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Gambogic acid grafted low molecular weight heparin micelles for targeted treatment in a hepatocellular carcinoma model with an enhanced anti-angiogenesis effect

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ABSTRACT

Gambogic acid (GA) is a potential anti-cancer agent with poor water-solubility, whereas heparin has anti-angiogenesis effects with good hydrophilicity. In this study, GA grafted low molecular weight heparin (GA-LMWH) was prepared and self-assembled into micelles in aqueous solution to improve the solubility and antitumor effects against hepatocellular carcinoma. The substitution of GA-LMWH is 27.5 ± 0.2%. The micelles had a mean size of 190.4 ± 10.8 nm, a low critical micelle concentration of 2.4 ± 0.2 μg mL^{-1}, and the highest area under the concentration-time curve and mean retention time in the liver compared to the heart, spleen, lung and kidney ($p < 0.05$). The targeting efficiency of micelles to the liver is 2.1-times higher than that of the GA solution. GA-LMWH micelles were administered intravenously and significantly improved liver function, decreased cell lesions in hepatic tissue, inhibited the expression of CD105 and prolonged survival time of hepatocellular carcinoma model compared with groups treated with normal saline or GA solution. These results suggest that GA-LMWH micelles may improve anti-cancer effects by targeting the delivery of GA to the liver and enhancing the anti-angiogenesis effect.

1. Introduction

Gambogic acid (GA) is an important effective component from the dry gum-resin of *Garcinia hanburyi* and possesses significant anticancer activity both *in vitro* and *in vivo* via multiple mechanisms (Kashyap et al., 2016; Wang and Chen, 2012; Yang et al., 2007). Additionally, the combination of GA with docetaxel or bortezomib (proteasome inhibitor) have synergistic antitumour effects (Zou et al., 2012; Liu et al., 2014), and GA reverses the multidrug resistance and increases the antitumour effects of adriamycin, docetaxel, verapamil and protopanaxadiol (Wang et al., 2014a,b). The results of a phase IIa study of GA injection indicated that GA exhibited a favorable safety profile when administered at 45 mg/m^2, but the percent of injection site reactions and phlebitis were relatively high (Chi et al., 2013). In addition to vascular irritation, the poor aqueous solubility and short half-life of GA *in vivo* restrict its clinical application. Many efforts have been undertaken to overcome these disadvantages of GA, such as chemical modifications (Sun et al., 2012; Zhang et al., 2012) and drug carriers. Carriers such as liposomes (Yu and Tang, 2016), electrosprayed particles (Yin et al., 2014), solid lipid nanoparticles (Huang et al., 2013), and polymeric nanosystems (Dahmani et al., 2016), have also been developed to improve the anticancer activity and reduce the side effects of GA. Moreover, GA has conjugated with a magnetic Fe_3O_4 colloidal suspension, which increased the water solubility of the drug and enhanced its chemotherapeutic efficiency for pancreatic cancer (Wang et al., 2011). The combination with other drug was also could enhance the cytotoxic effects and reduce the side-effect of GA. GA have synergistic antitumor effects with docetaxel (Zou et al., 2012), adriamycin, docetaxel, verapamil and protopanaxadiol (Wang et al., 2014a,b). The inhibitory effect of bortezomib (proteasome inhibitor) was enhanced many folds in synergism with GA (Liu et al., 2014).

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Polymeric micelles are nano-sized, spherical, supramolecular colloidal particles with a hydrophobic core and hydrophilic corona. The biocompatible polymeric corona causes reduced recognition of micelles by reticuloendothelial systems, thus providing long circulation of the loaded component in the blood stream. The nano-ranged size along with the long circulatory property allows polymeric micelles to eventually accumulate in any compromised tissue-vasculature sites, e.g. tumor via a passive targeting phenomenon commonly referred to as Enhanced Permeability and Retention (EPR) effect (Biswas et al., 2016). Some micelles have been developed for the delivery of GA, such as nanosized gambogic acid-mPEG2000 micelles (Cai et al., 2014), poloxamer 407/TPGS (D-α-Tocopheryl polyethylene glycol 1000 succinate) mixed micelles (Saxena and Hussain, 2012), and GA-loaded chitosan micelles (Zhu et al., 2008).

Heparin is a heterogeneous N- and O-sulphated glycosaminoglycan with a high anticoagulant activity that has been used for the treatment or prevention of thromboembolism in cancer patients. Previous studies have shown that heparin inhibits metastasis in animal cancer models (Yee et al., 2008). Low molecular weight heparin (LMWH) has fewer side effects and has replaced unfractionated heparin as an antithrombotic agent (Zed, 1999). Human clinical trials of LMWH in cancer have shown improved patient survival (Klerk et al., 2005), and the risk-effect ratio (in the case of an increased bleeding event) should be considered when using LMWH in patients with cancer (Chen et al., 2013). LMWH inhibits invasion, and metastasis of solid tumors *in vitro* (Zacharski, 2008), as well as angiogenesis, by tumour-derived adhesion factor, heparin EGF-like growth factor, hepatocyte growth factor/scatter factor, VEGF, and heparan sulphate proteoglycans (Shnoor et al., 2015). So LMWH may inhibit the angiogenesis without increased bleeding event by targeting delivery it to the tumor.

