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Cationic drug-derived nanoparticles for multifunctional delivery of anticancer siRNA

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ABSTRACT

Combined treatment of anticancer drugs and small interfering RNAs (siRNAs) have emerged as a new modality of anticancer therapy. Here, we describe a co-delivery system of anticancer drugs and siRNA in which anticancer drug-derived lipids form cationic nanoparticles for siRNA complexation. The anticancer drug mitoxantrone (MTO) was conjugated to palmitoleic acid, generating two types of palmitoleyl MTO (Pal-MTO) lipids: monopalmitoleyl MTO (mono-Pal-MTO) and dipalmitoleyl MTO (di-Pal-MTO). Among various lipid compositions of MTO, nanoparticles containing mono-Pal-MTO and di-Pal-MTO at a molar ratio of 1:1 (md11-Pal-MTO nanoparticles) showed the most efficient cellular delivery of siRNA, higher than that of Lipofectamine 2000. Delivery of red fluorescence protein-specific siRNA into B16F10-RFP cells using md11-Pal-MTO nanoparticles reduced the expression of RFP at both mRNA and protein levels, demonstrating silencing of the siRNA target gene. Moreover, delivery of Mcl-1-specific anticancer siRNA (siMcl-1) using md11-Pal-MTO enhanced antitumor activity in vitro, reducing tumor cell viability by 81% compared to a reduction of 68% following Lipofectamine 2000-mediated transfection of siMcl-1. Intratumoral administration of siMcl-1 using md11-Pal-MTO nanoparticles significantly inhibited tumor growth, reducing tumor size by 83% compared to untreated controls. Our results suggest the potential of md11-Pal-MTO multifunctional nanoparticles for co-delivery of anticancer siRNAs for effective combination therapy.

1. Introduction

Treatment regimes combining anticancer drugs and small interfering RNAs (siRNAs) have recently emerged as a new anticancer therapy modality. Although single siRNAs have been studied for treatment of various cancers [1–3], recent studies have reported significant improvement with combination therapy employing potential anticancer siRNAs and chemical drugs. [4–6] For example, co-treatment with the alkylating agent temozolomide and siRNA against O-methylguanine-O-methyltransferase has been used for glioma therapy [4]. Cisplatin has been used in combination with siRNA against Mcl-1 (myeloid cell leukemia sequence 1) for ovarian cancer therapy [5] and siRNA specific for NFE2-related factor 2 for lung cancer therapy [6]. In these studies, anticancer siRNAs and chemical drugs have typically been treated as separate formulations, with anticancer chemicals being dissolved in a suitable aqueous medium and siRNAs generally being complexed to transfection agents, such as cationic liposomes.

Co-delivery approaches in which anticancer siRNAs and chemicals are entrapped in the same nanocarriers have drawn recent attention [7–10]. These include mesoporous silica nanoparticles (NP) for co-delivery of anticancer Bcl-2 siRNA and doxorubicin [7], or for co-delivery of p-glycoprotein siRNA and doxorubicin [8]; a co-formulation of c-Myc siRNA and doxorubicin in liposomes, which was reported to significantly improve tumor growth inhibition [9]; and poly (lactide-co-glycolide) NP designed to co-deliver p-glycoprotein siRNA and paclitaxel [10]. In these approaches, siRNA and chemical drugs were physically co-encapsulated in nanocarriers.

In this report, we describe a novel delivery system for co-delivery of anticancer chemical drugs and siRNAs in which

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anticancer drug-derived lipid functions per se as a nanoparticle matrix for anticancer siRNA delivery [11]. We chose mitoxantrone (MTO) as an anticancer chemical drug because of its cationic property, which provides the potential for electrostatic interactions with negatively charged siRNAs. MTO was conjugated to hydrophobic palmitoleic acid to yield palmitoleyl MTO (Pal-MTO) lipids. Meanwhile, Mcl-1 has been reported as a member of the Bcl-2 family possessing an anti-apoptotic property [12]. Reduction of Mcl-1 protein expression contributes not only to the inhibition of cancer development, but also to increase sensitivity of chemotherapeutic agents to resistant tumor cells [13,14]. Thus we chose Mcl-1 as a target protein for combined anticancer therapy using MTO-derived nanoparticles.

