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Phosphonolipids as non-viral vectors for gene therapy

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Abstract – Several phosphonates with two fatty chains and different polar heads were synthesized and evaluated for their potential to transfer DNA into epithelial (COS-7) and hematopoïetic (K562) cell lines, and compared to commercially available references. In both cases, ammonium-phosphonates were particularly efficient. © Elsevier, Paris

non-viral transfection / phosphonolipids / ammoniums / polyamines

1. Introduction

Over the last decade, a wide expansion has occurred in the search for synthetic vectors able to complex with DNA, to carry the resulting 'lipoplex' through cell membranes, then to deliver the free DNA in (or close to) the nucleus, in order to replace a deficient gene [1, 2]. Although at present less efficient than the viral ones, the interest of synthetic vectors lies in the fact that the size of carried genetic material is not limited, and in the lack of immune response. The most common models are the cationic lipids roughly consisting of three parts i.e.:

(1) a cationic or polycationic center, able to bind to DNA,

(2) a lipidic part (generally two hydrocarbon chains between C_{12} and C_{20} or a cholesteryl moiety),

(3) a 'spacer' linking these two parts.

The first so-built cationic lipid was the DOTMA [3] consisting of a trimethylammonium moiety as polar head group and two oleyl chains as lipidic part, linked by a glycerol spacer. Combining different cationic heads with different spacers and lipidic parts led to many described cationic lipids, among them DOGS and DPPES [4, 5], DC-Chol [6], DMRIE [7], 'GL.67' [8], 'GAP-DLRIE' [9], BGSC and BGTC [10, 11]. The cationic part is generally a simple [3, 6] or functional [7, 9] ammonium, a polyamine [4, 8] or a guanidine [10, 11].

Considering these polar heads, we thought of a new spacer, more resembling the natural phospholipids of cell membranes, but without their hydrolytic instability and their long and tedious syntheses. We designed 'phosphonolipids' because of their relatively short syntheses, even if not modeling exactly the glycerophosphate structure of phospholipids. So, the aim of this work was to check the possible improvement in the efficiency of cationic lipids possessing a phosphonate spacer as compared to those quoted above. In this paper, we first report the synthesis of ammonium-phosphonates [12], guanidino-phosphonates, polyaminophosphonates and functional ammonium-phosphonates, having two hydrocarbon chains from C_{12} to C_{18}. Their efficiency as 'cytofectins' [7] was then checked on different cell lines, epithelial (COS-7) or hematopoïetic (K562) and compared with some published ones.

2. Chemistry

2.1. Ammonium-phosphonates

The general procedure of their syntheses is outlined in *figure 1*. Dioleylphosphite **1** ($R^1 = C_{18}H_{35}$) was obtained

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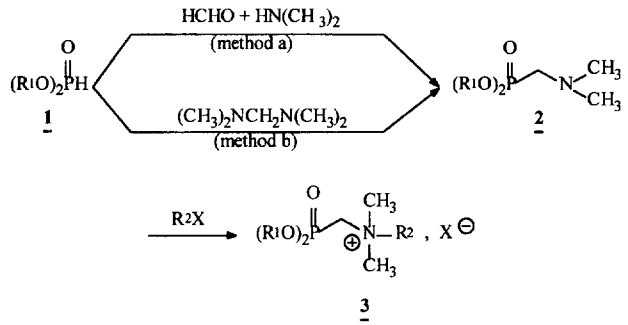


Figure 1.

by direct esterification of phosphorus trichloride by 3 equivalents of oleylalcohol, according to a procedure described for shorter alcohols [13]. C_{12}–C_{18} saturated dialkylphosphites **1** were synthesized by transesterification of commercial diphenylphosphite with an excess of the corresponding alcohol.

From dioleylphosphite **1** ($R^1 = C_{18}H_{35}$), the Field reaction [14] of formaldehyde (or paraformaldehyde) and dimethylamine led to the corresponding aminophosphonate **2** (method a). For the C_{12}–C_{18} saturated dialkylphosphites, the use of the aminal $(CH_3)_2NCH_2N(CH_3)_2$ was preferred (method b), for higher yields and lower hydrolytic degradation of the starting phosphite. Aminophosphonates **2** were then quaternarized to the ammonium **3** with alkyl iodides or functional bromides. Because of difficult – even impossible – distillation or recrystallisation, ammonium-phosphonates **3** were purified by repeated washings with non-polar solvents, in order to eliminate traces of starting materials. This method led to variable yields (see *table I*) but to satisfactory spectral data (^1H, ^{31}P and ^{13}C NMR) and microanalyses (see *table II*). For the salts **3**, replacement of the halide anion was possible either with sodium iodide/methylene chloride ($Br^- \rightarrow I^-$) or by exchange on DOWEX 1X2-200 ($I^- \rightarrow Cl^-$).

After a rapid evaluation [12] of these ammonium-phosphonates **3**, it appeared that the myristyl chain (C_{14}) generally gave the better results, whatever the tested cell lines. So, the other following phosphonates with different polar heads were all synthesized only from dimyristylphosphite **1** ($R = C_{14}H_{29}$).

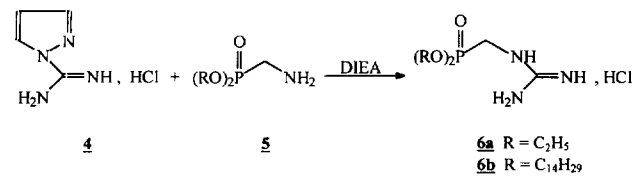


Figure 2.

2.2. Guanidino-phosphonates

Guanidino-phosphonates **6** were obtained from the guanidino-pyrazole **4** by adapting a method by Bernatowicz et al. [15] *(figure 2)*.

Although several methods are known for synthesis of **5** ($R = C_2H_5$) [16], we chose to adapt an elegant technique by Genêt et al. [17], i.e., Pd catalysed deallylation of the corresponding diallylamine **7** (obtained by a classical Field reaction) in presence of 2-mercaptobenzoïc acid as allyl acceptor *(figure 3)*.

Here also, repeated washings with diethylether decreased the yields (especially for **6b**) but allowed satisfactory analyses and spectral data *(tables I* and *II)*.

2.3. Polyaminophosphonates

Polyaminophosphonates **8** were obtained from a Field reaction of dimyristylphosphite with formaldehyde and appropriate triamine, first suitably protected as its bis-trifluoroacetyl derivative by mean of ethyl trifluoroacetate (which reacts only with terminal primary amines and lets unchanged the central secondary amine, according to [18] *(figure 4)*).

Deprotection by potassium carbonate in methanol [19] afforded the free triaminophosphonates **8**. Overall yields from the starting phosphite were fairly good (**8c**) to excellent (**8a**, **8b**) *(table I)*.

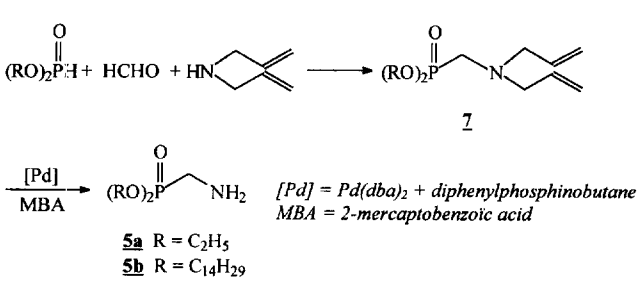
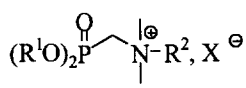
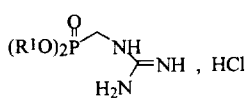
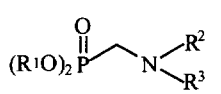
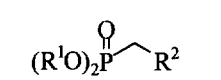


Figure 3.

