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A dual-targeting drug co-delivery system for tumor chemo- and gene combined therapy

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ABSTRACT

Regulation of gene expression using p53 is a promising strategy for treatment of numerous cancers, and chemotherapeutic drug dichloroacetate (DCA) induces apoptosis and growth inhibition in tumor, without apparent toxicity in normal tissues. Combining DCA and p53 gene could be an effective way to treat tumors. The progress towards broad applications of DCA/p53 combination requires the development of safe and efficient vectors that target to specific cells. In this study, we developed a DSPE-PEG-AA (1,2-distearoryl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol-2000)] ammonium salt-anisamide) modified reconstituted high-density lipoprotein-based DCA/p53-loaded nanoparticles (DSPE-PEG-AA/rHDL/DCA-PEI/p53 complexes), which was fabricated as a drug/gene dual-targeting co-delivery system for potential cancer therapy. Here, DCA-PEI was utilized to effectively condense the p53 plasmid, to incorporate the plasmid into rHDL and to act as an antitumor drug to inhibit tumor cell growth. The DSPE-PEG-AA/rHDL/DCA-PEI/p53 complexes exhibited desirable and homogenous particle size, neutral surface charge and low cytotoxicity for normal cells *in vitro*. The results of confocal laser scanning microscopy (CLSM) and flow cytometry confirmed that the scavenger receptor class B type I (SR-BI) and sigma receptor mediated dual-targeting function of the complexes inducing efficient cytoplasmic drug delivery and gene transfection in human lung adenocarcinoma cell line A549. And *in vivo* investigation on nude mice bearing A549 tumor xenografts revealed that DSPE-PEG-AA/rHDL/DCA-PEI/p53 complexes possessed specific tumor targeting and strong antitumor activity. The work described here demonstrated that the DSPE-PEG-AA/rHDL/DCA-PEI/p53 complexes might offer a promising tool for effective cancer therapy.

1. Introduction

Malignancy seriously threatens human's health. Currently, the commonly used clinical antitumor drugs have defects like poor efficacy, side effects and exorbitant prices. The small molecule dichloroacetate (DCA), a metabolic modulator with low price, has been used in humans for decades in the treatment of lactic acidosis and inherited mitochondrial diseases [1]. DCA inhibits mitochondrial pyruvate dehydrogenase kinase (PDK), shifts metabolism from glycolysis to glucose oxidation, increases mitochondrial hydrogen peroxide (H_2O_2), and activates Kv channels in all cancer cells, but not in normal cells. DCA induces apoptosis and inhibits tumor growth by reversing the metabolic-electrical remodeling (hyperpolarized mitochondria, activated NFAT1 and downregulated Kv1.5) in several cancer lines [2]. The mitochondria-NFAT-Kv axis and PDK are important therapeutic targets in cancer. Hence, the inexpensive and easily available DCA is a promising selective anticancer agent. But if treated with the chemotherapy drugs alone, advanced tumors are easy to form the tolerance to these drugs, and are vulnerable to relapse with extremely difficult retreatment. The limitations of traditional therapy make cancer new therapies (biological therapy, immunotherapy, gene therapy, and microbial treatment of the purified product) increasingly becoming the focus of clinical research hotspots.

Gene therapy represents a new and promising method for disease treatment by transmission of DNA that encodes therapeutic genes and/or consequent expression of therapeutically active proteins.

Abbreviations: DCA, dichloroacetate; DCA/p53, dichloroacetate/p53 gene; DSPE-PEG-AA, 1,2-distearoryl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol-2000)] ammonium salt-anisamide; PEI, polyethyleneimine; DCA-PEI, dichloroacetate-polyethyleneimine; rHDL, reconstituted high-density lipoprotein; CLSM, confocal laser scanning microscopy; SR-BI, scavenger receptor class B type I; PDK, pyruvate dehydrogenase kinase; ROS, reactive oxygen species; PEG, polyethylene glycol; FBS, fetal bovine serum; EPR, enhanced permeability and retention; DSPE-PEG-AA/rHDL/DCA-PEI/p53, 1,2-distearoryl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol-2000)] ammonium salt-anisamide/reconstituted high-density lipoprotein/dichloroacetate-polyethyleneimine/p53 gene; apo A-I, apolipoprotein A-I; DCA-PEI/p53, dichloroacetate-polyethyleneimine/p53 gene; Lipos/DCA-PEI/p53, liposome/dichloroacetate-polyethyleneimine/p53 gene; rHDL/DCA-PEI/p53, reconstituted high-density lipoprotein/dichloroacetate-polyethyleneimine/p53 gene; rHDL/DCA, reconstituted high-density lipoprotein/dichloroacetate; rHDL/DCA-PEI, reconstituted high-density lipoprotein/dichloroacetate-polyethyleneimine; rHDL/PEI/p53, reconstituted high-density lipoprotein/polyethyleneimine/p53 gene; EDC, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride; NHS, N-hydroxysuccinimide; GFP, green fluorescent protein; EPC, egg phosphatidylcholine; TEM, transmission electron microscope; DLS, dynamic light scattering.

