Cationic Lipophosphoramidates Containing a Hydroxylated Polar Headgroup for Improving Gene Delivery

ARTICLE *in* MOLECULAR PHARMACEUTICS · MAY 2015

Impact Factor: 4.38 · DOI: 10.1021/mp500807k · Source: PubMed

molecular pharmaceutics

Article

pubs.acs.org/molecularpharmaceutics

Cationic Lipophosphoramidates Containing a Hydroxylated Polar Headgroup for Improving Gene Delivery

Mathieu Berchel,*[,†,⊥] Tony Le Gall,[‡,⊥] Jean-Pierre Haelters,[†] Pierre Lehn,[‡] Tristan Montier,[‡,⊥,§,∥] and Paul-Alain Jaffrès[†,⊥]

[†]CEMCA, CNRS UMR 6521, SFR ScInBioS, Université Européenne de Bretagne, Université de Brest, Brest, France

[‡]Unité INSERM 1078, SFR ScInBioS, Université de Bretagne Occidentale, Université Européenne de Bretagne, Faculté de Médecine et des Sciences de la Santé, 22 avenue Camille Desmoulins, 29218 Brest, France

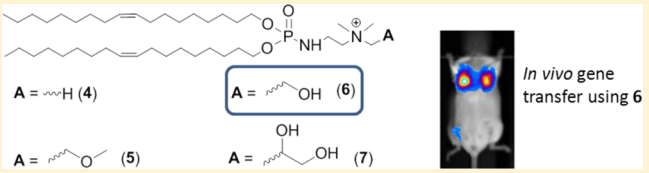
[§]CHRU de Brest, Service de Génétique Moléculaire et d'Histocompatibilité, 5 avenue du maréchal Foch, 29609 Brest Cedex, France

[∥]DUMG, Faculté de Médecine et des Sciences de la Santé, 22 avenue Camille Desmoulins, 29238 Brest, France

[⊥]Plateforme IBiSASynNanoVect, SFR ScInBioS, Université de Bretagne Occidentale, Université Européenne de Bretagne, 29238 Brest, Cedex 3, France

Supporting Information

ABSTRACT: The structure of the cationic moiety of amphiphiles is a key factor which directly influences their transfection efficacy. Accordingly, in the present work, we have synthesized three new lipophosphoramide-based amphiphilic compounds incorporating a methoxy **5**, hydroxyl **6**, or dihydroxyl **7** functional group in their cationic part. Gene delivery efficacies of these novel vectors were compared to our benchmark compound, the arsenolipophosphoramidate **KLN47**, and to its trimethylammonium (TMA) analogue **4**. We next studied the characteristics (size, ζ potential) of the nanometric assemblies formed (liposomes and lipid/DNA complexes), and the DNA binding ability of the cationic liposomes was characterized at the physicochemical level. *In vitro*, all of the cationic lipids evaluated were efficient not only to condense plasmids but also to transfect two types of human airway epithelial cells. Interestingly, *in vivo* administration to mice (via simple tail vein injection) showed that compound **6** was the most efficient in transfecting the lungs when compared to that of the other cationic lipids studied, including compound **KLN47**. All of these results suggest that a hydroxyethyldimethylammonium (HE-DMA) polar head could be a valuable alternative to a trimethylarsonium (TMAs) polar head and that they also invite further evaluation of the *in vivo* potential of compound **6** using more clinically relevant delivery procedures.



KEYWORDS: *amphiphiles, cationic lipids, DNA, gene delivery, liposomes*

INTRODUCTION

The treatment of inherited or acquired disorders by means of the delivery of a therapeutic gene to cells or tissues is dependent on the use of a carrier that can efficaciously and precisely transfect the genetic construct into the target cells. On the one hand, viral carriers are currently the most efficient systems for clinical applications. However, their use is impaired by drawbacks such as immunogenicity and oncogenicity, cost of production, and limited size of the nucleic acid sequence. On the other hand, chemical gene delivery systems (mostly cationic amphiphiles either lipidic or polymeric) have been extensively studied as promising alternatives for clinical purposes, for example, for the treatment of cystic fibrosis or cancers. Synthetic vectors have a series of beneficial features such as safety, ease of large-scale preparation at low cost, and ability to transfer larger DNA constructs.[1] In spite of the fact that numerous artificial cationic systems have been developed, the need of efficient nonviral gene carriers is still on demand. The efficiency of the delivery of genetic material by lipoplexes (aggregates of cationic lipid and pDNA) is strongly related to their chemical structures and their physical properties.[2,3] With the aim to improve the effectiveness of lipoplex-mediated delivery, many strategies which consist of modulating the molecular structure of cationic lipids were established during the last several years.[4] Basically, the molecular structure modifications have concerned the three building blocks of cationic lipids: (i) modification of the hydrophobic domain (size, nature, or symmetry),[5,6] (ii) modulation of the polar region mainly by the replacement of the common quaternary ammonium by guanidinium,[7] polyamines, and heterocycles (pyridinium and imidazolium),[8−10] trimethylphosphonium (TMP), or peptides,[11] and (iii) variation in the linker motif used to join the hydrophobic and hydrophilic moieties (ester, ether, amide, carbamate, phosphate, redox, or pH sensitive bonds).[12,13]

Received: September 17, 2014
Revised: April 29, 2015
Accepted: May 5, 2015
Published: May 5, 2015

DOI: 10.1021/mp500807k
Mol. Pharmaceutics 2015, 12, 1902−1910

Over the last years, our group has synthesized bioinspired cationic amphiphiles[14] (cationic lipophosphoramidates,[15] lipothiophosphoramidates,[16] and lipophosphates[17,18]) and helper lipids[19,20] which have demonstrated high transfection efficacies both in cell culture assays and in mouse experiments. Interestingly, the structure of these cationic amphiphiles could be easily tuned to include some functionalities that aimed to enhance transfection efficacies[5,21,22] or to provide some additional properties (e.g., fluorescence[23,24] and antibacterial action[25]). We previously found that modulation of the structure of the cationic building block, e.g., substitution of a trimethylammonium (TMA) by a TMP or a trimethylarsonium (TMAs) group, led to improvement of the transfection efficacies,[26] whereas the relative toxicity was reduced.[27,28] These observations were next confirmed by us and more recently by others.[29]

Regarding the biological properties of our TMAs-based vectors (transfection and antibacterial properties), we have recently suggested that the size of the cationic headgroup might have a key role; indeed, this parameter might directly impact the stability of the dipolar interactions in which the cationic moiety can be involved.[22] Accordingly, the high transfection efficacies observed with the TMAs-based vectors can be seen as a good compromise between the stability of the lipoplexes required during their extracellular trafficking (e.g., in the systemic circulation) and the desired instability after cell internalization in order to allow dismantling of the lipoplexes and release of the nucleic acid. Herein, we hypothesized that the functionalization of a TMA polar head could modulate the strength of interactions with anionic partners (e.g., phosphate groups of nucleic acid). We chose to direct our efforts toward the replacement of the cationic TMAs moiety of some lipophosphoramidate-based lipids by an ammonium group bearing a hydroxyethyl (HE) group. This choice was motivated by results from the literature. Indeed, incorporation of hydroxyl moieties into the positively charged constituent part of ammonium-based lipids was first investigated by Felgner and colleagues[30] who reported that DORIE (which has oleyl chains) and DMRIE (with tetradecyl chains) were more efficient than DOTMA (the analogue with a TMA polar head). Bennett et al. have also shown that lipids with a hydroxyl group in their cationic part were better than their methyl ether analogues for mouse lung transfection by intratracheal (i.t.) instillation.[31] More recently, Ding and colleagues reported that some cholesterol-based vectors with a cationic moiety bearing a HE group efficiently transfected the lungs of mice via i.t. instillation unlike their methyl ether analogues, while the contrary was observed in *in vitro* experiments.[32] All of these previous results led us to investigate the incorporation of a HE group in the polar region of lipophosphoramidates possessing a cationic ammonium moiety. Thus, in the present work, we undertook to synthesize three new cationic lipids characterized by a polar head bearing an ammonium group functionalized with one or two hydroxyl groups or a methoxyethyl (MOE) group. These lipids were formulated as lipoplexes and their gene transfer activity was evaluated *in vitro* (using human cell lines) and *in vivo* (following intravenous injection in mice). A luciferase-expressing plasmid (pDNA) was used to monitor the gene transfer efficacy in those different experiments. The TMA and TMAs analogues were also included for comparison purposes.

