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Research Paper

Design and Synthesis of N^4,N^9-Disubstituted Spermines for Non-viral siRNA Delivery – Structure-Activity Relationship Studies of siFection Efficiency *Versus* Toxicity

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Purpose. To study the effect of sequentially changing the chain length, oxidation level, and charge distribution in N^4,N^9-diacyl and N^4,N^9-dialkyl spermines on siRNA formulation, and then to compare their lipoplex transfection efficiency in cell lines.
Methods. Eight N^4,N^9-diacyl polyamines: N^4,N^9-[didecanoyl, dilauroyl, dimyristoyl, dimyristoleoyl, dipalmitoyl, distearoyl, dioleoyl and diretinoyl]-1,12-diamino-4,9-diazadodecane were synthesized. Their abilities to bind to siRNA and form nanoparticles were studied using a RiboGreen intercalation assay and particle sizing. Two diamides were also reduced to afford tetraamines N^4,N^9-distearyl- and N^4,N^9-dioleyl-1,12-diamino-4,9-diazadodecane. Delivery of fluorescein-labelled Label IT® RNAi Delivery Control was studied in FEK4 primary skin cells and in an immortalized cancer cell line (HtTA), and compared with TransIT-TKO.
Results. The design, synthesis, and structure-activity relationship studies of a series of N^4,N^9-disubstituted spermines as efficient vectors for non-viral siRNA delivery to primary skin and cancer cell lines is reported. These non-liposomal cationic lipids are promising siRNA carriers based on the naturally occurring polyamine spermine showing that C-18 is a better chain length as shorter chains are more toxic.
Conclusions. N^4,N^9-Distearoyl spermine and N^4,N^9-dioleoyl spermine are efficient siRNA formulation and delivery vectors, even in the presence of serum, comparable to TransIT-TKO. However, four positive charges distributed as in spermine was significantly more toxic.

KEY WORDS: lipopolyamines; N^4,N^9-dioleoyl spermine; NVGT; primary skin cells; siRNA delivery.

INTRODUCTION

The use of an efficient vector for poly-nucleic acid (gene or siRNA) delivery (1) is one of the determining factors for a successful therapy for difficult-to-treat diseases such as cancer (2,3), cystic fibrosis (4,5), and Duchenne muscular dystrophy (6). Among non-viral gene delivery systems, non-liposomal cationic lipids are promising, non-toxic gene carriers. The biological usefulness of spermine (1,12-diamino-4,9-diazadodecane) (7–10) and its conjugates, and the synthesis of the lipopolyamine dioctadecylamidoglycylspermine (DOGS, Transfectam), by Behr *et al.* (11) as an efficient non-viral transfection agent, encouraged us to focus on the synthesis of novel cationic lipids based on the naturally occurring polyamine spermine (12–15). In an effort to improve the efficiency and control of siRNA delivery, novel spermine based cationic lipid formulations have been investigated within our approach to molecular pharmaceutics for molecular medicine.

Methods to deliver poly-nucleic acids fall into two classes. The first employs genetically altered viruses that, in most cases, have had their genome altered or "gutted" to prevent viral replication, reduce cytotoxicity, and permit incorporation of the therapeutic transgene. These vectors can be extremely efficient at producing expression, with essentially only a single viral particle necessary to induce a measurable effect. Problems of low virus titre, an inability to transfect non-dividing cells, induction of strong immune responses, and significant toxicity must still be overcome (16). The second class of delivery systems is collectively referred to as non-viral and involves the use complexes with synthetic carrier molecules e.g. cationic lipids or polymers. Such non-viral delivery systems can potentially have considerable advantages over their viral counterparts e.g. greater control of their molecular composition for simplified manufacturing and analysis (17,18), and relatively lower immunogenicity (5,19–21), but these non viral systems are still significantly less efficient than viral systems.

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ABBREVIATIONS: EMEM, Earle's Minimal Essential Medium; FCS, foetal calf serum; HRMS, high-resolution mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

RNA interference (RNAi) is a naturally occurring phenomenon by which short RNA duplexes, short interfering RNA (siRNA), can reduce gene expression through enzymatic cleavage of a target mRNA mediated by the RNA induced silencing complex (RISC) (22–24). The essentially specific ability of synthetic siRNA to inhibit targeted genes, makes it an extremely powerful tool for functional genomics, it has attracted considerable interest recently (25). RNAi is commonly achieved by introducing chemically synthesized siRNA 19–22-mers into cells by transfection. However, many cells and cell lines are either refractory to or adversely affected by transfection, and the transient nature of this methodology means that more efficient (and less toxic) siRNA delivery vectors are required. The effect of the degree of saturation of cationic lipid analogues was investigated to identify the fusogenic and transfection ability of these compounds by Heyes *et al.* (26). The saturated cationic lipid (1,2-distearyloxy-*N*,*N*-dimethyl-3-aminopropane (DSDMA)) was more readily internalized to the cells, despite it having almost no ability of gene silencing (siRNA delivery). Whilst gene silencing increased with the degree of unsaturation, as a result of the increase in the fusogenic ability, lower transition temperature from L_α (lamellar organization) to H_{II} (two-dimensional hexagonal state), of the cationic lipid (26). In this study, we synthesized novel lipospermines in which the tetraamine spermine (the cationic moiety) and fatty chains (the lipophilic moiety) that have been reported to improve transfection efficiency by fusion with cellular membranes, are covalently bound together. We examine non-viral siRNA delivery efficiency and cell viability with the self-assembled nanoparticles of siRNA (lipoplexes) formed by these new cationic lipids.

