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RESEARCH PAPER

Aerosolized Montelukast Polymeric Particles—An Alternative to Oral Montelukast–Alleviate Symptoms of Asthma in a Rodent Model

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ABSTRACT

Purpose Cysteinyl leukotrienes (CysLTs) propagate inflammatory reactions that result from allergen exposure in asthma. Montelukast, a CysLT type-1 receptor antagonist, disrupts mediator–receptor interactions and minimizes inflammatory response. In this study, we have evaluated anti-asthmatic efficacy of inhalable montelukast-loaded large porous particulate formulations in ovalbumin-induced rat airway inflammation model that mimics asthma.

Methods The anti-inflammatory effects of a montelukast-loaded formulation were investigated in rats by measuring the total protein content, levels of injury markers and number of inflammatory cells in the bronchoalveolar lavage fluid (BALF). The histopathological studies assessed the morphological and structural changes that occur in asthmatic lungs. Animals were also challenged with methacholine to examine the airway hyper-reactivity.

Results Compared with healthy animals, asthmatic animals showed a 3.8- and 4.77-fold increase in the protein content and number of inflammatory cells in BALF, respectively. Intratracheal montelukast particles reduced the protein content by 3.3-fold and the number of inflammatory cells by 2.62-fold. Also, montelukast particles reduced the lactate dehydrogenase (LDH) and myeloperoxidase (MPO) levels by a 4.87- and 6.8-fold in BALF, respectively. Montelukast particles reduced the airway wall thickness by 2.5-fold compared with untreated asthmatic lungs. Further, particulate formulation protected the lungs against methacholine-induced bronchial provocation ($p < 0.05$).

Conclusions Respirable large porous particles containing montelukast alleviated allergen-induced inflammatory response in an animal model and prevented histological changes associated with asthma. Thus montelukast-loaded large porous polylactic acid (PLA) particles could be an aerosolized delivery approach for administration of currently available oral montelukast.

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ABBREVIATIONS

ANOVA	Analysis of variance
BALF	Bronchoalveolar lavage fluid
$CysLT_1$	Cysteinyl leukotriene type-1 receptor
EF_{50}	Tidal midexpiratory flow
HRP	Horseradish peroxidase
IT	Intratracheal
IV	Intravenous
LDH	Lactate dehydrogenase
MMAD	Mass median aerodynamic diameter
MPO	Myeloperoxidase
NAD	Nicotinamide adenine dinucleotide
OVA	Ovalbumin
PEI	Polyethylenimine
PLA	Poly (lactic acid)
PLGA	Poly (lactide-co- glycolic acid)
SD	Sprague–Dawley
TMBZ	3, 3′-5, 5′-Tetramethylbenzidine

INTRODUCTION

Bronchial inflammation, reversible airway obstruction, and airway hyper-reactivity are major pathological features of asthma in humans. In asthmatic patients, inflammatory cells migrate from blood stream to the lungs, bronchial smooth muscles contracts, mucus secretion increases but mucus clearance decreases. This series of pathological changes reduces airway lumen diameter in chronic conditions (1,2). Current therapeutic options for the management of asthma include inhaled corticosteroids and β_2 agonists. Inhaled corticosteroids are potent anti-inflammatory agents; however, they are non-specific and thus show major side effects such as growth retardation in children, osteoporosis,

reduction in bone mass, cataract formation, adrenal suppression, and oropharyngeal diseases (3–6). On the other hand, β_2 agonists are potent bronchodilators that only provide immediate relief from the respiratory obstruction without alleviating bronchial inflammation.

A growing body of data suggests that the underlying cause for asthma is allergen induced inflammation. The inflammatory mediators, especially cysteinyl leukotrienes (CysLTs), propagate inflammatory reactions when the lung is exposed to allergens. Upon binding the G protein-coupled CysLT membrane receptors of structural and inflammatory cells, CysLTs recruit and activate inflammatory cells, enhance vascular permeability, help develop edema, and exhibit bronchial smooth muscle contraction. Currently available CysLT type-1 receptor ($CysLT_1R$) antagonists such as montelukast function by interfering CysLTs–receptor interactions and constraining CysLTs mediated inflammatory pathways in asthma. Although a highly selective and a potent antagonist, oral montelukast is a second-line therapy for the management of inhaled corticosteroids resistant asthma in chronic asthmatic patients. Recent data suggest that montelukast, when used at higher doses, produce a series of secondary anti-inflammatory effect including inhibition of 5-lipooxygenase, histone acetyl transferase, and eosinophil adhesion. These pathways are distinct from those usually observed upon blockage of $CysLT_1R$ (7).

