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## **Arsonium-containing Lipophosphoramides, Poly-functional Nano-carriers for Simultaneous Antibacterial Action and Eukaryotic Cell Transfection**

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**Keywords:** antibacterial; gene delivery; multimodal therapy; nano-carrier; poly-functional.

Abbreviations: AA, antibacterial activity; BGTC, bis(guanidinium)-tren cholesterol; BSV, Brest synthetic vector; CF, cystic fibrosis; CFU, colony forming unit; CR, charge ratio; Ec, *Escherichia coli*; i.p., intraperitoneal; i.v., intravenous; MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration; LB, Luria Broth; LFM, lipofectamine; LX, lipoplex; MRSA, methicillin-resistant *Staphylococcus aureus*; Pa, *Pseudomonas aeruginosa*; pDNA, plasmid DNA; PEI, poly(ethylenimine); RPM, rotation per minute; RLU, relative light unit; RT, room temperature; Sa, *Staphylococcus aureus*; TE, transfection efficiency.

Gene therapy of diseases like cystic fibrosis (CF) would consist in delivering a gene medicine towards the lungs via the respiratory tract into the target epithelial cells. Accordingly, poly-functional nano-carriers are required in order to overcome the various successive barriers of such complex environment, among which the airway colonization with bacterial strains. In this work, the antibacterial effectiveness of a series of cationic lipids is investigated before evaluating its compatibility with gene transfer into human bronchial epithelial cells. Among the various compounds considered, some bearing a trimethyl-arsonium headgroup demonstrate very potent biocide effects towards clinically relevant bacterial strains. Contrary to cationic lipids exhibiting no or insufficient antibacterial potency, arsonium-containing lipophosphoramides can simultaneously inhibit bacteria while delivering DNA into eukaryotic cells, as efficiently and safely as in absence of bacteria. Moreover, such vectors can demonstrate antibacterial activity *in vitro* while retaining high gene transfection efficiency to the nasal epithelium as well as to the lungs in mice *in vivo*. Arsonium-containing amphiphiles are the first synthetic compounds shown able to achieve efficient gene delivery in presence of bacteria, a property particularly suitable for gene therapy strategies under infected conditions such as within the airways of CF patients.

## 1. Introduction

Gene therapy for the treatment of inherited diseases like CF is an active field of research. Several pre-clinical and clinical gene therapy studies using viral or non-viral vectors have been performed; they have pointed out the need of nucleic acid carriers displaying improved efficacy together with reduced side-effects.<sup>[1]</sup> Thus, smart and sophisticated systems continue to be developed, combining multiple properties in order to overcome the various successive barriers which hamper gene delivery.<sup>[2]</sup> When considering lung gene therapy for CF, a particular hurdle concerns the environmental colonization by multiple, usually multi-resistant, bacterial strains.<sup>[3]</sup> Indeed, it is well-established that chronic pulmonary infections affect the gene transfection mediated by viral or non-viral vectors.<sup>[4]</sup>

Over the last several years, numerous studies have reported diverse non-viral gene delivery systems capable of delivering DNA to the lungs *in vivo*. Among these synthetic vectors, cationic lipids are particularly attractive as it is possible to design and synthesize a great variety of reagents combining multiple favorable features such as simplicity of use, relatively high transfection activity, biodegradability, etc.<sup>[5]</sup> We ourselves have developed various phosphonolipids, lipophosphoramides, and lipothiophosphoramides with a view to reach the “best” compromise between efficacy and side-effects to the target cells or animals. A series of chemical variations were undertaken that involved the different parts constituting the basic chemical structure of cationic lipids, notably the cationic headgroup<sup>[6]</sup> and the spacer moiety.<sup>[7]</sup> The replacement of the trimethyl-ammonium by a trimethyl-phosphonium or -arsonium led to derivatives exhibiting significantly lower cytotoxicity.<sup>[6a,b]</sup> Recently, the modulation of fatty acid chains was investigated in the lipophosphoramide series; replacing oleyl<sup>[8]</sup> by linoleyl<sup>[9]</sup> or phytanyl<sup>[10]</sup> chains led to successive improvement regarding both transfection efficiency and biocompatibility in mice *in vivo*. Of note, all these derivatives were evaluated considering mainly their ability to deliver genes into mammalian cells.

However, other studies reported that cationic amphiphiles may also display some antibacterial activities.<sup>[11]</sup> Kichler et al. showed that cationic steroid antibiotics, which demonstrate DNA delivery properties, may also possess significant antibacterial potencies.<sup>[12]</sup> Lipidic derivatives of aminoglycosides have also been developed for gene transfection considering that these widely-used antibiotics bind nucleic acids with high affinity; while showing remarkable abilities as vehicles for the delivery of DNA<sup>[13]</sup> or RNA,<sup>[14]</sup> these compounds may also retain some antibacterial properties.<sup>[15]</sup> More recently, it was reported that a series of cationic sterol lipids can demonstrate some gene transfer and bactericidal activities.<sup>[16]</sup> It is noteworthy here that, in all these previous reports, the transfection properties and the antibacterial effects were investigated in independent experiments.

In the current study, we wonder whether one single compound could achieve at the same time (i) biocide effects towards bacterial cells together with (ii) efficient and safe gene transfer into human bronchial epithelial cells, under experimental conditions mimicking gene delivery within airways infected with a virulent antibiotic resistant strain (**Figure 1**). In order to address this question, we first investigate the antibacterial activity of numerous cationic lipids that were only used until now as transfection reagents; this series included a representative panel of our own cationic lipids further completed with some novel derivatives herein synthesized. The screening performed aimed at (i) unveiling structure–activity relationships and (ii) identifying compounds active at concentrations compatible with gene transfection experiments. Next, *in vitro* cell culture assays were performed to determine the ability of some selected compounds to display both antibacterial and gene transfection activities, either sequentially (i.e. the first then the second) or simultaneously (i.e., concomitantly, in one pot). Finally, investigations were conducted in mice in order to test whether the observations raised from *in vitro* studies could be further extended to *in vivo* experimental settings.

## 2. Results and Discussion

The chemical structure of the main synthetic phospholipids included in this study is reported in **Table 1** (see also Figure S1). The synthesis pathway used to obtain **1**, **6**, **8**, and **11** is summarized in **Figure 2**, the protocols being detailed in the Experimental Section. In addition, the crystal structure of compound **6** is described in Figure S2. These cationic lipids were formulated as liposomal solutions, their size and charge being on average ~200 nm and ~50 mV, respectively (Table S1).

### 2.1. Antibacterial Effectiveness of a Series of Cationic Lipids

All cationic liposomes were first evaluated as regards their biocide effect towards a series of Gram-positive and Gram-negative bacterial strains: three *S. aureus* (the laboratory strain RN4220,<sup>[17]</sup> the clinical strain Newman,<sup>[18]</sup> and the clinical methicillin-resistant *S. aureus* (MRSA) strain N315<sup>[19]</sup>), one *E. coli* (the laboratory strain MG1655<sup>[20]</sup>) and two *P. aeruginosa* (the clinical strains PA130709 and PA240709, each isolated from the sputum of a CF patient). Of note, these strains were chosen considering that they can be part of the microbiota colonizing the lungs of CF patients.<sup>[21]</sup> Some of them are resistant to numerous antibiotics (Table S2). In order to determine susceptibilities to cationic lipids, a liquid broth micro-dilution assay was employed as previously reported by ourselves<sup>[22]</sup> and others.<sup>[23]</sup> Table 1 summarizes the antibacterial activities found; the values reported herein correspond to minimal bactericidal concentrations (MBC), rather than minimal inhibitory concentrations (MIC), since, in each case, no bacterial growth occurred when aliquots of corresponding liquid cultures were spread onto non-selective nutritive agar plates. Whereas most derivatives are completely ineffective (i.e., MBC > 200  $\mu\text{M}$ ), some others display remarkable bactericidal activities, the lowest MBC being close to 1  $\mu\text{M}$ . These activities are mostly measured using *S. aureus* strains and, in some cases, the *E. coli* strain. Notably, **1**, **2**, and **3** retain their activities

against six different *S. aureus* strains (Table 1 and data not shown), especially towards one MRSA (N315<sup>[19]</sup>). The importance of this finding is further emphasized when considering previous similar reports.<sup>[24]</sup> For comparative purposes, the activities of benchmark compounds were determined under the same experimental conditions. First, well-known cationic transfection reagents such as Lipofectamine (LFM; Invitrogen), bis(guanidinium)-trencholesterol (BGTC),<sup>[25]</sup> or poly(ethylenimine) (PEI)<sup>[26]</sup> do not exhibit any antibacterial activity. The silver carbene **14** is active but mostly against Gram-negative strains, in agreement with literature results.<sup>[23]</sup> Finally, three commonly used antibiotics (kanamycin, ampicillin, and tetracyclin) are either totally ineffective (MBC > 200  $\mu\text{M}$ ) or active with MBC ranging from 1 up to 200  $\mu\text{M}$ . Taken together, these results highlight the antibacterial potential of several cationic lipophosphoramides showing that: (i) they are active at low concentrations (close to the MBC of the most efficient antibiotics tested); (ii) their MBC are in the same range of concentrations as those typically used for gene transfection (between 1 to 200  $\mu\text{M}$ ); (iii) importantly, in contrast to antibiotics, their activity appears independent of the antibiotic resistance profile as well as the virulence potential of the strains.

## 2.2. Structure–Antibacterial Activity Relationships

The various cationic lipids investigated exhibiting very different MBC, their chemical structure thus directly impact on their antibacterial activity. First, the polar headgroup is an obvious key parameter. Indeed, only the cationic lipids possessing a trimethyl-arsonium (**1-5**) or a trimethyl-phosphonium (**12**) headgroup can display such an activity; a trimethyl-ammonium polar head is on contrary clearly detrimental as observed when comparing the MBC of the **1/8**, **3/9** and **4/10** couples. The amphiphiles possessing a trimethyl-arsonium demonstrate the lowest MBC, being clearly more potent than their trimethyl-phosphonium analogs (**3 versus 12**). This might be ascribed to the size and the geometry of the cationic

polar head as these parameters can determine the strength of any dipolar interaction in which the headgroup might be involved (the trimethyl-arsonium polar head being indeed bigger than the trimethyl-phosphonium which is itself bigger than the trimethyl-ammonium<sup>[27]</sup>). A similar correlation was recently observed when comparing the transfection activity of arsonium-containing cationic lipids with that of ammonium analogs.<sup>[27,28]</sup> Of note, the incorporation of another type of cationic moiety (guanidinium in **11**) is detrimental for the bactericidal effect. However, it must be noted that the use of a trimethyl-arsonium headgroup, although necessary, is not sufficient to design an efficient antibacterial compound. Indeed, the arsonium-containing lipophosphoramidate **6**, which has two short ethyl chains, is completely devoid of any antibacterial activity.