MATERIALS AND METHODS

Materials

Chemicals, including spermine, acyl chlorides, carboxylic 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

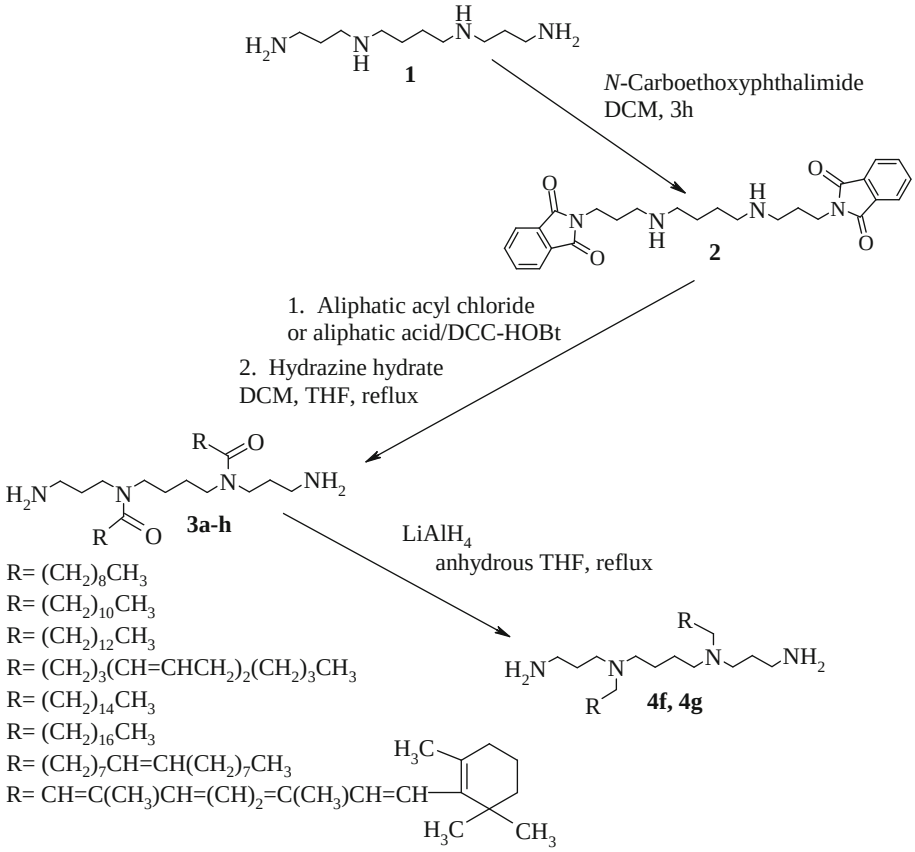
THF used in anhydrous conditions was distilled from sodium benzophenone ketyl. Glassware used in anhydrous conditions was baked for 1 h at 80°C, assembled hot and filled with anhydrous nitrogen before use. Ninhydrin used for detecting (poly)amines was prepared by dissolving 0.3 g ninhydrin in 100 ml 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). All the synthesized lipopolyamines were homogenous on silica gel TLC (CH_2Cl_2–MeOH-conc. aq. NH_3 25:10:1 *v*/*v*/*v*) and were characterized by nuclear magnetic resonance (NMR) spectroscopy, recorded in $CDCl_3$ unless otherwise noted using a Varian Mercury 400 (operating at 400 MHz for ^1H 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 ^1H. 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 and other NMR assignments follow from correlation spectroscopies; COSY, DEPT, HMQC, HMBC spectra were all recorded using automated programmes. 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. All lipopolyamines showed satisfactory HRMS data (reported in Da and within 5 ppm).

General Procedure for the Synthesis of N^4,N^9-Diacyl Spermines (4,9-Diacyl-1,12-diamino-4,9-diazadodecane 3a–h)

To a solution of 1,12-diamino-4,9-diazadodecane (spermine) **1** (e.g. 2.02 g, 10 mmol) in CH_2Cl_2 (30 ml), *N*-carboethoxyphthalimide (4.38 g, 20 mmol) was added. The reaction mixture was stirred at 20°C for 3 h then evaporated to dryness in vacuo and the residue was purified over silica gel (CH_2Cl_2:MeOH 10:1 then 2:1 *v*/*v*) to afford 1,12-diphthalimido-4,9-diazadodecane **2** (27) as a white solid m.p. 110–112°C, δ ^1H 1.39–1.43 (4H, m, H6, H7), 1.81 (4H, quintet, *J*=7, H2, H11), 2.52 (4H, t, *J*=7, H5, H8), 2.57 (4H, t, *J*=7, H3, H10), 3.57 (4H, t, *J*=7, H1, H12), 7.63–7.67 (4H, m, aromatic protons), 7.75–7.95 (4H, m, aromatic protons); δ ^{13}C 27.6, 28.7 (C2 and C11, C6 and C7), 35.7 (C1 and C12), 46.7 (C3 and C10), 49.5 (C5 and C8), 123.0 (aromatic C), 133.7 (aromatic C), 168.3 (4× C=O) ppm, MS *m*/*z* found 463.2319 (100%, $M+1)^+$, $C_{26}H_{31}N_4O_4$ requires $(M+1)^+$ 463.2340 (Δ 4.5 ppm). To a solution of 1,12-diphthalimido-4,9-diazadodecane **2** (2.31 g, 5 mmol) in CH_2Cl_2 (30 ml) and TEA (1.4 ml, 10 mmol), the appropriate acyl chloride (10 mmol) was added then the solution was heated under reflux for 18 h, cooled to 20°C, and evaporated to dryness in vacuo. The residue was dissolved in a mixture of CH_2Cl_2 and THF (30 ml, 1:1, *v*/*v*), treated with hydrazine hydrate (2 ml), and then heated under reflux for 4 h. After cooling to 20°C, the precipitate was removed by filtration, the filtrate was evaporated to dryness in vacuo, and the residue was purified by column chromatography over silica gel (CH_2Cl_2:MeOH 10:1 *v*/*v* then CH_2Cl_2:MeOH:NH_4OH 10:5:1 *v*/*v*/*v*) to afford the target compounds (Fig. 1):

