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COMMUNICATIONS

Cationic Lipophosphoramidates and Lipophosphoguanidines Are Very Efficient for in Vivo DNA Delivery

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Two new families of cationic lipids were designed and synthesized for gene delivery, namely "lipophosphoramidates" and "lipophosphoguanidines", whose efficiency was noteworthy. The most efficient have an arsonium cation as the polar head, and the unsaturated lipidic tails (e.g. oleyl) gave the better in vivo results (mice lungs).

INTRODUCTION

Although currently less efficient than viral vectors, synthetic vectors can encapsulate and, theoretically, carry out large genetic material (>100 kb) and do not lead to immunogenic response, hence a growing investment in the search for nonviral vectors with increased efficiency and lower toxicity. Among those, cationic lipids hold, together with polymers, the most important place (*1*). In these lipids, one must note that, with a few exceptions, the cationic or polycationic charge is always carried by nitrogen atoms. As an exception, precisely, we showed that in the case of cationic phosphonolipids, the replacement of an ammonium by a phosphonium or an arsonium polar head led to an increased efficiency (up to 7 times according to the cell lines tested) and decreased cytotoxicity (*2*).

Going on with our search for even more efficient vectors, we looked into the possibility of using the intrinsic properties of cells for designing synthetic vectors possessing a weak linker between the lipidic part and the polar cationic head, to split off the lipidic part after cell internalization. Indeed, the pH decrease, from more than 7 extracellular to 5−6 in endosomes and about 4 in some late lysosomes, is well established. Such a principle was recently described in novel cationic lipids incorporating an acid-sensitive acylhydrazone linker (*3*). Another example was the incorporation of an acid-labile vinyl ether linkage in the lipidic part of the cationic lipid (*4*). Something similar was recently described in the case of polymers: linear polyethyleneimine (PEI) reticulated with a linker including two ester bonds susceptible to hydrolysis at physiological conditions was more efficient and less toxic than reticular PEI (*5*).

In the field of phosphorylated compounds, the hydrolysis of the P(O)−N bond is well documented (*6*), and the

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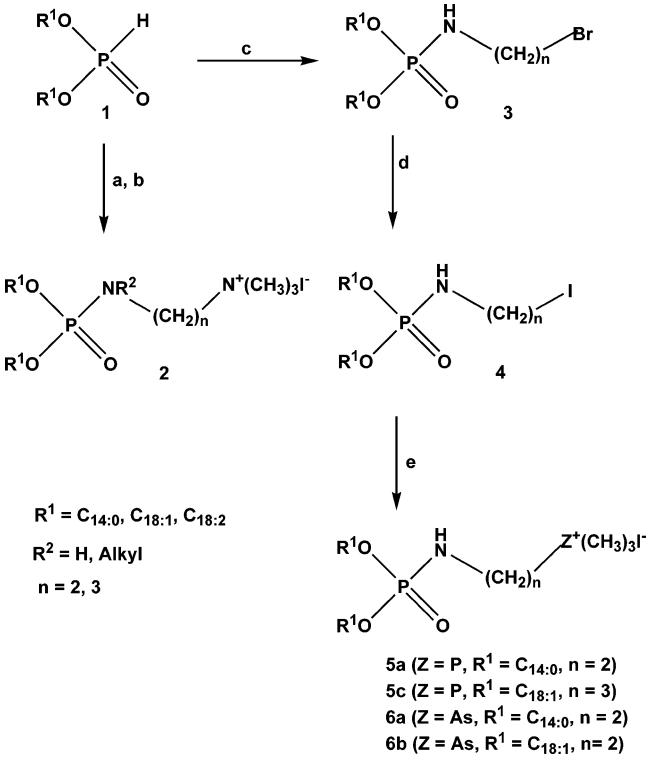
Scheme 1. Synthesis of Cationic Lipophosphoramidates[a]



[a] (a) $R^2HN(CH_2)_n$-$N(CH_3)_2/CCl_4$; (b) CH_3I, RT, 12 h; (c) $H_2N(CH_2)_nBr$,HBr/CCl_4; (d) NaI/acetone; (e) for Z = P: $(CH_3)_3P$/THF, 40 °C, 2 days; for Z = As: $(CH_3)_3As$, neat, 45 °C, 1 week.

dialkyl phosphite group is sometimes used as a protecting group for amines (*7*) and polyamines (*8*). Deprotection generally occurs easily in dilute mineral acids, but the P−N bond shows a relative stability in organic and Lewis acids (*7a*). Moreover and more significant, the enzymatic hydrolysis of the P−N bond of phosphoramidates by phosphoramidases active at pH 3.5−7.5 is well established (*9*).

