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(54) Title: NOVEL BIS-NITROGEN CONTAINING CATIONIC LIPIDS FOR OLIGONUCLEOTIDE DELIVERY

(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 and tolerability. The present invention employs bis-nitrogen containing cationic lipids to enhance the efficiency and tolerability of in vivo delivery of siRNA.

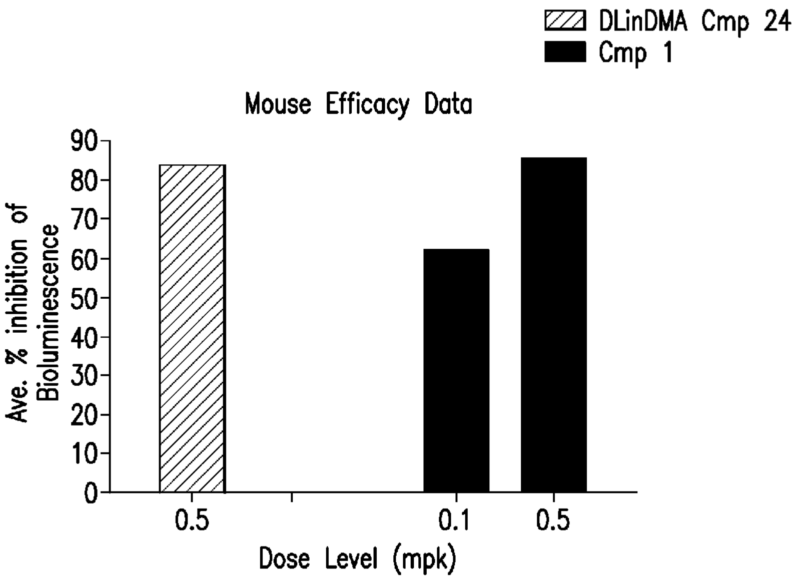

FIG.1

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).

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TITLE OF THE INVENTION

NOVEL BIS-NITROGEN CONTAINING 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 and tolerability. The present invention employs bis-nitrogen containing cationic lipids to enhance the efficiency and tolerability 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 and tolerability. The present invention employs bis-nitrogen containing cationic lipids to enhance the efficiency and tolerability of in vivo delivery of siRNA.

BRIEF DESCRIPTION OF THE FIGURES

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

FIGURE 2: LNP (Compound **1**) efficacy and tolerability in rat.

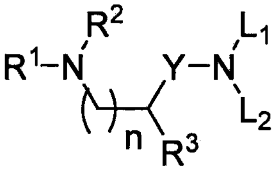
FIGURE 3: Absolute liver levels of cationic lipid (Compound **1**) in rat at 48 hours.

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:

n is 1 or 2;

Y is a bond, O(C=O), C=O, or NR'(C=O);

R^1 and R^2 are independently selected from H, $(C_1\text{-}C_4)$alkyl, heterocyclyl, and a polyamine, wherein said alkyl, heterocyclyl and polyamine are optionally substituted with one or more 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 heterocyclyl with 4-7 members optionally containing, in addition to the nitrogen, one or two additional heteroatoms selected from N, O and S, said monocyclic heterocyclyl is optionally substituted with one or more substituents selected from R';

R^3 is absent or can be taken together with R^1 to form a monocyclic heterocyclyl with 4-7 members optionally containing, in addition to the nitrogen, one or two additional heteroatoms selected from N, O and S, said monocyclic heterocyclyl is optionally substituted with one or more substituents selected from R';

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

R'' is selected from H and $(C_1\text{-}C_4)$alkyl, wherein said alkyl is optionally substituted with one or more substituents selected from halogen and OH;

L_1 is a $C_4\text{-}C_{22}$ alkyl or $C_4\text{-}C_{22}$ alkenyl, said alkyl and alkenyl are optionally substituted with one or more substituents selected from R'; and

L_2 is a $C_4\text{-}C_{22}$ alkyl or $C_4\text{-}C_{22}$ 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:

n is 1;

Y is a bond;

R^1 and R^2 are independently selected from H, $(C_1\text{-}C_4)$alkyl, heterocyclyl, and a polyamine, wherein said alkyl, heterocyclyl and polyamine are optionally substituted with one or more 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 heterocyclyl with 4-7 members optionally containing, in addition to the nitrogen, one or two additional heteroatoms selected from N, O and S, said monocyclic heterocyclyl is optionally substituted with one or more substituents selected from R';

R^3 is absent or can be taken together with R^1 to form a monocyclic heterocyclyl with 4-7 members optionally containing, in addition to the nitrogen, one or two additional heteroatoms selected from N, O and S, said monocyclic heterocyclyl is optionally substituted with one or more substituents selected from R';

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

R'' is selected from H and $(C_1\text{-}C_4)$alkyl, wherein said alkyl is optionally substituted with one or more substituents selected from halogen and OH;

L_1 is a $C_4\text{-}C_{22}$ alkyl or $C_4\text{-}C_{22}$ alkenyl, said alkyl and alkenyl are optionally substituted with one or more substituents selected from R'; and

L_2 is a $C_4\text{-}C_{22}$ alkyl or $C_4\text{-}C_{22}$ 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 third embodiment, the invention features a compound having Formula A, wherein:

n is 1;

Y is a O(C=O);

R^1 and R^2 are independently selected from H, $(C_1\text{-}C_4)$alkyl, heterocyclyl, and a polyamine, wherein said alkyl, heterocyclyl and polyamine are optionally substituted with one or more 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 heterocyclyl with 4-7 members optionally containing, in addition to the nitrogen, one or two additional heteroatoms selected from N, O and S, said monocyclic heterocyclyl is optionally substituted with one or more substituents selected from R';

R^3 is absent or can be taken together with R^1 to form a monocyclic heterocyclyl with 4-7 members optionally containing, in addition to the nitrogen, one or two additional heteroatoms selected from N, O and S, said monocyclic heterocyclyl is optionally substituted with one or more substituents selected from R';

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

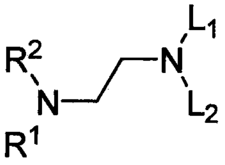
R" is selected from H and $(C_1\text{-}C_4)$alkyl, wherein said alkyl is optionally substituted with one or more substituents selected from halogen and OH;

L_1 is a $C_4\text{-}C_{22}$ alkyl or $C_4\text{-}C_{22}$ alkenyl, said alkyl and alkenyl are optionally substituted with one or more substituents selected from R'; and

L_2 is a $C_4\text{-}C_{22}$ alkyl or $C_4\text{-}C_{22}$ 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 another embodiment of this invention, the cationic lipids are illustrated by the Formula B:



B

wherein:

R^1 and R^2 are independently selected from H, $(C_1\text{-}C_4)$alkyl, heterocyclyl, and a polyamine, wherein said alkyl, heterocyclyl and polyamine are optionally substituted with one or more 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 heterocyclyl with 4-7 members optionally containing, in addition to the nitrogen, one or two additional heteroatoms selected from N, O and S, said monocyclic heterocyclyl is optionally substituted with one or more substituents selected from R';

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

R" is selected from H and $(C_1\text{-}C_4)$alkyl, wherein said alkyl is optionally substituted with one or more substituents selected from halogen and OH;

L_1 is a C_4-C_{22} alkyl or C_4-C_{22} alkenyl, said alkyl and alkenyl are optionally substituted with one or more substituents selected from R'; and

L_2 is a C_4-C_{22} alkyl or C_4-C_{22} alkenyl, said alkyl and alkenyl are optionally substituted with one or more substituents selected from R';

or any pharmaceutically acceptable salt or stereoisomer thereof.

