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Structural and Formulation Factors Influencing Pyridinium Lipid-based Gene Transfer

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Abstract

A series of pyridinium lipids containing a heterocyclic ring and a nitrogen atom were synthesized to determine the structure-activity relationship for gene delivery. Pyrylium chloroaluminate was synthesized by monoacylation of mesityl oxide and converted into pyrylium hexafluorophosphate, which was used as the key intermediate for reaction with different primary amines, to yield hydroxyethylpyridinium hexafluorophosphate and aminoethylpyridinium hexafluorophosphate. Acylation of these pyridinium salts with different types of fatty acid chlorides afforded the final pyridinium lipids, which were mixed with a co-lipid, such as L- α -dioleoylphosphatidylethanolamine (DOPE) and cholesterol (Chol) to prepare cationic liposomes by sonication. These liposomes were mixed with plasmid DNA encoding enhanced green fluorescent protein (pCMS-EGFP) or luciferase (pDNA3-Luc) and transfected into Chinese Hamster Ovary (CHO) cells. Several factors including hydrophobic anchor chain length, anchor chain type, configuration of double bond, linker type, co-lipid type, cationic lipid: co-lipid molar ratio, charge ratio (N/P), and cell type had significant influence on transfection efficiency and cytotoxicity. Pyridinium lipids with amide linker showed significantly higher transfection efficiency compared to their ester counterparts. Liposomes prepared at 1:1 molar ratio of pyridinium lipid and co-lipid showed higher transfection efficiency when either DOPE or cholesterol was used as a co-lipid to prepare cationic liposomes for complex formation with plasmid DNA at 3:1(+/-) charge ratio. Pyridinium liposomes based on hydrophobic anchor chain length of 16 showed higher transfection efficiency and lower cytotoxicity. The pyridinium lipid with trans-configuration of the double bond in fatty acid chain showed higher transfection efficiency than its counterpart with cis-configuration at the same fatty acid chain length. In the presence of serum, C16:0 and Lipofectamine significantly decreased their transfection efficiencies, which were completely lost at the serum concentration of 30% and higher, while C16:1 trans-isomer still had high transfection efficiency under these conditions. In conclusion, pyridinium lipids showed high transfection efficiency and have the potential to be used as transfection reagents in vitro and in vivo.

Keywords

pyridinium lipid; cationic liposomes; gene delivery; plasmid; transfection

INTRODUCTION

Cationic liposomes are the most commonly used synthetic carriers for delivery of oligonucleotides, siRNAs and plasmid DNA. The three basic components of a cationic lipid,

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which affect gene expression or silencing, include (i) a hydrophobic lipid anchor group, such as fatty acid chains of various lengths and unsaturation states, or cholesterol, which helps in forming liposomes and exchanging lipids in cell membrane (1), (ii) linker group, such as an ester, ether, amide, or carbamate, which binds the polar group to the lipid moiety and determines the stability and biodegradability of the cationic lipid (2), and (iii) a positively charged hydrophilic head group, such as mono- or poly- amine group, which interacts with pDNA via electrostatic interaction, leading to its condensation and enhanced the cellular uptake (3,4). Understanding of these structural parameters is essential for the rational design of efficient cationic lipid-based transfection reagents. Transfection efficiency of cationic liposomes greatly depends on (i) the extent of DNA condensation, (ii) increased cellular uptake due to ionic and/or hydrophobic interaction with negatively charged biological surfaces, and (iii) membrane fusion via transient membrane destabilization to achieve delivery into the cytoplasm while avoiding degradation in the lysosomal compartment (5,6).

Significant progress has been made in the design and function of cationic lipids since the introduction in 1987 of the transfection reagent Lipofectin, which is a 1:1 w/w mixture of the cationic lipid *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium (DOTMA) and DOPE (7). Many cationic lipids bearing either a single tertiary or quaternary ammonium head group or which contain protonatable polyamines linked to dialkyl or cholesterol anchors have been designed for transfection of a variety of cell types in culture (8–10). Commercially available cationic lipids used as liposomes or alone for gene delivery include DOTMA (Lipofectin); 2,3-dioleoyloxy-*N*-[2-spermine carboxamide] ethyl-*N,N*-dimethyl-1-propanammonium trifluoroacetate (DOSPA, Lipofectamine), 1,2-dioleoyl-3-Trimethylammonium-Propane (DOTAP), *N*-[1-(2,3-dimyristyloxy)propyl]-*N,N*-dimethyl-*N*-(2-hydroxyethyl) ammonium bromide (DMRIE), 3 β -[*N*-(*N,N'*-dimethylamino)ethane] carbamoyl] cholesterol (DC-Chol), and dioctadecyl amidoglyceryl spermine (DOGS, Transfectam) and dimethyldioctadecylammonium bromide (DDAB) (6,7,10–15). A few such compounds have also been used in initial clinical studies. However, many of the cationic lipids used in early clinical trials such as DC-Chol, DMRIE, and GL-67, although effective in vitro, have proven inefficient in vivo, especially when compared with viral vectors (16,17).

Various heterocyclic cationic lipids had been studied and used for gene delivery. Some of them showed higher transfection efficiency and lower cytotoxicity compared to cationic lipids with linear primary amines or polyamines as the head group (18). Pyridinium heterocyclic ring in the cationic amphiphile was first introduced in 1940s, which had antiseptic and antibiotic properties (19,20). Upon the invention of pyridinium-based cationic lipids, a series of these lipids have been developed and some of them have been reported to reach or surpass the transfection efficiency of commercial cationic lipid formulations, both in vitro and vivo, while maintaining low cytotoxicity (21–25).

In this study, we synthesized a series of pyridinium lipids containing a heterocyclic positively charged ring linked to different types of fatty acids via ester or amide spacers. Following purification and characterization of these cationic lipids using ^1H NMR, ^{13}C NMR and mass spectrometry, cationic liposomes were prepared using DOPE or cholesterol as a co-lipid, followed by determination of particle size and zeta potential. Reporter gene expression and cytotoxicity were determined after transfection of Chinese Hamster Ovary (CHO) cells, hepatocarcinoma (CRL1830) cells and prostate cancer (C4-2) cells with green fluorescent protein (GFP) or luciferase expression plasmid DNA after complex formation with cationic liposomes. Factors influencing gene transfer, including charge ratio (N/P) between cationic liposomes and pDNA, cationic lipid / co-lipid ratio, linker type, fatty acid carbon chain length, degree of saturation, configuration of double bond, the presence of serum and cell type were studied.

EXPERIMENTAL PROCEDURES

Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) was purchased from Avanti Polar Lipids (Alabaster, AL). Lipofectamine, DMEM and RPMI1640 were purchased from Invitrogen Corporation (Carlsbad, CA). Heat-inactivated fetal bovine serum (FBS) and penicillin (10000 units/mL penicillin G sodium)-streptomycin solution (10000 µg/mL streptomycin sulfate) in 0.85% saline were purchased from Mediatech, Inc. (Herndon, VA). Luciferase Assay Kit and Cell Culture Lysis Reagent 5X (CCLR) were procured from Promega Corporation (Madison, WI). QIAfilter plasmid maxi kit was purchased from Qiagen, Inc. (Valencia, CA); BCA protein assay kit from Pierce (Rockland, IL); pCMS-EGFP plasmid was procured from BD Biosciences Clontech (Palo Alto, CA) and pcDNA3-Luc plasmid was a kind gift from Expression Genetics, Inc. (Huntsville, AL). All reagents used for lipid synthesis were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA) and were used without further purification.

Synthesis of Pyridinium Lipids

General Methods—Routine thin layer chromatography (TLC) was performed on aluminum backed Uniplates. (Analtech, Newark, DE). ¹H NMR spectra were obtained on a Bruker AX 300 (Billerica, MA) or Varian Inova-500 (Palo Alto, CA) spectrometer. Chemical shifts for ¹H NMR were reported as parts per million (ppm) relative to tetramethylsilane (TMS) in deuterated chloroform (CDCl₃) as internal standard. ¹³C NMR spectra were obtained on a Bruker AX 300 spectrometer, operating at 75.5 MHz. To make sure the final results are reliable, we used both conventional mass and high-resolution mass (HRMS) spectrophotometers to characterize the synthesized pyridinium lipids. Conventional mass spectral data were collected on a Bruker ESQUIRE electrospray/ion trap instrument in positive and negative ion modes. High-resolution mass measurements were obtained using a Micromass Q-TOF2 mass spectrometer. HRMS samples were dissolved in 50% acetonitrile, 0.1% formic acid, and were infused by syringe (0.4 µl/min). To make the paper easier to be read we only listed the HRMS data.