N^4,N^9-didecanoyl spermine **3a** (90%) NMR δ ^1H 0.81 (6H, t, *J*=7, 2× CH_3 H10'), 1.21–1.32 (24H, m, 12× CH_2 H4'–H9'), 1.41–1.88 (12H, m, 6× CH_2 H2, H6, H3'), 2.09–2.41 (4H, m, H2'), 2.60–2.81 (8H, m, H1, NH_2), 3.11–3.51 (8H, m, H3, H5) ppm, δ ^{13}C 14.0 (C10'), 22.6–29.4 (C2, C6, C3'–C7', C9'), 31.8, 33.0, 39.1 (C8', C2', C1), 45.1, 47.3 (C3, C5), 172.9 (CON C1') ppm, HRMS *m*/*z*, found $[M+H]^+$511.4929, $C_{30}H_{63}N_4O_2$ requires 511.4951 (Δ ppm 4.3);

N^4,N^9-dilauroyl spermine **3b** (87%) NMR δ ^1H 0.81 (6H, t, *J*=7, 2× CH_3 H12'), 1.20–1.32 (32H, m, 16× CH_2 H4'–H11'), 1.53–1.77 (12H, m, H2, H6, H3'), 2.09–2.31 (4H, m,



Vector number	Acyl residue (R)	Chain name	C-chain number	Unsaturation level
3a	$(CH_2)_8CH_3$	Decanoyl	10	0
3b	$(CH_2)_{10}CH_3$	Lauroyl	12	0
3c	$(CH_2)_{12}CH_3$	Myristoyl	14	0
3d	$(CH_2)_3(CH{=}CHCH_2)_2(CH_2)_3CH_3$	Myristoleoyl	14	1, $\Delta^{5,8}$
3e	$(CH_2)_{14}CH_3$	Palmitoyl	16	0
3f	$(CH_2)_{16}CH_3$	Stearoyl	18	0
3g	$(CH_2)_7CH{=}CH(CH_2)_7CH_3$	Oleoyl	18	1, Δ^{9}
3h	$C_{19}H_{27}$	Retinoyl	20	5, Δ^{13}

Fig. 1. Syntheses of symmetrical N^4,N^9-diacylated **3** and N^4,N^9-dialkylated **4f** and **4g** spermines.

H2'), 2.60–2.76 (8H, m, H1, NH_2), 3.16–3.38 (8H, m, H3, H5) ppm, δ ^{13}C 14.0 (C12'), 22.6–29.6 (C2, C6, C3'–C9', C11'), 31.8, 36.7, 37.5 (C8' C2', C1), 46.7, 48.9 (C3, C5), 173.7 (CON C1') ppm, HRMS *m*/*z*, found $[M+H]^+$ 567.5561, $C_{34}H_{71}N_4O_2$ requires 567.5577 (Δ ppm 2.8);
N^4,N^9-dimyristoyl spermine **3c** (85%) NMR δ ^{1}H 0.85 (6H, t, *J*=7, 2× CH_3 H14'), 1.23–1.32 (40H, m, 20× CH_2 H4'–H13'), 1.58–1.68 (12H, m, H2, H6, H3'), 2.10–2.28 (4H, m, H2'), 2.60–2.74 (8H, m, H1, NH_2), 3.19–3.41 (8H, m, H3, H5) ppm, δ ^{13}C 14.1 (C14'), 22.6–29.6 (C2, C6, C3'–C11', C13'), 31.8, 36.8, 39.1 (C8', C2', C1), 46.5, 49.2 (C3, C5), 173.4 (CON C1') ppm, HRMS *m*/*z*, found $[M+H]^+$ 623.6170, $C_{38}H_{79}N_4O_2$ requires 623.6203 (Δ ppm 5.3);
N^4,N^9-dimyristoleoyl spermine **3d** (85%) NMR ($^2H_6$-DMSO) δ ^{1}H 0.86 (t, 6H, 2× CH_3, H14', *J*=8), 1.22–2.81 (br m, 52H, H1, H2, H6, H7, H11, H12, H2'–H8', H11'–H13'), 3.20–3.50 (br m, 8H, H3, H8, H10, H5), 5.31 (m, 4H, H9', H10'), 8.39 (br, s, 2× NH_2) ppm, δ ^{13}C ($^2H_6$-DMSO) 13.9 (C14'), 22.3–37.7 (C1, C2, C6, C7, C11, C12, C2'–C8', C11'–C13'), 41.9–47.4 (C3, C5, C8, C10), 129.5 (2× CH=CH, C9', C10'), 174.5 (CON, C1') ppm, HRMS *m*/*z*, found $[M+H]^+$ 619.5872, $C_{38}H_{75}N_4O_2$ requires 619.5890 (Δ ppm 2.9);
N^4,N^9-dipalmitoyl spermine **3e** (28) (80%) NMR δ ^{1}H 0.85 (6H, t, *J*=7, 2× CH_3 H16'), 1.22–1.25 (48H, m, 24× CH_2 H4'–H15'), 1.49-1.69 (12H, m, H2, H6, H3'), 2.10–2.30 (4H, m, H2'), 2.61–2.73 (8H, m, H1, NH_2), 3.22–

