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The antitumor effect of folic acid conjugated-*Auricularia auricular* polysaccharide-cisplatin complex on cervical carcinoma cells in nude mice

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ABSTRACT

A tumor-targeted, folic acid (FA) conjugated-*Auricularia auricular* polysaccharide (AAP) -cis-diaminedichloroplatinum (CDDP) complex (FA-AAP-CDDP) was used for cervical carcinoma chemotherapy. The drug delivery system was able to enhance the antitumor potency of CDDP, and to reduce the toxic side effects of CDDP. The kidney of mice treated by FA-AAP-CDDP complex had higher superoxide dismutase, catalase, and glutathione peroxidase activities, and lower malondialdehyde. FA-AAP-CDDP complex could induce more interleukin-2, interleukin-4, and interferon-γ in mice. In addition, the FA-AAP-CDDP complex significantly promoted the expression of Bax and caspase-3 protein, but inhibited the expression of Bcl-2 protein, which activated the mitochondrial apoptotic pathway of tumor cells in nude mice. Moreover, the FA-AAP-CDDP complex had a higher intratumoral accumulation, was lower in the kidneys. This study may provide a new direction for folate receptor targeted polymers to improve anti-tumor activity, but reduce side effects of CDDP.

1. Introduction

Cis-diaminedichloroplatinum (CDDP) is widely used in the treatment of various cancer, including solid tumors in the ovary [1], teste [2], head, and neck [3]. It may be used either alone or in combination with other drugs [4,5]. However, at the therapeutic dose, CDDP induces several adverse effects such as nephrotoxicity (mainly tubular necrosis) [6], bone marrow suppression (mainly aleucocytosis) [7], ototoxicity (cochlear damage) [8], and neurotoxicity (mainly peripheral sensory neuropathy) [9]. Due to rapid eliminate from body and low drug accumulation in tumor sites of CDDP. At the same time, CDDP is lack of selection to tumors, they destroy tumors as well as normal tissues [10]. It is also slightly soluble in water (2.53 g L^{-1} at 25 °C) which limits its efficacy [11]. Therefore, to overcome the above obstacles and enhance drug efficacy, a large number of nanoscale polymeric drug carriers made from natural biodegradable polysaccharides have been developed [12,13]. These polymer-drug complexes work better because of their enhanced permeability and retention (EPR) in tumor tissues, resulting in better theranostic efficacy and fewer side effects, which is attributing to the unique anatomical and pathophysiological characteristics of tumor blood vasculature [13]. Compared to normal blood vessels, tumor vasculatures generally exhibit defective vascular architecture and higher vascular permeability, thus macromolecules with molecular sizes >40–50 kDa could go across the blood vessel into tumor tissues but could not permeate into normal tissue, as the result, high tumor accumulation and less biodistribution in normal tissues could be attained [14].

Many studies have indicated that the folate receptor (FR) is widely over-expressed in a variety of tumors such as cancer of the uterus [15], lung [16], breast [17], and head [18], but has little or no expression in most normal tissues and organs [19]. Thus, as a widely-studied targeting ligand, folic acid (FA) is frequently used for targeting folate receptor overexpressed tumor cells because its advantages, including high binding affinity to FR, small sizes, fewer immunogenic effects *in vivo*, and is easier to be modified chemically [19]. It has been reported that various FA conjugated drug delivery systems, including liposomes [20],

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polysaccharides [21], baculovirus [22], and albumin [23], retain the ability to be internalized by FR using a receptor-mediated endocytosis process [24–26]. Among these drug delivery carriers, mushroom polysaccharides, as a class of natural polymers, may be promising due to their unique properties, such as nanometer size, good biocompatibility, lower systematic toxicity, excellent degradability, water solubility, and various biological activities [27,28]. Thus, mushroom polysaccharides possess better application value, compared with frequently-used polymer carrier, such as chitin [29], hemicellulose [30], α- and β-glucans [31], mannans [32], xylans [33], and galactans [34]. Finally, mushroom polysaccharides have several functional groups on the surface that can be easily conjugated with FA, such as amino/acetamido group, primary and secondary hydroxyl groups [35]. Therefore, FA-conjugated mushroom polysaccharides can be considered to be potential drug carriers [36,37].

Auricularia auricula (*A. auricula*) mushrooms, as the most important non-toxic edible mushrooms, are widely cultivated and consumed in Southeast Asian counties, such as China, Thailand, Korea, and Japan [38]. *A. auricula* fruiting bodies have been generally used as traditional food and folk drug for more than 1000 years in China [39]. *A. auricula* polysaccharides (AAP) showed various bioactivities, such as antitumor activity [40], antioxidant activity [41], and anticoagulant activity [42]. Xiong reported that AAP was used as negatively charged polyelectrolyte to prepare a novel nanoparticle polyelectrolyte complex with chitosan (AAP/LCS NP), possessing superior controlled-release property, suitable for the delivery of protein drugs [43]. Purified AAP has been used to deliver the hydrophilic drug doxorubicin hydrochloride (DOX) [44]. It was found that blank AAP-chitosan nanoparticles did not show any cytotoxic effects *in vitro*, while Dox-loaded AAP-chitosan nanoparticles increased the Dox cytotoxicity against MCF-7 cells which was attributed to a significantly increased cellular uptake, compared with free Dox [44]. Moreover, AAP is an environmentally friendly material [45]. In previous work from this laboratory, FA conjugated-*A. auricular* polysaccharide-cisplatin complex (FA-AAP-CDDP complex) and *A. auricular* polysaccharide-cisplatin complex (AAP-CDDP complex) were successfully synthesized and characterized [45]. The loading capacity and stability of FA-AAP-CDDP and AAP-CDDP complex were satisfactory. The FA-AAP-CDDP and AAP-CDDP complexes also were potentially suitable for delivering antitumor drugs.

