Novel neutral imidazole-lipophosphoramides for transfection assays†

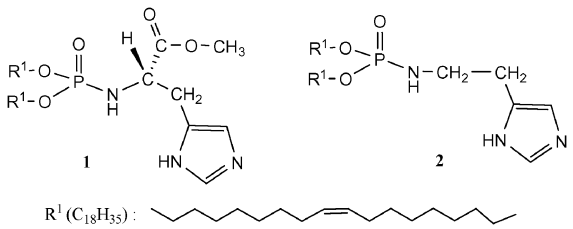
Mathieu Mével,[a] Cécile Neveu,[a] Cristine Gonçalves,[b] Jean-Jacques Yaouanc,*[a] Chantal Pichon,[b] Paul-Alain Jaffrès[a] and Patrick Midoux*[b]

Received (in Cambridge, UK) 27th March 2008, Accepted 7th May 2008
First published as an Advance Article on the web 16th June 2008
DOI: 10.1039/b805226c

New helper lipids, possessing an imidazole polar head, have been synthesized and included in formulations for transfection assays; these new helper lipids can improve the transfection by a factor of up to 100 compared to the use of DOPE as co-lipid.

Since the pioneering work of Felgner[1] and Behr[2] the molecular diversity of cationic lipids used as nonviral vectors for DNA delivery has become very broad.[3] Cationic lipids form electrostatic complexes (so-called lipoplexes) with plasmid DNA and help it to enter the cells according to endocytosis mechanisms.[4] They are usually formulated as liposomes with a neutral co-lipid like dioleoylphosphatidyl-ethanolamine (DOPE), cholesterol or dioleylphosphatidyl-choline (DOPC) both to decrease the amount of cationic lipids and to promote membrane fusion.[5] Although considerable efforts have been made in the design of various cationic lipids,[6] the synthesis of new co-lipids has been less explored. Structural modifications of DOPE itself (introduction of perfluoro chains or an alkyne group in the lipid chain) and of related structures derived from archae lipids have been performed.[7–9] Cationic lipophosphoramidates with a phosphonium or an arsonium group as polar head have proved their efficiency in transfection of cells both *in vitro* and *in vivo*.[10] We report herein the synthesis of novel neutral imidazole-lipophosphoramidates **1** and **2** (Scheme 1) and their incorporation into cationic lipophosphoramide formulations for transfection of mammalian cells.

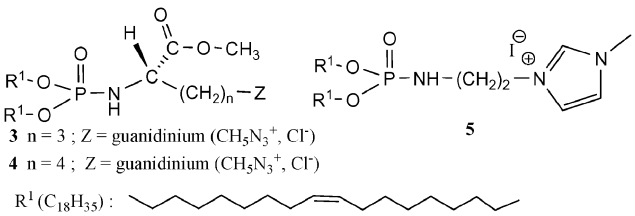
The lipophosphoramides **1** and **2** were synthesized according to a Todd–Atherton reaction involving dioleyl phosphite and either histidine methyl ester (to produce **1**) or histamine (to produce **2**). These lipophosphoramides are neutral at physiological pH (7.3) due to the pK_a of the imidazole heterocycle which varies from 5.5 to 6.2 for histidine methyl ester and histidine.[11] The protonation can thus occur in the acidic compartments of the cell (*e.g.* endosome), induce fusion of liposomes with endosome and favor the release of DNA in the cytosol.[12] Moreover, the modification of the protonation state of the co-lipid might induce structural changes that could also facilitate the liberation of the plasmid from the endosome.



Scheme 1 Neutral lipophosphoramidates **1** and **2**.

We have synthesized new lipophosphoramidates characterized by a cationic polar head composed of a natural α-amino-ester **3–4** (Scheme 2) or an *N*-3-methylimidazolium salt **5**.[13]

We formulated compounds **3–5** in the absence or in the presence of one co-lipid selected from among **1**, **2**, cholesterol (**Chol**) or **DOPE** in a 1 : 1 molar ratio. The transfection efficiencies of HEK293-T7 cells with 2.5 μg pCMVLuc plasmid obtained at the optimal lipid/DNA charge ratio are summarized in Fig. 1. It was observed that the arginine and homoarginine lipophosphoramidates **3** and **4** were more efficient when formulated with the neutral lipophosphoramidate **1** (Fig. 1; parts A and B). The luciferase activity was 350-fold higher than with the cationic lipid alone, or 100-fold higher than a formulation with DOPE. The luciferase expression reached the highest level (3.5×10^9 RLU/mg of protein) when **4** was formulated with **1**. **Chol** and the lipophosphoramidate **2** were much less efficient co-lipids for **3** and **4**, demonstrating that structural similarities (the presence of a carboxylic methyl ester) between the cationic lipid and the co-lipid might be an influencing factor. With the lipophosphoramidate **5**, DOPE or the lipophosphoramidate **2** produced the best formulations this time while the co-lipid **1** dramatically decreased the transfection efficiency (Fig. 1, part C). It is noticeable that the cationic lipid **5** and the co-lipid **2** share a related structural feature with the absence of the carboxylic methyl ester group in the α-position of the phosphoramide link. Compared to DOPE, there was no benefit of **1** or **2** with DOTAP (Fig. 1, part D).



