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Oral Delivery of Bavachinin-Loaded PEG-PLGA Nanoparticles for Asthma Treatment in a Murine Model

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T helper type 2 (Th2) cytokines, such as interleukin (IL)-4, IL-5, and IL-13, are the central therapeutic targets in the development of drugs against asthma. Recently, it was found that bavachinin, an essential small molecule in *Psoralea corylifolia*, has very efficient therapeutic function in asthma treatment in a murine model via the selective inhibition of Th2 cytokine production. However, the clinical potential of bavachinin is limited by its extremely low water solubility (<30 ng/mL). Here, we fabricated a nanoparticle delivery system for the oral administration of bavachinin to treat asthma, with the aim of solving its administration problem. In this study, bavachinin-loaded PEG₅₀₀₀-PLGA NPs were prepared through an emulsion-solvent evaporation technique, and their physical properties were carefully characterized. These NPs showed strong enrichment capability towards inflamed lung tissues. Through oral administration, these NPs showed very good anti-asthma therapeutic effects in murine asthma model, as demonstrated by a histological analysis of lung sections, enzyme-linked immunosorbent assay (ELISA) analysis of Th2 cytokine expressions, and fluorescence-activated cell sorting (FACS) analysis of Th1/Th2 cell differentiation. This system has the potential to be used for the oral delivery of bavachinin to treat asthma and may have potential for the oral delivery of other drugs that have limited pharmacokinetic efficacy.

KEYWORDS: Asthma, Bavachinin, Lung, Biodegradable Polymer, Oral Delivery.

INTRODUCTION

Asthma is a common chronic inflammatory disease of the airways.¹ It is associated with an immune response that is biased toward type 2 helper T cells (Th2), which preferentially produce Th2 type cytokines, including interleukin (IL)-4, -5, -9, and -13; these cytokines, in turn, drive eosinophilic inflammation and tissue damage, leading to airway hyper-responsiveness and airway infiltration of inflammatory cells.^{2–3} Currently, corticosteroids, such as β -agonists, are the main drugs used to treat asthma, but they are associated with serious side effects.^{4–6} Alternative treatments, with research focused on more specific strategies for treating asthma, are therefore urgently required. Among them, the inhibition of Th2 type

cytokines has emerged as a central therapeutic strategy in the development of new treatments for asthma,⁷ and its efficiency in the treatment of asthma has been confirmed in a number of clinical trials.^{8,9} This inhibition is mainly obtained by using biological macromolecules, such as humanized IL-4-specific and IL-4Ra-blocking monoclonal antibodies (pascolizumab, SB240683),¹⁰ IL-4 variant recombinant protein (pitrakinra),¹¹ and monoclonal antibodies against IL-13 (CAT-354 and AMG 317).¹² Inhibiting these cytokines by using small molecules is an effective alternative method with advantages such as low cost, higher stability and oral bio-availability compared with those of biological macromolecular drugs.

Psoralea corylifolia is a Chinese herb that is used to treat asthma in Chinese traditional medicine.¹³ Recently, it was found that bavachinin, an essential small molecule in *Psoralea corylifolia*, is very effective in treating asthma in a murine model and that it significantly reduces asthmatic inflammation and hyper-responsiveness via the selective

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inhibition of Th2 cytokine production.¹⁴ However, the clinical potential of bavachinin is limited by its extremely low water solubility (<30 ng/mL), which is much lower than even that of Taxol (1.6 µg/mL), and its rapid clearance.¹⁵ Therefore, with conventional oral administration, bavachinin has little chance to enter the blood system and reach the disease site. In a pilot animal study,¹⁴ bavachinin was administered orally by dissolving it in polyethylene glycol (PEG) 400/ethanol/water (4:1:5 v/v). The low-molecular weight liquid PEG 400 is an excellent solvent that can be used as a solubilizing agent for active substances and excipients that do not readily dissolve in water in animal studies. However, its potential toxicity may restrict its future clinical applications. Nanoparticles (NPs) may provide a highly feasible alternative solution for the delivery of drugs that have poor pharmacokinetic properties.^{16,17} By encapsulating drug molecules inside NPs, the profile of drug solubility, tissue distribution, and even toxicity and therapeutic effects can be modified or improved. Among the NPs, the drug-loaded poly (D,L-lactide-co-glycolide) (PLGA) NP technology is very interesting because of its excellent biocompatibility in medical applications.^{18–20} PLGA-encapsulated Taxol is now being tested in clinical trials for cancer chemotherapy.²¹

The oral administration of drugs has more favorable advantages compared to intravenous administration, so the development of an efficient oral delivery system for poorly soluble drugs would be interesting.^{22,23} Some researchers have found that the presence of a polyethylene glycol (PEG) chain coating on the surface of NPs can facilitate the rapid penetration of NPs through the gastrointestinal mucosal surface, the key barrier retarding NPs from entering the circulation system.^{24,25} Therefore, PEGylated NPs have the potential to form oral delivery systems.²⁶ Further, surface PEG modification can significantly increase the time that NPs circulate in the bloodstream.^{27–30}

In this paper, we used the copolymer PEG-PLGA to fabricate a NP delivery system, with the aim to test their capability for the oral delivery of bavachinin to treat asthma. In this study, bavachinin-loaded PEG-PLGA NPs were prepared through an emulsion-solvent evaporation technique, and their physical *in vitro* release and cytotoxicity properties were carefully characterized. An *in vivo* imaging experiment showed that the NPs have excellent targeting capability towards inflamed lung tissues. *In vivo* asthma treatment in a murine asthma model via the oral administration of these NPs, as evaluated by analyzing histological sections of the lungs, Th2 cytokine expression, and Th2 cell differentiation, clearly demonstrated the very strong efficacy of this strategy in asthma treatment.

