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CA 2458626 C 2011/05/03

(11)(21) **2 458 626**

(12) **BREVET CANADIEN
CANADIAN PATENT**

(13) **C**

(86) Date de dépôt PCT/PCT Filing Date: 2002/08/29

(87) Date publication PCT/PCT Publication Date: 2003/03/06

(45) Date de délivrance/Issue Date: 2011/05/03

(85) Entrée phase nationale/National Entry: 2004/02/25

(86) N° demande PCT/PCT Application No.: FR 2002/002959

(87) N° publication PCT/PCT Publication No.: 2003/018603

(30) Priorités/Priorities: 2001/08/29 (FR01/11199);
2001/11/16 (US60/331,452)

(51) Cl.Int./Int.Cl. *C07J 41/00* (2006.01),
A61K 31/575 (2006.01), *A61K 9/127* (2006.01)

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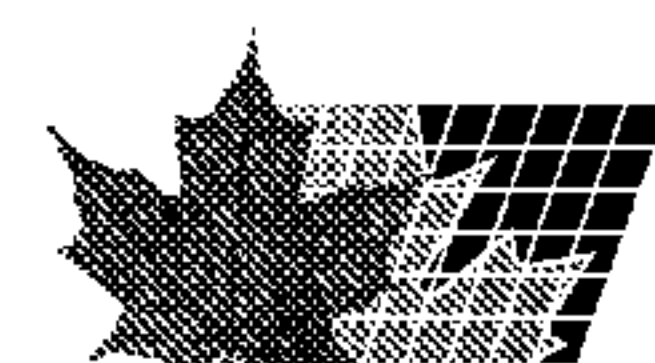
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(54) Titre : DERIVES LIPIDIQUES D'AMINOGLYCOSIDES POUR LA TRANSFECTION

(54) Title: LIPID DERIVATIVES OF AMINOGLYCOSIDES

(57) **Abrégé/Abstract:**

The invention concerns novel transfecting compounds consisting of an aminoglycoside bound to a lipid via a spacer, and their polyguanidyl derivatives. Said compounds are useful for in vitro, ex vivo or in vivo transfection of nucleic acids in different cell types.



(12) DEMANDE INTERNATIONALE PUBLIÉE EN VERTU DU TRAITÉ DE COOPÉRATION
EN MATIÈRE DE BREVETS (PCT)

(19) Organisation Mondiale de la Propriété
Intellectuelle
Bureau international



(43) Date de la publication internationale
6 mars 2003 (06.03.2003)

PCT

(10) Numéro de publication internationale
WO 03/018603 A1

(51) Classification internationale des brevets⁷ : C07J 41/00,
A61K 9/127, 31/575

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(21) Numéro de la demande internationale :
PCT/FR02/02959

(81) États désignés (*national*) : AE, AG, AL, AM, AT, AU, AZ,
BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ,
DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM,
HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK,
LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX,
MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI,
SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN,
YU, ZA, ZM, ZW.

(22) Date de dépôt international : 29 août 2002 (29.08.2002)

(25) Langue de dépôt : français

(26) Langue de publication : français

(30) Données relatives à la priorité :
01/11199 29 août 2001 (29.08.2001) FR
60/331,452 16 novembre 2001 (16.11.2001) US

(84) États désignés (*régional*) : brevet ARIPO (GH, GM, KE,
LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), brevet
eurasien (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), brevet
européen (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,
FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), brevet
OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML,
MR, NE, SN, TD, TG).

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Publiée :

- avec rapport de recherche internationale
- avant l'expiration du délai prévu pour la modification des
revendications, sera republiée si des modifications sont
reçues

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En ce qui concerne les codes à deux lettres et autres abrégia-
tions, se référer aux "Notes explicatives relatives aux codes et
abréviations" figurant au début de chaque numéro ordinaire de
la Gazette du PCT.

(54) Title: AMINOGLYCOSIDE LIPID DERIVATIVES FOR TRANSFECTION

(54) Titre : DERIVES LIPIDIQUES D'AMINOGLYCOSIDES_POUR LA TRANSFECTION

(57) Abstract: The invention concerns novel transfecting compounds consisting of an aminoglycoside bound to a lipid via a spacer, and their polyguanidyl derivatives. Said compounds are useful for *in vitro*, *ex vivo* or *in vivo* transfection of nucleic acids in different cell types.

(57) Abrégé : L'invention concerne des nouveaux composés transfectants qui sont constitués d'un aminoglycoside relié à un lipide par l'intermédiaire d'un espaceur, et leurs dérivés polyguanidylés. Ces composés sont utiles pour la transfection *in vitro*, *ex vivo* ou *in vivo* d'acides nucléiques dans différents types cellulaires.



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LIPID DERIVATIVES OF AMINOGLYCOSIDES

The present invention relates to novel compounds which allow the transfer of nucleic acids
5 into cells. More precisely, these novel compounds are lipid derivatives of aminoglycosides and their polyguanidylated derivatives. They are useful for the *in vitro*, *ex vivo* or *in vivo* transfection of nucleic acids into various cell types.

10 With the development of biotechnology it has become necessary to efficiently transfer nucleic acids into cells. This may involve transferring nucleic acids into cells *in vitro*, for example for producing recombinant proteins, or in the laboratory, for
15 studying the regulation of gene expression, cloning genes or any other manipulation involving DNA. It may also involve transferring nucleic acids into cells *in vivo*, for example for the creation of transgenic animals, the production of vaccines, labeling studies
20 or also therapeutic approaches. It may also involve transferring nucleic acids into cells *ex vivo*, in approaches for bone marrow transplantation or for immunotherapy, or other methods involving the transfer of genes into cells removed from an organism, with a
25 view to subsequently readministering them.

Today, several methods are proposed for the intracellular delivery of exogenous genetic material. One of them, in particular, is based on the use of nonviral vectors, which constitutes a very advantageous
30 alternative compared to viral methods, which are not entirely free of risks. These synthetic vectors have two main functions: to complex and compact the nucleic acid to be transfected, and to promote its passage across the plasma membrane and, optionally, across the
35 nuclear envelope.

Several families of synthetic vectors have thus been developed, such as for example polymers or biochemical vectors (consisting of a cationic protein
5 associated with a cellular receptor), but considerable progress has especially been accomplished with the development of lipofectants, and more particularly cationic lipids. It has thus been demonstrated that cationic lipids, due to their overall positive charge,
10 interfere spontaneously with DNA, which is negative overall, forming nucleolipid complexes capable of both protecting the DNA against nucleases and attaching to cell membranes for intracellular release of the DNA. Thus, most of the nonviral vectors developed to date
15 are based on amine protonation to allow binding to the DNA.

Various types of cationic lipid have been synthesized to date: lipids comprising a quaternary ammonium group (for example DOTMA, DOTAP, DMRIE, DLRIE,
20 etc.), lipopolyamines, such as for example DOGS, DC-chol or the lipopolyamines disclosed in patent application WO 97/18185, lipids which combine both a quaternary ammonium group and a polyamine, such as DOSPA, or lipids comprising various other cationic
25 entities derived from amines, in particular amidinium groups (for example ADPDE, ADODE or the lipids of patent EP 0 888 379 B1 or US 6,143,729, in particular BGTC).

Although the structure/activity relationships
30 of cationic lipids are not yet really understood, it is generally considered that the nature of the cationic component has a major impact on the transfection activity, although it is the nature and the physicochemical properties of the whole molecule (such
35 as the hydrophobicity/hydrophilicity balance, the interactions with colipids, etc.) which determine the

efficiency of a given cationic lipid in fine (see, for example, Felgner et al., *Am. Chem. Soc.*, 1996, pp. 177-190).

For this reason, in the past few years, a
5 great deal of effort has been spent in developing
cationic lipids which have novel cationic entities. The
object of the present invention is precisely to provide
novel transfecting compounds which are original by
virtue of their cationic component consisting of
10 aminoglycosides or of their polyguanidylated
derivatives, and which can be used effectively for the
in vitro, *ex vivo* or *in vivo* transfection of nucleic
acids. These novel compounds are particularly
advantageous because the aminoglycosides can interact
15 with three-dimensional substructures found in a large
variety of RNA molecules (Walter et al., *Curr. Opin.*
Chem. Biol., 1999, 3, pp. 694-704) and they therefore
constitute novel cationic groups which have structural
characteristics particularly suitable for synthesizing
20 a large variety of transfecting compounds.
Specifically, aminoglycosides are multifunctional
compounds containing up to 6 amino groups and several
hydroxyl functions. Selective acylation of one or more
of these groups has therefore made it possible to
25 obtain aminoglycosides which have the lipophilic
properties required for use in transfection. In
addition, aminoglycosides can be easily
polyguanidylated in a single step, which makes it
possible, for example, to synthesize transfecting
30 compounds having guanidinium groups in place of amines,
in their cationic component. Finally, these novel
transfecting compounds are also particularly
advantageous from a pharmaceutical point of view since
the aminoglycosides which constitute their cationic
35 component are natural compounds which are already
widely used in pharmacy as antibiotics, their

antibacterial activity in fact being derived from their interaction with prokaryotic rRNAs (Moazed et al., *Nature*, 1987, 327, pp. 389-394; Puhrohit et al., *Nature*, 1994, 370, pp. 659-662).

5 A first subject of the present invention is thus transfecting compounds, characterized in that they consist of an aminoglycoside linked to a lipid via a spacer, and their polyguanidylated derivatives.

10 In particular, the subject of the present invention is transfecting compounds of the general formula (I):



15 in which:

- A represents an aminoglycoside or its polyguanidylated derivative,
- Y represents a spacer, and
- L represents:

20 · either a radical $-(R_1)R_2$ with R_1 and R_2 which represent, independently of one another, a hydrogen atom or a fatty aliphatic chain or R_1 or R_2 is absent, it being understood that at least one of R_1 and R_2 represents a fatty aliphatic chain,

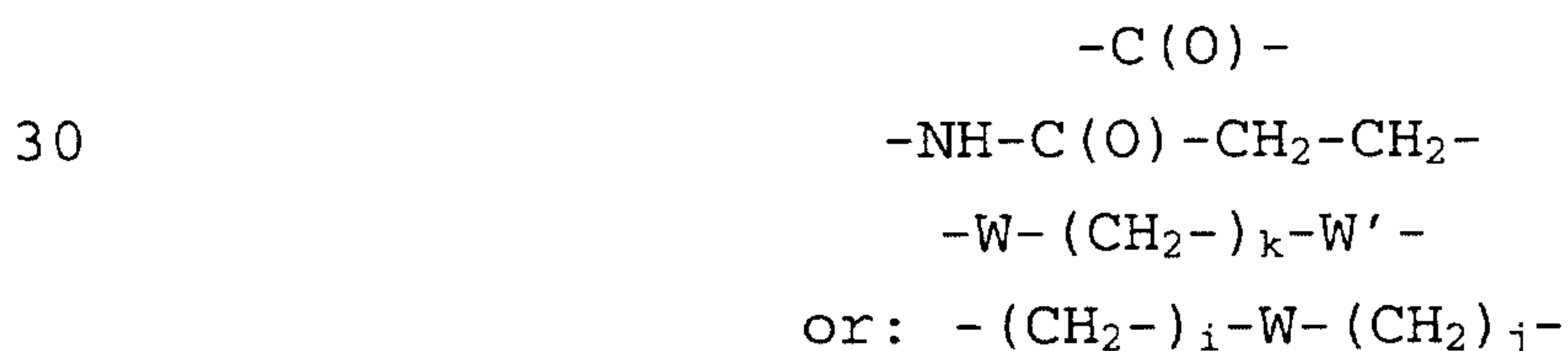
25 · or a radical $-N-R_3$ or $-O-R_3$, with R_3 which represents a steroidal derivative.

30 For the purpose of the present invention, the term "aminoglycoside" (or "aminosides") is intended to mean natural heterosides formed by the combining of a genin of the aminocyclitol group with generally several saccharides, at least one of which is an aminosugar (osamine). They may therefore be considered to be
35 pseudooligosaccharides having antibiotic properties. By way of example of suitable aminoglycosides, mention may

be made of amikacin, arbekacin, deoxyhydrostreptomycin, destomycin A, dibekacin, dihydrostreptomycin, gentamicin, gentamycin, hygromycin, isepamycin, kanamycin, micronomycin, paromomycin, ribostamycin, streptomycin, streptonicozide, neomycin, tobramycin, sisomycin or semi-synthetic aminosides.

The polyguanidylated derivative of an aminoglycoside according to the invention is the aminoglycoside for which the amino functions have been replaced with guanidinium functions. For example, the polyguanidylated derivative of kanamycin, or guanidinokanamycin, has been described by Baker et al. (*J. Org. Chem.*, 2000, 65, pp. 9054-9058).

According to the present invention, the term "spacer" is intended to mean any chemical group which makes it possible both to form the bond between the aminoglycoside or its polyguanidylated derivative and the lipid component of the molecule, and to distance these two components in order to reduce any undesired interaction between them. Consequently, the spacer is a difunctional chemical group which may, for example, consist of one or more chemical functions chosen from alkyls having 1 to 6 carbon atoms, ketone, ester, ether, amino, amide, amidine, carbamate or thiocarbamate functions, glycerol, urea, thiourea or aromatic rings. For example, the spacer may be chosen from the radicals of formula:



in which i, j and k are integers chosen between 1 and 6 inclusive, and W and W' are groups, which may be identical or different, chosen from ketone, ester,

ether, amino, amide, amidine, carbamate or thiocarbamate functions, glycerol, urea, thiourea or aromatic rings.

For the purpose of the present invention, the term "fatty aliphatic chains" is intended to mean saturated or unsaturated alkyl radicals containing 10 to 22 carbon atoms and optionally containing one or more hetero atoms, provided that said fatty aliphatic chains have lipid properties. Preferably, they are linear or branched alkyl radicals containing 10 to 22 carbon atoms and 1, 2 or 3 unsaturations. Preferably, said alkyl radicals comprise 10, 12, 14, 16, 18, 20 or 22 carbon atoms. Mention may be made more particularly of the aliphatic radicals $-(CH_2)_{11}CH_3$, $-(CH_2)_{13}CH_3$, $(CH_2)_{15}CH_3$ and $-(CH_2)_{17}CH_3$.

