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Research Paper

N^1,N^{12}-Diacyl Spermines: SAR Studies on Non-viral Lipopolyamine Vectors for Plasmid DNA and siRNA Formulation

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Purpose. To study the efficiency of novel synthetic N^1,N^{12}-diacyl spermines on DNA and siRNA binding, and to compare their transfection efficiency with viability in cell lines.
Methods. Five long chain N^1,N^{12}-diacyl lipopolyamines: N^1,N^{12}-[didecanoyl, dimyristoyl, dimyristoleoyl, distearoyl and dioleoyl]-1,12-diamino-4,9-diazadodecane were synthesized from the naturally occurring polyamine spermine. Their abilities to condense DNA and to form nanoparticles were characterized. Transfection efficiencies were studied in FEK4 primary skin cells and in an immortalized cancer cell line (HtTA), and compared with N^4,N^9-regioisomers. Also, the abilities of these novel compounds to bind to siRNA-forming nanoparticles were studied using a RiboGreen intercalation assay, and their abilities to deliver fluorescein-tagged siRNA into cells were quantified and compared with TransIT-TKO.
Results. By incorporating two long aliphatic chains and varying their acylation position, length, and oxidation state in a stepwise manner, we show efficient p and siRNA formulation and delivery to primary skin and cancer cell lines. Although two C14 chains (both saturated or both mono-*cis*-unsaturated) were efficient transfecting agents, they were highly toxic.
Conclusions. N^1,N^{12}-Dioleoyl spermine efficiently binds to and delivers pDNA and siRNA with high cell viability even in a primary skin cell line. It is a novel, efficient non-viral vector in the presence of serum.

KEY WORDS: gene delivery; primary skin cells; siRNA delivery; transfection; TransIT-TKO.

INTRODUCTION

Poly-nucleic acid delivery has many potential applications in medicine, and formulations to reach the goal of intracellular protein levels at therapeutic concentrations are moving even more towards utilising non-viral gene therapy (NVGT) (1). We have recently reported on the relevant literature in this fast-moving and competitive research area (2,3). Our non-viral gene delivery systems are centered upon formulations of poly-nucleic acids complexed with synthetic cationic lipids and lipopolyamines and composed of two long-carbon chains (or a steroid) (4) covalently bound to a polyamine, e.g. spermine (1,12-diamino-4,9-diazadodecane) (5,6). 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 (7,8) and siRNA delivery (9–23). Our non-viral vectors not only circumvent the drawbacks of viral vectors (limited payloads, immune responses), but they are also designed to have the advantages of simplicity of use based upon DNA and siRNA anion titration and ease of large-scale production. There is a clear need for designed "intelligent materials" (24) to achieve this nanoparticle self-assembly.

The essential requirements for gene delivery are the transport of DNA through the cell membrane and ultimately to the nucleus. A first step in gene formulation is DNA condensation through masking the negative charges of the phosphate backbone by titration-forming nanoparticles. The design of an efficient formula for the delivery of genetic material requires a detailed understanding of the barriers that hinder this process (20). These barriers include complex formation between the DNA and the lipopolyamine leading to DNA nanoparticle (lipoplex) formation by electrostatic charge neutralisation (at least to 90%) and overall packing as condensed DNA. Then, they are transported across cell-membranes, either by adsorptive endocytosis or mediated by cations, both routes leading to internalization of the lipoplex (25). The site of action of siRNA is in the cytosol, so there is 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.

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

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ABBREVIATIONS: EGFP, enhanced green fluo-rescent 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; NPC, nuclear pore complex; NVGT, non-viral gene therapy; pEGFP, plasmid enhanced green fluorescent protein; *t*-BOC, *t*-butoxycarbonyl.

type, length, position, and number of the hydrophobic anchors (5,6). 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. We have therefore designed a series of novel regioisomers of diacyl spermines in order to prepare lipoplex formulations (without any pre-preparation of liposomes) of the circular plasmid DNA encoding for enhanced green fluorescent protein (pEGFP) and a fluorescein-tagged siRNA for non-viral gene delivery and transfection of target cells as they are designed to form nanoparticles which will efficiently enter cells by endocytosis (Fig. 1).

Herein we report our investigations of the formulation and delivery of pEGFP, a 4.7 kbp plasmid, with a molecular weight of about 3.1 MDa (given an average of 330 Da per nucleotide, 660 Da per bp (26), carrying 9,400 negative charges, and a 21-mer of double stranded siRNA (27)). For our non-viral vectors, we vary the length and position of the two fatty chains covalently bound to spermine. Although we make changes to chain length from C18 (oleoyl and stearoyl) to C14 (myristoyl and myristoleoyl), and that is in itself significant, we also change the position of the amine groups and therefore the charge separation. These novel lipopolyamines are designed to incorporate the charge distribution of putrescine (1,4-diaminobutane), a moiety found in both spermine and spermidine. We report the synthesis and characterisation of pEGFP and fluorescent-tagged siRNA lipoplex nanoparticles, and we assess them by physicochemical techniques, e.g. ethidium bromide (EthBr) fluorescence quenching (28), and DNA gel shift assays, target cell pEGFP transfection (without and with serum—for nuclease activity), and toxicity (cell viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT assay) (29,30), and siRNA delivery with four 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) (5,6) and TransIT-TKO.

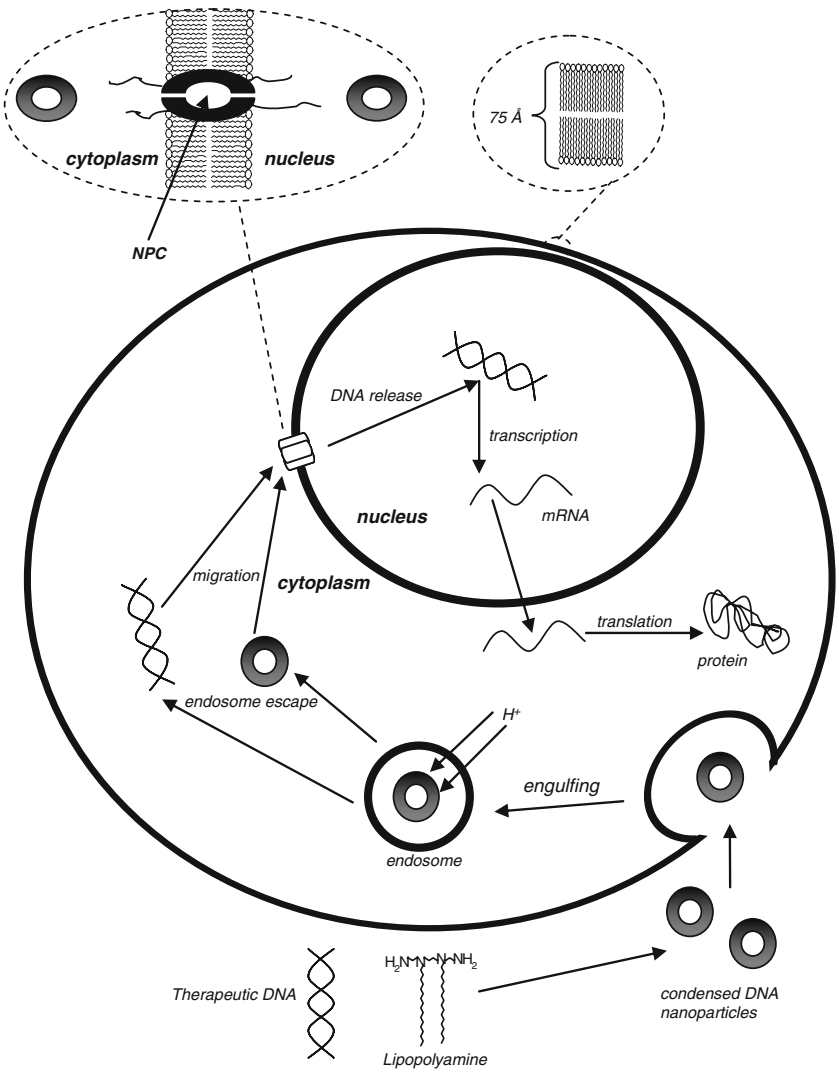

Fig. 1. The process of NVGT by endocytosis and the barriers that must be crossed by toroidal condensed DNA nanoparticles, illustrated for lipopolyamines in the formulation of lipoplexes leading to DNA delivery and ultimately to the goal of intracellular protein synthesis.