3.40 (8H, m, H3, H5) ppm, δ ^{13}C 14.1 (C16'), 22.6–29.6 (C2, C6, C3'–C13', C15'), 29.7, 31.8, 33.0 (C8', C2', C1), 46.5, 49.2 (C3, C5), 173.4 (CON C1') ppm, HRMS *m*/*z*, found $[M+H]^+$ 679.6796, $C_{42}H_{87}N_4O_2$ requires 679.6829 (Δ ppm 4.9);
N^4,N^9-distearoyl spermine **3f** (70%) NMR δ ^{1}H 0.91 (6H, t, *J*=7, 2× CH_3 H18'), 1.25–1.50 (56H, br m, 28× CH_2 H4'–H17'), 1.51–1.75 (12H, m, H2, H6, H3'), 2.12–2.29 (4H, m, H2'), 2.61–2.73 (8H, m, H1, NH_2), 3.24–3.48 (8H, m, H3, H5) ppm, δ ^{13}C 14.1 (C18'), 22.6–29.6 (C2, C6, C3'–C15', C17'), 31.9, 33.1, 39.1 (C8', C2', C1), 45.2, 47.3 (C3, C5), 173.0 (CON C1') ppm, HRMS *m*/*z*, found $[M+H]^+$ 735.7438, $C_{46}H_{95}N_4O_2$ requires 735.7455 (Δ ppm 2.3);
N^4,N^9-dioleoyl spermine **3g** (70%) NMR δ ^{1}H 0.91 (6H, t, *J*=7, 2× CH_3 H18'), 1.17–1.97 (52H, br m, 26× CH_2 H2, H6, H3'–H7', H12'–H17'), 2.03 (24H, app q, 4× allylic CH_2 H8', H11'), 2.57–2.68 (4H, m, H2'), 3.12–3.24 (12H, m, H1, H3, H5), 4.55–5.10 (br s, 2× NH_2), 5.32–5.41 (4H, m, 2× CH=CH H9', H10') ppm, δ ^{13}C 13.9 (C18'), 22.4–36.5 (C1, C2, C6, C2'–C8', C11'–C17'), 45.2 (C3), 47.2 (C5), 129.7 (CH = CH C9', C10'), 172.7 (CON C1') ppm, HRMS *m*/*z*, found $[M+H]^+$ 731.7142, $C_{46}H_{91}N_4O_2$ requires 731.7137 (Δ ppm 3.7);

To a solution of 1,12-diphthalimido-4,9-diazadodecane **2** (460 mg, 1 mmol) in CH_2Cl_2 (30 ml), DCC (4.10 g, 2 mmol), cat. HOBt (27 mg, 0.2 mmol) and then all *trans*-retinoic acid (150 mg, 0.5 mmol) were added and the solution was stirred at 20°C for 20 h, filtered to remove the urea and then the filtrate was evaporated to dryness in vacuo. The residue was deprotected (as above) with hydrazine hydrate (2 ml), and final column chromatographic purification (as above) gave:

all *trans*-N^4,N^9-diretinoyl spermine **3h** (60%) NMR δ ^{1}H 0.99 (12H, br s, H15''(CH_3)), 1.23–2.23 (42H, br m, overlapping, H2, H6, H3''(CH_3), H7'' (CH_3), H11''(CH_3), H12'', H13'', H14'', NH_2), 3.35–3.65 (12H, m, H1, H3, H5), 6.06–6.77 (12H, m, H2'', H4'', H5'', H6'', H8'', H9'') ppm, δ ^{13}C 12.7 (C3'' (CH_3), C7'' (CH_3)), 19.1–39.5 (C1, C2, C3, C5, C6, C11''(CH_3), C12'', C13'', C14'', C15'', C15''(CH_3)), 123.2–137.7 (C2''–C11''), 157.5 (CON C1'') ppm, HRMS *m*/*z*, found $[M+H]^+$ 767.6203, $C_{50}H_{79}N_4O_2$ requires 767.6198 (Δ ppm 0.7).

Synthesis of N^4,N^9-Dialkylated-1,12-diamino-4,9-diazadodecane (4f and 4g)

To a solution of **3f** or **3g** (1 mmol) in anhydrous THF (40 ml), an excess of lithium aluminium hydride (0.11 g, 3 mmol) was added and heated under reflux for 24 h then cooled to 20°C, cautiously quenched with wet EtOH (dropwise), then aq. 10% KOH (20 ml, caution!) was gradually added and the mixture was heated under reflux for another 30 min. The reaction mixture was cooled to 20°C, filtered and the filtrate was evaporated to dryness in vacuo. The residue was extracted with CH_2Cl_2 (2×20 ml) and the combined organic extracts were evaporated to dryness in vacuo. The residue was purified over silica gel (CH_2Cl_2:MeOH 10:1 *v*/*v* then CH_2Cl_2:MeOH:NH_4OH 10:5:1 *v*/*v*/*v*) to afford the target tetraamines (Fig. 1):

