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Asymmetrical N^4,N^9-Diacyl Spermines: SAR Studies of Non-Viral Lipopolyamine Vectors for Efficient siRNA Delivery with Silencing of EGFP Reporter Gene

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Running head: *Asymmetrical Lipopolyamines Deliver siRNA*

ABSTRACT: Our aim is to study the effects of varying the two acyl moieties in synthesized N^4,N^9-diacyl spermines on siRNA formulations and their delivery efficiency in cell lines. Six novel asymmetrical lipopolyamines: [N^4-cholesteryloxy-3-carbonyl-N^9-oleoyl-, N^4-decanoyl-N^9-oleoyl-, N^4-decanoyl-N^9-stearoyl-, N^4-lithocholoyl-N^9-oleoyl-, N^4-myristoleoyl-N^9-myristoyl-, and N^4-oleoyl-N^9-stearoyl]-1,12-diamino-4,9-diazadodecane were assessed for their abilities to bind to siRNA, studied using a RiboGreen intercalation assay, and to form nanoparticles. Their siRNA delivery efficiencies were quantified in FEK4 primary skin cells and in an immortalized cancer cell line (HtTA) using a fluorescein-tagged siRNA, and compared with formulations of N^4,N^9-dioleoyl-1,12-diamino-4,9-diazadodecane and of a leading transfecting agent, TransIT-TKO. Transfection was measured in terms of siRNA delivery and silencing of EGFP reporter gene in HeLa cells. By incorporating two different acyl moieties, changing their length and oxidation level in a controlled manner, we show efficient fluorescein-tagged siRNA formulation, delivery, and knock-down of EGFP reporter gene. N^4-Oleoyl-N^9-stearoyl spermine and N^4-myristoleoyl-N^9-myristoyl spermine are effective siRNA delivery vectors typically resulting in 89% cell delivery and gene silencing to 34% in the presence of serum, comparable with the results obtained with TransIT-TKO; adding a second lipid chain is better than incorporating a steroid moiety.

KEYWORDS: *N^4,N^9-dioleoyl spermine, lipopolyamine, primary skin cells, siRNA delivery, steroids*

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INTRODUCTION

Small (or short) interfering RNA (siRNA) delivery is a branch of polynucleic acid delivery that has many potential clinical applications and progress is being made towards RNA interference (RNAi) realising its potential and becoming an efficient medicine for the treatment of a wide range of diseases.[1,2] Following the discovery of gene silencing in petunia flowers [3,4] and then in animal cells,[5,6] siRNA is making significant progress in clinical trials, however, key obstacles still remain to be overcome.[7-20] siRNA is a 21-24 double stranded nucleic acid with a relatively large molecular weight (~13 kDa) and ~40 negative charges (from the 21 bp phosphates). These physicochemical properties ensure siRNA molecules have poor intracellular uptake and so the major challenge lies in the ability to deliver siRNA efficiently to its site of action, the cytoplasm. Due to the efficiency of RNase, siRNA undergoes fast enzymatic degradation in serum.[21] A wide variety of cationic lipids (lipoplexes) [21-26] and cationic polymers (polyplexes) [27,28] are being investigated as non-viral carriers for siRNA. They are more efficient than administering naked siRNA, but the in vitro/in vivo correlation of cationic lipid formulations is not good due to the various biological barriers and the interaction with serum components.[25] Formulation and stability studies of siRNA are therefore important, but few structure-activity relationship (SAR) studies have been reported. Such SAR studies are urgently required for non-viral vectors in siRNA delivery as there is no immediate correlation between the efficiency of a vector used for pDNA and for siRNA delivery.[1,18]

We are studying how novel lipopolyamines interact with a fluorescein-tagged siRNA in order to produce nanometre-sized particles suitable for transfecting cells with high efficiency and low toxicity. Our non-viral gene therapy (NVGT) delivery system focus is on the design of simple, practical formulations using lipopolyamines composed of two long-carbon chains or one chain and a steroid covalently bound to a polyamine e.g. the tetra-amine spermine (1,12-diamino-4,9-diazadodecane), a natural RNA binding agent.[29-31] The essential requirements for polynucleotide delivery are set out in detail in some of our recent research papers [32-36] Briefly these are: lipoplex mediated transport of siRNA through the cell membrane and thereby delivery to the cytosol. Overcoming the various lipid bilayer barriers requires the lipopolyamine to mask the polyanionic character of the siRNA as a nanoparticle (lipoplex) by electrostatic charge neutralisation, an important first step in NVGT. As the site of action of siRNA is in the cytosol, 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. The rationale for this series of novel asymmetrical cationic lipids is also set out in the previous and following papers.[33,36] We have chosen naturally occurring lipids and covalently bound them in different pairs to the naturally occurring polyamine spermine. Then, without any pre-preparation of liposomes, we titrate a fluorescein-tagged siRNA against the cationic lipids in order to investigate if they are suitable for efficient, non-toxic delivery to target

primary (hard-to-transfect) cells, by forming nanoparticles which will efficiently enter cells in NVGT. Such bio-nanoparticle lipoplex formulations leading to gene knock-down might be efficient in transfection, particularly if they work in the presence of serum nucleases (e.g. RNase) and if they show high cell viability. Such low toxicity might be especially predicted where the (asymmetrical) lipids of the formulation accurately mimic the constituents of human lipid bilayers. Herein we report our investigation on the formulation of siRNA with variation in the two lipid moieties regiospecifically covalently bound to the secondary amines of spermine.

We make sequential changes to chain length from C10 (decanoyl) to C18 (oleoyl), and we incorporate two steroid moieties, cholesteryl from essentially planar AB-cholesterol [37,38] and lithocholoyl [39,40] from the naturally occurring *cis*-AB bile acid steroid lithocholic acid. There is therefore the potential for different interactions with cellular bilayers and, subsequent to absorptive endocytosis, for fusion with and thereby weakening of the endosomal bilayer. The six new synthetic lipopolyamines spontaneously form lipoplexes with siRNA which are assessed by physico- and bio-chemical techniques: target cell transfection efficiency (in the presence of serum thereby demonstrating RNase protection) through both delivery of a fluorescein-tagged siRNA and silencing of EGFP reporter gene stably expressed in HeLa cells, using both primary and cancer cell lines, confocal microscopy, cell viability measured in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay.[41,42] We compare our results with those obtained with the non-liposomal lipopolyamine Lipogen® (N^4,N^9-dioleoyl spermine) [43,44] and also with a leading transfecting agent, TransIT-TKO.

