Pyridinium Cationic Lipids in Gene Delivery: A Structure−Activity Correlation Study

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Three series of pyridinium cationic lipids useful as nonviral gene delivery agents were prepared by reaction of pyrylium salts with aminodiols, followed by acylation with fatty acyl chlorides. On the basis of this set of compounds, we undertook a comprehensive structure−activity relationship study at the level of the linker, hydrophobic anchor, and counterion in order to identify the structural elements that generate the highest transfection efficiency for this new type of cationic lipid. The results revealed that when formulated with cholesterol at a 1:1 molar ratio, the 1-(1,3-dimyristoyloxyprop-2-yl)-2,4,6-trimethylpyridinium, under the form of hexafluorophosphate (**5AMyr**) or chloride (**5DMyr**), was able to transfect NCI-H23 lung carcinoma with efficiencies surpassing classic DOTAP-based formulations and with lower cytotoxicity. Subsequent tests on other malignancies yielded similarly promising results.

Introduction

The prospect of gene therapy gives a new perspective to medicine, allowing a revolutionary approach to treat diseases at the level where they are generated, namely, the living cell. When the cellular machinery is impaired because of a deficient gene, a functional gene incorporated into an appropriate vector may be delivered to the affected cells, tissues, or organs. After internalization, the DNA is transferred to the nucleus where the gene is integrated into the host genome, transcribed, and finally translated into the proteins needed to correct the cellular imbalance.[1,2] Despite the simple concept, the eventual success of this new form of therapy relies on the efficiency of the overall delivery process.[3]

Viral vectors are currently the most efficient systems for the transfer and expression (transfection) of foreign DNA into living cells.[4] However, their effectiveness is hampered by serious side effects, such as immunogenicity, difficulties associated with good manufacturing practice (GMP) production or storage, a limited size of the DNA that can be inserted into the virion, mutagenicity, and sometimes fatal toxicity.[5]

Cationic lipids have emerged[6] as safer alternatives to viral delivery. Having low immunogenicity and cytotoxicity, they also allow the use of plasmids of practically unlimited size and can be easily manufactured and stored in bulk quantities under GMP-compliant norms.[7−9] To bind and compact DNA efficiently, the cationic lipids usually must self-assemble first into cationic liposomes. Under this form they interact with the negatively charged plasmids to yield cationic lipids−DNA complexes (lipoplexes[10]) with differing sizes and shapes.[11−13] The characteristics of the lipoplexes are essential for their efficiency. Variables such as the lipid nature and composition of the parent cationic liposomes, the characteristics of the plasmid, or the method used to generate the lipoplexes are critical for achieving high levels of transfection.[11,14]

Despite tremendous synthetic efforts[7−9] that have generated several commercial cationic lipid transfection systems (DOTMA−Di-C14-amidine; Chart 1), there is still a need for improving in vivo efficiency and decreasing cytotoxicity. In this context, the heterocyclic cationic lipids newly introduced by several different groups[15−17] represent a promising alternative, displaying higher transfection efficiencies and a reduced cytotoxity when compared with their tetraalkylammonium congeners.

Among them, the pyridinium cationic lipids reported first by Engbert's group[16] and then also by ourselves[17] (DOTIM and SAINT; Chart 2) reached or surpassed the transfection efficiency of commercial cationic lipid formulations, while maintaining a low cytotoxicity.

Following our promising preliminary results, we report now a structure−activity relationship study aimed at identifying the most efficient structural variables at the level of the hydrophobic anchor, linker, and counterion for this type of pyridinium cationic lipid. Also, this study was meant to find the best liposomal formulation for these new cationic lipids.

Results and Discussion

1. Chemistry. The synthesis of the new cationic lipids is outlined in Scheme 1. It is based on the original synthetic strategy reported previously,[17,18] which involves the reaction of substituted pyrylium salts with primary amines[19] to generate the cationic head and the linker in a single step (Scheme 1). Mention must be made that various pyrylium salts can be employed,[19] allowing different designs for the polar head, for target-

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Chart 1

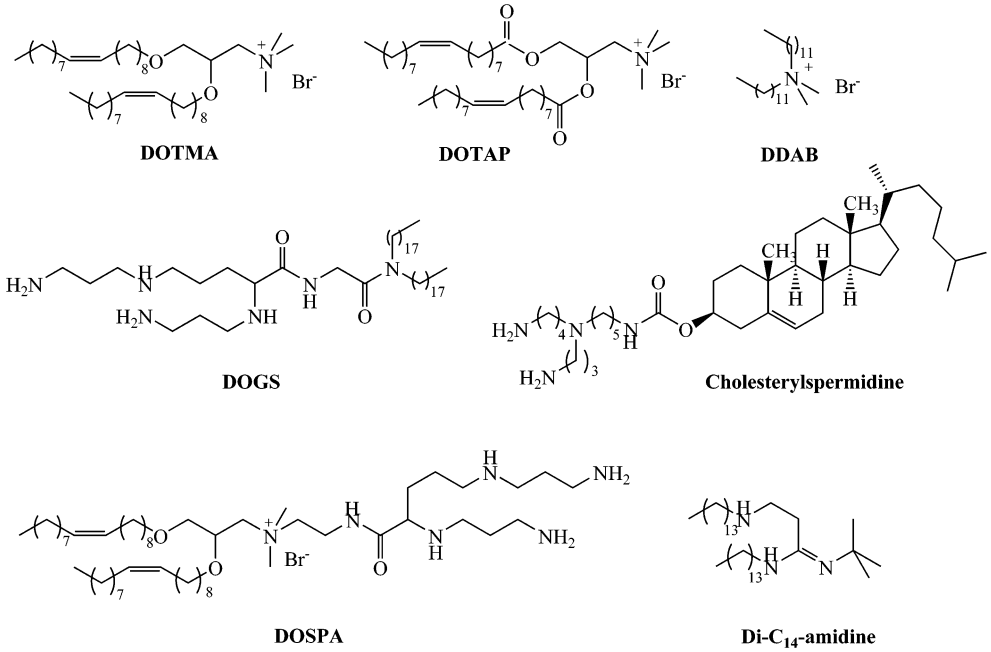
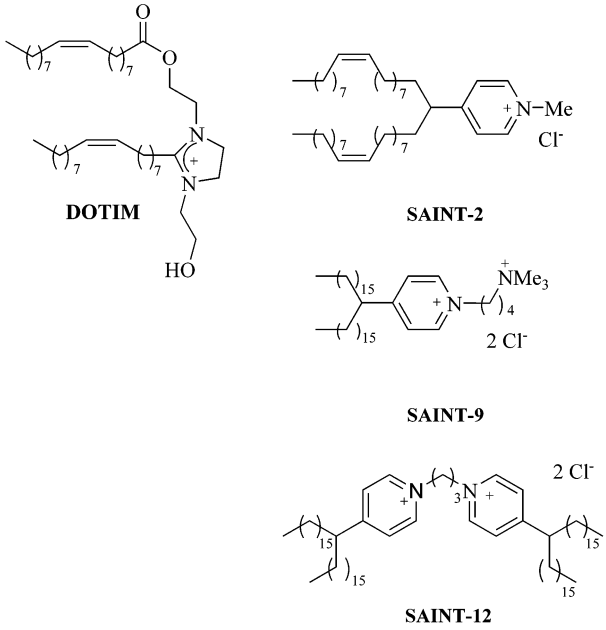


