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IL FARMACO 59 (2004) 869–878
IL FARMACO
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Original article

Synthesis, characterization and transfection activity of new saturated and unsaturated cationic lipids

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Received 9 April 2004; accepted 15 June 2004

Available online 30 September 2004

Abstract

We synthesized new cationic lipids, analogue to *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) and 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethylammonium bromide (DMRIE), in order to compare those containing a dodecyl chain with those having a relatively long chain with two or five double bonds, such as squalenyl and dihydrofarnesyl derivatives, or complex saturated structures, such as squalane derivatives. The fusogenic helper lipid dioleoylphosphatidylethanolamine (DOPE) was added to cationic lipids to form a stable complex. Liposomes composed of 50:50 w/w cationic lipid/DOPE were prepared and incubated with plasmidic DNA at various charge ratios and the diameter and zeta potential of the complexes were measured. The surface charge of the DNA/lipid complexes can be controlled by adjusting the cationic lipid/DNA ratio. Finally, we tested the in vitro transfection efficiency of the cationic lipid/DNA complexes using different cell lines. The transfection efficiency was highest for the dodecyloxy derivative containing a single hydroxyethyl group in the head, followed by the dodecyloxy and the farnesyloxy trimethylammonium derivatives. Instead the C27 squalenyl and C27 squalanyl derivatives resulted inactive.

Keywords: Cationic lipids; Transfection activity; Liposomes; Plasmidic DNA

1. Introduction

Over the last several years, various gene delivery systems have been investigated for gene therapy approaches of both inherited and acquired disease [1,2]. Different viral and non-viral systems are currently under intensive investigations [3]. Retroviral, adenoviral, and adeno-associated viral vectors produce highly efficient transfections regarding the number of transfected cells [4]. However drawbacks caused by the viral element, such as immune or toxic reactions, recombinations with the wild-type virus and insertional mutagenesis, have stimulated efforts to improve synthetic non-viral transfer systems based on cationic lipids/liposomes or cationic polymers [5,6].

Since the initial pioneering works of Felgner et al. in 1987 [7,8] and also of Beehr et al. in 1989 [9], many cationic lipid formulations have been described [10]. Cationic liposomes normally contain a cationic amphiphile and a helper lipid which is usually dioleoylphosphatidylethanolamine (DOPE). DOPE may be required for the generation of stable cationic liposomes, and also provides membrane fusion activity. The most common model for cationic lipids contain the following domains: (1) a cationic or polycationic centre able to bind to DNA; (2) a lipidic part (generally composed of two hydrocarbon chains between C12 and C20 or of a cholesteryl moiety); (3) a spacer linking these two parts [11].

The first cationic lipid was *N*-[1-(2,3-dioleoyloxy) propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) [7] which consisted of a trimethylammonium moiety and two oleoyl chains linked by a glycerol spacer. The cationic part is generally a simple [7,12] or functionalized ammonium as DMRIE [13,14], a polyamine [9,15] or guanidine [16,17]. The lipidic part consists of a flexible saturated fatty acid acyl chain or fatty acid chains containing a cis double bond (i.e. oleoyl) and/or a cholesterol ring [7,18]. Fatty acid chains give the lipoplexes membrane fluidity and high lipid-mixing ca-

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doi:10.1016/j.farmac.2004.06.007

pacity; in contrast, a cholesterol ring offers rigidity. More interestingly it has been reported that cationic cholesterol-based liposomes display a high membrane fusion capacity while being stable in the presence of various substances (ions, mucus) [12,19]. Liposomes containing the cationic cholesterol derivative were the first synthetic vectors to be used in clinical trials [20,21].

Cholesterol, as many steroid-like natural products is derived biogenetically through a complex mechanism that starting from the C30 acyclic precursor (3S)-2,3-oxidosqualene, leads to lanosterol [22]. In order to cyclize it to lanosterol, the semi-rigid squalene moiety containing all trans double bonds must assume a pre-boat–pre-chair–pre-boat–pre-chair conformation, similar to that found in cyclized molecule. Taking this mechanism into account we synthesized many inhibitors of cholesterol biosynthesis, based on azasqualene derivatives as acyclic molecules mimicking the natural substrate [23].

In this study, we developed new series of cationic lipids analogue to DOTMA and DMRIE by introducing one or more E double bonds in the hydrophobic chain, in order to obtain a semi-rigid hydrophobic anchor placed in between the flexible saturated chain and the cyclic rigid cholesterol moiety. We also wanted to evaluate the transfection efficiencies of the new unsaturated, squalene-like cationic lipids on a panel of normal and tumor cell lines, by comparison with analogues characterized by C12 saturated fatty acid chains and commercial cationic lipids.

2. Experimental procedures

The ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AC 200 instrument (Karlsruhe, Germany) in $CDCl_3$ solution at room temperature, with $SiMe_4$ as internal standard. Mass spectra were obtained on a Finnigan-MAT TSQ 700 spectrometer (San Jose, CA). Microanalyses were performed on an elemental analyzer 1106 (Carlo Erba Strumentazione, Milan, Italy). The reactions were monitored by thin-layer chromatography (TLC) on F_{254} silica gel precoated sheets (Merck, Milan, Italy); after development the sheets were exposed to iodine vapour. Flash-column chromatography was performed on 230–400 mesh silica gel (Merck). Petroleum ether refers to the fraction boiling in the 40–60 °C range. All solvents were distilled prior to flash chromatography.

Dioleoylphosphatidylethanolamine (DOPE) and Escort™ transfection reagent were purchased from Sigma Chemical Co. (St. Louis, MO), Lipofectin™ was from GIBCO BRL (Paisley, UK). All other reagents were of analytical grade.