Accordingly, the structural features of the hydrophobic domain also determine the level of antibacterial efficacy; indeed, lipophosphoramidates which only vary according to this chemical part displayed quite different activities. It is noteworthy here that the antibacterial potency of free fatty acids of various chain lengths and with different unsaturation degrees was reported earlier; they have been shown to be mainly effective against Gram-positive strains.<sup>[29]</sup> With regard to the cationic lipids investigated, it is noticeable that they all contain two aliphatic chains but, unlike free fatty acids, these chains are linked together to a cationic headgroup. Our results show some consistencies with those reports<sup>[29]</sup> as we observed that **2**, an arsonium-containing lipophosphoramidate bearing long di-unsaturated lipid chains (C18:2), was more potent than **3**, its mono-unsaturated analogue (C18:1), which was itself better than **5**, a fully saturated derivative (C18:0). Short saturated lipid chains (C14:0) can also greatly contribute to the antibacterial activity, depending on the polar headgroup and the linker they are associated with. Indeed, despite **1** and **8** are structurally close (they both incorporate two myristic chains and they only vary as regards their cationic group), they were highly efficient or completely ineffective, respectively. Altogether, this suggests that, unlike free fatty acids,



the lipid chains in cationic lipids may not be sufficient to provide, by themselves, an antibacterial potency; however, they are clearly essential and their chemical structure greatly modulate the antibacterial effectiveness.

Finally, the spacer moiety, which links together the polar headgroup and the lipid domain, also impacts the antibacterial potency depending on its chemical structure. Of note, **1** and **4** are two structural analogues that only differ as regards their linker: P(O)C phosphonate versus P(O)N phosphoramidate, respectively. As shown in Table 1, **4** was identified as a potent antibacterial compound against Gram-positive strains but, in all cases, it displayed MBC higher than those of **1**; when considering *E. coli* (a Gram-negative strain), **4** was inefficient (MBC > 200  $\mu\text{M}$ ) while **1** demonstrated the lowest MBC reached using this strain. The importance of the spacer moiety regarding the antibacterial potency is further evidenced when comparing the activity of the lipophosphoramidate **3** with that of the lipothiophosphoramidate **7**.<sup>[30]</sup> In our tests, these two derivatives interacted very differently with bacteria, as **3** demonstrated some antibacterial activities whereas **7** did not. Of note, changing the P(O)N functional group to a P(S)N decreases the polarity of the spacer moiety and, consequently, it extends the hydrophobic domain of the cationic lipid. In addition, it is likely that the lower polarity of the P(S)N functional group does not allow to form hydrogen bond networks as strong as the P(O)N. As observed when considering **6** at solid state (Figure S2), the P(O)N linkage should participate, via hydrogen bonds, in intermolecular interactions between neighbouring lipophosphoramidate entities, thus contributing to supramolecular organization as micelles or liposomes.

Overall, our results evidence that the whole chemical architecture of arsonium-containing lipophosphoramidates (i.e., the combination of a trimethyl-arsonium headgroup, a phosphoramidate spacer, and two short saturated or long unsaturated lipid chains) plays a critical role in their antibacterial ability. Interestingly, these results highlight that optimizing

the design of our cationic lipids for enhanced gene transfer activity led to a concomitant optimisation of their antibacterial potency.

### 2.3. Further Characterizations of Antibacterial Properties

Additional experiments unveiled deeper insights into the antibacterial properties of arsonium-containing cationic lipids (compounds hereafter termed arsenolipids). First, we observed that bactericidal effects were not immediate but delayed in time; indeed, they were preceded by bacterial growth inhibitions, i.e., arsenolipids were first bacteriostatic before being bactericidal (**Figure 3**). The cationic lipid dose and its exposure time with bacteria determined a balance between bacteriostatic and bactericidal effects. Schematically, for one given cationic lipid, the higher the dose, the shorter the time required to kill bacteria. As an example, a complete bactericidal effect was obtained after 2 h of incubation with  $100 \mu\text{g mL}^{-1}$  of **2** whereas the MBC dropped down to  $40 \mu\text{g mL}^{-1}$  for an incubation time of 15 h  $\frac{1}{2}$ ; furthermore, a minimum of  $20 \mu\text{g mL}^{-1}$  of **2** was required to obtain a bacteriostatic effect, a dose which became bactericidal after an incubation time as long as 32 h. Such antibacterial properties suggest that arsenolipids are not “detergent-like biocides” but, more likely, they might act through the alteration of vital cellular pathways. Additionally, the effect of the bacterial inoculum size was also considered as it determines the antibacterial potencies.<sup>[31]</sup> Not surprisingly, the higher the starting inoculum, the higher the cationic lipid dose required to obtain a complete bactericidal effect (**Figure 4**). However, we found that a potent arsenolipid is able to keep bacteriostatic/bactericidal effects up to very high bacterial densities. For instance, after 23 h at 37 °C,  $5 \mu\text{M}$  of **2** were sufficient to kill all bacteria when  $\sim 10^3$  colony forming unit (CFU)  $\text{mL}^{-1}$  were initially used, whereas the MBC was  $75 \mu\text{M}$  when starting with  $\sim 10^9$  CFU  $\text{mL}^{-1}$ , i.e., the MBC was multiplied by only 15 when the inoculum was multiplied by  $10^6$  (**Figure 4**). These results clearly emphasize the robustness of the antibacterial effects

mediated by the most potent arsenolipids. Importantly, they also indicate that such activities can persist regardless of the bacterial growth state (whether bacteria actively divide or not), a crucial property when expecting therapeutic efficacy at infectious sites where bacteria are not actively growing, especially *in vivo* (e.g., as in tuberculosis). The emergence of drug resistances in bacteria towards conventional antibiotics is a major public-health concern. In order to investigate whether or not arsonium-containing cationic lipids might select for drug-resistant isolates, the MRSA strain N315 was cultivated for serial passages on half-MIC of **2**, MIC values being re-evaluated every 24 h. As a positive control, the antibiotic norfloxacin was evaluated in parallel in the same way (Figure S3). While a strong increase in MIC of norfloxacin was already detected after 3 passages (reaching more than 20-fold the initial value after 10 days), the susceptibility of bacteria towards **2** did not change, even after 10 passages. This supports that arsonium-containing cationic lipids should not participate in the development of drug-resistant strains.

#### **2.4. Combined Antibacterial and Eukaryotic Cell Transfection Assays**

On the basis of these findings, *in vitro* experiments combining antibacterial and transfection assays were conducted thereafter. For these tests, cationic lipids were first mixed with a luciferase reporter plasmid DNA (pDNA) in order to obtain complexes called lipoplexes. Luciferase gene expression was used to report not only eukaryotic cell transfection efficiency but also cellular integrity (assuming that impaired cells should not be able to achieve “normal” gene expression). A sequential assay was first worked out in a view to easily and clearly determine, step by step, the ability of one single compound to display (1) antibacterial activity (2) DNA compaction and protection and finally (3) gene transfer into human bronchial epithelial cells. Next, a simultaneous assay was performed: one candidate compound was used

to test whether antibacterial and gene transfer activities could be obtained at the same time in one pot, under experimental conditions approaching real *in vivo* settings (Figure 1).

#### 2.4.1. Sequential Assay

As we have previously demonstrated the high transfection efficiency of several cationic lipids herein identified as potent antibacterials, we next wondered whether such compounds could achieve these two tasks sequentially: the antibacterial activity of lipoplexes was first determined (via incubation with bacteria for 24 h) before evaluating the transfection efficiency of these mixtures (by introducing them directly into the culture medium of eukaryotic cells). These experiments were carried out using **1**, **2** and **3**; the results obtained using **2** are shown in **Figure 5** (the complete set of results is available in Figures S5 and S6). Lipoplexes were prepared at different charge ratios (CR, +/-; which is the ratio of the positive charges provided by the vector polar headgroup to the negative charges provided by the DNA phosphates), from 0.5 to 4.0. As shown in Figure 5A, **2**-based lipoplexes displayed antibacterial activity as did corresponding liposomes, the MBC estimated here in 10% serum supplemented medium being similar to those previously measured in Luria Broth (LB; Table 1). Under identical conditions, LFM still didn't show any antibacterial activity. Panel B illustrates the compaction and protection of pDNA. When mixed with **2**, a full compaction was observed from CR 2; interestingly, at lower CR, pDNA was not deteriorated (despite it was only partially condensed). On the contrary, DNA mixed (or not) with LFM was degraded, as a consequence of bacterial growth (Figure S4). The addition of dextran sulfate further verified that the antibacterial activity of **2**-based lipoplexes can be obtained together with an efficient pDNA compaction and protection (Figure 5B and Figure S5). Finally, after 24 h at 37 °C, the transfection activity of "lipoplexes + bacteria" mixtures was examined: **2**-based lipoplexes were still able to transfect eukaryotic cells *in vitro*; their transfection efficiency was

actually the same as those measured with control lipoplexes (that were not mixed with any bacteria), even at CR $\frac{1}{2}$ . In the comparative experiment using LFM, the transfection efficiency was lost, likely due to the pDNA deterioration by bacteria (Figure 5C and Figure S6). Thus, one single arsenolipid can sequentially exert antibacterial activities and subsequently mediate gene transfer into eukaryotic cells, contrary to other cationic lipids devoid of any antibacterial activity such as LFM (Figure 5) or **10** (data not shown).

#### 2.4.2. Simultaneous Assay

Considering that **2** allows obtaining the best compromise between antibacterial activity and transfection efficiency, it was used for further *in vitro* evaluations in which prokaryotic and eukaryotic cells were cultured together, i.e., in one pot. Of note, the highly virulent clinical strain N315<sup>[19]</sup> was employed here. The luciferase activity (which reports the transfection efficiency) on one hand and the adenylate kinase activity (which measures not only cellular injuries but also bacterial growth; see Figure S7) on the other hand were determined at different time points of this experiment. **Figure 6** shows the results obtained with **2** or LFM when mixed with a pDNA at CR10; the results obtained using lipoplexes formed at 6 other CR (ranging from 4 to 9) are provided in Figure S8. Regarding the evaluations performed using LFM, the transfection efficiency progressively increased over time when bacteria were absent; in contrast, under conditions with bacteria, it first increased up to 21 h then no longer evolved during the rest of the experiment (Figure 6B). Such a transfection profile was obviously related to the progression of the infection, as the transfection inhibition was coincidental with the bacterial growth exponential phase (Figure 6A). These observations were roughly the same whatever the CR considered. Thus, despite LFM was a potent reagent for transfecting cells under sterile conditions, its inability to prevent bacterial growth clearly hampered its transfection

efficiency under infected conditions. This might result both from pDNA degradation and cellular injuries caused by the virulent bacteria used here. On contrary, **2**-based lipoplexes formed at CR10 mediated potent antibacterial activity (Figure 6A) together with very similar transfection profiles in the two compared experimental settings (with or without bacteria; Figure 6B). It is noteworthy that, at lower CR, **2** displayed either no or little antibacterial activity (CR4 to 6) or transient bacteriostatic effects (CR7 to 9); however, from CR5 and above, the transfection efficiencies measured at each time point in sterile versus contaminated conditions were of the same order of magnitude (Figure S8). Thus, in presence of bacteria, **2** is able to retain high transfection efficiency, even at doses that did not mediate any strong antibacterial effect. This might result from a protective effect towards both pDNA and eukaryotic cells against any bacterial-induced damages. Altogether, these findings emphasize that, in an infected environment, gene delivery can be more efficiently achieved by using a vector endowed with some antibacterial activity.

