Pergamon

Bioorganic & Medicinal Chemistry Letters 10 (2000) 891–894

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Synthesis and Evaluation of Vitamin D-Based Cationic Lipids for Gene Delivery In Vitro

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Received 21 December 1999; received in revised form 14 February 2000

Abstract—A new panel of steroidal cationic lipids has been synthesized for gene delivery. Using commercially available vitamin D_2 (calciferol) or vitamin D_3 (cholecalciferol) as hydrophobic motifs and a variety of cationic head groups as binding sites for negatively charged phosphate groups in DNA, we demonstrated that the transfection activity of the synthetic vitamin D-based cationic lipids **1d**, **2d** formulated with dioleoylphosphatidylethanolamine (DOPE) as a co-lipid is comparable to that of 3-(-[*N*-*N*′,*N*′-dimethylaminoethane)carbamoyl]cholesterol (DC-Chol). These synthetic lipids are effective in transfecting a variety of cell lines. These results suggest that vitamin D-based cationic lipids are useful transfection reagents for in vitro gene transfer studies. © 2000 Published by Elsevier Science Ltd.

Introduction

In 1991, Gao and Huang reported the synthesis and application of the cholesterol-based cationic lipid DC-Chol to deliver genes into mammalian cells.[1] Since then, considerable effort has been expended in synthesis of steroidal cationic lipids owing to their potential applications in gene therapy.[2–7] The molecular designs utilized often involve the conjugation of a hydrophilic amine or polyamine group with a hydrophobic cholesterol motif. These ventures led to the discovery of N5-cholesteryloxycarbonyl-5,10-diazatetradecane-1,14-diamine (GL-67) by Genzyme Inc., which has been proven to be effective for in vivo gene transfer via airway administration.[2] Another alternative in the design of the steroidal cationic lipids is use of a bile acid as a scaffold for gene delivery.[8] The steroidal skeleton of bile acids (A, B, C and D rings) is similar to that of cholesterol, but with polyhydroxyl groups on the steroid nucleus to give a relatively polar steroid tail. Previously, the routes of synthesis for steroidal cationic lipids have been focused on a combination of cationic amine groups with limited variations of the steroidal backbone (Chart 1). As a part of our ongoing study of cationic lipid-based gene delivery systems and cell targeting,[9] we were interested in knowing how the geometry of hydrophobic motif in cholesterol-based cationic lipids affects the outcome of transfection. Vitamin D analogues are secosteroids with a 9, 10 carbon–carbon bond breakage of B ring on the steroidal backbone,[10] which would serve as a hydrophobic domain in the cationic lipid-mediated gene transfection. Herein, we report our synthesis and evaluation of Vitamin D-based cationic lipids for gene delivery into cells.

Synthesis of vitamin D-based cationic lipids

The preparation of vitamin D-based cationic lipids was realized in a straightforward strategy as shown in Scheme 1.

Vitamin D_2 or D_3 was refluxed with 1,1′-carbonyldiimidazole (1.5 equiv) in the presence of triethylamine (2 equiv) in methylene dichloride for 2 h. After the usual workup, the reaction residue was purified by column chromatograpy to afford the corresponding imidazole carboxylic ester in ca. 90% yield. Imidazole carboxylic ester, which can be regarded as an analogue of chloroformate, was stirred with 2 equiv of the corresponding amine in dry methylene dichloride overnight. The reaction mixture was then diluted with chloroform. Imidazole and the excess amount of unreacted amine were easily removed by extraction with 5% potassium hydroxide aqueous solution. The organic phase was further washed with brine and dried over magnesium sulfate. After rotary evaporation, the residue was dried

