Bioorganic & Medicinal Chemistry Letters 21 (2011) 3734–3737

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

The synthesis of cholesterol-based cationic lipids with trimethylamine head and the effect of spacer structures on transfection efficiency

Bieong-Kil Kim [a,†], Yun-Ui Bae [b,†], Kyung-Oh Doh [b,†], Guen-Bae Hwang [a], Sung-Hye Lee [b], Hyungu Kang [a], Young-Bae Seu [a,*]

[a] School of Life Science & Biotechnology, Kyungpook National University, Sankyukdong, Bookgu, Daegu 702-701, Republic of Korea
[b] Department of Physiology, Yeungnam University College of Medicine, 317-1 Daemyung-dong, Daegu 705-717, Republic of Korea

ARTICLE INFO

Article history:
Received 11 February 2011
Revised 14 April 2011
Accepted 18 April 2011
Available online 24 April 2011

Keywords:
Cholesterol
Cationic liposome
Transfection
Gene transfer technique

ABSTRACT

Five cholesterol-based cationic lipids were newly synthesized based on cholest-5-en-3β-oxyethane-*N,N,N*-trimethylammonium bromide (Chol-ETA) structure where the cholesterol backbone is linked to cationic head via various lengths of ether-linked carbon spacer. The transfection efficiency of these compounds was increased in order of three (Chol-PRO) < four (Chol-BTA) < two (Chol-ETA) methylene unit in their spacer, and was decreased by an addition of isomethyl group to Chol-PRO spacer. In case of the presence of multiple bonds in the spacer, it required the more cationic lipids in liposome formulation than single bond in the spacer to present similar transfection efficiency.

By means of gene therapy, gene transfer technique using cationic liposomes has been continuously researched in the view of the fact that these non-viral vectors can avoid the in vivo problems such as immune response caused by viral vectors.[1–3] These cationic liposomes have been also synthesized in order to deliver various genetic materials including plasmid DNA and siRNA into cells in vitro.[4,5] For the effective delivery of anionic genetic materials, amphipathic cationic lipids generally consist of cationic head group, linker, and hydrophobic domain. Cationic head groups and polar linkers vary quite substantially, while hydrophobic regions are mostly composed of long chain fatty acids or cholesterol derivatives.[6] This molecular structure of cationic lipids is important factor influencing gene delivery. The relationship between structure of cationic lipid and transfection efficiency has been actively researched from various angles. However, it was very hard to understand structure-transfection activity relationship (SAR) for transfection due to involving numerous steps related to cellular delivery of DNA.[7]

Recently, Ghosh's group synthesized cholest-5-en-3β-oxyethane-*N,N,N*-trimethylammonium bromide (here called as Chol-ETA) where cholesterol lipid domain and trimethylamine head are linked to two methylene units via ether linker (Fig. 1). Although this structure is very simple, transfection efficiency of Chol-ETA was generally superior to complicated structures.[5,8–10] Not only the structure of hydrophobic domain and cationic head but also the linker between them is one of the important factor related to successful gene delivery according to the kind of bond[9,11] and spacer[8,12–14] in linker (Fig. 1). Therefore, we newly synthesized five compounds based on Chol-ETA, and investigated the effect of spacer structure on transfection efficiency (Scheme 1).

Six cholesterol-based cationic lipids have been synthesized based on Chol-ETA structure where the cholesterol backbone is linked to cationic head via a various types of ether-linked carbon spacer (Scheme 1). Compound **2** and **3** were synthesized by modifying the methods of Bajaj et al.[10] To an ice-cooled solution of cholesterol **1** in pyridine-chloroform (v/v, 1/1), *p*-toluenesulfonyl chloride was added and stirred for 12 h at room temperature. Cholesterol tosylate **2** tosylated from cholesterol **1** in practical yield was used in the next reaction without a purification process because it was sufficiently purified by precipitating as a result of thin-layer chromatography (TLC) and ^{1}H NMR analysis, and was unstable in a silica gel column. Cholesterol tosylate **2** and 7–10 equiv of appropriate diol in anhydrous dioxane were refluxed for 7 h to afford compound **3** in 70–87% yield. To link cationic head of trimethylamine, compound **3** was bromized by reacting with 1 equiv of carbon tetrabromide and 1.5 equiv of triphenylphosphine in methylene chloride in 91–100% yield. The last compounds were synthesized by reacting compound **4** with trimethylamine (ca. 4.3 mol/L in water) in dimethylsulfoxide/chloroform (v/v, 7/3) at room temperature for 24 h in 40–90% yield. Structures of all the synthetic intermediates and last compounds shown in

* Corresponding author. Tel.: +82 53 950 5380; fax: +82 53 955 5522.
E-mail address: ybseu@knu.ac.kr (Y.-B. Seu).
† Three authors contributed equally to this work.