## **2.5. *In vivo* Investigations**

Finally, we wondered whether the compatibility between the antibacterial and transfection activities of arsonium-containing lipophosphoramides could be further evidenced in an experimental setting using animals. Indeed, these reagents were evaluated earlier for *in vivo* gene transfer in mice, where they demonstrated high efficiencies together with acceptable toxicities for the host.<sup>[8,9,10]</sup> It is noteworthy that, in such applications, cationic lipids are used at concentrations (typically several hundred  $\mu\text{M}$ ) higher than those identified here as bactericidal. In an attempt to clearly address the potential of a dual-effectiveness in the context of *in vivo* gene delivery, the antibacterial and transfection activities of an arsonium-containing lipophosphoramide were studied sequentially: **2**-based lipoplexes were first incubated *in vitro* with bacteria for several hours before evaluating their *in vivo* transfection

efficiency. We assume that such an experimental setting mimick, at least in part, the sequential steps of *in vivo* gene delivery in a contaminated environment, as lipoplexes would first mix with bacteria before they can reach their target cells. First of all, we observed that 2-based lipoplexes were still able to efficiently inhibit bacterial growth and protect DNA; indeed, after several hours at 37 °C, a complete bactericidal effect was obtained and the DNA entrapped within the lipoplexes remained intact (not shown). Next, lipoplexes were administered in mice via either systemic or local delivery routes; non-invasive *in vivo* bioluminescence imaging revealed that luciferase activity was detectable around the lung area and from the nostril of animals, respectively (**Figure 7A**). Importantly, the luminescence signals collected were of the same order of magnitude whether lipoplexes were initially mixed with bacteria or not (Figure 7B). Thus, in presence of bacteria, 2-based lipoplexes can exhibit antibacterial activities while still retaining the required properties for efficient *in vivo* transfection, an important finding when considering lung gene therapy for diseases like CF. Ongoing *in vivo* experiments using animal models for septicaemia or chronic pulmonary infection will allow to further investigate the usefulness of arsonium-containing cationic lipids.

### 3. Conclusions

In summary, we have evidenced a particular class of cationic lipids, arsonium-containing lipophosphoramides, as efficient and robust antibacterial agents effective against Gram-positive, including multi-resistant, virulent clinical strains. The most potent compounds being active at relatively low concentrations, a balance can be found between (i) a selective biocide effect towards bacterial cells and (ii) an efficient and safe gene transfer into eukaryotic cells, leading to transgene expression. To the best of our knowledge, this is the first time that such a double simultaneous effect is demonstrated for one given compound. Such poly-functional nano-carriers may be suitable for a variety of applications. (i) For instance, they may help

preventing or fighting bacterial infections in various biological contexts; notably, they could be used to eradicate intracellular bacteria. (ii) They may also be useful for gene transfection studies under conditions where conventional antibiotics cannot be used. (iii) Finally, our findings further evidence the potential of arsenolipids for non-viral gene therapy in situations where bacterial infections might not be fully eradicated prior to gene transfer. Indeed, compounds able to retain their transfection activity under infected conditions (as demonstrated herein, *in vitro* as well *in vivo*) might be particularly suitable for gene therapy strategies in complex environments such as within the infected airways of CF patients. At present, arsonium-containing lipophosphoramides are evaluated in cocktails with the aim to obtain broader antibacterial spectrum. In conclusion, future sophisticated gene delivery systems may incorporate arsenolipids as “double-edged” active components for antibacterial as well as gene transfer applications.

#### 4. Experimental Section

*Chemicals and reagents:* The cationic lipids **2** (BSV4),<sup>[9]</sup> **3** (KLN47),<sup>[8]</sup> **4** (BSV98),<sup>[32]</sup> **5** (BSV82),<sup>[33]</sup> **7** (BSV21),<sup>[30]</sup> **9** (BSV36),<sup>[32]</sup> and **10** (GLB43),<sup>[34]</sup> as well as the silver complex **12**<sup>[23]</sup> were synthesised following reported methodologies. The other cationic lipids were synthesised as reported below. Lipofectamine (a commercial liposomal formulation of DOSPA/DOPE 3/1, w/w) was obtained from Invitrogen. DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) and PE-PEG5000 (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000]) were purchased from Avanti Polar Lipids.

*Chemical synthesis:* The cationic lipids were purified by flash chromatography (GRACE REVELERIS Flash Chromatography System) equipped with UV and DLS detectors allowing to attest the high purity (> 95%) of the purified compounds. These compounds were characterized by NMR (<sup>1</sup>H, <sup>31</sup>P, <sup>13</sup>C), including HMBC and HMQC experiments, and also by high-resolution mass spectrometry (HRMS). Pertinent spectroscopic and analytical data are



given below. **Caution:** care must be taken when handling trimethyl-arsine. All related experiments should be performed in a fume hood in a room that may be well-ventilated.

*Synthesis of O,O-Ditetradecyl-N-(2-N',N'-dimethylaminoethyl)phosphoramidate 14:* To ditetradecylphosphite (1.0 g, 2.1 mmol) and *N,N*-dimethylaminoethylenediamine (205 mg, 2.3 mmol) and bromotrichloromethane (230  $\mu$ L, 2.3 mmol) in anhydrous dichloromethane (10 mL), at 0 °C, di-isopropylethylamine (DIPEA) (404  $\mu$ L, 2.3 mmol) was slowly added. The reaction was further stirred for 1 h at 0 °C then 5 h at room temperature (RT). Solvent was evaporated in vacuo and the residue filtered on Celite. The filtrate was concentrated and purified by flash column chromatography on silica gel ( $\text{CH}_2\text{Cl}_2/\text{MeOH}$ , 9/1) to produce **14** as a viscous oil (1.1 g, 85% yield).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  0.83 (t,  $^3J_{\text{HH}} = 7.2$  Hz, 6H,  $\text{CH}_3$ -), 1.23 (m, 44H,  $-\text{CH}_2$ -), 1.59 (m, 4H,  $-\text{CH}_2$ -), 2.26 (s, 6H), 2.40 (t,  $^3J_{\text{HH}} = 5.7$  Hz, 2H,  $-\text{CH}_2\text{-N}$ -), 2.98 (m,  $^3J_{\text{HH}} = 5.7$  Hz, 2H,  $-\text{CH}_2\text{-NH}$ ), 3.25 (bs, 1H,  $-\text{NH}$ -), 3.95 (m,  $^3J_{\text{HH}} \sim ^3J_{\text{HP}} = 6.5$  Hz, 4H,  $-\text{CH}_2\text{-O}$ -).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  14.4, 23.0, 25.9, 29.6-30.8, 32.3, 38.9, 45.4, 59.9 and 66.7.  $^{31}\text{P}$  NMR ( $\text{CDCl}_3$ )  $\delta$  9.8. HRMS (MALDI-TOF)  $m/z$  calcd for  $\text{C}_{32}\text{H}_{69}\text{N}_2\text{O}_3\text{P}$  [(M+H) $^+$ ] 561.512; found 561.502.

*Synthesis of O,O-Ditetradecyl-N-(2-trimethylaminoethyl)phosphoramidate 8 (BSV61):* To compound **14** (500 mg, 0.9 mmol) in anhydrous dichloromethane (10 mL) was added methyl iodide (640 mg, 4.5 mmol) and the reaction was stirred overnight at RT. The crude was concentrated and the residue was purified by flash column chromatography on silica gel ( $\text{CH}_2\text{Cl}_2/\text{MeOH}$ , 9/1) to produce **8** as a colourless solid (582 mg, 92% yield).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  0.86 (t,  $^3J_{\text{HH}} = 7.2$  Hz, 6H,  $\text{CH}_3$ -), 1.24 (m, 44H,  $-\text{CH}_2$ -), 1.65 (m, 4H,  $-\text{CH}_2$ -), 3.47 (s, 9H), 3.55 (m,  $^3J_{\text{HH}} = 5.6$  Hz, 2H,  $-\text{CH}_2\text{-N}$ ), 3.87 (t,  $^3J_{\text{HH}} = 5.6$  Hz, 2H,  $-\text{CH}_2\text{-NH}$ ), 3.99 (m,  $^3J_{\text{HH}} \sim ^3J_{\text{HP}} = 6.5$  Hz, 4H,  $-\text{CH}_2\text{-O}$ -), 4.44 (bs, 1H,  $-\text{NH}$ -).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  14.4, 23.0, 25.9, 29.6-30.8, 32.3, 35.4, 54.1, 59.9 and 66.9.  $^{31}\text{P}$  NMR ( $\text{CDCl}_3$ )  $\delta$  8.7. HRMS (MALDI-TOF)  $m/z$  calcd for  $\text{C}_{33}\text{H}_{72}\text{N}_2\text{IO}_3\text{P}$  [(M $^+$ -I)] 575.528; found 575.531.

*Synthesis of O,O-Ditetradecyl-N-(2-aminoethyl)phosphoramidate 15:* To

ditetradecylphosphite (1.30 g, 2.7 mmol), ethylenediamine (1.6 g, 27.0 mmol) in anhydrous dichloromethane (10 mL) at 0 °C was added dropwise bromotrichloromethane (300 µL, 2.7 mmol). The reaction was stirred for 1 h at 0 °C then 5 h at RT. The crude was concentrated and the residue was taken up in dichloromethane, washed three times with water, brine, dried with MgSO<sub>4</sub>, filtered and concentrated to furnish **15** as a viscous oil (1.2 g, 85% yield). The oily product was engaged in the next step without further purification. <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 0.83 (t, <sup>3</sup>J<sub>HH</sub> = 7.2 Hz, 6H, CH<sub>3</sub>-), 1.23 (m, 44H, -CH<sub>2</sub>-), 1.59 (m, 4H, -CH<sub>2</sub>-), 2.78 (t, <sup>3</sup>J<sub>HH</sub> = 5.7 Hz, 2H, -CH<sub>2</sub>-NH<sub>2</sub>), 2.97 (m, <sup>3</sup>J<sub>HH</sub> = 5.7 Hz, 2H, -CH<sub>2</sub>-NH), 3.25 (bs, 1H, -NH-), 3.95 (m, <sup>3</sup>J<sub>HH</sub> ~ <sup>3</sup>J<sub>HP</sub> = 6.5 Hz, 4H, -CH<sub>2</sub>-O-). <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 14.4, 23.0, 25.9, 29.6-30.7, 32.3, 43.2, 44.1 and 66.8. <sup>31</sup>P NMR (CDCl<sub>3</sub>) δ 9.8. HRMS (MALDI-TOF) m/z calcd for C<sub>30</sub>H<sub>65</sub>N<sub>2</sub>O<sub>3</sub>P [(M+H)<sup>+</sup>] 533.481; found 533.472.

