Design, Syntheses, and Transfection Biology of Novel Non-Cholesterol-Based Guanidinylated Cationic Lipids

Joyeeta Sen and Arabinda Chaudhuri*

Division of Lipid Science and Technology, Indian Institute of Chemical Technology, Hyderabad-500 007, India

Received July 22, 2004

The design of efficacious cationic transfection lipids with guanidinium headgroups is an actively pursued area of research in nonviral gene delivery. Herein, we report on the design, syntheses, and gene transfection properties of six novel non-cholesterol-based cationic amphiphiles (**1**−**6**) with a single guanidinium headgroup in transfecting CHO, COS-1, MCF-7, A549, and HepG2 cells. The in vitro gene transfer efficiencies of lipids **1**−**6** were evaluated using both the reporter gene and the whole cell histochemical X-gal staining assays. The efficiencies of lipids **1**−**3**, in particular, were found to be about 2- to 4-fold higher than that of commercially available LipofectAmine in transfecting COS-1, CHO, A-549, and MCF-7 cells. However, the relative transfection efficiencies of lipids **1**−**3** and LipofectAmine were found to be comparable in HepG2 cells. Cholesterol was found to be a more efficacious co-lipid than dioleoyllphosphatidyl ethanolamine (DOPE). In general, lipids **1**−**3** containing the additional quaternized centers were observed to be more transfection efficient than lipids **4**−**6** with less positive headgroups. MTT-assay-based cell viability measurements in representative CHO cells revealed high (>75%) cell viabilities of lipids **1**−**6** across the lipid/DNA charge ratios 0.1:1 to 3:1. Electrophoretic gel patterns observed in DNase I protection experiments support the notion that enhanced degradation of DNA associated with lipoplexes of lipids **4**−**6** might play some role in diminishing their in vitro gene transfer efficacies. Size and global surface charge measurement by a dynamic laser light scattering instrument equipped with ζ-sizing capacity revealed the nanosizes and surface potentials of both the transfection efficient and the incompetent lipoplexes to be within the range of 200−600 nm and +3.4 to −34 mV, respectively. To summarize, given the feasibility of a wide range of structural manipulations in the headgroup regions of non-cholesterol-based cationic amphiphiles, our present findings are expected to broaden the potential of cationic amphiphiles with guanidinium headgroups for use in nonviral gene therapy.

Introduction

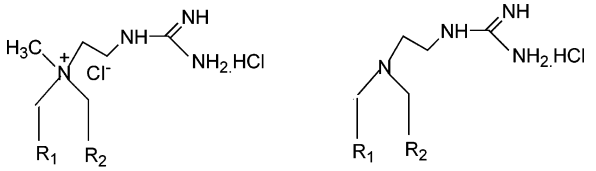
Design of safe and efficacious gene transfer reagents continues to remain as one of the key challenges in gene therapy, the therapeutic modality to combat myriads of inherited diseases, dreadful viral infections, and cancer. Viral vectors, although remarkably efficient in transfecting body cells, suffer from numerous biosafety-related disadvantages.[1−3] Conversely, cationic amphiphiles, 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 transfection vectors of choice,[4−14] our own work included.[15−19]

Design of an efficient cationic transfection lipid needs strategies to deal with a multitude of cellular barriers. Currently believed lipoplex (lipid/DNA complex)-mediated intracellular transfection pathways involve: (a) formation of lipid/DNA complexes and their initial binding to the cell surface; (b) endocytotic internalization of the lipid/DNA complexes; (c) trafficking in the endosome/lysosome compartment and escape of DNA from the endosome/lysosome compartment to the cell cytoplasm; (d) transport of the endosomally released DNA to the nucleus followed by its transgene expression.[20−23] Thus, rational design of efficient cationic transfection lipids are often based on covalent grafting of various structural elements (into the molecular architecture of the cationic lipids) capable of favorably modulating one or more of these mechanistic steps involved in lipofection pathways.[19,24−26] In 1996, a particularly elegant design of efficient cationic transfection lipid was pioneered by Vigneron et al.[27] The distinguishing features of the guanidinium functionality exploited in their design include: (a) the guanidinium group remains protonated over a much wider range of pH than other basic groups due to its remarkably high pK_a values (13.5); (b) it forms characteristic parallel zwitterionic hydrogen bonds N−H$^+$···O$^-$ with phosphate ions; (c) the guanidinium groups are also capable of forming hydrogen bonds with nucleic acid bases; (d) the guanidinium group of the arginyl residues plays a major role in DNA-binding proteins such as histones and protamines.[27] The rationales for using cholesterol as the hydrophobic tail were its membrane-compatible characteristics and its ability to facilitate the cellular uptake of various oligonucleotides and polar drugs.[27] It is pertinent to mention here that the design of an aliphatic-hydrocarbon-tail-based cationic lipid with an amidine headgroup, a headgroup structurally close to guanidine, was reported earlier by Ruysschaert et al. in 1994.[28]

* Author to whom correspondence should be addressed. Phone: 91-40-27193201. Fax: 91-40-27160757. E-mail: arabinda@iict.res.in.

10.1021/jm049417w CCC: $30.25
Published on Web 01/08/2005

Chart 1. Structures of Novel Non-Cholesterol-Based Guanidinylated Cationic Lipids **1**−**6**



1, $R_1 = R_2 = n\text{-}C_{13}H_{27}$;
2, $R_1 = R_2 = n\text{-}C_{15}H_{31}$;
3, $R_1 = R_2 = n\text{-}C_{17}H_{35}$

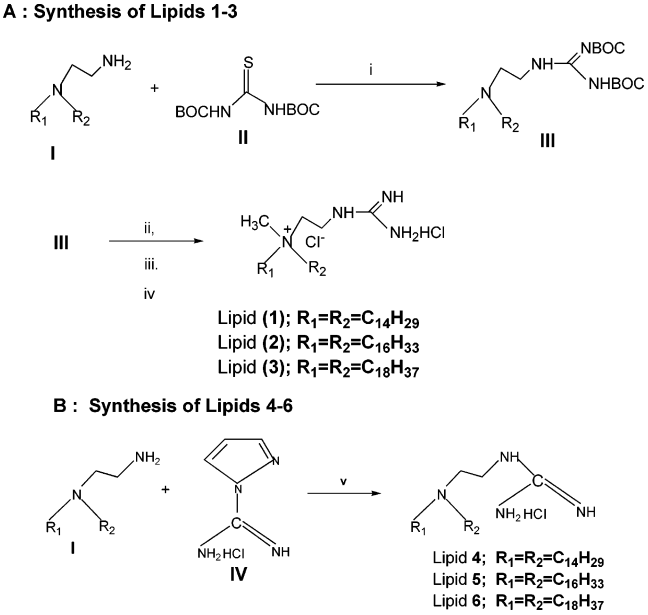
4, $R_1 = R_2 = n\text{-}C_{13}H_{27}$;
5, $R_1 = R_2 = n\text{-}C_{15}H_{31}$;
6, $R_1 = R_2 = n\text{-}C_{17}H_{35}$

Ever since the development of the above-mentioned cholesterol and aliphatic-hydrocarbon-tail-based cationic transfection lipids with guanidinium and amidine headgroups were reported, investigations aimed at broadening the scope of both cholesterol- and non-cholesterol-based cationic transfection lipids with guanidine or amidine headgroups have remained unabated.[29−45] Herein, we report on the design, syntheses, and gene transfection properties of six novel non-cholesterol-based nontoxic cationic amphiphiles (**1**−**6**, Chart 1) with a single guanidinium headgroup in transfecting CHO, COS-1, MCF-7, A549, and HepG2 cells. As delineated below, the efficiencies of lipids **1**−**3**, in particular, were found to be about 2- to 4-fold higher than that of commercially available LipofectAmine in transfecting COS-1, CHO, A-549, and MCF-7 cells. Given the feasibility of a wide range of structural manipulations in the headgroup regions of non-cholesterol-based cationic amphiphiles, present findings are expected to further broaden the potential of cationic amphiphiles with guanidinium headgroups for use in nonviral gene therapy.

