(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
29 November 2012 (29.11.2012)

WIPO | PCT

(10) International Publication Number
WO 2012/162210 A1

(51) International Patent Classification:
A61K 45/00 (2006.01) *G01N 33/92* (2006.01)
A61K 47/44 (2006.01)

(21) International Application Number:
PCT/US2012/038755

(22) International Filing Date:
21 May 2012 (21.05.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/490,300 26 May 2011 (26.05.2011) US

(71) Applicant *(for all designated States except US)*: **MERCK SHARP & DOHME CORP.** [US/US]; 126 East Lincoln Avenue, Rahway, New Jersey 07065-0907 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **DENG, Zhengwu James** [US/US]; 770 Sumneytown Pike, West Point, Pennsylvania 19486 (US). **LIAO, Hongbiao** [US/US]; 126 East Lincoln Avenue, Rahway, New Jersey 07065-0907 (US). **STANTON, Matthew, G.** [US/US]; 126 East Lincoln Avenue, Rahway, New Jersey 07065-0907 (US).

(74) Common Representative: **MERCK SHARP & DOHME CORP.**; 126 East Lincoln Avenue, Rahway, New Jersey 07065-0907 (US).

(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, QA, RO, RS, RU, RW, 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, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, 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, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

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

[Continued on next page]

(54) Title: RING CONSTRAINED CATIONIC LIPIDS FOR OLIGONUCLEOTIDE DELIVERY

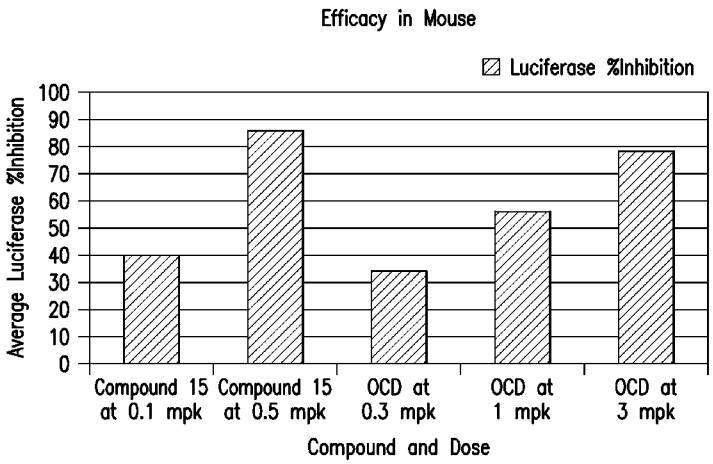


FIG.1

(57) Abstract: The instant invention provides for novel cationic lipids that can be used in combination with other lipid components such as cholesterol and PEG-lipids to form lipid nanoparticles with oligonucleotides. It is an object of the instant invention to provide a cationic lipid scaffold that demonstrates enhanced efficacy. The present invention employs ring constrained cationic lipids to enhance the efficiency of in vivodelivery of siRNA.

WO 2012/162210 A1

— *with sequence listing part of description (Rule 5.2(a))*

TITLE OF THE INVENTION

RING CONSTRAINED CATIONIC LIPIDS FOR OLIGONUCLEOTIDE DELIVERY

BACKGROUND OF THE INVENTION

The present invention relates to novel cationic lipids that can be used in combination with other lipid components such as cholesterol and PEG-lipids to form lipid nanoparticles with oligonucleotides, to facilitate the cellular uptake and endosomal escape, and to knockdown target mRNA both *in vitro* and *in vivo*.

Cationic lipids and the use of cationic lipids in lipid nanoparticles for the delivery of oligonucleotides, in particular siRNA and miRNA, have been previously disclosed. Lipid nanoparticles and use of lipid nanoparticles for the delivery of oligonucleotides, in particular siRNA and miRNA, has been previously disclosed. Oligonucleotides (including siRNA and miRNA) and the synthesis of oligonucleotides has been previously disclosed. (See US patent applications: US 2006/0083780, US 2006/0240554, US 2008/0020058, US 2009/0263407 and US 2009/0285881 and PCT patent applications: WO 2009/086558, WO2009/127060, WO2009/132131, WO2010/042877, WO2010/054384, WO2010/054401, WO2010/054405 and WO2010/054406, WO10105209). See also Semple S. C. et al., Rational design of cationic lipids for siRNA delivery, Nature Biotechnology, published online 17 January 2010; doi:10.1038/nbt.1602.

Other cationic lipids are disclosed in US patent applications: US 2009/0263407, US 2009/0285881, US 2010/0055168, US 2010/0055169, US 2010/0063135, US 2010/0076055, US 2010/0099738 and US 2010/0104629.

Traditional cationic lipids such as CLinDMA and DLinDMA have been employed for siRNA delivery to liver but suffer from non-optimal delivery efficiency along with liver toxicity at higher doses. It is an object of the instant invention to provide a cationic lipid scaffold that demonstrates enhanced efficacy. The present invention employs ring constrained cationic lipids to enhance the efficiency of in vivo delivery of siRNA.

SUMMARY OF THE INVENTION

The instant invention provides for novel cationic lipids that can be used in combination with other lipid components such as cholesterol and PEG-lipids to form lipid nanoparticles with oligonucleotides. It is an object of the instant invention to provide a cationic lipid scaffold that demonstrates enhanced efficacy. The present invention employs ring constrained cationic lipids to enhance the efficiency of in vivo delivery of siRNA.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1: LNP (Compound **15**) efficacy in mice.

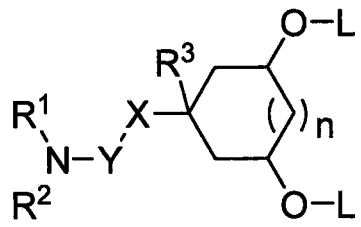
FIGURE 2: LNP (Compound **15**) efficacy in rat.

DETAILED DESCRIPTION OF THE INVENTION

The various aspects and embodiments of the invention are directed to the utility of novel cationic lipids useful in lipid nanoparticles to deliver oligonucleotides, in particular, siRNA and miRNA, to any target gene. (See US patent applications: US 2006/0083780, US 2006/0240554, US 2008/0020058, US 2009/0263407 and US 2009/0285881 and PCT patent applications: WO 2009/086558, WO2009/127060, WO2009/132131, WO2010/042877, WO2010/054384, WO2010/054401, WO2010/054405 and WO2010/054406). See also Semple S. C. et al., Rational design of cationic lipids for siRNA delivery, Nature Biotechnology, published online 17 January 2010; doi:10.1038/nbt.1602.

The cationic lipids of the instant invention are useful components in a lipid nanoparticle for the delivery of oligonucleotides, specifically siRNA and miRNA.

In a first embodiment of this invention, the cationic lipids are illustrated by the Formula A:



A

wherein:

R^1 and R^2 are independently selected from H, (C_1-C_6)alkyl, heterocycle, and polyamine, wherein said alkyl, heterocycle and polyamine are optionally substituted with one to three substituents selected from R', or R^1 and R^2 can be taken together with the nitrogen to which they are attached to form a monocyclic heterocycle with 4-7 members optionally containing, in addition to the nitrogen, one or two additional heteroatoms selected from N, O

and S, said monocyclic heterocycle is optionally substituted with one to three substituents selected from R';

R^3 is selected from H and $(C_1\text{-}C_6)$alkyl, said alkyl optionally substituted with one to three substituents selected from R';

R' is independently selected from halogen, R", OR", SR", CN, $CO_2R"$ and $CON(R")_2$;

R" is independently selected from H and $(C_1\text{-}C_6)$alkyl, wherein said alkyl is optionally substituted with halogen and OH;

n is 1, 2, 3, 4 or 5;

X is absent, O, NR", O(C=O), NR"(C=O), O(C=O)O, NR"(C=O)NR", O(C=O)NR", or NR"(C=O)O;

Y is absent or $(C_1\text{-}C_6)$alkyl; and

L is independently selected from $C_4\text{-}C_{24}$ alkyl and $C_4\text{-}C_{24}$ alkenyl, said alkyl and alkenyl are optionally substituted with one or more substituents selected from R';

or any pharmaceutically acceptable salt or stereoisomer thereof.

In a second embodiment, the invention features a compound having Formula A,

wherein:

R^1 and R^2 are each methyl;

R^3 is H;

n is 1;

X is absent;

Y is absent; and

L is independently selected from $C_4\text{-}C_{24}$ alkyl and $C_4\text{-}C_{24}$ alkenyl;

or any pharmaceutically acceptable salt or stereoisomer thereof.

In a third embodiment, the invention features a compound having Formula A, wherein:

R^1 and R^2 are each methyl;

R^3 is H;

n is 1;

X is O(C=O);

Y is methyl, ethyl or propyl; and

L is independently selected from C_4-C_{24} alkyl and C_4-C_{24} alkenyl;

or any pharmaceutically acceptable salt or stereoisomer thereof.

Specific cationic lipids are:

N,N-dimethyl-3,5-bis[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]cyclohexanamine (Compound 8);

Cis N,N-dimethyl-3,5-bis[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]cyclohexanamine (Compound 12);

3,5-bis((9Z,12Z)-octadeca-9,12-dienyloxy)cyclohexyl 4- (dimethylamino)butanoate (Compound 15);

(3*R*,5*R*)-*N*,*N*-dimethyl-3-[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yloxy]-5-(octyloxy)cyclohexanamine (Compound 18);

(3*R*,5*R*)-*N*,*N*-dimethyl-3-[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yloxy]-5-(octyloxy)cyclohexanamine (Compound 19);

(3*R*,5*S*)-*N*,*N*-dimethyl-3-[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yloxy]-5-(octyloxy)cyclohexanamine (Compound 20);

(3*S*,5*R*)-3-(decyloxy)-*N*,*N*-dimethyl-5-[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yloxy]cyclohexanamine (Compound 21); and

(3*R*,5*S*)-*N*,*N*-dimethyl-3-[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yloxy]-5-(octyloxy)cyclohexanamine (Compound 22);

or any pharmaceutically acceptable salt or stereoisomer thereof.

In another embodiment, the cationic lipids disclosed are useful in the preparation of lipid nanoparticles.

In another embodiment, the cationic lipids disclosed are useful components in a lipid nanoparticle for the delivery of oligonucleotides.

In another embodiment, the cationic lipids disclosed are useful components in a lipid nanoparticle for the delivery of siRNA and miRNA.