0960-894X/$ - see front matter Crown Copyright © 2011 Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2011.04.071

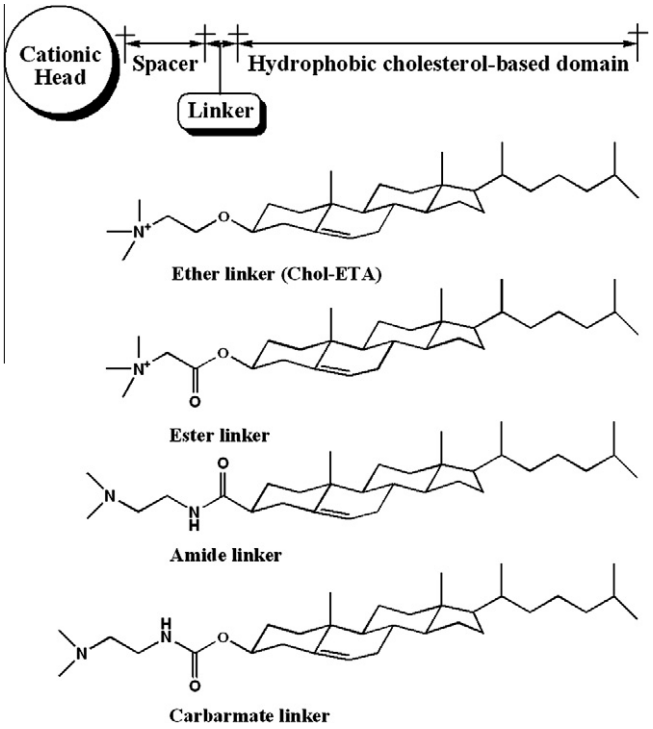
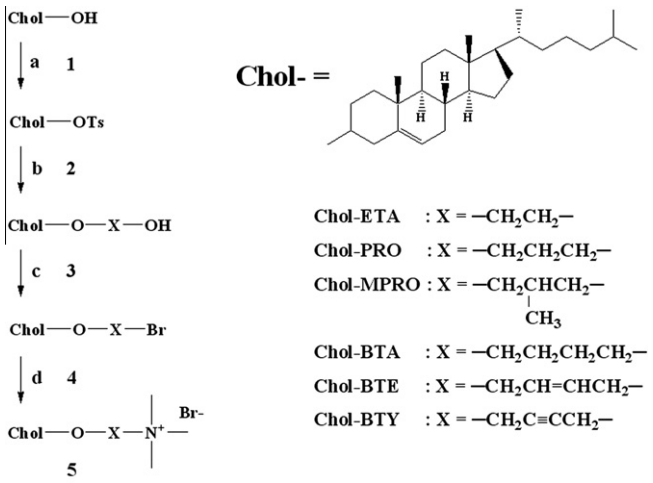


Figure 1. The common structure of cholesterol-based cationic lipids linked to cationic head via a various linker.



Scheme 1. Synthesis of Chol-ETA series. Reagents and conditions: (a) Cholesterol, *p*-toluenesulfonyl chloride, pyridine/$CHCl_3$ = 1/1 (yield: 100%); (b) X-diol/anhydrous dioxane, reflux (yield: 70–87%); (c) Carbon tetrabromide, triphenylphosphine/CH_2Cl_2 (yield: 91–100%); (d) Trimethylamine, DMSO/$CHCl_3$ = 7/3 (yield: 40–90%).

Scheme 1 were confirmed by ^{1}H NMR. The final compounds were characterized by FAB-MS to confirm the identity of the molecular ions.

The optimum transfection efficiency was shown with the addition of appropriate amount of helper lipids such as DOPE (1,2-dioleoyl-L-α-glycero-3-phosphatidyl-ethanolamine) and cholesterol in many cationic liposomes.[3,6,12] To form liposome formulation, lipid film made from appropriate weight ratio of cationic lipids and DOPE was briefly dispersed in an aqueous solution. The lipid film was hydrated at 4 °C overnight, followed by sonication at 60 °C for 20 min. Next, cationic liposome was passed 10 times through 200 nm polycarbonate membrane to remove overlarge liposomes.