Results and Discussion

Chemistry. The hydrophobic mixed primary−tertiary amine (**I**, Scheme 1, a readily available precursor in our laboratory prepared as described previously[19]) was reacted with 1 equiv each of *N*,*N*-di-*tert*-butyloxycarbonyl thiourea (**II**, Scheme 1, prepared by reacting thiourea with 2 equiv of Boc-anhydride (Boc = *tert*-butyloxycarbonyl) in the presence of 2 equiv of sodium hydride in anhydrous tetrahydrofuran as described previously[46]), mercuric chloride, and triethylamine in anhydrous *N*,*N*-dimethylformamide/dichloromethane under nitrogen atmosphere at 0 °C followed by the usual workup. The resulting tertiary amine intermediate **III** (Scheme 1) upon quaternization with excess methyl iodide followed by acid deprotection and chloride ion-exchange chromatography afforded lipids **1**−**3** (Scheme 1, part A). The mono-guanidinylated cationic lipids **4**−**6** containing a free tertiary amine center were prepared by reacting 1 equiv of the common mixed primary−tertiary amine intermediate **I** (Scheme 1, part B) with 1 equiv of 1H-pyrazole-1-carboxamidine hydrochloride (**IV**, Scheme 1, part B, prepared by reacting pyrazole with 1 equiv of cyanamide in anhydrous *p*-dioxan containing few drops of concentrated HCl) in the presence of 1 equiv of diisopropylethylamine in anhydrous *N*,*N*-dimethylformamide/dichloromethane. Lipids **4**−**6** were designed with a view to probe the role of an additional basic tertiary amine center, if any, in modulating the gene transfer properties of non-cholesterol-based guanidinylated cationic lipids. Structures of all of the synthetic intermediates and the target lipids shown in Scheme 1 were confirmed by ^{1}H NMR and LSIMS.

Scheme 1[a]



[a] Reagents and conditions: (i) $HgCl_2$ (1 equiv), TEA (1 equiv), DMF, N_2 atmospheric pressure, 0 °C; (ii) MeI; (iii) TFA/DCM (1:1); (iv) Cl ion-exchange resin; (v) DIEA (1 equiv), DMF/DCM.

Transfection Biology. Figure 1 summarizes the relative in vitro gene delivery efficacies of lipids **1**−**6** in transfecting CHO, COS-1, HepG2, 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 co-lipid, 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. The in vitro gene transfer efficiencies of lipids **1**−**6** were evaluated using both the reporter gene and the whole cell histochemical X-gal staining assays. In the reporter gene assay, the in vitro gene transfer efficiencies of lipids **1**−**3** with no free tertiary amine base centers were found to be remarkably efficient, about 2- to 4-fold higher than that of commercially available LipofectAmine in transfecting COS-1, CHO, A-549, and MCF-7 cells (Figure 1, parts A−D, respectively). However, the relative transfection efficiencies of lipids **1** and **2** and LipofectAmine were found to be comparable in HepG2 cells (Figure 1, part E). Interestingly, for the present lipids, cholesterol was found to be a more efficacious co-lipid than 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE). Representative transfection profiles using DOPE as a co-lipid are shown in Figure S7 of the Supporting Information. In general, the dicationic guanidinylated lipids **1**−**3** containing the additional quaternized centers were observed to be more transfection efficient than lipids **4**−**6** with less positive headgroups, and the optimal transfection properties were observed with the lipid/DNA charge ratio of 1:1 and 3:1 (Figure 1). Interestingly, only lipid **6** was found to be essentially incompetent in transfecting all five of the cells across all the lipid/DNA charge ratios (Figure 1). Similar transfection profiles

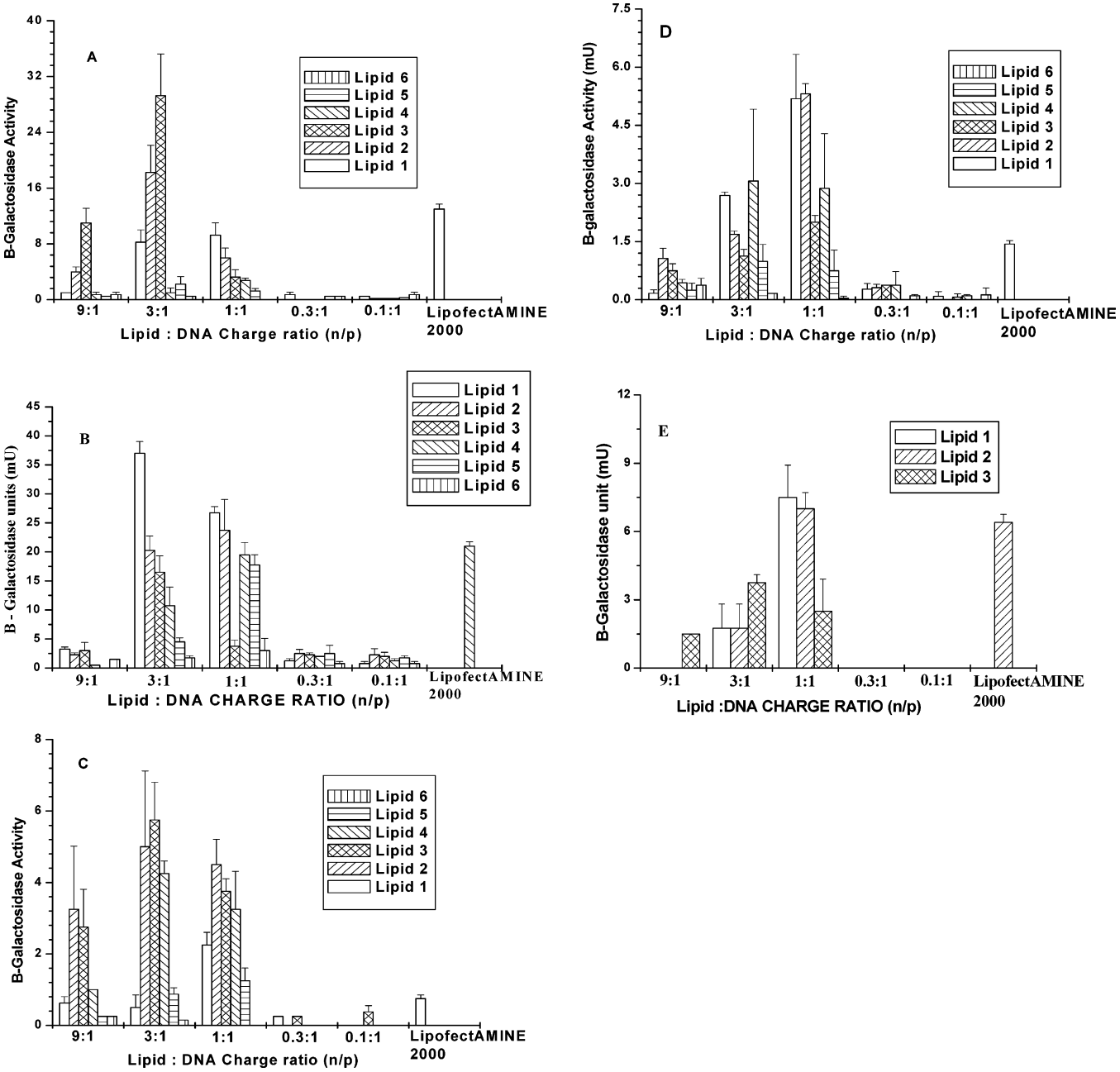


Figure 1. In vitro transfection efficiencies of lipids **1**−**6** in COS-1 (A), CHO (B), A-549 (C), MCF-7 (D), and HepG2 (E) cells using cholesterol as co-lipid (at a lipid/cholesterol mole ratio of 1:1). Units of β-galactosidase activity were plotted against the varying lipid to DNA (±) charge ratios. The transfection efficiencies of the lipids were compared to that of commercially available LipofectAmine 2000. Transfection experiments were performed as described in the text. The transfection values shown are the average of duplicate experiments performed on the same day.

for lipids **1**−**6** were also observed in the whole cell histochemical X-gal staining assay. A representative X-gal staining pattern is shown in Figure 2 for transfection efficient lipid **1** and transfection incompetent lipid **6** in CHO cells. An important point seems appropriate to bring at this point of discussion. Vigneron et al.[27] compared the transfection properties of their cholesterol-based bis-guanidinylated lipids with that of Lipofectin, one of the very first generations of liposomal transfection kits then commercially availlable. Given the usually higher in vitro transfection properties of LipofectAmine-2000 (compared to Lipofectin) and the superior transfection efficacies of lipids **1**−**3** to that of LipofectAmine-2000 (Figure 1), the non-cholesterol-based mono-guanidinylated cationic lipids described herein will further broaden the versatility of using guanidinylated cationic lipids in nonviral gene delivery.