Specific cationic lipids are:

N-[(11Z)-icos-11-en-1-yl]- N' N'–dimethyl-N-nonylethane-1,2-diamine (Compound **1**);

1-methylpiperidin-4-yl decyl[(9Z)-octadec-9-en-1-yl]carbamate (Compound **2**);

4-(dimethylamino)-N-[(9Z)-octadec-9-en-1-yl]-N-octylbutanamide (Compound **3**);

1-methylpiperidin-4-yl decyl[(9Z)-octadec-9-en-1-yl]carbamate (Compound **4**);

1-methylpiperidin-4-yl decyl[(9Z,12Z)-octadeca-9,12-dien-1-yl]carbamate (Compound **5**);

N,N-dimethyl-N'-[(9Z,12Z)-octadeca-9,12-dien-1-yl]-N'-octylpropane-1,3-diamine (Compound **6**);

N,N-dimethyl-N'-nonyl-N'-[(9Z,12Z)-octadeca-9,12-dien-1-yl]propane-1,3-diamine (Compound **7**);

(9Z,12Z)-N-[2-(pyrrolidin-1-yl)ethyl]-N-undecyloctadeca-9,12-dien-1-amine (Compound **8**);

(11Z)-N-decyl-N-[2-(pyrrolidin-1-yl)ethyl]icos-11-en-1-amine (Compound **9**);

N-decyl-10-{2-[(2-pentylcyclopropyl)methyl]cyclopropyl}-N-[2-(pyrrolidin-1-yl)ethyl]decan-1-amine (Compound **10**);

N-(8-{2-[(2-pentylcyclopropyl)methyl]cyclopropyl}octyl)-N-[2-(pyrrolidin-1-yl)ethyl]decan-1-amine (Compound **11**);

(11Z,14Z)-N-decyl-N-[2-(pyrrolidin-1-yl)ethyl]icosa-11,14-dien-1-amine (Compound **12**);

N-decyl-10-(2-octylcyclopropyl)-N-[2-(pyrrolidin-1-yl)ethyl]decan-1-amine (Compound **13**);

(9Z)-N-[(9Z)-octadec-9-en-1-yl]-N-[2-(pyrrolidin-1-yl)ethyl]octadec-9-en-1-amine (Compound **14**);

N,N-dimethyl-N',N'-di[(9Z)-octadec-9-en-1-yl]ethane-1,2-diamine (Compound **15**);

N,N-dimethyl-N',N'-di[(9Z,12Z)-octadeca-9,12-dien-1-yl]ethane-1,2-diamine (Compound **16**);

N,N-dimethyl-N'-[(9Z)-octadec-9-en-1-yl]-N'-octylethane-1,2-diamine (Compound **17**);

(9Z)-N-octyl-N-[2-(pyrrolidin-1-yl)ethyl]octadec-9-en-1-amine (Compound **18**);

N,N-dimethyl-N'-nonyl-N'-[(9Z,12Z)-octadeca-9,12-dien-1-yl]ethane-1,2-diamine (Compound **19**);

N-decyl-N',N'-dimethyl-N-[(9Z,12Z)-octadeca-9,12-dien-1-yl]ethane-1,2-diamine (Compound **20**);

N-decyl-N',N'-dimethyl-N-[(9Z)-octadec-9-en-1-yl]ethane-1,2-diamine (Compound **21**);

N,N-dimethyl-N'-nonyl-N'-[(9Z)-octadec-9-en-1-yl]ethane-1,2-diamine (Compound **22**); and

(9Z,12Z)-N-decyl-N-[2-(pyrrolidin-1-yl)ethyl]octadeca-9,12-dien-1-amine (Compound **23**).

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.

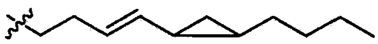
It is understood that one or more Si atoms can be incorporated into the cationic lipids of the instant invention by one of ordinary skill in the art to provide cationic lipids that are chemically stable and that can be readily synthesized by techniques known in the art from readily available starting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

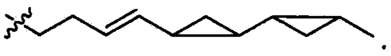
In the compounds of Formula A, the atoms may exhibit their natural isotopic abundances, or one or more of the atoms may be artificially enriched in a particular isotope having the same atomic number, but an atomic mass or mass number different from the atomic mass or mass number predominantly found in 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 (^1H) and deuterium (^2H). 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, n is 1 or 2.

In an embodiment of Formula A, n is 2.

In an embodiment of Formula A, n is 1.

In an embodiment of Formula A, n is 0.

In an embodiment of Formula A, Y is a bond, O(C=O), C=O, or NR'(C=O).

In an embodiment of Formula A, Y is a bond.

In an embodiment of Formula A, Y is a bond or O(C=O).

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

In an embodiment of Formula A, Y is C=O.

In an embodiment of Formula A or B, R^1 and R^2 are independently selected from H and $(C_1\text{-}C_4)$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 heterocyclyl with 4-7 members optionally containing, in addition to the nitrogen, one or two additional heteroatoms selected from N, O and S, said monocyclic heterocyclyl is optionally substituted with one to three substituents selected from R'.

In an embodiment of Formula A or B, 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 heterocyclyl with 4-7 members optionally containing, in addition to the nitrogen, one or two additional heteroatoms selected from N, O and S, said monocyclic heterocyclyl is optionally substituted with one to three substituents selected from R'.

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

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

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

In an embodiment of Formula A, R^3 is taken together with R^1 to form a monocyclic heterocyclyl.

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

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

In an embodiment of Formula A or B, R" is H.

In an embodiment of Formula A or B, L_1 is selected from C_4-C_{22} alkyl and C_4-C_{22} alkenyl, which are optionally substituted with halogen and OH.

In an embodiment of Formula A or B, L_1 is selected from C_4-C_{22} alkyl and C_4-C_{22} alkenyl.

In an embodiment of Formula A or B, L_1 is selected from C_{12}-C_{22} alkyl and C_{12}-C_{22} alkenyl.

In an embodiment of Formula A or B, L_1 is selected from C_{16}-C_{20} alkyl and C_{16}-C_{20} alkenyl.

In an embodiment of Formula A or B, L_1 is selected from C_{18} alkyl and C_{18} alkenyl.

In an embodiment of Formula A or B, L_2 is selected from C_4-C_{22} alkyl and C_4-C_{22} alkenyl, which are optionally substituted with halogen and OH.

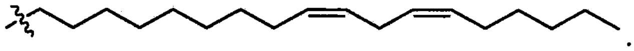
In an embodiment of Formula A or B, L_2 is selected from C_4-C_{22} alkyl and C_4-C_{22} alkenyl.

In an embodiment of Formula A or B, L_2 is selected from C_6-C_{18} alkyl and C_6-C_{18} alkenyl.

In an embodiment of Formula A or B, L_2 is selected from C_6-C_{18} alkyl.

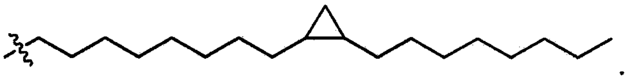
In an embodiment of Formula A or B, L_2 is selected from C_8-C_{10} alkyl.

In an embodiment of Formula A or B, L_1 is

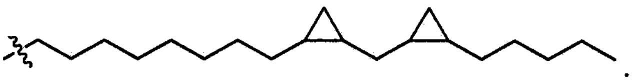


In an embodiment of Formula A or B, L_1 is

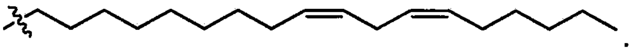
In an embodiment of Formula A or B, L_1 is



In an embodiment of Formula A or B, L_1 is



In an embodiment of Formula A or B, L_2 is



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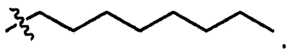
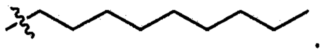
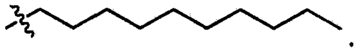


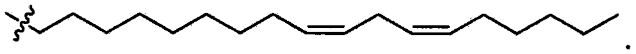
In an embodiment of Formula A or B, L_2 is



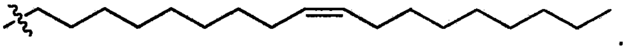
In an embodiment of Formula A or B, L_2 is



In an embodiment of Formula A or B, L_1 and L_2 are



In an embodiment of Formula A or B, L_1 and L_2 are



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

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

In an embodiment of Formula A or B, "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.

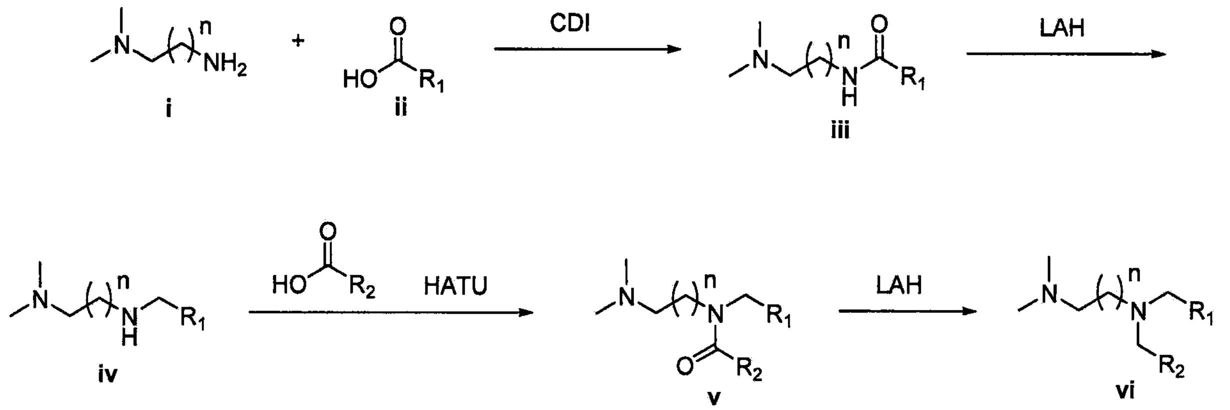
It will also be noted that the cationic lipids of the present invention are potentially internal salts or zwitterions, since under physiological conditions a deprotonated acidic moiety in the compound, such as a carboxyl group, may be anionic, and this electronic charge might then be balanced off internally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

