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Novel Lipophilic Chloroquine Analogues for a Highly Efficient Gene Transfer into Gynecological Tumors

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Abstract—Liposomal vectors based on cationic lipids have been proven to be an attractive alternative to viral vectors in gene therapy protocols with regard to safety and manufacturing concerns. In order to improve the transfection efficiency we have synthesized two novel carboxycholesteryl-modified chloroquine analogues. Due to their potential endosomal buffering capacity these compounds enable the efficient transfection of various gynecological tumors and therefore are promising reagents in gene therapy applications.

Introduction

During the last decade nonviral gene therapy using liposomal vectors in some respects has been proven to be advantageous in comparison to viral vectors. Liposomal vectors based on cationic lipids are much more easy to produce under cGMP conditions with fewer safety concerns. They are less toxic, nonimmunogenic and the introduced genes do not integrate into the host genome.

Since the first application of liposomal vectors for gene transfer,[1] a broad range of cationic lipids was synthesized on a mostly empirical basis.[2] While the lipidic part of these molecules usually comprises cholesterol or diglyceride-like structures, many modifications of the cationic headgroups were described. In general the cationic lipids have to be mixed with 30–70 molar percent of a proprietary helper lipid. Especially suitable is the neutral phospholipid 1,2-dioleyolglycerol-3-sn-phosphoethanolamine (DOPE) which is able to induce inverted hexagonal phases and therefore has fusogenic properties.[3] Due to electrostatic interactions the thus obtained positively charged bilayers form aggregates with plasmid DNA and other polyanions in a size range of 100–1000 nm.

Although the mechanism of cellular uptake of these aggregates (lipoplexes) is still unknown, there is evidence that they are internalized via an endocytotic pathway. Due to vacuolar H^+-ATPases the endosomal pH value drops to about 4–5 during maturation of the primary endosomes. Further there is evidence that this acidification is a prerequisite for the fusion of the endosomes with lysosomes.[4] To prevent the lysosomal degradation, the endocytosed aggregates should be able to buffer the endosomal pH drop. Until now the only cationic lipids which possess sufficient buffer capacity at physiological pH are lipopolyamines.[5] Unfavorably, the lipopolyamines are multi-functional molecules with only slight differences in the chemical reactivity of each function. Therefore their synthesis necessitates the use of orthogonal protecting group strategies and they have to be produced in multistep reactions. Another possibility to prevent endosomal acidification is the addition of weak bases to the cell culture medium. A 10–100 μM supplement of chloroquine could lead to a significant enhancement of the transfection efficiency[6] but obviously this method is limited to in vitro/ex vivo applications.

In order to combine the outstanding buffering properties of chloroquine-like compounds with lipidic structures for use in gene transfer experiments, we describe for the first time the synthesis of the carboxycholesteryl-modified chloroquine analogues CCQ22 (**3a**) and CCQ32 (**3b**).

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Materials and Methods

Unless otherwise stated all chemicals were purchased from Fluka (Buchs, Switzerland) and were of the highest available quality. The polyamine **1a** was purchased from Lancaster Synthesis GmbH (Mühlheim a. Main, Germany) while amine **1b** was synthesized by cyanoethylation of *N*,*N*-dimethyl-ethylenediamine and subsequent reduction with lithium tetrahydridoaluminate in tetrahydrofuran. DC-Chol was synthesized and formulated as described previously.[7] Cells were grown in accordance to standard ATCC conditions. The lipoplex preparation and the simultaneous determination of the transfection efficiency and cell viability was carried out as described previously.[8] Briefly, solutions of DOPE and the cationic lipids in chloroform were mixed in different molar ratios. The mixtures were evaporated in vacuo and the thus obtained lipid films were rehydrated with water to a final concentration of 1 mg/mL and sonified for 5 min in an ultrasonic bath. In a 96-well plate the aqueous liposomal dispersion was 2-fold serially diluted from 8 to 0.5 μg total lipid in 40 μL serum free medium. The lipids were then added to a solution of 0.5, 1, respectively, 1.5 μg of plasmid DNA (pRc/CMVlacZ)[9] in 40 μL serum free medium. After 30 min the thus obtained lipoplexes were added to the cells, which were grown in a 96-well plate to approximately 60–70% confluency and were supplemented with 80 μL medium containing 20% fetal calf serum. After 48 h the β-galactosidase expression [enzyme activity in mU per well] and the relative cell viability were determined according to the literature.[8]