In their original design of bis-guanidinium-tren-cholesterol (BGTC), Vigneron et al.,[27] the additional tertiary amine center between the polar bis-guanidinium head and the hydrophobic cholesterol tail in the lipid structure was grafted to ensure enhanced transfection efficacies through buffering of the acidic endosome. The presence of an additional tertiary amine center with pK_a values much lower than that of guanidine was expected to buffer (to some extent) the acidic environment of endosomes and thereby was expected to protect the lipoplex associated DNA against assault by lysosomal DNase. However, in our present study, lipids **4**−**6** with an additional tertiary amine base were,

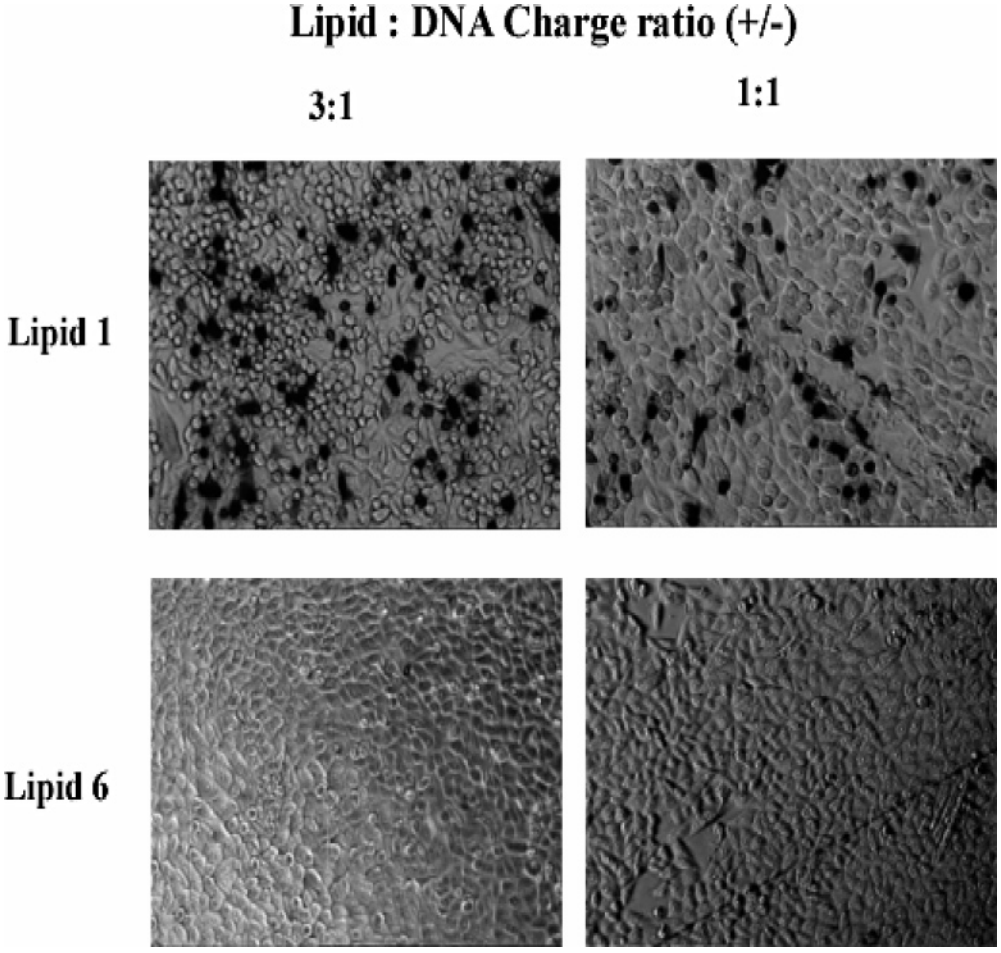


Figure 2. Histochemical whole cell staining of transfected CHO cells with X-gal with lipid **1** and lipid **6** at lipid/DNA charge ratios of 3:1 and 1:1. Cells expressing β-galactosidase were stained with X-gal as described in the text.

in general, found to be significantly less transfection efficient than their quaternized counterparts lipids **1**−**3** (Figure 1). Such relative transfection profiles of lipids **1**−**3** and **4**−**6** indirectly imply that the presence of an additional free tertiary amine center (with lower pK_a values) in the molecular structures of guanidinylated cationic lipids is perhaps not needed for non-cholesterol-based guanidinylated cationic lipids. Alternatively, endosomal buffering for guanidinylated cationic transfection lipids may not be as important a mechanistic event for non-cholesterol-based dicationic mono-guanidinylated cationic transfection lipids as was originally envisioned. for the cholesterol-based bis-guanidinylated cationic lipids.[27,33] Taken together, the results summarized in Figures 1 and 2 convincingly demonstrate the future potential of non-cholesterol-based mono-guanidinylated cationic transfection lipids for use in nonviral gene delivery.

MTT-based cell viabilities of both lipids **1**−**6** and LipofectAmine were evaluated in representative CHO cells across the entire range of lipid/DNA charge ratios used in the actual transfection experiments. Cell viabilities of both the lipids **1**−**3** and the lipids **4**−**6** were found to be remarkably high (more than 75%) particularly up to the lipid/DNA charge ratios of 3:1 (Figure 3). However, except for lipid **6**, high cell viabilities were somewhat compromised at high lipid/DNA charge ratios of 9:1 (Figure 3). Thus, the contrasting 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 lipids.

Nanosizes and Global Surface Charges of the Lipoplexes. Towards physicochemically characterizing the present lipoplexes, the nanosizes and the global surface charges of the representative lipoplexes prepared with lipid **1** (highly transfection competent) and lipid **6** (transfection incompetent) were measured using a dynamic laser light scattering instrument equipped with ζ-sizing capacity across the lipid/DNA charge ratios of 0.1:1 to 9:1 in the presence of Dulbecco's modified Eagle's medium (DMEM). The nanosizes of lipoplexes prepared from both the transfection efficient and the incompetent lipids (**1** and **6**, respectively) similarly increased with increasing lipid/DNA charge ratios within the range 230−590 nm (Table 1). Interestingly, in the presence of DMEM, although the global surface charges of lipoplex **1** became less negative as the lipid/DNA charge ratios increased from 0.1:1 to 3:1, that of the lipoplexes prepared with lipid **6** remained remarkably negative even at a lipid/DNA charge ratio of 3:1 (Table 1). Given that the biological membranes are also negatively charged, such significantly high negative surface potentials of lipoplex **6** may lead to some electrostatic barrier to its cellular uptake process. Flow cytometric studies will be needed in the future to gain better mechanistic insights into whether the completely transfection incompetent nature of lipid **6** originates from an impeded cellular uptake process.