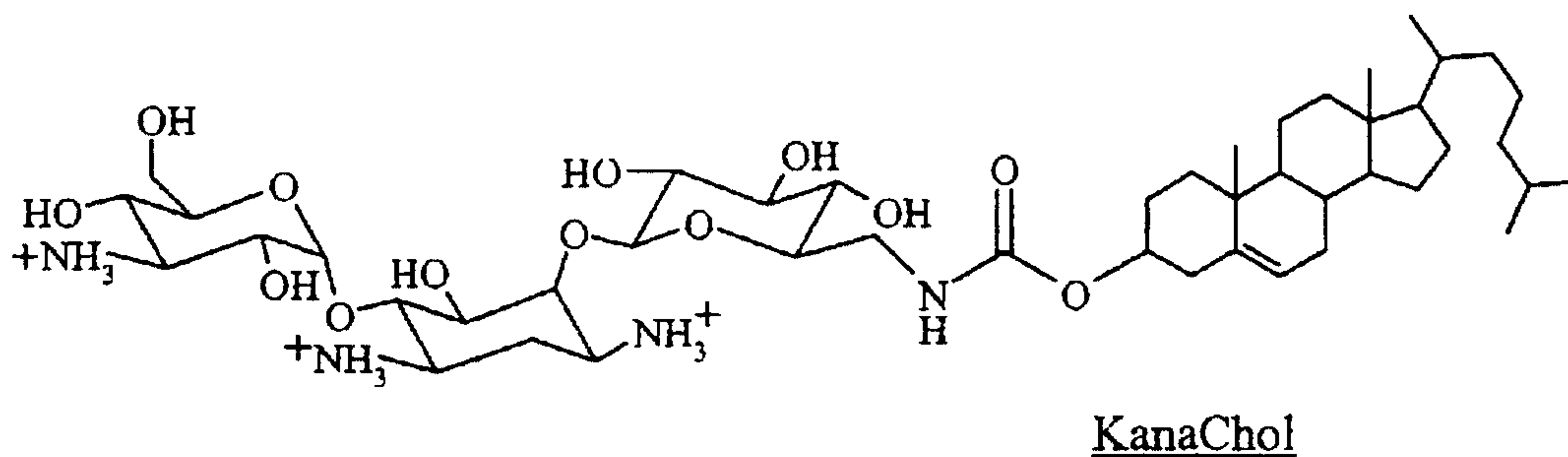
For the purpose of the present invention, the term "steroidal derivatives" is intended to mean polycyclic compounds of the cholestane type. These compounds may or may not be natural and are preferably chosen from cholesterol, cholestanol, 3- α -5-cyclo-5- α -cholestan-6- β -ol, cholic acid, cholesteryl formiate, cholestanyl formiate, 3- α -5-cyclo-5- α -cholestan-6- β -yl formiate, cholesterylamine, 6-(1,5-dimethylhexyl)-3a,5a-dimethylhexadecahydrocyclopenta[a]cyclopropa-[2,3]cyclopenta[1,2-f]naphthalen-10-ylamine or cholestanylamine.

The transfecting compounds, for which A and Y are as defined above in general formula (I) and L is a radical $-OR_3$ with R_3 which represents a steroidal derivative, constitute a subclass of transfecting compounds which are preferred for the purpose of the present invention.

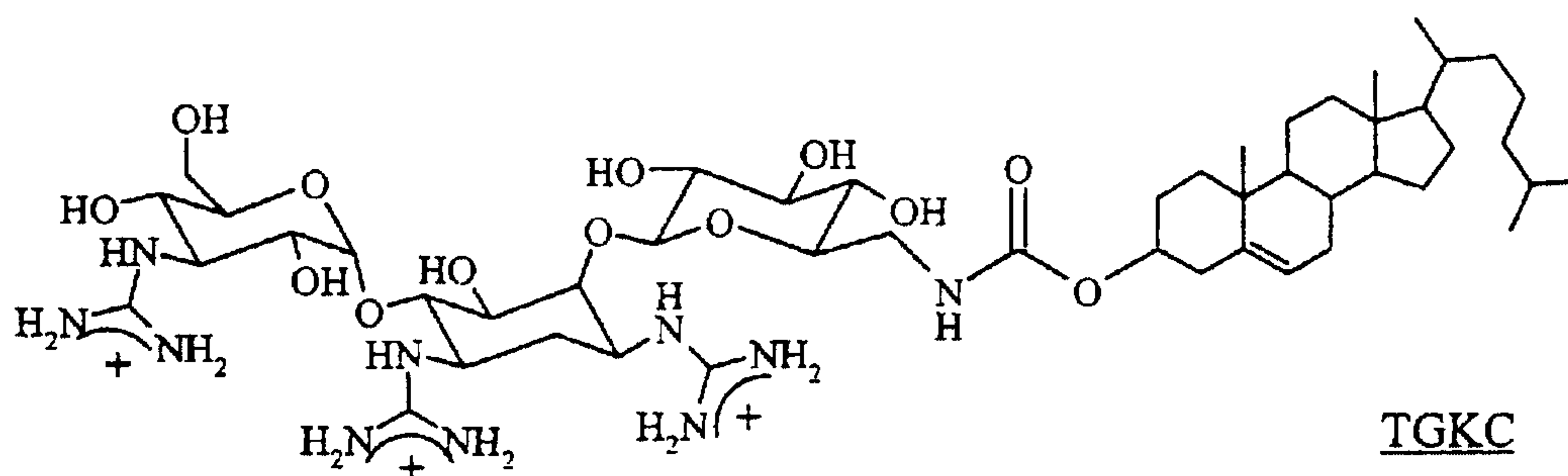
Even more preferably, the transfecting compounds according to the present invention are chosen from the compounds of general formula (I) for which A

and Y are as defined above and L is a radical $-OR_3$, with R_3 which represents a cholesterol radical.

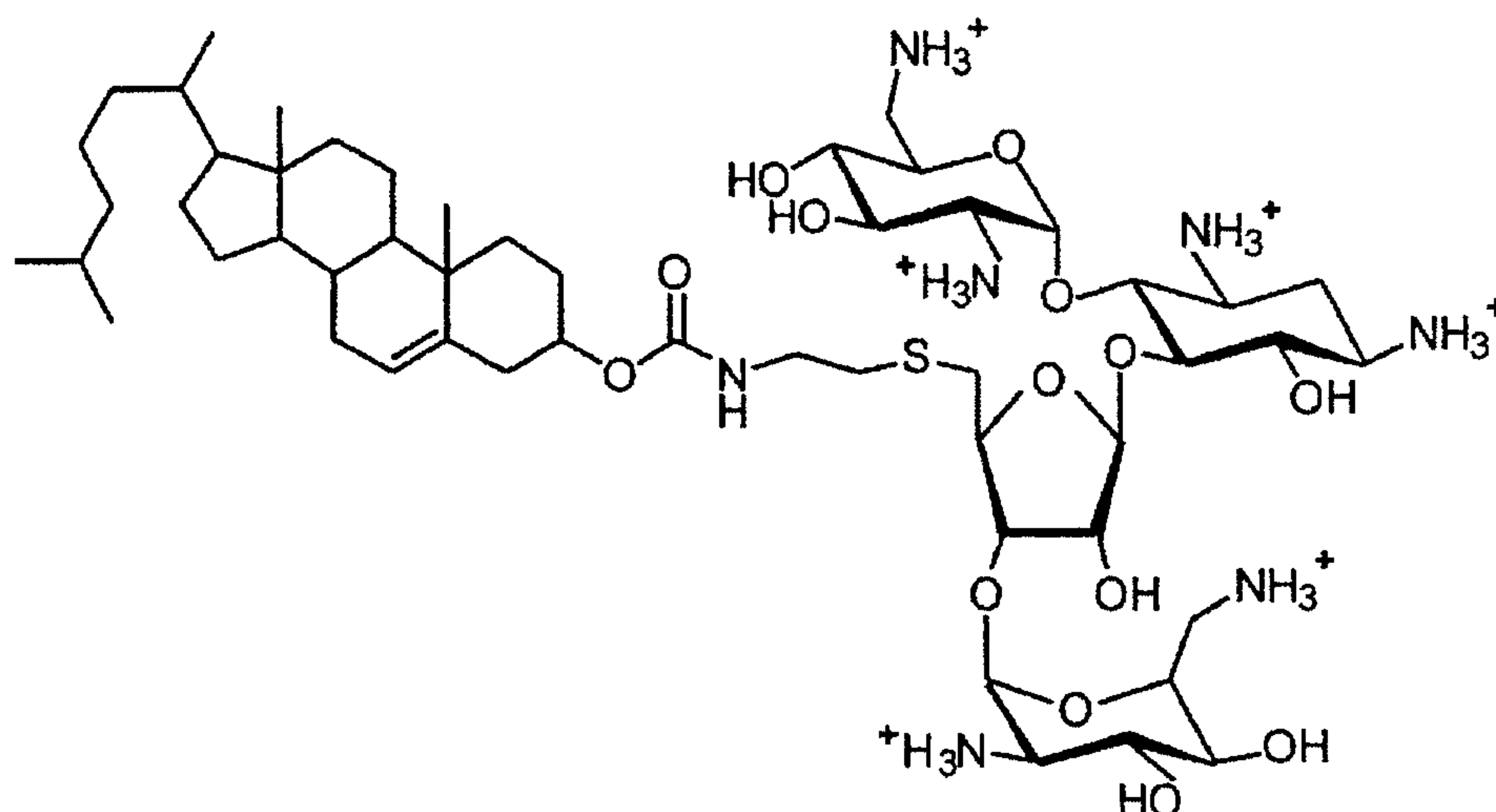
Transfecting compounds which are even more particularly preferred are in particular 3 β [6'-kanamycin-carbamoyl]cholesterol ("KanaChol") of formula:



3 β [6'-(1,3,3"-triguanidino)kanamycin-carbamoyl]-
10 cholesterol ("TGKC") of formula:



and (5"-aminoethylsulfanyl) neomycin
carbamoylcholesterol ("Neochol") of formula:

NeoChol

It is understood that the present invention also relates to the isomers of the products of general formula (I) when they exist, and also the mixtures thereof, or the salts thereof.

In particular, the compounds of the invention may be in the form of nontoxic and pharmaceutically acceptable salts. These nontoxic salts comprise salts with inorganic acids (for example hydrochloric, sulfuric, hydrobromic, phosphoric, nitric acid) or with organic acids (acetic, propionic, succinic, maleic, hydroxymaleic, benzoic, fumaric, methanesulfonic, trifluoroacetic or oxalic acid).

The compounds of general formula (I) according to the present invention are prepared by carrying out the following steps, in the order presented or according to any other known and equally suitable variant, using techniques of conventional organic synthesis, in solution or on solid supports, which are well known to those skilled in the art:

1) Production of the lipid component L

When the lipid component L of the compounds of general formula (I) is represented by a radical - (R₁)R₂ attached, for example, to a nitrogen atom of the spacer, the amine of formula HN(R₁)R₂ is first of all

formed. Said amine may be obtained by condensation of a carboxylic acid and an amine, one containing the substituent R_1 and the other the substituent R_2 , so as to form the corresponding amide, followed by reduction
5 of said amide thus obtained.

The formation of the amide is advantageously carried out by mixing the constituents and melting by heating to a temperature greater than the melting temperature of the substances involved, in general
10 between 20°C and 200°C, and then eliminating the water produced by dehydration of the medium; or more advantageously in the presence of a desiccating agent, such as, for example, phosphorus pentoxide or any other substance which can absorb water. This intermediate
15 amide may also be formed using a variant of this method or any other method of amide formation (such as, for example, coupling of the peptide type) involving carboxylic acids or their derivatives, conditions and reagents which are varied [R.C. Larock, Comprehensive
20 Organic Transformations, VCH editors] and well known to those skilled in the art.

Reduction of the amide obtained above to an amine of formula $\text{HN}(R_1)R_2$ may be carried out, for example, using a reducing agent such as lithium
25 aluminum hydride, or any other hydride or reducing agent which is effective in this case. The procedure is then preferably carried out in a nonprotic solvent (for example tetrahydrofuran or ethers), at a temperature lower than the boiling temperature of the solvent and
30 under a dry and/or inert atmosphere.

According to another variant, the lipid component denoted as $\text{NH}(R_1)R_2$ may be commercially available.

When the lipid component L of the compounds
35 of general formula (I) is represented by a radical $-\text{NR}_3$

or $-OR_3$, it is preferably chosen from commercially available products.

2) Grafting of the spacer Y

The spacer Y is then attached to the lipid
5 component L obtained in the preceding step, according to conventional techniques known to those skilled in the art. According to a preferred variant, an amide bond is formed by N-acylation of the lipid component L in a suitable solvent, such as dichloromethane,
10 chloroform, tetrahydrofuran or any other ether, at a temperature lower than the boiling temperature of the solvent, and under a dry and/or inert atmosphere. This reaction preferably takes place in the presence of an amine base, such as N,N-dimethylaminopyridine, or in
15 the presence of this base mixed with nonnucleophilic amine bases, such as triethylamine or ethyldiisopropylamine. Pyridine may also be used, alone or mixed with another base, diluted with one of the solvents mentioned or used, itself, as a solvent.

20 According to another variant, when the lipid component L represents a radical $-OR_3$, treatment, with phosgene, of the corresponding alcohol R_3OH gives a chloroformate (sometimes commercially available).

3) Grafting of the aminoglycoside

25 The grafting of the aminoglycoside onto the Y-L combination is carried out using the amine and/or hydroxyl functions carried by the aminoglycoside, whether they are present in the natural compound or originate from a modification produced according to the
30 conventional methods known to those skilled in the art.

According to a variant, an amide bond is formed by N-acylation of an amine function of the aminoglycoside with the Y-L combination suitably activated according to the conventional methods used to
35 create a peptide bond.

When the lipid component L of the compounds of general formula (I) is represented by the radical -OR₃, a preferred possibility consists in linking the components A and L via a carbamate bond obtained by
5 treating the aminoglycoside with the chloroformate, sometimes commercially available, of the alcohol R₃OH. This reaction takes place in a suitable solvent, such as dichloromethane, chloroform, tetrahydrofuran, dimethylformamide or any other ether, at a temperature
10 lower than the boiling temperature of the solvent, under a dry and/or inert atmosphere and in the presence of a nonnucleophilic amine base, such as triethylamine, ethyldiisopropylamine or pyridine.

In general, the aminoglycosides
15 preferentially used in the context of the invention are all commercially available.

4) Transformation of the amines into guanidines

The transformation of the amine functions of the aminoglycoside into guanidine functions may be
20 easily carried out according to the techniques known to those skilled in the art, in particular the techniques described, for example, by Baker et al. (*J. Org. Chem.*, 2000, 65, pp. 9054-8), Luedtke et al. (*J. Am. Chem.*, 2000, 122, pp. 12035-12036) or Iwanowicz et al. (*Synth.*
25 *Commun.* 1993, 23, pp. 1443-1445). Advantageously, the technique and the reagents described by Iwanowicz et al. (*Synth. Commun.* 1993, 23, pp. 1443-1445) are used.

Naturally, when the various substituents may interfere with the reaction, it is preferable to
30 protect them beforehand with radicals which are compatible and which can be introduced and eliminated without affecting the rest of the molecule. This is carried out according to the conventional methods known to those skilled in the art, and in particular
35 according to the methods described in T. W. Greene, *Protective Groups in Organic Synthesis*, Wiley-

Interscience, in McOmie, *Protective Groups in Organic Chemistry*, Plenum Press, or in P. J. Kocienski, *Protecting Groups*, Thieme.

Moreover, each step of the preparation
5 process may be followed, where appropriate, by steps for separating and purifying the compound obtained, according to any method known to those skilled in the art.