N^4,N^9-distearyl spermine **4f** (60%) NMR δ ^{1}H 0.86 (6H, t, *J*=7, 2× CH_3 H18'), 1.22–1.38 (56H, m, H4'–H17'), 1.40–1.68 (12H, m, H2, H6, H3'), 2.14–2.43 (4H, m, H2'), 2.53–2.69 (8H, m, H1, NH_2), 3.22–3.51 (12H, m, H1', H3, H5) ppm, δ ^{13}C 14.1 (C18'), 22.6–29.6 (C2, C6, C2'–C15', C17'), 29.6, 31.9 (C8', C1), 50.0, 51.9 (C3, C5), 54.1 (C1') ppm, HRMS *m*/*z*, found $[M+H]^+$ 707 7880, $C_{46}H_{99}N_4$ requires 707.7864 (Δ ppm 2.3);
N^4,N^9-dioleyl spermine **4g** (29) (50%) NMR δ ^{1}H 0.83 (6H, t, *J*=7, 2× CH_3 H18'), 1.21–1.99 (52H, m, H2, H6, H3'–H7', H12'–H17'), 2.15–2.73 (12H, m, H2', H8', H11'), 3.12–3.56 (12H, m, H1, H3, H5, H1'), 5.3 (4H, m, H9', H10') ppm, δ ^{13}C 14.0 (C18'), 22.6–33.6 (C1, C2, C6, C2'–C8', C11'–C17'), 45.2, 45.3 (C3, C5), 52.4 (C1'), 129.8 (CH = CH C9', C10') ppm, HRMS *m*/*z*, found $[M+H]^+$ 703.7511, $C_{46}H_{95}N_4$ requires 703.7484 (Δ ppm 3.8).

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$_{naked\ siRNA}$.

Lipoplex Particle Size

The particle size for the lipoplexes formed at an efficient 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 with 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 (NTA) software.

Cell Culture and siRNA Delivery in the Presence of Serum

Two cell lines were used in the transfection experiments, a human primary skin fibroblast cells FEK4 (30,31) derived from a foreskin explant, and a human cervix carcinoma HeLa derivative and transformed cell line (HtTA) (32,33). 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 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%).

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 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 (in the presence of serum). Then the media were replaced by 2 ml of fresh media and cultured for 44 h in full growth media at 37°C in 5% CO_2 before the assay.

Levels of fluorescein-labelled siRNA 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 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-distearoyl spermine **3f**, N^4,N^9-distearyl spermine **4f** complexed with 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 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (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 (DMSO). 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 (34,35).

Confocal Microscopy Visualization

Following the 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, Purification, and Analysis of Lipospermines

The practical synthetic process we have developed is outlined in Fig. 1. For the synthesis of N^4,N^9-diacylated spermines **3a–h**, firstly *N*-carboethoxyphthalimide (3 h at 20°C) was used to protect both the primary amino groups of the naturally occurring spermine (1,12-diamino-4,9-diazadodecane) **1** as 1,12-diphthalimido-4,9-diazadodecane **2** (27), then 2 equivalents of the acid chloride in the presence of triethylamine and the solution heated under reflux for 18 h, or 2 equivalents of DCC and catalytic HOBt and then the aliphatic all *trans*-retinoic acid were stirred in solution at 20°C for 20 h. After the acylation reaction, the phthalimide protecting groups were removed by heating under reflux for 4 h with hydrazine monohydrate in a mixture of DCM and THF, filtered and finally purified by column chromatography over silica gel (CH_2Cl_2:MeOH 10:1 *v*/*v* then CH_2Cl_2:MeOH:NH_4OH 10:5:1 *v*/*v*/*v*) to afford the target compounds. The yield of the target lipopolyamine conjugates, as their free bases, was typically 60%. To search for an increase in the efficiency of transfection by increasing the number of positive charges and therefore the siRNA binding affinity and/or the endosomal buffering capacity, N^4,N^9-dialkylated spermines distearyl **4f** and dioleyl **4g** were synthesized by reduction of the corresponding diacyl compounds **3f** and **3g** using lithium aluminium hydride. All the synthesized lipopolyamines were homogenous on silica gel thin-layer chromatography (CH_2Cl_2–MeOH-conc. aq. NH_3 25:10:1 *v*/*v*/*v*) and were characterized by ^1H and ^{13}C NMR nuclear magnetic resonance (NMR) spectroscopy, assignments follow from correlation spectroscopies.

RNA Binding (RiboGreen intercalation assay)

In Fig. 2, we show a measure of the siRNA binding ability of the synthesized N^4,N^9-diacyl lipopolyamines in a RiboGreen intercalation assay which is similar to the PicoGreen assay reported by Wagner *et al.* (36) and to our optimized ethidium bromide fluorescence quenching (37). These results from forming lipoplexes with siRNA show that all the synthesized lipospermines achieve more than 80% fluorescence quenching at N/P charge ratios below 3, except N^4,N^9-didecanoyl spermine which also achieved 85% fluorescence quenching at N/P=4.

Lipoplex Particle Size Measurements

The particle size characterization measurements were carried out on the lipoplexes at their optimum concentration

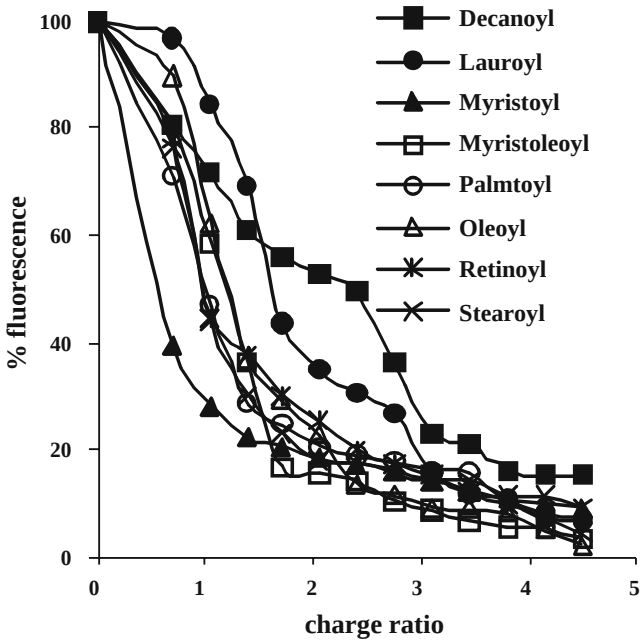


Fig. 2. Plot of RiboGreen intercalation assay of siRNA complexed with different lipospermines.

