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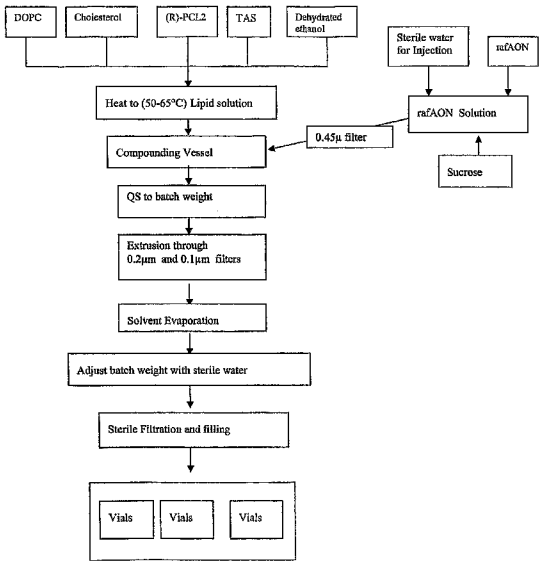
(54) **Title:** METHOD OF ADMINISTERING LIPOSOMES CONTAINING OLIGONUCLEOTIDES



(57) **Abstract:** The invention provides a method of treating a cellular proliferative disease, a method of inhibiting the growth of neoplastic cells and a method of reducing the expression of a raf sequence by administering an effective amount of a cationic liposomal composition. The cationic liposomal composition comprises c-raf Antisense Oligonucleotide (c-rafAON), a cationic cardiolipin analogue and, desirably, another lipid species. The preferred cationic cardiolipin analogue is 1,3-Bis-(1,2-bis-tetradecyloxy-propyl-3- dimethylethoxyammoniumbromide)-propane-2-ol (PCL-2). The preferred lipids include cholesterol, dioleoylphosphatidylethanolamine (DOPE), 1,2 Dioleoyl-sn-glycero-3- phosphocholine (DOPC), alpha tocopheryl acid succinate and phosphatidylcholine. The effective amount of the cationic liposomal composition is about 60 mg/m^2.

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METHOD OF ADMINISTERING LIPOSOMES CONTAINING OLIGONUCLEOTIDES

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Cross Reference to Related Applications

This Application claims the benefit of U.S. Provisional Application Number 60/701,775, filed on July 22, 2005, the disclosure of which is incorporated herein.

Field of the Inventions

This invention provides a method of treating a cellular proliferative disease and a method of inhibiting the growth of neoplastic cells by administering an effective amount of liposomal encapsulated oligonucleotides or combination of oligonucleotides. More specifically, the invention provides a method of reducing the expression of a raf sequence by administering an effective amount of cationic liposomal formulations of raf oligonucleotides, wherein said effective amount is about 60 mg/m^2.

Background of the Invention

Polynucleotides have potential use as therapeutic agents. For example, antisense oligonucleotides targeted to oncogenes have shown promise in neoplastic diseases, such as cancer. An exemplary agent, an antisense Oligonucleotide (AON) targeted to the mRNA of human c-raf oncogene, is called c-rafAON. c-rafAON, an antineoplastic agent used for the treatment of radiation resistant tumors, multiple myelomas and other cancers, acts by hybridizing with mRNA to inhibit translation, protein synthesis and tumor growth. Plasmids or other vectors containing the gene of interest also have therapeutic potential by being able to deliver transgenes of interest to patients.

While promising in their therapeutic potential, oligonucleotide agents cannot effectively penetrate cell membranes due to their negative charge. To address this issue, cationic liposomes have been used to improve cell transfection and increase therapeutic efficacy. *See* U.S. Patent Nos. 6,559,129 B1 and 6,333,314 B1. *See also* Zelphati *et.al.* J. Liposome Res., 7(1):31-49 (1997)). Several drawbacks, however, limit the usefulness of these liposomes as transfection agents. Due to the inflexibility of their cationic bilayer, many of these delivery systems exhibit poor chemical stability and inadequate drug loading.

Accordingly, these delivery agents are unsuitable for transfection with different types of polynucleotides.

A need also exists for an effective method of administering the polynucleotide species to oncogenes to inhibit the growth of neoplastic cells.

Brief Summary of the Invention

The invention provides a method of administering a cationic liposomal composition comprising c-raf Antisense Oligonucleotide (c-rafAON), a cationic cardiolipin analogue and, desirably, another lipid species. The preferred cationic cardiolipin analogue (CCLA) is 1,3-Bis-(1,2-bis-tetradecyloxy-propyl-3-dimethylethoxyammoniumbromide)-propane-2-ol (PCL-2) and the preferred additional lipids include cholesterol, dioleoylphosphatidylethanolamine (DOPE), 1,2 Dioleoyl-sn-glycero-3-phosphocholine (DOPC), alpha tocopheryl acid succinate and any other phosphatidylcholine.

The method of administration comprises administering an effective amount of the composition to inhibit the growth of neoplastic cells and/or reduce the expression of a raf sequence. Such administration includes the intravenous infusion of LErafAON once weekly for three consecutive weeks at dose levels of about 60 mg/m^2.

These and other advantages of the invention, as well as additional inventive features, will be apparent from the description of the invention provided herein.

Brief Description of the Figures

FIG. 1 illustrates the manufacturing process for LErafAON-ETU;

FIG. 2 illustrates the mean ± SEM plasma concentration- time profiles of rafAON following an IV administration of LErafAON-ETU or LErafAON (two vial) at a comparable dose to male CD2F1 mice;

FIG. 3 illustrates the mean ± SEM plasma concentration- time profiles of rafAON following an IV administration of LErafAON-ETU or LErafAON at a comparable dose to male cynomolgus monkeys on day 1;

FIG. 4 illustrate transfection efficiency of PCL-2-(CCLA)-based liposomes using β-galactosidase (β-gal) reporter gene assay for CHO, BALB/3T3 and $HepG_2$ cells and lucerifase reporter gene assay for MX-1 and A549 cells;

FIG. 5 illustrates the expression of lucerifase in both lung and heart tissues; and

FIG. 6 illustrates luceriface gene expression in lungs when delivered alone, by in vivo Gene SHUTTLE™ and by CCLA-based liposome.

Detailed Description of the Invention

In one embodiment, the invention provides a composition, comprising a cationic cardiolipin analogue (CCLA) and, desirably, another lipid species. Suitable cationic cardiolipin analogues for use in the inventive composition are described in international patent application PCT/US03/33099, which is incorporated herein by reference. A most preferred cationic cardiolipin analogue (CCLA) for inclusion in the inventive composition is 1,3-Bis-(1,2-bis-tetradecyloxy-propyl-3-dimethylethoxyammoniumbromide)-propane-2-ol (PCL-2), which is identified in PCT/US03/33099 as compound 19.

The use of novel cationic liposomes comprising cationic cardiolipin analogues, 1,3-Bis-(1,2-bis-tetradecyloxy-propyl-3-dimethylethoxyammoniumbromide)-propane-2-ol (PCL-2) can facilitate transfection of a wide variety of polynucleotide species and are effective in promoting transfection of primary cell cultures as well as transformed cells. Also, the inventive transfection agent is suitable for both *in vitro* and *in vivo* use. The inventive composition can also be used for the delivery of a variety of active agents including dermatological, cosmetic, and agricultural agents.

As noted herein, any suitable amount of cationic cardiolipin analogue (CCLA) can be used in the composition, desirably, the cationic cardiolipin analogue PCL-2 is present between about 0.3 mM and about 20 mM, such as between about 1 mM and about 15 mM, but the composition can include more or less cationic cardiolipin PCL-2 as desired. In certain preferred compositions, the cationic cardiolipin PCL-2 is present at about 0.35 mM.

Desirably, the composition further comprises at least one lipid in addition to the cationic cardiolipin analogue. The additional lipid(s) can be any desired lipid species suitable for forming the composition of interest, such as those described in PCT/US03/33099. Preferred lipids for inclusion in the inventive composition are selected from the group of lipids consisting of cholesterol, cholesterol derivatives, dioleoylphosphatidylethanolamine (DOPE), 1,2 Dioleoyl-sn-glycero-3-phosphocholine (DOPC), alpha tocopheryl acid succinate and any other phosphatidylcholine. Typically, the total lipid concentration of the composition is between about 1.0 mg/mL and about 60 mg/mL (such as at least about 5.0 mg/mL or at least about 10 mg/mL or even at least about

10 mg/mL and up to about 50 mg/mL, or up to about 40 mg/mL or even up to about 30 mg/mL) but can be higher or lower than these amounts as desired.

A most preferred composition in accordance with the present invention includes DOPC, cationic cardiolipin PCL-2, and cholesterol. In this composition, the cationic cardiolipin analogue, DOPC, and cholesterol can be present in any suitable proportion. However, such a composition preferably has a percent molar ratio of DOPC:PCL-2:cholesterol of between about (50-65):(25-35):(5-20). Also, desirably such a composition further includes D-alpha tocopheryl acid succinate. The D-alpha tocopheryl acid succinate can be present in any suitable amount in the composition, but desirably is present at between about 0.1 wt % and about 1 wt %, most preferably at about 0.2 wt%. However, the amount of D-alpha tocopheryl acid succinate can be more or less than these amounts, as desired.

Another preferred composition according to the invention includes cationic cardiolipin PCL-2 and cholesterol. Within such a composition, the cationic cardiolipin and cholesterol can be present in any suitable molar ratio. However, desirably the molar ratio of PCL-2 and cholesterol is between 1:3 and 6:1, such as between 1:3 and 3:1, or between 2:3 and 3:2, or even between 1:2 and 2:1.

Still another preferred composition according to the invention includes PCL-2 and DOPE. Within such a composition, the cationic cardiolipin and DOPE can be present in any suitable molar ratio. However, desirably the molar ratio of PCL-2 and DOPE is between 1:3 and 6:1, such as between 1:3 and 3:1, or between 2:3 and 3:2, or even between 1:2 and 2:1.

In addition to the cationic cardiolipin and other lipids, the composition can include stabilizers, absorption enhancers, antioxidants, phospholipids, biodegradable polymers and medicinally active agents among other ingredients. Suitable antioxidants include compounds such as ascorbic acid, tocopherol, and deteroxime mesylate. Suitable absorption enhancers include Na-salicylate-chenodeoxy cholate, Na deoxycholate, polyoxyethylene 9-lauryl ether, chenodeoxy cholate-deoxycholate and polyoxyethylene 9-lauryl ether, monolein, Na tauro-24,25-dihydrofusidate, Na taurodeoxycholate, Na glycochenodeoxycholate, oleic acid, linoleic acid, and linolenic acid. Polymeric absorption enhancers can also be included such as polyoxyethylene ethers, polyoxyethylene sorbitan esters, polyoxyethylene 10-lauryl ether, polyoxyethylene 16-lauryl ether, and azone (1-dodecylazacycloheptane-2-one).

In some embodiments, it is preferable for the inventive composition, especially liposomal composition to include a targeting agent, such as carbohydrate or a protein or other ligand that binds to a specific substrate, such as antibodies (or fragments thereof) or ligands that recognize cellular receptors. The inclusion of such agents (such as a carbohydrate or one or more proteins selected from groups of proteins consisting of antibodies, antibody fragments, peptides, peptide hormones, receptor ligands such as an antibody to a cellular receptor and mixtures thereof) can facilitate targeting the composition to a predetermined tissue or cell type.

In a preferred embodiment, the composition includes at least one sugar or biodegradable polymer. Examples of suitable sugars and polymers include sucrose, lactose, trehalose, dextrose, epichlorohydrin, branched hydrophilic polymers of sucrose, polyethylene glycols, polyvinyl alcohols, methoxypolyethylene glycol, ethoxypolyethylene glycol, polyethylene oxide, polyoxyethylene, polyoxypropylene, polyvinylpyrolidonepolypyrolidone, dextran, cellulose acetate, sodium alginate, N, N-diethylaminoacetate, block copolymers of polyoxyethylene and polyoxypropylene, polyvinyl pyrrolidone, polyoxyethylene X-lauryl ether wherein X is from 9 to 20, and polyoxyethylene sorbitan esters. A preferred sugar for inclusion in the inventive composition is sucrose. Where the sugar or polymer is present, it typically represents between about 1wt% and about 20 wt% of the composition, such as between about 5 wt% and about 30 wt%. Especially where the sugar is sucrose, a suitable amount of sugar for inclusion in the composition is 10-12 wt%. Additionally, the composition also can include carriers, such as physiologically acceptably carriers. Suitable carriers for physiological (e.g., medical, veterinary, experimental, etc.) uses are physiologically-compatible buffers (e.g., phosphate-buffered saline (PBS), HEPES, etc.), many of which are known to those of ordinary skill in the art.