Pyridinium amide cationic lipids **4–13** and pyridinium ester cationic lipids **14–18** were synthesized as described by Illies et al. (25) and their visual structures with minimized energy were simulated with the Chem3D Pro software, version 10.0 (ChemOffice, CambridgeSoft Corp.) using MM2 molecular mechanics routine toward a 0.1 rms gradient, which are shown in Table 1. Figure 1 outlines a synthesis route via the corresponding pyrylium salts. Mesityl oxide was monoacylated with fatty acid chloride in the presence of aluminum chloride to yield pyrylium chloroaluminate, which was subsequently converted into more stable hexafluorophosphate **1**, for using as a key intermediate to react with different primary amines, yielding aminoethylpyridinium hexafluorophosphate **2** and hydroxyethylpyridinium hexafluorophosphate **3**. Acylation of these pyridinium salts with different types of fatty acid chlorides afforded the final pyridinium amide cationic lipids **4–13** and pyridinium ester cationic lipids **14–18**, which were purified with chromatography on silica gel and recrystallized from ethanol.

Synthesis of Pyridinium Amide Cationic Lipids 4–13—Aminoethylpyridinium hexafluorophosphate **2**, compounds **4** and **5** were synthesized as described by Illies et al. (25) and the spectra were the same as those reported by these scientists.

4, 6-Dimethyl-2-tetradecyl-1-[2-(9Z)-hexadec-9-enoylamidoethyl] pyridinium hexafluorophosphate 6—Yield: 89.9 %; ¹H NMR (CDCl₃), δ, ppm: 7.41 (s, 1H, H-5 pyridinium), 7.38 (s, 1H, H-3 pyridinium), 6.75 (t, 1H, J=6.0 Hz), 5.33 (m, 2H, J= 5.4 Hz),

4.56 (*t*, 2H, $J=6.9$ Hz), 3.63 (*dt*, 2H, $J=6.9$ Hz, 6.6 Hz, CONHCH_2), 3.17 (*t*, 2H, $J=7.5$ Hz, CaPy-CH_2), 2.90 (*s*, 3H, CH_3 α -Pyridinium), 2.55 (*s*, 3H, CH_3 γ -Pyridinium), 2.18 (*t*, 2H, $J=7.5$ Hz, NHCOCH_2), 1.99 (4 H, *br*), 1.72 (*quint.*, 2H, $\text{CaPy-CH}_2\text{CH}_2$), 1.47 (*m*, 2H, $\text{NHCOCH}_2\text{CH}_2$), 1.25 (*s*, 38H, CH_2 from both of alkyl chains), 0.88 (*t*, 6H, CH_3 of alkyl chains); ^{13}C NMR (300 M, CDCl_3), 175.23 (C=O), 159.07, 158.14, 155.05 (C-2, C-4 and C-6 pyridium), 130.41, 130.28 (CH=CH), 128.68, 127.32 (C-3 and C-5 pyridium), 50.55 (CaPy-CH_2), 38.25 ($\text{C}\beta\text{Py-CH}_2$), 35.97, 33.25, 32.59, 32.57, 31.92, 31.75, 29.70, 29.69, 29.65, 29.64, 29.63, 29.63, 29.59, 29.39, 29.34, 29.29, 29.29, 29.24, 29.21, 29.06, 29.03, 28.84, 25.40, 22.67, 22.63, 21.73, 21.51, 14.05, 14.05. HRMS calcd for $\text{C}_{39}\text{H}_{71}\text{N}_2\text{O}^+$, $M_r = 583.5566$, found 583.5568.

4, 6-Dimethyl-2-tetradecyl-1-[2-(9E)-hexadec-9-enoylamidoethyl] pyridinium hexafluorophosphate 7—Yield: 81.5 %; ^1H NMR (300 M, CDCl_3), δ , ppm: 7.42 (*s*, 1H, H-5 pyridinium), 7.37 (*s*, 1H, H-3 pyridinium), 6.75 (*t*, 1H, $J=6.0$ Hz), 5.37 (*m*, 2H), 4.55 (*t*, 2H, $J=6.6$ Hz), 3.62 (*dt*, 2H, $J=6.6$ Hz, 6.3 Hz, CONHCH_2), 3.17 (*t*, 2H, $J=7.5$ Hz, CaPy-CH_2), 2.90 (*s*, 3H, CH_3 α -Pyridinium), 2.54 (*s*, 3H, CH_3 γ -Pyridinium), 2.17 (*t*, 2H, $J=7.5$ Hz, NHCOCH_2), 1.95 (4H, *br*), 1.72 (*quint.*, 2H, $\text{CaPy-CH}_2\text{CH}_2$), 1.46 (*quint.*, 2H, $\text{NHCOCH}_2\text{CH}_2$), 1.25 (*s*, 38H, CH_2 from both of alkyl chains), 0.87 (*t*, 6H, CH_3 of alkyl chains); ^{13}C NMR (300 M, CDCl_3), 174.67 (C=O), 158.47, 157.54, 154.51 (C-2, C-4, C-6 pyridinium), 129.37, 129.25 (CH=CH), 128.11, 126.72 (C-3 and C-5 pyridium), 49.97 (CaPy-CH_2), 37.64 ($\text{C}\beta\text{Py-CH}_2$), 36.38, 32.67, 31.35, 31.21, 29.17, 29.11, 29.09, 29.08, 29.07, 29.02, 28.82, 28.77, 28.72, 28.70, 28.69, 28.65, 28.60, 28.55, 28.49, 28.40, 26.66, 26.65, 24.83, 22.09, 22.06, 21.14, 20.92, 13.49, 13.48. HRMS calcd for $\text{C}_{39}\text{H}_{71}\text{N}_2\text{O}^+$, $M_r = 583.5566$, found 583.5562.

4, 6-Dimethyl-2-tetradecyl-1-(2-octadecanoylamidoethyl) pyridinium hexafluorophosphate 8—Yield: 87.5 %; ^1H NMR (CDCl_3), δ , ppm: 7.41 (*s*, 1H, H-5 pyridinium), 7.38 (*s*, 1H, H-3 pyridinium), 6.77 (*t*, 1H, $J=6.0$ Hz), 5.37 (*m*, 2H, $J=19.2$ Hz), 4.55 (*t*, 2H, $J=6.9$ Hz), 3.62 (*dt*, 2H, $J=6.9$ and 6.0 Hz, CONHCH_2), 3.17 (*t*, 2H, $J=7.2$ Hz, CaPy-CH_2), 2.90 (*s*, 3H, CH_3 α -Pyridinium), 2.54 (*s*, 3H, CH_3 γ -Pyridinium), 2.17 (*t*, 2H, NHCOCH_2), 1.95 (4H, *br*), 1.72 (*quint.*, 2H, $\text{CaPy-CH}_2\text{CH}_2$), 1.48 (*quint.*, 2H, $\text{NHCOCH}_2\text{CH}_2$), 1.25 (*s*, 50H, CH_2 from both of alkyl chains), 0.88 (*t*, 6H, CH_3 of alkyl chains); ^{13}C NMR (300 M, CDCl_3), 175.15 (C=O), 159.00, 158.06, 155.03 (C-2, C-4, C-6 pyridinium), 128.64, 127.24 (C-3 and C-5 pyridium), 50.49 (CaPy-CH_2), 38.17 ($\text{C}\beta\text{Py-CH}_2$), 35.94, 33.19, 31.89, 31.88, 29.67, 29.66, 29.66, 29.65, 29.64, 29.63, 29.63, 29.62, 29.61, 29.61, 29.60, 29.55, 29.49, 29.35, 29.33, 29.32, 29.31, 29.30, 29.26, 29.25, 29.22, 29.02, 25.37, 22.63, 22.63, 21.67, 21.42, 14.01, 14.01. HRMS calcd for $\text{C}_{41}\text{H}_{77}\text{N}_2\text{O}^+$, $M_r = 613.6036$, found 613.6036.

4, 6-Dimethyl-2-tetradecyl-1-(2-oleoylamidoethyl) pyridinium hexafluorophosphate 9—Yield: 62.3%; ^1H NMR (CDCl_3), δ , ppm: 7.41 (*s*, 1H, H-5 pyridinium), 7.38 (*s*, 1H, H-3 pyridinium), 6.77 (*t*, 1H, $J=6.0$ Hz, CONH), 5.33 (2H, *t*, HC=CH), 4.56 (*t*, 2H, $J=6.9$ Hz), 3.63 (*dt*, 2H, $J=6.9$ and 6.0 Hz, CONHCH_2), 3.17 (*t*, 2H, $J=7.5$ Hz, CaPy-CH_2), 2.90 (*s*, 3H, CH_3 α -Pyridinium), 2.55 (*s*, 3H, CH_3 γ -Pyridinium), 2.18 (*t*, 2H, NHCOCH_2), 1.99 (*q*, 4H), 1.72 (*quint.*, 2H, $\text{CaPy-CH}_2\text{CH}_2$), 1.47 (*quint.*, 2H, $\text{NHCOCH}_2\text{CH}_2$), 1.25 (*s*, 42H, CH_2 from both of alkyl chains), 0.88 (*t*, 6H, CH_3 of alkyl chains); ^{13}C NMR (300 M, CDCl_3), 175.20 (C=O), 159.07, 158.13, 155.05 (C-2, C-4, C-6 pyridinium), 129.95, 129.81 (CH=CH), 128.68, 127.31 (C-3 and C-5 pyridium), 50.55 (CaPy-CH_2), 38.24 ($\text{C}\beta\text{Py-CH}_2$), 35.96, 33.24, 31.91, 31.90, 29.78, 29.75, 29.68, 29.67, 29.66, 29.64, 29.63, 29.58, 29.51, 29.38, 29.33, 29.32, 29.29, 29.28, 29.28, 29.26, 29.25, 29.24, 29.18, 29.06, 27.23, 25.39, 22.66, 21.71, 21.49, 14.05, 14.05. HRMS calcd for $\text{C}_{41}\text{H}_{75}\text{N}_2\text{O}^+$, $M_r = 611.5879$, found 611.5872.