MATERIALS AND METHOD

Materials

PEG₅₀₀₀-PLGA (LA/GA ratio, 50:50; molecular weight, 45 kDa) was purchased from Jinan Daigang Biomaterial

Co., Ltd. (Jinan, China). Polyvinyl alcohol (PVA) was purchased from Sigma (St. Louis, MO, USA). 4',6-Diamidino-2-phenylindole (DAPI) was purchased from Roche (Mannheim, Germany). Paraformaldehyde and PBS were purchased from Sangon (Shanghai, China). Penicillin/streptomycin, fetal bovine serum, trypsin-EDTA, modified Eagle's medium (MEM) and Dulbecco's modification of Eagle's medium (DMEM) for cell culture were purchased from HyClone (South Logan, UT, USA). Deionized water was obtained using the Barnstead Nanopure water purification system from Thermo Scientific (Waltham, MA, USA). All other chemicals and reagents used were of analytical grade and obtained commercially unless stated otherwise.

Cells and Animals

The human colorectal adenocarcinoma cell line Caco-2, human cervical cancer cell line HeLa and murine embryonic fibroblast cell line NIH-3T3 cells were purchased from Saierbio (Tianjin, China). Caco-2 cells were cultured in MEM medium, and HeLa and NIH-3T3 cells were culture in DMEM medium. All cells were incubated in medium containing 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin at 37 °C in humidified air supplemented with 5% CO₂. BALB/c wild-type mice were purchased from the Academy of Military Science (Beijing, China) and maintained in a germ-free environment with free access to food and water. All animal procedures were conducted under a protocol approved by the Institutional Animal Care and Use Committee of Nankai University (Tianjin, China).

Preparation of Bavachinin-Loaded NPs and Fluorescence Labelled NPs

NPs were formulated using an emulsion-solvent evaporation technique. Briefly, the polymer solution was prepared by dissolving 30 mg of PEG₅₀₀₀-PLGA in 1.0 mL of chloroform in a glass vial with magnetic stirring. Then, 3.0 mg of bavachinin was added to the polymer solution. Following this, the loaded solution was added to 6 mL of 2% PVA and vortexed for 1 min. Then, the primary emulsion was sonicated using a micro-tip probe sonicator at an energy output of 100 W for 2 min over an ice bath. The formed organic solvent/water emulsion was gently stirred overnight at room temperature using a magnetic stirrer to evaporate the organic solvent. Subsequently, drug-loaded NPs were collected by centrifugation. After being washed three times with ultrapure water, the NPs were dried by lyophilization and stored at 4 °C. For preparation of bavachinin-loaded NPs with coumarin-6 or IR-780 labeling, the operation is similar with above, except that 3.0 µg of coumarin-6 or IR-780 was added together with 3.0 mg of bavachinin to the polymer solution and the loaded solution was then added to 6 mL of 2% PVA for emulsion formation.

Physical Characterization of NPs

The particle hydrodynamic diameter and Zeta potential were determined using dynamic laser light scattering (DLS; Zetasizer Nano ZS; Malvern Instruments, Malvern, UK), by dispersing NPs (~1.0 mg/mL) in ultrapure water prior to particle size determination. Transmission electron microscopy (TEM) was performed on the Tecnai G2 F20 system (FEI, Hillsboro, OR, USA), operating at 100 kV.

For the determination of drug-loading efficiency and encapsulation efficiency, the NPs were dissolved in acetonitrile, and the concentration of bavachinin encapsulated was analyzed using high-performance liquid chromatography (HPLC; LC-20AT, Shimadzu, Kyoto, Japan) with a C18 column. The HPLC was calibrated using a standard solution of bavachinin dissolved in acetonitrile. The drug loading efficiency (LE) and encapsulation efficiency (EE) were calculated according to the following formulae:

$$\text{LE (\%)} = \frac{\text{amount of bavachinin in NPs}}{\text{amount of NPs loading with bavachinin}}$$

$$\text{EE (\%)} = \frac{\text{amount of bavachinin in NPs}}{\text{initial amount of bavachinin}}$$

To determine the *in vitro* drug release behavior, 15 mg of the bavachinin-loaded NPs was re-suspended in 5 mL of phosphate-buffered saline (PBS) containing 0.1% Tween 80 (PBST) and gently shaken using a shaker set at 37 °C and 110 rpm. At each projected interval, the buffer was refreshed with the same volume of PBST following centrifugation at 12000 rpm for 10 min. The obtained supernatant was extracted with ether, and the solvent was changed to acetonitrile for analysis with HPLC. Release profiles were reported in terms of the cumulative bavachinin released versus time, and the values were expressed as the means $\pm$ SEM from 5 samples.

Cellular Uptake Evaluation

Caco-2 cells, which are widely used as *in vitro* model system for investigating oral uptake efficiency,³¹ as well as HeLa cells and NIH-3T3 cells were seeded on a 24-well cell culture plate covered with sterile coverslips at a density of 6×10^4 cells/well for 24 h. Then, the cell culture medium was replaced with fresh medium containing 100 $\mu\text{g/mL}$ bavachinin-loaded NPs labeled with coumarin-6 (green fluorescence) and incubated at 37 °C for 4 hours. The cells were washed 3–5 times with PBS and fixed with 100 μL of 10% formalin for 15 min and were stained with DAPI (blue fluorescence) for nuclei and Alexa Fluor 635-phalloidin (red fluorescence) for the cell cortex. After washing with PBS, the cells were mounted and visualized on a confocal laser scanning microscope (CLSM, TCS SP8, Leica, Wetzlar, Germany) sequentially using the 350 nm (blue, DAPI), 470–490 nm (green, coumarin-6), and 515–560 nm (red, Alexa Fluor 635-phalloidin) excitation filters. Each stain was performed on three individual groups, and the photographs in the figures are representative of each group.

