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A novel cationic lipid with intrinsic antitumor activity to facilitate gene therapy of TRAIL DNA

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ABSTRACT

Metformin (dimethylbiguanide) has been found to be effective for the treatment of a wide range of cancer. Herein, a novel lipid (1,2-di-(9*Z*-octadecenoyl)-3-biguanide-propane (DOBP)) was elaborately designed by utilizing biguanide as the cationic head group. This novel cationic lipid was intended to act as a gene carrier with intrinsic antitumor activity. When compared with 1,2-di-(9*Z*-octadecenoyl)-3-trimethylammonium-propane (DOTAP), a commercially available cationic lipid with a similar structure, the blank liposomes consisting of DOBP showed much more potent antitumor effects than DOTAP in human lung tumor xenografts, following an antitumor mechanism similar to metformin. Given its cationic head group, biguanide, DOBP could encapsulate TNF-related apoptosis-inducing ligand (TRAIL) plasmids into Lipid-Protamine-DNA (LPD) nanoparticles (NPs) for systemic gene delivery. DOBP-LPD-TRAIL NPs demonstrated distinct superiority in delaying tumor progression over DOTAP-LPD-TRAIL NPs, due to the intrinsic antitumor activity combined with TRAIL-induced apoptosis in the tumor. These results indicate that DOBP could be used as a versatile and promising cationic lipid for improving the therapeutic index of gene therapy in cancer treatment.

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1. Introduction

Gene therapy is an efficient therapeutic strategy for cancer treatment [1]. However, intravenous delivery of nucleic acid based bioactive agents is still a major challenge. Viral gene carriers have long been criticized for their potential risk of immunogenicity and toxicity [2]. Non-viral systems may offer safer profiles and hold great potential for systemic gene delivery, but favorable clinical outcomes are still a long way off, primarily due to ineffective delivery mechanisms [2,3].

The TNF-related apoptosis-inducing ligand (TRAIL) is a good candidate for cancer therapy due to its specific antitumor activity, which reduces risk to normal cells [4]. TRAIL has exhibited potent antitumor activity against a variety of tumors by inducing apoptosis in tumor cells, including human non-small-cell lung cancer (NSCLC) [4,5]. In addition to its strong antitumor activity, TRAIL presents no systemic toxicity in animals [6]. TRAIL-induced apoptosis is mediated by the death receptor pathway in tumor cells [7]. The binding of TRAIL to death domain-containing receptors on the cell surface will lead to the caspase-dependent irreversible apoptosis [7]. However, the application of TRAIL in cancer therapy is greatly limited by its short half-life *in vivo* [8]. Additionally, although TRAIL-targeted therapy holds great potential for cancer treatment, the clinical results of TRAIL have shown limitations, including inefficient delivery to tumor site, the complexity of the pathways, and resistance mechanisms [9]. Therefore, it is necessary to develop suitable nano-sized vehicles for TRAIL systemic delivery.

Metformin (dimethylbiguanide) is commonly used for patients with type II diabetes mellitus [10]. This cationic drug has well-established efficacy and a good safety profile, even at doses as high as 2 g/day, due to its very low toxicity [10]. More interestingly, metformin demonstrated significant antitumor activity against a wide range of cancers, including human NSCLC [10]. In our previous study, a cationic polymer of metformin (PolyMet) with intrinsic antitumor activity and low toxicity was designed and applied in siRNA delivery [11]. The biguanide groups of PolyMet can be used

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simultaneously as the cationic blocks for gene condensation and the chemotherapeutic agent for cancer treatment [11].

Designing carrier materials with intrinsic antitumor activity is an ideal strategy for cancer therapy. Therefore, we further hypothesized that biguanide could also be utilized as the cationic head group for a new cationic lipid, similar to 1,2-di-(9*Z*-octadecenoyl)-3-trimethylammonium-propane (DOTAP, a commercially available cationic lipid). Based on this rationale, we designed a DOTAP-like cationic lipid by changing the trimethylammonium of DOTAP to biguanide (Fig. 1A), namely 1,2-di-(9*Z*-octadecenoyl)-3-biguanide-propane (DOBP). DOBP was developed as a promising cationic lipid with intrinsic antitumor activity to facilitate gene therapy in cancer treatment. The biguanide group of DOBP acted similarly to metformin and perform antitumor activity by activating the AMPK signaling pathway [10]. And several studies have indicated that activation of AMPK signaling pathway could significantly sensitize TRAIL-induced apoptosis of human tumor cells [12–14]. Based on this rationale, we used a well-established Lipid-Protamine-DNA (LPD) formulation [15] to test the antitumor efficacy of the TRAIL plasmid DNA delivered by DOBP-LPD nanoparticles (NPs) (Fig. 1B) in a NSCLC model. The combined effect of DOBP and TRAIL was demonstrated.

2. Experimental section

2.1. Materials

Oleic acid, *N*-Boc-3-amino-glycerine, hydrochloride acid solution in 1,4-dioxane, dicyandiamide, protamine and cholesterol were purchased from Sigma-Aldrich. 1,2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP) was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). 1,2-distearoryl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethyleneglycol-2000)] ammonium salt (DSPE-PEG2000) was purchased from NOF America Corporation. DSPE-PEG-Anisamide (DSPE-PEG-AA) was synthesized based on the previous established protocols [16], and DSPE-PEG-AA was used to specifically target to H460 cells that overexpress sigma receptors for enhanced cellular uptake of liposomes and LPD NPs [17]. An expression vector containing the human TRAIL open reading frame was purchased from InvivoGen (San Diego, CA). Primers for PCR and qPCR were purchased from Integrated DNA.

