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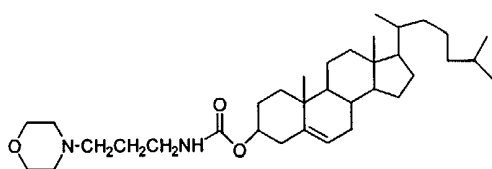
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(54) Title: MATERIALS AND METHODS RELATING TO GENE DELIVERY



(57) Abstract: Novel lipids for forming liposomes for use as nucleic acid delivery vehicles, e.g. for gene therapy, have structure with a steroid (cholesteryl) domain linked via a carbamoyl linkage to a head group which is positively charged in use (generally through protonation). The head group preferably comprises a nitrogen heterocycle, e.g. a piperazine or morpholine ring system.

Materials and Methods Relating to Gene Delivery

Field of the Invention

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The present invention relates to materials and methods involved in gene delivery. Particularly, but not exclusively, the present invention relates to new lipids derived from cholesterol which enhance the efficiency of gene delivery.

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Background of the Invention

Gene therapy represents a promising approach for the treatment of inherited or acquired diseases (Refs. 1-4). However, one of the most difficult hurdles in achieving effective gene therapy is the requirement for the use of efficient vehicles to deliver the gene of interest into target cells. A diverse spectrum of gene delivery vehicles ranging from replication incompetent viruses to DNA formulated with various delivery vehicles has been utilized (Refs. 5-7).

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Administration of DNA alone has also enabled successful gene transfer for a number of isolated applications but with a limited spectrum of organ-specific expression (Ref. 8). However, the majority of applications require the assistance of a delivery vehicle to facilitate gene transfer.

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Essentially, two approaches have been adopted to introduce exogenous DNA into cells. These are viral vector-based and plasmid DNA-based systems. Each system has its advantages and disadvantages, and all vehicles have been reported to achieve some level of gene

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delivery. Viral-based vectors have attracted most interests because of their expected high efficiency at mediating gene transfer (Refs. 9,10). Currently, the two most popular viral vectors for gene transfer are
5 replication defective retroviruses and adenoviruses. The retroviral vectors enable high degrees of gene transfer but can transduce only dividing cells (Ref. 11). Although adenoviral vectors can transfect non-dividing cells, they have been found to induce inflammatory and
10 immune responses and to therefore limit the duration of expression and efficacy of subsequent re-administrations (Refs. 12-14).

Most non-viral gene delivery systems are based on
15 cationic compounds. These include either cationic polymers or cationic lipids that spontaneously complex with a plasmid DNA construct by means of electrostatic interactions to yield a condensed form of DNA which shows increased stability toward nucleases. Several features
20 of the nonviral systems offer many advantages over viral systems, such as the ease of manufacture, safety, stability, lack of vector size limitations, low immunogenicity, and the potential to incorporate targeting ligand (Refs. 15-17).

25 Amongst the non-viral gene delivery systems, much interest has been paid to cationic liposomes, since these can potentially overcome problems associated with viral vectors. Several different cationic lipids, capable of
30 achieving reasonable level of gene delivery, have been synthesized (Refs. 18-26). These vary from lipids having monocation head groups, such as DOTMA (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride), DMRIE (dimyristoyl oxypropyl dimethyl hydroxyethyl
35 ammonium bromide), DOTAP (1,2-dioleoyl-3-trimethyl ammonium propane), and DC-chol (3- β -[N'-(N,N'-

dimethylaminoethane] carbamoyl cholesterol), to lipids having polycation head groups, such as DOSPA (2,3-dioleoyl-N[(sperminecarboxamino) ethyl]-N,N-dimethyl-1-propanaminium) and DOGS (Dioctadecyl amido glycidyl spermine). With the exception of DOGS, which is reported to have optimal gene delivery activity on its own (Ref. 28), most of the known cationic lipids are generally formulated as an aqueous liposome suspension with a helper lipid, e.g. DOPE (dioleoyl phosphatidylethanolamine) or cholesterol (Ref. 28).

The majority of previously disclosed cationic lipids are derivatives of long-chain fatty acids or cholesterol. The basic structure of most of those cationic lipids which are employed for gene delivery comprises four chemical functional domains, namely: (i) a positively charged head-group that allows interactions with negatively charged DNA, (ii) a spacer arm of varying length, (iii) a hydrophobic anchor group of either a cholesteryl or oleoyl residue which allows interactions with the cell membrane, and (iv) a linker bond that links the amino head group to the hydrophobic anchor residue (Ref. 27). Some of these cationic lipids exhibit a satisfactory level of gene delivery activities both *in vitro* and *in vivo*.

Although the uptake of cationic liposomes has been suggested to be via endocytosis and the endosomes (Refs. 30, 31), the exact mechanism of cationic lipid-mediated gene transfer has not been elucidated. Much of the recent development on new cationic lipid formulations has been empirical, and requires the systematic screening and testing of the formulations for optimal activity in the respective target cells/tissues.

Summary of the Invention

At its most general, the present invention provides novel lipids and liposomes which may enhance the delivery into cells of nucleic acid molecules *in vitro* and/or *in vivo*.

5 Further, the invention provides methods for preparing said lipids, and methods for their use in delivery of nucleic acids into cells.

Thus, in a first aspect, the present invention provides a
10 lipid having a steroid hydrophobic domain and a side chain including a heterocyclic ring. Preferably the lipid comprises a cholesterol hydrophobic domain, a carbamoyl linker bond and a head group which comprises a heterocyclic moiety and is positively charged and/or is
15 basic such that it can be protonated. In the latter case, the protonated form is generally a salt of an acid.

Preferably, the charged head group is or includes a nitrogen heterocycle. Particularly preferred are the
20 heterocycles morpholine, imidazole, pyridine or piperazine, optionally substituted.

Preferably a lipid of this aspect of the invention has a formula designated NCC1, NCC3, NCC4, NCC5, NCC6, NCC9 or
25 NCC10 as shown in Figure 2, or is a derivative and/or a protonated form thereof. Particularly preferred compounds are those designated NCC4 and NCC10, and derivatives and protonated forms thereof.

30 In a second aspect of the present invention, there is provided a lipid which comprises a cholesterol hydrophobic domain, a carbamoyl linker bond and a head group which comprises a primary or secondary aliphatic amine or polyamine, optionally protonated to render it
35 positively charged. Particularly preferred lipids of this embodiment have a formula designated NCC2, NCC7 or

NCC8 as shown in Figure 2, or are derivatives thereof.

A "derivative" of the lipid encompasses a lipid modified by adding or substituting one or more of its side groups, particularly on the steroid domain, without fundamentally
5 altering the essential structure and/or functional activity of the molecule, i.e. without significantly decreasing its efficacy in delivering nucleic acid into cells. For instance, the molecule may be modified by
10 substituting a hydrogen atom for a methyl group at one or more positions in the molecule. The degree and pattern of unsaturation of the steroid domain may be varied.

Preferred lipids of the present invention include groups, particularly involving nitrogen, which are protonated to
15 a significant extent under conditions of use.

The lipids as provided herein may be formulated into liposome preparations. Thus, the invention also
20 provides, in a further aspect, a liposome comprising one or more of the lipids described above. The liposomes are preferably prepared by mixing one or more of said lipids with a helper lipid such as DOPE or cholesterol, using standard methods known to those in the art ("Nonviral
25 vectors for gene therapy", Eds. Leaf Huang, Mien-Chie Hung and Ernst Wagner. Academic Press, 1999).

The lipids and liposomes of these aspects may be used in methods of nucleic acid delivery, e.g. gene delivery, as
30 described in further detail below.

In a further aspect, the present invention provides a method of producing a lipid of the first or second aspects, comprising the steps of reacting cholesteryl
35 chloroformate with an amine or a polyamine. The cholesteryl chloroformate may be dissolved in chloroform

in a first step, and an amine or polyamine may then be added in a second step. Alternatively, an amine chloroform solution may be added to a cholesterol chloroformate chloroform solution.

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In a preferred embodiment of this aspect of the invention, the amine or polyamine has a formula corresponding to the lipids shown in Figure 2 (replacing -CO.O-chol with H), or is a derivative or variant thereof, e.g. having one or more substituents on a heterocyclic ring and/or having less or more unsaturation.