The composition typically has a pH of between about 3 and about 8. However, the pH of the composition can vary considerably depending on its desired use. It is within the ordinary skill of the art to select a suitable pH for a desired use. Thus, in some embodiments, the pH of the composition can be adjusted to be acidic (e.g., between about 2 and about 6.9, such as between about 3 and about 6 or between about 4 and about 5). In other applications, it is desirable for the pH of the composition to be alkaline (e.g., between about 7.1 and about 11, or between about 8 and about 10, such as about 9). The pH of the

composition can be adjusted using suitable acidic or alkaline buffers as are known to those of ordinary skill in the art.

The composition can be of any suitable form, such as liposomal formulations, complexes, emulsions, suspensions, etc. Such formulations can be prepared by any suitable technique, depending on the type of composition, which are known to those of ordinary skill in the art. A preferred composition is a liposomal composition or other composition containing lipid vesicles. Such composition can include unilamellar or multilamellar vesicles, or mixtures thereof. Any suitable technique can be employed to produce such a liposomal formulation. Suitable techniques include the thin-film hydration method, reverse phase evaporation, ethanol injection, etc. For example, lipophilic liposome-forming ingredients can be dissolved or dispersed in a suitable solvent or combination of solvents and dried. Suitable solvents include any non-polar or slightly polar solvent, such as *t*-butanol, ethanol, methanol, chloroform, or acetone that can be evaporated without leaving a pharmaceutically unacceptable residue. Drying can be by any suitable means such as lyophilization, use of a rotary dryer, etc. Hydrophilic ingredients, such as some pharmaceutical agents, preservatives, and other agents, can be dissolved in polar solvents, including water, which can be mixed with the lipid phase either prior to drying or upon reconstitution. Mixing the dried lipophilic ingredients with the hydrophilic mixture can form liposomes. Mixing the polar solution with the dry lipid film can be by any means that strongly homogenizes the mixture. Vortexing, magnetic stirring and/or sonicating can effect the homogenization.

Where active agents are included in the liposomes they can be dissolved or dispersed in a suitable solvent and added to the liposome mixture prior to mixing. Typically hydrophilic active agents will be added directly to the polar solvent and hydrophobic active agents will be added to the nonpolar solvent used to dissolve the other ingredients but this is not required. The active agent could be dissolved in a third solvent or solvent mixture and added to the mixture of polar solvent with the lipid film prior to homogenizing the mixture.

The liposomes of the present invention can be multi or unilamellar vesicles depending on the particular composition and procedure to make them. Liposomes can be prepared to have substantially homogeneous sizes in a selected size range, such as about 1 micron or less, or about 500 nm or less, about 200 nm or less, or about 100 nm or less. Particle size has been shown to play a major role in liposome biodistribution and the route

of cell entry. Larger liposomes are distributed primarily to the reticuloendothelial (RES) system with negligible amounts in other tissues, whereas smaller liposomes are localized to other organs. Additionally, the clearance of multilamellar vesicles of heterogenous size distribution follows a biphasic pattern, with rapid clearance of larger liposomes and a slow rate of clearance of small liposomes. Limited information is available on the biodistribution of cationic liposomes containing oligonucleotides. Letsinger and colleagues previously reported that oligonucleotides complexed with cationic liposomes, approximately 2.0 microns in diameter, are transiently taken up by the lungs followed by rapid distribution to the liver. Recent studies demonstrated that endocytosis is the principal pathway for delivery of oligonucleotides via cationic liposomes. Preferably, the liposomal lamellar complex formulation of the present invention includes small liposomes so as to slow the rate of clearance of the active agent. One effective sizing method involves extruding an aqueous suspension of the liposomes through a series of polycarbonate membranes having a selected uniform pore size; the pore size of the membrane will correspond roughly with the largest sizes of liposomes produced by extrusion through that membrane. For physiological use, it is preferable for the liposomes to be extruded through 0.22 μm filters or less to effect sterilization of the formulation. Desirably, the mean size of the liposomes is between about 50 nm and about 250 nm, such as between about 100 nm and about 200 nm or between about 150 nm and 250 nm. Preferably, the size distribution of the liposomes is substantially uniform. Moreover, within a preparation, desirably, about 99% of the liposomes have a diameter less than about 500 nm (i.e., D99 less than about 500 nm) and, desirably, the D99 of a liposomal composition according to the invention is between about 170 nm and about 500 nm, such as between about 200 nm and about 350 nm or between about 250 nm and about 300 nm.

A preferred manufacturing protocol is represented as a flowchart in FIG. 1. This process involves dissolving: i) the lipid excipients, namely, DOPC, cholesterol, PCL-2 and D-Alpha tocopheryl acid succinate in dehydrated ethanol and ii) the active ingredient – a polynucleotide (indicated as a c-raf-1 antisense oligonucleotide in Figure 1), and sucrose in sterile water for injection. Ethanolic lipid solution is added to aqueous active solution to form liposomes. Following *quantum sufficit* (QS) to batch weight according to which the product weight is brought up to the desired weight depending on the batch size, liposomes are size reduced by extrusion three times through 0.2μ pore size polycarbonate membrane

filters and five times through 0.1μ pore size polycarbonate membrane filters to meet size specification. Ethanol added is removed from extruded liposomes by rotary evaporation under vacuum. After adjusting the product weight to the weight prior to solvent removal with sterile water for injection, the product is filtered through 0.22μ sterilizing filter, filled in sterile vials, stoppered and sealed. This process is used to manufacture the formulation at 10-20 kilogram scale.

The liposomal (or other lipid) composition or formulation can be in any desired form. For example, for pharmaceutical use, the composition can be ready for administration to a patient. Where such composition contains liposomes or other types of lipid vesicles, such formulations typically are in the form of vesicles in an aqueous medium (such as ethanol and water for injection). Alternatively, the formulation can be in dried or lyophilized form, in which instance, the composition preferably includes a cryoprotectant as well. Suitable cryoprotectants include, for example, sugars such as trehalose, maltose, lactose, sucrose, glucose, and dextran, with the most preferred sugars from a performance point of view being trehalose and sucrose. Other more complicated sugars can also be used, such as, for example, aminoglycosides, including streptomycin and dihydrostreptomycin.

A preferred active agent for inclusion within the lipid composition is a polynucleotide which can target desired sequences within cells, such as genes associated with a disease state (e.g., oncogenes or viral genes). Desirably, the polynucleotide for use in the inventive composition hybridizes to a specific human mRNA within cells and further inhibits gene expression as a result of hybridization to the targeted mRNA. For example, the polynucleotide can be targeted to an oncogene such as ras, raf cot, mos, myc, etc., and preferably is targeted to the human c-raf gene.

A preferred raf polynucleotide for use in the inventive composition is a 5 to 50-mer antisense oligodeoxyribonucleotide, preferably a 10-40-mer sequence, and more preferably a 15-25-mer sequence targeted to a specific gene of interest (*See*, e.g., U.S. Patent No. 6,126,965, disclosing a 15-mer anti c-raf-1 oligonucleotide having the sequence 5'-GTGCTCCATTGATGC-3'). Additional suitable anti-raf oligodeoxyribonucleotide sequences are known in the art and can be suitably used in the context of the present invention (*See*, e.g., U.S. Patent Nos. 6,090,626, 6,126,965, 6,333,314 and 6,410,518 and international patent application publication nos. WO 94/15645 and 94/23755). Where oligonucleotides are included in the composition, they preferably contain one or more

phosphothioate linkages preferably two phosphothioate linkages. Most preferably, oligonucleotides for inclusion in the inventive composition contain one phosphothioate linkage at each terminal end, but they can be present anywhere from one end to the other end (e.g., between the ends) of an oligonucleotide.

A most preferred composition contains a 15-mer anti c-raf-1 oligonucleotide having the sequence 5'-GTGCTCCATTGATGC-3', 1,2-dioleoyl-sn-Glycero-3-Phosphocholine (DOPC), novel positively charged phospholipids 1,3-Bis-(1,2-bis-tetradecyloxy-propyl-3-dimethylethoxyammoniumbromide)-propane-2-ol (PCL-2) and cholesterol as stabilizing agents, D-alpha tocopheryl acid succinate as an antioxidant, sucrose as a tonicity agent and ethanol and water for injection as solvents. This composition can be provided as a sterile liquid containing 2 mg/mL rafAON (50 mg rafAON per vial). Each vial is diluted 2-fold with 5% dextrose, USP to yield a 1 mg/mL rafAON liposome formulation. It may be further diluted up to 8-fold with 5% dextrose, USP prior to administration.

Desirably, where polynucleotides are included within the inventive composition, the respective charge ratio of cationic lipid: polynucleotide ranges from (1-4):1, such as between 2:1 and 3:1. Because PCL-2 has two positive charges, the overall lipid: drug molar amounts can be lower in the present invention than in previously reported cationic liposomal formulations. Desirably the respective total lipids to polynucleotide molar amounts are between about 10:1 and about 200:1, such as between about 20:1 and about 150:1 or between about 50:1 and about 100:1. Where the composition is liposomal, desirably at least about 40%, and more desirably at least about 50%, of the polynucleotide is complexed with cationic lipids in the inner core of the liposomes. Typically, between about 60% and about 80% of the polynucleotide is complexed with cationic lipids in the inner core of the liposomes and between about 40% and about 20% of the polynucleotide is on the outer surface of the membrane. However, in some embodiments, more (e.g., about 90% or even about 95%) of the polynucleotide can be complexed with cationic lipids in the inner core of the liposomes. The high intraliposomal entrapment imparts sustained release of c-rafAON from the formulation.

Where the polynucleotide within the composition targets an oncogene, the invention provides a method of inhibiting the growth of neoplastic cells using such a composition. In accordance with the inventive method, the composition is administered in an effective amount to such cells under conditions for the polynucleotide to enter the cells. Within the

cells, the polynucleotide inhibits the activity of the oncogene to which it is targeted, which results in the inhibition of growth of such neoplastic cells. In this context, "inhibition" does not require a complete cessation of the growth or proliferation of such cells. It is sufficient for the method to retard neoplastic growth. It is not a requirement that the inventive method completely eliminate neoplastic cells from the patient. Rather, it is sufficient for the inventive method to retard the growth or proliferation of the neoplastic cells within the patient. Preferably, however, the inventive method substantially inhibits the growth of the neoplastic cells, and, in some embodiments, the inventive method can result in tumor regression or elimination of neoplastic cells from the patient. The effective amount for such inhibition of a raf sequences is at least about 60 mg/m^2 such as between about 60 mg/m^2 to about 200 mg/m^2 once weekly, by intravenous infusion, for up to 3 consecutive weeks.

In accordance with the inventive method, the composition comprising the polynucleotide can be employed adjunctively with a second antineoplastic agent. Such a second antineoplastic agent can be, for example, a chemotherapeutic agent, a source of radiation, a gene therapy vector, or other antineoplastic agent. Specific examples of such chemotherapeutics include camptothecins (e.g., SN-38, irinotecan, etc.), doxorubicin, daunorubicin, methotrexate, adriamycin, tamoxifen, toremifene, cisplatin, epirubicin, docetaxel, paclitaxel, Gemzar, gemcitabine HCl, mitoxantrone, and other known chemotherapeutics useful for treatment of cancer. When used adjunctively, the inventive composition can be delivered prior to, concurrently with, or after the second antineoplastic agent. However employed, the inventive composition can enhance the efficacy of the second antineoplastic agent. Thus, the invention can be used to chemosensitize a tumor to the effect of chemotherapy. Similarly, the invention can be used to radiosensitize a tumor to the effects of radiation. In this respect, it is sufficient for the inventive method involving the use of a composition including the cationic cardiolipin analogue and the polynucleotide to contribute to the efficacy of the combined treatment regimen.

For delivery to a patient, the composition can be supplied in any suitable manner. For delivery to a tumor, for example, the composition can be formulated for injection directly into the tumor mass or infused through the circulation in the tumor. Alternatively, the composition can be delivered by injection (e.g., parenteral, intravenous, etc.) or adsorption (e.g., transdermal, transmucosal), as desired. Dermatological preparations can be applied suitable to skin or mucous tissues.

The following examples further illustrate the invention but, of course, should not be construed as in any way limiting its scope.

