Pharm Res (2013) 30:1362–1379
DOI 10.1007/s11095-013-0976-9

RESEARCH PAPER

Bioresponsive Deciduous-Charge Amphiphiles for Liposomal Delivery of DNA and siRNA

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Received: 26 September 2012 / Accepted: 4 January 2013 / Published online: 1 February 2013

ABSTRACT

Purpose Biolabile cationic lipids were developed for efficient intracellular delivery of DNA and siRNA.

Methods The compounds have been designed starting from the membrane lipid DOPC in a way they may loose their cationic charge when exposed to an acidic and/or enzymatic stimulus, such as those met during the journey of a lipoplex in biological media.

Results They demonstrated remarkable efficiency to deliver DNA in various cell lines (BHK-21, Calu-3, NCI-H292, and A549), with no significant cytotoxicity. Furthermore, two of the compounds (carbonate-based DOPC derivatives) revealed able to deliver small interfering RNA in U87Luc and A549Luc cancer cells and to mediate a selective 70–80% knockdown of the stably transfected luciferase gene.

Conclusions The results show that the described bioresponsive cationic lipids have high DNA and siARN delivery activity which is encouraging in view of delivering a therapeutic nucleic acid to pulmonary tissues *in vivo*.

KEY WORDS biodegradation · cationic lipid · nucleic acid delivery · RNA interference · transfection

Electronic supplementary material The online version of this article (doi:10.1007/s11095-013-0976-9) contains supplementary material, which is available to authorized users.

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INTRODUCTION

The use of cationic lipids for gene and oligonucleotide delivery has been introduced more than 20 years ago with two seminal papers by Behr (1) and Felgner (2). Since then a large number of synthetic cationic lipids have been described for DNA and oligonucleotide delivery purposes. Their efficiency however remains low when compared to that of viral carriers and the ultimate design of efficient liposomal formulations for gene delivery dedicated to clinical applications is still awaited.

To achieve DNA delivery, cationic lipid-based transfection particles have to cross various biological barriers such as the plasma membrane, the endosome membrane, and the nuclear envelope. The route for siRNA may be easier as the target for this nucleic acid, the RISC complex, is located in the cytosol. In any case, going through each one of these lipid membranes is critical for an efficient delivery process. The requirements for a particle to cross one or the other of those barriers have been separately addressed. However, synthetic delivery systems fully combining all the requirements for an efficient transfection are not yet available. Literature data suggest that lipoplexes enter cells mainly through the endocytic pathway (3,4) though recent reports suggest that direct fusion of lipoplexes with the plasma membrane might be responsible for the functional delivery of nucleic acids into the cell (5,6). The rapid endosome acidification following endocytosis (within 2–3 min) has been taken as an advantage in many delivery approaches involving the so-called "proton-sponge effect" (7), fusogenic lipids (8), or membrane-destabilizing peptides (9). The steps following the endosomal rupture are less clearly explained although it turned out that DNA traffic from the cytoplasm to the nucleus is an inefficient process (10) and the

rupture of the nuclear envelope during mitosis likely promotes nuclear importation of DNA in dividing cells.

Although formation of a complex between DNA and cationic lipid is important for cell entry and protection from attack by en-route DNAses, it also appears critical that, after DNA has entered the cell, it has to be released from the lipid for migration through the cytoplasm and expression in the nucleus. Otherwise, genetic material may stay transcriptionally inactive. The free energy of dissociation (ΔG) of DNA from cationic lipids is about 1 kT per nucleotide. Consequently, for a several kb plasmid, the extent of dissociation is negligible (11). In cells, DNA escape from the lipoplex probably is triggered by neutralization of the cationic lipids with cellular anionic lipids during fusion of endosome membrane with the lipoplex (12). It seems clear that decomplexation of DNA from the lipid carrier could be accelerated if the latter looses its cationic charge(s) in the course of the transfection process. To our knowledge, this particular point has been poorly investigated up to now. Grinstaff *et al.* have recently addressed this issue using "charge-reversal" amphiphiles (13–15). The global charge of the lipid carriers switched from +1 to −1 upon hydrolysis, and the compounds interestingly showed enhanced transfection efficiency when compared to parent DOTAP.

As a part of our ongoing research program focused on the development of biocompatible gene transfer reagents, we were pushed to design "deciduous-charge" cationic amphiphiles developed on the basis of the DOPC backbone. This work was especially motivated by previous studies that demonstrated the high potential of cationic DOPC phosphotriesters for DNA transfection (16). These phospholipid derivatives are especially interesting since they can be slowly metabolized by A_2 phospholipases and display only low toxicity (17). EDOPC, which is the lead compound in this series (see Fig. 1), displayed transfection activity similar to the golden standard transfer reagent Lipofectamine®. In recent studies, we thus developed new DOPC-based cationic lipids by connecting a detergent molecule to the phosphate moiety of the membrane phospholipid (18,19). These constructs were designed so that the detergent moiety is released inside the intracellular compartment for further facilitating the nucleic acid delivery process through destabilization of the endosome membrane. We were delighted to observe that these cationic detergent-lipid conjugates display remarkable ability to deliver siRNA into cancer cells. Initial acute toxicity issues were solved by varying the nature of the linker between the phospholipid anchor and the detergent moiety, so that the resulting cationic lipids can be entirely degraded into the endogenous membrane phospholipid DOPC along the delivery process.

Herein, we describe the synthesis and biological evaluation of another series of such deciduous-charge amphiphiles. These cationic DOPC conjugates were designed to loose their cationic charge upon hydrolysis either under a chemical or enzymatic stimulus, in a time-controlled manner. The compounds were expected to generate zwitterionic DOPC that is not able to form supramolecular assemblies with DNA, and a residue with mild surface-active properties (Scheme 1). Thus, the detergent moiety as introduced in our earlier communication was replaced by low molecular weight alcohols or carboxylic acids with various hydrophobicity, with a view to tentatively identifying structure-activity relationships (Fig. 1).

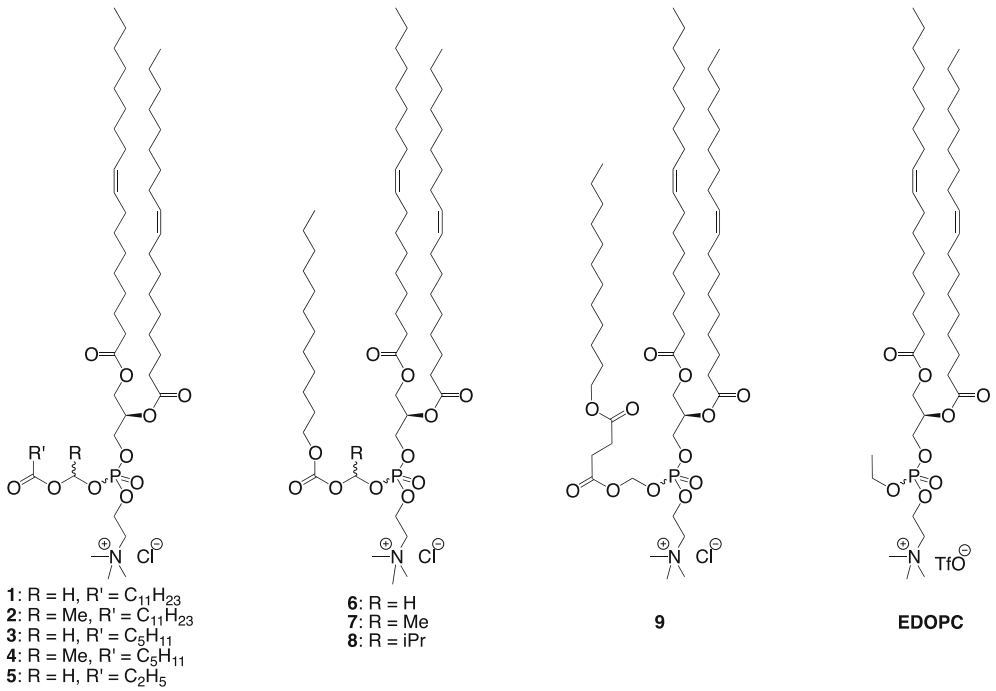
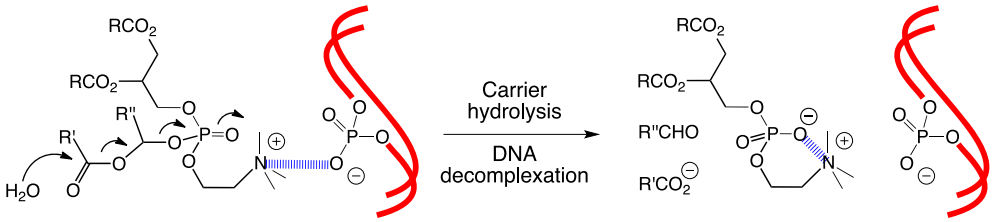


Fig. 1 Structure of the deciduous-charge cationic amphiphiles investigated in this work.



Scheme 1 Proposed mechanism for DNA decomplexation upon hydrolysis of the so-called deciduous-charge cationic lipids.

The physicochemical and biological behavior of the title compounds was examined. First, the ability of compounds **1-9** to bind DNA was investigated by usual agarose gel electrophoresis. The hydrolytic stability of the conjugates formulated in liposomes was determined by ^{31}P-NMR spectroscopy, and using an ethidium bromide intercalation assay when formulated in lipoplexes. Next, the ability of the conjugates to deliver a DNA plasmid encoding the luciferase protein into BHK-21 cells, and a small interfering RNA into U87Luc cells were examined. Finally, with the aim of assessing the further relevance of our lipoplex formulations for *in vivo* administration of therapeutic nucleic acid *via* the pulmonary route, the efficiency of compounds **1-9** to mediate DNA or siRNA transfection into various pulmonary cell lines (plasmid DNA: Calu3, NCI-H292 and A549 cells; siRNA: A549Luc cells) was evaluated.

MATERIALS AND METHODS

Materials

Unless otherwise stated, chemical reagents were purchased from Alfa Aesar (Bischeim, France) and used without purification. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and 3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide (ethidium bromide, EB) were from Sigma-Aldrich (Saint-Quentin Fallavier, France). When required, solvents were dried by standard procedures just before use (20). Thin layer chromatography (TLC) was performed on precoated plates (0.25 mm Silica Gel 60, F_{254}, Merck, Darmstadt, Germany). Products were purified by flash chromatography over silica gel (Silica Gel 60, 40–63 µm, Merck, Darmstadt, Germany). NMR spectra were recorded on Bruker 300 MHz Avance DPX or Bruker 400 MHz Avance III instruments. ^{1}H-, ^{13}C-, and ^{31}P-NMR chemical shifts δ are reported in ppm relative to their standard reference (^{1}H: $CHCl_3$ at 7.27 ppm; ^{13}C: $CDCl_3$ at 77.0 ppm; ^{31}P: H_3PO_4 at 0.0 ppm). IR spectra were recorded on a FT-IR Nicolet 380 spectrometer in the ATR mode and absorptions values ν are in wave numbers (cm^{-1}). Mass Spectra (MS) were recorded on an Agilent Technologies 6520 Accurate Mass Q-ToF instrument, using the electrospray ionization (ESI) mode. Mass data are reported in mass units (*m/z*). All synthesized cationic amphiphiles were periodically monitored for purity by thin-layer chromatography and repurified when required. BHK-21 cells (Syrian hamster kidney cells, CCL-10), Calu-3 cells (epithelial lung adenocarcinoma, HBT-55), U87 cells (glioblastoma, astrocytoma cell line derived from human malignant glioma; HTB-14), A549 cells (human lung carcinoma; CCL-185), and NCI-H292 cells (human lung mucoepidermoid carcinoma; CRL-1848) were obtained from ATCC-LGC (Molsheim, France). pCMV-Luc expression plasmid (5.5 kbp, BD Biosciences Clontech, Franklin Lakes, NJ, USA) was used as reporter gene to monitor DNA transfection activity (21). This plasmid encoded the *firefly luciferase* gene under the control of a strong CMV promoter. U87 and A549 cell lines were transformed to stably express the *Photinus pyralis* luciferase gene originating from the pGL3 plasmid (Clontech, Mountain View, CA) to assess siRNA delivery (22,23). The pGL3 plasmid encoded as well for a gene conferring resistance to the antibiotic G418. This antibiotic was thus used to select the transfected U87Luc and A549Luc cells. Luciferase gene silencing experiments were performed with an RNA duplex (siLuc) of the sense sequence: 5′-CUU ACG CUG AGU ACU UCG A. Control untargeted RNA duplex (sic) was of sequence: 5′-CGU ACG CGG AAU ACU UCG A. Both RNAs were from Eurogentec (Angers, France). DNA concentration refers to oligonucleotide phosphate concentration. siRNA concentrations refer to duplex. Fetal bovine serum (FBS), culture media (Dulbecco's Modified Eagle Medium, DMEM) and supplements were from GIBCO-BRL (Cergy-Pontoise, France). Lysis and luciferin solutions for monitoring luciferase activity were purchased from Promega (Charbonnières, France). Abbreviations: PE, petroleum ether; s, singlet; d, doublet; t, triplet; hept, heptuplet; br, broad.