In Vitro Cytotoxicity Assays

The cytotoxicity of the bavachinin-loaded NPs was evaluated by measuring the inhibition of cell growth of Caco-2 cells, HeLa cells and NIH-3T3 cells using a tetrazolium dye (MTT) assay. Typically, cells that were harvested in a logarithmic growth phase were seeded on 96-well plates at a density of 1×10^4 cells/well. Next, the cells were incubated with serial dilutions of bavachinin-loaded NPs for 24 h. Then, 10 μL of MTT solution (5.0 mg/mL) was added to each well. The plates were incubated with MTT for an additional 4 hours at 37 °C. After removing the supernatant, 100 μL of DMSO was added to fully dissolve the crystals. The absorbance at 490 nm, which is related to the number of metabolically active cells, was measured using a microplate reader (EXL-800; Bio-Tek, Winooski, VT, USA).

Construction of a Murine Asthma Model

Asthma was induced in female BALB/c wild-type mice (6 to 8 weeks old, 18–22 g) by an intraperitoneal injection of chicken OVA (75 μg , grade V, Sigma) mixed with 2.0 mg of aluminum hydroxide in 200 μL of PBS on days 0 and 7, followed by challenge with aerosolized OVA once a day for 7 consecutive days starting on day 14 to further increase sensitivity.

In Vivo Distribution of Bavachinin-Loaded NPs

To evaluate the NP distribution *in vivo*, the asthmatic or healthy mice, as the controls, were intravenously (*i.v.*) injected with bavachinin-loaded NPs labeled with IR-780 through the tail vein. Twenty-four hours later, the mice were euthanized, and the fluorescent signals in the organs were visualized using an IVIS Lumina Imaging system (Xenogen, MA, USA) with excitation at 710 nm and emission at 820 nm, and analyzed using the Living Image software ver. 3.2 (Xenogen, MA, USA).

In Vivo Asthma Treatment via Oral Administration of Bavachinin-Loaded NPs

For the *in vivo* asthma treatment, bavachinin-loaded NPs (50 mg/kg body weight based on bavachinin) were dissolved in PBS and given orally by gavage to the asthmatic mice once per day for a week. For the controls, the asthmatic mice were orally given free bavachinin (50 mg/kg body weight) dissolved in PEG 400/ethanol/water (4:1:5 v/v) or PBS only. The body weights of the mice were recorded every day. The mice were euthanized 7 days later, and their tissues and blood were harvested for histological analysis and cytokine determination.

Histological Analysis of Lung Sections

Immediately after sacrificing the mice, the lung tissues were harvested and flushed with PBS. After fixing with 4% paraformaldehyde overnight, the tissues were dehydrated and embedded in paraffin. Sections of 5 μm thickness

were made for hematoxylin and eosin (H&E) staining. The images were acquired with a Leica DM3000 microscope using a 320× objective.

Enzyme-Linked Immunosorbent Assay (ELISA) for Cytokines in Serum

The blood was collected to determine the concentrations of IL-4 and interferon (IFN)- γ in serum with ELISA kits (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. The concentrations were determined based on the absorbance at 450 nm, which was read with a microplate reader (Bio-Tek EXL-800), by comparison with the standard curve and using the data from PBS solution as the background.

Determination of the mRNA Levels of Th2 Cytokines in the Lungs

Lung tissues of the treated mice were collected to determine the mRNA levels of Th2 cytokines through quantitative real-time PCR. Here, the lung tissues were flushed with PBS, and the pulmonary lobule RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The mRNA concentrations were determined by real-time PCR using the Quantscript reverse transcription Kit (Tiangen, Beijing, China) and SYBR Premix Hotmaster Taq (Tiangen) according to the manufacturer's protocols.

Determining CD4⁺ T Cell Differentiation with Fluorescence-Activated Cell Sorting (FACS)

The spleens of the treated mice were collected to determine the Th1/Th2 differentiation of CD4⁺ T and were analyzed by fluorescence-activated cell sorting (FACS). Here, the spleens were flushed with PBS, and the lymphocytic cells were separated. The lymphocytic cells were stimulated with phorbomyristyl acetate (50 ng/mL; Sigma) and ionomycin (1.0 mg/mL; Sigma) in the presence of GolgiStop (BD Biosciences) for 4.5 h. Then, they were fixed and permeabilized for intracellular cytokine staining with antibodies (BD Biosciences, Franklin Lakes, NJ, USA), including anti-IL 4, 5, 13, and anti-IFN- γ . T cell ratios with different cytokine expression levels were analyzed by flow cytometry using a FACS Calibur flow cytometer (BD Biosciences) with the Cell Quest Pro/FlowJo.7.6.1 software.

Statistics

Statistical tests were performed using one-way ANOVA analysis using InStat version 3.06 software for Windows (GraphPad, San Diego, CA, USA). For all tests, *P* values <0.05 were considered statistically significant.

RESULTS

Physical Characterization of the Bavachinin-Loaded PEG-PLGA NPs

A PEGylated PLGA polymer, with the PEG chain covalently conjugated to the end of the PLGA chain, was

adopted to fabricate the bavachinin-loaded NPs. Because a 2- to 5 kDa PEG coating is thought to be very effective in mediating the rapid penetration of mucus^{32–34} and because a 5 kDa PEG coating is considered very helpful for obtaining a long blood circulation duration, we selected a PEG chain with an average molecular weight of 5 kDa to modify the PLGA polymer. The bavachinin-loaded PEG-PLGA NPs were prepared according to the optimized operation described in the methods section. The process parameters were assessed to determine the optimal preparation conditions, including the sonication time, PLGA content, surfactant content, solvent, and drug content, and the particles were prepared as described in the experimental section.