*Synthesis of O,O-Ditetradecyl-N-(2-guanidinoethyl)phosphoramidate 11 (BSV62):* Compound **15** (500 mg, 0.9 mmol) and 1H-pyrazole-1-carboxamidinium hydrochloride (151 mg, 1.0 mmol) were combined in absolute ethanol. DIPEA (181 µL, 1.0 mmol) was added and the reaction was heated to reflux overnight. Solvent was evaporated in vacuo, and the residue was taken up in chloroform, washed with water and brine, dried with MgSO<sub>4</sub>, filtered and concentrated to furnish **11** as a viscous oil (484 mg, 88% yield). <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 0.87 (t, <sup>3</sup>J<sub>HH</sub> = 7.2 Hz, 6H, CH<sub>3</sub>-), 1.29 (m, 44H, -CH<sub>2</sub>-), 1.61 (m, 4H, -CH<sub>2</sub>-), 3.06 (m, 2H, -CH<sub>2</sub>-NH<sub>2</sub>), 3.36 (m, 2H, -CH<sub>2</sub>-NH), 3.93 (m, <sup>3</sup>J<sub>HH</sub> ~ <sup>3</sup>J<sub>HP</sub> = 6.5 Hz, 4H, -CH<sub>2</sub>-O-), 4.65 (bs, 1H, -NH-), 7.20 (bs, 2H, -NH<sub>2</sub>), 7.88 (bs, 1H, =NH). <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 14.5, 23.0, 26.0, 29.7-30.8, 32.3, 41.1, 43.1, 67.4 and 158.7. <sup>31</sup>P NMR (CDCl<sub>3</sub>) δ 10.6. HRMS (MALDI-TOF) m/z calcd for C<sub>31</sub>H<sub>68</sub>ClN<sub>4</sub>O<sub>3</sub>P [(M<sup>+</sup> - Cl)] 575.502; found 575.400.

*Synthesis of O,O-Ditetradecyl-N-(2-bromoethyl)phosphoramidate 16a:* To ditetradecylphosphite (500 mg, 1.1 mmol), 2-bromoethylamine bromohydrate (237 mg, 1.2 mmol) and

bromotrichloromethane (230  $\mu$ L, 2.3 mmol) in anhydrous dichloromethane (5 mL) at 0  $^{\circ}$ C, was slowly added DIPEA (404  $\mu$ L, 2.3 mmol). The reaction was stirred for 1 h at 0  $^{\circ}$ C then 3 h at RT. Solvent was evaporated in vacuo, and ether was added (10 mL). The precipitate was removed by filtration on Celite. The filtrate was concentrated to produce **16a** as a viscous oil (590 mg, 90% yield) which was engaged in the next step without further purification.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  0.77 (t,  $^3J_{\text{HH}} = 7.2$  Hz, 6H,  $\text{CH}_3$ -), 1.16 (m, 44H,  $-\text{CH}_2$ -), 1.56 (m, 4H,  $-\text{CH}_2$ -), 3.18 (m,  $^3J_{\text{HH}} = 6.4$  Hz, 2H,  $-\text{CH}_2\text{-NH}$ ), 3.32 (t,  $^3J_{\text{HH}} = 6.4$  Hz, 2H,  $-\text{CH}_2\text{-Br}$ ), 3.71 (bs, 1H,  $-\text{NH}$ -), 3.87 (m,  $^3J_{\text{HH}} \sim ^3J_{\text{HP}} = 6.5$  Hz, 4H,  $-\text{CH}_2\text{-O}$ -).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  14.3, 21.6, 26.2, 29.5-30.7, 32.2, 33.7, 43.4 and 66.7.  $^{31}\text{P}$  NMR ( $\text{CDCl}_3$ )  $\delta$  9.6. HRMS (MALDI-TOF)  $m/z$  calcd for  $\text{C}_{30}\text{H}_{63}\text{BrNO}_3\text{P}$  [ $\text{M}^+$ ] 595.373; found 595.536.

*Synthesis of O,O-Diethyl-N-(2-bromoethyl)phosphoramidate 16b:* To diethylphosphite (1.0 g, 7.2 mmol), 2-bromoethylamine bromohydrate (1.6 g, 8.0 mmol) and bromotrichloromethane (230  $\mu$ L, 16.0 mmol) in anhydrous dichloromethane (10 mL) at 0  $^{\circ}$ C, was slowly added DIPEA (4.3 mL, 16.0 mmol). The reaction was stirred for 1 h at 0  $^{\circ}$ C then 3 h at RT. Solvent was evaporated in vacuo, and ether was added (20 mL). The precipitate was removed by filtration on Celite. The filtrate was concentrated to produce **16b** as a viscous oil (1.8 g, 95% yield). The oily product was engaged in the next step without further purification.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.30 (t,  $^3J_{\text{HH}} = 7.2$  Hz, 6H,  $\text{CH}_3$ -), 3.11 (bs, 1H,  $-\text{NH}$ -), 3.30 (m,  $^3J_{\text{HH}} = 6.0$  Hz, 2H,  $-\text{CH}_2\text{-NH}$ ), 3.46 (t,  $^3J_{\text{HH}} = 6.0$  Hz, 2H,  $-\text{CH}_2\text{-Br}$ ), 4.03 (m,  $^3J_{\text{HH}} \sim ^3J_{\text{HP}} = 6.5$  Hz, 4H,  $-\text{CH}_2\text{-O}$ -).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  16.3, 33.4, 43.1, and 62.3.  $^{31}\text{P}$  NMR ( $\text{CDCl}_3$ )  $\delta$  8.8. HRMS (MALDI-TOF)  $m/z$  calcd for  $\text{C}_6\text{H}_{15}\text{BrNO}_3\text{P}$  [ $(\text{M}+\text{H})^+$ ] 260.005; found 259.971.

*Synthesis of O,O-Ditetradecyl-N-(2-iodoethyl)phosphoramidate 17a:* Compound **16a** (375 mg, 6.8 mmol) and sodium iodide (1.4 g, 9.0 mmol) were combined in tetrahydrofurane (5 mL). The solution was refluxed overnight. After cooling, the volatiles were removed in vacuo and ether (10 mL) was added. The solution was filtered on Celite to remove sodium bromide

and the filtrate was concentrated to furnish compound **17a** as brown solid (331 mg, 82% yield) which was engaged in the next step without further purification.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  0.83 (t,  $^3J_{\text{HH}} = 7.2$  Hz, 6H,  $\text{CH}_3$ -), 1.23 (m, 44H), 1.63 (m, 4H,  $-\text{CH}_2$ -), 3.19-3.22 (m, 4H,  $-\text{CH}_2$ -), 3.59 (bs, 1H,  $-\text{NH}$ -), 3.97 (m,  $^3J_{\text{HH}} \sim ^3J_{\text{HP}} = 6.5$  Hz, 4H,  $-\text{CH}_2\text{-O}$ -).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  6.9, 14.4, 21.7, 25.9, 29.5-33.2, 44.2, and 66.9.  $^{31}\text{P}$  NMR ( $\text{CDCl}_3$ ) 8.5. HRMS (MALDI-TOF)  $m/z$  calcd for  $\text{C}_{30}\text{H}_{63}\text{INO}_3\text{P}$   $[(\text{M}+\text{H})^+]$  644.367; found 644.356.

*Synthesis of O,O-Diethyl-N-(2-iodoethyl)phosphoramidate 17b:* Compound **16b** (1.8 g, 6.8 mmol) and sodium iodide (1.4 g, 9.0 mmol) were combined in tetrahydrofuran (20 mL). The solution was refluxed overnight. After cooling, the volatiles were removed in vacuo and ether (20 mL) was added. The solution was filtered on Celite to remove sodium bromide and the filtrate was concentrated to furnish compound **16b** as a viscous oil (1.5 g, 72% yield) which was engaged in the next step without further purification.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  1.25 (t,  $^3J_{\text{HH}} = 7.2$  Hz, 6H,  $\text{CH}_3$ -), 3.12 (bs, 1H,  $-\text{NH}$ -), 3.30 (m,  $^3J_{\text{HH}} = 6.0$  Hz, 2H,  $-\text{CH}_2\text{-NH}$ ), 3.36 (m,  $^3J_{\text{HH}} = 6.0$  Hz, 2H,  $-\text{CH}_2\text{-I}$ ), 4.10 (m,  $^3J_{\text{HH}} \sim ^3J_{\text{HP}} = 6.5$  Hz, 4H,  $-\text{CH}_2\text{-O}$ -).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  6.9, 16.4, 43.9 and 62.9.  $^{31}\text{P}$  NMR ( $\text{CDCl}_3$ ) 8.6. HRMS (MALDI-TOF)  $m/z$  calcd for  $\text{C}_6\text{H}_{15}\text{INO}_3\text{P}$   $[(\text{M}+\text{H})^+]$  307.991; found 307.958.

*Synthesis of Ditetradecylphosphatidyl-2-aminoethyltrimethylarsonium Iodide 1 (BSV77):* Trimethylarsine (186 mg, 1.6 mmol) was added to a solution of compound **17a** (500 mg, 0.8 mmol) in anhydrous tetrahydrofuran (1 mL) placed in a Schlenk tube equipped with an efficient reflux condenser ( $-20$  °C). The solution was placed in an oil bath and heated at  $60$  °C for 48 h. The volatiles were removed in vacuo. The residue was dissolved in chloroform (2 mL) and purified by flash chromatography on silica gel ( $\text{CH}_2\text{Cl}_2/\text{MeOH}$ , 9/1) to produce compound **1** as a colourless solid (446 mg, 73% yield).  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  0.88 (t, 6H,  $^3J_{\text{HH}} = 7.2$  Hz, 6H,  $\text{CH}_3$ -), 1.26 (m, 44H), 1.66 (m, 4H,  $-\text{CH}_2$ -), 2.23 (s, 9H), 3.15 (t,  $^3J_{\text{HH}} = 7.5$  Hz, 2H,  $\text{CH}_2$ -), 3.52 (m,  $^3J_{\text{HH}} = 7.5$  Hz, 2H,  $\text{CH}_2\text{-NH}$ ), 4.38 (m,  $^3J_{\text{HH}} \sim ^3J_{\text{HP}} = 6.5$

Hz, 4H, -CH<sub>2</sub>-O-), 4.48 (bs, 1H). <sup>31</sup>P NMR (162 MHz, CDCl<sub>3</sub>): 8.86. <sup>13</sup>C NMR (300 MHz, CDCl<sub>3</sub>): 10.19, 14.45, 23.02, 25.93, 29.25-30.74, 32.26, 36.40, 67.50. HRMS (MALDI-TOF): m/z calcd. for C<sub>33</sub>H<sub>72</sub>AsINO<sub>3</sub>P [(M<sup>+</sup>-I)]: 636.446; found 636.488.

*Synthesis of Diethylphosphatidyl-2-aminoethyltrimethylarsonium Iodide 6 (BSV83):* Trimethylarsine (586 mg, 5.0 mmol) was added to a solution of compound **17b** (500 mg, 1.6 mmol) in anhydrous tetrahydrofuran (1 mL) placed in a Schlenk tube equipped with an efficient reflux condenser (-20 °C). The solution was placed in an oil bath heated at 60 °C for 48 h. The volatiles were removed in vacuo. The residue was dissolved in chloroform (2 mL) and purified by flash chromatography on silica gel (CH<sub>2</sub>Cl<sub>2</sub>/MeOH, 9/1) to produce compound **6** as a viscous oil (510 mg, 73% yield). <sup>1</sup>H NMR(CDCl<sub>3</sub>) δ 1.18 (t, <sup>3</sup>J<sub>HH</sub> = 7.2 Hz, 6H, CH<sub>3</sub>-), 2.22 (s, 9H), 2.22 (s, 9H), 3.13 (t, <sup>3</sup>J<sub>HH</sub> = 6.0 Hz, 2H, -CH<sub>2</sub>-), 3.48 (m, 2H, -CH<sub>2</sub>-), 4.03 (dd, <sup>3</sup>J<sub>HH</sub> ~ <sup>3</sup>J<sub>HP</sub> = 6.6 Hz, 4H, -CH<sub>2</sub>-O-), 4.71 (m, 1H, -NH-). <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 9.8, 16.3, 18.6, 36.0 and 63.0. <sup>31</sup>P (CDCl<sub>3</sub>) 8.9. HRMS (MALDI-TOF) m/z calcd for C<sub>9</sub>H<sub>24</sub>INO<sub>3</sub>PA [(M<sup>+</sup>- I)] 300.070; found 300.036.