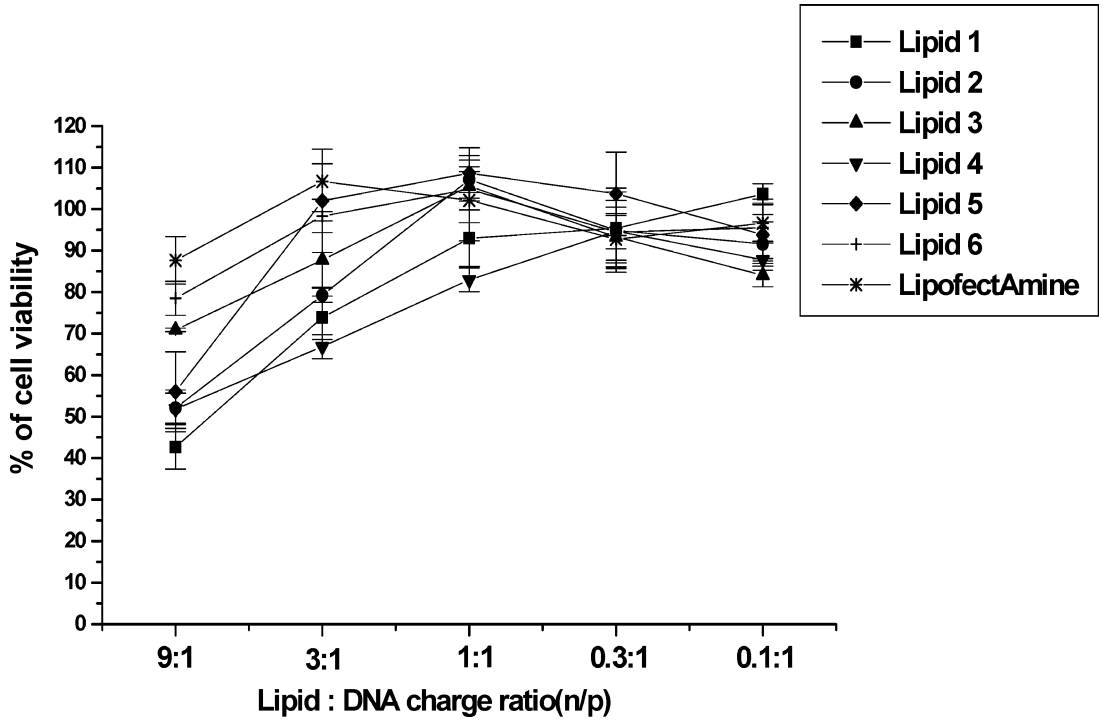


Figure 3. MTT-assay-based cellular cytotoxicities of lipids **1**−**6** and LipofectAmine against representative CHO cells. The cell viability values shown are the averages of duplicate experiments performed on the same day.

Table 1. Nanosizes (nm) and ζ Potential (mV) of Lipoplexes in the Presence of Plain DMEM[a]

		lipid/DNA charge ratio				
	sample code	9:1	3:1	1:1	0.3:1	0.1:1
sizes (nm)	lipid **1**	585.3 ± 3.1	408.7 ± 19.4	229.1 ± 1.8	216.2 ± 2.1	233.8 ± 4.8
	lipid **6**	514.4 ± 7.0	408.2 ± 11.3	274.7 ± 3.6	248.5 ± 3.8	290.4 ± 3.6
ζ potential(mV)	lipid **1**	1.7 ± 2.5	−1.5 ± 2.5	−3.2 ± 2.7	−29.8 ± 1.6	−34.1 ± 1.3
	lipid **6**	3.4 ± 3	−23.7 ± 3.6	−31.8 ± 2.2	−33.7 ± 0.8	−33.3 ± 0.8

[a] Sizes and ξ potentials were measured by laser light scattering technique using ζ sizer 3000A (Malvern Instruments, U.K.). Values shown are the averages obtained from 3 (size) and 10 (ζ potential) measurements.

Lipid/DNA Binding Interactions and Lipoplex Sensitivities to DNase I. Finally, with a view to characterize the electrostatic binding interactions between 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 the DNase I sensitivity assays. Representative electrophoretic gel patterns observed in the simple gel retardation assay for lipoplexes prepared from lipids **1**, **3**, **4**, and **6** are shown in Figure 4. The lipids 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). However, at lower lipid/DNA charge ratios of 3:1, more free DNA was found to be present in lipoplexes of lipids **6** compared to the amount of free DNA present in the lipoplexes prepared from lipids **1**, **3**, and **4** (Figure 4). Such gel patterns are consistent with the notion that relatively weaker lipid/DNA binding interactions might play some role in abolishing the in vitro gene transfer efficacies of lipid **6**. However, at a lower range of lipid/DNA charge ratios (1:1 to 0.1:1), all of the lipoplexes showed the presence of a significant amount of free DNA (Figure 4).

The presence of relatively higher amounts of free DNA in the lipoplexes prepared with relatively transfection inefficient lipids **4** and **6** (compared to those in lipoplexes **1** and **3**) was more convincingly demonstrated by monitoring the sensitivities of the lipoplexes upon

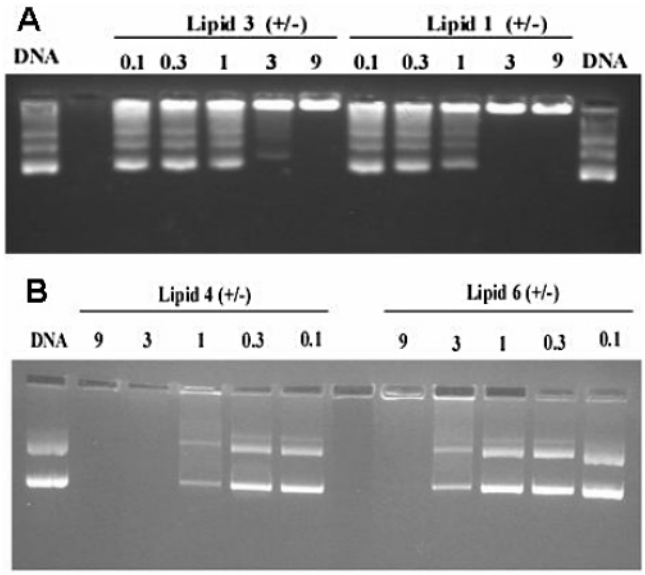


Figure 4. Electrophoretic gel patterns for lipoplex-associated DNA in gel retardation (part A) for lipids **1** and **3** and (part B) for lipids **4** and **6**. The lipid/DNA charge ratios are indicated at the top of each lane. The details of treatment are as described in the text.

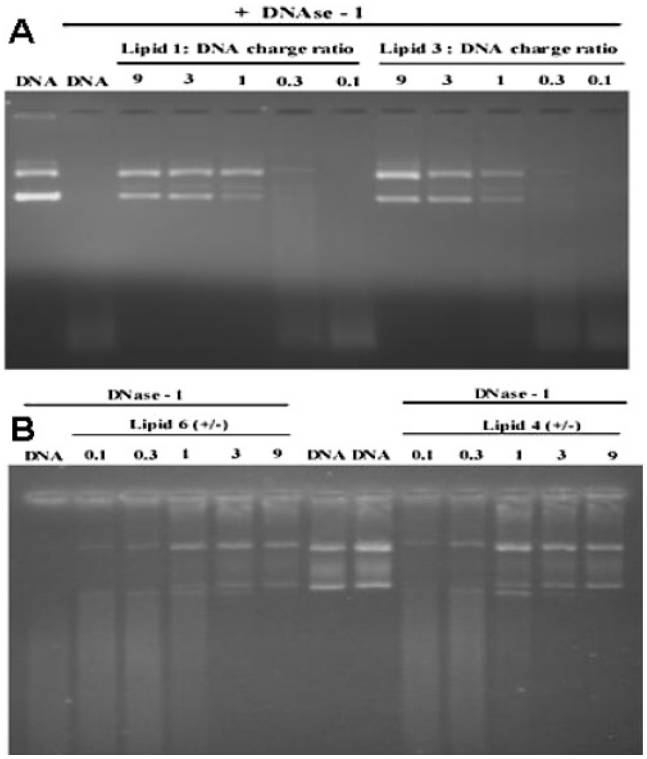


Figure 5. Electrophoretic gel patterns for lipoplex-associated DNA with DNase I sensitivity assay (part A) for lipids **1** and **3** and (part B) for lipids **4** and **6**. The lipid/DNA charge ratios are indicated at the top of each lane. The details of the treatment are as described in the text.

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 for representative lipoplexes prepared with lipids **1**, **3**, **4**, and **6** are shown in Figure 5. Band intensities of inaccessible and therefore undigested DNA associated with transfection incompetent lipoplexes **4** and **6** (lipoplex **6** in particular) were significantly less than those associated with transfection efficient lipoplexes **1** and **3** across the range of lipid/DNA charge ratios of 3:1 to 0:1:1 (Figure 5). Such gel patterns in DNase I sensitivity assays indicate that the plasmid DNA associated with lipids **4** and **6** (particularly lipid **6**) is likely to be more susceptible to degradation by cellular DNase I than the DNA complexed to lipids **1** and **3**. Thus, findings in our DNase I sensitivity assay are consistent with enhanced degradation of lipoplex-associated DNA by cellular DNase I playing some important role behind the severely compromised transfection efficacies of lipid **6** in particular.

