

Cation Substitution in Cationic Phosphonolipids: A New Concept To Improve Transfection Activity and Decrease Cellular Toxicity

Virginie Floch,^{*,†,§} Séverine Loisel,^{†,§} Erwann Guenin,[‡] Anne Cécile Hervé,[‡] Jean Claude Clément,[‡] Jean Jacques Yaouanc,[‡] Hervé des Abbayes,[‡] and Claude Férec[†]

Centre de Biogénétique, CHU, ETSBO, BP 454, 29275 Brest Cedex, France, and Laboratoire de Chimie Organique des Eléments de Transition, UBO, UMR CNRS 6521, UFR Sciences, BP 809, 29285 Brest Cedex, France

Received January 5, 2000

Cationic lipids have been shown to be an interesting alternative to viral vector-mediated gene delivery into *in vitro* and *in vivo* model applications. Prior studies have demonstrated that even minor structural modifications of the lipid hydrophobic domain or of the lipid polar domain result in significant changes in gene delivery efficiency. Previously, we developed a novel class of cationic lipids called cationic phosphonolipids and described the ability of these vectors to transfer DNA into different cell lines and *in vivo*. Up until now, in all new cationic lipids, nitrogen atoms have always carried the cationic or polycationic charge. Recently we have developed a new series of cationic phosphonolipids characterized by a cationic charge carried by a phosphorus or arsenic atom. In a second step, we have also examined the effects of the linker length between the cation and the hydrophobic domain as regards transfection activity. Transfection activities of this library of new cationic phosphonolipids were studied *in vitro* in different cell lines (HeLa, CFT1, K562) and *in vivo* using a luciferase reporter gene. A luminescent assay was carried out to assess luciferase expression. We demonstrated that cation substitution on the polar domain of cationic phosphonolipids (N → P or As) results in significant increase in transfection activity for both *in vitro* and *in vivo* assays and decrease of cellular toxicity.

Introduction

Cationic lipids have been shown to be an interesting alternative to viral vector-mediated gene delivery into *in vitro* and *in vivo* animal model applications. A large number of families of cationic vectors have now been synthesized.^{1,2} Cationic lipids can be defined as comprising three principal structural features: a cationic polynucleotide binding domain generally composed of a quaternary ammonium group, a polyamine, or a guanidine; a hydrophobic domain composed of two carbon chains (saturated or not) or a cholesteryl group; and a linker or spacer (ester, ether, amide, phosphate, or phosphonate) that binds the polar headgroup with the lipid moiety. Prior studies have demonstrated that even minor structural modifications of the lipid hydrophobic domain or of the lipid polar domain result in significant changes in gene delivery efficiency.^{3–7} Previously, we developed a novel class of cationic lipids called cationic phosphonolipids⁸ and described the ability of these vectors to transfer DNA into different cell lines.^{9–11} More recently, we have reported significant data about transfection efficiency of these new vectors in mouse lungs after intratracheal¹² or intravenous administration.¹³ Two recent studies^{14,15} reported transgene expression in pulmonary epithelial cells (type II alveolar epithelial cells) after systemic delivery of cationic lipid/DNA complexes. These data led us to think that intravenous delivery could be a potential mode of

administration for the gene therapy of lung diseases, particularly cystic fibrosis.

As well as improving transfection activity, minimizing the toxic side effects of cationic lipids is actually an important step in this field of research. Much effort has been devoted to resolving the toxicity problem of cationic lipid-mediated gene transfer.^{16–19} With the aim of developing efficient but less toxic cationic phosphonolipids, a new series of phosphonolipids was designed and synthesized. Up until now, several studies have been carried out to improve cationic lipids including different structural modifications.^{20–23} Nevertheless, in all these new cationic lipids, the cationic or polycationic charge has always been carried by nitrogen atoms, with the exception of the work of Haces and Ciccarone described in the patent literature.²⁴ On the basis of the results of Stekar et al.,²⁵ who demonstrated that nitrogen atom substitution by a phosphorus or arsenic atom in a lipid used as an antitumoral agent maintained biological activity but decreased cellular toxicity, we have developed a series of cationic arsenium and phosphonium phosphonolipids.²⁶ In a second step, we have also examined the effects of the linker length between the cation and the hydrophobic domain with regard to transfection activity and cellular toxicity. Transfection activities and toxic effects of this library of new cationic phosphonolipids were studied *in vitro* in different cell lines, and *in vivo* activity was also tested. We demonstrate that structural modifications in cationic phosphonolipids result in significant changes in both transfection activity and cellular toxicity.

* To whom correspondence should be addressed. Phone: 33 2 98 44 50 64. Fax: 33 2 98 01 81 24. E-mail: virginie.floch@univ-brest.fr.

[†] ETSBO.

[‡] UFR Sciences.

[§] The first two authors contributed equally to this work.

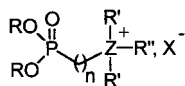


Figure 1. General structures of cationic phosphonolipids and synthesis: R = C₁₄H₂₉, C₁₈H₃₅; n = 1, 2, 3; R' = Me, ⁱPr; R'' = Me, Et, Pr, ⁱPr, Bu, C₂H₄N(Me)₃; X = halogen.

Table 1. Formula of Cationic Phosphonolipids Used

R	R'	R''	Z	n	X	compd
C ₁₈ H ₃₅	CH ₃	CH ₃	N	1	I	GLB43
C ₁₄ H ₂₉	CH ₃	CH ₃	N	1	I	GLB73
C ₁₄ H ₂₉	CH ₃	C ₂ H ₅	N	1	I	GLB391
C ₁₄ H ₂₉	CH ₃	C ₃ H ₇	N	1	I	GLB566
C ₁₄ H ₂₉	CH ₃	C ₄ H ₁₀	N	1	I	GLB570
C ₁₄ H ₂₉	CH ₃	CH ₃	N	2	I	ACH164
C ₁₈ H ₃₅	CH ₃	CH ₃	N	2	I	ACH194
C ₁₄ H ₂₉	CH ₃	CH ₃	N	3	I	ACH201
C ₁₈ H ₃₅	CH ₃	CH ₃	N	3	I	ACH204
C ₁₄ H ₂₉	CH ₃	CH ₃	P	1	I	EG176
C ₁₄ H ₂₉	CH ₃	C ₂ H ₅	P	1	I	EG175
C ₁₄ H ₂₉	CH ₃	C ₃ H ₇	P	1	I	EG202
C ₁₄ H ₂₉	CH ₃	C ₄ H ₁₀	P	1	I	EG203
C ₁₄ H ₂₉	CH ₃	<i>i</i> -C ₃ H ₇	P	1	I	EG172
C ₁₄ H ₂₉	<i>i</i> -C ₃ H ₇	CH ₃	P	1	I	EG300
C ₁₄ H ₂₉	<i>i</i> -C ₃ H ₇	<i>i</i> -C ₃ H ₇	P	1	I	EG315
C ₁₈ H ₃₅	CH ₃	CH ₃	P	1	I	EG343
C ₁₄ H ₂₉	CH ₃	CH ₃	P	2	I	EG308
C ₁₈ H ₃₅	CH ₃	CH ₃	P	2	I	EG316
C ₁₄ H ₂₉	CH ₃	CH ₃	P	3	I	EG327
C ₁₈ H ₃₅	CH ₃	CH ₃	P	3	I	EG353
C ₁₄ H ₂₉	CH ₃	CH ₃	As	1	I	EG373
C ₁₄ H ₂₉	CH ₃	CH ₃	As	2	I	EG371
C ₁₄ H ₂₉	CH ₃	CH ₃	As	3	I	EG356
C ₁₈ H ₃₅	CH ₃	CH ₃	As	2	I	EG372
C ₁₈ H ₃₅	CH ₃	CH ₃	As	3	I	EG363

Results

1. Cationic Phosphonolipid Synthesis. Figure 1 shows the general structure of cationic phosphonolipids. The different compounds tested in this report and the chemical modifications carried out on the different parts of the molecule are summarized in Table 1. Ammonium methylenephosphonates were obtained as previously described⁸ through the Mannich reaction of fatty phosphites with secondary amines in the presence of formaldehyde, followed by quaternarization of the intermediate dialkylaminophosphonates with a suitable alkyl halide (Figure 2). *N,N,N*-Trialkyldiaminophosphonates were also prepared according to this procedure, but their bis-quaternarization with alkyl halides failed, leading only to an ω -monoammonium salt which, prior to the second quaternarization, underwent *O*-alkyl bond cleavage by nucleophilic attack of the halide ion in an Arbuzov-like reaction. Fortunately, replacing alkyl halides by the powerful methylating agent, methyl trifluoromethanesulfonate, resulted in a clean and rapid double reaction at the two nitrogen atoms leading to the bis-ammonium derivatives.

Owing to the distinctive reactivity of phosphines and arsines with regard to amines, the Mannich reaction could not be extended to the synthesis of the phosphonium and arsonium analogues ($n = 1$). Thus, a different approach was envisioned, involving the ylide chemistry of tetraalkylphosphonium and -arsonium salts R₄Z⁺, X⁻ (Figure 3A). These salts, on reaction with *n*-butyllithium, gave readily the corresponding ylides which were directly condensed with a fatty chlorophosphate to yield, after acidification and metathesis, the expected onium methylenephosphonates in good yields.

Higher homologues ($n = 2, 3$) required the stepwise synthesis of fatty ω -bromoalkylphosphonates (Figure 3B): The Arbuzov reaction of ω, ω' -dibromoalkanes with triethyl phosphite²⁷ gave the corresponding diethyl ω -bromoalkylphosphonates which cannot however be easily transesterified with fatty alcohols. Thus the bis-trimethylsilyl esters obtained by treatment of diethyl ω -bromoalkylphosphonates with an excess of bromotrimethylsilane²⁸ were reacted with oxalyl chloride in the presence of a catalytic amount of DMF to yield the more reactive phosphonodichlorides according to the method of Bhongle,²⁹ which then could be esterified with a suited fatty alcohol. In the case of $n = 2$, according to the reaction conditions, the fatty vinylphosphonate could be formed to some extent during the esterification step. Treatment of this mixture with triethylamine in refluxing THF allowed the complete dehydrohalogenation to the vinyl species which proved to be a very convenient synthon, since the Michael 1,4-addition of secondary amines or acidic hydrogenophosphonium salts (Figure 3C) yielded quantitatively the corresponding onium salts (after an additional quaternarization step for the ammonium salt). Finally, arsonium ethylenephosphonates and onium propylenephosphonates (Z = N, P, As) were obtained (Figure 3D) through a classical S_N2 substitution of the corresponding ω -bromoalkylphosphonates by trimethylarsine, trimethylphosphine, and dimethylamine (in this latter case the ammonium salt was obtained after reaction with methyl iodide).

2. Polar Headgroup Substitution in Cationic Phosphonolipid Ammonium Compounds. The prototype GLB73 compound contains C_{14:0} alkyl chains and a polar headgroup comprising a quaternary trimethylammonium. The addition of alkyl groups with increasing chain length on the ammonium group has been described to modify transfection activity by increasing the cross-sectional area of the headgroup.³ To check this hypothesis, a series of cationic phosphonolipid analogues, each containing C_{14:0} aliphatic chains, and increasing alkyl chain lengths were synthesized. To compare the transfection activities of four cationic phosphonolipid analogues, K562, CFT1, and HT29 cells were assayed for luciferase expression 48 h after transfection. The results obtained using the chemiluminescent assay are presented in Figure 4. In our experimental conditions, and whatever the cell line used, the highest levels of plasmid transfection activity were observed with cationic phosphonolipids which incorporate a methyl or ethyl substitution on the quaternary ammonium group in the polar domain (GLB73 and GLB391). Moreover in vitro transfection results obtained using these two cationic phosphonolipids were significantly higher than with Lipofectin reagent ($p < 0.05$). When the propyl- or butylalkyl groups were used, the activity of the analogues significantly decreased as the alkyl chain length increased ($p < 0.05$). Furthermore we also studied the lengthening of the alkyl chain on phosphonium analogues without much more success (results not shown). Thus, after these first results we were inclined to use cationic phosphonolipids bearing a trimethylonium moiety.

3. Nitrogen Atom Substitution. Then, cationic phosphonolipid analogues each containing C_{14:0} aliphatic

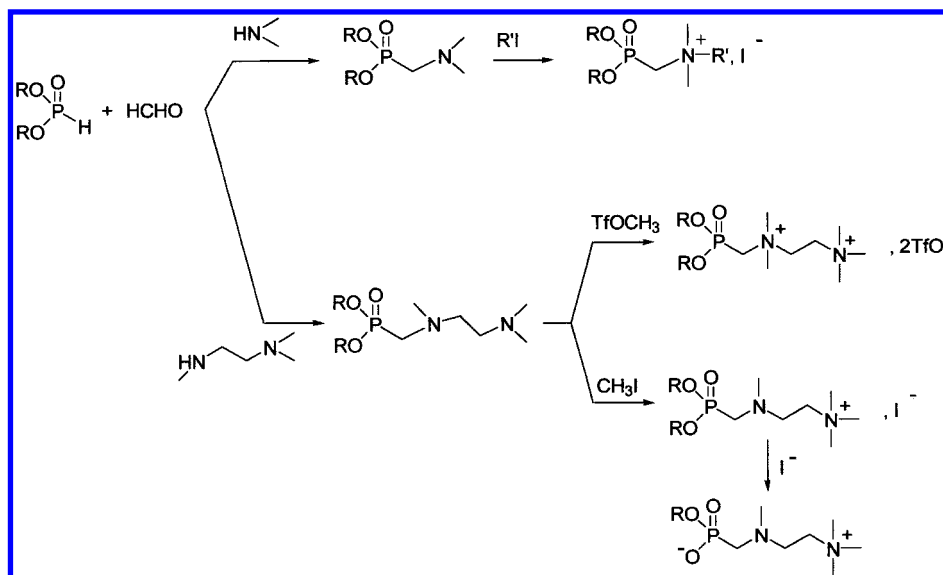


Figure 2. General synthesis of ammonium and diammonium methylenephosphonates: $R = C_{14}H_{29}, C_{18}H_{35}$.

chains and variable quaternary trimethylonium were synthesized. To check the influence on transfection activity of cation substitution in cationic phosphonolipids, a phosphonium or arsonium was introduced instead of the ammonium group. The transfection efficiency of two cationic phospholipid analogues containing a phosphonium or arsonium group was compared with the GLB73. Results obtained on K562 and HeLa cells are summarized in Figure 5. Using these two first GLB73 analogues (EG176 and EG 373), we show that substitution of the quaternary ammonium polar head by a phosphonium or arsonium salt led us to obtain a maintained transfection efficiency.

