Synthesis, Activity, and Structure−Activity Relationship Studies of Novel Cationic Lipids for DNA Transfer

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We have designed and synthesized original cationic lipids for gene delivery. A synthetic method on solid support allowed easy access to unsymmetrically monofunctionalized polyamine building blocks of variable geometries. These polyamine building blocks were introduced into cationic lipids. To optimize the transfection efficiency in the novel series, we have carried out structure−activity relationship studies by introduction of variable-length lipids, of variable-length linkers between lipid and cationic moiety, and of substituted linkers. We introduce the concept of using the linkers within cationic lipids molecules as carriers of side groups harboring various functionalities (side chain entity), as assessed by the introduction of a library composed of cationic entities, additional lipid chains, targeting groups, and finally the molecular probes rhodamine and biotin for cellular traffic studies. The transfection activity of the products was assayed in vitro on Hela carcinoma, on NIH3T3, and on CV1 fibroblasts and in vivo on the Lewis Lung carcinoma model. Products from the series displayed high transfection activities. Results indicated that the introduction of a targeting side chain moiety into the cationic lipid is permitted. A primary physicochemical characterization of the DNA/lipid complexes was demonstrated with this leading compound. Selected products from the series are currently being developed for preclinical studies, and the labeled lipopolyamines can be used to study the intracellular traffic of DNA/cationic lipid complexes.

Introduction

A great number of genes for which abnormal expression is linked to human diseases ares being cloned and identified. The ability to express such genes in humans might allow the prevention or even the cure of many important human diseases, which are either treated poorly or untreatable by the present therapies. However, unsufficient transgene expression in vivo impairs the development of effective gene therapy. Therefore, it would be of interest to develop compositions and delivery methods for gene therapy that provide a high level of transcription of the transgene and/or expression in a variety of cell and tissue types. Synthetic DNA delivery agents are of crucial interest for gene therapy as an alternative to viral vectors, since they display potentially less risks in terms of immunogenicity and propagation.[1,2] Thus, gene transfer using synthetic compounds is a rapidly increasing field.[3] So far the largest family of such agents is based on cationic lipids able to self-associate and form complexes with DNA inferring a compacted state to the plasmid. In the recent years, a variety of cationic lipids for gene transfer have been described.[3−8] Cationic lipids are composed of three elements as outlined in Figure 1.

Cationic lipids for DNA delivery can be classified into three different groups: (1) *Quaternary ammonium salt lipids* in which a lipid is linked through a spacer to a quaternary ammonium salt; examples of this class are DDAB and DMRIE[2,8] (see Figure 2). These cationic lipids have increased transfection activity when coformulated with the neutral lipid DOPE.[9] (2) *Lipoamines* in which a lipid is linked through a spacer to an amine or to a polyamine; examples of this class are DC-Chol[4] and DOGS[5] (the first coformulated with DOPE). (3) *Cationic lipids containing a quaternary ammonium salt*

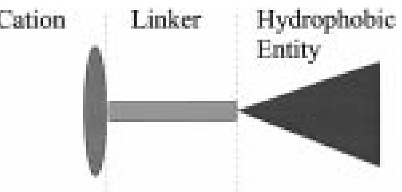

Figure 1. General composition of a cationic lipid for DNA delivery.

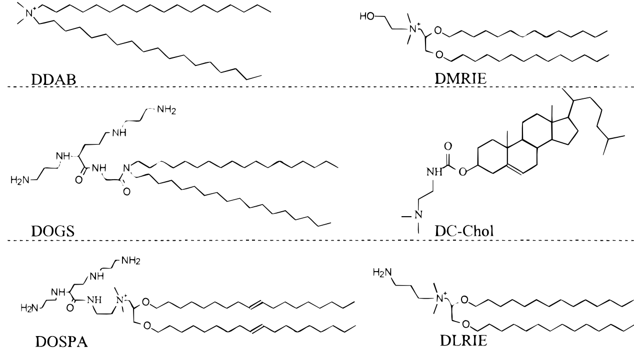

Figure 2. Representative cationic lipids for DNA delivery.

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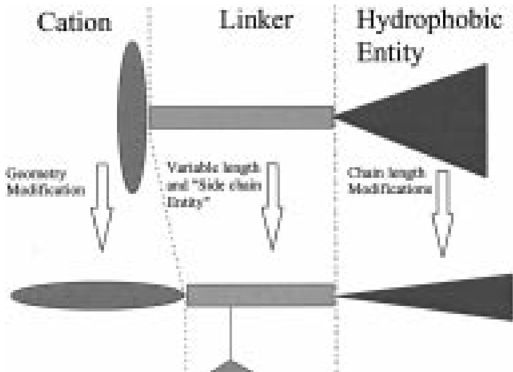

Figure 3. Structure−activity relationship studies on cationic lipids.

and a lipoamine; examples of this class are DOSPA[6] and DLRIE,[2,8] which combine the use of amines and a quaternary ammonium salt in the same molecule, and are also proposed in association with DOPE or other neutral lipids for transfection activity.

The main efforts of recent works have been focused on modifications of the lipid chains and of the substituents on the quaternary ammonium salts. These efforts led to the recently described DMRIE and DLRIE which are being studied in clinical trials. In the second and third groups, the polyamine component has usually been carboxyspermine as described by J. P. Behr in DOGS[5] and used also by others.[6] In contrast, modifications of the polyamine component have not been extensively reported (during completion of the manuscript for this paper, some geometrically differing polyamines have been described[7] but with a limited synthetic and diversity scope, as polyamines remained unfunctionalyzed and were directly linked to lipid-forming amide bonds). Similarly, replacement of the quaternary ammonium salt by other cationic entities and the introduction of different linkers have not been extensively and systematically studied. Ruysschaert and colleagues[10] have demonstrated that basic moieties such as amidines might confer a transfection activity when introduced into a lipid. We and others[11,12] have also used this concept to achieve increased transfection activity.

To obtain novel tools for increased in vitro and in vivo gene delivery, for tissue targeting and also for cellular traffic studies, we have implemented a step by step strategy by introduction of systematic modifications into the cationic lipid backbone. Subsequently, we have introduced a novel element, a "side chain entity", on the linker. Modifications were followed by in vitro biological assays. Molecules emerging from intermediate steps and having increased gene delivery properties as well as chemical versatility were selected as starting precursors for further structure−activity relationship (SAR) studies (see Figure 3). It was further tested to determine if the introduction of a "side chain entity" led to any transfection activity.

Our strategy included SAR studies focused on the following modifications: (1) introduction of a library of geometrically differing polyamines as the cation component, (2) introduction of a library of linkers with differing lengths, (3) modification of linker length and polyamine geometry, (4) variation in the length of the lipid moiety, (5) introduction of a novel "side chain entity" element into cationic lipids on the linker position, the group being either (a) a library of aliphatic, aromatic, cationic, or targeting moieties or (b) a library of molecular probes for vector trafficking studies.

Finally, we have assessed the transfection activity of several selected products in the in vivo Lewis lung carcinoma tumor model.

Results and Discussion

(1) Introduction of a Library of Geometrically Differing Polyamines as the Cation Component: Synthesis and in Vitro Transfecting Activity. To study the impact of polyamine polymorphism on gene transfer by lipopolyamines, we chose to prepare geometrically differing functionalized polyamines using the lipopolyamine DOGS[5] as a starting model. To avoid the tedious multistep reactions necessary to obtain monofunctionalized polyamines by solution methods,[13] we have developped a solid-phase synthesis which allowed a rapid preparation of a great number of differing monofunctionalized polyamines.[14]

In this approach, the alkylating reagent is covalently attached to the polymeric support through esterification. The principle is the use of solid-phase synthesis to obtain a "high dilution effect" at the proximity of the alkylating reagent, which prevents polyalkylation of the polyamine once the first alkylation has taken place. The symmetrical polyamine reacts with the alkylating reagent on the solid phase to yield an unsymmetrically monofunctionalized polyamine attached to the support.

