A Novel Tetraester Construct That Reduces Cationic Lipid-Associated Cytotoxicity. Implications for the Onset of Cytotoxicity†

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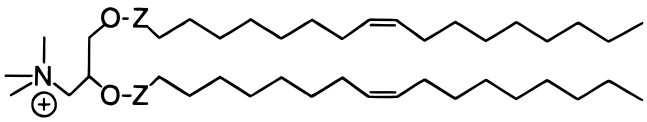
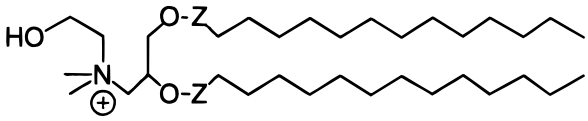
ABSTRACT: The preparation of cationic amphiphiles that induce minor cytotoxic response during polynucleotide delivery into mammalian cells has been limited by the conventional use of ester, amide, or carbamate linkages to tether either the polar or the hydrophobic domains. The deleterious effects of ammonium-based lipidic salts on cellular processes have been well-established. The present report is the first example of a linchpin tetraester construct that utilizes ester linkages to tether both the polar and hydrophobic domains. Dimyristoyl and dioleoyl analogues were prepared from pentaerythritol, *N*,*N*-dimethylglycine, and their corresponding fatty acyl groups via successive diesterifications followed by amine quaternization. The resultant cationic tetraesters were examined in transfection (luciferase) and cell proliferation (MTS) assays using NIH 3T3 and 16HBE14$_{o-}$ cells. The tetraesters exhibited transfection activity comparable to the well-studied lipids DOTAP and DC−cholesterol (DC−chol) in both cell lines. The tetraester construct afforded no cytotoxicity in NIH3T3 cells and provided a significant lowering of cytotoxicity relative to DC−chol in the 16HBE14$_{o-}$ cells. The expression of green fluorescent protein (GFP) in both cell lines also was examined using the lipid panel. Comparison of fluorescent and corresponding phase-contrast images confirmed the chemical cytotoxicity results and revealed that the cytotoxic response was not dependent on transgene expression. Phase-contrast micrographs of cells treated with the cationic lipid panel in the absence of GFP plasmid showed identical morphology to the GFP-transfected cells, suggesting that the onset of a lipid-mediated cytotoxic response might occur at a stage prior to endosomal encapsulation.

The remarkable but poorly understood ability of cationic amphiphiles to bind polynucleotides and facilitate gene transfer in vitro and in vivo has motivated numerous lipid structure−activity studies (*1*, *2*). As a consequence, the literature now reflects a diverse selection of cationic lipids that have been shown to enhance the intracellular delivery of exogenous polynucleotides to mammalian cells (*3*, *4*). The use of synthetic cationic lipids in this regard continues to emerge as a viable alternative to viral-mediated gene delivery. Since the initial lipid-mediated DNA-transfer experiments (*5*), cationic lipid design and technology have advanced to encompass successful intravenous delivery of DNA, albeit with far less efficiency than in vitro (*6*). Chart 1 depicts some of the better known cationic lipids used to transfect mammalian cells (*1*, *5*, *7*, *8*). The use of lipids **2**−**4** has progressed to the clinical level, and the outcome of their phase I studies has prompted much enthusiasm to continue optimization through lipid development and formulation (*9*−*11*). Guidelines for the rational design of cationic lipids have been elusive despite many analogue syntheses and transfections (*2*). Recent physicochemical investigations by our

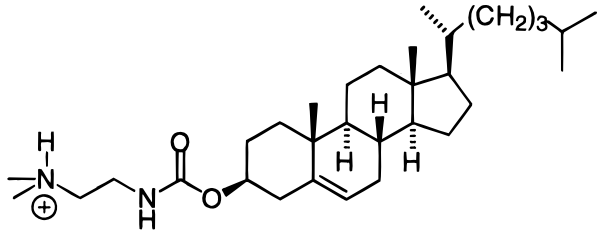
Chart 1: Cationic Amphiphiles for Polynucleotide Transfection



1: Z = CH_2 (**DOTMA**)
2: Z = C(O) (**DOTAP**)



3: Z = CH_2 (**DMRIE**)



4 (**DC-Chol**)

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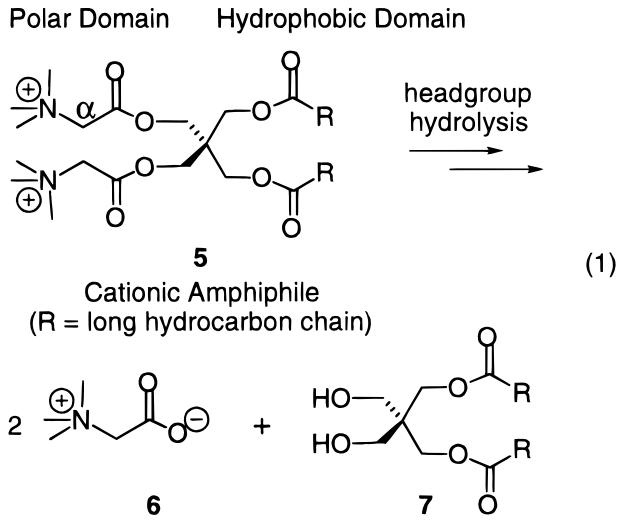
group and others have offered considerations into effective polynucleotide delivery (*12, 13*). However, most studies have been empirical, outpacing efforts to establish a molecular understanding of parameters influencing transfection efficiency (*14, 15*).

