

Synthesis and Evaluation of Cationic Lipids Bearing Cholesteryl Groups for Gene Delivery In Vitro

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A series of cationic lipids were designed and synthesized as vectors for gene delivery. The lipids contain a ketal moiety as a linker and a cholesteryl group as a hydrophobic tail. The framework of cholesteryl derivatives could increase the stability of liposomes by stabilizing the bilayers and their complexes with DNA to improve the transfection efficiency. The ketal bonds in lipids should easily degrade in an acidic environment in a cell (pH = 2–5) after transfection, resulting in little toxicity; in the neutral environment outside of cells (pH = 7), they should be stable as gene carriers. Gene transfer experiments in vitro with BL-6, 3LL, 293, 3T3, and Hela cells were performed. The results show that the gene transfection activity of three lipids is quite high, with least toxicity to cells under the experimental conditions.

With the rapid developments in the human genome sequencing project and the discovery of disease genes, gene therapy will be one of the major methods to cure diseases in the future. However, a great deal of progress still needs to be achieved for this to be possible. One of the key points is to prepare appropriate vectors for gene delivery.[1,2] Ideally, a gene vector should have the following properties: 1) it should protect the gene drug from inactivation by body fluids during travel from the site of administration to the site of action; 2) it should deliver the gene drug into the target cells with high efficiency; 3) it should be safe and easy to produce on a large scale; and 4) it should deliver the gene to the site where treatment is needed.[3]

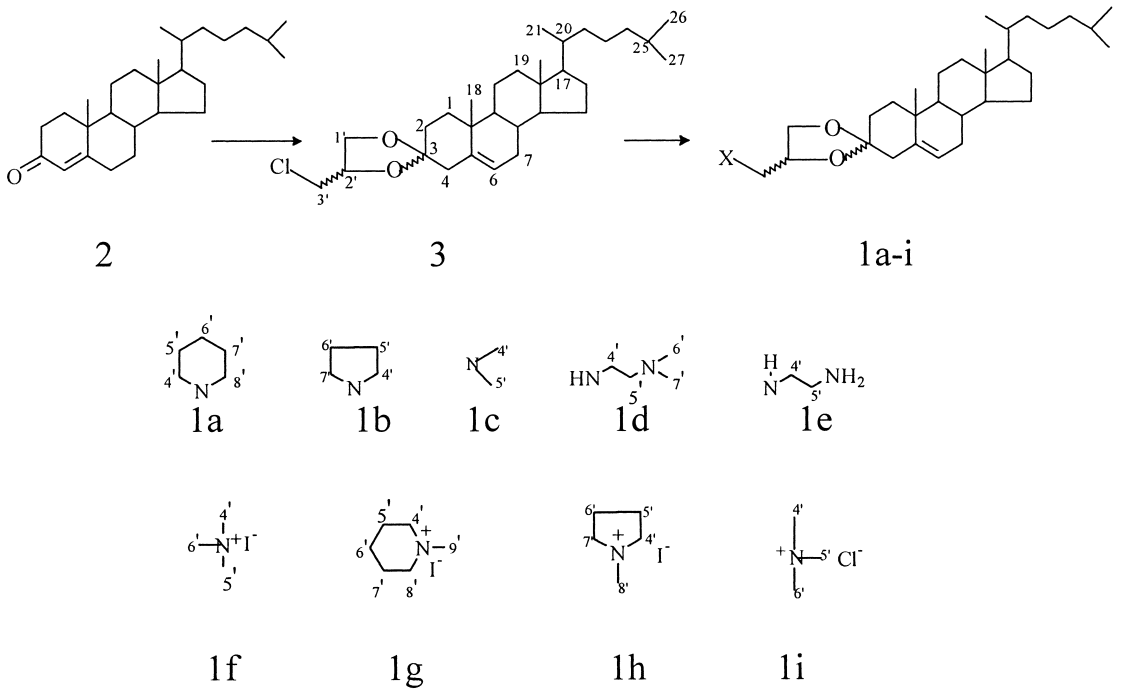
One of the vector systems developed during the past few years having great potential to satisfy these requirements is the cationic lipid-based delivery system.[4,5] Since the initial use of a such vector system in 1987 by Felgner and his colleagues,[6] many new cationic lipids have been synthesized, such as DOTMA[2,3-bis(oleoyloxy)propyl]trimethylammonium chloride analogues, complex alkylamine/alkanecarboxamides, cholesterol derivatives, and aromatic ring-based derivatives.[2,7] Several groups have been working on the establishment of a structure-function relationship for a cationic-based gene delivery system.[8–15] Some cationic lipids have been used in clinical trials for treating cancer[16–19] and cystic fibrosis.[20,21]

Cholesterol derivatives can increase the stability of liposomes by stabilizing the bilayers and their complexes with DNA to improve the transfection efficiency.[22] To date, two of the most efficacious cytofectins that have been reported are cholesterol derivatives: polyamine lipid 67[9] and CTAP.[23] From the standpoint of gene delivery, a good vector should be stable outside of cells and degradable after entering them. Most of the previously reported lipids use an ether or ester bond as a linker bond. However, the ether bond as a linkage is too stable in vivo, and may cause cytotoxicity. On the other hand, the ester bond is unstable under biological conditions.[24] Considering the acidic environment in a cell (pH = 2–5), the ketals should be easy to break up, resulting in little toxicity, while in the neutral environment outside of cells (pH = 7), they should be stable as gene carriers. We have designed and synthesized a series of novel cationic lipids **1a–h** with cholesteryl groups as a hydrophobic tail and ketal as a linker bond. The preparations of **1a–h** are outlined as follows (Scheme 1).