This method allowed the easy access to unsymmetrically monofunctionalized polyamines in one step starting from their symmetrical counterparts. The resulting products are fully protected by addition of exceeding di-*tert*-butyl dicarbonate or other protecting groups.[15] The building blocks are cleaved from the acid-sensitive 2-chlorotrityl chloride support with trifluoroethanol to give the fully protected functionalized polyamines bearing an acid function suitable for subsequent coupling with the lipid and amino acid component using BOP reagent.[16] This method seems to be very convenient for the synthesis of unsymmetrically monofunctionalized polyamines which could not be easily synthesized by conventional methods (such as branched shaped polyamines). We have thus obtained "linear-shaped", "globular-shaped", "T-shaped", and "branched-shaped" lipopolyamines (see Figure 4 and Table 1). The versatility of using amino acids as linkers has been demonstrated so far for other applications. Therefore, for the first study, Gly was chosen as linker between the functionalized polyamine and the lipid moiety.

The obtained lipopolyamines were assayed in vitro on human carcinoma Hela cells. Results presented in Table 1 show that although all the lipopolyamines displayed a high level of transfection efficiency in this model, the linear RPR-120535 leads to the highest level of luciferase expression, followed by the branched RPR-120525, the T-shaped RPR-126096, and finally the globular lipopolyamine. It should be pointed out that no significant differences in the cytotoxicity of the products could be observed by protein quantification (data not shown). Thus differences in activities are likely attributed to structural differences. From this first study emerged the linear lipopolyamine RPR-120535, which was used for further in vitro SAR studies.

Table 1. Impact of Polyamine Geometry on Expression of Luciferase in Transfected Human Hela Cells (see the Experimental Section)

N° RPR	Head Geometry	R_1	R_2	m	X	R_5	Hela cells [RLU]*
120525	Branched	H2N, N, N, NH2, H2N	H	1	$NHCH_2CO$	$(CH_2)_{17}CH_3$	$5.8\ 10^5$
120528	Globular	H2N, N, H2N	H	1	$NHCH_2CO$	$(CH_2)_{17}CH_3$	$1.3\ 10^5$
126096	« T » shape	H2N, N H	H2N	1	$NHCH_2CO$	$(CH_2)_{17}CH_3$	$5.5\ 10^5$
120535	linear	H2N, N H, H N	H	1	$NHCH_2CO$	$(CH_2)_{17}CH_3$	$2.4\ 10^6$

[a] RLU = luciferase activity in relative light units (RLU)/10 s for 10^3 plated cells (for substituents refer to the general structure in Figure 4).

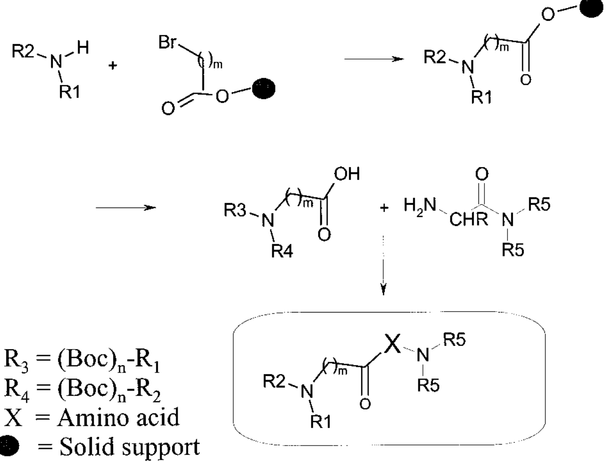

Figure 4. Solid phase synthesis of geometrically differing lipopolyamines.

(2) Introduction of Variable-Length Linkers. The linker in RPR-120535 contains five atoms between the polyamine and the lipid. To refine the transfection activity, we have introduced linkers with various numbers of atoms. The linker was modulated from two atoms in RPR-120534 up to nine atoms in RPR-128506. The method used for the synthesis was the same as for the first series (see the Experimental Section).

The products were tested on Hela cells, and the results in Table 2 suggest that all of the products possess a similar level of transfection efficiency precluding a clear selection. Additionally, no significant difference in the cytotoxicity of the products could be observed (data not shown). Thus, for chemical feasibility and versatility reasons, we selected RPR-120534 and RPR-120535 for further studies, which contain two and five atoms between the polyamine and the lipid, respectively.

(3) Modification of Linker Length and Polyamine Geometry. To further establish a clear activity ranking for our family of cationic lipids, we have synthesized cationic lipids containing geometrically differing polyamines and with two or five atoms linker (containing Gly or not), and we have compared their efficiency to transfect NIH3T3 mouse fibroblast cells instead of Hela cells, as in the latter no influence of the linker length could be detected (see Table 3).

Results obtained with the NIH3T3 fibroblasts suggest that a longer linker increases in general the gene delivery activity (see Table 3). This effect was significative for the globular-shaped couple 120527/120528 (the latter being about 20-fold more active) and for the linear-shaped couple 120535/12534 (25-fold increase in activity), and this effect was weak for the branched-shaped couple 120526/120525. The globular-shaped RPR-120527 and 120528 displayed lower transfection activities as compared to the linear, T-shaped, or branched spermine derivatives. These lower activities can be attributed in part to decreased p*K* values of the different amines in the globular compounds, since the shorter ethylene chains between the amines in the globular lipids RPR-120527 and 120528 probably have an impact on the total positive charges available for binding to DNA at physiological conditions, when compared with propylene or tetramethylene in spermine derivatives.

In this study RPR-120535, which includes a linker of five atoms and a linear polyamine, proved to be the most active product in vitro on HeLa human carcinoma cells

Table 2. Impact of Linker Length on Expression of Luciferase in Transfected Human Hela Cells (See the Experimental Section)

N° RPR	R_1	R_2	m	N° of atoms of linker	X	R_5	Hela cells [RLU]*
120534		H	1	2	-	$CH_2)_{17}CH_3$	2.6 10^6
122786		H	3	4	-	$(CH_2)_{17}CH_3$	1.5 10^6
120535		H	1	5	$NHCH_2CO$	$(CH_2)_{17}CH_3$	2.4 10^6
128506		H	1	9	$NH(CH_2)_5CO$	$(CH_2)_{17}CH_3$	3.5 10^6

[a] RLU = luciferase activity in relative light units (RLU)/10 s for 10^3 plated cells (for substituents refer to the general structure in Figure 4).

Table 3. Structure–Activity Relationship Studies on Linker Length/Polyamine Geometry[a]

N° RPR	R_1	R_2	Head Geometry	X	R_5	NIH3T3 cells [RLU]*
120526		H	Branched	-	$(CH_2)_{17}CH_3$	5.9 x10^7
120525		H	Branched	$NHCH_2CO$	$(CH_2)_{17}CH_3$	6.8 10^7
120527		H	Globular	-	$(CH_2)_{17}CH_3$	1.5 10^6
120528		H	Globular	$NHCH_2CO$	$(CH_2)_{17}CH_3$	2.0 10^7
120534		H	Linear	-	$(CH_2)_{17}CH_3$	2.0 10^6
120535		H	Linear	$NHCH_2CO$	$(CH_2)_{17}CH_3$	5.4 10^7

[a] Impact on expression of luciferase in transfected mouse fibroblasts NIH3T3 cells. *RLU = luciferase activity in relative light units (RLU)/10 s for 10^3 plated cells (for substituents refer to the general structure in Figure 4).

Table 4. Impact of the Length of the Lipid on Expression of Luciferase in Transfected Human Hela and Mouse Fibroblasts NIH3T3 Cells

N° RPR	R_1	R_2	X	R_5	Hela cells [RLU]*	NIH3T3 cells [RLU]*
120535	H_2N N H N H	H	$NHCH_2CO$	$(CH_2)_{17}CH_3$	$8.9\ 10^5$	$4.4\ 10^7$
122766	H_2N N H N H	H	$NHCH_2CO$	$(CH_2)_{13}CH_3$	$1.1\ 10^5$	$2.7\ 10^6$
122774	H_2N N H N H	H	$NHCH_2CO$	$CH_2)_{12}CH_3$	$2.3\ 10^4$	$2.0\ 10^5$
122767	H_2N N H N H	H	$NHCH_2CO$	$(CH_2)_{11}CH_3$	0	$2.6\ 10^3$

[a] RLU = luciferase activity in relative light units (RLU)/10 s for 10^3 plated cells (for substituents refer to the general structure in Figure 4).

and NIH3T3 mouse fibroblasts. In comparative studies, this lead compound RPR120535 was found to possess transfection efficiency similar to that of commercially available Transfectam and Lipofectamine on cell lines such as NIH3T3, 3LL mouse Lewis lung carcinoma, Caco-2 human colon carcinoma, and primary rabbit smooth muscle cells (in $\pm 2-5$-fold variation range depending on cell type, data not shown). The gene transfer efficiency and the chemical versatility conferred to the cationic lipid by the use of an α-amino acid as linker in RPR-120535 prompted us to use the latter as a model for further SAR studies.

