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Feature Article

Varying the Unsaturation in N^4,N^9-Dioctadecanoyl Spermines: Nonviral Lipopolyamine Vectors for More Efficient Plasmid DNA Formulation

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Purpose. The aim of the study is to analyze the effect of varying the degree of unsaturation in synthesized N^4,N^9-dioctadecanoyl spermines on DNA condensation and then to compare their transfection efficiency in cell culture.
Methods. The N^4,N^9-di-C18 lipopolyamines—saturated (stearoyl), C9-*cis*- (oleoyl), and C9,12-di-*cis*-(linoleoyl)—were synthesized from the naturally occurring polyamine spermine. The ability of these novel compounds to condense DNA and form nanoparticles was studied using ethidium bromide fluorescence quenching and nanoparticle characterization techniques. Transfection efficiency was studied in several primary skin cells (FEK4, FCP4, FCP5, FCP7, and FCP8) and in an immortalized cancer cell line (HtTA) and was compared with the commercially available nonliposomal transfection formulation Transfectam® (dioctadecylamidoglycyl spermine), which also contains two saturated C18 lipid chains.
Results. N^4,N^9-Dilinoleoyl spermine (C18, di-*cis*-9,12) is efficient at circular plasmid DNA (pEGFP) condensation and gives the most effective transfection in a series of primary skin cells and cancer cell lines at low charge ratios of 5.5 (± ammonium/phosphate).
Conclusions. The dienoic fatty acyl spermine conjugate N^4,N^9-dilinoleoyl spermine efficiently condenses DNA and achieves the highest transfection levels among the studied lipopolyamines in cultured cells.

KEY WORDS: FCP; FEK4; gene delivery; HtTA; lipopolyamine; N^4,N^9-dilinoleoyl spermine; N^4,N^9-dioleoyl spermine; N^4,N^9-distearoyl spermine; primary skin cells; transfection.

INTRODUCTION

As viral gene therapy continues to suffer significant problems with mammalian toxicity (1–3), the possibility of reaching the goal of intracellular protein levels at therapeutic concentrations moves even more toward utilizing nonviral gene therapy (NVGT). Within this broad term, we are concentrating on lipopolyamines composed of a lipophilic steroid attached either to a polyamine chain (4–8) or to a single or double long-carbon chain covalently bound to a polyamine, e.g., spermine (N^1,N^{12}-diamino-4,9-diazadodecane) (9–11). Other research groups are investing in a variety of alternative approaches classified under the NVGT umbrella, including the following: naked DNA (12), gene gun (bound to gold particles) (13,14), electroporation (15), polycation-mediated DNA delivery, and the use of a wide variety of cationic lipids (lipoplexes) (16), e.g., bolaamphiphiles (17), and cationic polymers (polyplexes) (18–20) [for reviews, see (21–25)]. For gene therapy to realize its potential and become an efficient medicine for the treatment of diseases such as cancer, cystic fibrosis, inflammation, or for vaccination, key obstacles must be overcome. The essential requirements for gene delivery are the transport of DNA through the cell membrane and ultimately to the nucleus. The design of an efficient formula for the delivery of genetic material requires a detailed understanding of the barriers that hinder this process. Thus, efficient formulations, lipoplexes (16,17), and polyplexes (18–20) must be able to deliver safely the required DNA across the various cellular barriers to the nucleus. Barriers to DNA delivery also include complex formation between the DNA and the lipopolyamine leading to DNA nanoparticle formation by electrostatic charge neutralization (to about 90% of the total negative charge) and overall packing as condensed DNA nanoparticles (26). So, a key first step in this method of gene formulation is masking the negative charges of the phosphate backbone. This titration with a lipopolyamine causes alleviation of charge repulsion between remote phosphates along the DNA helix leading to collapse into a more compact structure that facilitates cell entry.

For NVGT, except for naked (free, uncomplexed) DNA being trapped inside cells by direct association with the

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ABBREVIATIONS: DOGS, dioctadecylamidoglycyl spermine; DOPC, dioleoylphosphatidyl choline; DOPE, dioleoylphosphatidyl ethanolamine; EGFP, enhanced green fluorescent protein; EMEM, Earle's Minimal Essential Medium; EthBr, ethidium bromide; FCS, fetal calf serum; HRMS, high-resolution mass spectroscopy; NVGT, nonviral gene therapy; PE, phosphatidyl ethanolamine; PLL, poly-L-lysine.

chromatin during mitosis, the (prodrug) DNA must be formulated. Small molecule synthetic cationic lipids are one of the major gene carriers for NVGT, often classified as liposomal and nonliposomal nonviral delivery vectors. They condense DNA into nanoparticles that are readily endocytosed by cultured cells and facilitate endosomal escape leading to efficient delivery to the nucleus presumably crossing through the nuclear pore complex. After nuclear entry, the payload DNA should ultimately be able to give the desired protein through transcription and translation.