In another embodiment, the cationic lipids disclosed are useful components in a lipid nanoparticle for the delivery of siRNA.

The cationic lipids of the present invention may have asymmetric centers, chiral axes, and chiral planes (as described in: E.L. Eliel and S.H. Wilen, Stereochemistry of Carbon Compounds, John Wiley & Sons, New York, 1994, pages 1119-1190), and occur as racemates, racemic mixtures, and as individual diastereomers, with all possible isomers and mixtures thereof, including optical isomers, being included in the present invention. In addition, the cationic lipids disclosed herein may exist as tautomers and both tautomeric forms

are intended to be encompassed by the scope of the invention, even though only one tautomeric structure is depicted.

It is understood that substituents and substitution patterns on the cationic lipids of the instant invention can be selected by one of ordinary skill in the art to provide cationic lipids that are chemically stable and that can be readily synthesized by techniques known in the art, as well as those methods set forth below, from readily available starting materials. If a substituent is itself substituted with more than one group, it is understood that these multiple groups may be on the same carbon or on different carbons, so long as a stable structure results.

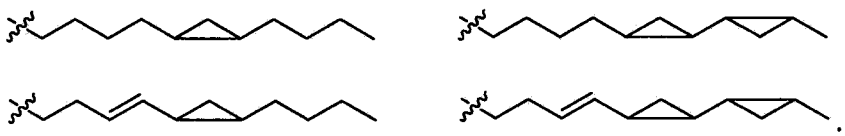
It is understood that one or more Si atoms can be incorporated into the cationic lipids of the instant invention by one of ordinary skill in the art to provide cationic lipids that are chemically stable and that can be readily synthesized by techniques known in the art from readily available starting materials.

In the compounds of Formula A, the atoms may exhibit their natural isotopic abundances, or one or more of the atoms may be artificially enriched in a particular isotope having the same atomic number, but an atomic mass or mass number different from the atomic mass or mass number predominantly found in nature. The present invention is meant to include all suitable isotopic variations of the compounds of Formula A. For example, different isotopic forms of hydrogen (H) include protium (^{1}H) and deuterium (^{2}H). Protium is the predominant hydrogen isotope found in nature. Enriching for deuterium may afford certain therapeutic advantages, such as increasing *in vivo* half-life or reducing dosage requirements, or may provide a compound useful as a standard for characterization of biological samples. Isotopically-enriched compounds within Formula A can be prepared without undue experimentation by conventional techniques well known to those skilled in the art or by processes analogous to those described in the Scheme and Examples herein using appropriate isotopically-enriched reagents and/or intermediates.

As used herein, "alkyl" means a straight chain, cyclic or branched saturated aliphatic hydrocarbon having the specified number of carbon atoms.

As used herein, "alkenyl" means a straight chain, cyclic or branched unsaturated aliphatic hydrocarbon having the specified number of carbon atoms including but not limited to diene, triene and tetraene unsaturated aliphatic hydrocarbons.

Examples of a cyclic "alkyl" or "alkenyl are:



As used herein, "heterocyclyl" or "heterocycle" means a 4- to 10-membered aromatic or nonaromatic heterocycle containing from 1 to 4 heteroatoms selected from the group consisting of O, N and S, and includes bicyclic groups. "Heterocyclyl" therefore includes, the following: benzoimidazolyl, benzofuranyl, benzofurazanyl, benzopyrazolyl, benzotriazolyl, benzothiophenyl, benzoxazolyl, carbazolyl, carbolinyl, cinnolinyl, furanyl, imidazolyl, indolinyl, indolyl, indolazinyl, indazolyl, isobenzofuranyl, isoindolyl, isoquinolyl, isothiazolyl, isoxazolyl, naphthpyridinyl, oxadiazolyl, oxazolyl, oxazoline, isoxazoline, oxetanyl, pyranyl, pyrazinyl, pyrazolyl, pyridazinyl, pyridopyridinyl, pyridazinyl, pyridyl, pyrimidyl, pyrrolyl, quinazolinyl, quinolyl, quinoxalinyl, tetrahydropyranyl, tetrazolyl, tetrazolopyridyl, thiadiazolyl, thiazolyl, thienyl, triazolyl, azetidinyl, 1,4-dioxanyl, hexahydroazepinyl, piperazinyl, piperidinyl, pyrrolidinyl, morpholinyl, thiomorpholinyl, dihydrobenzoimidazolyl, dihydrobenzofuranyl, dihydrobenzothiophenyl, dihydrobenzoxazolyl, dihydrofuranyl, dihydroimidazolyl, dihydroindolyl, dihydroisooxazolyl, dihydroisothiazolyl, dihydrooxadiazolyl, dihydrooxazolyl, dihydropyrazinyl, dihydropyrazolyl, dihydropyridinyl, dihydropyrimidinyl, dihydropyrrolyl, dihydroquinolinyl, dihydrotetrazolyl, dihydrothiadiazolyl, dihydrothiazolyl, dihydrothienyl, dihydrotriazolyl, dihydroazetidinyl, methylenedioxybenzoyl, tetrahydrofuranyl, and tetrahydrothienyl, and N-oxides thereof all of which are optionally substituted with one to three substituents selected from R".

As used herein, "polyamine" means compounds having two or more amino groups. Examples include putrescine, cadaverine, spermidine, and spermine.

As used herein, "halogen" means Br, Cl, F and I.

In an embodiment of Formula A, R^1 and R^2 are independently selected from H and $(C_1\text{-}C_6)$alkyl, wherein said alkyl is optionally substituted with one to three substituents selected from R', or R^1 and R^2 can be taken together with the nitrogen to which they are attached to form a monocyclic heterocycle with 4-7 members optionally containing, in addition to the nitrogen, one or two additional heteroatoms selected from N, O and S, said monocyclic heterocycle is optionally substituted with one to three substituents selected from R'.

In an embodiment of Formula A, R^1 and R^2 are independently selected from H, methyl, ethyl and propyl, wherein said methyl, ethyl and propyl are optionally substituted with one to three substituents selected from R', or R^1 and R^2 can be taken together with the nitrogen to which they are attached to form a monocyclic heterocycle with 4-7 members optionally containing, in addition to the nitrogen, one or two additional heteroatoms selected from N, O and S, said monocyclic heterocycle is optionally substituted with one to three substituents selected from R'.

In an embodiment of Formula A, R^1 and R^2 are independently selected from H, methyl, ethyl and propyl.

In an embodiment of Formula A, R^1 and R^2 are each methyl.

In an embodiment of Formula A, R^3 is selected from: H and methyl.

In an embodiment of Formula A, R^3 is H.

In an embodiment of Formula A, R' is R".

In an embodiment of Formula A, R" is independently selected from H, methyl, ethyl and propyl, wherein said methyl, ethyl and propyl are optionally substituted with one or more halogen and OH.

In an embodiment of Formula A, R" is independently selected from H, methyl, ethyl and propyl.

In an embodiment of Formula A, n is 1, 2, 3 or 4.

In an embodiment of Formula A, n is 1.

In an embodiment of Formula A, X is absent, O, NR", O(C=O), NR"(C=O), O(C=O)O, NR"(C=O)NR", O(C=O)NR", or NR"(C=O)O.

In an embodiment of Formula A, X is absent, or O(C=O).

In an embodiment of Formula A, X is absent.

In an embodiment of Formula A, X is O(C=O).

In an embodiment of Formula A, Y is absent, methyl, ethyl or propyl.

In an embodiment of Formula A, Y is absent, or propyl.

In an embodiment of Formula A, Y is absent.

In an embodiment of Formula A, L is independently selected from C_4-C_{24} alkyl and C_4-C_{24} alkenyl, which are optionally substituted with halogen and OH.

In an embodiment of Formula A, L is independently selected from C_4-C_{24} alkyl and C_4-C_{24} alkenyl.

In an embodiment of Formula A, L is independently selected from C_4-C_{24} alkenyl.

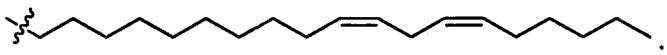
In an embodiment of Formula A, L is independently selected from C_{12}-C_{24} alkenyl.

In an embodiment of Formula A, when L is selected from an alkenyl, L is C_{12}-C_{24}, or C_{14}-C_{20}, or C_{16}-C_{20}.

In an embodiment of Formula A, when L is selected from an alkyl, L is C_{12}-C_{24}, or C_4-C_{12}, or C_8-C_{10}.

In an embodiment of Formula A, L is C_{18} alkenyl.

In an embodiment of Formula A, L is:



In an embodiment of Formula A, "heterocyclyl" is pyrolidine, piperidine, morpholine, imidazole or piperazine.

In an embodiment of Formula A, "monocyclic heterocyclyl" is pyrolidine, piperidine, morpholine, imidazole or piperazine.

In an embodiment of Formula A, "polyamine" is putrescine, cadaverine, spermidine or spermine.

In an embodiment, "alkyl" is a straight chain saturated aliphatic hydrocarbon having the specified number of carbon atoms.

In an embodiment, "alkenyl" is a straight chain unsaturated aliphatic hydrocarbon having the specified number of carbon atoms.

Included in the instant invention is the free form of cationic lipids of Formula A, as well as the pharmaceutically acceptable salts and stereoisomers thereof. Some of the isolated specific cationic lipids exemplified herein are the protonated salts of amine cationic lipids. The term "free form" refers to the amine cationic lipids in non-salt form. The encompassed pharmaceutically acceptable salts not only include the isolated salts exemplified for the specific cationic lipids described herein, but also all the typical pharmaceutically acceptable salts of the free form of cationic lipids of Formula A. The free form of the specific salt cationic lipids described may be isolated using techniques known in the art. For example,

the free form may be regenerated by treating the salt with a suitable dilute aqueous base solution such as dilute aqueous NaOH, potassium carbonate, ammonia and sodium bicarbonate. The free forms may differ from their respective salt forms somewhat in certain physical properties, such as solubility in polar solvents, but the acid and base salts are otherwise pharmaceutically equivalent to their respective free forms for purposes of the invention.

The pharmaceutically acceptable salts of the instant cationic lipids can be synthesized from the cationic lipids of this invention which contain a basic or acidic moiety by conventional chemical methods. Generally, the salts of the basic cationic lipids are prepared either by ion exchange chromatography or by reacting the free base with stoichiometric amounts or with an excess of the desired salt-forming inorganic or organic acid in a suitable solvent or various combinations of solvents. Similarly, the salts of the acidic compounds are formed by reactions with the appropriate inorganic or organic base.

