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**(54) IMPROVED AMINO LIPIDS AND METHODS FOR THE DELIVERY OF NUCLEIC ACIDS**

VERBESSERTE AMINOLIPIDE UND VERFAHREN ZUR FREISETZUNG VON NUKLEINSÄUREN

LIPIDES AMINÉS AMÉLIORÉS ET PROCÉDÉS D'ADMINISTRATION D'ACIDES NUCLÉIQUES

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**Description**

## BACKGROUND

5 Technical Field

[0001] The present invention relates to the field of therapeutic agent delivery using lipid particles. In particular, the present invention provides cationic lipids and lipid particles comprising these lipids, which are advantageous for the *in vivo* delivery of nucleic acids, as well as nucleic acid-lipid particle compositions suitable for *in vivo* therapeutic use. Additionally, the present invention provides methods of making these compositions, as well as relating to methods of introducing nucleic acids into cells using these compositions, *e.g.*, for the treatment of various disease conditions.

Description of the Related Art

15 [0002] Therapeutic nucleic acids include, *e.g.*, small interfering RNA (siRNA), micro RNA (miRNA), antisense oligonucleotides, ribozymes, plasmids, and immune stimulating nucleic acids. These nucleic acids act via a variety of mechanisms. In the case of siRNA or miRNA, these nucleic acids can down-regulate intracellular levels of specific proteins through a process termed RNA interference (RNAi). Following introduction of siRNA or miRNA into the cell cytoplasm, these double-stranded RNA constructs can bind to a protein termed RISC. The sense strand of the siRNA or miRNA is displaced from the RISC complex providing a template within RISC that can recognize and bind mRNA with a complementary sequence to that of the bound siRNA or miRNA. Having bound the complementary mRNA the RISC complex cleaves the mRNA and releases the cleaved strands. RNAi can provide down-regulation of specific proteins by targeting specific destruction of the corresponding mRNA that encodes for protein synthesis.

20 [0003] The therapeutic applications of RNAi are extremely broad, since siRNA and miRNA constructs can be synthesized with any nucleotide sequence directed against a target protein. To date, siRNA constructs have shown the ability to specifically down-regulate target proteins in both *in vitro* and *in vivo* models. In addition, siRNA constructs are currently being evaluated in clinical studies.

25 [0004] However, two problems currently faced by siRNA or miRNA constructs are, first, their susceptibility to nuclease digestion in plasma and, second, their limited ability to gain access to the intracellular compartment where they can bind RISC when administered systemically as the free siRNA or miRNA. These double-stranded constructs can be stabilized by incorporation of chemically modified nucleotide linkers within the molecule, for example, phosphothioate groups. However, these chemical modifications provide only limited protection from nuclease digestion and may decrease the activity of the construct. Intracellular delivery of siRNA or miRNA can be facilitated by use of carrier systems such as polymers, cationic liposomes or by chemical modification of the construct, for example by the covalent attachment of cholesterol molecules [reference]. However, improved delivery systems are required to increase the potency of siRNA and miRNA molecules and reduce or eliminate the requirement for chemical modification.

30 [0005] Antisense oligonucleotides and ribozymes can also inhibit mRNA translation into protein. In the case of antisense constructs, these single stranded deoxynucleic acids have a complementary sequence to that of the target protein mRNA and can bind to the mRNA by Watson-Crick base pairing. This binding either prevents translation of the target mRNA and/or triggers RNase H degradation of the mRNA transcripts. Consequently, antisense oligonucleotides have tremendous potential for specificity of action (*i.e.*, down-regulation of a specific disease-related protein). To date, these compounds have shown promise in several *in vitro* and *in vivo* models, including models of inflammatory disease, cancer, and HIV (reviewed in Agrawal, Trends in Biotech. 14:376-387 (1996)). Antisense can also affect cellular activity by hybridizing specifically with chromosomal DNA. Advanced human clinical assessments of several antisense drugs are currently underway. Targets for these drugs include the *bcl2* and apolipoprotein B genes and mRNA products.

35 [0006] Immune-stimulating nucleic acids include deoxyribonucleic acids and ribonucleic acids. In the case of deoxyribonucleic acids, certain sequences or motifs have been shown to illicit immune stimulation in mammals. These sequences or motifs include the CpG motif, pyrimidine-rich sequences and palindromic sequences. It is believed that the CpG motif in deoxyribonucleic acids is specifically recognized by an endosomal receptor, toll-like-Geceptor 9 (TLR-9), which then triggers both the innate and acquired immune stimulation pathway. Certain immune stimulating ribonucleic acid sequences have also been reported. It is believed that these RNA sequences trigger immune activation by binding to toll-like receptors 6 and 7 (TLR-6 and TLR-7). In addition, double-stranded RNA is also reported to be immune stimulating and is believe to activate via binding to TLR-3.

40 [0007] One well known problem with the use of therapeutic nucleic acids relates to the stability of the phosphodiester internucleotide linkage and the susceptibility of this linker to nucleases. The presence of exonucleases and endonucleases in serum results in the rapid digestion of nucleic acids possessing phosphodiester linkers and, hence, therapeutic nucleic acids can have very short half-lives in the presence of serum or within cells. (Zelphati, O., et al., Antisense. Res. Dev. 3:323-338 (1993); and Thierry, A.R., et al., pp147-161 in Gene Regulation: Biology of Antisense RNA and DNA

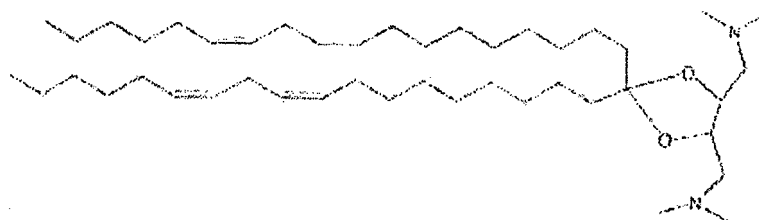


m, n, and p are either the same or different and independently either 0 or 1 with the proviso that m, n, and p are not simultaneously 0;

Y and Z are either the same or different and independently O, S, or NH.

[0014] In one embodiment, the amino lipid has the following structure:

[0015] In one embodiment, the amino lipid has the one of the following structures:



DLin-K<sup>2</sup>-DMA

or



DLin-K6-DMA.

[0016] In one embodiment, the present invention includes a lipid particle comprising an amino lipid according to any one of the above, wherein optionally and preferably, the particle further comprises a neutral lipid and a lipid capable of reducing particle aggregation; and wherein optionally and further preferably, wherein the lipid particle comprises:

- (i) DLin-K-C2-DMA;
- (ii) a neutral lipid selected from DSPC, POPC, DOPE, and SM;
- (iii) cholesterol; and
- (iv) a PEG-lipid,

in a molar ratio of about 20-60% DLin-K-DMA:5-25% neutral lipid :25-55% Chol:0.5-15% PEG-lipid.

[0017] In certain embodiments, the lipid particle further comprises a therapeutic agent; wherein optionally and preferably, the therapeutic agent is a nucleic acid; and wherein optionally and further preferably, the nucleic acid is a plasmid, or an immunostimulatory oligonucleotide.

[0018] In certain embodiments, the nucleic acid is selected from the group consisting of: a siRNA, a microRNA, an antisense oligonucleotide, and a ribozyme; optionally, wherein the nucleic acid is a siRNA.

[0019] In one embodiment, the present invention includes a pharmaceutical composition comprising the lipid particle of any one of the above and a pharmaceutically acceptable excipient, carrier, or diluent.

[0020] In one embodiment, the present invention provides an *in vitro* method of modulating the expression of a polypeptide by a cell, comprising providing to a cell the lipid particle of any one of the above; wherein optionally and preferably, the therapeutic agent is selected from an siRNA, a microRNA, an antisense oligonucleotide, and a plasmid capable of expressing an siRNA, a microRNA, or an antisense oligonucleotide, and wherein the siRNA, microRNA, or antisense RNA comprises a polynucleotide that specifically binds to a polynucleotide that encodes the polypeptide, or a complement thereof, such that the expression of the polypeptide is reduced; wherein optionally and further preferably, the therapeutic agent is a plasmid that encodes the polypeptide or a functional variant or fragment thereof, such that expression of the polypeptide or the functional variant or fragment thereof is increased.

[0021] In certain embodiments, the pharmaceutical composition is for use in a method of treating a disease or disorder characterized by over expression of a polypeptide in a subject, the method comprising providing the subject with the pharmaceutical composition, wherein the therapeutic agent is selected from an siRNA, a microRNA, an antisense oli-

gonucleotide, and a plasmid capable of expressing an siRNA, a microRNA, or an antisense oligonucleotide, and wherein the siRNA, microRNA, or antisense RNA comprises a polynucleotide that specifically binds to a polynucleotide that encodes the polypeptide, or a complement thereof.

**[0022]** In certain embodiments, the pharmaceutical composition is for use in a method of treating a disease or disorder characterized by underexpression of a polypeptide in a subject, wherein the therapeutic agent is a plasmid that encodes the polypeptide or a functional variant or fragment thereof.

In certain embodiments, the pharmaceutical composition is for use in a method of inducing an immune response in a subject, wherein the therapeutic agent is an immunostimulatory oligonucleotide.

In certain embodiments, the pharmaceutical composition for use as above, is provided to the patient in combination with a vaccine or antigen.

**[0023]** In one embodiment, the present invention includes a vaccine comprising a lipid particle as above and an antigen associated with a disease or pathogen.

**[0024]** In certain embodiments, said antigen is a tumor antigen.

**[0025]** In certain embodiments, said antigen is a viral antigen, a bacterial antigen, or a parasitic antigen.

## BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

### **[0026]**

Figure 1 is a diagram of a proposed mechanism of action for the membrane disruptive effects of cationic lipids and a structural diagram of DLinDMA divided into headgroup, linker and hydrocarbon chain domains. In isolation, cationic lipids and endosomal membrane anionic lipids such as phosphatidylserine adopt a cylindrical molecular shape, which is compatible with packing in a bilayer configuration. However, when cationic and anionic lipids are mixed together, they combine to form an ion pairs where the cross-sectional area of the combined headgroup is less than that of the sum of individual headgroup areas in isolation. The ion pair therefore adopts a molecular "cone" shape, which promotes the formation of inverted, non-bilayer phases such as the hexagonal H<sub>II</sub> phase illustrated. Inverted phases do not support bilayer structure and are associated with membrane fusion and membrane disruption (Hafez, I.M., et al., Gene Ther 8, 1188-1196 (2001) and Cullis, P.R., et al., Chem Phys Lipids 40, 127-144 (1986)).

Figures 2A-B are graphs depicting the *in vivo* silencing activity of nucleic acid-lipid particles comprising various cationic lipids. Figure 1A depicts the silencing activity of DLinDAP (▼), DLinDMA (▲), DLin-K-DMA (■) and DLin-K-C2-DMA (●) formulations in the mouse FVII model. All nucleic acid-lipid particles were prepared using the pre-formed vesicle (PFV) method and were composed of ionizable cationic lipid, DSPC, cholesterol and PEG-lipid (40/10/40/10 mol/mol) with a FVII siRNA-to-total lipid ratio of -0.05 (wt/wt). Data points are expressed as a percentage of PBS control animals and represent group mean (n=5) ± s.d, and all formulations were compared within the same study. Figure 2B demonstrates the influence of headgroup extensions on the activity of DLin-K-DMA. DLin-K-DMA (■) had additional methylene groups added between the DMA headgroup and the ketal ring linker to generate DLin-K-C2-DMA (●), DLin-K-C3-DMA (▲) and DLin-K-C4-DMA (▼). The activity of PFV formulations of each lipid was assessed in the mouse FVII model. Data points are expressed as a percentage of PBS control animals and represent group mean (n=4) ± s.d.

Figure 3 is a graph depicting the amount of residual FVII following administration of various dosages of the indicated nucleic acid-lipid particle formulations comprising encapsulated FVII siRNA to mice.

Figure 4 is a graph depicting the amount of residual FVII following administration of various dosages of the indicated nucleic acid-lipid particle formulations comprising encapsulated FVII siRNA to rats.

Figure 5 is a graph comparing the amount of residual FVII following administration of two nucleic acid-lipid particle formulations (DLin-K-C2-DMA or DLin-K-DMA) comprising encapsulated FVII siRNA to mice or rats.

Figure 6 is a graph comparing the amount of residual FVII following administration of various concentrations of three different nucleic acid-lipid particle formulations (DLin-K6-DMA, DLin-K-C2-DMA, and DLin-K-DMA) comprising encapsulated FVII siRNA to mice.

Figure 7 is a graph depicting the amount of residual FVII following administration of various dosages of the indicated nucleic acid-lipid particle formulations comprising encapsulated FVII si RNA to animals. C2 indicates DLin-K-C2-DMA; C3 indicates DLin-K-C3-DMA; and C4 indicates DLin-K-C4-DMA.

Figure 8 is a graph showing the amount of residual FVII following administration of various dosages of the nucleic acid-lipid particle formulations comprising the different indicated cationic lipids: DLinDAP (●), DLinDMA (▲), DLin-K-DMA (■), or DLIN-K-C2-DMA (◆).

Figures 9A-B illustrate the efficacy of KC2-SNALP formulations. Figure 9A is a graph showing the improved efficacy of KC2-SNALP versus a DLin-K-C2-DMA PFV formulation in mice. The *in vivo* efficacy of KC2-SNALP (O) was compared to that of the un-optimized DLin-KC2-DMA PFV formulation (●) in the mouse FVII model. Data points are expressed as a percentage of PBS control animals and represent group mean (n=5) ± s.d. Figure 9B depicts

the efficacy of KC2-SNALP in non-human primates. Cynomolgus monkeys (n = 3 per group) received either 0.03, 0.1, 0.3 or 1 mg/kg siTTR, or 1 mg/kg siApoB formulated in KC2-SNALP or PBS as 15 minute intravenous infusions (5 mL/kg) via the cephalic vein. Animals were sacrificed at 48 hours post-administration. *TTR* mRNA levels relative to *GAPDH* mRNA levels were determined in liver samples. Data points represent group mean  $\pm$  s.d. \* =  $P < 0.05$ ; \*\* =  $P < 0.005$ .

## DETAILED DESCRIPTION

**[0027]** The present invention is based, in part, upon the identification of novel cationic lipids that provide superior results when used in lipid particles for the *in vivo* delivery of a therapeutic agent. In particular, the present invention provides nucleic acid-lipid particle compositions (also referred to as formulations or liposomal formulations) comprising a cationic lipid according to the present invention that provide increased activity of the nucleic acid and significant tolerability of the compositions *in vivo*, which is expected to correlate with a significant increase in therapeutic index as compared to nucleic acid-lipid particle compositions previously described.

**[0028]** As described in the accompanying Examples, a rational design approach was employed for the discovery of novel lipids for use in next-generation lipid particle systems to deliver nucleic acids, including, *e.g.*, RNAi therapeutics. Using this approach, important structure-activity considerations for ionizable cationic lipids were described, and multiple lipids based on the DLinDMA structure were designed and characterized. Nucleic acid-lipid particles comprising the cationic lipid termed DLin-K-C2-DMA were shown to be well-tolerated in both rodent and non-human primates and exhibited *in vivo* activity at siRNA doses as low as 0.01 mg/kg in rodents, as well as silencing of a therapeutically significant gene (*TTR*) in non-human primates. Notably, the *TTR* silencing achieved in this work ( $ED_{50} \sim 0.3$  mg/kg), represents a significant improvement in activity relative to previous reports of LNP-siRNA mediated silencing in non-human primates. The efficacy observed in this study is believed to represent the highest level of potency observed for an RNAi therapeutic in non-human primates to date.

**[0029]** Accordingly, in certain embodiments, the present invention specifically provides for improved compositions for the delivery of siRNA molecules. It is shown herein that these compositions are effective in down-regulating the protein levels and/or mRNA levels of target proteins. The lipid particles and compositions of the present invention may be used for a variety of purposes, including the delivery of associated or encapsulated therapeutic agents to cells, both *in vitro* or *in vivo*. Accordingly, the present invention relates to methods of treating diseases or disorders in a subject in need thereof, by contacting the subject with a lipid particle of the present invention associated with a suitable therapeutic agent.

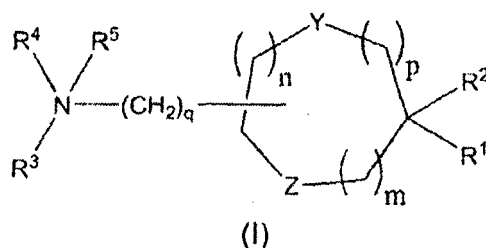
**[0030]** As described herein, the lipid particles of the present invention are particularly useful for the delivery of nucleic acids, including, *e.g.*, siRNA molecules and plasmids. Therefore, the lipid particles and compositions of the present invention may be used to modulate the expression of target genes and proteins both *in vitro* and *in vivo* by contacting cells with a lipid particle of the present invention associated with a nucleic acid that reduces target gene expression (*e.g.*, an siRNA) or a nucleic acid that may be used to increase expression of a desired protein (*e.g.*, a plasmid encoding the desired protein).

**[0031]** Various exemplary embodiments of the cationic lipids of the present invention, as well as lipid particles and compositions comprising the same, and their use to deliver therapeutic agents and modulate gene and protein expression are described in further detail below.

### A. Amino Lipids

**[0032]** The present invention provides novel amino lipids that are advantageously used in lipid particles of the present invention for the *in vivo* delivery of therapeutic agents to cells, including amino lipids having the following structures.

**[0033]** In one embodiment of the invention, the amino lipid has the following structure (I):



wherein

R<sup>1</sup> and R<sup>2</sup> are either the same or different and independently substituted C<sub>12</sub>-C<sub>24</sub> alkyl, optionally substituted C<sub>12</sub>-C<sub>24</sub> alkenyl, optionally substituted C<sub>12</sub>-C<sub>24</sub> alkynyl, or optionally substituted C<sub>12</sub>-C<sub>24</sub> acyl;

R<sup>3</sup> and R<sup>4</sup> are either the same or different and independently optionally substituted C<sub>1</sub>-C<sub>6</sub> alkyl, optionally substituted C<sub>1</sub>-C<sub>6</sub> alkenyl, or optionally substituted C<sub>1</sub>-C<sub>6</sub> alkynyl or R<sup>3</sup> and R<sup>4</sup> may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms chosen from nitrogen and oxygen;

R<sup>5</sup> is either absent or is hydrogen or C<sub>1</sub>-C<sub>6</sub> alkyl to provide a quaternary amine;

m, n, and p are either the same or different and independently either 0 or 1 with the proviso that m, n, and p are not simultaneously 0;

Y and Z are either the same or different and independently O, S, or NH; and,

q is 2.

**[0034]** "Alkyl" means a straight chain or branched, noncyclic or cyclic, saturated aliphatic hydrocarbon containing from 1 to 24 carbon atoms. Representative saturated straight chain alkyls include methyl, ethyl, n-propyl, n-butyl, n-pentyl, n-hexyl, and the like; while saturated branched alkyls include isopropyl, sec-butyl, isobutyl, *tert*-butyl, isopentyl, and the like. Representative saturated cyclic alkyls include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, and the like; while unsaturated cyclic alkyls include cyclopentenyl and cyclohexenyl, and the like.

**[0035]** "Alkenyl" means an alkyl, as defined above, containing at least one double bond between adjacent carbon atoms. Alkenyls include both *cis* and *trans* isomers. Representative straight chain and branched alkenyls include ethenyl, propenyl, 1-butenyl, 2-butenyl, isobutenyl, 1-pentenyl, 2-pentenyl, 3-methyl-1-butenyl, 2-methyl-2-butenyl, 2,3-dimethyl-2-butenyl, and the like.

**[0036]** "Alkynyl" means any alkyl or alkenyl, as defined above, which additionally contains at least one triple bond between adjacent carbons. Representative straight chain and branched alkynyls include acetylenyl, propynyl, 1-butyne, 2-butyne, 1-pentyne, 2-pentyne, 3-methyl-1-butyne, and the like.

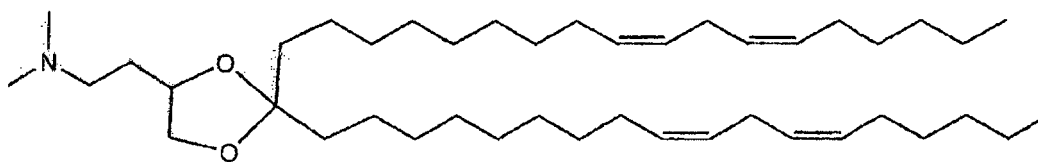
**[0037]** "Acyl" means any alkyl, alkenyl, or alkynyl wherein the carbon at the point of attachment is substituted with an oxo group, as defined below. For example, -C(=O)alkyl, -C(=O)alkenyl, and -C(=O)alkynyl are acyl groups.

**[0038]** "Heterocycle" means a 5- to 7-membered monocyclic, or 7- to 10-membered bicyclic, heterocyclic ring which is either saturated, unsaturated, or aromatic, and which contains from 1 or 2 heteroatoms independently selected from nitrogen, oxygen and sulfur, and wherein the nitrogen and sulfur heteroatoms may be optionally oxidized, and the nitrogen heteroatom may be optionally quaternized, including bicyclic rings in which any of the above heterocycles are fused to a benzene ring. The heterocycle may be attached via any heteroatom or carbon atom. Heterocycles include heteroaryls as defined below. Heterocycles include morpholinyl, pyrrolidinonyl, pyrrolidinyl, piperidinyl, piperizynyl, hydantoinyl, valerolactamyl, oxiranyl, oxetanyl, tetrahydrofuranyl, tetrahydropyranyl, tetrahydropyridinyl, tetrahydroimidinyl, tetrahydrothiophenyl, tetrahydrothiopyranyl, tetrahydropyrimidinyl, tetrahydrothiophenyl, tetrahydrothiopyranyl, and the like.

**[0039]** The terms "optionally substituted alkyl", "optionally substituted alkenyl", "optionally substituted alkynyl", "optionally substituted acyl", and "optionally substituted heterocycle" means that, when substituted, at least one hydrogen atom is replaced with a substituent. In the case of an oxo substituent (=O) two hydrogen atoms are replaced. In this regard, substituents include oxo, halogen, heterocycle, -CN, -OR<sup>x</sup>, -NR<sup>x</sup>R<sup>y</sup>, -NR<sup>x</sup>C(=O)R<sup>y</sup>, -NR<sup>x</sup>SO<sub>2</sub>R<sup>y</sup>, -C(=O)R<sup>x</sup>, -C(=O)OR<sup>x</sup>, -C(=O)NR<sup>x</sup>R<sup>y</sup>, -SO<sub>n</sub>R<sup>x</sup> and -SO<sub>n</sub>NR<sup>x</sup>R<sup>y</sup>, wherein n is 0, 1 or 2, R<sup>x</sup> and R<sup>y</sup> are the same or different and independently hydrogen, alkyl or heterocycle, and each of said alkyl and heterocycle substituents may be further substituted with one or more of oxo, halogen, -OH, -CN, alkyl, -OR<sup>x</sup>, heterocycle, -NR<sup>x</sup>R<sup>y</sup>, -NR<sup>x</sup>C(=O)R<sup>y</sup>, -NR<sup>x</sup>SO<sub>2</sub>R<sup>y</sup>, -C(=O)R<sup>x</sup>, -C(=O)OR<sup>x</sup>, -C(=O)NR<sup>x</sup>R<sup>y</sup>, -SO<sub>n</sub>R<sup>x</sup> and -SO<sub>n</sub>NR<sup>x</sup>R<sup>y</sup>.

**[0040]** "Halogen" means fluoro, chloro, bromo and iodo.

**[0041]** In certain embodiments, an amino lipid of the present invention has one of the following structures:



**DLin-K-C2-DMA**

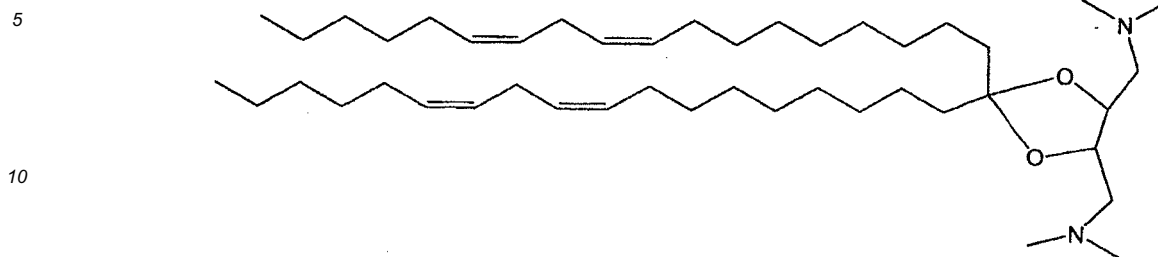
**C<sub>43</sub>H<sub>79</sub>NO<sub>2</sub>**

**Exact Mass: 641.61**

**Mol. Wt.: 642.09**

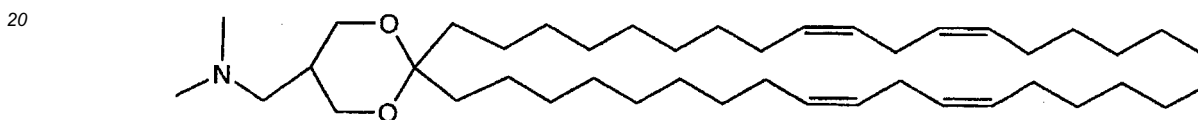
**C, 80.43; H, 12.40; N, 2.18; O, 4.98;**

[0042] In certain embodiments, an amino lipid of the present invention has one of the following structures:

DLin-K<sup>2</sup>-DMA;

15

or,



DLin-K6-DMA;

25

[0043] In some embodiments, the methods of the invention may require the use of protecting groups. Protecting group methodology is well known to those skilled in the art (*see, for example*, Protective Groups in Organic Synthesis, Green, T.W. et. al., Wiley-Interscience, New York City, 1999). Briefly, protecting groups within the context of this invention are any group that reduces or eliminates unwanted reactivity of a functional group. A protecting group can be added to a functional group to mask its reactivity during certain reactions and then removed to reveal the original functional group. In some embodiments an "alcohol protecting group" is used. An "alcohol protecting group" is any group which decreases or eliminates unwanted reactivity of an alcohol functional group. Protecting groups can be added and removed using techniques well known in the art.

30

35

[0044] The compounds of the present invention may be prepared by known organic synthesis techniques, including the methods described in more detail in the Examples. In general, the compounds of structure (I) above may be made by the following Reaction Schemes 1 or 2, wherein all substituents are as defined above unless indicated otherwise.

[0045] Compounds of structure (I) wherein m is 1 and p is 0 can be prepared according to Reaction Scheme 1: Ketone **1** and Grignard reagent **2**, wherein P is an alcohol protecting group such as trityl, can be purchased or prepared according to methods known to those of ordinary skill in the art. Reaction of **1** and **2** yields alcohol **3**. Deprotection of **3**, for example by treatment with mild acid, followed by bromination with an appropriate bromination reagent, for example phosphorous tribromide, yields **4** and **5** respectively. Treatment of bromide **5** with **6** yields the heterocyclic compound **7**. Treatment of **7** with amine **8** then yields a compound of structure (I) wherein m is 1 and R<sup>5</sup> is absent (**9**). Further treatment of **9** with chloride **10** yields compounds of structure (I) wherein m is 1 and R<sup>5</sup> is present.

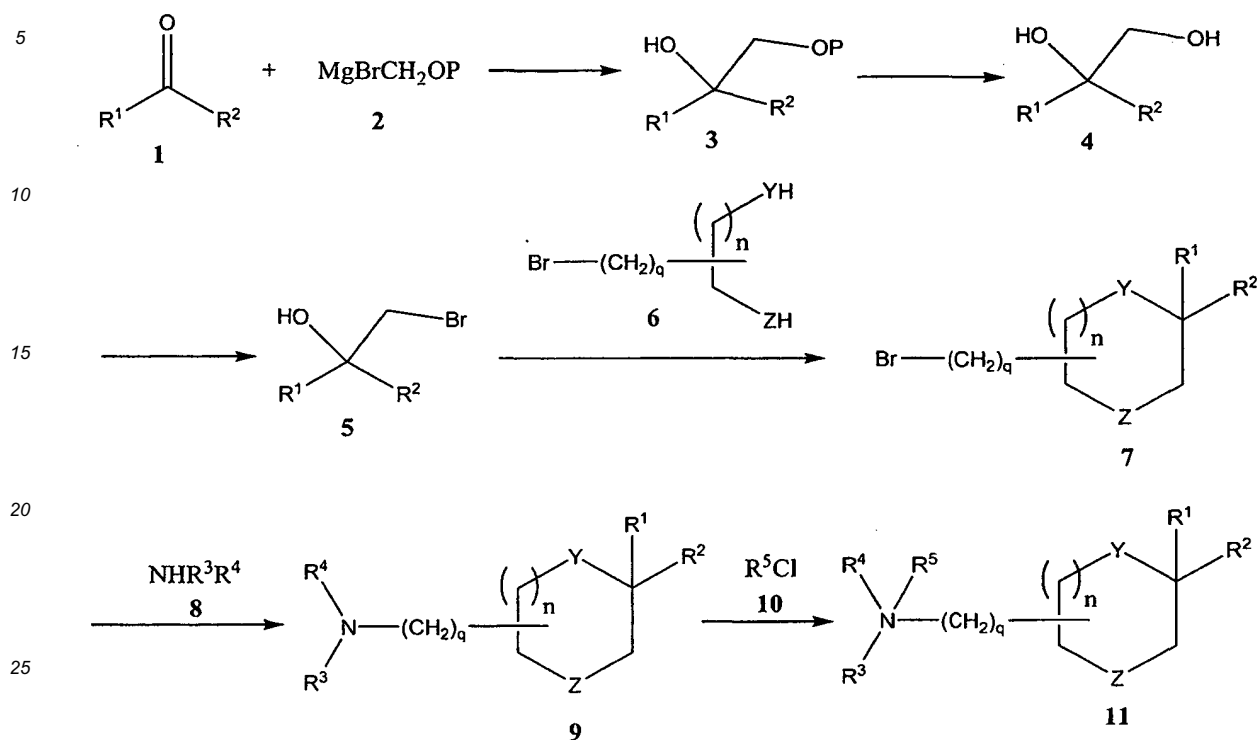
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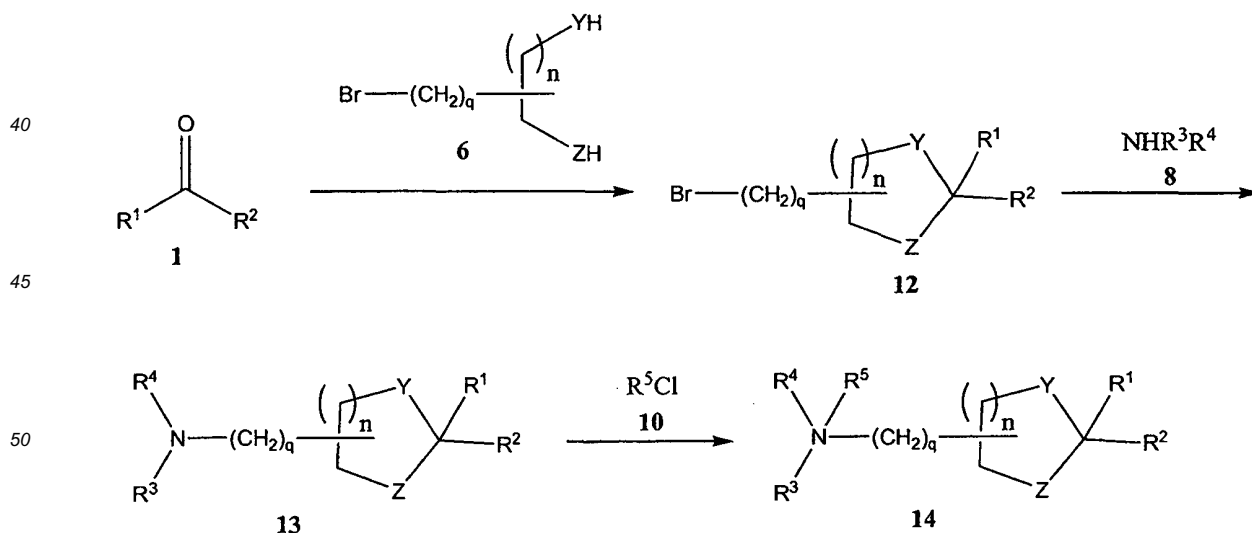
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## 1. Reaction Scheme 1



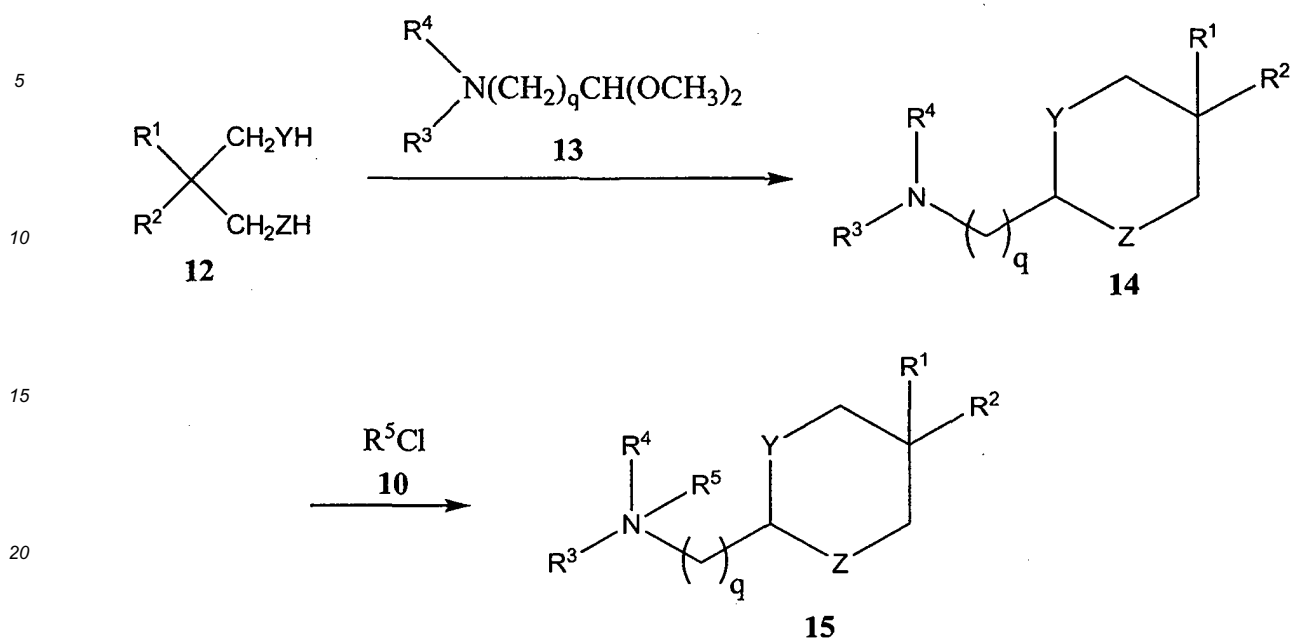
**[0046]** Compounds of structure (I) wherein  $m$  and  $p$  are 0 can be prepared according to Reaction Scheme 2. Ketone **1** and bromide **6** can be purchased or prepared according to methods known to those of ordinary skill in the art. Reaction of **1** and **6** yields heterocycle **12**. Treatment of **12** with amine **8** yields compounds of structure (I) wherein  $m$  is 0 and  $\text{R}^5$  is absent (**13**). Further treatment of **13** with **10** produces compounds of structure (I) wherein  $w$  is 0 and  $\text{R}^5$  is present.

## 2. Reaction Scheme 2



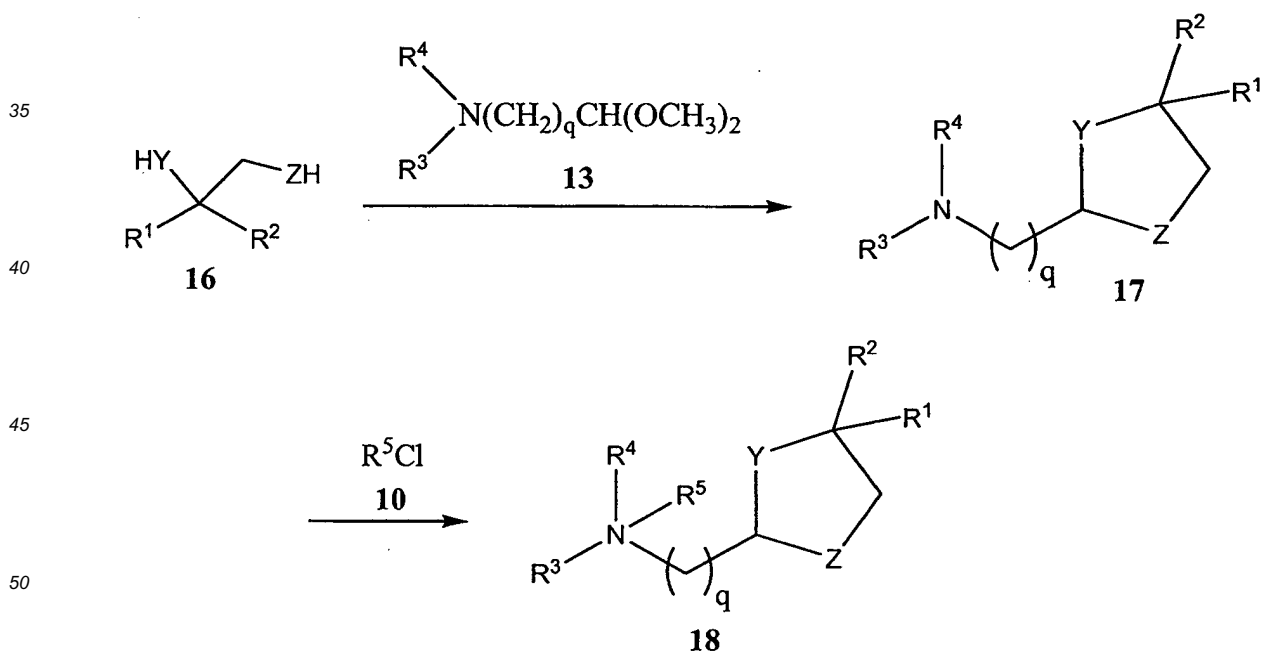
**[0047]** In certain embodiments where  $m$  and  $p$  are 1 and  $n$  is 0, compounds of this invention can be prepared according to Reaction Scheme 3. Compounds **12** and **13** can be purchased or prepared according to methods known to those of ordinary skill in the art. Reaction of **12** and **13** yields a compound of structure (I) where  $\text{R}^5$  is absent (**14**). In other embodiments where  $\text{R}^5$  is present, **13** can be treated with **10** to obtain compounds of structure **15**.