4. Nitrogen Atom Substitution, Linker, and Hydrophobic Chain Length Variation. To approach the chemical structure of natural phospholipids, cationic phosphonolipids including an ethylene or propylene linkage instead of methylene were synthesized. The influence of these linker length variations on quaternary trimethylonium (N^+ , P^+ , As^+) transfection activity was studied. Moreover, to study the influence of the hydrophobic chain length on transfection activity in vitro, results obtained with dioleoyl compounds were compared with their dimyristyl analogues. Gene transfer efficiencies of 17 cationic phosphonolipid analogues were compared 48 h after transfection using a chemiluminescent luciferase detection assay. The results obtained with the K562 nonadherent cell line are summarized in Figures 6. In our experimental conditions, and using quaternary trimethylammonium analogues (Figure 6A), the highest transfection activity was obtained when the methylene linkage was included between the hydrophobic and polar domains of the cationic phosphonolipid ($p = 0.003$). When the quaternary ammonium polar head was substituted by a phosphonium (Figure 6B) or an arsonium (Figure 6C), the best transfection activity was observed with an ethylene linkage ($p < 0.006$). Results suggest that lengthening both the atomic bulk of the headgroup cation and the linker length must be combined to improve the transfection activity of cationic phosphonolipids. The same results were obtained on adherent cell lines (data not shown). In our experimental conditions and whatever the quaternary trimethyl-

oniums (N^+ , P^+ , As^+) used, the highest transfection activities were always obtained with the dimyristyl analogues.

5. In Vitro Cytotoxicity of the Cationic Phosphonolipid Compounds. To test hypotheses pertaining to cation substitution decreasing cellular toxicity, nine dimyristyl cationic phosphonolipids including different oniums (N^+ , P^+ , As^+) and variable linker length were tested for transfection activity and cytotoxicity on the K562 cell line. Cationic phosphonolipids were evaluated for cytotoxicity index and luciferase expression at the same time. Each transfection experiment was carried out in duplicate (25 000 cells/well), one well contributing to the activity measurement, the other to the viability assay. An overlay of transfection activity versus cytotoxicity index obtained on the K562 cell line is presented in Figure 7. The lowest cytotoxicity index was obtained when the transfection assay was carried out using tetraalkylphosphonium or -arsonium compounds. Moreover an increase in linker length was correlated with a decrease in the cytotoxicity index. These data suggest that the combination of cation substitution and the growth in linker length lead to both good transfection efficiency and low cellular toxicity in vitro.

6. In Vivo Cytotoxicity of the Cationic Phosphonolipid Compounds. A last series of experiments was carried out in vivo to confirm the in vitro results of transfection activity improvement by cation substitution. Luciferase production in mouse lungs was measured 24 h after the intravenous administration of 18 different cationic phosphonolipids formulations, using an optimal lipid/DNA ratio previously determined.¹³ Results obtained are summarized in Figure 8. The luciferase levels observed after transfection using quaternary trimethylarsoniums or -phosphoniums are significantly higher than those obtained with the ammonium analogues. There is no significant difference between phosphonium and arsonium compound transfection activities. Besides the influence of cation substitution and the lengthening of linker on transfection activity, we also wanted to examine whether the hydrophobic moiety of cationic phosphonolipids plays an important role in in vivo transfection efficiency. Sub-

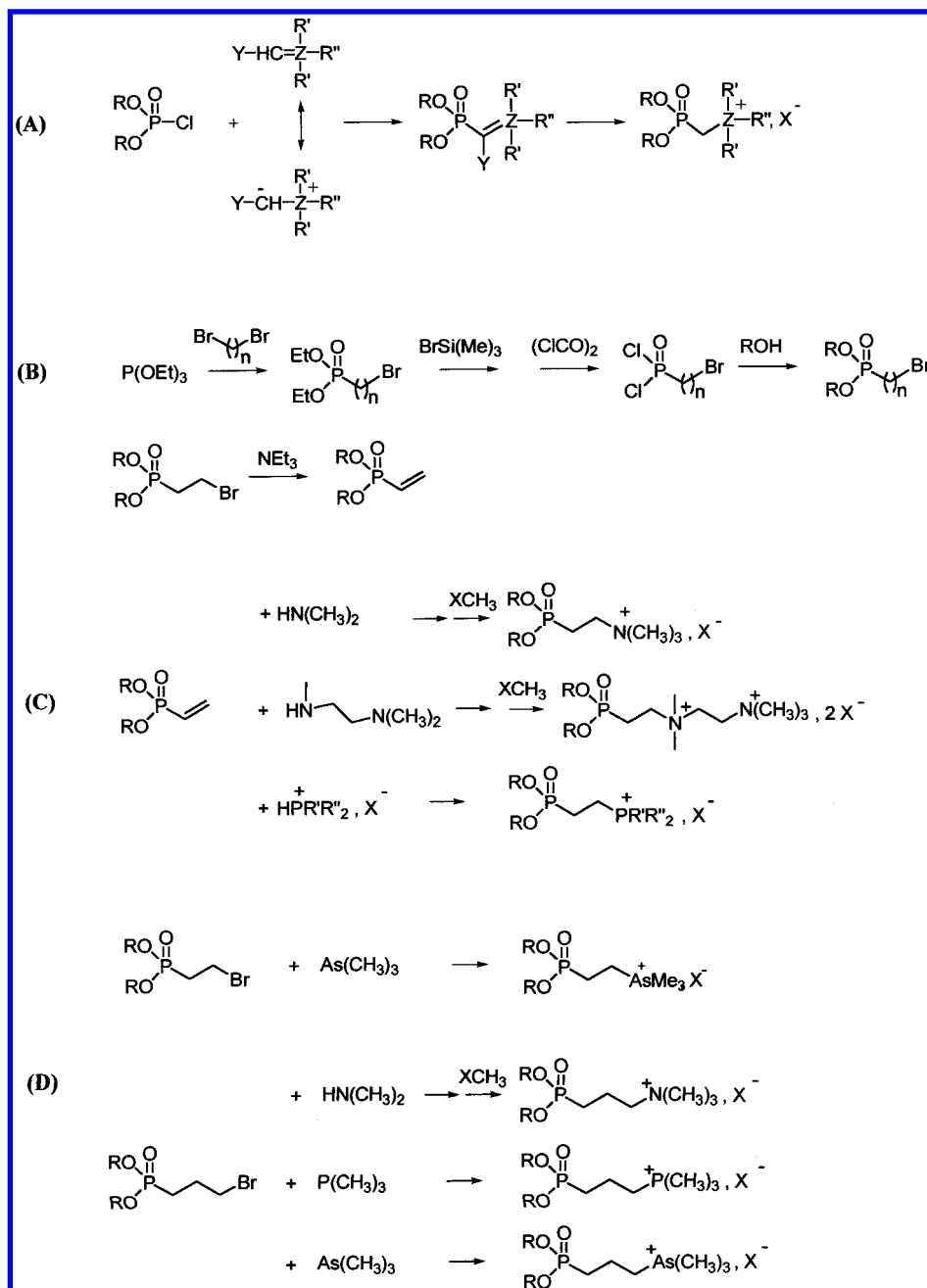


Figure 3. General synthesis of new cationic phosphonates: (A) R = C₁₄H₂₉, C₁₈H₃₅; Y = H, SiMe₃; R' = Me, ^tPr; R'' = Me, Et, Pr, ⁱPr, Bu; X = halogen; (B) R = C₁₄H₂₉, C₁₈H₃₅; n = 1, 2, 3; (C) R = C₁₄H₂₉, C₁₈H₃₅; R' = Me, ^tPr; R'' = Me, ⁱPr; X = halogen; (D) R = C₁₄H₂₉, C₁₈H₃₅; X = halogen.

sequently, we systematically examined the transfection activity of C_{14:0} compounds and those of their C_{18:1} analogues. Six different cationic phosphonolipids including variable hydrophobic chain length and ammonium, phosphonium, or arsonium polar headgroup were tested for in vivo transfection activity. Whatever the quaternary trimethylnium used, in vivo transfection efficiency of compounds including a C_{18:1} chain was higher than with the C_{14:0} analogues. Interestingly and as we previously reported,¹³ opposite results were observed in vitro (data not shown).

The inclusion of neutral lipids in cationic lipid formulations has been previously described as being an important factor for the in vitro transfection activity of cationic lipids.^{7,30} To test the importance of this parameter in vivo, three types of formulation were prepared for intravenous administration. These included lipoplex

with either cationic phosphonolipid alone or those containing equal amounts of cationic phosphonolipid and a neutral lipid, which was either DOPE or cholesterol. Depending on the type of quaternary trimethylnium, the inclusion of neutral lipid into cationic liposomes appears to have different effects on lipoplex transfection activity in vivo. For quaternary trimethylammoniums, inclusion of a neutral lipid decreased the level of gene expression in mouse lungs. Interestingly inclusion of cholesterol into quaternary trimethylphosphoniums or -arsoniums improved transfection efficiency. Compared to the effect of cholesterol, inclusion of DOPE into these last types of liposomes significantly decreased transfection activity ($p < 0.05$).

7. Determination of in Vivo Toxic Effects. In vivo toxic effects of transfection mediated by our cationic phosphonolipids or DOTMA were assessed using a

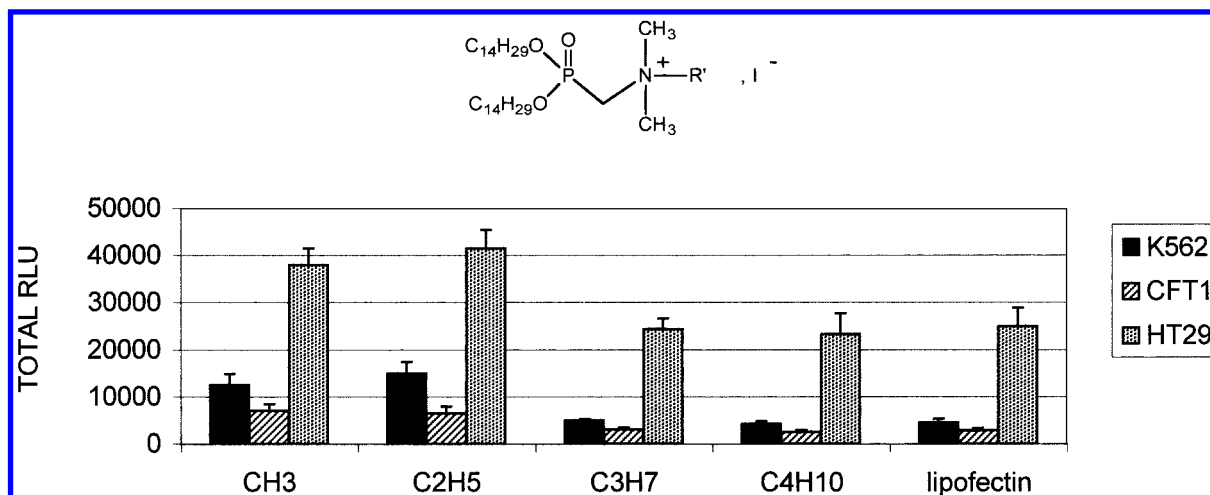


Figure 4. Influence of polar headgroup substitution in transfection activity of cationic phosphonolipid ammonium compounds. Transfections with various cationic phosphonolipid formulations were performed according to the procedures described in the Experimental Section, using HT29, K562, and CFT1 cells and pTG11033 plasmid DNA. Two days after transfection luciferase activity was determined using a luminescent assay. The total RLU values obtained by summing up the RLU values of 16 wells of the microtiter plate were plotted on the y-axis; each data point indicates the mean value of total RLU derived from 3 transfections and the standard error deviation of this mean.

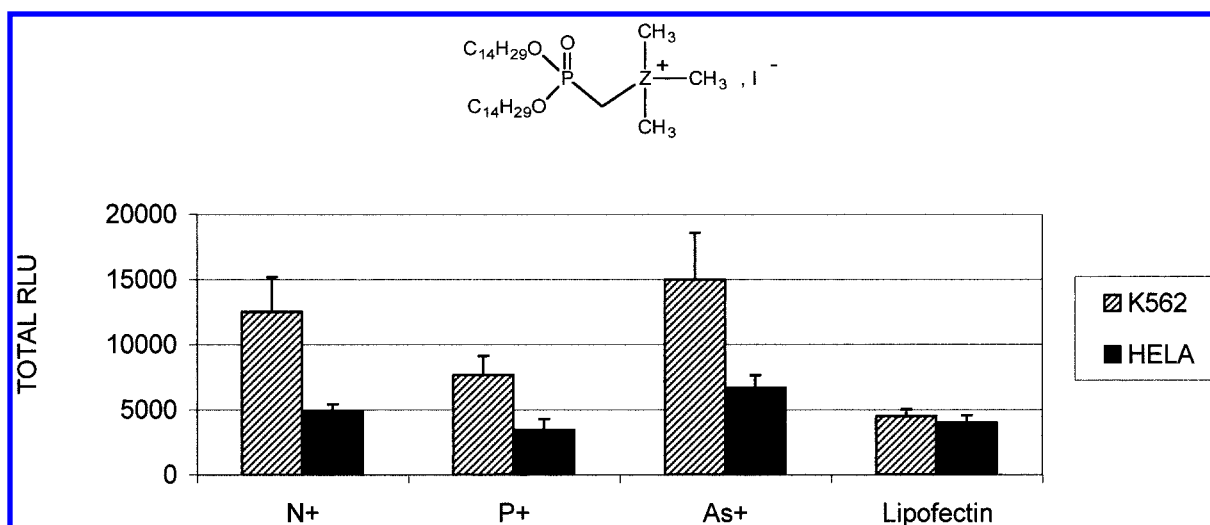


Figure 5. Influence of nitrogen atom substitution on transfection activity in vitro. Transfections with various quaternary trimethylammoniums (N⁺, P⁺, As⁺) were performed according to the procedures described in the Experimental Section, using K562 and HeLa cells and pTG11033 plasmid DNA. Two days after transfection luciferase activity was determined using a luminescent assay. The total RLU values obtained by summing up the RLU values of 16 wells of the microtiter plate were plotted on the y-axis; each data point indicates the mean value of total RLU derived from 3 transfections and the standard error deviation of this mean.

clinical biochemistry test. Serums concentrations of liver-specific enzymes including aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) were determined 24 h after mice were injected with 50 μ g of pTG11033 plasmid complexed with different cationic lipids. The results obtained are presented in Table 2. We reported that whatever the cationic lipids used transfection activity was linked to an increase of transaminase levels in comparison with the enzyme level obtained with the negative control (Figure 8). However combination of cation substitution and the growth in linker length in the cationic phosphonolipid structure led us to obtain good transfection efficiency and a decrease in transaminase levels. These chemical structure modifications decrease the toxicity index (TI) from 12.67 (GLB43) to 8.45 (EG372) and increase the transfection activity from 1814 RLU/mg protein (GLB43) to 3473 RLU/mg protein.

