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Research Paper

Very Long Chain N^4, N^9-Diacyl Spermines: Non-Viral Lipopolyamine Vectors for Efficient Plasmid DNA and siRNA Delivery

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Purpose. To study the effect of increasing the chain length over C-18 and varying the oxidation level in synthesized N^4,N^9-diacyl spermines on DNA and siRNA formulation, and then to compare their transfection efficiency in cell lines

Methods. The five novel very long chain N^4,N^9-diacyl polyamines: N^4,N^9-[diarachidoyl, diarachidonoyl, dieicosenoyl, dierucoyl and dinervonoyl]-1,12-diamino-4,9-diazadodecane were synthesized. The abilities of these novel compounds to condense DNA and to form nanoparticles were studied using ethidium bromide fluorescence quenching and nanoparticle characterization techniques. Transfection efficiency was studied in FEK4 primary skin cells and in an immortalized cancer cell line (HtTA), and compared with the non-liposomal transfection formulation Lipogen, N^4,N^9-dioleoyl-1,12-diamino-4,9-diazadodecane. Also, the abilities of these compounds to condense siRNA and to form nanoparticles were studied using a RiboGreen intercalation assay and their abilities to deliver siRNA into cells were studied in FEK4 and HtTA cells using fluorescein-labelled Label IT® RNAi Delivery Control, a sequenced 21-mer from Mirus.

Results. We show efficient pEGFP and siRNA formulation and delivery to primary skin and cancer cell lines.

Conclusions. Adding two C20 or C22 chains, both mono-*cis*-unsaturated, N^4,N^9-dieicosenoyl spermine and N^4,N^9-dierucoyl spermine, gave efficient siRNA delivery vectors, even in the presence of serum, comparable to TransIT-TKO and with excellent cell viability.

KEY WORDS: erucic acid; N^4, N^9-dioleoyl spermine; primary skin cells; siRNA delivery; very long chain fatty acid.

INTRODUCTION

The significant problems still associated with viral gene therapy, especially immunogenicity, mammalian toxicity, and the limited payload of DNA, ensure that the goal of intracellular protein levels at therapeutic concentrations moves even more towards utilising non-viral gene therapy (NVGT) (1). Within the broad term NVGT, except for naked (free) DNA being trapped inside cells during mitosis, the DNA must be formulated for delivery. Our NVGT focus is on lipopolyamines composed of two long-carbon chains (or a steroid) covalently bound to a polyamine e.g. spermine (1,12-diamino-4,9-diazadodecane) (2,3). Other research groups are investigating a variety of alternative approaches, including: naked polynucleotides, gene gun (bound to gold particles), electroporation, and the use of a wide variety of cationic lipids (lipoplexes) (4–8) and cationic polymers (polyplexes), for reviews see: (9–12). Structure-activity studies are required for non-viral vectors in siRNA delivery, as there is no immediate correlation between the efficiency of a vector used for both DNA and siRNA delivery (13–22).

Progress is being made towards gene therapy realising its potential and becoming an efficient medicine for the treatment of diseases such as inherited blindness, cancer, inflammation, for neuronal delivery (23) and for vaccination (24,25). However, key obstacles still remain to be overcome (26,27). 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. Efficient NVGT formulations should be able to deliver safely the required DNA across the various cellular barriers to the nucleus (28). These barriers require the lipopolyamine to condense the DNA as a nanoparticle (lipoplex) by electrostatic charge neutralisation (29). The site of action of siRNA is in the cytosol, so there in no requirement to enter the nucleus (as for pDNA), but the siRNA lipoplex must efficiently afford protection from the high enzyme activity of RNase which otherwise results in fast hydrolysis with no chance of a therapeutic endpoint.

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ABBREVIATIONS: EGFP, enhanced green fluorescent protein; EMEM, Earle's Minimal Essential Medium; EthBr, ethidium bromide; FCS, foetal calf serum; HRMS, high-resolution mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NVGT, non-viral gene therapy; pEGFP, plasmid encoding for enhanced green fluorescent protein.

Our aims are to design and develop efficient, non-toxic, non-viral vectors for *in vitro* and possible *in vivo* applications, using our novel spermine conjugates, based on change to the type, length, position, and number of the hydrophobic anchors (2,3). These cationic lipids (lipospermines) probably assist in the self-assembling of polycationic scaffolds, as well as facilitating absorptive endocytosis and/or fusion with cell membranes. They form spontaneous complexes (nanoparticles) with negatively charged poly-nucleic acids. Thus, these formed nanoparticles are suitable for gene or siRNA delivery (30). The complexes are monitored using ethidium bromide (EthBr) fluorescence quenching (31), transfection efficiency, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assays (32,33). We have therefore designed a series of novel very long chain lipopolyamines in order to prepare lipoplex formulations (without any pre-preparation of liposomes) of a circular plasmid DNA encoding for enhanced green fluorescent protein (pEGFP) and of a fluorescein-tagged siRNA in order to investigate if they are suitable for non-toxic transfection of target (primary) cells, by forming nanoparticles which will efficiently enter cells for NVGT by endocytosis. After entry, there are still various intracellular barriers that must be crossed by toroidal condensed DNA or siRNA nanoparticles. Such formulations of lipoplexes leading to DNA delivery and ultimately to the goal of intracellular protein synthesis, or to RNA knock-down, might be particularly useful in patients who do not find very long chain fatty acids (VLCFA) toxic, e.g. unsaturated fatty acids glyceryl trierucate and glyceryl trioleate in a 1:4 combination of rapeseed and olive oils commonly called Lorenzo's oil (34), whereas the build up of quantities of VLCFA in tissues and body fluids is normally toxic. These novel very long chain N^4, N^9-diacyl spermine vectors are designed to have simplicity of use based upon DNA condensation and siRNA binding both achieved by anion titration.

Herein we report our investigations on the formulations of pEGFP and siRNA with variation in the length of the two fatty chains regiospecifically covalently bound to spermine. We make sequential changes to chain length from C18 (oleoyl) to C24 (nervonoyl), through C20 (saturated arachidoyl, mono-unsaturated eicosenoyl, and tetra-unsaturated arachidonoyl) and C22 (erucoyl). In our experiments we have chosen to deliver a 4.7 kbp plasmid encoding for EGFP, with a molecular weight of about 3.1 MDa (given an average of 330 Da per nucleotide, 660 Da/bp (35), carrying 9,400 negative charges. We report the synthesis and characterisation of the nanoparticles, pEGFP transfection results, cell viability, and siRNA delivery with five new synthesized lipospermine formulations in both primary and cancer cell lines, and compare our results with those obtained with the non-liposomal lipospermine Lipogen® (N^4,N^9-dioleoyl spermine) (2,3) and TransIT-TKO.

MATERIALS AND METHODS

Materials

Chemicals, including spermine, very long chain fatty acids, reagents, solvents, and buffers, were routinely purchased from Sigma–Aldrich (Gillingham, UK) except where indicated, and cell culture materials were from Life Technologies (Paisley, Scotland).

General Details

THF used in anhydrous conditions was distilled from sodium benzophenone ketyl. Glassware used in anhydrous conditions was baked for 1 h at 80°C, assembled hot and filled with anhydrous nitrogen before use. Ninhydrin used for detecting (poly)amines was prepared by dissolving 0.3 g ninhydrin in 100 ml n-butanol. Column chromatography was performed over flash silica gel 60 (35–75 μm; Prolabo-Merck). Analytical TLC was performed using aluminium-backed plates coated with Kieselgel 60 F_{254} (Merck). ^{1}H and ^{13}C NMR spectra were recorded in d_6-DMSO using a Varian Mercury 400 (operating at 400 MHz for ^{1}H and 100.8 MHz for ^{13}C) spectrometer. Chemical shifts values are recorded in parts per million (ppm) on the δ scale. Coupling constants (J) are absolute values and recorded in Hz. Spectra were referenced internally using either the residual solvent resonance for ^{13}C or to TMS (0.0 ppm) for ^{1}H. Multiplicities are recorded as: s (singlet), t (triplet), m (multiplet), and br (broad). ^{13}C assignments were aided by 90° and 135° DEPT pulse sequences. High- and low-resolution FAB mass spectra (MS; positive ion mode, unless otherwise indicated) were measured on a VG AutoSpec Q spectrometer, using *m*-nitrobenzyl alcohol as the matrix. FAB HRMS were alternatively measured on a MicroTOF spectrometer, and on a Finnigan MAT 900 XLT which is a high resolution double focussing (EB) mass spectrometer with tandem ion trap. Mass spectrometric values are recorded in Da. All of the products are pure and homogeneous by TLC.