The first cellular barrier for the delivery of the DNA nanoparticles is the eukaryotic cell membrane, which is composed mainly of phospholipids (50–90% of total lipid content; most phospholipids are derivatives of diacyl-glycerol-3-phosphate), sterols (5–25%), and glycol lipids (usually less than 5%). The diacyl-glycerol chains (C14–C24) are derived from linear fatty acids with varying degrees of unsaturation (27). In NVGT, cationic lipids, which interact with the DNA payload, also mediate cell-membrane transport, typically through adsorptive endocytosis or mediated by cations (28–30), both routes leading to internalization of the DNA complex nanoparticles. Such endocytosis is via the clathrin-coated pits (250–300 nm) (31), followed by fusion of the early endosome and sorting to the late endosomal compartment, hence avoiding degradation in the lysosome. One of the important factors improving the release of free DNA or the lipoplex into the cytoplasm is the influence of the cationic lipid chain. Xu and Szoka (32) have reported that unsaturated hydrocarbon chains increase the transfection efficiency of the lipoplex by decreasing the rigidity of the bilayer and favoring a higher intermembrane transfer rate and lipid mixing, compared with their saturated counterparts. However, Roosjen *et al.* (33) recently reported that, in certain cases, saturated fatty chains afforded better results than unsaturated chains. Herein, we investigate the effects on DNA formulation of a circular plasmid with variation in the degree of unsaturation in the two C18 fatty chains in our lipospermines, the saturated N^4,N^9-distearoyl spermine, the alkenoic N^4,N^9-dioleoyl spermine, and the dienoic fatty acyl spermine conjugate N^4,N^9-dilinoleoyl spermine. We report the synthesis and characterization of the nanoparticles, summarize transfection results with the synthesized lipospermine formulations in a panel of both primary and cancer cell lines, and compare these results with those obtained with the commercially available nonliposomal lipospermine Transfectam® [dioctadecylamidoglycyl spermine (DOGS)] formulation (34,35).

MATERIALS AND METHODS

Materials

Chemicals, including spermine, acyl chlorides (linoleoyl, oleoyl, and stearoyl chlorides), solvents, buffers, and DNA were routinely purchased from Sigma-Aldrich, Dorset, UK, except where indicated. Transfectam® was from Promega (Southampton, UK), and cell culture materials were from Life Technologies (Paisley, Scotland).

Preparation of Plasmid DNA

We have chosen to deliver a 4.7-kbp plasmid encoding for enhanced green fluorescent protein (pEGFP), with a molecular weight of about 3.1 MDa (given an average of 330 Da per nucleotide, 660 Da per base pair (36), carrying 9400 negative charges). DNA plasmid pEGFP, purchased from Clontech (Basingstoke, UK), was transformed into *Escherichia coli* JM 109 bacterial strain (Promega) and was purified by a Qiagen Maxi kit (Qiagen, Chatsworth, CA, USA). DNA yields and purity were determined spectroscopically (OD_{260}/OD_{280} = 1.80–1.90 OD, optical density) and by agarose gel (1%) analysis.

Synthesis of Lipospermines N^4,N^9-Dilinoleoyl Spermine, N^4,N^9-Dioleoyl Spermine, and N^4,N^9-Distearoyl Spermine

Spermine was used as the starting material for the synthetic process, outlined in Fig. 1. The tetra-amine was protected on both the primary amino functional groups with ethyl trifluoroacetate (2.2 eq.) in methanol, and the reaction mixture was stirred for 18 h at 20°C. The solvent was evaporated to dryness *in vacuo* to form N^1,N^{12}-ditrifluoroacetyl-1,12-diamino-4,9-diazadodecane. Fatty acyl chloride (linoleoyl, oleoyl, and stearoyl; 2.2 eq) and triethylamine (2.5 eq) were added to the diprotected spermine solution in CH_2Cl_2 and methanol (1:1). The solution was stirred for 72 h at 20°C and then evaporated to dryness *in vacuo*. The residue was dissolved in CH_2Cl_2, and the solution was filtered and evaporated to dryness *in vacuo* to form N^4,N^9-[dilinoleoyl, dioleoyl (37), or distearoyl]-N^1,N^{12}-ditrifluoroacetyl-1,12-diamino-4,9-diazadodecane. For the removal of the ditrifluoroacetyl protecting groups, the tetra-amide was dissolved in methanol, and the pH of the solution was increased by saturating with ammonia gas, then it was left

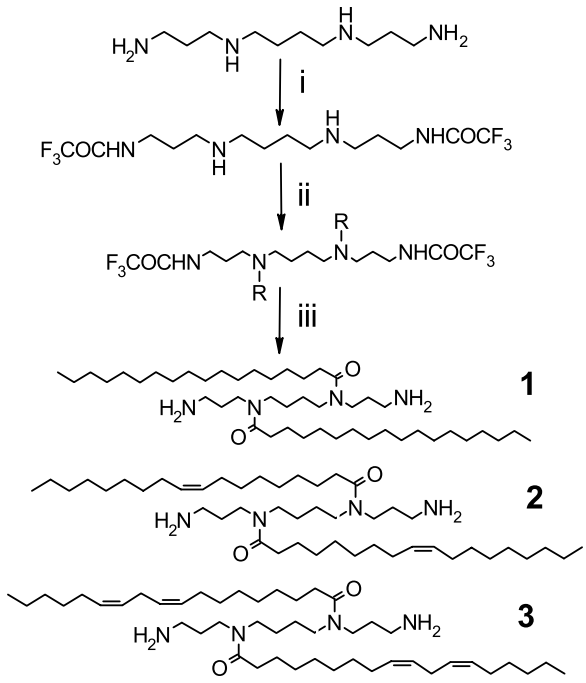

Fig. 1. Synthetic scheme of spermine-based cationic lipids: N^4,N^9-distearoyl spermine **1**, N^4,N^9-dioleoyl spermine **2**, and N^4,N^9-dilinoleoyl spermine **3**. Reagents: (i) ethyl trifluoroacetate, methanol, 18 h at 20°C; (ii) fatty acyl chloride, R = (stearoyl **1**, oleoyl **2**, and linoleoyl **3**), triethylamine, CH_2Cl_2, and methanol (1:1), 72 h at 20°C; (iii) methanol saturated with ammonia gas, 18 h at 20°C.