4, 6-Dimethyl-2-tetradecyl-1-[2-(9E)-Octadec-9-enoylamidoethyl] pyridinium hexafluorophosphate 10—Yield: 56.5 %; ^1H NMR (CDCl_3), δ , ppm: 7.42 (s, 1H, H-5 pyridinium), 7.37 (s, 1H, H-3 pyridinium), 6.76 (t, 1H, $J=6.3\text{ Hz}$), 5.37 (t, 2H, $\text{HC}=\text{CH}$, $J=19.2\text{ Hz}$), 4.57 (t, 2H, $J=6.9\text{ Hz}$), 3.63 (dt, 2H, $J=6.9$ and 6.3 Hz , CONHCH_2), 3.17 (t, 2H, $J=7.8\text{ Hz}$, CaPy-CH_2), 2.90 (s, 3H, CH_3 α -Pyridinium), 2.56 (s, 3H, CH_3 γ -Pyridinium), 2.18 (t, 2H, NHCOCH_2), 1.99 (br, 4H), 1.74 (quint., 2H, $\text{CaPy-CH}_2\text{CH}_2$), 1.46 (quint., 2H, $\text{NHCOCH}_2\text{CH}_2$), 1.25 (s, 42H, CH_2 from both of alkyl chains), 0.87 (t, 6H, CH_3 of alkyl chains); ^{13}C NMR (300 M, CDCl_3), 175.14 (C=O), 158.98, 158.06, 155.02 (C-2, C-4, C-6 pyridinium), 130.36, 130.21 (CH=CH), 128.63, 127.23 (C-3 and C-5 pyridium), 50.47 (CaPy- CH_2), 38.16 (C β Py- CH_2), 35.91, 33.17, 32.54, 32.51, 31.86, 31.84, 29.62, 29.63, 29.62, 29.61, 29.60, 29.59, 29.58, 29.53, 29.42, 29.34, 29.28, 29.23, 29.24, 29.20, 29.18, 29.15, 29.01, 28.98, 25.34, 22.60, 22.61, 21.65, 21.41, 13.99, 13.99. HRMS calcd for $\text{C}_{41}\text{H}_{75}\text{N}_2\text{O}^+$, $M_r = 611.5879$, found 611.5870.

4, 6-Dimethyl-2-tetradecyl-1-[2-(11Z)-eicos-11-enoylamidoethyl] pyridinium hexafluorophosphate 11—Yield: 79.8 %; ^1H NMR (CDCl_3), δ , ppm: 7.41 (s, 1H, H-5 pyridinium), 7.38 (s, 1H, H-3 pyridinium), 6.78 (t, 1H, $J=6.3\text{ Hz}$), 5.34 (m, 2H, $\text{HC}=\text{CH}$), 4.56 (t, 2H, $J=6.6\text{ Hz}$), 3.63 (dt, 2H, $J=6.6$ and 6.3 Hz , CONHCH_2), 3.17 (t, 2H, $J=7.8\text{ Hz}$, CaPy-CH_2), 2.90 (s, 3H, CH_3 α -Pyridinium), 2.55 (s, 3H, CH_3 γ -Pyridinium), 2.18 (t, 2H, NHCOCH_2), 1.99 (br, 4H), 1.72 (quint., 2H, $\text{CaPy-CH}_2\text{CH}_2$), 1.57–1.42 (m, 2H, $\text{NHCOCH}_2\text{CH}_2$), 1.25 (s, 46H, CH_2 from both of alkyl chains), 0.88 (t, 6H, CH_3 of alkyl chains); ^{13}C NMR (300 M, CDCl_3), 175.23 (C=O), 159.06, 158.13, 155.06 (C-2, C-4, C-6 pyridinium), 129.90, 129.90 (CH=CH), 128.69, 127.31 (C-3 and C-5 pyridium), 50.54 (CaPy- CH_2), 38.23 (C β Py- CH_2), 35.97, 33.24, 31.92, 31.90, 29.81, 29.80, 29.78, 29.69, 29.67, 29.65, 29.64, 29.59, 29.54, 29.52, 29.50, 29.39, 29.35, 29.32, 29.30, 29.29, 29.29, 29.28, 29.24, 29.06, 27.25, 27.23, 25.41, 24.78, 22.66, 21.72, 21.49, 14.05, 14.05. HRMS calcd for $\text{C}_{43}\text{H}_{79}\text{N}_2\text{O}^+$, $M_r = 639.6192$, found 639.6193.

4, 6-Dimethyl-2-tetradecyl-1-[2-(11E)-eicos-11-enoylamidoethyl] pyridinium hexafluorophosphate 12—Yield: 58.8 %; ^1H NMR (CDCl_3), δ , ppm: 7.41 (s, 1H, H-5 pyridinium), 7.38 (s, 1H, H-3 pyridinium), 6.78 (t, 1H, $J=6.0\text{ Hz}$), 5.38 (t, 2H, $\text{HC}=\text{CH}$), 4.57 (t, 2H, $J=6.6\text{ Hz}$), 3.64 (dt, 2H, $J=6.9$ and 6.0 Hz , CONHCH_2), 3.17 (t, 2H, $J=7.8\text{ Hz}$, CaPy-CH_2), 2.90 (s, 3H, CH_3 α -Pyridinium), 2.56 (s, 3H, CH_3 γ -Pyridinium), 2.18 (t, 2H, NHCOCH_2), 1.96 (br, 4H), 1.71 (quint., 2H, $\text{CaPy-CH}_2\text{CH}_2$), 1.48 (quint., 2H, $\text{NHCOCH}_2\text{CH}_2$), 1.25 (s, 42H, CH_2 from both of alkyl chains), 0.87 (t, 6H, CH_3 of alkyl chains); ^{13}C NMR (300 M, CDCl_3), 175.27 (C=O), 159.11, 158.16, 155.04 (C-2, C-4, C-6 pyridinium), 130.36, 130.36 (C=C), 128.68, 127.35 (C-3 and C-5 pyridium), 50.59 (CaPy- CH_2), 38.27 (C β Py- CH_2), 35.98, 33.28, 32.60, 31.92, 31.91, 29.70, 29.69, 29.69, 29.68, 29.68, 29.67, 29.67, 29.66, 29.65, 29.64, 29.59, 29.49, 29.49, 29.39, 29.35, 29.32, 29.30, 29.29, 29.19, 29.07, 25.40, 25.40, 22.66, 22.66, 21.74, 21.54, 14.05, 14.05. HRMS calcd for $\text{C}_{43}\text{H}_{79}\text{N}_2\text{O}^+$, $M_r = 639.6192$, found 639.6196.

4, 6-Dimethyl-2-tetradecyl-1-[2-(2-heptyl-undecanoyl)-amidoethyl] pyridinium hexafluorophosphate 13—Yield: 77.3 %; ^1H NMR (CDCl_3), δ , ppm: 7.41 (s, 1H, H-5 pyridinium), 7.37 (s, 1H, H-3 pyridinium), 6.78 (1H, t, 1H, $J=6.3\text{ Hz}$), 4.55 (t, 2H, $J=6.6\text{ Hz}$), 3.66 (dt, 2H, $J=6.6$ and 6.6 Hz , CONHCH_2), 3.22 (t, 2H, $J=6.9\text{ Hz}$, CaPy-CH_2), 2.93 (s, 3H, CH_3 α -Pyridinium), 2.55 (s, 3H, CH_3 γ -Pyridinium), 2.12 (quint., 1H, NHCOCH), 1.71 (quint., 2H, $\text{CaPy-CH}_2\text{CH}_2$), 1.46 (quint., 4H, $\text{NHCOCH}(\text{CH}_2)_2$), 1.25 and 1.23 (s, s, 46H, CH_2 from both of alkyl chains), 0.88–0.84 (m, 9H, CH_3 of alkyl chains); ^{13}C NMR (300 M, CDCl_3), 178.24 (C=O), 159.01, 158.01, 155.21 (C-2, C-4, C-6 pyridinium), 128.71, 127.25 (C-3 and C-5 pyridium), 50.51 (CaPy- CH_2), 47.17 ($\text{C}_9\text{H}_{19}\text{CHC}_7\text{H}_{15}$), 38.03 (C β Py- CH_2), 33.27, 32.68, 31.91, 31.89, 31.84, 29.72, 29.68, 29.67, 29.66, 29.65, 29.65, 29.61, 29.61, 29.60, 29.53, 29.42,

29.34, 29.33, 29.30, 29.71, 29.11, 29.06, 27.45, 27.38, 27.42, 26.77, 22.65, 22.62, 21.65, 21.44, 14.04, 14.04. HRMS calcd for $C_{41}H_{77}N_2O^+$, $Mr = 613.6035$, found 613.6037.