*Chloromethyl Dodecanoate (**10a**)*

Dodecanoyl chloride (3.30 g, 15.1 mmol) was added slowly to a mixture of paraformaldehyde (0.45 g, 15.1 mmol) and a catalytic amount of anhydrous $ZnCl_2$ (41 mg, 0.3 mmol) at −10°C under an argon atmosphere. The reaction mixture was stirred at this temperature for 1 h, and at room temperature for an additional period of 18 h. The crude reaction mixture was directly purified by flash column chromatography over silica gel (PE/Et_2O 95:5) to yield compound **10a** (2.60 g, 70%) as a clear oil. TLC R_f 0.40 (PE/Et_2O 95:5). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 5.67 (s, 2H), 2.34 (t, $^{3}J_{HH}$=7.5 Hz, 2H), 1.62 (m, 2H), 1.31–1.22 (m, 16H), 0.84 (t, $^{3}J_{HH}$=6.7 Hz, 3H). ^{13}C-

NMR (100 MHz, $CDCl_3$) δ 171.8, 68.7, 34.2, 32.1, 29.7 (2C), 29.6, 29.5, 29.3, 29.1, 24.7, 22.8, 14.3.

*1-Chloroethyl Dodecanoate (**10b**).*

This compound (4.05 g, 86%) was obtained as a clear oil from dodecanoyl chloride, acetaldehyde and $ZnCl_2$ following the same procedure as described for the preparation of **10a**. TLC R_f 0.40 (PE/Et_2O 95:5). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 6.53 (q, $^3J_{HH}$=5.8 Hz, 1H), 2.32 (t, $^3J_{HH}$=7.4 Hz, 2H), 1.76 (d, $^3J_{HH}$=5.8 Hz, 3H), 1.61 (m, 2H), 1.32–1.23 (m, 16H), 0.86 (t, $^3J_{HH}$=6.9 Hz, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 171.6, 34.4, 32.1, 29.8 (2C), 29.7, 29.6, 29.5, 29.4, 29.2, 25.4, 24.8, 22.9, 14.3.

*Chloromethyl Hexanoate (**10c**)*

This compound (2.60 g, 64%) was obtained as a clear oil from hexanoyl chloride, paraformaldehyde and $ZnCl_2$ following the same procedure as described for the preparation of **10a**. TLC R_f 0.25 (PE/Et_2O 98:2). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 5.68 (s, 2H), 2.36 (t, $^3J_{HH}$=7.2 Hz, 2H), 1.64 (m, 2H), 1.34–1.23 (m, 4H), 0.88 (t, $^3J_{HH}$=6.2 Hz, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 171.9, 69.8, 34.2, 31.3, 24.4, 22.4, 14.1.

*1-Chloroethyl Hexanoate (**10d**)*

This compound (3.35 g, 58%) was prepared from hexanoyl chloride, acetaldehyde and $ZnCl_2$ following the same procedure as described for the preparation of **10a**. TLC R_f 0.20 (PE/Et_2O 98:2). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 6.53 (q, $^3J_{HH}$=5.8 Hz, 1H), 2.32 (dd, $^3J_{HH}$=7.2 and 8.5 Hz, 2H), 1.76 (d, $^3J_{HH}$=5.8 Hz, 3H), 1.63 (m, 2H), 1.32–1.27 (m, 4H), 0.87 (t, $^3J_{HH}$=6.4 Hz, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 171.7, 80.8, 34.3, 31.3, 25.4, 24.4, 22.4, 14.1.

*Chloromethyl Propionate (**10e**)*

This compound (2.20 g, 66%) was prepared from propionyl chloride, paraformaldehyde and $ZnCl_2$ following the same procedure as described for the preparation of **10a**. TLC R_f 0.10 (PE/Et_2O 98:2). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 5.68 (s, 2H), 2.39 (q, $^3J_{HH}$=7.5 Hz, 2H), 1.15 (t, $^3J_{HH}$=7.5 Hz, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 172.6, 68.8, 27.6, 8.8.

*Chloromethyl Dodecyl Carbonate (**11a**)*

Dodecanol (3.00 g, 16.0 mmol) and anhydrous Et_3N (4.46 mL, 32.0 mmol) were dissolved in dry $CHCl_3$ (50 mL). The resulting mixture was cooled to 0°C under argon and chloromethylchloroformate (2.21 mL, 16.0 mmol) was added dropwise. The reaction mixture was stirred at room temperature for 1 h. Then it was diluted with CH_2Cl_2 (50 mL) and washed 2 times with 1 M $NaHCO_3$, and with brine. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by flash column chromatography over silica gel (PE/Et_2O 95:5) to yield **11a** (3.10 g, 69%) as a clear oil. TLC R_f 0.50 (PE/Et_2O 95:5). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 5.71 (s, 2H), 4.20 (t, $^3J_{HH}$=6.7 Hz, 2H), 1.67 (m, 2H), 1.35–1.23 (m, 18H), 0.86 (t, $^3J_{HH}$=6.7 Hz, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 153.6, 72.3, 69.6, 32.1, 29.8 (2C), 29.7, 29.6, 29.5, 29.3, 28.7, 25.8, 22.9, 14.3.

*1-Chloroethyl Dodecyl Carbonate (**11b**)*

This compound (2.45 g, 83%) was obtained as a clear oil from dodecanol, pyridine and chloroethylchloroformate, following the same procedure as described for the preparation of **11a**. TLC R_f 0.54 (PE/Et_2O 95:5). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 6.40 (q, $^3J_{HH}$=5.8 Hz, 1H), 4.17 (t, $^3J_{HH}$=6.7 Hz, 2H), 1.80 (d, $^3J_{HH}$=5.8 Hz, 3H), 1.68–1.63 (m, 2H), 1.33–1.23 (m, 18H), 0.85 (t, $^3J_{HH}$=6.3 Hz, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 153.1, 84.7, 69.3, 32.1, 29.8, 29.7, 29.6, 29.5, 29.3, 28.7, 25.8, 25.4, 22.9, 14.3.

*1-Chloro-2-methylpropyl dodecyl carbonate (**11c**)*

This compound (2.08 g, 65%) was obtained as a clear oil from dodecanol, pyridine and 1-chloro-2-methylpropyl chloroformate, following the same procedure as described for the preparation of **11a**. TLC R_f 0.62 (PE/Et_2O 95:5). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 6.16 (d, $^3J_{HH}$=4.6 Hz, 1H), 4.18 (dt, $^3J_{HH}$=3.4 and 6.7 Hz, 2H), 2.18 (dhept, $^3J_{HH}$=4.6 and 6.7 Hz, 1H), 1.68–1.65 (m, 2H), 1.34–1.24 (m, 18H), 1.05 (d, $^3J_{HH}$=6.7 Hz, 6H), 0.86 (t, $^3J_{HH}$=6.9 Hz, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 153.6, 92.9, 69.3, 35.5, 29.8 (2C), 29.7, 29.6, 29.5, 29.3, 28.7, 25.9, 25.8, 22.9, 17.4 (2C), 14.3.

*Chloromethyl Dodecyl Succinate (**12**)*

Dodecanol (5.0 g, 26.8 mmol), DMAP (163 mg, 1.34 mmol) and succinic anhydride (3.22 g, 32.21 mmol) were stirred in anhydrous toluene (50 mL) and the resulting reaction mixture was refluxed for 48 h under argon. The mixture was cooled down, extracted with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by flash column chromatography over silica gel eluting first with PE/Et_2O 70:30 to remove unreacted dodecanol, and finally with CH_2Cl_2/MeOH 95:5 to obtain 4-(dodecyloxy)-4-oxobutanoic acid (3.25 g, 43%). TLC R_f 0.50 (CH_2Cl_2/MeOH) 95:5). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.07 (t, $^3J_{HH}$=6.8 Hz, 2H), 2.67–2.58 (m, 4H), 1.59 (m, 2H), 1.32–1.23 (m, 18H), 0.86 (t, $^3J_{HH}$=6.9 Hz, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 178.0, 172.4, 65.3, 32.1, 29.8 (2C), 29.7 (2C), 29.6, 29.4, 29.1 (2C), 28.7, 26.1, 22.9, 14.3. A solution of the latter compound

(3.25 g, 11.3 mmol) in anhydrous CH_2Cl_2 (50 mL) was treated with oxalyl chloride (1.10 mL, 12.5 mmol) at 0°C under an argon atmosphere. After 30 min, the reaction mixture was allowed to warm to room temperature and was stirred for an additional period of 1 h. Volatiles were removed under reduced pressure, and the residue was coevaporated twice with anhydrous toluene. The expected acyl chloride derivative was recovered in quantitative yield and used in the next step without further purification. To dodecyl 4-chloro-4-oxobutanoate (275 mg, 0.90 mmol) were successively added paraformaldehyde (27 mg, 0.90 mmol) and anhydrous $ZnCl_2$ (2.5 mg, 2%mol.). The reaction mixture was heated at 35°C under an argon atmosphere for 18 h. After cooling to room temperature, the residue was purified by flash column chromatography over silica gel (PE/Et_2O 80:20) to yield compound **12** (150 mg, 50%) as a clear oil. TLC R_f 0.42 (PE/Et_2O 80:20). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 5.69 (s, 2H), 4.07 (t, $^3J_{HH}$=6.9 Hz, 2H), 2.70–2.62 (m, 4H), 1.61–1.58 (m, 2H), 1.28–1.24 (m, 18H), 0.86 (t, $^3J_{HH}$=6.7 Hz, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 172.1, 170.7, 68.9, 65.4, 32.1, 29.8 (2C), 29.7 (2C), 29.6, 29.5, 29.2, 28.9, 28.8, 26.1, 22.9, 14.3.

General Procedure for the Preparation of Cationic Lipids

To a solution of DOPC in anhydrous $CHCl_3$ was added a solution of the freshly prepared electrophiles **10a-e**, **11a-c** or **12** (8 eq.) in $CHCl_3$. The resulting reaction mixture was refluxed for 18 h under an argon atmosphere. Then it was cooled down at room temperature and concentrated under reduced pressure. Analytically pure compounds were obtained after purification by flash chromatography over silica gel (CH_2Cl_2/MeOH 98:2 to 90:10).