EXPERIMENTAL SECTION

Chemicals. Solvents and chemicals were purchased from Sigma-Aldrich. Molecular analyses were performed by mass spectrometry (BrukerAutoflex MALDI TOF-TOF III, LRF200CID) and NMR (^{1}H, ^{31}P, and ^{13}C) recorded on Bruker 500 MHz (Avance DRX500) or Bruker 400 (Avance DRX 400). Lipophosphoramidates **KLN47**, **2**, and **3** were synthesized as previously described.[33]

Synthesis of 2-(*O*,*O*-Dioleylphosphoramidoyl)-ethyltrimethylammonium (4). Compound **3** (0.75 mmol, 0.5 g) was dissolved in anhydrous dichloromethane (5.0 mL), and combined with methyl iodide in excess (212 mg, 1.50 mmol, 93 μL). The mixture was then agitated at RT overnight. Next, concentration of the crude product under reduced pressure led to a white waxy solid (608 mg, with a yield of 98%). Results from NMR analyses were as follows: (i) ^{1}H NMR (400 MHz, $CDCl_3$), δ 0.84 (t, 6H, J = 8.0 Hz), 1.23−1.28 (m, 44H), 1.66 (m, 4H), 1.97 (m, 8H), 3.46 (s, 9H), 3.84 (t, 2H, J = 6.0 H), 3.86 (m, 2H, J = 6.0 Hz),3.97 (m, 4H, ^2J = 8.0 Hz^3J = 6.0 Hz), 4.40 (bs, 1H), 5.32 (m, 4H). (ii) ^{31}P NMR (162 MHz, $CDCl_3$): δ 8.7 (s). (iii) ^{13}C NMR (300 MHz, $CDCl_3$): 13.5, 22.1, 24.8, 26.6, 28.7−32.8, 35.5, 54.1, 65.9, 66.5−66.7, 129.1−129.8. Finally, high resolution mass spectrometry (HRMS, MALDI TOF *m/z*) showed for $C_{41}H_{84}N_2O_3P$: calcd, 683.621 $[M]^+$; found, 683.581.

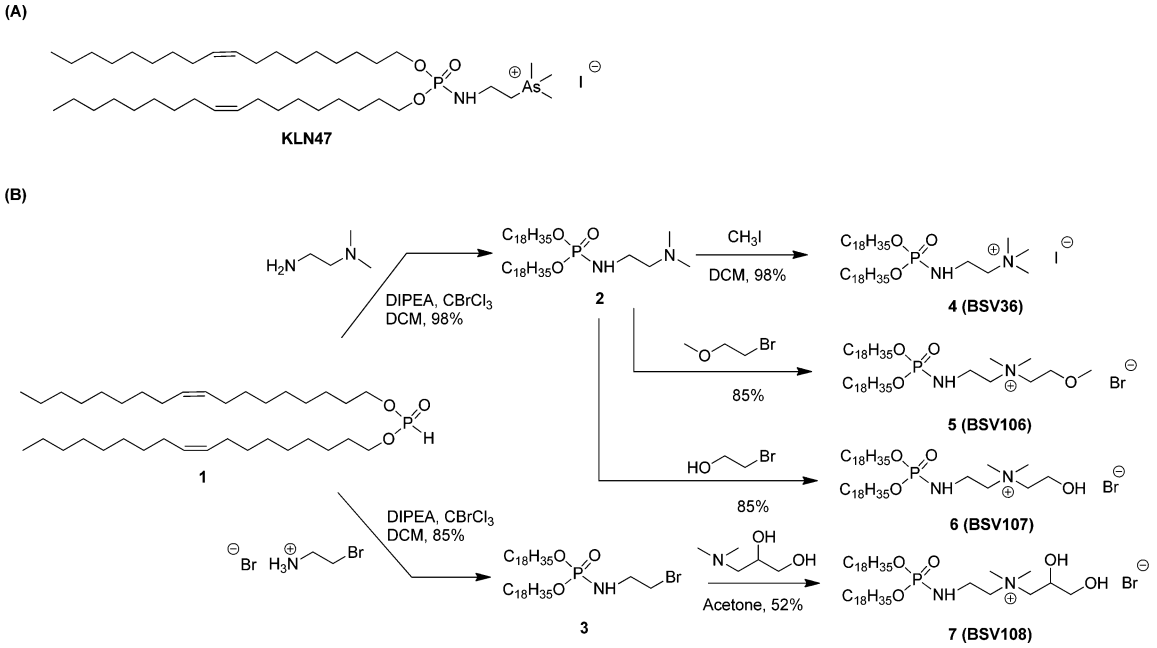
Synthesis Pathway of 2-(*O*,*O*-Dioleylphosphoramidoyl)-ethyl-3,3-dimethyl-3-ethylmethyl Ether Ammonium Bromide (5). Compound **3** (1.0 g, 1.72 mmol) and a large excess of 2-bromoethyl methyl ether (3 mL) were put into a Schlenk tube, and the combination was allowed to reflux overnight under nitrogen atmosphere. Excess of 2-bromoethyl methyl ether was removed under reduced pressure. The residue was purified using flash silica gel column chromatography (SGCC) with DCM/MeOH (0−10%) to obtain a colorless oil (1.0 g, 85% yield). Analytical data: (A) NMR, (i) ^{1}H NMR (400 MHz, $CDCl_3$), δ 0.87 (t, 6H, J = 8.0 Hz), 1.26 (m, 44H), 1.61 (m, 4H), 1.97 (m, 8H), 3.38 (s, 3H), 3.42 (s, 6H), 3.58 (m, 2H), 3.92 (m, 4H), 3.96 (m, 2H), 3.98 (m, 4H), 4.79 (m, 1H), 5.35 (m, 4H). (ii) ^{13}C NMR (300 MHz, $CDCl_3$): 14.1, 22.7, 25.6, 27.2, 29.2−29.7, 30.4, 31.9, 32.6, 36.1, 53.1, 59.2, 64.3, 65.8, 66.3, 67.0, 129.7−129.9. (iii) ^{31}P NMR (162 MHz, $CDCl_3$): δ 9.0 (s). (B) HRMS (see above) for $C_{43}H_{88}N_2O_4P$: calcd, 727.648 $[M]^+$; found, 727.641.