MATERIALS AND METHODS

Materials. Chemicals, 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). HeLa cells stably expressing EGFP were obtained from the Cell Service at Cancer Research UK (CRUK, London Research Institute, Clare Hall Laboratories, South Mimms, London, UK). siRNA for delivery was fluorescein-tagged siRNA Delivery Control (Label IT®, Mirus, Cambridge, UK) and siRNA against EGFP was purchased from Qiagen (Crawley, UK):

Sense strand: 5'-GCAAGCUGACCCUGAAGUUCAUTT-3',

Anti-sense strand: 5'-AUGAACUUCAGGGUCAGCUUGCCG-3',

Target DNA sequence: 5'-CGGCAAGCTGACCCTGAAGTTCAT-3'.

General Details. Glassware, silica gel column and analytical chromatography (typically CH_2Cl_2:MeOH:conc. aq. NH_3; 200:10:1 to 100:10:1 to 50:10:1 v/v/v), and ninhydrin or potassium permanganate used for the detection of polyamines, was as previously reported.[35] All the synthesized lipopolyamines were homogenous on silica gel thin-layer chromatography (TLC) (CH_2Cl_2-MeOH-conc. aq. NH_3 25:10:1, v/v/v) and showed satisfactory ^{1}H nuclear magnetic

resonance (NMR) and ^{13}C NMR spectroscopy as previously reported [35] and low- and high-resolution mass spectrometric (HRMS) Fast Atom Bombardment (FAB) data (positive ion mode, reported as *m/z* and within 5 ppm) using *m*-nitrobenzyl alcohol as the matrix. N^4,N^9-Dioleoyl spermine was prepared as previously reported.[43,44]

N^4-Cholesteryloxy-3-carbonyl-N^9-oleoyl-1,12-diamino-4,9-diazadodecane

MS, FAB found 879.8 (100%, M^++H), $C_{56}H_{102}N_4O_3$ requires (M^+) 878. HRMS, FAB found 879.8022 (M^++H), $C_{56}H_{103}N_4O_3$ requires (M^++H) 879.8025.

N^4-Decanoyl-N^9-oleoyl-1,12-diamino-4,9-diazadodecane

MS, FAB found 621.5 (100%, M^++H), $C_{38}H_{76}N_4O_2$ requires (M^+) 620. HRMS, FAB found 621.6036 (M^++H), $C_{38}H_{77}N_4O_2$ requires (M^++H) 621.6041.

N^4-Decanoyl-N^9-stearoyl-1,12-diamino-4,9-diazadodecane

MS, FAB found 623.4 (100%, M^++H), $C_{38}H_{78}N_4O_2$ requires (M^+) 622. HRMS, FAB found 623.6218 (M^++H), $C_{38}H_{79}N_4O_2$ requires (M^++H) 623.6192.

N^4-3α-Hydroxy-5β-cholan-24-carbonyl-N^9-oleoyl-1,12-diamino-4,9-diazadodecane

MS, FAB found 825.7 (100%, M^++H), $C_{52}H_{96}N_4O_3$ requires (M^+) 824. HRMS, FAB found 825.7545 (M^++H), $C_{52}H_{97}N_4O_3$ requires (M^++H) 825.7555.

N^4-Myristoleoyl-N^9-myristoyl-1,12-diamino-4,9-diazadodecane

MS, FAB found 621.6 (100%, M^++H), $C_{38}H_{76}N_4O_2$ requires (M^+) 620. HRMS, FAB found 621.6062 (M^++H), $C_{38}H_{77}N_4O_2$ requires (M^++H) 621.6041, FAB found 643.5843 (M^++Na), $C_{38}H_{76}N_4O_2Na$ requires (M^++Na) 643.5860.

N^4-Oleoyl-N^9-stearoyl-1,12-diamino-4,9-diazadodecane

MS, FAB found 733.6 (100%, M^++H), $C_{46}H_{92}N_4O_2$ requires (M^+) 732. HRMS, FAB found 733.7283 (M^++H), $C_{46}H_{93}N_4O_2$ requires (M^++H) 733.7293.

Particle Size. The average particle size for each lipoplex formed at a highly efficient *N/P* charge ratio of delivery was determined using a NanoSight LM10 (NanoSight Ltd, Salisbury, UK). All measurements were carried out on lipoplexes prepared from siRNA (25 pmol) in HEPES buffer (0.2 mL) at pH 7.4. Results were analysed with the Nanoparticle Tracking Analysis (NTA) software.

Cell Culture. Three cell lines were used in the transfection experiments, a human primary skin fibroblast cells FEK4 [45,46] derived from a foreskin explant, a human cervix carcinoma, HeLa derivative and transformed cell line (HtTA),[47,48] and HeLa cells stably expressing EGFP.[49,50] 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.[35]

The HeLa cell line stably expressing the red-shifted enhanced variant of wild-type GFP (EGFP) used here as a reporter protein was obtained from the Cell Service at Cancer Research UK (CRUK, London Research Institute, Clare Hall Laboratories, South Mimms, London, UK) and was

constructed by Dr Yilun Liu. Briefly, it was generated by the centrin protein (CEN) being subcloned into the expression vector pEGFP-C1 (Clontech, Cowley, UK), downstream of EGFP, under the control of the human CMV promoter. Stably transfected cells were selected with G418 and maintained as a polyclonal cell line, cultivated as described as described in the following paper.[36]

RNA Condensation (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.[35]

siRNA Transfection Experiments. For siRNA delivery we used fluorescein-tagged siRNA Delivery Control (Label IT®, Mirus). FEK4 and HtTA cells were seeded at 50,000 cells/well in 12-well plates in Eagle's Minimum Essential Medium (EMEM, 2 mL) containing fetal 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 fresh media (437.5 µL). The lipoplexes were 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 mins and then incubated with the cells (final volume of 0.5 mL) for 4 h at 37 °C in 5% CO_2 v/v in full growth medium. Then the media were replaced by fresh media (2 mL) for 44 h at 37 °C in 5% CO_2 v/v before the assay.

For EGFP gene silencing we used HeLa cells stably expressing EGFP, trypsinized at confluency 80-90%, and seeded at a density of 65,000 cells/well in 24-well plates. Cells were incubated for 24 h at 37 °C in 5% CO_2 v/v prior to transfection. The lipoplexes were prepared by mixing the specified amounts of the transfection reagent that resulted in best siRNA delivery in OptiMEM serum-free medium (50 µL), with 15 µL of siRNA (1 µM) in OptiMEM serum-free medium. The solutions were mixed for 2-3 s using a vortex mixer. On the day of transfection, the lipoplex solutions were added to wells containing Dulbecco's Modified Eagle's Medium (DMEM) (10% FCS) such that each well contained 15 pmol siRNA. The plates were then incubated for 48 h at 37 °C in 5% CO_2 v/v before the assay.