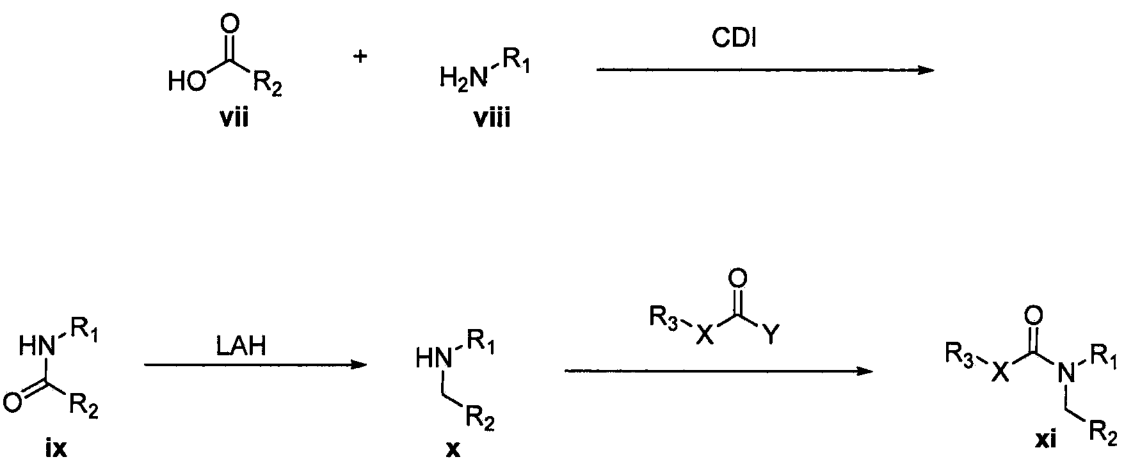
Compounds derived from **General Scheme 1** can be generally obtained from employing the following strategy. A diamine **i** is combined with a carboxylic acid **ii** and appropriate coupling agent to furnish an amino-secondary-amide intermediate **iii**. The carbonyl of this intermediate is subsequently reduced using LAH to furnish a new diamine **iv**. This compound is once again reacted with a carboxylic acid and coupling agent to provide an amino-tertiary-amide **v**. Reduction of the amide carbonyl yields the final diamine cationic lipid **vi**.

GENERAL SCHEME 1

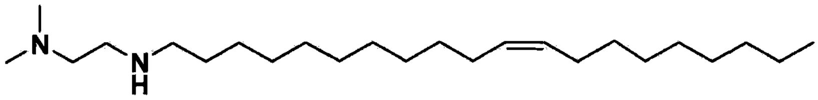


Compounds derived from **General Scheme 2** can be generally obtained from employing the following strategy. The amine intermediate **viii** undergoes standard amide coupling with a carboxylic acid **vii** followed by LAH reduction to yield a key secondary amine intermediate **x**. Finally, the amine is reacted with an acyl electrophile such as an activated carboxylic acid or chloroformate to furnish an amide or carbamates **xi**, respectively.

GENERAL SCHEME 2

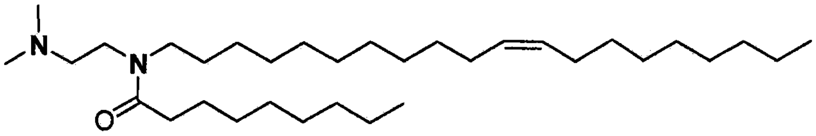


N'-[(11Z)-icos-11-en-1-yl]-N, N-dimethylethane-1, 2-diamine (Intermediate A)



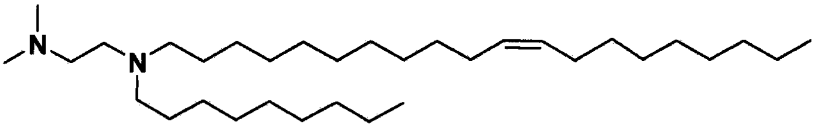
To a 500 mL DCM solution of oleic acid (20.0 g, 70.8 mmol) was added CDI (11.5 g, 70.8 mmol), and the solution stirred at RT for 1 hour, after which N,N-dimethylethane-1,2-diamine (6.24 g, 70.8 mmol) was added. After 1 hour the reaction was washed with 300 mL water, then with brine. The organic was dried over Na_2SO_4, filtrated and the solvent evaporated in vacuo to yield a colorless oil. The oil was taken up in 500 mL THF and cooled to 0°C in an ice bath. Lithium aluminium hydride 2.0 M solution in THF (53.2 mL, 106 mmol) was added slowly, dropwise. The ice bath was removed, and the reaction was allowed to gradually warm to RT over 2 hours. The reaction was then diluted with 100 mL more THF and was quenched by adding small portions of sodium sulfate dodecahydrate until fizzing stopped. Solids were filtered off using a Buchner funnel, and the filtrate was concentrated in vacuo to yield Intermediate **A** (20.5 g). LCMS (m + H): 339.5

N-[2-dimethylamine)ethyl]-N-[(11Z)-icos-11-en-1-yl]nonamide (Intermediate B)



To a 30 mL THF solution of N'-[(11Z)-icos-11-en-1-yl]-N, N-dimethylethane-1,2-diamine (2.50 g, 7.38 mmol) and nonanoic acid (1.29 g, 8.12 mmol) was added diisopropylethylamine (1.15 g, 8.86 mmol) and HATU (3.23 g, 8.49 mmol). The reaction stirred at RT for 1 hour, after which it was washed with 30 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 → 100% ethyl acetate) to give Intermediate **B** (3.02 g). LCMS (m + H): 479.7

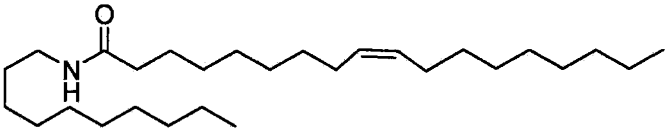
N-[(11Z)-icos-11-en-1-yl]- N' N'–dimethyl-N-nonylethane-1,2-diamine (Compound 1)



To 30 ml THF solution of N-[2-dimethylamine)ethyl]-N-[(11Z)-icos-11-en-1-yl]nonamide (3.02 g, 6.31 mmol) at 0°C was added lithium aluminum hydride 2.0 M solution in THF (6.31 mL, 12.6 mmol) slowly, dropwise. The reaction was stirred and allowed to gradually warm to

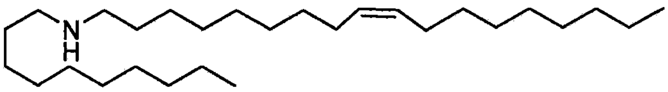
RT over 2 hours. The reaction was diluted in THF, and sodium sulfate dodecahydrate was added in small portions until fizzing stopped. Solids were removed by filtration through a Buchner funnel. The filtrate was concentrated to give N-[(11Z)-icos-11-en-1-yl]- N' N'–dimethyl-N-nonylethane-1,2-diamine (**1**) (1.89 g). HRMS (m + H): 463.4979. ^{1}H NMR δ (ppm)($CDCl_3$): 5.39 – 5.31 (2H, m), 2.56 – 2.53 (2H, m), 2.43 – 2.37 (6H, m) 2.24 – 2.22 (6H, m), 2.07 – 2.01 (4H, m), 1.81 (2H, m), 1.46 – 1.41 (4H, m), 1.40 – 1.20 (30H, m), 0.90 – 0.84 (6H, m).