EXAMPLE 1

This example demonstrates the construction of nine PCL-2-based cationic liposomes for gene transfer. These formulations are suitable for mixing with any polynucleotide of choice for evaluating transfection efficiency.

Cationic cardiolipin analogue, PCL-2, and either DOPE or Cholesterol quantities corresponding to each formulation (indicated in Table 1A & 1B) were dissolved in chloroform in a round bottom flask. The solvent was removed under reduced pressure using a rotary evaporator to form a lipid film. The lipid film was further dried under vacuum overnight, and then hydrated with 20 mM HEPES buffer, under nitrogen, at 25 – 40 °C. The bulk cationic liposomes were extruded through 0.2 μm pore size polycarbonate filter three times, and 0.1 μm pore size polycarbonate filter five times. The extruded cationic liposomes were characterized by size and pH, as indicated in Tables 1A & 1B.

Table 1A

Formulation Lot #	Molar Ratio		Lipid Concentration		Liposome Size (nm)		pH
	PCL-2/CHOL	PCL-2/DOPE	PCL-2 (mM)	Total Lipids (mg/mL)	Mean	D99	
PDM-GT-03-001	1 : 1		10	17.14	144.2	259.4	7.54
PDM-GT-03-002	3 : 2		10	15.85	173.8	290.2	7.45
PDM-GT-03-003	2 : 3		10	19.07	128.5	230.0	7.44
PDM-GT-03-004	2 : 1		10	15.20	163.3	311.4	7.64
PDM-GT-03-005		1 : 1	10	20.71	105.7	211.7	7.37
PDM-GT-03-006		2 : 1	10	16.99	101.6	182.0	7.28
PDM-GT-03-007		1 : 2	10	28.15	114.8	208.7	7.32
PDM-GT-03-008		3 : 2	10	18.23	109.2	196.0	7.46
PDM-GT-03-009		2 : 3	10	24.43	117.5	219.9	7.29

Cationic cardiolipin analogue, PCL-2, and either DOPE or Cholesterol quantities corresponding each formulation (indicated in Table 1B) were dissolved in chloroform in a round bottom flask. The solvent was removed under reduced pressure using a rotary evaporator to form a lipid film. The lipid film was further dried under vacuum overnight, and then hydrated with water, under nitrogen, at 25 – 40 °C. The bulk cationic liposomes were extruded through 0.2 μm pore size polycarbonate filter three times, and 0.1 μm pore size polycarbonate filter five times. The extruded cationic liposomes were characterized by size and pH, as illustrated below in Table IB.

Table 1B

Formulation Lot#	Molar Ratio	Lipid Concentration		Liposome Size (nm)		pH
	PCL-2/DOPE	PCL-2 (mg/mL)	Total Lipids (mg/mL)	Mean	D99	
001N0104	1:2	0.47	1 mg	115.6	224.6	4.3

EXAMPLE 2

This example demonstrates the construction of a PCL-2-(CCLA)-based cationic liposome composition containing an antisense oligonucleotide.

The composition is in the form of a lamellar complex having a percent molar ratio of 1,2 Dioleoyl-sn-glycero-3-phosphocholine (DOPC):cholesterol: and 1,3-Bis-(1,2-bistetradecyloxy-propyl-3-dimethylethoxyammonium bromide)-propane-2-ol (PCL-2), of 55:27:17 and having complexed therein c-rafAON (sequence sequence 5'-GTGCTCCATTGATGC-3') at total lipids:c-rafAON ratio of 90:1 and PCL-2:c-rafAON charge ratio of 2.2:1. This formulation also contains D-alpha tocopheryl acid succinate at 0.2 wt % and sucrose at 10-12 wt %.

c-rafAON (Lot ALH-01J-003-M) was obtained from Avecia Laboratories (MA, USA). DOPC, cholesterol, alpha tocopheryl acid succinate were obtained from Avanti Polar Lipids (Alabaster, AL, USA). PCL-2 was synthesized at NeoPharm Inc. Sucrose was obtained from Mallinckrodt (St. Louis, MO, USA).

To form this composition, the lamellar complex formulations were prepared by the thin film hydration method. The lipids (DOPC, PCL-2, cholesterol and D-alpha tocopheryl acid succinate) were dissolved in dehydrated alcohol. The lipid solution was evaporated to dryness using a rotary evaporator. After evaporation, the lipid residue was further dried overnight in a dessicator. Sucrose and c-raf AON were dissolved in de-ionized water. Then, the dried lipid residue was hydrated in the c-raf AON/sucrose solution to form a homogenous suspension. The size of the particles in the suspension was further reduced by extrusion through 0.8, 0.4, 0.2 and 0.1μm sized polycarbonate filters. The prototypes can also be prepared by reverse phase evaporation and ethanol injection methods.

The morphology of the formulation was determined by freeze-fracture microscopic method. Briefly, samples were quenched using sandwich technique and liquid nitrogen cooled propane at a cooling rate of 10,000 kelvin/second. The fracturing process was carried out in JEOL JED-9000 freeze-etching equipment and the exposed fracture planes were shadowed with platinum for 30 sec in an angle of 25-35 degree and with carbon for 35 sec (kV/60-70mA, 1/10-5 Torr). The platinum replicas were cleaned with concentrated, fuming HNO_3 for 24 to 36 hours, followed by repeating agitation with fresh chloroform/methanol (1:1 by volume) for at least five times. Subsequently, these cleaned replicas were examined with a JEOL 100 CX electron microscope.

The stability of the formulation was evaluated after storage at 2-8 °C and 25 °C for one month. The particle of the lamellar complex formulations was measured by dynamic light scattering technique (Nicomp Particle Sizer). The drug entrapment efficiency of the formulation was determined by ultrafiltration and/or passage through Centricon filters with 100,000 molecular weight cut off. The free and entrapped drug and lipid contents were determined by HPLC methods.

The freeze-fracture replicas revealed complexes of unilamellar liposomes of mean size of about 50-200 nm. The c-rafAON formulation stored at 2-8 °C or 25 °C was stable for one month. The mean particle size, entrapment efficiency, c-rafAON and DOPC concentrations were not significantly different from initial values after 1 month storage at 2-8 °C and 25 °C. These results are presented below in Table 2.

Table 2: One Month Stability data for GMP batch

Test	Specification	Initial	Storage Conditions	
			5°C (inverted)	25°C/60%RH (inverted)
Appearance	White Suspension	Conforms	Conforms	Conforms
pH	Report	5.0	5.1	5.0
rafAON	90-110%	107%	102.5%	103.0%
DOPC	70-110%	92.2%	93.8%	93.1%
Cholesterol	70-110%	93.5%	94.9%	94.7%
PCL-2	70-110%	87.1%	89.3%	90.8%
Mean Particle Size	< 400 nm	138.6nm	135.7nm	135.1nm
rafAON Entrapment	> 85%	100%	100.0%	100.0%

EXAMPLE 3

This example demonstrates the construction of a PCL-2-(CCLA)-based cationic liposome composition containing an antisense oligonucleotide.

The composition is a lamellar complex having a percent molar ratio of 1,2 Dioleoyl-sn-glycero-3-phosphocholine (DOPC):cholesterol: and 3-Bis-(1,2-bistetradecyloxy-propyl-3-dimethylethoxyammonium bromide)-propane-2-ol (PCL-2), of 60:31:9 and having complexed therein c-rafAON at total lipids:c-rafAON ratio of 195:1 and PCL-2:c-rafAON charge ratio of 2.4:1. This formulation also contains D-alpha tocopheryl acid succinate at 0.2 wt % and sucrose at 10 wt %.

c-rafAON (Lot ALH-01J-003-M) was obtained from Avecia Laboratories (MA, USA). DOPC and cholesterol were obtained from Avanti Polar Lipids (Alabaster, AL, USA) and alpha tocopheryl acid succinate from Sigma. PCL-2 was synthesized at NeoPharm Inc. Sucrose was obtained from Mallinckrodt (St. Louis, MO, USA).

The lamellar complex formulations were prepared by the thin film hydration method. The lipids (DOPC, PCL-2, cholesterol and D-Alpha Tocopheryl acid Succinate) were dissolved in dehydrated alcohol. Sucrose and c-raf AON were dissolved in de-ionized water. With constant stirring, the lipid solution was added to the c-rafAON solution to form multilamellar complexes. The size of the particles in the suspension was further reduced by extrusion through 0.8, 0.4, 0.2 and 0.1μm sized polycarbonate filters. The particle of the lamellar complex formulations was measured by dynamic light scattering technique (Nicomp Particle Sizer). The drug entrapment efficiency was determined by ultrafiltration and/or pass through Centricon filters with 100,000 molecular weight cut off, followed by the analysis of free c-rafAON in the filtrate by a HPLC method.

The release of c-rafAON from the lamellar complex formulation was determined by a reverse dialysis method. Briefly, 20 mL aliquot of the formulation was placed into 180 mL of stirred Phosphate Buffered Saline (PBS), pH 7.4 buffer, in which dialysis membrane tube (10,000 MW cutoff) containing 2 mL of the same PBS buffer was previously placed. At various time intervals, one dialysis tube is removed and analyzed for c-rafAON concentration.

The stability of the formulation was evaluated after storage at 2-8 °C for two months. The particle of the lamellar complex formulations was measured by dynamic light scattering technique (Nicomp Particle Sizer). The drug entrapment efficiency of the formulation was determined by ultrafiltration and/or passage through Centricon filters with 100,000 molecular weight cut off. The free and entrapped drug and lipid contents were determined by HPLC methods. It was observed that the lamellar complex formulation stored at 2-8 °C was stable for two months. The mean particle size, entrapment efficiency, c-rafAON and DOPC concentrations were not significantly different from initial values after 2 months of storage at 2-8°C. These results are presented in Tables 3A and 3B as follows:

Table 3A. Stability of lamellar complex formulation of c-rafAON

TIME (Month)	Storage conditions	Particle size (nm)		Entrapment Efficiency (%)
		Mean	99 percentile <	
Initial		162	294	98.8
2 month	2-8°C	162	287	98.7

Table 3B. Stability of lamellar complex formulation of c-rafAON

TIME (month)	Storage conditions	c-raf AON (% of initial concentration)	DOPC (% of initial concentration)	PCL-2 (% of initial concentration)
Initial		100	100	100
2 month	2-8°C	96	98	97

The physical and chemical stability of 8-fold diluted formulation samples in 5% Dextrose Solution was evaluated after storage at room and refrigerated temperatures (2-8 °C) for up to 48 hours. All stability samples were analyzed for appearance, pH, particle size, c-rafAON entrapment efficiency, c-rafAON concentration, DOPC concentration, cholesterol concentration and PCL2 concentration. The mean vesicle size was approximately 200 nm and entrapment efficiency of >99%. Less than 20 percent of c-rafAON was released from the formulation after 72 hours of dialysis. As presented in Table 3C, c-rafAON, DOPC, PCL-2, Cholesterol assay values, entrapment efficiency, pH and mean vesicle size were not significantly different from initial values after storage at 2-8°C or room temperature for 48 hours. The preparations were diluted in 5% Dextrose, USP.

Table 3C: Results of Liposomal rafAON (LErafAON) 8-fold diluted with 5% dextrose solution

Test	Initial	2-8 °C			Room Temperature		
		8 hr	24 hr	48 hr	8 hr	24 hr	48 hr
Appearance	White translucent suspension	White translucent suspension	White translucent suspension	White translucent suspension	White translucent suspension	White translucent suspension	White translucent suspension
pH	4.88	4.98	4.88	4.86	4.90	4.79	4.82
rafAON (mg/mL)	0.25 (100.0%)	0.23 (92.4%)	0.25 (101.0%)	0.24 (97.2%)	0.24 (97.2%)	0.24 (95.6%)	0.25 (100.0%)
DOPC (mg/mL)	2.02 (95.1%)	2.00 (94.0%)	2.01 (94.4%)	2.30 (108.0%)	1.93 (90.8%)	2.27 (107.0%)	2.26 (106.3%)
Cholesterol (mg/mL)	0.49 (97.0%)	0.47 (94.0%)	0.49 (98.8%)	0.53 (105.0%)	0.46 (92.6%)	0.53 (105.4%)	0.53 (105.2%)
(R)-PCL2 (mg/mL)	1.08 (96.1%)	1.07 (94.8%)	1.11 (99.0%)	1.02 (90.7%)	1.17 (103.8%)	1.17 (103.8%)	0.99 (87.6%)
Liposome Mean Size (D99)	150.1 nm (274.9)	145.8 nm (257.5)	147.4 nm (258.5)	144.6 nm (257.3)	147.5 nm (251.4)	147.6 nm (258.2)	148.1 nm (254.1)
Entrapment Efficiency (%)	100%	100%	100%	100%	100%	100%	100%

EXAMPLE 4

This example demonstrates the *in vivo* efficacy and low toxicity of a composition according to the present invention containing a c-raf-1 antisense oligonucleotide as compared to other liposomal compositions containing the same c-raf-1 antisense oligonucleotide.