2.1. Preparation of methanesulfonyl derivatives (2,7,9,11)

2.1.1. Dodecyl methanesulfonate (2, Scheme 1)

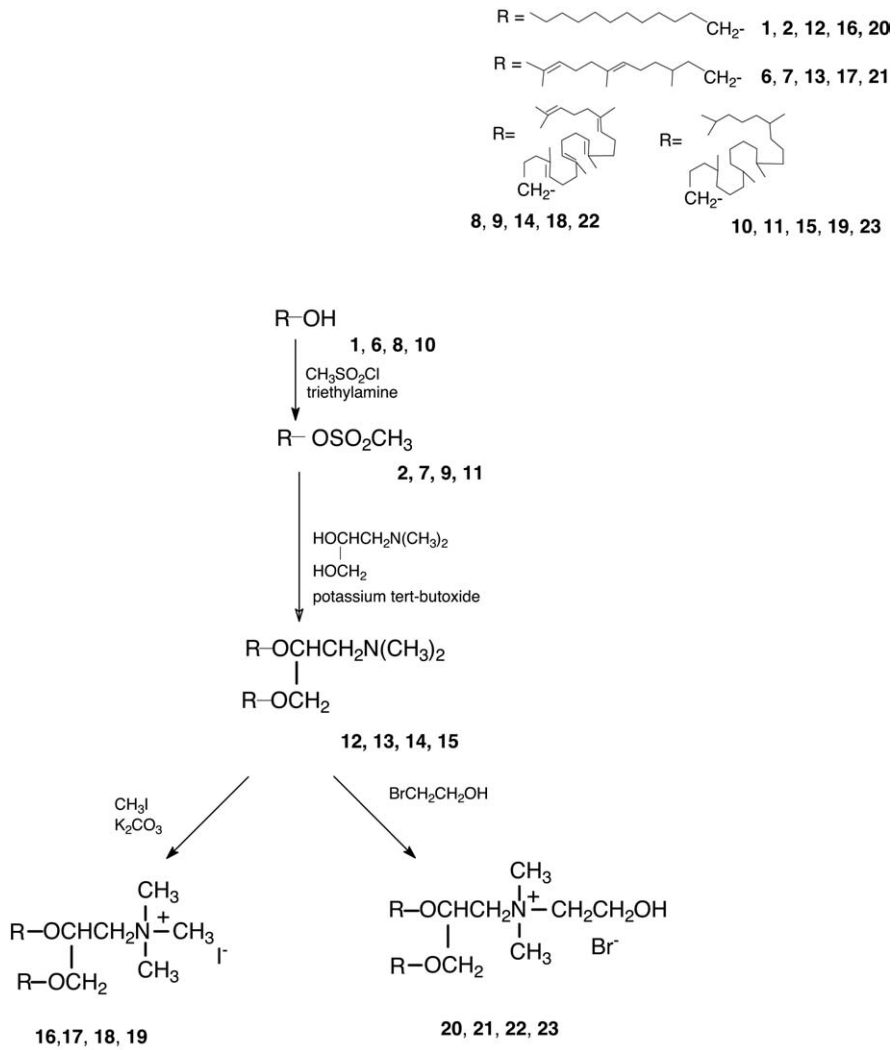
To dodecanol **1** (2 g, 10.7 mmol), dissolved in 20 ml of anhydrous dichloromethane, triethylamine (1.5 ml, 10.7 mmol) was added. The solution was cooled to 0 °C and methanesulfonyl chloride (1.3 ml, 16 mmol) was added dropwise under a dry nitrogen atmosphere. After stirring for 12 h at 0 °C, heptane was added and the white solid formed was filtered off. The solution was concentrated and then purified by flash chromatography with elution in petroleum ether/diethyl ether 95:5 v/v. The pure product **2** was obtained as a white solid (2.62 g, 93%). ^{1}H-NMR ($CDCl_3$) δ 1.1 (t, 3H, CH_3), 1.5 [m, 20H, (CH_2)$_{10}$], 3.25 (s, 3 H, CH_3SO_2), 4.4 (t, 2 H, CH_2OSO_2). MS–CI (isobutane) 265 (100). Satisfactory EI mass spectra has not been obtained. Anal. ($C_{13}H_{28}O_3S$) C, H, O, S.

2.1.2. (6E)-3,7,11-trimethyl-6,10-dodecadienyl methanesulfonate (7, Scheme 1)

Farnesol **3** (Scheme 2) (3 g, 13.5 mmol) was oxidized to (6E)-3,7,11-trimethyl-2,6,10-dodecatrienal as a mixture of 2E and 2Z isomers (**4**, Scheme 2) by reaction with pyridinium chlorochromate (5.8 g, 27 mmol) in 30 ml of anhydrous dichloromethane. The reaction was stirred for 1 h at room temperature under nitrogen atmosphere. The mixture was then evaporated under reduced pressure and the precipitate washed several times with diethyl ether; the ethereal phase was evaporated and purified by flash chromatography using petroleum ether as eluant. Yield 2.3 g, 78%.^{1}H-NMR ($CDCl_3$) δ 1.58–1.65 (m, 12 H, allylic CH_3), 2.00–2.12 (m, 8 H, allylic CH_2), 5.02–5.15 (t, 2 H, vinylic CH), 5.67 (m, 1 H, *E* and *Z* C=C*H*CHO), 9.94 (m, 1 H, *E* and *Z* C=CHC*H*O).MS-EI *m/z* 220 (15), 205 (20), 177 (18), 136 (40), 69 (100). Anal. ($C_{15}H_{24}O$) C, H, O.

(6*E*)-3,7,11-trimethyl-2,6,10-dodecatrienal **4** (2 g, 9 mmol), and $Pd(PPh_3)_4$ (104 mg, 0.09 mmol) were added to a mixture of acetic acid (0.52 ml, 9 mmol) and benzene (30 ml mixed under argon atmosphere). Bu_3SnH (2.9 ml, 10.8 mmol) was then added dropwise, and the mixture was allowed to react for 1 h at room temperature. The resulting brown solution was poured into iced water–dichloromethane (3 × 20 ml), washed with saturated brine (2 × 20 ml), dried with anhydrous magnesium sulfate and evaporated under reduced pressure. The product was purified by flash chromatography with petroleum ether/diethyl ether 99.5:0.5 v/v affording 1.3 g of (6*E*)-3,7,11-trimethyl-6,10-dodecadienal (**5**, Scheme 2) (65% yield). ^{1}H-NMR ($CDCl_3$) δ 0.90 [d, 3 H, CH(CH_3)], 1.25–1.50 [m, 3H, CH_2CH(CH_3)CH_2CHO], 1.55–1.60 (d, 9 H, allylic CH_3), 1.98–2.30 (m, 8 H, allylic CH_2 and CH_2CHO), 5.00–5.12 (m, 2 H, vinylic CH), 9.70 (t, 1 H, CHO). MS-EI *m/z* 222 (10), 189 (5), 179 (95), 69 (100). Anal. ($C_{15}H_{26}O$) C, H, O.

Aldehyde **5** was converted to the corresponding alcohol **6** by reaction with $NaBH_4$ (0.334 g, 8.9 mmol) in 30 ml of methanol. After stirring for 30 min at room temperature under a nitrogen atmosphere, the solvent was evaporated in vacuo and the solid washed with dichloromethane and evaporated under reduced pressure. The crude product was purified by flash chromatography with petroleum ether/diethyl



Scheme 1. Preparation of cationic lipids (16–23).

ether 99:1 v/v, giving (6*E*)-3,7,11-trimethyl-6,10-dodecadien-1-ol (**6**, Scheme 2) (1.09 g, 83%). ^{1}H-NMR ($CDCl_3$) δ 0.92 [d, 3 H, CH(CH_3)], 1.23–1.48 (m, 5H, $CH_2CH(CH_3)$ CH_2CH_2OH), 1.50–1.60 (m, 9 H, allylic CH_3), 2.00–2.25 (m, 6 H, allylic CH_2), 3.67 (m, 2 H, CH_2OH), 5.02–5.12 (m, 2 H, vinylic CH). MS-EI *m/z* 224 (20), 181 (100), 163 (60), 123 (90), 69 (95). Anal. ($C_{15}H_{28}O$) C, H, O.