Many efforts to develop cationic lipids and to improve transfection activity have focused on optimizing transgene expression (*16*). In addition to the requirement for greater expression, in vivo models focused on pulmonary delivery have revealed that dose-dependent immunological responses pose another principal challenge (*17*). The long-term administration of a cationic lipid−polynucleotide complex (lipoplex) has been shown in some cases to elicit inflammatory responses (*18*). Consequently, the development of cationic lipid constructs that minimize toxic side effects while maintaining an effective level of transgene expression has assumed the forefront of lipid-mediated gene-transfer research (*2*). We present herein a strategy to address the problem of lipid-associated cytotoxicity using a new synthesized class of tetraester-containing cationic lipids. Luciferase transfection experiments and a tetraazolium salt (MTS) cytotoxicity assay are used to evaluate the new lipids.

Cytotoxicity has been attributed to the inhibition of protein kinase C activity by cationic amphiphiles (*19*) after internalization of the lipoplex, presumably a consequence of amphiphile incorporation into the plasma membrane (*20*). In addition to the formation of transmembrane pores, the resultant disruptions of signal transduction and gene regulation processes impair cellular function. Silvius and Leventis proposed that one option to reduce cytotoxicity would be to attach the amphiphile hydrophobic domain via cleavable ester linkages (*7*). In this way, the cationic lipid may be biodegraded by endogenous esterases, thereby rendering the cationic DNA-binding domain nonamphiphilic. This action would be expected to lower the occurrence of cationic lipid in the plasma membrane. Indeed, this design consideration led to the development of DOTAP, a cationic lipid having an improved biocompatibility profile.

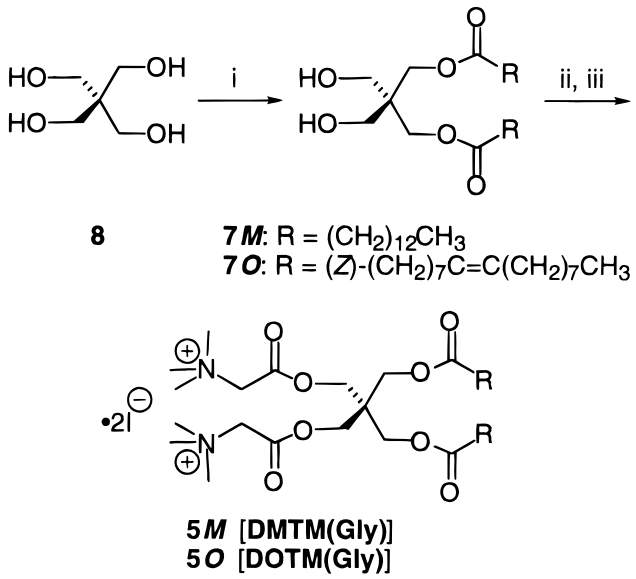
By building on this concept, we have designed a novel cationic amphiphile that uses ester linkages to attach the cationic DNA-binding domain (compound **5**, eq 1). The



(1)

polar domain ester linkages of tetraester **5** are more susceptible to nucleophilic attack and subsequent cleavage due to the proximal quaternary ammonium group. The dipole−dipole conflict between the α-carbon and the ester carbonyl activates the ester linkage toward hydrolysis. This effect parallels the chemistry of α-halogenated esters where it is known that the intrinsic dipole−dipole conflict enhances the rate of hydrolysis of α-halogenated esters relative to their nonhalogenated counterparts (e.g., α,α,α-trifluoroacetate hydrolysis occurs readily at pH 7.0 (*21*)). Our lipid utilizes a pentaerythritol ($C(CH_2OH)_4$) subunit as a linchpin to attach two cationic headgroups to a diacyl hydrophobic domain. We envision that headgroup hydrolysis would eliminate the cationic amphiphile, producing the zwitterionic amino acid derivative **6** and the uncharged diol diester **7**, species that are not expected to induce toxicity.

Scheme 1[a]



[a] i: RC(O)Cl (2.0 equiv), catalytic DMAP, pyidine, 0 °C. ii: *N*,*N*-dimethylglycine (3.0 equiv), pentafluorophenol (9.0 equiv), DCC (3.0 equiv), catalytic DMAP, DMF, room temperature. iii: CH_3I, room temperature.

MATERIALS AND METHODS

Reagents. Unless otherwise noted, all reagents were purchased from commercial suppliers and were used without further purification. (Dioleoylphosphatidyl)ethanolamine (DOPE) was obtained from Avanti (Alabaster, AL).

Analytical Methods. The progress of reactions was monitored by thin-layer chromatography (TLC) using Merck silica gel 60 F_{254} TLC plates (0.2-mm thickness). Visualization was accomplished by staining the plates with a 3% w/v phosphomolybdic acid/ethanol solution followed by charring on a hot plate. Silica gel 60 (230−400 mesh) was used for flash column chromatography. A Matteson Galaxy FTIR 3000 infrared spectrometer and a General Electric QE300 300-MHz nuclear magnetic resonance spectrometer were used to confirm the structure and purity of the compounds. Chemical shifts are reported in parts per million (δ) relative to residual chloroform (δ 7.26) in deuterated chloroform. Absorbance readings for cytotoxicity assays were performed on a Hewlett-Packard 8450A UV/vis spectrophotometer.

Syntheses. The cationic lipids **5** were prepared as shown in Scheme 1. Treatment of pentaerythritol (**8**, Aldrich Chemical Co., Milwaukee, WI) with 2 equiv of the appropri-

ate acyl chloride in pyridine containing a catalytic amount of 4-(*N*,*N*-dimethylamino)pyridine (DMAP) afforded mixtures of mono-, di-, tri-, and tetraacylated material. Purification by silica gel chromatography gave the dimyristoyl and dioleoyl derivatives **7*M*** and **7*O*** in 60 and 30% yield, respectively. Attachment of the *N*,*N*-dimethylglycine headgroup was accomplished using a DCC coupling protocol involving catalytic DMAP and pentafluorophenol (*22*). The resultant tetraesters were readily purified using column chromatography and were generally obtained in 35−50% yield. The final step of amine quaternization was conducted in methyl iodide at room temperature. Lipids **5*M*** and **5*O*** were purified by recrystallization from acetonitrile.