Preferably, the method includes the further step of purifying the newly synthesised lipid. This, in preferred embodiments, is achieved by: recrystallising the lipid with ethanol; recrystallising with ethanol and acetonitrile; recrystallising with methanol then ethanol; or evaporating the solvent under vacuum and dissolving the lipid in ethanol followed by salt formation with HCl gas, filtration and drying.

In still further aspects, the present invention provides methods of delivering nucleic acid into a cell, comprising the steps of: complexing said nucleic acid with a lipid or liposome as provided herein; and delivering said nucleic acid/cationic lipid complex or nucleic acid/liposome complex into the cell. Generally complexing occurs with lipid in a cationic state, e.g. due to protonation.

The nucleic acid may be a DNA or RNA molecule, and may be in the form of an expression vector such that, following delivery, it will be expressed by the cell. The delivery of the nucleic acid is preferably achieved by injection of the complex into the cell. The nucleic acid may be

delivered into the cell for a therapeutic, prognostic, diagnostic or prophylactic purpose, e.g. in a method of gene therapy, particularly for treating a cancer or a cardiovascular disease.

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Furthermore, the nucleic acid/lipid complex or nucleic acid/liposome complex may be formulated in a pharmaceutical composition, which may also comprise a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other material well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient (i.e. the nucleic acid/lipid or nucleic acid/liposome complex). The precise nature of the carrier or other material may depend on the route of administration, e.g. oral, intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraperitoneal routes.

20 Thus, in still further aspects, the present invention provides pharmaceutical compositions comprising one or more of the above lipids or liposomes complexed with a nucleic acid molecule, in combination with a pharmaceutically acceptable excipient.

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The pharmaceutical composition may be administered to a patient in a method of gene therapy. Administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated, and will be clear to one skilled in the art. Examples of the techniques and protocols which are generally applicable here can be found in

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Remington's Pharmaceutical Sciences, 16th edition, Osol, A. (ed), 1980.

A lipid/nucleic acid or liposome/nucleic acid complex
5 according to the present invention may be used to
introduce a particular gene or other nucleic acid
molecule into specifically-targetted cells, which will
depend on the condition to be treated, and the nucleic
acid may contain e.g. regulatory elements which are
10 switched on more or less selectively by these target
cells.

In a further aspect, the present invention provides the
use of a lipid as provided herein in the manufacture of a
15 delivery vehicle for delivering a nucleic acid molecule
into a cell, which use includes the step of complexing
said nucleic acid with a cationic lipid as provided
herein. The delivery vehicle thus constructed is, in a
preferred embodiment, in the form of a liposome. As
20 described above, preferably the liposome is prepared by
mixing the cationic lipid with a helper lipid such as
DOPE or cholesterol, using standard methods known to
those in the art.

25 These, and other, aspects of the invention will be
described in further detail below.

Brief Description of the Figures

30 Aspects and embodiments of the present invention will now
be illustrated, by way of example only, with reference to
the accompanying figures. Further aspects and
embodiments will be apparent to those skilled in the art.

All documents mentioned in this text are incorporated
35 herein by reference.

Figure 1. Schematic diagram showing the synthesis of preferred lipids of the present invention.

Figure 2. Chemical structures of the newly-synthesized lipids, NCC1-NCC10.

Figure 3. Comparison of the efficiencies of transfection of HepG2 cells *in vitro* mediated by the cationic lipids NCC1-NCC10. 3 μ g DNA was complexed with the cationic liposomes, which were formulated with the novel cationic lipids synthesized by the present inventors and described herein, and the helper lipid DOPE, at a molar ratio of 6:4 per transfection. The transfection activity values shown are expressed as mean values $\pm$ SD (N = 4).

Figure 4. Comparison of the efficiencies of transfection mediated by a variety of non-viral vectors on human HepG2 cells. The cationic liposomes derived from DC-chol, NCC4 and NCC10 contained DOPE at the molar ratio of 6:4. 3mg pCMV-Luciferase DNA was employed per transfection. Cells were harvested 24h following DNA-mediated gene transfer with the various non-viral reagents and the luciferase activities shown are mean values $\pm$ SD of triplicates.

Figure 5. Comparison of the efficiencies of transfection on human HepG2 cells following gene delivery with cationic liposomes and adenoviruses. The luciferase activities shown here were mean values $\pm$ SD of triplicates (N = 3).

Figure 6. Comparison of the efficiencies of transfection mediated by DC-chol, NCC4 and NCC10 on various human tumour cell lines. The cationic liposomes derived from DC-chol, NCC4 and NCC10 containing DOPE at the molar

ratio of 6:4. 3mg pCMV-Luciferase DNA was employed per transfection. The DNA-liposome complexes in 1ml lactate buffer were added to 5×10^5 cells per well in 6-well plates in the absence of serum. After 2h, 20% FBS was
5 added. The cells were harvested 24h after transfection. The luciferase activities shown are mean values $\pm$ SD of triplicates.

Figure 7. Effect of FBS on the efficiencies of
10 transfection mediated by cationic liposomes. Cationic liposomes prepared from NCC4, NCC10 or DC-chol at the charge ratios of 0.4, 1.0, 2.63 and 4.0 were employed to transfect pCMV-Luciferase DNA into human HepG2 cells in the presence or absence of FBS. The luciferase
15 activities shown are mean values $\pm$ SD of triplicates (N = 3).

Figure 8. Effect of mouse serum on the efficiencies of transfection mediated by cationic liposomes. Cationic
20 liposomes prepared from NCC4, NCC10 or DC-chol at the charge ratios of 0.4, 1.0, 2.63 and 4.0 were employed to transfect pCMV-Luciferase DNA into human HepG2 cells in the presence or absence of mouse serum. The mouse serum was obtained from normal BALB/c mice and pooled. The
25 luciferase activities shown are mean values $\pm$ SD of triplicates (N = 3).

Figure 9. NCC4 and NCC10 were less active at high FBS concentrations. Cationic liposomes prepared from NCC4,
30 NCC10 or DC-chol at the charge ratios of 2.6 and 4.0 were employed to transfect the pCMV-Luciferase DNA into human HepG2 cells in the presence or absence of FBS. The luciferase activities shown were means $\pm$ SD of triplicates (N=3).

Detailed Description

Chemical synthesis

Described herein are a series of novel cationic lipids
5 for gene delivery. Ten of these lipids, having the
structures as shown in Figure 2 were synthesized. They
are derivatives of cholesterol.

For all the syntheses, cholesteryl chloroformate was
10 allowed to react either with amines or polyamines to
produce the respective cationic lipids (Figure 1).

Essentially, two different protocols of synthesis were
employed:

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(1) Synthesis of $3\beta\{[4-(3\text{-aminopropyl})\text{ morpholine}]-$
 $\text{carbamoyl}\}$ cholesterol chloride (HCl salt of NCC4).

For this reaction, cholesteryl chloroformate (1g,
2.2mmol) was dissolved in 15ml of anhydrous chloroform
20 and stirred under N_2 . 4-(3-Aminopropyl) morpholine
(0.457ml, 2.2 mmol) was added to the reaction mixture
over a 20 min period. When the addition of 4-(3-
aminopropyl) morpholine was complete, the mixture was
further stirred at room temperature overnight. The
25 solvent was then evaporated under vacuum and the residue
was recrystallised with anhydrous ethanol and
acetonitrile. The desired product was obtained as a
white powder with an estimated yield of 49.2%. Proton
NMR (300MHz, $CDCl_3$): 5.66(s, 1H), 5.38(s, 1H), 4.43(bs,
30 1H), 4.12(bs, 4H), 3.31(t, 2H), 3.05(t, 6H), 2.36-0.62(m,
45H).

The HCl salts of NCC1, NCC2, NCC3, NCC6, NCC9 and NCC10
were similarly synthesized.

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(2) Synthesis of $3\beta\{[N, - \text{bis}(3\text{- aminopropyl})-1,3-$

propanediamine]-carbamoyl} cholesterol chloride, (HCl salt of NCC8).

N, N-bis (3-aminopropyl)-1,3-propanediamine (0.445ml, 2.2mmol) was dissolved in 10ml anhydrous chloroform and stirred under N₂. Cholesteryl chloroformate (1g, 2.2mmol)
5 was dissolved in 10ml anhydrous chloroform and added to the reaction mixture over a 30-min period. The mixture was further stirred at room temperature for 2 hrs. The solvent was evaporated under vacuum and the dry powder
10 was redissolved in 60ml of anhydrous ethanol and filtered. HCl vapor was passed into the solution until no further precipitate formed. The white powder obtained was then dried under vacuum. The yield for this synthesis is approximately 73.6%. Proton NMR (300MHz, CDCl₃): 5.44 (s, 1H), 4.40 (bs, 1H), 3.40-0.77 (m, 67H),
15 0.68 (s, 3H).