DLS and TEM were used to analyze the physical properties of these NPs. DLS analysis showed that the particles had a mean diameter of ~196 nm, a polydispersity of ~0.36 and a Zeta potential of ~−3.8 mV (Table I and Figs. 1(A, B)). The TEM images (Fig. 1(C)) show that these NPs had a spherical shape with a relatively smooth surface and moderate uniformity and an estimated particle size that was very consistent with that obtained from the DLS experiment.

The drug-loading/entrapment efficiency and *in vitro* release behavior of the bavachinin-loaded NPs were determined with HPLC. The NPs had a drug-loading efficiency of $8.22\% \pm 2.35\%$ and an encapsulation efficiency of $89.8\% \pm 7.83\%$ (Table I). The relatively high drug incorporation efficiency of bavachinin in PEG-PLGA NPs could be attributed to the super-hydrophobic nature of bavachinin, which results in minimal loss of the drug to the external aqueous phase.

The *in vitro* release of bavachinin-loaded NPs was analyzed in PBS buffer with the addition of Tween 80 to mimic the physiological condition and increase the water solubility of the released bavachinin (Fig. 1(D)). The data indicate that bavachinin was released from the NPs with a very well-controlled and sustained profile, which almost resembles a first-order release with a minimal initial burst, and the cumulative release reached 95.7% on day 16.

Cellular Uptake Evaluation of Bavachinin-Loaded PEG-PLGA NPs

We adopted Caco-2 cells, HeLa cells and NIH-3T3 cells to evaluate the cellular uptake efficiency of these NPs. Caco-2 cells can, to some extent, mimic the human small intestinal mucosa to predict the absorption of orally

Table I. Physical characteristics of bavachinin-loaded PEG₅₀₀₀-PLGA NPs.

NP	Size (nm)	EE (%)	LE (%)	PDI	Zeta potential (mV)
Bavachinin-loaded NPs	196	89.8	9.0	0.358	−3.8

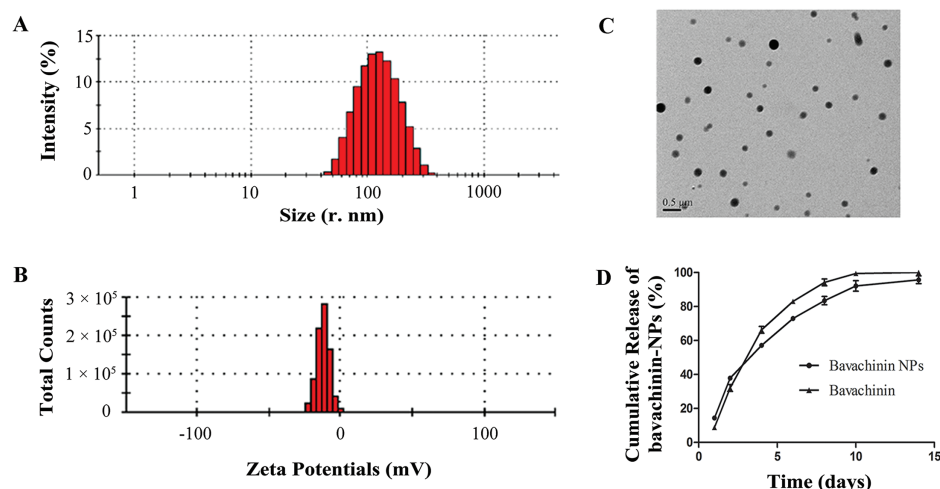


Figure 1. Physical characterization of bavachinin-loaded PEG₅₀₀₀-PLGA NPs. (A) Size distribution and (B) Zeta potential determined by DLS; (C) TEM image; (D) release profile of bavachinin-loaded NPs and free bavachinin (in solid form) in PBS buffer (pH 7.4) containing 0.1% tween 80 analyzed with HPLC.

administered drugs.²⁶ Here, the cells were incubated with bavachinin-loaded NPs that had been fluorescently labeled with coumarin-6 for convenience of monitoring, at a concentration of 100 $\mu\text{g/mL}$ at 37 °C for 4 hours. The intracellular uptake of the NPs was visualized with CLSM (Fig. 2). The CLSM images show strong green fluorescence of coumarin-6 in the cytoplasm around the nucleus (which was stained with blue fluorescence by DAPI) of most of the cells, which visually indicates that these cells exhibited strong absorption of these NPs.

Cytotoxicity Assay of the Bavachinin-Loaded PEG-PLGA NPs

To verify the non-cytotoxicity of the fabricated NPs, the MTT assay was carried out by incubating Caco-2, HeLa and NIH-3T3 cells with bavachinin-loaded NPs. As shown in Figure 3, the presence of the bavachinin-loaded NPs for 24 hours had no significant influence on the cell viabilities, even at concentrations up to 1.0 mg/mL of NPs, corresponding to 0.1 mg/mL of bavachinin, which suggests that these NPs had negligible cytotoxicity *in vitro*.

