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Note

Synthesis of cholesterol modified cationic lipids for liposomal drug delivery of antisense oligonucleotides

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Abstract

The paper describes a novel synthesis of cholest-5-en-3β-yl-6-aminohexyl ether (AH-Chol). AH-Chol was used to prepare positively charged liposomes. The liposomes consisted of phospholipon 90H and the cationic cholesterol derivative in an equimolar ratio. Liposome preparation was achieved by membrane homogenization after rehydration of a dry lipid film. Oligonucleotides (ODN) were adsorbed to the cationic liposomes very efficiently. At an ODN/liposome ratio of 1:5 (10:50 μg/ml) 84.2 ± 5.4% of the ODNs were bound to the liposomal membrane. Within the range of 1:40 and 1:100 charge neutralization occurred and the liposome dispersion showed an increase in particle size due to aggregation. Below or above this range of charge neutralization the ODN loaded liposome preparation was physically stable, no sedimentation, increase of vesicle size or vesicle aggregation occurred.

Keywords: Antisense oligonucleotides; Liposomes; Cholesterol; AH-Chol

1. Introduction

Over the past decade, antisense oligonucleotides (ODNs) have been investigated as potential inhibitors of the protein expression cascade. Most of these studies used ODNs in rather high concentrations, because oligonucleotides, being anionically charged macromolecules, do not penetrate into cells easily. For that reason cationic liposomes were investigated as DNA uptake enhancers, first for gene therapy and consequently also for antisense trials. The first cationic lipid DOTMA was introduced by Felgner in 1987 [1]. In the following years many derivatives of cationic lipids were synthesized, basically consisting of a quaternary ammonium head group and a lipophilic part. The lipophilic part of these molecules often represents non-saturated chained fatty acids like oleic acid. These residuals are able to destabilize cell membranes as well as endosomal membranes which seems to promote penetration enhancement. However, they also destabilize the liposomal membrane and reduce stability of the galenic formulation. In addition, due to the permanently charged ammonium group most of these preparations showed significant toxicity [2].

For that reason the aim of the present work was to design and to prepare a cationic lipid based on cholesterol, which is known to stabilize liposomal membranes to which a lipophilic aminohexyl chain is attached as chargeable headgroup (AH-Chol). The hexylspacer was linked to the cholesterol via an ether bond to prevent enzymatic cleavage of the lipid in biologic media.

Liposomes were prepared from this novel lipid compound in an equimolar ratio with saturated phosphatidylcholine (PC). Finally, we investigated the oligonucleotide binding to the pre-formed liposomes, the stability and size distribution of the ODN/liposome complex.

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2. Materials and methods

2.1. Chemicals

The following chemicals, 4-dimethylaminopyridine (DMAP), ethyl acetate (EtOAc); ethanol (EtOH), triethylamine (NEt_3), petroleum ether (PE), *p*-toluenesulfonic acid chloride (pTosCl), pyridine (py), methylenchloride (CH_2Cl_2), cholesterol, 6-amino-1-hexanol, dioxane, potassium carbonate (K_2CO_3), methanol (MeOH), were purchased from Merck (Darmstadt, Germany) and were used as obtained. The phosphodiester oligonucleotide (ODN) with the sequence 5′-ATGTTGCCCCATCATAAA-3′ (AS-2) used in this report was synthesized by MWG-Biotech (Ebersberg, Germany).

2.2. Synthesis of AH-chol

AH-Chol (Cholest-5-en-3β-yl 6-aminohexyl ether) was synthesized following a three-step procedure (Fig. 1). First cholesterol **1** was tosylated resulting in cholest-5-en-3β-yl *p*-toluenesulfonate **3**. This product reacted with (6-hydroxyhexyl)trifluoracetamide **2** to form the trifluoracetic acid protected amidohexyl ether **4**. Finally, AH-Chol was prepared by deprotection of compound **4** in methanol under mild basic conditions.

2.2.1. Synthesis of (6-hydroxyhexyl)trifluoracetamide (2)

To a solution of 100 g (0.85 mol) 6-amino hexan-1-ol in dichloromethane 142 g (1 mol, 1.2 equiv.) of ethyl trifluoracetate was added slowly. After stirring for 5 h, the mixture was concentrated in vacuo to 100 ml and was stored at 0ºC for 12 h resulting in colourless crystals. After filtration the precipitate was collected, washed with PE and was dried in vacuo to yield 174 g (96%) of the product. m.p. = 49–50°C; mol. wt. 213.20; $C_8H_{14}F_3NO_2$; TLC (I) R_f = 0.42 (CH_2Cl_2:EtOH 9:1 v/v); ^{1}H-NMR ($CDCl_3$) δ = 7.16 (bs, 1H, NH); 3.63 (t, J = 7.1 Hz, 2H, CH_2OH); 3.34 (q, J = 7.1 Hz, 2H, CH_2NH); 1.6–1.4 (m, 8H, CH_2). ^{13}C-NMR ($CDCl_3$) δ = 158.14–156.68 (q, CO); 121.58–110.15 (q, CF_3); 62.43 (t, CH_2OH), 39.75 (t, CH_2NH); 32.48/32.25/26.24/25.13 (t, CH_2).