The HCl salts of NCC5 and NCC7 were similarly synthesized.

20

Purification of lipids

Lipids NCC4 and NCC10 were purified by recrystallisation with ethanol and acetonitrile. Lipid NCC1 was purified by recrystallisation with methanol first, followed by
25 ethanol, while NCC2 was purified by recrystallisation with ethanol alone. Lipids NCC3, NCC4, NCC6 and NCC10 were recrystallised with ethanol-acetonitrile, while lipids NCC5, NCC7, NCC8 and NCC9 were purified by evaporating the solvent under vacuum and dissolving the
30 lipid in ethanol, followed by salt formation with HCl gas, filtration and drying.

Preparation of cationic liposomes

Cationic liposomes were prepared by mixing stocks of the various cationic lipids synthesized above with the helper lipid DOPE (dioleoyl phosphatidylethanolamine) at a molar ratio of 6:4 (cationic lipid:DOPE) under a gentle stream of N₂, and dried under vacuum overnight as described earlier (Ref. 32). The dried film of lipid was hydrated in 1ml of 20mM sterile HEPES buffer (pH7.8). For lipids that did not dissolve readily in HEPES, 0.7ml HEPES buffer was first added and the pH was then adjusted using 0.1N sterile HCl until all of the lipids had dissolved. The final volume was diluted to 1ml with the addition of extra HEPES buffer. The liposomes were vortexed for 1 min and hydrated overnight at 4°C. All the cationic liposomes preparations were further sonicated before use as described earlier (Ref. 32).

Preparation of liposomes and DNA complexes

The liposomes/DNA complexes were prepared as earlier described (Ref. 32). Briefly, various amounts of liposomes and DNA were mixed together in 1ml sodium lactated Ringer's buffer (B. Braun Melsungen AG, Melsungen, Germany) and the complexes were allowed to form for a minimum of 15 min at room temperature before being employed for gene delivery.

Transfection of cancer cell lines *in vitro*

The human cancer cell lines A549, HepG2, CNE-2, KZ2, and SW837 were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). The human breast cancer cell line MCF-7 was propagated in RPMI 1640 with 10 % FBS and 10µg/ml bovine insulin. For transfection, 1×10^5 cells were seeded into each well of a 6-well plate (Nunc, Denmark). After culturing for 12h, cells were washed twice with PBS. Freshly prepared liposome/DNA complexes were added into each well and incubated at room temperature for 15 min. For transfection performed in the presence of various concentrations of serum, FBS (Hyclone Laboratories, Logan, UT, USA) or mouse serum

(obtained from BALB/c mice) was added to the DNA-liposome solution to the final concentration as indicated in the text. 1ml of sodium lactated Ringer's buffer was then added into each well and incubated for an additional 2h at 37°C. At the end of 2h, 3ml of 20% FBS was added into each well and the cells were assayed after 24h.

Infection of HepG2 cells *in vitro*

Adenovirus containing the luciferase gene was obtained from Dr. Matt Cotton (IMP, Vienna, Austria). The adenoviruses were amplified using human 293 cells. For infection with the adenoviruses, 1×10^5 cells were seeded into each well of a 6-well plate 24h before infection and either 10, 100 or 1000 virus particles per cell were then added to each well. Cells were harvested 48h following infection. Luciferase activity and protein assays were performed as described below.

Gene transfer *in vivo*

The direct injection of DNA-liposome complexes into the spleens of mice has been previously described (REF. 32). Briefly, 20µg of pCMV-luciferase DNA was injected directly into the spleen of each mouse. Expression of the luciferase gene in the spleens of the recipient mice were assayed 24h following injection as previously described. The charge ratio (liposome:DNA) employed was 0.4.

Assay for Luciferase activity

Cells were harvested from 6-well plates 24h following transfection, washed, re-suspended in 120ml Tris-HCl (pH7.8), and freeze-thawed three times. Cell debris was discarded following centrifugation at 14,000g at 4°C for 10 min. and 100µl of the supernatant was employed for assaying luciferase activity using the Auto-Lumat LB952 luminometer (EG&G Berthold, Bad Wildbad, Germany). In

addition, 5 μ l of the supernatant was employed for the determination of protein concentration using the Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, CA, USA) with the Ultrospec 3000 UV/visible spectrophotometer (Pharmacia Biotech).

Determination of zeta potential and size of cationic liposomes

The zeta potential (electrokinetic potential) of the newly synthesized cationic liposomes was determined by the ZetaSizer 3000HS (Malvern Instruments, Worcestershire, GB). Zeta potential correlates with the net surface charge of the liposome complexes, and also reflects the stability of cationic liposomes in solution at various pHs. Calibration was established using the -50mV DTS50/50 standards from Malvern Instruments as recommended by the manufacturer. Experimental samples taken from the 1 ml liposome stock of the various lipids following sonication for 5 min (prepared as described under the preparation of cationic liposome above) were diluted in 20mM HEPES buffer (pH7.8) and measured 10 times at 144 volts and 25°C using a capillary cell to give a count rate of particle about 2500 KCps.

To measure the size of the liposomes, experimental samples taken from the 1 ml liposome stock of the various lipids following sonication for 5 min (prepared as described under the preparation of cationic liposome above) were diluted in 20mM HEPES buffer (pH7.8). Each sample was measured 10 times using a detector angle of 90 degree and the size obtained was analyzed using monomodal. The 204nm $\pm$ 6nm nanosphereTM Size standards polystyrene polymer (Duke Scientific Corp., CA, USA) was employed as standards to calibrate the instrument.

Results

A variety of amphipathic amines or polyamines were synthesized by acylation (Figure 1). These newly synthesized lipids were evaluated systematically for their abilities to effect gene delivery. Ten representative cationic lipids having different chemical structures and charges, as shown in Figure 2, have been chosen for study in details.

The present inventors have employed the cationic lipid DC-chol as a gene delivery vehicle.^{32,33} It has been obtained that the gene delivery activity of DC-chol is optimal when used in conjunction with the neutral lipid DOPE at the molar ratio of 6:4 (DC-chol: DOPE).¹⁹ When the newly synthesized cationic lipids were compared to DC-chol and DOPE at the molar ratio of 6:4 for their ability to deliver the reporter gene pCMV-Luciferase into human HepG2 cells, it was observed that NCC 3, 4, 5, 8 and 10 all gave significantly higher activities than DC-chol (Figure 3). The increase was even more pronounced for NCC4, NCC5 and NCC10 when compared to DC-chol. NCC4 and NCC10 gave an overall increase of more than 6- and 3-folds respectively in the luciferase activities when compared to DC-chol 24h following gene delivery into HepG2 cells (Figure 3). On the other hand, NCC2 and NCC6 demonstrated a reduction in luciferase activity in comparison to DC-chol under the same conditions following gene delivery to HepG2 cells (Figure 3). NCC4 and NCC10 were chosen to be further studied since they are relatively easy to be synthesized and demonstrated good level of gene expression following delivery.

When compared to LIPOFECTAMINE (Gibco-BRL, Gaithersburgh, USA) and the cationic polymer PEI, NCC4 and NCC10 gave more than 2-fold increase in the luciferase gene activity following introduction of the pCMV-luciferase DNA into HepG2 cells (Figure 4). Among the various viral vectors available for gene delivery, adenovirus is one of the most efficient. We have therefore compared the ability

of adenoviruses to infect the human HepG2 hepatoma cells to that of gene delivery mediated by the cationic liposomes containing NCC4, NCC10 and DC-chol (Figure 5). The infection conditions used have been optimized for adenovirus and different viral particles were employed for the infection (Figure 5). It could be demonstrated that NCC4 and NCC10 gave better or at least comparable level of gene expression to that obtained with 100 adenoviral particles.

The efficiencies of NCC4 and NCC10 to mediate gene transfer to various other tumor cell lines were further compared with that of DC-chol. These lines included KZ2 (melanoma), CNE2 (nasopharyngeal carcinoma), MCF7 (breast carcinoma), A549 (lung carcinoma), PA1 (ovarian carcinoma). For most of the cell lines studied, NCC4, NCC10 and DC-chol gave comparable results to that obtained with human hepatoma cell HepG2 (Figure 6). For the cell lines MCF-7 and SW837, DC-chol gave a higher level of gene expression in comparison to NCC4 (Figure 6). However, NCC10 gave the highest level of gene expression compared to NCC4 and DC-chol (Figure 6).