Synthesis of Pyridinium Ester Cationic Lipids 14–18—Hydroxyethylpyridinium hexafluorophosphate **3**, compounds **14** and **16** were synthesized as described method(25) and the spectra are the same as those reported in ref. 25.

4, 6-Dimethyl-2-tetradecyl-1-(2-pentadecanoylhydroxyethyl) pyridinium hexafluorophosphate 15—Yield: 72.0%; 1H NMR ($CDCl_3$), δ , ppm: 7.44 (s, 1H, H-5 pyridinium), 7.39 (s, 1H, H-3 pyridinium), 4.80 (t, 2H, $J=5.7$ Hz, $COOCH_2$), 4.47 (t, 2H, $J=5.7$ z), 3.04 (t, 2H, $J=7.8$ Hz, $CaPy-CH_2$), 2.87 (s, 3H, CH_3 δ -Pyridinium), 2.55 (s, 3H, CH_3 γ -Pyridinium), 2.25 (t, 2H, $J=7.5$ Hz, $COCH_2$), 1.79 (cv, 2H, $J=7.5$ Hz), 1.48 (cv, 2H, $COCH_2CH_2$), 1.25, 1.26 (s, 44H, CH_2 from both of alkyl chains), 0.88 (t, 6H, CH_3 of alkyl chains); ^{13}C NMR (300 M, $CDCl_3$), 173.00 (C=O), 158.63, 158.56, 155.43 (C-2, C-4, C-6 pyridinium), 128.81, 126.90 (C-3 and C-5 pyridium), 60.68($CaPy-CH_2$), 49.94($C\beta Py-CH_2$), 33.76, 33.38, 31.92, 29.68, 29.68, 29.67, 29.67, 29.66, 29.66, 29.65, 29.65, 29.64, 29.60, 29.59, 29.59, 29.58, 29.45, 29.36, 29.35, 29.34, 29.22, 29.19, 29.06, 28.37, 24.64, 22.67, 21.77, 21.63, 14.06, 14.06. HRMS calcd for $C_{38}H_{70}NO_2^+$, $Mr = 572.5406$, found 572.5404.

4, 6-Dimethyl-2-tetradecyl-1-(2-octadecanoylhydroxyethyl) pyridinium hexafluorophosphate 17—Yield: 70.1%; 1H NMR ($CDCl_3$), δ , ppm: 7.43 (s, 1H, H-5 pyridinium), 7.40 (s, 1H, H-3 pyridinium), 4.81 (t, 2H, $J=6.0$ Hz, $COOCH_2$), 4.49 (t, 2H, $J=6.0$ z), 3.06 (t, 2H, $J=7.5$ Hz, $CaPy-CH_2$), 2.89 (s, 3H, CH_3 α -Pyridinium), 2.57 (s, 3H, CH_3 γ -Pyridinium), 2.26 (t, 2H, $J=7.5$ Hz, $COCH_2$), 1.81 (*quint.*, 2H, $CaPy-CH_2CH_2$), 1.49 (*quint.*, 2H, $COCH_2CH_2$), 1.26, 1.27 (s, 50H, CH_2 from both of alkyl chains), 0.88 (t, 6H, CH_3 of alkyl chains); ^{13}C NMR (300 M, $CDCl_3$), 173.00 (C=O), 158.64, 158.55, 155.43 (C-2, C-4, C-6 pyridinium), 128.80, 126.90 (C-3 and C-5 pyridium), 60.68 ($CaPy-CH_2$), 49.95 ($C\beta Py-CH_2$), 33.76, 33.38, 31.92, 31.92, 29.70, 29.69, 29.69, 29.69, 29.68, 29.68, 29.67, 29.66, 29.65, 29.65, 29.64, 29.60, 29.58, 29.45, 29.35, 29.35, 29.34, 29.22, 29.19, 29.06, 28.37, 24.65, 24.65, 22.67, 22.67, 21.77, 21.63, 14.05, 14.05. HRMS calcd for $C_{41}H_{76}NO_2^+$, $Mr = 614.5875$, found 614.5880.

4, 6-dimethyl-2-tetradecyl-1-(2-oleoylhydroxyethyl) pyridinium hexafluorophosphate 18—Yield 69.5%; 1H NMR ($CDCl_3$), δ , ppm: 7.43 (s, 1H, H-5 pyridinium), 7.39 (s, 1H, H-3 pyridinium), 5.33 (*m*, 2H, CH from double bond in oleyl group), 4.80 (t, 2H, $J=6.0$ Hz, $COOCH_2$), 4.47 (t, 2H, $J=6.0$ Hz), 3.05 (t, 2H, $J=7.5$ Hz, $CaPy-CH_2$), 2.87 (s, 3H, CH_3 α -Pyridinium), 2.55 (s, 3H, CH_3 γ -Pyridinium), 2.25 (t, 2H, $J=7.8$ Hz, $COCH_2$), 1.99 (*q*, 4H), 1.79 (*quint.*, 2H, $CaPy-CH_2CH_2$), 1.48 (*quint.*, 2H, $COCH_2CH_2$), 1.26 (s, 44H, CH_2 from both of alkyl chains), 0.88 (t, 6H, CH_3 of alkyl chains); ^{13}C NMR (300 M, $CDCl_3$), 172.99 (C=O), 158.75, 158.62, 155.52 (C-2, C-4, C-6 pyridinium), 130.15, 129.59 ($CH=CH$), 128.76, 126.90 (C-3 and C-5 pyridium), 60.70 ($CaPy-CH_2$), 50.09 ($C\beta Py-CH_2$), 33.75, 33.42, 31.92, 31.90, 29.76, 29.68, 29.68, 29.67, 29.67, 29.65, 29.63, 29.57, 29.51, 29.44, 29.35, 29.34, 29.33, 29.29, 29.20, 29.19, 29.12, 29.07, 29.02, 28.41, 27.25, 27.16, 24.64, 22.66, 21.79, 14.04, 14.04. HRMS calcd for $C_{41}H_{74}NO_2^+$, $Mr = 612.5719$, found 612.5725.

Determination of Lipid Phase Transition Temperature and Melting Point—

Differential Scanning Calorimetry (DSC) studies were carried out to find the phase transition temperature and melting point with a DSC Q2000, TA Instruments (UK). About 5mg of pyridinium lipid or lipid mixture was scanned from 0 to 100 °C at a scan rate of 2°C / min. Data were analyzed using Universal Analysis software provided by TA instruments.

Preparation of Pyridinium Liposomes and Lipoplexes—A pyridinium lipid was dissolved in chloroform with a co-lipid, DOPE or Chol, at the molar ratio of 1:1, 2:1 and 4:1 to reach a final concentration of 1 mg/mL for the charged species. The solvent was removed by rotary evaporation and further dried under vacuum overnight to generate a thin lipid film. The lipid film was hydrated in sterile water at 60°C and vortexed to form cationic liposome suspension. Since lipid molecules form lipid bilayer upon solvent evaporation, no free lipids should appear in the aqueous solution. Therefore, we did not make any attempt to isolate free lipids which may be present in the liposome suspension. The particle size of these liposomes was further reduced by probe sonication (Sonication s-4000, MISONIX) at the amplitude of 60 for 5 min. Pyridinium liposomes were characterized for DNA condensation efficiency, particle size distribution, and ζ -potential as described previously (3). The particle size and ζ -potential of cationic liposomes and lipoplexes were determined at 25°C by dynamic light scattering using Malvern Zetasizer® (Malvern Instruments, UK). Briefly, DNA condensation was determined by 0.6% agarose gel electrophoresis at 10v/cm for 100min in 1×TBE buffer, followed by ethidium bromide (EtBr) staining.