MATERIALS AND METHODS

Materials

Chemicals, including spermine, acyl chlorides, 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

Glassware, ninhydrin used for the detection of polyamines, silica gel column and analytical chromatography were as previously reported (31). All the synthesized lipopolyamines were pure and 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) and ^{13}C NMR spectroscopy recorded 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. ^{13}C assignments were aided by 90° and 135° DEPT pulse sequences, and other NMR assignments follow from correlation spectroscopies; COSY, DEPT, HMBC, HMQC spectra were all recorded using automated programmes. All lipopolyamines showed satisfactory high- (HRMS) and low-resolution mass spectrometric FAB data (reported in Da and within 5 ppm).

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 (32), the target lipopolyamine displayed HRMS: found (*m/z* $[M+H]^+$) 731.7115, $C_{46}H_{91}N_4O_2$ requires 731.7142 (Δ3.7 ppm), consistent with the data previously reported (5,6).

N^4,N^9-Dimyristoleoyl-1,12-diamino-4,9-diazadodecane ***2***

Prepared via N^1,N^{12}-ditrifluoroacetyl-N^4,N^9-dimyristoleoyl-1,12-diamino-4,9-diazadodecane (706 mg, 87%, R_f=0.9 CH_2Cl_2:MeOH, 5:1, *v/v*) displayed MS, FAB$^+$ found 811.2 (100%, *m/z* $[M+H]^+$), $C_{42}H_{73}F_6N_4O_4$ requires 811, the target lipopolyamine displayed HRMS: found (*m/z* $[M+H]^+$) 619.5872, $C_{38}H_{75}N_4O_2$ requires 619.5890 (Δ2.9 ppm).

N^1,N^{12}-Ditrifluoroacetyl-N^4,N^9-di(t-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane

Ethyl trifluoroacetate (1.3 mL, 10.9 mmol, 2.2 eq.) in MeOH (10 mL) was added dropwise to spermine (1.0 g, 4.9 mmol) in MeOH in 0°C under anhydrous nitrogen; the reaction was stirred at 20°C for 24 h to form N^1,N^{12}-ditrifluoroacetyl-1,12-diamino-4,9-diazadodecane, which was taken into the next step without purification. Triethylamine (1.0 mL, 7.4 mmol, 1.5 eq.) followed by BOC-ON (2.7 g, 10.9 mmol, 2.2 eq.) were added to the diprotected spermine solution. The resulting yellow solution was stirred at 20°C for 24 h then evaporated to dryness in vacuo and the residue dissolved in EtOAc (25 mL). The organic phase was washed with 5% aq. NaOH (4×25 mL), water (2×25 mL), dried ($MgSO_4$) and concentrated. Column chromatography of the residue (CH_2Cl_2:MeOH; 120:1 to 100:1 to 50:1; *v/v*) gave the desired diamide-dicarbamate as an oil (2.5 g, 85%). R_f=0.9 (CH_2Cl_2:MeOH:conc. NH_3 aq; 50:10:1; *v/v/v*). ^{1}H NMR, $CDCl_3$: 1.48 (s, 18H, 2× $NCOOC(CH_3)_3$); 1.71–1.82 (m, 8H, H2, H6, H7, H11); 3.15–3.22 (m, 12H, H1, H3, H5, H8, H10, H12); 8.0 (br, N^1H, N^{12}H). ^{13}C NMR, $CDCl_3$: 25.8 (C6, C7); 27.1 (C2, C11); 28.2 (CH_3); 35.8 (C1, C12); 42.8 (C3, C10); 46.8 (C5, C8); 80.1 ($OC(CH_3)_3$); 116.0 ($NCOCF_3$, J=295); 157.0 (N^4-CO, N^9-CO); 157.8 (N^1-CO, N^{12}-CO, J=46). MS, FAB$^+$ found 594.6, (17%, M^++1), 56.8 (100%, $NH_2CH_2CH{=}CH_2$), $C_{24}H_{40}F_6N_4O_6$ requires (M^+) 594. HRMS *m/z*, FAB$^+$ found 595.2938, (M^++Na), $C_{24}H_{41}F_6N_4O_6$ requires (M^++1) 595.2925.

N^4,N^9-Di(t-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane

Potassium carbonate (295 mg, 2.1 mmol) was added to N^1,N^{12}-ditrifluoroacetyl-N^4,N^9-di(*t*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane (2.5 g, 4.2 mmol) in MeOH (10 mL) and water (600 μL), and the reaction mixture was heated under reflux (5 h), cooled to 20°C and then evaporated to dryness in vacuo. Water (30 mL) was added to the residue, which was extracted with $CHCl_3$ (3×25 mL). The combined organic phase was washed with water (15 mL), dried ($MgSO_4$) and concentrated. The aqueous layers were further basified with ice-cold 5% NaOH (10 mL) and extracted with $CHCl_3$ (4× 40 mL). After a water wash (25 mL), the combined organic extracts were evaporated to dryness in vacuo. Column chromatography of the oily residue (CH_2Cl_2:MeOH:conc. aq. NH_3; 200:10:1 to 100:10:1 to 50:10:1; *v/v/v*) gave the desired dicarbamate as an oil (1.0 g, 59%). R_f=0.4 (CH_2Cl_2:MeOH: conc. aq. NH_3; 50:10:1; *v/v/v*). ^{1}H NMR, $CDCl_3$: 1.40 (s, 18H, 2× $NCOOC(CH_3)_3$); 1.50–1.70 (m, 8H, H2, H6, H7, H11); 2.0 (br, 4H, NH_2); 2.63 (s, 4H, H1, H12) 3.10–3.22 (m, 8H, H3, H5, H8, H10). ^{13}C NMR, $CDCl_3$: 25.8 (C6, C7); 28.5 (CH_3); 32.0 (C2, C11); 39.2 (C1, C12); 44.2 (C3, C10); 46.6 (C5, C8); 79.8 (NCOOC); 149.3 (NCO). MS, FAB$^+$ found 402.7 (8%, M^++1), 56.8 (100%, $NH_2CH_2CH{=}CH_2$), $C_{20}H_{42}N_4O_4$ requires (M^+) 402. HRMS *m/z*, FAB$^+$ found 403.3275, (M^++1), $C_{20}H_{43}N_4O_4$ requires (M^++1) 403.3279.

N^1,N^{12}-Dioleoyl-N^4,N^9-di(t-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane

N^4,N^9-Di(*t*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane (500 mg, 1.2 mmol) was dissolved in pyridine (10 mL) under anhydrous nitrogen, and oleoyl chloride (0.9 mL, 2.7 mmol) was added. The reaction was continued for 16 h, the solution was then concentrated in vacuo. Purification of the crude compound was by column chromatography (CH_2Cl_2:MeOH; 100:1 to 50:1; *v/v*) to give an oil (420 mg, 36%). ^{1}H NMR, $CDCl_3$: 0.83 (t, 6H, H18′, J=8); 1.12–1.36 (m, 58H, H4′–H7′, H12′–H17′, 2× $NCOOC(CH_3)_3$); 1.41 (m, overlap, 8H, H6, H7, H3′); 1.58 (m, 4H, H2, H11); 1.96 (m, 8H, H8′, H11′); 2.14 (t, 4H, H2′, J=7); 3.16 (m, 12H, H1, H3, H5, H8, H10, H12); 5.29 (m, 4H, H9′, H10′); 6.75 (br, NH). ^{13}C NMR, $CDCl_3$: 14.1 (C18′); 28.4 ($NCOOC(CH_3)_3$); 22.7–31.9 (C2, C6, C7, C11, C3′–C8′, C11′–C17′); 35.3 (C2′); 36.8 (C1, C12); 43.2 (C3, C10); 46.6 (C5, C8); 79.7 (NCOOC); 129.8 (C9′, C10′); 156.3 (NCO); 173.2 (C1′). MS, FAB$^+$ found 931.1 (4%, M^++1), 57.1 (100%, $NH_2CH_2CH{=}CH_2$), 832.1 (M-BOC),

$C_{56}H_{106}N_4O_6$ requires (M^+) 930. HRMS *m/z*, FAB$^+$ found 953.8081, (M^++Na), $C_{56}H_{107}N_4O_6$ requires (M^++1) 931.8185.