One of the major disadvantages with current cationic liposomes as mediators for gene transfer is their low efficiency of transfection, especially when employed for *in vivo* gene delivery¹⁸. This observation has been demonstrated to be due to the inactivation of the DNA-liposome complexes *in vivo*³⁴⁻³⁶.

In the present study, we evaluated the ability of NCC4, NCC10 and DC-chol to withstand serum inactivation at various charge ratios using HepG2 cells in the presence of different concentrations of FBS and mouse serum. In the presence of FBS, NCC10 showed resistance to serum inactivation at all the serum concentrations tested (Figure 7). At the charge ratio 0.4, the efficiency of

transfection mediated by NCC10 was enhanced by more than 276 folds in the presence of 1% FBS. It is apparent that NCC10 became more sensitive at higher serum concentrations at the charge ratios of 0.4 and 1.0 (Figure 7). However, at the charge ratios of 2.6 and 4.0, the efficiency of transfection mediated by NCC10 was not affected by the increase in serum concentrations (Figure 7). In comparison to NCC10, NCC4 was basically ineffective in mediating gene transfer at the charge ratio of 0.4 (Figure 7). However, the efficiency of transfection of NCC4 markedly increased with the gradual increase in the charge ratios to 1.0, 2.6 and 4.0 (Figure 7). The efficiencies of gene delivery obtained with liposomes produced with DC-chol remained relatively low for all the conditions studied (Figure 7).

All the three lipids were tested for their ability to transfect the human cell line HepG2 at various charge ratios in the presence of 1% and 5% of mouse serum (Figure 8). Compared to FBS, it was apparent that mouse serum is a more potent inhibitor of cationic liposome-mediated gene delivery (Figure 8). Although the efficiency of transfection mediated by NCC4 increased with the increase in charge ratios in the presence of 1% mouse serum, NCC10 gave the best consistent resistance to mouse serum inactivation at all the conditions tested (Figure 8).

To study if the differences in the efficiencies of transfection obtained for NCC4, NCC10 and DC-chol in the presence of sera result from differences in the zeta potentials of the liposomes at the different serum concentrations, the zeta potentials of the liposomes prepared from the various lipids were determined. As shown in Table 1, the overall charge of all of the liposome preparations changed from a positive value to a negative value when measured in the presence of serum.

However, no significant difference in zeta potential could be obtained between NCC4, NCC10 and DC-chol at different concentrations of FBS (Table 1). Therefore, it is unlikely that the differences in the efficiencies of transfection obtained for NCC4, NCC10 and DC-chol in the presence of serum is due to differences in the zeta potentials of the liposomes.

Table 1. Determinations of the sizes and zeta potentials, in the absence or presence FBS, for cationic liposomes derived from NCC4, NCC10 and DC-chol.

<u>Liposomes</u>	<u>Size (nm)</u>	<u>Zeta potential (mV)</u>			
		<u>0% FBS</u>	<u>1% FBS</u>	<u>5% FBS</u>	<u>10% FBS</u>
DC-chol	127±2.5	41.1	-9.5	-19.9	-23.3
NCC4	129±1.7	15.5	-22.7	-21.8	-22.1
NCC10	138±2.9	27.3	-22.8	-22.8	-23.4

We have also compared the ability of the newly synthesized cationic lipids NCC4 and NCC10 with DC-chol in their ability to mediate gene transfer *in vivo*. DNA liposome complexes, prepared either from NCC4, NCC10 or DC-chol, at charge ratios of 0.4, 1.0, 2.6 as well as 4.0 were injected directly into the spleens of mice and assayed for luciferase activities one day following the injection. At the charge ratio of 0.4, both NCC4 and NCC10 gave the highest expression of the reporter gene pCMV-Luciferase (Table 2). The ability of the various cationic liposomes including NCC4, NCC10, DC-chol and DOTAP to mediated gene delivery were then compared under the pre-determined optimal conditions for each of the respective cationic liposome. The published optimal conditions for DOTAP³⁷ were followed. Under these conditions, it was observed that NCC4 and NCC10 were much better mediators for gene delivery in comparison to DOTAP and DC-chol (Table 2).

Table 2. Expression of luciferase in spleens of mice followed *in vivo* gene delivery with various formulations of cationic liposomes at different charge ratios.

5

RLU/mg					
Charge ratio	Liposome	organ	mouse1	mouse2	mouse3
0.4	NCC4	spleen	13416	110644	9038
	NCC10		8487	6605	5828
	DC-chol		7795	2973	2955
1.0	NCC4	spleen	570	3803	1470
	NCC10		2502	225	621
	DC-chol		145	547	37
2.6	NCC4	spleen	116	105	193
	NCC10		99	194	131
	DC-chol		44	133	53
4.0	NCC4	spleen	15	36	129
	NCC10		21	40	57
	DC-chol		182	203	62
0.4	DOTAP	spleen	672	272	120
3.0	DOTAP	spleen	401	495	4162

To try to understand why NCC4 and NCC10 did not produce a high level of *in vivo* gene expression following systemic delivery of the DNA/liposome complexes, we have repeated
5 our *in vitro* gene transfer experiments to include the serum concentration of 20% and 55% (Figure 9). At the liposome/DNA charge ratio of 2.6 and a serum concentration of 20%, NCC4 and NCC10 gave a marginally better level of gene expression in comparison to DC-chol
10 (Figure 9). At the liposome/DNA charge ratio of 4.0 and a serum concentration of 20%, NCC4 gave a high level of gene expression (Figure 9). However, at the liposome/DNA charge ratios of 2.6 and 4.0 and the serum concentration of 55%, it was demonstrated that the level of gene
15 expression obtained for NCC4 and NCC10 was low but still higher than that of DC-chol (Figure 9). A serum concentration of 55% would be similar to the serum concentration *in vivo*, this could therefore be one of the possible explanations for the low level of gene
20 expression obtained following systemic injection of the NCC4-, NCC10-DNA complexes. Therefore, it appears that there is a direct correlation of the ability of NCC4 and NCC10 to mediate gene transfer *in vitro* and *in vivo*.

25 Discussion

Structurally, there are four functional domains that could be identified in cationic lipids as described above. It has been reported that these four domains play
30 a role in determining their abilities to transfect cells as well as their toxicities to target cells.^{15,23,38} The anchor residues could be either cholesterol or diacyl chains.

Usually, it has been reported that lipids with cholesterol residues gave better efficiency of gene delivery and were relatively less toxic to target cells in comparison to lipids having diacyl residues in their hydrophobic anchor.²⁹ The linker within the cationic lipid could be in the form of a urea, amine, amide, ether or ester bond.²² The linker bond has been found to have some correlation with the stability of the cationic liposomes. It has been reported that when a carbamoyl bond is employed as the linker bond, the lipids derived are degradable and therefore would be potentially less toxic to the target cells both *in vitro* and *in vivo*.¹⁹

A spacer arm of 3-6 atoms between the amino group and the linker bond was reported to provide the optimal distance for efficient gene delivery activity.²³

The positively charged head group of a cationic lipid appears to be the most important domain in determining the overall efficiency of gene delivery characteristics for the particular cationic lipid. Lipids bearing linear amines or polyamines as positively charged head group exhibit good gene delivery activities.²³ This is especially true for cationic lipids that demonstrate an overall T-shape configuration.²³ Therefore, when the orientation of the amine or polyamine head group is structurally perpendicular in relation to the lipid anchor, the efficiency of the lipid to mediate DNA gene delivery will be enhanced.

We have synthesized a new series of cationic lipids using cholesterol and carbamoyl as the hydrophobic domain and linker bond respectively. This is to exploit the fact that lipids containing carbomoyl linker bond are degradable and could potentially be less toxic *in vivo*.