2. Material and methods

2.1. Synthesis of N-Boc-protected MTO

Anhydrous MTO dihydrochloride (26.0 mg, Sigma–Aldrich, St Louis, MO, USA) was dissolved in 5 mL anhydrous methanol and 2 mL triethylamine, and stirred for 1 h on ice with nitrogen purging. A solution of di-*tert*-butyl dicarbonate (43.6 mg) in 1 mL anhydrous tetrahydrofuran was gently added, and the mixture was stirred for 1 h. The reaction mixture was then stirred at room temperature for 10 h. After removal of the solvent, the residue was dissolved in 50 mL ethyl acetate and washed with a saturated potassium carbonate solution. After removal of moisture and solvent, the *N*-Boc-protected MTO was purified by silica gel column chromatography (hexane:ethyl acetate:methanol, 10:5:1).

2.2. Synthesis of mono-/di-Pal-N-Boc-protected MTO

Palmitoleic acid (15 mg), *N*,*N*′-dicyclohexylcarbodiimide (13 mg), and 4-(Dimethylamino)pyridine (8 mg) were dissolved in 10 mL anhydrous dichloromethane. The reaction mixture was stirred under a nitrogen atmosphere at room temperature for 2 h. A solution of *N*-Boc-protected MTO (25.1 mg) in 1 mL dichloromethane was then added to the reaction mixture. After stirring overnight, the reaction products were concentrated under vacuum. Next, the residue was redissolved in 50 mL ethyl acetate and extracted with a saturated potassium carbonate solution. After drying to remove moisture and solvent, purification of the residue by silica gel column chromatography (hexane:ethyl acetate:dichloromethane:methanol = 10:10:1:1) yielded mono- and di-Pal-*N*-Boc-protected MTO.

2.3. Synthesis and separation of mono-/di-Pal-MTO

Mono- (13.7 mg) and di-Pal-*N*-Boc-protected MTO (17.4 mg) were each reacted in 2 mL trifluoroacetic acid and 2 mL dichloromethane at room temperature for 2 h. After monitoring the completion of the reaction by thin layer chromatography, both mono- and di-Pal-MTO were concentrated under vacuum. Separation of mono- and di-Pal-MTO were performed by silica gel column chromatography using different solvents; the solvent for mono-Pal-MTO was a 1:8:2:0.1 (v/v) mixture of hexane: chloroform: methanol: ammonium hydroxide, and that for di-Pal-MTO was a 10:20:5:1:0.1 (v/v) mixture of hexane:ethyl acetate: dichloromethane: methanol: ammonium hydroxide. Mono- and di-Pal-MTO were characterized by ESI-MS (LCQ, Thermo Finnigan, San Jose, CA, USA), FT-NMR 500 MHz spectroscopy (AVANCE 500, Bruker, Germany), and CHNSO elemental analyzer (EA1110, CE Instruments, Milan, Italy).

2.4. Formation of Pal-MTO NP

For formation of NP, mono-Pal-MTO and di-Pal-MTO were dissolved in methanol and mixed at various molar ratios. The mixtures of mono-Pal-MTO and di-Pal-MTO were placed in a rotary evaporator, and methanol was removed under vacuum. The resulting thin film of Pal-MTO was hydrated with 1 mL of 20 mM HEPES buffer (pH 7.4). The solution was sonicated in a bath-type sonicator for about 10 min until a clear solution was formed.

2.5. Measurement of lipid film hydration

The extents of lipid film hydration were quantified by determining the fraction of lipid films used for the formation of nanoparticles. Mono-Pal-MTO and di-Pal-MTO were dissolved in methanol and mixed at various molar ratios. After removing methanol under vacuum, resulting thin films of Pal-MTO at various compositions were hydrated with distilled water. To quantify the extent of hydration, the colloidal nanoparticle solution resulting from the hydrated lipid films was separated from the adducts of nonhydrated lipid films by centrifugation at 12,000 rpm for 30min. The supernatant of nanoparticle solution and the lipid adduct precipitate were dehydrated under vacuum and redissolved in methanol. The fraction of hydrated lipid film-based Pal-MTO NP was determined by measuring the absorbance of each supernatant and precipitate at 660 nm.

2.6. Gel retardation assay

Formation of complexes between Pal-MTO NP and siRNA was confirmed by gel retardation assay. Nanoparticles of different formulations were mixed with scrambled siRNA in 20 mM HEPES buffer (pH 7.4) at N/P ratios from 0:1 to 20:1 and incubated for 20 min at room temperature. Each mixture was loaded onto a 20% polyacrylamide gel containing 0.5 mg/mL ethidium bromide, and separated by electrophoresis for 60 min at 100 V in Tris-borate-EDTA buffer. In some experiments, free MTO or md11-Pal-MTO NP were incubated at 37 °C with or without 50% of fetal bovine serum in phosphate-buffered saline (pH 7.4). After 6, 12, 24, and 48 h, the mixtures were loaded onto a 2.0% agarose gel and separated by electrophoresis. After electrophoresis, bands were visualized using a Gel Doc System (Bio-Rad Lab., Hercules, CA, USA).

