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Gene transfection activities of amphiphilic steroid–polyamine conjugates

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Abstract

The design and evaluation of a novel potent class of DNA delivery agents based on steroid–polyamine conjugates bearing a flexible linker are reported. The hydrophobic regions are based on steroids, i.e. chlolestane and lithocholic acid motifs. The linker, which couples a hydrophobic steroid and a hydrophilic polyamine, in this study can be regarded as a two-atom extension of the conventional carbamate linker. We found that the gene transfection activity of the steroid–polyamine conjugates is influenced by the polyamine chain length and steroid structure. Molecular modeling of the relevant amphiphilic molecules revealed low-energy structures in which the polyamine chains are folded rather than stretched. This work suggests a significant effect of space-filling, i.e. the shape and orientation of the hydrophilic and hydrophobic regions, upon the efficiency of gene transfection.

1. Introduction

Gene transfection across cell membranes is a fundamental technology for molecular–biological research and also for medicinal gene therapy [1]. Although the most efficient methods for transferring DNA across cell membranes involve the use of viral vectors, there are still arguments about risks in regard to immunogenicity and propagation [2,23]. In the past few years, a variety of non-viral gene-delivery systems have been investigated [3–9]. Although some success in getting DNA into cells has been achieved, gene delivery with non-viral vectors remains an inefficient process. More efficient DNA delivery systems are needed. Of all the non-viral DNA delivery systems, cationic lipids have been shown to be most promising in terms of efficiency and stability [10]. Cationic liposomes, composed of a cationic lipid and a neutral lipid, dioleoyl phosphatidylethanolamine (DOPE), are believed to interact with the negatively charged phosphate backbone of DNA, leading to a compacted structure. Lipofectin, a 1:1 mixture of the cationic lipid *N*-[(1,2,3-dioleoyloxy)-propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA, **1**) and DOPE was the first example of a non-viral DNA delivery agent [3,11]. Because there is no clear understanding of the mechanisms involved in the transfer of functionally active transfecting particles at the membrane surface, or within the cells, there is no basis for rational design of better chemical delivery agents. Among the synthetic DNA delivery agents, cationic lipids based on the cholesterol structure (e.g. 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]chlolesterol (DC-Chol, **2**) [5,12] and (3-β-(*N*4-spermine-carbamoyl)cholesterol **3**) [8] are promising candidates, and some are under clinical trial (Chart 1). The hydrophobic moiety of **2** and **3** is cholesterol, and the hydrophilic regions are poly-

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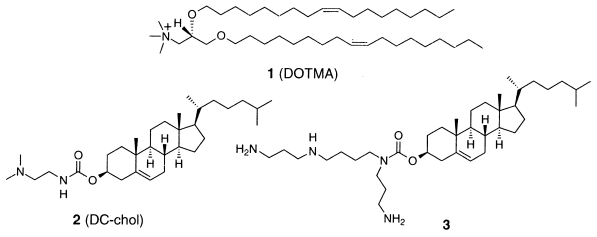

Chart 1.

amines. The linker which combines the hydrophilic and hydrophobic regions is a carbamate functionality, directly attached to the cholesterol skeleton. The linker is an important contributor to the conformational flexibility/rigidity of the whole molecules, and may constrain the possible orientations of the hydrophilic moiety with respect to the hydrophobic region [9]. In this paper, we report the design and evaluation of a novel class of DNA delivery agents based on steroid–polyamine conjugates bearing flexible linkers (Charts 2 and 3). These linkers, which couple the hydrophobic steroid and hydrophilic polyamine moieties, can be regarded as a two-atom extension of the conventional carbamate linker used previously (**2** and **3**). The steroid moieties are cholestane and lithocolic acid, of which the former has a *trans*-decaline structure and the latter, a *cis*-decaline structure. Thus the space-filling character of the hydrophobic unit is different in the two cases (Chart 3). In addition, the saturated steroid skeltones, cholestane and lithocolic