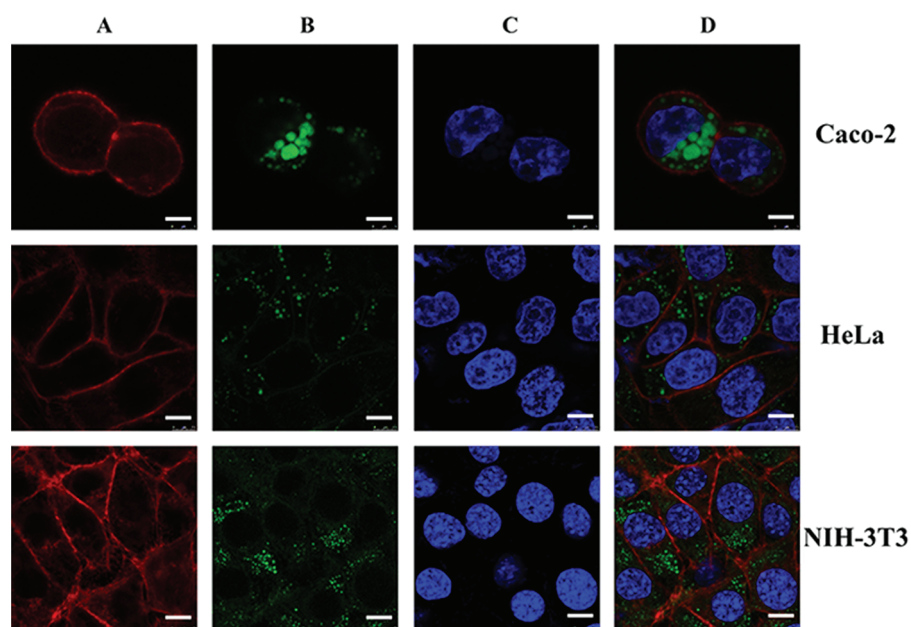


Figure 2. CLSM images of cells incubated with bavachinin-loaded PEG₅₀₀₀-PLGA NPs labeled with coumarin-6 at a concentration of 0.1 mg/mL at 37 °C for 4 h. The cell cortex was stained with Alexa Fluor 635-phalloidin (red fluorescence). Bavachinin-loaded NPs were labeled with coumarin-6 (green fluorescence). Nuclei were stained with DAPI (blue fluorescence). (A), (B) and (C) are the red fluorescence, green fluorescence and blue fluorescence channels, respectively. (D) is the merged channel. Scale bar = 10 μm .

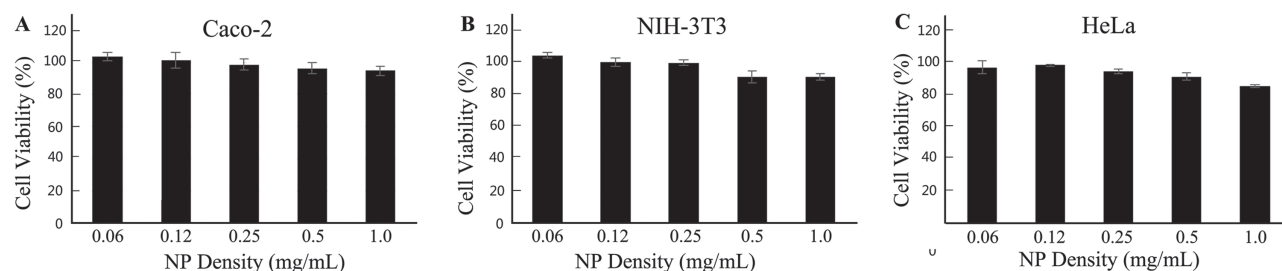


Figure 3. Cytotoxicity assays of Caco-2, NIH-3T3 and HeLa cells following incubation with various concentrations of bavachinin-loaded NPs.

In Vivo Distribution of the Bavachinin-Loaded PEG-PLGA NPs in a Murine Asthma Model

To evaluate the *in vivo* distribution of the bavachinin-loaded NPs, the mice were *i.v.* injected with bavachinin-loaded NPs that were pre-labeled with near-infrared (IR) probe IR-780. The distribution was sequentially monitored with an *in vivo* imaging system (Xenogen, MA, USA). Here, both asthmatic and healthy mice were used for comparison. Interestingly, after given the same dose of NPs, the asthmatic mice showed a significant increase in fluorescence intensity in the lungs compared with the healthy mice (Fig. 4). Further, in the asthmatic mice, the fluorescence intensity in the lung tissues was much higher than that in the liver or spleen, which indicated a very strong accumulation of the NPs in the lungs of the asthmatic mice. This situation was not found in the healthy mice. The synthesized bavachinin-loaded NPs seemed to be strongly enriched in the lungs of asthmatic mice.

In Vivo Treatment in a Murine Asthma Model via Oral Administration of Bavachinin-Loaded NPs

We studied the therapeutic potential of the oral administration of bavachinin-loaded NPs in a murine asthma model sensitized with OVA. NPs were administered via oral gavage at a dose of 50 mg/kg body weight (calculated based on bavachinin) once per day for a week,

starting from the last day of the challenge (from day 21 to day 27; Scheme 1). The asthmatic mice were also administered free bavachinin (50 mg/kg) dissolved in PEG 400/ethanol/water (4/1/5) or PBS only through oral gavage as the controls. On day 28, the mice were sacrificed, and their lungs, spleens, and peripheral blood were collected for further evaluation.

The formulation of PEG 400/ethanol/water (4/1/5) was used to increase the solubility of bavachinin in the control. As shown in Figure 5, although bavachinin formulated in PEG400/ethanol/water (4/1/5) was completely tolerated by the healthy mice, approximately 50% of the asthmatic mice died after being fed this formulation for one week through oral gavage, and their body weight decreased dramatically. Compared with this formulation, bavachinin-loaded NPs showed excellent safety, and the asthmatic mice fed these NPs orally all grew very well and showed no obvious body weight loss during the treatment process. The formulation of PEG 400/ethanol/water (4/1/5) seems to have some extent of toxicity.

