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Anchor-dependent lipofection with non-glycerol based cytofectins containing single 2-hydroxyethyl head groups[1]

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Abstract

Detailed structure–activity investigations aimed at probing the anchor chain length dependency for glycerol-based lipofectins have been reported previously. Herein, we report on the first detailed investigation on the anchor-dependent transfection biology of non-glycerol based simple monocationic cytofectins containing single 2-hydroxyethyl head group functionality using 11 new structural analogs of our previously published first generation of non-glycerol based transfection lipids (lipids **1**–**11**). The C-14 and C-16 analogs of DOMHAC (lipids **4** and **5**, respectively) were found to be remarkably efficient in transfecting COS-1 cells. In addition, the present anchor-dependency investigation also revealed that the C-14 analog of DOHEMAB (lipid **10**) is significantly efficient in transfecting both COS-1 and NIH3T3 cells. Our results also indicate that too strong lipid–DNA interactions might result in weaker transfection for non-glycerol based cationic lipids. In summary, the anchor-dependence investigations presented here convincingly demonstrate that non-glycerol based cationic lipids containing a single hydroxyethyl head group and hydrophobic C-14 or C-16 anchors are promising non-toxic cationic transfection lipids for future use in liposomal gene delivery. © 2002 Published by Elsevier Science B.V.

Keywords: Cytofectin; Cationic lipid; Lipofection; Lipid–DNA interaction; Transfection

Abbreviations: DOTMA, 1,2-dioleyl-3-*N*,*N*,*N*-trimethylaminopropane chloride; DMDHP, (±)-*N*,*N*-[bis(2-hydroxyethyl)]-*N*-[2,3-bis-(tetradecanoyloxy)propyl]ammonium chloride; DMRIE, 1,2-dimyristyloxypropyl-3-dimethyl-hydroethyl ammonium bromide; DOTAP, 1,2-dioleoyloxy-3-(trimethylamino)propane; DOPE, 1,2-dioleoyl-propyl-3-phosphatidylethanolamine; DOMHAC, *N*,*N*-di-n-octadecyl-*N*-methyl,*N*-(2-hydroxyethyl)ammonium chloride; DCMHAC, *N*,*N*-di-n-capryl-*N*-methyl,*N*-(2-hydroxyethyl)ammonium chloride; DDMHAC, *N*,*N*-di-n-decyl-*N*-methyl,*N*-(2-hydroxyethyl)ammonium chloride; DLMHAC, *N*,*N*-di-n-lauryl-*N*-methyl,*N*-(2-hydroxyethyl)-ammonium chloride; DMMHAC, *N*,*N*-di-n-myristyl-*N*-methyl,*N*-(2-hydroxyethyl)ammonium chloride; DHMHAC, *N*,*N*-di-n-hexadecyl-*N*-methyl,*N*-(2-hydroxyethyl)ammonium chloride; DOHEMAB, *N*,*N*-di[*O*-hexadecanoyl]hydroxyethyl-*N*-hydroxyethyl-*N*-methylammonium bromide; DCHEMAB, *N*,*N*-di[*O*-caproyl]hydroxyethyl-*N*-hydroxyethyl-*N*-methylammonium bromide; DDHEMAB, *N*,*N*-di[*O*-decanoyl]hydroxyethyl-*N*-hydroxyethyl-*N*-methylammonium bromide; DLHEMAB, *N*,*N*-di[*O*-lauroyl]hydroxyethyl-*N*-hydroxyethyl-*N*-methylammonium bromide; DMHEMAB, *N*,*N*-di[*O*-myristoyl]hydroxyethyl-*N*-hydroxyethyl-*N*-methylammonium bromide; MOOHAC, *N*-methyl-*N*-n-octadecyl-*N*-oleyl-*N*-hydroxyethylammonium chloride

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1. Introduction

Synthetic DNA delivery agents are gaining increasing importance in gene therapy as an alternative to viral vectors [1]. Robust manufacture, simplicity of handling techniques, low immunogenic response, and ability to form stable injectable complexes even with large DNA associated with cationic lipid mediated gene delivery are increasingly making them the vectors of choice among the arsenal of non-viral transfection vectors currently in use [2–10], ([11] and references therein), [12–31]. The term 'cytofectins' specifies this class of positively charged lipid molecules that can facilitate the functional entry of polynucleotides, macromolecules, and small molecules into living cells. Molecular architectures of cationic transfection lipids are, in general, composed of three segments namely, a hydrophobic anchor, a linker, and a head group. The contribution of each of these segments on the overall transfection is inadequately understood. Based on several studies it is apparent that the physical properties of the lipid–DNA complex, viz. size, surface charge and accessibility of the DNA in a complex have strong bearing on the transfection efficiency. Mapping transfection efficiency onto various segments of the cationic lipids is a challenging exercise.

A plethora of newer and more efficient cationic transfection lipids have been reported following the pioneering development of the glycerol based cationic lipid DOTMA [1] by Felgner et al. in 1987. Interestingly, the glycerol linker between the cationic head groups and the hydrophobic tails were retained in the molecular design of many of these subsequently developed transfection lipids such as, DOTAP [31], DMDHP [18], DMRIE [8], etc. Recently, we reported four efficient non-glycerol based non-toxic monocationic transfection lipids namely, DHDEAB, MOOHAC, DOMHAC and DOHEMAB [13], in which aliphatic hydrocarbon tails containing 16 or more carbon atoms were covalently linked to the cationic head groups either directly or via an ester group linker. Out of these four non-glycerol based lipids DHDEAB, the most efficient one, contains two 2-hydroxyethyl and two n-dexadecyl aliphatic hydrocarbon tails directly attached to the quaternized nitrogen atom. The in vitro transfection efficiency of DHDEAB in COS-1 cells was found to be 2–3-fold better than that of lipofectamine, one of the most extensively used commercially available transfection lipids. The remaining three transfection lipids of lesser transfection efficiency namely, MOOHAC, DOMHAC and DOHEMAB, contained only one 2-hydroxyethyl head group functionality covalently attached to the positively charged nitrogen atom.