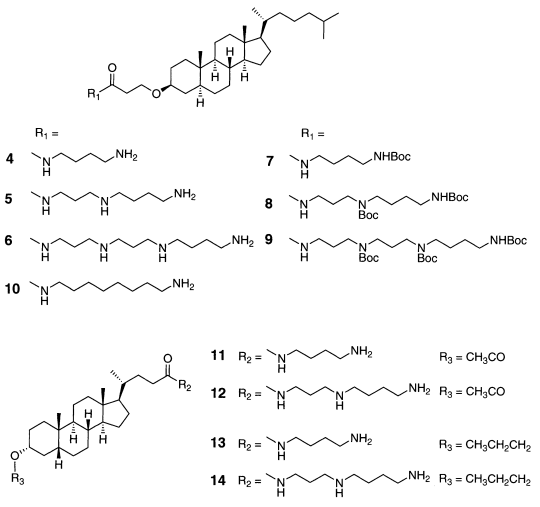

Chart 2.

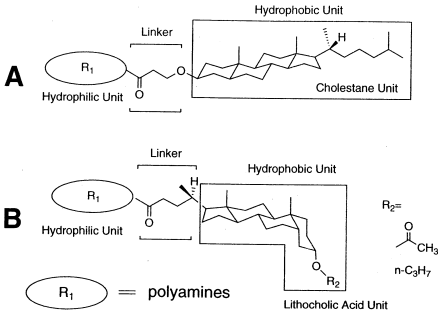

Chart 3.

acid exclude undesired autoxidation which can lead to cytotoxicity. We found that the gene transfection with the steroid–polyamine conjugates is influenced by polyamine chain length and steroid structure. Molecular modeling of the relevant amphiphilic molecules revealed low-energy structures in which the polyamine chains are folded rather than stretched. Extremely folded structures may decrease the efficiency as a DNA delivery vehicle. Our work suggests that the space-filling effect, i.e. shape and direction, of the hydrophilic and hydrophobic moieties influences the gene transfection.

2. Materials and methods

2.1. Materials

3β-[*N*-(Dimethylaminoethane)carbamoyl]cholesterol (DC-Chol) and 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE) were purchased from Sigma (St. Louis, MO). Lipofectin was purchased from Gibco (Grand Island, NY). Plasmid DNA (pGL3) was purchased from Promega (Madison, WI). The details of synthesis of the steroid–polyamine conjugates (Chart 1) will be reported elsewhere. Polyamines were synthesized as previously described [14]. Substitution of propanoic acid of the 3-hydroxyl group of cholestane was carried out through the addition reaction of acrylonitrile, acid-catalyzed solvolysis in ethanol, followed by alkaline hydrolysis. Amide bond formation was carried out with the conventional coupling reagents, DCC (1,3-dicyclohexylcarbodiimide) and *N*-hydroxysuccinimide.

2.2. Gene transfection activities of cholestane–polyamine conjugates

2.2.1. Liposome preparation

A solution of DOPE in chloroform was added to a solution of a steroid–polyamine conjugate in chloroform to give a mixture of DOPE/synthetic conjugate in a mole ratio of 3:2. The solvent was evaporated under reduced pressure to give a thin film of lipids. To this lipid film, phosphate-buffered saline (PBS) was added, and the sample was allowed to stand for 60 min under protection from sunlight. Then the samples were sonicated by using a bath-type sonicator (model 5210J, Branson) for 2–3 min, followed by a sonication with a probe-type sonicator (Sonifier 250, Branson) for 10 min to give small unilamellar vesicles (SUVs).