## 3. Reaction Scheme 3



25 **[0048]** In certain other embodiments where either  $m$  or  $p$  is 1 and  $n$  is 0, compounds of this invention can be prepared according to Reaction Scheme 4. Compound **16** can be purchased or prepared according to methods known to those of ordinary skill in the art and reacted with **13** to yield a compound of structure (I) where  $R^5$  is absent (**17**). Other embodiments of structure (I) where  $R^5$  is present can be prepared by treatment of **17** with **10** to yield compounds of structure **18**.

## 4. Reaction Scheme 4

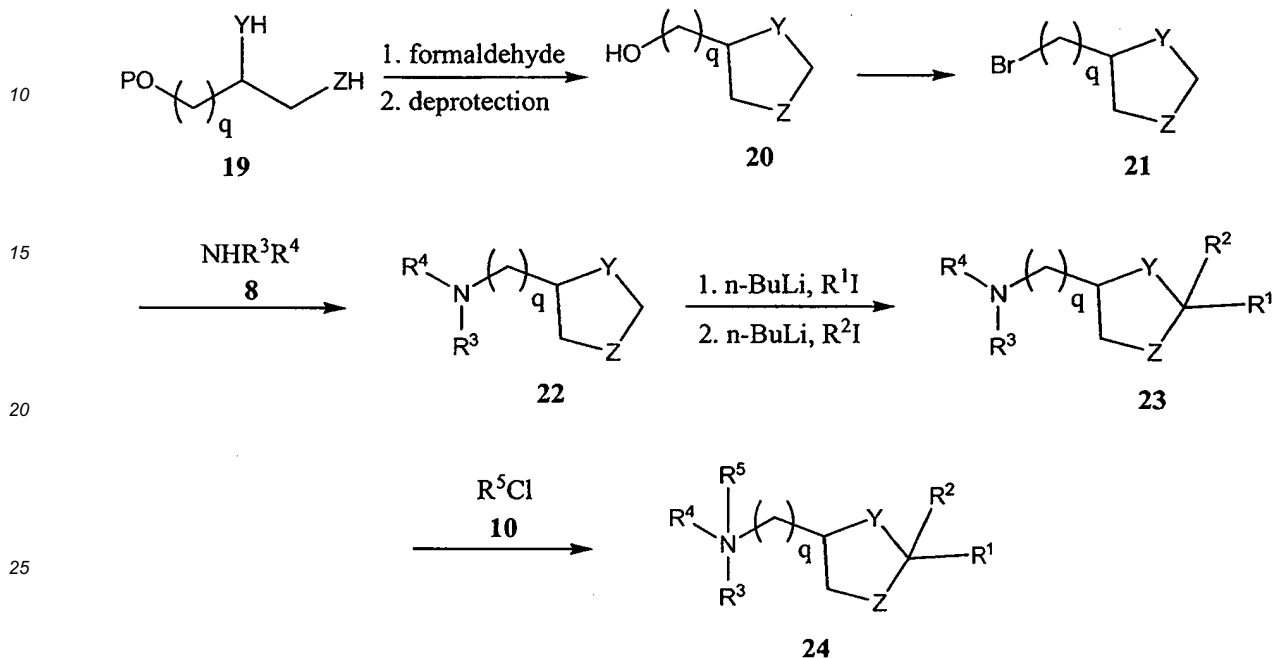


**[0049]** In certain specific embodiments of structure (I) where  $n$  is 1 and  $m$  and  $p$  are 0, compounds of this invention can be prepared according to Reaction Scheme 5. Compound **19** can be purchased or prepared according to methods known to those of ordinary skill in the art. Reaction of **19** with formaldehyde followed by removal of an optional alcohol protecting group (P), yields alcohol **20**. Bromination of **20** followed by treatment with amine **8** yields **22**. Compound **22**

can then be treated with n-butyl lithium and R<sup>1</sup>I followed by further treatment with n-butyl lithium and R<sup>2</sup>I to yield a compound of structure (I) where R<sup>5</sup> is absent (**23**). Further treatment of **23** with **10** yields a compound of structure (I) where R<sup>5</sup> is present (**24**).

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## 5. Reaction Scheme 5



**[0050]** In particular embodiments, the amino lipids of the present invention are cationic lipids. As used herein, the term "amino lipid" is meant to include those lipids having one or two fatty acid or fatty alkyl chains and an amino head group (including an alkylamino or dialkylamino group) that may be protonated to form a cationic lipid at physiological pH.

**[0051]** Other amino lipids would include those having alternative fatty acid groups and other dialkylamino groups, including those in which the alkyl substituents are different (e.g., N-ethyl-N-methylamino-, N-propyl-N-ethylamino- and the like). For those embodiments in which R<sup>11</sup> and R<sup>12</sup> are both long chain alkyl or acyl groups, they can be the same or different. In general, amino lipids having less saturated acyl chains are more easily sized, particularly when the complexes must be sized below about 0.3 microns, for purposes of filter sterilization. Amino lipids containing unsaturated fatty acids with carbon chain lengths in the range of C<sub>14</sub> to C<sub>22</sub> are preferred. Other scaffolds can also be used to separate the amino group and the fatty acid or fatty alkyl portion of the amino lipid. Suitable scaffolds are known to those of skill in the art.

**[0052]** In certain embodiments, amino or cationic lipids of the present invention have at least one protonatable or deprotonatable group, such that the lipid is positively charged at a pH at or below physiological pH (e.g. pH 7.4), and neutral at a second pH, preferably at or above physiological pH. It will, of course, be understood that the addition or removal of protons as a function of pH is an equilibrium process, and that the reference to a charged or a neutral lipid refers to the nature of the predominant species and does not require that all of the lipid be present in the charged or neutral form. Lipids that have more than one protonatable or deprotonatable group, or which are zwitterionic, are not excluded from use in the invention.

**[0053]** In certain embodiments, protonatable lipids according to the invention have a pKa of the protonatable group in the range of about 4 to about 11. Most preferred is pKa of about 4 to about 7, because these lipids will be cationic at a lower pH formulation stage, while particles will be largely (though not completely) surface neutralized at physiological pH around pH 7.4. One of the benefits of this pKa is that at least some nucleic acid associated with the outside surface of the particle will lose its electrostatic interaction at physiological pH and be removed by simple dialysis; thus greatly reducing the particle's susceptibility to clearance.

## B. Lipid Particles

**[0054]** The present invention also provides lipid particles comprising one or more of the amino lipids described above. Lipid particles include, but are not limited to, liposomes. As used herein, a liposome is a structure having lipid-containing

membranes enclosing an aqueous interior. Liposomes may have one or more lipid membranes. The invention contemplates both single-layered liposomes, which are referred to as unilamellar, and multi-layered liposomes, which are referred to as multilamellar. When complexed with nucleic acids, lipid particles may also be lipoplexes, which are composed of cationic lipid bilayers sandwiched between DNA layers, as described, *e.g.*, in Felgner, Scientific American.

**[0055]** The lipid particles of the present invention may further comprise one or more additional lipids and/or other components, such as cholesterol. Other lipids may be included in the liposome compositions of the present invention for a variety of purposes, such as to prevent lipid oxidation or to attach ligands onto the liposome surface. Any of a number of lipids may be present in liposomes of the present invention, including amphipathic, neutral, cationic, and anionic lipids. Such lipids can be used alone or in combination. Specific examples of additional lipid components that may be present are described below.

**[0056]** In certain embodiments, lipid particles of the present invention comprise an amino lipid described above, a non-cationic or neutral lipid, and a conjugated lipid that inhibits particle aggregation. In certain embodiments, lipid particles of the present invention comprise an amino lipid described above, a non-cationic or neutral lipid, a sterol, and a conjugated lipid that inhibits particle aggregation. In particular embodiments, these lipid particles further comprise a cationic lipid in addition to the amino lipid of the present invention.

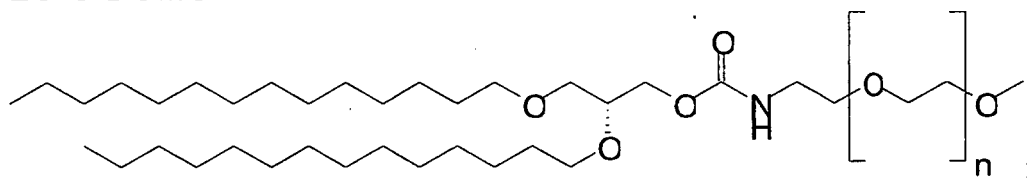
**[0057]** Additional components that may be present in a lipid particle of the present invention include bilayer stabilizing components such as polyamide oligomers (*see, e.g.*, U.S. Patent No. 6,320,017), peptides, proteins, detergents, lipid-derivatives, such as PEG coupled to phosphatidylethanolamine and PEG conjugated to ceramides (*see, U.S. Patent No. 5,885,613*).

**[0058]** Examples of lipids that reduce aggregation of particles during formation include polyethylene glycol (PEG)-modified lipids, monosialoganglioside Gm1, and polyamide oligomers ("PAO") such as (described in US Pat. No. 6,320,017). Other compounds with uncharged, hydrophilic, steric-barrier moieties, which prevent aggregation during formulation, like PEG, Gm1 or ATTA, can also be coupled to lipids for use as in the methods and compositions of the invention. ATTA-lipids are described, *e.g.*, in U.S. Patent No. 6,320,017, and PEG-lipid conjugates are described, *e.g.*, in U.S. Patent Nos. 5,820,873, 5,534,499 and 5,885,613. Typically, the concentration of the lipid component selected to reduce aggregation is about 1 to 15% (by mole percent of lipids).

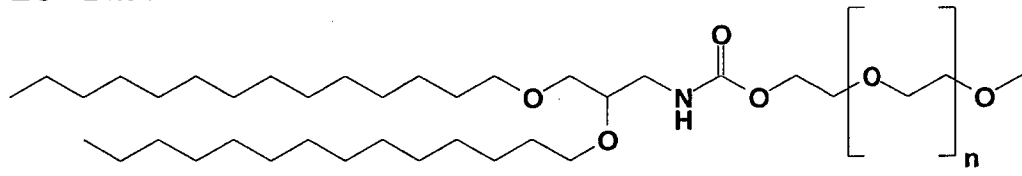
**[0059]** Specific examples of PEG-modified lipids (or lipid-polyoxyethylene conjugates) that are useful in the present invention can have a variety of "anchoring" lipid portions to secure the PEG portion to the surface of the lipid vesicle. Examples of suitable PEG-modified lipids include PEG-modified phosphatidylethanolamine and phosphatidic acid, PEG-ceramide conjugates (*e.g.*, PEG-CerC14 or PEG-CerC20) which are described in co-pending USSN 08/486,214, PEG-modified dialkylamines and PEG-modified 1,2-diaclyoxypropan-3-amines. Particularly preferred are PEG-modified diacylglycerols and dialkylglycerols.

**[0060]** In particular embodiments, a PEG-lipid is selected from:

PEG-C-DOMG

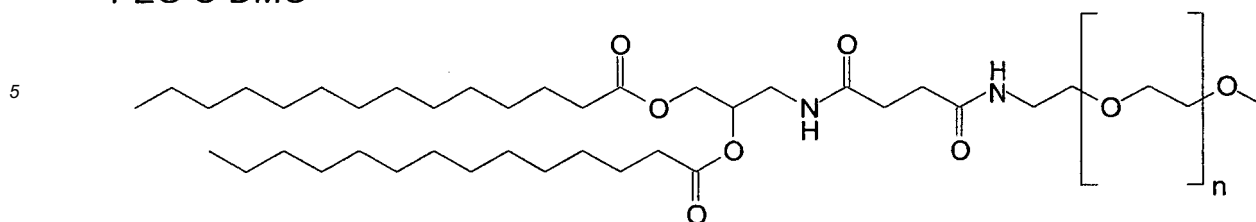


PEG- DMA



and

## PEG-S-DMG



**[0061]** In embodiments where a sterically-large moiety such as PEG or ATTA are conjugated to a lipid anchor, the selection of the lipid anchor depends on what type of association the conjugate is to have with the lipid particle. It is well known that mePEG (mw2000)-diastearoylphosphatidylethanolamine (PEG-DSPE) will remain associated with a liposome until the particle is cleared from the circulation, possibly a matter of days. Other conjugates, such as PEG-CerC20 have similar staying capacity. PEG-CerC14, however, rapidly exchanges out of the formulation upon exposure to serum, with a  $T_{1/2}$  less than 60 mins. in some assays. As illustrated in US Pat. Application SN 08/486,214, at least three characteristics influence the rate of exchange: length of acyl chain, saturation of acyl chain, and size of the steric-barrier head group. Compounds having suitable variations of these features may be useful for the invention. For some therapeutic applications it may be preferable for the PEG-modified lipid to be rapidly lost from the nucleic acid-lipid particle *in vivo* and hence the PEG-modified lipid will possess relatively short lipid anchors. In other therapeutic applications it may be preferable for the nucleic acid-lipid particle to exhibit a longer plasma circulation lifetime and hence the PEG-modified lipid will possess relatively longer lipid anchors.

**[0062]** It should be noted that aggregation preventing compounds do not necessarily require lipid conjugation to function properly. Free PEG or free ATTA in solution may be sufficient to prevent aggregation. If the particles are stable after formulation, the PEG or ATTA can be dialyzed away before administration to a subject.

**[0063]** The term "non-cationic lipid" refers to any amphipathic lipid as well as any other neutral lipid or anionic lipid. Non-cationic lipids used in the lipid particles, e.g., SNALP, of the present invention can be any of a variety of neutral uncharged, zwitterionic, or anionic lipids capable of producing a stable complex.

**[0064]** The term "neutral lipid" refers to any of a number of lipid species that exist either in an uncharged or neutral zwitterionic form at a selected pH. At physiological pH, such lipids include, for example, diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, cephalin, cholesterol, cerebrosides, and diacylglycerols.

**[0065]** The term "anionic lipid" refers to any lipid that is negatively charged at physiological pH. These lipids include, but are not limited to, phosphatidylglycerols, cardiolipins, diacylphosphatidylserines, diacylphosphatidic acids, N-dodecanoyl phosphatidylethanolamines, N-succinyl phosphatidylethanolamines, N-glutarylphosphatidylethanolamines, lysylphosphatidylglycerols, palmitoyloleyolphosphatidylglycerol (POPG), and other anionic modifying groups joined to neutral lipids. Such lipids include, for example diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, dihydrosphingomyelin, cephalin, and cerebrosides. The selection of neutral lipids for use in the particles described herein is generally guided by consideration of, e.g., liposome size and stability of the liposomes in the bloodstream. Preferably, the neutral lipid component is a lipid having two acyl groups, (i.e., diacylphosphatidylcholine and diacylphosphatidylethanolamine). Lipids having a variety of acyl chain groups of varying chain length and degree of saturation are available or may be isolated or synthesized by well-known techniques. In one group of embodiments, lipids containing saturated fatty acids with carbon chain lengths in the range of  $C_{14}$  to  $C_{22}$  are preferred. In another group of embodiments, lipids with mono or diunsaturated fatty acids with carbon chain lengths in the range of  $C_{14}$  to  $C_{22}$  are used. Additionally, lipids having mixtures of saturated and unsaturated fatty acid chains can be used. Preferably, the neutral lipids used in the present invention are DOPE, DSPC, POPC, or any related phosphatidylcholine. The neutral lipids useful in the present invention may also be composed of sphingomyelin, dihydrosphingomyelin, or phospholipids with other head groups, such as serine and inositol.

**[0066]** Non-limiting examples of non-cationic lipids include phospholipids such as lecithin, phosphatidylethanolamine, lysolecithin, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, egg sphingomyelin (ESM), cephalin, cardiolipin, phosphatidic acid, cerebrosides, dicetylphosphate, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleyolphosphatidylcholine (POPC), palmitoyloleyolphosphatidylethanolamine (POPE), palmitoyloleyolphosphatidylglycerol (POPG), dioleoylphosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dipalmitoylphosphatidylethanolamine (DPPE), dimyristoyl-phosphatidylethanolamine (DMPE), distearoyl-phosphatidylethanolamine (DSPE), monomethylphosphatidylethanolamine, dimethyl-phosphatidylethanolamine, dielaidoylphosphatidylethanolamine (DEPE), stearyloleyolphosphatidylethanolamine (SOPE), lysophosphatidylethanolamine, dilinoleyolphosphatidylcholine, and mixtures thereof. Other diacylphosphatidylcholine and diacylphosphatidylethanolamine phospholipids

can also be used. The acyl groups in these lipids are preferably acyl groups derived from fatty acids having C<sub>10</sub>-C<sub>24</sub> carbon chains, e.g., lauroyl, myristoyl, palmitoyl, stearoyl, or oleoyl.

**[0067]** Additional examples of non-cationic lipids include sterols such as cholesterol and derivatives thereof such as cholestanol, cholestanone, cholestenone, and coprostanol.

**[0068]** In some embodiments, the non-cationic lipid present in the lipid particle, e.g., SNALP, comprises or consists of cholesterol, e.g., a phospholipid-free SNALP. In other embodiments, the non-cationic lipid present in the lipid particle, e.g., SNALP comprises or consists of one or more phospholipids, e.g., a cholesterol-free SNALP. In further embodiments, the non-cationic lipid present in the SNALP comprises or consists of a mixture of one or more phospholipids and cholesterol.

**[0069]** Other examples of non-cationic lipids suitable for use in the present invention include nonphosphorous containing lipids such as, e.g., stearylamine, dodecylamine, hexadecylamine, acetyl palmitate, glycerolricinoleate, hexadecyl stearate, isopropyl myristate, amphoteric acrylic polymers, triethanolamine-lauryl sulfate, alkyl-aryl sulfate polyethoxy-lated fatty acid amides, dioctadecyldimethyl ammonium bromide, ceramide, sphingomyelin, and the like.

**[0070]** The non-cationic lipid typically comprises from about 13 mol % to about 49.5 mol %, about 20 mol % to about 45 mol %, about 25 mol % to about 45 mol %, about 30 mol % to about 45 mol %, about 35 mol % to about 45 mol %, about 20 mol % to about 40 mol %, about 25 mol % to about 40 mol %, or about 30 mol % to about 40 mol % of the total lipid present in the particle.

**[0071]** The sterol component of the lipid mixture, when present, can be any of those sterols conventionally used in the field of liposome, lipid vesicle or lipid particle preparation. A preferred sterol is cholesterol.

**[0072]** Other cationic lipids, which carry a net positive charge at about physiological pH, in addition to those specifically described above, may also be included in lipid particles of the present invention. Such cationic lipids include, but are not limited to, N,N-dioleoyl-N,N-dimethylammonium chloride ("DODAC"); N-(2,3-dioleoyloxy)propyl-N,N-N-triethylammonium chloride ("DOTMA"); N,N-distearoyl-N,N-dimethylammonium bromide ("DDAB"); N-(2,3-dioleoyloxy)propyl-N,N,N-trimethylammonium chloride ("DOTAP"); 1,2-Dioleoyloxy-3-trimethylaminopropane chloride salt ("DOTAP.Cl"); 3β-(N-(N',N'-dimethylaminoethane)-carbamoyl)cholesterol ("DC-Chol"), N-(1-(2,3-dioleoyloxy)propyl)-N-2-(sperminecarboxamido)ethyl)-N,N-dimethylammonium trifluoroacetate ("DOSPA"), dioctadecylamidoglycyl carboxyspermine ("DOGS"), 1,2-dioleoyl-sn-3-phosphoethanolamine ("DOPE"), 1,2-dioleoyl-3-dimethylammonium propane ("DODAP"), N,N-dimethyl-2,3-dioleoyloxypropylamine ("DODMA"), and N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide ("DMRIE"). Additionally, a number of commercial preparations of cationic lipids can be used, such as, e.g., LIPOFECTIN (including DOTMA and DOPE, available from GIBCO/BRL), and LIPOFECTAMINE (comprising DOSPA and DOPE, available from GIBCO/BRL). In particular embodiments, a cationic lipid is an amino lipid.

**[0073]** In numerous embodiments, amphipathic lipids are included in lipid particles of the present invention. "Amphipathic lipids" refer to any suitable material, wherein the hydrophobic portion of the lipid material orients into a hydrophobic phase, while the hydrophilic portion orients toward the aqueous phase. Such compounds include, but are not limited to, phospholipids, aminolipids, and sphingolipids. Representative phospholipids include sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine, or dilinoleoylphosphatidylcholine. Other phosphorus-lacking compounds, such as sphingolipids, glycosphingolipid families, diacylglycerols, and β-acyloxyacids, can also be used. Additionally, such amphipathic lipids can be readily mixed with other lipids, such as triglycerides and sterols.

**[0074]** Also suitable for inclusion in the lipid particles of the present invention are programmable fusion lipids. Such lipid particles have little tendency to fuse with cell membranes and deliver their payload until a given signal event occurs. This allows the lipid particle to distribute more evenly after injection into an organism or disease site before it starts fusing with cells. The signal event can be, for example, a change in pH, temperature, ionic environment, or time. In the latter case, a fusion delaying or "cloaking" component, such as an ATTA-lipid conjugate or a PEG-lipid conjugate, can simply exchange out of the lipid particle membrane over time. By the time the lipid particle is suitably distributed in the body, it has lost sufficient cloaking agent so as to be fusogenic. With other signal events, it is desirable to choose a signal that is associated with the disease site or target cell, such as increased temperature at a site of inflammation.

**[0075]** In certain embodiments, it is desirable to target the lipid particles of this invention using targeting moieties that are specific to a cell type or tissue. Targeting of lipid particles using a variety of targeting moieties, such as ligands, cell surface receptors, glycoproteins, vitamins (e.g., riboflavin) and monoclonal antibodies, has been previously described (see, e.g., U.S. Patent Nos. 4,957,773 and 4,603,044). The targeting moieties can comprise the entire protein or fragments thereof. Targeting mechanisms generally require that the targeting agents be positioned on the surface of the lipid particle in such a manner that the target moiety is available for interaction with the target, for example, a cell surface receptor. A variety of different targeting agents and methods are known and available in the art, including those described, e.g., in Sapra, P. and Allen, TM, Prog. Lipid Res. 42(5):439-62 (2003); and Abra, RM et al., J. Liposome Res. 12:1-3, (2002).

**[0076]** The use of lipid particles, i.e., liposomes, with a surface coating of hydrophilic polymer chains, such as polyethylene glycol (PEG) chains, for targeting has been proposed (Allen, et al., Biochimica et Biophysica Acta 1237: 99-108

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

**[0077]** Standard methods for coupling the target agents can be used. For example, phosphatidylethanolamine, which can be activated for attachment of target agents, or derivatized lipophilic compounds, such as lipid-derivatized bleomycin, can be used. Antibody-targeted liposomes can be constructed using, for instance, liposomes that incorporate protein A (see, Renneisen, et al., J. Bio. Chem., 265:16337-16342 (1990) and Leonetti, et al., Proc. Natl. Acad. Sci. (USA), 87:2448-2451 (1990). Other examples of antibody conjugation are disclosed in U.S. Patent No. 6,027,726. Examples of targeting moieties can also include other proteins, specific to cellular components, including antigens associated with neoplasms or tumors. Proteins used as targeting moieties can be attached to the liposomes via covalent bonds (see, Heath, Covalent Attachment of Proteins to Liposomes, 149 Methods in Enzymology 111-119 (Academic Press, Inc. 1987)). Other targeting methods include the biotin-avidin system.

**[0078]** In one exemplary embodiment, the lipid particle comprises a mixture of an amino lipid of the present invention, neutral lipids (other than an amino lipid), a sterol (e.g., cholesterol) and a PEG-modified lipid (e.g., a PEG-S-DMG, PEG-C-DOMG or PEG-DMA). In certain embodiments, the lipid mixture consists of or consists essentially of an amino lipid of the present invention, a neutral lipid, cholesterol, and a PEG-modified lipid. In further preferred embodiments, the lipid particle consists of or consists essentially of the above lipid mixture in molar ratios of about 20-70% amino lipid: 5-45% neutral lipid:20-55% cholesterol:0.5-15% PEG-modified lipid.

**[0079]** In particular embodiments, the lipid particle consists of or consists essentially of DLin-K-C2-DMA, DSPC, Chol, and either PEG-S-DMG, PEG-C-DOMG or PEG-DMA, e.g., in a molar ratio of about 20-60% DLin-K-C2-DMA: 5-25% DSPC:25-55% Chol:0.5-15% PEG-S-DMG, PEG-C-DOMG or PEG-DMA. In particular embodiments, the molar lipid ratio is approximately 40/10/40/10 (mol% DLin-K-C2-DMA/DSPC/Chol/PEG-S-DMG or DLin-K-C2-DMA/DSPC/Chol/PEG-C-DOMG or DLin-K-C2-DMA/DSPC/Chol/PEG-DMA) or 35/15/40/10 mol% DLin-K-C2-DMA/DSPC/Chol/PEG-S-DMG or DLin-K-C2-DMA/DSPC/Chol/PEG-C-DOMG or DLin-K-C2-DMA/DSPC/Chol/PEG-DMA. In another group of embodiments, the neutral lipid in these compositions is replaced with POPC, DOPE or SM.

**[0080]** In particular embodiments, the lipid particle consists of or consists essentially of DLin-K<sup>2</sup>-DMA, DSPC, Chol, and either PEG-S-DMG, PEG-C-DOMG or PEG-DMA, e.g., in a molar ratio of about 20-60% DLin-K<sup>2</sup>-DMA: 5-25% DSPC:25-55% Chol:0.5-15% PEG-S-DMG, PEG-C-DOMG or PEG-DMA. In particular embodiments, the molar lipid ratio is approximately 40/10/40/10 (mol% DLin-K<sup>2</sup>-DMA /DSPC/Chol/PEG-S-DMG or DLin-K<sup>2</sup>-DMA /DSPC/Chol/PEG-C-DOMG or DLin-K<sup>2</sup>-DMA /DSPC/Chol/PEG-DMA) or 35/15/40/10 mol% DLin-K<sup>2</sup>-DMA /DSPC/Chol/PEG-S-DMG or DLin-K<sup>2</sup>-DMA /DSPC/Chol/PEG-C-DOMG or DLin-K<sup>2</sup>-DMA /DSPC/Chol/PEG-DMA. In another group of embodiments, the neutral lipid in these compositions is replaced with POPC, DOPE or SM.

**[0081]** In particular embodiments, the lipid particle consists of or consists essentially of DLin-K6-DMA, DSPC, Chol, and either PEG-S-DMG, PEG-C-DOMG or PEG-DMA, e.g., in a molar ratio of about 20-60% DLin-K6-DMA: 5-25% DSPC:25-55% Chol:0.5-15% PEG-S-DMG, PEG-C-DOMG or PEG-DMA. In particular embodiments, the molar lipid ratio is approximately 40/10/40/10 (mol% DLin-K6-DMA /DSPC/Chol/PEG-S-DMG or DLin-K6-DMA /DSPC/Chol/PEG-C-DOMG or DLin-K6-DMA /DSPC/Chol/PEG-DMA) or 35/15/40/10 mol% DLin-K6-DMA /DSPC/Chol/PEG-S-DMG or DLin-K6-DMA /DSPC/Chol/PEG-C-DOMG or DLin-K6-DMA /DSPC/Chol/PEG-DMA. In another group of embodiments, the neutral lipid in these compositions is replaced with POPC, DOPE or SM.

### C. Therapeutic Agent-Lipid Particle Compositions and Formulations

**[0082]** The present invention includes compositions comprising a lipid particle of the present invention and an active agent, wherein the active agent is associated with the lipid particle. In particular embodiments, the active agent is a therapeutic agent. In particular embodiments, the active agent is encapsulated within an aqueous interior of the lipid particle. In other embodiments, the active agent is present within one or more lipid layers of the lipid particle. In other embodiments, the active agent is bound to the exterior or interior lipid surface of a lipid particle.

**[0083]** "Fully encapsulated" as used herein indicates that the nucleic acid in the particles is not significantly degraded after exposure to serum or a nuclease assay that would significantly degrade the free nucleic acid. In a fully encapsulated system, preferably less than 25% of particle nucleic acid is degraded in a treatment that would normally degrade 100% of free nucleic acid, more preferably less than 10% and most preferably less than 5% of the particle nucleic acid is degraded. Alternatively, full encapsulation may be determined by an Oligreen<sup>®</sup> assay. Oligreen<sup>®</sup> is an ultra-sensitive fluorescent nucleic acid stain for quantitating oligonucleotides and single-stranded DNA or RNA in solution (available

from Invitrogen Corporation, Carlsbad, CA). Fully encapsulated also suggests that the particles are serum stable, that is, that they do not rapidly decompose into their component parts upon *in vivo* administration.

**[0084]** Active agents, as used herein, include any molecule or compound capable of exerting a desired effect on a cell, tissue, organ, or subject. Such effects may be biological, physiological, or cosmetic, for example. Active agents may be any type of molecule or compound, including *e.g.*, nucleic acids, peptides and polypeptides, including, *e.g.*, antibodies, such as, *e.g.*, polyclonal antibodies, monoclonal antibodies, antibody fragments; humanized antibodies, recombinant antibodies, recombinant human antibodies, and Primatized™ antibodies, cytokines, growth factors, apoptotic factors, differentiation-inducing factors, cell surface receptors and their ligands; hormones; and small molecules, including small organic molecules or compounds.

**[0085]** In one embodiment, the active agent is a therapeutic agent, or a salt or derivative thereof. Therapeutic agent derivatives may be therapeutically active themselves or they may be prodrugs, which become active upon further modification. Thus, in one embodiment, a therapeutic agent derivative retains some or all of the therapeutic activity as compared to the unmodified agent, while in another embodiment, a therapeutic agent derivative lacks therapeutic activity.

**[0086]** In various embodiments, therapeutic agents include any therapeutically effective agent or drug, such as anti-inflammatory compounds, anti-depressants, stimulants, analgesics, antibiotics, birth control medication, antipyretics, vasodilators, anti-angiogenics, cytovascular agents, signal transduction inhibitors, cardiovascular drugs, *e.g.*, anti-arrhythmic agents, vasoconstrictors, hormones, and steroids.

**[0087]** In certain embodiments, the therapeutic agent is an oncology drug, which may also be referred to as an anti-tumor drug, an anti-cancer drug, a tumor drug, an antineoplastic agent, or the like. Examples of oncology drugs that may be used according to the invention include, but are not limited to, adriamycin, alkeran, allopurinol, altretamine, amifostine, anastrozole, araC, arsenic trioxide, azathioprine, bexarotene, biCNU, bleomycin, busulfan intravenous, busulfan oral, capecitabine (Xeloda), carboplatin, carmustine, CCNU, celecoxib, chlorambucil, cisplatin, cladribine, cyclosporin A, cytarabine, cytosine arabinoside, daunorubicin, cytoxan, daunorubicin, dexamethasone, dexrazoxane, doxorubicin, doxorubicin, DTIC, epirubicin, estramustine, etoposide phosphate, etoposide and VP-16, exemestane, FK506, fludarabine, fluorouracil, 5-FU, gemcitabine (Gemzar), gemtuzumabozogamicin, goserelin acetate, hydra, hydroxyurea, idarubicin, ifosfamide, imatinib mesylate, interferon, irinotecan (Camptostar, CPT-111), letrozole, leucovorin, leustatin, leuprolide, levamisole, litretinoin, megastrol, melphalan, L-PAM, mesna, methotrexate, methoxsalen, mithramycin, mitomycin, mitoxantrone, nitrogen mustard, paclitaxel, pamidronate, Pegademase, pentostatin, porfimer sodium, prednisone, rituxan, streptozocin, STI-571, tamoxifen, taxotere, temozolamide, teniposide, VM-26, topotecan (Hycamtin), toremifene, tretinoin, ATRA, valrubicin, velban, vinblastine, vincristine, VP16, and vinorelbine. Other examples of oncology drugs that may be used according to the invention are ellipticin and ellipticin analogs or derivatives, epothilones, intracellular kinase inhibitors and camptothecins.

#### 1. Nucleic Acid-Lipid Particles

**[0088]** In certain embodiments, lipid particles of the present invention are associated with a nucleic acid, resulting in a nucleic acid-lipid particle. In particular embodiments, the nucleic acid is partially or fully encapsulated in the lipid particle. As used herein, the term "nucleic acid" is meant to include any oligonucleotide or polynucleotide. Fragments containing up to 50 nucleotides are generally termed oligonucleotides, and longer fragments are called polynucleotides. In particular embodiments, oligonucleotides of the present invention are 20-50 nucleotides in length.

**[0089]** In the context of this invention, the terms "polynucleotide" and "oligonucleotide" refer to a polymer or oligomer of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars and intersugar (backbone) linkages. The terms "polynucleotide" and "oligonucleotide" also includes polymers or oligomers comprising non-naturally occurring monomers, or portions thereof, which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of properties such as, for example, enhanced cellular uptake and increased stability in the presence of nucleases.

**[0090]** Oligonucleotides are classified as deoxyribooligonucleotides or ribooligonucleotides. A deoxyribooligonucleotide consists of a 5-carbon sugar called deoxyribose joined covalently to phosphate at the 5' and 3' carbons of this sugar to form an alternating, unbranched polymer. A ribooligonucleotide consists of a similar repeating structure where the 5-carbon sugar is ribose.

**[0091]** The nucleic acid that is present in a lipid-nucleic acid particle according to this invention includes any form of nucleic acid that is known. The nucleic acids used herein can be single-stranded DNA or RNA, or double-stranded DNA or RNA, or DNA-RNA hybrids. Examples of double-stranded DNA include structural genes, genes including control and termination regions, and self-replicating systems such as viral or plasmid DNA. Examples of double-stranded RNA include siRNA and other RNA interference reagents. Single-stranded nucleic acids include, *e.g.*, antisense oligonucleotides, ribozymes, microRNA, and triplex-forming oligonucleotides.

**[0092]** Nucleic acids of the present invention may be of various lengths, generally dependent upon the particular form of nucleic acid. For example, in particular embodiments, plasmids or genes may be from about 1,000 to 100,000 nucleotide

residues in length. In particular embodiments, oligonucleotides may range from about 10 to 100 nucleotides in length. In various related embodiments, oligonucleotides, both single-stranded, double-stranded, and triple-stranded, may range in length from about 10 to about 50 nucleotides, from about 20 to about 50 nucleotides, from about 15 to about 30 nucleotides, from about 20 to about 30 nucleotides in length.

**[0093]** In particular embodiments, an oligonucleotide (or a strand thereof) of the present invention specifically hybridizes to or is complementary to a target polynucleotide. "Specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity such that stable and specific binding occurs between the DNA or RNA target and the oligonucleotide. It is understood that an oligonucleotide need not be 100% complementary to its target nucleic acid sequence to be specifically hybridizable. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target interferes with the normal function of the target molecule to cause a loss of utility or expression therefrom, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide 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 conducted. Thus, in other embodiments, this oligonucleotide includes 1, 2, or 3 base substitutions as compared to the region of a gene or mRNA sequence that it is targeting or to which it specifically hybridizes.

#### RNA Interference Nucleic Acids

**[0094]** In particular embodiments, nucleic acid-lipid particles of the present invention are associated with RNA interference (RNAi) molecules. RNA interference methods using RNAi molecules may be used to disrupt the expression of a gene or polynucleotide of interest. In the last 5 years small interfering RNA (siRNA) has essentially replaced antisense ODN and ribozymes as the next generation of targeted oligonucleotide drugs under development. siRNAs are RNA duplexes normally 21-30 nucleotides long that can associate with a cytoplasmic multi-protein complex known as RNAi-induced silencing complex (RISC). RISC loaded with siRNA mediates the degradation of homologous mRNA transcripts, therefore siRNA can be designed to knock down protein expression with high specificity. Unlike other antisense technologies, siRNA function through a natural mechanism evolved to control gene expression through non-coding RNA. This is generally considered to be the reason why their activity is more potent *in vitro* and *in vivo* than either antisense ODN or ribozymes. A variety of RNAi reagents, including siRNAs targeting clinically relevant targets, are currently under pharmaceutical development, as described, *e.g.*, in de Fougères, A. et al., *Nature Reviews* 6:443-453 (2007).