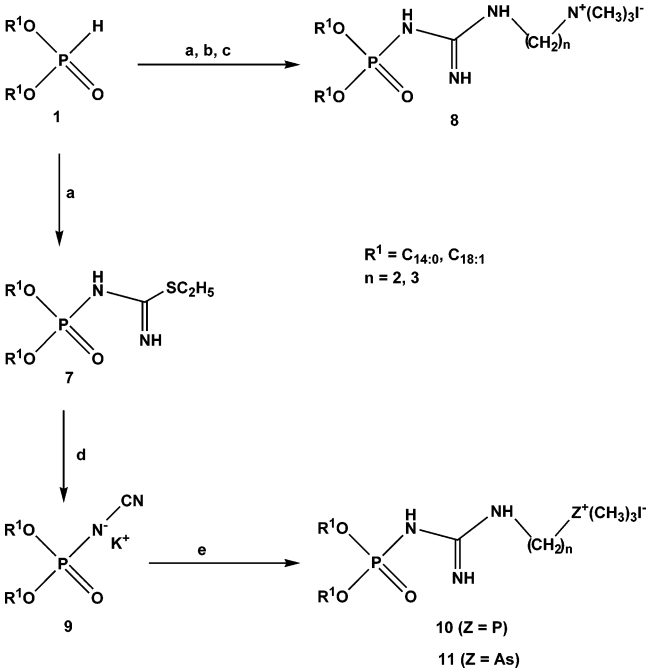
Taking these facts into account, we designed and synthesized two novel families of DNA monocationic carriers, namely "lipophosphoramidates" and "lipophosphoguanidines".

RESULTS

The main route leading to phosphoramidates is the reaction of a hydrogen phosphite with an amine, in the presence of CCl_4, under phase-transfer catalysis conditions (*10*) or not (*11*). We adapted these two procedures for reacting lipidic phosphites **1** with various diamines to get, after quaternisation with methyl iodide, a set of "lipophosphoramidates" **2** with an ammonium cation. Replacement of diamines by bromoalkylamines led to bromoalkylphosphoramidates **3**. After metathesis Br → I for increased reactivity, compounds **4** were reacted with trimethylphosphine or trimethylarsine, thus leading to lipophosphoramidates with trimethylphosphonium **5** or trimethylarsonium **6** cations, respectively (Scheme 1).

Hydrolysis of phosphoguanidines has been known for a long time because of many studies about conversion of phosphocreatine to creatinine and inorganic phosphate (*12*). With the same purpose as for lipophosphoramidates, we designed and synthesized cationic "lipophosphoguanidines". The first step of their synthesis was adapted for lipidic phosphites **1** from those of simple phosphoguanidines, i.e., the phosphorylation of 2-ethyl-2-thiopseudourea (*13*). Subsequent reaction with unsymmetrical diamines followed by quaternization of the terminal amine led to lipophosphoguanidines **8** with an ammonium cation (Scheme 2).

Scheme 2. Synthesis of Cationic Lipophosphoguanidines[a]



[a] (a) 2-Ethyl-2-pseudothiourea; (b) $H_2N(CH_2)_nN(CH_3)_2$; (c) CH_3I; (d) K_2CO_3/HgO; (e) $H_3N^+(CH_2)_nZ^+(CH_3)_3I^-$.

Dibenzyl (or diphenyl) phosphoryl isothioureas undergo ethanethiol elimination when reacted with sufficiently basic amines in the presence of HgO (*14*). In the case of lipophilic phosphoryl isothioureas **7**, replacement of amines by K_2CO_3 led to isolable potassium salts **9** which, upon reaction with aminoalkylphosphonium or arsonium hydrobromides led directly to "cationic lipophosphoguanidines" with a trimethyl phosphonium (**10**) or arsonium (**11**) cation, respectively (*15*).

