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Synthesis and Transfection Activity of New Cationic Phosphoramidate Lipids: High Efficiency of an Imidazolium Derivative

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*In an effort to enhance the gene-transfer efficiencies of cationic lipids and to decrease their toxicities, a series of new phosphoramidate lipids with chemical similarity to cell membrane phospholipids was synthesised. These lipids contained various cationic headgroups, such as arginine methyl ester, lysine methyl ester, homoarginine methyl ester, ethylenediamine, diaminopropane, guanidinium and imidazolium. Their transfection abilities, either alone or with the co-lipid DOPE, were evaluated in HEK293–T7 cells. We found that imidazolium lipophosphoramidate **7a**/DOPE lipoplexes gave the most efficient transfection with low toxicity (15%). The luciferase activity was 100 times higher than that obtained with DOTAP/DOPE lipoplexes. The size, ζ potential, pDNA–liposome interactions and cellular uptakes of the lipoplexes were determined. No definitive correlation between the ζ potential values and the transfection efficiencies could be established, but the uptake of lipoplexes by the cells was correlated with their final transfection efficiencies. Our results show that imidazolium phosphoramidate lipids constitute a potential new class of cationic lipids for gene transfer.*

Introduction

The challenge of finding efficient and safe carriers for the introduction of plasmid DNA (pDNA) encoding therapeutic genes into cells invites chemists and biologists to investigate the design of synthetic vectors. These vectors can be classified into two categories: polymeric vectors (polyethyleneimine, polylysine…) and monomolecular vectors (mainly cationic lipids). Cationic lipids generally consist of three parts: a lipid moiety, a cationic head and a spacer between these two parts. Molecular variations of these three constitutive parts have been widely investigated, and several reviews have compiled the different molecular structures of the cationic lipids.[1–5] The presence of two saturated or unsaturated aliphatic chains, as mimics of the membrane phospholipids, is one of their common structural features. The polar head is generally formed by a cationic function (ammonium, phosphonium, guanidinium, …) allowing interaction with the negatively charged phosphate group of pDNA and the production of a supramolecular complex known as lipoplex. These complexes are then able to enter into the cells by an adsorptive endocytosis mechanism and to deliver pDNA into the cytosol after destabilisation of the endosome membrane.[6,7]

A second crucial factor associated with the use of cationic lipids is their toxicity. In most cases, a compromise between transfection efficiency and toxicity is necessary. To decrease toxicity, the addition of a neutral co-lipid in the formulation is a widely used strategy. DOPE (dioleoylphosphatidylethanolamine) is the most common neutral co-lipid (zwitterionic phospholipid) associated with a large variety of cationic lipids. Its incorporation into the lipoplex decreases the concentration of cationic lipid and promotes fusion with the endosome membrane to facilitate the pDNA delivery into the cytosol.[8] Nevertheless, it is worth noting that, with certain cationic lipids, the addition of DOPE decreases the in vivo transfection efficiency, possibly by a mechanism involving lipoplex instability in the presence of lipoproteins.[9]

We have developed a first generation of cationic lipids characterised by a phosphonate function carrying the two aliphatic chains. Variations of the cationic headgroup have been evaluated, and factors influencing the transfection efficiencies of such vectors have been reported.[10] Surprisingly, the most efficient vector had an arsonium headgroup.[11,12] A second generation of lipids containing phosphoramidate functional groups rather than phosphonate ones was next designed. These cationic phosphoramidate lipids proved to be efficient in in vitro and in vivo transfection experiments.[13,14,15] In this series of vectors, one of the most efficient (in term of high transfection efficiency and low toxicity) was KLN47 (Scheme 1). These promising results encouraged us to investigate some new structural modifications of phosphoramidate lipids and to eval-

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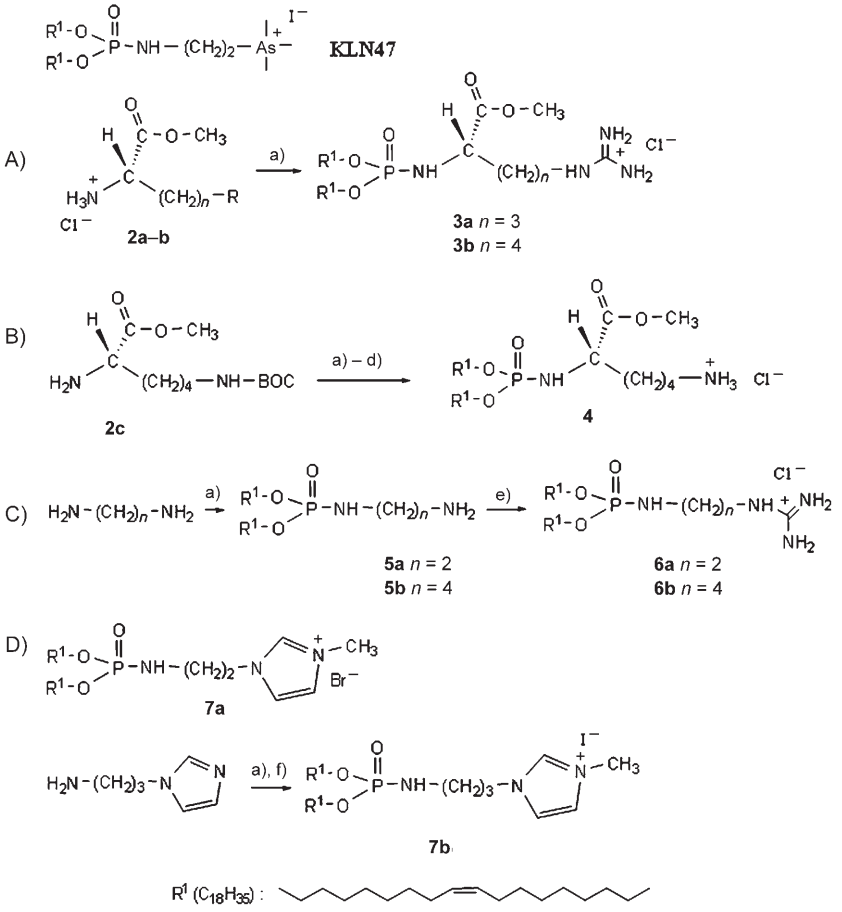

Scheme 1. Structures of the lipophosphoramidate derivatives and synthesis conditions: a) dioleylphosphite **1**, $CBrCl_3$ or CCl_4, DIPEA, MeOH or CH_2Cl_2 or $CHCl_3$, 0 °C to 20 °C; b) CF_3COOH, CH_2Cl_2, 2 h; c) K_2CO_3, NEt_3, CH_2Cl_2, d) HCl, Et_2O; e) 1*H*-pyrazole-1-carboxamidine.monohydrochloride, DIPEA, ethanol, 78 °C; f) CH_3I, excess, 20 °C, 16 h.

uate their influences on transfection efficiency. We focused our attention on the modification of the cationic headgroup while the phosphoramidate moiety including the aliphatic chains remained unchanged. The choice of $C_{18:1}$ aliphatic chains (oleic chains) linked to the phosphoramidate moiety was based on our previous works showing that vectors possessing aliphatic chains of this type were more efficient than those with shorter or saturated chains.

A series of new cationic phosphoramidate lipids was therefore synthesised, and their in vitro transfection activities (HEK293–T7 cells) were tested in formulations either alone or with the co-lipid DOPE. Plasmid-encoded luciferase or EGFP transgene expression were used as readouts to measure the transfection efficiencies. The physical properties of liposomes and lipoplexes were assessed by dynamic light scattering, ζ potential, dye exclusion and gel electrophoresis. The lipoplexes' toxicities were evaluated by cell viability with the aid of the colorometric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Moreover, the binding and the uptake of fluorescein-labelled pDNA complexed with a series of lipophosphoramide/DOPE liposomes were assessed by flow cytometry.