for 18 h at 20°C and then evaporated to dryness *in vacuo* to give a residue that was purified over silica gel (CH_2Cl_2–MeOH 5:3, v/v, then CH_2Cl_2–MeOH–conc. aq. NH_3 25:10:1, v/v/v) to afford the three desired lipopolyamine conjugates as their free bases. All three synthesized lipopolyamines were homogenous on silica gel thin-layer chromatography (CH_2Cl_2–MeOH–conc. aq. NH_3 25:10:1, v/v/v) and were characterized by ^{1}H-nuclear magnetic resonance (NMR; at 400 MHz) and ^{13}C-NMR spectroscopy (assignments follow from correlation spectroscopy) and by high-resolution mass spectroscopy (HRMS):

N^4,N^9-distearoyl spermine **1**, R_f = 0.41, NMR *inter alia* (C^2HCl_3) δ ^{1}H 0.91 ppm (t, 2 × CH_3), 1.25–1.75 ppm (br m, many × CH_2), 2.40–2.58 ppm (br s, 2 × NH_2), and 3.24–3.48 ppm (br m, many × NCH_2), δ ^{13}C 173 ppm (2 × CON), $C_{46}H_{95}N_4O_2$ found (*m*/*z* $[M + H]^+$) 735.7458, requires 735.7455 (Δ ppm −0.4), $C_{45}{}^{13}CH_{95}N_4O_2$ found 736.7500, requires 736.7489 (Δ ppm −1.5).

N^4,N^9-dioleoyl spermine **2**, R_f = 0.44, NMR *inter alia* (C^2HCl_3) δ ^{1}H 0.91 ppm (t, 2 × CH_3), 1.24–1.70 ppm (br m, many × CH_2), 2.03 ppm (app q, 4 × allylic CH_2), 4.55–5.10 ppm (br s, 2 × NH_2), and 5.32–5.41 ppm (m, 2 × CHCH), δ ^{13}C 130 ppm (2 × CHCH) and 173 ppm (2 × CON), $C_{46}H_{91}N_4O_2$ found (*m*/*z* $[M + H]^+$) 731.7115, requires 731.7142 (Δ ppm 3.7), $C_{45}{}^{13}CH_{91}N_4O_2$ found 732.7145, requires 732.7176 (Δ ppm 4.1).

N^4,N^9-dilinoleoyl spermine **3**, R_f = 0.49, NMR *inter alia* (C^2HCl_3) δ ^{1}H 0.89 ppm (t, 2 × CH_3), 1.25–2.00 ppm (br m, many × CH_2), 2.05 ppm (app q, 4 × allylic CH_2), 2.77 ppm (app t, 2 × doubly allylic CH_2), 5.29–5.42 ppm (m, 4 × CHCH), and 5.85–6.20 ppm (br s, 2 × NH_2), δ ^{13}C 128 and 130 ppm (4 × CHCH) and 174 ppm (2 × CON), $C_{46}H_{87}N_4O_2$ found (*m*/*z* $[M + H]^+$) 727.6846, requires 727.6829 (Δ ppm −2.3), $C_{45}{}^{13}CH_{87}N_4O_2$ found 728.6857, requires 728.6863 (Δ ppm 0.7).

DNA Condensation

DNA condensation was monitored using an ethidium bromide (EthBr) fluorescence quenching assay as described in detail earlier (11). Briefly, DNA (6 μg) was diluted to 3 ml with buffer [20 mM NaCl, 2 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 μM ethylenediaminetetraacetic acid (EDTA), pH 7.4] in a glass cuvette stirred with a microflea. EthBr solution (3 μl, 0.5 mg/ml) was added to the stirring solution and was allowed to equilibrate for 10 min. Separately, lipopolyamine aliquots [5 μl, according to the ammonium/phosphate (±) charge ratio required] were then added to the stirring solution, and the fluorescence was measured after 1-min equilibration using a Perkin-Elmer LS 50B luminescent spectrometer (λ_{excit} = 260 nm and λ_{emiss} = 600 nm with slit width 5 nm) while stirring using an electronic stirrer (Rank Bros. Ltd., Bottisham, Cambridge, England). The fluorescence was expressed as the percentage of the maximum fluorescence when EthBr was bound to the DNA in the absence of competition for binding and was corrected for background fluorescence of free EthBr in solution.

Also, each sample of plasmid DNA (0.5 μg), either free or complexed with the lipopolyamine, was analyzed by gel electrophoresis for about 60 min under 75 V/cm, through an agarose gel (1%) containing EthBr (1 μg/ml) in Tris–acetate–EDTA 1× (40 mM Tris–acetate and 1 mM EDTA) buffer. The (unbound) free DNA in the agarose gel was visualized under UV using GeneGenius (Syngene, Cambridge, UK).

Lipoplex Particle Size and ζ-Potential Measurements

The average particle size for the lipoplexes formed (at their optimum charge ratio of transfection), after mixing with a vortex mixer, was determined using a Malvern Zetasizer (Nano S, Malvern Instruments, Malvern, UK), and ζ-potential measurements were determined using a Malvern Zetasizer (Nano ZS, Malvern Instruments). All measurements were carried out on lipoplexes with 5 μg/ml plasmid DNA in HEPES buffer at pH 7.4 and 20°C.

Cell Culture and Transfection Experiments

Six cell lines were used in the transfection experiment; FEK4 (38), FCP4, FCP5, FCP7, and FCP8 cells are human primary fibroblasts derived from newborn foreskin explants (39). HtTA cells are a human cervical carcinoma, HeLa-derived and transformed cell line. Cells were cultured in Earle's Minimal Essential Medium (EMEM) supplemented with fetal calf serum (FCS), 15% in the case of FEK4 and FCP cells and 10% in the case of HtTA cells, penicillin and streptomycin (50 IU/ml each), glutamine (2 mM), and sodium bicarbonate (0.2%). Primary cells were passaged once a week and used between passages 7 and 15.