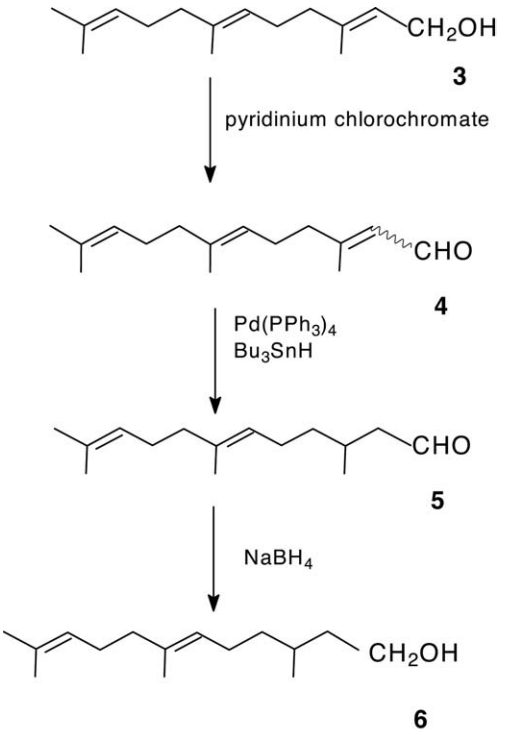
(6*E*)-3,7,11-trimethyl-6,10-dodecadien-1-ol **6** (1 g, 4.5 mmol) was transformed to the corresponding methanesulfonate **7** (Scheme 1) using the method described for compound **2**. The crude product was purified by flash chromatography (petroleum ether/diethyl ether, 98:2 v/v). Yield 1.08 g, 80%. ^{1}H-NMR ($CDCl_3$) δ 0.95 [d, 3 H, CH(CH_3)], 1.25–1.48 (m, 4H, $CH_2CH(CH_3)CH_2CH_2O$), 1.55–1.65 [m, 10 H, allylic CH_3 and $CH(CH_3)$], 2.2 (m, 6 H, allylic CH_2), 3.1 (s, 3 H, CH_3SO_2), 4.3 (m, 2 H, CH_2OSO_2), 5.1 (t, 2 H, vinylic CH). MS-EI *m/z* 302 (5), 259 (30), 123 (65), 81 (75), 69 (100). Anal. ($C_{16}H_{30}O_3S$) C, H, O, S.

*2.1.3. 1,1′,2-Trisnorsqualenyl methanesulfonate: (4E,8E,12E,16E)-4,8,13,17,21-pentamethyl-4,8,12,16,20-docosapentaenyl methanesulfonate (**9**, Scheme 1)*

1,1′,2-trisnorsqualene alcohol **8** (1 g, 2.6 mmol) previously prepared in our laboratory [24] was reacted with methanesulfonyl chloride (0.306 ml, 3.9 mmol) and triethylamine (0.362 ml, 2.6 mmol) in 20 ml of anhydrous dichloromethane and purified by flash chromatography with elution in petroleum ether/diethyl ether 97:3 v/v to give compound 9. Yield 0.6 g, 50%. ^{1}H-NMR ($CDCl_3$) δ 1.58–1.70 (m, 20 H, allylic CH_3 and $CH_2CH_2OSO_2$), 1.98–2.10 (m, 18 H, allylic CH_2), 2.98 (s, 3 H, CH_3SO_2), 4.19 (t, 2 H, CH_2OSO_2), 5.02–5.18 (m, 5 H, vinylic CH). MS-EI *m/z* 464 (5), 395 (10), 136 (45), 95 (100). Anal. ($C_{28}H_{48}O_3S$) C, H, O, S.

*2.1.4. 1,1′,2-Trisnorsqualanyl methanesulfonate: 4,8,13,17,21-pentamethyldocosyl methanesulfonate (**11**, Scheme 1)*

1,1′,2-trisnorsqualene alcohol **8** (1 g, 2.6 mmol) was added to a mixture of Pd/C (10% Pd, 200 mg) in 100 ml of



Scheme 2. Synthesis of (6E)-3,7,11-trimethyl-6,10-dodecandien1-ol (6) starting from farnesol (3).

absolute ethanol, under H_2 atmosphere. The reaction proceeded for 24 h under stirring under H_2 atmosphere, the catalyst was removed by filtration and the resulting solution was concentrated under reduced pressure. 1,1′,2-trisnorsqualane alcohol **10** was purified by flash chromatography (petroleum ether/diethyl ether, 98:2 v/v). Yield 67%, 0.825 g. The corresponding methanesulfonyl derivative **11** was obtained by procedure described above. The crude product was purified by flash chromatography (petroleum ether/diethyl ether 98:2 v/v) to give compound **11** (0.690 g, 70%).^{1}H-NMR ($CDCl_3$) δ 0.80–0.90 (m, 18 H, CH_3), 1.20–1.38 (m, 30 H, CH_2), 1.68–1.80 (m, 5 H, CH), 3.01 (s, 3H, CH_3–SO_2), 4.20 (t, 2 H, CH_2–O–SO_2). MS-EI *m/z* 474 (4), 351 (8), 125 (33), 97 (100). Anal. ($C_{28}H_{58}O_3S$) C, H, O, S.

2.2. Preparation of diethers (**12–15**)

For the preparation of compounds **12–15** the method described for the synthesis of DOTMA was followed, with minor modifications [7].

The methanesulfonates (**2, 7, 9** and **11**) (3.8 mmol), dissolved in xylene, were added to a mixture of 3-(dimethylamino)-1,2-propanediol (1.3 mmol) and potassium *tert*-butoxide (5.7 mmol) in 15 ml of xylene, under a nitrogen atmosphere. The reaction mixture was stirred under nitrogen at 50 °C for 30 min and then heated for 12 h under reflux. After the mixture had cooled to room temperature, heptane was added and washed with water and saturated brine; the organic layer was dried over anhydrous $MgSO_4$ and concentrated in vacuo to give a crude product, which was purified by flash chromatography. Compounds **12, 13** and **14** were purified by elution in diethyl ether, while compound **15** was purified by elution in diethyl ether/methanol 97:3 v/v.

2.2.1. N,N-*dimethyl-2,3-didodecyloxypropilamine (**12**, Scheme 1)*

0.346 g, 20% yield.^{1}H-NMR ($CDCl_3$) δ 0.86 (t, 6 H, CH_3), 1.27 (m, 36 H, CH_2), 1.56 (m, 4 H, 2 CH_2CH_2O), 2.24 [s, 6 H, $N(CH_3)_2$], 2.37 (d, 2H, CH_2N), 3.45–3.60 (m, 7 H, $CH_2OCHCH_2OCH_2$). MS-EI, *m/z* 455 (1), 396 (1), 270 (40), 58 (100). Anal. ($C_{29}H_{61}NO_2$) C, H, N,O.