Although montelukast has been used as an adjunctive therapy for asthma, studies concerning the safety and efficacy of inhalable montelukast are very limited. In an experimental asthma model, inhaled montelukast has been reported to inhibit CysLT induced bronchoconstriction (8). A double-blind placebo-controlled clinical trial suggest that inhaled montelukast, when administered with inhaled mometasone, significantly improved the lung functions and exhibited better control over the disease compared with mometasone alone (9). Thus far, there is no report concerning the development of controlled released formulation of montelukast that can be administered directly to the lung and that can reduce systemic exposure of the drug. We hypothesize that the montelukast encapsulated in respirable large porous polymeric particles produce sustained drug release and elicit potent anti-inflammatory effects in asthmatic rats. To test this hypothesis, we prepared PLA and PLGA based formulations of montelukast and incorporated polyethylenimine (PEI) to impart large porous characteristics to particles as reported previously (10). An ovalbumin-sensitized airway inflammation model was developed to evaluate the pharmacological efficacy of the montelukast-loaded large porous particles in rats. We have evaluated the allergen-induced inflammation, airway hyper-reactivity, and remodeling of asthmatic lungs upon treatment with plain montelukast, drug loaded particles, and sham particles without the drug.

MATERIALS AND METHOD

Materials

Poly (lactic acid) (PLA) was purchased from Lactel Absorbable Polymers (Pelham, AL). Montelukast sodium was a gift from Gyma Laboratories of America, Inc. (Westbury, NY). Male Sprague–Dawley® rats (250–350 g) were purchased from Charles River Laboratories (Wilmington, MA). Lactate dehydrogenase (LDH) and protein assay kits were obtained from Pointe Scientific, Inc. (Canton, MI) and Bio-Rad (Hercules, CA), respectively. Hema-3 manual staining kit was from Fisher Scientific (Pittsburgh, PA). Methacholine was purchased from Spectrum Chemicals (New Brunswick, NJ). Haematoxylin and eosin staining kit, periodic acid-Schiff staining kit and other analytical grade chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Methods

Preparation and Optimization of Montelukast-Loaded Particles

Montelukast loaded large porous particles were prepared using $W_1/O/W_2$ double-emulsion–solvent evaporation method as described earlier with few modifications (11). Briefly, an aliquot of the internal aqueous phase (0.5 ml, IAP, W_1) containing 3% PEI (porosigen) was first emulsified with 5 mL dichloromethane (DCM, organic phase, OP) containing 250 mg polymer, and 15 mg montelukast sodium dissolved in 0.5 mL methanol by a Branson Sonifier 450 (Branson Ultrasonics Corporation, Danbury, CT). The resulting primary emulsion (W_1/O) was homogenized with 1%*w/v* polyvinyl alcohol (PVA) solution (external aqueous phase, W_2) using an Ultra-Turrex T-25 basic homogenizer (IKEA, Wilmington, DE). The double emulsion ($W_1/O/W_2$) was then stirred overnight at room temperature for removal of DCM and hardening of the particles. Final formulations were washed thrice with water by centrifugation at 20,000 rpm for 15 min each at 4°C followed by lyophilization for 72 h to obtain free-flowing powdered formulations. Formulations were prepared in triplicates and stored at 4°C until further analyses.

Physical Characterization

The surface morphology of montelukast loaded particulate formulations was examined using a Hitachi S-3400N (Freehold, NJ) scanning electron microscope (SEM). *The volume-based mean diameters* were determined using a Malvern® Mastersizer 2000 (Malvern® Instruments Ltd., Worcestershire, UK) particle size analyzer as described earlier (11). Briefly, particles were suspended in water using a Hydro 2000MU sample dispersion unit and pumped into the particle size analyzer to measure volume-based mean diameter. *The zeta potential* of the

particles was measured in a Nano ZS90 Zetasizer (Malvern® Instruments Ltd., Worcestershire, UK) after dispersing the particles in 1× PBS buffer (11,12). *The tapped density* was determined according to the procedure described previously (12–14). An aliquot of particles was transferred to a 10 (±0.05) mL graduated cylinder and the initial volume was recorded and the density was calculated as the ratio of sample weight (g) and the volume (mL) occupied after tapping until no further change in the volume was observed. An eight-stage Marc-II Andersen Cascade Impactor (Westech Instruments Inc., Marietta, Georgia) was used to determine *the mass median aerodynamic diameter* (*MMAD*). The dry powder formulations were fired at 28.3 L/min into the impactor using a HandiHaler® (Boehringer Ingelheim, Ridgefield, CT) (11).