Ketalization of 3-chloro-1,2-propanediol with cholest-4-en-3-one **2** gave cholest-5-en-3-one 3- chloropropylene acetal **3**. The reaction of **3** with piperidine, pyrrolidine, dimethylamine, (asym)*N*,*N*-dimethylethylenediamine, ethylenediamine, respectively, gave **1a–e**. The reaction of **3** with trimethylamine at 120 °C for 24 h in an autoclave, or 80 °C for 7 d gave **1c**. The reaction of **1a–c** with iodomethane in $CHCl_3$/DMSO, respectively, gave **1f–h**.

Results

Transfection Activity. Four of the synthesized compounds (**1a**, **1b**, **1c**, **1f**,) were used to evaluate the activity in gene delivery. Standard transfection procedures were followed with BL-6 cells (murine melanoma). The data in Fig. 1A show that the transfection activity of these new cationic lipids is dependent on the cationic lipid to the DNA ratio. Using 1 μg of plasmid DNA per well, the transfection activities of **1b**, **1c**, and **1f** are comparable to that of DC-Chol.[25] The optimal transfection activity of these lipids follows the trend tertiary amine **1c** > quaternary amine **1f**. Meanwhile, tertiary amines, **1b** and **1c**, showed a constant activity in a broader range of cationic lipid to the DNA ratio, while DC-Chol, as well as our cationic lipid **1f** exhibited roughly bell-shaped lipid dose-response curves. The peak activity for **1b** and **1c** was seen at a ratio of 5:1 (nmol:μg), compared to that of **1f**, which exhibited the peak level at a ratio of 7.5:1 (nmol:μg). Compound **1a** did not exhibit any transfection activity under the experimental conditions. The data in Fig. 1B show the total amount of pro-



Scheme 1.

tein recovered from the cells. Protein recovery is commonly used as an indicator for toxicity.[26] It is evidenced from Fig. 1B that cationic lipids **1b**, **1c**, and **1f** appear to be the least toxic to cells under the experimental conditions.

Transfection Activity in Different Cell Lines. To determine whether these new cationic lipids are capable of transfecting other types of cells, four additional cell lines were selected to test the transfection activity of the cationic lipids. Because **1a** did not exhibit any transfection activity, we excluded its test in further experimentation. 3LL (lewis lung carcinoma), NIH3T3 (murine embryonic fibroblast), Hela (human cervical adenocarcinoma), and 293 (human emvryonic kidney) cells were transfected using the same lipid formations as those used in the experiments summarized in Fig. 1. In 3LL cells, the optimal transfection activity of these lipids showed **1c** > **1b** > **1f**. The peak activity for **1b**, **1c**, and **1f** was seen at a ratio of 5:1 (nmol:μg). Like in BL-6 cells, **1c** and **1b** showed a constant activity over a broader range for the cationic lipid to DNA ratio, while **1f** exhibited a roughly bell-shaped lipid dose response curve (Fig. 2A). The total proteins recovered from each well decreased with an increase in the amount of cationic lipid. Lipid **1f** appears to be the most toxic to cells, followed by that **1c** and **1b** appear to be the least toxic to cells (Fig. 2B). In 293 cells, the optimal transfection activity of these lipids showed **1c** > **1f** > **1b**. The peak activity for **1c** was seen at a ratio of 2.5:1 (nmol:μg), while **1b** and **1f** at 5:1 (nmol:μg). All three lipids showed a constant activity over a broader range for the cationic lipid to DNA ratio (Fig. 3A). The total proteins recovered from each well were very low (Fig. 3B). In 3T3 cells, the optimal transfecton activity of these lipids showed **1c** > **1f** > **1b**. The peak activity for **1c** and **1f** was seen at a ratio of 7.5:1 (nmol:μg). **1c** and **1b** showed a constant activity with an increase in the amount of cationic lipid up to 7.5 nmol (Fig. 4A). The total proteins recovered from each well showed that these lipids appear to be the least toxic to cells; they did not decrease along with an increase in the amount of cationic lipids (Fig. 4B). In Hela cells, the optimal transfection activity of these lipids showed **1c** > **1f** > **1b**. The peak activity for **1c** and **1b** was seen at a ratio of 5:1 (nmol:μg), while **1f** at 7.5:1 (nmol:μg). All three lipids showed a constant activity over a broader range for the cationic lipid to DNA ratio (Fig. 5A). The total protein recovery from each well decreased along with an increase in the amount of cationic lipids. **1c** appears to be the most toxic to cells (Fig. 5B).

Discussion

In the synthesis of the cationic lipids, demethylation is worth mentioning. The demethylation of *N*-methyl quaternary ammonium compounds via S_N2 displacement by Br^- or I^- in an alcohol solvent has been reported previously.[27–30] In this work, compound **3** and trimethylamine in methanol were heated at 120 °C for 24 h; alternatively, at 80 °C for 7 d, product **1c** was obtained. It was rationalized that the quaternary *N*,*N*,*N*-trimethyl ammonium salt was formed first. The ammonium salt is a typical surfactant which aggregates to form a micelle in methanol. One of the methyls in the ammonium salt was attacked by halide ions in a methanol solution, and the amine moiety was eliminated as a leaving group by a S_N2 substitution mechanism.

