

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
6 January 2011 (06.01.2011)

(10) International Publication Number
WO 2011/000106 A1

PCT

(51) International Patent Classification:

C07C 217/28 (2006.01) **C07D 317/28** (2006.01)
A61K 47/14 (2006.01) **C12N 15/113** (2010.01)
A61K 47/22 (2006.01) **A61K 31/713** (2006.01)
A61K 9/14 (2006.01) **C12N 15/88** (2006.01)
A61K 9/48 (2006.01)

(21) International Application Number:

PCT/CA2010/001029

(22) International Filing Date:

30 June 2010 (30.06.2010)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

61/222,462 1 July 2009 (01.07.2009) US
61/295,134 14 January 2010 (14.01.2010) US

(71) Applicant (for all designated States except US): **PROTI-VA BIOTHERAPEUTICS, INC.** [CA/CA]; 100-8900 Glenlyon Parkway, Burnaby, British Columbia V5J 5J8 (CA).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **HEYES, James** [CA/CA]; 47-870 West 7th Avenue, Vancouver, British Columbia V5Z 4C1 (CA). **PALMER, Lorne** [CA/CA]; 8076 Elliott Street, Vancouver, British Columbia V5S 2P2 (CA). **MASLOWSKI, Magdalena** [CA/CA]; 16070 95A Avenue, Surrey, British Columbia V4N 2P2 (CA).

MACLACHLAN, Ian [GB/CA]; 8040 Aves Terrace, Mission, British Columbia V4S 1E5 (CA).

(74) Agent: **KINGWELL, Brian G.**; Fetherstonhaugh, Suite 2200, 650 West Georgia Street, Vancouver, British Columbia V6B 4N8 (CA).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

(54) Title: IMPROVED CATIONIC LIPIDS AND METHODS FOR THE DELIVERY OF THERAPEUTIC AGENTS

(57) Abstract: The present invention provides compositions and methods for the delivery of therapeutic agents to cells. In particular, these include novel cationic lipids and nucleic acid-lipid particles that provide efficient encapsulation of nucleic acids and efficient delivery of the encapsulated nucleic acid to cells *in vivo*. The compositions of the present invention are highly potent, thereby allowing effective knock-down of a specific target protein at relatively low doses. In addition, the compositions and methods of the present invention are less toxic and provide a greater therapeutic index compared to compositions and methods previously known in the art.



WO 2011/000106 A1

IMPROVED CATIONIC LIPIDS AND METHODS FOR THE DELIVERY OF THERAPEUTIC AGENTS

CROSS-REFERENCE TO RELATED APPLICATIONS

5 [0001] This application claims priority to U.S. Provisional Application No. 61/222,462, filed July 1, 2009, and U.S. Provisional Application No. 61/295,134, filed January 14, 2010, the disclosures of which are hereby incorporated by reference in their entirety for all purposes.

BACKGROUND OF THE INVENTION

10 [0002] Therapeutic nucleic acids include, *e.g.*, small interfering RNA (siRNA), microRNA (miRNA), antisense oligonucleotides, ribozymes, plasmids, and immune-stimulating nucleic acids. These nucleic acids act via a variety of mechanisms. In the case of interfering RNA molecules such as siRNA and miRNA, these nucleic acids can down-regulate intracellular levels of specific proteins through a process termed RNA interference (RNAi). Following
15 introduction of interfering RNA into the cell cytoplasm, these double-stranded RNA constructs can bind to a protein termed RISC. The sense strand of the interfering RNA 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 interfering RNA. Having bound the complementary mRNA, the RISC complex cleaves the mRNA and releases the
20 cleaved strands. RNAi can provide down-regulation of specific proteins by targeting specific destruction of the corresponding mRNA that encodes for protein synthesis.

[0003] The therapeutic applications of RNAi are extremely broad, since interfering RNA 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
25 proteins in both *in vitro* and *in vivo* models. In addition, siRNA constructs are currently being evaluated in clinical studies.

[0004] However, two problems currently faced by interfering RNA 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
30 systemically as free interfering RNA molecules. These double-stranded constructs can be stabilized by the incorporation of chemically modified nucleotide linkers within the molecule, *e.g.*, phosphothioate groups. However, such chemically modified linkers provide only

limited protection from nuclease digestion and may decrease the activity of the construct.

Intracellular delivery of interfering RNA can be facilitated by the use of carrier systems such as polymers, cationic liposomes, or by the covalent attachment of a cholesterol moiety to the molecule. However, improved delivery systems are required to increase the potency of

interfering RNA molecules such as siRNA and miRNA and to reduce or eliminate the requirement for chemically modified nucleotide linkers.

[0005] In addition, problems remain with the limited ability of therapeutic nucleic acids such as interfering RNA to cross cellular membranes (*see, Vlassov et al., Biochim. Biophys. Acta*, 1197:95-1082 (1994)) and in the problems associated with systemic toxicity, such as complement-mediated anaphylaxis, altered coagulatory properties, and cytopenia (Galbraith *et al., Antisense Nucl. Acid Drug Des.*, 4:201-206 (1994)).

[0006] To attempt to improve efficacy, investigators have also employed lipid-based carrier systems to deliver chemically modified or unmodified therapeutic nucleic acids. Zelphati *et al. (J. Contr. Rel.*, 41:99-119 (1996)) describes the use of anionic (conventional) liposomes, pH sensitive liposomes, immunoliposomes, fusogenic liposomes, and cationic lipid/antisense aggregates. Similarly, siRNA has been administered systemically in cationic liposomes, and these nucleic acid-lipid particles have been reported to provide improved down-regulation of target proteins in mammals including non-human primates (Zimmermann *et al., Nature*, 441: 111-114 (2006)).

[0007] In spite of this progress, there remains a need in the art for improved lipid-therapeutic nucleic acid compositions that are suitable for general therapeutic use.

Preferably, these compositions would encapsulate nucleic acids with high-efficiency, have high drug:lipid ratios, protect the encapsulated nucleic acid from degradation and clearance in serum, be suitable for systemic delivery, and provide intracellular delivery of the

encapsulated nucleic acid. In addition, these nucleic acid-lipid particles should be well-tolerated and provide an adequate therapeutic index, such that patient treatment at an effective dose of the nucleic acid is not associated with significant toxicity and/or risk to the patient. The present invention provides such compositions, methods of making the compositions, and methods of using the compositions to introduce nucleic acids into cells, including for the treatment of diseases.

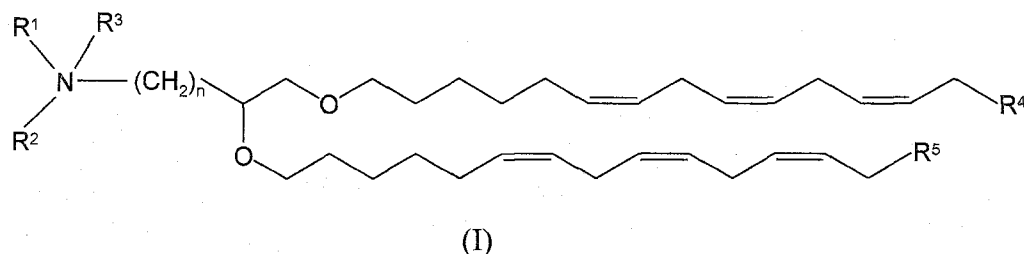
BRIEF SUMMARY OF THE INVENTION

[0008] The present invention provides novel cationic (amino) 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. The

present invention also provides methods of making these compositions, as well as methods of

introducing nucleic acids into cells using these compositions, *e.g.*, for the treatment of various disease conditions.

[0009] In one aspect, the present invention provides a cationic lipid of Formula I having the following structure:



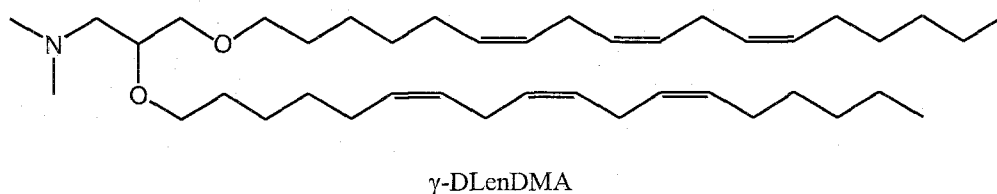
10 or salts thereof, wherein:

R^1 and R^2 are either the same or different and are independently an optionally substituted C_1 - C_6 alkyl, C_2 - C_6 alkenyl, or C_2 - C_6 alkynyl, or R^1 and R^2 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

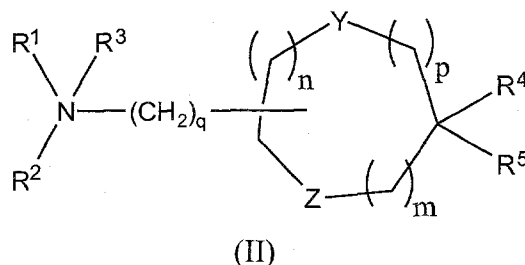
15 R^3 is either absent or is hydrogen (H) or a C_1 - C_6 alkyl to provide a quaternary amine;

R^4 and R^5 are either absent or present and when present are either the same or different and are independently an optionally substituted C_1 - C_{10} alkyl or C_2 - C_{10} alkenyl; and n is 0, 1, 2, 3, or 4.

20 [0010] In a particular embodiment, the cationic lipid of Formula I has the structure:



25 [0011] In another aspect, the present invention provides a cationic lipid of Formula II having the following structure:



or salts thereof, wherein:

R^1 and R^2 are either the same or different and are independently an optionally substituted C_1 - C_6 alkyl, C_2 - C_6 alkenyl, or C_2 - C_6 alkynyl, or R^1 and R^2 may join to form an

optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

R^3 is either absent or is hydrogen (H) or a C_1 - C_6 alkyl to provide a quaternary amine;

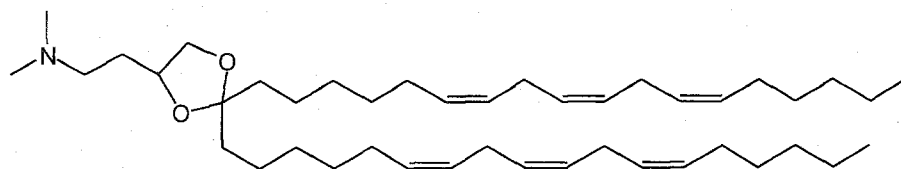
R^4 and R^5 are either the same or different and are independently an optionally substituted C_{12} - C_{24} alkyl, C_{12} - C_{24} alkenyl, C_{12} - C_{24} alkynyl, or C_{12} - C_{24} acyl, wherein at least one of R^4 and R^5 comprises at least three sites of unsaturation or a substituted C_{12} - C_{24} alkyl;

m, n, and p are either the same or different and are independently either 0, 1, or 2, with the proviso that m, n, and p are not simultaneously 0;

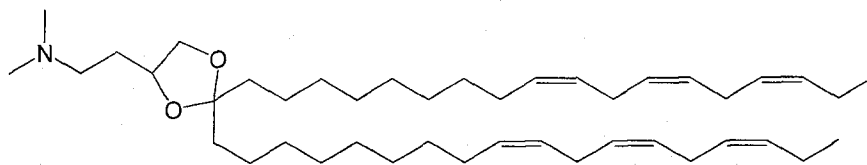
q is 0, 1, 2, 3, or 4; and

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

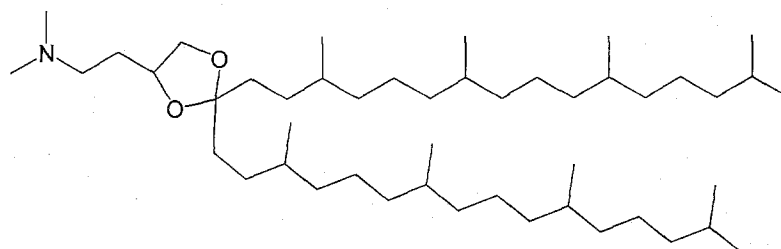
[0012] In particular embodiments, the cationic lipid of Formula II has a structure selected from the group consisting of:



γ -DLen-C2K-DMA

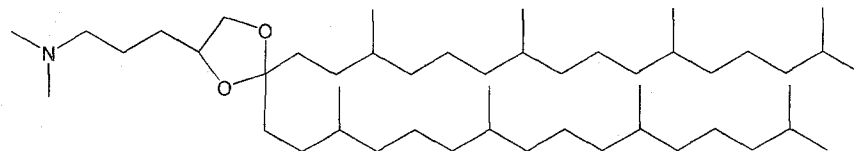


DLen-C2K-DMA



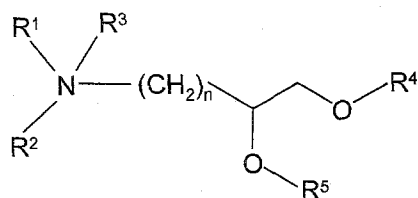
DPan-C2K-DMA

, and



DPan-C3K-DMA

[0013] In yet another aspect, the present invention provides a cationic lipid of Formula III having the following structure:



(III)

or salts thereof, wherein:

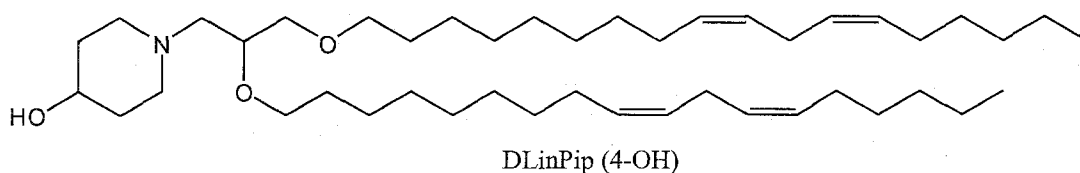
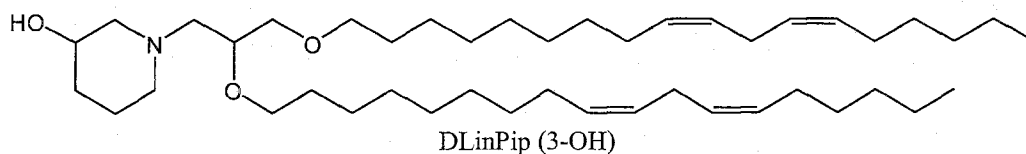
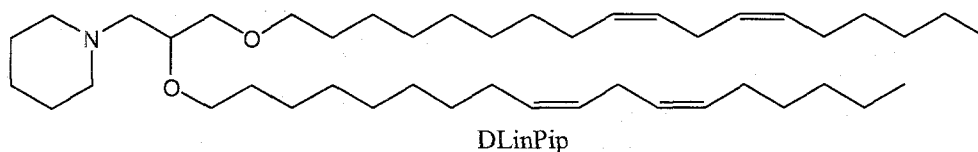
R^1 and R^2 are joined to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

R^3 is either absent or is hydrogen (H) or a C_1 - C_6 alkyl to provide a quaternary amine;

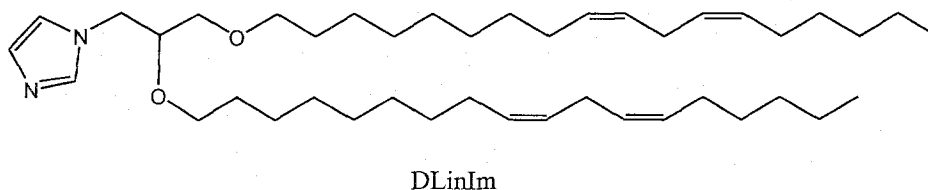
R^4 and R^5 are either the same or different and are independently an optionally substituted C_{12} - C_{24} alkyl, C_{12} - C_{24} alkenyl, C_{12} - C_{24} alkynyl, or C_{12} - C_{24} acyl; and

n is 0, 1, 2, 3, or 4.

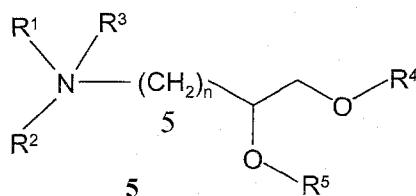
[0014] In particular embodiments, the cationic lipid of Formula III has a structure selected from the group consisting of:



and



[0015] In still yet another aspect, the present invention provides a cationic lipid of Formula IV having the following structure:



(IV)

or salts thereof, wherein:

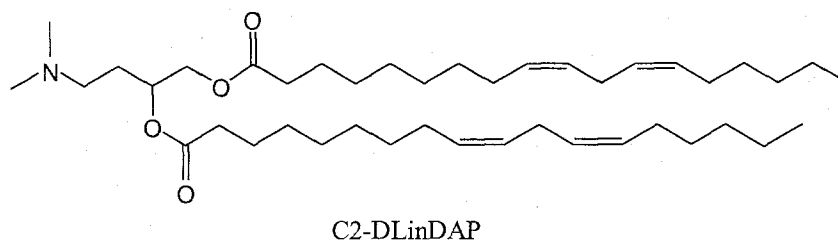
R^1 and R^2 are either the same or different and are independently an optionally substituted C_1 - C_6 alkyl, C_2 - C_6 alkenyl, or C_2 - C_6 alkynyl, or R^1 and R^2 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

R^3 is either absent or is hydrogen (H) or a C_1 - C_6 alkyl to provide a quaternary amine;

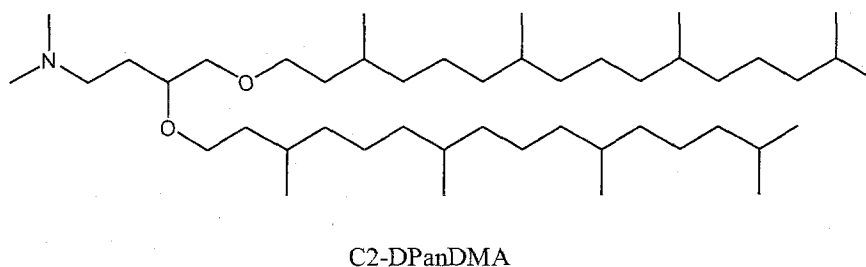
R^4 and R^5 are either the same or different and are independently an optionally substituted C_{12} - C_{24} alkyl, C_{12} - C_{24} alkenyl, C_{12} - C_{24} alkynyl, or C_{12} - C_{24} acyl; and

n is 2, 3, or 4.

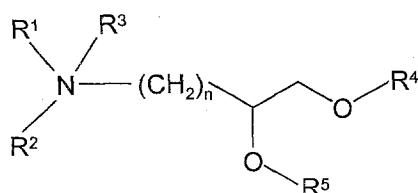
[0016] In particular embodiments, the cationic lipid of Formula IV has a structure selected from the group consisting of:



, and



[0017] In another aspect, the present invention provides a cationic lipid of Formula V having the following structure:



(V)

or salts thereof, wherein:

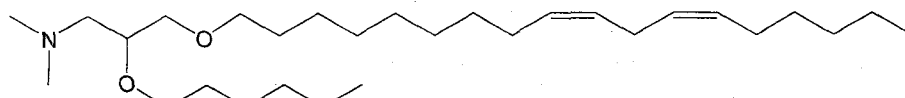
R^1 and R^2 are either the same or different and are independently an optionally substituted C_1 - C_6 alkyl, C_2 - C_6 alkenyl, or C_2 - C_6 alkynyl, or R^1 and R^2 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

R^3 is either absent or is hydrogen (H) or a C_1 - C_6 alkyl to provide a quaternary amine;

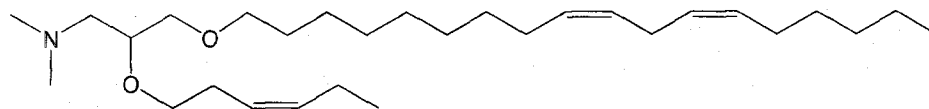
R^4 and R^5 are different and are independently an optionally substituted C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, or C_1 - C_{24} acyl; and

n is 0, 1, 2, 3, or 4.

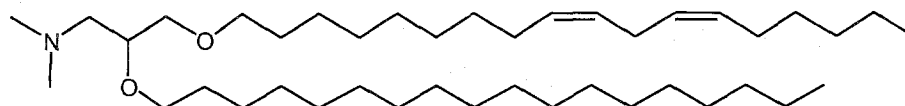
[0018] In particular embodiments, the cationic lipid of Formula V is an asymmetric lipid having a structure selected from the group consisting of:



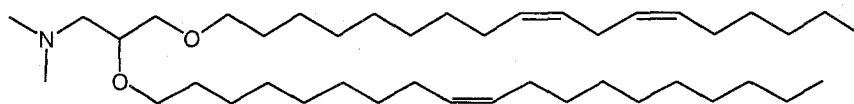
Linoleyl/ $C_6:0$ DMA



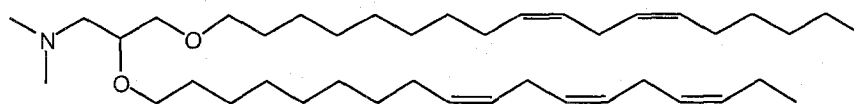
Linoleyl/ $C_6:1$ DMA



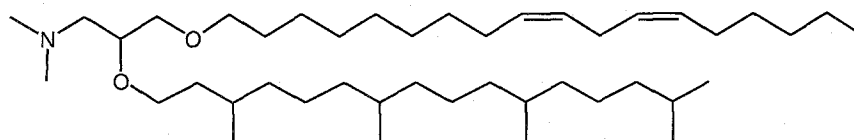
Linoleyl/Stearyl DMA



Linoleyl/Olel DMA

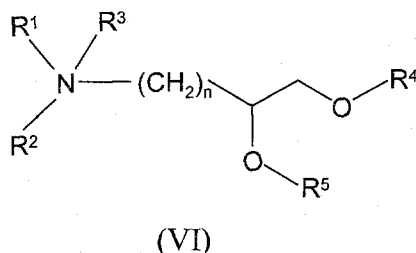


Linoleyl/Linolenyl DMA



Linoleyl/Phytanyl DMA

[0019] In yet another aspect, the present invention provides a cationic lipid of Formula VI having the following structure:



or salts thereof, wherein:

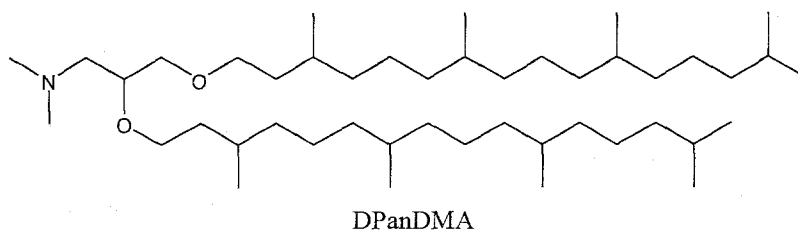
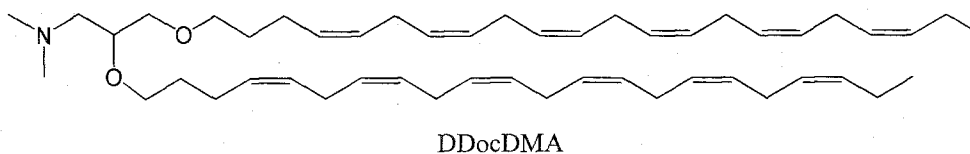
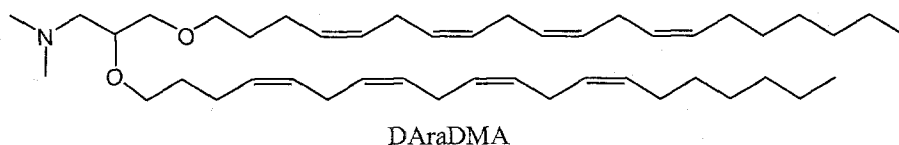
R^1 and R^2 are either the same or different and are independently an optionally substituted C_1 - C_6 alkyl, C_2 - C_6 alkenyl, or C_2 - C_6 alkynyl, or R^1 and R^2 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

R^3 is either absent or is hydrogen (H) or a C_1 - C_6 alkyl to provide a quaternary amine;

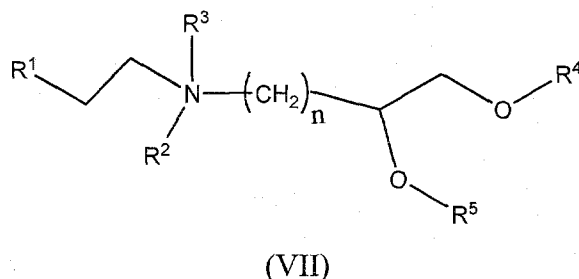
R^4 and R^5 are either the same or different and are independently an optionally substituted C_{12} - C_{24} alkyl, C_{12} - C_{24} alkenyl, C_{12} - C_{24} alkynyl, or C_{12} - C_{24} acyl, wherein at least one of R^4 and R^5 comprises at least four sites of unsaturation or a substituted C_{12} - C_{24} alkyl; and

n is 0, 1, 2, 3, or 4.

[0020] In particular embodiments, the cationic lipid of Formula VI has a structure selected from the group consisting of:



[0021] In still yet another aspect, the present invention provides a cationic lipid of Formula VII having the following structure:



5 or salts thereof, wherein:

R^1 is hydrogen (H) or $-(CH_2)_q-NR^6R^7R^8$, wherein:

R^6 and R^7 are either the same or different and are independently an optionally substituted C_1 - C_6 alkyl, C_2 - C_6 alkenyl, or C_2 - C_6 alkynyl, or R^6 and R^7 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

R^8 is either absent or is hydrogen (H) or a C_1 - C_6 alkyl to provide a quaternary amine; and

q is 0, 1, 2, 3, or 4;

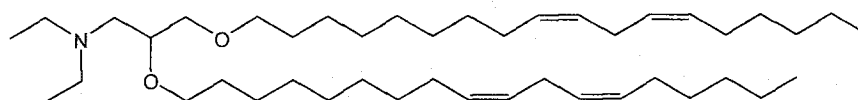
R^2 is an optionally substituted C_1 - C_6 alkyl, C_2 - C_6 alkenyl, or C_2 - C_6 alkynyl;

R^3 is either absent or is hydrogen (H) or a C_1 - C_6 alkyl to provide a quaternary amine;

R^4 and R^5 are either the same or different and are independently an optionally substituted C_{12} - C_{24} alkyl, C_{12} - C_{24} alkenyl, C_{12} - C_{24} alkynyl, or C_{12} - C_{24} acyl; and

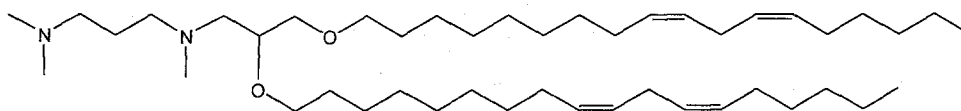
n is 0, 1, 2, 3, or 4.

[0022] In particular embodiments, the cationic lipid of Formula VII has a structure selected from the group consisting of:



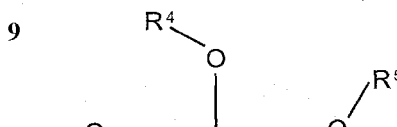
DLinDEA

and



2N-DLinDMA

[0023] In another aspect, the present invention provides a cationic lipid of Formula VIII having the following structure:



5

(VIII)

or salts thereof, wherein:

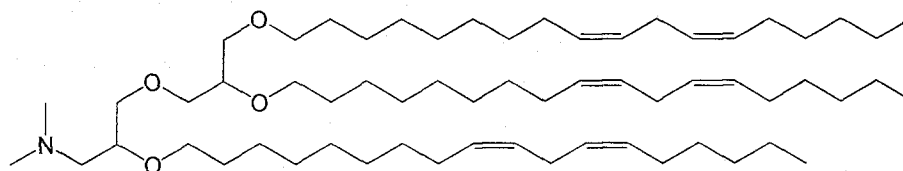
R^1 and R^2 are either the same or different and are independently an optionally substituted C_1 - C_6 alkyl, C_2 - C_6 alkenyl, or C_2 - C_6 alkynyl, or R^1 and R^2 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

R^3 is either absent or is hydrogen (H) or a C_1 - C_6 alkyl to provide a quaternary amine;

R^4 , R^5 , and R^6 are either the same or different and are independently an optionally substituted C_{12} - C_{24} alkyl, C_{12} - C_{24} alkenyl, C_{12} - C_{24} alkynyl, or C_{12} - C_{24} acyl; and n is 0, 1, 2, 3, or 4.

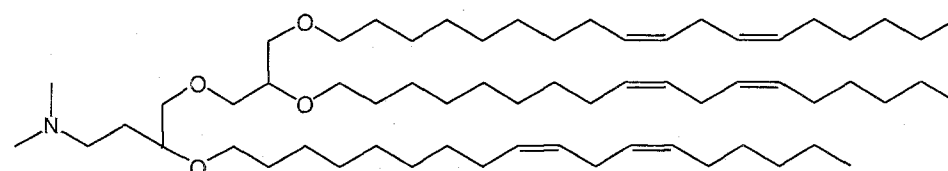
[0024] In particular embodiments, the cationic lipid of Formula VIII has a structure selected from the group consisting of:

20



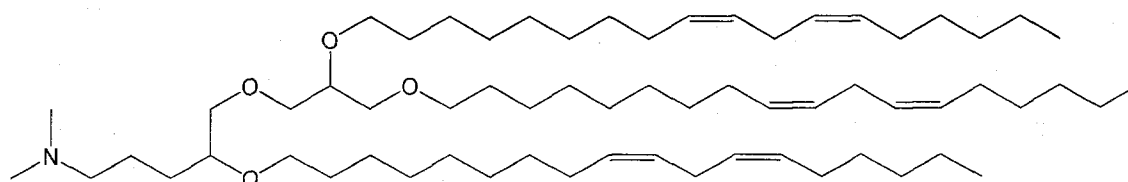
TLinDMA

25



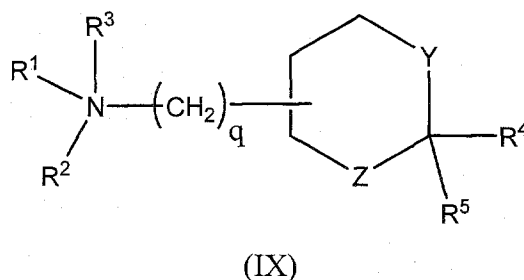
C2-TLinDMA

, and



C3-TLinDMA

[0025] In yet another aspect, the present invention provides a cationic lipid of Formula IX having the following structure:



5 or salts thereof, wherein:

R^1 and R^2 are either the same or different and are independently an optionally substituted C_1 - C_6 alkyl, C_2 - C_6 alkenyl, or C_2 - C_6 alkynyl, or R^1 and R^2 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

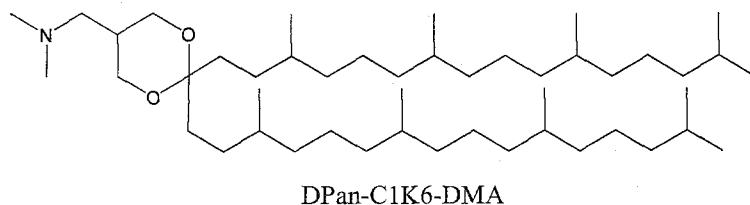
10 R^3 is either absent or is hydrogen (H) or a C_1 - C_6 alkyl to provide a quaternary amine;

R^4 and R^5 are either the same or different and are independently an optionally substituted C_{12} - C_{24} alkyl, C_{12} - C_{24} alkenyl, C_{12} - C_{24} alkynyl, or C_{12} - C_{24} acyl;

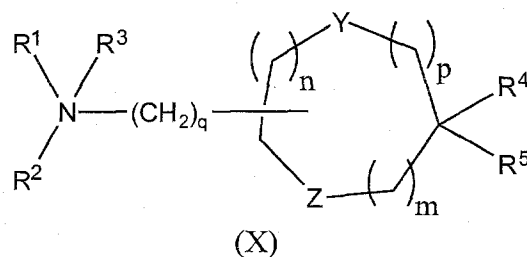
q is 0, 1, 2, 3, or 4; and

15 Y and Z are either the same or different and are independently O, S, or NH, with the proviso that if q is 1, R^1 and R^2 are not both methyl groups when R^4 and R^5 are both linoleyl moieties, Y and Z are both O, and the alkylamino group is attached to the '5' position of the 6-membered ring.

[0026] In a particular embodiment, the cationic lipid of Formula IX has the structure:



[0027] In still yet another aspect, the present invention provides a cationic lipid of Formula X having the following structure:



or salts thereof, wherein:

R^1 and R^2 are either the same or different and are independently an optionally substituted C_1 - C_6 alkyl, C_2 - C_6 alkenyl, or C_2 - C_6 alkynyl, or R^1 and R^2 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

R^3 is either absent or is hydrogen (H) or a C_1 - C_6 alkyl to provide a quaternary amine;

R^4 and R^5 are either the same or different and are independently an optionally substituted C_{12} - C_{24} alkyl, C_{12} - C_{24} alkenyl, C_{12} - C_{24} alkynyl, or C_{12} - C_{24} acyl, wherein at least one of R^4 and R^5 comprises at least one site of unsaturation in the *trans* (*E*) configuration;

m , n , and p are either the same or different and are independently either 0, 1, or 2, with the proviso that m , n , and p are not simultaneously 0;

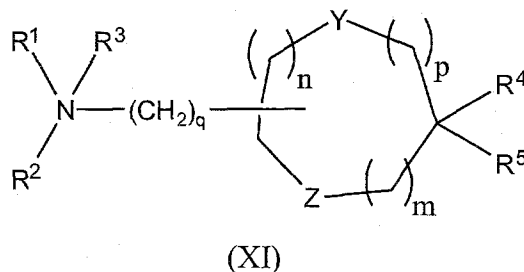
q is 0, 1, 2, 3, or 4; and

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

[0028] In a particular embodiment, the cationic lipid of Formula X has the structure:



[0029] In another aspect, the present invention provides a cationic lipid of Formula XI having the following structure:



or salts thereof, wherein:

R^1 and R^2 are joined to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

R^3 is either absent or is hydrogen (H) or a C_1 - C_6 alkyl to provide a quaternary amine;

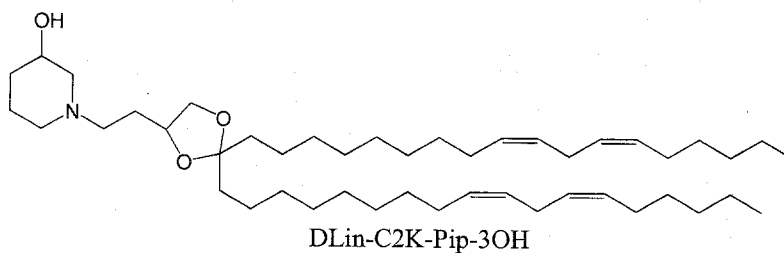
R^4 and R^5 are either the same or different and are independently an optionally substituted C_{12} - C_{24} alkyl, C_{12} - C_{24} alkenyl, C_{12} - C_{24} alkynyl, or C_{12} - C_{24} acyl;

m , n , and p are either the same or different and are independently either 0, 1, or 2, with the proviso that m , n , and p are not simultaneously 0;

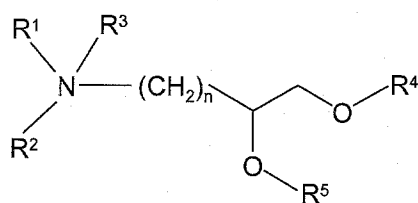
q is 0, 1, 2, 3, or 4; and

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

5 [0030] In a particular embodiment, the cationic lipid of Formula XI has the structure:



[0031] In yet another aspect, the present invention provides a cationic lipid of Formula XII having the following structure:



(XII)

or salts thereof, wherein:

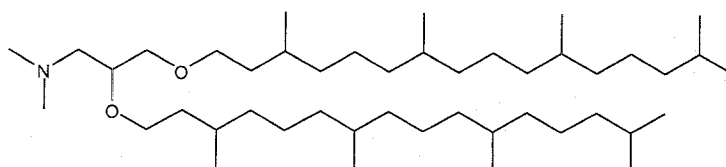
R¹ and R² are either the same or different and are independently an optionally substituted C₁-C₆ alkyl, C₂-C₆ alkenyl, or C₂-C₆ alkynyl, or R¹ and R² may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

R³ is either absent or is hydrogen (H) or a C₁-C₆ alkyl to provide a quaternary amine;

R⁴ and R⁵ are either the same or different and are independently a substituted C₁₂-C₂₄ alkyl; and

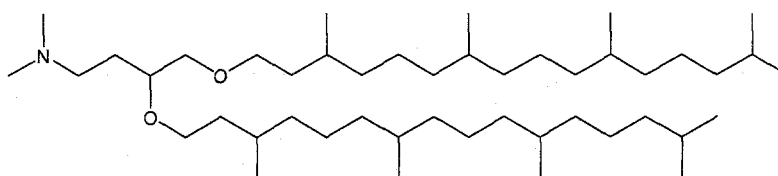
n is 0, 1, 2, 3, or 4.

[0032] In a particular embodiment, the cationic lipid of Formula XII has a structure selected from the group consisting of:



DPanDMA

and



C2-DPanDMA

[0033] In a further aspect, the present invention provides a lipid particle comprising one or more of the above cationic lipids of Formula I-XII or salts thereof. In certain embodiments, the lipid particle further comprises one or more non-cationic lipids such as neutral lipids. In certain other embodiments, the lipid particle further comprises one or more conjugated lipids capable of reducing or inhibiting particle aggregation. In additional embodiments, the lipid particle further comprises one or more active agents or therapeutic agents.

[0034] In certain embodiments, the non-cationic lipid component of the lipid particle may comprise a phospholipid, cholesterol (or cholesterol derivative), or a mixture thereof. In one particular embodiment, the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof. In certain other embodiments, the conjugated lipid component of the lipid particle may comprise a polyethyleneglycol (PEG)-lipid conjugate. In one particular embodiment, the PEG-lipid conjugate comprises a PEG-diacylglycerol (PEG-DAG) conjugate, a PEG-dialkyloxypropyl (PEG-DAA) conjugate, or a mixture thereof.

[0035] In some embodiments, the active agent or therapeutic agent comprises a nucleic acid. In certain instances, the nucleic acid comprises an interfering RNA molecule such as, *e.g.*, an siRNA, aiRNA, miRNA, Dicer-substrate dsRNA, shRNA, or mixtures thereof. In certain other instances, the nucleic acid comprises single-stranded or double-stranded DNA, RNA, or a DNA/RNA hybrid such as, *e.g.*, an antisense oligonucleotide, a ribozyme, a plasmid, an immunostimulatory oligonucleotide, or mixtures thereof.

[0036] In other embodiments, the active agent or therapeutic agent is fully encapsulated within the lipid portion of the lipid particle such that the active agent or therapeutic agent in the lipid particle is resistant in aqueous solution to enzymatic degradation, *e.g.*, by a nuclease or protease. In further embodiments, the lipid particle is substantially non-toxic to mammals such as humans.

[0037] In preferred embodiments, the present invention provides serum-stable nucleic acid-lipid particles (SNALP) comprising: (a) one or more nucleic acids such as interfering RNA molecules; (b) one or more cationic lipids of Formula I-XII or salts thereof; (c) one or more non-cationic lipids; and (d) one or more conjugated lipids that inhibit aggregation of particles.

[0038] In some embodiments, the present invention provides nucleic acid-lipid particles (e.g., SNALP) comprising: (a) one or more nucleic acids; (b) one or more cationic lipids of Formula I-XII or salts thereof comprising from about 50 mol % to about 85 mol % of the total lipid present in the particle; (c) one or more non-cationic lipids comprising from about 13 mol % to about 49.5 mol % of the total lipid present in the particle; and (d) one or more conjugated lipids that inhibit aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

[0039] In one aspect of this embodiment, the nucleic acid-lipid particle comprises: (a) a nucleic acid; (b) a cationic lipid of Formula I-XII or a salt thereof comprising from about 52 mol % to about 62 mol % of the total lipid present in the particle; (c) a mixture of a phospholipid and cholesterol or a derivative thereof comprising from about 36 mol % to about 47 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle.

This embodiment of nucleic acid-lipid particle is generally referred to herein as the "1:57" formulation. In one particular embodiment, the 1:57 formulation is a four-component system comprising about 1.4 mol % PEG-lipid conjugate (e.g., PEG2000-C-DMA), about 57.1 mol % cationic lipid of Formula I-XII or a salt thereof, about 7.1 mol % DPPC (or DSPC), and about 34.3 mol % cholesterol (or derivative thereof).

[0040] In another aspect of this embodiment, the nucleic acid-lipid particle comprises: (a) a nucleic acid; (b) a cationic lipid of Formula I-XII or a salt thereof comprising from about 56.5 mol % to about 66.5 mol % of the total lipid present in the particle; (c) cholesterol or a derivative thereof comprising from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. This embodiment of nucleic acid-lipid particle is generally referred to herein as the "1:62" formulation. In one particular embodiment, the 1:62 formulation is a three-component system which is phospholipid-free and comprises about 1.5 mol % PEG-lipid conjugate (e.g., PEG2000-C-DMA), about 61.5 mol % cationic lipid of Formula I-XII or a salt thereof, and about 36.9 mol % cholesterol (or derivative thereof).

[0041] Additional embodiments related to the 1:57 and 1:62 formulations are described in PCT Publication No. WO 09/127060 and U.S. Application No. 12/794,701, filed June 4, 2010, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0042] In other embodiments, the present invention provides nucleic acid-lipid particles (e.g., SNALP) comprising: (a) one or more nucleic acids; (b) one or more cationic lipids of

Formula I-XII or salts thereof comprising from about 2 mol % to about 50 mol % of the total lipid present in the particle; (c) one or more non-cationic lipids comprising from about 5 mol % to about 90 mol % of the total lipid present in the particle; and (d) one or more conjugated lipids that inhibit aggregation of particles comprising from about 0.5 mol % to about 20 mol % of the total lipid present in the particle.

[0043] In one aspect of this embodiment, the nucleic acid-lipid particle comprises: (a) a nucleic acid; (b) a cationic lipid of Formula I-XII or a salt thereof comprising from about 30 mol % to about 50 mol % of the total lipid present in the particle; (c) a mixture of a phospholipid and cholesterol or a derivative thereof comprising from about 47 mol % to about 69 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 1 mol % to about 3 mol % of the total lipid present in the particle. This embodiment of nucleic acid-lipid particle is generally referred to herein as the "2:40" formulation. In one particular embodiment, the 2:40 formulation is a four-component system which comprises about 2 mol % PEG-lipid conjugate (*e.g.*, PEG2000-C-DMA), about 40 mol % cationic lipid of Formula I-XII or a salt thereof, about 10 mol % DPPC (or DSPC), and about 48 mol % cholesterol (or derivative thereof).

[0044] In further embodiments, the present invention provides nucleic acid-lipid particles (*e.g.*, SNALP) comprising: (a) one or more nucleic acids; (b) one or more cationic lipids of Formula I-XII or salts thereof comprising from about 50 mol % to about 65 mol % of the total lipid present in the particle; (c) one or more non-cationic lipids comprising from about 25 mol % to about 45 mol % of the total lipid present in the particle; and (d) one or more conjugated lipids that inhibit aggregation of particles comprising from about 5 mol % to about 10 mol % of the total lipid present in the particle.

[0045] In one aspect of this embodiment, the nucleic acid-lipid particle comprises: (a) a nucleic acid; (b) a cationic lipid of Formula I-XII or a salt thereof comprising from about 50 mol % to about 60 mol % of the total lipid present in the particle; (c) a mixture of a phospholipid and cholesterol or a derivative thereof comprising from about 35 mol % to about 45 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 5 mol % to about 10 mol % of the total lipid present in the particle.

This embodiment of nucleic acid-lipid particle is generally referred to herein as the "7:54" formulation. In certain instances, the non-cationic lipid mixture in the 7:54 formulation comprises: (i) a phospholipid of from about 5 mol % to about 10 mol % of the total lipid present in the particle; and (ii) cholesterol or a derivative thereof of from about 25 mol % to about 35 mol % of the total lipid present in the particle. In one particular embodiment, the 7:54 formulation is a four-component system which comprises about 7 mol % PEG-lipid

conjugate (*e.g.*, PEG750-C-DMA), about 54 mol % cationic lipid of Formula I-XII or a salt thereof, about 7 mol % DPPC (or DSPC), and about 32 mol % cholesterol (or derivative thereof).

[0046] In another aspect of this embodiment, the nucleic acid-lipid particle comprises: (a) a nucleic acid; (b) a cationic lipid of Formula I-XII or a salt thereof comprising from about 55 mol % to about 65 mol % of the total lipid present in the particle; (c) cholesterol or a derivative thereof comprising from about 30 mol % to about 40 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 5 mol % to about 10 mol % of the total lipid present in the particle. This embodiment of nucleic acid-lipid particle is generally referred to herein as the “7:58” formulation. In one particular embodiment, the 7:58 formulation is a three-component system which is phospholipid-free and comprises about 7 mol % PEG-lipid conjugate (*e.g.*, PEG750-C-DMA), about 58 mol % cationic lipid of Formula I-XII or a salt thereof, and about 35 mol % cholesterol (or derivative thereof).

[0047] Additional embodiments related to the 7:54 and 7:58 formulations are described in U.S. Application No. _____, entitled “Novel Lipid Formulations for Delivery of Therapeutic Agents to Solid Tumors,” filed June 30, 2010, bearing Attorney Docket No. 020801-009610US, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0048] The present invention also provides pharmaceutical compositions comprising a lipid particle such as a nucleic acid-lipid particle (*e.g.*, SNALP) and a pharmaceutically acceptable carrier.

[0049] In another aspect, the present invention provides methods for introducing one or more therapeutic agents such as nucleic acids into a cell, the method comprising contacting the cell with a lipid particle described herein (*e.g.*, SNALP). In one embodiment, the cell is in a mammal and the mammal is a human.

[0050] In yet another aspect, the present invention provides methods for the *in vivo* delivery of one or more therapeutic agents such as nucleic acids, the method comprising administering to a mammal a lipid particle described herein (*e.g.*, SNALP). In certain embodiments, the lipid particles (*e.g.*, SNALP) are administered by one of the following routes of administration: oral, intranasal, intravenous, intraperitoneal, intramuscular, intra-articular, intralesional, intratracheal, subcutaneous, and intradermal. In particular embodiments, the lipid particles (*e.g.*, SNALP) are administered systemically, *e.g.*, via enteral or parenteral routes of administration. In preferred embodiments, the mammal is a human.

[0051] In a further aspect, the present invention provides methods for treating a disease or disorder in a mammal in need thereof, the method comprising administering to the mammal a therapeutically effective amount of a lipid particle (*e.g.*, SNALP) comprising one or more therapeutic agents such as nucleic acids. Non-limiting examples of diseases or disorders include a viral infection, a liver disease or disorder, and cancer. Preferably, the mammal is a human.

[0052] In certain embodiments, the present invention provides methods for treating a liver disease or disorder by administering a nucleic acid such as an interfering RNA (*e.g.*, siRNA) in nucleic acid-lipid particles (*e.g.*, SNALP), alone or in combination with a lipid-lowering agent. Examples of lipid diseases and disorders include, but are not limited to, dyslipidemia (*e.g.*, hyperlipidemias such as elevated triglyceride levels (hypertriglyceridemia) and/or elevated cholesterol levels (hypercholesterolemia)), atherosclerosis, coronary heart disease, coronary artery disease, atherosclerotic cardiovascular disease (CVD), fatty liver disease (hepatic steatosis), abnormal lipid metabolism, abnormal cholesterol metabolism, diabetes (including Type 2 diabetes), obesity, cardiovascular disease, and other disorders relating to abnormal metabolism. Non-limiting examples of lipid-lowering agents include statins, fibrates, ezetimibe, thiazolidinediones, niacin, beta-blockers, nitroglycerin, calcium antagonists, and fish oil.

[0053] In one particular embodiment, the present invention provides a method for lowering or reducing cholesterol levels in a mammal (*e.g.*, human) in need thereof (*e.g.*, a mammal with elevated blood cholesterol levels), the method comprising administering to the mammal a therapeutically effective amount of a nucleic acid-lipid particle (*e.g.*, a SNALP formulation) described herein comprising one or more interfering RNAs (*e.g.*, siRNAs) that target one or more genes associated with metabolic diseases and disorders. In another particular embodiment, the present invention provides a method for lowering or reducing triglyceride levels in a mammal (*e.g.*, human) in need thereof (*e.g.*, a mammal with elevated blood triglyceride levels), the method comprising administering to the mammal a therapeutically effective amount of a nucleic acid-lipid particle (*e.g.*, a SNALP formulation) described herein comprising one or more interfering RNAs (*e.g.*, siRNAs) that target one or more genes associated with metabolic diseases and disorders. These methods can be carried out *in vitro* using standard tissue culture techniques or *in vivo* by administering the interfering RNA (*e.g.*, siRNA) using any means known in the art. In preferred embodiments, the interfering RNA (*e.g.*, siRNA) is delivered to a liver cell (*e.g.*, hepatocyte) in a mammal such as a human.

[0054] Additional embodiments related to treating a liver disease or disorder using a lipid particle are described in, *e.g.*, PCT Application No. PCT/CA2010/000120, filed January 26,

2010, and U.S. Patent Publication No. 20060134189, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0055] In other embodiments, the present invention provides methods for treating a cell proliferative disorder such as cancer by administering a nucleic acid such as an interfering

5 RNA (*e.g.*, siRNA) in nucleic acid-lipid particles (*e.g.*, SNALP), alone or in combination with a chemotherapy drug. The methods can be carried out *in vitro* using standard tissue culture techniques or *in vivo* by administering the interfering RNA (*e.g.*, siRNA) using any

means known in the art. In preferred embodiments, the interfering RNA (*e.g.*, siRNA) is delivered to a cancer cell in a mammal such as a human, alone or in combination with a

10 chemotherapy drug. The nucleic acid-lipid particles and/or chemotherapy drugs may also be co-administered with conventional hormonal, immunotherapeutic, and/or radiotherapeutic agents.

[0056] Additional embodiments related to treating a cell proliferative disorder using a lipid particle are described in, *e.g.*, PCT Publication No. WO 09/082817, U.S. Patent Publication

15 No. 20090149403, PCT Publication No. WO 09/129319, and U.S. Provisional Application No. 61/245,143, filed September 23, 2009, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0057] In further embodiments, the present invention provides methods for preventing or treating a viral infection such as an Ebola virus infection by administering a nucleic acid such

20 as an interfering RNA (*e.g.*, siRNA) in nucleic acid-lipid particles (*e.g.*, SNALP), alone or in combination with the administration of conventional agents used to treat or ameliorate the viral condition or any of the symptoms associated therewith. The methods can be carried out

in vitro using standard tissue culture techniques or *in vivo* by administering the interfering

RNA using any means known in the art. In preferred embodiments, the interfering RNA

25 (*e.g.*, siRNA) is delivered to cells, tissues, or organs of a mammal such as a human that are infected and/or susceptible of being infected with the virus.

[0058] Additional embodiments related to preventing or treating a viral infection using a lipid particle are described in, *e.g.*, U.S. Patent Publication No. 20070218122, U.S. Patent

Publication No. 20070135370, U.S. Provisional Application No. 61/286,741, filed December

30 15, 2009, PCT Application No. PCT/CA2010/000444, entitled "Compositions and Methods for Silencing Hepatitis C Virus Expression," filed March 19, 2010, bearing Attorney Docket

No. 020801-008910PC, and U.S. Provisional Application No. 61/319,855, filed March 31,

2010, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0059] The lipid particles of the invention (*e.g.*, SNALP) comprising one or more cationic lipids of Formula I-XII or salts thereof are particularly advantageous and suitable for use in the administration of nucleic acids such as interfering RNA to a subject (*e.g.*, a mammal such as a human) because they are stable in circulation, of a size required for pharmacodynamic behavior resulting in access to extravascular sites, and are capable of reaching target cell populations.

[0060] Other objects, features, and advantages of the present invention will be apparent to one of skill in the art from the following detailed description and figures.

BRIEF DESCRIPTION OF THE DRAWINGS

[0061] Figure 1 shows the apparent pK_a values of exemplary SNALP formulations containing various cationic lipids described herein.

[0062] Figure 2 shows the apparent pK_a values of additional exemplary SNALP formulations containing various cationic lipids described herein.

[0063] Figure 3 shows a comparison of the plasma total cholesterol knockdown efficacy of exemplary SNALP formulations containing various cationic lipids described herein.

[0064] Figure 4 shows a comparison of the liver ApoB mRNA knockdown activity of exemplary SNALP formulations containing various cationic lipids described herein.

[0065] Figure 5 shows a comparison of the liver ApoB mRNA knockdown activity of additional exemplary SNALP formulations containing various cationic lipids described herein.

[0066] Figure 6 shows a dose response evaluation of three different doses of exemplary SNALP formulations containing various cationic lipids described herein on liver ApoB mRNA knockdown activity.

[0067] Figure 7 shows the tolerability of an exemplary γ -DLenDMA SNALP formulation.

[0068] Figure 8 shows a comparison of the liver ApoB mRNA knockdown activity of additional exemplary SNALP formulations containing various cationic lipids described herein.

[0069] Figure 9 shows a comparison of the liver ApoB mRNA knockdown activity of additional exemplary SNALP formulations containing various cationic lipids described herein.

[0070] Figure 10 shows a comparison of the liver ApoB mRNA knockdown activity of additional exemplary SNALP formulations containing various cationic lipids described herein.

[0071] Figure 11 shows a comparison of the tumor PLK-1 mRNA knockdown activity of exemplary SNALP formulations containing various cationic lipids described herein.

[0072] Figure 12 shows the effect of ApoE^{-/-} or C57 serum pre-incubation on silencing activity with exemplary SNALP formulations containing various cationic lipids described herein.

[0073] Figure 13 shows the effect of ApoE^{-/-} or C57 serum pre-incubation on silencing activity with additional exemplary SNALP formulations containing various cationic lipids described herein.

[0074] Figure 14 shows the effect of ApoE^{-/-} or C57 serum pre-incubation on silencing activity with additional exemplary SNALP formulations containing various cationic lipids described herein.

[0075] Figure 15 shows the *in vitro* serum protein binding to SNALP following pre-incubation with C57BL/6, APOE^{-/-}, or LDLr^{-/-} serum. (a) SNALP containing 0.1 mol% Biotin-PEG-DSPE were pre-incubated with mouse serum (C57BL/6 or APOE^{-/-}) for 1 hour at 37°C. Proteins were then extracted. For each serum type, 2 discrete SNALP preparations were used ('A' and 'B'). Lane 1: SNALP 'A' + C57BL/06 serum. Lane 2: SNALP 'B' + C57BL/6 serum. Lane 3: C57BL/6 serum control. Lane 4: SNALP 'A' + APOE^{-/-} serum. Lane 5: SNALP 'B' + APOE^{-/-} serum. Lane 6: APOE^{-/-} serum control. Major proteins were identified by LC/MS and Western blot analysis. (b) SNALP ligand blotting. SNALP containing 0.1 mol% Biotin-PEG-DSPE at equivalent lipid concentration were pre-incubated with APOE containing C57BL/6 serum (Blots 1, 4), APOE^{-/-} serum (Blots 2, 5), or in the absence of serum (Blots 3, 6) for 1 hour at 37°C. Binding to liver proteins on the C57BL/6 (Blots 1 to 3) and LDLr^{-/-} (Blots 4 to 6) was determined using streptavidin-HRP. APOE, apolipoprotein E; LDLr, low-density-lipoprotein receptor; SNALP, stable nucleic acid lipid particles.

[0076] Figure 16 shows the *in vitro* uptake and associated gene-silencing with SNALP by primary hepatocytes isolated from C57BL/6, APOE^{-/-}, and LDLr^{-/-} mice. (a, b) ³H-CHE labeled SNALP were pre-incubated with mouse serum (C57BL/6, APOE^{-/-} or LDLr^{-/-}) for 1 hour at 37°C. Hepatocytes were exposed to SNALP for either 4 hours (a) or 24 hours (b) at a concentration of 0.25 µg/mL siRNA. SNALP uptake was normalized to the total protein content. Error bars represent SD, n=3. (c, d) Unlabeled SNALP, pre-incubated in serum, were used to treat hepatocytes. Hepatocytes were exposed to SNALP for either 4 hours (c) or 24 hours (d) at a concentration of 0.25 µg/mL siRNA. The APOB and GAPDH mRNA concentrations were determined. Results expressed as % relative to a SNALP mismatch control. Error bars represent SD, n=3. APOE, apolipoprotein E; LDLr, low-density-lipoprotein receptor; SNALP, stable nucleic acid lipid particles.

[0077] Figure 17 shows the *in vivo* clearance, tissue biodistribution, and associated ApoB gene-silencing of SNALP in C57BL/6, APOE^{-/-}, and LDLr^{-/-} mice. (a) Clearance of ³H-CHE labeled SNALP in C57BL/6, APOE^{-/-}, and LDLr^{-/-} mice. Whole blood was obtained from tail nicks, and ³H counts were used to determine the percentage of injected dose remaining. (b) Biodistribution of ³H-CHE labeled SNALP in C57BL/6, APOE^{-/-}, and LDLr^{-/-} mice. Accumulation was determined 24 hours after injection. (c) *In vivo* knockdown of APOB mRNA. Liver ApoB and GAPDH mRNA were measured 48 hours following SNALP treatment. The ApoB:GAPDH ratio was determined and reported relative to PBS controls. All mice received a single intravenous administration of SNALP via the lateral tail vein. Error bars represent SD, n=3 for each experiment. APOB, apolipoprotein B; APOE, apolipoprotein E; LDLr, GAPDH, glyceraldehyde 3-phosphate dehydrogenase; low-density-lipoprotein receptor; PBS, phosphate-buffered saline; SNALP, stable nucleic acid lipid particles.

[0078] Figure 18 shows the effect of human serum components on *in vitro* APOB mRNA knockdown in primary hepatocytes. (a) Effect of human lipoprotein/SNALP pre-incubation on knockdown in APOE^{-/-} mouse hepatocytes. SNALP were pre-incubated with human lipoproteins or mouse or human sera for 1 hour at 37°C. APOE^{-/-} hepatocytes were then exposed to SNALP for 24 hours. (b) Gene-silencing in human primary hepatocytes. Human hepatocytes were exposed to SNALP (containing antiAPOB or the nontargeting antiLuc siRNA) pre-incubated with or without human serum at a siRNA concentration of 0.125 µg/mL. (c) Effect of APOE isoform on SNALP-mediated gene silencing in human hepatocytes. SNALP were pre-incubated with each of the 3 isoforms of human APOE. Human hepatocytes were then exposed to the SNALP for varying durations at a siRNA concentration of 0.125 µg/mL. The APOB and GAPDH mRNA concentrations were determined and expressed as a ratio relative to PBS control. Error bars represent SD, n=3. APOE, apolipoprotein E; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mRNA, messenger ribonucleic acid; PBS, phosphate-buffered saline; siRNA, small interfering ribonucleic acid; SNALP, stable nucleic acid lipid particles.

DETAILED DESCRIPTION OF THE INVENTION

I. Introduction

[0079] The present invention is based, in part, upon the discovery of novel cationic (amino) lipids that provide advantages when used in lipid particles for the *in vivo* delivery of an active or therapeutic agent such as a nucleic acid into a cell of a mammal. In particular, the present invention provides nucleic acid-lipid particle compositions comprising one or more of the

novel cationic lipids described herein that provide increased activity of the nucleic acid (*e.g.*, interfering RNA) and improved tolerability of the compositions *in vivo*, resulting in a significant increase in the therapeutic index as compared to nucleic acid-lipid particle compositions previously described.

5 [0080] In particular embodiments, the present invention provides novel cationic lipids that enable the formulation of improved compositions for the *in vitro* and *in vivo* delivery of interfering RNA such as siRNA. It is shown herein that these improved lipid particle compositions are effective in down-regulating (*e.g.*, silencing) the protein levels and/or mRNA levels of target genes. Furthermore, it is shown herein that the activity of these
10 improved lipid particle compositions is dependent on the presence of the novel cationic lipids of the invention.

[0081] The lipid particles and compositions of the present invention may be used for a variety of purposes, including the delivery of encapsulated or associated (*e.g.*, complexed) therapeutic agents such as nucleic acids to cells, both *in vitro* and *in vivo*. Accordingly, the
15 present invention further provides methods of treating diseases or disorders in a subject in need thereof by contacting the subject with a lipid particle that encapsulates or is associated with a suitable therapeutic agent, wherein the lipid particle comprises one or more of the novel cationic lipids described herein.

[0082] As described herein, the lipid particles of the present invention are particularly
20 useful for the delivery of nucleic acids, including, *e.g.*, interfering RNA molecules such as siRNA. Therefore, the lipid particles and compositions of the present invention may be used to decrease the expression of target genes and proteins both *in vitro* and *in vivo* by contacting cells with a lipid particle comprising one or more novel cationic lipids described herein, wherein the lipid particle encapsulates or is associated with a nucleic acid that reduces target
25 gene expression (*e.g.*, an siRNA). Alternatively, the lipid particles and compositions of the present invention may be used to increase the expression of a desired protein both *in vitro* and *in vivo* by contacting cells with a lipid particle comprising one or more novel cationic lipids described herein, wherein the lipid particle encapsulates or is associated with a nucleic acid that enhances expression of the desired protein (*e.g.*, a plasmid encoding the desired protein).

30 [0083] Various exemplary embodiments of the cationic lipids of the present invention, lipid particles and compositions comprising the same, and their use to deliver active or therapeutic agents such as nucleic acids to modulate gene and protein expression, are described in further detail below.

II. Definitions

[0084] As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

[0085] The term “interfering RNA” or “RNAi” or “interfering RNA sequence” as used herein includes single-stranded RNA (*e.g.*, mature miRNA, ssRNAi oligonucleotides, ssDNAi oligonucleotides), double-stranded RNA (*i.e.*, duplex RNA such as siRNA, Dicer-substrate dsRNA, shRNA, aiRNA, or pre-miRNA), a DNA-RNA hybrid (*see, e.g.*, PCT Publication No. WO 2004/078941), or a DNA-DNA hybrid (*see, e.g.*, PCT Publication No. WO 2004/104199) that is capable of reducing or inhibiting the expression of a target gene or sequence (*e.g.*, by mediating the degradation or inhibiting the translation of mRNAs which are complementary to the interfering RNA sequence) when the interfering RNA is in the same cell as the target gene or sequence. Interfering RNA thus refers to the single-stranded RNA that is complementary to a target mRNA sequence or to the double-stranded RNA formed by two complementary strands or by a single, self-complementary strand. Interfering RNA may have substantial or complete identity to the target gene or sequence, or may comprise a region of mismatch (*i.e.*, a mismatch motif). The sequence of the interfering RNA can correspond to the full-length target gene, or a subsequence thereof. Preferably, the interfering RNA molecules are chemically synthesized. The disclosures of each of the above patent documents are herein incorporated by reference in their entirety for all purposes.

[0086] Interfering RNA includes “small-interfering RNA” or “siRNA,” *e.g.*, interfering RNA of about 15-60, 15-50, or 15-40 (duplex) nucleotides in length, more typically about 15-30, 15-25, or 19-25 (duplex) nucleotides in length, and is preferably about 20-24, 21-22, or 21-23 (duplex) nucleotides in length (*e.g.*, each complementary sequence of the double-stranded siRNA is 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 nucleotides in length, preferably about 20-24, 21-22, or 21-23 nucleotides in length, and the double-stranded siRNA is about 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 base pairs in length, preferably about 18-22, 19-20, or 19-21 base pairs in length). siRNA duplexes may comprise 3' overhangs of about 1 to about 4 nucleotides or about 2 to about 3 nucleotides and 5' phosphate termini. Examples of siRNA include, without limitation, a double-stranded polynucleotide molecule assembled from two separate stranded molecules, wherein one strand is the sense strand and the other is the complementary antisense strand; a double-stranded polynucleotide molecule assembled from a single stranded molecule, where the sense and antisense regions are linked by a nucleic acid-based or non-nucleic acid-based linker; a double-stranded polynucleotide molecule with a hairpin secondary structure having self-complementary sense and antisense regions; and a circular single-stranded

polynucleotide molecule with two or more loop structures and a stem having self-complementary sense and antisense regions, where the circular polynucleotide can be processed *in vivo* or *in vitro* to generate an active double-stranded siRNA molecule. As used herein, the term “siRNA” includes RNA-RNA duplexes as well as DNA-RNA hybrids (see, e.g., PCT Publication No. WO 2004/078941).

[0087] Preferably, siRNA are chemically synthesized. siRNA can also be generated by cleavage of longer dsRNA (e.g., dsRNA greater than about 25 nucleotides in length) with the *E. coli* RNase III or Dicer. These enzymes process the dsRNA into biologically active siRNA (see, e.g., Yang *et al.*, *Proc. Natl. Acad. Sci. USA*, 99:9942-9947 (2002); Calegari *et al.*, *Proc. Natl. Acad. Sci. USA*, 99:14236 (2002); Byrom *et al.*, *Ambion TechNotes*, 10(1):4-6 (2003); Kawasaki *et al.*, *Nucleic Acids Res.*, 31:981-987 (2003); Knight *et al.*, *Science*, 293:2269-2271 (2001); and Robertson *et al.*, *J. Biol. Chem.*, 243:82 (1968)). Preferably, dsRNA are at least 50 nucleotides to about 100, 200, 300, 400, or 500 nucleotides in length. A dsRNA may be as long as 1000, 1500, 2000, 5000 nucleotides in length, or longer. The dsRNA can encode for an entire gene transcript or a partial gene transcript. In certain instances, siRNA may be encoded by a plasmid (e.g., transcribed as sequences that automatically fold into duplexes with hairpin loops).

[0088] As used herein, the term “mismatch motif” or “mismatch region” refers to a portion of an interfering RNA (e.g., siRNA) sequence that does not have 100 % complementarity to its target sequence. An interfering RNA may have at least one, two, three, four, five, six, or more mismatch regions. The mismatch regions may be contiguous or may be separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more nucleotides. The mismatch motifs or regions may comprise a single nucleotide or may comprise two, three, four, five, or more nucleotides.

[0089] The phrase “inhibiting expression of a target gene” refers to the ability of an interfering RNA (e.g., siRNA) to silence, reduce, or inhibit the expression of a target gene. To examine the extent of gene silencing, a test sample (e.g., a sample of cells in culture expressing the target gene) or a test mammal (e.g., a mammal such as a human or an animal model such as a rodent (e.g., mouse) or a non-human primate (e.g., monkey) model) is contacted with an interfering RNA (e.g., siRNA) that silences, reduces, or inhibits expression of the target gene. Expression of the target gene in the test sample or test animal is compared to expression of the target gene in a control sample (e.g., a sample of cells in culture expressing the target gene) or a control mammal (e.g., a mammal such as a human or an animal model such as a rodent (e.g., mouse) or non-human primate (e.g., monkey) model) that is not contacted with or administered the interfering RNA (e.g., siRNA). The expression of the target gene in a control sample or a control mammal may be assigned a value of 100%.

In particular embodiments, silencing, inhibition, or reduction of expression of a target gene is achieved when the level of target gene expression in the test sample or the test mammal relative to the level of target gene expression in the control sample or the control mammal is about 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, or 0%. In other words, the interfering RNA (*e.g.*, siRNA) silences, reduces, or inhibits the expression of a target gene by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% in a test sample or a test mammal relative to the level of target gene expression in a control sample or a control mammal not contacted with or administered the interfering RNA (*e.g.*, siRNA). Suitable assays for determining the level of target gene expression include, without limitation, examination of protein or mRNA levels using techniques known to those of skill in the art, such as, *e.g.*, dot blots, Northern blots, *in situ* hybridization, ELISA, immunoprecipitation, enzyme function, as well as phenotypic assays known to those of skill in the art.

[0090] An “effective amount” or “therapeutically effective amount” of an active agent or therapeutic agent such as an interfering RNA is an amount sufficient to produce the desired effect, *e.g.*, an inhibition of expression of a target sequence in comparison to the normal expression level detected in the absence of an interfering RNA. Inhibition of expression of a target gene or target sequence is achieved when the value obtained with an interfering RNA relative to the control is about 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, or 0%. Suitable assays for measuring expression of a target gene or target sequence include, *e.g.*, examination of protein or RNA levels using techniques known to those of skill in the art such as dot blots, northern blots, *in situ* hybridization, ELISA, immunoprecipitation, enzyme function, as well as phenotypic assays known to those of skill in the art.

[0091] By “decrease,” “decreasing,” “reduce,” or “reducing” of an immune response by an interfering RNA is intended to mean a detectable decrease of an immune response to a given interfering RNA (*e.g.*, a modified interfering RNA). The amount of decrease of an immune response by a modified interfering RNA may be determined relative to the level of an immune response in the presence of an unmodified interfering RNA. A detectable decrease can be about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, or more lower than the immune response detected in the presence of the unmodified interfering RNA. A decrease in the immune response to interfering RNA is typically measured by a decrease in cytokine production (*e.g.*, IFN γ ,

IFN α , TNF α , IL-6, IL-8, or IL-12) by a responder cell *in vitro* or a decrease in cytokine production in the sera of a mammalian subject after administration of the interfering RNA.

[0092] As used herein, the term “responder cell” refers to a cell, preferably a mammalian cell, that produces a detectable immune response when contacted with an immunostimulatory interfering RNA such as an unmodified siRNA. Exemplary responder cells include, *e.g.*, dendritic cells, macrophages, peripheral blood mononuclear cells (PBMCs), splenocytes, and the like. Detectable immune responses include, *e.g.*, production of cytokines or growth factors such as TNF- α , IFN- α , IFN- β , IFN- γ , IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, TGF, and combinations thereof. Detectable immune responses also include, *e.g.*, induction of interferon-induced protein with tetratricopeptide repeats 1 (*IFIT1*) mRNA.

[0093] “Substantial identity” refers to a sequence that hybridizes to a reference sequence under stringent conditions, or to a sequence that has a specified percent identity over a specified region of a reference sequence.

[0094] The phrase “stringent hybridization conditions” refers to conditions under which a nucleic acid will hybridize to its target sequence, typically in a complex mixture of nucleic acids, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes*, “Overview of principles of hybridization and the strategy of nucleic acid assays” (1993).

Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m , 50% of the probes are occupied at equilibrium). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization.

[0095] Exemplary stringent hybridization conditions can be as follows: 50% formamide, 5x SSC, and 1% SDS, incubating at 42°C, or, 5x SSC, 1% SDS, incubating at 65°C, with wash in 0.2x SSC, and 0.1% SDS at 65°C. For PCR, a temperature of about 36°C is typical for low stringency amplification, although annealing temperatures may vary between about 32°C and 48°C depending on primer length. For high stringency PCR amplification, a temperature of about 62°C is typical, although high stringency annealing temperatures can

range from about 50°C to about 65°C, depending on the primer length and specificity.

Typical cycle conditions for both high and low stringency amplifications include a denaturation phase of 90°C-95°C for 30 sec.-2 min., an annealing phase lasting 30 sec.-2 min., and an extension phase of about 72°C for 1-2 min. Protocols and guidelines for low

5 and high stringency amplification reactions are provided, e.g., in Innis *et al.*, *PCR Protocols, A Guide to Methods and Applications*, Academic Press, Inc. N.Y. (1990).

[0096] Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon
10 degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary “moderately stringent hybridization conditions” include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1X SSC at 45°C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and
15 wash conditions can be utilized to provide conditions of similar stringency. Additional guidelines for determining hybridization parameters are provided in numerous references, e.g., *Current Protocols in Molecular Biology*, Ausubel *et al.*, eds.

[0097] The terms “substantially identical” or “substantial identity,” in the context of two or more nucleic acids, refer to two or more sequences or subsequences that are the same or have
20 a specified percentage of nucleotides that are the same (*i.e.*, at least about 60%, preferably at least about 65%, 70%, 75%, 80%, 85%, 90%, or 95% identity over a specified region), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. This definition, when the context indicates, also
25 refers analogously to the complement of a sequence. Preferably, the substantial identity exists over a region that is at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or 60 nucleotides in length.

[0098] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and
30 reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[0099] A “comparison window,” as used herein, includes reference to a segment of any one of a number of contiguous positions selected from the group consisting of from about 5 to about 60, usually about 10 to about 45, more usually about 15 to about 30, in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith and Waterman, *Adv. Appl. Math.*, 2:482 (1981), by the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.*, 48:443 (1970), by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci. USA*, 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (*see, e.g., Current Protocols in Molecular Biology*, Ausubel *et al.*, eds. (1995 supplement)).

[0100] Non-limiting examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, *Nuc. Acids Res.*, 25:3389-3402 (1977) and Altschul *et al.*, *J. Mol. Biol.*, 215:403-410 (1990), respectively. BLAST and BLAST 2.0 are used, with the parameters described herein, to determine percent sequence identity for the nucleic acids of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). Another example is a global alignment algorithm for determining percent sequence identity such as the Needleman-Wunsch algorithm for aligning protein or nucleotide (*e.g.*, RNA) sequences.

[0101] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.,* Karlin and Altschul, *Proc. Natl. Acad. Sci. USA*, 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

[0102] The term “nucleic acid” as used herein refers to a polymer containing at least two deoxyribonucleotides or ribonucleotides in either single- or double-stranded form and includes DNA, RNA, and hybrids thereof. DNA may be in the form of, *e.g.*, antisense molecules, plasmid DNA, DNA-DNA duplexes, pre-condensed DNA, a PCR product,

vectors (P1, PAC, BAC, YAC, artificial chromosomes), expression cassettes, chimeric sequences, chromosomal DNA, or derivatives and combinations of these groups. RNA may be in the form of small interfering RNA (siRNA), Dicer-substrate dsRNA, small hairpin RNA (shRNA), asymmetrical interfering RNA (aiRNA), microRNA (miRNA), mRNA, tRNA, rRNA, viral RNA (vRNA), and combinations thereof. Nucleic acids include nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, and which have similar binding properties as the reference nucleic acid. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2'-O-methyl ribonucleotides, and peptide-nucleic acids (PNAs). Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.*, 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.*, 260:2605-2608 (1985); Rossolini *et al.*, *Mol. Cell. Probes*, 8:91-98 (1994)). "Nucleotides" contain a sugar deoxyribose (DNA) or ribose (RNA), a base, and a phosphate group. Nucleotides are linked together through the phosphate groups. "Bases" include purines and pyrimidines, which further include natural compounds adenine, thymine, guanine, cytosine, uracil, inosine, and natural analogs, and synthetic derivatives of purines and pyrimidines, which include, but are not limited to, modifications which place new reactive groups such as, but not limited to, amines, alcohols, thiols, carboxylates, and alkylhalides.

[0103] The term "gene" refers to a nucleic acid (*e.g.*, DNA or RNA) sequence that comprises partial length or entire length coding sequences necessary for the production of a polypeptide or precursor polypeptide.

[0104] "Gene product," as used herein, refers to a product of a gene such as an RNA transcript or a polypeptide.

[0105] The term "lipid" refers to a group of organic compounds that include, but are not limited to, esters of fatty acids and are characterized by being insoluble in water, but soluble in many organic solvents. They are usually divided into at least three classes: (1) "simple

lipids,” which include fats and oils as well as waxes; (2) “compound lipids,” which include phospholipids and glycolipids; and (3) “derived lipids” such as steroids.

[0106] The term “lipid particle” includes a lipid formulation that can be used to deliver an active agent or therapeutic agent, such as a nucleic acid (*e.g.*, an interfering RNA), to a target site of interest (*e.g.*, cell, tissue, organ, and the like). In preferred embodiments, the lipid particle of the invention is a nucleic acid-lipid particle, which is typically formed from a cationic lipid, a non-cationic lipid, and optionally a conjugated lipid that prevents aggregation of the particle. In other preferred embodiments, the active agent or therapeutic agent, such as a nucleic acid, may be encapsulated in the lipid portion of the particle, thereby protecting it from enzymatic degradation.

[0107] As used herein, the term “SNALP” refers to a stable nucleic acid-lipid particle. A SNALP represents a particle made from lipids (*e.g.*, a cationic lipid, a non-cationic lipid, and optionally a conjugated lipid that prevents aggregation of the particle), wherein the nucleic acid (*e.g.*, an interfering RNA) is fully encapsulated within the lipid. In certain instances, SNALP are extremely useful for systemic applications, as they can exhibit extended circulation lifetimes following intravenous (*i.v.*) injection, they can accumulate at distal sites (*e.g.*, sites physically separated from the administration site), and they can mediate silencing of target gene expression at these distal sites. The nucleic acid may be complexed with a condensing agent and encapsulated within a SNALP as set forth in PCT Publication No. WO 00/03683, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0108] The lipid particles of the invention (*e.g.*, SNALP) typically have a mean diameter of from about 30 nm to about 150 nm, from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, from about 70 nm to about 100 nm, from about 80 nm to about 100 nm, from about 90 nm to about 100 nm, from about 70 to about 90 nm, from about 80 nm to about 90 nm, from about 70 nm to about 80 nm, or about 30 nm, 35 nm, 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 105 nm, 110 nm, 115 nm, 120 nm, 125 nm, 130 nm, 135 nm, 140 nm, 145 nm, or 150 nm, and are substantially non-toxic. In addition, nucleic acids, when present in the lipid particles of the present invention, are resistant in aqueous solution to degradation with a nuclease. Nucleic acid-lipid particles and their method of preparation are disclosed in, *e.g.*, U.S. Patent Publication Nos. 20040142025 and 20070042031, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0109] As used herein, "lipid encapsulated" can refer to a lipid particle that provides an active agent or therapeutic agent, such as a nucleic acid (*e.g.*, an interfering RNA), with full encapsulation, partial encapsulation, or both. In a preferred embodiment, the nucleic acid is fully encapsulated in the lipid particle (*e.g.*, to form a SNALP or other nucleic acid-lipid particle).

[0110] The term "lipid conjugate" refers to a conjugated lipid that inhibits aggregation of lipid particles. Such lipid conjugates include, but are not limited to, PEG-lipid conjugates such as, *e.g.*, PEG coupled to dialkylxypropyls (*e.g.*, PEG-DAA conjugates), PEG coupled to diacylglycerols (*e.g.*, PEG-DAG conjugates), PEG coupled to cholesterol, PEG coupled to phosphatidylethanolamines, and PEG conjugated to ceramides (*see, e.g.*, U.S. Patent No. 5,885,613), cationic PEG lipids, polyoxazoline (POZ)-lipid conjugates (*e.g.*, POZ-DAA conjugates; *see, e.g.*, U.S. Provisional Application No. 61/294,828, filed January 13, 2010, and U.S. Provisional Application No. 61/295, 140, filed January 14, 2010), polyamide oligomers (*e.g.*, ATTA-lipid conjugates), and mixtures thereof. Additional examples of POZ-lipid conjugates are described in PCT Publication No. WO 2010/006282. PEG or POZ can be conjugated directly to the lipid or may be linked to the lipid via a linker moiety. Any linker moiety suitable for coupling the PEG or the POZ to a lipid can be used including, *e.g.*, non-ester containing linker moieties and ester-containing linker moieties. In certain preferred embodiments, non-ester containing linker moieties, such as amides or carbamates, are used.