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PII: S0960-894X(00)00127-X

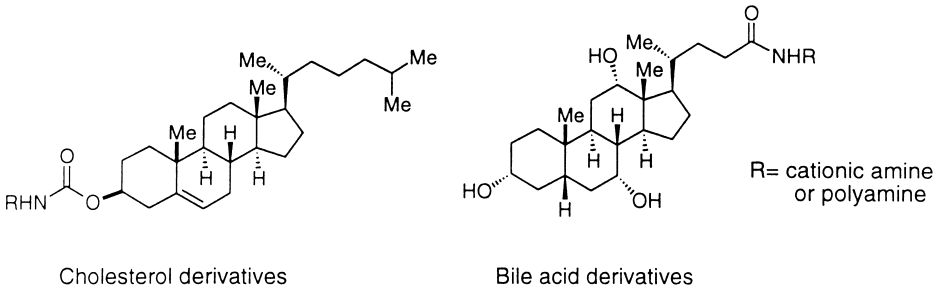
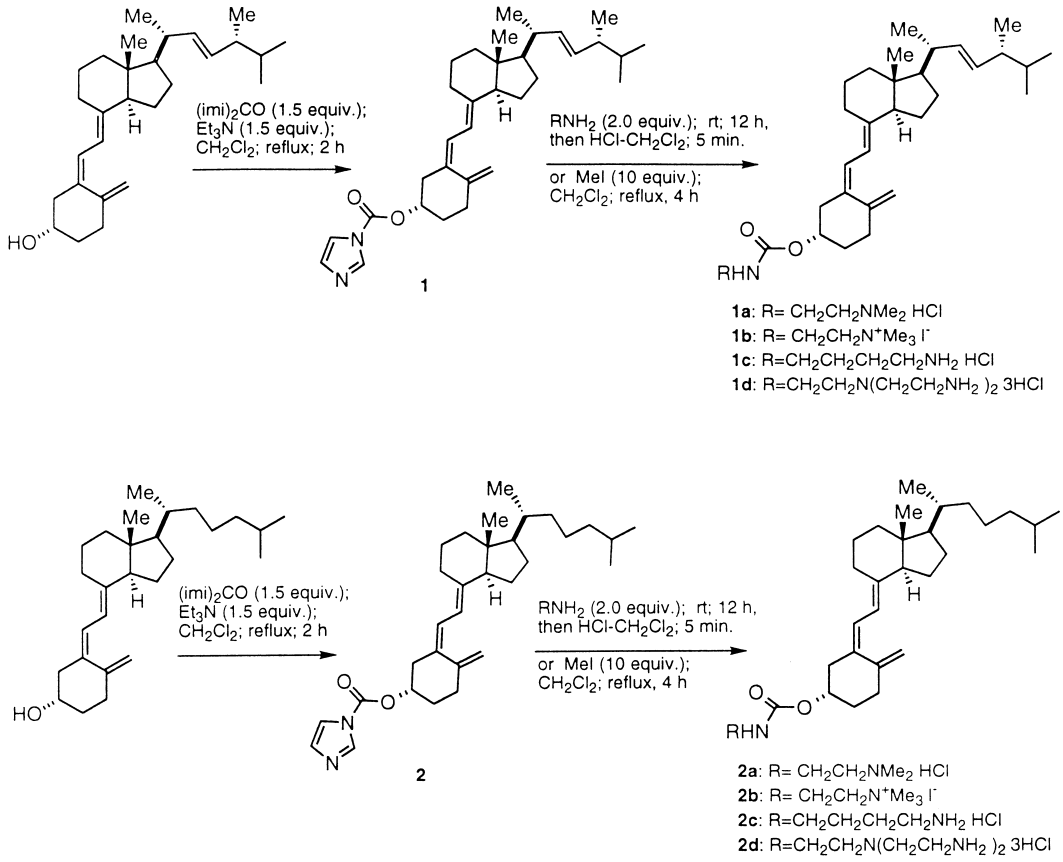


Chart 1.



Scheme 1.

under vacuum to afford the corresponding carbamate as a white foam in 95% yield. All compounds showed satisfactory purity by ^{1}H NMR analysis. The carbamates were protonated to yield the final cationic lipids **1a**, **1c–d**, **2a** and **2c–d**. The quaternary ammonium salts **1b**, **2b** were prepared by further quaternization of the tertiary amine precursors with 10 equiv of methyl iodide in refluxing methylene dichloride for 4 h. After removal of the solvent, the residue was recrystallized from acetonitrile to afford lipids **1b** and **2b** in ca. 88% yield. The structure of the new compounds was confirmed by ^{1}H NMR.[11]

