Bioorganic & Medicinal Chemistry Letters 22 (2012) 3837–3841

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Synthesis and biological activity of carbamate-linked cationic lipids for gene delivery in vitro

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ARTICLE INFO

Article history:
Received 24 October 2011
Revised 15 January 2012
Accepted 25 January 2012
Available online 2 February 2012

Keywords:
Carbamate-linked cationic lipids
DNA condensation
Gene delivery
Transfection efficiency
Cytotoxicity

ABSTRACT

We have introduced a convenient synthesis method for carbamate-linked cationic lipids. Two cationic lipids *N*-[1-(2,3-didodecylcarbamoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium iodide (DDCTMA) and *N*-[1-(2,3-didodecyl carbamoyloxy)propyl]-*N*-ethyl-*N*,*N*-dimethylammonium iodide (DDCEDMA), with identical length of hydrocarbon chains, alternative quaternary ammonium heads, carbamate linkages between hydrocarbon chains and quaternary ammonium heads, were synthesized for liposome-mediated gene delivery. Liposomes composed of DDCEDMA and DOPE in 1:1 ratio exhibited a lower zeta potential as compared to those made of pure DDCEDMA alone, which influences their DNA-binding ability. pGFP-N2 plasmid was transferred by cationic liposomes formed from the above cationic lipids into Hela and Hep-2 cells, and the transfection efficiency of some of cationic liposomes was superior or parallel to that of two commercial transfection agents, Lipofectamine2000 and DOTAP. Combined with the results of the agarose gel electrophoresis and transfection experiment, the DNA-binding ability of cationic lipids was too strong to release DNA from complex in the transfection, which could lead to relative low transfection efficiency and high cytotoxicity.

Cationic lipids have been favored for many potential advantages compared with viral vectors (such as ease of production, good repeatability and biodegradability, and wide range of clinical application and safety), and thus they have also attracted much attention as transfection reagents and carriers for gene therapy.[1] Since the pioneering work of Felgner et al.[2] on the use of a potential cationic lipid such as *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) in the study of complex transfection, many new cationic lipids with different structures have been synthesized for gene delivery.[3–6]

Cationic lipids are positively charged amphiphiles consisting of three basic chemical functional domains: a hydrophilic headgroup, a hydrophobic domain, and a linker bond that tethers the cationic headgroup and hydrophobic tail domain.[7] In general, the cationic headgroup is believed that electrostatic interaction between positive charge of cationic headgroup and polyanionic DNA might distort some cationic liposomes to form at least a partial bilayer wrapping on DNA molecules during the complex formation.[8] In the headgroup, the quaternary ammonium headgroup is by far the most frequently used in many of the established cationic lipids (such as DOTAP, DOTMA, DORIE) because of its lower steric hindrance around the nitrogen atom.[2–7] The hydrophobic domain of cationic lipids usually composed of saturated or unsaturated alkyl chains between C_{12} and C_{18} or of steroid groups determines the phase transition temperature and the fluidity of the bilayer, influences the stability of liposomes, protects the DNA from nucleases, and the release of DNA from complex. Cationic lipids containing two linear aliphatic chains have been widely investigated as the non-viral vectors, because they are capable of forming liposomes by themselves or with a helper lipid (such as DOPE, DOPC).[7] Linker bonds of cationic lipids commonly include ethers, esters, amides or carbamates, which have larger influence on the transfection efficiency, biodegradability and the stability of cationic lipids. The most frequently investigated aspect of linkers refers to a comparison between ether and ester type linkers, however, ether linker is reported to be chemically stable and non-biodegradable thereby leading to higher cytotoxicity; by contrast, ester is chemically not stable, but becomes prone to its biodegradation during systemic circulation.[9–11] Compared with those linkers, amides (as in DOGS) and carbamates (in DC-Chol) are not only chemically stable but also biodegradable.[12,13]

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0960-894X/$ - see front matter © 2012 Published by Elsevier Ltd.
doi:10.1016/j.bmcl.2012.01.097

Many research articles pertaining to pH-sensitive lipids for gene therapy are highlighted recently, and some of them detail the synthesis of cationic lipids containing variable length of hydrocarbon chains, alternative quaternary ammonium headgroups, and carbamate linker bonds between quaternary ammonium headgroups and hydrocarbon chains.[12–14] However, they used alkyl isocyanates as the reaction intermediates, which are relatively toxic and challenging from a purification perspective.[15] There are no published reports about the influence of formulations on the variability in gene expression into Hela cells. Therefore, the development of an environment-friendly and pragmatic method for carbamate synthesis represents the pivotal step, and we introduced environment-friendly raw material (*N*,*N*-carbonyldiimidazole) for the synthesis of carbamate-linked cationic lipids. The technological and synthesis conditions were optimized by studying the reaction condition by ESI-MS spectrometry. These carbamate-linked cationic lipids were also processed as liposomal delivery systems, which were evaluated for their transfection efficiency into Hela cells and compared with that of two commercial transfection agents, Lipofectamine2000 and DOTAP.

