iRNA agent, or siRNA agent, or precursor thereof) can include a single treatment or, for example, can include a series of treatments. It will also be appreciated that the effective dosage of a iRNA agent such as a siRNA agent used for treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result and become apparent from the results of diagnostic assays as described herein. For example, the subject can be monitored after administering a iRNA agent composition. Based on information from the monitoring, an additional amount of the iRNA agent composition can be administered.

Dosing is dependent on severity and responsiveness of the disease condition to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of

individual compounds, and can generally be estimated based on EC50s found to be effective in in vitro and in vivo animal models. In some embodiments, the animal models include transgenic animals that express a human gene, e.g., a gene that produces a target RNA. The transgenic animal can be deficient for the corresponding endogenous RNA.

5 In another embodiment, the composition for testing includes a iRNA agent that is complementary, at least in an internal region, to a sequence that is conserved between the target RNA in the animal model and the target RNA in a human.

The iRNA agent, e.g., a double-stranded iRNA agent, or siRNA agent, (e.g., a precursor, e.g., a larger iRNA agent which can be processed into a siRNA agent, or a DNA which encodes an iRNA agent, e.g., a double-stranded iRNA agent, or siRNA agent, or precursor thereof), can be provided in a powdered, crystallized or other finely divided form, with or without a carrier, e.g., a micro- or nano-particle suitable for inhalation or other pulmonary delivery. This can include providing an aerosol preparation, e.g., an aerosolized spray-dried composition. The aerosol composition can be provided in and/or dispensed by a metered dose delivery device.

10 The subject can be treated for a condition treatable by inhalation, e.g., by aerosolizing a spray-dried iRNA agent, e.g., a double-stranded iRNA agent, or siRNA agent, (e.g., a precursor, e.g., a larger iRNA agent which can be processed into a siRNA agent, or a DNA which encodes an iRNA agent, e.g., a double-stranded iRNA agent, or siRNA agent, or precursor thereof) composition and inhaling the aerosolized composition. The iRNA agent can be an siRNA. The composition can include a plurality of iRNA agents, e.g., specific for one or more different endogenous target RNAs. The method can include other features described herein.

A subject can be treated by, for example, administering a composition including an effective/defined amount of an iRNA agent, e.g., a double-stranded iRNA agent, or siRNA agent, (e.g., a precursor, e.g., a larger iRNA agent which can be processed into a siRNA agent, or a DNA which encodes an iRNA agent, e.g., a double-stranded iRNA agent, or siRNA agent, or precursor thereof), wherein the composition is prepared by a method that includes spray-drying, lyophilization, vacuum drying, evaporation, fluid bed drying, or a combination of these techniques.

30 In another aspect, the invention features a method that includes: evaluating a parameter related to the abundance of a transcript in a cell of a subject; comparing the evaluated parameter to a reference value; and if the evaluated parameter has a preselected relationship to the reference value (e.g., it is greater), administering a iRNA agent (or a



precursor, *e.g.*, a larger iRNA agent which can be processed into a siRNA agent, or a DNA which encodes a iRNA agent or precursor thereof) to the subject. In one embodiment, the iRNA agent includes a sequence that is complementary to the evaluated transcript. For example, the parameter can be a direct measure of transcript levels, a  
5 measure of a protein level, a disease or disorder symptom or characterization (*e.g.*, rate of cell proliferation and/or tumor mass, viral load).

In another aspect, the invention features a method that includes: administering a first amount of a composition that comprises an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be  
10 processed into a siRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, or precursor thereof) to a subject, wherein the iRNA agent includes a strand substantially complementary to a target nucleic acid; evaluating an activity associated with a protein encoded by the target nucleic acid; wherein the evaluation is used to determine if a second amount may be administered. In  
15 some embodiments the method includes administering a second amount of the composition, wherein the timing of administration or dosage of the second amount is a function of the evaluating. The method can include other features described herein.

In another aspect, the invention features a method of administering a source of a double-stranded iRNA agent (ds iRNA agent) to a subject. The method includes  
20 administering or implanting a source of a ds iRNA agent, *e.g.*, a siRNA agent, that (a) includes a double-stranded region that is 19-25 nucleotides long, for example, 21-23 nucleotides, (b) is complementary to a target RNA (*e.g.*, an endogenous RNA or a pathogen RNA), and, optionally, (c) includes at least one 3' overhang 1-5 nt long. In one embodiment, the source releases ds iRNA agent over time, *e.g.*, the source is a controlled  
25 or a slow release source, *e.g.*, a microparticle that gradually releases the ds iRNA agent. In another embodiment, the source is a pump, *e.g.*, a pump that includes a sensor or a pump that can release one or more unit doses.

In one aspect, the invention features a pharmaceutical composition that includes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, (*e.g.*, a precursor,  
30 *e.g.*, a larger iRNA agent which can be processed into a siRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, or precursor thereof) including a nucleotide sequence complementary to a target RNA, *e.g.*, substantially and/or exactly complementary. The target RNA can be a transcript of an endogenous human gene. In one embodiment, the iRNA agent (a) is 19-25 nucleotides

long, for example, 21-23 nucleotides, (b) is complementary to an endogenous target RNA, and, optionally, (c) includes at least one 3' overhang 1-5 nt long. In one embodiment, the pharmaceutical composition can be an emulsion, microemulsion, cream, jelly, or liposome.

5           In one example the pharmaceutical composition includes an iRNA agent mixed with a topical delivery agent. The topical delivery agent can be a plurality of microscopic vesicles. The microscopic vesicles can be liposomes. In some embodiments the liposomes are cationic liposomes.

          In another aspect, the pharmaceutical composition includes an iRNA agent, *e.g.*, a  
10 double-stranded iRNA agent, or siRNA agent (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be processed into a siRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, or precursor thereof) admixed with a topical penetration enhancer. In one embodiment, the topical penetration enhancer is a fatty acid. The fatty acid can be arachidonic acid, oleic acid, lauric acid, caprylic acid,  
15 capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C<sub>1-10</sub> alkyl ester, monoglyceride, diglyceride or pharmaceutically acceptable salt thereof.

          In one aspect, the invention features a pharmaceutical composition including an  
20 iRNA agent and a delivery vehicle. In one embodiment, the iRNA agent is (a) is 19-25 nucleotides long, for example, 21-23 nucleotides, (b) is complementary to an endogenous target RNA, and, optionally, (c) includes at least one 3' overhang 1-5 nucleotides long.

          In one embodiment, the delivery vehicle can deliver an iRNA agent, *e.g.*, a  
25 double-stranded iRNA agent, or siRNA agent, (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be processed into a siRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, or precursor thereof) to a cell by a topical route of administration. The delivery vehicle can be microscopic vesicles. In one example the microscopic vesicles are liposomes. In some embodiments the liposomes are cationic liposomes. In another example the microscopic vesicles are micelles. In one  
30 aspect, the invention features a pharmaceutical composition including an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, (*e.g.*, a precursor, *e.g.*, a larger iRNA agent which can be processed into a siRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, or precursor thereof) in an injectable dosage form. In one embodiment, the injectable dosage form of the

pharmaceutical composition includes sterile aqueous solutions or dispersions and sterile powders. In some embodiments the sterile solution can include a diluent such as water; saline solution; fixed oils, polyethylene glycols, glycerin, or propylene glycol.

In one aspect, the invention features a pharmaceutical composition including an  
5 iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, (*e.g.*, a precursor, *e.g.*,  
a larger iRNA agent which can be processed into a siRNA agent, or a DNA which  
encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, or  
precursor thereof) in oral dosage form. In one embodiment, the oral dosage form is  
10 selected from the group consisting of tablets, capsules and gel capsules. In another  
embodiment, the pharmaceutical composition includes an enteric material that  
substantially prevents dissolution of the tablets, capsules or gel capsules in a mammalian  
stomach. In some embodiments the enteric material is a coating. The coating can be  
acetate phthalate, propylene glycol, sorbitan monoleate, cellulose acetate trimellitate,  
hydroxy propyl methyl cellulose phthalate or cellulose acetate phthalate. In one  
15 embodiment, the oral dosage form of the pharmaceutical composition includes a  
penetration enhancer, *e.g.*, a penetration enhancer described herein.

In another embodiment, the oral dosage form of the pharmaceutical composition  
includes an excipient. In one example the excipient is polyethyleneglycol. In another  
example the excipient is precirol.

20 In another embodiment, the oral dosage form of the pharmaceutical composition  
includes a plasticizer. The plasticizer can be diethyl phthalate, triacetin dibutyl sebacate,  
dibutyl phthalate or triethyl citrate.

In one aspect, the invention features a pharmaceutical composition including an  
iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, (*e.g.*, a precursor, *e.g.*,  
25 a larger iRNA agent which can be processed into a siRNA agent, or a DNA which  
encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, or  
precursor thereof) in a rectal dosage form. In one embodiment, the rectal dosage form is  
an enema. In another embodiment, the rectal dosage form is a suppository.

In one aspect, the invention features a pharmaceutical composition including an  
30 iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, (*e.g.*, a precursor, *e.g.*,  
a larger iRNA agent which can be processed into a siRNA agent, or a DNA which  
encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, or  
precursor thereof) in a vaginal dosage form. In one embodiment, the vaginal dosage form

is a suppository. In another embodiment, the vaginal dosage form is a foam, cream, or gel.

In one aspect, the invention features a pharmaceutical composition including an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, (*e.g.*, a precursor, *e.g.*,  
5 a larger iRNA agent which can be processed into a siRNA agent, or a DNA which encodes an iRNA agent, *e.g.*, a double-stranded iRNA agent, or siRNA agent, or precursor thereof) in a pulmonary or nasal dosage form. In one embodiment, the iRNA agent is incorporated into a particle, *e.g.*, a macroparticle, *e.g.*, a microsphere. The particle can be produced by spray drying, lyophilization, evaporation, fluid bed drying,  
10 vacuum drying, or a combination thereof. The microsphere can be formulated as a suspension, a powder, or an implantable solid.

As used herein, the term "crystalline" describes a solid having the structure or characteristics of a crystal, *i.e.*, particles of three-dimensional structure in which the plane faces intersect at definite angles and in which there is a regular internal structure. The  
15 compositions of the invention may have different crystalline forms. Crystalline forms can be prepared by a variety of methods, including, for example, spray drying.

In one aspect the invention provides a method of modulating the expression of a target gene in a cell, comprising providing to said cell an iRNA agent of this invention. In one embodiment, the target gene is selected from the group consisting of Factor VII, Eg5, PCSK9, TPX2, apoB, SAA, TTR, RSV, PDGF beta gene, Erb-B gene, Src gene,  
20 CRK gene, GRB2 gene, RAS gene, MEKK gene, JNK gene, RAF gene, Erk1/2 gene, PCNA(p21) gene, MYB gene, JUN gene, FOS gene, BCL-2 gene, Cyclin D gene, VEGF gene, EGFR gene, Cyclin A gene, Cyclin E gene, WNT-1 gene, beta-catenin gene, c-MET gene, PKC gene, NFkB gene, STAT3 gene, survivin gene, Her2/Neu gene,  
25 topoisomerase I gene, topoisomerase II alpha gene, mutations in the p73 gene, mutations in the p21(WAF1/CIP1) gene, mutations in the p27(KIP1) gene, mutations in the PPM1D gene, mutations in the RAS gene, mutations in the caveolin I gene, mutations in the MIB I gene, mutations in the MTAI gene, mutations in the M68 gene, mutations in tumor suppressor genes, and mutations in the p53 tumor suppressor gene.