Scheme 2 Molecular structures of cationic lipids **3–5**.

[a] *CEMCA, UMR CNRS 6521, Université de Bretagne Occidentale, CNRS UMR 6521, 6 Avenue Le Gorgeu, 29238 Brest, France. E-mail: jean-jacques.yaouanc@univ-brest.fr; Fax: (+33) 298 017 001; Tel: (+33) 298 016 153*
[b] *Centre de Biophysique Moléculaire, CNRS UPR 4301 affiliated to Inserm and Université d'Orléans, Rue Charles Sadron, 45071 Orléans cedex 2, France. E-mail: patrick.midoux@cnrs-orleans.fr; Fax: (+33) 238 69 15 17; Tel: (+33) 238 25 55 95*

† Electronic supplementary information (ESI) available: General methods of the experimental section and supplementary data. See DOI: 10.1039/b805226c

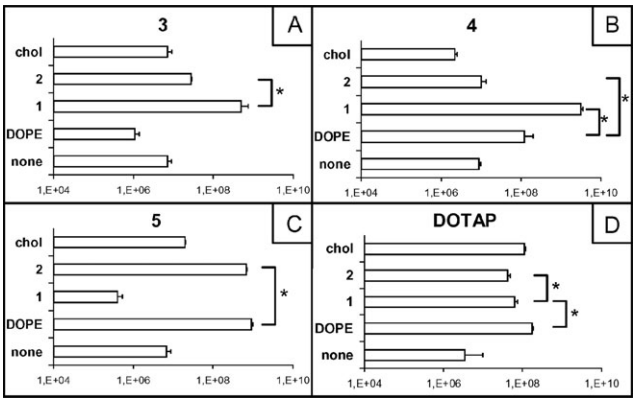


Fig. 1 Influence of **Chol**, **1**, **2** or DOPE on the transfection efficiency with cationic lipids: (A) **3**; (B) **4**; (C) **5**; (D) **DOTAP**. The luciferase activity was measured upon 48 h of culture and is expressed as Relative Light Unit per mg of protein (RLU/mg protein). The values shown are averages of three independent experiments. $*P < 0.005$.

Table 1 Influence of serum on the transfection efficiency

Ratio: RLU (50% serum)/RLU (without serum)					
Cat-lipids	Co-lipids				
	None	**DOPE**	**1**	**2**	**Chol**
4	0.17	1.25	1.82	ND	1.72
5	0.5	0.03	0.02	0.08	0.04
DOTAP	0.5	0.1	0.07	0.38	0.25

Among the best formulations, the transfection efficiency with **4**/**1** was higher ($P < 0.001$) than with **5**/**2** or **5**/DOPE. The cytotoxicity induced by **4**/**1** lipoplexes was low (5%) compared to that observed with **5**/**2** (45%) or **5**/**DOPE** (18%) lipoplexes (see ESI†). The numbers of cells transfected by **4**/**1** and **5**/**2** lipoplexes were 24% and 14%, respectively (see ESI†). In the presence of serum the transfection efficiency decreased when cationic lipids **5** or DOTAP were used whatever the nature of the co-lipid added (Table 1). In contrast, lipid **4** was less sensitive to serum especially when formulated with co-lipid **1**.

The size and the ξ potential of liposomes and lipoplexes were determined (see ESI†). Lipoplexes produced nano-objects with a size between 100 and 300 nm and a ξ potential ranging from -41 to $+62$ mV. Although no definitive correlation can be established between these data and the transfection efficiency, it appeared that the ξ potential of lipoplexes is close to 0 or slightly negative for the more efficient formulations.

The rational design of compounds **1** and **2** was based on their capacity to promote membrane destabilization in acid medium as the endosome lumen. When checked by FRET,[14] the fusion of **5**/DOPE liposomes with lipid bilayers containing phosphatidylcholine (PC) and phosphatidylserine (PS) was higher than with **5**/**2** ones at pH 7.4 (Fig. 2). In contrast, the fusion of **5**/**2** liposomes at pH 6 was higher than at pH 7.4 and this time close to that obtained with **5**/DOPE liposomes. The imidazole protonation of compound **2** at pH 6 is supposed to

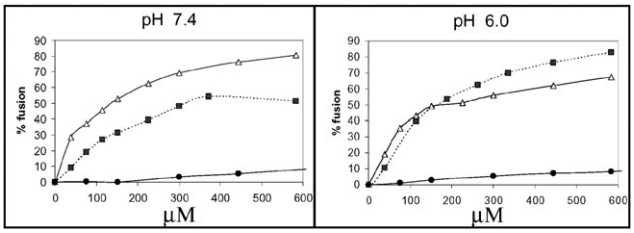


Fig. 2 Fusion of liposome with membrane models at pH 6.0 and pH 7.4. △: **5** with DOPE 1 : 1; ■: **5** with **2** 1 : 1; ●: PC/PS.