Detailed structure–activity investigations aimed at probing the anchor chain length dependency for glycerol-based lipofectins have been reported [6–8,18]. Herein, we report on the first detailed investigation on the anchor-dependent transfection biology of non-glycerol based simple monocationic cytofectins containing single 2-hydroxyethyl head group functionality using six new DOMHAC (lipids **1**–**6**, Chart 1) and five new DOHEMAB (lipids **7**–**11**, Chart 1) structural analogs. As delineated below, the C-14 and C-16 analogs of DOMHAC (lipids **4** and **5**, respectively, Chart 1) were found to be remarkably efficient in transfecting COS-1 cells. In addition, the present anchor-dependency investigation also revealed that the C-14 analog of DOHEMAB (lipid **10**, Chart 1) is significantly efficient in transfecting COS-1 and NIH3T3 (a hard-to-transfect primary cell line) cells. Our results also indicate that too strong lipid–DNA interactions might result in weaker transfection for non-glycerol based cationic lipids. In summary, the anchor-dependence investigations delineated in this paper convincingly demonstrate that non-glycerol based cationic lipids containing a single hydroxyethyl head group and hydrophobic C-14 or C-16 anchors are promising non-toxic cationic transfection lipids for future use in non-viral gene therapy.

2. Materials and methods

2.1. Materials

pCMV.SPORT-β-gal, cell culture media and fetal calf serum were purchased from Gibco BRL, Rockville, MD, USA. NP-40, ethidium bromide, antibiotics, agarose, *o*-nitrophenyl-β-D-galactopyranoside were purchased from Sigma, St. Louis, MO, USA. DNA molecular mass markers were purchased from Bangalore Genei, Bangalore, India. Cholesterol was purchased from Avanti Polar, AL, USA. COS-1 cell

line (SV 40 transformed African green monkey kidney, #ATCC CRL 1650) was obtained from ATCC, MD, USA. Unless otherwise stated, all reagents were of analytical grade purchased from local commercial suppliers and were used without further purification. Column chromatography was performed with silica gel (Acme Synthetic Chemicals, India, finer than 200 and 60–120 mesh).

2.2. Syntheses

Lipids **1**–**5** were synthesized following our previously published protocol [13] for the synthesis of DOMHAC (lipid **6**, Chart 1).

^{1}H-NMR (200 MHz, $CDCl_3$) of lipids **1--5**:

DCMHAC (**1**) δ/ppm = 0.90 [t, 6H, C$\underline{H}_3$-$(CH_2)_5$-]; 1.20–1.40 [m, 20H, -(C$\underline{H}_2$)$_5$-]; 1.60–1.75 [br, 4H, $CH_3(HOCH_2$-$CH_2)N^+(CH_2$-C$\underline{H}_2$-$)_2$]; 3.35 [s, 3H, C$\underline{H}_3(HOCH_2$-$CH_2)N^+(CH_2$-CH_2-$)_2$]; 3.40–3.58 [br, 2H, $CH_3(HO$-CH_2-$CH_2)N^+(C\underline{H}_2$-$CH_2$-$)_2$-]; 3.70–3.78 [br, 2H, CH_3 (HO-CH_2-C$\underline{H}_2)_2N^+(CH_2$-$CH_2$-$)_2$]; 4.07–4.13 [br, 2H, CH_3(HO-C$\underline{H}_2$-CH_2)-$N^+(CH_2$-CH_2-$)_2$].

DDMHAC (**2**) δ/ppm = 0.80–0.90 [t, 6H, C$\underline{H}_3$-$(CH_2)_7$-]; 1.15–1.45 [m, 28H, -(C$\underline{H}_2$)$_7$-]; 1.60–1.80 [br, 4H, $CH_3(HOCH_2$-$CH_2)N^+(CH_2$-C$\underline{H}_2$-$)_2$]; 3.35 [s, 3H, C$\underline{H}_3(HOCH_2$-$CH_2)N^+(CH_2$-CH_2-$)_2$]; 3.40–3.55 [br, 2H, $CH_3(HO$-CH_2-$CH_2)N^+(C\underline{H}_2$-$CH_2$-$)_2$-]; 3.65–3.78 [br, 2H, CH_3 (HO-CH_2-C$\underline{H}_2)_2N^+(CH_2$-$CH_2$-$)_2$]; 4.00–4.13 [br, 2H, CH_3(HO-C$\underline{H}_2$-CH_2)-$N^+(CH_2$-CH_2-$)_2$].

DLMHAC (**3**) δ/ppm = 0.81–0.97 [t, 6H, C$\underline{H}_3$-$(CH_2)_9$-]; 1.20–1.45 [m, 36H, -(C$\underline{H}_2$)$_9$-]; 1.60–1.80 [br, 4H, $CH_3(HOCH_2$-$CH_2)N^+(CH_2$-C$\underline{H}_2$-$)_2$]; 3.35 [s, 3H, C$\underline{H}_3(HOCH_2$-$CH_2)N^+(CH_2$-CH_2-$)_2$]; 3.41–3.58 [br, 2H, $CH_3(HO$-CH_2-$CH_2)N^+(C\underline{H}_2$-$CH_2$-$)_2$-]; 3.68–3.78 [br, 2H, CH_3 (HO-CH_2-C$\underline{H}_2)_2N^+(CH_2$-$CH_2$-$)_2$]; 4.05–4.17 [br, 2H, CH_3(HO-C$\underline{H}_2$-$CH_2)N^+$-$(CH_2$-CH_2-$)_2$].