N^1,N^{12}-Distearoyl-N^4,N^9-di(t-butoxycarbonyl)-1, 12-diamino-4,9-diazadodecane

N^4,N^9-Di(*t*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane (500 mg, 1.2 mmol) was dissolved in pyridine (10 mL) under anhydrous nitrogen, and stearoyl chloride (0.9 mL, 2.7 mmol) was added. The reaction was continued for 16 h; the solution was then concentrated in vacuo. Purification of the crude compound was by column chromatography (CH_2Cl_2:MeOH; 100:1 to 50:1; *v/v*) to give an oil (840 mg, 73%). ^{1}H NMR, $CDCl_3$: 0.96 (t, 6H, H18′, *J*=8); 1.29–1.33 (m, 56H, H4′–H17′); 1.40 (s, 18H, 2× NCOOC$(CH_3)_3$); 1.55–1.70 (m, 12H, H2, H6, H7, H11, H3′); 2.18 (m, 4H, H2′); 2.31 (m, 8H, H3, H5, H8, H10); 3.18 (m, 4H, H1, H12); 6.78 (br, NH). ^{13}C NMR, $CDCl_3$: 14.1 (C18′); 22.8–27.7 (C2, C6, C7, C11, C3′, C17′); 28.5 (OCH_3); 29.0–31.9 (C4′–C16′); 35.8 (C2′); 37.2 (C1, C12); 43.2 (C3, C10); 46.7 (C5, C8); 79.8 (NCOOC); 156.3 (NCO); 173.2 (C1′). MS, FAB$^+$ found 935.0 (8%, M^++1), 56.8 (100%, $NH_2CH_2CH{=}CH_2$), $C_{56}H_{110}N_4O_6$ requires (M^+) 934. HRMS *m/z*, FAB$^+$ found 935.8498, (M^++1), $C_{56}H_{111}N_4O_6$ requires (M^++1) 935.8516.

N^1,N^{12}-Dimyristoleoyl-N^4,N^9-di(t-butoxycarbonyl)-1, 12-diamino-4,9-diazadodecane

Myristoleoic acid (0.7 mL, 2.9 mmol), HOBt (70 mg, 0.6 mmol) and DCC (587 mg, 4.3 mmol) were added in the solvent of N^4,N^9-di(*t*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane (520 mg, 1.3 mmol) in CH_2Cl_2 (10 mL). The reaction was continued for 16 h; the solution was then filtered to remove the urea and concentrated in vacuo. Purification of the crude compound was by column chromatography (CH_2Cl_2: MeOH; 100:1 to 50:1; *v/v*) to give an oil (800 mg, 76%). ^{1}H NMR, $CDCl_3$: 0.85 (t, 6H, H14′, *J*=8); 1.13–1.35 (m, 42H, H4′–H7′, H12′, H13′, 2× NCOOC$(CH_3)_3$); 1.40–1.57 (m, overlap, 12H, H2, H6, H7, H11, H3′); 1.96 (m, 8H, H8′, H11′); 2.12 (t, 4H, H2′, *J*=6); 3.15 (m, 12H, H1, H3, H5, H8, H10, H12); 5.28 (m, 4H, H9′, H10′); 6.73 (br, NH). ^{13}C NMR, $CDCl_3$: 13.9 (C14′); 28.3 (2× NCOOC$(CH_3)_3$); 22.2–31.8 (C2, C6, C7, C11, C3′–C8′, C11′–C13′); 35.3 (C2′); 36.8 (C1, C12); 43.2 (C3, C10); 46.6 (C5, C8); 79.6 (NCOOC); 129.8 (C9′, C10′); 156.3 (NCO); 173.2 (C1′). MS, FAB$^+$ found 819.3 (16%, M^++1), 56.9 (100%, $NH_2CH_2CH{=}CH_2$), 719.2 (36%, M-BOC), $C_{48}H_{90}N_4O_6$ requires (M^+) 818. HRMS *m/z*, FAB$^+$ found 841.6768, (M^++Na), $C_{48}H_{90}N_4O_6$Na requires (M^++Na) 841.6753.

N^1,N^{12}-Dimyristoyl-N^4,N^9-di(t-butoxycarbonyl)-1, 12-diamino-4,9-diazadodecane

N^4,N^9-Di(*t*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane (500 mg, 1.2 mmol) was dissolved in pyridine (10 mL) under anhydrous nitrogen, and myristoyl chloride (0.9 mL, 2.7 mmol) was added. The reaction was continued for 16 h; the solution was then concentrated in vacuo. Purification of the crude compound was by column chromatography (CH_2Cl_2:MeOH; 100:1 to 50:1; *v/v*) to give an oil (824 mg, 81%). ^{1}H NMR, $CDCl_3$: 0.85 (t, 6H, H14′, *J*=7); 1.22–1.37 (m, 40H, H4′–H13′); 1.43 (s, 18H, 2× NCOOC$(CH_3)_3$); 1.55–1.59 (m, 8H, H6, H7, H3′); 1.61 (m, 4H, H2, H11); 2.15 (t, 4H, H2′, *J*=7); 2.28 (m, 8H, H3, H5, H8, H10); 3.18 (t, 4H, H1, H12, *J*=8); 6.71 (br, NH). ^{13}C NMR, $CDCl_3$: 14.1 (C14′); 22.6 (C13′); 25.2–27.7 (C2, C6, C7, C11, C3′); 28.4 (2× NCOOC$(CH_3)_3$); 29.0–31.8 (C4′–C12′); 35.3 (C2′); 36.9 (C1, C12); 43.3 (C3, C10); 46.7 (C5, C8); 79.7 (NCOOC); 156.3 (NCO); 173.3 (C1′). MS, FAB$^+$ found 823.0 (14%, M^++1), 56.9 (100%, $NH_2CH_2CH{=}CH_2$), $C_{48}H_{94}N_4O_6$ requires (M^+) 822. HRMS *m/z*, FAB$^+$ found 845.7095, (M^++Na), $C_{48}H_{94}N_4O_6$Na requires (M^++Na) 845.7066.

N^1,N^{12}-Didecanoyl-N^4,N^9-di(t-butoxycarbonyl)-1, 12-diamino-4,9-diazadodecane

N^4,N^9-Di(*t*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane (500 mg, 1.2 mmol) was dissolved in pyridine (10 mL) under anhydrous nitrogen, and decanoyl chloride (0.9 mL, 2.7 mmol) was added. The reaction was continued for 16 h; the solution was then concentrated in vacuo. Purification of the crude compound was by column chromatography (CH_2Cl_2:MeOH; 100:1 to 50:1; *v/v*) to give an oil (644 mg, 73%). ^{1}H NMR, $CDCl_3$: 0.83 (t, 6H, H10′, *J*=7); 1.21–1.36 (m, 28H, H6, H7, H4′–H9′); 1.41 (s, 18H, 2× NCOOC $(CH_3)_3$); 1.51–1.59 (m, 8H, H2, H11, H3′); 2.12 (t, 4H, H2′, *J*=8); 3.42 (m, 12H, H1, H3, H5, H8, H10, H12); 7.25 (br, NH). ^{13}C NMR, $CDCl_3$: 14.0 (C10′); 22.6 (C9′); 25.0 (C6, C7); 25.7 (C3′); 27.5 (C2, C11); 28.3 (CH_3); 29.0–29.4 (C4′–C7′); 31.7 (C8′); 35.3 (C1, C12); 36.8 (C2′); 43.2 (C3, C10); 46.6 (C5, C8); 77.0 (NCOOC); 156.3 (NCO); 173.4 (C1′). MS, FAB$^+$ found 711.4 (32%, M^++1), 57.0 (100%, $NH_2CH_2CH{=}CH_2$), 611.4 (38%, M-BOC), $C_{40}H_{78}N_4O_6$ requires (M^+) 710. HRMS *m/z*, FAB$^+$ found 733.5820, (M^++Na), $C_{40}H_{78}N_4O_6$Na requires (M^++Na) 733.5994.

N^1,N^{12}-Dioleoyl-1,12-diamino-4,9-diazadodecane ***3***

To a stirring solution of N^1,N^{12}-dioleoyl-N^4,N^9-di(*t*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane (420 mg, 0.5 mmol) in CH_2Cl_2 (180 mL), under nitrogen, at 20°C was added TFA (20 mL). After 2 h, the solution was concentrated in vacuo to yield the title compound as a white semi-solid (320 mg, 97%, di-TFA salt). ^{1}H NMR, d_6-DMSO: 0.96 (t, 6H, H18′, *J*=7); 1.22–1.64 (m, 52H, H2, H6, H7, H11, H3′–H7′, H12′–H17′); 2.00–2.55 (m, 14H, C2′, H8′, H11′, NH); 3.00 (m, 12H, H1, H3, H5, H8, H10, H12); 5.32 (m, 4H, H9′, H10′); 8.62 (br, NH). ^{13}C NMR, d_6-DMSO: 14.1 (C18′); 22.8–36.1 (C1, C2, C6, C7, C11, C12, C2′–C8′, C11′–C17′); 45.2 (C3, C10); 47.3 (C5, C8); 130.0 (C9′, C10′); 180.0 (C1′). MS, FAB$^+$ found 730.9 (100%, M^++1), $C_{46}H_{90}N_4O_2$ requires (M^+) 730.

