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Efficient down-regulation of PKC-α gene expression in A549 lung cancer cells mediated by antisense oligodeoxynucleotides in dendrosomes

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ABSTRACT

The completion of human genome project has increased our knowledge of the molecular mechanisms of many diseases, including cancer, thus providing new opportunities for gene therapy.

Antisense oligodeoxynucleotides (AsODN) possess great potential as sequence-specific therapeutic agents, which in contrast to classic treatments provide more efficient and target-specific approach to modulate disease-related genes. To be therapeutically effective, sufficient concentrations of intact AsODN must bypass membrane barriers and access the site of action. In this study, a dendrosome delivery strategy was designed to improve the encapsulation of AsODN in non-cationic liposomes to target PKC-α in lung cancer cells *in vitro*. Subcellular trafficking of fluorescently labeled AsODN was visualized using confocal microscopy. Uptake and expression of mRNA and target protein after AsODN delivery was measured by flow cytometry, qRT-PCR and Western blot analysis, respectively. Dendrosomes showed favorable physicochemical parameters: high encapsulation efficiency and uptake in serum-containing medium with no apparent cytotoxicity. AsODN encapsulated in dendrosome efficiently and specifically suppress the target gene at both mRNA and protein levels. Additional *in vivo* studies on the application of dendrosome as a delivery system for nucleic acid molecules may lead to improvement of this technology and facilitate the development of therapeutic antisense techniques.

1. Introduction

The overall goal of gene therapy is to cure or stabilize a disease process that results from the production of a mutant protein or overproduction of a normal protein. This goal is achieved by replacing the defective gene (oncogenes) or by reducing the overexpression of the target gene in order to reduce or inhibit the production of the disease-promoting protein (Hart, 2010).

Several strategies have been considered for manipulation of gene expression either on the transcriptional or on the translational level by nucleic acid-based therapeutics (*e.g.* aptamers, antisense oligonucleotides, ribozymes and siRNAs) (Chu et al., 2006; Crooke, 1999, 1998; Davis, 2002; Lewin and Hauswirth, 2001; Yang et al., 2011). The use of antisense oligodeoxynucleotide (AsODN) strategies in cancer, subject of the present investigation, is based on the potential of these compounds to down-regulate the oncogenic proteins and provided a chance to target diseases at their molecular level by selective inhibition of the expression of a single protein (Crooke, 1999, 1998).

Lung cancer is by far the leading cause of cancer death among both men and women. Non-small cell lung cancer (NSCLC) is the cause of death in more people in United States than breast, colorectal, and prostate cancer combined. Each year, more than 60,000 persons develop stages IIIB and IV NSCLC. Nearly all go on to die from metastatic spread (Cancer Facts and Figures, 2010).

Current available treatments of NSCLC are inadequate and hence there is a pressing need to develop appropriate treatments with effective results and fewer side-effects. This need has driven research on treatments of NSCLC that targets specific cellular signaling proteins associated with tumor growth.

One potential target in NSCLC is protein kinase C (PKC)-alpha, a signaling molecule with an important role in cell regulation and proliferation. This family of isozymes is composed of at least 11 different, but structurally related, serine/threonine kinases and is recognized as highly expressed in NSCLC (Lahn et al., 2004).

Several promising lipid and polymeric delivery systems have been proposed for gene therapy and many efforts have been

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undertaken to improve their efficiency (Kodama et al., 2006; Movassaghian et al., 2011; Perrie and Gregoriadis, 2000; Schäfer et al., 2010). However, development of a system considered satisfactory for clinical use has been elusive.

Among different available strategies, cationic liposomes have shown several advantages as gene delivery systems, including spontaneous transfection due to their net positive charge. However there are some disadvantages like cytotoxicity (Filion and Phillips, 1997; Sonawane et al., 2003; Wagner, 1999), lack of stability, activation of the complement system (induced secretion of cytokines such as TNF, IL-12 and IFNγ) and uptake by non-target tissues that have restricted their success (Esfand and Tomalia, 2001; Junghans et al., 2005; Lebedeva and Stein, 2001; Lysik and Wu-Pong, 2003). A reduction in toxicity and considerable improvement in the pharmacokinetics of cationic liposomal formulations for nucleic acid delivery systems can be achieved by switching to DNA encapsulation within non-cationic liposomes (Patil et al., 2004). However, poor entrapment efficiency of genetic material in non-cationic liposomes has limited their application (Junghans et al., 2001; Seeman, 2003; Vijayanathan et al., 2002). Lack of further development of these systems may be attributed to the inefficient association between anionic lipids and DNA molecules resulting from the repulsive electrostatic interaction between the negatively charged species (Patil and Rhodes, 2000; Perrie and Gregoriadis, 2000). This problem was addressed earlier by our group for plasmid DNA, through the development of a new class of formulations defined as dendrosomes (Movassaghian et al., 2011). Dendrosomes represent complexes of DNA with dendrimers incorporated into liposomes. To formulate dendrosomes, it is a prerequisite to condense the nucleic acids with polymer to form nano-sized particles, dendriplex. The size of the condensed DNA particles is a key determinant of successful *in vivo* application (Dauty et al., 2002). For this purpose, Polyamidoamine dendrimers (PAMAM) was chosen here due to its unique properties including a nanoscale molecular structure, with defined molecular weight and surface function (Esfand and Tomalia, 2001; Tomalia et al., 1990). These properties provided a platform for a systematic study, with few complications from heterogeneity and variable chemistry, commonly seen in other non-viral delivery agents, such as cationic lipids and polyethylenimine (Vijayanathan et al., 2002).

