Articles

Spacer-Arm Modulated Gene Delivery Efficacy of Novel Cationic Glycolipids: Design, Synthesis, and in Vitro Transfection Biology

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Design, syntheses and relative in vitro gene delivery efficacies of six novel cationic glycolipids **1**−**6** containing open-form galactosyl units in CHO, COS-1, MCF-7 and A549 cells are described. The results of the present structure−activity investigation convincingly demonstrate that the in vitro gene delivery efficacies of galactosylated cationic glycolipids are strikingly dependent on the absence of a spacer-arm between the open-form galactose and the positively charged nitrogen atom in their headgroup region. While the cationic glycolipids **1**−**3** with no headgroup spacer unit between the positively charged nitrogen and galactose showed high in vitro gene transfer efficacies in all four cells (lipids **1** and **2** with myristyl and palmityl tails, respectively, being the most efficacious), lipids **4**−**6** with five-carbon spacer units between the quaternized nitrogen and galactose heads were essentially transfection incompetent. The transfection inhibiting role of the five-carbon spacer unit in the headgroup region of the present novel class of cationic lipids was demonstrated by both β-galactosidase reporter gene expression and histochemical X-gal staining assays. Results of MTT assay-based cell viability measurements in representative MCF7 cells show that cell viabilities of lipoplexes (lipid:DNA complexes) prepared from all the lipids **1**−**6** are remarkably high. Thus, possibilities of differential cellular cytotoxicities playing any key role behind the strikingly contrasting transfection properties of lipids **1**−**3** with no spacer and lipids **4**−**6** with a spacer unit in the headgroup regions was ruled out. Electrophoresis gel patterns in DNase I sensitivity assays are consistent with more free DNA (accessible to DNase I) being present in lipoplexes of lipids **4**−**6** than in lipoplexes of lipids **1**−**3**. Thus, the results of our DNase I protection experiments support the notion that enhanced degradation of DNA associated with lipoplexes of lipids **4**−**6** may play an important role in abolishing their in vitro gene transfer efficacies.

Introduction

Gene therapy is gaining increasing importance as a therapeutic modality to combat myriads of inherited diseases, dreadful viral infections and cancer. A key challenge in the gene therapy approach is to develop safe and efficient vehicles for delivering therapeutic genes into body cells. Recombinant viral vectors, although remarkably efficient in transfecting body cells, suffer from numerous biosafety related disadvantages.[1−3] Viral vectors have low insert-size limit (for the therapeutic genes they can pack inside) and are capable of generating: (a) imflammatory and immunogenic responses against their structural components and (b) replication-competent viruses through recombination events with the host genome.[4−8] Conversely, cationic transfection lipids, because of their least immunogenic nature, robust manufacture, ability to deliver large pieces of DNA, and ease of handling and preparation techniques, are increasingly becoming the alternative nonviral vectors of choice for delivering genes into body cells.[9−24]

In our ongoing program on designing efficient novel cationic transfection lipids,[25−33] we recently demonstrated the potential of nonglycerol-based cationic glycolipids containing open-arabinose and -xylose sugar-heads for use in liposomal gene delivery. In the molecular structures of these novel cationic glycolipids, both the arabinose and xylose sugar-heads were directly linked to the positively charged nitrogen atoms of the cationic glycolipids without using any spacer in between.[32] With a view to probe the role of spacer-arm between the positively charged nitrogen atom and the sugar-head functionality in cationic glycolipid-mediated gene delivery, in the present investigation, we have designed and synthesized six novel cationic glycolipids **1**−**6** (Chart 1) with either having five-carbon spacer arms between the positively charged nitrogen and the galactose-heads (lipids **4**−**6**) or having no spacer-arms (lipids **1**−**3**). As described below, lipids **1**−**3** with no spacer unit in the headgroup region were found to be remarkably more efficient in transfecting multiple cells including CHO, COS-1, MCF-7 and A549 than lipids **4**−**6** containing five-carbon spacer arms. Herein, we report on the design, synthesis, physicochemical characterizations and the strikingly spacer-arm-dependent in vitro transfection biology of the novel cationic glycolipids **1**−**6**.

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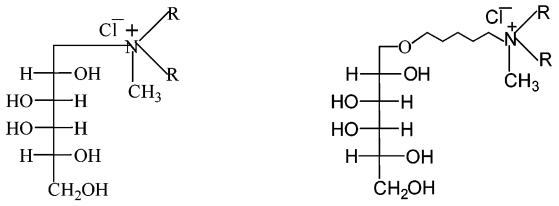
Chart 1. Structures of the New Cationic Glycolipids **1**−**6**



R = n-$C_{14}H_{29}$; Lipid 1
R = n-$C_{16}H_{33}$; Lipid 2
R = n-$C_{18}H_{37}$; Lipid 3

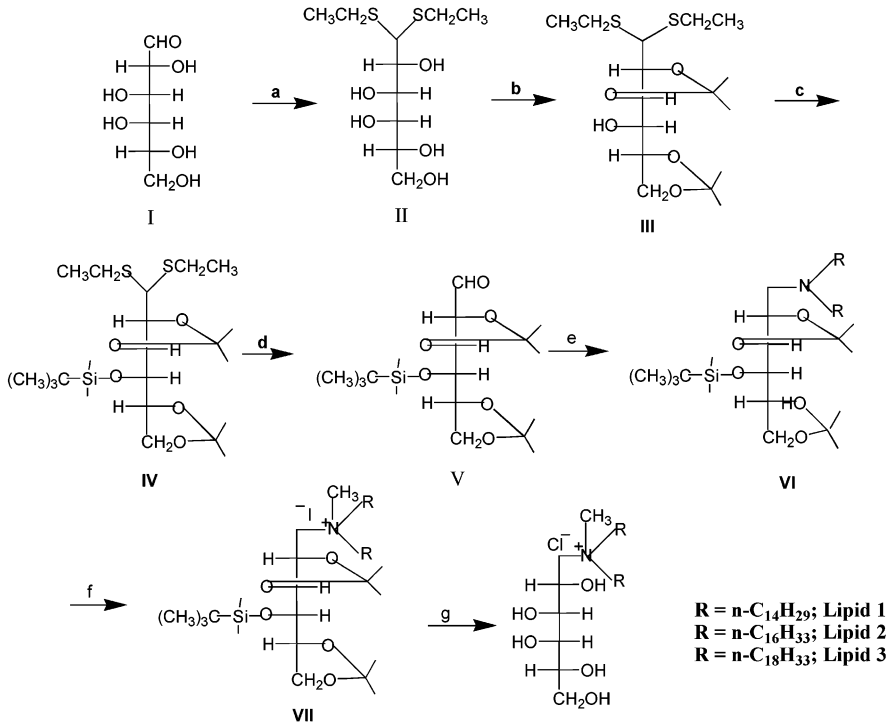
R = n-$C_{14}H_{29}$; Lipid 4
R = n-$C_{16}H_{33}$; Lipid 5
R = n-$C_{18}H_{37}$; Lipid 6

Results and Discussion

Chemistry. Cationic glycolipids **1**−**3** were synthesized by reacting the common intermediate 2,3:5,6-diisopropylidene-4-(*tert*-butyldimethylsilyl)-D-galactose (**V**, prepared conventionally in four steps from D-galactose as outlined in Scheme 1) with appropriate *N*,*N*-di-*n*-alkylamine followed by reduction with sodium cyanoborohydride (Scheme 1). The resulting tertiary amine intermediates **VI** (Scheme 1) upon quaternization with excess methyl iodide followed by acid deprotection and chloride ion exchange afforded lipids **1**−**3** (Scheme 1). Lipids **4**−**6** with a five-carbon spacer arm between the galactose functionality and the quaternized nitrogen atom were prepared by sequentially reacting the common primary amine intermediate **X** (prepared conventionally from intermediate **III**, the 2,3:5,6-diisopropylidene-D-galactose-thioacetal, in eight steps as depicted schematically in Scheme 2) with 2 equiv of the appropriate chain-length alkyl bromide, excess methyl iodide, acid deprotection and chloride exchange over Amberlyst A-26 (Scheme 2). Structures of all the synthetic intermediates shown in Schemes 1 and 2 were confirmed by ^{1}H NMR, LSIMS, and HRMS. However, the presence of multiple exchangeable hydroxyl functions in the headgroup regions of the final lipids (**1**−**6**) caused severe line broadening of the peaks in the ^{1}H NMR spectra, particularly in the range δ 3−5 ppm. Thus, the final lipids were characterized by the molecular ion peaks in their LSIMS.