In conclusion, we have demonstrated that the presently described non-cholesterol-based cationic amphiphiles particularly lipids **1**−**3** (Chart 1) containing a single guanidinium headgroup are remarkably efficacious in transfecting CHO, COS-1, MCF-7, and A549 cells with transfection efficiencies about 2- to 4-fold higher than that of widely used commercially available LipofectAmine-2000. For these new mono-guanidinylated cationic transfection lipids, cholesterol was found to be a more efficacious co-lipid than dioleoyllphosphatidyl ethanolamine (DOPE). In general, lipids **1**−**3** containing the additional quaternized centers (absent in lipids **4**−**6**) were observed to be remarkably transfection efficient. MTT-assay-based cell viability measurements in representative CHO cells revealed high (>75%) cell viabilities of lipids **1**−**6** across the lipid:DNA charge ratios 0.1:1 to 3:1. Electrophoretic gel patterns observed in DNase I protection experiments support the notion that enhanced degradation of DNA associated with lipoplexes of lipids **4**−**6** by cellular DNase I might possibly play some role in diminishing their in vitro gene transfer efficacies. Size and global surface charge measurement by a dynamic laser light scattering instrument equipped with ζ-sizing capacity revealed the nanosizes and surface potentials of both the transfection efficient and the incompetent lipoplexes to be within the range of 200−600 nm and +3.4 to −34 mV, respectively. Clearly, further cell biology experiments including cellular uptake studies, intracellular trafficking experiments aimed at understanding subcellular localizations of the lipid/DNA complexes, etc. need to be carried out in the future to gain better mechanistic insights into the origin of the contrasting transfection profiles of lipids **1**−**3** and **4**−**6**. To summarize, given the feasibility of a wide range of structural manipulations in the headgroup regions of non-cholesterol-based cationic amphiphiles, the present findings further broaden the potential of cationic amphiphiles with guanidinium headgroups for use in nonviral gene therapy.

Experimental Section

General Procedures and Materials. Fast atom bombardment mass spectrometry (FABMS) data were acquired by the liquid secondary ion mass spectrometry (LSIMS) technique using *meta*-nitrobenzyl alcohol as the matrix. LSIMS analysis was performed in the scan range 100−1000 amu at the rate of 3 scans/s. ^{1}H NMR spectra were recorded on a Varian FT 200 MHz, AV 300 MHz, or Varian Unity 400 MHz spectrometer. 1-Bromotetradecane, 1-bromohexadecane, 1-bromooctadecane, *n*-tetradecylamine, *n*-hexadecylamine, *n*-octadecylamine, pyrazole, and cyanamide were procured from Lancaster (Morecambe, England). *N*,*N*-Diisopropyl ethylamine was procured from Aldrich (USA). Unless otherwise stated, all of the reagents were purchased from local commercial suppliers and were used without further purification. The progress of the reactions was monitored by thin-layer chromatography on 0.25 mm silica gel plates. 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. LipofectAmine-2000 was purchased from Invitrogen Life Technologies. (USA). 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). DOPE and DOPC were purchased from Fluka (Switzerland). Unless otherwise stated, all of the other reagents purchased from local commercial suppliers were of analytical grades and were used without further purification. COS-1 (SV 40 transformed african green monkey kidney cells), CHO (Chinese hamster ovary), HepG2 (human hepatocarcinoma), MCF-7 (human breast adenocarcinoma cell), and A549 (human pulmonary carcinoma) cell lines were procured from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were grown at 37 °C in DMEM with 10% Fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO_2/95% air. Purity of all of the final lipids (**1**−**6**) was determined by analytical HPLC (Shimadzu model LC10A) using a PARTISIL 5 ODS-3 WCS analytical column (4.6 mm × 250 mm, Whatman Inc., Clifton, NJ) in two different mobile phases. One solvent system (A)

was methanol/acetonitrile/water/trifluoroacetic acid in the ratio 65:10:25:0.05 (v/v) for 15 min with a flow rate of 0.8 mL/min. The other mobile phase (B) was methanol/water/trifluoroacetic acid in the ratio 75:25:0.05 for 15 min with a flow rate of 0.8 mL/min. Peaks were detected by UV absorption at 219 nm. All of the target lipids (**1**−**6**) showed more than 95% purity. Typical retention times in mobile phase A were 3.56 min (lipid **1**), 3.59 min (lipid **2**), 3.61 min (lipid **3**), 3.57 min (lipid **4**), 3.62 min (lipid **5**), and 3.64 min (lipid **6**).

Synthesis of Lipids 1−3. Lipids **1**−**3** were synthesized following the procedures depicted schematically in Scheme 1 (part A). As representative details, synthetic procedures for lipid **2** are provided below.

Synthesis of Lipid 2. Step i. Synthesis of *N*,*N*-Di-*n*-hexadecyl-*N*-[2-(*N*′,*N*′-di-tertbutoxycarbonyl-guanidinyl]-ethylamine (III, Scheme 1, Part A). Mercury chloride (0.28 g, 1.0 mmol) was added to a mixture of *N*-2-aminoethyl-*N*,*N*-di-*n*-hexadecylamine (**I**, 0.49 g. 0.95 mmol), bis-*N*-Boc-thiourea (**II**, 0.26 g, 0.95 mmol, prepared by reacting 1 equiv of thiourea with 2 equiv of Boc-anhydride in the presence of 2 equiv of sodium hydride in anhydrous tetrahydrofuran as described previously[46]), and triethylamine (0.21 g, 2.1 mmol) dissolved in dry *N*,*N*-dimethylformamide (DMF, 5 mL) and dry dichloromethane (DCM, 2 mL) at 0 °C with continuous stirring. The resulting mixture was stirred at 0 °C under nitrogen for 40 min, diluted with ethyl acetate (20 mL), and filtered through a pad of Celite. The filtrate was sequentially washed with water (2 × 20 mL) and brine solution (2 × 20 mL), dried over anhydrous sodium sulfate, and filtered, and the solvent from the filtrate was removed by rotary evaporation. The residue upon column chromatographic purification with 60−120 mesh silica gel using 2−2.5% methanol/dichloromethane (v/v) as eluent afforded 0.51 g of the pure title compound **III** (71%, R_f = 0.8, 10% methanol/dichloromethane, v/v).

^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm): 0.9 (t, 6H, C*H*$_3$(CH$_2$)$_{13}$); 1.2−1.4 (bs, 56H, (C*H*$_2$)$_{14}$); 1.4−1.6 (2s, 18H, −CO−O−C(C*H*$_3$)$_3$); 2.4−2.7 (bm, 6H, N(C*H*$_2$CH$_2$)$_2$; NC*H*$_2$CH$_2$NH); 3.4−3.6 (m, 2H, NCH$_2$C*H*$_2$NH); 8.6 (t, 1H, CH$_2$N*H*); 11.4 (s, 1H, N*H*Boc).

Step ii. Synthesis of *N*,*N*-Di-*n*-hexadecyl-*N*-[2-(*N*′,*N*′-di-*tert*-butoxycarbonyl-guanidinyl]ethyl-*N*-methylammonium Iodide (Scheme 1, Part A). The intermediate **III** obtained above in step i was dissolved in 3 mL of dichloromethane/methanol (2:1, v/v), and 3 mL of methyl iodide was added. The solution was stirred at room temperature overnight. Solvent was removed on a rotary evaporator. The residue upon column chromatographic purification with 60−120 mesh size silica gel and 3% methanol in dichloromethane (v/v) as eluent afforded 0.47 g of the title compound (78% yield, R_f = 0.6, 10% methanol in dichloromethane, v/v).