Table I

No.	Code	Structure	R^1	R^2	X	Yield (%)	M.p. (°C)	Formula (MW)
3a	GLB.73		$C_{14}H_{29}$	CH_3	I	60	124	$C_{32}H_{69}INO_3P$ (673.79)
3b	GLB.84		$C_{14}H_{29}$	CH_3	Cl	60	122	$C_{32}H_{69}ClNO_3P$ (582.34)
3c	GLB.391		$C_{14}H_{29}$	C_2H_5	I	50	110	$C_{33}H_{71}INO_3P$ (687.81)
3d	GLB.43		$C_{18}H_{35}$	CH_3	I	47	119	$C_{40}H_{81}INO_3P$ (781.97)
3e	GLB.221		$C_{14}H_{29}$		I	38	96	$C_{33}H_{68}IN_2O_3P$ (698.80)
3f	GLB.145		$C_{14}H_{29}$		Br	62	70	$C_{34}H_{71}BrNO_3P$ (652.83)
3g	GLB.215		$C_{14}H_{29}$		I	29	109	$C_{34}H_{71}INO_3P$ (699.83)
3h	GLB.171		$C_{14}H_{29}$		Br	38	90	$C_{34}H_{69}BrNO_3P$ (650.81)
3i	GLB.205		$C_{14}H_{29}$		I	50	84	$C_{34}H_{69}INO_3P$ (697.81)
3j	GLB.574		$C_{14}H_{29}$		I	31	62	$C_{41}H_{87}INO_3P$ (800.03)
6b	GLB.457		$C_{14}H_{29}$	–	–	19	95	$C_{30}H_{65}ClN_3O_3P$ (582.30)
				R^2 R^3				
8a	GLB.397		$C_{14}H_{29}$	 	–	90	–	$C_{33}H_{72}N_3O_3P$ (589.93)
8b	GLB.403		$C_{14}H_{29}$	 	–	87	–	$C_{35}H_{76}N_3O_3P$ (617.99)
8c	GLB.387		$C_{14}H_{29}$	 	–	66	–	$C_{36}H_{78}N_3O_3P$ (632.01)
9	GLB.405		$C_{14}H_{29}$		–	27	88	$C_{33}H_{71}INO_4P$ (703.81)
10	GLB.253		$C_{14}H_{29}$		–	55	120	$C_{35}H_{76}IN_2O_3P$ (730.88)

2.4. Hydroxy and amino ammonium-phosphonates

Since DMRIE [7] and GAP-DLRIE [9] are functional ammoniums bearing an hydroxy or amino group, we synthesized also hydroxy **9** and amino **10** ammonium phosphonates, by a Field reaction from the corresponding functional secondary methylamines, then quaternarisation by methyl iodide *(figure 5)*.

Table II.