Results and Discussion

Synthesis of α-amino acid ester phosphoramidate lipids

With a view to obtaining cationic lipids with low cytotoxicities, we first investigated the use of natural amino acid esters as cationic polar heads. The chemical structures of the selected amino acid esters were dictated by the presence of a basic group in the side chain, to guarantee its protonation at the physiological pH of 7.4 (Scheme 1). This approach has already been explored, by Springer and co-workers, with cationic lipids possessing structures based on an amino glycerol backbone.[16] These lipids were each characterised by two lipid ether linkages and a polar head formed by an amino acid group attached through an amide bond. The main differences between our vectors and those reported by Springer are the natures of the links between the amino acid groups and lipid chains and the presence of phosphoramidate linkages. These phosphoramidate linkages allow the design of vectors with different shapes and efficacies. Among the available natural α-amino acid esters, we chose the methyl esters of arginine and lysine, which are each characterised by the presence of a basic functional group in the side chain (with pK_a values of 12.48 and 10.54 for arginine and lysine, respectively). Our study also included homoarginine methyl ester, because of its similarity with arginine (pK_a of the guanidinium group ~12.5). The attachment of the phosphoramidate groups on these α-amino acid esters was performed by a methodology based on the Todd–Atherton reaction.[17] This method uses a dialkylphosphite **1** carrying two unsaturated lipophilic chains, the amino acid methyl ester **2a** or **2b** as a hydrochlorate salt, bromotrichloromethane and diisopropylethylamine (DIPEA) acting as a base to generate the free α-amino acid esters in situ and to trap the produced HCl (Scheme 1 A). Similar conditions have been used in the past for the synthesis of amino acids functionalised with a diisopropylphosphoramidate function without any racemisation.[18,19] To avoid side reactions between the acidic function of the amino acid and the phosphite or phosphoramidate, these amino acids had to be protected as their ester forms. Indeed, attempts to engage the free carboxylic acid function in this reaction triggered side reactions characterised by the loss of some amount of lipophilic

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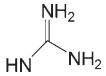
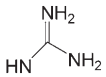
Table 1. Synthesis of lipophosphoramidate-amino acid esters **3a**, **3b** and **4**.

Substrate	Product number	R	*n*	DIPEA	Solvent	Product name	Yield % (mass g)[a]
2a (arginine methyl ester)	**3a**		3	3 equiv	MeOH	Arg	31 (1.26)
2b (homoarginine methyl ester)	**3b**		4	3 equiv	MeOH	H-Arg	30 (1.17)
2c (N-BOC lysine methyl ester)	**4**	$-NH_2$	4	2 equiv	$CHCl_3$	Lys	90 (3.50)

[a] Pure compounds.

chains, leading to complex mixtures. The postulated mechanism could involve a cyclisation via the carboxylate intermediate as a nucleophilic reagent and the phosphorus atom of the phosphoramidate functional group acting as the electrophile. This type of intramolecular cyclisation involving a phosphorus function has already been reported.[20,21]

By our synthesis strategy, new amphiphiles derived from arginine (Arg, **3a**) and homo-arginine (H-Arg, **3b**) were isolated in moderate yields (Scheme 1A and Table 1). This was probably related to side reactions between the solvent (methanol) and the bromodialkylphosphate generated in situ from dialkylphosphite under the Todd–Atherton coupling conditions. We investigated replacement of methanol with a less nucleophilic solvent, but in all cases difficulties associated with the solubilities of the precursors **2a** and **2b** ruled out these attempts. In spite of the moderate yields, these syntheses were achieved at a scale allowing isolation of 1 to 1.2 g of pure product in one batch, and further scaling up was also possible. To apply this methodology to lysine, protection of the amine function located on the side chain of the amino acid was required. The use of *N*-BOC-lysine (**2c**) was suitable for the Todd–Atherton coupling, providing (after deprotection with triflic acid and anion exchange with HCl) compound **4** (Lys; Scheme 1B and Table 1). The excellent yield (90%) might be explained by it having been possible in this case to perform the Todd–Atherton coupling in chloroform, which subsequently allowed the formation of side products to be avoided.

Synthesis of ω-amino- and ω-guanidinium alkylphosphoramidate lipids

The carboxylic ester group present in lipids **3a**, **3b** and **4** may have several effects on the vectorisation process. First of all, it might act as a hydrogen bond acceptor and might therefore play a role in the cohesion of the lipoplex. This carboxylic ester function could also be hydrolysed by chemical or biochemical processes and produces free carboxylic acid inside biological media. This could induce a strong structural modification of the lipoplex because one lipophilic chain could be lost. This reaction was observed when the synthesis of lipids was performed with amino acids containing an unprotected carboxylic acid function. Furthermore, such hydrolysis would transform the lipid from a cationic into a zwitterionic structure that could help the pDNA escape into the cytosol. In order to evaluate the influence of the carboxylic ester group on the transfection efficiency, we synthesised four other phosphoramidate lipids exhibiting some structural similarities with the previous ones but without the carboxylate ester function. Firstly, we attempted the synthesis of compound **6b** in one step starting from agmatine sulfate (4-aminobutyl-guanidine sulfate) under Todd–Atherton coupling conditions. Unfortunately, the solubility of that starting compound was very low in organic solvents, including methanol, so we synthesised two ω-aminophosphoramidates in the presence of large excesses of ethylenediamine and putrescine to avoid the formation of a diphosphoramidate (Scheme 1C). Next, the resulting compounds **5a** (EDA) and **5b** (BDA) were each treated with a guanidylation reactant (1*H*-pyrazole-1-carboxamidine monohydrochloride) in ethanol (Scheme 1C) to produce compounds **6a** (E-guanidino) and **6b** (B-guanidino) in 70% yields.

Synthesis of imidazolium alkylphosphoramidate lipids

The molecular diversity of our phosphoramidate was further increased through incorporation of an imidazole heterocyclic ring. Taking account of the fact that the imidazole ring present on the histidine side chain has a pK_a of 6.04, so that ~4% of the molecules are protonated at physiological pH, we decided to introduce an imidazolium group to guarantee a permanent cationic charge. The cationic phosphoramidate **7a** (imidazo) was synthesised as already reported,[22] while compound **7b** was prepared in two steps starting with treatment of the 3-aminopropylimidazole with dialkylphosphite under Todd–Atherton conditions, followed by imidazole quaternarisation in the presence of methyl iodide (Scheme 1D). It is worth noting that the structures of these imidazolium phosphoramidate lipids were very different from that of DOTIM, a 1,3-dialkylated imidazolinium derivative developed by Solodin et al.[23]

Transfection efficiency

The ability of each phosphoramide lipid to transfect mammalian cells was examined with HEK293–T7 cells and pCMVLuc, a plasmid encoding the luciferase gene. The plasmid DNA was complexed with liposomes formed with a phosphoramidate lipid either without DOPE (DOPE-free liposomes) or with one molar equivalent of DOPE (DOPE liposomes). DOTAP was used as a gold-standard transfection reagent. Surprisingly, we were