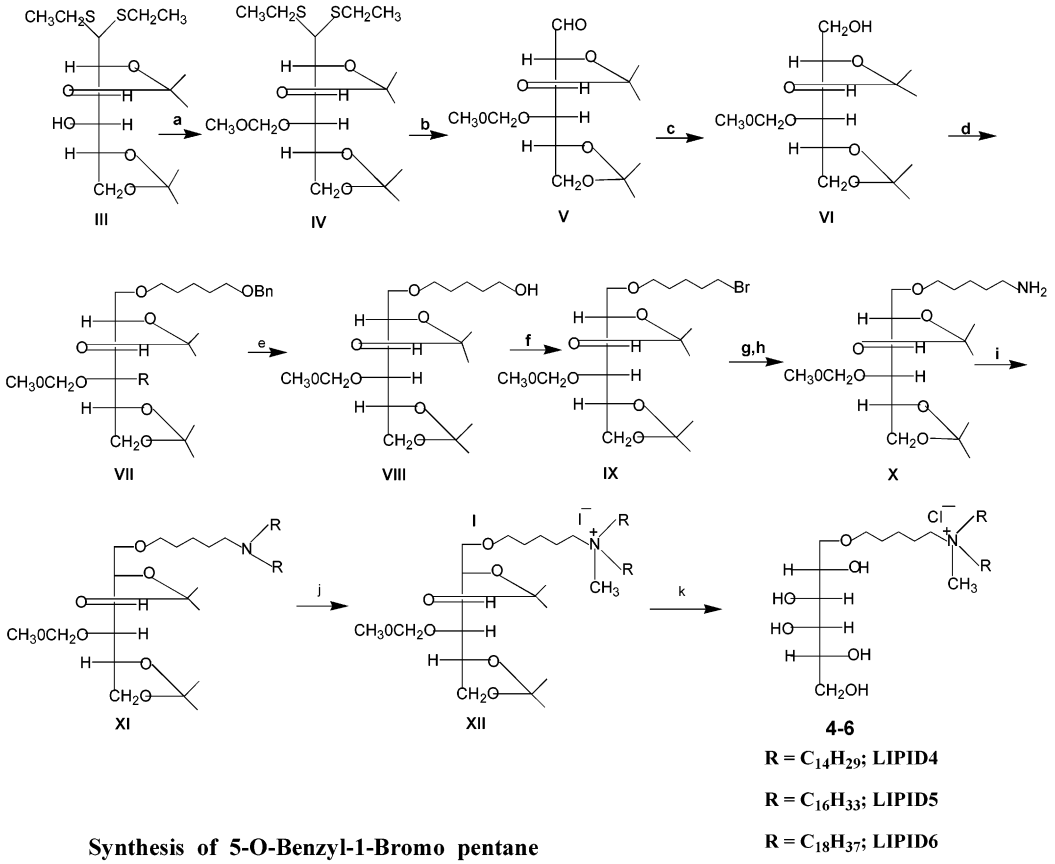
Transfection Biology. Figure 1 summarizes the relative in vitro gene delivery efficacies of lipids **1**−**6** in transfecting CHO, COS-1, MCF7 and A549 cells. In these in vitro transfection biology experiments, the cationic liposomes of lipids **1**−**6** were prepared in combination with an equimolar amount of cholesterol as the colipid and pCMV-SPORT-β-gal plasmid DNA was used as the reporter gene across the lipid:DNA charge ratios of 9:1 to 0.1:1. Lipids **1**−**3** with no spacer arms between the quaternized nitrogen atom and the galactose unit were found to be dramatically more efficient in transfecting all four cells than lipids **4**−**6** containing five-carbon headgroup spacer-arms. While lipids **1** and **2** were the most efficacious among the transfection-efficient glycolipids **1**−**3** with no headgroup spacer units, all three lipids **4**−**6** with a five-carbon spacer-arm in the headgroup region were essentially incompetent in transfecting any of the four cells across the entire lipid:DNA charge ratios of 0.1:1 to 9:1 (Figure 1). The optimal gene delivery efficiencies of lipids **1** and **2**, except in COS-1 cells, were comparable to that of DMRIE-C, a popular commercially available cationic transfection lipid (Figure 1). Lipids **1** and **2** showed their maximum cell transfection efficacies around lipid:DNA charge ratios of 0.3:1 to 3:1 (Figure 1). Such strikingly contrasting transfection profiles of glycolipids **1**−**3** and **4**−**6** were also observed in histochemical whole cell staining experiments with X-gal using the representa-

Scheme 1. Synthesis of Lipids **1**−**3**[a]



[a] Reaction conditions: (a) C_2H_5SH/concd HCl; (b) 2,2-DMP/PTSA/acetone; (c) $(CH_3)_3Si(CH_3)_2C1$/imidazole/dry DCM; (d) $HgCl_2$−HgO/acetone:water (8:2), 55 °C; (e) $(R)_2NH/Na_3BH_3CN$/dry DCM; (f) CH_3I/DCM; (g) trifluoroacetic acid/chloride ion exchange with Amberlyst A-26.

Scheme 2. Synthesis of Lipids **4**−**6**[a]



[a] Reaction conditions: (a) MOM-Cl/(^{i}Pr)$_2$N C_2H_5/dry DCM; (b) $HgCl_2/CdCO_3$/acetone:water (8:2), 55 °C; (c) $NaBH_4$, methanol, glacial acetic acid; (d)$BnO(CH_2)_5Br$, NaH, dry DMF, 80 °C (e) 10% Pd/C, H_2 (2 atm), ethyl acetate; (f) CBr_4/Ph_3P/imidazole/dry DCM; (g and h) NaN_3/dryDMF/80 °C; Ph_3P/THF/H_2O; (i) RBr, K_2CO_3, ethyl acetate, 70 °C, (j) CH_3I/DCM; (k) 6 N HCl/aqueous THF, Cl^- exchange with Amberlyst A-26; (l) BnBr, Ag_2O, DCM; (m) CBr_4/Ph_3P/imidazole/dry DCM.

tive CHO cells. Results of such histochemical staining experiments, once again, revealed that the percent of CHO cells transfected with lipids **1** and **2** are dramatically higher than that of CHO cells transfected with lipids **4**−**6** at both lipid:DNA charge ratios of 1:1 and 3:1 (Figure 2). Results of the histochemical whole cell staining experiments also demonstrated that lipid **2** is the most efficacious lipid among the three transfection efficient lipids **1**−**3** (Figure 2). Taken together, the results summarized in Figures 1 and 2 convincingly demonstrate that covalent grafting of spacer units between the quaternized nitrogen atom and the sugar functionalities in the headgroup region may seriously impede the in vitro gene delivery efficacies of cationic glycolipids.

MTT-based cell viability assays were performed in representative MCF7 cells across the entire range of lipid:DNA charge ratios used in the actual transfection experiments. Percent cell viabilities of both the glycolipids **1**−**3** and **4**−**6** were found to be remarkably high particularly up to lipid:DNA charge ratios of 3:1 in MCF7 cells (Figure 3). Thus, the dramatically opposite in vitro gene transfer efficacies of lipids **1**−**3** and **4**−**6** (Figures 1 and 2) are unlikely to originate from varying cell cytotoxicities of the present lipids. The global surface charge and sizes of the lipoplexes **1**−**3** and **4**−**6** across the varying lipid:DNA charge ratios (in the presence of DMEM) were measured using dynamic laser light scattering instrument equipped with zeta-sizing capacity. Interestingly, the lipoplex sizes and surface potentials of the most transfection efficient cationic glycolipid **2** were significantly larger and more negative compared to the rest of the glycolipids (Table 1). However, such lipoplex size and surface potentials measurement did not present any mechanistic insight to the possible origin behind their phenomenally opposite transfection profiles.

Lipid:DNA Binding Interactions and Lipoplex Sensitivities to DNase I. Finally, with a view to characterizing the electrostatic binding interactions between the plasmid DNA and the present cationic liposomes as a function of the lipid:DNA charge ratios, we performed both the conventional electrophoretic gel retardation assay and DNase I sensitivity assays. Results of the simple gel retardation assay revealed several interesting features. All the lipids (**1**−**6**) were capable of completely inhibiting the electrophoretic mobility of plasmid DNA from lipoplexes prepared at high lipid:DNA charge ratios of 9:1 (Figure 4, Parts A, C and E). However, at lower lipid:DNA charge ratios, more free DNA was found to be present in lipoplexes of lipids **4** and **5** compared to that in the lipoplexes prepared from lipids **1** and **2** (Figure 4, Parts A and C). Such gel patterns are consistent with the notion that

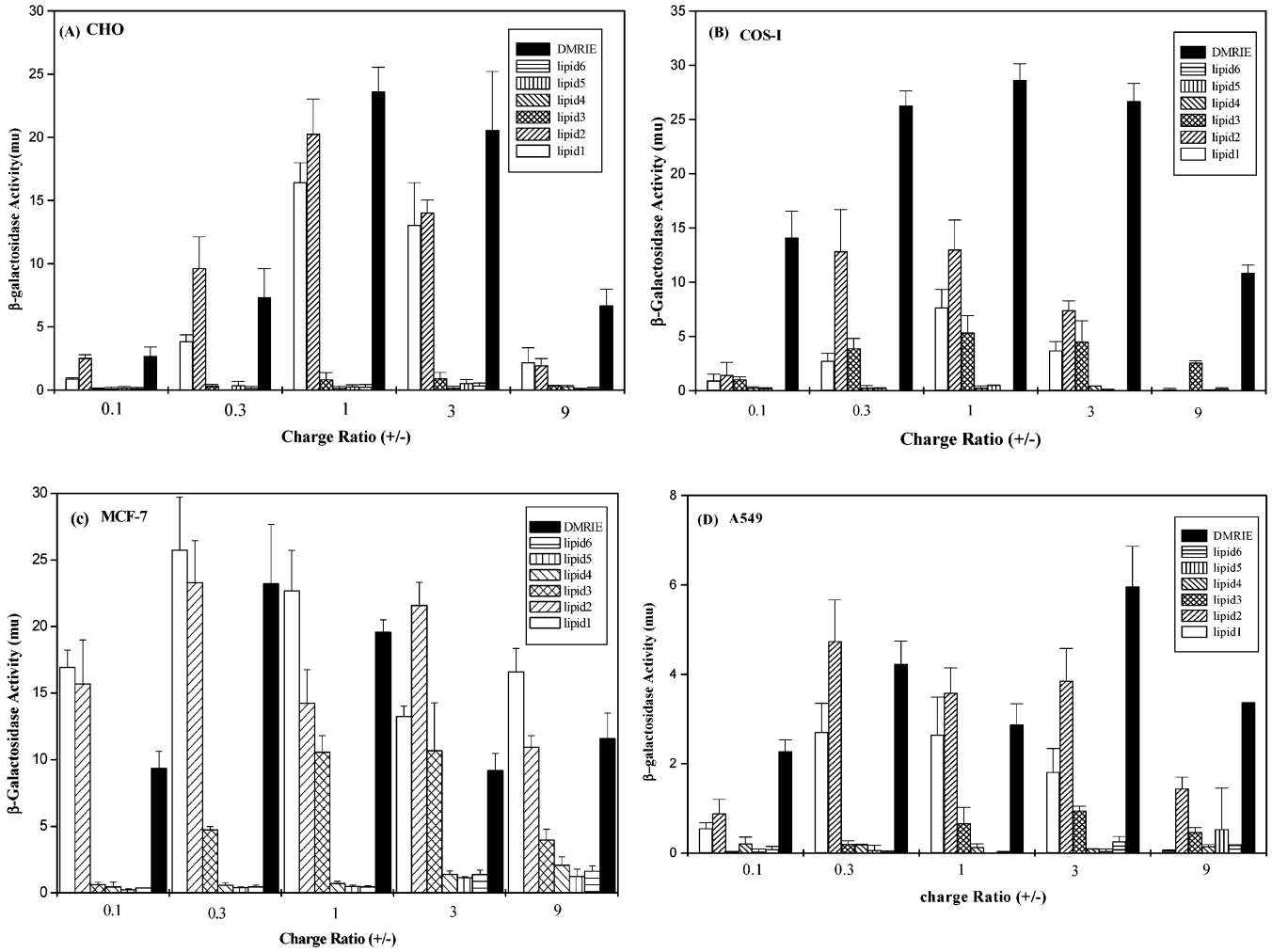


Figure 1. In vitro transfection efficiencies of lipids **1**−**6** in CHO (A), COS-1 (B), MCF-7 (C) and A549 (D) cells using cholesterol as colipid (at lipid:cholesterol mole ratio of 1:1). Units of β-galactosidase activity were plotted against the varying lipid to DNA (+/−) charge ratios. The *o*-nitrophenol formation (micromoles of *o*-nitrophenol produced per 10 min) was converted to β-galactosidase activity units using the standard curve obtained with pure (commercial) β-galactosidase. The transfection efficiencies of commercially available DMRIE are shown for comparison sake. The transfection values shown are average of triplicate experiments performed on the same day.

suboptimal lipid:DNA binding interactions could play an important role in abolishing the in vitro gene transfer efficacies of lipids **4** and **5** containing five-carbon spacer arms in their headgroup region. In agreement with this notion, a significant amount of free DNA was also found to be present at lower lipid:DNA charge ratios for lipoplexes made with lipid **3**, the weakest among the transfection efficient lipids **1**−**3** (Figure 4, Part E).