2.2.2. N,N-*Dimethyl-2,3-bis [(6E)-3,7,11-trimethyl-6,10-dodecadienyloxy]propylamine (**13**, Scheme 1)*

0.263 g, 13% yield. ^{1}H-NMR ($CDCl_3$) δ 0.90 [d, 6 H, 2 $CH(CH_3)$], 1.25–1.48 (m, 8 H, $2CH_2CH(CH_3)CH_2CH_2O$), 1.50–1.75 [m, 20 H, allylic CH_3 and 2 $CH(CH_3)$], 1.98–2.10 (m, 12 H, allylic CH_2), 2.28 [s, 6 H, $N(CH_3)_2$], 2.41 (d, 2 H, CH_2N), 3.46–3.62 (m, 7 H, $CH_2OCHCH_2OCH_2$), 5.00–5.12 (m, 4 H vinylic CH). MS-EI, *m/z* 532 (20), 462 (8), 395 (8), 308 (75), 123 (92), 109 (100). Anal. ($C_{35}H_{65}NO_2$) C, H, N,O.

2.2.3. N,N-*Dimethyl-2,3-bis[(4E,8E,12E,16E)-4,8,13,17,21-pentamethyl-4,8,12,16,20-docosapentaenyloxy]propylamine (**14**, Scheme 1)*

0.325 g, 10% yield. ^{1}H-NMR ($CDCl_3$) δ 1.35 (m, 4 H, 2 CH_2CH_2O), 1.58–1.70 (m, 36 H, allylic CH_3), 1.96–2.08 (m, 36 H, allylic CH_2), 2.27 [s, 6 H, $N(CH_3)_2$], 2.54 (d, 2 H, CH_2N), 3.45–3.62 (m, 7 H, $CH_2OCHCH_2OCH_2$), 5.00–5.15 (m, 10 H, vinylic CH). MS-EI, *m/z* 856 (16), 787 (14), 719 (25), 651 (28), 583 (85), 515 (22), 471 (38), 205 (100). Anal. ($C_{59}H_{101}NO_2$) C, H, N,O.

2.2.4. N,N-*Dimethyl-2,3-bis(4,8,13,17,21-pentamethyldocosyloxy)propylamine (**15**, Scheme 1)*

0.6 g, 18% yield. ^{1}H-NMR ($CDCl_3$) δ 0.82–0.90 (m, 36 H, CH_3), 1.25–1.60 (m, 60 H, CH_2), 1.70–1.80 (m, 10 H, CH), 2.27 [s, 6 H, $N(CH_3)_2$], 2.40 (d, 2 H, CH_2–N), 3.45–3.60 (m, 7 H, $CH_2OCHCH_2OCH_2$). MS-EI, *m/z* 876 (20), 861 (5), 764 (10), 480 (100). Anal. ($C_{59}H_{121}NO_2$) C, H, N,O.

2.3. Preparation of quaternary ammonium salts (**16–23**)

Compounds **16–19** were obtained by reaction of the corresponding diethers **12–15** (0.6 mmol) with methyl iodide (6 mmol) and potassium carbonate (12 mmol) in 20 ml of absolute ethanol. After 15 h under reflux, the reaction mixture was cooled and ethanol evaporated; water was then added and the mixture was extracted with dichloromethane (3 × 50 ml). The organic layers were pooled and washed with saturated brine, dried over anhydrous $MgSO_4$ and concentrated in vacuo.

*2.3.1. (2,3-Didodecyloxypropyl)trimethylammonium iodide (**16**, Scheme 1)*

0.329 g, 92% yield. ^{1}H-NMR ($CDCl_3$) δ 0.88 (t, 6 H, CH_3), 1.26 (m, 36 H, CH_2), 1.57 (m, 4 H, 2 CH_2CH_2O), 3.46–3.68 (m, 16 H, $N^+(CH_3)_3$ and $CH_2OCHCH_2OCH_2$), 4.06 (d, 2 H, CH_2N^+). MS-EI, *m/z* 455 (55), 411 (25), 397 (45), 300 (100). MS-CI (isobutane) 471 (100). Anal. ($C_{30}H_{64}INO_2$) C, H, I, N,O.

*2.3.2. {2,3-Bis[(6E)-3,7,11-trimethyl-6,10-dodecadienyloxy]propyl}trimethylammonium iodide (**17**, Scheme 1)*

0.234 g, 58% yield. ^{1}H-NMR ($CDCl_3$) δ 0.88 [d, 6 H, 2 $CH(CH_3)$], 1.22–1.47 (m, 8 H, 2 $CH_2CH(CH_3)CH_2CH_2O$), 1.50–1.75 [d, 20 H, allylic CH_3 and 2 $CH(CH_3)$], 1.96–2.18 (m, 12 H, allylic CH_2), 3.48–3.70 (m, 16 H, $N^+(CH_3)_3$ and $CH_2OCHCH_2OCH_2$), 4.08 (d, 2 H, CH_2–N^+), 5.02–5.13 (m, 4 H vinylic CH). MS-EI, *m/z* 547 (0.5), 532 (40), 463 (70), 308 (100). Anal. ($C_{36}H_{68}INO_2$) C, H, I, N,O.

*2.3.3. {2,3-Bis[(4E,8E,12E,16E)-4,8,13,17,21-pentamethyl-4,8,12,16,20-docosapentaenyloxy]propyl}trimethylammonium iodide (**18**, Scheme 1)*

0.419 g, 70% yield. ^{1}H-NMR ($CDCl_3$) δ 1.36 (m, 4 H, 2 CH_2-CH_2-O), 1.58–1.72 (m, 36 H, allylic CH_3), 1.95–2.10 (m, 36 H, allylic CH_2), 3.44–3.72 [m, 16 H, $N^+(CH_3)_3$ and $CH_2OCHCH_2OCH_2$), 4.08 (d, 2 H, CH_2N^+), 5.02–5.18 (m, 10 H vinylic CH). MS-CI (isobutane) 851 (100), 789 (22), 721 (16). A satisfactory EI mass spectrum has not been obtained. Anal. ($C_{60}H_{104}INO_2$) C, H, I, N,O.

*2.3.4. [2,3-Bis(4,8,13,17,21-pentamethyl-docosyloxy) propyl]trimethylammonium iodide (**19**, Scheme 1)*

0.357 g, 60% yield. ^{1}H-NMR ($CDCl_3$) δ 0.80–0.90 (m, 36 H, CH_3), 1.24–1.65 (m, 60 H, CH_2), 1.68–1.78 (m, 10 H, CH), 3.48 [s, 9 H, $N^+(CH_3)_3$], 3.59–3.78 (m, 7 H, $CH_2OCHCH_2OCH_2$), 4.05 (d, 2 H, CH_2N^+). MS-CI (isobutane) 892 (1), 877 (80), 776 (100). A satisfactory EI mass spectrum has not been obtained. Anal. ($C_{60}H_{124}INO_2$) C, H, I, N,O.

