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A cationic cytofectin with long spacer mediates favourable transfection in transformed human epithelial cells

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Abstract

The synthesis and transfection potential of a novel cationic cholesterol cytofectin with a dimethylamino head group and a long 12 atom, 15 Å spacer incorporating relatively polar amido and dicarbonyl hydrazine linkages are reported. Thus *N,N*-dimethylaminopropylamidosuccinylcholesterylformylhydrazide (MS09) in equimolar admixture with dioleoylphosphatidylethanolamine (DOPE) forms stable unilamellar liposomes (80–150 nm) which cluster into very effective transfecting, serum nuclease-resistant, lipoplexes with DNA (180–200 nm) at a liposome+/DNA− molar charge ratio of 2.8:1 (12:1, w/w). Gel retardation and ethidium displacement assays confirmed that DNA was fully liposome-associated and maximally compacted at this ratio. Transfection levels in three human transformed epithelial cell lines, as established by luciferase transgene activity, was found to be optimal at this charge ratio and in the following order: cervical carcinoma (HeLa) > oesophageal carcinoma (SNO) > hepatoblastoma (HepG2). Activity in the murine fibroblast line NIH-3T3 was comparable to that in HepG2 cells. MS09 lipoplexes achieved approximately three-times and two-times greater activity than Lipofectin® complexes in HeLa and SNO cells, respectively, whilst comparable levels were recorded in HepG2 and NIH-3T3 cells. MS09 lipoplexes were well tolerated by HepG2, HeLa and SNO cells with cell numbers found to be 80, 85 and 75% of untreated cultures, respectively, at the optimal transfection concentration. These lipoplexes also exhibited high activity in the presence of 10% foetal bovine serum (FBS) in HeLa (17% inhibition) and HepG2 (33% inhibition) cells.

Keywords: Cationic liposome; Transfection; Transformed epithelial cells

1. Introduction

Efforts to develop non-viral gene transfer vectors in mammalian systems with the design capability for whole organism applications are gaining impetus. This process is fuelled, in part, by a variety of safety and production difficulties associated with alternative viral methods and retroviral modalities in particular. Non-viral DNA transfer approaches are varied and include physical approaches such as electroporation (Meaking et al., 1995), the biolistic or gene gun method (Yang et al., 1999), and direct microinjection (Wolff et al., 1990), which deliver naked DNA into cells by penetration of the plasma membrane avoiding endosomes and lysosomes. A growing list of chemical methods is receiving evermore attention however. Amongst these, cationic polymers form a sizeable group that includes PEI (Boussif et al., 1995), poly-L-lysine (Wu and Wu, 1987), polybrene (Mumper et al., 1996), chitosan (Corsi et al., 2003) and oligoaminosiloxanes (Kichler et al., 2003). These polymers bind and compact DNA and transfect mammalian cells with varying degrees of efficiency.

Cationic liposomes, however, are arguably the most widely employed non-viral gene transfer vehicles. These submicron vesicles which form electrostatic complexes with DNA through interaction of their positively charged amphiphiles and the negatively charged phosphodiester backbone of the nucleic acid, are

Abbreviations: BCA, bicinchoninic acid; Chol-T, 3β[*N*-(*N*′,*N*′-dimethylaminopropane)-carbamoyl] cholesterol; DCC, dicyclohexylcarbodiimide; DC-Chol, dimethyl aminoethane carbamoyl cholesterol 3β[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl] cholesterol; DMF, dimethylformamide; DMSO, dimethylsulfoxide; DOSPER, 1,3-dioleoyloxy-2-(6-carboxyspermyl)-propylamide; DOPE, dioleoylphosphatidylethanolamine; DOTAP, *N*-(1-(2,3-dioleyloxy) propyl)-*N,N,N*-trimethylammonium methylsulfate; HEPES, 2-[4-(2-hydroxyethyl)-piperazinyl]-ethanesulfonic acid; HBS, HEPES buffered saline; MEM, minimum essential medium; MS04, cholesterylformylhydrazide; MS08, cholesterylformylhydrazidehemisuccinate; MS09, *N,N*-dimethylpropylamidosuccinylcholesterylformylhydrazide; NHS, *N*-hydroxysuccinimide; PBS, phosphate buffered saline; PEI, polyethyleneimine; RLU, relative light units; TEM, transmission electron microscopy

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readily prepared and are generally non-immunogenic or weakly so. Liposome–DNA complexes, termed lipoplexes (Felgner et al., 1997), facilitate the cellular uptake of genetic material by, as yet, ill-defined mechanisms which are believed to entail electrostatic interactions with the charged cell-surface residues and hydrophobic interactions with hydrophobic components of the cell membrane. This process is facilitated by a helper lipid, usually dioleoylphosphatidylethanolamine (DOPE) or cholesterol (Felgner et al., 1994 and Deshpande et al., 1998, respectively) which is included in the liposome formulation. Most cationic compounds intended for integration into liposomes embody a hydrophobic anchor which is embedded in the liposome bilayer. This apolar component may be cholesterol, as in the case of the well-known DC-Chol (Gao and Huang, 1991), or two closely apposed long hydrocarbon chains which are frequently integral components of unsaturated acyl groups as are found in DOTAP (Felgner et al., 1987) and DOSPER (Buchberger et al., 1996). The polar cationic head group is tethered to the anchor via a spacer which includes a linker. The biodegradable carbamoyl linker first used in DC-Chol has subsequently been employed extensively in related cytofectins of this class (Takeuchi et al., 1996; Singh et al., 2001a; Kisoon et al., 2002; Reynier et al., 2004) whilst ester (Lee et al., 2004), ether (Ghosh et al., 2000) and amide linkers (Hasegawa et al., 2002) have also been applied with success. The cationic components are varied but substituted aliphatic amines are favoured. The nature and length of the spacer element are important factors in accommodating cohesive charge–charge interactions in lipoplexes and influence their transfection potential. In this regard it has been reported recently that a single additional methylene in the spacer region of cationic cholesterol derivatives leads to potentiated transfection efficacy in a number of mammalian cell lines in vitro (Takeuchi et al., 1996; Reynier et al., 2004; Singh and Chaudhuri, 2004).