*General Procedure for the Synthesis of Diesters **7**.* To a solution of pentaerythritol (1.0 g, 7.3 mmol) and DMAP (ca. 10 mg) in freshly distilled pyridine (75 mL) at 0 °C was added dropwise the acyl chloride (14.7 mmol). The resultant clear solution was gradually warmed to room temperature over 4 h and then transferred to a separatory funnel containing CH_2Cl_2 (200 mL). The organic solution was extracted with 10% HCl, the layers were separated, and the organic phase was dried over Na_2SO_4. The solvents were removed by rotary evaporation. The residue was purified using silica gel flash chromatography, eluting with a gradient of CH_2Cl_2 to 19:1 CH_2Cl_2:MeOH, to give the desired diester products:

Dimyristoyl Diol 7M. $R_f = 0.25$, 19:1 MeOH:CH_2Cl_2; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.14 (s, 4H, CC*H*$_2$O−acyl), 3.57 (s, 4H, CC*H*$_2$OH), 2.32 (t, $J = 7$ Hz, 2H, OC(O)C*H*$_2$CH$_2$), 1.60 (m, 2H, OC(O)CH$_2$C*H*$_2$CH$_2$), 1.30 (m, 44H), 0.86 (t, $J = 7$ Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.4, 62.4, 62.3, 44.8, 31.8, 30.0, 29.9−28.7, 24.9, 24.7, 22.6, 14.0; FTIR (KBr) 3298, 2917, 2850, 1732, 1468, 1179 cm^{-1}; HRMS ($C_{33}H_{64}O_6$) calcd, 556.4703; found, 539.4679 [(M + H)$^+$ − H_2O].

Dioleoyl Diol 7O. $R_f = 0.35$, 19:1 MeOH:CH_2Cl_2; ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.35 (m, 4H), 4.10 (s, 4H, CC*H*$_2$O−acyl), 3.58 (s, 4H, CC*H*$_2$OH), 2.32 (t, $J = 7$ Hz, 2H, OC(O)C*H*$_2$CH$_2$), 2.00 (m, 8H), 1.61 (m, 2H, OC(O)CH$_2$C*H*$_2$CH$_2$), 1.28 (m, 40H), 0.86 (t, $J = 7$ Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.1, 129.8, 129.5, 62.3, 61.9, 44.5, 34.0, 31.8, 29.6−28.9, 27.0, 24.8, 22.5, 13.9; FTIR (KBr) 3451, 3004, 2922, 2855, 1742, 1466, 1170, 1053 cm^{-1}; HRMS ($C_{41}H_{76}O_6$) calcd, 664.5642; found, 647.5579 [(M + H)$^+$ − H_2O].

General Procedure for Attachment and Quaternization of the N,N-Dimethylglycine Headgroup. To a suspension of *N*,*N*-dimethylglycine (140 mg, 1.35 mmol) and the appropriate diester **7** (0.45 mmol) in DMF (6.8 mL) at room temperature was added pentafluorophenol (745 mg, 4.05 mmol). The mixture was warmed to 50 °C to effect complete dissolution of all solids. On cooling to room temperature, dicyclohexylcarbodiimide (300 mg, 1.45 mmol) and a catalytic amount of DMAP were added in 1 portion and stirred overnight. The reaction was diluted with Et_2O, and the precipitated urea was filtered. The filtrate was transferred to a separatory funnel and extracted successively with saturated NH_4Cl, water, and saturated $NaHCO_3$. The organic layer was dried over Na_2SO_4. The solvents were removed by rotary evaporation, and the residue was purified by column chromatography, eluting with a gradient of CH_2Cl_2 to 50:1 CH_2Cl_2:MeOH, to give the desired tetraesters:

Dimyristoyl Bis(N,N-dimethylglycyl) tetraester. $R_f = 0.23$, 9:1 CH_2Cl_2:MeOH; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.17 (s, 4H, (CH$_3$)$_2$NCH$_2$C(O)OC*H*$_2$), 4.11 (s, 4H, CC*H*$_2$O−acyl), 3.19 (s, 4H, (CH$_3$)$_2$NC*H*$_2$C(O)O), 2.35 (s, 12H, (C*H*$_3$)$_2$N), 2.30 (t, $J = 7$ Hz, 4H, OC(O)C*H*$_2$CH$_2$), 1.57 (m, 4H, OC(O)CH$_2$C*H*$_2$CH$_2$), 1.25 (s, 48H), 0.86 (t, $J = 7$ Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.4, 169.8, 62.4, 62.3, 56.2, 49.9, 44.9, 43.2, 34.8, 33.2, 32.1, 31.8, 29.9−28.5, 26.5, 25.5, 24.7, 22.5, 14.0; FTIR (KBr) 2922, 2853, 1746, 1467, 1284, 1148, 1064 cm^{-1}; HRMS ($C_{41}H_{78}N_2O_8$) calcd, 726.5758; found, 727.5815 (M + H)$^+$.

