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Combination therapy with epigenetic-targeted and chemotherapeutic drugs delivered by nanoparticles to enhance the chemotherapy response and overcome resistance by breast cancer stem cells

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ABSTRACT

Aberrant DNA hypermethylation is critical in the regulation of renewal and maintenance of cancer stem cells (CSCs), which represent targets for carcinogenic initiation by chemical and environmental agents. The administration of decitabine (DAC), which is a DNA hypermethylation inhibitor, is an attractive approach to enhancing the chemotherapeutic response and overcoming drug resistance by CSCs. In this study, we investigated whether low-dose DAC encapsulated in nanoparticles could be used to sensitize bulk breast cancer cells and CSCs to chemotherapy. *In vitro* studies revealed that treatment with nanoparticles loaded with low-dose DAC (NP_{DAC}) combined with nanoparticles loaded with doxorubicin (NP_{DOX}) better reduced the proportion of CSCs with high aldehyde dehydrogenase activity ($ALDH^{hi}$) in the mammospheres of MDA-MB-231 cells, and better overcame the drug resistance by $ALDH^{hi}$ cells. Subsequently, systemic delivery of NP_{DAC} significantly down-regulated the expression of DNMT1 and DNMT3b in a MB-MDA-231 xenograft murine model and induced increased caspase-9 expression, which contributed to the increased sensitivity of the bulk cancer cells and CSCs to NP_{DOX} treatment. Importantly, the combined treatment of NP_{DAC} and NP_{DOX} resulted in the lowest proportion of $ALDH^{hi}$ CSCs and the highest proportion of apoptotic tumor cells, and the best tumor suppressive effects in inhibiting breast cancer growth.

1. Introduction

Breast cancer is the leading cause of cancer related deaths in women worldwide [1]. Doxorubicin (DOX) is an anthracycline antibiotic and, as a first-in-class anti-cancer drug, has been used for treating breast cancer patients and can effectively prolong the patient's survival time [2,3]. Unfortunately, DOX treatment is not sufficient to kill cancer stem cells (CSCs). For example, accumulated evidence has established that CSC populations are more resistant to DOX than non-CSC populations, which enhances the risk for recurrence and metastasis after DOX treatment [4,5]. Therefore, determining how to eliminate CSCs is crucial for breast cancer treatment [5,6] Aberrant epigenetic modifications play key roles in tumorigenesis and drug resistance. Decitabine (DAC) is a DNA methyltransferase (DNMT) inhibitor that has been used as a cytotoxic agent in leukemia chemotherapies [7,8]. Previous research has shown that treatment with DAC at or near the maximally tolerated dose was only minimally effective, but it induced extensive toxicity in solid tumor patients [9,10]. Recent studies have demonstrated that low doses of DAC can inhibit the self-renewal capacity of CSCs and restore their sensitivity to anticancer drugs [11], thus suggesting that low-dose DAC may have broad applicability for cancer management [12]. However, many problems must be ruled out before using low-dose DAC for solid tumor treatment. For example, low bioavailability [13], poor stability [14], and serious side effects (severe myelosuppression) [15] limit its clinical applications against solid tumors. Nanotechnology provides an innovative alternative strategy for targeted drug delivery, which could enhance therapeutic efficacy and reduce adverse side effects [16–18]. Therefore, a promising strategy is to target both bulk tumor cells and CSCs by combining nanoparticle loaded with epigenetic-targeted and chemotherapy drugs to achieve therapeutic benefits and reduce the side effects.

In this study, nanoparticles were used to deliver low-dose DAC to the tumor to sensitize the cancer cells to chemotherapy with DOX-loaded nanoparticles (NP_{DOX}). We focused on the use of DAC-loaded nanoparticles (NP_{DAC}) combined with NP_{DOX} to treat bulk cancer cells and CSCs.

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We investigated whether treatment with NP_{DAC} and NP_{DOX} could effectively overcome the resistant CSCs, and whether it could inhibit breast tumor growth following systemic administration.

2. Materials and methods

2.1. Materials

The diblock copolymer of poly(ethylene glycol) with poly(*d,l*-lactide) ($MPEG_{5K}$-PLA_{11K}) was synthesized as previously described [19]. DAC was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). DOX was purchased from Zhejiang Hisun Pharmaceutical Co. Ltd. Dulbecco's modified Eagle's medium (DMEM) and DMEM/F12 were purchased from Gibco BRL (Eggenstein, Germany). Fetal bovine serum (FBS), 4,6-diamidino-2-phenylindole (DAPI), collagenase I, RNase A, heparin sulfate, and methyl thiazol tetrazolium (MTT) were purchased from Sigma Aldrich Co. (St. Louis, MO).

2.2. Characterizations

Particle size measurements were conducted as previously described [20]. Briefly, nanoparticles in water were analyzed by a Malvern Zetasizer Nano ZS90 apparatus at 25 °C. They were illuminated at a 633 nm wavelength radiation from a solid-state He-Ne laser and the scattered light was collected at an angle of 90°. The nanoparticles were analyzed in triplicate at a concentration of 1.0 mg/mL. The data were analyzed using Malvern Dispersion Technology Software 4.20. Transmission electron microscopy (TEM) observation was performed on a JEOL JEM-2100 F Transmission Electron Microscope (JEOL Co., Ltd., Tokyo, Japan) with an accelerating voltage of 200 kV.