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 v/v. Lipoplexes complexed with 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.[43,44] Then the plates were incubated for a further 4 h at 37 °C in an atmosphere of 5% CO_2 v/v. The percent viability was detected as previously reported.[35]

Flow Cytometry (FACS). For analysis of fluorescein-tagged siRNA by flow cytometry, fluorescence activated cell sorting (FACS), cell lines were trypsinized and resuspended in 15%

FCS EMEM. Cells were then centrifuged at 1,200 rpm for 5 min at 20 ^{o}C, washed twice by resuspending in PBS (1 mL/tube) and re-centrifuged at 1,200 rpm for 5 min at 20 ^{o}C. The collected cells were resuspended in PBS (500 μL/tube) and then transferred to a flow cytometer tube (Becton Dickinson, UK). Levels of fluorescein-tagged siRNA in the transfected cells were detected and corrected for background fluorescence of the control cells by FACS (Becton Dickinson FACS Vantage dual Laser Instrument, argon ion laser 488 nm). Typically 10,000 events were recorded. Fluorescein-tagged siRNA delivery efficiency was calculated based on the percentage of the fluorescent cell sorting events in the established range (M1) with correction of the background fluorescence of the control samples, λ_{ex} = 495 and λ_{em} = 518 nm.

For analysis of reduction of expression of EGFP by flow cytometry (FACS), HeLa cells stably expressing EGFP were trypsinized and resuspended in DMEM complete medium without phenol red. Cells were then centrifuged at 1,000 rpm for 5 min, washed twice by resuspending in PBS containing 0.1% BSA, and re-centrifuged at 1,000 rpm for 5 min. The collected cells were resuspended in PBS and then transferred to a flow cytometer tube. Cells (10,000-20,000 events) were analyzed using a FACSCanto flow cytometer (Becton Dickinson, UK), equipped with an argon ion laser at 488 nm for excitation, a Long Pass (LP) filter at 502 nm and a detector at 530 nm (range +/-15 nm) for fluorescence emission, EGFP expression was calculated as:

$$\%\,EGFP = \frac{EGFP\ fluorescence\ of\ transfected\ cells}{EGFP\ fluorescence\ of\ control\ cells} \times 100$$

Confocal Microscopy Cell Imaging. Cells were trypsinized at confluency 80-90% and were seeded at a density of 65,000 cells/well in 24-well plates that have round-glass cover slips (12 mm in diameter) and were incubated for 24 h prior to transfection which was carried out as described above. After that 48 h incubation, the cell culture media in each well were aspirated and the cells washed with PBS (3 x 0.5 mL). The cell membrane was then stained with wheat germ agglutinin (WGA) conjugated to Alexa Fluor® 555. The WGA-Alexa Fluor® 555 working solution was adjusted to a concentration of 5 μg/mL in Hank's balanced salt solution without phenol red. The cells were incubated for 10 min in the dye working solution at 37 °C, 5% CO_2 in the dark. The cells were then washed with PBS (3 x 0.5 mL) and then fixed with 4% paraformaldehyde in PBS for 20 min at 20 °C in the dark. The cover slips were then removed from each well, washed with PBS (2 x 0.5 mL), left to dry briefly in air, and then mounted on glass slides using Mowiol (polyvinyl alcohol) solution as the mounting media and left in the dark at 20 °C (18 h) to allow hardening of the mounting media. The cells were examined using a Carl Zeiss laser scanning microscope LSM 510 meta, with EGFP excitation 488 nm, emission 505-550 nm (band pass filter), Alexa Fluor® 555 excitation 543 nm, emission 560-615 nm (band pass filter).

RESULTS AND DISCUSSION

Synthesis of Lipopolyamines - Asymmetrical N^4,N^9-Disubstituted Spermines.

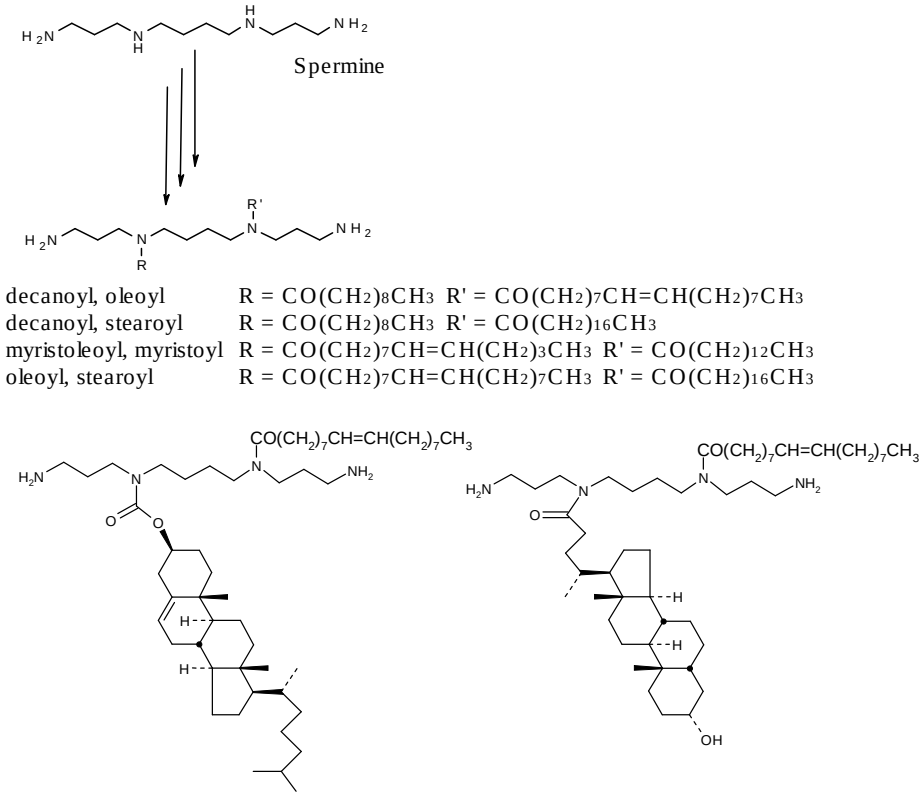

Figure 1. N^4,N^9-Disubstituted spermines asymmetrically acylated with aliphatic chains and steroids.