Thus, pharmaceutically acceptable salts of the cationic lipids of this invention include the conventional non-toxic salts of the cationic lipids of this invention as formed by reacting a basic instant cationic lipids with an inorganic or organic acid. For example, conventional non-toxic salts include those derived from inorganic acids such as hydrochloric, hydrobromic, sulfuric, sulfamic, phosphoric, nitric and the like, as well as salts prepared from organic acids such as acetic, propionic, succinic, glycolic, stearic, lactic, malic, tartaric, citric, ascorbic, pamoic, maleic, hydroxymaleic, phenylacetic, glutamic, benzoic, salicylic, sulfanilic, 2-acetoxy-benzoic, fumaric, toluenesulfonic, methanesulfonic, ethane disulfonic, oxalic, isethionic, trifluoroacetic (TFA) and the like.

When the cationic lipids of the present invention are acidic, suitable "pharmaceutically acceptable salts" refers to salts prepared form pharmaceutically acceptable non-toxic bases including inorganic bases and organic bases. Salts derived from inorganic bases include aluminum, ammonium, calcium, copper, ferric, ferrous, lithium, magnesium, manganic salts, manganous, potassium, sodium, zinc and the like. Particularly preferred are the ammonium, calcium, magnesium, potassium and sodium salts. Salts derived from pharmaceutically acceptable organic non-toxic bases include salts of primary, secondary and tertiary amines, substituted amines including naturally occurring substituted amines, cyclic amines and basic ion exchange resins, such as arginine, betaine caffeine, choline, N,N^1-dibenzylethylenediamine, diethylamin, 2-diethylaminoethanol, 2-dimethylaminoethanol, ethanolamine, ethylenediamine, N-ethylmorpholine, N-ethylpiperidine, glucamine, glucosamine, histidine, hydrabamine, isopropylamine, lysine, methylglucamine, morpholine,

piperazine, piperidine, polyamine resins, procaine, purines, theobromine, triethylamine, trimethylamine tripropylamine, tromethamine and the like.

The preparation of the pharmaceutically acceptable salts described above and other typical pharmaceutically acceptable salts is more fully described by Berg *et al.*, "Pharmaceutical Salts," *J. Pharm. Sci.,* 1977:66:1-19.

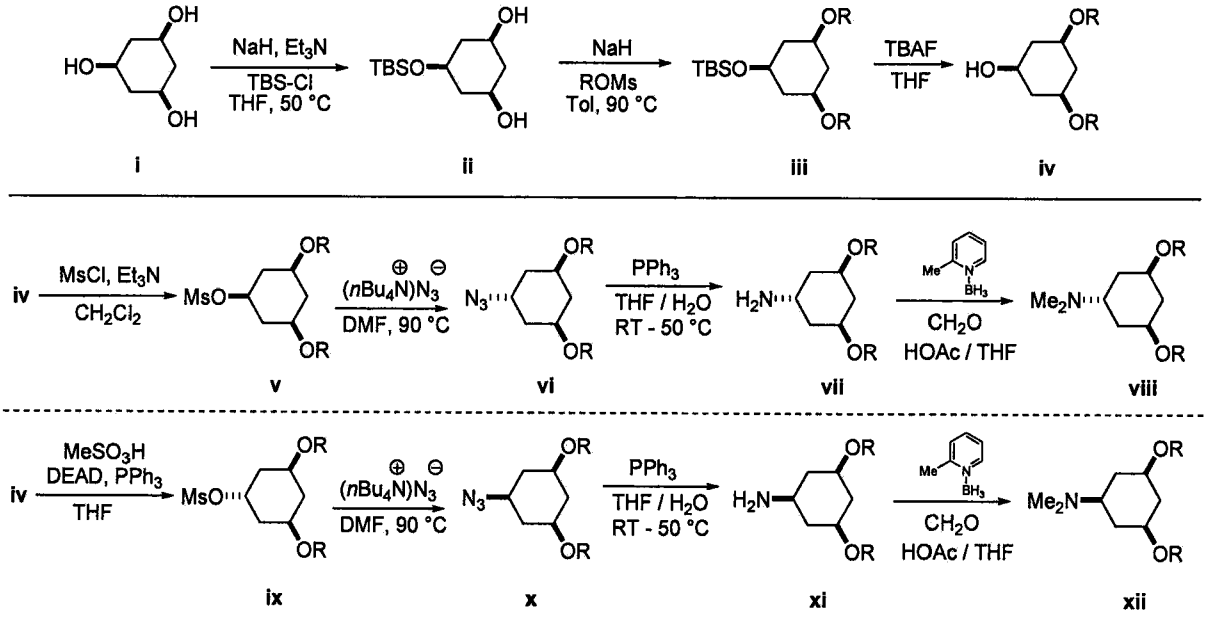
It will also be noted that the cationic lipids of the present invention are potentially internal salts or zwitterions, since under physiological conditions a deprotonated acidic moiety in the compound, such as a carboxyl group, may be anionic, and this electronic charge might then be balanced off internally against the cationic charge of a protonated or alkylated basic moiety, such as a quaternary nitrogen atom.

EXAMPLES

Examples provided are intended to assist in a further understanding of the invention. Particular materials employed, species and conditions are intended to be further illustrative of the invention and not limitative of the reasonable scope thereof. The reagents utilized in synthesizing the cationic lipids are either commercially available or are readily prepared by one of ordinary skill in the art.

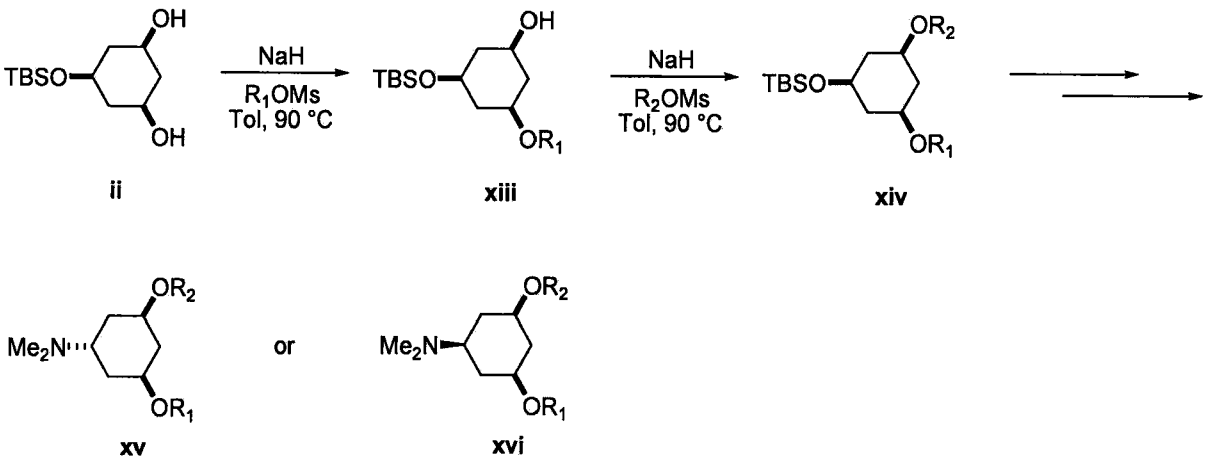
Synthesis of the novel cationic lipids bearing an amine on the cyclohexane is a linear process starting from 1,3,5-cyclohexanetriol **i** (illustrated in General Scheme 1, shown below). TBS protection of one of the alcohols furnishes intermediate **ii** and subsequent alkylation furnishes di-ether silyl protected intermediate, such as **iii**. TBAF induced desilylation affords hydroxyl di-ether intermediate **iv**. This intermediate represents a point of stereo-divergence and can be taken forward to the final targets following one of two similar routes (above and below dashed line). Thus **iv** undergoes stereo-retentive mesylation by treatment with MsCl to furnish **v**. Stereo-inverted azide **vi** is formed using (nBu$_4$N)N$_3$ in DMF. Reduction to the primary amine **vii** is accomplished with PPh_3. Finally the desired target **viii** is furnished by a reductive alkylation employing formaldehyde and 2-picoline borane as a reductant. The sequence leading to target **viii** is illustrated below in the scheme above the dashed line. Below the dashed line is depicted a slightly modified route to compound **xii**, which is isomeric to compound **viii**. Thus **vi** undergoes a stereo-invertive mesylation by employing $MeSO_3H$, DEAD and PPh_3 to afford intermediate **ix**. This mesylate can be used to furnish intermediates **x**, **xi**, and final target **xii** in a manner described above.

GENERAL SCHEME 1



Synthesis of asymmetric cyclohexyl based cationic lipids where R_1 does not equal R_2 is outlined in general scheme 2. Iterative alkylations of intermediate **ii** generates the asymmetric intermediate **xiv** through the mono-alkylated intermediate **xiii**. Intermediate **xiv** is carried on to asymmetric cationic lipids **xv** and **xvi** as described for general scheme 1 above.

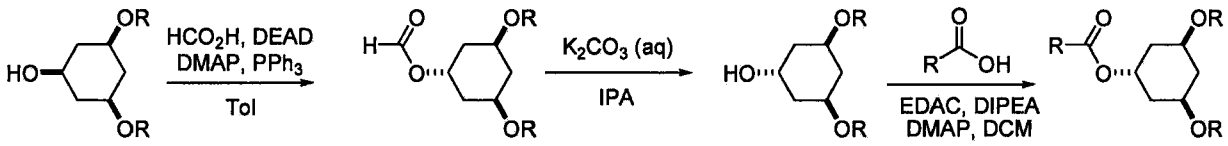
GENERAL SCHEME 2



Cationic lipids bearing a linker or spacer between the cyclohexane and a basic amine can be synthesized using a slightly modified route shown in General Scheme 3.

Mitsunobu inversion of the alcohol in **iv** with formic acid, followed by deprotection gives alcohol **xviii**. Esterification generates final compounds of type **xix**.