The present work studied the *in vivo* anti-tumor activities and toxicities of the AAP, CDDP, FA-AAP-CDDP, and AAP-CDDP complexes against xenograft sarcoma Hela cells in nude mice. The mechanism of the anti-tumor activities of the FA-AAP-CDDP were studied using an immunohistochemical method for monitoring the apoptosis-related proteins Bax, Bcl-2, and Caspase-3. Furthermore, the accumulation of CDDP, FA-AAP-CDDP, and AAP-CDDP complexes in kidneys and tumor sites were evaluated qualitatively and quantitatively.

2. Materials and methods

2.1. Materials

Unbranded dried *A. auricular* was purchased from a local Wal-Mart supermarket in Harbin (Heilongjiang, China). The AAP were extracted and purified as follows: The *A. auricular* was washed and dried at 60 °C in vacuum; then smashed to extremely fine particles (the particle diameter size was about less than 500 μm) using a crusher (FW177, Tianjin, China) and defatted by extraction with petroleum ether. The crude AAP were extracted using 0.1 mol L^{-1} NaOH solution, then some colored substances and phenolic compounds were removed by treating with 95% ethanol and kept overnight at 4 °C. The precipitates obtained were the AAP. The AAP were re-dissolved into distilled water and lyophilized (Vaco2, Zirbus, Duesseldorf, Germany). The polysaccharides were stored at −20 °C for up to 6 weeks. CDDP and FA were purchased from Sigma-Aldrich (St. Louis, MO, USA). The AAP-CDDP and FA-AAP-CDDP complexes were prepared using a "one-pot" method [45] by reacting AAP-CDDP with FA, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) or N-hydroxysuccinimide (NHS). The yield of AAP-CDDP and FA-AAP-CDDP was 55.4% and 42.5%. The FA content was measured to be 178.4 μg mg^{-1} of FA-AAP-CDDP. The superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), catalase (CAT), malondialdehyde (MDA), creatinine (CRE), urea nitrogen (UN), interleukin-2 (IL-2), interleukin-4 (IL-4), and interferon-γ (IFN-γ) measurement kits were purchased form Nanjing Jiancheng Bio-engineering Institute (Nanjing, Jiangsu, China). The rabbit monoclonal anti-Bcl-2, anti-Bax, anti-Caspase-3, and Harris hematoxylin were purchased from Boster Bio-Engineering Co., Ltd. (Wuhan, Hubei, China). All other chemicals were analytical grade and purchased from local suppliers.

2.2. Animal experiments

Female BALB/c mice, 6 weeks old, weighing 16–20 g, were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (SCXK(Beijing)2012-0001, Beijing, China). All animals were maintained under sterile conditions: temperature (22 ± 2 °C), light (12 h light/dark cycles), and humidity (50 ± 10%), and fed water and murine (Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) chow *ad libitum*. All animal experiments were done according to the Chinese National Institute of Health Guide for the Care and Use of Laboratory Animals, and approved using the "Guidelines of the Laboratory Protocol of Animal Handling" of Heilongjiang University of Chinese Medicine.

For all chemotherapy experiments, the BALB/c female mice were subcutaneously injected in their left flank with a tumor cell suspension (1×10^6 cells per mouse) in phosphate buffered saline (PBS, pH = 7.4) [46]. One week after injection, mice with 60–80 mm^3–tumors (one "ellipsoid" tumor per mouse, 6 mice in each group) received 0.2 mL of CDDP (5 mg kg^{-1}) [14], targeted or non-targeted FA-AAP-CDDP complex, or AAP-CDDP complex (equivalent CDDP concentration: 5 mg kg^{-1}), or FA + FA-AAP-CDDP (FA is the free folic acid, and the dose of folic acid is same as the folic acid content in FA-AAP-CDDP) via intravenous injection once a week, with a total of 4 injections administered (cumulative CDDP dose of 20 mg kg^{-1}). The tumor volume was measured using a vernier caliper (603, Harbin Measuring & Cutting Refco Group Ltd., Harbin, China), and then was calculated using the formula: $a \times b^2/2$, where "a" is the largest diameter, and "b" is the smallest diameter. A 0.9% NaCl solution was used as the control.

2.3. Tumor inhibition rate and organ index

All the mice were sacrificed by cervical dislocation 24 h after the last dose of the drugs. The tumors, heart, liver, spleen, and kidneys were dissected and weighed immediately. The tumor volume was calculated. Moreover, the toxicities of CDDP, targeted or non-targeted FA-AAP-CDDP complex, and AAP-CDDP complex were studied by monitoring the mice body weights. In addition, the tumor inhibition rates were calculated using the formula [47–49]: [(The mean tumor weight of the control group – The mean tumor weight of the treated group)/The mean tumor weight of the control group] × 100. The organ indexes for the heart, liver, spleen, and kidney were calculated using the formula: Organ index = [Average weight of the organ (mg)/Body weight (g)] × 1000.

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