2.2.2. Cell culture and transfection

NIH3T3 cells were cultured in DMEM (Gibco, Grand Island, NY) supplemented with 10% FBS (Bio-Whittaker; Walkerville, MD). Plasmid pGL3 DNA (5.4 μg) in TE buffer (the stock solution of the DNA was 1 mg/ml) was complexed with the above liposomes (prepared from 30 nmol of DOPE and 20 nmol of the synthetic conjugate in PBS) in SFM101 (Nissui, Japan) at ambient temperature by pipetting well, and the whole was incubated at 37°C for 15 min to allow formation of the DNA–liposome complex. Then this DNA–liposome complex was incubated with the cells (1×10^6 cells/dish) in the SFM101 medium in a culture dish (Corning; 60×15 mm) for 4 h at 37°C. The cells were washed and cultured in growth medium (DMEM) for another 40 h at 37°C, followed by luciferase assay after cell lysis.

2.2.3. Luciferase assay

The luciferase assay was carried out using a picagene luciferase assay kit (Toyo Ink, Tokyo). The cells were washed three times with PBS and lysed in a cell lysis buffer for 15 min at room temperature. The lysate was centrifuged at $12\,000\times g$ at 4°C for 1 min and the supernatant was subjected to luciferase assay. Light emission was measured with a luminometer (TD-20/20, Turner Designs; Sunnyvale, CA) and normalized to the amount of protein of each sample, determined with BCA assay.

2.3. Molecular modeling

Molecular dynamics and minimization were performed with the Insight/Discover 95 program suite (Molecular Simulation). Solvent was not included in the calculations, because there is no explicit model for bilayer interactions of these amphiphiles. The initial structure was minimized and was subject to equilibration at 298 K for 10 ps in molecular dynamics calculations. In the subsequent 10-ps molecular dynamics at 298 K, the structures were taken at 100-fs intervals. Among the structures, an energy minimum structure was equilibrated by running dynamics at 900 K for 10 ps. Data were collected from a subsequent 10-ps run at 900 K. A 1-fs time step was used throughout the calculations. A total of 100 frames was collected during the simulation. These 100 frames of conformations were subsequently subjected to annealing molecular dynamics to obtain equilibration at the lower temperature of 298 K for 10 ps. Data were recollected from a subsequent 10-ps run at 298 K. The structures were finally minimized with molecular mechanic calculations. Conformations in the neutral state were also calculated in a similar manner (data not shown).

3. Results

3.1. Gene transfection with cholestane–polyamine conjugate

We prepared three derivatives of cholestane–polyamine conjugates (**4**–**6**) and three corresponding *N*-Boc (*t*-butoxycarbonyl)-protected derivatives (**7**–**9**), in the latter, the Boc substitution diminished the basicity of the amine nitrogen atoms (Chart 2). The hydrophobic region is cholestane and the hydrophilic region is a polyamine in which the alkyl amino unit length has been increased. The polyamine moiety of **4** was diaminobutane, that of **5** was spermidine, and the corresponding polyamine of **6** was a non-natural isomer of spermine. Under the physiological conditions, the amine nitrogens of **4**–**6** are fully protonated because of the pK_a values of the relevant amines are around 10 [13]. Thus, **4** is monocationic, **5** is dicationic, and **6** is tricationic. Cationic liposomes prepared by mixing cholestane–polyamine conjugate

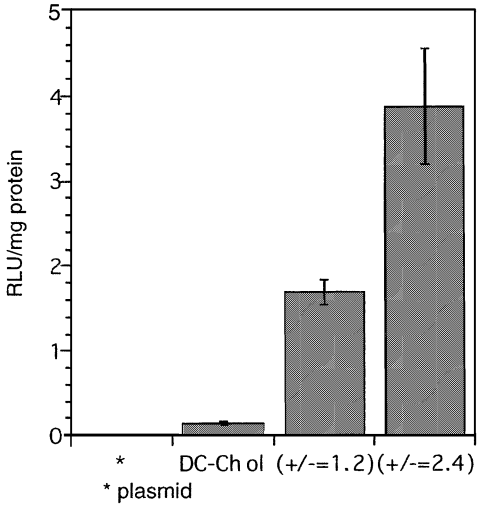

Fig. 1. Transfection activity of derivative **5** determined by luciferase assay. Luciferase activities of cell lysate obtained from NIH3T3 cells transfected using **5** at different doses of the lipids (25 and 50 nmol) were measured and compared to that from the cells using DC-Chol (50 nmol lipids). Transfection activity without lipids is shown as 'plasmid'. Each value is the mean ± S.E. ($n = 3$).

and DOPE in a mole ratio of 2:3 were used to transfect NIH3T3 cells with pGL3. The efficiency of the gene transfection was represented in terms of relative light unit (RLU) per mg protein in luciferase assay.