N^1,N^{12}-Distearoyl-1,12-diamino-4,9-diazadodecane ***4***

To a stirring solution of N^1,N^{12}-distearoyl-N^4,N^9-di(*t*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane (840 mg, 0.9 mmol) in CH_2Cl_2 (180 mL), under nitrogen, at 20°C was added TFA (20 mL). After 2 h, the solution was concentrated in vacuo to yield the title compound as a white solid (620 mg, 94%, di-TFA salt), m.p. 125–127°C. ^{1}H NMR, d_6-DMSO: 0.85 (t, 6H, H18′, *J*=8); 1.23–2.07 (m, 72H, H2, H6, H7, H11, H2′–H17′); 3.10 (m, 12H, H1, H3, H5, H8, H10, H12); 7.96, 8.61 (br, NH). ^{13}C NMR, d_6-DMSO: 13.7 (C18′); 21.9–29.1 (C2, C6, C7,

C11, C3′–C15′, C17′); 31.2–35.4 (C1, C12, C2′, C16′); 47.0 (C3, C10); 49.1 (C5, C8); 172.0 (C1′). MS, FAB^+ found 734.9 (100%, M^++1), $C_{46}H_{94}N_4O_2$ requires (M^+) 734. HRMS *m*/*z*, FAB^+ found 735.7441, (M^++1), $C_{46}H_{95}N_4O_2$ requires (M^++1) 735.7450.

N^1,N^{12}-Dimyristoleoyl-1,12-diamino-4,9-diazadodecane ***5***

To a stirring solution of N^1,N^{12}-dimyristoleoyl-N^4,N^9-di(*t*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane (800 mg, 1.0 mmol) in CH_2Cl_2 (180 mL), under nitrogen, at 20°C was added TFA (20 mL). After 2 h, the solution was concentrated in vacuo to yield the title compound as a white semi-solid (600 mg, 99%, di-TFA salt). ^{1}H NMR, d_6-DMSO: 0.96 (t, 6H, H14′, *J*=8); 1.38–2.04 (m, 46H, H2, H6, H7, H11, H11′–H13′, H3′–H8′, N^4H, N^9H); 2.24 (t, 4H, H2′, *J*=7); 2.98 (m, 8H, H3, H5, H8, H10); 3.32 (m, 4H, H1, H12); 5.40 (m, 4H, H9′, H10′); 8.20 (br, NH). ^{13}C NMR, d_6-DMSO: 13.2 (C14′); 22.0–35.9 (C2′–C8′, C11′–13′, C1, C2, C6, C7, C11, C12); 45.1 (C3, C10); 47.3 (C5, C8); 129.0 (C9′, C10′); 176.0 (C1′). MS, FAB^+ found 618.9 (100%, M^++1), $C_{38}H_{74}N_4O_2$ requires (M^+) 618. HRMS *m*/*z*, FAB^+ found 619.5885, (M^++1), $C_{38}H_{75}N_4O_2$ requires (M^++1) 619.5892.

N^1,N^{12}-Dimyristoyl-1,12-diamino-4,9-diazadodecane ***6***

To a stirring solution of N^1,N^{12}-dimyristoyl-N^4,N^9-di(*t*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane (824 mg, 1.0 mmol) in CH_2Cl_2 (180 mL), under nitrogen, at 20°C was added TFA (20 mL). After 2 h, the solution was concentrated in vacuo to yield the title compound as a white solid (620 mg, 99%, di-TFA salt), m.p. 92–94°C. ^{1}H NMR, d_6-DMSO: 0.91 (t, 6H, H14′, *J*=7); 1.22–1.37 (m, 40H, H4′–H13′); 1.55–1.59 (m, 8H, H6, H7, H3′); 1.61 (m, 4H, H2, H11); 2.15 (t, 4H, H2′, *J*=8); 2.28 (m, 8H, H3, H5, H8, H10); 2.40 (br, NH); 3.18 (m, 4H, H1, H12); 8.62 (br, NH). ^{13}C NMR, d_6-DMSO: 13.9 (C14′); 22.6 (C13′); 25.2–27.7 (C2, C6, C7, C11, C3′); 29.0–31.8 (C4′–C12′); 35.3 (C2′); 36.9 (C1, C12); 43.3 (C3, C10); 46.7 (C5, C8); 172.9 (C1′). MS, FAB^+ found 623.8 (100%, M^++1), $C_{38}H_{78}N_4O_2$ requires (M^+) 622. HRMS *m*/*z*, FAB^+ found 623.6199, (M^++1), $C_{38}H_{79}N_4O_2$ requires (M^++1) 623.6198.

N^1,N^{12}-Didecanoyl-1,12-diamino-4,9-diazadodecane ***7***

To a stirring solution of N^1,N^{12}-didecanoyl-N^4,N^9-di(*t*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane (644 mg, 0.9 mmol) in CH_2Cl_2 (180 mL), under nitrogen, at 20°C was added TFA (20 mL). After 2 h, the solution was concentrated in vacuo to yield the title compound as a white solid (455 mg, 98%, di-TFA salt), m.p. 67–69°C. ^{1}H NMR, d_6-DMSO: 0.83 (t, 6H, H10′, *J*=8); 1.21–1.59 (m, 36H, H2, H6, H7, H11, H3′–H9′); 2.02–2.14 (m, 14H, H3, H5, H8, H10, H2′, N^4H, N^9H); 3.42 (m, 4H, H1, H12); 7.25 (br, NH). ^{13}C NMR, $CDCl_3$: 14.0 (C10′); 22.6–29.4 (C2, C6, C7, C11, C3′–C7′, C9′); 31.7–36.8 (C1, C12, C2′, C8′); 43.2 (C3, C10); 46.6 (C5, C8); 173.4 (C1′). MS, FAB^+ found 511.6 (100%, M^++1), $C_{30}H_{62}N_4O_2$ requires (M^+) 510. HRMS *m*/*z*, FAB^+ found 511.4948, (M^++1), $C_{30}H_{63}N_4O_2$ requires (M^++1) 511.4946.

Amplification and Purification of Plasmid DNA (pEGFP)

DNA plasmid (26) encoding-enhanced green fluorescent protein (pEGFP) (Clontech) was transformed into *Escherichia coli* JM 109 bacterial strain (Promega) and grown on in Luria–Bertani (LB) broth and analysed as previously reported (31).

DNA Condensation (Ethidium Bromide Fluorescence Quenching Assay)

Each concentration of the pEGFP stock solutions (approximately 1 μg/μL, 1 mL) was determined spectroscopically (Milton Roy Spectronic 601 spectrometer, 1 cm path length, 3 mL cuvette) (26) with 6 μg (approximately 6 μL) pDNA diluted to 3 mL with buffer (20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) and condensed with lipopolyamine aliquots as previously reported (31).

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 as previously reported (31).

Lipoplex Particle Size

The average particle size for each lipoplex formed (at a highly efficient charge ratio of transfection) 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 RNAi Delivery Controls (Label IT®, Mirus) in HEPES buffer (0.2 mL) at pH 7.4 as previously reported (31).

ζ-Potential Measurements

The ζ-potential measurements for the lipoplexes formed at an efficient charge ratio of transfection, after mixing with a vortex mixer, were determined using a Delsa™Nano Zeta Potential (Beckman Coulter, Buckinghamshire, UK) as previously reported (31).

Cell Culture and Transfection Experiments

Two cell lines were used in the transfection experiments: human primary skin fibroblast cells FEK4 (33,34) derived from a foreskin explant and human cervix carcinoma, HeLa derivative and transformed cell line (HtTA) (35,36). The HtTA cells being stably transfected with a tetracycline-controlled transactivator (tTA) consisting of the tet repressor fused with the activating domain of virion protein 16 of the herpes simplex virus (HSV). Cells were cultured as previously reported (31).

DNA Transfection Experiments in the Absence or 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 Earle's Minimal Essential Medium (EMEM, 2 mL) containing foetal calf serum (FCS) for 24 h to reach a plate confluency of 50–60% on the day of transfection. Then the media were

replaced by 0.4 mL Opti-MEM (serum free media, Gibco BRL) 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 Opti-MEM (in the absence 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.