*Liposomal formulations:* All cationic lipids (except **7**) were formulated as liposomal solutions by the film hydration method, as previously described.<sup>[9]</sup> Lipids were mixed in chloroform then the solvent was evaporated. After drying, the film was hydrated for 12 h at 4 °C in 500 mL of 10 mM Hepes buffer, pH 7.4. It was vortexed and sonicated for 15 min at 20 °C at 37 kHz using an ultrasonic water bath (Bioblock Scientific). Liposomal solution of **7** was obtained by the ethanolic injection method, as previously reported.<sup>[30]</sup> Finally, the mean diameter and zeta potential of the liposomes were determined by dynamic light scattering, using a 3,000 Zetasizer (Malvern Instruments) at 25 °C with a measuring angle of 90° to the incident beam. Liposomal formulations were kept at 4 °C until subsequent use.

*Bacterial strains:* The various bacterial strains evaluated herein were as follows: three *S. aureus* (the laboratory strain RN4220,<sup>[17]</sup> and the clinical strains Newman<sup>[18]</sup> and MRSA

N315<sup>[19]</sup>), one *E. coli* (the laboratory strain MG1655<sup>[20]</sup>) and two *P. aeruginosa* (the clinical strains PA130709 and PA240709, that were isolated from CF patients during the course of this study). All bacterial routine handlings were conducted with Luria Bertani (LB) broth at 37 °C. A complete antibiotic resistance profile was established for each strain (see Table S2).

*Plasmid DNA:* Three different luciferase reporter pDNA were employed. For *in vitro* experiments, the pEGFP-Luc (6.4 Kb, Clontech) and the pGLuc (5.8 kb, New England Biolabs) were used. The pEGFP-Luc encodes a fusion of two reporter genes, the enhanced green fluorescent protein (EGFP; Abs/Em=488/507 nm) and the firefly *Photinus pyralis* luciferase; this pDNA allows evaluating the transfection activity via flow cytometry and luminometry. The pGLuc contains the “humanized” coding sequence for the secreted *Gaussia* Luciferase under the control of the cytomegalovirus promoter, for constitutive expression in mammalian cells; the gene transfer efficacy is readily detectable by dosing samples from the supernatant. For *in vivo* transfection experiments, we used the pTG11033 (9.6 kb, Transgene) which contains the firefly luciferase cDNA (under control of the strong cytomegalovirus immediate early, IE-CMV, promoter) and a HMG-1 intron (for enhanced transgene protein synthesis via increased mRNA export from the nucleus). All these pDNA were amplified in *E. coli* DH5 $\alpha$  bacteria, extracted by alkaline lysis, and purified using the Giga Prep Plasmid Purification kit (Qiagen), according to the manufacturer’s protocol. They were dissolved in endotoxin-free water and quantified by spectrophotometry at 260 nm. Only pDNA showing a  $A_{260}/A_{280}$  ratio between 1.8 and 2.0 were used.

*Antibacterial assays:* Minimal active antibacterial concentrations, either bacteriostatic (MIC) or bactericidal (MBC), were determined using a liquid broth micro-dilution method, as previously described.<sup>[22,23]</sup> Briefly, the reagents were first diluted in water then mixed with LB broth inoculated with one bacterial strain, in a final volume of 200  $\mu$ L per condition (i.e., 50  $\mu$ L of diluted reagent in water + 150  $\mu$ L of inoculated medium), in order to test concentrations

ranging from 1 to 200  $\mu\text{M}$ . Inoculums varied between  $10^7$  and  $10^8$  colony forming unit (CFU) per mL, in accordance with approved standards and previous studies.<sup>[31]</sup> After at least 24 h at 37 °C, bacterial growth was evaluated by considering medium turbidity (absorbance measurements at 650 nm). In order to discriminate between bacteriostatic or bactericidal effects, a sample of each liquid culture was spread onto nutritive non-selective agar plates. By performing this at several time points of the incubation, it was possible to estimate the minimal exposure time and concentration that were both required to obtain a complete bactericidal effect.

*Combined antibacterial and transfection assays – Sequential assay:* In this assay, lipoplexes were evaluated with regard to their abilities to kill a *S. aureus* strain and protect DNA while remaining efficient in transient transfection experiments using human bronchial epithelial cells. Lipoplexes were prepared by mixing the pEGFP-Luc to dilutions of cationic lipids, in order to form complexes characterized by increasing CR. These mixtures were kept at RT for 30 min then incubated with the bacterial strain RN4220 at 37 °C for 24 h. Agarose gel electrophoresis shift assays of lipoplexes were performed in order to evaluate the DNA compaction within the lipoplexes formed in each case; dextran sulfate was also used to recover free DNA from lipoplexes, thereby allowing assessing its integrity. Finally, lipoplexes were directly added dropwise into the antibiotic containing medium (EMEM) of 16HBE 14o-eukaryotic cells<sup>[35]</sup> that were seeded 24 h before in 96-well plates. After 24 h at 37 °C, cells were lysed using the Passive Lysis Buffer (Promega). For each supernatant, the luciferase activity was measured using the Firefly Luciferase Assay System (Promega) and the total protein content was quantified using the BC Assay kit (Interchim). Results were expressed as RLU (relative light units) per milligram of total protein.

*Combined antibacterial and transfection assays – Simultaneous assay:* In this assay, lipoplexes were evaluated by considering their simultaneous ability to inhibit or kill a *S.*

*aureus* strain and to deliver a reporter pDNA into human bronchial epithelial cells. Lipoplexes were prepared by mixing the pGLuc to dilutions of cationic lipids, in order to form complexes characterized by increasing CR. These mixtures were thereafter mixed the bacterial strain N315 then directly added dropwise into the antibiotic free medium (EMEM) of 16HBE 14o-eukaryotic cells that were seeded 24 h before in 96-well plates. During 55 h, samples of the supernatants (5  $\mu$ L each) were collected at regular time intervals in order to be assayed, as follows. The antibacterial activity was first estimated by considering variations of the media colour and further evaluated by measuring the adenylate kinase activity using the Toxilight kit (Lonza); results were expressed as a percentage of the maximal activity. The transfection efficiency was determined by evaluating the luciferase activity using the Gaussia Luciferase Assay System (Promega); results were expressed as RLU (relative light units) per  $\mu$ L.

*Animals:* Six to nine weeks old female Swiss mice (Janvier breeding center) were housed and maintained at the University animal facility; they were processed in accordance with the Laboratory Animal Care Guidelines (NIH publication #85–23 revised 1985) and with the agreement of the regional veterinary services (authorization FR; 29–024).

*Local in vivo administration:* Lipoplexes were prepared at RT by mixing, in water, cationic liposomes with PE-PEG5000 and pTG11033 at CR4. They were then mixed with  $10^7$  CFU  $\text{mL}^{-1}$  of the bacterial strain N315. After an overnight incubation at 37 °C, the bactericidal effect was verified by spreading an aliquot of the mixtures onto a nutritive non selective agar plate. These mixtures were then administered *in vivo* in mice via nasal deposition: animals were first anesthetized via ketamin/xylozain intraperitoneal (i.p.) injection (40 mg  $\text{kg}^{-1}$  animal); next, 100  $\mu$ L of complexes incorporating 70  $\mu$ g of pDNA were instilled via the right nostril of each animal within 30 min, using a catheter (< 0.5 mm outer diameter) connected to a 100  $\mu$ L syringe (Hamilton 1710RN) mounted on a pump (Harvard Apparatus). Once instilled, animals were placed in a box near a heat source until they recovered from



anesthesia. A total of 6 animals received 2-based lipoplexes that were previously mixed (3 mice) or not (3 mice) with bacteria; 2 mice received 70  $\mu\text{g}$  of naked (uncomplexed) pDNA.

*Systemic in vivo administration:* Lipoplexes were prepared at RT by mixing, in 10% serum 0.9% NaCl (a medium suitable for both bacterial growth and *in vivo* systemic injection), cationic liposomes with pTG11033 at CR4. They were then mixed with  $10^8$  CFU  $\text{mL}^{-1}$  of the bacterial strain N315. After an overnight incubation at 37 °C, the bactericidal effect was confirmed by spreading an aliquot of the mixtures onto a nutritive non selective agar plate. Next, these mixtures were intravenously administered *in vivo* in mice. Animals were first placed in a restrainer and 300  $\mu\text{L}$  of complexes incorporating 50  $\mu\text{g}$  of pDNA were injected into the tail vein of each animal within 5 to 10 sec., using a ½ inch 26-gauge needle and a 1 mL syringe. A total of 6 animals received 2-based lipoplexes that were previously mixed (3 mice) or not (3 mice) with bacteria; 2 mice received 50  $\mu\text{g}$  of naked (uncomplexed) pDNA.

*In vivo bioluminescence imaging of luciferase activity:* Mice received first an i.p. injection of luciferin (0.16  $\text{g kg}^{-1}$  animal; Interchim). Three minutes later, animals were anesthetized by isofluran inhalation (4% air-isofluran blend) or ketamin/xylazin i.p. injection (40  $\text{mg kg}^{-1}$  animal). Next, animals were laid inside the acquisition chamber of an *in vivo* imaging system equipped with a cooled, slow-scan, CCD camera (NightOwl, Berthold) and driven with associated software (WinLight 32, Berthold). Five minutes after luciferin injection, luminescence images were captured at 8 x 8 binning with exposure times ranging from 2 to 4 min, depending on the luminescence intensity. Finally, luminescence images were superimposed onto still images of each mouse. Signal intensities were quantified within the regions of interest as photons per pixel per second.

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BSV83\_ccdc\_deposit contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via [www.ccdc.cam.ac.uk/data\\_request/cif](http://www.ccdc.cam.ac.uk/data_request/cif).

### Supporting Information

Supporting Information is available online from the Wiley Online Library or from the author.