Chart 2



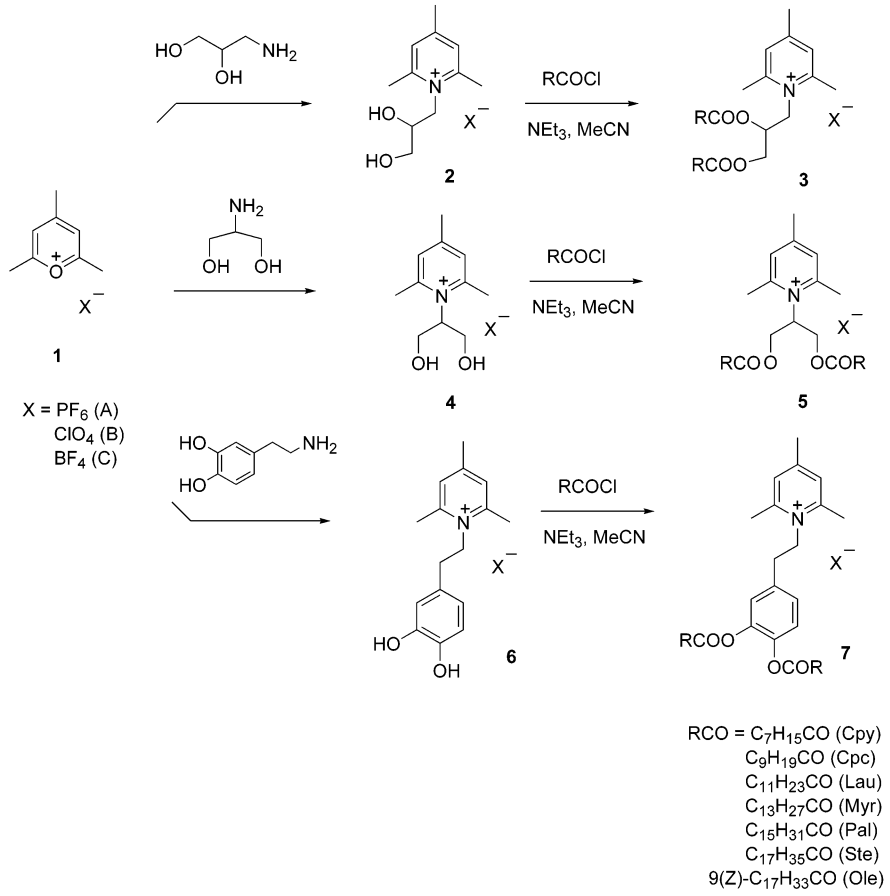
ing purposes or for inducing special optical properties (e.g., fluorescent cationic lipids as membrane markers[17]).

The choice of the appropriate amine allows the design of the linker. To investigate the influence of this structural element on the transfection efficiency of pyridinium cationic lipids, we decided to compare a flexible alkyl linker versus a flat, aromatic one by using aminopropanediol or dopamine as precursors. Another variable was allowed at this stage by using either 3-amino-1,2-propanediol or serinol in the alkyl linker set. The key intermediates **2**, **4**, and **6** with two hydroxy groups were obtained in high yields and were subsequently acylated with a wide range of fatty acyl chlorides in order to generate the corresponding cationic lipids **3**, **5**, and **7**. The pyridinium-based cationic lipids will be denoted by numbers followed by capital letters denoting the anion (**A** = PF_6^-, **B** = ClO_4^-, **C** = BF_4^-, **D** = Cl^-, **E** = I^-) and then by three letters indicating the fatty acid (**Cpy** = caprylic acid, **Cpc** = capric acid, **Lau** = lauric acid, **Myr** = myristic acid, **Pal** = palmitic acid, **Ste** = stearic acid, **Ole** = oleic acid). For the series bearing alkyl linkers, we varied the length of the hydrophobic anchor from 12 to 18 carbon atoms, by analogy with similar studies on cationic lipids bearing trimethylammonium polar heads.[20−22] The 10 cationic lipids **3ALau**−**3AOle** and **5ALau**−**5AOle** were thus obtained. For the dopamine-based series we also used shorter acyl chlorides (ranging from 8 to 18 carbon atoms) because of the additional contribution of the phenyl ring, yielding the seven pyridinium lipids **7ACpy**−**7AOle**. Attempts to synthesize lower homologues, derived from aminomethylcatechol or aminocatechol, failed because of the concurrent solvolytic reactions at the benzylic position in the former case or because of the instability of the aminocatechol (which is extremely sensitive to oxygen) in the latter case.

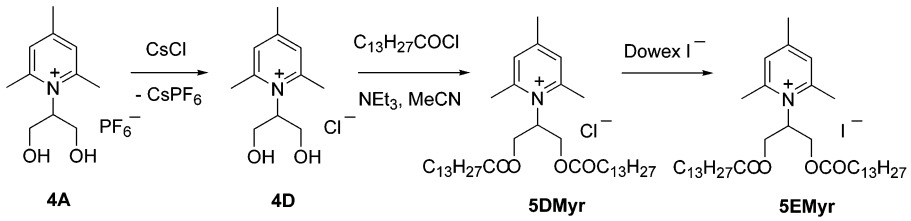
The hexafluorophosphate anion was selected as the first counterion in the study in order to avoid the difficulties associated with the perchlorates (hazardous) or tetrafluoroborates (too soluble). These last two anions were used only in the synthesis of lipid **5Myr** (following the same synthetic pathway) for studying the counterion effect on transfection. The chloride and iodide salts of **5Myr**, also used in the counterion study, were obtained via a different strategy, using anion exchange (Scheme 2). In the case of the chloride the anion exchange was accomplished with cesium chloride for the diol **4D**, taking advantage of the high solubility of this compound in water, the very low solubility of $CsPF_6$, and the subsequent step of acylation with acyl chloride that conserves the anion. The soluble **5DMyr** thus obtained was converted into the corresponding iodide **5EMyr** using classic anionic exchange on a Dowex resin.

The resulting cationic lipids considered in this study, together with their critical temperatures, are summarized in Table 1. As expected, the critical tempera-

Scheme 1. Synthetic Pathways for the New Cationic Lipids



Scheme 2. Preparation of Cationic Lipids with Chloride and Iodide Anions



ture (T_c), which is an important parameter for cationic lipids, decreases from the dopamine series to the two propanediol series, and among them it reaches a minimum for the 1,3-substituted compounds. Within the same series, the oleoyl derivative has the lowest T_c because of the steric hindrance induced by the cis double bond. This is clearly shown in the energy minimization models performed by the MM2 routine for saturated and unsaturated representatives from each series (Figure 1).