Formulations

The test formulation (LErafAON-ETU) was prepared as described in FIG. 1. Briefly, the lipid excipients, namely, DOPC, cholesterol, PCL-2 and D-Alpha tocopheryl acid succinate were dissolved in dehydrated ethanol and ii) the active ingredient a c-raf-1 antisense oligonucleotide (having the sequence 5'-GTGCTCCATTGATGC-3') and sucrose were dissolved in sterile water for injection. Ethanolic lipid solution was added to aqueous active solution to form liposomes. Following QS to batch weight, liposomes were extruded to meet size specifications and the ethanol was removed by rotary evaporation. After

adjusting the batch weight to pre-solvent removal weight, the product was filtered through 0.22μ sterilizing filter and filled in sterile vials, stoppered and sealed.

The comparator formulation (DDAB-LErafAON formulation) contained the same concentration of antisense oligonucleotide but was formulated using commercially available cationic lipid, dimethyldioctadecyl ammonium bromide (DDAB).

The manufacturing process for non-sonicated 2-vial LErafAON formulation involved separate batch preparations of lyophilized lipids and rafAON. For lyophilized lipids, lipid excipients, namely, Egg Phosphatidylcholine (Egg PC), Cholesterol and DimethylDodecylAmmonium Bromide (DDAB) was first dissolved in tertiary butyl alcohol, the solution was filtered through 0.22 μm sterilizing filter, filled into sterile vials and lyophilized. Lyophilized rafAON was manufactured by dissolving the drug substance in sterile water for injection, followed by its filtration through 0.22 μm sterilizing filter, filling in sterile vials and lyophilization. Immediately prior to administration, the rafAON lyophilized vial is reconstituted with 0.9% Sodium Chloride, USP. The reconstituted rafAON is then transferred into a vial of lyophilized lipids, hydrated and sonicated for 10 minutes.

Pharmacology Studies

The *in vivo* efficacy of the PCL-2 formulation, LErafAON-ETU, was compared to that of the DDAB formulation, LErafAON, in four studies.

1. *In Vivo* Efficacy of LErafAON-ETU and LErafAON in SCID Mice Bearing Human Prostate (PC-3) Tumors

The multiple dose therapeutic efficacies of the PCL-2 formulation LErafAON-ETU, and the control formulation, LErafAON, were evaluated in a SCID mouse xenograft model of human prostate cancer. PC-3 cells in the logarithmic growth phase were implanted subcutaneously (s.c.) in C.B.-17 SCID mice. Animals bearing tumors (50-125 mm^3) were randomized into different treatment groups (5-7 mice/group), and mice received iv administration of control vehicle, LErafAON at 12.5 or 25 mg/kg/day, or LErafAON-ETU at 12.5 or 25 mg/kg/day, on Days 1, 2, 4, 5, 7, 8, 10, 11, 13 and 14. Efficacy was evaluated

by comparing the % initial tumor volume (where Day 1 = 100%) of controls with LErafAON and LErafAON-ETU groups on Day 20.

Animals treated with 12.5 mg/kg/day of LErafAON or LErafAON-ETU did not show any tumor growth inhibition. However, animals treated with LErafAON or LErafAON-ETU at 25 mg/kg/day exhibited 15% and 44% tumor growth inhibition, respectively, compared to their respective controls (10% Sucrose). Both LErafAON and LErafAON-ETU were well tolerated, as no differences in body weight loss were observed.

The results of this study suggest that overall both the PCL-2 formulation (LErafAON-ETU) and the DDAB liposomal formulation (LErafAON) had comparable therapeutic efficacy against the human prostate tumor model in SCID mice.

2. *In Vivo* Efficacy of LErafAON-ETU and LErafAON in SCID Mice Bearing Human Breast (MDA-MB-231) Tumors

The multiple dose therapeutic efficacies of the PCL-2 formulation, LErafAON-ETU and the control formulation, LErafAON, were evaluated in a SCID mouse xenograft model of human breast cancer. MDA-MB-231 cells in the logarithmic growth phase were implanted s.c. in C.B.-17 female SCID mice. Animals bearing tumors (50-100 mm^3) were randomized into different treatment groups (8 mice/group), and received IV administration of control vehicle, or 25 mg/kg/day LErafAON or LErafAON-ETU on Days 1, 2, 4, 5, 7, 8, 10, 11, 13 and 14.

Maximal tumor growth inhibition was 65% on Day 12 and 59% on Day 15 for LErafAON and LErafAON-ETU, respectively, compared to the appropriate vehicle controls. No body weight loss was observed for any of the treatment groups.

The results suggest that LErafAON-ETU has equivalent therapeutic efficacy against the MDA-MB-231 human breast cancer tumor model as compared to the control LErafAON formulation.

3. *In Vivo* Efficacy of LErafAON-ETU and LErafAON and Their Combinations With Taxol® in SCID Mice Bearing Human Ovarian (SKOV-3) Tumors

A multiple dose therapeutic efficacy study of LErafAON-ETU and LErafAON alone and in combination with Taxol® was conducted in a SCID mouse xenograft model of human ovarian cancer. SKOV-3 cells in the logarithmic growth phase were implanted s.c. in C.B.-17 SCID mice. Treatment with controls or test articles was initiated when tumor volume reached 50-100 mm^3. Mice were randomized into treatment groups with 5-7 animals per group, then dosed intravenously with vehicle controls or with 25 mg/kg/day LErafAON or LErafAON-ETU on Days 1, 2, 4, 5, 7, 8, 10, 11, 12, and 13. Taxol® (25 mg/kg) was also administered intravenously to designated groups on Days 2, 5, and 9. Animals from combination therapy groups received Taxol® 2-3 hours after receiving the respective liposome formulation of rafAON.

LErafAON and LErafAON-ETU showed maximum tumor growth inhibition on Day 23 of 38% and 45%, respectively. Taxol® exhibited maximal tumor growth inhibition of 41% on Day 19. The combinations of Taxol® + LErafAON or Taxol® + LErafAON-ETU significantly regressed tumor growth by 90%. In addition, tumor growth to volumes greater than 400 mm^3 was delayed 13 days by Taxol® + LErafAON and 24 days by Taxol® + LErafAON-ETU.

The results of this study indicate that the PCL-2 formulation LErafAON-ETU has equivalent therapeutic efficacy as the control LErafAON against the SKOV-3 human ovarian cancer tumor model in SCID mice when administered as a single agent. However, in combination treatment with Taxol®, the PCL-2 formulation, LErafAON-ETU, resulted in superior tumor growth delay compared to the DDAB formulation (LErafAON).

4. *In Vivo* Efficacy of LErafAON-ETU and LErafAON and Their Combinations with Taxotere® in SCID Mice Bearing Human Prostate (PC-3) Tumors

A multiple dose therapeutic efficacy study of the PCL-2 formulation, LErafAON-ETU, and the control DDAB formulation, LErafAON, alone and in combination with sub-therapeutic doses of Taxotere® was conducted in a SCID mouse xenograft model of human

prostate cancer. PC-3 cells in the logarithmic growth phase were implanted s.c. in C.B.-17 SCID mice. Animals bearing tumors (50-100 mm^3) were randomized into different treatment groups (5-7 mice/group), and controls or test articles (25 mg/kg/day LErafAON or LErafAON-ETU) were administered intravenously for 5 consecutive days. Taxotere® was dosed to designated groups at 10 mg/kg on Day 2 and 5 mg/kg on Day 5. Efficacy was evaluated by comparing the % initial tumor volume (where Day 1 = 100%) of controls with treated groups on Day 21.

Tumor growth was inhibited 7% and 40% for mice dosed with LErafAON and LErafAON-ETU, respectively, compared to their controls. Taxotere® alone resulted in 83% tumor growth inhibition, whereas, in combination treatment, LErafAON + Taxotere® led to a similar tumor growth inhibition of 81%. Animals dosed with the PCL-2 formulation, LErafAON-ETU, + Taxotere® showed 99% tumor growth inhibition on Day 21.

The results of this study suggest that the PCL-2 formulation, LErafAON-ETU, in combination with Taxotere® has greater therapeutic efficacy in comparison to the control DDAB formulation LErafAON + Taxotere® combination.

Pharmacokinetics

The pharmacokinetics of the PCL-2 rafAON formulation, LErafAON-ETU, was compared to that of the control (DDAB) liposome formulation, LErafAON, in CD2F1 mice.

1. Bioanalytical Methods

Bioanalytical methods were developed to quantify total rafAON in mouse plasma and mouse tissues containing LErafAON or LErafAON-ETU, and were used to quantify rafAON in plasma and tissue samples from the single dose pharmacokinetics and tissue distribution study in mice. RafAON was extracted from mouse plasma samples by solid phase extraction (SPE), then quantified using LC-MS/MS. The method was found to be linear over the range 25-5000 ng/mL in mouse plasma. Tissues were homogenized mechanically to form a 5% (w/v) homogenate; this was then subjected to protein precipitation for sample preparation. Total rafAON was then quantified in samples by an LC-MS/MS method with linearity from 50-10,000 ng/mL tissue homogenate.

2. Pharmacokinetics and Tissue Distribution Study of LErafAON-ETU and LErafAON in Mice

A single dose pharmacokinetics and tissue distribution study of the control LErafAON and the PCL-2 formulation (LErafAON-ETU) was conducted following a 30 mg/kg intravenous dose to male CD2F1 mice. Three mice/group/time points were sacrificed at 5, 15 and 30 minutes and at 1, 2, 4, 8, 24, and 48 hours after formulation administration. Blood samples were processed for plasma, and plasma and tissues were assayed for rafAON concentrations.

The plasma C_{max} for animals dosed with LErafAON-ETU was approximately twice that for animals dosed with LErafAON. Total exposure (AUC_{0-48h} and $AUC_{0-\infty}$) was 2.3-fold higher for LErafAON-ETU than for LErafAON dosed mice. Clearance of LErafAON was 2.5-fold faster and volume of distribution (V_z) was almost 4-fold greater than for LErafAON-ETU as illustrated in FIG. 2 and Table 4A below.

Table 4A: Mean Pharmacokinetic Parameters for rafAON Following IV Administration of LErafAON-ETU or LErafAON (two-vial) to Male CD2F1 Mice

Formulation	Dose (mg/kg)	$t_{1/2}$ (h)	C_{max} (µg/mL)	V_{ss} (L/kg)	V_z (L/kg)	MRT (h)	CL (L/h/kg)	AUC_{0-48h} (µg•h/mL)	AUC_{0-inf} (µg•h/mL)
LErafAON ETU	27.7	4.22	16.6	4.79	8.31	3.51	1.37	20.3	20.3
LErafAON Two-vial	29.1	6.60	8.68[a]	10.1	32.1	2.99	3.37	8.70	8.64

[a] Extrapolated value at zero time-point is reported.

RafAON was rapidly distributed to the tissues following dose administration. The order of increasing tissue exposure over 48 hours was lungs > spleen > liver > kidneys > heart, for both formulations. RafAON concentrations were found to be greater in mice dosed with the PCL-2 formulation (LErafAON-ETU) in comparison to the DDAB formulation (LErafAON).

3. Pharmacokinetics and Tissue Distribution Study of LErafAON-ETU and LErafAON in Monkeys on Day 1

A comparative study was conducted following the first dose of LErafAON and LErafAON-ETU formulations to cynomolgus monkeys. Three male monkeys per group were dosed with 6.41 mg/kg body weight/dose LErafAON or 6.56 mg/kg body weight/dose LErafAON-ETU via a 15 minute constant rate IV infusion. Blood samples were collected to determine the plasma pharmacokinetic parameters of both formulation following first dose administration. The total rafAON concentration in plasma was determined using a validated LC-MS/MS method.

The C_{max} value of LErafAON-ETU was more than 10-fold greater than that of the original formulation, as illustrated in FIG. 3 and Table 4B. The AUC values of LErafAON-ETU were approximately 20-fold larger than those of LErafAON. LErafAON was eliminated more quickly from plasma in monkeys and had a greater volume of distribution (V_z), approximately 4 times that of the new formulation.