Preparation of lipid formulation and DNA/lipid complexes

Lipid formulations were prepared as previously described.[12] In brief, a mixture of the appropriate amount of cationic lipid and DOPE (molar ratio, 1:1) was dissolved in chloroform. After removal of chloroform under a stream of nitrogen gas, the resulting lipid films were dried under vacuum for 2 h to remove trace amounts of 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 (CMV) was diluted in serum free CHO-S-SFM medium 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 seven different cationic lipid concentrations: 1 nmol/125 μL, 2.5 nmol/125 μL, 5 nmol/125 μL, 7.5 nmol/125 μL, 10 nmol/

125 μL, 12.5 nmol/125 μL and 15 nmol/125 μL. DNA/lipid complexes in appropriate ratios were prepared by mixing equal volumes of diluted DNA solution and lipid suspension. The mixture (250 μL) was incubated for 5–10 min at room temperature before being added to cells.

Gene expression analysis

Cells were seeded at a density of 5×10^4 cells per well in 48-well plates 24 h before addition of DNA/lipid complexes. After removal of the medium, the cells were incubated with DNA/lipid mixture (250 μL/well) for 5 h followed by addition of 27.5 μL fetal bovine serum (FBS) to each well. The transfection solution was replaced with fresh medium containing 10% FBS 24 h post exposure to the DNA/lipid mixture. Cells were collected after an additional incubation for 24 h and a cell lysate was prepared by PBS washing (3 ×) and addition of 100 μL lysis buffer (0.1 M Tris–HCl, 0.1% Triton X-100, 2 mM EDTA, pH 7.8) per well. The cell lysates were collected, centrifuged in a microcentrifuge (12,000 rpm, 5 min, 4 °C) and the supernatant was used for measurement of luciferase activity using luciferase assay kit according to the established protocol.[12] Ten μL of cell lysate from transfected cells were used in each assay and luminescence was measured at 10 s.

Gene transfection in vitro

The transfection activity of cationic lipids was initially tested in BL-6 cells (murine melanoma). Data in Figure 1 shows that the transfection activity of these new cationic lipids is dependent on the cationic lipid to DNA ratio. Using 1 μg plasmid DNA per well, the optimal transfection activity of vitamin D_2-based cationic lipids follows the trend: polyamine **1d** >≈primary amine **1c** tertiary amine **1a** > quaternary amine **1b** (Fig. 1A). As is seen in Figure 1B, a very similar trend was obtained for vitamin D_3-based cationic lipids **2a–d**. With the exception of **1b** and **2b**, the transfection activity of these lipids is comparable to that of DC-Chol. Meanwhile, polyamines **1d**, **2d** showed a constant activity in a broader range of cationic lipid to DNA ratio while DC-Chol, as well as our mono cationic lipids **1a–c** and **2a–c**, exhibited roughly bell-shaped lipid dose–response curves. For these mono cationic lipids **1a–c** and **2a–c**, the peak activity occurred when 5 nmol lipid was used for each well, representing a calculated cationic lipid to DNA charge ratio of 1.7. On replacement of the hydrophobic steroidal moiety of DC-Chol with vitamin D_2 or D_3, the resultant tertiary amines **1a**, **2a** afforded a slight decrease of the transfection activity in BL-6 cells. It is worth noting that these new lipids with a slight difference in the hydrophobic motif did not exhibit obvious differences in their gene transfection activity.To determine whether these new cationic lipids were capable of transfecting other types of cells, similar transfection experiments were performed on four additional cell lines. 3LL (Lewis lung carcinoma), NIH3T3 (murine embryonic fibroblast), HeLa (human cervical adenocarcinoma) and 293 (human embryonic kidney) cells were transfected using same lipid formulations as those used in experiments summarized in Figure 1. For these experiments, we used 5.0 nmol cationic lipid and 1 μg plasmid DNA per well. Data summarized in Table 1 show the transfection activity of our eight new lipids. It is evident that the level of luciferase activity obtained in 293 cells is higher than that seen in the 3LL, NIH3T3, or HeLa cells, implying that different cell lines have different sensitivity in responding to the cationic lipid-based transfection reagents. Among the four cell lines examined, tertiary amines **1a**, **2a**, primary amines **1c**, **2c** and polyamines **1d**, **2d** gave a higher transfection activity than that of quaternary amines **1b**, **2b**. This observation is consistent with the results obtained from the transfection experiment using BL-6 cells.