Compound	^{1}H-NMR, δ ($CDCl_3$) [a]	^{31}P-NMR, δ ($CDCl_3$)	^{13}C-NMR, δ ($CDCl_3$)
3a/3b	0.86 (t, 6H, *J* = 6.7 Hz, C*H*$_3$ fatty chains), 1.24–1.31 (m, 44H, C*H*$_2$ fatty chains), 1.70 (q*, 4H, *J* = 7 Hz, C*H*$_2$ β-O), 3.68 (s, 9H, C*H*$_3$ α-N), 4.22 (q, 4H, *J* = 7 Hz, C*H*$_2$ α-O), 4.41 (d, 2H, *J* = 13 Hz, C*H*$_2$ α-P(O))	13 (s)	14.0 (s, *C*H$_3$ fatty chains); 22.5, 25.2, 29.0, 29.2, 29.4, 29.45, 29.50, 29.54 (s, *C*H$_2$ fatty chains); 30.2 (d, *J* = 6 Hz, *C*H$_2$ β-O); 31.7 (s, *C*H$_2$ fatty chains); 55.9 (d, *J* = 4 Hz, *C*H$_3$ α-N); 59.7 (d, *J* = 145 Hz, *C*H$_2$ α-P(O)); 68.1 (d, *J* = 7 Hz, *C*H$_2$ α-O)
3c	0.87 (t, 6H, *J* = 6.5 Hz, C*H*$_3$ fatty chains), 1.20–1.30 (m, 44H, C*H*$_2$ fatty chains), 1.45 (t, 3H, *J* = 7.2 Hz, C*H*$_3$ β-N), 1.71 (q*, 4H, *J* = 7.5 Hz, C*H*$_2$ β-O), (s, 9H, C*H*$_3$ α-N), 3.97 (q, 2H, *J* = 7.2 Hz, C*H*$_2$ α-N), 4.22 (q, 4H, *J* = 6.9 Hz, C*H*$_2$ α-O), 4.35 (d, 2H, *J* = 13.3 Hz, C*H*$_2$ α-P(O))	13 (s)	8.9 (s, *C*H$_3$ β-N); 13.7 (s, *C*H$_3$ fatty chains); 22.3, 25.0, 28.7, 28.9, 29.1, 29.2, 29.3 (s, *C*H$_2$ fatty chains); 30.0 (d, *J* = 5.6 Hz, *C*H$_2$ β-O); 31.5 (s, *C*H$_2$ fatty chains); 52.4 (s, *C*H$_3$ α-N); 57.0 (d, *J* = 146 Hz, *C*H$_2$ α-P(O)); 62.1 (s, *C*H$_2$ α-N); 67.7 (d, *J* = 6.4 Hz, *C*H$_2$ α-O)
3d	(t, 6H, *J* = 6.6 Hz, C*H*$_3$ fatty chains), 1.20–1.40 (m, 44H, C*H*$_2$ fatty chains), 1.65–1.80 (m, 4H, C*H*$_2$ β-O), 1.90–2.10 (m, 8H, C*H*$_2$ α-CH), (s, 9H, C*H*$_3$ α-N), 4.24 (q, 4H, *J* = 7 Hz, C*H*$_2$ α-O), 4.43 (d, 2H, *J* = 13 Hz, C*H*$_2$ α-P(O)), 5.35 (m, 4H, C*H*)	13 (s)	13.9 (s, *C*H$_3$ fatty chains); 22.5, 25.2, 27.0, 28.9, 29.05, 29.10, 29.2, 29.3, 29.6 (s, *C*H$_2$ fatty chains);30.2 (d, *J* = 5.8 Hz, *C*H$_2$ β-O); 31.7 (s, *C*H$_2$ fatty chains); 55.9 (d, *J* = 3.8 Hz, *C*H$_3$ α-N); 59.7 (d, *J* = 146 Hz, *C*H$_2$ α-P(O)); 68.1 (d, *J* = 7 Hz, *C*H$_2$ α-O); 129.5 (s, *C*H); 129.8 (s, *C*H)
3e	0.87 (t, 6H, *J* = 6.6 Hz, C*H*$_3$ fatty chains), 1.24–1.33 (m, 44H, C*H*$_2$ fatty chains), 1.73 (q*, 4H, *J* = 6.8 Hz, C*H*$_2$ β-O), 3.84 (s, 6H, C*H*$_3$ α-N), 4.25 (q, 4H, *J* = 6.9 Hz, C*H*$_2$ α-O), 4.64 (d, 2H, *J* = 13.3 Hz, C*H*$_2$ α-P(O)), 5.79 (s, 2H, C*H*$_2$ α-CN)	13 (s)	14.0 (s, *C*H$_3$ fatty chains); 22.6, 25.2, 29.0, 29.2, 29.4, 30.2, 31.8 (s, *C*H$_2$ fatty chains); 53.9 (s, *C*H$_3$ α-N); 54.2 (s, *C*H$_2$ α-CN); 58.1 (d, *J* = 145 Hz, *C*H$_2$ α-P(O)); 68.7 (d, *J* = 7 Hz, *C*H$_2$ α-O); 110.4 (s, *C*N)
3f/3g	0.88 (t, 6H, *J* = 6.6 Hz, C*H*$_3$ fatty chains), 1.16–1.35 (m, 44H, C*H*$_2$ fatty chains), 1.71 (q*, 4H, *J* = 7.2 Hz, C*H*$_2$ β-O), 3.54 (s, 6H, C*H*$_3$ α-N), (q, 4H, *J* = 6.7 Hz, C*H*$_2$ α-O), 4.37 (d, 2H, *J* = 13.2 Hz, C*H*$_2$ α-P(O)), 4.59 (d, 2H, *J* = 7 Hz, C*H*$_2$ α-N), 5.78–6.03 (m, 3H, *H* allyliques)	14 (s)	13.8 (s, *C*H$_3$ fatty chains); 22.3, 25.0, 28.8, 29.1, 29.2 (s, *C*H$_2$ fatty chains); 30.0 (d, *J* = 5.9 Hz, *C*H$_2$ β-O); 31.4 (s, *C*H$_2$ fatty chains); 51.9 (d, *J* = 3 Hz, *C*H$_3$ α-N); 56.2 (d, *J* = 145 Hz, *C*H$_2$ α-P(O)); 67.6 (d, *J* = 6.6 Hz, *C*H$_2$ α-O); 67.7 (d, *J* = 5 Hz, *C*H$_2$ α-N); 124.0 (s, allylic *C*H$_2$); 130.1 (s, allylic *C*H)
3h/3i	(t, 6H, *J* = 6.6 Hz, C*H*$_3$ fatty chains), 1.15–1.40 (m, 44H, C*H*$_2$ fatty chains), 1.69 (q*, 4H, *J* = 6.8 Hz, C*H*$_2$ β-O), 2.90 (s, 1H, C*H*), (s, 6H, C*H*$_3$ α-N), 4.21 (q, 4H, *J* = 7.0 Hz, C*H*$_2$ α-O), 4.56 (d, 2H, *J* = 13.1 Hz, C*H*$_2$ α-P(O)), 5.12 (s, 2H, C*H*$_2$ en α-N)	13 (s)	13.9 (s, *C*H$_3$ fatty chains); 22.4, 25.1, 28.9, 29.1, 29.30, 29.35, 29.40, 29.45 (s, *C*H$_2$ fatty chains); 30.1 (d, *J* = 5.9 Hz, *C*H$_2$ β-O); 31.7 (s, *C*H$_2$ fatty chains); 52.0 (s, *C*H$_3$ α-N); 56.5 (d, *J* = 145 Hz, *C*H$_2$ α-P(O)); 56.7 (d, *J* = 6.3 Hz, *C*H$_2$ α-N); 68.0 (d, *J* = 6.7 Hz, *C*H$_2$ α-O); 71.5 (s, *C*H); 81.9 (s, *C*)
3j	(t, 6H, *J* = 6.5 Hz, C*H*$_3$ fatty chains), 1.20–1.50 (m, 44H, C*H*$_2$ fatty chains), 1.71 (q*, 4H, *J* = 6.7 Hz, C*H*$_2$ β-O), 2.56 (s large, 2H, C*H*$_2$ β-N), 3.32 (s large, 2H, C*H*$_2$ α-NH$_2$), 3.55 (s, 6H, C*H*$_3$ α-N), 4.20–4.30 (m, 6H, C*H*$_2$ α-O and C*H*$_2$ α-P(O)), 6.50 (broad signal, 3H, N*H*$_2$)	13 (s)	14.1 (s, *C*H$_3$ fatty chains); 21.3 (s, *C*H$_2$ β-NH$_2$); 22.7, 25.5, 29.3, 29.4, 29.6, 29.70, 29.75 (s, *C*H$_2$ fatty chains); 30.5 (d, *J* = 5.3 Hz, *C*H$_2$ β-O); 31.9 (s, *C*H$_2$ fatty chains); 37.4 (s, *C*H$_2$ α-NH$_2$); 53.2 (s, *C*H$_3$ α-N); 57.8 (d, *J* = 146 Hz, *C*H$_2$ α-P(O)); 65.6 (s, *C*H$_2$ α-N); 68.4 (d, *J* = 6.5 Hz, *C*H$_2$ α-O)
3k	0.88 (t, 9H, *J* = 6.7 Hz, C*H*$_3$ fatty chains and C*H*$_3$ decyl chain), 1.20–1.50 (m, 58H, 44H C*H*$_2$ fatty chains and 14H C*H*$_2$ decyl chain), 1.60–1.80 (m, 6H, C*H*$_2$ β-O and C*H*$_2$ β-N), 3.59 (s, 6H, C*H*$_3$ α-N), 3.70–3.85 (m, 2H, C*H*$_2$ α-N), 4.24 (q, 4H, *J* = 6.9 Hz, C*H*$_2$ α-O), 4.40 (d, 2H, *J* = 13.2 Hz, C*H*$_2$ α-P(O))	14 (s)	13.9 (s, *C*H$_3$ fatty chains and *C*H$_3$ decyl chain); 22.5 (s, *C*H$_2$ fatty chains); 23.0 (s, *C*H$_2$ decyl chain); 25.3 (s, *C*H$_2$ fatty chains); 25.9 (s, *C*H$_2$ decyl chain); 29.0, 29.1, 29.2, 29.3, 29.4, 29.45, 29.50, 29.55 (s, *C*H$_2$ alkyl chain); 30.2 (d, *J* = 6.0 Hz, *C*H$_2$ β-O); 31.7 (s, *C*H$_2$ decyl chain); 31.8 (s, *C*H$_2$ fatty chains); 53.3 (d, *J* = 4.3 Hz, *C*H$_3$ α-N); 57.7 (d, *J* = 146 Hz, *C*H$_2$ α-P(O)); 66.5 (s, *C*H$_2$ α-N); 68.1 (d, *J* = 6.7 Hz, *C*H$_2$ α-O)
6a	0.86 (t, 6H, *J* = 6.8 Hz, C*H*$_3$ fatty chains), 1.20–1.40 (m, 44H, C*H*$_2$ fatty chains), 1.65 (q*, 4H, *J* = 6.6 Hz, C*H*$_2$ β-O), 3.69 (d, 2H, *J* = 12.6 Hz, C*H*$_2$ α-P(O)), 4.02 (q, 4H, *J* = 7.0 Hz, C*H*$_2$ α-O)	22 (s)	13.3 (s, *C*H$_3$ fatty chains); 21.5, 24.5, 28.1, 22.2, 28.45, 28.50, 28.55 (s, *C*H$_2$ fatty chains); 29.6 (d, *J* = 5.3 Hz, *C*H$_2$ β-O); 30.8 (s, *C*H$_2$ fatty chains); 36.3 (d, *J* = 154.6 Hz, *C*H$_2$ α-P(O)); 65.9 (d, *J* = 6.8 Hz, *C*H$_2$ α-O); 157.7 (d, *J* = 6.3 Hz, *C*=N)
8a	0.88 (t, 6H, *J* = 6.7 Hz, C*H*$_3$ fatty chains), 1.25–1.35 (m, 44H, C*H*$_2$ fatty chains), 1.66 (q*, 4H, *J* = 6.7 Hz, C*H*$_2$ β-O), 1.70–2.00 (s large, N*H*$_2$), 2.69 (t, 4H, *J* = 5.4 Hz, C*H*$_2$ α-N), 2.76 (t, 4H, *J* = 5.4 Hz, C*H*$_2$ α-N), 2.92 (d, 2H, *J* = 10.0 Hz, C*H*$_2$ α-P(O)), 4.04 (q, 4H, *J* = 7.0 Hz, C*H*$_2$ α-O)	25 (s)	13.9 (s, *C*H$_3$ fatty chains); 22.5, 25.4, 29.0, 29.2, 29.40, 29.45, 29.50 (s, *C*H$_2$ fatty chains); 30.5 (d, *J* = 5.4 Hz, *C*H$_2$ β-O); 31.8 (s, *C*H$_2$ fatty chains); 39.3 (s, *C*H$_2$ α-NH$_2$); 49.7 (d, *J* = 161.6 Hz, *C*H$_2$ α-P(O)); 58.0 (d, *J* = 7.0 Hz, *C*H$_2$ α-N);65.8 (d, *J* = 7.2 Hz, *C*H$_2$ α-O)