(4) Variable Length of the Lipid Moiety. The last classical element of the backbone to be studied was the lipid chain. Previous works have shown that a single fatty chain or cholesteryl in the absence of colipid DOPE prevented transfection activity and conferred undesirable cytotoxicity in lipopolyamines.[9] The optimal length of the double fatty chain might vary for different products. We have introduced lipid chains containing from 12 to 18 carbons and assayed in vitro the transfection efficacy of the resulting lipopolyamines on HeLa and NIH3T3 cell lines (see Table 4).

Results in Table 4 show that the hydrophobicity of the lipid moiety has a crucial effect on in vitro gene transfer on any cell line tested. A decrease in chain length from 18 to 12 is associated with a decrease in transfection ability and increased cytotoxicity (data not shown). This study confirmed that RPR-120535 is a potential candidate for an extensive physicochemical characterization and in vivo gene transfer studies. Moreover, the chemical versatility of the amino acid linker in RPR-120535 prompted us to synthesize libraries with a variety of subtituted linkers.

(5) Introduction of a Novel *"Side Chain Entity"* Element into the Linker Position of the Cationic Lipid: (a) A Library of Aliphatic, Aromatic, Cationic, or Targeting Moieties. We have introduced amino acids instead of Gly in the linker position of RPR-120535, which were substituted by a variety of functions such as aliphatic or aromatic chains, novel cationic or targeting entities through their side chains (see Table 5).

The results in Table 5 show that the presence of a guanidinium group in addition to a single amino group in RPR-120533 results in a weak transfection activity both on Hela and NIH3T3 cells. On the other hand, introduction of positively charged Arg or Lys, instead of Gly in RPR-120535, to give RPR-120531 and RPR-127888, led to products with significant but slightly decreased transfection ability on both cell lines, as compared to RPR-120535. When negatively charged Glu is introduced in the same position in RPR-126097, a transfecting activity similar to that of RPR-120535 is observed. It should be pointed out that the introduction of a carboxylate side chain in RPR-126097 adds a negative charge and consequently diminishes the net cationic charges available for DNA binding. In contrast, introduction of a cationic amino side chain in RPR-127888 and RPR-120531, which increased the net positive charge of the vector, resulted in decreased transfection efficiency. Thus, it seems most likely that there is an optimal net charge for maximal transfection efficiency of cationic lipid, and that increasing the net charge of the cationic head might not always be beneficial. Additional substitution on the amino acid side chain provided products with significant transfection activity on both cell lines (see RPR-121650 with modified guanidine group, RPR-122759 with modified Lys, and finally RPR-130605 with a polyunsaturated aliphatic side chain). RPR-130605 contains an arachidonic fatty acid side chain which might be considered as a membrane-targeting or anchoring agent due to the facility of insertion of fatty acids in cellular phospholipid bilayers.

Table 5. Impact of the Introduction of a "Side Chain Entity" in the Linker Position on the Expression of Luciferase in Human Hela and Mouse Fibroblasts NIH3T3 Cells

N° RPR	Cationic Entity	Linker	Lipid	Hela cells [RLU]*	NIH3T3 cells [RLU]*
120533	-	Arg	$N[(CH_2)_{17}CH_3]_2$	$4.7\ 10^3$	$8.7\ 10^3$
120531		Arg	$N[(CH_2)_{17}CH_3]_2$	$8.2\ 10^6$	$1.1\ 10^7$
121650		Arg(Z_2)	$N[(CH_2)_{17}CH_3]_2$	$1.2\ 10^6$	$7.8\ 10^7$
126097		Glu	$N[(CH_2)_{17}CH_3]_2$	$1.5\ 10^7$	$6.3\ 10^7$
127888		Lys	$N[(CH_2)_{17}CH_3]_2$	$7.0\ 10^5$	$1.0\ 10^7$
122759			$N[(CH_2)_{17}CH_3]_2$	$1.4\ 10^6$	$3.0\ 10^7$
130605			$N[(CH_2)_{17}CH_3]_2$	$4.6\ 10^5$	$8.2\ 10^6$
120535		**Gly**	$N[(CH_2)_{17}CH_3]_2$	$1.0\ 10^7$	$2.0\ 10^8$

[a] RLU = luciferase activity in relative light units (RLU)/10 s for 10^3 plated cells (see the Experimental Section).

On the basis of these results we have demonstrated that the introduction into cationic lipids of a linker bearing a side chain entity instead of glycine is indeed permitted for transfection in vitro. This posibility opens the field of specific targeting by introduction of peptides, glycosides, or other specific ligands.

(b) A Library of Molecular Probes for Vector Trafficking Studies. In view of investigating the intracellular fate of DNA/cationic lipid complexes, we have introduced two different molecular probes into the cationic lipids as the side chain entity. These products contain either a biotine side chain in RPR-122761 or a rhodamine in RPR-121653, which are well-known tools for microscopy studies using either a streptavidine-labeled ligand or directly the fluorescence of rhodamine (see Table 6).

In previous reports on this domain, the fate of the cationic lipid/DNA complexes was followed by addition of an additive labeled lipid to the complexes,[3,17–18] whereas we propose here the first directly labeled functional cationic lipids for such traffic studies. These labeled lipids were assayed on cell lines suitable for

Table 6. Impact of the Introduction of Molecular Probes as Side Chain Entities in the Linker Position on the Expression of Luciferase in Mouse Fibroblasts NIH3T3 and Monkey CV1 Cell Lines

N° RPR	Cationic Entity	Linker	Lipid	NIH3T3 cells [RLU]*	CV1 cells [RLU]*
120535		Gly	$N[(CH_2)_{17}CH_3]_2$	2.6 10^7	0.7 10^4
122761			$N[(CH_2)_{17}CH_3]_2$	5.4 10^6	1.6 10^5
121653			$N[(CH_2)_{17}CH_3]_2$	1.2 10^6	1.3 10^5

[a] RLU = luciferase activity in relative light units (RLU)/10 s for 10^3 plated cells (see the Experimental Section).

Table 7. Cationic Lipid-Mediated Luciferase Reporter Gene Transfer into Lewis Lung Carcinoma Tumors

expt	μg of DNA/tumor	cationic lipid no. RPR	cationic lipid nmol/μg of DNA	10^6 [RLU/tumor][a] mean ± SE	*n**
1	10	126 097	6	1.38 ± 0.38	6
	10	120 535	6	4.04 ± 0.99	6
2	10	126 097	6	2.19 ± 1.45	3
	10	130 605	6	0.66 ± 0.43	3

[a] RLU = luciferase activity in relative light units (10^6 RLU = 30 pg luciferase).

microscopy studies (mouse NIH3T3 and monkey CV1 fibroblasts). Both products showed significant in vitro gene delivery activity on both cell types (see Table 6) as compared to RPR-120535, which allowed subsequent intracellular traffic studies.[19]

(6) In Vivo Transfection on the Lewis Lung Carcinoma Tumor Model. We have selected RPR-120535 (with a linear geometry of the polyamine head and an unsubstituted linker), RPR-126097 (with a linear geometry of the polyamine head and a polyunsaturated side chain entity on linker), and RPR-130605 (linear geometry of the polyamine head and an anionic side chain entity on linker) to study and compare their gene transfer ability in the Lewis lung carcinoma tumor model[20] (3LL) in vivo. This model is widely used for evaluation of antitumoral activity, as it induces distant metastasis and allows to work on syngenic immunocompetent mice.[21] These tumors are developping rapidly and are highly necrotic. We injected cationic/DNA complexes directly into tumors grown subcutaneously.

Table 7 shows that the three products RPR-120535, 126097, and 130605 displayed significant transfection activity in 3LL tumor, whereas injection of naked DNA was unable to induce any luciferase activity above background (<10^4 RLU/tumor; data not shown), thus suggesting their interest as potential candidates for preclinical development. The presence of a "membrane-anchoring entity" such as arachidonyl group on the substituted linker in RPR-130605 did not enhance gene transfer in this in vivo model.