The presence of relatively higher amounts of free DNA in the lipoplexes prepared with transfection inefficient lipids **4**−**6** (compared to those in lipoplexes **1**−**3**) was more convincingly demonstrated by monitoring the sensitivities of the lipoplexes upon treatment with DNase I. After the free DNA digestion by DNase I, the total DNA (both digested and inaccessible DNA) was separated from the lipid and DNase I (by extracting with organic solvent) and loaded onto a 1% agarose gel. Resulting electrophoretic gel pattens in such DNase I protection experiments are summarized in Figure 4 (Parts B, D and F). Band intensities of inaccessible and therefore undigested DNA associated with transfection-incompetent lipoplexes **4**−**6** were significantly less than those associated with transfection efficient lipoplexes **1**−**3** essentially across the entire range of lipid:DNA charge ratios of 9:1 to 0:3:1 (Figure 4, Parts B, D and F). Such gel patterns in DNase I sensitivity assays indicate that the plasmid DNA associated with lipids **4**−**6** are likely to be more susceptible to degradation by cellular DNase I than the DNA complexed to lipids **1**−**3**. Thus, one of the factors abolishing the in vitro transfection efficacies of lipids **4**−**6** with five-carbon spacer arm units in the headgroup region could be higher degradation rates of the associated DNA by DNase I within the cell.

In conclusion, the results of the present structure−activity investigation involving the use of six novel cationic glycolipids (**1**−**6**) demonstrate that the in vitro gene delivery efficacies of galactosylated cationic glycolipids could be strikingly dependent on the presence or absence of a spacer-arm between the carbohydrate functionality and the positively charged nitrogen atom in the headgroup region. In general, while the glycolipids **1**−**3** with no-spacer arms in the headgroup region were highly efficacious in transfecting CHO, COS-1, MCF7 and A549 cells (lipids **1** and **2** with myristyl and palmityl tails, respectively, being the most efficient lipids), lipids **4**−**6** with a five-carbon spacer arm in the headgroup region were essentially incompetent in trans-

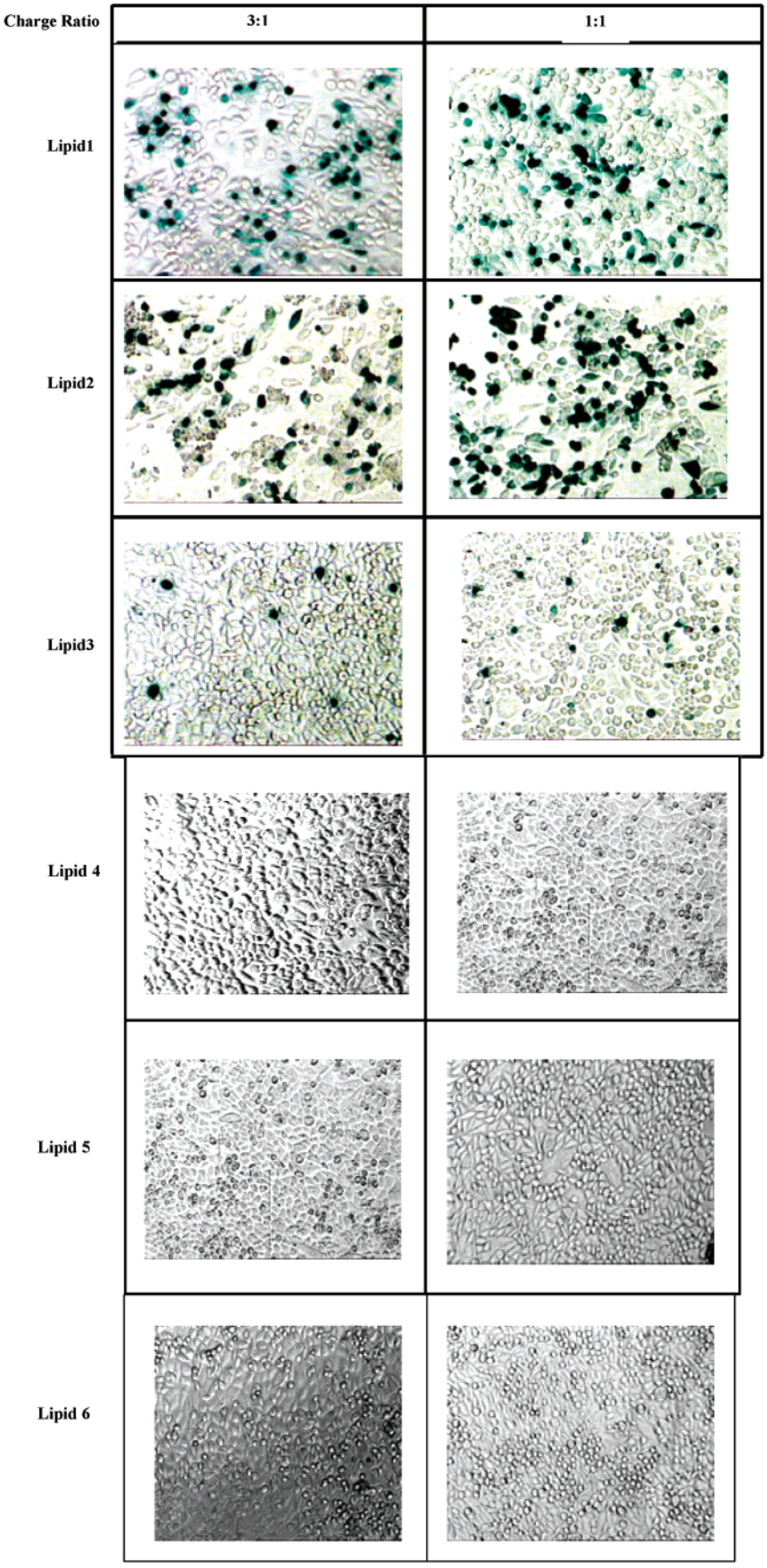


Figure 2. Histochemical whole cell staining of transfected CHO cells with X-gal with lipids **1**−**6** at lipid:DNA charge ratios of 3:1 and 1:1. Cells expressing β-galactosidase were stained with X-gal as described in the text.

fecting all the four cells. Thus, the results of our DNase I sensitivity assays indicate that relatively poor lipid:DNA interactions and therefore possibly more DNA degradation by DNase I within the cell may play an

Table 1.

lipids	lipid:DNA (±) 0.1:1	lipid:DNA (±) 0.3:1	lipid:DNA (±) 1:1	lipid:DNA (±) 3:1	lipid:DNA (±) 9:1
		A. Sizes of Lipoplexes (presence of DMEM)[a]			
1	259.5 ± 1.6	302.8 ± 1.6	258.9 ± 1.8	879.5 ± 66.3	1205.4 ± 83.6
2	549.3 ± 1.5	737.4 ± 45	1083 ± 44.6	1403 ± 121.1	1972 ± 169
3	282 ± 1.1	286.2 ± 2.8	288.1 ± 3.4	1067.9 ± 47.8	1514.5 ± 70.1
4	324.3 ± 6.8	370.3 ± 3.9	296.8 ± 7.9	562 ± 16.7	1056.9 ± 61.9
5	259.5 ± 15.9	242.4 ± 3.5	248.2 ± 6.8	1074 ± 160	832.9 ± 102
6	236.4 ± 14.8	254.7 ± 9.8	275.3 ± 7.4	1311 ± 42.3	1381.9 ± 77
		B. Zeta Potentials (ξ) of Lipoplexes[a] (presence of DMEM)			
1	−1.6 ± 2.7	−3.2 ± 2.2	−6.6 ± 1.6	−9.3 ± 3.9	17.6 ± 3
2	−34.4 ± 1.1	−34.4 ± 1.1	−33.4 ± 1.1	−27.8 ± 5.6	10.6 ± 4.9
3	−5.4 ± 4.5	−4.6 ± 2.6	−15.5 ± 5.9	−8.2 ± 3.1	6.2 ± 4.4
4	−3.4 ± 2	−8.0 ± 3.0	−5.9 ± 2.3	−12.9 ± 3.4	8.2 ± 1.4
5	−20.7 ± 5.0	−21.6 ± 3.1	−32.2 ±0.9	−35.8 ± 1.2	−15.8 ± 3.9
6	2.2 ± 2	−6.1 ± 4.1	−5.6 ± 3.1	−13 ± 3.6	8.3 ± 2

[a] Sizes and ξ potentials were measured by laser light scattering technique using Zetasizer 3000A (Malvern Instruments, UK). Values shown are the averages obtained from three (size) and 10 (zeta potential) measurements.