Table 4B: Pharmacokinetic Parameters (Mean ± SEM) for rafAON in Male Cynomolgus Monkeys after IV Administration of LErafAON-ETU or LErafAON on Day 1.

Formulation	Dose (mg/kg)	$t_{1/2}$ (h)	C_{max} (µg/mL)	V_{ss} (L/kg)	V_z (L/kg)	MRT (h)	CL (L/h/kg)	AUC_{0-8h} (µg•h/mL)	AUC_{0-inf} (µg•h/mL)
LErafAON	6.41 ± 0.00	0.879[a]	1.67 ± 0.42	5.87[a]	12.5[a]	0.595[a]	9.86[a]	0.583 ± 0.030	0.650[a]
LErafAON-ETU	6.56 ± 0.00	2.87[a]	19.8 ± 5.0	0.928[a]	3.19[a]	1.08[a]	0.702[a]	12.8[a]	13.1[a]
[a] No standard error of the mean (SEM) was calculated due to n=2 or n=1.									

4. Pharmacokinetics and Tissue Distribution Study of LErafAON-ETU in Monkeys on Days 1 and 22

A pharmacokinetic support to a four-cycle toxicity study of LErafAON-ETU was conducted in cynomolgus monkeys after IV infusion. Nine monkeys were randomly assigned to one of three test article-treated groups, as disclosed in Table 4C. Three male

monkeys per group were administered LErafAON-ETU as a 1-hour constant rate intravenous infusion according to the actual dose as indicated in Table 4D. Blood samples were collected on Days 1 (first dose) and 22 (fourth dose) and plasma samples were analyzed for rafAON concentrations by a validated LC-MS/MS method.

Table 4C: Dosages Assigned to Groups of Male Cynomolgus Monkeys

Group	Day 1 Dosage (mg/kg/dose)	Day 22 Dosage (mg/kg/dose)	Number of Animals
1	.673	.615	3
2	3.36	3.18	3
3	6.81	6.31	3

Mean C_{max} and AUC values increased with increasing dose on both days. The increase was approximately linear on Day 1 and whereas a more disproportionate increase in these parameters was noticed on Day 22. Mean distribution and clearance parameters were similar between all dose groups on both days. Mean C_{max} and $AUC_{0\text{-}6h\ post\ infusion}$ values, at the lowest dose level (.625 mg/kg), were comparable for both Days 1 and 22.

Table 4D: Mean (± SEM) Pharmacokinetic Parameters of rafAON on Days 1 and 22 in Male Cynomolgus Monkeys After Weekly Administration of LErafAON-ETU

Dose (mg/kg/cycle)	$t_{1/2}$ (h)	C_{max} (µg/mL)	V_{ss} (L/kg)	V_z (L/kg)	CL (L/h/kg)	$AUC_{0\text{-}6h}$[a] (µg•h/mL)	$AUC_{0\text{-}inf}$ (µg•h/mL)
Day 1							
0.673	4.98[b]	0.475 ± 0.067	4.70[b]	5.29[b]	0.736[b]	0.461 ± 0.072	0.915[b]
3.36	3.09 ± 1.09	1.54 ± 0.07	3.78 ± 0.88	5.83 ± 1.49	1.50 ± 0.29	2.07 ± 0.28	2.44 ± 0.53
6.81	3.81 ± 0.29	3.96 ± 0.76	3.75 ± 1.05	6.25 ± 1.52	1.11 ± 0.21	5.63 ± 1.43	6.71 ± 1.47
Day 22							
0.615	[c]	0.641 ± 0.090	[c]	[c]	[c]	0.530 ± 0.075	[c]
3.18	4.76[b]	6.60[b]	1.22[b]	2.69[b]	0.517[b]	6.54[b]	7.59[b]
6.31	3.70 ± 0.42	57.2 ± 29.1	1.10 ± 1.02	2.27 ± 1.81	0.366 ± 0.269	46.7 ± 22.5	48.0 ± 22.5

[a] Time refers to 0 h at start of infusion to 6 h post-infusion.

[b] No standard error of the mean (SEM) was calculated due to n=2 or n=1.

[c] Not calculated due to insufficient nonzero concentrations for all three animals.

Toxicology of LErafAON-ETU

1. Multiple Dose Toxicity in Mice

The toxicity of the PCL-2 rafAON formulation, LErafAON-ETU, was compared to that of the control liposome formulation, LErafAON, in CD2F1 mice.

A comparative multiple dose toxicity study of the control (LErafAON) and the PCL-2 formulation (LErafAON-ETU) was conducted in male and female CD2F1 mice. Animals were randomized into treatment groups (10/sex/group) and were dosed intravenously with 35 mg/kg/day LErafAON or LErafAON-ETU for 5 consecutive days. Control groups received either 10% sucrose or placebo liposomes. Groups of mice were sacrificed on either Day 8 or Day 29 of the study. Blood was collected for hematology and clinical chemistry evaluation and lung, liver, spleen, kidney and heart were taken form the animals for histopathology examination.

No significant body weight loss or clinical signs of toxicity were observed in mice dosed with the PCL-2 formulation (LErafAON-ETU). The mortality of animals dosed with LErafAON-ETU was 0% for both males and females, and was 100% for males and 40% for

females dosed with LErafAON. All males and 40% of the females dosed with LErafAON were found dead by Day 5, and all showed clinical signs of toxicity (hunched posture, rough coats, and dehydration). No statistically significant body weight loss was observed in the males, however females had 12% weight loss on Day 8, but recovered by Day 17. The females that survived dosing with LErafAON beyond Day 5 were lethargic, but recovered by Day 9. The results of this study are presented in Table 4E.

Table 4E: Toxicity of DDAB-Liposome or Cationic Cardiolipin Liposome-Based Antisense Oligonucleotide (AON) in Mice.

Test Article**	Dose (mg/kg/day)***	Number of Mice	%Survival Male	%Survival Female
DDAB Liposome-Based AON	35	10	0	60
Cationic Cardiolipin Liposome-Based AON	35	10	90****	100

*Dimethyldioctadecyl ammonium bromide
**Control- 100% Survival
***17.5 mg/kg, BID X 5
****Animal died due to technical error

Clinical pathology evaluation showed no significant differences in clinical chemistry between mice dosed with vehicle and those dosed with LErafAON or LErafAON-ETU with the exception that there was a significant increase in white blood count on Day 8 in both treated groups when compared to their respective controls. This value had returned to normal by Day 29.

Histopathological evaluations revealed that mice dosed with LErafAON that died during the study had pulmonary hemorrhage. These animals had grossly observed heart,

intestinal, liver and kidney lesions for which there was no corresponding microscopic observation.

Surviving animals that received the LErafAON had lesions on Day 8 in the spleen (erythropoiesis and reticuloendothelial cell vacuolation), lungs (acute diffuse hemorrhage, alveolar edema and necrosis, erythrophagocytosis and vacuolated macrophage/material accumulation) and liver (Kupffer cell hyperplasia and vacuolation, lipid granuloma and erythrophagocytosis). By Day 29, all of these findings had resolved with the exception of focal pigmented macrophage accumulation in the lung and lipid granuloma and erythrophagocytosis in the liver.

On Day 8, animals that received LErafAON-ETU had similar but less severe lesions in the spleen compared to those receiving LErafAON, only vacuolated macrophage/material accumulation in the lung and similar findings in the liver which were reduced in incidence and severity in this group compared to the LErafAON group. By Day 29, the only finding in the LErafAON-ETU group was lipid granuloma.

The results of this study indicate that administration of LErafAON-ETU appears to result in fewer lesions to the lungs, livers and spleens of CD2F1 mice than LErafAON. In addition, mice receiving LErafAON had significant mortality by Day 5 of the study, while those receiving LErafAON-ETU did not have any mortality.

2. Multiple Dose Toxicity in Cynomolgus Monkeys

A comparative repeated-dose toxicity study was LErafAON and LErafAON-ETU was conducted in male cynomolgus monkeys. Animals were randomized into treatment groups (3/group) and were dosed with approximately 6.25 mg/kg/day LErafAON or LErafAON-ETU on Days 1,2,4,5,8,9,11,12 and 15 via a 15-minute IV infusion. A single vehicle control group received a 5% dextrose solution.

Monkeys were observed daily for clinical signs of toxicity and were weighed weekly. Ophthalmic examination was conducted on Day 3 and at least 4 hours after dosing on Day 15. Electrocardiograms were conducted during Week 1 and on Day 15. Blood samples were collected on Day 4 and on Day 16 for hematology, clinical chemistry and coagulation determination. Blood samples were also collected on Days 4,5,12 and 16 to test for complement markers. Urine samples were collected overnight during Week 1 and on Days 15-16 for urinalysis. Monkeys administered test article were bled at scheduled time

points on Day 1, and plasma was separated and analyzed for drug concentration. Monkeys were sacrificed on Day 16 and a gross pathological examination was performed. The exams included macroscopic observations of external surfaces and orifices and of internal organs and body cavities. Selected organs were weighed and protocol-specified organs were processed for histopathological examination.

All animals survived to the scheduled necropsy. No clinical signs of acute toxicity were observed in the animals administered LErafAON or the control vehicle. All animals (3) receiving the LErafAON-ETU showed an acute adverse reaction on the first day of dosing that consisted of lethargy and pallor to the gums. One animal became limp and unresponsive to stimuli for several minutes. The onset of these findings was approximately 0.5 to 1 hour after dosing and they continued through the 2 hour observation point for 2 animals which appeared to have recovered by Day 2. These signs persisted through Day 3 for the most severely affected animal. No clinical signs of toxicity occurred on any other day of dosing, including Day 9, when one animal was mistakenly administered 2 doses of LErafAON-ETU.

One animal treated with LErafAON had localized subcutaneous abscess at a dose injection site. The lesion ruptured on Day 11 had appeared to be healing properly on Day 15 after being flushed with betadine/saline solution on Days 11 and 12. This lesion was considered possibly related to test article administration. Animals in both groups receiving test article had nodules near injection sites, and these lesions were considered possibly related to test article administration.

There was no treatment effect on body weights. There was no effect on body temperature or respiration rates. Treatment of animals with control vehicle and test articles did not lead to any changes in the ophthalmology and electrocardiography parameters examined. There were no treatment-related effects on urinalysis parameters.

Administration of LErafAON was associated with mild to moderate decreased red blood cell counts, hemoglobin, hematocrit and platelet count on Day 16. Administration of LErafAON-ETU was associated with minimal decrease in these parameters. Administration of LErafAON was associated with increased white blood cell count, neutrophil and reticulocyte counts while treatment with LErafAON-ETU was not. Only the change in white blood cell count was statistically significant. There was no effect on coagulation assay values (PT and PTT) after administration of rafAON formulations.

Both treatment groups had decreased mean albumin (A) values. Animals receiving LErafAON had increased globulin (G) and decreased A/G ratios which may have been related to localized injection site inflammation. Overall, the changes in hematology and clinical chemistry parameters were milder for animals in the LErafAON ETU dose group. These changes were consistent with the treatment-related inflammatory response at the injection sites.

Group mean increase in complement markers Bb, C3a, C4a and C5a were observed on Days 5 and 12. These increases were consistent with complement activation and were similar in magnitude for both groups. Lower mean complement CH50 (total complement activity) values on Day 5 and 12 were observed in animals administered LErafAON-ETU as compared to those administered LErafAON and were considered compound-related. However the Bb, C3a, C4a and CH50 values for both groups were comparable to baseline values and control group values on Day 16.

At necropsy, hard nodules near infusion sites were observed in all rafAON-treated animals. Microscopic examination of the injection site nodules was consistent with inflammation. In addition, increased absolute and relative liver, lung and spleen weights were observed in both treatment groups. Decreased absolute and relative thymus weights were observed in animals treated with LErafAON. Microscopic lesions considered to be compound-related were limited to histiocytic infiltration of the spleen and a mixed cell infiltration of the liver observed in all animals in each of the treated groups. The histiocytes contained a foamy cytoplasmic material that was believed to represent the lipid component of the compound formulation or related degradation products. The severities of these lesions were similar between treated groups and were considered to be of minimal toxicological significance.

LErafAON-ETU was shown to be equal or less toxic than the LErafAON sonicated formulation with the exception of clinical signs on Day 1.