Transfection optimizing medium (TOM), pCMVTnT-GFP, and pcDNA-Luc were obtained from Welgene (Korea) to test the ability of liposomes delivering plasmid DNA in COS-7 cell (african green monkey kidney cell). The day before transfection, COS-7 cells (4×10^4 per well) were seeded into 48-well plates. Thirty minutes before transfection, each well was briefly washed and added with 100 μl of TOM. Each liposome was diluted to 50 μl of TOM and mixed with pCMVTnT-GFP or pcDNA-Luc plasmid DNA (0.3 μg) in 50 μl of TOM. The lipoplexes, consisting of liposomes and DNA, were incubated for 10 min in room temperature, and then added to each well. The culture medium containing lipoplex was removed 4 h after the transfection, and fresh medium with 10% fetal bovine serum (FBS) was added to each well. After 18 h of further incubation, the medium was removed, and cells were washed twice with sterile phosphate buffered saline (PBS). The transfection efficiency was determined by means of a LMax II 384 luminometer (Molecular devices corp., USA) using a luciferase assay kit from Promega (USA). The protein content was quantified using a bicinchoninic acid (BCA) assay (PIERCE, USA). The luciferase efficiency was normalized by the protein content and expressed as relative light unit/μg of protein (RLU/μg protein). To observe GFP expression, fluorescence protein was observed on a Nikon ECLIPSE TE300 fluorescence microscope (Japan).

This study describes the synthesis and SAR of six cholesterol-based cationic lipids including Chol-ETA. These compounds were synthesized to observe the effect of change of a spacer structure

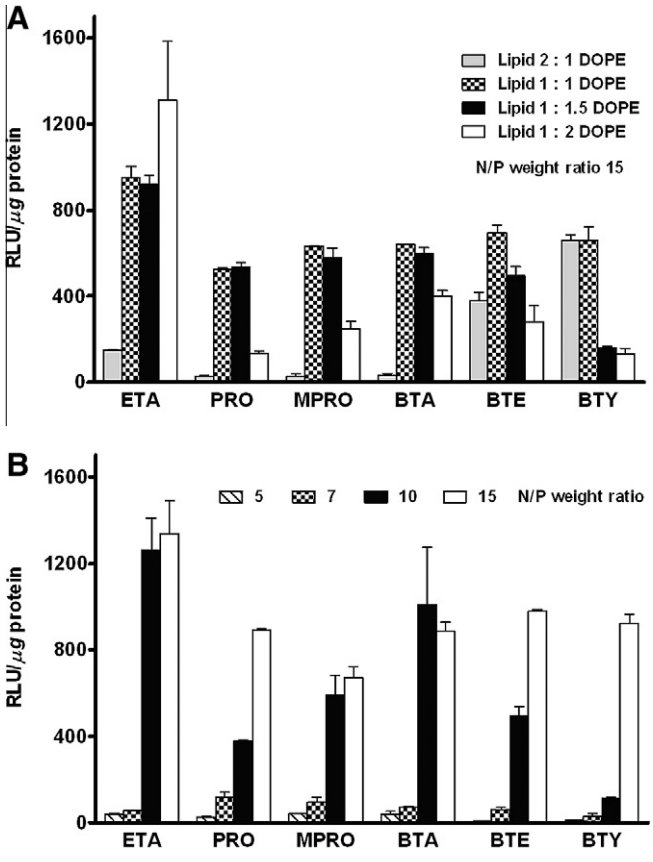


Figure 2. Transfection optimization of Chol-ETA series. (A) Transfection efficiencies of Chol-ETA series with various weight ratio of DOPE. The concentration of pcDNA-Luc was 0.3 μg/well, and liposoems were used at 15 N/P weight ratio. (B) Transfection efficiencies of Chol-ETA series using optimized lipid/DOPE formulations at various N/P weight ratios. Data are expressed as relative light unit (RLU)/μg total protein content as obtained from luciferase gene expression assay. Each bar value represents the mean ± SD of duplicate experiments performed on the same day.

connecting cationic head and hydrophobic domain on transfection efficiency. We used DOPE as co-lipid to enhance transfection efficiency, and sought the optimum transfection condition by varying the weight ratio of lipid:DOPE. We also measured the transfection efficiency at various liposome/DNA (N/P) weight ratios to find the optimum condition. In 15 N/P weight ratio, the optimum lipid:DOPE weight ratio was 1:1 for newly synthesized five compounds and 1:2 for Chol-ETA. In the optimum lipid:DOPE ratio, 15 N/P weight ratio showed the highest transfection efficiency in all liposomes except Chol-BTA (Fig. 2). Their transfection efficiency was compared with commercial cationic liposomes at optimal condition. These liposomes were as good as or better than commercial cationic liposomes for in vitro transfection in COS-7 cell (Fig. 3).

