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Single additional methylene group in the head-group region imparts high gene transfer efficacy to a transfection-incompetent cationic lipid

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Abstract **In combination with equimolar 1,2-dioleoyl-L-α-glycero-3-phosphatidyl ethanolamine, a novel cholesterol-based cationic lipid with β-alanine head-group (2) has been demonstrated to be strikingly more efficacious (10–24-fold) in transfecting CHO, COS-1 and HepG2 cells than its glycine analog (1) containing just one less methylene unit in its head-group region. Syntheses, characterizations and in vitro transfection biology of lipids 1 and 2 are described. Present findings demonstrate that even truly minor structural alterations, such as inclusion of just one additional methylene functionality in the polar head-group region, can convert an essentially transfection-incompetent cholesterol-based cationic amphiphile to a remarkably efficient cationic transfection lipid.**

Key words: DNA transfection; Cytofectin; Lipoplex; Cholesterol-based cationic amphiphile; Lipofection; Transgene expression

1. Introduction

Gene therapy is becoming increasingly important as a therapeutic modality to combat myriads of inherited diseases, dreadful viral infections and cancer. Designing safe and efficient carriers of therapeutic genes into body cells is one of the key challenges to ensure clinical success of gene therapy [1–3]. Recombinant retroviral vectors, one of the most powerful viral transfection vectors used for clinical applications in gene therapy, are remarkably efficient in transfecting body cells [4,5]. However, despite wide exploitation of retroviral gene transfer in many gene therapy trials, an alarming number of biosafety concerns and risks associated with the use of retroviral vectors are impeding their clinical success. Retroviral vectors, although generally replication-incompetent, are capable of: generating replication-competent virus through recombination events with the host genome; inducing inflammatory and adverse immunogenic responses against the structural components; producing insertional mutagenesis through random integration into the host genome etc. [6–9]. Additional major disadvantages of using viral vectors include their incapability of transfecting non-dividing cells, limited insert size and difficulty of large-scale production [4–9]. Conversely, because of their very low immunogenic nature, robust manufacture, ability to deliver large pieces of DNA, and ease of handling and preparation techniques, cationic liposomes are increasingly becoming the alternative non-viral vectors of choice in gene therapy [10–18].

One of the key parameters dictating DNA transfection efficiency in liposomal gene delivery is the molecular structure of the cationic lipid. Three major structural components of cationic transfection lipids (also known as cytofectins) include: a hydrophilic polar head-group region; a hydrophobic non-polar tail region consisting of either two long aliphatic hydrocarbon chains or a cholesterol unit and a small linker functionality connecting the hydrophobic tail and hydrophilic head-groups [19]. Investigations aimed at probing the role of each of these three structural components in modulating gene transfer efficacies of cytofectins [20–23] including our own [24–27] continue to be an intensively pursued area of research in non-viral gene delivery. Herein, we report on the syntheses, characterization and the phenomenally contrasting DNA transfection efficacies of two novel lipids namely, 2-(*N*-glycinyl)aminoethyl-cholesteryl ether (lipid **1**, Fig. 1) and 2-(*N*-β-alaninyl)aminoethyl-cholesteryl ether (lipid **2**, Fig. 1) bearing the same cholesterol hydrophobic skeleton, the same amide linker and differing by only *one* methylene unit in their polar head-group region. As delineated below, our present findings demonstrate that even truly minor structural alterations, such as inclusion of an additional single methylene functionality in the head-group region, can strikingly convert an essentially transfection-incompetent cholesterol-based cationic amphiphile to a remarkably efficient cationic transfection lipid.