In nature, RNA polyanionic charges are neutralised by polyammonium ion counterions. The high cellular concentrations of positively charged polyamines (e.g. spermine, spermidine) have higher affinity for RNA than for DNA wound around the basic proteins, histones.[29-31] The rational design and synthesis of six novel asymmetrical N^4,N^9-disubstituted spermines, comprised of different aliphatic chains and steroids, are set out in the preceding paper [35] as is their ready ability to fuse with plasma membranes. They were each prepared via the key intermediate N^1,N^{12}-ditrifluoroacetyl-N^4-*t*-butoxycarbonyl-1,12-diamino-4,9-diazadodecane.[35] The chain lengths were varied from 10 to 18, and the level of unsaturation along the chains was also controlled in our novel siFection vectors, obtained as their free bases, four containing two different long chains: N^4-decanoyl-N^9-oleoyl-1,12-diamino-4,9-diazadodecane, N^4-decanoyl-N^9-stearoyl-1,12-diamino-4,9-diazadodecane, N^4-myristoleoyl-N^9-myristoyl-1,12-diamino-4,9-diazadodecane, and N^4-oleoyl-N^9-stearoyl-1,12-diamino-4,9-diazadodecane, and the two steroid conjugates N^4-cholesteryloxy-3-carbonyl-N^9-oleoyl-1,12-diamino-4,9-diazadodecane and N^4-3α-hydroxy-5β-cholan-24-carbonyl-N^9-oleoyl-1,12-diamino-4,9-diazadodecane (Figure 1). These six target N^4,N^9-asymmetrical diacyl spermines were homogenous on silica gel TLC (CH_2Cl_2-MeOH-conc. aq. NH_3 25:10:1, v/v/v) and

all showed satisfactory HRMS data (within 5 ppm). We have therefore investigated them as potential non-viral vectors in our continuing SAR studies of pDNA versus siRNA delivery in order to investigate novel lipopolyamine molecules that have different lipid moieties on N^4,N^9-positions of the polyamine spermine backbone.[35]

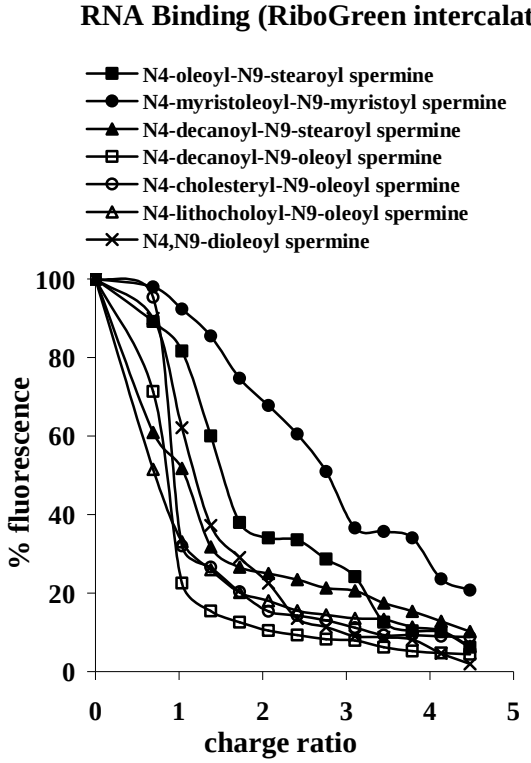

Figure 2. RiboGreen intercalation assay of siRNA complexed with different lipopolyamines.

The RiboGreen siRNA intercalation assay (Figure 2), comparable to measuring DNA condensation in an ethidium bromide fluorescence quenching assay,[51] was carried out on the 6 asymmetrical lipopolyamine vectors and the results compared with N^4,N^9-dioleoyl spermine. There is efficient siRNA binding as assessed by 90% fluorescence quenching at *N/P* charge ratios 3-4 for all the vectors except N^4-myristoleoyl-N^9-myristoyl spermine which shows 80% fluorescence quenching at *N/P* charge ratio 4.5. Therefore, as a result of neutralization of RNA phosphate negative charges by lipopolyamine ammonium positive charges, siRNA is efficiently condensed. *N/P* charge ratios were determined with 2.0 alkylammonium ion (*N*) charges on each cationic lipid and 40 phosphate anion (*P*) charges on ds-RNA of 21 bp where each terminal sugar residue is not phosphorylated in the synthesis. The results from siRNA binding, investigated using a RiboGreen fluorescence quenching assay, revealed that the majority of our synthetic lipopolyamines are able efficiently to condense siRNA to 10% fluorescence at *N/P* charge ratio 4; N^4-myristoleoyl-N^9-myristoyl spermine was able to quench the fluorescence by 80% at *N/P* charge ratio 4.5 (Figure 2).

siRNA Delivery and In Vitro Cytotoxicity.

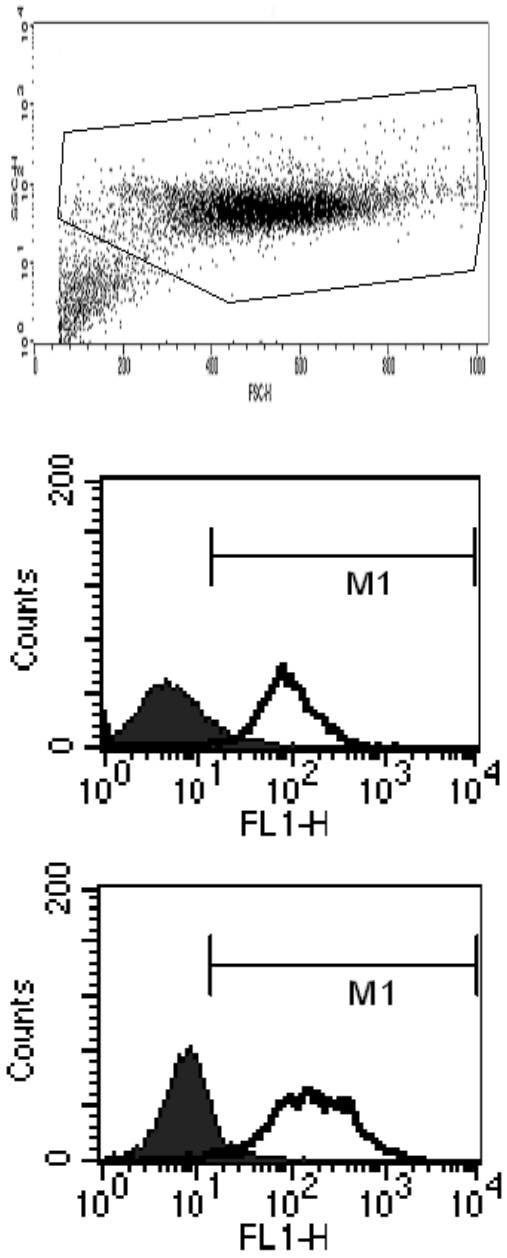

Figure 3. FACS analysis showing the live population gated (above), and of FEK4 (middle) and of HtTA (below) 48 h after delivery of fluorescein-tagged siRNA complexed with N^4-myristoleoyl-N^9-myristoyl spermine: ■ untransduced cells, □ fluorescein-positive cells.

