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Efficient nucleic acid transduction with lipoplexes containing novel piperazine- and polyamine-conjugated cholesterol derivatives

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ABSTRACT

To advance the use of cationic lipids for non-viral nucleic acid vector formulation, a panel of novel nitrogen heterocycle cholesteryl derivatives containing a biodegradable carbamate linker was synthesised. Optimally acting piperazine and cyclen compounds had nucleic acid-binding and lipoplex nanoparticle formation properties that were suitable for their use as non-viral vectors. It was found that the lipoplexes formed were capable of efficient non-toxic nucleic acid delivery to cells in culture. The chemical structure of individual cationic lipids, which is likely to influence lipoplex formation, affected efficiency of DNA or RNA transfection. The results indicated that the cyclen containing compound possessing two cholesteryl moieties resulted in efficient siRNA-mediated target gene silencing but was a poor reagent for DNA transfection.

Gene therapy has enormous potential for the management of many serious diseases. In addition to supplementing cellular genetic material, use of nucleic acids to activate RNA interference (RNAi) and silence pathology-causing gene expression has promising application.[1] However, achieving efficient and safe delivery of nucleic acids remains an important objective before the full potential of this approach is realized. Viral vectors have been extensively used, but complications of toxicity, mutagenesis and immunostimulation are problematic.[2] Non-viral vectors are usually chemically synthesized and have advantages of potential for large scale production and modification to alter biological properties. Nevertheless, improvement of nucleic acid delivery efficiency is important to achieve before widespread application of non-viral vectors is realized. DNA and RNA complexed to liposomes to form lipoplexes are commonly used for transfection of cells in culture and nucleic acid delivery in vivo.[3] Lipoplexes typically comprise nucleic acids bound to cationic constituents together with a helper/fusogenic lipid. Incorporation of cationic nucleic acid-binding cholesterol-based lipids into non-viral vectors has gained favor and this class of cationic lipid has been extensively studied.[4–11] Cationic cholesterol derivatives have three basic domains: a cholesteryl (hydrophobic) part, a cationic amine head group and a linker moiety.[8,10] Incorporation of a variety of polar head groups has been reported to bind DNA and achieve nucleic acid delivery to cells. These include linear polyamines such as spermine and spermidine,[12] dendritic amines,[13] piperazines,[14] heterocyclic and aromatic heterocyclic substituted amines.[15] Chemical properties of individual constituents as well as their formulation with other components of the lipoplexes influence transduction efficiency. To advance the favorable gene delivery properties of piperazine-containing formulations,[14] we have carried out analysis on a range of novel piperazine cholesteryl compounds and also extended the study to include carbamate-linked polyamine (cyclen) cholesterol derivatives. We have found that lipoplexes containing these lipids are efficient and non-toxic vectors for delivery of both DNA and synthetic RNAi effecters to cells in culture.

Substituted piperazine/cyclic polyamine (cyclen) derivatives were synthesized by reacting cholesteryl chloroformate with cyclic nitrogen containing compounds using a convenient acylation procedure (Fig. 1). Some of the compounds were not subjected to detailed analysis because of poor solubility or low DNA binding capacity. Briefly, synthesis reaction conditions were as follows. Cholesteryl chloroformate (1.0 mmol) in dichloromethane (10 cm^3) was added slowly (10 min) to a solution of amines/polyamines (1.2 mmol) in dichloromethane (10 cm^3) at 0 °C. The reaction solution was stirred for 10 min at 0 °C and then for a further 4–8 h at room temperature. Thereafter, the reaction mixture was washed with water (3 × 10 cm^3) and dried ($MgSO_4$) which afforded the product as a solid. The crude solid was subsequently purified