In comparison to the cationic lipid DC-chol, the main difference in the structure of a preferred group our newly synthesized cationic lipids is the presence of heterocycles as the amino group. Chemically, these
5 include morpholine (NCC4), imidazole (NCC3), pyridine (NCC6), and piperazine (NCC1, NCC5, NCC9, NCC10). To study the effect of these chemical changes on the overall efficiency of gene delivery of the cationic lipids, cationic lipids containing linear amine (NCC2) or
10 polyamine (NCC7 and 8) as head group were also synthesized and compared to lipids having heterocyclic head groups for their ability to act as gene delivery vehicles (Figure 2). Cationic liposomes prepared from the cationic lipids NCC1, NCC3, NCC4, NCC5 and NCC10 that
15 contain heterocycles as head groups gave better or similar efficiency of gene transfer in comparison to DC-chol. The only exception is NCC6 which gave a poorer efficiency in comparison to DC-chol (Figure 2).

20 It appears therefore that cationic lipids with linear primary amines or polyamines as the head group were less active than lipids having heterocycles as the head group in their structure with reference to their ability to deliver DNA into target cells. Within the group of
25 cationic lipids having heterocycles in their structure, lipids with piperazine (NCC1, 5, 9 and 10) and morpholine (NCC4) are relatively more active (Figure 3). Furthermore, NCC4 and NCC10 are the most active of the ten newly synthesized cationic lipids. Although NCC9 has
30 a piperazine group as the head group, it is not efficient in gene delivery. This may due to the fact that NCC9 contains two cholesteryl groups and as a result, it is possible that its overall structure might be too bulky to interact with DNA (Figure 2). In comparison, cationic
35 lipids with pyridine as their head group, for example

NCC6, are less active. The positively charged head group was generally believed to allow interactions between the cationic lipid and the negatively charged DNA, and also the cell membrane through charge/charge interactions.^{39,40}

5 The presence of nitrogen and oxygen atoms in the heterocyclic ring might further contribute to this charge/charge interaction of liposome and plasmid DNA and stabilize the binding between the cationic liposomes and DNA.

10

In our tests on the newly synthesized cationic lipids described herein, NCC4 and NCC10 were the most efficient gene delivery vehicles. This was also true when they were employed to transfect cell lines such as HepG2
15 (human liver cancer cell line) and KZ2 (human melanoma cell line) that are generally very difficult to transfect with other reagents including DC-chol, PEI, and LIPOFECTAMINE (Figure 6).

20 When first studied, the cationic lipid NCC5 gave high efficiency of transfection with HepG2. However, its activity decreased very sharply on storage. A likely explanation is that NCC5 not stable in such a formulation.

25

All the newly synthesized cationic lipids contain a carbamoyl bond, as in DC-chol. Therefore they should be hydrolyzed by esterases and degraded once administered into cells. Judging also from the appearance and the
30 amount of extractable protein recovered from the cells following transfection, the newly synthesized lipids exhibited no higher toxicity in comparison to DC-chol.

One of the major disadvantages with cationic liposomes as
35 a mediators for gene transfer is their low efficiency of

transfection, especially when employed for *in vivo* gene delivery.⁴¹ One of the explanations is that serum proteins interact with the membrane bilayers of liposomes and thus destabilize the DNA-liposome complexes *in vivo*.³⁴⁻³⁶ Attempts have been made to develop serum-resistant lipids and new formulations of DNA-liposome complexes to prevent the inactivation of cationic lipoplexes by serum.^{42,43} It has been demonstrated that the charge ratios of cationic liposomes to DNA play a critical role in determining the efficiency of gene delivery in the presence of serum.⁴⁴ It was also suggested that the role of charge ratio in serum sensitivity is dependent on the chemical structures of the cationic lipids. For example, the cationic lipid LIPOFECTAMINE is inactivated by serum at all the charge ratios studied. In contrast, cationic liposomes produced with the cationic lipid DOTAP and the helper lipid DOPE showed a lower charge ratio at which they could completely overcome the serum effect. In the present studies, NCC10 could overcome the serum effect at a lower charge ratio than NCC4. At a charge ratio of 0.4, the presence of FBS dramatically increased the gene expression of NCC10, whereas NCC4 was ineffective. However, when the charge ratio was increased from 0.4 to 2.6, the gene expression mediated by both NCC4 and NCC10 was substantially increased by inclusion of FBS in the transfection complexes (Figure 7). Compared to FBS, mouse serum is a more potent inactivator of cationic liposomes. The addition of 5% mouse serum completely inactivated the transfection activity of NCC4 and NCC10 (Figure 8). The mechanism by which a low concentration of serum could transiently increase the gene delivery activity of NCC4 and NCC10 is presently unknown. Nevertheless, it is possible that the uptake of the

liposome/DNA complexes into cells by endocytosis may be less active in the absence of serum.

The approach to generate new cationic lipids by systematically modifying the head group of a cationic lipid allow us to study the interactions between liposomes and cell membrane as well as their contributions towards the overall efficiency of gene delivery. A series of experiments has been performed to optimize the conditions for gene expression following intra-splenic injection of DNA/liposome complexes. The efficiency of gene delivery mediated by NCC4 and NCC10 at charge ratios of 0.4, 1.0, 2.6 as well as 4.0 was compared. At the charge ratio of 0.4, both NCC4 and NCC10 gave the highest level of gene expression of the reporter gene (Table 2). This is different from the cationic lipid DOTAP that is reported to have maximal efficiency of gene delivery at the charge ratio of 3.0. It was observed that both NCC4 and NCC10 gave higher efficiency of gene delivery into spleens when compared to DOTAP at its respective optimal conditions (Table 2).

We have observed that NCC4 and NCC10 can mediate efficient gene delivery *in vitro* and mediate efficient gene delivery followed intra-splenic injection *in vivo*. Moreover, it was demonstrated that both NCC4 and NCC10 could withstand serum inactivation *in vitro* by changing the DNA/lipid charge ratios. We have therefore attempted to achieve efficient gene delivery by systemic injection of NCC4 and NCC10 via the tail vein of mice. At the charge ratio of 0.4, both NCC4 and NCC10 demonstrate lower levels of gene expression following tail vein injection (data not shown). It can therefore be concluded that the conditions employed for intra-splenic

injection could not be directly employed for systemic gene delivery and the conditions established *in vitro* to demonstrate serum sensitivity (Figures 7 and 8) could not be applied directly *in vivo*. In addition, this suggests
5 that the mechanisms of serum inactivation of DNA-liposome complexes *in vitro* may be differ from that *in vivo*.

In vivo safety studies of cationic liposome, NCC4,
administered via intra-venous and intra-peritoneal
10 injections in mice

Introduction

15 Following on from the very promising pre-clinical data obtained for the cationic liposome, NCC4, a study was conducted to evaluate the safety of both intra-venous and intra-peritoneal administration of NCC4 into mice with lipid doses up to ten times the proposed starting dose
20 for future clinical trials in patients.

Materials and Methods

Mice

25 Balb/c mice (4 to 6 weeks old) were cared for and used in accordance with institutional guidelines. Mice were housed for 1 week before treatment. Healthy mice were injected with NCC4 or Ringer's buffer (B. Braun Melsungen
30 AG, Germany) under aseptic conditions.

Preparation of liposome

The liposome mixture was prepared by mixing NCC4 with DOPE (dioleoyl phosphatidylethanolamine) in a molar ratio of 6:4 under a gentle stream of N₂, dried under vacuum overnight and hydrated in sterile HEPES buffer (pH 7.8)¹.
5 The appropriate doses (320, 1600 or 3200 nmoles) of the liposome were prepared under aseptic conditions on the day of administration, by diluting in Ringer's buffer. The liposome mixture was then incubated at room temperature for 15 min before injection.

10

Acute toxicity test

Twenty mice were separated into four groups of five mice each. Each group of mice received either Ringer's
15 Buffer, or NCC4 (at doses 320 nmoles, 1600 nmoles or 3200 nmoles), respectively, in a single intra-venous injection of 0.5 ml. The dose of 320 nmole represents the proposed starting liposome dose for patients in our future phase I clinical trial. The other doses used represent 5- and
20 10-fold higher doses.

The mice were sacrificed (according to institutional guidelines) after 24 hours post-injection and their blood collected for analysis of liver and hematological
25 functions^{2,3} (Parkway Laboratory Services Ltd.). Liver function tests includes total serum protein, alkaline phosphatase, alanine aminotransferase (ALT), and aspartate aminotransferase (AST). Kidney function tests include urea, glucose and creatinine, while hematological
30 function tests include white and red blood cell counts, hemoglobin and platelet counts.

A similar experiment was repeated with twenty mice, except that the administration of the NCC4 liposome was
35 via the intra-peritoneal route.