The transduction of fluorescein-tagged siRNA into the cell lines FEK4 and HtTA was investigated and compared with a market leader, TransIT-TKO (Mirus). The practical concentrations (in a final volume of 0.5 mL) were determined experimentally by using ascending amounts of lipopolyamines until ~80% transfection was reached and there was not a further step-up in siRNA delivery efficiency at the next highest concentration. The gated flow cytometric

FACS analysis of FEK4 and HtTA cell lines 48 h after delivery of fluorescein-tagged siRNA clearly shows a high percentage of transduced fluorescein-positive cells (Figure 3). These are typical and representative FACS data, in this example tagged siRNA delivered complexed with the lipopolyamine N^4-myristoleoyl-N^9-myristoyl spermine. The monomodal distribution of cells showing a positive fluorescein signal, counted by FACS analysis, indicates siRNA delivery to ~90% of the gated population of live cells. The FEK4 primary cells show a mean fluorescence intensity (MFI) around 90 compared to the autofluorescence of the control (~4). From the (approaching normal) distribution, the majority of cells efficiently received larger amounts of the fluorescein-tagged siRNA. The MFI in the histogram of the HtTA cells is approximately twice that of the FEK4 primary cells (~200 vs. ~90 arbitrary fluorescence units respectively).

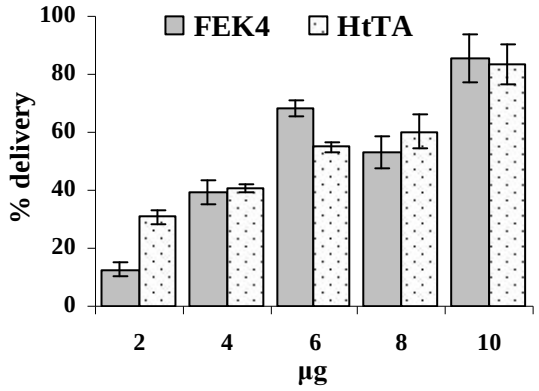

Figure 4. Lipofection of the primary skin cell line FEK4 and the cancer cell line HtTA after delivery of siRNA (12.5 pmol) complexed with N^4-decanoyl-N^9-oleoyl spermine at different ratios.

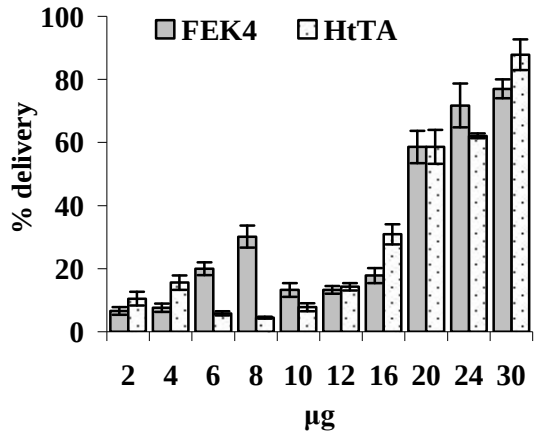

Figure 5. Lipofection of the primary skin cell line FEK4 and the cancer cell line HtTA after delivery of siRNA (12.5 pmol) complexed with N^4-decanoyl-N^9-stearoyl spermine at different ratios.

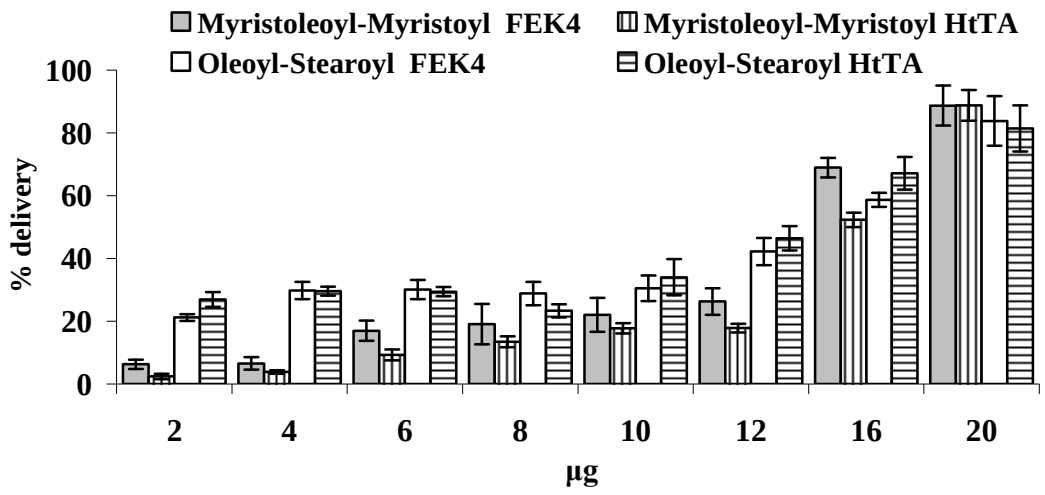

Figure 6. Lipofection of the primary skin cell line FEK4 and the cancer cell line HtTA after delivery of siRNA (12.5 pmol) complexed with N^4-myristoleoyl-N^9-myristoyl spermine and N^4-oleoyl-N^9-stearoyl spermine at different ratios.

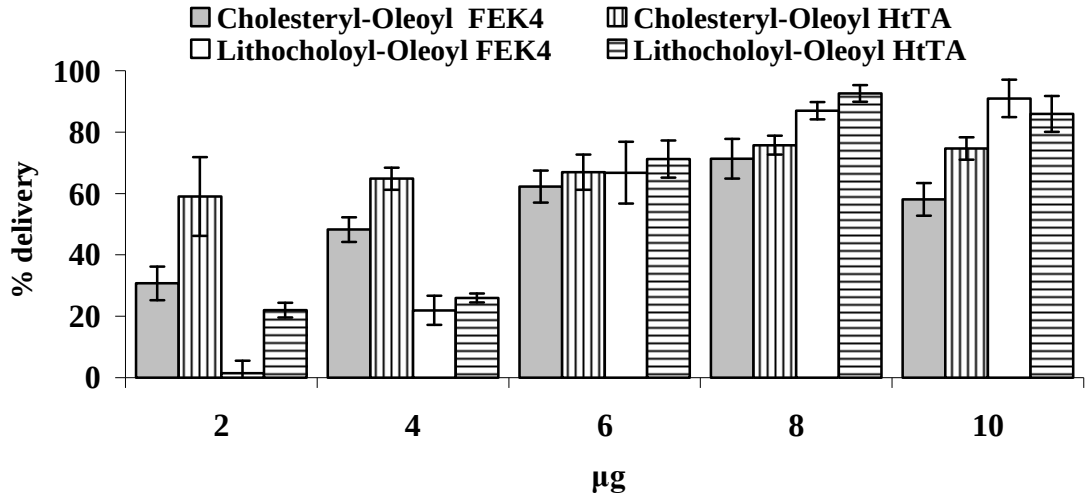

Figure 7. Lipofection of the primary skin cell line FEK4 and the cancer cell line HtTA after delivery of siRNA (12.5 pmol) complexed with N^4-cholesteryloxy-3-carbonyl-N^9-oleoyl spermine and N^4-lithocholoyl-N^9-oleoyl spermine at different ratios.