Histological Analysis

The lung sections were used for the histological analysis of the pathological tissue of the mice, and some representative images are shown in Figure 6. In the group of the asthmatic mice given PBS, the lung sections showed

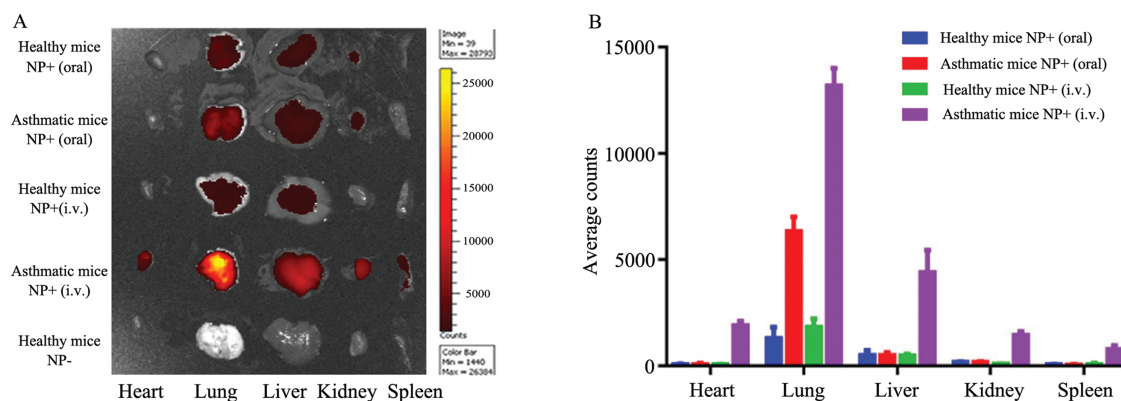
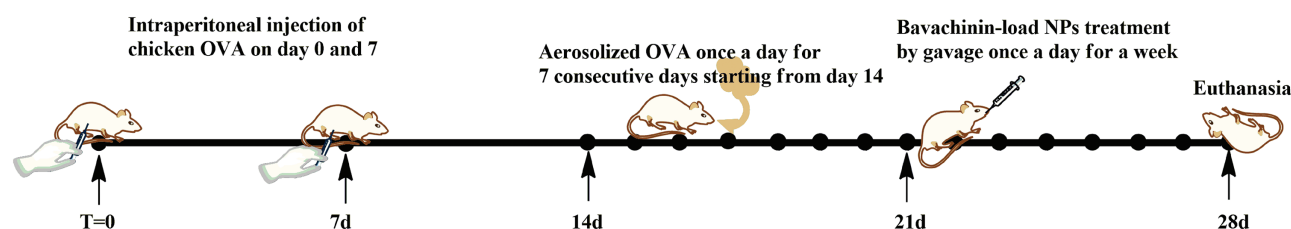


Figure 4. Distribution of bavachinin-loaded NPs in different organs. The asthmatic or healthy mice were *i.v.* or orally injected with IR-780 labeled bavachinin-loaded PEG₅₀₀₀-PLGA NPs, and the fluorescent signals in organs were visualized (A) and quantified (B) with an IVIS Lumina Imaging system by excitation at 710 nm and emission at 820 nm.



Scheme 1. Experimental schematic of asthma model construction and treatment with an oral gavage of bavachinin-loaded PEG₅₀₀₀-PLGA NPs. For the model construction, female BALB/c wild-type mice (6 to 8 weeks old) were intraperitoneally injected chicken OVA on days 0 and 7, followed by challenge with aerosolized OVA for 7 consecutive days. For treatment, bavachinin-loaded PEG₅₀₀₀-PLGA NPs (50 mg/kg body weight based on bavachinin) or free bavachinin (50 mg/kg) dissolved in PEG 400/ethanol/water (4/1/5 v/v) was given orally by gavage once a day for a week. Mice were euthanized at day 28, and their tissues and blood were harvested for further analysis.

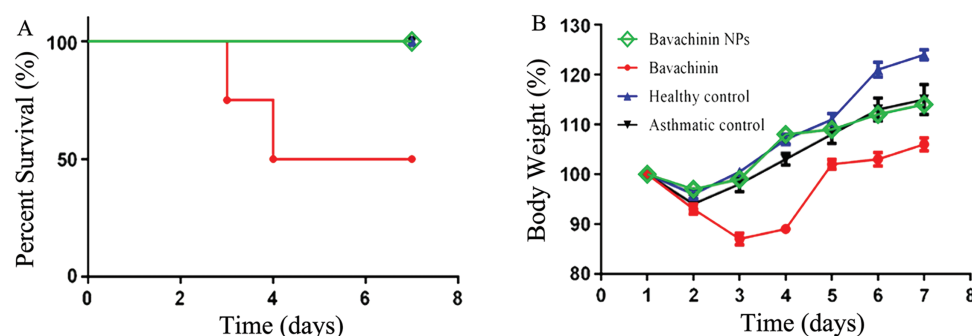


Figure 5. Survival rate (A) and body weight variation (B) of the asthmatic mice treated with oral gavage of bavachinin-loaded PEG₅₀₀₀-PLGA NPs or free bavachinin (50 mg/kg based on bavachinin) dissolved in PEG 400/ethanol/water (4/1/5) once a day for a week. Healthy mice without treatment were used as the healthy controls and asthmatic mice fed PBS were used as the asthma controls.

serious inflammatory cell infiltration in the bronchial membrane, vascular membrane, and surrounding lung tissue as well as increased bronchial wall thicknesses and airway smooth muscle areas. In the treatment groups,