(9Z)-N-decyoctadec-9-enamide (Intermediate C)



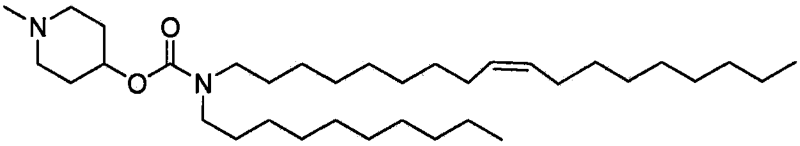
To a 500 mL DCM solution of) oleic acid (20.0 g, 70.8 mmol) was added CDI (11.48 g, 70.8 mmol), and the solution stirred for 1 hour at RT. Decylamine (11.14 g, 70.8 mmol) was added, and the reaction stirred another 16 hours at RT. The solution was washed with water and washed with brine. The organic layer was dried over Na_2SO_4, and purified by silica gel chromatography (0% ethyl acetate / hexane → 100% ethyl acetate) to give Intermediate **C** (13.0 g). LCMS (m + 1): 422.6

(9Z)-N-decyloctadec—en-1-amine (Intermediate D)



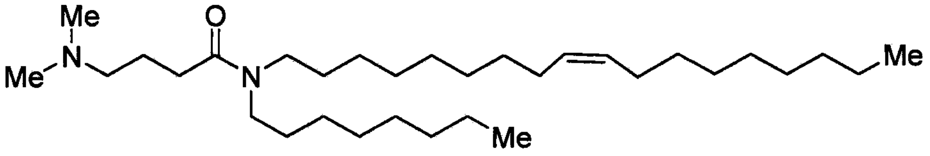
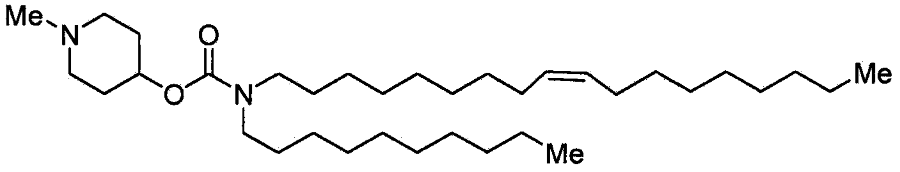
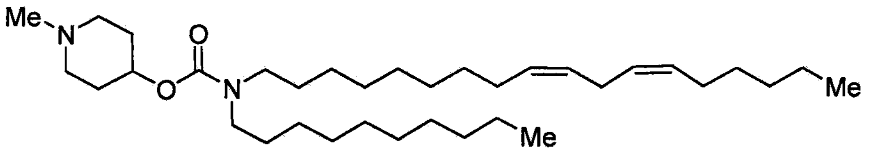
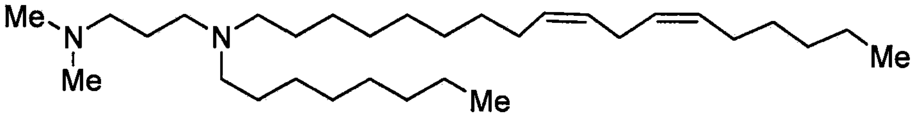
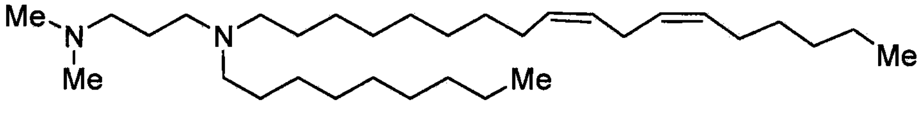
A 250 mL THF solution of (9Z)-N-decyoctadec-9-amide (28.1 g, 66.6 mmol) was cooled to 0°C in an ice bath. Litium aluminum hydride 2.0 M solution in THF (66.6 mL, 133 mmol) was added slowly, dropwise. The ice bath was removed, and the reaction was allowed to come to RT over 2 hours. The reaction was further diluted with 100 mL more of THF, and sodium sulfate dodecahydrate was added in small portions until fizzing stopped. Solids were removed using a Buchner funnel, and the filtrate was concentrated in vacuo to yield Intermediate **D** (26.7 g). LCMS (m + 1): 408.6

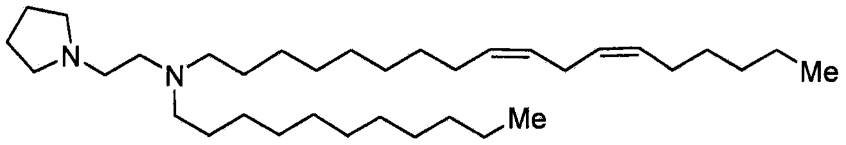
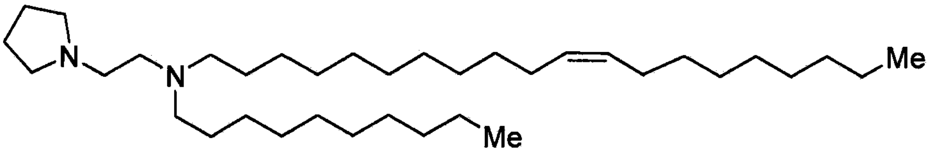
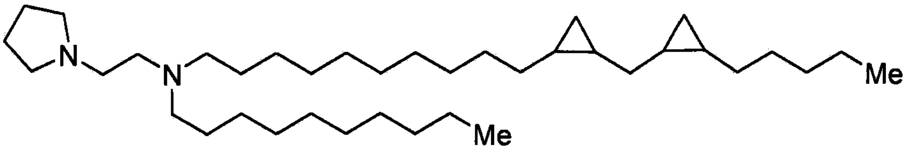
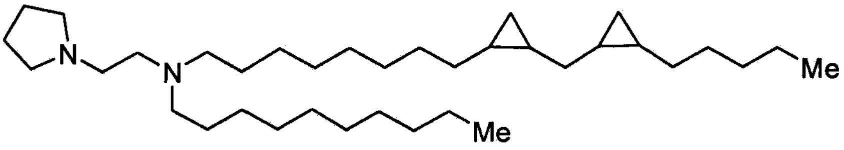
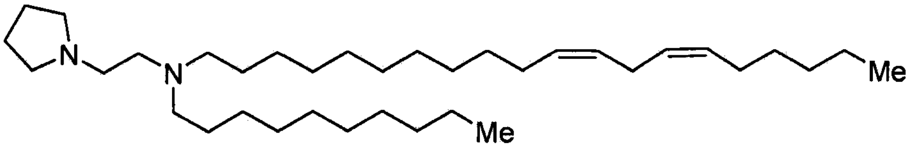
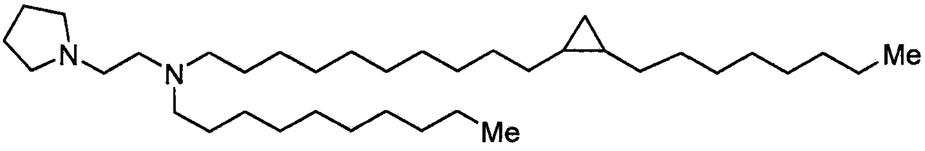
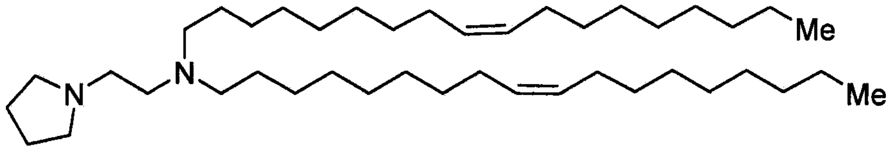
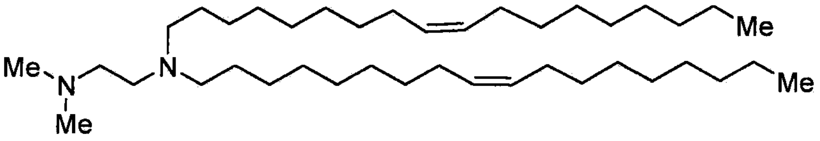
1-methylpiperidin-4-yl decyl[(9*Z*)-octadec-9-en-1-yl]carbamate (Compound 2)