**[0095]** While the first described RNAi molecules were RNA:RNA hybrids comprising both an RNA sense and an RNA antisense strand, it has now been demonstrated that DNA sense:RNA antisense hybrids, RNA sense:DNA antisense hybrids, and DNA:DNA hybrids are capable of mediating RNAi (Lamberton, J.S. and Christian, A.T., (2003) *Molecular Biotechnology* 24:111-119). Thus, the invention includes the use of RNAi molecules comprising any of these different types of double-stranded molecules. In addition, it is understood that RNAi molecules may be used and introduced to cells in a variety of forms. Accordingly, as used herein, RNAi molecules encompasses any and all molecules capable of inducing an RNAi response in cells, including, but not limited to, double-stranded polynucleotides comprising two separate strands, *i.e.* a sense strand and an antisense strand, *e.g.*, small interfering RNA (siRNA); polynucleotides comprising a hairpin loop of complementary sequences, which forms a double-stranded region, *e.g.*, shRNAi molecules, and expression vectors that express one or more polynucleotides capable of forming a double-stranded polynucleotide alone or in combination with another polynucleotide.

**[0096]** RNA interference (RNAi) may be used to specifically inhibit expression of target polynucleotides. Double-stranded RNA-mediated suppression of gene and nucleic acid expression may be accomplished according to the invention by introducing dsRNA, siRNA or shRNA into cells or organisms. siRNA may be double-stranded RNA, or a hybrid molecule comprising both RNA and DNA, *e.g.*, one RNA strand and one DNA strand. It has been demonstrated that the direct introduction of siRNAs to a cell can trigger RNAi in mammalian cells (Elshabir, S.M., et al. *Nature* 411:494-498 (2001)). Furthermore, suppression in mammalian cells occurred at the RNA level and was specific for the targeted genes, with a strong correlation between RNA and protein suppression (Caplen, N. et al., *Proc. Natl. Acad. Sci. USA* 98:9746-9747 (2001)). In addition, it was shown that a wide variety of cell lines, including HeLa S3, COS7, 293, NIH/3T3, A549, HT-29, CHO-K1 and MCF-7 cells, are susceptible to some level of siRNA silencing (Brown, D. et al. *TechNotes* 9(1):1-7, available at [http://www.dot.ambion.com/techlib/tn/91/912.html\(9/1/02\)](http://www.dot.ambion.com/techlib/tn/91/912.html(9/1/02))).

**[0097]** RNAi molecules targeting specific polynucleotides can be readily prepared according to procedures known in the art. Structural characteristics of effective siRNA molecules have been identified. Elshabir, S.M. et al. (2001) *Nature* 411:494-498 and Elshabir, S.M. et al. (2001), *EMBO* 20:6877-6888. Accordingly, one of skill in the art would understand that a wide variety of different siRNA molecules may be used to target a specific gene or transcript. In certain embodiments, siRNA molecules according to the invention are double-stranded and 16 - 30 or 18 - 25 nucleotides in length, including each integer in between. In one embodiment, an siRNA is 21 nucleotides in length. In certain embodiments, siRNAs have 0-7 nucleotide 3' overhangs or 0-4 nucleotide 5' overhangs. In one embodiment, an siRNA molecule has a two nucleotide 3' overhang. In one embodiment, an siRNA is 21 nucleotides in length with two nucleotide 3' overhangs (*i.e.*

they contain a 19 nucleotide complementary region between the sense and antisense strands). In certain embodiments, the overhangs are UU or dTdT 3' overhangs.

**[0098]** Generally, siRNA molecules are completely complementary to one strand of a target DNA molecule, since even single base pair mismatches have been shown to reduce silencing. In other embodiments, siRNAs may have a modified backbone composition, such as, for example, 2'-deoxy- or 2'-O-methyl modifications. However, in preferred embodiments, the entire strand of the siRNA is not made with either 2' deoxy or 2'-O-modified bases.

**[0099]** In one embodiment, siRNA target sites are selected by scanning the target mRNA transcript sequence for the occurrence of AA dinucleotide sequences. Each AA dinucleotide sequence in combination with the 3' adjacent approximately 19 nucleotides are potential siRNA target sites. In one embodiment, siRNA target sites are preferentially not located within the 5' and 3' untranslated regions (UTRs) or regions near the start codon (within approximately 75 bases), since proteins that bind regulatory regions may interfere with the binding of the siRNP endonuclease complex (Elshabir, S. et al. *Nature* 411:494-498 (2001); Elshabir, S. et al. *EMBO J.* 20:6877-6888 (2001)). In addition, potential target sites may be compared to an appropriate genome database, such as BLASTN 2.0.5, available on the NCBI server at [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov), and potential target sequences with significant homology to other coding sequences eliminated.

**[0100]** In particular embodiments, short hairpin RNAs constitute the nucleic acid component of nucleic acid-lipid particles of the present invention. Short Hairpin RNA (shRNA) is a form of hairpin RNA capable of sequence-specifically reducing expression of a target gene. Short hairpin RNAs may offer an advantage over siRNAs in suppressing gene expression, as they are generally more stable and less susceptible to degradation in the cellular environment. It has been established that such short hairpin RNA-mediated gene silencing works in a variety of normal and cancer cell lines, and in mammalian cells, including mouse and human cells. Paddison, P. et al., *Genes Dev.* 16(8):948-58 (2002). Furthermore, transgenic cell lines bearing chromosomal genes that code for engineered shRNAs have been generated. These cells are able to constitutively synthesize shRNAs, thereby facilitating long-lasting or constitutive gene silencing that may be passed on to progeny cells. Paddison, P. et al., *Proc. Natl. Acad. Sci. USA* 99(3):1443-1448 (2002).

**[0101]** ShRNAs contain a stem loop structure. In certain embodiments, they may contain variable stem lengths, typically from 19 to 29 nucleotides in length, or any number in between. In certain embodiments, hairpins contain 19 to 21 nucleotide stems, while in other embodiments, hairpins contain 27 to 29 nucleotide stems. In certain embodiments, loop size is between 4 to 23 nucleotides in length, although the loop size may be larger than 23 nucleotides without significantly affecting silencing activity. ShRNA molecules may contain mismatches, for example G-U mismatches between the two strands of the shRNA stem without decreasing potency. In fact, in certain embodiments, shRNAs are designed to include one or several G-U pairings in the hairpin stem to stabilize hairpins during propagation in bacteria, for example. However, complementarity between the portion of the stem that binds to the target mRNA (antisense strand) and the mRNA is typically required, and even a single base pair mismatch in this region may abolish silencing. 5' and 3' overhangs are not required, since they do not appear to be critical for shRNA function, although they may be present (Paddison et al. (2002) *Genes & Dev.* 16(8):948-58).

### MicroRNAs

**[0102]** Micro RNAs (miRNAs) are a highly conserved class of small RNA molecules that are transcribed from DNA in the genomes of plants and animals, but are not translated into protein. Processed miRNAs are single stranded ~17-25 nucleotide (nt) RNA molecules that become incorporated into the RNA-induced silencing complex (RISC) and have been identified as key regulators of development, cell proliferation, apoptosis and differentiation. They are believed to play a role in regulation of gene expression by binding to the 3'-untranslated region of specific mRNAs. RISC mediates down-regulation of gene expression through translational inhibition, transcript cleavage, or both. RISC is also implicated in transcriptional silencing in the nucleus of a wide range of eukaryotes.

**[0103]** The number of miRNA sequences identified to date is large and growing, illustrative examples of which can be found, for example, in: Griffiths-Jones S, et al. *NAR*, 2006, 34, Database Issue, D140-D144; Griffiths-Jones S. *NAR*, 2004, 32, Database Issue, D109-D111; and also at <http://microrna.sanger.ac.uk/sequences/>.

### Antisense Oligonucleotides

**[0104]** In one embodiment, a nucleic acid is an antisense oligonucleotide directed to a target polynucleotide. The term "antisense oligonucleotide" or simply "antisense" is meant to include oligonucleotides that are complementary to a targeted polynucleotide sequence. Antisense oligonucleotides are single strands of DNA or RNA that are 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 be used to target a specific, complementary (coding or non-coding) RNA. If binding takes place this DNA/RNA hybrid can be degraded by the enzyme RNase H. In particular embodiment, antisense oligonucleotides contain from about 10 to about 50 nucleotides, more preferably about 15 to about 30 nucleotides. The term also encompasses antisense oligonucleotides that may not be exactly complementary to the desired target gene. Thus, the invention

can be utilized in instances where non-target specific-activities are found with antisense, or where an antisense sequence containing one or more mismatches with the target sequence is the most preferred for a particular use.

**[0105]** Antisense oligonucleotides have been demonstrated to be effective and targeted inhibitors of protein synthesis, and, consequently, can be used to specifically inhibit protein synthesis by a targeted gene. The efficacy of antisense oligonucleotides for inhibiting protein synthesis is well established. For example, the synthesis of polygalacturonase and the muscarine type 2 acetylcholine receptor are inhibited by antisense oligonucleotides directed to their respective mRNA sequences (U. S. Patent 5,739,119 and U. S. Patent 5,759,829). Further, examples of antisense inhibition have been demonstrated with the nuclear protein cyclin, the multiple drug resistance gene (MDG1), ICAM-1, E-selectin, STK-1, striatal GABA<sub>A</sub> receptor and human EGF (Jaskulski et al., *Science*. 1988 Jun 10;240(4858):1544-6; Vasanthakumar and Ahmed, *Cancer Commun.* 1989;1 (4):225-32; Peris et al., *Brain Res Mol Brain Res.* 1998 Jun 15;57(2):310-20; U. S. Patent 5,801,154; U.S. Patent 5,789,573; U. S. Patent 5,718,709 and U.S. Patent 5,610,288). Furthermore, antisense constructs have also been described that inhibit and can be used to treat a variety of abnormal cellular proliferations, e.g. cancer (U. S. Patent 5,747,470; U. S. Patent 5,591,317 and U. S. Patent 5,783,683).

**[0106]** Methods of producing antisense oligonucleotides are known in the art and can be readily adapted to produce an antisense oligonucleotide that targets any polynucleotide sequence. Selection of antisense oligonucleotide sequences specific for a given target sequence is based upon analysis of the chosen target sequence and determination of secondary structure, T<sub>m</sub>, binding energy, and relative stability. Antisense oligonucleotides may be selected based upon their relative inability to form dimers, hairpins, or other secondary structures that would reduce or prohibit specific binding to the target mRNA in a host cell. Highly preferred target regions of the mRNA include those regions at or near the AUG translation initiation codon and those sequences that are substantially complementary to 5' regions of the mRNA. These secondary structure analyses and target site selection considerations can be performed, for example, using v.4 of the OLIGO primer analysis software (Molecular Biology Insights) and/or the BLASTN 2.0.5 algorithm software (Altschul et al., *Nucleic Acids Res.* 1997, 25(17):3389-402).

#### 25 Ribozymes

**[0107]** According to another embodiment of the invention, nucleic acid-lipid particles are associated with ribozymes. Ribozymes are RNA-protein complexes having specific catalytic domains that possess endonuclease activity (Kim and Cech, *Proc Natl Acad Sci USA.* 1987 Dec; 84(24):8788-92; Forster and Symons, *Cell.* 1987 Apr 24;49(2):211-20). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cech et al., *Cell.* 1981 Dec; 27(3 Pt 2):487-96; Michel and Westhof, *J Mol Biol.* 1990 Dec 5;216(3):585-610; Reinhold-Hurek and Shub, *Nature.* 1992 May 14;357(6374):173-6). This specificity has been attributed to the requirement that the substrate bind via specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

**[0108]** At least six basic varieties of naturally-occurring enzymatic RNAs are known presently. Each can catalyze the hydrolysis of RNA phosphodiester bonds *in trans* (and thus can cleave other RNA molecules) under physiological conditions. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of an enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

**[0109]** The enzymatic nucleic acid molecule may be formed in a hammerhead, hairpin, a hepatitis  $\delta$  virus, group I intron or RNaseP RNA (in association with an RNA guide sequence) or *Neurospora* VS RNA motif, for example. Specific examples of hammerhead motifs are described by Rossi et al. *Nucleic Acids Res.* 1992 Sep 11;20(17):4559-65. Examples of hairpin motifs are described by Hampel *et al.* (Eur. Pat. Appl. Publ. No. EP 0360257), Hampel and Tritz, *Biochemistry* 1989 Jun 13;28(12):4929-33; Hampel et al., *Nucleic Acids Res.* 1990 Jan 25;18(2):299-304 and U. S. Patent 5,631,359. An example of the hepatitis  $\delta$  virus motif is described by Perrotta and Been, *Biochemistry.* 1992 Dec 1;31(47):11843-52; an example of the RNaseP motif is described by Guerrier-Takada et al., *Cell.* 1983 Dec;35(3 Pt 2):849-57; *Neurospora* VS RNA ribozyme motif is described by Collins (Saville and Collins, *Cell.* 1990 May 18;61(4):685-96; Saville and Collins, *Proc Natl Acad Sci USA.* 1991 Oct 1;88(19):8826-30; Collins and Olive, *Biochemistry.* 1993 Mar 23;32(11):2795-9); and an example of the Group I intron is described in U. S. Patent 4,987,071. Important characteristics of enzymatic nucleic acid molecules used according to the invention are that they have a specific substrate binding site which is complementary to one or more of the target gene DNA or RNA regions, and that they have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule. Thus the ribozyme constructs need not be limited to specific motifs mentioned herein.

**[0110]** Methods of producing a ribozyme targeted to any polynucleotide sequence are known in the art. Ribozymes

may be designed as described in Int. Pat. Appl. Publ. No. WO 93/23569 and Int. Pat. Appl. Publ. No. WO 94/02595, and synthesized to be tested *in vitro* and *in vivo*, as described therein.

[0111] Ribozyme activity can be optimized by altering the length of the ribozyme binding arms or chemically synthesizing ribozymes with modifications that prevent their degradation by serum ribonucleases (see e.g., Int. Pat. Appl. Publ. No. WO 92/07065; Int. Pat. Appl. Publ. No. WO 93/15187; Int. Pat. Appl. Publ. No. WO 91/03162; Eur. Pat. Appl. Publ. No. 92110298.4; U. S. Patent 5,334,711; and Int. Pat. Appl. Publ. No. WO 94/13688, which describe various chemical modifications that can be made to the sugar moieties of enzymatic RNA molecules), modifications which enhance their efficacy in cells, and removal of stem II bases to shorten RNA synthesis times and reduce chemical requirements.

#### 10 Immunostimulatory Oligonucleotides

[0112] Nucleic acids associated with lipid particles of the present invention may be immunostimulatory, including immunostimulatory oligonucleotides (ISS; single- or double-stranded) capable of inducing an immune response when administered to a subject, which may be a mammal or other patient. ISS include, e.g., certain palindromes leading to hairpin secondary structures (see Yamamoto S., et al. (1992) J. Immunol. 148: 4072-4076), or CpG motifs, as well as other known ISS features (such as multi-G domains, see WO 96/11266).

[0113] The immune response may be an innate or an adaptive immune response. The immune system is divided into a more innate immune system, and acquired adaptive immune system of vertebrates, the latter of which is further divided into humoral cellular components. In particular embodiments, the immune response may be mucosal.

[0114] In particular embodiments, an immunostimulatory nucleic acid is only immunostimulatory when administered in combination with a lipid particle, and is not immunostimulatory when administered in its "free form." According to the present invention, such an oligonucleotide is considered to be immunostimulatory.

[0115] Immunostimulatory nucleic acids are considered to be non-sequence specific when it is not required that they specifically bind to and reduce the expression of a target polynucleotide in order to provoke an immune response. Thus, certain immunostimulatory nucleic acids may comprise a sequence corresponding to a region of a naturally occurring gene or mRNA, but they may still be considered non-sequence specific immunostimulatory nucleic acids.

[0116] In one embodiment, the immunostimulatory nucleic acid or oligonucleotide comprises at least one CpG dinucleotide. The oligonucleotide or CpG dinucleotide may be unmethylated or methylated. In another embodiment, the immunostimulatory nucleic acid comprises at least one CpG dinucleotide having a methylated cytosine. In one embodiment, the nucleic acid comprises a single CpG dinucleotide, wherein the cytosine in said CpG dinucleotide is methylated. In a specific embodiment, the nucleic acid comprises the sequence 5' TAACGTTGAGGGGCAT 3' (SEQ ID NO:2). In an alternative embodiment, the nucleic acid comprises at least two CpG dinucleotides, wherein at least one cytosine in the CpG dinucleotides is methylated. In a further embodiment, each cytosine in the CpG dinucleotides present in the sequence is methylated. In another embodiment, the nucleic acid comprises a plurality of CpG dinucleotides, wherein at least one of said CpG dinucleotides comprises a methylated cytosine. Exemplary immunostimulatory oligonucleotides are shown in Table 1.

[0117] In one specific embodiment, the nucleic acid comprises the sequence 5' TTCCATGACGTTCTGACGT 3' (SEQ ID NO:1). In another specific embodiment, the nucleic acid sequence comprises the sequence 5' TCCATGACGT-TCCTGACGT 3' (SEQ ID NO:31), wherein the two cytosines indicated in bold are methylated. In particular embodiments, the ODN is selected from a group of ODNs consisting of ODN #1, ODN #2, ODN #3, ODN #4, ODN #5, ODN #6, ODN #7, ODN #8, and ODN #9, as shown below.

Table 1. Exemplary Immunostimulatory Oligonucleotides (ODNs)

ODN NAME	ODN SEQ ID NO	ODN SEQUENCE (5'-3').
ODN 1 (INX-6295) human c-myc	SEQ ID NO: 2	5'-TAACGTTGAGGGGCAT-3
* ODN 1m (INX-6303)	SEQ ID NO: 4	5'-TAAZGTTGAGGGGCAT-3
ODN 2 (INX-1826)	SEQ ID NO: 1	5'-TCCATGACGTTCTGACGTT-3

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(continued)

	ODN NAME	ODN SEQ ID NO	ODN SEQUENCE (5'-3').
5	* ODN 2m (INX-1826m)	SEQ ID NO: 31	5'-TCCATGAZGTTCTGGAZGTT-3
10	ODN 3 (INX-6300)	SEQ ID NO: 3	5'-TAAGCATACGGGGTGT-3
15	ODN 5 (INX-5001)	SEQ ID NO: 5	5'-AACGTT-3
20	ODN 6 (INX-3002)	SEQ ID NO: 6	5'-GATGCTGTGTCTGGGGTCTCCGGGC-3'
25	ODN 7 (INX-2006)	SEQ ID NO: 7	5'-TCGTGTTTTGTGTTTTGTGTT-3'
30	ODN 7m (INX-2006m)	SEQ ID NO: 32	5'-TZGTZGTTTTGTZGTTTTGTZGTT-3'
35	ODN 8 (INX-1982)	SEQ ID NO: 8	5'-TCCAGGACTTCTCTCAGGTT-3'
40	ODN 9 (INX-G3139)	ISEQ ID NO: 9	5'-TCTCCCAGCGTGCGCCAT-3'
45	ODN 10 (PS-3082) murine Intracellular Adhesion Molecule-1	SEQ ID NO: 10	5'-TGCATCCCCCAGGCCACCAT-3
50	ODN 11 (PS-2302) human Intracellular Adhesion Molecule-1	SEQ ID NO: 11	5'-GCCCAAGCTGGCATCCGTCA-3'
55	ODN 12 (PS-8997) human Intracellular Adhesion Molecule-1	SEQ ID NO: 12	5'-GCCCAAGCTGGCATCCGTCA-3'
	ODN 13 (US3) human erb-B-2	SEQ ID NO: 13	5'-GGT GCTCACTGC GGC-3'
	ODN 14 (LR-3280)	SEQ ID NO: 14	5'-AACC GTT GAG GGG CAT-3'

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(continued)

	ODN NAME	ODN SEQ ID NO	ODN SEQUENCE (5'-3').
5	human c-myc		
10	ODN 15 (LR-3001) human c-myc	SEQ ID NO: 15	<b>5'-TAT GCT GTG CCG GGG TCT TCG GGC-3'</b>
15	ODN 16 (Inx-6298)	SEQ ID NO: 16	5'-GTGCCG GGGTCTTCGGGC-3'
20	ODN 17 (hIGF-1R) human Insulin Growth Factor 1 - Receptor	SEQ ID NO: 17	5'-GGACCCTCCTCCGGAGCC-3'
25	ODN 18 (LR-52) human Insulin Growth Factor 1 - Receptor	SEQ ID NO: 18	5'-TCC TCC GGA GCC AGA CTT-3'
30	ODN 19 (hEGFR) human Epidermal Growth Factor - Receptor	SEQ ID NO: 19	5'-AAC GTT GAG GGG CAT-3'
35	ODN 20 (EGFR) Epidermal Growth Factor - Receptor	SEQ ID NO: 20	5'-CCGTGGTCA TGCTCC-3'
40	ODN 21 (hVEGF) human Vascular Endothelial Growth Factor	SEQ ID NO: 21	5'-CAG CCTGGCTCACCG CCTTGG-3'
45	ODN 22 (PS-4189) murine Phosphokinase C - alpha	SEQ ID NO: 22	5'-CAG CCA TGG TTC CCC CCA AC-3'
50	ODN 23 (PS-3521)	SEQ ID NO: 23	5'-GTT CTC GCT GGT GAG TTT CA-3'
55	ODN 24 (hBcl-2) human Bcl-2	SEQ ID NO: 24	5'-TCT CCCAGCGTGCGCCAT-3'
	ODN 25 (hC-Raf-1)	SEQ ID NO: 25	5'-GTG CTC CAT TGA TGC-3'

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(continued)

ODN NAME	ODN SEQ ID NO	ODN SEQUENCE (5'-3').
human C-Raf-s		
ODN #26 (hVEGF-R1)  human Vascular Endothelial Growth Factor Receptor-1	SEQ ID NO: 26	5'- GAGUUCUGAUGAGGCCGAAAGGCCGAA AGUCUG-3'
ODN #27	SEQ ID NO: 27	5'-RRCGY-3'
ODN # 28 (INX-3280).	SEQ ID NO: 28	5'-AACGTTGAGGGGCAT-3'
ODN #29 (INX-6302)	SEQ ID NO: 29	5'-CAACGTTATGGGAGA-3'
ODN #30 (INX-6298)  human c-myc	SEQ ID NO: 30	5'-TAACGTTGAGGGGCAT-3'
<p>· "Z" represents a methylated cytosine residue.</p> <p>· Note: ODN 14 is a 15-mer oligonucleotide and ODN 1 is the same oligonucleotide having a thymidine added onto the 5' end making ODN 1 into a 16-mer. No difference in biological activity between ODN 14 and ODN 1 has been detected and both exhibit similar immunostimulatory activity (Mui et al., J Pharmacol. Exp. Ther. 298:1185-1192 (2001)).</p>		

**[0118]** Additional specific nucleic acid sequences of oligonucleotides (ODNs) suitable for use in the compositions and methods of the invention are described in U.S. Patent Appln. 60/379,343, U.S. patent application Ser. No. 09/649,527, Int. Publ. WO 02/069369, Int. Publ. No. WO 01/15726, U.S. Pat. No. 6,406,705, and Raney et al., Journal of Pharmacology and Experimental Therapeutics, 298:1185-1192 (2001). In certain embodiments, ODNs used in the compositions and methods of the present invention have a phosphodiester ("PO") backbone or a phosphorothioate ("PS") backbone, and/or at least one methylated cytosine residue in a CpG motif.

Nucleic Acid Modifications

**[0119]** In the 1990's, DNA-based antisense oligodeoxynucleotides (ODN) and ribozymes (RNA) represented an exciting new paradigm for drug design and development, but their application *in vivo* was prevented by endo- and exonuclease activity as well as a lack of successful intracellular delivery. The degradation issue was effectively overcome following extensive research into chemical modifications that prevented the oligonucleotide (oligo) drugs from being recognized by nuclease enzymes but did not inhibit their mechanism of action. This research was so successful that antisense ODN drugs in development today remain intact *in vivo* for days compared to minutes for unmodified molecules (Kurreck, J. 2003, Eur J Biochem 270:1628-44). However, intracellular delivery and mechanism of action issues have so far limited antisense ODN and ribozymes from becoming clinical products.

**[0120]** RNA duplexes are inherently more stable to nucleases than single stranded DNA or RNA, and unlike antisense ODN, unmodified siRNA show good activity once they access the cytoplasm. Even so, the chemical modifications developed to stabilize antisense ODN and ribozymes have also been systematically applied to siRNA to determine how much chemical modification can be tolerated and if pharmacokinetic and pharmacodynamic activity can be enhanced. RNA interference by siRNA duplexes requires an antisense and sense strand, which have different functions. Both are necessary to enable the siRNA to enter RISC, but once loaded the two strands separate and the sense strand is degraded

whereas the antisense strand remains to guide RISC to the target mRNA. Entry into RISC is a process that is structurally less stringent than the recognition and cleavage of the target mRNA. Consequently, many different chemical modifications of the sense strand are possible, but only limited changes are tolerated by the antisense strand.

5 [0121] As is known in the art, a nucleoside is a base-sugar combination. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

10 [0122] The nucleic acid that is used in a lipid-nucleic acid particle according to this invention includes any form of nucleic acid that is known. Thus, the nucleic acid may be a modified nucleic acid of the type used previously to enhance nuclease resistance and serum stability. Surprisingly, however, acceptable therapeutic products can also be prepared using the method of the invention to formulate lipid-nucleic acid particles from nucleic acids that have no modification to the phosphodiester linkages of natural nucleic acid polymers, and the use of unmodified phosphodiester nucleic acids (*i.e.*, nucleic acids in which all of the linkages are phosphodiester linkages) is a preferred embodiment of the invention.

#### 20 a. Backbone Modifications

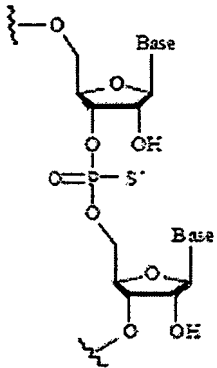
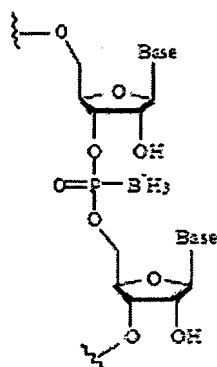
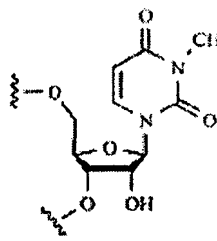
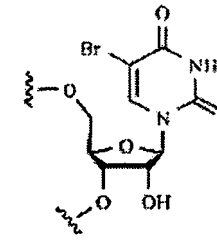
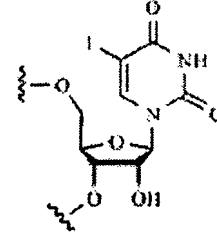
[0123] Antisense, siRNA, and other oligonucleotides useful in this invention include, but are not limited to, oligonucleotides containing modified backbones or non-natural internucleoside linkages. Oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. Modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides. Modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotri-esters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, phosphoroselenate, methylphosphonate, or O-alkyl phosphotriester linkages, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Particular non-limiting examples of particular modifications that may be present in a nucleic acid according to the present invention are shown in Table 2.

25 [0124] Various salts, mixed salts and free acid forms are also included. Representative United States patents that teach the preparation of the above linkages include, but are not limited to, U.S. Patent Nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050.

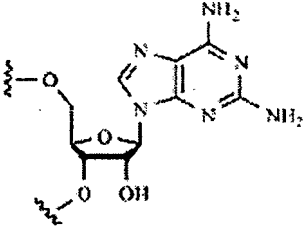
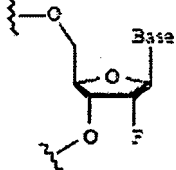
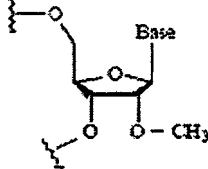
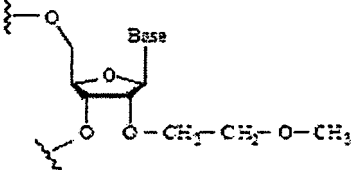
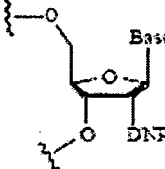
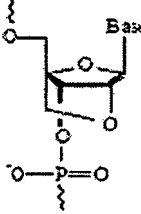
30 [0125] In certain embodiments, modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include, *e.g.*, those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH<sub>2</sub> component parts. Representative United States patents that describe the above oligonucleosides include, but are not limited to, U.S. Pat. Nos. 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439.

35 [0126] The phosphorothioate backbone modification (Table 2, #1), where a non-bridging oxygen in the phosphodiester bond is replaced by sulfur, is one of the earliest and most common means deployed to stabilize nucleic acid drugs against nuclease degradation. In general, it appears that PS modifications can be made extensively to both siRNA strands without much impact on activity (Kurreck, J., *Eur. J. Biochem.* 270:1628-44,2003). However, PS oligos are known to avidly associate non-specifically with proteins resulting in toxicity, especially upon *i.v.* administration. Therefore, the PS modification is usually restricted to one or two bases at the 3' and 5' ends. The boranophosphate linker (Table 2, #2) is a recent modification that is apparently more stable than PS, enhances siRNA activity and has low toxicity (Hall et al., *Nucleic Acids Res.* 32:5991-6000, 2004).

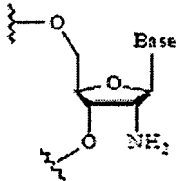
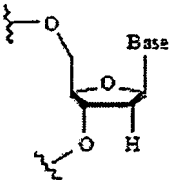
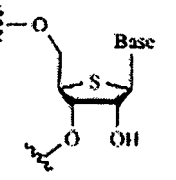
Table 2. Chemical Modifications Applied to siRNA and Other Nucleic Acids

#	Abbreviation	Name	Modification Site	Structure
1	PS	Phosphorothioate	Backbone	
2	PB	Boranophosphate	Backbone	
3	N3-MU	N3-methyl-uridine	Base	
4	5'-BU	5'-bromo-uracil	Base	
5	5'-IU	5'-iodo-uracil	Base	

(continued)

#	Abbreviation	Name	Modification Site	Structure
6	2,6-DP	2,6-diaminopurine	Base	
7	2'-F	2'-Fluoro	Sugar	
8	2'-OME	2'-O-methyl	Sugar	
9	2'-O-MOE	2'-O-(2-methoxyethyl)	Sugar	
10	2'-DNP	2'-O-(2,4-dinitrophenyl)	Sugar	
11	LNA	Locked Nucleic Acid (methylene bridge connecting the 2'-oxygen with the 4'-carbon of the ribose ring)	Sugar	

(continued)

#	Abbreviation	Name	Modification Site	Structure
12	2'-Amino	2'-Amino	Sugar	
13	2'-Deoxy	2'-Deoxy	Sugar	
14	4'-thio	4'-thio-ribonucleotide	Sugar	

**[0127]** Other useful nucleic acids derivatives include those nucleic acids molecules in which the bridging oxygen atoms (those forming the phosphoester linkages) have been replaced with -S-, -NH-, -CH<sub>2</sub>- and the like. In certain embodiments, the alterations to the antisense, siRNA, or other nucleic acids used will not completely affect the negative charges associated with the nucleic acids. Thus, the present invention contemplates the use of antisense, siRNA, and other nucleic acids in which a portion of the linkages are replaced with, for example, the neutral methyl phosphonate or phosphoramidate linkages. When neutral linkages are used, in certain embodiments, less than 80% of the nucleic acid linkages are so substituted, or less than 50% of the linkages are so substituted.

#### b. Base Modifications

**[0128]** Base modifications are less common than those to the backbone and sugar. The modifications shown in 0.3-6 all appear to stabilize siRNA against nucleases and have little effect on activity (Zhang, H.Y., et al., *Curr Top Med Chem* 6:893-900 (2006)).

**[0129]** Accordingly, oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C or m5c), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine.

**[0130]** Certain nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention, including 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2° C. (Sanghvi, Y. S., Croke, S. T. and Lebleu, B., eds., *Antisense Research and Applications* 1993, CRC Press, Boca Raton, pages 276-278). These may be combined, in particular embodiments, with 2'-O-methoxyethyl sugar modifications. United States patents that teach the preparation of certain of these modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. Pat. No. 3,687,808, as well as U.S. Pat. Nos. 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272;

5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; and 5,681,941.

### c. Sugar Modifications

**[0131]** Most modifications on the sugar group occur at the 2'-OH of the RNA sugar ring, which provides a convenient chemically reactive site (Manoharan, M., *Curr Opin Chem Biol* 8:570-9 (2004); Zhang, H.Y., et al., *Curr Top Med Chem* 6:893-900 (2006)). The 2'-F and 2'-OME are common and both increase stability, the 2'-OME modification does not reduce activity as long as it is restricted to less than 4 nucleotides per strand (Holen, T., et al., *Nucleic Acids Res* 31:2401-7 (2003)). The 2'-O-MOE is most effective in siRNA when modified bases are restricted to the middle region of the molecule (Prakash, T.P., et al., *J Med Chem* 48:4247-53 (2005)). Other modifications found to stabilize siRNA without loss of activity are shown in Table 2, 10-14.

**[0132]** Modified oligonucleotides may also contain one or more substituted sugar moieties. For example, the invention includes oligonucleotides that comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl, O-alkyl-O-alkyl, O-, S-, or N-alkenyl, or O-, S- or N-alkynyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C<sub>1</sub> to C<sub>10</sub> alkyl or C<sub>2</sub> to C<sub>10</sub> alkenyl and alkynyl. Particularly preferred are O[(CH<sub>2</sub>)<sub>n</sub>O]<sub>m</sub>CH<sub>3</sub>, O(CH<sub>2</sub>)<sub>n</sub>OCH<sub>3</sub>, O(CH<sub>2</sub>)<sub>2</sub>ON(CH<sub>3</sub>)<sub>2</sub>, O(CH<sub>2</sub>)<sub>n</sub>NH<sub>2</sub>, O(CH<sub>2</sub>)<sub>n</sub>CH<sub>3</sub>, O(CH<sub>2</sub>)<sub>n</sub>ONH<sub>2</sub>, and O(CH<sub>2</sub>)<sub>n</sub>ON[(CH<sub>2</sub>)<sub>n</sub>CH<sub>3</sub>]<sub>2</sub>, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C<sub>1</sub> to C<sub>10</sub> lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH<sub>3</sub>, OCN, Cl, Br, CN, CF<sub>3</sub>, OCF<sub>3</sub>, SOCH<sub>3</sub>, SO<sub>2</sub>CH<sub>3</sub>, ONO<sub>2</sub>, NO<sub>2</sub>, N<sub>3</sub>, NH<sub>2</sub>, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. One modification includes 2'-methoxyethoxy (2'-O--CH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta* 1995, 78, 486-504), *i.e.*, an alkoxyalkoxy group. Other modifications include 2'-dimethylaminoxyethoxy, *i.e.*, a O(CH<sub>2</sub>)<sub>2</sub>ON(CH<sub>3</sub>)<sub>2</sub> group, also known as 2'-DMAOE, and 2'-dimethylaminoethoxyethoxy (2'-DMAEOE).

**[0133]** Additional modifications include 2'-methoxy (2'-O-CH<sub>3</sub>), 2'-aminopropoxy (2'-OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugars structures include, but are not limited to, U.S. Pat. Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920.

**[0134]** In other oligonucleotide mimetics, both the sugar and the internucleoside linkage, *i.e.*, the backbone, of the nucleotide units are replaced with novel groups, although the base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Pat. Nos. 5,539,082; 5,714,331; and 5,719,262. Further teaching of PNA compounds can be found in Nielsen et al., *Science* 254, 1497-1500 (1991).

**[0135]** Particular embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular --CH<sub>2</sub>--NH--O--CH<sub>2</sub>--, --CH<sub>2</sub>--N(CH<sub>3</sub>)--O--CH<sub>2</sub>-- (referred to as a methylene (methylimino) or MMI backbone) --CH<sub>2</sub>--O--N(CH<sub>3</sub>)--CH<sub>2</sub>--, --CH<sub>2</sub>--N(CH<sub>3</sub>)--N(CH<sub>3</sub>)--CH<sub>2</sub>-- and --O--N(CH<sub>3</sub>)--CH<sub>2</sub>--CH<sub>2</sub>--(wherein the native phosphodiester backbone is represented as --O--P--O--CH<sub>2</sub>--) of the above referenced U.S. Pat. No. 5,489,677, and the amide backbones of the above referenced U.S. Pat. No. 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. Pat. No. 5,034,506.

### d. Chimeric Oligonucleotides

**[0136]** It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. Certain preferred oligonucleotides of this invention are chimeric oligonucleotides. "Chimeric oligonucleotides" or "chimeras," in the context of this invention, are oligonucleotides that contain two or more chemically distinct regions, each made up of at least one nucleotide. These oligonucleotides typically contain at least one region of modified nucleotides that confers one or more beneficial properties (such as, e.g., increased nuclease resistance, increased

uptake into cells, increased binding affinity for the RNA target) and a region that is a substrate for RNase H cleavage.