2. Biological Activity. 2.1. Influence of the Linker. The three series of cationic lipids newly synthesized were assessed for their transfection efficiency in vitro, using the experimental conditions optimized in a previous study.[23] Essentially the cationic lipids were mixed with cholesterol at a 1:1 molar ratio, dissolved in chloroform/methanol, and dried under vacuum to generate a lipid film. The dried lipid film was hydrated with sterile phosphate buffer isotonic saline with pH 7.4 (PBS) and sonicated to yield cationic liposomes. The resulting liposomal solution was allowed to react with a DNA solution to form the final cationic lipid−DNA complexes (lipoplexes), which were assessed for transfection efficiency using different cell cultures. We used a pGL3 plasmid (Promega; Madison, WI), encoding a firefly luciferase gene under the control of the constitutively active SV40 promoter as the reporter for the transfection efficiency. An optimal[23] electrostatic charge ratio cationic lipid/DNA of 2:1 was used in all preparations. We started with cholesterol (Chol) as colipid in the liposomal formulations because of the fact that it proved to be superior to dioleoylphosphatidylethanolamine (DOPE) for this type of pyridinium cationic lipid.[23] All liposomal preparations were sonicated for 30 min (twice at 15 min, with a 15 min pause between) at 65−68 °C for the new compounds and at 37 °C for the chosen reference cationic formulation DOTAP/Chol (1:1 molar ratio).[12,24] These conditions allowed us to generate a relatively homogeneous population of liposomes, with sizes ranging from 90 to 140 nm, as determined by dynamic light scattering (see Experimental Protocols). The ability of these new cationic lipids to generate relatively small cationic liposomes could be attributed to the relatively large curvature radius induced in the bilayer by the bulky pyridinium cationic head. We used the lung cancer cell line NCI-H23, which among several

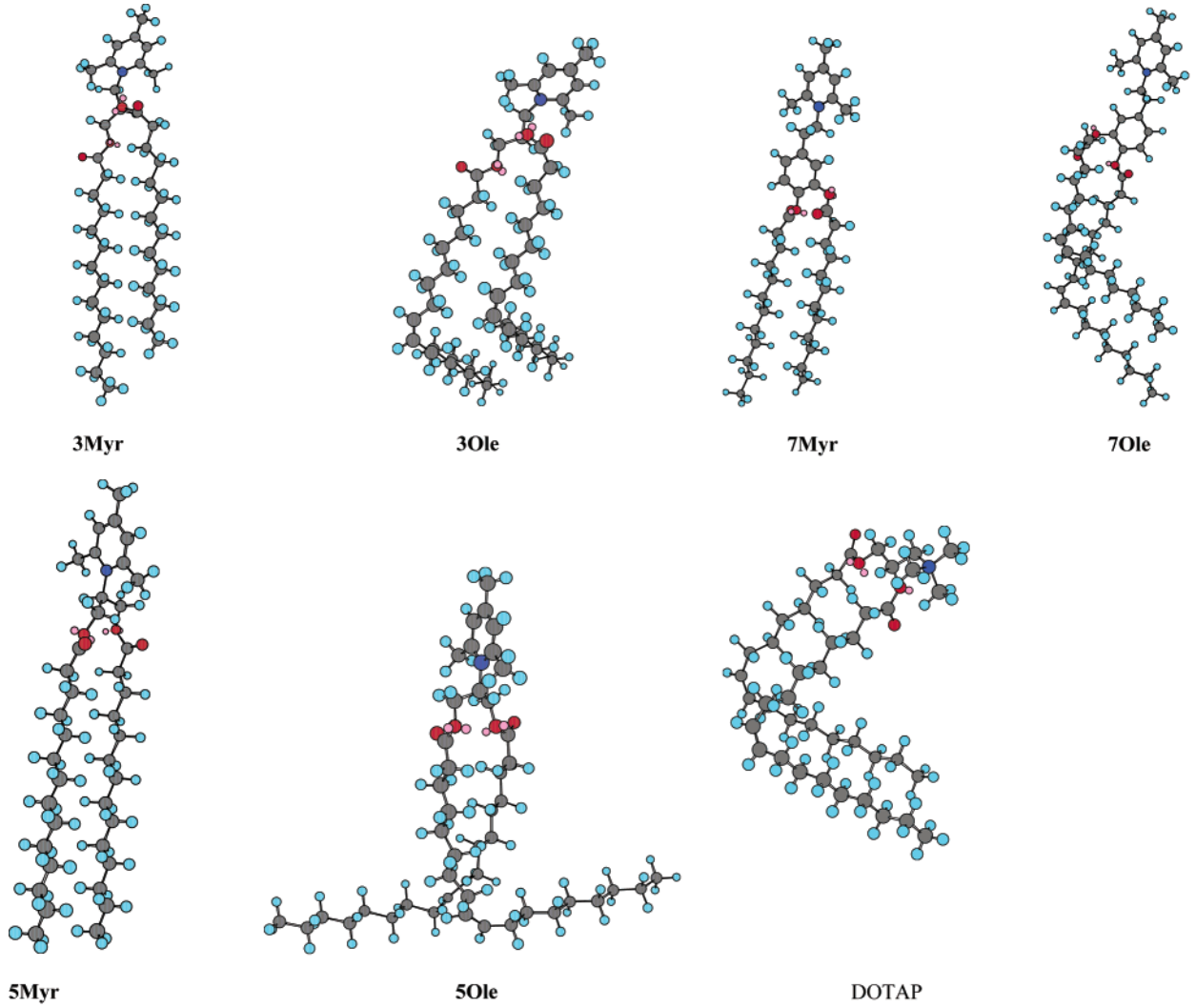


Figure 1. Structures of representative pyridinium cationic lipids and of tetramethylammonium lipid DOTAP used as reference. The energy minimization was performed with the Chem3DPro software (ChemOffice, CambridgeSoft Corp.) using MM2 molecular mechanics routine toward a 0.001 rms gradient.

Table 1. Cationic Lipids Analyzed in This Study and Their Critical Temperatures (T_c)

	T_c (°C)		
lipid	pure	with cholesterol (1:1 molar ratio)	with DOPE (1:1 molar ratio)
3ALau	114.0	106.7	
3AMyr	121.3	112.5	107.7
3APal	123.5	114.0	
3ASte	122.5	112.7	
3AOle	67.9	58.5	
5ALau	58.2	41.9	
5AMyr	67.8	64.8	22.4
5BMyr	60.2	58.4	
5CMyr	58.4	55.2	
5DMyr	76.3	81.2	
5EMyr	60.8	80.1	
5APal	75.5	61.0	
5ASte	79.6	66.1	
5AOle	17.0	31.0	
7ACpy	134.1	127.3	
7ACpc	140.0	129.2	
7ALau	140.7	126.9	
7AMyr	141.2	125.8	
7APal	140.0	124.1	
7ASte	138.8	123.9	
7AOle	106.8	97.3	

cultured cell lines tested proved to be the most sensitive toward these cationic lipids[23] as the primary cell culture for in vitro experiments (see Experimental Protocols). The results are summarized in Figure 2.

The data from Figure 2 clearly show the superiority of the aliphatic linker versus the aromatic ones. In the aromatic series (**7**), the best transfection efficiency was achieved by the lauroyl derivative **7ALau**, which was about 5 times more active than the decanoyl derivative **7ACpc** and about 10 times more efficient than the oleoyl derivative **7AOle**. The transfection is clearly correlated with the fluidity of the resulting cationic liposomes because the "stiffer" derivatives **7AMyr**−**7ASte** showed low transfection efficiencies (see also Table 1 for T_c). The same trend with respect to the hydrophobic anchor can be recognized in series **3** and **5**, which bear aliphatic, flexible linkers. The highest transfection peaks were with the myristoyl derivatives **3AMyr** and **5AMyr**; a secondary peak is due to the oleoyl derivative **3AOle**. Surprisingly, the 1,3-dioleoylderivative **5AOle** displayed low transfection efficiency, and various attempts to improve the transfection efficiency of this compound yielded consistently low results. This fact may be due to the unfavorable conformation adopted by these molecules in the lipid bilayer generated by the simultaneous action of the 1,3-linker and oleoyl fatty chains

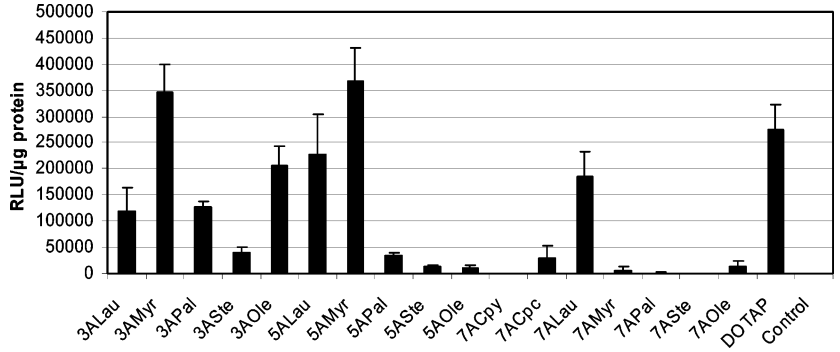


Figure 2. Transfection data for the three series of cationic lipids and DOTAP (conditioned with cholesterol as helper lipid, at 1:1 molar ratio).