The transfection efficiency of Chol-ETA having two methylene unit in their spacer was higher than others and there are no significant difference between three (Chol-PRO) and four (Chol-BTA) methylene units (Fig. 3). These results were also confirmed by green fluorescent protein (GFP) expression (Fig. 4). In aminoglycerol-based cationic lipids with diverse length of a spacer structure, transfection efficiency of the spacer length exceeding 6 carbons was lower than short counterparts, and binding with DNA was also weaker.[12] In cholesterol-based lipids, the spacer length presenting the highest transfection efficiency was three carbons in carbamate-linked cationic lipid but was two carbons in amide and ester-linked cationic lipids.[13,14] Elongation of the distance between cationic head and lipid domain from one oxyethylene units to four oxyethylene units represented to have negative effect on the transfection efficiency.[8] In this study, we found that two methylene unit in ether-linked carbon spacer of cationic lipid having cholesterol domain and trimethylamine cationic head is more feasible structure for in vitro transfection.

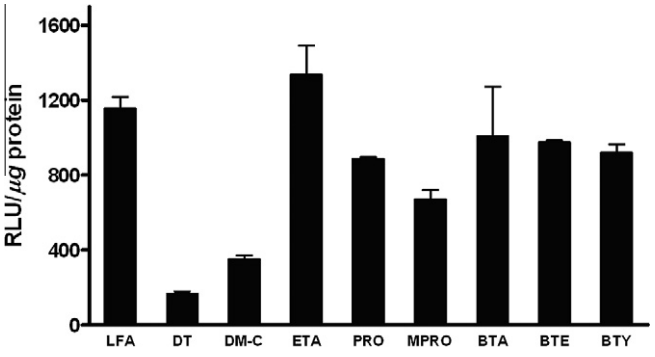


Figure 3. Comparision of transfection efficiencies using optimized Chol-ETA series with commercial cationic liposomes (LFA: Lipofectamine, DT: DOTAP, DM-C: DMRIE-C). Data are expressed as relative light unit (RLU)/μg total protein content as obtained from luciferase gene expression assay. Each bar value represents the mean ± SD of duplicate experiments performed on the same day.

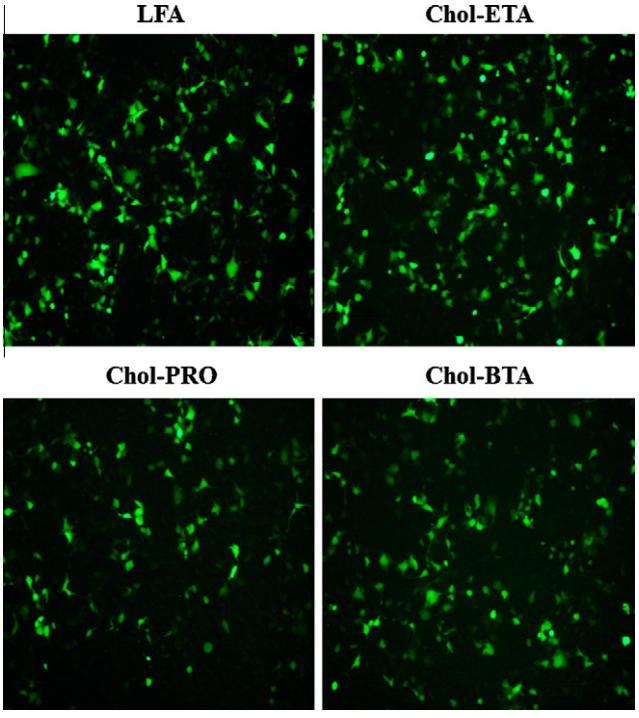


Figure 4. Expression of GFP using Lipofectamine (LFA), Chol-ETA, PRO and BTA. Plasmids pCMV-TnT (0.3 μg) were complexed with cationic liposomes in COS-7 cell. GFP expression was observed under fluorescence microscope.

