Kanamycin A-Derived Cationic Lipids as Vectors for Gene Transfection

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Cationic lipids nowadays constitute a promising alternative to recombinant viruses for gene transfer. We have recently explored the transfection potential of a new class of lipids based upon the use of aminoglycosides as cationic polar headgroups. The encouraging results obtained with a first cholesterol derivative of kanamycin A prompted us to investigate this family of vectors further, by modulating the constituent structural units of the cationic lipid. For this study, we have investigated the transfection properties of a series of new derivatives based on a kanamycin A scaffold. The results primarily confirm that aminoglycoside-based lipids are efficient vectors for gene transfection both in vitro and in vivo (mouse airways). Furthermore, a combination of transfection and physicochemical data revealed that some modifications of the constitutive subunits of kanamycin A-based vectors were associated with substantial changes in their transfection properties.

Introduction

Synthetic vectors are currently being developed as an alternative to recombinant viruses for gene delivery, in view of issues such as immunogenicity and large-scale production that confront the use of the latter.[1–4] Among these artificial vectors, cationic lipids are particularly attractive as they can be easily prepared and extensively characterised.[5–7] Indeed, their overall structures are relatively simple, each being basically composed of a cationic headgroup and a lipophilic moiety connected via a linker. Gene delivery by cationic lipids (termed lipofection) relies on their ability to interact with the negatively charged DNA through their cationic headgroups and to condense it into DNA/lipid aggregates (termed lipoplexes), which can be taken up by the target cells by an endocytosis mechanism involving electrostatic interactions between residual positive charges on the lipoplexes and negatively charged cell membrane residues.[8,9] The lipoplexes are thereby delivered to an early endosomal compartment, from which they must escape in order to avoid degradation by nucleases in the subsequent late endosomes and lysosomes. Finally, following endosomal escape, the DNA is then required to traffic to the nucleus to permit expression of the transgene. It should be noted that the processes of endosomal escape and nuclear uptake are still not fully understood and remain critical cellular barriers to lipofection.[10–14]

Although the transfection efficacy of a given cationic lipid depends on the properties of the self-assembled supramolecular DNA/lipid assemblies, cationic lipids offer the advantage that each of their basic constituent parts can be modified, thereby facilitating the elucidation of structure–activity relationships. In spite of some positive results, it is generally agreed that the efficiency of cationic lipids still needs to be significantly enhanced. This clearly requires the design of new, more efficient vectors, as well as a better understanding of the mechanisms underlying lipofection.[15–17]

We therefore set out to develop a new family of cationic lipids based upon the use of aminoglycosides as cationic headgroups. Aminoglycosides form a large family of polycationic compounds (each consisting of one six-carbon aminocyclitol moiety joined through a glycosidic linkage to one or several aminosugars) widely used as antibiotics. They are known to interact with the major groove of duplex RNA[18] or more generally with A-form nucleic acids.[19] Their antibacterial activity stems from their selective binding to the rRNA of bacteria, which results in impairment of protein synthesis.[20] Although interactions of aminoglycosides with DNA have been reported to be weaker than those with RNA,[21–23] neomycin B has been shown to interact with DNA and to protect it from nuclease activity.[22,24] These properties of aminoglycosides, together with their structural variety, make them good candidates for development of novel vectors for gene transfer. In addition, their multifunctionality provides a favourable scaffold for the synthesis of a variety of compounds. A preliminary study with a kanamycin A–cholesterol derivative, KanaChol (**1**), demonstrated the feasibility of using an aminoglycoside as a headgroup

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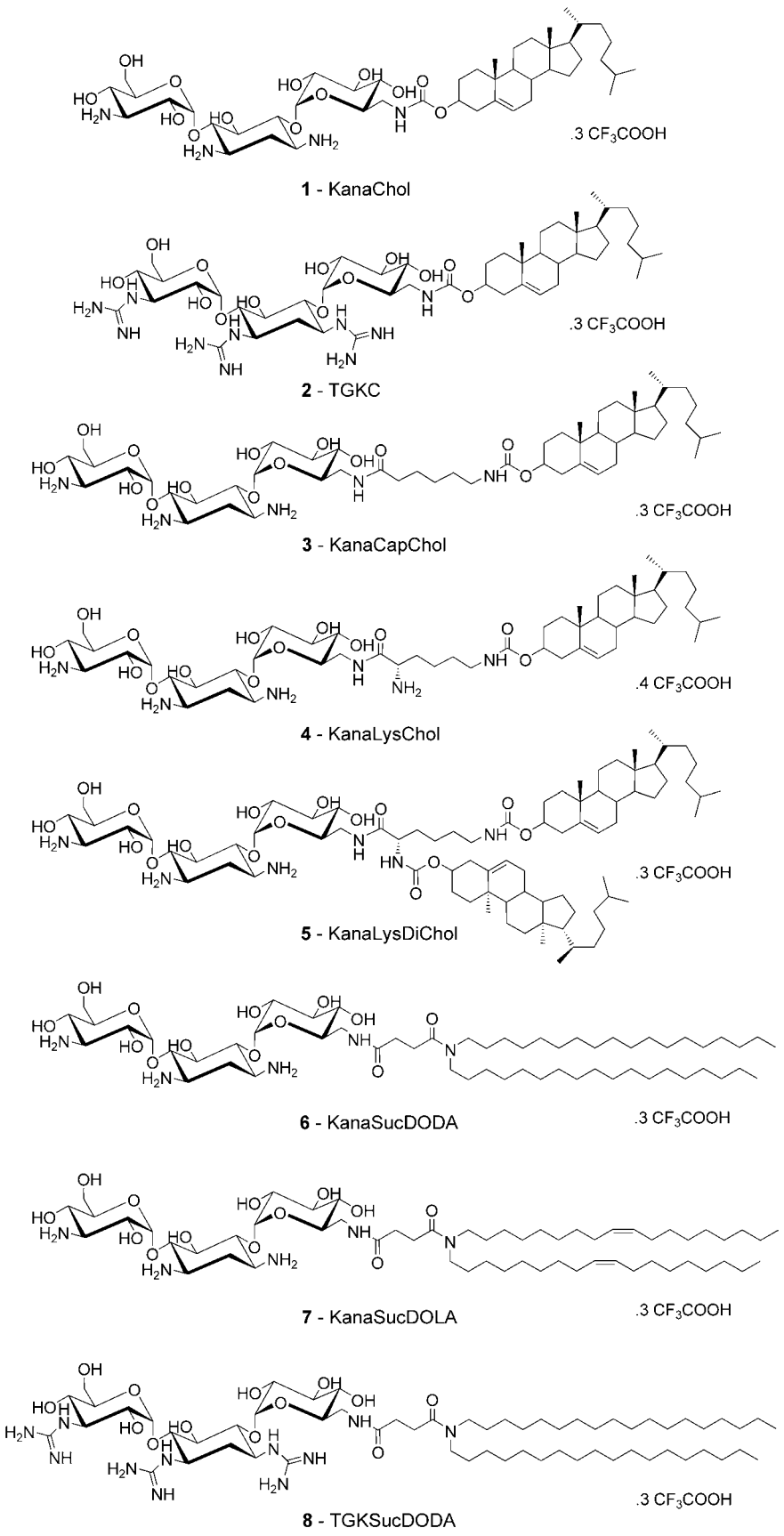
of a cationic lipid for gene transfer both in vitro and in vivo (mouse airways).[25] This prompted us to investigate the transfection potential of this new family of vectors further.