Another subject of the invention relates to
10 the compositions comprising a transfecting compound according to the invention and a nucleic acid. The respective amounts of each component may be easily adjusted by those skilled in the art depending on the transfecting compound used, on the nucleic acid and on
15 the desired applications (in particular on the type of cell to be transfected).

For the purpose of the invention, the term "nucleic acid" is intended to mean both a deoxyribonucleic acid and a ribonucleic acid. They may
20 be natural or artificial sequences, and in particular genomic DNA (gDNA), complementary DNA (cDNA), messenger RNA (mRNA), transfer RNA (tRNA) or ribosomal RNA (rRNA), hybrid sequences such as DNA/RNA chimeroplasts, or synthetic or semi-synthetic sequences, modified
25 oligonucleotides or unmodified oligonucleotides. These nucleic acids may be of human, animal, plant, bacterial, viral, etc. origin. They may be obtained using any technique known to those skilled in the art, in particular by screening libraries, by chemical
30 synthesis or by mixed methods including the chemical or enzymatic modification of sequences obtained by screening libraries. They may be modified chemically. In general, they contain at least 10, 20, 50 or 100 consecutive nucleotides, and preferably at least 200
35 consecutive nucleotides. Even more preferably, they contain at least 500 consecutive nucleotides.

With regard more particularly to the deoxyribonucleic acids, they may be single-stranded or double-stranded, as well as short oligonucleotides or longer sequences. In particular, the nucleic acids
5 advantageously consist of plasmids, vectors, episomes, expression cassettes, etc. These deoxyribonucleic acids may carry a prokaryotic or eukaryotic origin of replication which may or may not be functional in the target cell, one or more marker genes, sequences which
10 regulate transcription or replication, genes of therapeutic interest, antisense sequences which may or may not be modified, regions for binding to other cellular components, etc.

Preferably, the nucleic acid comprises one or
15 more genes of therapeutic interest under the control of regulatory sequences, for example one or more promoters and a transcriptional terminator which are active in the target cells.

For the purpose of the invention, the
20 expression "gene of therapeutic interest" is intended in particular to mean any gene encoding a protein product which has a therapeutic effect. The protein product thus encoded may in particular be a protein or a peptide. This protein product may be exogenous,
25 homologous or endogenous with respect to the target cell, i.e. a product which is normally expressed in the target cell when the latter does not show any pathological condition. In this case, the expression of a protein makes it possible, for example, to compensate
30 for insufficient expression in the cell or the expression of a protein which is inactive or weakly active due to a modification, or to overexpress the said protein. The gene of therapeutic interest may also encode a mutant of a cellular protein, which has
35 increased stability, modified activity, etc. The protein product may also be heterologous with respect

to the target cell. In this case, an expressed protein may, for example, add to or introduce deficient activity in the cell, making it possible for it to combat a pathological condition, or stimulate an immune
5 response.

Among the therapeutic products for the purpose of the present invention, mention may be made more particularly of enzymes, blood derivatives, hormones, lymphokines and cytokines and inhibitors
10 thereof or antagonists thereof (interleukins, interferons, TNF, interleukin 1 antagonists, soluble receptors for interleukin 1 and TNF α , etc.: FR 92/03120), growth factors, neurotransmitters or their precursors or synthetic enzymes, trophic factors
15 (BDNF, CNTF, NGF, IGF, GMF, aFGF, bFGF, NT3, NT5, HARP/pleitrophin, etc.) apolipoproteins (ApoAI, ApoAIV, ApoE, etc.: FR 93/05125), dystrophin or a minidystrophin (FR 91/11947), the CFTR protein associated with cystic fibrosis, tumor suppressor genes
20 (p53, Rb, Rap1A, DCC, k-rev, etc.: FR 93/04745), genes encoding factors involved in clotting (factors VII, VIII and IX), genes involved in DNA repair, suicide genes (thymidine kinase, cytosine deaminase), genes for hemoglobin or for other protein transporters, metabolic
25 enzymes, catabolic enzymes, etc.

The nucleic acid of therapeutic interest may also be an antisense sequence or gene or a DNA encoding an RNA with a ribozyme function, the expression of which in the target cell makes it possible to control
30 the expression of genes or the transcription of cellular mRNAs. Such sequences may, for example, be transcribed, in the target cell, into RNAs complementary to cellular mRNAs and thus block their translation into protein, according to the technique
35 described in patent EP 140 308. The therapeutic genes also comprise the sequences encoding ribozymes, which

are capable of selectively destroying target RNAs (EP 321 201).

As indicated above, the nucleic acid may also comprise one or more genes encoding an antigenic peptide capable of generating, in humans or animals, an immune response. In this particular embodiment, the invention allows the production of vaccines or the carrying out of immunotherapeutic treatments, applied to humans or to animals, in particular for treating or preventing infections, for example viral or bacterial infections, or cancers. They may in particular be antigenic peptides specific for the Epstein-Barr virus, for the HIV virus, for the hepatitis B virus (EP 185 573), for the pseudorabies virus, for the syncytia forming virus or for other viruses, or antigenic peptides specific for tumors (EP 259 212).

Preferentially, the nucleic acid also comprises sequences which allow the expression of the gene of therapeutic interest and/or of the gene encoding the antigenic peptide, in the desired cell or organ. They may be sequences which are naturally responsible for the expression of the gene in question when these sequences are capable of functioning in the infected cell. They may also be sequences of different origin (responsible for the expression of other proteins or even synthetic). In particular, they may be promoter sequences of eukaryotic or viral genes. For example, they may be promoter sequences derived from the genome of the cell intended to be infected. Similarly, they may be promoter sequences derived from the genome of a virus. In this regard, mention may be made, for example, of the E1A, MLP, CMV, RSV, etc. gene promoters. In addition, these expression sequences may be modified by adding activation sequences, regulatory sequences, etc. It may also be an inducible or repressible promoter.

Moreover, the nucleic acid may also comprise, in particular upstream of the gene of therapeutic interest, a signal sequence which directs the therapeutic product synthesized into the secretory pathways of the target cell. This signal sequence may be the natural signal sequence of the therapeutic product, but it may also be any other functional signal sequence or an artificial signal sequence. The nucleic acid may also comprise a signal sequence which directs the therapeutic product synthesized toward a particular compartment of the cell.

The compositions according to the invention may also comprise one or more adjuvants capable of associating with the transfecting compound/nucleic acid complexes and improving the transfecting power thereof. In another embodiment, the present invention therefore relates to compositions comprising a nucleic acid, a transfecting compound as described above and at least one adjuvant capable of associating with the transfecting compound/nucleic acid complexes and improving the transfecting power thereof. These adjuvants which make it possible to increase the transfecting power of the compounds according to the present invention are advantageously chosen from lipids (for example neutral lipids, in particular phospholipids), peptides (for example histone, nucleolin or protamine derivatives such as those described in WO 96/25508), proteins (for example proteins of the HMG type such as those described in WO 97/12051) and/or polymers (for example polymers which make it possible to turn the transfecting compound/nucleic acid formulations into "stealth" formulations, such as polyethylene glycol (PEG) introduced into the formulation on its own or in a form attached to a lipid in order to colloiddally stabilize the transfecting compound/nucleic acid formulations,

for example PEG-cholesterol). From this point of view, the compositions of the invention may comprise, as an adjuvant, one or more neutral lipids which in particular have the property of forming lipid
5 aggregates. The term "lipid aggregate" is a generic term which includes liposomes of all types (both unilamellar and multilamellar) and also micelles or more amorphous aggregates.

More preferentially, the neutral lipids used
10 in the context of the present invention are lipids containing two fatty chains. Particularly advantageously, use is made of natural or synthetic lipids which are zwitterionic or lacking ionic charge under physiological conditions. They may be chosen more
15 particularly from dioleoylphosphatidylethanolamine (DOPE), oleoylpalmitoylphosphatidylethanolamine (POPE), distearoyl-, dipalmitoyl- and dimirystoylphosphatidylethanolamines and also the derivatives thereof which are N-methylated 1 to 3
20 times, phosphatidylglycerols, diacylglycerols, glycosyldiacylglycerols, cerebroside (such as in particular galactocerebroside), sphingolipids (such as in particular sphingomyelins) or asialogangliosides (such as in particular asialoGM1 and asialoGM2).
25 According to a particularly preferred alternative, the lipid adjuvants used in the context of the present invention are chosen from DOPE, DOPC, cholesterol or the lipid derivatives of nonionic surfactants, such as PEG-cholesterol.

30 These various lipids may be obtained either by synthesis or by extraction from organs (for example the brain) or from eggs, using conventional techniques well known to those skilled in the art. In particular, the extraction of natural lipids may be carried out
35 using organic solvents (see also Lehninger, Biochemistry).

Preferentially, the compositions of the invention comprise from 0.01 to 20 equivalents of adjuvant per equivalent of nucleic acid in mol/mol, and more preferentially from 0.5 to 5 molar equivalents.

5 According to another alternative, the adjuvants cited above make it possible to improve the transfecting power of the compositions according to the present invention; in particular, the peptides, the proteins or certain polymers, such as polyethylene
10 glycol, may be conjugated to the transfecting compounds according to the invention, and not simply mixed. In particular, the PEG may be attached covalently to the lipid derivatives of aminoglycoside according to the present invention either at a remaining amine of the
15 aminoglycoside or at the hydroxyl radicals of the aminoglycoside.

According to a particularly advantageous embodiment, the compositions according to the present invention also comprise a targeting element which makes
20 it possible to direct the transfer of the nucleic acid. This targeting element may be an extracellular targeting element which makes it possible to direct the transfer of the nucleic acid toward certain desired cell types or tissues (tumor cells, hepatic cells,
25 hematopoietic cells, etc.). It may also be an intracellular targeting element which makes it possible to direct the transfer of the nucleic acid toward certain preferred cellular compartments (mitochondria, nucleus, etc.). The targeting element may be mixed with
30 the transfecting compounds according to the invention and with the nucleic acids and, in this case, the targeting element is preferably attached covalently to a fatty aliphatic chain (at least 10 carbon atoms) or to a polyethylene glycol. According to another
35 alternative, the targeting element is covalently attached to the transfecting compound according to the

invention, either on one of the chemical functions which make up the spacer Y, or at the end of the lipid component (for example at the end of R₁ and/or R₂ when they represent fatty aliphatic chains), or directly on
5 the aminoglycoside at one of the remaining amines or at the hydroxyl radicals. Finally, the targeting element may also be attached to the nucleic acid, as specified previously.

Among the targeting elements which can be
10 used in the context of the invention, mention may be made of sugars, peptides, proteins, oligonucleotides, lipids, neuromediators, hormones, vitamins or derivatives thereof. Preferentially, they are sugars, peptides, vitamins or proteins such as, for example,
15 antibodies or antibody fragments, cell receptor ligands or fragments thereof, receptors or receptor fragments. For example, they may be ligands for growth factor receptors, for cytokine receptors, for receptors of the cellular lectin type or for folate receptors, or
20 ligands containing an RGD sequence with an affinity for adhesion protein receptors such as integrins. Mention may also be made of transferrin receptors, HDL receptors and LDL receptors or the folate transporter. The targeting element may also be a sugar which makes
25 it possible to target lectins such as the receptors for asialoglycoproteins or for sialidized species, such as sialyl Lewis X, or an Fab antibody fragment or a single-chain antibody (ScFv).

A subject of the invention is also the use of
30 the transfecting compounds according to the present invention, for transferring nucleic acids into cells *in vitro*, *in vivo* or *ex vivo*. More precisely, a subject of the present invention is the use of the transfecting compounds as defined in the context of the invention,
35 for preparing a medicinal product intended to treat diseases, in particular diseases which result from a

deficiency in a protein or nucleic acid product. The polynucleotide contained in said medicinal product encodes said protein or nucleic acid product, or constitutes said nucleic acid product, capable of
5 correcting said diseases *in vivo* or *ex vivo*.

For uses *in vivo*, for example in therapy or for studying gene regulation or creating animal models of pathological conditions, the compositions according to the invention may be formulated with a view to
10 topical, cutaneous, oral, rectal, vaginal, parenteral, intranasal, intravenous, intramuscular, subcutaneous, intraocular, transdermal, intratracheal, intraperitoneal, etc. administration. Preferably, the compositions of the invention contain a vehicle which
15 is pharmaceutically acceptable for an injectable formulation, in particular for direct injection into the desired organ, or for topical administration (on skin and/or mucous membrane). They may in particular be sterile, isotonic solutions or dry compositions, in
20 particular lyophilized compositions, which, by adding, as appropriate, sterilized water or physiological saline, allow injectable solutes to be made up. The doses of nucleic acids used for the injection and also the number of administrations may be adjusted depending
25 on various parameters, and in particular depending on the method of administration used, on the pathological condition in question, on the gene to be expressed or on the desired duration of the treatment. With regard more particularly to the method of administration, it
30 may be either a direct injection into the tissues, for example into the tumors, or an injection into the circulatory system, or it may involve treating cells in culture followed by their reimplantation *in vivo*, by injection or transplantation. The relevant tissues in
35 the context of the present invention are, for example, muscles, skin, brain, lungs, trachea, liver, spleen,

bone marrow, thymus, heart, lymph, blood, bones, cartilage, pancreas, kidneys, bladder, stomach, intestines, testicles, ovaries, rectum, nervous system, eyes, glands, connective tissues, etc.

5 Another subject of the present invention relates to a method for transferring nucleic acids into cells, comprising the following steps:

- (1) bringing the nucleic acid into contact with a transfecting compound according to the present
10 invention, so as to form a complex, and
- (2) bringing the cells into contact with the complex formed in (1).

The invention also relates to a method for treating the human or animal body, comprising the
15 following steps:

- (1) bringing the nucleic acid into contact with a transfecting compound according to the present invention, so as to form a complex, and
- (2) bringing the cells of the human or animal body into
20 contact with the complex formed in (1).

The cells can be brought into contact with the complex by incubating the cells with said complex (for *in vitro* or *ex vivo* uses) or by injecting the complex into an organism (for *in vivo* uses). In
25 general, the amount of nucleic acid intended to be administered depends on very many factors, such as for example the disease to be treated or prevented, the very nature of the nucleic acid, the strength of the promoter, the biological activity of the product
30 expressed by the nucleic acid, the physical condition of the individual or of the animal (weight, age, etc.), the method of administration and the type of formulation. In general, the incubation is preferably carried out in the presence of, for example, from 0.01
35 to 1000 μg of nucleic acid per 10^6 cells. For administration *in vivo*, nucleic acid doses ranging from

0.01 to 50 mg may, for example, be used. The administration may be carried out as a single dose or a repeated dose with periods in between.

When the compositions of the invention also
5 contain one or more adjuvants as defined above, the adjuvant(s) is (are) mixed beforehand with the transfecting compound according to the invention and/or with the nucleic acid. Alternatively, the adjuvant(s) may be administered prior to administration of the
10 nucleolipid complexes.