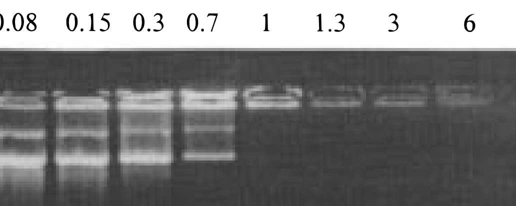

Figure 5. Gel retardation of RPR120535/DNA complexes as a function of lipopolyamine to plasmid DNA charge ratio.

(7) Physicochemical Characterization of DNA/RPR-120535 Complexes. Taking RPR-120535 as a model compound, we performed a primary characterization of lipopolyamine complexes with DNA. In a first approach we performed gel retardation experiments (see Figure 5). Complexes whose amine/phosphate charge ratios (±) ranged from 0 to 0.7 exhibited free DNA migrating normally into the gel, and the gel wells were fluorescent. Therefore in this zone were present both free DNA and RPR-120535/DNA complexes accessible to the ethidium bromide. For RPR-120535/DNA complexes with a charge ratio over 0.7, all DNA was completely associated to RPR-120535 and, consequently, retarded on the gel.

Subsequently, fluorescence experiments were performed by exposing RPR-120535/DNA complexes to ethidium bromide, whose fluorescence increases upon intercalation between DNA base pairs. The fluorescence intensity of each sample is depicted in Figure 6. A large and very sharp decrease of the fluorescence intensity was observed for RPR-120535/DNA charge ratios ranging from 0 to 1. The fluorescence signal was constant and remained low for lipid/DNA complexes

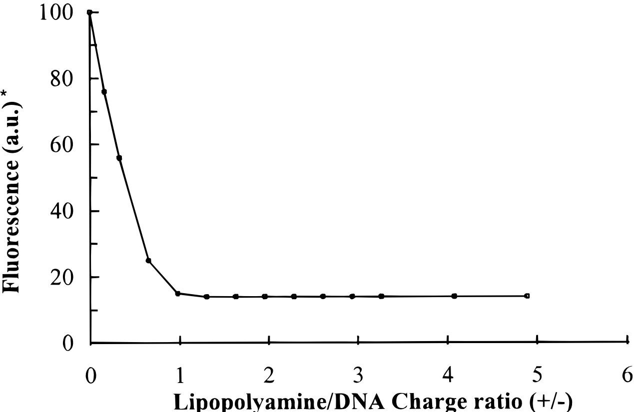

Figure 6. Ethidium bromide fluorescence of RPR120535/DNA complexes as a function of cationic lipid to DNA charge ratio. *a.u. = arbitrary units.

whose charge ratios were over 1. Therefore, plasmid DNA is accessible to ethidium bromide for RPR-120535/DNA charge ratios ranging from 0 to 1 while over the latter ratio the plasmid DNA was unaccessible to ethidium bromide intercalation.

Results from gel electrophoresis retardation and ethidium bromide exclusion suggest that plasmid DNA is likely in a compacted state when complexed with RPR-120535. This compacted state is probably responsible for DNA protection against degradation in the presence of DNAses we have observed (data not shown). Extensive physicochemical studies concerning the supramolecular structure and packaging of DNA/lipid complexes will be reported separately.[22]

Conclusions

We have demonstrated that the use of solid-phase synthesis is a powerful tool for the easy and quick synthesis of a variety of geometrically differing polyamines as lipopolyamine cationic head. The feasibility of the method was demonstrated by the synthesis of a variety of unsymmetrically functionalized polyamines on a solid-phase support and their introduction into lipopolyamines. The SAR studies demonstrate the potential of several products in in vitro as well as in in vivo models. The most active products do not necessitate additives such as DOPE to obtain optimal activity. Although the presence of a guanidinium group and an amine as cationic entities do not afford considerable transfection activity to cationic lipids, the guanidinium groups as a complement to polyamines contributed to a gain in the transfection efficiency at low cationic lipid/DNA charge ratio (data not shown). Most lipopolyamines studied so far bear T-shaped cationic heads. Interestingly, we present here original linear shaped polyamines which display advantageous DNA-complexing property and transfection efficiency. These interesting properties might result from the increased steric flexibility of a less constrained polyamine linked to the lipidic moiety by one of its extremities, allowing a constructive interaction with polyanionic DNA. However, one must be careful in such SAR studies since in vivo data do not necessarily correlate with in vitro results.[7] Advantages in terms of formulation and cytotoxicity of the leading product RPR-120535 were shown as compared to commercially available cationic lipids.

RPR-120531, RPR-120535, and RPR-130605 are currently being developed for preclinical studies, and RPR-121653 and RPR-122761 are being used as molecular probes to study the intracellular traffic of DNA/cationic lipid complexes.[19] The demonstration that introduction of a novel side chain entity by substitution of the linker in the lipopolyamine is permitted opens the field of specific targeting. The synthesis of a novel series of lipopolyamines having specific tissue/cell-targeted side chain entities such as peptides, sugars, or steroids is currently underway.

Experimental Section

Materials and Methods. Triethylamine, diisopropylethylamine, and arachidonic acid were purchased from Aldrich-France and used without further purification, Boc-amino acids, 2-chlorotrityl chloride resin, and BOP reagent were purchased from Neosystem, France. All solvents were analytically pure grade and were used without further purification.

Analytical and semipreparative HPLC were performed on a Merck-Hitachi gradient pump equipped with a AS-2000A autosampler, a L-6200A Intelligent pump, and a UV−vis detector L-4000 with tuneable wavelength set at 220 nm for analytical separations and 235 nm for semipreparative purifications. Preparative HPLC was performed on a Gilson gradient system equipped with two 305 intelligent pumps, a UV−vis Gilson 119 detector with double channel set at 220 and 254 nm, and a fraction collector Gilson 202.

Mobile phases were (A) H_2O (0.1% TFA) and (B) MeCN (0.08% TFA). Separation conditions are as follows: *Analytical:* column BU-300 aquapore Butyl 7m, 300A 300 × 4.6 mm from Perkin-Elmer, gradient H_2O/MeCN: 3 min [40/60], 3−20 min [0/100], 35 min [0/100]; flow, 1 mL/min, l = 220 nm. *Semipreparative:* biosil C18 HL 90-10 250-10 mm from Biorad, gradient H_2O/MeCN; 10 min [100/0], 10−45 min [0/100], 45−140 min [0/100]; flow, 4 mL/min, l = 220 nm. *Preparative:* column C4 Vydac-214 TP 1022 (10 mm) from Vydac, gradient H_2O/MeCN; 0−10 min [70/30], 10−80 min [0/100], 80−120 min [0/100]; flow, 18 mL/min, l = 220 and 254 nm. NMR and MS were carried out at the Analysis Department of Rhône Poulenc Rorer, Vitry sur Seine. ^{1}H NMR spectra were recorded on Brucker 300, 400, and 600 MHz spectrometers. Samples were dissolved in $CDCl_3$ or DMSO. Chemical shifts are in ppm relative to TMS internal standard. Mass spectra were carried out on a VG Autospec by LSIMS technique equipped with a cesium cannon, the matrix was a mixture of glycerol and thioglycerol or nitrobenzyl alcohol (FABMS), and a Perkin-Elmer Sciex API (III) MS. Yields of products [**1**−**5**] were calculated based on an initial loading of 0.6 mmol/g of solid support; the extent of this initial loading has a direct impact on the general yield. Thus, the yields vary depending on the quality and freshness of the starting chlorotrityl chloride resin.

The presence of polyamines in the cationic lipids makes elementary analysis not adequate as a purity criteria. The polyamines are highly hygroscopic, and they can adopt a different salt degree. As prove of our findings, we have performed a complete analysis of the leading product RPR-120535 (annexed document) including MS, IR, and ^{1}H NMR recorded with an Avance DMX 600 Brucker (2D DQF COSY and HOHAHA methods, t = 80 ms) and ^{13}C NMR (heteronuclear 2D techniques HMQC and HMBC with a delay of 50 ms in the reverse mode). Thus, we could unambiguously confirm the proposed structure, although the elementary analysis was not within the ±0.4% purity criteria (see the Experimental Section).