General Procedure for the Synthesis of N^4,N^9-Diacyl Spermines

A solution of ethyl trifluoroacetate (e.g. 0.2 ml, 1.5 mmol) in MeOH (10 ml) was added dropwise to spermine (147 mg, 0.7 mmol) in MeOH (10 ml) at 0°C under anhydrous nitrogen, the reaction was stirred at 20°C for 24 h. The solvent was removed in vacuo which gave a yellow oil. The residue was taken in to the next step without purification. The very long chain fatty acid (e.g. 500 mg, 1.6 mmol), N^1-hydroxybenzotriazole (HOBt; 27 mg, 0.2 mmol), and dicyclohexylcarbodiimide (DCC; 329 mg, 1.6 mmol) were added in CH_2Cl_2 (10 ml) and the mixture stirred at 20°C for 12 h. The solvent was removed in vacuo. The residue was dissolved in MeOH at 20°C, and the conjugate was deprotected by the addition of potassium carbonate (1.5 eq.) to a stirring solution of the protected polyamine in MeOH and water (50:3; *v*/*v*), and then the reaction mixture was heated under reflux for 5 h, cooled to 20°C and then concentrated in vacuo. Water (30 ml) was added to the residue which was extracted with $CHCl_3$ (3×25 ml). The combined organic phases were washed with water (15 ml), dried and concentrated. The aqueous layers were further basified with ice-cold 5% aq. NaOH (10 ml) and extracted with $CHCl_3$ (4×40 ml). After a water wash (25 ml), the combined organic extracts were concentrated in vacuo. Purification of the crude deprotected polyamine was by column chromatography (CH_2Cl_2/MeOH/conc. aq. NH_3 200:10:1 to 100:10:1 to 50:10:1; *v*/*v*/*v*).

N^4,N^9-Dioleoyl-1,12-diamino-4,9-diazadodecane 1

Prepared via N^1,N^{12}-ditrifluoroacetyl-N^4,N^9-dioleoyl-1,12-diamino-4,9-diazadodecane (36), the target lipopolyamine displayed HRMS: found (*m*/*z* $[M+H]^+$) 731.7115, $C_{46}H_{91}N_4O_2$ requires 731.7142 (Δ ppm 3.7), consistent with the data previously reported (2,3).

N^4,N^9-Diarachidoyl-1,12-diamino-4,9-diazadodecane 2

According to the General Procedure, a solution of ethyl trifluoroacetate (0.2 ml, 1.5 mmol) in MeOH (10 ml) was reacted with spermine (147 mg, 0.7 mmol) in MeOH (10 ml) at 0°C. Arachidic acid (500 mg, 1.6 mmol), HOBt (27 mg, 0.2 mmol) and DCC (329 mg, 1.6 mmol) were added in CH_2Cl_2 (10 ml). After the reaction (12 h), the conjugate was deprotected, concentrated in vacuo, and purified to yield the title compound as an oil (121 mg, 21%). ^{1}H NMR: 0.91 (t, 6H, H20', *J*=7); 1.12–2.40 (m, overlapping, 84H, H1, H2, H6, H7, H11, H12, H2'-H19'); 2.08 (br s, 4H, NH_2); 3.29–3.48 (m, overlapping, 8H, H3, H5, H8, H10). ^{13}C NMR: 14.1 (C20'); 22.7–35.8 (C1, C2, C6, C7, C11, C12, C2'-C19'); 41.9–49.3 (C3, C5, C8, C10); 178.9 (C1'). MS, FAB^+ found 791.8 (100%, M^++1), $C_{50}H_{102}N_4O_2$ requires (M^+) 790. HRMS *m*/*z*, FAB^+ found 791.8080, (M^++1), $C_{50}H_{103}N_4O_2$ requires (M^++1) 791.8076.

N^4,N^9-Dieicosenoyl-1,12-diamino-4,9-diazadodecane 3

According to the General Procedure, a solution of ethyl trifluoroacetate (0.2 ml, 1.5 mmol) in MeOH (10 ml) was reacted with spermine (148 mg, 0.7 mmol) in MeOH (10 ml) at 0°C. Eicosenoic acid (500 mg, 1.6 mmol), HOBt (27 mg, 0.2 mmol) and DCC (329 mg, 1.6 mmol) were added in CH_2Cl_2 (10 ml). After the reaction (12 h), the conjugate was deprotected, concentrated in vacuo, and purified to yield the title compound as an oil (70 mg, 12%). ^{1}H NMR: 0.87 (t, 6H, H20', *J*=8); 1.25–2.80 (m, overlapping, 76H, H1, H2, H6, H7, H11, H12, H2'-H10', H13'-H19'); 2.06 (br s, 4H, NH_2); 3.27–3.43 (m, overlapping, 8H, H3, H5, H8, H10); 5.33 (m, 4H, H11', H12'). ^{13}C NMR: 14.0 (C20'); 22.2–36.7 (C1, C2, C6, C7, C11, C12, C2'-C10', C13'-C19'); 38.8–40.1 (C3, C5, C8, C10); 129.7 (C11', C12'); 175.1 (C1'). MS, FAB^+ found 787.7, (2%, M^++1), 394.4 (100%), $C_{50}H_{98}N_4O_2$ requires (M^+) 786. HRMS *m*/*z*, FAB^+ found 787.7759, (M^++1), $C_{50}H_{99}N_4O_2$ requires (M^++1) 787.7763.

N^4,N^9-Diarachidonoyl-1,12-diamino-4,9-diazadodecane 4

According to the General Procedure, a solution of ethyl trifluoroacetate (0.2 ml, 1.5 mmol) in MeOH (10 ml) was reacted with spermine (151 mg, 0.7 mmol) in MeOH (10 ml) at 0°C. Arachidonic acid (500 mg, 1.6 mmol), HOBt (27 mg, 0.2 mmol) and DCC (329 mg, 1.6 mmol) were added in CH_2Cl_2 (10 ml). After the reaction (12 h), the conjugate was deprotected, concentrated in vacuo, and purified to yield the title compound as an oil (95 mg, 16%). ^{1}H NMR: 0.87 (t, 6H, H20', *J*=8); 1.25–2.83 (m, overlapping, 52H, H1, H2, H6, H7, H11, H12, H2'-H4', H7', H10', H13', H16'-H19'); 2.05 (br s, 4H, NH_2); 3.08–3.30 (m, overlapping, 8H, H3, H5, H8, H10); 5.31–5.41 (m, 16H, H5', H6', H8', H9', H11', H12', H14', H15'). ^{13}C NMR: 14.0 (C20'); 22.0–33.8 (C1, C2, C6, C7, C11, C12, C2'-C4', C7', C10', C13', C16'-C19'); 38.9–40.2 (C3, C5, C8, C10); 127.6–129.9 (C5', C6', C8', C9', C11', C12', C14', C15'); 175.0 (C1'). MS, FAB^+ found 775.6, (6%, M^++1), 178.8 (100%), $C_{50}H_{86}N_4O_2$ requires (M^+) 774. HRMS *m*/*z*, FAB^+ found 774.6828, (M^++1), $C_{50}H_{87}N_4O_2$ requires (M^++1) 775.6824.

N^4,N^9-Dierucoyl-1,12-diamino-4,9-diazadodecane 5

According to the General Procedure, a solution of ethyl trifluoroacetate (0.3 ml, 2.3 mmol) in MeOH (10 ml) was reacted with spermine (227 mg, 1.1 mmol) in MeOH (10 ml) at 0°C. Erucic acid (800 mg, 2.4 mmol), HOBt (41 mg, 0.3 mmol) and DCC (494 mg, 2.4 mmol) were added in CH_2Cl_2 (10 ml). After the reaction (12 h), the conjugate was deprotected, concentrated in vacuo, and purified to yield the title compound as an oil (496 mg, 52%). ^{1}H NMR: 0.84 (t, 6H, H22', *J*=8); 1.23–2.74 (m, overlapping, 84H, H1, H2, H6, H7, H11, H12, H2'-H12', H15'-H21'); 2.00 (br s, 4H, NH_2); 3.21–3.42 (m, overlapping, 8H, H3, H5, H8, H10); 5.31 (m, 4H, H13', H14'). ^{13}C NMR: 14.1 (C22'); 22.6–33.1 (C1, C2, C6, C7, C11, C12, C2'-C12', C15'-C21'); 42.7–47.4 (C3, C5, C8, C10); 129.8 (C13', C14'); 173.4 (C1'). MS, FAB^+ found 843.8, (100%, M^++1), $C_{54}H_{106}N_4O_2$ requires (M^+) 842. HRMS *m*/*z*, FAB^+ found 843.8379, (M^++1), $C_{54}H_{107}N_4O_2$ requires (M^++1) 843.8389.

