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Original article

Benzothiazole head group based cationic lipids: Synthesis and application for gene delivery

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ABSTRACT

A series of benzothiazole based lipids (**1**–**10**) containing different derivatives of benzothiazole in the head group region were synthesized to determine the structure–activity relationship for gene delivery. The liposomes formulated were mixed with plasmid DNA encoding green fluorescent protein (α5GFP) or β-galactosidase (*p*CMV-SPORT-β-gal) and transfected into B16F10 (Human melanoma cancer cells), CHO (Chinese hamster ovary), A-549 (Human lung carcinoma cells) and MCF-7 (Human breast carcinoma cells) types of cell lines. The efficiencies of lipids **9** and **10** in particular, were found to be comparable and even more when compared to that of LipofectAmine-2000. The transfection profiles of the efficient lipids are proved to be maintained even in the presence of serum. Thus, the benzothiazole head group based lipids developed have the potential to be used as transfection reagents in vitro and in vivo.

1. Introduction

The Field of cationic lipids, pioneered by Felgner [1,2], Behr [3,4], and Huang [5,6], has evolved exponentially, so that many different compounds have been reported [4,7–10]. They include diverse kinds of cationic lipids such as glycerol backbone [11], cholesterol based [6], aliphatic-hydrocarbon-tail based [12] and tocopherol based lipids [13,14]. Despite tremendous progresses, the therapeutic efficiency of cationic lipid-based transfection systems remains relatively low as compared to viral ones. This is largely due to incompatibilities between the lipid structure in the lipoplexes and the structure of the various physiological barriers encountered in vivo by the plasmid in the lipoplex on its way to the nucleus of target cells [15,16]. These incompatibilities can result in inefficient delivery of the required gene and in cytotoxic effects. Some of these problems are associated with the cationic nature of the vectors [17]. A solution found to circumvent these problems is to spread the positive charge of the cationic head by delocalizing it into a heterocyclic ring.

Upon the invention of pyridinium-based cationic lipids in 1940s, various heterocyclic cationic lipids had been studied and used for gene delivery. They include a series of these pyridinium heterocyclic ring based lipids [18,19], lipids with imidazolium polar heads [20], glycerol- and cholesterol-based positively charged heterocyclic amphiphiles [21,22] and Gemini lipids with aromatic backbone [23]. Heterocyclic cationic lipids containing imidazolium or pyridinium polar heads [19,20] have been reported to display higher transfection efficiencies and reduced cytotoxicity when compared with classical transfection systems.

In our continuous upsurge of designing novel and efficient vectors in the field of cationic lipid gene delivery, we exploited the positive charge delocalizing property of benzothiazole moiety in designing new series of lipids. Benzothiazole template is a privileged structure fragments in modern medicinal chemistry considering its broad pharmacological spectrum and affinity for various biotargets of these class heterocyclic compounds. Aminobenzothiazoles and related heterocycles represent a novel class of potent and selective antitumor agents which exhibit nanomolar inhibitory activity against a range of human breast, lung, colon, leukemia, CNS, melanoma, ovarian, renal and prostate cell lines

Abbrevations: Lipofect, LipofectAmine-2000; DMEM, Dulbecco's modified Eagle's medium; DMAP, 4-(dimethylamino)pyridine; FBS, fetal bovine serum; PBS, phosphate-buffered saline; ONPG, *O*-nitrophenyl-β-D-galactopyranoside.

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in vitro [24,25]. Benzothiazoles are used as organic NIR (Near-infrared) fluorophores and have been found to be the most useful [26]. Their physical properties, biodistribution, pharmacokinetics and applications for in vivo fluorescence imaging have been summarized [27,28].

To the best of our knowledge, there is no example using benzothiazole as the hydrophilic head group of cationic lipid for gene delivery. In the present study, we designed and synthesized a series of cationic lipids (**1**–**10**) with benzothiazole derivatives in the head group region of lipids. Their interactions with plasmid DNA and the properties of formed lipoplexes were examined. The in vitro transfection efficiencies towards four cell lines were investigated to study the SAR (structure activity relationship) of this type of cationic lipids in gene delivery. Results indicated that small changes in the lipid structure such as replacement of a nitro group with an amino group or methoxy group with a hydroxyl group or changes in the chain length attached (C_{16} and C_{18}) might lead to essential distinction in the in vitro transfection efficiencies. Lipids **9** and **10** with amine group as substituent have much higher reporter gene transfection efficiency than LipofectAmine-2000 in CHO and MCF-7 types of cells.