*1,2-Dioleoyl-sn-glycero-3-[(dodecanoyloxy)methyl]phosphocholine chloride (**1**)*. This compound (mixture of two diastereomers, 110 mg, 28%) was obtained as a waxy solid from DOPC and **10a** in $CHCl_3$ (5 mL). TLC R_f 0.20 (CH_2Cl_2/MeOH 90:10). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 5.67–5.56 (m, 2H), 5.36–5.28 (m, 4H), 5.23 (br s, 1H), 4.57 (br s, 2H), 4.32–4.11 (m, 6H), 3.49 (s, 9H), 2.39–2.27 (m, 6H), 1.97 (m, 8H), 1.62–1.57 (m, 6H), 1.27–1.23 (m, 56H), 0.85 (t, $^3J_{HH}$=6.6 Hz, 9H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 173.4, 173.0, 172.5, 130.2, 129.8, 91.7, 69.4, 69.3, 66.2, 65.7, 65.6, 62.3, 62.2, 61.6, 54.7, 34.3, 34.2, 34.1, 32.1, 29.9, 29.8, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 27.4, 27.3, 25.0, 24.7, 22.8, 21.4, 14.3. ^{31}P-NMR (162 MHz, $CDCl_3$) δ –3.94, –4.01. ESI-MS (*m/z*): calc. for $C_{57}H_{109}NO_{10}P$ 998.78, found 998.7.

*1,2-Dioleoyl-sn-glycero-3-[1-(dodecanoyloxy)ethyl]phosphocholine chloride (**2**)*. This compound (mixture of four diastereomers, 803 mg, 38%) was obtained as a waxy solid from DOPC and **10b** in $CHCl_3$ (5 mL). TLC R_f 0.26 (CH_2Cl_2/MeOH 90:10). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 6.41 (m, 1H), 5.35–5.19 (m, 5H), 4.53 (br s, 2H), 4.28–4.11 (m, 6H), 3.50 (s, 8H), 2.31–2.26 (m, 6H), 1.97 (m, 8H), 1.58–1.51 (m, 9H), 1.26–1.17 (m, 56H), 0.84 (t, $^3J_{HH}$=6.7 Hz, 9H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 173.5, 173.1, 172.1, 130.2, 129.8, 91.7, 91.7, 91.6, 91.5, 69.6, 69.5, 69.4, 66.7, 66.6, 65.6, 65.5, 62.1, 62.0, 61.8, 54.7, 34.3, 34.2, 32.1, 29.9, 29.8, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 27.4, 27.3, 25.0, 24.6, 22.8, 21.4, 14.3. ^{31}P-NMR (162 MHz, $CDCl_3$) δ –5.62, –5.72, –5.76, –5.84. ESI-MS (*m/z*): calc. for $C_{58}H_{111}NO_{10}P$ 1012.79, found 1012.7.

*1,2-Dioleoyl-sn-glycero-3-[(hexanoyloxy)methyl]phosphocholine chloride (**3**)*. This compound (mixture of two diastereomers, 100 mg, 28%) was obtained as a waxy solid from DOPC and **10c** in $CHCl_3$ (5 mL). TLC R_f 0.20 (CH_2Cl_2/MeOH 90:10). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 5.67–5.57 (m, 2H), 5.33–5.22 (m, 5H), 4.57 (br s, 2H), 4.34–4.13 (m, 6H), 3.49 (s, 9H), 2.40–2.27 (m, 6H), 1.93 (m, 8H), 1.65–1.53 (m, 6H), 1.27–1.24 (m, 44H), 0.85 (t, $^3J_{HH}$=6.6 Hz, 9H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 173.5; 173.0, 172.3, 130.2, 129.8, 83.1, 69.5, 69.4, 66.7, 66.6, 65.6, 65.3, 62.4, 62.3, 62.2, 61.6, 57.7, 34.3, 34.2, 34.0, 32.1, 31.3, 29.9, 29.7, 29.5, 29.4, 29.3, 29.2, 17.4, 27.3, 25.0, 24.3, 22.9, 22.4, 14.3, 14.0. ^{31}P-NMR (162 MHz, $CDCl_3$) δ –3.77, –3.94. ESI-MS (*m/z*): calc. for $C_{51}H_{97}NO_{10}P$ 914.69, found 914.6.

*1,2-Dioleoyl-sn-glycero-3-[1-(hexanoyloxy)ethyl]phosphocholine chloride (**4**)*. This compound (mixture of four diastereomers, 50.6 mg, 21%) was obtained as a waxy solid from DOPC and **10d** in $CHCl_3$ (4 mL). TLC R_f 0.23 (CH_2Cl_2/MeOH 90:10). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 6.43–6.37 (m, 1H), 5.34–5.27 (m, 4H), 5.21 (br s, 1H), 4.54 (br s, 2H), 4.30–4.13 (m, 6H), 3.48 (s, 9H), 2.32 (m, 4H), 1.99 (m, 8H), 1.56 (m, 9H), 1.31–1.24 (m, 44H), 0.86 (m, 9H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 173.5, 173.1, 172.5, 172.2, 130.3, 129.9, 91.8, 69.4, 66.3, 65.8, 62.5, 61.8, 61.6, 55.0, 50.1, 34.4, 34.3, 34.2, 32.1, 31.4, 29.9, 29.7, 29.4, 29.3, 29.2, 27.4, 27.3, 25.1, 24.4, 22.9, 22.4, 21.5, 14.3, 14.1. ^{31}P-NMR (162 MHz, $CDCl_3$) –5.67, –5.78, –5.84, –5.93. ESI-MS (*m/z*): calc. for $C_{52}H_{99}NO_{10}P$ 928.70, found 928.7.

*1,2-Dioleoyl-sn-glycero-3-[(propionyloxy)methyl]phosphocholine chloride (**5**)*. This compound (mixture of two diastereomers, 103 mg, 29%) was obtained as a waxy solid from DOPC and **10e** in $CHCl_3$ (6 mL). TLC R_f 0.17 (CH_2Cl_2/MeOH 90:10). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 5.66–5.62 (m, 2H), 5.32–5.29 (m, 4H), 5.23 (br s, 1H), 4.60 (br s, 2H), 4.30–4.14 (m, 6H), 3.48 (s, 9H), 2.41 (q, $^3J_{HH}$=7.5 Hz, 2H), 2.29 (q, $^3J_{HH}$=7.6 Hz, 4H), 1.99–1.95 (m, 8H), 1.57–1.54 (m, 4H), 1.31–1.11 (m, 40H), 1.13 (t, $^3J_{HH}$=7.37 Hz, 3H), 0.83 (t, $^3J_{HH}$=6.9 Hz, 6H). ^{13}C-NMR

(100 MHz, $CDCl_3$) δ 173.5, 173.1, 173.0, 130.2, 129.8, 83.2, 69.5, 69.4, 69.3, 66.7, 65.6, 65.5, 62.4, 62.3, 61.8, 61.7, 54.7, 34.3, 34.2, 32.1, 29.9, 29.7, 29.5, 29.4, 29.3, 29.2, 27.4, 27.3, 25.0, 22.8, 14.3, 8.8. ^{31}P-NMR (162 MHz, $CDCl_3$) δ –3.7, –3.8. ESI-MS (*m/z*): calc. for $C_{48}H_{91}NO_{10}P$ 872.64, found 872.6.

*1,2-Dioleoyl-sn-glycero-3-{[(dodecyloxycarbonyl)oxy]methyl}phosphocholine chloride (**6**)*. This compound (mixture of two diastereomers, 124 mg, 31%) was obtained as a waxy solid from DOPC and **11a** in $CHCl_3$ (6 mL). TLC R_f 0.23 (CH_2Cl_2/MeOH 90:10). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 5.63 (m, 2H), 5.32–5.28 (m, 4H), 5.22 (br s, 1H), 4.58 (br s, 2H), 4.28–4.09 (m, 8H), 3.51 (s, 9H), 2.29 (m, 4H), 1.99–1.95 (m, 8H), 1.65–1.56 (m, 6H), 1.27–1.16 (m, 58H), 0.85 (t, $^3J_{HH}$=6.5 Hz, 9H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 173.4, 173.1, 153.7, 130.2, 129.8, 86.1, 69.8, 69.4, 69.3, 66.8, 66.7, 65.6, 65.5, 62.4, 62.3, 61.6, 61.5, 54.8, 34.3, 34.2, 32.1, 29.9, 29.8, 29.7, 29.5, 29.4, 29.3, 29.2, 28.7, 27.4, 27.3, 25.8, 25.0, 22.9, 14.3. ^{31}P-NMR (162 MHz, $CDCl_3$) δ –3.4, –3.5. ESI-MS (*m/z*): calc. for $C_{58}H_{111}NO_{11}P$ 1028.79, found 1028.7.

*1,2-Dioleoyl-sn-glycero-3-{1-[(dodecyloxycarbonyl)oxy]ethyl}phosphocholine chloride (**7**)*. This compound (mixture of four diastereomers, 450 mg, 55%) was obtained as a waxy solid from DOPC and **11b** in $CHCl_3$ (12 mL). TLC R_f 0.28 (CH_2Cl_2/MeOH 90:10). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 6.28 (m, 1H), 5.35–5.26 (m, 4H), 5.20 (br s, 1H), 4.55 (br s, 2H), 4.32–4.07 (m, 8H), 3.51 (s, 9H), 2.33–2.26 (m, 4H), 1.96 (m, 8H), 1.67–1.56 (m, 9H), 1.26–1.22 (m, 58H), 0.85 (t, $^3J_{HH}$=6.9 Hz, 9H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 173.4, 173.1, 153.4, 130.2, 129.9, 95.2, 69.5, 69.4, 69.3, 66.3, 66.2, 65.7, 65.6, 65.5, 62.3, 61.7, 61.6, 61.5, 54.7, 34.3, 34.1, 32.1, 29.9, 29.8, 29.7, 29.5, 29.4, 29.3, 29.2, 28.7, 27.4, 27.3, 25.8, 25.0, 22.8, 21.5, 21.4, 14.3. ^{31}P-NMR (162 MHz, $CDCl_3$) δ –5.64, –5.79 (2P), –5.92. ESI-MS (*m/z*): calc. for $C_{59}H_{113}NO_{11}P$ 1042.80, found 1042.8.

*1,2-Dioleoyl-sn-glycero-3-{1-[(dodecyloxycarbonyl)oxy]-2-methylpropyl}phosphocholine chloride (**8**)*. This compound (mixture of four diastereomers, 120 mg, 27%) was obtained as a waxy solid from DOPC and **11c** in $CHCl_3$ (6 mL). TLC R_f 0.32 (CH_2Cl_2/MeOH 90:10). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 6.05–6.01 (m, 1H), 5.38–5.27 (m, 4H), 5.24–5.20 (br s, 1H), 4.55 (br s, 2H), 4.33–4.09 (m, 8H), 3.50 (s, 9H), 2.33–2.27 (m, 4H), 1.96 (m, 8H), 1.69–1.57 (m, 6H), 1.27–1.23 (m, 58H), 0.98 (d, $^3J_{HH}$=6.5 Hz, 6H), 0.85 (t, $^3J_{HH}$=6.7 Hz, 9H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 173.5, 173.2, 153.9, 130.3, 129.9, 100.3, 69.5, 69.4, 66.9, 65.8, 62.3, 61.8, 55.2, 55.1, 34.4, 34.2, 33.1, 32.1, 29.9, 29.8, 29.7, 29.5, 29.4, 29.3, 28.7, 27.5, 27.4, 25.8, 25.0, 22.8, 16.6, 16.0, 14.3. ^{31}P-NMR (162 MHz, $CDCl_3$) δ –4.69, –4.87, –4.92, –5.00. ESI-MS (*m/z*): calc. for $C_{61}H_{117}NO_{11}P$ 1070.84, found 1070.8.

