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Antitumor drug effect of betulinic acid mediated by polyethylene glycol modified liposomes

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ABSTRACT

Betulinic acid (BA), as a natural pentacyclic lupine-type triterpene, principally derives from bark of white birch, due to its potent pharmacological properties and low side-effect, which has been demonstrated a prominent efficiency on cancer therapy. However, the poor solubility and low bioavailability limit its pharmaceutical effect. Herein, we reported the rapid efficient synthesis of the polyethylene glycol modified (PEGylated) BA liposomes using ethanol injection technique for the first time. In the study, hydrophobic BA was encapsulated in the lipid bilayer of liposomes, meanwhile hydrophilic PEG layer covered the surface of liposomes. The mean diameter of PEGylated BA liposomes was 142 nm, which can effectively accumulate in the tumor tissues. *In vitro* drug release study showed that the PEGylated BA liposomes had a better sustained drug release effect than BA liposomes. The PEGylated BA liposomes also exhibited a better tumor inhibitory effect compared with those of free BA or BA liposomes *in vitro* and *in vivo* experiments. Therefore, the PEGylated BA liposomes could serve as a better alternative for the cancer therapy in future.

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1. Introduction

Cancer is a serious threat to human health, and current treatments for cancer are facing challenging obstacles. The traditional chemotherapy is limited by many defects, such as severe side effects, nonselective cytotoxicity and drug resistance [1]. Thus, it is essential to develop novel anticancer agents, which could improve the drug therapeutic efficacy. Natural products have been used to remedy human diseases for thousands of years and play an increasing roles in disease treatment [2,3]. Betulinic acid (3β-hydroxy-lup-20(29)-en-28-oic acid, BA, Fig. 1) is a plant-derived pentacyclic triterpenoid, which is widely distributed in natural plant e.g. bark of white birch (*Betula* species), and has been recognized to possess a variety of pharmacological properties [4]. BA and its derivatives have been shown to exert anti-inflammatory [5], anti-human immunodeficiency virus [6,7] and anticancer activities [8,9]. Significant antitumor activity of BA was first demonstrated in melanoma cell lines and later confirmed in melanoma xenograft mouse models [10]. Researches also showed that BA could delay tumor formation, decrease cell proliferation, invasion and angiogenesis in the MCF-7 xenograft tumor model. Meanwhile, a satisfactory safety for BA was supported *in vivo* studies [11]. However, due to the poor water solubility and dissolution rate of BA in the gastrointestinal tract, it is difficult to exert fully the anticancer activity of BA.

According to the Ostwald-Freundlich and Noyes-Whitney equations, the saturation, solubility, and dissolution rate of a drug can be increased by reducing the particle size to increase the interfacial surface area [12]. In the past decade, nanodrug delivery systems have shown the potential to carry drugs by decreasing their toxicity, sustaining their release, and increasing their efficacy, stability, and solubility [13], such as liposomes [14,15], polymersomes [16,17], polymeric nanoparticles [18,19] and dendrimers [20,21]. Among the most drug delivery systems, liposomes are a promising option for advantageous drug transport, which are self-assembled to spherical vesicles by a lipid bilayer and commonly used to encapsulate both hydrophilic and hydrophobic materials. As a nanodrug delivery, liposomes exhibited the well-established properties, including good biocompatibility, biodegradability, low toxicity, and controlled release of the entrapped drug, increased the therapeutic effect of the drug with minimizing side-effects [22]. Until now, many drugs have been successfully carried in liposomes. Researches showed that the bioavailability of paclitaxel liposomes increased 3.39-fold in comparison with free paclitaxel [23]. Pharmacokinetic assessments showed the advantages of systemic bioavailability of ursolic acid-loaded liposomes (AUC = 218.32 mg/L·h, $t_{1/2}$ = 7.61 h), which was 6-fold higher than that of free ursolic acid (AUC = 36.88 mg/L·h, $t_{1/2}$ = 0.78 h) [24]. However, the applications of conventional liposomes are still challenged by poor physical and chemical instabilities (aggregation or fusion, precipitation), low drug loading efficiency and relatively short blood circulation time [25]. Fortunately, surface modification of conventional liposomes with polymers could overcome above-mentioned shortcomings. Among the polymers, polyethylene glycol (PEG) is known to be one of the most successful choices for improving the disadvantage of conventional liposomes [26]. The amphiphilic PEG has a good water solubility, bio-inert, and non-toxic property that is widely used in the field

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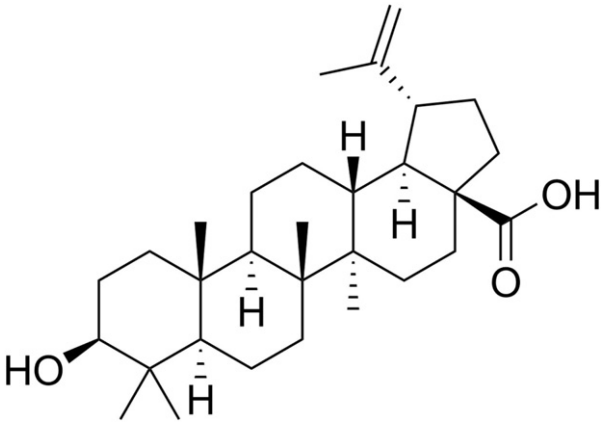
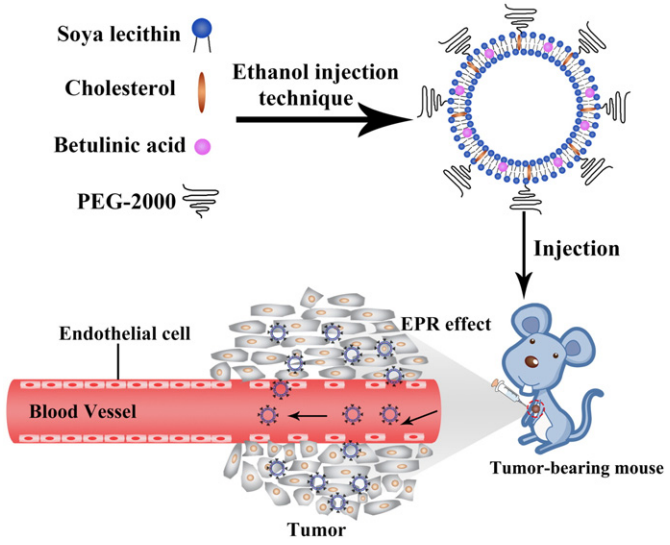