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not able to prepare liposomes with the lipophosphorimidate **7b** either in the absence or in the presence of DOPE. The transfection efficiency was determined by measuring the luciferase activity in cell lysates. Figure 1 summarises the transfection efficiency obtained with 2.5 μg pDNA at the optimal lipid/DNA charge ratio, which corresponds to the ratio between the number of positive charges of the liposomes and the number of negative charges of the pDNA phosphate, under the assumption that at pH 7.4 all the lipid headgroups were positive and all the pDNA phosphate functions were negative. Experiments conducted with lipoplexes made with DOPE-free liposomes revealed that the transfection efficiency was in the same range whatever the cationic lipid used, with the luciferase activity ranging from 10^7 to 5×10^7 RLU per mg protein. With DOPE-containing liposomes, the efficiency depended on the type of the cationic lipid used, within a ranking in the following order: imidazo (**7a**) ⩾ E-guanidino (**6a**) > DOTAP ~ Lys (**4**) ~ EDA (**5a**) > BDA (**5b**) ~ KLN47 > Arg (**3a**) > H-Arg (**3b**) > B-guanidino (**6b**). Thus, we first found that the transfection efficiency was dramatically improved by the insertion of an imidazolium headgroup (100-fold in relation to KLN47). Next, the highest luciferase activity with imidazo- (**7a**) and E-guanidino-lipoplexes (**6a**) was obtained at a lipid/DNA charge ratio of 2 whereas with the other types of lipoplexes it was obtained at a lipid/DNA charge ratio of 4. The imidazo-lipoplexes (**7a**) gave the highest luciferase activity (10^9 RLU per mg protein), ten and ~100 times higher than those of the E-guanidino- (**6a**) and DOTAP-lipoplexes, respectively. With reference to the liposomes developed by Springer and co-workers[16] composed of diether lipids with structures based on an amino-glycerol backbone and cholesterol as the colipid, we also found that the transfection efficiencies of Lys-lipoplexes (**4**) were superior to those of Arg-lipoplexes (**3a**). The efficacies of the imidazo-lipoplexes (**7a**) were 100 times higher when DOPE was present. In contrast, no great benefit was observed on addition of DOPE to the other cationic lipids, although it is widely accepted that inclusion of DOPE promotes hexagonal organisation, favouring membrane destabilisation.[24,25] Here, one may suppose that a lamellar/hexagonal transition phase might be favoured in imidazo (**7a**)/DOPE-liposomes. A higher transfection efficiency of imidazo-lipoplexes (**7a**) than of DOTAP/DOPE lipoplexes was also observed with HeLa cells. The luciferase activities were 8.6×10^6 and 10^5 RLU per mg protein with imidazo-lipoplexes (**7a**) and DOTAP/DOPE-lipoplexes, respectively.

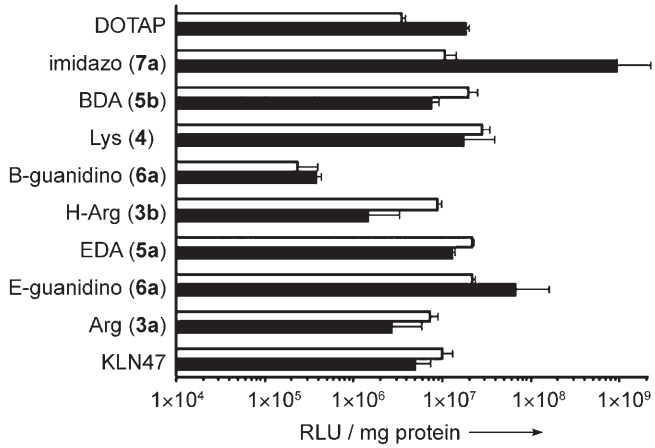

Figure 1. Transfection of HEK293–T7 cells with lipoplexes. Cells were transfected with pCMVLuc (2.5 μg) in complexation with various phosphoramidate liposomes made either with (■) DOPE (1:1, M/M) or without (□) DOPE. The luciferase activity expressed as Relative Light Units (RLUs) per mg protein was measured after 48 h of culture. The values shown are averages of three independent experiments. The lipid/DNA charge ratio was 2 for compounds **7a** and **6a** and 4 for the other compounds.

To ascertain the numbers of transfected cells, transfection was also performed with pCMV-EGFP, a plasmid DNA encoding the green fluorescent protein. As shown in Figure 2, 13, 12, 7 and 3% of the cells expressed EGFP upon transfection with imidazo (**7a**)/DOPE-, DOTAP/DOPE-, E-guanidino (**6a**)/DOPE- and KLN47/DOPE-lipoplexes, respectively. This percentage with the other lipoplexes was lower than 1%. Although imidazo (**7a**)/DOPE- and DOTAP/DOPE-lipoplexes yielded very similar percentages of transfected cells, the amount of EGFP produced inside the cells (which was proportional to the mean of the fluorescence intensity, MFI) was actually higher in cells transfected with the former than with the latter. These results are in agreement with the higher luciferase activity observed with imidazo (**7a**)/DOPE-lipoplexes than with the DOTAP/DOPE ones (Figure 1)

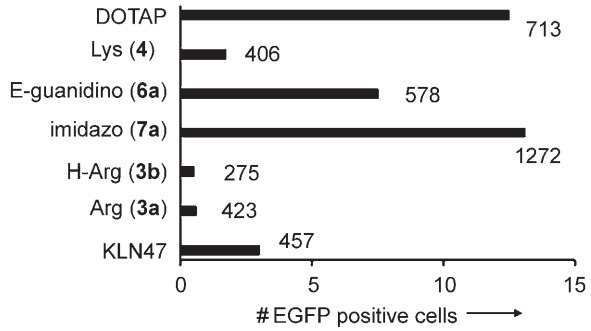

Figure 2. Ascertainment of the number of transfected HEK293–T7 cells. Cells were transfected with various cationic lipid/DOPE-liposomes in complexation with pCMV-EGFP (2.5 μg). After a culture time of 48 h, the number of cells expressing EGFP was measured by flow cytometry (λ_{ex} = 488 nm; λ_{em} = 520 nm). Each number indicates the mean fluorescence intensity (MFI) of the cells in arbitrary units. The values shown are each averages of three independent experiments. The lipid/DNA charge ratio was 2 for compounds **7a** and **6a** and 4 for the other compounds.

Interestingly, imidazo (**7a**)/DOPE-lipoplexes induced low toxicity (15%), the cell viability being comparable to those observed for the other lipoplexes (Figure 3). In contrast, lipoplexes containing the guanidinium phosphoramidate lipid (**6a**) displayed high cytotoxicity (62%, not shown). It should be stressed that a cholesterol derivative with a headgroup bearing two guanidinium groups,[26] as well as a non-cholesterol-based cationic amphiphile bearing a single guanidinium group,[27] have been described previously. A cell viability of >75% was claimed when CHO cells were transfected with the latter in as-

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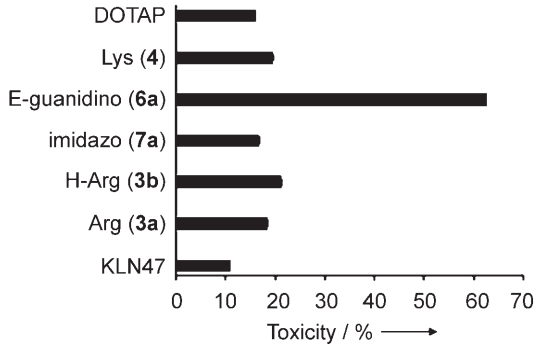

Figure 3. Cell viability after transfection. Colorimetric MTT assay-based percentage cell viabilities after transfection with the various lipoplexes. The lipid/DNA charge ratio was 2 for compounds **7a** and **6a** and 4 for the other compounds.

sociation with cholesterol as colipid at a positive charge ratio of 3, but high toxicity was in fact observed at higher charge ratios.

Physical parameters of liposomes and lipoplexes

Dynamic light scattering measurements of DOPE-free liposomes indicated that their diameters were between 130 and 240 nm (Table 2). In the presence of one molar equivalent DOPE, H-Arg- (**3b**) and Lys-liposomes (**4**) were bigger in size (+30% and +50%, respectively) than their DOPE-free counterparts. Slight decreases in size, by 16% and 13%, were noted for the BDA- (**5b**) and B-guanidino-liposomes (**6b**), respectively. There were no significant changes for the other cationic lipid/DOPE-liposomes. The size of DOTAP-liposomes, which was very small (35 nm) in the absence of DOPE, increased about sixfold in the presence of DOPE and was similar to those of the lipophosphoramidate/DOPE-liposomes. Phosphoramidate liposomes were positively charged, with ζ potential values ranging from 24 to 76 mV. The global charges of the Arg- (**3a**), H-Arg- (**3b**), Lys- (**4**), B-guanidino- (**6b**) and imidazo-liposomes (**7a**) were reduced by 15% to 20% in the presence of DOPE, while that of the EDA-liposomes (**5a**) was lowered by 38% (Table 2). Comparatively, DOTAP liposomes were less positively charged.