For transfection in the presence of serum, as in the above procedure, but on the day of transfection, the media were replaced by fresh EMEM (0.4 mL) containing FCS. The lipoplex was prepared as above and then incubated with the cells 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 enhanced green fluorescent protein (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) as previously reported (31).

siRNA 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 non-targeting siRNA1 (50 ng) (Dharmacon, Thermo Fisher Scientific Biosciences) or complexed with lipopolyamines at different ratios in TE buffer (50 μL, 10 mM Tris–HCl, 1 mM EDTA, pH 7.5, in DEPC-treated water) using FLUOstar Optima Microplate Reader (BMG-LABTECH), λ_{ex}=480 nm, λ_{em}=520 nm as previously reported (31,37).

siRNA Delivery Experiments

For the siRNA delivery, we use fluorescein-labelled RNAi Delivery Controls (Label IT®, Mirus). FEK4 and HtTA cells were seeded at 50,000 cells/well in 12-well plates in EMEM (2 mL) containing FCS 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-labelled siRNA in the transfected cells were detected as previously reported (31,37).

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^1,N^{12}-dioleoyl spermine complexed with pEGFP or siRNA, were added in the same way as in the transfection protocol. After incubation for 44 h, the media were replaced with fresh media (90 μL) and sterile filtered MTT solution (10 μL, 5 mg/mL) to reach a final concentration of 0.5 mg/mL (29). Then the plates were incubated for a further 4 h at 37°C in an atmosphere of 5% *v*/*v* CO_2. The percent viability was detected as previously reported (31,37).

Confocal Microscopy Visualization

Following the siRNA delivery 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

Spermine was used as the starting material for the synthesis of the seven desired lipopolyamines: N^4,N^9-dioleoyl spermine **1**, N^4,N^9-dimyristoleoyl spermine **2**, N^1,N^{12}-dioleoyl spermine **3**, N^1,N^{12}-distearoyl spermine **4**, N^1,N^{12}-dimyristoleoyl spermine **5**, N^1,N^{12}-dimyristoyl spermine **6**, and N^1,N^{12}-didecanoyl spermine **7** (Fig. 2).

The tetra-amine was chemo-selectively protected on both the primary amino functional groups with ethyl trifluoroacetate (2.2 eq.) in methanol (38,39). Fatty acyl chloride (oleoyl or myristoleoyl) (2.2 eq.) and triethylamine (2.5 eq.) were then added to the diprotected spermine solution in CH_2Cl_2 and methanol (1:1, *v*/*v*). Deprotection by treatment with potassium carbonate in aq. methanol (40,41) and flash column chromatography afforded the two target N^4,N^9-lipospermines, 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*) (4–6). Alternatively, *t*-BOC protection of the two secondary amines was achieved with BOC-ON, which yielded the diamide-dicarbamate (39), and potassium carbonate catalyzed deprotection of the primary amines afforded N^4,N^9-di-*t*-BOC spermine (39,42). Acylation of the two primary amines in pyridine, followed by *t*-BOC deprotection using TFA in CH_2Cl_2 (1:9 *v*/*v*) afforded the five target N^1,N^{12}-lipospermines as their di-trifluoroacetate salts. The structures of these novel target N^1,N^{12}-lipospermines were established by ^{1}H- (at 400 MHz) and ^{13}C- (at 100.8 MHz) NMR spectroscopy and confirmed by HRMS. The characteristic ^{1}H NMR signals included δ_H 0.8–0.9 methyl protons, 1.2–1.3 many methylene

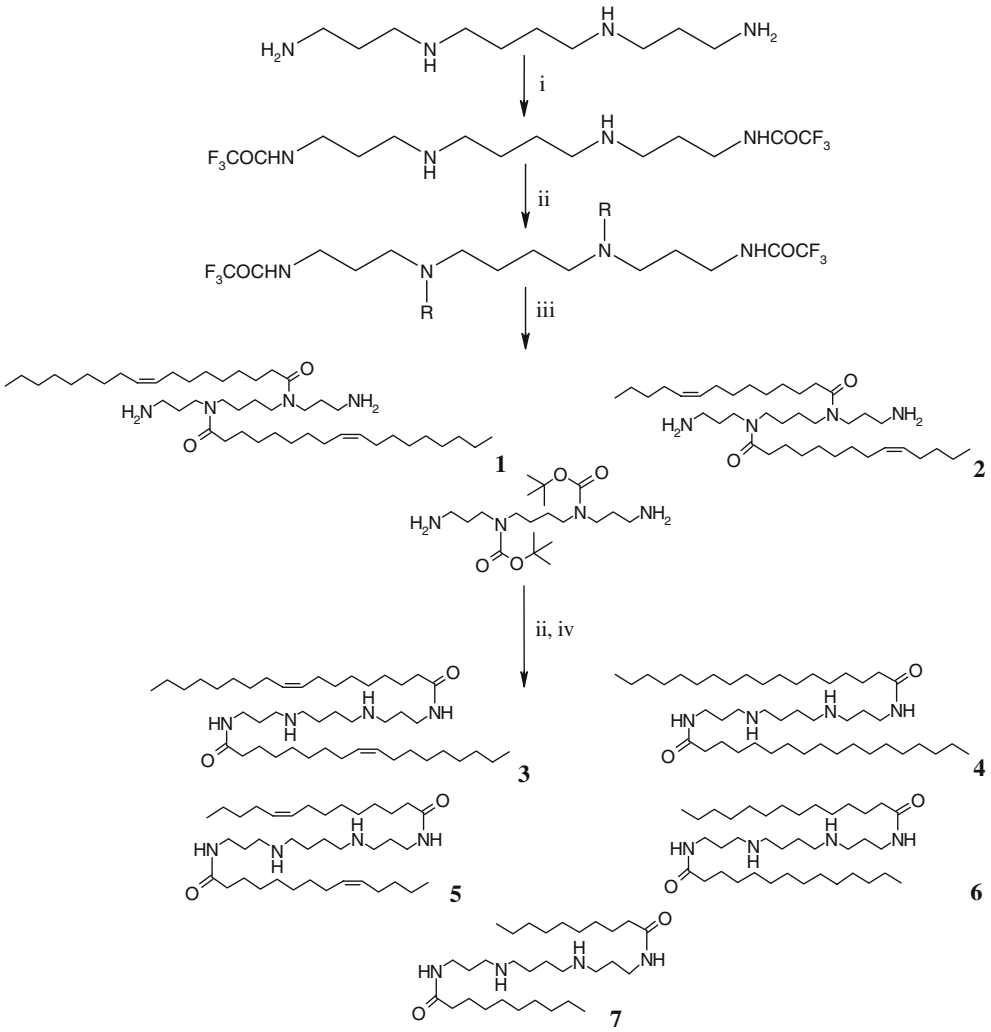

Fig. 2. Synthetic scheme for spermine-based cationic lipids. Reagents: *i* ethyl trifluoroacetate, 24 h at 20°C; *ii* fatty acyl chloride, fatty acid (with DCC and cat. HOBt) or BOC-ON (R=oleoyl, myristoleoyl, or *t*-BOC), pyridine, 16 h at 20°C; *iii* potassium carbonate in aq. methanol, reflux (5 h); *iv* TFA:CH_2Cl_2 (1:9 *v/v*), 2 h at 20°C.

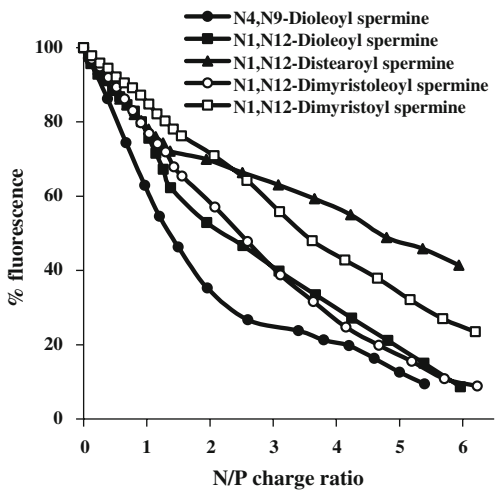

Fig. 3. Plot of EthBr fluorescence quenching assay of pEGFP complexed with different lipospermines.

chain protons, and 5.3–5.4 ppm for the alkene protons (where required). Relative to spermine (free base in $CDCl_3$) where δ_C C3 47.6 and C5 49.8 (Δ2.2 ppm), whose chemical shifts are predicted to decrease by 2 ppm on being α- to a protonated ammonium ion due to a slight decrease in deshielding of the carbon atoms in the vicinity of the ammonium ion, C3 was found to resonate typically at 43.2–45.2 and C5 at 46.6–47.3 (Δ2.1–3.4 ppm) where C3 was predicted (in $CDCl_3$) to be at 45.6 and C5 47.7 (Δ2.1 ppm) (43). Other spermine resonances

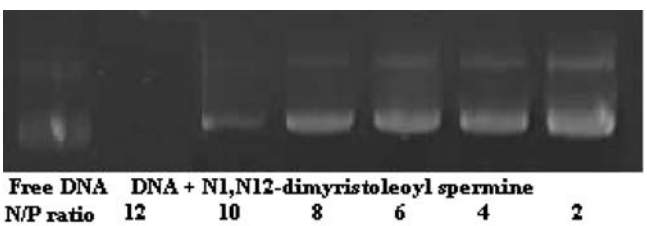

Fig. 4. Agarose gel (1%) assay of fluorescent EthBr intercalated in pEGFP, lipoplex formation with N^1,N^{12}-dimyristoyl spermine **6** at *N*/*P* ratios 2–12 progressively retards gel permeation.