**[0137]** In one embodiment, a chimeric oligonucleotide comprises at least one region modified to increase target binding affinity. Affinity of an oligonucleotide for its target is routinely determined by measuring the  $T_m$  of an oligonucleotide/target pair, which is the temperature at which the oligonucleotide and target dissociate; dissociation is detected spectrophotometrically. The higher the  $T_m$ , the greater the affinity of the oligonucleotide for the target. In one embodiment, the region of the oligonucleotide which is modified to increase target mRNA binding affinity comprises at least one nucleotide modified at the 2' position of the sugar, most preferably a 2'-O-alkyl, 2'-O-alkyl-O-alkyl or 2'-fluoro-modified nucleotide. Such modifications are routinely incorporated into oligonucleotides and these oligonucleotides have been shown to have a higher  $T_m$  (*i.e.*, higher target binding affinity) than 2'-deoxyoligonucleotides against a given target. The effect of such increased affinity is to greatly enhance oligonucleotide inhibition of target gene expression.

**[0138]** In another embodiment, a chimeric oligonucleotide comprises a region that acts as a substrate for RNase H. Of course, it is understood that oligonucleotides may include any combination of the various modifications described herein

**[0139]** Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such conjugates and methods of preparing the same are known in the art.

**[0140]** Those skilled in the art will realize that for *in vivo* utility, such as therapeutic efficacy, a reasonable rule of thumb is that if a thioated version of the sequence works in the free form, that encapsulated particles of the same sequence, of any chemistry, will also be efficacious. Encapsulated particles may also have a broader range of *in vivo* utilities, showing efficacy in conditions and models not known to be otherwise responsive to antisense therapy. Those skilled in the art know that applying this invention they may find old models which now respond to antisense therapy. Further, they may revisit discarded antisense sequences or chemistries and find efficacy by employing the invention.

**[0141]** The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including Applied Biosystems. Any other means for such synthesis may also be employed; the actual synthesis of the oligonucleotides is well within the talents of the routineer. It is also well known to use similar techniques to prepare other oligonucleotides such as the phosphorothioates and alkylated derivatives.

#### Characteristic of Nucleic Acid-Lipid Particles

**[0142]** In certain embodiments, the present invention relates to methods and compositions for producing lipid-encapsulated nucleic acid particles in which nucleic acids are encapsulated within a lipid layer. Such nucleic acid-lipid particles, incorporating siRNA oligonucleotides, are characterized using a variety of biophysical parameters including: (1) drug to lipid ratio; (2) encapsulation efficiency; and (3) particle size. High drug to lipid ratios, high encapsulation efficiency, good nuclease resistance and serum stability and controllable particle size, generally less than 200 nm in diameter are desirable. In addition, the nature of the nucleic acid polymer is of significance, since the modification of nucleic acids in an effort to impart nuclease resistance adds to the cost of therapeutics while in many cases providing only limited resistance. Unless stated otherwise, these criteria are calculated in this specification as follows:

Nucleic acid to lipid ratio is the amount of nucleic acid in a defined volume of preparation divided by the amount of lipid in the same volume. This may be on a mole per mole basis or on a weight per weight basis, or on a weight per mole basis. For final, administration-ready formulations, the nucleic acid:lipid ratio is calculated after dialysis, chromatography and/or enzyme (*e.g.*, nuclease) digestion has been employed to remove as much of the external nucleic acid as possible;

Encapsulation efficiency refers to the drug to lipid ratio of the starting mixture divided by the drug to lipid ratio of the final, administration competent formulation. This is a measure of relative efficiency. For a measure of absolute efficiency, the total amount of nucleic acid added to the starting mixture that ends up in the administration competent formulation, can also be calculated. The amount of lipid lost during the formulation process may also be calculated. Efficiency is a measure of the wastage and expense of the formulation; and

Size indicates the size (diameter) of the particles formed. Size distribution may be determined using quasi-elastic light scattering (QELS) on a Nicomp Model 370 sub-micron particle sizer. Particles under 200 nm are preferred for distribution to neo-vascularized (leaky) tissues, such as neoplasms and sites of inflammation.

#### Pharmaceutical Compositions

**[0143]** The lipid particles of present invention, particularly when associated with a therapeutic agent, may be formulated as a pharmaceutical composition, *e.g.*, which further comprises a pharmaceutically acceptable diluent, excipient, or carrier, such as physiological saline or phosphate buffer, selected in accordance with the route of administration and

standard pharmaceutical practice.

**[0144]** In particular embodiments, pharmaceutical compositions comprising the lipid-nucleic acid particles of the invention are prepared according to standard techniques and further comprise a pharmaceutically acceptable carrier. Generally, normal saline will be employed as the pharmaceutically acceptable carrier. Other suitable carriers include, e.g., water, buffered water, 0.9% saline, 0.3% glycine, and the like, including glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, etc. In compositions comprising saline or other salt containing carriers, the carrier is preferably added following lipid particle formation. Thus, after the lipid-nucleic acid compositions are formed, the compositions can be diluted into pharmaceutically acceptable carriers such as normal saline.

**[0145]** The resulting pharmaceutical preparations may be sterilized by conventional, well known sterilization techniques. The aqueous solutions can then be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, etc. Additionally, the lipidic suspension may include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as  $\alpha$ -tocopherol and water-soluble iron-specific chelators, such as ferrioxamine, are suitable.

**[0146]** The concentration of lipid particle or lipid-nucleic acid particle in the pharmaceutical formulations can vary widely, i.e., from less than about 0.01 %, usually at or at least about 0.05-5% to as much as 10 to 30% by weight and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected. For example, the concentration may be increased to lower the fluid load associated with treatment. This may be particularly desirable in patients having atherosclerosis-associated congestive heart failure or severe hypertension. Alternatively, complexes composed of irritating lipids may be diluted to low concentrations to lessen inflammation at the site of administration. In one group of embodiments, the nucleic acid will have an attached label and will be used for diagnosis (by indicating the presence of complementary nucleic acid). In this instance, the amount of complexes administered will depend upon the particular label used, the disease state being diagnosed and the judgement of the clinician but will generally be between about 0.01 and about 50 mg per kilogram of body weight, preferably between about 0.1 and about 5 mg/kg of body weight.

**[0147]** As noted above, the nucleic acid-lipid particles of the invention may include polyethylene glycol (PEG)-modified phospholipids, PEG-ceramide, or ganglioside  $G_{M1}$ -modified lipids or other lipids effective to prevent or limit aggregation. Addition of such components does not merely prevent complex aggregation. Rather, it may also provide a means for increasing circulation lifetime and increasing the delivery of the lipid-nucleic acid composition to the target tissues.

**[0148]** The present invention also provides lipid-therapeutic agent compositions in kit form. The kit will typically be comprised of a container that is compartmentalized for holding the various elements of the kit. The kit will contain the particles or pharmaceutical compositions of the present invention, preferably in dehydrated or concentrated form, with instructions for their rehydration or dilution and administration. In certain embodiments, the particles comprise the active agent, while in other embodiments, they do not.

#### D. Methods of Manufacture

**[0149]** The methods and compositions of the invention make use of certain cationic lipids, the synthesis, preparation and characterization of which is described below and in the accompanying Examples. In addition, the present invention provides methods of preparing lipid particles, including those associated with a therapeutic agent, e.g., a nucleic acid. Generally, any method of preparing nucleic acid-lipid particles may be used according to the present invention by using one or more of the lipids of the present invention in the resulting nucleic acid-lipid particles. Examples of suitable methods are known in the art and described, e.g., in U.S. Patent Application Publication No. 2006/0134189.

**[0150]** In one embodiment, a mixture of lipids is combined with a buffered aqueous solution of nucleic acid to produce an intermediate mixture containing nucleic acid encapsulated in lipid particles wherein the encapsulated nucleic acids are present in a nucleic acid/lipid ratio of about 3 wt% to about 25 wt%, preferably 5 to 15 wt%. The intermediate mixture may optionally be sized to obtain lipid-encapsulated nucleic acid particles wherein the lipid portions are unilamellar vesicles, preferably having a diameter of 30 to 150 nm, more preferably about 40 to 90 nm. The pH is then raised to neutralize at least a portion of the surface charges on the lipid-nucleic acid particles, thus providing an at least partially surface-neutralized lipid-encapsulated nucleic acid composition.

**[0151]** As described above, several of these cationic lipids are amino lipids that are charged at a pH below the  $pK_a$  of the amino group and substantially neutral at a pH above the  $pK_a$ . These cationic lipids are termed titratable cationic lipids and can be used in the formulations of the invention using a two-step process. First, lipid vesicles can be formed at the lower pH with titratable cationic lipids and other vesicle components in the presence of nucleic acids. In this manner, the vesicles will encapsulate and entrap the nucleic acids. Second, the surface charge of the newly formed vesicles can be neutralized by increasing the pH of the medium to a level above the  $pK_a$  of the titratable cationic lipids

present, *i.e.*, to physiological pH or higher. Without intending to be bound by any particular theory, it is believed that the very high efficiency of nucleic acid encapsulation is a result of electrostatic interaction at low pH. At acidic pH (e.g. pH 4.0) the vesicle surface is charged and binds a portion of the nucleic acids through electrostatic interactions. When the external acidic buffer is exchanged for a more neutral buffer (e.g., pH 7.5) the surface of the lipid particle or liposome is neutralized, allowing any external nucleic acid to be removed. More detailed information on the formulation process is provided in various publications (e.g., US Patent 6,287,591 and US Patent 6,858,225). Particularly advantageous aspects of this process include both the facile removal of any surface adsorbed nucleic acid and a resultant nucleic acid delivery vehicle which has a neutral surface. Liposomes or lipid particles having a neutral surface are expected to avoid rapid clearance from circulation and to avoid certain toxicities which are associated with cationic liposome preparations. It is further noted that the vesicles formed in this manner provide formulations of uniform vesicle size with high content of nucleic acids. Additionally, the vesicles have a size range of from about 30 to about 150 nm, more preferably about 30 to about 90 nm. Additional details concerning these uses of such titratable cationic lipids in the formulation of nucleic acid-lipid particles are provided in US Patent 6,287,591 and US Patent 6,858,225.

**[0152]** In certain embodiments, the mixture of lipids includes at least two lipid components: a first amino lipid component of the present invention that is selected from among lipids which have a pKa such that the lipid is cationic at pH below the pKa and neutral at pH above the pKa, and a second lipid component that is selected from among lipids that prevent particle aggregation during lipid-nucleic acid particle formation. In particular embodiments, the amino lipid is a novel cationic lipid of the present invention.

**[0153]** In certain embodiments of preparing the nucleic acid-lipid particles of the invention, the mixture of lipids is typically a solution of lipids in an organic solvent. This mixture of lipids can then be dried to form a thin film or lyophilized to form a powder before being hydrated with an aqueous buffer to form liposomes. Alternatively, in a preferred method, the lipid mixture can be solubilized in a water miscible alcohol, such as ethanol, and this ethanolic solution added to an aqueous buffer resulting in spontaneous liposome formation. In most embodiments, the alcohol is used in the form in which it is commercially available. For example, ethanol can be used as absolute ethanol (100%), or as 95% ethanol, the remainder being water. This method is described in more detail in US Patent 5,976,567.

**[0154]** In one exemplary embodiment, the mixture of lipids is a mixture of cationic amino lipids, neutral lipids (other than an amino lipid), a sterol (e.g., cholesterol) and a PEG-modified lipid (e.g., a PEG-S-DMG, PEG-C-DOMG or PEG-DMA) in an alcohol solvent. In certain embodiments, the lipid mixture consists essentially of a cationic amino lipid, a neutral lipid, cholesterol and a PEG-modified lipid in alcohol, more preferably ethanol. In certain embodiments, the first solution consists of the above lipid mixture in molar ratios of about 20-70% amino lipid: 5-45% neutral lipid:20-55% cholesterol:0.5-15% PEG-modified lipid. In other embodiments, the first solution consists essentially of DLin-K-C2-DMA, DSPC, Chol and PEG-S-DMG, PEG-C-DOMG or PEG-DMA, more preferably in a molar ratio of about 20-60% DLin-K-C2-DMA: 5-25% DSPC:25-55% Chol:0.5-15% PEG-S-DMG, PEG-C-DOMG or PEG-DMA. In another group of embodiments, the neutral lipid in these compositions is replaced with POPC, DOPE or SM.

**[0155]** In certain embodiments in accordance with the invention, the lipid mixture is combined with a buffered aqueous solution that may contain the nucleic acids. The buffered aqueous solution of is typically a solution in which the buffer has a pH of less than the pK<sub>a</sub> of the protonatable lipid in the lipid mixture. Examples of suitable buffers include citrate, phosphate, acetate, and MES. A particularly preferred buffer is citrate buffer. Preferred buffers will be in the range of 1-1000 mM of the anion, depending on the chemistry of the nucleic acid being encapsulated, and optimization of buffer concentration may be significant to achieving high loading levels (see, e.g., US Patent 6,287,591 and US Patent 6,858,225). Alternatively, pure water acidified to pH 5-6 with chloride, sulfate or the like may be useful. In this case, it may be suitable to add 5% glucose, or another non-ionic solute which will balance the osmotic potential across the particle membrane when the particles are dialyzed to remove ethanol, increase the pH, or mixed with a pharmaceutically acceptable carrier such as normal saline. The amount of nucleic acid in buffer can vary, but will typically be from about 0.01 mg/mL to about 200 mg/mL, more preferably from about 0.5 mg/mL to about 50 mg/mL.

**[0156]** The mixture of lipids and the buffered aqueous solution of therapeutic nucleic acids is combined to provide an intermediate mixture. The intermediate mixture is typically a mixture of lipid particles having encapsulated nucleic acids. Additionally, the intermediate mixture may also contain some portion of nucleic acids which are attached to the surface of the lipid particles (liposomes or lipid vesicles) due to the ionic attraction of the negatively-charged nucleic acids and positively-charged lipids on the lipid particle surface (the amino lipids or other lipid making up the protonatable first lipid component are positively charged in a buffer having a pH of less than the pK<sub>a</sub> of the protonatable group on the lipid). In one group of preferred embodiments, the mixture of lipids is an alcohol solution of lipids and the volumes of each of the solutions is adjusted so that upon combination, the resulting alcohol content is from about 20% by volume to about 45% by volume. The method of combining the mixtures can include any of a variety of processes, often depending upon the scale of formulation produced. For example, when the total volume is about 10-20 mL or less, the solutions can be combined in a test tube and stirred together using a vortex mixer. Large-scale processes can be carried out in suitable production scale glassware.

**[0157]** Optionally, the lipid-encapsulated therapeutic agent (e.g., nucleic acid) complexes which are produced by

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

**[0158]** Extrusion of liposome compositions through a small-pore polycarbonate membrane or an asymmetric ceramic membrane results in a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired liposome complex size distribution is achieved. The liposomes may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in liposome size. In some instances, the lipid-nucleic acid compositions which are formed can be used without any sizing.

**[0159]** In particular embodiments, methods of the present invention further comprise a step of neutralizing at least some of the surface charges on the lipid portions of the lipid-nucleic acid compositions. By at least partially neutralizing the surface charges, unencapsulated nucleic acid is freed from the lipid particle surface and can be removed from the composition using conventional techniques. Preferably, unencapsulated and surface adsorbed nucleic acids are removed from the resulting compositions through exchange of buffer solutions. For example, replacement of a citrate buffer (pH about 4.0, used for forming the compositions) with a HEPES-buffered saline (HBS pH about 7.5) solution, results in the neutralization of liposome surface and nucleic acid release from the surface. The released nucleic acid can then be removed via chromatography using standard methods, and then switched into a buffer with a pH above the pKa of the lipid used.

**[0160]** Optionally the lipid vesicles (*i.e.*, lipid particles) can be formed by hydration in an aqueous buffer and sized using any of the methods described above prior to addition of the nucleic acid, according to the preformed vesicle (PFV) method. As described above, the aqueous buffer should be of a pH below the pKa of the amino lipid. A solution of the nucleic acids can then be added to these sized, preformed vesicles. To allow encapsulation of nucleic acids into such "pre-formed" vesicles the mixture should contain an alcohol, such as ethanol. In the case of ethanol, it should be present at a concentration of about 20% (w/w) to about 45% (w/w). In addition, it may be necessary to warm the mixture of preformed vesicles and nucleic acid in the aqueous buffer-ethanol mixture to a temperature of about 25° C to about 50° C depending on the composition of the lipid vesicles and the nature of the nucleic acid. It will be apparent to one of ordinary skill in the art that optimization of the encapsulation process to achieve a desired level of nucleic acid in the lipid vesicles will require manipulation of variable such as ethanol concentration and temperature. Examples of suitable conditions for nucleic acid encapsulation are provided in the Examples. Once the nucleic acids are encapsulated within the preformed vesicles, the external pH can be increased to at least partially neutralize the surface charge. Unencapsulated and surface adsorbed nucleic acids can then be removed as described above.

**[0161]** In other embodiments, nucleic acid lipid particles are prepared via a continuous mixing method, *e.g.*, a process that includes providing an aqueous solution comprising a nucleic acid such as an siRNA, in a first reservoir, and providing an organic lipid solution in a second reservoir, and mixing the aqueous solution with the organic lipid solution such that the organic solution mixes with the aqueous solution so as to substantially instantaneously produce a liposome encapsulating the nucleic acid. This process and the apparatus for carrying out this process are described in detail in U.S. Patent Application Publication No. 2004/0142025.

**[0162]** In another embodiment, nucleic acid lipid particles are produced via a direct dilution process that includes forming a liposome solution and immediately and directly introducing the liposome solution to a collection vessel containing a controlled amount of dilution buffer. In certain embodiments, the collection vessel includes one or more elements configured to stir the contents of the collection vessel to facilitate dilution. In other embodiment, a third reservoir containing dilution buffer is fluidly coupled to a second mixing region. In this embodiment, the liposome solution formed in the first mixing region is immediately and directly mixed with the dilution buffer in the second mixing region. Processes and apparatus for carrying out these direct dilution methods are described in further detail in U.S. Patent Application Publication No. 2007/0042031.

#### E. Method of Use

**[0163]** The lipid particles of the present invention may be used to deliver a therapeutic agent to a cell, *in vitro* or *in vivo*. In particular embodiments, the therapeutic agent is a nucleic acid, which is delivered to a cell using a nucleic acid-lipid particles of the present invention. While the following description of various methods of using the lipid particles and

related pharmaceutical compositions of the present invention are exemplified by description related to nucleic acid-lipid particles, it is understood that these methods and compositions may be readily adapted for the delivery of any therapeutic agent for the treatment of any disease or disorder that would benefit from such treatment.

5 **[0164]** In certain embodiments, the present invention relates to methods for introducing a nucleic acid into a cell. Preferred nucleic acids for introduction into cells are siRNA, immune-stimulating oligonucleotides, plasmids, antisense oligonucleotides, and ribozymes. These methods may be carried out by contacting the particles or compositions of the present invention with the cells for a period of time sufficient for intracellular delivery to occur.

10 **[0165]** The compositions of the present invention can be adsorbed to almost any cell type. Once adsorbed, the nucleic acid-lipid particles can either be endocytosed by a portion of the cells, exchange lipids with cell membranes, or fuse with the cells. Transfer or incorporation of the nucleic acid portion of the complex can take place via any one of these pathways. Without intending to be limited with respect to the scope of the invention, it is believed that in the case of particles taken up into the cell by endocytosis the particles then interact with the endosomal membrane, resulting in destabilization of the endosomal membrane, possibly by the formation of non-bilayer phases, resulting in introduction of the encapsulated nucleic acid into the cell cytoplasm. Similarly in the case of direct fusion of the particles with the cell plasma membrane, when fusion takes place, the liposome membrane is integrated into the cell membrane and the contents of the liposome combine with the intracellular fluid. Contact between the cells and the lipid-nucleic acid compositions, when carried out *in vitro*, will take place in a biologically compatible medium. The concentration of compositions can vary widely depending on the particular application, but is generally between about 1  $\mu\text{mol}$  and about 10 mmol. In certain embodiments, treatment of the cells with the lipid-nucleic acid compositions will generally be carried out at physiological temperatures, 15 (about 37°C) for periods of time from about 1 to 24 hours, preferably from about 2 to 8 hours. For *in vitro* applications, the delivery of nucleic acids can be to any cell grown in culture, whether of plant or animal origin, vertebrate or invertebrate, and of any tissue or type. In preferred embodiments, the cells will be animal cells, more preferably mammalian cells, and most preferably human cells.

20 **[0166]** In one group of embodiments, a lipid-nucleic acid particle suspension is added to 60-80% confluent plated cells having a cell density of from about  $10^3$  to about  $10^5$  cells/mL, more preferably about  $2 \times 10^4$  cells/mL. The concentration of the suspension added to the cells is preferably of from about 0.01 to 20  $\mu\text{g/mL}$ , more preferably about 1  $\mu\text{g/mL}$ .

25 **[0167]** Typical applications include using well known procedures to provide intracellular delivery of siRNA to knock down or silence specific cellular targets. Alternatively applications include delivery of DNA or mRNA sequences that code for therapeutically useful polypeptides. In this manner, therapy is provided for genetic diseases by supplying deficient or absent gene products (*i.e.*, for Duchenne's dystrophy, see Kunkel, et al., Brit. Med. Bull. 45(3):630-643 (1989), and for cystic fibrosis, see Goodfellow, Nature 341:102-103 (1989)). Other uses for the compositions of the present invention include introduction of antisense oligonucleotides in cells (see, Bennett, et al., Mol. Pharm. 41:1023-1033 (1992)).

30 **[0168]** Alternatively, the compositions of the present invention can also be used for deliver of nucleic acids to cells *in vivo*, using methods which are known to those of skill in the art. With respect to application of the invention for delivery of DNA or mRNA sequences, Zhu, et al., Science 261:209-211 (1993), describes the intravenous delivery of cytomegalovirus (CMV)-chloramphenicol acetyltransferase (CAT) expression plasmid using DOTMA-DOPE complexes. Hyde, et al., Nature 362:250-256 (1993), describes the delivery of the cystic fibrosis transmembrane conductance regulator (CFTR) gene to epithelia of the airway and to alveoli in the lung of mice, using liposomes. Brigham, et al., Am. J. Med. Sci. 298:278-281 (1989), describes the *in vivo* transfection of lungs of mice with a functioning prokaryotic gene encoding the intracellular enzyme, chloramphenicol acetyltransferase (CAT). Thus, the compositions of the invention can be used in the treatment of infectious diseases.

35 **[0169]** For *in vivo* administration, the pharmaceutical compositions are preferably administered parenterally, *i.e.*, intraarticularly, intravenously, intraperitoneally, subcutaneously, or intramuscularly. In particular embodiments, the pharmaceutical compositions are administered intravenously or intraperitoneally by a bolus injection. For one example, see Stadler, et al., U.S. Patent No. 5,286,634. Intracellular nucleic acid delivery has also been discussed in Straubinger, et al., METHODS IN ENZYMOLOGY, Academic Press, New York. 101:512-527 (1983); Mannino, et al., Biotechniques 6:682-690 (1988); Nicolau, et al., Crit. Rev. Ther. Drug Carrier Syst. 6:239-271 (1989), and Behr, Acc. Chem. Res. 26:274-278 (1993). Still other methods of administering lipid-based therapeutics are described in, for example, Rahman et al., U.S. Patent No. 3,993,754; Sears, U.S. Patent No. 4,145,410; Papahadjopoulos et al., U.S. Patent No. 4,235,871; Schneider, U.S. Patent No. 4,224,179; Lenk et al., U.S. Patent No. 4,522,803; and Fountain et al., U.S. Patent No. 4,588,578.

40 **[0170]** In other methods, the pharmaceutical preparations may be contacted with the target tissue by direct application of the preparation to the tissue. The application may be made by topical, "open" or "closed" procedures. By "topical," it is meant the direct application of the pharmaceutical preparation to a tissue exposed to the environment, such as the skin, oropharynx, external auditory canal, and the like. "Open" procedures are those procedures which include incising the skin of a patient and directly visualizing the underlying tissue to which the pharmaceutical preparations are applied. This is generally accomplished by a surgical procedure, such as a thoracotomy to access the lungs, abdominal laparotomy

to access abdominal viscera, or other direct surgical approach to the target tissue. "Closed" procedures are invasive procedures in which the internal target tissues are not directly visualized, but accessed via inserting instruments through small wounds in the skin. For example, the preparations may be administered to the peritoneum by needle lavage. Likewise, the pharmaceutical preparations may be administered to the meninges or spinal cord by infusion during a lumbar puncture followed by appropriate positioning of the patient as commonly practiced for spinal anesthesia or metrazamide imaging of the spinal cord. Alternatively, the preparations may be administered through endoscopic devices.

**[0171]** The lipid-nucleic acid compositions can also be administered in an aerosol inhaled into the lungs (see, Brigham, et al., *Am. J. Sci.* 298(4):278-281 (1989)) or by direct injection at the site of disease (Culver, *Human Gene Therapy*, MaryAnn Liebert, Inc., Publishers, New York. pp.70-71 (1994)).

**[0172]** The methods of the present invention may be practiced in a variety of hosts. Preferred hosts include mammalian species, such as humans, non-human primates, dogs, cats, cattle, horses, sheep, and the like.

**[0173]** Dosages for the lipid-therapeutic agent particles of the present invention will depend on the ratio of therapeutic agent to lipid and the administering physician's opinion based on age, weight, and condition of the patient.

**[0174]** In one embodiment, the present invention relates to a method of modulating the expression of a target polynucleotide or polypeptide. These methods generally comprise contacting a cell with a lipid particle of the present invention that is associated with a nucleic acid capable of modulating the expression of a target polynucleotide or polypeptide. As used herein, the term "modulating" refers to altering the expression of a target polynucleotide or polypeptide. In different embodiments, modulating can mean increasing or enhancing, or it can mean decreasing or reducing. Methods of measuring the level of expression of a target polynucleotide or polypeptide are known and available in the arts and include, e.g., methods employing reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemical techniques. In particular embodiments, the level of expression of a target polynucleotide or polypeptide is increased or reduced by at least 10%, 20%, 30%, 40%, 50%, or greater than 50% as compared to an appropriate control value.

**[0175]** For example, if increased expression of a polypeptide desired, the nucleic acid may be an expression vector that includes a polynucleotide that encodes the desired polypeptide. On the other hand, if reduced expression of a polynucleotide or polypeptide is desired, then the nucleic acid may be, e.g., an antisense oligonucleotide, siRNA, or microRNA that comprises a polynucleotide sequence that specifically hybridizes to a polynucleotide that encodes the target polypeptide, thereby disrupting expression of the target polynucleotide or polypeptide. Alternatively, the nucleic acid may be a plasmid that expresses such an antisense oligonucleotide, siRNA, or microRNA.

**[0176]** In one particular embodiment, the present invention relates to a method of modulating the expression of a polypeptide by a cell, comprising providing to a cell a lipid particle that consists of or consists essentially of DLin-K-C2-DMA, DSPC, Chol and PEG-S-DMG, PEG-C-DMG or PEG-DMA, e.g., in a molar ratio of about 20-60% DLin-K-C2-DMA: 5-25% DSPC:25-55% Chol:0.5-15% PEG-S-DMG, PEG-C-DMG or PEG-DMA, wherein the lipid particle is associated with a nucleic acid capable of modulating the expression of the polypeptide. In particular embodiments, the molar lipid ratio is approximately 40/10/40/10 (mol% DLin-K-C2-DMA/DSPC/Chol/PEG-S-DMG). In another group of embodiments, the neutral lipid in these compositions is replaced with POPC, DOPE or SM. In other embodiments, the cationic lipid is replaced with DLin-K<sup>2</sup>-DMA or DLin-K<sub>6</sub>-DMA.

**[0177]** In particular embodiments, the therapeutic agent is selected from an siRNA, a microRNA, an antisense oligonucleotide, and a plasmid capable of expressing an siRNA, a microRNA, or an antisense oligonucleotide, and wherein the siRNA, microRNA, or antisense RNA comprises a polynucleotide that specifically binds to a polynucleotide that encodes the polypeptide, or a complement thereof, such that the expression of the polypeptide is reduced.

**[0178]** In other embodiments, the nucleic acid is a plasmid that encodes the polypeptide or a functional variant or fragment thereof, such that expression of the polypeptide or the functional variant or fragment thereof is increased.

**[0179]** In related embodiments, the present invention relates to a method of treating a disease or disorder characterized by overexpression of a polypeptide in a subject, comprising providing to the subject a pharmaceutical composition of the present invention, wherein the therapeutic agent is selected from an siRNA, a microRNA, an antisense oligonucleotide, and a plasmid capable of expressing an siRNA, a microRNA, or an antisense oligonucleotide, and wherein the siRNA, microRNA, or antisense RNA comprises a polynucleotide that specifically binds to a polynucleotide that encodes the polypeptide, or a complement thereof.

**[0180]** In one embodiment, the pharmaceutical composition comprises a lipid particle that consists of or consists essentially of DLin-K-C2-DMA, DSPC, Chol and PEG-S-DMG, PEG-C-DMG or PEG-DMA, e.g., in a molar ratio of about 20-60% DLin-K-C2-DMA: 5-25% DSPC:25-55% Chol:0.5-15% PEG-S-DMG, PEG-C-DMG or PEG-DMA, wherein the lipid particle is associated with the therapeutic nucleic acid. In particular embodiments, the molar lipid ratio is approximately 40/10/40/10 (mol% DLin-K-C2-DMA/DSPC/Chol/PEG-S-DMG). In another group of embodiments, the neutral lipid in these compositions is replaced with POPC, DOPE or SM. In other embodiments, the cationic lipid is replaced with DLin-K<sup>2</sup>-DMA or DLin-K<sub>6</sub>-DMA.

**[0181]** In another related embodiment, the present invention relates to a method of treating a disease or disorder characterized by underexpression of a polypeptide in a subject, comprising providing to the subject a pharmaceutical composition of the present invention, wherein the therapeutic agent is a plasmid that encodes the polypeptide or a

functional variant or fragment thereof.

**[0182]** In one embodiment, the pharmaceutical composition comprises a lipid particle that consists of or consists essentially of DLin-K-C2-DMA, DSPC, Chol and PEG-S-DMG, PEG-C-DOMG or PEG-DMA, *e.g.*, in a molar ratio of about 20-60% DLin-K-C2-DMA: 5-25% DSPC:25-55% Chol:0.5-15% PEG-S-DMG or PEG-DMA, wherein the lipid particle is associated with the therapeutic nucleic acid. In particular embodiments, the molar lipid ratio is approximately 40/10/40/10 (mol% DLin-K-C2-DMA/DSPC/Chol/PEG-S-DMG). In another group of embodiments, the neutral lipid in these compositions is replaced with POPC, DOPE or SM. In other embodiments, the cationic lipid is replaced with DLin-K<sup>2</sup>-DMA or DLin-K6-DMA.

**[0183]** The present invention further relates to a method of inducing an immune response in a subject, comprising providing to the subject the pharmaceutical composition of the present invention, wherein the therapeutic agent is an immunostimulatory oligonucleotide. In certain embodiments, the immune response is a humoral or mucosal immune response. In one embodiment, the pharmaceutical composition comprises a lipid particle that consists of or consists essentially of DLin-K-C2-DMA, DSPC, Chol and PEG-S-DMG, PEG-C-DOMG or PEG-DMA, *e.g.*, in a molar ratio of about 20-60% DLin-K-C2-DMA: 5-25% DSPC:25-55% Chol:0.5-15% PEG-S-DMG, PEG-C-DOMG or PEG-DMA, wherein the lipid particle is associated with the therapeutic nucleic acid. In particular embodiments, the molar lipid ratio is approximately 40/10/40/10 (mol% DLin-K-C2-DMA/DSPC/Chol/PEG-S-DMG, PEG-C-DOMG or PEG-DMA). In another group of embodiments, the neutral lipid in these compositions is replaced with POPC, DOPE or SM. In other embodiments, the cationic lipid is replaced with DLin-K<sup>2</sup>-DMA or DLin-K6-DMA.

**[0184]** In further embodiments, the pharmaceutical composition is provided to the subject in combination with a vaccine or antigen. Thus, the present invention itself provides vaccines comprising a lipid particle of the present invention, which comprises an immunostimulatory oligonucleotide, and is also associated with an antigen to which an immune response is desired. In particular embodiments, the antigen is a tumor antigen or is associated with an infective agent, such as, *e.g.*, a virus, bacteria, or parasite.

**[0185]** A variety of tumor antigens, infectious agent antigens, and antigens associated with other disease are well known in the art and examples of these are described in references cited herein. Examples of antigens suitable for use in the present invention include, but are not limited to, polypeptide antigens and DNA antigens. Specific examples of antigens are Hepatitis A, Hepatitis B, small pox, polio, anthrax, influenza, typhus, tetanus, measles, rotavirus, diphtheria, pertussis, tuberculosis, and rubella antigens. In a preferred embodiment, the antigen is a Hepatitis B recombinant antigen. In other aspects, the antigen is a Hepatitis A recombinant antigen. In another aspect, the antigen is a tumor antigen. Examples of such tumor-associated antigens are MUC-1, EBV antigen and antigens associated with Burkitt's lymphoma. In a further aspect, the antigen is a tyrosinase-related protein tumor antigen recombinant antigen. Those of skill in the art will know of other antigens suitable for use in the present invention.

**[0186]** Tumor-associated antigens suitable for use in the subject invention include both mutated and non-mutated molecules that may be indicative of single tumor type, shared among several types of tumors, and/or exclusively expressed or overexpressed in tumor cells in comparison with normal cells. In addition to proteins and glycoproteins, tumor-specific patterns of expression of carbohydrates, gangliosides, glycolipids and mucins have also been documented. Exemplary tumor-associated antigens for use in the subject cancer vaccines include protein products of oncogenes, tumor suppressor genes and other genes with mutations or rearrangements unique to tumor cells, reactivated embryonic gene products, oncofetal antigens, tissue-specific (but not tumor-specific) differentiation antigens, growth factor receptors, cell surface carbohydrate residues, foreign viral proteins and a number of other self proteins.

**[0187]** Specific embodiments of tumor-associated antigens include, *e.g.*, mutated antigens such as the protein products of the Ras p21 protooncogenes, tumor suppressor p53 and BCR-abl oncogenes, as well as CDK4, MUM1, Caspase 8, and Beta catenin; overexpressed antigens such as galectin 4, galectin 9, carbonic anhydrase, Aldolase A, PRAME, Her2/neu, ErbB-2 and KSA, oncofetal antigens such as alpha fetoprotein (AFP), human chorionic gonadotropin (hCG); self antigens such as carcinoembryonic antigen (CEA) and melanocyte differentiation antigens such as Mart 1/Melan A, gp100, gp75, Tyrosinase, TRP1 and TRP2; prostate associated antigens such as PSA, PAP, PSMA, PSM-P1 and PSM-P2; reactivated embryonic gene products such as MAGE 1, MAGE 3, MAGE 4, GAGE 1, GAGE 2, BAGE, RAGE, and other cancer testis antigens such as NY-ESO1, SSX2 and SCP1; mucins such as Muc-1 and Muc-2; gangliosides such as GM2, GD2 and GD3, neutral glycolipids and glycoproteins such as Lewis (y) and globo-H; and glycoproteins such as Tn, Thompson-Freidenreich antigen (TF) and sTn. Also included as tumor-associated antigens herein are whole cell and tumor cell lysates as well as immunogenic portions thereof, as well as immunoglobulin idiotypes expressed on monoclonal proliferations of B lymphocytes for use against B cell lymphomas.

**[0188]** Pathogens include, but are not limited to, infectious agents, *e.g.*, viruses, that infect mammals, and more particularly humans. Examples of infectious virus include, but are not limited to: Retroviridae (*e.g.*, human immunodeficiency viruses, such as HIV-1 (also referred to as HTLV-III, LAV or HTLV-III/LAV, or HIV-III); and other isolates, such as HIV-LP; Picornaviridae (*e.g.*, polio viruses, hepatitis A virus; enteroviruses, human Coxsackie viruses, rhinoviruses, echoviruses); Calciviridae (*e.g.*, strains that cause gastroenteritis); Togaviridae (*e.g.*, equine encephalitis viruses, rubella viruses); Flaviviridae (*e.g.*, dengue viruses, encephalitis viruses, yellow fever viruses); Coronaviridae (*e.g.*, coronaviruses);

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Rhabdoviridae (e.g., vesicular stomatitis viruses, rabies viruses); Coronaviridae (e.g., coronaviruses); Rhabdoviridae (e.g., vesicular stomatitis viruses, rabies viruses); Filoviridae (e.g., ebola viruses); Paramyxoviridae (e.g., parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); Orthomyxoviridae (e.g., influenza viruses); Bungaviridae (e.g., Hantaan viruses, bunga viruses, phleboviruses and Nairo viruses); Arenaviridae (hemorrhagic fever viruses); Reoviridae (e.g., reoviruses, orbiviruses and rotaviruses); Birnaviridae; Hepadnaviridae (Hepatitis B virus); Parvoviridae (parvoviruses); Papovaviridae (papilloma viruses, polyoma viruses); Adenoviridae (most adenoviruses); Herpesviridae herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV), herpes virus; Poxviridae (variola viruses, vaccinia viruses, pox viruses); and Iridoviridae (e.g., African swine fever virus); and unclassified viruses (e.g., the etiological agents of Spongiform encephalopathies, the agent of delta hepatitis (thought to be a defective satellite of hepatitis B virus), the agents of non-A, non-B hepatitis (class 1 =internally transmitted; class 2=parenterally transmitted (i.e., Hepatitis C); Norwalk and related viruses, and astroviruses).