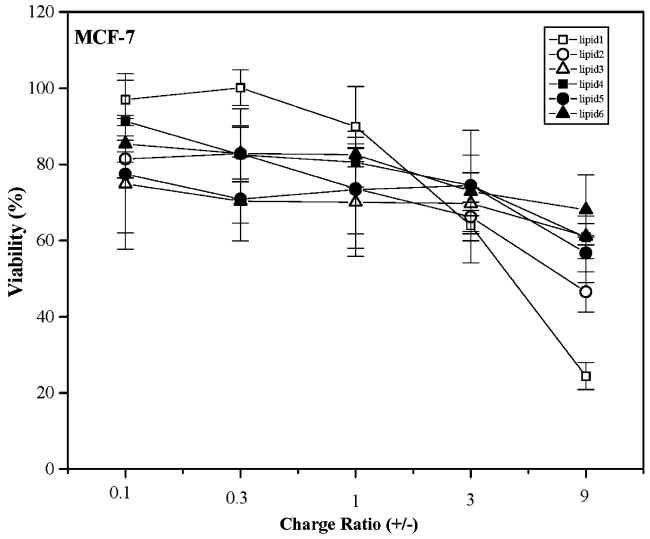


Figure 3. MTT assay-based cellular cytotoxicities of lipid **1**−**6** against representative MCF-7 cells. The percent cell viability values shown are average of triplicate experiments performed on the same day.

important role behind the seriously compromised transfection efficacies of glycolipids **4**−**6** with a five-carbon spacer unit in the headgroup region. Clearly, further cell biology experiments, including cellular uptake studies and intracellular trafficking experiments aimed at understanding subcellular localizations of the lipid:DNA complexes, etc., need to be carried out in the future for gaining better mechanistic insight into the origin of the diametrically opposite transfection profiles of lipids **1**−**3** and **4**−**6**. Investigations toward this end are currently in progress in our laboratories.

Experimental Section

General Procedures and Materials. High-resolution mass spectometric (HRMS) analysis was performed on a Micromass AUTOSPEC-M mass spectrometer (Manchester, UK) with OPUS V 3.1X data system. Data were acquired by liquid secondary ion mass spectrometry (LSIMS) using *m*-nitrobenzyl alcohol as the matrix. ^{1}H NMR spectra were recorded on a Varian FT 200 MHz, AV 300 MHz or Varian Unity 500 MHz instrument. Elemental analyses (C, H, N) were performed on Perkin-Elmer 240c in the microanalytical laboratory of the University of Hyderabad. 1-Bromohexadecane, *n*-hexadecylamine, boron trifluoride etherate, sodium cyanoborohydride, *tert*-butyldimethylsilyl chloride, tetra-*n*-butylammonium fluoride (1 M solution in THF) and Amberlyst A-26 were purchased from Lancaster (Morecambe, UK). Carbon tetrabromide and ethanethiol were purchased from Fluka (Switzerland) and *N*,*N*-diisopropylethylamine were procured from Aldrich (Milwaukee, WI). Unless otherwise stated, all other reagents were purchased from local commercial suppliers and were used without further purification. Column chromatography was performed with silica gel (Acme Synthetic Chemicals, India, 60−120 mesh). *p*-CMV-SPORT-β-gal plasmid was a generous gift from Dr. Nalam Madhusudhana Rao of Centre for Cellular and Molecular Biology, Hyderabad, India. Cell culture media, fetal bovine serum, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), poly(ethylene glycol) 8000, *o*-nitrophenyl-β-D-galactopyranoside and cholesterol were purchased from Sigma (St. Louis, MO). NP-40, antibiotics and agarose were purchased from Hi-media, India. COS-1 (SV 40 transformed african green monkey kidney cell), and CHO (Chinese hamster ovarian cell), MCF-7 (human brest adenocarcinoma cell) and A549 (human pulmonary carcinoma cell) cell lines were procured from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS in a humidified atomosphere containing 5% CO_2/95% air. Purity of lipids **2** and **5** was confirmed by elemental analyses (C, H, N), and lipids **1**, **3**, **4** and **6** were found to be more than 95% pure by reversed phase HPLC analysis (Shimadzu Model LC10A) using two different mobile phases (A: pure methanol and B: 95:5, v/v, methanol/water), PARTISIL 5 ODS-3 WCS analytical column (4.6 × 250 mm, Whatman Inc., Clifton, NJ) and a flow rate of 1.6−1.8 mL/min. Typical retention times in mobile phase A were 1.09 min (lipid **1**); 0.87 min (lipid **3**); 1.21 min (lipid **4**) and 1.00 min (lipid **6**).

Syntheses of Lipids 1−6. As representative details, syntheses and spectral characterization of lipids **2** and **5** and all their synthetic intermediates shown in Schemes 1 and 2 are provided below. Lipids **1** and **3** and lipids **4** and **6** were synthesized following essentially the same protocols adopted for preparing lipids **2** and **5**, respectively.

Synthesis of Lipid 2 (Scheme 1). Step a: Synthesis of D-Galactose-diethyl-dithioacetal (II). In a 250 mL round-bottomed flask, D-galactose **I** (20 g, 111 mmol) was dissolved in concentrated HCl (50 mL). The reaction mixture was stirred for 10 min in ice−salt bath. Ethanethiol (16.4 mL, 222 mmol) was added dropwise to the cold solution. The reaction mixture was stirred in ice−salt bath for 1 h, stirred at 0 °C for 5 h and kept at 4 °C for 12 h. The mixture was dissolved in methanol (1000 mL), and the solution was neutralized by addition of lead carbonate. The salts were filtered, and the filtrate was concentrated. The resulting solid upon drying under vacuum afforded pure compound **II** (24 g, 75% yield). mp: 138−140 °C (lit: 140−142 °C).[34]

Step b: Synthesis of 2,3:5,6-Diisopropylidene-D-galactose-diethyl-dithioacetal (III). In a 250 mL two necked round-bottom flask, 2,2-dimethoxypropane (24 mL, 209 mmol) was added dropwise at 0 °C to a solution of **II** (10 g, 34.9 mmol) and PTSA (2.63 g, 13.8 mmol) in dry acetone (50 mL) under

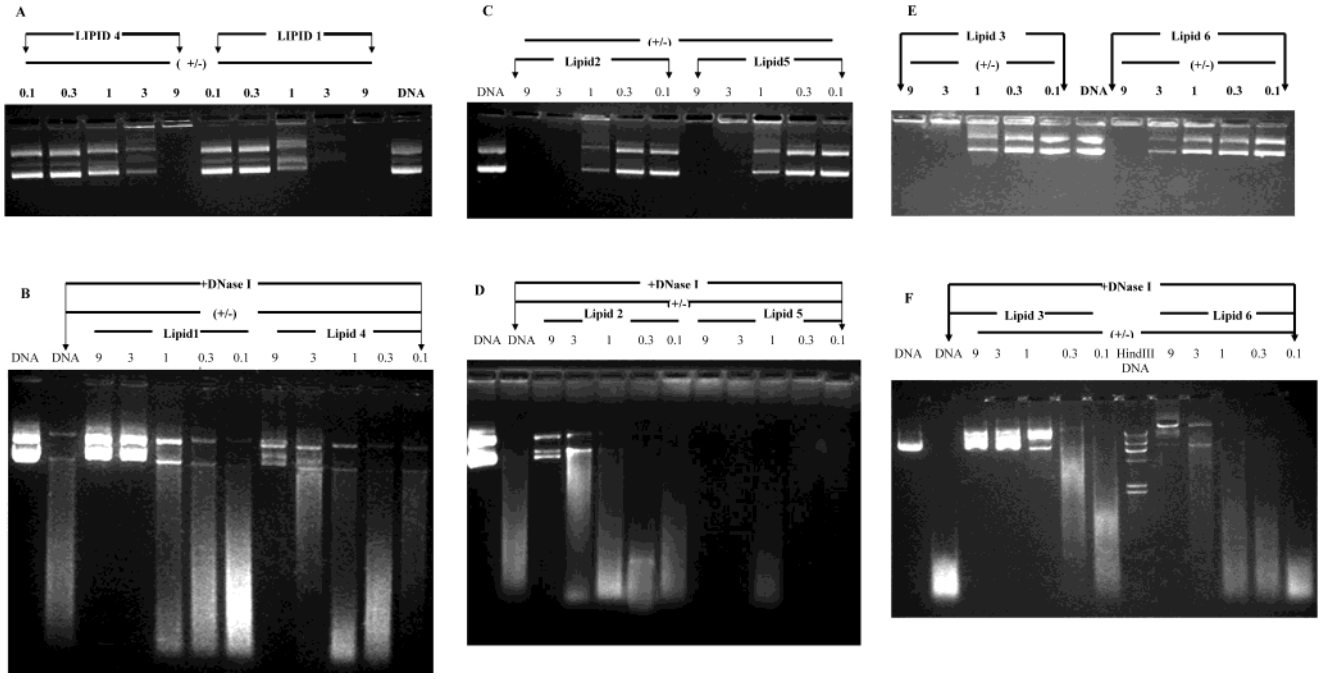


Figure 4. Electrophoretic gel patterns for lipoplex-associated DNA in gel retardation (Parts A, C and E) and DNase I sensitivity assays (Parts B, D and F). The lipid:DNA charge ratios are indicated at the top of each lane. The details of treatment are as described in the text.

nitrogen atmosphere. The reaction mixture was stirred for 2 h, neutralized with $NaHCO_3$ and filtered. The filtrate was dried over anhydrous Na_2SO_4 and concentrated in a rotary evaporator. The residue upon 60−120 mesh silica gel column using 4% ethyl acetate in hexane as eluent afforded the pure liquid title compound **III** (6.5 g, 58% yield, $R_f = 0.6$, 30% ethyl acetate/hexane). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 1.20−1.29 (m, 6H, −S−CH_2−CH_3), 1.30−1.42 (4s, 12H, −2{COC(CH_3)$_2$-OC}, 2.00−2.20 (brs, 1H, O*H*), 2.70 (q, 4H, SCH_2CH$_3$), 3.70−3.80 (m, 3H, H-5, H-6 and H-6′), 4.00 (d, H-1), 4.05−4.10 (m, 2H, H-2 and H-3), 4.29−4.35 (dd, 1H, H-1)

Step c: Synthesis of 2,3:5,6-Diisopropylidene-4-(*tert*-butyl-dimethylsilyloxy)galactose-dithioketal (IV). In a 50 mL two neck round-bottom flask, a mixture of compound **III** (2.23 g, 6.80 mmol) and imidazole (2.78 g, 40.8 mmol) was dissolved in dry dichloromethane (10 mL) and stirred at 0 °C. TBDMSCl (3.0 g, 20.4 mmol) dissolved in dichloromethane was added to the cold reaction mixture. A white precipitate appeared after addition of TBDMSCl was completed. The temperature of the reaction mixture was slowly raised to room temperature, and the stirring was continued for 1 h. The mixture was concentrated on a rotary evaporator, and the residue upon column chromatographic purification (using 60−120 mesh size silica gel and 1% ethyl acetate/hexane, v/v, as eluent) afforded the title compound **IV** as a colorless liquid (1.94 g, 66% yield, R_f = 0.8, 20:80 ethylacetae:hexane). ^{1}H NMR (200 MHz, $CDCl_3$): 0.00−0.10 (6H, Si(CH_3)$_2$), 0.90−1.00 (m, 9H, (CH_3)$_3$Si), 1.20−1.30 (m, 6H, 2SCH$_2$CH_3), 1.35−1.50 (m, 12H, 2{COC(CH_3)$_2$OC}, 2.60−2.80 (m, 4H, 2SCH_2CH$_3$), 3.65−3.90 (m, 3H, H-5, H-6 and H-6′), 4.00−4.20 (m, 3H, H-1,H-2, and H-3), 4.30 (dd, 1H, H-4).