N^4,N^9-Dinervonoyl-1,12-diamino-4,9-diazadodecane 6

According to the General Procedure, a solution of ethyl trifluoroacetate (20 μl, 0.2 mmol) in MeOH (10 ml) was reacted with spermine (26 mg, 0.13 mmol) in MeOH (10 ml) at 0°C. Nervonic acid (100 mg, 0.3 mmol), HOBt (2 mg, 0.01 mmol) and DCC (62 mg, 0.3 mmol) were added in CH_2Cl_2 (10 ml). After the reaction (12 h), the conjugate was deprotected, concentrated in vacuo, and purified to yield the title compound as an oil (50 mg, 43%). ^{1}H NMR: 0.85 (t, 6H, H24', *J*=8); 1.23–2.81 (m, overlapping, 92H, H1, H2, H6, H7, H11, H12, H2'-H14', H17'-H23'); 1.97 (br s, 4H, NH_2); 3.16–3.35 (m, overlapping, 8H, H3, H5, H8, H10); 5.76 (m, 4H, H15', H16'). ^{13}C NMR: 14.0 (C24'); 22.1–31.4 (C1, C2, C6, C7, C11, C12, C2'-C14', C17'-C23'); 38.8–40.1 (C3, C5, C8, C10); 129.7 (C15', C16'); 174.5 (C1'). MS, FAB^+ found 898.8, (1%, M^++1), 100.8 (100%), $C_{58}H_{114}N_4O_2$ requires (M^+) 898. HRMS *m*/*z*, FAB^+ found 899.9031, (M^++1), $C_{58}H_{115}N_4O_2$ requires (M^++1) 899.9015.

Amplification and Purification of Plasmid DNA

DNA plasmid encoding EGFP (Clontech) was transformed into *Escherichia coli* JM 109 bacterial strain (Promega). The transformed cells were grown in larger quantities of Luria–Bertani broth supplemented with 125 mg/l ampicillin. pEGFP was produced in large-scale using HiSpeed plasmid purification Maxi kit (Qiagen) according to the manufacturer's protocol. DNA yields and purity were determined spectroscopically (OD_{260}/OD_{280}=1.80 to 1.90 OD, optical density) and by agarose gel (1%) analysis.

DNA Condensation (Ethidium Bromide Fluorescence Quenching Assay)

Each concentration of the DNA stock solutions (approximately 1 μg/μl, 1 ml) was determined spectroscopically

(Milton Roy Spectronic 601 spectrometer, 1 cm path length, 3 ml cuvette) (35) and 6 μg (approximately 6 μl) of DNA was diluted to 3 ml with buffer [20 mM NaCl, 2 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 μM ethylene diamine tetraacetic acid (EDTA), pH 7.4] in a glass cuvette stirred with a micro-flea. Immediately prior to analysis, EthBr solution (3 μl, 0.5 mg/ml) was added to the stirring solution and allowed to equilibrate for 10 min. Separately each lipopolyamine, aliquots (5 μl), according to the ammonium/phosphate (N/P, ±) charge ratio required, were then added to the stirring solution and the fluorescence measured after 1 min equilibration using a Perkin–Elmer LS 50B luminescent spectrometer (λ_{ex}=260 nm and λ_{em}=600 nm with slit width 5 nm) while stirring using an electronic stirrer (Rank Bros. Ltd.) (31). The total lipopolyamine solution added to the DNA solution did not exceed 5% of the total volume of the solution, so no correction was made for sample dilution. 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.

Gel Electrophoresis

Each sample of plasmid DNA (0.5 μg), either free or complexed with different lipopolyamine concentrations, was analyzed by gel electrophoresis for about 1 h 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

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 NanoSight LM10 (NanoSight Ltd, Salisbury, UK). All measurements were carried out on lipoplexes prepared from 1 μg pEGFP or 25 pmol of siRNA (fluorescein-labelled Label IT® RNAi Delivery Control, Mirus) in HEPES buffer (0.2 ml) at pH 7.4 and 20°C. Results were analysed with the Nanoparticle Tracking Analysis software.

ζ-Potential Measurements

The ζ-potential measurements for the lipoplexes formed at their optimum charge ratio of transfection, after mixing with a vortex mixer, were determined using a Delsa™Nano Zeta Potential (Beckman Coulter, Buckinghamshire, UK). All measurements were carried out on lipoplexes with 3 μg/ml pDNA in HEPES buffer at pH 7.4 in a sample volume of 2 ml.

Cell Culture and Transfection Experiments

Two cell lines were used in the transfection experiments, a human primary skin fibroblast cells FEK4 (37) derived from a foreskin explant, and a human cervix carcinoma HeLa derivative and transformed cell line (HtTA) (38). The HtTA cells being stably transfected with a tetracycline-controlled transactivator consisting of the tet repressor fused with the activating domain of virion protein 16 of the herpes simplex virus. Cells were cultured in Earle's minimal essential medium (EMEM) supplemented with foetal calf serum (FCS; 15% in the case of FEK4 and 10% in the case of HtTA cells), penicillin and streptomycin (50 IU/ml each), glutamine (2 mM), and sodium bicarbonate (0.2%).

Transfection Experiments in the Presence of Serum

For the transfection (gene delivery) and the resultant gene activity (transfection efficiency), FEK4 and HtTA cells were seeded at 50,000 cells/well in 12-well plates in EMEM (2 ml) containing FCS (15% in the case of FEK4 and 10% in the case of HtTA cells) for 24 h to reach a plate confluency of 50–60% on the day of transfection. Then the media were replaced by fresh EMEM (0.4 ml) containing FCS for 4 h at 37°C in 5% CO_2. The lipoplex was prepared by mixing pEGFP (1 μg in 50 μl) with the cationic lipopolyamine in Opti-MEM (typically 10 μg in 50 μl) according to the N/P charge ratio, at 20°C for 30 min, and then incubated with the cells (final volume of 0.5 ml) for 4 h at 37°C in 5% CO_2 in full growth medium (in the presence of serum). Then the cells were washed and cultured for a further 44 h in full growth medium at 37°C in 5% CO_2 before the assay.

Levels of EGFP (positive cells) 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. At the end of that 44 h, cells were washed twice with PBS (1 ml/well) to remove FCS and dead cells. Adherent cells were trypsinised (0.25% *w*/*v*, 0.5 ml/well) and incubated for 5 min at 37°C in 5% CO_2 *v*/*v*, followed by checking by microscopy to ensure at least 85% of the cells were detached. FCS EMEM (1 ml, 15% *v*/*v*) was added to each well to stop trypsinisation, and the cell suspension was then transferred to FACS polystyrene test tubes (12×75 mm, Falcon) and centrifuged (Falcon 6/300 MSE, Sanyo) at 1,200 rpm for 5 min at 20°C. Cells were re-suspended in PBS (1 ml/tube) and re-centrifuged at 1,200 rpm for 5 min at 20°C. The cell suspension for FACS analysis was obtained by dissolving the pellet in PBS (500 μl/tube). An untransfected cell sample was used as a control. CELLQuest v.1.0 software (Becton Dickinson Biosciences) was used to analyse the FACS data. Typically, 10,000 events were acquired. Recordings were made at green fluorescence (FL1) and data were expressed as histograms. Only a subset of the data obtained from healthy cells (the major population) was analysed through a gate setting. This gating was determined from the dot plots between forward-scattered light (FSC) and side-scattered light (SSC). FSC is a parameter proportional to cell size and SSC indicates the cell granularity or internal complexity. In the histogram of events at different fluorescence intensity control group, the fluorescence intensity range (M_1) was set as a constant range throughout the experiments. For EGFP detection, the percentage of fluorescent cells sorting events in the established range (M_1) was

reported with correction for the background fluorescence of the control sample with λ_{ex}=495 nm and λ_{em}=518 nm.