increase the fusogenic character of **5**/**2** liposomes in acidic medium. As in the case of the transfection efficiency, this property may also depend on the type of the cationic lipid/co-lipid association. Although the fusion was higher with **4**/**1** liposomes than **4**/DOPE ones, the fusion with the former was indeed lower at pH 6 than at pH 7.4 (see ESI†). Membrane destabilization might occur this time according to the proton sponge hypothesis.[15]

In conclusion, these novel neutral imidazole-lipophosphoramides improve significantly the transfection efficiency of cationic lipophosphoramides, some combinations being significantly better than DOPE as co-lipid. These results underline the benefit of co-lipids with an imidazole head group and demonstrate the necessity to design neutral and cationic lipids jointly to produce optimized formulations.

We thank L. Lebègue for his technical help, Brest Métropole Océane, Conseil Régional de Bretagne and Vaincre La Mucoviscidose for their financial support.

Notes and references

1. P. L. Felgner, T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold and M. Danielsen, *Proc. Natl. Acad. Sci. U. S. A.*, 1987, **84**, 7413–7417.
2. J. P. Behr, B. Demeneix, J. P. Loeffler and J. Perez-Mutul, *Proc. Natl. Acad. Sci. U. S. A.*, 1989, **86**, 6982–6986.
3. (*a*) A. D. Miller, *Angew. Chem., Int. Ed. Engl.*, 1988, **37**, 1768–1785; (*b*) I. Tranchant, B. Thompson, C. Nicolazzi, N. Mignet and D. Scherman, *J. Gene Med.*, 2004, **6**, S24–S35.
4. (*a*) I. S. Zuhorn, R. Kalicharan and D. Hoekstra, *J. Biol. Chem.*, 2002, **277**, 18021–18028; (*b*) I. S. Zuhorn, J. B. Engberts and D. Hoekstra, *Eur. Biophys. J.*, 2007, **36**, 349–362.
5. (*a*) C. R. Safinya, *Curr. Opin. Struct. Biol.*, 2001, **11**, 440–448; (*b*) R. Koynova, L. Wang and R. C. MacDonald, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 14373–14378.
6. (*a*) B. Martin, M. Sainlos, A. Aissaoui, N. Oudrhiri, M. Hauchecorne, J. P. Vigneron, J. M. Lehn and P. Lehn, *Curr. Pharm. Des.*, 2005, **11**, 375–394; (*b*) M. Rajesh, J. Sen, M. Srujan, K. Mukherjee, B. Sreedhar and A. Chaudhuri, *J. Am. Chem. Soc.*, 2007, **129**, 11404–11420.
7. J. Gaucheron, C. Boulanger, C. Santaella, N. Sbirrazzuoli, O. Boussif and P. Vierling, *Bioconjugate Chem.*, 2001, **12**, 949–963.
8. S. Fletcher, A. Ahmad, E. Perouzel, M. R. Jorgensen and A. D. Miller, *Org. Biomol. Chem.*, 2006, **4**, 196–199.
9. M. Brard, C. Lainé, G. Réthoré, I. Laurent, C. Neveu, L. Lemiègre and T. Benvegnu, *J. Org. Chem.*, 2007, **72**, 8267–8279.
10. (*a*) M. Mével, T. Montier, F. Lamarche, P. Delépine, T. Le Gall, J. J. Yaouanc, P. A. Jaffrès, D. Cartier, P. Lehn and J. C. Clément, *Bioconjugate Chem.*, 2007, **18**, 1604–1611; (*b*) E. Picquet,

K. Le Ny, P. Delépine, T. Montier, J. J. Yaouanc, D. Cartier, H. Des Abbayes, C. Férec and J. C. Clément, *Bioconjugate Chem.*, 2005, **16**, 1051–1053.
11. D. H. Sachs, A. N. Schechter and J. S. Cohen, *J. Biol. Chem.*, 1971, **246**, 6576–6580.
12. V. V. Kumar, C. Pichon, M. Refregiers, B. Guérin, P. Midoux and A. Chaudhuri, *Gene Ther.*, 2003, **10**, 1206–1215.
13. M. Mével, G. Breuzard, J. J. Yaouanc, J. C. Clément, P. Lehn, C. Pichon, P. A. Jaffrès and P. Midoux, *ChemBioChem*, 2008, **9**, 1462–1471.
14. D. K. Struck, D. Hoekstra and R. E. Pagano, *Biochemistry*, 1981, **20**, 4093–4099.
15. N. Sonawane, F. J. Szoka and A. Verkman, *J. Biol. Chem.*, 2003, **278**, 44826–44831.