In this work we therefore studied the transfection properties of a series of lipids in which the kanamycin A headgroup is linked to various spacer and lipophilic subunits. It was also expected that such a study might shed some light on the factors underlying their transfection activity. We first designed KanaChol analogues bearing various linkers between the kanamycin A headgroup and the cholesterol moiety. Indeed, the length and nature of the spacer subunit may affect the physicochemical and biological properties of the lipoplexes. This led us to synthesise KanaCapChol (**3**), KanaLysChol (**4**) and KanaLysDiChol (**5**),[26] the structures of which are shown in Scheme 1. Next, we also synthesised two aliphatic derivatives of kanamycin A: KanaSucDODA (**6**) and KanaSucDOLA (**7**), which have distearyl and dioleyl chains, respectively. The synthesis of KanaSucDOLA is briefly described in the Experimental Section, whereas that of the other compounds has already been reported.[26] Here, we reasoned that long C_{18} aliphatic chains should be quite appropriate for counterbalancing the hydrophilicity and bulkiness of the aminoglycosidic headgroup, while also providing vectors with different properties from those of cholesterol. Finally, we also prepared TGKSucDODA, a fully guanidylated derivative of KanaSucDODA, in order to crosscheck our findings with KanaChol and its fully guanidinylated derivative TGKC (**2**), that guanidinylation resulted in a decrease in transfection activity (unlike what we had previously observed with other cationic cholesterol derivatives bearing guanidinium groups, such as BGTC).[27,28]



Scheme 1. Structures of the lipid derivatives of kanamycin A.

Here we report the results of in vitro transfection experiments with this series of kanamycin A-derived cationic lipids **1**–**8** (Scheme 1). The most efficient vectors were subsequently found to mediate transfection of mouse airways in vivo. We also describe the results of physicochemical studies that offer some insight into the properties underlying the transfection activity of kanamycin A-derived lipids.

Results

In vitro transfection by cholesterol derivatives

For in vitro transfection, the novel cholesterol derivatives of kanamycin A were formulated as liposomes with the colipid DOPE, as we had previously shown that KanaChol was more efficient when formulated as KanaChol/DOPE liposomes.[25] In addition, the novel compounds were found to be notably less water-soluble than KanaChol, presumably because their longer spacer subunits (derived from 6-aminocaproic acid or lysine) affected the hydrophobicity/hydrophilicity balance; consequently, addition of DOPE was required to obtain homogenous dispersions.

The transfection efficiency of cationic lipids being in general highly dependent both on the charge ratio of the lipoplexes and on the cell type used, the transfection activities of these vectors were therefore evaluated in five different mammalian cell lines and the optimal charge ratio was assessed by dose-response curves in each case, as shown in Figure 1. To determine the theoretical charge ratios of the lipoplexes, we considered 1 μg of DNA to be equal to 3 nmol of negative charges and assumed that the three amine groups of kanamycin A were protonated at the neutral pH of lipoplex formation, as previously discussed for KanaChol.[25] The dose-response curves (Figure 1) showed that the overall transfection efficiencies of

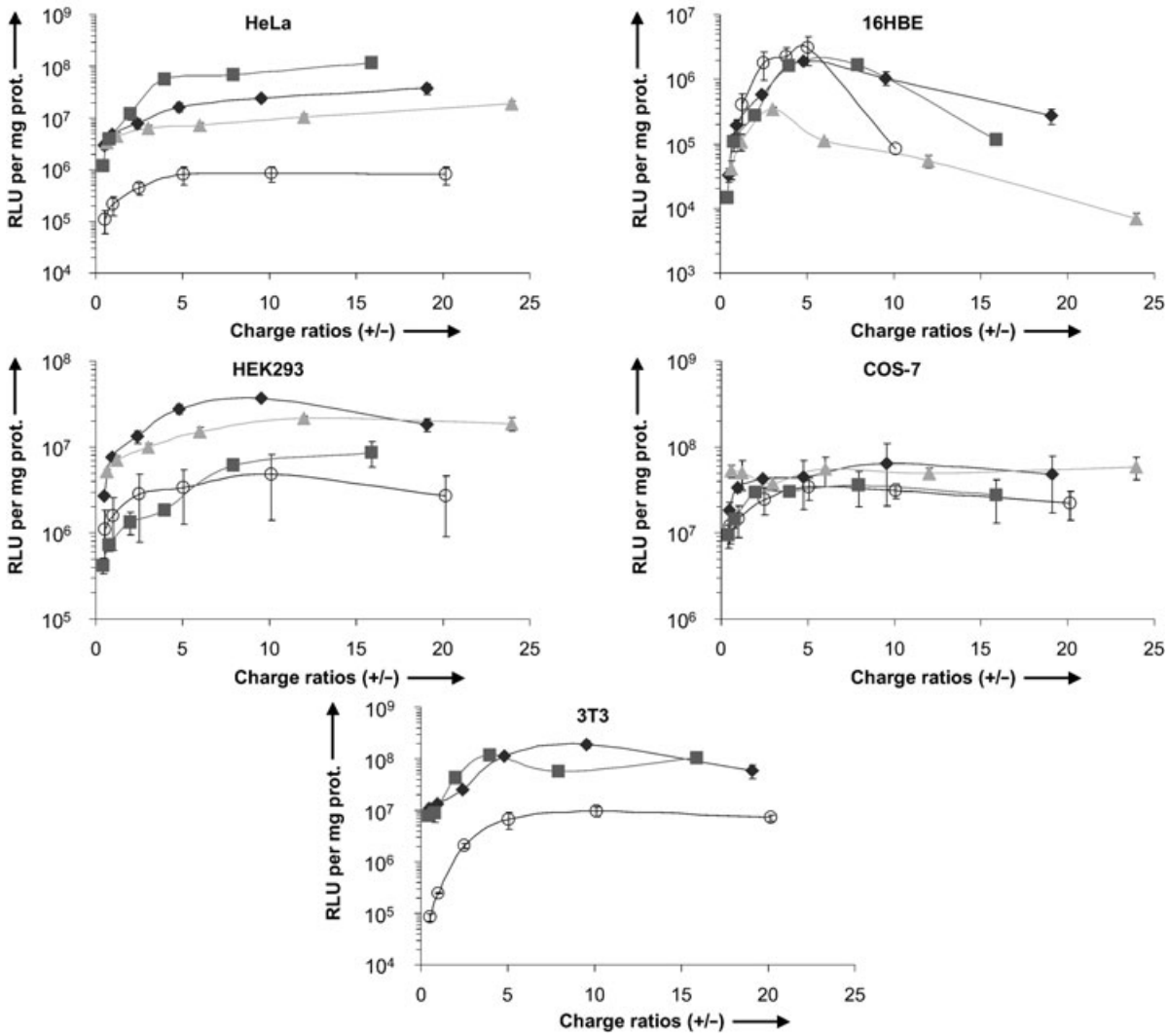


Figure 1. Dose-response curves of the in vitro transfection activity of cholesterol derivatives of kanamycin A formulated as liposomes with DOPE (molar ratio 1:1): (○) = KanaChol/DOPE, (◆) = KanaCapChol/DOPE, (▲) = KanaLysChol/DOPE, and (■) = KanaLysDiChol/DOPE. Luciferase reporter gene expression is indicated as a function of the charge ratio of the lipoplexes. Cell lines were transfected as described in the Experimental Section using lipoplexes prepared by mixing luciferase-expressing plasmid DNA (5 μg) with the required amounts of lipid. Data are expressed as relative lights units (RLU) per mg of cell protein (mean ± SD with $n \geq 3$).