The disclosures of each of the above patent documents are herein incorporated by reference in their entirety for all purposes.

[0111] The term "amphipathic lipid" refers, in part, 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. Hydrophilic characteristics derive from the presence of polar or charged groups such as carbohydrates, phosphate, carboxylic, sulfato, amino, sulfhydryl, nitro, hydroxyl, and other like groups. Hydrophobicity can be conferred by the inclusion of apolar groups that include, but are not limited to, long-chain saturated and unsaturated aliphatic hydrocarbon groups and such groups substituted by one or more aromatic, cycloaliphatic, or heterocyclic group(s). Examples of amphipathic compounds include, but are not limited to, phospholipids, aminolipids, and sphingolipids.

[0112] Representative examples of phospholipids include, but are not limited to, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine, and dilinoleoylphosphatidylcholine. Other compounds

lacking in phosphorus, such as sphingolipid, glycosphingolipid families, diacylglycerols, and β -acyloxyacids, are also within the group designated as amphipathic lipids. Additionally, the amphipathic lipids described above can be mixed with other lipids including triglycerides and sterols.

5 [0113] 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, cerebroside, and diacylglycerols.

10 [0114] The term “non-cationic lipid” refers to any amphipathic lipid as well as any other neutral lipid or anionic lipid.

[0115] 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-
15 glutarylphosphatidylethanolamines, lysylphosphatidylglycerols, palmitoyl-oleoylphosphatidylglycerol (POPG), and other anionic modifying groups joined to neutral lipids.

[0116] The term “hydrophobic lipid” refers to compounds having apolar groups that include, but are not limited to, long-chain saturated and unsaturated aliphatic hydrocarbon
20 groups and such groups optionally substituted by one or more aromatic, cycloaliphatic, or heterocyclic group(s). Suitable examples include, but are not limited to, diacylglycerol, dialkylglycerol, N-N-dialkylamino, 1,2-diacyloxy-3-aminopropane, and 1,2-dialkyl-3-aminopropane.

[0117] The term “fusogenic” refers to the ability of a lipid particle, such as a SNALP, to
25 fuse with the membranes of a cell. The membranes can be either the plasma membrane or membranes surrounding organelles, *e.g.*, endosome, nucleus, *etc.*

[0118] As used herein, the term “aqueous solution” refers to a composition comprising in whole, or in part, water.

[0119] As used herein, the term “organic lipid solution” refers to a composition comprising
30 in whole, or in part, an organic solvent having a lipid.

[0120] “Distal site,” as used herein, refers to a physically separated site, which is not limited to an adjacent capillary bed, but includes sites broadly distributed throughout an organism.

[0121] “Serum-stable” in relation to nucleic acid-lipid particles such as SNALP means that
35 the particle is not significantly degraded after exposure to a serum or nuclease assay that

would significantly degrade free DNA or RNA. Suitable assays include, for example, a standard serum assay, a DNase assay, or an RNase assay.

[0122] “Systemic delivery,” as used herein, refers to delivery of lipid particles that leads to a broad biodistribution of an active agent such as an interfering RNA (*e.g.*, siRNA) within an organism. Some techniques of administration can lead to the systemic delivery of certain agents, but not others. Systemic delivery means that a useful, preferably therapeutic, amount of an agent is exposed to most parts of the body. To obtain broad biodistribution generally requires a blood lifetime such that the agent is not rapidly degraded or cleared (such as by first pass organs (liver, lung, *etc.*) or by rapid, nonspecific cell binding) before reaching a disease site distal to the site of administration. Systemic delivery of lipid particles can be by any means known in the art including, for example, intravenous, subcutaneous, and intraperitoneal. In a preferred embodiment, systemic delivery of lipid particles is by intravenous delivery.

[0123] “Local delivery,” as used herein, refers to delivery of an active agent such as an interfering RNA (*e.g.*, siRNA) directly to a target site within an organism. For example, an agent can be locally delivered by direct injection into a disease site such as a tumor or other target site such as a site of inflammation or a target organ such as the liver, heart, pancreas, kidney, and the like.

[0124] The term “mammal” refers to any mammalian species such as a human, mouse, rat, dog, cat, hamster, guinea pig, rabbit, livestock, and the like.

[0125] The term “cancer” refers to any member of a class of diseases characterized by the uncontrolled growth of aberrant cells. The term includes all known cancers and neoplastic conditions, whether characterized as malignant, benign, soft tissue, or solid, and cancers of all stages and grades including pre- and post-metastatic cancers. Examples of different types of cancer include, but are not limited to, liver cancer, lung cancer, colon cancer, rectal cancer, anal cancer, bile duct cancer, small intestine cancer, stomach (gastric) cancer, esophageal cancer; gallbladder cancer, pancreatic cancer, appendix cancer, breast cancer, ovarian cancer; cervical cancer, prostate cancer, renal cancer (*e.g.*, renal cell carcinoma), cancer of the central nervous system, glioblastoma, skin cancer, lymphomas, choriocarcinomas, head and neck cancers, osteogenic sarcomas, and blood cancers. Non-limiting examples of specific types of liver cancer include hepatocellular carcinoma (HCC), secondary liver cancer (*e.g.*, caused by metastasis of some other non-liver cancer cell type), and hepatoblastoma. As used herein, a “tumor” comprises one or more cancerous cells.

III. Novel Cationic Lipids

[0126] The present invention provides, *inter alia*, novel cationic (amino) lipids that can advantageously be used in the lipid particles described herein for the *in vitro* and/or *in vivo* delivery of therapeutic agents such as nucleic acids to cells. The novel cationic lipids of the invention have the structures set forth in Formulas I-XII herein, and include the (R) and/or (S) enantiomers thereof.

[0127] In some embodiments, the cationic lipid comprises a racemic mixture. In other embodiments, the cationic lipid comprises a mixture of one or more diastereomers. In certain embodiments, the cationic lipid is enriched in one enantiomer, such that the cationic lipid comprises at least about 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% enantiomeric excess. In certain other embodiments, the cationic lipid is enriched in one diastereomer, such that the cationic lipid comprises at least about 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% diastereomeric excess. In certain additional embodiments, the cationic lipid is chirally pure (*e.g.*, comprises a single optical isomer). In further embodiments, the cationic lipid is enriched in one optical isomer (*e.g.*, an optically active isomer), such that the cationic lipid comprises at least about 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% isomeric excess. The present invention provides the synthesis of the cationic lipids of Formulas I-XII as a racemic mixture or in optically pure form.

[0128] The terms “cationic lipid” and “amino lipid” are used interchangeably herein to include those lipids and salts thereof having one, two, three, or more fatty acid or fatty alkyl chains and a pH-titratable amino head group (*e.g.*, an alkylamino or dialkylamino head group). The cationic lipid is typically protonated (*i.e.*, positively charged) at a pH below the pK_a of the cationic lipid and is substantially neutral at a pH above the pK_a . The cationic lipids of the invention may also be termed titratable cationic lipids.

[0129] The term “salts” includes any anionic and cationic complex, such as the complex formed between a cationic lipid disclosed herein and one or more anions. Non-limiting examples of anions include inorganic and organic anions, *e.g.*, hydride, fluoride, chloride, bromide, iodide, oxalate (*e.g.*, hemioxalate), phosphate, phosphonate, hydrogen phosphate, dihydrogen phosphate, oxide, carbonate, bicarbonate, nitrate, nitrite, nitride, bisulfite, sulfide, sulfite, bisulfate, sulfate, thiosulfate, hydrogen sulfate, borate, formate, acetate, benzoate, citrate, tartrate, lactate, acrylate, polyacrylate, fumarate, maleate, itaconate, glycolate, gluconate, malate, mandelate, tiglate, ascorbate, salicylate, polymethacrylate, perchlorate, chlorate, chlorite, hypochlorite, bromate, hypobromite, iodate, an alkylsulfonate, an arylsulfonate, arsenate, arsenite, chromate, dichromate, cyanide, cyanate, thiocyanate,

hydroxide, peroxide, permanganate, and mixtures thereof. In particular embodiments, the salts of the cationic lipids disclosed herein are crystalline salts.

[0130] The term “alkyl” includes a straight chain or branched, noncyclic or cyclic, saturated aliphatic hydrocarbon containing from 1 to 24 carbon atoms. Representative saturated straight chain alkyls include, but are not limited to, methyl, ethyl, *n*-propyl, *n*-butyl, *n*-pentyl, *n*-hexyl, and the like, while saturated branched alkyls include, without limitation, isopropyl, *sec*-butyl, isobutyl, *tert*-butyl, isopentyl, and the like. Representative saturated cyclic alkyls include, but are not limited to, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, and the like, while unsaturated cyclic alkyls include, without limitation, cyclopentenyl, cyclohexenyl, and the like.

[0131] The term “alkenyl” includes 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, but are not limited to, ethylenyl, propylenyl, 1-butenyl, 2-butenyl, isobutylenyl, 1-pentenyl, 2-pentenyl, 3-methyl-1-butenyl, 2-methyl-2-butenyl, 2,3-dimethyl-2-butenyl, and the like.

[0132] The term “alkynyl” includes 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, without limitation, acetylenyl, propynyl, 1-butylnyl, 2-butylnyl, 1-pentylnyl, 2-pentylnyl, 3-methyl-1 butynyl, and the like.

[0133] The term “acyl” includes any alkyl, alkenyl, or alkynyl wherein the carbon at the point of attachment is substituted with an oxo group, as defined below. The following are non-limiting examples of acyl groups: -C(=O)alkyl, -C(=O)alkenyl, and -C(=O)alkynyl.

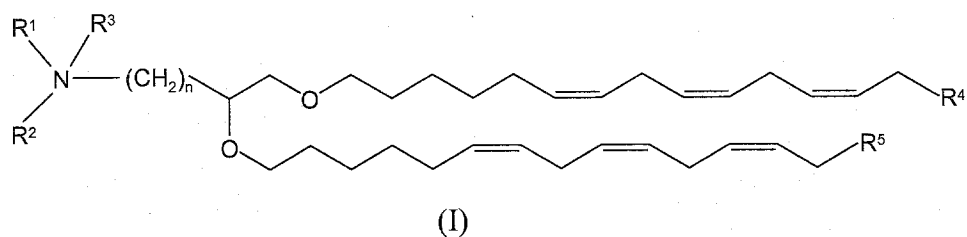
[0134] The term “heterocycle” includes a 5- to 7-membered monocyclic, or 7- to 10-membered bicyclic, heterocyclic ring which is either saturated, unsaturated, or aromatic, and which contains from 1 or 2 heteroatoms independently selected from nitrogen, oxygen and sulfur, and wherein the nitrogen and 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, but are not limited to, heteroaryls as defined below, as well as morpholinyl, pyrrolidinonyl, pyrrolidinyl, piperidinyl, piperizynyl, hydantoinyl, valerolactamyl, oxiranyl, oxetanyl, tetrahydrofuranyl, tetrahydropyranyl, tetrahydropyridinyl, tetrahydroprimidinyl, tetrahydrothiophenyl, tetrahydrothiopyranyl, tetrahydropyrimidinyl, tetrahydrothiophenyl, tetrahydrothiopyranyl, and the like.

[0135] The terms “optionally substituted alkyl”, “optionally substituted alkenyl”, “optionally substituted alkynyl”, “optionally substituted acyl”, and “optionally substituted

heterocycle” mean 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, but are not limited to, oxo, halogen, heterocycle, -CN, -OR^x, -NR^xR^y, -NR^xC(=O)R^y, -NR^xSO₂R^y, -C(=O)R^x, -C(=O)OR^x, -C(=O)NR^xR^y, -SO_nR^x, and -SO_nNR^xR^y, wherein n is 0, 1, or 2, R^x and R^y are the same or different and are independently hydrogen, alkyl, or heterocycle, and each of the alkyl and heterocycle substituents may be further substituted with one or more of oxo, halogen, -OH, -CN, alkyl, -OR^x, heterocycle, -NR^xR^y, -NR^xC(=O)R^y, -NR^xSO₂R^y, -C(=O)R^x, -C(=O)OR^x, -C(=O)NR^xR^y, -SO_nR^x, and -SO_nNR^xR^y. The term “optionally substituted,” when used before a list of substituents, means that each of the substituents in the list may be optionally substituted as described herein.

[0136] The term “halogen” includes fluoro, chloro, bromo, and iodo.

[0137] In one aspect, the present invention provides a cationic lipid of Formula I having the following structure:



or salts thereof, wherein:

R¹ and R² are either the same or different and are independently an optionally substituted C₁-C₆ alkyl, C₂-C₆ alkenyl, or C₂-C₆ alkynyl, or R¹ and R² may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

R³ is either absent or is hydrogen (H) or a C₁-C₆ alkyl to provide a quaternary amine;

R⁴ and R⁵ are either absent or present and when present are either the same or different and are independently an optionally substituted C₁-C₁₀ alkyl or C₂-C₁₀ alkenyl; and n is 0, 1, 2, 3, or 4.

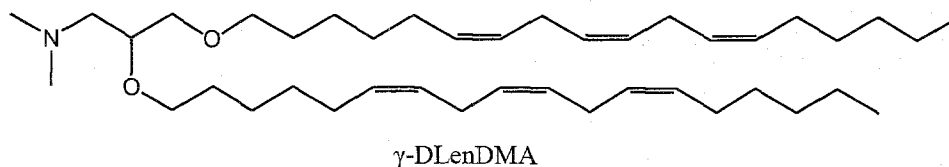
[0138] In some embodiments, R¹ and R² are independently an optionally substituted C₁-C₄ alkyl, C₂-C₄ alkenyl, or C₂-C₄ alkynyl. In a preferred embodiment, R¹ and R² are both methyl groups. In another preferred embodiment, R⁴ and R⁵ are both butyl groups. In yet another preferred embodiment, n is 1. In other embodiments, R³ is absent when the pH is above the pK_a of the cationic lipid and R³ is hydrogen when the pH is below the pK_a of the cationic lipid such that the amino head group is protonated. In an alternative embodiment, R³ is an

optionally substituted C₁-C₄ alkyl to provide a quaternary amine. In further embodiments, R⁴ and R⁵ are independently an optionally substituted C₂-C₆ or C₂-C₄ alkyl or C₂-C₆ or C₂-C₄ alkenyl.

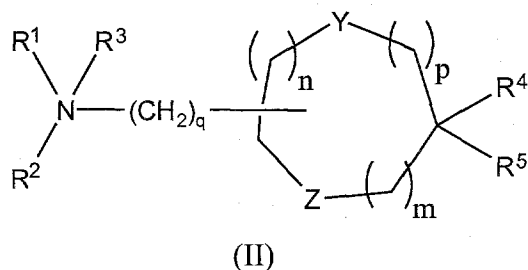
[0139] In an alternative embodiment, the cationic lipid of Formula I comprises ester linkages between the amino head group and one or both of the alkyl chains. In some embodiments, the cationic lipid of Formula I forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula I is the oxalate (e.g., hemioxalate) salt thereof, which is preferably a crystalline salt.

[0140] Although each of the alkyl chains in Formula I contains *cis* double bonds at positions 6, 9, and 12 (i.e., *cis,cis,cis*-Δ⁶,Δ⁹,Δ¹²), in an alternative embodiment, one, two, or three of these double bonds in one or both alkyl chains may be in the *trans* configuration.

[0141] In a particularly preferred embodiment, the cationic lipid of Formula I has the structure:



[0142] In another aspect, the present invention provides a cationic lipid of Formula II having the following structure:



or salts thereof, wherein:

R¹ and R² are either the same or different and are independently an optionally substituted C₁-C₆ alkyl, C₂-C₆ alkenyl, or C₂-C₆ alkynyl, or R¹ and R² may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

R³ is either absent or is hydrogen (H) or a C₁-C₆ alkyl to provide a quaternary amine;

R⁴ and R⁵ are either the same or different and are independently an optionally substituted C₁₂-C₂₄ alkyl, C₁₂-C₂₄ alkenyl, C₁₂-C₂₄ alkynyl, or C₁₂-C₂₄ acyl, wherein at least one of R⁴ and R⁵ comprises at least three sites of unsaturation or a substituted C₁₂-C₂₄ alkyl;

m, n, and p are either the same or different and are independently either 0, 1, or 2, with the proviso that m, n, and p are not simultaneously 0;

q is 0, 1, 2, 3, or 4; and

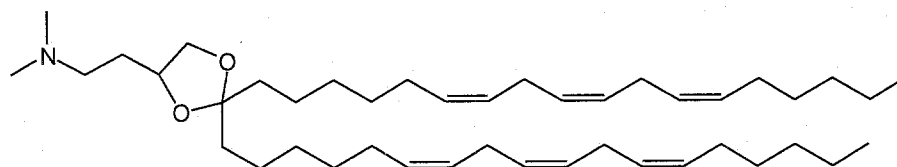
Y and Z are either the same or different and are independently O, S, or NH.

- 5 [0143] In some embodiments, R¹ and R² are independently an optionally substituted C₁-C₄ alkyl, C₂-C₄ alkenyl, or C₂-C₄ alkynyl. In a preferred embodiment, R¹ and R² are both methyl groups. In another preferred embodiment, q is 2. In other embodiments, R³ is absent when the pH is above the pK_a of the cationic lipid and R³ is hydrogen when the pH is below the pK_a of the cationic lipid such that the amino head group is protonated. In an alternative
- 10 embodiment, R³ is an optionally substituted C₁-C₄ alkyl to provide a quaternary amine. In further embodiments, R⁴ and R⁵ are independently an optionally substituted C₁₂-C₂₀ or C₁₄-C₂₂ alkyl, C₁₂-C₂₀ or C₁₄-C₂₂ alkenyl, C₁₂-C₂₀ or C₁₄-C₂₂ alkynyl, or C₁₂-C₂₀ or C₁₄-C₂₂ acyl.
- [0144] In embodiments where at least one of R⁴ and R⁵ comprises a branched alkyl group (e.g., a substituted C₁₂-C₂₄ alkyl group), the branched alkyl group may comprise a C₁₂-C₂₄
- 15 alkyl having at least 1-6 (e.g., 1, 2, 3, 4, 5, 6, or more) C₁-C₆ alkyl substituents. In particular embodiments, the branched alkyl group comprises a C₁₂-C₂₀ or C₁₄-C₂₂ alkyl with 1-6 (e.g., 1, 2, 3, 4, 5, 6) C₁-C₄ alkyl (e.g., methyl, ethyl, propyl, or butyl) substituents. Preferably, the branched alkyl group comprises a phytanyl (3,7,11,15-tetramethyl-hexadecanyl) moiety. In other preferred embodiments, R⁴ and R⁵ are both phytanyl moieties.
- 20 [0145] In alternative embodiments, at least one of R⁴ and R⁵ comprises a branched acyl group (e.g., a substituted C₁₂-C₂₄ acyl group). In certain instances, the branched acyl group may comprise a C₁₂-C₂₄ acyl having at least 1-6 (e.g., 1, 2, 3, 4, 5, 6, or more) C₁-C₆ alkyl substituents. In particular embodiments, the branched acyl group comprises a C₁₂-C₂₀ or C₁₄-C₂₂ acyl with 1-6 (e.g., 1, 2, 3, 4, 5, 6) C₁-C₄ alkyl (e.g., methyl, ethyl, propyl, or butyl)
- 25 substituents. Preferably, the branched acyl group comprises a phytanoyl (3,7,11,15-tetramethyl-hexadecanoyl) moiety.
- [0146] In embodiments where at least one of R⁴ and R⁵ comprises at least three sites of unsaturation, the double bonds present in one or both alkyl chains may be in the *cis* and/or *trans* configuration. In some embodiments, R⁴ and R⁵ are independently selected from the
- 30 group consisting of a dodecatrienyl moiety, a tetradectrienyl moiety, a hexadecatrienyl moiety, an octadecatrienyl moiety, an icosatrienyl moiety, and a phytanyl moiety, as well as acyl derivatives thereof (e.g., linolenoyl, γ -linolenoyl, phytanoyl, *etc.*). In certain instances, the octadecatrienyl moiety is a linolenyl moiety or a γ -linolenyl moiety. In preferred
- embodiments, R⁴ and R⁵ are both linolenyl moieties or γ -linolenyl moieties. In particular
- 35 embodiments, R⁴ and R⁵ independently comprise a backbone of from about 16 to about 22

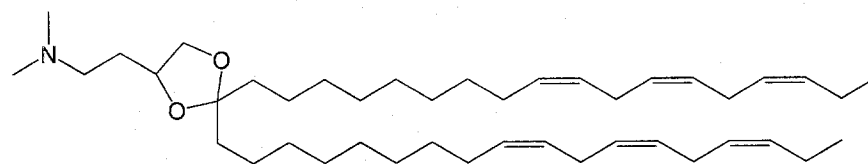
carbon atoms, and one or both of R^4 and R^5 independently comprise at least three, four, five, or six sites of unsaturation.

[0147] In some embodiments, the cationic lipid of Formula II forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula II is the oxalate (e.g., hemioxalate) salt thereof, which is preferably a crystalline salt.

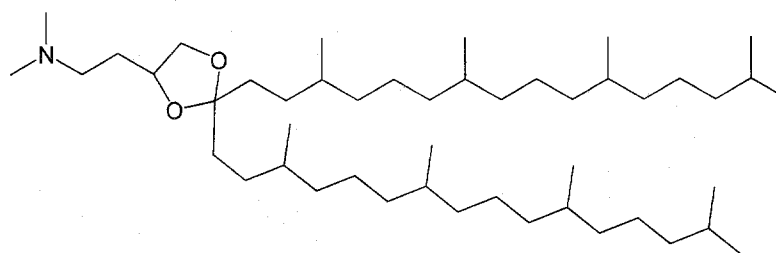
[0148] In a particularly preferred embodiment, the cationic lipid of Formula II has a structure selected from the group consisting of:



γ -DLen-C2K-DMA

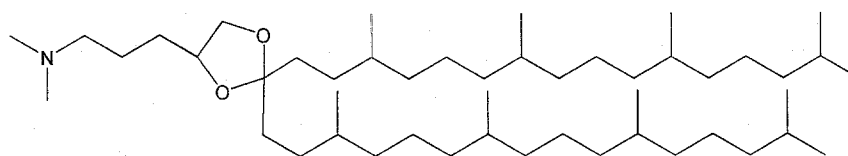


DLen-C2K-DMA



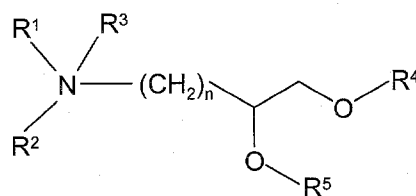
DPan-C2K-DMA

, and



DPan-C3K-DMA

[0149] In yet another aspect, the present invention provides a cationic lipid of Formula III having the following structure:



(III)

or salts thereof, wherein:

R^1 and R^2 are joined to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

R^3 is either absent or is hydrogen (H) or a C_1 - C_6 alkyl to provide a quaternary amine;

R^4 and R^5 are either the same or different and are independently an optionally substituted C_{12} - C_{24} alkyl, C_{12} - C_{24} alkenyl, C_{12} - C_{24} alkynyl, or C_{12} - C_{24} acyl; and

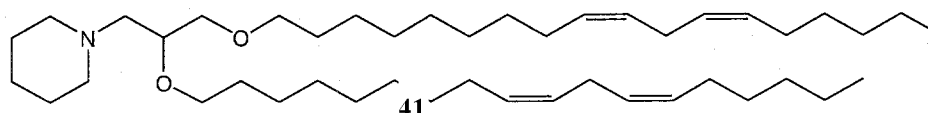
n is 0, 1, 2, 3, or 4.

[0150] In some embodiments, R^1 and R^2 are joined to form a heterocyclic ring of 5 carbon atoms and 1 nitrogen atom. In certain instances, the heterocyclic ring is substituted with a substituent such as a hydroxyl group at the ortho, meta, and/or para positions. In a preferred embodiment, n is 1. In other embodiments, R^3 is absent when the pH is above the pK_a of the cationic lipid and R^3 is hydrogen when the pH is below the pK_a of the cationic lipid such that the amino head group is protonated. In an alternative embodiment, R^3 is an optionally substituted C_1 - C_4 alkyl to provide a quaternary amine. In further embodiments, R^4 and R^5 are independently an optionally substituted C_{12} - C_{20} or C_{14} - C_{22} alkyl, C_{12} - C_{20} or C_{14} - C_{22} alkenyl, C_{12} - C_{20} or C_{14} - C_{22} alkynyl, or C_{12} - C_{20} or C_{14} - C_{22} acyl.

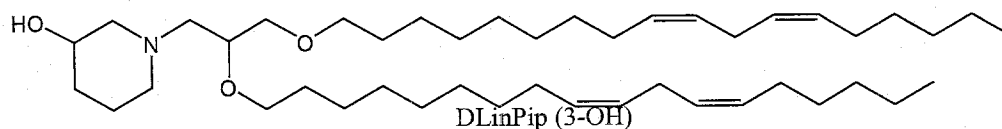
[0151] In certain embodiments, R^4 and R^5 are independently selected from the group consisting of a dodecadienyl moiety, a tetradecadienyl moiety, a hexadecadienyl moiety, an octadecadienyl moiety, an icosadienyl moiety, a dodecatrienyl moiety, a tetradectrienyl moiety, a hexadecatrienyl moiety, an octadecatrienyl moiety, an icosatrienyl moiety, and a branched alkyl group as described above (*e.g.*, a phytanyl moiety), as well as acyl derivatives thereof (*e.g.*, linoleoyl, linolenoyl, γ -linolenoyl, phytanoyl, *etc.*). In some instances, the octadecadienyl moiety is a linoleyl moiety. In other instances, the octadecatrienyl moiety is a linolenyl moiety or a γ -linolenyl moiety. In particular embodiments, R^4 and R^5 are both linoleyl moieties, linolenyl moieties, γ -linolenyl moieties, or phytanyl moieties.

[0152] In some embodiments, the cationic lipid of Formula III forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula III is the oxalate (*e.g.*, hemioxalate) salt thereof, which is preferably a crystalline salt.

[0153] In a particularly preferred embodiment, the cationic lipid of Formula III has a structure selected from the group consisting of:

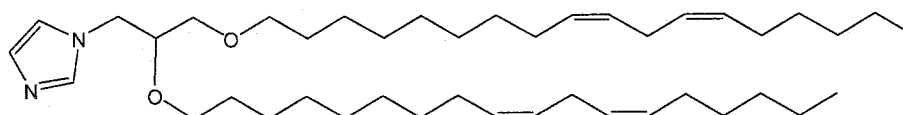


DLinPip



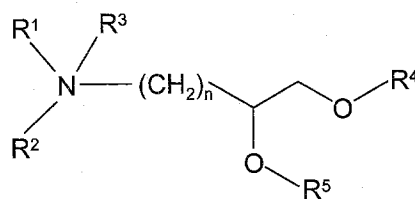
DLinPip (4-OH)

, and



DLinIm

10 **[0154]** In still yet another aspect, the present invention provides a cationic lipid of Formula IV having the following structure:



or salts thereof, wherein:

R¹ and R² are either the same or different and are independently an optionally substituted C₁-C₆ alkyl, C₂-C₆ alkenyl, or C₂-C₆ alkynyl, or R¹ and R² may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

R³ is either absent or is hydrogen (H) or a C₁-C₆ alkyl to provide a quaternary amine;

R⁴ and R⁵ are either the same or different and are independently an optionally substituted C₁₂-C₂₄ alkyl, C₁₂-C₂₄ alkenyl, C₁₂-C₂₄ alkynyl, or C₁₂-C₂₄ acyl; and

n is 2, 3, or 4.

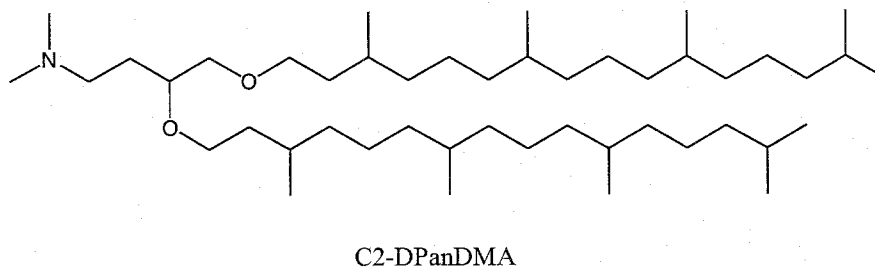
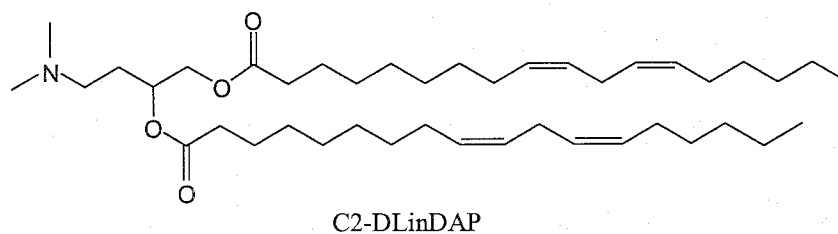
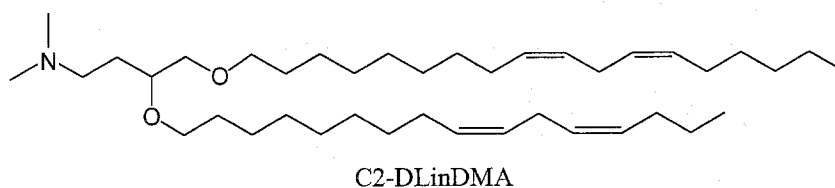
[0155] In some embodiments, R¹ and R² are independently an optionally substituted C₁-C₄ alkyl, C₂-C₄ alkenyl, or C₂-C₄ alkynyl. In a preferred embodiment, R¹ and R² are both methyl groups. In another preferred embodiment, n is 2. In other embodiments, R³ is absent when the pH is above the pK_a of the cationic lipid and R³ is hydrogen when the pH is below the pK_a of the cationic lipid such that the amino head group is protonated. In an alternative embodiment, R³ is an optionally substituted C₁-C₄ alkyl to provide a quaternary amine. In

further embodiments, R^4 and R^5 are independently an optionally substituted C_{12} - C_{20} or C_{14} - C_{22} alkyl, C_{12} - C_{20} or C_{14} - C_{22} alkenyl, C_{12} - C_{20} or C_{14} - C_{22} alkynyl, or C_{12} - C_{20} or C_{14} - C_{22} acyl.

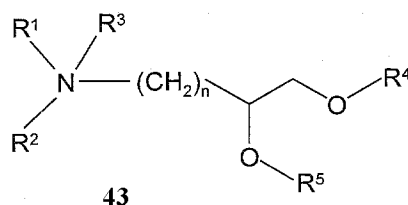
[0156] In certain embodiments, R^4 and R^5 are independently selected from the group consisting of a dodecadienyl moiety, a tetradecadienyl moiety, a hexadecadienyl moiety, an octadecadienyl moiety, an icosadienyl moiety, a dodecatrienyl moiety, a tetradectrienyl moiety, a hexadecatrienyl moiety, an octadecatrienyl moiety, an icosatrienyl moiety, and a branched alkyl group as described above (*e.g.*, a phytanyl moiety), as well as acyl derivatives thereof (*e.g.*, linoleoyl, linolenoyl, γ -linolenoyl, phytanoyl, *etc.*). In some instances, the octadecadienyl moiety is a linoleyl moiety. In other instances, the octadecatrienyl moiety is a linolenyl moiety or a γ -linolenyl moiety. In particular embodiments, R^4 and R^5 are both linoleyl moieties, linolenyl moieties, γ -linolenyl moieties, or phytanyl moieties.

[0157] In some embodiments, the cationic lipid of Formula IV forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula IV is the oxalate (*e.g.*, hemioxalate) salt thereof, which is preferably a crystalline salt.

[0158] In a particularly preferred embodiment, the cationic lipid of Formula IV has a structure selected from the group consisting of:



[0159] In another aspect, the present invention provides a cationic lipid of Formula V having the following structure:



(V)

or salts thereof, wherein:

R^1 and R^2 are either the same or different and are independently an optionally substituted C_1 - C_6 alkyl, C_2 - C_6 alkenyl, or C_2 - C_6 alkynyl, or R^1 and R^2 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

R^3 is either absent or is hydrogen (H) or a C_1 - C_6 alkyl to provide a quaternary amine;

R^4 and R^5 are different and are independently an optionally substituted C_1 - C_{24} alkyl, C_2 - C_{24} alkenyl, C_2 - C_{24} alkynyl, or C_1 - C_{24} acyl; and

n is 0, 1, 2, 3, or 4.

[0160] In some embodiments, R^1 and R^2 are independently an optionally substituted C_1 - C_4 alkyl, C_2 - C_4 alkenyl, or C_2 - C_4 alkynyl. In a preferred embodiment, R^1 and R^2 are both methyl groups. In another preferred embodiment, n is 1. In other embodiments, R^3 is absent when the pH is above the pK_a of the cationic lipid and R^3 is hydrogen when the pH is below the pK_a of the cationic lipid such that the amino head group is protonated. In an alternative embodiment, R^3 is an optionally substituted C_1 - C_4 alkyl to provide a quaternary amine. In further embodiments, R^4 and R^5 are different and are independently an optionally substituted C_4 - C_{20} alkyl, C_4 - C_{20} alkenyl, C_4 - C_{20} alkynyl, or C_4 - C_{20} acyl.

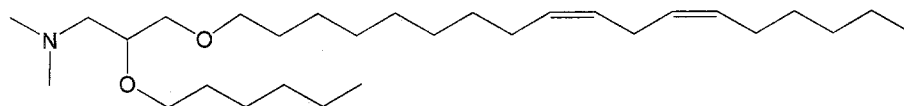
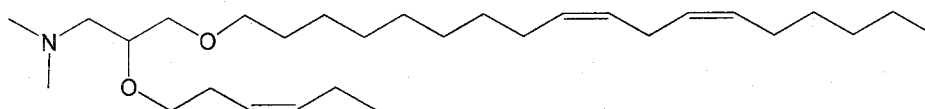
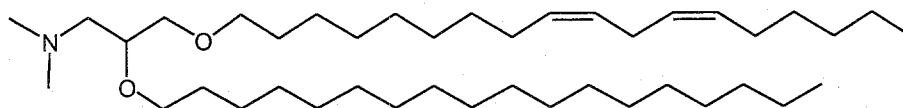
[0161] In some embodiments, R^4 is an optionally substituted C_{12} - C_{24} alkyl, C_{12} - C_{24} alkenyl, C_{12} - C_{24} alkynyl, or C_{12} - C_{24} acyl, and R^5 is an optionally substituted C_4 - C_{10} alkyl, C_4 - C_{10} alkenyl, C_4 - C_{10} alkynyl, or C_4 - C_{10} acyl. In certain instances, R^4 is an optionally substituted C_{12} - C_{20} or C_{14} - C_{22} alkyl, C_{12} - C_{20} or C_{14} - C_{22} alkenyl, C_{12} - C_{20} or C_{14} - C_{22} alkynyl, or C_{12} - C_{20} or C_{14} - C_{22} acyl, and R^5 is an optionally substituted C_4 - C_8 or C_6 alkyl, C_4 - C_8 or C_6 alkenyl, C_4 - C_8 or C_6 alkynyl, or C_4 - C_8 or C_6 acyl.

[0162] In other embodiments, R^4 is an optionally substituted C_4 - C_{10} alkyl, C_4 - C_{10} alkenyl, C_4 - C_{10} alkynyl, or C_4 - C_{10} acyl, and R^5 is an optionally substituted C_{12} - C_{24} alkyl, C_{12} - C_{24} alkenyl, C_{12} - C_{24} alkynyl, or C_{12} - C_{24} acyl. In certain instances, R^4 is an optionally substituted C_4 - C_8 or C_6 alkyl, C_4 - C_8 or C_6 alkenyl, C_4 - C_8 or C_6 alkynyl, or C_4 - C_8 or C_6 acyl, and R^5 is an optionally substituted C_{12} - C_{20} or C_{14} - C_{22} alkyl, C_{12} - C_{20} or C_{14} - C_{22} alkenyl, C_{12} - C_{20} or C_{14} - C_{22} alkynyl, or C_{12} - C_{20} or C_{14} - C_{22} acyl.

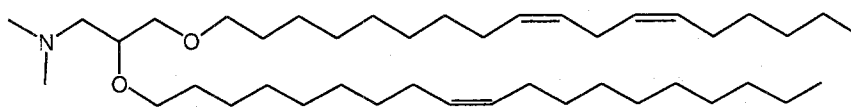
[0163] In particular embodiments, R^4 is a linoleyl moiety, and R^5 is a C_6 alkyl moiety, a C_6 alkenyl moiety, an octadecyl moiety, an oleyl moiety, a linolenyl moiety, a γ -linolenyl moiety, or a phytanyl moiety. In other embodiments, one of R^4 or R^5 is a phytanyl moiety.

[0164] In some embodiments, the cationic lipid of Formula V forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula V is the oxalate (*e.g.*, hemioxalate) salt thereof, which is preferably a crystalline salt.

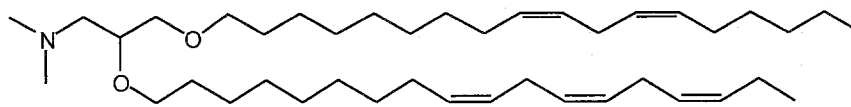
[0165] In a particularly preferred embodiment, the cationic lipid of Formula V is an asymmetric lipid having a structure selected from the group consisting of:

Linoleyl/C₆:0 DMALinoleyl/C₆:1 DMA

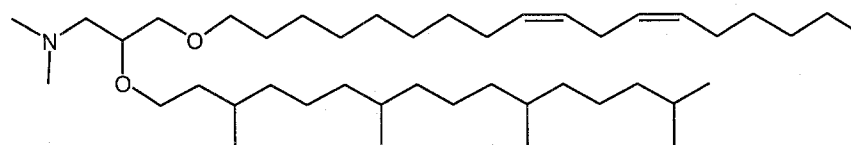
Linoleyl/Stearyl DMA



Linoleyl/Oleyl DMA

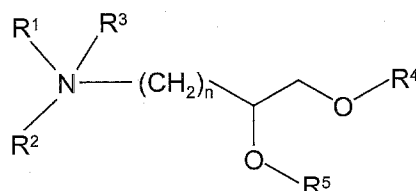


Linoleyl/Linolenyl DMA



Linoleyl/Phytanyl DMA

[0166] In yet another aspect, the present invention provides a cationic lipid of Formula VI having the following structure:



(VI)

or salts thereof, wherein:

R^1 and R^2 are either the same or different and are independently an optionally substituted C_1 - C_6 alkyl, C_2 - C_6 alkenyl, or C_2 - C_6 alkynyl, or R^1 and R^2 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

R^3 is either absent or is hydrogen (H) or a C_1 - C_6 alkyl to provide a quaternary amine;

R^4 and R^5 are either the same or different and are independently an optionally substituted C_{12} - C_{24} alkyl, C_{12} - C_{24} alkenyl, C_{12} - C_{24} alkynyl, or C_{12} - C_{24} acyl, wherein at least one of R^4 and R^5 comprises at least four sites of unsaturation or a substituted C_{12} - C_{24} alkyl; and

n is 0, 1, 2, 3, or 4.

[0167] In some embodiments, R^1 and R^2 are independently an optionally substituted C_1 - C_4 alkyl, C_2 - C_4 alkenyl, or C_2 - C_4 alkynyl. In a preferred embodiment, R^1 and R^2 are both methyl groups. In another preferred embodiment, n is 1. In other embodiments, R^3 is absent when the pH is above the pK_a of the cationic lipid and R^3 is hydrogen when the pH is below the pK_a of the cationic lipid such that the amino head group is protonated. In an alternative embodiment, R^3 is an optionally substituted C_1 - C_4 alkyl to provide a quaternary amine. In

further embodiments, R^4 and R^5 are independently an optionally substituted C_{12} - C_{20} or C_{14} - C_{22} alkyl, C_{12} - C_{20} or C_{14} - C_{22} alkenyl, C_{12} - C_{20} or C_{14} - C_{22} alkynyl, or C_{12} - C_{20} or C_{14} - C_{22} acyl.

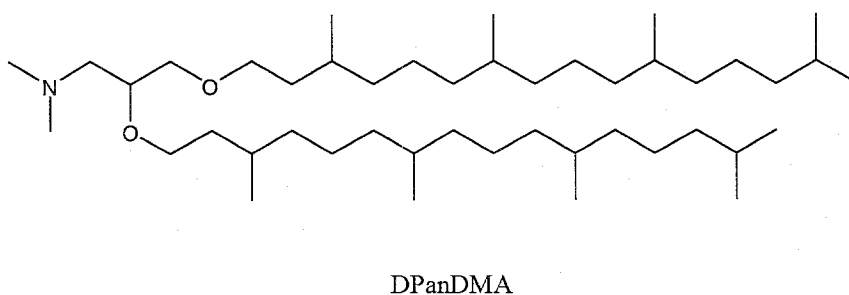
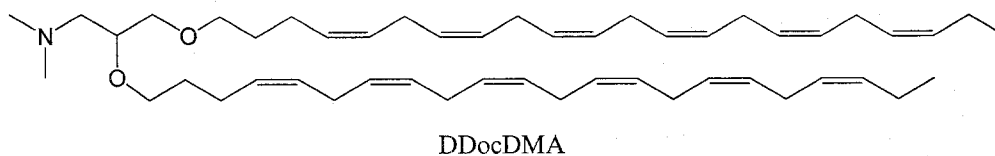
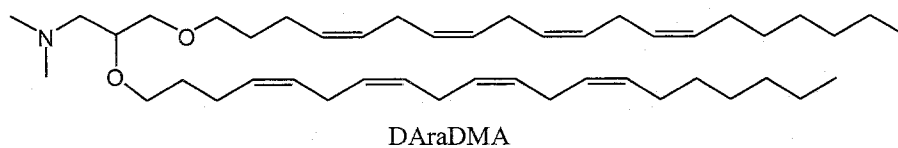
[0168] In embodiments where at least one of R^4 and R^5 comprises a branched alkyl group (e.g., a substituted C_{12} - C_{24} alkyl group), the branched alkyl group may comprise a C_{12} - C_{24} alkyl having at least 1-6 (e.g., 1, 2, 3, 4, 5, 6, or more) C_1 - C_6 alkyl substituents. In particular embodiments, the branched alkyl group comprises a C_{12} - C_{20} or C_{14} - C_{22} alkyl with 1-6 (e.g., 1, 2, 3, 4, 5, 6) C_1 - C_4 alkyl (e.g., methyl, ethyl, propyl, or butyl) substituents. Preferably, the branched alkyl group comprises a phytanyl (3,7,11,15-tetramethyl-hexadecanyl) moiety.

[0169] In alternative embodiments, at least one of R^4 and R^5 comprises a branched acyl group (e.g., a substituted C_{12} - C_{24} acyl group). In certain instances, the branched acyl group may comprise a C_{12} - C_{24} acyl having at least 1-6 (e.g., 1, 2, 3, 4, 5, 6, or more) C_1 - C_6 alkyl substituents. In particular embodiments, the branched acyl group comprises a C_{12} - C_{20} or C_{14} - C_{22} acyl with 1-6 (e.g., 1, 2, 3, 4, 5, 6) C_1 - C_4 alkyl (e.g., methyl, ethyl, propyl, or butyl) substituents. Preferably, the branched acyl group comprises a phytanoyl (3,7,11,15-tetramethyl-hexadecanoyl) moiety.

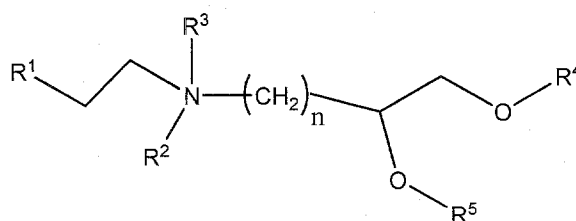
[0170] In embodiments where at least one of R^4 and R^5 comprises at least four sites of unsaturation, the double bonds present in one or both alkyl chains may be in the *cis* and/or *trans* configuration. In a particular embodiment, R^4 and R^5 independently comprise four, five, or six sites of unsaturation. In some instances, R^4 comprises four, five, or six sites of unsaturation and R^5 comprises zero, one, two, three, four, five, or six sites of unsaturation. In other instances, R^4 comprises zero, one, two, three, four, five, or six sites of unsaturation and R^5 comprises four, five, or six sites of unsaturation. In a preferred embodiment, both R^4 and R^5 comprise four, five, or six sites of unsaturation. In particular embodiments, R^4 and R^5 independently comprise a backbone of from about 18 to about 24 carbon atoms, and one or both of R^4 and R^5 independently comprise at least four, five, or six sites of unsaturation.

[0171] In some embodiments, the cationic lipid of Formula VI forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula VI is the oxalate (e.g., hemioxalate) salt thereof, which is preferably a crystalline salt.

[0172] In a particularly preferred embodiment, the cationic lipid of Formula VI has a structure selected from the group consisting of:



[0173] In still yet another aspect, the present invention provides a cationic lipid of Formula VII having the following structure:



(VII)

or salts thereof, wherein:

R^1 is hydrogen (H) or $-(CH_2)_q-NR^6R^7R^8$, wherein:

R^6 and R^7 are either the same or different and are independently an optionally substituted C_1 - C_6 alkyl, C_2 - C_6 alkenyl, or C_2 - C_6 alkynyl, or R^6 and R^7 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

R^8 is either absent or is hydrogen (H) or a C_1 - C_6 alkyl to provide a quaternary amine; and

q is 0, 1, 2, 3, or 4;

R^2 is an optionally substituted C_1 - C_6 alkyl, C_2 - C_6 alkenyl, or C_2 - C_6 alkynyl;

R^3 is either absent or is hydrogen (H) or a C_1 - C_6 alkyl to provide a quaternary amine;

R^4 and R^5 are either the same or different and are independently an optionally substituted C_{12} - C_{24} alkyl, C_{12} - C_{24} alkenyl, C_{12} - C_{24} alkynyl, or C_{12} - C_{24} acyl; and

n is 0, 1, 2, 3, or 4.

[0174] In some embodiments, R^2 is an optionally substituted C_1 - C_4 alkyl, C_2 - C_4 alkenyl, or C_2 - C_4 alkynyl. In other embodiments, R^3 is absent when the pH is above the pK_a of the cationic lipid and R^3 is hydrogen when the pH is below the pK_a of the cationic lipid such that the amino head group is protonated. In an alternative embodiment, R^3 is an optionally substituted C_1 - C_4 alkyl to provide a quaternary amine. In certain embodiments, R^4 and R^5 are independently an optionally substituted C_{12} - C_{20} or C_{14} - C_{22} alkyl, C_{12} - C_{20} or C_{14} - C_{22} alkenyl, C_{12} - C_{20} or C_{14} - C_{22} alkynyl, or C_{12} - C_{20} or C_{14} - C_{22} acyl.

[0175] In further embodiments, R^6 and R^7 are independently an optionally substituted C_1 - C_4 alkyl, C_2 - C_4 alkenyl, or C_2 - C_4 alkynyl. In other embodiments, R^8 is absent when the pH is above the pK_a of the cationic lipid and R^8 is hydrogen when the pH is below the pK_a of the cationic lipid such that the amino head group is protonated. In an alternative embodiment, R^8 is an optionally substituted C_1 - C_4 alkyl to provide a quaternary amine.

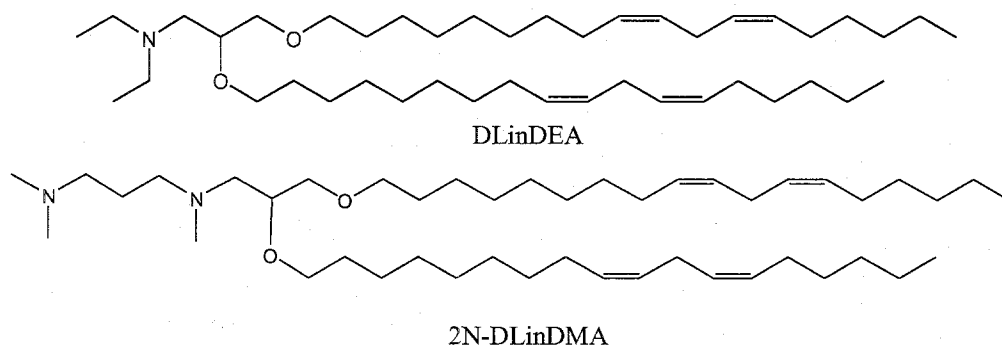
[0176] In a preferred embodiment, R^1 is hydrogen and R^2 is an ethyl group. In another preferred embodiment, R^6 and R^7 are both methyl groups. In certain instances, n is 1. In certain other instances, q is 1.

[0177] In certain embodiments, R^4 and R^5 are independently selected from the group consisting of a dodecadienyl moiety, a tetradecadienyl moiety, a hexadecadienyl moiety, an octadecadienyl moiety, an icosadienyl moiety, a dodecatrienyl moiety, a tetradectrienyl

moiety, a hexadecatrienyl moiety, an octadecatrienyl moiety, an icosatrienyl moiety, and a branched alkyl group as described above (*e.g.*, a phytanyl moiety), as well as acyl derivatives thereof (*e.g.*, linoleoyl, linolenoyl, γ -linolenoyl, phytanoyl, *etc.*). In some instances, the octadecadienyl moiety is a linoleyl moiety. In other instances, the octadecatrienyl moiety is a linolenyl moiety or a γ -linolenyl moiety. In particular embodiments, R^4 and R^5 are both linoleyl moieties, linolenyl moieties, γ -linolenyl moieties, or phytanyl moieties.

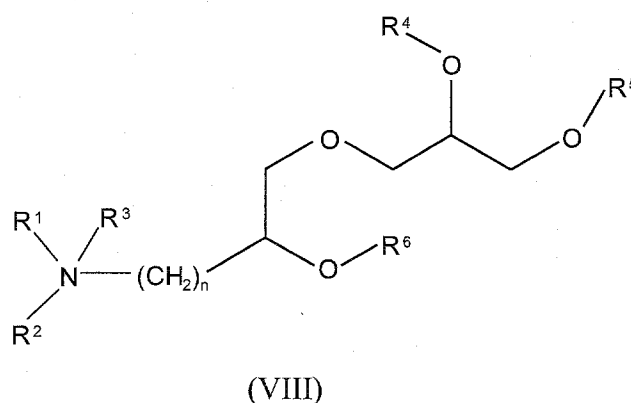
[0178] In some embodiments, the cationic lipid of Formula VII forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula VII is the oxalate (*e.g.*, hemioxalate) salt thereof, which is preferably a crystalline salt.

[0179] In a particularly preferred embodiment, the cationic lipid of Formula VII has a structure selected from the group consisting of:



and

[0180] In another aspect, the present invention provides a cationic lipid of Formula VIII having the following structure:



or salts thereof, wherein:

R^1 and R^2 are either the same or different and are independently an optionally substituted C_1 - C_6 alkyl, C_2 - C_6 alkenyl, or C_2 - C_6 alkynyl, or R^1 and R^2 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

R^3 is either absent or is hydrogen (H) or a C_1 - C_6 alkyl to provide a quaternary amine;

R^4 , R^5 , and R^6 are either the same or different and are independently an optionally substituted C_{12} - C_{24} alkyl, C_{12} - C_{24} alkenyl, C_{12} - C_{24} alkynyl, or C_{12} - C_{24} acyl; and

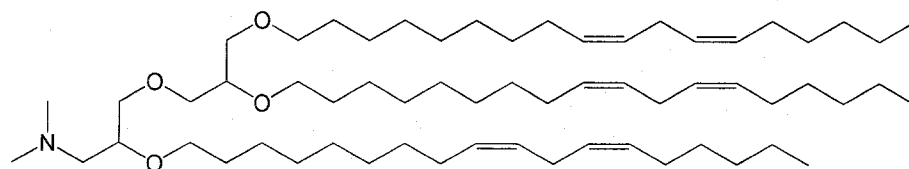
n is 0, 1, 2, 3, or 4.

[0181] In some embodiments, R^1 and R^2 are independently an optionally substituted C_1 - C_4 alkyl, C_2 - C_4 alkenyl, or C_2 - C_4 alkynyl. In a preferred embodiment, R^1 and R^2 are both methyl groups. In one particular embodiment, n is 1. In another particular embodiment, n is 2. In yet another particular embodiment, n is 3. In other embodiments, R^3 is absent when the pH is above the pK_a of the cationic lipid and R^3 is hydrogen when the pH is below the pK_a of the cationic lipid such that the amino head group is protonated. In an alternative embodiment, R^3 is an optionally substituted C_1 - C_4 alkyl to provide a quaternary amine. In further embodiments, R^4 , R^5 , and R^6 are independently an optionally substituted C_{12} - C_{20} or C_{14} - C_{22} alkyl, C_{12} - C_{20} or C_{14} - C_{22} alkenyl, C_{12} - C_{20} or C_{14} - C_{22} alkynyl, or C_{12} - C_{20} or C_{14} - C_{22} acyl.

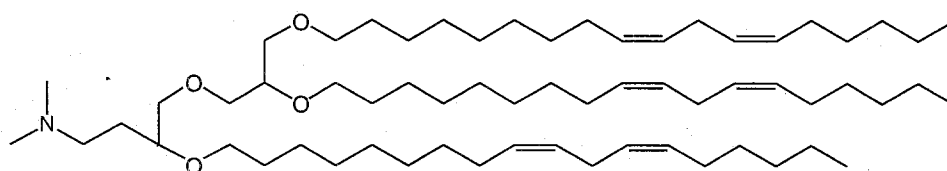
[0182] In certain embodiments, R^4 , R^5 , and R^6 are independently selected from the group consisting of a dodecadienyl moiety, a tetradecadienyl moiety, a hexadecadienyl moiety, an octadecadienyl moiety, an icosadienyl moiety, a dodecatrienyl moiety, a tetradectrienyl moiety, a hexadecatrienyl moiety, an octadecatrienyl moiety, an icosatrienyl moiety, and a branched alkyl group as described above (*e.g.*, a phytanyl moiety), as well as acyl derivatives thereof (*e.g.*, linoleoyl, linolenoyl, γ -linolenoyl, phytanoyl, *etc.*). In some instances, the octadecadienyl moiety is a linoleyl moiety. In other instances, the octadecatrienyl moiety is a linolenyl moiety or a γ -linolenyl moiety. In particular embodiments, R^4 , R^5 , and R^6 are all linoleyl moieties, linolenyl moieties, γ -linolenyl moieties, or phytanyl moieties.

[0183] In some embodiments, the cationic lipid of Formula VIII forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula VIII is the oxalate (*e.g.*, hemioxalate) salt thereof, which is preferably a crystalline salt.

[0184] In a particularly preferred embodiment, the cationic lipid of Formula VIII has a structure selected from the group consisting of:

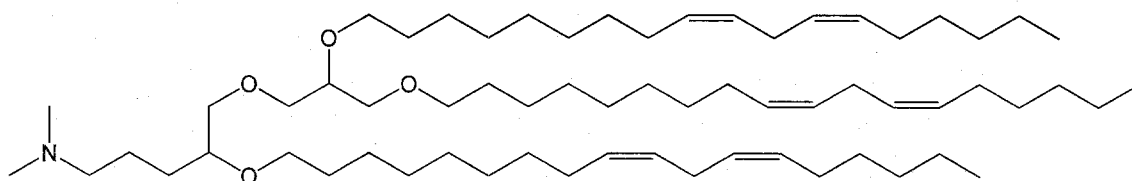


TLinDMA



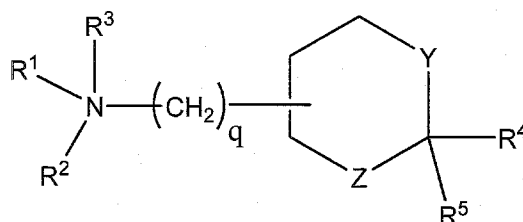
C2-TLinDMA

, and



C3-TLinDMA

5 [0185] In yet another aspect, the present invention provides a cationic lipid of Formula IX having the following structure:



(IX)

or salts thereof, wherein:

10 R^1 and R^2 are either the same or different and are independently an optionally substituted C_1 - C_6 alkyl, C_2 - C_6 alkenyl, or C_2 - C_6 alkynyl, or R^1 and R^2 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

15 R^3 is either absent or is hydrogen (H) or a C_1 - C_6 alkyl to provide a quaternary amine;

R^4 and R^5 are either the same or different and are independently an optionally substituted C_{12} - C_{24} alkyl, C_{12} - C_{24} alkenyl, C_{12} - C_{24} alkynyl, or C_{12} - C_{24} acyl;

q is 0, 1, 2, 3, or 4; and

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

20 wherein if q is 1, R^1 and R^2 are both methyl groups, R^4 and R^5 are both linoleyl moieties, and Y and Z are both O, then the alkylamino group is attached to one of the two carbons adjacent to Y or Z (*i.e.*, at the '4' or '6' position of the 6-membered ring).

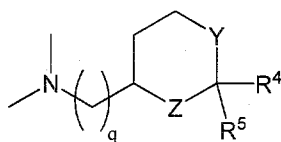
[0186] In some embodiments, R^1 and R^2 are independently an optionally substituted C_1 - C_4 alkyl, C_2 - C_4 alkenyl, or C_2 - C_4 alkynyl. In a preferred embodiment, R^1 and R^2 are both methyl groups. In another preferred embodiment, q is 2. In a particular embodiment, Y and Z are

25

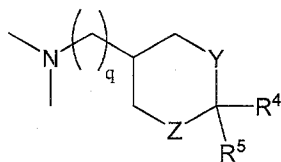
both oxygen (O). In other embodiments, R^3 is absent when the pH is above the pK_a of the cationic lipid and R^3 is hydrogen when the pH is below the pK_a of the cationic lipid such that the amino head group is protonated. In an alternative embodiment, R^3 is an optionally substituted C_1 - C_4 alkyl to provide a quaternary amine. In further embodiments, R^4 and R^5 are independently an optionally substituted C_{12} - C_{20} or C_{14} - C_{22} alkyl, C_{12} - C_{20} or C_{14} - C_{22} alkenyl, C_{12} - C_{20} or C_{14} - C_{22} alkynyl, or C_{12} - C_{20} or C_{14} - C_{22} acyl.

[0187] In other embodiments, R^4 and R^5 are independently selected from the group consisting of a dodecadienyl moiety, a tetradecadienyl moiety, a hexadecadienyl moiety, an octadecadienyl moiety, an icosadienyl moiety, a dodecatrienyl moiety, a tetradectrienyl moiety, a hexadecatrienyl moiety, an octadecatrienyl moiety, an icosatrienyl moiety, and a branched alkyl group as described above (*e.g.*, a phytanyl moiety), as well as acyl derivatives thereof (*e.g.*, linoleoyl, linolenoyl, γ -linolenoyl, phytanoyl, *etc.*). In some instances, the octadecadienyl moiety is a linoleyl moiety. In other instances, the octadecatrienyl moiety is a linolenyl moiety or a γ -linolenyl moiety. In particular embodiments, R^4 and R^5 are both linoleyl moieties, linolenyl moieties, γ -linolenyl moieties, or phytanyl moieties.

[0188] The alkylamino head group of Formula IX may be attached to the '4' or '5' position of the 6-membered ring as shown below in an exemplary embodiment wherein R^1 and R^2 are both methyl groups:

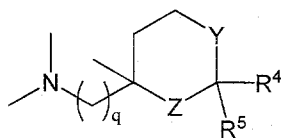


Head Group at '4' Position



Head Group at '5' Position

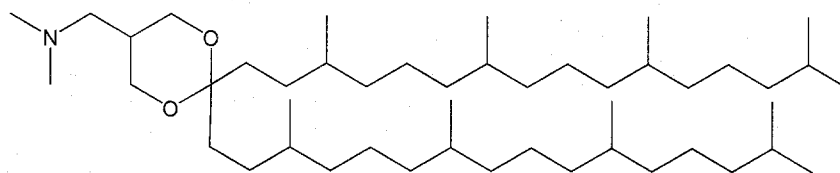
[0189] In further embodiments, the 6-membered ring of Formula IX may be substituted with 1, 2, 3, 4, or 5 independently selected C_1 - C_6 alkyl, C_2 - C_6 alkenyl, C_2 - C_6 alkynyl, C_1 - C_6 alkoxy, or hydroxyl substituents. In one particular embodiment, the 6-membered ring is substituted with 1, 2, 3, 4, or 5 independently selected C_1 - C_4 alkyl (*e.g.*, methyl, ethyl, propyl, or butyl) substituents. An exemplary embodiment of a cationic lipid of Formula IX having a substituted 6-membered ring (methyl group attached to the '4' position) and wherein R^1 and R^2 are both methyl groups is shown below:



[0190] In particular embodiments, the cationic lipids of Formula IX may be synthesized using 2-hydroxymethyl-1,4-butanediol and 1,3,5-pentanetriol (or 3-methyl-1,3,5-pentanetriol) as starting materials.

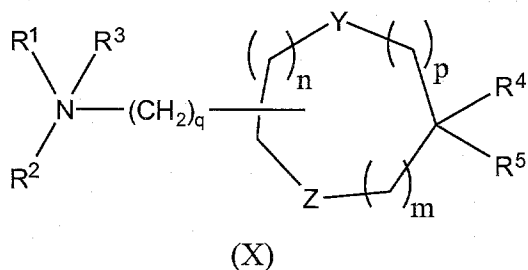
5 [0191] In some embodiments, the cationic lipid of Formula IX forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula IX is the oxalate (*e.g.*, hemioxalate) salt thereof, which is preferably a crystalline salt.

10 [0192] In a particularly preferred embodiment, the cationic lipid of Formula IX has the structure:



DPan-C1K6-DMA

[0193] In still yet another aspect, the present invention provides a cationic lipid of Formula X having the following structure:



20 or salts thereof, wherein:

R^1 and R^2 are either the same or different and are independently an optionally substituted C_1 - C_6 alkyl, C_2 - C_6 alkenyl, or C_2 - C_6 alkynyl, or R^1 and R^2 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

25 R^3 is either absent or is hydrogen (H) or a C_1 - C_6 alkyl to provide a quaternary amine;

R^4 and R^5 are either the same or different and are independently an optionally substituted C_{12} - C_{24} alkyl, C_{12} - C_{24} alkenyl, C_{12} - C_{24} alkynyl, or C_{12} - C_{24} acyl, wherein at least one of R^4 and R^5 comprises at least one site of unsaturation in the *trans* (*E*) configuration;

m, n, and p are either the same or different and are independently either 0, 1, or 2, with the proviso that m, n, and p are not simultaneously 0;

q is 0, 1, 2, 3, or 4; and

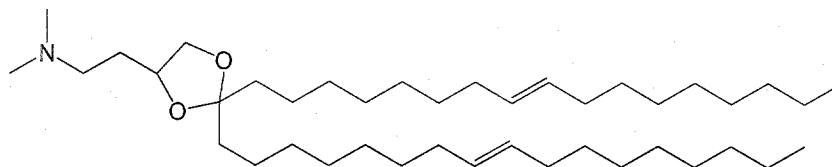
Y and Z are either the same or different and are independently O, S, or NH.

5 [0194] In some embodiments, R^1 and R^2 are independently an optionally substituted C_1 - C_4 alkyl, C_2 - C_4 alkenyl, or C_2 - C_4 alkynyl. In a preferred embodiment, R^1 and R^2 are both methyl groups. In another preferred embodiment, q is 2. In other embodiments, R^3 is absent when the pH is above the pK_a of the cationic lipid and R^3 is hydrogen when the pH is below the pK_a of the cationic lipid such that the amino head group is protonated. In an alternative
10 embodiment, R^3 is an optionally substituted C_1 - C_4 alkyl to provide a quaternary amine. In further embodiments, R^4 and R^5 are independently an optionally substituted C_{12} - C_{20} or C_{14} - C_{22} alkyl, C_{12} - C_{20} or C_{14} - C_{22} alkenyl, C_{12} - C_{20} or C_{14} - C_{22} alkynyl, or C_{12} - C_{20} or C_{14} - C_{22} acyl.

[0195] In certain embodiments, at least one of R^4 and R^5 further comprises one, two, three, four, five, six, or more sites of unsaturation in the *cis* and/or *trans* configuration. In some
15 instances, R^4 and R^5 are independently selected from any of the substituted or unsubstituted alkyl or acyl groups described herein, wherein at least one or both of R^4 and R^5 comprises at least one, two, three, four, five, or six sites of unsaturation in the *trans* configuration. In one particular embodiment, R^4 and R^5 independently comprise a backbone of from about 12 to about 22 carbon atoms (e.g., 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or 22 carbon atoms), and
20 one or both of R^4 and R^5 independently comprise at least one, two, three, four, five, or six sites of unsaturation in the *trans* configuration. In some preferred embodiments, at least one of R^4 and R^5 comprises an (*E*)-heptadeceyl moiety. In other preferred embodiments, R^4 and R^5 are both (*E*)-8-heptadeceyl moieties.

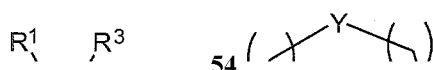
[0196] In some embodiments, the cationic lipid of Formula X forms a salt (preferably a
25 crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula X is the oxalate (e.g., hemioxalate) salt thereof, which is preferably a crystalline salt.

[0197] In a particularly preferred embodiment, the cationic lipid of Formula X has the structure:



DHep-C2K-DMA

[0198] In another aspect, the present invention provides a cationic lipid of Formula XI having the following structure:



(XI)

5 or salts thereof, wherein:

R^1 and R^2 are joined to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

10 R^3 is either absent or is hydrogen (H) or a C_1 - C_6 alkyl to provide a quaternary amine;

R^4 and R^5 are either the same or different and are independently an optionally substituted C_{12} - C_{24} alkyl, C_{12} - C_{24} alkenyl, C_{12} - C_{24} alkynyl, or C_{12} - C_{24} acyl;

m, n, and p are either the same or different and are independently either 0, 1, or 2, with the proviso that m, n, and p are not simultaneously 0;

15 q is 0, 1, 2, 3, or 4; and

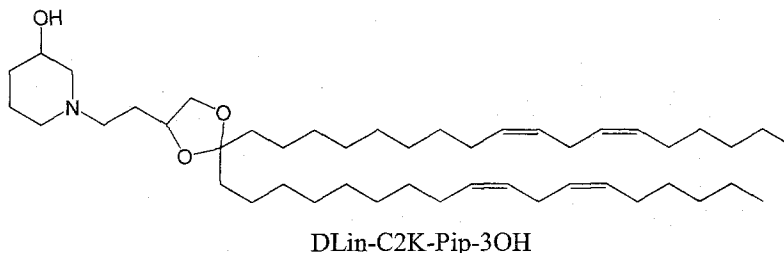
Y and Z are either the same or different and are independently O, S, or NH.

[0199] In some embodiments, R^1 and R^2 are joined to form a heterocyclic ring of 5 carbon atoms and 1 nitrogen atom. In certain instances, the heterocyclic ring is substituted with a substituent such as a hydroxyl group at the ortho, meta, and/or para positions. In a preferred
 20 embodiment, q is 2. In other embodiments, R^3 is absent when the pH is above the pK_a of the cationic lipid and R^3 is hydrogen when the pH is below the pK_a of the cationic lipid such that the amino head group is protonated. In an alternative embodiment, R^3 is an optionally substituted C_1 - C_4 alkyl to provide a quaternary amine. In further embodiments, R^4 and R^5 are independently an optionally substituted C_{12} - C_{20} or C_{14} - C_{22} alkyl, C_{12} - C_{20} or C_{14} - C_{22} alkenyl,
 25 C_{12} - C_{20} or C_{14} - C_{22} alkynyl, or C_{12} - C_{20} or C_{14} - C_{22} acyl.

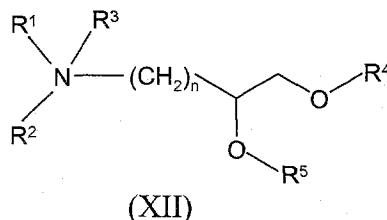
[0200] In certain embodiments, R^4 and R^5 are independently selected from the group consisting of a dodecadienyl moiety, a tetradecadienyl moiety, a hexadecadienyl moiety, an octadecadienyl moiety, an icosadienyl moiety, a dodecatrienyl moiety, a tetradectrienyl moiety, a hexadecatrienyl moiety, an octadecatrienyl moiety, an icosatrienyl moiety, and a
 30 branched alkyl group as described above (e.g., a phytanyl moiety), as well as acyl derivatives thereof (e.g., linoleoyl, linolenoyl, γ -linolenoyl, phytanoyl, etc.). In some instances, the octadecadienyl moiety is a linoleyl moiety. In other instances, the octadecatrienyl moiety is a linolenyl moiety or a γ -linolenyl moiety. In particular embodiments, R^4 and R^5 are both linoleyl moieties, linolenyl moieties, γ -linolenyl moieties, or phytanyl moieties.

[0201] In some embodiments, the cationic lipid of Formula XI forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula XI is the oxalate (*e.g.*, hemioxalate) salt thereof, which is preferably a crystalline salt.

5 [0202] In a particularly preferred embodiment, the cationic lipid of Formula XI has the structure:



[0203] In yet another aspect, the present invention provides a cationic lipid of Formula XII having the following structure:



or salts thereof, wherein:

R^1 and R^2 are either the same or different and are independently an optionally substituted C_1 - C_6 alkyl, C_2 - C_6 alkenyl, or C_2 - C_6 alkynyl, or R^1 and R^2 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

R^3 is either absent or is hydrogen (H) or a C_1 - C_6 alkyl to provide a quaternary amine;

R^4 and R^5 are either the same or different and are independently a substituted C_{12} - C_{24} alkyl; and

n is 0, 1, 2, 3, or 4.

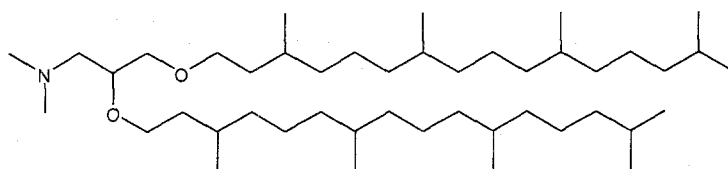
[0204] In some embodiments, R^1 and R^2 are independently an optionally substituted C_1 - C_4 alkyl, C_2 - C_4 alkenyl, or C_2 - C_4 alkynyl. In a preferred embodiment, R^1 and R^2 are both methyl groups. In one particular embodiment, n is 1. In another particular embodiment, n is 2. In other embodiments, R^3 is absent when the pH is above the pK_a of the cationic lipid and R^3 is hydrogen when the pH is below the pK_a of the cationic lipid such that the amino head group is protonated. In an alternative embodiment, R^3 is an optionally substituted C_1 - C_4 alkyl to provide a quaternary amine.

[0205] In embodiments where at least one of R^4 and R^5 comprises a branched alkyl group (e.g., a substituted C_{12} - C_{24} alkyl group), the branched alkyl group may comprise a C_{12} - C_{24} alkyl having at least 1-6 (e.g., 1, 2, 3, 4, 5, 6, or more) C_1 - C_6 alkyl substituents. In particular embodiments, the branched alkyl group comprises a C_{12} - C_{20} or C_{14} - C_{22} alkyl with 1-6 (e.g., 1, 2, 3, 4, 5, 6) C_1 - C_4 alkyl (e.g., methyl, ethyl, propyl, or butyl) substituents. Preferably, the branched alkyl group comprises a phytanyl (3,7,11,15-tetramethyl-hexadecanyl) moiety. In particular embodiments, R^4 and R^5 are both phytanyl moieties.

[0206] In alternative embodiments, at least one of R^4 and R^5 comprises a branched acyl group (e.g., a substituted C_{12} - C_{24} acyl group). In certain instances, the branched acyl group may comprise a C_{12} - C_{24} acyl having at least 1-6 (e.g., 1, 2, 3, 4, 5, 6, or more) C_1 - C_6 alkyl substituents. In particular embodiments, the branched acyl group comprises a C_{12} - C_{20} or C_{14} - C_{22} acyl with 1-6 (e.g., 1, 2, 3, 4, 5, 6) C_1 - C_4 alkyl (e.g., methyl, ethyl, propyl, or butyl) substituents. Preferably, the branched acyl group comprises a phytanoyl (3,7,11,15-tetramethyl-hexadecanoyl) moiety. In particular embodiments, R^4 and R^5 are both phytanoyl moieties.

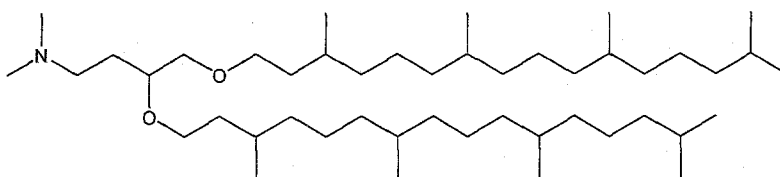
[0207] In some embodiments, the cationic lipid of Formula XII forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula XII is the oxalate (e.g., hemioxalate) salt thereof, which is preferably a crystalline salt.

[0208] In a particularly preferred embodiment, the cationic lipid of Formula XII has a structure selected from the group consisting of:



DPanDMA

and



C2-DPanDMA

[0209] The compounds of the invention may be prepared by known organic synthesis techniques, including the methods described in the Examples. In some embodiments, the synthesis of the cationic lipids of the invention may require the use of protecting groups. Protecting group methodology is well known to those skilled in the art (see, e.g., 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 the 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

5 removed to reveal the original functional group. In certain instances, an "alcohol protecting group" is used. An "alcohol protecting group" is any group which decreases or eliminates the unwanted reactivity of an alcohol functional group. Protecting groups can be added and removed using techniques well known in the art.

[0210] In certain embodiments, the cationic lipids of the present invention have at least one
10 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 be understood by one of ordinary skill in the art 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
15 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.

[0211] In certain other embodiments, protonatable lipids according to the invention have a pK_a of the protonatable group in the range of about 4 to about 11. Most preferred is a pK_a of
20 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 of around pH 7.4. One of the benefits of this pK_a 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
25 susceptibility to clearance.

IV. Active Agents

[0212] Active agents (e.g., therapeutic agents) include any molecule or compound capable of exerting a desired effect on a cell, tissue, organ, or subject. Such effects may be, e.g., biological, physiological, and/or cosmetic. Active agents may be any type of molecule or
30 compound including, but not limited to, nucleic acids, peptides, polypeptides, small molecules, and mixtures thereof. Non-limiting examples of nucleic acids include interfering RNA molecules (e.g., siRNA, Dicer-substrate dsRNA, shRNA, aiRNA, and/or miRNA), antisense oligonucleotides, plasmids, ribozymes, immunostimulatory oligonucleotides, and mixtures thereof. Examples of peptides or polypeptides include, without limitation,

antibodies (*e.g.*, polyclonal antibodies, monoclonal antibodies, antibody fragments; humanized antibodies, recombinant antibodies, recombinant human antibodies, and/or Primatized™ antibodies), cytokines, growth factors, apoptotic factors, differentiation-inducing factors, cell-surface receptors and their ligands, hormones, and mixtures thereof.

- 5 Examples of small molecules include, but are not limited to, small organic molecules or compounds such as any conventional agent or drug known to those of skill in the art.

[0213] In some embodiments, 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
10 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 is a prodrug that lacks therapeutic activity, but becomes active upon further modification.

A. Nucleic Acids

[0214] In certain embodiments, lipid particles of the present invention are associated with a
15 nucleic acid, resulting in a nucleic acid-lipid particle (*e.g.*, SNALP). In some embodiments, the nucleic acid is fully encapsulated in the lipid particle. As used herein, the term “nucleic acid” includes any oligonucleotide or polynucleotide, with fragments containing up to 60 nucleotides generally termed oligonucleotides, and longer fragments termed polynucleotides. In particular embodiments, oligonucleotides of the invention are from about 15 to about 60
20 nucleotides in length. Nucleic acid may be administered alone in the lipid particles of the invention, or in combination (*e.g.*, co-administered) with lipid particles of the invention comprising peptides, polypeptides, or small molecules such as conventional drugs.

[0215] 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-
25 occurring bases, sugars and intersugar (backbone) linkages. The terms “polynucleotide” and “oligonucleotide” also include 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, reduced immunogenicity, and increased stability in the
30 presence of nucleases.

[0216] Oligonucleotides are generally 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.

[0217] The nucleic acid that is present in a nucleic acid-lipid 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 are described herein and include, *e.g.*, structural genes, genes including control and termination regions, and self-replicating systems such as viral or plasmid DNA. Examples of double-stranded RNA are described herein and include, *e.g.*, siRNA and other RNAi agents such as Dicer-substrate dsRNA, shRNA, aiRNA, and pre-miRNA. Single-stranded nucleic acids include, *e.g.*, antisense oligonucleotides, ribozymes, mature miRNA, and triplex-forming oligonucleotides. In further embodiments, the nucleic acids are double-stranded DNA. Examples of double-stranded DNA include, *e.g.*, DNA-DNA hybrids comprising a DNA sense strand and a DNA antisense strand as described in PCT Publication No. WO 2004/104199, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0218] Nucleic acids of the 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 about 100,000 nucleotide residues in length. In particular embodiments, oligonucleotides may range from about 10 to about 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 60 nucleotides, from about 15 to about 60 nucleotides, from about 20 to about 50 nucleotides, from about 15 to about 30 nucleotides, or from about 20 to about 30 nucleotides in length.

[0219] In particular embodiments, an oligonucleotide (or a strand thereof) of the invention specifically hybridizes to or is complementary to a target polynucleotide sequence. The terms "specifically hybridizable" and "complementary" as used herein 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. In preferred embodiments, an oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target sequence interferes with the normal function of the target sequence 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, the oligonucleotide may include 1, 2, 3, or more base substitutions as compared to the region of a gene or mRNA sequence that it is targeting or to which it specifically hybridizes.