Usually when lipoplexes are formed at high positive charge ratios, both lipids and pDNA undergo topological transformation into compact quasi-spherical particles, in which the complexes have an ordered multilamellar structure. In these, the sizes of the phosphoramidate lipoplexes (formed at their lipid/DNA charge ratio optimal for transfection) were bigger (20% to 67%) than those of the corresponding liposomes (Table 2). In contrast, the sizes of the imidazo- (**7a**) and B-guanidino-lipoplexes (**6b**) did not increase. With regard to the global surface charge, the ζ potential values of KLN47-, Arg- (**3a**), H-Arg- (**3b**), Lys- (**4**), B-guanidino- (**6b**) and DOTAP-lipoplexes were highly positive and close to those of their corresponding DNA-free liposomes (Table 2). This suggests that the plasmid DNA was surrounded by sufficient cationic lipids to neutralise the negative charge of the DNA completely and to provide a complex with a net positive charge. In contrast, E-guanidino (**6a**)/DOPE-, imidazo- (**7a**), EDA- (**5a**) and BDA-lipoplexes (**5b**) were negative, suggesting either 1) that the liposomes did not completely surround the plasmid, or 2) that the pDNA and cationic lipids were organised in a different way within those lipoplexes, thus providing complex with net negative charges. From the transfection efficiency data (Figure 1), it is interesting to notice that imidazo- (**7a**) and E-guanidino-lipoplexes (**6a**), which gave the highest transfection efficiencies, had negative ζ potentials. This indeed contrasts with the fact that efficient transfection is usually correlated with a high association of positively charged lipoplexes with negatively charged residues on the cell surface.

Liposome–DNA interactions

SYBR Green I was used to investigate DNA–liposome interactions. This nonfluorescent molecule becomes fluorescent once intercalated between the base pairs of plasmid DNA. Conversely, the fluorescence intensity drops, due to dye exclusion, when DNA condensation occurs in the presence of cationic liposomes. As shown in Figure 4, the dye exclusion depended on the liposome types. The fluorescence intensities decreased

Table 2. Sizes and ζ potentials of liposomes and lipoplexes.

	Size [nm]				ζ [mV]			
	Liposomes		Lipoplexes		Liposomes		Lipoplexes	
	−DOPE[a]	+DOPE[a]	−DOPE[a]	+DOPE[a]	−DOPE[a]	+DOPE[a]	−DOPE[a]	+DOPE[a]
KLN47	210±4	214±4	229±23	255±18	62±2	61±2	60±1	65±1
Arg (**3a**)	165±21	169±22	218±29	203±42	76±6	65±2	64±11	62±6
H-Arg (**3b**)	143±20	211±3	239±31	220±23	70±2	53±3	75±2	50±3
Lys (**4**)	129±37	218±6	170±32	240±28	69±5	56±1	63±4	53±1
E-guanidino (**6a**)	177±22	189±12	231±7	226±51	63±4	64±1	21±5	−17±2
imidazo (**7a**)	244±56	269±29	236±20	231±41	69±2	56±1	−1.2±0.2	−10±4
EDA (**5a**)	212±26	202±18	250±23	223±12	39±4	24±2	−26±2	−18±4
BDA (**5b**)	205±10	171±20	239±26	228±18	40±3	37±6	−9±2	−14±4
B-guanidino (**6b**)	193±12	168±40	219±8	186±14	71±2	62±6	42±6	39±2
DOTAP	35±3	200±25	77±23	125±10	20±4	38±2	31±1	37±1
			243±20	293±10				

[a] With DOPE, the lipid/DOPE molar ratio was 1:1. Lipoplexes were formed at the lipid/DNA charge ratio which gave the highest transfection efficiency.

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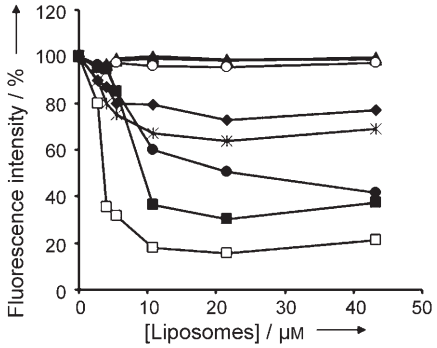

Figure 4. DNA–liposome interactions: pDNA (1 μg) in the presence of SYBR Green I was added to solutions containing various amount of liposomes (cationic lipid/DOPE molar ratio of 1:1). After 30 min at 20 °C, the fluorescence intensities were measured (λ_{exc} = 488 nm; λ_{em} = 520 nm) and the fractions of dye exclusion were calculated from the fluorescence intensities observed in the absence and in the presence of liposomes. Arg (**3a**) (△); H-Arg (**3b**) (▲); imidazo (**7a**) (●); E-guanidino (**6a**) (□); Lys (**4**) (○); DOTAP (■); EDA (**5a**) (◆); BDA (**5b**) (*).

drastically when the amounts of E-guanidino (**6a**)/DOPE- and DOTAP/DOPE-liposomes increased, indicating that they interacted strongly with the plasmid DNA. Indeed, 50% of the dye was excluded when 1 μM DNA was added to 5 μM and 10 μM (corresponding to lipid/DNA charge ratios of 0.8 and 1.7, respectively) of E-guanidino (**6a**)/DOPE- and DOTAP/DOPE-liposomes, respectively. Half of the dye was excluded with 20 μM imidazo (**7a**)/DOPE-liposomes, indicating that these liposomes interacted significantly less with the plasmid DNA. BDA (**5b**)/DOPE- and EDA (**5a**)/DOPE-liposomes led to 35% and 30% dye exclusion at 30 μM. In conclusion, the strengths of the liposome–DNA interactions were in the following order: E-guanidino (**6a**) > DOTAP > imidazo (**7a**) > BDA (**5a**) ~ EDA (**5b**). In contrast, no significant dye exclusion was observed in the presence of Arg (**3a**)/DOPE-, H-Arg (**3b**)/DOPE- and Lys (**4**)/DOPE-liposomes, suggesting the absence of tight complexes with pDNA.

The gel retardation experiments confirmed that E-guanidino (**6a**)/DOPE- and DOTAP/DOPE-liposomes formed strong lipoplexes (data not shown). Indeed, no migration of the pDNA was observed in the presence of 20 μM of those liposomes. Conversely, a small amount of pDNA was released and migrated into the agarose gel in the presence of 20 μM imidazo (**7a**)/DOPE-liposomes. In contrast, pDNA migration was observed with the other DOPE liposomes. These data are in agreement with dye exclusion experiments. It is notable that the elongation of the spacer by a methylene group between the lipid moiety and the cationic head of H-Arg (**3b**) did not increase the interaction with pDNA in relation to Arg (**3a**). The carboxylic ester groups present in the Arg (**3a**), H-Arg (**3b**) and Lys (**4**) phosphoramidate lipids did not seem to give any benefit for DNA–liposome association. Theoretically, they would be expected to impair the interactions between the phosphate groups of the nucleic acid and the basic function of the cationic lipids. Indeed, the E-guanidinium phosphoramidate lipid (**6a**), without a carboxylic ester group, gave liposomes that interacted more strongly with the DNA than their Arg (**3a**) counterparts. Similarly, the EDA (**5a**) and BDA (**5b**) phosphoramidate liposomes interacted more strongly with the DNA than their Lys (**4**) counterpart.