the novel cholesterol derivatives of kanamycin A (formulated as liposomes with DOPE) were higher than those of KanaChol/DOPE liposomes with all cell lines except for 16HBE cells. In this latter case, the decline in transfection at high charge ratios (i.e., at high lipid/DNA ratios) suggests that the lipids may have some intrinsic toxic effects not observed with the other cell lines. Increases in activity of up to tenfold between KanaChol and KanaCapChol were even observed with HeLa, HEK293 and 3T3 cells. The data shown in Figure 1 also indicate that, although some slight variations were observed, the incorporation of an additional amino group (KanaLysChol) or cholesterol subunit (KanaLysDiChol) did not significantly affect the overall transfection activity when compared to KanaCapChol.

As concerns the underlying mechanisms of the globally improved transfection efficacy of KanaCapChol, KanaLysChol and KanaLysDiChol versus KanaChol, it is noteworthy that the common element for these three vectors in comparison with KanaChol (in which the headgroup and cholesterol moieties are adjacent) is a longer spacer. It is therefore likely that the incorporation of this linker subunit induces this overall increase in efficiency. The introduction of a spacer arm may be associated with a modification of the hydrophilicity/hydrophobicity balance of the vectors, all three compounds being more lipophilic than KanaChol, although the fact that similar activities were obtained with KanaCapChol, KanaLysChol and KanaLysDiChol (which differ greatly in terms of lipophilicity) suggests that factors other than the hydrophilicity/hydrophobicity balance may underlie the increased activity of these lipids. Thus, we also studied the behaviour of the different cholesterol derivatives of kanamycin A in Langmuir monolayers. Measurement of the surface pressure versus the molecular area at constant temperature is a method widely used for the study of the physical properties of lipids.[29] Here, we first observed that all the cholesterol derivatives of kanamycin A were able to form monolayers and that the introduction of a longer spacer resulted in an increase in the mean molecular area, as shown for KanaCapChol versus KanaChol in Figure 2. In addition, comparison of the slopes of the surface pressure/area isotherms observed with KanaChol and KanaCapChol indicated that the introduction of a spacer unit resulted in more fluid lipidic layers (Figure 2). These data suggest that the incorporation of a longer spacer unit into the structures of cholesterol derivatives of kanamycin A may provide the lipoplexes with physicochemical properties beneficial for their transfection activity.

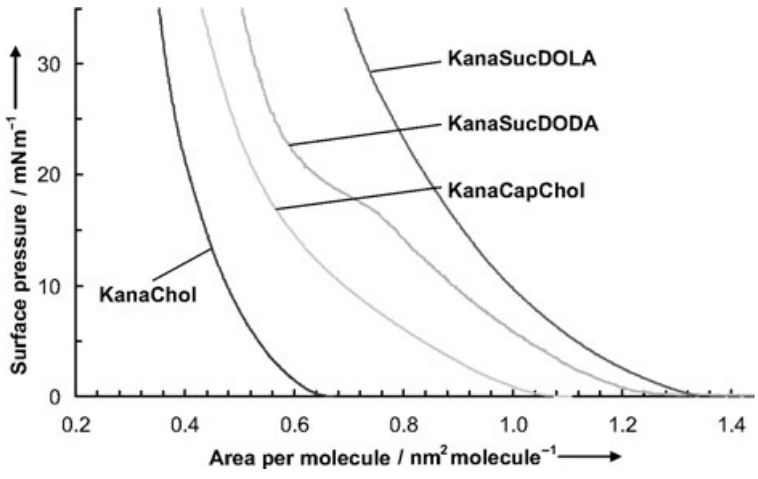


Figure 2. Surface pressure/area isotherms (20 °C) for KanaChol, KanaCapChol, KanaSucDODA and KanaSucDOLA in tridistilled water.

In vitro transfection by aliphatic derivatives

We next evaluated the transfection properties of KanaSucDODA and KanaSucDOLA, which are kanamycin A-based cationic lipids with aliphatic hydrophobic moieties. Here, attention was first paid to the self-assembling properties of these compounds in aqueous media: that is, their ability to form vesicles in the absence of the helper lipid DOPE. Indeed, the bulkiness of the kanamycin A headgroup may hinder the stability of the lipid bilayers formed by such vectors formulated as vesicles. Recent work on a monostearoylamide derivative of kanamycin A[30] indeed showed that it spontaneously formed micelles in aqueous medium. We expected, however, that our aliphatic derivatives of kanamycin A should display different behaviour, as their hydrophobic parts are each composed of two chains. We therefore studied the sizes of the assemblies formed by KanaSucDOLA and KanaSucDODA (formulated without DOPE under the same conditions as used for the preparation of DOPE-containing liposomes) by dynamic light scattering (DLS) and also examined the morphology of KanaSucDOLA assemblies by transmission electron microscopy (TEM). The results clearly showed the presence of vesicles with a size range from 25 to 45 nm, which is very similar to the size of the liposomes obtained with KanaSucDODA/DOPE or KanaChol/DOPE.[25] The ultimate evidence for the formation of vesicles was the direct observation of circular structures of KanaSucDOLA with a size distribution in agreement with the DLS measurements by TEM (data not shown).

Liposomal formulations (with and without DOPE) of KanaSucDODA and KanaSucDOLA were next used for transfection experiments with two cell lines (HeLa and 16HBE). In addition, in the case of KanaSucDODA, we also performed transfections with two other cell lines (HEK293 and COS-7) in order to investigate the influence of the unsaturated co-lipid DOPE further. As discussed above, dose-response curves were established for each cell line. As shown in Figure 3, both vectors were efficient for gene transfection into all cell lines tested and their optimal transfection activities were only slightly dependent on their formulations. However, the influence of DOPE varied according to the nature of the lipophilic portion. Indeed, in the case of KanaSucDODA, addition of DOPE did in general slightly increase the activity of the vector. In contrast, in the case of KanaSucDOLA, the presence of DOPE resulted in a slight decrease in transfection efficiency (best observed on HeLa cells). It is notable that similar effects linked to the presence of the neutral co-lipid were observed in preliminary experiments with aliphatic derivatives of aminoglycosides