DMMHAC (**4**) δ/ppm = 0.90 [t, 6H, C$\underline{H}_3$-$(CH_2)_{11}$-]; 1.20–1.45 [m, 44H, -(C$\underline{H}_2$)$_{11}$-]; 1.60–1.80 [br, 4H, $CH_3(HOCH_2$-$CH_2)N^+(CH_2$-C$\underline{H}_2$-$)_2$]; 3.35 [s, 3H, C$\underline{H}_3(HOCH_2$-$CH_2)N^+(CH_2$-CH_2-$)_2$]; 3.41–3.58 [br, 2H, $CH_3(HO$-CH_2-$CH_2)N^+(C\underline{H}_2$-$CH_2$-$)_2$-]; 3.70–3.80 [br, 2H, CH_3 (HO-CH_2-C$\underline{H}_2)_2N^+(CH_2$-$CH_2$-$)_2$]; 4.07–4.13 [br, 2H, CH_3(HO-C$\underline{H}_2$-$CH_2)N^+(CH_2$-CH_2-$)_2$].

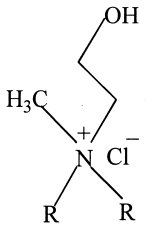
DHMHAC (**5**) δ/ppm = 0.90 [t, 6H, C$\underline{H}_3$-$(CH_2)_{13}$-]; 1.20–1.45 [m, 52H, -(C$\underline{H}_2$)$_{13}$-]; 1.60–1.80 [br, 4H, $CH_3(HOCH_2$-$CH_2)N^+(CH_2$-C$\underline{H}_2$-$)_2$]; 3.35 [s, 3H, C$\underline{H}_3(HOCH_2$-$CH_2)N^+(CH_2$-CH_2-$)_2$]; 3.41–3.58 [br, 2H, $CH_3(HO$-CH_2-$CH_2)N^+(C\underline{H}_2$-$CH_2$-$)_2$-]; 3.70–3.80 [br, 2H, CH_3 (HO-CH_2-C$\underline{H}_2)_2N^+(CH_2$-$CH_2$-$)_2$]; 4.07–4.13 [br, 2H, CH_3(HO-C$\underline{H}_2$-$CH_2)N^+(CH_2$-CH_2-$)_2$].

Lipids **7**–**10** were synthesized following our previously published protocol [13] for the synthesis of DOHEMAB (lipid **11**, Chart 1).

^{1}H-NMR (200 MHz, $CDCl_3$) of lipids **7**-**10**:

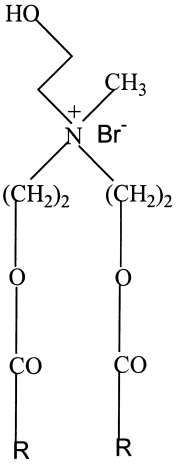
DCHEMAB (**7**) δ/ppm = 0.88 [t, 6H, C$\underline{H}_3$-$(CH_2)_4$]; 1.20–1.40 [m, 16H, -(C$\underline{H}_2$)$_4$-]; 1.50–1.70 [m, 4H, $CH_3(HOCH_2$-$CH_2)N^+(CH_2$-CH_2-O-CO-CH_2-C$\underline{H}_2$-$)_2$]; 2.22–2.40 [t, 4H, $CH_3(HOCH_2$-$CH_2)N^+$-$(CH_2$-CH_2-O-CO-C$\underline{H}_2$-CH_2-$)_2$]; 3.43 [S, 3H, C$\underline{H}_3$-$(HOCH_2$-$CH_2)N^+(CH_2$-CH_2-O-CO-CH_2-CH_2-$)_2$];

DOMHAC Analogs



Where R = C_8H_{17}, **DCMHAC**, **1**
R = $C_{10}H_{21}$, **DDMHAC**, **2**
R = $C_{12}H_{25}$, **DLMHAC**, **3**
R = $C_{14}H_{29}$, **DMMHAC**, **4**
R = $C_{16}H_{33}$, **DHMHAC**, **5**
R = $C_{18}H_{37}$, **DOMHAC**, **6**

DOHEMAB Analogs:



Where R=C_7H_{15}, **DCHEMAB**, **7**
R=C_9H_{19}, **DDHEMAB**, **8**
R=$C_{11}H_{22}$, **DLHEMAB**, **9**
R=$C_{13}H_{27}$, **DMHEMAB**, **10**
R=$C_{15}H_{31}$, **DOHEMAB**, **11**

Chart 1. DOMHAC and DOHEMAB analogs used in the study.

3.90–4.22 [m, 9H, $CH_3(\underline{H}OC\underline{H}_2\text{-}CH_2)N^+(CH_2\text{-}CH_2\text{-}O\text{-}CO\text{-}CH_2\text{-}CH_2\text{-})_2$]; 4.58–4.65 [br, t, 4H, $CH_3\text{-}(HOCH_2\text{-}CH_2)N^+(CH_2\text{-}C\underline{H}_2\text{-}O\text{-}CO\text{-}CH_2\text{-}CH_2\text{-})_2$].

DDHEMAB (8) δ/ppm = 0.88 [t, 6H, $C\underline{H}_3\text{-}(CH_2)_6$]; 1.20–1.42 [m, 24H, $\text{-}(C\underline{H}_2)_6\text{-}$]; 1.50–1.75 [m, 4H, $CH_3(HOCH_2\text{-}CH_2)N^+(CH_2\text{-}CH_2\text{-}O\text{-}CO\text{-}CH_2\text{-}C\underline{H}_2\text{-})_2$]; 2.22–2.40 [2t, 4H, $CH_3(HOCH_2\text{-}CH_2)N^+\text{-}(CH_2\text{-}CH_2\text{-}O\text{-}CO\text{-}C\underline{H}_2\text{-}CH_2\text{-})_2$]; 3.43 [S, 3H, $C\underline{H}_3\text{-}(\text{-}HOCH_2\text{-}CH_2)N^+(CH_2\text{-}CH_2\text{-}O\text{-}CO\text{-}CH_2\text{-}CH_2\text{-})_2$]; 3.90–4.20 [m, 9H, $CH_3(\underline{H}OC\underline{H}_2\text{-}CH_2)N^+(CH_2\text{-}CH_2\text{-}O\text{-}CO\text{-}CH_2\text{-}CH_2\text{-})_2$]; 4.55–4.65 [br, t, 4H, $CH_3\text{-}(HOCH_2\text{-}CH_2)N^+(CH_2\text{-}C\underline{H}_2\text{-}O\text{-}CO\text{-}CH_2\text{-}CH_2\text{-})_2$].