In Vitro Transfection—CHO cells were seeded in 24-well plates, and maintained in 0.5ml of RPMI 1640 medium containing 10% fetal bovine serum (FBS) under 5% CO₂ at 37°C for 24h. The cell number was about 4×10^4 cells per well. GFP or luciferase expression plasmid (pCMS-EGFP or pDNA3-Luc) and cationic liposomes (different formulations) were diluted to 25 μ l by Opti-MEM® I Medium, respectively, and incubated for 5min at room temperature. Lipoplexes were prepared by mixing plasmid and liposomes in equal volume, and incubated for 20min at room temperature to allow complex formation. Immediately before transfection, the media was removed, and cells were washed gently with sterile Phosphate Buffered Saline (PBS). Lipoplexes were added to each well, diluted to 0.5 ml by Opti-MEM® I Medium, and the plate was incubated under 5% CO₂ at 37°C for 6h. Following transfection, the culture medium was replaced with RPMI 1640 medium containing 10% FBS. GFP gene expression was observed under fluorescence microscopy (Olympus, Japan).

For luciferase expression, the cells were collected and lyzed. The cell lysate were measured for luciferase gene expression by Luciferase Assay Kit (Promega, Madison, WI). The total protein concentration was measured by bicinchoninic acid (BCA) Protein Assay Reagent (Pierce, Rockford, IL). Luciferase activity was normalized by the total protein content and expressed as relative light unit (RLU) per μ g of protein (RLU/ μ g protein).

To determine whether the presence of serum in the culture media affects the transfection efficiency of pyridinium lipids, we used RPMI 1640 culture medium containing 0, 5, 10, 15, 20, 30 and 40% FBS for transfection of CHO cells with pyridinium lipid-based lipoplexes and their transfection efficiencies were determined.

To determine the effect of cell types on gene transfer, two more cell lines were used to determine transfection efficiency and cytotoxicity. Hepatocarcinoma cells (CRL 1830) were seeded with 0.5mL of DMEM medium containing 10% FBS under 5% CO₂ at 37°C. Prostate cancer cells (C4-2) cells seeded with RPMI 1640 medium containing 10% FBS under 5% CO₂ at 37°C. The cell number was about 4×10^4 cells per well for 24 well plate for both of them. Luciferase expression and cytotoxicity were measured at the end of the transfection.

Cytotoxicity Assay—The cytotoxicity of lipoplexes at 48h post-transfection was determined using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay in CHO cells. Briefly 5 mg/mL stock solution of MTT in PBS was diluted for 10 times with complete growth medium. Four hours before the end of transfection study, cell culture medium was removed and replaced by this MTT medium to achieve a final concentration of 0.5 mg/mL MTT. The plate was then incubated at 37°C in 5% CO₂ for 4 h. The medium was removed,

blotted dried, and 200 μ l of DMSO was added to each well to dissolve the formazan crystals. Finally the plate was read spectrophotometrically at 560 nm using a microplate reader (Bio-Rad, Hercules, CA). The relative cell viability was calculated as $[\text{Abs}]_{\text{sample}}/[\text{Abs}]_{\text{control}} \times 100\%$.

Statistical Analysis—Data were expressed as the mean $\pm$ standard deviation (SD). The difference between any two groups was determined by ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

Synthesis of Pyridinium Lipids

The goal of this study was to synthesize a series of pyridinium lipids to determine the structure-activity relationship for enhanced gene delivery. All of these cationic lipids contain a heterocyclic pyridinium ring linked to a single alkyl chain fatty acid of different alkyl chain lengths (C14, C15, C16, C18 and C20), types (cis-trans isomers) and branched chain via ester and amide spacers. We synthesized not only the saturated and unsaturated even number carbon chain length but also carbon chain length of 15 (odd number) saturated analog and branched carbon chain saturated analog. Synthesis scheme of pyridinium lipids is shown in Figure 1. Briefly, mesityl oxide was monoacylated to yield pyrylium chloroaluminates, which were converted into pyrylium hexafluorophosphates (**1**) by hexafluorophosphoric acid. Pyrylium hexafluorophosphate was used as a key intermediate for reaction with different primary amines to form a pyridinium heterocyclic ring with different linkers in the final structure. Acylation of these pyridinium salts with different lengths and types of fatty acid chlorides afforded the final pyridinium lipids. Intermediate and final products of these pyridinium lipids were characterized by ^1H NMR, ^{13}C NMR and high-resolution mass spectrometry (HRMS), and these results are included in the methods section.

The structures with minimized energy of these lipids are listed in Table 1. Two side chains are much closer in energy minimized structures of unsaturated lipids than those of saturated lipids, which may facilitate the formation of lipid bilayers in liposomes. The melting points of these lipids are listed in Table 1. More than half of these pyridinium lipids have melting points of more than 60°C. Increase in fatty acid chain length increased the melting point. Saturated lipids have higher melting points than unsaturated lipids except branched one. For unsaturated lipids, melting point of trans-isomer is higher than that of cis-isomer of the same fatty acid chain length. Lipid phase transition temperatures of pure pyridinium lipids and lipid mixture are listed in Table 2. The lipids with amide linker showed lower phase transition temperature compared to lipids with ester linker. Increase in hydrophobic carbon chain length increased the phase transition temperature. At the same carbon chain length the phase transition temperature of trans isomer was higher than that of cis isomer. Phase transition temperature was higher when formulated with cholesterol than that when formulated with DOPE. Higher phase transition temperature makes liposome preparation difficult by rehydration of lipid films and extrusion. Therefore, probe sonication was used to produce small unilamellar vesicles (SUV) at 60°C. Sterile water was used as the suspension medium in cationic liposome preparation so as to prevent cationic liposome aggregation in long term storage in electrolytical buffer.

Preparation and Characterization of Lipoplexes

Transfection efficiency of lipoplexes is known to depend on the particle size and surface charge of liposomes and lipoplexes (26,27). Therefore, we determined the mean particle size, particle size distribution (PSD) and ζ -potential of pyridinium liposomes prepared using different co-lipids and molar ratios of cationic lipid / co-lipid. The mean particle size of these liposomes

was about 97.98 ± 4.31 nm, with the polydispersity index (PDI) of 0.32 ± 0.03 , and ζ -potential was 41.33 ± 4.10 mV (Table 3). This suggests little difference in particle size and ζ -potential among different liposome preparations. Pyridinium liposomes were stable and no aggregation was observed at 4 °C during the experimental period. Lipoplexes were formed by simple mixing pyridinium liposomes and plasmid DNA. The degree of DNA condensation increased with the increase in charge ratio. However, migration of some free DNA was seen when lipoplexes were prepared at 0.5/1 to 2.5/1(+/-). At the charge ratio of 3:1 (+/-) or above plasmid DNA was completely condensed in all the formulations, as evidenced by no band shift upon agarose gel electrophoresis of lipoplexes (data not shown). Upon lipoplex formation, the particle size, polydispersity index and ζ -potential of lipoplexes were determined and found to be 124.33 ± 2.08 nm, 0.17 ± 0.02 and 28.07 ± 5.12 mV, respectively. Particle size distribution of lipoplexes (<200nm) was more than 90% at 0h and more than 80% after 10h. There was no significant change in the particle size, particle size distribution and ζ -potential even at 10h post-incubation at room temperature, suggesting these lipoplexes are fairly stable on storage.

Factors Influencing Transfection Efficiency

The effect of various structure and formulation parameters on in vitro transfection efficiency of pyridinium lipids in CHO cells was examined as a function of linker (ester vs. amide), hydrophobic carbon chain length (14, 16 and 18) and type (single vs. double), configuration of double bond (cis-isomer vs. trans-isomer), co-lipid type (DOPE vs. Chol), cationic lipid/co-lipid molar ratio (1:1, 2:1 and 4:1) and cationic lipid/DNA (+/-) charge (1:1, 3:1 and 5:1) ratio. Lipofectamine is one of the most commonly used transfection reagents and thus we selected this as the positive control for screening of our pyridinium lipids. Our aim was to optimize our own cationic liposome formulations, not the transfection conditions of Lipofectamine. Therefore, we formulated Lipofectamine/plasmid complexes at 3/1 (+/-) charge ratio, which is in the range of the recommended conditions by the manufacturer. Transfection efficiency was reported as relative light units (RLU) per μ g of total protein content.

Charge Ratios—Pyridinium lipid (C16:0, amide linker) was mixed with Chol at the cationic lipid / co-lipid molar ratio of 1:1 to prepare cationic liposomes, and lipoplexes were prepared by mixing with luciferase plasmid at the charge ratio of 1:1, 3:1 and 5:1 (+/-). Luciferase gene expression (Figure 2A) and cytotoxicity (Figure 2B) in CHO cells were determined at 48h post-transfection. Luciferase gene expression levels increased with increase in lipid/DNA charge ratios from 1:1 to 5:1, while cell viability decreased slightly with increase in charge ratio to 5:1(+/-). Compared to Lipofectamine, much higher transfection efficiency was observed at the charge ratios of 3:1 and 5:1(+/-) groups but there was no significant difference between 3:1 and 5:1. Further, there was no significant difference in cytotoxicity between pyridinium liposomes and Lipofectamine.