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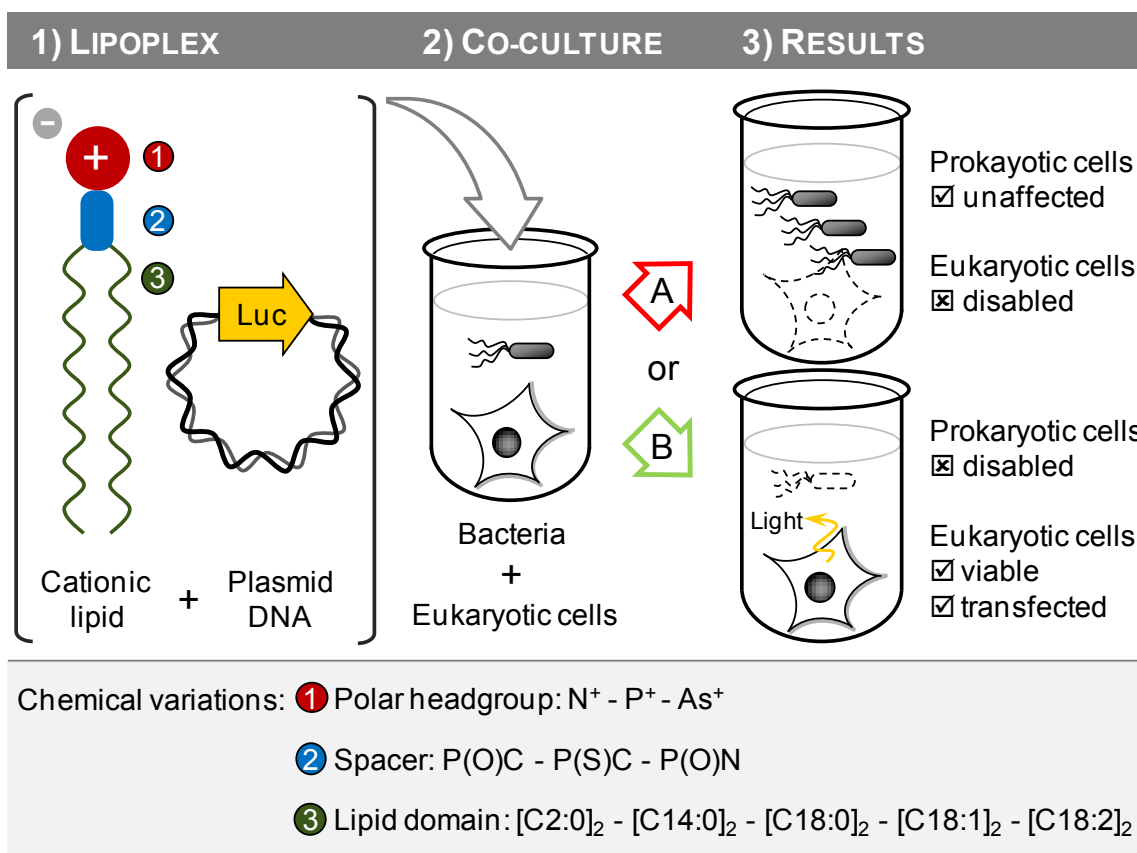
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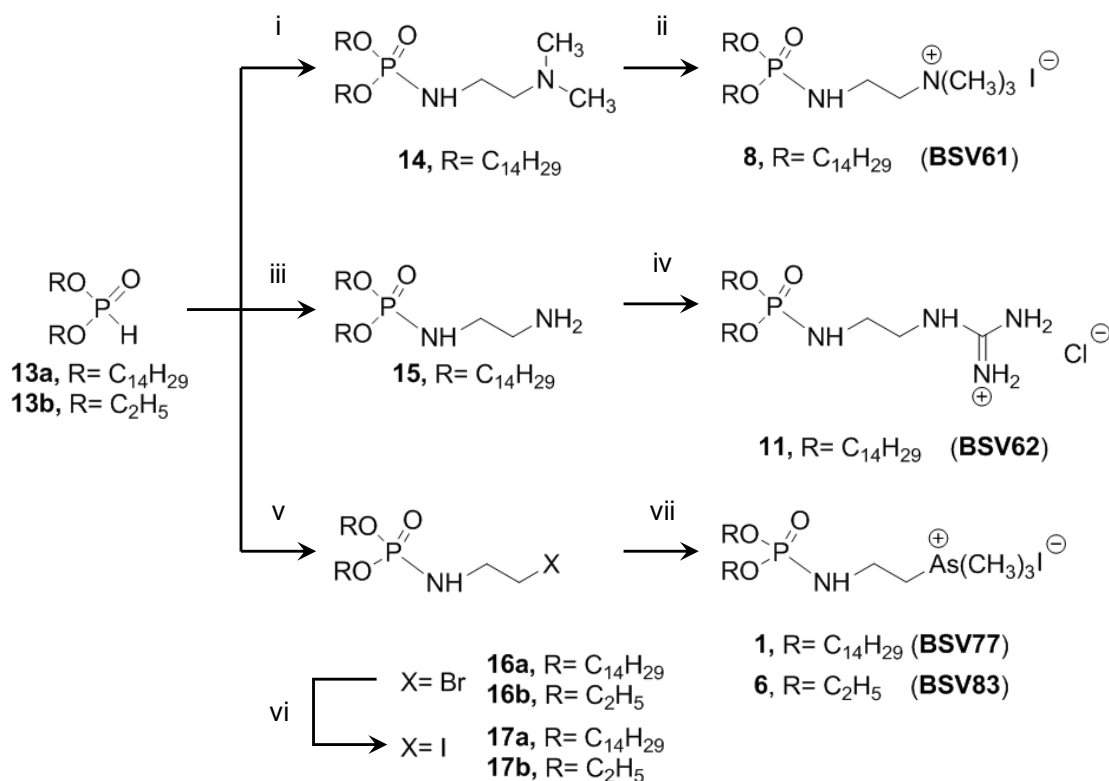
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**Figure 1.** Schematic illustration of the purpose of this study. Cationic lipids displaying chemical variations according to three structural parts are mixed with a luciferase (Luc) reporter plasmid DNA. The lipoplexes obtained are evaluated as regard their effects towards bacteria and eukaryotic cells incubated together. This protocol may allow distinguishing between at least two types of complexes: **(A)** lipoplexes exhibiting no or insufficient antibacterial effectiveness cannot prevent bacterial growth which in turn results in eukaryotic cell disabling. **(B)** On contrary, when prepared with cationic lipids incorporating suitable structural components, complexes could be able to achieve simultaneously, i.e. at the same time and in one pot, (i) biocide effect towards prokaryotic cells together with (ii) efficient and secured gene transfer into eukaryotic cells, leading to reporter gene expression.

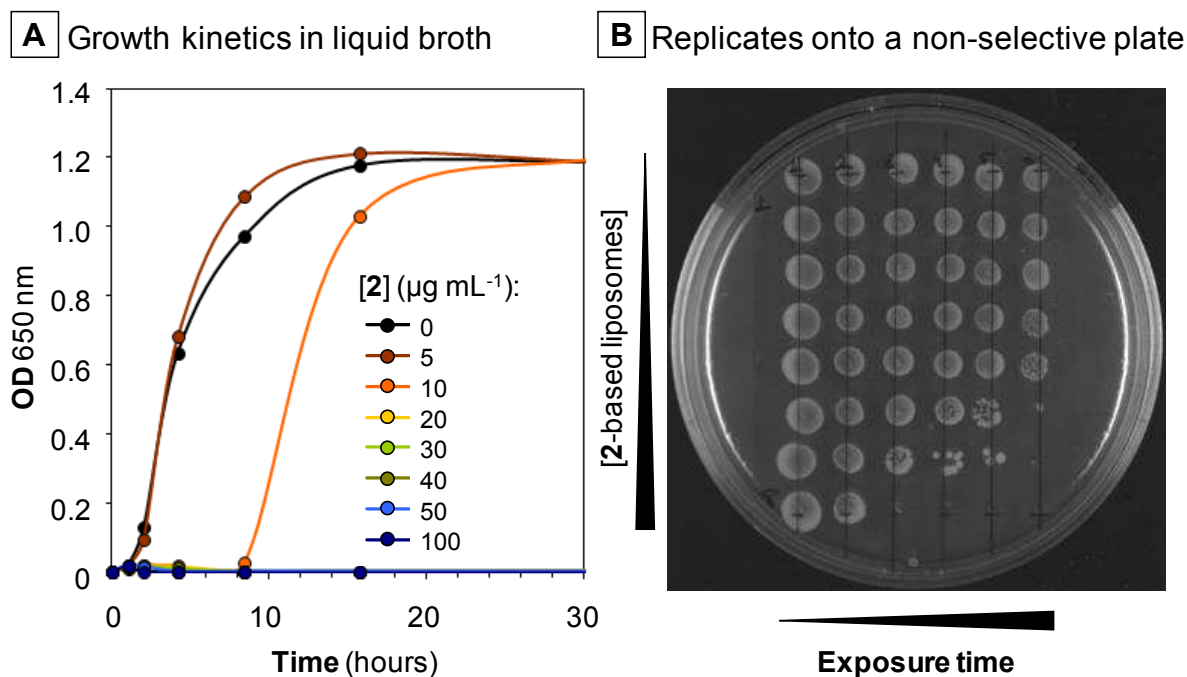


**Figure 2.** Reaction scheme for the synthesis of cationic lipids **1**, **6**, **8**, and **11**. Reagents and conditions: (i) H<sub>2</sub>N(CH<sub>2</sub>)<sub>2</sub>N(CH<sub>3</sub>)<sub>2</sub>, CBrCl<sub>3</sub>, DIPEA, CH<sub>2</sub>Cl<sub>2</sub>, 0 °C for 1 h then RT for 5 h, 85%. (ii) CH<sub>3</sub>I, CH<sub>2</sub>Cl<sub>2</sub>, reflux, O/N, 92%. (iii) H<sub>2</sub>N(CH<sub>2</sub>)<sub>2</sub>NH<sub>2</sub>, CBrCl<sub>3</sub>, DIPEA, CH<sub>2</sub>Cl<sub>2</sub>, 0 °C for 1 h then RT for 5 h, 85%. (iv) 1*H*-pyrazole-1-carboxamide hydrochloride, EtOH, reflux, O/N, 88%. (v) HBr.H<sub>2</sub>N(CH<sub>2</sub>)<sub>2</sub>Br, CBrCl<sub>3</sub>, DIPEA, CH<sub>2</sub>Cl<sub>2</sub>, 0 °C for 1 h then RT for 3 h, 90% for R= C<sub>14</sub>H<sub>29</sub> and 95% for R= C<sub>2</sub>H<sub>5</sub>. (vi) NaI, THF, reflux, O/N, 72% for R= C<sub>14</sub>H<sub>29</sub> and 82% for R= C<sub>2</sub>H<sub>5</sub>. (vii) AsMe<sub>3</sub>, THF, 60 °C for 48 h, 82% for R= C<sub>14</sub>H<sub>29</sub> and 73% for R= C<sub>2</sub>H<sub>5</sub>.

**Table 1.** Chemical structure, numbering, and antibacterial effect of synthetic cationic lipids and benchmark compounds.