All our siRNA delivery experiments are performed in the presence of serum. From our typical delivery results with these novel lipopolyamines (Figures 4, 5, 6, and 7) we conclude that delivery increases with lipopolyamine *N/P* charge ratio in both cell lines. High efficiency (71-93%) of siRNA delivery was achieved on optimisation, N^4-oleoyl-N^9-stearoyl spermine, N^4-myristoleoyl-N^9-myristoyl spermine, and N^4-decanoyl-N^9-oleoyl spermine being comparable with N^4,N^9-dioleoyl spermine. Therefore, whilst siRNA delivery is dose dependent, the balance of optimised delivery with toxicity (cell viability) will also have to be considered (Figure 8).

The highest efficiency of siRNA delivery into primary skin cell line FEK4 cells was found with N^4-myristoleoyl-N^9-myristoyl spermine (89%), N^4-lithocholoyl-N^9-oleoyl spermine (87%), N^4-decanoyl-N^9-oleoyl spermine (86%), N^4-oleoyl-N^9-stearoyl spermine (84%), N^4-decanoyl-N^9-stearoyl spermine (77%), and the lowest with N^4-cholesteryloxy-3-carbonyl-N^9-oleoyl spermine (71%). The results of siRNA delivery into cancer HtTA cells follow a similar pattern from highest efficiency with N^4-lithocholoyl-N^9-oleoyl spermine (93%) to lowest with N^4-cholesteryloxy-3-carbonyl-N^9-oleoyl spermine (76%). Incorporating two asymmetrical chains or other lipid moieties, our siRNA delivery results (Figure 8, histograms, carried out in triplicate on 3 separate experiments, n = 9) show that our synthesized asymmetrical lipopolyamines are efficient delivery vectors that work both in primary (hard-to-transfect) cell lines and even in the presence of serum i.e. of hydrolytic RNases. The siRNA delivery efficiencies of the tested asymmetrical lipopolyamines, except N^4-cholesteryloxy-3-carbonyl-N^9-oleoyl spermine, are comparable with the results obtained with a market leader TransIT-TKO, a commercially available reagent (92% FEK4 and 93% HtTA).

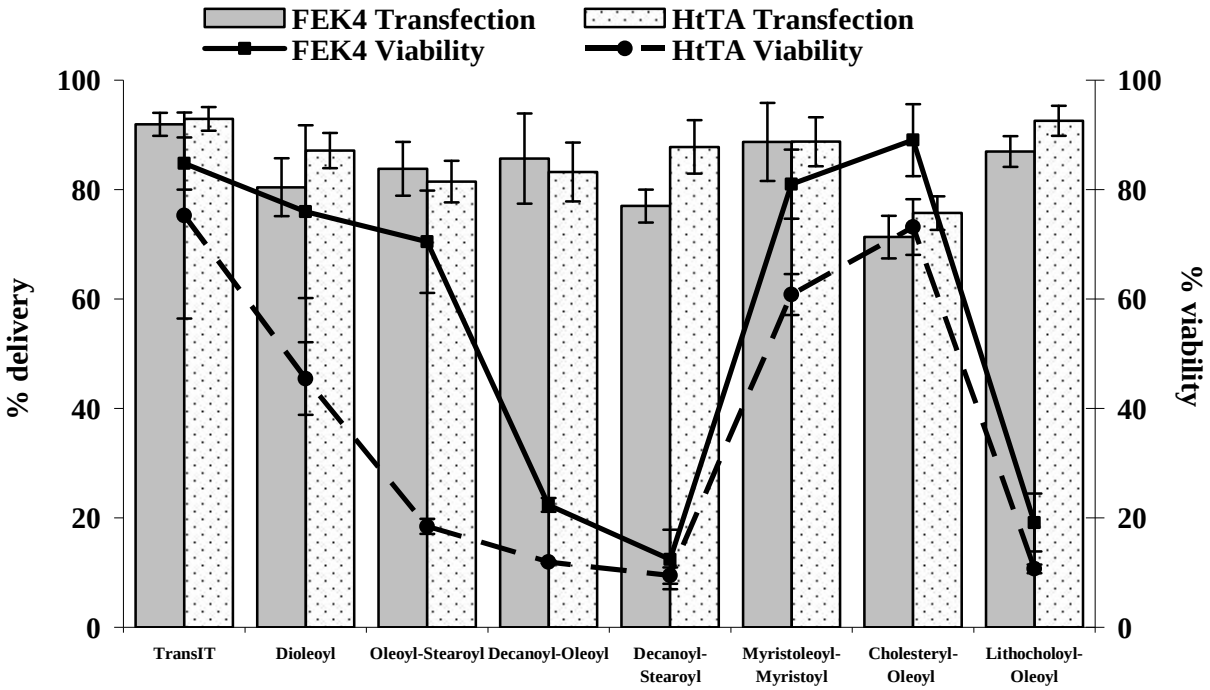

Figure 8. Lipofection and cytotoxicity effects of siRNA (12.5 pmol) complexed with: TransIT-TKO (4 μL), Dioleoyl (N^4,N^9-dioleoyl spermine) (8 μg, N/P = 44), Oleoyl-Stearoyl (N^4-oleoyl-N^9-stearoyl spermine) (20 μg, N/P = 109), Decanoyl-Oleoyl (N^4-decanoyl-N^9-oleoyl spermine) (10 μg, N/P = 65), Decanoyl-Stearoyl (N^4-decanoyl-N^9-stearoyl spermine) (30 μg, N/P = 193), Myristoleoyl-Myristoyl (N^4-myristoleoyl-N^9-myristoyl spermine) (20 μg, N/P = 129), Cholesteryl-Oleoyl (N^4-cholesteryloxy-3-carbonyl-N^9-oleoyl spermine) (8 μg, N/P = 36), and Lithocholoyl-Oleoyl (N^4-lithocholoyl-N^9-oleoyl spermine) (8 μg, N/P = 39) on the primary skin cell line FEK4 and the HeLa derived cancer cell line HtTA (mean ± S.D., n = 9).