*1,2-Dioleoyl-sn-glycero-3-{4-[(dodecyloxy)-4-oxobutanoyloxy]methyl}phosphocholine chloride (**9**)*. This compound **9** (mixture of two diastereomers, 110 mg, 26%) was obtained as a waxy solid from DOPC and **12** in $CHCl_3$ (8 mL). TLC R_f 0.16 (CH_2Cl_2/MeOH 90:10). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 5.65–5.62 (m, 2H), 5.33–5.29 (m, 4H), 5.23–5.21 (m, 1H), 4.58 (br s, 2H), 4.47 (m, 1H), 4.32–4.13 (m, 6H), 4.03 (t, $^3J_{HH}$=7.2 Hz, 2H), 3.49 (s, 9H), 2.68–2.60 (m, 4H), 2.31–2.27 (m, 4H), 2.00–1.95 (m, 8H), 1.61–1.56 (m, 6H), 1.27–1.23 (m, 58H), 0.85 (t, $^3J_{HH}$=6.9 Hz, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 173.5, 173.1, 172.5, 171.2, 130.2, 129.9, 83.2, 83.2, 69.4, 66.7, 65.5, 65.2, 62.4, 61.7, 61.6, 54.8, 34.3, 34.2, 32.1, 29.9, 29.8, 29.7, 29.5, 29.4, 29.3, 29.2, 28.9, 28.7, 28.6, 27.4, 26.1, 25.0, 22.9, 14.3. ^{31}P-NMR (162 MHz, $CDCl_3$) δ –3.35, –3.52. ESI-MS (*m/z*): calc. for $C_{61}H_{115}NO_{12}P$ 1084.82, found 1084.8.

Methods

Preparation of Liposomes

For the hydrolysis rate measurements involving ^{31}P-NMR spectroscopy, liposomes were prepared by a solvent injection technique (24). A dry lipid film (10 μmol) was dissolved in *i*-PrOH (200 μL) and then injected with a syringe (flow rate: 600 μL/min) under stirring (stirring speed: 400 rpm) in the middle of the appropriate aqueous buffer medium (either Hepes 10 mM, pH7.4 or AcOK/AcOH 10 mM, pH4.5), at 22°C.

Preparation of Lipoplexes

Typically, the appropriate volume of a freshly prepared solution of cationic lipid (2 mM in EtOH) was deposited at the bottom of a 1.5 mL polyethylene tube and dried under vacuum. Then, pCMV-Luc DNA (60 μM in 4.5% glucose, 40 μL) or siRNA (siLuc or sic, 100 nM in 4.5% glucose, 40 μL) was added to the resulting lipid film. After stirring by vortex for 30 s, the preparation was allowed to stand at room temperature for 30 min before use.

Dynamic Light Scattering Measurements

The average particle size of liposomes and lipoplexes was measured by photon correlation spectroscopy using a Zetasizer nanoZS apparatus (Malvern Instruments, Paris, France). All measurements were performed on undiluted freshly prepared liposomes or lipoplexes (*vide supra*) at 25°C and in triplicate. Data were analyzed using the multimodal number distribution software supplied with the instrument and expressed as mean (± SD).

DNA Retardation Assay

Freshly prepared lipoplexes at the desired N/P ratio were analyzed by electrophoresis through a 1% agarose gel. The gel was run in a 40 mM Tris-acetate-EDTA buffer, pH 8.0 (TAE) and the DNA was stained using an ethidium bromide solution (EB, 0.5 μg/mL).

Ethidium Bromide Intercalation Assay

In a 96-well microplate, EB (0.28 μg) and salmon sperm DNA (2.4 μg) were first mixed in 10 mM Hepes pH 7.4 or 10 mM acetate buffer pH 4.5 (67 μL), and kept at room temperature for 2 min. Then, an ethanolic solution of cationic lipid (1.45 mM) was added to the DNA/EB mixture in order to get a N/P ratio of 3. Subsequently, fluorescence spectra were recorded for the complex solution as a function of time on a Gemini XPS Fluorescence Microplate Reader (Molecular Devices, Saint-Grégoire, France) with excitation and emission wavelengths at 546 and 600 nm, respectively. The emission intensities of the initial DNA/EB complex (I_{100}) and of the cationic lipid/DNA/EB solution (I_0) were taken as the corresponding references for relative intensity 100% (F_{100}) and 0% (F_0), respectively. Further, the emission of the DNA/EB complex at 600 nm (I) was measured and the fluorescence percentage was calculated according to the following equation: $F(\%) = ((I - I_0)/(I_{100} - I_0)) \times 100$.

Cell Culture

All cell lines were grown in culture flasks at 37°C in a 5% CO_2 humidified chamber. BHK-21 cells were grown in DMEM-F12 medium containing FBS (10%), penicillin (100 units/mL), and streptomycin (100 μg/mL). A549 and Calu-3 cells were grown in DMEM-F12 medium containing FBS (10%), penicillin (100 units/mL), streptomycin (100 μg/mL), and Hepes (5 mM). NCI-H292 cells were grown in RPMI 1640 supplemented with FBS (10%), sodium pyruvate (1 mM), L-glutamine (2 mM), penicillin (100 units/mL), and streptomycin (100 μg/mL). U87Luc cells were grown in DMEM supplemented with FBS (10%), penicillin (100 units/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM). Selection of U87Luc and A549Luc cells was performed by adding G418 (0.8 mg/mL) to the culture medium. At confluence, cells were released with trypsin (0.5% in PBS), centrifuged (4 ° C, 5 min, 120 g) counted and transferred into 96-well plates (Becton-Dickinson) in 100 μL culture medium at a density of 6,000 cells/well for DNA delivery experiments, and at a density of 8,000 cells/well for siRNA delivery experiments. Plates were maintained at 37°C in a 5% CO_2 humidified chamber for 24 h before transfection experiment.

DNA Transfection Experiments

Freshly prepared DNA lipoplexes (10 μL, *i.e.* 0.2 μg DNA/well) were added in triplicate to the wells of the cell culture plates by dilution with the cell culture serum-containing medium. Cells were then let to grow in the incubator without further handling. Luciferase gene expression was assessed 24 h later using a commercial kit according to the manufacturer's protocol (Promega, Charbonnières, France). Briefly, the cell culture medium was carefully removed. The adherent cells were then washed with PBS (100 μL) and treated with the kit lysis buffer (20 μL) for 15 min. The solution was then diluted with PBS (150 μL) and luciferase content was measured by monitoring light production during 1 s from 5 μL aliquot of each sample upon addition of the luciferin substrate (35 μL) using a luminometer (Berthold Centro LB960 XS, Thoiry, France). Value for each sample is the mean of triplicate determination (± SD).

siRNA Delivery Experiments

Freshly prepared siRNA lipoplexes (10 μL, *i.e.* 1 pmol RNA) were added in triplicate to the wells of the cell culture plates (final RNA concentration 10 nM). Cells were then let to grow in the incubator without further handling. Luciferase gene expression was assessed 48 h later as described above, except PBS volume added was different (200 μL). The content of luciferase protein in each sample was reported as the residual luciferase activity when compared to non-treated cells (100%). Value for each sample is the mean of triplicate determination (± SD).

Quantification of Cell Internalized Material

The night before experiment, BHK-21 cells were seeded into 96-well plates at a density of 8,000 cells per well in 0.1 mL of cell culture medium. A freshly prepared 2 mM ethanolic solution of each cationic lipid (3 μL, 6 nmol) was placed at the bottom of a 1.5 mL polyethylene tube and dried under vaccum. A solution of carboxyfluorescein 5′-labeled oligonucleotide of sequence TCGCCCTCGCCCTCG (2 μg) in 4.5% glucose (0.1 mL) was then added to the lipid film. The tube was vigorously vortexed for 30 s and 10 μL aliquots were added into the wells by dilution with the cell culture medium containing 10% FBS (0.1 mL). After a 4-h incubation period, the supernatant was carefully removed and the adherent cells were washed with PBS containing heparin (0.1 mL, 10 mg/mL). The cells were then treated with 150 mM phosphate buffer, pH 7.8 containing 0.2% Triton X-100® and 0.2% SDS (80 μL) at 37°C for 30 min, for lysis. Fluorescence was measured using a Xenius XM spectrofluorimeter (SAFAS, Monaco). Values were corrected by subtracting the value obtained from cells treated with the naked fluorescent DNA and were given relative to the applied quantity of labeled DNA (n=4, mean ± SD).

Lipid Metabolization and MS Quantification

The night before experiment, BHK-21 cells were seeded into 12-well plates at a density of 100,000 cells per well in 1 mL of cell culture medium. A freshly prepared 2 mM ethanolic solution of each cationic lipid (4 µL, 8 nmol) was placed at the bottom of a 1.5 mL polyethylene tube and dried under vacuum. A solution of pCMV-Luc (2.64 µg) in 4.5% (w/v) glucose (100 µL) was added to the lipid film (N/P 1). The mixture was vortexed for 30 s and then placed into wells by dilution with the cell culture medium containing 10% FBS (1 mL). After a 4-h incubation period in a cell incubator to allow lipoplexes to settle down, the medium (1 mL) was removed. The cells were washed with PBS (1 mL) and further incubated for 20 h with fresh cell culture medium (1 mL). For lipid extraction, the cell culture medium was carefully removed and the cells were recovered from the plate in 1 mL PBS by scrapping with a rubber policeman. Each cell suspension (1 mL) was recovered, placed into a glass vial and was extracted by addition of $CHCl_3$/MeOH (2 mL, 2:1 v/v), followed by vigorous agitation (30 s) and phase separation by centrifugation (500 RCF, 10 min). Part of the clear organic layer (600 µl) was transferred into a glass vial, immediately frozen on dry ice and stored at –20°C until mass spectrometry analysis. Quantification of residual cationic lipid was achieved by Q-ToF MS analysis using a spiking technique and Flow Injection Analysis (FIA). Extract (1 µl) was injected and the molecular peak area corresponding to the cationic lipid was determined. Then, a spiking injection of the cationic lipid allowed precise determination of the amount of cationic lipid in the extract.

Lactate Dehydrogenase Assay

Lactate dehydrogenase (LDH) cell release was used to assess the cytotoxicity of the formulations. Typically, at the end of each transfection experiment, an aliquot of each culture supernatant was transferred into a 96-well assay plate and LDH activity was measured using a commercial kit (Cytotoxicity Detection Kit Plus, Roche Applied Science) according to the manufacturer's instructions. LDH activity was expressed as the percentage of the maximal LDH release obtained after treatment of the cells with the kit lysis solution (5 µL). Value for each sample is the mean of triplicate determination (± SD). LDH release values of less than 10% were considered as non-significant.

Statistical Analysis

All data are presented as the mean ± standard deviation (SD). Statistical significance between treatments was assessed by the student's t-test. Data were considered as statistically significant for p value less then 0.05 (***$p<0.001$, **$p<0.01$, *$p<0.05$).