The strength of salt bridges underpinning DNA–cytofectin interactions will be affected by the proximity of the cationic and anionic components and the immediate molecular environment. In examining interactions between the double stranded DNA and the cationic surface of the unilamellar liposomes the concept of relative surface exposure should be considered. It has been calculated that the accessible area in B-DNA is rather polar with phosphate oxygens accounting for 45% of this surface (Alden and Kim, 1979). Furthermore the DNA molecules are extensively hydrated with hydration being more strongly held around phosphate bridges. The DNA hydration shells may adversely affect the formation of ion pairs with the cationic liposome bilayer by restricting convergence of charge centres. The resulting longer range electrostatic interactions may then be more easily destabilized by ions and polyions in cell culture medium and serum. It therefore seems reasonable to assume that amphiphiles with long, somewhat polar spacers which favour extension of cationic heads into the aqueous milieu may facilitate interaction with the hydration shell, thereby bringing the cationic heads on amphiphiles into closer proximity to the anionic centres on the DNA.

To explore this possibility we have therefore prepared a cholesteryl derivative bearing a cationic dimethylamino head group which is connected to the fused ring system via a relatively polar 12 atom, 15 Å spacer. Thus *N,N*-dimethylaminopropylamidosuccinylcholesterylformylhydrazide (MS09, Fig. 1) which we have formulated with equimolar amounts of DOPE into stable unilamellar liposomes forms tight lipoplexes with plasmid DNA that are resistant to assault by serum nucleases and that are well tolerated by the transformed human epithelial cell lines SNO (oesophageal), HepG2 (hepatocyte-derived), HeLa (cervical) and the murine NIH-3T3 fibroblast line. We report here high transfection activity as measured by the expression of the luciferase gene in the pGL3 vector, in the human lines examined in this study. We show also that these levels compare very favourably with those achieved by the commercial transfection agent Lipofectin®.

2. Materials and methods

2.1. Chemicals and reagents

Cholesterylchloroformate, NHS, dimethylaminopropylamine, DCC, DOPE and succinic anhydride were obtained from Sigma–Aldrich (St. Louis, MI). Hydrazine, pyridine, DMF, silica gel $60F_{254}$ chromatography plates, HEPES, glutaraldehyde, OsO_4, propylene oxide, lead citrate and all organic solvents were purchased from Merck (Darmstadt, Germany). The components of Spurr's resin were purchased from TAAB Laboratories (UK). BSA and pBR322 DNA were from Roche (Mannheim, Germany). Agarose (ultrapure DNA grade) was from BioRad (Richmond, CA). Trypsin–EDTA and penicillin–streptomycin mixtures were supplied by Whittaker Bioproducts (Walkersville, MD). Foetal calf serum was sourced from Delta Bioproducts (Johannesburg, South Africa). Minimum essential medium (MEM) with Earle's salts and Lipofectin® were provided by Gibco BRL (Inchinnan, Scotland). The luciferase assay kit and the pGL3 control vector was purchased from Promega Corporation (Madison, WI). All plastic ware was from Bibby-Sterilin (Staffordshire, England). All other reagents were of analytical grade and ultrapure water (Milli-Q50) was used throughout.

2.2. Synthesis of cholesterol derivatives

2.2.1. Cholesterylformylhydrazide (MS04)

A solution of cholesterylchloroformate (1.13 g, 2.5 mmol) in $CHCl_3$ (5 ml) at 0 °C was added, with stirring, to a solution of hydrazine (240 mg, 7.5 mmol) in $CHCl_3$:MeOH (3:0.6 ml). After 24 h at room temperature the solution was concentrated in vacuo to a crystalline mass which was recrystallized from $CHCl_3$:MeOH (4:1, v/v) to afford 917 mg of product (83% yield). mp: 225–227 °C.

2.2.2. Cholesterylformylhydrazidehemisuccinate (MS08)

A solution of MS04 (89 mg, 0.2 mmol) and succinic anhydride (20 mg, 0.2 mmol) in DMF:pyridine (1:1, v/v, 2 ml) was maintained at room temperature overnight. Solvent was then evaporated under reduced pressure and the product was obtained as white crystals from absolute ethanol. Yield: 58 mg (53%). mp: 195–196 °C.