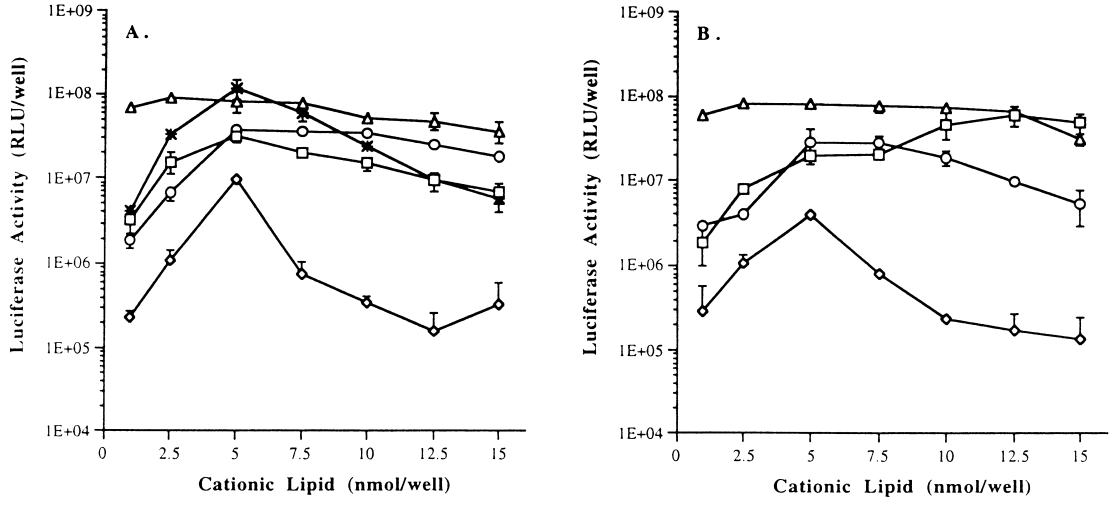


Figure 1. Effect of cationic lipid to DNA ratio on the transfection activity of cationic lipids. Cells (BL-6) were transfected with 1 μg of pCMV-Luc plasmid DNA with various amounts of cationic lipdis. Curves in A represent the level of luciferase gene expression in cells transfected with cationic lipids **1a** (□), **1b** (◇), **1c** (○), **1d** (△) and DC-Chol (∗) at 1, 2.5, 5, 7.5, 10, 12,5 or 15 nmol/well, respectively. Curves in B represent the level of luciferase gene expression in cells transfected with cationic lipids **2a** (□), **2b** (◇), **2c** (○), and **2d** (△) at 1,2.5, 5, 7.5, 10, 12.5 or 15 nmol/well, respectively. Data represent mean ± SD ($n = 3$).

Table 1. Transfection activity of vitamin D-based cationic lipids in different cell lines

Cell line	RLU/well[a]							
	1a	**1b**	**1c**	**1d**	**2a**	**2b**	**2c**	**2d**
3LL	8.0×10^5	4.0×10^3	6.1×10^5	5.9×10^5	2.2×10^5	5.3×10^3	8.2×10^5	1.5×10^5
NIH3T3	2.7×10^5	1.8×10^5	2.1×10^6	1.9×10^6	1.2×10^5	5.7×10^5	7.8×10^6	6.9×10^5
HeLa	2.6×10^6	3.8×10^4	9.3×10^5	5.4×10^5	2.9×10^5	1.8×10^4	1.7×10^6	1.4×10^6
293	3.9×10^6	1.6×10^6	6.3×10^7	4.8×10^7	1.1×10^6	5.6×10^5	4.7×10^7	3.2×10^7

[a]Different cells (5×10^4) were seeded in 48-well plate 24 h before the addition of DNA/lipid complexes (1 μg DNA/5 nmol cationic lipid per well). The level of luciferase gene expression is expressed as mean of relative light units (RLU) per well of triplicate experiments ($n = 3$).