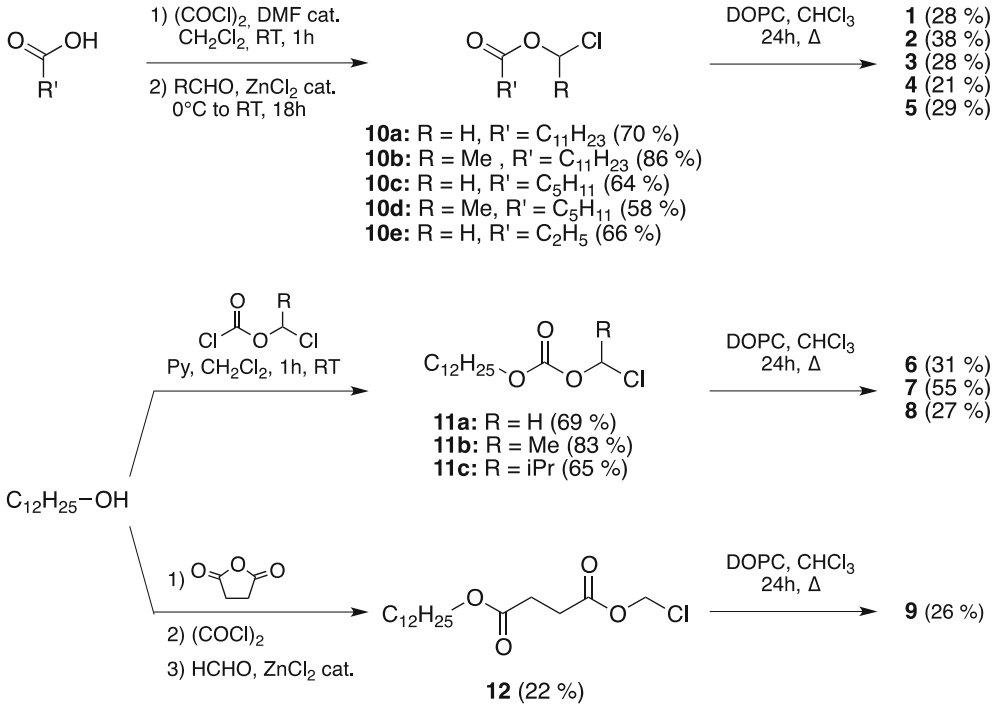
RESULTS AND DISCUSSION

Lipid Design

The cationic lipids described herein result from the transformation of the membrane phospholipid DOPC into phosphotriester compounds. These conjugates were designed so they may display various degradation kinetics depending on the chemical nature of the linker installed between DOPC and a "pendant" alkyl moiety (alcohol or carboxylic acid). Upon chemical or enzymatic hydrolysis, the compounds are supposed to restore DOPC which, as a zwitterionic amphiphile, cannot preserve the previously established electrostatic interactions with the polyphosphate backbone of a nucleic acid (Scheme 1). Consequently, lipoplexes prepared from these conjugates are expected to disassemble under hydrolytic conditions (*e.g.* under a pH or an enzyme stimulus) and release the free nucleic acid. So the core issue is that this hydrolysis preferentially takes place intracellularly, *i.e.* once the lipoplexes have penetrated into the target cells.

Very few publications describe the synthesis of phosphotriester derivatives of phosphocholines (PCs) (16,25). These compounds were prepared by direct *O*-alkykation of PCs with simple alkyl triflates. Besides, though some ketal phosphates have been previously described, they only relate to phosphosugar compounds and thus involve nucleophilic displacement of a chloride (26), bromide (27), or triflate (28) on a cyclic ketal substrate. Compounds **1-9** described herein were prepared by *O*-alkylation of DOPC with 1-chloroalkyl esters and carbonates, as shown in Scheme 2.

In order to access compounds **1** to **5**, various chloroalkylesters have been prepared following a previously reported procedure (29). Briefly, acyl chlorides, quantitatively obtained by treatment of the corresponding carboxylic acid with oxalyl chloride, were reacted with aldehydes (HCHO, CH_3CHO or *i*-PrCHO) in the presence of a catalytic amount of $ZnCl_2$, at low temperature (–20°C-0°C). Purification of the crude reaction mixtures over silica gel provided the expected 1-chloroalkylesters **10a-e** in good to excellent yields (58–86%). Conjugates **1-5** were then obtained by reacting DOPC with the corresponding 1-chloroalkylesters in refluxing $CHCl_3$ for 24 h. They were prepared in 21–38% yield and significant amount of unreacted DOPC (> 50%) was recovered during the purification step. Attempts for improving the reaction yield invariably failed or introduced outstanding separation difficulties so the pure compounds could not be obtained satisfactorily. Following a similar synthetic pathway, preparation of compounds **6-8** involved 1-alkyl-1-chloroalkylcarbonates **11a-c**. These carbonates were obtained in good yield upon reaction between dodecyl alcohol with the corresponding 1-chloroalkychlorocarbonate in the presence of dry pyridine, according to a usual procedure (30). Phosphotriesters **6-8** were then synthesized straightforwardly by reacting



Scheme 2 Synthetic route to the deciduous-charge cationic amphiphiles **1**-**9**.

11a-c with DOPC as outlined above. Once again, moderate conversion was a key parameter for convenient purification of the compounds, and a significant amount of unreacted DOPC was recovered after silica gel column chromatography. Finally, succinate derivative **9** was prepared in 4 steps. First, dodedacanol was reacted with succinic anhydride in the presence of a catalytic amount of DMAP in refluxing toluene. The resulting hemisuccinate was quantitatively converted into the corresponding acid chloride, which was then reacted with a stoichiometric amount of paraformaldehyde, in the presence of a catalytic amount of $ZnCl_2$, to yield 1-chloromethylester **12**. The latter compound was conjugated to DOPC in chloroform to afford title compound **9** in 26% yield, together with unreacted DOPC (57%).

Hydrolytic Properties of the Conjugates

As a first step for evaluating the properties of compounds **1**-**9**, we examined their hydrolytic stability under neutral and acidic conditions in a model experiment involving ^{31}P-NMR measurements. Phosphotriesters **1**-**9** and phosphodiester DOPC display ^{31}P chemical shifts differing by 5–6 ppm in aqueous media allowing a precise monitoring of the hydrolysis reaction. The cationic lipids were formulated into liposomes at pH7.4 and pH4.5, and incubated at 37°C. Periodical acquisition of ^{31}P-NMR spectra allowed determination of the time $t_{1/2}$ required for 50% hydrolysis (Table I).

As anticipated, most of the cationic conjugates (**1**-**6** and **9**) displayed lowered stability from neutral to acidic pH. Surprisingly, this was not the case for carbonates **7** and **8** as these two compounds were hydrolyzed at the same rate whatever the pH value. This suggests that stability of the conjugates cannot be analyzed only taking into account the chemical reactivity of the acetal moiety but should rather be interpreted with regard to the respective sensitivity of the neighboring carbonate and ester moieties towards hydrolysis. Indeed hydrolysis rate depends on the reaction mechanism (specific base-catalyzed, water-catalyzed or specific acid-catalyzed) (31), but also on

Table I Hydrolytic Stability of Amphiphiles **1**-**9**

Compound		1	2	3	4	5	6	7	8	9	EDOPC
$t_{1/2}$ (h)	pH7.4	173	10	280	5.4	320	385	29	497	319	–[a]
	pH4.5	94	2.1	179	1.3	170	108	27	498	150	–[a]

Compounds formulated into liposomes were incubated at 37°C and hydrolysis was monitored by ^{31}P-NMR measurements. Time required for 50% hydrolysis ($t_{1/2}$) was calculated from the theoretical curve fitting with the experimental data

[a] No hydrolysis was detectable after 10 days

steric and electronic effects at the acetal center. Throughout the whole series of compounds, we can observe that the introduction of a methyl substituent on the acetal linker invariably provokes an increase in the hydrolysis rate, both under acidic and physiological pH (**2**, **4**, and **7** *vs* **1**, **3**, and **6**, respectively). At the opposite, data obtained for derivative **8** indicate that the introduction of an *iso*-propyl substituent at the ketal moiety strongly decreases the hydrolysis rate. This observation may be explained either by a more hydrophobic environment established in the acetal region or some steric hindrance, which may hamper the approach of the ionic species (H_3O^+ or OH^-) or water molecule that is required for the hydrolysis to occur, whatever the mechanism involved. Finally, in liposome preparations based on EDOPC no trace of hydrolysis could be detected over a 10-day period.

Lipoplexes Formation and Characterization

The ability of cationic lipids **1**-**9** to interact electrostatically with nucleic acid to form lipoplexes was assessed by conventional electrophoretic DNA retardation assay in which a full retard of plasmid DNA is observed at a charge ratio (lipid ammonium/DNA phosphate ratio, N/P) corresponding to electroneutrality. Data indicate that the introduction of a hydrophobic substituent onto the phosphate group of DOPC, whatever its length (C_2 to C_{12}), has no significant effect on the ability of the ammonium headgroup of the lipid carrier to interact electrostatically with the DNA polyphosphate backbone (see Supplementary Material, Fig. S1). All the compounds investigated herein fully retarded DNA at N/P between 1 and 1.2. Same was observed for siRNA lipoplexes (see Supplementary Material, Fig. S2).

The size of lipoplexes has been reported to be determinant for *in vitro* transfection experiments as the cell uptake of lipoplexes and the transfection efficiency was found to increase together with the lipoplex size (32). Thus, the hydrodynamic diameter of pCMV-Luc complexes with cationic vectors **1**-**9** was determined by dynamic laser light scattering measurements (at N/P 3). In all the cases, lipoplexes were observed as a uniform population of complexes with a narrow distribution and a diameter in the range of 315–820 nm (Table II). In comparison, lipoplexes prepared from EDOPC were slightly larger (902 nm). Intriguingly, the 3 compounds incorporating a non-substituted acetal bridge (**1**, **6**, and **9**) led to the formation of negatively charged lipoplexes (–26.0, –26.4, and –18.0 mV, resp.) whereas all the other cationic lipids investigated herein led to positively charged particles (+43.5 to +62.9 mV). Such negative charges contrast with the results from the gel retardation assay. Experiments on hydrolytic stability previously showed that these three lipids were far more stable than compounds **2**, **4** and **7**. The loss of superficial cationic headgroups is thus not likely. Alkyl substitution at the acetal position is expected to introduce some steric hindrance and to increase hydrophobicity in the direct neighborhood of the phosphate group. This may alter solvation of the polar headgroup of the compounds by water molecules (33) and affect the distribution of ions in the surrounding interfacial region with modification of zeta potential (ζ). However, if this was the reason for the large variation of zeta potential observed, lipoplexes prepared from compound **3** should give a negative ζ value as well. This is not the case. Furthermore, ζ measured for liposomes prepared from lipids **1**, **6**, and **9** was highly positive (+ 50.9± 2.8 mV, 52.7±1.5 mV, 49.8±3.5 mV, resp.). Additional investigation will be required to elucidate the origin of the negative charge of lipoplexes based on compounds **1**, **6**, and **9**.

Table II Particle Size and Zeta Potential of Lipoplexes Obtained From Lipids **1**-**9** and EDOPC, and pCMV-Luc as Measured by Dynamic Light Scattering

Compound	Particle size (nm)	Zeta potential (mV)
1	535.3 ± 52.9	−26.0 ± 1.1
2	669.8 ± 17.7	+46.4 ± 1.9
3	656.7 ± 42.1	+55.4 ± 2.5
4	484.5 ± 40.6	+49.0 ± 1.1
5	464.8 ± 56.6	+51.1 ± 1.9
6	315.4 ± 46.4	−26.4 ± 1.3
7	819.3 ± 79.8	+62.9 ± 0.1
8	721.8 ± 65.7	+48.5 ± 1.8
9	364.3 ± 34.6	−18.0 ± 0.9
EDOPC	901.7 ± 18.8	+43.5 ± 0.8

Lipoplexes were freshly prepared in 4.5% glucose, at charge ratio N/P 3 and 25°C. Data reported are the mean of three independent measurements ± SD

Ethidium Bromide Intercalation Assay

As cationic lipids **1**-**9** display various kinetics of chemical hydrolysis and all efficiently bind to DNA, the next step in our investigations was to determine how fast the cationic lipids are hydrolyzed when formulated into lipoplexes. To do so, we looked at the behavior of DNA lipoplexes in the presence of ethidium bromide (EB), an intercalating agent widely used as a fluorescent probe for monitoring nucleic acid condensation and protection. The kinetics of hydrolysis of the cationic lipids into DOPC within the lipoplexes is expected to control DNA decomplexation and release. Lipoplexes were thus prepared at physiological pH (pH7.4, 10 mM Hepes buffer) or under slightly acidic conditions (pH4.5, 10 mM acetate buffer) at N/P 3, in the presence of EB. The fluorescence of EB was then measured as a function of time (Fig. 2), according to a previously described procedure (34). As EB fluorescence is quenched by water molecules, fluorescence increase may be directly correlated to EB intercalation in between nucleic acid base pairs and,