3. Toxicity of Weekly IV Infusions of LErafAON-ETU in Male Cynomolgus Monkeys

Male cynomolgus monkeys (3/group) were administered doses of approximately 0.625, 3.125 or 6.25 mg/kg LErafAON-ETU once per day on Days 1, 8, 15 and 22. Compound was administered as a 1 hour infusion on each day. Animals were sacrificed on

Day 26. Assessment of toxicity was based on mortality, clinical observations (including qualitative food consumption), body weights, physical and ophthalmic and electrocardiographic examination, clinical pathology and anatomic pathology. Blood was analyzed for complement markers.

All monkeys survived to their schedules sacrifice. Clinical signs consisting of tremors near the end of the 1-hour dosing period were observed in 2/3 monkeys given 6.25 mg/kg on Day 1 only. No other clinical signs were observed during the study in any animals. There were no compound-related effects on body weight, food consumption, ophthalmic examinations or electrocardiogram examinations. Gross observations at necropsy showed no remarkable findings, including at the infusion/catheterization sites. There were no apparent compound-related changes in organ weights.

The preliminary findings showed that there were no compound-related changes in any hematology or clinical chemistry parameters examined with the possible exception of mildly increased mean eosinophil counts at the 6.25 mg/kg dose level, primarily due to 1 animal.

Complement markers C3 and Bb increased at 10 minutes after each administration in all dose groups, but only after the first and last administration of LErafAON-ETU. Complement markers C5a and CH50 were not affected by compound administration. All complement markers had returned to baseline values before each dose administration.

Administration of LErafAON-ETU to monkeys once weekly for 4 weeks at doses of approximately 0.625, 3.125 and 6.25 mg/kg/dose was tolerated by the animals. With the exception of tremor on Day 1 in the high dose group, there were no adverse clinical signs and no effects on body weight or food consumption.

EXAMPLE 5

This example demonstrates that the compositions of the present invention represent a novel cationic cardiolipin platform for safe and enhanced in vitro and in vivo delivery.

A. Materials and Methods

1. Preparation of NeoPhectin®-AT (PCL-2-(CCLA)-Based Liposomes)

Cholesterol and DOPE (dioleoylphosphatidylethanolamine) used as helper lipids were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Several CCLA-based liposomes were prepared from CCLA and helper lipids at different molar ratio using thin film hydration method. In brief, CCLA, DOPE or cholesterol were dissolved in chloroform in a round bottom flask. The solvent was removed under reduced pressure using a rotatory evaporator to form a lipid film, and further dried under vacuum overnight. The lipid film was hydrated with 10% sucrose in RNase-free water under nitrogen, at 25-40°C. RNase-free water was obtained from Sigma (St. Louis, MO). The bulk cationic liposomes were extruded through 0.2 μm pore size polycarbonate filter three times and 0.1 μm pore size polycarbonate filter five times. The extruded liposomes were sterilized by filtering through 0.22 μm sterile filter unit (Millipak® 20 positively charged). The prepared liposomes (NeoPhectin-AT ™) had a mean particle size of 110-120 nm. The size of the liposomes was characterized using a light scattering particle sizer (Nicomp Model 380, Santa Barbara, CA).

2. Preparation of Cell Culture

A549 (human lung cancer), PC-3 (human prostate cancer), SK-OV-3 (human ovarian cancer), MX-1 and MDA-MB-231 (human breast cancer) cells were obtained from the National Cancer Institute (Fredrick, MD) and maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (HI-FBS). CHO (Chinese Hamster Ovary), BALB/3T3 (murine embryonic fibroblasts) and $HepG_2$ (human hepatocellular carcinoma) were purchased from American Type Culture Collection (Manassas, VA). CHO cells were maintained in Kaighn's modification of Ham's F12 medium (F12K) with 10% HI-FBS. BALB/3T3 cells were maintained in Dulbecco's Modified Eagle's Medium with 10% calf serum. $HepG_2$ cells were maintained in Eagle's Minimum Essential Medium with 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids and 10% HI-FBS. All culture medium and reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA).

B. Liposome Studies

1. In vitro efficiency

One day prior to transfection, CHO (25 000 cells/well), BALB/3T3 (25 000 cells/well), MX-1 (50 000 cells/well), and A549 (25 000 cells/well) were seeded in 96-well plates and $HepG_2$ (200 000 cells/well) in 24-well plates, and cultured overnight in a 5% CO_2 incubator. The cells were then washed with phosphate-buffered saline to remove the residual serum before adding the transfection mixture.

The transfection efficiency of PCL-2-(CCLA)-based liposomes was determined using β-galactosidase (β-gal) reporter gene assay for CHO, BALB/3T3 and $HepG_2$ cells and luciferase reporter gene assay for MX-1 and A549 cells. The pSV-β-galactosidase control vectors were purchased from Promega (Madison, WI) and the gWIZ luciferase vectors were purchased from Gene Therapy Systems (San Diego, CA). Total amount of DNA plasmids was 0.2 μg/well in 96-well plates and 1 μg/well in 24-well plates. The plasmids were diluted with OptiMEM I Reduced Serum Medium (Invitrogen, Carlsbad, CA) in cell culture tubes. Equal volume of OptiMEM I medium was used to dilute CCLA-based liposome. Appropriate amount of CCLA-based liposome was resuspended in OptiMEM I medium to obtain CCLA/DNA (+/-) charge ratio of 8:1 and diluted in series to 4:1, 2:1, 1:1 and 1:2 with OptiMEM I medium and incubated at room temperature for 5 minutes. DNA was then added drop by drop to CCLA-based liposome dilution and incubated at room temperature for additional 30 minutes. After incubation, the DNA/CCLA-based liposome complex was added to the cells and incubated further for 4-6 hours. After transfection, the residual DNA/CCLA-based liposome complex was washed off and the cells were replenished with regular culture medium and incubated up to 24 hours.

β-gal reporter gene assay was performed using β-gal Reporter Gene Assay System (Promega, Madison, WI) according to the manufacturer's protocol. The optical density (OD) of the sample was read at 414 nm using a plate reader (Thermo Electron, Franklin, MA). The amount of β-gal expression was calculated based on the exogenous β-gal standard. The expression of luciferase was measured using Luciferase Assay System (Promega, Madison, WI). The assay reagent was mixed with 20 μl of cell lysate and the relative light unit (RLU) was determined by a luminometer (Thermo Electron, Franklin, MA).

To determine the optimal combination for the CCLA-based liposome transfection, two most commonly used helper lipids, cholesterol and DOPE, were formulated with CCLA at different molar ratio and screened for their transfection efficiency *in vitro* using β-gal reporter gene assay. The evaluation results showed that the formulation composed of CCLA: DOPE at molar ratio of 1:2 had high transfection among the cell lines tested.

In CHO, BALB/3T3, A549 and MX-1 cells, the peak gene expression from reporter genes was obtained at 2:1 of CCLA/DNA (+/-) charge ratio, whereas in $HepG_2$ cells, the peak activity was at charge ratio of 1:1, as illustrated in FIG. 3.

2. In vivo efficacy

The transfection efficiency of PCL-2-(CCLA)-based liposome in male Balb/c mice was determined using Luciferase reporter gene assay. The gWIZ luciferase vectors (50 μg/mouse) were gently mixed to the surface of CCLA-based liposome. The DNA/CCLA-based liposome complex was injected to mice intravenously through tail vein within 8 hours of the preparation. Balb/c mice were obtained from Charles River Laboratories (Portage, MI). Equal quantity of DNA was delivered with In Vivo GeneSHUTTLE™, a DOTAP-based formulation (QBiogene, Carlsbad, CA) following the manufacturer's recommended protocol. The animals were sacrificed 24 hours after the injection. The lung and heart tissues were quickly removed and frozen in an ethanol/dry ice bath. The tissues were then homogenized in Cell Culture Lysis Reagent (Promega, Madison, WI) using an Autogizer (Tomtec, Hamden, CT). The homogenate was centrifuged at 12 000 rpm (16 000g) for 10 minutes at 4°C and the supernatant was transferred to a separate tube. A 20 μL aliquot from the supernatant was used to measure the luciferase activity. The protein concentration of each tissue sample was determined using RC DC protein assay according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA).

Several formulations composed of CCLA and cholesterol or DOPE at different molar ratios, were screened. Interestingly, the molar ratio of CCLA: DOPE at 1:2, the same composition to the *in vitro* formulation, showed better transfection activity than CCLA with cholesterol. When different charge ratios of CCLA/DNA (+/-) were tested with 50 μg of luciferase reporter gene, the maximum expression of luciferase was observed at 5:1 (+/-) both in lung and heart tissues, as illustrated in FIG. 5. In contrast, no expression was observed in the mice treated with DNA or CCLA-based liposome alone.

The transfection efficiency of CCLA-based liposomes was also compared with another commercially available cationic liposome (In Vivo GeneSHUTTLE™) in male Balb/c mice. The DOTAP-based In Vivo GeneSHUTTLE™ is one of very few commercially available liposome used for *in vivo* experiments. The luciferase reporter gene (50 μg/mouse) was delivered with either the optimal charge ratio (+/- 5:1) of CCLA-based liposomes or the In Vivo GeneSHUTTLE™ using the manufacturer's recommended condition. Our results showed that the luciferase gene expression in lungs, if delivered by CCLA-based liposome, was approximately 7 fold higher than by In Vivo GeneSHUTTLE™, as illustrated in FIG. 6. The calculated charge ratio of DOTAP to DNA was also approximately 5:1.

3. In vivo toxicity

The toxicity of PCL-2-(CCLA)-based liposome was determined in male Balb/c mice. The single-dose toxicity of the CCLA-based liposome was evaluated by injecting mice with 100 mg/kg of CCLA-based liposome via the tail vein. The DOTAP-based In Vivo GeneSHUTTLE™ was included for comparative analysis. The multiple-dose toxicity was also examined by injecting mice with 50, 75 or 100 mg/kg of the CCLA-based liposome via the tail vein for 3 consecutive days. Three animals were used in each group. The mortality, body weight and clinical signs of toxicity were monitored daily for 14 days.

No mortality was observed in CCLA liposome-treated group whereas 66.6% mortality was recorded for the In Vivo GeneSHUTTLE™-treated group, as indicated in Table 5A. In the multiple-dose toxicity study, mice were injected with 50, 75 or 100 mg/kg of CCLA-based liposome for 3 consecutive days. No mortality was seen in the groups injected with 50, and 75 mg/kg/day, whereas 33.3% mice died 3 days after receiving 100 mg/kg/day of CCLA-based liposome, as indicated in Table 5A. There was no significant body weight loss in 50 and 75 mg/kg treated groups and only up to 10.1% weight loss occurred in mice treated with 100 mg/kg/day of CCLA-based liposome. The surviving animals completely recovered 10 days after the initial treatment. Our results indicated that the toxicity of CCLA-based liposome was significantly lower than In Vivo GeneSHUTTLE™.

Table 5A: Toxicity studies of the CCLA-based liposome

Single-dose toxicity (I.V. x 1)

Transfection reagents	Dose (mg/kg)	Mortality rate (%)
CCLA-based liposome	100	0
In Vivo GeneSHUTTLE™	100	66.7

Multiple-dose toxicity (I.V. x 3)

Transfection reagents	Cumulative Dose (mg/kg/day)	Mortality rate (%)
CCLA-based liposome	50	0
	75	0
	100	33.3

EXAMPLE 6

A dose escalation study was conducted in order to determine the Maximum Tolerated Dose (MTD) of LErafAON-ETU in patients. LErafAON-ETU was administered as an IV infusion once weekly for three consecutive weeks. Patients were eligible to receive repeated Treatment Cycles (3 weeks each) until the occurrence of unacceptable toxicity or disease progression. Dose levels of 7.5, 15, 30, 60, 120, 240 and 480 mg/m^2 were planned to be administered until a cohort experienced a Dose Limiting Toxicity (DLT). The results for 7.5, 15,30, and 60 mg/m^2 doses are shown in Tables 6A-6D.