2. Materials and methods

2.1. Materials

Amberlyst A-26 was purchased from Lancaster (Morecambe, UK). Carbonyl diimidazole (CDI), *N*,*N*-dimethylethylenediamine and cholesterol chloroformate were procured from Fluka, Switzerland. Column chromatography was performed with silica gel (Acme Synthetic Chemicals, India, 60–120 mesh). Cell culture media, fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), polyethylene glycol 8000, *o*-nitrophenyl-β-D-galactopyranoside (ONPG), β-galactosidase enzyme and 1,2-dioleoyl-L-α-glycero-3-phosphatidyl ethanolamine (DOPE) were purchased from Sigma (St. Louis, MO, USA). NP40, antibiotics and agarose were purchased from Hi-media, India. DC-Chol was synthesized as re-

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; FBS, fetal bovine serum; CDI, carbonyl diimidazole; DCM, dichloromethane; TFA, trifluoroacetic acid; DOPE, 1,2-dioleoyl-L-α-glycero-3-phosphatidyl ethanolamine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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ported previously [16]. Unless otherwise stated all the other reagents purchased from local commercial suppliers were of analytical grade and were used without further purification. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a Varian FT 200 MHz, AV 300 MHz or Varian Unity 400 MHz. The FABMS analyses were performed on a Micromass AUTOSPEC-M mass spectrometer (Manchester, UK) with OPUS V3, 1× data system. Data were acquired by liquid secondary ion mass spectrometry (LSIMS) using *meta*-nitrobenzyl alcohol as the matrix.

2.2. Syntheses

The synthetic procedures for preparing lipids **1** and **2** are depicted schematically in Fig. 1. Detailed experimental procedures are delineated below for lipid **2** as a representative example. Lipid **1** was synthesized following essentially the same procedure.

Step a: Synthesis of *N*-BOC-β-alanine-cholesteryl ethylamide (II, Fig. 1). *N*-BOC-β-alanine (88 mg, 0.47 mmol) was dissolved in dry dichloromethane (DCM, 1 ml) in a two-necked 25 ml round-bottomed flask under nitrogen atmosphere and CDI (76 mg, 0.47 mmol) dissolved in dry DCM (1 ml) was added dropwise. After stirring the reaction mixture at room temperature for 3 h, 2-aminoethyl cholesteryl ether, **I** (200 mg, 0.47 mmol, a readily available cholesterol derivative in our laboratory prepared conventionally from the corresponding primary alcohol in three steps) dissolved in dry DCM (2 ml) was added and stirring was continued at room temperature for 19 h. Column chromatographic purification (using 60–120 mesh size silica and 15–20% v/v acetone in hexane as the eluent) of the residue afforded lipid **2** as a white solid (259 mg, 93%, $R_f = 0.4$, 1:4 v/v, acetone:hexane).

^{1}H NMR (*N*-BOC-β-alanine-cholesteryl ethylamide, lipid **2**, 200 MHz, $CDCl_3$): $\delta = 0.60$–2.30 [m, 43H, cholesteryl skeleton], 1.40 [s, 9H, $(CH_3)_3COCONH$-], 2.35 [t, 2H, BOC-NH-CH_2-CH_2-CO-], 3.10 [m, 1H, $H_{3\alpha}$(Chol)], 3.30–3.75 [m, 6H, -CO-NH-CH_2-CH_2-O-+BOC-NH-CH_2-CH_2-CO-], 5.15 [bs, 1H, BOC-N*H*-CH_2-CH_2-CO-], 5.30 [m, 1H, H_6(Chol)], 5.95 [brs, 1H, -CON*H*-CH_2-CH_2-].

^{1}H NMR (*N*-BOC-glycine-cholesteryl ethylamide, lipid **1**, 300 MHz, $CDCl_3$): $\delta = 0.60$–2.35 [m, 43H, cholesteryl skeleton], 1.40 [s, 9H, $(CH_3)_3COCONH$-], 3.15 [m, 1H, $H_{3\alpha}$(Chol)], 3.40–3.60 [m, 4H, -CO-NH-CH_2-CH_2-O-], 3.85 [d, 2H, BOC-NH-CH_2-CO-], 5.10 [bs, 1H, BOC-N*H*-], 5.40 [m, 1H, H_6(Chol)], 6.40 [brs, 1H, -CON*H*-CH_2-CH_2-].