Since the first cationic lipid DOTMA was employed as a gene vector in 1987, many cationic lipids have been synthesized and shown to be active in vitro and some in vivo. Most of these reported lipids had an ether or ester bond as a linker bond. Compared to the structure of these preexisting cationic lipids, cationic lipids designed and synthesized in present work have a unique structure of ketal as a linker bond, which is bio-

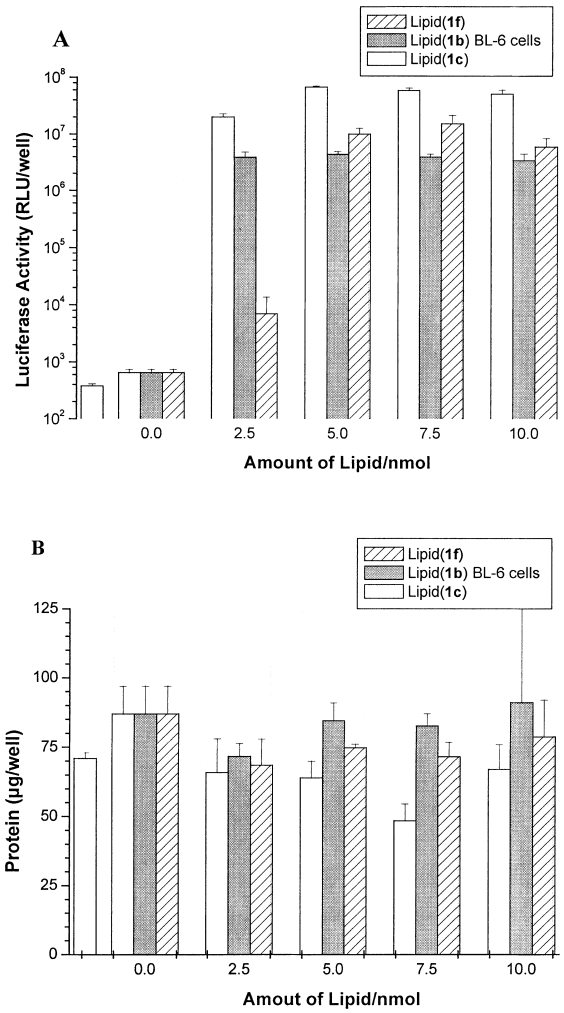


Fig. 1. Effect of cationic Lipid to DNA ratio on the transfection activity. Cells (BL-6) were transfected with 1 μg of PCMV Luc plamid DNA various amounts of cationic lipids. The level of luciferase gene expression (A) and total protein recovery (B) in cells transfected with cationic lipids **1b**, **1c**, and **1f** at 2.5, 5.0, 7.5, and 10.0 nmol/well, respectively. Data represent mean ± SD (n = 3).

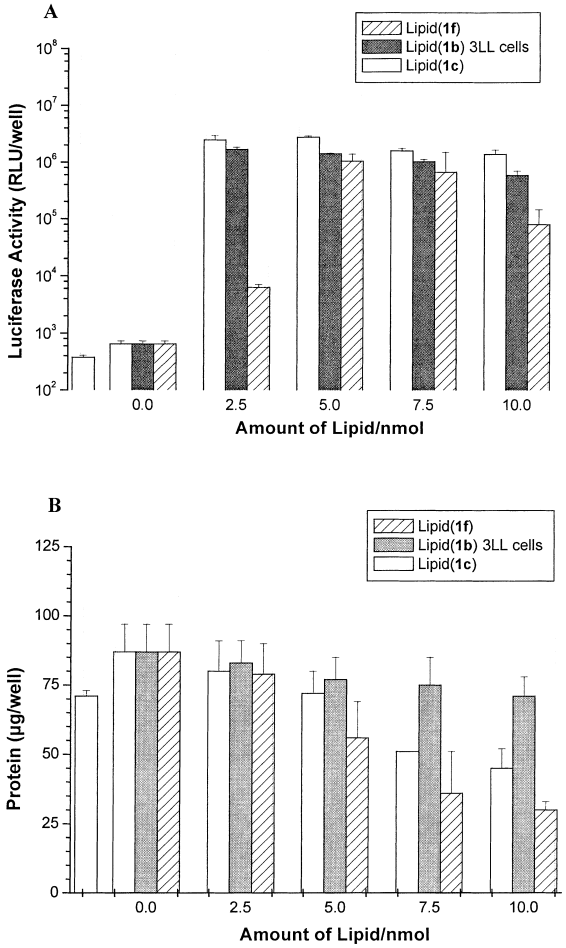


Fig. 2. Effect of cationic Lipid to DNA ratio on the transfection activity. Cells (3LL) were transfected with 1 μg of PCMV Luc plamid DNA various amounts of cationic lipids. The level of luciferase gene expression (A) and total protein recovery (B) in cells transfected with cationic lipids **1b**, **1c**, and **1f** at 2.5, 5.0, 7.5, and 10.0 nmol/well, respectively.

degradable.[31] As a gene vector they are stable outside of cells and are degradable after entering cells. An in vitro experiment verified that they had good transfection activity and low toxicity. The head group of a cationic lipid is also important for a gene vector. In vitro transfection showed that three (**1b**, **1c**, **1f**) of the tested four lipids bearing a cholesteryl group as a hydrophobic tail and a ketal as a linker bond exhibit good transfection activity, while **1a** does not exhibit any transfection activity. This may be due to a steric effect. Piperidine does not effectively bind with DNA, but pyrrolidine does well. Tertiary amine **1c** gave a higher transfection activity than that of quaternary amines **1f**. This observation is consistent with results observed by other groups.[32] Although the luciferase activity obtained in five cells tested implies that different cell lines have different sensitivity in responding to a cationic lipid-based transfection reagent, our cationic lipids, especially **1c**, have good transfection activity in any cell, and showed a constant activity over a broader range of the cationic lipid to DNA ratio.