Table 6A

Cohort #1: 7.5 mg/m² once per week for 21 days

Patient #; Initials; Age; Sex; Dx;	Start Date	Dose (mg/m² x # of infusions)	Related AEs Graded >= 2 and SAEs	Best Response & Cycle(s) Assessed	Date of Progression & Cycle Progressed	Comments/ Reason Patient Stopped Treatment Other Than PD
#0101, B-F; 61 yoF; Ovarian Carcinoma	11/15/04	7.5 x 6	Anemia (Gr2) Cycle 1 SAE (Pain) Cycle 1	PD Cycle 2	12/21/04 Cycle 2	—
#0102, LJS; 62 yoF; Liposarcoma	11/30/04	7.5 x 11	Fatigue (Gr2) Cycle 2	SD Cycle 2	2/15/05 Cycle 4	—
#0103; ALC; 61 yoF; Ovarian Ca	12/9/04	7.5 x 18	Acute Infusion Reaction (Gr2) Cycle 1 Diarrhea (Gr3) Cycle 1	SD Cycle 2-4	4/14/05 Cycle 6	—

Table 6B

Cohort #2: 15 mg/m² once per week for 21 days

Patient #; Initials; Age; Sex; Dx;	Start Date	Dose (mg/m² x # of infusions)	Related AEs Graded >= 2 and SAEs	Best Response & Cycle(s) Assessed	Date of Progression & Cycle Progressed	Comments/ Reason Patient Stopped Treatment Other Than PD
#0201; NAB; 68 yoF; Adenocarcinoma	1/18/05	15 x 19	Acute IRR (Gr 2) Cycle 1	Stable Disease, Cycle 2,4, 6	6/9/05 Cycle 7	—

Table 6C

Cohort #3: 30 mg/m^2 once per week for 21 days

Patient #; Initials; Age; Sex; Dx;	Start Date	Dose (mg/m^2 x # of infusions)	Related AEs Graded >= 2 and SAEs	Best Response & Cycle(s) Assessed	Date of Progression & Cycle Progressed	Comments/ Reason Patient Stopped Treatment Other Than PD
#0301; LPC; 69 yoF; Non-Non-Small Cell Lung Ca	2/16/05	30 x 12	Acute IRR (G2) Cycle 1; Acute IRR (G 2) Cycle 2	Stable Disease Cycle 2-4	—	Off Study 5/11/05 after cycle 4 due to worsening performance status
#0302; DAS; 48 yoM; Melanoma	3/15/05	30 x 6	Acute IRR (G2) Cycle 1	Progressive Disease Cycle 2	4/22/05 Cycle 2	—
#0303; GRH; 61 yoM; Retroperitoneal Sarcoma	3/17/05	30 x 12	Acute IRR (G2) Cycle 1	Stable Disease Cycle 2	6/8/05 Cycle 4	—

Table 6D

Cohort #4: 60 mg/m^2 once per week for 21 days

Patient #; Initials; Age; Sex; Dx;	Start Date	Dose (mg/m^2 x # of infusions)	Related AEs Graded >= 2 and SAEs	Best Response & Cycle(s) Assessed	Date of Progression & Cycle Progressed	Comments/ Reason Patient Stopped Treatment Other Than PD
#401: PAT; 58 yoM; Renal Cell Ca	10/05/2005 Screening	N/A	SAE (Hematuria) G2 Screening	N/A	N/A	Never received treatment. Screen Failure.
#0402; AJF; 71 yoF; Colorectal adenoca	1/19/06	60 x 3	—	—	2/9/06 Cycle 1	—

No dose limiting toxicities were experienced at 15 mg/m^2. A review of infusion times, for patients treated at 15 and 30 mg/m^2, showed that infusion times of 60 minutes and 90 minutes were not tolerated. Similarly, an infusion time of 180 minutes presented some adverse effects for patients. Accordingly, a solution was sought to adjust infusion time to prevent or minimize infusion related reactions. As shown in Table 6E, drug exposure was first calculated to determine the maximum tolerable rate.

Table 6E

Dose Level	1 Hour Rate	1.5 Hour Rate	3.0 Hour Rate
7.5 mg/m^2	7.5 mg/m^2/hr	5 mg/m^2/hr	2.5 mg/m^2/hr
15 mg/m^2	15 mg/m^2/hr	**10 mg/m^2/hr**	5 mg/m^2/hr
30 mg/m^2	30 mg/m^2/hr	20 mg/m^2/hr	**10 mg/m^2/hr**
60 mg/m^2	60 mg/m^2/hr	40 mg/m^2/hr	20 mg/m^2/hr
90 mg/m^2	90 mg/m^2/hr	60 mg/m^2/hr	30 mg/m^2/hr
120 mg/m^2	120 mg/m^2/hr	80 mg/m^2/hr	40 mg/m^2/hr

The calculations, presented in Table 6E, showed that a dose of 10 mg/m^2/hr is well tolerated. Thus, subsequent doses at 30 mg/m^2/hr with premedication were administered over 90 minutes in the following crescendo [titration] fashion:

10 mg/m^2/hr for the first 30 minutes

20 mg/m^2/hr for the next 30 minutes

30 mg/m^2/hr for the last 30 minutes

With this protocol, no dose limiting toxicities occurred. Thus, another dose at 60 mg/m^2/hr, with premedication, was administered according to the protocol below. No dose limiting toxicities occurred at this dose level.

Protocol

10 mg/m^2/hr for the first 30 minutes

30 mg/m^2/hr for the next 30 minutes

80 mg/m^2/hr for the last 30 minutes

All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to,") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as

appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

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What Is Claimed Is:

1. A method of treating a cellular proliferative disease, comprising administering to a patient in need of such treatment a pharmaceutical composition comprising an effective amount of antisense raf oligonucleotide encapsulated liposomes wherein the effective amount is about 60 mg/m^2.

2. A method of inhibiting the growth of neoplastic cells comprising administering an effective amount of antisense raf oligonucleotide encapsulated liposomes to neoplastic cells under conditions such that said antisense raf oligonucleotide enters said cells, whereby the antisense oligonucleotide inhibits the expression of a raf oncogene within said neoplastic cells and wherein the effective amount is about 60 mg/m^2.

3. A method of reducing expression of a raf sequence in a target cell comprising administering an effective amount of antisense raf oligonucleotide encapsulated liposomes, wherein the effective amount is about 60 mg/m^2.

4. The method of claim 1, wherein the disease comprises cancer.

5. The method of claim 3, wherein the target cell is in vivo.

6. The method of any of claims 1-3, wherein the liposomes are comprised of 1,3-Bis-(1,2-bis-tetradecyloxy-propyl-3-dimethylethoxyammoniumbromide)-propane-2-ol (PCL-2) or analogue thereof and further comprising at least one lipid selected from the group of lipids consisting of cholesterol, dioleoylphosphatidylethanolamine (DOPE), 1,2 Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and any other phosphatidylcholine.

7. The method of claim 6, wherein the liposomes have a percent molar ratio of DOPC:cholesterol:PCL-2 of between about (50-65):(25-35):(5-20).

8. The method of claim 6, wherein the liposomes have a percent molar ratio of DOPC:cholesterol:PCL-2 of between about (50-65):(25-35):(5-20).

9. The method of claim 6, further comprising D-alpha tocopheryl acid succinate.

10. The method of claim 9, wherein the D-alpha tocopheryl acid succinate is present at between about 0.1 wt % and about 1 wt %.

11. The method of claim 6, wherein the liposomes consist of PCL-2 or analogues thereof and cholesterol.

12. The method of claim 11, wherein the molar ratio of PCL-2 and cholesterol is between 1:3 and 6:1.

13. The method of claim 6, wherein the liposomes consist of PCL-2 or analogues thereof and DOPE.

14. The method of claim 13, wherein the molar ratio of PCL-2 or analogues thereof and DOPE is between 1:3 and 6:1.

15. The method of claim 13, wherein the molar ratio of PCL-2 or analogues thereof and DOPE is 1:2.

16. The method of claim 6, wherein each of said liposomes comprises a total lipid concentration of about 1.0 mg/mL to about 60 mg/mL.

17. The method of claim 6, wherein the concentration of PCL-2 is about 0.3mM to about 20mM.

18. The method of claim 6, wherein a portion of the PCL-2 is present within said liposomes.

19. The method of claim 6, wherein a portion of the PCL-2 is part of an emulsion.

20. The method of claim 6, wherein the mean size of each of said liposomes is between about 50 nm and about 200 nm.

21. The method of claim 6, wherein the liposomes are unilamellar vesicles.

22. The method of claim 6, wherein the liposomes are multilamellar vesicles.

23. The method of claim 6, wherein the liposomes are a mixture of multilamellar and unilamellar vesicles.

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24. The method of claim 6, wherein the raf antisense oligonucleotide is about 5 to about 50 nucleotides long.

25. The method of claim 6, wherein the raf antisense oligonucleotide is about 10 to about 40 nucleotides long.

26. The method of claim 6, wherein the raf antisense oligonucleotide is about 15 to about 25 nucleotides long.

20 27. The method of claim 6, further comprising a sugar.

28. The method of claim 6, wherein each of said liposomes further comprise at least one active agent.

29. The method of claim 28, wherein the active agent is selected from a group consisting of a radiation agent, a chemotherapeutic agent and an anticancer drug.

30. The method of claim 6, wherein the respective charge ratio of PCL-2: oligonucleotide ranges from 1.0: 1 to 4.0: 1.

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31. The method of claim 6, wherein the oligonucleotide hybridizes to a human mRNA.

32. The method of claim 31, wherein the oligonucleotide is sufficiently complementary to the human mRNA.

33. The method of claim 31, wherein the oligonucleotide inhibits the expression of the gene upon binding to the mRNA.

34. The method of claim 31, wherein the mRNA is an oncogene.

35. The method of claim 34, wherein the oncogene is raf.

36. The method of claim 34, wherein the oncogene is c-raf-1.

37. The method of claim 6, wherein the composition is administered adjunctively with a second antineoplastic agent.

38. The method of claim 37, wherein the composition is administered prior to, concurrently with, or after the second antineoplastic agent.

39. The method of claim 37, wherein the second antineoplastic agent is a chemotherapeutic agent.

40. The method of claim 39, wherein the chemotherapeutic agent is docetaxel.

41. The method of claim 6, wherein the composition is administered adjunctively with radiation.

42. The method of claim 6, wherein the composition is administered intravenously at an adjusted rate to minimize size effects.

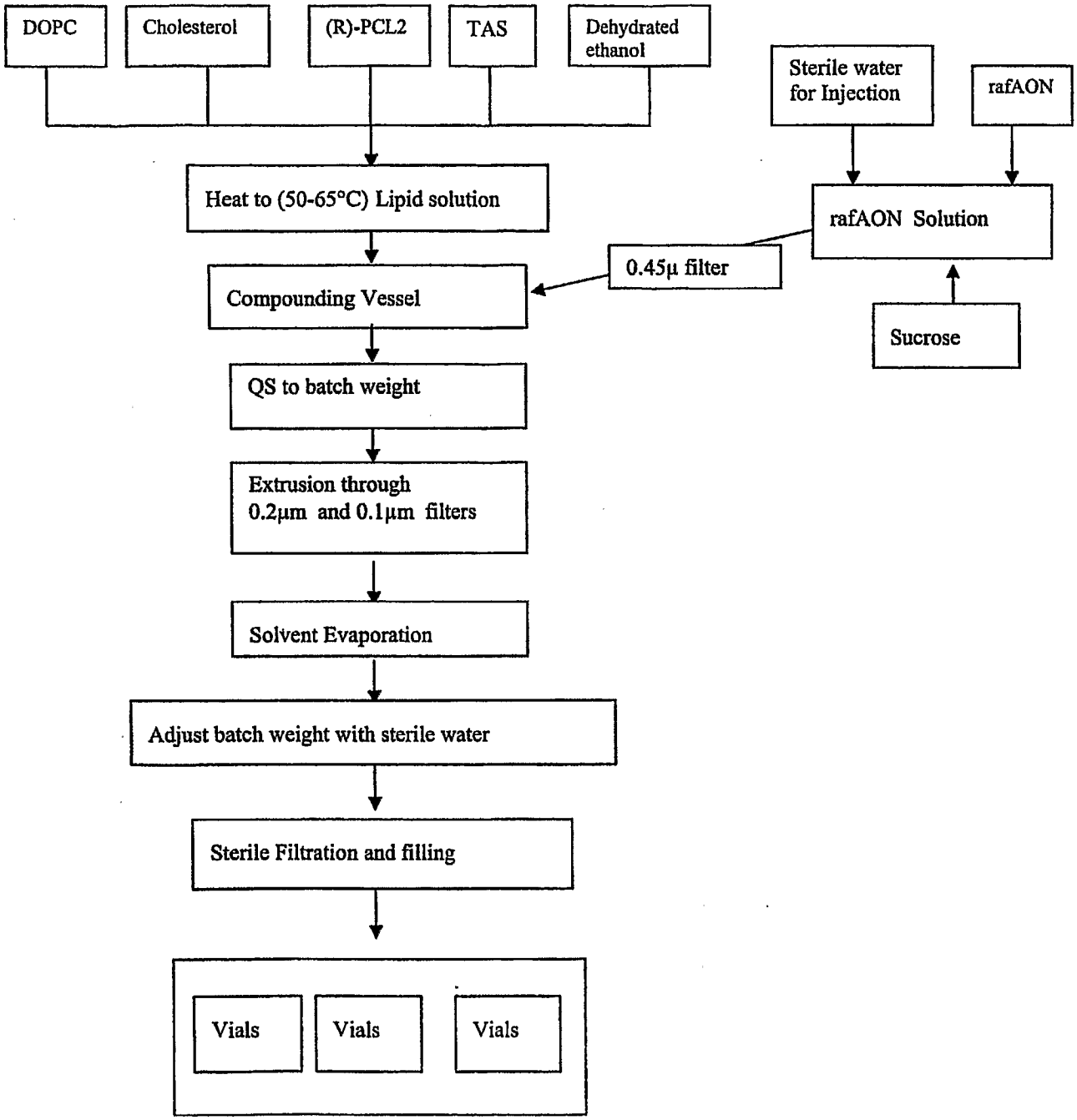

DOPC
Cholesterol
(R)-PCL2
TAS
Dehydrated ethanol
Sterile water for Injection
rafAON
Heat to (50-65°C) Lipid solution
rafAON Solution
0.45µ filter
Compounding Vessel
Sucrose
QS to batch weight
Extrusion through 0.2µm and 0.1µm filters
Solvent Evaporation
Adjust batch weight with sterile water
Sterile Filtration and filling
Vials
Vials
Vials