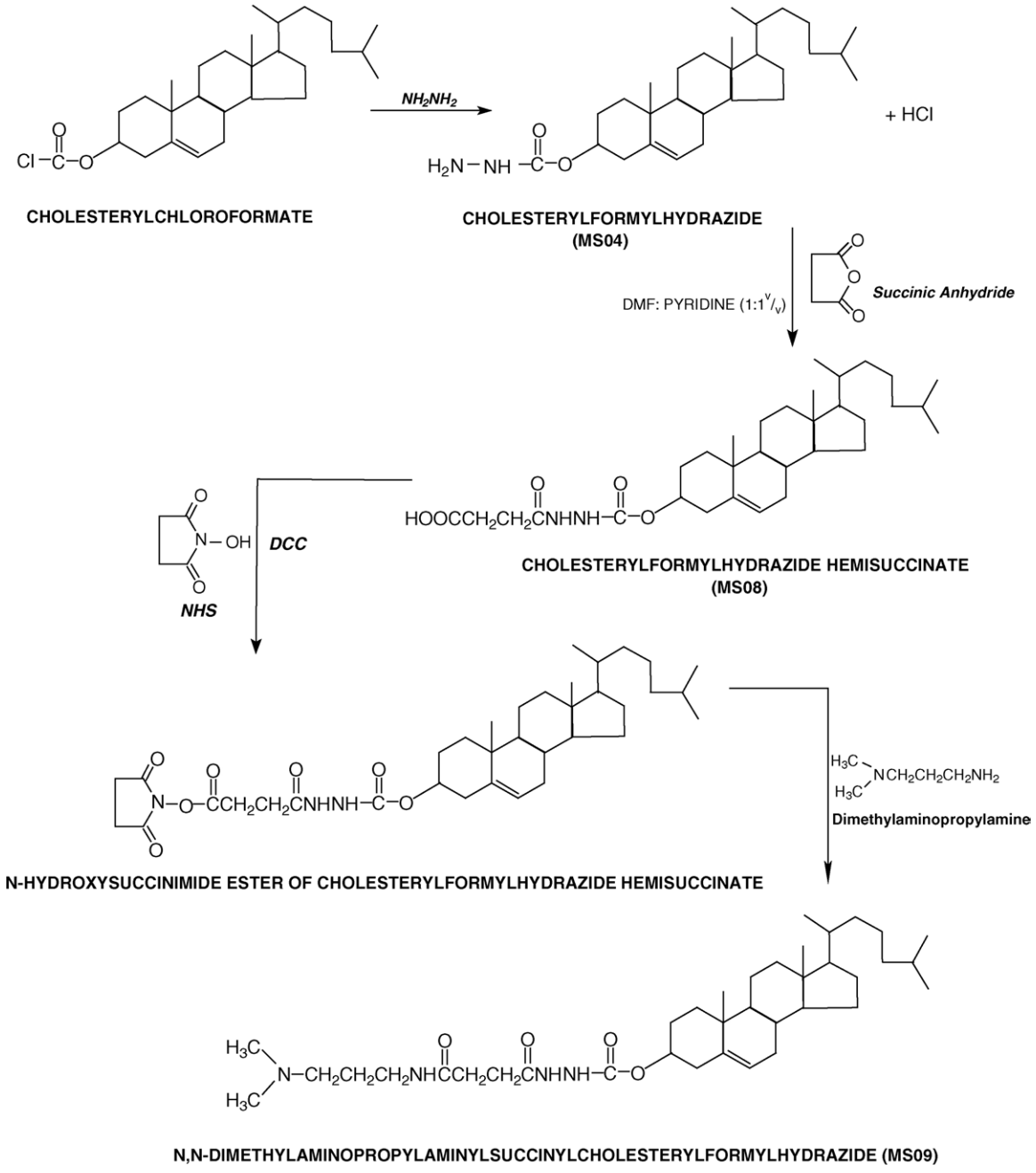

Fig. 1. Scheme outlining the synthesis of *N,N*-dimethylaminpropylamidosuccinylcholesterylformylhydrazide (MS09) from cholesteryl chloroformate.

2.2.3. N-hydroxysuccinimide ester of cholesterylformylhydrazidehemisuccinate

MS08 (82 mg, 0.15 mmol), DCC (62 mg, 0.3 mmol) and *N*-hydroxysuccinimide (35 mg, 0.3 mmol) were dissolved in DMF (1 ml) and the progress of the reaction was monitored by TLC on silica gel $60F_{254}$ plates developed in $CHCl_3$:MeOH (9:1, v/v). After 48 h the dicyclohexylurea crystals were removed by filtration and the crude product obtained by evaporation of the solvent in vacuo. The product was dissolved in $CHCl_3$ and this was extracted with water to remove excess *N*-hydroxysuccinimide. After evaporation of the solvent the residue was extracted with petroleum ether (60–80 °C) to remove traces of DCC. Finally the product was obtained as white crystals from EtOH. Yield: 53 mg (55%).

2.2.4. N,N-dimethylpropylamidosuccinylcholesterylformylhydrazide (MS09)

The *N*-hydroxysuccinimide ester of cholesterylformylhydrazidehemisuccinate (53 mg, 0.083 mmol) and dimethylaminopropylamine (36 mg, 0.35 mmol) were dissolved in water:pyridine:DMF (13:7:10, v/v/v, 1.5 ml). The reaction was monitored by TLC in $CHCl_3$:MeOH (95:5, v/v) and the product was purified on four 10 cm × 20 cm $60F_{254}$ TL plates devel-

oped in the same solvent system. Yield: 20 mg (38%). mp: 155–156 °C.

2.3. Plasmid DNA

The 5256 bp pGL3 control vector (Promega, Madison, WI) used in this study encodes the *Photinus pyralis* luciferase gene (luc^+) flanked by SV40 promoter and enhancer sequences which afford strong expression in a range of mammalian cell types. The 4363 bp pBR322 plasmid (Roche Diagnostics, Mannheim, Germany) was used in the dye displacement assay only.

2.4. Liposome preparation and transmission electron microscopy

Liposomes were prepared by a method adapted from that of Gao and Huang (1991). Briefly, MS09 (2 μmol) and DOPE (2 μmol) were dissolved in $CHCl_3$ (1 ml). The lipids were deposited as a thin film on the inner wall of a test tube by rotary evaporation of the solvent at 20 °C. The thin film was rehydrated in sterile HBS (20 mM HEPES, 150 mM NaCl, pH 7.5, 1 ml) overnight and finally the suspension was vortexed before sonicating for 5 min on a Transsonic bath-type sonicator. The resulting liposomes were stored at 4 °C. Size and lamellarity of liposomes were established by TEM. In brief, liposome suspensions (50 μl) were mixed with a 5% (w/v) BSA solution in Tris–HCl (100 mM, pH 7.2) and treated with 25% (w/v) glutaraldehyde (50 μl). Gels were diced and treated with OsO_4 (24 h). After dehydration, gels were treated successively with propylene oxide, propylene oxide:Spurr's resin (1:1, v/v) and finally Spurr's resin. After staining in uranyl acetate and lead citrate, sections were viewed in a Jeol 1010 electron microscope at 60 kV.

2.5. Lipoplex characterization

2.5.1. Transmission electron microscopy

Lipoplex suspensions (50 μl), which were prepared by adding liposomes (4 μmol lipid/ml HBS) to plasmid DNA (1 μg/μl HBS) at 20 °C and allowing to mature for 20 min, were placed on parafilm sheets and mixed with 0.5% w/v uranyl acetate (50 μg). After 3 min, the mixtures were transferred to the matt surface of formvar coated copper grids. Discs were air dried overnight and viewed in a Jeol 1010 transmission electron microscope at 60 kV.

2.5.2. Gel retardation assay

Liposome:DNA mixtures in HBS (10 μl) were incubated at 20 °C for 30 min and mixed with 3 μl gel loading buffer (50% glycerol, 0.5% bromophenol blue, 0.5% xylene cyanol, 72 mM Tris–HCl, 60 mM NaH_2PO_4 and 20 mM EDTA at pH 7.5) before loading onto a 1% agarose gel in a Mini-Sub® apparatus (Bio-Rad, Richmond, CA). Electrophoresis was conducted for 90 min in TPE running buffer (36 mM Tris–HCl, 30 mM NaH_2PO_4, 10 mM EDTA, pH 7.5) at 50 V (Consort E455 power supply, Turnhout, Belgium). The gel was stained with ethidium bromide (1 μg/ml running buffer) for 30 min and viewed under transillumination at 300 nm and images captured on a Gene Genius Bioimaging System (Syngene, Cambridge, UK).