Results

As outlined in Figure 1, the nucleophilic aromatic substitution of 4,7-dichloroquinoline with the polyamines **1a**(**b**) in molten phenol yields the chloroquine analogues **2a**(**b**). Presumably because of the lower sterical hindrance the primary amino group reacts nearly exclusively.[10] Due to the strong electron withdrawal of the aromatic nitrogen the electrophilicity of the ring bound secondary amine is highly decreased and the reaction with cholesterolchloroformate leads to the acylation of the aliphatic secondary amine. For the same reason it is not possible to acylate chloroquine itself. The cationic lipids CCQ22 (**3a**) and CCQ32 (**3b**) were obtained in 25–30% overall yield after purification by flash chromatography with dichloromethane/methanol as eluent. The purity of the compounds was further determined by HPLC analysis. Due to the strong UV absorption of the aromatic ring system they can be easily detected at a wavelength of 220 nm. This is advantageous in comparison to hitherto described cationic lipids which contain only poor chromophores and are hardly detectable by UV absorption.

In order to determine the suitability of the synthesized cationic lipids for the transfection of eucaryotic cells, they were mixed with the neutral phospholipid DOPE in different molar ratios. For lipoplex formation aqueous liposomal dispersions of the lipids were mixed with plasmid DNA (pRc/CMVlacZ) in serum free medium and were used for the transfection of three gynecological tumor cell lines (Hey, SKBR3, SKOV3) in the presence of 10% serum.

The transfection efficiency and cell viability in comparison to the well known lipid DC-Chol was optimized simultaneously according to a novel 96-well assay, which uses β-galactosidase as reporter gene and acidic phosphatase as a relative measure of the cell viability.[8] The results are shown in Figures 2 and 3. The cationic lipid CCQ22 shows superior transfection efficiency in comparison to DC-Chol on all tested cell lines. Especially suitable are mixtures with 60–80 molar percent of DOPE which enabled an approximately 4–5-fold higher gene expression compared to DC-Chol.

Concomitantly, the novel lipid CCQ22 facilitate a significantly higher cell viability. Relative to the untreated

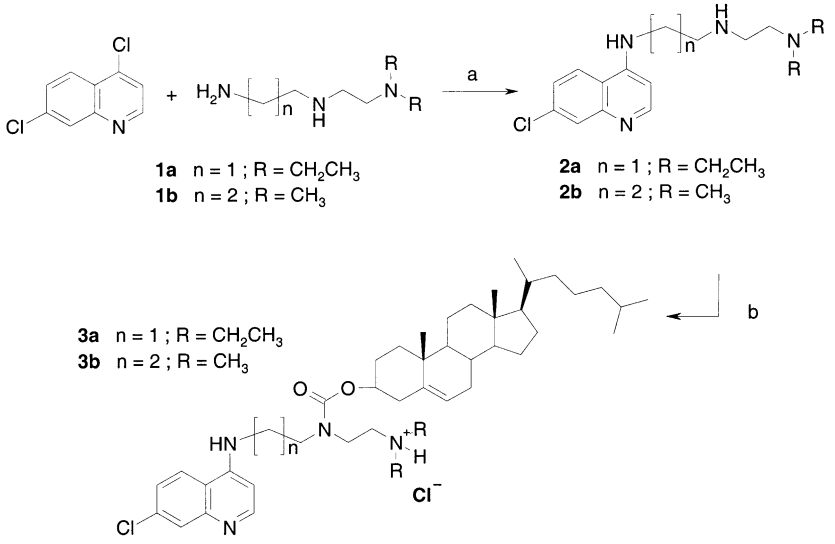

Figure 1. Synthesis of the carboxycholesteryl-modified chloroquine-analogues CCQ22 (**3a**) and CCQ32 (**3b**): (a) phenol, 125 °C, 24 h; (b) cholesterolchloroformate, dichloromethane, triethylamine, 4 h.