DNA uptake

It is widely accepted that the main entry route of lipoplexes into the cells is endocytosis. To evaluate plasmid DNA internalisation as a function of the natures of the cationic headgroups of liposomes, HEK293–T7 cells were incubated for 2 h at 4 °C or 37 °C in the presence of lipoplexes made with fluorescein-labelled pDNA and of DOPE-containing liposomes. The cell fluorescence intensities were then measured by flow cytometry. Cells incubated at 4 °C were harvested by scraping without trypsin treatment to measure lipoplexes bound on the cell surface, while upon incubation at 37 °C, cells were harvested with trypsin to discard lipoplexes bound on the cell surface and to measure F-pDNA internalised into the cells. As shown in Figure 5A, the means of cell fluorescence intensity (MFI) of cells

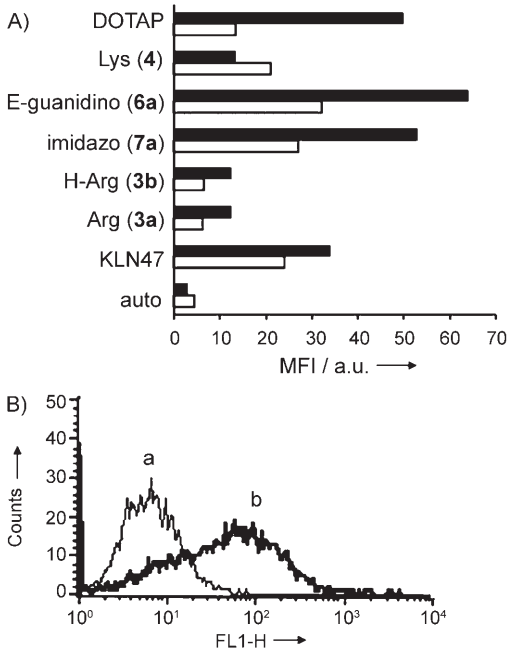

Figure 5. Uptake of lipoplexes. A) HEK293–T7 cells were incubated for 4 h either at (■) 37 °C or (□) 4 °C with various lipoplexes made with fluorescein-labelled DNA (2.5 μg) and cationic lipid/DOPE liposomes (1:1, m/m). After having been washed, the cells were harvested as indicated in the text, and the mean cell-associated fluorescence intensity (MFI) was measured by flow cytometry (λ_{ex} = 488 nm; λ_{em} = 520 nm). The lipid/DNA charge ratio was 2 for compounds **7a** and **6a** and 4 for the other compounds. B) Histograms of cells incubated at 37 °C in a) the absence and b) the presence of **7a**-lipoplexes.

incubated at 4 °C showed that the binding of E-guanidino- (**6a**), imidazo- (**7a**) and KLN47-lipoplexes to the cell surfaces was around three to four times stronger than with the other lipoplexes. These data indicated that there was no obvious correlation between the binding of the lipoplexes onto HEK293–T7 cells and their ζ potential values. The binding was indeed

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strong both with negative lipoplexes (imidazo (**7a**) and E-guanidino (**6a**)) and with positive lipoplexes (KLN47 and DOTAP).

In relation to cells incubated at 4 °C, the MFIs of the cells incubated at 37 °C had increased twofold with E-guanidino- (**6a**) and imidazo-lipoplexes (**7a**) and fourfold with DOTAP-lipoplexes (Figure 5A). This indicated that those lipoplexes were readily taken up by the cells. As shown by flow cytometry histograms, the majority of the cells internalised imidazo-lipoplexes (**7a**, Figure 5B). In contrast, we observed only weak cellular uptake with KLN47-, Arg- (**3a**), H-Arg- (**3b**) and Lys (**4**) lipoplexes. According to their weak interaction with pDNA as revealed by dye exclusion and electrophoresis gel retardation, disassembly of these latter lipoplexes in the extracellular environment or at the cell membrane level might explain their low internalisation by the cells. The transfection efficiency was correlated with the uptake of pDNA. Indeed, lipoplexes that were taken up well (imidazo- (**7a**), E-guanidino- (**6a**) and DOTAP lipoplexes) were also those that displayed high transfection efficiencies (Figure 1). Different endocytosis mechanisms can occur depending on the size of those lipoplexes. For instance, it has been reported that lipoplexes with a size of ~200 nm are taken up by clathrin-mediated endocytosis, but that bigger ones or aggregates are taken up by caveolae-mediated endocytosis or macropinocytosis.[6] The differences in their transfection efficiencies might thus be related to their propensity to form aggregates once in culture medium. Difference could also occur in their capacity to deliver the plasmid into the cytosol and/or the nucleus.

Conclusions

Phosphoramidate lipids have been proposed in order to develop a family of cationic lipids having some chemical similarity to the phospholipids present in the cell membranes, to decrease lipoplex cytotoxicity. Cationic phosphoramidate lipids with a polar head made of an ammonium, a phosphonium or an arsonium group have proved to be efficient in transfecting cells both in vitro and in vivo. Here, a series of new cationic phosphoramidate lipids characterised either by a permanent cationic charge (guanidinium or imidazolium groups) or by an acquired charge at physiological pH (amino group) was synthesised. The other cationic lipids were synthesised from α,ω-diaminoalkanes or α-amino-ω-imidazolyl alkanes. We basically found that the incorporation of an imidazolium headgroup (compound **7a**) resulted in a 100-fold improvement in the transfection efficiency relative to that of the arsonium lipophosphoramidate KLN47. Cationic liposomes made up of equimolar amounts of compound **7a** and DOPE formed negatively charged lipoplexes, yielding the most efficient transfection of HEK293–T7 cells, with low toxicity. With regard to the other derivatives, no benefit was obtained from the presence of carboxylic methyl ester groups in the arginine (**3a**), homoarginine (**3b**) and lysine (**4**) polar heads. These lipophosphoramidates did not form tight complexes with DNA, and transfections were equal to or less efficient than with those observed with the analogous compounds (**6a**, **6b**, **5a**, **5b**) without carboxylic methyl ester groups. Comparison between lipoplexes made with compounds **3a** versus **3b**, **5a** versus **5b** and **6a** versus **6b** shows that the transfection efficiency was better when the spacer between the phosphoramidate moiety and the polar head was short. Our results indicate that the uptake of lipoplexes by the target cells was clearly correlated with their final transfection efficiencies. The absence of any evident correlation between the ζ potentials of the lipoplexes and their transfection efficiencies, as well as their capacity to bind to and be taken up by the cells, suggests that more physicochemical and biological characterisations of lipoplexes will have to be conducted. Although the guanidinium counterpart of compound **7a** also exhibited high transfection efficiency, compound **6a** induced very high cytotoxicity, suggesting that imidazolium group is less harmful for the cells than guanidinium. Compound **7a**, which is more effective in vitro than KLN47, the in vivo efficiency of which has been demonstrated, might be expected also to be more effective in vivo. In conclusion, the design of cationic lipids with a permanent cationic charge contributed by an as yet not greatly developed imidazolium group might be worth consideration for the design of new cationic lipids for nonviral gene delivery.

Experimental Section

All reagents were purchased from Sigma (St. Quentin Fallavier, France) unless stated otherwise. Solvents were freshly distilled on appropriate drying agents (CH_2Cl_2 was distilled over P_2O_5, DIPEA was distilled over NaOH), and reactions were run under nitrogen. All compounds were fully characterised by ^1H (500 MHz), ^{13}C (100 MHz) and ^{31}P (121.49 MHz) NMR spectroscopy (Bruker AC 300 and Advance DRX 500 spectrometers). Coupling constants (*J*) are given in Hertz. When needed, ^{13}C heteronuclear HMQC and HMBC were used to establish structures unambiguously. Mass spectroscopy analyses were performed by CRMPO (Université de Rennes 1, Rennes, France) with a MS/MS high-resolution Micromass ZABSpec-TOF. Commercial compounds [Boc-lysine methyl ester hydrochloride (**2c**, Novabiochem, VWR International S.A.S, Fontenay sous Bois, France), ethylenediamine, diaminobutane, pyrazolecarboxamide hydrochloride, 3-aminopropylimidazole] were used as received. The other amino esters **2a**–**b** were synthesised from amino acids.[28] Dioleylphosphite (**1**) was synthesised by the reported method.[29] DOTAP was purchased from Sigma and DOPE from Fluka.

General procedure for the synthesis of phosphoramidates from α-aminomethyl esters 3a and 3b: Phosphite **1** (2.91 g, 5 mmol) and arginine or homoarginine methyl ester dihydrochloride (1.3 g, or 1.4 g, 5 mmol) were dissolved in methanol (20 mL). $CBrCl_3$ (550 μL, 5.5 mmol) was then added under nitrogen at 0 °C, followed by slow addition of DIPEA (2.6 mL, 15 mmol). The reaction mixture was stirred for 2 h at 0 °C and for one night at 20 °C. After purification by silica column chromatography ($CHCl_3$/MeOH 100:0 to 80:20) these compounds were obtained as orange oils (yields: **3a** = 31 %, **3b** = 30 %).