2.5.3. Nuclease digestion assay

Liposome:DNA mixtures were incubated in HBS (20 μl) for 20 min at 20 °C, and thereafter, foetal bovine serum was added to a final concentration of 10%. Samples were incubated for a further 4 h at 37 °C before receiving EDTA and SDS to final concentrations of 10 mM and 0.5% (w/v), respectively. After a further 20 min at 55 °C loading buffer (3 μl) was added to mixtures before subjecting to electrophoresis as described in Section 2.5.2.

2.5.4. Ethidium displacement assay

Lipoplex formation was also followed by the displacement of intercalated ethidium cation upon association of plasmid DNA with liposomes in a Shimadzu RF-551 spectrofluorometric detector (excitation and emission wavelengths of 520 and 600 nm, respectively). The assay was adapted from that described by Tros de Ilarduya et al. (2002). A solution of ethidium bromide (ETBr, 1 μg) in HBS (500 μl) was used to provide a baseline reading of fluorescence (0%). Thereafter, pBR322 DNA (6 μg) was added and this solution was used to set the instrument to 100% fluorescence. Aliquots of MS09 liposomes (10 μg) were then added and readings taken after mixing at each step until 90 μg MS09 liposomes had been added.

2.6. Cell lines and their maintenance

HeLa, HepG2, SNO and NIH-3T3 cells were obtained from Highveld Biological (Pty) Ltd., Kelvin, South Africa. Cells were propagated at 37 °C in 25 cm^2 flasks in 5 ml of MEM containing 10% (v/v) foetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin sulphate, 10 mM $NaHCO_3$ and 20 mM HEPES (pH 7.5). Cells were split in a ratio of 1:3 (1:2 HepG2) every 3–4 days, and stored in a biofreezer (−80 °C) in complete medium containing 10% (v/v) DMSO.

2.7. Growth inhibition assays

HeLa, HepG2, SNO and NIH-3T3 cells were separately trypsinized and seeded into 24-well plates at the following densities: 2.0×10^4, 1.8×10^4, 1.8×10^4 and 2.1×10^4 per well, respectively. Cells were incubated for 24 h to allow attachment and growth to semi-confluency. Lipoplexes were assembled in HEPES (10 μl) and incubated at 20 °C for 20 min. Growth medium was removed from cells and replaced with serum free medium (0.5 ml). Lipoplexes were then added to wells and plates were incubated at 37 °C for 4 h. The medium was then replaced by complete medium and cells were incubated at 37 °C for a further 48 h. Adherent cells were quantified by the method of Schellekens and Stitz (1980). In brief, cells were washed twice with PBS and stained with 200 μl crystal violet solution (0.5%, w/v crystal violet; 0.8%, w/v sodium chloride; 5%, v/v formaldehyde; 50%, v/v, ethanol) for 20 min. Stain was removed and wells washed extensively with water. The multi-well plates were

then dried for 24 h and the stain was extracted from fixed cells into 2-methoxyethanol (0.5 ml) over a period of 36 h with gentle rocking (20 revolutions/min) on a Stuart Scientific STR 6 platform shaker Surrey, UK). Absorbance values of extracts were measured at 550 nm in a Novaspec spectrophotometer (Biochrom, Cambridge, UK).

2.8. Transfections

Cells were seeded into 24-well plates at a density of 2.3×10^4 (HeLa), 1.9×10^4 (HepG2), 1.8×10^4 (SNO) and 2.0×10^4 (NIH-3T3) in complete medium and grown to semi-confluency. MS09 liposome–DNA complexes were assembled as described in Section 2.7 while Lipofectin®–DNA complexes were prepared according to the manufacturers of the transfecting agent. After washing cells with PBS serum free medium (0.5 ml) was introduced into wells. Transfection complexes were then added to wells and after 4 h incubation at 37 °C the medium was removed, and replaced with complete growth medium and cells cultured for a further 48 h. In some experiments transfection complexes were introduced to cells in complete medium at the outset.

2.9. Luciferase assay

The medium was aspirated and cells washed with PBS (2 ml × 0.5 ml). Cell lysis was effected by cell culture lysis reagent (80 μl/well) over 15 min. Lysates were collected and centrifuged (12,000 × *g*) to pellet cellular debris. To 20 μl of the supernatant was added luciferase assay reagent (100 μl). After mixing, relative light units (RLU) of each sample were measured over a 10 s period in a Lumac Biocounter M1500 (Landgraaf, Netherlands). Protein content of supernatants was determined using the BCA assay (Smith et al., 1985) and luciferase activity was expressed as RLU/mg protein.

3. Results

3.1. Chemical syntheses and spectral analyses

The synthesis of the target compound MS09 was achieved from cholesterylchloroformate in four steps (Fig. 1). The hydrazide MS04, which was obtained in high yield, was prepared to afford a convenient amino functionality for extension by subsequent succinylation (MS08). The hemisuccinate was then coupled to *N,N*-dimethylaminopropylamine through an amide linkage following carboxyl group activation by *N*-hydroxysuccinimide. In the process, a 12 atom spacer with polar amido and 1,2-dicarbonyl hydrazine linkages was constructed between the basic dimethylamino head group and the hydrophobic cholesteryl skeleton. Structures of cholesteryl derivatives were confirmed by infrared (IR) spectrophotometry on a Nicolet Impact 420 spectrophotometer, ^{1}H (300 MHz) NMR on a Gemini 300 instrument and electrospray time of flight mass spectrometry on a Waters APIQ-TOF Ultima instrument. The results were as follows:

3.1.1. Cholesterylformylhydrazide (MS04)

IR (film) 3416 (b, N–H), 2929 (st, C–H), 1731 (m, C=O), 1495 (m, C=C) cm^{-1}. ^{1}H NMR (DMSO d^6) δ 0.66 (s, 3H, C–CH_3), 0.86 (d, 6H, CH–CH_3), 0.91 (d, 3H, CH–CH_3), 0.99 (s, 3H, C–CH_3), 3.38 (bs, 2H, NH_2), 4.38 (m, 1H, Chol–$H_{3\alpha}$), 5.33 (d, 1H, Chol–H_6), 7.93 (s, 1H, NH). Ms, *m*/*z*, ES-TOF 445.44 [M + H^+], 467.39 [M + Na^+].