In conclusion, a series of novel cationic lipids bearing a ketal as a linker bond non-viral vector were designed and synthesized for gene delivery. Transfection results suggest that they are useful transfection reagents for in vitro gene transfer.

Experimental

Cholesterol was purified by recrystallization in binary solvents of $CHCl_3$/MeOH. All other reagents were purified by distillation. 3-Chloro-1,2-propanediol was used a non-chiral reagent. Each of these compounds, **1a–h** and **3**, is a mixture of diastereoisomers. The melting points were determined on a Yanaco melting apparatus and not corrected. ^{1}H NMR was recorded with a Bruker DMX-500 spectrometer using $CDCl_3$ as a solvent; the chemical shifts were given in ppm downfield from TMS. IR spectra were recorded on a Bruker Vector 220 Infrared Spectrometer using a KBr pellet. An elemental analysis was performed on a Perkin-Elmer 240C analytical instrument. MS were recorded with a Hewlett Packard HP 5988A instrument (70 eV).

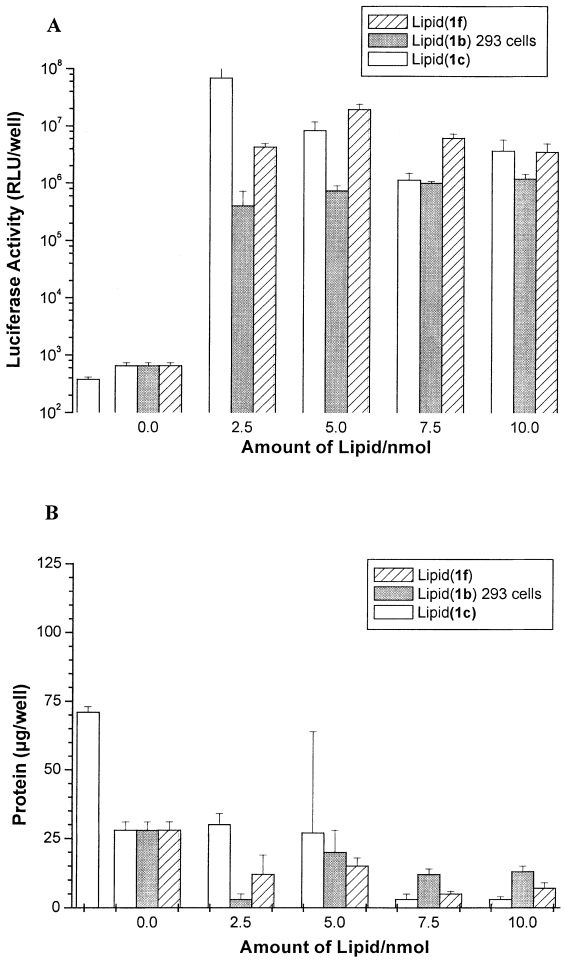


Fig. 3. Effect of cationic Lipid to DNA ratio on the transfection activity. Cells (293) were transfected with 1 μg of PCMV Luc plamid DNA various amounts of cationic lipids. The level of luciferase gene expression (A) and total protein recovery (B) in cells transfected with cationic lipids **1b**, **1c** and **1f**, at 2.5, 5.0, 7.5, and 10.0 nmol/well, respectively.