30 Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

5 Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

As used herein, the term "subject in need thereof" refers to a subject diagnosed with or exhibiting one or more conditions associated with a disease or condition treatable by administration of ligand conjugated oligonucleotide of the invention, a subject who has been diagnosed with or exhibited one or more conditions treatable by administration of ligand conjugated oligonucleotide in the past, or a subject who has been deemed at risk of developing one or more conditions associated with a disease or condition treatable by administration of ligand conjugated oligonucleotide in the future due to hereditary or environmental factors. In certain embodiments of the invention, the subject in need thereof is suffering from a disease or condition such as, but not limited to respiratory and/or pulmonary disease or condition, male infertility, viral infection, a uterine disorder, an endometrial disorder or condition, cancer, primary cancer and/or metastatic cancer.

20 In some embodiments, a subject in need thereof refers to a subject with a pulmonary condition having clinically abnormal spirometry values. Examples of spirometry parameters which can indicate the need of a subject include, but are not restricted to forced expiration volume (FEV<sub>i</sub>), forced vital capacity (FVC), forced expiratory flow (FEF<sub>25-75</sub>) and the like. In some embodiments of the invention administration of the ligand conjugated oligonucleotide to the subject results in an improvement in one or more of the spirometric parameters.

#### Pulmonary administration of ligand conjugated oligonucleotide

Pulmonary administration may be accomplished by suitable means known to those in the art. Pulmonary administration of ligand conjugated oligonucleotide requires dispensing of the biologically active substance from a delivery device into the oral cavity of a subject during inhalation. For purposes of the present invention, compositions comprising ligand conjugated oligonucleotide are administered via inhalation of dry powder formulation of the invention, via a dry powder inhaler delivery device. Such

delivery devices are well known in the art and include, but are not limited to, metered dose and pre-metered dry powder inhalers, or any other appropriate delivery mechanisms that allow for dispensing of a solid or dry powder form.

## 5 Dry Powder Inhaler (DPI) Devices

According to some aspects of one embodiment, the dry powder formulation comprising ligand conjugated oligonucleotide, or biologically active portion thereof, is delivered to a subject through a dry powder inhaler (DPI). A DPI is used to deliver an agent, such as ligand conjugated oligonucleotide, in a solid or dry powder form using a subject's inspiration to deliver the dry powder to the lungs, instead of a mist. A DPI is used to breathe in (inhale) the ligand conjugated oligonucleotide so that it goes directly into the subject's lungs. A DPI is a propellant-free device, wherein the agent to be delivered is blended with suitable carriers known in the art. The unit dose of agent used in a DPI device is often a dry powder blister disc or hard capsule. A DPI produces

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15  
20

dispersible and stable dry powder formulations which are inhaled, including spray drying, spray-freeze drying, and micronized milling formulations. DPI devices have been used to deliver macromolecular agents, including insulin, interferon (IFN), and growth hormone (GH). Examples of DPI devices include, but are not limited to, the following:

The AIR® inhaler (Alkermes) which includes a small, breath-activated system that delivers porous powder from a capsule (see WO 99/66903 and WO 00/10541). The porous particles have an aerodynamic diameter of 1 -5  $\mu$ m and are prepared by spray drying. The AIR™ inhaler has been used to deliver albuterol, epinephrine, insulin, and hGH. The TurboHaler® (AstraZeneca) is also a DPI which may be used in the methods of the invention and is described in EP patent 0799067, incorporated by reference herein.

25 This DPI device is an inspiratory flow-driven, multidose dry-powder inhaler with a multi-dose reservoir that provides up to 200 doses of the drug formulation and dose ranges from a few micrograms to 0.5 mg. Examples of the TurboHaler™ include Pulmicort® (also Pulmicort® TurboHaler®), Oxis® (formoterol) and Symbicort® (budesonide/formoterol).

30 Eclipse™ (Aventis) represents a breath actuated reusable capsule device capable of delivering up to 20 mg of formulation. The powder is sucked from the capsule into a vortex chamber where a rotating ball aids in powder disaggregation as the subject inhales (see U.S. Pat. No. 6,230,707 and WO9503846).

Another DPI device which may be used in the methods and compositions of the invention includes the Ultrahaler® (Aventis), as described in U.S. Pat. No. 5,678,538 and WO2004026380.

Another DPI device, which may be used in the methods and compositions of the invention includes the Bang Olufsen breath actuated inhaler, which is a disposable breath actuated inhaler using blister strips with up to sixty doses (see EP 1522325).

An active DPI (also usable as an MDI— described below) described in WO 94/19042 (Bespak) employs multiple, carbon fiber brush, setaceous electrodes to disperse powders and aerosols into fine/particles/mists. As the patient inhales, 1 to 10 kvolts is passed through the electrodes to disperse the powder/aerosol. A breath sensor is employed to initiate the electric discharge.

The HandiHaler® (Boehringer Ingelheim GmbH) is a single dose DPI device, which can deliver up to 30 mg of formulated drug in capsules (see WO2004024156). An example of this device is Spiriva® (tiotropium bromide). The PADD DPI (Britannia Pharmaceuticals) is a pressurized aerosol dry powder delivery device utilizing a novel formulation comprised of surface active phospholipids, dipalmitoyl phosphatidyl choline (DPPC) and phosphatidyl glycerol (PG), prepared in the form of a fine powder. The PADD device offers the highest payload possible with a propellant powered device, (see U.S. Pat. No. 6,482,391). Another DPI device, which may be used in the methods and compositions of the invention includes the Pulvinal® inhaler (Chiesi) which is a breath-actuated multidose (100 doses) dry powder inhaler (see U.S. Pat. No. 5,351,683). The Pulvinal inhaler has been used to deliver respiratory drugs such as salbutamol (Butovent® Pulvinal®), beclomethasone (Clenil® Pulvinal®) as well as budesonide and formoterol.

Another DPI device which may be used in the methods and compositions of the invention includes NEXT DPITM, which features multidose capabilities, moisture protection, dose counting and doses only when proper aspiratory flow is reached (see EP1196146, U.S. Pat. No. 6,528,096, WO0178693, WO0053158).

The DirectHaler™ (Direct-Haler A/S) may also be used in the methods and compositions of the invention (see U.S. Pat. No. 5,797,392). This single dose, premetered, pre-filled, disposable DPI device made from polypropylene resembles a straw, and has been used to deliver formulations of budesonide and formoterol. The Accuhaler/Diskus™ (GlaxoSmithKline) is a disposable small DPI device using doses in double foil blister strips (see GB2242134), which has been used to deliver flutacasonone

propionate/salmeterol xinafoate, flutacasonone propionate, salmeterol xinafoate, and salbutamol.

In addition, the methods may include the FlowCaps® (Hovione), a capsule- based, re-fillable, reusable, pen-shaped, moisture-proof passive dry-powder inhaler (see U.S. Pat. No. 5,673,686).

In one embodiment, the DPI device used in the invention is a multi-dose device such as the Clickhaler® (Innovata PLC), (see U.S. Pat. No. 5,437,270), used to treat asthma and COPD with a variety of drugs, including salbutamol (Asmasal®), beclomethasone (Asmabec®), and procaterol hydrochloride (Meptin®) as well as budesonide and formoterol. Another DPI device suitable for use with the invention includes the Duohaler® (Innovata PLC) (see WO0139823). Duohaler® is actually ideally suited for the delivery of fixed combination therapy with additional compositions/drugs for CF, asthma, COPD and the like.

In one embodiment, the DPI device used in the invention is an S2 unit dose (Innovata PLC), which is a re-useable or disposable single-dose DPI for the delivery of a wide range of therapeutics in high concentrations (see AU3320101).

Yet another DPI device which may be used in the methods and compositions of the invention includes Taifun® DPI (LAB International) which is a multiple-dose (up to 200) DPI device that is breath actuated and flow rate independent (see U.S. Pat. No. 6,132,394). In one embodiment, the DPI device used in the invention is MedTone® (Mannkind Corp., see WOO 107107) which comprises an intake section, a mixing section, and a mouthpiece. The mouthpiece is connected by a swivel joint to the mixing section. The intake chamber comprises a piston with a tapered piston rod and spring, and one or more bleedthrough orifices to modulate the flow of air through the device. The mixing section holds a capsule with holes containing a dry powder medicament, and further opens and closes the capsule when the intake section is at a certain angle to the mouthpiece. The mixing section is a Venturi chamber to impart a cyclonic flow to air passing through the mixing chamber. The mouthpiece includes a tongue depressor, and a protrusion to contact the lips of the user to tell the user that the DPI is in the correct position. Technosphere® Insulin System, used for the treatment of diabetes, consists of a dry-powder Technosphere® formulation (see US2004096403) of insulin and MedTone® inhaler through which the powder is inhaled into the deep lung. The powder formulation of the drug to be delivered in microparticles has a size range between 0.5 and ten microns, preferably in the range of two to five microns, formed of a material releasing drug at a pH



of greater than 6.4.). In the Technosphere device, a dry powder insulin formulation containing insulin complexed to 3,6-di(fumaryl4-aminobutyl)-2,5-diketopiperazine (hereinafter fumaryl diketopiperazine or FDKP) is used. The use of diketopiperazines for drug delivery is known in the art (see for example U.S. Pat. Nos. 5,352,461 ; U.S. Pat. No. 5,503,852; U.S. Pat. No. 6,071,497; and U.S. Pat. No. 6,331,318). Pulmonary drug delivery using diketopiperazine and other microparticles is disclosed in U.S. Pat. No. 6,428,771. Particularly advantageous devices for powder delivery are disclosed in U.S. Patent No. 7,464,706 and in U.S. Pat. No. 6,923,175.

Another DPI device which may be used in the methods and compositions of the invention includes Xcelovair™ (Meridica/Pfizer) which features pre-metered, hermetically sealed doses in a fine particle fraction delivery to achieve up to 50% fine particle mass.

Yet another DPI device which may be used in the methods and compositions of the invention includes MicroDose® DPI (Microdose Technologies) which is a small electronic DPI device that uses piezoelectric vibrator (ultrasonic frequencies) to deaggragate the drug powder (small or large molecules, neat chemical or mixtures of drug and lactose up to 3 mg drug) in an aluminum blister (single or multiple dose) (see U.S. Pat. No. 6,026,809).