3.1.2. Cholesterylformylhydrazidehemisuccinate (MS08)

IR (film) 3452 (b, N–H), 2934 (m, C–H), 1675 (m, C=O), 1567 (w, C=C) cm^{-1}. ^{1}H NMR (DMSO d^6) δ 0.64 (s, 3H, C–CH_3), 0.83 (d, 6H, CH–CH_3), 0.89 (d, 3H, CH–CH_3), 0.96 (s, 3H, C–CH_3), 4.3 (Chol–$H_{3\alpha}$), 5.34 (d, 1H, Chol–H_6). Ms, *m*/*z*, ES-TOF 545.09 [M + H^+], 567.63 [M + Na^+].

3.1.3. N,N-dimethylaminopropylamidosuccinylcholesterylformylhydrazide (MS09)

IR (film) 3441 (b, N–H), 2945 (m, C–H), 1634 (m, C=O), 1557 (w, C=C) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 0.65 (s, 3H, C–CH_3), 0.84 (d, 6H, CH–CH_3), 0.88 (d, 3H, CH–CH_3), 0.98 (s, 3H, C–CH_3), 2.26 (s, 6H, N–CH_3), 3.28 (q, CH_2–CH_2–NH), 4.48 (m, 1H, Chol–$H_{3\alpha}$), 5.35 (bs, 1H, Chol–H_6). Ms, *m*/*z*, ES-TOF 629.82 [M + H^+].

3.2. Ultrastructure of liposomes and lipoplexes

Liposomes prepared from MS09 and DOPE in a 1:1 molar ratio at a concentration of 4 μmol total lipid per milliliter of HBS were stable for several months when stored at 4 °C. Embedded and sectioned samples appeared unilamellar and predominantly spherical or oval in the 80–150 nm size range when viewed by TEM (Fig. 2A). Although some size dispersity of liposomes was observed, on addition of plasmid DNA, lipoplexes of more uniform size were generated. At a Lip+/DNA− molar charge ratio of 2.8 globular aggregates (180–200 nm) comprised of smaller vesicles not unlike those reported by Percot et al. (2004) were observed by TEM after negative staining (Fig. 2B). At a marginally higher charge ratio of 3.3 particles were somewhat larger (280–350 nm) and spherical liposome-like structures (50–70 nm) were clearly discernible in the aggregates (Fig. 2C).

3.3. Liposome–plasmid association

The formation of electrostatic complexes between the cationic liposomes and pGL3 plasmid DNA was followed by electrophoresis retardation assays on agarose gels. Fig. 3 shows the effect an increasing Lip+/DNA− charge ratio has on the migration of pGL3 plasmid DNA. The amount of liposome-associated DNA retained in the wells increases with increasing liposome concentration until at a liposome/DNA ratio of 12:1 (w/w), corresponding to a +/− charge ratio of 2.8 all the DNA was liposome-bound. At this ratio, lipoplexes appear to contain clusters of unilamellar vesicles, and in consequence only about half the cationic charges are exposed to the DNA (liposome outer leaflet). This would give an effective +/− charge ratio of

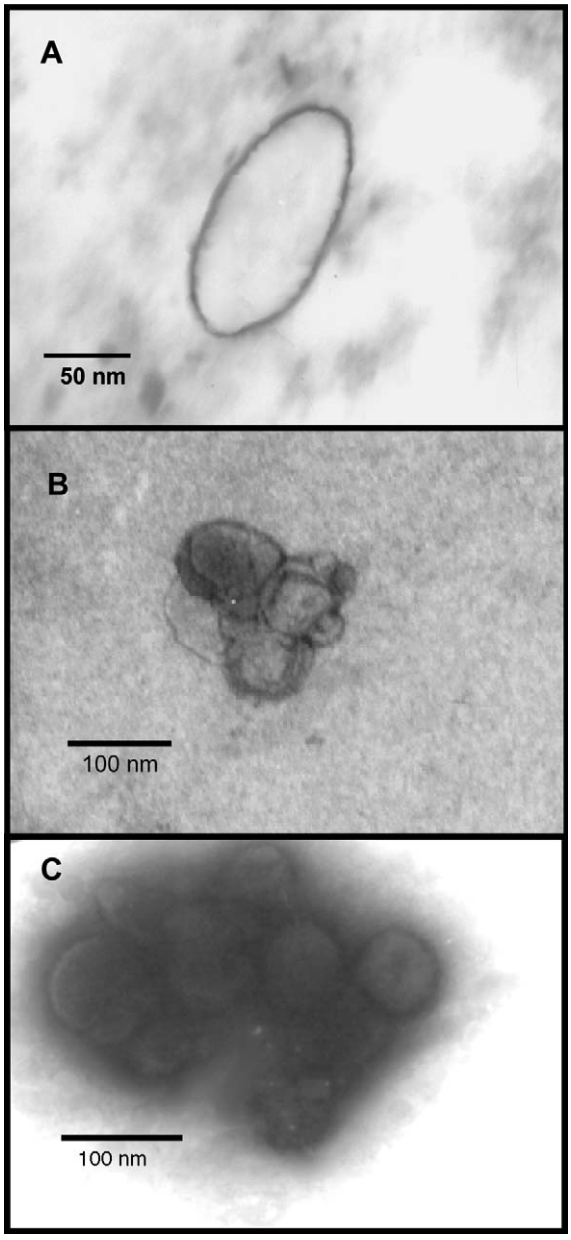

Fig. 2. Transmission electron micrographs (TEMs) of unilamellar liposomes prepared from MS09 and DOPE in 1:1 molar ratio, (A); lipoplexes at a liposome: pGL3 DNA molar charge ratio of 2.8:1 (B) and 3.3:1 (C).