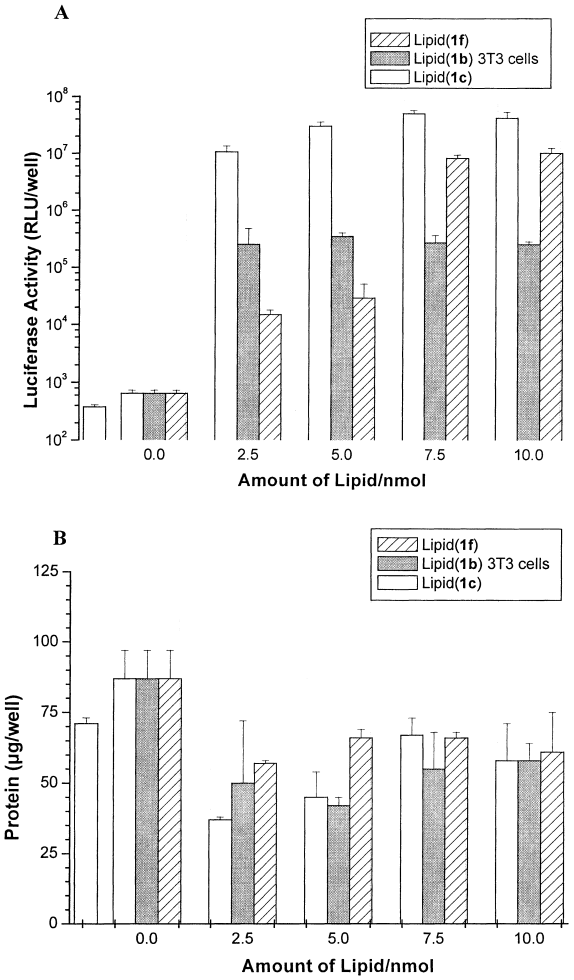


Fig. 4. Effect of cationic Lipid to DNA ratio on the transfection activity. Cells (3T3) were transfected with 1 μg of PCMV Luc plamid DNA various amounts of cationic lipids. The level of luciferase gene expression (A) and total protein recovery (B) in cells transfected with cationic lipids **1b**, **1c**, and **1f** at 2.5, 5.0, 7.5, and 10.0 nmol/well, respectively.

Cholest-5-en-3-one 3-Chloropropylene Acetal (3). A solution containing cholest-4-en-3-one **2** (10 g, 26 mmol), was prepared with high purity (>99%) via the oxidation of cholesterol by cyclohexone and recrystallization from methanol; 3-chloro-1,2-propanediol (4 mL, 47 mmol), benzene (100 mL, solvent), and *p*-toluenesulfonic acid (catalyst) were refluxed with **2** for 7 h using a water separator to remove the produced water. The reaction was monitored by TLC (GF254 silica gel plates), and neutralized by a K_2CO_3 solution (5%). The resulting solution was washed twice with water and the organic layer was separated. After removal of the solvent, the residue was isolated by a silica-gel column chromatograph using mixed solvents of petroleum ether and ethyl acetate (50:1 v/v) as an eluent; **3** was obtained (20% yield). Mp 98–100 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.69 (s, 3H, H-19), 0.86 (d, J = 2.2 Hz, 3H, H-26), 0.88 (d, J = 2.2 Hz, 3H, H-27), 0.92 (d, J = 6.5 Hz, 3H, H-21), 1.05 (s, 3H, H-18), 1–2 (strong coupling, 28H, CH_2, CH), 3.46 (m, 1H, H-3′), 3.59 (m, 1H, H-1′), 3.91, 3.93 (m, 1H, H-3′ integral ratio 25:75), 4.11, 4.13 (m, 1H, H-1′ integral ratio 25:75), 4.33 (m, 1H, H-2′), and 5.34 (t, J = 2.5 Hz, 1H, H-6); IR (KBr) 2947, 1097, and 1116 cm^{-1}; EIMS *m/z* 478 (0.07), 476 (0.19), 384 (0.08), 269 (0.13), 229 (0.25), 197 (0.21), 147 (100), 119 (2.26), 95 (3.89), 69 (3.86), 55 (14.57), and 43 (8.08); Anal. Calcd for $C_{30}H_{49}ClO_2$: C 75.50, H 10.37%; found C 75.67, H 10.26%.

Cholest-5-en-3-one 3-Piperidinopropylene Acetal (1a). Compund **3** (3 g, 6.3 mmol) dissolved in piperidine (10 mL) was refluxed for 8 h. After filtration and removal of excess piperidine, the residue was extracted with water and chloroform. The organic layer was separated by a silica-gel column chromatography, eluted with chloroform, followed by $CHCl_3$/MeOH (20:1 v/v). **1a** was separated and recrystallized in absolute ethanol (65% yield). Mp 148–150 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.68 (s, 3H, H-19), 0.86 (d, J = 2.2 Hz, 3H, H-26), 0.87 (d, J = 2.2 Hz, 3H, H-27), 0.92 (d, J = 6.5 Hz, 3H, H-21), 1.02 (s, 3H, H-18), 1–2 (strong coupling, 34H, CH_2, CH), 2.46 (br.s, 4H, H-4′, H-8′), 2.55 (m, 1H, H-3′), 2.65 (m, 1H, H-3′), 3.59, 3.61 (m, 1H, H-1′ integral ratio 12:88), 4.05, 4.10 (m, 1H, H-1′ integral ratio 88:12), 4.33 (m, 1H, H-2′), and 5.34 (t, J = 2.5 Hz, 1H, H-6); IR (KBr) 2936, 1364, and 1091 cm^{-1}; EIMS *m/z* 525 (0.08), 384 (0.07), 269 (0.07), 229