For the preparation of compounds **20–23**, the diethers **12–15** (0.6 mmol) were reacted with 2-bromoethanol (3 mmol) in 15 ml of acetonitrile, under reflux for 12 h. After evaporation of the solvent, the crude product was treated as described for compounds **16–19**.

*2.3.5. (2,3-Didodecyloxypropyl) (2-hydroxyethyl)dimethylammonium bromide (**20**, Scheme 1)*

0.306 g, 88% yield. ^{1}H-NMR ($CDCl_3$) δ 0.89 (t, 6 H, CH_3), 1.28 (m, 36 H, CH_2), 1.56 (m, 4 H, 2 CH_2CH_2O), 2.87 (s, 1 H, OH), 3.48–3.69 [m, 13 H, $N^+(CH_3)_2$ and $CH_2OCHCH_2OCH_2$), 3.87 (t, 2 H, CH_2OH), 4.06–4.16 (m, 4 H, $CH_2N^+CH_2$). MS-EI, *m/z* 455 (10), 300 (8), 270 (100). Anal. ($C_{31}H_{66}BrNO_3$) C, H, Br, N,O.

*2.3.6. [2,3-Bis (6E)-(3,7,11-trimethyl-6,10-dodecadienyloxy)propyl](2-hydroxyethyl) dimethylammonium bromide (**21**, Scheme 1)*

0.224 g, 57% yield. ^{1}H-NMR ($CDCl_3$) δ 0.90 [d, 6 H, $CH(CH_3)$], 1.23–1.48 (m, 8 H, 2 $CH_2CH(CH_3)CH_2CH_2O$), 1.50–1.72 [d, 20 H, allylic CH_3 and 2 $CH(CH_3)$], 1.98–2.18 (m, 12 H, allylic CH_2), 3.17 (s, 1 H, OH), 3.45–3.67 (m, 13 H, $(CH_3)_2N^+$ and $CH_2OCHCH_2OCH_2$), 3.93 (t, 2 H, CH_2OH), 4.04–4.12 (m, 4 H, $CH_2N^+CH_2$), 5.00–5.12 (m, 4 H vinylic CH). MS-EI, *m*/z 532 (25), 531(14), 494 (55), 424 (62), 308 (60), 294 (20), 270 (100). Anal. ($C_{37}H_{70}BrNO_3$) C, H, Br, N, O.

*2.3.7. {2,3-Bis[(4E,8E,12E,16E)-4,8,13,17,21-pentamethyl-4,8,12,16,20-docosapentaenyloxy]propyl}(2-hydroxyethyl)dimethylammonium bromide (**22**, Scheme 1)*

0.571 g, 97% yield. ^{1}H-NMR ($CDCl_3$) δ 1.35 (m, 4 H, 2 CH_2CH_2O), 1.50–1.68 (m, 36 H, allylic CH_3), 1.95–2.10 (m, 36 H, allylic CH_2), 2.45 (s, 1 H, OH), 3.35–3.60 [m, 13 H, $(CH_3)_2N^+$ and $CH_2OCHCH_2OCH_2$], 3.88 (t, 2 H, CH_2OH), 4.02–4.13 (m, 4 H, $CH_2N^+CH_2$), 5.00–5.14 (m, 10 H vinylic CH). MS-CI, *m/z* 901 (10), 857 (100). Anal. ($C_{61}H_{106}BrNO_3$) C, H, Br, N,O.

*2.3.8. [2,3-Bis (4,8,13,17,21-pentamethyldocosyloxy) propyl](2-hydroxyethyl)dimethylammonium bromide (**23**, Scheme 1)*

0.558 g, 93% yield. ^{1}H-NMR ($CDCl_3$) δ 0.80–0.92 (d, 36 H, CH_3), 1.22–1.60 (m, 60 H, CH_2), 1.62–1.75 (m, 10 H, CH), 2,45 (s, 1H, OH), 3.38–3.62 [m, 13 H, $(CH_3)_2N^+$ and $CH_2OCHCH_2OCH_2$], 3.88 (t, 2 H, CH_2–OH), 4.05–4.15 (m, 4 H, $CH_2N^+CH_2$). MS-CI, *m/z* 877 (100), 863 (15). Anal. ($C_{61}H_{126}BrNO_3$) C, H, Br, N, O.

2.4. Preparation of liposomes

Compounds (**16–23**) were prepared either alone or in combination with the neutral colipid DOPE (1:1 w/w). The cationic lipids and DOPE were dissolved in chloroform and an appropriate volume of solution was transferred to glass vials. The solvent was evaporated under reduced pressure to give the dried lipid films. Residual traces of chloroform were removed by placing vials under high vacuum overnight. The lipid films were re-suspended in sterile pyrogen-free distilled water at a concentration of 1 mg/ml by vortexing for 1 min at room temperature or sonicating in a bath type sonicator (Bransonic 12) to clarity (in the case of the less soluble compounds **18, 19, 22** and **23**).

We further prepared other two different types of liposomes by adding a solution of Tween 80 in acetone. The first kind of liposomes were obtained by adding Tween 80, in order to obtain a mixture of cationic lipid/DOPE/Tween 80 1:1:1 w/w. The other liposomes were prepared by adding Tween 80 to the cationic liposomes in a 1:1 ratio.

Liposome size was monitored by photon correlation spectroscopy using a Coulter Model N4SD submicron particle analyzer (Coulter Electronics Inc., Hialeah, FL).

The zeta potential of the different preparations was also evaluated (Coulter DELSA model 440, Coulter Electronics Inc.). Light scattering data were collected for 60 s at four angles simultaneously using a frequency range of 500 Hz and a current range equal to or lower than the conductivity of the sample.

2.5. Preparation of DNA/cationic lipid complexes

Plasmid DNA and the different cationic lipid/DOPE (1:1 w/w) preparations were mixed together by vortexing at room temperature and the complex formation was allowed to proceed for 30 min at room temperature. Different amounts of cationic liposomes were used in order to obtain complexes at ratio of 1:2, 1:1, 2:1, 3:1, 5:1 and 8:1 lipid/DNA (ratio w/w).The size and the zeta potential values of the complexes thus prepared were determined as detailed above.