Dioleoyl Bis(N,N-dimethylglycyl) Tetraester. $R_f = 0.38$, 9:1 CH_2Cl_2:MeOH; ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.34 (m, 4H), 4.15 (s, 4H, (CH$_3$)$_2$NCH$_2$C(O)OC*H*$_2$), 4.10 (s, 4H, CC*H*$_2$O−acyl), 3.17 (s, 4H, (CH$_3$)$_2$NC*H*$_2$), 2.33 (s, 12H, (C*H*$_3$)$_2$N), 2.27 (t, $J = 7$ Hz, 4H, OC(O)C*H*$_2$CH$_2$), 2.00 (m, 8H), 1.58 (m, 4H, OC(O)CH$_2$C*H*$_2$CH$_2$), 1.26 (m, 40H), 0.86 (t, $J = 7$ Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.0, 170.1, 130.1, 129.7, 104.5, 62.1, 61.8, 45.2, 41.0, 34.0, 30.0−28.8, 27.1, 24.7, 22.8, 14.2; FTIR (KBr) 3003, 2923, 2855, 2773, 1744, 1465, 1147, 1064 cm^{-1}; HRMS ($C_{49}H_{90}N_2O_8$) calcd, 834.6697; found, 835.6810 (M + H)$^+$.

The *N*,*N*-dimethylamino tetraester was dissolved in excess iodomethane, and the resulting solution was stirred at room temperature for 8 h. Iodomethane was removed under reduced pressure (CAUTION: a fume hood is required) to afford the corresponding crude bisiodide salt. Aqueous workup using 5% $NaHCO_3$ resulted in headgroup hydrolysis to afford the diols **7**. Purification was accomplished by repeated recrystallization from acetonitrile:

Tetraester 5M. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.73 (s, 4H, (CH$_3$)$_3$NC*H*$_2$), 4.32 (s, 4H, (CH$_3$)$_3$NCH$_2$C(O)OC*H*$_2$), 4.18 (s, 4H, CC*H*$_2$O−acyl), 3.60 (s, 18H, (C*H*$_3$)$_3$N), 2.30 (t, $J = 7$ Hz, 4H, OC(O)C*H*$_2$CH$_2$), 1.60 (m, 4H, OC(O)CH$_2$C*H*$_2$-CH$_2$), 1.25 (s, 48H), 0.86 (t, $J = 7$ Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.4, 163.3, 65.2, 63.7, 61.2, 54.9, 49.9, 48.6, 47.7, 43.2, 34.6, 31.2, 29.8−28.9, 24.8, 22.8, 14.5; FTIR (KBr) 3011, 2917, 2850, 1743, 1472, 1247, 1191, 1123, 1021 cm^{-1}; HRMS ($C_{43}H_{84}N_2O_8I_2$) calcd, 1010.4321; found, 883.5215 (M^{2+} + I$^-$).

Tetraester 5O. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.73 (s, 4H, (CH$_3$)$_3$NC*H*$_2$), 5.34 (m, 4H), 4.33 (s, 4H, (CH$_3$)$_3$NCH$_2$C-(O)OC*H*$_2$), 4.18 (s, 4H, CC*H*$_2$O−acyl), 3.60 (s, 18H, (C*H*$_3$)$_3$N), 2.31 (t, $J = 7$ Hz, 4H, OC(O)C*H*$_2$CH$_2$), 1.60 (m, 4H, OC(O)CH$_2$C*H*$_2$CH$_2$), 1.27 (m, 40H), 0.86 (t, $J = 7$ Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.7, 164.0, 129.8, 129.4, 65.2, 63.2, 61.3, 54.6, 42.3, 33.9, 31.7, 29.7, 29.5, 29.3, 29.1, 29.0, 27.0, 24.6, 22.4, 22.4, 13.8; FTIR (KBr) 3362, 3007, 2923, 2853, 1740, 1466, 1255, 1197, 1017 cm^{-1}; HRMS ($C_{51}H_{96}N_2O_8I_2$) calcd, 1118.5260; found 991.6212 (M^{2+} + I$^-$).

Liposome Formulation. The cationic lipid (1.0 μmol) and DOPE (1.0 μmol) were added as chloroform solutions to a 1.9-mL sample vial. The chloroform was evaporated using a stream of dry argon at room temperature. The resulting thin lipid films were placed under vacuum for 2−3 h to ensure that all traces of solvent were removed. Sterile water (1.0 mL) was then added to hydrate the lipid thin films, and the resultant suspension was vigorously mixed (vortex) at room temperature with occasional warming in a 60 °C water bath. The resultant 1.0 mM lipid suspension was used within 2 h of hydration.

Cell Culture. NIH 3T3 cells were obtained from ATCC (CRL 1658) and grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY) with 10% fetal calf serum (GIBCO) in a humidified 10% CO_2 incubator at 37 °C. Human bronchial epithelial cells (16HBE14$_{o-}$) were cultured in Eagle's modified essential medium (MEM; GIBCO) supplemented with 10% fetal bovine serum (GIBCO), 1% glutamine (GIBCO), 1% penicillin, and streptomycin (GIBCO) and grown in a 5% humidified CO_2 incubator at 37 °C. Both cell lines were subcultured into sterile, untreated flasks.

Transfection Experiments. The NIH 3T3 cells were plated at 50 000 cells per well on a standard 24-well plate (Corning, Corning, NY) 24 h prior to transfection. Cells were approximately 80% confluent at the time of transfection. The HBE cells were plated at 50 000 cells/well on a 24-well tissue culture plate coated with fibronectin, vitrogen (collagen), and bovine serum albumin as previously described (*23*) and were transfected as subconfluent monolayers 24 h prior to transfection. The growth media was removed via aspiration and each well was washed once with 0.5 mL of buffered saline and overlaid with only MEM.