According to another advantageous alternative, the tissues may be subjected to chemical or physical treatment intended to improve the transfection. In the case of physical treatment, this
15 may use electrical impulses as in the case of electrotransfer, or mechanical forces as in the case of "sodoporation".

The present invention thus provides a particularly advantageous method for transferring
20 nucleic acids *in vivo*, in particular for the treatment of diseases, comprising the *in vivo* or *in vitro* administration of a nucleic acid encoding a protein, or possibly being transcribed into a nucleic acid, which is capable of correcting said disease, said nucleic
25 acid being associated with a transfecting compound according to the invention under the conditions defined above.

The transfecting compounds of the invention are particularly useful for transferring nucleic acids
30 into primary cells or into established lines. They may be fibroblast cells, muscle cells, nerve cells (neurons, astrocytes, glial cells), hepatic cells, hematopoietic cells (lymphocytes, CD34 cells, dendritic cells, etc.), epithelial cells, etc., in differentiated
35 form or pluripotent form (precursors).

Another subject of the present invention also relates to the transfection kits which comprise one or more transfecting compounds according to the invention and mixtures thereof. Such kits may be in the form of
5 packaging containing compartments which can take various containers, such as for example vials or tubes. Each of these containers comprises the various elements required to carry out the transfection, individually or mixed: for example one or more transfecting compound(s)
10 according to the invention, one or more nucleic acid(s), one or more adjuvant(s), cells, etc.

Besides the arrangements above, the present invention also comprises other characteristics and/or advantages which will emerge from the following
15 examples and figures, which should be considered as illustrating the invention without limiting the scope thereof. In particular, the Applicant provides, with no limitation being implied, an operating protocol and also reaction intermediates which can be used for
20 preparing the transfecting compounds according to the invention. Of course, it is within the scope of those skilled in the art to use this protocol or intermediate products as a basis for developing similar processes with a view to producing these same compounds.

25 **FIGURES**

Figure 1: Reaction scheme for the synthesis of (5"-aminoethylsulfanyl) neomycin carbamoylcholesterol trifluoroacetate (cf. example 3). a) Boc_2O , Et_3N , $\text{DMF}/\text{H}_2\text{O}$, 60°C , 88%. b) 2,4,6-Triisopropylbenzenesulfonyl
30 chloride, pyridine, 50%. c) $\text{HS}-\text{CH}_2-\text{CH}_2-\text{NH}_2$, NaOEt/EtOH , 46%. d) Cholesteryl chloroformate, Et_3N , THF/DMF , 52%. e) Trifluoroacetic acid, $T < 0^\circ\text{C}$, 40 min, 98%.

Figure 2: dose-response curves of the *in vitro* transfection activity of the transfecting compound
35 "KanaChol". The expression of the reporter gene is indicated as a function of the charge ratio of the

lipoplexes formed with the plasmid DNA. The HeLa, HEK293 and COS cells were transfected in accordance with the protocol described in example 4 using lipoplexes prepared by mixing 5 μ g of plasmid DNA
5 expressing luciferase with the required amount of lipid. The data are expressed in relative light units (RLU) per mg of cellular proteins.

Figure 3: dose-response curves of the in vitro transfection activity of the KanaChol/DOPE (molar ratio
10 1:1) liposomes. The expression of the reporter gene is indicated as a function of the charge ratio of the lipoplexes formed with the plasmid DNA. The HeLa, HEK293 and COS cells were transfected in accordance with the protocol described in example 5 using
15 lipoplexes prepared by mixing 5 μ g of plasmid DNA expressing luciferase with the required amount of lipid to form lipoplexes characterized by the charge ratio indicated. The data are expressed in relative light units (RLU) per mg of cellular proteins.

20 **Figure 4:** cytotoxicity of the transfecting compound KanaChol. HeLa cells were transfected in accordance with the protocol described in example 4 using lipoplexes prepared by mixing 5 μ g of plasmid DNA expressing luciferase with the amount of lipid required
25 to form lipoplexes characterized by the charge ratio indicated. At 48 hours post-transfection, the cells were recovered and the toxicity was quantified using the total amount of cellular proteins in the cell lysate as an index of the number of cells, cell death
30 producing a decrease in the extractable proteins. The total cellular proteins in the cell lysate is indicated as a function of the charge ratio of the lipoplexes. The data are expressed as concentration of extractable cellular proteins in the cell lysate (at fixed volume).

35 **Figure 5:** cytotoxicity of the KanaChol/DOPE (molar ratio 1:1) liposomes. HeLa cells were transfected in

accordance with the protocol described in example 5 using lipoplexes prepared by mixing 5 μ g of plasmid DNA expressing luciferase with the amount of lipid required to form lipoplexes characterized by the charge ratio indicated. At 48 hours post-transfection, the cells were recovered and the toxicity was quantified using the total amount of cellular proteins in the cell lysate as an index of the number of cells, cell death producing a decrease in the extractable proteins. The total cellular proteins in the cell lysate is indicated as a function of the charge ratio of the lipoplexes. The data are expressed as concentration of extractable cellular proteins in the cell lysate (at fixed volume).

Figure 6: Expression of luciferase in various cell lines transfected either with the transfecting compound KanaChol or with the KanaChol/DOPE (1:1) liposomes. The various cell lines were transfected in accordance with the protocol described in example 6 using either KanaChol/DNA lipoplexes or KanaChol/DOPE/DNA lipoplexes (with a positive charge ratio of 3-5) prepared by mixing 5 μ g of plasmid DNA expressing luciferase and the required amount of lipid to obtain a positive charge ratio of 3-5. The data are expressed in relative light units (RLU) per mg of cellular proteins.

Figure 7: Expression of luciferase in various cell lines transfected with the TGKC/DOPE (molar ratio 1:1) liposomes. The various cell lines were transfected in accordance with the protocol described in example 7 using TGKC/DOPE/DNA lipoplexes (with a positive charge ratio of 3-5) prepared by mixing 5 μ g of plasmid DNA expressing luciferase and the required amount of lipid to obtain a positive charge ratio of 3-5. The data are expressed in relative light units (RLU) per mg of cellular proteins.

Figure 8: Expression of luciferase in various cell lines (HEK293, NIH 3T3, HeLa, COS, 16HBE) transfected

with the NeoChol transfecting compound. Expression of the reporter gene is indicated at various charge ratios for the lipoplexes formed with the plasmid DNA. The various cell lines were transfected in accordance with the protocol described in example 8 using NeoChol/DNA lipoplexes prepared by mixing 5 µg of plasmid DNA expressing luciferase. The data are expressed in relative light units (RLU) per mg of cellular proteins.

Figure 9: Expression of luciferase in various cell lines (HEK293, NIH 3T3, HeLa, COS, 16HBE) transfected with the NeoChol/DOPE liposomes (molar ratio 1:1). Expression of the reporter gene is indicated at various charge ratios for the lipoplexes formed with the plasmid DNA. The various cell lines were transfected in accordance with the protocol described in example 8 using NeoChol/DOPE/DNA lipoplexes prepared by mixing 5 µg of plasmid DNA expressing luciferase. The data are expressed as relative light units (RLU) per mg of cellular proteins.

Figure 10: Expression of the CAT gene in the airways of mice following instillation of KanaChol/DOPE/DNA lipoplexes stabilized with Chol-PEG (hybrid molecule for which a polyethylene glycol is covalently attached to cholesterol) or NeoChol/DOPE/DNA stabilized with Chol-PEG. These liposomes, characterized by a positive charge ratio of 3.75 for KanaChol/DOPE/DNA lipoplexes stabilized with Chol-PEG or 3.3 for NeoChol/DOPE/DNA lipoplexes stabilized with Chol-PEG and a cholesterol-PEG/DNA weight/weight ratio of 2, were used to deliver a total dose of 100 µg of plasmid DNA expressing the CAT gene. Intranasal instillation was performed, and then the trachea and lungs of the mice treated were removed in order to measure the CAT expression according to the method described in example 9. The data are expressed in ng of CAT protein / 100 mg of total cellular proteins.

Figure 11: Schematic representation of the plasmid pCMV-Luc.

Figure 12: Schematic representation of the plasmid pCIK-CAT.

5 **EXAMPLES**

The reagents and catalysts such as triethylamine, trifluoroacetic acid, p-toluenesulfonic acid, benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), dicyclohexylcarbodiimide
10 (DCC), carbon disulfide, palladium-on-charcoal, tetradecylamine, di-tert-butyl dicarbonate, 4-dimethylaminopyridine, diisopropylethylamine, N-benzyloxycarbonyloxy-5-norbornene-2,3-dicarboximide, 2-(trimethylsilyl)ethyl p-nitrophenyl carbonate and
15 cholesteryl chloroformate are commercially available, as is Kanamycin A. In addition, N,N'-bis-tert-butoxycarbonylthiourea was prepared in accordance with the procedure published in *Synth. Commun.* (1993), 23, pp. 1443-1445 and neomycin amine was prepared according
20 to Tor et al. (*J. Am. Chem. Soc.* 2000, 122, 980-981).

The proton nuclear magnetic resonance (¹H NMR) spectra were recorded on a Bruker 200 MHz spectrometer. The chemical shifts are expressed in ppm (parts per million) and the multiplicities are
25 expressed using the usual abbreviations.

The plasmids used are:

- pCMV-Luc described in the publication by Patel et al. (BBRC, 2001, 6, pp. 536-543), which contains the luc gene encoding
30 luciferase under the control of the CMV immediate early promoter of the cytomegalovirus. This plasmid is represented in figure 11. It was constructed by subcloning the CMV promoter (from the pCDNA3
35 plasmid of Invitrogen) upstream of the luc

gene in the plasmid pGL2-Basic (Promega). It is 6255 bp in size.

- pCIK-CAT (for the transfections in vivo) obtained from D. Giel (Oxford, UK), which is described by Pitard et al. (*J. Gene Med.* 2001, 3, 478-487). It was constructed by subcloning the E. coli CAT gene in the plasmid pCI (Promega). This plasmid is represented in figure 12. It is 4697 bp in size.

Example 1: Synthesis of 3 β [6'-kanamycin-carbamoyl]cholesterol ("KanaChol")

a) Synthesis of [(1,3,3"-triamino)-6'-N-(benzyloxycarbonyl)]kanamycin A

- 15 N-Benzyloxycarbonyloxy-5-norbornene-2,3-dicarboximide (5.18 g; 16.5 mmol) and triethylamine (1.67 g; 16.5 mmol) are added to a solution of kanamycin A in the form of free base (8 g; 16.5 mmol) in a mixture of dimethyl sulfoxide (800 ml) and water
20 (80 ml). After stirring overnight at room temperature, a considerable precipitate has formed and analysis of the reaction mixture by TLC (thin-layer chromatography) reveals the presence of unreacted products. Water (640 ml) is then added again until the mixture becomes
25 homogeneous, followed by N-benzyloxycarbonyloxy-5-norbornene-2,3-dicarboximide (2.6 g; 8.3 mmol). After 4 days of stirring, the solvents are evaporated off under vacuum and the residue is chromatographed on a silica column with an eluent ranging from a
30 dichloromethane/methanol/ammonia (5/4/1) mixture to a methanol/ammonia (9/1) mixture. By evaporating off the solvents from the fractions concerned, the expected compound is recovered (7.15 g; 70%).

b) Synthesis of [(1,3,3"-triazatrimethylsilylethoxycarbonyl)]-[6'-N-(benzyloxycarbonyl)]kanamycin A ("Kana-tri[Teoc]-mono[Cbz]")

5 Triethylamine (0.25 g; 3 equivalents;
2.42 mmol) and mixed 2-(trimethylsilyl)ethyl p-
nitrophenyl carbonate (0.92 g; 4 equivalents;
3.23 mmol) were added to a solution of Kana-mono[Cbz]
10 (0.5 g; 0.81 mmol) in a water (7 ml)/dioxane (16 ml)
mixture. The mixture is stirred at 55°C for 48 hours
and, after having been concentrated, the suspension
obtained is poured into butanol presaturated with water
(60 ml). After several washes with a 1N sodium
15 hydroxide solution (800 ml) and with a saturated sodium
chloride solution, the butanolic phase is evaporated to
dryness. The desired product is obtained in the form of
a yellow powder (0.73 g; 86%) by adding a mixture of
methanol and acetone to the residue and concentrating
the solution thus obtained.

20 c) Synthesis of [(1,3,3"-triazatrimethylsilylethoxycarbonyl)]kanamycin A ("Kana-tri[Teoc]-NH₂")

 Palladium-on-charcoal (Pd/C at 10%; 0.3 g) is
added to a degassed solution of Kana-tri[Teoc]-
25 mono[Cbz] (0.3 g; 0.29 mmol) in methanol (25 ml) and a
stream of hydrogen is bubbled into the mixture for
1 hour. The latter is again stirred overnight under a
hydrogen atmosphere, and the suspension is then
filtered over celite and evaporated to dryness. The
30 desired product in the form of a white solid (0.23 g;
90%) is thus obtained.

d) Synthesis of [(1,3,3"-triazatrimethylsilylethoxycarbonyl)]-[6'-N-(3-oxocarbonylcholesteryl)]kanamycin A ("Kana-tri[Teoc]-Chol")

Triethylamine (1.5 equivalents; 0.017 g; 0.16 mmol) and cholesteryl chloroformate (1.5 equivalents; 0.074 g; 0.16 mmol) are added to a solution of Kana-tri[Teoc]-NH₂ (0.1 g; 0.11 mmol) in a mixture of tetrahydrofuran (5 ml) and dimethylformamide (2 ml). The mixture is stirred at room temperature for 48 hours before being concentrated under reduced pressure until only the dimethylformamide remains. Adding water to this solution causes a white precipitate to form, which is filtered (0.12 g; 83%).

e) Synthesis of 1,3,3"-triamino-6'-N-(3-oxocarbonylcholesteryl)kanamycin A trifluoroacetate ("Kanachol")

The Kana-tri[Teoc]-Chol obtained in the previous step (0.070 g; 0.053 mmol) is suspended for 45 minutes in trifluoroacetic acid maintained at 0°C in an ice bath. After having been returned to room temperature, the mixture is concentrated to dryness. The residue is taken up in methanol and again concentrated to dryness. By repeating this procedure several times, the Kanachol is obtained in the form of a white solid (0.063 g; 97%).

¹H NMR (CD₃OD): δ (ppm) 5.32 (m, 1H, anomeric H); 5.09 (m, 1H, H of cholesterol); 4.97 (m, 1H, anomeric H); 4.30 (m, 1H, H of cholesterol); 3.85-2.98 (m, 16H, diverse protons of the glycosidic rings); 2.30-0.81 (m, 44H, diverse protons of the glycosidic rings and of the cholesterol nucleus); 0.63 (s, 3H, CH₃ of cholesterol).