1. siRNA

5 [0220] The siRNA component of the nucleic acid-lipid particles of the present invention is capable of silencing the expression of a target gene of interest. Each strand of the siRNA duplex is typically about 15 to about 60 nucleotides in length, preferably about 15 to about 30 nucleotides in length. In certain embodiments, the siRNA comprises at least one modified nucleotide. The modified siRNA is generally less immunostimulatory than a corresponding
10 unmodified siRNA sequence and retains RNAi activity against the target gene of interest. In some embodiments, the modified siRNA contains at least one 2'OMe purine or pyrimidine nucleotide such as a 2'OMe-guanosine, 2'OMe-uridine, 2'OMe-adenosine, and/or 2'OMe-cytosine nucleotide. The modified nucleotides can be present in one strand (*i.e.*, sense or antisense) or both strands of the siRNA. In some preferred embodiments, one or more of the
15 uridine and/or guanosine nucleotides are modified (*e.g.*, 2'OMe-modified) in one strand (*i.e.*, sense or antisense) or both strands of the siRNA. In these embodiments, the modified siRNA can further comprise one or more modified (*e.g.*, 2'OMe-modified) adenosine and/or modified (*e.g.*, 2'OMe-modified) cytosine nucleotides. In other preferred embodiments, only uridine and/or guanosine nucleotides are modified (*e.g.*, 2'OMe-modified) in one strand (*i.e.*,
20 sense or antisense) or both strands of the siRNA. The siRNA sequences may have overhangs (*e.g.*, 3' or 5' overhangs as described in Elbashir *et al.*, *Genes Dev.*, 15:188 (2001) or Nykänen *et al.*, *Cell*, 107:309 (2001)), or may lack overhangs (*i.e.*, have blunt ends).

[0221] In particular embodiments, the selective incorporation of modified nucleotides such as 2'OMe uridine and/or guanosine nucleotides into the double-stranded region of either or
25 both strands of the siRNA reduces or completely abrogates the immune response to that siRNA molecule. In certain instances, the immunostimulatory properties of specific siRNA sequences and their ability to silence gene expression can be balanced or optimized by the introduction of minimal and selective 2'OMe modifications within the double-stranded region of the siRNA duplex. This can be achieved at therapeutically viable siRNA doses
30 without cytokine induction, toxicity, and off-target effects associated with the use of unmodified siRNA.

[0222] The modified siRNA generally comprises from about 1% to about 100% (*e.g.*, about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 35%, 40%, 45%, 50%,

55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%) modified nucleotides in the double-stranded region of the siRNA duplex. In certain embodiments, one, two, three, four, five, six, seven, eight, nine, ten, or more of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides. In certain other embodiments, some or all of the modified nucleotides in the double-stranded region of the siRNA are 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more nucleotides apart from each other. In one preferred embodiment, none of the modified nucleotides in the double-stranded region of the siRNA are adjacent to each other (*e.g.*, there is a gap of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 unmodified nucleotides between each modified nucleotide).

10 **[0223]** In some embodiments, less than about 50% (*e.g.*, less than about 49%, 48%, 47%, 46%, 45%, 44%, 43%, 42%, 41%, 40%, 39%, 38%, 37%, or 36%, preferably less than about 35%, 34%, 33%, 32%, 31%, or 30%) of the nucleotides in the double-stranded region of the siRNA comprise modified (*e.g.*, 2'OMe) nucleotides. In one aspect of these embodiments, less than about 50% of the uridine and/or guanosine nucleotides in the double-stranded region of one or both strands of the siRNA are selectively (*e.g.*, only) modified. In another aspect of these embodiments, less than about 50% of the nucleotides in the double-stranded region of the siRNA comprise 2'OMe nucleotides, wherein the siRNA comprises 2'OMe nucleotides in both strands of the siRNA, wherein the siRNA comprises at least one 2'OMe-guanosine nucleotide and at least one 2'OMe-uridine nucleotide, and wherein 2'OMe-guanosine nucleotides and 2'OMe-uridine nucleotides are the only 2'OMe nucleotides present in the double-stranded region. In yet another aspect of these embodiments, less than about 50% of the nucleotides in the double-stranded region of the siRNA comprise 2'OMe nucleotides, wherein the siRNA comprises 2'OMe nucleotides in both strands of the modified siRNA, wherein the siRNA comprises 2'OMe nucleotides selected from the group consisting of 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, and mixtures thereof, and wherein the siRNA does not comprise 2'OMe-cytosine nucleotides in the double-stranded region. In a further aspect of these embodiments, less than about 50% of the nucleotides in the double-stranded region of the siRNA comprise 2'OMe nucleotides, wherein the siRNA comprises 2'OMe nucleotides in both strands of the siRNA, wherein the siRNA comprises at least one 2'OMe-guanosine nucleotide and at least one 2'OMe-uridine nucleotide, and wherein the siRNA does not comprise 2'OMe-cytosine nucleotides in the double-stranded region. In another aspect of these embodiments, less than about 50% of the nucleotides in the double-stranded region of the siRNA comprise 2'OMe nucleotides, wherein the siRNA comprises 2'OMe nucleotides in both strands of the modified siRNA, wherein the siRNA comprises 2'OMe nucleotides selected from the group consisting of

2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, and mixtures thereof, and wherein the 2'OMe nucleotides in the double-stranded region are not adjacent to each other.

[0224] In other embodiments, from about 1% to about 50% (*e.g.*, from about 5%-50%,

- 5 10%-50%, 15%-50%, 20%-50%, 25%-50%, 30%-50%, 35%-50%, 40%-50%, 45%-50%, 5%-45%, 10%-45%, 15%-45%, 20%-45%, 25%-45%, 30%-45%, 35%-45%, 40%-45%, 5%-40%, 10%-40%, 15%-40%, 20%-40%, 25%-40%, 25%-39%, 25%-38%, 25%-37%, 25%-36%, 26%-39%, 26%-38%, 26%-37%, 26%-36%, 27%-39%, 27%-38%, 27%-37%, 27%-36%, 28%-39%, 28%-38%, 28%-37%, 28%-36%, 29%-39%, 29%-38%, 29%-37%, 29%-36%, 30%-40%, 30%-39%, 30%-38%, 30%-37%, 30%-36%, 31%-39%, 31%-38%, 31%-37%, 31%-36%, 32%-39%, 32%-38%, 32%-37%, 32%-36%, 33%-39%, 33%-38%, 33%-37%, 33%-36%, 34%-39%, 34%-38%, 34%-37%, 34%-36%, 35%-40%, 5%-35%, 10%-35%, 15%-35%, 20%-35%, 21%-35%, 22%-35%, 23%-35%, 24%-35%, 25%-35%, 26%-35%, 27%-35%, 28%-35%, 29%-35%, 30%-35%, 31%-35%, 32%-35%, 33%-35%, 34%-35%, 30%-34%, 31%-34%, 32%-34%, 33%-34%, 30%-33%, 31%-33%, 32%-33%, 30%-32%, 31%-32%, 25%-34%, 25%-33%, 25%-32%, 25%-31%, 26%-34%, 26%-33%, 26%-32%, 26%-31%, 27%-34%, 27%-33%, 27%-32%, 27%-31%, 28%-34%, 28%-33%, 28%-32%, 28%-31%, 29%-34%, 29%-33%, 29%-32%, 29%-31%, 5%-30%, 10%-30%, 15%-30%, 20%-34%, 20%-33%, 20%-32%, 20%-31%, 20%-30%, 21%-30%, 22%-30%, 23%-30%, 24%-30%, 25%-30%, 25%-29%, 25%-28%, 25%-27%, 25%-26%, 26%-30%, 26%-29%, 26%-28%, 26%-27%, 27%-30%, 27%-29%, 27%-28%, 28%-30%, 28%-29%, 29%-30%, 5%-25%, 10%-25%, 15%-25%, 20%-29%, 20%-28%, 20%-27%, 20%-26%, 20%-25%, 5%-20%, 10%-20%, 15%-20%, 5%-15%, 10%-15%, or 5%-10%) of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides. In one aspect of these
- 25 embodiments, from about 1% to about 50% of the uridine and/or guanosine nucleotides in the double-stranded region of one or both strands of the siRNA are selectively (*e.g.*, only) modified. In another aspect of these embodiments, from about 1% to about 50% of the nucleotides in the double-stranded region of the siRNA comprise 2'OMe nucleotides, wherein the siRNA comprises 2'OMe nucleotides in both strands of the siRNA, wherein the
- 30 siRNA comprises at least one 2'OMe-guanosine nucleotide and at least one 2'OMe-uridine nucleotide, and wherein 2'OMe-guanosine nucleotides and 2'OMe-uridine nucleotides are the only 2'OMe nucleotides present in the double-stranded region. In yet another aspect of these embodiments, from about 1% to about 50% of the nucleotides in the double-stranded region of the siRNA comprise 2'OMe nucleotides, wherein the siRNA comprises 2'OMe
- 35 nucleotides in both strands of the modified siRNA, wherein the siRNA comprises 2'OMe

nucleotides selected from the group consisting of 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, and mixtures thereof, and wherein the siRNA does not comprise 2'OMe-cytosine nucleotides in the double-stranded region. In a further aspect of these embodiments, from about 1% to about 50% of the nucleotides in the double-stranded region of the siRNA comprise 2'OMe nucleotides, wherein the siRNA comprises 2'OMe nucleotides in both strands of the siRNA, wherein the siRNA comprises at least one 2'OMe-guanosine nucleotide and at least one 2'OMe-uridine nucleotide, and wherein the siRNA does not comprise 2'OMe-cytosine nucleotides in the double-stranded region. In another aspect of these embodiments, from about 1% to about 50% of the nucleotides in the double-stranded region of the siRNA comprise 2'OMe nucleotides, wherein the siRNA comprises 2'OMe nucleotides in both strands of the modified siRNA, wherein the siRNA comprises 2'OMe nucleotides selected from the group consisting of 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, and mixtures thereof, and wherein the 2'OMe nucleotides in the double-stranded region are not adjacent to each other.

[0225] In certain embodiments, the siRNA component of the nucleic acid-lipid particles of the present invention (*e.g.*, SNALP) comprises an asymmetric siRNA duplex as described in PCT Publication No. WO 2004/078941, which comprises a double-stranded region consisting of a DNA sense strand and an RNA antisense strand (*e.g.*, a DNA-RNA hybrid), wherein a blocking agent is located on the siRNA duplex. In some instances, the asymmetric siRNA duplex can be chemically modified as described herein. Other non-limiting examples of asymmetric siRNA duplexes are described in PCT Publication No. WO 2006/074108, which discloses self-protected oligonucleotides comprising a region having a sequence complementary to one, two, three, or more same or different target mRNA sequences (*e.g.*, multivalent siRNAs) and one or more self-complementary regions. Yet other non-limiting examples of asymmetric siRNA duplexes are described in PCT Publication No. WO 2009/076321, which discloses self-forming asymmetric precursor polynucleotides comprising a targeting region comprising a polynucleotide sequence complementary to a region of one, two, three, or more same or different target mRNA sequences (*e.g.*, multivalent siRNAs); a first self-complementary region; and a second self-complementary region, wherein the first and second self-complementary regions are located one at each end of the targeting region and both self-complementary regions form stem-loop structures, wherein the first self-complementary region is capable of being cleaved by a RNase III endoribonuclease that is not a class IV DICER endoribonuclease, and wherein both self-complementary regions comprise a nucleotide sequence that is complementary to a region of the target gene

sequence, but wherein a portion of the target sequence present in the targeting region does not have a complementary sequence in either of the self-complementary regions. The disclosures of each of the above patent documents are herein incorporated by reference in their entirety for all purposes.

- 5 [0226] Additional ranges, percentages, and patterns of modifications that may be introduced into siRNA are described in U.S. Patent Publication No. 20070135372, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

a) Selection of siRNA Sequences

[0227] Suitable siRNA sequences can be identified using any means known in the art.

- 10 Typically, the methods described in Elbashir *et al.*, *Nature*, 411:494-498 (2001) and Elbashir *et al.*, *EMBO J.*, 20:6877-6888 (2001) are combined with rational design rules set forth in Reynolds *et al.*, *Nature Biotech.*, 22(3):326-330 (2004).

- [0228] As a non-limiting example, the nucleotide sequence 3' of the AUG start codon of a transcript from the target gene of interest may be scanned for dinucleotide sequences (*e.g.*,
15 AA, NA, CC, GG, or UU, wherein N = C, G, or U) (*see, e.g.*, Elbashir *et al.*, *EMBO J.*, 20:6877-6888 (2001)). The nucleotides immediately 3' to the dinucleotide sequences are identified as potential siRNA sequences (*i.e.*, a target sequence or a sense strand sequence). Typically, the 19, 21, 23, 25, 27, 29, 31, 33, 35, or more nucleotides immediately 3' to the dinucleotide sequences are identified as potential siRNA sequences. In some embodiments,
20 the dinucleotide sequence is an AA or NA sequence and the 19 nucleotides immediately 3' to the AA or NA dinucleotide are identified as potential siRNA sequences. siRNA sequences are usually spaced at different positions along the length of the target gene. To further enhance silencing efficiency of the siRNA sequences, potential siRNA sequences may be analyzed to identify sites that do not contain regions of homology to other coding sequences,
25 *e.g.*, in the target cell or organism. For example, a suitable siRNA sequence of about 21 base pairs typically will not have more than 16-17 contiguous base pairs of homology to coding sequences in the target cell or organism. If the siRNA sequences are to be expressed from an RNA Pol III promoter, siRNA sequences lacking more than 4 contiguous A's or T's are selected.

- 30 [0229] Once a potential siRNA sequence has been identified, a complementary sequence (*i.e.*, an antisense strand sequence) can be designed. A potential siRNA sequence can also be analyzed using a variety of criteria known in the art. For example, to enhance their silencing efficiency, the siRNA sequences may be analyzed by a rational design algorithm to identify sequences that have one or more of the following features: (1) G/C content of about 25% to

about 60% G/C; (2) at least 3 A/Us at positions 15-19 of the sense strand; (3) no internal repeats; (4) an A at position 19 of the sense strand; (5) an A at position 3 of the sense strand; (6) a U at position 10 of the sense strand; (7) no G/C at position 19 of the sense strand; and (8) no G at position 13 of the sense strand. siRNA design tools that incorporate algorithms that assign suitable values of each of these features and are useful for selection of siRNA can be found at, *e.g.*, <http://ihome.ust.hk/~bokcmho/siRNA/siRNA.html>. One of skill in the art will appreciate that sequences with one or more of the foregoing characteristics may be selected for further analysis and testing as potential siRNA sequences.

[0230] Additionally, potential siRNA sequences with one or more of the following criteria can often be eliminated as siRNA: (1) sequences comprising a stretch of 4 or more of the same base in a row; (2) sequences comprising homopolymers of Gs (*i.e.*, to reduce possible non-specific effects due to structural characteristics of these polymers; (3) sequences comprising triple base motifs (*e.g.*, GGG, CCC, AAA, or TTT); (4) sequences comprising stretches of 7 or more G/Cs in a row; and (5) sequences comprising direct repeats of 4 or more bases within the candidates resulting in internal fold-back structures. However, one of skill in the art will appreciate that sequences with one or more of the foregoing characteristics may still be selected for further analysis and testing as potential siRNA sequences.

[0231] In some embodiments, potential siRNA sequences may be further analyzed based on siRNA duplex asymmetry as described in, *e.g.*, Khvorova *et al.*, *Cell*, 115:209-216 (2003); and Schwarz *et al.*, *Cell*, 115:199-208 (2003). In other embodiments, potential siRNA sequences may be further analyzed based on secondary structure at the target site as described in, *e.g.*, Luo *et al.*, *Biophys. Res. Commun.*, 318:303-310 (2004). For example, secondary structure at the target site can be modeled using the Mfold algorithm (available at http://mfold.burnet.edu.au/rna_form) to select siRNA sequences which favor accessibility at the target site where less secondary structure in the form of base-pairing and stem-loops is present.

[0232] Once a potential siRNA sequence has been identified, the sequence can be analyzed for the presence of any immunostimulatory properties, *e.g.*, using an *in vitro* cytokine assay or an *in vivo* animal model. Motifs in the sense and/or antisense strand of the siRNA sequence such as GU-rich motifs (*e.g.*, 5'-GU-3', 5'-UGU-3', 5'-GUGU-3', 5'-UGUGU-3', *etc.*) can also provide an indication of whether the sequence may be immunostimulatory.

Once an siRNA molecule is found to be immunostimulatory, it can then be modified to decrease its immunostimulatory properties as described herein. As a non-limiting example, an siRNA sequence can be contacted with a mammalian responder cell under conditions such that the cell produces a detectable immune response to determine whether the siRNA is an

immunostimulatory or a non-immunostimulatory siRNA. The mammalian responder cell may be from a naïve mammal (*i.e.*, a mammal that has not previously been in contact with the gene product of the siRNA sequence). The mammalian responder cell may be, *e.g.*, a peripheral blood mononuclear cell (PBMC), a macrophage, and the like. The detectable immune response may comprise production of a cytokine or growth factor such as, *e.g.*, TNF- α , IFN- α , IFN- β , IFN- γ , IL-6, IL-8, IL-12, or a combination thereof. An siRNA molecule identified as being immunostimulatory can then be modified to decrease its immunostimulatory properties by replacing at least one of the nucleotides on the sense and/or antisense strand with modified nucleotides. For example, less than about 30% (*e.g.*, less than about 30%, 25%, 20%, 15%, 10%, or 5%) of the nucleotides in the double-stranded region of the siRNA duplex can be replaced with modified nucleotides such as 2'OMe nucleotides. The modified siRNA can then be contacted with a mammalian responder cell as described above to confirm that its immunostimulatory properties have been reduced or abrogated.

[0233] Suitable *in vitro* assays for detecting an immune response include, but are not limited to, the double monoclonal antibody sandwich immunoassay technique of David *et al.* (U.S. Patent No. 4,376,110); monoclonal-polyclonal antibody sandwich assays (Wide *et al.*, in Kirkham and Hunter, eds., *Radioimmunoassay Methods*, E. and S. Livingstone, Edinburgh (1970)); the "Western blot" method of Gordon *et al.* (U.S. Patent No. 4,452,901); immunoprecipitation of labeled ligand (Brown *et al.*, *J. Biol. Chem.*, 255:4980-4983 (1980)); enzyme-linked immunosorbent assays (ELISA) as described, for example, by Raines *et al.*, *J. Biol. Chem.*, 257:5154-5160 (1982); immunocytochemical techniques, including the use of fluorochromes (Brooks *et al.*, *Clin. Exp. Immunol.*, 39:477 (1980)); and neutralization of activity (Bowen-Pope *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:2396-2400 (1984)). In addition to the immunoassays described above, a number of other immunoassays are available, including those described in U.S. Patent Nos. 3,817,827; 3,850,752; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; and 4,098,876. The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0234] A non-limiting example of an *in vivo* model for detecting an immune response includes an *in vivo* mouse cytokine induction assay as described in, *e.g.*, Judge *et al.*, *Mol. Ther.*, 13:494-505 (2006). In certain embodiments, the assay that can be performed as follows: (1) siRNA can be administered by standard intravenous injection in the lateral tail vein; (2) blood can be collected by cardiac puncture about 6 hours after administration and processed as plasma for cytokine analysis; and (3) cytokines can be quantified using sandwich ELISA kits according to the manufacturer's instructions (*e.g.*, mouse and human

IFN- α (PBL Biomedical; Piscataway, NJ); human IL-6 and TNF- α (eBioscience; San Diego, CA); and mouse IL-6, TNF- α , and IFN- γ (BD Biosciences; San Diego, CA)).

[0235] Monoclonal antibodies that specifically bind cytokines and growth factors are commercially available from multiple sources and can be generated using methods known in the art (*see, e.g., Kohler et al., Nature*, 256: 495-497 (1975) and Harlow and Lane, ANTIBODIES, A LABORATORY MANUAL, Cold Spring Harbor Publication, New York (1999)). Generation of monoclonal antibodies has been previously described and can be accomplished by any means known in the art (Buhring *et al.*, in Hybridoma, Vol. 10, No. 1, pp. 77-78 (1991)). In some methods, the monoclonal antibody is labeled (*e.g., with any composition detectable by spectroscopic, photochemical, biochemical, electrical, optical, or chemical means*) to facilitate detection.

b) Generating siRNA Molecules

[0236] siRNA can be provided in several forms including, *e.g., as one or more isolated small-interfering RNA (siRNA) duplexes, as longer double-stranded RNA (dsRNA), or as siRNA or dsRNA transcribed from a transcriptional cassette in a DNA plasmid. In some embodiments, siRNA may be produced enzymatically or by partial/total organic synthesis, and modified ribonucleotides can be introduced by in vitro enzymatic or organic synthesis. In certain instances, each strand is prepared chemically. Methods of synthesizing RNA molecules are known in the art, e.g., the chemical synthesis methods as described in Verma and Eckstein (1998) or as described herein.*

[0237] An RNA population can be used to provide long precursor RNAs, or long precursor RNAs that have substantial or complete identity to a selected target sequence can be used to make the siRNA. The RNAs can be isolated from cells or tissue, synthesized, and/or cloned according to methods well known to those of skill in the art. The RNA can be a mixed population (obtained from cells or tissue, transcribed from cDNA, subtracted, selected, *etc.*), or can represent a single target sequence. RNA can be naturally occurring (*e.g., isolated from tissue or cell samples*), synthesized *in vitro* (*e.g., using T7 or SP6 polymerase and PCR products or a cloned cDNA*), or chemically synthesized.

[0238] To form a long dsRNA, for synthetic RNAs, the complement is also transcribed *in vitro* and hybridized to form a dsRNA. If a naturally occurring RNA population is used, the RNA complements are also provided (*e.g., to form dsRNA for digestion by E. coli RNase III or Dicer*), *e.g., by transcribing cDNAs corresponding to the RNA population, or by using RNA polymerases. The precursor RNAs are then hybridized to form double stranded RNAs*

for digestion. The dsRNAs can be directly administered to a subject or can be digested *in vitro* prior to administration.

[0239] Methods for isolating RNA, synthesizing RNA, hybridizing nucleic acids, making and screening cDNA libraries, and performing PCR are well known in the art (*see, e.g.,*

5 Gubler and Hoffman, *Gene*, 25:263-269 (1983); Sambrook *et al., supra*; Ausubel *et al., supra*), as are PCR methods (*see, U.S. Patent Nos. 4,683,195 and 4,683,202; PCR Protocols: A Guide to Methods and Applications* (Innis *et al., eds*, 1990)). Expression libraries are also well known to those of skill in the art. Additional basic texts disclosing the general methods of use in this invention include Sambrook *et al., Molecular Cloning, A Laboratory Manual*
10 (2nd ed. 1989); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al., eds.*, 1994). The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0240] Preferably, siRNA are chemically synthesized. The oligonucleotides that comprise the siRNA molecules of the invention can be synthesized using any of a variety of techniques
15 known in the art, such as those described in Usman *et al., J. Am. Chem. Soc.*, 109:7845 (1987); Scaringe *et al., Nucl. Acids Res.*, 18:5433 (1990); Wincott *et al., Nucl. Acids Res.*, 23:2677-2684 (1995); and Wincott *et al., Methods Mol. Bio.*, 74:59 (1997). The synthesis of oligonucleotides makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end and phosphoramidites at the 3'-end. As a non-limiting example,
20 small scale syntheses can be conducted on an Applied Biosystems synthesizer using a 0.2 μ mol scale protocol. Alternatively, syntheses at the 0.2 μ mol scale can be performed on a 96-well plate synthesizer from Protogene (Palo Alto, CA). However, a larger or smaller scale of synthesis is also within the scope of this invention. Suitable reagents for oligonucleotide synthesis, methods for RNA deprotection, and methods for RNA purification are known to
25 those of skill in the art.

[0241] siRNA molecules can also be synthesized via a tandem synthesis technique, wherein both strands are synthesized as a single continuous oligonucleotide fragment or strand separated by a cleavable linker that is subsequently cleaved to provide separate fragments or strands that hybridize to form the siRNA duplex. The linker can be a polynucleotide linker or
30 a non-nucleotide linker. The tandem synthesis of siRNA can be readily adapted to both multiwell/multiplate synthesis platforms as well as large scale synthesis platforms employing batch reactors, synthesis columns, and the like. Alternatively, siRNA molecules can be assembled from two distinct oligonucleotides, wherein one oligonucleotide comprises the sense strand and the other comprises the antisense strand of the siRNA. For example, each
35 strand can be synthesized separately and joined together by hybridization or ligation

following synthesis and/or deprotection. In certain other instances, siRNA molecules can be synthesized as a single continuous oligonucleotide fragment, where the self-complementary sense and antisense regions hybridize to form an siRNA duplex having hairpin secondary structure.

5

c) Modifying siRNA Sequences

[0242] In certain aspects, siRNA molecules comprise a duplex having two strands and at least one modified nucleotide in the double-stranded region, wherein each strand is about 15 to about 60 nucleotides in length. Advantageously, the modified siRNA is less immunostimulatory than a corresponding unmodified siRNA sequence, but retains the capability of silencing the expression of a target sequence. In preferred embodiments, the degree of chemical modifications introduced into the siRNA molecule strikes a balance between reduction or abrogation of the immunostimulatory properties of the siRNA and retention of RNAi activity. As a non-limiting example, an siRNA molecule that targets a gene of interest can be minimally modified (e.g., less than about 30%, 25%, 20%, 15%, 10%, or 5% modified) at selective uridine and/or guanosine nucleotides within the siRNA duplex to eliminate the immune response generated by the siRNA while retaining its capability to silence target gene expression.

[0243] Examples of modified nucleotides suitable for use in the invention include, but are not limited to, ribonucleotides having a 2'-O-methyl (2'OMe), 2'-deoxy-2'-fluoro (2'F), 2'-deoxy, 5-C-methyl, 2'-O-(2-methoxyethyl) (MOE), 4'-thio, 2'-amino, or 2'-C-allyl group. Modified nucleotides having a Northern conformation such as those described in, e.g., Saenger, *Principles of Nucleic Acid Structure*, Springer-Verlag Ed. (1984), are also suitable for use in siRNA molecules. Such modified nucleotides include, without limitation, locked nucleic acid (LNA) nucleotides (e.g., 2'-O, 4'-C-methylene-(D-ribofuranosyl) nucleotides), 2'-O-(2-methoxyethyl) (MOE) nucleotides, 2'-methyl-thio-ethyl nucleotides, 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy-2'-chloro (2'Cl) nucleotides, and 2'-azido nucleotides. In certain instances, the siRNA molecules described herein include one or more G-clamp nucleotides. A G-clamp nucleotide refers to a modified cytosine analog wherein the modifications confer the ability to hydrogen bond both Watson-Crick and Hoogsteen faces of a complementary guanine nucleotide within a duplex (see, e.g., Lin *et al.*, *J. Am. Chem. Soc.*, 120:8531-8532 (1998)). In addition, nucleotides having a nucleotide base analog such as, for example, C-phenyl, C-naphthyl, other aromatic derivatives, inosine,azole carboxamides, and nitroazole derivatives such as 3-nitropyrrole, 4-nitroindole, 5-nitroindole, and 6-nitroindole

(see, e.g., Loakes, *Nucl. Acids Res.*, 29:2437-2447 (2001)) can be incorporated into siRNA molecules.

[0244] In certain embodiments, siRNA molecules may further comprise one or more chemical modifications such as terminal cap moieties, phosphate backbone modifications, and the like. Examples of terminal cap moieties include, without limitation, inverted deoxy abasic residues, glyceryl modifications, 4',5'-methylene nucleotides, 1-(β -D-erythrofuransyl) nucleotides, 4'-thio nucleotides, carbocyclic nucleotides, 1,5-anhydrohexitol nucleotides, L-nucleotides, α -nucleotides, modified base nucleotides, *threo*-pentofuransyl nucleotides, acyclic 3',4'-seco nucleotides, acyclic 3,4-dihydroxybutyl nucleotides, acyclic 3,5-dihydroxypentyl nucleotides, 3'-3'-inverted nucleotide moieties, 3'-3'-inverted abasic moieties, 3'-2'-inverted nucleotide moieties, 3'-2'-inverted abasic moieties, 5'-5'-inverted nucleotide moieties, 5'-5'-inverted abasic moieties, 3'-5'-inverted deoxy abasic moieties, 5'-amino-alkyl phosphate, 1,3-diamino-2-propyl phosphate, 3-aminopropyl phosphate, 6-aminoethyl phosphate, 1,2-aminododecyl phosphate, hydroxypropyl phosphate, 1,4-butanediol phosphate, 3'-phosphoramidate, 5'-phosphoramidate, hexylphosphate, aminohexyl phosphate, 3'-phosphate, 5'-amino, 3'-phosphorothioate, 5'-phosphorothioate, phosphorodithioate, and bridging or non-bridging methylphosphonate or 5'-mercapto moieties (see, e.g., U.S. Patent No. 5,998,203; Beaucage *et al.*, *Tetrahedron* 49:1925 (1993)). Non-limiting examples of phosphate backbone modifications (*i.e.*, resulting in modified internucleotide linkages) include phosphorothioate, phosphorodithioate, methylphosphonate, phosphotriester, morpholino, amidate, carbamate, carboxymethyl, acetamidate, polyamide, sulfonate, sulfonamide, sulfamate, formacetal, thioformacetal, and alkylsilyl substitutions (see, e.g., Hunziker *et al.*, *Nucleic Acid Analogues: Synthesis and Properties*, in *Modern Synthetic Methods*, VCH, 331-417 (1995); Mesmaeker *et al.*, *Novel Backbone Replacements for Oligonucleotides*, in *Carbohydrate Modifications in Antisense Research*, ACS, 24-39 (1994)). Such chemical modifications can occur at the 5'-end and/or 3'-end of the sense strand, antisense strand, or both strands of the siRNA. The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0245] In some embodiments, the sense and/or antisense strand of the siRNA molecule can further comprise a 3'-terminal overhang having about 1 to about 4 (*e.g.*, 1, 2, 3, or 4) 2'-deoxy ribonucleotides, modified (*e.g.*, 2'OMe) and/or unmodified uridine ribonucleotides, and/or any other combination of modified (*e.g.*, 2'OMe) and unmodified nucleotides.

[0246] Additional examples of modified nucleotides and types of chemical modifications that can be introduced into siRNA molecules are described, *e.g.*, in UK Patent No. GB

2,397,818 B and U.S. Patent Publication Nos. 20040192626, 20050282188, and 20070135372, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0247] The siRNA molecules described herein can optionally comprise one or more non-nucleotides in one or both strands of the siRNA. As used herein, the term “non-nucleotide” refers to any group or compound that can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their activity. The group or compound is abasic in that it does not contain a commonly recognized nucleotide base such as adenosine, guanine, cytosine, uracil, or thymine and therefore lacks a base at the 1'-position.

[0248] In other embodiments, chemical modification of the siRNA comprises attaching a conjugate to the siRNA molecule. The conjugate can be attached at the 5' and/or 3'-end of the sense and/or antisense strand of the siRNA via a covalent attachment such as, *e.g.*, a biodegradable linker. The conjugate can also be attached to the siRNA, *e.g.*, through a carbamate group or other linking group (*see, e.g.*, U.S. Patent Publication Nos. 20050074771, 20050043219, and 20050158727). In certain instances, the conjugate is a molecule that facilitates the delivery of the siRNA into a cell. Examples of conjugate molecules suitable for attachment to siRNA include, without limitation, steroids such as cholesterol, glycols such as polyethylene glycol (PEG), human serum albumin (HSA), fatty acids, carotenoids, terpenes, bile acids, folates (*e.g.*, folic acid, folate analogs and derivatives thereof), sugars (*e.g.*, galactose, galactosamine, N-acetyl galactosamine, glucose, mannose, fructose, fucose, *etc.*), phospholipids, peptides, ligands for cellular receptors capable of mediating cellular uptake, and combinations thereof (*see, e.g.*, U.S. Patent Publication Nos. 20030130186, 20040110296, and 20040249178; U.S. Patent No. 6,753,423). Other examples include the lipophilic moiety, vitamin, polymer, peptide, protein, nucleic acid, small molecule, oligosaccharide, carbohydrate cluster, intercalator, minor groove binder, cleaving agent, and cross-linking agent conjugate molecules described in U.S. Patent Publication Nos. 20050119470 and 20050107325. Yet other examples include the 2'-O-alkyl amine, 2'-O-alkoxyalkyl amine, polyamine, C5-cationic modified pyrimidine, cationic peptide, guanidinium group, amidinium group, cationic amino acid conjugate molecules described in U.S. Patent Publication No. 20050153337. Additional examples include the hydrophobic group, membrane active compound, cell penetrating compound, cell targeting signal, interaction modifier, and steric stabilizer conjugate molecules described in U.S. Patent Publication No. 20040167090. Further examples include the conjugate molecules described in U.S. Patent Publication No. 20050239739. The type of conjugate used and the extent of

conjugation to the siRNA molecule can be evaluated for improved pharmacokinetic profiles, bioavailability, and/or stability of the siRNA while retaining RNAi activity. As such, one skilled in the art can screen siRNA molecules having various conjugates attached thereto to identify ones having improved properties and full RNAi activity using any of a variety of well-known *in vitro* cell culture or *in vivo* animal models. The disclosures of the above-described patent documents are herein incorporated by reference in their entirety for all purposes.

d) Target Genes

[0249] The siRNA component of the nucleic acid-lipid particles described herein can be used to downregulate or silence the translation (*i.e.*, expression) of a gene of interest. Genes of interest include, but are not limited to, genes associated with viral infection and survival, genes associated with metabolic diseases and disorders (*e.g.*, liver diseases and disorders), genes associated with tumorigenesis or cell transformation (*e.g.*, cancer), angiogenic genes, immunomodulator genes such as those associated with inflammatory and autoimmune responses, receptor ligand genes, and genes associated with neurodegenerative disorders.

[0250] In particular embodiments, the present invention provides a cocktail of two, three, four, five, six, seven, eight, nine, ten, or more siRNA molecules that silences the expression of multiple genes of interest. In some embodiments, the cocktail of siRNA molecules is fully encapsulated in a lipid particle such as a nucleic acid-lipid particle (*e.g.*, SNALP). The siRNA molecules may be co-encapsulated in the same lipid particle, or each siRNA species present in the cocktail may be formulated in separate particles.

[0251] Genes associated with viral infection and survival include those expressed by a host (*e.g.*, a host factor such as tissue factor (TF)) or a virus in order to bind, enter, and replicate in a cell. Of particular interest are viral sequences associated with chronic viral diseases. Viral sequences of particular interest include sequences of Filoviruses such as Ebola virus and Marburg virus (*see, e.g.*, Geisbert *et al.*, *J. Infect. Dis.*, 193:1650-1657 (2006)); Arenaviruses such as Lassa virus, Junin virus, Machupo virus, Guanarito virus, and Sabia virus (Buchmeier *et al.*, *Arenaviridae: the viruses and their replication*, In: *FIELDS VIROLOGY*, Knipe *et al.* (eds.), 4th ed., Lippincott-Raven, Philadelphia, (2001)); Influenza viruses such as Influenza A, B, and C viruses, (*see, e.g.*, Steinhauer *et al.*, *Annu Rev Genet.*, 36:305-332 (2002); and Neumann *et al.*, *J Gen Virol.*, 83:2635-2662 (2002)); Hepatitis viruses (*see, e.g.*, Hamasaki *et al.*, *FEBS Lett.*, 543:51 (2003); Yokota *et al.*, *EMBO Rep.*, 4:602 (2003); Schlomai *et al.*, *Hepatology*, 37:764 (2003); Wilson *et al.*, *Proc. Natl. Acad. Sci. USA*, 100:2783 (2003); Kapadia *et al.*, *Proc. Natl. Acad. Sci. USA*, 100:2014 (2003); and *FIELDS VIROLOGY*, Knipe *et*

al. (eds.), 4th ed., Lippincott-Raven, Philadelphia (2001)); Human Immunodeficiency Virus (HIV) (Banerjea *et al.*, *Mol. Ther.*, 8:62 (2003); Song *et al.*, *J. Virol.*, 77:7174 (2003); Stephenson, *JAMA*, 289:1494 (2003); Qin *et al.*, *Proc. Natl. Acad. Sci. USA*, 100:183 (2003)); Herpes viruses (Jia *et al.*, *J. Virol.*, 77:3301 (2003)); and Human Papilloma Viruses (HPV) (Hall *et al.*, *J. Virol.*, 77:6066 (2003); Jiang *et al.*, *Oncogene*, 21:6041 (2002)).

[0252] Exemplary Filovirus nucleic acid sequences that can be silenced include, but are not limited to, nucleic acid sequences encoding structural proteins (*e.g.*, VP30, VP35, nucleoprotein (NP), polymerase protein (L-pol)) and membrane-associated proteins (*e.g.*, VP40, glycoprotein (GP), VP24). Complete genome sequences for Ebola virus are set forth in, *e.g.*, Genbank Accession Nos. NC_002549; AY769362; NC_006432; NC_004161; AY729654; AY354458; AY142960; AB050936; AF522874; AF499101; AF272001; and AF086833. Ebola virus VP24 sequences are set forth in, *e.g.*, Genbank Accession Nos. U77385 and AY058897. Ebola virus L-pol sequences are set forth in, *e.g.*, Genbank Accession No. X67110. Ebola virus VP40 sequences are set forth in, *e.g.*, Genbank Accession No. AY058896. Ebola virus NP sequences are set forth in, *e.g.*, Genbank Accession No. AY058895. Ebola virus GP sequences are set forth in, *e.g.*, Genbank Accession No. AY058898; Sanchez *et al.*, *Virus Res.*, 29:215-240 (1993); Will *et al.*, *J. Virol.*, 67:1203-1210 (1993); Volchikov *et al.*, *FEBS Lett.*, 305:181-184 (1992); and U.S. Patent No. 6,713,069. Additional Ebola virus sequences are set forth in, *e.g.*, Genbank Accession Nos. L11365 and X61274. Complete genome sequences for Marburg virus are set forth in, *e.g.*, Genbank Accession Nos. NC_001608; AY430365; AY430366; and AY358025. Marburg virus GP sequences are set forth in, *e.g.*, Genbank Accession Nos. AF005734; AF005733; and AF005732. Marburg virus VP35 sequences are set forth in, *e.g.*, Genbank Accession Nos. AF005731 and AF005730. Additional Marburg virus sequences are set forth in, *e.g.*, Genbank Accession Nos. X64406; Z29337; AF005735; and Z12132. Non-limiting examples of siRNA molecules targeting Ebola virus and Marburg virus nucleic acid sequences include those described in U.S. Patent Publication No. 20070135370 and U.S. Provisional Application No. 61/286,741, filed December 15, 2009, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0253] Exemplary Arenavirus nucleic acid sequences that can be silenced include, but are not limited to, nucleic acid sequences encoding nucleoprotein (NP), glycoprotein (GP), L-polymerase (L), and Z protein (Z). Complete genome sequences for Lassa virus are set forth in, *e.g.*, Genbank Accession Nos. NC_004296 (LASV segment S) and NC_004297 (LASV segment L). Non-limiting examples of siRNA molecules targeting Lassa virus nucleic acid sequences include those described in U.S. Provisional Application No. 61/319,855, filed

March 31, 2010, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0254] Exemplary host nucleic acid sequences that can be silenced include, but are not limited to, nucleic acid sequences encoding host factors such as tissue factor (TF) that are known to play a role in the pathogenesis of hemorrhagic fever viruses. The mRNA sequence of TF is set forth in Genbank Accession No. NM_001993. Those of skill in the art will appreciate that TF is also known as F3, coagulation factor III, thromboplastin, and CD142. Non-limiting examples of siRNA molecules targeting TF nucleic acid sequences include those described in U.S. Provisional Application No. 61/319,855, filed March 31, 2010, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0255] Exemplary Influenza virus nucleic acid sequences that can be silenced include, but are not limited to, nucleic acid sequences encoding nucleoprotein (NP), matrix proteins (M1 and M2), nonstructural proteins (NS1 and NS2), RNA polymerase (PA, PB1, PB2), neuraminidase (NA), and haemagglutinin (HA). Influenza A NP sequences are set forth in, *e.g.*, Genbank Accession Nos. NC_004522; AY818138; AB166863; AB188817; AB189046; AB189054; AB189062; AY646169; AY646177; AY651486; AY651493; AY651494; AY651495; AY651496; AY651497; AY651498; AY651499; AY651500; AY651501; AY651502; AY651503; AY651504; AY651505; AY651506; AY651507; AY651509; AY651528; AY770996; AY790308; AY818138; and AY818140. Influenza A PA sequences are set forth in, *e.g.*, Genbank Accession Nos. AY818132; AY790280; AY646171; AY818132; AY818133; AY646179; AY818134; AY551934; AY651613; AY651610; AY651620; AY651617; AY651600; AY651611; AY651606; AY651618; AY651608; AY651607; AY651605; AY651609; AY651615; AY651616; AY651640; AY651614; AY651612; AY651621; AY651619; AY770995; and AY724786. Non-limiting examples of siRNA molecules targeting Influenza virus nucleic acid sequences include those described in U.S. Patent Publication No. 20070218122, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0256] Exemplary hepatitis virus nucleic acid sequences that can be silenced include, but are not limited to, nucleic acid sequences involved in transcription and translation (*e.g.*, En1, En2, X, P) and nucleic acid sequences encoding structural proteins (*e.g.*, core proteins including C and C-related proteins, capsid and envelope proteins including S, M, and/or L proteins, or fragments thereof) (*see, e.g.*, FIELDS VIROLOGY, *supra*). Exemplary Hepatitis C virus (HCV) nucleic acid sequences that can be silenced include, but are not limited to, the 5'-untranslated region (5'-UTR), the 3'-untranslated region (3'-UTR), the polyprotein translation initiation codon region, the internal ribosome entry site (IRES) sequence, and/or

nucleic acid sequences encoding the core protein, the E1 protein, the E2 protein, the p7 protein, the NS2 protein, the NS3 protease/helicase, the NS4A protein, the NS4B protein, the NS5A protein, and/or the NS5B RNA-dependent RNA polymerase. HCV genome sequences are set forth in, *e.g.*, Genbank Accession Nos. NC_004102 (HCV genotype 1a), AJ238799 (HCV genotype 1b), NC_009823 (HCV genotype 2), NC_009824 (HCV genotype 3), NC_009825 (HCV genotype 4), NC_009826 (HCV genotype 5), and NC_009827 (HCV genotype 6). Hepatitis A virus nucleic acid sequences are set forth in, *e.g.*, Genbank Accession No. NC_001489; Hepatitis B virus nucleic acid sequences are set forth in, *e.g.*, Genbank Accession No. NC_003977; Hepatitis D virus nucleic acid sequence are set forth in, *e.g.*, Genbank Accession No. NC_001653; Hepatitis E virus nucleic acid sequences are set forth in, *e.g.*, Genbank Accession No. NC_001434; and Hepatitis G virus nucleic acid sequences are set forth in, *e.g.*, Genbank Accession No. NC_001710. Silencing of sequences that encode genes associated with viral infection and survival can conveniently be used in combination with the administration of conventional agents used to treat the viral condition.

Non-limiting examples of siRNA molecules targeting hepatitis virus nucleic acid sequences include those described in U.S. Patent Publication Nos. 20060281175, 20050058982, and 20070149470; U.S. Patent No. 7,348,314; and PCT Application No. PCT/CA2010/000444, entitled "Compositions and Methods for Silencing Hepatitis C Virus Expression," filed March 19, 2010, bearing Attorney Docket No. 020801-008910PC, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0257] Genes associated with metabolic diseases and disorders (*e.g.*, disorders in which the liver is the target and liver diseases and disorders) include, but are not limited to, genes expressed in dyslipidemia, such as, *e.g.*, apolipoprotein B (APOB) (Genbank Accession No. NM_000384), apolipoprotein CIII (APOC3) (Genbank Accession Nos. NM_000040 and NG_008949 REGION: 5001..8164), apolipoprotein E (APOE) (Genbank Accession Nos. NM_000041 and NG_007084 REGION: 5001..8612), proprotein convertase subtilisin/kexin type 9 (PCSK9) (Genbank Accession No. NM_174936), diacylglycerol O-acyltransferase type 1 (DGAT1) (Genbank Accession No. NM_012079), diacylglycerol O-acyltransferase type 2 (DGAT2) (Genbank Accession No. NM_032564), liver X receptors such as LXR α and LXR β (Genbank Accession No. NM_007121), farnesoid X receptors (FXR) (Genbank Accession No. NM_005123), sterol-regulatory element binding protein (SREBP), site-1 protease (S1P), 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMG coenzyme-A reductase); and genes expressed in diabetes, such as, *e.g.*, glucose 6-phosphatase (*see, e.g.*, Forman *et al.*, *Cell*, 81:687 (1995); Seol *et al.*, *Mol. Endocrinol.*, 9:72 (1995), Zavacki *et al.*, *Proc. Natl. Acad. Sci. USA*, 94:7909 (1997); Sakai *et al.*, *Cell*, 85:1037-1046 (1996); Duncan

et al., *J. Biol. Chem.*, 272:12778-12785 (1997); Willy *et al.*, *Genes Dev.*, 9:1033-1045 (1995); Lehmann *et al.*, *J. Biol. Chem.*, 272:3137-3140 (1997); Janowski *et al.*, *Nature*, 383:728-731 (1996); and Peet *et al.*, *Cell*, 93:693-704 (1998)).

[0258] One of skill in the art will appreciate that genes associated with metabolic diseases and disorders (*e.g.*, diseases and disorders in which the liver is a target and liver diseases and disorders) include genes that are expressed in the liver itself as well as and genes expressed in other organs and tissues. Silencing of sequences that encode genes associated with metabolic diseases and disorders can conveniently be used in combination with the administration of conventional agents used to treat the disease or disorder. Non-limiting examples of siRNA molecules targeting the APOB gene include those described in U.S. Patent Publication Nos. 20060134189, 20060105976, and 20070135372, and PCT Publication No. WO 04/091515, the disclosures of which are herein incorporated by reference in their entirety for all purposes. Non-limiting examples of siRNA molecules targeting the APOC3 gene include those described in PCT Application No. PCT/CA2010/000120, filed January 26, 2010, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Non-limiting examples of siRNA molecules targeting the PCSK9 gene include those described in U.S. Patent Publication Nos. 20070173473, 20080113930, and 20080306015, the disclosures of which are herein incorporated by reference in their entirety for all purposes. Exemplary siRNA molecules targeting the DGAT1 gene may be designed using the antisense compounds described in U.S. Patent Publication No. 20040185559, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Exemplary siRNA molecules targeting the DGAT2 gene may be designed using the antisense compounds described in U.S. Patent Publication No. 20050043524, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0259] Genes associated with tumorigenesis or cell transformation (*e.g.*, cancer or other neoplasia) include, for example, genes involved in p53 ubiquitination, c-Jun ubiquitination, histone deacetylation, cell cycle regulation, transcriptional regulation, and combinations thereof. Non-limiting examples of gene sequences associated with tumorigenesis or cell transformation include serine/threonine kinases such as polo-like kinase 1 (PLK-1) (Genbank Accession No. NM_005030; Barr *et al.*, *Nat. Rev. Mol. Cell Biol.*, 5:429-440 (2004)) and cyclin-dependent kinase 4 (CDK4) (Genbank Accession No. NM_000075); ubiquitin ligases such as COP1 (RFWD2; Genbank Accession Nos. NM_022457 and NM_001001740) and ring-box 1 (RBX1) (ROC1; Genbank Accession No. NM_014248); tyrosine kinases such as WEE1 (Genbank Accession Nos. NM_003390 and NM_001143976); mitotic kinesins such as Eg5 (KSP, KIF11; Genbank Accession No. NM_004523); transcription factors such as

forkhead box M1 (FOX M1) (Genbank Accession Nos. NM_202002, NM_021953, and NM_202003) and RAM2 (R1 or CDCA7L; Genbank Accession Nos. NM_018719, NM_001127370, and NM_001127371); inhibitors of apoptosis such as XIAP (Genbank Accession No. NM_001167); COP9 signalosome subunits such as CSN1, CSN2, CSN3, CSN4, CSN5 (JAB1; Genbank Accession No. NM_006837); CSN6, CSN7A, CSN7B, and CSN8; and histone deacetylases such as HDAC1, HDAC2 (Genbank Accession No. NM_001527), HDAC3, HDAC4, HDAC5, HDAC6, HDAC7, HDAC8, HDAC9, *etc.*

[0260] Non-limiting examples of siRNA molecules targeting the PLK-1 gene include those described in U.S. Patent Publication Nos. 20050107316 and 20070265438; and PCT

Publication No. WO 09/082817, the disclosures of which are herein incorporated by reference in their entirety for all purposes. Non-limiting examples of siRNA molecules targeting the Eg5 and XIAP genes include those described in U.S. Patent Publication No. 20090149403, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Non-limiting examples of siRNA molecules targeting the CSN5 gene include those described in PCT Publication No. WO 09/129319, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Non-limiting examples of siRNA molecules targeting the COP1, CSN5, RBX1, HDAC2, CDK4, WEE1, FOXM1, and RAM2 genes include those described in U.S. Provisional Application No. 61/245,143, filed September 23, 2009, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0261] Additional examples of gene sequences associated with tumorigenesis or cell transformation include translocation sequences such as MLL fusion genes, BCR-ABL (Wilda *et al.*, *Oncogene*, 21:5716 (2002); Scherr *et al.*, *Blood*, 101:1566 (2003)), TEL-AML1, EWS-FLI1, TLS-FUS, PAX3-FKHR, BCL-2, AML1-ETO, and AML1-MTG8 (Heidenreich *et al.*, *Blood*, 101:3157 (2003)); overexpressed sequences such as multidrug resistance genes (Nieth *et al.*, *FEBS Lett.*, 545:144 (2003); Wu *et al.*, *Cancer Res.* 63:1515 (2003)), cyclins (Li *et al.*, *Cancer Res.*, 63:3593 (2003); Zou *et al.*, *Genes Dev.*, 16:2923 (2002)), beta-catenin (Verma *et al.*, *Clin Cancer Res.*, 9:1291 (2003)), telomerase genes (Kosciulek *et al.*, *Mol Cancer Ther.*, 2:209 (2003)), c-MYC, N-MYC, BCL-2, growth factor receptors (*e.g.*, EGFR/ErbB1 (Genbank Accession Nos. NM_005228, NM_201282, NM_201283, and NM_201284; *see also*, Nagy *et al. Exp. Cell Res.*, 285:39-49 (2003)), ErbB2/HER-2 (Genbank Accession Nos. NM_004448 and NM_001005862), ErbB3 (Genbank Accession Nos. NM_001982 and NM_001005915), and ErbB4 (Genbank Accession Nos. NM_005235 and NM_001042599)), and mutated sequences such as RAS (Tuschl and Borkhardt, *Mol. Interventions*, 2:158 (2002)). Non-limiting examples of siRNA molecules targeting the EGFR gene include those

described in U.S. Patent Publication No. 20090149403, the disclosure of which is herein incorporated by reference in its entirety for all purposes. siRNA molecules that target VEGFR genes are set forth in, *e.g.*, GB 2396864; U.S. Patent Publication No. 20040142895; and CA 2456444, the disclosures of which are herein incorporated by reference in their
5 entirety for all purposes.

[0262] Silencing of sequences that encode DNA repair enzymes find use in combination with the administration of chemotherapeutic agents (Collis *et al.*, *Cancer Res.*, 63:1550 (2003)). Genes encoding proteins associated with tumor migration are also target sequences of interest, for example, integrins, selectins, and metalloproteinases. The foregoing examples
10 are not exclusive. Those of skill in the art will understand that any whole or partial gene sequence that facilitates or promotes tumorigenesis or cell transformation, tumor growth, or tumor migration can be included as a template sequence.

[0263] Angiogenic genes are able to promote the formation of new vessels. Angiogenic genes of particular interest include, but are not limited to, vascular endothelial growth factor (VEGF) (Reich *et al.*, *Mol. Vis.*, 9:210 (2003)), placental growth factor (PGF), VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), and the like. siRNA molecules that target VEGFR genes are set forth in, *e.g.*, GB 2396864; U.S. Patent Publication No. 20040142895; and CA 2456444, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0264] Immunomodulator genes are genes that modulate one or more immune responses.

20 Examples of immunomodulator genes include, without limitation, growth factors (*e.g.*, TGF- α , TGF- β , EGF, FGF, IGF, NGF, PDGF, CGF, GM-CSF, SCF, *etc.*), interleukins (*e.g.*, IL-2, IL-4, IL-12 (Hill *et al.*, *J. Immunol.*, 171:691 (2003)), IL-15, IL-18, IL-20, *etc.*), interferons (*e.g.*, IFN- α , IFN- β , IFN- γ , *etc.*), and TNF. Fas and Fas ligand genes are also immunomodulator target sequences of interest (Song *et al.*, *Nat. Med.*, 9:347 (2003)). Genes
25 encoding secondary signaling molecules in hematopoietic and lymphoid cells are also included in the present invention, for example, Tec family kinases such as Bruton's tyrosine kinase (Btk) (Heinonen *et al.*, *FEBS Lett.*, 527:274 (2002)).

[0265] Cell receptor ligand genes include ligands that are able to bind to cell surface receptors (*e.g.*, cytokine receptors, growth factor receptors, receptors with tyrosine kinase activity, G-protein coupled receptors, insulin receptor, EPO receptor, *etc.*) to modulate (*e.g.*,
30 inhibit) the physiological pathway that the receptor is involved in (*e.g.*, cell proliferation, tumorigenesis, cell transformation, mitogenesis, *etc.*). Non-limiting examples of cell receptor ligand genes include cytokines (*e.g.*, TNF- α , interferons such as IFN- α , IFN- β , and IFN- γ , interleukins such as IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-
35 13, IL-15, IL-17, IL-23, IL-27, chemokines, *etc.*), growth factors (*e.g.*, EGF, HB-EGF,

VEGF, PEDF, SDGF, bFGF, HGF, TGF- α , TGF- β , BMP1-BMP15, PDGF, IGF, NGF, β -NGF, BDNF, NT3, NT4, GDF-9, CGF, G-CSF, GM-CSF, GDF-8, EPO, TPO, *etc.*), insulin, glucagon, G-protein coupled receptor ligands, *etc.*

[0266] Templates coding for an expansion of trinucleotide repeats (*e.g.*, CAG repeats) find use in silencing pathogenic sequences in neurodegenerative disorders caused by the expansion of trinucleotide repeats, such as spinobulbar muscular atrophy and Huntington's Disease (Caplen *et al.*, *Hum. Mol. Genet.*, 11:175 (2002)).

[0267] In addition to its utility in silencing the expression of any of the above-described genes for therapeutic purposes, the siRNA described herein are also useful in research and development applications as well as diagnostic, prophylactic, prognostic, clinical, and other healthcare applications. As a non-limiting example, the siRNA can be used in target validation studies directed at testing whether a gene of interest has the potential to be a therapeutic target. The siRNA can also be used in target identification studies aimed at discovering genes as potential therapeutic targets.

e) Exemplary siRNA Embodiments

[0268] In some embodiments, each strand of the siRNA molecule comprises from about 15 to about 60 nucleotides in length (*e.g.*, about 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 nucleotides in length, or 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length). In one particular embodiment, the siRNA is chemically synthesized. The siRNA molecules of the invention are capable of silencing the expression of a target sequence *in vitro* and/or *in vivo*.

[0269] In other embodiments, the siRNA comprises at least one modified nucleotide. In certain embodiments, the siRNA comprises one, two, three, four, five, six, seven, eight, nine, ten, or more modified nucleotides in the double-stranded region. In particular embodiments, less than about 50% (*e.g.*, less than about 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, or 5%) of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides. In preferred embodiments, from about 1% to about 50% (*e.g.*, from about 5%-50%, 10%-50%, 15%-50%, 20%-50%, 25%-50%, 30%-50%, 35%-50%, 40%-50%, 45%-50%, 5%-45%, 10%-45%, 15%-45%, 20%-45%, 25%-45%, 30%-45%, 35%-45%, 40%-45%, 5%-40%, 10%-40%, 15%-40%, 20%-40%, 25%-40%, 30%-40%, 35%-40%, 5%-35%, 10%-35%, 15%-35%, 20%-35%, 25%-35%, 30%-35%, 5%-30%, 10%-30%, 15%-30%, 20%-30%, 25%-30%, 5%-25%, 10%-25%, 15%-25%, 20%-25%, 5%-20%, 10%-20%, 15%-20%, 5%-15%, 10%-15%, or 5%-10%) of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides.

[0270] In further embodiments, the siRNA comprises modified nucleotides including, but not limited to, 2'-O-methyl (2'OMe) nucleotides, 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, locked nucleic acid (LNA) nucleotides, and mixtures thereof. In preferred embodiments, the siRNA comprises 2'OMe nucleotides (*e.g.*, 2'OMe purine and/or pyrimidine nucleotides) such as, *e.g.*, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, 2'OMe-cytosine nucleotides, or mixtures thereof. In one particular embodiment, the siRNA comprises at least one 2'OMe-guanosine nucleotide, 2'OMe-uridine nucleotide, or mixtures thereof. In certain instances, the siRNA does not comprise 2'OMe-cytosine nucleotides. In other embodiments, the siRNA comprises a hairpin loop structure.

[0271] In certain embodiments, the siRNA comprises modified nucleotides in one strand (*i.e.*, sense or antisense) or both strands of the double-stranded region of the siRNA molecule. Preferably, uridine and/or guanosine nucleotides are modified at selective positions in the double-stranded region of the siRNA duplex. With regard to uridine nucleotide modifications, at least one, two, three, four, five, six, or more of the uridine nucleotides in the sense and/or antisense strand can be a modified uridine nucleotide such as a 2'OMe-uridine nucleotide. In some embodiments, every uridine nucleotide in the sense and/or antisense strand is a 2'OMe-uridine nucleotide. With regard to guanosine nucleotide modifications, at least one, two, three, four, five, six, or more of the guanosine nucleotides in the sense and/or antisense strand can be a modified guanosine nucleotide such as a 2'OMe-guanosine nucleotide. In some embodiments, every guanosine nucleotide in the sense and/or antisense strand is a 2'OMe-guanosine nucleotide.

[0272] In certain embodiments, at least one, two, three, four, five, six, seven, or more 5'-GU-3' motifs in an siRNA sequence may be modified, *e.g.*, by introducing mismatches to eliminate the 5'-GU-3' motifs and/or by introducing modified nucleotides such as 2'OMe nucleotides. The 5'-GU-3' motif can be in the sense strand, the antisense strand, or both strands of the siRNA sequence. The 5'-GU-3' motifs may be adjacent to each other or, alternatively, they may be separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more nucleotides.

[0273] In some embodiments, a modified siRNA molecule is less immunostimulatory than a corresponding unmodified siRNA sequence. In such embodiments, the modified siRNA molecule with reduced immunostimulatory properties advantageously retains RNAi activity against the target sequence. In another embodiment, the immunostimulatory properties of the modified siRNA molecule and its ability to silence target gene expression can be balanced or optimized by the introduction of minimal and selective 2'OMe modifications within the siRNA sequence such as, *e.g.*, within the double-stranded region of the siRNA duplex. In

certain instances, the modified siRNA is at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% less immunostimulatory than the corresponding unmodified siRNA. It will be readily apparent to those of skill in the art that the immunostimulatory properties of the modified siRNA molecule and the corresponding unmodified siRNA molecule can be determined by, for example, measuring INF- α and/or IL-6 levels from about two to about twelve hours after systemic administration in a mammal or transfection of a mammalian responder cell using an appropriate lipid-based delivery system (such as the SNALP delivery system disclosed herein).

5 [0274] In other embodiments, a modified siRNA molecule has an IC₅₀ (*i.e.*, half-maximal inhibitory concentration) less than or equal to ten-fold that of the corresponding unmodified siRNA (*i.e.*, the modified siRNA has an IC₅₀ that is less than or equal to ten-times the IC₅₀ of the corresponding unmodified siRNA). In other embodiments, the modified siRNA has an IC₅₀ less than or equal to three-fold that of the corresponding unmodified siRNA sequence.

15 In yet other embodiments, the modified siRNA has an IC₅₀ less than or equal to two-fold that of the corresponding unmodified siRNA. It will be readily apparent to those of skill in the art that a dose-response curve can be generated and the IC₅₀ values for the modified siRNA and the corresponding unmodified siRNA can be readily determined using methods known to those of skill in the art.

20 [0275] In another embodiment, an unmodified or modified siRNA molecule is capable of silencing at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% of the expression of the target sequence relative to a negative control (*e.g.*, buffer only, an siRNA sequence that targets a different gene, a scrambled siRNA sequence, *etc.*).

25 [0276] In yet another embodiment, a modified siRNA molecule is capable of silencing at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% of the expression of the target sequence relative to the corresponding unmodified siRNA sequence.

30 [0277] In some embodiments, the siRNA molecule does not comprise phosphate backbone modifications, *e.g.*, in the sense and/or antisense strand of the double-stranded region. In other embodiments, the siRNA comprises one, two, three, four, or more phosphate backbone modifications, *e.g.*, in the sense and/or antisense strand of the double-stranded region. In preferred embodiments, the siRNA does not comprise phosphate backbone modifications.

[0278] In further embodiments, the siRNA does not comprise 2'-deoxy nucleotides, *e.g.*, in the sense and/or antisense strand of the double-stranded region. In yet further embodiments, the siRNA comprises one, two, three, four, or more 2'-deoxy nucleotides, *e.g.*, in the sense and/or antisense strand of the double-stranded region. In preferred embodiments, the siRNA does not comprise 2'-deoxy nucleotides.

[0279] In certain instances, the nucleotide at the 3'-end of the double-stranded region in the sense and/or antisense strand is not a modified nucleotide. In certain other instances, the nucleotides near the 3'-end (*e.g.*, within one, two, three, or four nucleotides of the 3'-end) of the double-stranded region in the sense and/or antisense strand are not modified nucleotides.

[0280] The siRNA molecules described herein may have 3' overhangs of one, two, three, four, or more nucleotides on one or both sides of the double-stranded region, or may lack overhangs (*i.e.*, have blunt ends) on one or both sides of the double-stranded region. In certain embodiments, the 3' overhang on the sense and/or antisense strand independently comprises one, two, three, four, or more modified nucleotides such as 2'OMe nucleotides and/or any other modified nucleotide described herein or known in the art.

[0281] In particular embodiments, siRNAs are administered using a carrier system such as a nucleic acid-lipid particle. In a preferred embodiment, the nucleic acid-lipid particle comprises: (a) one or more siRNA molecules; (b) a cationic lipid of Formula I-XII or a salt thereof; and (c) a non-cationic lipid (*e.g.*, DPPC, DSPC, DSPE, and/or cholesterol). In certain instances, the nucleic acid-lipid particle may further comprise a conjugated lipid that prevents aggregation of particles (*e.g.*, PEG-DAA).

2. Dicer-Substrate dsRNA

[0282] As used herein, the term "Dicer-substrate dsRNA" or "precursor RNAi molecule" is intended to include any precursor molecule that is processed *in vivo* by Dicer to produce an active siRNA which is incorporated into the RISC complex for RNA interference of a target gene.

[0283] In one embodiment, the Dicer-substrate dsRNA has a length sufficient such that it is processed by Dicer to produce an siRNA. According to this embodiment, the Dicer-substrate dsRNA comprises (i) a first oligonucleotide sequence (also termed the sense strand) that is between about 25 and about 60 nucleotides in length (*e.g.*, about 25-60, 25-55, 25-50, 25-45, 25-40, 25-35, or 25-30 nucleotides in length), preferably between about 25 and about 30 nucleotides in length (*e.g.*, 25, 26, 27, 28, 29, or 30 nucleotides in length), and (ii) a second oligonucleotide sequence (also termed the antisense strand) that anneals to the first sequence under biological conditions, such as the conditions found in the cytoplasm of a cell. The

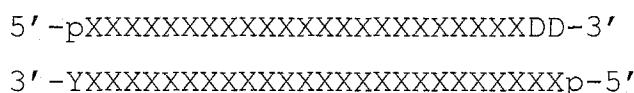
second oligonucleotide sequence may be between about 25 and about 60 nucleotides in length (*e.g.*, about 25-60, 25-55, 25-50, 25-45, 25-40, 25-35, or 25-30 nucleotides in length), and is preferably between about 25 and about 30 nucleotides in length (*e.g.*, 25, 26, 27, 28, 29, or 30 nucleotides in length). In addition, a region of one of the sequences, particularly of the antisense strand, of the Dicer-substrate dsRNA has a sequence length of at least about 19 nucleotides, for example, from about 19 to about 60 nucleotides (*e.g.*, about 19-60, 19-55, 19-50, 19-45, 19-40, 19-35, 19-30, or 19-25 nucleotides), preferably from about 19 to about 23 nucleotides (*e.g.*, 19, 20, 21, 22, or 23 nucleotides) that are sufficiently complementary to a nucleotide sequence of the RNA produced from the target gene to trigger an RNAi response.

[0284] In a second embodiment, the Dicer-substrate dsRNA has several properties which enhance its processing by Dicer. According to this embodiment, the dsRNA has a length sufficient such that it is processed by Dicer to produce an siRNA and has at least one of the following properties: (i) the dsRNA is asymmetric, *e.g.*, has a 3'-overhang on the antisense strand; and/or (ii) the dsRNA has a modified 3'-end on the sense strand to direct orientation of Dicer binding and processing of the dsRNA to an active siRNA. According to this latter embodiment, the sense strand comprises from about 22 to about 28 nucleotides and the antisense strand comprises from about 24 to about 30 nucleotides.

[0285] In one embodiment, the Dicer-substrate dsRNA has an overhang on the 3'-end of the antisense strand. In another embodiment, the sense strand is modified for Dicer binding and processing by suitable modifiers located at the 3'-end of the sense strand. Suitable modifiers include nucleotides such as deoxyribonucleotides, acyclonucleotides, and the like, and sterically hindered molecules such as fluorescent molecules and the like. When nucleotide modifiers are used, they replace ribonucleotides in the dsRNA such that the length of the dsRNA does not change. In another embodiment, the Dicer-substrate dsRNA has an overhang on the 3'-end of the antisense strand and the sense strand is modified for Dicer processing. In another embodiment, the 5'-end of the sense strand has a phosphate. In another embodiment, the 5'-end of the antisense strand has a phosphate. In another embodiment, the antisense strand or the sense strand or both strands have one or more 2'-O-methyl (2'OMe) modified nucleotides. In another embodiment, the antisense strand contains 2'OMe modified nucleotides. In another embodiment, the antisense strand contains a 3'-overhang that is comprised of 2'OMe modified nucleotides. The antisense strand could also include additional 2'OMe modified nucleotides. The sense and antisense strands anneal under biological conditions, such as the conditions found in the cytoplasm of a cell. In addition, a region of one of the sequences, particularly of the antisense strand, of the Dicer-substrate dsRNA has a sequence length of at least about 19 nucleotides, wherein these

nucleotides are in the 21-nucleotide region adjacent to the 3'-end of the antisense strand and are sufficiently complementary to a nucleotide sequence of the RNA produced from the target gene. Further, in accordance with this embodiment, the Dicer-substrate dsRNA may also have one or more of the following additional properties: (a) the antisense strand has a right shift from the typical 21-mer (*i.e.*, the antisense strand includes nucleotides on the right side of the molecule when compared to the typical 21-mer); (b) the strands may not be completely complementary, *i.e.*, the strands may contain simple mismatch pairings; and (c) base modifications such as locked nucleic acid(s) may be included in the 5'-end of the sense strand.

[0286] In a third embodiment, the sense strand comprises from about 25 to about 28 nucleotides (*e.g.*, 25, 26, 27, or 28 nucleotides), wherein the 2 nucleotides on the 3'-end of the sense strand are deoxyribonucleotides. The sense strand contains a phosphate at the 5'-end. The antisense strand comprises from about 26 to about 30 nucleotides (*e.g.*, 26, 27, 28, 29, or 30 nucleotides) and contains a 3'-overhang of 1-4 nucleotides. The nucleotides comprising the 3'-overhang are modified with 2'OMe modified ribonucleotides. The antisense strand contains alternating 2'OMe modified nucleotides beginning at the first monomer of the antisense strand adjacent to the 3'-overhang, and extending 15-19 nucleotides from the first monomer adjacent to the 3'-overhang. For example, for a 27-nucleotide antisense strand and counting the first base at the 5'-end of the antisense strand as position number 1, 2'OMe modifications would be placed at bases 9, 11, 13, 15, 17, 19, 21, 23, 25, 26, and 27. In one embodiment, the Dicer-substrate dsRNA has the following structure:



wherein "X" = RNA, "p" = a phosphate group, "X" = 2'OMe RNA, "Y" is an overhang domain comprised of 1, 2, 3, or 4 RNA monomers that are optionally 2'OMe RNA monomers, and "D" = DNA. The top strand is the sense strand, and the bottom strand is the antisense strand.

[0287] In a fourth embodiment, the Dicer-substrate dsRNA has several properties which enhance its processing by Dicer. According to this embodiment, the dsRNA has a length sufficient such that it is processed by Dicer to produce an siRNA and at least one of the following properties: (i) the dsRNA is asymmetric, *e.g.*, has a 3'-overhang on the sense strand; and (ii) the dsRNA has a modified 3'-end on the antisense strand to direct orientation of Dicer binding and processing of the dsRNA to an active siRNA. According to this

embodiment, the sense strand comprises from about 24 to about 30 nucleotides (*e.g.*, 24, 25, 26, 27, 28, 29, or 30 nucleotides) and the antisense strand comprises from about 22 to about 28 nucleotides (*e.g.*, 22, 23, 24, 25, 26, 27, or 28 nucleotides). In one embodiment, the Dicer-substrate dsRNA has an overhang on the 3'-end of the sense strand. In another embodiment, the antisense strand is modified for Dicer binding and processing by suitable modifiers located at the 3'-end of the antisense strand. Suitable modifiers include nucleotides such as deoxyribonucleotides, acyclonucleotides, and the like, and sterically hindered molecules such as fluorescent molecules and the like. When nucleotide modifiers are used, they replace ribonucleotides in the dsRNA such that the length of the dsRNA does not change. In another embodiment, the dsRNA has an overhang on the 3'-end of the sense strand and the antisense strand is modified for Dicer processing. In one embodiment, the antisense strand has a 5'-phosphate. The sense and antisense strands anneal under biological conditions, such as the conditions found in the cytoplasm of a cell. In addition, a region of one of the sequences, particularly of the antisense strand, of the dsRNA has a sequence length of at least 19 nucleotides, wherein these nucleotides are adjacent to the 3'-end of antisense strand and are sufficiently complementary to a nucleotide sequence of the RNA produced from the target gene. Further, in accordance with this embodiment, the Dicer-substrate dsRNA may also have one or more of the following additional properties: (a) the antisense strand has a left shift from the typical 21-mer (*i.e.*, the antisense strand includes nucleotides on the left side of the molecule when compared to the typical 21-mer); and (b) the strands may not be completely complementary, *i.e.*, the strands may contain simple mismatch pairings.

[0288] In a preferred embodiment, the Dicer-substrate dsRNA has an asymmetric structure, with the sense strand having a 25-base pair length, and the antisense strand having a 27-base pair length with a 2 base 3'-overhang. In certain instances, this dsRNA having an asymmetric structure further contains 2 deoxynucleotides at the 3'-end of the sense strand in place of two of the ribonucleotides. In certain other instances, this dsRNA having an asymmetric structure further contains 2'OMe modifications at positions 9, 11, 13, 15, 17, 19, 21, 23, and 25 of the antisense strand (wherein the first base at the 5'-end of the antisense strand is position 1). In certain additional instances, this dsRNA having an asymmetric structure further contains a 3'-overhang on the antisense strand comprising 1, 2, 3, or 4 2'OMe nucleotides (*e.g.*, a 3'-overhang of 2'OMe nucleotides at positions 26 and 27 on the antisense strand).

[0289] In another embodiment, Dicer-substrate dsRNAs may be designed by first selecting an antisense strand siRNA sequence having a length of at least 19 nucleotides. In some instances, the antisense siRNA is modified to include about 5 to about 11 ribonucleotides on

the 5'-end to provide a length of about 24 to about 30 nucleotides. When the antisense strand has a length of 21 nucleotides, 3-9, preferably 4-7, or more preferably 6 nucleotides may be added on the 5'-end. Although the added ribonucleotides may be complementary to the target gene sequence, full complementarity between the target sequence and the antisense siRNA is not required. That is, the resultant antisense siRNA is sufficiently complementary with the target sequence. A sense strand is then produced that has about 22 to about 28 nucleotides. The sense strand is substantially complementary with the antisense strand to anneal to the antisense strand under biological conditions. In one embodiment, the sense strand is synthesized to contain a modified 3'-end to direct Dicer processing of the antisense strand. In another embodiment, the antisense strand of the dsRNA has a 3'-overhang. In a further embodiment, the sense strand is synthesized to contain a modified 3'-end for Dicer binding and processing and the antisense strand of the dsRNA has a 3'-overhang.

[0290] In a related embodiment, the antisense siRNA may be modified to include about 1 to about 9 ribonucleotides on the 5'-end to provide a length of about 22 to about 28 nucleotides. When the antisense strand has a length of 21 nucleotides, 1-7, preferably 2-5, or more preferably 4 ribonucleotides may be added on the 3'-end. The added ribonucleotides may have any sequence. Although the added ribonucleotides may be complementary to the target gene sequence, full complementarity between the target sequence and the antisense siRNA is not required. That is, the resultant antisense siRNA is sufficiently complementary with the target sequence. A sense strand is then produced that has about 24 to about 30 nucleotides. The sense strand is substantially complementary with the antisense strand to anneal to the antisense strand under biological conditions. In one embodiment, the antisense strand is synthesized to contain a modified 3'-end to direct Dicer processing. In another embodiment, the sense strand of the dsRNA has a 3'-overhang. In a further embodiment, the antisense strand is synthesized to contain a modified 3'-end for Dicer binding and processing and the sense strand of the dsRNA has a 3'-overhang.

[0291] Suitable Dicer-substrate dsRNA sequences can be identified, synthesized, and modified using any means known in the art for designing, synthesizing, and modifying siRNA sequences. In particular embodiments, Dicer-substrate dsRNAs are administered using a carrier system such as a nucleic acid-lipid particle (*e.g.*, SNALP). In a preferred embodiment, the nucleic acid-lipid particle comprises: (a) one or more Dicer-substrate dsRNA molecules; (b) a cationic lipid of Formula I-XII or a salt thereof; and (c) a non-cationic lipid (*e.g.*, DPPC, DSPC, DSPE, and/or cholesterol). In certain instances, the nucleic acid-lipid particle may further comprise a conjugated lipid that prevents aggregation of particles (*e.g.*, PEG-DAA).

[0292] Additional embodiments related to the Dicer-substrate dsRNAs of the invention, as well as methods of designing and synthesizing such dsRNAs, are described in U.S. Patent Publication Nos. 20050244858, 20050277610, and 20070265220, and U.S. Application No. 12/794,701, filed June 4, 2010, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

3. shRNA

[0293] A “small hairpin RNA” or “short hairpin RNA” or “shRNA” includes a short RNA sequence that makes a tight hairpin turn that can be used to silence gene expression via RNA interference. The shRNAs of the invention may be chemically synthesized or transcribed from a transcriptional cassette in a DNA plasmid. The shRNA hairpin structure is cleaved by the cellular machinery into siRNA, which is then bound to the RNA-induced silencing complex (RISC).

[0294] The shRNAs of the invention are typically about 15-60, 15-50, or 15-40 (duplex) nucleotides in length, more typically about 15-30, 15-25, or 19-25 (duplex) nucleotides in length, and are preferably about 20-24, 21-22, or 21-23 (duplex) nucleotides in length (*e.g.*, each complementary sequence of the double-stranded shRNA is 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 nucleotides in length, preferably about 20-24, 21-22, or 21-23 nucleotides in length, and the double-stranded shRNA is about 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 base pairs in length, preferably about 18-22, 19-20, or 19-21 base pairs in length). shRNA duplexes may comprise 3' overhangs of about 1 to about 4 nucleotides or about 2 to about 3 nucleotides on the antisense strand and/or 5'-phosphate termini on the sense strand. In some embodiments, the shRNA comprises a sense strand and/or antisense strand sequence of from about 15 to about 60 nucleotides in length (*e.g.*, about 15-60, 15-55, 15-50, 15-45, 15-40, 15-35, 15-30, or 15-25 nucleotides in length), preferably from about 19 to about 40 nucleotides in length (*e.g.*, about 19-40, 19-35, 19-30, or 19-25 nucleotides in length), more preferably from about 19 to about 23 nucleotides in length (*e.g.*, 19, 20, 21, 22, or 23 nucleotides in length).

[0295] Non-limiting examples of shRNA include a double-stranded polynucleotide molecule assembled from a single-stranded molecule, where the sense and antisense regions are linked by a nucleic acid-based or non-nucleic acid-based linker; and a double-stranded polynucleotide molecule with a hairpin secondary structure having self-complementary sense and antisense regions. In preferred embodiments, the sense and antisense strands of the shRNA are linked by a loop structure comprising from about 1 to about 25 nucleotides, from about 2 to about 20 nucleotides, from about 4 to about 15 nucleotides, from about 5 to about

12 nucleotides, or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more nucleotides.

[0296] Additional shRNA sequences include, but are not limited to, asymmetric shRNA precursor polynucleotides such as those described in PCT Publication Nos. WO 2006/074108 and WO 2009/076321, the disclosures of which are herein incorporated by reference in their entirety for all purposes. For example, PCT Publication No. WO 2006/074108 discloses self-protected oligonucleotides comprising a region having a sequence complementary to one, two, three, or more same or different target mRNA sequences (*e.g.*, multivalent shRNAs) and one or more self-complementary regions. Similarly, PCT Publication No. WO 2009/076321 discloses self-forming asymmetric precursor polynucleotides comprising a targeting region comprising a polynucleotide sequence complementary to a region of one, two, three, or more same or different target mRNA sequences (*e.g.*, multivalent shRNAs); a first self-complementary region; and a second self-complementary region, wherein the first and second self-complementary regions are located one at each end of the targeting region and both self-complementary regions form stem-loop structures, wherein the first self-complementary region is capable of being cleaved by a RNase III endoribonuclease that is not a class IV DICER endoribonuclease, and wherein both self-complementary regions comprise a nucleotide sequence that is complementary to a region of the target gene sequence, but wherein a portion of the target sequence present in the targeting region does not have a complementary sequence in either of the self-complementary regions.

[0297] Suitable shRNA sequences can be identified, synthesized, and modified using any means known in the art for designing, synthesizing, and modifying siRNA sequences. In particular embodiments, shRNAs are administered using a carrier system such as a nucleic acid-lipid particle (*e.g.*, SNALP). In a preferred embodiment, the nucleic acid-lipid particle comprises: (a) one or more shRNA molecules; (b) a cationic lipid of Formula I-XII or a salt thereof; and (c) a non-cationic lipid (*e.g.*, DPPC, DSPC, DSPE, and/or cholesterol). In certain instances, the nucleic acid-lipid particle may further comprise a conjugated lipid that prevents aggregation of particles (*e.g.*, PEG-DAA).