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The gene p53 resides in normal cells, and has relationships with human tumors. The wild-type p53 gene is a tumor suppressor gene, and the main function is to arrest the cell cycle or induce apoptosis. There are three key steps in the p53-induced apoptosis signaling pathways: (1) induces oxidation reduction related gene transcription; (2) forms reactive oxygen species (ROS); (3) degrades mitochondrial oxidative, and ultimately activates caspase leading to apoptosis [3]. It is found that one of the p53 anticancer mechanisms is similar to DCA inducing mitochondrial depolarization and reversing the metabolic-electrical remodeling. Recent studies show that the p53 signaling pathway regulation may be attributable to the ability of p53 to decrease activity of glutathione peroxidase and content of glutathione, but facilitate the trigger of ROS. And then, mitochondrial membrane potential becomes lower, which causes endometrial cardiolipin oxidation and bax shift. Subsequently, cytochrome C and apoptosis inducing factors are released into the cytoplasm to activate the mitochondrial pathway and cause apoptosis of tumor cells [4,5]. Therefore, it is reasonable to presume that DCA and p53 could be combined to act synergistically to fight cancer, which is proposed for the first time.

Efficient delivery of genetic material and drug to the targeted cells of a patient without apparent toxicity and side effects in cancer therapy requires an ideal delivery vector. Synthetic lipoproteins offer an excellent tool for targeting-delivery of biological/chemical agents (chemotherapeutics, siRNAs, photosensitizers, and imaging contrast agents) into various cell types [6]. Tumor conditions have been implicated in high levels of SR-BI expression, thus SR-BI being a potential target for cancer theranostics. Acton et al. firstly provided the evidence for SR-BI as a receptor of lipoproteins [7]. So reconstituted high-density lipoprotein (rHDL) has been most widely used in the area of oncology, where the development of rHDL nanoparticles has taken advantage of the overexpression of SR-BI receptor in cancer cells. These rHDL nanoparticles are totally natural and non-immunogenic. Methods to synthesize biomimetic HDLs have been explored and data demonstrate that this type of delivery vehicle may be highly beneficial for SR-BI targeting and efficacious systemic delivery [8,9]. The recent studies have shown that cholesterol-conjugated siRNA or DNA molecules could be assembled into rHDL nanoparticles and delivered to cells expressing SR-BI receptors [10,11]. Interestingly, a diverse set of human tumors also overexpress sigma receptors which suggested the prospect of using sigma-receptor binding ligands (*e.g.*, anisamide) for the diagnosis and targeted therapy of a variety of tumors [12]. So rHDL formulation was protected by PEG tethered with an anisamide ligand for binding to the sigma receptor overexpressed tumor cells, which offers a dual tumor-targeting drug delivery system.