Example 2: Synthesis of 3β [6'-(1,3,3"-triguanidino)kanamycin-carbamoyl]cholesterol ("TGKC")

a) Synthesis of [1,3,3"-triazaz(N,N'-bis-tert-butoxycarbonyldiaminomethylene)-6'-N-(3-oxocarbonylcholesteryl)kanamycin A ("Kana-tri[GuanBoc]-Chol")

Triethylamine (30 equivalents; 0.36 g; 3.63 mmol), N,N'-bis-tert-butoxycarbonylthiourea (3.5

equivalents; 0.117 g; 0.42 mmol) and mercuric chloride (3.5 equivalents; 0.115 g; 0.42 mmol) are added successively to a solution of Kanachol, obtained according to the protocol of the example above (0.15 g; 5 0.12 mmol), in dimethylformamide (10 ml). The mixture is stirred at room temperature and under an inert atmosphere for 48 hours. The solution is then poured into a mixture of water and ethyl acetate and the aqueous phase is extracted 4 times with ethyl acetate. 10 After washing with a saturated sodium chloride solution and drying over sodium sulfate, the pooled organic fractions are concentrated to dryness. The desired product is obtained in the form of a white powder (0.1 g; 51%).

15 b) Synthesis of the triflate salt of [1,3,3"-
triazaz(N,N'-bisdiaminomethylene)-[6'-N-(3-
oxocarbonylcholesteryl)kanamycin A ("Kana-tri[Guan]-
Chol" or "KTGC")

The Kana-tri[GuanBoc]-Chol obtained in the 20 previous step (0.060 g; 0.037 mmol) is suspended in trifluoroacetic acid maintained in an ice bath. After 2.5 hours at 0°C and then a return to room temperature, the mixture is concentrated to dryness. The red-brown residue is taken up in methanol and then again 25 concentrated to dryness. This operation is repeated 3 times. Pentane is then added and the mixture is subjected to sonication so as to obtain a fine suspension, which is then centrifuged. 0.030 g of pure KTGC is thus recovered (yield: 60%).

30 ¹H NMR (D₂O): δ (ppm) 5.48 (m, 1H, anomeric H); 5.05 (m, 1H); 3.97 (m, 1H, H of cholesterol); 3.70-2.60 (m, diverse protons of the glycosidic rings); 2.18-0.80 (m, diverse protons of the glycosidic rings and of the cholesterol nucleus).

35 Example 3: Synthesis of (5"-aminoethylsulfanyl)
neomycin carbamoylcholesterol ("NeoChol")

The amino glycoside neomycin is grafted onto the Y-L assembly using an amine function originating from a modification carried out according to Tor et al. (J. Am. Chem. Soc. 2000, 122, 980-981). The reaction scheme for the synthesis of NeoChol is given in figure 1.

a) Synthesis of N-hexa-tert-butyloxycarbonyl NeoChol

10

Triethylamine (14 ml; 0.10 mmol; 1.2 equivalents) and cholesteryl chloroformate (43.1 mg; 0.10 mmol; 1.2 equivalents) are added to a solution of neomycin amine (102 mg, 0.08 mmol, 1 equivalent), prepared according to Tor et al. (J. Am. Chem. Soc. 2000, 122, 980-981), in a mixture of tetrahydrofuran (6 ml) and dimethylformamide (2 ml). The reaction mixture gradually becomes cloudy and is stirred overnight. The volatile solvents are then evaporated off under vacuum and the residue is chromatographed on a silica column with a 90/5/5 dichloromethane/methanol/ethyl acetate mixture as eluent. By evaporating the solvents from the fractions concerned, the expected product is recovered in the form of a white solid.

25

Rf = 0.45 (dichloromethane/methanol 9/1; thin layer chromatography)

¹H NMR(200 MHz, CDCl₃): δ (ppm) 6.15 (broad s); 5.90 (m, 1 H); 5.35 (m, 2H); 5.36 (m, 1H, Chol); 5.25-4.86 (m, 5H); 4.60-3.20 (m, 15H); 3.10 (m, 1H); 2.86 (m, 2H); 2.69 (m, 2H); 2.50-1.72 (m, 10H); 1.72-0.84 (m, 87H); 0.71 (s, 3H, CH₃ Chol).

35 **Mass** (MALDI-TOF): [MNa⁺] 1708.98.

B) Synthesis of (5"-aminoethylsulfanyl) neomycin carbamoylcholesterol trifluoroacetate

The N-hexa-tert-butyloxycarbonyl NeoChol obtained in the previous step (60 mg; 0.035 mmol; 1 equivalent) is suspended in trifluoroacetic acid (2 ml) placed in an ice bath. After 50 minutes at 0°C, the mixture is concentrated to dryness. The residue is taken up in methanol and is then again concentrated to dryness. This operation is repeated three times and the solids residue is then dissolved in water and lyophilized; NeoChol is thus recovered in the form of a cottony white powder (59.6 mg, 95.2%).

¹H NMR (200 MHz, [D4]methanol): δ 6.05 (broad s, 1H); 5.46-5.31 (m, 3H); 4.43-4.29 (m, 4H); 4.21-3.82 (m, 5H); 3.67-3.11 (m, 13H); 2.89-2.60 (m, 3H); 2.58-2.25 (m, 3H); 2.20-1.73 (m, 7H); 1.72-0.84 (m, 33H); 0.71 (s, 3H, CH₃ Chol).

20

Mass (MALDI-TOF): [MH⁺] 1086.83; [MNa⁺] 1108.80.

Example 4: Dose-response curves of the *in vitro* transfection of the transfecting compound KanaChol in aqueous medium

25

The aim of this example is to illustrate the ability of the transfecting compounds according to the invention to transfect cells *in vitro*.

This study was carried out for lipoplexes comprising various amounts of KanaChol: 6.6, 13.2, 33, 66 or 132 nmol of KanaChol per 5 µg of DNA. In order to determine the theoretical mean charge ratio, it was considered that 1 µg of DNA represents 3 nmol of negative charges and that 3 amine groups of kanamycin are protonated at neutral pH during the formation of the lipoplexes and the transfection. In fact,

35

electrophoretic gel shift studies for the kanamycin complexed with nucleic acids suggest that the mean positive charge of the KanaChol is rather 2-3.

Preparation of the KanaChol/DNA lipoplexes:

5 the KanaChol obtained according to example 1 is dissolved in a 20 mM Hepes buffer solution (pH 7.4) at a positive charge concentration of 20 mM (considering that 3 amine functions are protonated at neutral pH). The lipoplexes are then prepared in accordance with the
10 protocol described by Vigneron et al. (*PNAS*, 93, pp. 9682-9686, Sept. 1996) or by Patel et al. (*Biochem. and Biophys. Res. Comm.*, 281, pp. 536-543, 2001).

Cell culture: HeLa cells (American type Culture Collection (ATCC) Rockville, MD, USA) derived
15 from a human cervical epithelium carcinoma are cultured in the presence of a medium of the MEM (minimum essential medium) type supplemented with 10% of fetal calf serum and antibiotics. The medium and the additives come from Gibco/BRL Life Technologies
20 (Gaithersburg, MD, USA). The cells are cultured in flasks at 37°C and at 5% of carbon dioxide in an incubator. The same procedure is used for the HEK293 and COS cells.

Transfection: one day before transfection,
25 the HeLa cells are transferred into 6-well plates with approximately 250,000 cells per well. These dilutions represent approximately 80% confluencing after 24 hours. For the transfection, the cells are washed twice and incubated at 37°C with 500 μ l of medium
30 without serum. 500 μ l of complexes containing 5 μ g of plasmid DNA are added to each well (the complexes are prepared at least 30 minutes before being added to the wells).

After six hours at 37°C, the plates without
35 serum are supplemented with 10% (v/v) of FCS ("Fetal Calf Serum").

All of the plates are placed at 37°C and at 5% of carbon dioxide for 24 hours.

This protocol is similar to those described by Vigneron et al. (*PNAS*, 93, pp. 9682-9686, Sept. 1996) or by Patel et al. (*Biochem. and Biophys. Res. Comm.*, 281, pp. 536-543, 2001).

Determination of luciferase activity:

Briefly, the transfected cells are washed twice with 500 μ l of PBS (phosphate buffer) and then lysed with 250 μ l of reagent (25 mM triphosphate, pH 7.8 / 8 mM MgCl₂ / 1 mM dithiothreitol / 15% glycerol / 1% triton X-100). The lysate is then clarified by centrifugation (15 minutes at 15°C) in a microcentrifuge.

A 20 μ l aliquot of supernatant of the centrifuged lysate is diluted in 100 μ l of lysis buffer, to which 4 μ l of 25 mM ATP (Sigma) and 20 μ l of 25 mM luciferin (Sigma) are added. The samples are then placed in a Lumet LB9501 luminometer (Berthold, Nashua, NH) and the light emission integration value is measured for 10 seconds.

The luciferase activity is thus expressed in Relative Light Units ("RLU") and standardized with the sample protein concentration obtained using a Biorad kit.

This protocol is similar to that described by Vigneron et al. (*PNAS*, 93, pp. 9682-9686, Sept. 1996) or by Patel et al. (*Biochem. and Biophys. Res. Comm.*, 281, pp. 536-543, 2001).

The results for the 3 cell lines summarized in figure 2 show high expression levels with optimum transfection efficiency for the lipoplexes which have a theoretical mean charge ratio of approximately 5. The transfection is highest in the COS cell line.

A control was also carried out with complexes formed between kanamycin (not attached to a cholesterol derivative) and the DNA: no transfection was observed.

These data therefore demonstrate that the KanaChol makes it possible to carry out efficient transfection of DNA into cells *in vitro*. These results are also coherent with the hypothesis that the
5 transfection is related to the electrostatic interaction between the positive lipoplexes and the cell surface, which is negative overall.

Figure 2 also shows that the transfection efficiency decreases for very high charge ratios
10 (greater than approximately 7). This decrease may reflect a certain toxic effect of cationic lipids in general, and therefore of the KanaChol in particular, when they are used in large amounts.

Thus, the cytotoxicity of the KanaChol was
15 also measured at various charge ratios with the DNA. The toxicity was quantified using the total amount of cellular proteins in the cell lysate per well as an index of the number of cells. A decrease in the total amount of cellular proteins in the cell lysate per well
20 corresponds to a decrease in the number of cells and, consequently, reflects the existence of a certain toxicity of the KanaChol/DNA lipoplexes with respect to the cells.

Determination of the protein level: the
25 protein level was determined using the Biorad kit according to a protocol identical to that described by Oudrhiri et al. (*PNAS*, 94, pp. 1651-1656, 1997), and in accordance with the manufacturer's instructions.

The results summarized in figure 4 show a
30 decrease in the total number of extractable cellular proteins at high charge ratio, i.e. an increase in the cytotoxicity in parallel with the increase in the KanaChol concentrations. Thus, the decrease in the transfection activity at high charge ratios in the 3
35 cell lines tested may be related to the existence of a

certain toxicity of the KanaChol used in very large amounts.

Example 5: Dose-response curves of the in vitro transfection of the KanaChol/DOPE liposomes

5 The aim of this example is to illustrate the ability of the transfecting compounds according to the invention to transfect cells *in vitro*, when they are formulated in the form of liposomes with neutral lipids.

10 Cationic lipids are often formulated in the form of liposomes with neutral lipid DOPE. It is in fact thought that the use of DOPE, due to its fusogenic properties, would aid transfection by facilitating the exit of the lipoplexes from the endocytic vesicles in
15 which they are internalized. It has thus been shown by Felgner et al. (*J. Biol. Chem.*, 1994, 269, pp. 2550-2561), for a series of cationic lipids, that formulating them with 50% of DOPE makes it possible to increase the transfection efficiency 2- to 5-fold
20 compared to formulations without zwitterionic lipid.

 For this reason, KanaChol/DOPE (1:1 molar) liposomes were prepared in the following way: a mixture of KanaChol and DOPE (molar ratio 1:1) in chloroform is evaporated under vacuum and resuspended in a 20 mM
25 Hepes buffer solution (pH 7.4). The final concentration of lipids is 5 mg/ml. The mixture is then sonicated for 10 minutes using a Branson Sonifier 450 ultrasound machine equipped with a Sonifier Cell Disruptor B-30 terminal, so as to obtain liposomes. The resulting
30 solution is cooled to room temperature before filtration through a 0.22 μ m filter (Millex GS, Millipore).

 In the same way as the example above, this study was carried out for liposomes comprising various
35 amounts of KanaChol, so as to measure the transfection

efficiency at variable theoretical mean charge ratios, in the 3 cell lines HeLa, HEK293 and COS.

The results summarized in figure 3 show high levels of luciferase expression for the 3 cell lines. These transfection levels are entirely comparable with those obtained with the formulations of KanaChol alone (without DOPE), which may be explained by the fact that the 3 cell lines used are very easily transfectable. On the other hand, it is observed that there is no clear decrease in the transfection activity at high charge ratios when KanaChol/DOPE/DNA liposomes are used.

Thus, the cytotoxicity of the KanaChol/DOPE liposomes was also measured at various charge ratios with the DNA, according to a protocol similar to that described in the example above. The data summarized in figure 5 indicate that these liposomal formulations show no apparent toxicity at high charge ratios. It therefore appears that the KanaChol, used in liposomal formulation, is a transfection vector which is both efficient and nontoxic.

Example 6: Spectra for the *in vitro* transfection activity of the transfecting compound KanaChol and of the KanaChol/DOPE liposomes

The aim of this example is to illustrate the ability of the transfecting compounds according to the invention to transfect other cell types *in vitro*, whether they are used alone or in the form of a liposome with DOPE.

The *in vitro* transfection experiments carried out in examples 4 and 5 were again carried out in a similar way, but with other less conventional cell lines (species and specificity of each cell line are indicated in figure 6). The KanaChol/DNA and KanaChol/DOPE/DNA formulations are used at charge ratios of between 3 and 5 (optimum values as defined in

examples 4 and 5). The results obtained are summarized in the table of figure 6.

It appears that the KanaChol/DOPE (molar ratio 1:1) formulation induces high transfection efficiency for all the cell lines, the KanaChol used alone (without DOPE) being less efficient overall, in particular in the cell lines 16HBE and HepG2. It therefore appears that the use of DOPE would have a beneficial effect on transfection efficiency.

10 **Example 7: In vitro transfection activity of the TGKC/DOPE liposomes**

The aim of this example is to illustrate the ability of the polyguanidylated transfecting compounds according to the invention (formulated in the form of liposomes) to transfect cells *in vitro*.

TGKC/DOPE (molar ratio 1:1) formulations obtained in a similar way to the protocol described in example 5 are thus used to transfect various cell lines (COS, HeLa, NIH3T3 and MITC) *in vitro*, according to transfection methods similar to those described in the previous examples. The charge ratio of the TGKC/DOPE/DNA lipoplexes was fixed between 3 and 5, i.e. at values which had previously proved to be optimal with the KanaChol. Naturally, in order to determine the theoretical mean charge ratio, it was considered that the 3 guanidinium groups of the TGKC are positively charged at neutral pH (due to the high pKa of guanidinium functions).