In another embodiment, the DPI device used in the invention is Nektar Pulmonary Inhaler® (Nektar) which creates an aerosol cloud suitable for deep lung delivery (see AU4090599, U.S. Pat. No. 5,740,794), using compressed gas to aerosolize the powder. The Nektar Pulmonary Inhaler® is used in Exubera® inhalable insulin (Pfizer, Sanofi-Aventis, and Nektar), as well as to administer tobramycin, leuprolide, and single chain antibodies.

Also included in the invention is the Nektar Dry Powder Inhaler® (Nektar) which is used in combination with Nektar Pulmonary Technology® (see US2003094173). The Nektar DPI is ideal for large payloads (2-50 mg) and a variety of molecular sizes, and has been used to deliver tobramycin inhalation powder for lung infections in Cystic Fibrosis and amphotericin B for treatment of fungal infection. Also included in the invention is the active DPI Oriel™ (see WOO 168169).

In addition, EasyHaler® (Orion Pharma), a multidose dry powder inhaler for lung and nasal delivery may be used in the methods and compositions of the invention (see WO02102444). The EasyHaler® includes Beclomet EasyHaler®/Atomide EasyHaler®

(beclomethasone dipropionate) and Buventol EasyHaler®/Salbu EasyHaler® (salbutamol).

Also included in the invention is the Jethaler® (Pulmotec) which utilizes the MAG (mechanical aerosol generation from a highly compressed solid) technology for CFC-free dry-powder inhalation. The JetHaler® has been used to deliver budesonide (Budesonidratiopharm®).

Yet another DPI device which may be used in the methods and compositions of the invention includes AccuBreathe™ single dose DPI (Respirics) (see WO03035137, U.S. Pat. No. 6,561,186). Also included in the invention is the AcuBreather™ multidose DPI (Respirics) which uses an aclar/ PVC moisture protected blister cartridge capable of holding 25-50 mg of powder (30 dose and 15 dose devices respectively) and are capable of holding and delivering two different drug formulations simultaneously (see U.S. Pat. No. 6,561, 186), using i-Point™ technology for drug release. Also included in the invention is the Twisthaler® (Schering-Plough), capable of 14-200 actuations (U.S. Pat. No. 5,829,434), packaged with a desiccant. Products including this DPI device include the Asmanex Twisthaler (mometasone furoate).

Another DPI device which may be used in the methods and compositions of the invention includes the multidose SkyeHaler® DPI (SkyePharma) (see U.S. Pat. No. 6,182,655, WO97/20589), for dosing from 200 ug to 5 mg. This DPI is device is included in Foradil Certihaler® (formoterol fumarate). Also included in the invention is the refiUable, multidose Novolizer® (Meda AB) dry powder inhaler (U.S. Pat. No. 5,840, 279, U.S. Pat. No. 6,071,498, WO9700703).

Another DPI device which may be used in the methods and compositions of the invention includes the Blister Inhaler™ (Meda AB), which is a refiUable, multi-dose, breath activated, dry powder inhaler with dose counter (U.S. Pat. No. 5,881,719, WO9702061), able to deliver moisture-sensitive compounds (e.g. proteins and peptides). Other DPI devices include the SpinHaler® (Aventis and Rhone-Poulenc Rorer); the unit dose DPI (Bespak; a single unit dose device; see US Pat. No. US6945953), theDiskHaler® (GlaxoSmithKline; a multidose device for local lung delivery— see U.S. Pat. No. 5,035,237), Rotohaler® (GlaxoSmithKline) (see U.S. Pat. No. 5,673,686, U.S.Pat. No. 5,881,721); LABHaler® (LAB International; a breath-actuated disposable single dose dry powder delivery device); AirMaXTM (Ivax; a multiple dose reservoir inhaler; see U.S. Pat. No. 5,503,144); Aerolizer™ (Novartis); see U.S. Pat. No. 6,488,027, U.S. Pat. No. 3,991, 761); Rexam DPI (Rexam Pharma; see U.S. Pat.

No.5,651 , 359 and EP0707862; bead inhaler multiple dose (Valois; WO0035523, U.S. Pat.No. 6,056,169; a multiple dose DPI pulmonary delivery device on license from Elan/Dura/Quadrant); Aspirair® (Ventura; WO 02/089880; a single dose, breath activated DPI); and Gyrohaler® (Ventura; GB2407042; a passive disposable DPI).

5 Other examples of commercially available dry powder inhalers suitable for use in accordance with the methods herein include the Spinhaler® powder inhaler (Fisons) and the Ventolin® Rotahaler® (GlaxoSmithKline). See also the dry powder delivery devices described in WO 93/00951 , WO 96/09085, WO 96/32152, and U.S. Pat. Nos 5,458,135, 5,785,049, and 5,993,783, herein incorporated by reference. In one embodiment, the  
10 invention provides a dry powder inhaler (DPI) device for pulmonary administration of ligand conjugated oligonucleotide to a subject, wherein the DPI device comprises a reservoir comprising an inhalable powder or dry powder composition comprising the ligand conjugated oligonucleotide, and a means for introducing the inhalable powder or dry powder composition into the subject via inhalation. The invention also provides an  
15 inhalable powder which comprises the ligand conjugated oligonucleotide and is administered to the subject via a dry powder inhaler (DPI).

The DPI device used in the invention may be either a single dose or a multidose inhaler. In addition, the DPI device used in the invention may also be either pre-metered or device-metered.

20

#### Metered Dose Inhaler (MDI) Device

In one embodiment, the ligand conjugated oligonucleotide, including an enzymatically active portion thereof, is delivered to a subject through metered dose inhaler (MDI) device. An MDI device uses a propellant to deliver reproducible metered  
25 drug dose to the lung and/or airways, and comprises a drug or agent, propellants (e.g. hydrofluoroalkanes (HFA)), surfactants (e.g. phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol, lysophosphatidyl choline, phosphatidic acid, triglycerides, monoglycerides, soy lecithin, fatty acids, and alkyl-polyglycosides), and solvents. An MDI device is often a compact pressurized dispenser, including a canister,  
30 metering valve, and spacer. The dose administered by an MDI device is generally in mg and ranges in volume from about 25 to 100 mL. Additionally, MDI devices are advantageous as they are tamper- proof.

Examples of CFC-free MDI products include Albuterol® HFA (Ivax), Atrovent®-HFA (Boehringer-Ingelheim), Proventil®-HFA (3M), Flovent®-HFA (GSK), Qvar®

(3M), Ventolin® HFA(GSK), Xopenex® HFA (3M/Sepracor), Salamol Easi-Breathe® CFC-Free (Ivax), Berotec® (Boehringer-Ingelheim), Berodual® (Boehringer-Ingelheim), Intal® Forte (Rhone/Aventis), and Seretide® EvoHaler® (GSK).

Examples of MDI devices include, but are not limited to, the following:

5 In one embodiment, the invention provides an MDI device for pulmonary administration of ligand conjugated oligonucleotide to a subject, wherein the MDI device is anAutoHaler® (3M) (see U.S. Pat. No. 6,120,752). Examples of AutoHaler® devices being used to deliver therapeutic agents include Aerobid® (flunisolide), Alupent® (metaproterenol sulphate), Atrovent®/Atovent®-HFA (ipratropium bromide),  
10 Combivent® (albuterol sulfate/ipratropium bromide), MaxAir® AutoHaler® (pirbuterol acetate), Proventil®- HFA (albuterol sulphate), Qvar® (beclomethasone dipropionate) and Xopenex® HFA (levalbuterol hydrochloride).

Another MDI device which may be used in the methods and compositions of the invention includes the breath-activated MD Turbo™ (Accentia Bio), which transforms  
15 metered-dose inhalers into a breath-activated, dose-counting inhaler.

In one embodiment, the invention provides an MDI device for pulmonary administration of ligand conjugated oligonucleotide to a subject, wherein the MDI device is the continuous inhalation flow device WatchHaler® (Activaero GmbH).

The portable drug delivery system EZ Spacer® (AirPharma) may also be used in  
20 the methods and compositions of the invention. In another embodiment, the Asmair® (Bang and Olufsen Medicom AS) MDI. In yet another embodiment, the invention includes an Active DPI/MPI device (Bespak) (see W09419042). In still another embodiment, the invention provides an MDI device for pulmonary administration of ligand conjugated oligonucleotide to a subject, wherein the MDI device is a device for  
25 delivering metered aerosols comprising an active ingredient in solution in a propellant consisting of a hydrofluoroalkane (HFA) (see WOO 149350; Chiesi).

Other examples of MDI devices which may be used in the invention include MDI inhalers described in U.S. Pat. No. 6,170,717 (GlaxoSmithKline); EasiBreath® MDI (Ivax; W0193933, U.S. Pat. No. 5,447,150); MDI breath coordinated inhaler and breath  
30 actuated inhaler (Kos; CA2298448 and WO2004082633); Tempo™ (MAP Pharma; U.S. Pat. No. 6,095,141 , U.S. Pat. No. 6,026, 808 and U.S. Pat. No. 6,367,471); Xcelovent™ (Meridica/Pfizer; W09852634; a breath operated device that also has a dose counter feature); and Increased dosage MDI (Nektar see WO2004041340; a device

capable of delivering 2 mg to 5 mg of a formulated drug using HFA propellants) and a MDI described in WO03053501 (Vectura).

Thus, the invention also includes a metered dose inhaler (MDI) device for pulmonary administration of ligand conjugated oligonucleotide to a subject, the MDI  
5 device comprising a pressurized canister comprising an aerosol comprising the ligand conjugated oligonucleotide and a propellant, and a means for introducing the aerosol into the subject via inhalation. Formulations of ligand conjugated oligonucleotide for use in the methods of the invention is formulated in dry powder formulation suitable for inhalation. Suitable preparations include all dry powder formulation preparations so long  
10 as the particles comprising the protein composition are delivered in a size range consistent with that described for the delivery device, e.g., a dry powder form of the formulation.

Thus, a liquid formulation comprising ligand conjugated oligonucleotide, or enzymatically active portion thereof, intended for use in the methods of the present  
15 invention may either be used as a liquid solution or suspension in the delivery device or first be processed into a dry powder form using lyophilization or spray-drying techniques well known in the art. Powder comprising a ligand conjugated oligonucleotide such as a plant expressed recombinant human ligand conjugated oligonucleotide, may also be prepared using other methods known in the art, including crystallization or precipitation  
20 (see, for example, dry powder microspheres (PROMAXX; Baxter) described in U.S. Pat. No. 5,525,519; U.S. Pat. No. 5,599, 719; U.S. Pat. No. 5,578,709; U.S. Pat. No. 5,554,730; U.S. Pat. No. 6,090,925; U.S. Pat. No. 5,981,719; U.S. Pat. No. 6,458,387, each of which is incorporated herein by reference).