**[0189]** Also, gram negative and gram positive bacteria serve as antigens in vertebrate animals. Such gram positive bacteria include, but are not limited to Pasteurella species, Staphylococci species, and Streptococcus species. Gram negative bacteria include, but are not limited to, Escherichia coli, Pseudomonas species, and Salmonella species. Specific examples of infectious bacteria include but are not limited to: Helicobacter pylori, Borelia burgdorferi, Legionella pneumophila, Mycobacteria sps (e.g., M. tuberculosis, M. avium, M. intracellulare, M. kansasii, M. goodii), Staphylococcus aureus, Neisseria gonorrhoeae, Neisseria meningitidis, Listeria monocytogenes, Streptococcus pyogenes (Group A Streptococcus), Streptococcus agalactiae (Group B Streptococcus), Streptococcus (viridans group), Streptococcus faecalis, Streptococcus bovis, Streptococcus (anaerobic sps.), Streptococcus pneumoniae, pathogenic Campylobacter sp., Enterococcus sp., Haemophilus influenzae, Bacillus anthracis, corynebacterium diphtheriae, corynebacterium sp., Erysipelothrix rhusiopathiae, Clostridium perfringens, Clostridium tetani, Enterobacter aerogenes, Klebsiella pneumoniae, Pasteurella multocida, Bacteroides sp., Fusobacterium nucleatum, Streptobacillus moniliformis, Treponema pallidum, Treponema pertense, Leptospira, Rickettsia, and Actinomyces israelii.

**[0190]** Additional examples of pathogens include, but are not limited to, infectious fungi that infect mammals, and more particularly humans. Examples of infectious fungi include, but are not limited to: Cryptococcus neoformans, Histoplasma capsulatum, Coccidioides immitis, Blastomyces dermatitidis, Chlamydia trachomatis, Candida albicans. Examples of infectious parasites include Plasmodium such as Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale, and Plasmodium vivax. Other infectious organisms (i.e., protists) include Toxoplasma gondii.

### EXAMPLES

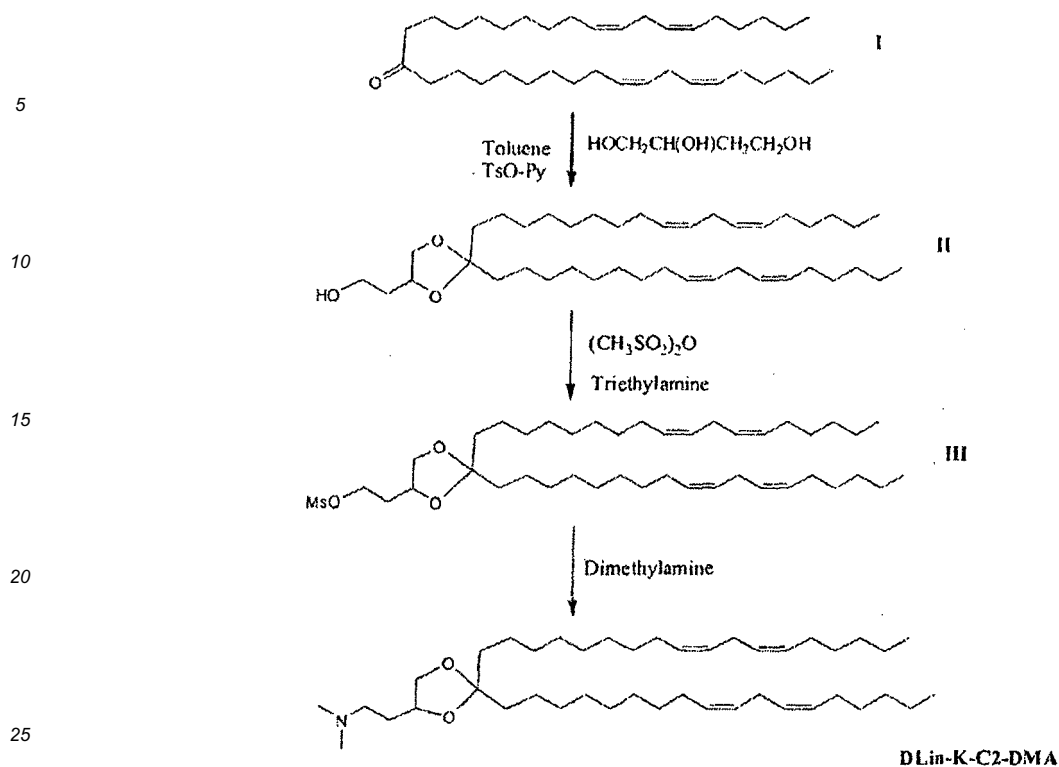
#### EXAMPLE 3

#### SYNTHESIS OF 2,2-DILINOLEYL-4-(2-DIMETHYLAMINOETHYL)-[1,3]-DIOXOLANE (**DLIN-K-C2-DMA**)

**[0191]** DLin-K-C2-DMA was synthesized as shown in the schematic diagram and description below.

#### Synthesis of 2,2-Dilinoleyl-4-(2-hydroxyethyl)-[1,3]-dioxolane (**II**)

**[0192]** A mixture of dilinoleyl ketone (**I**, previously prepared as described in Example 1, 527 mg, 1.0 mmol), 1,3,4-butanetriol (technical grade, ca. 90%, 236 mg, 2 mmol) and pyridinium p-toluenesulfonate (50 mg, 0.2 mmol) in 50 mL of toluene was refluxed under nitrogen overnight with a Dean-Stark tube to remove water. The resulting mixture was cooled to room temperature. The organic phase was washed with water (2 x 30 mL), brine (50 mL), and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Evaporation of the solvent resulted in a yellowish oily residual (0.6 g). The crude product was purified by column chromatography on silica gel (230-400 mesh, 100 mL) with dichloromethane as eluent. This afforded 0.5 g of pure **II** as colourless oil. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 5.25-5.48 (8H, m, 4 x CH=CH), 4.18-4.22 (1H, m, OCH), 4.08 (1H, dd, OCH), 3.82 (2H, t, OCH<sub>2</sub>), 3.53 (1H, t, OCH), 2.78 (4H, t, 2 x C=C-CH<sub>2</sub>-C=C), 2.06 (8H, q, 4 x allylic CH<sub>2</sub>), 1.77-1.93 (2H, m, CH<sub>2</sub>), 1.52-1.68 (4H, m, 2 x CH<sub>2</sub>), 1.22-1.45 (32H, m), 0.86-0.94 (6H, t, 2 x CH<sub>3</sub>) ppm.



30 Synthesis of 2,2-Dilinoleyl-4-(2-methanesulfonylethyl)-[1,3]-dioxolane (III)

35 **[0193]** To a solution of 2,2-dilinoleyl-4-(2-hydroxyethyl)-[1,3]-dioxolane (II, 500 mg, 0.81 mmol) and dry triethylamine (218 mg, 2.8 mmol) in 50 mL of anhydrous  $\text{CH}_2\text{Cl}_2$  was added methanesulfonyl anhydride (290 mg, 1.6 mmol) under nitrogen. The resulting mixture was stirred at room temperature overnight. The mixture was diluted with 25 mL of  $\text{CH}_2\text{Cl}_2$ . The organic phase was washed with water (2 x 30 mL), brine (50 mL), and dried over anhydrous  $\text{Na}_2\text{SO}_4$ . The solvent was evaporated to afford 510 mg of yellowish oil. The crude product was used in the following step without further purification.

40 Synthesis of 2,2-Dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA)

45 **[0194]** To the above crude material (III) under nitrogen was added 20 mL of dimethylamine in THF (2.0 M). The resulting mixture was stirred at room temperature for 6 days. An oily residual was obtained upon evaporation of the solvent. Column chromatography on silica gel (230-400 mesh, 100 mL) with 0-5% methanol gradient in dichloromethane as eluent resulted in 380 mg of the product **DLin-K-C2-DMA** as pale oil.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$ : 5.27-5.49 (8, m, 4x  $\text{CH}=\text{CH}$ ), 4.01-4.15 (2H, m, 2 x OCH), 3.49 (1 H, t OCH), 2.78 (4H, t, 2 x  $\text{C}=\text{C}-\text{CH}_2-\text{C}=\text{C}$ ), 2.34-2.54 (2H, m,  $\text{NCH}_2$ ), 2.30 (6H, s, 2 x  $\text{NCH}_3$ ), 2.06 (8H, q, 4 x allylic  $\text{CH}_2$ ), 1.67-1.95 (2H, m,  $\text{CH}_2$ ), 1.54-1.65 (4H, m, 2 x  $\text{CH}_2$ ), 1.22-1.45 (32H, m), 0.90 (6H, t, 2 x  $\text{CH}_3$ ) ppm.

50 EXAMPLE 6

SYNTHESIS OF 2,2-DILINOLEYL-5-DIMETHYLAMINOMETHYL-[1,3]-DIOXANE (DLIN-K6-DMA)

**[0195]** DLin-K6-DMA was synthesized as described and shown in the schematic diagram below.

55 Synthesis of 2,2-Dilinoleyl-5-hydroxymethyl)-[1,3]-dioxane (II)

**[0196]** A mixture of dilinoleyl ketone (I, previously prepared as described in Example 1, 1.05 g, 2.0 mmol), 2-hydroxymethyl-1,3-propanediol (475 mg, 4 mmol) and pyridinium p-toluenesulfonate (100 mg, 0.4 mmol) in 150 mL of toluene was refluxed under nitrogen overnight with a Dean-Stark tube to remove water. The resulting mixture was cooled

to room temperature. The organic phase was washed with water (2 x 60 mL), brine (60 mL), and dried over anhydrous  $\text{Na}_2\text{SO}_4$ . Evaporation of the solvent resulted in pale oil (1.2 g). The crude product was purified by column chromatography on silica gel (230-400 mesh, 100 mL) with 0-1 % methanol gradient in dichloromethane as eluent. This afforded 1.0 g of pure **II** as colourless oil.

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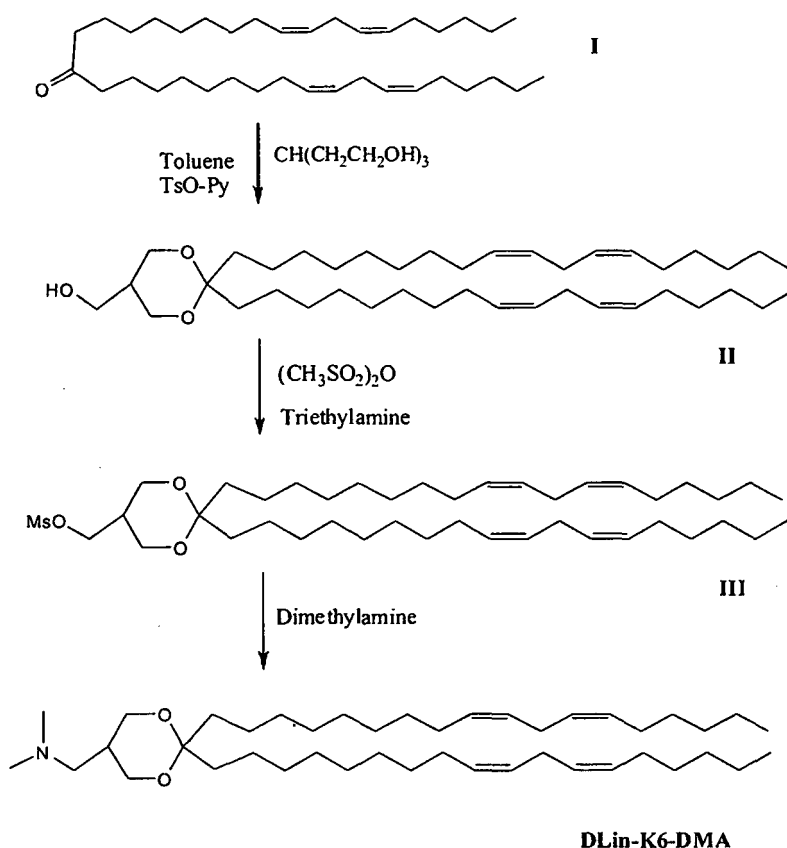
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### 35 Synthesis of 2,2-Dilinoleyl-5-methanesulfonylmethyl-[1,3]-dioxane (III)

**[0197]** To a solution of 2,2-dilinoleyl-5-hydroxymethyl-[1,3]-dioxane (**II**, 1.0 g, 1.6 mmol) and dry triethylamine (430 mg, 4.2 mmol) in 120 mL of anhydrous  $\text{CH}_2\text{Cl}_2$  was added methanesulfonyl anhydride (600 mg, 3.3 mmol) under nitrogen. The resulting mixture was stirred at room temperature overnight. The organic phase was washed with water (2 x 60 mL), brine (60 mL), and dried over anhydrous  $\text{Na}_2\text{SO}_4$ . The solvent was evaporated to afford 1.1 g of pale oil. The crude product was used in the following step without further purification.

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### Synthesis of 2,2-Dilinoleyl-5-dimethylaminomethyl-[1,3]-dioxane (DLin-K6-DMA)

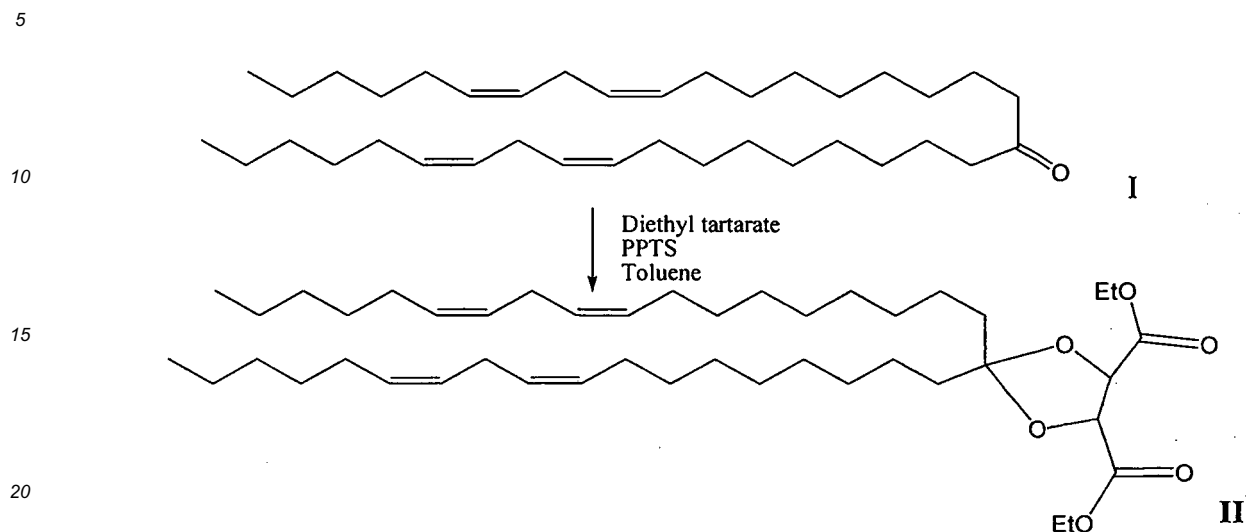
**[0198]** To the above crude material (**III**, 1.1 g) under nitrogen was added 20 mL of dimethylamine in THF (2.0 M). The resulting mixture was stirred at room temperature for 7 days. An oily residual was obtained upon evaporation of the solvent. Column chromatography on silica gel (230-400 mesh, 100 mL) with 0-30% ethyl acetate gradient in hexanes as eluent resulted in 260 mg of the product **DLin-K<sup>6</sup>-DMA** as pale oil.  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$ : 5.24-5.51 (8, m, 4 x  $\text{CH}=\text{CH}$ ), 4.04 (2H, dd, 2 x OCH), 3.75 (2H, dd OCH), 2.7-2.9 (2H, br,  $\text{NCH}_2$ ), 2.78 (4H, t, 2 x  $\text{C}=\text{C}-\text{CH}_2-\text{C}=\text{C}$ ), 2.57 (6H, s, 2 x  $\text{NCH}_3$ ), 1.95-2.17 (9H, q, 4 x allylic  $\text{CH}_2$  and CH), 1.67-1.95 (2H, m,  $\text{CH}_2$ ), 1.54-1.65 (4H, m, 2 x  $\text{CH}_2$ ), 1.22-1.45 (32H, m), 0.90 (6H, t, 2 x  $\text{CH}_3$ ) ppm.

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### EXAMPLE 13

### 55 SYNTHESIS OF 2,2-DILINOLEYL-4,5-BIS(DIMETHYLAMINO METHYL)-[1,3]-DIOXOLANE (DLIN -K<sup>2</sup>-DMA)

**[0199]** DLin-K<sup>2</sup>-DMA was synthesized as described and shown in the schematic diagrams below.

Synthesis of D-Lin-K-diethyltartarate (II)**[0200]**

25 **[0201]** A mixture of D-Lin-Ketone (I, 1 gram, 1.9 mmol), Diethyl-D-tartarate (412 mg, 2 mmol) and Pyridinium p-tolene sulfonate (250 mg, 1 mmol) in 25 mL of toluene was refluxed under nitrogen for two days with a Dean-stark tube to remove water. The resulting mixture was cooled to room temperature. The organic phase was washed with water NaHCO<sub>3</sub> and brine (2 X 50 mL) and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Evaporation of the solvent resulted in yellowish oily residue. Column chromatography on silica gel (230-400 mesh, 500 mL) eluted with 0-10% ether gradients in hexanes as eluent afforded 400 mg of pure D-Lin-diethyltartarate (II).

30 <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ: 5.27-5.46 (8H, m), 4.67 (2H, s), 4.20-4.30 (1 H, t), 2.75 (4H, t), 2.02-2.09 (8H, m) 1.62-1.72 (4H, m), 1.2-1.47 (32 H, m), 0.87-0.90 (6H, t) ppm.

Synthesis of D-Lin-K-diethyldiol (III)

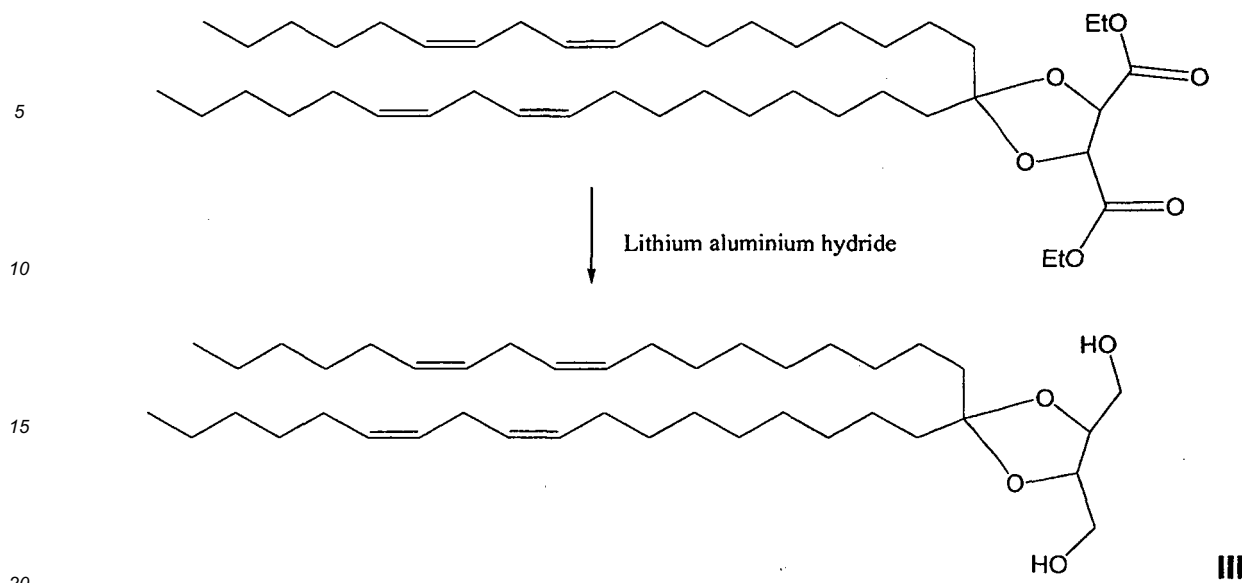
35 **[0202]** To a solution of Lithiumaluminiumhydride (32 mg, 1 mmol) in dry THF a solution of D-Lin-K-diethyltartarate (II, 600mg, 0.85 m mol) was added in dry THF at 0° C under argon atmosphere and then the reaction was stirred for four hours at room temperature. The reaction mixture was quenched with ice cold water and then filtered through celite and the evaporation of solvent gave crude reduced alcohol. Column chromatography on silica gel (230-400 mesh, 500 mL) eluted with 10-40% ethyl acetate gradients in hexanes as eluent afforded 350 mg of pure D-Lin-diethyltartarate (III).

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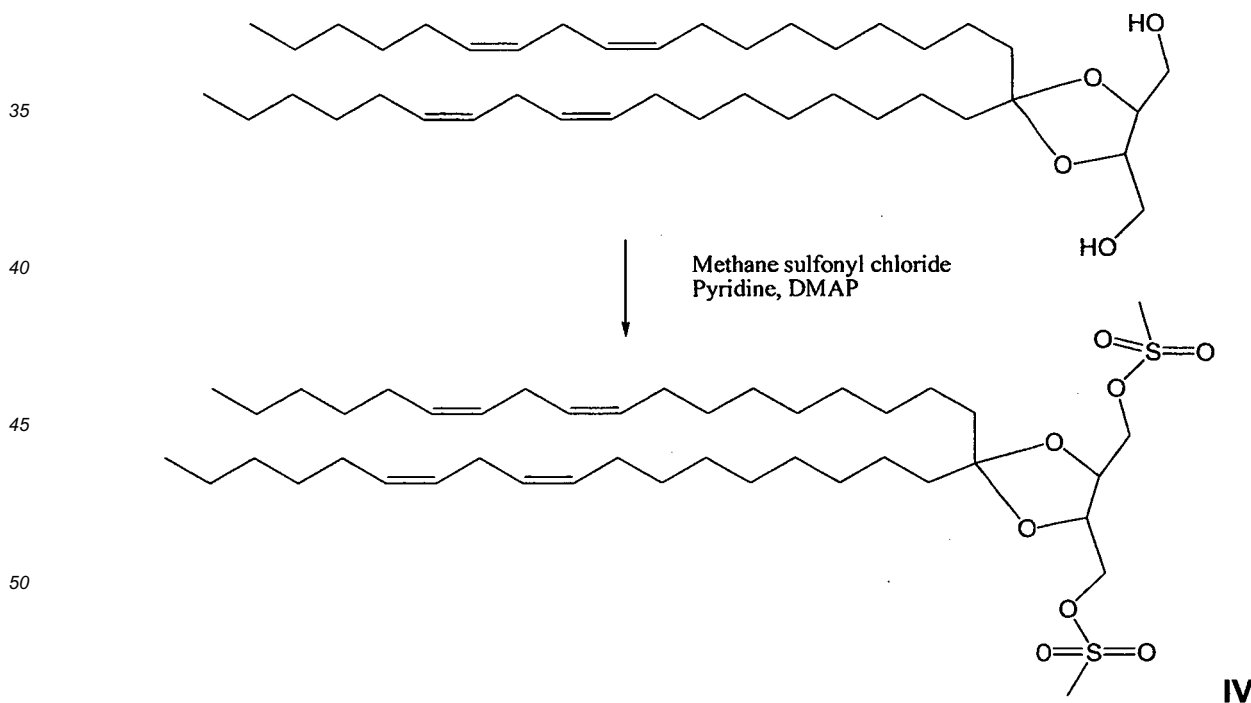
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$^1\text{H NMR}$  (300 MHz,  $\text{CDCl}_3$ )  $\delta$ : 5.27-5.46 (8H, m), 3.95 (2H, t), 3.65-3.85 (4H, dd), 2.75 (4H, t), 2.02-2.09 (8H, m) 1.62-1.72 (4H, m), 1.2-1.47 (32 H, m), 0.87-0.90 (6H, t) ppm.

25 Synthesis of D-Lin-K-diethyltartarate (IV)

[0203] To a mixture of D-Lin-K-diethyltartarate (III) alcohol (570 mg, 0.95 mmol) in dry dichloromethane pyridine (275 mg, 3.85 mmol) and 4-(Dimethylamino)pyridine (122mg, 1 mmol) was added under argon atmosphere to this solution a solution of methane sulfonyl chloride (500 mg, 2.5 mmol) was slowly added and stirred over night.



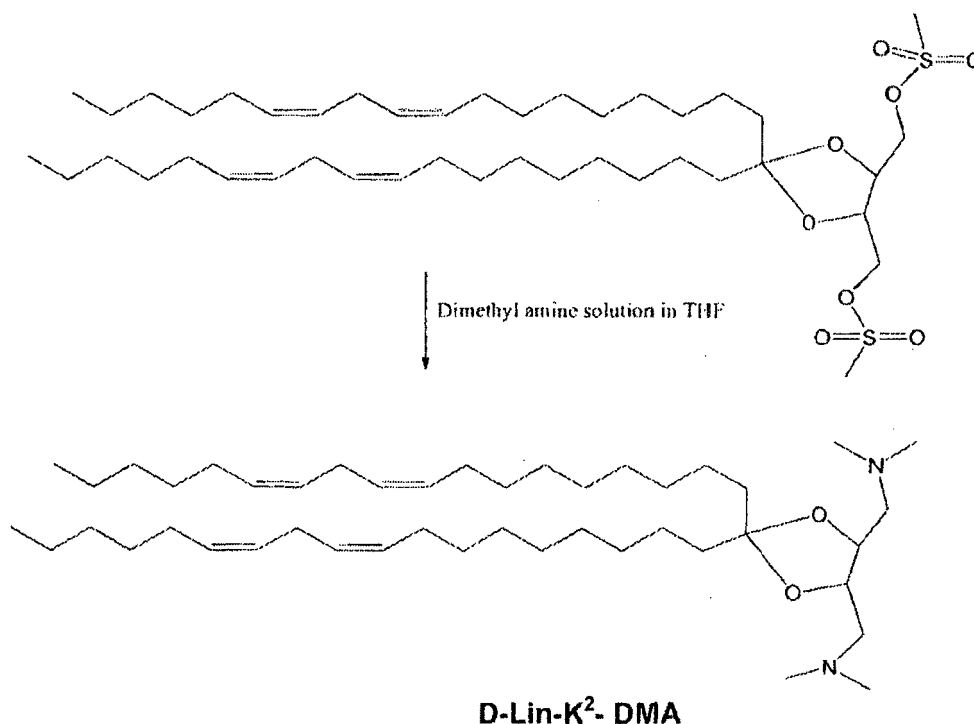
[0204] The organic phase was washed with water and brine (2 X 50 mL) then solvent was evaporated to give yellowish oil residue. Purified over Column chromatography on silica gel (230-400 mesh, 500 mL), eluted with 10-40% ethyl acetate gradients in hexanes as eluent, afforded 300 mg of pure D-Lin-K-diethyltartarate (IV).

55

$^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$ : 5.27-5.46 (8H, m), 4.35 (4H, d), 4.12-4.17 (2H, t), 3.08(6H, s), 2.75 (4H, t), 2.02-2.09 (8H, m) 1.62-1.72 (4H, m), 1.2-1.47 (32 H, m), 0.87-0.90 (6H, t) ppm.

#### Synthesis of **D-Lin-K<sup>2</sup>-DMA**

**[0205]** Anhydrous dimethyl amine solution in THF was added to the reaction vessel containing (300 mg) of D-Lin-diethyltartarate (**IV**) at room temperature for 5 min. the reaction flask was then sealed and the mixture stirred at room temperature for 6 days. Evaporation of the solvent left 300 mg of residual. The crude product was purified by column chromatography on silica gel(230-400 mesh, 500 mL) eluted with 0-10% Methanol gradients in chloroform as eluent afforded 50 mg of pure **D-Lin-K<sup>2</sup>-DMA**.



$^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$ : 5.27-5.46 (8H, m), 3.72-3.80 (2H, t), 2.75 (4H, t), 2.49 (4H, d), 2.30 (12H, s), 2.02-2.09 (8H, m) 1.62-1.72 (4H, m), 1.2-1.47 (32 H, m), 0.87-0.90 (6H, t) ppm.

#### EXAMPLE 16

##### INFLUENCE OF CATIONIC LIPID ON IN VIVO GENE SILENCING

**[0206]** It is well established that *in vivo* RNAi silencing of specific hepatocyte proteins can be achieved following intravenous (i.v.) administration of siRNA's encapsulated in, or associated with, select nanoparticles designed for intracellular delivery. One of the most active, and well characterized of these is a stable nucleic acid lipid particle (SNALP) containing the cationic lipid 1,2-dilinoyleoxy-3-dimethylaminopropane (DLinDMA). In this Example, rational design in combination with *in vivo* screening were applied to systematically modify the structure of DLinDMA and identify molecular features that enhance or reduce cationic lipid potency. More than 30 lipids were synthesized and incorporated into nucleic acid-lipid particles, *i.e.*, lipid nanoparticles (LN) encapsulating an siRNA (LN-siRNA) targeting Factor VII (FVII), a blood clotting component synthesized and secreted by hepatocytes that is readily measured in serum. LN-siRNA systems were prepared using the same process, lipid molar ratios and particle size to minimize effects on activity resulting from formulation characteristics other than the cationic lipid. Each formulation was administered as a single bolus injection over a range of doses enabling an estimate of the siRNA dose required to reduce FVII serum protein concentrations by 50% after 24 h ( $\text{ED}_{50}$ ).

**[0207]** The studies described herein were performed using the following materials and methods.

MATERIALS AND METHODSLipids

5 **[0208]** Cationic lipids were synthesized as described in the previous Examples. Distearoylphosphatidylcholine (DSPC) was purchased from Northern Lipids (Vancouver, Canada). Cholesterol was purchased from Sigma Chemical Company (St. Louis, Missouri, USA) or Solvay Pharmaceuticals (Weesp, The Netherlands).

10 **[0209]** The synthesis of N-[(methoxy poly(ethylene glycol)<sub>2000</sub>carbamy]-1,2-dimyristyloxylpropyl-3-amine (PEG-C-DMA) and N-[(methoxy poly(ethylene glycol)<sub>2000</sub>succinimidyl]-1,2-dimyristyloxylpropyl-3-amine (PEG-S-DMA) were as described by Hayes et. al., J. Control Release 112:280-290 (2006). R-3-[(ω-methoxy-poly(ethylene glycol)2000)carbonyl]-1,2-dimyristyloxylpropyl-3-amine (PEG-C-DOMG) was synthesized as described herein and by Akinc et. al., Nat. Biotechnol. 26:561-56 (2008). These three PEG-lipids were interchangeable in the formulation without impacting activity (data not shown), Therefore, throughout the text, they are referred to generally as PEG-lipid for purposes of clarity.

15 Synthesis of siRNA

**[0210]** All siRNAs and 2'-OMe oligoribonucleotides were synthesized by Alnylam as described in John et al. (Nature advance online publication, 26 September 2007 (DOI:10.1038/nature06179)). Oligonucleotides were characterized by electrospray mass spectrometry and anion exchange HPLC.

20 **[0211]** Sequences of siRNAs used in these studies were as follows:

si-FVII sense, 5'GGAUCAUCUCAAGUCUJUACTT 3' (SEQ ID NO:33);  
 si-FVII antisense, 5'- GUAAGACUUGAGAUGCCTT -3' (SEQ ID NO:34);  
 si-Luc sense, 5'-cuuAcGcuGAGuAcuucGATT-3' (SEQ ID NO:35);  
 25 si-Luc antisense, 5'-UCGAAGuACUcAGCGuAAGTT-3' (SEQ ID NO:36),

with lower-case letters denoting 2'-O-Me-modified nucleotides; and underlined letters denoting 2'-F-modified nucleotides. All siRNAs contained phosphorothioate linkages between the two thymidines (T) at the 3' end of each strand.

30 Preformed vesicle method to formulate nucleic acid-lipid particles

**[0212]** Nucleic acid-lipid particles were made using the preformed vesicle (PFV) method, essentially as described in Maurer *et al.* (Biophys J., 2001). Cationic lipid, DSPC, cholesterol and PEG-lipid were solubilised in ethanol at a molar ratio of 40/10/40/10, respectively. The lipid mixture was added to an aqueous buffer (50mM citrate, pH 4) with mixing to a final ethanol and lipid concentration of 30% (vol/vol) and 6.1 mg/mL respectively and allowed to equilibrate at room temperature for 2 min before extrusion. The hydrated lipids were extruded through two stacked 80 nm pore-sized filters (Nuclepore) at 22°C using a Lipex Extruder (Hope, M.J. et al. Biochim. Biophys. Acta 812:55-65 (1985)) until a vesicle diameter of 70-90 nm, as determined by Nicomp analysis, was obtained. This generally required 1-3 passes. The FVII siRNA (solubilised in a 50mM citrate, pH 4 aqueous solution containing 30% ethanol) was added to the pre-equilibrated (35°C) vesicles, at a rate of ~5mL/min with mixing. After a final target siRNA/lipid ratio of 0.06 (wt/wt) was achieved, the mixture was incubated for a further 30 min at 35°C to allow vesicle reorganization and encapsulation of the FVII siRNA. The ethanol was then removed and the external buffer replaced with PBS (155mM NaCl, 3mM Na<sub>2</sub>HPO<sub>4</sub>, 1 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.5) by either dialysis or tangential flow diafiltration.

45 Particle Size Analysis

**[0213]** The size distribution of liposomal siRNA formulations was determined using a NICOMP Model 380 Sub-micron particle sizer (PSS NICOMP, Particle Sizing Systems, Santa Barbara, CA). Mean particle diameters were generally in the range 50-120 nm, depending on the lipid composition used. Liposomal siRNA formulations were generally homogeneous and had standard deviations (from the mean particle size) of 20-50 nm, depending on the lipid composition and formulation conditions used.

Measurement of free siRNA by ion exchange chromatography

55 **[0214]** Anion exchange chromatography using either DEAE Sepharose columns or commercial centrifugal devices (Vivapure D Mini columns) was used to measure the amount of free siRNA in the sample. For the DEAE Sepharose columns, siRNA-containing formulations were eluted through the columns (~2.5 cm bed height, 1.5 cm diameter) equilibrated with HBS (145 mM NaCl, 20 mM HEPES, pH 7.5). An aliquot of the initial and eluted sample were assayed for

lipid and siRNA content by HPLC and A260, respectively. The percent encapsulation was calculated based on the change in siRNA to lipid ratios between the pre and post column samples. For the Vivapure centrifugal devices, an aliquot (0.4 mL, <1.5 mg/mL siRNA) of the siRNA-containing formulation was eluted through the positively charged membrane by centrifugation (2000 xg for 5 min). Aliquots of the pre and post column samples were analyzed as described above to determine the amount of free siRNA in the sample.

#### Determination of siRNA Concentration

**[0215]** siRNA concentration was determined by measuring the absorbance at 260 nm after solubilization of the lipid. The lipid was solubilized according to the procedure outlined by Bligh and Dyer (Bligh, et al., Can. J. Biochem. Physiol. 37:911-917 (1959). Briefly, samples of liposomal siRNA formulations were mixed with chloroform/methanol at a volume ratio of 1:2.1:1 (aqueous sample:methanol:chloroform). If the solution was not completely clear (*i.e.*, a single, clear phase) after mixing, an additional 50-100 mL (volume recorded) of methanol was added and the sample was remixed. Once a clear monophasic solution was obtained, the sample was assayed at 260 nm using a spectrophotometer. siRNA concentration was determined from the A260 readings using a conversion factor of approximately  $45 \mu\text{g/mL} = 1.0 \text{ OD}$ , using a 1.0 cm path length. The conversion factor in the chloroform/methanol/water monophasic solution varies ( $35\text{-}50 \mu\text{g/mL} = 1.0 \text{ OD}$ ) for each lipid composition and is determined empirically for each novel lipid formulation using a known amount of siRNA.

#### Determination of Lipid Concentrations and Ratios

**[0216]** Cholesterol, DSPC, PEG-lipid, and the various cationic lipids were measured against reference standards using a Waters Alliance HPLC system consisting of an Alliance 2695 Separations Module (autosampler, HPLC pump, and column heater), a Waters 2424 Evaporative Light Scattering Detector (ELSD), and Waters Empower HPLC software (version 5.00.00.00, build number 1154; Waters Corporation, Milford, MA, USA). Samples (15  $\mu\text{L}$ ) containing 0.8 mg/mL total lipid in 90% ethanol were injected onto a reversed-phase XBridge C18 column with 2.5  $\mu\text{m}$  packing, 2.1 mm x 50 mm (Waters Corporation, Milford, MA, USA) heated at 55°C and chromatographed with gradient elution at a constant flow rate of 0.5 mL/min. The mobile phase composition changed from 10 mM  $\text{NH}_4\text{HCO}_3$ :methanol (20:80) to THF:10 mM  $\text{NH}_4\text{HCO}_3$ :methanol (16:4:80) over 16 minutes. The gas pressure on the ELSD was set at 25 psi, while the nebulizer heater-cooler set point and drift tube temperature set point were set at 100% and 85°C respectively. Measured lipid concentrations (mg/mL) were converted to molar concentrations, and relative lipid ratios were expressed as mol% of the total lipid in the formulation.

#### Determination of Encapsulation Efficiency

**[0217]** Trapping efficiencies were determined after removal of external siRNA by tangential flow diafiltration or anion exchange chromatography. siRNA and lipid concentrations were determined (as described above) in the initial formulation incubation mixtures and after tangential flow diafiltration. The siRNA-to-lipid ratio (wt/wt) was determined at both points in the process, and the encapsulation efficiency was determined by taking the ratio of the final and initial siRNA-to-lipid ratio and multiplying the result by 100 to obtain a percentage.