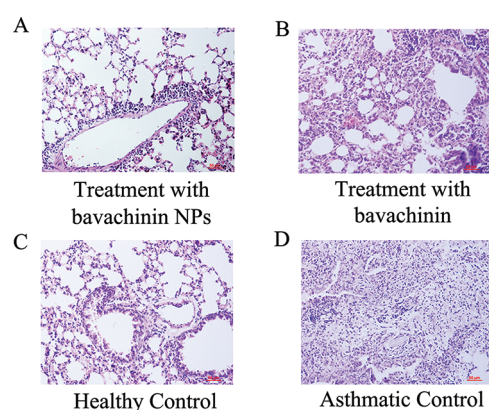


Figure 6. H&E staining of lung tissues. Histological analysis of lungs from the asthmatic mice treated with (A) bavachinin-loaded PEG₅₀₀₀-PLGA NPs (50 mg/kg based on bavachinin) and (B) bavachinin dissolved in PEG 400/ethanol/water (4/1/5) through oral gavage once a day for one week. The lung sections from healthy mice (C) and the asthmatic mice fed with PBS (D) were adopted as controls. One representative picture of H&E-stained lung sections for each group is shown, and the images are shown with 200-fold magnification.

the asthmatic mice were orally given bavachinin-loaded NPs (50 mg/kg) or bavachinin (50 mg/kg) formulated in PEG400/ethanol/water (4/1/5) and showed a significant reduction of these symptoms. The bronchial epithelium and vessel walls were almost close to the normal values found in the healthy control group. Histological analysis revealed a significant reduction of pathological tissue injury. The histological result indicated that the oral administration of bavachinin-loaded NPs exhibited good therapeutic function in the treatment of asthma, with the same efficiency as the formulation aided with PEG 400/ethanol/water (4/1/5).

IL-4 and IFN- γ Expression Levels in Peripheral Blood

The cytokine expression levels of these treated mice in the peripheral blood were analyzed with an ELISA, and the results are shown in Figure 7. Compared with the healthy control group, the asthmatic group had much higher IL-4 expression. After treatment with bavachinin-loaded NPs, IL-4 expression was significantly reduced to a normal level. In contrast, the asthmatic group had a relatively low expression level of IFN- γ compared with the healthy mice. After NP treatment, the IFN- γ level increased significantly to a normal level. These results indicated that bavachinin-loaded NPs can efficiently modulate the

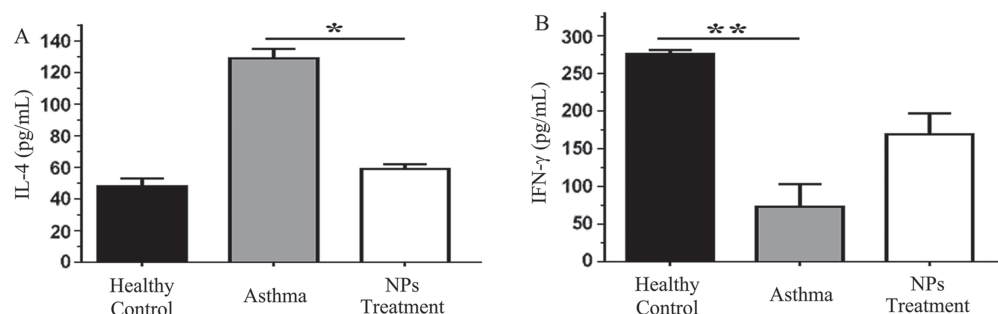


Figure 7. IL-4 and IFN- γ concentrations in the peripheral blood determined by ELISA. The asthmatic mice were treated with an oral gavage of bavachinin-loaded PEG₅₀₀₀-PLGA NPs (50 mg/kg based on bavachinin) once a day for a week, and the IL-4 and IFN- γ concentration in the peripheral blood were determined by ELISA. Asthmatic mice fed PBS and healthy mice without treatment were used as the controls. The results are presented as the mean $\pm$ SE from 5 animals. * P < 0.05 between the asthma control and NP treatment group; ** P < 0.01 between the healthy controls and the asthma controls.

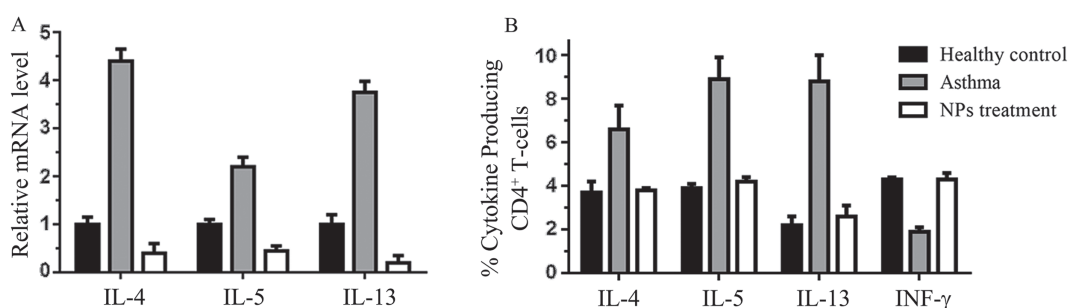


Figure 8. RT-PCR analysis of the Th1/Th2 cytokine mRNAs in the lungs and FACS analysis of the Th1/Th2 balance in the spleens. Asthmatic mice were treated with oral gavage of bavachinin-loaded PEG₅₀₀₀-PLGA NPs (50 mg/kg based on bavachinin) once a day for a week. The mRNA levels of Th2 cytokines in the lung tissues were analyzed with RT-PCR (A) and Th1/Th2 cell differentiation in the spleens was analyzed with FACS analysis (B). Asthmatic mice treated with oral gavage of PBS and healthy mice without treatment were used as the controls. The results are presented as the mean $\pm$ SE from 5 animals.

disordered expression of IL-4 and of IFN- γ in the murine asthma model.

Cytokine Expression Alteration in the Lung Tissues Checked with RT-PCR

The local Th2 cytokine level in the lung tissues of the asthmatic mice treated with bavachinin-loaded NPs was also checked by quantifying the mRNA concentrations with RT-PCR. As shown in Figure 8(A), the group treated with bavachinin-loaded NPs showed a marked down regulation of the IL-4, IL-5, and IL-13 mRNA levels in the lung tissues compared with both the healthy control group and the asthma group without treatment.