Type of Linkers—Pyridinium lipids (C16:0 with ester and amide linkers) were used to prepare liposomes with DOPE and Chol at the cationic lipid / co-lipid molar ratio of 1:1. Following transfection of CHO cells with lipoplexes (3:1, +/-), GFP positive CHO cells were visualized under fluorescence microscopy at 48h post-transfection. The cationic lipids with amide linkers significantly increased transfection efficiency in all liposomal formulations in CHO cells compared to their counterparts with ester linker, as evidenced by much higher levels of green fluorescence (Figure 3).

Cationic Lipid:co-lipid Molar Ratios—Pyridinium lipid with C16:0 fatty acid chain and amide linker was used to formulate liposomes with cholesterol at cationic lipid / co-lipid molar ratio of 1:1, 2:1 and 4:1, and lipoplexes were prepared at the charge ratio of 3:1 (+/-). At 48 h post-transfection, luciferase gene expression (Figure 4A) and cytotoxicity (Figure 4B) were measured. Pyridinium liposomes prepared at 1:1 and 2:1 cationic lipid/co-lipid molar ratios

showed higher transfection efficiency compared to 4:1 molar ratio and Lipofectamine. Cell viability slightly decreased when the molar ratio of pyridinium lipid to Chol increased but no difference compared to Lipofectamine. So we decided to select 1:1 cationic lipid/co-lipid molar ratio to prepare liposomes for further studies.

Type of co-lipids—Pyridinium lipids with amide linker but different fatty acid chain (C16:0 and C18:0) were used to prepare liposomes by mixing with DOPE and Chol at the molar ratio of 1:1, and lipoplexes were prepared at the charge ratio of 3:1 (+/-). After 48 h post-transfection, luciferase gene expression (Figure 5A) and cytotoxicity (Figure 5B) were measured. Chol as a co-lipid showed higher transfection efficiency in C16:0 amide lipid, on the contrary DOPE as a co-lipid showed higher transfection efficiency in C18:0 amide lipid. Both of the formulations showed higher transfection efficiency than Lipofectamine/pDNA complexes formulated at 3:1 (+/-) charge ratio. No difference in cytotoxicity between pyridinium liposomes and Lipofectamine.

Alkyl Chain Length—Pyridinium lipids with amide linker and different alkyl chain length (C14:0, C16:0 and C18:0) were used to formulate liposomes with cholesterol at cationic lipid / co-lipid molar ratio of 1:1. At 48h post-transfection of CHO cells with lipoplexes, luciferase level was determined at 3:1 (+/-) charge ratio (Figure 6A). Cytotoxicity data was shown in Figure 6B. Pyridinium lipid with C14:0 and C16:0 showed much higher transfection efficiency compared to C18:0 and also higher than Lipofectamine at 3:1 (+/-) ratio. There was no difference in cytotoxicity compared to Lipofectamine for all the formulations except C14:0 lipid with amide linker, which showed the lowest level of cell viability. We also found that odd number (C15:0) and C18:0 branched carbon chain analogs could not increase the transfection efficiency (data not shown).

Saturation of Alkyl Chain—Pyridinium lipids (C16:0 and C16:1) were used to prepare cationic liposomes using Chol as a colipid at the cationic lipid to colipid molar ratio of 1:1. At 48 h post-transfection of CHO cells with lipoplexes (3:1, +/-), luciferase gene expression (Figure 7A) and cytotoxicity (Figure 7B) were measured. The trans-isomer showed slightly higher transfection efficiency than saturated lipid, and much higher transfection efficiency than its counterpart cis-isomer. There was no significant difference in cell viability between pyridinium lipids and Lipofectamine.

Configuration of Alkyl Chain—Pyridinium lipids with different alkyl chain length (C16:1, C18:1 and C20:1) and different configuration (cis and trans) of double bond were used to formulate cationic liposomes with DOPE at the cationic lipid to colipid molar ratio of 1:1. At 48 h post-transfection of CHO cells with lipoplexes (3:1, +/-), luciferase gene expression (Figure 8A) and cytotoxicity (Figure 8B) were measured. The liposomes formulated with trans configuration lipids showed higher transfection efficiency compared to their counterpart lipids with cis configuration at the same fatty acid chain length. C16:1 trans isomer has much higher transfection efficiency than C18:1 and C20:1 trans isomers at the same type of formulations. There was no significant difference in cell viability between all formulations and Lipofectamine.

Influence of Serum on Gene Transfer—Since serum is known to interfere with the transfection of lipoplexes, we transfected CHO cells with the lipoplexes prepared using two most potent pyridinium lipid-based liposome formulations {C16:0, amide: cholesterol at 1:1 molar ratio (5CH1) and C16:1, amide, trans: DOPE at 1:1 molar ratio (7D1)} in the presence of serum (0, 5, 10, 15, 20, 30 and 40%). As shown in Figure 9, the formulation 7D1 showed significantly higher transfection efficiency compared to the formulation 5CH1 and Lipofectamine (formulated at 3:1, +/- charge ratio) at each serum concentration. The

transfection efficiencies were decreased with increase in serum concentration. At the serum concentration of 30% and higher, 5CH1 and Lipofectamine lost their transfection ability when formulated at 3/1 (+/-) charge ratio, while 7D1 still kept high transfection efficiency. The luciferase gene expression in 7D1 treated cells at the serum concentration of 40% showed similar level compared to those of 5CH1 and Lipofectamine in the serum free medium.

Different Cell Lines—Two pyridinium lipid formulations (5CH1 and 7D1) based on cationic /co-lipid molar ratio of 1:1 and complex formation at 3:1 (+/-) charge ratio were tested in two additional cell lines, CRL 1830 and C4-2. At 48 h post-transfection, luciferase gene expression and cytotoxicity in CRL 1830 (Figure 10) and C4-2 (Figure 11) were measured. Both formulations worked very well in these two cell lines as evidence by more than 5 –10 times of luciferase level compared to Lipofectamine when formulated at 3/1 (+/-) charge ratio. There was no significant difference in cell viability between all formulations and Lipofectamine. These results suggest that the order of magnitude to which pyridinium lipid based formulations exceeds Lipofectamine 3/1- (+/-) formulation is cell type dependent. Nevertheless, in all cases, much higher transfection efficiency was obtained when transfection was carried out using pyridinium lipid with C16 fatty acid chain and amide linker.

DISCUSSION

The structural and formulation factors, such as lipid/DNA ratio, co-lipid type, and cationic lipid/co-lipid molar ratio have been shown to influence gene transfer efficiency both in vitro and in vivo (8,26,28). Pyridinium lipids have been shown to display higher transfection efficiency in cells that are not easily transfected by other cationic lipids, such as lipofectin, and transfect relatively a large number of cells (21). Therefore, here we report the effect of these parameters on the transfection efficiency of a series of pyridinium lipids.

The lower phase transition temperature makes the lipid molecule movement (lateral or flip-flop movement) easier within the liposomal bilayer compared to higher phase transition temperature. Generally, the phase transition temperature is influenced by the lipid structure and the liposome composition. In our study, some of newly synthesized pyridinium lipids have the higher phase transition temperature, especially for the lipids with ester linker, compared to commonly used lipids. The longer the fatty acid chain length the higher the phase transition temperature is; the higher the degree of saturation the higher the phase transition temperature is; and the colipid will lower the phase transition temperature (Table 2). The high phase transition temperature makes liposome preparation difficult by extrusion even at 65°C. It needs much higher energy to allow the lipid molecules to move and disperse into aqueous solution to form bilayer structures. Therefore, we chose probe sonication method to prepare liposome suspension. Also, the higher phase transition temperature may cause phase inversion difficult, further influence membrane transport of lipids and gene transfer.

In general, transfection efficiency increases with increase in the charge ratio of lipoplexes until it becomes too toxic to the cells. To determine the structure-activity relationship of pyridinium lipid, we first determined the effect of cationic lipid / plasmid charge ratio on the transfection efficiency to find the best charge ratio for further evaluations. Based on our transfection data, lipoplexes prepared at the charge ratio of 3:1 (+/-) showed much higher transfection efficiency compared to 1:1 (+/-), but no significant difference compared to 5:1(+/-). There was no significant difference between pyridinium lipids and Lipofectamine in cytotoxicity. Therefore, the charge ratio was fixed to 3:1 (+/-) in all the following experiments.

