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Mitochondria and nuclei dual-targeted heterogeneous hydroxyapatite nanoparticles for enhancing therapeutic efficacy of doxorubicin

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ABSTRACT

Dual-targeted nanoparticles have been increasingly used to realize greater anti-proliferation effect by attacking double key sites of tumor cells. In order to retain nuclei inhibition effect and enhance DOX-induced apoptosis by mitochondrial pathway simultaneously, hyaluronic acid (HA) modified hydroxyapatite (HAP) nanoparticles (HAP-HA), the functional calcium-based tumor targeting nanoparticles, have been developed. In this nanosystem, HA acts as an active tumor-targeting ligand to bind the CD44 receptors which are overexpressed on the surface of tumor cells while HAP can load and deliver DOX to both nuclei and mitochondria of tumor cells. In this study, DOX-loaded HAP-HA nanoparticles (DOX/HAP-HA) exhibited satisfactory drug loading efficiency which was up to 214.55 ± 51.05 μg mg^{-1} and showed a uniform nano-scaled particle size. The mitochondrial and nuclei targetability of DOX/HAP-HA was confirmed by confocal laser scanning microscopy analyses. Besides, western blot assay demonstrated that DOX/HAP-HA could markedly enhance mitochondrial cytochrome C leakage and thereby activate apoptotic cascade associated with it. In addition, *in vivo* anti-tumor efficacy and toxicity evaluation of DOX/HAP-HA indicated that DOX/HAP-HA was more effective and less harmful compared to other groups. DOX/HAP-HA might be a new promising targeted delivery system for effective cancer therapy.

1. Introduction

Mitochondria play an important role in suppression of tumor growth since they could determine the aberrant energetic metabolism of malignant cells and regulate cell death by apoptosis and necrosis [1]. Moreover, nuclei control the activities of cells by regulating gene expression, suggesting that interfering with nuclei could also effectively inhibit tumor cell proliferation [2]. Therefore, enhanced antitumor effect could be potentially achieved by combining anticancer agents acting on mitochondria and nuclei. However, there were still some defects of combination therapy: increased toxicity from combination chemotherapeutics of several drugs without survival benefit may occur in some cases [3]; and clinical convenience of treatment and patient compliance would sometimes be challenged by the drug incompatibility for that patients were required to establish multiple vein passages in dosing. To address these dilemmas, we proposed a novel strategy that enabling single anticancer drug to influence on both mitochondria and nuclei of the tumor cells to achieve efficient anticancer therapy by miscellaneous mechanisms. In this way, reinforced anticancer efficiency, as with the combination therapy, would be realized by single drug chemotherapy while the risk of increased toxicity and complex incompatibility of combination therapy could also be avoided.

Doxorubicin (DOX) inhibits the progression of topoisomerase II, which relaxes supercoils in DNA for transcription in nuclei [4–6]. It was also found that DOX could lead to cardiac cells injury by damaging myocardium mitochondria, resulting in serious intracellular biochemical reactions disorder and fatal cardiotoxicity [7,8]. This was because selective accumulation and redox cycling of DOX had taken place in mitochondria of cardiac cells, which subsequently generated reactive oxygen and nitrogen species (ROS and RNS) [9,10]. Therefore, DOX theoretically has the potential to act on multiple key sites in the tumor cell by miscellaneous mechanisms (i.e. the mitochondrial injury mechanism and nuclei damage mechanism), which is expected to exhibit enhanced antitumor activity. However, free DOX always enters the nuclei of tumor cells rapidly without tarrying in the mitochondria [11,12], which weakens the potential of DOX in mitochondrial pathway treatment of cancer. In addition, DOX-induced cardio-, liver and renal toxicity

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as its common side effects also hampered its application in clinical cancer chemotherapy [13–20]. Hence, we designed a nano-sized hydroxyapatite-based tumor targeting nanocarrier to facilitate the simultaneous delivery of DOX into both mitochondria and nuclei of tumor cells, aiming at improving the antitumor efficiency and reducing the side effects of DOX.

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HAP) nanoparticles, a hydroxylated calcium phosphate-based material, identified as a preferable drug carrier due to its prominent biocompatibility, non-toxicity and non-mutagenicity as well as its excellent drug-loading capacity [21–24]. Moreover, HAP nanoparticles was also found to show significant prohibitory effects on the metabolic viability of several types of tumor cells such as hepatic cancer, colon cancer, osteosarcoma and melanoma [25–28]. Yuan et al. described that HAP nanoparticles could enter into the nuclei to impede the proliferation of tumor cells [29]. Particularly, HAP could also enter into the mitochondria of tumor cells and induce apoptosis through changing mitochondrial membrane potential, leading to leakage of the cytochrome C, thus decreasing expression of apoptosis-related protein such as Bax, Bcl-2 and increasing expression of caspase-3 and -9 [29–32].