Transfection activity of liposomes with the cholestane–spermidine conjugate **5** was examined and compared with that of DC-Chol (**2**) (Fig. 1). Liposomes with the cholestane–spermidine conjugate (**5**) showed higher transfection activity than DC-Chol (**2**) with both amounts of the lipid mixture (25 and 50 nmol, mixture of **5** and DOPE). In the case of **5**, 25 and 50 nmol of the lipids with 5.4 μg of plasmid DNA corresponds to values of the charge ratio (+/−) of 1.2 and 2.4, respectively, when the two amine nitrogen atoms of **5** are fully protonated. As shown in Fig. 1, the efficiency of transfection with 50 nmol of the lipids was more than twice that with 25 nmol of the lipids. Therefore, an excess amount of the liposomes with respect to the plasmid DNA is important for the efficiency of transfection, probably because a large amount of the cationic liposomes can encapsulate the plasmid DNA to give a more compacted complex, or perhaps because all the cationic centers of the lipid may not be available to interact with the negatively charged DNA molecule.

The effect of the length of the polyamine chains on the gene transfection was also studied (Fig. 2). The amount of lipids was fixed at 50 nmol. While the transfection efficiency of the derivative **6** bearing a long polyamine chain is comparable to that of lipo-

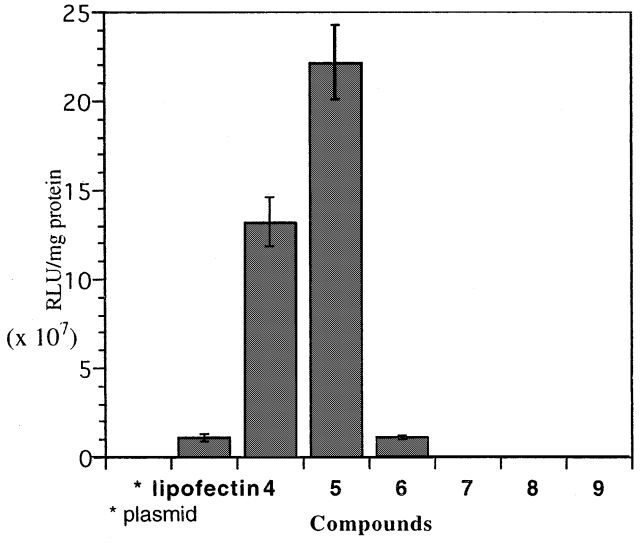

Fig. 2. Effects of the number of positive charges and chain length of compounds on transfection activity. Transfection activities of compounds **4**–**9** and lipofectin (**1**) were measured. The dose of the lipids was fixed at 50 nmol except for the case of plasmid only. Each value is the mean ± S.E. ($n = 3$).

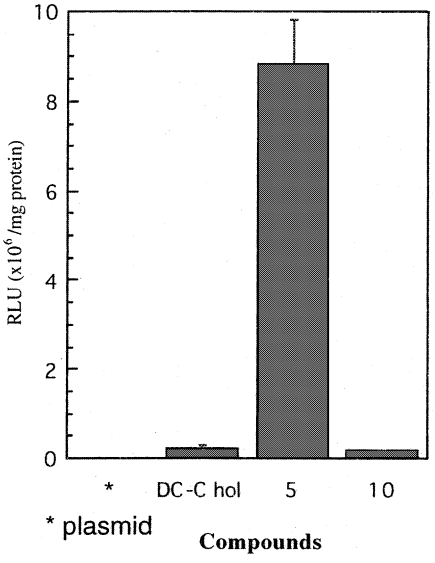

Fig. 3. Effects of the position of positive charges on transfection activity. Transfection activity of compound **10** was compared with those of DC-Chol (**2**) and compound **5**. The dose of the lipids was fixed at 50 nmol except for the case of plasmid only. Each value is the mean ± S.E. ($n = 3$).