^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm): 0.9 (t, 6H, C*H*$_3$(CH$_2$)$_{14}$); 1.2−1.3 (m, 52H, CH$_3$(C*H*$_2$)$_{13}$); 1.4−1.6 (2s, 18H, −CO−O−C(C*H*$_3$)$_3$); 1.65 (m, 4H, N$^+$(CH$_2$C*H*$_2$)$_2$); 3.3 (s, 3H, N$^+$C*H*$_3$); 3.4 (m,4H, N$^+$(C*H*$_2$CH$_2$)$_2$); 3.6 (m, 2H, N$^+$C*H*$_2$CH$_2$NH); 3.8 (m, 2H, N$^+$CH$_2$C*H*$_2$NH); 8.4 (t, 1H, CH$_2$N*H*); 11.3 (s, 1H, N*H*Boc).

Steps iii and iv. Synthesis of *N*,*N*-Di-*n*-hexadecyl-*N*-[2-guanidinyl]ethyl-*N*-methylammonium Chloride (Lipid 2, Scheme 1, Part A). The intermediate obtained in step ii was dissolved in dry DCM (2 mL), and trifluoroacetic acid (TFA, 2 mL) was added to the solution at 0 °C. The resulting solution was left stirred at room temperature overnight to ensure complete deprotection. Excess TFA was removed by flushing nitrogen to give the title compound as a trifluoroacetate salt. Column chromatographic purification using 60−120 mesh size silica gel and 12−14% (v/v) methanol/chloroform as eluent followed by chloride ion exchange chromatography over amberlyst A-26 chloride ion exchange resin afforded 0.31 g of the pure lipid **2** (94% yield, R_f = 0.3, 10% methanol in chloroform, v/v).

^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm): 0.9 (t, 6H, C*H*$_3$(CH$_2$)$_{14}$); 1.2−1.3 (m, 52H, CH$_3$(C*H*$_2$)$_{13}$); 1.5−1.7 (m, 4H, N$^+$(CH$_2$C*H*$_2$)$_2$); 3.0 (s, 3H, N$^+$C*H*$_3$); 3.1 (m, 4H, N$^+$(C*H*$_2$CH$_2$)$_2$); 3.5 (m, 2H, N$^+$C*H*$_2$CH$_2$NH); 3.7 (m, 2H, N$^+$CH$_2$C*H*$_2$NH); 7.4 (bs, 4H, N*H*$_2^+$); 8.7 (bs, 1H, CH$_2$N*H*). LSIMS (lipid **2**) *m*/*z*: 565 [M$^+$] (Calcd for $C_{36}H_{77}N_4$, 22%).

***N*,*N*-Di-*n*-tetradecyl-*N*-[2-guanidinyl]ethyl-*N*-methylammonium Chloride (Lipid 1).** ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm): 0.9 (t, 6H, C*H*$_3$(CH$_2$)$_{14}$); 1.2−1.3 (m, 44H, CH$_3$(C*H*$_2$)$_{11}$); 1.5−1.7 [m, 4H, N$^+$(CH$_2$C*H*$_2$)$_2$); 3.0 (s, 3H, N$^+$C*H*$_3$); 3.1 (m, 4H, N$^+$(C*H*$_2$CH$_2$)$_2$); 3.5 (m, 2H, N$^+$C*H*$_2$CH$_2$NH); 3.7 (m, 2H, N$^+$CH$_2$C*H*$_2$NH); 7.4 (bs, 4H, N*H*$_2^+$); 8.7 (bs, 1H, CH$_2$N*H*). LSIMS (lipid **1**) *m*/*z*: 510 [M + 1$^+$] (Calcd for $C_{32}H_{69}N_4$, 82%).

***N*,*N*-Di-*n*-octadecyl-*N*-[2-guanidinyl]ethyl-*N*-methylammonium Chloride (Lipid 3).** ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm): 0.9 (t, 6H, C*H*$_3$(CH$_2$)$_{15}$); 1.2−1.3 (m, 60H, CH$_3$(C*H*$_2$)$_{15}$); 1.5−1.7 (m, 4H, N$^+$(CH$_2$C*H*$_2$)$_2$); 3.0 (s, 3H, N$^+$C*H*$_3$); 3.1 (m, 4H, N$^+$(C*H*$_2$CH$_2$)$_2$); 3.5 (m, 2H, N$^+$C*H*$_2$CH$_2$NH); 3.7 (m, 2H, N$^+$CH$_2$C*H*$_2$NH); 7.4 (bs, 4H, N*H*$_2^+$); 8.7 (bs, 1H, CH$_2$N*H*). LSIMS (lipid **3**) *m*/*z*: 621 [M$^+$] (Calcd for $C_{40}H_{85}N_4$, 40%).

Synthesis of Lipids 4−6. Lipids **4**−**6** were synthesized following the procedures depicted schematically in Scheme 1 (part B). As representative details, synthetic procedures for lipid **5** are provided below.

Synthesis of Lipid 5. Step v. Synthesis of *N*,*N*-Di-*n*-hexadecyl-*N*-[2-guanidinyl]ethylamine (Lipid 5). Dry *N*,*N*-dimethylformamide was added to *N*-2-aminoethyl-*N*,*N*-di-*n*-hexadecylamine (**I**, 0.20 g, 0.39 mmol), 1H-pyrazole-1-carboxamidine hydrochloride (0.06 g, 0.39 mmol, prepared by reacting 1 equiv of pyrazole with 1 equiv of cyanamide in anhydrous *p*-dioxan containing a few drops of concentrated HCl), and *N*,*N*-di-isopropyl-*N*-ethyl amine (DIEA, 0.051 g, 68 μL, 0.39 mmol) to produce a final concentration of approximately 2 M reagents. The mixture was stirred at room temperature under nitrogen for 14 h. The reaction mixture was then taken in 100 mL of ethyl acetate, washed sequentially with water (2 × 100 mL) and brine solution (2 × 100 mL), dried over anhydrous magnesium sulfate, and filtered. Ethyl acetate was removed from the filtrate on a rotary evaporator. Silica gel column chromatographic purification of the resulting residue using 60−120 mesh silica gel size and 7−9% methanol in chloroform (v/v) as the eluent afforded 0.13 g of pure lipid **5** (55% yield, R_f = 0.7−0.8 using 20% methanol chloroform as the TLC developer).

^{1}H NMR (200 MHz, $CDCI_3$) δ (ppm): 0.9 (t, 6H, C*H*$_3$(CH$_2$)$_{13}$); 1.2−1.4 (m, 52H, (C*H*$_2$)$_{13}$); 1.5 (m, 4H, N(CH$_2$C*H*$_2$)$_2$); 2.4 (t, 4H, N(C*H*$_2$CH$_2$)$_2$); 2.6 (t, 2H, N(C*H*$_2$CH$_2$NH)); 3.25 (t, 2H, N(CH$_2$-C*H*$_2$NH)); 7.4 (bm, 4H, N*H*$_2^+$); 8.1 (bs, 1H, CH$_2$N*H*). LSIMS (lipid **5**) *m*/*z*: 551 [M$^+$] (Calcd for $C_{35}H_{75}N_4$, 100%).

***N*,*N*-Di-*n*-tetradecyl-*N*-[2-guanidinyl]ethylamine (Lipid 4).** ^{1}H NMR (200 MHz, $CDCI_3$) δ (ppm): 0.9 (t, 6H, C*H*$_3$-(CH$_2$)$_{11}$); 1.2−1.4 (m, 44H, (C*H*$_2$)$_{11}$); 1.5 (m, 4H, N(CH$_2$C*H*$_2$)$_2$); 2.4 (t, 4H, N(C*H*$_2$CH$_2$)$_2$); 2.6 (t, 2H, N(C*H*$_2$CH$_2$NH)); 3.25 [t, 2H, N(CH$_2$C*H*$_2$NH)]; 7.4 [bm, 4H, N*H*$_2$ $^+$]; 8.1 [bs, 1H, −CH$_2$N*H*]. LSIMS (lipid **4**) *m*/*z*: 496 [M + 1$^+$] (Calcd for $C_{31}H_{67}N_4$, 100%).