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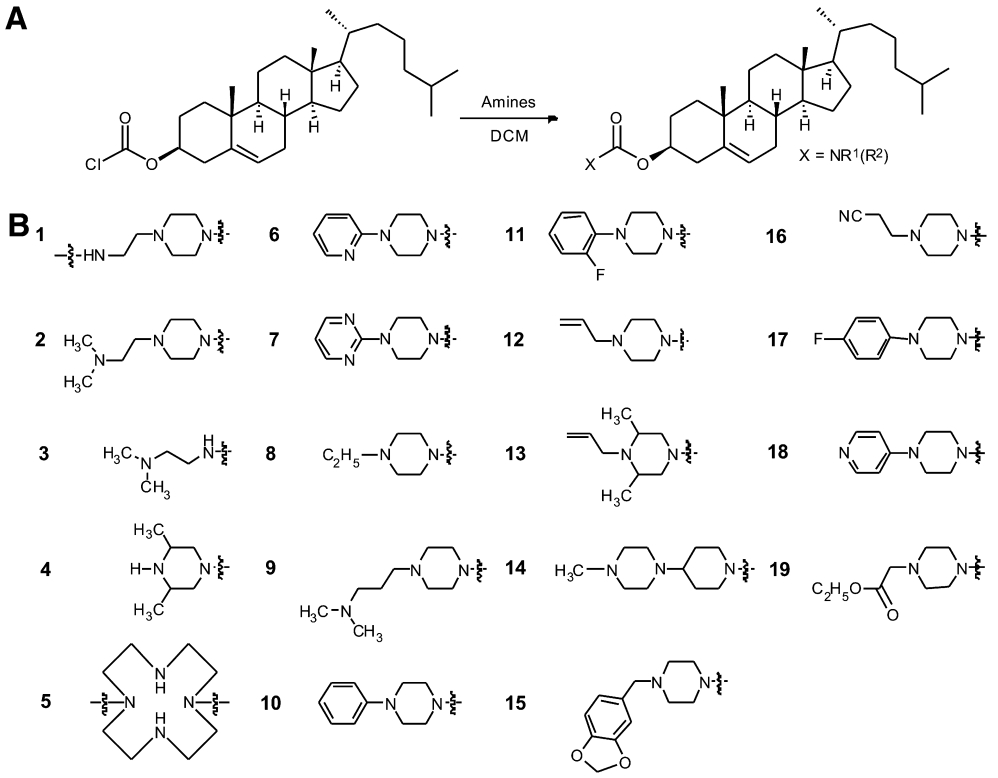

Figure 1. Cationic cholesterol derivatives **1–19** used in this study. (A) Schematic representation of the chemical synthesis. (B) Structures of moieties (X) attached to cholesterol derivatives **1–19**.

by silica gel column chromatography using 2–10% methanol-dichloromethane as eluant. This gave the desired conjugated compounds **1–19** in good to excellent yields. All compounds were characterized by NMR, IR and mass spectroscopy and gave acceptable