RNA Binding (RiboGreen Intercalation Assay)

RiboGreen solution (Invitrogen, 50 µl diluted 1 to 20) was added to each well of a 96-well plate (opaque bottom) containing free siRNA (50 ng) or complexed with lipospermines at different ratios in TE buffer (50 µl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5, in DEPC (diethyl pyrocarbonate)-treated water) using FLUOstar Optima Microplate Reader (BMG-LABTECH), λ_{ex}=480 nm and λ_{em}=520 nm. The amount of siRNA available to interact with the probe was calculated by subtracting the values of residual fluorescence (RiboGreen without siRNA) from those obtained for each measurement, and expressed as a percentage of the control that contained naked siRNA only, according to the following formula: % free siRNA = 100 × RiboGreen fluorescence$_{complexes}$/RiboGreen fluorescence$_{naked\ siRNA}$.

RNA Transfection Experiments

For the siRNA delivery we used fluorescein-labelled Label IT® RNAi Delivery Control (Mirus). FEK4 and HtTA cells were seeded at 50,000 cells/well in 12-well plates in EMEM (2 ml) containing FCS (15% in the case of FEK4 and 10% in the case of HtTA cells) for 24 h to reach a plate confluency of 50–60% on the day of transfection. Then the media were replaced by 437.5 µl fresh media. The lipoplex was prepared by mixing siRNA (12.5 pmol in 12.5 µl) with the different amounts of the cationic lipopolyamines in Opti-MEM (typically 2–20 µg in 50 µl) at 20°C for 30 min and then incubated with the cells (final volume of 0.5 ml) for 4 h at 37°C in 5% CO_2 in full growth medium. Then the media were replaced by 2 ml of fresh media for 44 h at 37°C in 5% CO_2 before the assay.

Levels of fluorescein-tagged siRNA in the transfected cells were detected and corrected for background fluorescence of the control cells using a FACS machine (using same protocol as above for pEGFP). The transfection efficiency was calculated based on the percentage of fluorescein-positive cells in the total number of cells with λ_{ex}=495 nm and λ_{em}=518 nm.

In Vitro Cytotoxicity (MTT) Assay

FEK4 and HtTA cells were seeded in 96-well plates at 8,000 cells/well and incubated for 24 h at 37°C in 5% CO_2. Lipoplexes e.g. N^4,N^9-dierucoyl spermine **5** complexed with pEGFP DNA or siRNA were added in the same way as in the transfection protocol. After incubation for 44 h, the media were replaced with 90 µl of fresh media and 10 µl of sterile filtered MTT solution (5 mg/ml) to reach a final concentration of 0.5 mg/ml. Then the plates were incubated for a further 4 h at 37°C in 5% CO_2 *v*/*v*. After incubation, the media and the unreacted dye were aspirated and the formed blue formazan crystals were dissolved in 200 µl/well of dimethyl sulfoxide. The colour produced was measured using a plate-reader (VERSAmax) at λ=570 nm. The percent viability relative to control wells containing cells without added DNA or lipopolyamine is calculated by (test absorbance/control absorbance) ×100 (39).

Confocal Microscopy Visualization

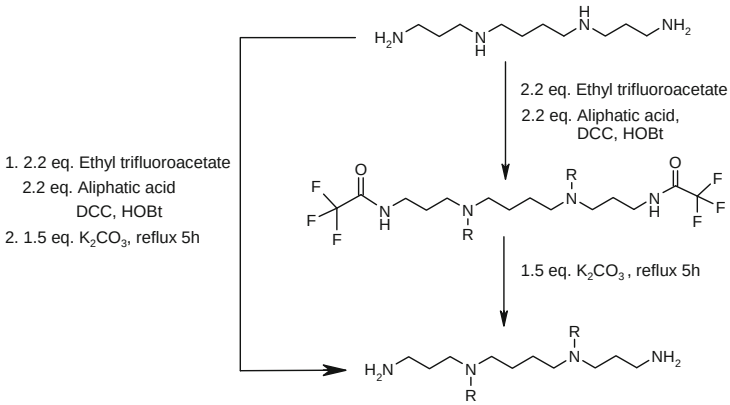
Following the DNA and siRNA transfection protocols, cells were seeded on a sterile cover-slip at the bottom of each well. After 48 h, media were aspirated and cells were fixed with freshly prepared 4% formaldehyde solution in PBS (1 ml/well) for 15 min at 37°C. After formaldehyde fixing, cells adhering to cover-slips were labelled with labelling solution according to the manufacturer's protocol. Labelling solution contained both Alexa Fluor 594 wheat germ agglutinin for cell membrane labelling, λ_{ex}=591 nm and λ_{em}=618 nm, and Hoechst 33342 for nuclei labelling, λ_{ex}=350 nm and λ_{em}=461 nm, mixed in one solution purchased from Invitrogen (Image-iT live plasma membrane and nuclear labelling kit), cell labelling with Alexa Fluor WGA (5 µg/ml) and Hoechst 33342 (2 µl, 2 µM). After that, labelled cells were mounted using mounting liquid (20 µl, Mowiol, Merck) and left for 16 h. Then, mounted cover-slips were viewed on a confocal laser scanning microscope (LSM510META, Zeiss, Jena, Germany) under the 60× oil immersion objective, with filters: red λ_{ex}=543 nm and λ_{em}=560–615 nm, blue λ_{ex}=405 nm and λ_{em}=420–480 nm, and green λ_{ex}=488 nm and λ_{em}=505–530 nm.

RESULTS AND DISCUSSION

Synthesis of Lipospermines (N^4,N^9-Disubstituted Spermine Derivatives)

As naturally occurring long chain fatty acid esters of glycerol (and the corresponding amides) readily partition into plasma membranes, some structurally similar compounds, which incorporate spermine not (amino)glycerol as the backbone, were designed, synthesized, and investigated as potential DNA and siRNA vectors. In nature, DNA is condensed by positively charged polyamines (e.g. spermine, spermidine) working together with histones, poly-basic proteins. We have previously synthesized and characterized two analogues of N^4,N^9-dioleoyl spermine **1** with diacyl chains on both the N^4,N^9 positions of spermine (2,3). However, the database of such compounds needs to be enriched, so potential vectors for pDNA and siRNA delivery have been designed and prepared. Specifically, molecules with very long aliphatic chains conjugated with spermine were synthesized (Fig. 1), the length of the chains was varied from 18 to 24 and the level of unsaturation along the chains was controlled as well.

Fig. 1 shows the steps in the reaction for the synthesis of these N^4,N^9-disubstituted spermines. In 1995, two research groups independently reported the selective protection of a primary amine as its trifluoroacetamide by reaction with ethyl trifluoroacetate (40,41). When we developed this strategy in our homologation of polyamines for the rapid, practical synthesis of lipospermines and related lipoplexes (42), we assumed that the higher nucleophilicity of the secondary amines is masked by correspondingly powerful steric effects. So this facile and specific introduction of trifluoroacetyl, using ethyl trifluoroacetate, makes it a superior protecting group to carbobenzoxy (Z, CBZ) and to *tert*-butoxycarbonyl (Boc) for the purpose of gram scale protection of polyamines (42). Thus, trifluoroacetyl is the protecting group of choice, over Z



Vector number	Acyl residue (R)	Chain name	C-chain length	Unsaturation level
1	$CO(CH_2)_7CH{=}CH(CH_2)_7CH_3$	Oleoyl	18	1, Δ^9
2	$CO(CH_2)_{18}CH_3$	Arachidoyl	20	0
3	$CO(CH_2)_9CH{=}CH(CH_2)_7CH_3$	Eicosenoyl	20	1, Δ^{11}
4	$CO(CH_2)_3(CH{=}CHCH_2)_4(CH_2)_3CH_3$	Arachidonoyl	20	4, $\Delta^{5,8,11,14}$
5	$CO(CH_2)_{11}CH{=}CH(CH_2)_7CH_3$	Erucoyl	22	1, Δ^{13}
6	$CO(CH_2)_{13}CH{=}CH(CH_2)_7CH_3$	Nervonoyl	24	1, Δ^{15}

Fig. 1. Synthesis of symmetrical N^4,N^9-disubstituted spermines.

and Boc, for practical routes to polyamine amides (2,3,42). Therefore, using this strategy, we chemo-selectively protected the two primary amines of spermine then, without purification, aliphatic acyl chains were added. DCC was used to form the amide bond. DCC converts the carboxylic acid group into a reactive acylating agent which then undergoes a nucleophilic acyl substitution with the amine. In this reaction, we used HOBt as a catalyst to help in the formation of the amide bond. DCC was then converted into dicyclohexylurea which is insoluble in CH_2Cl_2, precipitated and was removed by filtration. Thus, the tetra-amine spermine (203 mg, 1 mmol) in MeOH/CH_2Cl_2 (1:1; *v*/*v*) was protected on both the primary amino functional groups by reaction with ethyl trifluoroacetate (0.3 ml, 2.2 eq.) in MeOH, under anhydrous nitrogen, 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 as a yellow oily residue. This residue was taken into the next step without purification. The required very long chain fatty acid (e.g. erucic etc.; 2.2 eq.), DCC (2.2 eq.), and catalytic HOBt (0.2 eq.) were added to the diprotected spermine solution in MeOH/CH_2Cl_2 (1:1; *v*/*v*). The solution was stirred for 12 h at 20°C and then evaporated to dryness in vacuo to afford N^4,N^9-dierucoyl-N^1, N^{12}-ditrifluoroacetyl-1,12-diamino-4,9-diazadodecane, in a comparable route used for the dioleoyl analogue (36).