Synthesis Scheme for 2-(*O*,*O*-Dioleylphosphoramidoyl)-ethyl-3,3-dimethyl-3-hydroxyethylammonium Bromide (6). Compound **3** (1.0 g, 1.72 mmol) and a large excess of 2-iodoethanol (3 mL) were placed in a Schlenk tube, and the mix was next subjected to heating at reflux for 2 days under nitrogen atmosphere. The use of low pressure allowed then to remove the excess of 2-bromodoethanol. The residual substance was finally purified by flash SGCC (DCM/MeOH; 0−10%MeOH) to obtain an uncolored oil (1.0 g, 85%). Analytical data: (A) NMR, (i) ^{1}H NMR (400 MHz, $CDCl_3$), δ 0.85 (t, 6H, J = 8.0 Hz), 1.22 (m, 44H), 1.59 (m, 4H), 1.92 (m, 8H), 3.34 (s, 6H), 3.35 (m, 2H), 3.59 (m, 4H), 3.88 (m, 4H), 4.02 (m, 2H), 4.81 (m, 1H), 5.03 (bs, 1H), 5.26 (m, 4H). (ii) ^{13}C NMR (300 MHz, $CDCl_3$): 14.1, 22.6, 25.5, 27.2, 29.2−32.6, 35.8, 53.2, 55.9, 65.1, 66.4, 67.1−67.2, 129.6−129.9. (iii) ^{31}P NMR (162 MHz, $CDCl_3$): δ 9.9 (s). (B) HRMS for $C_{42}H_{86}N_2O_4P$: calcd, 713.633 $[M]^+$; found, 713.499.

Synthesis of 2-(*O*,*O*-Dioleylphosphoramidoyl)-ethyl-3,3-dimethyl-2,3-dihydroxypropyl Ammonium Bromide

DOI: 10.1021/mp500807k
Mol. Pharmaceutics 2015, 12, 1902−1910

Scheme 1. (A) Molecular Structure of Compound KLN47 and (B) Synthetic Routes to Cationic Lipophosphoramides 4, 5, 6, and 7



(7). Compound **3** (500 mg, 0.71 mmol) and 3-(dimethylamino)-1,2-propanediol (85 mg, 0.71 mmol) in anhydrous acetone (5 mL) were put into a Schlenk tube. The mixture was placed under N_2 atmosphere and heated at reflux overnight. After a concentration step, the crude substance was purified by flash SGCC (DCM/MeOH ; 0−10% of MeOH) to afford a colorless waxy solid (300 mg, 52% yield). Analytical results: (A) NMR, (i) ^{1}H NMR (400 MHz, $CDCl_3$), δ 0.85 (t, 6H, J = 8.0 Hz), 1.24−1.27 (m, 44H), 1.61−1.63 (m, 4H), 1.95−1.99 (m, 8H), 3.42 (s, 6H), 3.67 (m, 2H), 3.68 (m, 2H), 3.74 (m, 2H), 3.91−3.96 (m, 4H), 3.99 (m, 1H), 4.40 (m, 1H), 4.73 (m, 1H), 5.28 (m, 4H). (ii) ^{13}C NMR (300 MHz, $CDCl_3$): 14.0, 22.5, 25.5, 27.1, 29.1−30.3, 31.8, 32.5, 35.7, 43.7, 50.2, 59.2−53.1, 64.2, 65.2, 66.3, 67.0, 67.4, 129.6−130.3. (iii) ^{31}P NMR (162 MHz, $CDCl_3$): δ 8.9 (s). (B) HRMS for $C_{43}H_{88}N_2O_5P$: calcd, 743.643 $[M]^+$; found, 743.682.

Liposomal Solutions. The liposomal solutions were prepared by hydration of a lipid film as previously reported[17] (hydration for 12 h at 4 °C; vortex during 1 min; sonication 3 times for10 min each). The concentration of the final liposomal solution was 1.0 mM for cell culture experiments and 10 mM for experiments with mice.

DNA. We utilized the plasmids pGL3-Ctrl (5.6 kb; Promega), pEGFPLuc (6.4 kb; Clontech), and pGM144 (3.7 kb; UK CF gene therapy consortium)[34] for conducting physicochemical studies, *in vitro* transfection experiments, and *in vivo* transfection experiments, respectively. These different plasmids were produced and purified as previously reported.[6,12,15,17,18,20,21,25]

DNA Compaction. The ability of the various cationic lipids to condense pDNA was investigated by exploiting the property of DNA to fluoresce when it is mixed with ethidium bromide (EtBr). The DNA condensation capacity of any cationic lipid does indeed correlate with its ability to expel EtBr from it, which results in a fluorescence decrease.[6,12,15,17,18,20,21,25] In addition, for each cationic lipid to be studied, various quantities were mixed with a fixed amount of pDNA, and then DNA retardation (due to the electrostatic interaction between DNA and cationic lipid) was assessed by electrophoresis (1% agarose gel).

Cells. For *in vitro* gene transfection experiments (see below), we used the A549 cell line (alveolar type II cells derived from human lung cancer cells: ATCC No. CCl-185) as well as 16HBE 14o-cells (immortalized human bronchial epithelial cells).[35] Both cell lines were grown and maintened as usually recommended.[6,12,15,17,18,20,21,25]

Gene Transfer into Cell Lines. The *in vitro* transfection properties (including efficiency and cytotoxicity) were determined using procedures that we as well as others have previously published.[6,12,15,17,18,20,21,25,36] Transfection efficiencies were measured in RLU per mg of total cell protein. Cell viability was assessed by the use of the Vialight kit (Lonza) as recommended by the producer. Data were reported in %, relative to control (i.e., nontransfected) cells.

Animals. For *in vivo* gene transfer experiments (see below), female Swiss mice (6 to 9-weeks old) were obtained from Janvier Breeding Center (Le Genest-Saint-Isle, France) and housed at the animal facility of Brest University. Experiments were performed in accordance with the ARRIVE guidelines (NIH publication #85-23, revised 1985)[37] and also with the agreement of the regional veterinary services (authorization FR; 29-024).

***In Vivo* Gene Transfer in Mice.** The procedures used were as we have previously described,[15,20,21] with some modifications. Briefly, lipid/DNA complexes were formed at RT in isotonic saline at a charge ratio of 4 (CR4). Each animal received (via its tail vein) 200 μL of complexes, incorporating 25 or 50 μg (see below) of the luciferase-expressing plasmid pGM144. Next, from 24 h and then each day up to day 4 after injection, noninvasive bioluminescence imaging was performed in living anesthetized animals (NightOwl II imaging system, Berthold). Bioluminescence emission, expressed in photons/pixel/s, was quantified within the areas of interest. Concerning the evaluation of side-effects, we undertook to measure the

DOI: 10.1021/mp500807k
Mol. Pharmaceutics 2015, 12, 1902−1910

activities of the liver enzymes ALT and AST in the mouse plasma collected at 24 h as well as a few days after administration. These *in vivo* toxicity measurements were performed using the Elitech kit (Elitech) together with the Selectra E apparatus (Elitech), according to the producer's protocol, the results being expressed in IU/L.

Statistical Test. The Mann–Whitney–Wilcoxon (U) test was used to statistically compare the transfection efficiency of our lipids. Differences were considered significant for $\alpha < 5\%$ (nondirectional test).

RESULTS AND DISCUSSION

Synthesis of Cationic Lipids. Lipophosphoramidate **KLN47** was used as a reference and prepared as previously described.[33] The synthetic routes to prepare the cationic lipophosphoramidates **4** (BSV36), **5** (BSV106), **6** (BSV107), and **7** (BSV108) are depicted in Scheme 1. The synthesis was performed by using a three-step procedure starting from *O*,*O*-dioleylphosphite **1**. The first step consisted in the formation of the neutral lipophosphoramidate **2**, which was synthesized from **1** and *N*,*N*-dimethylethylenediamine under mild and efficient conditions according to the Atherton–Todd reaction.[38] Next, the tertiaryamine was quaternized with methyl iodide, 2-bromoethyl methyl ether, or bromoethanol to obtain the cationic amphiphiles **4**, **5**, and **6**, respectively, in nearly quantitative yields. The compound **7** was synthesized using a similar procedure from phosphite **1** and bromoethylamino-phosphoramidate followed by quaternization with the commercially available 3-(dimethylamino)-1,2-propanediol to furnish the bis-hydroxy derivative **7** in modest yield. Finally, the new synthesized compounds were purified and fully characterized as indicated in the Experimental Section.