Discussion

Besides improving transfection activity, the development of new cationic lipid compounds with low cellular toxicity while maintaining an efficient transgene expression could be one of the next challenges of nonviral vector-mediated gene transfer. In this way series of new cationic phosphonolipids compounds have been synthesized and screened in in vitro and in vivo experiments.

In a first set of experiments, structure–activity relationship studies were carried out by the introduction of variable alkyl groups on the polar domain. Then, we studied the influence of cation substitution on the polar domain (N \rightarrow P or As) and also extended our study to compounds with ethylene and propylene linkages instead of methylene, to increase the chemical stability and approach the chemical structure of natural phosphonolipids.

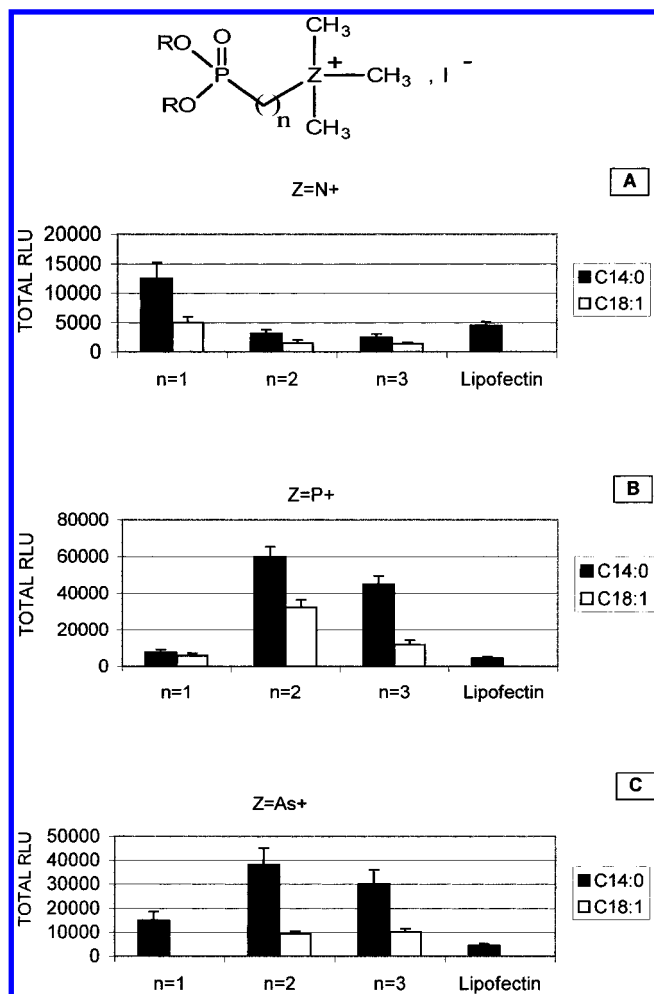


Figure 6. Influence of nitrogen atom substitution, linker, and hydrophobic chain length variation on transfection activity in vitro. Transfections with various dioleoyl (C_{18:1}) or dimyristyl (C_{14:0}) quaternary trimethyloniums (N⁺, P⁺, As⁺) including methylene, ethylene, or propylene linkages were performed according to the procedures described in the Experimental Section, using K562 cells and pTG11033 plasmid DNA. Two days after transfection luciferase activity was determined using a luminescent assay. The total RLU values obtained by summing up the RLU values of 16 wells of the microtiter plate were plotted on the y-axis; each data point indicates the mean value of total RLU derived from 3 transfections and the standard error deviation of this mean.

The decision to use tetraalkylammonium phosphonolipids for this study was based on previous observations that tertiary aminophosphonolipids were not efficient as transfection agents.³¹ This observation is in agreement with those of other authors.³ While polyamine-based cationic lipids have often been described as more efficient than monocationic ones, in our experiment cationic phosphonolipids including a diamine, triamine, or guanidine group in the polar domain were always less active than their tetraalkylammonium analogues.³¹ In this previous work transfection activities of GLB43 and GLB73 compounds were compared to those obtained with LipofectAMINE and Transfectam (i.e. DOGS); in our experimental conditions cationic phosphonolipids led us to obtain higher transfection activity than with polycationic reagents.³¹ As we reported previously¹³ we demonstrated that dimyristyl cationic phosphonolipids were more efficient than dioleoyl analogues for in vitro

experiments. This observation is in agreement with those of other authors.^{7,4}

A first set of structural modifications was carried out in order to improve transfection efficiency. According to Felgner and Wheeler observations,^{7,20} transfection activity could be improved by the introduction of a hydroxyethyl or amine on the quaternary ammonium, by influencing lipid surface hydration, effective charge of the molecule, or hydrogen bond formation. These observations were not in agreement with our previous ones. In a previous series of studied compounds, the best transfection activity was observed with a cationic phosphonolipid which incorporates a quaternary trimethylammonium group in the polar domain.^{9,10}

Here, we report the transfection activity results of a second series of cationic phosphonolipids synthesized in order to analyze the influence of the addition of alkyl groups of increasing chain length to the ammonium group. Bennet et al. reported that increasing the polar domain cross-sectional area by substitution of a methyl by an ethyl group in an analogue of the DOTMA correlated with improving levels of plasmid transfection activity.³ The same observation was reported by Felgner et al. using analogues of DORIE.⁷ Our results are not in agreement with these studies: cationic phosphonolipids that incorporate a quaternary ethyltrimethylammonium group in their polar domain are not significantly more efficient than their trimethyl analogues ($p > 0.05$). Moreover, increasing chain length to the ammonium group by including a propyl or butyl group decreased transfection activity, probably due to a steric effect. To test hypotheses pertaining to the steric effect, cationic phosphonolipids including isopropyl groups were synthesized. Addition of isopropyl groups that increase the cross-sectional area of the headgroup correlated with a decrease in transfection activity (data not shown).

Much effort has been devoted to resolving the toxicity problem of cationic lipid-mediated gene transfer. For example novel pyridinium surfactants for nontoxic in vitro gene delivery and cationic lipids derived from sphingosine have been synthesized.^{18,19} Recently we have shown that transfection efficiency with low toxicity can be obtained in vitro using glycine betaine amphiphile derivatives.¹⁷ Silvius and Leventis³² proposed that one way of decreasing cellular toxicity would be to use ester linkages between the two domains of the cationic lipid compound. Following this hypothesis Aberle et al. have recently synthesized a novel tetraester construct that reduces cationic lipid-associated cytotoxicity.¹⁶ It is important to underline that, up until now in all cationic lipid structures synthesized, the cationic charge has always been localized on the nitrogen atom.

1

Stekar et al. reported that for Edelfosine and Miltefosine (two antineoplastically neutral zwitterionic phospholipids), the substitution of the quaternary ammonium polar head by a phosphonium or arsonium results in a maintained biological activity and a decreased cellular toxicity. On the basis of this concept, we have designed a new panel of cationic phosphonolipids that uses a phosphorus or arsenic atom to carry the cationic charge of the compound. Our results demonstrate that, whatever the cation used (P⁺ or As⁺) and the cell line

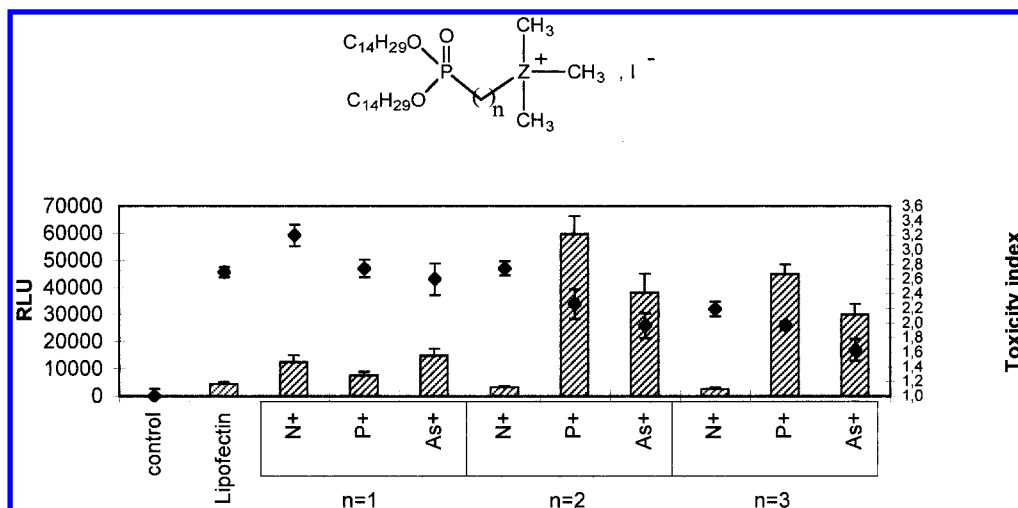


Figure 7. In vitro cytotoxicity of various quaternary trimethylonium (N^+ , P^+ , As^+) compounds. Luciferase activity results observed 48 h after transfection of K562 cells are summarized in bar graph forms. Each data point indicates the mean value of total RLU derived from 3 transfections and the standard error deviation of this mean. Toxicity index, depicted by the solid line, is plotted according to the right vertical axis, determined as described in the Experimental Section.

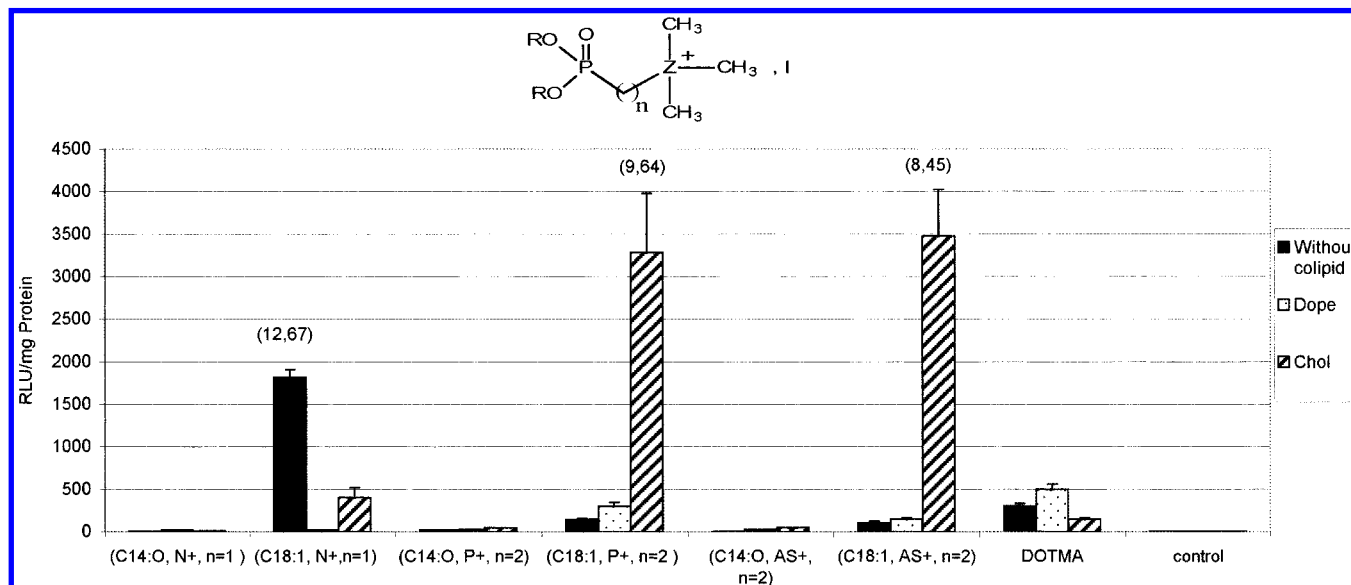


Figure 8. Influence of chemical modifications and formulation of cationic phosphonolipids on transfection efficiency in vivo. Luciferase activity in mouse lungs was assayed 24 h after intravenous injection of 50 μg of pTG11033 plasmid complexed with different cationic phosphonolipids formulated with or without colipid. In the control group, mice received only naked DNA (50 μg). The data are presented as mean \pm SEM ($n = 8$). TI, toxicity index, determined as described in the Experimental Section.