For the transfection (gene delivery) and the resultant gene activity (transfection efficiency), cells were seeded in 6-well plates at a density of 1 × 10^5 cells/well (in 4 ml EMEM with FCS) for 24 h to reach a 50–60% confluency on the day of transfection. The complex was prepared by mixing pEGFP (2 μg/ml/well) with each lipopolyamine in Opti-MEM (serum-free media, Gibco BRL, Berlin, Germany) according to the charge ratio at 20°C for 30 min and then incubated with the cells for 4 h at 37°C in 5% CO_2. Then the cells were washed and cultured for a further 44 h in growth medium at 37°C in 5% CO_2.

Levels of EGFP in the transfected cells were detected and corrected for background fluorescence of the control cells using a fluorescence-activated cell sorting (FACS) machine (Becton Dickinson FACS Vantage dual Laser Instrument, argon ion laser 488 nm). The transfection efficiency was calculated based on the percentage of the cells that expressed EGFP (positive cells) in the total number of cells.

In Vitro Cytotoxicity

Briefly, FEK4, FCPs, and HtTA cells were seeded in 96-well plates at 8000 cells/well and incubated for 24 h at 37°C in 5% CO_2. Lipoplexes with pEGFP were added at the same concentration as in the transfection protocol (2 μg/ml). Cytotoxicity was evaluated after incubation for 44 h; sterile-filtered 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (10 μl, 5 mg/ml; Sigma-Aldrich, UK) was added to reach a final MTT concentration of 0.5 mg/ml. The color produced was measured using a plate

reader (VERSAmax™) at λ = 570 nm. The percent viability related to control wells containing untreated cells (without naked DNA and without lipopolyamine–DNA lipoplex) is calculated by [OD (measured)/OD (untreated cells)] × 100 (40). The same protocol was followed for the commercially available reagent Transfectam® (34,35).

RESULTS

Synthesis of Lipospermines

The tetra-amine spermine was used as the starting material for the synthesis of the three desired lipopolyamines: N^4,N^9-distearoyl spermine **1**, N^4,N^9-dioleoyl spermine **2**, and N^4,N^9-dilinoleoyl spermine **3** (Fig. 1). The tetra-amine was protected on both the primary amino functional groups with ethyl trifluoroacetate (2.2 eq.) in methanol. Each corresponding fatty acyl chloride (stearoyl, oleoyl, or linoleoyl) was used as the acylating agent together with triethylamine in CH_2Cl_2 and methanol (1:1). Deprotection in methanol saturated with ammonia gas and flash column chromatography afforded the three target lipospermines, homogenous on silica gel thin-layer chromatography, N^4,N^9-dilinoleoyl spermine R_f = 0.49, N^4, N^9-dioleoyl spermine R_f = 0.44, and N^4,N^9-distearoyl spermine R_f = 0.41 (CH_2Cl_2–MeOH–conc. aq. NH_3 25:10:1, v/v/v), and fully characterized by ^{1}H-NMR (at 400 MHz) and ^{13}C-NMR spectroscopy and by HRMS.

DNA Condensation

In Fig. 2, we show the DNA condensation ability of the synthesized lipopolyamine formulations in comparison with the commercially available Transfectam® in an EthBr fluorescence quenching assay. Three cationic lipid formulations (N^4,N^9-dilinoleoyl spermine, N^4,N^9-dioleoyl spermine, and Transfectam®) show similar DNA condensing ability. They are able to condense DNA completely [defined as more than 90% EthBr fluorescence quenching (26,41)] at N/P charge ratio 2, whereas N^4,N^9-distearoyl spermine is only able to displace around 80% of EthBr at the same charge ratio.

The binding of our three synthetic lipopolyamines with polyanionic DNA was also studied by analysis of the electrophoretic mobility of the circular plasmid DNA within an agarose gel (1%). All four spermine conjugates (including Transfectam®) were able to condense pEGFP DNA efficiently (as a result of the interactions between the lipopolyamine ammonium ions and the DNA phosphate charges) at their optimized respective charge ratios (N/P) of transfection (see Table I). All these conjugates completely inhibited the electrophoretic mobility of circular plasmid DNA from lipoplexes at these optimized N/P charge ratios.

Table I. Particle Size and Polydispersity of pEGFP Complexes with the Studied Lipopolyamines

Lipospermine	Charge ratio (N/P)	Lipoplex diameter (nm)	Polydispersity
N^4,N^9-Distearoyl spermine	15.0	217 (21)	0.46
N^4,N^9-Dioleoyl spermine	2.5	366 (73)	0.32
N^4,N^9-Dilinoleoyl spermine	5.5	71 (2.5)	0.19
Transfectam®	5.9	62 (3.2)	0.22

Each lipoplex diameter value represents mean ± SD.

Lipoplexes Particle Size and ζ-Potential Measurements

The particle size and ζ-potential characterization measurements were carried out on the lipoplexes at their optimum N/P charge ratio of transfection (see Table I). Particle size characterization by dynamic light scattering showed that the average particle size of N^4,N^9-dilinoleoyl spermine was considerably smaller (71 nm) than those of N^4,N^9-distearoyl spermine and N^4,N^9-dioleoyl spermine (Table I). The surface charge, as determined by ζ-potential measurements, was +43 and +32 mV for N^4,N^9-dilinoleoyl spermine (at N/P charge ratio of 5.5) and N^4,N^9-dioleoyl spermine (at N/P charge ratio of 2.5) lipoplexes, respectively (Fig. 3). Transfectam® lipoplex shows a ζ-potential of +20 mV (at N/P charge ratio of 6) in phosphate-buffered saline solution (Remy and Behr, personal communication).