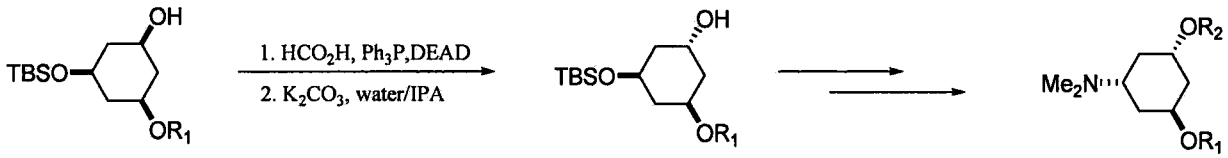
GENERAL SCHEME 3



iv **xvii** **xviii** **xix**

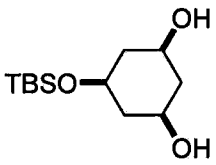
Cationic lipids of type **xxi** were prepared by inversion of the alcohol in intermediate **xiii** via Mitsunobu chemistry. Conversion to final products **xxi** was accomplished as described in General Schemes 1 and 2.

GENERAL SCHEME 4



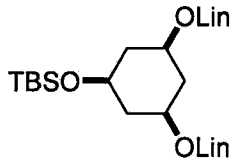
xiii **xx** **xxi**

5-{[*tert*-butyl(dimethyl)silyl]oxy}cyclohexane-1,3-diol (2)



To 80 ml THF solution of cyclohexane-1,3,5-triol (1.96 g, 14.8 mmol) was added triethylamine (2.27 mL, 98 mmol) and TBS-Cl (2.45 g, 16.3 mmol) at 20 °C. After which, sodium hydride (0.59 g, 14.8 mmol) was added in and the reaction was stirred at 45 °C for 16 hours. The reaction was cooled to 10 °C and filtrated. The solution was concentrated to white solid, which was washed by 50 mL of hexane twice to give title compound (3.6 g). ^{1}H NMR δ (ppm)($CDCl_3$): 3.77-3.85 (3 H, m), 2.10-2.02 (5 H, m), 1.60-1.53 (3 H, m), 0.89 (9 H, s), 0.085 (6 H, s).

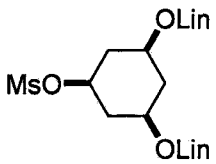
3,5-bis[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]cyclohexanol (4)



To 60 ml toluene solution of 5-{[tert-butyl(dimethyl)silyl]oxy}cyclohexane-1,3-diol **(2)** (1.50 g, 6.09 mmol) was added Linoleyl methane sulfonate (6.29 g, 18.3 mmol) and 60% sodium hydride (0.97 g, 24.4 mmol). The reaction was stirred at 100 °C for 24 hours. After cooled to ambient temperature, the reaction was diluted with 300 mL dichloromethane and washed by 2 x 100 mL of saturated NH_4Cl solution, $NaHCO_3$ solution, brine, respectively. The organic was dried over Na_2SO_4, filtrated and purified by silica gel chromatography (0% ethyl acetate / hexane → 15% ethyl acetate / hexane) to give (3,5-bis[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]cyclohexyloxy)(tert-butyl)dimethylsilane (**3**) (4.4 g).

To 35 ml THF solution of (3,5-bis[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]cyclohexyloxy)(tert-butyl)dimethylsilane (**3**) (2.3 g, 3.09 mmol) was added 1M tetrabutylammonium fluoride solution in THF (6.19 mL, 6.19 mmol). The reaction was stirred at ambient temperature for 4 hours, after which solvent was evaporated. The residue was redissolved in 200 mL dichloromethane and washed by 2 x 100 mL of saturated NH_4Cl solution, $NaHCO_3$ solution, brine, respectively. The organic was dried over Na_2SO_4, filtrated and purified by silica gel chromatography (0% ethyl acetate / hexane → 50% ethyl acetate / hexane) to give the title compound (**4**) (1.2 g). ^1H NMR δ (ppm)($CDCl_3$): 5.42-5.30 (8 H, m), 3.64-3.60 (1 H, m), 3.46-3.42 (4 H, m), 3.29-3.22 (2 H, m), 2.79-2.76 (4 H, m), 2.33-2.26 (3 H, m), 2.08-2.02 (8 H, m), 1.58-1.52 (6 H, m), 1.39-1.25 (34 H, m), 0.91-0.87 (6 H, m).

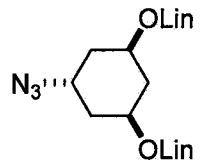
***Cis* 3,5-bis[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]cyclohexyl methanesulfonate (5)**



To 100 ml dichloromethane solution of 3,5-bis[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]cyclohexanol (4) (7.5 g, 11.9 mmol) at 0 °C was added methane sulfonyl chloride (1.86 mL, 23.9 mmol) and triethyl amine (3.66 mL, 26.2 mmol). The reaction was stirred from 0

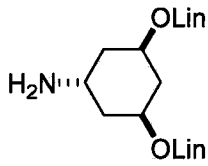
°C to ambient temperature for 16 hours. After which, the reaction was diluted with 150 mL of dichloromethane, and washed by 2 x 100 mL of $NaHCO_3$ solution, water and brine, respectively. The organic was dried over Na_2SO_4 and filtrated. After evaporation of solvent, the residue was purified by silica gel chromatography (1% ethyl acetate / hexane → 45% ethyl acetate / hexane) to give the title compound (7.5 g). MS 707.9 (M+1).

1-azido-3,5-bis[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]cyclohexane (6)



To 3 ml DMF solution of all *Cis* 3,5-bis[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]cyclohexyl methanesulfonate (5) (1.3 g, 1.84 mmol) was added tetrabutylammonium azide (1.56 g, 5.52 mmol). The reaction was stirred at 90 °C for 16 hours. The reaction was diluted with 100 mL dichloromethane and washed by 50 mL of $NaHCO_3$ solution, water, brine. The organic was dried over Na_2SO_4, filtrated and purified by silica gel chromatography (0 % ethyl acetate/ hexane → 20 % ethyl acetate / hexane) to give title compound (1.1 g). HRMS 656.6333 (M+1).

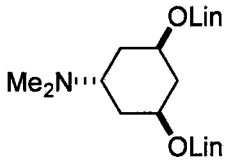
3,5-bis[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]cyclohexanamine (7)



To 100 ml THF solution of 1-azido-3,5-bis[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]cyclohexane (6) (6.5 g, 9.94 mmol) was added triphenyl phosphine (5.21 g, 19.9 mmol). After stirred at 20 °C for 3 hours, 3 mL of water was added into reaction. The reaction was further stirred at 50 °C for 16 hours. The reaction was concentrated and the residue was dissolved with 200 mL dichloromethane and washed by 100 mL of $NaHCO_3$ solution, water, brine. The organic was dried over Na_2SO_4, filtrated and purified by silica gel chromatography (0 % → 15 % methanol / dichloromethane) to give title compound (4.4 g). HRMS 628.6035 (M+1). ^1H NMR δ (ppm)($CDCl_3$): 5.41-5.30 (8 H, m), 3.69-3.63 (2 H, m), 3.53-3.39 (5 H,

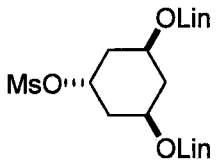
m), 2.79-2.76 (4 H, m), 2.41-2.38 (2 H, m), 2.07-2.02 (8 H, m), 1.88-1.85 (2 H, m), 1.56-1.52 (4 H, m), 1.45-1.20 (36 H, m), 0.93-0.88 (6 H, m).

N,N-dimethyl-3,5-bis[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]cyclohexanamine (8)



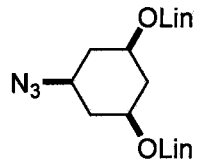
To 50 ml solution acetic acid / THF 1:4 of 3,5-bis[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]cyclohexanamine (7) (2.70 g, 4.30 mmol) was added 37% formaldehyde solution (3.20 mL, 43.0 mmol) followed by 2-picoline borane (1.52 g, 14.2 mmol). After 5 hours at ambient temperature, the reaction was diluted with 300 mL dichloromethane and washed by 2 x 100 mL of $NaHCO_3$ solution, water, brine, respectively. The organic was dried over Na_2SO_4, filtrated and purified by silica gel chromatography (0 % → 12 % methanol / dichloromethane) to give title compound (8) (2.54 g). HRMS 656.6339 (M+1). ^1H NMR δ (ppm)($CDCl_3$): 5.39-5.30 (8 H, m), 3.59-3.54 (2 H, m), 3.49-3.39 (5 H, m), 2.79-2.76 (4 H, m), 2.48-2.32 (2 H, m), 2.24-2.19 (8H, m), 2.06-2.02 (8 H, m), 1.56-1.51 (4 H, m), 1.38-1.19 (34 H, m), 0.91-0.87 (6 H, m).

3,5-bis[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]cyclohexyl methanesulfonate (9)



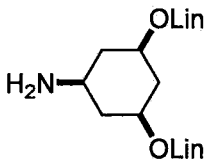
To 50 ml THF solution of 3,5-bis[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]cyclohexanol (4) (2.0 g, 3.18 mmol) at 0 °C was added triphenyl phosphine (2.50 g, 9.54 mmol), followed by DMAP (1.03 g, 8.43 mmol). Methanesulfonic acid (0.43 mL, 6.68 mmol) and DEAD (4.57 g, 10.5 mmol) was added into the reaction slowly in sequence at 0 °C. The reaction was stirred from 0 °C to ambient temperature for 24 hours. After which, the reaction was filtrated. The solution was concentrated and diluted with 150 mL of hexane. The hexane solution was filtrated and washed by 2 x 100 mL of $NaHCO_3$ solution, water and brine, respectively. The organic was dried over Na_2SO_4 and filtrated. Evaporation of solvent gave the title compound (2.22 g). MS 729.8 (M+Na).

Cis 1-azido-3,5-bis[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]cyclohexane (10)



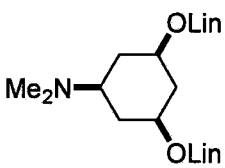
To 4 ml DMF solution of 3,5-bis[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]cyclohexyl methanesulfonate (9) (2.22 g, 3.14 mmol) was added tetrabutylammonium azide (2.68 g, 9.42 mmol). The reaction was stirred at 90 °C for 3 hours. The reaction was diluted with 100 mL dichloromethane and washed by 50 mL of $NaHCO_3$ solution, water, brine. The organic was dried over Na_2SO_4, filtrated and purified by silica gel chromatography (0 % ethyl acetate/ hexane → 25 % ethyl acetate / hexane) to give title compound (10) (1.92 g). MS 676.8 (M+Na).