We added the isomethyl group in the spacer to know whether spatial factor causes steric inhibition in lipoplex conformation. The addition of isomethyl group in the spacer, Chol-MPRO, decreased transfection efficiency when compared with Chol-PRO. In addition, the presence of multiple bonds in the spacer did not make much difference in the transfection efficiencies at the optimum condition (Fig. 3: Chol-BTA, Chol-BTE, Chol-BTY). The transfection efficiency in 2:1 cationic lipid:DOPE weight ratio (as in a large amount of cationic lipid) was increased in order of single bond (Chol-BTA) < double bond (Chol-BTE) < triple bond (Chol-BTY). The transfection efficiency in 1:2 cationic lipid:DOPE weight ratio (as in a small amount of cationic lipid) was increased in order of single bond > double bond > triple bond (Fig. 2A). In other words, although the best transfection efficiencies were similar, the more cationic lipids in the liposome formulation were required for the optimal transfection condition according to the increase of π-bond from single bond to triple bond (Fig. 2B). It can be attributed to the fact that the face of an electron-rich π system (e.g. benzene, ethylene) noncovalently interact with an adjacent cation including simple inorganic ions (e.g. Li^+, Na^+ and K^+), protonated amines (RNH_3^+), quaternary ammoniums, sulfoniums and carbocations, known as cation-π interaction[15,16] which may inhibit charge interaction between a cationic liposome with plasmid DNA.

In summary, five compounds were newly synthesized based on Chol-ETA to investigate the effect of various spacer structures on the transfection efficiency. These compounds showed sufficient transfection efficiency when compared with commercial cationic liposomes in COS-7 cell. The transfection efficiency of cationic lipid with two methylene unit in their spacer was highest than the others. The addition of isomethyl group in the spacer decreased transfection efficiency when compared with its counterpart, Chol-PRO. Also, the structure including multiple bonds in their spacer required the more cationic lipids in the liposome preparation than single bond to present similar transfection efficiency.

Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (No.2010-0024376).

References and notes

1. Li, Z.; Düllmann, J.; Schiedlmeier, B.; Schmidt, M.; von Kalle, C.; Meyer, J.; Forster, M.; Stocking, C.; Wahlers, A.; Frank, O.; Ostertag, W.; Kühlcke, K.; Eckert, H. G.; Fehse, B.; Baum, C. *Science* **2002**, *296*, 497.
2. Marshall, E. *Science* **2002**, *298*, 510.
3. Woods, N. B.; Muessig, A.; Schmidt, M.; Flygare, J.; Olsson, K.; Salmon, P.; Trono, D.; von Kalle, C.; Karlsson, S. N. B. *Blood* **2003**, *101*, 1284.
4. Ren, T.; Liu, D. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1247.
5. Bajaj, A.; Mishra, S. K.; Kondaiah, P.; Bhattacharya, S. *Biochim. Biophys. Acta* **2008**, *1778*, 1222.
6. Miller, A. D. *Curr. Med. Chem.* **2003**, *10*, 1195.

7. Zabner, J.; Fasbender, A. J.; Moninger, T.; Poellinger, K. A.; Welsh, M. J. *J. Biol. Chem.* **1995**, *270*, 18997.
8. Ghosh, Y. K.; Visweswariah, S. S.; Bhattacharya, S. *Bioconjug. Chem.* **2002**, *13*, 378.
9. Ghosh, Y. K.; Visweswariah, S. S.; Bhattacharya, S. *FEBS Lett.* **2000**, *473*, 341.
10. Bajaj, A.; Kondiah, P.; Bhattacharya, S. *J. Med. Chem.* **2007**, *50*, 2432.
11. Kim, B. K.; Doh, K. O.; Nam, J. H.; Kang, H.; Park, J. G.; Moon, I. J.; Seu, Y. B. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 2986.
12. Yingyongnarongkul, B. E.; Radchatawedchakoon, W.; Krajarng, A.; Watanapokasin, R.; Suksamrarn, A. *Bioorg. Med. Chem.* **2009**, *17*, 176.
13. Takeuchi, K.; Ishihara, M.; Kawaura, C.; Noji, M.; Furuno, T.; Nakanishi, M. *FEBS Lett.* **1996**, *397*, 207.
14. Fichert, T.; Regelin, A.; Massing, U. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 787.
15. Ma, J. C.; Dougherty, D. A. *Chem. Rev.* **1997**, *97*, 1303.
16. Vijay, D.; Sastry, G. N. *Phys. Chem. Chem. Phys.* **2008**, *10*, 582.