Melting point, phase transition temperature, biodegradability and transfection efficiency are known to depend on the structure of the linker that binds to the polar headgroup to the hydrophobic lipid chains (5). Therefore, we determined the effect of amide and ester linkers

on transfection efficiency and cytotoxicity. We used these linkers because they are biodegradable and thus could reduce the cytotoxicity of pyridinium lipids (25). As shown in Figure 3, the lipid with amide linker showed higher transfection efficiency compared to their counterpart with the ester linker regardless of the fatty acid chain length. The high transfection efficiency of amide linker lipids may be caused by their lower phase transition temperature (Table 2) which makes liposomal bilayer structure more stable in aqueous solution during transfection process and storage.

The molar ratio of a cationic lipid to co-lipid is known to affect the phase transition temperature of cationic liposomes and the structure of lipoplexes. Therefore, we prepared cationic liposomes using pyridinium lipid and cholesterol at the molar ratio of 1:1, 2:1 and 4:1, as cholesterol has been reported to be superior to DOPE for pyridinium lipids (23). Luciferase gene expression (Figure 4A) and cytotoxicity (Figure 4B) were influenced by the molar ratio of pyridinium lipid to Chol, with the highest transfection efficiency for the liposomes prepared at 1:1 cationic lipid/co-lipid molar ratio. Increase in the amount of Chol slightly decreased cytotoxicity of pyridinium liposomes. So we fixed cationic lipid / co-lipid molar ratio to 1:1 in other experiments. These results are in good agreements with the work of Illies et al. (23).

DOPE and Chol are the two most commonly used co-lipids in cationic liposome preparation. The incorporation of fusogenic lipids such as DOPE improves the endosomal release by membrane fusion between the liposomal and endosomal bilayers (29,30). DOPE has been shown to be a major driving force in promoting transfection efficiency of SAINT-2 cationic lipids, as it gets transformed from its lamellar phase in water to the hexagonal phase when dispersed in a physiological salt solution (31). The use of Chol as a co-lipid has been reported to show enhanced in vivo gene expression, as Chol-containing liposomes efficiently interact with the cell membrane in the presence of serum (32). Based on our data, the use of both DOPE and Chol as co-lipids showed higher transfection efficiency of pyridinium lipids compared to Lipofectamine when lipoplexes were prepared at 3/1 (+/-) charge ratio. The selection of co-lipid was dependent on the type of cationic lipids.

The hydrophobic chain length influences the shape of amphiphiles and supramolecular associations generated through the self-assembling process (25). Therefore, we synthesized a series of pyridinium lipids with different length of fatty acids to determine the influence of alkyl chain length on gene transfer. Pyridinium lipids with C14:0 and C16:0 fatty acid and amide linker formulated with Chol showed higher transfection efficiency compared to C18:0 lipid with the same linker (Figure 6A). Since C14:0 was more toxic than C16:0 but had similar transfection efficiency, we only synthesized C16:1, C18:1 and C20:1 for further evaluation. As shown in Figure 8A, C16:1 showed higher transfection efficiency compared to C18:1 and C20:1. Illies et al. (2006) reported similar results (25). Van der Woude et al. (21) also reported significant reduction in transfection efficiency and cytotoxicity with elongation of the alkyl chain length to C18:0 (SAINT-5) relative to that of C16:0 (SAINT-1). Srilakshmi et al. (33) demonstrated that the transfection efficiency of cationic lipids was anchor-dependent when they studied the cationic lipids containing hydrophobic anchor chain ranging from C12 to C18. In their study, the lipid containing 16-carbon chain showed better transfection efficiency among these compounds, while lipids containing less than 14-carbon anchor chain did not show any transfection, indicating that a minimum of 14-carbon anchor length is essential for imparting transfection properties to their cationic lipids (34). Cationic lipids containing less than 14-carbon anchor chain do not form stable lipoplexes that are required for efficient cellular uptake of plasmid DNA. Non-condensed plasmid DNA present in the lipoplexes either gets degraded by the serum proteins and/or enters into a nonproductive pathway (34). On the contrary, the carbon anchor chain will promote strong interaction between DNA and cationic lipid by hydrophobic strength besides electrostatic interaction, which will decrease the release of DNA from cationic liposomes, leading to further decrease in gene expression (22). A balance

between the stability of the liposome in the medium and endosomal DNA release into the cytoplasm is important for efficient transfection efficiency (35).

The degree of un-saturation and configuration of hydrophobic anchor chain can affect the transfection efficiency and cytotoxicity (21). The degree of un-saturation in lipid structure affects the fusogenicity of the lipid (L_{α} / H_{II} transition) (36,37). Increase in the chain length of saturated hydrophobic anchors increases the phase transition temperature: 45.5, 63.5 and 73.4 for C14:0, C16:0 and C18:0 of pyridinium lipids with ester linkages; and 38.9, 39.3 and 45.4 for C14:0, C16:0 and C18:0 of pyridinium lipids with amide linkers (Table 2). The increase in phase transition temperature decreases fusogenicity, leading to decrease in transfection efficiency (38). Double bond in the fatty acid carbon chain will also decrease the melting point (Table 1). To investigate the relevance of cis versus trans orientation, we synthesized new lipids with one double bond (cis or trans) in the hydrophobic carbon chain. As apparent in Figure 7A and Figure 8A trans-orientation (C16:1, C18:1 and C20:1) enhances the transfection efficiency compared to cis-oriented alkyl chains of the same length. This trend is in good agreement with the previous report by Van der Woude et al. (21). Based on our results shown in Figure 5A, Figure 7A and Figure 8A, we conclude that the trans-isomer worked better than cis and saturated lipids. Since the position and number of double bonds in these unsaturated fatty acid chains may also influence the transfection efficiency of these pyridinium lipids, we plan to synthesize some new compounds to determine the effect of position but number of double bonds in the fatty acid chain on transfection efficiency and cytotoxicity.

The stability of the lipoplexes in the presence of serum is important for their in vivo efficacies (39,40). Increase in serum concentration significantly decreased the transfection efficiency (Figure 9). Serum contains nucleases and other enzymes, which would degrade plasmid DNA, and also contains proteins, such as albumin, which would inhibit the cellular association of the lipoplex (41). However, our lipid trans-isomer C16:1 showed excellent performance even at the serum concentration of 30% and above which Lipofectamine totally lost its transfection ability when lipoplexes were formulated at 3/1 (+/-) charge ratio. This finding suggests that C16:1 trans-isomer-based liposomes can be used for in vivo gene delivery. Gopal et al. (22) also demonstrated good transfection efficiency of glutamide-containing pyridinium lipids in the presence of serum.

A major issue of interest is obviously the understanding mechanism as to why pyridinium lipid could improve transfection efficiency. Positively charged pyridinium headgroup plays an important role in improving the transfection efficiency of pyridinium lipids. The antimicrobial activity of alkyl pyridinium salts has been shown to be associated with the cell leakage, and increased with increase in the number of pyridinium headgroups (42). Multiheaded pyridinium amphiphiles have been shown to be more active as compared to their trimethylammonium counterparts (43). Woude et al. reported that alkyl pyridinium cationic lipid (SAINT-2) could increase the membrane permeability although this effect is not high to assure the lower cytotoxicity compared to DOTMA (21). Tucker et al. reported that incubation of cells with alkyl pyridinium sponge toxins could make transient pores on the cell membrane to facilitate cDNA transmembrane movement (44). Transient permeabilization of cells with pyridinium cationic lipids was further proven by the evidence of more efficient tissue penetration of pyridinium-based lipoplexes compared to those formed by DOTAP in vivo (24). The pore on the cell surface membrane resulted in increased intracellular Ca^{2+} , collapsed membrane potentials and reduced input resistance (44,45). DOSPA is a polycation which is the component of Lipofectamine. It has been reported that polycation could form positively charged loops fixed on DNA molecules. These loops interact with negatively charged lipids on plasma membranes, which probably induce effective non-specific endocytosis and subsequent cell transformation (46). Pore-forming ability combined with ionic interaction, membrane

association and endocytosis may explain why some of our newly synthesized pyridinium cationic lipids showed relatively higher transfection efficiency compared to Lipofectamine.

In conclusion, we have demonstrated that a number of structural and formulation parameters including hydrophobic carbon chain lengths, double bond configuration in the unsaturated aliphatic hydrocarbon tails, lipid / colipid ratios, lipid / DNA charge ratios and cell types play important roles in gene transfer and cytotoxicity of these pyridinium lipids. Important findings include: (a) higher transfection efficiencies of pyridinium lipids with the amide linkers compared to their ester counterparts; (b) higher transfection efficiency at 1:1 molar ratio of pyridinium lipid and co-lipid compared to 2:1 and 4:1; (c) efficient transfection with C16 chain length pyridinium lipids, (d) better transfection efficiencies in the presence and absence of serum, compared to that of Lipofectamine, a widely used commercially available liposomal transfection kit, (e) higher transfection efficiencies of pyridinium lipids with the trans orientation in the hydrophobic anchor chain compared to those with cis orientation; and (f) C16:1 trans-isomer pyridinium lipids were more efficient in transfection efficiency in the presence of high concentration of serum compared with Lipofectamine when lipoplexes were prepared at 3/1 (+/-) charge ratio.