Cis 3,5-bis[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]cyclohexanamine (11)



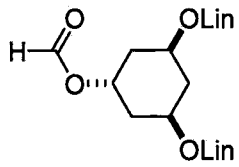
To 100 ml THF solution of all *Cis* 1-azido-3,5-bis[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]cyclohexane (10) (3.1 g, 4.74 mmol) was added triphenyl phosphine (2.49 g, 9.48 mmol). After stirred at 20 °C for 5 hours, 3 mL of water was added into reaction. The reaction was further stirred at 50 °C for 16 hours. The reaction was concentrated and the residue was dissolved with 200 mL hexane and washed by 100 mL of $NaHCO_3$ solution, water, brine. The organic was dried over Na_2SO_4, filtrated and concentrated to dryness to give title compound (11) (2.7 g). MS 629.8 (M+1).

Cis N,N-dimethyl-3,5-bis[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]cyclohexanamine (12)



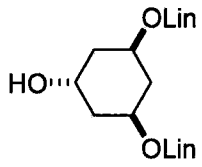
To 50 ml solution acetic acid / THF 1:9 of all *Cis* 3,5-bis[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]cyclohexanamine (11) (2.10 g, 3.34 mmol) was added 37% formaldehyde solution (2.49 mL, 33.4 mmol) followed by 2-picoline borane (1.07 g, 10.0 mmol). After 16 hours at ambient temperature, the reaction was diluted with 300 mL hexane and washed by 2 x 100 mL of $NaHCO_3$ solution, water, brine, respectively. The organic was dried over Na_2SO_4, filtrated and purified by silica gel chromatography (0 % → 12 % methanol / dichloromethane) to give title compound (12) (1.81 g). HRMS 656.6344 (M+1). ^{1}H NMR δ (ppm)($CDCl_3$): 5.39-5.30 (8 H, m), 3.47-3.43 (4 H, m), 3.24-3.19 (2 H, m), 2.79-2.75 (4 H, m), 2.43-2.40 (1 H, m), 2.29 (6H, s), 2.15-2.12 (2H, m), 2.07-2.02 (8 H, m), 1.57-1.54 (8 H, m), 1.39-1.29 (32 H, m), 0.90-0.87 (6 H, m).

3,5-bis((9Z,12Z)-octadeca-9,12-dienyloxy)cyclohexyl formate (13)



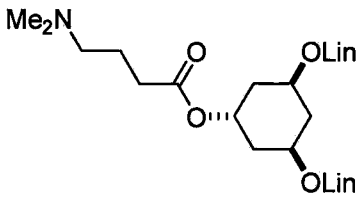
In a 500mL RBF, 3,5-bis((9Z,12Z)-octadeca-9,12-dienyloxy)cyclohexanol (4) (6.3 g, 10.0 mmol) in THF (50 mL) was added triphenylphosphine (5.25g, 20.0 mmol). After cooling in ice-water bath, formic acid (0.76 mL, 20.0 mmol) was added. Diethyl azodicarboxylate 40% solution in toluene (7.3 mL, 16.0 mmol) was charged via syringe pump over 45 minutes. The mixture was allowed to warm to room temperature and stirred overnight. The mixture was concentrated down to about 20 mL and diluted with MTBE (50 mL). The precipitated solid was filtered out and the filtrate was concentrated down to oil. The crude product (oil) was purified by silica gel chromatography (15% MTBE in Hexane) to obtain 5.9 g of **13** as clear oil (90% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.29 (s, 1H), 5.33-5.45 (m, 9H), 3.41-3.57 (m , 6H), 2.79-2.81 (t, J = 6.6 Hz, 4H), 2.51-2.53 (m, 1H), 2.22-2.25 (m, 1H), 2.06-2.10 (dd, *J* = 13.5, 6.5 Hz, 8H), 1.54-1.58 (m, 3H), 1.32-1.40 (m, 36H), 0.90-0.93 (t, *J* = 3.4 Hz, 3H).

3,5-bis((9Z,12Z)-octadeca-9,12-dienyloxy)cyclohexanol (14)



In a 3N 250mL RBF equipped with overhead agitator, to 3,5-bis((9Z,12Z)-octadeca-9,12-dienyloxy)cyclohexyl formate (**13**) (5.9 g, 9.0 mmol) in IPA (40 mL) was added 15% potassium carbonate solution (45 mL). The mixture was agitated at 50 °C for 24 hours. The mixture was then concentrated down under vacuum to about 60 mL and extracted with MTBE (100 mL). The organic layer was washed with water (100 mL) and dried over Na_2SO_4. After filtration and concentration, the crude product was purified by silica gel chromatography (30% MTBE in Hexane) to obtain 5.0 g of **14** as clear oil (89% yield). ^1H NMR (400 MHz, $CDCl_3$) δ 5.30-5.41 (m, 8H), 4.32-4.33 (s, 1H), 3.59-3.67 (m, 2H), 3.40-3.52 (m, 4H), 2.76-2.79 (t, J = 6.5 Hz, 4H), 2.46-2.49 (m, 1H), 2.02-2.12 (m, 10H), 1.51-1.57 (m, 4H), 1.24-1.41 (m, 36H), 0.87-0.91 (t, J = 6.8 Hz, 3H).

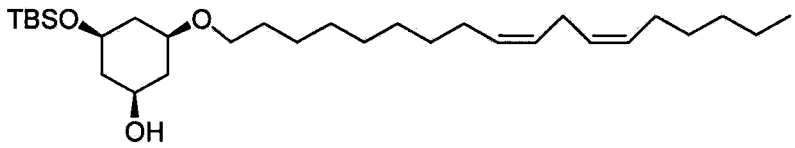
3,5-bis((9Z,12Z)-octadeca-9,12-dienyloxy)cyclohexyl 4- (dimethylamino)butanoate (15)



In a 250mL RBF, to 3,5-bis((9Z,12Z)-octadeca-9,12-dienyloxy)cyclohexanol (**14**) (2.5 g, 4.0 mmol) in methylene chloride (25 mL) was added 4-(dimethylamino)butyric acid hydrochloride (0.8 g, 4.8 mmol) and DIPEA (2.1 mL, 11.9 mmol) and 4-dimethylaminopyridine (0.1 g, 0.8 mmol). After cooled in ice-water bath, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (0.9 g, 4.8 mmol) was added. The mixture was allowed to warm to room temperature and stirred overnight. The mixture was added with water (50 mL). The organic layer was washed with 10% brine (50 mL) and dried over Na_2SO_4. After filtration and concentration, the crude product was purified by silica gel chromatography (5% MeOH in CH_2Cl_2) to obtain 2.6 g of **15** as clear oil (89% yield). ^1H NMR (400 MHz, $CDCl_3$) δ 5.28-5.41 (m, 9H), 3.37-3.51 (m, 6H), 2.75-2.79 (t, J = 6.6 Hz, 4H), 2.46-2.49 (m, 1H), 2.26-2.35 (m, 4H), 2.22 (s, 6H), 2.15-2.19 (m, 2H), 2.02-2.07 (dd, J =

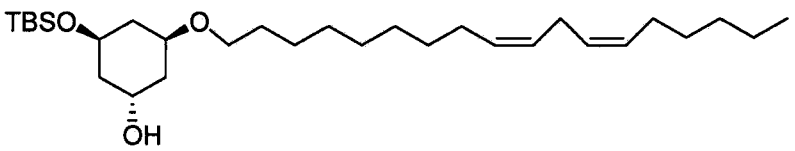
13.7, 6.8 Hz, 8H), 1.74-1.82 (m, 2H), 1.50-1.55 (m, 3H), 1.29-1.39 (m, 36H), 0.87-0.90 (t, J = 6.8 Hz, 3H).

(1*R*,3*R*,5*S*)-3-{[*tert*-butyl(dimethyl)silyl]oxy}-5-[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yloxy]cyclohexanol (16)



In a 3N 1L RBF equipped with overhead agitator and reflux condenser, Compound (**2**) (37.5 g, 152 mmol) suspended in toluene (800 mL) was added sodium hydride 60% (6.1 g, 152 mmol) and triethylamine (2.1 mL, 15.2 mmol), followed by linoleyl mesylate (52.4 g, 152 mmol). The mixture was agitated at 75 °C for 18 hour. After cooling, the mixture was concentrated down to about 100 mL and diluted with MTBE (300 mL). The mixture was quenched with 7% $NaHCO_3$ (200 mL) and the aqueous layer was separated. The organic layer was washed with water (200 mL) and dried over Na_2SO_4. After filtration and concentration, the crude product was purified by silica gel chromatography (30% MTBE in Hexane) to obtain 39.1 g of **16** as clear oil (52% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.31-5.39 (m, 4H), 3.59-3.63 (m , 2H), 3.41-3.45 (t, J = 6.8 Hz, 2H), 3.21-3.24 (m, 1H), 2.75-2.80 (t, J = 6.4 Hz, 2H), 2.25-2.28 (m, 1H), 2.12-2.16 (m, 2H), 2.02-2.07 (dd, J = 13.6, 6.8 Hz, 4H), 1.50-1.54 (m, 3H), 1.26-1.37 (m, 18H), 0.88 (m, 15H), 0.05 (s, 6H).

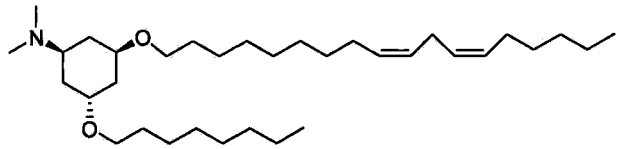
(1*S*,3*R*,5*S*)-3-{[*tert*-butyl(dimethyl)silyl]oxy}-5-[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yloxy]cyclohexanol (Compound 17)



In a 500mL RBF, Compound (**16**) (39.1 g, 79.0 mmol) in THF (300 mL) was added triphenylphosphine (41.4 g, 158.0 mmol). After cooled in ice-water bath, formic acid (5.96 mL, 158.0 mmol) was added. DEAD 40% solution in toluene (50.4 mL, 111.0 mmol) was charged via syringe pump over 45 minutes. The mixture was allowed to warm to room temperature and stirred overnight. The mixture was concentrated down to about 100 mL and

diluted with MTBE (150 mL). The precipitated solid was filtered out and the filtrate was concentrated down to oil. The crude product (oil) was purified by silica gel chromatography (10% MTBE in Hexane) to obtain 36.6 g of intermediate as clear oil. In another 3N 250mL RBF equipped with overhead agitator, the purified intermediate in IPA (200 mL) was added 15% potassium carbonate solution (360 mL). The mixture was agitated at 50 °C for 12 hours. The mixture was then concentrated down under vacuum and extracted with MTBE (200 mL). The organic layer was washed with water (200 mL) and dried over Na_2SO_4. After filtration and concentration, the crude product was purified by silica gel chromatography (30% MTBE in Hexane) to obtain 33.5 g of **17** as clear oil (86% yield over two steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.29-5.39 (m, 4H), 4.28-4.29 (m, 1H), 3.99-4.04 (m, 1H), 3.60-3.65 (m, 1H), 3.40-3.48 (m, 2H), 2.76-2.79 (t, J = 6.4 Hz, 2H), 2.25-2.28 (m, 1H), 2.02-2.10 (m, 5H), 1.93-1.97 (m, 1H), 1.53-1.56 (m, 3H), 1.26-1.37 (m, 18H), 0.89 (m, 15H), 0.07 (s, 6H).