Cationic lipid−DNA complexes were prepared 15−40 min prior to transfection. The pGL3-control vector encoding for firefly luciferase (Promega, Madison, WI) was slowly added to a diluted (DMEM or MEM) quantity of the cationic lipid:DOPE suspension in a polystyrene tube (Falcon No. 2058), and the lipid−DNA complex was diluted to a final volume of 800 μL. Typically (e.g., using DOTAP), 24 μL of the lipid suspension was used to complex 4.0 μg of the plasmid DNA, yielding a 2:1 cationic lipid:DNA phosphate molar ratio. Immediately on DNA addition, the suspension was vortexed and allowed to incubate for 15 min at room temperature. A 200-μL aliquot of the resultant lipid−DNA suspension was added to each well (1.0 μg of DNA/well, $n = 4$). The treated cells were then incubated for 4 h at 37 °C. Control wells were treated with 200 μL of medium without supplement. At this time, 500 μL of the appropriate growth media including 10% FCS was added to all wells, and the cells were cultured for 48 h prior to lysis and analysis.

Luciferase Assay. Relative luciferase activity was determined by using the enhanced luciferase assay kit and a Moonlight 2010 luninometer (Analytical Luminescence Laboratories, Sparks, MD). Concentrated luciferase lysis buffer (233.3 μL) was applied to each well. Removal of growth media was not necessary prior to application of the lysis buffer. This technique enhances reproducibility by avoiding the possibility of cell loss during media removal. Luciferase light emissions from 31.1 μL of the lysate were measured over a 10-s period, and the results were expressed as a function of assumed total lysate volume of 933.3 μL. Activity has been expressed as relative light units, which are a function of assay conditions, luciferase concentration, luminometer photomultiplier tube sensitivity, and background. The results are summarized as the mean ($n = 4$) and standard deviation of the total luciferase light units (RLU) obtained from cells lysed after transfection of 1.0 μg of DNA.

Cytotoxicity Determination. Cell viability was determined by evaluating the lactate dehydrogenase activity of post-transfected cells prior to lysis using the CellTiter 96 aqueous nonradioactive cell proliferation assay (Promega). The tetraazolium salt solution (Owen's reagent; 100 μL) was added to each well, and the 24-well plate was then gently agitated to ensure complete mixing. After incubation for an additional 3−4 h, each well was assayed for formazan production by removing an aliquot of the medium and diluting with 9 parts phosphate-buffered saline (PBS) solution. The dilution was conducted in a 1.5-mL methyl acrylate UV/vis disposable cell, and the relative amount of formazan was determined by taking an absorbance reading at 490 nm using PBS as the reference. The mean ($n = 4$) of the absorbance values was calculated and compared to the mean absorbance value for nontransfected cells. The final results were expressed in terms of relative cytotoxicity. Relative cytotoxicity was calculated by subtracting from one the ratio of mean absorbance value for transfected cells over mean absorbance value for untreated cells. A relative cytotoxicity value of zero implies no difference from untreated cells, no measurable cytotoxicity. The maximum value of one implies total cell death for treated cells, no measurable formazan production indicating significant toxicity. Negative values may reflect enhanced formazan production as a consequence of cell growth relative to untreated cells.

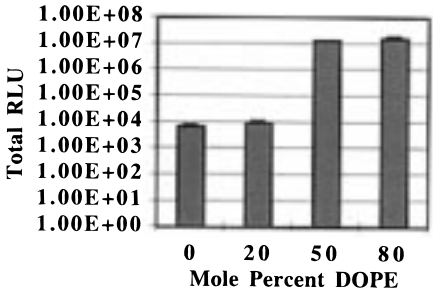


FIGURE 1: Transfection activity of DMTM(Gly):DOPE liposomes. DNA transfections were performed using NIH 3T3 cells. Lipoplexes were formulated at a 2:1 molar charge ratio (lipid charge to DNA phosphate charge). The results are summarized as the mean ($n = 4$) and standard deviation of total luciferase light units (RLU) obtained from cells lysed after administration of 1 μg of DNA.

Phase Contrast and Fluorescence Microscopy. Fluorescence experiments were conducted by transfecting the pEGFP-C1 C−terminal protein fusion vector (Clontech, Palo Alto, CA) into NIH 3T3 and 16HBE14$_{o-}$ cells. The transfection protocol was identical to that of luciferase expression experiments. Cells were grown for 48 h post-transfection, fixed with 3.7% formaldehyde, and viewed on a Zeiss ICM 405 inverted microscope with high-resolution long-working distance objectives. Cells were photographed on the fluorescein channel and with phase-contrast optics with a 40× water objective, using T-Max 400 (Kodak, Rochester, NY) film.

RESULTS

Luciferase Transfection. Many transfection lipids have shown improved activity on co-formulation with DOPE (*24*). To determine if the pentaerythritol lipids require this action, a DOPE titration using the dimyristoyl lipid DMTM(Gly) was conducted. The binary lipid mixtures were treated with pGL3-control, plasmid DNA encoding the firefly luciferase gene. NIH 3T3 transfection results from administration of the DMTM(Gly) lipoplexes are depicted in Figure 1. The data reveal that an equimolar amount of DMTM(Gly) and DOPE affords near optimal transfection activity, with a larger