***N*, *N*-Di-*n*-octadecyl-*N*-[2-guanidinyl]ethylamine (Lipid 6).** ^{1}H NMR (200 MHz, $CDCI_3$) δ (ppm): 0.9 (t, 6H, C*H*$_3$-(CH$_2$)$_{15}$); 1.2−1.4 (m, 60H, (C*H*$_2$)$_{15}$); 1.5 (m, 4H, N(CH$_2$C*H*$_2$)$_2$); 2.4 (t, 4H, N(C*H*$_2$CH$_2$)$_2$); 2.6 (t, 2H, N(C*H*$_2$CH$_2$NH)); 3.25 (t, 2H, N(CH$_2$C*H*$_2$NH)); 7.4 (bm, 4H, N*H*$_2^+$); 8.1 (bs, 1H, CH$_2$N*H*). LSIMS (lipid **6**) *m*/*z*: 607 [M$^+$] (Calcd for $C_{39}H_{83}N_4$, 100%).

Preparation of Liposomes. The cationic lipid and cholesterol in a 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 to 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 earlier.[15] 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 20 000 cells (for CHO, MCF-7, A549, HepG2) and 15 000 cells (for COS-1) per well in a 96-well plate 18−24 h before the transfection. Plasmid DNA (0.3 μg) 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 phosphate-buffered saline (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 2× substrate solution (1.33 mg/mL of *o*-nitrophenyl-β-D-galactopyranoside (ONPG), 0.2 M sodium phosphate (pH 7.3), and 2 mM magnesium chloride) to the lysate in a 96-well plate. Adsorption 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 out in duplicate, and the transfection efficiency values shown in Figures 1 are the average of duplicate experiments performed on the same day. Each transfection experiment was repeated three times, and the day to day variation in the average transfection 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.[15] 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.

ξ Potential and Size Measurements. The sizes and the surface charges (ξ potentials) of liposomes and lipoplexes were measured by photon correlation spectroscopy and electrophoretic mobility on a ξ sizer 3000HS_A (Malvern, U.K.). 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 ± 5 nm polystyrene polymer (Duke Scientific Corp. Palo Alto, CA). The diameters of liposomes and lipoplexes were calculated by using the automatic mode. The ξ 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. Measurements were done 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 was assessed by a gel retardation assay on a 1% agarose gel (prestained with ethidium bromide) across the varying lipid/DNA charge 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. Loading buffer (4 μL, 6×) (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 amounts of cationic lipids (using indicated lipid/DNA charge ratios in Figure 5) 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 1μg/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 (25 μL) on a 1% agarose gel (prestained with ethydium 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.[16] 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 of 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).

Acknowledgment. Financial support received from the Department of Biotechnology, Government of India (to A.C.) is gratefully acknowledged. J.S. sincerely thanks the University Grant Commission (UGC), Government of India, for her doctoral research fellowship. We thank Shiva Prasad for helping us to take the gel pictures.

Supporting Information Available: Reverse phase HPLC chromatograms for the lipids **1**−**6** in two mobile phases with details of the HPLC parameters and representative reduced transfection profiles of lipids **1**−**6** in CHO, HepG2, and A-549 cells. This information is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Verma, I. M.; Somina, M. Gene Therapy—Promises, Problems and Prospects. *Nature* **1997**, *389*, 239−242.
(2) Anderson, W. F. Human Gene Therapy. *Nature* **1998**, *392*, 25−30.
(3) Yla-Herttuala, S.; Martin, J. F. Cardiovascular Gene Therapy. *Lancet* **2000**, *355*, 213−222.
(4) Ilies, M. A.; Seitz, W. A.; Ghiviriga, I.; Johnson, B. H.; Miller, A.; Thompson, E. B.; Balaban, A. T.; Pyridinium Cationic Lipids in Gene Delivery: A Structure−Activity Correlation Study. *J. Med. Chem.* **2004**, *15*, 3744−3754.
(5) Lee, Y.; Koo, H.; Lim, Y. B.; Lee, Y.; Mo, H.; Park, J. S.; New Cationic Lipids for Gene Transfer with High Efficiency and Low Toxicity: T-Shape Cholesterol Ester Derivatives. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2637−2641.
(6) Wang, L.; MacDonald, R. C.; New Strategy for Transfection: Mixtures of Medium-Chain and Long Chain Cationic Lipids Synergistically Enhance Transfection. *Gene Ther.* **2004**, *11*, 1358−1362.
(7) Niculescu-Duvaz, D.; Heyes, J.; Springer, C. J. Structure−Activity Relationship in Cationic Lipid mediated Gene Transfection. *Curr. Med. Chem.* **2003**, *10*, 1233−1261 and the references therein.
(8) Bell, P. C.; Bergsma, I. P.; Bras, W.; Stuart, M. C. A.; Rowan, M. C.; Feiters, M. C.; Engberts, J. B. F. N. Transfection Mediated by Gemini Surfactants: Engineered Escape from Endosomal Compartment. *J. Am. Chem. Soc.* **2003**, *125*, 1551−1558.
(9) Kirby, A. J.; Carmilleri, P.; Engberts, J. B. F. N.; Feiters, M. C.; Nolte, R. J. M.; Soderman, O.; Bergsma, M.; Bell, P. C.; Fielden, M. L.; Garcia Rodriguez. C. L.; Guedat, P.; Kremer, A.; McGregor, C.; Perrin, C.; Ronsin, G.; Van Eijik, M. C. P. Gemini Surfactants: New Synthetic Vectors for Gene Transfection. *Angew. Chem., Int. Ed.* **2003**, *42*, 1448−1457.
(10) Leclercq, F.; Cohen-Ohana, M.; Mignet, N.; Sbarbati, A.; Herscovici, J.; Scherman, D.; Byk, G.; Design, Synthesis and Evaluation of Gadolinium Cationic Lipids as Tools for Biodistribution Studies of Gene Delivery Complexes. *Bioconjugate Chem.* **2003**, *14*, 112−119.
(11) Fabio, K.; Gaucheron, J.; Giorgio, C. D.;.Vierling, P. Novel Galactosylated Polyamine Bolaamphiphiles for Gene Delivery. *Bioconjugate Chem.* **2003**, *14*, 358−367.

