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Research paper

# Targeted delivery of nucleic acids into xenograft tumors mediated by novel folate-equipped liposomes

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ABSTRACT

Folate receptors (FR) are cellular markers highly expressed in various cancer cells. Here, we report on the synthesis of a novel folate-containing lipoconjugate (FC) built of 1,2-di-*O*-ditetradecyl-*rac*-glycerol and folic acid connected via a PEG spacer, and the evaluation of the FC as a targeting component of liposomal formulations for nucleic acid (NA) delivery into FR expressing tumor cells. FR-targeting liposomes, based on polycationic lipid 1,26-bis(cholest-5-en-3β-yloxycarbonylamino)-7,11,16,20-tetraazahexacosan tetrahydrochloride (2X3), lipid helper dioleoylphosphatidylethanolamine (DOPE) and novel FC, formed small compact particles in solution with diameters of 60 ± 22 nm, and were not toxic to cells. Complexes of NAs with the liposomes were prepared at various nitrogen to phosphate ratios (N/P) to optimize liposome/cell interactions. We showed that FR-mediated delivery of different nucleic acids mediated by 2X3-DOPE/FC liposomes occurs *in vitro* at low N/P (1/1 and 2/1); under these conditions FC-containing liposomes display 3–4-fold higher transfection efficiency in comparison with conventional formulation. Lipoplexes formed at N/P 1/1 by targeted liposomes and cargo (Cy7-labeled siRNA targeting MDR1 mRNA) *in vivo* efficiently accumulate in tumor (~15–18% of total amount), and kidneys (71%), and were retained there for more than 24 h, causing efficient downregulation of p-glycoprotein expression (to 40% of control) in tumors. Thus, FC containing liposomes provide effective targeted delivery of nucleic acids into tumor cells *in vitro* and in xenograft tumors *in vivo*.

## 1. Introduction

In the last few decades, there has been an increasing interest in the use of therapeutic nucleic acids (NAs) in the treatment of hereditary and acquired diseases [1–3]. According to their chemical nature, nucleic acids are polyanions, and the surface of eukaryotic cells is also negatively charged. Therefore, naked, unprotected NAs are unable to penetrate through the cell membranes into the cells. To solve this problem, several new viral and non-viral (liposomes, polymers, and nanoparticles) delivery systems have been created [4–9]. Viral delivery systems have shown high transfection activity, but their use is limited by immunogenicity, toxicity, and oncogenicity [10,11]. In contrast, liposome-based delivery systems are devoid of such limitations [12]. These systems have many potential advantages, including their significant simplicity and ease of production, good repeatability and biodegradability, potential commercial value, wide range of clinical applications and safety, and thus are preferable to other non-viral vectors [13–16].

An important requirement of modern liposome-based delivery systems is the ability to transfer NAs only into the target cells, allowing an increase in the efficiency of such systems and the avoidance of delivery into cells that are not therapeutic targets. This will have a positive effect on the biosafety of the delivery systems and allow a reduction in detrimental side effects. Targeted delivery can be achieved by equipping the liposomes with ligands for the receptors specifically expressed on the surface of abnormal cells but not on the surface of healthy ones [14,17,18].

The overexpression of folate receptor (FR) was observed on the surface of various epithelial cancer cells including cancers of the ovary, uterus, lung, kidney, breast, colon, prostate, and brain [19–21]. Folic acid (FA) is a water-soluble vitamin (B9) that is essential for the synthesis of nucleotides and thereby for the growth and development of all types of cells. In humans, there are three types of FRs: membrane-bound FR-α, FR-β, and FR-γ secreted by hematopoietic cells [20].

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Overexpression of FR-α was found in over 90% of ovarian carcinomas and at rather high frequency in other epithelial cancers; FR-β is overexpressed in myeloid leukemia [22] and activated macrophages [23,24]. In contrast, normal tissues lack overexpression of FRs [25].