The results summarized in figure 7 clearly show that the TGKC/DOPE liposomes make it possible to efficiently transfect the various cell lines tested.

Example 8: In vitro transfection activity spectra for the NeoChol transfecting compound and for the NeoChol/DOPE liposomes

The aim of this example is to illustrate the ability of the transfecting compounds according to the invention to transfect various cell types *in vitro*, whether they are used alone or in the form of a liposome with DOPE.

5

The *in vitro* transfection experiments performed in examples 4, 5, 6 and 7 were carried out in a similar manner using the NeoChol/DNA and NeoChol/DOPE/DNA formulations at various charge ratios (between 1.3 and 26 for NeoChol/DNA and between 0.66 and 26 for NeoChol/DOPE/DNA) and in a series of 5 cell lines (HeLa, NIH 3T3, HEK293, COS and 16HBE). The lipoplexes and the liposomes were prepared in a manner similar to the protocol described in examples 4 and 5 (estimating that 5 amino functions are protonated for NeoChol at neutral pH). The results obtained are summarized in figures 8 and 9.

The results summarized in figure 8 clearly show that the NeoChol/DNA formulations make it possible to efficiently transfect the various cell lines tested.

It appears that the NeoChol/DOPE formulation (molar ratio 1:1) induces a high transfection efficiency for all the cell lines, the NeoChol used alone (without DOPE) appearing overall to be a little less efficient, in particular in the 16HBE cell line (except for the high charge ratios). It therefore appears that the use of DOPE would have a beneficial effect on the transfection efficacy.

Example 9: *In vivo* gene transfer into the airways of mice using the KanaChol/DOPE/Chol-PEG liposomes

This example illustrates the ability of the transfecting compounds according to the invention to transfect DNA into cells *in vivo*.

The *in vivo* gene transfer was carried out with 50 μ l (per dose) of transfecting medium containing the KanaChol/DOPE/DNA or NeoChol/DOPE/DNA lipoplexes stabilized with Chol-PEG, in the airways of female
5 BALB/c mice weighing 30 g (intranasal administration). Each animal received 3 doses, approximately 4 hours apart, i.e. a total amount of 100 μ g of pCIK-CAT plasmid DNA.

The formulations used were prepared in the
10 following way: KanaChol/DOPE liposomes are prepared according to the protocol described in example 6. A solution of steric stabilizer Chol-PEG (a cholesterol attached to a polyethylene glycol chain containing approximately 100 oxyethylene units) is then added
15 immediately before mixing with the pCIK-CAT plasmid DNA in aqueous solution. The DNA concentration is thus approximately 0.6 mg/ml and the positive charge ratio is 3.75. The final Chol-PEG/DNA ratio is then 2 (weight/weight). The use of Chol-PEG allows the
20 production of colloiddally stable solutions which makes it possible to have very high transfection efficiency in the airways of mice *in vivo* via intranasal instillation of the liposomal formulations. The NeoChol/DOPE/Chol-PEG formulations are prepared in the
25 same way, but the positive charge ratio used is 3.3 for these formulations.

The reporter gene used in the present case is the CAT ("Escherichia Coli chloramphenicol acetyltransferase") gene. The administration into the
30 airways is carried out by intranasal instillation. 48 hours post-transfection, the animals are killed and the lungs and trachea are removed separately for analysis. The tissue pieces are placed in a TEN buffer (40 mM of Tris-HCl, 1 mM of EDTA and 150 mM of sodium chloride,
35 pH 7.8) and agitated on ice for approximately 30 seconds using an Ultra-Turrax T25 agitator (Fisher

Bioblock Scientific, Strasbourg, France). The cells are then lysed with 3 freezing-thawing cycles and a clear supernatant is obtained by centrifugation. The CAT concentration is determined using a CAT ELISA assay
5 carried out in accordance with the manufacturer's (Boehringer Mannheim) instructions. The CAT levels are expressed in ng of CAT protein per 100 mg of total proteins, the protein concentration being determined using a Bio-Rad assay.

10 The table of figure 10 shows the levels of expression of the CAT gene obtained in the homogenates of trachea and of lung. The expression of the CAT gene is highest in the lung. Figure 10 also indicates that very low levels of expression are obtained after
15 administration of identical doses of "naked" DNA (i.e. nonformulated DNA). These results suggest that, under the conditions of the experiment, the KanaChol/DOPE and NeoChol/DOPE liposomes allow a transfection efficiency *in vivo*, in airways of mice, which is greater than that
20 obtained with naked DNA. The expression levels obtained using the NeoChol/DOPE liposomes are of the same order of magnitude as those obtained using the KanaChol/DOPE liposomes. These results are also entirely similar to those which had been obtained with
25 BGTC/DOPE formulations (described in application WO 97/31935), these being formulations which had proved to be most particularly efficient and advantageous for transfecting airways (Densmore et al., *J. of Gene Med.*, 1999, Vol 1(4)).

CLAIMS

1. Transfecting compound of general formula
(I):

5 A-Y-L (I)

in which:

- A represents an aminoglycoside chosen from amikacin, arbekacin, dibekacin, gentamycin, isepamycin, kanamycin, 10 micromycin, paromomycin, neomycin and tobramycin,
 - Y represents a spacer comprising one or more chemical functions chosen from -C(O)-, -C(O)-N- and -C(O)-NH-, and
 - 15 - L represents:
 - a radical -(R₁)R₂ in which R₁ and R₂ represent, independently of one another, a hydrogen atom or a fatty aliphatic chain, wherein at least one of 20 R₁ and R₂ represents a fatty aliphatic chain chosen from alkyl radicals containing 14, 16, 18, 20 or 22 carbon atoms and optionally 1, 2 or 3 unsaturations, or
 - 25 · a radical -N-R₃ or -O-R₃, in which R₃ represents a polycyclic compound of the cholestane type,
- or an isomer or a salt thereof.

2. Transfecting compound according to claim 30 1, characterized in that said aminoglycoside is chosen from kanamycin, paromomycin, neomycin and tobramycin.

3. Transfecting compound according to claim 3, characterized in that said spacer is chosen from the radicals of formula:

35 -C(O)- and
-W-(CH₂-)_k-W'-

in which k is an integer chosen between 1 and 6 inclusive, and W and W' are groups, which may be identical or different, chosen from -C(O)-, -C(O)-N- and -C(O)-NH-.

5 4. Transfecting compound according to claim 1 or 2, characterized in that said aminoglycoside is chosen from dibekacin, kanamycin, paromomycin, neomycin and tobramycin.

10 5. Transfecting compound according to any one of claims 1 to 4, characterized in that said fatty aliphatic chain(s) is (are) chosen from the aliphatic radicals $-(CH_2)_{11}CH_3$, $-(CH_2)_{13}CH_3$, $(CH_2)_{15}CH_3$ and $-(CH_2)_{17}CH_3$.

15 6. Transfecting compounds according to any one of claims 1 to 5, characterized in that said polycyclic compound of the cholestane type is chosen from cholesterol, cholestanol, 3- α -cyclo-5- α -cholestan-6- β -ol, cholic acid, cholesteryl formiate, cholestanyl formiate, 3- α -5-cyclo-5- α -cholestan-6- β -yl formiate, 20 cholesterylamine, and cholestanylamine.

7. Transfecting compound according to claim 1, characterized in that it is 3 β [6'-kanamycin-carbamoyl]cholesterol.

25 8. Transfecting compound according to claim 1, characterized in that it is 3 β [6'-(1,3,3"-triguanidino)kanamycin-carbamoyl]cholesterol.

9. Transfecting compound according to claim 1, characterized in that it is (5"-aminoethylsulfanyl) neomycin carbamoylcholesterol.

30 10. Composition, characterized in that it comprises a transfecting compound according to any one of claims 1 to 9, and a nucleic acid.

11. Composition according to claim 10, characterized in that the nucleic acid is a 35 deoxyribonucleic acid or a ribonucleic acid.

12. Composition according to claim 10 or 11, characterized in that said nucleic acid comprises one or more genes of therapeutic interest under the control of regulatory sequences.

5 13. Composition according to any one of claims 10 to 12, characterized in that said nucleic acid is an antisense sequence or gene.

10 14. Composition according to any one of claims 10 to 13, characterized in that it also comprises one or more adjuvants.

15 15. Composition according to claim 14, characterized in that said one or more adjuvants are chosen from lipids, peptides, proteins and polymers.

15 16. Composition according to claim 15, characterized in that said one or more adjuvants are chosen from neutral lipids.

20 17. Composition according to claim 16, characterized in that said neutral lipid is chosen from natural and synthetic lipids which are zwitterionic or lacking ionic charge under physiological conditions.

25 18. Composition according to claim 17, characterized in that said neutral lipid is chosen from dioleoylphosphatidylethanolamine (DOPE), oleoyl-palmitoylphosphatidylethanolamine (POPE), distearoyl-, dipalmitoyl- and dimirystoylphosphatidylethanolamines and also the derivatives thereof which are N-methylated 1 to 3 times, phosphatidylglycerols, diacylglycerols, glycosyldiacylglycerols, cerebrosides, sphingolipids, asialogangliosides, DOPC and cholesterol.

30 19. Composition according to claim 17, characterized in that said neutral lipid is chosen from galactocerebrosides, sphingomyelins, asialoGM1 and asialoGM2.

35 20. Composition according to claim 15, characterized in that said adjuvant is polyethylene glycol (PEG) or PEG-cholesterol.

21. Composition according to any one of claims 10 to 20, characterized in that it also comprises an extracellular or intracellular targeting element.

5 22. Composition according to claim 21, characterized in that said targeting element is chosen from sugars, peptides, proteins, oligonucleotides, lipids, neuromediators, hormones, vitamins and derivatives thereof.

10 23. Composition according to claim 21 or 22, characterized in that said targeting element is covalently attached to a fatty alkyl chain containing at least 10 carbon atoms or to a polyethylene glycol (PEG).

15 24. Composition according to claim 21, characterized in that said targeting element is covalently attached either to the transfecting compound according to any one of claims 1 to 9, or to the nucleic acid.

20 25. Composition according to any one of claims 10 to 24, characterized in that it also comprises a vehicle which is pharmaceutically acceptable for an injectable formulation.

25 26. Composition according to any one of claims 10 to 24, characterized in that it also comprises a vehicle which is pharmaceutically acceptable for administration on the skin, on mucous membranes or both.

30 27. Use of a transfecting compound according to any one of claims 1 to 9, for transferring nucleic acids in vitro.

35 28. Use of a transfecting compound according to any one of claims 1 to 9 or of a composition according to any one of claims 10-26, for manufacturing a medicinal product intended to treat diseases.

29. Use according to claim 28, characterized in that said composition is mixed with one or more adjuvant as defined in any one of claims 15 to 20, a targeting element as defined in any one of claims 21 to 23 or both.

30. Method for transferring nucleic acids into cells in vitro, characterized in that it comprises the following steps:

(1) bringing the nucleic acid into contact with a transfecting compound as defined in any one of claims 1 to 9, so as to form a complex, and

(2) bringing the cells into contact with the complex formed in (1).

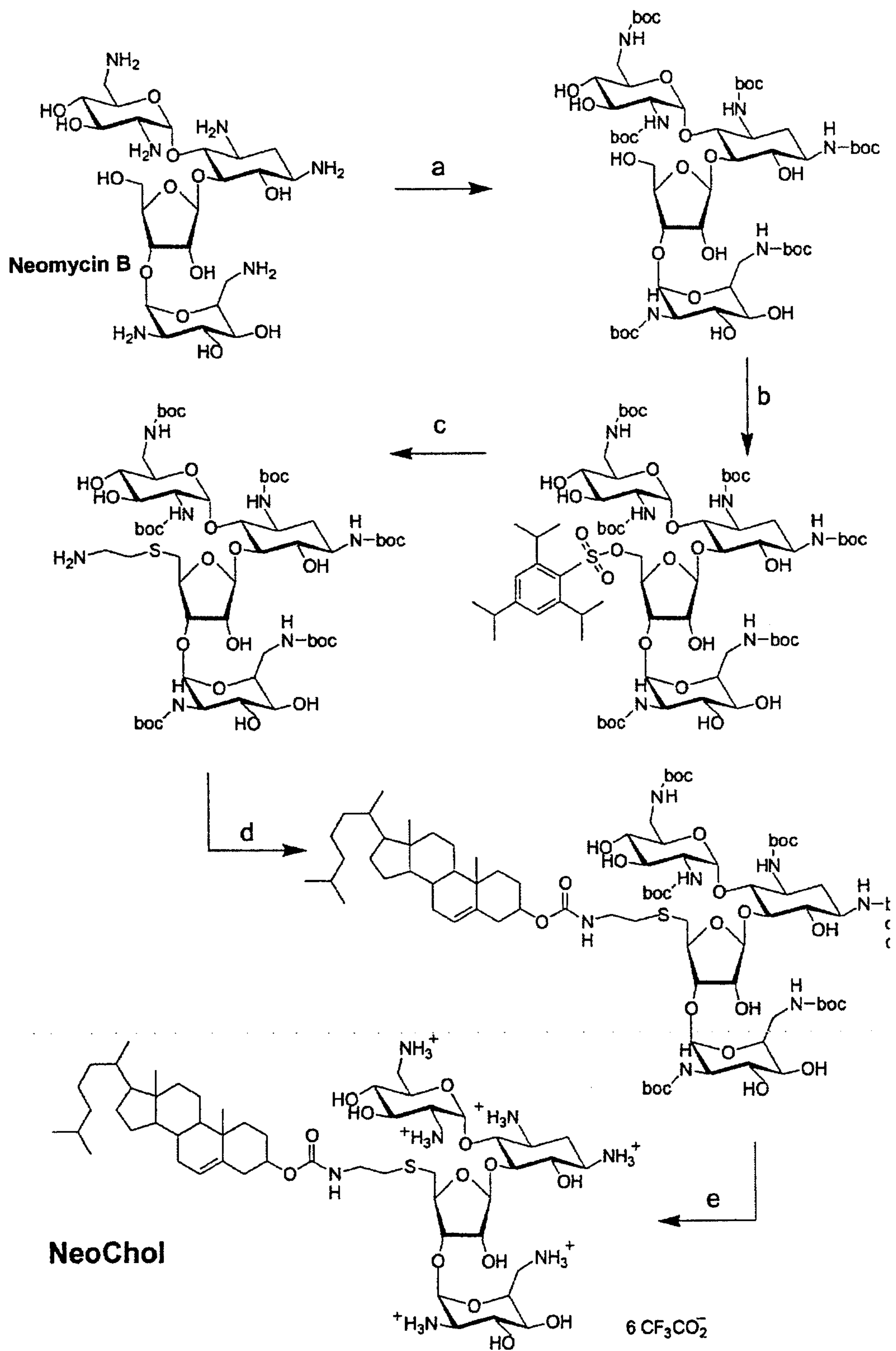
31. Method for transferring nucleic acids into cells in vitro according to claim 30, characterized in that said complex or said nucleic acid are mixed beforehand with one or more adjuvants as defined in any one of claims 15 to 20, a targeting element as defined in any one of claims 21 to 23, or both.