Formulation and Characterization of Liposomes and Lipid/pDNA Complexes. Liposomal solutions of cationic lipid **KLN47** and compounds **4**, **5**, **6**, and **7** studied herein were obtained as outlined above in the Experimental Section. The biophysical characteristics of these liposomal formulations (size, ζ potential) were studied using, respectively, DLS, and electrophoresis. Their size was in the same range as that observed for the arsenolipophosphoramidate **KLN47** (90–160 nm), except for the liposomes prepared from compound **5** (with a methoxyethyl (MOE) group in the polar head) which exhibited a larger particle size (351 nm). The polydispersity indexes indicated a rather narrow distribution (see Table 1, Supporting Information). With respect to the zeta potential measurements, all compounds studied (**KLN47**, **4**, **5**, **6**, and **7**) exhibited the same range of values with a net positive charge as expected for cationic lipids (+30 to + 47 mV). Next, we also investigated the same characteristics for the cationic lipid/pDNA complexes studied, these assemblies being formed at (±) CRs of 0.5, 1.0, 2.0, 4.0, 6.0, and 8.0 by adding pDNA (20 μg) to a variable volume of liposomal solution in sterile water. The theoretical (±) CR is defined as the ratio of positive charges to negative charges provided, respectively, by the cationic lipid and the phosphate groups of the pDNA (with 1 μg of DNA corresponding to 3 nmol of negative charges, whereas each cationic lipid molecule contributes one permanent positive charge). As shown in Supporting Information, the sizes of the lipoplexes varied between 150 to 455 nm. Such size heterogeneity might be related to the structure of the lipid–pDNA assembly.[39] As expected, the zeta potential was negative for CR 0.5 and 1.0, whereas it became clearly positive for CR ≥ 2.0. No significant difference was observed between the lipoplexes whatever the structure of the cationic moieties of our lipids.

DNA Condensation Assay. Ability of compounds **4**, **5**, **6**, and **7** to condense pDNA and to form lipoplexes was assessed by ethidium bromide exclusion and pDNA retardation assays at different CRs (0.5 to 8.0) (Figure 1). The agarose gel

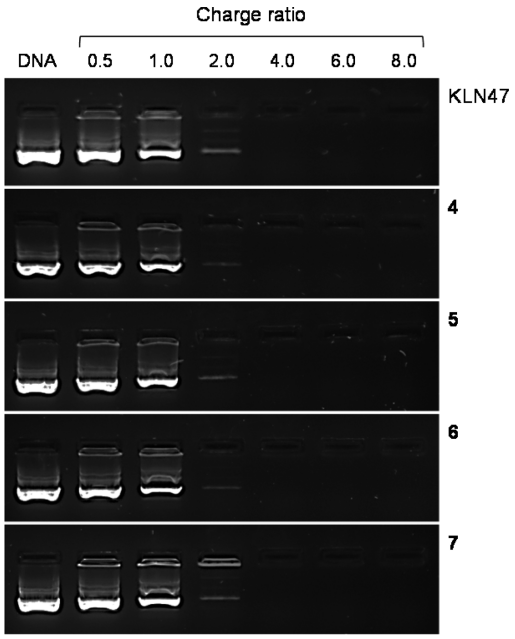


Figure 1. DNA condensation ability of cationic lipids. Lipoplexes were formed at CR ranging from 0.5 to 8.0 by mixing a fixed quantity of pDNA with augmenting amounts of a given compound. Next, DNA retardations were assessed by agarose gel electrophoresis.

electrophoresis retardation assays showed that all compounds did efficiently compact pDNA at CR 2.0 or above since a strong decrease in fluorescence was noticed from that CR. The absence of free pDNA was observed in each case at CR 4.0. Overall, all of the compounds studied yielded very similar pDNA retardation profiles. These data show that the structure of the cationic polar heads used herein did not induce a significantly different behavior with respect to the compaction of pDNA.

***In Vitro* Transfection Assay.** We thereafter examined the efficiency of gene transfection into cultured cells of lipoplexes characterized by different CRs (0.5 to 8.0). The above pDNA retardation assays suggested that the CR 2.0 and 4.0 were the best conditions for transfection. Because our research efforts are chiefly directed toward treatment of the pulmonary complications of cystic fibrosis (CF), we decided to work here with two cell lines derived from human airway epithelial cells, i.e., the A549 and 16HBE 14o-cell lines. As **KLN47** has been shown to efficiently transfect both cell lines, it was used here as a benchmark.[22] Regarding the transfection ability of **KLN47**, **4**, **5**, **6**, and **7**, we first explored the impact of the lipoplex CR on gene transfer efficiency since lipofection is supposed to require interactions between positive lipoplexes and negative plasma membrane residues. As shown in Figure 2, all of the cationic formulations studied mediated significant luciferase levels in both cell lines. The best CR was either 2.0 or 4.0, and only weak differences were observed between these two CRs (Figure 2). However, it is noteworthy that, for A549 cells, lower luciferase levels were obtained, especially for our reference

DOI: 10.1021/mp500807k
Mol. Pharmaceutics 2015, 12, 1902–1910

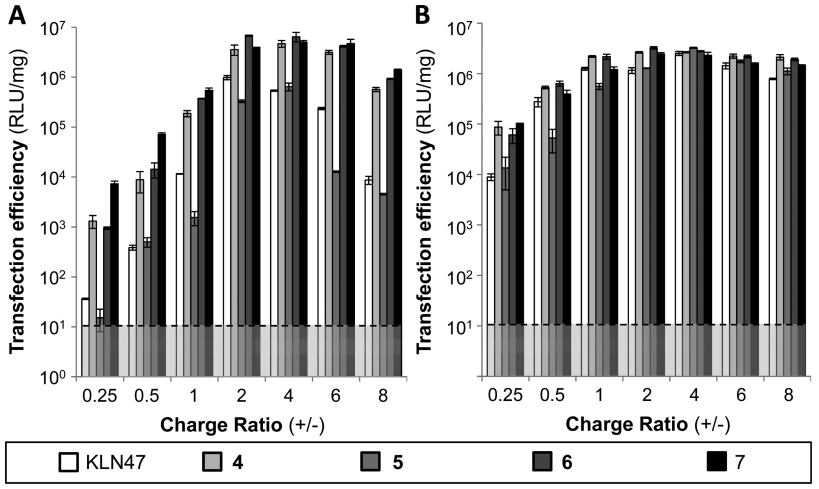


Figure 2. *In vitro* gene transfection efficiency into A549 (A) and 16HBE 14o$^-$ cells (B) of lipoplexes formed at different CRs. Transfection was performed as indicated in the Experimental Section. Luciferase levels were measured at 48 h after deposition of the lipoplexes onto cells. Transfection efficiency is given as RLU/mg of total protein, with a dashed line indicating the positivity threshold (mean with n = 3).