Where the liquid formulation is lyophilized prior to use in the delivery methods of  
25 the invention, the lyophilized composition may be milled to obtain the finely divided dry powder consisting of particles within the desired size range noted above. Where spray-drying is used to obtain a dry powder form of the liquid formulation, the process is carried out under conditions that result in a substantially amorphous finely divided dry powder consisting of particles within the desired size range noted above. Similarly, if the  
30 starting formulation is already in a lyophilized form, the composition can be milled to obtain the dry powder form for subsequent preparation as an aerosol or other preparation suitable for pulmonary inhalation. Where the starting formulation is in its spray-dried form, the composition has preferably been prepared such that it is already in a dry powder form having the appropriate particle size for dispensing as an aqueous or nonaqueous

solution or suspension or dry powder form in accordance with the pulmonary administration methods of the invention. For methods of preparing dry powder forms of formulations, see, for example, WO 96/32149, WO 97/41833, WO 98/29096, and U.S. Pat. Nos. 5,976,574, 5,985,248, and 6,001,336; herein incorporated by reference. The

5 resulting dry powder form of the composition is then placed within an appropriate delivery device for subsequent preparation as an aerosol or other suitable preparation that is delivered to the subject via pulmonary inhalation. Where the dry powder form of the formulation is to be prepared and dispensed as an aqueous or non- aqueous solution or suspension, a metered-dose inhaler, or other appropriate delivery device is used. A

10 pharmaceutically effective amount of the dry powder form of the composition is administered in an aerosol or other preparation suitable for pulmonary inhalation. The amount of dry powder form of the composition placed within the delivery device is sufficient to allow for delivery of a pharmaceutically effective amount of the composition to the subject by inhalation. Thus, the amount of dry powder form to be placed in the

15 delivery device will compensate for possible losses to the device during storage and delivery of the dry powder form of the composition. Following placement of the dry powder form within a delivery device, the properly sized particles as noted above are suspended in an aerosol propellant. The pressurized nonaqueous suspension is then released from the delivery device into the air passage of the subject while inhaling. The

20 delivery device delivers, in a single or multiple fractional dose, by pulmonary inhalation a pharmaceutically effective amount of the composition to the subject's lungs. The aerosol propellant may be any conventional material employed for this purpose, such as a chlorofluorocarbon, a hydrochloro- fluorocarbon, a hydrofluorocarbon, or a hydrocarbon, including trichlorofluoromethane, dichlorodifluoro-methane, dichlorotetrafluoromethane, dichlorodifluoro-methane, dichlorotetrafluoroethanol, and 1,1,1,2-tetra-fluoroethane, or

25 combinations thereof. A surfactant may be added to the formulation to reduce adhesion of the protein-containing dry powder to the walls of the delivery device from which the aerosol is dispensed. Suitable surfactants for this intended use include, but are not limited to, sorbitan trioleate, soya lecithin, and oleic acid. Devices suitable for pulmonary

30 delivery of a dry powder form of a protein composition as a nonaqueous suspension are commercially available. Examples of such devices include the Ventolin metered-dose inhaler (Glaxo Inc., Research Triangle Park, N.C.) and the Intal Inhaler (Fisons, Corp., Bedford, Mass.). See also the aerosol delivery devices described in U.S. Pat. Nos. 5,522,378, 5,775,320, 5,934,272 and 5,960,792, herein incorporated by reference. Where

the solid or dry powder form of the formulation is to be delivered as a dry powder form, a dry powder inhaler or other appropriate delivery device is preferably used. The dry powder form of the formulation is preferably prepared as a dry powder aerosol by dispersion in a flowing air or other physiologically acceptable gas stream in a conventional manner. Examples of dry powder inhalers suitable for use in accordance with the methods herein are described above.

When a formulation comprising a ligand conjugated oligonucleotide is processed into a solid or dry powder form for subsequent delivery as an aerosol, it may be desirable to have carrier materials present that serve as a bulking agent or stabilizing agent. In this manner, the present invention discloses stabilized lyophilized or spray-dried formulations comprising ligand conjugated oligonucleotide for use in the methods of the present invention. These compositions may further comprise at least one bulking agent, at least one agent in an amount sufficient to stabilize the protein during the drying process, or both. By "stabilized" is intended the ligand conjugated oligonucleotide thereof retains its monomeric or multimeric form as well as its other key properties of quality, purity, and potency following lyophilization or spray-drying to obtain the solid or dry powder form of the composition.

Preferred carrier materials for use as a bulking agent include glycine, mannitol, alanine, valine, or any combination thereof, most preferably glycine. The bulking agent is present in the formulation in the range of 0% to about 10% (w/v), depending upon the agent used. When the bulking agent is glycine, it is present in the range of about 0% to about 4%, preferably about 0.25% to about 3.5%, more preferably about 0.5% to 3.0%, even more preferably about 1.0% to about 2.5%, most preferably about 2.0%. When the bulking agent is mannitol, it is present in the range of about 0% to about 5.0%, preferably about 1.0% to about 4.5%, more preferably about 2.0% to about 4.0%, most preferably about 4.0%. When the bulking agent is alanine or valine, it is present in the range of about 0% to about 5.0%, preferably about 1.0% to about 4.0%, more preferably about 1.5% to about 3.0%, most preferably about 2.0%.

Preferred carrier materials for use as a stabilizing agent include any sugar or sugar alcohol or any amino acid. Preferred sugars include sucrose, trehalose, raffinose, stachyose, sorbitol, glucose, lactose, dextrose or any combination thereof, preferably sucrose. When the stabilizing agent is a sugar, it is present in the range of about 0% to about 9.0% (w/v), preferably about 0.5% to about 5.0%, more preferably about 1.0% to about 3.0%, most preferably about 1.0%. When the stabilizing agent is an amino acid, it is

present in the range of about 0% to about 1.0% (w/v), preferably about 0.3% to about 0.7%, most preferably about 0.5%. These stabilized lyophilized or spray-dried compositions may optionally comprise methionine, ethylenediaminetetracetic acid (EDTA) or one of its salts such as disodium EDTA or other chelating agent, which  
5 protect ligand conjugated oligonucleotide against methionine oxidation. Methionine is present in the stabilized lyophilized or spray-dried formulations at a concentration of about 0 to about 10.0 mM, preferably about 1.0 to about 9.0 mM, more preferably about 2.0 to about 8.0 mM, even more preferably about 3.0 to about 7.0 mM, still more preferably about 4.0 to about 6.0 mM, most preferably about 5.0 mM. EDTA is present at  
10 a concentration of about 0 to about 10.0 mM, preferably about 0.2 mM to about 8.0 mM, more preferably about 0.5 mM to about 6.0 mM, even more preferably about 0.7 mM to about 4.0 mM, still more preferably about 0.8 mM to about 3.0 mM, even more preferably about 0.9 mM to about 2.0 mM, most preferably about 1.0 mM.

The composition of the invention can be formulated with addition ingredients.

15 These can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel™, or corn starch; a lubricant such as magnesium stearate or Sterotes™; a  
glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin;  
20 or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring. When the dosage unit form is a capsule, it can contain, in addition to material of the above type, a liquid carrier. In addition, dosage unit forms can contain various other materials which modify the physical form of the dosage unit, for example, coatings of sugar, shellac, or other enteric agents.

25 The stabilized lyophilized or spray-dried compositions may be formulated using a buffering agent, which maintains the pH of the formulation within an acceptable range when in a liquid phase, such as during the formulation process or following reconstitution of the dried form of the composition. In some embodiments the pH is in the range of about pH 4.0 to about pH 8.5, about pH 4.5 to about pH 7.5, about pH 5.0 to about pH  
30 6.5, about pH 5.6 to about pH 6.3, and about pH 5.7 to about pH 6.2. Suitable pH's include about 4.0, about 4.5, about 5.0, about 5.1, about 5.2, about 5.3, about 5.4, about 5.5, about 5.6, about 5.7, about 5.8, about 5.9, about 6.0, about 6.1, about 6.2, about 6.3, about 6.4, about 6.5, about 6.6, about 6.7, about 6.8, about 6.9, about 7.0, about 7.1, about 7.2, about 7.3, about 7.4, about 7.5, about 7.7, about 7.8, about 7.9, about 8.0, about 8.2,



about 8.4, about 8.6, about 8.8, about 9.0. In one particular embodiment, the pH is about 7.0 to 8.2. Suitable buffering agents include, but are not limited to, citrate buffer, phosphate buffer, succinate buffer, more particularly a sodium citrate/citric acid.

Alternatively imidazole or histidine or other base/acid that maintains pH in the range of about pH 4.0 to about 8.5 can be used. Buffers are chosen such that they are compatible with the drying process and do not affect the quality, purity, potency, and stability of the protein during processing and upon storage.

Any of the formulations comprising human ligand conjugated oligonucleotide contemplated for use in the methods of the invention may be formulated with at least one surfactant. For pulmonary intracellular administration of the ligand conjugated oligonucleotide, the surfactant can be in an amount sufficient to enhance absorption of the inhaled particles comprising ligand conjugated oligonucleotide to obtain an absorbable composition for use in pulmonary inhalation in accordance with the methods described herein. Any surfactant that enhances absorption of a formulation comprising ligand conjugated oligonucleotide thereof in the manner disclosed herein may be used to obtain these absorbable protein-containing formulations. Surfactants suitable for use in enhancing absorption of the inhaled ligand conjugated oligonucleotide include, but are not limited to, polyoxyethylene sorbitol esters such as polysorbate 80 (Tween 80) and polysorbate 20 (Tween 20); polyoxypropylene- polyoxyethylene esters such as Poloxamer 188; polyoxyethylene alcohols such as Brij 35; a mixture of polysorbate surfactants with phospholipids such as phosphatidylcholine and derivatives (dipalmitoyl, dioleoyl, dimyristyl, or mixed derivatives such as 1-palmitoyl, 2-olcoyl, etc.), dimyristolglycerol and other members of the phospholipid glycerol series; lysophosphatidylcholine and derivatives thereof; mixtures of polysorbates with lysolecithin or cholesterol; a mixture of polysorbate surfactants with sorbitan surfactants (such as sorbitan monoleate, dioleate, trioleate or others from this class); poloxamer surfactants; bile salts and their derivatives such as sodium cholate, sodium deoxycholate, sodium glycodeoxycholate, sodium taurocholate, etc.; mixed micelles of ligand conjugated oligonucleotide with bile salts and phospholipids; Brij surfactants (such as Brij 35- PEG923) lauryl alcohol, etc.). The amount of surfactant to be added is in the range of about 0.005% to about 1.0% (w/v), preferably about 0.005% to about 0.5%, more preferably about 0.01% to about 0.4%, even more preferably about 0.03% to about 0.3%, most preferably about 0.05% to about 0.2%.