Steps b and c. *N*-BOC-β-alanine-cholesteryl ethylamide, **II** (intermediate product obtained from step **a**, 170 mg, 0.28 mmol) was taken in a 25 ml round-bottomed flask and dissolved in 1.30 ml of trifluoroacetic acid (TFA):DCM (1:4, v/v). After stirring the reaction at room temperature for 70 min, the TFA–DCM mixture was completely removed with a stream of nitrogen gas. Column chromatographic purification of the residue (using 60–120 mesh size silica and 7–8% v/v methanol in chloroform as the eluent) followed by chloride ion exchange in Amberlyst A-26 using methanol as the eluent afforded lipid **2** as a white solid (140 mg, 92%, $R_f = 0.3$, 10% v/v methanol in chloroform).

^{1}H NMR (lipid **2**, 300 MHz, $CDCl_3$): $\delta = 0.60$–2.40 [m, 43H, cholesteryl skeleton], 2.85 [bs, 2H, $^+NH_3$-CH_2-CH_2-CO-], 3.15 [m, 1H, $H_{3\alpha}$(Chol)], 3.30–3.85 [m, 6H, -NH-CH_2-CH_2-O-+$^+NH_3$-CH_2-CH_2-CO-], 5.30 [s, 1H, H_6(Chol)], 7.80–8.10 [brs, 4H, -CON*H*-CH_2-CH_2-+$^+NH_3$-CH_2-CH_2-CO-].

FABMS (LSIMS): *m*/*z*: 501 [M^+] for $C_{32}H_{57}N_2O_2$.

^{1}H NMR (lipid **1**, 300 MHz, $CDCl_3$): $\delta = 0.60$–2.40 [m, 43H, cholesteryl skeleton], 3.15 [m, 1H, $H_{3\alpha}$(Chol)], 3.30–3.60 [m, 4H, -NH-CH_2-CH_2-O-], 4.00 [brs, 2H, $^+NH_3$-CH_2-CO-], 5.30 [s, 1H, H_6(Chol)], 8.55 [brs, 1H, -CON*H*-CH_2-CH_2-].

FABMS (LSIMS): *m*/*z*: 487 [M^+] for $C_{31}H_{55}N_2O_2$.

2.3. Cells and cell culture

CHO (Chinese hamster ovary), COS-1 (SV 40 transformed African green monkey kidney cells) and HepG2 (human hepatocarcinoma) cell lines were procured from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, 50 μg/ml penicillin, 50 μg/ml streptomycin and 20 μg/ml kanamycin in a humidified atmosphere containing 5% CO_2.

2.4. Preparation of plasmid DNA

pCMV-SPORT-β-gal plasmid was a generous gift from Dr. Nalam Madhusudhana Rao (Centre for Cellular and Molecular Biology, Hyderabad, India). Plasmid was amplified in *Escherichia coli* DH5α, isolated by alkaline lysis and finally purified by PEG-8000 precipitation as described previously [28]. The purity of plasmid was checked by the A_{260}/A_{280} ratio (around 1.9) and 1% agarose gel electrophoresis.

2.5. Preparation of liposome

Cationic lipids (**1** and **2**) and DOPE (at 1:1 molar ratio) dissolved in chloroform were dried under a stream of nitrogen gas, vacuum desiccated for a minimum period of 4 h and hydrated in HEPES buffer pH 7.4 overnight at a final cationic lipid concentration of 1 mM. The hydrated lipid films were vortexed and then sonicated to clarity using a Ti-probe sonicator. Liposomes of DC-Chol were prepared by the ethanol injection method. Briefly, 70 μl of an ethanolic solution of DC-Chol and DOPE (at 3:2 molar ratio) was rapidly injected into 930 μl of HEPES buffer, pH 7.4 under vortexing to give a final cationic lipid concentration of 1 mM.