Encapsulation Efficiency

Montelukast was extracted in methanol from the particles. Briefly, ~10 mg particles was dispersed in 20 mL methanol and stirred overnight. The extracted drug was quantified using a fluorescence spectrophotometer at 350 nm excitation and 392 nm emission in a 96-well microtiter plate (Synergy MX Microplate Reader, Biotech, Winnoski, VT).

In Vitro Release Studies

Drug release profiles were evaluated in a simulated lung fluid (SLF) prepared according to Moss formula (15) plus 0.1% Tween 80 at pH 7.4 as reported earlier (6). Briefly, ~10 mg of lyophilized particles were suspended in 1 mL SLF and stirred at 300 rpm at 37±1°C. An aliquot of the samples was collected after centrifugation over a period of 4 days at various time intervals. The amount of drug liberated from the particles was measured in a fluorescence spectrophotometer.

Development of Ovalbumin Induced Airway Inflammation Model and Treatment with Various Formulations

We developed an ovalbumin sensitized/challenged model of airway inflammation to evaluate the anti-asthmatic effect of the formulations. Briefly, ~300 g male Sprague–Dawley® (SD) rats (Charles River Laboratories, Charlotte, NC) were divided into five groups, four rats in each. The animals were sensitized with intra-peritoneal injections of 100 μg ovalbumin, dispersed in 5 mg aluminum hydroxide, on three days – 0, 7 and 14th day (16,17). Twenty four hours after last ovalbumin-alum injection, animals were anesthetized with isoflurane to receive (i) saline, (ii) plain montelukast oral, (iii) plain montelukast intratracheal (IT), (iv) blank particles without montelukast IT, and (v) montelukast loaded large porous particles IT. The animals were treated once a day for 3 days (on day 15, 16 and 17) at a dose of 0.5 mg/kg montelukast or particles containing equivalent amount of the drug. Each day, 1 h after dosing with plain montelukast or particles, animals were challenged with 100 μL (1 mg/mL) intratracheal ovalbumin solution. The formulations and ovalbumin were administered using a microsprayer for rats (PennCentury®, Philadelphia) as reported previously (11). A sixth group of animals was used as negative control that received an intraperitoneal injection of PBS but received no treatment. On day 18, animals were anesthetized and sacrificed for collection of bronchoalveolar lavage (BAL) fluid and lung tissue as described below. The study design for development of ovalbumin–induced rat airway inflammation model, treatments, and sample collection schedules are summarized in Fig. 1. All animal studies were performed in compliance with the NIH Guideline for the Care and Use of Laboratory Animals under an approved protocol (AM-02004).

Collection of BAL Fluid and Lung Tissue from Animals

On Day 18, 24 h after the last ovalbumin challenge, animals were anesthetized with a cocktail of ketamine and xylazine, and sacrificed for collection of lungs and BAL fluid according to our previously reported procedure (11,12). Briefly, a tracheotomy was performed and a small syringe containing 5 mL cold phosphate buffered saline (PBS) was instilled into the lungs. The fluid was withdrawn after 30 s of PBS instillation, and the procedure was repeated twice. Collected BAL fluid was placed on the ice and centrifuged at 500× g for 10 min to separate suspended cells and supernatant. The supernatant was stored at −20°C for subsequent analysis.

For histological evaluations, trachea and lungs were exposed by tracheotomy and thoracotomy, respectively. A thin plastic tube, connected to a reservoir containing 10% formalin in PBS placed at 20 cm height, was positioned into the trachea and the lungs were inflated by perfusing with the liquid for 20 min. Lungs were then excised from the chest cavity and placed in 10% formalin for a day and then in 30% sucrose solution for 2 days. Left lungs were embedded in TissueTek® (Sakura Finetek, Torrance, CA) and stored overnight at −80°C. Later, 25 μm lung sections were prepared by slicing lung blocks with a Cryostat (Leica Microsystems Inc., Buffalo Grove, IL). The staining of slides is discussed below.