FRs bind FA with high affinity, and FR-α has a 10-fold higher affinity for folate than FR-β. After the binding of FA or its conjugates to membrane-bound FRs, the ligand/receptor complex penetrates the cell by receptor-mediated endocytosis. The average rate of FA penetration into a cancer cell overexpressing FR is $1–2 \times 10^5$ molecules/cell/h. Moreover, when FA or its conjugates bind to FRs, they are endocytosed and released from the receptor, and the receptors are recycled to the cell surface for re-use within 30 min [26]. It should be noted that the expression status of FR-α correlates with the de-differentiation of tumor cells as well as with poor prognosis and resistance to chemotherapy [27]. Overall, as a targeting ligand, FA has important advantages over other ligands such as low immunogenicity, rapid penetration of tumor cells, simple chemical structure, and high specificity towards tumors [17,28].

Therefore, folate modification has been employed in various delivery systems including liposomes [29–31], polymers [32–35] and dendrimers [36,37], to facilitate the uptake of anticancer agents into cancer cells. These agents include doxorubicin [29,32,33,35], antibodies [30], and nucleic acids [28,29,31,34,37]. It has been reported that most of the folate-containing carriers demonstrate a significant increase in the accumulation of the drugs in the cancer cells *in vitro* [32,33,36] and *in vivo* [30,31,34,35,37].

Cationic liposomes have several features that restrict their wide application, for example, their rapid clearance by the reticuloendothelial system (RES) [38]. To overcome this problem and to prolong circulation time of liposomes *in vivo*, polyethylene glycol (PEG) is often used in cationic liposomes [39,40]. Moreover, the threshold size for extravasation of targeted nano-sized carriers in tumors is approximately 400 nm in diameter, although nanocarriers with diameters < 200 nm are preferred. The surface charge of nanocarriers is another important parameter; both highly positively- and highly negatively-charged nanocarriers are susceptible to rapid clearance by RES [41]. Thus, it is important to design nanocarriers that meet the criteria of size and zeta potential (neutral or slightly negative/positive), and that efficiently bind NAs.

To address these problems, we designed and synthesized a new folate-containing lipoconjugate (FC) as the component of a targeted liposome-based delivery system (**F**). FC-based delivery vector has a small size (100–200 nm) and slightly positive zeta potential, and provides targeted delivery of NA into tumor cells expressing folate receptors, e.g., human epidermoid carcinoma (KB-3-1) and human embryonic kidney 293 (HEK 293) cells *in vitro*. We determined optimal compositions of lipoplexes of **F** formulation and NA for delivery into tumor cells, and demonstrated efficient accumulation of siRNA in xenograft tumors, mediated by FC-based liposomal formulation **F**.

## 2. Materials and methods

### 2.1. Protocol of folate lipoconjugate (FC) synthesis and characterization

Protocol of folate lipoconjugate (FC) synthesis and characterization is in Section S1, Supplementary materials

### 2.2. Liposome preparation

All liposome formulations were prepared by hydrating thin lipid films [42]. Briefly, a solution of polycationic lipid 1,26-bis (cholest-5-en-3β-yloxycarbonylamino)-7,11,16,20-tetraazahexacosan tetrahydrochloride (2X3) [43] in a mixture of $CHCl_3$–$CH_3OH$ (1:1) was added to a solution of dioleoylphosphatidylethanolamine (DOPE, Avanti Polar Lipids) in $CHCl_3$ at a molar ratio of 1:2, and gently stirred. A solution of FC (2 M%) in $CHCl_3$–$CH_3OH$ (1:1) was added to the 2X3-DOPE mixture, and organic solvents were removed *in vacuo*. The obtained lipid film was dried for 4 h at 0.1 Torr to remove residual organic solvents and was hydrated in deionized water (MilliQ) at 4 °C overnight. The resulting liposomal dispersion was sonicated for 15 min at 70–75 °C in a bath-type sonicator (Bandelin Sonorex Digitec DT 52H, Germany), flushed with argon and stored at 4 °C.