32. Method for transferring nucleic acids into cells in vitro according to claim 30, characterized in that one or more adjuvants as defined in any one of claims 15 to 20 are administered to the cells beforehand.

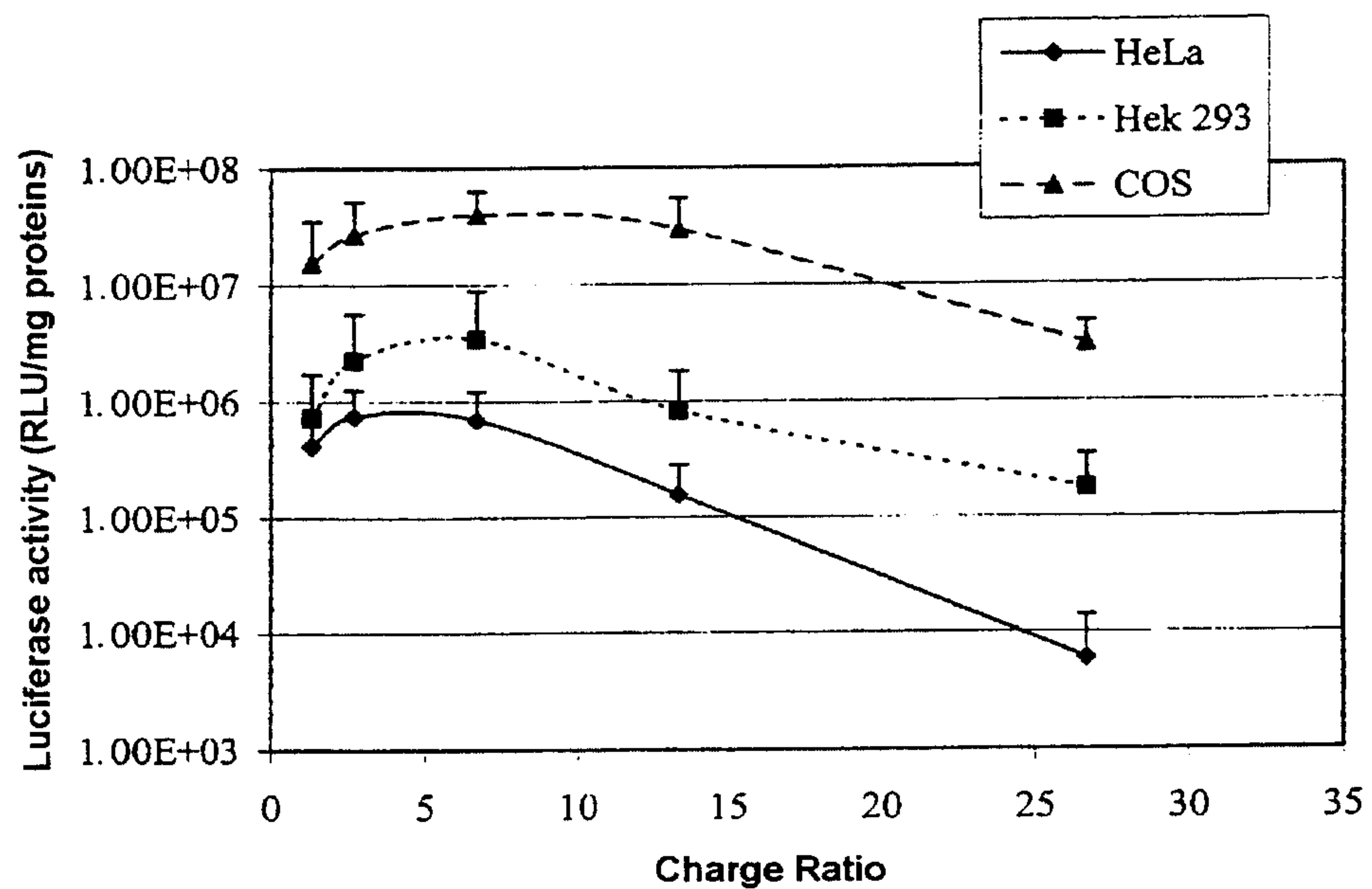
33. Method for transferring nucleic acids into cells in vitro according to any one of claims 30 to 32, characterized in that the cells are also subjected to chemical or physical treatment.

34. Transfection kit, characterized in that it comprises one or more transfecting compounds according to any one of claims 1 to 8.

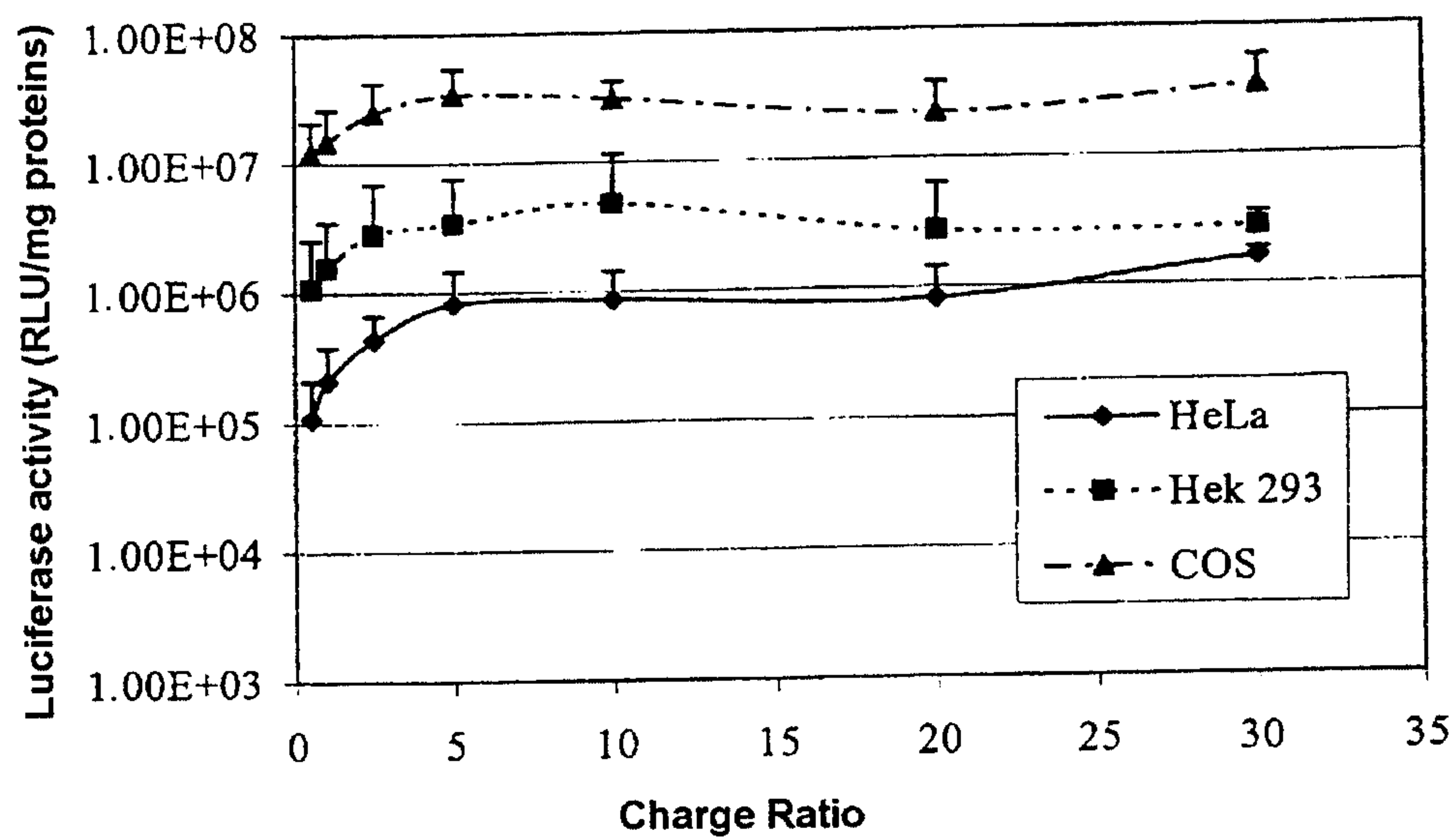
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FIG.1

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FIG. 2**KanaChol/DNA formulations at various charge ratios**

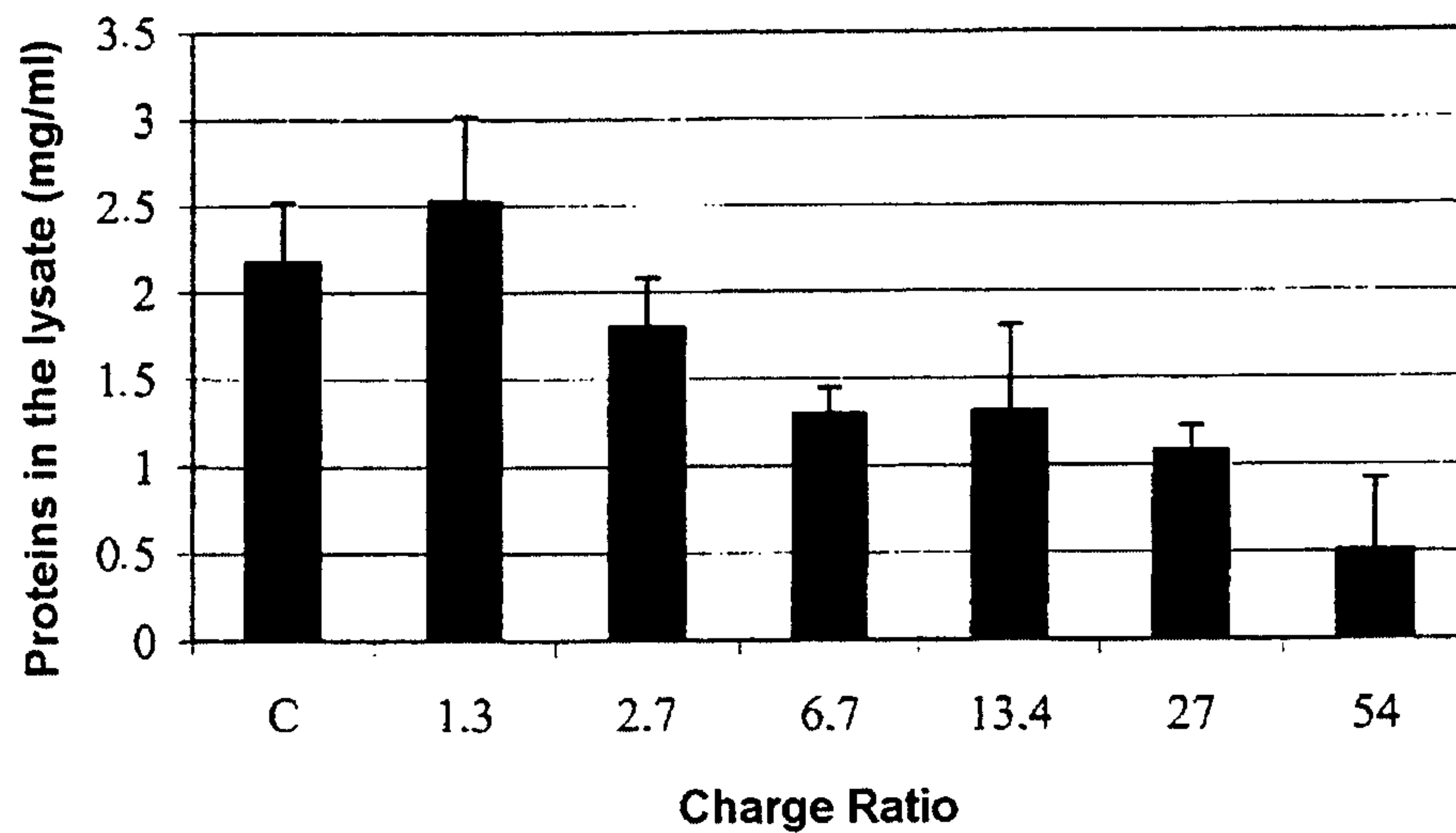
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FIG. 3**KanaChol/DOPE/DNA formulations at various charge ratios**

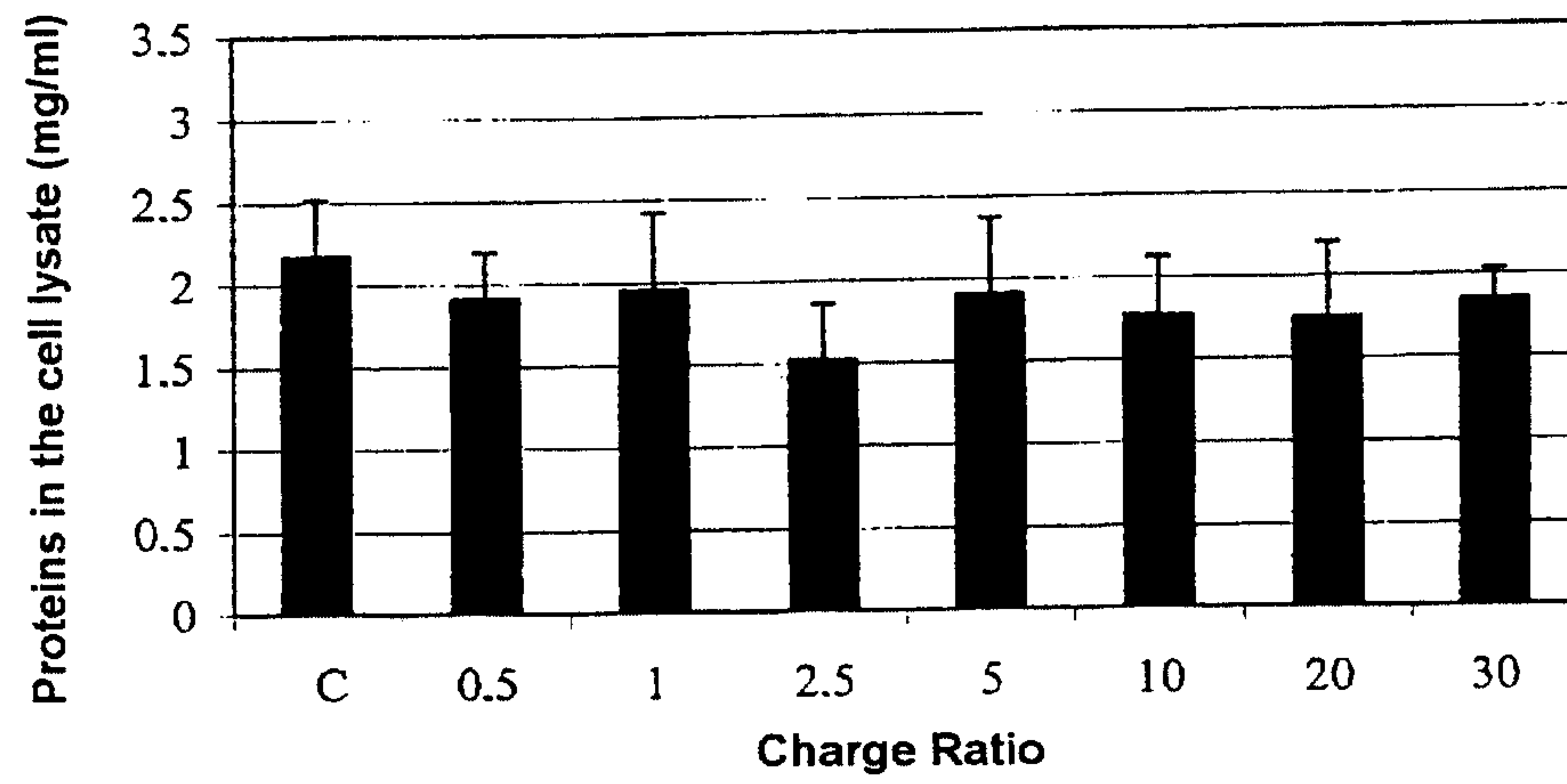
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FIG. 4