#### In vivo screening of cationic lipids for FVII activity

**[0218]** FVII activity was evaluated in FVII siRNA-treated animals at 24 hours after intravenous (bolus) injection in C57BL/6 mice or 48 hours after intravenous (bolus) injection in SD rats. Six to 8 week old, female C57BL/6 mice were obtained from Charles River Laboratories and acclimated for one week prior to use in studies. Animals were held in a pathogen-free environment and all procedures involving animals were performed in accordance with the guidelines established by the Canadian Council on Animal Care.

**[0219]** LN-siRNA systems containing Factor VII siRNA were diluted to the appropriate concentrations in sterile phosphate buffered saline immediately prior to use, and the formulations were administered intravenously via the lateral tail vein in a total volume of 10 mL/kg. After 24 h, animals were anaesthetised with Ketamine/Xylazine and blood was collected by cardiac puncture and processed to serum (Microtainer Serum Separator Tubes; Becton Dickinson, Franklin Lakes, NJ, USA). Serum was tested immediately or stored at -70°C for later analysis for serum Factor VII levels.

**[0220]** FVII was measured using a commercially available kit (Biophen FVII Kit™; Aniera Corp., Mason, OH), following the manufacturer's instructions at a microplate scale. FVII reduction was determined against untreated control animals, and the results were expressed as % Residual FVII. Five dose levels (0.1, 0.3, 0.5, 1.0, and 3.0 mg/kg) were typically used.

Pharmacokinetic and liver analysis

**[0221]** A fluorescently labeled siRNA (Cy-3 labeled luciferase siRNA, Alnylam Pharmaceuticals) was used to measure the siRNA content in plasma and liver after iv administration of LN-siRNA systems. The measurements were done by first extracting the Cy3-siRNA from the protein-containing biological matrix and then analyzing the amount of Cy-3 label in the extract by fluorescence. Two extraction methods were used, a chloroform/methanol mixture for the plasma samples and a commercial phenol/chloroform mixture (Trizol® reagent) with the tissue samples.

**[0222]** For plasma, blood was collected in EDTA-containing Vacutainer tubes and centrifuged at 1000 xg for 10 min at 4-8°C to isolate the plasma. The plasma was transferred to an eppendorf tube and either assayed immediately or stored in a -30°C freezer. An aliquot of the plasma (100 µL maximum) was diluted to 500 µL with PBS (145 mM NaCl, 10 mM phosphate, pH 7.5), methanol (1.05 mL) and chloroform (0.5 mL) was added, and the sample vortexed to obtain a clear, single phase solution. Water (0.5 mL) and chloroform (0.5 mL) was then added and the resulting emulsion sustained by mixing periodically for a minimum of 3 minutes. The mixture was centrifuged at 3000 rpm for 20 minutes and the top aqueous phase containing the Cy-3-label transferred to a new test tube. The fluorescence of the solution was measured using an SLM Fluorimeter at an excitation wavelength of 550 nm (2 nm bandwidth) and emission wavelength of 600 nm (16 nm bandwidth). A standard curve was generated by spiking aliquots of plasma from untreated animals with the Cy-3-siRNA containing formulation (0 to 15 µg/mL), and the sample processed as indicated above.

**[0223]** For liver, sections (400 - 500 mg) of tissue from saline-perfused animals was accurately weighed and homogenized in 1 mL of Trizol using Fastprep tubes. An aliquot of the homogenate (typically equivalent to 50 mg of tissue) was transferred to an eppendorf tube and additional Trizol added to 1 mL final. Chloroform (0.2 mL) was added, and the solution was mixed and incubated for 2-3 min before being centrifuged for 15 min at 12,000 xg. An aliquot (0.5 mL) of the top Cy-3-containing aqueous phase was diluted with 0.5 mL of PBS and the fluorescence of the sample measured as described above.

Measurement of FVII protein in serum

**[0224]** Serum Factor VII levels were determined using the colorimetric Biophen VII assay kit (Anaira, USA). Briefly, serially diluted pooled control serum (200% - 3.125%) and appropriately diluted plasma samples from treated animals were analyzed using the Biophen VII kit according to manufacturer's instructions in 96-well, flat bottom, non-binding polystyrene assay plates (Corning, Corning, NY) and absorbance at 405nm was measured. A calibration curve was generated using the serial diluted control serum and used to determine levels of Factor VII in serum from treated animals.

Determination of Tolerability

**[0225]** The tolerability of empty DLin-K-C2-DMA lipid particles (DLin-K-C2-DMA/DSPC/Chol/PEG-C-DOMG (40/10/40/10)) was evaluated by monitoring weight change, cageside observations, clinical chemistry and, in some instances, hematology in femal Sprague Dawley rats and female C57BL/6 mice. Animal weights were recorded prior to treatment and at 24 hours after intravenous treatment with various dosages. Data was recorded as % Change in Body Weight. In addition to body weight measurements, clinical chemistry panel, including liver function markers, was obtained at each dose level at 24 hours post-injection using an aliquot of the serum collected for FVII analysis. Samples were sent to the Central Laboratory for Veterinarians (Langley, BC) for analysis. In some instances, additional animals were included in the treatment group to allow collection of whole blood for hematology analysis.

In situ determination of pKa using TNS

**[0226]** In situ pKa measurements were made using the pH sensitive fluorescent probe TNS, using a modification of the approach previously published in Bailey, A.L. and Cullis, P.R., Biochemistry 33:12573-12580 (1994).

RESULTS

**[0227]** Initial studies were performed in eight to 10 week old, female C57BL/6 mice in two stages. In the first stage, the activity associated with the benchmark lipid DLinDMA was compared to the activity associated with modified forms of DLinDMA. This stage resulted in the identification of DLin-K-DMA as having increased activity as compared to DLinDMA. Therefore, in the second stage, the activities of modified forms of DLinDMA were compared to the activity of DLin-K-DMA. Dose response curves were used to estimate an ED<sub>50</sub> for each formulation, which is defined as the siRNA dose required to reduce the concentration of serum FVII protein by 50%, and is ~1.0 mg/kg for the DLinDMA benchmark formulation. The ED<sub>50</sub> for formulations with poor activity is expressed as a range, e.g. 12-25 mg/kg, indicating that a 50% reduction in serum FVII protein levels occurs between these siRNA doses. If a formulation showed good activity

(ED<sub>50</sub> < 2 mg/kg), then the dose response was repeated over a narrower dose range and head-to-head with DLinDMA for greater comparative accuracy.

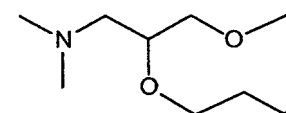
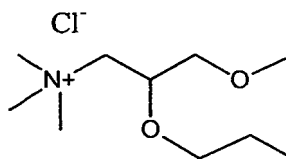
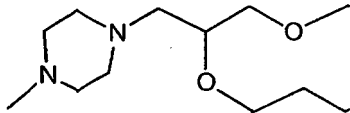
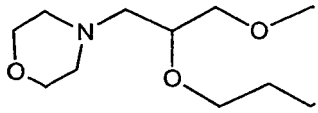
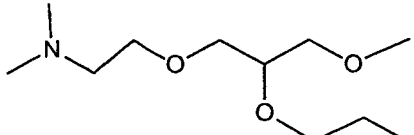
#### Screen 1a: Headgroup modifications to DLinDMA and *in vivo* FVII activity

**[0228]** For the purposes of this study, DLinDMA was divided into three key structural domains that were modified separately, including the headgroup, the linker, and the hydrocarbon chains. The dimethylaminopropane headgroup is hydrophilic and contains a tertiary amine function with an apparent pKa (*in situ*) of pH 6.4. Consequently, DLinDMA is almost completely charged at pH 4, the pH at which the LN-siRNA systems are formed through electrostatic interaction with siRNA. Whereas, at pH 7.4, ~5-10% of DLinDMA molecules are charged; therefore, the cationic charge density at the surface of these nanoparticles in the circulation is relatively low. In contrast, at endosomal pH's (~pH 5), the surface charge density is increased significantly, which is expected to promote the formation of ion pairs with anionic phospholipids and disrupt the endosomal membrane (Hafez, I.M. and Cullis, P.R., *Adv. Drug Deliv. Rev.* 4:139-148 (2001) and Xu, Y. and Szoka, F.C., *Biochemistry* 35:5616-5623 (1996)).

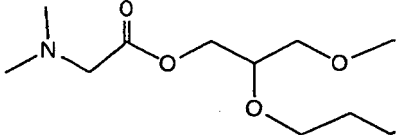
**[0229]** The headgroup modifications made to DLinDMA are shown in Table 3, and the first three were designed to alter the nature of the positive charge. DLinTMA contains a quaternary amino group and is permanently charged, and showed reduced activity with an estimated ED<sub>50</sub> between 2 - 5 mg/kg. A similar decrease in activity was observed when the dimethylamine function was replaced by a piperazine moiety (DLinMPZ, ED<sub>50</sub> 2 - 5 mg/kg), and reduced more significantly when substituted by a morpholino group (DLinMA, ED<sub>50</sub> 12-25 mg/kg).

**[0230]** One rationale for making the remaining two modifications shown in Table 3 was to compare the activities of two lipids with similar headgroup structures but with different rates of metabolism *in vivo*. Lipid degradation was not measured *in vivo* for any of the lipids screened here, but it is expected that an ethoxy group (DLin-EG-DMA) would be more resistant to enzymatic cleavage than an ester group (DLinDAC) (Martin, B. et al., *Curr. Pharm. Des* 11:375-394 (2005)); none the less, both lipids exhibited similar activity.

Table 3. Headgroup modifications to DLinDMA

Abbreviated Name	Chemical Name	ED <sub>50</sub> (mg/kg)	Headgroup Modification
DLinDMA (Benchmark)	1,2-Dilinoleyloxy-3-dimethylaminopropane	-1	
DLinTMA.Cl	1,2-Dilinoleyloxy-3-trimethylaminopropane chloride	2-5	
DLinMPZ	1,2-Dilinoleyloxy-3-(N-methylpiperazino) propane	2-5	
DLinMA	1,2-Dilinoleyloxy-3-morpholinopropane	12-25	
DLin-EG-DMA	1,2-Dilinoleyloxy-3-(2-N,N-dimethylamino) ethoxypropane	5-12	

(continued)

Abbreviated Name	Chemical Name	ED <sub>50</sub> (mg/kg)	Headgroup Modification
DLinDAC	1,2-Dilinoleyloxy-3-(dimethylamino)acetoxypropane	5-12	

Screen 1b: Linker modifications to DLinDMA and *in vivo* FVII activity

**[0231]** DLinDMA has two unsaturated hydrocarbon chains joined to the dimethylaminopropane headgroup through two ethoxy linkages. In a bilayer structure, the linker region resides at the membrane interface, an area of transition between the hydrophobic membrane core and hydrophilic headgroup surface. The approach to linker modification of DLinDMA was to introduce linker groups expected to exhibit different rates of chemical or enzymatic stability and spanning a range of hydrophilicity. The epoxy moiety is considered to be more resistant to degradation than most other types of chemical bonds *in vivo*, which may be why these lipids have been found to be less well tolerated than cationic lipids with ester linkages for example (Martin, B. et al., *Curr. Pharm. Des* 11:375-394 (2005)). A variety of these rationally designed lipids were made, characterized, and tested, including those shown in Table 4.

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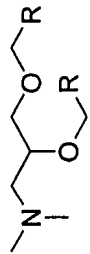
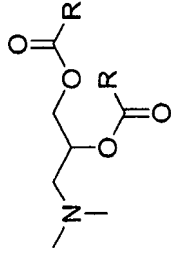
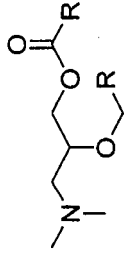
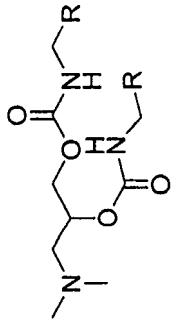
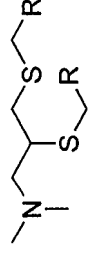
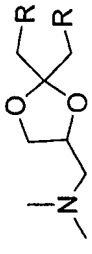

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Table 4. Linker modifications to DLinDMA

Abbreviated Name	Chemical Name	ED <sub>50</sub> (mg/kg)	Structure
DLinDMA (Benchmark)	1,2-Dilinoleoyloxy-3-dimethylaminopropane	~1	
DLinDAP	1,2-Dilinoleoyl-3-dimethylaminopropane	40-50	
DLin-2-DMAP	1-Linoleoyl-2-linoleoyloxy-3-dimethylaminopropane	5-12	
DLin-C-DAP	1,2-Dilinoleylcarbamoyloxy-3-dimethylaminopropane	12-25	
DLin-S-DMA	1,2-Dilinoleylthio-3-dimethylaminopropane	12-25	
DLin-K-DMA	2,2-Dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane	~0.4	
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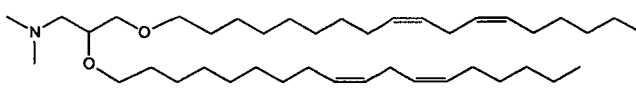
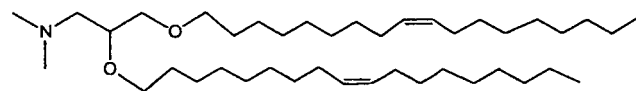
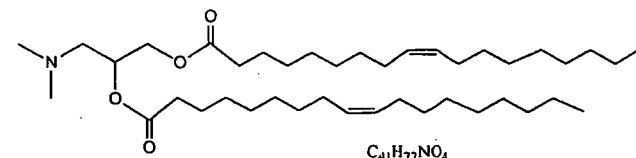
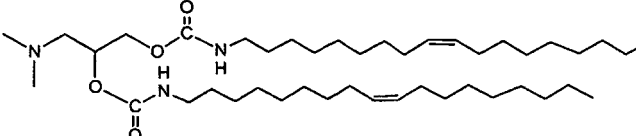
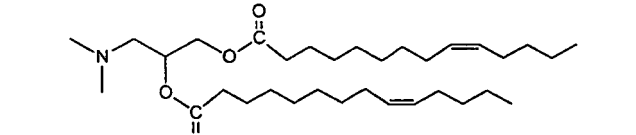
**[0232]** The first modification listed in Table 4 is DLinDAP, in which esters replace the ethoxy linkers of DLinDMA. Remarkably, nucleic acid lipid particles comprising Factor VII siRNA and containing DLinDAP showed significantly reduced *in vivo* activity as compared to those containing DLinDMA (ED<sub>50</sub> 12 - 25 mg/kg), despite its very similar structure to DLinDMA. Further, nucleic acid-lipid particles based on DLin-2-DMAP, a lipid with one ethoxy linkage and one ester linkage, yielded activity intermediate between DLinDAP- and DLinDMA-based nucleic acid-lipid particles. Nucleic acid-lipid particles based on lipids containing carbamate (DLin-C-DAP) or thioether (DLin-S-DMA) linkages also resulted in dramatically reduced *in vivo* activity.

**[0233]** The final modification was to insert a ketal ring linker, which introduced interesting structural changes to the lipid molecule. First, the ketal is known to be more acid labile than ethoxy linkers (Martin, B. et al., *Curr. Pharm. Des* 11:375-394 (2005)), which may decrease its half-life in the endocytic pathway. Second, the hydrocarbon chains now bond to the linker group through a single carbon. Interestingly, the introduction of a ketal ring linker into DLinDMA resulted in nucleic acid-lipid particles that were ~2.5-fold more potent in reducing serum FVII protein levels relative to the DLinDMA benchmark, with an ED<sub>50</sub> (i.e., dose to achieve 50% gene silencing) of ~0.4 mg/kg versus 1 mg/kg, respectively (Figure 2).

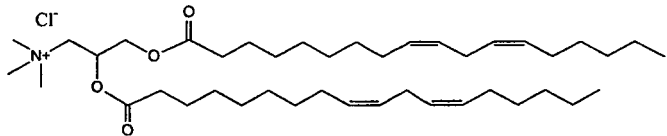
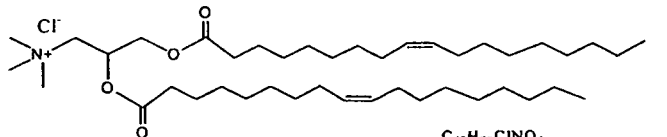
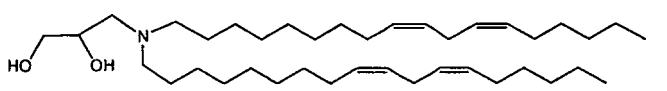
#### Screen 1c: Reduce unsaturation of DLinDMA hydrocarbon chains, miscellaneous modifications and *in vivo* FVII activity

**[0234]** A variety of other cationic lipids containing modifications as compared to DLinDMA were tested in the Factor VII knockdown system. For example, the propensity of lipid molecules to adopt inverted non-bilayer phases is known to increase with increasing hydrocarbon chain unsaturation (Cullis, P.R., et al., *Chem. Phys. Lipids* 40:127-144 (1986)). Given the hypothesis that formation of these non-bilayer phases is responsible for endosome disruption and release of siRNA into the cytoplasm and the observation that SNALP activity *in vitro* also increases with increasing unsaturation (Heyes, J. et al., *J. Control Release* 107:276-287 (2005)), it was of interest to see what happened to LN-siRNA potency *in vivo* when DLinDMA, containing two C18:2 chains was replaced by DODMA, with two C18:1 chains. These cationic lipids and the results of these experiments are shown in Table 5.

Table 5. Miscellaneous modifications to headgroup, linker and hydrocarbon chains

Abbreviated Name	ED <sub>50</sub> (mg/kg)	Modification
DLinDMA (Benchmark)	~1	 1,2-Dilinoleyloxy-3-dimethylaminopropane
DODMA	2-5	 1,2-Dioleoyloxy-3-dimethylaminopropane
DODAP	>25	 C <sub>31</sub> H <sub>77</sub> N <sub>3</sub> O <sub>4</sub> 1,2-Dioleoyl-3-dimethylaminopropane
DO-C-DAP	>10	 1,2-Dioleoylcarbamoyloxy-3-dimethylaminopropane
DMDAP	>10	 1,2-Diyristoleoyl-3-trimethylaminopropane chloride

(continued)

Abbreviated Name	ED <sub>50</sub> (mg/kg)	Modification
DLinTAP.Cl	>25	 1,2-Dilinoleyloxy-3-trimethylaminopropane chloride
DOTAP.Cl	>25	 1,2-Dioleoyl-3-trimethylaminopropane chloride C <sub>42</sub> H <sub>80</sub> ClNO <sub>4</sub>
DLinAP	5-12	 3 (N,N-Dilinoleylamino)-1,2-propanediol

**[0235]** As shown in Table 5, DODMA was 2 to 5-fold less active than the more unsaturated DLinDMA, and substituting ether linkages for esters (DODAP) decreased activity more than 25-fold.

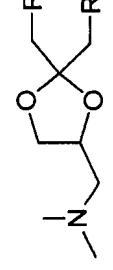
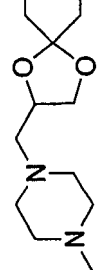
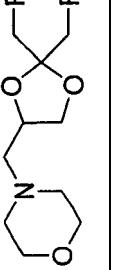
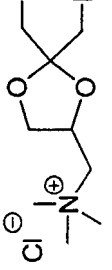
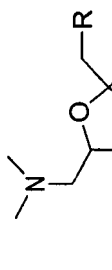
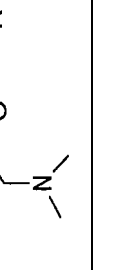
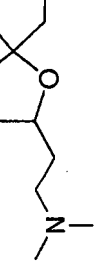
**[0236]** Carbamate linked C18:1 chains (DO-C-DAP) were also an inactive combination at 10 mg/kg, the maximum dose tested. DMDAP was synthesized to determine if the shorter C14:1 hydrocarbon chains might enable the ester-linked lipid through enhanced lipid mixing with the target endosomal membrane (Mui, B. et al., *Biochim. Biophys. Acta* 1467:281-292 (2000)); however, no activity was observed for DMDAP-LN-siRNA in the FVII model up to a maximum dose of 10 mg/kg. The permanently charged, ester linked lipids DLinTAP and DOTAP were of interest, because the latter lipid is one of the most commonly used cationic lipids for transfection. However, in the LN-siRNA model, neither of these ester-linked lipids showed any signs of activity, (ED<sub>50</sub> > 25 mg/kg). The last lipid represented a radical structural change to DLinDMA, in which the dimethylpropane headgroup was reversed and the hydrocarbon chains bond directly to the amino nitrogen, leaving a dihydroxy headgroup; however, DLinAP showed poor activity with an ED<sub>50</sub> in the 5-12 mg/kg range.

**[0237]** In summary, the incremental modifications to DLinDMA successfully identified DLin-K-DMA as a cationic lipid that is significantly more potent than DLinDMA when tested head-to-head in the same *in vivo* model and LN-siRNA formulation.

#### Screen 2a: Headgroup modifications to DLin-K-DMA and *in vivo* FVII activity

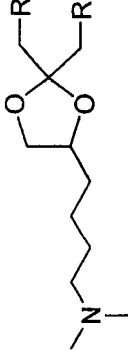
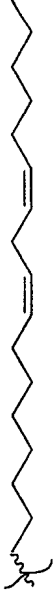
**[0238]** Given the importance of positive charge in the mechanism of action hypothesis guiding the lipid design, the effects of structural changes in the amine-based headgroup were investigated in the context of DLin-K-DMA as the new benchmark lipid. A series of headgroup modifications were made, characterized, and tested to explore the effect of size, acid dissociation constant, and number of ionizable groups (Table 6).

Table 6. Headgroup modifications to DLin-K-DMA

Abbreviated Name	Chemical Name	ED <sub>50</sub> (mg/kg)	Modification
DLin-K-DMA (Benchmark)	2,2-Dilinoleyl-4-dimethylamino methyl-[1,3]-dioxolane	~0.4	
DLin-K-MPZ	2,2-Dilinoleyl-4-N-methyl piperazino-[1,3]-dioxolane	-1.5	
DLin-K-MA	2,2-Dilinoleyl-4-N-morpholino-[1,3]-dioxolane	>15	
DLin-K-TMA.Cl	2,2-Dilinoleyl-4-trimethylamino-[1,3]-dioxolane Chloride	>5 <sup>a</sup>	
DLin-K <sup>2</sup> -DMA	2,2-Dilinoleyl-4,5-bis(dimethylamino methyl)-[1,3]-dioxolane	-0.4	
DLin-K-C2-DMA	2,2-Dilinoleyl -4-(2-dimethyl aminoethyl)-[1,3]-dioxolane	~0.1	
DLin-K-C3-DMA	2,2-Dilinoleyl -4-(3-dimethyl aminopropyl)-[1,3]-dioxolane	~0.6	

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(continued)

Abbreviated Name	Chemical Name	ED <sub>50</sub> (mg/kg)	Modification
DLin-K-C4-DMA	2,2-Dilinoleyl 4-(4-dimethyl aminobutyl)-[1,3]-dioxolane	>3	
<p style="text-align: center;">   <b>R =</b> </p>			
<p><sup>a</sup> No activity observed at 5 mg/kg and lethal at next dose of 15 mg/kg</p>			

**[0239]** DLin-K-DMA contains a chiral carbon at position 4 of the ketal ring structure. Therefore, the two optically pure (+) and (-) enantiomers were synthesized and their activities compared to that of the racemic mixture. All three formulations exhibited indistinguishable dose responses, each with an  $ED_{50} \sim 0.3$  mg/kg.

**[0240]** The first three modifications listed in Table 6 were also applied to DLinDMA in screen 1, the introduction of piperazino (DLin-K-MPZ) and morpholino (DLin-K-MA) amino moieties to modify the characteristics of the ionizable positive charge, and also converting the tertiary dimethylamine into the permanently charged quaternary amine of DLin-K-TMA. Although all these modifications significantly decreased activity, it is interesting to note that DLin-K-MPZ, with an  $ED_{50} \sim 1.5$ , was almost as active as DLinDMA but approximately 5-fold less active than DLin-K-DMA, and the same modification to DLinDMA reduced its activity by a similar factor. Furthermore, the morpholino amine function made DLin-K-MA inactive at the maximum dose tested (15 mg/kg) similar to DLinMA, which has an  $ED_{50}$  of 12 - 25 mg/kg. Another observation of note is that DLin-K-TMA (permanent positive charge) is toxic. No activity was observed at 5 mg/kg, but animals experienced significant weight loss (data not shown) and did not survive the next dose at 15 mg/kg.

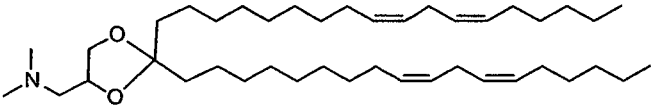
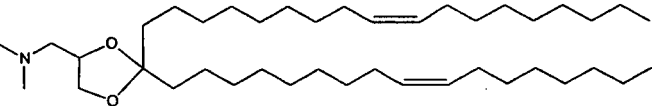
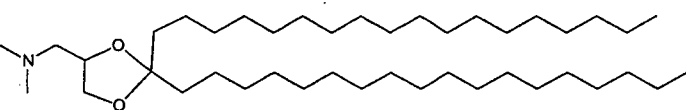
**[0241]** The modification abbreviated as DLin-K<sup>2</sup>-DMA denotes the presence of two dimethylamine moieties. Within error, this lipid had the same activity as DLin-K-DMA, despite the larger headgroup.

**[0242]** As an additional parameter, the distance between the demethylamino group and the dioxolane linker was varied by introducing additional methylene groups. The remaining three lipids were closely related in structure, and were synthesized to determine what effect distancing the positive charge from the dioxolane ring had on activity. This parameter can affect both the  $pK_a$  of the amine headgroup as well as the distance and flexibility of the charge presentation relative to the lipid bilayer interface. Inserting a single additional methylene group into the headgroup (DLin-K-C2-DMA) produced a dramatic increase in potency relative to DLin-K-DMA. The  $ED_{50}$  for this lipid was  $\sim 0.1$  mg/kg, making it 4-fold more potent than DLin-K-DMA and 10-fold more potent than the DLinDMA benchmark when compared head-to-head in the FVII model (Figure 2A). Additional methylene groups decreased activity with a significant reduction occurring between DLin-K-C3-DMA ( $ED_{50} \sim 0.6$  mg/kg) and DLin-K-C4-DMA ( $ED_{50} > 3$  mg/kg) (Figure 2B).

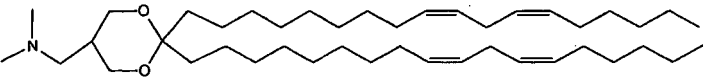
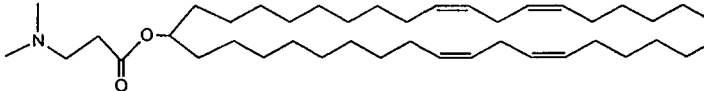
#### Screen 2b: Modifications to the headgroup, linker and hydrocarbon chains of DLin-K-DMA and in vivo FVII activity

**[0243]** A number of additional structural modifications made to DLin-K-DMA are presented in Table 7. The first three in the series confirmed the importance of hydrocarbon chain unsaturation for *in vivo* activity. A progressive decrease in  $ED_{50}$ 's from  $\sim 0.3$ , to  $\sim 1.0$  and  $\sim 8.0$  mg/kg was observed going from DLin-K-DMA (C18:2) to DO-K-DMA (C18:1) and DS-K-DMA (C18:0), respectively. The next modification (DLin-K6-DMA) demonstrated that the 5-membered ketal ring of DLin-K-DMA could be substituted by a 6-membered dioxalane ring structure without loss of activity. The final lipid shown in Table 7 represented a more radical modification. DLin-M-DMA does not have a ketal ring linker, but the hydrocarbon chains still bond directly with a single carbon. Interestingly, this lipid remained relatively active with an  $ED_{50} \sim 0.7$  mg/kg.

Table 7. Miscellaneous modifications to headgroup, linker and hydrocarbon chains of DLin-K-DMA

Abbreviated Name	$ED_{50}$ (mg/kg)	Modification
DLin-K-DMA (Benchmark)	$\sim 1$	 2,2-Dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane
DO-K-DMA	$\sim 1.0$	 2,2-Dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane
DS-K-DMA	$\sim 8.0$	 2,2-Distearoyl-4-dimethylaminomethyl-[1,3]-dioxolane

(continued)

Abbreviated Name	ED <sub>50</sub> (mg/kg)	Modification
DLin-K6-DMA	~0.3	 2,2-Dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane
DLin-M-DMA	~0.7	 Dilinoleylmethyl-3-dimethylaminopropionate

#### Comparison of the *In Vivo* Activity of Nucleic Acid-Lipid Formulations in Mice and Rats

**[0244]** The ability of various nucleic acid-lipid formulations comprising different cationic lipids was further explored in mice and rats. Each of the tested nucleic acid-lipid formulations was prepared as described above using PEG-C-DOMG as the PEG-lipid. The formulations initially tested (which included either DLin-K-DMA, DLin-K-MPZ, DLin-K-C2-DMA, or DLin-K-C4-DMA) reduced residual FVII levels in both mice (Figure 3) and rats (Figure 4). However, the DLin-K-C2-DMA formulation showed a remarkably enhanced ability to reduce FVII levels in both mice and rats. The activity of the DLin-K-C2-DMA formulation was approximately 2-3-fold greater than the DLin-K-DMA formulation in mice, and approximately 10-20-fold greater than the DLin-K-DMA formulation in rats. A comparison of FVII reduction in mice and rats using the DLin-K-DMA formulation or the DLin-K-C2-DMA formulation is shown in Figure 5. Formulations having DLin-K-C4-DMA or DLin-K-MPZ (MPZ) as the cationic lipid showed similar activity to each other and to the DLinDMA formulation.

**[0245]** The liposomal formulation having DLin-K6-DMA as the cationic lipid was also tested in comparison to DLin-K-C2-DMA and DLin-K-DMA. The DLin-K6-DMA formulation reduced FVII levels in mice similarly to DLin-K-DMA, as shown in Figure 6.

#### Pharmacokinetics and liver accumulation of cationic LN-siRNA formulations

**[0246]** The correlation between levels of siRNA delivered to the liver and FVII reduction was determined by encapsulating Cy-3 labeled siRNA in the selection of LN-siRNA systems covering a spectrum of *in vivo* activities shown in Table 8. Cy-3 fluorescence was measured in plasma and liver tissue 0.5 and 3.0 h post injection. The plasma data indicated a wide variety of clearance rates at the early time point, but for the majority of the formulations, 20-50% of the injected siRNA dose was recovered in the liver within 0.5 h, whether they were highly active or exhibited poor activity. The most active formulations, DLinDMA and DLin-K-DMA, showed relatively high levels of siRNA in the liver at 0.5 h, 50% and 32% respectively. All formulations showed a decrease in liver Cy-3 levels after 3 h, which presumably reflected metabolism. This study suggests that gross delivery to the liver alone does not explain differences in activity.

Table 8. Plasma and liver concentrations of Cy-3 siRNA for a selection of active and inactive cationic lipid-containing LN-siRNA systems

Lipid	0.5 h (% injected dose)		3.0 h (% injected dose)		ED <sub>50</sub> (mg/kg)
	Plasma	Liver	Plasma	Liver	
DLin-K-DMA	1.1	32.0	0.4	4.0	0.3
DLinDMA	15.3	50.0	0.7	17.0	1.0
DLinMPZ	20.3	52.0	0.4	37.5	2-5
DLinAP	86.2	11.5	23.1	5.0	5-12
DLin-2-DMAP	17.5	20.5	8.8	2.5	5-12
DLinDAC	27.1	29.0	0.3	6.5	12-25
DLinDAP	46.6	20.5	3.3	16.5	12-25
DLin-C-DAP	69.4	28.5	19.0	13.5	12-25
DLin-S-DMA	10.7	2.5	5.4	0	12-25
DLinMA	20.2	10.5	0.4	4.5	12-25

Tolerability of DLin-K-C2-DMA-containing LN-siRNA systems

**[0247]** Rats administered the liposomal formulation containing DLin-K-C2-DMA showed a dose-dependent loss of weight. Rats administered 91 mg/kg appeared normal and had normal livers. Rats administered 182 mg/kg showed slower movement and a scruffy coat. Their livers were slightly pale, and one of three livers showed some slight mottling. Of the rats administered 364 mg/kg, one died, and they showed hunched, slower movement, quinting eyes, scruffy coats, piloerection, red/orange uring, with pale and some mottling livers. Rats showed significant increases in ALT/AST, as low as 182 mg/kg lipid.

**[0248]** Histopathology results for livers obtained from rats treated with 91 mg/kg ("5" mg/kg) were normal. The livers of rats treated with 182 mg/kg ("10" mg/kg) showed mild to moderate hepatocellular necrosis, centrilobular, and hepatocellular vacuolization. One of the livers of the surviving rats treated with 364 mg/kg ("20" mg/kg) showed moderate hepatocellular necrosis, centrilobular, and the other showed diffuse, mild to moderate hepatocellular necrosis (not concentrated in centrilobular region) with mild inflammation.

**[0249]** Mice treated with the liposomal formulation of DLin-K-C2-DMA also showed a dose-dependent loss of weight, although no mice died. Mice also showed a greater than 10-fold increase in ALT-AST at approximately 1100 mg/kg lipid. However, the mice showed no obvious clinical signs, except at greater than 1300 mg/kg, where the mouse exhibited hunched, slower movement and a scruffy coat.

**[0250]** The introduction of a ketal linker did not appear to impart any significant toxicity issues in mice and, in fact, the LN systems containing DLin-K-DMA and encapsulating FVII siRNA were extraordinarily well tolerated in mice. The data presented in Table 9 are from a study designed to determine appropriate dosing ranges, and extreme, single doses of lipid and siRNA were achieved. The toxicity criteria measured were % change in body weight and serum levels of the liver enzyme markers, ALT and AST.

Table 9. Key tolerability parameters for DLin-K-DMA-containing LN-siRNA systems in mice at extreme doses

siRNA (mg/kg)	Total Lipid (mg/kg)	% Change Body Weight	ALT (IU/L)	AST (IU/L)
0 - 10	0 - 164	0	45	90
46	750	-5.0	174	340
61	1000	-4.5	448	816
76	1250	-5.5	1771	4723
92	1500	-6.0	4723	2094

Compared to saline controls, no changes in blood chemistry or body weight were observed up to an siRNA dose of 10 mg/kg, which for this formulation was > 30-fold greater than the ED<sub>50</sub> dose.

**[0251]** A massive siRNA dose of 46 mg/kg (150-fold more than the ED<sub>50</sub>) was administered before significant toxicity signs were measured. This siRNA dose translated to a total lipid dose of 750 mg/kg at the siRNA-to-lipid ratio of 0.06 (wt/wt). Even at these levels, the increases in serum ALT and AST were relatively modest (< 10-fold normal), and it is not until siRNA doses exceeded 61 mg/kg that severe (> 10-fold) increases are observed. The maximum dose tested was 92 mg siRNA/kg, equivalent to 1500 mg total lipid/kg. Animals lost 6% body weight but no deaths occurred at any of the doses tested.

Characterization of Nucleic Acid-Lipid Particles

**[0252]** Characteristics of selected nucleic acid- lipid formulations are summarized in Table 10, wherein C2 indicates that the cationic lipid is DLin-K-C2-DMA; C4 indicates that the cationic lipid is DLin-K-C4-DMA; and MPZ indicates that the cationic lipid is DLin-K-MPZ. Each of the formulations described below contained PEG-C-DOMG as the PEG-lipid.

Table 10. Characteristics of Formulations Comprising Various Amino Lipids

Formulation	Final Lipid Ratio (mol%)				Particle Size (nm)	Final D/L Ratio (wt/wt)*	% Free siRNA
	Cat	DSPC	Chol	PEG-C-DOMG			
C2	37.4	11.5	41.3	9.8	72 ± 23	0.037	7.8
C4	37.9	9.6	42.8	9.7	64 ± 15	0.058	1.2
MPZ	ND	ND	ND	ND	71 ± 21	0.056**	5.1

\* Based on encapsulated material; \*\* estimated from DSPC, Chol, PEG-C-DOMG results

Apparent pKa's of key cationic lipids measured in situ in LN-siRNA formulations

**[0253]** Two important parameters underlying the lipid design are the  $pK_a$  of the ionizable cationic lipid and the abilities of these lipids, when protonated, to induce a non-bilayer (hexagonal  $H_{II}$ ) phase structure when mixed with anionic lipids. The  $pK_a$  of the ionizable cationic lipid determines the surface charge on the LNP under different pH conditions. The charge state at physiologic pH (e.g., in circulation) can influence plasma protein adsorption, blood clearance and tissue distribution behavior (Semple, S.C., et al., *Adv. Drug Deliv Rev* 32:3-17 (1998)), while the charge state at acidic pH (e.g., in endosomes) can influence the ability of the LNP to combine with endogenous anionic lipids to form endosomolytic non-bilayer structures (Hafez, I.M., et al., *Gene Ther* 8:1188-1196 (2001)). Consequently, the ability of these lipids to induce  $H_{II}$  phase structure in mixtures with anionic lipids is a measure of their bilayer destabilizing capacity and relative endosomolytic potential.