for transfection (38). Particle size characterization by laser diffraction showed that the nanoscale particle size of the formed siRNA complexes ranged from 90 nm (N^4,N^9-dimyristoyl spermine **3c** and N^4,N^9-dimyristoleoyl spermine **3d**) both with C-14 chains to 170 nm (N^4,N^9-didecanoyl spermine **3a**) with C10 chain length. These lipoplex nanoparticles were ranged around 140 nm in diameter (Table I) which is typical for such formulations e.g. Schiffelers *et al.*, made polyplexes of PEI–PEG with siRNA of 90–120 nm, (39) and Ishida *et al.* prepared cationic lipoplexes typically of 280–308 nm (40). In gene therapy, the particle size of the final pDNA formulation is also an important factor in improving gene delivery (41,42). On the relationship between particle size and transfection efficiency, there are no definite limits to the nanoparticle sizes that are suitable for transfection (43). Nanoparticles have relatively higher intracellular uptake than microparticles (44). Also, on the nanoscale, smaller-size polyplexes are more able to enter cells and thereby increase the efficiency of transfection (45).

Table I. Particle size (mean±S.D., n=9) of siRNA lipoplexes

Lipospermine	siRNA-Lipoplex diameter (nm)
N^4,N^9-didecanoyl spermine **3a**	170 (29)
N^4,N^9-dilauroyl spermine **3b**	150 (21)
N^4,N^9-dimyristoyl spermine **3c**	90 (24)
N^4,N^9-dimyristoleoyl spermine **3d**	90 (14)
N^4,N^9-dioleoyl spermine **3f**	110 (12)

siRNA Delivery and *In Vitro* Cytotoxicity

The transduction of fluorescein-tagged siRNA 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) (46). The practical concentrations (in a final volume of 0.5 ml) were experimentally determined by using ascending amounts of lipospermines until we reached around 80% transfection and there was not a further step-up in transfection efficiency at the next highest concentration. From our typical results, Fig. 3, we conclude that for these lipopolyamines e.g. N^4,N^9-didecanoyl spermine **3a** and N^4,N^9-dimyristoleoyl spermine **3d**, transfection efficiency increases with lipopolyamine ratio. The gated flow cytometric FACS analysis, Fig. 4, of FEK4 and HtTA cell lines after 48 h transfection of fluorescein-tagged siRNA complexed with another representative lipopolyamine, N^4,N^9-diretinoyl spermine **3h**, clearly shows a high percentage of live transduced fluorescein-positive cells. The siFection results (histograms Fig. 5) indicate that the transfection ability of the tested lipospermines except N^4,N^9-dilauroyl spermine **3b** are comparable with the results obtained with a market leader TransIT, a commercially available reagent (92% FEK4 and 93% HtTA). The highest transfection of primary skin cell line FEK4 cells was found with N^4,N^9-

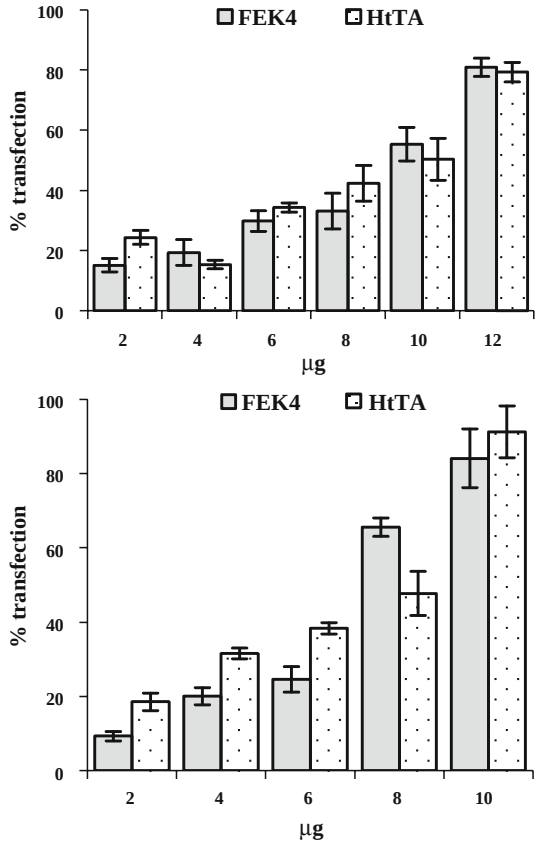


Fig. 3. Lipofection of the primary skin cell line FEK4 and the cancer cell line HtTA transfected with siRNA (12.5 pmol) complexed with increasing concentrations of N^4,N^9-didecanoyl spermine **3a** (*upper*) and N^4,N^9-dimyristoleoyl spermine **3d** (*lower*).