Tumour angiogenesis is a critical step in tumor growth and metastasis, and inhibiting tumor angiogenesis has achieved a great progress in cancer treatment in clinical. The strategy of combined anti-angiogenesis drugs with chemotherapeutics for cancer treatment could inhibit the proliferation and invasion of the tumor, and it has been used in clinical (Stark et al., 2013; Sandler et al., 2006; Slamon et al., 2001). In order to enhance the antitumor efficiency of GA, LMWH was selected not only as the hydrophilic polymeric corona of micelles but also as an anti-angiogenesis drug for combination therapy with GA. GA grafted LWMH (GA-LWMH) were prepared through an amido linkage. GA was wrapped in LWMH, which served as a hydrophilic shell, to increase the solubility, prolong the half-life, and enhance the targeting effect of GA to tumor tissues. Furthermore, LWMH is not only a drug carrier but is also enriched in tumor and inhibits angiogenesis and tumor growth; therefore, we hypothesize that GA-LWMH promotes the anti-tumour activity of gambogic acid *in vivo*.

2. Materials and methods

2.1. Materials

Gambogic acid (purity ≥ 99.0%) was extracted and isolated from gamboge, the resin from trees of the family *Clusiaceae*, in our laboratory. Low molecular weight heparin (LMWH, 175 U mg^{-1}, Mw 4000–8000) was purchased from BoMei reagent company (Hefei, China). 1-ethyl-(3-dimethylaminopropyl)-carbodiimide (EDCI), 1-hydroxy benzotriazole (HOBT), 4-dimethyl amine pyridine (DMAP), sodium periodate, sodium cyanoborohydride and tyramine were from the Aldrich Company (St. Louis, MO). Fluorescein isothiocyanate (FITC) and sodium hydrogen sulphite were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). The CD105 antibody was obtained from Bioss Biological Technology Co., LTD (Beijing, China). All other chemicals and solvents were of reagent grade or higher.

2.2. Animals

Kunming mice (male, weight, 20–25 g; age, 7–8 weeks) and male H_{22} tumour-bearing mice were supplied by the Experimental Animal Center of Anhui Medical University (Anhui, China) and were allowed to acclimatization for at least 1–2 weeks before experimentation. They were fed a standard diet and water. All animal experiments were performed in accordance with the Animal Management Rules of the Ministry of Health of the People's Republic of China (Document No.55, 2001) and the guidelines for the Care and Use of Laboratory Animals of our university.

2.3. Cell

H_{22} cells were extracted from the ascites of H_{22} tumour-bearing mice and maintained in ascitic form by sequential passages into the peritoneal cavities of male Kunming mice by weekly intraperitoneally transplanting 1×10^6 mL^{-1} tumor cells in 0.4 mL (Yang et al., 2014).

2.4. Synthesis of GA-LMWH

The synthetic route is shown in Fig. 1. GA (189 mg, 0.3 mmol), HOBT (49 mg, 0.36 mmol) and EDCI (69 mg, 0.36 mmol) were dissolved in 2 mL of a methylene chloride solution while stirring with a magnetic stirrer for 1 h in an ice water bath to form a reactive intermediate ester. Then, 0.2 mL of an ethylenediamine solution was added and stirred at room temperature until the reaction was complete as assessed using a thin layer chromatography test. The mixture was precipitated in distilled water, and a light yellow resulting product, GA-NH_2, was obtained after washing three times with a mixture of methylene chloride/distilled water (1:1, v/v) and drying with anhydrous sodium sulphate.

LMWH (2.3 mg) was dissolved in 4.0 mL of formamide in a 60 °C water bath for 10 min. After cooling to room temperature, EDCI (14.42 mg) and a proper amount of DMAP were mixed with the LMWH solutions while stirring with a magnetic stirrer for 15 min in an ice water bath to form a reactive intermediate ester, followed by the addition of GA-NH_2 dissolved in DMF (2.0 mL). The resulting solutions were stirred at room temperature. After the reaction, one volume of distilled water was added, and the solution was then adjusted to pH 3.5 with HCl (1 mol mL^{-1}). The precipitates were washed three times and extracted with a methylene chloride solution before drying with anhydrous sodium sulphate overnight. The crude product was further purified by dialyzing against distilled water using a dialysis membrane (Molecular Weight Cut Off 3500) for 48 h and then lyophilized.

2.5. Synthesis of GA-LMWH-FITC

LMWH (80 mg) was dissolved in sodium bicarbonate buffer, and the pH was adjusted to 7.5. Then, tyramine (55 mg) and sodium cyanoborohydride (10 mg) were added with stirring (Harenberg et al., 2002). Additional sodium cyanoborohydride (10 mg) was added after reaction for 24 h at room temperature, and the reaction continued for 24 h. After dialysis, LMWH-tyramine was collected by lyophilization. LMWH-tyramine was dissolved in 7.0 mL of sodium bicarbonate buffer and adjusted pH to 8.5. Then, 8 mg of FITC was added and reacted for 6 h at 25 °C in the dark. LMWH-tyramine-FITC was precipitated and washed with ethanol until no fluorescence remained in the supernatant. Then, LMWH-tyramine-FITC was obtained by nitrogen blow. GA was bound to LMWH-FITC