DLHEMAB (9) δ/ppm = 0.88 [t, 6H, $C\underline{H}_3\text{-}(CH_2)_8$]; 1.20–1.40 [m, 32H, $\text{-}(C\underline{H}_2)_8\text{-}$]; 1.50–1.65 [m, 4H, $CH_3(HOCH_2\text{-}CH_2)N^+(CH_2\text{-}CH_2\text{-}O\text{-}CO\text{-}CH_2\text{-}C\underline{H}_2\text{-})_2$]; 2.25–2.42 [2t, 4H, $CH_3(HOCH_2\text{-}CH_2)N^+\text{-}(CH_2\text{-}CH_2\text{-}O\text{-}CO\text{-}C\underline{H}_2\text{-}CH_2\text{-})_2$]; 3.45 [S, 3H, $C\underline{H}_3\text{-}(HOCH_2\text{-}CH_2)N^+(CH_2\text{-}CH_2\text{-}O\text{-}CO\text{-}CH_2\text{-}CH_2\text{-})_2$]; 3.82–4.35 [m, 9H, $CH_3(\underline{H}OC\underline{H}_2\text{-}CH_2)N^+(CH_2\text{-}CH_2\text{-}O\text{-}CO\text{-}CH_2\text{-}CH_2\text{-})_2$]; 4.48–4.68 [br, t, 4H, $CH_3\text{-}(HOCH_2\text{-}CH_2)N^+(CH_2\text{-}C\underline{H}_2\text{-}O\text{-}CO\text{-}CH_2\text{-}CH_2\text{-})_2$].

DMHEMAB (10) δ/ppm = 0.88 [t, 6H, $C\underline{H}_3\text{-}(CH_2)_{10}\text{-}$]; 1.20–1.40 [m, 40H, $\text{-}(C\underline{H}_2)_{10}\text{-}$]; 1.50–1.65 [m, 4H, $CH_3(HOCH_2\text{-}CH_2)N^+(CH_2\text{-}CH_2\text{-}O\text{-}CO\text{-}CH_2\text{-}C\underline{H}_2\text{-})_2$]; 2.25–2.40 [2t, 4H, $CH_3(HOCH_2\text{-}CH_2)N^+(CH_2\text{-}CH_2\text{-}O\text{-}CO\text{-}C\underline{H}_2\text{-}CH_2\text{-})_2$]; 3.43 [S, 3H, $C\underline{H}_3(HOCH_2\text{-}CH_2)N^+(CH_2\text{-}CH_2\text{-}O\text{-}CO\text{-}CH_2\text{-}CH_2\text{-})_2$]; 3.85–4.20 [m, 9H, $CH_3(\underline{H}OC\underline{H}_2\text{-}CH_2)N^+\text{-}(CH_2\text{-}CH_2\text{-}O\text{-}CO\text{-}CH_2\text{-}CH_2\text{-})_2$]; 4.55–4.62 [br, t, 4H, $CH_3(HOCH_2\text{-}CH_2)N^+(CH_2\text{-}C\underline{H}_2\text{-}O\text{-}CO\text{-}CH_2\text{-}CH_2\text{-})_2$].

2.3. Liposome preparation

The mixtures of cationic amphiphiles and the colipid (cholesterol) in the appropriate ratios were dissolved in chloroform in a glass vial. The chloroform was removed with a thin flow of moisture free nitrogen and the dried film of lipid left in the vial was then kept under high vacuum for 8 h. One ml of autoclaved sterile deionized water was added to the vacuum dried lipid film and the mixture was allowed to swell for 15 h (overnight). The vial was then vortexed for 2–3 min at room temperature and occasionally shaken in 45°C water bath to produce multilamellar vesicles (MLV). Small unilamellar vesicles (SUV) were then prepared by sonicating the MLV placed in an ice bath for 3–4 min until clarity using a Branson 450 sonifier at 100% duty cycle and 25 W output power.

2.4. Preparation of plasmid DNA

pCMV.SPORT-β-gal plasmid DNA was prepared by alkaline lysis procedure and purified by PEG-8000 precipitation according to Maniatis and coworkers [32]. The plasmid preparations showing OD_{260}/OD_{280} more than 1.8 were used.

2.5. Transfection assays

COS-1 and NIH3T3 cells were seeded at a density of 15 000 cells/well in a 96-well plate 18 h before the transfection. Plasmid (0.15 μg) was complexed with varying amounts of lipid (0.05–4.3 nmol) in 13 μl of plain DMEM medium for 30 min. The charge ratios were varied from 0.1:1 to 9:1 (±) over this range of the lipid. The complex was diluted to 100 μl with plain DMEM and added to the wells. After 3 h of incubation, 100 μl of DMEM with 10% FCS was added to the cells. The medium was changed to 10% complete medium after 24 h and the reporter gene activity was estimated after 48 h. The cells were washed twice with PBS and lysed in 50 μl of lysis buffer (0.25 M Tris–HCl (pH 8.0) and 0.5% NP40). Care was taken to ensure complete lysis. The β-galactosidase activity per well was estimated by adding 50 μl of 2× substrate solution (1.33 mg/ml of ONPG, 0.2 M sodium phosphate (pH 7.15) and 2 mM magnesium chloride) to the lysate in a 96-well plate. Absorption at 405 nm was converted to β-galactosidase units by using calibration curve constructed with pure commercial β-galactosidase enzyme. The values of β-galactosidase units in replicate plates assayed on the same day varied by less than 30%. The transfection efficiency values reported were average values from four replicate transfection plates assayed on the same day. Each transfection experiment was repeated three times on three different days and the day-to-day variation in average transfection efficiency values for identically treated replicate transfection plates was 2–3-fold and was dependent on the cell density and conditions of the cells.