***O*,*O*-Dioleyl-*N*-(L-arginine methyl ester)phosphoramidate 3**: ^1H NMR (DMSO): $\delta = 0.83$ (t, $^3J_{H,H} = 6.6$ Hz, 6H; CH_3), 1.23 (m, 44H; CH_2), 1.53 (m, 4H; CH_2 β-O), 1.96 (m, 8H; CH_2 α-CH=CH), 1.52 (m, 2H), 1.65 (m, 2H), 3.10 (m, 2H), 3.60 (m, CH), 3.61 (s, 3H; OCH_3), 3.95 (m, $^3J_{H,H} = {}^3J_{P,H} = 6.4$ Hz, 4H; CH_2 α-O), 5.31 (m, 4H; CH=CH), 5.42 (t, 1H; NH), 7.80 ppm (m, 1H; NH); ^{13}C NMR (DMSO): $\delta = 13.8$ (s; 2CH_3), 22.0 to 31.3 (s; 28CH_2), 25.1 (s; CH_2), 29.0 (d, $^3J_{P,C} =$

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6.2 Hz; 2 CH_2 β-O), 30.0 (s; CH_2), 39.9 (s; CH_2), 51.6 (s; OCH_3), 53.6 (s; CH), 65.3 (d, $^2J_{P,C}$ = 6.6 Hz; 2 CH_2 α-O), 129.5 (s; CH=CH), 129.9 (s; CH=CH), 156.8 (s; C=NH_2), 173.5 ppm (s; CO_2); ^{31}P NMR (DMSO): δ = 8.9 ppm (s); ESI for $C_{43}H_{86}N_4O_5P$: calcd 769.63359 $[M+H]^+$; found: 769.6338.

***O,O*-Dioleyl-*N*-(L-homoarginine methyl ester)phosphoramidate (3 b)**: ^{1}H NMR (DMSO): δ = 0.83 (t, $^3J_{H,H}$ = 6.6 Hz, 6 H; CH_3), 1.23 (m, 44 H; CH_2), 1.53 (m, 4 H; CH_2 β-O), 1.45 (m, 2 H), 1.50 (m, 2 H), 1.65 (m, 2 H), 1.96 (m, 8 H; CH_2 α-CH=CH), 3.06 (m, 2 H), 3.54 (m, CH), 3.62 (s, 3 H; OCH_3), 3.95 (m, $^3J_{H,H}$ = $^3J_{P,H}$ = 6.4 Hz, 4 H; CH_2 α-O), 5.31 (m, 4 H; CH=CH), 5.38 (m, 1 H; NH), 7.65 ppm (m, 1 H; NH); ^{13}C NMR (DMSO): δ = 13.8 (s; 2 CH_3), 22.0 to 31.3 (s; 28 CH_2), 21.3 (s; CH_2), 27.8 (s; CH_2), 29.0 (d, $^3J_{P,C}$ = 6.2 Hz; 2 CH_2 β-O), 29.4 (s; CH_2), 40.7 (s; CH_2), 51.4 (s; OCH_3), 54.5 (s; CH), 65.3 (d, $^2J_{P,C}$ = 6.6 Hz; 2 CH_2 α-O), 129.5 (s; CH=CH), 129.9 (s; CH=CH), 156.8 (s; C=NH_2), 173.5 ppm (s; CO_2); ^{31}P NMR (DMSO): δ = 8.8 ppm (s); ESI for $C_{44}H_{88}N_4O_5P$: calcd 783.64924 $[M+H]^+$; found: 783.6484.

Synthesis of *O,O*-dioleyl-*N*-(L-lysine methyl ester)phosphoramidate (4): Phosphite **1** (2.91 g, 5 mmol) and Boc-lysine methyl ester hydrochloride (1.48 g, 5 mmol) were dissolved in $CHCl_3$ (20 mL). $CBrCl_3$ (550 μL, 5.5 mmol) was then added under nitrogen at 0 °C, followed by slow addition of DIPEA (1.74 mL, 10 mmol). The reaction mixture was stirred for 2 h at 0 °C and for one night at 20 °C. After evaporation under reduced pressure, the crude product was dissolved in ether. After filtration, the residue was concentrated in vacuo to produce the N-BOC-protected phosphoramidate. This compound was then stirred in a CH_2Cl_2/CF_3CO_2H mixture (5 mL/5 mL) over 2 h at 20 °C. After concentration in vacuo, the residue was dissolved in CH_2Cl_2 and stirred in the presence of an excess of K_2CO_3 (1.5 g) and one drop of NEt_3. The solution was stirred for 2 h at 20 °C. After filtration, an excess of HCl in solution in diethyl ether (2 N/4 mL) was added, and the reaction mixture was stirred for 1 h at 20 °C. Evaporation of the solvents produced compound **4** as an orange oil. (yield = 70 %). ^{1}H NMR (DMSO): δ = 0.83 (t, $^3J_{H,H}$ = 6.6 Hz, 6 H; CH_3), 1.23 (m, 44 H; CH_2), 1.35 (m, 2 H), 1.53 (m, 4 H; CH_2 β-O), 1.55 (m, 2 H); 1.60 (m, 2 H), 1.96 (m, 8 H; CH_2 α-CH=CH), 2.70 (t; $^3J_{H,H}$ = 7.4 Hz, 2 H), 3.55 (m, CH), 3.71 (s, 3 H; OCH_3), 3.95 (m, $^3J_{H,H}$ = $^3J_{P,H}$ = 6.4 Hz, 4 H; CH_2 α-O), 5.31 (m, 4 H; CH=CH), 5.35 ppm (m, 1 H; NH); ^{13}C NMR (DMSO): δ = 13.8 (s; 2 CH_3), 22.0 to 31.3 (s; 28 CH_2), 25.0 (s; CH_2), 26.5 (s; CH_2), 28.8 (s; CH_2), 29.0 (d, $^3J_{P,C}$ = 6.2 Hz; 2 CH_2 β-O), 38.5 (s; CH_2), 51.6 (s; OCH_3), 53.9 (s; CH), 65.3 (d, $^2J_{P,C}$ = 6.6 Hz; 2 CH_2 α-O), 129.5 (s; CH=CH), 129.9 (s; CH=CH), 173.6 ppm (s; CO_2); ^{31}P NMR (DMSO): δ = 8.7 ppm (s); ESI for $C_{43}H_{86}N_2O_5P$, $[M+H]^+$; calcd: 741.62744; found: 741.6262.

Synthesis of ω-aminoalkylphosphoramidates: Phosphite **1** (2.91 g, 5 mmol) and ethylenediamine or diaminobutane (3.34 mL or 4.17 mL, 50 mmol) were dissolved in CH_2Cl_2 (10 mL). $CBrCl_3$ (500 μL, 5 mmol) was then added, and the solution was stirred for one night. After washing with water (2×50 mL), the organic phase was dried over anhydrous magnesium sulfate, filtered and evaporated to give the desired compound as a yellow oil (yields: **5 a** = 85 %, **5 b** = 76 %).

***O,O*-Dioleyl-*N*-ethylenediaminephosphoramidate (5 a)**: ^{1}H NMR ($CDCl_3$): δ = 0.86 (t, $^3J_{H,H}$ = 6,.6 Hz, 6 H; CH_3), 1.26 (m, 44 H; CH_2), 1.65 (m, 4 H; CH_2 β-O), 1.99 (m, 8 H; CH_2 α-CH=CH), 2.64 (m, 2 H; NH_2), 2.78 (t, $^3J_{H,H}$ = 5.5 Hz, 2 H), 2.94 (m, 2 H), 3.33 (m, 1 H; NH), 3.98 (m, $^3J_{H,H}$ = $^3J_{P,H}$ = 6.4 Hz, 4 H; CH_2 α-O), 5.32 ppm (m, 4 H; CH=CH); ^{13}C NMR ($CDCl_3$): δ = 14.1 (s; 2 CH_3), 22.6 to 32.0 (s; 28 CH_2), 30.3 (d, $^3J_{P,C}$ = 6.2 Hz; 2 CH_2 β-O), 42.7 (s; CH_2), 43.7 (s; CH_2), 66.1 (d, $^2J_{P,C}$ = 6.6 Hz; 2 CH_2 α-O), 129.6 (s; CH=CH), 129.7 ppm (s; CH=CH); ^{31}P NMR ($CDCl_3$): δ = 10.0 ppm (s); ESI for $C_{38}H_{78}N_2O_3P$; calcd: 641.57501 $[M+H]^+$; found: 641.5739.