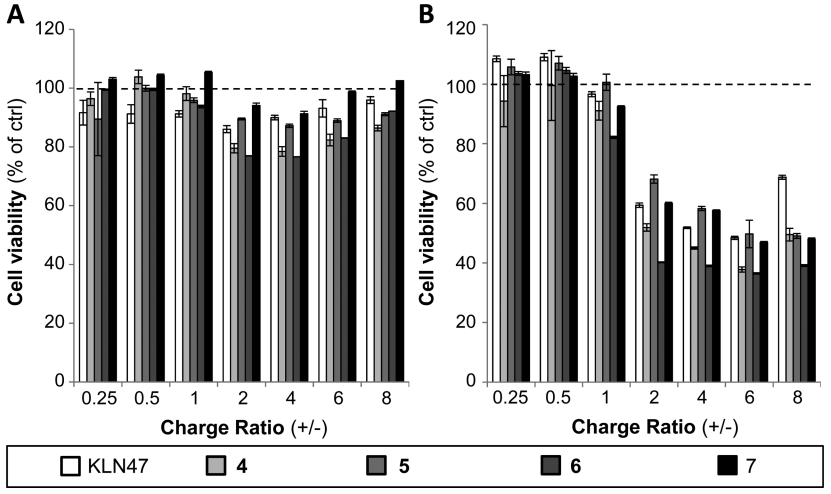


Figure 3. Viability of A549 (A) and 16HBE 14o$^-$cells (B) following transfection with lipoplexes formed at different CR. Untransfected cells were used as controls (100% viability; dashed line) (mean with n = 3).

vector **KLN47** and the MOE-bearing compound **5**. Additionally, the transfection efficacies of these two lipids strongly decreased at higher CRs (CR 6.0 or 8.0). Besides, compounds **4**, **6**, and **7** exhibited the higher transfection efficacies whatever the cell line and the CRs tested. Altogether, these results indicate that the functionalization of a TMA polar headgroup by the substitution of one methyl group in compound **4** with one HE group (**6**) slightly improved the transfection efficacy at the optimal CRs. Moreover, with one cell line, the cationic lipid **6** was almost 10-fold more efficient than our benchmark compound **KLN47**, which bears a TMAs polar head (statistically significant difference assessed using the Mann−Whitney−Wilcoxon test).These results are interesting since TMAs-based cationic lipids are much more expensive to produce than their ammonium equivalents.

Cytotoxicity. We also performed measurements of viability of the cultured cells to assess the cytotoxicity of gene transfer using the compounds studied herein. Thus, we evaluated the cytotoxicity of the cationic lipids **KLN47**, **4**, **5**, **6**, and **7** by measuring the ATP content of the cells. As shown in Figure 3, the cellular toxicity appeared to be dose-dependent, as it increased with the CR and thus with the amount of cationic lipid used. However, it was also dependent on the cell line. Indeed, a cell viability around 80% was measured for the A549 cell line, whereas the16HBE cells appeared to be more sensitive to transfection since at CR > 1.0, the viability dropped to about 40−50%. Although no significant difference was highlighted between the cationic lipids at low CR, the most toxic cationic lipid was compound (**4**) with the TMA headgroup. The introduction of one HE group into the polar head moiety (**6**) slightly reduced the toxicity, whereas the incorporation of a DHPr group (**7**) produced one of the less toxic derivatives in the present series of compounds. In agreement with previous studies, the benchmark compound **KLN47** belongs to the less toxic cationic lipids.[26] This strongly suggests, as we have already hinted to elsewhere,[22] that the use of a hindered cationic polar head (by the functionalization of an ammonium cationic group or the use of a bigger atom such as arsonium instead of nitrogen) was an effective strategy to decrease the cytotoxicity.

In vivo transfection experiments were performed in Swiss mice by intravenous (i.v.) injection. The cationic lipid **KLN47** was again used here as reference. We have indeed previously reported that, when injected via the tail vein, lipophosphoramidate-based lipoplexes can allow luciferase reporter gene

DOI: 10.1021/mp500807k
Mol. Pharmaceutics 2015, 12, 1902−1910

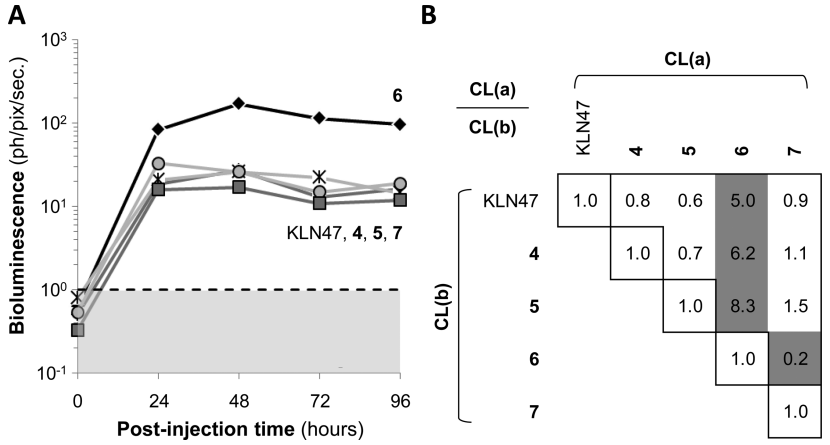


Figure 4. *In vivo* bioluminescence in the mouse following intravenous injection of the different types of lipid/pDNA complexes, the lipoplexes having been formed at CR4 and incorporating 50 μg of pDNA. (A) Kinetics of *in vivo* bioluminescence. Signals are expressed in the ph/pix/s unit. A dashed line indicates the threshold of positivity. (B) Pairwise comparisons of bioluminescence. Each value corresponds to the ratio of mean intensities calculated over 4 days postinjection for one cationic lipid (CL) versus another one. Luciferase intensities were compared over this period using the Mann–Whitney–Wilcoxon test. Pairs for which (1) the ratio was either >2.0 or <0.5 and (2) the difference was statistically significant (Figure S2 in the Supporting Information) are shown in gray.

transfection into the lungs, as shown by *in vivo* bioluminescence imaging, luciferase acitivity measurements in lung homogenates, and also by the immunohistochemistry for the identification of the transfected airway cells.[14,21] Thus, to begin to explore the *in vivo* gene transfer capability of our vectors, we used here such an easy administration method as well as an optimized CpG-free plasmid allowing high and sustained luciferase expression (pGM144 from the UK CF gene therapy consortium)[34] Accordingly, *in vivo* bioluminescence imaging was used to observe luciferase reporter gene expression, repeated imaging being performed for 4 days after lipoplex administration. Lung homogenates were also prepared and assayed for luciferase activity. Considering the present *in vitro* evaluations (see above Figure 2) and also previous studies, we used positively charged lipoplexes characterized by a CR of 4.0 obtained by mixing, in 0.9% NaCl, a constant amount of pDNA (50 μg) with the required amount of cationic lipid, the formed lipoplexes being next administered to mice via their tail vein. As a control, other mice received the same quantity of noncomplexed pDNA, and as expected, the use of such naked pDNA did not lead to luciferase expression.

In contrast, significant luciferase expression was observed with the reference **KLN47**-based lipoplexes (Figure 4),[26] in the mouse lungs (no detectable bioluminescent signal originating from any other areas of the animals). All the different cationic lipids **4**, **5**, **6**, and **7** studied herein were also efficient to transfect pDNA *in vivo* (Figure 4). Compound **6**-based lipoplexes appeared actually the most effective; on average, they allowed achieving a 5-fold higher luciferase expression *in vivo* when compared to that of any other (Figure 4).