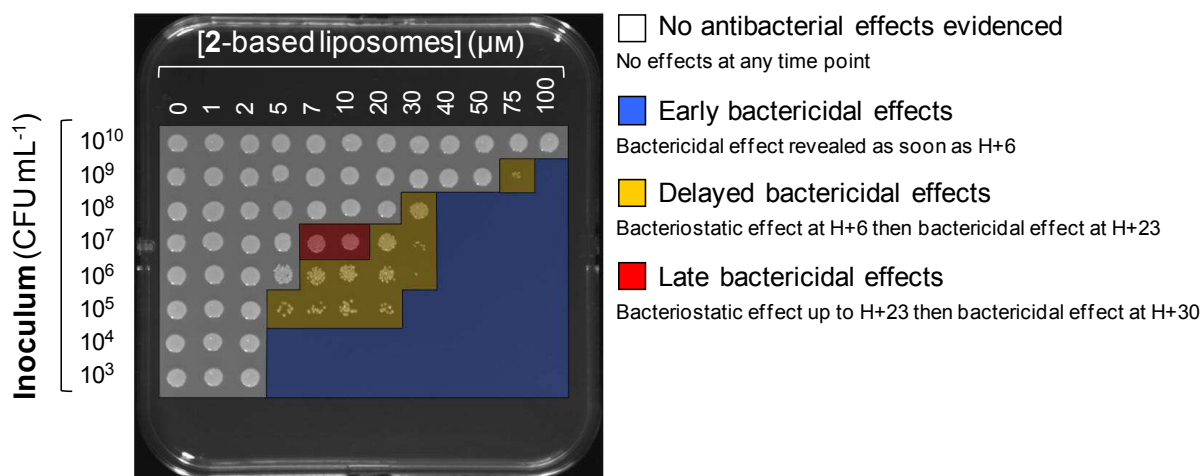
Cationic compound molecular structure <sup>a)</sup> (lipid chain code)-linker-headgroup, counter ion	Number (name)	Sa <sup>b)</sup> RN4220	Sa <sup>b)</sup> Newman	Sa <sup>b)</sup> N315	Ec <sup>b)</sup> MG1655	Pa <sup>b)</sup> 240709
(C <sub>14</sub> H <sub>29</sub> O) <sub>2</sub> -P(O)-NH-(CH <sub>2</sub> ) <sub>2</sub> -As <sup>+</sup> (CH <sub>3</sub> ) <sub>3</sub> , I <sup>-</sup>	<b>1</b> (BSV77)	1 <sup>c)</sup>	5	5	75	>200
(C <sub>18</sub> H <sub>33</sub> O) <sub>2</sub> -P(O)-NH-(CH <sub>2</sub> ) <sub>2</sub> -As <sup>+</sup> (CH <sub>3</sub> ) <sub>3</sub> , I <sup>-</sup>	<b>2</b> (BSV4)	1-5	10	10	200	>200
(C <sub>18</sub> H <sub>35</sub> O) <sub>2</sub> -P(O)-NH-(CH <sub>2</sub> ) <sub>2</sub> -As <sup>+</sup> (CH <sub>3</sub> ) <sub>3</sub> , I <sup>-</sup>	<b>3</b> (KLN47)	5	20	20	>200	>200
(C <sub>14</sub> H <sub>29</sub> O) <sub>2</sub> -P(O)-(CH <sub>2</sub> ) <sub>3</sub> -As <sup>+</sup> (CH <sub>3</sub> ) <sub>3</sub> , I <sup>-</sup>	<b>4</b> (BSV98)	30	40	40	>200	>200
(C <sub>18</sub> H <sub>37</sub> O) <sub>2</sub> -P(O)-NH-(CH <sub>2</sub> ) <sub>2</sub> -As <sup>+</sup> (CH <sub>3</sub> ) <sub>3</sub> , I <sup>-</sup>	<b>5</b> (BSV82)	40	50	200	>200	>200
(C <sub>2</sub> H <sub>5</sub> O) <sub>2</sub> -P(O)-NH-(CH <sub>2</sub> ) <sub>2</sub> -As <sup>+</sup> (CH <sub>3</sub> ) <sub>3</sub> , I <sup>-</sup>	<b>6</b> (BSV83)	>200	>200	>200	>200	>200
(C <sub>18</sub> H <sub>35</sub> O) <sub>2</sub> -P(S)-NH-(CH <sub>2</sub> ) <sub>2</sub> -As <sup>+</sup> (CH <sub>3</sub> ) <sub>3</sub> , I <sup>-</sup>	<b>7</b> (BSV21)	>200	>200	>200	>200	>200
(C <sub>14</sub> H <sub>29</sub> O) <sub>2</sub> -P(O)-NH-(CH <sub>2</sub> ) <sub>2</sub> -N <sup>+</sup> (CH <sub>3</sub> ) <sub>3</sub> , I <sup>-</sup>	<b>8</b> (BSV61)	>200	>200	>200	>200	>200
(C <sub>18</sub> H <sub>35</sub> O) <sub>2</sub> -P(O)-NH-(CH <sub>2</sub> ) <sub>2</sub> -N <sup>+</sup> (CH <sub>3</sub> ) <sub>3</sub> , I <sup>-</sup>	<b>9</b> (BSV36)	>200	>200	>200	>200	>200
(C <sub>14</sub> H <sub>29</sub> O) <sub>2</sub> -P(O)-NH-CH <sub>2</sub> -N <sup>+</sup> (CH <sub>3</sub> ) <sub>3</sub> , I <sup>-</sup>	<b>10</b> (GLB43)	>200	>200	>200	>200	>200
(C <sub>14</sub> H <sub>29</sub> O) <sub>2</sub> -P(O)-NH-(CH <sub>2</sub> ) <sub>2</sub> -N <sup>+</sup> H=C(NH <sub>2</sub> ) <sub>2</sub> , Cl <sup>-</sup>	<b>11</b> (BSV62)	>200	>200	>200	>200	>200
(C <sub>18</sub> H <sub>35</sub> O) <sub>2</sub> -P(O)-NH-(CH <sub>2</sub> ) <sub>2</sub> -P <sup>+</sup> (CH <sub>3</sub> ) <sub>3</sub> , I <sup>-</sup>	<b>12</b> (BSV99)	10	50	70	200	nd
(C <sub>18</sub> H <sub>35</sub> O) <sub>2</sub> -P(O)-NH-(CH <sub>2</sub> ) <sub>3</sub> -P <sup>+</sup> (CH <sub>3</sub> ) <sub>3</sub> , I <sup>-</sup>	<b>13</b> (BSV100)	>200	>200	>200	>200	nd
Lipofectamine <sup>®</sup> (Invitrogen)	-	>200	>200	>200	>200	>200
DDYSA <sup>d)</sup>	<b>14</b>	200	200	200	100	75
Ampicillin sodium salt	-	1	1	100	25	>200
Kanamycin A monosulfate	-	25	10	>200	5	100
Tetracyclin hydrochloride	-	1	1	1	5	200

<sup>a)</sup> see Figure S1 for the detailed chemical structure of cationic lipids; <sup>b)</sup> Sa, *S. aureus*; Ec, *E. coli*; Pa, *P. aeruginosa*; <sup>c)</sup> Susceptibilities reported as MBC in  $\mu\text{mol per L}$  ( $\mu\text{M}$ ), as determined using the liquid broth micro-dilution assay. Bacterial inoculums ranged from  $10^6$  to  $10^7$  colony forming unit (CFU) per mL. MBC were determined using liposomal formulations prepared at different times (median values, with  $n \geq 3$ ); <sup>d)</sup> DDYSA: (1,3-Dimethyl-4,5-Dichloroimidazole-2-Ylidene)- Silver(I) Acetate. BSV, Brest Synthetic Vector; nd, not determined. Of note, the results obtained using PA240709 (shown here) and PA130709 (not shown) were very similar.

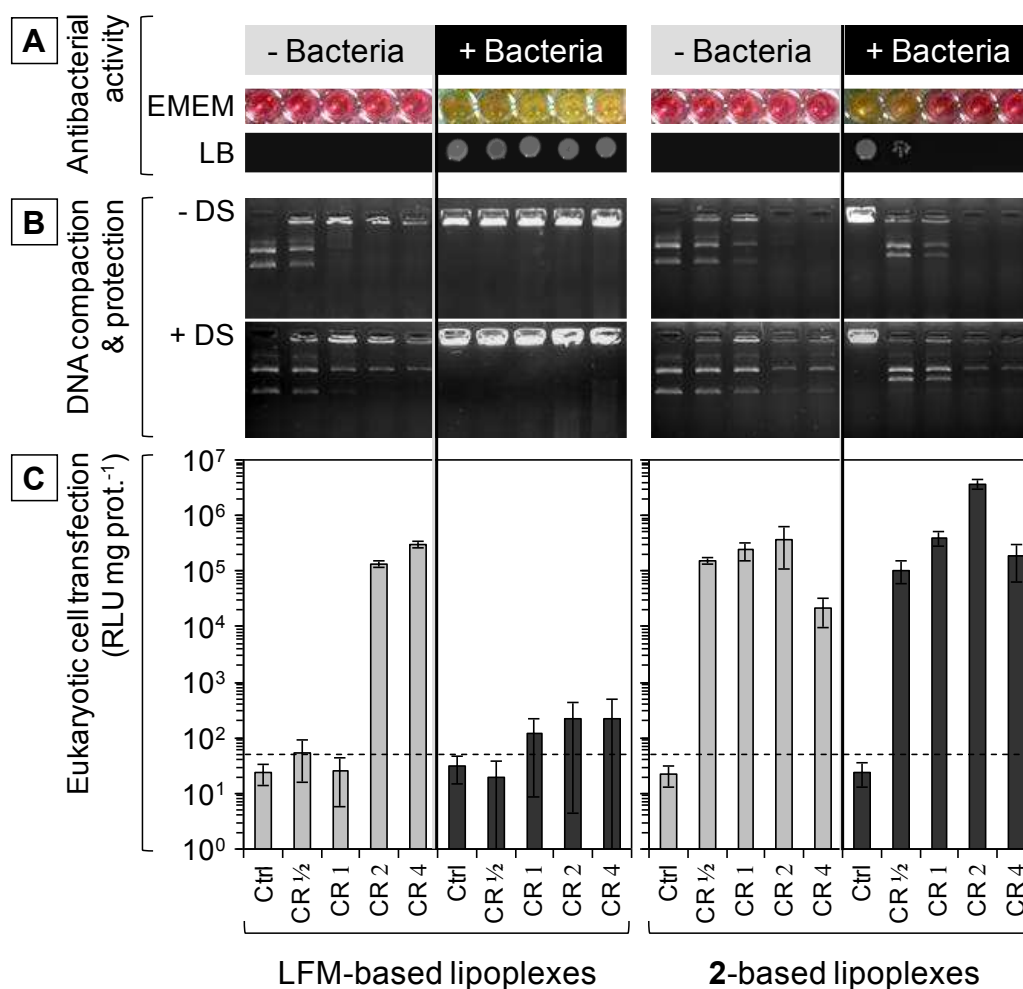


**Figure 3.** Liposomes prepared with an arsonium-containing lipophosphoramidate can exhibit potent bactericidal but also bacteriostatic effects. Different quantities of **2**-based liposomes were diluted in LB broth inoculated with the *S. aureus* strain RN4220. These mixtures were incubated at 37 °C for several hours. At different time points, bacterial growths were assessed spectroscopically by measuring the absorption at 650 nm (**A**); samples were dotted onto a nutritive non-selective agar plate. After an overnight incubation at 37 °C, growths on solid medium (**B**) allowed to distinguish between either a bacteriostatic or a bactericidal effect as a result of the (i) exposure time with **2** and (ii) its dose in liquid broth. Of note, a bactericidal effect was obtained after 32 h of exposure with 20  $\mu\text{g mL}^{-1}$  of **2** (not shown). The 6 time points in **B** are circled in **A**.