[0298] Additional embodiments related to the shRNAs of the invention, as well as methods of designing and synthesizing such shRNAs, are described in U.S. Patent Application No. 12/794,701, filed June 4, 2010, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

4. aiRNA

[0299] Like siRNA, asymmetrical interfering RNA (aiRNA) can recruit the RNA-induced silencing complex (RISC) and lead to effective silencing of a variety of genes in mammalian cells by mediating sequence-specific cleavage of the target sequence between nucleotide 10 and 11 relative to the 5' end of the antisense strand (Sun *et al.*, *Nat. Biotech.*, 26:1379-1382 (2008)). Typically, an aiRNA molecule comprises a short RNA duplex having a sense strand and an antisense strand, wherein the duplex contains overhangs at the 3' and 5' ends of the antisense strand. The aiRNA is generally asymmetric because the sense strand is shorter on both ends when compared to the complementary antisense strand. In some aspects, aiRNA molecules may be designed, synthesized, and annealed under conditions similar to those used for siRNA molecules. As a non-limiting example, aiRNA sequences may be selected and generated using the methods described above for selecting siRNA sequences.

[0300] In another embodiment, aiRNA duplexes of various lengths (*e.g.*, about 10-25, 12-20, 12-19, 12-18, 13-17, or 14-17 base pairs, more typically 12, 13, 14, 15, 16, 17, 18, 19, or 20 base pairs) may be designed with overhangs at the 3' and 5' ends of the antisense strand to target an mRNA of interest. In certain instances, the sense strand of the aiRNA molecule is about 10-25, 12-20, 12-19, 12-18, 13-17, or 14-17 nucleotides in length, more typically 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides in length. In certain other instances, the antisense strand of the aiRNA molecule is about 15-60, 15-50, or 15-40 nucleotides in length, more typically about 15-30, 15-25, or 19-25 nucleotides in length, and is preferably about 20-24, 21-22, or 21-23 nucleotides in length.

[0301] In some embodiments, the 5' antisense overhang contains one, two, three, four, or more nontargeting nucleotides (*e.g.*, "AA", "UU", "dTdT", *etc.*). In other embodiments, the 3' antisense overhang contains one, two, three, four, or more nontargeting nucleotides (*e.g.*, "AA", "UU", "dTdT", *etc.*). In certain aspects, the aiRNA molecules described herein may comprise one or more modified nucleotides, *e.g.*, in the double-stranded (duplex) region and/or in the antisense overhangs. As a non-limiting example, aiRNA sequences may comprise one or more of the modified nucleotides described above for siRNA sequences. In a preferred embodiment, the aiRNA molecule comprises 2'OMe nucleotides such as, for example, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, or mixtures thereof.

[0302] In certain embodiments, aiRNA molecules may comprise an antisense strand which corresponds to the antisense strand of an siRNA molecule, *e.g.*, one of the siRNA molecules described herein.

[0303] In particular embodiments, aiRNAs are administered using a carrier system such as a nucleic acid-lipid particle (*e.g.*, SNALP). In a preferred embodiment, the nucleic acid-lipid

particle comprises: (a) one or more aiRNA molecules; (b) a cationic lipid of Formula I-XII or a salt thereof; and (c) a non-cationic lipid (*e.g.*, DPPC, DSPC, DSPE, and/or cholesterol). In certain instances, the nucleic acid-lipid particle may further comprise a conjugated lipid that prevents aggregation of particles (*e.g.*, PEG-DAA).

- 5 [0304] Suitable aiRNA sequences can be identified, synthesized, and modified using any means known in the art for designing, synthesizing, and modifying siRNA sequences. Additional embodiments related to the aiRNA molecules of the invention are described in U.S. Patent Publication No. 20090291131 and PCT Publication No. WO 09/127060, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

10 5. miRNA

- [0305] Generally, microRNAs (miRNA) are single-stranded RNA molecules of about 21-23 nucleotides in length which regulate gene expression. miRNAs are encoded by genes from whose DNA they are transcribed, but miRNAs are not translated into protein (non-coding RNA); instead, each primary transcript (a pri-miRNA) is processed into a short stem-loop structure called a pre-miRNA and finally into a functional mature miRNA. Mature miRNA molecules are either partially or completely complementary to one or more messenger RNA (mRNA) molecules, and their main function is to downregulate gene expression. The identification of miRNA molecules is described, *e.g.*, in Lagos-Quintana *et al.*, *Science*, 294:853-858; Lau *et al.*, *Science*, 294:858-862; and Lee *et al.*, *Science*, 294:862-864.

- 20 [0306] The genes encoding miRNA are much longer than the processed mature miRNA molecule. miRNA are first transcribed as primary transcripts or pri-miRNA with a cap and poly-A tail and processed to short, ~70-nucleotide stem-loop structures known as pre-miRNA in the cell nucleus. This processing is performed in animals by a protein complex known as the Microprocessor complex, consisting of the nuclease Drosha and the double-stranded RNA binding protein Pasha (Denli *et al.*, *Nature*, 432:231-235 (2004)). These pre-miRNA are then processed to mature miRNA in the cytoplasm by interaction with the endonuclease Dicer, which also initiates the formation of the RNA-induced silencing complex (RISC) (Bernstein *et al.*, *Nature*, 409:363-366 (2001)). Either the sense strand or antisense strand of DNA can function as templates to give rise to miRNA.

- 30 [0307] When Dicer cleaves the pre-miRNA stem-loop, two complementary short RNA molecules are formed, but only one is integrated into the RISC complex. This strand is known as the guide strand and is selected by the argonaute protein, the catalytically active RNase in the RISC complex, on the basis of the stability of the 5' end (Preall *et al.*, *Curr.*

Biol., 16:530-535 (2006)). The remaining strand, known as the anti-guide or passenger strand, is degraded as a RISC complex substrate (Gregory *et al.*, *Cell*, 123:631-640 (2005)). After integration into the active RISC complex, miRNAs base pair with their complementary mRNA molecules and induce target mRNA degradation and/or translational silencing.

5 [0308] Mammalian miRNA molecules are usually complementary to a site in the 3' UTR of the target mRNA sequence. In certain instances, the annealing of the miRNA to the target mRNA inhibits protein translation by blocking the protein translation machinery. In certain other instances, the annealing of the miRNA to the target mRNA facilitates the cleavage and degradation of the target mRNA through a process similar to RNA interference (RNAi).
10 miRNA may also target methylation of genomic sites which correspond to targeted mRNA. Generally, miRNA function in association with a complement of proteins collectively termed the miRNP.

[0309] In certain aspects, the miRNA molecules described herein are about 15-100, 15-90, 15-80, 15-75, 15-70, 15-60, 15-50, or 15-40 nucleotides in length, more typically about 15-30, 15-25, or 19-25 nucleotides in length, and are preferably about 20-24, 21-22, or 21-23 nucleotides in length. In certain other aspects, miRNA molecules may comprise one or more modified nucleotides. As a non-limiting example, miRNA sequences may comprise one or more of the modified nucleotides described above for siRNA sequences. In a preferred embodiment, the miRNA molecule comprises 2'OMe nucleotides such as, for example,
20 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, or mixtures thereof.

[0310] In particular embodiments, miRNAs are administered using a carrier system such as a nucleic acid-lipid particle (*e.g.*, SNALP). In a preferred embodiment, the nucleic acid-lipid particle comprises: (a) one or more miRNA molecules; (b) a cationic lipid of Formula I-XII or a salt thereof; and (c) a non-cationic lipid (*e.g.*, DPPC, DSPC, DSPE, and/or cholesterol).
25 In certain instances, the nucleic acid-lipid particle may further comprise a conjugated lipid that prevents aggregation of particles (*e.g.*, PEG-DAA).

[0311] In other embodiments, one or more agents that block the activity of an miRNA targeting an mRNA of interest are administered using a lipid particle of the invention (*e.g.*, a nucleic acid-lipid particle such as SNALP). Examples of blocking agents include, but are not
30 limited to, steric blocking oligonucleotides, locked nucleic acid oligonucleotides, and Morpholino oligonucleotides. Such blocking agents may bind directly to the miRNA or to the miRNA binding site on the target mRNA.

[0312] Additional embodiments related to the miRNA molecules of the invention are described in U.S. Patent Publication No. 20090291131 and PCT Publication No. WO

09/127060, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

6. Antisense Oligonucleotides

[0313] In one embodiment, the nucleic acid is an antisense oligonucleotide directed to a target gene or sequence of interest. The terms “antisense oligonucleotide” or “antisense” 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. Antisense RNA oligonucleotides prevent the translation of complementary RNA strands by binding to the RNA. Antisense DNA oligonucleotides can be used to target a specific, complementary (coding or non-coding) RNA. If binding occurs, this DNA/RNA hybrid can be degraded by the enzyme RNase H. In a particular embodiment, antisense oligonucleotides comprise from about 10 to about 60 nucleotides, more preferably from 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.

[0314] 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 (*see*, U.S. Patent Nos. 5,739,119 and 5,759,829).

Furthermore, examples of antisense inhibition have been demonstrated with the nuclear protein cyclin, the multiple drug resistance gene (MDR1), ICAM-1, E-selectin, STK-1, striatal GABAA receptor, and human EGF (*see*, Jaskulski *et al.*, *Science*, 240:1544-6 (1988); Vasanthakumar *et al.*, *Cancer Commun.*, 1:225-32 (1989); Peris *et al.*, *Brain Res Mol Brain Res.*, 15;57:310-20 (1998); and U.S. Patent Nos. 5,801,154; 5,789,573; 5,718,709 and 5,610,288). Moreover, antisense constructs have also been described that inhibit and can be used to treat a variety of abnormal cellular proliferations, *e.g.*, cancer (*see*, U.S. Patent Nos. 5,747,470; 5,591,317; and 5,783,683). The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0315] 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_m , 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.*, 25:3389-402 (1997)).

7. Ribozymes

[0316] 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 (*see*, Kim *et al.*, *Proc. Natl. Acad. Sci. USA.*, 84:8788-92 (1987); and Forster *et al.*, *Cell*, 49:211-20 (1987)). 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 (*see*, Cech *et al.*, *Cell*, 27:487-96 (1981); Michel *et al.*, *J. Mol. Biol.*, 216:585-610 (1990); Reinhold-Hurek *et al.*, *Nature*, 357:173-6 (1992)). 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.

[0317] At least six basic varieties of naturally-occurring enzymatic RNA molecules 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.

[0318] The enzymatic nucleic acid molecule may be formed in a hammerhead, hairpin, hepatitis δ 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 in, *e.g.*, Rossi *et al.*, *Nucleic Acids Res.*, 20:4559-65 (1992). Examples of hairpin motifs are described in, *e.g.*, EP 0360257, Hampel *et al.*, *Biochemistry*, 28:4929-33 (1989); Hampel *et al.*, *Nucleic Acids Res.*, 18:299-304 (1990); and U.S. Patent No. 5,631,359. An example of the hepatitis δ virus motif is described in, *e.g.*, Perrotta *et al.*, *Biochemistry*, 31:11843-52 (1992). An example of the RNaseP motif is described in, *e.g.*, Guerrier-Takada *et al.*, *Cell*, 35:849-57 (1983). Examples of the Neurospora VS RNA ribozyme motif is described in, *e.g.*, Saville *et al.*, *Cell*, 61:685-96 (1990); Saville *et al.*, *Proc. Natl. Acad. Sci. USA*, 88:8826-30 (1991); Collins *et al.*, *Biochemistry*, 32:2795-9 (1993). An example of the Group I intron is described in, *e.g.*, U.S. Patent No. 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. The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0319] Methods of producing a ribozyme targeted to any polynucleotide sequence are known in the art. Ribozymes may be designed as described in, *e.g.*, PCT Publication Nos. WO 93/23569 and WO 94/02595, and synthesized to be tested *in vitro* and/or *in vivo* as described therein. The disclosures of these PCT publications are herein incorporated by reference in their entirety for all purposes.

[0320] 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.*, PCT Publication Nos. WO 92/07065, WO 93/15187, WO 91/03162, and WO 94/13688; EP 92110298.4; and U.S. Patent No. 5,334,711, which describe various chemical modifications that can be made to the sugar moieties of enzymatic RNA molecules, the disclosures of which are each herein incorporated by reference in their entirety for all purposes), modifications which enhance their efficacy in cells, and removal of stem II bases to shorten RNA synthesis times and reduce chemical requirements.

8. Immunostimulatory Oligonucleotides

[0321] Nucleic acids associated with the 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 such as a human. ISS include, *e.g.*, certain palindromes leading to hairpin secondary structures (*see*, Yamamoto *et al.*, *J. Immunol.*, 148:4072-6 (1992)), or CpG motifs, as well as other known ISS features (such as multi-G domains; *see*; PCT Publication No. WO 96/11266, the disclosure of which is herein incorporated by reference in its entirety for all purposes).

[0322] 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 sequence 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.

[0323] 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 the CpG dinucleotide is methylated. 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 the CpG dinucleotides comprises a methylated cytosine. Examples of immunostimulatory oligonucleotides suitable for use in the compositions and methods of the present invention are described in PCT Publication Nos. WO 02/069369, WO 01/15726, and WO 09/086558; U.S. Patent No. 6,406,705; and Raney *et al.*, *J. Pharm. Exper. Ther.*, 298:1185-92 (2001), the disclosures of which are herein incorporated by reference in their entirety for all purposes. In certain embodiments, the oligonucleotides used in the compositions and methods of the invention have a phosphodiester ("PO") backbone or a phosphorothioate ("PS") backbone, and/or at least one methylated cytosine residue in a CpG motif.

B. Other Active Agents

[0324] In certain embodiments, the active agent associated with the lipid particles of the invention may comprise one or more therapeutic proteins, polypeptides, or small organic molecules or compounds. Non-limiting examples of such therapeutically effective agents or drugs include oncology drugs (*e.g.*, chemotherapy drugs, hormonal therapeutic agents,

immunotherapeutic agents, radiotherapeutic agents, *etc.*), lipid-lowering agents, anti-viral drugs, anti-inflammatory compounds, antidepressants, stimulants, analgesics, antibiotics, birth control medication, antipyretics, vasodilators, anti-angiogenics, cytovascular agents, signal transduction inhibitors, cardiovascular drugs such as anti-arrhythmic agents, hormones, vasoconstrictors, and steroids. These active agents may be administered alone in the lipid particles of the invention, or in combination (*e.g.*, co-administered) with lipid particles of the invention comprising nucleic acid such as interfering RNA.

[0325] Non-limiting examples of chemotherapy drugs include platinum-based drugs (*e.g.*, oxaliplatin, cisplatin, carboplatin, spiroplatin, iproplatin, satraplatin, *etc.*), alkylating agents (*e.g.*, cyclophosphamide, ifosfamide, chlorambucil, busulfan, melphalan, mechlorethamine, uramustine, thiotepa, nitrosoureas, *etc.*), anti-metabolites (*e.g.*, 5-fluorouracil (5-FU), azathioprine, methotrexate, leucovorin, capecitabine, cytarabine, floxuridine, fludarabine, gemcitabine, pemetrexed, raltitrexed, *etc.*), plant alkaloids (*e.g.*, vincristine, vinblastine, vinorelbine, vindesine, podophyllotoxin, paclitaxel (taxol), docetaxel, *etc.*), topoisomerase inhibitors (*e.g.*, irinotecan (CPT-11; Camptosar), topotecan, amsacrine, etoposide (VP16), etoposide phosphate, teniposide, *etc.*), antitumor antibiotics (*e.g.*, doxorubicin, adriamycin, daunorubicin, epirubicin, actinomycin, bleomycin, mitomycin, mitoxantrone, plicamycin, *etc.*), tyrosine kinase inhibitors (*e.g.*, gefitinib (Iressa[®]), sunitinib (Sutent[®]; SU11248), erlotinib (Tarceva[®]; OSI-1774), lapatinib (GW572016; GW2016), canertinib (CI 1033), semaxinib (SU5416), vatalanib (PTK787/ZK222584), sorafenib (BAY 43-9006), imatinib (Gleevec[®]; STI571), dasatinib (BMS-354825), leflunomide (SU101), vandetanib (Zactima[™]; ZD6474), *etc.*), pharmaceutically acceptable salts thereof, stereoisomers thereof, derivatives thereof, analogs thereof, and combinations thereof.

[0326] Examples of conventional hormonal therapeutic agents include, without limitation, steroids (*e.g.*, dexamethasone), finasteride, aromatase inhibitors, tamoxifen, and goserelin as well as other gonadotropin-releasing hormone agonists (GnRH).

[0327] Examples of conventional immunotherapeutic agents include, but are not limited to, immunostimulants (*e.g.*, Bacillus Calmette-Guérin (BCG), levamisole, interleukin-2, alpha-interferon, *etc.*), monoclonal antibodies (*e.g.*, anti-CD20, anti-HER2, anti-CD52, anti-HLA-DR, and anti-VEGF monoclonal antibodies), immunotoxins (*e.g.*, anti-CD33 monoclonal antibody-calicheamicin conjugate, anti-CD22 monoclonal antibody-pseudomonas exotoxin conjugate, *etc.*), and radioimmunotherapy (*e.g.*, anti-CD20 monoclonal antibody conjugated to ¹¹¹In, ⁹⁰Y, or ¹³¹I, *etc.*).

[0328] Examples of conventional radiotherapeutic agents include, but are not limited to, radionuclides such as ⁴⁷Sc, ⁶⁴Cu, ⁶⁷Cu, ⁸⁹Sr, ⁸⁶Y, ⁸⁷Y, ⁹⁰Y, ¹⁰⁵Rh, ¹¹¹Ag, ¹¹¹In, ^{117m}Sn, ¹⁴⁹Pm,

¹⁵³Sm, ¹⁶⁶Ho, ¹⁷⁷Lu, ¹⁸⁶Re, ¹⁸⁸Re, ²¹¹At, and ²¹²Bi, optionally conjugated to antibodies directed against tumor antigens.

[0329] Additional oncology drugs that may be used according to the invention include, but are not limited to, alkeran, allopurinol, altretamine, amifostine, anastrozole, araC, arsenic trioxide, bexarotene, biCNU, carmustine, CCNU, celecoxib, cladribine, cyclosporin A, cytosine arabinoside, cytoxan, dexrazoxane, DTIC, estramustine, exemestane, FK506, gemtuzumab-ozogamicin, hydrea, hydroxyurea, idarubicin, interferon, letrozole, leustatin, leuprolide, litretinoin, megastrol, L-PAM, mesna, methoxsalen, mithramycin, nitrogen mustard, pamidronate, Pegademase, pentostatin, porfimer sodium, prednisone, rituxan, streptozocin, STI-571, taxotere, temozolamide, VM-26, toremifene, tretinoin, ATRA, valrubicin, and velban. 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.

[0330] Non-limiting examples of lipid-lowering agents for treating a lipid disease or disorder associated with elevated triglycerides, cholesterol, and/or glucose include statins, fibrates, ezetimibe, thiazolidinediones, niacin, beta-blockers, nitroglycerin, calcium antagonists, fish oil, and mixtures thereof.

[0331] Examples of anti-viral drugs include, but are not limited to, abacavir, aciclovir, acyclovir, adefovir, amantadine, amprenavir, arbidol, atazanavir, atripla, didanosine, edoxudine, efavirenz, emtricitabine, enfuvirtide, entecavir, entry inhibitors, famciclovir, fixed dose combinations, fomivirsen, fosamprenavir, foscarnet, fosfonet, fusion inhibitors, ganciclovir, ibacitabine, imunovir, idoxuridine, imiquimod, indinavir, inosine, integrase inhibitors, interferon type III (*e.g.*, IFN- λ molecules such as IFN- λ 1, IFN- λ 2, and IFN- λ 3), interferon type II (*e.g.*, IFN- γ), interferon type I (*e.g.*, IFN- α such as PEGylated IFN- α , IFN- β , IFN- κ , IFN- δ , IFN- ϵ , IFN- τ , IFN- ω , and IFN- ζ), interferon, lamivudine, lopinavir, loviride, MK-0518, maraviroc, moroxydine, nelfinavir, nevirapine, nexavir, nucleoside analogues, oseltamivir, penciclovir, peramivir, pleconaril, podophyllotoxin, protease inhibitors, reverse transcriptase inhibitors, ribavirin, rimantadine, ritonavir, saquinavir, stavudine, synergistic enhancers, tenofovir, tenofovir disoproxil, tipranavir, trifluridine, trizivir, tromantadine, truvada, valaciclovir, valganciclovir, vicriviroc, vidarabine, viramidine, zalcitabine, zanamivir, zidovudine, pharmaceutically acceptable salts thereof, stereoisomers thereof, derivatives thereof, analogs thereof, and mixtures thereof.

V. Lipid Particles

[0332] In certain aspects, the present invention provides lipid particles comprising one or more of the cationic (amino) lipids or salts thereof described herein. In some embodiments, the lipid particles of the invention further comprise one or more non-cationic lipids. In other
5 embodiments, the lipid particles further comprise one or more conjugated lipids capable of reducing or inhibiting particle aggregation. In additional embodiments, the lipid particles further comprise one or more active agents or therapeutic agents such as therapeutic nucleic acids (*e.g.*, interfering RNA such as siRNA).

[0333] Lipid particles include, but are not limited to, lipid vesicles such as liposomes. As
10 used herein, a lipid vesicle includes a structure having lipid-containing membranes enclosing an aqueous interior. In particular embodiments, lipid vesicles comprising one or more of the cationic lipids described herein are used to encapsulate nucleic acids within the lipid vesicles. In other embodiments, lipid vesicles comprising one or more of the cationic lipids described herein are complexed with nucleic acids to form lipoplexes.

[0334] The lipid particles of the invention typically comprise an active agent or therapeutic
15 agent, a cationic lipid, a non-cationic lipid, and a conjugated lipid that inhibits aggregation of particles. In some embodiments, the active agent or therapeutic agent is fully encapsulated within the lipid portion of the lipid particle such that the active agent or therapeutic agent in the lipid particle is resistant in aqueous solution to enzymatic degradation, *e.g.*, by a nuclease
20 or protease. In other embodiments, the lipid particles described herein are substantially non-toxic to mammals such as humans. The lipid particles of the invention typically have a mean diameter of from about 30 nm to about 150 nm, from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 to about 90 nm. The lipid particles of the invention also typically
25 have a lipid:therapeutic agent (*e.g.*, lipid:nucleic acid) ratio (mass/mass ratio) of from about 1:1 to about 100:1, from about 1:1 to about 50:1, from about 2:1 to about 25:1, from about 3:1 to about 20:1, from about 5:1 to about 15:1, or from about 5:1 to about 10:1.

[0335] In preferred embodiments, the lipid particles of the invention are serum-stable nucleic acid-lipid particles (SNALP) which comprise an interfering RNA (*e.g.*, siRNA,
30 Dicer-substrate dsRNA, shRNA, aiRNA, and/or miRNA), a cationic lipid (*e.g.*, one or more cationic lipids of Formula I-XII or salts thereof as set forth herein), a non-cationic lipid (*e.g.*, mixtures of one or more phospholipids and cholesterol), and a conjugated lipid that inhibits aggregation of the particles (*e.g.*, one or more PEG-lipid conjugates). The SNALP may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more unmodified and/or modified interfering
35 RNA molecules. Nucleic acid-lipid particles and their method of preparation are described

in, e.g., U.S. Patent Nos. 5,753,613; 5,785,992; 5,705,385; 5,976,567; 5,981,501; 6,110,745; and 6,320,017; and PCT Publication No. WO 96/40964, the disclosures of which are each herein incorporated by reference in their entirety for all purposes.

[0336] In the nucleic acid-lipid particles of the invention, the nucleic acid may be fully encapsulated within the lipid portion of the particle, thereby protecting the nucleic acid from nuclease degradation. In preferred embodiments, a SNALP comprising a nucleic acid such as an interfering RNA is fully encapsulated within the lipid portion of the particle, thereby protecting the nucleic acid from nuclease degradation. In certain instances, the nucleic acid in the SNALP is not substantially degraded after exposure of the particle to a nuclease at 37°C for at least about 20, 30, 45, or 60 minutes. In certain other instances, the nucleic acid in the SNALP is not substantially degraded after incubation of the particle in serum at 37°C for at least about 30, 45, or 60 minutes or at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, or 36 hours. In other embodiments, the nucleic acid is complexed with the lipid portion of the particle. One of the benefits of the formulations of the present invention is that the nucleic acid-lipid particle compositions are substantially non-toxic to mammals such as humans.

[0337] The term “fully encapsulated” indicates that the nucleic acid in the nucleic acid-lipid particle is not significantly degraded after exposure to serum or a nuclease assay that would significantly degrade free DNA or RNA. In a fully encapsulated system, preferably less than about 25% of the nucleic acid in the particle is degraded in a treatment that would normally degrade 100% of free nucleic acid, more preferably less than about 10%, and most preferably less than about 5% of the nucleic acid in the particle is degraded. “Fully encapsulated” also indicates that the nucleic acid-lipid particles are serum-stable, that is, that they do not rapidly decompose into their component parts upon *in vivo* administration.

[0338] In the context of nucleic acids, full encapsulation may be determined by performing a membrane-impermeable fluorescent dye exclusion assay, which uses a dye that has enhanced fluorescence when associated with nucleic acid. Specific dyes such as OliGreen[®] and RiboGreen[®] (Invitrogen Corp.; Carlsbad, CA) are available for the quantitative determination of plasmid DNA, single-stranded deoxyribonucleotides, and/or single- or double-stranded ribonucleotides. Encapsulation is determined by adding the dye to a liposomal formulation, measuring the resulting fluorescence, and comparing it to the fluorescence observed upon addition of a small amount of nonionic detergent. Detergent-mediated disruption of the liposomal bilayer releases the encapsulated nucleic acid, allowing it to interact with the membrane-impermeable dye. Nucleic acid encapsulation may be

calculated as $E = (I_o - I)/I_o$, where I and I_o refer to the fluorescence intensities before and after the addition of detergent (*see, Wheeler et al., Gene Ther.*, 6:271-281 (1999)).

[0339] In other embodiments, the present invention provides a nucleic acid-lipid particle (*e.g.*, SNALP) composition comprising a plurality of nucleic acid-lipid particles.

5 [0340] In some instances, the SNALP composition comprises nucleic acid that is fully encapsulated within the lipid portion of the particles, such that from about 30% to about 100%, from about 40% to about 100%, from about 50% to about 100%, from about 60% to about 100%, from about 70% to about 100%, from about 80% to about 100%, from about 90% to about 100%, from about 30% to about 95%, from about 40% to about 95%, from
10 about 50% to about 95%, from about 60% to about 95%, from about 70% to about 95%, from about 80% to about 95%, from about 85% to about 95%, from about 90% to about 95%, from about 30% to about 90%, from about 40% to about 90%, from about 50% to about 90%, from about 60% to about 90%, from about 70% to about 90%, from about 80% to about 90%, or at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%,
15 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% (or any fraction thereof or range therein) of the particles have the nucleic acid encapsulated therein.

[0341] In other instances, the SNALP composition comprises nucleic acid that is fully encapsulated within the lipid portion of the particles, such that from about 30% to about 100%, from about 40% to about 100%, from about 50% to about 100%, from about 60% to
20 about 100%, from about 70% to about 100%, from about 80% to about 100%, from about 90% to about 100%, from about 30% to about 95%, from about 40% to about 95%, from about 50% to about 95%, from about 60% to about 95%, from about 70% to about 95%, from about 80% to about 95%, from about 85% to about 95%, from about 90% to about 95%, from about 30% to about 90%, from about 40% to about 90%, from about 50% to about 90%, from
25 about 60% to about 90%, from about 70% to about 90%, from about 80% to about 90%, or at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% (or any fraction thereof or range therein) of the input nucleic acid is encapsulated in the particles.

[0342] Depending on the intended use of the lipid particles of the invention, the proportions
30 of the components can be varied and the delivery efficiency of a particular formulation can be measured using, *e.g.*, an endosomal release parameter (ERP) assay.

[0343] In particular embodiments, the present invention provides a lipid particle (*e.g.*, SNALP) composition comprising a plurality of lipid particles described herein and an antioxidant. In certain instances, the antioxidant in the lipid particle composition reduces,
35 prevents, and/or inhibits the degradation of a cationic lipid present in the lipid particle. In

instances wherein the active agent is a therapeutic nucleic acid such as an interfering RNA (e.g., siRNA), the antioxidant in the lipid particle composition reduces, prevents, and/or inhibits the degradation of the nucleic acid payload, e.g., by reducing, preventing, and/or inhibiting the formation of adducts between the nucleic acid and the cationic lipid. Non-

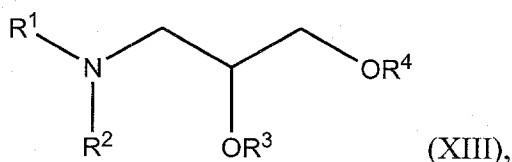
5 limiting examples of antioxidants include hydrophilic antioxidants such as chelating agents (e.g., metal chelators such as ethylenediaminetetraacetic acid (EDTA), citrate, and the like), lipophilic antioxidants (e.g., vitamin E isomers, polyphenols, and the like), salts thereof; and mixtures thereof. If needed, the antioxidant is typically present in an amount sufficient to prevent, inhibit, and/or reduce the degradation of the cationic lipid and/or active agent present
10 in the particle, e.g., at least about 20 mM EDTA or a salt thereof, or at least about 100 mM citrate or a salt thereof. An antioxidant such as EDTA and/or citrate may be included at any step or at multiple steps in the lipid particle formation process described in Section VI (e.g., prior to, during, and/or after lipid particle formation).

[0344] Additional embodiments related to methods of preventing the degradation of
15 cationic lipids and/or active agents (e.g., therapeutic nucleic acids) present in lipid particles, compositions comprising lipid particles stabilized by these methods, methods of making these lipid particles, and methods of delivering and/or administering these lipid particles are described in U.S. Provisional Application No. 61/265,671, entitled "SNALP Formulations Containing Antioxidants," filed December 1, 2009, the disclosure of which is herein
20 incorporated by reference in its entirety for all purposes.

A. Cationic Lipids

[0345] Any of the novel cationic lipids of Formula I-XII or salts thereof as set forth herein may be used in the lipid particles of the present invention (e.g., SNALP), either alone or in combination with one or more other cationic lipid species or non-cationic lipid species.

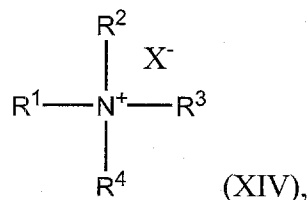
25 [0346] In some embodiments, a mixture of cationic lipids or salts thereof can be included in the lipid particles of the present invention. In these embodiments, the mixture of cationic lipids includes a cationic lipid of Formulas I-XII together with one or more additional cationic lipids. Other cationic lipids suitable for use in combination with the cationic lipids of Formulas I-XII include cationic lipids of Formula XIII having the following structure (or
30 salts thereof):



wherein R^1 and R^2 are independently selected and are H or C_1 - C_3 alkyls, R^3 and R^4 are independently selected and are alkyl groups having from about 10 to about 20 carbon atoms, and at least one of R^3 and R^4 comprises at least two sites of unsaturation. In some instances, R^1 and R^2 are both methyl groups. In certain instances, R^3 and R^4 are both the same, *i.e.*, R^3 and R^4 are both linoleyl (C_{18}), *etc.* In certain other instances, R^3 and R^4 are different, *i.e.*, R^3 is tetradectrienyl (C_{14}) and R^4 is linoleyl (C_{18}). In a preferred embodiment, the cationic lipid of Formula XIII is symmetrical, *i.e.*, R^3 and R^4 are both the same. In another preferred embodiment, both R^3 and R^4 comprise at least two sites of unsaturation. In some embodiments, R^3 and R^4 are independently selected from the group consisting of dodecadienyl, tetradecadienyl, hexadecadienyl, linoleyl, and icosadienyl. In a preferred embodiment, R^3 and R^4 are both linoleyl. In some embodiments, R^3 and R^4 comprise at least three sites of unsaturation and are independently selected from, *e.g.*, dodecatrienyl, tetradectrienyl, hexadecatrienyl, linolenyl, and icosatrienyl. In particular embodiments, the cationic lipid of Formula XIII comprises 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), or mixtures thereof.

[0347] In some embodiments, the cationic lipid of Formula XIII forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula XIII is the oxalate (*e.g.*, hemioxalate) salt thereof, which is preferably a crystalline salt.

[0348] Other cationic lipids suitable for use in combination with the cationic lipids of Formulas I-XII include cationic lipids of Formula XIV having the following structure (or salts thereof):



wherein R^1 and R^2 are independently selected and are H or C_1 - C_3 alkyls, R^3 and R^4 are independently selected and are alkyl groups having from about 10 to about 20 carbon atoms, and at least one of R^3 and R^4 comprises at least two sites of unsaturation. In certain instances, R^3 and R^4 are both the same, *i.e.*, R^3 and R^4 are both linoleyl (C_{18}), *etc.* In certain other instances, R^3 and R^4 are different, *i.e.*, R^3 is tetradectrienyl (C_{14}) and R^4 is linoleyl (C_{18}). In a preferred embodiment, the cationic lipid of Formula XIV is symmetrical, *i.e.*, R^3 and R^4 are both the same. In another preferred embodiment, both R^3 and R^4 comprise at least two sites of unsaturation. In some embodiments, R^3 and R^4 are independently selected from the group

consisting of dodecadienyl, tetradecadienyl, hexadecadienyl, linoleyl, and icosadienyl. In a preferred embodiment, R^3 and R^4 are both linoleyl. In some embodiments, R^3 and R^4 comprise at least three sites of unsaturation and are independently selected from, *e.g.*, dodecatrienyl, tetradectrienyl, hexadecatrienyl, linolenyl, and icosatrienyl.

5 [0349] In some embodiments, the cationic lipid of Formula XIV forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula XIV is the oxalate (*e.g.*, hemioxalate) salt thereof, which is preferably a crystalline salt.

[0350] The synthesis of cationic lipids such as DLinDMA and DLenDMA, as well as
10 additional cationic lipids falling within the scope of Formulas XIII and XIV, is described in U.S. Patent Publication No. 20060083780, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0351] In addition to the cationic lipids of Formulas XIII and XIV, other cationic lipids suitable for use in combination with one or more cationic lipids of Formulas I-XII include,
15 but are not limited to, 1,2-dioeylecarbamoxyloxy-3-dimethylaminopropane (DO-C-DAP), 1,2-dimyristeoyl-3-dimethylaminopropane (DMDAP), 1,2-dioeyle-3-trimethylaminopropane chloride (DOTAP.Cl), dilinoleylmethyl-3-dimethylaminopropionate (DLin-M-K-DMA; also known as DLin-M-DMA), N,N-dioeyle-N,N-dimethylammonium chloride (DODAC), 1,2-dioeyleoxy-N,N-dimethylaminopropane (DODMA), 1,2-distearyleoxy-N,N-
20 dimethylaminopropane (DSDMA), N-(1-(2,3-dioeyleoxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1-(2,3-dioeyleoxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), 3-(N(N',N'-dimethylaminoethane)-carbamoyle)cholesterol (DC-Chol), N-(1,2-dimyristyleoxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE), 2,3-dioeyleoxy-N-
25 [2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), dioctadecylamidoglycyl spermine (DOGS), 3-dimethylamino-2-(cholest-5-en-3-beta-oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane (CLinDMA), 2-[5'-(cholest-5-en-3-beta-oxy)-3'-oxapentoxy]-3-dimethyle-1-(cis,cis-9',1-2'-octadecadienoxy)propane (CpLinDMA), N,N-dimethyl-3,4-dioeyleoxybenzylamine (DMOBA), 1,2-N,N'-
30 dioeylecarbamyl-3-dimethylaminopropane (DOcarbDAP), 1,2-N,N'-dilinoeylecarbamyl-3-dimethylaminopropane (DLincarbDAP), 1,2-dilinoeylecarbamoxyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-dilinoeyleoxy-3-(dimethylamino)acetoxyp propane (DLin-DAC), 1,2-dilinoeyleoxy-3-morpholinopropane (DLin-MA), 1,2-dilinoeyle-3-dimethylaminopropane (DLinDAP), 1,2-dilinoeylethio-3-dimethylaminopropane (DLin-S-
35 DMA), 1-linoeyle-2-linoeyleoxy-3-dimethylaminopropane (DLin-2-DMA), 1,2-

5 dimethylamino)ethoxypropane (DLin-EG-DMA), and mixtures thereof.

10 61/334,104, entitled "Novel Cationic Lipids and Methods of Use Thereof," filed May 12, 2010, and PCT Publication Nos. WO 2010/054401, WO 2010/054405, WO 2010/054406, and WO 2010/054384, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

15 M-K-DMA, as well as additional cationic lipids, is described in PCT Publication No. WO 2010/042877, the disclosure of which is incorporated herein by reference in its entirety for all purposes.

20 DOAP, and DLin-EG-DMA, as well as additional cationic lipids, is described in PCT Publication No. WO 09/086558, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

25 herein incorporated by reference in its entirety for all purposes.

30 commercial preparations of cationic lipids can be used, such as, *e.g.*, LIPOFECTIN[®] (including DOTMA and DOPE, available from GIBCO/BRL); LIPOFECTAMINE[®] (including DOSPA and DOPE, available from GIBCO/BRL); and TRANSFECTAM[®] (including DOGS, available from Promega Corp.).

[0357] In some embodiments, the cationic lipid comprises from about 45 mol % to about 90 mol %, from about 45 mol % to about 85 mol %, from about 45 mol % to about 80 mol %, 35

from about 45 mol % to about 75 mol %, from about 45 mol % to about 70 mol %, from about 45 mol % to about 65 mol %, from about 45 mol % to about 60 mol %, from about 45 mol % to about 55 mol %, from about 50 mol % to about 90 mol %, from about 50 mol % to about 85 mol %, from about 50 mol % to about 80 mol %, from about 50 mol % to about 75 mol %, from about 50 mol % to about 70 mol %, from about 50 mol % to about 65 mol %, from about 50 mol % to about 60 mol %, from about 55 mol % to about 65 mol % or from about 55 mol % to about 70 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0358] In certain preferred embodiments, the cationic lipid comprises from about 50 mol % to about 58 mol %, from about 51 mol % to about 59 mol %, from about 51 mol % to about 58 mol %, from about 51 mol % to about 57 mol %, from about 52 mol % to about 58 mol %, from about 52 mol % to about 57 mol %, from about 52 mol % to about 56 mol %, or from about 53 mol % to about 55 mol % (or any fraction thereof or range therein) of the total lipid present in the particle. In particular embodiments, the cationic lipid comprises about 50 mol %, 51 mol %, 52 mol %, 53 mol %, 54 mol %, 55 mol %, 56 mol %, 57 mol %, 58 mol %, 59 mol %, 60 mol %, 61 mol %, 62 mol %, 63 mol %, 64 mol %, or 65 mol % (or any fraction thereof or range therein) of the total lipid present in the particle. In certain other embodiments, the cationic lipid comprises (at least) about 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, or 90 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0359] In additional embodiments, the cationic lipid comprises from about 2 mol % to about 60 mol %, from about 5 mol % to about 50 mol %, from about 10 mol % to about 50 mol %, from about 20 mol % to about 50 mol %, from about 20 mol % to about 40 mol %, from about 30 mol % to about 40 mol %, or about 40 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0360] Additional percentages and ranges of cationic lipids suitable for use in the lipid particles of the present invention are described in PCT Publication No. WO 09/127060, U.S. Application No. 12/794,701, filed June 4, 2010, and U.S. Application No. _____, entitled "Novel Lipid Formulations for Delivery of Therapeutic Agents to Solid Tumors," filed June 30, 2010, bearing Attorney Docket No. 020801-009610US, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0361] It should be understood that the percentage of cationic lipid present in the lipid particles of the invention is a target amount, and that the actual amount of cationic lipid present in the formulation may vary, for example, by ± 5 mol %. For example, in the 1:57 lipid particle (e.g., SNALP) formulation, the target amount of cationic lipid is 57.1 mol %,

but the actual amount of cationic lipid may be ± 5 mol %, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol % of that target amount, with the balance of the formulation being made up of other lipid components (adding up to 100 mol % of total lipids present in the particle). Similarly, in the 7:54 lipid particle (*e.g.*, SNALP) formulation, the target amount of cationic lipid is 54.06 mol %, but the actual amount of cationic lipid may be ± 5 mol %, ± 4 mol %, ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol % of that target amount, with the balance of the formulation being made up of other lipid components (adding up to 100 mol % of total lipids present in the particle).

B. Non-Cationic Lipids

[0362] The non-cationic lipids used in the lipid particles of the invention (*e.g.*, SNALP) can be any of a variety of neutral uncharged, zwitterionic, or anionic lipids capable of producing a stable complex.

[0363] 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, cerebroside, dicetylphosphate, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoyl-phosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE), palmitoyloleoyl-phosphatidylglycerol (POPG), dioleoylphosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dipalmitoyl-phosphatidylethanolamine (DPPE), dimyristoyl-phosphatidylethanolamine (DMPE), distearoyl-phosphatidylethanolamine (DSPE), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, dielaidoyl-phosphatidylethanolamine (DEPE), stearylloleoyl-phosphatidylethanolamine (SOPE), lysophosphatidylcholine, dilinoleoylphosphatidylcholine, 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 C₁₀-C₂₄ carbon chains, *e.g.*, lauroyl, myristoyl, palmitoyl, stearyl, or oleoyl.

[0364] Additional examples of non-cationic lipids include sterols such as cholesterol and derivatives thereof. Non-limiting examples of cholesterol derivatives include polar analogues such as 5 α -cholestanol, 5 β -coprostanol, cholesteryl-(2'-hydroxy)-ethyl ether, cholesteryl-(4'-hydroxy)-butyl ether, and 6-ketcholestanol; non-polar analogues such as 5 α -cholestane,

cholestenone, 5 α -cholestanone, 5 β -cholestanone, and cholesteryl decanoate; and mixtures thereof. In preferred embodiments, the cholesterol derivative is a polar analogue such as cholesteryl-(4'-hydroxy)-butyl ether. The synthesis of cholesteryl-(2'-hydroxy)-ethyl ether is described in PCT Publication No. WO 09/127060, the disclosure of which is herein

5 incorporated by reference in its entirety for all purposes.

[0365] In some embodiments, the non-cationic lipid present in the lipid particles (*e.g.*, SNALP) comprises or consists of a mixture of one or more phospholipids and cholesterol or a derivative thereof. In other embodiments, the non-cationic lipid present in the lipid particles (*e.g.*, SNALP) comprises or consists of one or more phospholipids, *e.g.*, a cholesterol-free
10 lipid particle formulation. In yet other embodiments, the non-cationic lipid present in the lipid particles (*e.g.*, SNALP) comprises or consists of cholesterol or a derivative thereof, *e.g.*, a phospholipid-free lipid particle formulation.

[0366] Other examples of non-cationic lipids suitable for use in the present invention include nonphosphorous containing lipids such as, *e.g.*, stearylamine, dodecylamine,
15 hexadecylamine, acetyl palmitate, glycerolricinoleate, hexadecyl stearate, isopropyl myristate, amphoteric acrylic polymers, triethanolamine-lauryl sulfate, alkyl-aryl sulfate polyethyloxyated fatty acid amides, dioctadecyldimethyl ammonium bromide, ceramide, sphingomyelin, and the like.

[0367] In some embodiments, the non-cationic lipid comprises from about 10 mol % to
20 about 60 mol %, from about 20 mol % to about 55 mol %, from about 20 mol % to about 45 mol %, from about 20 mol % to about 40 mol %, from about 25 mol % to about 50 mol %, from about 25 mol % to about 45 mol %, from about 30 mol % to about 50 mol %, from about 30 mol % to about 45 mol %, from about 30 mol % to about 40 mol %, from about 35 mol % to about 45 mol %, from about 37 mol % to about 42 mol %, or about 35 mol %, 36
25 mol %, 37 mol %, 38 mol %, 39 mol %, 40 mol %, 41 mol %, 42 mol %, 43 mol %, 44 mol %, or 45 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0368] In embodiments where the lipid particles contain a mixture of phospholipid and cholesterol or a cholesterol derivative, the mixture may comprise up to about 40 mol %, 45
30 mol %, 50 mol %, 55 mol %, or 60 mol % of the total lipid present in the particle.

[0369] In some embodiments, the phospholipid component in the mixture may comprise from about 2 mol % to about 20 mol %, from about 2 mol % to about 15 mol %, from about 2 mol % to about 12 mol %, from about 4 mol % to about 15 mol %, or from about 4 mol % to about 10 mol % (or any fraction thereof or range therein) of the total lipid present in the
35 particle. In certain preferred embodiments, the phospholipid component in the mixture

comprises from about 5 mol % to about 10 mol %, from about 5 mol % to about 9 mol %, from about 5 mol % to about 8 mol %, from about 6 mol % to about 9 mol %, from about 6 mol % to about 8 mol %, or about 5 mol %, 6 mol %, 7 mol %, 8 mol %, 9 mol %, or 10 mol % (or any fraction thereof or range therein) of the total lipid present in the particle. As a non-limiting example, a 1:57 lipid particle formulation comprising a mixture of phospholipid and cholesterol may comprise a phospholipid such as DPPC or DSPC at about 7 mol % (or any fraction thereof), *e.g.*, in a mixture with cholesterol or a cholesterol derivative at about 34 mol % (or any fraction thereof) of the total lipid present in the particle. As another non-limiting example, a 7:54 lipid particle formulation comprising a mixture of phospholipid and cholesterol may comprise a phospholipid such as DPPC or DSPC at about 7 mol % (or any fraction thereof), *e.g.*, in a mixture with cholesterol or a cholesterol derivative at about 32 mol % (or any fraction thereof) of the total lipid present in the particle.

[0370] In other embodiments, the cholesterol component in the mixture may comprise from about 25 mol % to about 45 mol %, from about 25 mol % to about 40 mol %, from about 30 mol % to about 45 mol %, from about 30 mol % to about 40 mol %, from about 27 mol % to about 37 mol %, from about 25 mol % to about 30 mol %, or from about 35 mol % to about 40 mol % (or any fraction thereof or range therein) of the total lipid present in the particle. In certain preferred embodiments, the cholesterol component in the mixture comprises from about 25 mol % to about 35 mol %, from about 27 mol % to about 35 mol %, from about 29 mol % to about 35 mol %, from about 30 mol % to about 35 mol %, from about 30 mol % to about 34 mol %, from about 31 mol % to about 33 mol %, or about 30 mol %, 31 mol %, 32 mol %, 33 mol %, 34 mol %, or 35 mol % (or any fraction thereof or range therein) of the total lipid present in the particle. In other embodiments, the cholesterol component in the mixture comprises about 36, 37, 38, 39, 40, 41, 42, 43, 44, or 45 mol % (or any fraction thereof or range therein) of the total lipid present in the particle. Typically, a 1:57 lipid particle formulation comprising a mixture of phospholipid and cholesterol may comprise cholesterol or a cholesterol derivative at about 34 mol % (or any fraction thereof), *e.g.*, in a mixture with a phospholipid such as DPPC or DSPC at about 7 mol % (or any fraction thereof) of the total lipid present in the particle. Typically, a 7:54 lipid particle formulation comprising a mixture of phospholipid and cholesterol may comprise cholesterol or a cholesterol derivative at about 32 mol % (or any fraction thereof), *e.g.*, in a mixture with a phospholipid such as DPPC or DSPC at about 7 mol % (or any fraction thereof) of the total lipid present in the particle.

[0371] In embodiments where the lipid particles are phospholipid-free, the cholesterol or derivative thereof may comprise up to about 25 mol %, 30 mol %, 35 mol %, 40 mol %, 45 mol %, 50 mol %, 55 mol %, or 60 mol % of the total lipid present in the particle.

[0372] In some embodiments, the cholesterol or derivative thereof in the phospholipid-free lipid particle formulation may comprise from about 25 mol % to about 45 mol %, from about 25 mol % to about 40 mol %, from about 30 mol % to about 45 mol %, from about 30 mol % to about 40 mol %, from about 31 mol % to about 39 mol %, from about 32 mol % to about 38 mol %, from about 33 mol % to about 37 mol %, from about 35 mol % to about 45 mol %, from about 30 mol % to about 35 mol %, from about 35 mol % to about 40 mol %, or about 30 mol %, 31 mol %, 32 mol %, 33 mol %, 34 mol %, 35 mol %, 36 mol %, 37 mol %, 38 mol %, 39 mol %, 40 mol %, 41 mol %, 42 mol %, 43 mol %, 44 mol %, or 45 mol % (or any fraction thereof or range therein) of the total lipid present in the particle. As a non-limiting example, a 1:62 lipid particle formulation may comprise cholesterol at about 37 mol % (or any fraction thereof) of the total lipid present in the particle. As another non-limiting example, a 7:58 lipid particle formulation may comprise cholesterol at about 35 mol % (or any fraction thereof) of the total lipid present in the particle.

[0373] In other embodiments, the non-cationic lipid comprises from about 5 mol % to about 90 mol %, from about 10 mol % to about 85 mol %, from about 20 mol % to about 80 mol %, about 10 mol % (*e.g.*, phospholipid only), or about 60 mol % (*e.g.*, phospholipid and cholesterol or derivative thereof) (or any fraction thereof or range therein) of the total lipid present in the particle.

[0374] Additional percentages and ranges of non-cationic lipids suitable for use in the lipid particles of the present invention are described in PCT Publication No. WO 09/127060, U.S. Application No. 12/794,701, filed June 4, 2010, and U.S. Application No. _____, entitled "Novel Lipid Formulations for Delivery of Therapeutic Agents to Solid Tumors," filed June 30, 2010, bearing Attorney Docket No. 020801-009610US, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0375] It should be understood that the percentage of non-cationic lipid present in the lipid particles of the invention is a target amount, and that the actual amount of non-cationic lipid present in the formulation may vary, for example, by ± 5 mol %. For example, in the 1:57 lipid particle (*e.g.*, SNALP) formulation, the target amount of phospholipid is 7.1 mol % and the target amount of cholesterol is 34.3 mol %, but the actual amount of phospholipid may be ± 2 mol %, ± 1.5 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol % of that target amount, and the actual amount of cholesterol may be ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol % of that target amount,

with the balance of the formulation being made up of other lipid components (adding up to 100 mol % of total lipids present in the particle). Similarly, in the 7:54 lipid particle (*e.g.*, SNALP) formulation, the target amount of phospholipid is 6.75 mol % and the target amount of cholesterol is 32.43 mol %, but the actual amount of phospholipid may be ± 2 mol %, ± 1.5 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol % of that target amount, and the actual amount of cholesterol may be ± 3 mol %, ± 2 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol % of that target amount, with the balance of the formulation being made up of other lipid components (adding up to 100 mol % of total lipids present in the particle).

C. Lipid Conjugates

[0376] In addition to cationic and non-cationic lipids, the lipid particles of the invention (*e.g.*, SNALP) may further comprise a lipid conjugate. The conjugated lipid is useful in that it prevents the aggregation of particles. Suitable conjugated lipids include, but are not limited to, PEG-lipid conjugates, POZ-lipid conjugates, ATTA-lipid conjugates, cationic-polymer-lipid conjugates (CPLs), and mixtures thereof. In certain embodiments, the particles comprise either a PEG-lipid conjugate or an ATTA-lipid conjugate together with a CPL.

[0377] In a preferred embodiment, the lipid conjugate is a PEG-lipid. Examples of PEG-lipids include, but are not limited to, PEG coupled to dialkyloxypropyls (PEG-DAA) as described in, *e.g.*, PCT Publication No. WO 05/026372, PEG coupled to diacylglycerol (PEG-DAG) as described in, *e.g.*, U.S. Patent Publication Nos. 20030077829 and 2005008689, PEG coupled to phospholipids such as phosphatidylethanolamine (PEG-PE), PEG conjugated to ceramides as described in, *e.g.*, U.S. Patent No. 5,885,613, PEG conjugated to cholesterol or a derivative thereof, and mixtures thereof. The disclosures of these patent documents are herein incorporated by reference in their entirety for all purposes.

[0378] Additional PEG-lipids suitable for use in the invention include, without limitation, mPEG2000-1,2-di-O-alkyl-*sn*3-carbomoylglyceride (PEG-C-DOMG). The synthesis of PEG-C-DOMG is described in PCT Publication No. WO 09/086558, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Yet additional suitable PEG-lipid conjugates include, without limitation, 1-[8'-(1,2-dimyristoyl-3-propanoxy)-carboxamido-3',6'-dioxaoctanyl]carbomoyl- ω -methyl-poly(ethylene glycol) (2KPEG-DMG). The synthesis of 2KPEG-DMG is described in U.S. Patent No. 7,404,969, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0379] PEG is a linear, water-soluble polymer of ethylene PEG repeating units with two terminal hydroxyl groups. PEGs are classified by their molecular weights; for example, PEG

2000 has an average molecular weight of about 2,000 daltons, and PEG 5000 has an average molecular weight of about 5,000 daltons. PEGs are commercially available from Sigma Chemical Co. and other companies and include, but are not limited to, the following:

monomethoxypolyethylene glycol (MePEG-OH), monomethoxypolyethylene glycol-

succinate (MePEG-S), monomethoxypolyethylene glycol-succinimidyl succinate (MePEG-S-NHS), monomethoxypolyethylene glycol-amine (MePEG-NH₂), monomethoxypolyethylene glycol-tresylate (MePEG-TRES), monomethoxypolyethylene glycol-imidazolyl-carbonyl (MePEG-IM), as well as such compounds containing a terminal hydroxyl group instead of a terminal methoxy group (*e.g.*, HO-PEG-S, HO-PEG-S-NHS, HO-PEG-NH₂, *etc.*). Other

PEGs such as those described in U.S. Patent Nos. 6,774,180 and 7,053,150 (*e.g.*, mPEG (20 KDa) amine) are also useful for preparing the PEG-lipid conjugates of the present invention. The disclosures of these patents are herein incorporated by reference in their entirety for all purposes. In addition, monomethoxypolyethyleneglycol-acetic acid (MePEG-CH₂COOH) is particularly useful for preparing PEG-lipid conjugates including, *e.g.*, PEG-DAA conjugates.

[0380] The PEG moiety of the PEG-lipid conjugates described herein may comprise an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain instances, the PEG moiety has an average molecular weight of from about 750 daltons to about 5,000 daltons (*e.g.*, from about 1,000 daltons to about 5,000 daltons, from about 1,500 daltons to about 3,000 daltons, from about 750 daltons to about 3,000 daltons, from about 750 daltons to about 2,000 daltons, *etc.*). In other instances, the PEG moiety has an average molecular weight of from about 550 daltons to about 1000 daltons, from about 250 daltons to about 1000 daltons, from about 400 daltons to about 1000 daltons, from about 600 daltons to about 900 daltons, from about 700 daltons to about 800 daltons, or about 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, or 1000 daltons. In preferred embodiments, the PEG moiety has an average molecular weight of about 2,000 daltons or about 750 daltons.

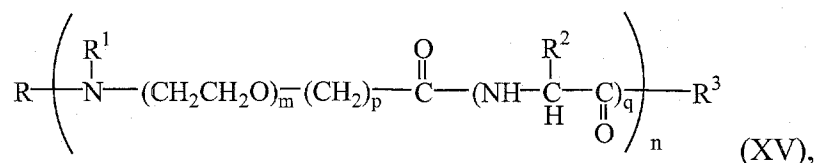
[0381] In certain instances, the PEG can be optionally substituted by an alkyl, alkoxy, acyl, or aryl group. The PEG can be conjugated directly to the lipid or may be linked to the lipid via a linker moiety. Any linker moiety suitable for coupling the PEG to a lipid can be used including, *e.g.*, non-ester containing linker moieties and ester-containing linker moieties. In a preferred embodiment, the linker moiety is a non-ester containing linker moiety. As used herein, the term "non-ester containing linker moiety" refers to a linker moiety that does not contain a carboxylic ester bond (-OC(O)-). Suitable non-ester containing linker moieties include, but are not limited to, amido (-C(O)NH-), amino (-NR-), carbonyl (-C(O)-), carbamate (-NHC(O)O-), urea (-NHC(O)NH-), disulphide (-S-S-), ether (-O-), succinyl (-

(O)CCH₂CH₂C(O)-), succinamidyl (-NHC(O)CH₂CH₂C(O)NH-), ether, disulphide, as well as combinations thereof (such as a linker containing both a carbamate linker moiety and an amido linker moiety). In a preferred embodiment, a carbamate linker is used to couple the PEG to the lipid.

- 5 [0382] In other embodiments, an ester containing linker moiety is used to couple the PEG to the lipid. Suitable ester containing linker moieties include, *e.g.*, carbonate (-OC(O)O-), succinoyl, phosphate esters (-O-(O)POH-O-), sulfonate esters, and combinations thereof.

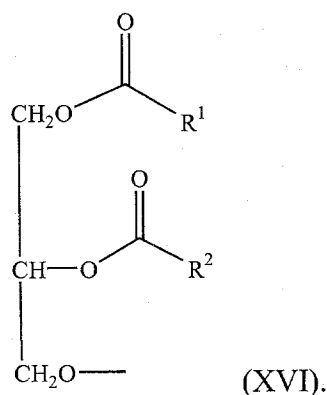
- [0383] Phosphatidylethanolamines having a variety of acyl chain groups of varying chain lengths and degrees of saturation can be conjugated to PEG to form the lipid conjugate. Such
 10 phosphatidylethanolamines are commercially available, or can be isolated or synthesized using conventional techniques known to those of skilled in the art. Phosphatidyl-ethanolamines containing saturated or unsaturated fatty acids with carbon chain lengths in the range of C₁₀ to C₂₀ are preferred. Phosphatidylethanolamines with mono- or diunsaturated fatty acids and mixtures of saturated and unsaturated fatty acids can also be used. Suitable
 15 phosphatidylethanolamines include, but are not limited to, dimyristoyl-phosphatidylethanolamine (DMPE), dipalmitoyl-phosphatidylethanolamine (DPPE), dioleoylphosphatidylethanolamine (DOPE), and distearoyl-phosphatidylethanolamine (DSPE).

- [0384] The term "ATTA" or "polyamide" includes, without limitation, compounds
 20 described in U.S. Patent Nos. 6,320,017 and 6,586,559, the disclosures of which are herein incorporated by reference in their entirety for all purposes. These compounds include a compound having the formula:

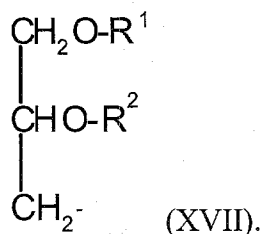


- wherein R is a member selected from the group consisting of hydrogen, alkyl and acyl; R¹ is
 25 a member selected from the group consisting of hydrogen and alkyl; or optionally, R and R¹ and the nitrogen to which they are bound form an azido moiety; R² is a member of the group selected from hydrogen, optionally substituted alkyl, optionally substituted aryl and a side chain of an amino acid; R³ is a member selected from the group consisting of hydrogen, halogen, hydroxy, alkoxy, mercapto, hydrazino, amino and NR⁴R⁵, wherein R⁴ and R⁵ are
 30 independently hydrogen or alkyl; n is 4 to 80; m is 2 to 6; p is 1 to 4; and q is 0 or 1. It will be apparent to those of skill in the art that other polyamides can be used in the compounds of the present invention.

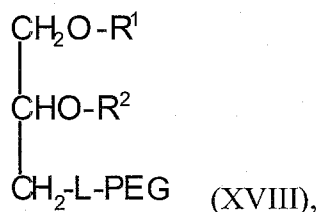
[0385] The term “diacylglycerol” or “DAG” includes a compound having 2 fatty acyl chains, R^1 and R^2 , both of which have independently between 2 and 30 carbons bonded to the 1- and 2-position of glycerol by ester linkages. The acyl groups can be saturated or have varying degrees of unsaturation. Suitable acyl groups include, but are not limited to, lauroyl (C_{12}), myristoyl (C_{14}), palmitoyl (C_{16}), stearoyl (C_{18}), and icosoyl (C_{20}). In preferred embodiments, R^1 and R^2 are the same, *i.e.*, R^1 and R^2 are both myristoyl (*i.e.*, dimyristoyl), R^1 and R^2 are both stearoyl (*i.e.*, distearoyl), *etc.* Diacylglycerols have the following general formula:



10 [0386] The term “dialkyloxypropyl” or “DAA” includes a compound having 2 alkyl chains, R^1 and R^2 , both of which have independently between 2 and 30 carbons. The alkyl groups can be saturated or have varying degrees of unsaturation. Dialkyloxypropyls have the following general formula:



15 [0387] In a preferred embodiment, the PEG-lipid is a PEG-DAA conjugate having the following formula:



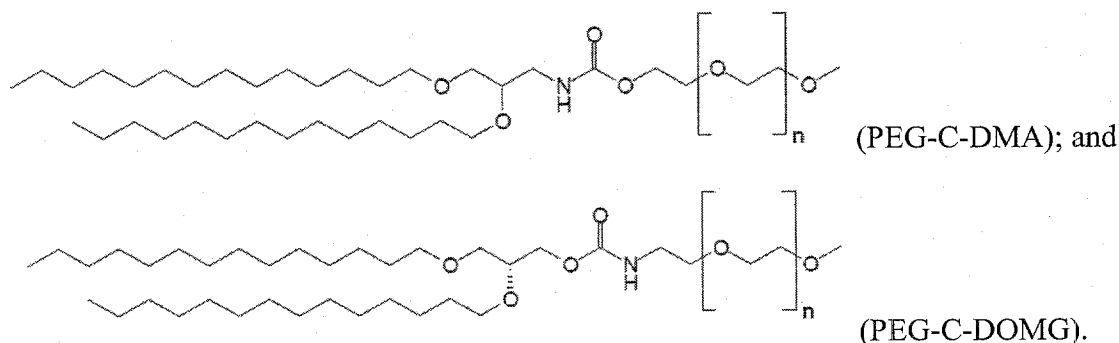
wherein R^1 and R^2 are independently selected and are long-chain alkyl groups having from about 10 to about 22 carbon atoms; PEG is a polyethyleneglycol; and L is a non-ester containing linker moiety or an ester containing linker moiety as described above. The long-

chain alkyl groups can be saturated or unsaturated. Suitable alkyl groups include, but are not limited to, decyl (C₁₀), lauryl (C₁₂), myristyl (C₁₄), palmityl (C₁₆), stearyl (C₁₈), and icosyl (C₂₀). In preferred embodiments, R¹ and R² are the same, *i.e.*, R¹ and R² are both myristyl (*i.e.*, dimyristyl), R¹ and R² are both stearyl (*i.e.*, distearyl), *etc.*

5 [0388] In Formula XVIII above, the PEG has an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain instances, the PEG has an average molecular weight of from about 750 daltons to about 5,000 daltons (*e.g.*, from about 1,000 daltons to about 5,000 daltons, from about 1,500 daltons to about 3,000 daltons, from about 750 daltons to about 3,000 daltons, from about 750 daltons to about 2,000 daltons, *etc.*). In
10 other instances, the PEG moiety has an average molecular weight of from about 550 daltons to about 1000 daltons, from about 250 daltons to about 1000 daltons, from about 400 daltons to about 1000 daltons, from about 600 daltons to about 900 daltons, from about 700 daltons to about 800 daltons, or about 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, or 1000 daltons. In preferred embodiments, the PEG has an average
15 molecular weight of about 2,000 daltons or about 750 daltons. The PEG can be optionally substituted with alkyl, alkoxy, acyl, or aryl groups. In certain embodiments, the terminal hydroxyl group is substituted with a methoxy or methyl group.

[0389] In a preferred embodiment, "L" is a non-ester containing linker moiety. Suitable non-ester containing linkers include, but are not limited to, an amido linker moiety, an amino
20 linker moiety, a carbonyl linker moiety, a carbamate linker moiety, a urea linker moiety, an ether linker moiety, a disulphide linker moiety, a succinamidyl linker moiety, and combinations thereof. In a preferred embodiment, the non-ester containing linker moiety is a carbamate linker moiety (*i.e.*, a PEG-C-DAA conjugate). In another preferred embodiment, the non-ester containing linker moiety is an amido linker moiety (*i.e.*, a PEG-A-DAA
25 conjugate). In yet another preferred embodiment, the non-ester containing linker moiety is a succinamidyl linker moiety (*i.e.*, a PEG-S-DAA conjugate).

[0390] In particular embodiments, the PEG-lipid conjugate is selected from:



[0391] The PEG-DAA conjugates are synthesized using standard techniques and reagents known to those of skill in the art. It will be recognized that the PEG-DAA conjugates will contain various amide, amine, ether, thio, carbamate, and urea linkages. Those of skill in the art will recognize that methods and reagents for forming these bonds are well known and readily available. *See, e.g.,* March, ADVANCED ORGANIC CHEMISTRY (Wiley 1992); Larock, COMPREHENSIVE ORGANIC TRANSFORMATIONS (VCH 1989); and Furniss, VOGEL'S TEXTBOOK OF PRACTICAL ORGANIC CHEMISTRY, 5th ed. (Longman 1989). It will also be appreciated that any functional groups present may require protection and deprotection at different points in the synthesis of the PEG-DAA conjugates. Those of skill in the art will recognize that such techniques are well known. *See, e.g.,* Green and Wuts, PROTECTIVE GROUPS IN ORGANIC SYNTHESIS (Wiley 1991).

[0392] Preferably, the PEG-DAA conjugate is a PEG-didecyloxypropyl (C₁₀) conjugate, a PEG-dilauryloxypropyl (C₁₂) conjugate, a PEG-dimyristyloxypropyl (C₁₄) conjugate, a PEG-dipalmitoyloxypropyl (C₁₆) conjugate, or a PEG-distearoyloxypropyl (C₁₈) conjugate. In these embodiments, the PEG preferably has an average molecular weight of about 750 or about 2,000 daltons. In one particularly preferred embodiment, the PEG-lipid conjugate comprises PEG2000-C-DMA, wherein the "2000" denotes the average molecular weight of the PEG, the "C" denotes a carbamate linker moiety, and the "DMA" denotes dimyristyloxypropyl. In another particularly preferred embodiment, the PEG-lipid conjugate comprises PEG750-C-DMA, wherein the "750" denotes the average molecular weight of the PEG, the "C" denotes a carbamate linker moiety, and the "DMA" denotes dimyristyloxypropyl. In particular embodiments, the terminal hydroxyl group of the PEG is substituted with a methyl group. Those of skill in the art will readily appreciate that other dialkyloxypropyls can be used in the PEG-DAA conjugates of the present invention.

[0393] In addition to the foregoing, it will be readily apparent to those of skill in the art that other hydrophilic polymers can be used in place of PEG. Examples of suitable polymers that can be used in place of PEG include, but are not limited to, polyvinylpyrrolidone, polymethyloxazoline, polyethyloxazoline, polyhydroxypropyl methacrylamide, polymethacrylamide and polydimethylacrylamide, polylactic acid, polyglycolic acid, and derivatized celluloses such as hydroxymethylcellulose or hydroxyethylcellulose.

[0394] In addition to the foregoing components, the lipid particles (*e.g.*, SNALP) of the present invention can further comprise cationic poly(ethylene glycol) (PEG) lipids or CPLs (*see, e.g.,* Chen *et al.*, *Bioconj. Chem.*, 11:433-437 (2000); U.S. Patent No. 6,852,334; PCT Publication No. WO 00/62813, the disclosures of which are herein incorporated by reference in their entirety for all purposes).

[0395] Suitable CPLs include compounds of Formula XIX:



wherein A, W, and Y are as described below.

[0396] With reference to Formula XIX, "A" is a lipid moiety such as an amphipathic lipid, a neutral lipid, or a hydrophobic lipid that acts as a lipid anchor. Suitable lipid examples include, but are not limited to, diacylglycerols, dialkylglycerols, N,N-dialkylamines, 1,2-diacyloxy-3-aminopropanes, and 1,2-dialkyl-3-aminopropanes.

[0397] "W" is a polymer or an oligomer such as a hydrophilic polymer or oligomer. Preferably, the hydrophilic polymer is a biocompatible polymer that is nonimmunogenic or possesses low inherent immunogenicity. Alternatively, the hydrophilic polymer can be weakly antigenic if used with appropriate adjuvants. Suitable nonimmunogenic polymers include, but are not limited to, PEG, polyamides, polylactic acid, polyglycolic acid, polylactic acid/polyglycolic acid copolymers, and combinations thereof. In a preferred embodiment, the polymer has a molecular weight of from about 250 to about 7,000 daltons.

[0398] "Y" is a polycationic moiety. The term polycationic moiety refers to a compound, derivative, or functional group having a positive charge, preferably at least 2 positive charges at a selected pH, preferably physiological pH. Suitable polycationic moieties include basic amino acids and their derivatives such as arginine, asparagine, glutamine, lysine, and histidine; spermine; spermidine; cationic dendrimers; polyamines; polyamine sugars; and amino polysaccharides. The polycationic moieties can be linear, such as linear tetralysine, branched or dendrimeric in structure. Polycationic moieties have between about 2 to about 15 positive charges, preferably between about 2 to about 12 positive charges, and more preferably between about 2 to about 8 positive charges at selected pH values. The selection of which polycationic moiety to employ may be determined by the type of particle application which is desired.

[0399] The charges on the polycationic moieties can be either distributed around the entire particle moiety, or alternatively, they can be a discrete concentration of charge density in one particular area of the particle moiety *e.g.*, a charge spike. If the charge density is distributed on the particle, the charge density can be equally distributed or unequally distributed. All variations of charge distribution of the polycationic moiety are encompassed by the present invention.

[0400] The lipid "A" and the nonimmunogenic polymer "W" can be attached by various methods and preferably by covalent attachment. Methods known to those of skill in the art can be used for the covalent attachment of "A" and "W." Suitable linkages include, but are not limited to, amide, amine, carboxyl, carbonate, carbamate, ester, and hydrazone linkages.

It will be apparent to those skilled in the art that "A" and "W" must have complementary functional groups to effectuate the linkage. The reaction of these two groups, one on the lipid and the other on the polymer, will provide the desired linkage. For example, when the lipid is a diacylglycerol and the terminal hydroxyl is activated, for instance with NHS and DCC, to form an active ester, and is then reacted with a polymer which contains an amino group, such as with a polyamide (*see, e.g.*, U.S. Patent Nos. 6,320,017 and 6,586,559, the disclosures of which are herein incorporated by reference in their entirety for all purposes), an amide bond will form between the two groups.

[0401] In certain instances, the polycationic moiety can have a ligand attached, such as a targeting ligand or a chelating moiety for complexing calcium. Preferably, after the ligand is attached, the cationic moiety maintains a positive charge. In certain instances, the ligand that is attached has a positive charge. Suitable ligands include, but are not limited to, a compound or device with a reactive functional group and include lipids, amphipathic lipids, carrier compounds, bioaffinity compounds, biomaterials, biopolymers, biomedical devices, analytically detectable compounds, therapeutically active compounds, enzymes, peptides, proteins, antibodies, immune stimulators, radiolabels, fluorogens, biotin, drugs, haptens, DNA, RNA, polysaccharides, liposomes, virosomes, micelles, immunoglobulins, functional groups, other targeting moieties, or toxins.

[0402] In some embodiments, the lipid conjugate (*e.g.*, PEG-lipid) comprises from about 0.1 mol % to about 2 mol %, from about 0.5 mol % to about 2 mol %, from about 1 mol % to about 2 mol %, from about 0.6 mol % to about 1.9 mol %, from about 0.7 mol % to about 1.8 mol %, from about 0.8 mol % to about 1.7 mol %, from about 0.9 mol % to about 1.6 mol %, from about 0.9 mol % to about 1.8 mol %, from about 1 mol % to about 1.8 mol %, from about 1 mol % to about 1.7 mol %, from about 1.2 mol % to about 1.8 mol %, from about 1.2 mol % to about 1.7 mol %, from about 1.3 mol % to about 1.6 mol %, from about 1.4 mol % to about 1.5 mol %, or about 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, or 2 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0403] In other embodiments, the lipid conjugate (*e.g.*, PEG-lipid) comprises from about 0 mol % to about 20 mol %, from about 0.5 mol % to about 20 mol %, from about 2 mol % to about 20 mol %, from about 1.5 mol % to about 18 mol %, from about 2 mol % to about 15 mol %, from about 4 mol % to about 15 mol %, from about 2 mol % to about 12 mol %, from about 5 mol % to about 12 mol %, or about 2 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0404] In further embodiments, the lipid conjugate (*e.g.*, PEG-lipid) comprises from about 4 mol % to about 10 mol %, from about 5 mol % to about 10 mol %, from about 5 mol % to

about 9 mol %, from about 5 mol % to about 8 mol %, from about 6 mol % to about 9 mol %, from about 6 mol % to about 8 mol %, or about 5 mol %, 6 mol %, 7 mol %, 8 mol %, 9 mol %, or 10 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

5 [0405] Additional examples, percentages, and/or ranges of lipid conjugates suitable for use in the lipid particles of the invention are described in PCT Publication No. WO 09/127060, U.S. Patent Application No. 12/794,701, filed June 4, 2010, U.S. Application No. _____, entitled "Novel Lipid Formulations for Delivery of Therapeutic Agents to Solid Tumors," filed June 30, 2010, bearing Attorney Docket No. 020801-009610US, U.S. Provisional
10 Application No. 61/294,828, filed January 13, 2010, U.S. Provisional Application No. 61/295, 140, filed January 14, 2010, and PCT Publication No. WO 2010/006282, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0406] It should be understood that the percentage of lipid conjugate (*e.g.*, PEG-lipid) present in the lipid particles of the invention is a target amount, and that the actual amount of
15 lipid conjugate present in the formulation may vary, for example, by ± 2 mol %. For example, in the 1:57 lipid particle (*e.g.*, SNALP) formulation, the target amount of lipid conjugate is 1.4 mol %, but the actual amount of lipid conjugate may be ± 0.5 mol %, ± 0.4 mol %, ± 0.3 mol %, ± 0.2 mol %, ± 0.1 mol %, or ± 0.05 mol % of that target amount, with the balance of the formulation being made up of other lipid components (adding up to 100
20 mol % of total lipids present in the particle). Similarly, in the 7:54 lipid particle (*e.g.*, SNALP) formulation, the target amount of lipid conjugate is 6.76 mol %, but the actual amount of lipid conjugate may be ± 2 mol %, ± 1.5 mol %, ± 1 mol %, ± 0.75 mol %, ± 0.5 mol %, ± 0.25 mol %, or ± 0.1 mol % of that target amount, with the balance of the formulation being made up of other lipid components (adding up to 100 mol % of total lipids
25 present in the particle).

[0407] One of ordinary skill in the art will appreciate that the concentration of the lipid conjugate can be varied depending on the lipid conjugate employed and the rate at which the lipid particle is to become fusogenic.

[0408] By controlling the composition and concentration of the lipid conjugate, one can
30 control the rate at which the lipid conjugate exchanges out of the lipid particle and, in turn, the rate at which the lipid particle becomes fusogenic. For instance, when a PEG-DAA conjugate is used as the lipid conjugate, the rate at which the lipid particle becomes fusogenic can be varied, for example, by varying the concentration of the lipid conjugate, by varying the molecular weight of the PEG, or by varying the chain length and degree of saturation of
35 the alkyl groups on the PEG-DAA conjugate. In addition, other variables including, for

example, pH, temperature, ionic strength, *etc.* can be used to vary and/or control the rate at which the lipid particle becomes fusogenic. Other methods which can be used to control the rate at which the lipid particle becomes fusogenic will become apparent to those of skill in the art upon reading this disclosure. Also, by controlling the composition and concentration of the lipid conjugate, one can control the lipid particle (*e.g.*, SNALP) size.

VI. Preparation of Lipid Particles

[0409] The lipid particles of the present invention, *e.g.*, SNALP, in which an active agent or therapeutic agent such as an interfering RNA (*e.g.*, siRNA) is entrapped within the lipid portion of the particle and is protected from degradation, can be formed by any method known in the art including, but not limited to, a continuous mixing method, a direct dilution process, and an in-line dilution process.

[0410] In particular embodiments, the cationic lipids may comprise lipids of Formulas I-XII or salts thereof, alone or in combination with other cationic lipids. In other embodiments, the non-cationic lipids are egg sphingomyelin (ESM), distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), dipalmitoyl-phosphatidylcholine (DPPC), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, 14:0 PE (1,2-dimyristoyl-phosphatidylethanolamine (DMPE)), 16:0 PE (1,2-dipalmitoyl-phosphatidylethanolamine (DPPE)), 18:0 PE (1,2-distearoyl-phosphatidylethanolamine (DSPE)), 18:1 PE (1,2-dioleoyl-phosphatidylethanolamine (DOPE)), 18:1 trans PE (1,2-dielaidoyl-phosphatidylethanolamine (DEPE)), 18:0-18:1 PE (1-stearoyl-2-oleoyl-phosphatidylethanolamine (SOPE)), 16:0-18:1 PE (1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE)), polyethylene glycol-based polymers (*e.g.*, PEG 2000, PEG 5000, PEG-modified diacylglycerols, or PEG-modified dialkyloxypropyls), cholesterol, derivatives thereof, or combinations thereof.

[0411] In certain embodiments, the present invention provides nucleic acid-lipid particles (*e.g.*, SNALP) produced via a continuous mixing method, *e.g.*, a process that includes providing an aqueous solution comprising a nucleic acid (*e.g.*, interfering RNA) in a first reservoir, providing an organic lipid solution in a second reservoir (wherein the lipids present in the organic lipid solution are solubilized in an organic solvent, *e.g.*, a lower alkanol such as ethanol), and mixing the aqueous solution with the organic lipid solution such that the organic lipid solution mixes with the aqueous solution so as to substantially instantaneously produce a lipid vesicle (*e.g.*, liposome) encapsulating the nucleic acid within the lipid vesicle. This process and the apparatus for carrying out this process are described in detail in U.S.

Patent Publication No. 20040142025, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0412] The action of continuously introducing lipid and buffer solutions into a mixing environment, such as in a mixing chamber, causes a continuous dilution of the lipid solution with the buffer solution, thereby producing a lipid vesicle substantially instantaneously upon mixing. As used herein, the phrase “continuously diluting a lipid solution with a buffer solution” (and variations) generally means that the lipid solution is diluted sufficiently rapidly in a hydration process with sufficient force to effectuate vesicle generation. By mixing the aqueous solution comprising a nucleic acid with the organic lipid solution, the organic lipid solution undergoes a continuous stepwise dilution in the presence of the buffer solution (*i.e.*, aqueous solution) to produce a nucleic acid-lipid particle.

[0413] The nucleic acid-lipid particles formed using the continuous mixing method typically have a size of from about 30 nm to about 150 nm, from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, from about 70 nm to about 100 nm, from about 80 nm to about 100 nm, from about 90 nm to about 100 nm, from about 70 to about 90 nm, from about 80 nm to about 90 nm, from about 70 nm to about 80 nm, less than about 120 nm, 110 nm, 100 nm, 90 nm, or 80 nm, or about 30 nm, 35 nm, 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 105 nm, 110 nm, 115 nm, 120 nm, 125 nm, 130 nm, 135 nm, 140 nm, 145 nm, or 150 nm (or any fraction thereof or range therein). The particles thus formed do not aggregate and are optionally sized to achieve a uniform particle size.