Thus, these *in vivo* experiments demonstrated that the TMAs-based **KLN47** was indeed an efficient vector *in vivo* but that the new vector **6** was even better with a bioluminescence signal increased by a factor 5. Two conclusions might be drawn from these results: (1) The replacement of one methyl group of a TMA polar head by a HE moiety can increase the transfection efficacy; (2) the HE-DMA polar head may thus constitute an alternative to the TMAs polar head. Of note, the HE moiety produced a more efficient vector than its MOE (**5**) or DHPr analogues (**7**). Similar observations were previously noted by Bennett et al., who compared the hydration and the transfection properties of HE functionalized lipids and of their MOE analogues.[31] Besides the high transfection efficacies observed with compound **6**, all the other cationic lipids tested herein showed almost similar luciferase expression *in vivo* with a maximum of bioluminescence observed 24 h after injection, that maximum level being maintained up to 96 h (sustained expression). These positive *in vivo* bioluminescence results were corroborated by measuring the luciferase activity in lung homogenates prepared 4 days after intravenous injection (Figure 5). Here too, compound **6** was the most potent

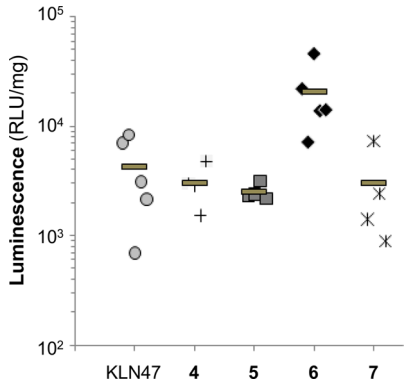


Figure 5. Luciferase activity in lung homogenates. Luminescence was evaluated in the lung homogenates obtained from lungs harvested 4 days after intravenous injection (50 μg pDNA injected). Values are expressed as RLU/mg protein (mean with $n \geq 4$).

cationic lipid among the compounds tested (statistically significant differences assessed using the Mann–Whitney–Wilcoxon test). Thus, these results suggest that compound **6** with its HE-DMA headgroup might have a great potential for *in vivo* transfection. It should, however, be stressed here that, as previously reported, lung transfection using lipoplexes by tail vein injection has some particular features.[21,40] Indeed, after intravenous tail vein injection, the positively charged DNA complexes may interact with blood components and the resulting large aggregates be next entrapped into the pulmonary microcirculation (the first blood microvasculature encountered

DOI: 10.1021/mp500807k
Mol. Pharmaceutics 2015, 12, 1902–1910

after i.v. injection), an accumulation favoring their dismantling and subsequent local gene transfection (in particular into the alveolar airway cells which are close to the pulmonary circulation).[21,40] Nevertheless, despite these limitations, our results suggest that compound **6** might be quite useful for *in vivo* applications of lipofection and that they thus invite one to further explore its *in vivo* potential, either for local gene delivery (e.g., by aerosol into the airways) or via systemic administration, the latter probably requiring, ideally, the use of long-lived DNA complexes equipped with motifs allowing targeted transfection via specific ligand−cell receptor recognition.[41−43]

Besides evaluation of the transfection efficacies, we also investigated the *in vivo* toxicity of our lipoplexes by quantifying the activity in the mouse plasma of the liver enzymes ALT and AST, the increase of which is a marker of hepatocellular toxicity.[21,44] As shown in Figure 6, all the formulations

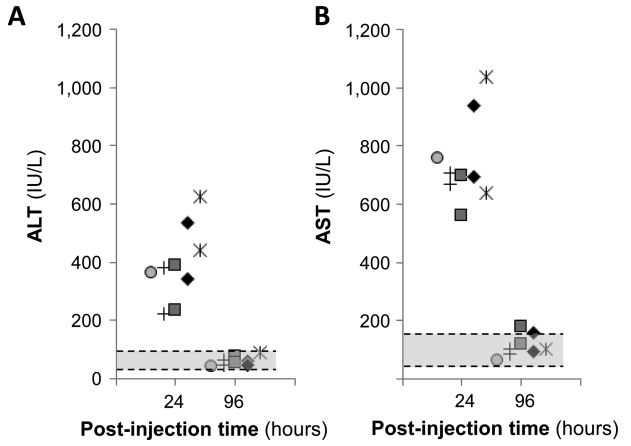


Figure 6. Evaluation of *in vivo* side-effects. ALT (A) and AST (B) plasma activities were quantified at early (24 h) and late (96 h) stages after intravenous injection (50 μg pDNA injected). Activities are given as IU/L. Mice injected with naked pDNA were used as controls (Ctrl). The dashed lines indicate the threshold (min and max) values in each case. The symbol code is the same as that in Figure 5.

administered induced ALT and AST responses which were transient since at 96 h values <200 UI/L of AST and ALT were measured, while, interestingly, high bioluminescence signals were still detected (Figure 6). Noteworthy, compound **6**, which was the most effective cationic lipid, had a very similar hepatotoxicity pattern in comparison with that of the other compounds.

Hours after Injection. Since **6** yielded the highest efficiency over all of the other cationic lipids evaluated herein, another series of experiments was performed in order to investigate whether, when injected at a lower dose, this compound would still exhibit such a superiority, while being less toxic (than at previous dose). Thus, **6**-based lipoplexes formed at CR 4.0 but incorporating half the quantity of vector and pDNA (25 μg of pDNA instead of 50 μg previously) were intravenously administered in mice. **KLN47**-based lipoplexes were prepared in the same manner and used for comparison. The results (see Supporting Information Figure SI1) showed that **6**-based lipoplexes still mediated clearly positive transfection activity, although the intensities measured here were lower than those when higher quantities of pDNA were used (Figure 5). Under those conditions, the transfection efficacies observed with **6** were almost one-log higher than those with **KLN47**-based lipoplexes. Here, the animals showed no obvious clinical signs during the days after injection, and none of them died. Additionally, the quantification of ALT and AST also confirmed the reduction of the side-effects (levels of ALT and AST lower than 50% being measured 24 h after injection as compared with the previous experiment and normal values being recovered at 96 h postinjection). Thus, increasing the transfection efficiency of cationic lipids appears to be interesting as it allows for decreasing both the vector and DNA doses to be administered and thereby increasing the tolerance and facilitating repeated administrations.

CONCLUSIONS

We report the synthesis of three new lipophosphoramidates possessing identical structures except for their cationic polar headgroup which incorporated a HE, a DHPr, or a MOE function. All of these cationic lipids demonstrated good capacity to compact DNA. *In vitro* transfection experiments showed that these compounds were able to efficiently and safely deliver a pDNA into human bronchial epithelial cell lines. Intravenous experiments in mice indicated that a derivative bearing a hydroxyethyl moiety (compound **6**) was the most efficient, mediating reporter gene (luciferase) expression at least 5-times above that of all other compounds studied, including our TMAs-based reference cationic lipid **KLN47**. In addition, production of the HE-DMA polar headgroup is also cost-effective. Thus, HE-DMA-based cationic lipids might constitute an interesting alternative to TMAs-based ones for *in vivo* lipofection.

ASSOCIATED CONTENT

Supporting Information

NMR spectra of the new synthesized products; size and zeta potential (ζ) of liposomes and lipoplexes; *in vivo* transfection activity following i.v. injection of **6**- and **KLN47**-based lipoplexes at half the usual dose. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/mp500807k.

AUTHOR INFORMATION

Corresponding Author

*Phone: +33 0-298016286. Fax: +33 0-298017001. E-mail: mathieu.berchel@univ-brest.fr.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank the "Services Communs RMN-RPE and mass spectrometry"of the University of Western Britanny in Brest. We are also pleased to acknowledge the support of "Association Française contre les Myopathies" (AFM, Evry, France), as well as "Vaincre La Mucoviscidose" (Paris, France), "Association de Biogenétique et de Transfusion Sanguine Gaetan Saleun" (Brest, France), Biogenouest, and "Conseil Régional de Bretagne".