2.2.2. Synthesis of cholest-5-en-3β-yl p-toluenesulfonate (3)

One hundred grams (0.26 mol) cholesterol was dissolved in 100 ml dry triethylamine and 5 ml dry pyridine under an argon atmosphere. Then 0.15 g (1.3 mmol) DMAP and 74 g (0.39 mol, 1.5 equiv.) *p*-toluolsulfonic acid chloride was added and the mixture was stirred at room temperature over night. The mixture was evaporated and partitioned between ether and 0.1 N HCl and was extracted several times with 0.1 N HCl. The organic phase was washed with a diluted solution of $NaHCO_3$ and the combined organic phases were dried over Na_2SO_4. Crystallization from acetone yielded 123.9 g (88%) of compound **3** as colourless needles, m.p. = 134°C; mol. wt. 540.86; $C_{34}H_{52}O_3S$; TLC (II) R_f = 0.65 (PE:ether 3:1 v/v); ^{1}H-NMR ($CDCl_3$) δ = 7.77 (d, J = 8 Hz, 2H, arom.); 7.30 (d, J = 8 Hz, 2H, arom.); 5.27 (d, J = 5.2 Hz, 1H, 6H); 4.35–4.23 (m, 1H, 3H); 2.42 (s, 3H, CH_3-arom.), ESI$^+$-MS (Da/e) 558.2 $(M + NH_4)^+$.

2.2.3. Synthesis of cholest-5-en-3β-yl 6-trifluoracetic acid amidohexyl ether (4)

Samples of 20.25 g (95 mmol, 4 equiv.) of alcohol **2** and 12.87 g (23.8 mmol) of cholesteryltosylate **3** were dissolved in dry dioxane under argon atmosphere and boiled for 22 h. The reaction was then evaporated to a gum and partitioned between CH_2Cl_2 and water. The organic layer was dried (Na_2SO_4), evaporated to dryness, and the residue was purified by vacuum flash chromatography (10–50% EtOAc in PE) yielding 7.80 g (42%) of a colourless crystalline product after evaporation upon standing. m.p. = 55°C; mol. wt. 581.85; $C_{35}H_{58}F_3NO_2$; TLC (I/II) R_f = 0.31 (PE:EA 9:1 v/v); ^{1}H-NMR ($CDCl_3$) δ = 6.41 (bs, 1H, NH); 5.31 (d, J = 5.1 Hz, 1H, 6H); 3.42 (t, J = 6.5 Hz, 2H, OCH_2); 3.33 (q, J = 6.7 Hz, 2H, NCH_2); 3.15–3.02 (m, 1H, 3H).

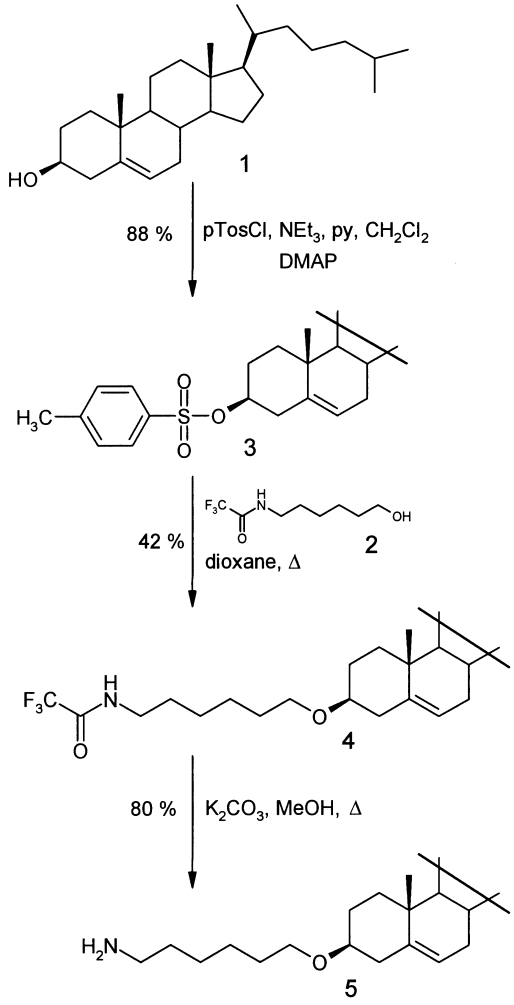

Fig. 1. Synthesis of cholest-5-en-3β-yl-6-aminohexyl ether (AH-Chol) (**5**).