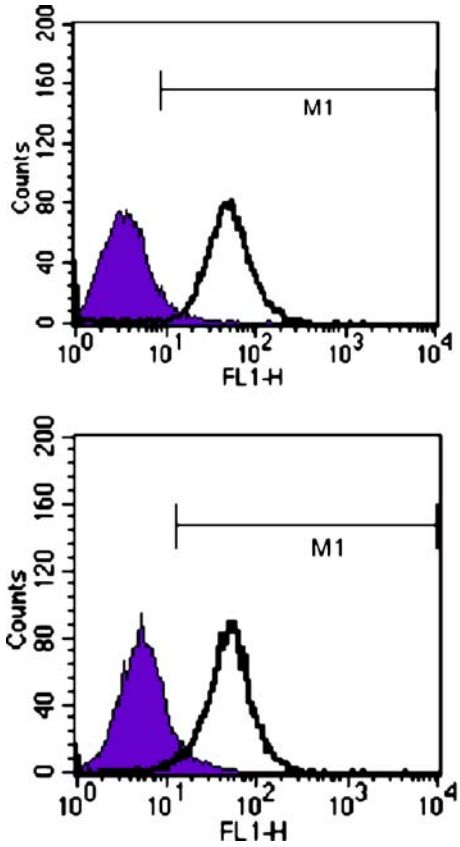


Fig. 4. Gated FACS analysis of FEK4 (*upper*) and HtTA (*lower*) after 48 h transfection of fluorescein-tagged siRNA complexed with N^4,N^9-diretinoyl spermine **3h**: *shaded area* untransduced cells, *unshaded area* fluorescein-positive cells.

dimyristoyl spermine **3c** (90%) and N^4,N^9-dimyristoleoyl spermine **3d** (84%), then N^4,N^9-dipalmitoyl spermine **3e** (85%) then N^4,N^9-dioleoyl spermine **3g** (80%) and the lowest was N^4,N^9-dilauroyl spermine **3b** (57%). The transfection results of siRNA delivery into cancer HtTA cells (Fig. 5) also follows this pattern from highest transfection ability with N^4,N^9-dimyristoyl spermine **3c** (92%) to lowest with N^4,N^9-dilauroyl spermine **3b** (59%).

The cell viability (MTT assay) results (lines Fig. 5) indicate that there is not a large difference in the viability of FEK4 (84%) and HtTA (75%) cells between the commercially available TransIT-TKO and N^4,N^9-distearoyl spermine **3f** and N^4,N^9-dioleoyl spermine **3g** while all the other shorter lipospermines are more toxic to both cell lines. The cytotoxicity of the siRNA lipoplexes is high, with C-14 (saturated or unsaturated) chains being the most toxic, but C-10, C-12, and C-16 conjugates are also toxic. The vitamin A derived all *trans*-N^4,N^9-diretinoyl spermine **3h** was investigated as it has two C-20 chains, and might therefore show a further improvement over di-C-18 (distearoyl **3f** and dioleoyl **3g**) which are themselves less toxic than dipalmitoyl **3e** (C-16). However, diretinoyl spermine **3h** was significantly more toxic to both cell lines than either of the two C-18 compounds, and its all *trans*-shape and structure with its terminal trimethylated cyclohexene ring mean that it is closer in lipid volume to C-12 and C-14 than to C-18.

We considered that by more closely mimicking the positive charge distribution of the naturally occurring tetraamine spermine i.e. mimicking spermine in one of its biologically important roles, that of stabilising condensed DNA wrapped around histones, so with more positive charges we might increase siRNA binding affinity. Or, by increasing the number of basic sites from 2 (diacyl-diamine) to four (tetraamine), we might produce a more efficient proton sponge effect when the early-endosomal pH starts to drop from cytosolic 7.4 towards 5.5 on intracellular acidification, as spermine has 3.8 positive charges at pH=7.4 and can clearly support 4.0 positive charges in acidic milieu. We therefore reduced the two amide functional groups of our two most effective siRNA delivery agents that showed the highest cell viability, to form the analogous tetraamines containing two tertiary amines.

Comparisons between the two C18 acyl lipospermines, the saturated N^4,N^9-distearoyl spermine **3f** and the unsaturated N^4,N^9-dioleoyl spermine **3g** and their alkyl analogues N^4,N^9-distearyl spermine **4f** and N^4,N^9-dioleyl spermine **4g** (Fig. 6) show that there is not a large difference in the transfection efficiency, neither gain nor loss, on doubling the number of positive charges in the conjugate. N^4,N^9-Distearoyl spermine **3f** shows transfection efficiencies of 72% and 71% (FEK4 and HtTA respectively), N^4,N^9-distearyl spermine **4f** 88% and 85%, N^4,N^9-dioleoyl spermine **3g** 80% and 87%, and N^4,N^9-dioleyl spermine **4g** 91% and 87%, but the cell viabilities drop from around 75% to 10–27%. Therefore, the two alkyl analogues **4f** and **4g** are much more toxic (Fig. 6) to both cell lines.

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 (red), and Hoechst 33342 (2 μM) for nuclei labelling