2.6. Exclusion of ethidium bromide (EtBr) from DNA by the cationic lipids

The extent of EtBr binding to the DNA was monitored by the changes in the fluorescence. EtBr fluorescence was monitored in Hitachi 4500 fluorimeter by setting the excitation wavelength at 518 nm and emission wavelength at 585 nm. To one ml of TE buffer (pH 8.0), 0.78 nmol of DNA and 2.5 nmol of EtBr were added. The change in fluorescence was monitored after adding small volumes of lipids **1–6** to the EtBr:DNA complex. Arbitrary fluorescence values were recorded after allowing sufficient time for equilibration. The order of addition of EtBr or lipid to DNA did not alter the final values, indicating that the equilibrium does not depend on the order of addition and reaches in minutes. Percent fluorescence was calculated considering the fluorescence value in the absence of lipid as 100.

2.7. Toxicity assay

Cytotoxic effects of the lipoplexes made from lipids **1–11** were tested on COS-1 cell lines using the MTT assay as described previously [13]. The treatment protocols were identical for both cytotoxicity and transfection assays.

3. Results and discussion

3.1. Transfection biology for lipids *1–6*

The transfection efficiencies of the cationic lipids **1–6** were tested using the pCMV-β-gal plasmid carrying the β-galactosidase reporter gene. Since cationic transfection lipids work efficiently with colipids, preferably DOPE or cholesterol, we initially tested the transfection efficiencies of the lipid formulations containing the cationic lipids **1–6** using both cholesterol and DOPE as colipids at 1:1 mole ratios, and lipids **1–6** showed remarkably higher reporter gene activity with cholesterol as colipid compared to DOPE (data not shown). Lipid/DNA charge ratios between 1 and 3 produced maximal reporter gene activity (Fig. 1A,B). Above 3:1 charge ratio and up to 9:1, the transfection efficiency was poor as seen by the decrease in β-gal activity (Fig. 1A,B). In the present study we have compared the transfection efficiencies of the six lipids **1–6** with that of lipofectamine, one of the most widely used commercially available cationic transfection lipids. In COS-1 cells lipids **4–6** gave transfection comparable to lipofectamine (Fig. 1A). Lipid **5** containing C-16 hydrophobic chains was better among the monohydroxyethyl head group containing compounds, while lipids **1–3**

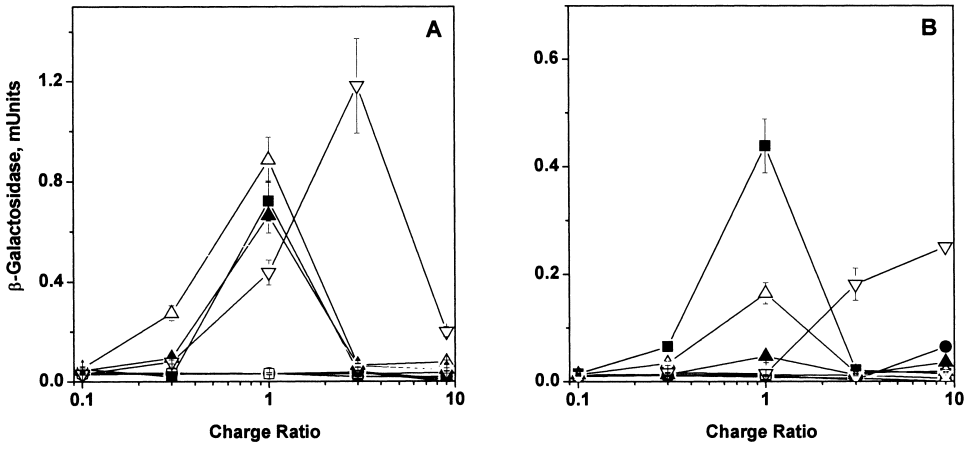


Fig. 1. Transfection efficiencies of lipids **1–6** in COS-1 cells (A) and in NIH3T3 cells (B) using cholesterol as the colipid (at 1:1 mole ratio of lipid to cholesterol). The transfection efficiencies of the lipids **1** (○), **2** (●), **3** (□), **4** (■), **5** (△) and **6** (▲) were compared with that of lipofectamine (▽). Units of β-galactosidase activity were plotted against the varying lipid to DNA (±) charge ratios. The *o*-nitrophenol formation (μmol of *o*-nitrophenol produced/min) was converted to units using standard curve obtained with pure (commercial) β-galactosidase. All lipids were tested on the same day and the data presented are an average of three experiments ($n=3$). DNA (0.3 μg) was complexed with lipid at various charge ratios in a volume of 50 μl for 30 min and added to cells after diluting the medium to 100 μl. The incubation of lipoplex with cells was allowed for 30 min before adding another 100 μl of DMEM medium. The reporter gene activity was assayed after 48 h by lysing the cells with NP40 (0.5%) containing PBS.

did not show any transfection, indicating that minimum 14-carbon anchor length is essential for imparting transfection properties to our novel mono-hydroxyethyl head group containing monocationic lipids (Fig. 1A).

In general, the presence of membrane reorganizing unsaturated hydrophobic alkenyl chains in the molecular architecture of cationic lipids is known to enhance their transfection efficiencies. However, in COS-1 cells, lipids **4**–**6** with no unsaturated anchors and MOOHAC, an unsaturated alkenyl anchor containing monocationic transfection lipid with a single hydroxyethyl head group previously reported by us [13], were found to be equally transfection efficient (Fig. 1A). Such superior transfection properties of myristyl analogs compared to palmitoyl, stearyl and oleyl derivatives have also been previously reported for cationic transfection lipids with different head groups [15,19,28].

Interestingly, unlike in COS-1 cells, lipid **4** was observed to be the only lipid that showed significant (better than lipofectamine) transfection properties in NIH3T3 cells (Fig. 1B). The cell biological basis of higher transfection by lipid **4** in NIH3T3 cells is not clear. Whether or not the significant transfection property of lipid **4** has any bearing with the efficient cellular uptake of the relevant lipoplexes is still an open question. Very recently, we have observed that lipid **4** is also remarkably efficient in transfecting HepG2 cells (G.V. Srilakshmi et al., unpublished results). Since, in principle, ligands specific to liver cell receptors can be covalently tagged to the surface of lipid **4**, such a surface-modified lipid **4** is likely to find future applications as an efficient transfection vector in targeted gene therapy of liver cancer.