BAL Fluid Analysis

Total Protein Content. The protein content in the BAL fluid was determined using a Biorad® DC protein assay kit (Hercules, CA). An aliquot of BAL fluid (5 μL) was added into a 96-well plate containing the assay mixture (25 μL alkaline copper tartrate solution +200 μL dilute Folin reagent). Reaction mixture was stirred, incubated in the dark for 15 min, and the absorbance was measured at 570 nm using Synergy MX

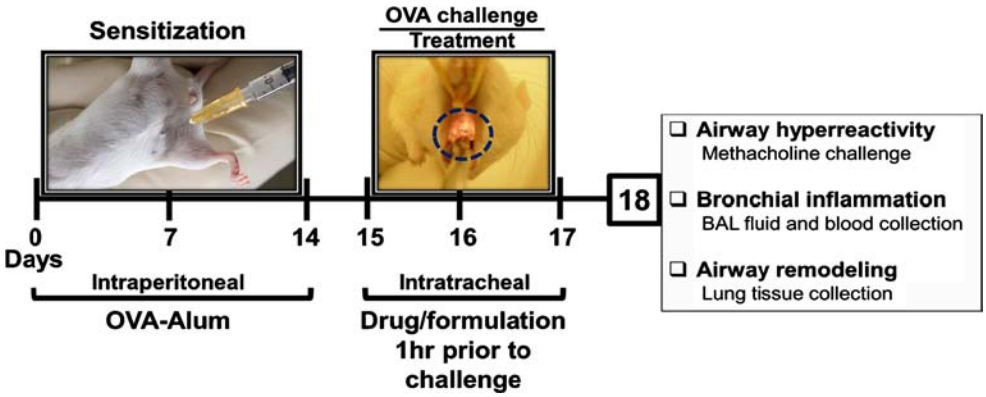

Fig. 1 Scheme for development of ovalbumin (OVA)–induced airway inflammation model using Sprague–Dawley® rats. Animals were sensitized with OVA-alum injections on day 0, 7 and 14th followed by allergen challenge on day 15, 16 and 17th. Plain drug or formulations were administered once-a-day 1 h before the OVA challenge, and bronchoalveolar lavage (BAL) fluid and lung tissue samples were collected for further studies.

Microplate Reader (Biotech, Winnoski, VT). Bovine serum albumin was used as a standard for calibration.

Lactate Dehydrogenase (LDH) and Myeloperoxidase (MPO) Activity. LDH levels in BALF, obtained on day 18, were quantified using an LDH kit from Pointe Scientific, Inc. (Canton, MI). Briefly, 50 µL plasma or 100 µL BAL fluid was added into 1 mL reaction mixture containing lactate and NAD (Nicotinamide adenine dinucleotide). LDH activity was measured as the change in absorbance per minute at 340 nm. To measure myeloperoxidase (MPO) activity (16), 50 µL BAL samples was added to 40 µl of 0.3 mM hydrogen peroxide solution. TMBZ (3,3′-,5,5′-Tetramethylbenzidine, 10 µL, 1.6 mM) solution, prepared in dimethyl sulfoxide, was then added to the above assay mixture to initiate the reaction, which was stopped after 2 min by adding 10 µL of 0.18 M sulfuric acid. Solutions prepared by serial dilution of horseradish peroxidase were used to construct the standard curve. The absorbance was measured in a microtiter plate reader at 450 nm (Synergy MX Microplate Reader, Biotech, Winnoski, VT).

Inflammatory Cells. To count inflammatory cells, BALF was centrifuged to obtain the cell pellet, which was suspended in 5 mL PBS. An aliquot of cell suspension (100 µl) was mixed with an equal volume of 0.4% Trypan blue solution. The number of cells was counted using a hemocytometer. Differential leukocyte counting was performed by staining slides containing smeared cells with a Hema-3 manual staining kit (Fisher Scientific, Pittsburgh, PA). Briefly, dried slides were dipped into three different solutions sequentially each six times (1 s each) starting with a fixative solution, followed by xanthene solution, and finally thiazine solution. Slides were washed with distilled water and air-dried for viewing under a light microscope.

Histological Examination of Lungs

The lung histology and the airway wall thickness were assessed after staining lung sections with haematoxylin and eosin (H&E) reagents. The wall thickness was measured using ImageJ software (National Institutes of Health, Bethesda, MD). For each section, the thickness of 8–10 airways was measured and reported as mean ± SD.