In the present study, dendrosomes are investigated for delivery of short single strand oligodeoxynucleotides (AsODN) which target PKC-α in NSCLC and are shown to down-regulate production of target protein.

Our studies indicate that the encapsulating oligonucleotides in dendrosomes form stable nanoparticles in a highly reproducible manner that promoted efficient cellular uptake and most importantly displayed no non-specific toxicity.

2. Materials and methods

2.1. Materials

Lipids:1,2-di-(9-octadecenoyl)-sn-glycero-3-phosphoethanolamine (DOPE), L-α-phosphatidylcholine (PC chicken, 95%) and cholesterol were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Fifth generation poly-amidoamine dendrimers (PAMAM, G5, ethylene diamine core), heparin sodium salt from porcine intestinal mucosa (Grade I-A, ≥198 USP units/mg) and Sepharose® CL-4B were purchased from Sigma–Aldrich (St. Louis, MO, USA). An RNAqueous®-4PCR Kit for Isolation of DNA-free RNA was purchased from Ambion Applied Biosystem, Life Technologies Co. (Carlsbad, CA, USA). LightCycler® 480 SYBR Green I Master was obtained from Roche Applied Science (Indianapolis, IN, USA). Dulbecco's modified Eagle's minimum essential medium (DMEM) and 0.25% Trypsin/EDTA 1X were purchased from Mediatech, Inc. (Manassas, VA, USA). HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was obtained from MP Biomedical (Solon, OH, USA). A DNase I kit was purchased from Ambion, Inc. (Austin, TX, USA). Cell Titer-Blue® was purchased from Promega (Madison, WI, USA). Quant-IT™ OliGreen® ssDNA Reagent, LysoTracker® Red and Hoechst 33342 were obtained from Molecular Probes® Invitrogen Life Technologies Co. (Carlsbad, CA, USA). All other reagents were of analytical grade.

2.2. Antisense oligodeoxynucleotides

A 20-mer phosphorothioate modified AP1261 antisense oligodeoxynucleotide (5′–3′) TCCATGACGAAGTACAGCCG against a 3′-untranslated region of protein kinase C-α and 5′-FAM conjugated AsODN were synthesized by Invitrogen Life Technologies Co. (Carlsbad, CA, USA). A scrambled oligonuclotides (ScODN) with the same number of bases, but a mismatch sequence (SC1261: CGAGCACGCAGTATCACTAG) was employed as the control here.

2.3. Cell culture

The A549 lung cancer cell line (Non-Small Cell Lung Carcinoma, NSCLC) and NIH/3T3 (mouse fibroblast) were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in DMEM, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 incubator.

2.4. Preparation of dendriplexes

Dendrimer was diluted to an appropriate concentration in HBG buffer (20 mM HEPES – in 5% buffered glucose, pH 7.4) and stored at 4 °C until use. The dendrimer/AsODN complexes (dendriplex) were prepared by adding a fixed amount of AsODN to varying amounts of dendrimer diluted separately in equal volumes of HBG buffer. The AsODN solution was transferred to the polymer solution, mixed by vigorous pipetting and followed by 30 min incubation at room temperature. Dendrimer to AsODN ratio (named N/P) was based on the calculation of the electrostatic charge present on each component: the number of terminal NH_2 groups on PAMAM dendrimer (N) *versus* the number of phosphate groups in the AsODN (P). Dendriplexes were prepared at (N/P) ratios of 0.5:1–20:1. The final concentration of AsODN was 10 μg/ml.

2.5. Preparation of the dendrosome

Dendrosomes were prepared by incubation of the dendriplex with a dispersion of freshly prepared liposomes. The thin film hydration method was used for liposome preparation. Briefly, for each formulation, a mixture of lipids (PC/DOPE/Cholesterol at 43:43:14 molar ratio) in a chloroform solution was dried to form a thin film in a round-bottomed flask using a rotary evaporator under vacuum. The residual solvent was removed in a freeze-drier overnight. The freeze-dried matrix was hydrated with HBG buffer to a final lipid concentration of 15 mg/ml followed by sonication for 2–5 min in an ultrasound bath system. Prior to addition of dendriplex, the vesicles were extruded 10 times through a 200 nm polycarbonate membrane using a Mini-Extruder (Avanti Polar Lipids, Alabaster, USA). The liposomes were then incubated with dendriplex for 1 h at room temperature and the resulted dendrosomes were stored at 4 °C until further analysis or use, not more than one month.