Step d: Synthesis of 1-Aldehydo-2,3:5,6-diisopropylidene-4-(*tert*-butyldimethylsilyloxy)-D-galactose (V). Compound **IV** (0.99 g, 2.23 mmol) was deprotected by refluxing in 50 mL of 80/20 acetone/water (v/v) with mercuric chloride (1.8 g, 6.6 mmol) and red mercuric oxide (1.7 g, 8.24 mmol) for exactly 1.5 h. The reaction mixture was cooled and filtered. The filtrate was concentrated, the resulting residue was dissolved in $CHCl_3$ and the chloroform extract was washed with 1 N KI (3 × 50 mL) followed by brine solution (2 × 50 mL). The organic layer was separated, dried over anhydrous Na_2SO_4 and filtered. The filtrate upon concentration in a rotavapor afforded the crude aldehyde (**V**) as a liquid (0.6 g, 85%, R_f = 0.6, 30:70 ethyl acetate:hexane).

Step e: Synthesis of 1-Deoxy-1-[di-*n*-hexadecyl(amino)]-2,3:5,6-diisopropylidene-4-(*tert*-butyldimethylsilyloxy)-D-galactose (VI). The crude aldehyde **V** prepared above (0.3 g, 1.0 mmol) and *N*,*N*-di-*n*-hexadecylamine (0.4 g 1.1 mmol) were dissolved in 10 mL of dichloromethane and was stirred for 0.5 h under nitrogen in room temperature. Sodium cyanoborohydride (0.1 g, 1.6 mmol) was added to the reaction mixture, and the stirring was continued further for 24 h under nitrogen atmosphere. The reaction mixture was concentrated, and the residue upon column chromatographic purification (using 60−120 mesh size silica gel and 1.8−2% ethyl acetate in hexane as eluent) afforded the title compound **VI** as a semisolid (0.19 g, 24% yield, R_f = 0.8, 20:80 ethyl acetate: hexane). ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 0.10 (S, 6H, 2SCH_3), 0.90−1.00 (m, 15H), 1.15−1.35 (m, 56H), 1.36−1.40 (4S, 12H, 2{COC(CH_3)$_2$OC}), 2.40−2.65 (m, 5H, CH^{1}H^{2}N(CH$_2$-CH$_2$), 2.70−2.90 (m, 1H, CH^{1}H^{2}N(CH$_2$CH$_2$), 3.58−3.72 (m, 2H, H-5 and H-6′), 3.83−3.97 (m, 3H, H-3, H-4 and H-6), 4.00−4.20 (m, 1H, H-2).

Step f: Synthesis of 1-Deoxy-1-[di-*n*-hexadecyl(methyl)amino]-2,3:5,6-diisopropylidene-4-(*tert*-butyldimethylsilyloxy)-D-galactose (VII). Compound **VI** (0.12 g, 0.14 mmol) was treated with excess methyl iodide (3 mL) and stirred for 3 h at room temperature. The reaction mixture was concentrated, and the residue upon column chromatographic purification (using 60−120 mesh size silica gel and 2.5% methanol in chloroform as eluent) afforded the title compound **VII** as a white solid (0.073 g, 60% yield, R_f = 0.5, 5% methanol/chloroform). ^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) 0.10 (S, 6H, Si(CH_3)$_2$), 0.80−1.00 (m, 15H, SiC(CH_3)$_3$ and CH_3(CH$_2$)$_{14}$, 1.10−1.50 (m, 64H), 1.60−1.90 (m, 4H), 3.50 (S, 3H, CH_3N^+), 3.58−4.40 (m, 12H, N(CH_2)$_3$, H-2, H-3, H− 4, H-5, H-6 and H-6′). FABMS (LSIMS): *m*/*z*: 839 $[M]^+ + 1$ for $C_{51}H_{104}0_5NSi$.

Step g: Synthesis of 1-Deoxy-1-[di-*n*-hexadecyl-(methyl)amino])-D-galactitol (lipid 2, Scheme 1). Compound **VII** (0.073 g, 0.086 mmol) was dissolved in neat trifluoroacetic acid (2 mL) and stirred at room temperature for 48 h. Excess trifluoroacetic acid was removed with nitrogen flow, and the residue upon column chromatographic purification (using 60−120 mesh size silica gel and 6−8% methanol in chloroform as eluent) followed by chloride ion exchange in Amberlyst A-26 (using methanol as eluent) afforded target lipid **1** as a white solid (0.025 g, 45% yield, R_f = 0.1, 1:9 methanol;chloroform). ^{1}H NMR (200 MHz,$CDCl_3$): δ (ppm)

0.80 (t,6H, $(CH_3)_2(CH_2)_n$) 1.10−1.80 (m, 56H, 2 $(CH_3)(CH_2)_{14}$-CH_2N); 3.10 (S, 3H, N^+CH_3); 3.20−5.20 (m, 12H, $N(CH_2)_2$, N−H-1, H-1′, H-2, H-3, H− 4, H-5, H-6 and H-6′). FABMS (LSIMS) *m/z*: 645 $[M]^+ + 1$ for $C_{39}H_{82}0_5N$. HRMS (LSIMS) *m/z*: calcd (for $C_{39}H_{82}O_5N$ the quaternary ammonium ion, 100%) 644.6193, found 644.6172.

Synthesis of 1-Deoxy-1-[di-*n*-tetradecyl(methyl)amino]-D-galactitol (lipid 1, Scheme 1). The title lipid **1** (white solid) was synthesized following the same synthetic procedure as described above for preparing lipid **2** using *N*,*N*-di-*n*-tetradecylamine in step e with an overall yield of 44%. All the isolated intermediates gave spectroscopic data in agreement with their assigned structures shown in Scheme 1. ^{1}H NMR (200 MHz,-$CDCl_3$) of intermediate **VI** (Scheme 1 where R = *n*-$C_{14}H_{29}$): δ (ppm) 0.10 (S, 6H, 2SiCH_3), 0.90−1.00 (m, 15H), 1.10−1.30 (m, 48H), 1.36−1.50 (4S, 12H, 2{COC$(CH_3)_2$OC}), 2.40−2.65 (m, 5H, $CH^1H^2N(CH_2CH_2)$, 2.70−2.90 (m, 1H, $CH^1H^2N(CH_2$-$CH_2)$, 3.60−4.10 (m, 6H, H-2, H-3, H-4, H-5, H-6 and H-6′). ^{1}H NMR (200 MHz,$CDCl_3$) of intermediate **VII** (Scheme 1 where R = $C_{14}H_{29}$): δ (ppm) 0.10 (S, 6H, Si$(CH_3)_2$, 0.80−1.00 (m, 15H, SiC$(CH_3)_3$ and $CH_3(CH_2)_{14}$, 1.18−1.32 (m, 56H), 1.65−1.90 (m, 4H), 3.50 (S, 3H, CH_3N^+), 3.60−3.80 (m, 6H, $N(CH_2)_3$), 3.81−4.40 (m, 6H, H-2, H-3, H− 4, H-5, H-6 and H-6′). FABMS (LSIMS): *m/z*: 783 $[M]^+ + 1$ for $C_{47}H_{96}0_5NSi$. ^{1}H NMR (200 MHz, $CDCl_3$+DMSO) of lipid **1**: δ (ppm) 0.90 (t, 6H, $(CH_3)_2$-$(CH_2)_n$) 1.15−1.40 (m, 44H), 1.50−1.90 (m, 4H), 3.20−3.90 (m, 9H, N^+CH_3, $N(CH_2)_2$, N-H-1, H-1′), 4.30−5.40 (m, 6H, H-2, H-3, H− 4, H-5, H-6 and H-6′). FABMS (LSIMS) *m/z*: 588 $[M]^+ + 1$ for $C_{35}H_{74}0_5N$.

Synthesis of 1-Deoxy-1-[di-*n*-octadecyl(methyl)amino]-D-galactitol (lipid 3, Scheme 1). The title lipid **3** (white solid) was synthesized following the same synthetic synthetic procedure as described above for preparing lipid **2** using *N*,*N*-di-*n*-octadecylamine with an overall yield of 44%. All the isolated intermediates gave spectroscopic data in agreement with their assigned structures shown in Scheme 1. ^{1}H NMR (300 MHz,-$CDCl_3$) of intermediate **VI** (Scheme 1 where R = *n*-$C_{18}H_{37}$): δ (ppm) 0.10 (s, 6H, 2SCH_3), 0.80−1.00 (m, 15H), 1.10−1.30 (m, 64H), 1.36−1.50 (4S, 12H, 2{COC$(CH_3)_2$OC}), 2.40−2.65 (m, 5H, $CH^1H^2N(CH_2CH_2)$, 2.70−2.90 (m, 1H, $CH^1H^2N(CH_2CH_2)$, 3.55−4.10 (m, 6H, H-2, H-3, H-4, H-5, H-6 and H-6′). FABMS (LSIMS) *m/z*: 879 $[M]^+ + 1$ for $C_{54}H_{109}0_5NSi$. ^{1}H NMR (200 MHz,$CDCl_3$) of intermediate **VII** (Scheme 1 where R = $C_{18}H_{37}$): δ (ppm) 0.10 (S, 6H, Si$(CH_3)_2$, 0.80−1.00 (m, 15H, SiC$(CH_3)_3$ and 2C$H_3(CH_2)_{14}$), 1.20−1.50 (m, 72H), 1.55−1.90 (m, 4H), 3.50 (S, 3H, CH_3N^+), 3.60−4.40 (m, 12H, $N(CH_2)_3$), H-2, H-3, H− 4, H-5, H-6 and H-6′). FABMS (LSIMS) *m/z*: 895 $[M]^+ + 1$ for $C_{55}H_{112}O_5NSi$. ^{1}H NMR (200 MHz,$CDCl_3$) of lipid **3**: δ (ppm) 0.90 (t, 6H, $(CH_3)_2(CH_2)_n$) 1.10−1.80 (m, 64H) 3.10 (S, 3H, N^+CH_3), 3.20−4.60 (m, 12H, $N(CH_2)_2$, N-H-1, H-1′, H-2, H-3, H− 4, H-5, H-6 and H-6′). FABMS (LSIMS) *m/z*: 701 $[M]^+ + 1$ for $C_{43}H_{90}0_5N$.