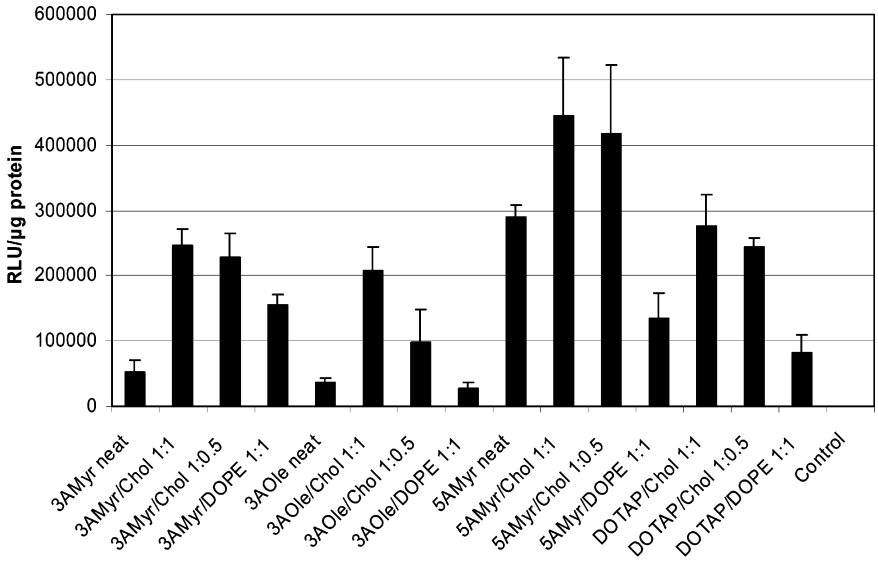


Figure 3. Transfection efficiency of lead pyridinium cationic lipids and DOTAP (as reference) with different helper lipids.

(Figure 1). As a general observation, the oleoyl derivatives were always inferior to their myristoyl congeners for these lipids bearing pyridinium polar heads, a feature observed also by Massing et al.[22] for their DOTAP-like quaternary ammonium lipids conditioned with cholesterol as colipid.

2.2. Influence of the Helper Lipid. Following these preliminary observations, we selected the lead compounds **3AMyr**, **3AOle**, and **5AMyr** for further investigations concerning the specific properties of the cationic liposomes they generate and their transfection ability. We started by checking the influence of the commonly used helper lipids cholesterol and DOPE in the transfection efficiency of pyridinium cationic liposomes, against DOTAP as reference. The ability of our lipids to act without any helper lipid was also tested in the same experiment, and the results are summarized in Figure 3.

The data of Figure 3 confirm the previous finding[23] that even when no helper lipid was used, the pyridinium cationic lipids alone were able to transfect the NCI-H23 lung cancer cell line. The liposomes generated from pure cationic lipids were generally very polydisperse and relatively unstable. Cholesterol at a 1:1 molar ratio to cationic lipids proved to be the best formulation for this type of cationic lipid, irrespective of the structure of the linker or hydrophobic anchor. The average diameter of the corresponding liposomes remained around 110 nm (88−132 nm), and the small differences in size did not seem to affect significantly the transfection efficiency. The vesicles were stable at room temperature, and no aggregation was observed. Halving the molar ratio of cholesterol to lipid generated a decrease in the average size of the cationic vesicles (e.g., from 132 to 89 nm for **3AMyr**, from 113 to 103 nm for **3AOle**, and from 95 to 88 nm for **5AMyr**), which translated into a slight decrease in the transfection efficiency. These findings can be explained considering the differences between the cross-sectional areas of polar head (A_p) and hydrophobic anchor (A_{np}) for the cationic lipids (see Figure 1) and cholesterol.[25,26] In the case of the former ones, A_p is close to or exceeds A_{np} (considering also the hydration shell of bulky pyridinium polar head), while cholesterol has $A_{np} < A_p$, so vesicles of higher curvature can be generated with less cholesterol. However, smaller sizes did not translate into higher transfection efficiencies, since cholesterol plays an essential role in the fluidity of the liposomes and lipoplexes. In previous optimization studies, we had also found that the 1:1 molar ratio to cationic lipids seems to be optimal for cholesterol-based liposomes because any further increase of this ratio makes these liposomes and lipoplexes less efficient.[23] On the other hand, DOPE was less effective than cholesterol, irrespective of the structure of cationic lipid, thus confirming the conclusion of our preliminary study[23] and also the conclusions of Massing et al.[22] The same trend in terms of the influence of the helper lipid

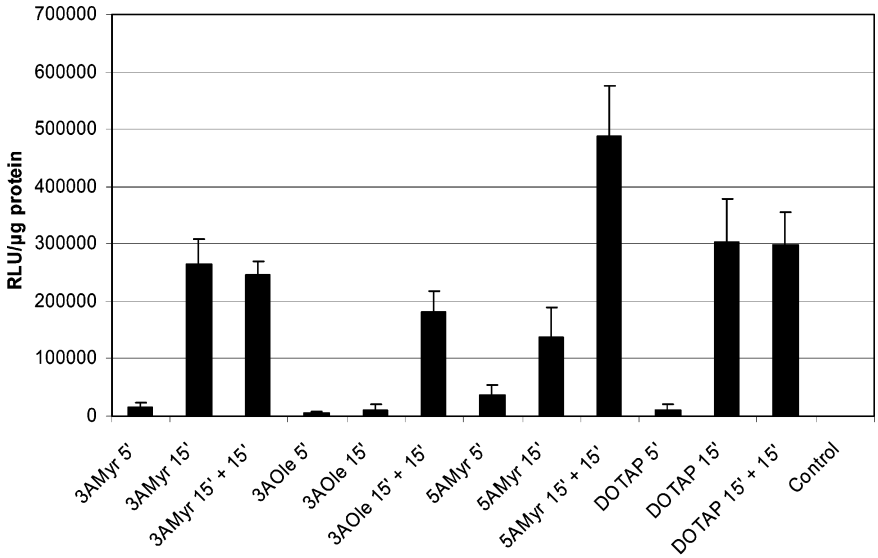


Figure 4. Influence of the sonication time on the cationic lipids **3AMyr**, **3AOle**, and **5AMyr** versus DOTAP as standard. Al preparations were done with cholesterol as helper lipid at 1:1 molar ratio.

and its molar ratio was found for DOTAP, similar to observations of other research groups.[12]

2.3. Influence of the Sonication Time. To check the best method for generating liposomes, we studied the influence of the sonication time on liposome size, coupled with a biological assessment of the transfection efficiency of the corresponding lipoplexes and using the same DNA amount as in the previous studies presented above (Figure 4). The results showed that pyridinium cationic lipids required slightly more sonication time (usually twice at 15 min with a 15 min relaxation time between) than DOTAP in order to form efficient liposomes. This can be attributed to the bulkier, less hydrophilic pyridinium polar head compared to the tetramethylammonium one, as well as to the less hydrophilic counterion (PF_6^- versus Cl^-). The average diameter of the cationic liposomes after 5, 15, and 15 + 15 min of sonication were 366, 130, and 138 nm for **3AMyr**/Chol 1:1; 130, 110, and 118 nm for **3AOle**/Chol 1:1; 131, 195, and 123 nm for **5AMyr**/Chol 1:1; and 150, 117, and 122 nm for **DOTAP**/Chol 1:1, respectively. However, the transfection trend remained the same, namely, **5AMyr**/Chol 1:1 was the most efficient liposomal preparation when optimally conditioned. Changing the hydration medium from PBS to 5% glucose[12] did not improve the transfection efficiency of the tested cationic lipids (data not shown).