fectin (**1** (DOTMA) and DOPE), the analogues **4** and **5** were much more effective than **6**, **5** being the most effective. Thus, an optimal length of the polyamine chain exists for efficient gene transfection, at least under the conditions of this experiment.

The basicity of the amino nitrogen atoms of the polyamine chains is also crucial for the gene transfection activity, because none of the N-Boc-protected analogues **7**, **8** and **9** showed gene transfection activity [14].

The importance of the two amine nitrogen atoms of **5** for high transfection activity is confirmed by the fact that the carbon analogue **10**, in which the secondary amine (–NH–) of **5** is replaced with a methylene (–CH_2–) group, has a significantly reduced transfection efficiency, comparable with that of DC-Chol (**2**) (Fig. 3). This suggests that two amino nitrogen atoms with three or four methylene units are important for efficient gene transfection.

3.2. Gene transfection with lithocholic acid–polyamine conjugate

We prepared four derivatives of the lithocholic acid–polyamine conjugate (**11**–**14**) (Chart 2). The hydrophobic region is based on lithocholic acid and the hydrophilic region is a polyamine with an alkyl amino unit. Diaminobutane and spermidine, which are effective in the case of the cholestane–polyamine conjugates, were used as the polyamine moiety. The butanoic acid side chain attached to the steroid nucleus can be used as a flexible linker (Chart 3 [6]. The hydroxy group at the C-3 of lithocholic acid was protected with an acetyl group (**11** and **12**), or a propyl group (**13** and **14**). The cationic liposomes were prepared and the luciferase assay (NIH3T3 cells with pGL3) was carried out in the same manner as described for the cholestane–polyamine conjugates. Transfection activities of the liposomes with lithocholic acid–polyamine conjugates **11**–**14** were examined and compared to that of DC-Chol (**2**) (Fig. 4). In the case of short polyamine conjugates (**11** and **13**), transfection activity was significantly decreased. The transfection activities of longer polyamine conjugates (**12** and **14**) were increased as compared with the corresponding short–polyamine analogues **11** and **13**, respectively. The transfection activity of **14** was comparable to that of DC-Chol (**2**). Therefore, the

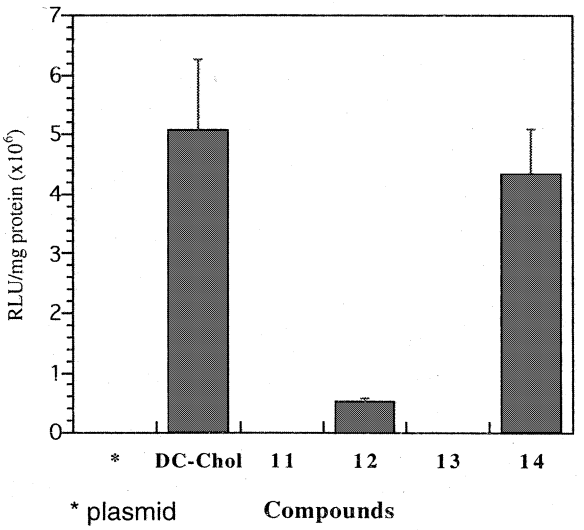

Fig. 4. Effects of the space-filling of the hydrophobic region and polyamine chain length on transfection activity. Transfection activities of compounds **11**–**14** were measured in comparison with that of DC-Chol (**2**). The dose of the lipids was fixed at 50 nmol except for the case of plasmid only. Each value is the mean ± S.E. ($n = 3$).

lithocholic acid–polyamine conjugate showed transfection activity, though it was lower than that of the corresponding cholestane–polyamine conjugate. These results suggest a significant effect of the shape of the steroid moiety upon gene transfection activity.