Synthesis of Lipid 5 (Scheme 2). Step a: Synthesis of 2,3:5,6-Diisopropylidene-4-methoxymethyl-D-galactose-diethyl-dithioacetal (IV). In a 50 mL two-necked round-bottom flask, compound **III** (6.30 g, 17.24 mmol) was dissolved in dry dichloromethane (15 mL) at 0 °C. Diisopropylethylamine (6.0 mL, 34.2 mmol) was added dropwise to the solution, the mixture stirred for 1 h at 0 °C and methoxymethyl cholride (1.56 mL, 54.6 mmol) added dropwise to the reaction mixture under stirring. The reaction mixture was stirred for 6 h at room temparature, diluted with chloroform (100 mL) and washed with H_2O (50 mL). The organic layer was separated and dried over anhydrous Na_2SO_4 and concentrated in a rotary evaporator to afford the crude product which upon purification on 60−120 mesh size silica gel column using 2% ethyl acetate in hexane as eluent afforded pure compound **IV** as a pale yellow liquid (4.92 g, 79% yield, R_f = 0.8, 30% ethyl acetate/hexane). ^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) = 1.25 (m, 6H, SCH_2CH_3), 1.30−1.42 (4s, 12H, 2{COC$(CH_3)_2$OC}, 2.70 (q, 4H, 2SCH_2CH_3), 3.39 (s, 3H, OCH_3), 3.60 (dd, 1H, H-5), 3.65−3.80 (m, 2H, H-6, 6′), 4.00 (d, 1H, H-1), 4.02−4.39 (m, 3H, H-2, H-3 and H-4), 4.60 (s, 2H, OCH_2O).

Step b: Synthesis of 2,3:5,6-Diisopropylidene-4-methoxymethyl-D-galactal (V). Compound **IV** (4.50 g, 10.97 mmol) and $CdCO_3$ (34.07 g, 197.5 mmol) were suspended in 60 mL of a mixture of acetone and water (8:2, v/v), and the reaction mixture was heated at 50 °C. $HgCl_2$ (15.9 g, 58.8 mmol) dissolved in 10 mL of acetone was added over a period of 5 min. The reaction mixture was stirred for 2 h at 50 °C. The reaction mixture was cooled and filtered through fresh $CdCO_3$. The filtrate was concentrated, and the residue obtained was dissolved in $CHCl_3$ (100 mL) and washed first with 1 N aqueous KI (500 mL) and finally with brine solution (100 mL). The organic layer was separated and dried over anhydrous Na_2SO_4 and concentrated to afford aldehyde **V** as a liquid (2.66 g, 85% yield, R_f = 0.7,1:1 ethyl acetate:hexane).

Step c: Synthesis of 2,3:5,6-Di-*O*-isopropylidene-4-methoxymethyl-galactitol (VI). Crude aldehyde **V** (2.60 g, 14.1 mmol) was dissolved in 10 mL of methanol, and the solution was cooled to 0 °C. $NaBH_4$ (0.33 g, 8.51 mmol) was added in portions to the cold solution. The reaction mixture was stirred at 0 °C for 1 h and stirred at room temperature for 3 h. The solvent was evaporated, and the residue was dissolved in 10 mL water, neutralized with glacial acetic acid and extracted with ethyl acetate (2 × 10 mL). The combined organic extract was washed with brine solution, dried over anhydrous Na_2SO_4 and concentrated on rotary evaparator. The residue upon chromatography over 60−120 mesh silica gel column using 20% ethyl acetate/hexane as eluent afforded pure compound **VI** as a colorless liquid (1.57 g, 60% yield, R_f = 0.5, 40:60 ethyl acetate/hexane). ^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) = 1.20−1.40 (4s, 12H, 2{COC$(CH_3)_2$OC})), 2.39−2.50 (brs, 1H, O*H*), 3.30 (s, 3H, OCH_3), 3.40−3.80 (m, 6H, H-2, H-3, H-4, H-5, H-6 and H-6′), 3.85−4.10 (m, 2H, H-1, H-1′), 4.60 (s, 2H, OCH_2O). FABMS (LSIMS): *m/z*: 307 $[M]^+ + 1$ for $C_{14}H_{26}O_7$.

Step d: Synthesis of 5-Benzyloxypentyl-2,3:5,6-di-*O*-isopropylidene-4-methoxymethy-D-galactoside (VII). In a 50 mL two neck round-bottom flask, compound **VI** (1.54 g, 5 mmol) dissolved in 10 mL of dry DMF was added at 0 °C to a suspension of NaH (0.6 g, 12.6 mmol) in 10 mL of dry DMF. The reaction mixture was stirred at 0 °C for 1 h. 5-O-benzyl-1-bromo-pentane (1.55 g, 6 mmol) was added to the reaction mixture and kept under stirring initially for 1 h at 0 °C and then at room temperature for 6 h and finally at at 80 °C for 48 h. The reaction mixture was then diluted with $CHCl_3$ (100 mL) and washed with H_2O (3 × 50 mL). The organic layer was separated, dried over anhydrous Na_2SO_4 and concentrated by rotary evaporator. The residue upon chromatographic purification on a 60−120 mesh size silica gel column using 3.5−5% ethyl acetate/hexane as the eluent afforded compound **VII** as a colorless liquid (1.3 g, 53.6% yield, R_f = 0.6, 30:70 ethyl acetate/hexane). ^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) = 1.30−1.48 (4s, 12H, 2{COC$(CH_3)_2$OC}, 1.50−1.89 (m, 6H), 3.40 (s, 3H, OCH_3), 3.41−3.58 (m, 6H), 3.60−3.80 (m, 4H, H-3, H-5, H-6, H-6′), 4.00−4.02 (m, 2H, H-2 and H-4), 4.50 (s, 2H, OCH_2-Ph) 4.70 (s, 2H, OCH_2O), 7.20−7.40 (m, 5H, aromatic). FABMS (LSIMS): *m/z*: 467 [loss of CH_3] for $C_{26}H_{42}O_8$.

Step e: Synthesis of 5-Hydroxypentyl-2,3:5,6-di-*O*-isopropylidene-4-methoxymethyl-D-galactoside (VIII). Compound **VII** (0.9 g, 1.87 mmol) dissolved in ethyl acetate (10 mL) was added to the 10% Pd/C (200 mg) suspended in ethyl acetate (5 mL), and the reaction mixture was stirred at room temp for 20 h under H_2 (2 atm). The mixture was filtered using Celite, and the filtrate was dried over anhydrous Na_2-SO_4 and concentrated. The crude product upon column chromatograpphy over 60−120 mesh size silica gel using 20:80 ethyl acetate:hexane as eluent afforeded pure compound **VIII** as a colorless liquid (0.71 g, 97% yield, R_f = 0.6, 1:1 ethyl acetate/hexane. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 1.30−1.40 (m, 12H, 2{COC$(CH_3)_2$OC}, 1.45−1.60 (m, 6H), 1.65−1.80 (brs, 1H, O*H*), 3.30 (s, 3H, OCH_3), 3.40−3.80 (m, 10H), 3.90−4.10 (m, 2H, H-2 and4), 4.60 (s, 2H, OCH_2).

Step f: Synthesis of 5-Bromopentyl-2,3:5,6-di-*O*-isopropylidene-4-methoxymethyl-D-galactoside (IX). In a 50 mL two-neck round-bottom flask, imidazole (0.24 g, 3.54 mmol)

and triphenylphosphine (0.93 g, 3.55 mmol) were dissolved in dry dichloromethane under nitrogen atmosphere. Compound **VIII** (0.7 g, 1.78 mmol), dissolved in dry dichloromethane, was added dropwise followed by addition of CBr_4 (1.17 g, 3.54 mmol) to the reaction mixture. The reaction mixture was stirred at 0 °C for 1 h, the temperature gradually raised to room temp and the reaction mixture was further stirred at room temperature for 12 h. The reaction mixture was concentrated, and the residue upon column chromatographic purification (using 60−120 mesh size silica gel and 10:90 ethyl acetate:hexane as eluent) afforded compound **IX** as a colorless liquid (0.60 g, 75% yield, $R_f = 0.4$, 20:80 ethyl acetate/hexane). ^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) = 1.30−1.45 (4s, 12H, 2{COC(*CH*$_3$)$_2$OC}, 1.45−1.70 (m, 4H),1.80−2.00 (m, 2H), 3.39 (s, 3H, O−CH_3), 3.40−3.80 (m, 10H), 4.00−4.20 (m, 2H, H-2 and H-4), 4.70 (s, 2H, OC*H*$_2$O). FABMS (LSIMS): *m*/*z*: 455 $[M]^+$ for $C_{19}H_{35}O_7Br$.