[0414] In another embodiment, the present invention provides nucleic acid-lipid particles (*e.g.*, SNALP) produced via a direct dilution process that includes forming a lipid vesicle (*e.g.*, liposome) solution and immediately and directly introducing the lipid vesicle solution into a collection vessel containing a controlled amount of dilution buffer. In preferred aspects, the collection vessel includes one or more elements configured to stir the contents of the collection vessel to facilitate dilution. In one aspect, the amount of dilution buffer present in the collection vessel is substantially equal to the volume of lipid vesicle solution introduced thereto. As a non-limiting example, a lipid vesicle solution in 45% ethanol when introduced into the collection vessel containing an equal volume of dilution buffer will advantageously yield smaller particles.

[0415] In yet another embodiment, the present invention provides nucleic acid-lipid particles (*e.g.*, SNALP) produced via an in-line dilution process in which a third reservoir containing dilution buffer is fluidly coupled to a second mixing region. In this embodiment,

the lipid vesicle (*e.g.*, liposome) solution formed in a first mixing region is immediately and directly mixed with dilution buffer in the second mixing region. In preferred aspects, the second mixing region includes a T-connector arranged so that the lipid vesicle solution and the dilution buffer flows meet as opposing 180° flows; however, connectors providing shallower angles can be used, *e.g.*, from about 27° to about 180° (*e.g.*, about 90°). A pump mechanism delivers a controllable flow of buffer to the second mixing region. In one aspect, the flow rate of dilution buffer provided to the second mixing region is controlled to be substantially equal to the flow rate of lipid vesicle solution introduced thereto from the first mixing region. This embodiment advantageously allows for more control of the flow of dilution buffer mixing with the lipid vesicle solution in the second mixing region, and therefore also the concentration of lipid vesicle solution in buffer throughout the second mixing process. Such control of the dilution buffer flow rate advantageously allows for small particle size formation at reduced concentrations.

[0416] These processes and the apparatuses for carrying out these direct dilution and in-line dilution processes are described in detail in U.S. Patent Publication No. 20070042031, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0417] The nucleic acid-lipid particles formed using the direct dilution and in-line dilution processes typically have a size of from about 30 nm to about 150 nm, from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, from about 70 nm to about 100 nm, from about 80 nm to about 100 nm, from about 90 nm to about 100 nm, from about 70 to about 90 nm, from about 80 nm to about 90 nm, from about 70 nm to about 80 nm, less than about 120 nm, 110 nm, 100 nm, 90 nm, or 80 nm, or about 30 nm, 35 nm, 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 105 nm, 110 nm, 115 nm, 120 nm, 125 nm, 130 nm, 135 nm, 140 nm, 145 nm, or 150 nm (or any fraction thereof or range therein). The particles thus formed do not aggregate and are optionally sized to achieve a uniform particle size.

[0418] If needed, the lipid particles of the invention (*e.g.*, SNALP) can be sized by any of the methods available for sizing liposomes. The sizing may be conducted in order to achieve a desired size range and relatively narrow distribution of particle sizes.

[0419] Several techniques are available for sizing the particles to a desired size. One sizing method, used for liposomes and equally applicable to the present particles, is described in U.S. Patent No. 4,737,323, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Sonicating a particle suspension either by bath or probe sonication produces a progressive size reduction down to particles of less than about 50 nm in size.

Homogenization is another method which relies on shearing energy to fragment larger particles into smaller ones. In a typical homogenization procedure, particles are recirculated through a standard emulsion homogenizer until selected particle sizes, typically between about 60 and about 80 nm, are observed. In both methods, the particle size distribution can be monitored by conventional laser-beam particle size discrimination, or QELS.

[0420] Extrusion of the particles through a small-pore polycarbonate membrane or an asymmetric ceramic membrane is also an effective method for reducing particle sizes to a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired particle size distribution is achieved. The particles may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in size.

[0421] In some embodiments, the nucleic acids present in the particles are precondensed as described in, *e.g.*, U.S. Patent Application No. 09/744,103, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0422] In other embodiments, the methods may further comprise adding non-lipid polycations which are useful to effect the lipofection of cells using the present compositions. Examples of suitable non-lipid polycations include, hexadimethrine bromide (sold under the brand name POLYBRENE[®], from Aldrich Chemical Co., Milwaukee, Wisconsin, USA) or other salts of hexadimethrine. Other suitable polycations include, for example, salts of poly-L-ornithine, poly-L-arginine, poly-L-lysine, poly-D-lysine, polyallylamine, and polyethyleneimine. Addition of these salts is preferably after the particles have been formed.

[0423] In some embodiments, the nucleic acid to lipid ratios (mass/mass ratios) in a formed nucleic acid-lipid particle (*e.g.*, SNALP) will range from about 0.01 to about 0.2, from about 0.05 to about 0.2, from about 0.02 to about 0.1, from about 0.03 to about 0.1, or from about 0.01 to about 0.08. The ratio of the starting materials (input) also falls within this range. In other embodiments, the particle preparation uses about 400 µg nucleic acid per 10 mg total lipid or a nucleic acid to lipid mass ratio of about 0.01 to about 0.08 and, more preferably, about 0.04, which corresponds to 1.25 mg of total lipid per 50 µg of nucleic acid. In other preferred embodiments, the particle has a nucleic acid:lipid mass ratio of about 0.08.

[0424] In other embodiments, the lipid to nucleic acid ratios (mass/mass ratios) in a formed nucleic acid-lipid particle (*e.g.*, SNALP) will range from about 1 (1:1) to about 100 (100:1), from about 5 (5:1) to about 100 (100:1), from about 1 (1:1) to about 50 (50:1), from about 2 (2:1) to about 50 (50:1), from about 3 (3:1) to about 50 (50:1), from about 4 (4:1) to about 50 (50:1), from about 5 (5:1) to about 50 (50:1), from about 1 (1:1) to about 25 (25:1), from about 2 (2:1) to about 25 (25:1), from about 3 (3:1) to about 25 (25:1), from about 4 (4:1) to

about 25 (25:1), from about 5 (5:1) to about 25 (25:1), from about 5 (5:1) to about 20 (20:1), from about 5 (5:1) to about 15 (15:1), from about 5 (5:1) to about 10 (10:1), or about 5 (5:1), 6 (6:1), 7 (7:1), 8 (8:1), 9 (9:1), 10 (10:1), 11 (11:1), 12 (12:1), 13 (13:1), 14 (14:1), 15 (15:1), 16 (16:1), 17 (17:1), 18 (18:1), 19 (19:1), 20 (20:1), 21 (21:1), 22 (22:1), 23 (23:1), 24 (24:1), or 25 (25:1), or any fraction thereof or range therein. The ratio of the starting materials (input) also falls within this range.

[0425] As previously discussed, the conjugated lipid may further include a CPL. A variety of general methods for making SNALP-CPLs (CPL-containing SNALP) are discussed herein. Two general techniques include the “post-insertion” technique, that is, insertion of a CPL into, for example, a pre-formed SNALP, and the “standard” technique, wherein the CPL is included in the lipid mixture during, for example, the SNALP formation steps. The post-insertion technique results in SNALP having CPLs mainly in the external face of the SNALP bilayer membrane, whereas standard techniques provide SNALP having CPLs on both internal and external faces. The method is especially useful for vesicles made from phospholipids (which can contain cholesterol) and also for vesicles containing PEG-lipids (such as PEG-DAAAs and PEG-DAGs). Methods of making SNALP-CPLs are taught, for example, in U.S. Patent Nos. 5,705,385; 6,586,410; 5,981,501; 6,534,484; and 6,852,334; U.S. Patent Publication No. 20020072121; and PCT Publication No. WO 00/62813, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

VII. Kits

[0426] The present invention also provides lipid particles (*e.g.*, SNALP) in kit form. In some embodiments, the kit comprises a container which is compartmentalized for holding the various elements of the lipid particles (*e.g.*, the active agents or therapeutic agents such as nucleic acids and the individual lipid components of the particles). Preferably, the kit comprises a container (*e.g.*, a vial or ampoule) which holds the lipid particles of the invention (*e.g.*, SNALP), wherein the particles are produced by one of the processes set forth herein. In certain embodiments, the kit may further comprise an endosomal membrane destabilizer (*e.g.*, calcium ions). The kit typically contains the particle compositions of the invention, either as a suspension in a pharmaceutically acceptable carrier or in dehydrated form, with instructions for their rehydration (if lyophilized) and administration.

[0427] The lipid particles of the present invention can be tailored to preferentially target particular tissues, organs, or tumors of interest. In some instances, the 1:57 lipid particle (*e.g.*, SNALP) formulation can be used to preferentially target the liver (*e.g.*, normal liver tissue). In other instances, the 7:54 lipid particle (*e.g.*, SNALP) formulation can be used to

preferentially target solid tumors such as liver tumors and tumors outside of the liver. In preferred embodiments, the kits of the invention comprise these liver-directed and/or tumor-directed lipid particles, wherein the particles are present in a container as a suspension or in dehydrated form.

- 5 [0428] In certain other instances, it may be desirable to have a targeting moiety attached to the surface of the lipid particle to further enhance the targeting of the particle. Methods of attaching targeting moieties (*e.g.*, antibodies, proteins, *etc.*) to lipids (such as those used in the present particles) are known to those of skill in the art.

VIII. Administration of Lipid Particles

- 10 [0429] Once formed, the lipid particles of the invention (*e.g.*, SNALP) are useful for the introduction of active agents or therapeutic agents (*e.g.*, nucleic acids such as interfering RNA) into cells. Accordingly, the present invention also provides methods for introducing an active agent or therapeutic agent such as a nucleic acid (*e.g.*, interfering RNA) into a cell. In some instances, the cell is a liver cell such as, *e.g.*, a hepatocyte present in liver tissue. In
15 other instances, the cell is a tumor cell such as, *e.g.*, a tumor cell present in a solid tumor. The methods are carried out *in vitro* or *in vivo* by first forming the particles as described above and then contacting the particles with the cells for a period of time sufficient for delivery of the active agent or therapeutic agent to the cells to occur.

- [0430] The lipid particles of the invention (*e.g.*, SNALP) can be adsorbed to almost any
20 cell type with which they are mixed or contacted. Once adsorbed, the 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 active agent or therapeutic agent (*e.g.*, nucleic acid) portion of the particle can take place via any one of these pathways. In particular, when fusion takes place, the particle membrane is integrated into the cell membrane and the
25 contents of the particle combine with the intracellular fluid.

- [0431] The lipid particles of the invention (*e.g.*, SNALP) can be administered either alone or in a mixture with a pharmaceutically acceptable carrier (*e.g.*, physiological saline or phosphate buffer) selected in accordance with the route of administration and standard pharmaceutical practice. Generally, normal buffered saline (*e.g.*, 135-150 mM NaCl) will be
30 employed as the pharmaceutically acceptable carrier. Other suitable carriers include, *e.g.*, water, buffered water, 0.4% saline, 0.3% glycine, and the like, including glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc.* Additional suitable carriers are described in, *e.g.*, REMINGTON'S PHARMACEUTICAL SCIENCES, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). As used herein, "carrier" includes any and all

solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human.

[0432] The pharmaceutically acceptable carrier is generally added following lipid particle formation. Thus, after the lipid particle (*e.g.*, SNALP) is formed, the particle can be diluted into pharmaceutically acceptable carriers such as normal buffered saline.

[0433] The concentration of particles in the pharmaceutical formulations can vary widely, *i.e.*, from less than about 0.05%, usually at or at least about 2 to 5%, to as much as about 10 to 90% 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, particles composed of irritating lipids may be diluted to low concentrations to lessen inflammation at the site of administration.

[0434] The pharmaceutical compositions of the present invention may be sterilized by conventional, well-known sterilization techniques. Aqueous solutions can 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 can 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, and calcium chloride. Additionally, the particle suspension may include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as α -tocopherol, and water-soluble iron-specific chelators, such as ferrioxamine, are suitable.

[0435] In some embodiments, the lipid particles of the invention (*e.g.*, SNALP) are particularly useful in methods for the therapeutic delivery of one or more nucleic acids comprising an interfering RNA sequence (*e.g.*, siRNA). In particular, it is an object of this invention to provide *in vitro* and *in vivo* methods for treatment of a disease or disorder in a mammal (*e.g.*, a rodent such as a mouse or a primate such as a human, chimpanzee, or monkey) by downregulating or silencing the transcription and/or translation of one or more target nucleic acid sequences or genes of interest. As a non-limiting example, the methods of the invention are useful for *in vivo* delivery of interfering RNA (*e.g.*, siRNA) to the liver

and/or tumor of a mammalian subject. In certain embodiments, the disease or disorder is associated with expression and/or overexpression of a gene and expression or overexpression of the gene is reduced by the interfering RNA (*e.g.*, siRNA). In certain other embodiments, a therapeutically effective amount of the lipid particle may be administered to the mammal. In some instances, an interfering RNA (*e.g.*, siRNA) is formulated into a SNALP, and the particles are administered to patients requiring such treatment. In other instances, cells are removed from a patient, the interfering RNA is delivered *in vitro* (*e.g.*, using a SNALP described herein), and the cells are reinjected into the patient.

A. *In vivo* Administration

[0436] Systemic delivery for *in vivo* therapy, *e.g.*, delivery of a therapeutic nucleic acid to a distal target cell via body systems such as the circulation, has been achieved using nucleic acid-lipid particles such as those described in PCT Publication Nos. WO 05/007196, WO 05/121348, WO 05/120152, and WO 04/002453, the disclosures of which are herein incorporated by reference in their entirety for all purposes. The present invention also provides fully encapsulated lipid particles that protect the nucleic acid from nuclease degradation in serum, are non-immunogenic, are small in size, and are suitable for repeat dosing.

[0437] For *in vivo* administration, administration can be in any manner known in the art, *e.g.*, by injection, oral administration, inhalation (*e.g.*, intranasal or intratracheal), transdermal application, or rectal administration. Administration can be accomplished via single or divided doses. The pharmaceutical compositions can be administered parenterally, *i.e.*, intraarticularly, intravenously, intraperitoneally, subcutaneously, or intramuscularly. In some embodiments, the pharmaceutical compositions are administered intravenously or intraperitoneally by a bolus injection (*see, e.g.*, U.S. Patent No. 5,286,634). Intracellular nucleic acid delivery has also been discussed in Straubinger *et al.*, *Methods Enzymol.*, 101:512 (1983); Mannino *et al.*, *Biotechniques*, 6:682 (1988); Nicolau *et al.*, *Crit. Rev. Ther. Drug Carrier Syst.*, 6:239 (1989); and Behr, *Acc. Chem. Res.*, 26:274 (1993). Still other methods of administering lipid-based therapeutics are described in, for example, U.S. Patent Nos. 3,993,754; 4,145,410; 4,235,871; 4,224,179; 4,522,803; and 4,588,578. The lipid particles can be administered by direct injection at the site of disease or by injection at a site distal from the site of disease (*see, e.g.*, Culver, HUMAN GENE THERAPY, MaryAnn Liebert, Inc., Publishers, New York. pp.70-71(1994)). The disclosures of the above-described references are herein incorporated by reference in their entirety for all purposes.

[0438] In embodiments where the lipid particles of the present invention (*e.g.*, SNALP) are administered intravenously, at least about 5%, 10%, 15%, 20%, or 25% of the total injected dose of the particles is present in plasma about 8, 12, 24, 36, or 48 hours after injection. In other embodiments, more than about 20%, 30%, 40% and as much as about 60%, 70% or 80% of the total injected dose of the lipid particles is present in plasma about 8, 12, 24, 36, or 48 hours after injection. In certain instances, more than about 10% of a plurality of the particles is present in the plasma of a mammal about 1 hour after administration. In certain other instances, the presence of the lipid particles is detectable at least about 1 hour after administration of the particle. In certain embodiments, the presence of a therapeutic agent such as a nucleic acid is detectable in cells of the lung, liver, tumor, or at a site of inflammation at about 8, 12, 24, 36, 48, 60, 72 or 96 hours after administration. In other embodiments, downregulation of expression of a target sequence by an interfering RNA (*e.g.*, siRNA) is detectable at about 8, 12, 24, 36, 48, 60, 72 or 96 hours after administration. In yet other embodiments, downregulation of expression of a target sequence by an interfering RNA (*e.g.*, siRNA) occurs preferentially in liver cells (*e.g.*, hepatocytes), tumor cells, or in cells at a site of inflammation. In further embodiments, the presence or effect of an interfering RNA (*e.g.*, siRNA) in cells at a site proximal or distal to the site of administration or in cells of the lung, liver, or a tumor is detectable at about 12, 24, 48, 72, or 96 hours, or at about 6, 8, 10, 12, 14, 16, 18, 19, 20, 22, 24, 26, or 28 days after administration. In additional embodiments, the lipid particles (*e.g.*, SNALP) of the invention are administered parenterally or intraperitoneally.

[0439] The compositions of the present invention, either alone or in combination with other suitable components, can be made into aerosol formulations (*i.e.*, they can be “nebulized”) to be administered via inhalation (*e.g.*, intranasally or intratracheally) (*see*, Brigham *et al.*, *Am. J. Sci.*, 298:278 (1989)). Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

[0440] In certain embodiments, the pharmaceutical compositions may be delivered by intranasal sprays, inhalation, and/or other aerosol delivery vehicles. Methods for delivering nucleic acid compositions directly to the lungs via nasal aerosol sprays have been described, *e.g.*, in U.S. Patent Nos. 5,756,353 and 5,804,212. Likewise, the delivery of drugs using intranasal microparticle resins and lysophosphatidyl-glycerol compounds (U.S. Patent 5,725,871) are also well-known in the pharmaceutical arts. Similarly, transmucosal drug delivery in the form of a polytetrafluoroethylene support matrix is described in U.S. Patent No. 5,780,045. The disclosures of the above-described patents are herein incorporated by reference in their entirety for all purposes.

[0441] Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. In the practice of this invention, compositions are preferably administered, for example, by intravenous infusion, orally, topically, intraperitoneally, intravesically, or intrathecally.

[0442] Generally, when administered intravenously, the lipid particle formulations are formulated with a suitable pharmaceutical carrier. Many pharmaceutically acceptable carriers may be employed in the compositions and methods of the present invention. Suitable formulations for use in the present invention are found, for example, in REMINGTON'S PHARMACEUTICAL SCIENCES, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). A variety of aqueous carriers may be used, for example, water, buffered water, 0.4% saline, 0.3% glycine, and the like, and may include glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc.* Generally, normal buffered saline (135-150 mM NaCl) will be employed as the pharmaceutically acceptable carrier, but other suitable carriers will suffice. These compositions can be sterilized by conventional liposomal sterilization techniques, such as filtration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, *etc.* These compositions can be sterilized using the techniques referred to above or, alternatively, they can be produced under sterile conditions. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration.

[0443] In certain applications, the lipid particles disclosed herein may be delivered via oral administration to the individual. The particles may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, pills, lozenges, elixirs, mouthwash, suspensions, oral sprays, syrups, wafers, and the like (*see, e.g.,* U.S. Patent Nos. 5,641,515, 5,580,579, and 5,792,451, the disclosures of which are herein incorporated by reference in their entirety for all purposes). These oral dosage forms may also contain the following: binders, gelatin; excipients, lubricants, and/or flavoring agents. When the unit

dosage form is a capsule, it may contain, in addition to the materials described above, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. Of course, any material used in preparing any unit dosage form should be pharmaceutically pure and substantially non-toxic in the amounts employed.

5 [0444] Typically, these oral formulations may contain at least about 0.1% of the lipid particles or more, although the percentage of the particles may, of course, be varied and may conveniently be between about 1% or 2% and about 60% or 70% or more of the weight or volume of the total formulation. Naturally, the amount of particles in each therapeutically useful composition may be prepared in such a way that a suitable dosage will be obtained in
10 any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

15 [0445] Formulations suitable for oral administration can consist of: (a) liquid solutions, such as an effective amount of a packaged therapeutic agent such as nucleic acid (*e.g.*, interfering RNA) suspended in diluents such as water, saline, or PEG 400; (b) capsules, sachets, or tablets, each containing a predetermined amount of a therapeutic agent such as nucleic acid (*e.g.*, interfering RNA), as liquids, solids, granules, or gelatin; (c) suspensions in
20 an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, sucrose, mannitol, sorbitol, calcium phosphates, corn starch, potato starch, microcrystalline cellulose, gelatin, colloidal silicon dioxide, talc, magnesium stearate, stearic acid, and other excipients, colorants, fillers, binders, diluents, buffering agents, moistening agents, preservatives, flavoring agents, dyes, disintegrating agents, and pharmaceutically
25 compatible carriers. Lozenge forms can comprise a therapeutic agent such as nucleic acid (*e.g.*, interfering RNA) in a flavor, *e.g.*, sucrose, as well as pastilles comprising the therapeutic agent in an inert base, such as gelatin and glycerin or sucrose and acacia emulsions, gels, and the like containing, in addition to the therapeutic agent, carriers known in the art.

30 [0446] In another example of their use, lipid particles can be incorporated into a broad range of topical dosage forms. For instance, a suspension containing nucleic acid-lipid particles such as SNALP can be formulated and administered as gels, oils, emulsions, topical creams, pastes, ointments, lotions, foams, mousses, and the like.

[0447] When preparing pharmaceutical preparations of the lipid particles of the invention,
35 it is preferable to use quantities of the particles which have been purified to reduce or

eliminate empty particles or particles with therapeutic agents such as nucleic acid associated with the external surface.

[0448] The methods of the present invention may be practiced in a variety of hosts.

Preferred hosts include mammalian species, such as primates (*e.g.*, humans and chimpanzees as well as other nonhuman primates), canines, felines, equines, bovines, ovines, caprines, rodents (*e.g.*, rats and mice), lagomorphs, and swine.

[0449] The amount of particles administered will depend upon the ratio of therapeutic agent (*e.g.*, nucleic acid) to lipid, the particular therapeutic agent (*e.g.*, nucleic acid) used, the disease or disorder being treated, the age, weight, and condition of the patient, and the

judgment 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, or about 10^8 - 10^{10} particles per administration (*e.g.*, injection).

B. *In vitro* Administration

[0450] For *in vitro* applications, the delivery of therapeutic agents such as nucleic acids (*e.g.*, interfering RNA) 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 are animal cells, more preferably mammalian cells, and most preferably human cells (*e.g.*, tumor cells or hepatocytes).

[0451] Contact between the cells and the lipid particles, when carried out *in vitro*, takes place in a biologically compatible medium. The concentration of particles varies widely depending on the particular application, but is generally between about 1 μ mol and about 10 mmol. Treatment of the cells with the lipid particles is generally carried out at physiological temperatures (about 37°C) for periods of time of from about 1 to 48 hours, preferably of from about 2 to 4 hours.

[0452] In one group of preferred embodiments, a lipid particle suspension is added to 60-80% confluent plated cells having a cell density of from about 10^3 to about 10^5 cells/ml, more preferably about 2×10^4 cells/ml. The concentration of the suspension added to the cells is preferably of from about 0.01 to 0.2 μ g/ml, more preferably about 0.1 μ g/ml.

[0453] To the extent that tissue culture of cells may be required, it is well-known in the art.

For example, Freshney, *Culture of Animal Cells, a Manual of Basic Technique*, 3rd Ed., Wiley-Liss, New York (1994), Kuchler *et al.*, *Biochemical Methods in Cell Culture and Virology*, Dowden, Hutchinson and Ross, Inc. (1977), and the references cited therein provide a general guide to the culture of cells. Cultured cell systems often will be in the form of monolayers of cells, although cell suspensions are also used.

[0454] Using an Endosomal Release Parameter (ERP) assay, the delivery efficiency of the SNALP or other lipid particle of the invention can be optimized. An ERP assay is described in detail in U.S. Patent Publication No. 20030077829, the disclosure of which is herein incorporated by reference in its entirety for all purposes. More particularly, the purpose of an ERP assay is to distinguish the effect of various cationic lipids and helper lipid components of SNALP or other lipid particle based on their relative effect on binding/uptake or fusion with/destabilization of the endosomal membrane. This assay allows one to determine quantitatively how each component of the SNALP or other lipid particle affects delivery efficiency, thereby optimizing the SNALP or other lipid particle. Usually, an ERP assay measures expression of a reporter protein (*e.g.*, luciferase, β -galactosidase, green fluorescent protein (GFP), *etc.*), and in some instances, a SNALP formulation optimized for an expression plasmid will also be appropriate for encapsulating an interfering RNA. In other instances, an ERP assay can be adapted to measure downregulation of transcription or translation of a target sequence in the presence or absence of an interfering RNA (*e.g.*, siRNA). By comparing the ERPs for each of the various SNALP or other lipid particles, one can readily determine the optimized system, *e.g.*, the SNALP or other lipid particle that has the greatest uptake in the cell.

C. Cells for Delivery of Lipid Particles

[0455] The compositions and methods of the present invention are used to treat a wide variety of cell types, *in vivo* and *in vitro*. Suitable cells include, but are not limited to, hepatocytes, hematopoietic precursor (stem) cells, fibroblasts, keratinocytes, endothelial cells, skeletal and smooth muscle cells, osteoblasts, neurons, quiescent lymphocytes, terminally differentiated cells, slow or noncycling primary cells, parenchymal cells, lymphoid cells, epithelial cells, bone cells, and the like.

[0456] In particular embodiments, an active agent or therapeutic agent such as a nucleic acid (*e.g.*, an interfering RNA) is delivered to cancer cells (*e.g.*, cells of a solid tumor) including, but not limited to, liver cancer cells, lung cancer cells, colon cancer cells, rectal cancer cells, anal cancer cells, bile duct cancer cells, small intestine cancer cells, stomach (gastric) cancer cells, esophageal cancer cells, gallbladder cancer cells, pancreatic cancer cells, appendix cancer cells, breast cancer cells, ovarian cancer cells, cervical cancer cells, prostate cancer cells, renal cancer cells, cancer cells of the central nervous system, glioblastoma tumor cells, skin cancer cells, lymphoma cells, choriocarcinoma tumor cells, head and neck cancer cells, osteogenic sarcoma tumor cells, and blood cancer cells.

[0457] *In vivo* delivery of lipid particles such as SNALP encapsulating a nucleic acid (e.g., an interfering RNA) is suited for targeting cells of any cell type. The methods and compositions can be employed with cells of a wide variety of vertebrates, including mammals, such as, e.g. canines, felines, equines, bovines, ovines, caprines, rodents (e.g., mice, rats, and guinea pigs), lagomorphs, swine, and primates (e.g. monkeys, chimpanzees, and humans).

D. Detection of Lipid Particles

[0458] In some embodiments, the lipid particles of the present invention (e.g., SNALP) are detectable in the subject at about 1, 2, 3, 4, 5, 6, 7, 8 or more hours. In other embodiments, the lipid particles of the present invention (e.g., SNALP) are detectable in the subject at about 8, 12, 24, 48, 60, 72, or 96 hours, or about 6, 8, 10, 12, 14, 16, 18, 19, 22, 24, 25, or 28 days after administration of the particles. The presence of the particles can be detected in the cells, tissues, or other biological samples from the subject. The particles may be detected, e.g., by direct detection of the particles, detection of a therapeutic nucleic acid such as an interfering RNA (e.g., siRNA) sequence, detection of the target sequence of interest (i.e., by detecting expression or reduced expression of the sequence of interest), or a combination thereof.

1. Detection of Particles

[0459] Lipid particles of the invention such as SNALP can be detected using any method known in the art. For example, a label can be coupled directly or indirectly to a component of the lipid particle using methods well-known in the art. A wide variety of labels can be used, with the choice of label depending on sensitivity required, ease of conjugation with the lipid particle component, stability requirements, and available instrumentation and disposal provisions. Suitable labels include, but are not limited to, spectral labels such as fluorescent dyes (e.g., fluorescein and derivatives, such as fluorescein isothiocyanate (FITC) and Oregon Green™; rhodamine and derivatives such as Texas red, tetra-rhodamine isothiocyanate (TRITC), etc., digoxigenin, biotin, phycoerythrin, AMCA, CyDyes™, and the like; radiolabels such as ^3H , ^{125}I , ^{35}S , ^{14}C , ^{32}P , ^{33}P , etc.; enzymes such as horse radish peroxidase, alkaline phosphatase, etc.; spectral colorimetric labels such as colloidal gold or colored glass or plastic beads such as polystyrene, polypropylene, latex, etc. The label can be detected using any means known in the art.

2. Detection of Nucleic Acids

[0460] Nucleic acids (e.g., interfering RNA) are detected and quantified herein by any of a number of means well-known to those of skill in the art. The detection of nucleic acids may

proceed by well-known methods such as Southern analysis, Northern analysis, gel electrophoresis, PCR, radiolabeling, scintillation counting, and affinity chromatography. Additional analytic biochemical methods such as spectrophotometry, radiography, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), and hyperdiffusion chromatography may also be employed.

[0461] The selection of a nucleic acid hybridization format is not critical. A variety of nucleic acid hybridization formats are known to those skilled in the art. For example, common formats include sandwich assays and competition or displacement assays.

Hybridization techniques are generally described in, e.g., "Nucleic Acid Hybridization, A Practical Approach," Eds. Hames and Higgins, IRL Press (1985).

[0462] The sensitivity of the hybridization assays may be enhanced through the use of a nucleic acid amplification system which multiplies the target nucleic acid being detected. *In vitro* amplification techniques suitable for amplifying sequences for use as molecular probes or for generating nucleic acid fragments for subsequent subcloning are known. Examples of techniques sufficient to direct persons of skill through such *in vitro* amplification methods, including the polymerase chain reaction (PCR), the ligase chain reaction (LCR), Q β -replicase amplification, and other RNA polymerase mediated techniques (e.g., NASBATM) are found in Sambrook *et al.*, In *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (2000); and Ausubel *et al.*, *SHORT PROTOCOLS IN MOLECULAR BIOLOGY*, eds., Current Protocols, Greene Publishing Associates, Inc. and John Wiley & Sons, Inc. (2002); as well as U.S. Patent No. 4,683,202; PCR Protocols, A Guide to Methods and Applications (Innis *et al.* eds.) Academic Press Inc. San Diego, CA (1990); Arnheim & Levinson (October 1, 1990), *C&EN* 36; *The Journal Of NIH Research*, 3:81 (1991); Kwoh *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:1173 (1989); Guatelli *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:1874 (1990); Lomell *et al.*, *J. Clin. Chem.*, 35:1826 (1989); Landegren *et al.*, *Science*, 241:1077 (1988); Van Brunt, *Biotechnology*, 8:291 (1990); Wu and Wallace, *Gene*, 4:560 (1989); Barringer *et al.*, *Gene*, 89:117 (1990); and Sooknanan and Malek, *Biotechnology*, 13:563 (1995). Improved methods of cloning *in vitro* amplified nucleic acids are described in U.S. Pat. No. 5,426,039. Other methods described in the art are the nucleic acid sequence based amplification (NASBATM, Cangene, Mississauga, Ontario) and Q β -replicase systems. These systems can be used to directly identify mutants where the PCR or LCR primers are designed to be extended or ligated only when a select sequence is present. Alternatively, the select sequences can be generally amplified using, for example, nonspecific PCR primers and the amplified target region later probed for a specific sequence indicative of a mutation. The

disclosures of the above-described references are herein incorporated by reference in their entirety for all purposes.

[0463] Nucleic acids for use as probes, *e.g.*, in *in vitro* amplification methods, for use as gene probes, or as inhibitor components are typically synthesized chemically according to the solid phase phosphoramidite triester method described by Beaucage *et al.*, *Tetrahedron Letts.*, 22:1859 1862 (1981), *e.g.*, using an automated synthesizer, as described in Needham VanDevanter *et al.*, *Nucleic Acids Res.*, 12:6159 (1984). Purification of polynucleotides, where necessary, is typically performed by either native acrylamide gel electrophoresis or by anion exchange HPLC as described in Pearson *et al.*, *J. Chrom.*, 255:137 149 (1983). The sequence of the synthetic polynucleotides can be verified using the chemical degradation method of Maxam and Gilbert (1980) in Grossman and Moldave (eds.) Academic Press, New York, *Methods in Enzymology*, 65:499.

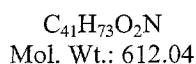
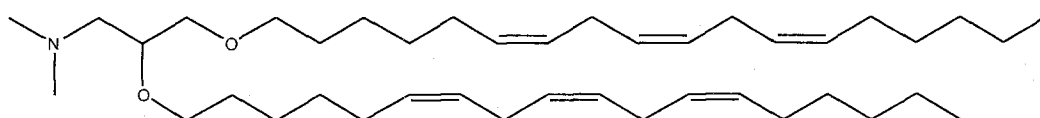
[0464] An alternative means for determining the level of transcription is *in situ* hybridization. *In situ* hybridization assays are well-known and are generally described in Angerer *et al.*, *Methods Enzymol.*, 152:649 (1987). In an *in situ* hybridization assay, cells are fixed to a solid support, typically a glass slide. If DNA is to be probed, the cells are denatured with heat or alkali. The cells are then contacted with a hybridization solution at a moderate temperature to permit annealing of specific probes that are labeled. The probes are preferably labeled with radioisotopes or fluorescent reporters.

IX. Examples

[0465] The present invention will be described in greater detail by way of specific examples. The following examples are offered for illustrative purposes, and are not intended to limit the invention in any manner. Those of skill in the art will readily recognize a variety of noncritical parameters which can be changed or modified to yield essentially the same results.

Example 1. Synthesis of 1,2-Di- γ -linolenyloxy-*N,N*-dimethylaminopropane (γ -DLenDMA).

[0466] γ -DLenDMA (Compound 1) having the structure shown below was synthesized as described below.



[0467] A 250 mL round bottom flask was charged with 3-(dimethylamino)-1,2-propanediol (0.8 g, 6.7 mmol), tetrabutylammonium hydrogen sulphate (1 g), gamma linolenyl mesylate (cis-6,9,12-octadecatriene sulphonic acid) (5 g, 14.6 mmol), and 30 mL toluene. After stirring for 15 minutes, the reaction was cooled to 0-5°C. A solution of 40% sodium

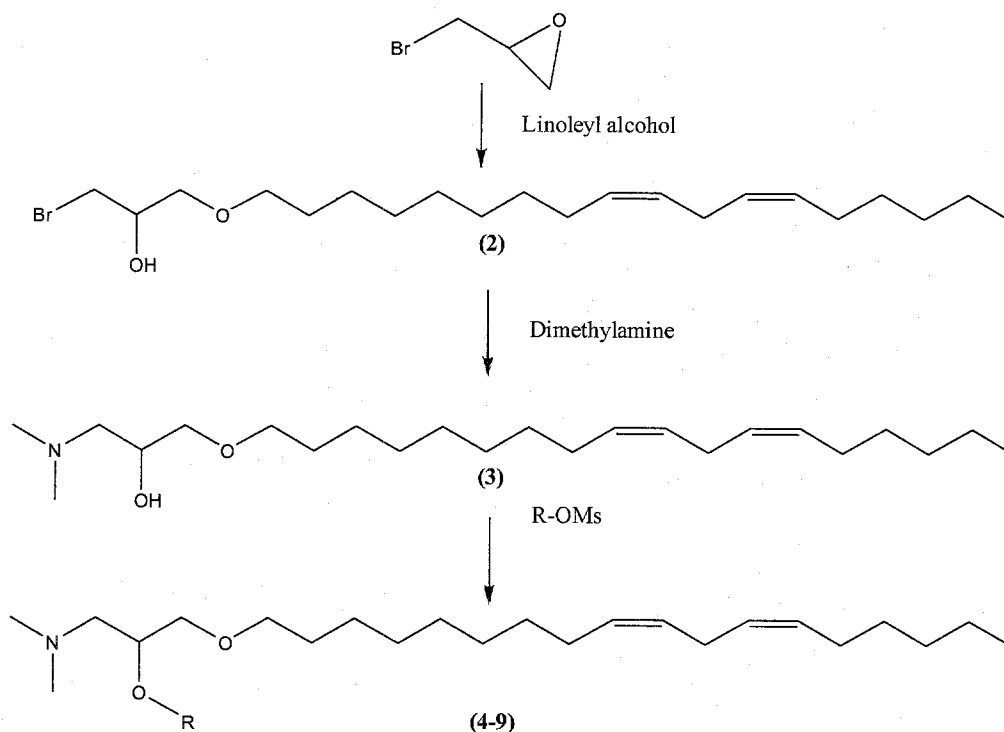
5 hydroxide (15 mL) was added slowly. The reaction was left to stir for approximately 48 hours. An additional 15 mL of toluene was then added to the reaction vessel, along with 40% sodium hydroxide (15 mL). After the reaction was stirred for an additional 12 hours, water (50 mL) and isopropyl acetate (50 mL) were added and stirred for 15 minutes. The mixture was then transferred to a 500 mL separatory funnel and allowed to separate. The lower
10 aqueous phase was run off and the organic phase was washed with saturated sodium chloride (2 × 50 mL). Since the aqueous and organic phases resulting from the saturated sodium chloride washes could not be completely separated after 20 minutes, the lower aqueous phase (slightly yellow) was run off and back extracted with chloroform (~45 mL). The organic phase was dried with MgSO₄, filtered, and the solvent evaporated.

15 [0468] The crude product, an orange liquid, was purified on column chromatography using silica gel (60g) with 0-3% methanol gradient in dichloromethane to yield 3.19 g. The product was further purified via column chromatography on silica gel (50 g) with 10-30% ethyl acetate gradient in hexanes to yield 1.26 g pure product.

Example 2. Synthesis of Asymmetric Cationic Lipids.

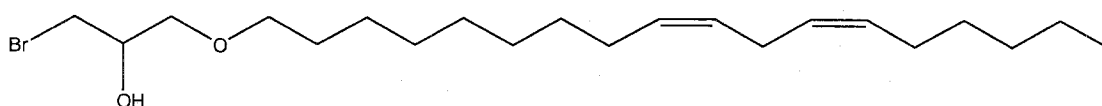
20 [0469] Asymmetric cationic lipids (Compounds 4-9) having the structures shown below were synthesized as shown in the following schematic diagram.

Scheme 1



R = oleyl = Compound 4;
 R = phytanyl = Compound 5;
 R = linolenyl = Compound 6;
 R = stearyl = Compound 7;
 R = hexanyl = Compound 8;
 R = cis-3-hexenyl = Compound 9.

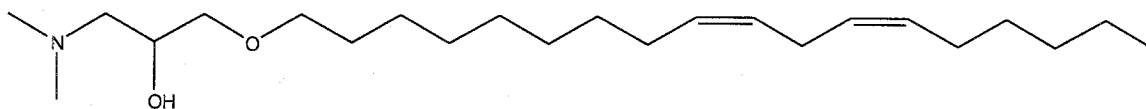
Step 1: Synthesis of 2-Hydroxy-3-linoleyloxypropylbromide (Compound 2):



Chemical Formula: $C_{21}H_{39}O_2Br$
 Molecular Weight: 403.44

[0470] A 500 ml round bottom flask was charged with epibromohydrin (5.8 g, 42 mmol), linoleyl alcohol (15g, 56 mmol), a stir bar, and then flushed with nitrogen. Anhydrous DCM (250 ml) was added via cannula, followed by $BF_3 \cdot Et_2O$ (0.53 ml, 4.2 mmol), and stirred at 40°C for 3 hours. Progress of reaction was monitored by TLC to ensure no epoxide was remaining. The reaction mixture was transferred to a 500 ml separatory funnel with DCM (2 × 50 ml), washed with $NaHCO_3$ (2 × 200 ml), water (200 ml), brine (200 ml), dried with $MgSO_4$, and solvent evaporated. The crude product was purified using column chromatography on silica gel (300 g) with 5-15% ether gradient in hexanes to yield 12.6 g.

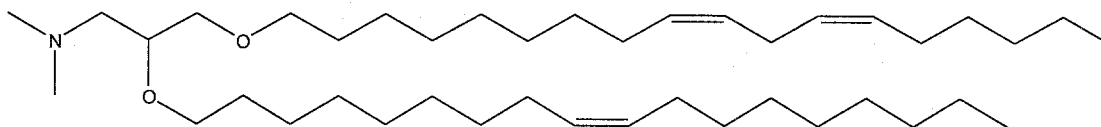
Step 2: Synthesis of 2-Hydroxy-3-linoleyloxv-N,N-dimethylpropylamine (Compound 3):



$C_{23}H_{45}O_2N$
Molecular Weight: 367.61

[0471] A 500 mL round bottom flask was charged with Compound 2 (2-Hydroxy-3-linoleyloxypropylbromide) (10 g, 25 mmol) and a stir bar. After flushing with nitrogen, a 2.0 M solution of dimethylamine in methyl alcohol (250 mL) was added via cannula. The resulting mixture was stirred at room temperature for 48 hours. The progress of the reaction was monitored using TLC. The reaction solution was evaporated and the crude product (9.1 g) used without further purification.

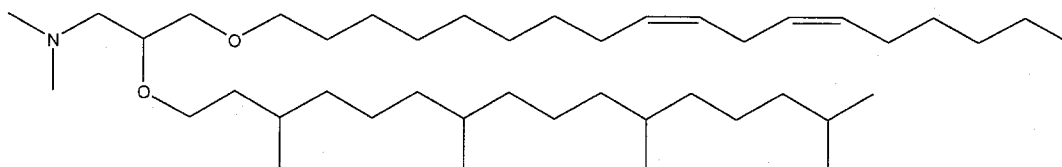
10 Synthesis of Linoleyl/Oleyl DMA (Compound 4):

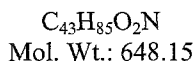


$C_{41}H_{79}O_2N$
Mol. Wt.: 618.08

[0472] A 250 mL round bottom flask was charged with 2-Hydroxy-3-linoleyloxy-*N,N*-dimethylpropylamine (1.5 g, 4.1 mmol), oleyl methane sulfonate (2.14 g, 6.2 mmol), toluene (30 mL), and a stir bar. The resulting mixture was cooled to 0-5°C. While stirring, a solution of 40% NaOH (15 mL) was added, followed by 1.0 M solution of tertbutyl ammonium hydroxide ((TBAH) 1.5 mL, 1.5 mmol). The reaction was stirred at room temperature for 60 hours and progress of reaction was monitored using TLC. Water (50 mL) and isopropyl acetate (50 mL) were added and the resulting solution stirred for 10 minutes. The mixture was quantitatively transferred to a 500 mL separatory funnel with isopropyl acetate (10 mL), and the lower aqueous phase was run off. The organic phase was washed with water (2 × 50 mL), dried with $MgSO_4$, filtered, and solvent removed. During the second wash, ethanol (25 mL) was added to ensure complete separation of the two phases. The aqueous phases were pooled and back extracted with chloroform. The crude product (4.1 g) was purified by column chromatography on silica gel (60 g) with 0-3% methanol gradient in dichloromethane to yield 0.45 g.

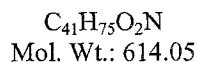
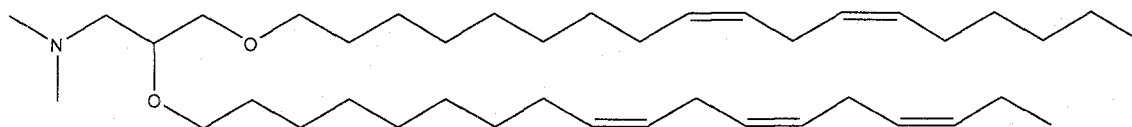
Synthesis of Linoleyl/Phytanyl DMA (Compound 5):





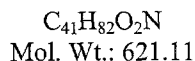
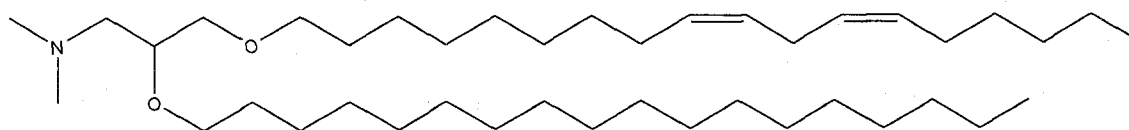
[0473] Compound 5 was synthesized analogously to Compound 4, but substituting oleyl mesylate for phytanyl methane sulfonate (2.26 g, 6.2 mmol) in the alkylation step. Final yield obtained: 0.46 g.

Synthesis of Linoleyl/Linolenyl DMA (Compound 6):



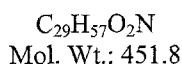
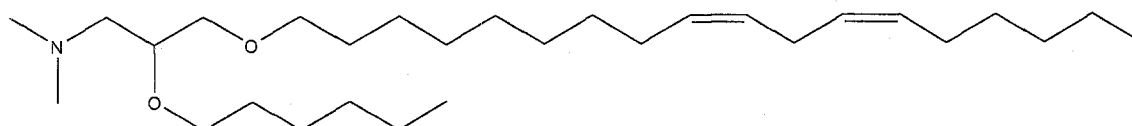
[0474] Compound 6 was synthesized analogously to Compound 4, but substituting oleyl mesylate for linolenyl methane sulfonate (2.12 g, 6.2 mmol) in the alkylation step. Final yield obtained: 0.49 g.

Synthesis of Linoleyl/Stearyl DMA (Compound 7):



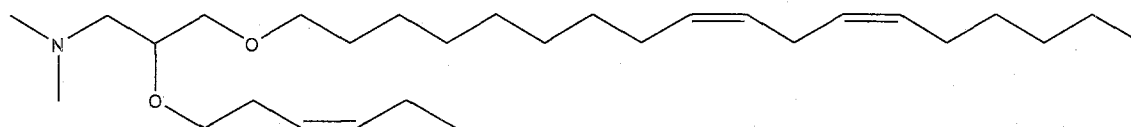
[0475] Compound 7 was synthesized analogously to Compound 4, but substituting oleyl mesylate for stearyl methane sulfonate (2.16 g, 6.2 mmol) in the alkylation step. Final yield obtained: 0.77g.

Synthesis of Linoleyl/C₆:0 DMA (Compound 8):



[0476] Compound 8 was synthesized analogously to Compound 4, but substituting oleyl mesylate for hexanoyl methane sulfonate (1.12 g, 6.2 mmol) in the alkylation step. Final yield obtained: 0.18 g.

Synthesis of Linoleyl/ C₆:1 DMA (Compound 9):



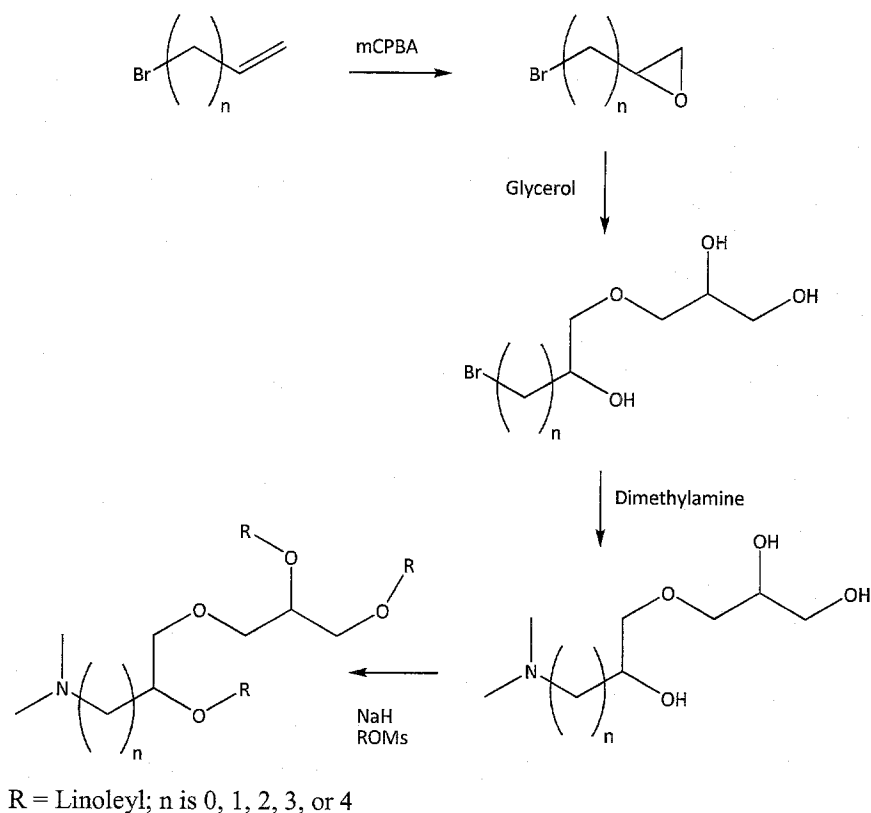
$C_{29}H_{57}O_2N$
Mol. Wt.: 449.8

[0477] A 100 mL round bottom flask was charged with a stir bar, NaH (0.4 g, 17 mmol), and 35 mL benzene. Subsequently, 2-Hydroxy-3-linoleyloxy-*N,N*-dimethylpropylamine (1.20 g, 3.3 mmol) was added followed immediately by *cis*-3-hexenyl methane sulfonate (0.76 g, 4.3 mmol). The reaction was flushed with nitrogen and stirred overnight at 50°C. Progress of the reaction was monitored via TLC. The reaction mixture was transferred to a 250 mL separatory funnel and diluted with benzene to a final volume of 100 mL. The reaction was quenched with ethanol (60 mL) and then washed with water (100 mL). The lower aqueous phase was run off and the reaction mixture washed again with ethanol (60 mL) and water (100 mL). The organic phase was dried with $MgSO_4$, filtered, and solvent removed. The crude product (0.83 g) was purified by column chromatography on silica gel (30 g) with 0-3% methanol gradient in dichloromethane to yield 0.17 g.

Example 3. Synthesis of Cationic Lipids of the TLinDMA Family.

[0478] The following diagram provides a general scheme for synthesizing members of the C(n)-TLinDMA family of cationic lipids:

Scheme 2



[0479] TLinDMA (1-(2,3-linoleyloxypropoxy)-2-(linoleyloxy)-(N,N-dimethyl)-propyl-3-amine) (Compound 12) was synthesized as follows:

5 Synthesis of Compound 10:

[0480] A 1000 ml round bottom flask was charged with epibromohydrin (5 g, 37 mmol), glycerol (10 g, 110 mmol), a stir bar and then flushed with nitrogen. Anhydrous chloroform (350 mL) was added via cannula, followed by $\text{BF}_3 \cdot \text{Et}_2\text{O}$ (0.5 mL, 3.7 mmol) and refluxed for 3 hours under nitrogen. The reaction mixture was cooled and subsequently stirred at room temperature overnight. Upon completion of the reaction, the reaction mixture was concentrated and the crude product (15 g) was purified via column chromatography using silica gel (150 g).

Synthesis of Compound 11:

[0481] A 500 mL round bottom flask was charged with Compound 10 (3.8 g, 17 mmol) and a stir bar. After flushing with nitrogen, dimethylamine in a 2.0 M methyl alcohol solution (170 mL) was added via cannula. The resulting mixture was stirred at room temperature for 48 hours. The progress of the reaction was monitored using TLC. The crude product was used without further purification.

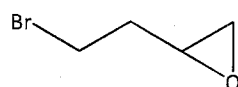
Synthesis of TLinDMA (Compound 12):

[0482] A 100 mL round bottom flask was charged with a stir bar, NaH (0.6 g, 24 mmol), and 25 mL benzene. Subsequently, Compound 11 (0.4 g, 2 mmol) was added followed

immediately by linoleyl methane sulfonate (2.8 g, 8 mmol). The reaction was flushed with nitrogen and refluxed overnight. Progress of the reaction was monitored via TLC. The reaction mixture was transferred to a 250 mL separatory funnel and diluted with benzene to a final volume of 50 mL. The reaction was quenched with ethanol (30 mL) and then washed with water (50 mL). The lower aqueous phase was run off and the reaction mixture was washed again with ethanol (30 mL) and water (50 mL). The organic phase was dried with MgSO_4 , filtered, and solvent removed. The crude product (2.3 g) was purified via column chromatography on silica gel (60 g) with 0-3% methanol gradient in dichloromethane.

[0483] C2-TLinDMA (Compound 16) was synthesized as follows:

10 Synthesis of Compound 13:



Chemical Formula: $\text{C}_4\text{H}_7\text{BrO}$

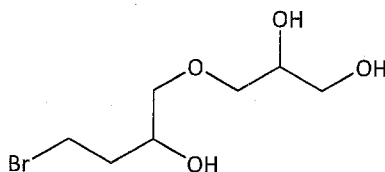
Exact Mass: 150.0

Molecular Weight: 151.0

Elemental Analysis: C, 31.82; H, 4.67; Br, 52.92; O, 10.60

[0484] A solution of 4-bromo-1-butene (11.5 g, 85 mmol) in CH_2Cl_2 (anh., 120 ml) was prepared under nitrogen in a 1000 ml RBF with a magnetic stirrer. In a separate flask, a solution of 3-chloroperbenzoic acid (77%, MW 173, 44.05 g, 196 mmol) in CH_2Cl_2 (anh., 250 ml) prepared and added to the reaction mixture by cannula. The reaction was stirred for 3 days, and then concentrated. The product (oil/white solid mixture) was re-dissolved in THF (300 mL) and a solution of 4% sodium dithionite (180 mL) added to remove excess peracid. The mixture (now cloudy) was stirred for 20 minutes and then EtOAc (750 mL) added. The mixture was transferred to a separating funnel and the organic was washed with water (100 mL), sat. NaHCO_3 (2 x 300 mL, EFFERVESCENCE), water again (300 mL) and brine (300 mL). The solution was concentrated and the product purified by chromatography.

Synthesis of Compound 14:

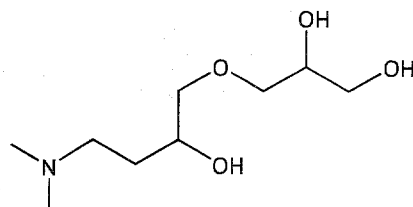


Chemical Formula: $\text{C}_7\text{H}_{15}\text{BrO}_4$

Exact Mass: 242.0

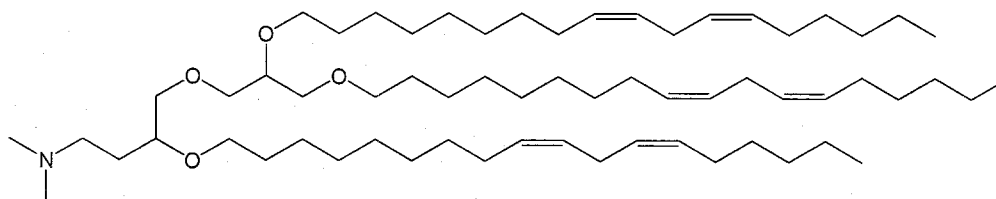
Molecular Weight: 243.1

[0485] A 250 ml round bottom flask was charged with Compound 13 (1.3 g, 9 mmol), glycerol (2.5 g, 27 mmol), a stir bar and then flushed with nitrogen. Anhydrous chloroform (100 mL) was added via cannula, followed by $\text{BF}_3 \cdot \text{Et}_2\text{O}$ (0.15 mL, 1.1 mmol) and refluxed for 3 hours under nitrogen. The reaction mixture was subsequently stirred at room temperature overnight.

Synthesis of Compound 15:

Chemical Formula: $C_9H_{21}NO_4$
 Exact Mass: 207.1
 Molecular Weight: 207.3

[0486] A 50 mL round bottom flask was charged with Compound 14 (0.3 g, 1.2 mmol) and a stir bar. After flushing with nitrogen, dimethylamine in a 2.0 M methyl alcohol solution (25 mL) was added via syringe. The resulting mixture was stirred at room temperature for 48 hours. The progress of the reaction was monitored using t.l.c. The reaction mixture was concentrated and the crude product used without further purification.

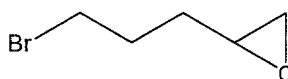
Synthesis of C2-TLinDMA (Compound 16):

Chemical Formula: $C_{63}H_{117}NO_4$
 Exact Mass: 951.9
 Molecular Weight: 952.6

[0487] A 100 mL round bottom flask was charged with a stir bar, NaH (0.6 g, 24 mmol), and 25 mL benzene. Compound 15 (0.37 g, 1.8 mmol) was added followed immediately by linoleyl methane sulfonate (2.8 g, 8 mmol). The reaction was refluxed overnight and progress of the reaction was monitored via t.l.c. The reaction mixture was transferred to a 250 mL separatory funnel and diluted with benzene to a final volume of 50 mL. The reaction was quenched with ethanol (30 mL) and then washed with water (50 mL). The lower aqueous phase was run off and the reaction mixture washed again with ethanol (30 mL) and water (50 mL). The organic phase was dried with $MgSO_4$, filtered, and solvent removed. The crude product, 2.5 g, was purified using column chromatography on silica gel (60 g), eluted with 0-3% methanol gradient in DCM.

[0488] C3-TLinDMA (Compound 20) was synthesized as follows:

Synthesis of Compound 17:

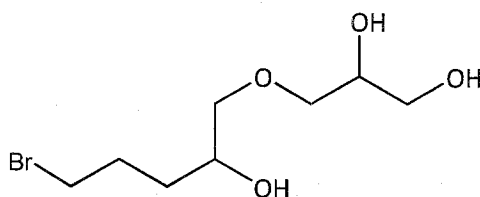
Chemical Formula: C_5H_9BrO

Exact Mass: 164.0

Molecular Weight: 165.0

Elemental Analysis: C, 36.39; H, 5.50; Br, 48.42; O, 9.69

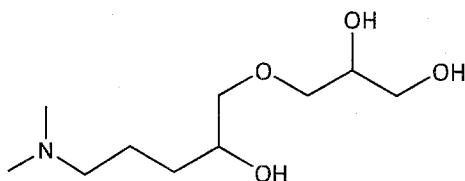
[0489] A solution of 5-bromo-1-pentene (85 mmol) in CH_2Cl_2 (anh., 120 ml) is prepared under nitrogen in a 1000 ml RBF with a magnetic stirrer. In a separate flask, a solution of 3-chloroperbenzoic acid (77%, MW 173, 44.05 g, 196 mmol) in CH_2Cl_2 (anh., 250 ml) is prepared and added to the reaction mixture by cannula. The reaction is stirred for 3 days, and then concentrated. The product (oil/white solid mixture) is re-dissolved in THF (300 mL) and a solution of 4% sodium dithionite (180 mL) added to remove excess peracid. The mixture (now cloudy) is stirred for 20 minutes and then EtOAc (750 mL) added. The mixture is transferred to a separating funnel and the organic is washed with water (100 mL), sat. $NaHCO_3$ (2 x 300 mL, EFFERVESCENCE), water again (300 mL) and brine (300 mL). The solution is concentrated and the product purified by chromatography.

Synthesis of Compound 18:Chemical Formula: $C_8H_{17}BrO_4$

Exact Mass: 256.0

Molecular Weight: 257.1

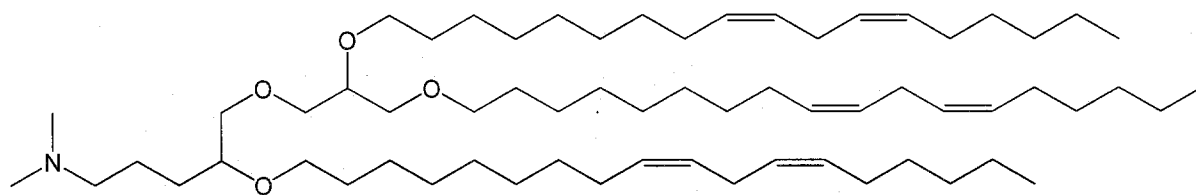
[0490] A 250 ml round bottom flask is charged with Compound 17 (1.3 g, 9 mmol), glycerol (2.5 g, 27 mmol), a stir bar and then flushed with nitrogen. Anhydrous chloroform (100 mL) is added via cannula, followed by $BF_3 \cdot Et_2O$ (0.15 mL, 1.1 mmol) and refluxed for 3 hours under nitrogen. The reaction mixture is subsequently stirred at room temperature overnight.

Synthesis of Compound 19:Chemical Formula: $C_{10}H_{23}NO_4$

Exact Mass: 221.2

Molecular Weight: 221.3

[0491] A 50 mL round bottom flask is charged with Compound 18 (0.3 g, 1.2 mmol) and a stir bar. After flushing with nitrogen, dimethylamine in a 2.0 M methyl alcohol solution (25 mL) is added via syringe. The resulting mixture is stirred at room temperature for 48 hours. The progress of the reaction is monitored using t.l.c. The reaction mixture is concentrated and the crude product used without further purification.

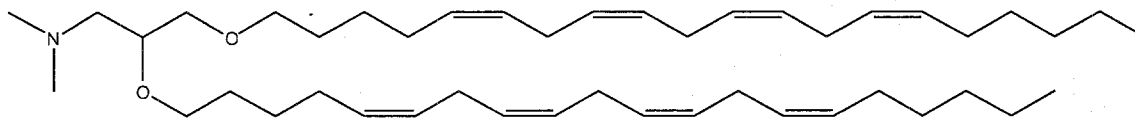
Synthesis of Compound 20:

Chemical Formula: $C_{64}H_{119}NO_4$
 Exact Mass: 965.9
 Molecular Weight: 966.6

[0492] A 100 mL round bottom flask is charged with a stir bar, NaH (0.6 g, 24 mmol), and 25 mL benzene. Compound 19 (0.37 g, 1.8 mmol) is added followed immediately by linoleyl methane sulfonate (2.8 g, 8 mmol). The reaction is refluxed overnight and progress of the reaction monitored via t.l.c. The reaction mixture is transferred to a 250 mL separatory funnel and diluted with benzene to a final volume of 50 mL. The reaction is quenched with ethanol (30 mL) and then washed with water (50 mL). The lower aqueous phase is run off and the reaction mixture washed again with ethanol (30 mL) and water (50 mL). The organic phase is dried with $MgSO_4$, filtered, and solvent removed. The crude product, 2.5 g, is purified using column chromatography on silica gel (60 g), eluted with 0-3% methanol gradient in DCM.

Example 4. Synthesis of 1,2-Diarachidonyloxy-(N,N-dimethyl)-propyl-3-amine (DAraDMA).

[0493] DAraDMA (Compound 21) having the structure shown below was synthesized as described below.



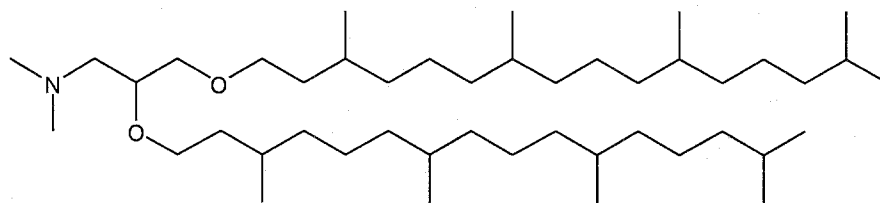
$C_{45}H_{77}O_2N$
 Mol. Wt.: 664.1

[0494] A 250 mL round bottom flask was charged with 3-(dimethylamino)-1,2-propanediol (0.3 g, 2.5 mmol), tetrabutylammonium hydrogen sulphate (0.4 g), arachidonyl methane sulfonate (2 g, 5.4 mmol), and 30 mL toluene. After stirring for 15 minutes, the reaction was cooled to 0-5°C. A solution of 40% sodium hydroxide (15 mL) was added slowly. The reaction was left to stir for approximately 48 hours. After 48 hours of stirring, water (50 mL) and isopropyl acetate (50 mL) were added and stirred for 15 minutes. The mixture was then transferred to a 500 mL separatory funnel, quantitatively transferred with (2 × 5 mL toluene) and allowed to separate. The lower aqueous phase was run off and the organic phase was washed with saturated sodium chloride (50 mL). Approximately 20 minutes were allowed

for phase separation. The two aqueous phases were separately and consecutively back extracted with chloroform (50 mL). The organic phase was subsequently dried with MgSO_4 , filtered, and the solvent evaporated. The crude product (2.6 g) was purified on column chromatography using silica gel (60 g) with 0-2 % methanol gradient in dichloromethane.

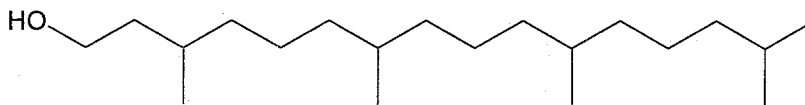
5 **Example 5. Synthesis of 1,2-Diphytanyloxy-3-(*N,N*-dimethyl)-propylamine (DPanDMA).**

[0495] DPanDMA (Compound 24) having the structure shown below was synthesized as described below.



$\text{C}_{45}\text{H}_{93}\text{NO}_2$
Exact Mass: 679.72
Mol. Wt.: 680.23
C, 79.46; H, 13.78; N, 2.06; O, 4.70

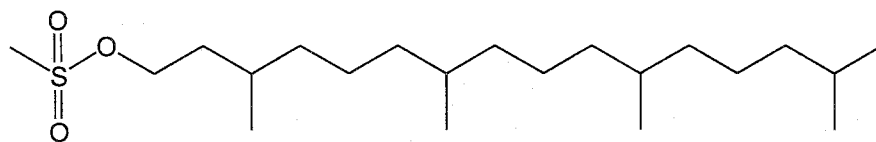
10 **Step 1: Synthesis of Phytanol (Compound 22):**



$\text{C}_{20}\text{H}_{42}\text{O}$
Exact Mass: 298.32
Mol. Wt.: 298.55
C, 80.46; H, 14.18; O, 5.36

[0496] Phytol (21.0 g, 70.8 mmol), ethanol (180 mL) and a stir bar were added to a 500 mL round bottom flask. Raney Nickel 2800 (as purchased, a 50% by weight solution in water if used as purchased, Nickel > 89% metal present) (6.8 g, 51.5 mmol) was added, and the flask sealed and flushed with hydrogen. A 12" needle was used to bubble hydrogen through the solution for 10 minutes. The reaction was stirred for 5 days, using a balloon as a hydrogen reservoir. Hydrogen was also bubbled through the reaction mixture at 24 h and 48 h, 5 minutes each time. The metal catalyst was then removed by filtering through Celite. The ethanolic solution was concentrated, and 200 mL of DCM added to the resulting oil. The solution was washed with water (2 x 100 mL), dried over MgSO_4 , and concentrated. TLC indicated formation of the phytanol product, yield 20.0 g.

Step 2: Synthesis of Phytanyl Mesylate (Compound 23):



C₂₁H₄₄O₃S
 Exact Mass: 376.30
 Mol. Wt.: 376.64
 C, 66.97; H, 11.78; O, 12.74; S, 8.51

[0497] Phytanol (**22**) (20.0 g, 66.7 mmol), triethylamine (18.6 mL, 133 mmol), and a stir bar were added to a 1000 mL round bottom flask. The flask was sealed and flushed with nitrogen. Anhydrous DCM (250 mL) was added, and the mixture cooled to -15°C (ice and NaCl). Mesyl Chloride (10.4 mL, 133 mmol) was added slowly via syringe over a 30 minute period, and the reaction stirred at -15°C for a further 1.5 hours. At this point TLC showed that the starting material had been used up. The solution was diluted with DCM (250 mL) and washed with saturated NaHCO₃ (2 x 200 mL). The organic phase was then dried (MgSO₄), filtered, and concentrated (rotovap). The crude product was purified by column chromatography. Yield: 21.5 g, 85.7%.

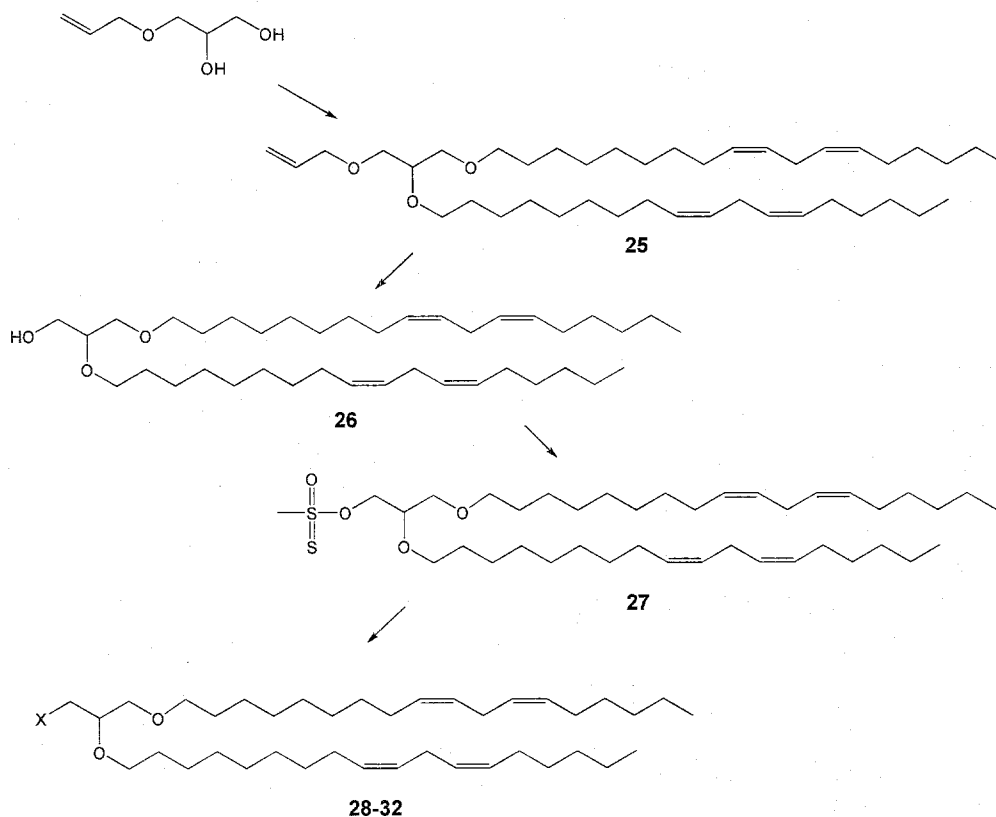
Synthesis of DPanDMA (Compound **24**):

[0498] Sodium hydride (2.5 g, 100 mmol) was added to a 250 mL round bottom flask, along with benzene (40 mL) and a stir bar. In a 50 mL beaker, a solution was made from the *N,N*-Dimethyl-3-aminopropane-1,2-diol (1.42 g, 12 mmol) and benzene (60 mL). This was added to the reaction vessel and the reaction stirred for 10 minutes (effervescence). Phytanyl Mesylate (**23**) (10.52 g, 28 mmol) was added and the flask fitted with a condenser, flushed with nitrogen, and heated to reflux. After 18 hours, the flask was removed from the heat and allowed to cool. The volume was made up to 200 mL with benzene. EtOH was added slowly to quench unreacted sodium hydride. Once quenching was complete, the reaction mixture was washed twice with EtOH/H₂O, in a ratio to the benzene of 1:1:0.6 benzene:water:ethanol. The aqueous phases were combined and extracted with CHCl₃ (2 x 100 mL). Finally, the organic phase was dried (MgSO₄), filtered, and concentrated (rotovap). Purification by column chromatography yielded DPanDMA as a pale yellow oil (6.1 g, 8.97 mmol, 74.7%).

Example 6. Synthesis of Novel Lipids with Alternate Head Groups.

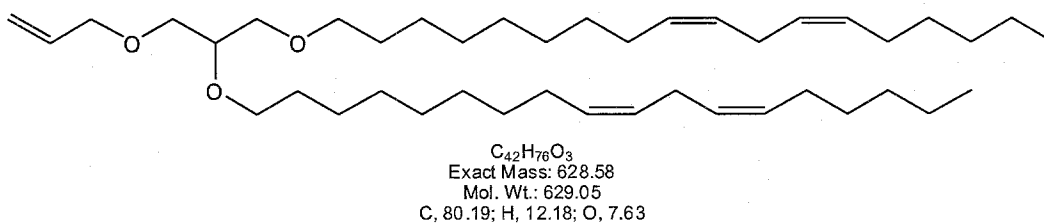
[0499] Novel lipids with alternate head groups (Compounds **28-33**) having the structures shown below were synthesized as shown in the following schematic diagram.

Scheme 3



- 28: DLinPip: X = Piperidino
 29: DLinPip-3OH: X = 3-Hydroxypiperidino
 30: DLinPip-4OH: X = 4-Hydroxypiperidino
 31: DLinDEA: X = Diethylamino
 32: 2N-DLinDMA: X = $(\text{CH}_3)_2\text{NCH}_2\text{CH}_2\text{N}(\text{CH}_3)$
 33: DLinIm: X = Imidazole

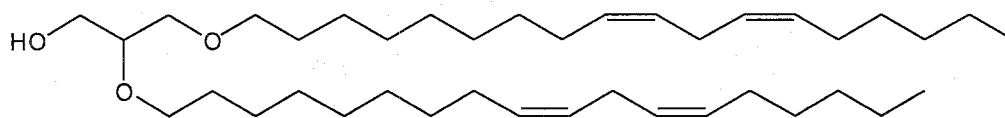
Step 1: Synthesis of 1,2-Dilinoleyloxy-3-allyloxypropane (Compound 25):



[0500] In a 2000 mL round bottom flask equipped with a large magnetic stir bar, 3-allyloxypropane-1,2-diol (5.94 g, 45 mmol), and tetrabutylammonium hydrogen sulfate (6 g, 18 mmol) were added. A solution of linoleyl mesylate (33.8 g, 98 mmol) in toluene (150 mL) was also made and added. The mixture was cooled to 0-5°C with an ice bath. 50% sodium hydroxide (70 mL) was then slowly added, rinsing in with deionized water (20 mL). The resulting mixture is stirred at room temperature, under nitrogen for 40 hours. Deionized water (200 mL) and isopropyl acetate (200 mL) were added and the mixture stirred vigorously for a further 10-15min. The mixture was transferred to a 1000-mL separating funnel and allowed to separate. The aqueous phase was removed and the organic phase washed with saturated sodium chloride solution (2 x 150 mL). The organic phase was dried

with magnesium sulfate (40 g), filtered, and concentrated to obtain a neat orange oil. The oil was purified by column chromatography. Final yield: 19.5 g (69%).

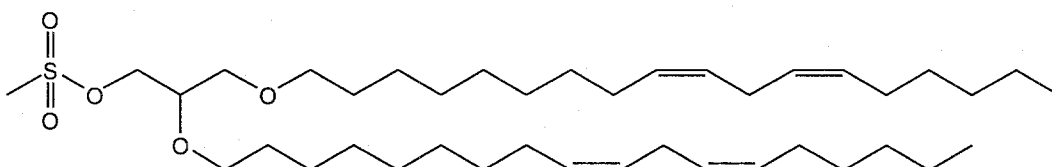
Step 2: Synthesis of 1,2-Dilinoleylglycerol (Compound 26):



$C_{39}H_{72}O_3$
Exact Mass: 588.55
Mol. Wt.: 588.99
C, 79.53; H, 12.32; O, 8.15

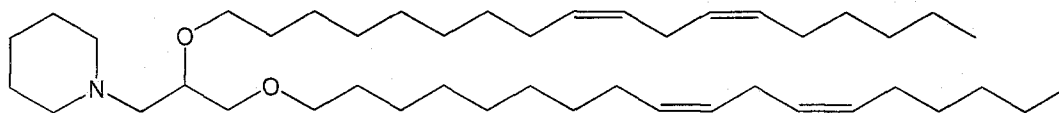
- 5 [0501] 1,2-Dilinoleyloxy-3-allyloxypropane (**25**) (18 g, 28.6 mmol) was dissolved in ethanol (150 mL) in a 500 mL round bottom flask with a stir bar. Trifluoroacetic acid (15 mL) and tetrakis (triphenylphosphine) palladium(0) (5.0 g, 4.3 mmol) were added and the flask flushed with nitrogen. A water condenser was fitted and the flask wrapped tin foil to reduce exposure to light. The reaction was refluxed under nitrogen overnight. The solution
10 was concentrated to an orange oil, then redissolved in DCM (250 mL), and washed with water (2 x 200 mL) and saturated brine (1 x 200 mL). The aqueous phases were combined and extracted with DCM (2 x 100 mL). Organic phases were combined, dried over $MgSO_4$ and concentrated. The crude product was purified by column chromatography to yield 1,2-dilinoleylglycerol as a colorless oil (11.0 g, 18.7 mmol, 65.3%).

15 Step 3: Synthesis of 1,2-Dilinoleyl-3-methanesulfonylglycerol (Compound 27):



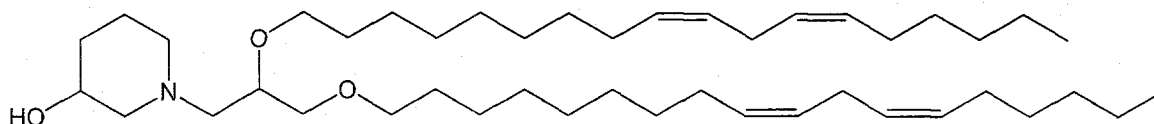
$C_{40}H_{74}O_5S$
Exact Mass: 666.53
Mol. Wt.: 667.08
C, 72.02; H, 11.18; O, 11.99; S, 4.81

- [0502] 1,2-Dilinoleylglycerol (**26**) (11.0 g, 18.7 mmol), triethylamine (5.04 mL, 36 mmol), and a stir bar were added to a 250 mL round bottom flask. The flask was sealed and flushed with nitrogen. Anhydrous DCM (75 mL) was added, and the mixture cooled to $-15^{\circ}C$ (Ice
20 and NaCl). Mesyl Chloride (2.80 mL, 36 mmol) was added via syringe over a 5 minute period, and the reaction stirred at $-15^{\circ}C$ for 1 hour. At this point TLC showed that the starting material had been consumed. The solution was diluted with DCM (120 mL) and washed with saturated $NaHCO_3$ (2 x 150 mL). The organic phase was then dried ($MgSO_4$), filtered, concentrated, and the product purified by column chromatography (10.6 g, 85%).

Synthesis of 1,2-Dilinoleyloxy 3-piperidinopropylamine (DLinPip) (Compound 28):

Chemical Formula: $C_{44}H_{81}NO_2$
 Exact Mass: 655.6
 Molecular Weight: 656.1
 Elemental Analysis: C, 80.54; H, 12.44; N, 2.13; O, 4.88

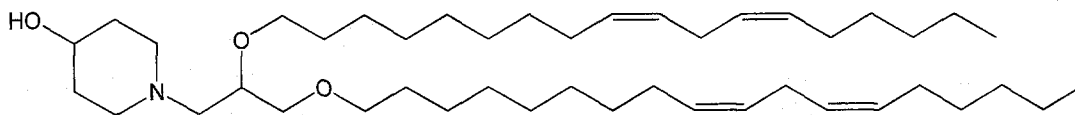
[0503] 1,2-Dilinoleyl-3-methanesulfonylglycerol (**27**) (1.0 g, 1.5 mmol) and a stir bar were added to a 50 mL RBF and flushed with nitrogen. Ethanol (15 mL) and piperidine (1.28 g, 1.48 mL, 15 mmol) were added and the reaction stirred for 48 h at 50°C. The reaction mixture was concentrated (by rotovap) to a volume of 5-10 mL, then ethyl acetate (50 mL) added (a precipitate formed). The mixture was washed with $NaHCO_3$ (2 x 50 mL), and water (2 x 50 mL). The precipitate was washed out. The aqueous phase was extracted once with DCM (30 mL), then the organics combined, dried ($MgSO_4$), and concentrated to yield a yellow oil, which was purified by column chromatography (500 mg, 51%).