ζ-Potential is an important parameter helping to predict the stability of the formulation as well as the ability of the positively charged particles to interact with cell membranes

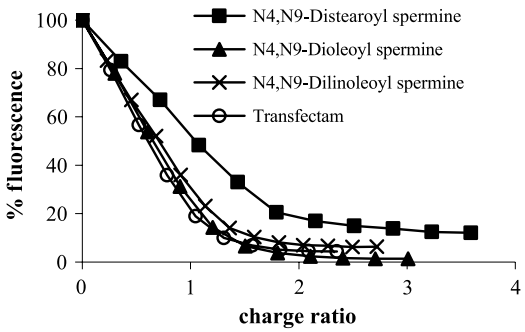

Fig. 2. Plot of EthBr displacement assay of calf thymus DNA complexed with N^4,N^9-dilinoleoyl spermine, N^4,N^9-dioleoyl spermine, N^4,N^9-distearoyl spermine, and Transfectam®.

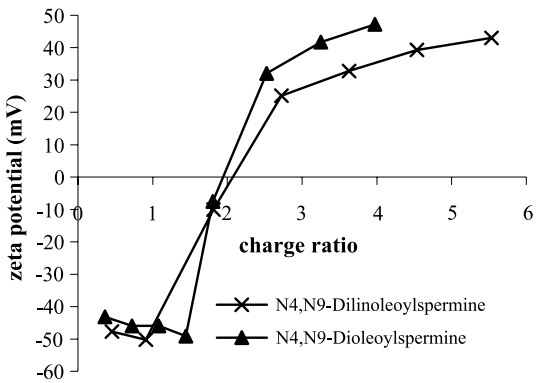

Fig. 3. ζ-Potential of N^4,N^9-dilinoleoyl spermine and N^4,N^9-dioleoyl spermine complexed with plasmid encoding for enhanced green fluorescent protein (pEGFP) at different N/P charge ratios.

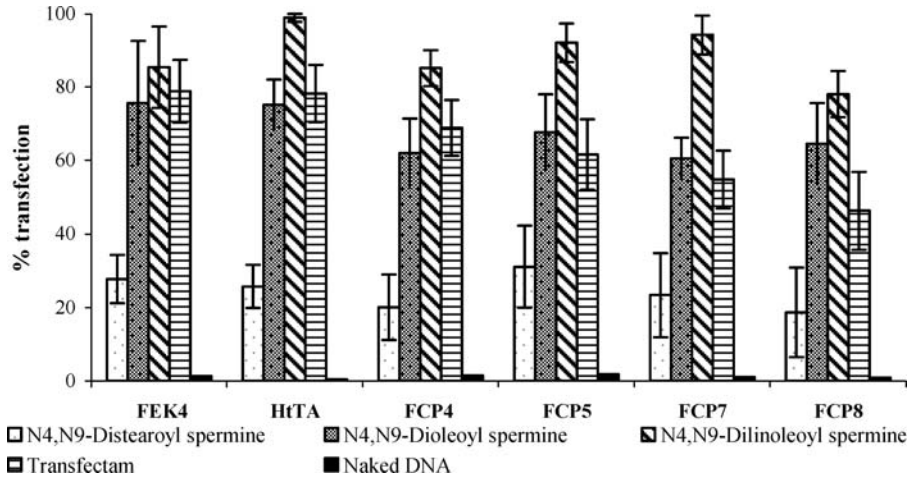

Fig. 4. Lipofection of the primary skin cell lines (FEK4, FCP4, FCP5, FCP7, and FCP8) and the cancer cell line HtTA transfected with pEGFP complexed with N^4,N^9-distearoyl spermine, N^4,N^9-dioleoyl spermine, N^4,N^9-dilinoleoyl spermine, and Transfectam® (at their respective N/P ratios for best transfection). The data represent three different experiments (three replicates each), and the error bars represent SD.

(Remy and Behr, Personal Communication), (42). ζ-Potential depends on several factors, including pH, ionic charge, ion size, and concentration of ions in solution (43). The formed nanoparticles are considered to be stable when they have pronounced ζ-potential values, either positive or negative, but the tendency to aggregate is higher when the ζ-potential is close to zero. Charge neutrality ($\zeta = 0$) occurred at an N/P charge ratio of about 2 for both lipopolyamines N^4,N^9-dilinoleoyl spermine and N^4,N^9-dioleoyl spermine (comparable with our EthBr results; Fig. 2). Ciani *et al.* (43) found neutralization N/P charge ratios of 6 and 2 with intact liposomes. Similarly, also wor-king with liposomes, Lobo *et al.* (44) and Wiethoff *et al.* (45) reported that neutralization charge ratios of cationic lipid–DNA complexes occurred at N/P charge ratios of 3.5, 2.5, and 1.5.

Transfection Experiments

The transduction of EGFP into a series of primary skin cell lines (FEK4, FCP4, FCP5, FCP7, and FCP8) and a cancer cell line (HeLa-derived HtTA) was investigated. The optimum concentrations (and corresponding N/P charge ratios) for transfection were experimentally determined to be N^4,N^9-distearoyl spermine (33.4 μg/ml, N/P = 15.0), N^4,N^9-dioleoyl spermine (5.5 μg/ml, N/P = 2.5), N^4,N^9-dilinoleoyl spermine (12.1 μg/ml, N/P = 5.5), and Transfectam® (15.0 μg/ml, N/P = 5.9). The results indicate the improved transfection ability of N^4,N^9-dilinoleoyl spermine, greater than 85% in many of the studied primary cell lines (80% for FCP8 cells) and 99% in the case of the cancer cell line HtTA compared to the saturated N^4,N^9-distearoyl spermine (18–32%), the mono-unsaturated N^4,N^9-dioleoyl spermine (62–75%), and the commercially available Transfectam® (46–79%; Fig. 4). As a negative control, naked (uncomplexed circular) pEGFP DNA typically gave 1–2% transfection of these cell lines. Also, the shift in the EGFP positive cells compared to the untransfected cells in FACS analysis (Fig. 5) shows the high efficiency of N^4,N^9-dilinoleoyl spermine by transfecting both primary (e.g., FEK4) and cancer (HtTA) cell lines.