2.6. Cell cultures

Cell lines that are derived from different origin were used for this study. HEK 293 (human foetal kidney), CHO (chinese hamster ovary), A431 (human epidermoid carcinoma) and KB (human nasopharyngeal carcinoma) cell lines were purchased from ATCC (Rockville, MD) and IGROV1 (human ovarian carcinoma) cell line was purchased from J. Benard (Institute G. Roussy, Villejuif, France). All cell lines were maintained in RPMI 1640 (Gibco BRL, Paisley, UK) supplemented with 5% FCS, 2 mM L-glutamine and gentalyn (100 U/ml) at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.7. Transfection

Cells (1×10^5) were seeded in 12-well plates one day before transfection at a density resulting in 40–50% confluence on the day of transfection. Before transfection, cells were washed once with PBS, and 1 ml of fresh medium without FCS was added. Supercoiled pCAT basic vector (Promega, Madison, WI) (2.5 μg), used as carrier for DNA, was co-transfected with CMV-Luc (0.5 μg), containing the luciferase gene downstream from a CMV promoter (a gift from Dr. Maria Zajac-Kaye, Bethesda, MD) using 5 μl of each cationic liposome (Lipofectin™, Escort™ or the new synthesized products). After 10 min incubation at room temperature, the mixture was added dropwise to the cells followed by overnight incubation at 37 °C in a 5% CO_2 atmosphere. Medium was replaced with fresh medium containing 10% FCS and incubation continued for an additional 48 h. Cell lysates were prepared using 100 μl of Reporter Lysis Buffer (Promega) per well. To determine transfection efficiency, 100 μl of Luciferase Assay Buffer (Promega) was added to 10 μg of cell lysate and luciferase activity was evaluated using a TD Luminometer (Promega). The final values of luciferase are reported in terms of RLU/mg total protein. We also evaluated the transfection efficiency of complexes obtained by incubating DNA and cationic lipid/DOPE/Tween 80 1:1:1 or DNA and cationic lipid/Tween 80 1:1.

3. Results

3.1. Chemistry

Different cationic lipids based on an hydrophobic and a cationic portion, linked by an ethereal bond similar to the commercially-available reagent DOTMA [7], were prepared. As shown in Scheme 1, in order to evaluate the importance of chain length and number of insaturations present in the hydrophobic portion for transfection capacity, we developed various compounds with dodecyl, 2,3-dihydrofarnesyl, trisnorsqualenyl and trisnorsqualanyl ethereal chains, bearing methyl and 2-hydroxyethyl substituents linked to the nitrogen atom.

The preparation of the compounds is shown in Scheme 1; initially the methanesulfonyl derivatives of dodecanol **2**, 1,1′,2-trisnorsqualene alcohol **9**, 1,1′,2-trisnorsqualane alcohol **11** and of a derivative of farnesol, obtained by selective reduction of the double bond vicinal to the alcohol group **7** [25], were prepared. For this purpose, the different alcohols **1, 6, 8** and **10** were reacted with methanesulfenyl chloride in the presence of triethylamine.

For the preparation of the farnesol derivative it was not possible to use farnesol directly, because the double bond in position 2 with respect to the alcohol function renders it too reactive with methanesulphenyl chloride and various degradation products are formed; we consequently reduced this double bond (Scheme 2).

Reaction between 3-(dimethylamino)-1,2-propanediol and the different methanesulfonates **2, 7, 9** and **11** in the presence potassium *tert*-butoxide then afforded the diethers **12–15**. Transformation to the corresponding quaternary ammonium salts was achieved by reacting the diethers with methyl iodide [23] in the case of derivatives **16–19** or with 2-bromoethanol [13] in the case of derivatives **20–23**. The compounds were characterized by ^{1}H-NMR and MS spectra.

3.2. Preparation and characterization of liposomes

The cationic lipids were dissolved in chloroform and mixed in 1:1 ratio (w/w) with the colipid DOPE and the dried film was resuspended by vortexing or by sonicating it to clarity. In the preparations of lipid vesicles with the compounds containing the derivative of squalene and squalane in the hydrophobic chains (compounds **18, 19, 22** and **23**) it was difficult to resuspend these derivatives in water, due to their low solubility. In the case of these same derivatives, to prepare liposomes it was necessary to sonicate in a bath-type sonicator.

The different preparations showed mean diameters of 320–400 nm for the vortexed liposomes and of 250–300 nm for the sonicated ones.

Cationic liposomes displayed a net positive zeta potential value ranging from +40 to +55 mV both for the cationic liposomes alone and for their mixture with DOPE. These values were similar to those observed for commercially available cationic lipids such as Lipofectin™ and Escort™.

In order to improve the solubility of compounds **18, 19, 22** and **23**, we prepared other liposomes composed either of cationic lipid/DOPE/Tween 80 in a ratio of 1:1:1 or of cationic lipid/Tween 80 1:1 w/w. We observed an increased solubility only for the preparation composed of cationic liposomes and Tween 80. There was no difference in terms of size and charge.

3.3. Complexes with DNA

Complexes between plasmidic DNA and various amounts of the different preparations of cationic lipids, in 1:1 mixture with DOPE, were prepared and analyzed. The mean diameter of the complexes was determined using photon correlation spectroscopy.

At low lipid/DNA ratios, the complexes were small with a mean diameter similar to that of the corresponding vesicles alone. The size of the vesicles increased on increasing the amount of lipid used (lipid/DNA ratio 2:1 and 5:1), then the complexes were smaller for higher excesses of lipid (8:1). Fig. 1 shows the size of the complexes with different excesses of lipids **16, 17, 20** and **21**.

We further evaluated the zeta potential values of these complexes: results are in Fig. 2. We observed that the complexes showed negative zeta potentials until 5:1 lipid/DNA ratio, while we obtained a net positive value for excesses of lipids over DNA of 8:1.

The results were satisfactory for all the cationic lipids, except for the squalene and squalane derivatives **18, 19, 22** and **23**. For these, it was not possible to establish the exact concentration in the mixtures, given their poor solubility in water. On account of this problem, we did not succeed in verifying for what lipid excess the mixture incubated with DNA would have had a positive zeta potential.

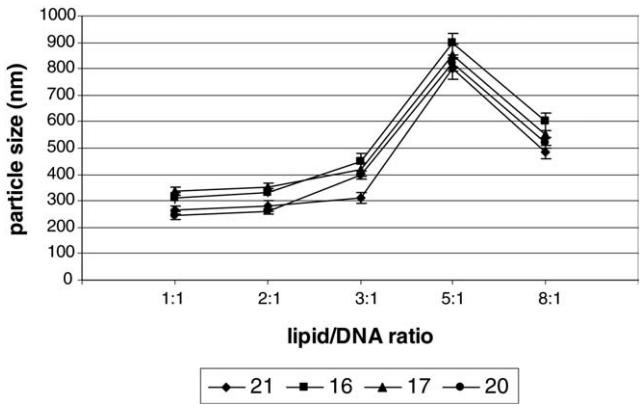


Fig. 1. Size of complexes between DNA and lipid. Cationic lipid vesicles were prepared as 1:1 (w/w) of cationic lipid:DOPE and mixed with DNA at various ratios. Error bars represent standard deviations.