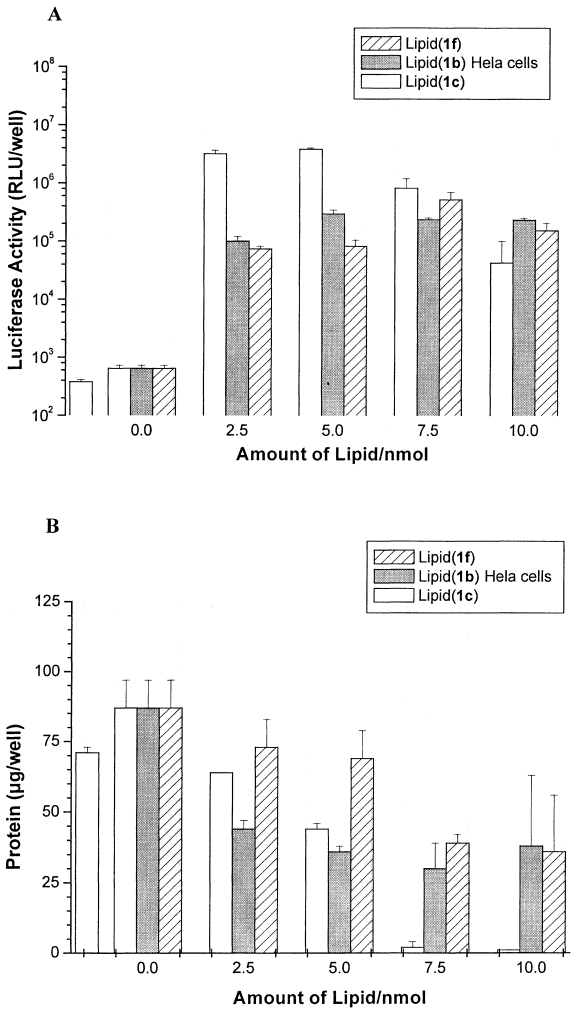


Fig. 5. Effect of cationic Lipid to DNA ratio on the transfection activity. Cells (Hela) were transfected with 1 μg of PCMV Luc plamid DNA various amounts of cationic lipids. The level of luciferase gene expression (A) and total protein recovery (B) in cells transfected with cationic lipids **1b**, **1c**, and **1f** at 2.5, 5.0, 7.5, and 10.0 nmol/well, respectively.

(0.28), 196 (0.81), 142 (3.17), 98 (100), 69 (3.27), and 55 (5.56); Anal. Calcd for $C_{35}H_{59}NO_2$: C 79.92, H 11.33, N 2.66%; found C 79.64, H 11.19, N 2.83%.

Cholest-5-en-3-one 3-(1-Pyrrolidinyl)propylene Acetal (1b). Compound **3** (3 g, 6.3 mmol) dissolved in pyrrolidine (10 mL) was refluxed for 6 h. After removing excess pyrrolidine, the residue was extracted with water and $CHCl_3$. The organic layer was separated by a silica-gel column with $CHCl_3$/MeOH (20:1 v/v) to give **1b** (76% yield). Mp 104–106 °C; ^{1}H MNR (500 MHz, $CDCl_3$) δ 0.68 (s, 3H, H-19), 0.86 (d, J = 2.2 Hz, 3H, H-26), 0.87 (d, J = 2.2 Hz, 3H, H-27), 0.92 (d, J = 6.5 Hz, 3H, H-21), 1.01 (s, 3H, H-18), 1–2 (strong coupling 32H, CH_2, CH), 2.40 (br.s, 4H, H-4′, H-7′), 2.43 (m, 1H, H-3′), 2.51 (m, 1H, H-3′), 3.55, 3.64 (m, 1H, H-1′ integral ratio 41:59), 4.08, 4.10 (m, 1H, H-1′ integral ratio 59:41), 4.28 (m, 1H, H-2′), and 5.33 (t, J = 2.5 Hz, 1H, H-6); IR (KBr) 3440, 2939, 1374, and 1095 cm^{-1}; EIMS *m/z* 511 (0.08), 384 (0.08), 229 (0.21), 178 (2.95), 128 (4.59), 84 (100), and 55 (6.54); Anal. Calcd for $C_{34}H_{57}NO_2$: C 79.78, H 11.22, N 2.74%; found C 79.51, H 11.07, N 2.56%.

Cholest-5-en-3-one 3-(Dimethylamino)propylene Acetal (1c). An ethanol solution of compound **3** (3 g, 6.3 mmol) and dimethylamine (4 g, 89 mmol) in autoclave was heated at 120 °C for 10 h. After ethanol was removed, the residue was extracted with water and $CHCl_3$. The organic layer was chromatographed over a silica-gel column with $CHCl_3$/MeOH (15:1), giving **1c** (60% yield). Mp 140–141 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.68 (s, 3H, H-19), 0.86 (d, J = 2.2 Hz, 3H, H-26), 0.88 (d, J = 2.2 Hz, 3H, H-27), 0.92 (d, J = 6.5 Hz, 3H, H-21), 1.04 (s, 3H, H-18), 1–2 (strong coupling, 28H, CH_2, CH), 2.27 (s, 6H, H-4′ and H-5′), 2.35 (m, 1H, H-3′), 2.48 (m, 1H, H-3′), 3.57, 3.62 (m, 1H, H-1′ integral ratio 59:41), 4.04, 4.11 (m, 1H, H-1′ integral ratio 41:59), 4.24 (m, 1H, H-2′), and 5.34 (t, J = 2.5 Hz, 1H, H-6); IR (KBr) 2936, 2884, 2867, 2766, 1380, 1365, and 1099 cm^{-1}; EIMS *m/z* 485 (0.10), 382 (0.13), 269 (0.15), 229 (0.37), 156 (2.93), 102 (11.10), 84 (7.79), 58 (100), and 43 (5.39); Anal. Calcd for $C_{32}H_{55}NO_2$: C 79.10, H 11.43, N 2.88%; found C 78.87, H 11.19, N 3.05%.

A methanol solution of compound **3** (2 g, 4.2 mmol) and trimethylamine (5.9 g, 0.1 mol) in an autoclave was heated at 120 °C for 24 h. Upon removing methanol, the residue was separated with a silica-gel column; interestingly, a demethylation product **1c**, rather than **1i**, was obtained as the product. Alternatively, when the reaction was carried out at 80 °C for 7 d, the same results were obtained.