We have previously reported the use of aqueous ammonia or methanol saturated with ammonia gas (18 h at 20°C) to remove selectively the di-trifluoroacetyl protecting

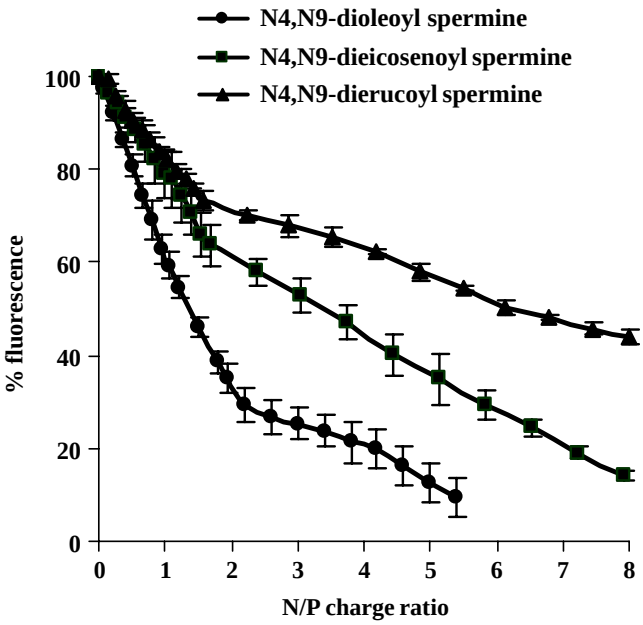


Fig. 2. Plot of EthBr fluorescence quenching assay of pEGFP complexed with different lipospermines **1**, **3**, and **5**.

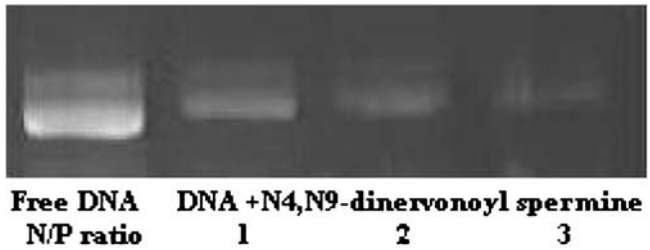


Fig. 3. Typical 1% agarose gel of fluorescent EthBr intercalated in pEGFP, assay of DNA gel permeation on complexation (lipoplex formation) with N^4,N^9-dinervonoyl spermine **6** at N/P charge ratios 1–2–3.

Table I. Particle Size (mean ± SD, n=9) and Zeta Potential of pEGFP and siRNA Lipoplexes

Lipospermine	DNA-lipoplex N/P ratio	DNA-lipoplex diameter (nm)	DNA-lipoplex ζ-potential (+) mV	siRNA-lipoplex N/P ratio	siRNA-lipoplex diameter (nm)	siRNA-lipoplex ζ-potential (+) mV
N^4,N^9-dioleoyl spermine **1**	2.5	150 (12)	2.2			
N^4,N^9-dieicosenoyl spermine **3**	10	170 (22)	16.7	19	170 (31)	7.5
N^4,N^9-diarachidonoyl spermine **4**	8	170 (42)		29		8.1
N^4,N^9-dierucoyl spermine **5**	4	210 (31)	3.6	27	180 (28)	5.5
N^4,N^9-dinervonoyl spermine **6**	4	190 (30)		25	110 (21)	

groups in our synthesis of polyamine conjugates with regiochemical control (2,3,42). The yield for this deprotection step was increased to 80% by using K_2CO_3 (1.5 eq.) in MeOH/water (50:3; *v*/*v*) and the mixture heated under reflux (5 h) (43). The combined $CHCl_3$ extracts were concentrated and purified to homogeneity over silica gel (CH_2Cl_2/MeOH/ conc. aq. NH_3 200:10:1 to 100:10:1 to 50:10:1; v/v/v) to afford the six desired synthetic lipopolyamine conjugates as their free bases, N^4,N^9-symmetrical diacyl spermines: N^4,N^9-dioleoyl spermine **1**, N^4,N^9-diarachidoyl spermine **2**, N^4,N^9-dieicosenoyl spermine **3**, N^4,N^9-diarachidonoyl spermine **4**, N^4,N^9-dierucoyl spermine **5**, and N^4,N^9-dinervonoyl spermine **6** (Fig. 1). The target vectors were homogenous on silica gel thin-layer chromatography e.g. N^4,N^9-dioleoyl spermine **1** R_f=0.44 (CH_2Cl_2/MeOH/ conc. aq. NH_3 25:10:1; *v*/*v*/*v*) and solutions of ninhydrin or potassium permanganate were used to visualize the plates. The products were fully characterized by ^{1}H NMR at 400 MHz and ^{13}C NMR spectroscopy at 100 MHz. NMR assignments follow from correlation spectroscopy and all lipopolyamines showed satisfactory HRMS data (reported in Da and within 5 ppm), as typified by N^4,N^9-dioleoyl spermine **1** found (*m*/*z* $[M+H]^+$) 731.7115, $C_{46}H_{91}N_4O_2$ requires 731.7142 (Δ ppm 3.7). The characteristic ^{1}H NMR signals included: δ 0.8–0.9 ppm methyl protons, 1.2–1.3 ppm many methylene chain protons, and 5.3–5.8 ppm the alkene protons (where required), whilst the characteristic ^{13}C NMR signals included: δ 13.9–14.1 ppm methyls, 22.0–39.1 ppm methylenes, 38.8–40.2 or 41.9–49.3 ppm C3, C5, C8, C10, 127.6–129.9 *cis*-alkenes (where required), and 172.7–175.1 ppm the amide carbonyl groups. With these new target lipopolyamines in hand, we can now investigate if the length and oxidation level of the aliphatic chain influences the transfection efficiency with pDNA and then compare this efficiency with toxicity. Similar studies will then be undertaken for siRNA delivery.

DNA Condensation

In Fig. 2, we show the DNA condensation ability of the synthesized lipopolyamines in an EthBr fluorescence quenching assay. N^4,N^9-Dioleoyl spermine **1** has the best DNA condensing ability, 90% EthBr fluorescence quenched at N/P charge ratio 5.5, while N^4,N^9-dieicosenoyl spermine **3** shows such a result at N/P=8, and N^4,N^9-dierucoyl spermine **5** is only able to achieve around 60% fluorescence quenching at the same N/P ratio. The gel electrophoresis results (Fig. 3) show, by inhibiting the electrophoretic mobility of pDNA, that N^4, N^9-dinervonoyl spermine **6** was able to condense pEGFP efficiently (as a result of neutralization of DNA phosphate negative charges by the ammonium positive charges).

Lipoplex Particle Size and Zeta-Potential Measurements

The particle size and ζ-potential characterization measurements were carried out on the lipoplexes at their optimum N/P charge ratio for transfection (see: Table I).