Table II *(continued).*

8b	0.89 (t, 6H, J = 6.6 Hz, CH_3 fatty chains), 1.25–1.35 (m, 44H, CH_2 fatty chains), 1.61 (q*, 4H, J = 6.7 Hz, CH_2 β-N), 1.67 (q*, 4H, J = 6.9 Hz, CH_2 β-O), 1.80 (s large, NH), 2.65 (t, 4H, J = 6.9 Hz, CH_2 α-N), 2.76 (q, 4H, J = 6.7 Hz, CH_2 α-N), 2.85 (d, 2H, J = 10.4 Hz, CH_2 α-P(O)), 4.06 (q, 4H, J = 6.9 Hz, CH_2 α-O)	26 (s)	14.0 (s, CH_3 fatty chains); 22.5, 25.4, 29.1, 29.2, 29.40, 29.45, 29.50, 29.55, 29.6 (s, CH_2 fatty chains); 30.3 (s, CH_2 β-N); 30.5 (d, J = 5.7 Hz, CH_2 β-O); 31.8 (s, CH_2 fatty chains); 39.6 (s, CH_2 α-NH_2); 49.8 (d, J = 163.8 Hz, CH_2 α-P(O)); 52.6 (d, J = 8.7 Hz, CH_2 α-N); 65.7 (d, J = 7.2 Hz, CH_2 α-O)
8c	0.88 (t, 6H, J = 6.8 Hz, CH_3 fatty chains), 1.20–1.30 (m, 44H, CH_2 fatty chains), 1.30–1.50 (m, 4H, CH_2 β-N), 1.58 (q*, 4H, J = 6.8 Hz, CH_2 β-O), 1.65 (q*, 2H, J = 7.0 Hz, CH_2 β-N), 2.56 (t, 2H, J = 6.7 Hz, CH_2 α-N), 2.65 (t, 2H, J = 6.9 Hz, CH_2 α-N), 2.69 (t, 2H, J = 6.8 Hz, CH_2 α-N), 2.73 (t, 4H, J = 6.7 Hz, CH_2 α-N), 2.84 (d, 2H, J = 10.4 Hz, CH_2 α-P(O)), 4.04 (q, 4H, J = 6.8 Hz, CH_2 α-O)	26 (s)	14.1 (s, CH_3 fatty chains); 22.6 (s, CH_2 fatty chains); 24.2 (s, CH_2 β-N); 25.5, 29.2, 29.3, 29.50, 29.55 (s, CH_2 fatty chains); 30.5 (s, CH_2 β-N); 30.6 (d, J = 5.8 Hz, CH_2 β-O); 31.0 (s, CH_2 β-N); 31.9 (s, CH_2 fatty chains); 39.8 (s, CH_2 α-N); 41.8 (s, CH_2 α-N); 49.8 (d, J = 162.6 Hz, CH_2 α-P(O)); 52.7 (d, J = 7.3 Hz, CH_2 α-N); 55.0 (d, J = 9.8 Hz, CH_2 α-N); 65.8 (d, J = 7.0 Hz, CH_2 α-O)
9	0.87 (t, 6H, J = 7.0 Hz, CH_3 fatty chains), 1.20–1.40 (m, 44H, CH_2 fatty chains), 1.71 (q*, 4H, J = 6.8 Hz, CH_2 β-O), 1.79 (s large, 1H, OH), 3.61 (s, 6H, CH_3 α-N), 4.00–4.10 (m, 2H, CH_2 α-N), 4.20 (q, 6H, J = 6.8 Hz, CH_2 α-O), 4.28 (d, 2H, J = 13.3 Hz, CH_2 α-P(O))	13 (s)	14.1 (s, CH_3 fatty chains); 22.7, 25.4, 29.1, 29.3, 29.5, 29.60, 29.62, 29.64, 29.67 (s, CH_2 fatty chains); 30.4 (d, J = 5.7 Hz, CH_2 β-O); 31.9 (s, CH_2 fatty chains); 54.3 (s, CH_3 α-N); 56.0 (s, CH_2 α-N); 59.0 (d, J = 154 Hz, CH_2 α-P(O)); 68.1 (d, J = 6.7 Hz, CH_2 α-O); 68.7 (s, CH_2 α-O and β-N)
10	0.86 (t, 6H, J = 6.7 Hz, CH_3 fatty chains), 1.20–1.28 (m, 44H, CH_2 fatty chains), 1.64 (q*, 4H, J = 6.8 Hz, CH_2 β-O), 2.51 (s, 3H, CH_3 α-N), 2.79 (d, 2H, J = 11.1 Hz, CH_2 α-P(O)), 3.10 (s, 2H, CH_2 α-N central), 3.51 (s, 9H, CH_3 α-N), 3.96 (t, 2H, J = 4.8 Hz, CH_2 α-N terminal), 4.00 (q, 4H, J = 6.9 Hz, CH_2 α-O)	23 (s)	13.6 (s, CH_3 fatty chains); 22.1, 24.9, 28.6, 28.7, 28.9, 29.0, 29.1 (s, CH_2 fatty chains); 30.0 (d, J = 5.7 Hz, CH_2 β-O); 31.3 (s, CH_2 fatty chains); 44.0 (d, J = 13.2 Hz, CH_3 α-N central); 52.4 (d, J = 164 Hz, CH_2 α-P(O)); 52.7 (d, J = 6.0 Hz, CH_2 α-N central); 53.7 (s, CH_3 α-N terminal); 61.9 (s, CH_2 α-N terminal); 65.5 (d, J = 7.0 Hz, CH_2 α-O)

[a] q*: quintuplet.

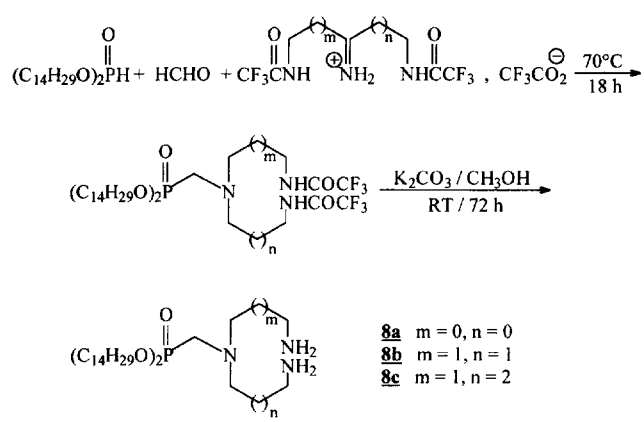


Figure 4.

In the case of the diamine, only the terminal nitrogen was quaternarized, even in presence of an excess of CH_3I, leading to the amine-ammonium **10**. As in the other cases, repeated washings with non-polar solvents allowed to achieve satisfactory spectral data.