Cholest-5-en-3-one 3-[2-(Dimethylaminoethylamino)]propylene Acetal (1d). Compound **3** (3 g, 6.3 mmol) dissolved in (asym) *N*,*N*-dimethylethylenediamine (10 mL) was refluxed for 24 h. After removing excess (asym) *N*,*N*-dimethylethylenediamine, the residue was separated by a silica-gel column chromatography using $CHCl_3$/MeOH (10:1 v/v) as an eluent, and recrystallized in petroleum ether; **1d** was obtained (50% yield). Mp 119–120 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.68 (s, 3H, H-19), 0.86 (d, J = 2.2 Hz, 3H, H-26), 0.88 (d, J = 2.2 Hz, 3H, H-27), 0.92 (d, J = 6.5 Hz, 3H, H-21), 1.02 (s, 3H, H-18), 1–2 (strong coupling, 28H, CH_2, CH), 2.22 (s, 6H, H-6′, and H-7′), 2.41 (t, J = 6.2 Hz, 2H, H-5′), 2.72 (m, 3H, H-3′, and H-4′), 2.76 (m, 1H, H-3′), 3.63, 3.67 (m, 1H, H-1′ integral ratio 47:53), 4.02, 4.05 (m, 1H, H-1′ integral ratio 53:47), 4.24 (m, 1H, H-2′), and 5.34 (t, J = 2.5 Hz, 1H, H-6); IR (KBr) 2946, 2819, 2769, 1332, 1137, and 1104 cm^{-1}; EIMS *m/z* 485 (0.16), 384 (0.06), 269 (0.08), 229 (0.32), 156 (7.85), 102 (32.35), 84 (20.27), 58 (100), and 43 (8.48); Anal. Calcd for $C_{34}H_{60}N_2O_2$: C 77.20, H 11.46, N 5.30%; found C 76.96, H 11.16, N 5.55%.

Cholest-5-en-3-one 3-(2-Aminoethylamino)propylene Acetal (1e). A mixture of compound **3** (2 g, 4.2 mmol) and ethylenediamine (10 mL) was refluxed for 10 h under nitrogen. After removing excess ethylenediamine, the residue was separated by a silica-gel column chromatography using $CHCl_3$/MeOH (8:1) as an eluent to give **1e** (56% yield). Mp 98–101 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.67 (s, 3H, H-19), 0.86 (d, J = 2.2 Hz, 3H, H-26), 0.87 (d, J = 2.2 Hz, 3H, H-27), 0.91 (d, J = 6.5 Hz, 3H, H-21), 1.02 (s, 3H, H-18), 1–2 (strong coupling 28H, CH_2, CH), 2.42 (t, J = 6.3 Hz, 2H, H-5′), 2.70 (m, 3H, H-3′, and H-4′), 2.72 (m, 1H, H-3′), 3.65, 3.68 (m, 1H, H-1′ integral ratio 52:48), 4.07, 4.11 (m, 1H, H-1′ integral ratio 48:52), 4.25 (m, 1H, H-2′), and 5.33 (t, J = 2.5 Hz, 1H, H-6); IR (KBr) 3441, 2938, 2884, and 1096 cm^{-1}; EIMS *m/z* 470 (4.92), 384 (0.69), 229 (0.89), 211 (30.45), 156 (30.96), 113 (100), and 55 (17.6); Anal. Calcd for $C_{32}H_{56}N_2O_2$: C 76.75, H 11.27, N 5.59%; found C 76.48, H 11.15, N 5.72%.

Cholest-5-en-3-one 3-(Trimethylammonio)propylene Acetal Iodide (1f). A $CHCl_3$/MeOH (1:1, v/v) solution of compound **1c** (1 g, 2.1 mmol) and iodomethane (1 mL, 16 mmol) was stirred in darkness for 24 h. After removing the solvent, **1f** could be directly crystallized in a mixed solution of $CHCl_3$ and absolute ethanol (75% yield). Mp >300 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.68 (s, 3H, H-19), 0.86 (d, J = 2.2 Hz, 3H, H-26), 0.88 (d, J = 2.2 Hz, 3H, H-27), 0.92 (d, J = 6.5 Hz, 3H, H-21), 1.04 (s, 3H, H-18), 1–2 (strong coupling, 28H, CH_2, CH), 3.44 (m, 1H, H-3′), 3.54 (s, 9H, H-5′, H-6′, H-7′), 3.72 (m, 1H, H-3′), 4.31 (m, 1H, H-1′), 4.43 (m, 1H, H-1′), 4.72 (m, 1H, H-2′), and 5.33 (t, J = 2.5 Hz, 1H, H-6); IR (KBr) 2939, 2884, and 1113 cm^{-1}; Anal. Calcd for $C_{33}H_{58}INO_2$: C 63.13, H 9.33, N 2.23%; found C 63.39, H 9.17, N 2.15%.