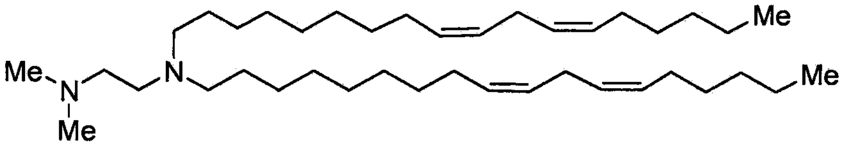
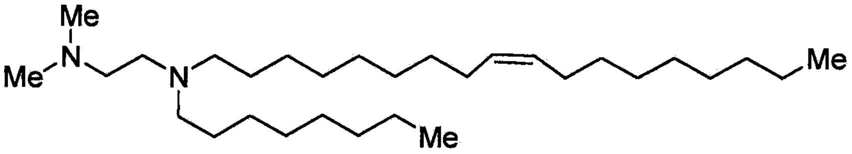
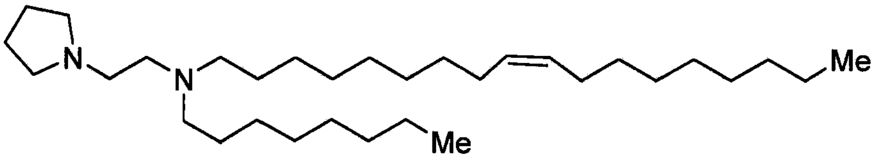
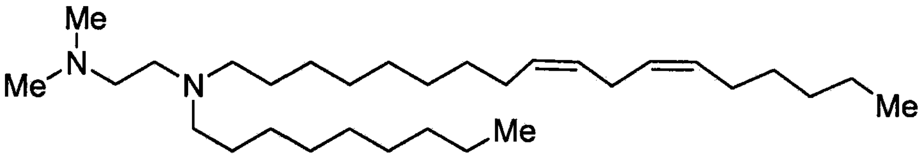
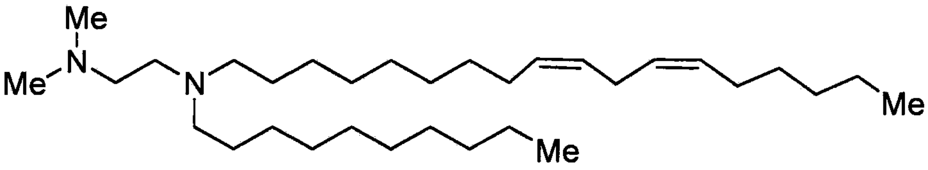
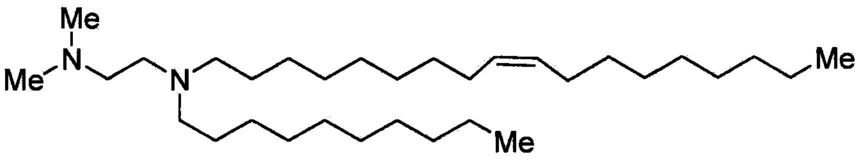
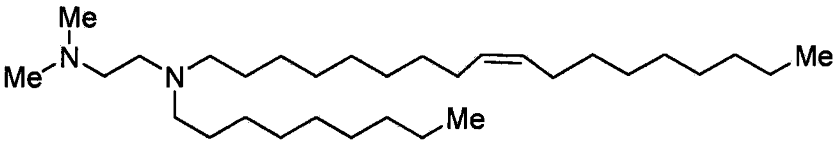
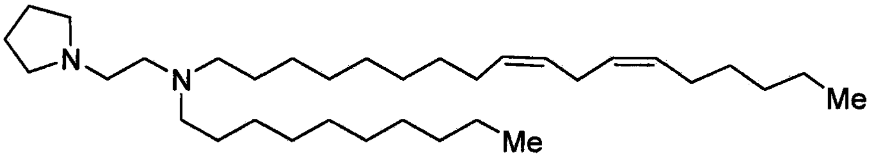


To a solution of 4-hydroxy-1-methylpiperidine (0.52 mL, 4.41 mmol) in 25 mL DCM was added triethylamine (1.03 mL, 7.36 mmol) at 0°C. 4-Nitrophenyl chloroformate (0.89 g, 4.41 mmol) in 3 mL of DCM was added dropwise. The cold bath was removed, and the reaction was warmed to RT over 4 hours. (9Z)-N-decyloctadec—en-1-amine was added, and the reaction was heated to 30°C for 19 hours. The reaction was cooled to RT, washed with water, washed with brine, dried over Na_2SO_4, and purified by silica gel chromatography (0% acetone / hexane → 15% acetone/hexane with 2% TEA) to yield compound (**2**) (0.90 g). HRMS (m + H): 549.5345. ^{1}H NMR δ (ppm)($CDCl_3$): 5.36 – 5.32 (2H, m), 4.73 – 4.71 (1H, m), 3.18 (4H, br s), 2.59 (2H, m), 2.29 (5H, m), 2.02 – 1.99 (4H, m), 1.94 – 1.90 (2H, m), 1.77 – 1.69 (2H), 1.55 – 1.50 (4H), 1.39 – 1.20 (38H, m), 0.88 – 0.86 (6H, m).

Compounds **3-23** were prepared in a manner analogous to that described for compounds 1 and 2 above:

Compound #	Structure
3	
4	
5	
6	
7	

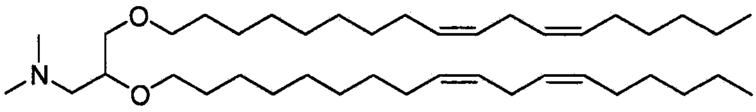
8	
9	
10	
11	
12	
13	
14	
15	

16	
17	
18	
19	
20	
21	
22	
23	

Compound #	IUPAC Name	Predicted HRMS (M+H)	Measured HRMS (M+H)
3	4-(dimethylamino)-*N*-[(9*Z*)-octadec-9-en-1-yl]-*N*-octylbutanamide	493.5091	493.5082
4	1-methylpiperidin-4-yl decyl[(9*Z*)-octadec-9-en-1-yl]carbamate	549.5354	549.5345
5	1-methylpiperidin-4-yl decyl[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yl]carbamate	547.5197	547.5190
6	*N*,*N*-dimethyl-*N'*-[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yl]-*N'*-octylpropane-1,3-diamine	463.4986	463.4965
7	*N*,*N*-dimethyl-*N'*-nonyl-*N'*-[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yl]propane-1,3-diamine	477.5142	477.5120
8	(9*Z*,12*Z*)-*N*-[2-(pyrrolidin-1-yl)ethyl]-*N*-undecyloctadeca-9,12-dien-1-amine	517.5461	517.5453
9	(11*Z*)-*N*-decyl-*N*-[2-(pyrrolidin-1-yl)ethyl]icos-11-en-1-amine	533.5768	533.5762
10	*N*-decyl-10-{2-[(2-pentylcyclopropyl)methyl]cyclopropyl}-*N*-[2-(pyrrolidin-1-yl)ethyl]decan-1-amine	559.5925	559.5921
11	*N*-(8-{2-[(2-pentylcyclopropyl)methyl]cyclopropyl}octyl)-*N*-[2-(pyrrolidin-1-yl)ethyl]decan-1-amine	531.5612	531.5611
12	(11*Z*,14*Z*)-*N*-decyl-*N*-[2-(pyrrolidin-1-yl)ethyl]icosa-11,14-dien-1-amine	531.5539	531.5610
13	*N*-decyl-10-(2-octylcyclopropyl)-*N*-[2-(pyrrolidin-1-yl)ethyl]decan-1-amine	547.5852	547.5941
14	(9*Z*)-*N*-[(9*Z*)-octadec-9-en-1-yl]-*N*-[2-(pyrrolidin-1-yl)ethyl]octadec-9-en-1-amine	615.6551	615.6570
15	*N*,*N*-dimethyl-*N'*,*N'*-di[(9*Z*)-octadec-9-en-1-yl]ethane-1,2-diamine	589.6394	589.6413
16	*N*,*N*-dimethyl-*N'*,*N'*-di[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yl]ethane-1,2-diamine	585.6081	585.6099
17	*N*,*N*-dimethyl-*N'*-[(9*Z*)-octadec-9-en-1-yl]-*N'*-octylethane-1,2-diamine	451.4986	451.4992
18	(9*Z*)-*N*-octyl-*N*-[2-(pyrrolidin-1-yl)ethyl]octadec-9-en-1-amine	477.5142	477.5148

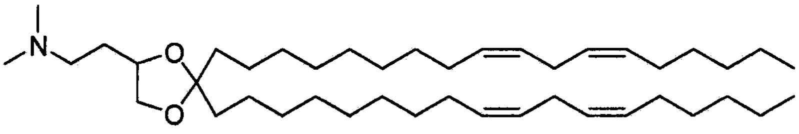
19	*N*,*N*-dimethyl-*N'*-nonyl-*N'*-[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yl]ethane-1,2-diamine	463.4986	463.4979
20	*N*-decyl-*N'*,*N'*-dimethyl-*N*-[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yl]ethane-1,2-diamine	477.5142	477.5138
21	*N*-decyl-*N'*,*N'*-dimethyl-*N*-[(9*Z*)-octadec-9-en-1-yl]ethane-1,2-diamine	479.5299	479.5294
22	*N*,*N*-dimethyl-*N'*-nonyl-*N'*-[(9*Z*)-octadec-9-en-1-yl]ethane-1,2-diamine	465.5142	465.5138
23	(9*Z*,12*Z*)-*N*-decyl-*N*-[2-(pyrrolidin-1-yl)ethyl]octadeca-9,12-dien-1-amine	503.5299	503.5291

Compound 24 is DLinDMA as described in *J. Controlled Release*, 2005, *107*, 276-287, US 2006/0083780 A1, and US 2006/0008910 A1.