**[0254]** The fluorescent probe 2-(p-toluidino)-6-naphthalene sulfonic acid (TNS), which exhibits increased fluorescence in a hydrophobic environment, can be used to assess surface charge on lipid bilayers. Titrations of surface charge as a function of pH can then be used to determine the apparent  $pK_a$  (hereafter referred to as  $pK_a$ ) of constituent lipids (Cullis, P.R., et al., *Chem Phys Lipids* 40:127-144 (1986)). Using this approach, the  $pK_a$  values for nucleic acid-lipid particles containing various cationic lipids were determined and are summarized in Table 11. The relative ability of the protonated form of certain ionizable cationic lipids to induce  $H_{II}$  phase structure in anionic lipids was ascertained by measuring the bilayer-to-hexagonal  $H_{II}$  transition temperature ( $T_{BH}$ ) in equimolar mixtures with distearoylphosphatidylserine (DSPS) at pH 4.8, using  $^{31}P$  NMR (Cullis, P.R. and de Kruijff, B., *Biochim Biophys Acta* 513:31-42 (1978)) and differential scanning calorimetric (DSC) analyses (Expand, R.M. et al., *Biochemistry* 28:9398-9402 (1989)). Both techniques gave similar results.

**[0255]** The data presented in Table 11 indicate that the highly active lipid DLin-K-C2-DMA has  $pK_a$  and  $T_{BH}$  values that are theoretically favorable for use in siRNA delivery systems. The  $pK_a$  of 6.4 indicates that LNPs based on DLin-KC2-DMA have limited surface charge in circulation, but will become positively charged in endosomes. Further, the  $T_{BH}$  for DLin-K-C2-DMA is 7 °C lower than that for DLinDMA, suggesting that this lipid has improved bilayer destabilizing capacity. However, the data also demonstrate that  $pK_a$  and  $T_{BH}$  do not fully account for the *in vivo* activity of lipids used in LNPs. For example, DLin-K-C3-DMA and DLin-K-C4-DMA have identical  $pK_a$  and  $T_{BH}$  values, yet DLin-KC4-DMA is more than 5-fold less active *in vivo*. Moreover, DLin-K-C2-DMA and DLin-K-C4-DMA, which have very similar  $pK_a$  and  $T_{BH}$  values, exhibit a >30-fold difference in *in vivo* activity. Thus, while the biophysical parameters of  $pK_a$  and  $T_{BH}$  are useful for guiding lipid design, the results presented in Table 11 support the strategy of testing variants of lead lipids, even ones with very similar  $pK_a$  and  $T_{BH}$  values.

Table 11.  $pK_a$ 's of key cationic lipids measured in situ in preformed vesicles using TNS fluorescence titrations

Cationic Lipid	Apparent $pK_a$	$H_{II}$ transition temperature (°C)	FVII ED <sub>50</sub> (mg/kg)
DLin-K-C3-DMA	6.8	18	~0.6
DLin-K-C4-DMA	6.8	18	>3.0
DLinDMA	6.4	27	~1.0
DLin-K-C2-DMA	6.4	20	~0.1
DLin-K6-DMA	6.2	n.d.	~0.3
DLin-K-MPZ	6.2	n.d.	~1.5
DLinDAP	5.7	26	>25
DLin-K-DMA (racemic mixture)	5.6	n.d.	~0.3
DLin-K-MA	5.6	n.d.	>15
DLin-K-DMA	5.6	19	~0.4

**[0256]** As shown above, the potency of LN-siRNA systems containing 40 mole % DLin-K-C2-DMA was such that as little as ~100 picomoles of encapsulated siRNA administered as a single i.v. bolus to a mouse was sufficient to knockdown serum concentrations of FVII protein by 50% within 24 h of injection.

**[0257]** In this study, the ratios of lipid components and siRNA-to-lipid were kept constant for all formulations, so that any differences in surface charge could be attributed to cationic lipid  $pK_a$ . The siRNA-to-lipid ratio used in the activity screen was 0.06 wt/wt, which means that positive charge was in excess of negative charge. Charge neutralization for formulations containing 40 mole% monobasic cationic lipid occurs at a ratio of approximately 0.17 wt/wt. Consequently, assuming one cationic lipid forms an ion pair with each negative charge on the siRNA backbone, then approximately 35% of the total cationic lipid was associated with siRNA inside the nanoparticle and, therefore, could not contribute to surface charge. Interestingly, increasing the siRNA-to-lipid ratio above ~0.08 (wt/wt) in a 40/10/40/10 formulation de-

creased the potency of LN-siRNA systems (data not shown). A similar response has been reported for siRNA delivered *in vivo* using lipidoid nanoparticles (A. Akinc, M. et al., Mol. Ther. (2009) and may reflect the importance of free cationic lipid (lipid not associated with siRNA) and/or the total cationic lipid dose injected.

5 [0258] One of the most striking observations was the dependence of activity on the extent of hydrocarbon chain unsaturation. For both DLinDMA and DLin-K-DMA, there was a significant decrease in potency for each drop in the number of double bonds. Without wishing to be bound by theory, it is proposed that (active) synthetic cationic lipid inserted into the endosomal membrane and endogenous anionic phospholipids (such as phosphatidylserine) form ion pairs (Hafez, I.M., et al., Gene Ther. 8:1188-1196 (2001) and Xu, Y. and Szoka, F.C., Biochemistry 35:5616-5623 (1996)).  
10 The resulting charge neutralization effectively reduces the cross-sectional area of the combined headgroups, which corresponds to a substantial decrease in their intrinsic radius of curvature. Applying the molecular shape arguments employed to describe lipid polymorphism, this means the cationic and anionic lipids go from a cylindrical shape they adopt in isolation to a cone shape formed by the neutral ion pair. Cylindrical shaped lipids are compatible with bilayer structure, whereas cone shaped lipids are not Cullis, P.R. et al., Chem Phys. Lipids 40:127-144 (1986) and Hafez, I.M. and Cullis, P.R., Adv. Drug Deliv. Rev. 47:139-148 (2001)), they prefer to adopt inverted lipid phases, such as the hexagonal H<sub>II</sub> phase, that disrupt bilayer structure. As a consequence, the endosome membrane is lysed, enabling  
15 siRNA to access the cytoplasm where it can engage RISC and cleave FVII mRNA.

[0259] The results described herein are consistent with the shape concept introduced above, because adding cis double bonds to a given chain length increased the cross-sectional area swept out by the terminal methyl groups, thus promoting cone-like geometry. The propensity to adopt non-bilayer structures when paired with anionic phospholipids is also  
20 a plausible rationale for why the ketal-containing lipid family is so active. Both hydrocarbon chains bond through a single carbon into the ketal ring linker, the tetrahedral bond angle will tend to splay the chains apart favoring a cone shape.

#### EXAMPLE 17

#### 25 EFFICACY AND TOLERABILITY OF THE DLIN-K- C2-DMA SNALP FORMULATION

[0260] The efficacy and tolerability of nucleic acid lipid particles comprising DLin-K-C2-DMA was further validated in the context of nucleic acid-lipid particles formulated for delivery of siRNA *in vivo*, termed KC2-SNALP. These particles comprise a different ratio of lipids than the PFV-prepared nucleic acid-lipid particles described in Example 16.

30 [0261] siRNA were encapsulated in SNALP using a controlled step-wise dilution method process described by Jeffs et al. (Pharm Res 22:362-372 (2005)) The lipid constituents of KC2-SNALP were DLin-KC2-DMA (cationic lipid), di-palmitoylphosphatidylcholine (DPPC; Avanti Polar Lipids, Alabaster, AL), synthetic cholesterol (Sigma, St. Louis, MO) and PEG-C-DMA used at a molar ratio of 57.1:7.1:34.3:1.4, respectively. Upon formation of the loaded particles, SNALP were dialyzed against PBS and filter sterilized through a 0.2 μm filter before use. Mean particle sizes were 75-85 nm  
35 and 90-95% of the siRNA was encapsulated within the lipid particles. The final lipid-to-siRNA ratio in formulations used for *in vivo* testing was approximately 6.5:1 (wt:wt).

[0262] The KC2-SNALP formulation showed a marked improvement in potency in the mouse FVII model as compared to the DLin-K-C2-DMA formulation described in Example 16. The measured ED<sub>50</sub> decreased from ~0.1 mg/kg for the DLin-K-C2-DMA nucleic acid-lipid formulation described in Example 16 to ~0.02 mg/kg for the KC2-SNALP formulation  
40 (Figure 8A). KC2-SNALP was also found to exhibit similar potency in rats (data not shown).

[0263] In addition to efficacy, tolerability is another critical attribute of a suitable nucleic acid-lipid particle delivery system for human use, so the single-dose tolerability of KC2-SNALP was studied in rats. Doses near the efficacious dose level were found to be very well tolerated (data not shown); therefore, single-dose escalation studies were conducted starting at doses ~50-fold higher (1 mg/kg) than the observed ED<sub>50</sub> of the formulation. To understand formulation toxicity  
45 in the absence of any toxicity or pharmacologic effects resulting from target silencing, these experiments were conducted using the non-targeting control siRNA sequence directed against luciferase described in Example 16. Clinical signs were observed daily, and body weights, serum chemistry, and hematology parameters were measured at 72 hours post-dose. As shown in Table 12, KC2-SNALP was very well tolerated at the high dose levels examined (relative to the observed ED<sub>50</sub> dose) with no dose-dependent, clinically significant changes in key serum chemistry or hematology parameters.  
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Table 12. Clinical chemistry and hematology parameters in rats

Vehicle	siRNA dose (mg/kg)	ALT (U/L)	AST (U/L)	Total Bilirubin (mg/dL)	BUN (mg/dL)	RBC (x 10 <sup>6</sup> /μL)	Hemo-globin (g/dL)	WBC (x 10 <sup>3</sup> /μL)	PLT (x 10 <sup>3</sup> /μL)
PBS		56 ± 16	109 ± 31	2 ± 0	4.8 ± 0.8	5.5 ± 0.3	11.3 ± 0.4	11 ± 3	166 ± 177
KC2-SNALP	1	58 ± 22	100 ± 14	2 ± 0	4.4 ± 0.6	5.6 ± 0.2	11.6 ± 0.6	13 ± 2	1000 ± 272
KC2-SNALP	2	73 ± 9	81 ± 10	2.2 ± 0.4	4.3 ± 0.6	5.9 ± 0.3	11.6 ± 0.3	13 ± 4	1271 ± 269
KC2-SNALP	3	87 ± 19	100 ± 30	2 ± 0	5.0 ± 0.8	6.0 ± 0.2	11.9 ± 0.4	15 ± 2	958 ± 241

EXAMPLE 18

## IN vivo EFFICACY AND TOLERABILITY OF KC2-SNALP IN PRIMATES

5 **[0264]** Given the promising activity and safety profile observed in rodents in the studies described in Example 17, studies were performed in non-human primates to investigate the translation of DLin-KC2-DMA activity in higher species. For these studies, transthyretin (TTR), a hepatic gene of high therapeutic interest, was targeted.

10 **[0265]** Cynomolgus monkeys were treated with a single 15 minute intravenous infusion of KC2-SNALP-formulated siTTR at siRNA doses of 0.03, 0.1, 0.3 and 1 mg/kg. Control animals received a single 15 minute intravenous infusion of PBS or KC2-SNALP-formulated ApoB siRNA at a dose of 1 mg/kg. All siRNAs were synthesized by Alnylam and were characterized by electrospray mass spectrometry and anion exchange HPLC. The sequences for the sense and antisense strands of FVII, ApoB, and Control siRNAs have been reported (Akinc, A. et al., Nat. Biotechnol 26:561-569 (2008)). The sequences for the sense and antisense strands of the TTR siRNA were as follows:

15 siTTR sense: 5'- GuAAccAAGAGuAuuccAuTT-3' (SEQ ID NO:37); and  
siTTR antisense: 5'- AUGGAAuACUCUUGGUuACTT-3' (SEQ ID NO:38),

with 2'-O-Me modified nucleotides shown in lower case. siRNAs were generated by annealing equimolar amounts of complementary sense and antisense strands.

20 **[0266]** Tissues were harvested at 48 hours post-administration, and liver mRNA levels of TTR were determined. A clear dose response was obtained with an apparent ED<sub>50</sub> of ~0.3 mg/kg (Figure 8B). A toxicological analysis indicated that the treatment was well tolerated at the dose levels tested, with no treatment-related changes in animal appearance or behavior. No dose-dependent, clinically significant alterations in key clinical chemistry or hematological parameters were observed (Table 13).

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Table 13. Clinical chemistry, and hematology parameters in NHPs

Treatment	siRNA dose (mg/kg)	ALT (U/L)	AST (U/L)	Total Bilirubin (mg/dL)	BUN (mg/dL)	RBC (x 10 <sup>6</sup> /μL)	Hemo-globin (g/dL)	WBC (x 10 <sup>3</sup> /μL)	PLT (x 10 <sup>3</sup> /μL)
PBS	-	54 ± 25	51 ± 27	0.3 ± 0.1	27 ± 4	4.6 ± 0.5	13.8 ± 0.6	17.6 ± 3.0	515 ± 70
siApoB	1	42 ± 11	49 ± 12	0.3 ± 0.1	23 ± 3	6.0 ± 0.2	14.2 ± 0.9	13.6 ± 3.7	508 ± 49
siTTR	0.03	57 ± 11	47 ± 12	0.1 ± 0	15 ± 4	4.8 ± 0.4	11.5 ± 0.9	10.9 ± 2.2	495 ± 105
siTTR	0.1	50 ± 22	63 ± 47	0.13 ± 0.1	20 ± 3	5.0 ± 0.0	11.1 ± 0.4	12.9 ± 3.3	528 ± 22
siTTR	0.3	67 ± 8	66 ± 18	0.1 ± 0	21 ± 6	5.1 ± 0.2	11.0 ± 0.5	11.1 ± 5.5	529 ± 72
siTTR	1	47 ± 5	43 ± 7	0.13 ± 0.1	19 ± 1	4.9 ± 0.1	10.9 ± 0.4	10.7 ± 1.6	477 ± 34

**[0267]** In summary, a rational design approach was employed for the discovery of novel lipids for use in next-generation LNP systems to deliver RNAi therapeutics. Using this approach, important structure-activity considerations for ionizable cationic lipids were described, and multiple lipids based on the DLinDMA structure were designed and characterized. A SNALP formulation of the best performing lipid (DLfn-K-C2-DMA) was well-tolerated in both rodent and non-human primates and exhibited *in vivo* activity at siRNA doses as low as 0.01 mg/kg in rodents, as well as silencing of a therapeutically significant gene (TTR) in non-human primates. Notably, the TTR silencing achieved in this work (ED<sub>50</sub> ~ 0.3 mg/kg), represents a significant improvement in activity relative to previous reports of LNP-siRNA mediated silencing in non-human primates. The efficacy observed in this study, to our knowledge, represents the highest level of potency observed for an RNAi therapeutic in non-human primates to date, and highlights the considerable progress that has been made in both RNAi and delivery technologies.

## SEQUENCE LISTING

**[0268]**

<110> Tekmira Pharmaceutical Corporation The University of British Columbia Hope, Michael J. Semple, Sean C. Chen, Jianxin Madden, Thomas D. Cullis, Pieter R. Ciufolini, Marco A.

<120> IMPROVED AMINO LIPIDS AND METHODS FOR THE DELIVERY OF NUCLEIC ACIDS

<130> 480208.461PC

<140> PCT

<141> 2009-10-09

<150> US 61/104,219

<151> 2008-10-09

<150> US 61/104,212

<151> 2008-10-09

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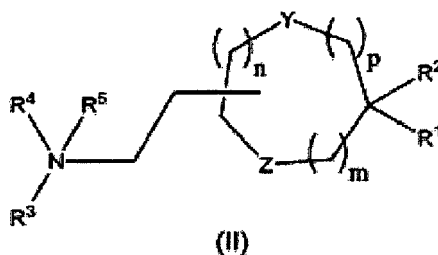
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20 **Claims**

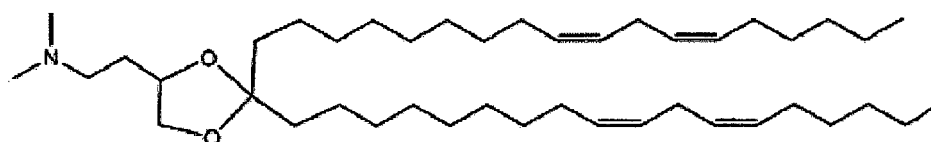
1. An amino lipid having the following structure (II):



or salts thereof, wherein

35  $R^1$  and  $R^2$  are either the same or different and independently substituted  $C_{12}$ - $C_{24}$  alkyl, optionally substituted  $C_{12}$ - $C_{24}$  alkenyl, optionally substituted  $C_{12}$ - $C_{24}$  alkynyl, or optionally substituted  $C_{12}$ - $C_{24}$  acyl;  
 $R^3$  and  $R^4$  are either the same or different and independently optionally substituted  $C_1$ - $C_6$  alkyl, optionally substituted  $C_1$ - $C_6$  alkenyl, or optionally substituted  $C_1$ - $C_6$  alkynyl, or  $R^3$  and  $R^4$  may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms chosen from nitrogen and oxygen;  
 40  $R^5$  is either absent or is hydrogen or  $C_1$ - $C_6$  alkyl to provide a quaternary amine;  
 $m$ ,  $n$ , and  $p$  are either the same or different and independently either 0 or 1 with the proviso that  $m$ ,  $n$ , and  $p$  are not simultaneously 0;  
 $Y$  and  $Z$  are either the same or different and independently O, S, or NH.

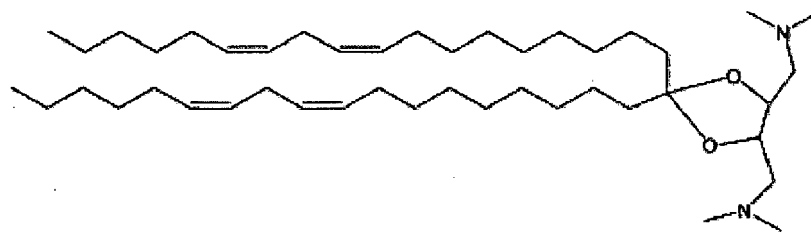
45 2. The amino lipid of claim 1, having the structure:



55 DLin-K-C2-DMA.

3. An amino lipid having one of the following structures:

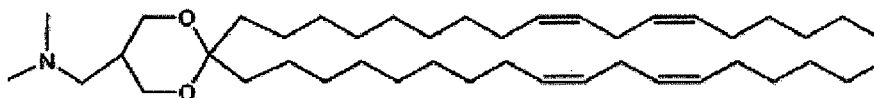
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10

DLin-K<sup>2</sup>-DMA or

15



20

DLin-K6-DMA.

25

4. A lipid particle comprising an amino lipid of any one of claims 1 to 3, wherein optionally and preferably, the particle further comprises a neutral lipid and a lipid capable of reducing particle aggregation; and wherein optionally and further preferably, wherein the lipid particle comprises:

30

- (i) DLin-K-C2-DMA;
- (ii) a neutral lipid selected from DSPC, POPC, DOPE, and SM;
- (iii) cholesterol; and
- (iv) a PEG-lipid,

in a molar ratio of about 20-60% DLin-K-DMA:5-25% neutral lipid :25-55% Chol:0.5-15% PEG-lipid.

35

5. The lipid particle of claim 4 further comprising a therapeutic agent; wherein optionally and preferably, the therapeutic agent is a nucleic acid; and wherein optionally and further preferably, the nucleic acid is a plasmid, or an immunostimulatory oligonucleotide.

40

6. The lipid particle of claim 5, wherein the nucleic acid is selected from the group consisting of: a siRNA, a microRNA, an antisense oligonucleotide, and a ribozyme; optionally, wherein the nucleic acid is a siRNA.

45

7. A pharmaceutical composition comprising the lipid particle of any one of claims 4, 5 or 6 and a pharmaceutically acceptable excipient, carrier, or diluent.

50

8. An *in vitro* method of modulating the expression of a polypeptide by a cell, comprising providing to a cell the lipid particle of any one of claims 4-7; wherein optionally and preferably, the therapeutic agent is selected from an siRNA, a microRNA, an antisense oligonucleotide, and a plasmid capable of expressing an siRNA, a microRNA, or an antisense oligonucleotide, and wherein the siRNA, microRNA, or antisense RNA comprises a polynucleotide that specifically binds to a polynucleotide that encodes the polypeptide, or a complement thereof, such that the expression of the polypeptide is reduced; wherein optionally and further preferably, the therapeutic agent is a plasmid that encodes the polypeptide or a functional variant or fragment thereof, such that expression of the polypeptide or the functional variant or fragment thereof is increased.

55

9. The pharmaceutical composition of claim 7 for use in a method of treating a disease or disorder **characterized by** over expression of a polypeptide in a subject, the method comprising providing the subject with the pharmaceutical composition, wherein the therapeutic agent is selected from an siRNA, a microRNA, an antisense oligonucleotide, and a plasmid capable of expressing an siRNA, a microRNA, or an antisense oligonucleotide, and wherein the siRNA, microRNA, or antisense RNA comprises a polynucleotide that specifically binds to a polynucleotide that encodes the polypeptide, or a complement thereof.

10. The pharmaceutical composition of claim 7 for use in a method of treating a disease or disorder **characterized by** underexpression of a polypeptide in a subject, wherein the therapeutic agent is a plasmid that encodes the polypeptide or a functional variant or fragment thereof.

5 11. The pharmaceutical composition of claim 7 for use in a method of inducing an immune response in a subject, wherein the therapeutic agent is an immunostimulatory oligonucleotide.

12. The pharmaceutical composition for use in claim 11, wherein the pharmaceutical composition is provided to the patient in combination with a vaccine or antigen.

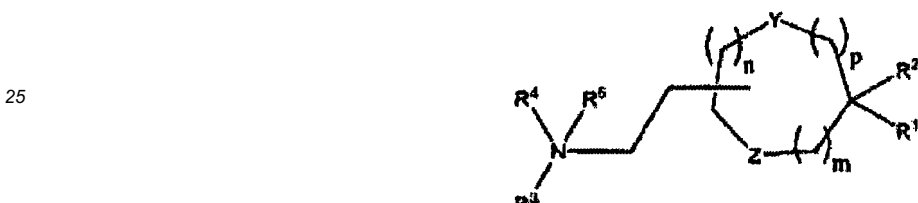
10 13. A vaccine comprising the lipid particle of claim 5 and an antigen associated with a disease or pathogen.

14. The vaccine of claim 13, wherein said antigen is a tumor antigen.

15 15. The vaccine of claim 13, wherein said antigen is a viral antigen, a bacterial antigen, or a parasitic antigen.

### Patentansprüche

20 1. Aminolipid mit der folgenden Struktur (II):



30 oder Salzen davon, wobei

R<sup>1</sup> und R<sup>2</sup> entweder dasselbe oder jeweils ein unterschiedliches und unabhängig substituiertes C<sub>12</sub>-C<sub>24</sub>-Alkyl, optional substituiertes C<sub>12</sub>-C<sub>24</sub>-Alkenyl, optional substituiertes C<sub>12</sub>-C<sub>24</sub>-Alkynyl oder optional substituiertes C<sub>12</sub>-C<sub>24</sub>-Acyl sind;

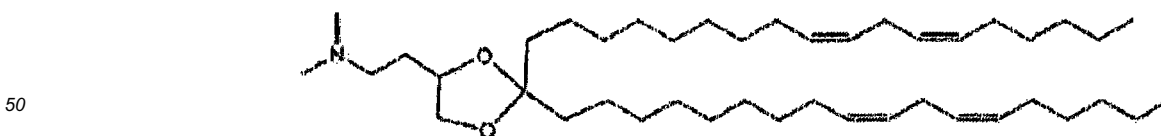
35 R<sup>3</sup> und R<sup>4</sup> entweder dasselbe oder jeweils ein unterschiedliches und unabhängig optional substituiertes C<sub>1</sub>-C<sub>6</sub>-Alkyl, optional substituiertes C<sub>1</sub>-C<sub>6</sub>-Alkenyl oder optional substituiertes C<sub>1</sub>-C<sub>6</sub>-Alkynyl sind oder R<sup>3</sup> und R<sup>4</sup> sich verbinden können, um einen optional substituierten heterozyklischen Ring aus 4 bis 6 Kohlenstoffatomen und 1 oder 2 aus Stickstoff und Sauerstoff ausgewählten Heteroatomen zu bilden;

R<sup>5</sup> entweder fehlt oder Wasserstoff oder C<sub>1</sub>-C<sub>6</sub>-Alkyl ist, um ein quartäres Amin bereitzustellen;

40 m, n, und p entweder dasselbe oder unterschiedlich und unabhängig entweder 0 oder 1 sind mit der Maßgabe, dass m, n, und p nicht gleichzeitig 0 sind;

Y und Z entweder dasselbe oder verschieden und unabhängig O, S, oder NH sind.

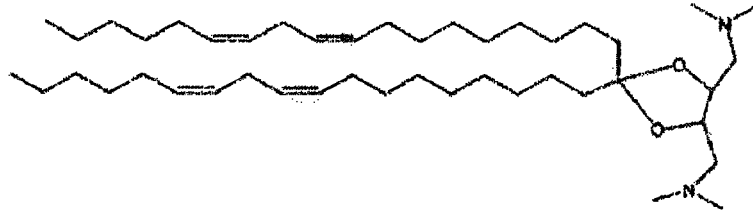
45 2. Aminolipid nach Anspruch 1, mit der Struktur:



DLin-K-C2-DMA.

55 3. Aminolipid mit einer der folgenden Strukturen:

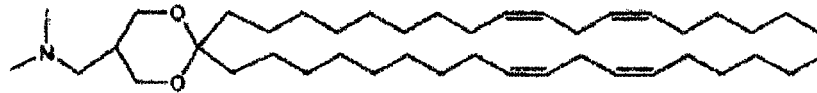
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10

DLin-K<sup>2</sup>-DMA oder

15



20

DLin-K6-DMA.

25

4. Ein Lipidpartikel umfassend ein Aminolipid nach einem beliebigen der Ansprüche 1 bis 3, wobei, optional und vorzugsweise, das Partikel ferner ein neutrales Lipid und ein Lipid umfasst, das zur reduzierenden Partikelaggregation imstande ist; und wobei, optional und ebenfalls vorzugsweise, das Lipidpartikel umfasst:

30

- (i) DLin-K-C2-DMA;
- (ii) ein aus DSPC, POPC, DOPE und SM ausgewähltes neutrales Lipid;
- (iii) Cholesterin; und
- (iv) ein PEG-Lipid,

in einem Molverhältnis von etwa 20-60 % DLin-K-DMA:5-25 % neutrales Lipid:25-55% Chol:0,5-15 % PEG-Lipid.

35

5. Das Lipidpartikel nach Anspruch 4, ferner umfassend ein Therapeutikum; wobei, optional und vorzugsweise, das Therapeutikum eine Nukleinsäure ist; und wobei, optional und ebenfalls vorzugsweise, die Nukleinsäure ein Plasmid oder ein immunstimulierendes Oligonukleotid ist.

40

6. Das Lipidpartikel nach Anspruch 5, wobei die Nukleinsäure ist aus der Gruppe ausgewählt wird, die aus Folgendem besteht: einer siRNA, einer microRNA, einem Antisense-Oligonukleotid und einem Ribozym; optional, wobei die Nukleinsäure eine siRNA ist.

45

7. Pharmazeutische Zusammensetzung des Lipidpartikel nach einem beliebigen der Ansprüche 4, 5 oder 6 und pharmazeutisch annehmbaren Hilfsstoff, Träger oder Verdünner.

50

8. *In-vitro*-Verfahren zur Modulation der Expression eines Polypeptids durch eine Zelle, umfassend die Bereitstellung, für eine Zelle, des Lipidpartikels nach einem beliebigen der Ansprüche 4-7; wobei, optional und vorzugsweise, das Therapeutikum aus einer siRNA, einer microRNA, einem Antisense-Oligonukleotid und einem Plasmid mit Befähigung zur Expression einer siRNA, einer microRNA, oder eines Antisense-Oligonukleotids ausgewählt wird und wobei die siRNA, microRNA, oder Antisense-RNA ein Olynukeotid umfasst, das sich spezifisch an ein Polynukleotid bindet, das das Polypeptid oder ein Komplement davon codiert, sodass die Expression des Polypeptids reduziert wird; wobei, optional und ebenfalls vorzugsweise, das Therapeutikum ein Plasmid ist, das das Polypeptid oder eine funktionelle Variante ein funktionelles Fragment davon codiert, sodass die Expression des Polypeptids oder der funktionellen Variante oder des funktionelles Fragments davon erhöht wird.

55

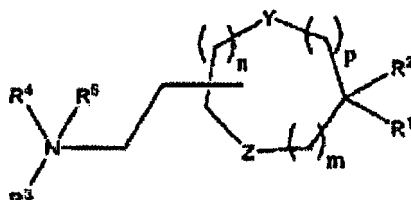
9. Pharmazeutische Zusammensetzung nach Anspruch 7 zur Verwendung bei einem Verfahren zur Behandlung einer Erkrankung oder Leiden, die oder das durch Überexpression eines Polypeptids bei einem Probanden gekennzeichnet ist, wobei das Verfahren die Versorgung des Probanden mit der pharmazeutischen Zusammensetzung umfasst,

wobei das Therapeutikum aus siRNA, einer micro A, einem Antisense-ligonukleotid und einem Plasmid mit Befähigung zur Expression einer siRNA, einer microRNA, oder eines Antisense-Oligonukleotids ausgewählt wird und wobei die siRNA, microRNA, oder Antisense-RNA ein Polynukleotid umfasst, das sich spezifisch an ein Polynukleotid bindet, das das Polypeptid oder ein Komplement davon codiert.

10. Pharmazeutische Zusammensetzung nach Anspruch 7 zur Verwendung bei einem Verfahren zur Behandlung einer Erkrankung oder eines Leidens, die oder das durch Unterexpression eines Polypeptids bei einem Probanden gekennzeichnet ist, wobei das Therapeutikum ein Plasmid ist, das das Polypeptid oder eine funktionelle Variante oder ein funktionelles Fragment davon codiert.
11. Pharmazeutische Zusammensetzung nach Anspruch 7 zur Verwendung bei einem Verfahren zur Induzierung einer Immunantwort bei einem Probanden, wobei das Therapeutikum ein immunstimulierendes Oligonukleotid ist.
12. Pharmazeutische Zusammensetzung zur Verwendung in Anspruch 11, wobei die pharmazeutische Zusammensetzung für den Patienten in Kombination mit einem Vakzin oder Antigen bereitgestellt wird.
13. Vakzin umfassend das Lipidpartikel nach Anspruch 5 und ein mit einer Erkrankung oder einem Pathogen assoziiertes Antigen.
14. Vakzin nach Anspruch 13, wobei das Antigen ein Tumorantigen ist.
15. Vakzin nach Anspruch 13, wobei das Antigen ein virales Antigen, ein bakterielles Antigen oder ein parasitäres Antigen ist.

## Revendications

1. Lipide aminé présentant la structure suivante (II) :



ou sels correspondants, dans lequel

$R_1$  et  $R_2$  sont identiques ou différents et indépendamment un groupe alkyle en  $C_{12}-C_{24}$  substitué, un groupe alcényle en  $C_{12}-C_{24}$  éventuellement substitué, un groupe alcynyle en  $C_{12}-C_{24}$  éventuellement substitué, ou un groupe acyle en  $C_{12}-C_{24}$  éventuellement substitué ;

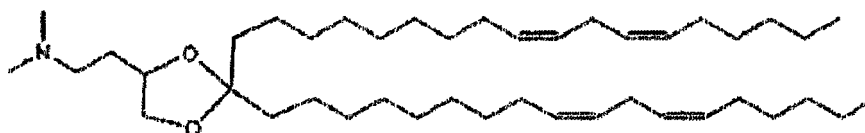
$R_3$  et  $R_4$  sont identiques ou différents et indépendamment un groupe alkyle en  $C_1-C_6$  éventuellement substitué, un groupe alcényle en  $C_1-C_6$  éventuellement substitué, ou un groupe alcynyle en  $C_1-C_6$  éventuellement substitué, ou  $R_3$  et  $R_4$  peuvent se joindre pour former un hétérocycle éventuellement substitué de 4 à 6 atomes de carbone et 1 ou 2 hétéroatomes choisis parmi l'azote et l'oxygène ;

$R_5$  est soit absent, soit de l'hydrogène ou un groupe alkyle en  $C_1-C_6$  pour fournir une amine quaternaire ;

$m$ ,  $n$  et  $p$  sont identiques ou différents et valent indépendamment 0 ou 1, sous réserve que  $m$ ,  $n$  et  $p$  ne valent pas simultanément 0 ;

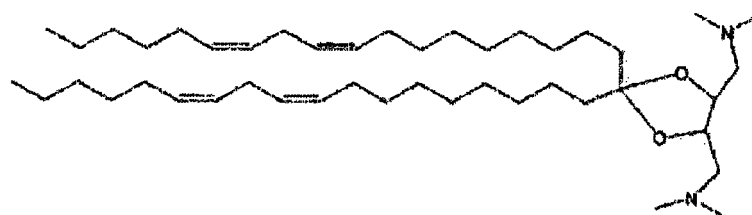
$Y$  et  $Z$  sont identiques ou différents et indépendamment O, S ou NH.

2. Lipide aminé selon la revendication 1, présentant la structure :

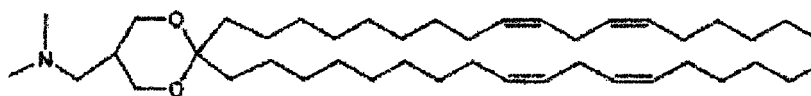


DLin-K-C2-DMA.

3. Lipide aminé présentant l'une des structures suivantes :



DLin-K2-DMA ou



DLin-K6-DMA.

4. Particule lipidique comprenant un lipide aminé selon l'une quelconque des revendications 1 à 3, ladite particule comprenant, éventuellement et de préférence, un lipide neutre et un lipide capable de réduire l'agrégation de particules ; et ladite particule lipidique comprenant, éventuellement et encore de préférence :

- (i) DLin-K-C2-DMA;
- (ii) un lipide neutre choisi parmi DSPC, POPC, DOPE et SM ;
- (iii) du cholestérol ; et
- (iv) un lipide dérivé du PEG,
- (v) selon un rapport molaire d'environ 20 à 60 % de DLin-K-DMA sur 5 à 25 % de lipide neutre sur 25 à 55 % de cholestérol sur 0,5 à 15 % de lipide dérivé du PEG.

5. Particule lipidique selon la revendication 4, comprenant en outre un agent thérapeutique ; dans laquelle, éventuellement et de préférence, l'agent thérapeutique est un acide nucléique ; et dans laquelle, éventuellement et encore de préférence, l'acide nucléique est un plasmide ou un oligonucléotide immunostimulateur.

6. Particule lipidique selon la revendication 5, dans laquelle l'acide nucléique est choisi dans le groupe constitué d'un ARNc, d'un microARN, d'un oligonucléotide antisens et d'un ribozyme ; l'acide nucléique étant, éventuellement, un ARNc.

7. Composition pharmaceutique comprenant la particule lipidique selon l'une quelconque des revendications 4, 5 et 6, et excipient, support ou diluant pharmaceutiquement acceptable.

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8. Procédé *in vitro* de modulation de l'expression d'un polypeptide par une cellule, consistant à fournir à une cellule la particule lipidique selon l'une quelconque des revendications 4 à 7 ; dans lequel, éventuellement et de préférence, l'agent thérapeutique est choisi parmi un ARN*ic*, un microARN, un oligonucléotide antisens et un plasmide capable d'exprimer un ARN*ic*, un microARN ou un oligonucléotide antisens, et dans lequel l'ARN*ic*, le microARN ou l'ARN antisens comprend un polynucléotide qui se lie de manière spécifique à un polynucléotide codant le polypeptide, ou un complément correspondant, de telle sorte que l'expression du polypeptide soit réduite ; et dans lequel, éventuellement et encore de préférence, l'agent thérapeutique est un plasmide qui code le polypeptide, ou une variante ou un fragment fonctionnel correspondant, de telle sorte que l'expression du polypeptide ou de la variante ou du fragment fonctionnel correspondant soit augmentée.
  9. Composition pharmaceutique selon la revendication 7 destinée à être utilisée dans un procédé de traitement d'une maladie ou d'un trouble **caractérisé par** une surexpression d'un polypeptide chez un sujet, le procédé consistant à fournir au sujet la composition pharmaceutique, dans laquelle l'agent thérapeutique est choisi parmi un ARN*ic*, un microARN, un oligonucléotide antisens et un plasmide capable d'exprimer un ARN*ic*, un microARN ou un oligonucléotide antisens, et dans laquelle l'ARN*ic*, le microARN ou l'ARN antisens comprend un polynucléotide qui se lie de manière spécifique à un polynucléotide codant le polypeptide, ou un complément correspondant.
  10. Composition pharmaceutique selon la revendication 7, destinée à être utilisée dans un procédé de traitement d'une maladie ou d'un trouble **caractérisé par** la sous-expression d'un polypeptide chez un sujet, dans laquelle l'agent thérapeutique est un plasmide codant le polypeptide ou une variante ou un fragment fonctionnel correspondant.
  11. Composition pharmaceutique selon la revendication 7, destinée à être utilisée dans un procédé d'induction d'une réponse immunitaire chez un sujet, dans laquelle l'agent thérapeutique est un oligonucléotide immunostimulateur.
  12. Composition pharmaceutique destinée à être utilisée selon la revendication 11, la composition pharmaceutique étant fournie au patient en combinaison avec un vaccin ou un antigène.
  13. Vaccin comprenant la particule lipidique selon la revendication 5 et un antigène associé à une maladie ou un pathogène.
  14. Vaccin selon la revendication 13, dans lequel ledit antigène est un antigène tumoral.
  15. Vaccin selon la revendication 13, dans lequel ledit antigène est un antigène viral, un antigène bactérien ou un antigène parasitaire.