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Figure 1.
Synthesis scheme of pyridinium lipids.

Figure 2.

Effect of charge ratios on transfection efficiency (A) and cytotoxicity (B) at 48 h after transfection of lipoplexes. Pyridinium lipid **5** (C16:0, amide linker, trans-isomer) was mixed with cholesterol (Chol) at the cationic lipid / co-lipid ratio of 1:1 to prepare cationic liposomes, and lipoplexes were prepared by mixing with luciferase plasmid at the charge ratio of 1:1, 3:1 and 5:1 (+/-). Luciferase gene expression and cytotoxicity in CHO cells were determined after 48h post-transfection. The dose of pcDNA3-Luc plasmid was 0.2 μ g /well for 4 $\times$ 10⁴ cells.

Figure 3.

Comparison of gene expression between amide and ester linker. Lipid **5** (C16:0, amide linker) and lipid **16** (C16:0, ester linker) was used to prepare cationic liposomes with co-lipid DOPE and cholesterol at the molar ratio of 1:1. Lipoplexes were formed by mixing with luciferase plasmid at the charge ratio of 3:1 (+/-). GFP gene expression was observed at 48 h after transfection under fluorescence microscopy and normal light as control. The dose of pCMS-EGFP plasmid was 0.4 μ g /well for 4×10^4 cells.

Figure 4.

Effect of cationic lipid /co-lipid molar ratios on transfection efficiency (A), cytotoxicity (B). Lipid **5** (C16:0, amide linker) was used to prepare cationic liposomes with co-lipid cholesterol (Chol) at the molar ratio of 1:1, 2:1 and 4:1. Lipoplexes were formed by mixing with luciferase plasmid at the charge ratio of 3:1 (+/-). Luciferase gene expression and cytotoxicity in CHO cells were determined at 48h after transfection. The dose of pcDNA3-Luc plasmid was 0.2 μ g / well for 4×10^4 cells.

Figure 5.

Effect of co-lipid type on transfection efficiency (A) and cytotoxicity (B). Lipids **5** (C16:0, amide linker) and **8** (C18:0, amide linker) were used to prepare cationic liposomes with co-lipid DOPE and cholesterol (Chol) at the molar ratio of 1:1. Lipoplexes were formed by mixing with luciferase plasmid at the charge ratio of 3:1 (+/-). Luciferase gene expression and cytotoxicity in CHO cells were determined at 48h after transfection. The dose of pcDNA3-Luc plasmid was 0.2μg /well for 4×10^4 cells.

Figure 6.

Effect of fatty acid chain length on transfection efficiency (A) and cytotoxicity (B). Lipids **4** (C14:0, amide linker), **5** (C16:0, amide linker) and **8** (C18:0, amide linker) were used to prepare cationic liposomes with co-lipid cholesterol (Chol) at the molar ratio of 1:1. Lipoplexes were formed by mixing with luciferase plasmid at 3:1(+/-) charge ratio. Luciferase gene expression and cytotoxicity in CHO cells were determined at 48h after transfection. The dose of pcDNA3-Luc plasmid was 0.2μg /well for 4×10^4 cells.

Figure 7.

Effect of degree of saturation on transfection efficiency (A) and cytotoxicity (B). Lipids **5** (C16:0, amide linker), **6** (C16:1, amide linker, cis-isomer) and **7** (C16:1, amide linker, trans-isomer) were used to prepare cationic liposomes with co-lipid cholesterol (Chol) at the molar ratio of 1:1. Lipoplexes were formed by mixing with luciferase plasmid at the charge ratio of 3:1(+/-). Luciferase gene expression and cytotoxicity in CHO cells were determined at 48h after transfection. The dose of pcDNA3-Luc plasmid was 0.2 μ g /well for 4×10^4 cells.

Figure 8.

Effect of alkyl chain configuration on transfection efficiency (A) and cytotoxicity (B). Lipids **6** (C16:1, amide linker, cis-isomer), **7** (C16:1, amide linker, trans-isomer), **9** (C18:1, amide linker, cis-isomer), **10** (C18:1, amide linker, trans-isomer), **11** (C20:1, amide linker, cis-isomer) and **12** (C20:1, amide linker, trans-isomer) were used to prepare cationic liposomes with co-lipid DOPE at the molar ratio of 1:1. Lipoplexes were formed by mixing with luciferase plasmid at the charge ratio of 3:1(+/-). Luciferase gene expression and cytotoxicity in CHO cells were determined at 48h after transfection. The dose of pcDNA3-Luc plasmid was 0.2μg /well for 4×10^4 cells.

Figure 9.

Influence of serum on gene transfer. Lipids **5** (C16:0, amide linker) and **7** (C16:1, amide linker, trans-isomer) were used to prepare cationic liposomes with co-lipid DOPE and cholesterol at the molar ratio of 1:1. Lipoplexes were formed by mixing with luciferase plasmid at the charge ratio of 3:1(+/-). Luciferase gene expression and cytotoxicity in CHO cells were determined at 48h after transfection. The dose of pcDNA3-Luc plasmid was 0.2µg /well for 4×10^4 cells.

Figure 10.

Transfection efficiency (**A**) and cytotoxicity (**B**) in hepatocarcinoma cells. Lipids **5** (C16:0, amide linker) and **7** (C16:1, amide linker, trans-isomer) were used to prepare cationic liposomes with co-lipid DOPE and cholesterol at the molar ratio of 1:1. Lipoplexes were formed by mixing with luciferase plasmid at the charge ratio of 3:1(+/-). Luciferase gene expression and cytotoxicity in CHO cells were determined at 48h after transfection. The dose of pcDNA3-Luc plasmid was 0.2μg /well for 4×10^4 cells.

Figure 11.

Transfection efficiency (**A**) and cytotoxicity (**B**) in prostate cancer cells. Lipids **5** (C16:0, amide linker) and **7** (C16:1, amide linker, trans-isomer) were used to prepare cationic liposomes with co-lipid DOPE and cholesterol at the molar ratio of 1:1. Lipoplexes were formed by mixing with luciferase plasmid at the charge ratio of 3:1(+/-). Luciferase gene expression and cytotoxicity in CHO cells were determined at 48h after transfection. The dose of pcDNA3-Luc plasmid was 0.2 μg /well for 4×10^4 cells.

Table 1
Minimized Structures and Melting Points of Pyridinium Lipids

Compound ID	Amide	M.P.	Compound ID	Ester	M.P.
4		73.6 °C	14		72.3 °C
5		80.7 °C	15		81.7 °C
8		83.8 °C	16		77.1 °C
13		-16.3 °C	17		74.4 °C

Compound ID	Amide	M.P.	Compound ID	Ester	M.P.
6		36.8 °C			
7		63.5 °C			

Compound ID	Amide	M.P.	Compound ID	Ester	M.P.
9		50 °C	18		48.7°C

Compound ID	Amide	M.P.	Compound ID	Ester	M.P.
10		64.8 °C			

Compound ID	Amide	M.P.	Compound ID	Ester	M.P.
11		50.4 °C			

Compound ID	Amide	M.P.	Compound ID	Ester	M.P.
12		69.4 °C			

Compound ID	Amide	M.P.	Compound ID	Ester	M.P.

Table 2

Determination of Lipid Phase Transition Temperature (°C) By Differential Scanning Calorimetry (DSC)

Type of hydrophobic anchor chain	Linker		Lipid with amide linker/Co-lipid (molar ratio 1:1)	
	Ester	Amide	DOPE	CHOL
C14:0	45.5–47.5	38.9–44.9	18.2–21.4	32.9–35.2
C16:0	63.5–66.1	39.3–41.4	37.8–40.0	40.2–42.1
C16:1, cis		29.7–31.1		
C16:1, trans		35.7–36.7		
C18:0	73.4–74.1	45.4–48.7	24.2–26.3	42.1–46.5
C18:1, cis		31.4–32.1		
C18:1, trans		34.1–37.9		
C20:1, cis		36.7–39.6		
C20:1, trans		37.4–41.3		

Values are “onset to peak temperature”.

N/A: no significant phase transition temperature was observed.

Table 3Particle Size and ζ -potential of Pyridinium Cationic Liposome and Lipoplexes

	Pyridinium cationic liposomes	Lipoplexes (0h)	Lipoplexes (10h)
Particle size (nm)	97.98 + 4.31	124.33 + 2.08	128.33 + 1.53
PDI	0.32 + 0.03	0.17 + 0.02	0.15 + 0.02
PSD (<200nm)	>95%	>90%	>80%
ζ -potential (mV)	41.33 + 4.10	28.07 + 5.12	31.17 + 5.50

PDI: polydispersion index, PSD: particle size distribution

Values are mean $\pm$ SD