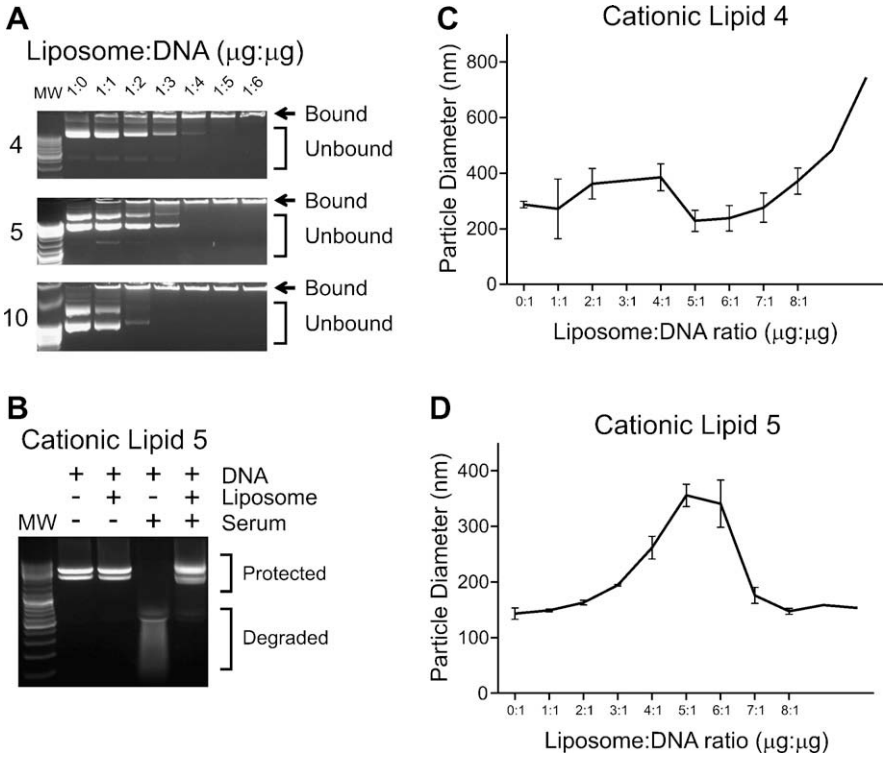

Figure 2. Representative analysis of properties of lipoplexes formulated with individual piperazine cholesteryl derivatives. (A) Mobility shift assay to determine DNA binding by liposomes. Varying ratios of liposome:DNA (μg:μg) were combined then subjected to agarose gel electrophoresis. Bound uncharged nucleic acid was retarded in the wells, while uncomplexed DNA retained electrophoretic mobility. (B) Nuclease protection of liposomes was determined by incubating naked and complexed DNA in the presence of DNase-containing serum. This was followed by electrophoresis to assess evidence for nucleic acid degradation. Lipoplex particle size analysis of formulations containing cationic cholesterol derivative **4** (C) and **5** (D). Malvern particle size analysis was carried out to determine the lipoplex particle sizes at varying liposome: DNA (μg:μg) ratios.

data. Cationic lipid **5** was formulated with dioleylphosphatidyl ethanolamine (DOPE) at a 7.2:4 molar ratio, while all of the other lipids were mixed at a ratio of 6:4. Formation of complexes with DNA was carried out using standard methodology.[16]

Initially, mobility shift analysis was carried out to determine the DNA binding capabilities of the soluble cholesteryl amine-containing derivatives, (Fig. 2A). Substituted piperazine-containing aromatic (**10**) and heteroaromatic (**6**,**7**) rings were found to be insoluble. DNA was incubated at varying ratios (μg:μg) to cationic lipid prior to electrophoresis. The ratio at which retardation of the DNA occurred was used as an indicator of the point at which neutralization of the DNA charge was achieved. This charge neutralizing ratio varied with different compounds and ranged from 3:1 (e.g., lipid **10**) to 9:1 (e.g., lipid **4**) for cationic lipids with decreasing DNA binding capabilities (Table 1). The DC-Chol (lipid **3**) standard was capable of neutralizing DNA charge at a ratio of 5:1, which is similar to previously reported DNA binding capacity.[5] Stability of the DNA within the particle formulations was assessed after exposure to serum containing DNases and representative analysis is shown in Figure 2B. Our findings were that formulation of the DNA within the lipoplexes was protected against degradation by nucleases, which is important for use of nucleic acid complexes for gene delivery. To characterize the physical properties of the lipoplex nanoparticles further, formulations were subjected to Malvern particle size analysis. Formulations were prepared without extrusion and under conditions where DNA charge was neutralized at minimum lipid concentration. The average particle diameter ranged from 150 to 300 nm (Table 1). Sizes also varied according to the amount of lipid incorporated into the complexes (Fig. 2C and D). With formulations containing lipid **4**, increasing the ratio of lipid:DNA from 0:1 to 10:1 resulted in a steady increase of lipoplex diameter from 280 to 750 nm. Conversely, increasing the content of lipid **5** within the lipoplex resulted in an increase of particle diameter size to 350 nm at a ratio of 5:1 and thereafter, the particle size diameter diminished to 150 nm with lipid:DNA ratios above 7:1. These particle sizes are predicted to be suitable for use in vivo, where diameters of below 150 nm are needed to penetrate certain membrane fenestrations and to avoid consumption by the reticuloendothelial system.[10]

To determine DNA transfection efficiency of lipoplexes, *enhanced green fluorescent protein* (*eGFP*) and *luciferase* reporter gene transfection to cultured cells was used. Comparison revealed that incorporation of cationic lipid **4** into lipoplex compounds achieved the best transfection efficiency, which was also similar to that of DC-Chol (Fig. 3A and B). Interestingly, considerable variation was observed with formulations that contained different cholesteryl piperazine and cyclen compounds. To characterize RNA transfection properties of lipoplexes, formulations were prepared that contained anti-hepatitis B virus (HBV) siRNA. The siRNA duplex comprised 5′ UUGAAGUAUGCCUCAAGGUCG 3′ antisense and 5′ ACCUUGAGGCAUACUUCAATT 3′ sense strands, which targeted HBV coordinates 1700–1720 (Genbank Accession No. J02203). Cells