2.2. Synthesis of DOBP

2.2.1. Synthesis of 1,2-di-(9Z-octadecenoyl)-3-amino-propane (DOAP)

Oleic acid (3 mmol) was dissolved in 20 mL of dichloromethane, then *N*-Boc-3-amino-glycerine (1 mmol), EDC (3 mmol) and DMAP (3 mmol) were added. The reaction mixture was stirred at 25 °C for 24 h under nitrogen. Post-reaction, *N*-Boc-DOAP was obtained and purified by silica column chromatography (petroleum ether/ethyl acetate = 20:1). The Boc protection of the product was deprotected using hydrochloride acid solution in 1,4-dioxane, and DOAP (chloride salt) was obtained with a yield of 85%. The chemical structure of DOAP (chloride salt) was confirmed by nuclear magnetic resonance spectroscopy (NMR, 400 MHz ^{1}H NMR, BRUKER AV-400).

2.2.2. Synthesis of 1,2-di-(9Z-octadecenoyl)-3-biguanide-propane (DOBP)

DOAP (chloride salt, 1 mmol) was dissolved in 20 mL of 1,4-dioxane, and dicyandiamide (2 mmol) in 2 mL of DMF was added. $FeCl_3$ (1 mmol) was utilized as catalyst. The reaction mixture was stirred at 70 °C for 48 h under nitrogen. The reaction mixture was washed with 5% hydrochloride acid to remove the $FeCl_3$ and free dicyandiamide, and dried with anhydrous sodium sulfate. Sodium sulfate was filtered, and the solvent was evaporated under vacuum. DOBP (chloride salt) was purified by silica column chromatography (dichloromethane/methanol = 30:1) with a yield of 30%. The chemical structure of DOBP (chloride salt) was confirmed by nuclear magnetic resonance spectroscopy (NMR, 400 MHz ^{1}H NMR, BRUKER AV-400).

2.3. Preparation of liposome and LPD NPs

DOBP or DOTAP and cholesterol (1:1, mol/mol) were dissolved in chloroform and the solvent was evaporated under reduced pressure. The lipid film was hydrated overnight with distilled water to make the final concentration 10 mM DOBP or DOTAP and 10 mM cholesterol. The DOBP-liposome (Lipo-DOBP) and the DOTAP-liposome (Lipo-DOTAP) were sequentially extruded through 400 nm, 200 nm, 100 nm and 50 nm polycarbonate membranes (Millipore, Billerica, MA), respectively. The polyplex core of the LPD NPs was prepared by mixing 26 μg protamine in100 μL 5% glucose with equal volume of 50 μg plasmid in 5% glucose. The mixture was incubated at room temperature for 10 min before 60 μL of Lipo-DOBP or Lipo-DOTAP (10 mmol/L each) were added. PEGylation and targeting modification were achieved by adding 15 mol% DSPE-PEG and DSPE-PEG-AA in the formulations with further performed at 60 °C for 15 min. The size and zeta potential were measured by using Zetasizer (Nano ZS, Malvern Co., UK). The morphology of the LPD NPs was observed by JEOL 100CX II transmission electron microscopy (TEM) (JEOL, Japan). NPs were negatively stained with 2% uranyl acetate. To investigate the stability of LPD NPs under serum condition, LPD NPs were incubated in PBS (pH 7.4) supplemented with 5% of fetal bovine serum (FBS) for 12 h at 37 °C, and the mean diameter of the NPs was determined at timed intervals.

2.4. Cell culture

H460 human NSCLC cells and MRC-5 human fetal lung fibroblast cells were originally obtained from American Type Culture Collection (ATCC). The cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (100 μg/mL) in a humidified atmosphere of 5% CO_2 at 37 °C.

2.5. In vitro cytotoxicity of blank liposome

The *in vitro* cytotoxicity of metformin, Lipo-DOTAP and Lipo-DOBP was investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in H460 cells and MRC-5. Briefly, cells were incubated in 100 μL of complete culture medium in 96-well plates at a density of 5000 cells/well for 24 h. After pre-incubation, the cells were exposed to serial dilutions of metformin, Lipo-DOTAP and Lipo-DOBP at different concentrations for 4 h, and washed and further incubated for 48 h. The cells without any treatment were utilized as control.

2.6. In vitro apoptosis and necrosis assay

H460 cells were seeded into a 6-well plate (1.0×10^6 cells/well) for 24 h. After pre-incubation, the cells were exposed to Lipo-DOTAP (20 and 50 μM), Lipo-DOBP (20 and 50 μM) and PEI (1.25 and 12.5 μM) for 6 h. Then, the cells were washed with PBS and trypsinized. Apoptosis and necrosis analysis was evaluated according to the manufacturer's protocols (Beckon Dickinson, CA). Annexin V-FITC (FL1)/PI (FL2) fluorescence signals were analyzed by flow cytometry on a BD FACSAria instrument (Beckon Dickinson, CA).

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