Several examples of cationic lipids **2**, **5**, and **6** were submitted to transfection activity assays (*16*): in vitro (HeLa, CHO cell lines or CF nasal polyps primary cultures) and in vivo (mice lung). For in vitro experiments with HeLa cell line and CF primary culture, the luciferase activity was measured 2 days after transfection, using a chemiluminescent assay. For CHO, the percentage of GFP^+ cells was measured by Facs flow analysis. Whatever cell or technique used (*17*), the activity with lipid **6b** ($R^1 = C_{18:1}$, $n = 2$) or **8a** ($R^1 = C_{14:0}$, $n = 3$), for instance, was systematically 20−25% greater than DOTAP or Lipofectine, two commercial references, and corresponded to 25−30% of GFP^+ viable cells. More significant were the results of in vivo assays on mice lung (Figure 1) (the lipid-to-DNA charge ratio used was 4, as this ratio had been previously shown to be the optimal (*18*)). Lipids **5a** ($R^1 = C_{14:0}$, $n = 2$), **5c** ($R^1 = C_{18:1}$, $n = 3$), **6a** ($R^1 = C_{14:0}$, $n = 2$), and **6b** exhibit a luciferase expression up to 3600 times higher than DOTAP (*19*).

As checked on a model compound (**2**, $R^1 = C_2H_5$, $R^2 = CH_3$, $n = 3$, for solubility reasons) the acid-catalyzed hydrolysis of the P−N bond is not very fast (4 days for 100% P−N hydrolysis at pH = 2). So, the total hydrolysis of the P−N bond seems unnecessary to get the high levels of in vivo observed efficiency, since the highest expression levels were reached within 24 h (in vivo) or 48 h (in vitro).

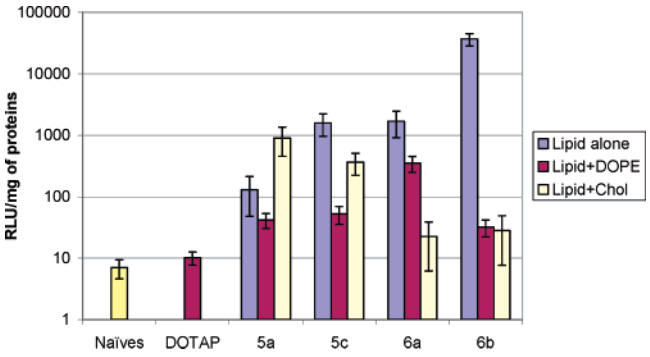


Figure 1.

But it may also be presumed that these noteworthy in vivo results are due to an enzymatic activity (which remains to be proved), accelerating the rate of the P−N bond hydrolysis, which does not work in vitro. Since this relevant efficiency seems accompanied by a weak cytotoxicity, a more complete biological evaluation is currently under investigation from our group.

ACKNOWLEDGMENT

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(16) Cationic lipids were dissolved in chloroform in glass vials. Chloroform was then evaporated by vacuum, resulting in a dry lipid film. Sterile pyrogen-free distilled water was added in appropriate amounts to the lipid. The vials were then sealed and stored overnight at 4 °C. The resulting solution was sonicated for 10 min in a bath sonicator. The plasmid and lipid were then combined and incubated for 30 min at room temperature to form the lipoplexes.

(17) For a preliminary in vitro and ex vivo biological evaluation of lipophosphoramidates, see Montier, T., Delépine, P., Marianowski, R., Le Ny, K., Le Bris, M., Gillet, D., Potard, G., Mondine, P., Frachon, I., Yaouanc, J. J., Clément, J. C., Des Abbayes, H., and Férec, C. (2004) CFTR transgene expression in primary ΔF 508 epithelial cell cultures from human nasal polyps following gene transfer with cationic phosphonolipids. *Mol. Biotechnol. 26,* 193−205.

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(19) For in vivo mice lung assays, the following protocol was implemented: Five-week-old female Swiss mice were used. Once anesthetized by ether inhalation, each mouse received 200 μL of the lipoplex solution containing 50 μg of plasmid encoding luciferase as an intravenous bolus in the tail vein. Lungs were harvested 24 h after injection. Each tissue was crushed in a mortar cooled by liquid nitrogen and incubated with lysis buffer (Promega) for 30 min. A centrifugation at 10 000*g* was performed, and supernatant luciferase activity was assayed in a 'MLX microtiter plate luminometer' (Dynex technologies) using a 'luciferase assay system' (Promega). Five mice were used for each time point. The total protein concentration of these supernatants was measured with the 'Coomassie Plus Protein Assay Kit' (Pierce). Results are expressed in RLU per mg of total proteins.

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