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Chinese Journal of Natural Medicines 2015, **13**(12): 0915–0924

Chinese Journal of Natural Medicines

Improved anti-tumor activity and safety profile of a paclitaxel-loaded glycyrrhetinic acid-graft-hyaluronic acid conjugate as a synergistically targeted drug delivery system

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Available online 20 Dec., 2015

[ABSTRACT] The present study was designed to develop and evaluate glycyrrhetinic acid-graft-hyaluronic acid (HGA) conjugate for intravenous paclitaxel (PTX) delivery. Lyophilized PTX-loaded self-assembled HGA nanoparticles (PTX/HGAs) were prepared and characterized by dynamic light scattering measurements. Hemolysis test, intravenous irritation assessment, and *in vitro* and *in vivo* pharmacodynamic studies were carried out. B16F10 and HepG2 cells were used in the cell apoptosis analysis. The mouse MDA-MB-231 xenograft model was used for the evaluation of *in vivo* anticancer activity of the drugs, by the analysis of tumor growth and side effects on other tissues. PTX/HGAs showed high stability and good biocompability. Compared with PTX plus GA plus HA solution, PTX/HGAs displayed obvious superiority in inducing the apoptosis of the cancer cells. Following systemic administration, PTX/HGAs efficiently suppressed tumor growth, with mean tumor inhibition ratio (TIR) being 65.08%, which was significantly higher than that of PTX plus GA plus HA treatment. In conclusion, PTX/HGAs demonstrated inhibitory effects tumor growth without unwanted side effects, suggesting that HGA conjugates hold a great potential as a delivery carrier for cancer chemotherapeutics to improve therapeutic efficacy and minimize adverse effects.

[KEY WORDS] Hyaluronic acid; Glycyrrhetinic acid; Conjugate; Synergistically targeted delivery; Antitumor activity

[CLC Number] R965 **[Document code]** A **[Article ID]** 2095-6975(2015)12-0915-10

Introduction

Paclitaxel (PTX) is the first-line chemotherapeutic drug for the treatment of breast, ovarian, non-small cell lung carcinoma and so on [1]. Increasing studies have recently proven that PTX could inhibit hepatocyte cell proliferation *in vitro* [2]. The intravenous route of administration is preferred for the commercial agent of PTX (Taxol®), which requires dissolving the paclitaxel in cremophor EL (polyoxyethylated castor oil, CrEL) and ethanol [3]. However, the clinical performance of Taxol has been relatively disappointing because of the side effects such as neutropenia and peripheral neuropathy [4]. It is well known that CrEL may lead to nonlinear pharmacokinetics and decreased unbound drug fraction, limiting tumor penetration [5-6].

In the past few years, several drug delivery systems have been developed, based on one of the useful functions: (a) passive targeting, (b) active targeting, and (c) stimuli sensitivity [7]. However, only partial amounts of drug can reach its target site. To overcome this problem, several different targeted mechanisms have been combined in novel drug delivery systems. As a biodegradable glycosaminoglycan, hyaluronic acid (HA) plays an important role in designing a potential carrier to target several solid tumors [8]-[9]. Most HA-based nanocarriers exhibit superior intracellular targeting effect characterized by the over-expression of its main receptor-CD44 in the tumors [10]. It is generally believed that

[Received on] 04-Sep.-2015

[Research funding] This work was supported by the National Natural Science Foundation of China (No. 81173006), Project Program of State Key Laboratory of Natural Medicines, China Pharmaceutical University (No. JKGQ201107), the Jiangsu Natural Science Foundation of China (No. BK20131312) and the 12th of Six Talent Peak Foundation of Jiangsu Province.

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These authors have no conflict of interest to declare.

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glycyrrhetinic acid (GA), the main bioactive compound extracted from licorice, is widely used as the targeted moiety in drug delivery systems [11]. Rohilla *et al*. considered that vehicles modified with GA could be selectively distributed into the liver, taking advantage of the interaction between GA and the abundant receptors on hepatocyte membranes [12].

We have successfully synthesized an amphiphilic HA derivative conjugated with GA (HGA), which could be developed to self-assemble nanoparticles for delivery of PTX [13]. HGA nanoparticles could be considered as a novel PTX carrier, to not only reduce the adverse effects by eliminating CrEL from its formulation, but also show a synergistic targeting effect on tumor cells with the combination of HA and GA. As reported previously, HGA nanoparticles with less negative surface charge could readily load PTX with high efficiency up to 31.16% (wt.) [13], leading to reducing cost of final formulation and increasing the drug accumulation in tumors. In the present study, we further evaluated the tumor targeting behavior of the PTX-loaded HGA nanoparticles (PTX/HGAs), at the cellular and whole animal levels, as well as the safety profile of HGA conjugate and PTX/HGAs *in vivo*. The apoptosis studies *in vitro* were determined by flow cytometry using different cancer cells including mouse melanoma cells (B16F10) and human hepatocellular carcinoma cells (HepG2). Biological effects of the PTX/HGAs were evaluated in BALB/c nude mice bearing breast cancer xenograft tumors, including evaluations of therapeutic efficacy, body weight changes andhistological examinations. The control systems used in the present study were saline and/or physical mixture of PTX, GA, and HA (PTX plus GA plus HA.)