Table I. Particle Size (mean±SD, n=9) pEGFP and siRNA Complexes with Selected Lipopolyamines

Lipospermine	DNA-lipoplex N/P charge ratio	DNA-lipoplex diameter (nm)	siRNA-lipoplex diameter (nm)
N^4,N^9-dioleoyl spermine **1**	2.5	150 (12)	110 (12)
N^1,N^{12}-dioleoyl spermine **3**	3	170 (30)	150 (28)
N^1,N^{12}-distearoyl spermine **4**	30	210 (35)	
N^1,N^{12}-dimyristoleoyl spermine **5**	12	250 (49)	170 (34)
N^1,N^{12}-dimyristoyl spermine **6**	12	190 (39)	

are found as expected: C1 36–38, C2 24–26, C6 23–24 ppm. The characteristic ^{13}C NMR signals also included 13.2–14.1 methyls, 21.9–36.8 ppm methylenes (a long alkyl chain typically starts 14.1, 22.8, 32.1, 29.5), 129.0–130.0 *cis*-alkenes C9′ and C10′ (where required), and 172.0–180.0 ppm the amide carbonyl groups.

DNA Condensation

In Fig. 3, we show the DNA condensation ability of the synthesized lipopolyamines in an EthBr fluorescence quenching assay. The results show that N^1,N^{12}-dioleoyl spermine **3** and N^1,N^{12}-dimyristoleoyl spermine **5** have the best DNA condensing ability, more than 90% EthBr fluorescence quenching at N/P charge ratio 6, where DNA is defined as condensed (44), and N^1,N^{12}-dimyristoyl spermine **6** shows 75% fluorescence quenching, but N^1,N^{12}-distearoyl spermine **4** only achieves 55% at the same N/P ratio. Typical gel electrophoresis data (Fig. 4) show that N^1,N^{12}-dimyristoyl spermine **6** was able to condense pEGFP DNA progressively, as a result of neutralization of DNA phosphate negative charges on titration with the two ammonium positive charges on the lipopolyamine, to its optimised N/P charge ratio of transfection (N/P=12) at which it inhibited the electrophoretic mobility of plasmid DNA.

Lipoplex Particle Size and ζ-Potential

The particle size measurements were carried out on the lipoplexes at a concentration which gave a high transfection efficiency. Particle size characterization by laser diffraction showed that the nanoscale particle size of the formed DNA complexes ranged from 170 nm (N^1,N^{12}-dioleoyl spermine **3**) to 250 nm (N^1,N^{12}-dimyristoleoyl spermine **5**), compared with N^4,N^9-dioleoyl spermine **1** pEGFP lipoplex nanoparticles of 150 nm (Table I). Two siRNA lipoplexes were measured: 150 nm (N^1,N^{12}-dioleoyl spermine **3**) and 170 nm (N^1,N^{12}-dimyristoleoyl spermine **5**), compared with N^4,N^9-dioleoyl spermine **1** siRNA lipoplex nanoparticles of 110 nm (Table I). Two representative ζ-potential measurements for the lipoplexes formed (at their optimum charge ratio of transfection with 3 μg/mL pEGFP) were measured with a Delsa™Nano Zeta Potential instrument showing N^4,N^9-dioleoyl spermine **1** +2.17 mV (at N/P=2.5) and N^1,N^{12}-dioleoyl spermine **3** +3.69 mV (at N/P=3).

pEGFP Transfection and *In Vitro* Cytotoxicity

In preliminary experiments, we readily established that saturated lipid chains were more toxic than unsaturated ones, and that shorter chain analogues were more toxic than longer ones. We therefore did not make any further detailed studies of N^1,N^{12}-didecanoyl spermine **7**. The transduction of EGFP into a primary skin cell line FEK4 and a cancer cell line (HeLa-derived HtTA) was investigated in presence and absence of serum (Fig. 5), and we found that there was no significant difference in the transfection levels in the presence of serum for N^4,N^9-dioleoyl spermine **1** (Lipogen) and N^1,N^{12}-dioleoyl spermine **3**. Encouraged by these positive results, all our subsequent transfection 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

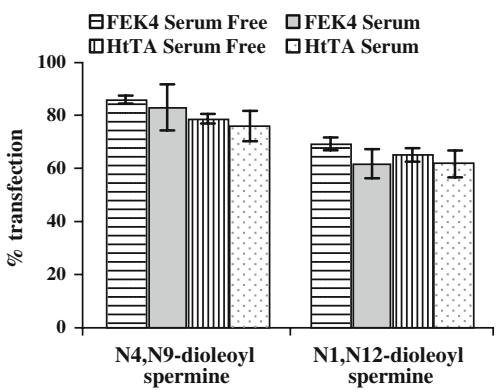

Fig. 5. Lipofection effects of pEGFP (1 μg) complexed with Lipogen (N^4,N^9-dioleoyl spermine **1** (2.77 μg) and N^1,N^{12}-dioleoyl spermine **3** (3.32 μg) on the primary skin cell line FEK4 and the HeLa-derived cancer cell line HtTA in the absence and presence of serum.

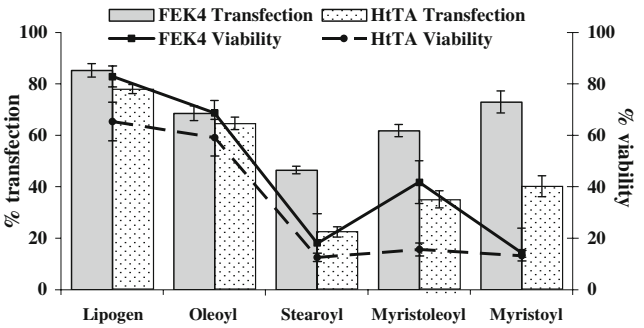

Fig. 6. Lipofection and cytotoxicity effects of pEGFP (1 μg) complexed with Lipogen (N^4,N^9-dioleoyl spermine **1**) (2.77 μg), Oleoyl (N^1,N^{12}-dioleoyl spermine **3**) (3.32 μg), Stearoyl (N^1,N^{12}-distearoyl spermine **4**) (33.36 μg), Myristoleoyl (N^1,N^{12}-dimyristoleoyl spermine **5**) (11.24 μg) and Myristoyl (N^1,N^{12}-dimyristoyl spermine **6**) (11.31 μg) on the primary skin cell line FEK4 and the HeLa-derived cancer cell line HtTA.

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. Therefore, the optimum amounts (and corresponding *N*/*P* charge ratios) for transfection with pEGFP (1 μg in 50 μL) were found to be N^4,N^9-dioleoyl spermine **1** (Lipogen, 2.77 μg, *N*/*P*=2.5), N^1,N^{12}-dioleoyl spermine **3** (3.32 μg, *N*/*P*=3), N^1,N^{12}-distearoyl spermine **4** (33.36 μg, *N*/*P*=30.0), N^1,N^{12}-dimyristoleoyl spermine **5** (11.24 μg, *N*/*P*=12.0), and N^1,N^{12}-dimyristoyl spermine **6** (11.31 μg *N*/*P*=12.0). The results (Fig. 6, histograms) indicate that N^1,N^{12}-dioleoyl spermine **3**, which typically shows 70% and 65% transfection ability, and 70% and 60% cell viability for FEK4 and HtTA cells, respectively (Fig. 6, lines), is the best of all the tested N^1,N^{12}-diacyl spermine vectors and can be compared with its N^4,N^9-regioisomer (Lipogen **1**). N^1,N^{12}-Distearoyl spermine **4**, the saturated analogue of N^1,N^{12}-dioleoyl spermine **3**, whilst maintaining the same (C-18) chain length, was not a good transfecting agent and was far too toxic. Shortening the acyl chains to C14 gave mixed pEGFP transfection results with certainly no improvement over C-18, and the toxicity remained high, with the saturated analogue N^1,N^{12}-dimyristoyl spermine **6** more toxic than the unsaturated N^1,N^{12}-dimyristoleoyl spermine **5**.

siRNA Delivery and *In Vitro* Cytotoxicity

The RiboGreen intercalation assay was performed on two selected non-viral delivery vectors from the N^1,N^{12}-diacyl spermine series and compared with the corresponding N^4,N^9-regioisomers. These vectors were both from the unsaturated series as they showed the highest cell viability in the pEGFP transfection assays. The siRNA binding results from the RiboGreen intercalation assay show that efficient fluorescence quenching occurs by *N*/*P* charge ratio 4 for all four lipopolyamines (Fig. 7). However, the N^4,N^9-regioisomer pattern is preferred, as N^4,N^9-dioleoyl spermine (Lipogen) **1** and N^4,N^9-dimyristoleoyl spermine **2** achieved 80% fluorescence quenching by *N*/*P*=2.5.