General Procedures. Method 1: Synthesis of Monofunctionalized Polyamines. (A) Anchorage of the Acidic Function to the Polymeric Support. Briefly, *O*-chlorotrityl chloride resin (5 g, 1.2 mmol of Cl/g of resin from Nova) was placed in the solid-phase synthesis flask and 50 mL of CH_2Cl_2 was added, followed by bromoacetic acid (for products **1**−**3**) (1.05 g, 7 mmol) or acrylic acid for product **5** (0.5 g, 7 mmol)

and DIEA (0.95 mL, 7.5 mmol). The flask was placed into a motor flask shaker and was shaken for 2 h at room temperature. The solution was then filtered, and the resin beads were washed alternatively with CH_2Cl_2 and ^iPrOH (3 × 50 mL) and MeOH (2 × 50 mL) and dried on N_2.

(B) Reaction of the Polyamine with the Bromoacetyl Resins. Molar excesses of 10-fold of polyamines were dissolved in 50 mL of DMF or CH_2Cl_2, added to the flask, and agitated for 2 h. The solvent was filtered, and the resin was washed alternatively with CH_2Cl_2 and ^iPrOH (3 × 50 mL). Kaiser tests (cold and 110 °C) are positive.

(C) Protection of the Amine Groups of the Functionalized Polyamine on the Solid Support. Di-*tert*-butyl dicarbonate (48 mmol) and DIEA (50 mmol) were dissolved in dichloromethane (50 mL) and added to the flask shaker; the reaction was left overnight at room temperature under shaking. The solvent was filtrated, and the resin was washed alternatively with CH_2Cl_2 and ^iPrOH (10 × 50 mL), MeOH (2 × 50 mL), and ether (2 × 50 mL) and dried on N_2. The Kaiser test was negative.

(D) Cleavage of the Protected Polyamino Acids from the Resin. The resin was placed in a 250 mL round-bottomed flask equipped with a magnetic stirrer. A solution composed of 50 mL of CH_2Cl_2 and 25 mL of CF_3CH_2OH was added and stirred for 2 h at room temperature. The solution was filtered and the resin washed with 100 mL of CH_2Cl_2. The organic fractions were collected and evaporated. The crude products were purified by flash chromatography on SiO_2 with $CHCl_3$/MeOH (9:1 or 8:2). The fractions containing the good products were identified by TLC and characterized by mass spectroscopy and NMR.

Method 2: Synthesis of Lipopolyamines. The protected carboxyl-functionalized polyamines **1**−**5** obtained by method 1 were reacted with amine components with BOP reagent. Briefly, to a stirred solution of the amino component (1 mmol) in MeCN or DMF were added BOP reagent (1.1 mmol), the acid component (1.1 mmol), and DIEA (3 mmol) at room temperature. After 15 min the pH was checked for basicity (in cases where the pH was lower than pH = 9, more DIEA was added) and the reaction mixture left at room temperature for 1 h. The reaction was followed by fluorescamine test. When the test was negative, the solvent was evaporated, and the crude product was dissolved in EtOAc (100 mL) and washed with $KHSO_4$, $NaHCO_3$, and saturated NaCl. The final Boc-protected products were deprotected using trifluoroacetic acid for 1 h and purified by HPLC. Z or ClZ protecting groups were cleaved as previously described.[23]

BocNH(CH$_2$)$_3$NBoc(CH$_2$)$_4$NBoc(CH$_2$)$_3$NBocCH$_2$CO$_2$H (1). Product **1** was synthesized using method 1: yield 40%; TLC R_f = 0.9 ($CHCl_3$/MeOH, 8:2); HPLC t_R = 4.22 min (H_2O/MeCN: 3 min [40/60], 3−20 min [0/100], 35 min [0/100]), ^1H NMR (400 MHz, $(CD_3)_2SO$-d_6 with some drops of CD_3COOD-d_4, δ in ppm) 1.40 (4 s, 36H, C(CH$_3$)$_3$), 1.46 (mt, 4H, CH$_2$CH$_2$ central butylene), 1.64 and 1.74 (2 mts, 2H each, CH$_2$ central propylene), 2.96 (t, J = 7 Hz, 2H, CH$_2$NCOO), 3.15 (mt, 8H, CH$_2$NCH$_2$), 3.23 (t, J = 7.5 Hz, 2H, CH$_2$NCOO), 3.83 (s, 2H, OCONCH$_2$COO);MH$^+$ 661.

BocNH(CH$_2$)$_3$NBoc(CH$_2$)$_4$N[(CH$_2$)$_3$NHBoc]CH$_2$CO$_2$H (2). During the synthesis of product **1**, product **2** was isolated after SiO_2 purification with a yield of 20%: TLC R_f = 0.5 ($CHCl_3$/MeOH, 8:2); ^1H NMR (400 MHz, $(CD_3)_2SO$-d_6, δ in ppm) 1.30−1.60 (mt, 4H, (CH$_2$)$_2$ centrals butylene), 1.40 (s, 27H, C(CH$_3$)$_3$), 1.56 (mt, 4H, CH$_2$ propylenes), 2.68 and 3.11 (respectively t broad and t, J = 7 Hz, 4H each, NCH$_2$ butylene and NCH$_2$ propylenes), 2.90 and 2.96 (2 q, J = 7 Hz, 2H each, BocNHCH$_2$ propylenes), 3.18 (s, 2H, NCH$_2$COO); MH$^+$ 561.

{BocNH(CH$_2$)$_3$}$_2$N(CH$_2$)$_4$N{(CH$_2$)$_3$NHBoc}(CH$_2$)$_3$NBocCH$_2$CO$_2$H (3). Product **3** was synthesized using method 1: yield 35%; TLC R_f = 0.2 ($CHCl_3$/MeOH, 8:2); ^1H NMR (400 MHz, $(CD_3)_2SO$-d_6 with some drops of CD_3COOD-d_4, temperature of 433 K, δ in ppm) 1.42 (s, 36H, C(CH$_3$)$_3$), 1.56 (mt, 4H, CH$_2$CH$_2$ central butylene), from 1.65 to 1.85 (mt, 8H, CH$_2$ central propylenes), 2.76 (mt, 12H, CH$_2$N(CH$_2$)$_2$), 3.06 (t, J = 6.5 Hz, 6H, OCON CH$_2$), 3.29 (mt, 2H, NCH$_2$), 3.86 (s, 2H, OCONCH$_2$COO); MH$^+$ 775.

{BocNH(CH$_2$)$_2$}$_2$N(CH$_2$)$_2$NBocCH$_2$CO$_2$H 4. Product **4** was synthesized using method 1: yield 29%; TLC R_f = 0.55 ($CHCl_3$/MeOH, 8:2); ^1H NMR (400 MHz, $(CD_3)_2SO$-d_6 temperature of 393 K, δ in ppm) 1.44 (s, 27H, C(CH$_3$)$_3$), 2.58 (t, J = 6.5 Hz, 4H, CH$_2$NCH$_2$), 2.66 (t, J = 7 Hz, 2H, NCH$_2$), 3.04 (q, J = 6.5 Hz, 4H, OCONCH$_2$), 3.28 (t, J = 7 Hz, 2H, OCONCH$_2$), 3.76 (s, 2H, OCONCH$_2$COO), 6.06 (mf, 2H, CONH), MH$^+$ 505.

BocNH(CH$_2$)$_3$NBoc(CH$_2$)$_4$NBoc(CH$_2$)$_3$NBocCH$_2$CH$_2$CO$_2$H (5). Product **5** was synthesized using method 1: yield 40%; TLC R_f = 0.63 ($CHCl_3$/MeOH, 8:2); ^1H NMR (300 MHz, $(CD_3)_2SO$-d_6, δ in ppm) 1.43 (m, 40H, C(CH$_3$)$_3$ and (CH$_2$)$_2$ of butyl), from 1.55 to 1.80 (m, 4H, CH$_2$ of propyl), 2.45 (t, J = 7 Hz, 2H, CH$_2$COO), 2.93 (q, J = 7 Hz, 2H, NCH$_2$), from 3.05 to 3.50 (m, 12H, NCH$_2$), 6.82 (bb, 1H, NHCO); MH$^+$ 675.

RPR-120525. Product **3** was coupled with glycyldioctadecylamide using method 2 and purified by HPLC using semipreparative method: t_R = 38.72 min: ^1H NMR (400 MHz, $(CD_3)_2SO$-d_6. T = 386 K, δ in ppm) 0.90 (t, J = 7 Hz, 6H, CH$_3$), 1.30 (mt, 60H, CH$_2$ central/lipid chains), 1.55 (mt, 4H, 1 CH$_2$ from every lipid chain), 1.65 (mt, 4H, CH$_2$CH$_2$ central/butyl), 1.97 (mt, 8H, CH$_2$ central/propyls); from 2.80 to 3.05−3.06 and 3.28 (mt, 2 t, J = 7.5 Hz, 18H − 2H and 4H, NCH$_2$), 3.80 (s, 2H, NCH$_2$CON), 4.03 (d, J = 5.5 Hz, 2H, CONCH$_2$CON), from 6.00 to 9.00 (mt, NH$_2$ and NH), 8.27 (mt, 1H, CONH); MH$^+$ 935.