Synthesis of 1,2-Dilinoleyloxy 3-(3'-hydroxypiperidino)-propylamine (DLinPip-3OH) (Compound 29):

Chemical Formula: $C_{44}H_{81}NO_3$
 Exact Mass: 671.6
 Molecular Weight: 672.1
 Elemental Analysis: C, 78.63; H, 12.15; N, 2.08; O, 7.14

[0504] 1,2-Dilinoleyl-3-methanesulfonylglycerol (**27**) (1.0 g, 1.5 mmol) and a stir bar were added to a 50 mL RBF and flushed with nitrogen. Ethanol (15 mL) and 3-hydroxypiperidine (1.52 g, 15 mmol, 10-fold excess) were added and the reaction stirred for 48 h at 50°C. The reaction mixture was concentrated (by rotovap), then ethyl acetate (30 mL) added (a precipitate formed). The mixture was washed with $NaHCO_3$ (2 x 30 mL), and water (2 x 30 mL) and brine (30 mL). The precipitate was washed out. The organic was dried ($MgSO_4$) and concentrated to yield a yellow oil, which was purified by column chromatography (450 mg, 45%).

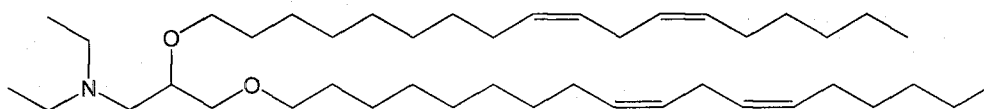
Synthesis of 1,2-Dilinoleyloxy 3-(4'-hydroxypiperidino)-propylamine (DLinPip-4OH) (Compound 30):



Chemical Formula: $C_{44}H_{81}NO_3$
 Exact Mass: 671.6
 Molecular Weight: 672.1
 Elemental Analysis: C, 78.63; H, 12.15; N, 2.08; O, 7.14

[0505] 1,2-Dilinoleyl-3-methanesulfonyl glycerol (**27**) (1.0 g, 1.5 mmol) and a stir bar were added to a 50 mL RBF and flushed with nitrogen. Ethanol (15 mL) and 4-hydroxypiperidine (1.52 g, 15 mmol, 10-fold excess) were added and the reaction stirred for 48 h at 50°C. The reaction mixture was concentrated (by rotovap), then ethyl acetate (30 mL) added (a precipitate formed). The mixture was washed with $NaHCO_3$ (2 x 30 mL), and water (2 x 30 mL) and brine (30 mL). The precipitate was washed out. The organic was dried ($MgSO_4$) and concentrated to yield a yellow oil, which was purified by column chromatography (470 mg, 47%).

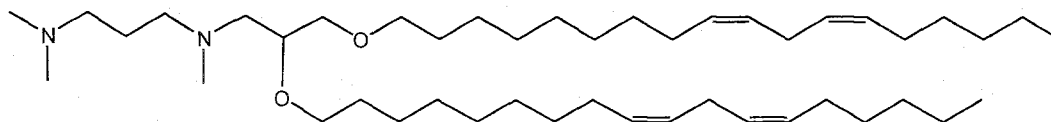
10 Synthesis of 1,2 Dilinoleyloxy 3-(N,N dimethyl)-propylamine (DLinDEA) (Compound 31):



Chemical Formula: $C_{43}H_{81}NO_2$
 Exact Mass: 643.6
 Molecular Weight: 644.1
 Elemental Analysis: C, 80.18; H, 12.68; N, 2.17; O, 4.97

[0506] 1,2-Dilinoleyl-3-methanesulfonyl glycerol (**27**) (1.0 g, 1.5 mmol) and a stir bar were added to a 50 mL RBF and flushed with nitrogen. Ethanol (15 mL) and diethylamine (1.55 mL, 15 mmol) were added and the reaction stirred for 48 h at 40°C. The reaction mixture was concentrated (by rotovap), then ethyl acetate (30 mL) added (a precipitate formed). The mixture was washed with $NaHCO_3$ (2 x 30 mL), and water (2 x 30 mL) and brine (30 mL). The precipitate was washed out. The organic was dried ($MgSO_4$) and concentrated to yield a yellow oil, which was purified by column chromatography (590 mg, 61%).

20 Synthesis of N1-((2,3-linoleyloxy)propyl)-N1,N3,N3-trimethylpropane-1,3-diamine (2N-DLinDMA) (Compound 32):

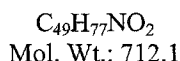
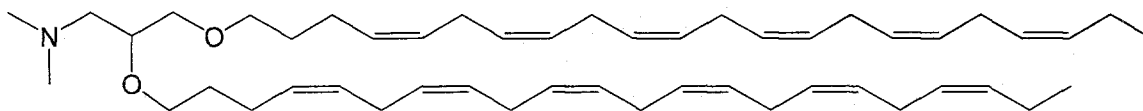


$C_{46}H_{86}N_2O_2$
 Exact Mass: 686.67
 Mol. Wt.: 687.18
 C, 78.65; H, 12.61; N, 4.08; O, 4.66

[0507] 1,2-Dilinoley1-3-methanesulfonylglycerol (**27**) (1.50 g, 2.25 mmol) and a stir bar were put in a 100 mL round-bottom flask. A solution of *N,N,N'*-trimethyl-1,3-propanediamine (2.75 g, 23.7 mmol) in methanol (30 mL) was added, then the mixture heated to reflux for 48 hours. The reaction mixture was diluted with CHCl₃ (50 mL), and washed with 1M NaOH (50 mL). Separation was aided by further addition of MeOH. The reaction was washed 2 more times with dH₂O (25 mL). The aqueous phases were extracted with CHCl₃ (20 mL), and all organics combined. The organic was dried over MgSO₄, filtered, concentrated, and purified by column chromatography to yield a yellow oil (610 mg, 39%).

Example 7. Synthesis of 1,2-Didocosahexaenyloxy-(*N,N*-dimethyl)-propyl-3-amine (DDocDMA).

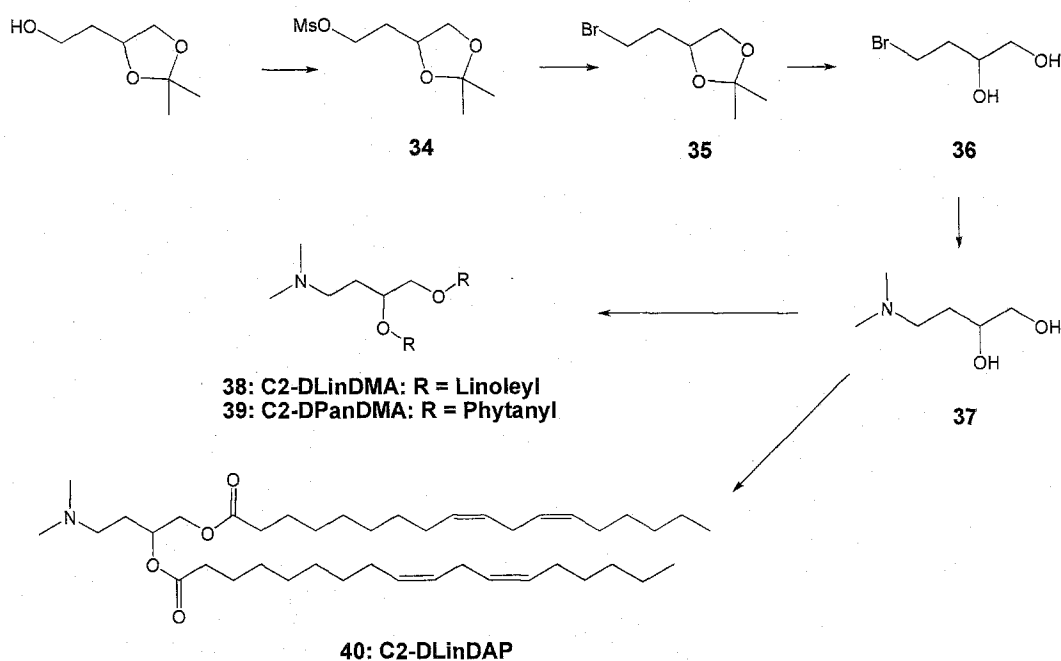
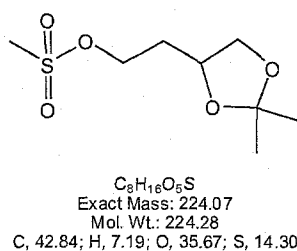
[0508] DDocDMA (Compound **33**) having the structure shown below was synthesized as described below.



[0509] A 250 mL round bottom flask was charged with 3-(dimethylamino)-1,2-propanediol (0.28 g, 2.36 mmol), tetrabutylammonium hydrogen sulphate (0.4 g), docosahexaenyl methane sulfate (2.0 g, 5.1 mmol), and 30 mL toluene. After stirring for 15 minutes, the reaction was cooled to 0-5°C. A solution of 40% sodium hydroxide (15 mL) was added slowly. The reaction was left to stir for approximately 40 hours. After 40 hours of stirring, water (50 mL) and isopropyl acetate (50 mL) were added and stirred for 15 minutes. The mixture was then transferred to a 500 mL separatory funnel, quantitatively transferred with (2 × 5 mL toluene) and allowed to separate. The lower aqueous phase was run off and the organic phase was washed with saturated sodium chloride (50 mL). Approximately 20 minutes were allowed for phase separation. The two aqueous phases were separately and consecutively back extracted with chloroform (50 mL). The organic phase was subsequently dried with MgSO₄, filtered, and the solvent evaporated. The crude product (4.8 g) was purified on column chromatography using silica gel (60 g) with 0-2 % methanol gradient in dichloromethane.

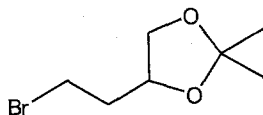
Example 8. Synthesis of Novel C2 Lipids.

[0510] Novel C2 lipids (Compounds **38-40**) having the structures shown below were synthesized as shown in the following schematic diagram.

Scheme 4**Step 1: Synthesis of 4-(2-Methanesulfonylethyl)-2,2-dimethyl-1,3-dioxolane (34):**

- 5 [0511] 4-(2-Hydroxyethyl)-2,2-dimethyl-1,3-dioxolane (25 g, 170 mmol), triethylamine (55.9 mL, 400 mmol), and a stir bar were added to a 1000 mL round bottom flask. The flask was sealed and flushed with nitrogen. Anhydrous DCM (600 mL) was added, and the mixture cooled to approx -5°C (ice and NaCl). Mesyl chloride (19.9 mL, 255 mmol, 1.5 eq) was added slowly via syringe over a 60 minute period, and the reaction stirred at -5°C for a
- 10 further 1.5 hours. At this point TLC showed that the starting material had been consumed. The solution was diluted with DCM (350 mL), divided into two (~500 mL) portions, and each portion worked up as follows: the solution was transferred to a 1000-mL separating funnel and washed with saturated $NaHCO_3$ (2 x 200 mL). The organic phase was then dried ($MgSO_4$), filtered, and concentrated (rotovap). The crude product was purified by column
- 15 chromatography. Final yield: 32.0 g, 84.1%.

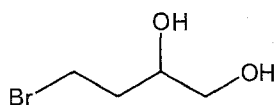
Step 2: Synthesis of 4-(2-Bromoethyl)-2,2-dimethyl-1,3-dioxolane (Compound 35):



$C_7H_{13}BrO_2$
 Exact Mass: 208.01
 Mol. Wt.: 209.08
 C, 40.21; H, 6.27; Br, 38.22; O, 15.30

[0512] Magnesium bromide etherate (40 g, 130 mmol) and a stir bar were added to a 2000 mL round bottom flask and flushed with nitrogen. A solution of 4-(2-methanesulfonylethyl)-2,2-dimethyl-1,3-dioxolane (**34**) (17.5 g, 78 mmol) in anhydrous diethyl ether (900 mL) was added via cannula, and the suspension stirred overnight. The ether was first decanted into a beaker. Water (200 mL) and ether (300 mL) were added to the precipitate and stirred for 5 minutes. The precipitate was dissolved, and the ether phase was then collected and added to the ether solution from the reaction. The organic phase was then washed, concentrated to about 500 mL, washed with water, dried over anhydrous Mg_2SO_4 , filtered, and concentrated to yield a yellow oil (16.0 g). This was purified by flash chromatography to yield 10.6 g of product (50.7 mmol, 65%).

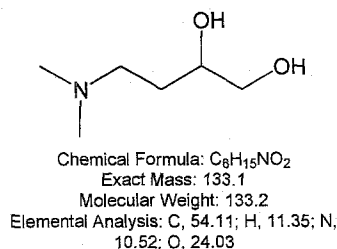
Step 3: Synthesis of 4-Bromobutane-1,2-diol (Compound 36):



$C_4H_9BrO_2$
 Exact Mass: 167.98
 Mol. Wt.: 169.02
 C, 28.42; H, 5.37; Br, 47.28; O, 18.93

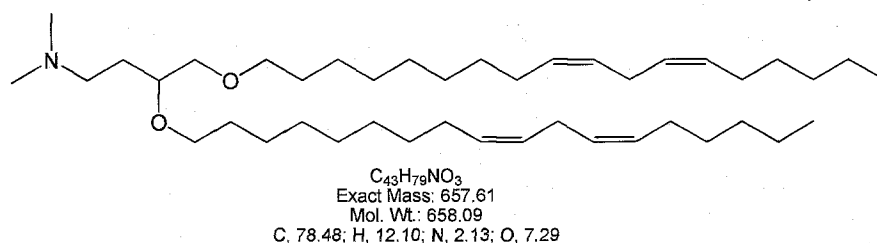
[0513] 4-(2-Bromoethyl)-2,2-dimethyl-1,3-dioxolane (**35**) (9 g, 43 mmol) was added to a 500 mL RBF with a stirbar. 100 mL of MeOH:H₂O:HCl in a ratio of (60:20:5) were added. After 30 minutes, sat. NaHCO₃ (~75 mL) was added (effervescence), until pH paper indicated solution was basic. At this point the mixture was slightly cloudy. Ether (300 mL) was added (while stirring) and the cloudiness disappeared. The reaction mixture was transferred to a 1000 mL sep funnel and the 2 phases separated. The extraction of the aqueous phase was repeated two more times (2 x 300 mL ether). Organics were combined, dried over $MgSO_4$ and concentrated to yield a colorless oil (7.0 g), which was purified by column chromatography.

Step 4: Synthesis of 4-(Dimethylamino)-1,2-butanediol (Compound 37):



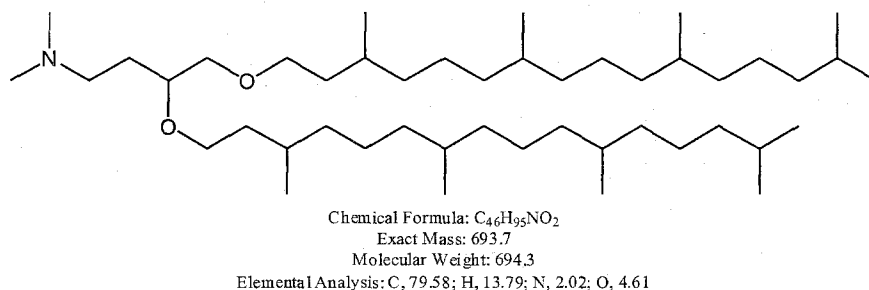
[0514] 4-Bromobutane-1,2-diol (**36**) (1 g, 6.0 mmol) was added to a 50mL RBF with a stir bar, sealed, and flushed with nitrogen. 30 mL of Dimethylamine (2.0M solution in MeOH) was delivered by cannula and the reaction stirred overnight. TLC indicated all the starting material had disappeared. The solvent (and DMA) were removed by evaporation and the crude product used without further purification.

Synthesis of 1,2-Dilinoleyloxy-(*N,N*-dimethyl)-butyl-4-amine (C2-DLinDMA) (**38**):



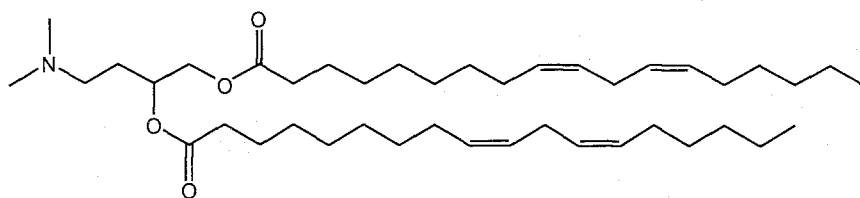
[0515] 4-(Dimethylamino)-1,2-butanediol (**37**) (1.3 g, 3.4 mmol), linoleyl mesylate (2.0 g, 5.8 mmol), tetrabutylammonium hydrogen sulphate (0.5 g, 1.5 mmol), toluene (30 mL), and a stir bar were added to a 100 mL RBF. 30 mL of 40% NaOH was made and added to the reaction mixture. The resulting mixture was stirred at room temperature, under nitrogen for 60 hours. Deionized water (50 mL) and isopropyl acetate (50 mL) were added and the mixture stirred vigorously for a further 10-15 min. The mixture was transferred to a 250 mL separating funnel and allowed to separate and the aqueous phase removed. The organic phase was washed twice with water (2 x 30 mL) using MeOH to aid the separation, and the organic phase was dried ($MgSO_4$), filtered, and concentrated to obtain a dark yellow oil. The oil was purified by column chromatography.

Synthesis of 1,2-Diphytanyloxy-(*N,N*-dimethyl)-butyl-4-amine (C2-DPanDMA) (**39**):



[0516] Sodium hydride (360 mg, 15 mmol), benzene (40 mL), and a stir bar were added to a 50 mL round bottom flask. 4-(Dimethylamino)-1,2-butanediol (**37**) (200 mg, 1.5 mmol) was added and the reaction stirred for 10 minutes (effervescence). Phytanyl Mesylate (**23**), (1.07 g, 2.92 mmol) was then added and the flask fitted with a condenser, flushed with nitrogen, and heated to reflux. After 18 hours, the flask was allowed to cool to room temperature. The volume was made up to 40 mL with benzene. EtOH was added slowly to quench unreacted sodium hydride. Once quenching was complete, the reaction mixture was washed twice with an EtOH/H₂O, in a ratio to the benzene of 1:1:0.6 benzene:water:ethanol. The aqueous washes were combined and extracted with CHCl₃ (2 x 20 mL). Finally, the organics were combined, dried (MgSO₄), filtered, and concentrated (rotovap). Purification by column chromatography yielded a pale yellow oil (250 mg, 0.145 mmol, 25%).

Synthesis of 1,2-Dilinoleoyloxy-(*N,N*-dimethyl)-butyl-4-amine (C2-DLinDAP) (**40**):



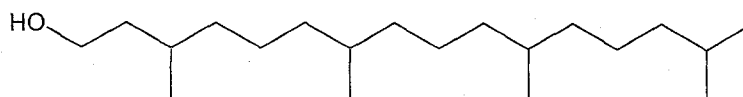
Chemical Formula: C₄₂H₇₅NO₄
 Exact Mass: 657.6
 Molecular Weight: 658.0
 Elemental Analysis: C, 76.66; H, 11.49; N, 2.13; O, 9.73

[0517] A flask containing 4-(Dimethylamino)-1,2-butanediol (**37**) (crude, 266 mg, 2 mmol (max)), TEA (0.84 mL, 6 mmol), and DMAP (24 mg, 0.2 mmol) was flushed with nitrogen before the addition of anhydrous CH₂Cl₂ (50 mL). Linoleoyl chloride (1.2 g, 4 mmol) was added and the solution stirred overnight. The solution was rinsed into a 250 mL separatory funnel with DCM (~ 70 mL) and washed with water (2 x 50 mL). The organic was dried (MgSO₄), concentrated, and purified by chromatography.

Example 9. Synthesis of Novel Phytanyl Cationic Lipids.

[0518] DPan-C2K-DMA (Compound **47**), DPan-C1K6-DMA (Compound **50**), and DPan-C3K-DMA (Compound **54**) having the structures shown below were synthesized as shown in the following schematic diagram.

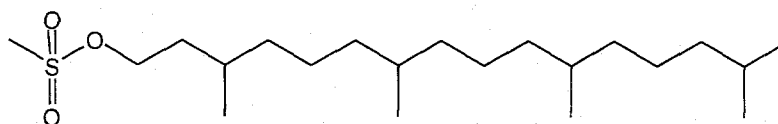
Synthesis of Phytanol (Compound **22**):



C₂₀H₄₂O
 Exact Mass: 298.32
 Mol. Wt.: 298.55
 C, 80.46; H, 14.18; O, 5.36

[0519] Phytol (21.0 g, 70.8 mmol), ethanol (180 mL) and a stir bar were added to a 500 mL round bottom flask. Raney Nickel 2800 (as purchased, a 50% by weight solution in water if used as purchased, Nickel > 89% metal present) (6.8 g, 51.5 mmol) was added, and the flask sealed and flushed with hydrogen. A 12" needle was used to bubble hydrogen through the solution for 10 minutes. The reaction was stirred for 5 days, using a balloon as a hydrogen reservoir. Hydrogen was also bubbled through the reaction mixture at 24 h and 48 h, 5 minutes each time. The metal catalyst was then removed by filtering through Celite. The ethanolic solution was concentrated, and 200 mL of DCM added to the resulting oil. The solution was washed with water (2 x 100 mL), dried over MgSO₄, and concentrated. TLC indicated formation of the phytanol product, yield 20.0 g.

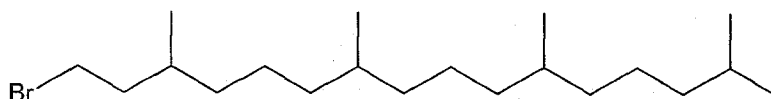
Synthesis of Phytanyl Mesylate (Compound 23):



C₂₁H₄₄O₃S
Exact Mass: 376.30
Mol. Wt.: 376.64
C, 66.97; H, 11.78; O, 12.74; S, 8.51

[0520] Phytanol (22) (20.0 g, 66.7 mmol), triethylamine (18.6 mL, 133 mmol) and a stir bar were added to a 1000 mL round bottom flask. The flask was sealed and flushed with nitrogen. Anhydrous DCM (250 mL) was added, and the mixture cooled to -15°C (Ice and NaCl). Mesyl Chloride (10.4 mL, 133 mmol) was added slowly via syringe over a 30 minute period, and the reaction stirred at -15°C for a further 1.5 hours. At this point TLC showed that the starting material had been used up. The solution was diluted with DCM (250 mL) and washed with saturated NaHCO₃ (2 x 200 mL). The organic phase was then dried (MgSO₄), filtered and concentrated (rotovap). The crude product was purified by column chromatography. Yield 21.5 g, 85.7%.

Synthesis of Phytanyl Bromide (Compound 41):

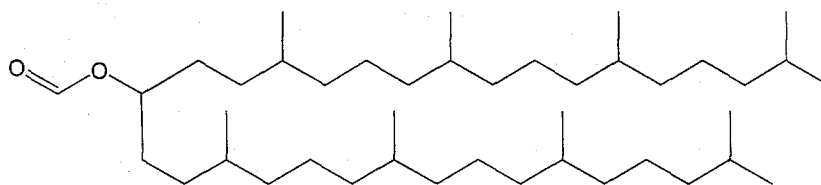


Chemical Formula: C₂₀H₄₁Br
Exact Mass: 360.2
Molecular Weight: 361.4
Elemental Analysis: C, 66.46; H, 11.43; Br, 22.11

[0521] Magnesium bromide etherate (17 g, 55 mmol) and a stir bar were added to a 500 mL round bottom flask. The flask was sealed and flushed with nitrogen and anhydrous diethyl ether (200 mL) added via cannula. A solution of phytanyl mesylate (23) (10.9 g, 28.9 mmol

(FW = 377)) in anhydrous ether (50 mL) was also added via cannula, and the suspension stirred overnight. The following morning a precipitate had formed on the side of the flask. Chilled water (200 mL) was added (ppt dissolved) and the mixture transferred to a 1000-mL separating funnel. After shaking, the organic phase was separated. The aqueous phase was then extracted with ether (2 x 150 mL) and all ether phases combined. The ether phase was washed with water (2 x 150 mL), brine (150 mL) and dried over anhydrous Mg_2SO_4 . The solution was filtered, concentrated, and purified by flash chromatography. Final yield 9.5 g (26.3 mmol, 91.1%).

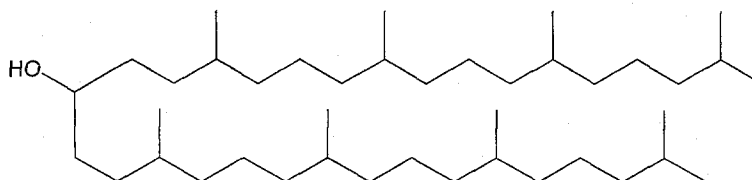
Synthesis of Compound 42:



Chemical Formula: $\text{C}_{42}\text{H}_{84}\text{O}_2$
 Exact Mass: 620.65
 Molecular Weight: 621.12
 Elemental Analysis: C, 81.22; H, 13.63; O, 5.15

[0522] Magnesium turnings (720 mg, 30 mmol), a crystal of iodine, and a stirbar were added to a 500 mL round-bottom flask. The flask was flushed with nitrogen and anhydrous diethyl ether (200 mL) added via cannula. A solution of phytanyl bromide (41) (9.5 g, 26.3 mmol) in anhydrous ether (20 mL) was added and the resulting cloudy mixture refluxed overnight. The mixture was cooled to RT and, without removing the subaseal or condenser, ethyl formate (2.2 g, 2.41 mL, 30 mmol) added via syringe and 12" needle. The addition was made dropwise, directly into the reaction mixture, and the cloudy suspension again stirred overnight. R.M. was transferred to a 500-mL sep. funnel with ether (50 mL), and washed with 10% H_2SO_4 (100 mL – the cloudy R.M. now clarified upon shaking), water (2 x 100 mL) and brine. The organic was dried over anhydrous Mg_2SO_4 , filtered, and concentrated. Yield (crude) was 8 g. TLC indicated that the majority of product was the diphytanylmethyl formate, which was purified by chromatography (0 – 6% ether in hexane).

Synthesis of Compound 43:

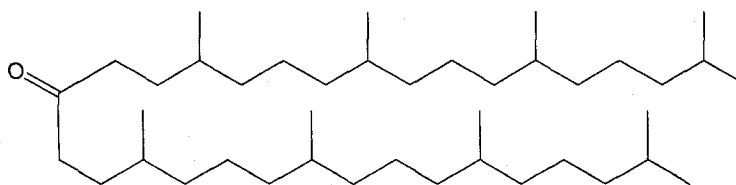


Chemical Formula: $\text{C}_{41}\text{H}_{84}\text{O}$
 Exact Mass: 592.65
 Molecular Weight: 593.11
 Elemental Analysis: C, 83.03; H, 14.28; O, 2.70

[0523] The purified formate (**42**) (5.5 g, 8.86 mmol) was then transferred to a 1000 mL round bottom flask with stirbar and 90% EtOH (500 mL) and KOH (2.0 g, 35.7 mmol) added. The reaction mixture was clear, and was stirred overnight. The following day the mixture was concentrated by rotovap to 50% of its volume and then poured into 200 mL of 5% HCl.

5 The aqueous phase was extracted with ether (3 x 100 mL). The combined ether extracts were washed with water (3 x 200 mL), dried (MgSO₄), and concentrated. TLC (DCM) revealed reaction to have gone cleanly to completion, and the product (5.5 g, 100%) was used without further purification.

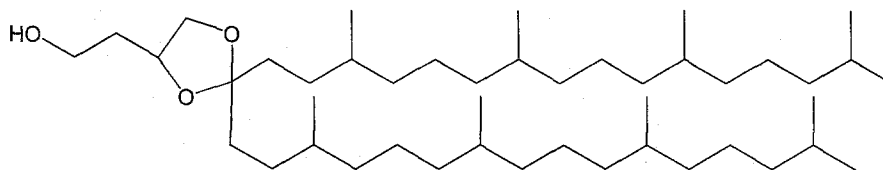
Synthesis of Compound 44:



Chemical Formula: C₄₁H₈₂O
Exact Mass: 590.6
Molecular Weight: 591.1
Elemental Analysis: C, 83.31; H, 13.98; O, 2.71

10 [0524] To a mixture of Compound **43** (5.5 g, 9.3 mmol), pyridinium chlorochromate (PCC) (5.5 g, 25.5 mmol) and anhydrous sodium carbonate (0.6 g, 5.66 mmol) in DCM were added. The resulting suspension was stirred for 1 h, but TLC indicated still some starting material (SM) remaining. The suspension was stirred another hour, and appeared to have progressed slightly, but not to completion. Further PCC (1.0 g) and sodium carbonate (0.2 g) were added
15 and the reaction stirred overnight. Reaction had now gone to completion. Ether (300 mL) was then added to the mixture and the resulting brown suspension filtered through a pad of silica (300 mL), washing the pad with ether (3 x 100 mL). The ether phases were combined, concentrated, and purified to yield 5.0 g (90%) of ketone.

20 Synthesis of Compound 45:

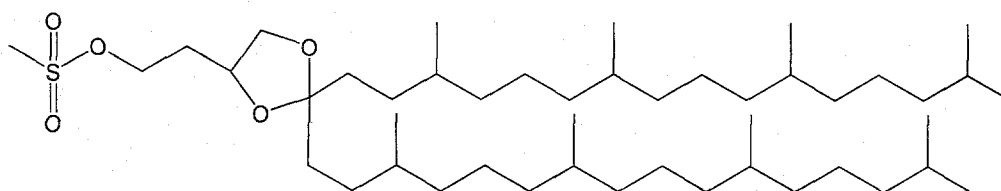


Chemical Formula: C₄₅H₉₀O₃
Exact Mass: 678.69
Molecular Weight: 679.19
Elemental Analysis: C, 79.58; H, 13.36; O, 7.07

[0525] A 100 mL round bottom flask was charged with Compound **44** (1.4 g, 2.4 mmol), 1, 2, 4-butanetriol (0.51 g, 4.8 mmol), pyridinium p-toluenesulfonate (0.06 g, 0.24 mmol), and a stir bar. The reaction vessel was flushed with nitrogen and anhydrous toluene (30 mL) added

via cannula. The flask was equipped with a Dean-Stark tube and condenser and flushed with nitrogen. The reaction was refluxed under nitrogen overnight and progress of the reaction monitored via TLC. After refluxing for three hours, reaction solution deposited in the Dean-Stark tube was removed via syringe (20 mL) and the reaction vessel immediately replenished with fresh toluene (20 mL). This was repeated every hour, for a total of three times, and then left to reflux mildly overnight. After cooling to room temperature, the reaction mixture was transferred to a 250 mL separatory funnel with toluene (2×5 mL), washed with 5% aqueous Na_2CO_3 (2×50 mL), water (50 mL), and dried over MgSO_4 . Evaporation of the solvent gave 1.67 g of crude product which was purified via column chromatography on silica gel (50 g) using dichloromethane as eluent. Yield: 1.4 g, 2.06 mmol, 86%.

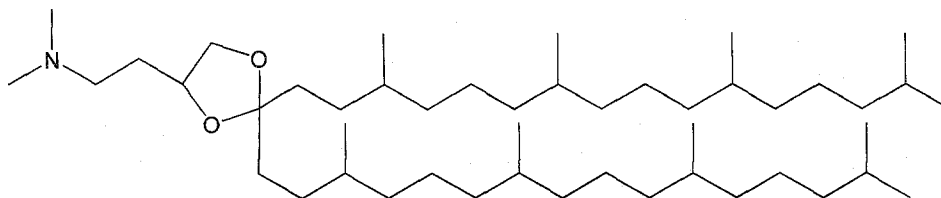
Synthesis of Compound 46:



Chemical Formula: $\text{C}_{46}\text{H}_{92}\text{O}_5\text{S}$
 Exact Mass: 756.67
 Molecular Weight: 757.28
 Elemental Analysis: C, 72.96; H, 12.25; O, 10.56; S, 4.23

[0526] A 100 mL round bottom flask was charged with Compound 45 (1.4 g, 2.06 mmol) and a stir bar. The vessel was flushed with nitrogen and DCM (25 mL) added. Subsequently, triethylamine (0.72 g, 7.1 mmol, 0.99 mL) was added via syringe and the resulting solution cooled to -15°C (NaCl , ice). In a separate 50 mL round bottom flask, a solution of methanesulfonic anhydride (0.74 g, 4.1 mmol) and DCM (20 mL) was prepared. This solution was added drop wise to the above solution over a 30 minute period. The reaction vessel was maintained at -15°C . The reaction mixture was stirred at room temperature overnight and monitored via TLC. The reaction mixture was then diluted with DCM (25 mL), and washed with NaHCO_3 (2×30 mL), then dried over anhydrous MgSO_4 . The crude product (1.7g) was used in the next step without further purification.

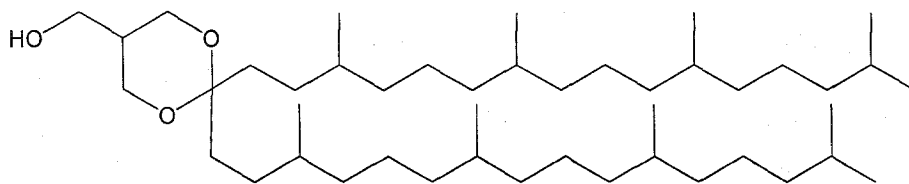
Synthesis of DPan-C2K-DMA (Compound 47):



Chemical Formula: $C_{47}H_{95}NO_2$
 Exact Mass: 705.74
 Molecular Weight: 706.26
 Elemental Analysis: C, 79.93; H, 13.56; N, 1.98; O, 4.53

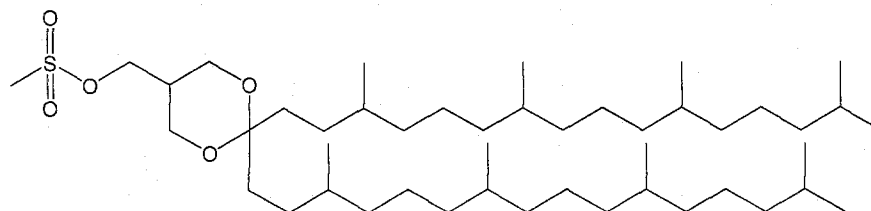
[0527] A 500 mL round bottom flask was charged with crude Compound 46 (1.7 g, 2.5 mmol) and a stir bar. The reaction vessel was flushed with nitrogen and dimethylamine in THF (2.0 M, 65 mL) subsequently added via syringe. The resulting mixture was stirred for three days at room temperature. The reaction was concentrated and the crude product purified by column chromatography using silica gel (40 g) with a gradient of 0-5% methanol in dichloromethane.

Synthesis of Compound 48:



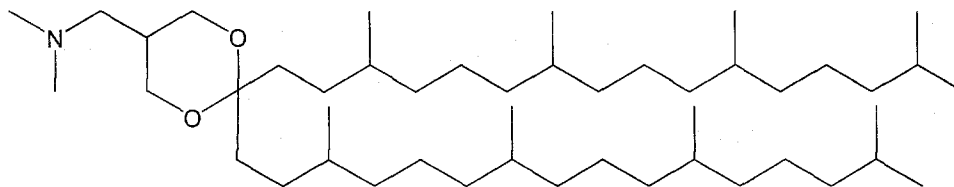
Chemical Formula: $C_{45}H_{90}O_3$
 Exact Mass: 678.69
 Molecular Weight: 679.19
 Elemental Analysis: C, 79.58; H, 13.36; O, 7.07

[0528] A 100 mL round bottom flask was charged with Compound 44 (1.2 g, 2.1 mmol), 2-hydroxymethyl-1, 3-propanediol (0.45 g, 4.2 mmol), pyridinium p-toluenesulfonate (0.05 g, 0.21 mmol), and a stir bar. The reaction vessel was flushed with nitrogen and anhydrous toluene (45 mL) subsequently added via cannula. The flask was equipped with a Dean-Stark tube and condenser and flushed with nitrogen. The reaction was refluxed under nitrogen overnight and progress of the reaction monitored via TLC. After refluxing for three hours, reaction solution deposited in the Dean-Stark tube was removed via syringe (20 mL) and the reaction vessel immediately replenished with fresh toluene (20 mL). This was repeated every hour, for a total of three times, and then left to reflux mildly overnight. After cooling to room temperature, the reaction mixture was transferred to a 250 mL separatory funnel with toluene (2 × 5 mL), washed with 5% aqueous Na_2CO_3 (2 × 50 mL), water (50 mL), and dried over $MgSO_4$. Evaporation of the solvent gave 1.44 g of crude product which was then purified via column chromatography on silica gel (35 g) with 0-3% methanol gradient in dichloromethane.

Synthesis of Compound 49:

Chemical Formula: C₄₆H₉₂O₅S
 Exact Mass: 756.67
 Molecular Weight: 757.28
 Elemental Analysis: C, 72.96; H, 12.25; O, 10.56; S, 4.23

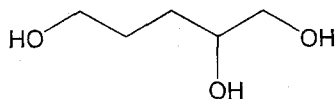
[0529] A 250 mL round bottom flask was charged with Compound 48 (1.2 g, 1.8 mmol) and a stir bar. The vessel was flushed with nitrogen and DCM (25 mL) added. Subsequently, triethylamine (0.62 g, 6.1 mmol, 0.85 mL) was added via syringe and the resulting solution cooled to -15°C (NaCl, ice). In a separate 50 mL round bottom flask, a solution of methanesulfonyl anhydride (0.67 g, 3.7 mmol) and DCM (20 mL) was prepared. This solution was added drop wise to the above solution over a 30 minute period. The reaction vessel was maintained at -15°C during the addition. The reaction mixture was stirred at room temperature overnight and monitored via TLC. The reaction mixture was then diluted with DCM (25 mL) and washed with NaHCO₃ (2× 30 mL), then dried over anhydrous MgSO₄. The crude product (1.6 g) was used in the following step without further purification.

Synthesis of DPan-C1K6-DMA (Compound 50):

Chemical Formula: C₄₇H₉₅NO₂
 Exact Mass: 705.74
 Molecular Weight: 706.26
 Elemental Analysis: C, 79.93; H, 13.56; N, 1.98; O, 4.53

[0530] A 250 mL round bottom flask was charged with crude Compound 49 (1.6 g, 2.1 mmol) and a stir bar. The reaction vessel was flushed with nitrogen and dimethylamine in THF (2.0 M, 60 mL) subsequently added via syringe. The resulting mixture was stirred for six days at room temperature. After solvent was evaporated, the crude product was purified using column chromatography on silica gel (30 g) with 0-30% ethyl acetate gradient in hexanes.

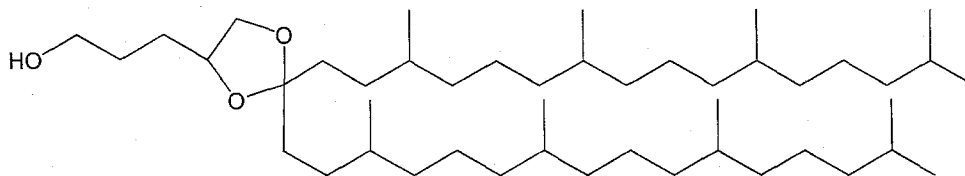
Synthesis of Compound 51:



Chemical Formula: $C_5H_{12}O_3$
 Exact Mass: 120.08
 Molecular Weight: 120.15
 Elemental Analysis: C, 49.98; H, 10.07; O, 39.95

[0531] A 50 mL round bottom flask was charged with (R)- γ -hydroxymethyl- γ -butyrolactone (1.0 g, 8.6 mmol), flushed with nitrogen, and sealed with a rubber septum. Anhydrous THF (40 mL) was subsequently added via syringe. The (R)- γ -hydroxymethyl- γ -butyrolactone solution was then added drop wise under nitrogen to a prepared solution containing $LiAlH_4$ (3.5 g, 92 mmol) in 160 mL anhydrous THF. During the addition, the reaction vessel was maintained at 0° C. The resulting suspension was stirred at room temperature overnight. The reaction mixture was cooled to 0° C and brine (10-22 mL) added very slowly using a Pasteur pipette. The mixture was stirred under nitrogen at room temperature overnight. The white solid was filtered and washed with THF (3 $\times$ 25 mL). The organics were combined and concentrated. After solvent was removed, the crude product seemed to contain water along with an oily residue; therefore, the crude product was azeotroped within ethanol (100 mL) resulting in a yellow oil. The crude product (0.45 g) was used in the next step without further purification.

Synthesis of Compound 52:

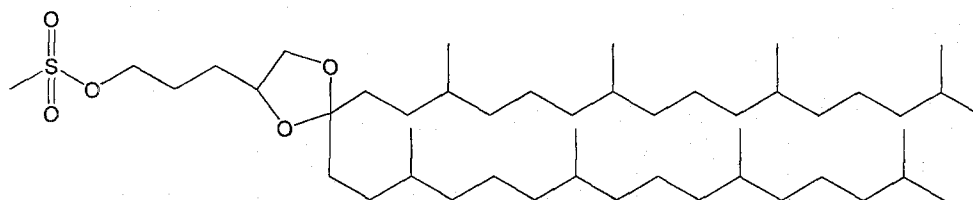


Chemical Formula: $C_{46}H_{92}O_3$
 Exact Mass: 692.70
 Molecular Weight: 693.22
 Elemental Analysis: C, 79.70; H, 13.38; O, 6.92

[0532] A 100 mL round bottom flask was charged with Compound 44 (1.0 g, 1.8 mmol), Compound 51 (crude, 0.450 g, 3.6 mmol), pyridinium p-toluenesulfonate (0.05 g, 0.24 mmol), and a stir bar. The reaction vessel was flushed with nitrogen and anhydrous toluene (45 mL) subsequently added via cannula. The flask was equipped with a Dean-Stark tube and condenser and flushed with nitrogen. The reaction was refluxed under nitrogen overnight and progress of reaction monitored via TLC. After refluxing for three hours, reaction solution deposited in the Dean-Stark tube was removed via syringe (20 mL) and the reaction vessel immediately replenished with fresh toluene (20 mL). This was repeated every hour, for a total of five times, and then left to reflux mildly overnight. After cooling to room

temperature, the reaction mixture was transferred to a 250 mL separatory funnel with toluene (2 × 5 mL), washed with 5% aqueous Na₂CO₃ (2 × 50 mL), water (50 mL), and dried over MgSO₄. Evaporation of the solvent gave 1.13 g of crude product which was then purified via column chromatography on silica gel (30 g) using dichloromethane as eluent. Yield, 1.0 g.

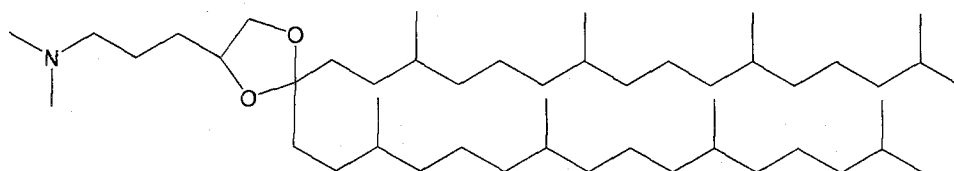
5 Synthesis of Compound 53:



Chemical Formula: C₄₇H₉₄O₅S
 Exact Mass: 770.68
 Molecular Weight: 771.31
 Elemental Analysis: C, 73.19; H, 12.28; O, 10.37; S, 4.16

[0533] A 250 mL round bottom flask was charged with Compound 52 (1.0 g, 1.44 mmol) and a stir bar. The vessel was flushed with nitrogen and DCM (25 mL) added. Subsequently, triethylamine (0.51 g, 5 mmol, and 0.7 mL) was added via syringe and the resulting solution cooled to -15°C (NaCl, ice). In a separate 50 mL round bottom flask, a solution of methanesulfonic anhydride (0.54 g, 3.0 mmol) and anhydrous DCM (20 mL) was prepared. This solution was added drop wise to the above solution over a 30 minute period. The reaction vessel was maintained at -15°C. The reaction mixture was stirred at room temperature overnight and monitored via TLC. The reaction mixture was then diluted with DCM (25 mL) and washed with NaHCO₃ (2 × 30 mL), then dried over anhydrous MgSO₄. The crude product (1.2 g) was used in the next step without further purification.

Synthesis of DPan-C3K-DMA (Compound 54):

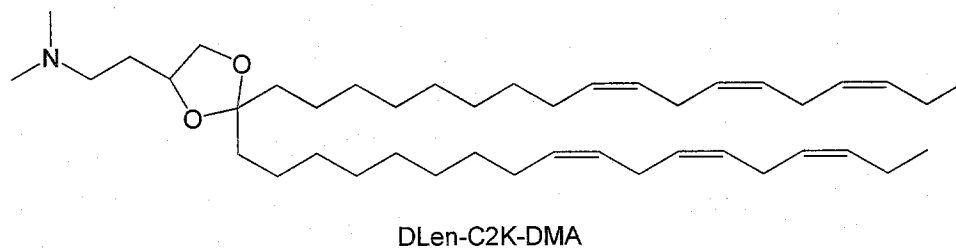


Chemical Formula: C₄₈H₉₇NO₂
 Exact Mass: 719.75
 Molecular Weight: 720.29
 Elemental Analysis: C, 80.04; H, 13.57; N, 1.94; O, 4.44

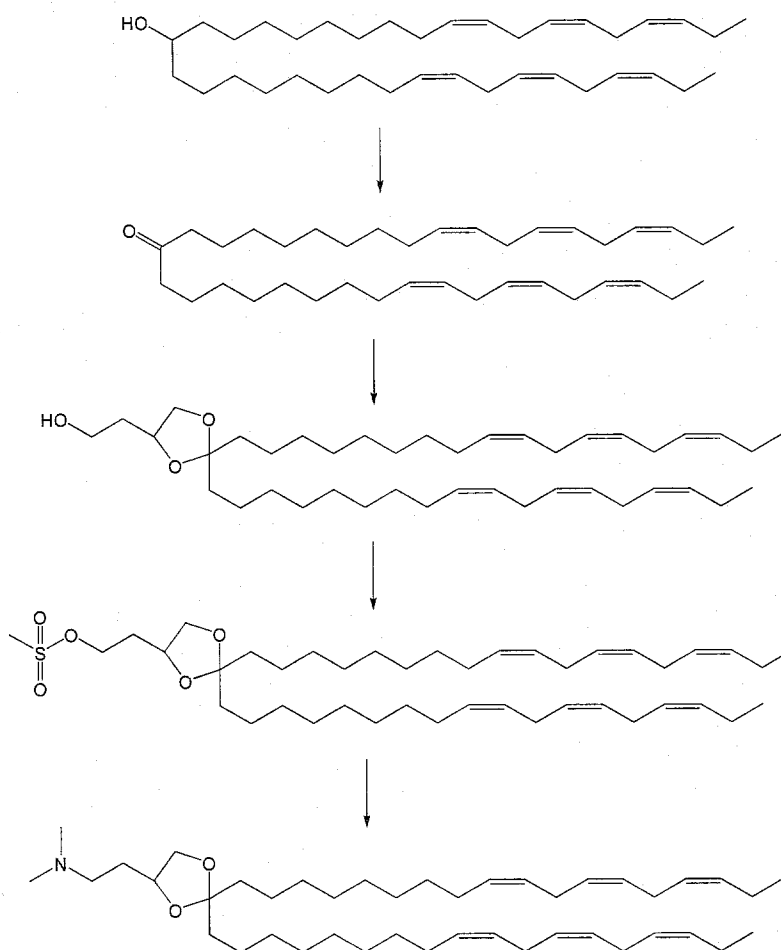
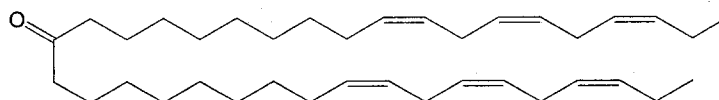
[0534] A 100 mL round bottom flask was charged with crude Compound 53 (1.2 g, 1.6 mmol) and a stir bar. The reaction vessel was flushed with nitrogen and dimethylamine in THF (2.0 M, 45 mL) subsequently added via syringe. The resulting mixture was stirred for four days at room temperature. After solvent was evaporated, the crude product was purified using column chromatography on silica gel (30 g) with 0-30% ethyl acetate gradient in hexanes.

Example 10. Synthesis of DLen-C2K-DMA.

[0535] DLen-C2K-DMA (Compound 58) having the structure shown below was synthesized as described in Scheme 5 below.



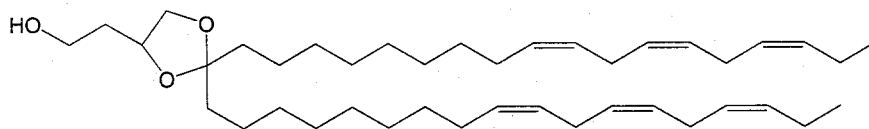
5

Scheme 5**Synthesis of dilinolenyl ketone (Compound 55):**

[0536] To a 1000 mL RBF containing a solution of dilinolenyl methanol (6.0 g, 11.4 mmol) in anh. DCM (200 mL) was added pyridinium chlorochromate (7.39 g, 34.2 mmol), anh. sodium carbonate (1.0 g, 5.66 mmol) and a stirbar. The resulting suspension was stirred under nitrogen at RT for 3 h, after which time TLC indicated all SM to have been consumed. Ether (300 mL) was then added to the mixture and the resulting brown suspension filtered

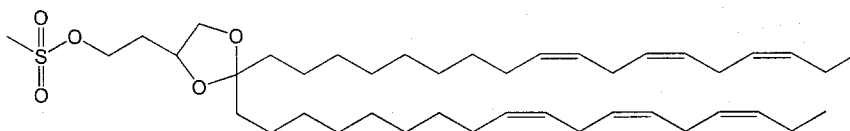
through a pad of silica (300 mL), washing the pad with ether (3 x 100 mL). The ether phases were combined, concentrated and purified to yield 4.2 g (8.0 mmol, 70%) of the ketone.

Synthesis of linolenyl ketal (Compound 56):



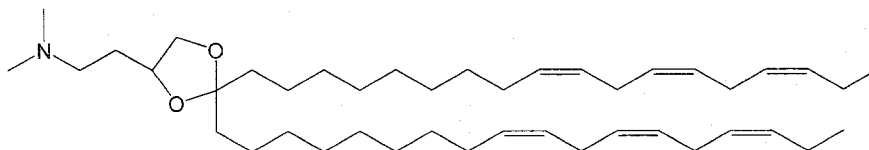
- 5 [0537] A 100 mL RBF was charged with dilinolenyl ketone (Compound 55) (4.2 g, 8.2 mmol), 1,2,4-butanetriol (3.4 g, 32 mmol), PPTS (200 mg, 0.8 mmol) and a stir bar. The flask was flushed with nitrogen and anhydrous toluene (60 mL) added. The reaction vessel was fitted with a Dean Stark tube and condenser and brought to reflux and the reaction was left overnight. After cooling to room temperature, the reaction mixture diluted with toluene
10 (50 mL), and washed with 5% aq. Na_2CO_3 (2×50 mL), water (50 mL), dried (MgSO_4) and purified by chromatography to yield 3.0 g (4.9 mmol, 59%) of the ketal.

Mesylate formation (Compound 57):



- [0538] A 250 mL RBF was charged with the ketal (Compound 56) (3.0 g, 4.9 mmol), TEA (2.2 mL, 15.6 mmol) and a stir bar. The flask was flushed with nitrogen, anh. DCM (20 mL) added, and the solution cooled to -15°C . In a separate 50 mL flask, a solution of MsCl (9.7 mmol, 2 eqv.) in anhydrous DCM (30 mL) was prepared, then transferred to the reaction vessel by syringe over 20 minutes. The reaction was stirred for 90 minutes at -15°C , at which point starting material had been consumed. The reaction mixture was diluted with a further
20 50 mL of DCM, washed with NaHCO_3 (2×50 mL), dried (MgSO_4) and purified by chromatography. Final yield 3.1 g, 4.5 mmol, 92%.

Synthesis of DLen-C2K-DMA (Compound 58):

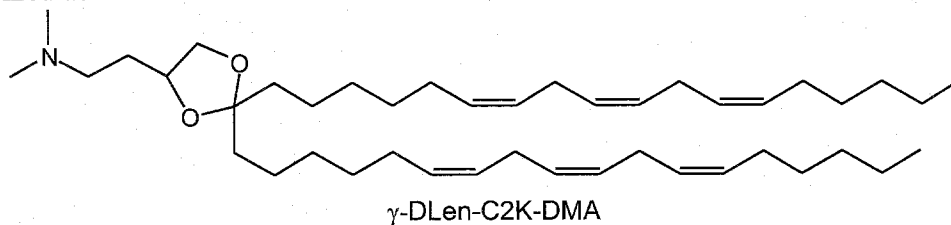
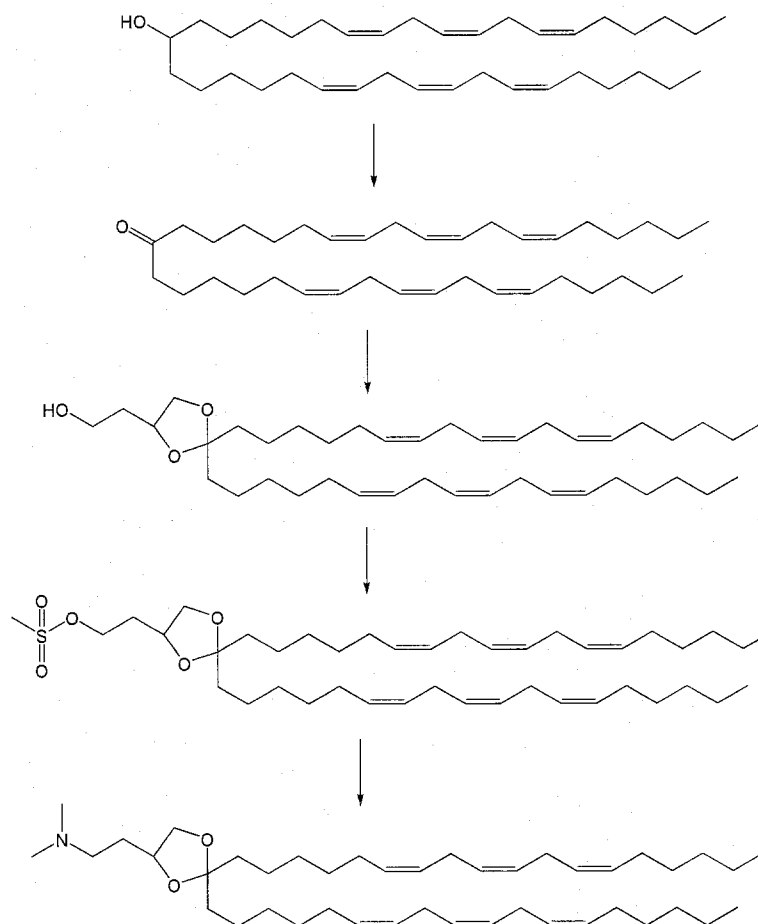
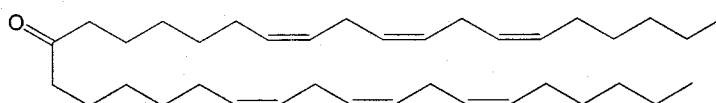


- [0539] A 250 mL RBF was charged with the mesylate (Compound 57) (3.0 g, 4.35 mmol), isopropanol (25 mL) and a stir bar. The flask was flushed with nitrogen, sealed, and a 2.0 M solution of dimethylamine in methanol (120 mL) added via cannula. The reaction was stirred at room temperature for 3 days. The solution was concentrated and purified by chromatography. Final yield 2.49 g, 3.9 mmol, 90%.

Example 11. Synthesis of γ -DLen-C2K-DMA.

[0540] γ -DLen-C2K-DMA (Compound **62**) having the structure shown below was synthesized as described in Scheme 6 below.

5

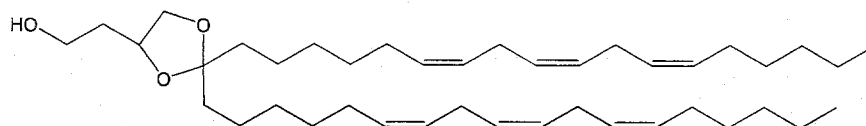
**Scheme 6**10 **Synthesis of di- γ -linolenyl ketone (Compound **59**):**

[0541] To a 1000 mL RBF containing a solution of di- γ -linolenyl methanol (6.0 g, 11.4 mmol) in anh. DCM (200 mL) was added pyridinium chlorochromate (7.39 g, 34.2 mmol), anh. sodium carbonate (1.0 g, 5.66 mmol) and a stirbar. The resulting suspension was stirred under nitrogen at RT for 3 h, after which time TLC indicated all SM to have been consumed. Ether (300 mL) was then added to the mixture and the resulting brown suspension filtered

15

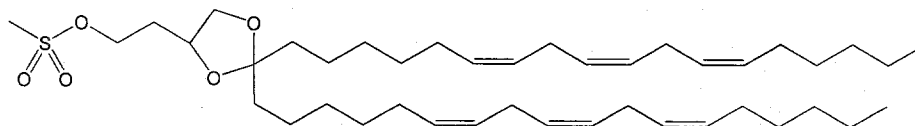
through a pad of silica (300 mL), washing the pad with ether (3 x 100 mL). The ether phases were combined, concentrated and purified to yield 5.5 g (10.5 mmol, 92%) of ketone.

Synthesis of γ -linolenyl ketal (Compound 60):



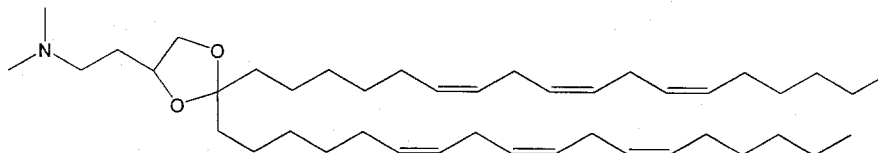
- 5 [0542] A 100 mL RBF was charged with di- γ -linolenyl ketone (Compound 59) (2.14 g, 4.1 mmol), 1,2,4-butanetriol (1.7 g, 16.0 mmol), PPTS (100 mg, 0.4 mmol) and a stir bar. The flask was flushed with nitrogen and anhydrous toluene (30 mL) added. The reaction vessel was fitted with a Dean Stark tube and condenser and brought to reflux and the reaction was left overnight. After cooling to room temperature, the reaction mixture was washed with 5%
10 aq. Na_2CO_3 (2×50 mL), water (50 mL), dried (MgSO_4) and purified by chromatography to yield 1.34 g (2.2 mmol, 53%) of the ketal.

Mesylate formation (Compound 61):



- [0543] A 250 mL RBF was charged with the ketal (Compound 60) (1.34 g, 2.19 mmol),
15 TEA (1 mL, 7.1 mmol) and a stir bar. The flask was flushed with nitrogen, anh. DCM (10 mL) added, and the solution cooled to -15°C . In a separate 50 mL flask, a solution of MsCl (342 μL , 4.4 mmol, 2 eqv.) in anhydrous DCM (15 mL) was prepared, then transferred to the reaction vessel by syringe over 20 minutes. The reaction was stirred for 90 minutes at -15°C , at which point starting material had been consumed. The reaction mixture was diluted with a
20 further 50 mL of DCM, washed with NaHCO_3 (2×50 mL), dried (MgSO_4) and purified by chromatography. Final yield 1.31 g, 1.90 mmol, 87%.

Synthesis of γ -DLen-C2K-DMA (Compound 62):



- [0544] A 250 mL RBF was charged with the mesylate (Compound 61) (1.31 g, 1.9 mmol),
25 isopropanol (10 mL) and a stir bar. The flask was flushed with nitrogen, sealed, and a 2.0 M solution of dimethylamine in methanol (60 mL) added via cannula. The reaction was stirred

at room temperature for 3 days. The solution was concentrated and purified by chromatography. Final yield 1.1 g, 1.72 mmol, 91%.

Example 12. Lipid Encapsulation of siRNA.

[0545] All siRNA molecules used in these studies were chemically synthesized and annealed using standard procedures.

[0546] In some embodiments, siRNA molecules were encapsulated into serum-stable nucleic acid-lipid particles (SNALP) composed of the following lipids: (1) the lipid conjugate PEG2000-C-DMA (3-N-[(methoxypoly(ethylene glycol)2000)carbamoyl]-1,2-dimyristyloxypropylamine); (2) one or more cationic lipids or salts thereof (*e.g.*, cationic lipids of Formula I-XII of the invention and/or other cationic lipids described herein); (3) the phospholipid DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine) (Avanti Polar Lipids; Alabaster, AL); and (4) synthetic cholesterol (Sigma-Aldrich Corp.; St. Louis, MO) in the molar ratio 1.4:57.1:7.1:34.3, respectively. In other words, siRNA molecules were encapsulated into SNALP of the following “1:57” formulation: 1.4% PEG2000-C-DMA; 57.1% cationic lipid; 7.1% DPPC; and 34.3% cholesterol. It should be understood that the 1:57 formulation is a target formulation, and that the amount of lipid (both cationic and non-cationic) present and the amount of lipid conjugate present in the formulation may vary. Typically, in the 1:57 formulation, the amount of cationic lipid will be 57.1 mol % $\pm$ 5 mol %, and the amount of lipid conjugate will be 1.4 mol % $\pm$ 0.5 mol %, with the balance of the 1:57 formulation being made up of non-cationic lipid (*e.g.*, phospholipid, cholesterol, or a mixture of the two).

[0547] In other embodiments, siRNA were encapsulated into SNALP composed of the following lipids: (1) the lipid conjugate PEG750-C-DMA (3-N-[(Methoxypoly(ethylene glycol)750)carbamoyl]-1,2-dimyristyloxypropylamine); (2) one or more cationic lipids or salts thereof (*e.g.*, cationic lipids of Formula I-XII of the invention and/or other cationic lipids described herein); (3) the phospholipid DPPC; and (4) synthetic cholesterol in the molar ratio 6.76:54.06:6.75:32.43, respectively. In other words, siRNA were encapsulated into SNALP of the following “7:54” formulation: 6.76 mol % PEG750-C-DMA; 54.06 mol % cationic lipid; 6.75 mol % DPPC; and 32.43 mol % cholesterol. Typically, in the 7:54 formulation, the amount of cationic lipid will be 54.06 mol % $\pm$ 5 mol %, and the amount of lipid conjugate will be 6.76 mol % $\pm$ 1 mol %, with the balance of the 7:54 formulation being made up of non-cationic lipid (*e.g.*, phospholipid, cholesterol, or a mixture of the two).

[0548] For vehicle controls, empty particles with identical lipid composition may be formed in the absence of siRNA.

Example 13. pK_a Measurements of SNALP Formulations Containing Novel Cationic Lipids.

[0549] This example demonstrates the determination of pK_a values of 1:57 SNALP formulations containing various novel cationic lipids described herein with an siRNA targeting apolipoprotein B (APOB).

[0550] 1:57 SNALP formulations containing encapsulated APOB siRNA were prepared as described in Section VI above with the following cationic lipids: (1) DLinDMA; (2) DLenDMA; (3) γ -DLenDMA ("g-DLenDMA"); (4) C2-DLinDAP; (5) DLinPip (4-OH); (6) DLinPip; (7) DLinPip (3-OH); (8) DLinDEA; (9) DDocDMA; (10) DAraDMA; (11) TLinDMA; (12) DLin-C2K Pip (3OH); (13) DHep-C2K-DMA; (14) DPanDMA; (15) C2-DPanDMA ("DPan-C2-DMA"); (16) DPan-C2K-DMA; (17) DPan-C3K-DMA; (18) DPan-K6-DMA ("DPan-C1K6-DMA"); and (19) C2-TLinDMA.

[0551] The apparent pK_a of the cationic lipids present in these SNALP formulations was determined using a 2-(*p*-toluidinyl)-naphthalene-6-sodium sulfonate (TNS) assay. TNS is a negatively-charged indicator of membrane potential that is electrostatically attracted to positively charged membranes (*see*, Bailey and Cullis, *Biochemistry*, 33 12573-80 (1994)). Subsequent adsorption to the lipid membrane results in the immediate environment of the TNS becoming more lipophilic, removing the water molecules that otherwise quench TNS fluorescence. As a result, TNS measures the surface potential of the particle, wherein the more positive the surface potential, the greater the level of fluorescence. The surface pK_a values of each SNALP formulation were determined by varying the local pH in the presence of TNS. By plotting fluorescence versus pH, the pK_a of the cationic lipid can be estimated in the particle as the pH where fluorescence equals 50% of total fluorescence.

[0552] The results of the TNS assays are shown in Figures 1-2. Figure 2 (top panel) shows the following 1:57 SNALP pK_a values: DLinDMA ~5.8 ; DDocDMA, DAraDMA ~5.65; TLinDMA ~5.35; DLin-C2K Pip (3OH), DHep-C2K-DMA ~6.05; and C2:DPanDMA ~6.1. Figure 2 (bottom panel) shows the following 1:57 SNALP pK_a values: DLinDMA ~5.8; DPanDMA ~ 5.5; C2-DPanDMA ~6.1; DPan-C2K-DMA ~5.95; DPan-K6-DMA ~5.5; and DPan-C3K-DMA ~6.25. The 1:57 SNALP pK_a value for C2-TLinDMA was ~5.85.

Example 14. Characterization of SNALP Formulations Containing Novel Cationic Lipids.

[0553] This example demonstrates the efficacy of 1:57 SNALP formulations containing various novel cationic lipids described herein with an siRNA targeting ApoB in a mouse liver model. The APOB siRNA sequence used in this study is provided in Table 1.

Table 1

siRNA	APOB siRNA Sequence	% 2'OMe-Modified	% Modified in DS Region
ApoB-10164	5'-AGU <u>G</u> UCA <u>U</u> CACAC <u>U</u> GAAUACC-3' (SEQ ID NO:1) 3'-GU <u>U</u> CACAGUAGU <u>G</u> <u>U</u> GACUUAU-5' (SEQ ID NO:2)	7/42 = 16.7%	7/38 = 18.4%

Column 1: The number after "ApoB" refers to the nucleotide position of the 5' base of the sense strand relative to the human APOB mRNA sequence NM_000384. Column 2: 2'OMe nucleotides are indicated in bold and underlined. The 3'-overhangs on one or both strands of the siRNA molecule may alternatively comprise 1-4 deoxythymidine (dT) nucleotides, 1-4 modified and/or unmodified uridine (U) ribonucleotides, or 1-2 additional ribonucleotides having complementarity to the target sequence or the complementary strand thereof. Column 3: The number and percentage of 2'OMe-modified nucleotides in the siRNA molecule are provided. Column 4: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA molecule are provided.

[0554] 1:57 SNALP formulations containing encapsulated APOB siRNA were prepared as described in Section VI above with the following cationic lipids: (1) DLinDMA; (2) DLin-K-C2-DMA ("C2K"); (3) DLin-K-C3-DMA ("C3K"); (4) DLin-K-C4-DMA ("C4K"); (5) DLin-K6-DMA; (6) DLin-C2-DMA; (7) DLenDMA; (8) γ -DLenDMA ("g-DLenDMA"); (9) DLin-K-DMA; (10) DLinMorph; (11) Linoleyl/Oleyl DMA ("Lin/Ol"); (12) Linoleyl/Linolenyl DMA ("Lin/Len"); (13) Linoleyl/Phytanyl DMA ("Lin/Pan"); (14) Linoleyl/Stearyl DMA ("Lin/Str"); and (15) Linoleyl/C6:1 DMA ("Lin/C6:1").

[0555] Each SNALP formulation was administered by intravenous (IV) injection at 0.1 mg/kg into female Balb/c mice (n = 3 per group). Plasma total cholesterol and/or liver ApoB mRNA levels were evaluated at 48 hours after SNALP administration. For dose response studies, SNALP formulations were administered by IV injection at 0.01 mg/kg, 0.03 mg/kg, or 0.1 mg/kg into female Balb/c mice (n = 3 per group). Liver ApoB mRNA levels were evaluated at 48 hours after SNALP administration.

[0556] Figures 3-5 show a comparison of the plasma total cholesterol knockdown efficacy and/or the liver ApoB mRNA knockdown activity of each of these SNALP formulations (Error bars = SD). Figure 6 shows a dose response evaluation of three different doses of SNALP formulations containing either DLinDMA, DLin-K-C2-DMA ("C2K"), DLenDMA, or γ -DLenDMA on liver ApoB mRNA knockdown activity (Error bars = SD).

[0557] These figures illustrate that a SNALP formulation containing γ -DLenDMA was unexpectedly more potent in silencing ApoB expression compared to a SNALP formulation containing either DLinDMA or DLenDMA and produced *in vivo* results similar to that of a SNALP formulation containing DLin-K-C2-DMA ("C2K"). These figures also illustrate that a SNALP formulation containing an asymmetric cationic lipid such as Linoleyl/Linolenyl DMA ("Lin/Len") displayed greater ApoB silencing activity compared to a SNALP formulation containing DLinDMA.

Example 15. Tolerability of γ -DLenDMA SNALP Formulation.

[0558] The tolerability of the 1:57 γ -DLenDMA SNALP formulation was evaluated with a single IV bolus injection of SNALP at either 10 mg/kg, 15 mg/kg, or 20 mg/kg in Balb/c mice (n = 3 per group). Liver enzyme levels were measured at 48 hours post-injection.

- 5 Figure 7 shows that both DLenDMA and γ -DLenDMA SNALP formulations did not significantly elevate liver enzyme levels compared to the PBS control.

Example 16. Characterization of Additional SNALP Formulations Containing Novel Cationic Lipids.

- 10 [0559] This example demonstrates the efficacy of additional 1:57 SNALP formulations containing various novel cationic lipids described herein with an siRNA targeting ApoB in a mouse liver model. The APOB siRNA sequence used in these studies is provided in Table 1.

- [0560] 1:57 SNALP formulations containing encapsulated APOB siRNA at a 6:1 lipid:drug (L:D) ratio were prepared with the following cationic lipids: (1) DLinDMA; (2) Linoleyl/Linolenyl DMA ("Lin/LenDMA"); (3) DPanDMA; (4) TLinDMA; (5) Linoleyl/C6:0 DMA
15 ("Lin/6:0"); (6) C2-DPanDMA; (7) DLin-C2K-Pip (3OH); and (8) DHep-C2K-DMA.

- [0561] Each SNALP formulation was administered by intravenous (IV) injection at 0.1 mg/kg into female Balb/c mice (n = 3 per group). Livers were collected at 48 hours after SNALP administration and liver ApoB mRNA levels were evaluated by performing an ApoB/GAPDH QG assay. Table 2 provides a characterization of the SNALP formulations
20 used in this *in vivo* study.

Table 2

SNALP	Size (nm)	Poly	Encapsulation %
1:57 DLinDMA	74.96 77.88	0.023 0.054	79
1:57 Lin/LenDMA	81.12 98.21	0.023 0.010	63
1:57 DPanDMA	64.84 65.44	0.063 0.033	78
1:57 TLinDMA	71.98 73.12	0.037 0.069	51
1:57 Lin/6:0	101.7 97.64	0.095 0.119	78
1:57 C2-DPanDMA	95.09 98.26	0.058 0.052	90
1:57 DLin-C2K-Pip (3OH)	68.92 73.27	0.093 0.067	73
1:57 DHep-C2K-DMA	75.29 79.89	0.034 0.037	89

1:57 DLinDMA (TFU)	82.23	0.039	89
--------------------	-------	-------	----

Columns 2 & 3: The bottom value in each entry corresponds to the particle size and polydispersity observed 10 days after the formulation was prepared.

[0562] Figure 8 illustrates, *inter alia*, that: (1) DHep-C2K-DMA, which contains double bonds in the *trans* configuration thought to reduce potency, was unexpectedly comparable to DLinDMA with respect to silencing activity; (2) C2-DPanDMA, which contains saturated fatty alkyl chains thought to decrease potency, was unexpectedly more potent compared to DLinDMA and substantially more potent compared to DPanDMA with respect to silencing activity; (3) TLinDMA displayed silencing activity that was comparable to DLinDMA; and (4) Lin/LenDMA had more potent silencing activity than DLinDMA. A similar study with 1:57 SNALP containing C2-TLinDMA showed that C2-TLinDMA (49% knockdown) was more potent than DLinDMA (25% knockdown) with respect to ApoB silencing activity.

[0563] In another study, 1:57 SNALP formulations containing encapsulated APOB siRNA at a 6:1 L:D ratio were prepared with the following cationic lipids: (1) DLinDMA; (2) C2-DPanDMA; (3) DPan-C2K-DMA; (4) DPan-C3K-DMA; and (5) DPan-C1K6-DMA.

[0564] Each SNALP formulation was administered by intravenous (IV) injection at 0.1 mg/kg into female Balb/c mice (n = 3 per group). Livers were collected at 48 hours after SNALP administration and liver ApoB mRNA levels were evaluated by performing an ApoB/GAPDH QG assay. Table 3 provides a characterization of the SNALP formulations used in this *in vivo* study.

Table 3

	Size (nm)	Poly	Encapsulation %
1:57 DLinDMA	76.63	0.033	81
1:57 C2-DPanDMA	85.80	0.003	88
1:57 DPan-C2K-DMA	79.06	0.020	87
1:57 DPan-C3K-DMA	93.46	0.002	90
1:57 DPan-C1K6-DMA	72.78	0.031	77

[0565] Figure 9 illustrates that C2-DPanDMA, DPan-C2K-DMA, and DPan-C3K-DMA, which contains saturated fatty alkyl chains thought to decrease potency, were unexpectedly more potent compared to DLinDMA with respect to silencing activity. C2-DPanDMA had the greatest activity of all the phytanyl-containing cationic lipids tested.

Example 17. Characterization of Additional SNALP Formulations Containing Novel Cationic Lipids.

[0566] This example demonstrates the efficacy of additional 1:57 SNALP formulations containing various novel cationic lipids described herein with an siRNA targeting ApoB in a mouse liver model. The APOB siRNA sequence used in these studies is provided in Table 1.

[0567] 1:57 SNALP formulations containing encapsulated APOB siRNA were prepared as described in Section VI above with the following cationic lipids: (1) DLin-C2K-DMA (“C2K”); (2) γ -DLen-C2K-DMA (“g-DLen-C2K-DMA”); and (3) DLen-C2K-DMA.

[0568] For dose response studies, SNALP formulations were administered by IV injection at 0.01 mg/kg, 0.033 mg/kg, or 0.1 mg/kg into female Balb/c mice (n = 3 per group). Liver ApoB mRNA levels were evaluated at 48 hours after SNALP administration by a branched DNA assay (QuantiGene assay) to assess ApoB mRNA relative to the housekeeping gene GAPDH.

[0569] Figure 10 shows a comparison of the liver ApoB mRNA knockdown activity of each of these SNALP formulations at three different doses (Error bars = SD), as well as the KD50 values obtained for each of these formulations. In particular, Figure 10 shows that a SNALP formulation containing g-DLen-C2K-DMA displayed similar ApoB silencing activity at all three doses and an identical KD50 value compared to a SNALP formulation containing C2K. Furthermore, Figure 10 shows that a SNALP formulation containing DLen-C2K-DMA displayed considerable potency in silencing ApoB mRNA expression.

Example 18. Characterization of Tumor-Directed SNALP Formulations Containing Novel Cationic Lipids.

[0570] This example demonstrates the efficacy of tumor-directed SNALP formulations containing various novel cationic lipids described herein with an siRNA targeting polo-like kinase 1 (PLK-1) in a mouse distal tumor model. The PLK-1 siRNA sequence used in this study is provided in Table 4.

Table 4

siRNA	PLK-1 siRNA Sequence	% 2'OMe-Modified	% Modified in DS Region
PLK1424 2/6	5' -AGA <u>U</u> CACCC <u>U</u> CCU <u>U</u> AAA <u>U</u> AU-3' (SEQ ID NO:1) 3' -C <u>U</u> U <u>C</u> <u>U</u> AGUGGGAGGAAUUU <u>AU</u> -5' (SEQ ID NO:2)	9/42 = 21.4%	7/38 = 18.4%

Column 1: The number after “PLK” refers to the nucleotide position of the 5' base of the sense strand relative to the start codon (ATG) of the human PLK-1 mRNA sequence NM_005030. Column 2: 2'OMe nucleotides are indicated in bold and underlined. The 3'-overhangs on one or both strands of the siRNA molecule may alternatively comprise 1-4 deoxythymidine (dT) nucleotides, 1-4 modified and/or unmodified uridine (U) ribonucleotides, or 1-2 additional ribonucleotides having complementarity to the target sequence or the complementary strand thereof. Column 3: The number and percentage of 2'OMe-modified nucleotides in the siRNA molecule are provided. Column 4: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA molecule are provided.

[0571] 7:54 SNALP formulations containing encapsulated PLK-1 siRNA were prepared with the following cationic lipids: (1) DLinDMA; (2) TLinDMA; (3) DLin-C1K-DMA (“DLin-K-DMA”); (4) DPanDMA; (5) DPan-C2-DMA (“C2-DPanDMA”); (6) DPan-C2K-DMA; (7) DPan-C3K-DMA; and (8) DPan-C1K6-DMA.

[0572] Each SNALP formulation was administered by intravenous (IV) injection at 3 mg/kg into Scid mice containing Hep3B tumors (n = 4 per group). Tumor tissue was collected at 24 hours after SNALP administration and tumor PLK-1 mRNA levels were evaluated by performing a PLK-1/GAPDH QG assay. Table 5 provides a characterization of the SNALP formulations used in this *in vivo* study.

Table 5

Mol %				Composition		Initial Encaps.	Finished Product	
PEG-lipid	Cationic	Chol	DPPC	PEG-lipid	Cationic Lipid	(%)	Z-Avg (nm)	Encaps. (%)
6.76 54.06 32.43 6.75				PEG750-C-DMA	DLinDMA	75	74	0.08
					TLinDMA	70	126	0.07
					DLin-C1K-DMA	56	84	0.05
					DPanDMA	75	76	0.08
					DPan-C2-DMA	86	81	0.06
					DPan-C2K-DMA	84	76	0.08
					DPan-C3K-DMA	86	81	0.06
					DPan-C1K6-DMA	71	80	0.06

[0573] Figure 11 illustrates that all of the cationic lipids tested displayed similar potencies in silencing PLK-1 expression. Interestingly, there was no difference in silencing activity between DPanDMA and C2-DPanDMA using the tumor-directed 7:54 SNALP formulation, compared to the considerable difference observed in the liver with 1:57 SNALP.