In this study, we successfully developed a dual-targeting co-delivery system (DSPE-PEG-AA/rHDL/DCA-PEI/p53 complexes) simultaneously transporting chemical drug (DCA) and therapeutic gene (p53) into the tumor cells (Scheme 1). This system was prepared by condensing p53 with DCA-PEI and coated with a layer of lipids, apolipoprotein A-I (apo A-I) and DSPE-PEG-AA. We took the cationic polymer/DNA complexation approach to construct the DNA-loaded rHDL nanoparticles with very similar surface properties of the natural HDL nanoparticles. DCA-PEI was firstly synthesized and then served to condense the p53 plasmid to form DCA-PEI/p53 complexes. The complexes were assembled into the core of lipoplexes to formulate DCA/p53-loaded nanoparticles (Lipos/DCA-PEI/p53 complexes). Then, the functional protein apo A-I was introduced to assemble DCA/p53-loaded rHDL nanoparticles (rHDL/DCA-PEI/p53 complexes) [6]. Finally, the rHDL/DCA-PEI/p53 complexes were protected by PEG tethered with an anisamide ligand to construct the drug co-delivery system (DSPE-PEG-AA/rHDL/DCA-PEI/p53 complexes), which could target sigma and SR-BI receptors of tumor cells. Subsequently, the buffering capacity, physicochemical characteristics and the amidase triggered drug (DCA) release profile of DCA-PEI/pDNA complexes were investigated in detail. Meanwhile, the DSPE-PEG-AA/rHDL/DCA-PEI mediated p53 expression and the inhibitory effects of DSPE-PEG-AA/rHDL/DCA-PEI/p53 complexes on cancer cells were also explored *in vitro*. *In vivo*, the biodistribution and anti-tumor efficacy of DSPE-PEG-AA/rHDL/DCA-PEI/p53 complexes complexes in A549 tumor xenograft nude mouse models were investigated to further demonstrate their potential clinical application in tumor therapy.

2. Materials and methods

2.1. Materials

Branched polyethyleneimine (PEI, 1.8K), dichloroacetate (DCA) and amidase were purchased from Sigma-Aldrich (St. Louis, MO, USA). N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were obtained from Aladdin Reagent Database Inc. (Shanghai, China). The reporter plasmid (pEGFP-C3, 4.7 kb) encoding enhanced green fluorescent protein (GFP) and plasmid pCMV-NeoBam-p53wt (wt-p53, 8.4 kb) encoding wild type p53 (p53) driven by CMV promoter purchased from Addgene were propagated in DH-5a *Escherichia coli* and purified by Endo Free Plasmid Maxi Kit (Qiagen, Germany). Egg phosphatidylcholine (EPC) and cholesterol were purchased from NOF (Tokyo, Japan). Recombinant human apolipoprotein A-I was isolated from human plasma fraction IV which was a gift from Tonglu Biological Pharmaceutical Co. Ltd. (Anhui, China) as described by Settasatian et al [13]. And 1,2-distearoryl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol-2000)] ammonium salt-anisamide (DSPE-PEG-AA) was a generous gift from Dr. Leaf Huang (Division of Molecular Pharmaceutics, Eshelman School of Pharmacy, University of North Carolina). Other materials were purchased from Sigma-Aldrich unless otherwise stated.

2.2. Cell culture and animal model

Human lung adenocarcinoma cell line A549 and human lung bronchial epithelial cell line BEAS-2B were purchased from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, USA), 100 U/ml penicillin and 100 mg/ml streptomycin in a humidified atmosphere of 95% air/5% CO_2 incubator at 37 °C. All experiments were performed on cells in the logarithmic phase of growth. Female BALB/c nude mice (7 weeks old) were maintained at standard conditions with free access to food and water. All animal experiments were conducted in strict accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The A549 tumor-bearing nude mice models were established by subcutaneous inoculation of the A549 cell suspension on the right hind flank of nude mice.

2.3. Synthesis of DCA-PEI

DCA-PEI was prepared by an amide formation reaction between activated carboxyl groups of DCA and primary amine groups of PEI. Firstly, DCA (90.55 mg, 0.60 mmol) was dissolved in 2 ml of DMF. Then EDC (230.28 mg, 1.20 mmol) and NHS (207.16 mg, 1.79 mmol) were added to the clear solution of DCA and stirred under nitrogen for 2 h to activate the carboxyl groups of DCA at room temperature. Secondly, the above solution was added dropwise to PEI 1.8K (129.00 mg, 3.00 mmol) dissolved in mixed solvent of DMF/H_2O (2: 1, v/v), and the reaction was kept for 24 h at room temperature with stirring. Subsequently, the reaction mixture was further purified by dialysis in deionized water (DW) (MWCO 1000, 3 × 2 l) for 2 days. Finally, the resulting solution was lyophilized to obtain DCA-PEI as a white floc powder. The DCA-PEI was stored at −20 °C until further use and then characterized by ^{1}H NMR.