2.6. Transfection procedure

Cells were seeded at a density of 20 000 cells/well (for CHO and HepG2) or 15 000 cells/well (for COS-1) in a 96 well plate usually 18–24 h before transfection. 0.30 μg of plasmid DNA (diluted to 50 μl with plain DMEM) was complexed with varying amounts of cationic liposomes (diluted to 50 μl with plain DMEM) for 15–30 min. The molar ratios (lipid:DNA) were varied from 0.5:1 to 8:1. Cells were washed twice with phosphate-buffered saline (PBS), pH 7.40 (100 μl each) and the lipid:DNA complex was added to the cells. After incubation for 3 h in a humidified atmosphere containing 5% CO_2 at 37°C, 100 μl of DMEM containing 20% FBS was added to the cells. The medium was changed to complete medium containing 10% FBS after 24 h and the reporter gene activity was assayed after 48 h. Cells were washed once with PBS buffer, pH 7.40 (100 μl) and lysed with 50 μl of lysis buffer (0.25 M Tris–HCl, pH 8.0 and 0.5% NP40). 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 7.30 and 2 mM magnesium chloride) to the cell lysate in a 96 well plate. Absorption of the product *ortho*-nitrophenol at 405 nm was converted to absolute β-galactosidase units using a calibration curve constructed with pure (commercial) β-galactosidase enzyme. The transfection values reported are the average values of two replicate experiments performed in the same plate on the same day. Each transfection experiment was performed three times on three different days. The day to day variation in transfection efficiency was mostly within two- to three-fold and was dependent on the cell density and condition of the cells.

2.7. X-gal staining

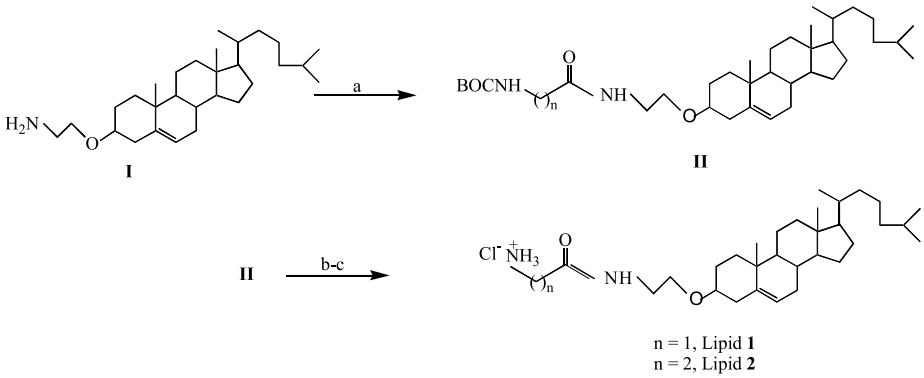
Cells expressing β-galactosidase were histochemically stained with the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as described previously [29]. Briefly, 48 h after transfection with lipoplexes in 96 well plates, the cells were washed two times (2×100 μl) with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and fixed with 0.5% glutaraldehyde in PBS (225 μl). After 15 min incubation at room temperature, the cells were washed again with PBS three times (3×250 μl) and subsequently stained with 1.0 mg/ml X-gal in PBS containing 5.0 mM $K_3[Fe(CN)_6]$, and 5.0 mM $K_4[Fe(CN)_6]$ and 1 mM $MgSO_4$ for 2–4 h at 37°C. Blue colored cells were identified by light microscope (Leica, Germany). A minimum of 100 cells were counted to determine the percentage of cells expressing β-galactosidase.

2.8. Cell viability assay

Cytotoxicity of the cationic lipids was assessed using the MTT reduction assay as described earlier [30]. The cytotoxicity assay was performed in 96 well plates by maintaining the ratio of number of cells to amount of cationic lipid constant as in transfection experiments. Briefly, 3 h after the addition of lipoplexes, MTT (5 mg/ml PBS) was added to cells and incubated for 3–4 h at 37°C in a CO_2 incubator. Results are expressed as percent viability = [A_{550}(treated cells)−background]/[A_{550}(untreated cells)−background]×100.

2.9. Size and surface charge measurements

The sizes and the global surface charges (ζ potentials) of liposomes and lipoplexes in HEPES buffer, pH 7.4, or plain DMEM were mea-



Reagents: (a) N-BOC-amino acid/CDI/DCM; (b) TFA:DCM (1:4); (c) Cl^- exchange with Amberlyst A-26.

Fig. 1. Synthesis of lipids **1** and **2**.

sured by photon correlation spectroscopy and electrophoretic mobility with a Zetasizer 3000HSA (Malvern Instruments, UK). The system was calibrated using the 199 ± 6 nm Nanosphere™ Size Standard (Duke Scientific, Palo Alto, CA, USA) and DTS 0050 standard from Malvern.