ζ-Potential is an important parameter helping to predict the stability of the formulation as well as the ability of the positively charged nanoparticles to interact with cell membranes. ζ-Potential depends on several factors, including: pH, ionic charge, ion size, and concentration of ions in solution. 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-

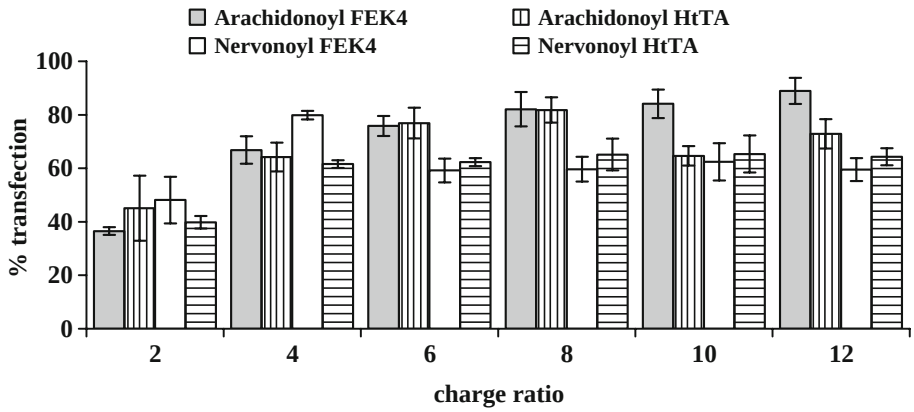


Fig. 4. Lipofection of the primary skin cell line FEK4 and the cancer cell line HtTA transfected with pEGFP (1 μg) complexed with N^4,N^9-diarachidonoyl spermine **4** and N^4,N^9-dinervonoyl spermine **6** at different N/P charge ratios.

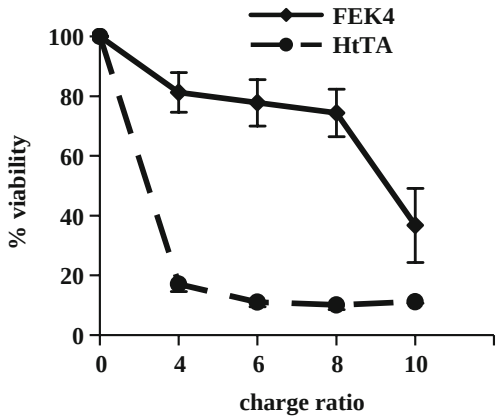


Fig. 5. Cytotoxicity effect of pEGFP (1 μg) complexed with N^4,N^9-diarachidonoyl spermine **4** at different N/P ratios on the primary skin cell line FEK4 and the cancer cell line HtTA.

potential is close to zero (3). The ζ-potential measurements on the lipoplexes, at their optimum concentrations of transfection, show that all values are positive (Table I), therefore there is a net positive charge on the surface. For DNA lipoplexes, ζ-potentials range from +2.2 mV (N^4,N^9-dioleoyl spermine **1**) to +16.7 mV (N^4,N^9-dieicosenoyl spermine **3**) (44–46), while for siRNA lipoplexes, ζ-potentials ranged from +5.5 mV (N^4,N^9-dierucoyl spermine **5**) to +8.1 mV (N^4,N^9-diarachidonoyl spermine **4**); the measured ζ-potential for naked DNA is -1.0 mV, for siRNA -0.3, and for the siRNA lipoplex with TransIT-TKO +2.6 mV.

pEGFP Transfection Experiments and *In Vitro* Cytotoxicity

The transduction of EGFP into a primary skin cell line (FEK4) and a cancer cell line (HeLa-derived HtTA) was investigated. All our polynucleotide delivery experiments are performed in the presence of serum. The optimum concentrations (in a final volume of 0.5 ml) and their corresponding N/P charge ratios for transfection were experimentally determined by using ascending N/P ratios of lipospermines from 2, 4, 6 etc. until we reached around 80% transfection and there was not a further step-up in transfection efficiency at the next highest N/P ratio. Results in Fig. 4 show that the transfection efficiency is related to the N/P ratio. On the other hand, the toxicity also increases as the N/P ratio is increased (Fig. 5). So the optimum concentrations (and corresponding N/P charge ratios) for transfection were found to be N^4,N^9-dioleoyl spermine **1** (2.8 μg, N/P=2.5), N^4,N^9-diarachidoyl spermine **2** (4.8 μg, N/P=4.0), N^4,N^9-dieicosenoyl spermine **3** (11.9 μg, N/P=10.0), N^4,N^9-diarachidonoyl spermine **4** (9.4 μg, N/P=8.0), N^4,N^9-dierucoyl spermine **5** (5.1 μg, N/P=4.0), and N^4,N^9-dinervonoyl spermine **6** (5.4 μg, N/P=4.0). The results (Fig. 6) indicate that the transfection ability of the different lipospermines (at their optimum pDNA delivery N/P ratios) on both cell lines lie in the range 70–85% except for N^4,N^9-diarachidoyl spermine **2** (5–10%). The cell viability (MTT assay) results indicate that while there is not a large difference in the viability of FEK4 cells typically (60–80%), the viability of HtTA cancer cells varies from N^4,N^9-dioleoyl spermine **1** (65%), N^4,N^9-dieicosenoyl spermine **3** and N^4,N^9-dinervonoyl spermine **6** (50%), N^4,N^9-dierucoyl spermine **5** (25%), and finally N^4,N^9-diarachidonoyl spermine **4** (10%).

siRNA Delivery and *In Vitro* Cytotoxicity

The transduction of fluorescein-tagged siRNA (RNAi Delivery Control, Mirus) into a primary skin cell line FEK4 and a cancer cell line (HeLa-derived HtTA) was investigated and compared with a market leader TransIT-TKO (Mirus) which works in the presence of serum. Therefore, to rise to this challenge, all our polynucleotide delivery experiments are performed in the presence of serum. The delivered siRNA is a 21 nucleotide "target" sequence with UG-3′ overhangs:

GAGGCUCAACUGGCUGACCUG
GUCUCCGAGUUGACCGACUGG

The Label IT RNAi Delivery Control is a fluorescein-labelled RNA duplex that has the same length, charge, and configuration as standard siRNA. The sequence of the duplex (above) is inert and is not known to affect any cellular events. The non-enzymatic Label IT labelling reagent attaches to any

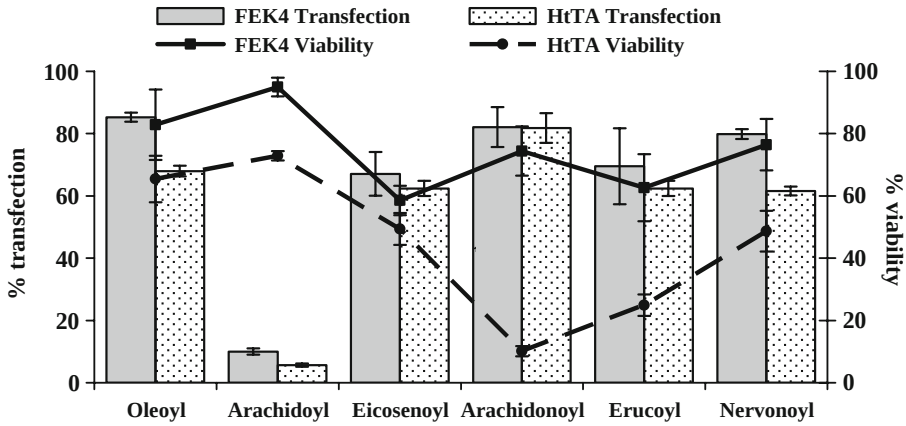


Fig. 6. Lipofection and cytotoxicity effects of pEGFP (1 μg) complexed with oleoyl (N^4,N^9-dioleoyl spermine **1**) (2.8 μg), arachidoyl (N^4,N^9-diarachidoyl spermine **2**) (4.8 μg), eicosenoyl (N^4,N^9-dieicosenoyl spermine **3**) (11.9 μg), arachidonoyl (N^4,N^9-diarachidonoyl spermine **4**) (9.4 μg), erucoyl (N^4,N^9-dierucoyl spermine **5**) (5.1 μg) and nervonoyl (N^4,N^9-dinervonoyl spermine **6**) (5.4 μg) on the primary skin cell line FEK4 and on the HeLa derived cancer cell line HtTA.