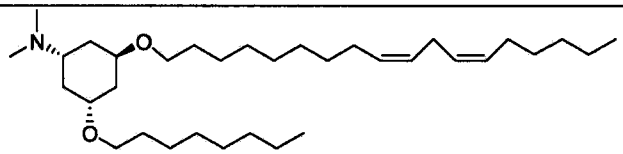
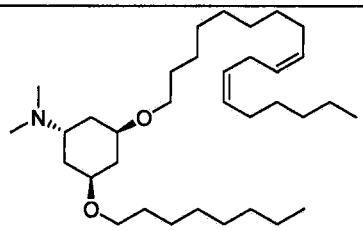
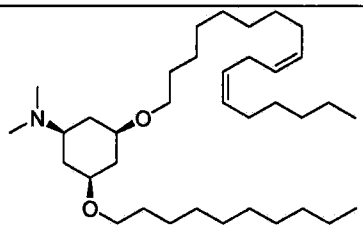
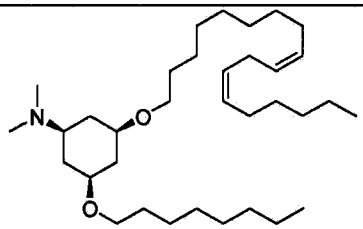
(3*R*,5*R*)-*N*,*N*-dimethyl-3-[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yloxy]-5-(octyloxy)cyclohexan (Compound 18)



Compound **18** was prepared in a manner analogous as that described for compound **7**. MS 520.6 (M+H). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.30-5.41 (m, 4H), 3.73-3.78 (m, 1H), 3.50-3.57 (m, 1H), 3.32-3.49 (m, 4H), 2.72-2.79 (t, J = 6.7 Hz, 2H), 2.67-2.73 (m, 1H), 2.28 (s, 6H), 2.18-2.27 (m, 2H), 1.97-2.07 (m,5H), 1.49-1.57 (m, 4H), 1.13-1.48 (m, 28H), 0.87-0.91 (m, 6H).

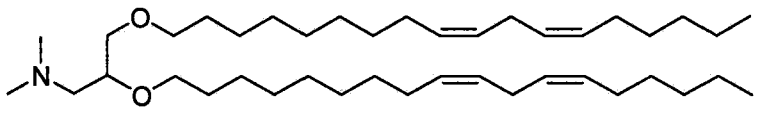
Compounds **19-22** were prepared according to General Schemes 1, 2 and 4 as described for Compounds **7**, **8**, **11**, **12**, **15** and **18** above.

Table 1.

Compound #	Name	Structure	M+H	LCMS
19	**(3*R*,5*R*)-*N*,*N*-dimethyl-3-[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yloxy]-5-(octyloxy)cyclohexanamine**		520.9	520.6
20	**(3*R*,5*S*)-*N*,*N*-dimethyl-3-[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yloxy]-5-(octyloxy)cyclohexanamine**		521.0	520.6
21	**(3*S*,5*R*)-3-(decyloxy)-*N*,*N*-dimethyl-5-[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yloxy]cyclohexanamine**		548.0	a
22	**(3*R*,5*S*)-*N*,*N*-dimethyl-3-[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yloxy]-5-(octyloxy)cyclohexanamine**		520.9	520.6

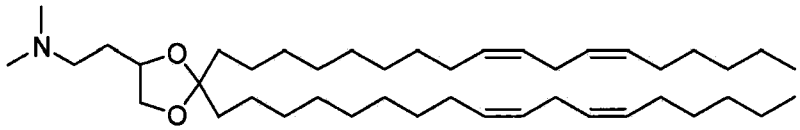
[a] NMR data for **(3*S*,5*R*)-3-(decyloxy)-*N*,*N*-dimethyl-5-[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yloxy]cyclohexanamine (Cmpd 21)** - ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.30-5.39 (m, 4H), 3.42-3.48 (m, 4H), 3.17-3.25 (m, 2H), 2.75-2.78 (t, J = 6.4 Hz, 2H), 2.31-2.43 (m, 2H), 2.31 (s, 6H), 2.14-2.17 (m, 2H), 2.02-2.07 (m, 4H), 1.51-1.57 (m, 4H), 1.19-1.38 (m, 31H), 0.86-0.91 (t, J = 6.8 Hz, 3H)

Compound **23** is DLinDMA as described in *J. Controlled Release*, **2005**, *107*, 276-287, US 2006/0083780 A1, and US 2006/0008910 A1.



(23)

Compound **24** is DLinKC2DMA as described in *Nature Biotechnology*, **2010**, *28*, 172-176, WO 2010/042877 A1, WO 2010/048536 A2, WO 2010/088537 A2, and WO 2009/127060 A1.



(24)

LNP COMPOSITIONS

The following lipid nanoparticle compositions (LNPs) of the instant invention are useful for the delivery of oligonucleotides, specifically siRNA and miRNA:

Cationic Lipid / Cholesterol / PEG-DMG 56.6/38/5.4;

Cationic Lipid / Cholesterol / PEG-DMG 60/38/2;

Cationic Lipid/ Cholesterol / PEG-DMG 67.3/29/3.7;

Cationic Lipid / Cholesterol / PEG-DMG 49.3/47/3.7;

Cationic Lipid / Cholesterol / PEG-DMG 50.3/44.3/5.4;

Cationic Lipid / Cholesterol / PEG-C-DMA / DSPC 40/48/2/10;

Cationic Lipid / Cholesterol / PEG-DMG / DSPC 40/48/2/10; and

Cationic Lipid / Cholesterol / PEG-DMG / DSPC 58/30/2/10.

LNP process description:

The Lipid Nano-Particles (LNP) are prepared by an impinging jet process. The particles are formed by mixing lipids dissolved in alcohol with siRNA dissolved in a citrate buffer. The mixing ratio of lipids to siRNA are targeted at 45-55% lipid and 65-45% siRNA. The lipid solution contains a novel cationic lipid of the instant invention, a helper lipid (cholesterol) , PEG (e.g. PEG-C-DMA, PEG-DMG) lipid, and DSPC at a concentration of 5-

15 mg/mL with a target of 9-12 mg/mL in an alcohol (for example ethanol). The ratio of the lipids has a mole percent range of 25-98 for the cationic lipid with a target of 35-65, the helper lipid has a mole percent range from 0-75 with a target of 30-50, the PEG lipid has a mole percent range from 1-15 with a target of 1-6, and the DSPC has a mole precent range of 0-15 with a target of 0-12. The siRNA solution contains one or more siRNA sequences at a concentration range from 0.3 to 1 .0 mg/mL with a target of 0.3 -0.9 mg/mL in a sodium citrate buffered salt solution with pH in the range of 3.5-5. The two liquids are heated to a temperature in the range of 15-40°C, targeting 30-40°C, and then mixed in an impinging jet mixer instantly forming the LNP. The teeID has a range from 0.25 to 1.0 mm and a total flow rate from 10 -600 mL/min. The combination of flow rate and tubing ID has effect of controlling the particle size of the LNPs between 30 and 200 nm. The solution is then mixed with a buffered solution at a higher pH with a mixing ratio in the range of 1:1 to 1:3 vol:vol but targeting 1:2 vol:vol. This buffered solution is at a temperature in the range of 15-40°C, targeting 30-40°C. The mixed LNPs are held from 30 minutes to 2 hrs prior to an anion exchange filtration step. The temperature during incubating is in the range of 15-40°C, targeting 30-40°C. After incubating the solution is filtered through a 0.8 um filter containing an anion exchange separation step. This process uses tubing IDs ranging from 1 mm ID to 5 mm ID and a flow rate from 10 to 2000 mL/min. The LNPs are concentrated and diafiltered via an ultrafiltration process where the alcohol is removed and the citrate buffer is exchanged for the final buffer solution such as phosphate buffered saline. The ultrafiltration process uses a tangential flow filtration format (TFF). This process uses a membrane nominal molecular weight cutoff range from 30 -500 KD. The membrane format can be hollow fiber or flat sheet cassette. The TFF processes with the proper molecular weight cutoff retains the LNP in the retentate and the filtrate or permeate contains the alcohol; citrate buffer; final buffer wastes. The TFF process is a multiple step process with an initial concentration to a siRNA concentration of 1 -3 mg/mL. Following concentration, the LNPs solution is diafiltered against the final buffer for 10 -20 volumes to remove the alcohol and perform buffer exchange. The material is then concentrated an additional 1-3 fold. The final steps of the LNP process are to sterile filter the concentrated LNP solution and vial the product.

Analytical Procedure:

1) siRNA concentration

The siRNA duplex concentrations are determined by Strong Anion-Exchange High-Performance Liquid Chromatography (SAX-HPLC) using Waters 2695 Alliance system

(Water Corporation, Milford MA) with a 2996 PDA detector. The LNPs, otherwise referred to as RNAi Delivery Vehicles (RDVs), are treated with 0.5% Triton X-100 to free total siRNA and analyzed by SAX separation using a Dionex BioLC DNAPac PA 200 (4 × 250 mm) column with UV detection at 254 nm. Mobile phase is composed of A: 25 mM $NaClO_4$, 10 mM Tris, 20% EtOH, pH 7.0 and B: 250 mM $NaClO_4$, 10 mM Tris, 20% EtOH, pH 7.0 with liner gradient from 0-15 min and flow rate of 1 ml/min. The siRNA amount is determined by comparing to the siRNA standard curve.