2. Results and discussion

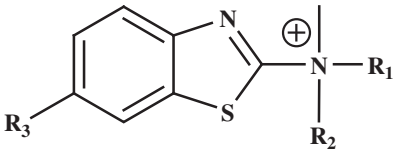
The present work illustrates the synthesis of lipids **1**–**10** with benzothiazole derivatives in the head group region and their physicochemical characteristics. The results of in vitro transfection experiments performed on four different types of cell lines to assess the transfection efficiencies of lipids **1**–**10** are reported. In addition, the inverted fluorescent microscope experiments in support of the results obtained in in vitro transfection studies are reported. A study of cytotoxicity in all the four types of cell lines used for transfection experiments and serum compatibility of transfection efficient lipids (**5**, **6**, **9** and **10**) in four types of cell lines are also reported.

2.1. Chemistry

The synthesized lipids **1**–**10** (Chart 1) described herein show some common structural features which include (a) presence of derivatives of benzothiazoles in the head group region and (b) the presence of *n*-hexadecyl or *n*-octadecyl group in the hydrophobic region. The details of the synthetic procedures for all the novel transfection lipids shown in Chart 1 are described in the Experimental section. As outlined in Scheme 1 (A, B, C), the reactions involved in preparing these new lipids are straightforward. Cationic lipids **1**–**4**, **7** and **8** were synthesized by the *N*-alkylation reaction with derivatives of 2-amino benzothiazoles available commercially to yield the respective intermediate tertiary amines. The resulting tertiary amine intermediates upon quaternization with excess methyliodide followed by chloride ion exchange over Amberlyst-26 yielded lipids **1**–**4**, **7** and **8** (Scheme 1 (A, B, C)). Lipids **5** and **6** were synthesized from lipids **3** and **4** respectively by demethylation of methoxy group present on these lipids. Similarly, lipids **9** and **10** were yielded respectively by reduction of nitro group present on lipids **7** and **8**. Structures of all the synthetic intermediates and final lipids shown in Scheme 1 (A, B, C) are confirmed by ^{1}H NMR and molecular ion peaks in their ESI mass spectra.

2.2. Liposomal formation

Liposomes could be conveniently prepared from a mixture of lipid with varying amounts of cholesterol, 1,2-dioleoyl-*sn*-glycero-phosphoethanolamine (DOPE) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) as colipids. Out of various combinations of mole ratios of lipid to colipid (0.5:0.5, 1:1, 1:2, 1:3, and 1:4) tried, it is found that a 1:1 molar ratio of lipid and colipid is found to form optically transparent suspensions. For the present lipids **1**–**10**, cholesterol was found to be a more efficacious colipid when compared to DOPC and DOPE (data not shown). This may be due to the greater steric requirements of the benzothiazole polar head, which favor cholesterol over DOPE and DOPC as reported earlier [17]. The beneficial effect of cholesterol for the fluidity of bilayers made out of lipids with an elevated critical temperature is well documented [29]. Liposomes were prepared under sterile conditions and were sonicated for 5 min at room temperature before transfection experiments. The vesicular suspensions were found to be stable even after two months if stored at 4 °C (stability studies, Supporting information).



Lipid 1: R_1,R_2 = $C_{16}H_{33}$; R_3=H

Lipid 2: R_1,R_2 = $C_{18}H_{37}$; R_3=H

Lipid 3: R_1,R_2 = $C_{16}H_{33}$; R_3=OCH_3

Lipid 4: R_1,R_2 = $C_{18}H_{37}$; R_3=OCH_3

Lipid 5: R_1,R_2 = $C_{16}H_{33}$; R_3=OH

Lipid 6: R_1,R_2 = $C_{18}H_{37}$; R_3=OH

Lipid 7: R_1,R_2 = $C_{16}H_{33}$;R_3=NO_2

Lipid 8: R_1,R_2= $C_{18}H_{37}$; R_3=NO_2

Lipid 9: R_1,R_2 = $C_{16}H_{33}$; R_3=NH_2

Lipid 10: R_1,R_2 = $C_{18}H_{37}$; R_3=NH_2

Chart 1. Structure of lipids **1**–**10**.

2.3. Transfection biology

2.3.1. In vitro transfection studies

The relative in vitro gene delivery efficacies of lipids **1**–**10** in CHO, B16F10, A-549 and MCF-7 cells across the lipid:DNA charge ratios of 8:1 to 1:1 using cholesterol as colipid are summarized in Figs. 1–4. *p*CMV-SPORT-β-gal plasmid DNA was used as the reporter gene. The transfection efficiencies of the lipids **1**–**10** were compared with that of the LipofectAmine-2000. The results of Figs. 1–4 summarize the following transfection profiles. The transfection results reveal that there exists a difference in transfection profiles of benzothiazole based lipids **1**–**10** based on hydrophobic group. When comparing similar pairs across all experiments, it is found that among the pairs 1/2, 3/4, and 9/10, lipids with C_{16} performs better than lipids with C_{18} nearly 70% of time, and among the pairs 5/6 and 7/8, lipids with C_{16} and C_{18} perform nearly 50% of time better than each other.

Among lipids **1**–**10**, lipids **5**, **6** with hydroxyl group and lipids **9**, **10** with amino group substituents respectively at the 6-position of