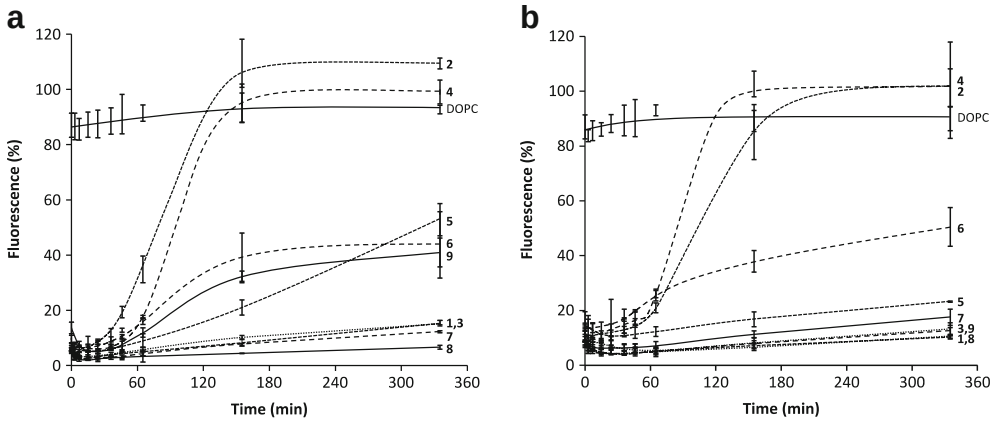


Fig. 2 Hydrolysis of the deciduous-charge amphiphiles **1**-**9** in lipoplex formulations. DNA release was monitored by an ethidium bromide intercalation assay. Picture shows the variation of EB fluorescence intensity as a function of time, at pH7.4 (**a**) and 4.5 (**b**), and at 37°C ($n = 3$, mean ± SD). The DOPC annotated curves refer to DNA preparations containing DOPC instead of a cationic lipid; DNA is thus fully accessible for EB intercalation (positive control).

consequently, to cationic lipid hydrolysis and DNA release. Among all the lipids under evaluation, compounds **2** and **4** offered the fastest kinetics of nucleic acid release from the lipoplexes. This result is fully consistent with those obtained in the previous ^{31}P-NMR experiments and is another confirmation that installation of a methyl group on the acetal moiety in the ester series (**2** *vs.* **1,** and **4** *vs.* **3**) decreases the hydrolytic stability of the compound. As already observed in absence of DNA, the introduction of the more hydrophobic and bulky *iso*-propyl group at the same place (compound **8**) drastically slowed down the hydrolytic cleavage of the lipid, and DNA release was the slowest that was measured in the series of compounds. Cationic lipids **1**, **3**, and **7** all displayed similar DNA decondensation abilities with *ca.* 10–15% DNA release after a 5 h incubation period at 37°C. Noteworthy, compound **7** which displayed intermediate hydrolytic susceptibility when formulated as liposomes (see Table I) appeared significantly more resistant within lipoplexes. Compound **6** showed intermediate resistance and 40–45% DNA was exposed to EB intercalation after 5 h regardless of the pH conditions. Finally, compounds **5** and **9** displayed marked sensitivity to pH conditions and the corresponding lipoplexes hydrolyzed 2-3 times more quickly at pH7.4 than under acidic conditions (pH4.5). The same experiments were conducted with siRNA lipoplexes and gave similar results (see Supplementary Material, Fig. S4). Based on these data, we may expect differences in transfection efficiency among the various lipoplexes.

Plasmid DNA Transfection

Cationic *O*-alkyl phosphocholine derivatives have been extensively studied in terms of physical chemistry and biological properties, and transfection efficiency was reported for some of them, including EDOPC (8,11,16,17,35,36). So we investigated the ability of the deciduous-charge carriers developed herein to mediate DNA transfection, and EDOPC was used as a reference. Furthermore, this cationic lipid proved to be resistant towards chemical hydrolysis (*vide supra*), and intracellular degradation was reported to result exclusively from phospholipase A_2 activity, thus specifically producing the corresponding cationic lysoPC derivative (17). We started the biological evaluation of the compounds using the BHK-21 cell line, and pCMV-Luc, a DNA plasmid encoding for the *firefly luciferase* gene under the control of a strong promoter (Fig. 3a). Compounds **2** and **4** that displayed higher sensitivity to hydrolysis were the least efficient to transfect cells with pDNA. This may be explained assuming that these compounds are too early hydrolyzed, *i.e.* probably before the lipoplexes can enter the cells. In such a situation, DOPC formation triggers DNA release outside the cells which definitely prevents further internalization. All the other lipid carriers investigated herein showed a high transfection efficiency. Except for compound **9** (and compound **5** at electroneutrality), they all compared very favorably to EDOPC and appeared up to two orders of magnitude more powerful for delivering pDNA into the cells. Noteworthy, in a previous report by MacDonalds *et al.*, EDOPC was shown to be superior to the gold standard cationic lipid Lipofectamine® for transfecting BHK-21 cells (16). Also important to note is the fact that, among the lipoplex formulations evaluated, transfection efficiency reached its optimum at an N/P ratio close to electroneutrality. Proof was made that it was not a result of particle aggregation that would favor sedimentation (see Supplementary Material, Table S1). This is particularly interesting since the use of an excess of cationic lipid to mediate transfection is usually accompanied with increased toxicity. From these results however, no clear relationship appears between transfection efficiency and the previously determined hydrolytic stability of the compounds. This is not really surprising considering that hydrolysis by intracellular enzymes is likely to supersede pure chemical hydrolysis, with substrate

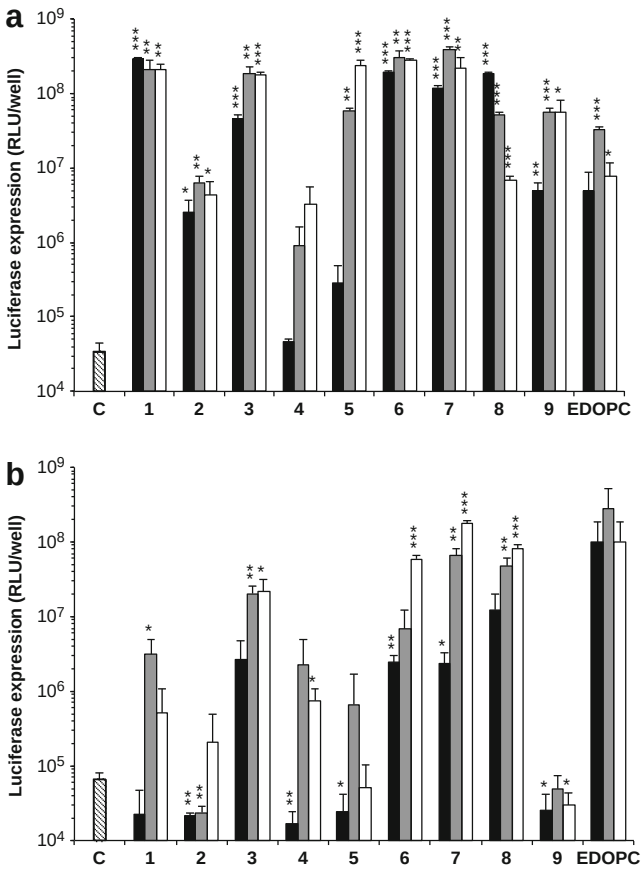


Fig. 3 Expression of luciferase in BHK-21 cells treated with pCMV-Luc pDNA complexed with the deciduous-charge amphiphiles **1**-**9** or EDOPC (**a**), and **1**-**9**/DOPE or EDOPC/DOPE (1:1 mol) (**b**), in the presence of 10% FBS. Lipoplexes were prepared at various N/P charge ratios (1.0: *black*; 3.0: *grey*; 5.0: *white*). Control (C) refers to basal luminescence measured in untreated cells. Data shown are representative of a triplicate determination (mean ± SD). Statistically significant differences when compared to untreated cells (**c**): ***$p < 0.001$, **$p < 0.01$ or *$p < 0.05$.

Table III Uptake of Transfection Particles by BHK-21 Cells and Lipid Carrier Metabolization

Compound	Cell uptake[a] (%)	Intracellular residual cationic lipid[b] (%)
1	5.1 ± 0.5	3.82 ± 0.03
2	1.8 ± 0.6	0.16 ± 0.01
3	8.3 ± 0.8	2.90 ± 0.09
4	2.5 ± 1.1	0.06 ± 0.01
5	5.0 ± 1.1	2.15 ± 0.01
6	6.3 ± 2.1	3.42 ± 0.06
7	4.4 ± 1.2	2.15 ± 0.02
8	5.1 ± 0.3	3.37 ± 0.12
9	4.4 ± 0.9	2.76 ± 0.08
EDOPC	5.6 ± 2.3	3.11 ± 0.02

Particles were obtained from lipids **1**-**9** and EDOPC, and pCMV-Luc as described in Materials and Methods. Lipoplexes were freshly prepared in 4.5% glucose, at charge ratio N/P 3 and 25°C. Cell uptake refers to the fraction of transfection material which was internalized over a 4-h incubation at 37°C. Intracellular residual cationic lipid corresponds to the amount of lipid found inside the cells after an incubation period of 24 h

[a] Determined by fluorescence measurements

[b] Determined by Q-ToF MS analysis of cell extracts

selectivity that cannot be anticipated (19,37). In order to gain a better insight into the fate of the transfection particles, two kinds of experiments were conducted. First, DNA was replaced with a fluorescent oligonucleotide and the lipoplexes were incubated with cells for 4 h before the latter were washed for removing non-internalized material. Then, the amount of internalized lipoplexes was quantified by fluorescence measurement (Table III). Besides, lipoplexes prepared from pCMV-Luc were incubated with cells for 4 h. Then they were washed for removing non-internalized material, and incubated for an additional 20-h period with fresh culture medium. At the end of incubation, supernatant was discarded and the cells were transfered into a glass vial. The lipid content was extracted with $CHCl_3$/MeOH, and residual cationic lipid was quantified by Q-ToF MS analysis. Cell uptake measurements did confirm that lipoplexes prepared from lipids **2** and **4** are less efficiently internalized by cells than the others, by a two-fold factor or more. This is in support of the idea of their premature degradation due to rapid hydrolysis of the lipid matrix in the extracellular compartment. Cell uptake for the other lipoplexes appeared rather similar from one lipid to another (4.4–6.3%), except for lipopexes prepare from compound **3** (8.3%). Correlatively, faster hydrolysis of compounds **2** and **4** was reflected by the very low amount of residual cationic lipid measured inside the cells after the 4-h incubation. Moreover, worth to note that for most of the lipids, intracellular $t_{1/2}$ was less than 4 h, and definitely does not match with $t_{1/2}$ measured for hydrolytic stability in buffered saline solution (see Table I).

As the so-called helper lipids enhance the cell transfection activity of most cationic lipids, we then investigated the influence of the most popular of them, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), in our preparations. The role of DOPE in transfection assays has been extensively described (38,39). The mechanism of action of this lipid is related to its capacity to induce a bilayer phase transition from the liquid-crystalline lamellar phase L_α to the inverted hexagonal H_{II} phase. It is speculated that such a transition facilitates the disruption of endosomes and the release of the nucleic acid payload into the cytosol. We thus evaluated the efficiency of deciduous-charge cationic lipid/DOPE mixtures (1:1 molar ratio) to condense and deliver pDNA to BHK-21 cells. No significant difference was observed in DNA condensation by the mixture of lipids when compared to the pure lipids (see Supplementary Material, Fig. S3). Considering transfection of BHK-21 cells, the results obtained showed that, when associated with

DOPE, efficiency of most of the compounds at N/P 1 was reduced by up to four orders of magnitude and cationic lipid **9** was unable to assist DNA transfection when mixed with DOPE (Fig. 3b). Carbonate deciduous-charge lipids (compounds **6**, **7**, and **8**) revealed a little bit less sensitive to DOPE addition than ester-based compounds and transfection efficiency was intermediately affected at the higher charge ratio (N/P 5). In the particular case of EDOPC, addition of DOPE in lipoplexes improved transfection efficiency by a *ca.* fifteen-fold factor. Noteworthy, MacDonald *et al.* have previously reported that no mixture of EDOPC with DOPE was as effective as the pure cationic lipid, though the 1:1 mixture (the most common ratio used with other cationic lipid transfection reagents) was nearly as effective as pure EDOPC (17). The reason why our results differ from those of the litterature could not be determined. Interestingly, introduction of DOPE into the lipoplexes resulted in systematic increase in the ζ potential measured for the particles (see Supplementary Material, Table S2). Particularly compounds **1**, **6**, and **9**, which formed negatively charged lipoplexes in the absence of DOPE (see Table II), gave positively charged particles when mixed with the presumed helper lipid. Despite this, the transfection efficiency was found much lower in the presence of DOPE, which was rather unexpected. The slight variations in the size of the lipoplexes upon DOPE mixing do not suggest any obvious explanation for this.