1.4 at complete DNA retardation (Fig. 3, lane 8). Similar arguments have been advanced before to explain the apparent excess positive charge in cluster complexes of this nature at full DNA retardation (Cao et al., 2000; Kisoon et al., 2002; Percot et al., 2004). This association was also explored in an ethidium displacement experiment. On intercalation of the planar aromatic cationic fluorophore into the naked DNA a marked enhancement of fluorescence, a result of steric protection from molecular oxygen induced quenching, is noted (Even-Chen and Barenholz, 2000). Cationic liposome preparations are known to displace DNA-associated ethidium (Xu et al., 1999) in a process which is usually complete within 30 s (Xu and Szoka, 1996). Thus on stepwise addition of MS09 liposomes a steady decrease in fluorescence was observed (Fig. 4) until a point of inflection was

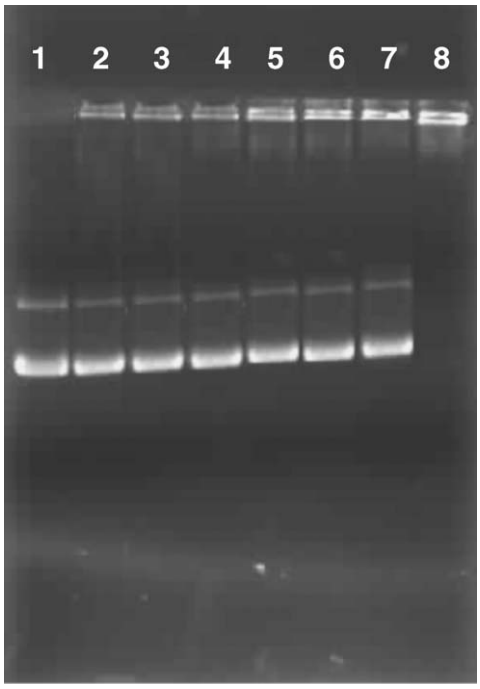

Fig. 3. Gel retardation study of MS09 cationic liposome:DNA complexes. Incubation mixtures (10 μl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained varying amounts of cationic liposomes in lanes 1–8 (0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 μg) and pGL3 DNA (0.5 μg).

reached at a liposome:DNA ratio of 12:1 (w/w) which approximated 60% displacement and corresponded with the complete association of DNA with liposomes as determined in the gel retardation assay (Fig. 3). A further increase in MS09 up to a ratio of 20:1 resulted in additional, but modest, reduction in fluorescence suggesting that the plasmid DNA did not undergo significant additional condensation.

The integrity of the DNA in serum-containing medium is crucial in gene delivery systems. Of particular concern is the possible degradation of the nucleic acid by serum nucleases. Results presented in Fig. 5 confirm that complete protection from digestion in medium containing 10% foetal bovine serum is afforded to liposome-bound DNA over a wide liposome:DNA range (4:1–14:1, w/w). It has already been suggested that electro-

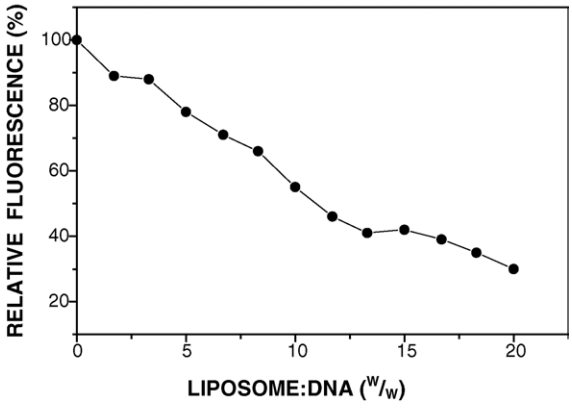

Fig. 4. Ethidium intercalation displacement assay for MS09 cationic liposomes at varying liposome:DNA (w/w) ratios.

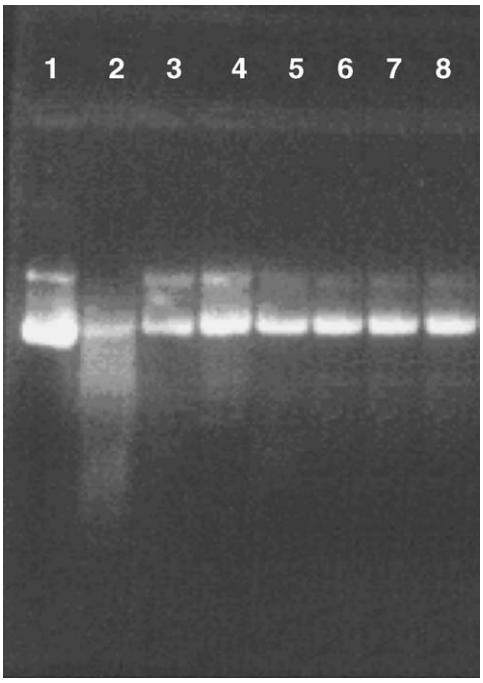

Fig. 5. Serum nuclease protection assay for MS09 lipoplexes. pBR322 DNA (1 μg) was incubated with varying amounts of MS09 liposomes (0, 4, 6, 8, 10, 12, 14 μg, lanes 2–8) and exposed to 10% FBS for 4 h whereupon mixtures were applied to a 1% agarose gel. Uncomplexed and untreated pBR322 DNA is shown in lane 1.

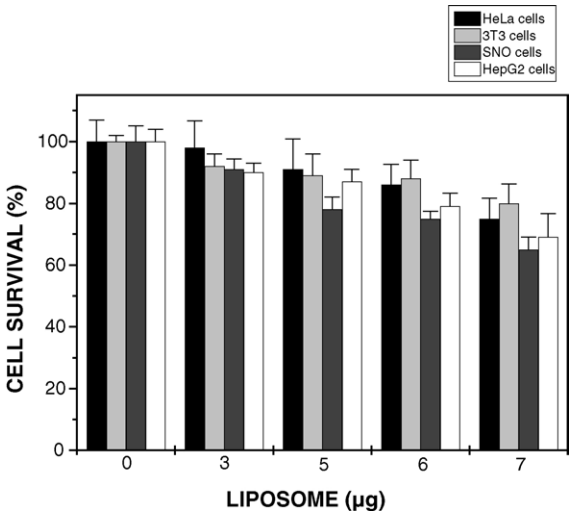

Fig. 6. Growth inhibition studies of MS09 liposome:pGL3 DNA complexes in three transformed human epithelial lines (HeLa, SNO, HepG2) and one murine fibroblast line (NIH-3T3). Cells in multiwell plates bathed in 0.5 ml MEM were exposed to lipoplexes containing 0.5 μg DNA and varying amounts of MS09 liposomes as indicated. Data are presented as means ± S.D. ($n = 4$).

static forces between positively charged cationic liposomes and negatively charged DNA lead to the formation of highly organized supramolecular structures where the DNA is condensed and protected against nuclease degradation (Pitard, 2002).