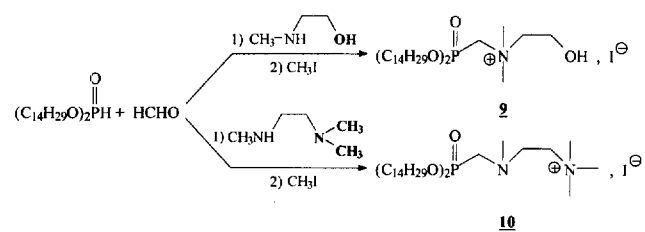


Figure 5.

3. Biological results and discussion

The in vitro gene transfer was targeted at two cell lines: COS-7, a monkey kidney adherent one, and K562, a human hematopoïetic non-adherent line and at primary CD34+ cells (for their culture, see experimental part). In a first series of experiments, several ammonium-phosphonates **3** were checked and compared with four commercially available cationic reagents: two ammoniums: Lipofectin (i.e. DOTMA/DOPE 1:1 (w:w)) [20] and LipofectACE (i.e. DDAB/DOPE 1:2.5 (w:w)) [21], and

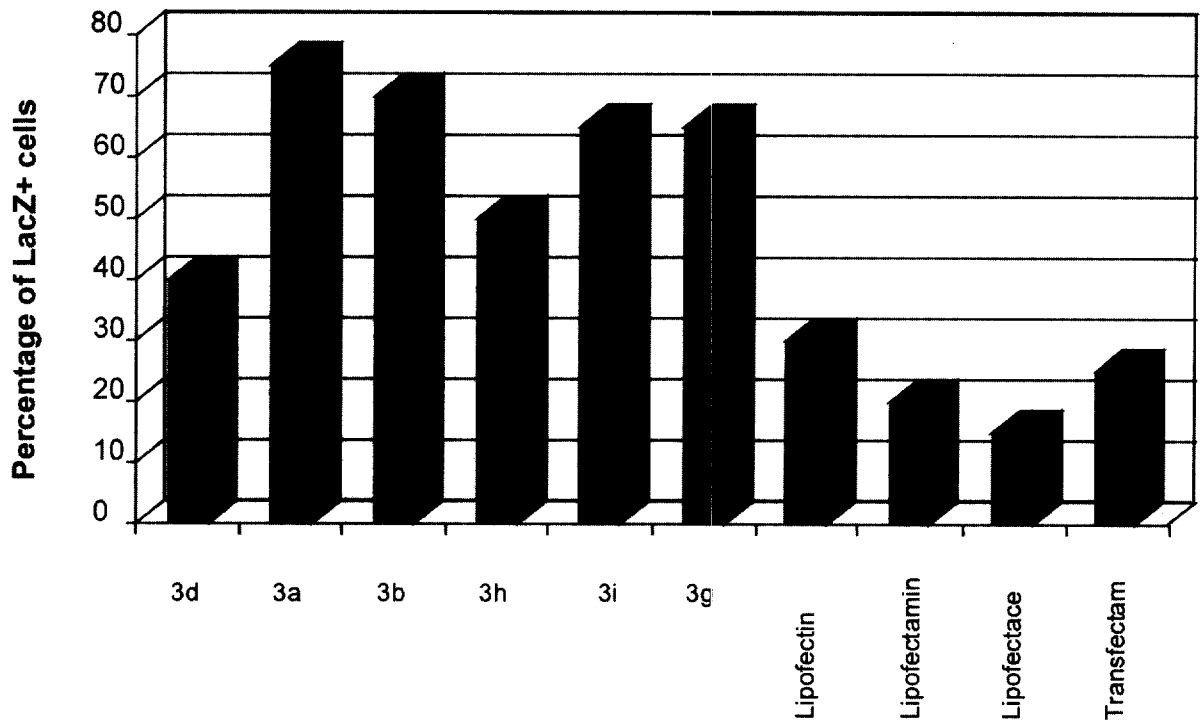


Figure 6. Percentage of LacZ+ cells.

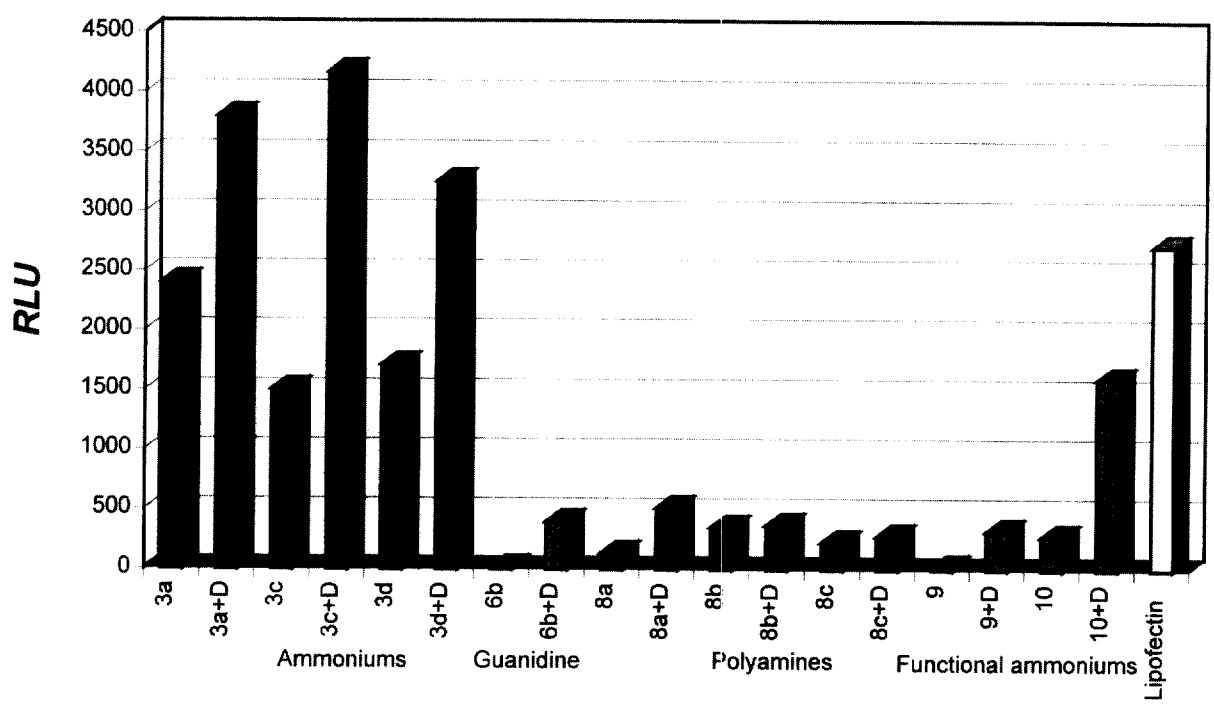


Figure 7. Transfection activity on COS-7 cell line.