The transduction of fluorescein-tagged siRNA into a primary skin cell line FEK4 and a cancer cell line (HeLa-derived HtTA) was investigated in the presence of serum (RNase). We aimed for cellular delivery efficiency about 80% (or

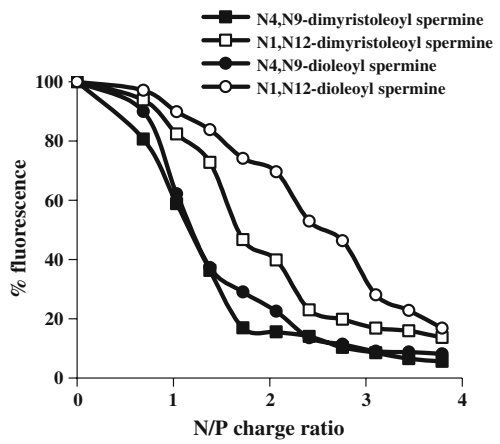

Fig. 7. Plot of RiboGreen intercalation assay of siRNA complexed with different lipospermines.

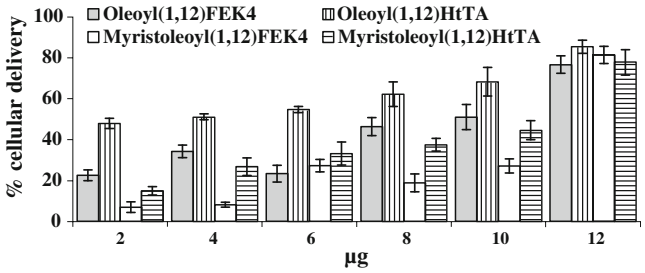

Fig. 8. Cellular delivery to the primary skin cell line FEK4 and the cancer cell line HtTA of fluorescein-tagged siRNA (12.5 pmol) complexed with N^1,N^{12}-dioleoyl spermine **3** and N^1,N^{12}-dimyristoleoyl spermine **5** at increasing concentrations.

higher), but also with a view to minimal toxicity (high cell viability), minimum effective concentrations. These optimum delivery concentrations were experimentally determined by using ascending amounts of lipopolyamines (Fig. 8). Flow cytometric FACS analysis of both cell lines, gated for live cells (Fig. 9), after 48 h transduction with fluorescein-tagged siRNA complexed with N^1,N^{12}-dioleoyl spermine **3**, clearly shows high siRNA delivery efficiency. The siFection of FEK4 primary cells (hard to transfect) and a cancer cell line (HtTA) were compared with a market leader TransIT-TKO (Mirus). The siRNA delivery results (Fig. 10, histograms) show that N^4,N^9-dioleoyl spermine **1** (Lipogen, 80% FEK4 and 87% HtTA), N^1,N^{12}-dioleoyl spermine **3** (77% FEK4 and 85% HtTA), N^4,N^9-dimyristoleoyl spermine **2** (84% FEK4 and 91% HtTA) and N^1,N^{12}-dimyristoleoyl spermine **5** (81% FEK4 and 78% HtTA) are

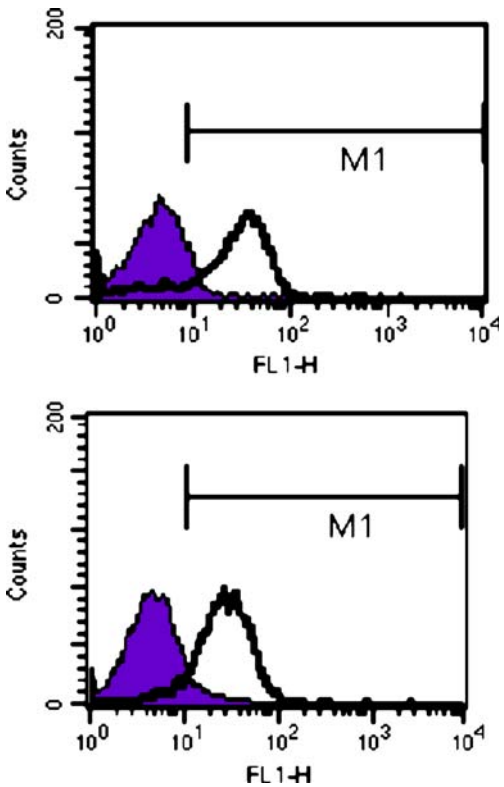

Fig. 9. Gated FACS analysis of FEK4 (*above*) and HtTA (*below*) after 48 h transfection of fluorescein-tagged siRNA complexed with N^1,N^{12}-dioleoyl spermine **3** (12 μg): *shaded in violet* untransduced cells, *shaded in white* fluorescein-positive cells.

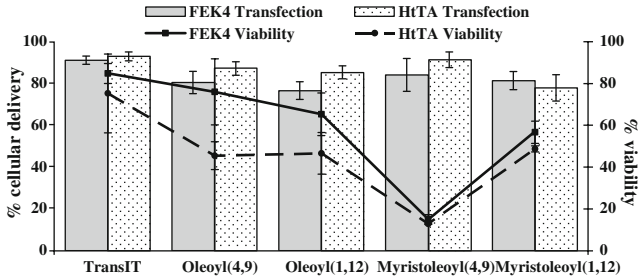

Fig. 10. Cellular delivery and cytotoxicity effects of siRNA (12.5 pmol) complexed with TransIT-TKO (4 μL), Oleoyl (4,9) (Lipogen, N^4,N^9-dioleoyl spermine **1**) (8.0 μg), Oleoyl (1,12) (N^1, N^{12}-dioleoyl spermine **3**) (12.0 μg), Myristoleoyl (4,9) (N^4,N^9-dimyristoleoyl spermine **2**) (10.0 μg) and Myristoleoyl (1,12) (N^1, N^{12}-dimyristoleoyl spermine **5**) (12.0 μg) on the primary skin cell line FEK4 and the HeLa-derived cancer cell line HtTA.

just lower in efficiency for both cell lines than TransIT-TKO (91% FEK4 and 93% HtTA). The cell viability (MTT assay) results (Fig. 10, lines) show that N^4,N^9-dioleoyl spermine **1** (Lipogen) and N^1,N^{12}-dioleoyl spermine **3** have better viability of FEK4 cells (76% and 65% respectively) over HtTA cells (46% and 47% respectively); next is N^1,N^{12}-dimyristoleoyl spermine **5**. Whilst N^4,N^9-dimyristoleoyl spermine **2** achieves high siFection results, it is too toxic. However, N^1,N^{12}-dioleoyl spermine **3** and N^1,N^{12}-dimyristoleoyl spermine **5** are efficient new non-viral siRNA delivery vectors (Figs. 8, 9, 10 and 11), displaying satisfactory cell viability.

In this study, we have investigated changes to the position, length and degree of unsaturation of the symmetrical diacyl fatty chain formulations of lipospermine on pDNA and siRNA binding and cellular delivery. The lipid moiety in our cationic lipids interacts with the phospholipid bilayer of the cell membrane, and that facilitates cell entry, either in crossing the membrane bilayer and/or in helping to weaken the endosomal bilayer and thereby aiding escape into the cytosol (Fig. 1). DNA must now traffic to the nucleus and cross the nuclear membrane, which is thought to occur through the nuclear pore complex (NPC) or by direct association with the chromatin during mitosis. After nuclear entry, the payload DNA should successfully be able to give the desired protein through transcription and translation (Fig. 1). As siRNA has its site of action in the cytosol, escape from the endosome is therefore a key parameter, without the need to traffic to the nucleus.