FIG. 1

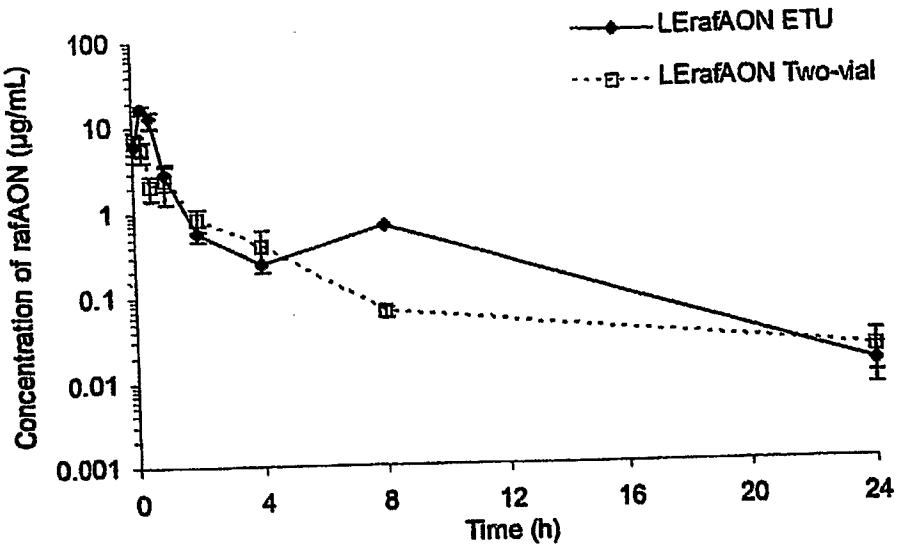

LErafAON ETU
LErafAON Two-vial
Concentration of rafAON (µg/mL)
100
10
1
0.1
0.01
0.001
0
4
8
12
16
20
24
Time (h)

FIG. 2

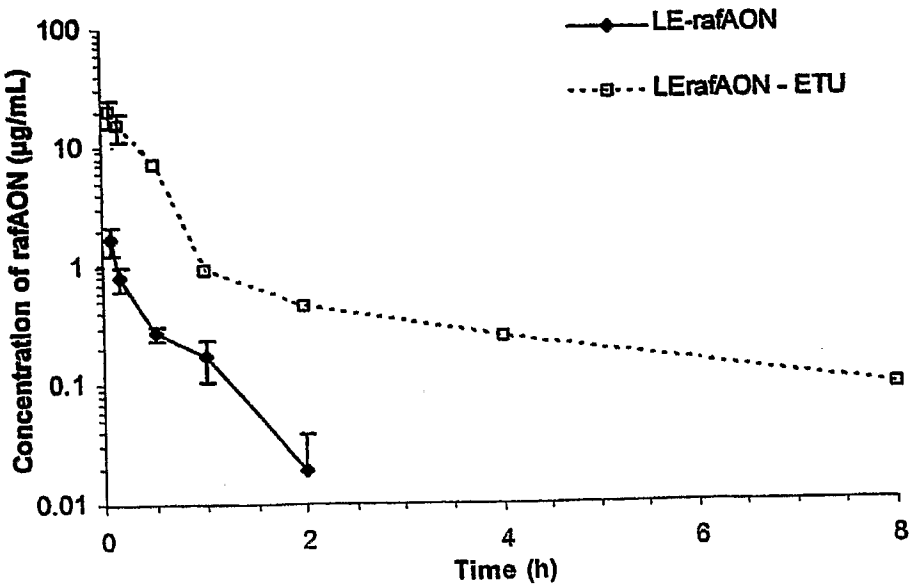

LE-rafAON
LErafAON - ETU
Concentration of rafAON (µg/mL)
100
10
1
0.1
0.01
0
2
4
6
8
Time (h)

FIG. 3

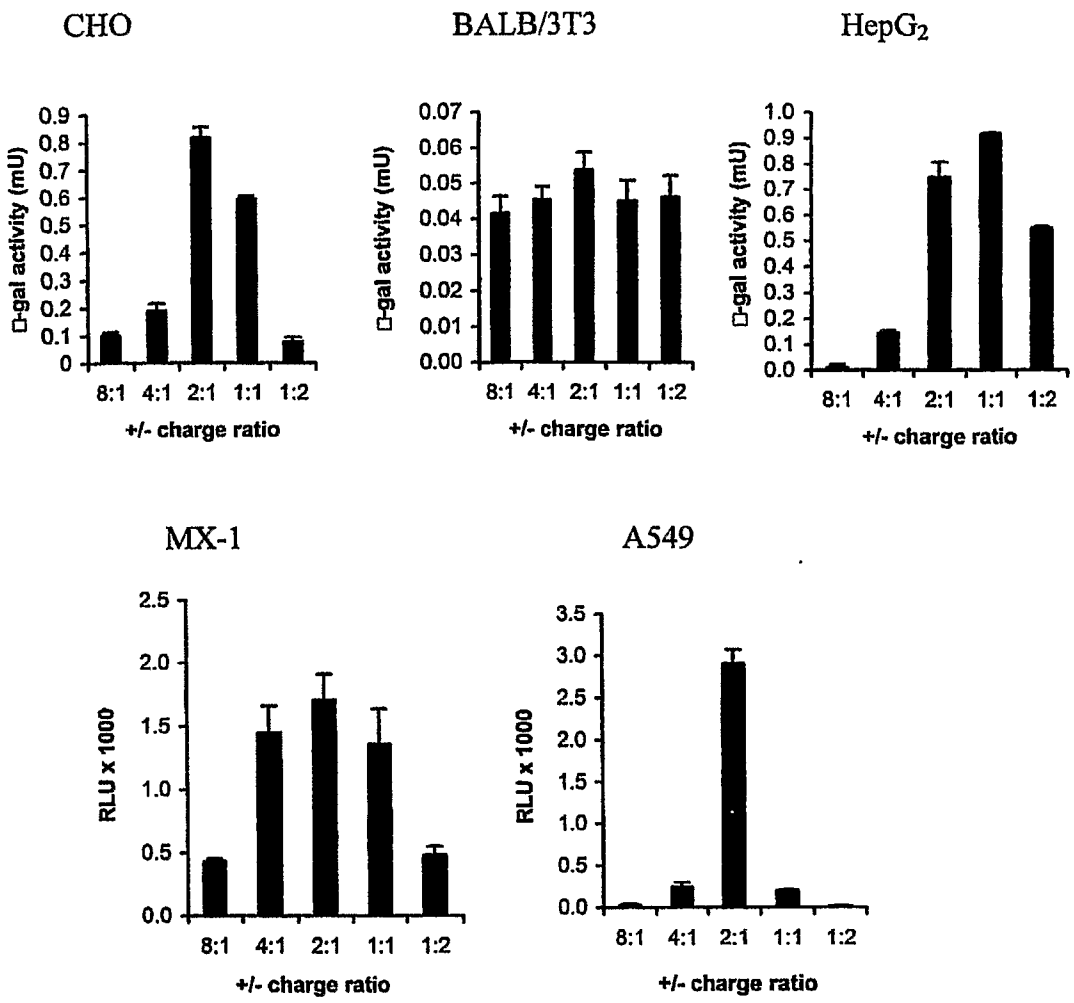
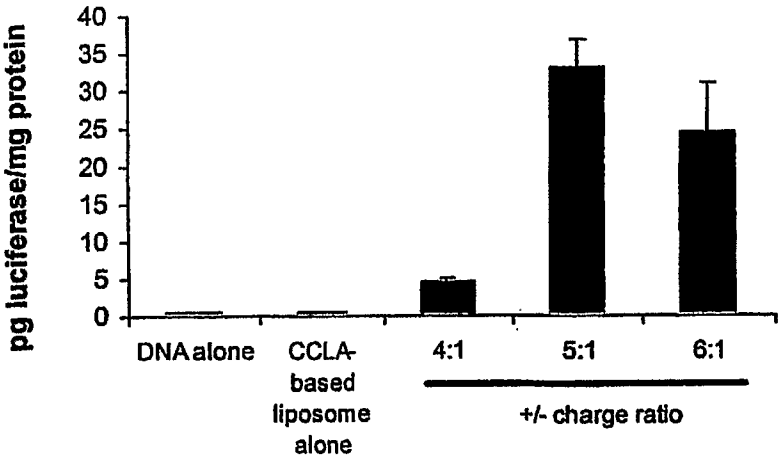

CHO
BALB/3T3
HepG2
MX-1
A549
□-gal activity (mU)
RLU x 1000
8:1 4:1 2:1 1:1 1:2
+/- charge ratio

FIG. 4

Lung



Heart

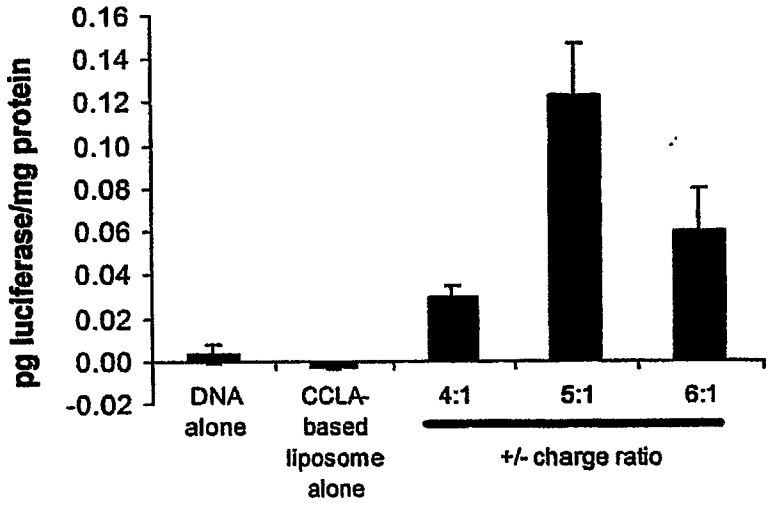


FIG. 5

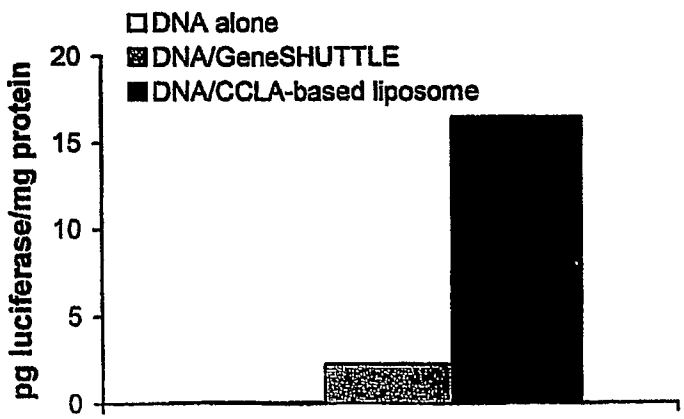


FIG. 6