2) Encapsulation rate

Fluorescence reagent SYBR Gold is employed for RNA quantitation to monitor the encapsulation rate of RDVs. RDVs with or without Triton X-100 are used to determine the free siRNA and total siRNA amount. The assay is performed using a SpectraMax M5*e* microplate spectrophotometer from Molecular Devices (Sunnyvale, CA). Samples are excited at 485 nm and fluorescence emission was measured at 530 nm. The siRNA amount is determined by comparing to the siRNA standard curve.

Encapsulation rate = (1- free siRNA/total siRNA) ×100%

3) Particle size and polydispersity

RDVs containing 1 μg siRNA are diluted to a final volume of 3 ml with 1 × PBS. The particle size and polydispersity of the samples is measured by a dynamic light scattering method using ZetaPALS instrument (Brookhaven Instruments Corporation, Holtsville, NY). The scattered intensity is measured with He–Ne laser at 25°C with a scattering angle of 90°.

4) Zeta Potential analysis

RDVs containing 1 μg siRNA are diluted to a final volume of 2 ml with 1 mM Tris buffer (pH 7.4). Electrophoretic mobility of samples is determined using ZetaPALS instrument (Brookhaven Instruments Corporation, Holtsville, NY) with electrode and He–Ne laser as a light source. The Smoluchowski limit is assumed in the calculation of zeta potentials.

5) Lipid analysis

Individual lipid concentrations are determined by Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) using Waters 2695 Alliance system (Water Corporation, Milford MA) with a Corona charged aerosol detector (CAD) (ESA Biosciences, Inc, Chelmsford, MA). Individual lipids in RDVs are analyzed using an Agilent Zorbax SB-C18 (50 × 4.6 mm, 1.8 μm particle size) column with CAD at 60 °C. The mobile phase is composed of A: 0.1% TFA in H_2O and B: 0.1% TFA in IPA. The gradient changes from 60%

mobile phase A and 40% mobile phase B from time 0 to 40% mobile phase A and 60% mobile phase B at 1.00 min; 40% mobile phase A and 60% mobile phase B from 1.00 to 5.00 min; 40% mobile phase A and 60% mobile phase B from 5.00 min to 25% mobile phase A and 75% mobile phase B at 10.00 min; 25% mobile phase A and 75% mobile phase B from 10.00 min to 5% mobile phase A and 95% mobile phase B at 15.00 min; and 5% mobile phase A and 95% mobile phase B from 15.00 to 60% mobile phase A and 40% mobile phase B at 20.00 min with flow rate of 1 ml/min. The individual lipid concentration is determined by comparing to the standard curve with all the lipid components in the RDVs with a quadratic curve fit. The molar percentage of each lipid is calculated based on its molecular weight.

Utilizing the above described LNP process, specific LNPs with the following ratios were identified:

Nominal composition:

Cationic Lipid / Cholesterol / PEG-DMG 60/38/2

Cationic Lipid / Cholesterol / PEG-DMG / DSPC 58/30/2/10

Luc siRNA

5'-iB-**A***U***AAGG***C**U**A***U***GAAGAGA***U***ATT**-iB 3' (SEQ.ID.NO.:1)

3'-UU*U***A***U***U***C***C**GA***U***A***C***U***U***C***U***C**UAU-5' (SEQ.ID.NO.:2)

AUGC – Ribose

iB – Inverted deoxy abasic

UC – 2' Fluoro

AGT – 2' Deoxy

AGU – 2' OCH_3

Nominal composition

Cationic Lipid /Cholesterol/PEG-DMG 60/38/2

Cationic Lipid / Cholesterol / PEG-DMG / DSPC 40/48/2/10

Cationic Lipid / Cholesterol / PEG-DMG / DSPC 58/30/2/10

ApoB siRNA

5'-iB-CUUU*AA*C*AA*UUCCU*GAAA*U**TsT**-iB-3' (SEQ ID NO.:3)

3'-UsU*GAAA*U*UG*UU*AAGGA*CUsUsUsA-5' (SEQ ID NO.:4)

AUGC – Ribose

iB – Inverted deoxy abasic

UC – 2' Fluoro

AGT – 2' Deoxy

AGU – 2' OCH_3

UsA – phophorothioate linkage

Oligonucleotide synthesis is well known in the art. (See US patent applications: US 2006/0083780, US 2006/0240554, US 2008/0020058, US 2009/0263407 and US 2009/0285881 and PCT patent applications: WO 2009/086558, WO2009/127060, WO2009/132131, WO2010/042877, WO2010/054384, WO2010/054401, WO2010/054405 and WO2010/054406). The siRNAs disclosed and utilized in the Examples were synthesized via standard solid phase procedures.

EXAMPLE 1

Mouse In Vivo Evaluation of Efficacy

LNPs utilizing compounds in the nominal compositions described immediately above were evaluated for in vivo efficacy. The siRNA targets the mRNA transcript for the firefly (Photinus pyralis) luciferase gene (Accession # M15077). The primary sequence and chemical modification pattern of the luciferase siRNA is displayed above. The in vivo luciferase model employs a transgenic mouse in which the firefly luciferase coding sequence is present in all cells. ROSA26- LoxP-Stop-LoxP-Luc (LSL-Luc) transgenic mice licensed from the Dana Farber Cancer Institute are induced to express the Luciferase gene by first removing the LSL sequence with a recombinant Ad-Cre virus (Vector Biolabs). Due to the organo-tropic nature of the virus, expression is limited to the liver when delivered via tail vein injection. Luciferase expression levels in liver are quantitated by measuring light output, using an IVIS imager (Xenogen) following administration of the luciferin substrate (Caliper Life Sciences). Pre-dose luminescence levels are measured prior to administration of the RDVs. Luciferin in PBS (15mg/mL) is intraperitoneally (IP) injected in a volume of 150 μL. After a four minute incubation period mice are anesthetized with isoflurane and placed in the IVIS imager. The RDVs (containing siRNA) in PBS vehicle were tail vein injected n a volume of 0.2 mL. Final dose levels ranged from 0.1 to 0.5 mg/kg siRNA. PBS vehicle alone was dosed as a control. Mice were imaged 48 hours post dose using the method described above. Changes in luciferin light output directly correlate with luciferase mRNA levels and represent an indirect measure of luciferase siRNA activity. In vivo efficacy results are expressed as % inhibition of luminescence relative to pre-dose luminescence levels. Systemic administration of the luciferase siRNA RDVs decreased luciferase expression in a dose dependant manner. Greater efficacy was observed in mice dosed with Compound **15** containing RDVs than with

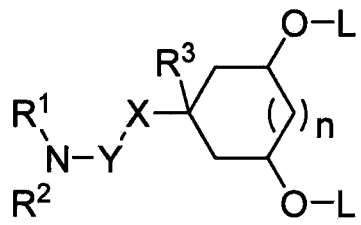
the RDV containing the octyl-CLinDMA (OCD) cationic lipid (Figure 1). OCD is known and described in WO2010/021865.

Rat In Vivo Evaluation of Efficacy and Toxicity

LNPs utilizing compounds in the nominal compositions described above, were evaluated for in vivo efficacy and increases in alanine amino transferase and aspartate amino transferase in Sprague-Dawley (Crl:CD(SD) female rats (Charles River Labs). The siRNA targets the mRNA transcript for the ApoB gene (Accession # NM 019287). The primary sequence and chemical modification pattern of the ApoB siRNA is displayed above. The RDVs (containing siRNA) in PBS vehicle were tail vein injected in a volume of 1 to 1.5 mL. Infusion rate is approximately 3 ml/min. Five rats were used in each dosing group. After LNP administration, rats are placed in cages with normal diet and water present. Six hours post dose, food is removed from the cages. Animal necropsy is performed 24 hours after LNP dosing. Rats are anesthetized under isoflurane for 5 minutes, then maintained under anesthesia by placing them in nose cones continuing the delivery of isoflurane until ex-sanguination is completed. Blood is collected from the vena cava using a 23 guage butterfly venipuncture set and aliquoted to serum separator vacutainers for serum chemistry analysis. Punches of the excised caudate liver lobe are taken and placed in RNALater (Ambion) for mRNA analysis. Preserved liver tissue was homogenized and total RNA isolated using a Qiagen bead mill and the Qiagen miRNA-Easy RNA isolation kit following the manufacturer's instructions. Liver ApoB mRNA levels were determined by quantitative RT-PCR. Message was amplified from purified RNA utilizing a rat ApoB commercial probe set (Applied Biosystems Cat # RN01499054_m1). The PCR reaction was performed on an ABI 7500 instrument with a 96-well Fast Block. The ApoB mRNA level is normalized to the housekeeping PPIB (NM 011149) mRNA. PPIB mRNA levels were determined by RT-PCR using a commercial probe set (Applied Biosytems Cat. No. Mm00478295_m1). Results are expressed as a ratio of ApoB mRNA/ PPIB mRNA. All mRNA data is expressed relative to the PBS control dose. Serum ALT and AST analysis were performed on the Siemens Advia 1800 Clinical Chemistry Analyzer utilizing the Siemens alanine aminotransferase (Cat# 03039631) and aspartate aminotransferase (Cat# 03039631) reagents. Similar efficacy was observed in rats dosed with Compound **15** containing RDV than with the RDV containing the cationic lipid DLinKC2DMA (Cmpd **24**) (Figure 2).

WHAT IS CLAIMED IS:

1. A cationic lipid of Formula A:



A

wherein:

R^1 and R^2 are independently selected from H, $(C_1\text{-}C_6)$alkyl, heterocycle, and polyamine, wherein said alkyl, heterocycle and polyamine are optionally substituted with one to three substituents selected from R', or R^1 and R^2 can be taken together with the nitrogen to which they are attached to form a monocyclic heterocycle with 4-7 members optionally containing, in addition to the nitrogen, one or two additional heteroatoms selected from N, O and S, said monocyclic heterocycle is optionally substituted with one to three substituents selected from R';

R^3 is selected from H and $(C_1\text{-}C_6)$alkyl, said alkyl optionally substituted with one to three substituents selected from R';

R' is independently selected from halogen, R", OR", SR", CN, $CO_2R"$ and $CON(R")_2$;

R" is independently selected from H and $(C_1\text{-}C_6)$alkyl, wherein said alkyl is optionally substituted with halogen and OH;

n is 1, 2, 3, 4 or 5;

X is absent, O, NR", O(C=O), NR"(C=O), O(C=O)O, NR"(C=O)NR", O(C=O)NR", or NR"(C=O)O;

Y is absent or (C_1-C_6)alkyl; and

L is independently selected from C_4-C_{24} alkyl and C_4-C_{24} alkenyl, said alkyl and alkenyl are optionally substituted with one or more substituents selected from R';

or any pharmaceutically acceptable salt or stereoisomer thereof.