The cell viability (MTT assay) results for the siRNA lipoplexes (Figure 8, lines) indicate that there is not a large difference in the cell viability between the commercially available TransIT-TKO, FEK4 (85%) and HtTA (75%) cells, and N^4-myristoleoyl-N^9-myristoyl spermine and N^4-cholesteryloxy-3-carbonyl-N^9-oleoyl spermine, next is N^4-oleoyl-N^9-stearoyl spermine, while N^4-decanoyl-N^9-stearoyl spermine, N^4-decanoyl-N^9-oleoyl spermine, and N^4-lithocholoyl-N^9-oleoyl spermine lipopolyamines are more toxic to both cell lines.

Lipoplex Particle Size Measurements. The particle size of the formulation is also an important factor in improving delivery.[52] Particle size characterization measurements, by laser/NTA, were carried out on selected lipoplexes at their most efficient concentration for delivery, varying the amount of cationic lipid/siRNA 12.5 pmol. The nanoscale of the formed siRNA lipoplexes ranged from (mean ± S.D., n = 9): N^4,N^9-dioleoyl spermine (110 ± 23 nm, at 8 μg, N/P = 44), N^4-decanoyl-N^9-stearoyl spermine (130 ± 36 nm, at 30 μg, N/P = 193), N^4-decanoyl-N^9-oleoyl spermine (150 ± 35 nm, at 10 μg, N/P = 65), to N^4-myristoleoyl-N^9-myristoyl spermine (170 ± 38 nm, at 20 μg, N/P = 129) and N^4-oleoyl-N^9-stearoyl spermine (170 ± 46 nm, at 20 μg, N/P = 109), values around a particle size of 155 nm, in the range 130-170 nm. These lipoplex nanoparticle sizes compare with those from pEGFP of N^4-decanoyl-N^9-oleoyl spermine (90 ± 18 nm) to N^4-myristoleoyl-N^9-myristoyl spermine (210 ± 37 nm) values around a pDNA lipoplex particle size of 165 nm.[35] These nanoscale self-assembled siRNA lipoplexes of asymmetrical N^4,N^9-diacyl spermines transduce target cell lines efficiently. The N/P ratios of the siRNA lipoplex nanoparticles that were determined to show optimum delivery (Figure 8) are all significantly above N/P = 4.5, the charge ratio above which our synthetic asymmetrical lipospermines achieved almost complete siRNA binding (Figure 2). Therefore, optimal siRNA delivery efficiency is not only a matter of siRNA binding and associated charge neutralisation, but also of total lipospermine content in the formulae, resulting in a net positive charge and an excess of acyl lipids.

The length and type of the aliphatic chains (the hydrophobic domain) incorporated into cationic lipids significantly affects their pDNA transfection efficiency,[17, 32-35, 44] but this factor is not yet understood for siRNA delivery. This series of novel non-viral vectors is designed to mimic the composition of cell membrane phospholipid bilayers with their high percentage of asymmetrical fatty chains. We are taking advantage of having short- and long-fatty chains in the same molecule, or a saturated and an unsaturated fatty chain, or a fatty acyl chain with a steroid, mixing these different hydrophobic domains in order to facilitate inter-membrane mixing leading to endosomal escape [53] which will deliver the siRNA directly into the cytosol. Experimental evidence for this is that the di-C14 vector N^4-myristoleoyl-N^9-myristoyl spermine was one of the best in this series. By incorporating two different acyl moieties, and changing their length and oxidation level in a stepwise manner, we have demonstrated some structurally different examples of non-viral lipopolyamine vectors capable of siRNA lipoplex self-assembly and effecting efficient

siRNA delivery. A common moiety in these effective siRNA delivery vectors is a *cis*-mono-unsaturated alkyl chain, e.g. the oleoyl group (C18), a result possibly related to the issues of hydrophobic moiety hydration or packing.[52-54] By incorporating two different lipid moieties, we have shown that all our synthesized lipopolyamines afford siRNA delivery results varying in range, and at different *N/P* charge ratios, and also most of the tested asymmetrical diacyl spermines are not toxic except N^4-lithocholoyl-N^9-oleoyl spermine and those containing saturated C10 acyl chains, e.g. N^4-decanoyl-N^9-stearoyl spermine. Therefore, by considering a combination of the siRNA lipoplex delivery efficiency and cell viability (Figure 8) we see that N^4-oleoyl-N^9-stearoyl spermine and N^4-myristoleoyl-N^9-myristoyl spermine are as effective as TransIT-TKO, typically resulting in 89% cell delivery in the presence of serum i.e. of RNase. We have shown that adding a second lipid chain is better than incorporating a steroid.

siRNA Mediated Gene Silencing. We postulate that the lipid moieties in our cationic lipids interact with the phospholipid bilayer of the cell membrane, facilitating cell entry either in crossing the membrane bilayer and/or in helping to weaken the endosomal bilayer and thereby aiding siRNA either as a nanoparticle or now free (uncomplexed) from the condensing lipopolyamine to escape into the cytosol and silence target genes. These self-assembled non-liposomal formulations of siRNA will have different shapes (volumes) according to the lipid substituent pattern. The longer chain non-steroidal asymmetrical lipopolyamines are remarkably non-toxic and capable of delivering siRNA to primary cell lines in the presence of serum and with high efficiencies. These are important new siRNA delivery vectors, but siRNA delivery does not necessarily equate with target gene silencing. Therefore, HeLa cells stably expressing EGFP were used to test the ability of lipoplexes of selected synthesized asymmetrical lipopolyamines to mediate gene silencing, comparable with recently reported HeLa705 cells containing an aberrant luciferase gene [55] and C6 glioma cells stably expressing GFP.[56] The three lipopolyamines that showed the best balance between highly efficient fluorescein-tagged siRNA delivery and the associated cell viability (Figure 8) were chosen to test their abilities to knock-down EGFP reporter gene. HeLa cell transfection with siRNA, i.e. delivery and functional knock-down, using a siRNA against EFGP (siEGFP) was comparable to that achieved with TransIT-TKO (24%) as a positive control (Figure 9). The EGFP expression in the transfected HeLa cells was significantly reduced (from 100%) to 23%, 34%, and 34% for Dioleoyl (10 μg cationic lipid), Myristoleoyl-Myristoyl and Oleoyl-Stearoyl respectively (each at 25 μg cationic lipid) (Figure 9). The MFIs of siRNA tagged with the fluorescent dye Alexa Fluor 647 and delivered with Dioleoyl, Myristoleoyl-Myristoyl, and Oleoyl-Stearoyl to HeLa cells stably expressing EGFP, under the same conditions as used in the gene silencing experiments, were 606, 421, and 951 fluorescence units respectively. Using a scrambled siRNA as a negative control (siNC, Figure 9), we have also demonstrated that the gene silencing achieved with all four vectors is due to sequence-specific EGFP gene knock-down and not due to other off-target or cationic lipid-related effects, e.g. cell toxicity.