***O,O*-Dioleyl-*N*-diaminopropanephosphoramidate (5 b)**: ^{1}H NMR (CD_3OD): δ = 0.89 (t, $^3J_{H,H}$ = 6.6 Hz, 6 H; CH_3), 1.29 (m, 44 H; CH_2), 1.52 (m, 2 H), 1.53 (m, 2 H), 1.66 (m, 4 H; CH_2 β-O), 2.00 (m, 8 H; CH_2 α-CH=CH), 2.69 (t, 2 H), 2.89 (m, $^3J_{H,H}$ = 5.5 Hz, 2 H), 3.98 (m, $^3J_{H,H}$ = $^3J_{P,H}$ = 6.4 Hz, 4 H; CH_2 α-O), 5.32 ppm (m, 4 H; CH=CH); ^{13}C NMR (CD_3OD): δ = 14.5 (s; 2 CH_3), 23.7 to 33.6 (s; 28 CH_2), 30.0 (s; 2 CH_2), 30.8 (d, $^3J_{P,C}$ = 6.2 Hz; 2 CH_2 β-O), 41.9 (s; CH_2), 42.0 (s; CH_2), 67.5 (d, $^2J_{P,C}$ = 6.6 Hz; 2 CH_2 α-O), 130.8 (s; CH=CH), 130.9 ppm (s; CH=CH); ^{31}P NMR (CD_3OD): δ = 9.7 ppm (s); ESI for $C_{40}H_{82}N_2O_3P$ calcd: 669.60631 $[M+H]^+$; found: 669.6067.

Synthesis of guanidinium phosphoramidates 6 a and 6 b: Compounds **5 a** or **5 b** (1.92 g or 2 g, 3 mmol) and pyrazolecarboxamide hydrochloride (440 mg, 3 mmol) were dissolved in absolute ethanol (30 mL). DIPEA (523 μL, 3 mmol) was then added, and the reaction mixture was heated at reflux overnight. After concentration in vacuo, the residue was dissolved in CH_2Cl_2 (30 mL) and washed with water (2×50 mL), and the organic phase was dried over anhydrous magnesium sulfate, filtered and evaporated to give the desired compound (yield = 70 %).

***O,O*-Dioleyl-*N*-(2-guanidinylethylene)phosphoramidate (6 a)**: ^{1}H NMR (CD_3OD): δ = 0.84 (t, $^3J_{H,H}$ = 6.6 Hz, 6 H; CH_3), 1.24 (m, 44 H; CH_2), 1.55 (m, 4 H; CH_2 β-O), 1.96 (m, 8 H; CH_2 α-CH=CH), 2.85 (m, 2 H), 3.14 (m, 2 H), 3.87 (m, $^3J_{H,H}$ = $^3J_{P,H}$ = 6.4 Hz, 4 H; CH_2 α-O), 4.96 (m, 1 H; NH), 5.32 (m, 4 H; CH=CH), 7.54 ppm (m, 1 H; NH); ^{13}C NMR (CD_3OD): δ = 14.1 (s; 2 CH_3), 22.6 to 32.0 (s; 28 CH_2), 26.6 (d, $^3J_{P,C}$ = 6.2 Hz; 2 CH_2 β-O), 39.9 (s; CH_2), 42.0 (s; CH_2), 65.4 (d, $^2J_{P,C}$ = 6.6 Hz; 2 CH_2 α-O), 129.6 (s; CH=CH), 129.7 (s; CH=CH), 157.8 ppm (s; C=NH_2); ^{31}P NMR (CD_3OD): δ = 9.9 ppm (s); ESI for $C_{39}H_{80}N_4O_3P$: calcd: 683.59681 $[M+H]^+$; found: 683.5967.

***O,O*-Dioleyl-*N*-(3-guanidinylpropylene)phosphoramidate (6 b)**: ^{1}H NMR (CD_3OD): δ = 0.89 (t, $^3J_{H,H}$ = 6.6 Hz, 6 H; CH_3), 1.29 (m, 44 H; CH_2), 1.53 (m, 2 H), 1.55 (m, 2 H), 1.62 (m, 4 H; CH_2 β-O), 2.00 (m, 8 H; CH_2 α-CH=CH), 2.91 (m, 2 H), 3.17 (m, 2 H), 3.95 (m, $^3J_{H,H}$ = $^3J_{P,H}$ = 6.4 Hz, 4 H; CH_2 α-O), 5.34 ppm (m, 4 H; CH=CH); ^{13}C NMR (CD_3OD): δ = 14.5 (s; 2 CH_3), 26.7 to 33.6 (s; 28 CH_2), 26.9 (d, $^3J_{P,C}$ = 6.2 Hz; 2 CH_2 β-O), 28.0 (s; 2 CH_2), 41.5 (s; CH_2), 42.1 (s; CH_2), 67.6 (d, $^2J_{P,C}$ = 6.6 Hz; 2 CH_2 α-O), 130.9 (s: CH=CH), 131.4 (s; CH=CH), 157.0 ppm (s; C=NH_2); ^{31}P NMR (CD_3OD): δ = 10.3 ppm (s).

Synthesis of *O,O*-dioleyl-*N*-[3*N*-(*N*-methylimidazolium iodide)propylene]phosphoramidate (7 b): Phosphite **1** (2.91 g, 5 mmol) and 3-aminopropylimidazole (597 μL, 5 mmol) were dissolved in $CHCl_3$ (20 mL). CCl_4 (530 μL, 5.5 mmol) was then added under nitrogen at 0 °C, followed by slow addition of DIPEA (870 μL, 5 mmol). The reaction mixture was stirred for 2 h at 0 °C and for one night at 20 °C. After evaporation under reduced pressure, the product was dissolved in diethyl ether, and the ammonium salts were removed by filtration. After concentration in vacuo, the residue was dissolved in CH_3I (3 mL), and the solution was stirred overnight at 20 °C. After evaporation of the volatiles, compound **7 b** was obtained as an orange oil (yield = 80 %). ^{1}H NMR (CD_3OD): δ = 0.89 (t, $^3J_{H,H}$ = 6.6 Hz, 6 H; CH_3), 1.28 (m, 44 H; CH_2), 1.65 (m, 4 H; CH_2 β-O), 2.00 (m, 8 H; CH_2 α-CH=CH), 2.03 (m, 2 H), 2.94 (m, 2 H), 3.67 (s, 3 H; CH_3), 3.97 (m, $^3J_{H,H}$ = $^3J_{P,H}$ = 6.4 Hz, 4 H; CH_2 α-O), 4.30 (t, 2 H), 5.33 (m, 4 H; CH=CH), 7.57 (s; CH), 7.64 ppm (s; CH); ^{13}C NMR (CD_3OD): δ = 14.5 (s; 2 CH_3), 23.7 to 33.6 (s; 28 CH_2), 26.7 (d, $^3J_{P,C}$ = 6.2 Hz; 2 CH_2 β-O), 28.3 (s; CH_2), 36.5 (s; CH_3), 38.5 (s; CH_2), 48.0 (s; CH_2), 67.9 (d, $^2J_{P,C}$ = 6.6 Hz; 2 CH_2 α-O), 123.8 (s; CH), 125.1 (s; CH), 130.7 (s; CH=CH), 130.9 ppm (s; CH=CH); ^{31}P NMR (CD_3OD): δ = 9.9 ppm (s); ESI for $C_{43}H_{83}N_3O_3P$: calcd 720.61721 $[M+H]^+$; found: 720.6143.