The extent of goblet cell hyperplasia in rat lungs was evaluated by staining lung sections with periodic acid-Schiff (PAS) reagent as recommended by the vendor (Sigma-Aldrich, St. Louis, MO). Briefly, the lung sections were first rehydrated followed by incubation with periodic acid solution for 5 min. After washing with DI water, sections were incubated for 15 min with Schiff's reagent. Followed by another round of thorough washing, lung sections were covered with glass coverslips using the mounting medium (Calbiochem FluorSave, EMD Millipore, Billerica, MA). Sections were viewed and images were generated using a bright-field microscope (1X-81, Olympus).

Methacholine Provocation Test—Measurement of Airway Hyperreactivity

The airway hyperreactivity in rats was measured non-invasively using a custom-made head-out body plethysmography chamber after methacholine challenge. The chamber was equipped with a pneumotachograph and a differential low pressure transducer (DLP) 2.5 differential pressure transducer type 381 (Hugo Sachs Electronik, Harvard Apparatus, Holliston, MA) connected to a bridge amplifier (ADInstruments Inc., Colorado Springs, CO). Data was recorded using the Powerlab® 16/30 system with LabChart Pro 7.1 software (ADInstruments Inc., Colorado Springs, CO). Tidal midexpiratory flow (EF_{50}), a parameter representing flow at midexpiratory tidal volume, was measured after methacholine challenge as reported earlier (18). Briefly, a spontaneously breathing anesthetized rat was placed in the head-out body plethysmography chamber and the respiratory airflow was monitored for 20 min to acclimatize the animal and obtain a baseline. Rats were exposed to different concentrations of methacholine solution (0 to 40 mg/ml) for 1 min *via* inhalation using a PulmoNeb® nebulizer (Devilbiss Healthcare, Somerset, PA). The respiratory airflow and tidal

volume were measured for 5 min after the challenge. Assuming 100% as the baseline, minimum EF_{50} were recorded and expressed as the percentage of baseline. The extent of airway hypersensitivity was assessed by comparing methacholine dose–response curves for treated and untreated asthmatic animals.

Statistical Analysis

The data have been presented as mean ± standard deviation. Means were compared using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test using GraphPad Prism 5 (version 5.01, GraphPad Software, Inc.). A p-value ≤ 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Physicochemical Properties and Drug Release Profile of the Particles

Physical properties of inhaled particles dictate the aerodynamic behavior, particle deposition and retention in the respiratory tract. Thus, we have evaluated the size, surface morphology, tapped density, aerodynamic diameter, drug encapsulation efficiency, zeta potential, and *in vitro* drug release profiles of drug loaded large porous particles (Fig. 1 and Table I). The particles were near-perfect spheres with porous surface. Similar to our earlier studies, PEI produced particles with numerous pores on the surface (10,19). PEI increases the osmotic pressure of the IAP which pulls water toward the particle core by creating aqueous channels. When process of lyophilization removes water from the channels, particles become porous (Fig. 2a). The volume-based mean diameter—the diameter of a sphere that has the same volume as the test particle – of the particles was 7.72±0.67 μm, which was little above the respirable size. But the aerodynamic diameter – calculated as mass median aerodynamic diameter (MMAD) – of the particles went down to 2.51±0.12 μm, a ~2-fold reduction compared with the volume diameter. This observation is consistent with the established fact that the density of porous particles is smaller than nonporous particles and aerodynamic diameter is proportional to particle density (20). Indeed, the tapped density of the particles was 0.10 g/mL, much smaller than unit density (Table I) and thus MMAD was well within the size range for respirable particles. The zeta potential for the montelukast-loaded particles was positive because of PEI, a

Table I Physicochemical Properties of Montelukast-Loaded Large Porous Particles

Volume-based mean diameter	7.7 2±0.67 μm
Tapped density	0.1025±0.002 g/ml
MMAD	2.51±0.12 μm
Zeta potential	7.61±1.97 mV
Drug entrapment efficiency	82.52±0.41%
Cumulative drug release	31.10±1.27%