3.2. Transfection biology for lipids 7–11

The conformational flexibility/rigidity of a transfection lipid as well as optimal contact between the cationic head group and the negatively charged phosphate of the DNA in lipoplexes are likely to be modulated by the linker functionality present in the molecular architecture of the transfection lipid [17]. With a view to introduce a more biodegradable linker group, an ester functionality has been synthetically incorporated in between the hydrophobic anchor and the positively charged nitrogen atoms in lipids **7**–**11** (Chart 1) using a two-carbon spacer unit.

Transfection efficiencies of these lipids **7**–**11** were tested in COS-1 and NIH3T3 cells using the pCMV-β-Gal reporter gene across the lipid/DNA charge ratios range 0.1–9 using cholesterol (in 1:1 mole ratio with respect to the cationic lipid) as the colipid. Once again, striking C-14 anchor dependency was observed in both COS-1 and NIH3T3 cells (Fig. 2A,B). The lipids **7**–**9** and **11** did not show any transfection in both COS-1 and NIH3T3 cells whereas lipid **10** showed higher transfection efficiency to lipofectamine in NIH3T3 cells and somewhat less than

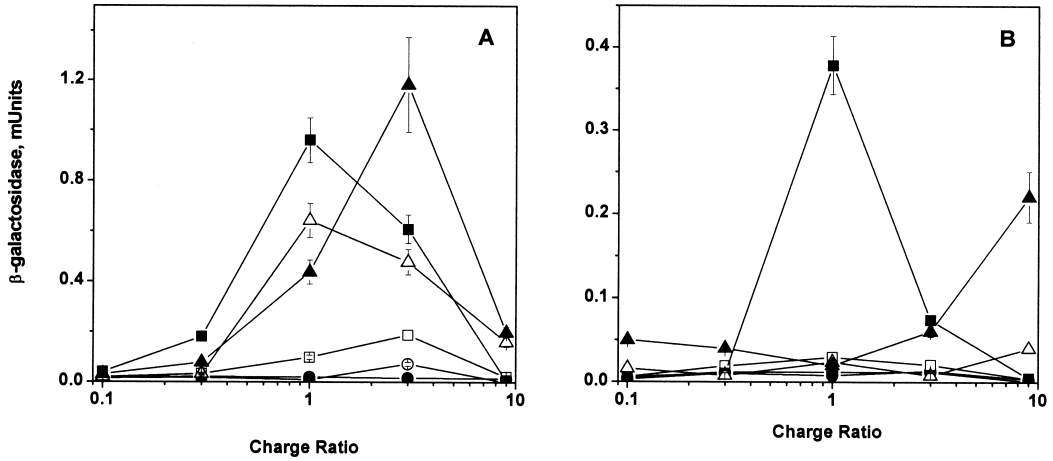


Fig. 2. Transfection efficiencies of lipids **7**–**11** in COS-1 cells (A) and in NIH3T3 cells (B) using cholesterol as the colipid (at 1:1 mole ratio of lipid to cholesterol). The transfection efficiencies of the lipids **7** (○), **8** (●), **9** (□), **10** (■) and **11** (△) were compared with that of lipofectamine (▲). Experimental details are described in Fig. 1.

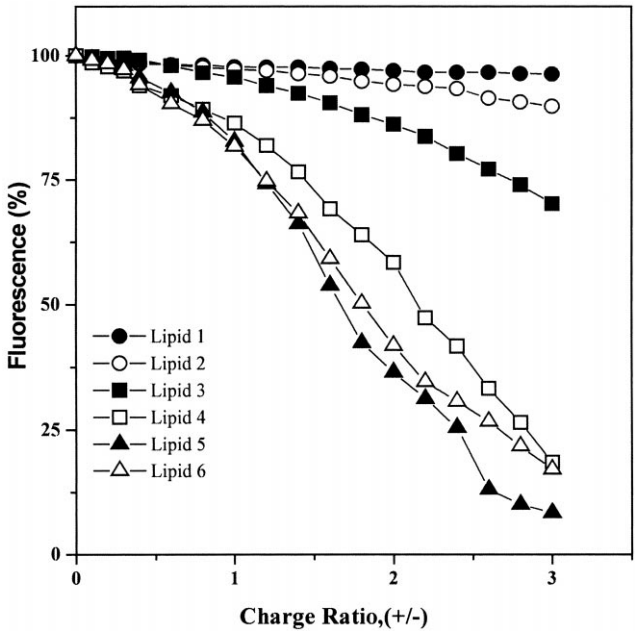


Fig. 3. EtBr exclusion from lipid–DNA complex. Decrease in fluorescence of EtBr was used to assess the interaction of DNA with lipids **1**–**6**. DNA:EtBr complex was titrated with increasing amounts of lipid. The order of addition of lipid or EtBr to DNA did not alter the observed profiles. Fluorescence in the absence of lipid was taken to be 100. The data presented are an average of two independent experiments ($n=2$).

lipofectamine in COS-1 cells (Fig. 2A,B). The optimal lipid to DNA charge ratio for lipid **10** was observed to be 1:1 in both COS-1 and NIH3T3 cells (Fig. 2A,B). As in the case of lipid **4** mentioned above, whether or not the significant transfection property of lipid **10** has any relationship with the efficient cellular uptake of the relevant lipoplexes cannot be ascertained from our present findings. Potential biodegradability and comparable transfection efficiency to lipofectamine in both COS-1 and NIH3T3 cells, makes lipid **10** a promising cationic lipid.