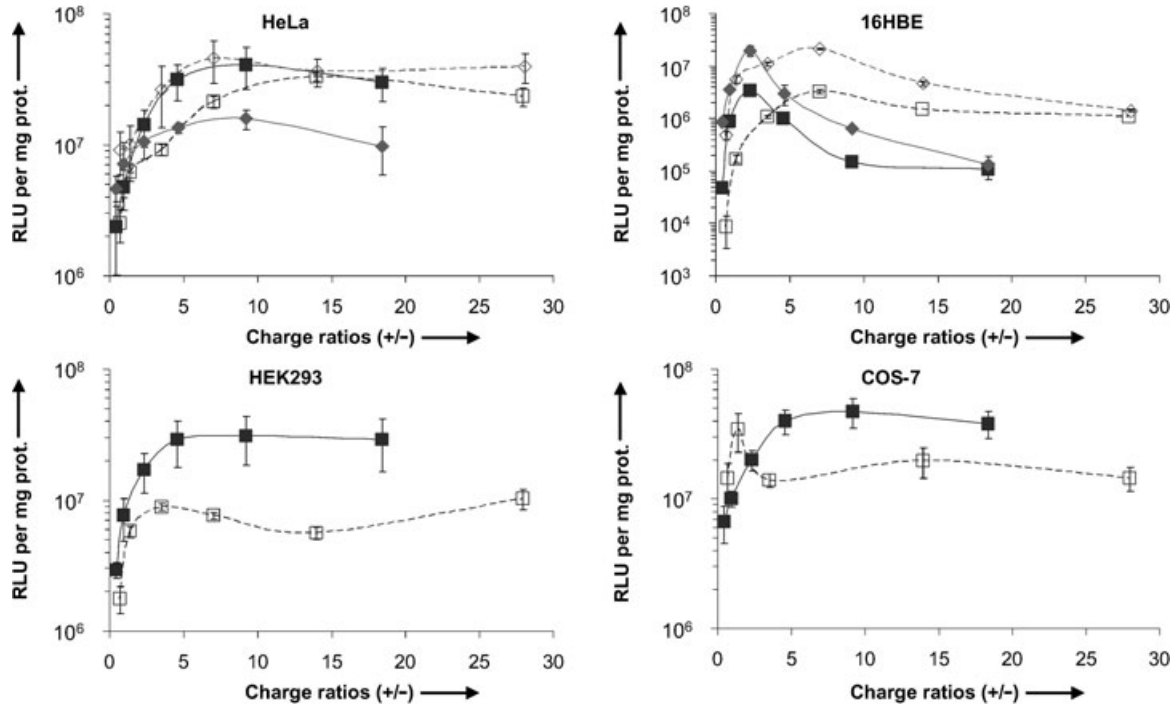


Figure 3. Dose-response curves of the in vitro transfection activities of aliphatic derivatives of kanamycin A formulated as liposomes with and without DOPE (molar ratio 1:1): (□ and dashed line) = KanaSucDODA, (■) = KanaSucDODA/DOPE, (◇ and dashed line) = KanaSucDOLA, and (◆) = KanaSucDOLA/DOPE. Luciferase reporter gene expression is indicated as a function of the charge ratio of the lipoplexes. Cell lines were transfected as described in the Experimental Section through the use of lipoplexes prepared by mixing luciferase-expressing plasmid DNA (5 μg) with the required amounts of lipid. Data are expressed as relative lights units (RLUs) per mg of cell protein (mean ± SD with $n \geq 3$).

other than kanamycin A, independently of the nature of the headgroup.[31]

Such opposing effects of the addition of DOPE to KanaSucDODA and KanaSucDOLA may have several explanations. Firstly, a well-known drawback of cationic lipids is some level of cytotoxicity, which can limit transgene expression by compromising the cellular processes of transcription and translation. Here, as shown in Figure 4, the addition of DOPE does not seem to modify the cytotoxicity of KanaSucDOLA (Figure 4B), whereas it appears to decrease the cytotoxicity of KanaSucDODA (Figure 4B) in a manner similar to that observed with KanaChol (Figure 4A). This, however, does not explain the generally negative effect of the addition of DOPE on the transfection efficiency of KanaSucDOLA, so it may also be necessary to take some physicochemical considerations into account. The unsaturated dioleyl chains of KanaSucDOLA might indeed give rise to a lower phase-transition temperature (gel to liquid crystal phase transition) in relation to the distearyl derivative KanaSucDODA. Accordingly, this phase transition could be observed (as a shoulder) on the isotherm of KanaSucDODA at 20 °C, while it was not observed in the case of the unsaturated KanaSucDOLA, in which the chains remain in a fluid state at that same temperature (Figure 2). This was confirmed by differential scanning calorimetry (DSC) studies. As shown in Figure 5, scanning of KanaSucDODA and KanaSucDOLA vesicles (without DOPE) from 0 to 60 °C enabled us to estimate the phase-transition temperatures (T_ms) of the two compounds. For the distearyl derivative KanaSucDODA, the T_m was about 34 °C, whereas for the unsaturated derivative KanaSucDOLA, no phase transition could be observed, this latter result being in good agreement with the results obtained with other dioleyl cationic lipids, for which the T_ms were found to be below 0 °C.[32,33] Altogether, one may hypothesise that the fluidity induced by unsaturation may provide the KanaSucDOLA vector with part of the fusogenic activity of DOPE.[5] Thus, in the case of KanaSucDODA, the T_m of which is close to the physiological temperature (37 °C) at which the transfection experiments were performed, the addition of DOPE might lower the T_ms of the resulting liposomes and lipoplexes and thereby provide them with favourable fusogenic properties. In contrast, in the case of KanaSucDOLA, the T_m of which is already low, addition of DOPE might not change the physicochemical behaviour of the lipoplexes. Moreover, the presence of the neutral DOPE here may only lead to a decrease in the positive charge densities on the surfaces of the liposomes, resulting in decreased interactions with the negatively charged DNA. It is also noteworthy that KanaSucDOLA alone seems to be more efficient than KanaSucDODA, whether formulated with or without DOPE. This finding, which further emphasises that the nature of the hydrophobic subunit and the fluidity of the lipid layers of the resulting lipoplexes are important factors in gene transfection, is in good agreement with previous studies.[34,35] However, a different effect of the addition of DOPE has been reported in studies of monovalent cationic lipids.[35] Here, the size and multivalency of the aminoglycoside headgroup may thus also govern the overall transfection efficiency.

Finally, similar physicochemical considerations can be made in comparison of the transfection properties of the cholesterol and aliphatic derivatives of kanamycin A. Indeed, as shown in Figure 2, the profiles of the isotherms indicate that the aliphatic derivatives produce more fluid lipid layers than KanaChol. This may be correlated with the increased efficiency of KanaSucDOLA and KanaSucDODA in relation to KanaChol. As discussed above for the cholesterol derivatives, the presence of a spacer subunit—the succinyl group—is probably contributing to the increased fluidity of the lipidic layers formed by KanaSucDOLA and KanaSucDODA.

In vitro transfection by aliphatic derivatives of guanidinylated kanamycin A

In our previous study with KanaChol, we also evaluated the transfection activity of its fully guanidinylated analogue TGKC.[25] In this work we therefore also investigated the transfection efficiency of the guanidylated KanaSucDODA derivative TGKSucDODA (**8**, formulated as liposomes with DOPE, since better transfection results had been obtained with KanaSucDODA/DOPE liposomes than with KanaSucDODA alone, as indicated above). For comparative purposes, the transfection activities of TGKSucDODA and its parent vector KanaSucDODA were evaluated in two cell lines (HeLa and COS-7) that we had previously already used when comparing TGKC and KanaChol.[25]

Figure 6 shows that—as previously observed for KanaChol and its guanidinylated analogue TGKC—the transfection activity of the KanaSucDODA/DOPE formulation was clearly higher than that of the TGKSucDODA/DOPE formulation, the guanidinylated analogue giving rise to luciferase levels at least ten times lower. The same trend was observed in the absence of DOPE with HeLa cells (data not shown). These results confirm our previous findings with the KanaChol/TGKC homologues, showing that cationic lipids with fully guanidinylated aminoglycoside headgroups were less efficient than their amino counterparts. Moreover, TGKSucDODA/DOPE liposomes were actually slightly more efficient than TGKC/DOPE liposomes. This is in agreement with the results obtained with the corresponding parent amino vectors (see above). Therefore, aliphatic chain derivatives appear to be more efficient than unspaced cholesterol derivatives, irrespective of the nature of the functional groups (amines or guanidiniums) present in the aminoglycoside-based headgroup.