3. Results and discussion

Fig. 2A–C summarizes the efficacies of lipids **1** and **2** (used as cationic liposomes prepared in combination with an equimolar amount of DOPE as co-lipid) in transfecting CHO, COS-1 and HepG2 cells across the increasing lipid:DNA molar ratios 0.5:1.0–8.0:1.0 (using pCMV-SPORT-β-gal plasmid as the reporter gene). Although lipids **1** and **2** structurally differ from each other by only one CH_2 unit in the headgroup region (all the other structural components being exactly the same), the in vitro gene delivery efficacies of lipid **2** were found to be strikingly superior to those of lipid **1** in all three cell lines (Fig. 2A–C). While the transfection efficiencies of lipid **2** at lipid:DNA molar ratios of 2:1 or 1:1 were comparable to or better than those of DC-Chol (one of the most popular, easy-to-synthesize and extensively used cholesterol-based cationic transfection lipids in liposomal gene delivery), lipid **1** was found to be about 10–24-fold less efficient (essentially transfection-inefficient) in transfecting all three cell lines (Fig. 2A–C). In addition, with a view to measuring the relative percentage profiles of the transfected cells for lipids **1**, **2** and DC-Chol, the representative CHO cells were histochemically stained with X-gal after 48 h of transfection using the optimal lipid:DNA molar ratio of 2:1. Such histochemical staining of whole cells showed that the percentages of transfected CHO cells with lipid **2** were significantly higher than those with lipids **1** and DC-Chol (Fig. 3), a result consistent with the relative transfection efficacy profiles of these lipids obtained by quantitative estimation of the expressed β-galactosidase (Fig. 2A–C).

Interestingly, while lipid **2** showed markedly higher transfection efficacies than lipid **1** in all three cell lines, the relative transfection properties of lipid **2** and DC-Chol were found to be cell-dependent (Fig. 2A–C). The origin of such cell-dependent relative transfection profiles of lipid **2** and DC-Chol remains elusive at this point of investigation. The overall transfection efficiencies of both lipids **2** and DC-Chol in COS-1 and CHO cells were observed to be significantly higher than those in HepG2 cells (Fig. 2A–C). Such relatively weak transfection behavior of lipid **2** and DC-Chol in HepG2 cells may partly originate from the lower cell proliferating rate of HepG2 cells compared to that of COS-1 and CHO cells. Taken together, the relative transfection properties of lipids **1** and **2** in COS-1, CHO and HepG2 cells summarized in Fig. 2A–C convincingly demonstrate that even truly minor structural variations are also capable of adversely impeding the gene delivery efficacies of cholesterol-based cationic transfection lipids.

With a view to gaining insight into whether the phenomenally different in vitro DNA transfection properties of lipids **1** and **2** were due to their varying inherent toxicity profiles, MTT-based cell viability assays were performed in HepG2 cells across the entire range of lipid:DNA molar ratios used

Table 1
Sizes and ζ potentials of liposomes and lipoplexes[a]

Lipid	Lipid:DNA (molar ratio)					
	1:0	0.5:1	1:1	2:1	4:1	8:1
	Size (nm)					
1	231 ± 2.1	705.2 ± 29.1	654.8 ± 24.8	733.3 ± 23.8	940.7 ± 10.7	1000.6 ± 21.8
2	116.3 ± 3.1	518.7 ± 12.6	373.7 ± 3.7	446.6 ± 15.5	690.9 ± 7.4	1638.6 ± 133.5
	Zeta potential (mV)					
1	28.7 ± 2.2	−34.8 ± 1.3	−32.5 ± 1.1	−33.8 ± 1.1	−33.3 ± 1	−32.9 ± 0.8
2	22.8 ± 3.2	−36.7 ± 0.8	−36.5 ± 0.6	−36.4 ± 0.8	−33.4 ± 1	−23.6 ± 2

[a]Sizes and ζ potentials of liposomes and lipoplexes (in HEPES buffer, pH 7.40 and plain DMEM, respectively) were measured by laser light scattering using a Zetasizer 3000A (Malvern Instruments, UK). Values shown are the averages of three (for size) and 10 measurements (for ζ potential).