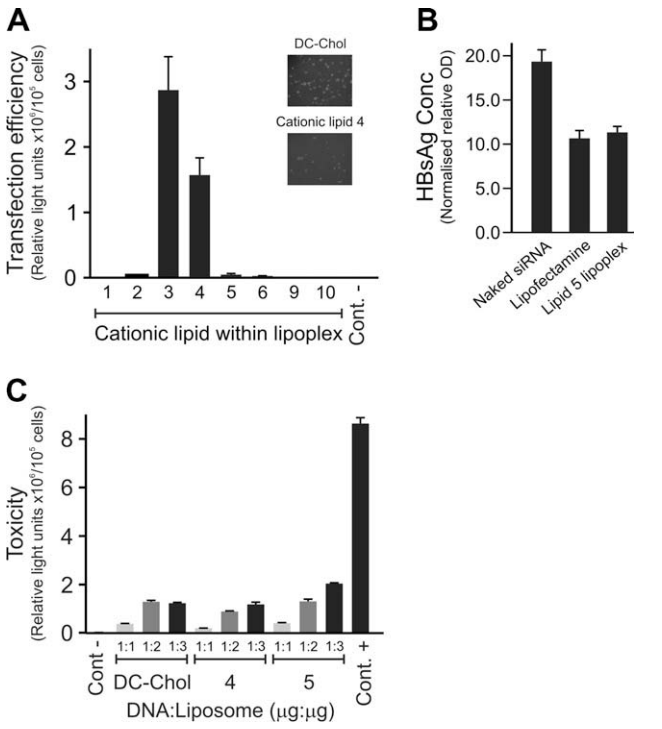

Figure 3. Analysis of nucleic acid transfection efficiency and toxicity of individual lipoplexes. (A) Comparison of luciferase reporter gene activity following transfection of Huh-7 cells with lipoplexes formulated with each of the indicated piperazine cholesterol derivatives. Representative fluorescence microscopy fields to detect eGFP-expressing Huh-7 cells following transfection with lipoplexes containing DC-Chol or cationic lipid **5** are shown in the inset. (B) Inhibition of HBV surface antigen secretion from Huh-7 cells following siRNA transfection. Cells were initially transfected with a replication-competent HBV plasmid and treated with the siRNA formulations 24 h thereafter. Controls included naked siRNA and a commercially available transfection reagent (Lipofectamine 2000). (C) Toxicity analysis of lipoplexes containing DC-Chol, cationic lipids **5** and **6** which were formulated at varying ratios of DNA to liposome.

Table 1
Minimum liposome to DNA ratio, with particle diameters for selected lipoplexes, at which nucleic acid charge neutralization occurs within the formulations.

Cationic lipid	Optimal DNA binding ratio (μg:μg)	Lipoplex particle diameter (nm)
1	No binding	
2	5:1	
3	5:1	240
4	5:1	158.8
5	5:1	524.2
8	4:1	
9	7:1	
10	3:1	766.8

were initially transfected with a HBV replication-competent plasmid[17] and thereafter with formulations containing anti HBV siRNA. Knockdown of HBV surface antigen (HBsAg), a marker of viral replication in culture, was determined using ELISA assay.[18] Compared to mock transfected cells, the siRNA delivered within lipoplex **5** achieved significant inhibition of secretion of HBsAg from the transfected cultured cells (Fig. 3C). Silencing efficiency was similar to that achieved with a commercially available transfection reagent (Lipofectamine 2000).

To assess unintended off target effects of the formulations, toxicity was assessed using the Cytotox Glo kit (Promega, WI, USA). Cell injury induces release of proteases that cleave 3 amino acids from a luciferin precursor to generate a mature luciferase substrate and emitted light is used as an index of cell toxicity. Under standard conditions of transfection, formulations were essentially non-toxic, but with addition of more lipids, toxicity increased. Representative toxicity assessment of formulations containing lipids **4** and **5** was similar to that observed for the control DC-Chol formulations (Fig. 3D). Induction of a non-specific interferon response, as assessed by activation of *interferon-β* gene expression, was also determined. We observed that transfected siRNA did not induce activation of this gene (not shown).