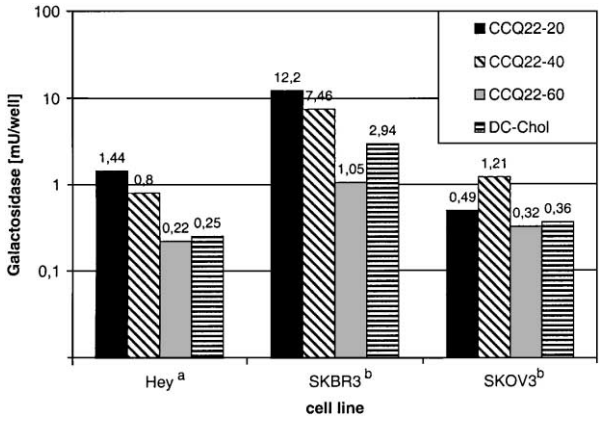

Figure 2. Transfection efficiency of different liposomal formulations of compound **3a** in comparison to DC-Chol on gynecological cell lines. The efficiency is measured as gene expression of the reporter gene product β-galactosidase (mU enzyme activity per well). CCQ22-20 means 20 mol% **3a** and 80 mol% DOPE: (a) 1 μg DNA + 1 μg lipid per well; (b) 0.5 μg DNA + 2 μg lipid; DC-Chol was used in mixture with DOPE according to the literature.

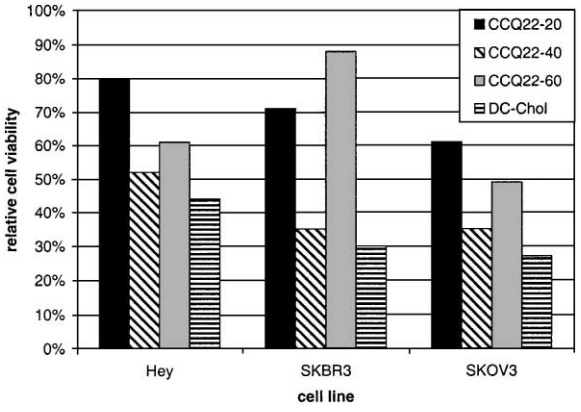

Figure 3. Cell viability 48 h after transfection in relation to untreated cells; conditions as in Figure 2.

control approximately 60–70% of the cells are viable at the conditions of the highest gene expression, while DC-Chol yielded only about 30–50% viable cells. Similar or even better results were obtained for the cationic lipid CCQ32 (data not shown).

Discussion

The novel cationic lipids can be synthesized in two steps starting from inexpensive, readily available chemicals. Further, they can be purified to homogeneity by simple flash chromatography on a multigram scale. Due to the heteroaromatic system of the compounds the HPLC analysis is sensitively performed by UV detection at 220 nm. Both lipids CCQ22 and CCQ32 contain two basic functionalities with significantly different pK values. The terminal tertiary amino group has a pK of approximately 10 and therefore is nearly completely protonated at a physiological pH of 7.4. Thus it should strongly interact with polyanions like DNA. On the other hand the basicity of the 4-aminoquinoline like structure is much lower (8.2 for 4-amino-7-chloroquinoline). For this reason, the ring nitrogen is not fully protonated and should be able to buffer the endosomal compartment. Compared to the well described cationic lipid DC-Chol, which is currently used in first clinical trials, the newly synthesized lipids enable a significantly higher gene transfer into the tested gynecological tumor cells. Due to the lower toxicity of the lipids **3a(b)** the lipoplexes can be left in contact with the cells during the whole 48 h incubation time. Thus the novel lipids are promising reagents for the treatment of different gynecological cancers. Further in vitro optimization of the transfection efficiency and first in vivo studies are subject to current investigations.

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