Results and Discussion

The safety profile of both intra-venous and intra-
5 peritoneal routes of administration of the NCC4 liposome
was examined in separate sets of experiments. Three
groups (of five mice) were given single injections of
NCC4 at a range of doses (diluted in Ringer's buffer):
320 nmol (which represents the proposed starting liposome
10 dose for patients in our future phase I clinical trial),
as well as 5- and 10-fold higher doses, respectively.
Another group of mice was injected with Ringer's buffer
only, as control. The mice were sacrificed and their
blood collected to assay for liver and renal functions.

15 From biochemical assays, hepatic and renal functions were
found to be normal in all groups of mice, whether
injected via intra-venous or intra-peritoneal routes. In
particular, levels of serum protein and enzymes (alkaline
20 phosphatase, alanine aminotransferase and aspartate
aminotransferase) were within the normal value range,
suggesting that all these groups of mice had normal liver
function. Creatinine, urea and glucose levels in these
mice showed normal kidney functions. The various blood
25 counts indicate normal hematological values in these
mice.

These findings indicate that the cationic liposome NCC4
is a safe gene delivery system by both the intra-venous
30 and intra-peritoneal routes of administration even at a
dosage that is as high as 10 times the proposed dose for
future clinical trials in patients.

Design of functional analogues

35 In a further aspect, the present invention provides the

use of the above cationic lipids in methods of designing or screening for further useful cationic lipids.

Accordingly, the present invention provides a method of
5 designing functional analogues of the cationic lipids as provided herein, which are effective in delivering nucleic acid molecules into cells, said method comprising:

- 10 (i) analysing a cationic lipid which is effective in delivering nucleic acid molecules into cells, to determine the groups which are essential and important for the activity, to define a core structure; and,
- (ii) using the core structure to design and/or screen candidate mimetics having the functional
15 activity.

The designing of functional analogues of known pharmaceutically active compounds is a known approach to the development of pharmaceuticals based on a "lead"
20 compound. This might be desirable where the active compound is difficult or expensive to synthesise or where it is unsuitable for a particular method of administration. Compound design, synthesis and testing may be used to avoid randomly screening large numbers of
25 molecules for a target property.

There are several steps commonly taken in the design of a functional analogue of a compound having a given target property. Firstly, the particular parts of the compound
30 that are critical and/or important in determining the target property are determined. These parts or residues constituting the active region of the compound are sometimes known as its "pharmacophore".

35 Once the pharmacophore has been found, its structure is

modelled to according its physical properties, e.g. stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g. spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

10 A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can conveniently be selected so that the compound is easy to synthesise, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the functional activity of the lead compound. The compound or compounds found by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final compounds for further testing or optimisation, e.g. *in vivo* or clinical testing.

Functional analogues of this type, together with their use in nucleic acid delivery, form a further aspect of the invention.

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CLAIMS:

1. A lipid having a steroid hydrophobic domain and a side chain including a heterocyclic ring.
5
2. A lipid according to claim 1 wherein the heterocyclic ring is part of a polycyclic system.
3. A lipid according to claim 1 or 2 wherein the
10 heterocyclic ring includes a ring nitrogen atom.
4. A lipid according to any preceding claim wherein the heterocyclic ring is positively charged or can be protonated to render it positively charged.
15
5. A lipid according to claim 4 which is positively charged owing to protonation.
6. A lipid having a steroid hydrophobic domain linked
20 to a side chain which comprises a primary and/or a secondary aliphatic amine group or a salt in which said amine group is protonated.
7. A lipid according to any preceding claim wherein the
25 side chain includes a carbamoyl linkage.
8. A lipid according to claim 1 having a steroid hydrophobic domain linked via a carbamoyl linkage to a head group which comprises a nitrogen heterocycle which
30 is positively charged or includes at least one basic nitrogen atom such that it can be protonated or a salt in which a said basic nitrogen atom is protonated.

9. A lipid according to any preceding claim wherein the steroid domain is a cholesteryl group or derivative thereof.

5 10. A lipid according to claim 9 wherein the steroid domain is a 3- β -cholesteryl group or derivative thereof.

11. A lipid according to any preceding claim wherein the side chain includes a carbamoyl linkage attached directly
10 to the steroid nucleus and orientated -N-CO-O-[steroid].

12. A lipid according to any preceding claim wherein a spacer arm is interposed between the carbamoyl linkage and the heterocyclic ring or amine group, the spacer arm
15 comprising a chain of 3-6 atoms.

13. A lipid according to any preceding claim wherein the heterocyclic ring comprises a saturated or unsaturated nitrogen heterocycle selected from 5 and 6 membered rings
20 containing 1 or 2 N atoms and optionally one O or S atom.

14. A lipid according to claim 13 wherein the heterocycle is selected from piperazine, imidazole, morpholine and pyridine.
25

15. A lipid selected from NCC1-NCC10 as shown in Figure 2 and salts and derivatives thereof.

16. NCC4 as shown in Figure 2 or a salt and/or a
30 derivative thereof.

17. NCC10 as shown in Figure 2 or a salt and/or a derivative thereof.

18. A liposome comprising one or more of the lipids according to any preceding claim.

5 19. A liposome according to claim 18 which also includes a helper liquid.

20. A complex of a nucleic acid and a lipid or liposome according to any preceding claim.

10

21. A complex according to claim 20 in which the nucleic acid is an expression vector.

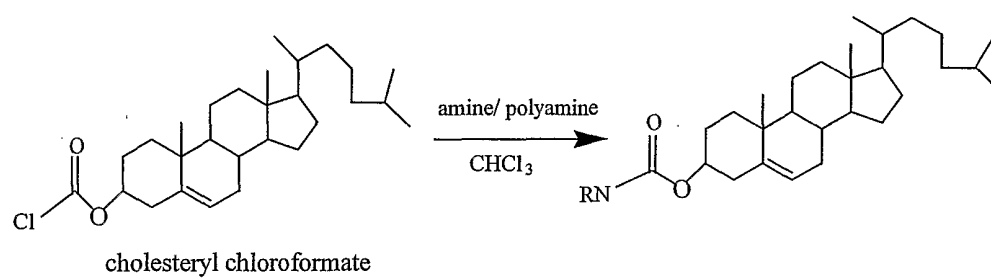
15 22. A pharmaceutical composition containing a complex according to claim 20 or 21.

20 23. Use of a complex according to claim 20 or claim 21 or a lipid or liposome according to any of claims 1 - 19 in manufacturing a composition for use in a method of gene therapy.

24. Use according to claim 23 wherein the therapy is for a cancer or a cardiovascular disease.

25 25. A method of producing a lipid according to any of claims 1 to 17 comprising reacting a sterol chloroformate with a primary or secondary amine $R_1 R_2 NH$ where R_1 comprises said heterocyclic ring or ring system or said side chain amine group and R_2 is H or alkyl (preferably methyl, ethyl or propyl) or R_1 and R_2 are linked to form a
30 heterocyclic ring.