In vivo gene transfection into mouse airways

We previously reported that KanaChol/DOPE liposomes were efficient for gene transfection into mouse airways in vivo.[25] This prompted us to test

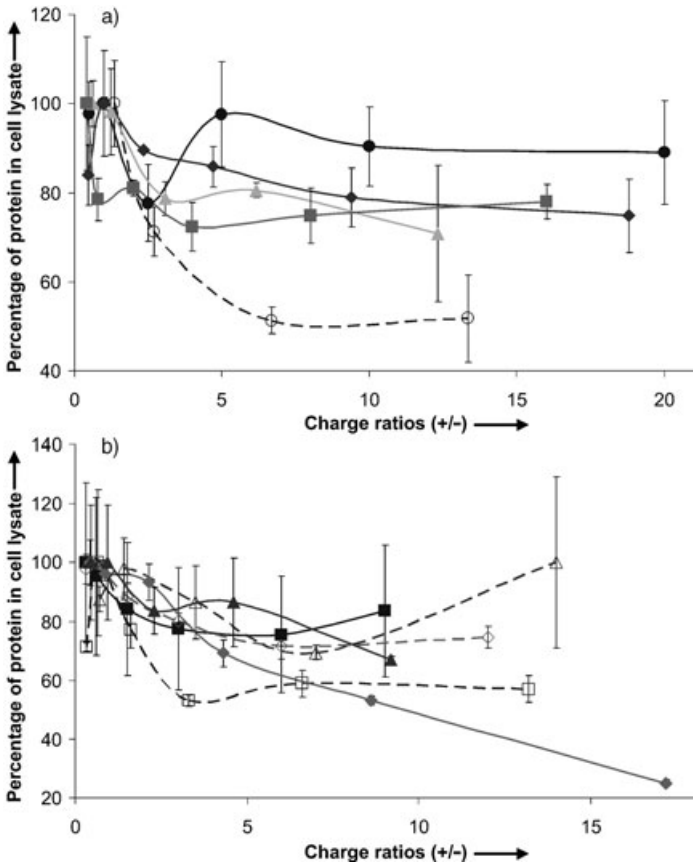


Figure 4. Cytotoxicity of kanamycin A derivatives. a) Cholesterol derivatives of kanamycin A: (○ and -----) = KanaChol, (●) = KanaChol/DOPE, (◆) = KanaCapChol/DOPE, (▲) = KanaLysChol/DOPE, and (■) = KanaDiLysChol/DOPE. b) Aliphatic derivatives of kanamycin A: (□ and -----) = KanaSucDODA, (■) = KanaSucDODA/DOPE, (◇ and dashed line) = TGKSucDODA, (◆) = TGKSucDODA/DOPE, (△ and dashed line) = KanaSucDOLA, and (▲) = KanaSucDOLA/DOPE. HeLa cells were transfected as described in the Experimental Section through the use of plasmid DNA (5 μg) mixed with the amounts of lipids required to form lipoplexes characterised by the indicated charge ratios. At 48 h post-transfection, cells were harvested (for monitoring of luciferase expression) and toxicity was quantified by use of the total amount of cell protein in the cell lysate as an index of cell number, cell death leading to a decrease in extractable protein. Total cell protein in the cell lysate is indicated as a function of the charge ratio of the lipoplexes. Data are expressed as percentages of the concentration of extractable cell protein in the cell lysate in relation to the quantity obtained for untransfected cells (fixed volume, mean ± SD with $n = 3$).

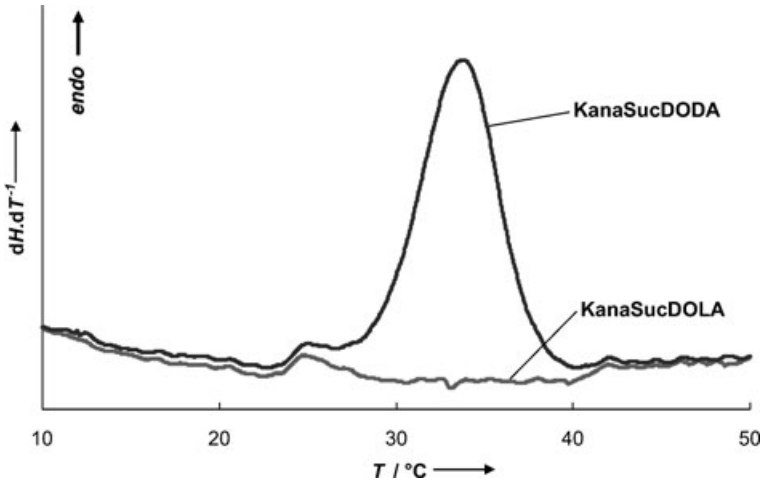


Figure 5. Differential scanning calorimetry of aliphatic derivatives of kanamycin A: KanaSucDODA and KanaSucDOLA upscanned from 0 to 60 °C.