2.4. Influence of the Counterion. Another important element in the structure of cationic lipids is the counterion of the positively charged polar head. It is known that counterions play an important role in cationic liposome properties, influencing the cationic lipid hydration shell and therefore the cross-sectional area of the polar head (A_p). Moreover, the interaction of cationic liposomes with DNA is driven not only by the electrostatic attraction between the positively charged lipid headgroups and the negatively charged phosphate groups on the DNA but also by the entropically favored release of the counterions of both DNA and cationic lipids.[7,27] The counterions also have a great impact on the intrinsic cytotoxicity of the cationic lipid. Consequently we investigated the influence of the counterion on the transfection efficiency using **5Myr**/Chol 1:1 hydrated with PBS and sonicated for two sequences of 15 min each as the favored liposomal preparation. The lipids **5Myr** bearing different anions such as hexafluorophosphate (**5AMyr**), perchlorate (**5BMyr**), tetrafluoroborate (**5CMyr**), chloride (**5DMyr**), and iodide (**5EMyr**) were synthesized according to Schemes 1 and 2. Data from Table 1 show that the critical temperature of the lipid is considerably influenced by the counterion, increasing in the order $BF_4^- < ClO_4^- < I^- < PF_6^- < Cl^-$. This is also valid for the corresponding mixtures with cholesterol at 1:1 molar ratio. However, the transfection efficiency of the liposomal preparations based on **5Myr** with these counterions did not follow the same trend (Figure 5). One can observe that the perchlorate **5BMyr** yielded the most efficient lipoplexes, followed by the hexafluorophosphate **5AMyr**, chloride **5DMyr**, tetrafluoroborate **5CMyr**, and iodide **5EMyr**.

To assess the cytotoxicity associated with every anion, a WST-1[28] viability assay was conducted in parallel. The results, summarized in Figure 6, showed that the minimum cytotoxicity was obtained with the hexafluorophosphate **5AMyr**, chloride **5DMyr**, and tetrafluoroborate **5CMyr** whereas the perchlorate **5BMyr** and iodide **5EMyr** were more cytotoxic. We emphasize that the optimum effect transfection/cytotoxicity is attained with hexafluorophosphate and chloride and that **5Myr** conditioned with these anions surpassed DOTAP in transfection efficiency, simultaneously displaying a lower cytotoxic effect. Mention must be made that extending the duration of cell exposure to lipoplexes generated from **5AMyr** and **5DMyr** from 1 to 3 h translates into higher transfection efficiencies without any visible cytotoxic effect (data not shown).

2.5. Influence of the Cell Line. It is well-known that specific cell types and cell lines may vary in their ability to be transfected. In addition to the NCI-H23 lung cancer cell line, we therefore tested our optimized formulation of **5AMyr**/Chol 1:1 versus DOTAP/Chol 1:1 as reference on four other cell lines from tumors representing cancers of great importance to human health, as well as different tissues of origin: breast carcinomas MCF-7, MDA-Mb231, prostate carcinoma DU-145, and glioma SWB-95 (Figure 7).

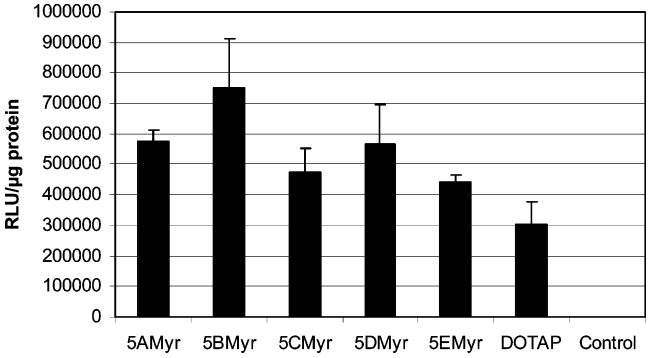


Figure 5. Transfection of NCI-H23 lung cancer cells with lipids **5Myr** bearing different counterions PF_6^- (**A**), ClO_4^- (**B**), BF_4^- (**C**), Cl^- (**D**), and I^- (**E**) and with DOTAP (Cl^-). All the preparations were done with cholesterol at 1:1 molar ratio to the cationic lipid, hydrated with PBS, and sonicated for two sequences of 15 min each. The resulted cationic liposomes were allowed to interact with the same amount of DNA to form the final lipoplexes.

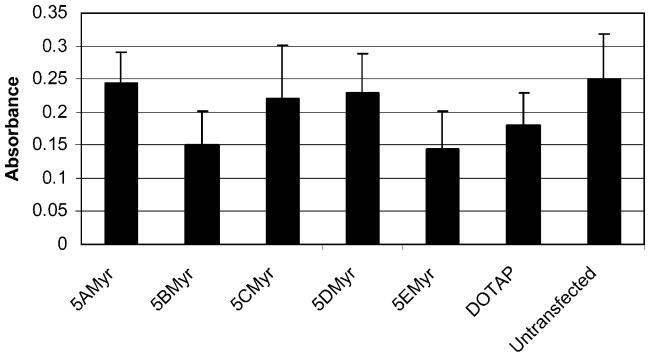


Figure 6. Viability assay results for the cancer cell line NCI-H23 transfected with **5AMyr**−**5EMyr** and DOTAP (Cl^-) based lipoplexes. The other characteristics were the same as in the transfection experiment represented in Figure 5.

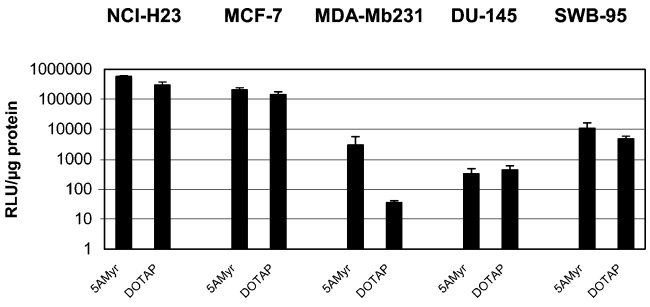


Figure 7. Transfection efficiency of optimized **5AMyr**-based lipoplexes versus DOTAP-based lipoplexes on different tumor cell lines. Note the log scale. Values for **5AMyr** and **DOTAP** were 576 000, 303 000 (NCI-H23, from Figure 4), 206 000, 143 000 (MCF-7), 2950, 36 (MDA-Mb231), 327, 442 (DU-145), 10934, and 4810 (SWB-95).