Example 19. Role of ApoE in the *In Vitro* Uptake of SNALP by Hepatocytes.

[0574] This example demonstrates the requirement of ApoE for the *in vitro* uptake and knockdown of 1:57 SNALP formulations on ApoE^{-/-} primary hepatocytes.

[0575] 1:57 SNALP formulations containing encapsulated ApoB siRNA were prepared as described in Section V above with the following cationic lipids: (1) DLinDMA; (2) DLin-K-C2-DMA (“C2K”); (3) DLin-K-C3-DMA (“C3K”); (4) DLin-K-C4-DMA (“C4K”); (5) DLin-K6-DMA; (6) DLin-C2-DMA; (7) DLenDMA; (8) γ -DLenDMA (“g-DLenDMA”); and (9) DLin-K-DMA.

[0576] ApoE^{-/-} primary hepatocytes were isolated and plated at 2.5×10^4 cells/well. Each SNALP formulation was pre-incubated with 50% C57 (ApoE-containing) serum or ApoE^{-/-} serum at siRNA concentrations of 4000-62.5 nM for 1 hr at 37°C. Samples were then diluted to 40-0.625 nM on the hepatocytes for 24 hr. After 24 hr, cells were lysed and ApoB and GAPD mRNA levels were measured via bDNA (QG) assay.

[0577] Figures 12-14 show that most of the SNALP formulations tested required ApoE for their activity. In particular, ApoE^{-/-} serum pre-incubation or no pre-incubation resulted in similar levels of silencing activity, but the addition of C57 serum resulted in a significant increase in silencing activity. SNALP containing either DLenDMA or γ -DLenDMA showed the greatest levels of silencing activity when ApoE-containing serum was present. SNALP containing either DLin-C3K-DMA or DLin-C4K-DMA were less dependent on ApoE compared to other formulations tested. In fact, similar activities were observed for SNALP containing DLin-C4K-DMA in both C57 and ApoE^{-/-} serum.

Example 20. Apolipoprotein E-Mediated Autogenous Targeting of Stable Nucleic Acid-Lipid Particles (SNALP).

ABSTRACT

[0578] Using stable nucleic acid-lipid particles (SNALP) optimized for delivery to the liver, we have previously demonstrated potent and long-lasting hepatocellular gene silencing; however, the precise mechanisms contributing to hepatocellular activity have not been fully delineated. Here we show that liver-directed SNALP take advantage of endogenous blood components that bind to receptors expressed on the surface of target cell populations, facilitating SNALP uptake via receptor-mediated endocytosis. Liver-directed SNALP formulations are remodeled in the blood compartment, acquiring endogenous apolipoprotein E in the process and causing cells that express members of the low-density lipoprotein-receptor family of cell-surface receptors to accumulate SNALP. This mechanism is clearly differentiated from active targeting technologies that rely on exogenous targeting ligands, and from passive targeting approaches that rely on the inherent physicochemical properties of the delivery system to influence distribution and uptake. We propose the term 'autogenous targeting' to describe the active targeting of drug delivery systems by the *in vivo* adoption of endogenous targeting ligands.

INTRODUCTION

[0579] Small interfering RNAs (siRNA) are powerful, target-specific molecules designed to suppress gene expression through the endogenous cellular process of RNA interference (RNAi) [1]. Since this fundamental gene-silencing mechanism was first recognized, tremendous progress has been made in developing siRNA as a novel class of therapeutic agent for a broad spectrum of disease. However, the primary barrier to realizing the potential of siRNA-based drugs is the difficulty delivering large, unstable siRNA molecules safely, while still facilitating disease-site targeting and intracellular delivery of the nucleic acid [2-4]. A number of groups have investigated alternative nucleotide chemistry as a way to

improve the pharmacologic properties of siRNA, while others have examined encapsulation in lipid-based carriers like liposomes or lipid nanoparticles, methods that have been used successfully for small-molecule drug-delivery. Typically, lipidic systems function as drug carriers, where the solubilized drug is encapsulated in the internal aqueous space and

5 enclosed by liposomal lamellae. Such formulations can be used to overcome a drug's non-ideal properties, such as limited solubility, serum stability, circulation half-life,

biodistribution or target tissue selectivity. Previous experience with conventional small-molecule drugs has shown that the drugs that benefit the most from liposomal delivery are those that have an intracellular site of action, are chemically labile, and are subject to

10 enzymatic degradation [5]. Furthermore, when siRNA-based drugs are successfully delivered to a target cell via a liposomal delivery system, the pharmacokinetics, biodistribution, and intracellular delivery of the siRNA payload becomes determined by the physicochemical properties of the carrier rather than the nucleotide sequence or chemical modification pattern of the siRNA duplex. By using this kind of delivery system, 'active' targeting technologies
15 can be incorporated that make delivery to disease sites and uptake by target cells more efficient.

[0580] Active targeting refers to processes that aim to increase the accumulation, retention, or internalization of a drug by attaching specific ligands to the surface of the delivery system.

This differs from passive 'disease site targeting' or 'enhanced permeability and retention' effects (EPR), which result in the accumulation of appropriately designed carriers in target
20 sites such as tumor tissue [6-7]. Active targeting has been applied to liposomal small-

molecule drug formulations with some success and has generally improved the therapeutic index of the liposomal drug when measured in preclinical studies. However, despite the apparent advantages associated with these targeting systems, certain challenges must be

25 addressed before they can be successfully applied to nucleic acid delivery. One challenge is with the process of attaching the targeting ligand to the delivery system, where ligands are chemically coupled to preformed liposomes, or, alternatively, where ligand-lipid conjugates are incorporated in the first stages of the formulation process. These processes are limited by suboptimal coupling efficiencies, negative impacts on nucleic encapsulation efficiency, or
30 suboptimal presentation of targeting ligands. Another challenge is, when targeting ligands are used for nucleic acid delivery, they may cause the adaptive arm of the immune system to react as if the targeting ligands were foreign antigens [8]. This could manifest as antibody responses to the carrier, which, at best, may attenuate the potency of subsequent doses and, at worst, may result in dose-limiting toxicities or hypersensitivity reactions.

[0581] To address these challenges, we developed an approach to targeted delivery that does not rely on decorating the delivery system with exogenous targeting ligands, but instead exploits an endogenous pathway that facilitates receptor-mediated hepatocellular uptake. Previously, we reported that stable nucleic acid-lipid particles (SNALP) were an effective systemic vehicle for delivering siRNA to the murine and non-human primate liver, and we demonstrated therapeutic effects in silencing either endogenously expressed hepatocellular transcripts or exogenous viral genes [2, 9-10]. Here we describe the mechanism by which SNALP are remodeled in the blood and acquire endogenous apolipoprotein E (APOE) in the process, which, in turn, facilitates highly efficient hepatocellular accumulation of SNALP via low-density lipoprotein-receptor-mediated endocytosis.

MATERIALS AND METHODS

[0582] **Materials.** Hanks Buffered Salt Solutions (HBSS), human recombinant insulin, ethylenediaminetetracetic acid (EDTA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Williams' Medium E (WME), (N-morpholino)propanesulfonic acid (MOPS) buffer and hepatocyte wash buffer were purchased from Invitrogen Canada Inc. (Burlington, ON, Canada). WME contained 58 µg/mL of human recombinant insulin, 1 nM dexamethasone, and 0.1% BSA, except where specified. Rat-tail collagen, BSA, cholesterol, and dexamethasone were purchased from Sigma-Aldrich (Oakville, ON, Canada). Human high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very-low-density lipoprotein (VLDL) were purchased from Biomedical Technologies Inc (Stoughton, MA, USA). Human intermediate-density lipoprotein (IDL) was purchased from Athens Research and Technology (Athens, Georgia, USA). Human recombinant APOE2, APOE3, and APOE4 isolated from *S. frugiperda* were purchased from VWR Canlab (Edmonton, AB, Canada). ³H-CHE was purchased from Perkin-Elmer (Boston, MA, USA). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and biotinylated poly(ethyleneglycol) distearoylphosphatidylethanolamine (Biotin-PEG-DSPE), were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Streptavidin-agarose beads were purchased from ProZyme (San Leandro, CA, USA). 1,2-Dilinoleyloxy-*N,N*-dimethyl-3-aminopropane (DLinDMA) and *N*-[(methoxy poly(ethylene glycol)₂₀₀₀) carbamyl]-1,2-dimyristyloxypropyl-3-amine (PEG-C-DMA) were synthesized as previously described [11-12]. All tissue culture incubations were carried out at 37°C in an atmosphere of 5% CO₂. C57BL/6 (wild type), LDLr knockout (LDLr^{-/-}) and APOE knockout (APOE^{-/-}) mice (The Jackson Laboratory, JAX® Mice and Services, Bar Harbor, ME, USA) were subjected to a 1-week acclimation period prior to use. Animal studies were performed in accordance with the Canadian Council on Animal Care (CCAC) guidelines and followed

protocols approved by the Institutional Animal Care and Use Committee at Tekmira Pharmaceuticals Corporation (Burnaby, BC, Canada).

[0583] SNALP Formulation. SNALP were prepared using the method previously described [10,12]. In brief, an ethanolic solution of lipids was mixed with an aqueous solution of nucleic acid. The resulting SNALP were subsequently concentrated, diafiltered against 20 wash volumes of phosphate-buffered saline (PBS) using a cross-flow ultrafiltration cartridge (GE Healthcare, Piscataway, NJ, USA) and, finally, sterile-filtered through Acrodisc 0.8/0.2 µm Supor filters (Pall Corp., Ann Arbor, MI).

[0584] SNALP-Serum and APOE Isoform Pre-Incubations. SNALP and the relevant serum from C57BL/6, APOE^{-/-} or LDLr^{-/-} mice were mixed at a ratio of 7 µg siRNA per 100 µL of serum (provides for optimal levels of activity as measured by target 'knock down') and incubated for 1 hour at 37°C. The resulting pre-incubated SNALP were diluted with PBS and serum to final concentrations of 29.3 µg/mL siRNA and 41.3 % serum.

[0585] APOE isoforms were prepared in PBS at 50 µg/mL. SNALP were diluted to 50 µg/mL siRNA. Equal volumes (5 µL) of SNALP and APOE solutions were then combined and incubated for 1 hour at 37°C.

[0586] *In Vitro* Serum Protein Binding to Biotinylated SNALP. SNALP were prepared containing 0.1 mol% Biotin-PEG-DSPE and pre-incubated with the serum as described above. Streptavidin-agarose beads (50 µL) were prewashed with PBS containing 150 mM NaCl (2 x 500 µL) followed by PBS containing 1M NaCl (2 x 1 mL). SNALP (120 nmol total lipid) was added to the beads and incubated for 1 hour at room temperature. The beads were then washed with PBS (150mM NaCl, 3 x 500 µL). Proteins bound to the SNALP were visualized by PAGE analysis using a precast 10% NuPAGE Bis/Tris gel (Invitrogen,

Burlington, ON, Canada) run in 1 x MOPS buffer using the XCell SureLock Gel System (Invitrogen, Burlington, ON, Canada). The agarose beads were first suspended in a solution of 4X LDS Sample Buffer (8.75 µL, Invitrogen), 10X Reducing Reagent (3.5 µL, Invitrogen) and distilled water (7.75 µL). Proteins were denatured by heating the suspension for 10 minutes at 70°C, and 20 µL of the resulting solution was loaded onto the gel, which was run at 200 V for 50 minutes. Proteins on the gel were stained with a Coomassie Blue solution.

Proteins were identified by LC/MS using an Agilent 1100 Nano-HPLC and an ABI 4000 QTrap system. Separations were accomplished with a reverse-phase column (Phenomenex Jupiter Proteo, 4µm, 90 Å, 75 µm x 150 mm) and 0-80% acetonitrile gradient.

[0587] Isolation and Identification of Proteins Binding SNALP. The identity of human and mouse serum proteins as visualized by PAGE was confirmed by LC/MS. Proteins were isolated and stained with Coomassie Brilliant Blue G250. Gel slices were cut into 1-mm

cubes and washed overnight in methanol / water / acetic acid (50:45:5). The samples were dehydrated with acetonitrile (ACN) and dried in a vacuum centrifuge. The protein in the gel pieces were reduced with 10 mM dithiothreitol (DTT) for 30 minutes, the DTT solution was removed, and the proteins were alkylated with 100 mM iodoacetamide for 30 minutes. The samples were dehydrated with ACN, rehydrated with NH_4HCO_3 , and again dehydrated with ACN. The gel was rehydrated with a trypsin solution (20 ng/ μl , 50mM NH_4HCO_3 , 10% ACN) and incubated overnight at 37°C. Proteins were extracted twice with ACN / water / formic acid (50 / 45 / 5), then evaporated to dryness and resuspended in ACN / water / formic acid (3 / 95.5 / 1.5) for analysis by HPLC-ESI-MS/MS.

[0588] Mass Spectrometry. HPLC-ESI-MS/MS was performed using an Agilent 1100 Nano-HPLC and an ABI 4000 QTrap system. Separations were accomplished with a reverse-phase column (Phenomenex Jupiter Proteo, 4 μm , 90 Å, 75 μm x 150 mm) and an ACN gradient (0 – 80%) with a flow rate of 300 nl/min. Electrospray voltage was 1850 V. A positive ion data-dependent MS/MS analysis was performed with an Enhanced MS spectrum of the 400-1600 m/z range followed by an Enhanced Resolution scan to assign charge states. For each scan cycle, the top five +2 and +3 ions were subjected to collisional-induced dissociation, and mass spectra were collected with an Enhanced Product Ion scan. The fragment mass spectra were searched against IPI_Human or IPI_Mouse databases (<http://www.ebi.ac.uk/IPI/IPIhelp.html>), as appropriate, using Mascot (Matrix Science) and X!Tandem (<http://www.thegpm.org/TANDEM/>) search engines.

[0589] In Vitro LDLr-Ligand Binding Studies. Livers from C57BL/6 and LDLr^{-/-} mice were homogenized and cellular membranes purified based on the methods of Kovanen *et al.* [13]. Proteins were extracted from the resulting homogenate and concentrated on a DEAE-Sepharose column according to the method of Schneider *et al.* [14]. Protein concentrations were determined using the Pierce BCA Assay.

[0590] Aliquots containing 400 μg of protein in 1 x Laemmli buffer were separated by 4-12% SDS-PAGE (150V for 1.5 hours). The proteins were then transferred to Hybond-C nitrocellulose membranes using a Mini Trans Blot System (Bio-Rad, Mississauga, ON, Canada) (200 mA for 2 hours). Membranes were subsequently blocked with Ligand Blot Buffer A (LBB 'A') (20 mM Tris, 90 mM NaCl, pH 8) containing 5% BSA.

[0591] SNALP (4.45 μg siRNA) containing 0.1 mol% Biotin-PEG-DSPE were pre-incubated with serum then added to Ligand Blot Buffer B (LBB 'B') (20 mM Tris, 200 mM NaCl, pH 8) containing 5% BSA and used to probe membrane protein extracts isolated from C57BL/6 and LDLr^{-/-} livers. After 2 hours at 37°C the blots were immediately washed 3 times with LBB 'B', followed by soaking for 3 periods of 10 minutes with LBB 'B'. The

blots were finally incubated for 1 hour at room temperature with Streptavidin-HRP diluted 1/80,000 in LBB 'A' containing 5% BSA. After immediately washing 3 times with LBB 'B' followed by 3 10-minute washes with LBB 'B', bound SNALP was visualized using the Amersham ECL Western Blotting System (GE Healthcare, Piscataway, NJ, USA).

- 5 **[0592] *In Vitro* SNALP Uptake by Primary Hepatocytes from Wild-Type and Knockout Mice.** Primary hepatocytes from C57BL/6, APOE^{-/-}, and LDLr^{-/-} mice were isolated using a collagenase perfusion method. In brief, mice were euthanized by intraperitoneal injection of ketamine/xylazine, a catheter was inserted into the left ventricle of the heart, and the portal vein was severed. Livers were initially perfused with a solution of
- 10 HBSS (with no calcium or magnesium salts) containing insulin (2.9 µg/mL), EDTA (0.5 mM), and HEPES (10 mM) at a rate of 10 mL/min for 3 minutes, followed by a solution of 100 U/mL of Collagenase II in HBSS (10 mL/min for 3 minutes). Livers were then excised and submersed in WME (15 mL) in a petri dish. The hepatocytes were extracted on ice using a cell scraper and isolated by centrifugation at 50 x g for 5 minutes at 4°C. The supernatant
- 15 was removed and the pellet resuspended in Hepatocyte Wash Medium (30 mL). The suspension was passed consecutively through 100 µm and 70 µm cell strainers (BD Biosciences, Bedford, MA, USA). The washing/resuspension process was repeated a further 2 times, and the final resuspension was with WME containing 58 µg/mL human recombinant insulin, 1 nM dexamethasone, and 10% FBS, rather than Hepatocyte Wash Medium.
- 20 Hepatocytes were counted using a haemocytometer and added to 12-well plates precoated with rat-tail collagen at 3x10⁵ cells/well. The cells were incubated for 4 hours, washed with HBSS (no salts), and fresh WME was added. Cells were then incubated overnight.
- [0593]** Preparations of ³H-CHE-labeled SNALP that had been pre-incubated in respective sera were diluted to siRNA concentrations of 0.25 µg/mL with WME; 1mL SNALP per well
- 25 was added to primary hepatocytes. Cells were incubated for either 4 hours or 24 hours, washed 3 times with ice-cold HBSS (no salts), and lysed with PBS containing 0.1% Triton X-100. Radioactivity of the lysates was determined by liquid scintillation counting using Picofluor 15 and a Beckman LS6500 (Beckman Instruments, CA, USA), and protein content was determined using the Pierce BCA Assay (Thermo Scientific, Waltham, MA, USA).
- 30 Uptake was determined by normalizing radioactivity to the amount of protein per well (n=3).
- [0594] *In Vitro* Silencing of APOB in Primary Hepatocytes (Human/Mouse).** Primary hepatocytes from C57BL/6, APOE^{-/-}, and LDLr^{-/-} mice were seeded in 96-well Primaria plates (BD Biosciences, Bedford, MA, USA) at 2.5x10⁴ cells/well and incubated overnight. Human hepatocytes were purchased preseeded in 96-well plates at 5x10⁴ cells/well (BD
- 35 Biosciences, Bedford, MA, USA). SNALP, pre-incubated as described above, were diluted

to relevant concentrations in WME, added to the hepatocytes, and incubated for up to 24 hours. For time points less than 24 hours, cells were then washed with HBSS, fresh media was applied, and the plate was returned to the incubator for the remainder of the 24 hours. After 24 hours, all cells were lysed using the Quantigene Lysis Mixture and APOB mRNA knockdown was determined using the Quantigene Assay (Panomics, CA, USA). Levels of APOB mRNA were compared to those of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene (n=3).

[0595] Clearance and Biodistribution. SNALP containing tritiated cholesteryl hexadecyl ether (^3H -CHE) at 1 $\mu\text{Ci}/\text{mg}$ total lipid were prepared as described above. SNALP were administered to C57BL/6, APOE $^{-/-}$, and LDLr $^{-/-}$ mice via lateral tail-vein injection at a siRNA dose of 1 mg/kg in a 200 μL injection volume. At appropriate time points, 20 μL of blood was obtained from a tail nick, and ^3H -CHE was determined in whole blood by liquid scintillation counting using Picofluor 15 and a Beckman LS6500 (Beckman Instruments, CA, USA). At the 24-hour time point, mice were euthanized and tissues were collected and homogenized in a FastPrep Homogenizer (Thermo Scientific, Waltham, MA, USA). Liver (100 μL) and other tissue (200 μL) homogenates were assayed for radioactivity by liquid scintillation counting with Picofluor 40. The data are presented as % injected dose/organ (n=3).

[0596] SNALP-Mediated Silencing of APOB mRNA and Protein Expression in Knockout Mice. C57BL/6, APOE $^{-/-}$, and LDLr $^{-/-}$ mice were treated with SNALP as described above. After 48 hours, mice were euthanized and livers were collected into RNALater solution (Sigma-Aldrich, Oakville, ON, Canada). A portion of each liver was homogenized and APOB mRNA silencing was measured by comparing to the GAPDH housekeeping gene using the Quantigene Assay (Panomics, CA, USA) (n=3).

[0597] SNALP-Human Lipoprotein Pre-Incubation. Lipoproteins were first prepared in PBS at the concentrations typically found in human serum (HDL: 1.5 mg/mL, LDL: 0.65 mg/mL, IDL: 0.075 mg/mL, VLDL: 0.15 mg/mL). SNALP were diluted with PBS to 100, 50, 25, 12.5, and 6.25 $\mu\text{g}/\text{mL}$ siRNA. Equal volumes (5 μL) of SNALP and lipoprotein solutions were combined prior to incubation for 1 hour at 37°C.

RESULTS

[0598] *In Vitro* Serum Protein Binding. It is understood that many drug delivery systems, including liposomes, bind a complex array of proteins upon exposure to plasma and that liposomes with different membrane compositions have markedly different clearance and biodistribution properties [15]. An objective of this study was to determine whether any part

of SNALP pharmacology is related to the amount and type of protein associated with SNALP upon exposure to blood.

[0599] To identify plasma proteins that bind to SNALP, we developed an *in vitro* Streptavidin/Biotin pull-down procedure. SNALP containing 0.1 mol% Biotin-PEG-DSPE were incubated with mouse serum. Subsequent incubation with streptavidin-agarose beads allowed for recovery and washing of SNALP, along with any SNALP-associated proteins, and for analysis by SDS-PAGE. The identities of adsorbed proteins were established by LC/MS and confirmed by Western blotting. When SNALP were incubated with C57BL/6 mouse serum, they associated with a number of distinct plasma proteins, most predominantly APOE and APOA-I (Figure 15a). The identity of APOE was further corroborated by the results of a pull-down experiment following incubation with APOE^{-/-} mouse serum, in which there was no evidence of an APOE-comigrating band adsorbing to SNALP. As with C57BL/6 serum, APOA-I from APOE^{-/-} serum associated with SNALP, but APOA-IV and, to a lesser degree, APOB48 also associated with the SNALP particle following incubation in APOE^{-/-} serum. Incubation with LDLr^{-/-} mouse serum facilitated the adsorption of both APOE and APOA-I.

[0600] ***In Vitro* LDLr Ligand Blotting.** The 34 kDa glycoprotein APOE, found predominantly in VLDL, HDL, and chylomicrons, is a high-affinity ligand for LDLr and other members of the LDLr family of cell-surface receptors [16], some of which are abundant in the liver. To assess whether SNALP-associated APOE has the potential to facilitate LDLr-mediated binding, we conducted a ligand blotting experiment to determine if SNALP-bound APOE would be capable of binding to immobilized LDLr. Detergent extracts of liver membrane proteins from LDLr-expressing C57BL/6 mice and LDLr^{-/-} mice were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were probed using biotinylated SNALP that had been pre-incubated in C57BL/6 or APOE^{-/-} serum. Membrane-bound SNALP were visualized by adding streptavidin-HRP and ECL. Pre-incubation in APOE-containing serum potentiated the binding of biotinylated SNALP to immobilized LDLr (Figure 15b, panel 1). When SNALP were pre-incubated in serum derived from APOE^{-/-} mice (Figure 15b, panel 2), or if the pre-incubation step was omitted entirely (Figure 15b, panel 3), binding of SNALP to the LDLr was not observed. The role of LDLr was further substantiated when we isolated liver membrane proteins from LDLr^{-/-} mice and performed a comparable analysis. Consistent with the LDL receptor being the major APOE-binding protein in liver-membrane extracts, no SNALP binding to liver membrane extracts from LDLr^{-/-} mice was observed (Figure 15b, panels 4 to 6).

[0601] In Vitro Uptake and Gene Silencing in C57BL/6, APOE^{-/-}, and LDLr^{-/-}

Primary Hepatocytes. To elucidate the mechanism of SNALP activity in hepatocytes, we conducted *in vitro* uptake and gene-silencing studies using primary hepatocytes from C57BL/6, APOE^{-/-}, and LDLr^{-/-} mice. Hepatocytes were cultured in serum-free conditions.

5 To facilitate interaction with serum proteins, SNALP were pre-incubated in C57BL/6, APOE^{-/-}, or LDLr^{-/-} serum before use. Hepatocytes were exposed to radio-labelled SNALP for 4 hours (Figure 16a) or 24 hours (Figure 16b). Since cells that were exposed to SNALP for 4 hours were subsequently incubated in fresh media for a further 20 hours, both treatment groups were allowed equal time for gene silencing to manifest.

10 **[0602]** SNALP exposed to APOE-containing sera (from either C57BL/6 or LDLr^{-/-} mice) were taken up by LDLr-expressing hepatocytes (from either C57BL/6 or APOE^{-/-} mice) to a greater degree than in LDLr^{-/-} hepatocytes. Uptake of SNALP following pre-incubation with APOE^{-/-} serum was low in all hepatocyte types. While LDLr^{-/-} hepatocytes took up little SNALP at 4 hours (Figure 16a), prolonged exposure resulted in APOE-dependent SNALP
15 accumulation comparable to that achieved in LDLr^{+/+} hepatocytes (Figure 16b). This suggests that an APOE-dependent mechanism, possibly involving other members of the LDL receptor family, may facilitate SNALP uptake in LDLr^{-/-} cells. In aggregate, the results of this experiment support a role for both APOE and LDLr in hepatocellular SNALP uptake.

[0603] We conducted a parallel study to assess APOE-dependent gene silencing in primary
20 hepatocytes from C57BL/6, APOE^{-/-}, and LDLr^{-/-} mice (Figures 16c and 16d). C57BL/6 hepatocytes, which express LDLr and actively secrete APOE protein, exhibited potent gene silencing independent of the pre-incubation conditions or the time point of analysis.

Hepatocytes derived from APOE^{-/-} mice (expressing functional LDLr) exhibited gene-silencing levels comparable to C57BL/6 cells when exogenous APOE was added to the tissue
25 culture media. Finally, consistent with the SNALP uptake studies, we observed little gene silencing in LDLr^{-/-} hepatocytes exposed to SNALP for 4 hours; however, we observed significant knockdown in LDLr^{-/-} hepatocytes after 24 hours of continuous exposure to SNALP in tissue culture media complemented with exogenous APOE. These results support the hypothesis that hepatocellular SNALP uptake is potentiated by an APOE-dependent
30 LDLr-mediated uptake mechanism that can, in part, be complemented by other APOE-dependent mechanisms in the absence of LDLr expression.

[0604] Plasma Clearance and Biodistribution in APOE^{-/-} and LDLr^{-/-} Mice. To further explore the role of APOE and LDLr in SNALP pharmacology, we characterized the blood clearance and biodistribution of ³H-CHE-labeled SNALP following intravenous
35 administration in C57BL/6, APOE^{-/-}, and LDLr^{-/-} mice. Figure 17a illustrates the plasma

clearance properties of ^3H -CHE-labeled SNALP after a single intravenous administration of 1 mg/kg SNALP siRNA. In C57BL/6 mice, the plasma clearance half-life of SNALP was approximately 30 minutes, while clearance of the same SNALP in APOE $^{-/-}$ mice was considerably slower (half-life of approximately 4 hours). This supports the hypothesis that APOE participates in, or otherwise facilitates, the rapid clearance of this SNALP formulation from the blood. When administered to LDLr $^{-/-}$ mice, SNALP exhibited clearance properties that were intermediate between those of C57BL/6 and APOE $^{-/-}$ mice. When measured 4 hours after administration, approximately 30% of the injected dose remained in the circulation of LDLr $^{-/-}$ mice, whereas at the same time point only 5% of the injected dose remained in circulation of C57BL/6 mice. These results suggest a role for both the LDLr and APOE in the plasma clearance of SNALP.

[0605] To further characterize the influence of APOE and LDLr on SNALP pharmacology, we conducted a biodistribution study in C57BL/6, APOE $^{-/-}$, and LDLr $^{-/-}$ mice. Figure 17b shows the distribution of SNALP 24 hours after intravenous administration. At 24 hours, 53% of the total injected dose had accumulated in the liver of C57BL/6 mice, while considerably less SNALP (19%) was observed in the liver of APOE $^{-/-}$ mice. LDLr $^{-/-}$ mice accumulated an intermediate amount of SNALP in the liver (37%), consistent with the intermediate rate of clearance (Figure 17a).

[0606] **SNALP Efficacy in C57BL/6, APOE $^{-/-}$, and LDLr $^{-/-}$ Mice.** The implications of SNALP clearance and biodistribution were explored by conducting a correlative analysis of SNALP efficacy, as measured by APOB gene silencing following delivery of anti-APOB siRNA. In a dose-response study, C57BL/6, APOE $^{-/-}$ and LDLr $^{-/-}$ mice were administered intravenous doses of siRNA ranging from 0.25 to 18 mg/kg. APOB mRNA was measured in the liver 48 hours after SNALP administration. In C57BL/6 mice, SNALP were highly active, yielding 71% knockdown even at the lowest administered dose of 0.25 mg/kg (Figure 17c). In APOE $^{-/-}$ mice, silencing of APOB mRNA was only observed at the highest dose (18 mg/kg), while LDLr $^{-/-}$ mice yielded intermediate results. Significant gene silencing was observed at all siRNA doses, further emphasizing the role of both APOE and LDLr in the hepatocellular uptake of SNALP.

[0607] **Human Lipoproteins Can Act as an APOE Donor Potentiating SNALP Activity in Murine APOE $^{-/-}$ Hepatocytes.** To examine the interaction between SNALP and human lipoproteins and assess the potential role of human APOE in SNALP pharmacology, we exposed SNALP to a variety of human lipoprotein species and examined the impact on SNALP potency in primary hepatocytes obtained from APOE $^{-/-}$ mice. SNALP were incubated with purified human lipoproteins at the nominal lipoprotein concentrations found

in normal human serum [17-21]. The resulting APOB gene-silencing activity was determined in murine APOE^{-/-} hepatocytes and compared to that of SNALP incubated with C57BL/6 mouse serum, APOE^{-/-} mouse serum, or human whole serum (Figure 18a).

[0608] Pre-incubation of SNALP with either C57BL/6 mouse serum or human whole serum resulted in maximal potency as measured by APOB gene silencing, while pre-incubation in APOE^{-/-} mouse serum yielded minimal activity. When SNALP were pre-incubated with human lipoproteins, the resulting activity correlated with the relative APOE content of the lipoprotein species in question. APOE-poor IDL and LDL facilitated relatively modest knockdown of APOB expression in cultured hepatocytes. The APOE-rich lipoproteins, VLDL and HDL, were more effective, yielding the most pronounced gene-silencing when combined as a cocktail in the pre-incubation step.

[0609] **SNALP-Mediated APOB Gene Silencing in Cultured Human Hepatocytes.** To assess the potential for autogenous targeting to human cells, we assessed the relative potency of anti-APOB SNALP in cultured human hepatocytes. Notably, in the absence of exogenous APOE, cultured human hepatocytes exhibited a transfection profile more similar to that of murine APOE^{-/-} hepatocytes than C57BL/6 cells (Figure 18b). SNALP potency was fully constituted following pre-incubation in APOE-containing serum, while it was dramatically reduced in the 'No Serum' samples. This suggests that under the conditions of this study the cultured human hepatocytes did not secrete sufficient APOE-containing lipoproteins to complement serum-free media. Notably, the murine hepatocytes used in this study were freshly isolated primary cells prepared in our own laboratory, while the human hepatocytes were purchased from a vendor following isolation by cadaveric liver perfusion.

[0610] **Purified APOE Isoforms Potentiate SNALP-Mediated APOB Gene Silencing in Cultured Human Hepatocytes.** Our final study examined the effect of individual APOE isoforms on SNALP-mediated RNA interference in cultured human hepatocytes. The APOE gene is polymorphic in humans, coding for 3 main isoforms (E2, E3, and E4). Although the isoforms only differ at amino acid positions 112 and 158, the physiological effects associated with each isoform can be profound. Presence of the E4 isoform has been strongly linked to atherosclerosis [22] and Alzheimer's disease [23]. SNALP were pre-incubated individually with either individual APOE isoforms or whole serum then used to transfect human hepatocytes at a siRNA concentration of 0.125 µg/mL siRNA. The differences in activity between APOE isoforms were marginal, although the pattern APOE4 > APOE3 > APOE2 appeared to be conserved at all SNALP exposure times (Figure 18c). This hierarchy loosely correlates with the reported affinities of APOE isoforms for the LDLr. APOE3 and APOE4 bind to the LDLr with equal affinity and more strongly than the APOE2 isoform [24]. Again,

SNALP that were not complemented with purified recombinant APOE or APOE-containing serum were unable to mediate significant APOB knockdown in human hepatocytes.

DISCUSSION

[0611] We previously described a number of SNALP formulations optimized for delivery to the liver that achieved potent, enduring silencing of target genes [2, 9, 10]. In this study, we sought to delineate the mechanism by which liver-directed SNALP formulations achieve high levels of potency and answer the specific question of how SNALP are taken up by their target cells. We hypothesized that the mechanism responsible for hepatocellular uptake of SNALP may involve elements of particle remodeling and/or interaction with blood components. Previous reports suggest that, following contact with blood, neutral liposomes rapidly associate with a variety of serum proteins [15] including apolipoproteins and the more abundant opsonizing proteins of the complement system. Other investigators have reported that the exchangeable apolipoproteins APOE and APOA-I are able to associate with liposomes [25, 26]. Further, APOE, but not APOA-I or APOA-IV, can enhance liposomal uptake in cultured HepG2 (hepatocellular carcinoma) cells [27]. Yan et al. suggested that the hepatocellular uptake of empty liposomes may involve an interaction between liposomally associated APOE and LDLr [28].

[0612] In our characterization of the uptake mechanism of liver-directed SNALP, we began by identifying the major serum proteins binding to the particles. We developed a method for collecting and analyzing SNALP-bound proteins with SDS-polyacrylamide gel electrophoresis (Figure 15a) and LC/MS. The major SNALP binding proteins in normal or LDLr^{-/-} serum are serum albumin, APOA-I, and APOE. In the absence of APOE, in APOE^{-/-} serum, SNALP bound APOA-IV and APOB48 in addition to binding serum albumin and APOA-I. This suggests that, of these apolipoprotein species, APOE has the highest affinity for SNALP, and that SNALP may have a limited capacity for binding apolipoproteins. In the absence of APOE, the available capacity for apolipoprotein binding may be utilized by APOA-IV and APOB48, both of which are more abundant in the serum of APOE knockout mice than the serum of normal mice. As APOE has a high affinity for the LDLr [16], we developed an assay to examine whether the SNALP/serum protein complexes were capable of binding to immobilized LDLr extracted from hepatocytes (Figure 15b). This analysis confirmed that SNALP bind to LDLr in an APOE-dependent manner.

[0613] To further understand the significance of these observations, we conducted SNALP uptake and efficacy experiments in primary murine hepatocytes (Figure 16). By collecting fresh primary hepatocytes directly from wild-type and knockout mice, we were able to examine the specific consequence of removing either LDLr or APOE from the experimental

system. These studies showed a high degree of SNALP uptake and SNALP siRNA-mediated gene silencing in those cells that express LDLr (C57BL/6 and APOE^{-/-} hepatocytes), provided SNALP are exposed to a source of APOE (supplied as exogenous APOE during serum pre-incubation, or endogenously when secreted by APOE-expressing cells directly into the culture medium). In instances where either APOE or LDLr were removed from the system, SNALP uptake and gene silencing activity was reduced.

[0614] LDLr^{-/-} hepatocytes accumulate significant levels of SNALP in an APOE-dependant manner (Figure 16), albeit at a rate that was substantially reduced compared to LDLr-expressing cells. This suggests that other receptors, possibly including other members of the LDLr family, can facilitate APOE-mediated SNALP uptake in non-LDLr-expressing cells. Wolfrum et al. postulated that once siRNA-cholesterol conjugates had bound to APOE-containing HDL, hepatocellular uptake could be mediated via scavenger receptor BI (SR-BI) [29], an APOE-binding receptor broadly expressed in the liver [30, 31] that also seems a likely candidate for participation in SNALP uptake.

[0615] When our results are considered in conjunction with the serum protein binding assay (Figure 15a), it would appear that, consistent with the results of liposomal uptake studies performed by Bisgaier et al. [27], neither APOA-I, APOA-IV, nor APOB48 (which bind to SNALP in the absence of APOE) are major contributors to hepatocellular SNALP uptake.

[0616] The results of our *in vitro* analysis are also consistent with the results of subsequent *in vivo* studies. SNALP uptake by the liver is most rapid in wild-type mice expressing both the LDL receptor and APOE, as reflected by the rapid blood clearance and the high level of SNALP accumulation. The resulting efficacy, assessed by siRNA-mediated APOB gene silencing, confirms that liver uptake involved the hepatocytes, rather than the liver macrophages alone. Accumulation and activity of SNALP in the livers of APOE and LDLr knockout mice was comparatively diminished, highlighting the impact of the LDLr/APOE pathway. Nonetheless, a significant amount of SNALP was shown to accumulate in the liver of these groups, highlighting the requirement for efficient intracellular delivery of SNALP as a precursor to mediating RNAi.

[0617] In spite of the compelling data acquired from murine studies, the extent to which this uptake pathway would be available for exploitation by SNALP in other species, particularly humans, is unclear. Our final panel of experiments (Figure 18) aimed to address this question, with encouraging results. Pre-incubation of SNALP with human serum components appeared to be equally successful in effecting SNALP uptake and activity in both murine and human hepatocytes. Time-course studies revealed potent APOE-dependent

gene silencing in human hepatocytes. Importantly, SNALP activity was independent of APOE isoform.

[0618] Taken together, these results demonstrate that an endogenous uptake pathway is available and can be readily exploited to direct hepatocellular SNALP uptake. Liver-directed SNALP formulations are remodeled in the blood compartment, accumulating endogenous ApoE, which serves to target SNALP for accumulation by cells that express members of the LDL-receptor family of cell-surface receptors. By its very nature, this targeting mechanism removes many of the hurdles presented by active targeting strategies that must incorporate exogenous targeting ligands. Cumbersome and expensive manufacturing processes could be circumvented, as could potential immunogenic responses to exogenously supplied targeting ligands, which may manifest in their most benign form as attenuated potency in multiple dosing regimes or as more serious dose-limiting toxicities, including the development of hypersensitivity reactions. This approach is clearly differentiated from active targeting technologies that rely on exogenous targeting ligands, and from passive targeting approaches that rely on the inherent physicochemical properties of the delivery system to influence distribution and uptake. We propose the term ‘autogenous targeting’ to describe the active targeting of drug delivery systems by the *in vivo* adoption of *endogenous* targeting ligands.

[0619] An understanding of APOE-mediated SNALP uptake is of considerable utility in terms of further advancing this technology. Here we have described some novel techniques which we have used to improve our understanding of the parameters that may influence the activity of liver-directed SNALP formulations [2, 9, 32].

[0620] The LDL receptor family contains several members, all related by common structural and functional domains, including the cysteine-rich ligand-binding domains that bind ApoB and ApoE. The most prominent members of the LDL receptor family are the LDL receptor, the LDL receptor related protein (LRP1), megalin (LRP2 or gp330), the VLDL receptor, LR11 (SorLA), apolipoprotein E receptor type 2 (apoER2, LRP3, 4, 5, and 6), and LRP1B [33]. These receptors are expressed in tissues as diverse as liver, neuron, smooth muscle, adrenal gland, kidney, lung, eye, testes, and ovary. Members of the family are implicated in disease processes such as atherosclerotic plaque formation, β -amyloid production in Alzheimer’s disease, and growth and proliferation of various tumor types.

REFERENCES

1. Elbashir, SM, Harborth, J, Lendeckel, W, Yalcin, A, Weber, K, and Tuschl, T (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411: 494-498.

2. Zimmermann, TS, *et al.* (2006). RNAi-mediated gene silencing in non-human primates. *Nature* 441: 111-114.
3. de Fougerolles, A, Vornlocher, HP, Maraganore, J, and Lieberman, J (2007). Interfering with disease: a progress report on siRNA-based therapeutics. *Nat Rev Drug Discov* 6: 443-453.
4. Behlke, MA (2006). Progress towards *in vivo* use of siRNAs. *Mol Ther* 13: 644-670.
5. Tardi, P, Choice, E, Masin, D, Redelmeier, T, Bally, M, and Madden, TD (2000). Liposomal encapsulation of topotecan enhances anticancer efficacy in murine and human xenograft models. *Cancer Res* 60: 3389-3393.
- 10 6. Mayer, LD, Bally, MB, and Cullis, PR (1990). Strategies for optimizing liposomal doxorubicin. *J Liposome Res* 1: 463-480.
7. Seymour, LW (1992). Passive tumor targeting of soluble macromolecules and drug conjugates. *Crit Rev Ther Drug Carrier Syst* 9: 135-187.
8. Heidel, JD, *et al.* (2007). Administration in non-human primates of escalating
15 intravenous doses of targeted nanoparticles containing ribonucleotide reductase subunit M2 siRNA. *Proc Natl Acad Sci U S A* 104: 5715-5721.
9. Morrissey, DV, *et al.* (2005). Potent and persistent *in vivo* anti-HBV activity of chemically modified siRNAs. *Nat Biotechnol* 23: 1002-1007.
10. Judge, AD, Bola, G, Lee, AC, and MacLachlan, I (2006). Design of noninflammatory
20 synthetic siRNA mediating potent gene silencing *in vivo*. *Mol Ther* 13: 494-505.
11. Heyes, J, Hall, H, Vicky, T, Lenz, R, and MacLachlan, I (2006). Synthesis and characterisation of novel poly(ethylene glycol)-lipid conjugates suitable for use in drug delivery. *J Control Release* 112: 280-290.
12. Heyes, J, Palmer, L, Bremner, K, and MacLachlan, I (2005). Cationic lipid saturation
25 influences intracellular delivery of encapsulated nucleic acids. *J Control Release* 107: 276-287.
13. Kovanen, PT, Brown, MS, and Goldstein, JL (1979). Increased binding of low density lipoprotein to liver membranes from rats treated with 17 alpha-ethinyl estradiol. *J Biol Chem* 254: 11367-11373.
- 30 14. Schneider, WJ, Beisiegel, U, Goldstein, JL, and Brown, MS (1982). Purification of the low density lipoprotein receptor, an acidic glycoprotein of 164,000 molecular weight. *J Biol Chem* 257: 2664-2673.
15. Chonn, A, Semple, SC, and Cullis, PR (1992). Association of blood proteins with large unilamellar liposomes *in vivo*. Relation to circulation lifetimes. *J Biol Chem*
35 267: 18759-18765.

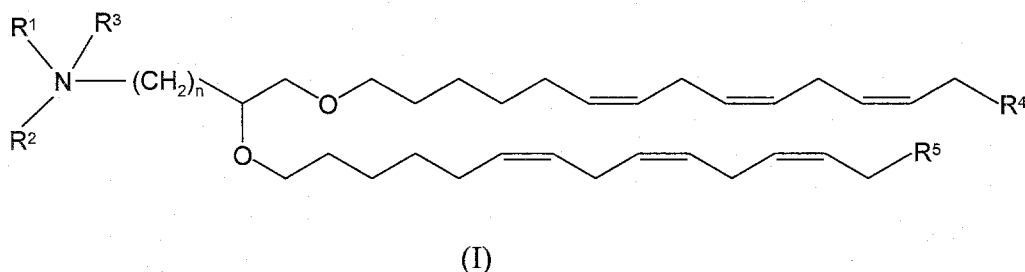
16. Innerarity, TL, Pitas, RE, and Mahley, RW (1979). Binding of arginine-rich (E) apoprotein after recombination with phospholipid vesicles to the low density lipoprotein receptors of fibroblasts. *J Biol Chem* 254: 4186-4190.
17. Fredrickson, DS, Levy, RI, and Lees, RS (1967). Fat transport in lipoproteins--an
5 integrated approach to mechanisms and disorders. *N Engl J Med* 276: 34-42 contd.
18. Fredrickson, DS, Levy, RI, and Lees, RS (1967). Fat transport in lipoproteins--an integrated approach to mechanisms and disorders. *N Engl J Med* 276: 94-103 contd.
19. Fredrickson, DS, Levy, RI, and Lees, RS (1967). Fat transport in lipoproteins--an integrated approach to mechanisms and disorders. *N Engl J Med* 276: 148-156 contd.
- 10 20. Fredrickson, DS, Levy, RI, and Lees, RS (1967). Fat transport in lipoproteins--an integrated approach to mechanisms and disorders. *N Engl J Med* 276: 215-225 contd.
21. Fredrickson, DS, Levy, RI, and Lees, RS (1967). Fat transport in lipoproteins--an integrated approach to mechanisms and disorders. *N Engl J Med* 276: 273-281 concl.
22. Davignon, J, Gregg, RE, and Sing, CF (1988). Apolipoprotein E polymorphism and
15 atherosclerosis. *Arteriosclerosis* 8: 1-21.
23. Corder, EH, *et al.* (1993). Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science* 261: 921-923.
24. Hatters, DM, Peters-Libeu, CA, and Weisgraber, KH (2006). Apolipoprotein E structure: insights into function. *Trends Biochem Sci* 31: 445-454.
- 20 25. Mendez, AJ, He, JL, Huang, HS, Wen, SR, and Hsia, SL (1988). Interaction of rabbit lipoproteins and red blood cells with liposomes of egg yolk phospholipids. *Lipids* 23: 961-967.
26. Rensen, PC, Schiffelers, RM, Versluis, AJ, Bijsterbosch, MK, Van Kuijk-Meuwissen, ME, and Van Berkel, TJ (1997). Human recombinant apolipoprotein E-enriched
25 liposomes can mimic low-density lipoproteins as carriers for the site-specific delivery of antitumor agents. *Mol Pharmacol* 52: 445-455.
27. Bisgaier, CL, Siebenkas, MV, and Williams, KJ (1989). Effects of apolipoproteins A-IV and A-I on the uptake of phospholipid liposomes by hepatocytes. *J Biol Chem* 264: 862-866.
- 30 28. Yan, X, *et al.* (2005). The role of apolipoprotein E in the elimination of liposomes from blood by hepatocytes in the mouse. *Biochem Biophys Res Commun* 328: 57-62.
29. Wolfrum, C, *et al.* (2007). Mechanisms and optimization of in vivo delivery of lipophilic siRNAs. *Nat Biotechnol* 25: 1149-1157.

30. Landschulz, KT, Pathak, RK, Rigotti, A, Krieger, M, and Hobbs, HH (1996). Regulation of scavenger receptor, class B, type I, a high density lipoprotein receptor, in liver and steroidogenic tissues of the rat. *J Clin Invest* 98: 984-995.
31. Rudling, MJ, Reihner, E, Einarsson, K, Ewerth, S, and Angelin, B (1990). Low
5 density lipoprotein receptor-binding activity in human tissues: quantitative importance of hepatic receptors and evidence for regulation of their expression in vivo. *Proc Natl Acad Sci USA* 87: 3469-3473.
32. Geisbert, TW, *et al.* (2006). Postexposure protection of guinea pigs against a lethal ebola virus challenge is conferred by RNA interference. *J Infect Dis* 193: 1650-1657.
- 10 33. Schneider, WJ (2008). Lipoprotein receptors. In: Vance, DE and JE Vance eds. *Biochemistry of lipids, lipoproteins and membranes*, 5 ed. Elsevier: Amsterdam, London. pp 555-578.

[0621] It is to be understood that the above description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those of skill in the art upon reading the
15 above description. The scope of the invention should, therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled. The disclosures of all articles and references, including patent applications, patents, PCT publications, and Genbank Accession Nos., are incorporated herein by reference for all
20 purposes.

WHAT IS CLAIMED IS:

1. A cationic lipid of Formula I having the following structure:



or salts thereof, wherein:

R^1 and R^2 are either the same or different and are independently an optionally substituted C_1 - C_6 alkyl, C_2 - C_6 alkenyl, or C_2 - C_6 alkynyl, or R^1 and R^2 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

R^3 is either absent or is hydrogen (H) or a C_1 - C_6 alkyl to provide a quaternary amine;

R^4 and R^5 are either absent or present and when present are either the same or different and are independently an optionally substituted C_1 - C_{10} alkyl or C_2 - C_{10} alkenyl; and

n is 0, 1, 2, 3, or 4.

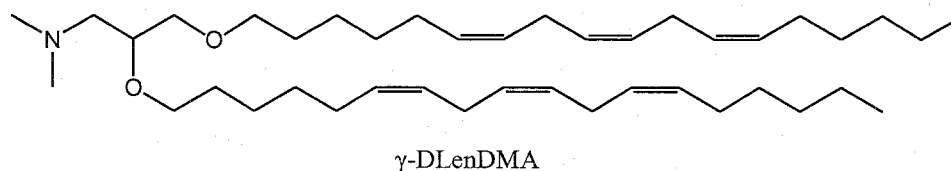
2. The cationic lipid of claim 1, wherein R^4 and R^5 are both butyl groups.

3. The cationic lipid of claim 1 or 2, wherein R^1 and R^2 are both methyl groups.

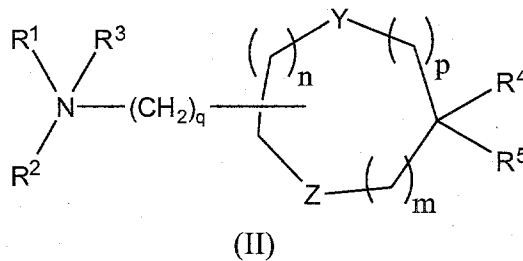
4. The cationic lipid of any one of claims 1-3, wherein R^3 is absent.

5. The cationic lipid of any one of claims 1-4, wherein n is 1.

6. The cationic lipid of claim 1, having the structure:



7. A cationic lipid of Formula II having the following structure:



or salts thereof, wherein:

R¹ and R² are either the same or different and are independently an optionally substituted C₁-C₆ alkyl, C₂-C₆ alkenyl, or C₂-C₆ alkynyl, or R¹ and R² may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

R³ is either absent or is hydrogen (H) or a C₁-C₆ alkyl to provide a quaternary amine;

R⁴ and R⁵ are either the same or different and are independently an optionally substituted C₁₂-C₂₄ alkyl, C₁₂-C₂₄ alkenyl, C₁₂-C₂₄ alkynyl, or C₁₂-C₂₄ acyl, wherein at least one of R⁴ and R⁵ comprises at least three sites of unsaturation or a substituted C₁₂-C₂₄ alkyl;

m, n , and p are either the same or different and are independently either 0, 1, or 2, with the proviso that m, n , and p are not simultaneously 0;

q is 0, 1, 2, 3, or 4; and

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

8. The cationic lipid of claim 7, wherein the substituted C₁₂-C₂₄ alkyl comprises a C₁₂-C₂₄ alkyl having at least 1-6 C₁-C₆ alkyl substituents.

9. The cationic lipid of claim 7, wherein R⁴ and R⁵ are independently selected from the group consisting of a dodecatrienyl moiety, a tetradecatrienyl moiety, a hexadecatrienyl moiety, an octadecatrienyl moiety, an icosatrienyl moiety, and a phytanyl moiety.

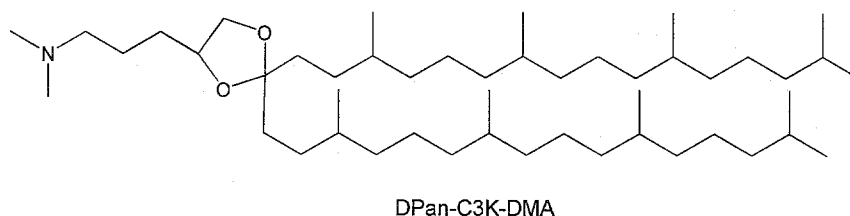
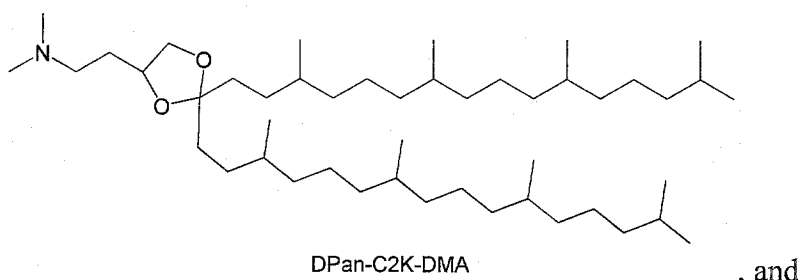
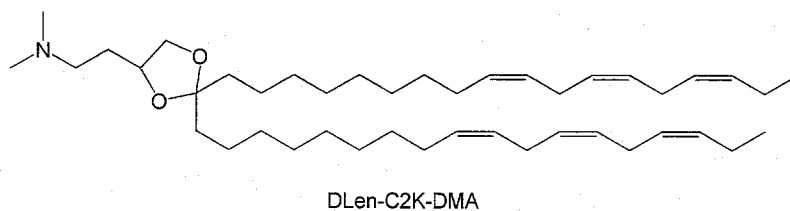
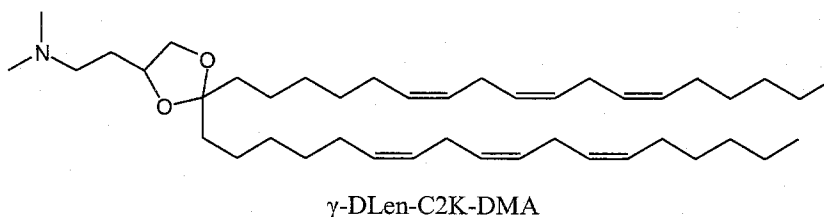
10. The cationic lipid of claim 9, wherein the octadecatrienyl moiety is a linolenyl moiety or a γ -linolenyl moiety.

11. The cationic lipid of claim 7, wherein R⁴ and R⁵ are both linolenyl moieties, γ-linolenyl moieties, or phytanyl moieties.

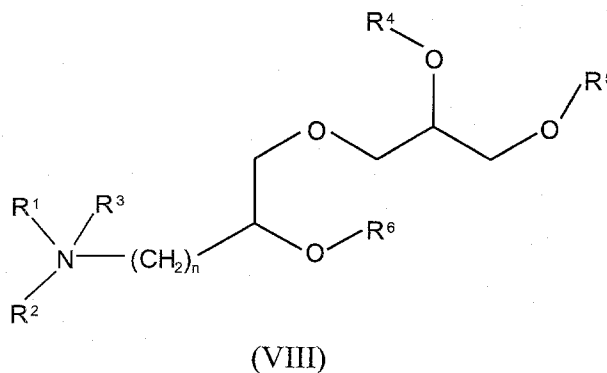
12. The cationic lipid of any one of claims 7-11, wherein R^1 and R^2 are both methyl groups.

13. The cationic lipid of any one of claims 7-12, wherein q is 2.

14. The cationic lipid of claim 7, having a structure selected from the group consisting of:



15. A cationic lipid of Formula VIII having the following structure:



or salts thereof, wherein:

R^1 and R^2 are either the same or different and are independently an optionally substituted C_1 - C_6 alkyl, C_2 - C_6 alkenyl, or C_2 - C_6 alkynyl, or R^1 and R^2 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

R^3 is either absent or is hydrogen (H) or a C_1 - C_6 alkyl to provide a quaternary amine;

R^4 , R^5 , and R^6 are either the same or different and are independently an optionally substituted C_{12} - C_{24} alkyl, C_{12} - C_{24} alkenyl, C_{12} - C_{24} alkynyl, or C_{12} - C_{24} acyl; and n is 0, 1, 2, 3, or 4.

16. The cationic lipid of claim 15, wherein R^4 , R^5 , and R^6 are independently selected from the group consisting of a dodecadienyl moiety, a tetradecadienyl moiety, a hexadecadienyl moiety, an octadecadienyl moiety, an icosadienyl moiety, a dodecatrienyl moiety, a tetradectrienyl moiety, a hexadecatrienyl moiety, an octadecatrienyl moiety, an icosatrienyl moiety, and a phytanyl moiety.

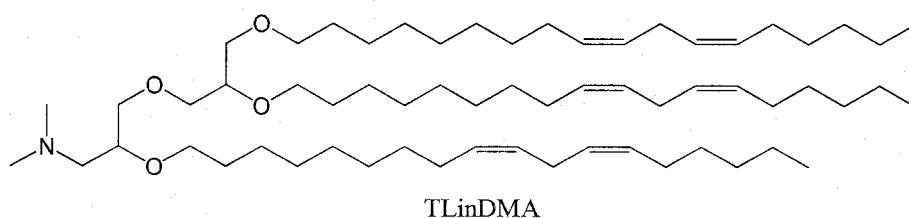
17. The cationic lipid of claim 16, wherein the octadecadienyl moiety is a linoleyl moiety.

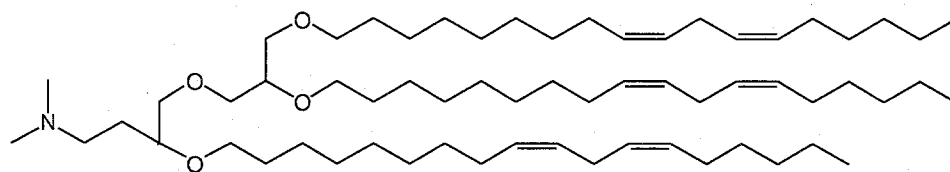
18. The cationic lipid of claim 17, wherein R^4 , R^5 , and R^6 are linoleyl moieties.

19. The cationic lipid of claim 16, wherein the octadecatrienyl moiety is a linolenyl moiety or a γ -linolenyl moiety.

20. The cationic lipid of any one of claims 15-19, wherein R^1 and R^2 are both methyl groups.

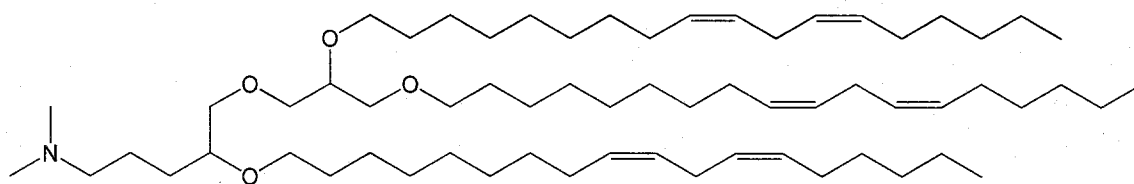
21. The cationic lipid of claim 15, having a structure selected from the group consisting of:





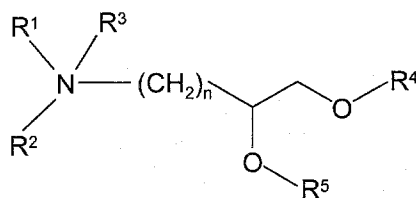
C2-TLinDMA

, and



C3-TLinDMA

22. A cationic lipid of Formula XII having the following structure:



(XII)

or salts thereof, wherein:

R^1 and R^2 are either the same or different and are independently an optionally substituted C_1 - C_6 alkyl, C_2 - C_6 alkenyl, or C_2 - C_6 alkynyl, or R^1 and R^2 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

R^3 is either absent or is hydrogen (H) or a C_1 - C_6 alkyl to provide a quaternary amine;

R^4 and R^5 are either the same or different and are independently a substituted C_{12} - C_{24} alkyl; and

n is 0, 1, 2, 3, or 4.

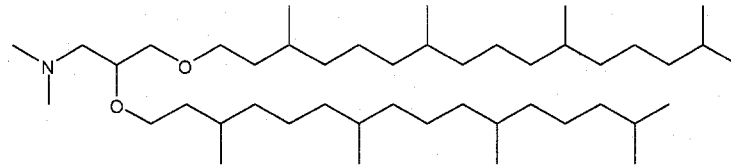
23. The cationic lipid of claim 22, wherein the substituted C_{12} - C_{24} alkyl comprises a C_{12} - C_{24} alkyl having at least 1-6 C_1 - C_6 alkyl substituents.

24. The cationic lipid of claim 22, wherein R^4 and R^5 are both phytanyl moieties.

25. The cationic lipid of any one of claims 22-24, wherein R^1 and R^2 are both methyl groups.

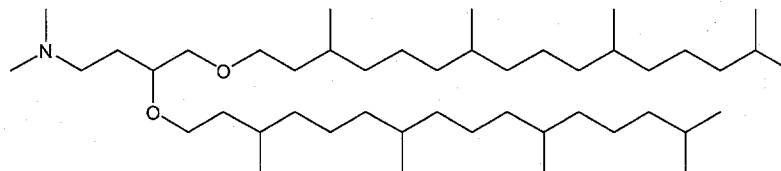
26. The cationic lipid of any one of claims 22-25, wherein n is 2.

27. The cationic lipid of claim 22, having a structure selected from the group consisting of:



DPanDMA

and



C2-DPanDMA

28. A lipid particle comprising a cationic lipid of any one of claims 1-27.

29. The lipid particle of claim 28, wherein the particle further comprises a non-cationic lipid.

30. The lipid particle of claim 29, wherein the non-cationic lipid is selected from the group consisting of a phospholipid, cholesterol, or a mixture of a phospholipid and cholesterol.

31. The lipid particle of claim 30, wherein the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof.

32. The lipid particle of claim 30, wherein the cholesterol is a cholesterol derivative.

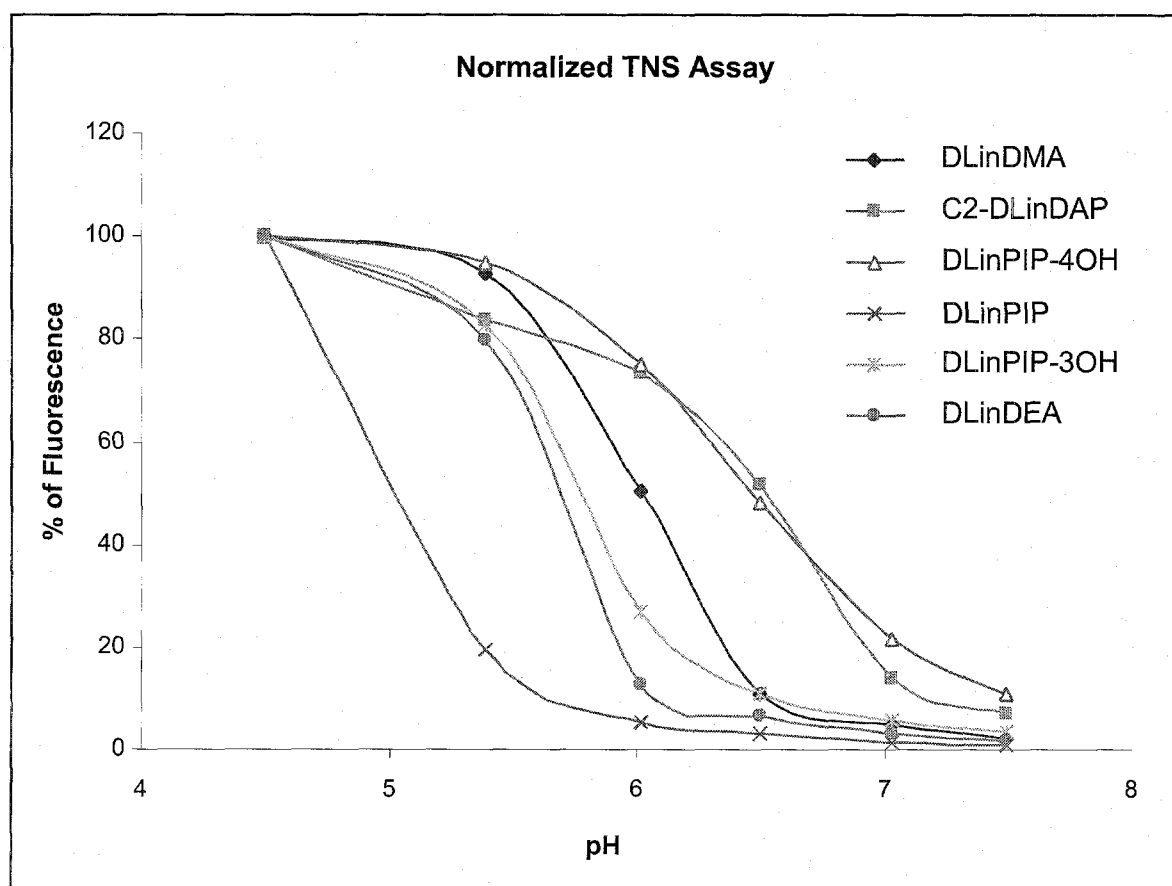
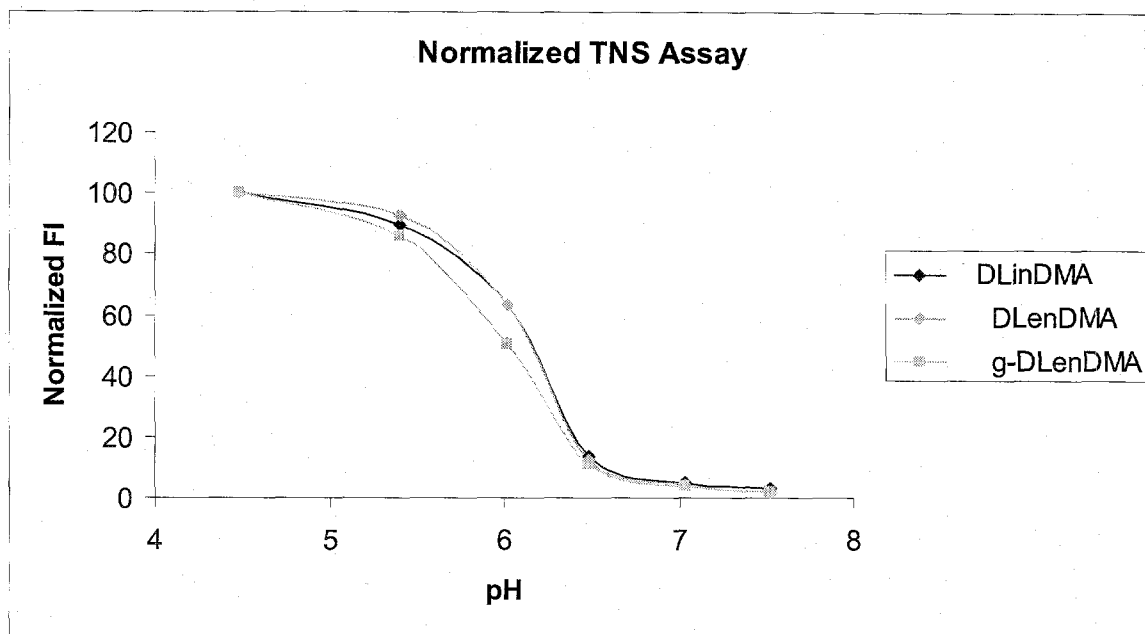
33. The lipid particle of any of claims 28 or 29, wherein the particle further comprises a conjugated lipid that inhibits aggregation of particles.

34. The lipid particle of claim 33, wherein the conjugated lipid that inhibits aggregation of particles comprises a polyethyleneglycol (PEG)-lipid conjugate.

- 1 35. The lipid particle of claim 34, wherein the PEG-lipid conjugate
2 comprises a PEG-diacylglycerol (PEG-DAG) conjugate, a PEG-dialkyloxypropyl (PEG-
3 DAA) conjugate, or a mixture thereof.
- 1 36. The lipid particle of any of claims 28, 29, or 33, wherein the particle
2 further comprises a therapeutic agent.
- 1 37. The lipid particle of claim 36, wherein the therapeutic agent is a
2 nucleic acid.
- 1 38. The lipid particle of claim 37, wherein the nucleic acid is an interfering
2 RNA.
- 1 39. The lipid particle of claim 38, wherein the interfering RNA is selected
2 from the group consisting of a small interfering RNA (siRNA), an asymmetrical interfering
3 RNA (aiRNA), a microRNA (miRNA), a Dicer-substrate dsRNA, a small hairpin RNA
4 (shRNA), and mixtures thereof.
- 1 40. The lipid particle of claim 38, wherein the interfering RNA is an
2 siRNA.
- 1 41. The lipid particle of claim 36, wherein the therapeutic agent is not
2 substantially degraded after incubation of the particle in serum at 37°C for 30 minutes.
- 1 42. The lipid particle of claim 36, wherein the therapeutic agent is fully
2 encapsulated in the particle.
- 1 43. The lipid particle of claim 37, wherein the particle has a lipid:nucleic
2 acid mass ratio of from about 5:1 to about 15:1.
- 1 44. The lipid particle of any of claims 28, 29, 33, or 36, wherein the
2 particle has a median diameter of from about 30 nm to about 150 nm.
- 1 45. A pharmaceutical composition comprising a lipid particle of any of
2 claims 28, 29, 33, or 36 and a pharmaceutically acceptable carrier.
- 1 46. A method for introducing a therapeutic agent into a cell, the method
2 comprising:
3 contacting the cell with a lipid particle of claim 36.

- 1 47. The method of claim 46, wherein the cell is in a mammal.
- 1 48. A method for the *in vivo* delivery of a therapeutic agent, the method
2 comprising:
3 administering to a mammal a lipid particle of claim 36.
- 1 49. The method of claim 48, wherein the administration is selected from
2 the group consisting of oral, intranasal, intravenous, intraperitoneal, intramuscular, intra-
3 articular, intralesional, intratracheal, subcutaneous, and intradermal.
- 1 50. The method of claim 48, wherein the mammal is a human.
- 1 51. A method for treating a disease or disorder in a mammal in need
2 thereof, the method comprising:
3 administering to the mammal a therapeutically effective amount of a lipid
4 particle of claim 36.
- 1 52. The method of claim 51, wherein the disease or disorder is selected
2 from the group consisting of a viral infection, a liver disease or disorder, and cancer.
- 1 53. The method of claim 51, wherein the mammal is a human.