2.7. Particle size and zeta potential measurements

The sizes and polydispersity index (PDI) values of various Pal-MTO NP (40 μM) with or without siRNA (100 nM) complexation were determined using a dynamic light scattering method. The samples were diluted with 20 mM HEPES buffer (pH 7.4), and the hydrodynamic diameters of the particles were measured with an ELS-8000 dynamic light scattering instrument (Photal, Osaka, Japan) using dynamic He–Ne laser (10 mW) light scattering at an angle of 90° at 24.1 °C. Zeta potentials were determined using laser Doppler microelectrophoresis at an angle of 22°. A software package (ELS-8000 software) supplied by the manufacturer was used to analyze the data.

2.8. Cryogenic-transmission electron microscopy (Cryo-TEM)

The morphology of Pal-MTO NP with or without complexed siRNA was observed by cryo-TEM. For Pal-MTO nanoparticles alone, nanoparticles were dispersed in PBS. For complexes, siRNAs were complexed to Pal-MTO NP at an N/P ratio of 10:1. Both samples were prepared as a thin liquid film supported on a cryo-grid. Cryo-TEM images were recorded at approximately −170 °C (acceleration voltage, 120 kV) with a Tecnai 12 electron microscope (Philips, Eindhoven, Netherlands) equipped with a Multiscan 600W CCD camera (Gantan Inc., Warrendale, PA, USA).

2.9. Cellular uptake of siRNA

Pal-MTO NP-mediated cellular uptake of siRNA was studied by confocal microscopy providing Z-stack images and flow cytometry. Human epithelial carcinoma KB cells (American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and 100 units/mL penicillin plus 100 μg/mL streptomycin (Sigma–Aldrich, St Louis, MO, USA). Cells were seeded onto 48-well plates at a density of 5×10^4 cells/well. The following day, the cells were treated with 20 nM of fluorescent dsRNA (Block-iT™ Fluorescent Oligo, Invitrogen, Carlsbad, CA, USA) complexed with Lipofectamine 2000 (Invitrogen) or with various Pal-MTO nanoparticles at a weight ratio of nanoparticles: dsRNA of 10:1. After incubation for 12 h, the cells were fixed in 4% paraformaldehyde in PBS for 15 min, and stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). The fixed cells were then observed under a confocal laser-scanning microscope (LSM 5 Exciter, Carl Zeiss, Inc., Jena, Germany). For flow cytometry, one day before transfection, KB cells were seeded onto 24-well plates. And On day 0, the cells were incubated with fluorescent dsRNA in naked form or as a complex with L2K or various Pal-MTO nanoparticles. After incubation for 12 h, the cells were detached and analyzed using a BD FACSCalibur system with Cell Quest Pro software (BD Bioscience, San Jose, CA, USA).

2.10. Assessment of target mRNA reduction by reverse transcription polymerase chain reaction

The siRNA-mediated reduction of target expression was tested at the mRNA level using reverse transcription polymerase chain reaction (RT-PCR). One day before transfection, mouse B16F10-RFP cells were seeded onto 12-well plates at a density of 2×10^5 cells/well. After reaching approximately 70%–80% confluence, siRNA complexes were added to the cells. siRNAs used were luciferase-specific siGL2 (control) or red fluorescence protein-specific siRFP (Samchully Pharm. Co., Seoul, South Korea) mixed with md11-Pal-MTO nanoparticles at an N/P ratio of 10:1, or with Lipofectamine 2000, according to the manufacturer's instruction. The final concentration of siRNA was adjusted to 50 nM. After 24 h, total RNA was isolated using the TRIzol™ reagent (Invitrogen) and reverse transcribed into cDNA using AccuPower RT PreMix (Bioneer, South Korea). The primers for RFP were 5′-AGG ACG GCG GCG TGG CGA CC-3′ (sense) and 5′-TGG TGG CGG CCC TCG GTG CG-3′ (antisense). The PCR products (367-bp for RFP) were separated on 1% agarose gels. RFP mRNA levels were normalized to those of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).