The results showed that transfection occurred on all cell types. Using **5AMyr**/Chol 1:1 in NCI-H23 cells resulted in 1.5−2 times greater expression of luciferase than using DOTAP/Chol 1:1 (see Figure 4). The above combination also performed better in MCF-7, MDA-Mb231, and SWB-95 cells (Figure 7). The differences were statistically significant at $p < 0.05$ or better in all cases. Actual values are given in the caption of Figure 7. Interestingly, the glioma SWB-95, known as a cell line relatively hard to transfect, showed significant transfection. The breast carcinoma MDA-Mb231, as well as the prostate carcinoma DU-145, showed relatively modest levels of transfection with both **5AMyr**/Chol and DOTAP/Chol. The high sensitivity of NCI-H23 lung carcinoma to pyridinium lipoplexes may be associated with the existence of a polyamine transporter[29] that is particularly effective for pyridinium compounds.[23,30] Mention must be made that normal cell lines are very hard to transfect under the same conditions with either **5AMyr**/Chol or DOTAP/Chol because of a much lower metabolic and division activity than that of tumor cells. This fact is important for future in vivo studies using apoptotic plasmids, where selective killing of tumor cells is essential.

These findings were confirmed by a different experiment using a plasmid expressing the gene for the enhanced green fluorescent protein (GFP). After transfection, the cultures were evaluated for expression of the gene by fluorescence microscopy. Examples of these transfected cells showed clearly the superiority of **5AMyr**/Chol on DOTAP/Chol (see Supporting Information).

Conclusions

Three series of new pyridinium cationic lipids bearing different linkers and hydrophobic anchors were synthesized using an original approach based on the high-yield reaction of pyrylium salts with aminodiols that allows the simultaneous generation of the polar head and linker in a single step.[17] A structure−activity relationship study was conducted for identifying the most effective structural parameters and their influence on transfection efficiency using the lung cancer cell line NCI-H23. The best transfection efficiencies were obtained with compounds having an aliphatic linker and myristoyl fatty chains, the compound **5AMyr** being the most effective (about twice more effective than DOTAP). We identified cholesterol at a molar ratio of 1:1 as the optimum helper lipid for these pyridinium cationic lipids. Mention must be made that DOTAP/Chol 1:1 formulation was used successfully for in vivo experiments[31] and that this formulation was also evaluated in gene therapy on human subjects.[32]

We also studied the effect of the counterion on transfection and cytotoxicity, and we found that **5Myr**/Chol 1:1 was more effective and less cytotoxic than DOTAP/Chol 1:1 when hexafluorophosphate or chloride anions were used as counterions for the pyridinium polar head. Tests on other tumor cell lines using different plasmids showed that the transfection efficiency with pyridinium cationic lipids was cell-dependent and that **5AMyr**/Chol 1:1 performed better than DOTAP/Chol 1:1 on the majority of these cell lines. The most susceptible remained the lung carcinoma NCI-H23, and this fact may be linked to the existence in these cells of a polyamine transporter that is particularly effective for pyridinium compounds. This finding gives good hopes of using the new pyridinium cationic lipids as gene transfer agents in the treatment of lung cancer and cystic fibrosis.

Considering also the success of several studies using the pyridinium moiety in the generation of membrane-impermeable inhibitors and activators of carbonic anhydrase[33,34] (which confers isozyme selectivity) with applications as anticancer agents,[35] this study demon-

strates once again the usefulness of the pyridinium group as a versatile moiety in drug targeting.[36]

Experimental Protocols

1. Chemistry. 1.1. General. Melting points for the diols and the phase transition temperature T_c for the cationic lipids were determined by differential scanning calorimetry (DSC), using a TA-Instruments Q100 DSC and a heating rate of 5 °C/min. The IR spectra were recorded on a Nicolet Avatar 360 FTIR spectrophotometer in the range 650−4000 cm^{-1} using a ZnSe attenuated total reflectance (ATR) accessory. The compounds were dissolved in a small amount of solvent (MeOH for the diols, $CHCl_3$ for the lipids), and the resulting solutions were left to evaporate to dryness on the surface of the ZnSe crystals of the ATR accessory. The NMR spectra were recorded at ∼303 K with a Varian Inova spectrometer equipped with a 5 mm indirect detection probe operating at 500 MHz for ^1H NMR and at 125 MHz for ^{13}C NMR. Chemical shifts are reported as δ values, using TMS as internal standard for proton spectra and the solvent resonance for carbon spectra. Assignments were made on the basis of signal intensity, selective decoupling, COSY ($^1H-^1H$), and HETCOR ($^1H-^{13}C$) sequences. Elemental analyses were performed by combustion, using a Perkin-Elmer 2400 series II CHNS analyzer.

Racemic 3-amino-1,2-propanediol, 2-amino-1,3-propanediol, triethylamine, acetic anhydride, acetic acid, acyl chlorides, and other solvents were from Acros. TLC was performed on silica gel 60-F_{254} plates (Merck), and the sample eluted with MeOH/$CHCl_3$ 20:80 (v/v). The pyrylium salts were prepared according to the literature.[19,37,38] CAUTION: Since perchlorates may explode when they are heated or upon impact in dry form, they should be stored moist with water.

1.2. General Procedure for the Preparation of the Pyridinium Diols 2, 4, and 6. The aminodiol (10 mmol) was dissolved by stirring in anhydrous ethanol (30 mL). Next, the corresponding pyrylium salt (12 mmol of hexafluorophosphate, perchlorate, tetrafluoroborate) was added, followed immediately by the addition of 12 mmol of triethylamine. In the case of dopamine, commercially available as hydrochloride, the quantity of triethylamine was doubled. The resulting mixture was refluxed for 15 min, and then glacial acetic acid (25−30 mmol) was added and the reflux was continued for 1−3 h (TLC control). After this period, concentrated aqueous ammonia (2 mL) was added and the mixture was heated for 5 min in order to convert any unreacted pyrylium salt into the corresponding pyridine, which is soluble in diethyl ether. The final solution was cooled and poured under stirring into anhydrous diethyl ether (200−300 mL). In the case of aliphatic aminodiols, the resulting insoluble heavier oily layer was separated and washed with two additional portions (20 mL each) of diethyl ether. After a final separation, the oily layer was taken in a few milliliters of hot isopropyl alcohol, treated with charcoal, filtered, and allowed to cool slowly when crystallization occurred. In the case of dopamine the precipitated product was filtered, dried, and washed with water to eliminate the ammonium salts. Yields were in the range of 50−85%. The products were recrystallized from methanol or 2-propanol.

1.3. General Procedure for the Preparation of the Pyridinium Lipids 3, 5, and 7. The pyridinium diol **2**, **4**, or **6** (2 mmol) was dissolved by stirring in 15−30 mL of anhydrous acetonitrile. Triethylamine (0.56 mL, 4 mmol) was added, followed by dropwise addition of acid chloride RCOCl (4.4 mmol) when the color became yellow, and triethylamine hydrochloride started to precipitate. The suspension was stirred for 15 min at room temperature and then refluxed for another 3−5 h. The solvent was evaporated (rotavapor) under reduced pressure, and the residue was extracted with 15 mL of distilled water and 15 mL of chloroform. The aqueous layer was separated, extracted with 15 mL of chloroform, and discarded. The combined chloroform extracts were shaken with 15 mL of distilled water, dried over sodium sulfate, and evaporated under reduced pressure. Final purification was effected by flash chromatography on silica gel 60 (40−60 μM) with a solvent mixture of chloroform and methanol (80:20 v/v), followed by recrystallization from ethanol.