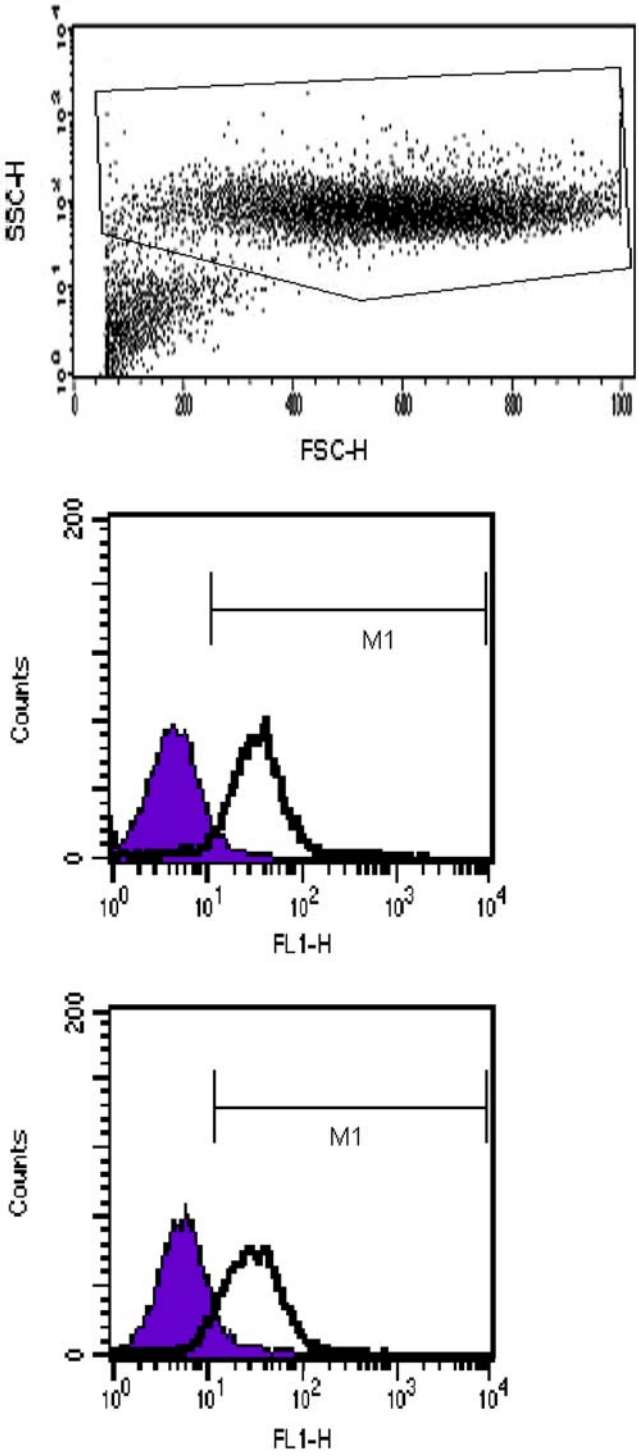


Fig. 7. FACS analysis showing the live population gated (*above*), and of FEK4 (*middle*) and of HtTA (*below*) after 48 h transfection of fluorescein-tagged siRNA complexed with N^4,N^9-dierucoyl spermine **5**: ■ untransduced cells, □ fluorescein-positive cells.

reactive heteroatom on any nucleotide residue of DNA or RNA. This direct covalent attachment is made possible by a reactive alkylating agent with strong nucleic acid binding capability facilitated via electrostatic interactions. As the Label IT labelling technology will bind to any nucleotide, we cannot be sure where the fluorophore was attached exactly, but because the reaction is controlled we know that there are 1–2 labels per duplex.

The optimum concentrations for transfection (in a final volume of 0.5 ml) were experimentally determined by using ascending amounts of lipospermines, as above for pDNA, and high transfection efficiency is immediately seen from our FACS analyses in both cell lines (Fig. 7) with gating to measure mainly live cells. The siRNA transfection efficiency results (carried out in triplicate on three separate experiments, $n=9$, histograms in Fig. 8) indicate that the transfection ability of N^4,N^9-dieicosenoyl spermine **3**, N^4,N^9-diarachidonoyl spermine **4**, and N^4,N^9-dierucoyl spermine **5** for both cell lines are comparable (e.g. N^4,N^9-dierucoyl spermine **5** FEK4 88.6±7.2%, HtTA 84.9±4.4%) to the results of TransIT (91% FEK4 and 93% HtTA) even in the presence of serum. The longer C-24 N^4,N^9-dinervonoyl spermine **6** has lower transfection levels and even lower are those for the saturated fat derived N^4,N^9-diarachidoyl spermine **2**.

The cell viability (MTT assay) results (lines in Fig. 8) indicate that there is no significant difference in the viability of FEK4 [85%, $p=0.15$ analysis of variance (ANOVA)] and HtTA (75%, $p=0.42$ ANOVA) cells between the commercially available TransIT-TKO and all the tested lipospermines except N^4,N^9-diarachidonoyl spermine **4** which is more toxic to both cell lines and N^4,N^9-dinervonoyl spermine **6** which is significantly less toxic ($p<0.01$ ANOVA).

The RiboGreen intercalation assay (Fig. 9) was performed on the three most efficient siRNA delivery vectors. Thus, siRNA was complexed with N^4,N^9-dieicosenoyl spermine **3**, N^4,N^9-diarachidonoyl spermine **4**, and N^4,N^9-dierucoyl spermine **5**. The results show that efficient siRNA binding, as assessed by fluorescence quenching, is occurring by N/P charge ratio 2 for N^4,N^9-dierucoyl spermine **5**, by charge ratio 3 for N^4,N^9-diarachidonoyl spermine **4**, and at charge ratio 4.5 for N^4,N^9-dieicosenoyl spermine **3**.

We have investigated the biological effects of increasing the length of the symmetrical diacyl fatty chain formulation of lipospermine on pDNA condensation and cellular delivery. The results from pEGFP condensation (Fig. 2), investigated using the EthBr fluorescence quenching assay, revealed that of our synthetic lipopolyamines N^4,N^9-dioleoyl spermine **1** (Lipogen®) was able to condense DNA to less than 20% EthBr fluorescence at N/P charge ratio 3.5 (47); however, N^4,N^9-dieicosenoyl spermine **3** and N^4,N^9-dierucoyl spermine **5** were not able to quench the EthBr fluorescence by 80% even at N/P charge ratio 6 (Fig. 2). The particle size of the final pDNA formulation is also an important factor in improving gene delivery (48,49). Results for pDNA showed particle size around 190 nm, and for siRNA around 145 nm (Table I), e.g. N^4,N^9-dierucoyl spermine **5** showed a pDNA lipoplex particle size of 210 nm (mean value ± SD 31, $n=9$) and an siRNA lipoplex particle size of 180 nm (mean value ± SD 28, $n=9$). On the relationship between particle size and transfection efficiency, there are no definite limits to the nanoparticle sizes that are suitable for transfection (50). Nanoparticles have relatively higher intracellular uptake than microparticles (51). Also, on the nanoscale, smaller-size polyplexes are more able to enter cells and thereby increase the efficiency of transfection (52).

In crossing the cell membrane bilayer (i.e. cell entry) and/or in helping to weaken the endosomal bilayer and

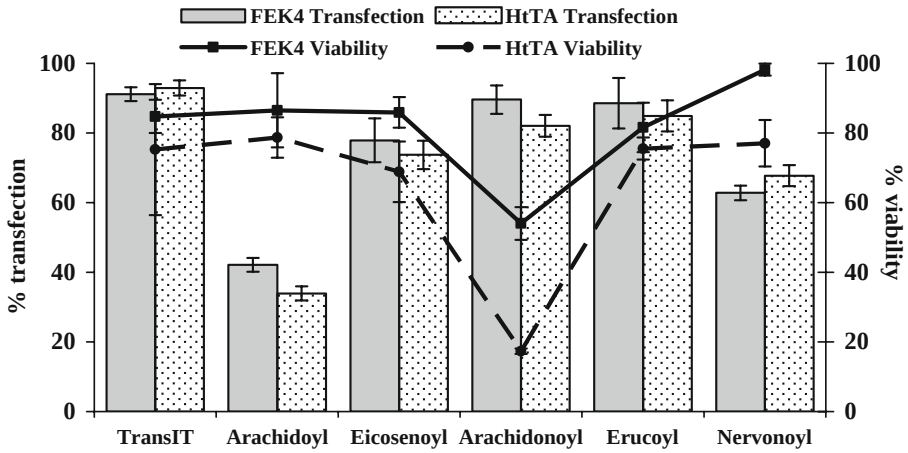


Fig. 8. Lipofection and cytotoxicity effects of siRNA (12.5 pmoles) complexed with TransIT- TKO (4 μl), arachidoyl (N^4,N^9-diarachidoyl spermine **2**; 6.0 μg), eicosenoyl (N^4,N^9-dieicosenoyl spermine **3**; 4.0 μg), arachidonoyl (N^4,N^9-diarachidonoyl spermine **4**; 6.0 μg), erucoyl (N^4,N^9-dierucoyl spermine **5**; 6.0 μg) and nervonoyl (N^4,N^9-dinervonoyl spermine **6**; 6.0 μg) on the primary skin cell line FEK4 and on the HeLa derived cancer cell line HtTA.

