International Journal of Pharmaceutics 528 (2017) 163–171

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Liposomal andrographolide dry powder inhalers for treatment of bacterial pneumonia via anti-inflammatory pathway

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ARTICLE INFO

Article history:
Received 4 April 2017
Received in revised form 18 May 2017
Accepted 1 June 2017
Available online 2 June 2017

Keywords:
Andrographolide
Bacterial pneumonia
Dry powder inhaler
Inflammation
Liposome
NF-κB

ABSTRACT

Andrographolide (AG) is a chemical entity from traditional Chinese herbs and its oral pills have been applied to the treatment of respiratory inflammation. Here we report pulmonary delivery of liposomal AG dry powder inhalers (LADPIs) for treatment of *Staphylococcus aureus*-induced pneumonia. AG liposomes were prepared with the injection method and then freeze-dried for preparation of LADPIs. AG liposomes were small and stable with a mean size of 77.91 nm and a zeta potential of −56.13 mV. Liposomes were well recovered after re-hydration of LADPIs that were suitable for pulmonary delivery with a mass mean aerodynamic diameter (MMAD) of 4.87 μm and a fine particle fraction (FPF) of 23.03%. However, the MMAD and FPF of AG powders were 10.14 μm and 8.37%, respectively. The *in vitro* anti-*S. aureus* effects of AG powders and LADPIs were investigated, but were not found. They were intratracheally sprayed into the rat lungs for treatment of *S. aureus* pneumonia. Surprisingly, LADPIs showed a stronger anti-*S. aureus* pneumonic effect *in vivo*, than AG at a ten-fold dose or than an antibiotic, penicillin. LADPIs significantly decreased many pro-inflammatory cytokines including TNF-α, IL-1. Furthermore, the phosphorylation of IκB-α in the nuclear factor-κB (NF-κB) pathway was also remarkably inhibited. AG regulated the immune reaction to maintain the antibacterial effect while downregulating inflammatory response so that AG showed a strong effect on bacterial pneumonia. LADPIs are a promising pulmonary delivery medicine for the treatment of bacterial pneumonia.

1. Introduction

Treatment of bacterial pneumonia has become one of the greatest challenges in the 21st century with the emergence of highly resistant strains of bacteria and patients with acute or chronic pulmonary infections where the use of antibiotics is widespread (Loebinger and Wilson, 2012; Thuong et al., 2016). Bacterial pneumonia can be caused by various bacteria, such as *Staphylococcus aureus* (Toshinori et al., 2016), *Klebsiella pneumonia* (Rosen et al., 2016), and *Pseudomonas aeruginosa* (Sami et al., 2014). *S. aureus* is an important human pathogen and a leading cause of nosocomial infections, especially for bacterial pneumonia (Francis et al., 2005; Rubinstein et al., 2008). Furthermore, antibiotic therapies may cause toxic reactions (Jamali et al., 2014), dual infections (Song et al., 2015), allergic responses (Rebecca et al., 2006), and more importantly, bacterial resistances. Methicillin-resistant strains of *S. aureus* are frequently identified as the cause of outbreaks of clinical infections (Hageman et al., 2006; Klein et al., 2007). Therefore, it is necessary to find effective non-toxic pharmaceutical ingredients against bacterial pneumonia with no or weak drug resistance. Scientists are turning attention to natural products (Muluye et al., 2014; Wu et al., 2014).

Andrographolide (AG), a natural diterpenoid, is the major active ingredient of *Andrographis paniculata*. AG shows multiple pharmacological functions involving anti-inflammation (Ji et al., 2016), immune stimulation (Singha et al., 2003), antibacterial (Wen et al., 2014a), and antiviral activities (Panraksa et al., 2017). AG is a prescribed drug in China for preventing and treating common cold, influenza, bacterial and viral infections on upper respiratory tracts (Peng et al., 2016; Wang et al., 2016b). AG formulations are currently available in the market, such as dripping pills, and

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http://dx.doi.org/10.1016/j.ijpharm.2017.06.005
0378-5173/

tablets. However, AG is insoluble in water, leading to the poor solubility and low oral bioavailability.

Liposomes, as phospholipid vesicles that can entrap and deliver drugs (Narayan et al., 2016), have the advantages of tissue targeting and controlled release after administration (Liang et al., 2017). They have been broadly applied in medical (Liu et al., 2017), food and cosmetic formulations (Zhao and Temelli, 2017). Moreover, liposomes have become the non-parenteral dosage form for pulmonary drug delivery (Chen et al., 2012; Liu et al., 2015).

Pulmonary drug delivery is a non-invasive approach with a lot of advantages such as a large absorptive area and easily permeable membrane. Dry powder inhalers (DPIs) are one effective pulmonary delivery system that directly transports drugs into the deep sites of the lung (Muralidharan et al., 2014; Zhu et al., 2015). The aerodynamic diameter of DPIs must be in the size range of 1–5 μm in order to efficiently deposit in the lower respiratory tract (Simon et al., 2016).

In this study, we prepared inhalable liposomal AG dry powder inhalers (LADPIs) for the treatment of bacterial pneumonia induced by *S. aureus* and explored the anti-bacterial mechanisms.