3.3. Molecular modeling of cationic amphiphiles

We carried out molecular modeling of *N*-protonated structures of the amphiphilic compounds, **2** (DC-Chol), cholestane–polyamine conjugates **4**–**6** and lithocholic acid–polyamine conjugates **12** and **14** with simulated annealing molecular dynamics and energy minimization by using consistent valence force field (CVFF). The objective was to look for any conformations accessible to these cationic amphiphiles regardless of the environment. The effect of solvent molecules was neglected. The amphiphiles were defined to be in their *N*-protonated states, i.e. the nitrogen-protonated structures (except the amide nitrogen atom) were calculated. Superposition of 100 frames from the simulation is shown in Fig. 5. Molecular modeling of the cholestane–polyamine conjugates **4**–**6** based on the annealing molecular dynamics and minimization predicted a low-energy structure in which the polyamine tails are folded rather than

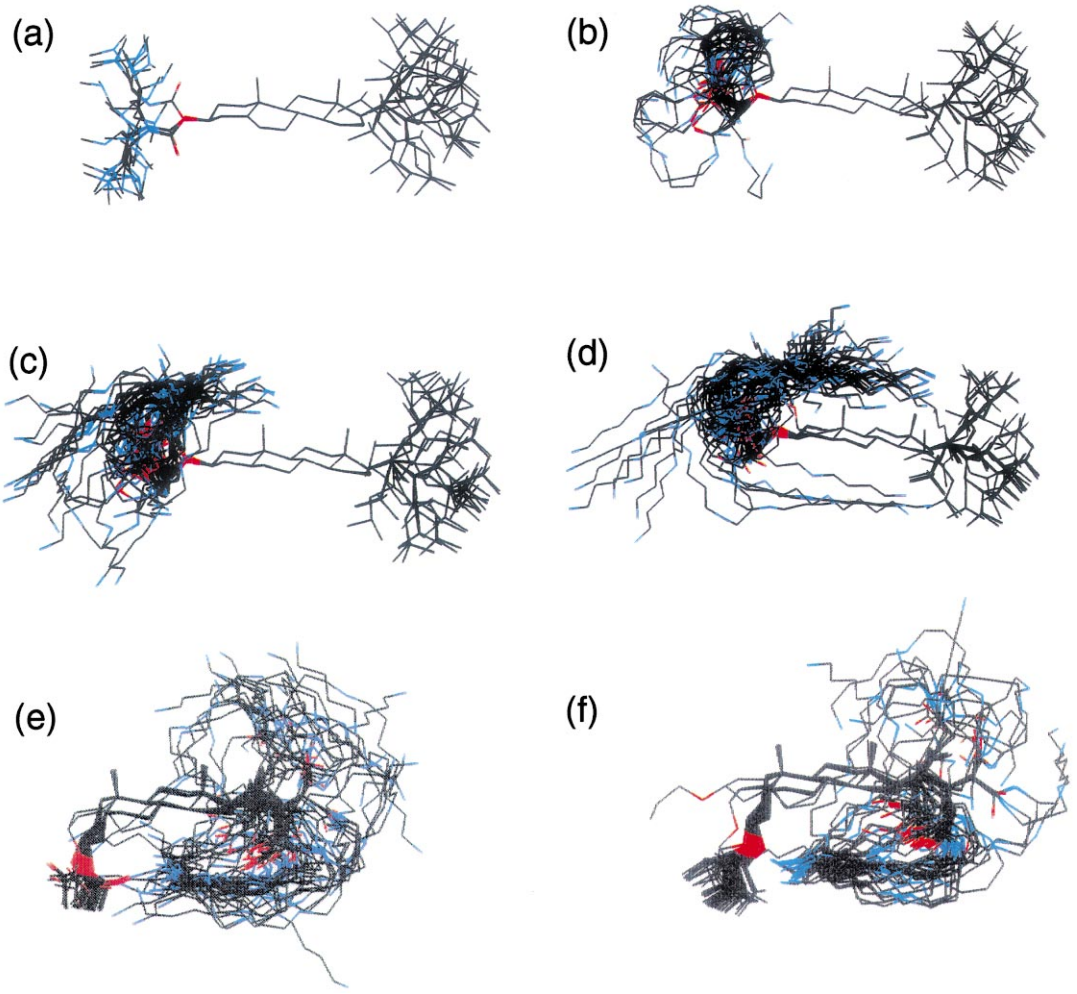

Fig. 5. Molecular dynamics simulations for each of cationic lipids in the *N*-protonated state. These representations consist of superposition of 100 frames from the simulation. Red, oxygen atom; blue, nitrogen atom. Hydrogen atoms are omitted for clarity. (a) **2**-H^+ (+1); (b) **4**-H^+ (+1); (c) **5**-$2H^+$ (+2); (d) **6**-$3H^+$ (+3); (e) **12**-$2H^+$ (+2); (f) **14**-$2H^+$ (+2).

stretched, particularly in the case of **6**, which bears a long polyamine chain (Fig. 5b–d). The accessible space of the hydrophilic tail of **2** (DC-Chol) is rather limited, and the structures are stretched (Fig. 5a). Apparently, the corresponding space of the hydrophilic tail of **4** is larger, supporting a flexible linker (Fig. 5b). The structures with a folded polyamine tail were also predicted in the cases of free (neutral) amphiphiles (**4**, **5** and **6**) (data not shown). The lithocholic acid–polyamine analogues **12** and **14** are also apt to take folded conformations, probably due to the *cis*-decaline structure of the lithocholic acid moiety. These simulations also support a flexible linker of these lithocholic acid–polyamine conjugates.

4. Discussion