Steps g and h: Synthesis of 5-Aminopentyl-2,3:5,6-di-*O*-isopropylidene-4-methoxymethyl-D-galactoside (X). In a 25 mL round-bottomed flask, sodium azide (0.25 g, 3.97 mmmol) was added to a solution of compound **IX** (0.600 g, 1.31 mmol) in dry DMF under nitrogen atmosphere. The reaction mixture was stirred at 70 °C for 16 h. The reaction mixture was diluted with $CHCl_3$ (50 mL) and washed with water (3 × 50 mL). The organic layer was separated, dried over anhydrous Na_2SO_4 and filtered. Removal of the solvent from the filtrate on rotary evaporator afforded pure compound **X** as a colorless liquid (0.41 g, 73% yield, $R_f = 0.4$, 20:80 ethyl acetate/hexane). ^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) = 1.30−1.45 (4s, 12H, 2{COC(*CH*$_3$)$_2$OC}, 1.45−1.70 (m, 4H), 1.80−2.00 (m, 2H), 3.20 (t, 2H C*H*$_2$N$_3$), 3.39 (s, 3H, OC*H*$_3$), 3.40−3.80 (m, 8H), 4.00−4.20 (m, 2H, H-2 and H-4),4.70 (s, 2H, OC*H*$_2$). FABMS (LSIMS): *m*/*z*: 440 $[M]^+$ + Na for $C_{19}H_{35}O_7N_3$. IR: V_{max} = 2097.3 cm^{-1} (N_3).

In a 25 mL round-bottom flask, a mixture of the azide intermediate obtained above in step g (0.4 g, 0.97 mmol) and triphenylphosphine (0.38 g, 1.43 mmol) dissolved in tetrahydrofuran (5 mL) was stirred at room temparature. After 10 min, 3 drops of water was added and the reaction mixture was kept under stirring for 16 h. The reaction mixture was then concentrated on rotary evaporator, and column chromatographic purification (using 60−120 mesh size silica gel and 8−9% methanol in chloroform as the eluent) of the residue afforded the title compound **X** as a colorless liquid (0.32 g, 84% yield, R_f = 0.4, 10:90 methanol/chloroform). ^{1}H NMR (200 MHz, $CDCl_3$):δ (ppm) =1.20−1.40 (4s, 12H, 2{COC(*CH*$_3$)$_2$OC}, 1.40−1.80 (m, 6H), 2.80−3.00 (m, 2H, C*H*$_2$NH$_2$), 3.30 (s, 3H, OC*H*$_3$), 3.39−3.90 (m, 8H, CH_2O, H-1, H-1′, H-3, H-5, H-6, H-6′), 3.90−4.19 (m, 2H, H-2, H-4), 4.60 (s, 2H, OC*H*$_2$O).

Step i: Synthesis of 5-(*N*,*N*-Di-*n*-hexadecylamino)-pentyl-2,3:5,6-di-*O*-isopropylidene-4-methoxymethyl-D-galactoside (XI). In a 50 mL round-bottom flask, a mixture of *n*-hexadecyl bromide (0.6 g, 2.00 mmol), compound **X** (0.31 g, 0.79 mmol) and K_2CO_3 (0.17 g, 1.19 mmol) in ethyl acetate (30 mL) was stirred at 70 °C for 24 h. Another 0.5 mL of *n*-hexadecyl bromide and 0.05 g of K_2CO_3 were added to the reaction mixture, and stirring was continued for additional 48 h at 70 °C. The reaction mixture was diluted with 60 mL of ethyl acetate and washed with water (2 × 30 mL). The organic layer was separated, dried over Na_2SO_4, filtered and concentrated. The residue upon column chromatographic purification (using 60−120 mesh size silica gel and 5:95 acetone:hexane as eluent) afforded pure compound **XI** as a liquid (0.45 g, 66% yield, R_f = 0.6, 30:70 acetone/hexane). ^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) = 0.90 (t, 6H, C*H*$_3$(CH$_2$)$_{14}$), 1.10−1.90 (m, 74H) 2.20−2.40 (m, 6H (CH$_2$)$_3$N), 3.39 (S, 3H, OCH$_3$), 3.40−3.80 (m, 8H, H-1, H-1′, H-3, H-5, H-6 and H-6′, OC*H*$_2$), 4.00−4.20 (m, 2H, H-2 and H-4), 4.70 (s, OC*H*$_2$O). FABMS (LSIMS): *m*/*z*: 841 $[M]^+$ + 1 for $C_{51}H_{101}O_7N$.

Step j: Synthesis of 5-[(*N*,*N*-Di-*n*-hexadecyl-*N*-methyl)amino]pentyl-2,3:5,6-di-*O*-isopropylidene-4-methoxymethyl-D-galactoside (XII). In a 25 mL round-bottom flask, methyl iodide (3 mL) was added to compound **XI** (0.3 g, 0.35 mmol) dissolved in dichloromethane (5 mL), and the reaction mixture was stirred at room temp for 3 h. The reaction mixture was concentrated, and the residue upon column chromatographic purification (using 60−120 mesh size silica gel and 20:80 methanol:chloroform as eluent) afforded compound **XII** as an yellowish solid (0.3 g, 98% yield, R_f = 0.6,1:1 acetone/hexane). ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm) = 0.8 (t, 6H, C*H*$_3$(CH$_2$)$_{14}$), 1.10−1.80 (m, 74H), 3.30 (s, 3H, C*H*$_3$N$^+$-(CH$_2$)$_3$), 3.34 (s, 3H, OC*H*$_3$) 3.40−3.78 (m, 14H, H-1, H-1′, H-3, H-5, H-6, H-6′, OC*H*$_2$, N$^+$ (C*H*$_2$)$_3$), 4.00−4.10 (m, 2H, H-2 and H-4), 4.60 (s, OC*H*$_2$O). FABMS (LSIMS): *m*/*z*: 855 $[M]^+$ for $C_{54}H_{104}O_7N$.

Step k: Synthesis of 5-[(*N*,*N*-Di-*n*-hexadecyl-*N*-methyl)amino]-1-*O*-pentyl-D-galactose (lipid 5, Scheme 2). Compound **XII** (0.3 g, 0.35 mmol) was dissolved in tetrahydrofuran (5 mL) containing 6 N HCl (2 mL). The mixture was stirred at 50 °C for 5−6 h. The excess tetrahydrofuran was removed on rotary evaporator, and the residue upon column chromatographic purification (using a 60−120 silica gel mesh size, 8−9% methanol:chloroform as eluent) followed by chloride ion exchange (using Amberlyst A-26 with methanol as the eluent) afforded the pure title lipid **5** as a colorless solid (0. 11 g, 42% yield, R_f = 0.2, 10:90 methanol/chloroform). ^{1}H NMR (200 MHz, $CDCl_3$): 0.90 (t, 6H, −CH_2−C*H*$_3$), 1.00−1.60 (m, 62H), 3.40−5.20 (m, 19H). FABMS (LSIMS): *m*/*z*: 731 $[M]^+$ + 1 for $C_{44}H_{92}O_6N$ HRMS (LSIMS) *m*/*z*: Calcd (for $C_{44}H_{92}O_6N$ the 4° ammonium ion,100%) 730.6924, found 730.6889.

Synthesis of *O*-Benzyl-5-bromo-1-pentanol. Step l: 5-*O*-Benzyl-1-pentanol. The precursor to the title reagent used in step d was prepared from the corresponding 1,5-pentanediol (2.0 mL) following a previously described monobenzylation procedure of symmetric diol.[35] The residue upon purification by column chromatography (using 60−120 silica gel mesh size, 20:80 ethyl acetate/hexane as a elutent) afforded pure 5-*O*-benzyl-1-pentanol, as a colorless liquid (1.41 g, 75% yield, R_f = 0.4,3:7 ethyl acetate/hexane). ^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm)= 1.28−1.7 (m, 6H, OCH$_2$(C*H*$_2$)$_3$CH$_2$OH), 1.9 (s,1H, OH), 3.4 (t, 2H, HOC*H*$_2$CH$_2$), 3.48 (t, 2H, C*H*$_2$OBn), 4.4 (s, 2H, OC*H*$_2$Ph), 7.2−7.3 (m, 5H, aromatic).

Step m. In a 50 mL two-neck round-bottom flask, imidazole (0.68 g, 10 mmol) and triphenylphosphine (2.63 g, 10 mmol) were dissolved in dry dichloromethane under nitrogen atmosphere. To the resulting solution was added dropwise the compound obtained in step l (1.30 g, 6.7 mmol) dissolved in dry dichloromethane followed by addition of CBr_4 (3.32 g, 32 mmol). The reaction mixture was stirred at 0 °C for 1 h, and the temperature was gradually raised to room temperature. The reaction mixture was kept under stirring at room temperature for 6 h. The reaction mixture was then concentrated, and the residue upon column chromatographic purification using 60−120 mesh silica gel and 3% ethyl acetate in hexane as eluent afforded the title reagent (*O*-benzyl-5-bromo-1-pentanol used in step f) as a colorless liquid (0.95 g, 55.2% yield, R_f = 0.7 with 15:85 ethyl acetate: hexane). ^{1}H NMR (200 MHz, $CDCl_3$): δ (ppm) = 1.2−1.4 (m, 6H, OCH$_2$(C*H*$_2$)$_3$-CH$_2$Br), 3.3−3.5 (m, 4H, OC*H*$_2$(CH$_2$)$_3$C*H*$_2$Br), 4.45 (s, OC*H*$_2$-Ph), 7.2−7.4 (m, 5H, aromatic).

Step k: Synthesis of 5-(*N*,*N*-Di-*n*-tetradecyl-*N*-methylamino)-1-*O*-pentyl-D-galactose (lipid 4, Scheme 2). The title lipid **4** (white solid) was synthesized following the same synthetic procedure as described above for preparing lipid **5** using *n*-tetradecyl bromide in step i with an overall yield of 34%. All the isolated intermediates gave spectroscopic data in agreement with their assigned structures shown in Scheme 2. ^{1}H NMR (200 MHz, $CDCl_3$) of intermediate **XI** (Scheme 2, where R = *n*-$C_{14}H_{29}$). δ (ppm) = 0.90 (t, 6H, C*H*$_3$(CH$_2$)$_{12}$), 1.10−1.50 (m, 62H), 1.55−1.70 (m, 4H), 2.20−2.40 (m, 6H (C*H*$_2$)$_3$N), 3.39 (S, 3H, OCH$_3$), 3.40−3.80 (m, 8H, H-1,H-1′, H-3, H-5, H-6 and H-6′, OCH$_2$), 4.00−4.20 (m, 2H, H-2 and H-4), 4.70 (s, 2H, OC*H*$_2$O). FABMS (LSIMS): *m*/*z*: 783 $[M]^+$ + 1 for $C_{47}H_{93}O_7N$. ^{1}H NMR (200 MHz, $CDCl_3$) of intermediate **XII** (Scheme 2, where R = *n*-$C_{14}H_{29}$: δ (ppm) = 0.90 (t, 6H, C*H*$_3$(CH$_2$)$_{14}$), 1.20−1.30 (m, 44H), 1.35−1.45 (m, 12H), 1.60−1.80 (m, 4H), 3.35 (s, 3H, C*H*$_3$N$^+$(CH$_2$)$_3$), 3.40 (s, 3H, OC*H*$_3$) 3.45−3.80 (m, 14H, H-1, H-1′, H-3, H-5, H-6, H-6′, OC*H*$_2$, N$^+$(C*H*$_2$)$_3$), 4.00−4.10

(m, 2H, H-2 and H-4), 4.60 (s,2H OCH_2O). FABMS (LSIMS): *m/z*: 799 $[M]^+ + 1$ for $C_{48}H_{96}O_7N$. ^1H NMR (200 MHz, $CDCl_3$+DMSO): (lipid **4**) δ (ppm) = 0.90 (t, 6H, CH_2CH_3), 1.00−1.80 (m, 54H), 3.20−3.90 (m, 13H), 4.32−5.30 (m, 6H). FABMS (LSIMS): *m/z*: 675 $[M]^+ + 1$ for $C_{40}H_{84}O_6N$.