The nucleic acid-binding lipid components of lipoplex vectors have an influence on their transfection efficiency and the overall behavior of liposome-derived non-viral vectors. Identification of new cationic compounds and optimization of formulations for use in therapy is therefore an important objective to advance

therapeutic nucleic acid transfer. In this report, we have shown that cationic cholesteryl piperazines and cyclen derivatives, which were synthesized using uncomplicated protocols, can be conveniently formulated in lipoplex non-viral particles. Properties of DNA binding, nanoparticle size, protection from nuclease degradation and efficiency of nucleic acid delivery were found to be useful for achieving efficient nucleic acid transfer. Vectors containing the cationic lipids functioned well as transfecting reagents and were capable of delivery of either plasmid DNA or siRNA. Efficiency was achieved which is similar to that of standard and commercially available reagents, however the transfection efficiency of formulations differed according to the type of cationic cholesterol derivative contained within the vector. This observation is likely to be a result of particular properties of the 2 types of nucleic acid tested. Although both DNA and RNA have the same anionic charge to mass ratio, their behavior within lipoplexes is different. Neutralization of anionic phosphates of circular duplex plasmid DNA leads to controlled condensation during the formation of the lipoplex. However, because of their small size, siRNA interaction with cationic lipids may be different and lead to partial encapsulation, irregularly sized particles and poor stability of lipoplexes. Our observations indicate that cationic compound **4** favors an ordered condensation of plasmid DNA, which does not occur with lipid **5**. Conversely, siRNA lipoplexes containing compound **5** are capable of good RNA transfection efficiency. This interesting distinction is likely to result from differing interaction between plasmid DNA or siRNA with each of the cationic cholesteryl piperazines. Cationic lipid **5** contains two cholesteryl moieties, which are covalently linked by a aza-macrocycle.[19] However, lipid **4** has a more conventional structure with single cholesterol attached to DNA binding cationic head group. Dual cholesterol moieties within the lipoplex may favor controlled interaction of the smaller siRNAs to form a lipoplex that is capable of efficient delivery of the siRNAs to their cytoplasmic site of action. The promising properties of the transfection reagents which we have observed have prompted us to initiate assessment of their use for nucleic acid delivery in vivo.

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References and notes

1. Dykxhoorn, D. M.; Palliser, D.; Lieberman, J. *Gene Ther.* **2006**, *13*, 541.
2. Raty, J. K.; Lesch, H. P.; Wirth, T.; Yla-Herttuala, S. *Curr. Drug Saf.* **2008**, *3*, 46.
3. Kostarelos, K.; Miller, A. D. *Adv. Genet.* **2005**, *53PA*, 69.
4. Belmont, P.; Aissaoui, A.; Hauchecorne, M.; Oudrhiri, N.; Petit, L.; Vigneron, J. P.; Lehn, J. M.; Lehn, P. *J. Gene Med.* **2002**, *4*, 517.
5. Gao, X.; Huang, L. *Biochem. Biophys. Res. Commun.* **1991**, *179*, 280.
6. Hasegawa, S.; Hirashima, N.; Nakanishi, M. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1299.
7. Hattori, Y.; Ding, W. X.; Maitani, Y. *J. Control Release* **2007**, *120*, 122.
8. Karmali, P. P.; Chaudhuri, A. *Med. Res. Rev.* **2007**, *27*, 696.
9. Lee, Y.; Koo, H.; Lim, Y. B.; Lee, Y.; Mo, H.; Park, J. S. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2637.
10. Martin, B.; Sainlos, M.; Aissaoui, A.; Oudrhiri, N.; Hauchecorne, M.; Vigneron, J. P.; Lehn, J. M.; Lehn, P. *Curr. Pharm. Des.* **2005**, *11*, 375.
11. Singh, R. S.; Chaudhuri, A. *FEBS Lett.* **2004**, *556*, 86.
12. Blagbrough, I. S.; Geall, A. J.; Neal, A. P. *Biochem. Soc. Trans.* **2003**, *31*, 397.
13. Haensler, J.; Szoka, F. C., Jr. *Bioconjug. Chem.* **1993**, *4*, 372.
14. Gao, H.; Hui, K. M. *Gene Ther.* **2001**, *8*, 855.
15. Ilies, M. A.; Seitz, W. A.; Caproiu, M. T.; Wentz, M.; Garfield, R. E.; Balaban, A. T. *Eur. J. Org. Chem.* **2003**, 2645.
16. Singh, M.; Kisoon, N.; Ariatti, M. *Drug Deliv.* **2001**, *8*, 29.
17. Nassal, M. *J. Virol.* **1992**, *66*, 4107.
18. Carmona, S.; Ely, A.; Crowther, C.; Moolla, N.; Salazar, F. H.; Marion, P. L.; Ferry, N.; Weinberg, M. S.; Arbuthnot, P. *Mol. Ther.* **2006**, *13*, 411.
19. Venkata Ramana, A.; Watkinson, M.; Todd, M. H. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 3007.