For preparation of rhodamine B-labeled liposomes 1 M% of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Avanti Polar Lipids) was added to the organic solution of lipids, followed by the procedure described above.

### 2.3. Oligonucleotides and plasmids

The 25-mer oligodeoxyribonucleotide with an aminohexyl linker at the 3′ end (5′-TAC AGT GGA ATT GTA TGC CTA TTA T-3′) was synthesized using the phosphoramidite method and purified by HPLC (Institute of Chemical Biology and Fundamental Medicine, SB RAS, Novosibirsk, RF). Fluorescein isothiocyanate (FITC) was attached to the amino group of the linker as previously described [44]. The purity of 5′-fluorescein-labeled oligodeoxyribonucleotide (here and after FITC-ODN), analyzed by electrophoresis in 20% PAAM/8 M urea gel, was 95–98%. FITC-ODN solution was stored at −20 °C until required.

For the plasmid DNA (pDNA) transfection experiments, pEGFP-C2 plasmid (Clontech, Heidelberg, Germany) was used.

### 2.4. siRNAs

The sense and antisense strands of siRNAs were synthesized, isolated and characterized as described previously [45]. The 2′-*O*-methylated siRNAs used in this study are as follows: siMDR, homologous to the region 411–431 nt of human MDR1 gene mRNA (sense strand 5′-GCGCGAGGUCGGGAUmGGAUCU-3′; antisense strand 5′-GCGCGAG GUCGGGAUmGGAUCU-3′); siScr with no significant homology to any known mouse, rat or human mRNA sequence (sense strand 5′-GCUUG AAGUCUUUmAAUUmAAGG-3′; antisense strand 5′-UUmAAUUmAAA GACUUCmAAGCGG-3′) (2′-*O*-methyl-modified C and U nucleotides are designated as Cm and Um). Fluorescein was attached to the 5′-end of the siScr antisense strand by the reaction of FITC with the primary amino group of aminohexyl linker introduced at the 5′-end of the oligoribonucleotide, based on standard solid-phase synthesis. Cyanine7 (Cy7) was attached to the 3′-end of the antisense strand of siMDR equipped with a 3′-aminohexyl linker according to the manufacturer's protocol, using Cy7 *N*-hydroxysuccinimide esters (Biotech Industry Ltd, Russia) in 0.1 M Tris buffer (pH 8.4). Isolation of the oligoribonucleotides and their conjugates was accomplished by electrophoresis in 12% PAAM/8M urea gel. The purified oligoribonucleotides were characterized by electrophoretic mobility in 12% PAAM/8M urea gel and by MALDI-TOF-MS or by LC-ESI-MS. siRNAs (50 μM) were annealed in a buffer containing 30 mM HEPES-KOH (pH 7.4), 100 mM sodium acetate and 2 mM magnesium acetate, by heating at 90 °C for 5 min, followed by cooling to room temperature. The siRNA preparations were stored at −20 °C until required.

### 2.5. Preparation of the complexes of cationic liposomes and nucleic acids

Prior to use, the cationic liposome and nucleic acid complexes were formed in a serum-free Opti-MEM (Invitrogen, USA) (unless another is specified) by vigorous mixing of equal volumes of liposomes and respective nucleic acid solutions in Opti-MEM taken at appropriate concentrations (correspond to a final concentration in the well of 1 μM for FITC-ODN, 2 μg/ml for pEGFP-C2, and 0.2 μM for FITC-siRNA); the resulting mixtures were incubated for 20 min at room temperature. NA/liposome complexes were formed at N/P ratios of 1/2, 1/1, 2/1, and 4/1 for FITC-ODN transfection; 1/1, 2/1, 4/1, 6/1, 8/1, and 10/1 for pEGFP-C2 transfection; 1/1, 2/1, 4/1, and 6/1 for FITC-siRNA transfection.