26. A method according to claim 25 wherein the sterol chloroformate is cholesteryl chloroformate.

**Figure 1**

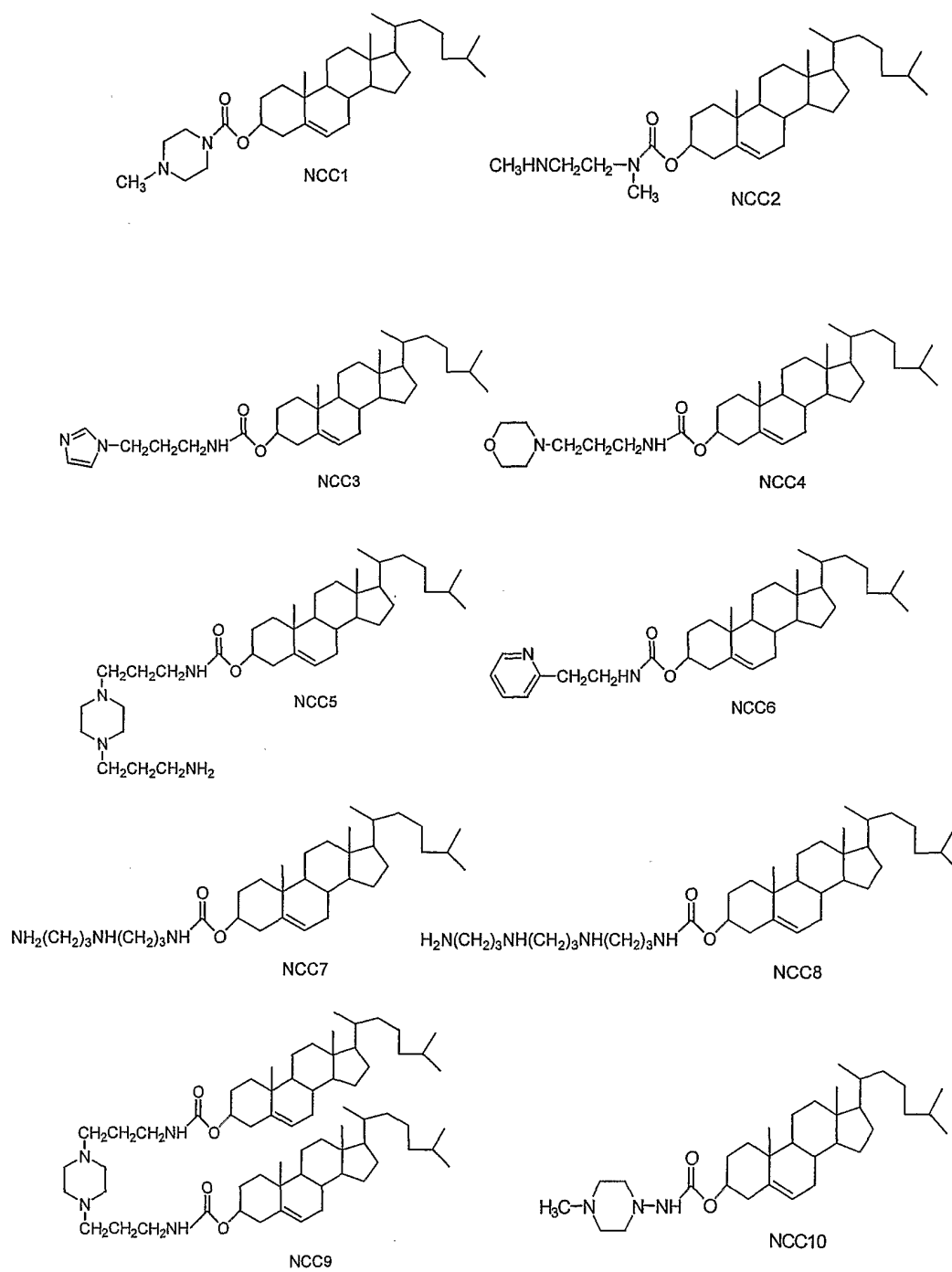
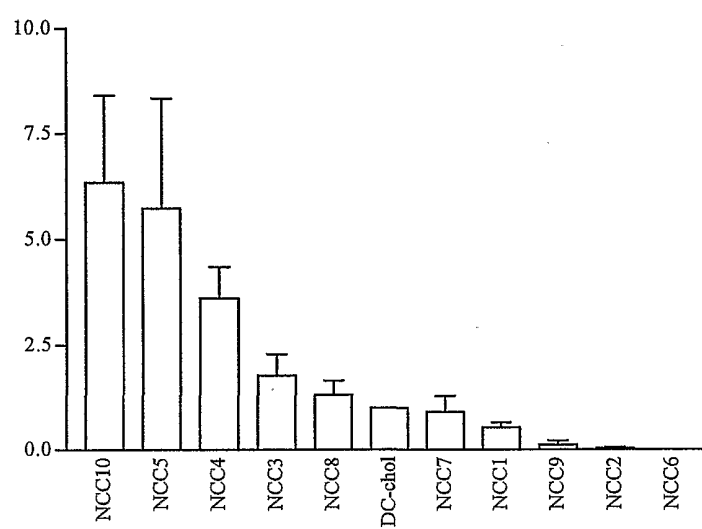
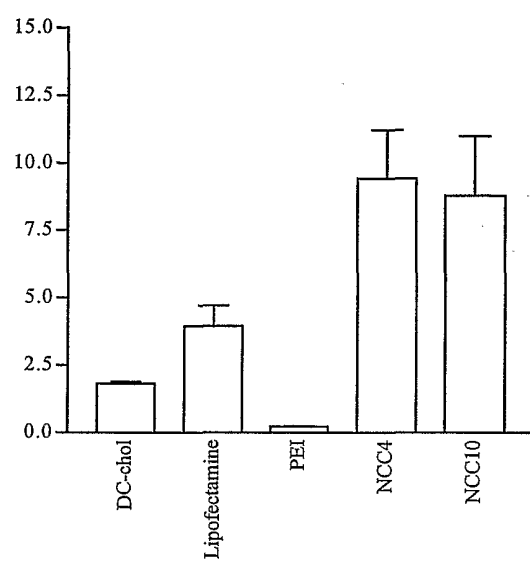
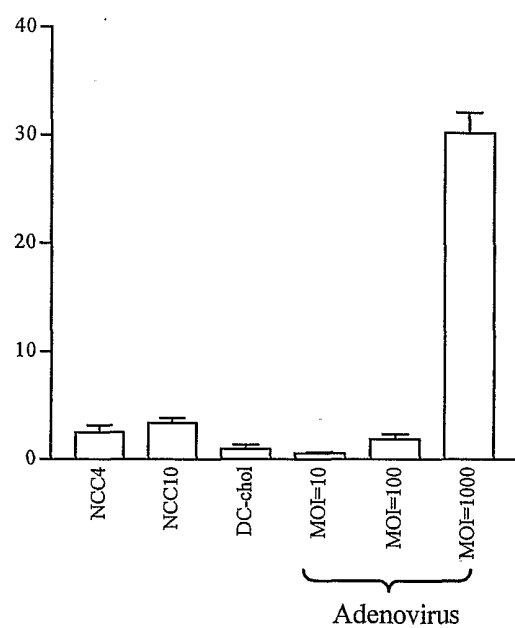