Table 2. Measurement of Toxicity in Vivo^a

cationic lipids	without co-lipid				with DOPE				with cholesterol			
	ASAT	TI	ALAT	TI	ASAT	TI	ALAT	TI	ASAT	TI	ALAT	TI
(C _{14:0} , N^+ , $n = 1$)	80 (4)	1.12	25 (3)	1.08	130 (10)	1.83	31 (12)	1.34	75 (10)	1.05	24 (8)	1.04
(C _{18:1} , N^+ , $n = 1$)	900 (50)	12.67	250 (10)	10.86	125 (11)	1.76	30 (2)	1.3	225 (15)	3.16	70 (10)	3.04
(C _{14:0} , P^+ , $n = 2$)	120 (27)	1.69	55 (10)	2.39	145 (20)	2.04	39 (9)	1.69	174 (34)	2.45	34 (6)	1.47
(C _{18:1} , P^+ , $n = 2$)	250 (35)	3.52	35 (9)	1.52	646 (45)	9.09	65 (12)	2.82	685 (20)	9.64	184 (4)	8
(C _{14:0} , AS^+ , $n = 2$)	79 (5)	1.11	26 (6)	1.13	155 (23)	2.18	34 (23)	1.47	190 (43)	2.67	41 (3)	1.78
(C _{18:1} , AS^+ , $n = 2$)	225 (45)	3.16	35 (7)	1.52	303 (45)	4.26	36 (7)	1.56	600 (25)	8.45	172 (9)	7.47
DOTMA	657 (42)	9.25	70 (6)	3.04	275 (12)	3.87	74 (23)	3.2	255 (46)	3.59	37 (10)	1.6
control	71 (5)	1	23 (4)	1	71 (5)	1	23 (4)	1	71 (5)	1	23 (4)	1

^a Toxic effects of lipoplexes after intravenous administration were assessed by determination of transaminase levels in transfected mice serums. Data represent mean (SEM) from 6 animals. TI: toxicity index = transfected mice transaminase levels (ASAT or ALAT)/control mice transaminase levels (ASAT or ALAT).

tested, transfection activity is maintained and in some cases significantly improved. We demonstrate that both optimal transfection efficiency and minimal cell toxicity are obtained when substitution of cation is combined with lengthening of the linker. The improvement of

transfection activity by cation substitution is also confirmed by in vivo experiments. In these in vivo tests and as we have already reported,¹³ compounds including C_{18:1} chains in their hydrophobic moiety are more efficient than their C_{14:0} analogues. Our observations

concerning liposome formulation could suggest that the function of the neutral lipid is different in vivo from in vitro and depends on the nature of the quaternary trimethylonium used. These observations are in agreement with those of other authors.^{33–35} Our observations concerning in vivo toxicity of 21 formulations suggest that although transfection activity was linked to an increase of transaminase levels, structural modifications of cationic phosphonolipids can decrease the toxicity index observed after intravenous administration of lipoplexes. In fact, we demonstrate that combination of cation substitution and growth in linker length in the cationic phosphonolipid structure leads us to obtain good transfection efficiency and a decrease in the toxicity index. Studies designed to address the role of plasmid DNA in toxic effects are under investigation.

In conclusion, the present in vitro and in vivo results lead us to think that cation substitution in cationic lipids could be a good concept to improve transfection activity and decrease the cellular toxicity of this type of nonviral vectors.

Experimental Section

1. Synthesis of Cationic Phosphonolipids. Unless otherwise noted, all reagents were purchased from commercial suppliers and were used without further purification. Solvents were freshly distilled on appropriate dryers, THF on sodium/benzophenone, diethyl ether on calcium hydride, and DMF on phosphorus pentoxide, and stored under nitrogen atmosphere. All the reactions with phosphine and arsine were run under nitrogen atmosphere using the Schlenk technique.

The ammonium compounds noted GLB43, GLB73, GLB391, GLB566, and GLB570 were prepared as previously described.⁸ Procedures were given with $R = C_{14}H_{29}$ and $R' = R'' = CH_3$ (see Figure 3) and were the same for the other compounds. Typically, all the reactions were followed by a counterion metathesis.

All compounds were purified by successive recrystallizations from diethyl ether at -20°C . They were fully characterized by ^1H (400 MHz), ^{13}C (100 MHz), and ^{31}P (121.49 MHz) NMR spectroscopy. ^1H , ^{13}C , and ^{31}P spectra were recorded at 298 K, on Bruker AC 300 and Bruker Advance DRX 400 spectrometers. Samples were dissolved in CDCl_3 . Chemical shifts δ are in ppm, relative to TMS internal standard for ^1H and ^{13}C , H_3PO_4 as external reference for ^{31}P . Coupling constants J are given in hertz (Hz). Thermotrope properties were studied by DSC and fusion temperatures ($\Delta H_{\text{fusion}} \approx 90 \text{ J g}^{-1}$) were determined using a DSC 92 SETARAM calorimeter. The compounds obtained were highly hygroscopic which makes elementary analysis not adequate as a purity criteria. However, when needed, ^{13}C NMR heteronuclear HMQC and HMBC were used to unambiguously establish the proposed structures.

A. General Procedure for Onium Methylenephosphonates. The phosphonium and arsonium salts were prepared according to well-known procedures by reacting in THF trialkylphosphine and trimethylarsine with a suited alkyl halogenide, alkyl iodide, and bromomethyltrimethylsilane, respectively.

Ditetradecyl (Trimethylphosphonio)methylphosphonate Iodide, EG176. A suspension of tetramethylphosphonium iodide (2.18 g, 10 mmol) in freshly distilled THF (10 mL) was treated dropwise, at -10°C under a nitrogen atmosphere, with a commercial 2.5 M *n*-butyllithium solution in hexane (4.0 mL). The resulting solution was then added dropwise, at -10°C , under nitrogen atmosphere, to a solution of chloroditetradecyl phosphate (2.55 g, 5 mmol) in 5 mL of THF and the mixture stirred 2 h at room temperature. The solution was acidified (pH = 1) with 1 M ethereal hydrogen chloride, concentrated under vacuum, the residue taken up in 20 mL CH_2Cl_2 and washed twice by 10 mL of water. The counterion metathesis was achieved by stirring this CH_2Cl_2 solution with

10 mL of a saturated sodium iodide aqueous solution. Drying on MgSO_4 , filtration, evaporation of the solvent gave the crude product which was recrystallized from cold diethyl ether (-20°C): yield 76%; ^{31}P NMR 16.7 (d, $\text{P}=\text{O}$, $J_{\text{P-P}} = 12.7$) 25.7 (d, P^+ , $J_{\text{P-P}} = 12.7$); ^1H NMR 0.87 (t, 6H, $J_{\text{H-H}} = 6.8$, CH_3) 1.24 (m, 44H, CH_2) 1.68 (m, 4H, $\text{CH}_2\text{CH}_2\text{O}$) 2.34 (d, 9H, $J_{\text{P-H}} = 14.4$, $\text{P}(\text{CH}_3)_3$) 3.59 (dd, 2H, $J_{\text{P-H}} = 19.2$ and 16.6, $\text{P}-\text{CH}_2-\text{P}$) 4.19 (dt, 4H, $J_{\text{H-H}} = J_{\text{P-H}} = 6.8$, CH_2O); ^{13}C NMR 10.6 (d, $J_{\text{P-C}} = 55.9$, CH_3) 14.0 (CH_3) 22.1 (dd, $J_{\text{O-P-C}} = 133.1$, $J_{\text{P-C}} = 47.5$, $\text{P}-\text{CH}_2-\text{P}$) from 22.5–31.9 several singlets (CH_2) 29.9 (d, $J_{\text{P-C}} = 6.3$, $\text{CH}_2\text{CH}_2\text{O}$) 67.7 (d, $J_{\text{P-C}} = 6.7$, CH_2O); mp = 57°C .

Ditetradecyl (dimethylethylphosphonio)methylphosphonate iodide, EG175: yield 72%; ^{31}P NMR 16.9 (d, $\text{P}=\text{O}$, $J_{\text{P-P}} = 12.4$) 30.8 (d, P^+ , $J_{\text{P-P}} = 12.4$); ^1H NMR 0.87 (t, 6H, $J_{\text{H-H}} = 6.8$, CH_3) 1.24 (m, 44H, CH_2) 1.35 (t, 3H, $J_{\text{H-H}} = 7.6$, $\text{CH}_3\text{CH}_2\text{P}^+$) 1.68 (m, 4H, $\text{CH}_2\text{CH}_2\text{O}$) 2.28 (d, 9H, $J_{\text{P-H}} = 13.9$, $\text{P}(\text{CH}_3)_2$) 2.72 (m, 2H, $\text{CH}_3\text{CH}_2\text{P}^+$) 3.64 (dd, 2H, $J_{\text{P-H}} = 17.6$ and 16.5, $\text{P}-\text{CH}_2-\text{P}$) 4.19 (dt, 4H, $J_{\text{H-H}} = J_{\text{P-H}} = 6.8$, CH_2O); ^{13}C NMR 5.9 (d, $J_{\text{P-C}} = 5.29$, $\text{CH}_3\text{CH}_2\text{P}^+$) 8.1 (d, $J_{\text{P-C}} = 51.8$, $\text{P}^+(\text{CH}_3)_2$) 17.3 (d, $J_{\text{P-C}} = 51.9$, $\text{CH}_3\text{CH}_2\text{P}^+$) 14.0 (CH_3) 20.5 (dd, $J_{\text{O-P-C}} = 132.3$, $J_{\text{P-C}} = 46.0$, $\text{P}-\text{CH}_2-\text{P}$) from 22.5–31.9 several singlets (CH_2) 29.9 (d, $J_{\text{P-C}} = 6.3$, $\text{CH}_2\text{CH}_2\text{O}$) 67.7 (d, $J_{\text{P-C}} = 6.7$, CH_2O).

Ditetradecyl (dimethylpropylphosphonio)methylphosphonate iodide, EG202: yield 68%; ^{31}P NMR 17.2 (d, $\text{P}=\text{O}$, $J_{\text{P-P}} = 12.2$) 28.4 (d, P^+ , $J_{\text{P-P}} = 12.2$); ^1H NMR 0.87 (t, 6H, $J_{\text{H-H}} = 6.8$, CH_3) 1.15 (dt, 3H, $J_{\text{H-H}} = 7.6$, $J_{\text{P-H}} = 1.4$, $\text{CH}_3\text{CH}_2\text{CH}_2\text{P}^+$) 1.24 (m, 44H, CH_2) 1.30 (m, 2H, $\text{CH}_3\text{CH}_2\text{CH}_2\text{P}^+$) 1.68 (m, 4H, $\text{CH}_2\text{CH}_2\text{O}$) 2.28 (d, 6H, $J_{\text{P-H}} = 13.9$, $\text{P}(\text{CH}_3)_2$) 2.63 (m, 2H, $\text{CH}_3\text{CH}_2\text{CH}_2\text{P}^+$) 3.61 (dd, 2H, $J_{\text{P-H}} = 17.7$ and 15.6, $\text{P}-\text{CH}_2-\text{P}$) 4.19 (dt, 4H, $J_{\text{H-H}} = J_{\text{P-H}} = 6.8$, CH_2O); ^{13}C NMR 8.6 (dd, $J_{\text{P-C}} = 51.1$, $J_{\text{P-C}} = 2.7$, $\text{P}^+(\text{CH}_3)_2$) 14.0 (CH_3) 15.2 (d, $J_{\text{P-C}} = 18.0$, $\text{CH}_3\text{CH}_2\text{CH}_2\text{P}^+$) 15.5 (d, $J_{\text{P-C}} = 4.2$, $\text{CH}_3\text{CH}_2\text{CH}_2\text{P}^+$) 20.8 (dd, $J_{\text{O-P-C}} = 133.2$, $J_{\text{P-C}} = 48.0$, $\text{P}-\text{CH}_2-\text{P}$) 24.8 (s, $\text{CH}_3\text{CH}_2\text{CH}_2\text{P}^+$) from 22.5–31.9 several singlets (CH_2) 29.9 (d, $J_{\text{P-C}} = 6.3$, $\text{CH}_2\text{CH}_2\text{O}$) 67.7 (d, $J_{\text{P-C}} = 6.7$, CH_2O).

Ditetradecyl (butyldimethylphosphonio)methylphosphonate iodide, EG203: yield 60%; ^{31}P NMR 16.8 (d, $\text{P}=\text{O}$, $J_{\text{P-P}} = 12.0$) 28.7 (d, P^+ , $J_{\text{P-P}} = 12.0$); ^1H NMR 0.87 (t, 6H, $J_{\text{H-H}} = 6.8$, CH_3) 0.99 (t, 3H, $J_{\text{H-H}} = 7.0$, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{P}^+$) 1.24 (m, 44H, CH_2) 1.30 (m, 2H, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{P}^+$) 1.56 (m, 2H, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{P}^+$) 1.68 (m, 4H, $\text{CH}_2\text{CH}_2\text{O}$) 2.26 (d, 6H, $J_{\text{P-H}} = 14.0$, $\text{P}(\text{CH}_3)_2$) 2.63 (m, 2H, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{P}^+$) 3.61 (dd, 2H, $J_{\text{P-H}} = 17.8$ and 16.3, $\text{P}-\text{CH}_2-\text{P}$) 4.19 (dt, 4H, $J_{\text{H-H}} = J_{\text{P-H}} = 6.8$, CH_2O); ^{13}C NMR 8.1 (dd, $J_{\text{P-C}} = 54.3$, $J_{\text{P-C}} = 2.8$, $\text{P}^+(\text{CH}_3)_2$) 13.0 (s, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{P}^+$) 14.0 (CH_3) 16.2 (dd, $J_{\text{O-P-C}} = 132.0$, $J_{\text{P-C}} = 48.1$, $\text{P}-\text{CH}_2-\text{P}$) 22.9 (s, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{P}^+$) 23.0 (d, $J_{\text{P-C}} = 4.3$, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{P}^+$) 23.2 (d, $J_{\text{P-C}} = 17.0$, $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{P}^+$) from 22.5–31.9 several singlets (CH_2) 29.9 (d, $J_{\text{P-C}} = 6.3$, $\text{CH}_2\text{CH}_2\text{O}$) 67.7 (d, $J_{\text{P-C}} = 6.7$, CH_2O).

Ditetradecyl (dimethylisopropylphosphonio)methylphosphonate iodide, EG172: yield 75%; ^{31}P NMR 16.7 (d, $\text{P}=\text{O}$, $J_{\text{P-P}} = 11.8$) 35.7 (d, P^+ , $J_{\text{P-P}} = 11.8$); ^1H NMR 0.87 (t, 6H, $J_{\text{H-H}} = 6.8$, CH_3) 1.24 (m, 44H, CH_2) 1.32 (dd, 6H, $J_{\text{H-H}} = 7.2$, $J_{\text{P-H}} = 12.9$, $(\text{CH}_3)_2\text{CHP}^+$) 1.68 (m, 4H, $\text{CH}_2\text{CH}_2\text{O}$) 2.20 (d, 6H, $J_{\text{P-H}} = 13.8$, $\text{P}(\text{CH}_3)_2$) 3.26 (m, 1H, $(\text{CH}_3)_2\text{CHP}^+$) 3.73 (dd, 2H, $J_{\text{P-H}} = 18.0$ and 16.6, $\text{P}-\text{CH}_2-\text{P}$) 4.19 (dt, 4H, $J_{\text{H-H}} = J_{\text{P-H}} = 6.8$, CH_2O); ^{13}C NMR 5.6 (dd, $J_{\text{P-C}} = 52.9$, $J_{\text{P-C}} = 2.5$, $\text{P}^+(\text{CH}_3)_2$) 14.0 (CH_3) 15.4 (d, $J_{\text{P-C}} = 1.9$, $(\text{CH}_3)_2\text{CHP}^+$) 18.7 (dd, $J_{\text{O-P-C}} = 133.5$, $J_{\text{P-C}} = 46.8$, $\text{P}-\text{CH}_2-\text{P}$) 22.2 (dd, $J_{\text{P-C}} = 48.0$, $J_{\text{P-C}} = 2.9$, $(\text{CH}_3)_2\text{CHP}^+$) from 22.5–31.9 several singlets (CH_2) 29.9 (d, $J_{\text{P-C}} = 6.3$, $\text{CH}_2\text{CH}_2\text{O}$) 67.7 (d, $J_{\text{P-C}} = 6.7$, CH_2O). Anal. Calcd: C, 56.81; H, 10.24; P, 8.62. Found: C, 56.81; H, 10.42; P, 8.53.