ABBREVIATIONS

TMA, trimethylammonium; TMP, trimethylphosphonium; TMAs, trimethylarsonium; HE, hydroxyethyl; HE-DMA, hydroxyethyl-dimethylammonium; DHPr, dihydroxypropyl; DHPr-DMA, dihydroxypropyl-dimethylammonium; MOE, methoxyethyl; MOE-DMA, methoxyethyl-dimethylammonium; SGCC, silica gel column chromatography; HRMS, high

DOI: 10.1021/mp500807k
Mol. Pharmaceutics 2015, 12, 1902−1910

resolution mass spectrometry; RT, room temperature; DLS, dynamic light scattering; CR, (±) charge ratio; i.v., intravenous; i.t., intratracheal; pDNA, plasmid DNA; RLU, relative light units; ALT, alanine aminotransferase; AST, aspartate aminotransferase; IU/L, International Units per Liter

REFERENCES

(1) Zhang, S. B.; Zhao, Y. N.; Wang, B. Hybrids of nonviral vectors for gene delivery. *Bioconjugate Chem.* **2010**, *21*, 1003−1009.

(2) Ma, B.; Zhang, S.; Jiang, H.; Zhao, B.; Lv, H. Lipoplexes morphologies and their influence on transfection efficiency in gene delivery. *J. Controlled Release* **2007**, *123*, 184−194.

(3) Resina, B. S.; Prévot, P.; Thierry, A. R. Physico-chemical characteristics of lipoplexes influence cell uptake mechanism and transfection efficacy. *PLoS One* **2009**, *4*, e6057−e6058.

(4) Mintzer, M. A.; Simanek, E. E. Nonviral vectors for gene delivery. *Chem. Rev.* **2009**, *109*, 259−302.

(5) Zhi, D.; Zhang, S.; Wang, B.; Zhao, Y.; Yang, B.; Yu, S. Transfection efficiency of cationic lipids with different hydrophobic domains in gene delivery. *Bioconjugate Chem.* **2010**, *21*, 563−577.

(6) Le Corre, S. S.; Berchel, M.; Belmadi, N.; Denis, C.; Haelters, J. P.; Le Gall, T.; Lehn, P.; Montier, T.; Jaffrès, P. A. Cationic lipophosphoramidates with two different lipid chains: synthesis and evaluation as gene carriers. *Org. Biomol. Chem.* **2014**, *12*, 1463−1475.

(7) Aissaoui, A.; Oudrhiri, N.; Petit, L.; Hauchecorne, M.; Kan, E.; Sainlos, M.; Julia, S.; Navarro, J.; Vigneron, J. P.; Lehn, J. M. Progress in gene delivery by cationic lipids: guanidinium-cholesterol based systems as an example. *Curr. Drug Targets* **2002**, *3*, 1−16.

(8) Van Der Woude, I.; Wagenaar, A.; Meekel, A. A. P.; Ter Beest, M. B. A.; Ruiters, M. H. J.; Engberts, J. B. F. N.; Hoekstra, D. Novel pyridinium surfactant for efficient, nontoxic in vitro gene delivery. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 1160−1165.

(9) Mével, M.; Breuzard, G.; Yaouanc, J. J.; Clément, J. C.; Lehn, P.; Pichon, C.; Jaffrès, P. A.; Midoux, P. Synthesis and transfection activity of new cationic phosphoramidate lipids: high efficiency of an imidazolium derivative. *ChemBioChem* **2008**, *9*, 1462−1471.

(10) Midoux, P.; Pichon, C.; Yaouanc, J. J.; Jaffrès, P. A. Chemical vectors for gene delivery: a current review on polymers, peptides and lipids containing histidine or imidazole as nucleic acid carriers. *Br. J. Pharmacol.* **2009**, *157*, 166−178.

(11) Chen, J. X.; Wang, H. Y.; Quan, C. Y.; Xu, X. D.; Zhang, X. Z.; Zhuo, R. X. Amphiphilic cationic lipopeptides with RGD sequences as gene vectors. *Org. Biomol. Chem.* **2010**, *8*, 3142−3148.

(12) Fraix, A.; Le Gall, T.; Berchel, M.; Denis, C.; Lehn, P.; Montier, T.; Jaffrès, P. A. Cationic lipophosphoramidates with two disulfide motifs: synthesis, behaviour in reductive media and gene transfection activity. *Org. Biomol. Chem.* **2013**, *11*, 1650−1658.

(13) Donkuru, McD.; Wettig, S. D.; Verrall, R. E.; Badea, I.; Foldvari, M. Designing pH-sensitive gemini nanoparticles for non-viral gene delivery into keratinocytes. *J. Mater. Chem.* **2012**, *22*, 6232−6244.

(14) Montier, T.; Benvegnu, T.; Jaffres, P. A.; Yaouanc, J. J.; Lehn, P. Progress in cationic lipids mediated gene transfection: a series of bio-inspired lipids as an example. *Curr. Gene. Ther.* **2008**, *17*, 296−312.

(15) Le Gall, T.; Loizeau, D.; Picquet, E.; Carmoy, N.; Yaouanc, J. J.; Burel Deschamps, L.; Delépine, P.; Giamarchi, P.; Jaffrès, P. A.; Lehn, P.; Montier, T. A novel cationic lipophosphoramide with diunsaturated lipid chains: synthesis, physicochemical properties and transfection activities. *J. Med. Chem.* **2010**, *53*, 1496−1508.

(16) Fraix, A.; Montier, T.; Carmoy, N.; Loizeau, D.; Burel-Deschamps, L.; Le Gall, T.; Giamarchi, P.; Couthon-Gourvès, H.; Haelters, J. P.; Lehn, P.; Jaffrès, P. A. Cationic lipo-thiophosphoramidates for gene delivery: synthesis, physico-chemical characterization and gene transfection activity - comparison with lipophosphoramidates. *Org. Biomol. Chem.* **2011**, *9*, 2422−2432.

(17) Le Corre, S. S.; Belmadi, N.; Berchel, M.; Le Gall, T.; Haelters, J. P.; Lehn, P.; Montier, T.; Jaffrès, P. A. Cationic dialkylarylphosphates: a new family of bio-inspired cationic lipids for gene delivery. *Org. Biomol. Chem.* **2015**, *13*, 1122−1132.

(18) Le Corre, S. S.; Berchel, M.; Le Gall, T.; Haelters, J. P.; Lehn, P.; Montier, T.; Jaffrès, P. A. Cationic trialkylphosphates: synthesis and transfection efficacies compared to phosphoramidate analogues. *Eur. J. Org. Chem.* **2014**, 8041−8048.

(19) Mével, M.; Neveu, C.; Gonçalves, C.; Yaouanc, J. J.; Pichon, C.; Jaffrès, P. A.; Midoux, P. Novel neutral imidazole-lipophosphoramides for transfection assays. *Chem. Commun.* **2008**, 3124−3126.

(20) Le Gall, T.; Barbeau, J.; Barrier, S.; Berchel, M.; Lemiègre, L.; Jeftic, J.; Meriadec, C.; Artzner, F.; Gill, D. R.; Hyde, S. C.; Férec, C.; Lehn, P.; Jaffrès, P. A.; Benvegnu, T.; Montier, T. Effects of a novel archaeal tetraether-based colipidon the *in vivo* gene transfer activity of two cationic amphiphiles. *Mol. Pharmaceutics* **2014**, *11*, 2973−2988.