1/22

**FIG. 1**

2/22

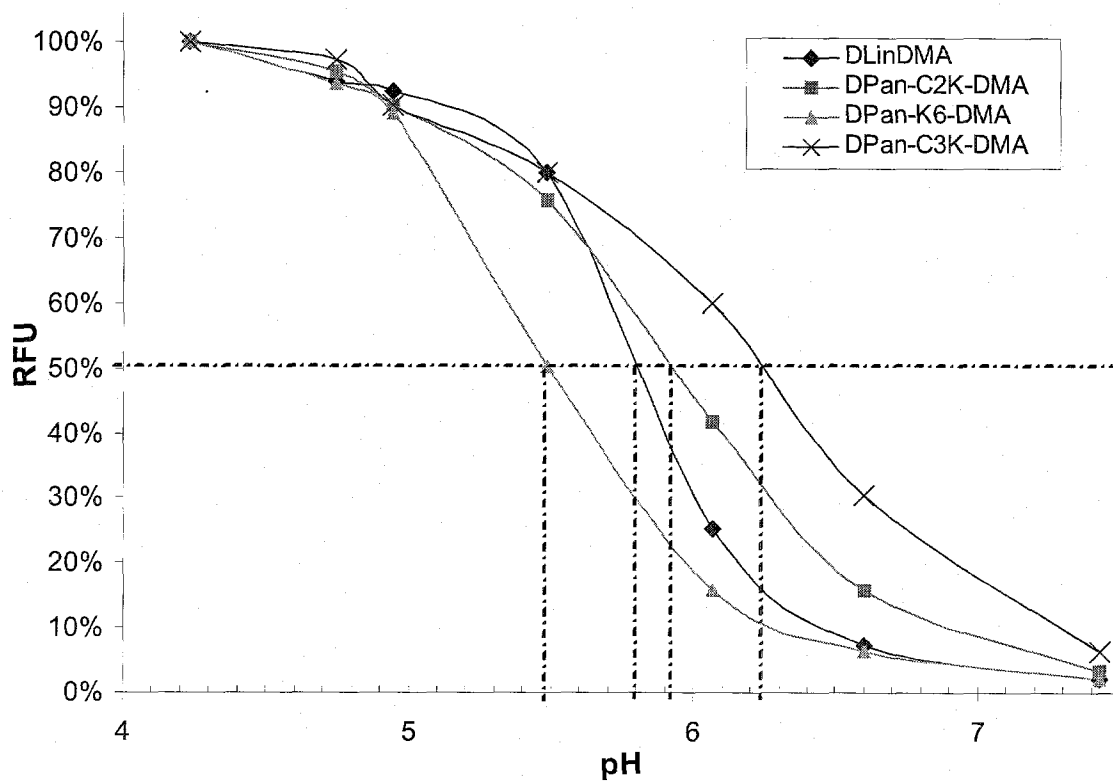
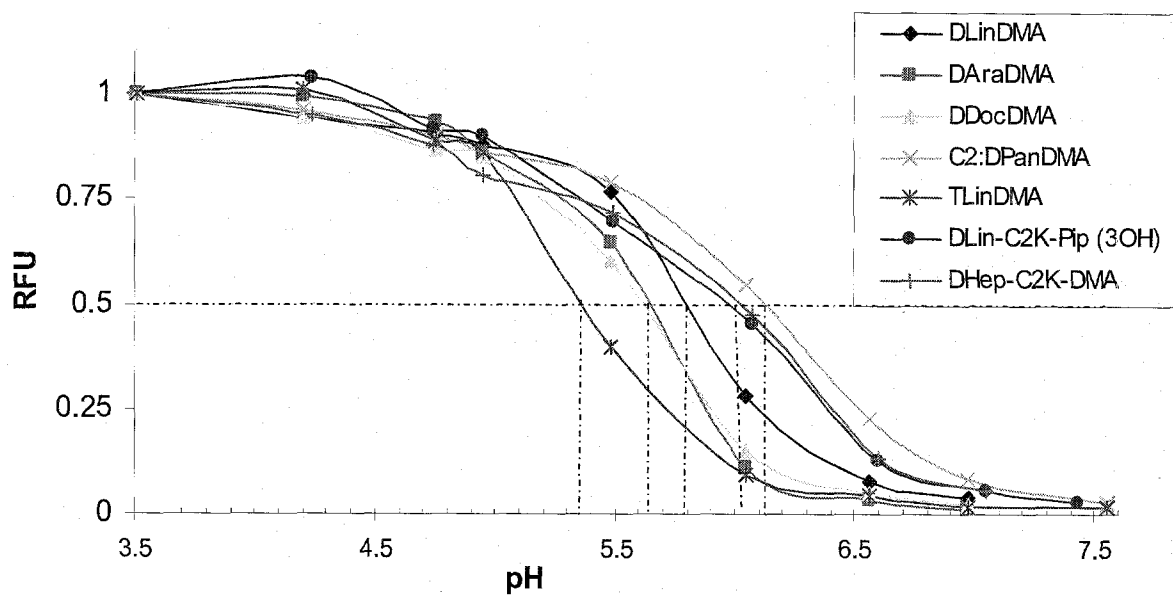


FIG. 2

3/22

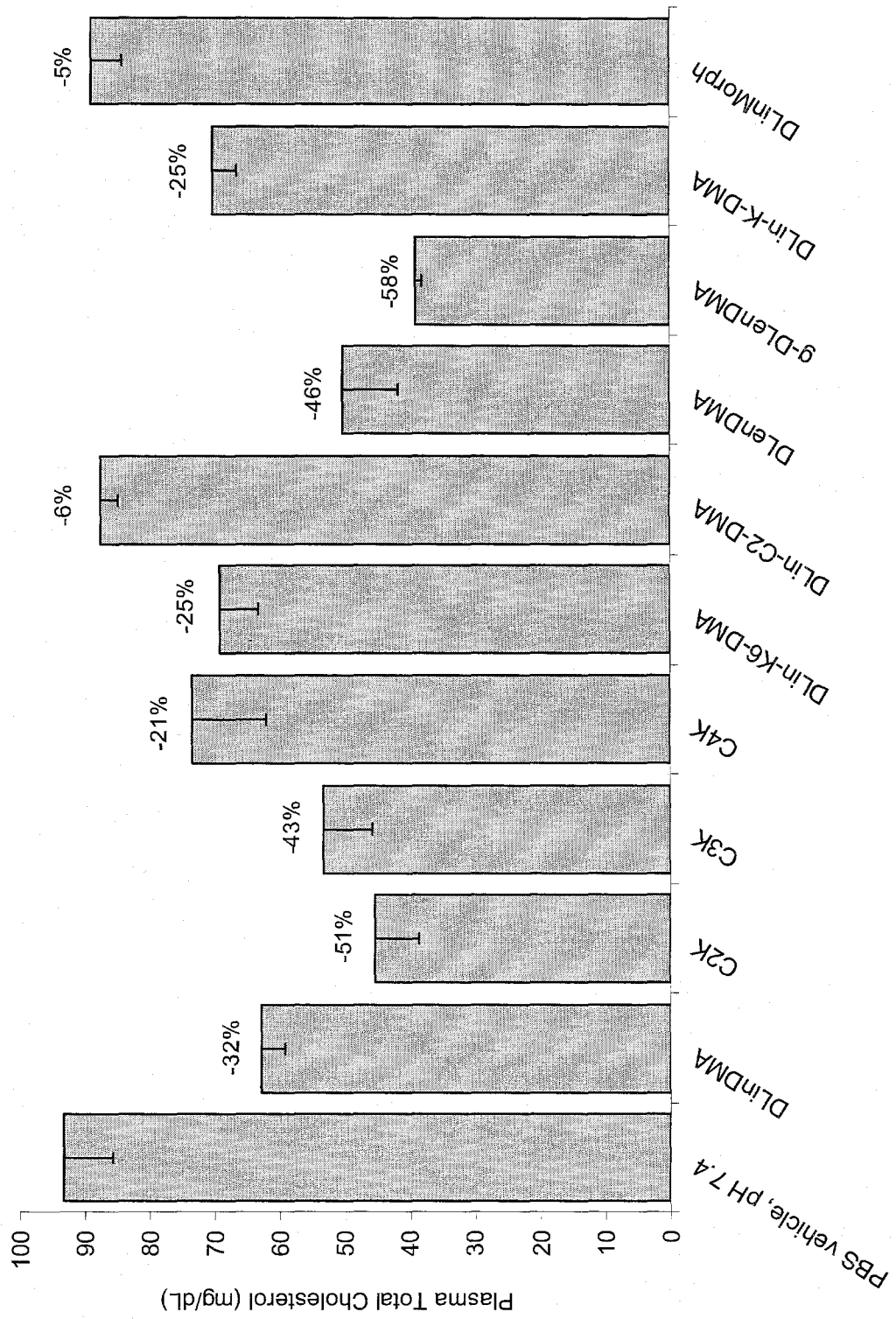
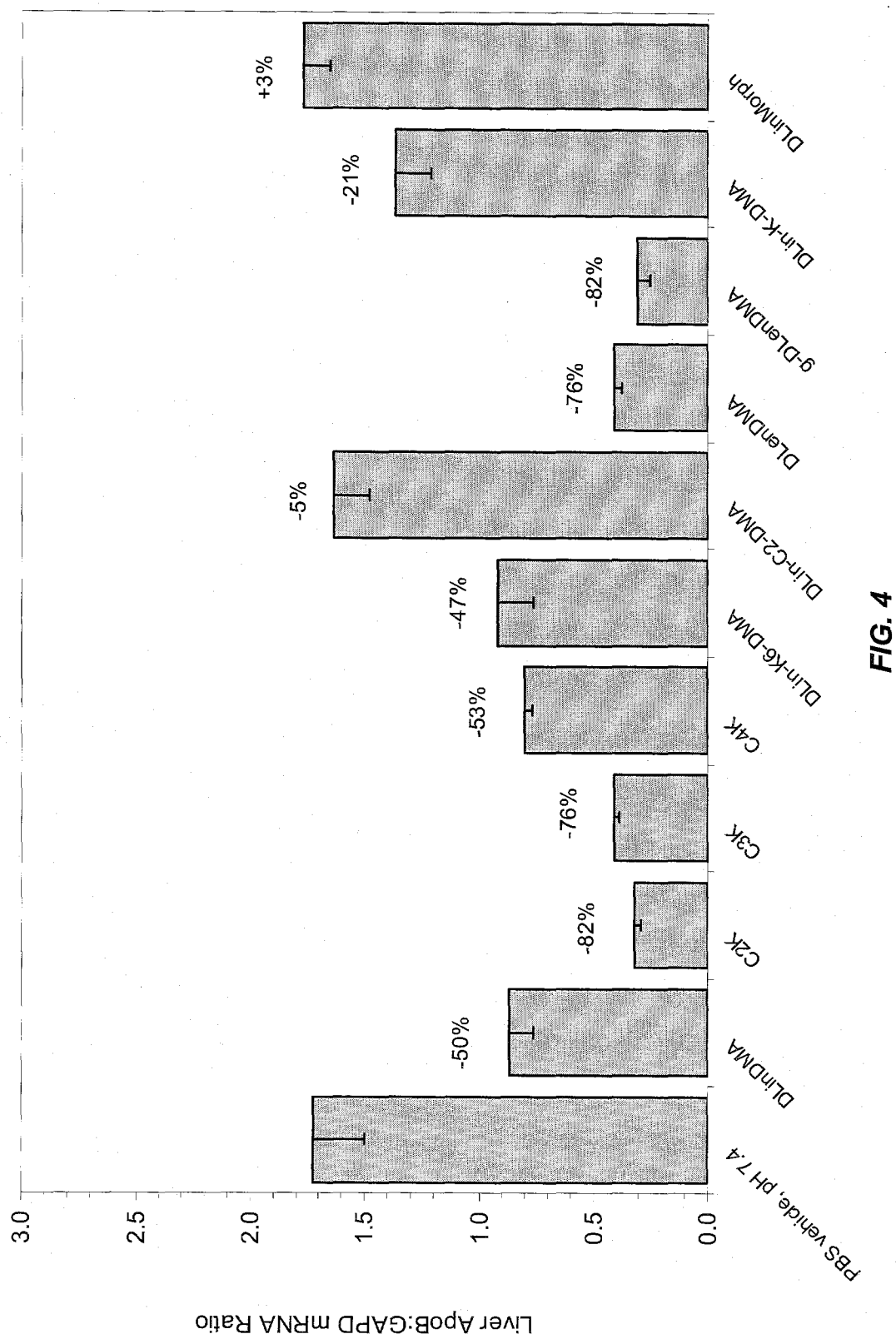


FIG. 3

4/22



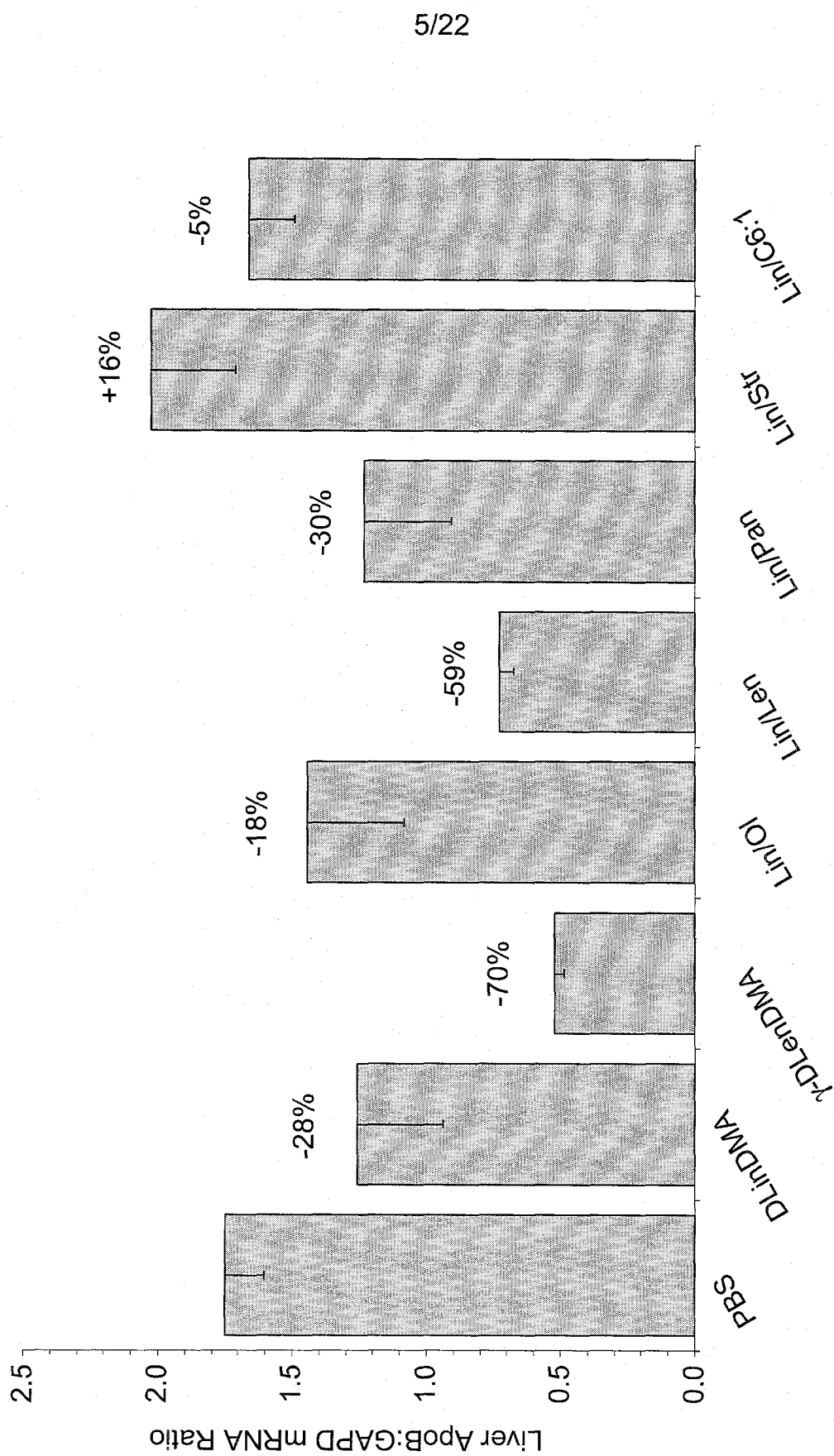


FIG. 5

6/22

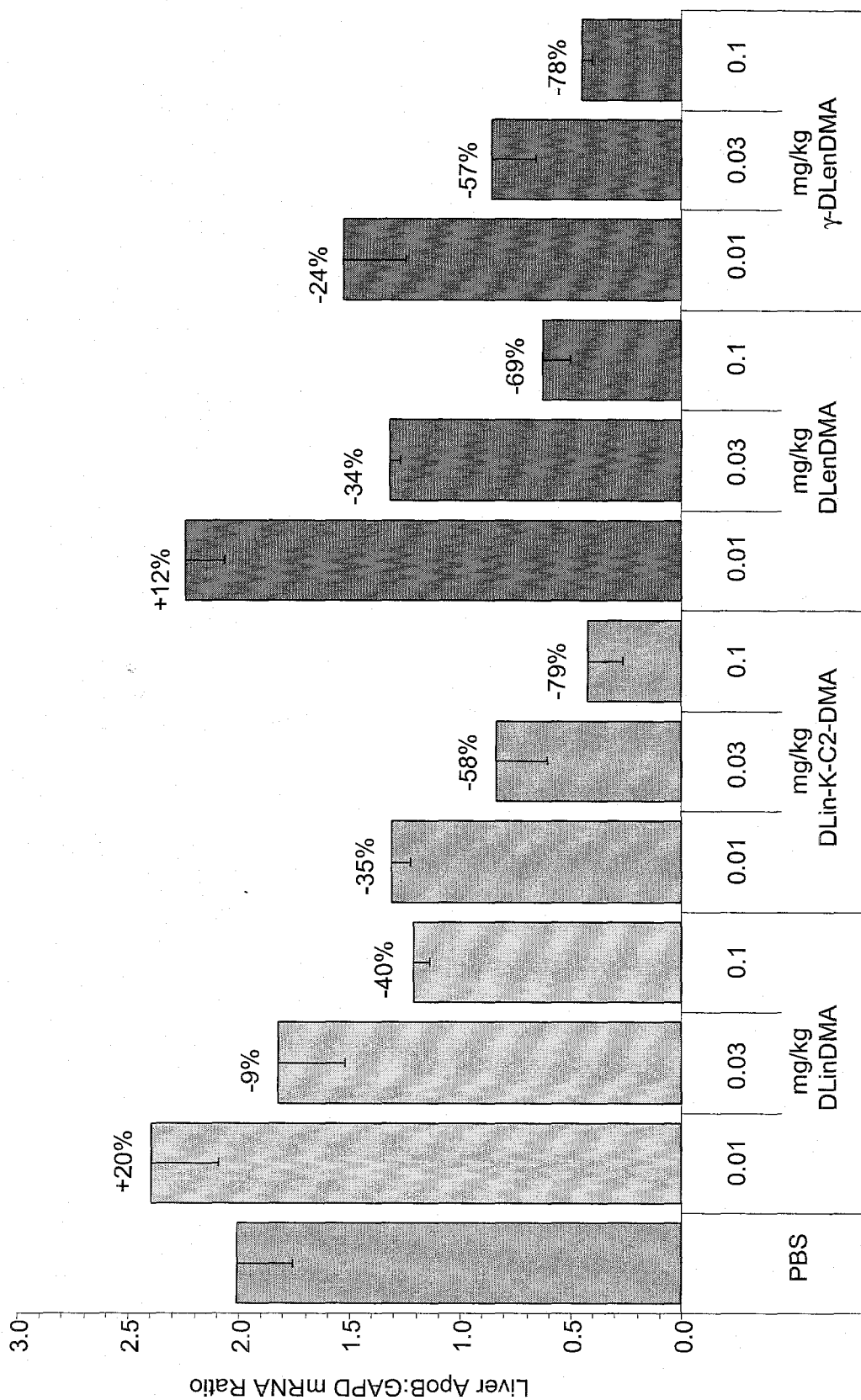


FIG. 6

7/22

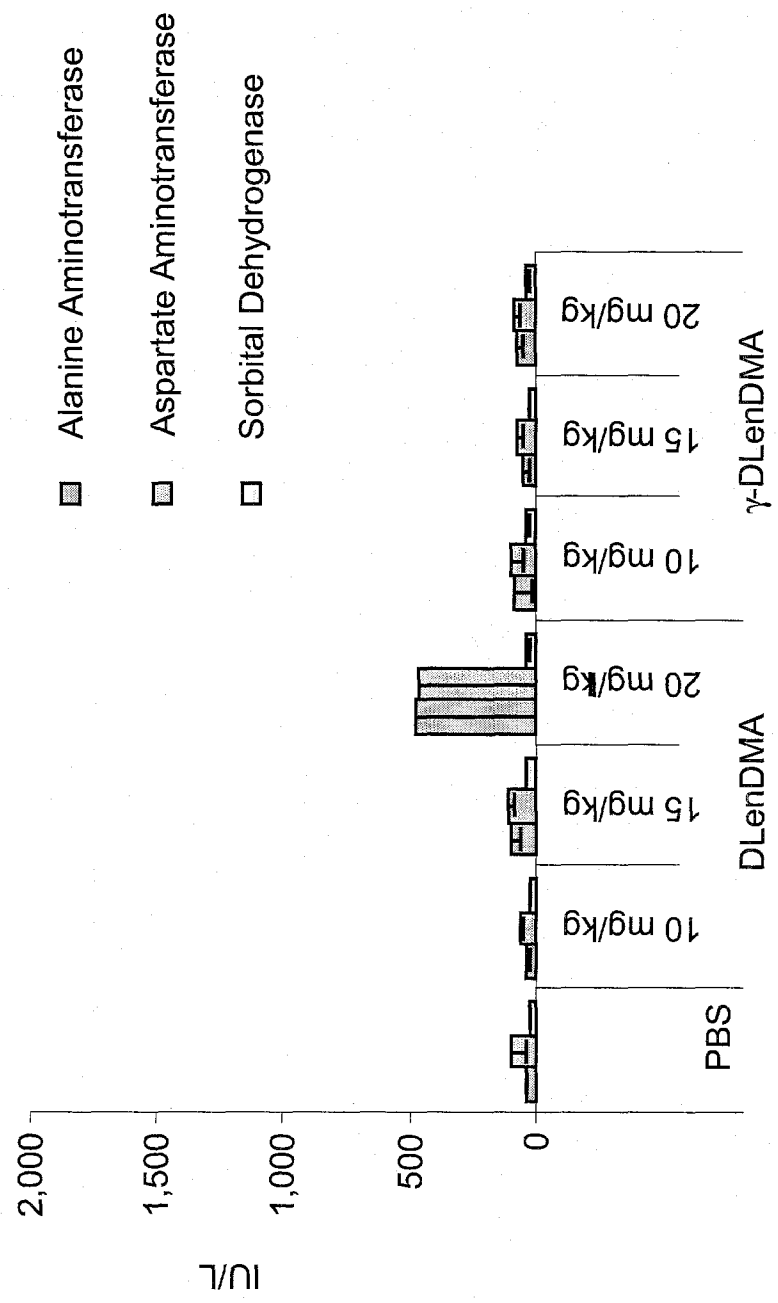


FIG. 7

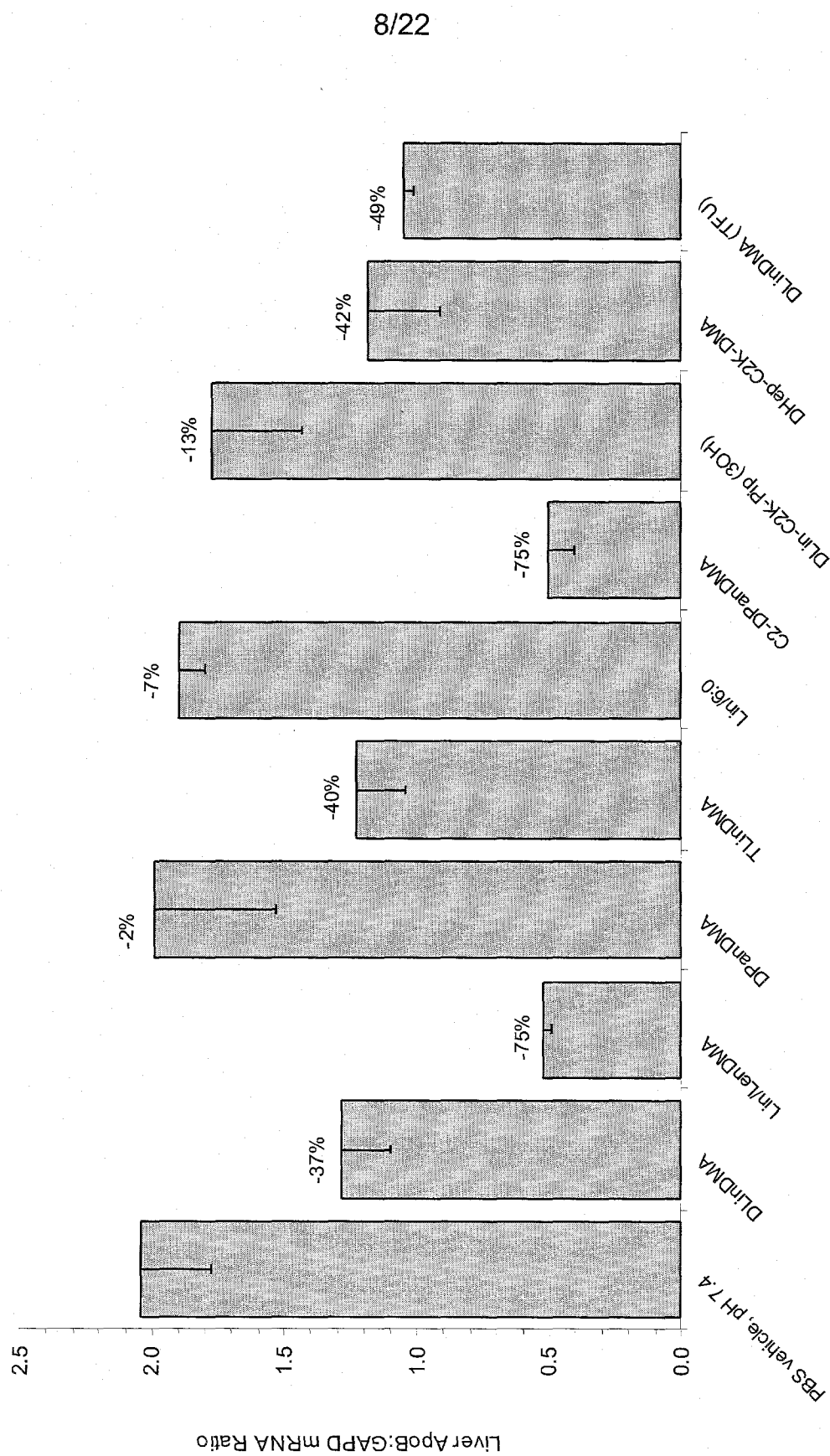


FIG. 8

9/22

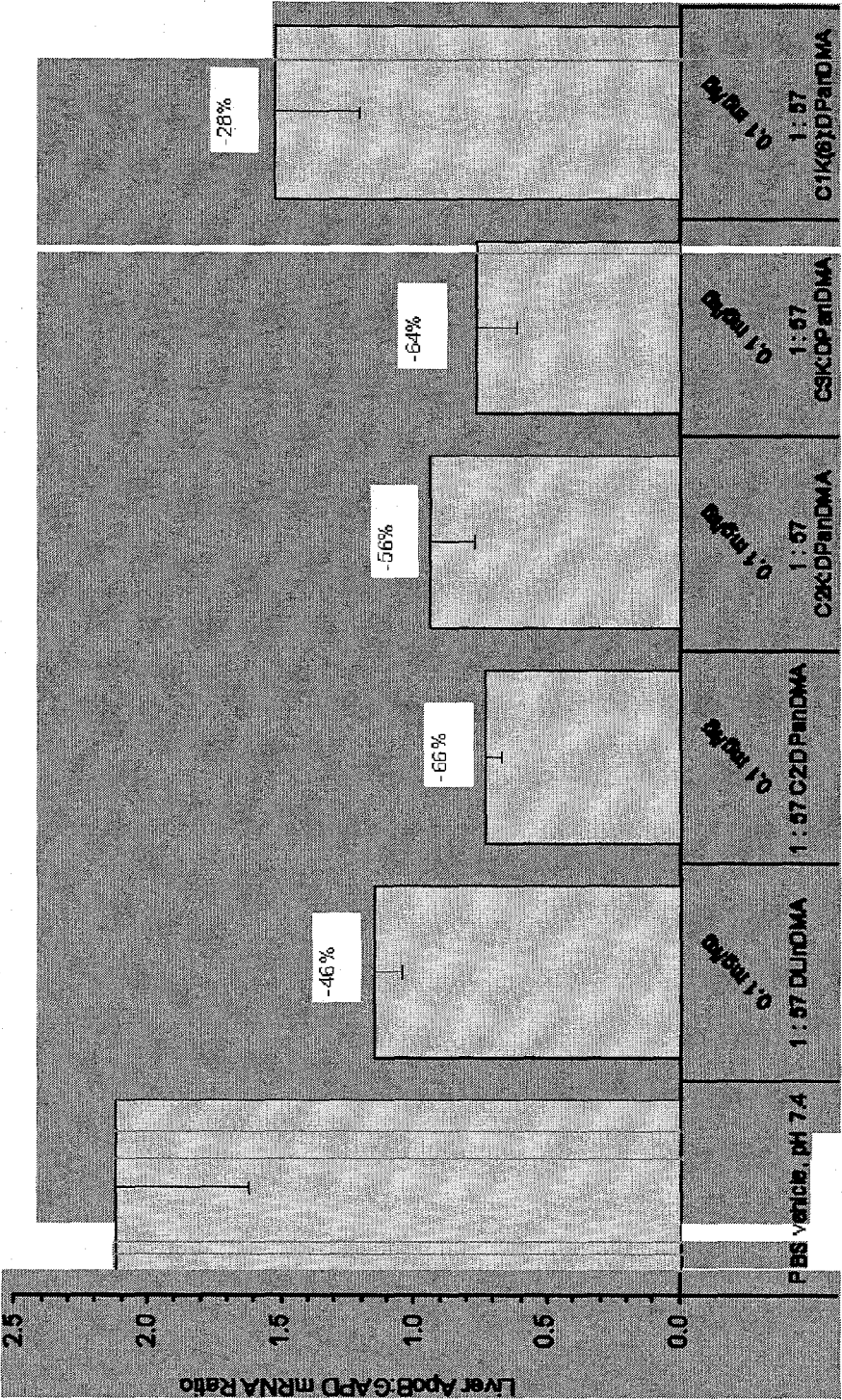


FIG. 9

10/22

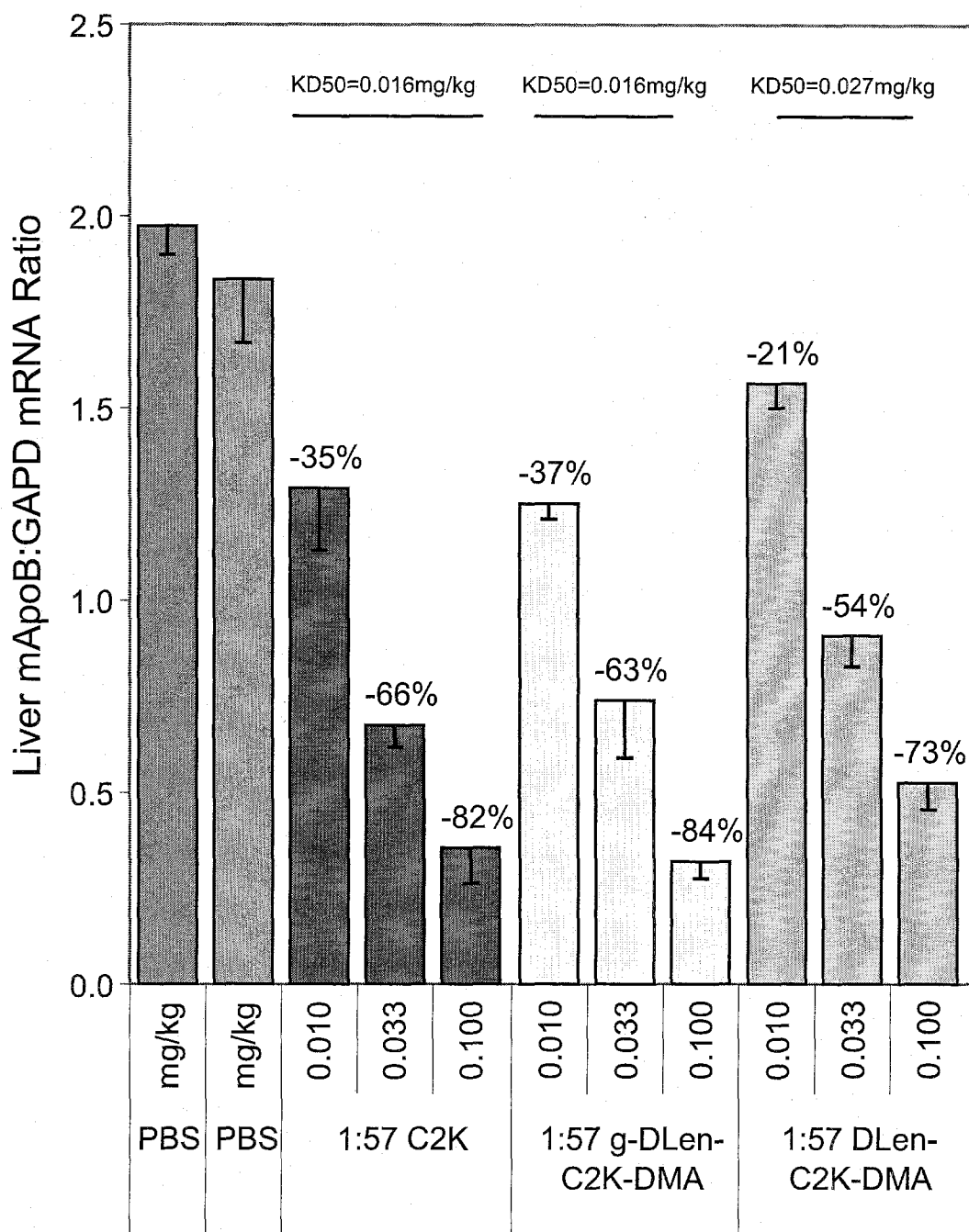
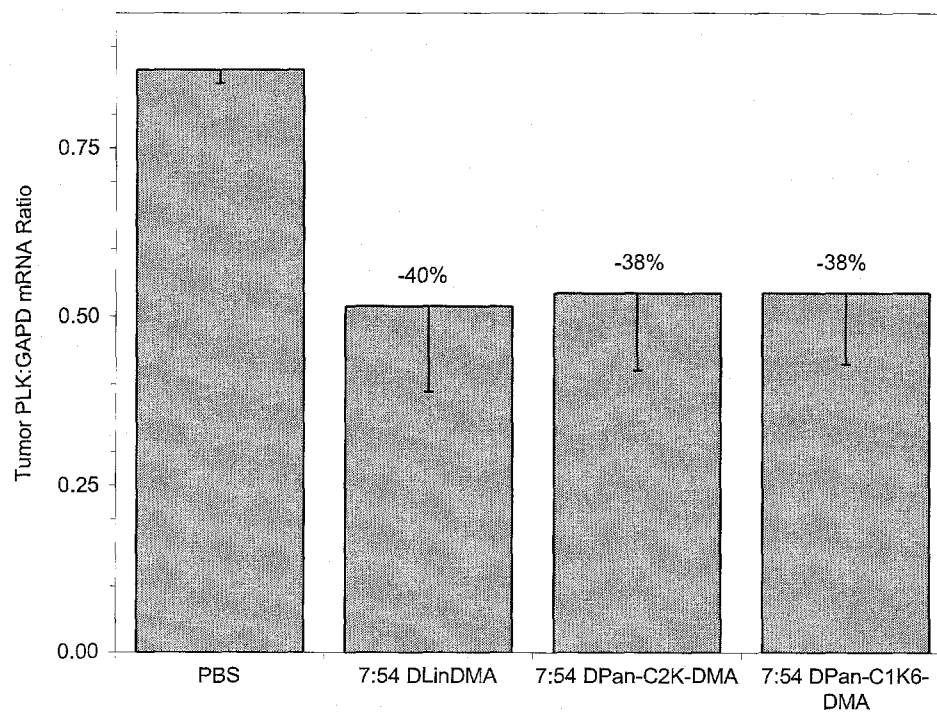
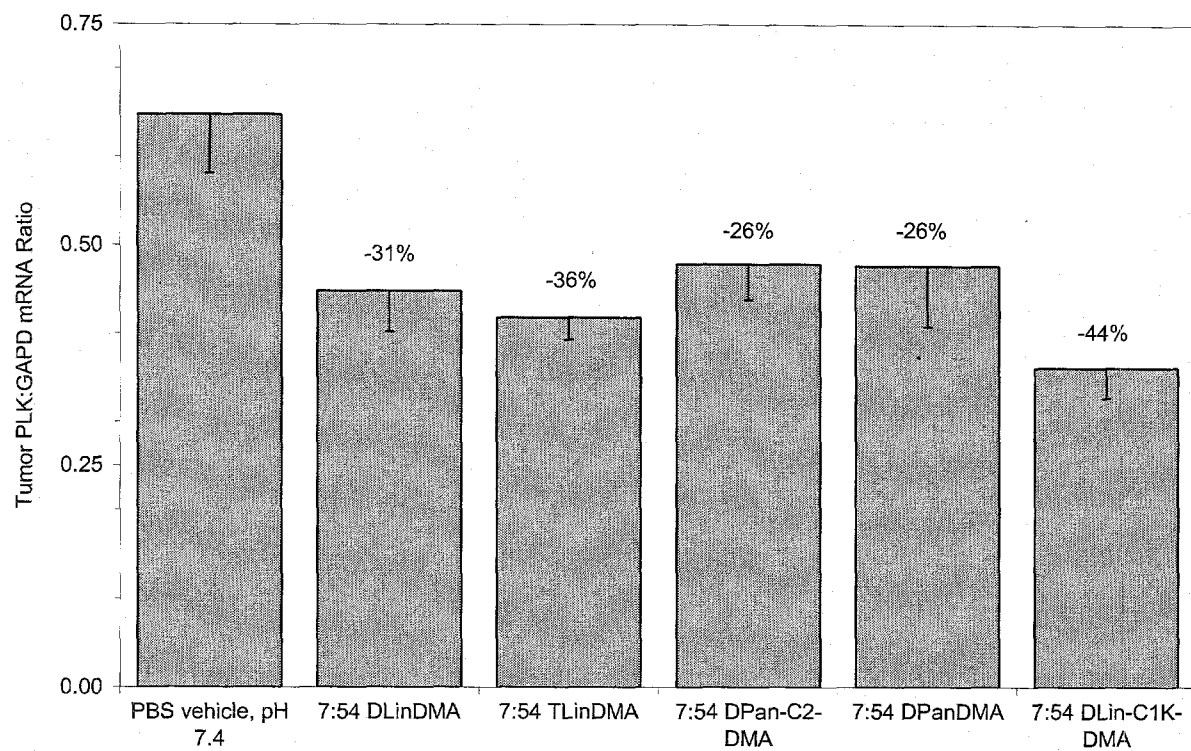


FIG. 10

11/22

**FIG. 11**

12/22

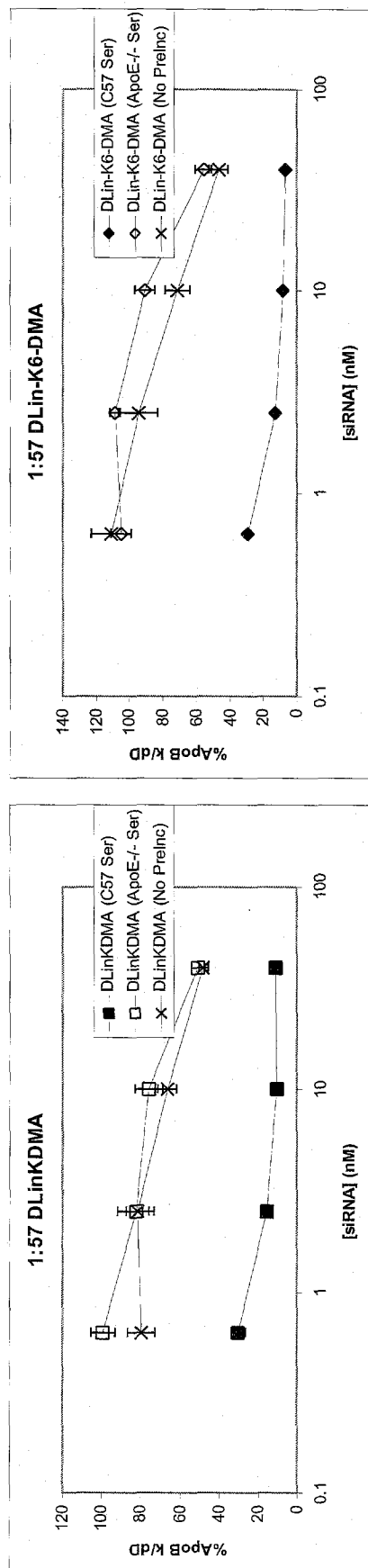
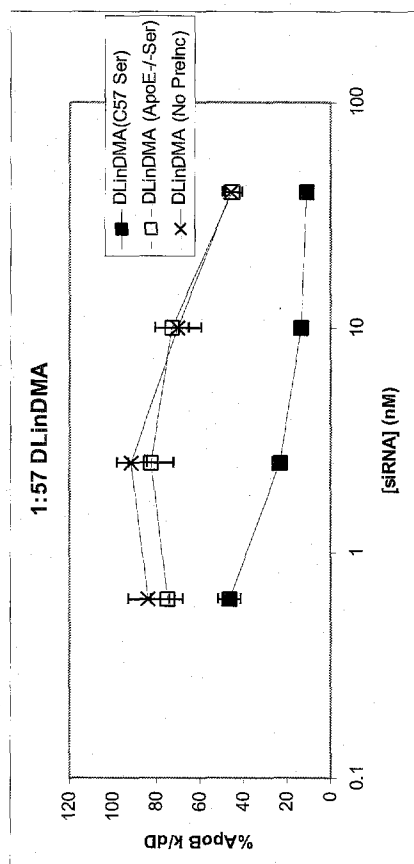


FIG. 12

13/22

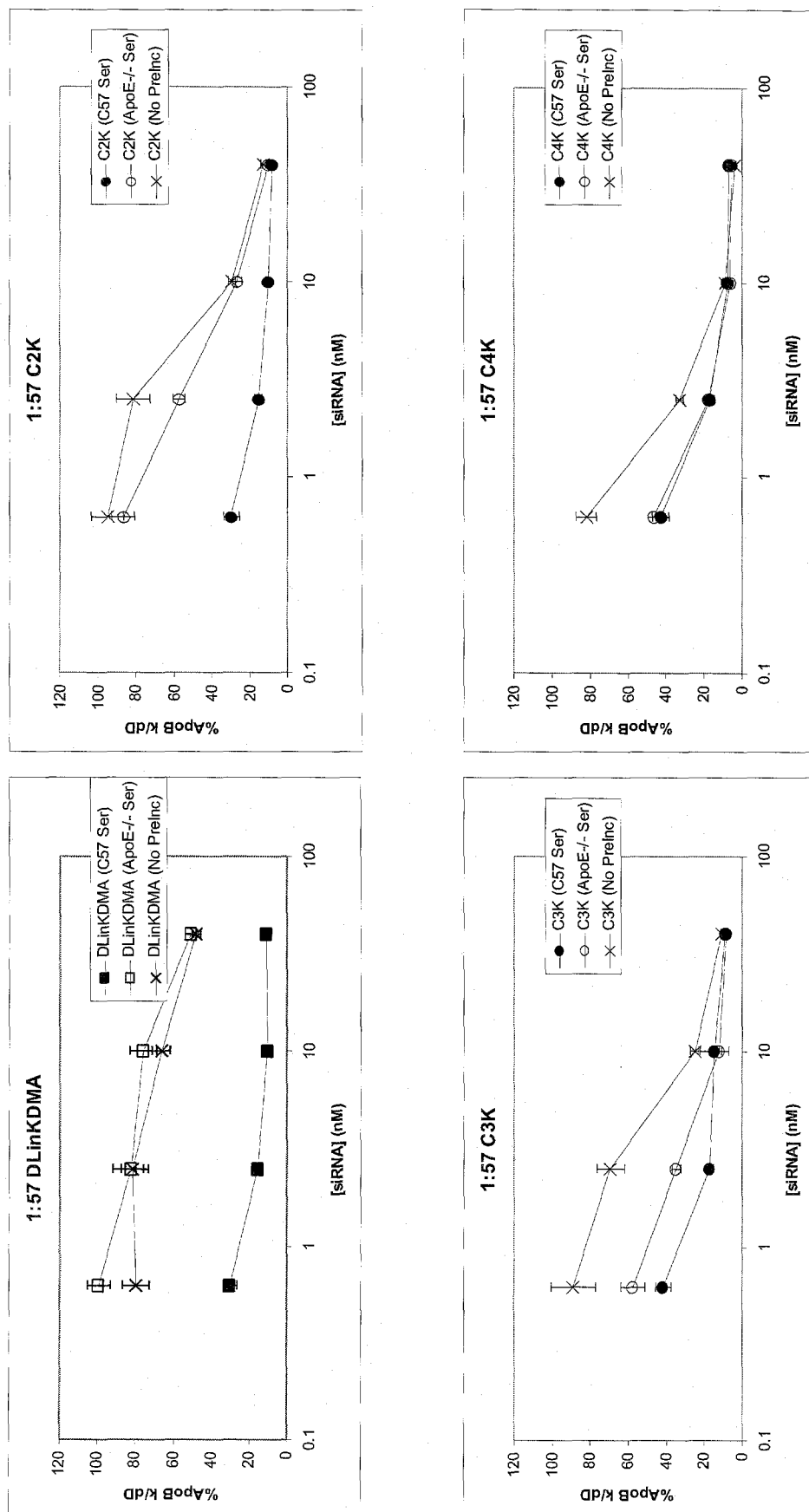


FIG. 13

14/22

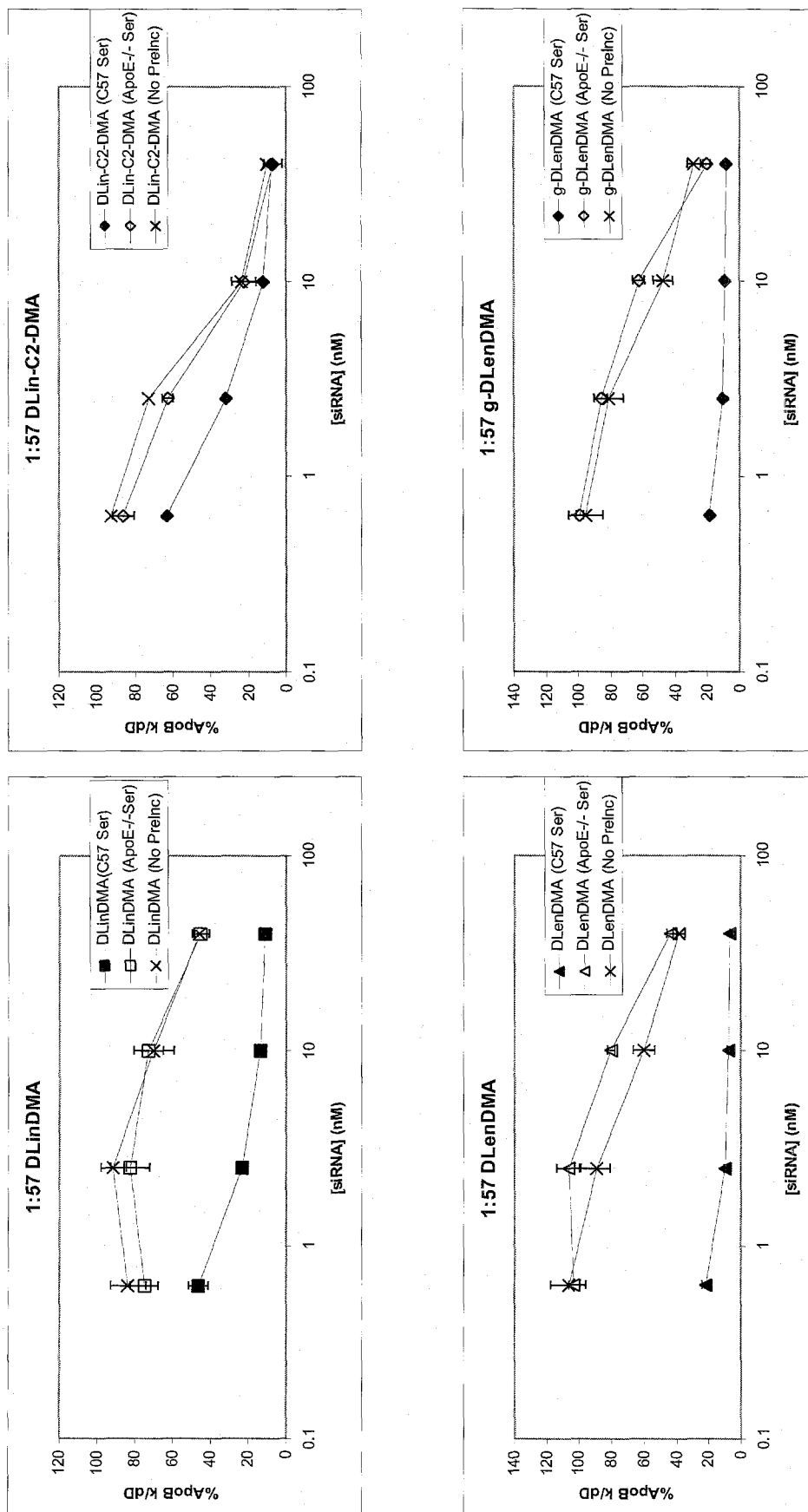


FIG. 14

15/22

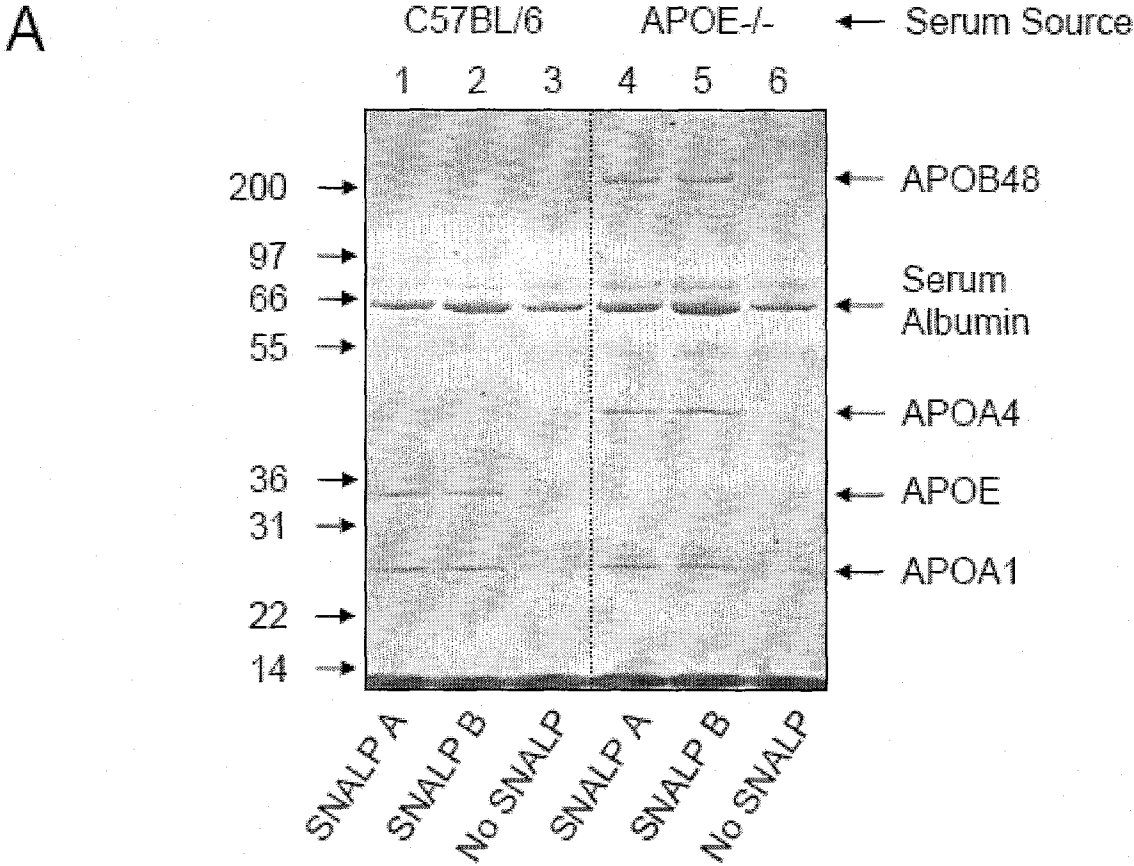


FIG. 15a

16/22

B

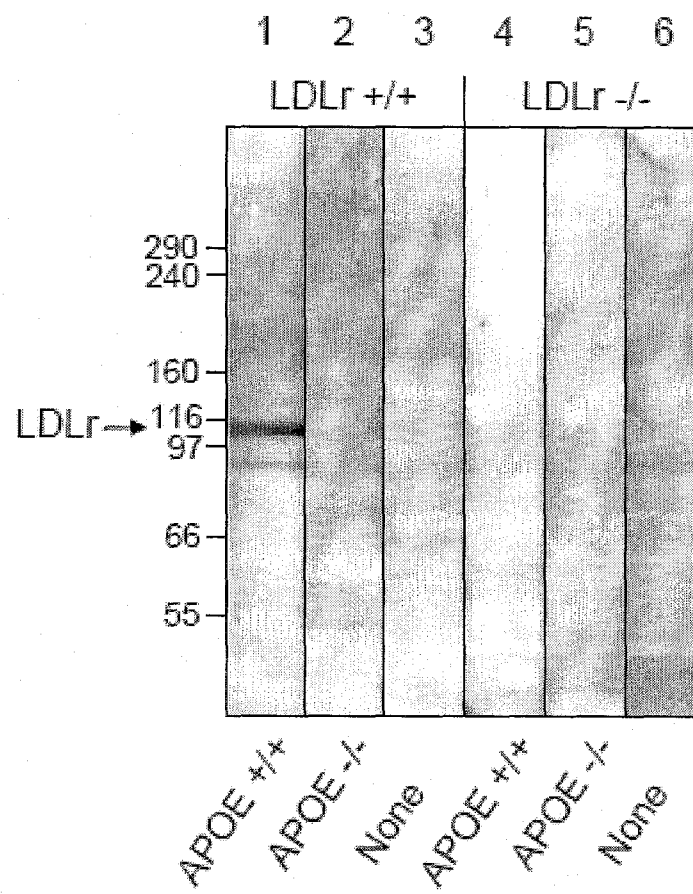
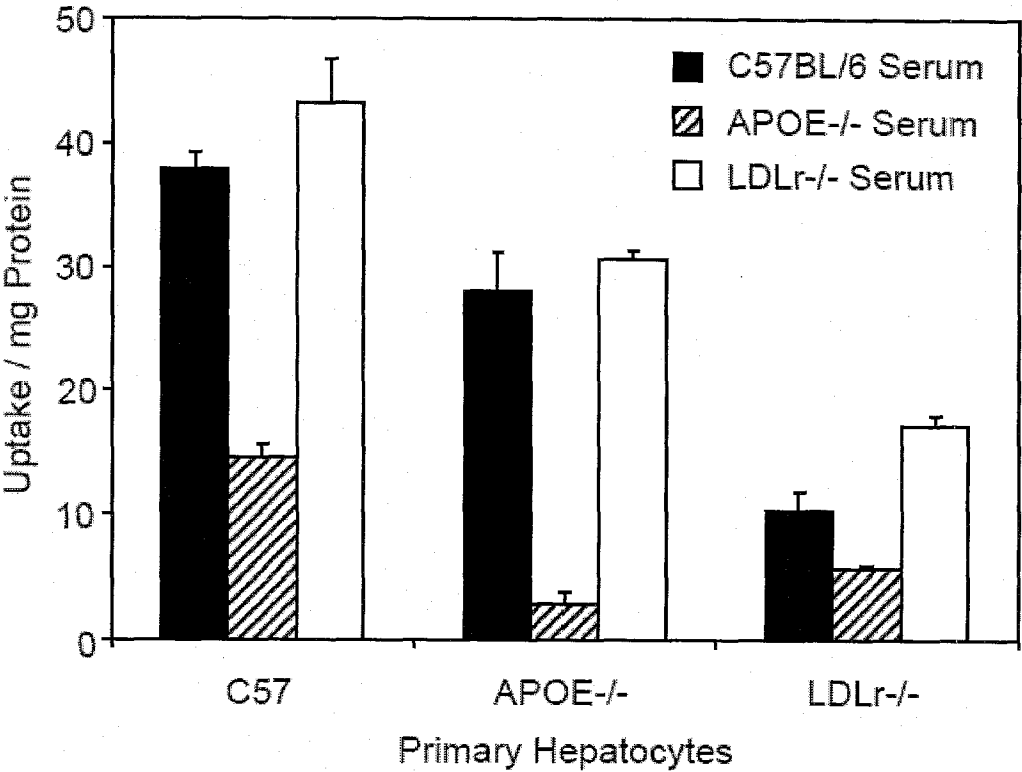


FIG. 15b

17/22

A



B

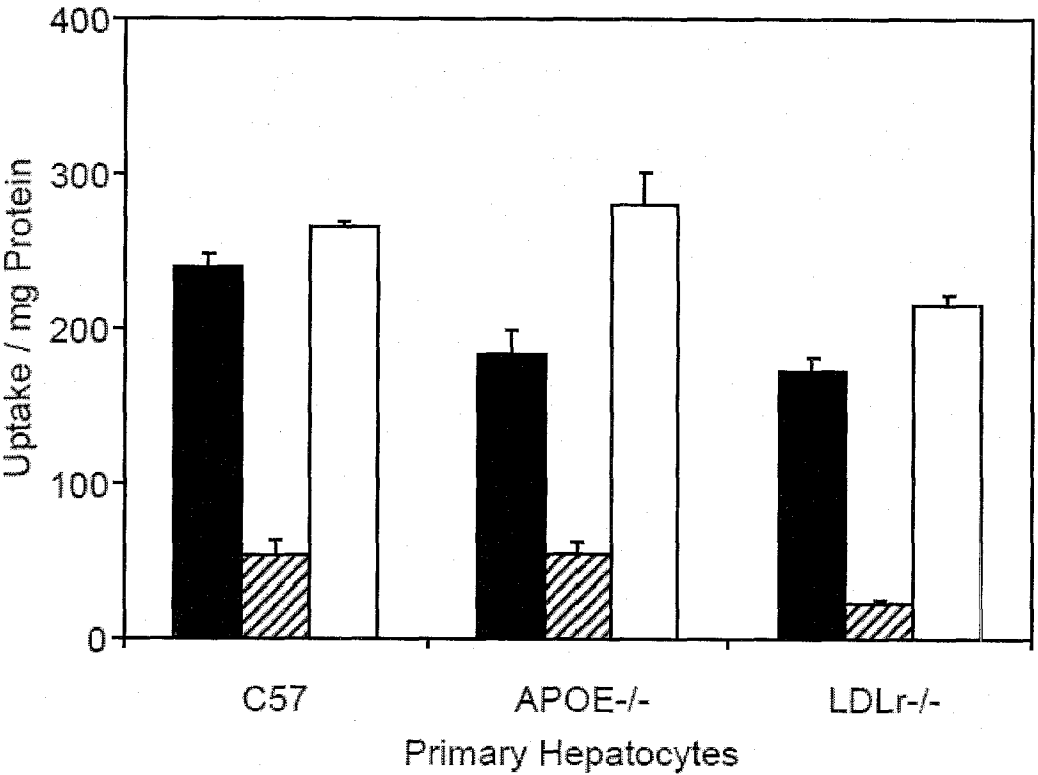


FIG. 16a-b

18/22

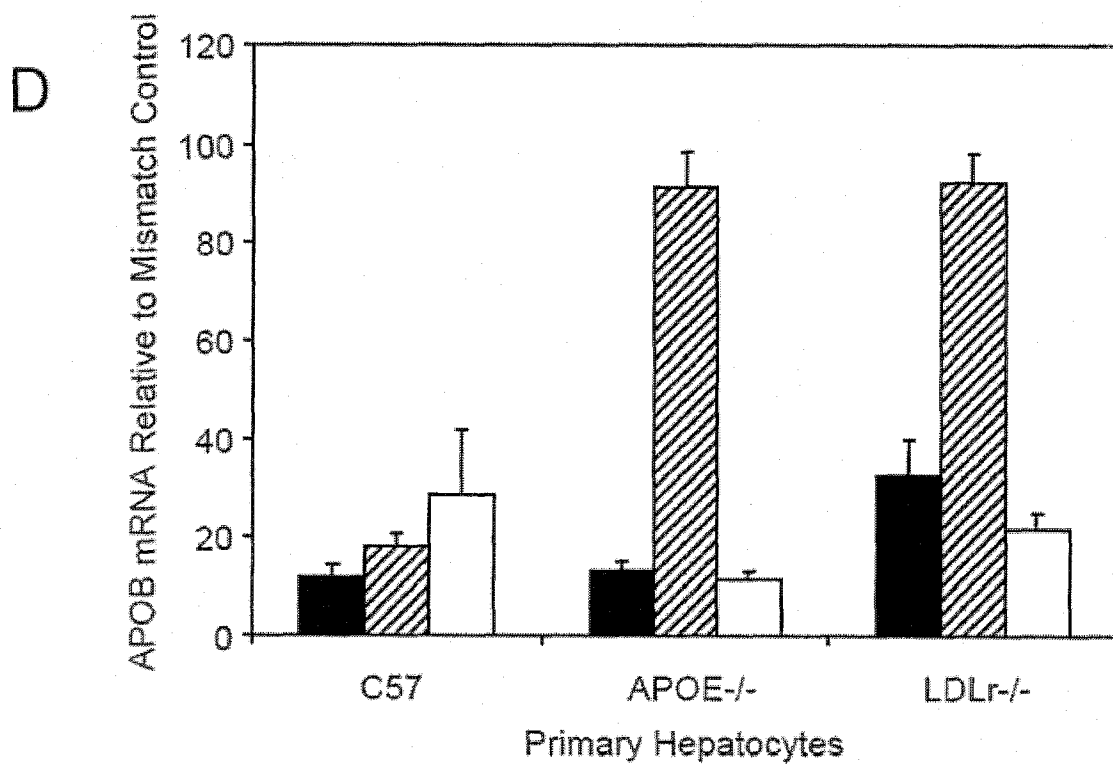
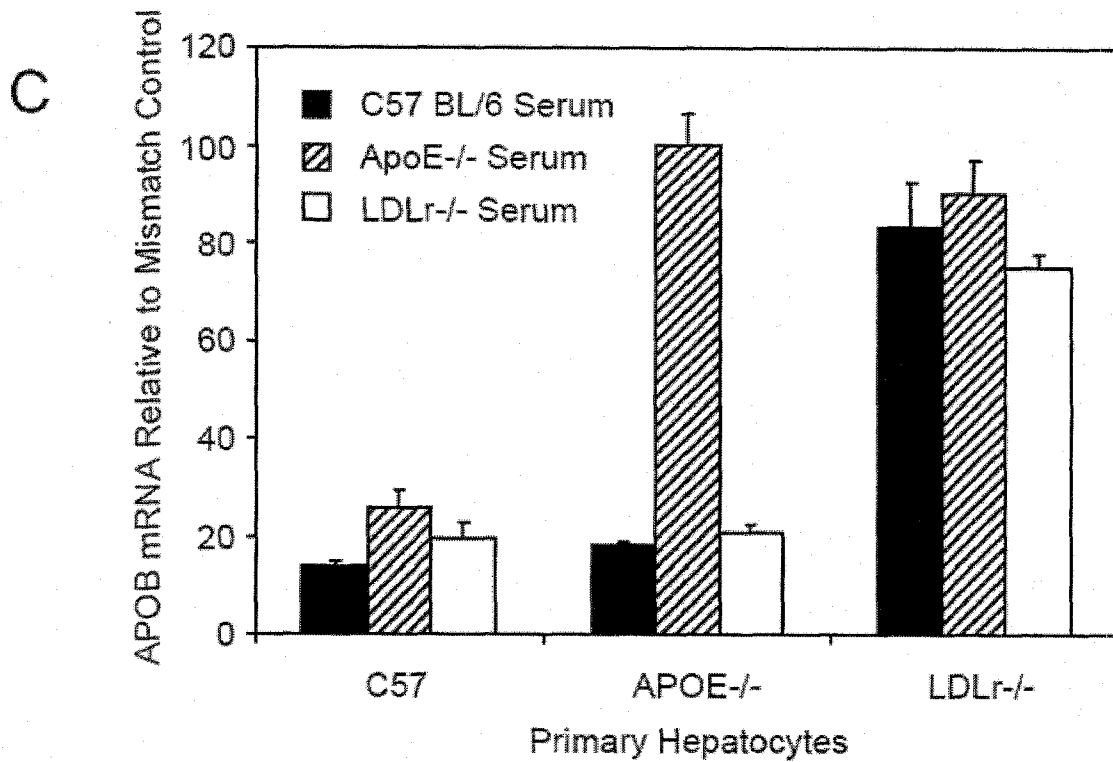


FIG. 16c-d

19/22

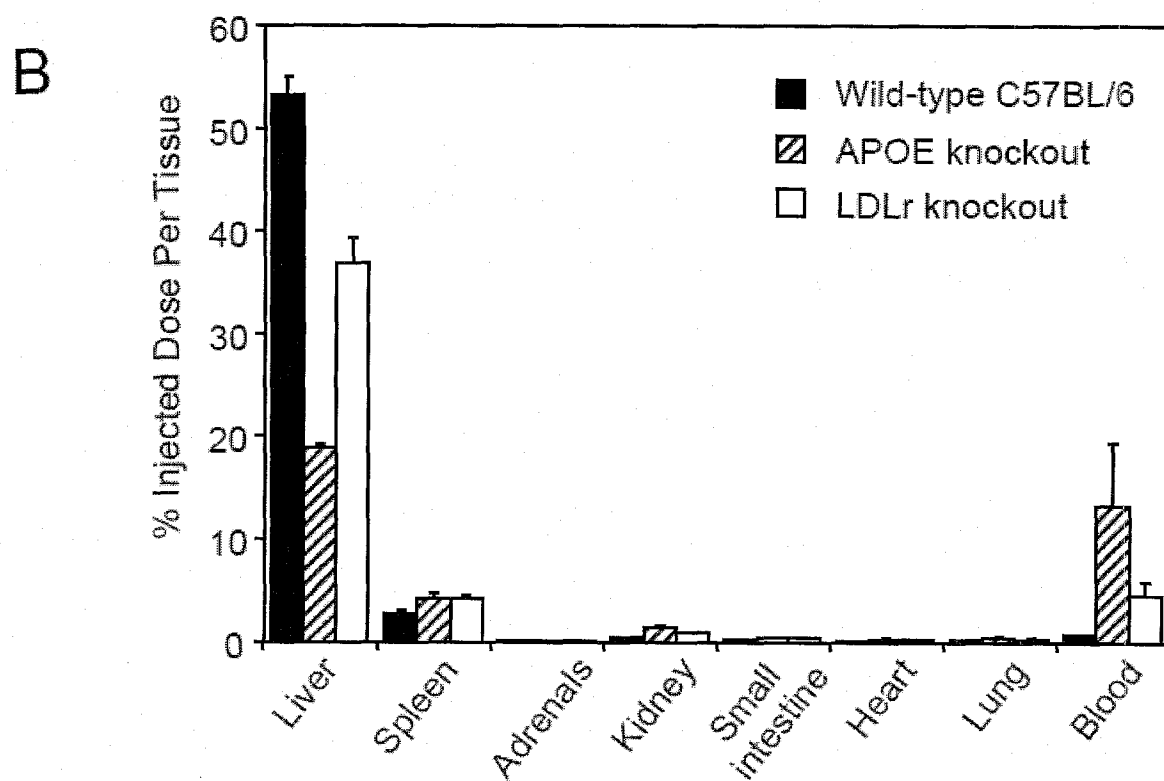
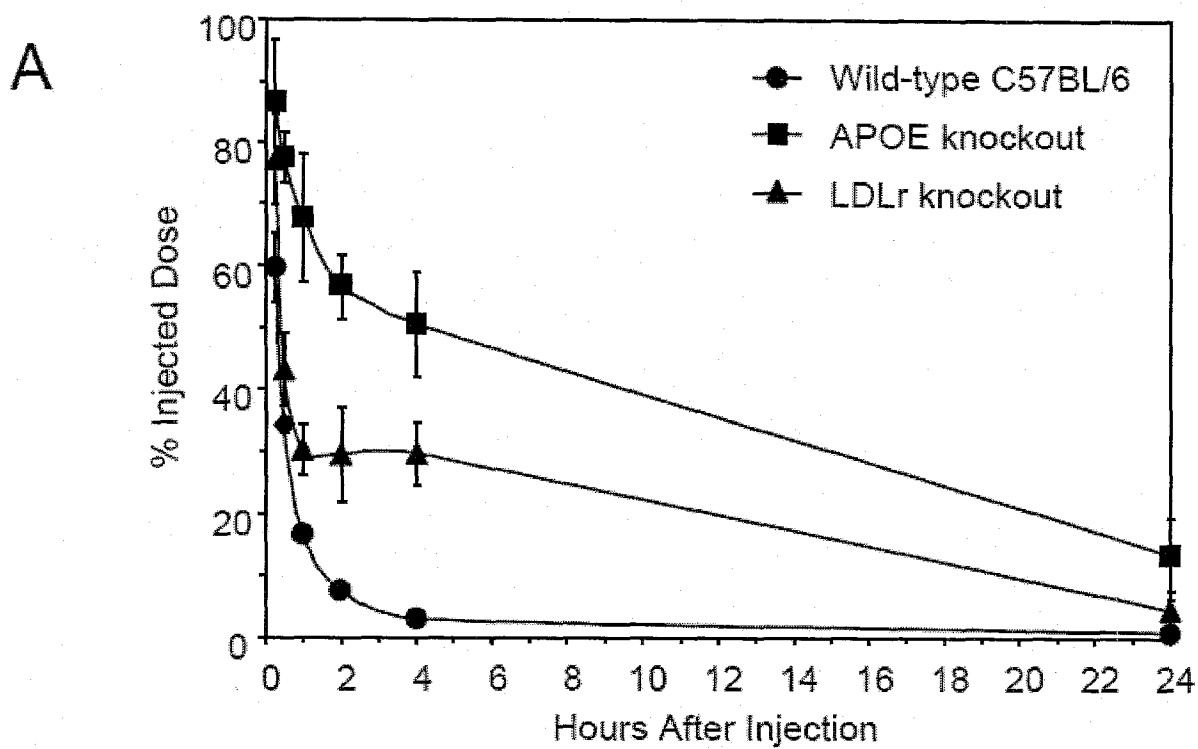


FIG. 17a-b

20/22

C

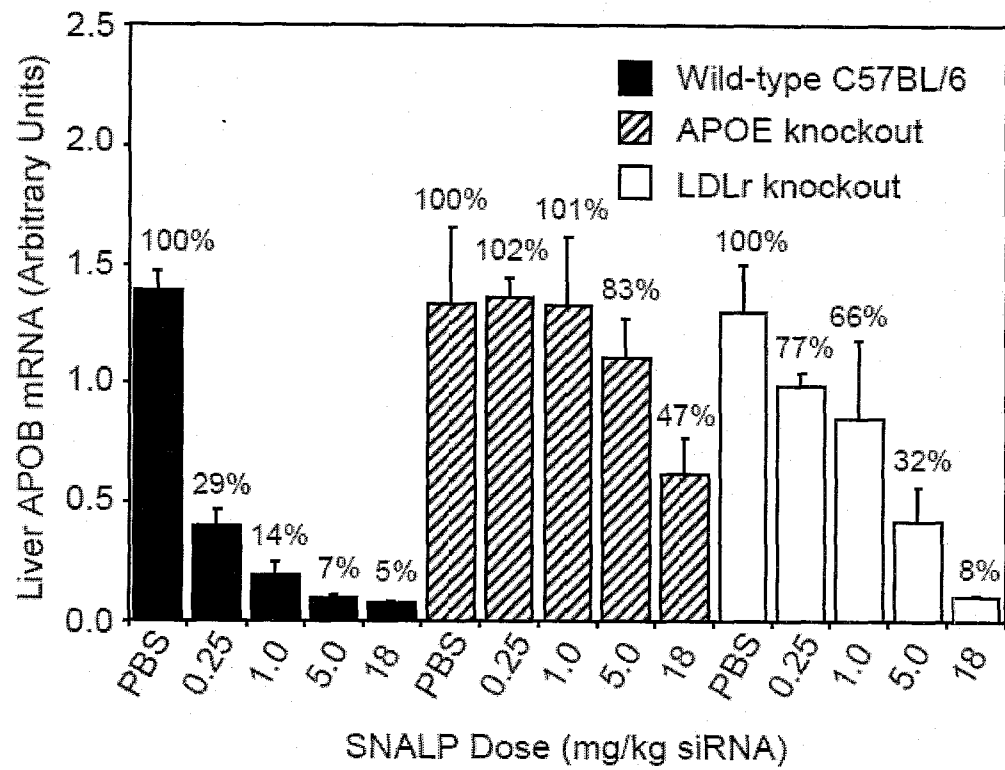


FIG. 17c

21/22

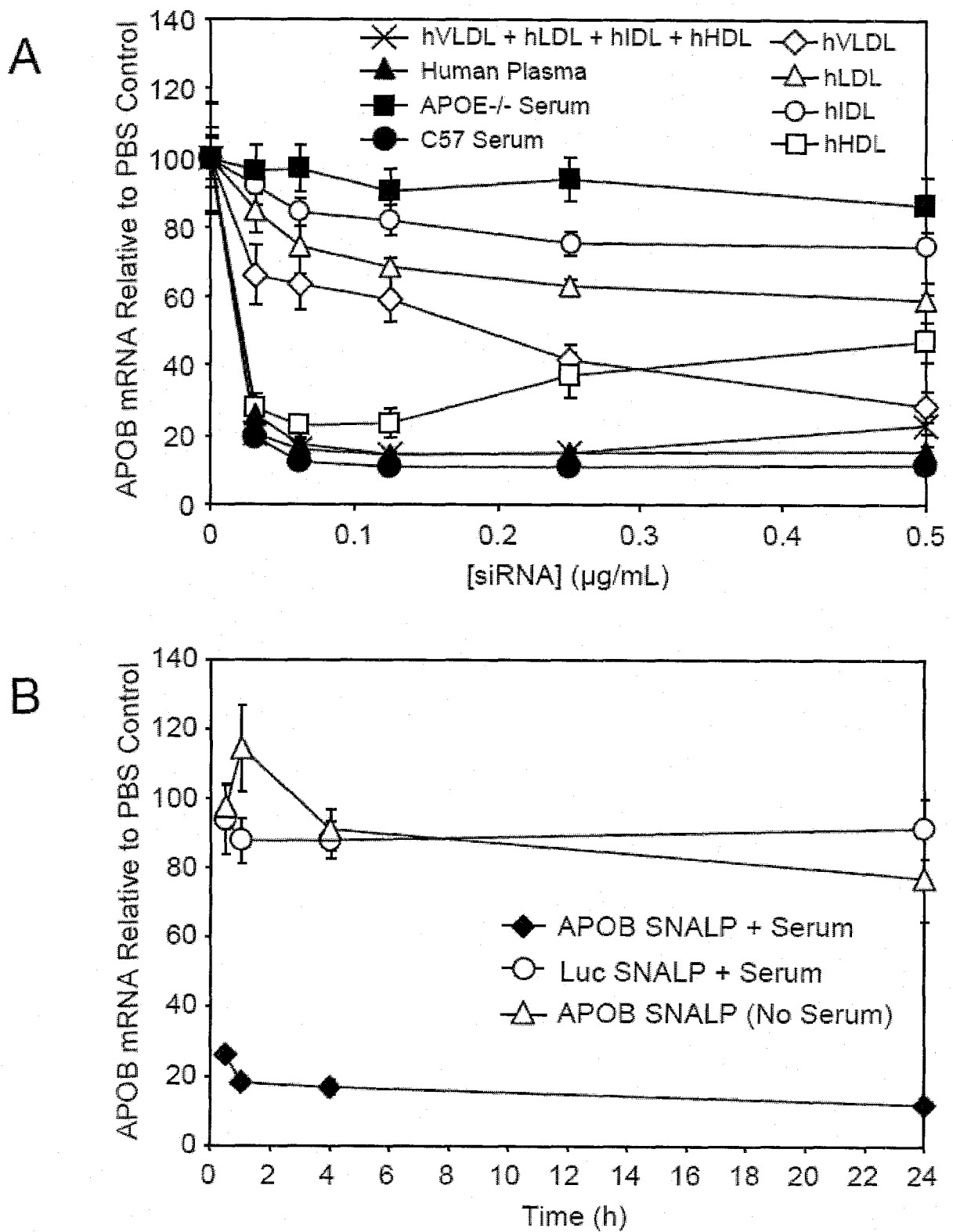


FIG. 18a-b

22/22

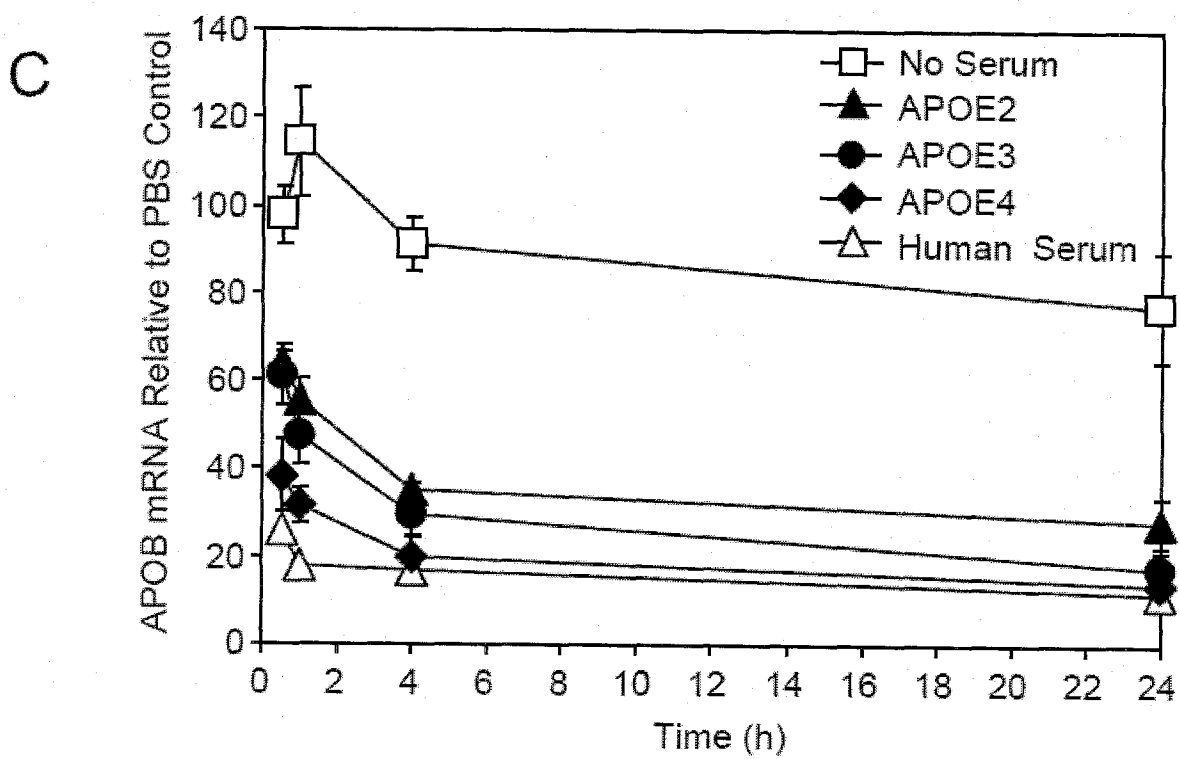


FIG. 18c

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2010/001029

A. CLASSIFICATION OF SUBJECT MATTER

IPC: *C07C 217/28* (2006.01) , *A61K 47/14* (2006.01) , *A61K 47/22* (2006.01) , *A61K 9/14* (2006.01) ,
A61K 9/48 (2006.01) , *C07D 317/28* (2006.01) (more IPCs on the last page)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: *C07C 217/28* (2006.01) , *A61K 47/14* (2006.01) , *A61K 47/22* (2006.01) , *A61K 9/14* (2006.01) ,
A61K 9/48 (2006.01) , *C07D 317/28* (2006.01) (more IPCs on the last page)

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

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
Canadian Patent Database, STN (CAS Registry, CAPLUS); search terms: cationic, lipids

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 2010/054401 A1 (MANOHARAN et al) 14 May 2010 (14-05-2010) (see the whole document, especially Table 1; page 158, compound 005-31)	7-14, 28-53
P,X	WO 2009/132131 A1 (MANOHARAN et al) 29 October 2009 (29-10-2009) (see the whole document, especially page 21, formula (I))	7, 8, 28-53
X	JAEGER et al., "Preparation and Characterization of Glycerol-Based Cleavable Surfactants and Derived Vesicles" J. Am. Chem. Soc. 1989, 111, 3001-3006 (compounds 1a, 1b, 1c, 1d)	7, 8, 12, 28

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

[X] See patent family annex.

* Special categories of cited documents :	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

19 August 2010 (19-08-2010)

Date of mailing of the international search report

12 October 2010 (12-10-2010)

Name and mailing address of the ISA/CA
Canadian Intellectual Property Office
Place du Portage I, C114 - 1st Floor, Box PCT
50 Victoria Street
Gatineau, Quebec K1A 0C9
Facsimile No.: 001-819-953-2476

Authorized officer

May Ling Nung (819) 997-2939

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2010/001029

C12N 15/113 (2010.01) , *A61K 31/713* (2006.01) , *C12N 15/88* (2006.01)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2010/001029

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WANG et al., "Preparation, Properties, and Applications of Vesicle-Forming Cleavable Surfactants with a 1,3-Dioxane Ring" Journal of Colloid and Interface Science, 1995, 173, 49-54. (compounds 4c, 4d)	7, 8, 13, 28
X	YAMAMURA et al., "Synthesis and Properties of Destructible Double-Chain Cationic Surfactants with a 1,3-Dioxolane Ring" Kagaku to Kogyo (Osaka, Japan) 1994, 68(3), 127-131. (Scheme 1, compounds 4, 5)	7, 8, 28
X	US 6,670,332 B1 (WHEELER, C J) 30 December 2003 (30-12-2003) (see the whole document)	22, 23, 28, 36, 37, 41-43, 45-53
X	US 6,649,780 B1 (EIBL et al) 18 November 2003 (18-11-2003) (see the whole document)	22, 23, 25, 28-32, 36-53
X	MOSS et al., "Dynamics of Liposomes Constructed from Phytanyl Lipids" Tetrahedron Letters, vol. 31, no. 52, pp 7559-7562. (see the whole document)	22-25, 27, 28
X	ARPICCO et al., "Synthesis, Characterization and Transfection Activity of New Saturated and Unsaturated Cationic Lipids" Farmaco 2004, 59(11), 869-878. (see the whole document, especially Scheme 1, compounds 15, 19, 23)	22, 23, 25, 28-37, 41-43, 45-53
X	GAUCHERON et al., "Transfection with Fluorinated Lipoplexes Based on Fluorinated Analogues of DOTMA, DMRIE and DPPEs" Biochimica et Biophysica Acta 1564 (2002) 349-358. (see the whole document, especially scheme 1)	22, 23, 25, 28-30, 36, 37, 41-43, 45-53
X	MOSS et al., "Relation of Surfactant Monomer Structure to Flip-Flop Dynamics in Surface-Differentiated Synthetic Bilayer Membranes" J. Am. Chem. Soc. 1990, 112, 6391-6392. (Chart I, compound 6)	22, 23, 25, 28
X	WETZER et al., "S-Layer Reconstitution at Phospholipid Monolayers" Langmuir 1998, 14, 6899-6906. (Table I, compound DPTAP)	22, 23, 25, 28
X	CAS REGISTRY (Online) STN INTERNATIONAL, COLUMBUS, OHIO, US Registry Numbers: 832075-06-6; 784136-07-8; 733738-92-6.	22, 25

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/CA2010/001029

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	WO 2009/129387 (DANDE et al) 22 October 2009 (22-10-2009) (see the whole document, especially the claims)	22-53

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/CA2010/001029**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons :

1. ☒ Claim Nos. : 46-53

because they relate to subject matter not required to be searched by this Authority, namely :

Claims 46-53 are directed to a method for treatment of the human or animal body by surgery or therapy which the International Search Authority is not required to search. However, this Authority has carried out a search based on the alleged effect or purpose/use of the product defined in said claims.

2. ☒ Claim Nos. : 7-13, 22-26, 28

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

See extra sheet

3. ☐ Claim Nos. :

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows :

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Continuation of Box II.2

Present claims 7-13, 22-26, and 28 relate to compounds defined by reference to broad terms, namely “optionally substituted” or “substituted, without definite indication of what the nature of the substituent can be. The search on these claims revealed a very large number of documents relevant to the issue of novelty of compounds of said claims, especially when the substituent is a terminal alkyl group or an oxo substituent on the carbon atom immediately next to the oxygen atoms (alkyl acyl). Some of these documents are listed in the search report, however, the list is by no means exhaustive.

Additionally, present claims 7-13 relate to an extremely large number of possible compounds. Support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, a complete search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the compounds of formula (II) with Y and Z equal to an oxygen atom.

The search on the formulation and uses of said compounds of claims 7-13, 22-26, and 28 has been limited in the same way.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2010/001029

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO2010054401A1	14-05-2010	WO2010054384A1 WO2010054405A1 WO2010054406A1	14-05-2010 14-05-2010 14-05-2010
WO2009132131A1	29-10-2009	WO2009132131A1	29-10-2009
US6670332B1	30-12-2003	CA2237316A1 EP0863749A2 JP2000502061T JP4430135B2 JP2008247906A US2004171572A1 WO9719675A2 WO9719675A3	05-06-1997 16-09-1998 22-02-2000 10-03-2010 16-10-2008 02-09-2004 05-06-1997 02-10-1997
US6649780B1	18-11-2003	US6649780B1	18-11-2003
WO2009129387A2	22-10-2009	US2009263407A1 US2009285881A1 US2010055168A1 US2010055169A1 US2010063135A1 US2010076055A1 US2010099738A1 US2010104629A1 WO2009129385A1 WO2009129387A3 WO2009129395A1 WO2010030730A1 WO2010030739A1	22-10-2009 19-11-2009 04-03-2010 04-03-2010 11-03-2010 25-03-2010 22-04-2010 29-04-2010 22-10-2009 27-05-2010 22-10-2009 18-03-2010 18-03-2010