2. Materials and methods

2.1. Materials

AG was provided by Sichuan Wenlong Pharmaceutical Co., Ltd., China. Soybean lecithin (SPC > 90%) was purchased from Shanghai Taiwei medicine Co., Ltd., China. Cholesterol was purchased from Sinopharm Reagent Co., Ltd. (Shanghai, China). Luria-Bertani broth and powdered agar were purchased from Beijing Aobox Biotechnology Ltd. Other reagents were of analytic grade. Pure water prepared with Heal Force Pure Water System was always used.

2.2. Animals

Male Sprague-Dawley (SD) rats (190–200 g) were provided by Vital River Experimental Animal Technology Co., Ltd. (Beijing, China). The handling and surgical process involving animals were conducted in strict accordance with the Guidelines for the Use of Laboratory Animals of Beijing Institute of Radiation Medicine (BIRM) and the animal experiments were approved by the animal subject review committee. Peripheral blood was collected via tail veins before sacrifice of the animals, then the lung bronchoalveolar lavage fluids (BALFs) were collected and the lung tissues were excised followed by hematoxylin and eosin (H&E) staining. The animals were always under humane care.

2.3. Preparation of AG liposomes

A preparation method of liposomes was referred to (Wang et al., 2016a). Simply, soybean lecithin and cholesterol (6:1, mol/mol) were dissolved in ethanol with AG. The solution was slowly injected into phosphate buffered solutions (PBS, pH 6.5) under agitation and liposomes were formed. Ethanol was removed under vacuum. The primitive liposomes were sonicated under ice bath for 5 min and then extruded through 0.22-μm filters.

2.4. Characterization of AG liposomes

AG liposomes were observed under a Hitachi H-7965 80-kV transmission electron microscope (TEM) after negative staining with 2% sodium phosphotungstate solution as in our previous research (Du et al., 2016). A dynamic light scattering method was applied on Zetasizer Nano ZS (Malvern, UK) at 25 °C to measure the sizes, size distribution and zeta potentials of AG liposomes.

2.5. Preparation of AG powders and LADPIs

AG raw materials were 180-mesh sieved to obtain AG powders. Liposomal AG DPIs (LADPIs) were prepared after mannitol was added into AG liposomes and the suspensions were further freeze-dried for 36 h on a lyophilizer (LGJ-30F, Beijing Songyuan Huaxing Technology Develop Co., Ltd., China).

2.6. Characterization of AG powders and LADPIs

Repose angles of powders were measured with the fixed funnel method (Ranjot et al., 2016). Briefly, AG powders or LADPIs were dropped through a funnel tip to a culture dish with 5-cm height difference. Repose angles can be calculated according to Eq. (1).

$$\tan\theta = \frac{h}{r} \tag{1}$$

where θ is the repose angle, h is the pile height, and r is the radius of pile bottom. Tapped density was calculated with the graduated flask method. The volume diameters of AG powders and LADPIs were measured on a particle size analyzer (BT2001, Bettersize Instruments Ltd., Dandong, China) based on the laser light diffraction method. If 50% of the particles are less than one volume diameter, the volume diameter is defined as the geometric mean diameter (D_{50}). The mass mean aerodynamic diameter (MMAD) of powders was calculated according to Eq. (2).

$$d_a = d_g\sqrt{\frac{\rho}{\rho_0}\times\chi} \tag{2}$$

where d_a is the MMAD, d_g is D_{50}, ρ is the tapped density (g/cm), ρ_0 is the standard density (1 g/mL), and χ is the dynamic shape factor that is 1 in case of spherical particles ($\chi = 1$ in this study).

Surface morphology of LADPIs was observed using a scanning electron microscope (SEM, S-4800, Hitachi, Japan). The rehydrated suspension of LADPIs was investigated using the TEM method mentioned above.

The dissolution of AG powders was measured in simulated lung fluids (SLFs). Because dialysis bags adsorbed the released AG, we selected the direct dissolution method. Briefly, AG powders were put into the SLF (50 mL) and then stirred at 100 rpm and 37 °C. At the predetermined time points, we stopped stirring and withdrew the supernatant (1 mL) that was then 0.45-μm filtered. AG in the filtrate was measured with the high performance liquid chromatographic (HPLC) method in Section 2.7.

2.7. Measurement of AG encapsulation and loading efficiencies in LADPIs

LADPIs were rehydrated with water and the liposomes were separated through a Sephadex G-25 gel column with water as the eluent. The collected samples were dissolved in methanol and the encapsulated AG was determined using an HPLC method on the Shimadzu LC-10A instrument at 30 °C. An Eclipse Plus C18 HPLC column (4.6 mm × 250 mm, 5 μm) was used. The mobile phase was 55% methanol in water at the flow rate of 1 mL/min. The measurement wavelength was 223 nm. The total AG was also determined after the LADPIs were dissolved in methanol. Encapsulation efficiency (EE) and drug loading efficiency (DL) were calculated with Eqs. (3) and (4).

$$\mathrm{EE}(\%) = \frac{\text{Encapsulated AG}}{\text{Total AG}}\times 100\% \tag{3}$$

$$\mathrm{DL}(\%) = \frac{\text{Total AG}}{\text{LADPIs}}\times 100\% \tag{4}$$