Th2/Th1 Differentiation Analysis with FACS

To test the impact of NP treatment on CD4⁺ T cell differentiation, i.e., the Th1 versus Th2 balance, splenocytes from treated mice were isolated and activated with PMA/ionomycin. Then, the cells were stained intracellularly with anti-cytokine antibodies. The percentages of IL-4-, IL-5-, IL-13-, and IFN- γ -produced CD4⁺ T cells were analyzed by flow cytometry. As shown in Figure 8(B), the percentage of IL-4-produced T cells in the asthmatic mice was markedly higher than that in the

healthy mice, but after treatment with bavachinin-loaded NPs, the percentage decreased significantly to a level that was very close to that observed in healthy mice. The percentages of IL-5- and IL-13-expressing T cells showed the same trends (Fig. 8(B)). The percentage of IFN- γ -expressing T cells in treated asthmatic mice increased to a percentage close to that of the untreated asthmatic mice.

DISCUSSION

In this work, we used the PEG₅₀₀₀-PLGA copolymer to fabricate bavachinin-loaded NPs, with the aim of finding an oral delivery system for bavachinin, a very promising therapeutic agent for asthma that is limited by its extremely poor water solubility. It has extremely low water solubility (approximately 30 ng/mL), which is even lower than that of Taxol (1.6 μ g/mL). The particles were prepared according to the emulsion-solvent evaporation technique, and their treatment potential was evaluated through *in vitro* and *in vivo* experiments.

This particulate system can well solve the solubility problem associated with bavachinin. Different from our published procedure, where a formula of PEG 400/ethanol/water (4/1/5) was used to increase the water

solubility of bavachinin, this system showed greatly improved safety. The formula of PEG 400/ethanol/water (4/1/5) seems to have potential toxicity. As shown in Figure 4, although bavachinin formulated in PEG 400/ethanol/water was completely tolerated by healthy mice after one week of administration, 50% of the asthmatic mice died after being fed this formulation for one week, and their body weight decreased dramatically. This result may indicate that bavachinin formulated in PEG 400/ethanol/water is not appropriate for the clinical use of bavachinin. Compared with this formulation, bavachinin-loaded PEG-PLGA NPs showed much higher biocompatibility, and the asthmatic mice treated with these NPs all grew very well and showed no obvious side effects.

Further, bavachinin-loaded NPs had a very good capability of targeting the inflamed lung tissues, as shown by the *in vivo* distribution of the NPs in the asthmatic mice. In the asthmatic mice, the amount of bavachinin-loaded NPs in the lung was much higher than that observed in the other organs, including liver, spleen and kidney, and the enrichment was much more obvious in the asthmatic mice than in the healthy mice. These NPs had the ability to be selectively accumulated in the inflamed lungs, which is very interesting and should be very useful for enhancing their therapeutic effects and further reducing drug toxicity. The inflamed lungs may exhibit vasculature leakage resulting from inflammation, which can lead to NP accumulation due to the enhanced permeability of the capillary endothelium effect (EPR effect), which is well known in cancer treatment with the targeted delivery of drugs to tumor sites.^{35,36} PEG₅₀₀₀ modification can significantly elongate the circulation half-life of NPs *in vivo*, which may further enhance this effect.

The prepared bavachinin-loaded NPs showed excellent therapeutic function, as demonstrated in the OVA-induced murine asthma model. After treatment with bavachinin-loaded NPs via oral administration, the asthmatic mice clearly showed a significant suppression of the pathogenesis of allergen-induced airway inflammation in the lung tissues, as assessed by the histological analysis of the lung sections. ELISA analysis also showed that the Th1/Th2 cytokines in the peripheral blood of these mice were clearly modulated, where IL-4 expression was efficiently suppressed and IFN- γ expression was moderately elevated. RT-PCR analysis also showed that the mRNA levels of the local Th1/Th2 cytokines in the lung tissues were modulated. mRNAs of IL-4, IL-5 and IL-13 were reduced to those of normal mice, whereas that of INF- γ increased. FACS analysis showed that treatment with these NPs also modulated the Th1 versus Th2 differentiation balance. The percentages of IL-4-, IL-5-, and IL-13-expressing T cells were significantly reduced (Fig. 8(B)). These results showed that an oral gavage of bavachinin-loaded NPs can efficiently suppress systemic or local Th2 cytokine expression and Th2 cell differentiation while slightly increasing Th1 cytokine expression

and Th1 cell differentiation. Th2 cytokines, including IL-4, -5, -9, -13 and -33, play critical roles in the pathogenesis of asthma. The elevated expression of these cytokines in the serum is well known to be associated with asthmatic symptoms because they drive eosinophilic inflammation and tissue damage, which lead to airway hyperresponsiveness and airway infiltration of inflammatory cells. Blocking Th2 cytokine production and modulating Th1/Th2 differentiation have proven to be an efficient therapeutic strategy for clinical asthma treatment. The efficient modulation of the Th1/Th2 balance by bavachinin-loaded NPs could explain the effectiveness of these NPs in suppressing asthma symptoms.

CONCLUSION

In this work, we fabricated bavachinin-loaded PEG₅₀₀₀-PLGA NPs, with the aim of orally delivering bavachinin to treat asthma. These NPs had very good biocompatibility and exhibited specific targeting capability to the inflamed lung tissues. Through oral administration, these NPs showed very good anti-asthma therapeutic effects in a murine allergic asthma model, as assessed by analysis of histological sections, local and systemic cytokine expression, and T cell differentiation. These results indicated that these NPs could be used as an oral delivery system for bavachinin in asthma treatment. This strategy may also have the potential to be applied for other drugs that have poor pharmacokinetic properties.

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