The formulation of the invention may include a suitable dosage according to the disorder being treated. In one embodiment, the formulation of the invention comprises a dose of about 0.01 mg to 10 mg of ligand conjugated oligonucleotide. Alternatively, the formulation of the invention comprises a dose of about 0.1 mg to 5 mg; about 1 mg to 5 mg; about 2.5 mg to 5 mg, about 2.0 to 4.5 mg, about 2.2 to 4.0 mg, about 2.0 to 3.0 mg, about 2.2 to 3.0 mg, about 2.3 to 3.0 mg, about 2.4 to 2.8 mg, about 2.4 to 2.6 mg; or about 2.5 mg of the ligand conjugated oligonucleotide or enzymatically active portion thereof. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

Thus, in some embodiments, the dosage regimen includes, but is not limited to a single dose of the dry powder formulation of the invention, of 1.0 to 10 mg ligand conjugated oligonucleotide, administered daily, a single dose of 2.0 to 5 mg ligand conjugated oligonucleotide, administered daily, a single dose of 2.0- 3.0 mg ligand conjugated oligonucleotide, administered daily, a plurality of doses, each dose comprising 1.0-3.0 mg ligand conjugated oligonucleotide, the doses administered at least twice, 2-3 times, 2-4 times or 2-6 times daily, a plurality of doses, each dose comprising 1.0-3.0 mg ligand conjugated oligonucleotide, the doses administered once every 36 hours, once every 36-48 hours, once every 36-72 hours, once every 2-3 days, once every 2-4 days, once every 2-5 days, or once every week, a plurality of doses, each dose comprising 1.0-3.0 mg ligand conjugated oligonucleotide, the doses administered once every 36 hours, once every 36-48 hours, once every 36-72 hours, once every 2-3 days, once every 2-4 days, once every 2-5 days, or once every week.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The formulation can be formulated as a solution, microemulsion, dispersion, liposome, or other ordered structure suitable to high drug concentration before processing into a dry powder. Sterile inhalable solutions can be prepared by incorporating the active compound (i.e., siRNA, antisense, microRNA, shRNA) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those

enumerated above. The proper fluidity of a solution can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prolonged action of inhalable compositions can be brought about by including in the composition an agent that delays  
5 absorption, for example, monostearate salts and gelatin.

In one embodiment, the ligand conjugated oligonucleotide or active portion for use in the methods of the invention is incorporated into a pharmaceutical formulation as described in Examples 2- 5. Supplementary active compounds can also be incorporated into the compositions for pulmonary delivery. In certain embodiments, a ligand  
10 conjugated oligonucleotide or active portion for use in the methods of the invention is coformulated with and/or coadministered with one or more additional therapeutic agents mentioned hereinabove. For example, ligand conjugated oligonucleotide may be coformulated and/or coadministered with one or more additional compositions that reduce actin inhibition (e.g. magnesium or potassium salts), and/or one or more chemical agents  
15 that inhibit mucus production (such as antiinflammatory agents, bronchodilators and/or mucus secretion blockers, as described in US 7,763,610) or any combination thereof. Furthermore, the ligand conjugated oligonucleotide of the invention may be used in combination with two or more of the foregoing therapeutic agents. Such combination therapies may advantageously utilize lower dosages of the administered therapeutic  
20 agents, thus avoiding possible side effects, complications or low level of response by the patient associated with the various monotherapies.

The formulations of the invention may include a "therapeutically effective amount" or a "prophylactically effective amount" of a ligand conjugated oligonucleotide. A "therapeutically effective amount" refers to an amount effective, at dosages and for  
25 periods of time necessary, to achieve the desired therapeutic result. A therapeutically effective amount of the ligand conjugated oligonucleotide may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the ligand conjugated oligonucleotide or active portion thereof to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or  
30 detrimental effects of the ligand conjugated oligonucleotide are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically, since a prophylactic dose is used in subjects prior to or at

an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

The invention also pertains to packaged formulations or kits for pulmonary administration of a ligand conjugated oligonucleotide, e.g., conjugated siRNA. In one embodiment of the invention, the kit comprises a ligand conjugated oligonucleotide, such as conjugated siRNA, and instructions for pulmonary administration of the ligand conjugated oligonucleotide, wherein the ligand conjugated oligonucleotide is in a dry powder formulation suitable for inhalation. The instructions may describe when, e.g., at day 1, day 4, week 0, week 2, week 4, etc., the different doses of ligand conjugated oligonucleotide shall be administered via inhalation to a subject for treatment.

Another aspect of the invention pertains to kits containing a dry powder formulation comprising a ligand conjugated oligonucleotide, such as conjugated siRNA, and a pharmaceutically acceptable carrier, and one or more formulations each comprising an additional therapeutic agent, and a pharmaceutically acceptable carrier.

The package or kit alternatively can contain the ligand conjugated oligonucleotide and it can be promoted for use, either within the package or through accompanying information, for the uses or treatment of the disorders described herein. The packaged formulations or kits further can include a second agent (as described herein) packaged with or copromoted with instructions for using the second agent with a first agent (as described herein).

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures in vitro, in cell cultures or experimental animals. The data obtained from these in vitro and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl, et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p. 1).

Dosage amount and interval may be adjusted individually, for example, to provide serum and cell levels of the active ingredient which are sufficient to induce or suppress the biological effect (minimal effective concentration, MEC). The MEC will vary for each preparation, but can be estimated from in vitro data. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. Detection assays can be used to determine plasma concentrations.

Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

5           The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

          Compositions of some embodiments of the invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or  
10           more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which  
15           notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions comprising a preparation of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate  
20           container, and labeled for treatment of an indicated condition, as is further detailed above.

          The invention is further illustrated by the following examples, which should not be construed as further limiting. The contents of all references, pending patent applications and published patents, cited throughout this application are hereby expressly incorporated by reference.

25

## EXAMPLES

### **Example 1. RNA Synthesis and Duplex Annealing**

#### **1. Oligonucleotide Synthesis:**

5 All oligonucleotides were synthesized on an AKTAoligopilot synthesizer or an ABI 394 synthesizer. Commercially available controlled pore glass solid support (dT-CPG, 500Å, Prime Synthesis) and RNA phosphoramidites with standard protecting groups, 5'-*O*-dimethoxytrityl N<sup>6</sup>-benzoyl-2'-*t*-butyldimethylsilyl-adenosine-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite, 5'-*O*-dimethoxytrityl-N<sup>4</sup>-acetyl-2'-*t*-butyldimethylsilyl-cytidine-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite, 5'-*O*-dimethoxytrityl-N<sup>2</sup>-isobutryl-2'-*t*-butyldimethylsilyl-guanosine-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite, and 5'-*O*-dimethoxytrityl-2'-*t*-butyldimethylsilyl-uridine-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite (Pierce Nucleic Acids Technologies) were used for the oligonucleotide synthesis unless otherwise specified. The 15 2'-F phosphoramidites, 5'-*O*-dimethoxytrityl-N<sup>4</sup>-acetyl-2'-fluro-cytidine-3'-*O*-N,N'-diisopropyl-2-cyanoethyl-phosphoramidite and 5'-*O*-dimethoxytrityl-2'-fluro-uridine-3'-*O*-N,N'-diisopropyl-2-cyanoethyl-phosphoramidite were purchased from (Promega). All phosphoramidites were used at a concentration of 0.2M in acetonitrile (CH<sub>3</sub>CN) except for guanosine which was used at 0.2M concentration in 10% THF/ANC (v/v).

20 Coupling/recycling time of 16 minutes was used. The activator was 5-ethyl thiotetrazole (0.75M, American International Chemicals), for the PO-oxidation Iodine/Water/Pyridine was used and the PS-oxidation PADS (2 %) in 2,6-lutidine/ACN (1:1 v/v) was used. .

Ligand conjugated strands were synthesized using solid support containing the corresponding ligand. For example, the introduction of carbohydrate moiety/ligand (for 25 e.g., GalNAc) at the 3'-end of a sequence was achieved by starting the synthesis with the corresponding carbohydrate solid support. Similarly a cholesterol moiety at the 3'-end was introduced by starting the synthesis on the cholesterol support. In general, the ligand moiety was tethered to *trans*-4-hydroxyprolinol via a tether of choice as described in the previous examples to obtain a hydroxyprolinol-ligand moiety. The hydroxyprolinol-30 ligand moiety was then coupled to a solid support via a succinate linker or was converted to phosphoramidite via standard phosphitylation conditions to obtain the desired carbohydrate conjugate building blocks. See Examples 1-11 for details. Fluorophore labeled siRNAs were synthesized from the corresponding phosphoramidite or solid support, purchased from Biosearch Technologies. The oleyl lithocholic (GalNAc)<sub>3</sub>

polymer support made in house at a loading of 38.6  $\mu\text{mol}/\text{gram}$ . The Mannose (Man)<sub>3</sub> polymer support was also made in house at a loading of 42.0  $\mu\text{mol}/\text{gram}$ .

Conjugation of the ligand of choice at desired position, for example at the 5'-end of the sequence, was achieved by coupling of the corresponding phosphoramidite to the growing chain under standard phosphoramidite coupling conditions unless otherwise specified. An extended 15 min coupling of 0.1M solution of phosphoramidite in anhydrous CH<sub>3</sub>CN in the presence of 5-(ethylthio)-1*H*-tetrazole activator to a solid bound oligonucleotide. Oxidation of the internucleotide phosphite to the phosphate was carried out using standard iodine-water as reported (1) or by treatment with *tert*-butyl hydroperoxide/acetonitrile/water (10: 87: 3) with 10 min oxidation wait time conjugated oligonucleotide. Phosphorothioate was introduced by the oxidation of phosphite to phosphorothioate by using a sulfur transfer reagent such as DDTT (purchased from AM Chemicals), PADS and or Beaucage reagent The cholesterol phosphoramidite was synthesized in house, and used at a concentration of 0.1 M in dichloromethane. Coupling time for the cholesterol phosphoramidite was 16 minutes.

## 2. Deprotection- I (Nucleobase Deprotection)

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

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

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

## 4. Analysis

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

## 5. HPLC Purification

The ligand conjugated oligonucleotides were purified reverse phase preparative HPLC. The unconjugated oligonucleotides were purified by anion-exchange HPLC on a TSK gel column packed in house. The buffers were 20 mM sodium phosphate (pH 8.5) in 10% CH<sub>3</sub>CN (buffer A) and 20 mM sodium phosphate (pH 8.5) in 10% CH<sub>3</sub>CN, 1M NaBr (buffer B). Fractions containing full-length oligonucleotides were pooled, desalted, and lyophilized. Approximately 0.15 OD of desalted oligonucleotides were diluted in water to 150 µl and then pipetted in special vials for CGE and LC/MS analysis. Compounds were finally analyzed by LC-ESMS and CGE.

## 6. siRNA preparation

For the preparation of siRNA, equimolar amounts of sense and antisense strand were heated in 1xPBS at 95°C for 5 min and slowly cooled to room temperature. Integrity of the duplex was confirmed by HPLC analysis.

The siRNA and conjugate preparation were prepared according to the protocols disclosed in PCT/US2012/065601 and PCT/US2008/085577.