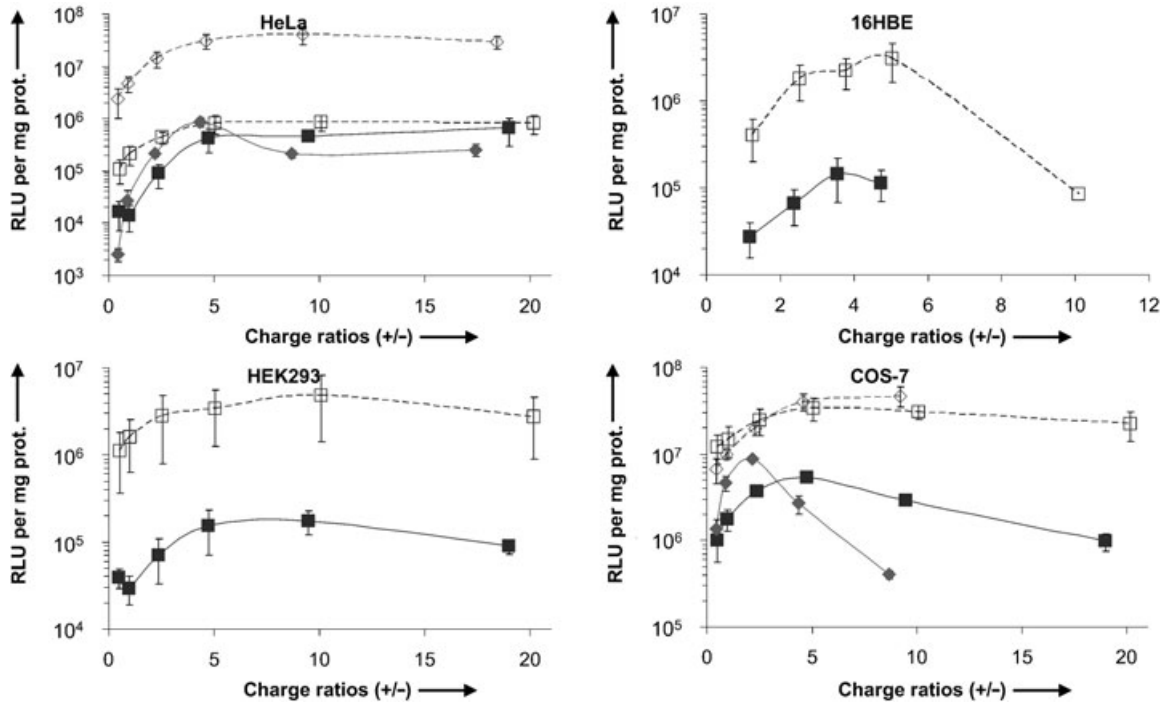


Figure 6. Dose-response curves of the in vitro transfection activities of guanidinylated derivatives of kanamycin A and their amino analogues, formulated as liposomes with DOPE (molar ratio 1:1): (□ and -----) = KanaChol/DOPE, (■) = TGKC/DOPE, (◇ and -----) = KanaSucDODA/DOPE, and (◆) = TGKSucDODA/DOPE. Luciferase reporter gene expression is indicated as a function of the charge ratios of the lipoplexes. Cell lines were transfected as described in the Experimental Section through the use of lipoplexes prepared by mixing luciferase-expressing plasmid DNA (5 μg) with the required amounts of lipid. Data are expressed as relative light units (RLUs) per mg of cell protein (mean ± SD with $n \geq 3$).

some of the new derivatives (under the same conditions) to ascertain their capacity to mediate gene transfection in vivo. Here we chose to use novel cholesterol derivatives, since cationic cholesterol vectors have been reported to be both more stable and more efficient for in vivo transfection of epithelial cells.[5] Furthermore, it has also been reported that cationic lipids incorporating longer spacer units gave better results in vivo, as they may provide enhanced interactions of the lipoplexes with the surface mucosal tissue.[36] We therefore selected the cholesterol derivatives KanaCapChol and KanaLysChol for experiments involving gene transfection into mouse airway epithelium in vivo.

Chol-PEG-stabilised KanaCapChol/DOPE/DNA and KanaLysChol/DOPE/DNA lipoplexes were prepared and administered as described in the Experimental Section. Addition of cholesterol–poly(ethylene glycol) (Chol–PEG) has been shown to allow the preparation of colloidally stable lipoplexes at the high DNA concentrations required for efficient gene transfer to the mouse airways by intranasal instillation.[37–39]

The levels of transgene expression (CAT reporter gene) obtained with each vector are indicated in Table 1. Comparison both with uncomplexed plasmid DNA ("naked DNA") and with BGTC (a vector that has been thoroughly studied[27,28,39,40]) shows that the two vectors were efficient under our conditions. Indeed, both vectors were able to mediate, especially in the lungs, levels of CAT expression of the same order of magnitude as previously obtained on administration of BGTC-based lipoplexes by intranasal instillation. However, the beneficial effects of the longer spacers observed in vitro with KanaCapChol and KanaLysChol were not found in vivo, as only KanaLysChol yielded CAT levels higher than those obtained with KanaChol.[25] Such results are not surprising, since in vitro experiments may not predict transfection outcomes under the widely varying and complex conditions of any in vivo administration.[40–42] Confirmation of relatively subtle differences in the transfection activities of two efficient vectors would therefore require many more in vivo experiments. Accordingly, as the CAT levels measured here were of the same order of magnitude as those obtained with KanaChol, these differences in activity may presumably be related to the experimental variations inherent in the intranasal instillation technique (which is

Table 1. CAT expression in mouse airways after intranasal instillation of Chol–PEG stabilised lipoplexes of KanaCapChol/DOPE and KanaLysChol/DOPE.

Type of vector	CAT expression in trachea [ng per 100 mg protein]	CAT expression in lungs [ng per 100 mg protein]
KanaCapChol/DOPE	0.09 ± 0.10	1.55 ± 0.41
KanaLysChol/DOPE	0.90 ± 1.24	8.34 ± 7.82
"naked" DNA	0.03 ± 0.05	0.15 ± 0.16

Chol-PEG-stabilised KanaCapChol/DOPE/DNA and KanaLysChol/DOPE/DNA lipoplexes (characterised by charge ratios of 3.75 and Chol–PEG/DNA (*w*/*w*) ratios of 2) were used to deliver a total dose of 100 μg of CAT-expressing plasmid DNA. Intranasal instillation was performed, and tracheas and lungs of the treated mice were harvested, processed and assayed for CAT expression as previously described.[39] Data are expressed as ng of CAT protein per 100 mg of total cell protein (mean ± SD with $n = 8$ for KanaCapChol/DOPE and $n = 14$ for KanaLysChol/DOPE).

highly convenient, but the lipoplexes may be lost in the nasal passages as previously reported[43]). Overall, however, these results do confirm that aminoglycoside-based lipids can mediate gene transfer in vivo.

Discussion

The results reported here demonstrate that kanamycin A-based lipids incorporating significant spacer and/or aliphatic lipid moieties can mediate efficient gene transfection. They also confirm that guanidinylation of the amine groups of the kanamycin A headgroup decreases their transfection activity.

The mechanisms underlying this negative effect of the guanidinylation of kanamycin A remain unclear, however. Changing the ammonium groups into guanidinium groups clearly affects the interaction of the headgroup with the DNA. However, the resulting effects can hardly be predicted. Indeed, on the one hand, the guanidinium group presents a more delocalised positive charge than the ammonium group, which might reduce the electrostatic attraction of the vector with anions.[44] On the other hand, both the strong basicities of the guanidinium groups (pK_a around 13.6[45]) and their planar structures (which allow the formation of chelating hydrogen bonds) are likely to reinforce the interactions between the headgroup and the DNA.[27,46] Accordingly, recent studies showed that the binding of guanidinium and ammonium groups to phosphate could be characterised by different thermodynamic profiles.[47,48]

In particular, as concerns aminoglycosides, it is noteworthy that the affinity of their derivatives[49] for the HIV RRE (Rev Response Element) RNA sequence was increased upon guanidinylation (as a result of the semirigid preorganisation of the cationic units). We therefore also performed oligodeoxynucleotide melting experiments with kanamycin A and its tetraguanidinylated analogue[49] as headgroup models. The results indicated that the guanidinylated analogue of kanamycin A induces a stronger stabilisation of a 25-mer duplex than the aminoglycoside (ΔT_m of 13.3 ± 0.7 °C for 10 molar equivalents of the tetraguanidinylated analogue in comparison with 6.3 ± 0.7 °C for kanamycin A under the same conditions). This might be related to the fact that the aminoglycoside, unlike its guanidinylated analogue, is not fully protonated at the pH (7.2) used for the melting study, as suggested by the basicities of the different amino groups of kanamycin A (pK_a: 9.03, 8.16, 7.42 and 6.19[50]).