Fig. 1. Molecular structure of BA.

of pharmaceutics for proteins and drug carriers [27]. PEGylated liposomes were regarded as stealth liposomes, which significantly extended blood circulating time of nanocarriers, decreased interaction with plasma proteins and reduced uptake by the reticuloendothelial system [28]. Meanwhile, liposomes coated with PEG also improved the accumulation of nanocarriers in tumor tissue by exerting the enhanced permeability and retention (EPR) effect [29]. Therefore, PEGylated liposomes provide an attractive platform to improve the therapeutic effects of a variety of drugs.

At present, the preparation and antitumor efficiency of PEG-modified betulinic acid liposomes were researched for the first time (Scheme 1). In the study, the PEGylated BA liposomes were prepared to improving its bioavailability by taking advantage of its small size and surface effects. The preparation formulation of PEGylated BA liposomes was optimized by orthogonal experiment to obtain the optimal mass ratios of soybean lecithin, cholesterol, BA, and polyethylene glycol. Morphological observation of the liposomes was performed using the transmission electron microscope and atomic force microscopy. Moreover, the particle size distribution of the vesicles was determined by dynamic light scattering method. Furthermore, the antitumor efficacy of the carriers was investigated on U14 tumor-bearing mice.



Scheme 1. Schematic illustration of the synthetic route to the PEGylated BA liposome and cancer therapy.

2. Materials and methods

2.1. Materials

Betulinic acid was purchased from Nanjing Zelang Medical Technology Co., Ltd Nanjing, China). Soya lecithin (SPC, PC-98) was purchased from Shenyang Tianfeng Biological Pharmaceutical Co, Ltd (Shenyang, China). Cholesterol (Chol) was purchased from Tianjin Damao chemical instruments supply station (Tianjin, China). PEG-2000 was purchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Sephadex G-75 was purchased from Beijing biodee Biotechnology Co., Ltd (Beijing, China). All the chemicals and solvents used in the study were of analytical and chromatographic grade, and deionized water was used throughout the experiments.

2.2. Preparation of conventional and PEGylated BA liposomes

In this investigation, liposomes were prepared by the ethanol injection technique, as described previously [30]. Briefly, soya lecithin, cholesterol and betulinic acid were mixed in a mass ratio of 50:6.2:5. The mixture was well dissolved in absolute ethanol (4 mL) as the lipid phase. Tween-80 (10 μL) and PEG-2000 (5 mg) were solubilized in 10 mL of phosphate-buffered saline (PBS; pH 6.5, 0.01 M) in a water bath at 43 °C as the aqueous phase. Then, the lipid phase was added dropwise into the aqueous phase under magnetic stirring. To remove residual ethanol, the emulsion was stirred gently for one hour. Finally, homogeneous and translucent blue opalescent solution was obtained. Eventually, PEG decorated liposomes (PEGylated BA-Lips) were obtained and the concentration of BA encapsulated in liposomes was 0.5 mg/mL. BA liposomes (without PEG, BA-Lips) were prepared using the same method. All the liposome suspensions were kept at 4 °C for further analysis.

2.3. Determination of encapsulation efficiency

The amount of BA encapsulated in liposomes was determined by high performance liquid chromatography (HPLC, Agilent, USA). Sephadex gel column chromatography was used to separate non-entrapped BA from liposomes by protein purification system (Bio-Rad, USA). A gel chromatographic column filled with Sephadex G-75 was used to separate the liposomal nanoparticle systems. One milliliter of BA liposomal dispersion was slowly injected into the chromatographic column and eluted with deionized water at detection wavelength of 210 nm. Free BA remained in the gel, and then the eluted liposomes were collected at 7.2 min and analyzed by HPLC.

The eluted liposomes were dissolved in the same amount methanol for demulsification, and then centrifuged at 10,000 rpm for 30 min. Finally, the supernatant was filtered with an organic membrane (0.45 μm) before being assayed via the HPLC. An Agilent ZORBA × 300SB-C18 (4.6 mm × 250 mm, 5 μm) was used for HPLC analysis at room temperature. The mobile phase was consisted of methanol and phosphate buffer (90:10, v/v, pH 3.0) and the flow rate was maintained at 0.8 mL/min. The injection volume was 10 μL and the determine wavelength was 210 nm according to the absorption peak wavelength of BA. The standard curves of BA had a good linear relationship in a range from 5 to 100 $\mu g \cdot mL^{-1}$ ($r = 0.9999$). The percentage of encapsulating efficiency (EE) was calculated according to the following equation:

$$EE\ (\%) = (W_{en}/W_{total}) \times 100\%$$

where W_{en} is the amount of BA encapsulated in liposomes, and W_{total} is the initial amount of BA added in the preparation, respectively.

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