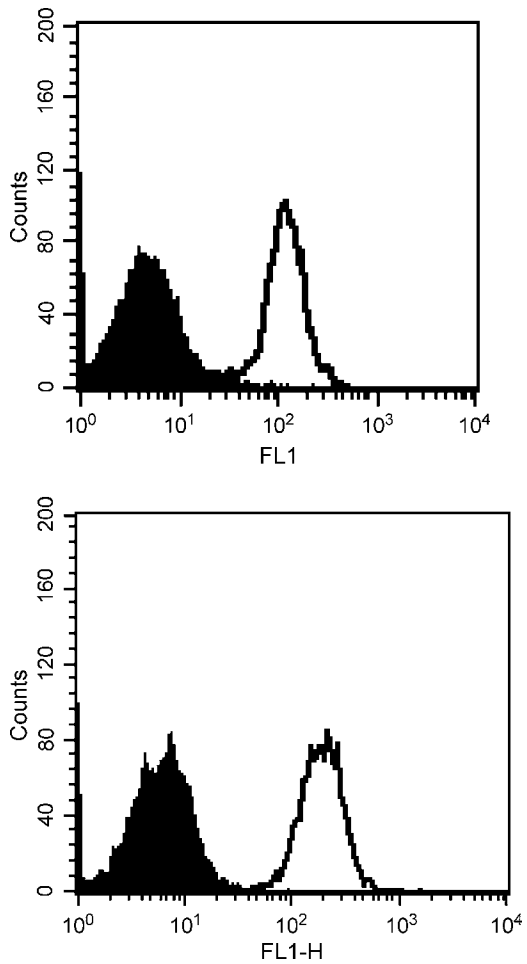

Fig. 5. Fluorescence-activated cell sorting analysis of FEK4 (above) and HtTA (below) cells after 48-h transfection of pEGFP complexed with N^4,N^9-dilinoleoyl spermine: ■, untransduced cells; □, EGFP positive cells.

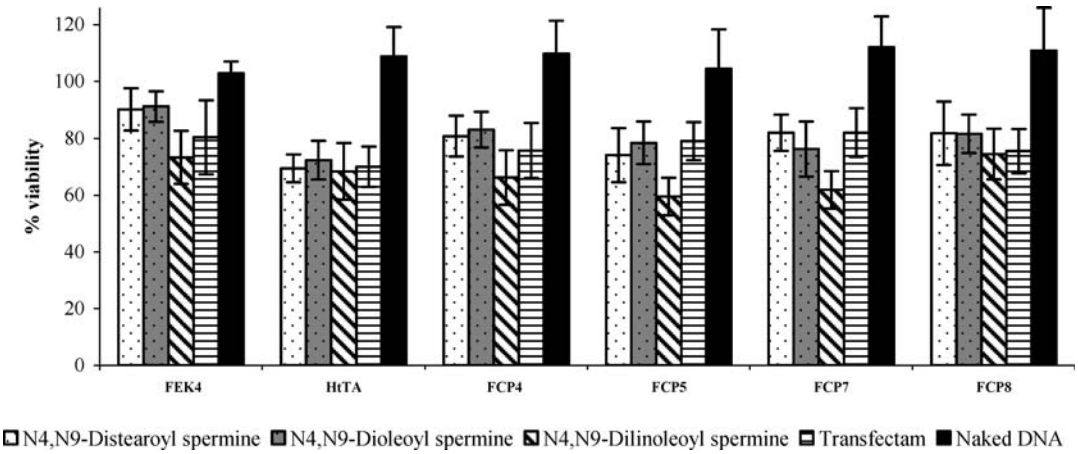

Fig. 6. Cytotoxicity effect of pEGFP (2 μg/ml) free (naked DNA) or complexed with N^4,N^9-distearoyl spermine (33.4 μg/ml), N^4,N^9-dioleoyl spermine (5.5 μg/ml), N^4,N^9-dilinoleoyl spermine (12.1 μg/ml), and Transfectam® (15.0 μg/ml) in the tested cells.

In Vitro Cytotoxicity

Cell viability [MTT assay (46)] results indicate that there is no significant difference in lipoplex toxicity in the case of HtTA and FCP8 primary skin cells for all four studied lipopolyamines (Fig. 6). Only small differences were observed across the other four cell lines, often with percentage cell viability about 80%. N^4,N^9-Dilinoleoyl spermine typically shows 70% cell viability in most of the studied cell lines, and such a value has recently been reported to be acceptable for a safe DNA delivery vector (47).