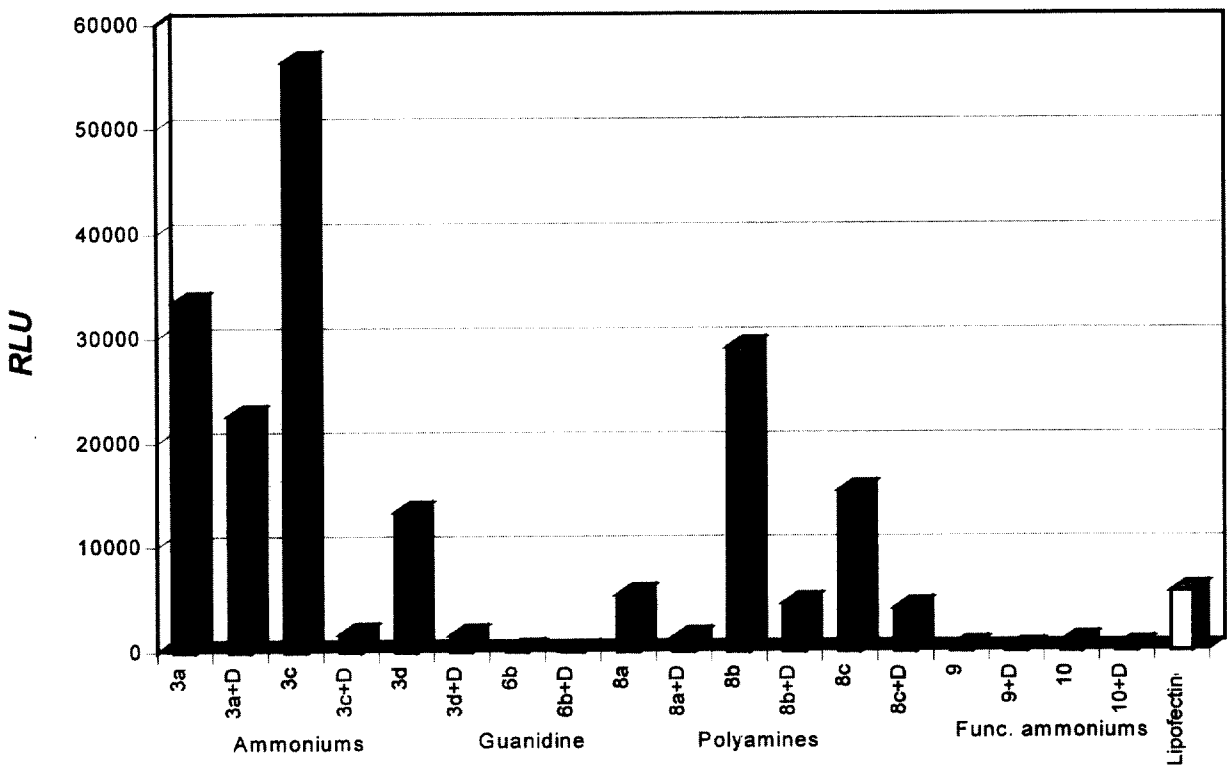


Figure 8. Transfection activity on K562 cell lines.

two polyamines: LipofectAMINE (i.e. DOSPA/DOPE 3:1 (w:w)) [22] and Transfectam (i.e. DOGS) [4], by the FACS-Gal analysis of the LacZ+ percentage in K562 cells transfected with a pCMV LacZ plasmid (see experimental part) *(figure 6)*.

In that preliminary experiment, ammonium-phosphonates were used alone, without the neutral lipid DOPE.

Among them, at least six exhibited an activity (in term of LacZ+ cells) greater than the four commercial references. Among these, the activity of Lipofectin was a little above the three others and was thus selected as reference for two other series of experiments including as well the guanidino-phosphonate **6b** (GLB.457), the polyamines **8a**, **8b**, **8c** (GLB.397, GLB.403, GLB.387) and the functional ammoniums **9** (GLB.405) and **10** (GLB.253). For these two other series of experiments, we used a luminescent detection of β-galactosidase (see experimental section for details), and each phosphonolipid was checked alone or in 1:1 mixture (w:w) with DOPE. Here again, several phosphonolipids were more efficient than Lipofectin, either on adherent COS-7 *(figure 7)* or on non-adherent K562 cells *(figure 8)*.

From these two histograms, it is obvious than phosphonolipids were systematically more effective with DOPE in COS-7, whereas the opposite was noticed in K562, where the phosphonolipids were far more efficient without DOPE. This was particularly striking for compound **3c** (GLB.391) which was the most outstanding in the two series of experiments, with (COS-7) or without (K562) DOPE.

Since the activity of three simple ammonium **3a** (GLB.73), **3c** (GLB.391) and **3d** (GLB.43) was greater than that of Lipofectin, it is obvious that the insertion of a phosphonate spacer in place of the glycerol of DOTMA brought about to a marked enhancement of the transfection properties, on COS-7 cells. On the other hand, the results of the other families i.e., guanidine **6b**, triamines **8a**, **8b**, **8c**, and functional ammoniums **9** and **10** were rather disappointing.

With non-adherent K562 cells, simple ammonium kept their superiority, but triamines **8a**, **8b**, **8c** also exhibited

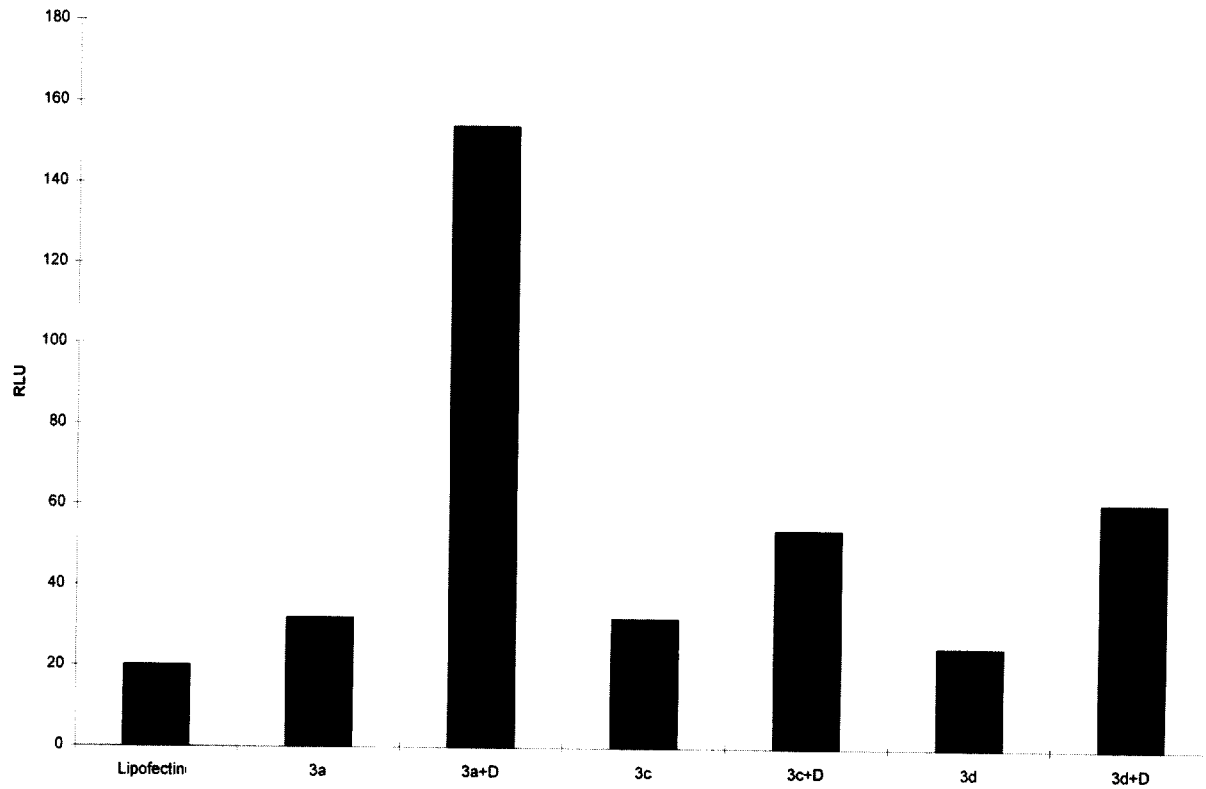


Figure 9. Transfection activity on CD34+ primary cells.

an interesting activity. On the other hand, guanidine **6b** and functional ammoniums **9** and **10** gave particularly low results.