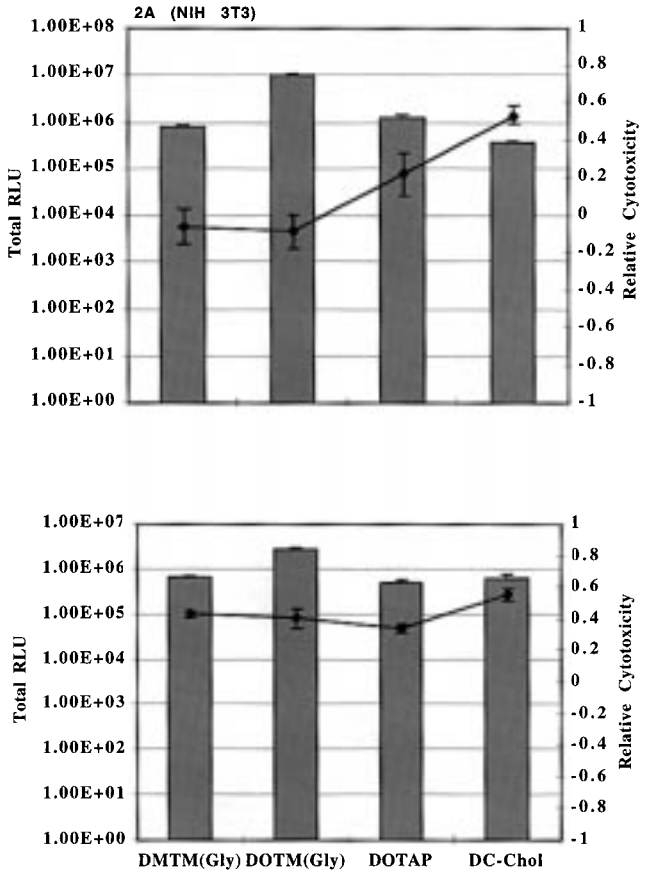


FIGURE 2: Luciferase transfection results summarized in bar graph form as the mean ($n = 4$) and standard deviation of total luciferase light units (RLU, left vertical axis) obtained from NIH 3T3 (A) or 16HBE14$_{o-}$ (B) cells lysed after administration of 1 μg of DNA. Relative cytotoxicity, depicted by the solid line, is plotted according to the right vertical axis, calculated as described in the Material and Methods section. With the exception of DOTAP, 1:1 cationic lipid:DOPE liposomes at a 2:1 molar charge ratio (lipid charge to DNA phosphate charge) were used for lipoplex formation. DOTAP was used without a co-lipid.

percentage of DOPE giving only negligible improvement. The results for DOTM(Gly):DOPE optimization were similar (data not shown). We were gratified that the pentaerythritol construct with its labile headgroup attachments facilitated DNA transfection comparable to DOTAP (*vida infra*).

Regarding the chemical stability of lipids **5**, it should be noted that although aqueous dispersions (vortex mixed, pH = 7−7.4) of **5** were stable at room temperature for at least 24 h, the onset of *headgroup* cleavage to afford diols **7** was observed within hours at pH = 8.

Cytotoxicity Assay. To test whether the novel tetraester motifs of lipids **5*M*** and **5*O*** do in fact ameliorate lipid-induced cytotoxicity, a cell viability assay was performed. The relative cytotoxicity for lipids **5** and the popular transfection lipids DOTAP and DC−chol was determined by measuring the lactate dehydrogenase activity for lipoplex-treated cells (*25*). The comparative assay was performed using NIH 3T3 and 16HBE14$_{o-}$ cells, and the results are plotted in parts A and B of Figure 2, respectively. A requisite for evaluation of cytotoxicity is the concurrent determination of relative transfection activity. Thus, the cationic lipids were evaluated for relative cytotoxicity immediately prior to the determination of luciferase activity, each treated well contributing toward both activity and cytotoxicity measurements.

Figure 2 shows an overlay of transfection activity vs relative cytotoxicity for lipids **5**, DOTAP, and DC−cholesterol. As might be expected from consideration of previous hydrophobic domain studies (*1, 26*), variation in the transfection activity for the pentaerythritol derivatives was influenced by side-chain composition. The dioleoyl analogue DOTM(Gly) showed greater transfection activity than the dimyristoyl analogue in both cell lines. DOTM-(Gly) was also a more active transfection lipid in comparison to DOTAP and DC−cholesterol, reaching an order of magnitude greater expression in both cell lines. The dimyristoyl lipid DMTM(Gly) exhibited transfection activities similar to DOTAP and DC−chol.

The MTS relative cytotoxicity data for the cationic lipid panel is illustrated in Figure 2. A reading of zero on the cytotoxicity scale (right vertical axis) indicates that the treated cells have comparable metabolic (dehydrogenase) activity relative to the untreated control cells and implies the cationic lipid agent does not impair cellular function. The differences in relative cytotoxicity were most pronounced in the NIH 3T3 cells (Figure 2A). DMTM(Gly) and DOTM(Gly) did not induce a cytotoxic response, whereas DC−chol was found to be significantly more cytotoxic, impairing near 60% of the treated cells. In the HBE cell line (Figure 2B), all the lipids induced a cytotoxic response. The pentaerythritol constructs exhibited comparable cytotoxicity to DOTAP and slightly improved cytotoxicity relative to DC−chol.

Green Fluorescent Protein (GFP) Transfection. To probe the relationships between cellular uptake, expression, and cytotoxicity, we conducted a transfection study using the GFP reporter gene. Fluorescent microscopy of cells treated with GFP-encoded plasmid has been shown to be a sensitive tool for determining the occurrence of transgene expression (*15*). Micrograph images of GFP transfection in NIH 3T3 cells using DOTM(Gly) and DC−chol are given in Figure 3. Parts A and D of Figures 3 identify the cells that are expressing green fluorescent protein using DOTM(Gly) and DC−chol, respectively. In agreement with the luciferase assay, both lipids were active in facilitating gene transfer, as indicated by the presence of fluorescent cells. Parts B and E of Figure 3 are phase-contrast images of the same field of view shown by the respective fluorescent images. The phase-contrast image for DOTM(Gly) (Figure 3B) showed normal cellular morphology, consistent with the low cytotoxicity response measured by the MTS assay. However, the phase-contrast image for cells treated with DC−chol (Figure 3E) showed dense regions of large vacuoles, indicative of cationic lipid-induced cytotoxicity (*27*). Cells treated with the cationic lipid formulations from the tested panel (mock transfections, Figure 3C and 3F, no plasmid present) showed very similar morphology to cells transfected with their corresponding GFP lipoplexes. HBE phase-contrast pictures depicted some cellular debris and abnormal cellular morphology for all the cationic lipids examined (data not shown), a result consistent with the MTS assay for cytotoxicity.