The impact of DNA transfection on cell viability was then assessed by a LDH release assay (40). Cell death by necrosis is systematically accompanied by damage to cell membrane and subsequent release of cytoplasmic material into the extracellular compartment. Loss of cell viability can be directly monitored by measuring the activity of the cytosolic enzyme LDH in the culture supernatant before measuring luciferase activity. As shown on Fig. 4, the lipoplex formulations did not significantly perturb overall the membrane integrity of the cells (LDH release not exceeding 5–6% except in the case of lipid **8**), although a general trend was that lipids in the carbonate series (compounds **6**, **7**, and **8)** appeared a little bit more aggressive towards the BHK-21 cell line than lipids in the ester series (compounds **1-5** and **9**). All in all, in this first set of experiments, the more favorable ratio between pDNA transfection efficiency and cytotoxicity was observed for compound **1**.

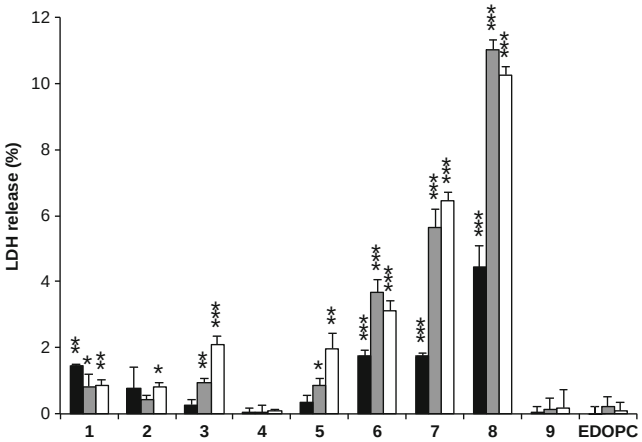


Fig. 4 Lipoplex cytotoxicity as determined by a LDH release assay. Cytotoxicity was evaluated on BHK-21 cells after 24 h incubation with lipoplexes prepared from pDNA and the deciduous-charge amphiphiles **1**-**9**, and EDOPC, in the presence of 10% FBS. Lipoplexes were prepared at various N/P charge ratios (1.0: *black*; 3.0: *grey*; 5.0: *white*). Basal LDH is set at 0%, and 100% represents the total LDH released after cell lysis. Data shown are representative of a triplicate determination (mean ± SD). Statistically significant differences when compared to untreated cells (0%): ***$p < 0.001$, **$p < 0.01$ or *$p < 0.05$.

Our ongoing research programs being focused on therapeutic gene delivery to pulmonary tissues, we then explored the potential of the deciduous-charge lipids to transfect various pulmonary cell lines. Indeed, lung disorders such as cystic fibrosis, chronic obstructive pulmonary disease (COPD) and asthma are among the more representative causes of mortality and morbidity worldwide according to the World Health Organization (WHO) (http://www.who.int/respiratory/en/), and identifying powerful and cost-effective treatments is a matter of high priority. In this case, local pulmonary gene delivery may be especially advantageous as it reduces systemic side effects and do not lead to en-route interactions with serum proteins. As a significant consequence, the required dose of therapeutic nucleic acid can be substantially reduced. Depending on the respiratory disease and the therapeutic goal, the target cells in the lung can vary from epithelial cells to fibroblasts, macrophages or endothelial cells. Targeting epithelial cells is especially attractive as these cells constitute one of the first line of defense in the lung, and are affected in many chronic lung diseases. Furthermore, this cell type is among the ones that are directly accessible to nucleic acid-containing nanoparticles *via* inhalation or instillation. Nevertheless, in the airway, nanoparticles have to overcome specific non-cellular barriers, such as the mucus layer that protects the conducting airways from the nose to the terminal bronchioles, or the surfactant that covers the alveoli. Respiratory mucus has the physiological function to trap inhaled particles which are then transported by ciliated cells towards the oropharynx where they are expelled by the nose or swallowed (mucociliary clearance). Mucus is a viscoelastic layer that essentially consists in a three-dimensional network of cross-linked negatively charged glycosylated proteins (mucins) incorporating electrolytes, albumin, lysozyme, DNA and some other minor components (41). In addition to difficulties related to their diffusion through the viscoelastic mucus layer, positively charged lipoplexes may interact electrostatically with mucus components leading to surface charge neutralization (42), particle aggregation, and nucleic acid release (43). Considering these points, we conducted the following evaluation on three representative cell lines: A549 cells (alveolar, human lung carcinoma), Calu-3 cells (bronchial, epithelial lung adenocarcinoma), and NCI-H292

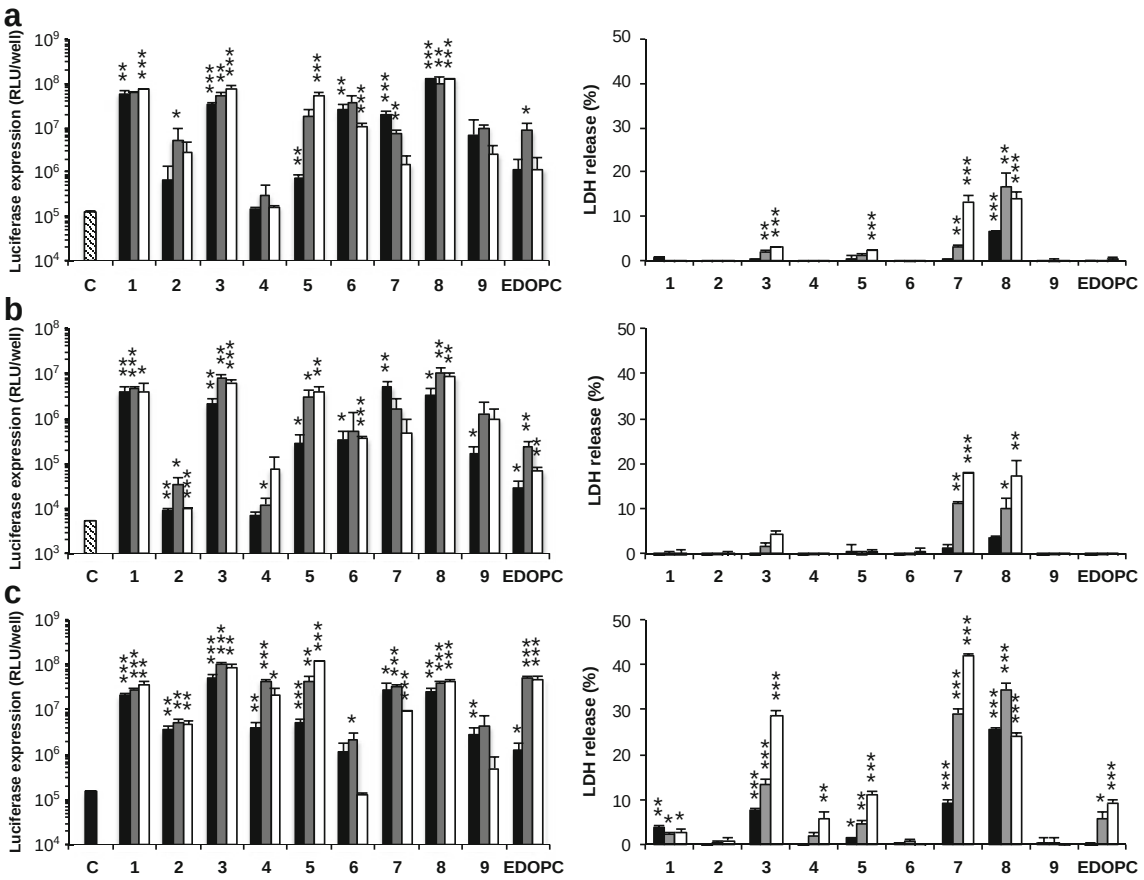


Fig. 5 Expression of luciferase (*left*) and LDH release (*right*) in A549 (**a**), Calu-3 (**b**), and NCI-H292 (**c**) cells treated with pCMV-Luc pDNA complexed with the deciduous-charge amphiphiles **1**-**9** or EDOPC, in the presence of 10% FBS. Lipoplexes were prepared at various N/P charge ratios (1.0: *black*; 3.0: *grey*; 5.0: *white*). Control (C) refers to basal luminescence measured in untreated cells. Basal LDH is set at 0%, and 100% represents the total LDH released after cell lysis. Data shown are representative of a triplicate determination (mean ± SD).

mucus-secreting cells (bronchial, human lung mucoepidermoid carcinoma). The transfection results obtained with the deciduous-charge cationic lipids are showed in Fig. 5. It can be observed that almost all these new derivatives are better candidates than reference EDOPC to deliver luciferase gene in respiratory cells though there are some differences from one cell line to the other. As previously observed on the BHK-21 cell line, the less robust cationic lipids **2** and **4** failed to promote transfection of A549 and Calu-3 cells but offered intermediate efficacy on the NCI-H292 cell line. More consistently, compounds **1**, **3**, and **8** systematically led to the higher cell transfection levels, whatever the cell line considered. In addition, transfection appears only poorly sensitive to the charge ratio with these compounds and maximum efficacy most of the time was achieved at N/P 1. At the opposite, transfection efficiency of compound **5** was improved from N/P 1 to N/P 5 by one to two orders of magnitude. Compounds **6**, **7**, and **9** offered good to intermediate performances. All in all, A549, Calu-3 and NCI-H292 cells were satisfactorily transfected by the title compounds. As NCI-H292 cells are mucus-secreting cells, our results indicate that mucus is not necessarily a critical barrier for efficient transfection with these carriers through the airway route. Interpretation of the data on the basis of structural features of the cationic lipids reveals that varying the length of the alkyl chain in the ester series (compounds **1**, **3**, and **5**) has no drastic effect on transfection efficiency except for the compound with the very short chain (compound **5**) which required a higher charge ratio for reaching maximum efficiency. There is also a general trend according to which introduction of an alkyl group on the ketal bridge in the ester series (**1** *vs.* **2**, and **3** *vs.* **4**) is definitely detrimental to the transfection efficiency of the compounds. On the opposite, in the carbonate series, increasing steric hindrance or hydrophobicity at this position (H, Me, *i*-Pr for **6** to **8**, resp.) revealed rather beneficial and improved transfection efficiency by up to two orders of magnitude in some cases.