1.4. Anion Exchange Procedure for Obtaining the Pyridinium Diol 4D. The pyridinium propanediol hexafluorophosphate **4A** (0.68 g, 2 mmol) was dissolved under gentle heating in 1 mL of distilled water and treated with a warm solution obtained by dissolving 0.42 g of cesium chloride (2.5 mmol) in 1 mL of distilled water. Precipitation of cesium hexafluorophosphate occurred instantaneously and was finalized by slowly cooling the suspension at room temperature and then to 0°C. The precipitate was filtered off and the resulting solution evaporated to dryness to yield the crude pyridinium chloride, which was used directly into the next step.

1.5. Anion Exchange Procedure for Obtaining the Pyridinium Lipid 5EMyr. An amount of 10 g of DOWEX anion-exchange resin 1X8 (in chloride form) was suspended in doubly distilled water and allowed to completely swell overnight. The next day the resin was packed in a small column and treated with a solution of 25 g of potassium iodide in 80 mL of doubly distilled water. The resin gradually turned yellow when eluted because of iodide binding. Then the resin was washed with distilled water until no halide anions were detected in the eluted fractions ($AgNO_3$ test). Then 60 mL of 95% EtOH was added in order to change the polarity of the medium, followed by addition of the sample (**5DMyr**, 70 mg) dissolved in 3 mL of EtOH 95%. Isocratic elution with 95% EtOH afforded the desired **5EMyr**, which was concentrated and recrystallized from absolute ethanol. Yield was 53 mg (69%).

1-(2,3-Dihydroxypropyl)-2,4,6-trimethylpyridinium hexafluorophosphate 2A: mp 136.6 °C. Anal. ($C_{11}H_{18}NO_2^+PF_6^-$) C, H, N.

1-(2,3-Dilauroyloxypropyl)-2,4,6-trimethylpyridinium hexafluorophosphate 3ALau: T_c = 114.0 °C. Anal. ($C_{35}H_{62}NO_4^+PF_6^-$) C, H, N.

1-(2,3-Dimyristoyloxypropyl)-2,4,6-trimethylpyridinium hexafluorophosphate 3AMyr: T_c = 121.3 °C. Anal. ($C_{39}H_{70}NO_4^+PF_6^-$) C, H, N.

1-(2,3-Dipamitoyloxypropyl)-2,4,6-trimethylpyridinium hexafluorophosphate 3APal: T_c = 123.5 °C. Anal. ($C_{43}H_{78}NO_4^+PF_6^-$) C, H, N.

1-(2,3-Distearoyloxypropyl)-2,4,6-trimethylpyridinium hexafluorophosphate 3ASte: T_c = 122.5 °C. Anal. ($C_{47}H_{86}NO_4^+PF_6^-$) C, H, N.

1-(2,3-Dioleoyloxypropyl)-2,4,6-trimethylpyridinium hexafluorophosphate 3AOle: T_c = 67.9 °C. Anal. ($C_{47}H_{82}NO_4^+PF_6^-$) C, H, N.

1-(1,3-Dihydroxypropane-2-yl)-2,4,6-trimethylpyridinium hexafluorophosphate 4A: mp 110.9 °C. Anal. ($C_{11}H_{18}NO_2^+PF_6^-$) C, H, N.

1-(1,3-Dilauroyloxypropane-2-yl)-2,4,6-trimethylpyridinium hexafluorophosphate 5ALau: T_c = 58.2 °C. Anal. ($C_{35}H_{62}NO_4^+PF_6^-$) C, H, N.

1-(1,3-Dimyristoyloxypropane-2-yl)-2,4,6-trimethylpyridinium hexafluorophosphate 5AMyr: T_c = 67.8 °C. Anal. ($C_{39}H_{70}NO_2^+PF_6^-$) C, H, N.

1-(1,3-Dimyristoyloxypropane-2-yl)-2,4,6-trimethylpyridinium perchlorate 5BMyr: T_c = 60.2 °C. Anal. ($C_{39}H_{70}NO_4^+ClO_4^-$) C, H, N.

1-(1,3-Dimyristoyloxypropane-2-yl)-2,4,6-trimethylpyridinium tetrafluoroborate 5CMyr: T_c = 58.4 °C. Anal. ($C_{39}H_{70}NO_4^+BF_4^-$) C, H, N.

1-(1,3-Dimyristoyloxypropane-2-yl)-2,4,6-trimethylpyridinium chloride 5DMyr: T_c = 76.3 °C. Anal. ($C_{39}H_{70}NO_4^+Cl^-$) C, H, N.

1-(1,3-Dimyristoyloxypropane-2-yl)-2,4,6-trimethylpyridinium iodide 5EMyr: T_c = 60.8 °C. Anal. ($C_{39}H_{70}NO_4^+I^-$) C, H, N.

1-(1,3-Dipamitoyloxypropane-2-yl)-2,4,6-trimethylpyridinium hexafluorophosphate 5APal: T_c = 75.5 °C. Anal. ($C_{43}H_{78}NO_4^+PF_6^-$) C, H, N.

1-(1,3-Distearoyloxypropane-2-yl)-2,4,6-trimethylpyridinium hexafluorophosphate 5ASte: T_c = 79.6 °C. Anal. ($C_{47}H_{86}NO_4^+PF_6^-$) C, H, N.

1-(1,3-Dioleoyloxypropane-2-yl)-2,4,6-trimethylpyridinium hexafluorophosphate 5AOle: T_c = 17.0 °C. Anal. ($C_{47}H_{82}NO_4^+PF_6^-$) C, H, N.

1-(3,4-Dihydroxyphenylethyl)-2,4,6-trimethylpyridinium hexafluorophosphate 6A: mp 218−220°. Anal. ($C_{16}H_{20}NO_2^+PF_6^-$) C, H, N.

1-(3,4-Dioctanoyloxyphenylethyl)-2,4,6-trimethylpyridinium hexafluorophosphate 7ACpy: T_c = 134.1 °C. Anal. ($C_{32}H_{48}NO_4^+PF_6^-$) C, H, N.

1-(3,4-Didecanoyloxyphenylethyl)-2,4,6-trimethylpyridinium hexafluorophosphate 7ACpc: T_c = 140.0 °C. Anal. ($C_{36}H_{56}NO_4^+PF_6^-$) C, H, N.

1-(3,4-Dilauroyloxyphenylethyl)-2,4,6-trimethylpyridinium hexafluorophosphate 7ALau: T_c = 140.7 °C. Anal. ($C_{40}H_{64}NO_4^+PF_6^-$) C, H, N.

1-(3,4-Dimyristoyloxyphenylethyl)-2,4,6-trimethylpyridinium hexafluorophosphate 7AMyr: T_c = 141.2 °C. Anal. ($C_{44}H_{72}NO_4^+PF_6^-$) C, H, N.

1-(3,4-Dipalmitoyloxyphenylethyl)-2,4,6-trimethylpyridinium hexafluorophosphate 7APal: T_c = 140.0 °C. Anal. ($C_{48}H_{80}NO_4^+PF_6^-$) C, H, N.

1-(3,4-Distearoyloxyphenylethyl)-2,4,6-trimethylpyridinium hexafluorophosphate 7ASte: T_c = 138.8 °C. Anal. ($C_{52}H_{88}NO_4^+PF_6^-$) C, H, N.

1-(3,4-Dioleoyloxyphenylethyl)-2,4,6-trimethylpyridinium hexafluorophosphate 7AOle: T_c = 106.8 °C. Anal. ($C_{52}H_{84}NO_4^+PF_6^-$) C, H, N.