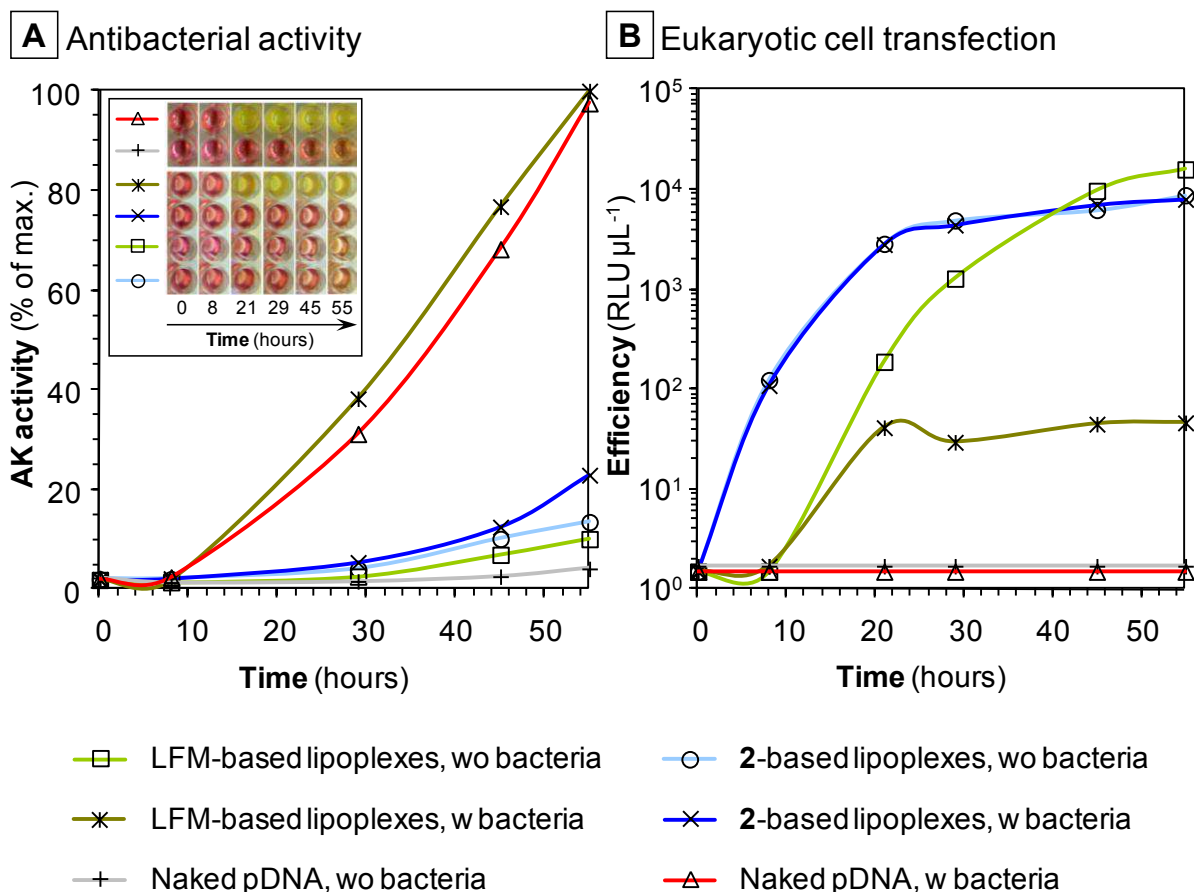




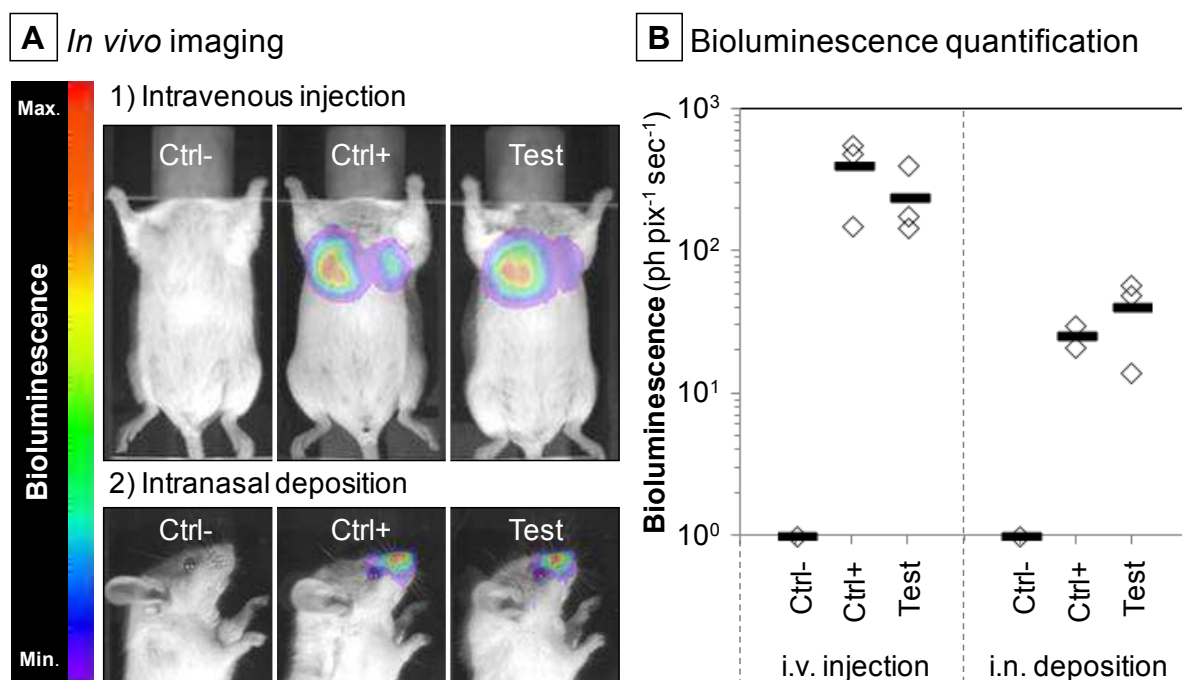
**Figure 4.** Liposomes prepared with an arsonium-containing lipophosphoramidate can exhibit biocide effects up to very high bacterial density and regardless of bacterial growth state. Different inoculums of the *S. aureus* strain RN4220 (ranging from  $10^{10}$ , i.e. pure O/N starved culture, to  $10^3$   $\text{CFU mL}^{-1}$ ) were mixed in LB broth with increasing quantities of **2**-based liposomes. These mixtures were then incubated at 37 °C for several hours. Plating an aliquot of each condition collected at three time points allowed evaluating the bacteriostatic and bactericidal effects of **2** according to (i) the number of bacterial cells initially present and (ii) their ability to continue growing.



**Figure 5.** Lipoplexes prepared with an arsonium-containing lipophosphoramidate can sequentially kill bacteria and then transfect eukaryotic cells *in vitro*. A constant amount of pDNA was mixed with increasing quantities of a cationic lipid (either LFM or **2**) in order to obtain lipoplexes characterized with different charge ratios (CR). These complexes were then diluted in EMEM (Eagle's minimal essential medium supplemented with 10% serum) inoculated with the *S. aureus* strain RN4220. After 24 h at 37 °C, the colour of the culture media (indicative of its pH) was considered as a marker of bacterial growth (pink, neutral pH, no growth; yellow, acidic pH, growth); bacterial growths were ascertained by replicating an aliquot of each condition onto a nutritive non-selective LB plate (A). Agarose gel electrophoresis was used to assess pDNA entrapment within lipoplexes; the dismantling of these complexes (using dextran sulfate) allowed verifying pDNA integrity (B). Finally, human bronchial epithelial cells were used to evaluate the transfection efficiency of each mixture, in the unit of relative light units (RLU) per mg of proteins (C). Ctrl, control (naked pDNA); DS, dextran sulfate.



**Figure 6.** Lipoplexes prepared with an arsonium-containing lipophosphoramidate can both inhibit bacteria and transfect eukaryotic cells in one pot. During 55 hours, 2-based lipoplexes (formed here at CR10) were assayed for their simultaneous ability to (1) inhibit the growth of the bacterial strain N315 and to (2) transfect human bronchial epithelial cells both cultivated in EMEM (without any antibiotics). LFM-based lipoplexes were used as controls. Antibacterial activity was monitored by considering the adenylate kinase (AK) activity (**A**) together with variations of the medium colour (inset in **A**). Transfection efficiency was quantitated as RLU per  $\mu\text{L}$  of supernatant (**B**). w, with; wo, without.

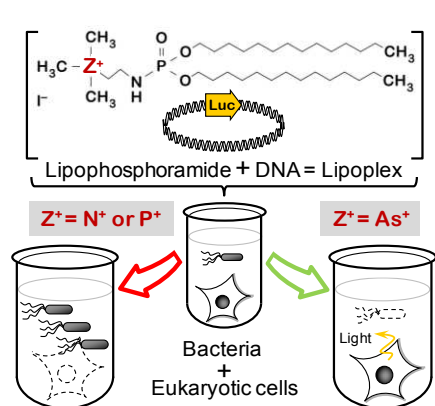


**Figure 7.** Lipoplexes prepared with an arsonium-containing lipophosphoramidate can both kill bacteria *in vitro* while retaining high gene transfection efficiency in mice *in vivo*. **2**-based lipoplexes (formed at CR4) were first mixed with a large inoculum ( $10^7$  to  $10^8$  CFU mL<sup>-1</sup>) of the bacterial strain N315. After 12 h at 37 °C, a complete bactericidal effect was obtained (not shown). These mixtures were thereafter administrated in mice via intravenous (i.v.) injection or intranasal (i.n.) deposition in order to determine their ability to transfect the lungs and the nasal epithelium, respectively. Lipoplexes that were not mixed with any bacteria were used to determine the transfection efficiency reference level. Luciferase gene expression in mice was evaluated 24 h post-administration (**A**). Transfection efficiencies were determined via bioluminescence quantification within the regions of interest in the unit of ph pix<sup>-1</sup> sec<sup>-1</sup> (**B**). Ctrl-, negative control (mice administrated with naked DNA); Ctrl+, positive control (mice administrated with lipoplexes that were not incubated with bacteria); Test (mice administrated with lipoplexes pre-incubated with bacteria).

## Arsonium-containing Lipophosphoramides, Poly-functional Nano-carriers for Simultaneous Antibacterial Action and Eukaryotic Cell Transfection

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Contrary to compounds exhibiting no ( $Z^+=N^+$ ) or insufficient ( $Z^+=P^+$ ) antibacterial effectiveness, arsonium ( $Z^+=As^+$ )-containing lipophosphoramides can simultaneously inhibit bacteria while safely and efficiently delivering DNA into eukaryotic cells, thereby leading to luciferase (Luc) reporter gene expression. Such poly-functional nano-carriers may therefore be particularly suitable for gene therapy

strategies under infected conditions such as within the airways of cystic fibrosis patients.