Cytotoxicity of the KanaChol/DNA formulations at various charge ratios



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Fig. 5**Cytotoxicity of the KanaChol/DOPE/DNA formulations at various charge ratios**

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FIG. 6

Expression of luciferase (RLU/mg proteins) in various mammalian cell lines transfected with KanaChol or KanaChol/DOPE (molar ratio 1:1)

Cell line	Species/ Tissue type	KanaChol	KanaChol/ DOPE
NIH 3T3	Mouse fibroblasts	4.5×10^5	4.3×10^6 ($\pm 3 \times 10^6$)
A549	Human lung carcinoma	2×10^5 ($\pm 0.3 \times 10^5$)	8.5×10^6 ($\pm 6 \times 10^6$)
16HBE	Human bronchial epithelium	3.4×10^3 ($\pm 1.7 \times 10^3$)	3×10^6 ($\pm 0.4 \times 10^6$)
HepG2	Human hepatoblastoma	5×10^4 ($\pm 4 \times 10^4$)	5.2×10^6 ($\pm 3.2 \times 10^6$)
MITC	Human thymic myoid cells	3.2×10^6 ($\pm 0.3 \times 10^6$)	1.5×10^7 ($\pm 0.5 \times 10^7$)

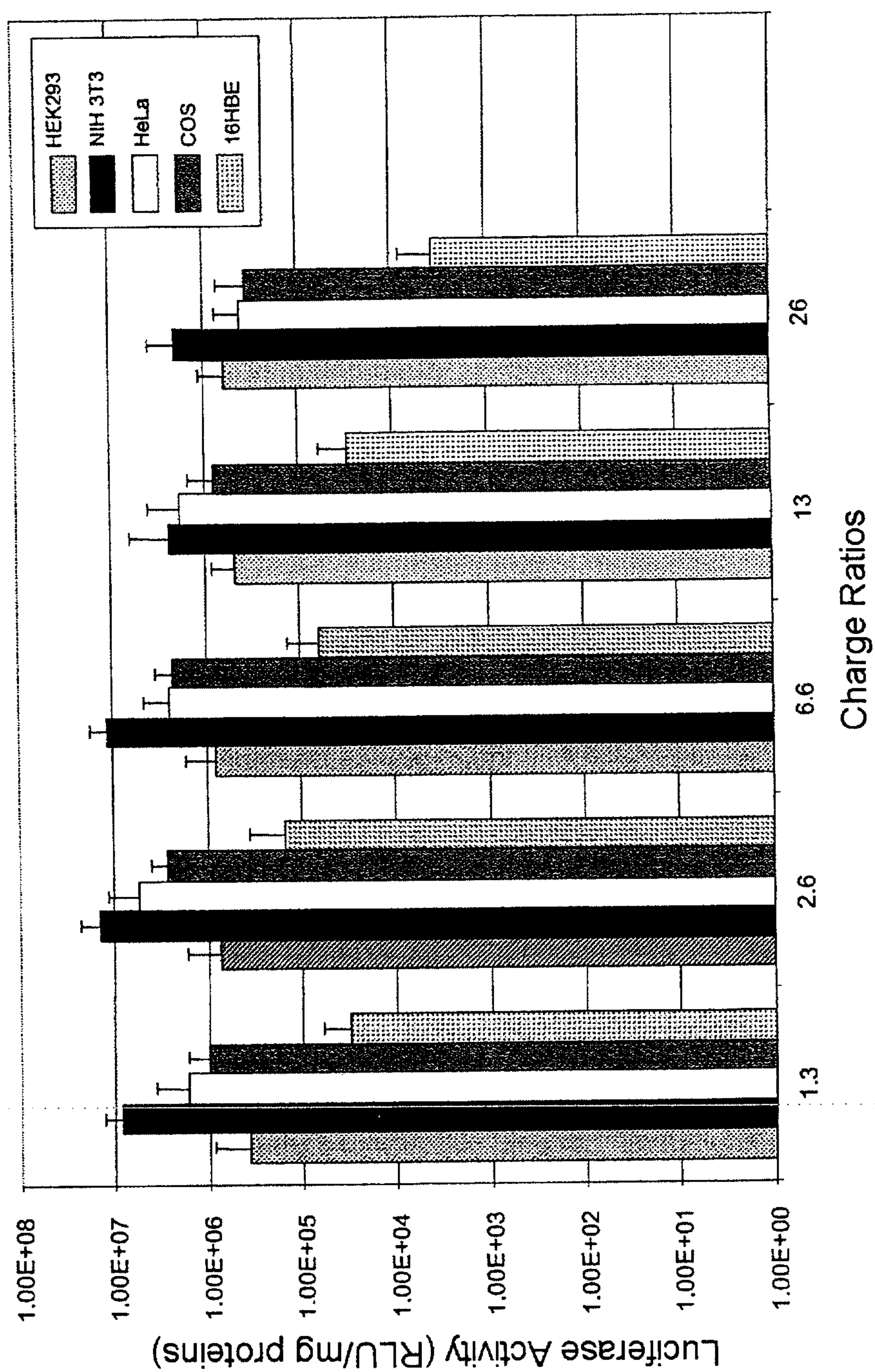
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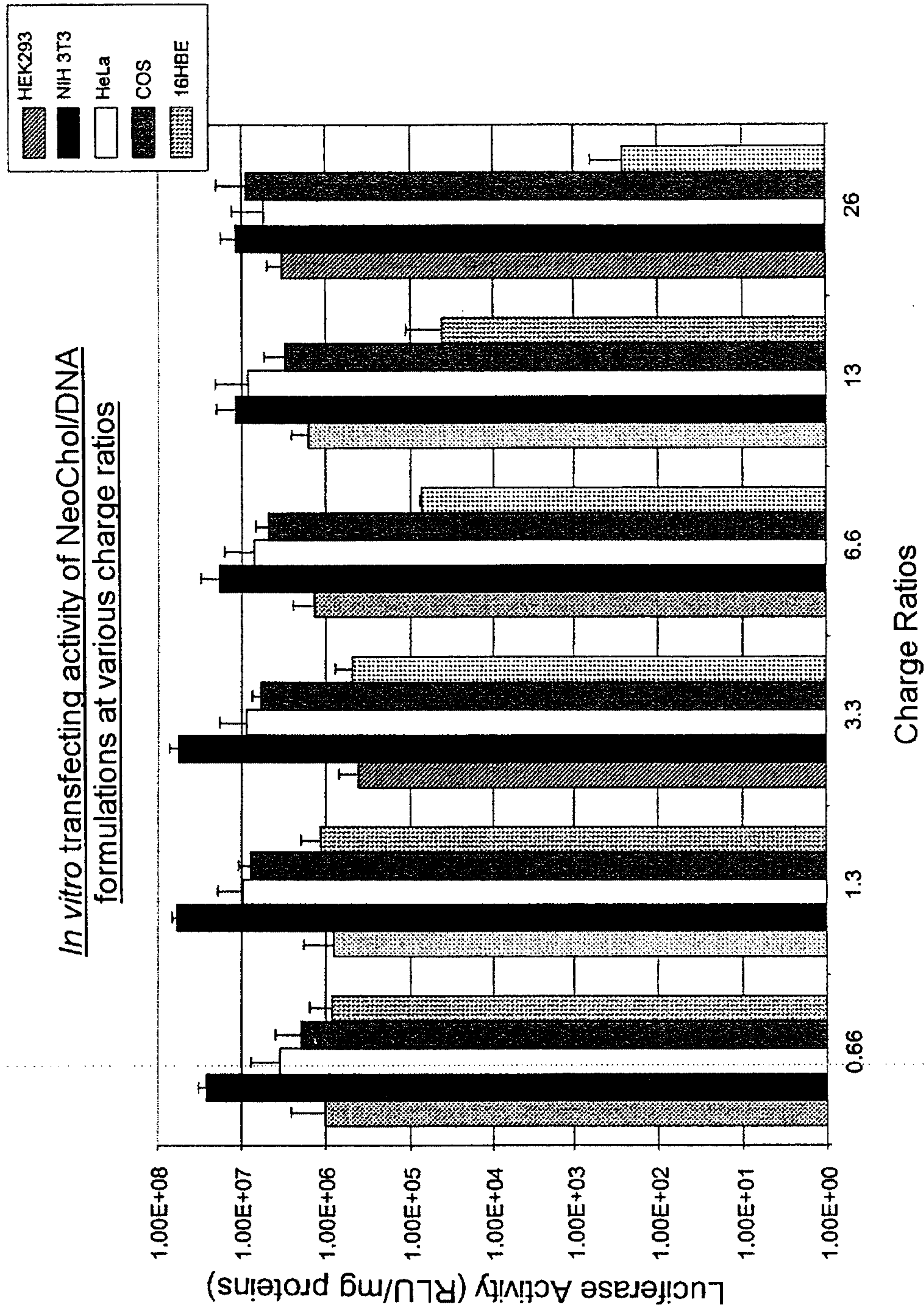
FIG. 7**Expression of luciferase in mammalian cell lines
transfected with TGKC/DOPE liposomes**

Cell line	Luciferase Activity (RLU / mg proteins)
COS	$4.5 \times 10^6 (\pm 1 \times 10^6)$
HeLa	$5 \times 10^5 (\pm 2.5 \times 10^5)$
NIH 3T3	$5.2 \times 10^5 (\pm 2 \times 10^5)$
MITC	$5.6 \times 10^6 (\pm 1 \times 10^6)$

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In vitro transfecting activity of NeoChol/DNA formulations at various charge ratios





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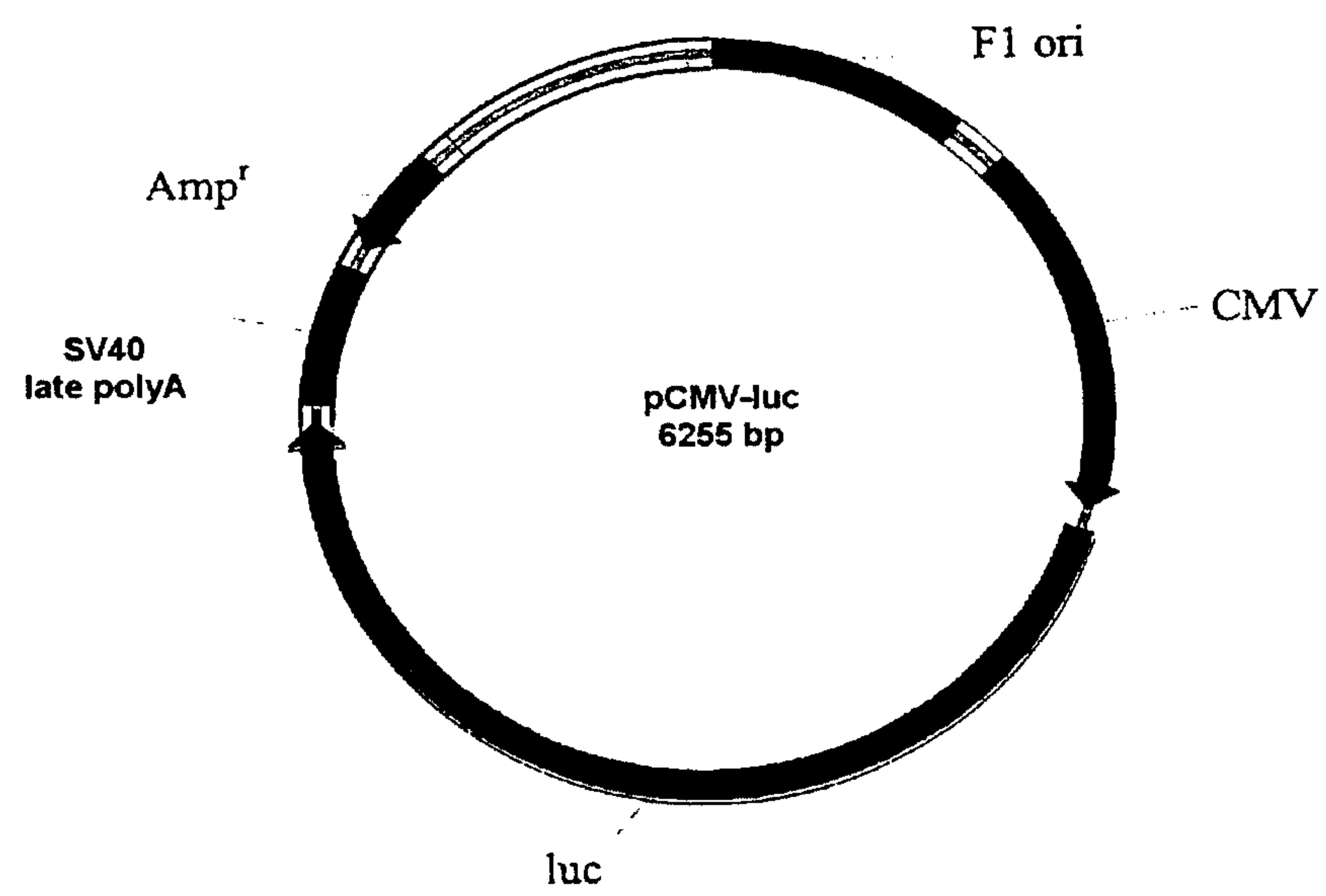
FIG. 10

CAT expression in airways of mice following intranasal instillation of KanaChol/DOPE/DNA or NeoChol/DOPE/DNA lipoplexes stabilized with Chol-PEG

Type of vector	CAT expression in the trachea (ng/100 mg proteins)	CAT expression in the lungs (ng/100 mg proteins)
KanaChol/DOPE	0.8 ± 0.9	6.1 ± 3.5
NeoChol/DOPE (1/1)	3.7 ± 6	10 ± 8
Nonformulated DNA	0.03 ± 0.05	0.14 ± 0.16

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FIG. 11



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FIG. 12