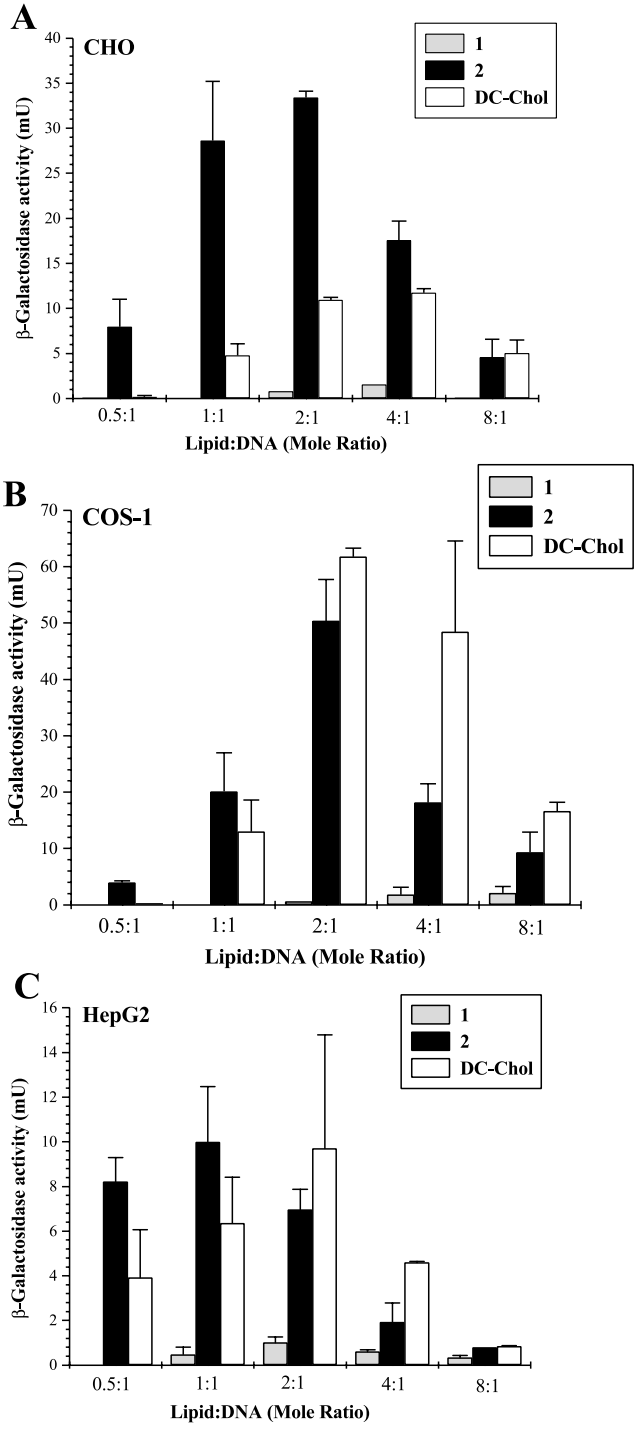


Fig. 2. A–C: Transfection efficiencies of cationic lipids **1**, **2** and DC-Chol in CHO (A), COS-1 (B) and HepG2 (C) cells. The β-galactosidase activities in each well was converted to absolute β-galactosidase milliunits using a standard curve obtained with pure (commercial) β-galactosidase. All the lipids were tested on the same day and the data shown are the average value of two replicate experiments performed on the same day ($n=2$). Each transfection experiment was performed three times on three different days.