DISCUSSION

Cationic lipid-associated cytotoxicity has been a persistent challenge during polynucleotide delivery. Transgene delivery and expression constitute a complex process that includes steps involving lipoplex formulation, cellular internalization,

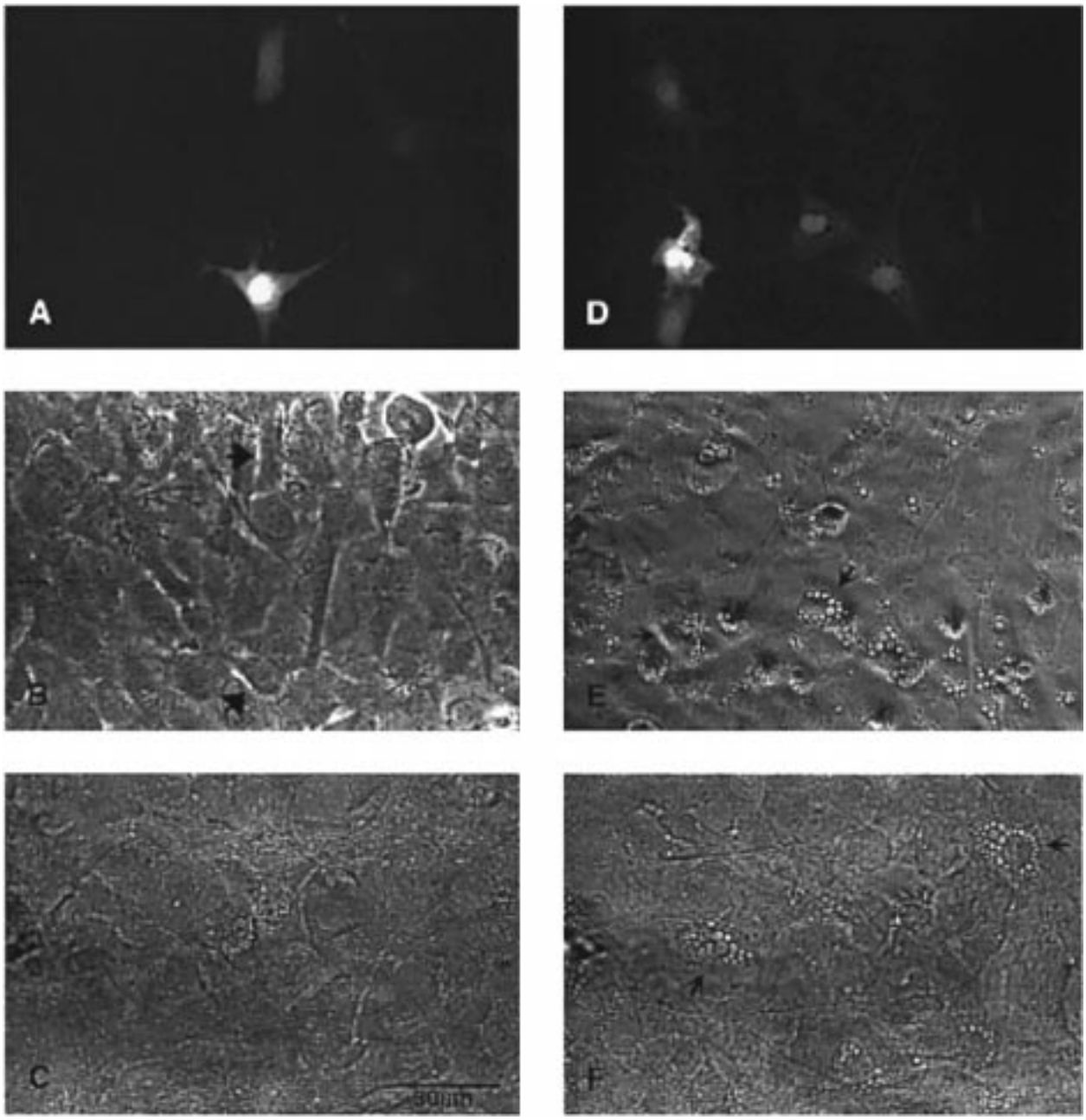


FIGURE 3: Transfection of the pEGFP-C1 terminal fusion vector into NIH 3T3 cells using DOTM(Gly)−DOPE (A, B) and DC−chol−DOPE (C, D) cationic lipid formulations. The plasmid was administered at 1.0 μg. The formulation of DOTM(Gly)−DOPE was effective for transfections, as evidenced by the fluorescent cells (A), two of which are designated by arrows in the accompanying phase micrograph (B). Phase microscopy (B) demonstrated that the cellular morphology was similar to that of mock-transfected controls (C) lacking the DNA plasmid. Cells trasfected with DC−chol−DOPE (a commercially available cationic lipid) were fluorescent (D); however, numerous large vacuoles were present both in experimental transfectants (→) (E) as well as in mock-transfected controls (→) (F), suggesting that the cationic lipids were the source of intracellular vacuoles.

endosome escape, and nuclear localization (*14*). Studies have shown that cationic lipid translocation to the cytoplasmic membrane occurs after lipoplex uptake as measured by fluorescent labeling techniques (*28*). Cellular processes are inhibited by the incorporation of positively charged lipids in the plasma membrane (*19*), and ultimately, this incorporation leads to cell dysfunction and potentially cell death. To address this toxicity issue, we prepared a novel cationic lipid construct containing a novel tetraester motif to accelerate biodegradation of the charged species. We envisioned that enhanced clearance of the cationic lipid might alleviate cytotoxicity. This consideration is based in part on observations by Hofland et al. (*29*), who noted in DNA transfection studies a strong correlation between cytotoxicity and excess uncomplexed cationic lipid.