3.4. Cell growth inhibition studies

Cytotoxicity may present an important yet sometimes overlooked obstacle to effective non-viral gene transfer. This parameter was measured in all four cell lines studied, under the conditions selected for gene transfer experiments. Lipoplexes were generally well tolerated over the entire lipid concentration range of 8.5–20 μM with maximum growth inhibitions of 20% for HeLa, 25% for NIH-3T3, 35% for SNO and 31% for HepG2 cells recorded at the highest lipid concentration (Fig. 6). It has been suggested that toxicity attributed to cationic lipids arises mostly from the increasing cell membrane permeability and creation of transmembrane pores (Lasic, 1997), while the interaction with anionic lipids such as cardiolipin found in mitochondrial membranes would adversely affect the basic energy reactions of the cell (Dass and Burton, 1999).

3.5. Transfection activity of MS09 lipoplexes

The transfection activity of MS09 lipoplexes was measured in three human transformed epithelial cell lines (HeLa, HepG2, SNO) and one murine fibroblast line (NIH 3T3) at various Lip+/DNA− charge ratios in the presence or absence of 10% FBS. In all cases maximum activity was obtained at a liposome: DNA ratio of 12:1 (w/w) (Fig. 7A–D), at which all the plasmid DNA was liposome-bound (Fig. 3) and near maximally condensed (Fig. 4). Highest levels were achieved in HeLa cells followed by SNO cells, whilst the lowest levels of luciferase activity were recorded in HepG2 and NIH 3T3 cells. The presence of 10% FBS in the initial 4 h incubation of lipoplexes with cells reduced luciferase activity in the cell lines by varying degrees. In the human lines, transfection activities at the optimal Lip+/DNA− ratio were reduced to 83, 67 and 40% in HepG2, HeLa and SNO cells, respectively. In all cases the inhibitory effect was less apparent at Lip+/DNA− ratios above and below the optimum. In all transfection experiments with MS09 lipoplexes, 0.5 μg of pGL3 DNA was used in each well while the liposome concentration was varied. Lipofectin®, which was used as a comparator in transfection studies, displayed greatest transfection activity in the cell lines studied when combined with pGL3 DNA at a ratio of 8:1 (w/w). Unlike MS09 liposomes however, highest levels were achieved in the murine NIH 3T3 line (Fig. 8). Although the plasmid concentration adopted in the Lipofectin® experiments was twice that used in the MS09 studies, the MS09 lipoplexes achieved approximately three-times and two-times greater activity than Lipofectin® in HeLa cells and oesophageal SNO cells, respectively, whilst comparable levels were noted for HepG2 and NIH 3T3 cells.

4. Discussion

It has been known for some time that the distance between the cationic head group and the hydrophobic anchor is a crucial factor in the design of cytofectins (Remy et al., 1995; Wheeler et al., 1996). The dimethylamino head group, which is a feature of several cationic cholesterol transfecting agents including DC-Chol (Gao and Huang, 1991), cholesteryl-3β-oxysuccinamidoethylenedimethylamine, cholesteryl-3β-carboxyamidoethylenedimethylamine (Farhood et al., 1992), chole-

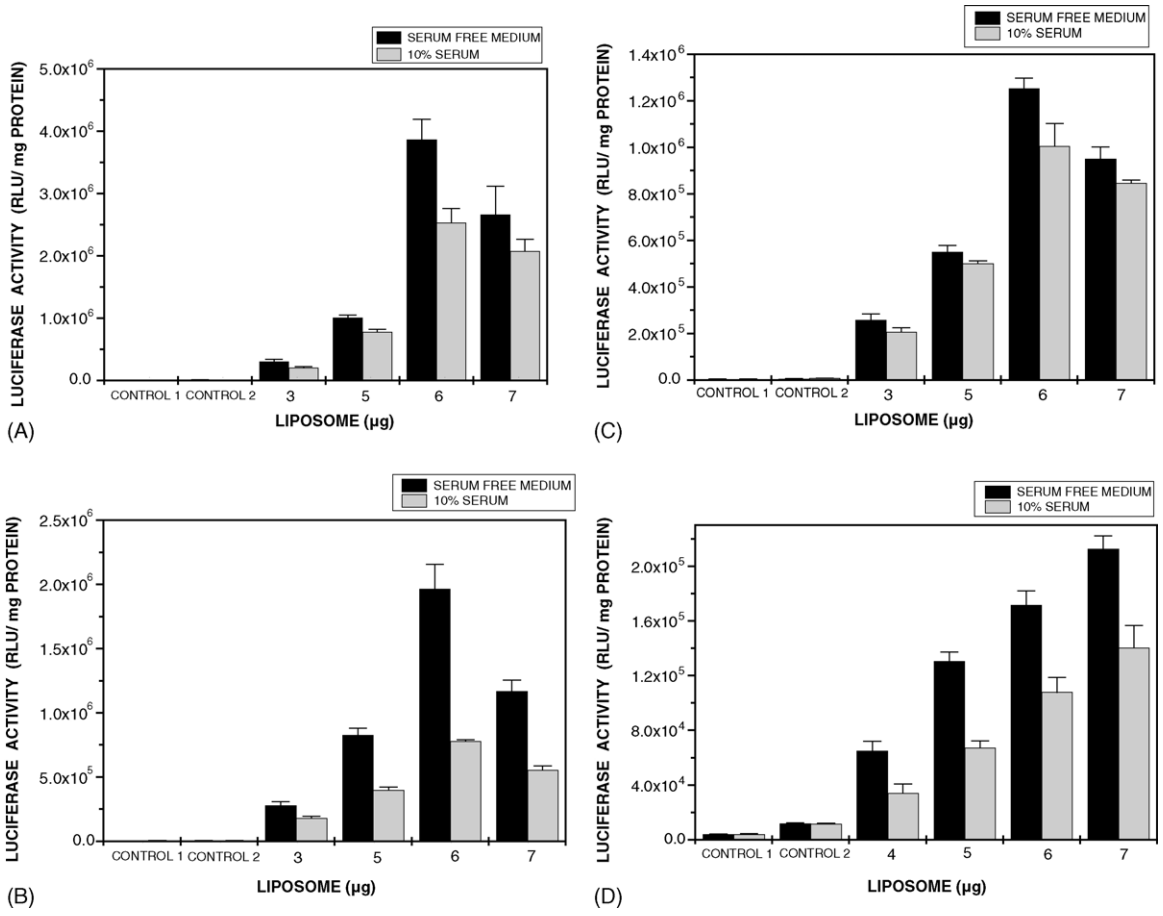

Fig. 7. Transfection studies of MS09 liposome:pGL3 DNA complexes, with and without 10% FBS in HeLa cells (A), SNO cells (B), HepG2 cells (C) and NIH-3T3 cells (D). Experiments were conducted in multiwell dishes and cells were exposed to 0.5 μg pGL3 DNA complexed to varying amounts of MS09 liposomes (3, 5, 6, 7 μg). Control 1 shows endogenous luminescence of unexposed cells while control 2 shows luciferase activity of cells exposed to pGL3 DNA alone. Data are presented as mean ± S.D. ($n = 4$).