Ditetradecyl (diisopropylmethylphosphonio)methylphosphonate iodide, EG300: yield 81%; ^{31}P NMR 17.1 (d, $\text{P}=\text{O}$, $J_{\text{P-P}} = 11.4$) 41.9 (d, P^+ , $J_{\text{P-P}} = 11.4$); ^1H NMR 0.87 (t, 6H, $J_{\text{H-H}} = 6.8$, CH_3) 1.24 (m, 44H, CH_2) 1.41 and 1.47 two diastereotopic groups (dd, 6H, $J_{\text{H-H}} = 7.2$, $J_{\text{P-H}} = 13.0$, $(\text{CH}_3)_2\text{CHP}^+$) 1.68 (m, 4H, $\text{CH}_2\text{CH}_2\text{O}$) 2.14 (d, 3H, $J_{\text{P-H}} = 12.8$, PCH_3) 3.17 (m, 2H, $((\text{CH}_3)_2\text{CH})_2\text{P}^+$) 3.51 (dd, 2H, $J_{\text{P-H}} = 18.9$ and

15.2, P-CH₂-P) 4.19 (dt, 4H, $J_{\text{H-H}} = J_{\text{P-H}} = 6.8$, CH₂O); ¹³C NMR 3.0 (dd, $J_{\text{P-C}} = 50.2$, $J_{\text{P-C}} = 2.8$, P⁺CH₃) 14.0 (CH₃) 16.5 and 16.2 two diastereotopic groups (d, $J_{\text{P-C}} = 2.2$ and 2.5, (CH₃)₂CHP⁺) 16.8 (dd, $J_{\text{O-P-C}} = 134.1$, $J_{\text{P-C}} = 42.5$, P-CH₂-P) 22.4 (dd, $J_{\text{P-C}} = 45.3$, $J_{\text{P-C}} = 2.7$, ((CH₃)₂CH)₂P⁺) from 22.5–31.9 several singlets (CH₂) 29.9 (d, $J_{\text{P-C}} = 6.3$, CH₂-CH₂O) 67.7 (d, $J_{\text{P-C}} = 6.7$, CH₂O).

Ditetradecyl (triisopropylphosphonio)methylphosphonate iodide, EG315: yield 63%; ³¹P NMR 17.4 (d, P=O, $J_{\text{P-P}} = 9.8$) 45.7 (d, P⁺, $J_{\text{P-P}} = 9.8$); ¹H NMR 0.87 (t, 6H, $J_{\text{H-H}} = 6.8$, CH₃) 1.24 (m, 44H, CH₂) 1.51 (dd, 18H, $J_{\text{H-H}} = 7.2$, $J_{\text{P-H}} = 13.8$ ((CH₃)₂CH)₃P⁺) 1.68 (m, 4H, CH₂CH₂O) 3.21 (m, 3H, ((CH₃)₂CH)₃P⁺) 3.46 (dd, 2H, $J_{\text{P-H}} = 20.2$ and 12.2, P-CH₂-P) 4.19 (dt, 4H, $J_{\text{H-H}} = J_{\text{P-H}} = 6.8$, CH₂O); ¹³C NMR 14.0 (CH₃) 14.7 (dd, $J_{\text{O-P-C}} = 133.0$, $J_{\text{P-C}} = 39.8$, P-CH₂-P) 17.2 (d, $J_{\text{P-C}} = 1.9$, ((CH₃)₂CH)₃P⁺) 21.6 (d, $J_{\text{P-C}} = 29.1$, ((CH₃)₂CH)₃P⁺) from 22.5–31.9 several singlets (CH₂) 29.9 (d, $J_{\text{P-C}} = 6.3$, CH₂-CH₂O) 67.7 (d, $J_{\text{P-C}} = 6.7$, CH₂O).

Dioleoyl (trimethylphosphonio)methylphosphonate iodide, EG343: yield 68%; ³¹P NMR 16.9 (d, P=O, $J_{\text{P-P}} = 12.8$) 25.8 (d, P⁺, $J_{\text{P-P}} = 12.8$); ¹H NMR 0.87 (t, 6H, $J_{\text{H-H}} = 6.8$, CH₃) 1.24 (m, 44H, CH₂) 1.68 (m, 4H, CH₂CH₂O) 2.01 (m, 8H, CH₂CH=CHCH₂) 2.37 (dd, 2H, $J_{\text{P-H}} = 18.0$ and 17.9, P-CH₂-P) 4.19 (dt, 4H, $J_{\text{H-H}} = J_{\text{P-H}} = 6.8$, CH₂O) 5.36 (m, 4H, CH=CH); ¹³C NMR 10.6 (dd, $J_{\text{P-C}} = 55.5$, $J_{\text{P-C}} = 2.5$, P⁺(CH₃)₃) 14.0 (CH₃) 22.0 (dd, $J_{\text{O-P-C}} = 130.2$, $J_{\text{P-C}} = 48.5$, P-CH₂-P) from 22.5–31.9 several singlets (CH₂) 29.9 (d, $J_{\text{P-C}} = 6.3$, CH₂-CH₂O) 67.7 (d, $J_{\text{P-C}} = 6.7$, CH₂O) 130.1 and 129.8 (CH=CH).

Ditetradecyl (Trimethylarsonio)methylphosphonate Iodide, EG373. This compound was prepared as above, starting from trimethylsilylmethylenetriethylarsonium bromide. The TMS stabilizing group was simply removed during the metathesis step: yield 60%; ³¹P NMR 19.5 (s, P=O); ¹H NMR (300 MHz) 0.87 (t, 6H, $J_{\text{H-H}} = 6.8$, CH₃) 1.24 (m, 44H, CH₂) 1.68 (m, 4H, CH₂CH₂O) 2.41 (s, 9H, As(CH₃)₃) 3.61 (d, 2H, $J_{\text{P-H}} = 16.4$, P-CH₂-As) 4.19 (dt, 4H, $J_{\text{H-H}} = J_{\text{P-H}} = 6.8$, CH₂O); ¹³C NMR 9.4 (s, As(CH₃)₃) 14.0 (CH₃) 21.5 (d, $J_{\text{O-P-C}} = 136.7$, P-CH₂-As) from 22.5–31.9 several singlets (CH₂) 29.9 (d, $J_{\text{P-C}} = 6.3$, CH₂CH₂O) 67.7 (d, $J_{\text{P-C}} = 6.7$, CH₂O); mp = 56 °C. Anal. Calcd: C, 49.87; H, 9.55; As, 9.58; P, 3.95. Found: C, 50.00; H, 9.51; As, 9.58; P, 3.95.

B. General Procedure for Fatty ω-Bromoalkylphosphonates and Vinylphosphonates. Phosphodichlorides were obtained according to literature procedures^{27–29} by a three-step reaction from diethyl ω-bromoalkylphosphonates.

Ditetradecyl 2-Bromoethylphosphonate. To 2-bromoethylphosphonic dichloride (2.26 g, 10 mmol) in 10 mL of diethyl ether was added dropwise a solution of tetradecanol (4.29 g, 20 mmol) and diisopropylethylamine (5.20 g, 40 mg) in 10 mL of diethyl ether, at –10 °C, under nitrogen atmosphere and the mixture stirred 16 h at –10 °C. After filtration the filtrate was concentrated under vacuum, the residue taken up in 20 mL CH₂Cl₂ and washed twice by 10 mL of an aqueous hydrochloric acid solution (10%). Drying on MgSO₄, filtration, evaporation of the solvent gave the ditetradecyl 2-bromoethylphosphonate: yield 90%; ³¹P NMR 25.3 (s, P=O); ¹H NMR 0.86 (t, 6H, $J_{\text{H-H}} = 6.7$, CH₃) 1.24 (m, 44H, CH₂) 1.68 (m, 4H, CH₂CH₂O) 2.38 (m, 2H, O=PCH₂) 3.52 (m, 2H, CH₂Br) 4.09 (dt, 4H, $J_{\text{H-H}} = J_{\text{P-H}} = 6.8$, CH₂O); ¹³C NMR 14.1 (CH₃) 23.9 (BrCH₂) 30.4 (d, $J_{\text{P-C}} = 138.7$, O=PCH₂) from 22.5–31.9 several singlets (CH₂) 30.5 (d, $J_{\text{P-C}} = 6.3$, CH₂CH₂O) 67.5 (d, $J_{\text{P-C}} = 6.7$, CH₂O).

Dioleoyl 2-bromoethylphosphonate: yield 90%; ³¹P NMR 25.9 (s, P=O); ¹H NMR 0.86 (t, 6H, $J_{\text{H-H}} = 6.7$, CH₃) 1.24 (m, 44H, CH₂) 1.68 (m, 4H, CH₂CH₂O) 1.98 (m, 8H, CH₂CH=CHCH₂) 2.41 (m, 2H, O=PCH₂) 3.58 (m, 2H, CH₂Br) 4.09 (dt, 4H, $J_{\text{H-H}} = J_{\text{P-H}} = 6.8$, CH₂O) 5.37 (m, 4H, CH=CH); ¹³C NMR 14.1 (CH₃) 24.0 (BrCH₂) 30.1 (d, $J_{\text{P-C}} = 138.7$, O=PCH₂) from 22.5–31.9 several singlets (CH₂) 30.5 (d, $J_{\text{P-C}} = 6.3$, CH₂-CH₂O) 67.5 (d, $J_{\text{P-C}} = 6.7$, CH₂O) 129.3 and 129.1 (CH=CH).

Ditetradecyl 3-bromopropylphosphonate: yield 90%; ³¹P NMR 30.8 (s, P=O); ¹H NMR 0.86 (t, 6H, $J_{\text{H-H}} = 6.7$, CH₃) 1.24 (m, 44H, CH₂) 1.68 (m, 4H, CH₂CH₂O) 1.89 (m, 2H, CH₂CH₂CH₂) 2.15 (m, 2H, O=PCH₂) 3.77 (t, 2H, $J_{\text{H-H}} = 6.5$,

CH₂Br) 4.09 (dt, 4H, $J_{\text{H-H}} = J_{\text{P-H}} = 6.8$, CH₂O); ¹³C NMR 14.1 (CH₃) 24.0 (d, $J_{\text{P-C}} = 142.7$, O=PCH₂) 25.8 (d, $J_{\text{P-C}} = 4.4$, CH₂CH₂CH₂) 33.2 (d, $J_{\text{P-C}} = 18.3$, BrCH₂) from 22.5–31.9 several singlets (CH₂) 30.5 (d, $J_{\text{P-C}} = 6.3$, CH₂CH₂O) 67.5 (d, $J_{\text{P-C}} = 6.7$, CH₂O).

Dioleoyl 3-bromopropylphosphonate: yield 90%; ³¹P NMR 31.0 (s, P=O); ¹H 0.86 (t, 6H, $J_{\text{H-H}} = 6.7$, CH₃) 1.24 (m, 44H, CH₂) 1.68 (m, 4H, CH₂CH₂O) 1.91 (m, 2H, CH₂CH₂CH₂) 1.98 (m, 8H, CH₂CH=CHCH₂) 2.15 (m, 2H, O=PCH₂) 3.46 (t, 2H, $J_{\text{H-H}} = 6.7$, CH₂Br) 4.09 (dt, 4H, $J_{\text{H-H}} = J_{\text{P-H}} = 6.8$, CH₂O) 5.37 (m, 4H, CH=CH); ¹³C NMR 14.1 (CH₃) 23.8 (d, $J_{\text{P-C}} = 142.7$, O=PCH₂) 25.5 (d, $J_{\text{P-C}} = 3.2$, CH₂CH₂CH₂) 33.8 (d, $J_{\text{P-C}} = 18.1$, BrCH₂) from 22.5–31.9 several singlets (CH₂) 30.5 (d, $J_{\text{P-C}} = 6.3$, CH₂CH₂O) 67.5 (d, $J_{\text{P-C}} = 6.7$, CH₂O) 129.1 and 129.3 (CH=CH).

Ditetradecyl Vinylphosphonate. Ditetradecyl 2-bromoethylphosphonate (5.82 g, 10 mmol) was dissolved in 10 mL of THF. Triethylamine (5.06 g, 50 mmol) was then added and the mixture was refluxed for 24 h. After filtration the filtrate was concentrated under vacuum, the residue was taken up in 20 mL CH₂Cl₂ and washed twice by 10 mL of an aqueous hydrochloric acid solution (10%). Drying on MgSO₄, filtration, evaporation of the solvent gave the ditetradecyl vinylphosphonate: yield 100%; ³¹P NMR 17.4 (s, P=O); ¹H NMR 0.86 (t, 6H, $J_{\text{H-H}} = 6.7$, CH₃) 1.24 (m, 44H, CH₂) 1.68 (m, 4H, CH₂-CH₂O) 3.98 (dt, 4H, $J_{\text{H-H}} = J_{\text{P-H}} = 6.8$, CH₂O) 6.14 (m, 3H, CH=CH₂); ¹³C NMR 14.1 (CH₃) from 22.7–32.7 several singlets (CH₂) 30.5 (d, $J_{\text{P-C}} = 6.3$, CH₂CH₂O) 65.2 (d, $J_{\text{P-C}} = 6.7$, CH₂O) 125.7 (d, $J_{\text{P-C}} = 184.2$, O=PCH=) 134.8 (s, =CH₂).