In terms of cytotoxicity (Fig. 5, right), carbonates **7** and **8** that regularly stand as good transfection agents provoked significant LDH release in the different cell lines investigated. This effect was especially important with NCI-H292

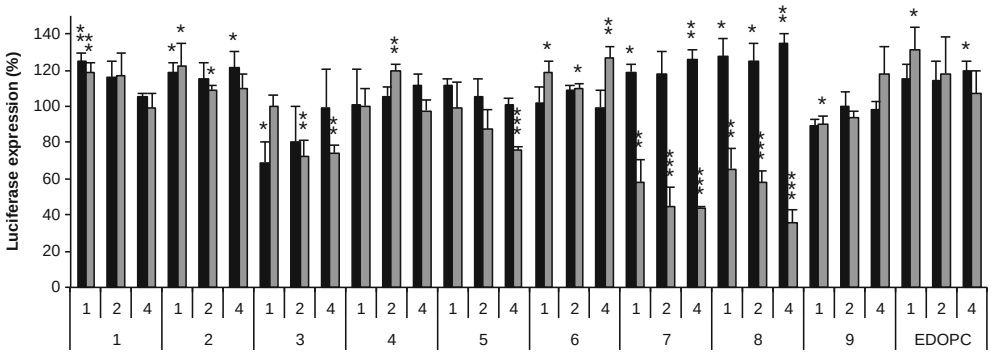


Fig. 6 Efficiency of cationic lipids **1**-**9**, as compared to reference EDOPC, to assist siRNA delivery in U87 cells that were stably transformed to express the luciferase protein. The culture medium contained 10% FBS. Each lipid was mixed with either untargeting (control) siRNA (sic: *black*) or luc-targeting siRNA (siLuc: *grey*) and added to cells. Luciferase activity was measured 48 h later and plotted relative to untreated cells (100% luciferase expression, data not shown). Lipoplex were prepared from 1.0 pmol siRNA and 1 nmol, 2 nmol, or 4 nmol of cationic lipid. Luciferase activity was measured as indicated in Methods. Data shown are representative of a triplicate determination (mean ± SD).

cells for which up to 30–40% LDH release was observed. NCI-H292 cells were invariably more sensitive to the cationic lipids investigated herein than the other cell lines. Whether this hypersensitivity might be related to mucus secretion or not was not investigated. Compounds **1** and **3** that displayed similar transfection efficiency were non cytotoxic (LDH < 5%), at least in the A549 and Calu-3 cell lines.

siRNA Delivery

Next, we examined the capacity of the cationic lipids **1**-**9** to deliver siRNA into cells. First, we used the U87 epithelial-like cell line (human glioblastoma-astrocytoma) that has been stably transformed to express a luciferase gene (U87Luc cells) (22). The delivery of a specific siRNA (siLuc) into the cytosol of these cells induces reduction of luciferase expression, when compared to a mismatched siRNA (sic) used as a negative control. The siRNA lipoplexes were added to the cells in culture medium and luciferase activity was measured after a 48 h incubation period. Knockdown of the luciferase expression in the U87Luc cells mediated by the nine deciduous-charge cationic lipids is shown in Fig. 6. Particularly interesting to note is that carbonate derivatives **7** and **8** were able to silence luciferase expression down to 40–30%, in a dose-dependent manner. On the other hand, derivatives **1** and **3** that revealed especially powerful to deliver pDNA into cells (*vide supra*) failed to efficiently deliver siRNA as the luciferase protein was nearly fully expressed (> 75%) when compared to the negative control. All the other compounds in the series revealed unable to significantly deliver siRNA to U87Luc cells, as it was the case for EDOPC. No explanation for these results could be obtained considering the size and zeta potential of the particles since all the lipoplexes did show comparable properties (ζ: between +54.3 and +64.0 mV; size: between 453.2 and 674.9 nm), except those prepared from **6** for which ζ was out of the previous range (+2.3 mV) (see Supplementary Material, Table S3). Cytotoxicity of the siRNA formulations was determined in parallel (Fig. 7). The formulations leading to substantial transfection activity systematically provoked measurable LDH release. However,

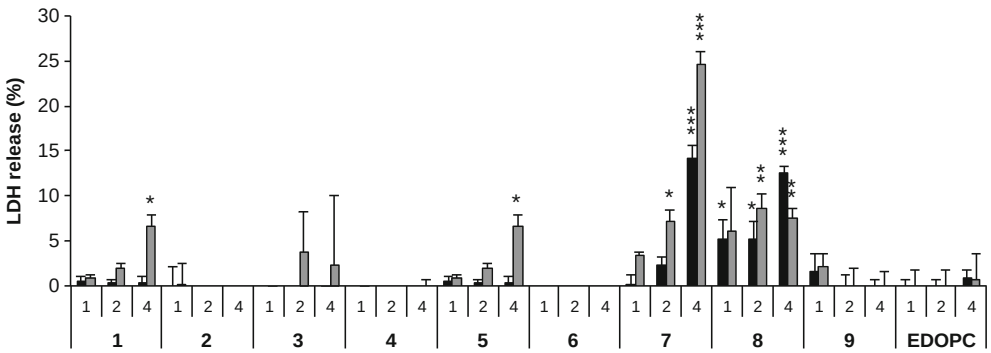


Fig. 7 Lipoplexes cytotoxicity on U87Luc cells as determined by the LDH release assay. Cytotoxicity was evaluated after 48 h incubation in the presence of lipoplexes prepared from 1.0 pmol of siRNA (sic: *black*; siLuc: *grey*), and increasing amount of lipid (1, 2, and 4 nmol, corresponding to N/P 25, 50, and 100, resp.). Basal LDH production is set at 0%, and 100% represents the total LDH released after cell lysis. Data shown are representative of a triplicate determination (mean ± SD).

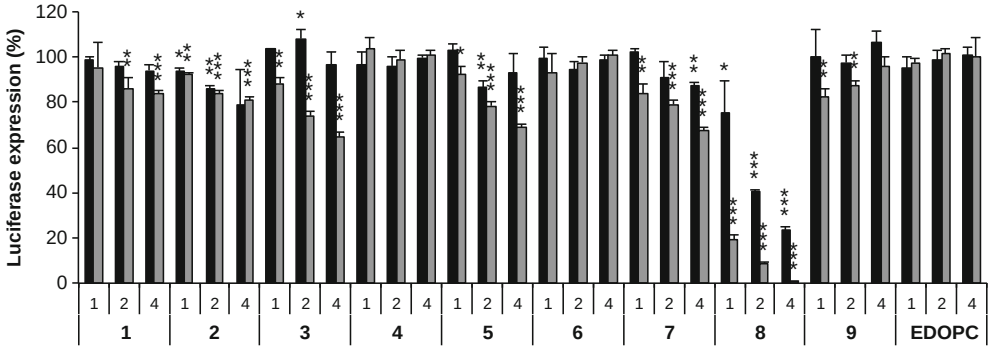


Fig. 8 Efficiency of cationic lipids **1**-**9**, as compared to reference EDOPC, to assist siRNA delivery in A549Luc cells. The culture medium contained 10% FBS. Each lipid was mixed with either untargeting (control) siRNA (*black*) or luc-targeting siRNA (*grey*) and added to cells. Luciferase activity was measured 48 h later and plotted relative to untreated cells (100% luciferase expression, data not shown). Lipoplex were prepared from 1.0 pmol siRNA and 1 nmol, 2 nmol, or 4 nmol of cationic lipid (corresponding to N/P 25, 50, and 100, resp.). Luciferase activity was measured as indicated in Methods. Data shown are representative of a triplicate determination (mean ± SD).

except with the carbonate compound **7** at the higher dosage, this release was less than 5–8% which can be considered as negligible.

Still with the view of a possible use of the deciduous-charge cationic lipids to deliver therapeutic nucleic acid to airway epithelium, we finally evaluated the ability of the compounds to help the delivery of siRNA in alveolar cells. A549Luc cells (23) were thus treated with sic and siLuc lipoplexes and luciferase expression was measured (Fig. 8) together with LDH release (Fig. 9). Similarly to what was observed on U87Luc cells, compound **8** proved to be the most effective cationic lipid in the series in delivering siRNA to the alveolar cells. More than 80% luciferase knockdown was achieved at the lower N/P ratio, with only reduced cytotoxicity. Increasing the amount of cationic lipid resulted in improved luciferase silencing but was however detrimental to cell viability as more than 15% LDH release was measured at the intermediate charge ratio. Cationic lipid **7** that had comparable efficiency to that of **8** on U87Luc cells was only poorly active on A549Luc cells. On the other hand, lipid **3** had some statistically significant activity on the alveolar cells what was not observed on U87Luc cells. From the results obtained on the two different cell lines, it may be tentatively concluded that the more effective deciduous-charge cationic lipids for siRNA delivery are the carbonate-based compounds.

CONCLUSION

A series of new cationic lipids resulting from the conjugation of DOPC with alcohols and carboxylic acids through a biodegradable linker have been developed to enhance endosome escape of lipoplexes during nucleic acid delivery. Hydrolytic stability of the compounds was evaluated when formulated into liposomes and lipoplexes. Interestingly, respective hydrolysis rates not only depend on the structure of the biodegradable spacer and on the incubation conditions, but also on the type of supramolecular assembly. For compound **7** especially, hydrolysis was faster in liposomes than in lipoplexes suggesting that DNA might modify hydration

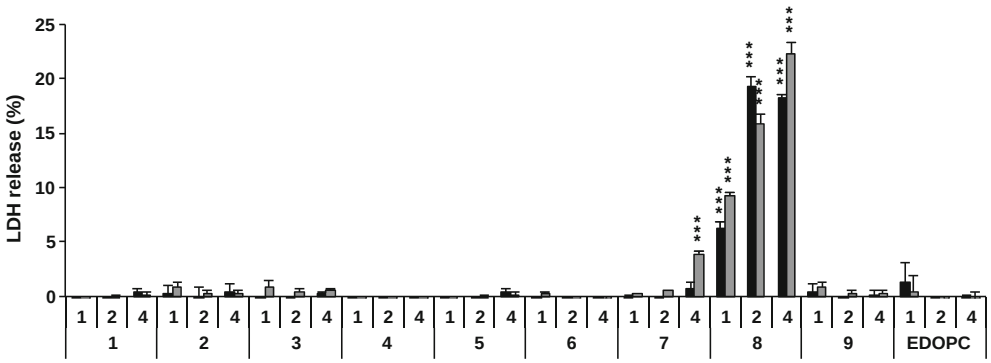


Fig. 9 Lipoplexes cytotoxicity on A549Luc cells as determined by the LDH release assay. Cytotoxicity was evaluated after 48 h incubation in the presence of lipoplexes prepared from 1.0 pmol untargeting (control) siRNA (*black*) or luc-targeting siRNA (*grey*) and increasing amount of lipid. Basal LDH production is set at 0%, and 100% represents the total LDH released after cell lysis. Data shown are representative of a triplicate determination (mean ± SD).

of the lipid polar headgroup thus reducing accessibility to hydrolysis reagents from the aqueous media. Transfection efficiency of the compounds has been evaluated in various cell lines. All together the findings presented herein show that most of the DOPC cationic derivatives efficiently assist transfection of cells with pDNA. Furthermore, the so-called deciduous-charge amphiphiles offer transfection efficiency enhanced by up to a *ca.* 60-fold factor when compared to the parent compound EDOPC. Of special interest is compound **1** that consistently proved more efficient than the other compounds evaluated herein for delivering pDNA into cells, though forming negatively charged transfection particles. This might represent a major advantage for *in vivo* transfection, giving the opportunity to reduce fatal recruitment of negatively charged serum proteins through electrostatic interactions. Finally, compounds **7** and **8** proved potent as well for delivering siRNA into cells, provoking up to 80% of gene knockdown, whereas the other deciduous-charge compounds and EDOPC were totally inactive. The question as to why the compounds behave so differently remains partly unanswered. However it appears that efficiency of the compounds does not correlate with their hydrolytic susceptibility in buffered saline solution, under model conditions as those used herein. Data on the intracellular degradation of the compounds failed to provide a clear picture of the input of the biodegradability on the transfection efficiency. Whether the by-products (other than DOPC) formed upon lipid intracellular degradation may be involved in the course of the transfection process was not checked and might provide additional insight for better comprehension of the activity of the deciduous-charge cationic lipids described herein. However, the high water solubility of these by-products probably would make very complicated the investigation of this hypothesis in so far as modelling and control of their local distribution appear highly unreliable.

ACKNOWLEDGMENTS AND DISCLOSURES

This work was supported by the Agence Nationale pour la Recherche (ANR, France).

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