We found that novel synthetic cationic amphiphiles based on cholestane–polyamine conjugates with a flexible linker are efficient vehicles for gene transfection into NIH3T3 cells. A similar result was obtained in the case of COS-7 cells. Space-filling (i.e. shape) of the rigid hydrophobic steroid moieties and the flexible hydrophilic polyamine moieties has a significant impact on the gene transfection activity. Molecular modeling suggests that the linear polyamines can fold to take a compact structure. Effects of hydrophilic chain lengths of cationic cholesterol derivatives on gene transfection have been studied [4,12]. The effect of the hydrophobic groups of cytofectins,

flexible linear alky and alkeny chains on gene transfection was also studied [15]. The gene transfection process in vitro is proposed to involve the initial electrostatic interaction of cationic liposomes with negatively charged DNA to form a plasmid–liposome complex [16,17] the delivery of this complex into the target cells through endocytosis, and the endosomal release of DNA into the cytoplasm [18]. At present, we cannot identify the critical process that is sensitive to the polyamine chain length and the shape of the hydrophobic steroid moiety. The stability of cationic liposomes and the liposome–DNA complexes may be relevant. As a rule of thumb, it appears that addition of protonation sites in terms of the numbers of the amino nitrogen atoms does not necessarily enhance gene transfection of the present series of steroid–polyamine conjugates [7,13,19–21]. One possible reason is that the predicted self-folded conformations of the cationic amphiphiles (for example **6**) disfavor effective interaction with the negatively charged plasmid DNA. The effects of the polyamine chain lengths of the DC-Chol analogues were studied [22], which demonstrated that the longer the polyamine chain lengths, the more active in terms of gene transfection in vitro. This observation is consistent with the nature of the rigid carbamate linker of DC-Chol.

Therefore, this work suggests a significant effect of space-filling, i.e. the shape and direction, of the hydrophilic and hydrophobic moieties upon gene transfection.

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