RPR-120526. Product **3** was coupled with dioctadecylamine using method 2 and purified by HPLC using semipreparative method: t_R = 38 min; ^1H NMR (400 MHz, $(CD_3)_2SO$-d_6, T = 386 K, δ in ppm) 0.88 (t, J = 7 Hz, 6H, CH$_3$), 1.29 (mt, 60H, CH$_2$ central/lipid chains), 1.52 (mt, 4H, 1 CH$_2$ from each lipid chain), 1.68 (mt, 4H, CH$_2$CH$_2$ centra/butyl), from 1.90 to 2.10 (mt, 8H, CH$_2$ central/propyl), 2.90 from 2.95 to 3.15−3.18 and 3.15 (t, mt and 2 t broad, J = 7.5 Hz, 24H, NCH$_2$), 4.02 (s, 2H: NCH$_2$CON); MH$^+$ 878.

RPR-120527. Product **4** was coupled with dioctadecylamine using method 2 and purified by HPLC using semipreparative method: t_R = 100.1 min; MH$^+$ 708.

RPR-120528. Product **4** was coupled with glycyldioctadecylamide using method 2 and purified by HPLC using semipreparative method: t_R = 122 min; ^1H NMR (400 MHz, $(CD_3)_2SO$-d_6, T = 386 K, δ in ppm) 0.87 (t, J = 7 Hz, 6H, CH$_3$), from 1.15 to 1.35 (mt, 60H, CH$_2$ central/lipid chains), 1.45 and 1.55 (2 mts, 2H each, 1 CH$_2$ from each lipid chain), 2.64 (t, J = 5.5 Hz, 4H, CH$_2$NCH$_2$), 2.75 (t, J = 6 Hz, 2H, NCH$_2$), 2.95 (t, J = 5.5 Hz, 4H, NCH$_2$), 3.08 (t, J = 6 Hz, 2H, NCH$_2$), 3.25 (mt, 4H, NCH$_2$ lipid chains), 3.88 (s, 2H, NCH$_2$CON), 4.06 (d, J = 5 Hz, 2H, CONCH$_2$CON), 7.75 (mf broad/residual, NH), 8.68 (t residual, J = 5 Hz, CONH); MH$^+$ 765.

RPR-120531. Product **1** was coupled with Arg(Z$_2$)dioctadecylamide using method 2. The final deprotected product was purified using HPLC preparative method: t_R = 13.83 min (analytical method); ^1H NMR (400 MHz, $(CD_3)_2SO$-d_6, T = 386 K, δ in ppm) 0.90 (t, J = 7 Hz, 6H, CH$_3$), 1.28 (mt, 60H, CH$_2$ lipid chains), from 1.40 to 1.80 (mt, 12H, CH$_2$), 1.93 (mt, 4H, CH$_2$ central/propyls), from 2.80 to 3.10 (mt, 16H, NCH$_2$ and NCH$_2$ lipid chains), 3.42 (mt, 2H, CH$_2$N amide), 3.77 (mt, 2H, NCH$_2$CON), 4.67 (mt, 1H, NCHCON), from 6.80 to 7.50 (mf broad, 2H, NH$_2$), 7.78−7.92−8.80 and 9.03 (mt and 3mfs, 1H − 2H − 4H and 1H, CONH − NH and NH$_2$); MH$^+$ 920.

RPR-120534. Product **1** was coupled to dioctadecylamine using method 2 and purified using HPLC semipreparative method: t_R = 15.2 min (analytical method); ^1H NMR (400 MHz, mixture of $^2/_3$CF$_3$COOD and $^1/_3$CD$_3$COOD-d_4, δ in ppm) 0.78 (t, J = 7 Hz, 6H, CH$_3$), 1.20 (mt, 60H, CH$_2$ central/lipid chains), 1.52 (mt, 4H, 1 CH$_2$ from each lipid chain), 1.80 (mt, 4H, CH$_2$CH$_2$ central/butyl), 2.23 and 2.32 (2 mts, 2H each, CH$_2$ central/propyls), from 3.10 to 3.40 (3 mts, 16H, NCH$_2$), 4.15 (s, 2H, NCH$_2$CON); MH$^+$ 764.

RPR-120535. Product **1** was coupled to glycyldioctadecylamide using method 2 and purified by HPLC using preparative method: t_R = 15.35 min (analytical method); ^1H NMR (400 MHz, $(CD_3)_2SO$-d_6, T = 300 K, δ in ppm) 0.83 (t, J = 7 Hz,

6H, CH_3), 1.23 (mt, 60H, CH_2 central/lipid chains), 1.43 and 1.53 (2 mts, 2H each, 1 CH_2 from each lipid chain), 1.63 (mt, 4H, CH_2CH_2 central/butyl), 1.96 (mt, 4H, CH_2 central/propyls), 2.93–3.00 and 3.22 (3 mts, 16H, NCH_2), 3.83 (s, 2H, NCH_2-CON), 4.03 (s, 2H, $CONCH_2CON$); MH^+ 821. Anal. ($C_{50}H_{104}N_6O_2$, acetate salt) C, calcd 65.6, found 64.1; H, calcd 11.4, found 12.05; N, calcd 7.9, found 8.16.

RPR-121650. The lipid is synthesized as RPR-120531, but Z groups were not cleaved. The product is purified by HPLC using the preparative method: t_R = 17.75 min (analytical method); ^1H NMR (400 MHz, $(CD_3)_2SO$, δ in ppm) 0.87 (t, J = 7 Hz, 6H, CH_3), 1.25 (mt, 60H, CH_2 central/lipid chains), 1.40 and 1.57 (2 mts, 2H each, 1 CH_2 each lipid chain), 1.65 (mt, 8H, CH_2CH_2 central/butyl), 1.95 (mt, 4H, CH_2 central/propyls), from 2.85 to 3.05 (mt, 14H, NCH_2), 3.23 (t, J = 7.5 Hz, 2H, NCH_2), 3.75 (s, 2H, NCH_2CON), 3.85 and 3.95 (2 mts, 1H each, CH_2NC), 4.67 (mt, 1H, CONCHCON), 5.07 and 5.25 (AB and s, J = 13.5 Hz, 2H each, $NCOOCH_2Ar$), from 7.25 to 7.45 (mt, 10H, H aromatics), 7.95–8.85–9.00 and 9.20 (4 mfs, H exchangeables); MH^+ 1188.

RPR-122759, RPR-121653, and RPR-122761. Product **1** was coupled to Lys(ClZ)dioctadecylamide using method 2 without deprotection. The product was used for the synthesis of RPR-122759, RPR-121653, and RPR-122761. **RPR-122759** was obtained after cleavage using TFA and purification by HPLC using preparative method: t_R = 16.79 min (analytical method); ^1H NMR (400 MHz, $(CD_3)_2SO$-d_6, 373K, δ in ppm) 0.91 (t, J = 7 Hz, 6H, CH_3 lipid chains), 1.31 (mt, 60H, $(CH_2)_{15}$ central/lipid chains), from 1.30 to 1.75 (mt, 10H, 1 CH_2 from each lipid chain, $(CH_2)_3$ central Lys), 1.72 (mt, 4H, $(CH_2)_2$ central/butyl), 1.95 (mt, 4H, CH_2 propyls), 2.98–3.06 and from 2.90 to 3.50 (2 mts and mf, 18H overall, NCH_2 Lys − NCH_2 butyl − NCH_2 propyls and NCH_2 lipid chains), 3.59 (s, 2H, NCH_2CON), 4.75 (q, J = 7 Hz, 1H, CONCHCON Lys), 5.16 (s, 2H, $COOCH_2Ar$), 6.85 (mf, 1H, OCONH), from 7.35 to 7.55 (mt, 5H, H aromatics), 8.15 (mf, 1H, CONH Lys); MH^+ 1060.