In conclusion, we have synthesized a new panel of steroidal cationic lipids and evaluated their in vitro transfection activity. Among the lipids tested, polyamines **1d**, **2d** exhibited superior transfection activity in a broader range of lipid to DNA ratios in BL-6 cells. Transfection results obtained from the additional cell lines suggest that these new vitamin D-based cationic lipids are useful transfection reagents for in vitro gene transfer studies.

Acknowledgements

Assistance from Professor Joseph E. Knapp for critical reading of this manuscript is acknowledged. This work was supported in part by a grant from NIH (CA 72925) and research contract with Targeted Genetics Corporation.

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11. Compound **1**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.11 (s, 1H, imidazole ring), 7.49 (s, 1H, imidazole ring), 7.06 (s, 1H, imidazole ring), 6.25 (d, $J = 11.2$ Hz, 1H), 6.02 (d, $J = 11.2$ Hz, 1H), 5.20 (m, 1H), 5.19 (m, 2H), 5.11(s, 1H), 4.89 (s, 1H), 2.86–2.65 (m, 2H), 2.60–2.40 (m, 2H), 2.30 (m, 1H), 2.20–1.80 (m, 5H), 1.70–1.60 (m, 4H), 1.60–1.40 (m, 4H), 1.40–1.20 (m, 3H), 1.02 (d, $J = 6.6$ Hz, 3H), 0.91 (d, $J = 7$ Hz, 3H), 0.83 (d, $J = 6.6$ Hz, 3H), 0.82 (d, $J = 6.6$ Hz, 3H), 0.56 (s, 3H). Compound **2**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.12 (s, 1H, imidazole ring), 7.41 (s, 1H, imidazole ring), 7.06 (s, 1H, imidazole ring), 6.26 (d, $J = 11.2$ Hz, 1H), 6.03 (d, $J = 11.2$ Hz, 1H), 5.20 (m, 1H), 5.12 (s, 1H), 4.90 (s, 1H), 2.85–2.70 (m, 2H), 2.60–2.40 (m, 2H), 2.30 (m, 1H), 2.20–1.80 (m, 5H), 1.80–1.60 (m, 3H), 1.60–1.40 (m, 4H), 1.40–1.20 (m, 6H), 1.20–1.05 (m, 3H), 0.92 (d, $J = 6$ Hz, 3H), 0.87 (d, $J = 6.6$ Hz, 6H), 0.55 (s, 3H). Compound **1b**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.20 (d, $J = 11.2$ Hz, 1H), 6.02 (m, 1H), 6.01(d, $J = 11.2$ Hz, 1H), 5.19 (m, 2H), 5.05 (s, 1H), 4.85 (s, 1H), 4.82 (m, 1H), 3.83 (m, 2H), 3.79 (m, 2H), 3.47 (s, 9H, N^+Me_3), 2.80 (m, 1H), 2.57 (m, 1H), 2.35 (m, 1H), 2.19 (m, 1H), 2.10–1.80 (m, 6H), 1.70 (m, 4H), 1.50 (m, 4H), 1.30 (m, 3H), 1.01 (d, $J = 6.6$ Hz, 3H), 0.91 (d, $J = 6.7$ Hz, 3H), 0.83 (d, $J = 6.6$ Hz, 3H), 0.82 (d, $J = 6.6$ Hz, 3H), 0.55 (s, 3H). Compound **2b**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.21 (d, $J = 11.2$ Hz, 1H), 6.01 (d, $J = 11.2$ Hz, 1H), 5.99 (m, 1H), 5.05 (s, 1H), 4.83 (s, 1H), 4.78 (m, 1H), 3.83 (m, 2H), 3.71 (m, 2H), 3.47 (s, 9H, N^+Me_3), 2.80 (m, 1H), 2.60 (m, 1H), 2.40 (m, 2H), 2.20 (m, 1H), 2.00–1.80 (m, 5H), 1.70–1.60 (m, 3H), 1.60–1.45 (m, 4H), 1.40–1.20 (m, 6H), 1.20–1.10 (m, 3H), 0.92 (d, $J = 6$ Hz, 3H), 0.86 (d, $J = 6.6$ Hz, 6H), 0.53 (s, 3H).
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