DISCUSSION

In this study, we have investigated the change in the degree of unsaturation of the di-C18 fatty chain formulation of lipospermine on DNA condensation and cellular delivery. The results from pEGFP condensation, investigated by EthBr fluorescence quenching assay, revealed that two of our synthetic lipopolyamines and the commercial formulation Transfectam® were able to condense DNA to less than 10% EthBr fluorescence, where DNA is defined as condensed (41); however, N^4,N^9-distearoyl spermine had not reduced (quenched) the EthBr fluorescence to 10% by N/P charge ratio 3.5 (Fig. 2). Particle size of the final gene formulation is also an important factor in improving gene delivery (48,49). Particle size results (Table I) showed larger particles with both N^4,N^9-dioleoyl spermine and N^4,N^9-distearoyl spermine formulations over those obtained with N^4,N^9-dilinoleoyl spermine and Transfectam®. These results are in concordance with the improvement in transfection achieved with N^4,N^9-dilinoleoyl spermine over our other two formulations. On the other hand, although Transfectam® has the smallest particle size (62 nm), the saturation of the di-C18 fatty chains could possibly contribute to the lower transfection efficiency of this spermine conjugate in comparison to our formulation results with N^4,N^9-dilinoleoyl spermine. Gao and Huang (51) reported that the addition of poly-L-lysine (PLL) or protamine to DC-Chol liposomes reduces the size of the complex as well as its heterogeneity in all ratios of lipid/DNA that improve transfection efficiency. Noguchi *et al.* (16), also working with protamine, reported that smaller-sized particles transfect cells efficiently. The incorporation of protamine as a nuclear localization signal to liposomes incorporating a cationic cholesterol derivative reduced the size of the formed particles from 0.4–1.8 μm, for the DNA–liposome complex, to 0.1–0.8 μm, for the DNA–protamine–liposome complex. On the relationship between particle size and transfection efficiency, there are no definite limits to the nanoparticle size that are suitable for transfection (51). Nanoparticles have relatively higher intracellular uptake than microparticles (52). Also, on the nanoscale, smaller-size polyplexes are more able to enter cells and thereby increase the efficiency of transfection (53). Similarly, in 2004, Rejman *et al.* (54) showed with fluorescently labeled nanospheres (latex beads) that size itself can determine the pathway of entry. Ogris *et al.* (55) reported larger particles (1000 nm) exceeding the transfection efficiency achieved with smaller particles (40 nm) using DNA/transferrin–polyethylenimine complexes at physiological salt concentration. With a different DNA condensation system, they also reported that small toroid structures of DNA/transferrin–PLL complexes (80–100 nm) showed high transfection efficiency as their diameters are in the range of the coated pits involved in the endocytosis process (56).

We have previously reported (5–10) the importance of the substituents in the lipid moiety conjugated to the cationic polyamine to achieve improvements in DNA condensation efficiency for nonliposomal formulations where the lipid moiety must be considered in shape (volume) and substituent pattern, as well as the polyamine moiety and its pK_a values. The design and synthesis of novel cationic lipids based on the tetra-amine spermine, as nonliposomal formulations, where the lipid moiety is a long carbon chain, were largely instigated by Behr *et al.* (34) and Remy *et al.* (35) with their design and preparation of the highly efficient lipopolyamine DOGS (Transfectam®). A leading nonviral vector following from such structure–activity considerations is RPR120535 (57) where the di-C18 saturated alkyl chains are substituents pendant from a diamide at one end of the tetra-amine spermine, used as the cationic moiety to take advantage of DNA binding by polyamines (58). Unsaturated chains have

also been incorporated as the lipophilic moiety in lipopolyamine vectors.

We (11) and others (37) have used two covalently bound oleoyl chains in nonliposomal formulations for improved transfection efficiency by fusion with cellular membranes. In liposomal formulations, dioleoylphosphatidyl ethanolamine (DOPE) is often used as a helper lipid for its fusogenic ability as it has the characteristics of nonbilayer forming activity leading to destabilization of the lipid bilayer (59). Oleoyl and oleyl chains have also been bound as esters and ethers in liposomal formulations: DOSPER [1,3-dioleoyloxy-2-(6-carboxyspermine)] (60), DOSPA-DOPE (3:1 w/w, Lipofectamine™), and DOTMA-DOPE (1:1 w/w, Lipofectin®) (Fig. 7). Nonliposomal cationic-lipid delivery vectors combine both the characteristics of cationic and of helper lipids. So a factor that is important for both facilitating cell