RPR121653 was obtained after deprotection of the ClZ group as previously described[23] and reaction of the partially deprotected intermediate (77 mg, 60 mmol) with tetramethyl rhodamine isothiocyanate (30 mg, 68 mmol) in 3 mL of MeOH and in the presence of DIEA (64 mL) overnight. The solution is evaporated in vacuo, and the obtained red colored solid is treated with 4 mL of TFA during 1 h, the TFA is evaporated, and the product is purified by HPLC using semipreparative method: t_R = 61.55 min (semipreparative method); MH^+ 1335.

RPR-122761 was obtained after deprotection of the ClZ group as previously described[23] and coupling of the partially deprotected intermediate with biotine in DMF using method 2. The product was purified by HPLC using semipreparative method: t_R = 13.12 min (analytical method); MH^+1118.

RPR-122766. The lipid was synthesized as RPR-120535 but using glycylditetradecylamide instead of glycyldioctadecylamide. The product was purified by HPLC using semipreparative method: t_R = 9.54 min (analytical method); ^1H NMR (400 MHz, $(CD_3)_2SO$-d_6, 393 K, δ in ppm) 0.90 (t, J = 7 Hz, 6H, CH_3 lipid chains), 1.31 (mt, 44H, $(CH_2)_{11}$ central/lipid chains), 1.58 (mt, 4H, 1 CH_2 from each lipid chain), 1.76 (mt, 4H, $(CH_2)_2$ central/butyl), 2.00 (mt, 4H, CH_2 central/propyls), 2.98 and 3.08 (mt and t, J = 7 Hz, 12H, NCH_2 butyl and NCH_2 propyls), 3.30 (t, J = 7 Hz, 4H, NCH_2 lipid chains), 3.65 (s, 2H, NCH_2CON), 4.06 (d, J = 4 Hz, 2H, $CONCH_2CON$ Gly), 8.10 (mf, 1H, CONH Gly); MH^+ 709.

RPR-122767. The lipid was synthesized as RPR-120535 but using glycyldidodecylamide instead of glycyldioctadecylamide. The product is purified by HPLC using semipreparative method: t_R = 9.92 min (analytical); ^1H NMR (400 MHz, $(CD_3)_2SO$-d_6, 403K, δ in ppm) 0.93 (t, J = 7 Hz, 6H, CH_3 lipid chains), 1.33 (mt, 36H, $(CH_2)_9$ central/lipid chains), 1.58 (mt, 4H, 1 CH_2 from each lipid chain), 1.75 (mt, 4H, $(CH_2)_2$ central/butyl), 1.95 and 2.00 (2 mts, 2H each, CH_2 central/propyls); 2.98 and 3.00 (2 mts, 12H, NCH_2 butyl and NCH_2 propyls), 3.30 (t, J = 7 Hz, 4H, NCH_2 lipid chains), 3.58 (s, 2H, NCH_2CON), 4.05 (s, 2H, $CONCH_2CON$ Gly); MH^+ 653.

RPR-122774. The lipid was synthesized as RPR-120535 but using glycylditridecyldecylamide instead of glycyldioctadecylamide. The product is purified by HPLC using the semipreparative method: t_R = 10.64 min (analytical); ^1H NMR (400 MHz, $(CD_3)_2SO$-d_6, 393 K, δ in ppm) 0.91 (t, J = 7 Hz, 6H, CH_3 lipid chains), 1.33 (mt, 40H, $(CH_2)_{10}$ central/lipid chains), 1.58 (mts, 4H, 1 CH_2 from each lipid chain), 1.75 (mt, 4H, $(CH_2)_2$ central/butyl), 2.00 (mt, 4H, CH_2 central/propyls), 2.98 and 3.08 (2 t, J = 7 Hz, 12H, NCH_2 butyl and NCH_2 propyls), 3.32 (t, J = 7 Hz, 4H, NCH_2 lipid chains), 3.65 (s, 2H, NCH_2CON), 4.06 (d, J = 4 Hz, 2H, $CONCH_2CON$ Gly), 8.60 (s large, 1H, CONH Gly); MH^+ 681.

RPR-122786. In a 500 mL round-bottomed flask are poured spermine (1.8 g, 9 mmol), methanol (60 mL), and $NaCNBH_3$ (0.138 g, 2.3 mmol). The solution is vigorously stirred, and 35.5 mL of a solution composed of 5.5 mL of succinic semialdehyde 15% and 30 mL of methanol is added dropwise during 2 h. The reaction mixture is left for additional 2 h and the amines protected with di-*tert*-butyl dicarbonate as usual. The solution is evaporated and the crude product redissolved in ethyl acetate (100 mL) and extracted with saturated $NaHCO_3$ (3 × 100 mL). The aqueous phase is washed with ether (3 × 50 mL) and 0/5 M $KHSO_4$ is added until pH = 3. Turbidity is observed, and the aqueous phase is extracted with ethyl acetate (3 × 100 mL). The organic layer is dried on $MgSO_4$ and evaporated in vacuo. The product, $BocNH(CH_2)_3NBoc(CH_2)_4NBoc(CH_2)_3NBoc(CH_2)_3CO_2H$, was coupled with dioctadecylamine using method 2. The obtained product is purified by HPLC using semipreparative conditions: t_R = 15.04 min.(analytical); ^1H NMR (400 MHz, $(CD_3)_2SO$-d_6, 383 K, δ in ppm) 0.85 (t, J = 7 Hz, 6H, CH_3 lipid chains), 1.22 (mt, 60H, $(CH_2)_{15}$ central lipid chains), 1.48 (mf, 4H, 1 CH_2 from each lipid chain), 1.72 (mt, 4H, $(CH_2)_2$ central/butyl), 1.88 (mt, 2H, CH_2 central NH-pentanoyl), 1.99 (mt, 4H, CH_2 propyls), 2.42 (t, J = 7 Hz, 2H: $COCH_2$ from NH-pentanoyl), 2.96–3.03 and 3.22 (3 mts, 18H, NCH_2 from NH-pentanoyl − NCH_2 butyl − NCH_2 propyls and NCH_2 lipid chains); MH^+ 792.

RPR-126096. The lipid was synthesized as RPR-120535 but using product **2** instead of product **1**. The product is purified by HPLC using preparative method: t_R = 13.6 min (analytical); ^1H NMR (400 MHz, $(CD_3)_2SO$-d_6 with some drops CD_3COOD-d_4, δ in ppm) 0.87 (t, J = 7 Hz, 6H, CH_3 lipid chains), 1.28 (mt, 60H, $(CH_2)_{15}$ central lipid chains), 1.46 and 1.54 (2 mts, 2H each, 1 CH_2 from each lipid chain), 1.63 (mt, 4H, $(CH_2)_2$ central/butyl), 1.91 (mt, 4H, CH_2 propyls), from 2.85 to 3.15 (mt, 12H, NCH_2 butyl and NCH_2 propyls), 3.24 (mt, 4H, NCH_2 lipid chains), 3.76 (mf, 2H, NCH_2CON), 4.05 (s broad, 2H, $CONCH_2CON$ from Gly); MH^+ 821.

RPR-126097. The lipid was synthesized as RPR-120535 but using Glu(Bz)dioctadecylamide instead of Gly-dioctadecylamide. The Bz group was cleaved as previously described.[23] The product is purified by HPLC using preparative method: t_R = 14.64 min (analytical); ^1H NMR (400 MHz, $(CD_3)_2SO$-d_6, 383 K, δ in ppm) 0.90 (t, J = 7 Hz, 6H, CH_3 lipid chains), 1.30 (mt, 60H, $(CH_2)_{15}$ central lipid chains), 1.56 (mf, 4H, 1 CH_2 from each lipid chain), from 1.60 to 2.00 (mt, 2H, CH_2 central Glu), 1.73 (mt, 4H, $(CH_2)_2$ central/butyl), 1.98 (mt, 4H, CH_2 propyls), 2.32 (t, J = 7 Hz, 2H, $COCH_2$Glu), 3.00–3.06 and 3.45 (t and 2 mts, J = 7 Hz, 16H, NCH_2 butyl − NCH_2 propyls and NCH_2 lipid chains), 3.65 (s broad, 2H, NCH_2CON), 4.85 (mt, 1H, CONCHCON Glu), 8.19 (s broad, 1H, CONH Glu); MH^+ 893.