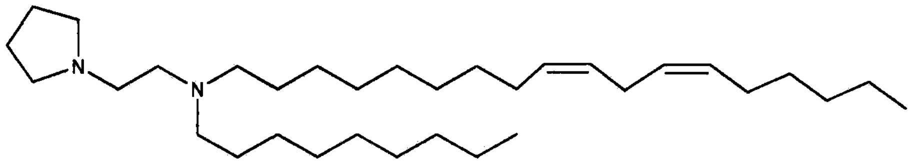
(24)

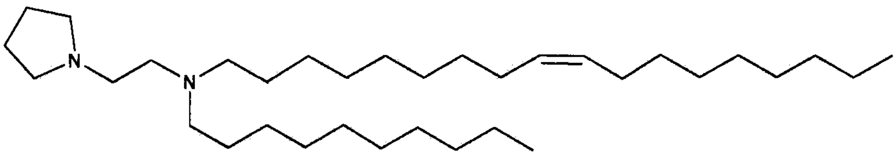
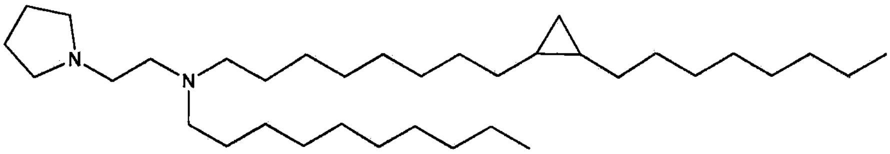
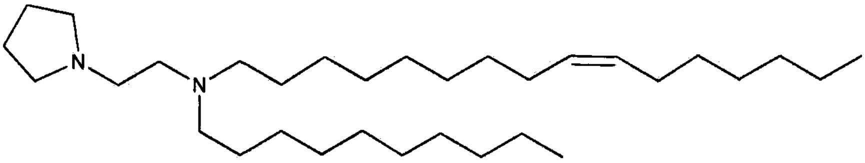
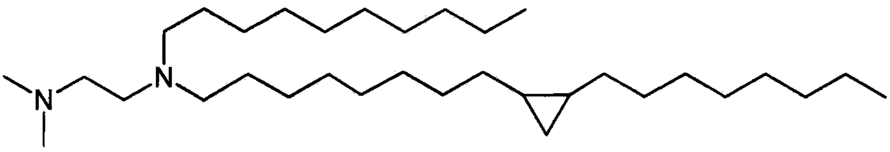
Compound 25 is DLinKC2DMA as described in *Nature Biotechnology*, 2010, *28*, 172-176, WO 2010/042877 A1, WO 2010/048536 A2, WO 2010/088537 A2, and WO 2009/127060 A1.



(25)

Compounds 26-30 were prepared in a manner analogous to that described for compounds 1 and 2 above:

Compound #	**Structure**
26	

27	
28	
29	
30	

Compound#	Name	Predicted HRMS (M+H)	Measured HRMS (M+H)
26	(9Z,12Z)-N-nonyl-N-[2-(pyrrolidin-1-yl)ethyl]octadeca-9,12-dien-1-amine		N/A[1]
27	(9Z)-N-decyl-N-[2-(pyrrolidin-1-yl)ethyl]octadec-9-en-1-amine		N/A[2]
28	N-[8-(2-octylcyclopropyl)octyl]-N-[2-(pyrrolidin-1-yl)ethyl]decan-1-amine	519.5612	519.5613
29	(9Z)-N-decyl-N-[2-(pyrrolidin-1-yl)ethyl]hexadec-9-en-1-amine	477.5142	477.5141
30	N-decyl-N',N'-dimethyl-N-[8-(2-octylcyclopropyl)octyl]ethane-1,2-diamine		N/A[3]

1. No HRMS data available. Compound confirmed by HPLC.

1. No HRMS data available. 1H NMR d (ppm)(400 MHz, CDCl3): 5.36-5.34 (m, 2H), 2.61-2.51 (m, 8H), 2.44-2.40 (m, 4H), 2.04-1.97 (m, 4H), 1.79-1.76 (m, 4H), 1.46-1.41 (m, 4H), 1.26 (m, 36H), 0.88 (t, J = 6.68 Hz, 6H).

3. No HRMS data available. LC/MS (M+H) = 493.8.

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.

$$\text{Encapsulation rate} = (1 - \text{free siRNA/total siRNA}) \times 100\%$$

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***CU***A***U***GAAGAGA***U***ATT**-iB 3' (SEQ.ID.NO.:1)
3'-UU*U*A*UUCC*GA*U*A*CUUCUC*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 Compound **1-23**, 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.

Similar efficacy was observed in mice dosed with Compound **1** containing RDVs than with the RDV containing the DLinDMA (Compound **24**) 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 gauge 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 and improved tolerability were observed in rats dosed with Compound **1** containing RDV than with the RDV containing the cationic lipid DLinKC2DMA (Compound **25**, Figure 2).

Determination of Cationic Lipid Levels in Rat Liver

Liver tissue was weighed into 20-ml vials and homogenized in 9 v/w of water using a GenoGrinder 2000 (OPS Diagnostics, 1600 strokes/min, 5min). A 50 μL aliquot of each tissue homogenate was mixed with 300 μL of extraction/protein precipitating solvent

(50/50 acetonitrile/methanol containing 500 nM internal standard) and the plate was centrifuged to sediment precipitated protein. A volume of 200 μL of each supernatant was then transferred to separate wells of a 96-well plate and 10 μl samples were directly analyzed by LC/MS-MS.

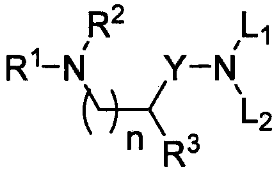
Standards were prepared by spiking known amounts of a methanol stock solution of Compound **1** or OCD (OCD is known and described in WO2010/021865) into untreated rat liver homogenate (9 vol water/weight liver). Aliquots (50 μL) each standard/liver homogenate was mixed with 300 μL of extraction/protein precipitating solvent (50/50 acetonitrile/methanol containing 500 nM internal standard) and the plate was centrifuged to sediment precipitated protein. A volume of 200 μL of each supernatant was transferred to separate wells of a 96-well plate and 10 μl of each standard was directly analyzed by LC/MS-MS.

Absolute quantification versus standards prepared and extracted from rat liver homogenate was performed using an Aria LX-2 HPLC system (Thermo Scientific) coupled to an API 4000 triple quadrupole mass spectrometer (Applied Biosystems). For each run, a total of 10 μL sample was injected onto a BDS Hypersil C8 HPLC column (Thermo, 50 x 2mm, 3 μm) at ambient temperature (Figure 3).

Mobile Phase A: 95% H2O/5% methanol/10 mM ammonium formate/0.1%formic acid *Mobile Phase B:* 40% methanol/60% n-propanol/10 mM ammonium formate/0.1%formic acid The flow rate was 0.5 mL/min and gradient elution profile was as follows: hold at 80% A for 0.25 min, linear ramp to 100% B over 1.6 min, hold at 100% B for 2.5 min, then return and hold at 80% A for 1.75 min. Total run time was 5.8 min. API 4000 source parameters were CAD: 4, CUR: 15, GS1: 65, GS2: 35, IS: 4000, TEM: 550, CXP: 15, DP: 60, EP: 10.