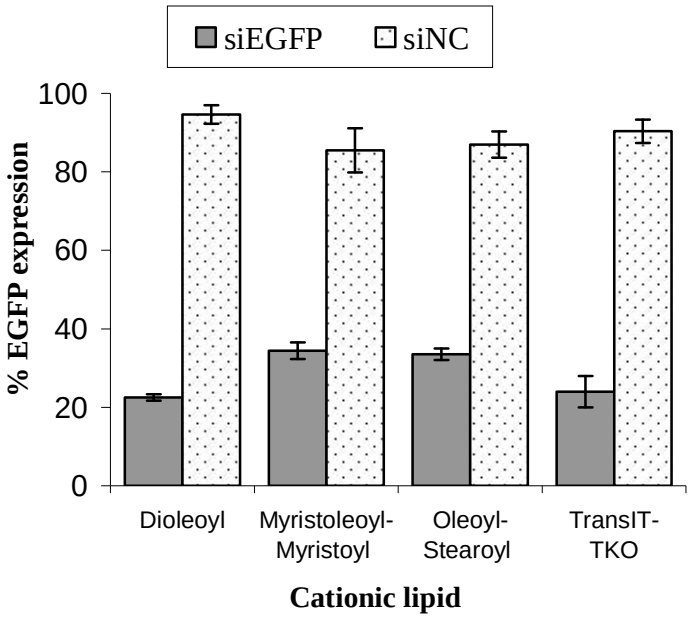

Figure 9. Silencing of EGFP expression in a HeLa cell line that stably expresses EGFP, 48 h post-transfection with siRNA against EGFP (siEGFP, 15 pmol/well, 15 nM) complexed with Dioleoyl (N^4,N^9-dioleoyl spermine, 10 μg), Myristoleoyl-Myristoyl (N^4-myristoleoyl-N^9-myristoyl spermine, 25 μg), Oleoyl-Stearoyl (N^4-oleoyl-N^9-stearoyl spermine, 25 μg), and TransIT-TKO (4 μL/well) as a positive control. The data obtained with siNC are a negative control (mean ± S.D., n = 6).

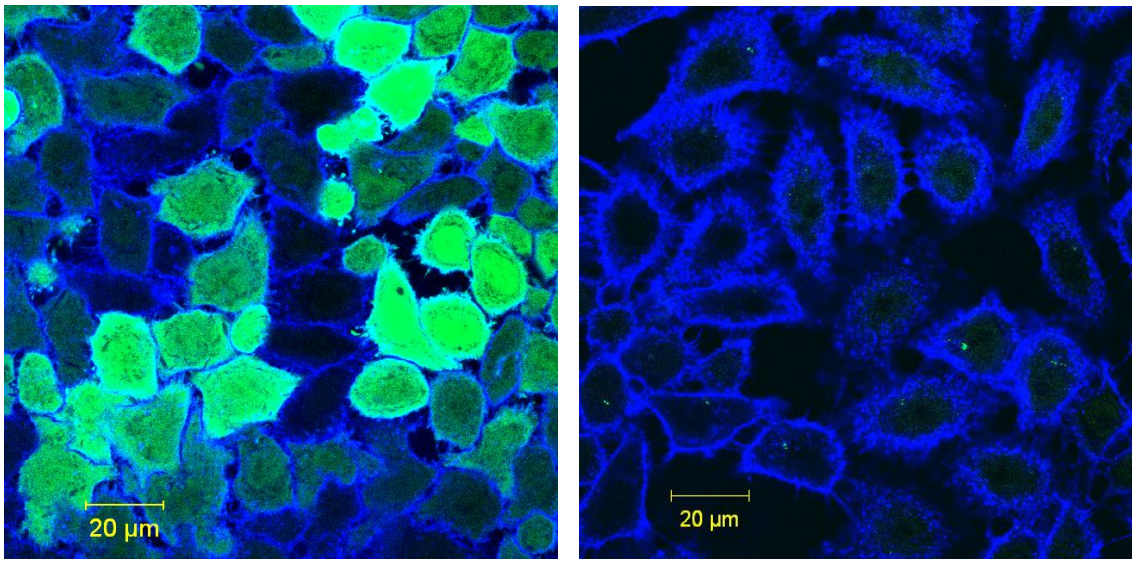

Figure 10. Confocal photomicrographs of HeLa cells stably expressing EGFP, showing cell membranes stained with WGA-Alexa Fluor® 555 (blue) and EGFP fluorescence (green). Representative pictures are shown of untransfected HeLa cells (control, left) and HeLa cells 48 h post-transfection with siRNA against EGFP delivered with N^4-myristoleoyl-N^9-myristoyl spermine (gene silencing to 34%, right) (LSM 510 meta, under the 60× oil immersion objective).

Using confocal laser scanning microscopy, these HeLa cells are shown to be transfected successfully with siRNA against EGFP. As the cells biosynthesize EGFP by transcription and translation, the diminution in the green fluorescence is quantifiable for post-transcriptional siRNA activity by gene silencing. siRNA lipoplexes of N^4-myristoleoyl-N^9-myristoyl spermine showed loss of cytosolic green fluorescence from EGFP to 34% (Figure 10, cf Figure 3). The cell membranes are labeled with WGA-Alexa Fluor® 555 (shown in blue). The presence of an unsaturation center in at least one of the fatty acid chains is a common feature for effective gene knock-down with our vectors. We conclude that the delivery and knock-down results obtained with these asymmetrical lipopolyamines, values comparable to those obtained with TransIT-TKO and N^4,N^9-dioleoyl spermine, can be attributed to the fusogenic ability of the unsaturated fatty acid chains (which have at least one *cis*-double bond) that favors (L_α to H_{II}) phase transition, facilitating both membrane fusion and endosomal escape.[44,49,57] Such lipopolyamines are efficient non-viral vectors in both siRNA lipoplex delivery and gene silencing. As more and better vectors remain critical requirements for clinical siRNA delivery,[18] these SAR study results should find ready application.

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ABBREVIATIONS USED

DMEM, Dulbecco's Modified Eagle's Medium; EGFP, enhanced green fluorescent protein; EMEM, Eagle's Minimum Essential Medium; FCS, fetal calf serum; HRMS, high-resolution mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NTA, Nanoparticle Tracking Analysis; NVGT, non-viral gene therapy; WGA, wheat germ agglutinin.

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