RPR-127888. Product RPR-122759 is deprotected as previously reported to give RPR-127888. The product is purified by HPLC using semipreparative method: t_R = 11.76 min (analytical); ^1H NMR (400 MHz, $(CD_3)_2SO$-d_6, 393 K, δ in ppm) 0.91 (t, J = 7 Hz, 6H, CH_3 lipid chains), 1.31 (mt, 60H, $(CH_2)_{15}$ central/lipid chains), from 1.35 to 1.75 (mt, 10H, 1 CH_2 from each lipid chain, $(CH_2)_3$ central/Lys), 1.75 (mt, 4H, $(CH_2)_2$ central/butyl), 2.00 (mt, 4H, CH_2 propyls), 2.82–2.98–3.06 and from 3.10 to 3.50 (2 t − mt and 2mfs, J = 7 Hz, 18H, NCH_2 Lys − NCH_2 butyl − NCH_2 propyls and NCH_2 lipid chains),

3.62 (s, 2H, NCH_2CON), 4.73 (q, $J = 7$ Hz, 1H, CONCHCON Lys), 8.18 (d, $J = 7$ Hz, 1H, CONH Lys); MH^+ 892.

RPR-128506. The lipid was synthesized as RPR-120535 but using 6-(aminocaproyl)dioctadecylamide instead of glycyldiocadecylamine. The product was purified by HPLC using semipreparative method: $t_R = 13.94$ min (analytical); ^1H NMR (300 MHz, $(CD_3)_2SO$-d_6, δ in ppm) 0.87 (t, $J = 7$ Hz, 6H, CH_3 lipid chains), 1.28 (mt, 60H, $(CH_2)_{15}$ central/lipid chains), 1.48 (mt, 10H, 1 CH_2 from each lipid chain and $(CH_2)_3$ central/aminohexanoyl), 1.65 (mt, 4H, $(CH_2)_2$ central/butyl), 1.95 (mt, 4H, CH_2 propyls), 2.27 (t, $J = 7$ Hz, 2H, $COCH_2$ aminohexanoyl), from 2.85 to 3.30 (mts, 18H, NCH_2 aminohexanoyle − NCH_2 butyl − NCH_2 propyls and NCH_2 lipid chains), 3.70 (s broad, 2H, NCH_2CON), from 7.90 to 9.10 (mfs exchangeables); MH^+ 877.

RPR-130605. The lipid was synthesized as RPR-121653 but using arachidonic acid instead of rhodamine−isothiocyanate, which was coupled to the intermediate semiprotected product using method 2. The product was purified by HPLC using the semipreparative method: $t_R = 20.67$ min; ^1H NMR (400 MHz, $(CD_3)_2SO$-d_6 some drops of CD_3COOD-d_4, 393 K, δ in ppm) 0.90 (t, $J = 7$ Hz, 6H, CH_3 lipid chains), 0.91 (t, $J = 7$ Hz, 3H, arachidonyl), 1.31 (mt, 60H, $(CH_2)_{15}$ central/lipid chains), from 1.35 to 1.75 (mt, 18H, 1 CH_2 from each lipid − $(CH_2)_3$ central and CH_2 central/arachidonyl and $(CH_2)_3$ central/Lys), 1.75 (mt, 4H, $(CH_2)_2$ central/butyl), 2.02 (mt, 4H, CH_2 propyls), 2.10 (mt, 6H, $COCH_2$ and two $=CCH_2$ from arachidonyl), 2.80−2.97−3.06 and from 3.10 to 3.50 (mt − t − mt and 2 mfs, $J = 7$ Hz, 24H, $=CCH_2C=$ from arachidonyl − NCH_2 Lys − NCH_2 butyl − NCH_2 propyls and NCH_2lipid chains), 3.62 (s, 2H, NCH_2CON), 4.73 (dd, $J = 8$ and 5 Hz, 1H, CONCHCON Lys), 5.38 (mt, 8H, CH=CH from arachidonyl); MH^+ 1177.

***In Vitro* Transfections.** 5×10^4 cells were plated in 24-well plates in Dubelcco Modified Eagle Medium (DMEM) for NIH3T3 and Hela cells and in Minimum Essential Medium (MEM) for CV1 cells at 37 °C in a humidified atmosphere containing 5% CO_2 so that the growth was exponential. Cells were transfected after 24 h of culture at 50/70% confluency. Immediately prior to transfection, cells were washed twice with 0.5 mL of serum free medium and then supplemented with 0.5 mL of serum free medium. Plasmid DNA containing the luciferase gene under the dependency of the cytomegalovirus (CMV) immediate early promoter purified according to standard procedure was mixed with a variable quantity of cationic lipid in 75 mM NaCl final concentration. The stock solution of cationic lipid (5 mM) was in aqueous solution. Four ratios of cationic lipid per DNA were studied, varying from 2 to 12 nmol of cationic lipid/mg of DNA. For each condition a triplicate determination was performed (1 mg of plasmid DNA/well). Extemporaneous mixing of DNA with cationic lipids was added dropwise to the cells. After 2 h of transfection, fetal calf serum was added (to a 10% final concentration). The cells were then incubated at 37 °C for an additional period of 44 h up to the evaluation of the efficiency of gene transduction.

For each cationic lipid, we have reported in the tables the luciferase activity observed at the optimal cationic lipid/DNA transfection ratio (generally 6 nmol of cationic lipid/mg of DNA).

***In Vivo* Gene Transfer.** Lewis lung carcinoma tumors were passaged as explant form mouse to mouse. Typically, tumor fragments were inoculated subcutaneously into the flank of adult C57/BL6 mice. Tumors were injected with the plasmid-containing solution one week after inoculation, when the tumors reached a size around 100 mm^3. Plasmid used was pXL 2621 derived from pGL2 control vector (Promega), in wich the SV40 promoter has been replaced by the Mlu*I*/Hin*dIII* fragment of the CMV promoter extracted from pCDNA3 (in vitrogen). Briefly, plasmid was dissolved in a NaCl 150 mM, D-glucose 5%, Mes pH 6.2, 5 mM solution, to a final concentration of 0.1 mg/mL. Lipopolyamines were prepared as a 20 mM stock solution in ethanol, and the required amount of this solution was added to the plasmid-containing preparation. After at least 30 min of incubation at room temperature, 100 mL of this solution was injected directly into the tumors using a Hamilton syringe. Evaluation of luciferase activity was assessed in tumor homogenates 48 h after DNA injection.

Luciferase Assay for *in Vitro* and *in Vivo* Assays. Luciferase activity (Promega assay system) was monitored using a Berthold luminometer (10 s monitoring, 10^6 relative light units (RLU) equivalent to 40 pg of luciferase). Tumor or cell samples were homogenized in Promega cell lysis buffer and, after centrifugation, 10 mL of supernatant was mixed with 50 mL of luciferase assay substrate (Promega). Protein was determined using Pierce assay system.

Physicochemical Characterization. Agarose Gel Electrophoresis. RPR120535/DNA samples containing 1 mg of DNA were loaded on a 0.8% agarose gel.

Fluorescence Studies. Fluoresence measurements were carried out on a Perkin-Elmer LS50 B spectrofluorophotometer, with slits of excitation and emission of 5 nm. Ethidium bromide was added to solutions containing complexes prepared at 10 mg of DNA/ml followed by the addition of ethidium bromide to a final concentration of 5 mM. All sample readings were recorded using an excitation wavelength of 260 nm and emission wavelength of 590 nm, and values were expressed as arbitrary fluorescence units. Measurements were conducted in a medium containing 150 mM NaCl.

Abbreviations: Boc, *tert*-butoxycarbonyl; BOP, (benzotriazolyloxy)tris(dimethylamino)phosphonium hexafluorophosphate; DC-Chol, 3β-[*N*-(*N*,*N*-dimethylamino)ethyl]carbamoyl] cholesterol; DDAB, dimethyldioctadecylammonium bromide; DIEA, diisopropylethylamine; DLRIE, (±)-*N*-(3-aminopropyl)-*N*,*N*-dimethyl-2,3-bis(dodecyloxy)-1-propanaminium bromide; DMRIE, 1,2-dimyristoyloxypropyl-3-dimethylhydroxyethylammonium bromide; DNA, deoxyribonucleic acid; DOGS, dioctadecylamine−glycyne−spermine; DOPE, dioleoylphosphatidylethanolamine; DOSPA, 2,3-dioleoyloxy-*N*-(2-(sperminecarboxamido)ethyl)-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate; LARLU, luciferase activity in relative light units; Z, benzyloxycarbonyl.

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