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Cells and cell culture: Human Embryo Kidney HEK293–T7 cells (kindly given by Drs. L. Huang and M. Brisson, University of Pittsburgh, Pittsburgh, PA)[30] and human epithelial ovary carcinoma HeLa cells (CCL21, ATCC, Rockville, MD) were cultured in DMEM and MEM, respectively. Culture media contained Fetal Bovine Serum (FBS; 10%), L-glutamine (2 mM; Life Technologies), sodium pyruvate (1 mM; Life Technologies), penicillin (100 units mL^{-1}; Life Technologies) and streptomycin (100 Units mL^{-1}; Life Technologies) and geneticin (400 $\mu g\,mL^{-1}$; for HEK293–T7 cells). Cells were mycoplasma-free, as evidenced by the bis-benzimidazole (Hoechst 33258, Molecular Probes) method.[31]

Plasmids: pTG11033 (pCMV-Luc; 9514 bp; kindly donated by Transgene S.A., Strasbourg, France) and pCMV-EGFP were plasmids encoding the firefly luciferase gene and the jellyfish *Aequorea victoria* green fluorescent protein (EGFP) under the control of the human cytomegalovirus promoter, respectively. Supercoiled plasmid DNA was isolated by a standard alkaline lysis method, and purification was carried out with the QIAGEN Mega Kit (QIAGEN, Courtaboeuf, France). The pCMV-Luc was labelled with the Label IT fluorescein nucleic acid labelling kit (MIRUS, Madison, WI, USA) at 1:1 (reagent/pDNA) *w/w* ratio according to the manufacturer's instructions.

Liposomes: A lipid film was prepared in a sterile balloon (10 mL) under nitrogen by drying an ethanol mixture of one cationic lipid (10.8 mM, 0.5 mL) and DOPE (10.8 mM, 0.5 mL; molar ratio of 1:1). The film was hydrated in sterile HEPES buffer (10 mM, 1 mL) at pH 7.4, vortexed for 3 min and kept at 4 °C. After 2 h, the mixture was vortexed and sonicated for 15 min at 37 kHz with a Bioblock ultrasonic bath (Bioblock Scientific, Illkirch, France).

Lipoplexes: Liposomes (5.4 mM, 9 μL, 0.0486 μmol positive charge or 18 μL, 0.0972 μmol positive charge), made either with a cationic lipid alone or with a mixture of a cationic lipid and DOPE (molar ratio of 1:1), were diluted in HEPES buffer (10 mM, 200 μL) at pH 7.4. After 15 min, pCMV-Luc (7.5 μg, 1.19×10^{-6} μmol, 0.0227 μmol negative charge) in HEPES buffer (10 mM, 20 μL) at pH 7.4 was added to the liposome solution, and the mixture was incubated for 30 min at room temperature. The lipoplex solution was made up to 1.5 mL with serum-free medium, and the NaCl concentration was adjusted to 0.15 M with a 5 M NaCl solution.

DNA–liposome interactions: Liposomes (5.4 mM, 4 μL) were diluted in HEPES buffer (10 mM, 500 μL) at pH 7.4 and serial dilutions were performed. pCMV-Luc (1 μg) in HEPES buffer (10 mM, 20 μL) at pH 7.4 mixed with SYBR Green I (Molecular Probes; final dilution 1/10000) was added to the liposome solution. After 30 min at 20 °C, the fluorescence intensity was measured with a Shimadzu 5000 spectrofluorimeter ($\lambda_{ex}=480$ nm; $\lambda_{em}=520$ nm). DNA/liposome complex formation was evaluated by dye exclusion by the following relationship: $(F_{sample}-F_{blank})/(F_{DNAonly}-F_{blank})$ where F_{sample} and $F_{DNAonly}$ were the fluorescence intensities of SYBR Green I in the presence of DNA and liposomes and in the presence of DNA without liposomes, respectively. F_{blank} was the fluorescence intensity of SYBR Green I in the absence of DNA and liposomes.

Size and ζ potential measurements: Liposomes and lipoplexes were diluted in HEPES buffer (10 mM, 1 mL) at pH 7.4. Their ζ potentials were measured by electrophoretic mobility with ZetaSizer 3000 (Malvern Instruments, Orsay, France). The following parameters were set up: viscosity, 0.891 cP; dielectric constant, 79; temperature, 25 °C; F(Ka), 1.50 (Smoluchowsky); maximum voltage of the current, 15 V. The system was calibrated with DTS 5050 standard (Malvern). Measurements were carried out ten times with the zero-field correction. The ζ potential was calculated by use of the Smoluchowsky approximation. The sizes of liposomes and lipoplexes was measured by quasi-elastic laser light scattering (QELS) with the ZetaSizer 3000 instrument in HEPES buffer (10 mM) at pH 7.4 with a sample refractive index of 1.59 and a viscosity of 0.89. The system was calibrated with the 200 ± 5 nm polystyrene polymer (Duke Scientific Corps Palo Alto, CA). The diameters of liposomes and lipoplexes were calculated in the automatic mode.

Transfections: Two days prior to transfection, cells were seeded at 1×10^5 cells per 2 cm^2 in culture medium (1 mL) in a 24-well plate. At the time of the experiment, cell cultures were 80% confluent. Cells were washed twice with serum-free culture medium before incubation with lipoplexes. The lipoplex solution (0.5 mL, 2.5 μg pDNA) was then added in each well, and cells were incubated at 37 °C. After 4 h, the medium was removed, and cells were cultured 48 h at 37 °C in complete culture medium.

Luciferase assay: For measuring luciferase gene expression, the luminescence activity was monitored according to De Wet et al.[32] The medium was discarded, and cells were washed three times with PBS. The homogenisation buffer (200 μL)—containing Tris phosphate (25 mM, pH 7.8), $MgCl_2$ (8 mM), DTT (1 mM), EDTA (1 mM), Triton X-100 (1%), and glycerol (15%)—was poured into each well, and tissue culture plates were kept for 15 min at 20 °C. The solution was recovered and spun down (5 min at 800 g). ATP (2 mM) in the homogenisation buffer without Triton X-100 (95 μL) was added to the supernatant (60 μL), and the solution was shaken with a vortex. The luminescence was recorded for 4 s in a Lumat LB 9501 luminometer (Berthold, Wildbach, Germany) after addition of luciferin (167 mM) in water (150 μL). Measurements were done in duplicate. The number of RLUs of 1 $pg\,mL^{-1}$ of luciferase under these conditions was 2000. The data shown correspond to the number of relative light units (RLUs) per mg proteins. Proteins were determined on each sample by a modified bicinchoninic acid (BCA) colorimetric assay.[33,34]

Measurement of EGFP-positive cells: Forty-eight hours after transfection with pCMV-EGFP lipoplexes, cells were harvested by treatment with trypsin and washed in complete medium, and the pellet was suspended in sheath fluid. The cell-associated fluorescence intensity was then measured by flow cytometry (FACSort, Becton Dickinson; $\lambda_{ex}=488$ nm; $\lambda_{em}=520$ nm).

Toxicity assay: The cell viability was evaluated by the colorometric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[35] MTT (5 $mg\,mL^{-1}$ in PBS, 50 μL) was added to the cell culture 48 h after transfection, and the system was incubated for 4 h at 37 °C. MTT converted into an insoluble dye in living cells was then solubilised with acidic isopropanol. The absorbance was measured at 570 nm and expressed as a percentage of the absorbance measured for untransfected cells cultured under the same conditions as those used for transfected cells.

Plasmid DNA uptake: HEK293–T7 cells were incubated at 4 °C or 37 °C for 4 h with lipoplexes made with a fluorescein-labeled pCMV-Luc. After having been washed, the cells incubated at 4 °C were harvested by scraping, while cells incubated at 37 °C were harvested with trypsin. The cell-associated fluorescence intensity was then measured by flow cytometry (FACSort, Becton Dickinson; $\lambda_{ex}=488$ nm; $\lambda_{em}=520$ nm).

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