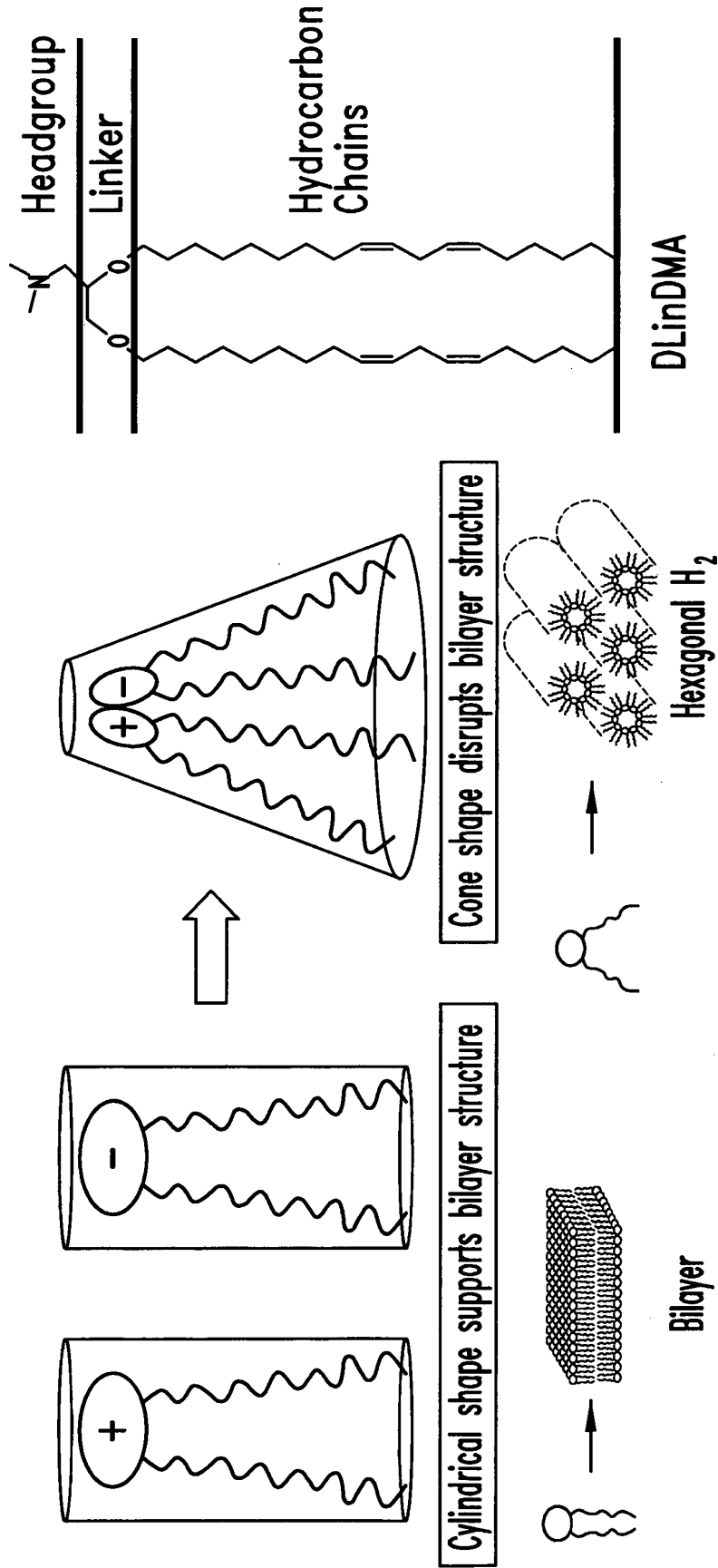


FIG. 1

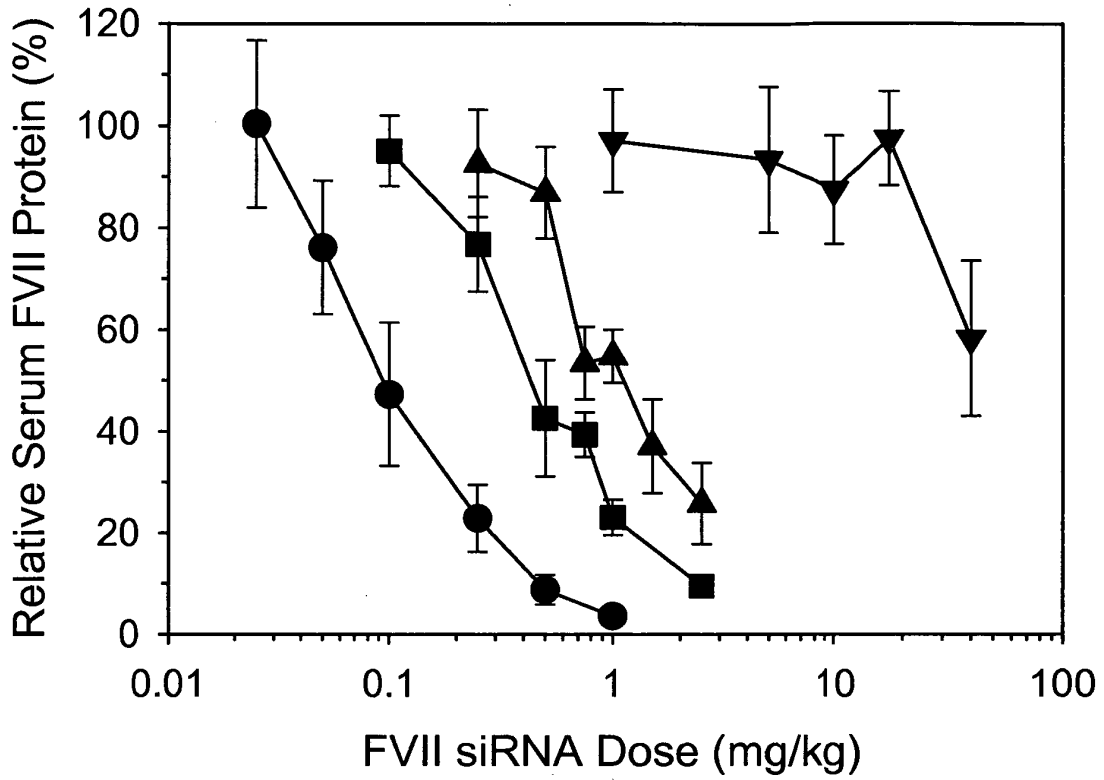


FIG. 2A

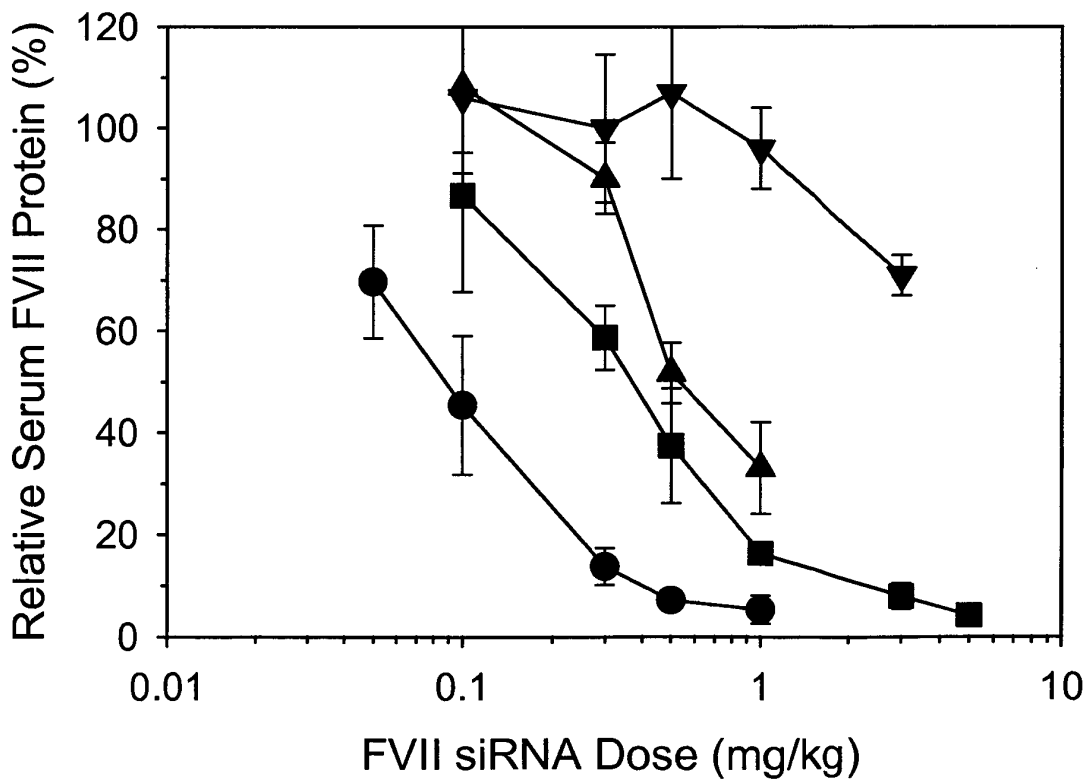


FIG. 2B

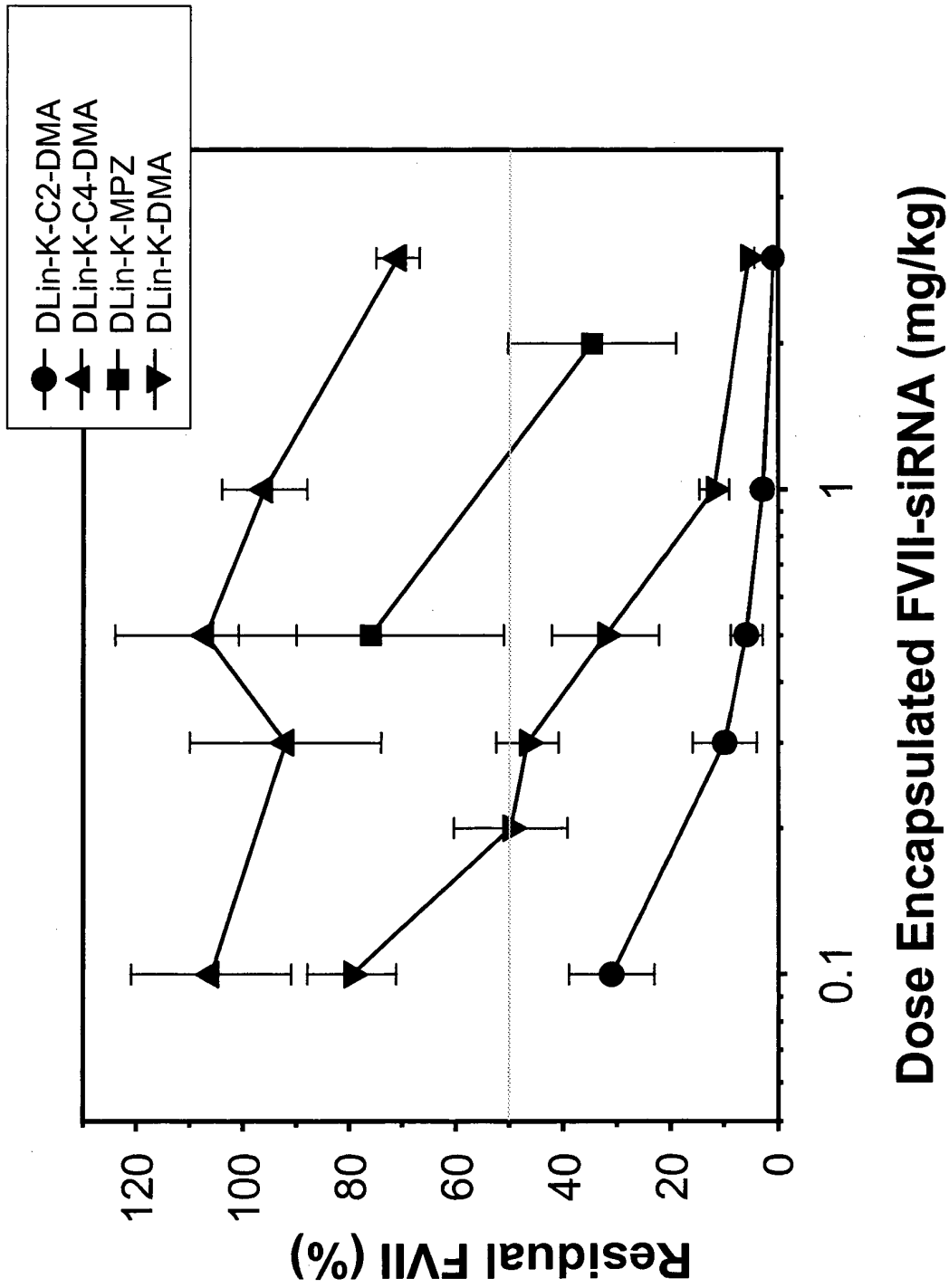


FIG. 3

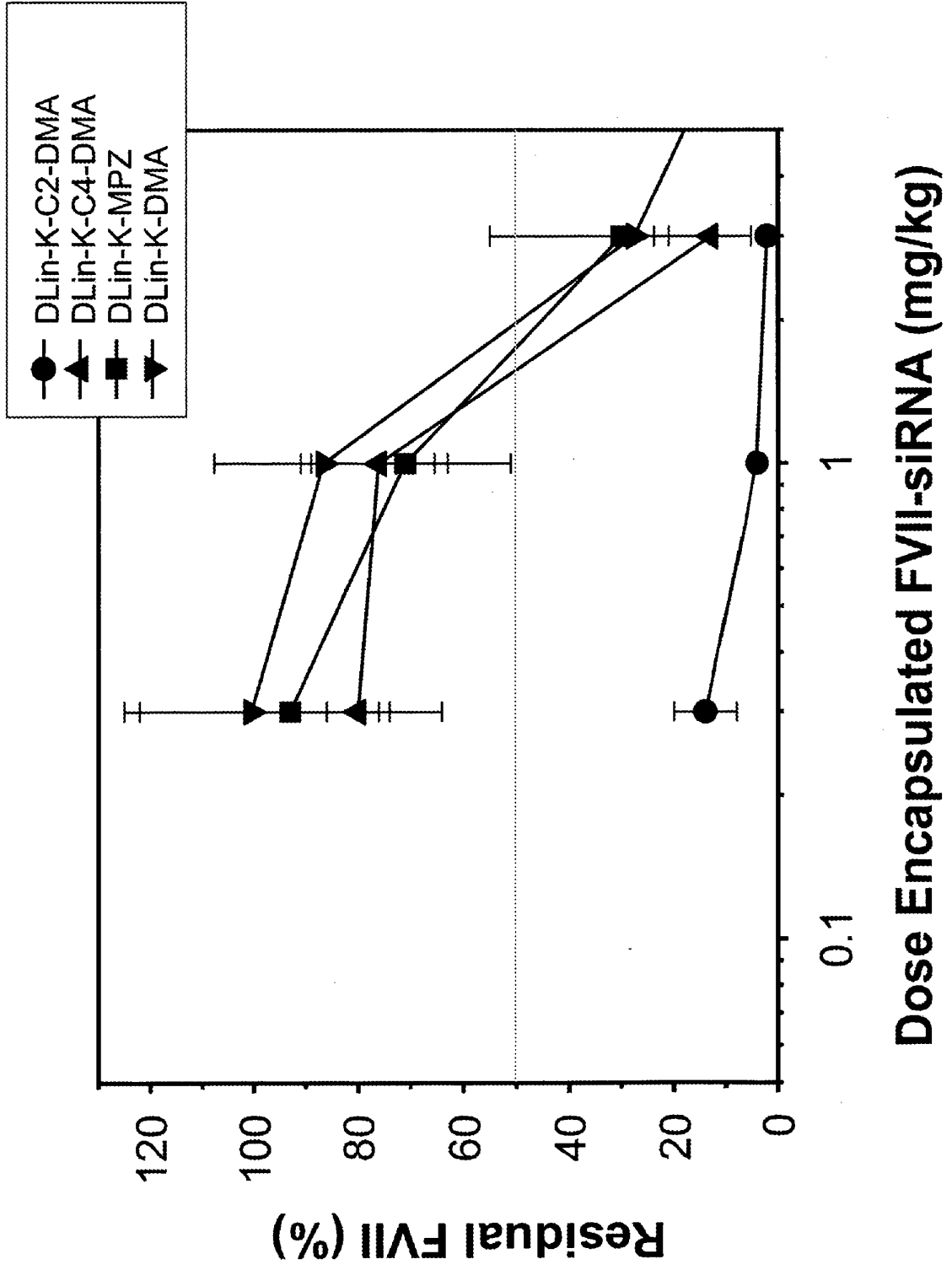
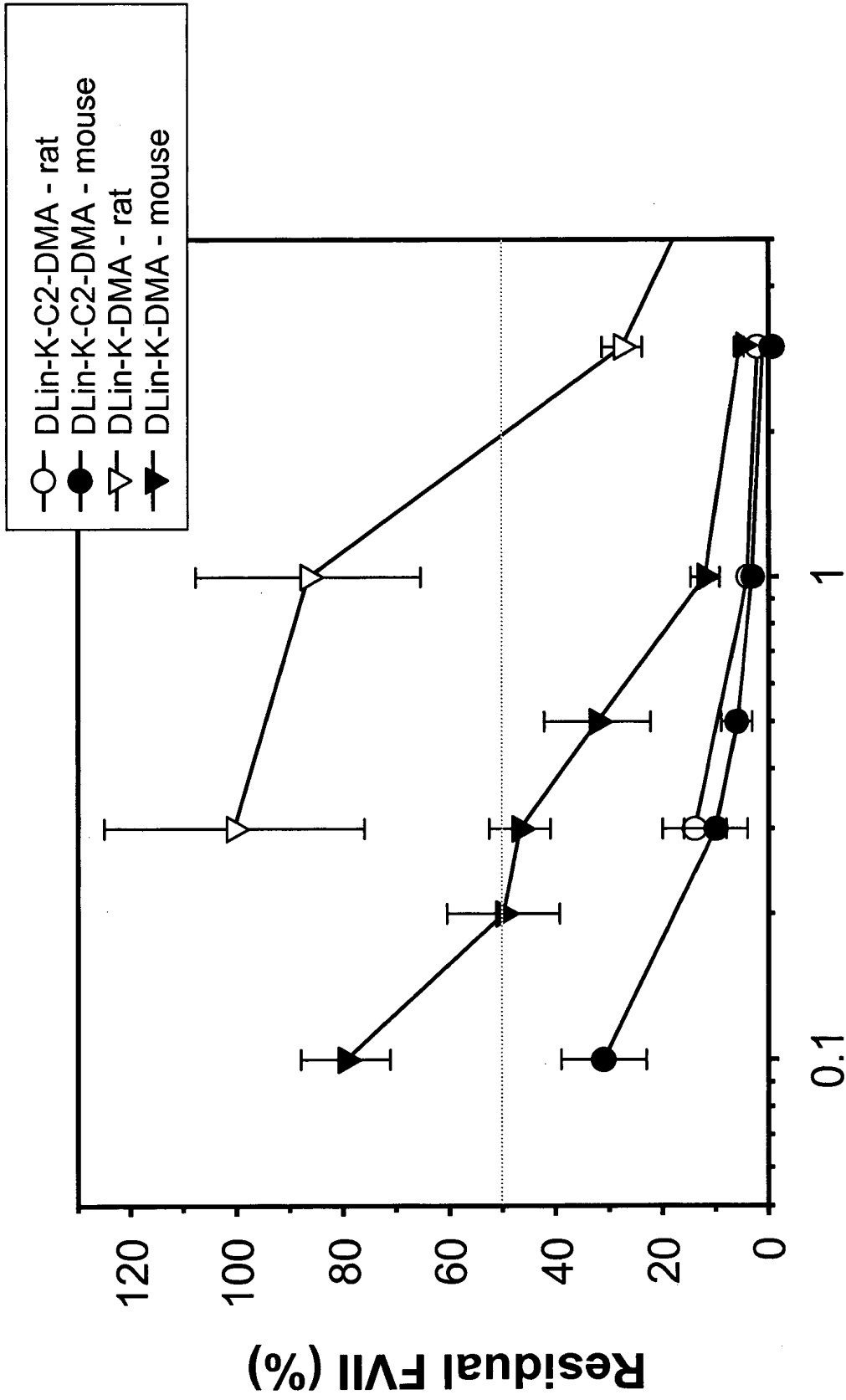


FIG. 4



**Dose Encapsulated FVII-siRNA (mg/kg)**

*FIG. 5*

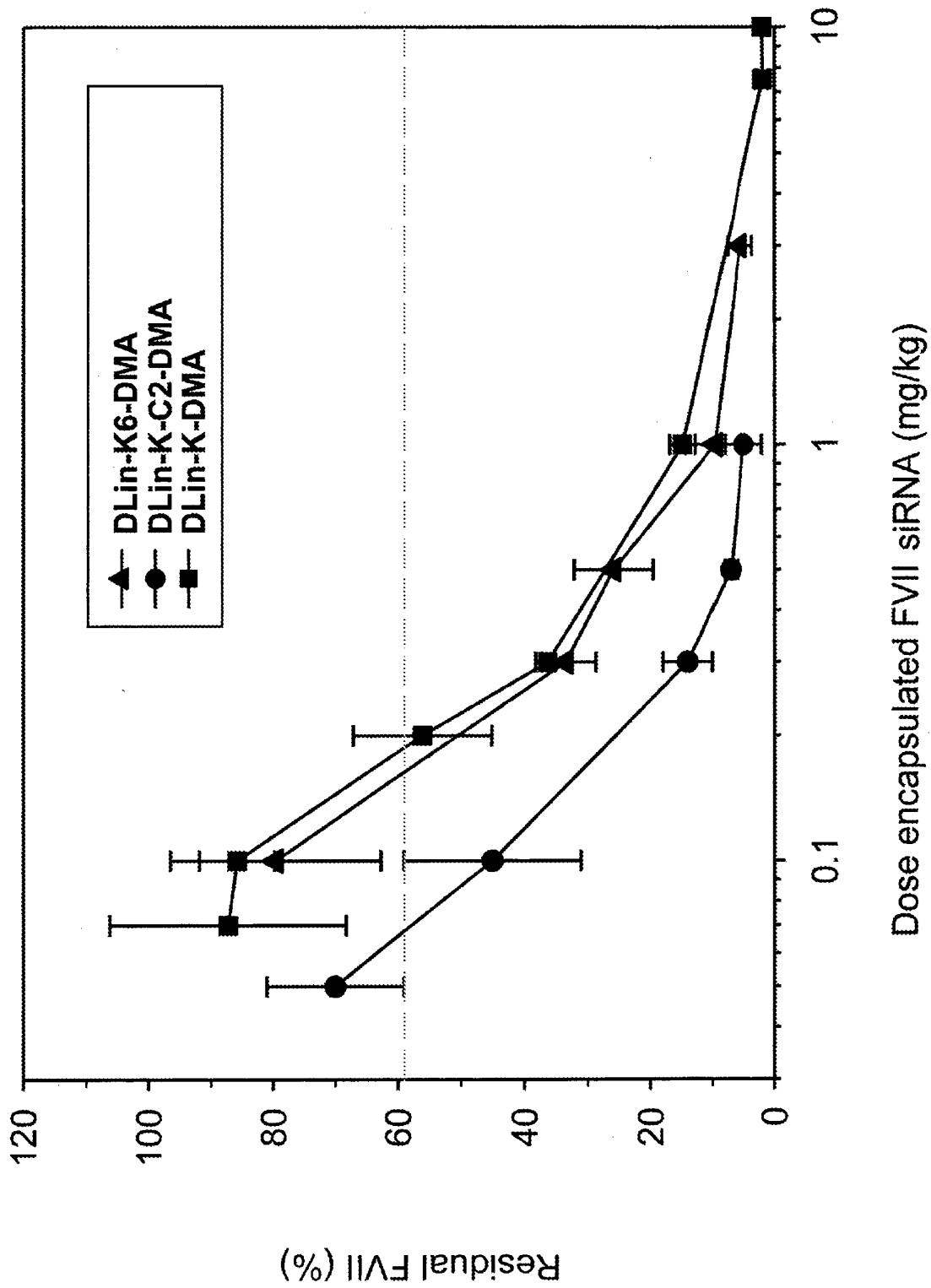


FIG. 6

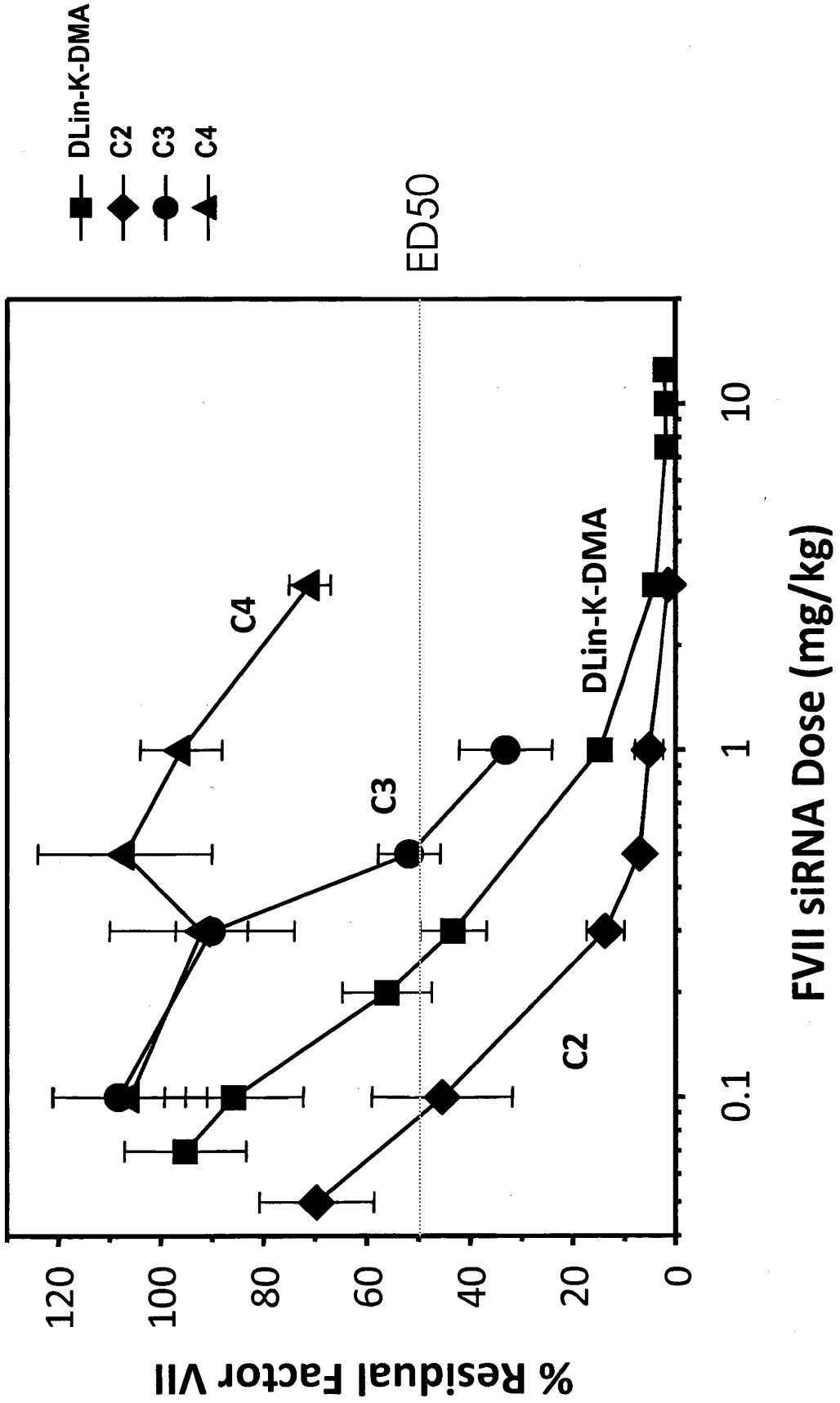


FIG. 7

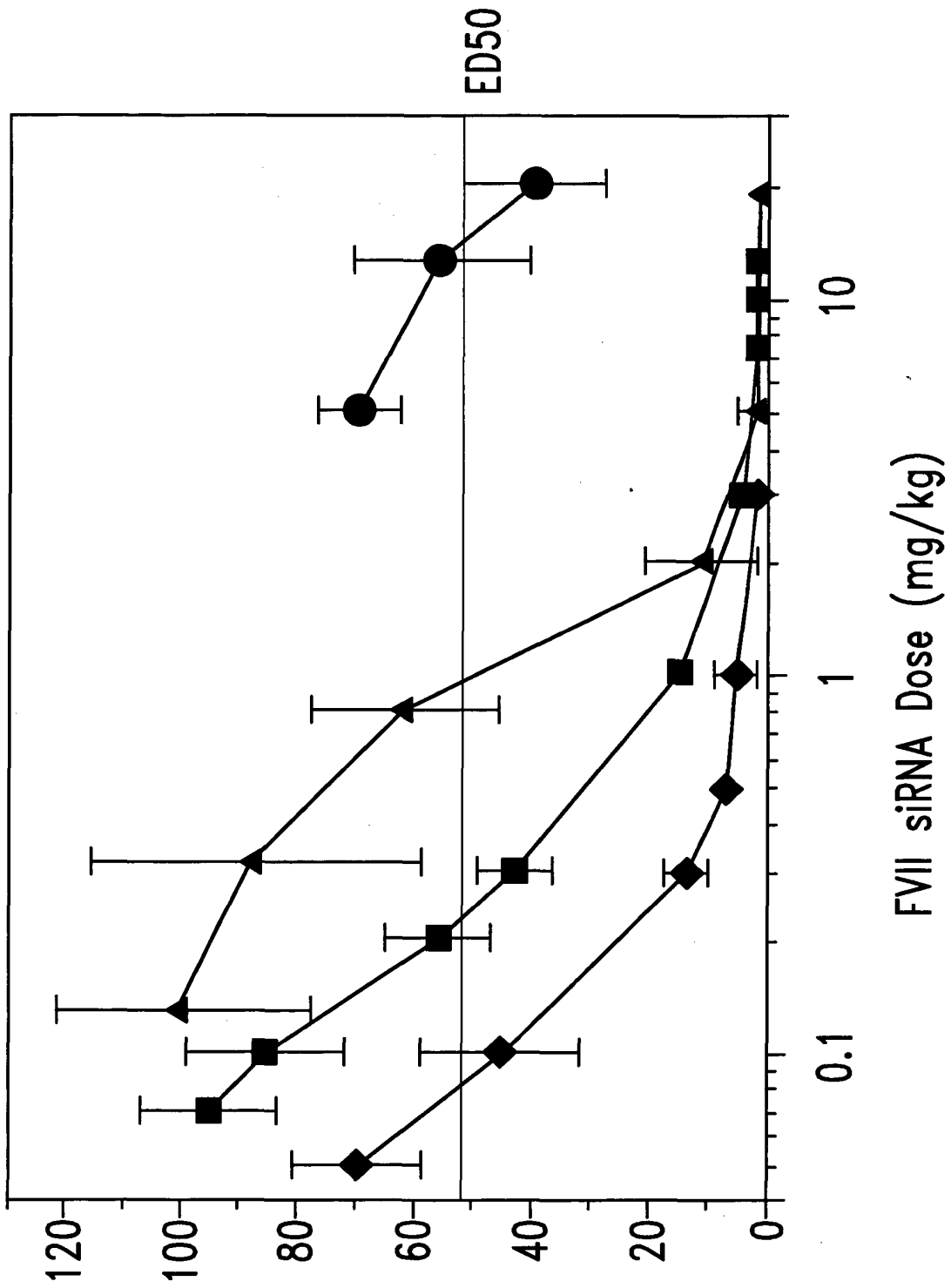


FIG. 8

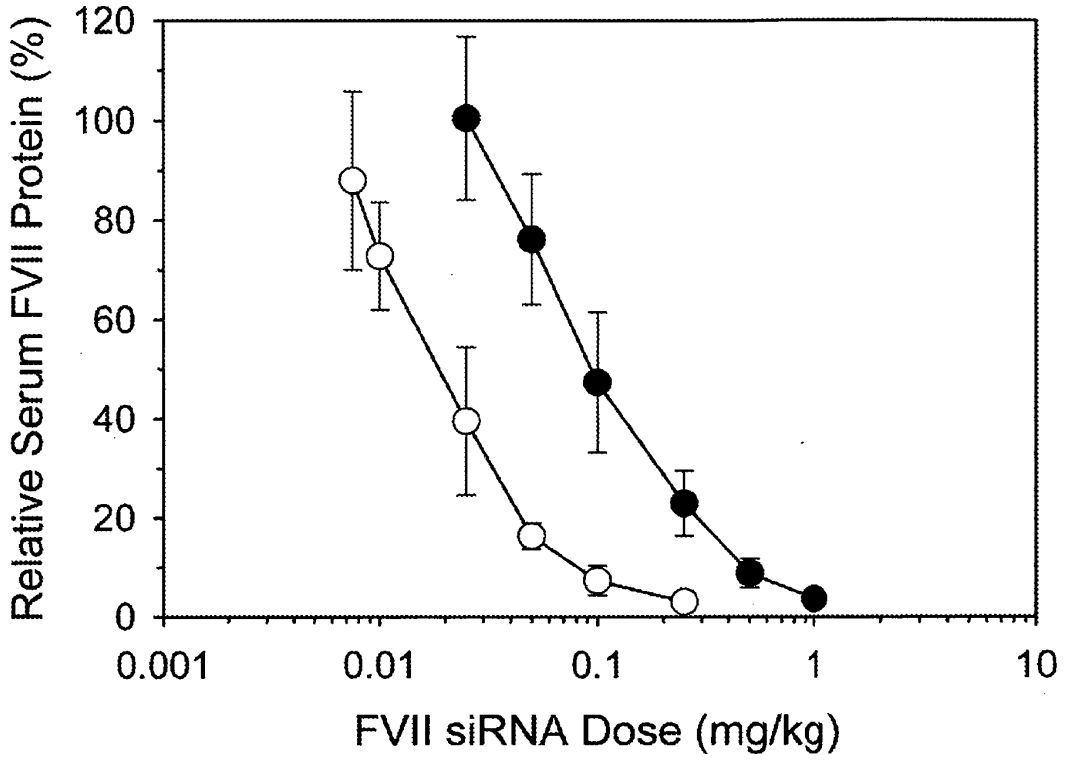


FIG. 9A

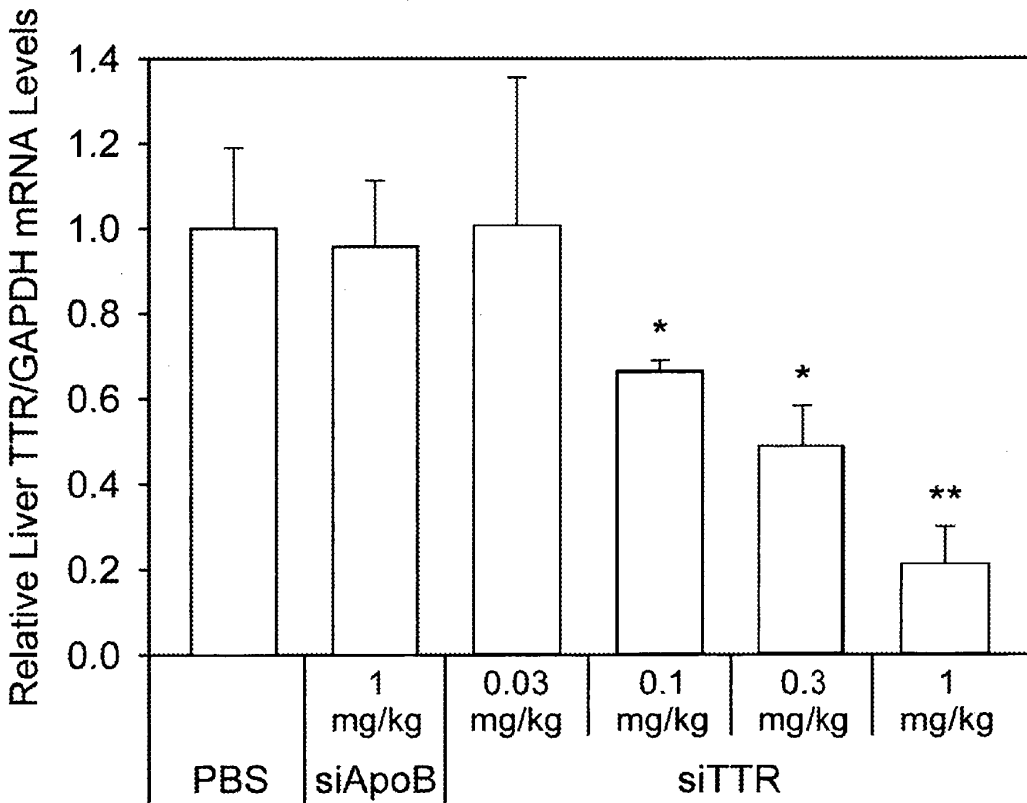


FIG. 9B

## REFERENCES CITED IN THE DESCRIPTION

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