The position, length and degree of unsaturation play an important role in their design and formulation where the lipid moiety must be considered in shape (volume) and substituent pattern, as well as the polyamine moiety and its pK_a values (4–6,45–47). The first key step in this gene formulation is DNA condensation into nanoparticles by masking the negative charges of the phosphate backbone (44). 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 pEGFP condensation results (Fig. 3), investigated using the EthBr fluorescence quenching assay, revealed that of our synthetic lipopolyamines N^1,N^{12}-dioleoyl spermine **3** and N^1, N^{12}-dimyristoleoyl spermine **5** were able to condense pDNA to less than 10% EthBr fluorescence at *N*/*P* charge ratio 6, while N^1,N^{12}-dimyristoyl spermine **6** and N^1,N^{12}-distearoyl spermine **4** show only 75% and 55% condensation, respectively, at that *N*/*P* ratio. The lipid chains make a significant contribution to the pDNA condensation; the differences are clear (Fig. 3) with respect to position and unsaturation, where pDNA condensation efficiency follows: saturated—1,12<unsaturated—1,12<unsaturated—4,9 regioisomers. Thus, N^4,N^9-dioleoyl spermine **1** (Lipogen) is the best pDNA condensing agent at *N*/*P* charge ratios 4 and 5. The RiboGreen siRNA intercalation assay results showed efficient fluorescence quenching for the four chosen lipopolyamines (Fig. 7), the N^4,N^9-regioisomeric pattern being preferred, achieving 80% fluorescence quenching by *N*/*P*=2.5, which only occurred at *N*/*P*=4 for the N^1,N^{12}-analogues. Particle size of the final pDNA formulation is also an important factor in improving gene delivery (48,49). Particle size results (Table I) for pDNA nanoparticle lipoplexes were around 205 nm, and for two siRNA lipoplexes were 150 and 170 nm. On the relationship between particle size and transfection efficiency, there are no definite limits to the nanoparticle size that is suitable for transfection (50). Nanoparticles have relatively higher intracellular uptake than microparticles (22). Also, on the nanoscale, smaller-size

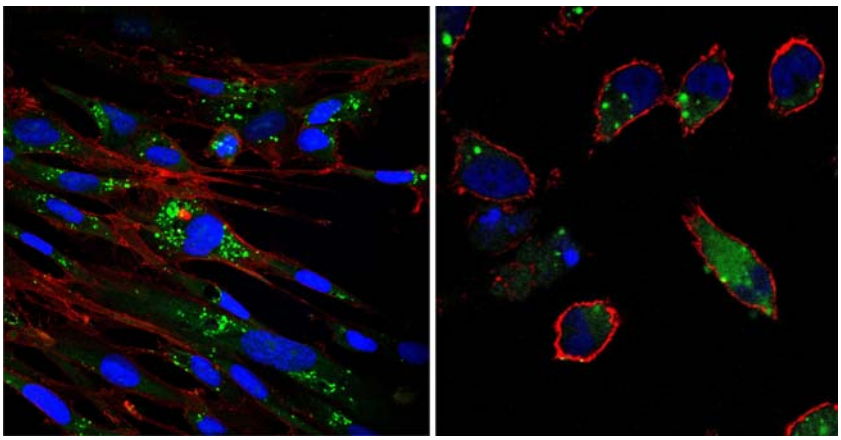

Fig. 11. The FEK4 primary cell line (*left*) and HtTA cell line (*right*) showing delivery of a fluorescein-siRNA tag inside the cells, delivered with N^1,N^{12}-dioleoyl spermine **3**. The tag fluoresces green, the nuclei fluoresce blue from the Hoechst 33342, and the cell lipid bilayers fluoresce red from the Alexa Fluor 594 (LSM510META, under the ×60 oil immersion objective).

polyplexes are more able to enter cells and thereby increase the efficiency of transfection (51).

From the combination of both the transfection and viability results (Fig. 6), we found that N^1,N^{12}-dioleoyl spermine **3** (transfection efficiency FEK4 70%, HtTA 65% and cell viability FEK4 70%, HtTA 60%) was a better vector than the other three N^1,N^{12}-analogues based on the two factors of transfection efficiency and cell viability. It was superior to the saturated analogue N^1,N^{12}-distearoyl spermine **4** and the shorter analogues, unsaturated N^1,N^{12}-dimyristoleoyl spermine **5** and saturated N^1,N^{12}-dimyristoyl spermine **6** and comparable to its N^4,N^9-regiosisomer N^4,N^9-dioleoyl spermine **1** (Lipogen), which displayed transfection efficiency FEK4 85%, HtTA 78% and cell viability FEK4 83%, HtTA 65%. Based on these DNA transfection and cell viability results, we investigated the change in the position and length of the symmetrical diacyl fatty chain formulation of lipospermine on siRNA binding (Fig. 7) and cellular delivery (Figs. 8, 9, 10 and 11). Following from a similar combination of both the siRNA delivery and the cell viability results, we found that the two dioleoyl lipospermine regioisomers **1** and **3** are of comparable activity, only slightly below that achieved with TransIT and more efficient than the two dimyristoleoyl regioisomeric analogues **2** and **5** (Fig. 10).

Whilst in the earlier part of these studies, we delivered pDNA and measured transfection by assaying the produced EGFP, in the latter part we have presented preliminary evidence for efficient siRNA delivery using a fluorescently tagged probe, a ds RNA 21-mer designed not to interfere with normal cell protein biosynthesis (31). Barriers to efficient siRNA delivery include excretion through lysosomes, serum nuclease (RNase) digestion, non-specific distribution (*in vivo*), and tissue barriers that must be crossed, e.g. into the cell and out of the endosome. To support further our gated FACS evidence (Fig. 9) that there is siRNA delivery to the cells, we have carried out confocal laser scanning microscopy studies on both cell lines (Fig. 11). Using 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. 11) successful delivery of the (green) fluorescent tag to both FEK4 and HtTA cell-lines with N^1,N^{12}-dioleoyl spermine **3**. We have also previously reported a successful protein (β-actin) knockdown experiment in HEK 293 cells (30% as assayed by Western blot protein analysis) where the siRNA was delivered with N^4,N^9-dioleoyl spermine **1** (Lipogen) (52).

The DNA compacting properties of polyamines (especially spermine) are well-known, hence the use of spermine as the cationic part in several synthetic DNA carriers. Clement and his research group recently reported the use lipophosphoramidate as the lipidic part of lipospermines for gene delivery (53). Due to their high net charge at physiological pH, polyamines effectively charge neutralize the phosphodiester backbone of DNA (54). Many pK_a measurements have been made on spermidine, spermine, and their derivatives (55,56), which all indicate their high number of positive charges at physiological pH. Spermidine typically shows pK_a values of 11.1, 10.0 and 8.6 or 10.9, 9.8 and 8.4 (57) and spermine pK_1 10.9, pK_2 10.1, pK_3 8.9 and pK_4 8.1 (43) and 10.9, 10.0, 8.8 and 8.4 (58). However, in preparing the N^4,N^9-regioisomeric analogues, we have separated the two primary amines from the (significant base-weakening) transient charges on the secondary amines, and the primary amines may therefore be assumed to have pK_a values typical of primary amines, 10.6–10.9, i.e. N^4,N^9-diacyl analogues will carry two positive charges at physiological pH (7.4). Moving the diacyl chains to the N^1,N^{12}-position means that these analogues will still carry two positive charges at pH=7.4, but their pK_a values are likely to follow those of putrescine (1,4-diaminobutane) (10.8 and 9.4 or 10.7 and 9.2) (59,60), and we assume that there will not be any significant effect from the γ-amide functional groups. Thus, these novel conjugates are dibasic for *N*/*P* charge ratio calculations, and their efficient charge neutralisation by phosphate anion titration will be a function of steric more than electronic effects when compared with the 12 atoms (10 C and 2 N) separating the two positive charges in the N^4,N^9-series. We conclude from the above results that N^1,N^{12}-dioleoyl spermine **3** is the best of N^1,N^{12}-substituted series for both DNA and siRNA delivery. It is comparable with its commercially available N^4,N^9-regioisomer N^4,N^9-dioleoyl spermine **1** (Lipogen) for DNA transfection efficiency where Lipogen **1** achieves typically 81% transfection and N^1,N^{12}-dioleoyl spermine **3** achieves 67% (both with good cell viability), and for siRNA delivery, typically 83% with Lipogen **1** and 81% with our novel analogue **3** (with similar cell viability).

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