Dioleoyl vinylphosphonate: yield 100%; ³¹P NMR 17.7 (s, P=O); ¹H NMR 0.86 (t, 6H, $J_{\text{H-H}} = 6.7$, CH₃) 1.24 (m, 44H, CH₂) 1.68 (m, 4H, CH₂CH₂O) 1.98 (m, 8H, CH₂CH=CHCH₂) 3.98 (dt, 4H, $J_{\text{H-H}} = J_{\text{P-H}} = 6.8$, CH₂O) 5.37 (m, 4H, CH=CH) 6.12 (m, 3H, CH=CH₂); ¹³C NMR 14.1 (CH₃) from 22.7–32.7 several singlets (CH₂) 30.5 (d, $J_{\text{P-C}} = 6.3$, CH₂CH₂O) 65.2 (d, $J_{\text{P-C}} = 6.7$, CH₂O) 125.7 (d, $J_{\text{P-C}} = 184.4$, O=PCH=) 129.3 and 129.5 (CH=CH) 134.4 (s, =CH₂).

C. General Procedure for Ammonium and Phosphonium Ethylenephosphonates. 2-(Ditetradecyloxyphosphoryl)-N,N,N-trimethylethanaminium Iodide, ACH164. 5 mL of a commercial solution of dimethylamine (2 M in THF, 10 mmol) was added to a flask containing ditetradecyl vinylphosphonate (2.5 g, 5 mmol) in 10 mL of ethyl alcohol. The mixture was stirred for 24 h and then concentrated to dryness: the residue was taken up in 20 mL of diethyl ether and methyl iodide (1.42 g, 10 mmol) was added. After stirring one night the precipitated was filtered and washed twice by 10 mL diethyl ether. The product was isolated as a yellow powder: yield 75%; ³¹P NMR 23.7 (s, P=O); ¹H NMR (300 MHz) 0.87 (t, 6H, $J_{\text{H-H}} = 6.7$, CH₃) 1.27 (m, 44H, CH₂) 1.67 (m, 4H, CH₂CH₂O) 2.38 (m, 2H, O=PCH₂) 3.49 (s, 9H, (CH₃)₃N) 3.77 (m, 2H, CH₂N) 4.09 (dt, 4H, $J_{\text{H-H}} = J_{\text{P-H}} = 6.8$, CH₂O); ¹³C NMR 14.0 (CH₃) 20.5 (d, $J_{\text{O-P-C}} = 138.5$, O=P-CH₂) from 22.6–31.9 several singlets (CH₂) 30.5 (d, $J_{\text{P-C}} = 6.2$, CH₂-CH₂O) 53.7 (N(CH₃)₃) 61.9 (CH₂N) 67.7 (d, $J_{\text{P-C}} = 6.7$, CH₂O); mp = 110 °C. Anal. Calcd: C, 54.76; H, 10.44; N, 1.94; P, 4.28. Found: C, 54.89; H, 10.57; N, 1.94; P, 4.30.

2-(Dioleoyloxyphosphoryl)-N,N,N-trimethylethanaminium iodide, ACH194: yield 65%; ³¹P NMR 23.9 (s, P=O); ¹H NMR (300 MHz) 0.87 (t, 6H, $J_{\text{H-H}} = 6.7$, CH₃) 1.27 (m, 44H, CH₂) 1.67 (m, 4H, CH₂CH₂O) 1.99 (m, 8H, CH₂CH=CHCH₂) 2.34 (m, 2H, O=PCH₂) 3.50 (s, 9H, (CH₃)₃N) 3.76 (m, 2H, CH₂N) 4.09 (dt, 4H, $J_{\text{H-H}} = J_{\text{P-H}} = 6.8$, CH₂O) 5.32 (m, 4H, CH=CH); ¹³C NMR 14.0 (CH₃) 21.4 (d, $J_{\text{O-P-C}} = 138.8$, O=P-CH₂) from 22.6–31.9 several singlets (CH₂) 30.5 (d, $J_{\text{P-C}} = 6.2$, CH₂CH₂O) 53.8 (N(CH₃)₃) 62.1 (CH₂N) 67.1 (d, $J_{\text{P-C}} = 6.6$, CH₂O) 129.9 and 129.7 (CH=CH).

Ditetradecyl 2-(Trimethylphosphonio)ethylphosphonate Iodide, EG308. Acidic phosphonium salts were prepared by acidification of trialkylphosphine, with a 1 M hydrogenchloride solution in diethyl ether or with a stoichiometric addition of trifluoroacetic acid. Hydrogenotrimethylphosphonium trifluoroacetate (1.14 g, 6 mmol) and ditetradecyl vinylphosphonate (2.5 g, 5 mmol) were dissolved in 20 mL of THF. The

mixture was refluxed for 16 h. After usual workup the product was isolated as a yellow powder: yield 75%; ^{31}P NMR 27.2 (d, $\text{P}=\text{O}$, $J_{\text{P-P}} = 12.7$) 29.1 (d, P^+ , $J_{\text{P-P}} = 12.7$); ^1H NMR 0.86 (t, 6H, $J_{\text{H-H}} = 6.7$, CH_3); 1.24 (m, 44H, CH_2) 1.68 (m, 4H, $\text{CH}_2\text{-CH}_2\text{O}$) 2.17 (m, 2H, $\text{CH}_2\text{P}=\text{O}$) 2.29 (d, 9H, $J_{\text{P-H}} = 14.1$, $\text{P}(\text{CH}_3)_3$) 2.84 (m, 2H, CH_2P^+) 3.98 (dt, 4H, $J_{\text{H-H}} = J_{\text{P-H}} = 6.8$, CH_2O); ^{13}C NMR 9.7 (d, $J_{\text{P-C}} = 54.1$, $\text{P}(\text{CH}_3)_3$) 14.1 (CH_3) 17.9 (dd, $J_{\text{P-C}} = 143.9$, $J_{\text{P-C}} = 4.2$, $\text{O}=\text{PCH}_2$) 17.7 (dd, $J_{\text{P-C}} = 53.7$, $J_{\text{P-C}} = 4.0$, P^+CH_2) from 22.7–32.7 several singlets (CH_2) 30.5 (d, $J_{\text{P-C}} = 6.3$, $\text{CH}_2\text{CH}_2\text{O}$) 65.2 (d, $J_{\text{P-C}} = 6.7$, CH_2O); mp = 91 °C. Anal. Calcd: C, 56.24; H, 10.15; P, 8.79. Found: C, 55.96; H, 10.26; P, 9.13.

Diolelyl 2-(trimethylphosphonio)ethylphosphonate iodide, EG316: yield 70%; ^{31}P NMR 27.6 (d, $\text{P}=\text{O}$, $J_{\text{P-P}} = 37.7$) 29.5 (d, P^+ , $J_{\text{P-P}} = 37.7$); ^1H NMR 0.86 (t, 6H, $J_{\text{H-H}} = 6.7$, CH_3); 1.24 (m, 44H, CH_2) 1.68 (m, 4H, $\text{CH}_2\text{CH}_2\text{O}$) 1.94 (m, 8H, $\text{CH}_2\text{CH}=\text{CHCH}_2$) 2.10 (m, 2H, $\text{CH}_2\text{P}=\text{O}$) 2.25 (d, 9H, $J_{\text{P-H}} = 14.2$, $\text{P}(\text{CH}_3)_3$) 2.72 (m, 2H, CH_2P^+) 3.98 (dt, 4H, $J_{\text{H-H}} = J_{\text{P-H}} = 6.8$, CH_2O) 5.28 (m, 4H, $\text{CH}=\text{CH}$); ^{13}C NMR 9.1 (d, $J_{\text{P-C}} = 54.0$, $\text{P}(\text{CH}_3)_3$) 14.1 (CH_3) 17.9 (dd, $J_{\text{P-C}} = 143.4$, $J_{\text{P-C}} = 3.9$, $\text{O}=\text{PCH}_2$) 17.9 (dd, $J_{\text{P-C}} = 53.5$, $J_{\text{P-C}} = 4.2$, P^+CH_2) from 22.7–32.7 several singlets (CH_2) 30.5 (d, $J_{\text{P-C}} = 6.3$, $\text{CH}_2\text{CH}_2\text{O}$) 65.2 (d, $J_{\text{P-C}} = 6.7$, CH_2O) 129.3 and 129.1 ($\text{CH}=\text{CH}$). Anal. Calcd: C, 54.23; H, 9.84. Found: C, 53.88; H, 9.84.

D. General Procedure for Ammonium and Phosphonium Propylenephosphonates and Arsonium Ethylene- and Propylenephosphonates. 3-(Ditetradecyloxyphosphoryl)-N,N,N-trimethyl-1-propanaminium iodide, ACH201. Ditetradecyl 3-bromopropylphosphonate (2.98 g, 5 mmol) was dissolved in 10 mL of DMF. 1.25 mL (10 mmol) of a commercial dimethylamine aqueous solution (40%) was then added and the mixture was refluxed for 2 h. After evaporation the residue was taken up in 20 mL CH_2Cl_2 and washed twice by 10 mL of a saturated aqueous NaHCO_3 solution. The organic phase was then evaporated and the residue was dissolved in 20 mL diethyl ether. Methyl iodide (1.42 g, 10 mmol) was added and the solution was stirred for one night at room temperature. The ammonium salt was purified as already described. A yellowish powder was obtained: yield 75%; ^{31}P NMR 29.3 (s, $\text{P}=\text{O}$); ^1H NMR 0.87 (t, 6H, $J_{\text{H-H}} = 6.8$) 1.25 (m, 44H, CH_2) 1.66 (m, 4H, $\text{CH}_2\text{CH}_2\text{O}$) 1.87 (td, 2H, $J_{\text{H-H}} = 7.0$, $J_{\text{P-H}} = 17.8$, $\text{O}=\text{PCH}_2$) 2.10 (m, 2H, $\text{O}=\text{PCH}_2\text{CH}_2\text{CH}_2\text{N}^+$) 3.46 (s, 9H, $\text{N}^+(\text{CH}_3)_3$) 3.86 (m, 2H, CH_2N^+) 4.01 (dt, 4H, $J_{\text{H-H}} = J_{\text{P-H}} = 6.8$, CH_2O); ^{13}C NMR 14.1 (CH_3) 17.1 ($\text{O}=\text{PCH}_2\text{CH}_2\text{CH}_2\text{N}^+$) 21.5 (d, $J_{\text{P-C}} = 143.2$, $\text{O}=\text{PCH}_2$) from 22.6–31.8 several singlets (CH_2) 30.5 (d, $J_{\text{P-C}} = 6.3$, $\text{CH}_2\text{CH}_2\text{O}$) 53.7 ($\text{N}^+(\text{CH}_3)_3$) 66.2 (d, $J_{\text{P-C}} = 6.7$, CH_2O) 66.2 (CH_2N^+); mp = 79 °C. Anal. Calcd: C, 56.73; H, 10.50; N, 1.95; P, 4.30. Found: C, 56.90; H, 10.52; N, 2.07; P, 4.38.

3-(Diolelyoxyphosphoryl)-N,N,N-trimethyl-1-propanaminium iodide, ACH204: yield 70%; ^{31}P NMR 29.5 (s, $\text{P}=\text{O}$); ^1H NMR 0.87 (t, 6H, $J_{\text{H-H}} = 6.8$) 1.25 (m, 44H, CH_2) 1.66 (m, 4H, $\text{CH}_2\text{CH}_2\text{O}$) 1.94 (m, 8H, $\text{CH}_2\text{CH}=\text{CHCH}_2$) 1.95 (m, 2H, $\text{O}=\text{PCH}_2$) 2.13 (m, 2H, $\text{O}=\text{PCH}_2\text{CH}_2\text{CH}_2\text{N}^+$) 3.46 (s, 9H, $\text{N}^+(\text{CH}_3)_3$) 3.88 (m, 2H, CH_2N^+) 4.01 (dt, 4H, $J_{\text{H-H}} = J_{\text{P-H}} = 6.8$, CH_2O) 5.35 (m, 4H, $\text{CH}=\text{CH}$); ^{13}C NMR 14.1 (CH_3) 17.0 ($\text{O}=\text{PCH}_2\text{CH}_2\text{CH}_2\text{N}^+$) 21.1 (d, $J_{\text{P-C}} = 143.4$, $\text{O}=\text{PCH}_2$) from 22.6–31.8 several singlets (CH_2) 30.5 (d, $J_{\text{P-C}} = 6.3$, $\text{CH}_2\text{CH}_2\text{O}$) 53.8 ($\text{N}^+(\text{CH}_3)_3$) 66.2 (d, $J_{\text{P-C}} = 6.7$, CH_2O) 66.2 (CH_2N^+) 129.7 and 129.9 ($\text{CH}=\text{CH}$).

Ditetradecyl 3-(Trimethylphosphonio)propylphosphonate Iodide, EG327. Ditetradecyl 3-bromopropylphosphonate (2.98 g, 5 mmol) was dissolved in 10 mL of THF. 10 mL of a 1 M commercial trimethylphosphine solution were then added and the mixture was refluxed for 72 h. After usual workup the product was isolated as a yellow powder: yield 82%; ^{31}P NMR 29.6 (d, $J_{\text{P-P}} = 5.9$, P^+) 27.6 (d, $J_{\text{P-P}} = 5.9$, $\text{P}=\text{O}$); ^1H NMR 0.86 (t, 6H, $J_{\text{H-H}} = 6.7$, CH_3) 1.24 (m, 44H, CH_2) 1.68 (m, 4H, $\text{CH}_2\text{CH}_2\text{O}$) 1.69 (m, 2H, $\text{O}=\text{PCH}_2\text{CH}_2\text{CH}_2\text{P}^+$) 1.96 (m, 2H, $\text{O}=\text{PCH}_2$) 2.21 (d, 9H, $J_{\text{P-H}} = 14.1$, $\text{P}^+(\text{CH}_3)_3$) 2.76 (m, 2H, CH_2P^+) 3.98 (dt, 4H, $J_{\text{H-H}} = J_{\text{P-H}} = 6.8$, CH_2O); ^{13}C NMR 9.0 (d, $J_{\text{P-C}} = 54.1$, $\text{P}^+(\text{CH}_3)_3$) 14.1 (CH_3) 15.3 ($\text{O}=\text{PCH}_2\text{CH}_2\text{CH}_2\text{P}^+$) 24.0 (dd, $J_{\text{P-C}} = 52.1$, $J_{\text{P-C}} = 15.1$, P^+CH_2) 25.4 (dd, $J_{\text{P-C}} = 138.7$, $J_{\text{P-C}} = 17.1$, $\text{O}=\text{PCH}_2$) from 22–32.7

several singlets (CH_2) 30.5 (d, $J_{\text{P-C}} = 6.0$, $\text{CH}_2\text{CH}_2\text{O}$) 65.2 (d, $J_{\text{P-C}} = 6.7$, CH_2O); mp = 80 °C. Anal. Calcd: C, 56.20; H, 10.24; P, 8.62. Found: C, 56.04; H, 10.30; P, 8.80.