2. Preparation and Evaluation of Liposomes. Dioleoylphosphatidylethanolamine (DOPE), cholesterol (Chol) and *N*-(2,3-dioleoyloxypropyl)-*N*,*N*,*N*-trimethylammonium chloride (DOTAP) were purchased from Sigma (St. Louis, MO). The purity of the lipids was checked by TLC and also by differential scanning calorimetry (DSC) using a Q100 TA Instruments DSC apparatus (New Castle, DE).

For the preparation of liposomes a protocol optimized for this type of compound[23] was used. The cationic lipid (with or without helper lipid) was dissolved in chloroform/methanol (2:1, v/v) to reach a final concentration of 1 mM for the charged species. From this stock solution an amount of 50 μL was transferred to a 1 mL microcentrifuge tube (USA Scientific, Catalog No. 1415-2500), and the solvent was evaporated in a SpeedVac evaporator under vacuum for 1 h. Traces of organic solvent were removed by drying the lipid film in a vacuum desiccator for 3 h over Drierite. The dried film was hydrated overnight after adding 500 μL of sterile phosphate buffer isotonic saline, pH 7.4 (PBS) (GibcoBRL/Invitrogen, Carlsbad, CA), purging the tube with sterile nitrogen, and vortexing it for 1 min. Liposomes were generated next day by sonicating the tube in a water bath sonicator (Branson, model 1210) for 5−30 min at 65−68 °C for formulations based on lipids **3**, **5**, and **7** in a mixture with cholesterol and at 37 °C for DOTAP/cholesterol. After sonication the preparations were allowed to reach 22 °C and assessed on the specified cells within 30 min from the preparation. The size of the lipoplexes was determined in parallel by dynamic light scattering, using a DynaPro particle sizer (Protein Solutions; Piscataway, NJ) and employing the Dynamics data collection and analysis software. In each experiment the liposomal preparation (20 μL) was diluted with PBS to a final volume of 200 μL. From this solution an aliquot (15 μL) was introduced in a quartz cuvette precleaned with distilled water and methanol and dried to eliminate any contaminants. The cuvette was sealed with Parafilm, and the mean average diameter of the vesicles was determined at 25 °C as the result of at least three reliable readings.

3. Preparation of Lipoplexes. Two plasmids were used in this study: (1) pGL3 (Promega, Madison, WI), encoding a firefly luciferase gene under control of the constitutively active SV40 promoter and (2) pEGFP-C1 (BD Biosciences Clontech, Palo Alto, CA), encoding the enhanced green fluorescent protein (GFP). Each plasmid was amplified in *Escherichia coli* and purified using a Genopure Plasmid MaxiKit (Roche, Indianapolis, IN).

For generating the lipoplexes, 2.8 μL of plasmid DNA solution (0.5 μg/μL) was diluted with 100 μL of Optimem (Gibco/Invitrogen) to make plasmid DNA stock solutions. A liposome solution was prepared separately by diluting 40 μL of the initial liposomal stock solution (0.1 mM in cationic lipid) with Optimem in order to reach a final volume of 50 μL. To this liposome solution 50 μL of the DNA stock solution was added, and after the sample was mixed, the tube was incubated for 30 min at 22 °C. The content was then diluted with 500 μL of Optimem (final volume of the lipoplex stock solution: 600 μL).

4. Cell Transfection. The lipoplexes were tested for their ability to transfect five cancer cell lines: two breast carcinomas (MCF-7 and MDA-Mb231), a lung carcinoma (NCI-H23), a prostate carcinoma (DU-145), and a brain glioma (SWB-95). The cells were maintained in 10% fetal bovine serum (FBS) enriched medium at 37 °C in a humidified atmosphere of 95% air/5% CO_2. The following media were used: RPMI 1640 (CellGro, Houston, TX) for NCI-H23, Iscove's modified Dulbecco's medium (Gibco/Invitrogen) for MCF-7, Dulbecco's modified Eagle medium (Gibco/Invitrogen) for MDA-Mb231, minimum essential medium (Gibco/Invitrogen) for DU-145, and minimum essential medium Eagle α modification (Sigma; St. Louis, IL) for the SWB-95 cell line. Twenty-four hours prior to transfection, the cells were transferred to 96-well microtiter plates (Costar, catalog no. 3596) at a density of 20 000 cells/well. Each well received 100 μL of appropriate medium, and the plate was incubated under the same conditions as above. All experiments were done in triplicate or quadruplicate. The error bar represents 1 standard deviation from the average value.

Immediately before transfection the medium was removed, and the cells from each well were briefly washed with 200 μL of sterile PBS. After removal of the PBS solution, each well received 100 μL of lipoplex stock solution, and the plate was returned to the incubator for 1 h. An additional 100 μL of medium was added to each well, and the plate was incubated for a further 24 h.

5. Luciferase and Protein Content Assay. Twenty-four hours after transfection the medium was aspirated and the wells were washed briefly with 200 μL of PBS. After removal of PBS the cells were lysed by adding 100 μL of 1X reporter lysis buffer (Promega) to each well and incubating the plate at 37 °C for 10 min. The cell lysate was collected and used for luciferase and protein assays.

For the luciferase assay, 20 μL of cell lysate was transferred to a test tube and assessed directly by means of a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA) using a luciferase assay kit from Promega.

The protein content was quantified using a bicinchoninic acid (BCA) assay (Pierce; Rockford, IL). The BCA assay was prepared as specified in its manufacturer's instructions. An amount of 40 μL of cell lysate was treated with 1 mL of BCA reagent in an acryl cuvette, and the solution was incubated for 1 h at 37 °C. The light absorption was then read at 562 nm by means of a Beckman DU-600 UV−vis spectrometer (Palo Alto, CA), and the protein content was estimated by comparison to bovine serum albumin (BSA) standards. The luciferase activity was normalized by the protein content and expressed as relative luciferase units per microgram of protein (RLU/μg protein).

6. Viability Assay. To quantify the relative cytotoxicity of the nonviral cationic vectors, a WST-1 standard viability method[28] was performed in parallel with the luciferase and BCA assays. Five wells of cells for each liposomal preparation were set up in parallel and transfected identically as in the pGL3 assay. Twenty-four hours after transfection, 20 μL of WST-1 tetrazolium dye solution (Roche) was added to each well (still containing the serum and the liposomal preparation). A blank was prepared by mixing 100 μL of Optimem, 100 μL of FBS, and 20 μL of tetrazolium dye solution, and the plate was incubated at 37 °C in the CO_2 incubator. After 3 h the colorimetric measurement was performed at 450 nm (with a reference wavelength 595 that was subtracted) by means of a Vmax kinetic microplate reader (Molecular Devices, Sunny-

vale, CA). The value corresponding to the blank was deducted from the value corresponding to each well.

7. Green Fluorescent Protein Assay. In the alternative screening method, intact cells were transfected in the same way using the GFP plasmid and a four-well tissue chamber/slide with removable walls (Miles Scientific, Naperville, IL). Twenty-four hours after transfection, the medium was removed and the cells were briefly washed with PBS and fixed with a 4% solution of formaldehyde in PBS for 5 min in the dark. Then the formaldehyde solution was discarded, and the cells were washed again with PBS. After removal of the PBS and plate walls, the preparations were investigated by means of a Nikon Eclipse E800 fluorescent microscope with a mounted CoolSnap FX camera (Roper Scientific, Tucson, AZ) using white polarized light with an excitation filter for 480/30 nm and an emission filter for 535/40 nm (Chroma Technologies, filter set 31001).

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Supporting Information Available: The complete characterization of the new compounds, the results of transfection experiments using the GFP plasmid, and elemental analysis results. This material is available free of charge via the Internet at http://pubs.acs.org.

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