3.4. In vitro transfection efficiency

The transfection efficiency of the different cationic lipids in mixture with DOPE was evaluated on two normal and three malignant cell lines with different transfection capacities: HEK 293 (human foetal kidney), CHO (chinese hamster ovary), A431 (human epidermoid carcinoma), KB (human nasopharyngeal carcinoma) and IGROV1 (human ovarian carcinoma) cell lines. We compared the capabilities of our cationic lipids to those of the commercially-available Lipofectin™ and Escort™.

As shown in Fig. 3, two groups of cell lines could be distinguished within the five cell lines tested. HEK and CHO cells belong at the first group; they showed high transfection efficiency with dodecyl and farnesyl derivatives **16, 20** and **17** but also with the commercial cationic lipids, although the latter had a transfection capability about 10-fold lower. The carcinoma cell lines belong to the second group; they showed a transfection efficiency with commercial cationic lipid in an order of magnitude between 1 and 10, while the transfection capability reached 10 and 10^2 of magnitude with the dodecyl and farnesyl derivatives **16, 20** and **17**. This increment was particularly evident in IGROV1 cells, which showed a transfection efficiency of a magnitude around 10^3. Note that with compound **20**, the dodecyloxypropyl hydroxyethyldimethyl ammonium derivative, all five cell lines were found to be transfected with an efficiency from four to one thousand times higher than that of Lipofectin™.

The squalene and squalene derivatives did not provide satisfactory results in that, owing to their poor solubility in an aqueous environment, it was not possible to incubate them with known quantities of DNA. We carried out other transfection experiments preparing liposomes composed of cationic lipid/DOPE/Tween 80 1:1:1 and cationic lipid/Tween 80 1:1, but none of these compounds showed a better transfection efficiency in all cell lines tested (data not shown).

4. Discussion

Cationic lipid molecules contain four different functional domains: a positively charged head group, a spacer of va-

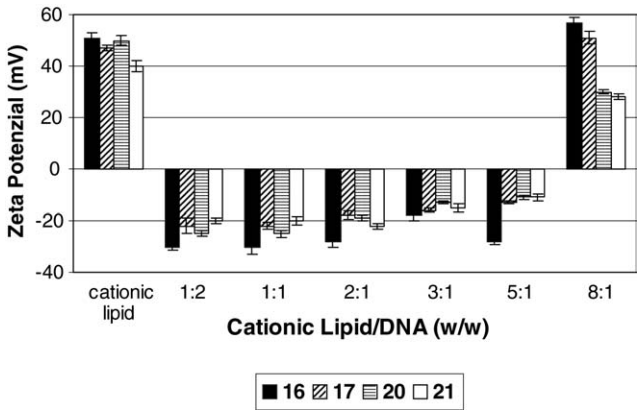


Fig. 2. Zeta potentials of cationic lipids alone and cationic lipid:DNA complexes. Error bars represent standard deviations.

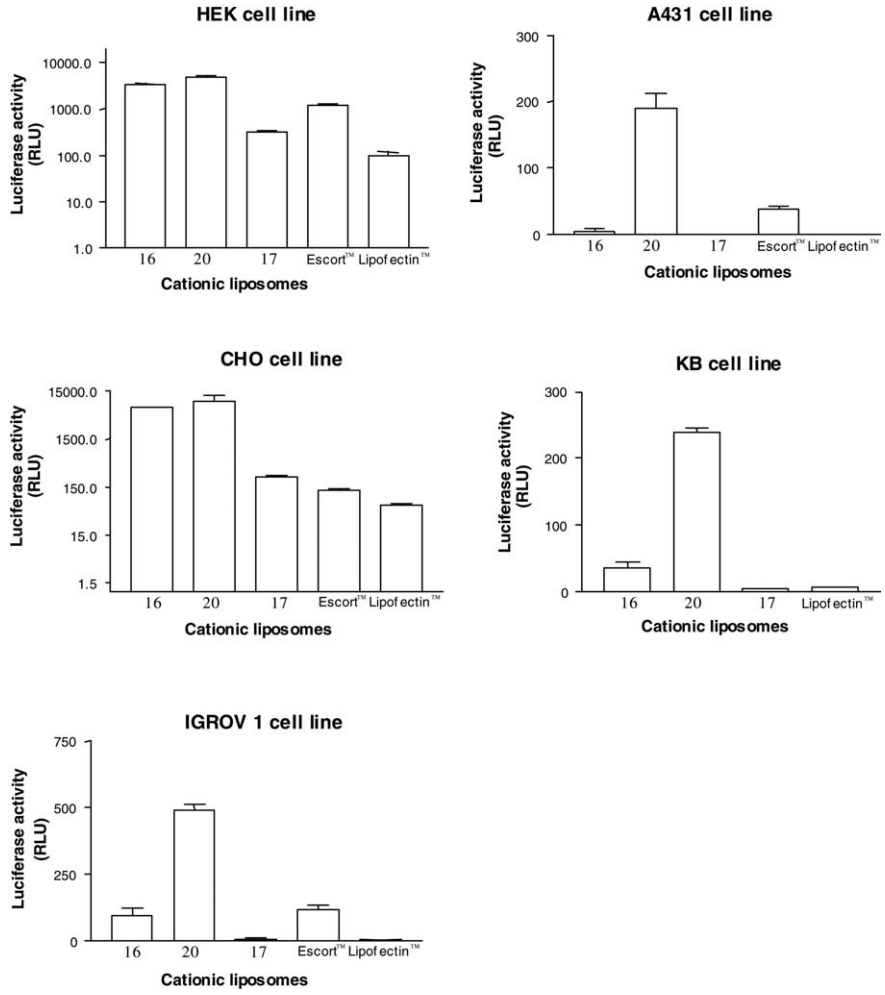


Fig. 3. Evaluation of the transfection efficiency. Cells were transiently transfected as reported in Materials and methods. For KB cells the transfection efficiency with Escort™ was not evaluated. Data (mean ± S.D.) are representative of three independent experiments for each cell line.

rying length, a linker bond and a hydrophobic anchor. Taking the cationic lipid series as a model [7,13], the positively charged head group consisted of a quaternary ammonium with substitution of methyl or hydroxyethyl groups responsible for interactions between liposome-DNA complex and cell membrane. A spacer arm containing only one atom spacer, and the hydrophobic portion made up of an alkoxy chain, are important for stability, biodegradability and transfection activity.