Cholest-5-en-3-one 3-(*N*-Methylpiperidimio)propylene Acetal Iodide (1g). A $CHCl_3$/MeOH (1:1, v/v) solution of compound **1a** (1.9 mmol) and iodomethane (1 mL, 16 mmol) was stirred in darkness for 24 h at room temperature. After removing the solvent, **1g** was directly crystallized in a mixed solution of $CHCl_3$ and absolute ethanol (70% yield). Mp 280 °C (decomp); ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.68 (s, 3H, H-19), 0.86 (d, J = 2.2 Hz, 3H, H-26), 0.87 (d, J = 2.2 Hz, 3H, H-27), 0.92 (d, J = 6.5 Hz, 3H, H-21), 1.02 (s, 3H, H-18), 1–2 (strong coupling, 30H, CH_2, CH), 2.37 (m, 4H, H-5′, and H-7′), 3.00 (br.s, 3H, H-9′), 3.30 (br.s, 6H, H-4′, H-8′, and H-3′), 3.74 (m, 1H, H-1′), 4.06 (m, 1H, H-1′), 4.18 (br.s, 1H, H-2′), and 5.73 (br.s, 1H, H-6); IR (KBr) 2936, 2868, 1366, 1331, 1112, and 1089 cm^{-1}; Anal. Calcd for $C_{36}H_{62}INO_2$: C 64.74, H 9.38, N 2.10%; found C 64.50, H 9.12, N 2.26%.

Cholest-5-en-3-one 3-(*N*-Methylpyrrolidimio)propylene Acetal Iodide (1h). A mixture of compound **1b** (1.95 mmol) and iodomethane (1 mL) in $CHCl_3$/DMSO (1:1, v/v) binary solvents was stirred for 24 h in the dark. After removing the solvents, **1h** was crystallized from $CHCl_3$/MeOH solvents (63% yield). Mp 264 °C (decomp); ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.68 (s, 3H, H-19), 0.86 (d, J = 2.2 Hz, 3H, H-26), 0.87 (d, J = 2.2 Hz, 3H, H-27), 0.92 (d, J = 6.5 Hz, 3H, H-21), 1.02 (s, 3H, H-18), 1–2 (strong coupling, 28H, CH_2, CH), 2.39 (m, 4H, H-5′, and H-6′), 3.02 (br.s, 3H, H-8′), 3.35 (br.s, 6H, H-4′, H-7′, and H-3′), 3.76 (m, 1H, H-1′), 4.10 (m, 1H, H-1′), 4.18 (br.s, 1H, H-2′), and 5.62 (br.s, 1H, H-6); IR (KBr) 3439, 2938, 1376, and 1112 cm^{-1}; Anal. Calcd for $C_{35}H_{60}INO_2$: C 64.30, H 9.25, N 2.14%; found C 64.57, H 9.43, N 2.11%.

Preparation of Lipid Formulation and DNA/Lipid Complexes. Lipid formulations were prepared as previously described.[33] In brief, a mixture of appropriate amounts of cationic lipid and DOPE (molar ratio 1:1) was dissolved in chloroform. After removing chloroform under a stream of nitrogen gas, the resulting lipid films were dried under a vacuum for 2 h to remove trace amounts of the organic solvent, and then hydrated in phosphate buffered saline (PBS pH = 7.4) at a cationic lipid concentration of 5 mM. Hydration proceeded for 2 h at room temperature and the lipid suspension was then sonicated for 5 min using a bath sonicator.

pCMV-Luc plasmid DNA containing the firefly luciferase gene driven by the cytomegalovirus immediate early promoter (CMR) was diluted in a serum-free CHO-S-SFM medium (life Technology inc.) to give a DNA concentration at 1 μg/125 μL. The cationic lipid suspension (5 mM) was diluted with Hank's balanced salt solution (HBSS) to generate different cationic lipid concentrations: 2.5 nmol/125 μL; 5 nmol/125 μL; 7.5 nmol/125 μL; 10 nmol/125 μL. DNA/lipid complexes in appropriate ratios were prepared by mixing equal volumes of a diluted DNA solution and lipid suspension. The mixture (250 μL) was incubated for 5–10 min at room temperature before being added to cells.

Cell Culture. Five cell lines derived from different origins were used for this study. Murine melanoma BL-6 cells were cultured in a RPMI Medium supplemented with 10% fetal bovine sencm (FBS). Human emlryonic kidney (293 cells), Hela cells, NIH3T3, and 3LL were cultured in a DMEM medium with 10% FBS.

Transfection In Vitro. For a standard transfection, cells were seeded at a density of 5×10^4 cells per well in 48-well plates 24 h before the addition of DNA/lipid complexes. After removing the medium, the cells were incubated with a DNA/lipid mixture (250 μL/well) for 5 h, followed by the addition of 27.5 μL of fetal bovine serum (FBS) to each well. The transfection solution was replaced with a fresh medium containing 10% FBS 24 h post exposure to the DNA/lipid mixture. Cells were collected after additional incubation for 24 h, and a cell lysate was prepared by PBS washing (3×) and the addition of 100 μL of lysis a buffer (0.1 M tris-HCl 0.1 TritonX-100, 2 mM EDTA, pH = 7.80) per well. The cell lysates were collected and centrifuged in a microcentrifuger (12,000 rpm, 5 min, 4 °C), and the supernatant was used for measuring the luciferase activity. A luciferase assay kit purchased from promega (Madison, WI) was used in a Luminometerc Autolumat according to the established protocol.[33] Tenal of cell lysate from transfected cells was used in each assay, and the luminescence was measured for 10 s. The protein concentration of the supernatant was determined by a standard protein assay using a protein assay reagent purchased from Biorad (Hercules, CA)

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