### Example 2. Evaluating Microsprayer Delivery of GalNAc Conjugates *In Vivo*

Experimental design

- Two rodent specific GalNAc-ESC conjugates

» TTR: AD-57727

strand	Seq 5'-3'
sense	AfsasCfaGfuGfuUfCfUfuGfcUfcUfaUfaAfL96
antis	usUfsaUfaGfaGfcAfagaAfcAfcUfgUfususu

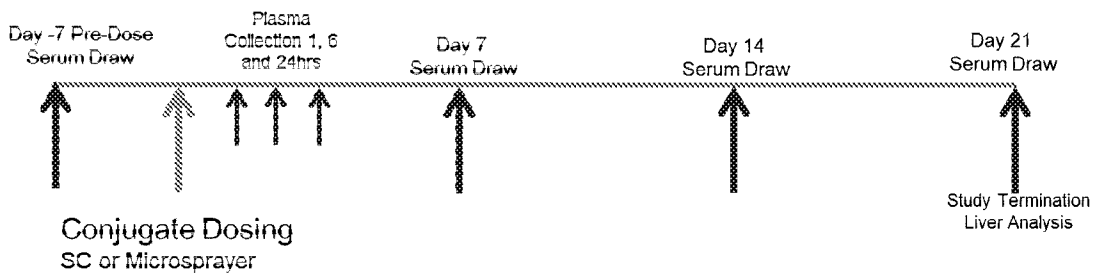
» FactorVII: AD-60347

strand	Seq 5'-3'
S	CfsasGfgAfuCfaUfCfUfcAfaGfuCfuUfaCfL96
AS	gsUfsaAfgAfcUfuGfagaUfgAfuCfcUfgsgsc

Lowercase nucleotides (a, u, g, c) are 2'-O-methyl nucleotides; Nf (e.g., Af) is a 2'-fluoro nucleotide; s is a phosphothiorate linkage; L96 indicates a GalNAc<sub>3</sub> ligand.



- Dose response: 0.3, 1.0 and 3.0 mg/kg
- CD57 females; N=4
- Endpoints (all analysis at Alnylam):
  - » Serum TTR or Factor VII levels @ day -7 (pre-dose), 7 d, 14 d, 21 d
  - » Plasma siRNA] @ 1hr, 6hr, 24hr
  - » Liver mRNA] and siRNA] at harvest (day 21)



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Potent GalNAC conjugated siRNAs targeting transthyretin (TTR) and Factor VII (FVII) were selected for efficacy evaluation following lung delivery by Microsprayer. GalNac conjugated duplexes were either injected subcutaneously or delivered via Microsprayer into C57BL/6 mice (N=4 per group) at dose levels 3, 1 or .3mg/kg or with an equal volume of 1x Dulbecco's Phosphate-Buffered Saline (DPBS) (Life Technologies, Cat# 14040133). Plasma was collected 1, 6, 24 hrs post dose and analyzed by qPCR. Sera was collected pre-dose (day -7), day 7, day 14 and day 21. Circulating FVII activity levels were determined utilizing a Biophen FVII chromogenic assay from Aniara (Cat #A221304). Circulating TTR levels were determined with an ELISA kit acquired from Alpco Diagnostics (Cat #41-PALMS-E01). Livers were snap frozen at Day 21 for mRNA and siRNA analysis.

Figures 1,2 and 3 show that Microsprayer dosing leads to comparable silencing observed with SC administration at the dose levels examined. ESC GalNac-siRNA conjugates show comparable efficacy and duration in mouse liver when administered by Microsprayer®-mediated intra-tracheal delivery via lung to that observed with SC administration. The data support that efficient systemic exposure of GalNac-siRNA conjugates and delivery to liver can be achieved via lung.

**Example 3. Nebulization of ligand conjugated siRNA with Pari eFlow® device*****Droplet size and analytical integrity***

**Methods:** A 150 mg/ml solution of ligand conjugated siRNA (in 2 mls of PBS) is filled into the Pari eFlow® electronic device and run until nebulization is completed and all aerosol is collected and allowed to condense in a polypropylene tube. Aliquots of material post nebulization are analyzed to determine geometric droplet size distribution by laser diffraction (Malvern MasterSizerX) under standard conditions. Aliquots of material pre and post nebulization are analyzed to determine analytical integrity by a stability using anion exchange HPLC methodology.

**Biological Activity:** A 25 mg/ml solution of ligand conjugated siRNA (in 1 ml of PBS) is prepared, 100 µl is removed (pre-nebulization aliquot) prior to nebulization with the Pari eFlow® electronic device, and 500 µl of the nebulized solution is collected after condensing by passage over an ice bath into a chilled 50 ml conical tube (post-nebulization aliquot). Serial dilutions of both aliquots are tested in our *in vitro* transfection/infection plaque assay as previously described with the exception that siRNA is complexed with lipofetamine-2000.

**Example 4. Inhalable siRNAs: ligand conjugated siRNA**

To investigate the *in vivo* effects of aerosolization and delivery by inhalation of siRNAs targeting a target gene as well as the pharmacokinetic properties of inhaled siRNAs, a double-blind, randomized, placebo-controlled, evaluation study in human adult subjects is performed. The study measured routine bloods and clinical observations, inflammatory biomarkers, tolerability and plasma pharmacokinetics. As used in this specification “inhalation” refers to administration of a dosage form that is formulated and delivered for topical treatment of the pulmonary epithelium. As described above, an inhalable dosage form comprise particles of respirable size, i.e., particles that are sufficiently small to pass through the mouth or nose and larynx upon inhalation and into the bronchi and alveoli of the lungs.

In the study, ascending doses of aerosolized ligand conjugated siRNA or placebo are administered once daily by inhalation for 3 consecutive days to 4 cohorts of 12 subjects each with 8 subjects receiving ligand conjugated siRNA and 4 subjects receiving placebo in each cohort for a total of 48 subjects. Ligand conjugated siRNA maximum solubility concentration in the finished product is 150 mg/mL. Therefore, a 150 mg/ml solution of

ligand conjugated siRNA is diluted to the appropriate concentration and filled into the Pari eFlow® electronic device and run until nebulization is completed.

Blood samples evaluated for pharmacokinetics (PK) included pre dose and post dose at 2, 5, 15, and 30 minutes, 1 hour and 24 hours on Day 0 and post third dose at 2, 5, 15, and 30 minutes, 1 hour and 24 hours after the third dose (13 samples per subject). Urine collection for PK included: pre dose and post third dose at 0-6 hours, 6-12 hours and 12-24 hours.

Plasma ligand conjugated siRNA concentrations, and derived parameters ( $C_{pre}$ ,  $C_{max}$ ,  $t_{max}$ ,  $t_{1/2}$ ,  $CL/F$ ,  $V_d/F$ ,  $AUC_{last}$ ) are evaluated for PK.

ligand conjugated siRNA has previously been evaluated for toxicity by inhalation administration in rats and monkeys at doses as high as 36 mg/kg/day and 30 mg/kg/day, respectively. The highest dose to be administered in the single dose part of the current study is 210 mg/day (or 3 mg/kg, assuming 70 kg body weight). On a mg/kg basis, this dose is approximately 10 fold lower than the doses given previously to rats and monkeys. The initial doses in this study are 7.0 mg, 21.0 mg and 70.0 mg providing a safety margin of about 300 fold, 100-fold and 30 fold, respectively.

Dose levels for the multiple dose part of the study are 7.0 mg, 21.0 mg, 70.0 mg and 210 mg, given as a daily delivered dose (DD) for three consecutive days.

The highest dose to be administered in the single dose part of the current study is chosen at 210 mg/day (or 3 mg/kg, assuming 70 kg body weight).

Study drug exposure duration in the multiple dose part of the study is chosen to be 3 days, with once daily dosing, based on the intended therapeutic dosing duration which is likely to be short due to the acute nature of RSV infections.

#### Pulmonary Function Tests

PFT are conducted at screening to identify healthy volunteers with respect to capacities and flow-rates. PFT provides an objective method for assessing the mechanical and functional properties of the lungs and chest wall. PFT measures:

Lung capacities *e.g.*, Slow Vital Capacity (SVC) and Force Vital Capacity (FVC), which provide a measurement of the size of the various compartments within the lung

Volume parameters (*e.g.*, FEV1) and flow-rates (*e.g.*, FEF25-75), which measure maximal flow within the airways

Serial evaluation of pulmonary function after inhalation of ligand conjugated siRNA or placebo are conducted. Additional PFT testing is conducted on Day 0 at pre-dose (about -

30 min) and at 30 min and 2 h, 6 h, and 12 h on Days 1, 1 and 2 at the same time as pre-dose on Day 0.

PFT provides lung capacities and flow-rates. The SVC is the volume of gas slowly inhaled when going from complete expiration to complete inhalation. The FVC is the volume expired when going from complete inhalation to complete exhalation as hard and fast as possible. The FEV1 is the amount expired during the first second of the FVC maneuver. The Forced Expiratory Flow (FEF25-75) is the average expiratory flow over the middle half of the FVC. SVC, FVC, FEV1 and FEF25-75 is measured according the ATS/ERS guidelines. In this study, FEV1 is the main parameter.

As shown in Figure 16, no significant change in lung function is seen on aerosol administration of ligand conjugated siRNA.

#### Plasma

For single dosing, blood samples are collected for the analysis of ligand conjugated siRNA in plasma at pre dose and post dose (post nebulization) at 2, 5, 15, and 30 minutes, 1 hour and 24 hours on Day 0 (7 samples per volunteer).

For multi-dosing, blood samples are collected for analysis of ligand conjugated siRNA in plasma at pre-dose and at 2, 5, 15 and 30 min, 1 h, and 24 h post first-dose on Day 0 (post nebulization), and at 2, 5, 15, 30 min, 1 h, and 24 h after the third dose (post dose nebulization of third dose).

Blood samples of 5 mL each are taken via an indwelling intravenous catheter or by direct venipuncture into tubes containing K3EDTA as the anticoagulant. In case of sampling through the intravenous catheter, the first 1 mL of blood is discarded in order to prevent any dilution of blood with heparin used to flush the catheter.

We claim:

1. An inhalable formulation comprising a ligand-conjugated oligonucleotide and particles of a physiologically acceptable pharmacologically-inert carrier.

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2. The inhalable formulation of claim 1, wherein said ligand-conjugated oligonucleotide is a multivalent N-Acetylgalactosamine conjugated oligonucleotide.

3. The inhalable formulation of claim 1, wherein said physiologically acceptable pharmacologically-inert carrier is a dry powder.

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4. The inhalable formulation of claim 3, wherein said dry powder carrier is selected from the group consisting of (a) at least one crystalline sugar selected from the group consisting of glucose, arabinose, maltose, saccharose, dextrose, and lactose; and (b) at least one polyalcohol selected from the group consisting of mannitol, maltitol, lactitol, and sorbitol.

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5. The inhalable formulation of claim 3, wherein said carrier is in a form of finely divided particles having a mass median diameter (MMD) in the range of 0.5 to 10 microns.

20

6. The inhalable formulation of claim 3, wherein said carrier is in a form of finely divided particles having a mass median diameter (MMD) in the range of 1.0 to 6.0 microns.

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7. The inhalable formulation of claim 3, wherein said carrier is in a form of coarse particles having a mass diameter of 50 to 500 microns.