Diolelyl 3-(trimethylphosphonio)propylphosphonate iodide, EG353: yield 70%; ^{31}P NMR 29.7 (d, $J_{\text{P-P}} = 5.9$, P^+) 27.1 (d, $J_{\text{P-P}} = 5.9$, $\text{P}=\text{O}$); ^1H NMR 0.86 (t, 6H, $J_{\text{H-H}} = 6.7$, CH_3) 1.24 (m, 44H, CH_2) 1.68 (m, 4H, $\text{CH}_2\text{CH}_2\text{O}$) 1.70 (m, 2H, $\text{O}=\text{PCH}_2\text{CH}_2\text{CH}_2\text{P}^+$) 1.98 (m, 2H, $\text{O}=\text{PCH}_2$) 2.18 (d, 9H, $J_{\text{P-H}} = 14.0$, $\text{P}^+(\text{CH}_3)_3$) 2.67 (m, 2H, CH_2P^+) 3.98 (dt, 4H, $J_{\text{H-H}} = J_{\text{P-H}} = 6.8$, CH_2O) 5.32 (m, 4H, $\text{CH}=\text{CH}$); ^{13}C NMR 9.2 (d, $J_{\text{P-C}} = 54.5$, $\text{P}^+(\text{CH}_3)_3$) 14.1 (CH_3) 15.5 (d, $J_{\text{P-C}} = 3.2$, $\text{O}=\text{PCH}_2\text{CH}_2\text{CH}_2\text{P}^+$) 24.2 (dd, $J_{\text{P-C}} = 52.6$, $J_{\text{P-C}} = 14.5$, P^+CH_2) 25.5 (dd, $J_{\text{P-C}} = 141.0$, $J_{\text{P-C}} = 16.8$, $\text{O}=\text{PCH}_2$) from 22–32.7 several singlets (CH_2) 30.5 (d, $J_{\text{P-C}} = 6.0$, $\text{CH}_2\text{CH}_2\text{O}$) 65.2 (d, $J_{\text{P-C}} = 6.7$, CH_2O) 129.8 and 129.4 ($\text{CH}=\text{CH}$).

Ditetradecyl 2-(Trimethylarsonio)ethylphosphonate Iodide, EG371. Ditetradecyl 2-bromoethylphosphonate (2.91 g, 5 mmol) and trimethylarsine (1.2 g, 10 mmol) were heated neat for 1 week at 45 °C in a sealed tube. After evaporation of the excess of trimethylarsine and usual purification the product was isolated as a powder: yield 88%; ^{31}P NMR 28.7 (s, $\text{P}=\text{O}$); ^1H NMR 0.87 (t, 6H, $J_{\text{H-H}} = 6.8$, CH_3) 1.24 (m, 44H, CH_2) 1.68 (m, 4H, $\text{CH}_2\text{CH}_2\text{O}$) 2.25 (s, 9H, $\text{As}^+(\text{CH}_3)_3$) 2.34 (td, 2H, $J_{\text{H-H}} = 6.7$, $J_{\text{P-H}} = 17.2$, CH_2As^+) 2.95 (td, 2H, $J_{\text{H-H}} = 6.7$, $J_{\text{P-H}} = 20.8$, $\text{O}=\text{PCH}_2$) 4.19 (dt, 4H, $J_{\text{H-H}} = J_{\text{P-H}} = 6.8$, CH_2O); ^{13}C NMR 10.5 ($\text{As}^+(\text{CH}_3)_3$) 14.0 (CH_3) 20.1 (d, $J_{\text{P-C}} = 143.8$, $\text{O}=\text{PCH}_2$) 21.2 (d, $J_{\text{P-C}} = 5.8$, CH_2As^+) from 22.5–31.9 several singlets (CH_2) 29.9 (d, $J_{\text{P-C}} = 6.3$, $\text{CH}_2\text{CH}_2\text{O}$) 67.7 (d, $J_{\text{P-C}} = 6.7$, CH_2O); mp = 80 °C. Anal. Calcd: C, 52.30; H, 9.68; As, 9.64; P, 3.97. Found: C, 51.91; H, 9.56; As, 9.64; P, 4.10.

Diolelyl 2-(trimethylarsonio)ethylphosphonate iodide, EG372: yield 63%; ^{31}P NMR 29.0 (s, $\text{P}=\text{O}$); ^1H NMR 0.87 (t, 6H, $J_{\text{H-H}} = 6.8$, CH_3) 1.24 (m, 44H, CH_2) 1.68 (m, 4H, $\text{CH}_2\text{CH}_2\text{O}$) 1.96 (m, 8H, $\text{CH}_2\text{CH}=\text{CHCH}_2$) 2.25 (s, 9H, $\text{As}^+(\text{CH}_3)_3$) 2.28 (td, 2H, $J_{\text{H-H}} = 6.7$, $J_{\text{P-H}} = 17.1$, CH_2As^+) 2.94 (td, 2H, $J_{\text{H-H}} = 6.7$, $J_{\text{P-H}} = 20.4$, $\text{O}=\text{PCH}_2$) 4.19 (dt, 4H, $J_{\text{H-H}} = J_{\text{P-H}} = 6.8$, CH_2O) 5.29 (m, 4H, $\text{CH}=\text{CH}$); ^{13}C NMR 10.5 ($\text{As}^+(\text{CH}_3)_3$) 14.0 (CH_3) 20.0 (d, $J_{\text{P-C}} = 143.6$, $\text{O}=\text{PCH}_2$) 20.7 (d, $J_{\text{P-C}} = 4.8$, CH_2As^+) from 22.5–31.9 several singlets (CH_2) 29.9 (d, $J_{\text{P-C}} = 6.3$, $\text{CH}_2\text{CH}_2\text{O}$) 67.7 (d, $J_{\text{P-C}} = 6.7$, CH_2O) 129.3 and 129.5 ($\text{CH}=\text{CH}$).

Ditetradecyl 3-(Trimethylarsonio)propylphosphonate Iodide, EG356. Ditetradecyl 3-bromopropylphosphonate (2.98 g, 5 mmol) and trimethylarsine (1.2 g, 10 mmol) were heated neat for 1 week at 45 °C in a sealed tube. After evaporation of the excess of trimethylarsine and usual purification the product was isolated as a powder: yield 90%; ^{31}P NMR 29.8 (s, $\text{P}=\text{O}$); ^1H NMR 0.87 (t, 6H, $J_{\text{H-H}} = 6.8$) 1.25 (m, 44H, CH_2) 1.66 (m, 4H, $\text{CH}_2\text{CH}_2\text{O}$) 1.89 (m, 2H, $\text{O}=\text{PCH}_2\text{CH}_2\text{CH}_2\text{As}^+$) 1.94 (m, 2H, CH_2As^+) 2.18 (s, 9H, $\text{As}^+(\text{CH}_3)_3$) 2.89 (m, 2H, $\text{O}=\text{PCH}_2$) 4.01 (dt, 4H, $J_{\text{H-H}} = J_{\text{P-H}} = 6.8$, CH_2O); ^{13}C NMR 8.3 ($\text{As}^+(\text{CH}_3)_3$) 14.0 (CH_3) 16.7 (d, $J_{\text{P-C}} = 4.4$, $\text{O}=\text{PCH}_2\text{CH}_2\text{CH}_2\text{As}^+$) 25.4 (d, $J_{\text{P-C}} = 18.8$, CH_2As^+) 25.8 (d, $J_{\text{P-C}} = 141.0$, $\text{O}=\text{PCH}_2$) from 22.5–31.9 several singlets (CH_2) 29.9 (d, $J_{\text{P-C}} = 6.3$, $\text{CH}_2\text{CH}_2\text{O}$) 67.77 (d, $J_{\text{P-C}} = 6.7$, CH_2O); mp = 50 °C.

Diolelyl 3-(trimethylarsonio)propylphosphonate iodide, EG363: yield 60%; ^{31}P NMR 29.6 (s, $\text{P}=\text{O}$); ^1H NMR 0.87 (t, 6H, $J_{\text{H-H}} = 6.8$) 1.25 (m, 44H, CH_2) 1.66 (m, 4H, $\text{CH}_2\text{CH}_2\text{O}$) 1.92 (m, 2H, $\text{O}=\text{PCH}_2\text{CH}_2\text{CH}_2\text{As}^+$) 1.97 (m, 2H, CH_2As^+) 1.98 (m, 8H, $\text{CH}_2\text{CH}=\text{CHCH}_2$) 2.18 (s, 9H, $\text{As}^+(\text{CH}_3)_3$) 2.92 (m, 2H, $\text{O}=\text{PCH}_2$) 4.01 (dt, 4H, $J_{\text{H-H}} = J_{\text{P-H}} = 6.8$, CH_2O) 5.29 (m, h, $\text{CH}=\text{CH}$); ^{13}C NMR 9.2 ($\text{As}^+(\text{CH}_3)_3$) 14.0 (CH_3) 16.9 (d, $J_{\text{P-C}} = 4.4$, $\text{O}=\text{PCH}_2\text{CH}_2\text{CH}_2\text{As}^+$) 26.0 (d, $J_{\text{P-C}} = 15.0$, CH_2As^+) 25.8 (d, $J_{\text{P-C}} = 141.2$, $\text{O}=\text{PCH}_2$) from 22.5–31.9 several singlets (CH_2) 29.9 (d, $J_{\text{P-C}} = 6.3$, $\text{CH}_2\text{CH}_2\text{O}$) 67.8 (d, $J_{\text{P-C}} = 6.7$, CH_2O) 129.1 and 130 ($\text{CH}=\text{CH}$).

2. Cell Lines and Plasmid DNA. For the in vitro experiments we used the CFT-1, HT29, HeLa and K562 cell lines. CFT-1 cells are SV40 large T-transformed CF-tracheal cells obtained from a CF fetus after therapeutic abortion.³⁶ They were grown in MEM/Ham-F-12 (50/50) medium supplemented with 10% of fetal calf serum (FCS), 0.2 mM glutamine, 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin and 1% fungizone.

HT29 and HeLa cells (obtained from ATCC) were maintained DMEM or MEM, respectively, and supplemented with 10% fetal calf serum (FCS), 0.2 mM glutamine, 100 U/mL penicillin, 100 U/mL streptomycin and 1% fungizone. The human hematopoietic nonadherent cell line K562 was obtained from ATCC (no. ccl 243 ATCC, Rockville, MD) and was maintained in RPMI-1640 medium supplemented with 10% FCS, 0.2 mM glutamine, 100 U/mL penicillin, 100 U of streptomycin and 1% fungizone. Cells concentrations were maintained between 10^5 /mL and 10^6 /mL. All cells were maintained in 5% CO₂ and at 37 °C.

Plasmid pTG11033 (a gift from TRANSGENE Strasbourg France) contains the luciferase gene under the control of cytomegalovirus immediate early gene 1 (IE1-CMV) promoter, intron 1 of the *HMGCoAR* (hydroxymethyl-glutaryl-CoA reductase) gene and the simian virus 40 (SV40) poly(A) signal.

Plasmids were amplified in the DH5 α strain of *Escherichia coli*, isolated by alkaline lysis and purified with Qiagen Endo-free Giga-prep Kit (Qiagen SA, Cortaboef, France) according to the manufacturer's protocol. Plasmids were dissolved in endotoxin-free water and stored at -20 °C. DNA was quantified by spectrophotometry at an optical density of 260 nm and was free of major protein contamination with a A260/280 ratio of between 1.8 and 2.0. The amount of endotoxin present was determined using a chromogenic limulus amoebocyte clotting assay (QCL-1000 kit, Biowhittaker, France) and values were <10 endotoxin units (EU)/mg DNA.

3. Preparation of Cationic Phosphonolipid/DNA Complexes. The cationic phosphonolipids and DOTMA used in this report were synthesized by our group.^{8,26} Their structures are presented in Table 1. Commercialized cationic reagents was also used: Lipofectin (DOTMA/DOPE 1:1 (w:w)). Each of the cationic phosphonolipids was prepared alone or in combination with the neutral lipid DOPE or cholesterol (Sigma, Saint Quentin Fallavier, France). The cationic lipid to colipid ratio used was 1:1 (w:w). The phosphonolipids were formulated by mixing chloroform solutions of the different lipids in glass vials, then removing the chloroform by rotary evaporation to produce dried lipid films. Sterile pyrogen-free DI water was then added and the vials were sealed and stored overnight at 4 °C. Small unilamellar vesicles (suv) were prepared by sonicating the compounds for 10 min in a sonicator (Prolabo, Paris, France). To prepare the cationic phosphonolipid/DNA complexes, plasmid DNA was first diluted with sterile pyrogen-free DI water and added to the lipid solution. The lipoplexes were kept 30 min at room temperature before being administered into animals or used for in vitro transfections.

4. In Vitro Transfection and Reporter Gene Assay. Transfection activity of the cationic lipid/DNA complexes in vitro was assessed using different cell lines. Cells were seeded onto a 96-well tissue culture plate at 20 000/well (16 wells/lipid tested) 24 h before transfection and incubated overnight in a humidified 5% CO₂ atmosphere at 37 °C. Transfection of the cells was performed as described by Felgner et al.⁷ with the following modifications. Cationic phosphonolipids used for transfection of adherent cells were formulated with DOPE (1/1, w:w), but according to previous results¹⁰ cationic phosphonolipids formulated without colipid were used for transfection assay on nonadherent cells. Appropriate amounts of the cationic lipids and the plasmid vector pTG11033 in OptiMEM were complexed and 100 μ L was added to each well. After 2.5 h at 37 °C, the cells were supplemented with 200 μ L of appropriate growth medium. Following a further 48 h at 37 °C, the cells were assayed for luciferase expression using a chemiluminescent assay (Promega). Assays were carried out as described by the manufacturer. The results were expressed in total relative light units (TRLU) obtained for 16 wells.