The use of ester, amide, and carbamate linkages to tether polar and hydrophobic domains is common practice; however, the present report is the first example of a linchpin tetraester construct. In this study, we used pentaerythritol as the linchpin. The headgroup of the tetraester lipids **5** is a quaternized *N*,*N*-dimethylglycine. The proximal location of the ammonium moiety to the polar domain ester linkage leads to an intrinsically activated ester prone to facile hydrolysis.

We have determined that the pentaerythritol lipids DOTM(Gly) and DMTM(Gly) are capable of facilitating the

intracellular delivery of plasmid DNA at levels comparable to the well-investigated compounds DOTAP and DC−cholesterol (Figures 1 and 2). We have also conducted a cell proliferation assay to measure the relative cytotoxicities of the cationic lipid panel. The MTS assay, a widely used method to evaluate transfection associated cytotoxicity (*2*, *27*, *30*), measures mitochondrial lactate dehydrogenase activity through tetraazolium salt bioreduction. The low relative cytotoxicity responses determined for the pentaerythritol lipids in NIH 3T3 and HBE cell lines (Figure 2) suggest that the use of intrinsically activated functional groups may be a useful strategy to reduce cationic lipid-induced toxicity. The α-ammonium headgroup design was shown to be sufficiently stable during lipoplex preparation and delivery, but the enhanced biocompatiblity of this design over the more conventional gene-transfer agents is presumably a result of its rapid degradation in the biological milieu. Although no intracellular degradation studies have yet been conducted for any cationic transfection lipid, the cytotoxicity results support the ester biocompatibility concept put forth by Silvius and Leventis (*7*).

The cytotoxicity performance of DC−chol may be an issue of incompatibility with our chosen in vitro models. Post-transfection histochemical studies involving DC−chol formulations show marginal cytotoxic response in human clinical trials (*11*). Furthermore, Templeton et al. have shown that lipoplex formulations containing cholesterol and DOTAP demonstrated superior in vivo activity over DOTAP:DOPE lipoplexes, but their findings were reversed in tissue culture (*31*). Thus, one might infer that the cholesterol unit or the carbamate linkage of DC−chol confers lipoplex stability in vivo but may be a limitation in vitro (*32*). To test this notion, we are currently examining the pentaerythritol lipids in studies of murine lung transfection.

Since a clear correlation between luciferase expression and cationic lipid-induced cytotoxicity was not evident from the results in Figure 2, we envisioned that the use of a GFP assay would allow us to more closely examine a potential link between transfection and cationic lipid-induced cytotoxicity. A previous study had noted a lack of correlation between transfection activity and cytotoxicity (*2*). By comparing fluorescent images with the corresponding phase contrast images, we sought to ascertain if cytotoxicity is limited to those cells expressing GFP. Comparison of the fluorescent and phase-contrast images (Figure 3) revealed that the cationic lipid-induced cytotoxic response was not limited to those cells expressing GFP. Vacuolization was noted in cells that did *and* that did not express GFP. Thus, the onset of cytotoxicity precedes gene expression. Phase-contrast images of cells transfected with DC−chol (Figure 3E) depicted significantly greater vacuolization than cells transfected with DOTM(Gly) (Figure 3B), DOTAP, or DMTM(Gly) (images not shown). The association of abnormal vacuolization to cellular dysfunction has been reported (*27*). Since the percentage of transduced cells is known to exceed the number of cells that express the transgene product (*15*), it is not surprising that the toxicity response is more widespread than the expression response. The cytotoxicity results for the mock transfected cells using DC−chol (Figure 3F) show that exposure to a typical cationic liposome preparation results in a toxic response, presumably a consequence of cationic lipid incorporation in the plasma membrane facilitated by lipid mixing or fusion (*33*). These cytotoxicity observations are significant in light of ongoing discussion that the release of cationic lipid from its lipoplex during endosomal disruption may be the primary source of cytotoxicity. Much evidence supports the endosomal pathway for lipoplex entry, thereby also supporting the probability of extensive lipid release on endosomal disruption (*14*). However, since removal of DNA in the mock transfection experiments did not alter the extent of vacuolization, we infer a similar onset of cytotoxicity for both the liposome and lipoplex aggregates, beginning with lipid mixing or fusion events at the stage of initial cellular contact. Although cationic liposome interactions with the plasma membrane are expected to differ somewhat from the lipoplex interactions with the membrane, the former aggregate being more inclined to engage in lipid mixing and fusion due to composition and size considerations (*34*, *35*), lipoplex preparations typically contain sufficient excess unbound cationic lipid that similar contributions to early lipid mixing may occur.

In conclusion, we have developed a tetraester cationic lipid construct that undergoes facile hydrolysis to ameliorate lipid-associated cytotoxicity. The modular design, based on pentaerythritol, enables variable headgroup and hydrophobic domain composition, affording unprecedented flexibility for analogue synthesis. Members of this new lipid class were shown to facilitate reporter gene transfection at levels comparable to the transfection standards DOTAP and DC−chol. Using a cell proliferation assay, we observed that the pentaerythritol lipids were less cytotoxic than DC−chol in both NIH 3T3 and $16HBE14_{o-}$ cells. This result was visually confirmed using phase-contrast microscopy. The comparable transfection activities and lower cytotoxicities observed for the tetraester cationic lipids underscore the useful strategy of devising nontoxic lipids by enhancing their biocompatibility.

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