8. The inhalable formulation of claim 7, wherein said coarse particles have a mass diameter of 150 to 400 microns.

30

9. The inhalable formulation of any one of claims 3-8, further comprising, as an active ingredient, a magnesium salt.

10. The inhalable formulation of claim 3, further comprising one or more additive materials selected from the group consisting of an amino acid, a water soluble surface active agent, a lubricant, and a glidant.

5 11. A dry powder inhaler device comprising the inhalable formulation of any one of claims 3-10 and a means for introducing the inhalable formulation into the airways of a subject by inhalation.

10 12. The dry powder inhaler device of claim 11, wherein said device is a single dose or a multidose inhaler.

13. The dry powder inhaler device of claim 11, wherein said device is pre-metered or device-metered.

15 14. The inhalable formulation of claim 1, wherein said physiologically acceptable pharmacologically-inert carrier is an inert liquid carrier.

20 15. The inhalable formulation of claim 14, wherein said liquid carrier is selected from the group consisting of water, an aqueous alcoholic solution, perfluorocarbon and saline.

16. The inhalable formulation of claim 14, further comprising, as an active ingredient, a magnesium salt.

25 17. The inhalable formulation of claim 14, further comprising one or more additive materials selected from the group consisting of a surfactant, a mucolytic agent, an adsorption enhancer, and a lubricant.

30 18. The inhalable formulation of claim 14, wherein said ligand-conjugated oligonucleotide is formulated in liposomes.

19. A liquid inhaler device, comprising the inhalable formulation of any one of claims 14-18 and a means for introducing the inhalable formulation into the airways of a subject by inhalation.

20. The liquid inhaler device of claim 19, wherein said device is a single dose or a multidose inhaler.

5           21. The liquid inhaler device of claim 19, wherein said device is pre-metered or device-metered.

22. The liquid inhaler device of claim 19, wherein said device is a metered dose inhaler or a nebulizer.

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23. The liquid inhaler device of claim 19, wherein said inhalable formulation is provided for inhalation in particles ranging from about 1 to 10 microns in size.

15           24. The liquid inhaler device of claim 19, wherein said inhalable formulation is provided for inhalation in particles ranging from about 2 to 5 microns in size.

25. The inhalable formulation of claim 1, wherein said oligonucleotide is selected from the group consisting of a siRNA, a shRNA, an antisense, and a miRNA.

20           26. A method for reducing or inhibiting the expression of an aberrant protein in a subject, comprising administering to the subject in need thereof an effective amount of the the inhalable formulation of any one of claims 1-10, 14-18, and 25.

25           27. The method of claim 26, wherein said subject is suffering from a disease or condition selected from the group consisting of male infertility, metastatic cancer, a viral, bacterial, fungal or protozoan infection, sepsis, atherosclerosis, diabetes, delayed type hypersensitivity, and a uterine disorder.

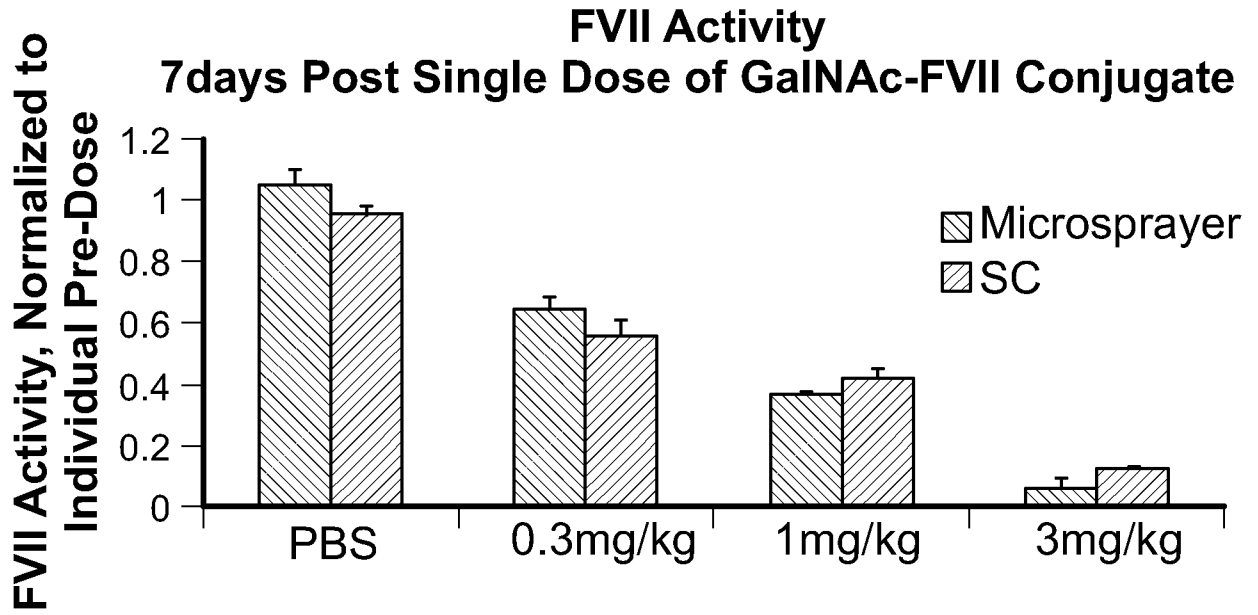


FIG. 1A

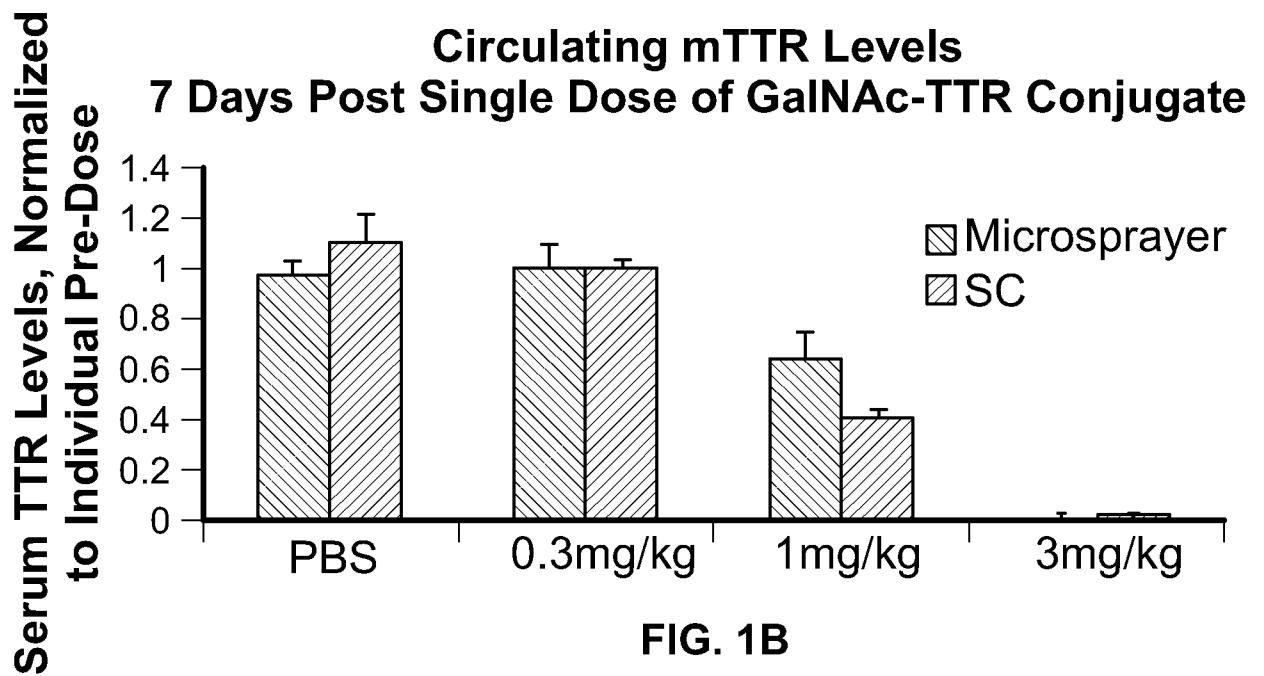
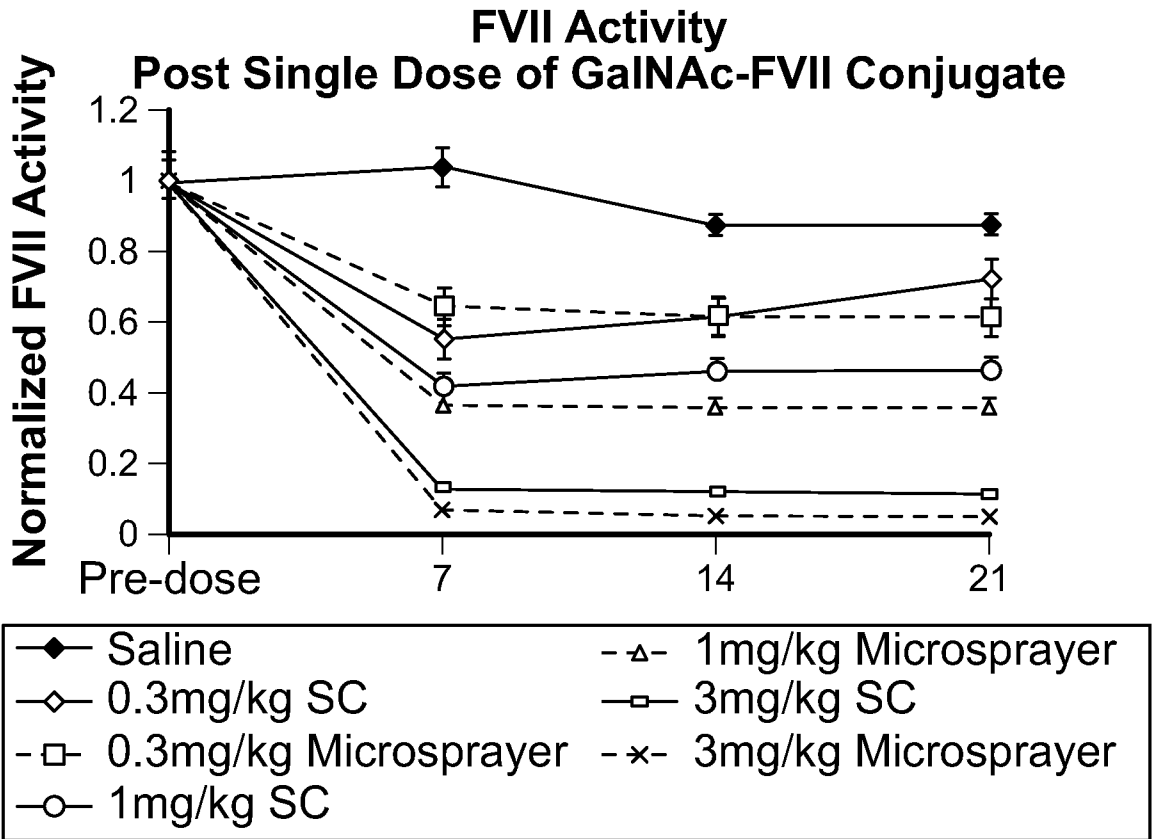
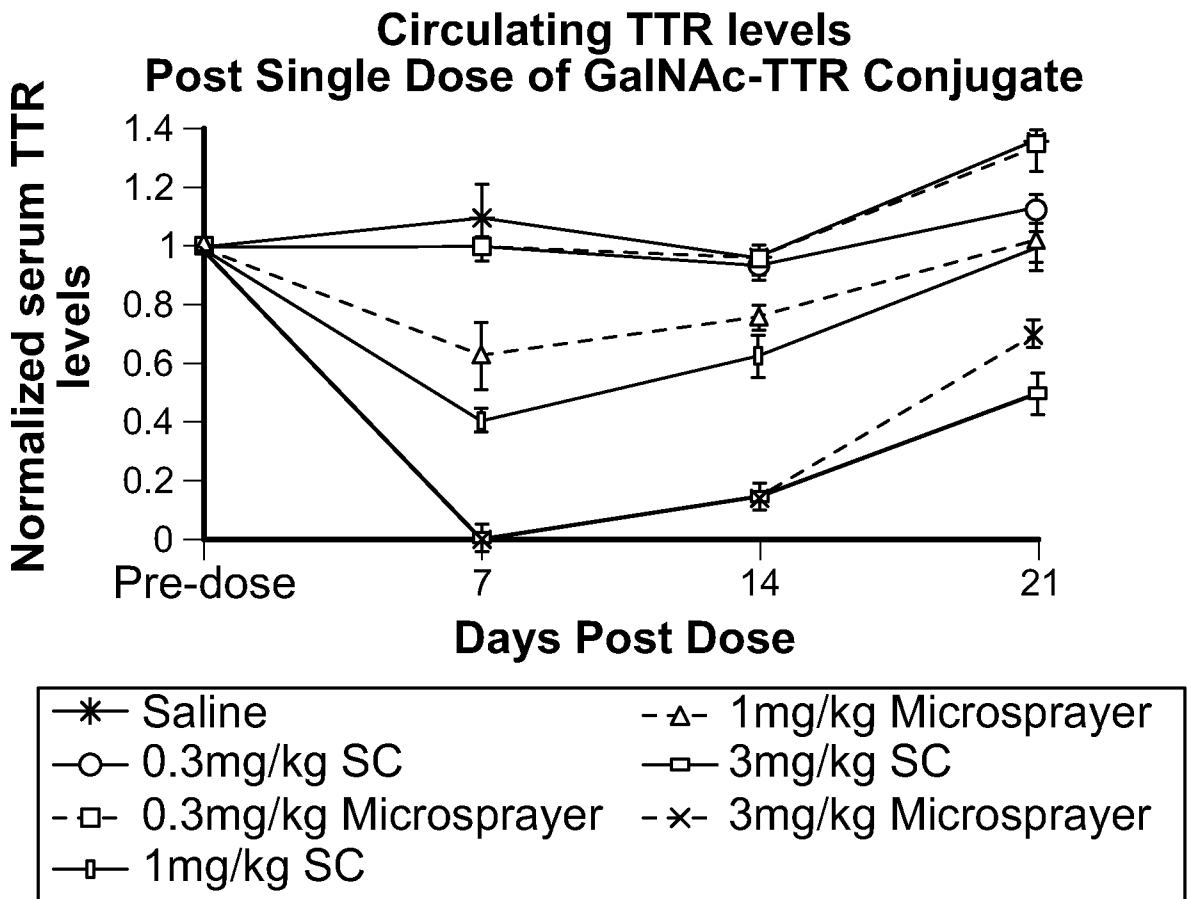