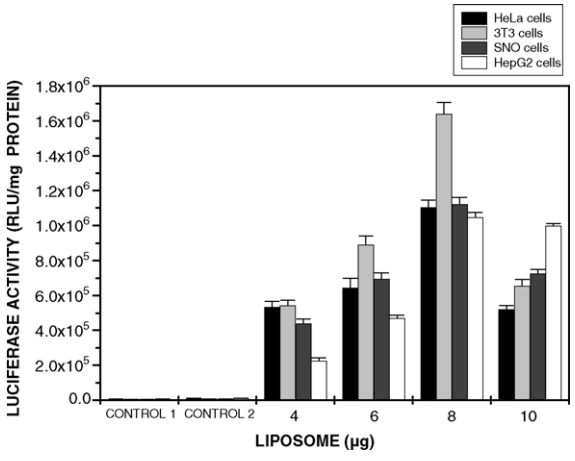

Fig. 8. Transfection studies of Lipofectin®:pGL3 DNA complexes in three transformed human epithelial lines (HepG2, SNO, HeLa) and one murine fibroblast line (NIH-3T3). Cells in multiwell plates exposed to varying amounts of Lipofectin® (4, 6, 8, 10 μg) and pGL3 DNA (1 μg). In control 1, cells were unexposed while in control 2, cells were exposed to pGL3 DNA alone. Data are presented as mean ± S.D. ($n = 4$).

steryl-3β-carboxyamidopropanedimethylamine (Takeuchi et al., 1996), cholesteryl-3β-oxysuccinamidopropanedimethylamine (Singh et al., 2001a) and cholesteryl-3β-oxycarboxyamidopropanedimethylamine (Chol-T) (Takeuchi et al., 1996; Singh et al., 2001b), has been selected for the work reported herein. In this study, a cationic cholesterol derivative MS09, in which the dimethylamino function and the hydrophobic cholesteryl moiety are separated by a considerably greater distance, has been prepared and screened in three transformed human epithelial cell lines and one murine cell line. We have designed this derivative to explore the possibility of improving the electrostatic interaction between cationic and anionic centres in the cytofectin and DNA components of lipoplexes by increasing the spacer length to 12 atoms. The spacer incorporates hydrophilic features (amido and 1,2-dicarbonyl hydrazine linkages) to promote extension and stability in the hydrated environment surrounding the nucleic acid and liposome. In support of this notion, it has already been suggested that liposomal formulations with good surface hydration will lead to increased transfection activity (Bennett et al., 1996). The derivative was formulated into stable unilamellar liposomes with DOPE, a colipid which has been reported to assist in the formation of liposomes, that have significantly increased transfection efficiency,

and which tends to reduce the cytotoxicity of cationic surfactants (Sternberg et al., 1994). Lipoplexes were well-defined aggregates in appearance (TEM) with a substructure of globular vesicles (50–70 nm) (Fig. 2B and C). In earlier studies with lipoplexes containing Chol-T, a cationic cholesterol cytofectin with a much shorter propylcarbamoyl spacer/linker, we had observed similar aggregates (Moodley et al., 2002). It seems fair to conclude from these observations and those of others (Cao et al., 2000; Percot et al., 2004) that cationic liposomes comprising DOPE in equimolar admixture with monocationic cholesterol derivatives embodying substituted amino head groups and carbamoyl linkers readily assemble with DNA into compact clusters which increase in size as the Lip+/DNA− ratio is raised.

It is noteworthy that in this study maximum transgene activity was achieved in four unrelated cell types by MS09 lipoplexes of the same Lip+/DNA− molar charge ratio (2.8:1), a ratio which coincided with complete association of DNA with liposomes (Fig. 3) and maximal compaction of the nucleic acid (Fig. 4). Protection against serum nuclease digestion, however, was clearly conferred on liposome-associated plasmid DNA even at a far lower liposome:DNA ratio (4:1, w/w,1:1 molar charge) (Fig. 5).

Importantly, MS09-derived lipoplexes were well tolerated by human hepatoblastoma, cervical carcinoma and oesophageal carcinoma cells at the lipoplex level resulting in the highest luciferase activity, with cell numbers at 80, 85 and 75% of untreated cultures, respectively (Fig. 6). This favourable biocompatibility of MS09 lipoplexes with human lines was further underscored particularly in the hepatoblastoma (HepG2) and cervical carcinoma (HeLa) cells where transfection levels in the presence of 10% FBS resulted in only moderate reduction of transfection activity. It has been reported that the efficiency of liposome-mediated gene expression is often adversely affected (Yang and Huang, 1997) while it has also been reported that in some cases a total inhibition of transfection may result after incubation with serum for as little as 2 h (Nchinda et al., 2002). Of interest also was the finding that MS09 lipoplexes were rather more effective in transfecting HeLa and SNO cells than Lipofectin® although in experiments with the commercial transfecting agent DNA levels were doubled. Moreover, MS09 lipoplexes showed their highest transfection activity in HeLa cells whilst Lipofectin® appeared to be most effective in NIH-3T3 cells. It has been suggested, however, that HeLa cells may be more easily transfected than fibroblasts due to the fact that after translocation of the DNA into the nucleus, it disappears rapidly in fibroblasts, but remains for longer periods in HeLa cells (Coonrod et al., 1997; Zhdanov et al., 2002).

In summary, we have prepared a new cholesterol-based cationic derivative with a relatively long 12 atom spacer which displays favourable transfection characteristics in selected human cancer lines when formulated with DOPE into unilamellar liposomes. Low cytotoxicity and good tolerance of serum by lipoplexes in cervical (HeLa) and hepato (HepG2) carcinoma cells vindicate the design criteria adopted for *N,N*-dimethylpropylamidosuccinylcholesterylformylhydrazide (MS09). These findings augur well for the further development of this system which is currently being investigated in our laboratory for possible inclusion in transfection systems with targeting capabilities.

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