3.3. Lipid–DNA interactions

Intercalation-induced fluorescence increase and competition with cationic lipids to bind to DNA has made EtBr an excellent tool to study cationic lipid–DNA interactions. To assess the representative lipid–DNA interactions of the presently described mono-hydroxyethyl head group containing transfection lipids, we have titrated the EtBr:pCMV β-gal complex with increasing amounts of cationic lipids **1**–**6**. The data in Fig. 3 shows that lipids **1**–**3** interact poorly with DNA as seen by their relatively poor ability to exclude ethidium bromide from DNA. Lipids **4**–**6** interact with DNA equally well (Fig. 3) though lipid **4** is marginally weaker than the other two. The decrease in EtBr fluorescence at charge ratios of 3:1 was greater than 80% with lipids **4**–**6**, and greater than 30% with lipid **3**, whereas there was no significant decrease in florescence with lipids **1** and **2**. Given their shorter hydrophobic anchor lengths, lipids **1**–**3** may not form stable lipoplexes that are required for uptake of DNA by the cells. DNA that is not present in a lipid complex either may get degraded by the serum proteins and/or may enter into a non-productive pathway.

3.4. Toxicity assay

An MTT-based viability assay was performed to assess the cytotoxicity of the six lipid formulations (**1**–**6**) at various charge ratios with COS-1 cells. Lipids **3** and **6** showed least cytotoxicity even at a 9:1 lipid/DNA charge ratio (Fig. 4). For lipids **1**, **4** and **5** at a 3:1 charge ratio the toxicity varied from 15–25%

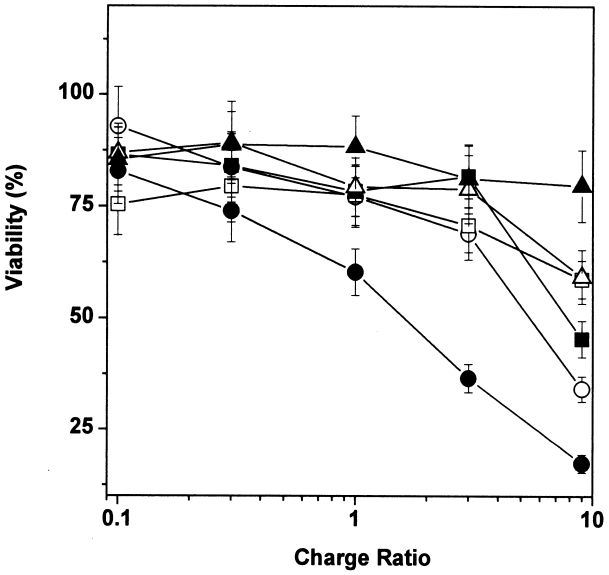


Fig. 4. Cytotoxicity (viability) of lipids **1**–**6** on COS-1 cells. Toxicities were assessed in COS-1 cells using MTT assay. The absorption obtained with reduced formazon with cells in the absence of lipids was taken to be 100. The data presented are an average of three independent experiments ($n=3$). Lipids: **1** (○), **2** (●), **3** (□), **4** (■), **5** (△), **6** (▲).

of the treated cells. Lipid **2** at a 3:1 charge ratio showed the highest toxicity with 40% of the treated cells being affected. The toxicity of lipid **2** (with shorter alkyl chains) may originate from its probable detergent-like cell-lysing activity. The MTT based viability assay was also performed to assess the cytotoxicity of the five lipid formulations (**7**–**11**) at various charge ratios with COS-1 cells. The lipid–DNA complexation and treatment were identical to the transfections (vide supra). Lipids **8**–**11** showed low cytotoxicity even at a 9:1 charge ratio (Fig. 5). For lipids **7**–**11** at a 3:1 charge ratio, the toxicity varied from 10–20% of the treated cells being affected. Lipid **7** at a 3:1 charge ratio showed the highest toxicity with 25% of the treated cells being affected. The toxicity of lipid **7** with its shorter alkyl chains may, like lipid **2**, originate from its probable detergent-like cell-lysing activity.

In summary, the transfection efficiencies of the C-14 and C-16 analogs of DOMHAC (lipids **4** and **5**, respectively, Chart 1) were found to be remarkably efficient in transfecting COS-1 cells (comparable to lipofectamine). Additionally, the present anchor-dependency investigation also revealed that the C-14 analog of DOHEMAB (lipid **10**, Chart 1) is significantly efficient in transfecting COS-1 and NIH3T3 cells. To conclude, the anchor-dependence investigations delineated in this study convincingly demonstrate that non-glycerol based cationic lipids containing a single hydroxyethyl head group and hydrophobic C-14 or C-16 anchors are promising non-toxic cationic transfection lipids for future use in non-viral gene therapy.

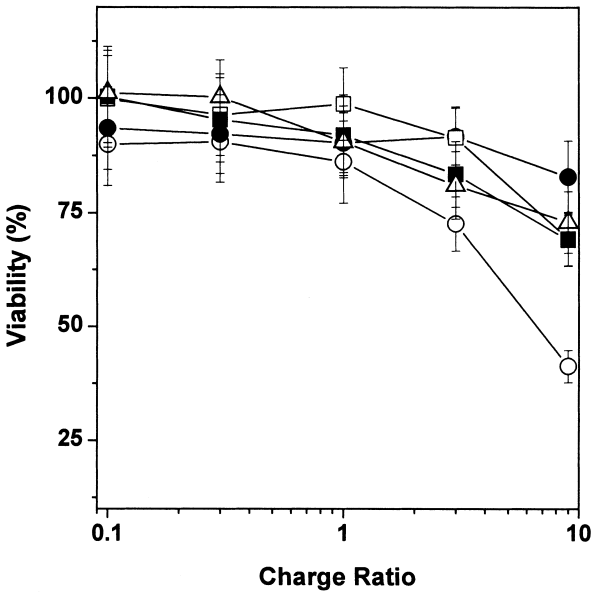


Fig. 5. Cytotoxicity (viability) of lipids **7**–**11** on COS-1 cells. Toxicities were assessed in COS-1 cells using MTT assay. The absorption obtained with reduced formazon with cells in the absence of lipids was taken to be 100. The data presented are an average of three independent experiments ($n=3$). Lipids: **7** (○), **8** (●), **9** (□), **10** (■), **11** (△).