2.3. Preparation of NP_{DOX} and NP_{DAC}

NP_{DAC} was prepared by a double emulsion-solvent evaporation technique. Briefly, an aqueous solution of DAC (5 mg) in 200 μL water was emulsified by sonication (450 W for 2 min) over an ice bath in 2 mL of chloroform containing 30 mg of $MPEG_{5K}$-PLA_{11K}. This primary emulsion was further emulsified in 8 mL water by sonication (450 W for 2 min) over an ice bath to form a water-in-oil-in-water emulsion. The organic solvents were removed using a rotary evaporator.

NP_{DOX} was prepared by a single-emulsion technique. Briefly, 10 mg desalted DOX and 100 mg $MPEG_{5K}$-PLA_{11K} were dissolved in 1.5 mL ethyl acetate. This primary solution was further emulsified in 8.5 mL water by sonication (450 W for 2 min) over an ice bath to form an oil-in-water emulsion. Ethyl acetate was then evaporated from the mixture.

2.4. Mammosphere culture

Single-cell suspensions of MDA-MB-231 cells (1000 cells/mL) were cultured in suspension in serum-free DMEM-F12 supplemented with B27 (1:50, Invitrogen, Carlsbad, CA), 20 ng/mL hEGF (BD Biosciences, San Jose, CA), 0.4% low-endotoxin bovine serum albumin (BSA, Sangon Biotech, China), and 5 mg/mL insulin (Sigma-Aldrich, St. Louis, MO). To propagate mammospheres *in vitro*, mammospheres were collected by gentle centrifugation, dissociated into single cells, and then cultured to generate mammospheres of the next generation.

2.5. Analysis of ALDH expression by flow cytometry

Cells were pre-blocked with 3% BSA and stained with ALDH substrate using the ALDEFLUOR kit (Stem Cell Technologies). The cells were acquired and analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). The data were collected from three independent experiments.

2.6. Cellular uptake

$ALDH^{hi}$ mammosphere cells (4×10^5) were seeded into 24-well plates with ultralow attachment surface and cultured for 12 h. They were then treated for 4 h with different formulations of equivalent DAC and DOX. The concentrations of DAC and NP_{DAC} (equivalent DAC) were 1 μg/mL, and the concentrations of DOX and NP_{DOX} (equivalent DOX) were also 1 μg/mL. After removing the media by centrifugation at 250 g for 5 min, the cells were washed twice with cold phosphate buffered saline (PBS, 0.01 M, pH 7.4). Then, the fluorescence of the DOX in $ALDH^{hi}$ was analyzed using FlowJo Software. For the quantitative determination of DAC, the sorted $ALDH^{hi}$ cells were dissolved in DMSO, and DAC concentrations were measured by high-performance liquid chromatography (HPLC).

HPLC analyses were performed using a Waters HPLC system consisting of a Waters 1525 binary pump, Waters 2475 fluorescence detector, 1500 column heater, and a Symmetry C18 column. For DAC detection, HPLC-grade acetonitrile and 0.01 mol/L ammonium acetate in water solution (95:5, v/v) were used as the mobile phase at 30 °C with a flow rate of 1.0 mL/min. The UV detector was set to 244 nm for absorption and linked to Breeze software for data analysis.

2.7. Cell apoptosis analysis on $ALDH^{hi}$ cells

Mammosphere cells were treated with trypsin (0.05% with 0.02% ethylene diamine tetraacetic acid, Gibco, Canada), washed twice with PBS, and then stained with the ALDEFLUOR kit. The $ALDH^{hi}$ cells were sorted by MoFlo Astrios (Beckman Coulter). Equal numbers of live cells were plated in ultralow attachment plates for 6 h, and then exposed to DOX (50 nmol/L), NP_{DOX} (DOX equivalent 50 nmol/L), DAC (100 nmol/L), and NP_{DAC} (DAC equivalent 100 nmol/L) separately or together for 96 h. Next, the cells were harvested, washed, and re-suspended in cold staining buffer (PBS, 0.01 mol/L, pH 7.4). Apoptotic cells were stained with the Annexin V-FITC apoptosis detection kit I (BD Biosciences, San Jose, USA), and then detected by flow cytometry.

2.8. Orthotopic xenograft model and tumor suppression study

Female NOD/SCID mice were obtained from Beijing HFK Bioscience Co., Ltd., and used at 6-8 weeks old. All animals received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals. The procedures were approved by the University of Science and Technology of China Animal Care and Use Committee.

The xenograft tumor model was generated by the subcutaneous injection of 2×10^5 MDA-MB-231 cells diluted in matrigel (1:3 with PBS, BD Biosciences) into the mammary fat pad of the mice. When the tumor size reached 60–80 mm^3, the mice were randomly divided into eight groups. Mice were grouped for treatment as follows: PBS, nanoparticle, free DOX (1 mg/kg), NP_{DOX} (DOX equivalent 1 mg/kg), free DAC (1 mg/kg), NP_{DAC} (equivalent DAC, 1 mg/kg), DOX/DAC (DOX, 1 mg/kg; DAC, 1 mg/kg), and NP_{DOX}/NP_{DAC} (equivalent DAC, 1 mg/kg; equivalent DAC, 1 mg/kg) by *i.v.* injection every other day beginning at 14 day. Mice were injected 5 times in total and tumors were collected after the last injection. The perpendicular diameter of tumor was measured by caliper every other day beginning at 13 day. The tumor volume was calculated based on the following equation: tumor volume $= 1/2 \times \text{length} \times \text{width}^2$.

2.9. Analysis of the proportion of CSCs in tumor

At the end of study period (25 days), the animals were sacrificed and the tumor tissues were excised. After digestion into single cells, the cells were stained with ALDH substrate as described above, and analyzed using a FACSCalibur flow cytometer.