Step k: Synthesis of 5-(*N*,*N*-Di-*n*-octadecyl-*N*-methylamino)-1-*O*-pentyl-D-galactose (lipid 6, Scheme 2). The title lipid **6** (white solid) was synthesized following the same synthetic synthetic procedure as described above for lipid **5** using *n*-octadecyl bromide with an overall yield of 35%. All the isolated intermediates gave spectroscopic data in agreement with their assigned structures shown in Scheme 2. ^1H NMR (200 MHz, $CDCl_3$) of intermediate **XI** (Scheme 2, where R = *n*-$C_{18}H_{37}$): δ (ppm): = 0.90 (t, 6H, $CH_3(CH_2)_{12}$), 1.10−1.60 (m, 82H), 2.20−2.50 (m, 6H $(CH_2)_3N$), 3.39 (s, 3H, OCH_3), 3.40−3.90 (m, 8H, H-1,H-1′, H-3, H-5, H-6 and H-6′, OCH_2), 4.00−4.20 (m, 2H, H-2andH-4), 4.70 (s, 2H OCH_2O). FABMS (LSIMS): *m/z*: 897 $[M]^+ + 1$ for $C_{55}H_{109}O_7N$. ^1H NMR (200 MHz, $CDCl_3$) of intermediate **XII** in Scheme 2, where R = $C_{18}H_{37}$, δ (ppm) = 0.90 (t, 6H, $CH_3(CH_2)_{14}$), 1.10−1.90 (m,82H), 3.30 (S, 3H,$CH_3N^+(CH_2)_3$), 3.39 (3H, OCH_3) −3.80 (m, 14H, H-1, H-1′, H-3, H-5, H-6, H-6′, OCH_2, $N^+(CH_2)_3$), 4.00−4.10 (m, 2H, H-2 and H-4), 4.60 (s, 2H OCH_2O). FABMS (LSIMS): *m/z*: 911 $[M]^+ + 1$ for $C_{56}H_{112}O_7N$. ^1H NMR (200 MHz, $CDCl_3$): (lipid **6**) δ (ppm) = 0.90 (t, 6H, CH_2CH_3), 1.00−2.00 (m, 70H), 3.00−5.00 (m, 19H). FABMS (LSIMS): *m/z*: 787 $[M]^+ + 1$ for $C_{48}H_{100}O_6N$.

Preparation of Liposomes. The cationic lipid and cholesterol in 1:1 mole ratio was dissolved in a mixture of chloroform and methanol (3:1, v/v) in a glass vial. The solvent was removed with a thin flow of moisture-free nitrogen gas, and the dried lipid film was then kept under high vacuum for 8 h. Sterile deionized water (5 mL) was added to the vacuum-dried lipid film, and the mixture was allowed to swell overnight. The vial was then vortexed for 2−3 min at room temperature to produce multilamellar vesicles (MLVs). MLVs were then sonicated in an ice bath until clarity using a Branson 450 sonifier at 100% duty cycle and 25 W output power to produce small unilamellar vesicles (SUVs).

Preparation of Plasmid DNA. pCMV-SPORT-β-gal plasmid DNA was prepared by alkaline lysis procedure and purified by PEG-8000 precipitation according to the protocol described by Maniatis and co-workers.[36] The plasmid preparations showing a value of OD_{260}/OD_{280} more than 1.8 were used.

Transfection Biology. Cells were seeded at a density of 20000 cells (for CHO, MCF-7, A549) and 15000 cells (for COS-1) per well in a 96-well plate 18−24 h before the transfection. 0.3 μg of plasmid DNA was complexed with varying amounts of lipids (0.09−8.1 nmol) in plain DMEM medium (total volume made up to 100 μL) for 30 min. The charge ratios were varied from 0.1:1 to 9:1 (+/−) over these ranges of the lipids. The complexes were then added to the cells. After 3 h of incubation, 100 μL of DMEM with 20% FBS 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 (100 μL each) and lysed in 50 μL of lysis buffer [0.25 M Tris-HCl pH 8.0, 0.5% NP40]. Care was taken to ensure complete lysis. The β-galactosidase activity per well was estimated by adding 50 μL of 2X-substrate solution [1.33 mg/mL of ONPG, 0.2 M sodium phosphate (pH 7.3) and 2 mM magnesium chloride] to the lysate in a 96-well plate. Adsoption at 405 nm was converted to β-galactosidase units using a calibration curve constructed with pure commercial β-galactosidase enzyme. The values of β-galactosidase units in triplicate experiments assayed on the same day varied by less than 20%. The transfection experiment was carried in duplicate and the transfection efficiency values shown in Figure 1 are the average of triplicate experiments performed on the same day. Each transfection experiment was repeated two times and the day to day variation in average tansfection efficiency was found to be within 2-fold. The transfection profiles obtained on different days were identical.

Toxicity Assay. Cytotoxicities of the lipids **1**−**6** were assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay as described earlier.[32] The cytotoxicity assay was performed in 96-well plates by maintaining the same ratio of number of cells to amount of cationic lipid, as used in the transfection experiments. MTT was added 3 h after addition of cationic lipid to the cells. Results were expressed as percent viability = [A_{540}(treated cells) − background/A_{540}(untreated cells) − background] × 100.

Zeta Potential (ξ) and Size Measurements. The sizes and the surface charges (zeta potentials) of liposomes and lipoplexes were measured by photon correlation spectroscopy and electrophoretic mobility on a Zeta sizer $3000HS_A$ (Malvern UK). The sizes were measured in deionized water with a sample refractive index of 1.59 and a viscosity of 0.89. The system was calibrated by using the 200 nm ± 5 nm polystyrene polymer (Duke Scientific Corps., Palo Alto, CA). The diameters of liposomes and lipoplexes were calculated by using the automatic mode. The zeta potential was measured using the following parameters: viscosity, 0.89 cP; dielectric constant, 79; temperature, 25 °C; *F*(Ka), 1.50 (Smoluchowski); maximum voltage of the current, *V*. The system was calibrated by using DTS0050 standard from Malvern, UK. Measurements were performed 10 times with the zero field correction. The potentials were calculated by using the Smoluchowski approximation.

DNA Binding Assay. The DNA binding ability of the cationic lipids **1** and **6** (Figure 4, Parts A, C, and E) were assessed by their gel retardation assay on a 1% agarose gel (prestained with ethidium bromide) across the varying lipid: DNA charger ratios of 0.1:1 to 9:1. pCMV-β-gal (0.30 μg) was complexed with the varying amount of cationic lipids in a total volume of 20 μL in Hepes buffer, pH 7.40 and incubated at room temperature for 20−25 min. 4 μL of 6X loading buffer (0.25% bromophenol blue in 40% (w/v) sucrose in H_2O) was added to it, and the resulting solution (24 μL) was loaded on each well. The samples were electrophoresed at 80 V for 45 min, and the DNA bands were visualized in the Gel documentation unit.

DNase 1 Sensitivity Assay. Briefly, in a typical assay, pCMV-β-gal (1000 ng) was complexed with the varying amount of cationic lipids (using indicated lipid:DNA charge ratios in Figure 4, Parts B, D and F) in a total volume of 30 μL in Hepes buffer, pH 7.40, and incubated at room temperature for 30 min on a rotary shaker. Subsequently, the complexes were treated with 10 μL of DNase I (at a final concentration of 1 μg/mL) in the presence of 20 mM $MgCl_2$ and incubated for 20 min at 37 °C. The reactions were then halted by adding EDTA (to a final concentration of 50 mM) and incubated at 60 °C for 10 min in a water bath. The aqueous layer was washed with 50 μL of phenol:chloroform:isoamyl alcohol (25:24:1mixture, v/v) and centrifuged at 10 000*g* for 5 min. The aqueous supernatants were separated, loaded (15 μL for lipids **1, 2, 4** and **5** and 30 μL for lipids **3** and **6**) on a 1% agarose gel (prestained with ethidium bromide) and electrophoresed at 100 V for 1 h.

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.[37] Briefly, 48 h after transfection with lipoplexes in 96-well plates, the cells were washed two times (2 × 100 μL) with phosphate-buffered saline (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 were stained with 1.0 mg/mL X-gal in PBS containing 5.0 mM $K_3[Fe(CN)_6]$, 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).

Abbreviations

MOM-Cl, methoxymethyl chloride; PTSA, *p*-toluenesulfonic acid; TBAF, tetra-*n*-butylammonium fluoride; TBDMSCl, *tert*-butyldimethylsilyl chloride; DMP, 2,2-dimethoxypropane; Chol, cholesterol; CBr_4, carbon tet-

rabromide DCM, dichloromethane; DMAP, 4-(*N*,*N*-dimethylamino)pyridine; DMEM, Dulbecco's Modified Eagles Medium; DMF, *N*,*N*-dimethylformamide; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; FBS, fetal bovine serum; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; PBS, phosphate-buffered saline; TFA, trifluoroacetic acid.

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Supporting Information Available: Reverse phase HPLC chromatograms for the lipids **1**, **3**, **4** and **6** in two mobile phases A and B with details of used HPLC parameters and actual elemental analysis results (C, H, N) for lipids **2** and **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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