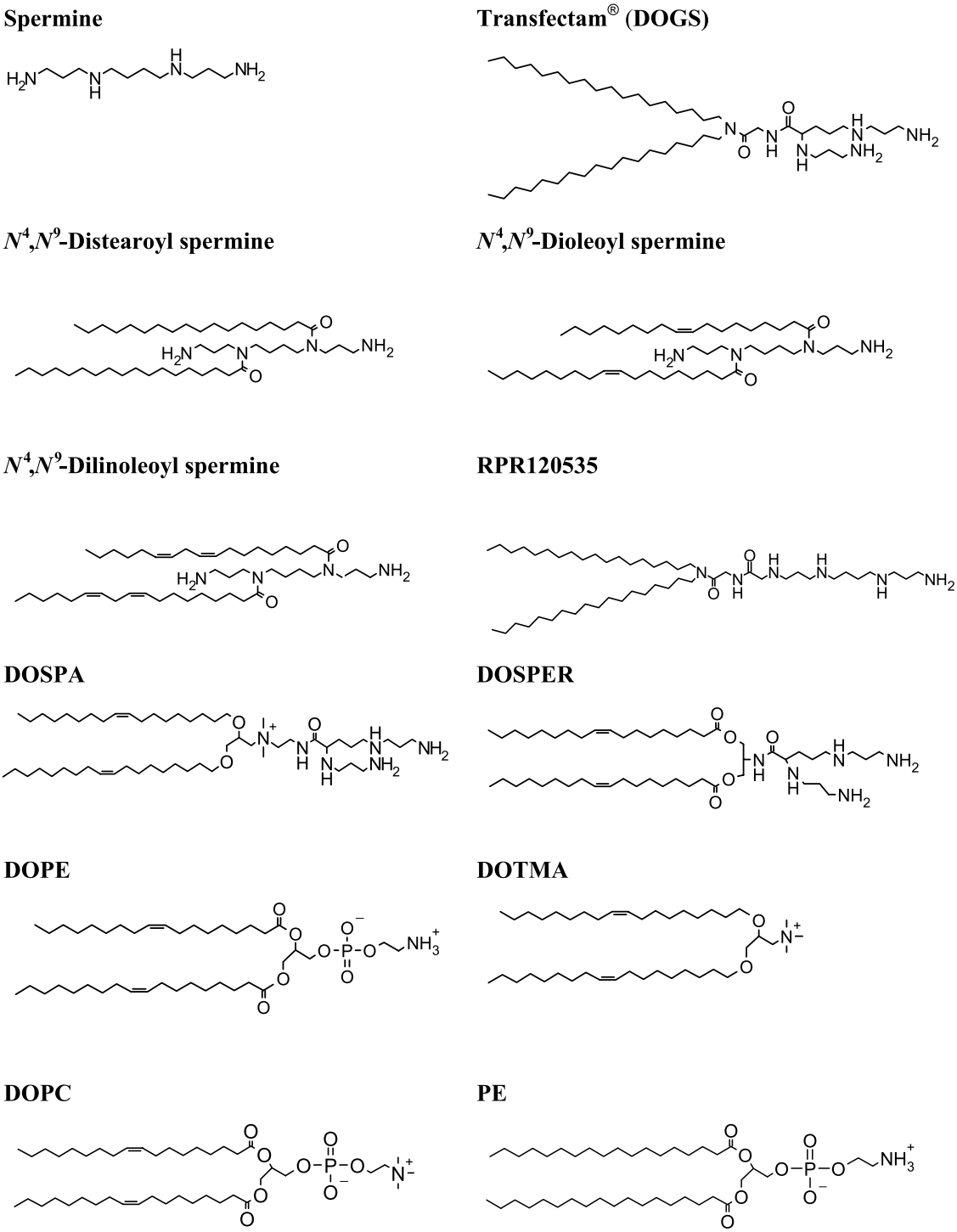

Fig. 7. Chemical structures of spermine and spermine conjugates together with DOTMA (cationic) and neutral helper lipids and gene delivery formulation excipients.

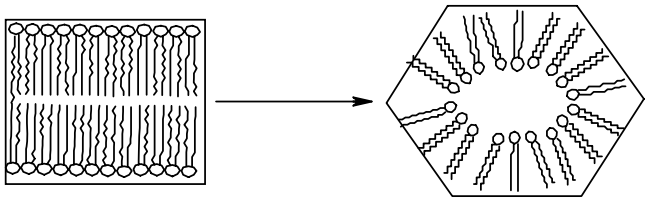

Fig. 8. Typical diagram of the change in lipid phase structure from the lamellar L_α phase to the inverted hexagonal H_{II} phase.

entry and the efficient intracellular release of free DNA (or lipoplexes) into the cytoplasm is the influence of the cationic lipid chain. The lipid moiety in our cationic lipids interacts with the phospholipid bilayer of the cell membrane and, either in crossing the membrane bilayer or in helping to weaken the endosomal bilayer, thereby aids the escape into the cytosol.

Cationic lipids in aqueous solutions exhibit different polymorphic phases. The two most important organizational forms are $L\alpha$ (lamellar organization with fluid hydrocarbon chains) and H_{II} (two-dimensional hexagonal state; Fig. 8) (61). Farhood *et al.* (59) have reported that the replacement of DOPE with a trimethylated structural analog dioleoylphosphatidyl choline (DOPC; Fig. 7), in cationic liposomal formulations, abolishes most of the transfection activity of the lipoplex, as DOPE exhibits a high tendency to form the inverted hexagonal (H_{II}) phase particularly at acidic pH.

Lipids that are cylindrical in solution shape prefer bilayer formations, whereas lipids with large head groups and small diameter hydrocarbon chains (inverted cones) form micellar structures (61). Lipids with small head groups and long hydrocarbon chains, e.g., (saturated) phosphatidyl ethanolamine (PE) and DOPE (Fig. 7), because of their solution cone shape, favor phase transition to an inverted hexagonal phase, as a result of steric factors (62). Also, increasing the degree of unsaturation of the acyl chain (especially in the *cis*-configuration) favors hexagonal phase formation ($L_\alpha \rightarrow H_{II}$ phase transition) and potentially improves the fusogenic characteristics of the lipid. The actual significance of maximizing such fusogenic characteristics in NVGT remains to be proven. Gaucheron *et al.* (63), in their detailed study on fluorinated lipospermines, speculated that the improved transfection with the unsaturated over the saturated counterparts could be a result of "their greater ability to promote membrane fusion with and destabilization of the endosomal membrane allowing optimal DNA release in the cytosol" and "related to the fluidification effect of the CC bonds." Vierling *et al.* (64) have also reported that unsaturated fluorinated lipids showed lower lamellar phase transition temperature than the corresponding fluorinated saturated lipids. The fusogenic characteristics of our synthetic nonliposomal lipopolyamines were taken under consideration to account for the observed difference in gene delivery. The two factors head group and chain length are held constant among our investigated compounds. The degree of unsaturation of the C18 fatty chain may be a key factor that explains the increase in the transfection efficiency of the lipoplex. This may be a result of the improved fusogenic characteristics of N^4,N^9-dilinoleoyl spermine with its (di-*cis*-configuration) dienoic fatty acyl residue, more than the monounsaturated N^4,N^9-dioleoyl spermine and the saturated N^4,N^9-distearoyl spermine. In conclusion, in this study, we have designed and synthesized novel gene carriers and characterized N^4,N^9-dilinoleoyl spermine as a promising DNA delivery formulation with its increased degree of unsaturation in both of the lipospermine fatty chains.

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