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References

[1] P.L. Felgner, T.G. Gadek, M. Holm, R. Roman, H.W. Chan, M. Wenz, J.P. Northorp, G.M. Ringold, M. Danielsen, Proc. Natl. Acad. Sci. USA 84 (1987) 7413–7417.
[2] X. Gao, L. Huang, Biochem. Biophys. Res. Commun. 179 (1991) 280–285.
[3] J.P. Behr, B. Demeneix, J.P. Loeffler, J. Perez-Mutul, Proc. Natl. Acad. Sci. USA 86 (1989) 6982–6986.
[4] P. Hawley-Nelson, V. Ciccarone, G. Gebeyehu, J. Jesse, P.L. Felgner, Focus 15 (1993) 73–79.
[5] E.R. Lee, J. Marshall, C.S. Siegel, C. Jiang, N.S. Yew, M.R. Nichols, J.B. Nietupsky, R.J. Ziegler, M.B. Lane, K.X. Wang, N.C. Wan, R.K. Scheule, D.J. Harris, A.E. Smith, H. Cheng, Hum. Gene Ther. 7 (1996) 1701–1717.
[6] A.E. Regelin, S. Fankhaenel, L. Gurtesch, C. Prinz, G.V. Kiedrowski, U. Massing, Biochim. Biophys. Acta 1464 (2000) 151–164.
[7] T. Ren, Y.K. Song, G. Zhang, D. Liu, Gene Ther. 7 (2000) 764–768.
[8] J.H. Felgner, R. Kumar, C.N. Sridhar, C.J. Wheeler, Y.-J. Tsai, R. Border, P. Ramsey, M. Martin, P.L. Felgner, J. Biol. Chem. 269 (1994) 2550–2561.
[9] D. Luo, W.M. Saltzman, Nat. Biotechnol. 18 (2000) 33–37.
[10] S. Li, L. Huang, Gene Ther. 7 (2000) 31–34.
[11] A.D. Miller, Angew. Chem. Int. Engl. Edn. 37 (1998) 1768–1785.
[12] S. Kawakami, A. Sato, M. Nishikawa, F. Yamashita, M. Hashida, Gene Ther. 7 (2000) 292–299.

[13] R. Banerjee, P.K. Das, G.V. Srilakshmi, A. Chaudhuri, N.M. Rao, J. Med. Chem. 42 (1999) 4292–4299.
[14] G.H. Camerol, M.J. Moghddam, V.J. Bender, R.G. Whittaker, M. Mott, T.J. Lockett, Biochim. Biophys. Acta 1417 (1999) 37–50.
[15] J. Wang, X. Guo, Y. Xu, L. Barron, F.C. Szoka, J. Med. Chem. 41 (1998) 2207–2215.
[16] G. Byk, C. Dubertret, V. Escriou, M. Frederic, G. Jaslin, R. Rangaara, B. Pitard, J. Crouzet, P. Wils, B. Schwartz, D. Scherman, J. Med. Chem. 41 (1998) 224–235.
[17] G. Byk, B. Wetzer, M. Frederic, C. Dubertret, B. Pitard, G. Jaslin, D. Scherman, J. Med. Chem. 43 (2000) 4377–4387.
[18] M.J. Bennett, A.M. Aberle, R.P. Balasubramaniam, J.G. Malone, R.W. Malone, M.H. Nantz, J. Med. Chem. 40 (1997) 4069–4078.
[19] V. Floch, G.Le. Bolc'h, C. Gable-Guillaume, N.Le. Bris, J.-J. Yaouanc, H.Des. Abbayes, C. Fe'rec, J.-C. Cle'ment, Eur. J. Med. Chem. 33 (1998) 923–934.
[20] S. Kawakami, F. Yamashita, M. Nishikawa, Y. Takakura, M. Hashida, Biochem. Biophys. Res. Commun. 252 (1998) 78–83.
[21] A.M. Aberle, F. Tablin, J. Zhu, N.J. Walker, D.C. Gruenert, M.H. Nantz, Biochemistry 37 (1998) 6533–6540.
[22] Y. Liu, L.C. Mounkes, H.D. Liggitt, C.S. Brown, I. Solodin, T.D. Heath, R.J. Debs, Nat. Biotechnol. 15 (1997) 167–173.
[23] N.S. Templeton, D.D. Lasic, P.M. Frederik, H.H. Strey, D.D. Roberts, G.N. Pavlakis, Nat. Biotechnol. 15 (1997) 647–652.
[24] N.J. Caplen, E.W. Alton, P.G. Middleton, J.R. Dorin, B.J. Stevension, X. Gao, S.R. Durham, P.K. Jeffery, M.E. Hodson, C. Coutelle, L. Huang, D.J. Porteous, R. Williamson, D.M. Geddes, Nat. Med. 1 (1995) 39–46.
[25] X. Gao, L. Huang, Biochemistry 35 (1996) 1027–1036.
[26] H.E.J. Hofland, L. Shephard, S.M. Sullivan, Proc. Natl. Acad. Sci. USA 93 (1996) 7305–7309.
[27] C.J. Wheeler, P.L. Felgner, Y.J. Tsai, J. Marshall, L. Sukhu, G. Doh, J. Hartikka, J. Nietupski, M. Manthorpe, M. Nichols, M. Plewe, X. Liang, J. Norman, A. Smith, S.H.A. Cheng, Proc. Natl. Acad. Sci. USA 93 (1996) 11454–11459.
[28] I. Solodin, C. Brown, M. Bruno, C. Chow, E.-H. Jang, R. Debs, T. Heath, Biochemistry 34 (1995) 13537–13544.
[29] Y. Liu, D. Liggitt, G. Tu, W. Zhong, K. Gaensler, R. Debs, J. Biol. Chem. 270 (1995) 24864–24870.
[30] E.W. Alton, P.G. Middleton, N.J. Caplen, S.N. Smith, D.M. Steel, F.M. Munkonge, P.K. Jeffery, B.J. Stevension, G. McLachlan, J.R. Dorin, D.J. Porteous, Nat. Genet. 5 (1993) 135–142.
[31] R. Leventis, J.R. Silvius, Biochim. Biophys. Acta 1023 (1990) 124–132.
[32] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.