These results suggest that TGKC and TGKSucDODA might bind to plasmid DNA more tightly than their amino parent compounds. The resulting higher stabilities of the lipoplexes formed by these guanidinylated lipids might therefore be the cause of their decreased transfection activities. It has indeed been shown that, after their uptake into the target cells by endocytosis, efficient decomplexation of the lipoplexes is needed to permit transcription of the DNA.[6,10] Direct microinjection of lipoplexes into the nucleus showed that complexation with the cationic lipid hinders transgene transcription;[10,51] moreover, in a recent study, inhibition of transcription was associated with an increased affinity of polylysine oligomers for DNA.[52] Finally, the strong basicity of the guanidinium group may also affect the buffering capacity of the cationic lipid in the endosome and thereby hinder endosomal escape of the lipoplexes. High buffering capacities in endosomes have indeed been shown to be associated with high transfection activities of several synthetic vectors, including cationic lipids[53] and the cationic polymer polyethylenimine (PEI).[54]

This work also allowed the identification of some critical factors in gene transfection by kanamycin A-based lipids. The substitution of cholesterol by aliphatic chains and the incorporation of substantial spacer subunits resulted in significant increases in in vitro transfection efficiencies, which seem in both cases to be correlated with gains in the fluidities of the lipid layers formed by the vectors. The fluidities of the lipoplex bilayers therefore appear to be critical factors. In contrast, other factors appear to affect the transfection potentials to lesser extents. On comparison of the activities of KanaCapChol, KanaLysChol and KanaLysDiChol, for instance, neither changes of the vector geometry nor of its hydrophilicity/hydrophobicity balance seem to modify its efficiency significantly. The transfection process may thus be affected by the properties of the bioassemblies formed by the lipoplexes, rather than by the individual properties of the vector.

Interestingly, fluidity may play a critical role at various stages of the transfection process. Modifications of the lipidic layers' properties can indeed affect the morphologies of the lipoplexes as well as their interactions with the various cellular components encountered during their uptake by endocytosis. Here, the sizes of the lipoplexes, as evaluated by DLS, seem to be in the same range for all vectors. However, better characterisation of the supramolecular organisation of the lipoplexes would require additional studies, such as electron microscopy studies and X-ray scattering experiments. On the other hand, modifications of the stabilities of the lipoplexes through increased fluidity might affect the protection of the DNA against (chemical or enzymatic) degradation as well as their interactions with the cellular membranes along the endocytosis pathway. More fluid lipid layers may in particular facilitate endosomal escape of the lipoplexes and subsequent DNA release into the cytosol. Indeed, according to a recent model,[55] destabilisation of the endosomal membrane requires formation of neutral ion pairs (between the cationic lipids and the anionic phospholipids of the cytoplasm-facing endosomal monolayer) and diffusion of the anionic lipids into the DNA complexes: two processes enhanced by increased fluidity of the lipoplex lipid layers. It is also noteworthy that aminoglycosides have been reported to destabilise negatively charged membranes;[56,57] the aminoglycoside headgroup is therefore probably also contributing to the efficiency of the vectors. In future studies, it will therefore be interesting to investigate the interaction of liposomes and lipoplexes formed by aminoglycoside-derived cationic lipids with negatively charged model membranes, particularly in order to evaluate their ability to form inverted hexagonal phases, which have been reported to play a key role in endosomal escape of the DNA complexes.[33,58]

In conclusion, this work confirms the transfection activities of cationic lipids characterised by headgroups consisting of

aminoglycosides. It also provides some insight into the structure–activity relationships of this novel and promising class of cationic lipids.

Experimental Section

Synthesis of KanaSucDOLA: All commercially available chemicals were reagent grade and were used without further purification. Analytical TLC was performed with 0.2 mm silica-coated aluminium sheets, and visualisation was achieved by use of UV light or by spraying either with a solution of ninhydrin (0.3% in weight in *n*-butanol containing 3% acetic acid in volume) or with an iodine solution (0.1 M in 10% aqueous sulfuric acid solution). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 300 instrument.

KanaSucDOLA was obtained from the coupling of *N*-succinyl-dioleylamine and a previously described intermediate of kanamycin A protected on all the amino functions except 6′-*N*.[26] Briefly, oleylamine was acylated with oleyl chloride and the resulting amide was reduced with $LiAlH_4$ in order to obtain dioleylamine.[59] Further acylation of the dioleylamine with succinic anhydride yielded *N*-succinyl-dioleylamine. The resulting acid was coupled to {1,3,3′′-tris-*N*-(trimethylsilyl-ethoxycarbonyl)}kanamycin A[26] in the presence of EDC and HOAt, and a final deprotection step with trifluoroacetic acid yielded the desired compound, KanaSucDOLA (**7**). R_f = 0.32 (CH_2Cl_2/MeOH/NH_4OH 5:4:1). ^{1}H NMR (300 MHz, CD_3OD/$CDCl_3$, 25 °C): δ = 5.32 (m, 4H; H-9 and H-10 from oleyl chains), 5.22 (d app, J = 3.3 Hz, 1H; anomeric proton), 5.05 (d app, J = 3.3 Hz, 1H; anomeric proton), 3.96–3.38 (m, 17H), 3.33–3.24 (m, 4H), 3.15 (t, J = 9.3 Hz, 1H), 2.64–2.50 (m, 5H), 2.00 (m, 8H; H-8 and H-11 from oleyl chains), 1.58–1.46 (m, 5H), 1.30–1.24 (m, 44H), 0.86 ppm (t app, J = 6.7 Hz, 6H; $-CH_3$ from oleyl chains); ^{13}C NMR (75 MHz, CD_3OD/$CDCl_3$, 25 °C): δ = 174.11, 172.10, 129.80, 129.76, 129.58, 129.56, 100.38, 98.73, 73.55, 72.78, 72.09, 71.91, 71.11, 68.63, 66.02, 60.52, 55.59, 49.96, 46.37, 40.20, 33.51, 32.41, 31.76, 30.42, 29.59, 29.56, 29.53, 29.49, 29.42, 29.35, 29.25, 29.16, 29.12, 29.08, 29.03, 28.97, 28.61, 27.93, 27.60, 27.51, 27.00, 26.90, 26.77, 24.84, 22.49, 13.64 ppm; MS: *m/z* MALDI-TOF $[M+Na]^+$: 1106.8.

Preparation of cationic lipid formulations: For preparation of cationic liposomes composed of lipid (aliphatic or cholesterol) derivatives of kanamycin A and the neutral colipid dioleoylphosphatidylethanolamine (DOPE), a mixture of cationic lipid and DOPE (molar ratio 1:1) in $CHCl_3$ was evaporated under vacuum and resuspended in a Hepes buffer solution (20 mM, pH 7.4). The final total lipid concentration was 5 mg mL^{-1}. The same method was used for the preparation of liposomal formulations of aliphatic derivatives without DOPE, the final cationic lipid concentration in this case being 5 mg mL^{-1}. The mixture was sonicated for 10 min with a sonicator probe (sonifier cell disruptor B-30 terminal equipped with a Branson sonifier 450) to form liposomes. The resulting solution was allowed to cool to room temperature before filtration through a 0.22 μ filter (Millex GS, Millipore).