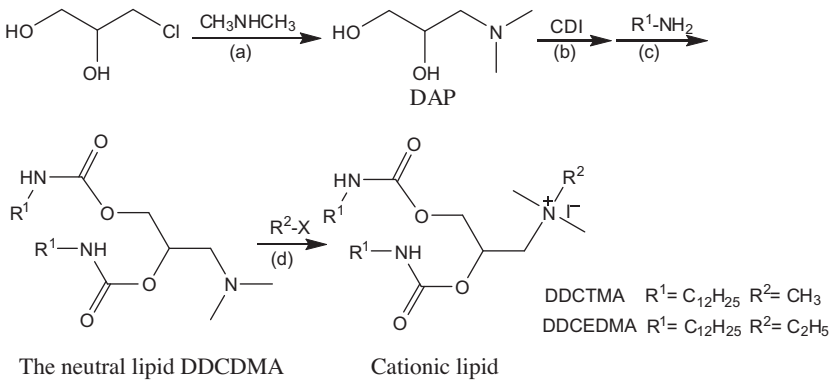
Compounds containing carbamate-linked are stable in the neutral pH condition but liable to acid-catalyzed hydrolysis under appropriate circumstances.[12,13] Because of their special chemical properties, carbamate-linked lipids have been widely used in drug delivery to achieve high delivery efficiencies.[16,17] The most adopted approach for the preparation of carbamate-linked compounds is an addition reaction taking place on isocyanate and alcoholic hydroxyl group.[12] However, isocyanates are powerful irritants to the mucous membranes of the eyes and respiratory tracts. The presently known methods for the synthesis of isocyanates usually introduce highly toxic phosgene, cyanic compounds, or explosive azides. Thereby this method is not suitable for the syntheses of most cationic lipids. We synthesized carbamate-linked cationic lipids by using environment-friendly CDI as a raw material (Scheme 1). The particularity of CDI is its ability to react with alcohol, carboxylic acid and amine groups giving rise to esters, ketone, carbamates and ureas.[18,19] The advantages of this method include the mild reaction conditions which minimize subsidiary reaction, the lack of formation of amine hydrochloride when using acid chlorides, and circumventing lengthy purification steps. Moreover, imidazole, the by-product obtained both when CDI reacts with hydroxyl and carboxylic acid and after the reaction of carbonyl imidazole with alcohol or amine, is easily removed from the reaction mixture by an acidic wash.

In initial experiments, to the magnetic stirred solution of CDI in toluene at +40 °C with a dry N_2 inlet, the solution of laurylamine in toluene was added dropwise over 1 h and the reaction mixture was stirred at 40 °C for 2 h, and then the solution of DAP in toluene was added dropwise over 1 h and the obtained reaction mixture was stirred for 2 h at the same temperature. The molecular formula of DDCDMA is $C_{31}H_{63}N_3O_4$ and the calculated molecular weight is 541.48 Da. However, positive electrospray mass spectra of DDCDMA-1 (*m*/*z* calcd for [M+H], 542.49; [M+Na], 564.48) indicate that the preparing process cannot meet the requirements as shown in Figure 1A. It may be because the amino groups exhibit high nucleophilicity and react with CDI to generate some compounds containing uramido.[20] Consequently, we changed the reaction condition, namely, DAP was first reacted with CDI, and then laurylamine was added. The main peaks at *m*/*z* 542.10 represented $[M+H]^+$ and *m*/*z* 564.10 represented $[M+Na]^+$, which was in accordance with target intermediate DDCDMA (Fig. 1B). Another peak at *m*/*z* 330.95 represented a single-substitution product (Fig. 1D) in the acquisition. Thus, the crude product needs further purification. In the previous literature, it was purified by gel column, and eluted with chloroform/methanol (4:1 vol/vol) to yield the desired compounds.[13] In this paper, it was purified through recrystallization from the alkane solution to obtain purified DDCDMA as shown in Figure 1C.[21] Toward studying gene transfer efficiencies of lipids bearing different headgroups, cationic lipids (DDCTMA and DDCEDMA) were also synthesized following the synthetic route as shown in Scheme 1.

Agarose gel electrophoresis was used to assess DNA binding at different conditions. In order to determine the effect of cationic lipid headgroup structures, DOPE, and cationic lipid to pGFP-N2 plasmid weight ratios (N/P ratios), pGFP-N2 plasmid complexes were prepared by adjusting the stoichiometry of cationic liposomes and plasmid (N/P, 0.5:1, 1:1, 1.5:1, 2:1, 3:1, 4:1, 6:1 and 8:1), using liposomes prepared from cationic lipid (DDCTMA or DDCEDMA) and DOPE at identical molar ratios.

The results of the gel retardation assay indicated that the DNA-binding ability of cationic liposomes increased with an increase in the N/P ratio (Fig. 2). As shown in Figure 2A, the mobility of plasmid DNA was not observed when cationic lipid with methyl group (DDCTMA) was complexed with DNA at the N/P ratios of 3:1 and above. Effective DNA condensation was achieved with cationic liposomes containing an ethyl group (DDCEDMA) at the N/P ratios of 4:1 and above, since no mobility of plasmid DNA was observed at these weight ratios (Fig. 2C). This might be due to the smaller steric hindrance of the trimethyl head, which may combine with DNA easily. Cationic lipid headgroup is closely related to their DNA-binding ability.[22]

When mixed with DOPE, obvious differences were found in the DNA condensation capability of cationic liposomes (DDCTMA/DOPE = 1:1 and DDCEDMA/DOPE = 1:1) as shown in Figure 2. At the N/P ratios of 6:1 and above, the plasmid DNA was completely complexed with DDCTMA/DOPE = 1:1, whereas DDCEDMA/



Scheme 1. Synthetic routes of cationic lipid. Reagents and conditions: (a) 2.5 equiv 33% dimethylamine in aqueous solution, 1.0 equiv sodium hydroxide, 4 h at 50 °C, (70%); (b) 2.1 equiv CDI in toluene solution, 3 h at 40 °C with N_2; (c) 2.1 equiv alkylamine in toluene solution, 3 h at 40 °C with N_2, (50–60%); (d) 40 equiv halogenated hydrocarbons, 24 h, 70–80 °C (90–95%).