(21) Lindberg, M.; Carmoy, N.; Le Gall, T.; Fraix, A.; Berchel, M.; Lorilleux, C.; Couthon-Gourvès, H.; Bellaud, P.; Fautrel, A.; Jaffrès, P. A.; Lehn, P.; Montier, T. The gene transfection properties of a lipophosphoramidate derivative with two phytanyl chains. *Biomaterials* **2012**, *33*, 6240−6253.

(22) Berchel, M.; Le Gall, T.; Couthon-Gourvès, H.; Haelters, J.-P.; Montier, T.; Midoux, P.; Lehn, P.; Jaffrès, P.-A. Lipophosphonates/lipophosphoramidates: a family of synthetic vectors efficient for gene delivery. *Biochimie* **2012**, *94*, 33−41.

(23) Berchel, M.; Haelters, J. P.; Couthon-Gourvès, H.; Deschamps, L.; Midoux, P.; Lehn, P.; Jaffrès, P. A. Modular construction of fluorescent lipophosphoramidates by click chemistry. *Eur. J. Org. Chem.* **2011**, 6294−6303.

(24) Berchel, M.; Haelters, J. P.; Afonso, D.; Maroto, A.; Deschamps, L.; Giamarchi, P.; Jaffrès, P. A. functionalized phospholipid molecular platform: use for production of cationic fluorescent lipids. *Eur. J. Org. Chem.* **2014**, 1074−1083.

(25) Le Gall, T.; Berchel, M.; Le Hir, S.; Fraix, A.; Salaün, J. Y.; Férec, C.; Lehn, P.; Jaffres, P.-A.; Montier, T. Arsonium-containing lipophosphoramides, poly-functional nano-carriers for simultaneous antibacterial action and eukaryotic cell transfection. *Adv. Healthcare Mater.* **2013**, *2*, 1513−1524.

(26) Laurent, V.; Fraix, A.; Montier, T.; Cammas-Marion, S.; Ribault, C.; Benvegnu, T.; Jaffrès, P. A.; Loyer, P. Highly efficient gene transfer into hepatocyte-like HepaRG cells: New means for drug metabolism and toxicity studies. *Biotechnol. J.* **2010**, *5*, 314−320.

(27) Floch, V.; Loisel, S.; Guenin, E.; Hervé, A. C.; Clément, J. C.; Yaouanc, J. J.; Des Abbayes, H.; Férec, C. Cation substitution in cationic phosphonolipids: a new concept to improve transfection activity and decrease cellular toxicity. *J. Med. Chem.* **2000**, *43*, 4617−4628.

(28) Guénin, E.; Hervé, A. C.; Floch, V.; Loisel, S.; Yaouanc, J. J.; Clément, J. C.; Férec, C.; Des Abbayes, H. Cationic phosphonolipids containing quaternary phosphonium and arsonium groups for DNA transfection with good efficiency and low cellular toxicity. *Angew. Chem., Int. Ed.* **2000**, *39*, 629−631.

(29) Ornelas-Megiatto, C.; Wich, P. R.; Fréchet, J. M. J. Polyphosphonium polymers for siRNA delivery: An efficient and nontoxic alternative to polyammonium carriers. *J. Am. Chem. Soc.* **2012**, *134*, 1902−1905.

(30) Felgner, J. H.; Kumar, R.; Sridhar, C. N.; Wheeler, C. J.; Tsai, Y. J.; Border, R.; Ramsey, P.; Martin, M.; Felgner, P. L. Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. *J. Biol. Chem.* **1994**, *269*, 2550−2561.

(31) Bennett, A. M.; Aberle, R. P.; Balasubramaniam, J. G.; Malone, R. W.; Nantz, M. H. Cationic lipid-mediated gene delivery to murine lung: correlation of lipid hydration with in vivo transfection activity. *J. Med. Chem.* **1997**, *40*, 4069−4078.

(32) Ding, W.; Hattori, Y.; Higashiyama, K.; Maitani, Y. Hydroxyethylated cationic cholesterol derivatives in liposomevectors promote gene expression in the lung. *Int. J. Pharm.* **2008**, *354*, 196−203.

(33) Picquet, E.; Le Ny, K.; Delépine, P.; Montier, T.; Yaouanc, J. J.; Cartier, D.; des Abbayes, H.; Férec, C.; Clément, J. C. Cationic lipophosphoramidates and lipophosphoguanidines are very efficient for in vivo DNA delivery. *Bioconjugate Chem.* **2005**, *16*, 1051−1053.

DOI: 10.1021/mp500807k
Mol. Pharmaceutics 2015, 12, 1902−1910

(34) Hyde, S. C.; Pringle, I. A.; Abdullah, S.; Lawton, A. E.; Davies, L. A.; Varathalingam, A.; Nunez-Alonso, G.; Green, A. M.; Bazzani, R. P.; Sumner-Jones, S. G.; Chan, M.; Li, H.; Yew, N. S.; Cheng, S. H.; Boyd, A. C.; Davies, J. C.; Griesenbach, U.; Porteous, D. J.; Sheppard, D. N.; Munkonge, F. M.; Alton, E. W.; Gill, D. R. CpG-free plasmids confer reduced inflammation and sustained pulmonary gene expression. *Nat. Biotechnol.* **2008**, *26*, 549−551.
(35) Cozens, A. L.; Yezzi, M. J.; Kunzelmann, K.; Ohrui, T.; Chin, L.; Eng, K.; Finkbeiner, W. E.; Widdicombe, J. H.; Gruenert, D. C. CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells*Am. J. Respir. Cell Mol. Biol.* **1994**, *10*, 38−47.
(36) Kilkenny, C.; Browne, W. J.; Cuthill, I. C.; Emerson, M.; Altman, D. G. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol.* **2010**, *8*, e1000412.
(37) Felgner, P. L.; Gadek, T. R.; Holm, M.; Roman, R.; Chan, H. W.; Wenz, M.; Northrop, J. P.; Ringold, G. M.; Danielsen, M. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 7413−7417.
(38) Le Corre, S. S.; Berchel, M.; Couthon-Gourvès, H.; Haelters, J. P.; Jaffrès, P. A. Atherton-Todd reaction: mechanism, scope and applications. *Beilstein J. Org. Chem.* **2014**, *10*, 1166−1196.
(39) Safinya, C. R. Structures of lipid-DNA complexes: supramolecular assembly and gene delivery. *Curr. Opin. Struct. Biol.* **2001**, *11*, 440−448.
(40) Li, S.; Tseng, W. C.; Stolz, D. B.; Wu, S. P.; Watkins, S. C.; Huang, L. Dynamic changes in the characteristics of cationic lipidic vectors after exposure to mouse serum: implications for intravenous lipofection. *Gene Ther.* **1999**, *6*, 585−594.
(41) Ogris, M.; Wagner, E. To be targeted: is the magic bullet concept a viable option for synthetic nucleic acid therapeutics? *Hum. Gene Ther.* **2011**, *22*, 799−807.
(42) Guo, X.; Huang, L. Recent advances in nonviral vectors for gene delivery. *Acc. Chem. Res.* **2012**, *45*, 971−979.
(43) Lin, G.; Zhang, H.; Huang, L. Smart polymeric nanoparticles for cancer gene delivery. *Mol. Pharmaceutics* **2015**, *12*, 314−321.
(44) Morille, M.; Montier, T.; Legras, P.; Carmoy, N.; Brodin, P.; Pitard, B.; Benoît, J. P.; Passirani, C. Long-circulating DNA lipid nanocapsules as new vector for passive tumor targeting. *Biomaterials* **2010**, *31*, 321−329.

DOI: 10.1021/mp500807k
Mol. Pharmaceutics 2015, 12, 1902−1910