The poor result of guanidinium-phosphonate **6b** is rather surprising, insofar as guanidinium-cholesterol cationic lipids proved to be efficient for transfection into various mammalian cell lines [10, 11]. With these results in mind, the best simple ammonium-phosphonates **3a**, **3c** and **3d** were successfully checked on other non-adherent hematopoïetic TF1 [24] and on cells in primary culture (CD34+), as shown on *figure 9*.

The transfection efficacy of cationic lipids is known to be strongly dependent on the lipid/DNA ratio. So, using our transfection protocol on K562 cells, determination of the best lipid/DNA ratio was carried out. *Figures 10* and *11* show the optimal ratio for the ammonium **3c**, without or with DOPE (**3c** + D). In a general way, whatever the lipid used among those described above, the optimal ratio was contained between 3 and 7 (μg lipid/μg DNA).

The entity DNA/cationic lipid may be considered as a drug since its introduction in the cell should temporarily restore a deficient functionality. In cystic fibrosis for instance, mutations in a gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) – an anionic channel regulated by intracellular ATP – induce an abnormally processed protein, virtually absent from cell membrane [25]. Complementation of respiratory epithelium with CFTR c DNA has been achieved both in vitro [26] and in vivo [27]. We turned that facts to good account for checking successfully the ammonium-phosphonates **3** in vitro on human tracheal CFT-1 cells [23] and in vivo in mouse lung [28].

In conclusion, the aim of this work was to design a new easy to build spacer and to check the possible improvement on the resulting new synthetic vectors compared to commercially available cationic lipids, on adherent and non-adherent cell lines. Many so-built 'phosphonolipids' exhibited an activity higher than four commercial references. This was particularly true for simple ammonium-phosphonates **3** (and to a lesser extent for some polyaminophosphonates **8** in the case of cells in suspension).

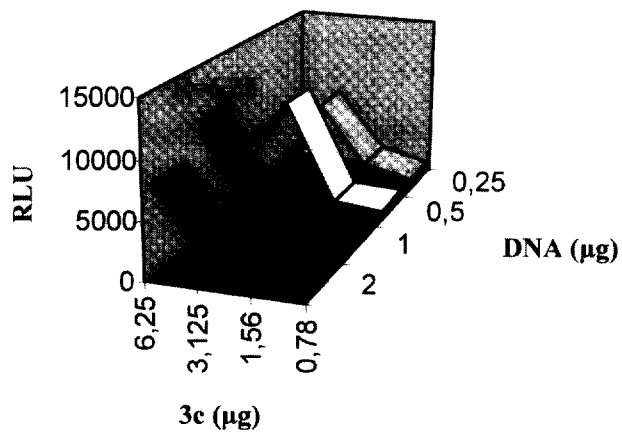


Figure 10. Optimal lipid/DNA ratio for **3c**.

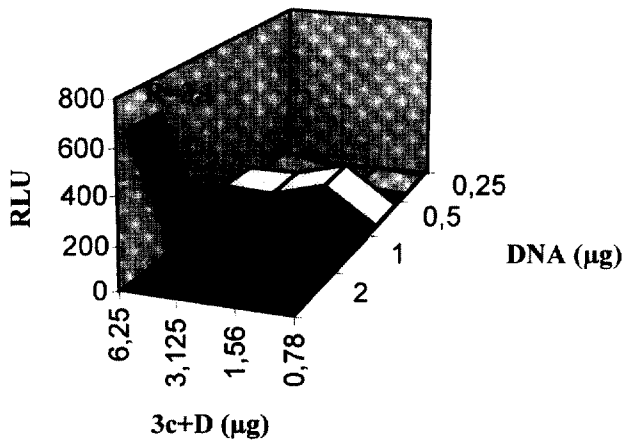


Figure 11. Optimal lipid/DNA ratio for **3c** + D.

The preliminary screening of various polar heads brought to the fore some mismatches in the different families of phosphonolipids and allowed a selection of the most efficient of them for a more complete evaluation. Since synthetic vectors appear more and more promising for gene therapy, many of the phosphonolipids described above are valuable potent agents for that purpose.

4. Experimental protocols

4.1. Chemistry

The structure of all the compounds were supported by their NMR spectra (^1H, ^{13}C were obtained on a Bruker AC 300 and Bruker Avance DRX 400, δ in ppm, tetramethylsilane internal reference. ^{31}P on the same spectrometers or on Jeol FX 100, with H_3PO_4 as external reference). Melting points were determined on a Köfler hotstage apparatus and are uncorrected. Elemental analyses were performed by the Service Central d'Analyse du CNRS (Vernaison, France) and were within ±0.4% of the calculated values.

*4.1.1. Dimethylaminomethylphosphonic acid ditetradecylester **2a***

Ditetradecylphosphite (0.61 g, 1.3 mmol) and tetramethyl diaminomethane (0.53 mL, 3.9 mmol) were heated under nitrogen at 80 °C for 5 h. Left over aminal and dimethylamine were discarded at 80 °C in vacuo. Phosphonate **2a** was obtained as white solid (95% yield) pure enough (NMR) for further transformation.

*4.1.2. Dimethylaminomethylphosphonic acid dioleylester **2d***

To a mixture of 1.06 g (1.8 mmol) of dioleylphosphite and 0.23 mL (1.8 mmol) of dimethylamine (40% w. aqueous solution) in 10 mL THF was slowly added 0.14 mL of HCHO (37% w. aqueous solution). The solution was then warmed to 70 °C for 4 h, then THF eliminated in vacuo. After drying ($MgSO_4$) in CH_2Cl_2 solution and usual work up, aminophosphonate **2d** was isolated as a yellowish oil (70% yield).

*4.1.3. Ammonium-phosphonates **3a–j**: general procedure*

Aminophosphonate **2a** or **2d** was reacted with an excess of alkylhalide R^2X, in CH_2Cl_2, at RT for 18 to 72 h according to R^2. Solvent was eliminated in vacuo and the crude ammonium washed several times with diethyloxide or hexanes up to complete elimination of the residual starting materials then dried in vacuo (see yields in *table I* and pertinent NMR data in *table II*).

*4.1.4. Di(prop-2-enyl)aminomethylphosphonic acid ditetradecylester **7b***

To a mixture of ditetradecylphosphite (0.31 g, 0.7 mmol) and diallylamine (0.1 mL, 0.8 mmol) in 5 mL THF was added 0.07 mL (0.8 mmol) of a 37% aqueous HCHO. The reaction mixture was stirred at 70 °C for 18 h, then THF discarded in vacuo, the residue dissolved in CH_2Cl_2, dried ($MgSO_4$). After usual work-up, aminophosphonate **7b** was obtained as a yellowish solid (100% crude yield).

*4.1.5. Aminomethylphosphonic acid ditetradecylester **5b***

From 0.53 g (0.9 mmol) of the crude phosphonate **7b**, the typical procedure for deallylation of Genêt et al. [17], then several washings (diethyloxide) of the oily residue led to 0.18 g (41% yield) of **5b**.

*4.1.6. Guanidinomethylphosphonic acid ditetradecylester **6b***

To a mixture of 0.16 g (1.1 mmol) of aminophosphonate **5b** an 0.15 g (1.0 mmol) of pyrazolylguanidinium chloride **4** (prepared according to Bernatowicz's method [15]) in 8 mL DMF was added 0.18 mL (1.0 mmol) of diisopropylethylamine, and the reaction mixture stirred at RT. A white precipitate was gradually formed and collected by filtration after 3 days. Several washings (THF) then drying in vacuo led to spectroscopically pure **6b** (49% yield).