FIG. 1B

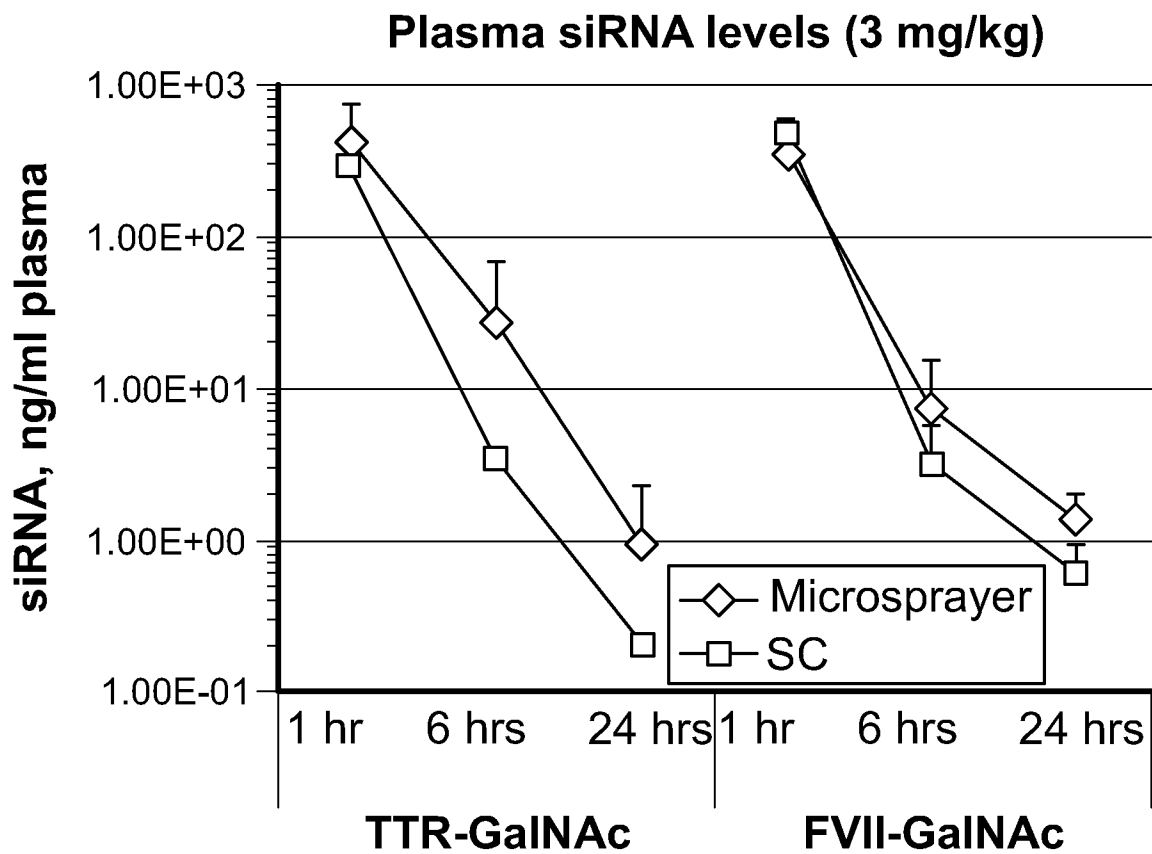
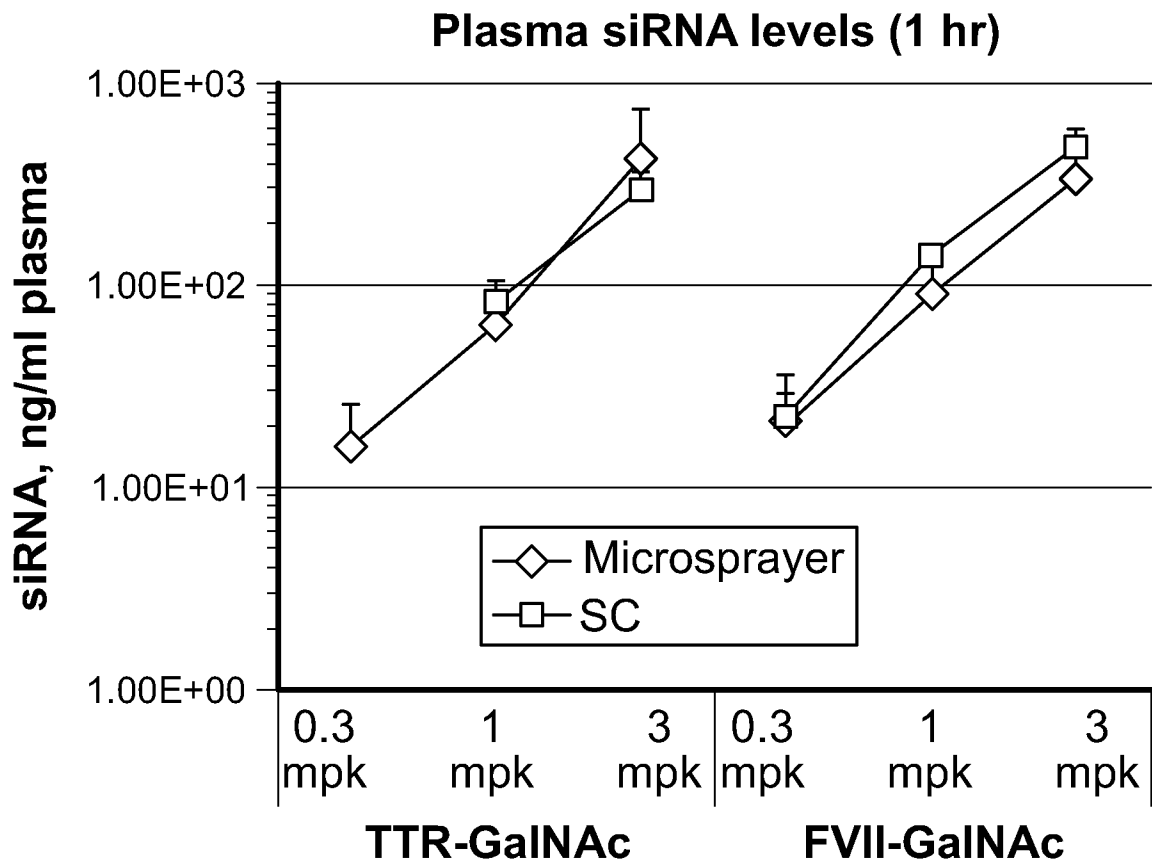




**FIG. 2A**



**FIG. 2B**



**FIG. 3**

**INTERNATIONAL SEARCH REPORT**

International application No  
PCT/US2015/054526

**A. CLASSIFICATION OF SUBJECT MATTER**  
 INV. A61K47/48 A61K31/713  
 ADD.  
 According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**  
 Minimum documentation searched (classification system followed by classification symbols)  
 A61K

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

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

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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X	WO 2014/089313 A1 (ALNYLAM PHARMACEUTICALS [US]) 12 June 2014 (2014-06-12) pages 56,64 -----	1-27
X	US 2013/273164 A1 (MINKO TAMARA [US] ET AL) 17 October 2013 (2013-10-17) paragraphs [0104], [0107], [0111] -----	1-27

Further documents are listed in the continuation of Box C.

See patent family annex.

\* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search

10 February 2016

Date of mailing of the international search report

22/02/2016

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No PCT/US2015/054526
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