Materials and Methods

Materials

Hyaluronic acid (10 KDa) was obtained from Shandong Freda Biochem Co., Ltd., (Shandong, China). Paclitaxel (PTX) and glycyrrhetinic acid (GA) were purchased from Chongqing Melian Pharmaceuticals Co., Ltd., (Chongqing, China) and Nanjing Zelang Medicine Technology Co., Ltd., (Jiangsu, China), respectively. Anhydrous dimethylformamide (DMF), anhydrous formamide, anhydrous dichloromethane (DCM), and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) were obtained from Shanghai Lingfeng Chemical Reagent Co., Ltd., (Shanghai, China) and Sigma Chemical Co., (St. Louis, MO, USA), respectively. N-Hydroxysuccinimide (NHS), N, N-dicyclohexyl carbodiimide (DCC) and pyrene were from Sinopharm Chemical Reagent Co., Ltd., (Nanjing, China). DeadEnd Fluorometric TUNEL System and mouse specific HRP/DAB detection IHC kit were purchased from Promega (Madison, WI, USA) and Abcam (Cambridge, MA, USA), respectively. All other chemicals were of analytical grade and used without further purification.

Animals

B16F10 and HepG2 cells were obtained from Institute of Biochemistry and Cell Biology (Shanghai, China). Rabbits (1.8−2.0 kg) were purchased from the Qinglongshan Animal Breeding Center (Nanjing, China). MDA-MB-231 tumor-bearing mice (20−25 g) were obtained from Experimental Animal Center of China Pharmaceutical University (Nanjing, China). Animals were cared for in accordance with the National Institute of Health (NIH) guidelines for the care and use of laboratory animals (NIH publication 80−23, revised in 1996). Animals were housed under a 12-h light/dark cycle (lights on a.m.), allowed food and water ad libitum, and acclimatized for 2 weeks. All animal experiments complied with the requirements of the National Act on the Use of Experimental Animals (China). The animals used for the experiment were treated according to the protocols evaluated and approved by Ethics Committee of China Pharmaceutical University.

Synthesis and characterization of HGA conjugates

The HGA conjugate was synthesized as described in our previous report [13]. Briefly, GA was converted to aminoethyl GA (GA-NH_2) in the presence of DCC and NHS, which was ready for reaction with the carboxylic acids of HA. GA-NH_2 was then chemically conjugated to the backbone of HA after the addition of EDC and NHS. The degree of substitution (DS, defined as the number of GA per HA molecule) was in the range of 5.32%−20.18%, by varying the molar ratio of GA-NH_2 to thecarboxylic acid of HA. HGA synthesis was verified by ^{1}H NMR and FI-TR spectra.

Preparation and characterization of PTX-loaded HGA nanoparticles

PTX-loaded HGA nanoparticles were prepared by a simple dialysis method. In brief, the PTX solution at the concentration of 30 mg·mL^{-1} in ethanol was added into the HGA solution at 6 mg·mL^{-1} with stirring, and the solution was ultrasonicated for 30 min in ice-bath by an ultrasonicator (JY92-2D, Ningbo Scentz Biotechnology Co., Ltd., Ningbo, China). The resultant solution was dialyzed against an excess amount of distilled water with a dialysis bag (Molecular Weight Cut Off 1 000) overnight, filtered through a 0.8-μm microporous membrane, and lyophilized with lactose, sucrose or mannnitol.

The amount of PTX in the nanoparticles was measured using high performance liquid chromatography (HPLC, Shimadzu LC-2010 system, Kyoto, Japan) with UV detection at 227 nm. The drug-loading (DL) and entrapment efficiency (EE) of PTX were calculated as follows:

$$DL(\%) = \frac{\text{weight of PTX in nanoparticles}}{\text{weight of PTX in nanoparticles} + \text{weight of HGA fed initially}} \times 100 \quad (1)$$

$$EE(\%) = \frac{\text{weight of PTX in nanoparticles}}{\text{weight of PTX fed initially}} \times 100 \quad (2)$$

Hemolysis test and intravenous irritation assessment

As reported previously, the concentration of rabbit red blood cells (RBC) and the incubating times were fixed [14-15] (Table

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