The fatty acid chains, which are usually monounsaturated (oleoyl, C18) or shorter saturated (C16 or C14), are generally important since they are responsible for membrane fluidity and good lipid mixing within the bilayer. Felgner et al. [13] have shown that, as the length of the saturated aliphatic chain increases from 14 to 16 or 18 carbon atoms, the transfection activity of the resulting cationic lipids progressively declines. By contrast, it has also been shown that the highest activity is either for palmitoyl or for oleoyl chains. In cationic lipids, increasing the number of double bonds in the fatty acid chain corresponds to a decrease of both the phase transition temperature and the bilayer stiffness of the resulting vesicles [27,28]. Bilayer rigidity may interferes with membrane fusion, which is the postulated step in the DNA delivery mechanism [11]. Lipid fusion is very important for the escape of DNA from the endosome to the nucleus. In the preliminary formulation study we determined the phase transition temperature of the different cationic ammonium lipids by DSC. The C12 saturated derivatives showed an endothermic transition characterized by a sharp peak, respectively, at 53.37 and 79.77 °C. By introducing one or more double bonds in the fatty chain we expected to obtain a decrease of phase-transition temperature. Indeed, the bis unsaturated C15 dihydro farnesyl derivative showed a peak at lower temperature (29.75 °C). The C27 squalenyl ammonium derivative, characterized by the presence of four *E* double bonds showed a broad peak, from 30 to 42 °C, at DSC. The proximity of the phase transition temperature to 37 °C, temperature of the cells during transfection, may also influence the efficiency of the fusion step.

Cationic lipids form a stable complex with the fusogenic helper lipid DOPE, which has been reported to have a high tendency to form an inverted hexagonal phase (H_{II}) at acidic pH [29]. Formation of the H_{II} by DOPE probably destabilizes the endosomes, resulting in an efficient escape from lysosomal degradation and cytoplasmatic release of DNA [30]. The preparation of MLV cationic liposome was strongly affected

by the solubility of each lipid; thus whereas the preparation of liposomes containing a short lipophilic tail, such as **16** and **20**, and the farnesyl derivatives (**17**, **21**) was easy, those incorporating the highly lipophilic derivatives (**18**, **19**, **22**, and **23**) were little soluble in water. For these compounds, long sonication times were necessary. By adding DOPE to liposomes we obtained particles whose diameter was inversely related to the amount of DOPE. DOPE probably interacts with cationic lipids, forming a stable complex through ion pair formation between the negatively charged phosphate on DOPE and the quaternary ammonium group on the cationic lipid, as well as favourable intermolecular hydrogen bond interactions [31,32].

The MLV liposomes composed of a 50:50 w/w cationic lipid/DOPE were mixed with plasmid DNA at various charge ratios and the diameter and zeta potential of the resulting complexes (lipoplexes) were measured. As expected, the absolute value of the mean diameter of the lipoplexes was a function of the excess of lipid/DNA ratio, varying from 300 nm (lipid/DNA ratio 1:1) to a maximum of 750–900 nm at liposome/DNA ratio of 5:1, and returning to a smaller size (500–600 nm) at a lipid/DNA ratio 8:1.

The surface charge of a DNA/lipid complex is expected to influence its interaction with various biological components as well as its distribution, access and entry to the target cells. As observed using light scattering analysis, the lipoplex zeta potential changed from negative to positive when the mixing charge ratio changes from excess negative to excess positive. For instance the zeta potential changed from a negative value (–25 mV) for a 1:2 lipid/DNA ratio to a positive ratio (+55 mV) for a 8:1 lipid/DNA ratio. The surface charge of the DNA/lipid complexes can therefore be controlled by adjusting the ratio of cationic lipid to DNA ratio.

To improve the water solubility of squalene and squalane cationic lipids, we decided to use the neutral surfactant Tween 80 with or without DOPE as helper co-lipid. It has been found that the inclusion of small amounts of Tween 80 into the lipid formulation (such as DC-cholesterol) provides better stability for DNA/lipid complexes, as well as high transfection activity [33].

Similarly to DOPE, the addition of Tween 80 to cationic lipids progressively reduced the diameter of the particles. In addition, due to the absence of electric charge, the addition of Tween 80 to the lipid did not change the absolute value of the original positive charge. Thus, if we only considered the problems related to physical stability of the colloidal formulation, the surfactant Tween 80 behaved like the helper lipid DOPE.

Lastly, we tested the in vitro transfection efficiency of the cationic lipid/DNA complexes using different formulations: (1) cationic lipid/DOPE 1:1 w/w; (2) cationic lipid/Tween 80 1:1 w/w; (3) cationic lipid/DOPE: Tween 80 1:1:1.

For comparison we also tested the commercial lipids Escort™ (DOTAP/DOPE; 1:1) and Lipofectin™ (DOTMA/DOPE; 1:1). The differently formulated lipid/DNA complexes were co-transfected with a plasmid containing a luciferase gene placed downstream from a cytomegalovirus promoter. The different transfection capacities were evaluated on five types of cell lines: HEK, CHO, A431, IGROV1 and KB.

Initially we tested the transfection efficiencies of all the novel transfection lipids using a 1:1 w/w mixture with DOPE. Interestingly, the amphipile having both a C12 saturated chain and a single hydroxyethyl group in the head group showed the highest activity compared with the commercial Lipofectin™, followed by the C12 ammonium analogue of DOTMA. If the hydrophobic moiety was increased to a C15 chain containing two double bonds, as in the farnesyl derivative (**17**), the activity, albeit less, was still satisfactory. C27 fully saturated squalane derivatives (**19** and **23**), or those containing three double bonds (**18** and **22**), retained no activity. The addition of Tween 80, as in formulations 2 and 3, slightly increased the acqueous solubility but did not increase transfection activity.

Thus, for our series of lipids, Tween 80 was not able to replace DOPE as a helper lipid. Indeed, when the most active cationic lipid was complexed with Tween 80, the activity dramatically decreased. Taking Lipofectin (RLU=1) as reference, **20** had RLU=1016 when formulated as a 1:1 DOPE complex, RLU=7 when 1 mg of Tween 80 was added (formulation 3) and RLU was negative when DOPE is replaced by the surfactant.

These finding suggest the fundamental role of DOPE in the activity of all synthesized lipid compounds, as has already been reported [32]. A proper DOPE association with the cationic lipid may increase DNA cell uptake, facilitate the escape of DNA from endosomes and increase the ability of DNA to dissociate from the lipid complex. We also showed that the activity may vary depending on the target cells, which may include differences in cell surface composition that effect complex binding, and differences in the complex uptake mechanism. The results also showed that, for best transfection activity, a shorter and saturated alkyl chain has a much more beneficial effect than a longer unsaturated fatty chain.

The C12 cationic lipid derivatives **16** and **20** have been reported in a patent, with no description of synthesis or biological activity [34]. Compound **20** was also cited, within a list of different lipoplexes, in a review not reporting any detailed investigations [35].

The C15 farnesil derivative, having an unusual branched structure, albeit less active than the C12 saturated lipids, is very interesting, being more active than commercial lipids, such as DOTMA or ESCORT™ in almost all cell lines tested. Starting from this lead, further work will be necessary to better elucidate the structural–relationship, taking into consideration new series of lipid derivatives.

Acknowledgements

This work was supported by MIUR 40–60%.

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