Figure 2

**Figure 3**

**Figure 4**

**Figure 5**

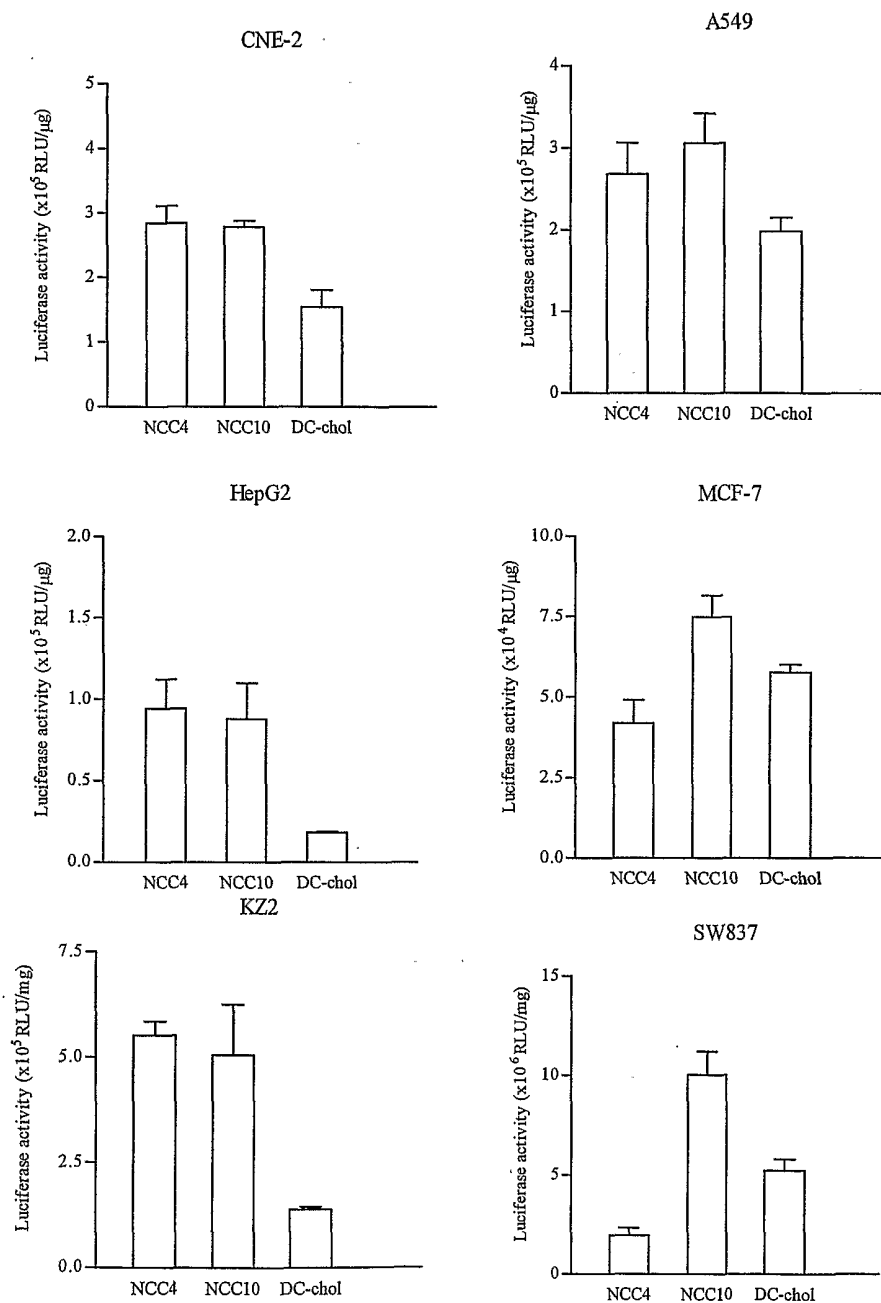


Figure 6

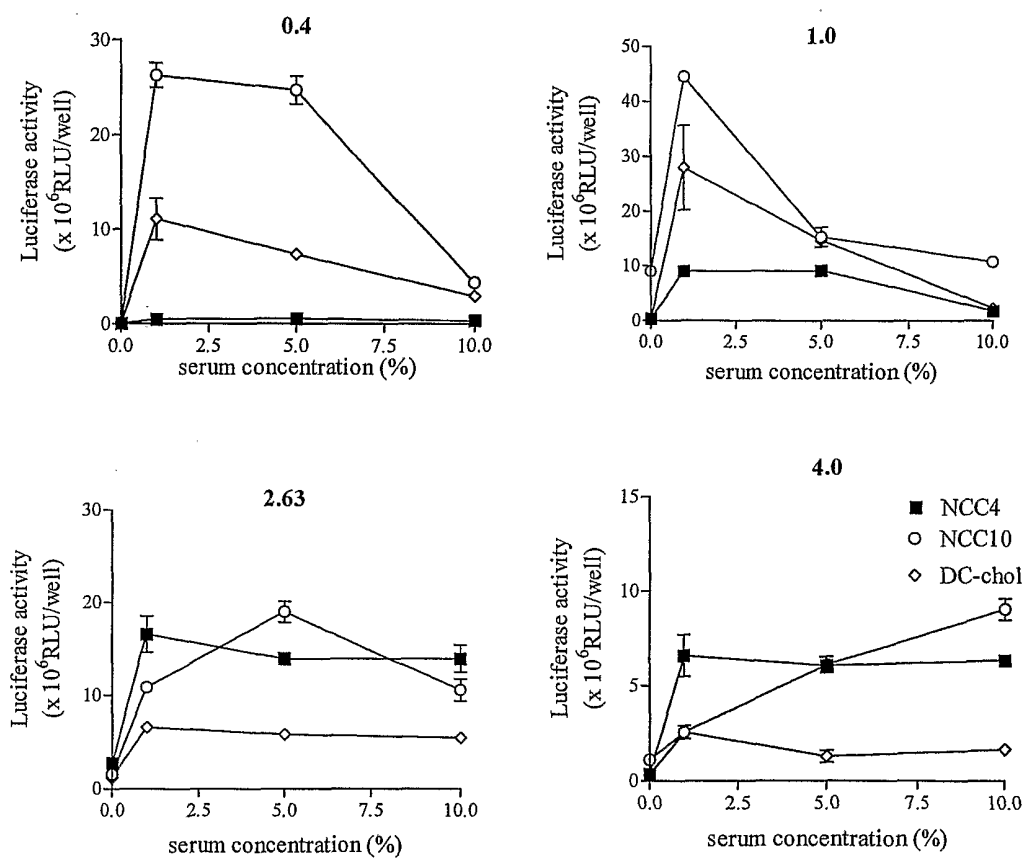


Figure 7

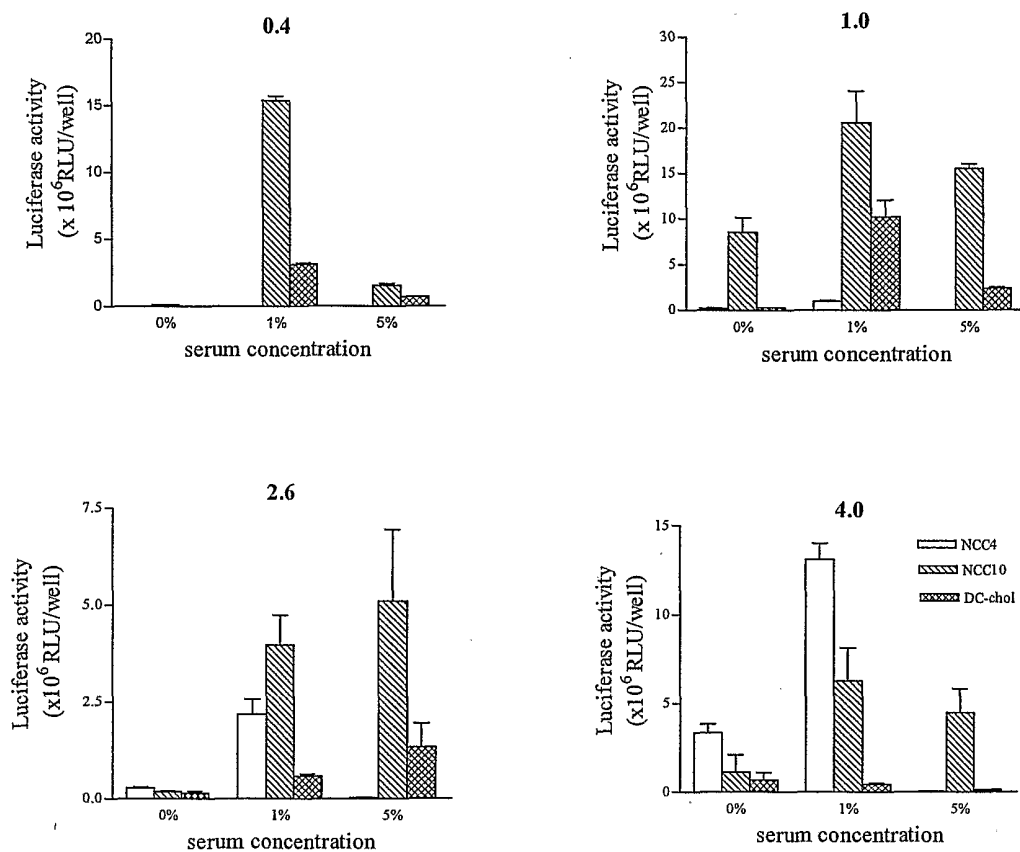
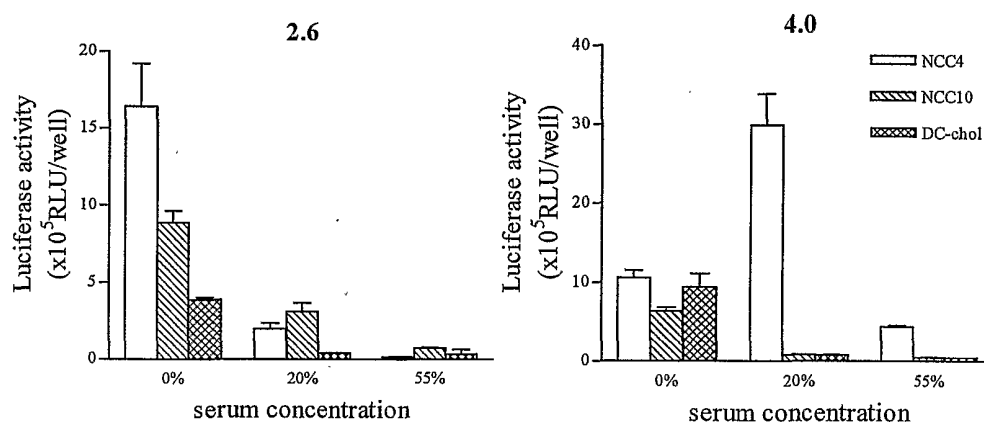


Figure 8

**Figure 9**