WHAT IS CLAIMED IS:

1. A cationic lipid of Formula A:



A

wherein:

n is 1 or 2;

Y is a bond, O(C=O), C=O, or NR'(C=O);

R^1 and R^2 are independently selected from H, $(C_1\text{-}C_4)$alkyl, heterocyclyl, and a polyamine, wherein said alkyl, heterocyclyl and polyamine are optionally substituted with one or more 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 heterocyclyl with 4-7 members optionally containing, in addition to the nitrogen, one or two additional heteroatoms selected from N, O and S, said monocyclic heterocyclyl is optionally substituted with one or more substituents selected from R';

R^3 is absent or can be taken together with R^1 to form a monocyclic heterocyclyl with 4-7 members optionally containing, in addition to the nitrogen, one or two additional heteroatoms selected from N, O and S, said monocyclic heterocyclyl is optionally substituted with one or more substituents selected from R';

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

R" is selected from H and $(C_1\text{-}C_4)$alkyl, wherein said alkyl is optionally substituted with one or more substituents selected from halogen and OH;

L_1 is a C_4-C_{22} alkyl or C_4-C_{22} alkenyl, said alkyl and alkenyl are optionally substituted with one or more substituents selected from R'; and

L_2 is a C_4-C_{22} alkyl or C_4-C_{22} 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:

n is 1;

Y is a bond;

R^1 and R^2 are independently selected from H, $(C_1\text{-}C_4)$alkyl, heterocyclyl, and a polyamine, wherein said alkyl, heterocyclyl and polyamine are optionally substituted with one or more 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 heterocyclyl with 4-7 members optionally containing, in addition to the nitrogen, one or two additional heteroatoms selected from N, O and S, said monocyclic heterocyclyl is optionally substituted with one or more substituents selected from R';

R^3 is absent or can be taken together with R^1 to form a monocyclic heterocyclyl with 4-7 members optionally containing, in addition to the nitrogen, one or two additional heteroatoms selected from N, O and S, said monocyclic heterocyclyl is optionally substituted with one or more substituents selected from R';

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

R'' is selected from H and $(C_1\text{-}C_4)$alkyl, wherein said alkyl is optionally substituted with one or more substituents selected from halogen and OH;

L_1 is a $C_4\text{-}C_{22}$ alkyl or $C_4\text{-}C_{22}$ alkenyl, said alkyl and alkenyl are optionally substituted with one or more substituents selected from R'; and

L_2 is a $C_4\text{-}C_{22}$ alkyl or $C_4\text{-}C_{22}$ alkenyl, said alkyl and alkenyl are optionally substituted with one or more substituents selected from R';

or any pharmaceutically acceptable salt or stereoisomer thereof.

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

wherein:

n is 1;

Y is a O(C=O);

R^1 and R^2 are independently selected from H, $(C_1\text{-}C_4)$alkyl, heterocyclyl, and a polyamine, wherein said alkyl, heterocyclyl and polyamine are optionally substituted with one or more 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 heterocyclyl with 4-7 members optionally containing, in addition to the nitrogen, one or two additional heteroatoms selected from N, O and S, said monocyclic heterocyclyl is optionally substituted with one or more substituents selected from R';

R^3 is absent or can be taken together with R^1 to form a monocyclic heterocyclyl with 4-7 members optionally containing, in addition to the nitrogen, one or two additional heteroatoms selected from N, O and S, said monocyclic heterocyclyl is optionally substituted with one or more substituents selected from R';

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

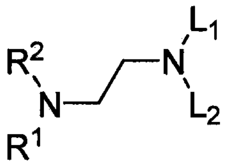
R'' is selected from H and $(C_1\text{-}C_4)$alkyl, wherein said alkyl is optionally substituted with one or more substituents selected from halogen and OH;

L_1 is a C_4-C_{22} alkyl or C_4-C_{22} alkenyl, said alkyl and alkenyl are optionally substituted with one or more substituents selected from R'; and

L_2 is a C_4-C_{22} alkyl or C_4-C_{22} alkenyl, said alkyl and alkenyl are optionally substituted with one or more substituents selected from R';

or any pharmaceutically acceptable salt or stereoisomer thereof.

4. A cationic lipid of Formula B:



B

wherein:

R^1 and R^2 are independently selected from H, (C_1-C_4)alkyl, heterocyclyl, and a polyamine, wherein said alkyl, heterocyclyl and polyamine are optionally substituted with one or more 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 heterocyclyl with 4-7 members optionally containing, in addition to the nitrogen, one or two additional heteroatoms selected from N, O and S, said monocyclic heterocyclyl is optionally substituted with one or more substituents selected from R';

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

R" is selected from H and (C_1-C_4)alkyl, wherein said alkyl is optionally substituted with one or more substituents selected from halogen and OH;

L_1 is a C_4-C_{22} alkyl or C_4-C_{22} alkenyl, said alkyl and alkenyl are optionally substituted with one or more substituents selected from R'; and

L_2 is a C_4-C_{22} alkyl or C_4-C_{22} alkenyl, said alkyl and alkenyl are optionally substituted with one or more substituents selected from R';

or any pharmaceutically acceptable salt or stereoisomer thereof.

5. A cationic lipid which is:

N-[(11Z)-icos-11-en-1-yl]- N' N'–dimethyl-N-nonylethane-1,2-diamine;
1-methylpiperidin-4-yl decyl[(9Z)-octadec-9-en-1-yl]carbamate;
4-(dimethylamino)-N-[(9Z)-octadec-9-en-1-yl]-N-octylbutanamide;
1-methylpiperidin-4-yl decyl[(9Z)-octadec-9-en-1-yl]carbamate;
1-methylpiperidin-4-yl decyl[(9Z,12Z)-octadeca-9,12-dien-1-yl]carbamate;
N,N-dimethyl-N'-[(9Z,12Z)-octadeca-9,12-dien-1-yl]-N'-octylpropane-1,3-diamine;
N,N-dimethyl-N'-nonyl-N'-[(9Z,12Z)-octadeca-9,12-dien-1-yl]propane-1,3-diamine;
(9Z,12Z)-N-[2-(pyrrolidin-1-yl)ethyl]-N-undecyloctadeca-9,12-dien-1-amine;

(11Z)-N-decyl-N-[2-(pyrrolidin-1-yl)ethyl]icos-11-en-1-amine;
N-decyl-10-{2-[(2-pentylcyclopropyl)methyl]cyclopropyl}-N-[2-(pyrrolidin-1-yl)ethyl]decan-1-amine;
N-(8-{2-[(2-pentylcyclopropyl)methyl]cyclopropyl}octyl)-N-[2-(pyrrolidin-1-yl)ethyl]decan-1-amine;
(11Z,14Z)-N-decyl-N-[2-(pyrrolidin-1-yl)ethyl]icosa-11,14-dien-1-amine;
N-decyl-10-(2-octylcyclopropyl)-N-[2-(pyrrolidin-1-yl)ethyl]decan-1-amine;
(9Z)-N-[(9Z)-octadec-9-en-1-yl]-N-[2-(pyrrolidin-1-yl)ethyl]octadec-9-en-1-amine;
N,N-dimethyl-N',N'-di[(9Z)-octadec-9-en-1-yl]ethane-1,2-diamine;
N,N-dimethyl-N',N'-di[(9Z,12Z)-octadeca-9,12-dien-1-yl]ethane-1,2-diamine;
N,N-dimethyl-N'-[(9Z)-octadec-9-en-1-yl]-N'-octylethane-1,2-diamine;
(9Z)-N-octyl-N-[2-(pyrrolidin-1-yl)ethyl]octadec-9-en-1-amine;
N,N-dimethyl-N'-nonyl-N'-[(9Z,12Z)-octadeca-9,12-dien-1-yl]ethane-1,2-diamine;
N-decyl-N',N'-dimethyl-N-[(9Z,12Z)-octadeca-9,12-dien-1-yl]ethane-1,2-diamine;
N-decyl-N',N'-dimethyl-N-[(9Z)-octadec-9-en-1-yl]ethane-1,2-diamine;
N,N-dimethyl-N'-nonyl-N'-[(9Z)-octadec-9-en-1-yl]ethane-1,2-diamine;
(9Z,12Z)-N-decyl-N-[2-(pyrrolidin-1-yl)ethyl]octadeca-9,12-dien-1-amine;
(9Z,12Z)-N-nonyl-N-[2-(pyrrolidin-1-yl)ethyl]octadeca-9,12-dien-1-amine;
(9Z)-N-decyl-N-[2-(pyrrolidin-1-yl)ethyl]octadec-9-en-1-amine;
N-[8-(2-octylcyclopropyl)octyl]-N-[2-(pyrrolidin-1-yl)ethyl]decan-1-amine;
(9Z)-N-decyl-N-[2-(pyrrolidin-1-yl)ethyl]hexadec-9-en-1-amine; and
N-decyl-N',N'-dimethyl-N-[8-(2-octylcyclopropyl)octyl]ethane-1,2-diamine;

or any pharmaceutically acceptable salt or stereoisomer thereof.