thereby aiding escape into the cytosol, the lipid moiety in our cationic lipids interacts with the phospholipid bilayer (53). DNA either as a nanoparticle or now free (uncomplexed) from the condensing lipopolyamine must then traffic to the nucleus and cross the nuclear membrane which is thought to occur through the nuclear pore complex or by direct association with the chromatin in mitosis (33). After nuclear entry, the payload DNA should successfully yield the desired protein through transcription and translation. The first key step in this gene formulation is DNA condensation into nanoparticles by 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. The lipid chains make a significant contribution to this DNA compaction (Fig. 2) (42,54) and this is observable in the agarose gel permeation assay (Fig. 3). Heparan sulfate and other cell-surface polyanionic (sulfated) glycosaminoglycans are also involved in the cell binding of cationic lipoplexes and polyplexes leading to non-specific endocytosis. They interact with the net positive charge on the cationic lipoplexes and inhibit cation-mediated gene transfer, probably directing the lipoplexes into intracellular compartments that do not support transcription. Thus, the mechanisms of the uptake and the intracellular fate of lipoplexes are still obscure, but glycosaminoglycans may act as cellular receptors for cationic ligands, rather than only as passive cation binding sites (55–59).

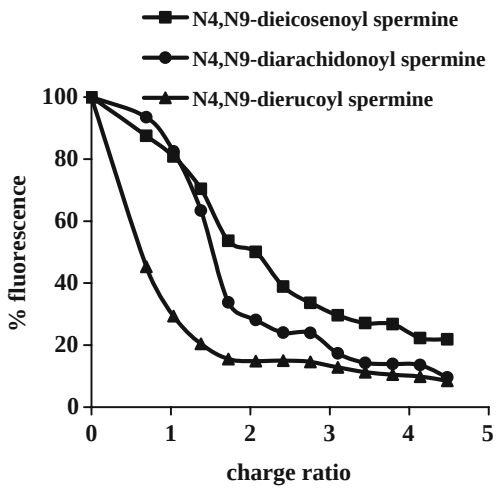


Fig. 9. Plot of RiboGreen intercalation assay of siRNA complexed with different lipospermines.

We have previously reported the importance of the substituents in the lipid moiety conjugated to the cationic polyamine to achieve improvements in DNA condensation efficiency for non-liposomal 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 (2,3,54,60–62). The design and synthesis of novel cationic lipids based on the tetra-amine spermine, as non-liposomal formulations, where the lipid moiety is long carbon chains, were largely instigated by Behr *et al.* (32) and Remy *et al.* (33) with their design and preparation of the highly efficient lipopolyamine dioctadecylglycyl spermine (Transfectam®). It is generally agreed that the length and type of the aliphatic chains incorporated into cationic lipids significantly affect their transfection efficiency. Thus, vectors are often prepared in a series differing in their hydrophobic domain. Kirby, Camilleri, and co-workers identified C18 chains to be optimal in a series of gemini surfactants according to the order C18 > C16 > C14 (63), which was also reported by Scherman and co-workers with a series of linear polyamine-based vectors (64).

It has been proposed that a shorter chain length may facilitate intermembrane mixing, an important factor in endosomal escape.(53) Variation in the acyl chain, longer than the oleoyl group (C18), has led to higher levels of transfection, a result possibly related to the issues of hydrophobic moiety hydration (53,65,66). Lindner and co-workers reported the use of very long chain lipids e.g. erucoyl and compared them with other shorter chains in their designed cationic lipids (67), while Cullis and co-workers have studied the variation of the length of the acyl chains contained in the hydrophobic anchor from octanoyl to myristoyl to arachidoyl (68–70).

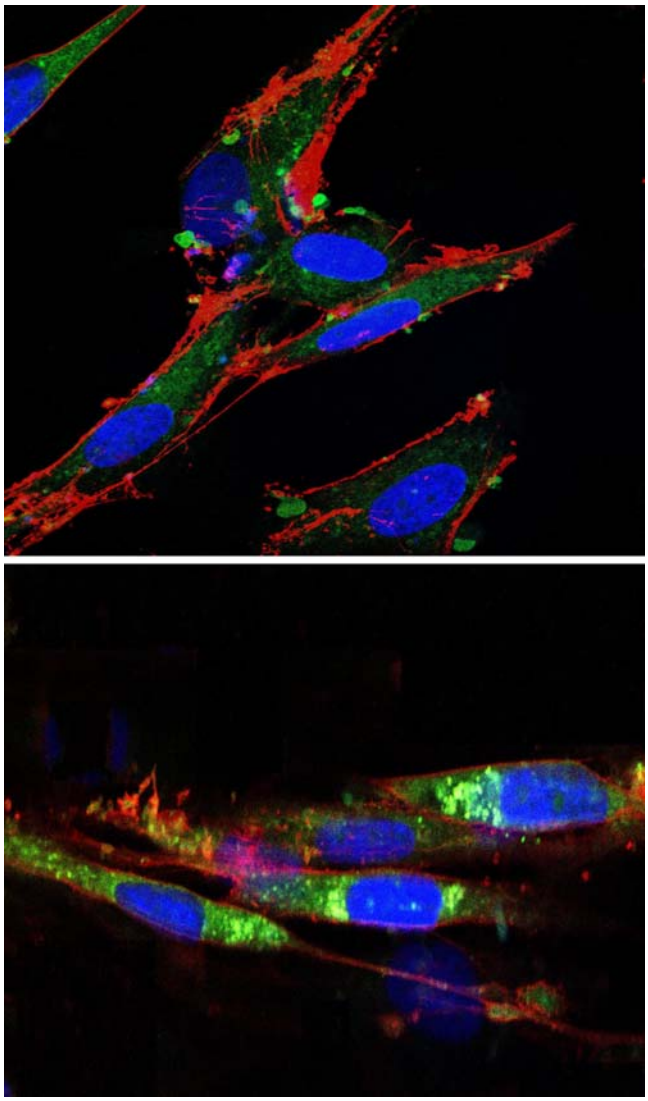


Fig. 10. The FEK4 primary cell line transfected with pEGFP (*upper*) showing cytosolic green fluorescence from EGFP, delivered with N^4,N^9-dieicosenoyl spermine **3**, and transfected with fluorescein-labelled siRNA (*lower*) showing cytosolic green fluorescence from fluorescein, delivered with N^4,N^9-dierucoyl spermine **5**. The nuclei fluoresce blue from the Hoechst 33342 and the cell lipid bilayers fluoresce red from the Alexa Fluor 594 (LSM510META, Zeiss, Jena, Germany, under the 60× oil immersion objective).

Using confocal laser scanning microscopy with one labelling solution (Invitrogen) containing both Alexa Fluor 594 wheat germ agglutinin (5 μg/ml) for cell membrane labelling, and Hoechst 33342 (2 μM) for nuclei labelling, we have shown (Fig. 10) that FEK4 cells were successfully transfected with pDNA as the cells biosynthesized EGFP by transcription and translation. Successful delivery of fluorescent-siRNA to the cytosol was also shown (Fig. 10).

Incorporating two aliphatic chains and then stepwise increasing their length, our siRNA delivery results (Fig. 8) show that of our synthesized very long chain lipospermines, N^4,N^9-dieicosenoyl spermine **3** and N^4,N^9-dierucoyl spermine **5** are two new, efficient delivery vectors that work in primary cell lines and in the presence of serum. They demonstrate efficiencies comparable to the results obtained with TransIT, N^4,N^9-dieicosenoyl spermine **3** typically 78% FEK4 and 74% HtTA, and N^4,N^9-dierucoyl spermine **5** typically 89% FEK4 and 85% HtTA along with cell viability at least as high as TransIT (>80%). These novel very long chain lipopolyamines are remarkably non-toxic and capable of delivering siRNA to hard-to-transfect primary cell lines in the presence of serum and with efficiency equalling a market leader, TransIT-TKO. They are two important new siRNA delivery vectors.

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