*2.2.4. Synthesis of cholest-5-en-3β-yl 6-aminohexyl ether (ah-chol) (***5**)

A sample of 5.1 g (8.76 mmol) of **4** was dissolved in 100 ml of methanol, 12.11 g (87.6 mmol, 10 equiv.), K_2CO_3 was added and the suspension was heated for 24 h. The reaction mixture was evaporated to a gum and partitioned between CH_2Cl_2 and water. AH-Chol was extracted four times with CH_2Cl_2 at pH 12. The sampled organic liquids were dried over Na_2SO_4/K_2CO_3 3:1, filtrated and evaporated to obtain 3.41 g (80%) of the pure wax-like product. Mol. wt. 485.84; $C_{33}H_{51}NO$; ^{1}H-NMR ($CDCl_3$) δ = 5.31 (d, J = 5.1 Hz, 1H, 6H); 3.42 (t, J = 6.7 Hz, 2H, OCH_2); 2.66 (t, J = 6.9 Hz, 2H, NCH_2); 3.15–3.02 (m, 1H, 3H), ESI$^+$-MS (Da/e) 486.4 $(M + H)^+$.

2.3. Preparation of liposomes

Liposomes were prepared using a film method followed by extrusion. The liposome preparation consisted of equimolar ratios of AH-Chol and phosphatidylcholine (PC) (Phospholipon™ 90H, Nattermann, Germany). The lipids were dissolved (10 mmol) with 15.0 ml chloroform (Merck, Darmstadt, Germany) in a round-bottom flask. The solvent was evaporated under reduced pressure (Rotavapor, Büchi, Switzerland) and the resulting film was dried under vacuum for at least 2 h. Subsequently, the lipid film was rehydrated at 65°C with purified water for about 1 h. Alternatively, reconstitution was performed with phosphate buffered (10 mM) isotonic mannitol solution pH 5.5 or phosphate buffered saline (PBS) pH 7.4. Glass beads were added to facilitate homogenization, resulting a multilamellar large vesicle (MLV) suspension. This raw suspension was processed in 1 ml steps and 20 cycles with a LiposoFast homogenizer (Avestin, Germany) using a 400 nm polycarbonate membrane.

Oligonucleotide loading to pre-formed liposomes was achieved by adsorption of the anionic ODNs to the cationically charged liposomes. Briefly, 100 μl of the specified ODN concentration was mixed in a 1.5 ml reaction tube at room temperature with an equal amount of the liposome preparation at different ratios. The amount of unbound ODNs was measured after ultracentrifugation (100 000 × *g*, 2 h, Beckman Optima L-80, USA) by SAX-HPLC analysis of the supernatant as published before [3] and the ODN binding was calculated from this value with respect to the initial ODN concentration.

2.4. Characterization of liposomes

The vesicle diameter was determined by photon correlation spectroscopy (PCS). The PCS instrument consisted of a BI-200 SM Goniometer Vers. 2.0 connected to a BI-2030 AT Digital Correlator (Brookhaven Instruments, Holtsville, NY). Measurements were taken at a 90° scattering angle and at 25°C.

The surface charge (zeta potential) of the liposomes was determined from the electrophoretic mobility. Measurements were performed using a Lazer Zee Meter™ Model 501 (PenKem, Bedford Hills, NY). All samples were adjusted to a final concentration of 100 μg/ml in deionized water. The measured zeta potential was calculated and corrected for a standard reference temperature of 20°C.

3. Results and discussion

3.1. Synthesis of cholest-5-en-3β-yl 6-aminohexyl ether (ah-chol)

As described in Fig. 1, cholesteryl tosylate **3** was synthesized according to a modified procedure of Kosower and Winstein [4]. Deprotection of the intermediate amine using known methods produced cholest-5-en-3β-yl 6-aminohexyl ether **5** in a three-step synthesis with an overall yield of 30%. This modified synthesis is a major improvement in comparison to the six step synthesis described by Shen et al. [5,6], and shortened the chemical synthesis described by Carroll et. al. [7] significantly.