5. Intravenous Gene Delivery and Luciferase Expression in Mouse Tissues. Cationic phosphonolipid/pTG11033 plasmid DNA complexes (formulated with a lipid to DNA charge ratio of 4) were delivered by a single injection of 200 μ L in the tail vein of 6-week-old female Swiss mice, and each animal received 50 μ g of plasmid DNA. The animals were killed 24 h after injection and mouse tissues were immediately

frozen on dry ice and stored at -70 °C until examined. Luciferase activity was assayed using a chemiluminescent kit (Promega). The extraction of luciferase from mouse tissues was carried out as described by Thierry et al.³⁷ The total protein concentration of the tissue extract was determined using the Bio-Rad protein assay. The luciferase activity of each sample was normalized to the relative light unit (RLU)/mg of extracted protein.

6. Determination of Cell Toxicity. The relative cytotoxicity of the different lipid/DNA complexes were determined as the number of cells surviving the transfection experiment measured using a chemiluminescent assay: CYTOLITE assay (Pakard) as specified by the manufacturer. 24 h before the assay, the cells were plated in a 96-wells plate (25 000 cells/well). Cells were treated for transfection as described above and incubated for an additional 48-h period. After this time the cytotoxicity assay was carried out as specified by the manufacturer. The amount of relative light units (RLU) formed was proportional to the number of living cells. Nontransfected cells were used as control. The final results were expressed in toxicity index. This toxicity index was the calculated ratio of number of living cells in the "control well" over the number of living cells in the "transfected well". A toxicity index of 1 shows no differences between control and transfected cells implying no cytotoxicity. The cytotoxicity index increased as the toxicity of the cationic lipid tested increased.

7. Measurement of Clinical Biochemistry Parameters. Serum samples were obtained by separation of the coagulated whole blood of animal 24 h after intravenous administration of lipoplexes. Serum concentration of alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured by automated analyzer ARS (Dade Behring) in an independent laboratory (Laboratoire d'Analyses Medicales Floch, Brest, France). The results are also expressed as toxicity index. This toxicity index was the calculated ratio of the "control mouse" transaminase level over the transaminase level of the "injected mouse". The toxicity index increased as the toxicity of the cationic lipid tested increased.

8. Statistical Analysis. Results are expressed as relative light units (RLU) means \pm standard error of the mean (SEM). After ANOVA analysis, the statistical significance of the data has been determined with a Student's unpaired *t*-test as appropriate (banferroni principle). Probability (*p*) < 0.05 was considered significant.

9. Abbreviations: DOPE, dioleoylphosphatidylethanolamine; DORIE, 1,2-dioleoyloxypropyl-3-dimethylhydroxyethylammonium bromide; DOTMA, 1,2-dioleoyloxypropyl-3-trimethylammonium bromide; THF, tetrahydrofuran; DMF, *N,N*-dimethylformamide; DSC, differencial scanning calorimetry; s, singlet; d, doublet; t, triplet; dd, double doublets; dt, double triplets; m, multiplet.

Acknowledgment. This work was supported by INSERM (CRI No. 9607), by AFLM Association Française de Lutte contre la Mucoviscidose, by A.F.S. Agence Française du Sang, and by Association de Transfusion Sanguine Gaëtan Saleun. We thank Transgene for the pTG11033 plasmid.

References

- Miller, A. D. Cationic liposomes for gene therapy. *Angew. Chem., Int. Ed.* **1998**, *37*, 1768–1785.
- Gao, X.; Huang, L. Cationic liposome-mediated gene transfer. *Gene Ther.* **1995**, *2*, 710–722.
- Bennett, M. J.; Aberle, A. M.; Balasubramaniam R. P.; Malone, J. G.; Nantz, M. H.; Malone, R. W. Considerations for the design of improved cationic amphiphile-based transfection reagents. *J. Liposome Res.* **1996**, *6*, 545–565.
- Balasubramaniam, R. P.; Bennett, M. J.; Aberle, A. M.; Malone, J. G.; Nantz, M. H.; Malone, R. W. Structural and functional analysis of cationic transfection lipids: the hydrophobic domain. *Gene Ther.* **1996**, *3*, 163–172.

- (5) Lee, E. R.; Marshall, J.; Siegel, C. S.; Jiang, C.; Yew, N. S.; Nichols, M. R.; Nietupski, J. B.; Ziegler, R. J.; Lane, M. B.; Wang, K. X.; Wan, N. C.; Scheule, R. K.; Harris, D. J.; Smith, A. E.; Cheng, S. H. Detailed analysis of structure and formulations of cationic lipids for efficient gene transfer to the lung. *Hum. Gene Ther.* **1996**, *7*, 1701–1717.
- (6) Aberle, A. M.; Bennet, M. J.; Malone, R. W.; Nantz, M. H. The counterion influence on cationic lipid-mediated transfection of plasmid DNA. *Biochim. Biophys. Acta* **1996**, *1299*, 81–83.
- (7) Felgner, J. N.; Kummur, R.; Sridhar, C. N.; Wheeler, C.; Tsai, Y. K. J. K.; Border, R.; Ramsay, P.; Martin, M.; Felgner, P. Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. *J. Biol. Chem.* **1994**, *269*, 2550–2561.
- (8) Le Bolc'h, G.; Le Bris, N.; Yaouanc, J. J.; Clément, J. C.; des Abbayes, H. Cationic phosphonolipids as non viral vectors for DNA transfection. *Tetrahedron Lett.* **1995**, *36*, 6681–6684.
- (9) Audrézet, M. P.; Le Bolc'h, G.; Floch, V.; Yaouanc, J. J.; Clément, J. C.; Des Abbayes, H.; Mercier, B.; Paul, A.; Férec, C. Novel cationic phosphonolipids agents for gene transfer to a cystic fibrosis cell line. *J. Liposome Res.* **1997**, *7*, 273–300.
- (10) Floch, V.; Le Bolc'h, G.; Audrézet, M. P.; Yaouanc, J. J.; Clément, J. C.; Des Abbayes, H.; Mercier, B.; Abgrall, J. F.; Férec, C. Cationic phosphonolipids as non viral vectors for DNA transfection in hematopoietic cell lines and CD34⁺ cells. *Blood Cells Mol. Dis.* **1997**, *23*, 69–87.
- (11) Floch, V.; Audrézet, M. P.; Guillaume, C.; Gobin, E.; Le Bolc'h, G.; Clément, J. C.; Yaouanc, J. J.; des Abbayes, H.; Mercier, B.; Leroy, J. P.; Abgrall, J. F.; Férec, C. Transgene expression kinetics after transfection with cationic phosphonolipids in hematopoietic non adherent cells. *Biochim. Biophys. Acta* **1998**, *1371*, 53–70.
- (12) Guillaume-Gable, C.; Floch, V.; Mercier, B.; Audrézet, M. P.; Gobin, E.; Le Bolc'h, G.; Yaouanc, J. J.; Clément, J. C.; Des Abbayes, H.; Leroy, J. P.; Morin, V.; Férec, C. Cationic phosphonolipids as non viral gene transfer agents in lungs of mice. *Hum. Gene Ther.* **1998**, *9*, 2309–2319.
- (13) Floch, V.; Delépine, P.; Guillaume, C.; Loisel, S.; Chassé, S.; Le Bolc'h, G.; Gobin, E.; Leroy, J. P.; Férec, C. Systemic administration of cationic phosphonolipids/DNA complexes and the relationship between formulation and lung transfection efficiency. *Biochim. Biophys. Acta* **2000**, *1464*, 95–103.
- (14) Griesenbach, U.; Chonn, A.; Cassady, R.; Hannam, V.; Ackerley, C.; Post, M.; Tanswell, A. K.; Olek, K.; O'Brodovich, H.; Tsui, L.-C. Comparison between intratracheal and intravenous administration of liposome-DNA complexes for cystic fibrosis lung gene therapy. *Gene Ther.* **1998**, *5*, 181–188.
- (15) Goula, D.; Benoist, C.; Mantero, S.; Merlo, G.; Levi, G.; Deme-neix, B. A. Polyethylenimine-based intravenous delivery of transgenes to mouse lung. *Gene Ther.* **1998**, *5*, 1291–1295.
- (16) Aberle, A. M.; Tablin, F.; Zhu, J.; Walker, N.; Gruenert, D. C.; Nantz, M. A novel tetraester construct that reduces cationic lipid-associated cytotoxicity. Implications for the onset of cytotoxicity. *Biochemistry* **1998**, *37*, 6533–6540.
- (17) Floch, V.; Legros, N.; Loisel, S.; Guillaume, C.; Guilbot, J.; Benvegnu, T.; Ferrieres, V.; Plusquellec, D.; Férec, C. New biocompatible cationic amphiphiles derivative from glycine betaine: a novel family of efficient non viral gene transfer agents. *Biochem. Biophys. Res. Commun.* **1998**, *251*, 360–365.
- (18) Van Der Woude, I.; Wagenaar, A.; Meekel, A. A. P.; Ter Beest, M. B. A.; Ruiters, M. H. J.; Engberts, J. B. F. N.; Hoekstra, D. Novel pyridinium surfactants for efficient, non toxic in vitro gene delivery. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 1160–1165.
- (19) Paukku, T.; Lauraeus, S.; Huhtaniemi, I.; Kinnunen, P. K. J. Novel cationic liposomes for DNA-transfection with high efficiency and low toxicity. *Chem. Phys. Lipids* **1997**, *63*, 251–258.
- (20) Wheeler, C. J.; Sukhu, L.; Yang, G.; Tsai, Y.; Bustamente, C.; Felgner, P.; Norman, J.; Manthorpe, M. Converting an alcohol to an amine in a cationic lipid dramatically alters the co-lipid requirement, cellular transfection activity and the ultrastructure of DNA-cytfectin complexes. *Biochim. Biophys. Acta* **1996**, *1280*, 1–11.
- (21) Solodin, E. J.; Brown, C. S.; Bruno, M. S.; Chow, C. Y.; Jang, E.; Debs, R. J.; Heath, T. D. A novel series of amphiphilic imidazolium compounds for in vitro and in vivo gene delivery. *Biochemistry* **1995**, *34*, 3537–3544.
- (22) Byk, G.; Dubertret, C.; Escriviou, V.; Frederic, M.; Jaslin, G.; Rangara, R.; Pitard, B.; Crouzet, J.; Wils, P.; Schwartz, B.; Scherman, D. Synthesis, activity, and structure–activity relationship studies of novel cationic lipids for DNA transfer. *J. Med. Chem.* **1998**, *41*, 224–235.
- (23) Cooper, R. G.; Etheridge, C. J.; Stewart, L.; Marshall, J.; Rudginsky, S.; Cheng, S. H.; Miller, A. D. Polyamine analogues of 3 β -[N-(N,N-dimethylaminoethane)carbamoyl]-cholesterol (DC-Chol) as agents for gene delivery. *Chem. Eur. J.* **1998**, *4*, 137–151.
- (24) Haces, A.; Ciccarone, V. Highly packed polycationic ammonium, sulfonium and phosphonium lipids. U.S. Patent 94/14475 (Life Technologies Inc.).
- (25) Stekar, J.; Nössner, G.; Kutscher, B.; Engel, J.; Hilgard, P. Synthesis, antitumor activity, and tolerability of phospholipids containing nitrogen homologues. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 238–240.
- (26) Guenin, E.; Herve, A. C.; Floch, V.; Loisel, S.; Yaouanc, J. J.; Clément, J. C.; Férec, C.; Des Abbayes, H. Cationic phosphonolipids containing quaternary phosphonium and arsonium for DNA transfection with good efficiency and low cellular toxicity. *Angew. Chem., Int. Ed. Engl.* **2000**, *39*, 629–631.
- (27) Balczewski, P.; Pietrzykowski, W. M. A free radical approach to functionalization of phosphonates utilizing novel 2- and 3-phosphonyl radicals. *Tetrahedron* **1996**, *52*, 13681–13694.
- (28) McKenna, C. E.; Higa, M. T.; Cheung, N. H.; McKenna, M. C. The facile dealkylation of phosphonic acid dialkyl esters by bromotrimethylsilane. *Tetrahedron Lett.* **1977**, *2*, 155–158.
- (29) Bhongle, N. N.; Notter, R. H.; Turcotte, J. G. Expedient and high-yield synthesis of alkylphosphonyl dichlorides under mild, neutral conditions: reaction of bis(trimethylsilyl)alkyl phosphonates with oxalyl chloride/dimethylformamide. *Synth. Commun.* **1987**, *17*, 1071–1076.
- (30) Farhood, H.; Serbina, N.; Huang, L. The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. *Biochim. Biophys. Acta* **1995**, *1235*, 289–295.
- (31) Floch, V.; Le Bolc'h, G.; Gable-Guillaume, C.; Le Bris, N.; Yaouanc, J. J.; Des Abbayes, H.; Férec, C.; Clément, J. C. Phosphonolipids as non viral vectors for gene therapy. *Eur. J. Med. Chem.* **1998**, *33*, 923–934.
- (32) Leventis, R.; Silvius, J. R. Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles. *Biochim. Biophys. Acta* **1990**, *1023*, 124–32.
- (33) Liu, Y.; Mounkes, L. C.; Liggitt, H. D.; Brown, C. S.; Solodin, I.; Heath, T. D.; Debs, R. Factors influencing the efficiency of cationic liposome mediated intravenous gene delivery. *Nat. Biotechnol.* **1997**, *15*, 167–174.
- (34) Song, Y. K.; Liu, F.; Chu, S.; Liu, D. Characterization of cationic liposome mediated gene transfer in vivo by intravenous administration. *Hum. Gene Ther.* **1997**, *8*, 1585–1594.
- (35) Smyth-Templeton, N.; Lasic, D. D.; Frederik, P. M.; Strey, H. H.; Roberts, D. D.; Pavlakis, G. N. Improved DNA: liposome complexes for increased systemic delivery and gene expression. *Nat. Biotechnol.* **1997**, *15*, 647–652.
- (36) Lemnaouar, M.; Chastre, E.; Paul, A.; Mergey, M.; Veiddere, D.; Cherqui, G.; Barbry, P.; Simon-Bouy, B.; Fanen, P.; Gespach, C.; Picard, J. Oncogene-mediated propagation of tracheal epithelial cells from two cystic fibrosis fetuses with different mutations. Characterization of CFT-1 and CFT-2 cells in culture. *Eur. J. Clin. Invest.* **1993**, *23*, 151–160.
- (37) Thierry, A. R.; Lunardi-Iskandar, Y.; Bryant, J. L.; Rabinovich, P.; Gallo, R. C.; Mahan, L. C. Systemic gene therapy: Biodistribution and long-term expression of a transgene in mice. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 9742–9746.

JM000006Z