2. A cationic lipid of Formula A according to Claim 1, wherein:

R^1 and R^2 are each methyl;

R^3 is H;

n is 1;

X is absent;

Y is absent; and

L is independently selected from C_4-C_{24} alkyl and C_4-C_{24} alkenyl;

or any pharmaceutically acceptable salt or stereoisomer thereof.

3. A cationic lipid of Formula A according to Claim 1, wherein:

R^1 and R^2 are each methyl;

R^3 is H;

n is 1;

X is O(C=O);

Y is methyl, ethyl or propyl; and

L is independently selected from C_4-C_{24} alkyl and C_4-C_{24} alkenyl;

or any pharmaceutically acceptable salt or stereoisomer thereof.

4. A cationic lipid which is:

N,N-dimethyl-3,5-bis[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]cyclohexanamine (Compound 8);
Cis N,N-dimethyl-3,5-bis[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]cyclohexanamine (Compound 12);
3,5-bis((9Z,12Z)-octadeca-9,12-dienyloxy)cyclohexyl 4- (dimethylamino)butanoate (Compound 15);
(3*R*,5*R*)-*N*,*N*-dimethyl-3-[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yloxy]-5-(octyloxy)cyclohexanamine (Compound 18);
(3*R*,5*R*)-*N*,*N*-dimethyl-3-[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yloxy]-5-(octyloxy)cyclohexanamine (Compound 19);
(3*R*,5*S*)-*N*,*N*-dimethyl-3-[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yloxy]-5-(octyloxy)cyclohexanamine (Compound 20);
(3*S*,5*R*)-3-(decyloxy)-*N*,*N*-dimethyl-5-[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yloxy]cyclohexanamine (Compound 21); and
(3*R*,5*S*)-*N*,*N*-dimethyl-3-[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yloxy]-5-(octyloxy)cyclohexanamine (Compound 22);

or any pharmaceutically acceptable salt or stereoisomer thereof.

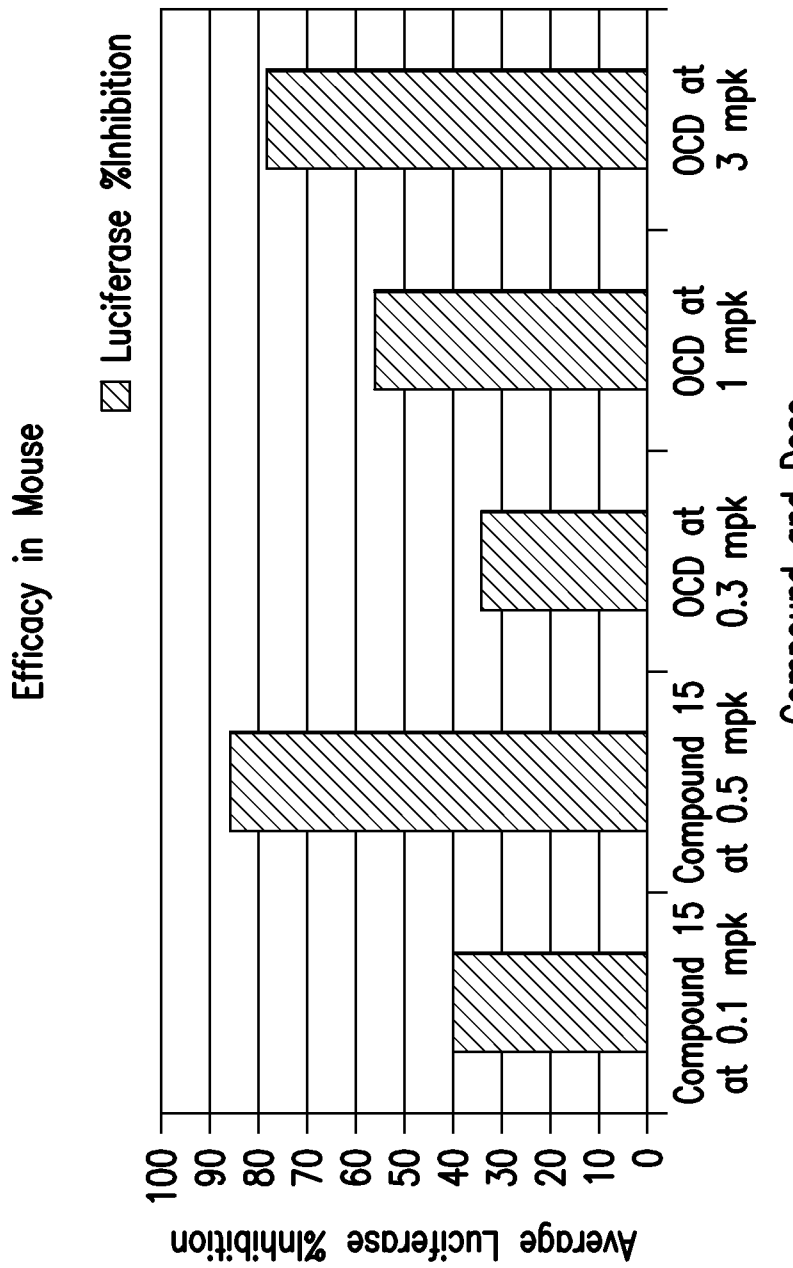

Efficacy in Mouse
Luciferase %Inhibition
Average Luciferase %Inhibition
100
90
80
70
60
50
40
30
20
10
0
Compound 15 at 0.1 mpk
Compound 15 at 0.5 mpk
OCD at 0.3 mpk
OCD at 1 mpk
OCD at 3 mpk
Compound and Dose

FIG. 1

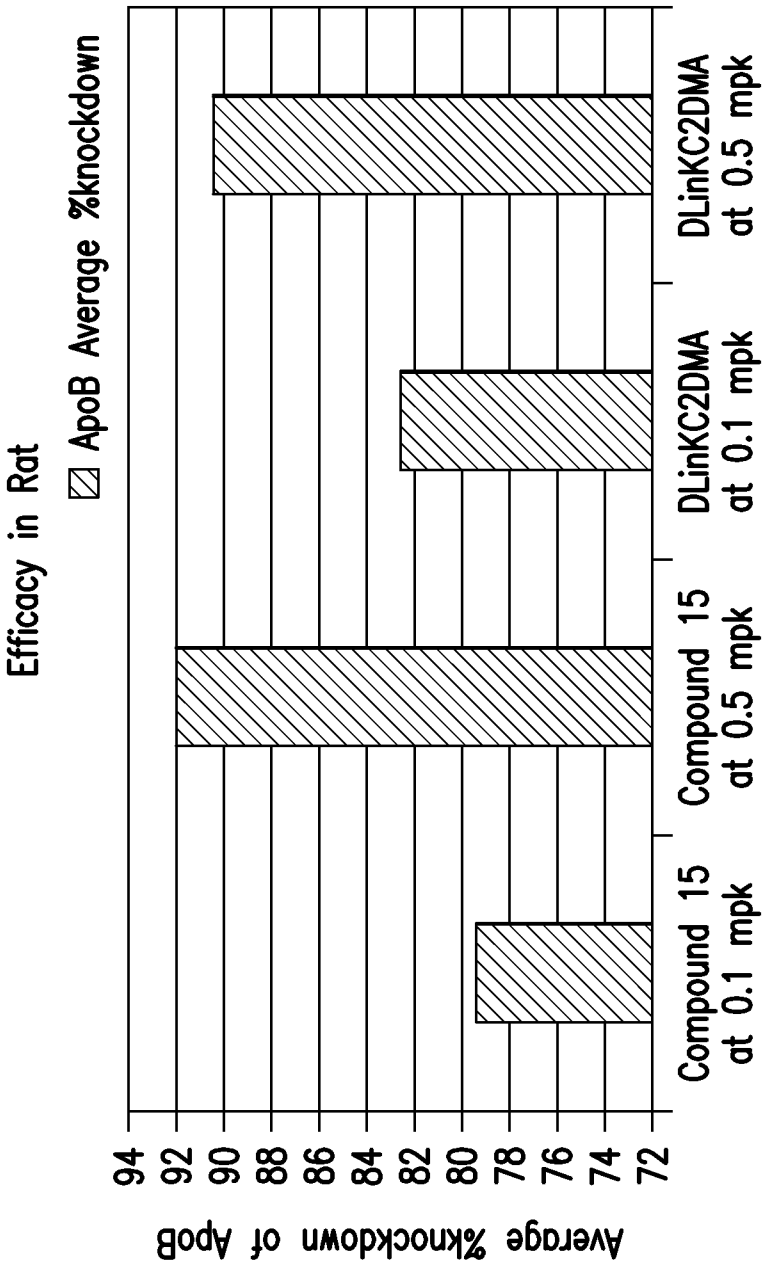

Efficacy in Rat
ApoB Average %knockdown
Average %knockdown of ApoB
94
92
90
88
86
84
82
80
78
76
74
72
Compound 15 at 0.1 mpk
Compound 15 at 0.5 mpk
DLinKC2DMA at 0.1 mpk
DLinKC2DMA at 0.5 mpk
Compound and Dose

FIG.2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/38755

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 45/00; A61K 47/44; G01N 33/92 (2012.01)
USPC - 424/283.1
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(8): A61K 45/00; A61K 47/44; G01N 33/92 (2012.01)
USPC: 424/283.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
IPC(8): G01N 33/92; C12N 15/11 (2012.01)
USPC: 436/71, 514/44A

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST, SureChem, PubChem, Dialog
Cationic lipid, cyclohexyl

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2011/0076335 A1 (YAWORSKI et al.) 31 March 2011 (31.03.2011) para[0391], [0448]	1-4
Y	US 2009/0285881 A1 (DANDE et al.) 19 November 2009 (19.11.2009) para[0005]-para[0011]	1-4
Y	US 2009/0163705 A1 (MANOHARAN et al .) 25 June 2009 (25.06.2009) para[0009]-para[0013]	1-4
Y	Majeti et al. Enhanced Intravenous Transgene Expression in Mouse Lung Using Cyclic-Head Cationic Lipids. Chemistry & Biology, Vol. 11, 427-437, April, 2004. page 427, Summary	1-4

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 July 2012 (13.07.2012)	06 AUG 2012
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)