(12) Ewert, K.; Ahmed, A.; Evans, H. M.; Schmidt, H. W.; Safinya, C. R.; Efficient Synthesis and Cell-Transfection Properties of a New Multivalent Cationic Lipid for Non-Viral Gene Delivery. *J. Med. Chem*. **2002**, *45*, 5023−5029.
(13) Guenin, E.; Herve, A. C.; Floch, V.; Loisel, S.; Yaounac, J. J.; Clement, J. C.; Ferec, C.; des Abbayas, H. Cationic Phosphonolipids containing Quaternary Phosphonium and Arsonium Groups for DNA Transfection with Good Efficiency and Low Cellular Toxicity. *Angew. Chem., Int. Ed.* **2000**, *39*, 629−631.
(14) Kawakami, S.; Sato, A.; Nishikawa, M.; Yamashita, F.; Hashida, M. Mannose Receptor Mediated Gene Tranfer into Macrophases Using Novel Mannosylated Cationic Liposomes. *Gene. Ther.* **2000**, *7*, 292−299.
(15) Karmali, P. P.; Kumar, V. V.; Chaudhuri, A. Design, Syntheses, and In Vitro Gene Delivery efficacies of Novel Mono-, Di-, and Tri-Lysinated Cationic Lipids: A Structure Activity Investigation. *J. Med. Chem.* **2004**, *47*, 2123-2132.
(16) Mahidhar, Y. V., Rajesh, M.; Chaudhuri, A. Strikingly Spacer-Arm Modulated Gene Delivery Efficacies of Novel Cationic Glycolipids: Design, Synthesis and In Vitro Transfection Biology. *J. Med. Chem.* **2004**, *47*, 3938−3948.
(17) Majeti, B. K.; Singh, R. S.; Yadav, S. K.; Reddy, S. B.; Ramkrishna, S.; Diwan, P. V.; Madhavendra, S. S.; Chaudhuri, A. Enhanced Intravenous Transgene Expression in Mouse Lung Using Cyclic-Head Cationic Lipids. *Chem. Biol.* **2004**, *11,* 427−437.
(18) Singh, R. S.; Chaudhuri, A. Single Additional Methylene Group in the Head Group Region Imparts High Gene Transfer Efficacy to a Transfection-Incompetent Cationic Lipid. *FEBS Lett.* **2004**, *556*, 86−90.
(19) Kumar, V. V.; Pichon, C.; Refregiers, M.; Guerin, B.; Midoux, P.; Chaudhuri, A. Single Histidine Residue in Head Group Region is Sufficient to Impart Remarkable Gene Transfection Properties to Cationic Lipids: Evidence for Histidine-mediated Membrane Fusion at Acidic pH. *Gene. Ther.* **2003**, *10*, 1206−1215.
(20) Zabner, J.; Fasbender, A. J.; Moninger, T.; Poellinger, K. A.; Welsh, M. J. Cellular and Molecular Barriers to Bene Transfer by a Cationic Lipid. *J. Biol. Chem.* **1995**, *270*, 18997−19007.
(21) Friend, D. S.; Papahadjopoulos, D.; Debs, R. J.; Endocytosis and Intracellular Processing Accompanying Transfection Mediated by Cationic Liposomes. *Biochim. Biophys. Acta* **1996**, *1278*, 41−50.
(22) Xu, Y.; Szoka, Jr. F. C. Mechanism of DNA Release from Cationic Liposome/ DNA Complexes used in Cell Transfection. *Biochemistry* **1996**, *35*, 5616−5623.
(23) Bally, M. B.; Harvie, P.; Wong, M. P. F.; Kong, S.; Wasan, E. K.; Reimer, D. L. Biological Barriers to Cellular Delivery of Lipid-based DNA Carriers. *Adv. Drug Deliv. Rev.* **1999**, *38*, 291−315.
(24) Zhu, J.; Munn, R. J.; Nantz, M. H. Self-Cleaving Ortho Ester Lipids: A Class of pH-Vulnerable Ampliphiles. *J. Am. Chem. Soc.* **2000**, *122*, 2645−2646.
(25) Budker, V.; Gurevich, V.; Hagstrom, J. E., Bortzov, F.; Wolff, J. A. pH-sensitive Cationic Liposomes: A New Synthetic Virus-Like Vector. *Nat. Biotechnol.* **1996**, *14*, 760−764.
(26) Reddy, J. A.; Abburi, C.; Hofland, H.; Howard, S. J.; Vlahov, I.; Wils, P.; Leamon, C. P. Folate-targeted, Cationic Liposome-Mediated Gene Transfer into Disseminated Peritoneal Tumors. *Gene Ther.* **2002**, *9*, 1542−1550.
(27) Vigneron, J.-P.; Oudrhiri, N.; Fauquet, M.; Vergely, L.; Bradley, J.c.; Basseville, M.; Lehn, P.; Lehn, J. M. Guanidinium−Cholesterol Cationic Lipids: Efficient Vectors for the Transfection of Eukaryotic Cells. *Proc. Natl. Acad. Sci., U.S.A.* **1996**, *93*, 9682−9686.
(28) Ruysschaert, J. M., el Ouahabi, A, Willeaume, V., Huez, G., Fuks, R., Vandenbranden, M., Di Stefano, P. A Novel Cationic Amphiphile for Transfection of Mammalian cells. *Biochem. Biophys. Res. Commun.* **1994**, *203*, 1622−1628.
(29) Oudrhiri, N.; Vigneron, J. P.; Peuchmaur, M.; Leclerc, T.; Lehn, J. M.; Lehn, P. Gene Transfer by Guanidinium-Cholesterol Cationic Lipids into Airway Epithelial Cells in Vitro and in Vivo. *Proc. Natl. Acad. Sci., U.S.A.* **1997**, *94*, 1651−1656.
(30) Pitard, B.; Oudrhiri, N.; Vigneron, J. P.; Hauchecorne, M.; Aguerre, O.; Toury, R.; Airiau, M.; Ramasawmy, R.; Scherman, D.; Crouzet, J.; Lehn, J. M.; Lehn, P. Structural Characteristics of Supramolecular Assemblies Formed by Guanidinium-Cholesterol Reagents for Gene Transfection. *Proc. Natl. Acad. Sci., U.S.A.* **1999**, *96*, 2621−2626.
(31) Pitard, B.; Oudrhiri, N.; Lambert, O.; Vivien, E.; Masson, C.; Wetzer, B.; Hauchecorne, M.; Scherman, D.; Rigaud, J. L.; Vigneron, J. P.; Lehn, J. M.; Lehn, P. Sterically Stabilized BGTC-Based Lipoplexes: Structural Features and Gene Transfection into the Mouse Airways in Vivo. *J. Gene. Med.* **2001**, *3*, 478−487.
(32) Densmore, C. L.; Giddings, T. H.; Waldrep, J. C.; Kinsey, B. M.; Knight, V. Gene Transfer by Guanidinium−Cholesterol: Dioleoylphosphatidyl−Ethanolamine Liposome−DNA Complexes in Aerosol. *J. Gene Med.* **1999**, *1*, 251−264.
(33) Aissaoui, A.; Oudrhiri N.; Petit, L.; Hauchecorne, M.; Kan, E.; Sainlos, M.; Julia, S.; Navarro, J.; Vigneron, J. P.; Lehn J. M.; Lehn, P. Progress in Gene Delivery by Cationic Lipids: Guanidinium−Cholesterol-Based Systems as an Example. *Curr. Drug Targets* **2002**, *3*, 1−16.
(34) Tran, P. L.; Vigneron, J.-P.; Pericat, D.; Dubois, S.; Cazals, D.; Hervy, M.; DeClerck, Y. A. Degott, C.; Auclair, C. Gene Therapy for Hepatocellular Carcinoma using Non-Viral Vectors Composed of Bis-Guanidinium-Tren-Cholesterol and Plasmids Encoding the Tissue Inhibitors of Metalloproteinases TIMP-2 and TIMP-3. *Cancer Gene Ther.* **2003**, *10*, 435−444.
(35) Luton, D.; Oudrhiri, N.; deLagausie, P.; Aissaoui, A.; Hauchecorne, M.; Julia, S.; Ouru, J. F.; Aigrain, Y.; Peuchmaur, M.; Vigneron, J.-P.; Lehn, J. M.; Lehn, P. Gene Transfection into Fetal Sheep Airways in Utero using Guanidinium−Cholesterol Cationic Lipids. *J. Gene Med.* **2004**, *6*, 328−336.
(36) Patel, M.; Vivien,E.; Hauchcorne,M.; Oudrhiri, N.; Ramaswamy, R.; Vigneron, J.-P.; Lehn, J.-M. Efficient Gene Transfection by Bisguanylated diacetylene lipid formulations. *Biochem. Biophys. Res. Commun.* **2001**, *281*, 536−543.
(37) Solodin, I.; Heath. T, D.; Synthesis of Novel Cationic Lipids with a Guanidine Group. Cationic Lipids *Synlett* **1996**, 617−618.
(38) El Ouahabi, A.; Pector , V.; Fuks, R.; Vandenbranden, M.; Ruysschaert, J.M.; Double Long-Chain Amidine Liposome-Mediated Self-Replicating RNA Transfection. *Acta Clin. Belg.* **1996**, *51*, 194.
(39) Megabios Corporation World Patent WO9514381, 1995.
(40) Gilead Sciences, Inc. World Patent WO9601840, 1996.
(41) Rhone-Poulenc Rorer, S. A. World Patent WO9617823, 1996.
(42) Byk, G.; Soto, J.; Mattler, C.; Frederic, M.; Scherman, D. Novel Non-Viral Vectors for Gene Delivery: Synthesis of a Second Generation Library of Mono-Functionalized Poly-(Guanidinium) Amines and Their Introduction into Cationic Lipids. *Biotech. Bioeng. (Com. Chem)* **1998**, *61*, 81−87.
(43) Rhone-Poulenc Rorer, S. A. French Patent FR9706549, 1998.
(44) Frederic, M.; Scherman, D.; Byk, G. Introduction of Cyclic Guanidines into Cationic Lipids for Non-Viral Gene Delivery. *Tetrahedron Lett.* **2000**, *41*, 675−679.
(45) Byk, G.; Scherman, D.; Novel Cationic Lipids for Gene Delivery and Gene Therapy. *Expert Opin. Ther. Pat.* **1998**, *8*, 1125−1141.
(46) Iwanowicz, E. J.; Poss, M. A.; Lin, J. Preparation of *N*,*N*′-Bis-*tert*-Butoxycarbonylthiourea. *Synth. Commun.* **1993**, *23*, 1443−1445.

JM049417W