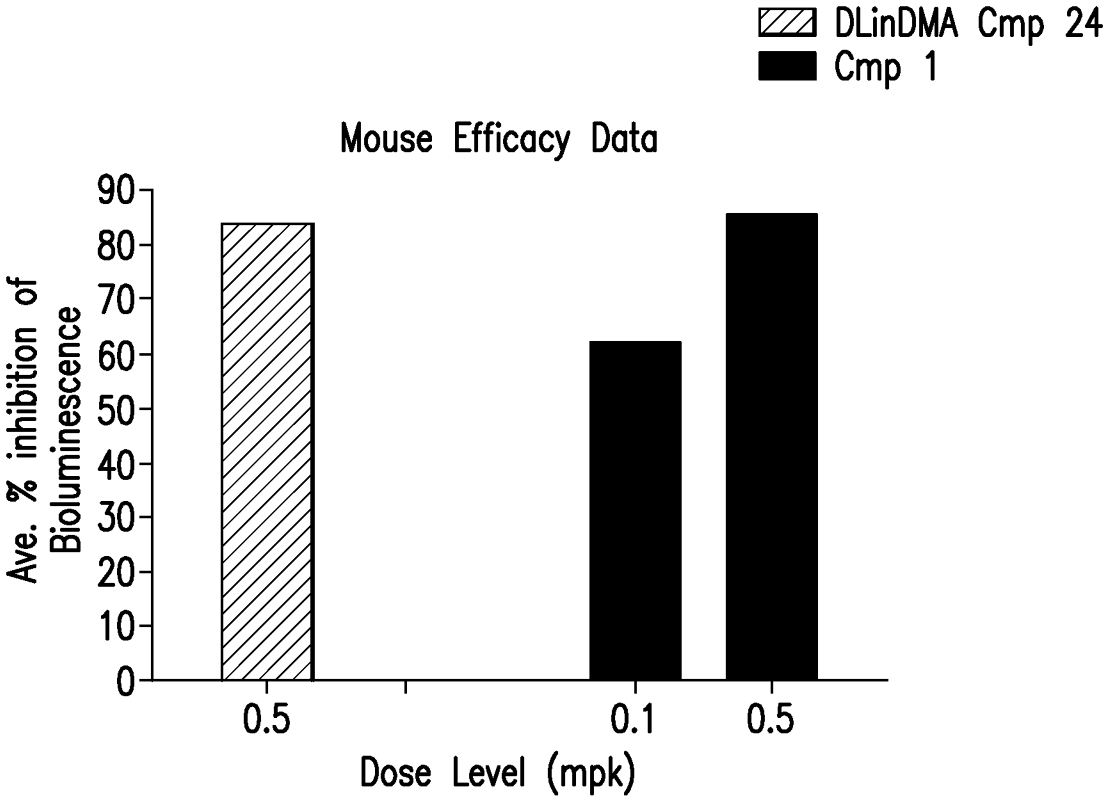

DLinDMA Cmp 24
Cmp 1
Mouse Efficacy Data
Ave. % inhibition of Bioluminescence
90
80
70
60
50
40
30
20
10
0
0.5
0.1
0.5
Dose Level (mpk)

FIG.1

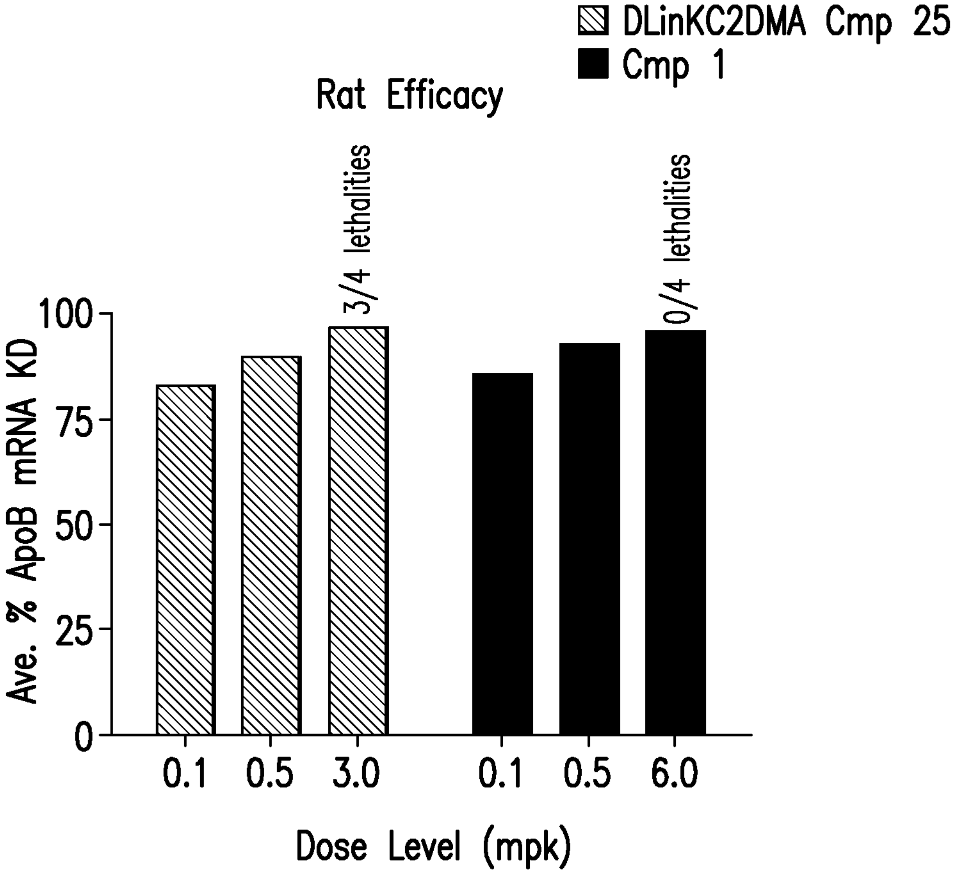

▨ DLinKC2DMA Cmp 25
■ Cmp 1
Rat Efficacy
3/4 lethalities
0/4 lethalities
Ave. % ApoB mRNA KD
100
75
50
25
0
0.1
0.5
3.0
0.1
0.5
6.0
Dose Level (mpk)

FIG.2

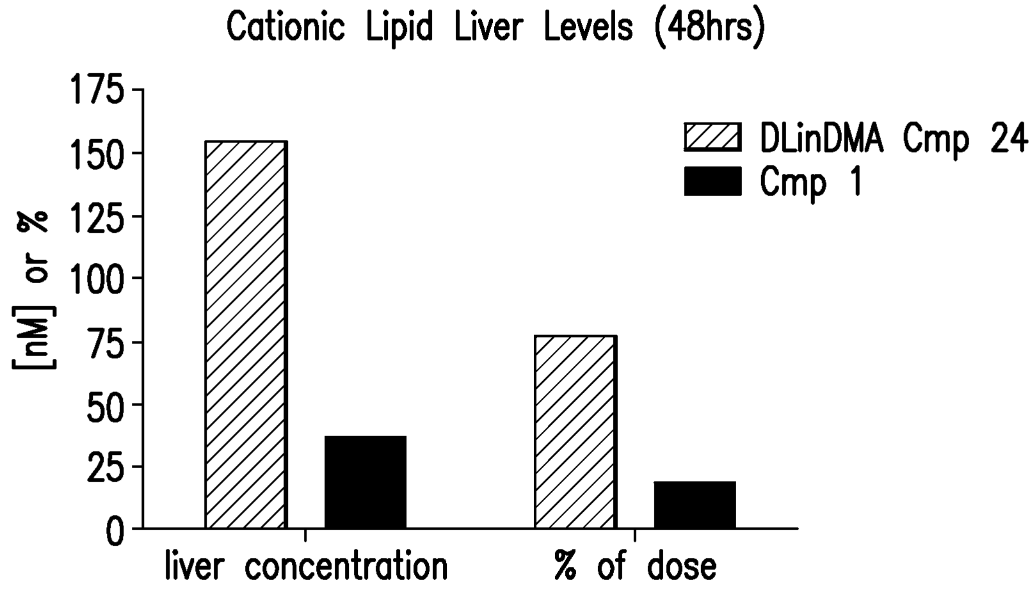

Cationic Lipid Liver Levels (48hrs)
[nM] or %
175
150
125
100
75
50
25
0
DLinDMA Cmp 24
Cmp 1
liver concentration
% of dose

FIG.3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/46990

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C11C 3/00; C12N 15/00; C12N 15/11 (2012.01)
USPC - 554/58
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)
USPC: 554/58

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 435/455, 514/44A (keyword limited; terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST (USPT, PGPB, EPAB, JPAB), Google Patents/Scholar
Search Terms Used: Cationic lipid, lipopolyamine, diamine, nitrogen, transfection, carbamate, piperidine

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 6,281,371 B1 (Klosel et al.) 28 August 2001 (28.08.2001) col 7, ln 45-56, col 8, ln 15-21, col 9, ln 5-15, col 12, ln 5-10,	1, 2, 4 --------- 3, 5
Y	US 6,171,612 B1 (Byk et al.) 09 January 2001 (09.01.2001) abstract, col 4,	3
Y	US 2003/0229037 A1 (Massing et al.) 11 December 2003 (11.12.2003) abstract, para [0069]	5

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

Date of the actual completion of the international search 19 September 2012 (19.09.2012)	Date of mailing of the international search report 01 OCT 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)