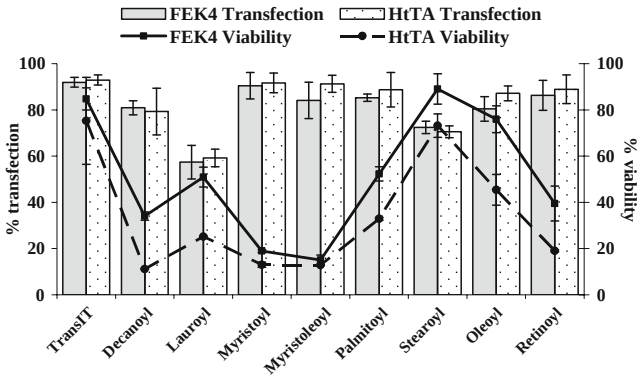


Fig. 5. Lipofection and cytotoxicity effects of siRNA (12.5 pmol) complexed with *TransIT-TKO* (4 μl), *decanoyl* (N^4,N^9-didecanoyl spermine **3a**; 12.0 μg), *lauroyl* (N^4,N^9-dilauroyl spermine **3b**; 4.0 μg), *myristoyl* (N^4,N^9-dimyristoyl spermine **3c**; 12.0 μg), *myristoleoyl* (N^4,N^9-dimyristoleoyl spermine **3d**; 10.0 μg), *palmitoyl* (N^4,N^9-dipalmitoyl spermine **3e**; 12.0 μg), *stearoyl* (N^4,N^9-distearoyl spermine **3f**; 8.0 μg), *oleoyl* (N^4,N^9-dioleoyl spermine **3g**; 8.0 μg), and *retinoyl* (N^4,N^9-diretinoyl spermine **3h**; 12.0 μg) on the primary skin cell line FEK4 and on the HeLa derived cancer cell line HtTA. The data represent three different experiments (three replicates each) and the *error bars* show the S.D.

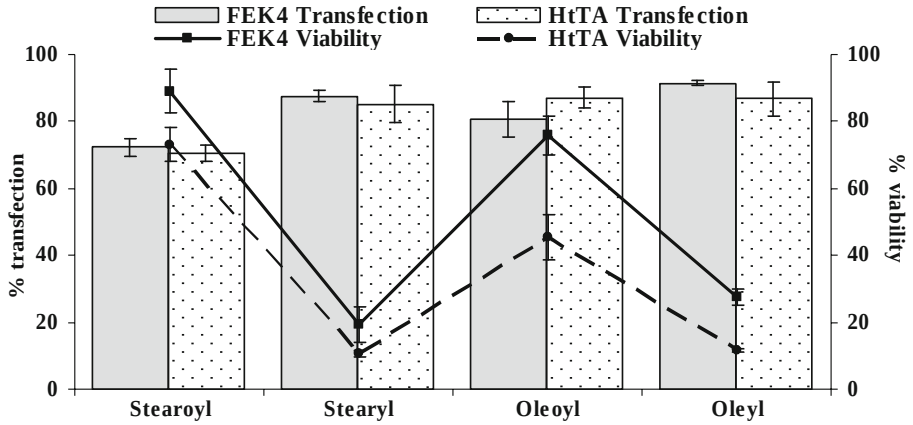


Fig. 6. Lipofection and cytotoxicity effects of siRNA (12.5 pmol) complexed with *stearoyl* (N^4,N^9-distearoyl spermine **3f**; 8.0 μg), *stearyl* (N^4,N^9-distearyl spermine **4f**; 6.0 μg), *oleoyl* (N^4,N^9-dioleoyl spermine **3g**; 8.0 μg), and *oleyl* (N^4,N^9-dioleyl spermine **4g**; 4.0 μg) on the primary skin cell line FEK4 and the HeLa derived cancer cell line HtTA.

(blue), we have shown (Fig. 7) that N^4,N^9-distearoyl spermine **3f** successfully delivered fluorescent-siRNA to the cytosol of FEK4 cells.

These short chain synthetic lipospermines have the ability to deliver siRNA into FEK4 and HtTA cell lines. Whilst increasing the fatty acyl chain length over C-14 does not lead to any further increase in the transfection efficiency (over 92%), it does decrease cytotoxicity (46). The novel N^4, N^9-diretinoyl spermine **3h** lipopolyamine has a similarly long (C-20) carbon chain which does deliver siRNA, but it is too toxic to be considered efficient. Increasing the number of positive charges in the conjugate from 2 to 4 resulted in significantly more toxic compounds. As shorter chain lipopolyamines are too cytotoxic, the most efficient lipopolyamines for siRNA delivery were N^4,N^9-distearoyl spermine **3f** and N^4,N^9-dioleoyl spermine **3g**. These are practical non-liposomal siFection vectors, comparable with TransIT.

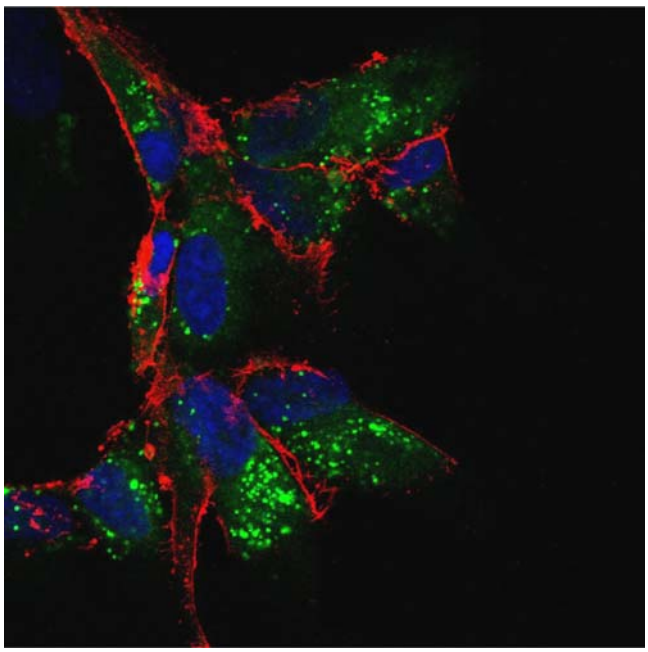


Fig. 7. The FEK4 primary cell line transfected with fluorescein-labelled siRNA showing cytosolic green fluorescence from fluorescein, delivered with N^4,N^9-distearoyl spermine **3f**. The cell lipid bilayers fluoresce red from the Alexa Fluor 594 and the nuclei fluoresce blue from the Hoechst 33342 (LSM510META, Zeiss, Jena, Germany, under the ×60 oil immersion objective).

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