Furthermore, highly hydrophilic hyaluronic acid (HA) was designed to coat on the surface of HAP nanoparticles by covalent bond in order to overcome some defects of HAP nanoparticles, such as lacking of active targeting effect, burst drug release, ease of aggregation and low water-solubility [22,33,34]. HA, a nonsulfated glycosaminoglycan, was widely utilized in biomedical applications due to its biocompatibility and water-soluble properties [35,36]. Additionally, HA is also a promising candidate polymer for targeting CD44-overexpressing tumor cells [37,38]. Maiolino et al. reported that HA modification resulted in a 9.4-fold increase of the nanoparticle internalized by MDA-MB-231 cells [39]. Besides, hydrophilic and negative property of HA could assist nanoparticles in evading the uptake by immune cells. Thus, HA was also expected to facilitate tumor targeting delivery of HAP nanoparticles, thereby enhance the *in vivo* efficacy of DOX.

Overall, HA modified HAP nanoparticles (HAP-HA) were developed to enhance the tumor-targeted delivery of DOX and improve its antitumor efficacy by mitochondrial and nuclei dual-targeted strategy. As displayed in Scheme 1, we hypothesized that HAP-HA would promote the targeted delivery of DOX to liver tumor through synergistic mechanism of enhanced permeability and retention effect (EPR effect) and HA-mediated specific tumor-targeting as well as reduced reticuloendothelial system (RES) uptake due to hydrophilic HA. HAP-HA could enhance accumulation of DOX in tumor site and facilitate its transmembrane transport. In the endosome, DOX was released effectively due to the pH-sensitive properties of CaP-based formulations. Subsequently, the tumor cells were killed when DOX and HAP entered the nuclei and mitochondria. Herein, physicochemical characteristics of HAP-HA and DOX/HAP-HA were characterized in detail. Further to this, capacity cellular uptake and intracellular distribution behaviors of DOX/HAP-HA were explored by confocal laser scanning microscope to detect the mitochondrial and nuclei target ability of them. Meanwhile, the mitochondrial damage caused by DOX/HAP-HA was tested by western blot assay and mitochondria swelling test. Finally, the tumor-targeted capacity, biodistribution and anti-tumor efficacy of DOX/HAP-HA were investigated in Heps xenograft mouse models to further explore their potential clinical application in anti-tumor therapy.

2. Materials and methods

2.1. Materials

Hyaluronic acid (HA, MW 7800 Da) was purchased from Shandong Freda Biological Chemical Co., Ltd. (Shandong, China). Hydroxyapatite (HAP) was offered by Beijing DK nano technology Co., Ltd.(Beijing, China). Doxorubicin (DOX) was from Shenzhen Main Luck Pharmaceuticals Inc. (Shenzhen, China). 3-aminopropyl-triethoxysilane (APS) was obtained from Shanghai Yaohua chemical plant (Shanghai, China). Dimethylformamide (DMF) and Formamide (FM) were from Shanghai Lingfeng Chemical Reagent Co., Ltd. (Shanghai, China). Dicyclohexylcarbodiimide (DCC) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). *N*-Hydroxysuccinimide (NHS), 4′,6-diamidino-2-phenylindole (DAPI), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) and Janus green B (JB) were all from Aladdin Reagent Database Inc. (Shanghai, China). DiR was supplied by Beijing Fanbo biochemical Co., Ltd. (Beijing, China). DMEM medium and penicillin-streptomycin solution were purchased from HyClone (Logan City, USA). Fetal bovine serum (FBS) was from Zhejiang tianhang Biological technology stock Co., Ltd. (Zhejiang, China). Tissue Mitochondria Isolation Kit was provided by Beyotime Biotechnology Co., Ltd (Shanghai, China). All other chemicals were of analytical grade and were used without further purification.

2.2. Cell culture

Human hepatoma cell (HepG2) was exploited for cell studies. HepG2 were cultured in DMEM medium containing 10% fetal bovine serum (FBS), 100 U/mL of penicillin and 100 μg/mL of streptomycin at 37 °C using a humidified 5% CO_2 incubator (311, Thermo scientific, USA).

2.3. Animals

Kunming mice (KM mice) and New Zealand rabbits were purchased from Qinglongshan breading farm (Nanjing, China). All animal experiments were conducted under a protocol approved by China Pharmaceutical University Institutional Animal Care and Use Committee. In order to obtain xenograft Heps tumor xenograft mice, approximately 10^7 of Heps cells (murine liver cancer cell) were incubated subcutaneously in the flank region of the mice. Tumor volume (V) was determined by measuring length (L) and width (W), and calculated as formula:

$$V = \frac{1}{2} L \times W^2 \quad (1)$$

2.4. Synthesis and characterization of HAP-HA

The synthetic scheme of HAP-HA is illustrated in Fig. 1A. Firstly, HAP nanoparticles having primary amino groups (HAAPS) were synthesized by coupling amino of APS with HAP. APS (0.221 g) in 100 mL aqueous alcohol solution (volume ratio of ethanol and water was 9: 1) was reacted with 1.0 g HAP nanoparticles for 3 h. Then the pH of the mixture was adjusted to 9–10 with $NH_3 \cdot H_2O$ followed by stirring for another 3 h. Subsequently, the system was filtered by vacuum suction and the powder was dried at room temperature and then cured at 130 °C for 2 h. The powder was washed by ethanol thoroughly to remove excessive APS and dried by vacuum before being dispersed in the aqueous solution by the