3.2. Preparation of liposomes

Liposomes were prepared from the synthesized AH-Chol and a commercially available lipid, phospholipon 90H (PC), in equimolar ratios. In the first step a standard film method was applied followed by a reconstitution of the dry lipids in an aqueous phase, resulting a MLV raw dispersion. The size distribution of this MLV preparation was determined to range from 1.5 to 3 μm (data not shown).

The raw dispersion was homogenized using a lab scale membrane homogenizer as published previously [8]. An effective diameter of about 400–450 nm was achieved for the pure liposome preparation using a 400 nm polycarbonate membrane. This preparation was physically stable, no sedimentation or increase in vesicle size was observed at 4°C within 3 months.

3.3. Characterization of oligonucleotide loaded liposomes

Liposomes consisting of PC / AH-Chol 1:1 showed a high adsorption capacity for ODNs. At a ODN/liposome ratio 1:5 (10:50 μg/ml) 84.2% (SD 5.4%, n = 3), and at an increased ratio of 1:25 (10:250 μg/ml), 99.8% (SD 0.5%, n = 3) of the ODNs were adsorbed to the lipid membrane in purified water. This adsorption profile was found to be pH and salt dependent. Equivalent results were obtained for the adsorption in purified water as well as in phosphate-buffered isotonic mannitol solution (pH 5.5). A slightly reduced adsorption capacity was observed for PBS (pH 7.4) buffer. The pK_a value for AH-Chol was calculated to be 10.61 (±0.1) using the ACD pKa program (http://www.acdlabs.-com). Therefore, overall protonization at physiological pH

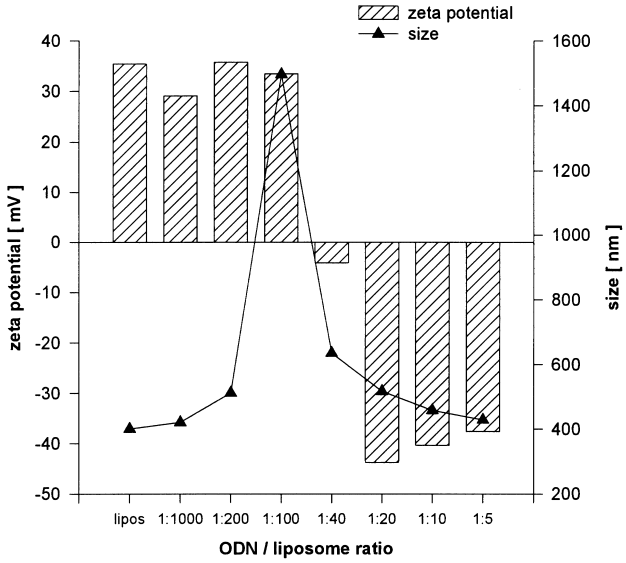

Fig. 2. Vesicle size and zeta potential of oligonucleotide loaded preparations determined in purified water at different ODN/liposome ratios (w/w). Data representing mean values: size measurements $n = 3$, zeta potential $n = 10$.

can be predicted from this pKa value facilitating high ODN adsorption.

Fig. 2 shows that the overall positive charge of the liposomes is neutralized between a ODN/liposome ratio ranging from 1:100 to 1:40. This neutralization is also indicated by a vesicle aggregation, which did not occur at higher ODN concentrations and negative zeta potentials.

From this charge titration profile including the weight and molecular ratio of ODNs as well as the number of the phosphodiester groups of an oligonucleotide, it can be calculated that about 10% of the chargeable AH-Chol amino group are present at the surface of the liposomes. This is in concordance with EM pictures showing an oligolamellar structure of the 400 nm liposomes (picture not shown). Therefore, most of the charged lipids are present in the inner side of the liposome and are not available for the adsorption of the ODNs. In our case this inner structure seems to be beneficial for the galenic stability of the ODN/liposome preparation and changes in vesicle size were not observed after adsorption of ODNs, with the exception of the charge neutralization range. In contrast to these results many cationic lipids, and also derivatives based on cholesterol like DC-Chol, showed changes in the vesicle arrangement after the adsorption of ODNs or plasmid DNA [9,10]. However, also the preparation method of the ODN/lipid complex as well as the structure of pre-formed liposomes seems to be crucial for the physical stability of an ODN drug formulation.

Concluding our results a cationically-charged derivative of cholesterol was synthesized and a liposome formulation with high ODN adsorption capacity and improved stability was prepared. Further studies will focus on the cytotoxicity as well as on antisense effects of this promising formulation.

Acknowledgements

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