Plasmids: The plasmid pCMV-Luc used for in vitro transfection experiments has been described previously.[60] Plasmid pCIK-CAT, which was used for the in vivo studies, was obtained from D. Gill (Oxford, UK). Briefly, pCIK-CAT was constructed by subcloning the *Escherichia coli* chloramphenicol acetyltransferase (CAT) gene (equipped with a Kozak translation sequence) into a pCI backbone (Promega). Both plasmids were amplified in *E. coli* and were purified by standard techniques (QIAGEN EndoFree plasmid mega kit).

Preparation of lipoplexes: For in vitro transfection experiments, lipoplexes were prepared as described previously.[27,60] Preparation of colloidally stable lipoplexes for in vivo gene transfection into mouse airways (by intranasal instillation) has also been described previously.[39] Schematically, stable kanamycin A derivative/DOPE/DNA lipoplexes were obtained in hypotonic medium by addition of the steric stabiliser Chol-PEG (kindly provided by C. Masson, Paris, France, and in which a PEG chain of approximately 100 oxyethylene units is linked to an hydrophobic anchor composed of a cholesterol molecule), to the kanamycin A derivative/DOPE liposomes (in 20 mM Hepes buffer) immediately prior to mixing with pCIK-CAT DNA in water.

Dynamic light scattering: The size distributions of liposomes and lipoplexes were evaluated by dynamic light scattering (at an angle of 90°) by use of a laser light scattering apparatus (Autosizer 4700; Malvern Instruments, Orsay, France). Lipoplex samples were prepared at DNA concentrations of 10 μg mL^{-1} and at charge ratios of 6 (+/−). Mean particle diameters were determined by multimodal fit analysis.

Surface pressure/area isotherms: All the surface pressure/area isotherms were measured by use of a Langmuir–Wilhelmy film balance (NIMA 611 A) equipped with a temperature controller set at 20 °C. Lipid solutions were prepared in a chloroform/methanol mixture (95:5) at a concentration of 1 mg mL^{-1}. By use of a microsyringe, drops of lipid solutions were spread on tridistilled water contained in a trough made of Teflon. After total evaporation of the organic solvent (approximately 10 min), the surface layer was compressed by use of two symmetrical barriers to determine the isotherms, the surface pressure being measured by the Wilhelmy method.[29]

Differential scanning calorimetry (DSC): Liposomes studied by DSC were prepared by a slightly different method from that used for the transfection studies. Chloroform solutions of the aliphatic derivatives of kanamycin A were evaporated under vacuum and resuspended in Hepes buffer solutions (20 mM, pH 7.4) in order to obtain final lipid concentrations of 7 mM. The mixtures were heated at 60 °C and were then vortexed for 10 min. The heating and vortexing process was repeated three times in order to obtain multilamellar vesicles, which were used for DSC measurements. Thermograms were measured on a DSC-7 scanning calorimeter (Perkin–Elmer); samples were prepared with the liposome solutions (50 μL) and were scanned at a rate of 5 °C min^{-1}.

Thermal denaturation experiments: The oligodeoxynucleotides were purchased from Eurogentec (PAGE purified). They consist of a 25-mer duplex derived from a recognition sequence of the methyl transferase M.*Taq*I (5′-TGAGATCCAGTTCGTAGTAACCCAC and 5′-GTGGGTTACTACGAACTGGATCTCA).[61,62] Thermal denaturation measurements were performed with a Uvikon XL spectrophotometer. The temperatures of the two six-cell holders were regulated by an electrical thermosystem and controlled through a temperature sensor immersed in a reference cell containing appropriate buffer. The samples were studied between 18 and 90 °C with a temperature change rate of 0.2 °C min^{-1}. T_m values were obtained by calculating the first derivatives of the melting curves. The melting profiles were monitored at 260 nm and subtracted from the absorbance at 500 nm, which was used as internal base line. ΔT_m corresponds to the difference between the T_m values obtained in the presence and in the absence of a ligand. To form the duplex, equimolar amounts of the complementary oligodeoxynucleotide strands (1 μM) in HEPES buffer (20 mM, pH 7.2) containing sodium chloride (10 mM) were incubated at 90 °C for 5 min; the duplex

was then slowly cooled to room temperature and stored at 4 °C for 12 h. Kanamycin A (sulfate) or its tetraguanidinylated analogue (trifluoroacetate)[49] were added to the duplex at different molar ratios (1.5, 5 and 10), and the solutions were allowed to equilibrate at 4 °C for 3 h before measurements were made.

Cell and culture conditions: The in vitro transfection activities of the different reagents were evaluated in transient transfection experiments with a variety of mammalian cell lines. The cell lines tested were as follows: the HeLa cell line (derived from a human epithelioid cervical carcinoma), COS-7 cells (produced by Simian Virus-40 transformation of monkey kidney cells), HEK293 cells (which are adenovirus-transformed human embryo kidney cells), the 16HBE cell line (kindly provided by D. Gruenert, University of Vermont, Burlington, VT, USA, and of human bronchial epithelium origin[63]) and 3T3 cells (which are mouse fibroblasts).

All cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum and antibiotics, and routinely maintained at 37 °C in a humidified 5% CO_2/95% air-containing atmosphere.

In vitro transfection, luciferase assay and determination of cytotoxicity: In vitro transfection experiments were performed as previously described,[27,60] except that the transfection medium was not replaced with fresh culture medium until harvesting of the cells (at 48 h post-transfection) for monitoring of luciferase activity. The luciferase assay has also been previously described.[27] Data for luciferase activity were expressed as relative light units (RLUs) per mg of cell protein, the protein concentration being determined by use of the Bio-Rad protein assay. Cellular toxicities of the lipid formulations were quantified by use of the total amount of cell protein in the cell lysate (per well) as an index of the cell number; cytotoxicity data are expressed as the percentage of the concentration of extractable cell protein in the cell lysate (of fixed volume) from transfected wells in comparison to untransfected cells.

In vivo gene delivery to mouse airways: Female BALB/c mice (30 g body weight) were purchased from Charles River (Saint-Aubin-Les-Elbeuf, France). Intranasal administration of the lipoplexes was conducted as previously described.[39] Schematically, the mice were briefly anaesthetised with halothane (Belamont, Paris, France) and instilled intranasally with Chol–PEG-stabilised lipoplexes (50 μL) characterised by a charge ratio of 3.75 and a Chol–PEG/DNA (*w*/*w*) ratio of 2; each animal received three doses (about 4 h apart), a total amount of 100 μg of pCIK-CAT being administered. All animal procedures were performed in compliance with current French legislation

CAT expression in vivo: At 48 h after instillation, the animals were killed by i.p. overdoses of pentobarbital and the lungs and tracheas were removed for analysis. CAT expression in vivo was evaluated as previously described.[39] Briefly, tissue pieces were placed in TEN buffer (40 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, pH 7.8) and disrupted on ice for about 30 s by use of an Ultra-Turrax T25 homogeniser (Fischer Bioblock Scientific, Strasbourg, France). Cells were lysed by three freeze-thaw cycles and the supernatant was obtained by centrifugation. CAT concentration was determined by a CAT ELISA assay performed according to the manufacturer's instructions (Boehringer Mannheim). CAT levels were expressed as ng of CAT protein per 100 mg of total protein, the protein concentration being determined by the Bio-Rad assay.

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