in the actual transfection experiments. Cell viabilities of both lipid **1** and **2** were found to be remarkably high (>80% cell viability) up to a lipid:DNA charge ratio of 4:1 (Fig. 4). Thus, the contrasting transfection behaviors of lipids **1** and **2** (Fig. 2) are unlikely to originate from varying cell cytotoxicities of the lipids. With a view to probing the role of sizes and global surface charges of the lipoplexes (if any) in modulating the transfection properties of lipids **1** and **2**, we next measured the ζ potentials and particle sizes of the lipoplexes made from lipids **1** and **2** across the varying lipid:DNA molar ratios in the presence of DMEM using a dynamic laser light scattering instrument equipped with ζ-sizing capacity. Interestingly, ζ potentials of the lipoplexes prepared from both lipid **1** and **2** were found to remain essentially unchanged around −30 to −35 mV as the lipid:DNA charge ratios were increased from 0.5:1 to 4:1 (Table 1). Thus, the surface potential characteristics summarized in Table 1 convincingly demonstrate that the lipoplex global surface charge is unlikely to play any dominant role in influencing transfection efficacies of the presently described cholesterol-based cationic lipids. Surprisingly, both liposomes and lipoplexes prepared from lipid **2** having the additional methylene unit in its head-group region were measured to be about 1.5–2.0-fold smaller than those prepared from lipid **1** (Table 1). Thus, relatively poor endocytotic cellular uptake of these larger lipoplexes of lipid **1**

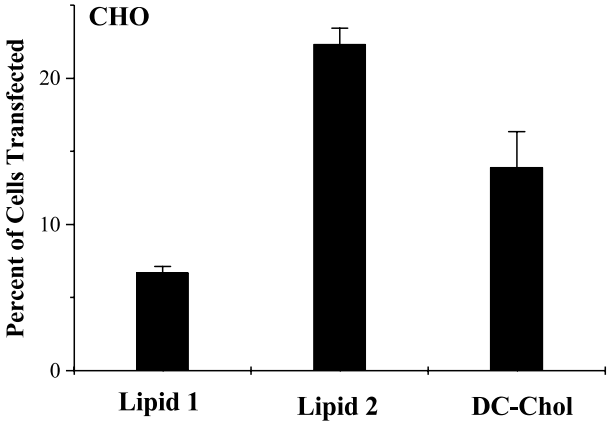


Fig. 3. Histochemical X-gal staining of transfected CHO cells with lipids **1**, **2** and DC-Chol at a lipid:DNA molar ratio of 2:1. Cells expressing β-galactosidase were stained with X-gal as described in the text. A minimum of 100 cells were counted to estimate the percent of cells transfected. The values shown are averages of two independent measurements.

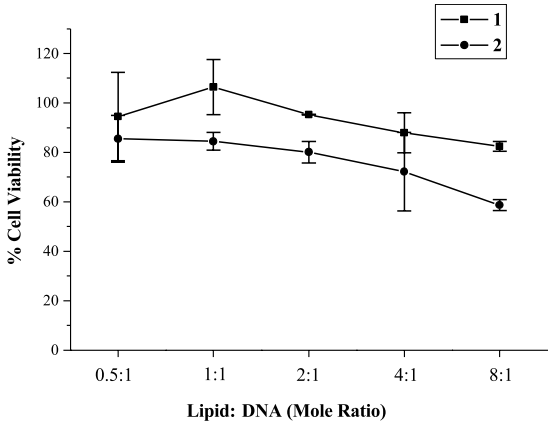


Fig. 4. Representative cell viabilities of cationic lipids **1** and **2** in HepG2 cells using the MTT-based assay. The toxicity assays were performed as described in the text. The data presented are average values of duplicate experiments ($n=2$). Results were expressed as % viability = $[A_{550}$(treated cells)−background$]/[A_{550}$(untreated cells)−background$]\times 100$.

(650–950 nm, Table 1) could be one of the impeding parameters responsible for making lipid **1** essentially transfection-incompetent. However, why liposomes and lipoplexes of lipid **2** with one extra methylene unit in its molecular structure are smaller than those of lipid **1** remains an open question at this point of the investigation.

In summary, the present findings demonstrate that even truly minor structural modification, such as inclusion of an additional single methylene unit in the head-group region, can impart remarkable gene transfer property to an essentially transfection-incompetent cholesterol-based cationic lipid. High DNA transfection efficacies of lipid **2** in multiple cells will now provide the impetus for undertaking detailed structure–activity studies involving libraries of cholesterol-based cationic lipids with β-alanine analogs in their head-group regions.

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