*4.1.7. Triaminophosphonates **8a, 8b, 8c**: general procedure*

Triamines 2.2, 3.3 or 3.4 (spermidine) were bistrifluoroacetylated with an excess $CF_3CO_2C_2H_5$, according to the O'Sullivan's procedure [18]. The crude white solids obtained were purified by repeated washings with CH_2Cl_2 then ethylacetate (yields 90% (2.2); 93% (3.3); 73% (3.4)).

Bistrifluoroacetylated triamines (as their trifluoroacetate salts) were reacted (1.1 mmol) with 0.46 g (1.0 mmol) ditetradecylphosphite and 0.08 mL HCHO (37% aqueous solution) in THF solution. After 18 h at 70 °C, the solvent was evaporated and the residue taken up from an aqueous basic solution with CH_2Cl_2. Usual work-up led to diprotected triaminophosphonates with 100% yields (2.2; 3.3) or 91% yield (3.4).

Deprotection was achieved with an excess Na_2CO_3 in a mixture methanol:water (70:30) at RT for 3 days and led to free triamines-phosphonates **8a** (100% yield), **8b** (94% yield) and **8c** (100% yield).

*4.1.8. N-(2-Hydroxyethyl)-N-methylaminomethylphosphonic acid ditetradecylester, methiodide **9***

To a mixture of ditetradecylphosphite (0.53 g; 1.1 mmol) and N-methylethanolamine (0.092 mL; 1.1 mmol) in 10 mL THF, was added 0.1 mL (1.3 mmol) of HCHO (37% aqueous solution). The reaction mixture was stirred for 18 h at 70 °C, then THF discarded in vacuo and the residue dissolved in CH_2Cl_2 and dried ($MgSO_4$). The crude white oil (73% yield) was then reacted overnight with an excess CH_3I in CH_2Cl_2. After evaporation of CH_2Cl_2 and left over CH_3I, the ammonium-phosphonate **9** was precipitated in diethyl oxide and washed several times with the same solvent, then dried in vacuo, thus leading to pure **9** (m.p. = 88 °C; 27% yield).

*4.1.9. [(2-Dimethylaminomethyl)methylamino]methylphosphonic acid ditetradecylester, methiodide **10***

From ditetradecylphosphite (0.47 g; 1.0 mmol), N, N, N'-trimethylethylediamine (0.125 mL; 1.0 mmol) and 37% HCHO (aqueous solution) (0.1 mL; 1.1 mmol), the use of the same procedure as for **9**, led, after several washings with diethyl oxide, to pure **10** (m.p. = 120 °C; 59% overall yield).

4.2. Biology

4.2.1. Cell-culture

K562 cells: The human hematopoietic non-adherent cell line K562 was obtained from American Type Culture Collection (No. ccl 243 ATCC, Rockville, MD, USA) and was maintained in RPMI-1640 medium (Gibco BRL, Cergy Pontoise, France) supplemented with 10% fetal calf serum (FCS), 0.2 mM glutamine, 100 U/mL of penicillin, 100 U of streptomycin and 1% fungizone.

COS-7 cells: The monkey kidney adherent cell line COS-7 was obtained from American Type Culture Collection (No. crl 1651 ATCC, Rockville, MD, USA) and was maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Cergy Pontoise, France) supplemented with 10% fetal calf serum (FCS), 0.2 mM glutamine, 100 U/mL of penicillin, 100 U of streptomycin and 1% fungizone.

CD34+ cells: Cryopreserved peripheral blood progenitor cells(PBPCs) were used for this study. After informed consent of patients, cells were collected by apheresis during hematopoïetic recovery after chemotherapy and recombinant human GCSF administration. CD34+ cells were selected from harvested PBPC s by immunoaffinity absorption columns (Ceprate LC; Cell pro Inc., Bothell, WA, USA). Enriched CD34+ cells were cultured in 50 mL culture flasks at 10^6 cells/5 mL in IMDM medium containing 20% fetal calf serum (Gibco BRL), in presence of recombinant human (Rh) stem cell factor (Genzyme) recombinant human 113 and recombinant human 116 (Genzyme).

4.2.2. Plasmid

The plasmid used was pCMVLacZ, containing the LacZ gene encoding β-galactosidase under the control of the cytomegalovirus (CMV) promoter. It was grown in Epicurian coli (Stratagene), extracted and purified on QUIAGEN-500 columns (Coger). The structure and purity were checked after enzymatic digestion with Eco RI by electrophoresis in a 0.7% agarose gel followed by

ethidium bromide staining to detect DNA. DNA concentration was determined by absorbance at 260 nm.

4.2.3. Cationic lipids

Each of the cationic phosphonolipids was prepared alone or in combination with the neutral lipid DOPE (Sigma, Saint Quentin Fallavier, France). The phosphonolipids were formulated by mixing chloroform solutions of the different lipids in glass vials, then chloroform was removed by rotary evaporation to produce dried lipids films. One mL of sterile pyrogen-free DI water was added per mg of lipid and the vials were sealed and stored one night at +4 °C. Small unilamellar vesicles (suv) were prepared by sonicating the compounds for 10 min in a sonicator (Prolabo, Paris, France) [29].

4.2.4. Transfection protocol

Transfection activity of the cationic lipid: DNA complexes in vitro was assessed using K562 and COS-7 cell lines and primary CD34+ cells. Cells were seeded onto a 96-well tissue culture plate at 20 000 per well. Adherent cells (COS-7) were seeded 24 h before transfection and incubated overnight in a humidified 5% CO_2 atmosphere at 37 °C. Non-adherent cells (K562, CD34+) were seeded 1 h before transfection. Transfection of the cells was performed essentially as described by Felgner et al. [30] with the following modifications. Appropriate amounts of the cationic lipids and the plasmid vector pCMV β-Gal in OptiMEM were complexed and 100 mL were added to each well. After 2 h and 30 min at 37 °C, the cells were supplemented with 200 mL of appropriate growth medium. Following a further 72 h at 37 °C, the cells were assayed for expression of β-Gal using a chemiluminescent assay.

4.2.5. Luminescent detection of β-galactosidase

β-Galactosidase activity was measured with a luminescent β-galactosidase detection kit (Clontech).The cells were lysed in 50 mL of lysis buffer (Clontech), 25 mL of the supernatant were mixed to 50 mL of reaction buffer (Clontech) in a microtiter plate. The plate was incubated at room temperature for 90 min. β-Galactosidase activity in the supernatant was quantified with a luminometer (LMR Dynex) to integrate light emission over a 5 s reaction period. The results were expressed in RLU (relative light unit).

4.2.6. Analysis of β-Gal expression in viable cells using a FACS-Gal assay

To investigate gene expression in the cells, a FACS-Gal assay [31] was also used. According to this flow cytometry method, enzyme activity is measured by using a fluorogenic substrate (fluorescein di-β-D-galactopyranoside) (FDG) hydrolyzed by β-galactosidase inside cells. For this assay 1×10^5 cells in a 35-mm tissue culture plate were transfected with an optimal phosphonolipid-to-DNA ratio. The FACS-Gal assays were done basically as described elsewhere [32]. Briefly, aliquots of cells were suspended in 50 mL of growth medium and warmed at 37 °C. FDG (2 mM in 98% distilled water) (Sigma) was warmed at 37 °C and 50 mL were added to each 50 mL aliquot of cells. The cells and FDG were rapidly mixed and immediately incubated in a 37 °C bath for 1 min. The cells were removed from the water bath, 1 mL or more of ice cold growth medium or phosphate-buffered saline (PBS) was added to the cells and incubated on ice until analyzed on a FACS can (Becton–Dickinson). With this fluorescent assay, the percentage of positive cells (LacZ+) was evaluated by comparison with a control transfected with the same quantity of lipid-DNA complex (in this control the DNA used was pBR322).

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