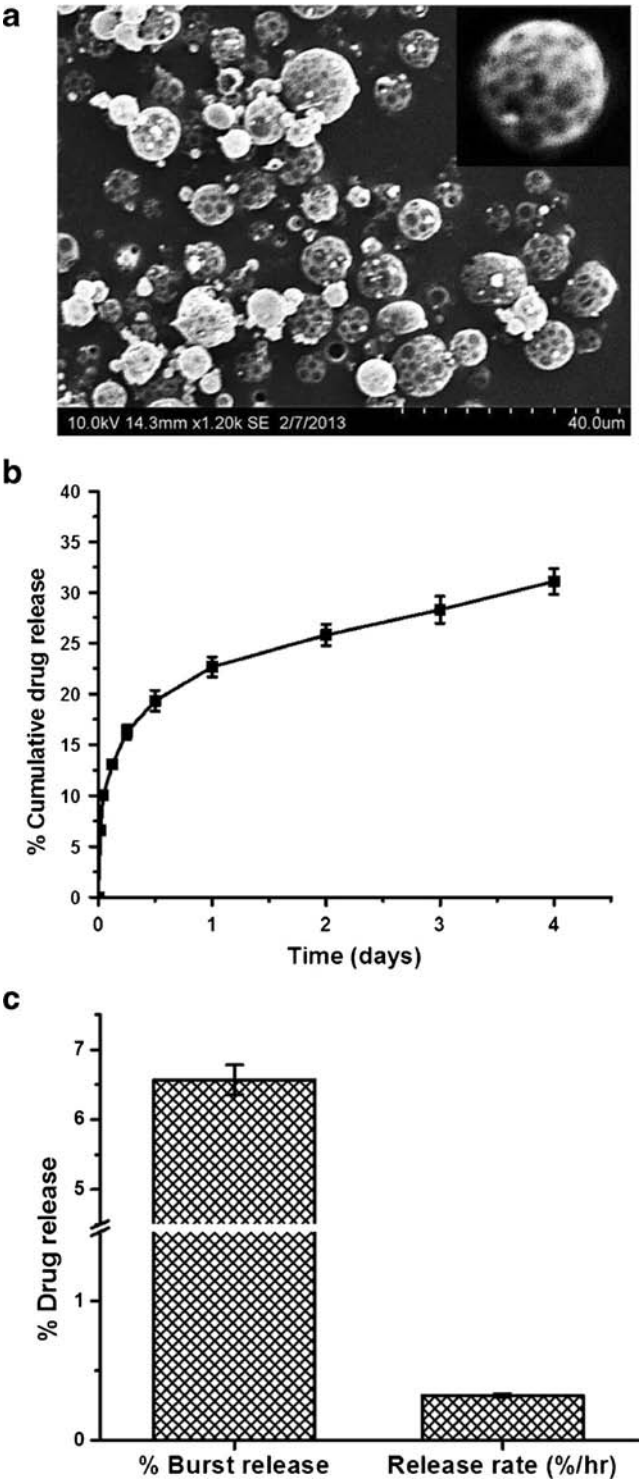

Fig. 2 **(a)** Surface characteristics, **(b)** *In vitro* release profiles, and **(c)** % burst release and release rate of drug from montelukast-loaded large porous particles.

positively charged compound used as a porosigen. Although PLA produces negatively charged particles, PEI (that has relocated from particle core to the surface) may have neutralized the negative functional groups of lactic acid and thus turned them to positively charged particles.

The drug payload for the particles was of 82.52±0.41%, which was remarkably high (Table I). The increased entrapment stems from the hydrophobicity of the drug. The drug release from the particles showed a biphasic pattern, first a small initial burst release phase followed by a slower sustained release phase. While the burst release occurred during the first 30 min, the drug continued to be released for 4 days with a cumulative release of 31.10±1.27% (Figs. 2b and c). The surface pores facilitated the burst release but the drug within the particle core came out to the media at a continuous fashion. Overall, the particles that we have used in biological studies, described below, had favorable physical properties and release profiles for inhalational delivery.

Assessment of Injury Markers in BALF

The lung Inflammation, a complex process, involves various endogenous mediators and inflammatory cells. Although inflammation, a protective mechanism, removes invading microorganisms, engulfs foreign particles, destroys cells, and cures wounds, an unwanted inflammatory response can invigorate release of pro-inflammatory chemicals and cause cell injury and death. To quantify the injury markers produced in response to ovalbumin exposure, we first assayed the total protein content in the BAL fluid. The total protein content was increased by ~3.5 fold in the lungs of asthmatic animals compared with sham animals (Fig. 3a), indicating a strong inflammatory response to allergen exposure. Oral montelukast failed to reduce the levels of inflammatory markers. But plain montelukast IT and montelukast-loaded particles IT reduced the total protein content in BALF by several fold. In both cases, the reduction in protein content was statistically significant compared with ovalbumin sensitized untreated rats ($p<0.01$). This observation is consistent with the hypothesis that inhaled montelukast particles elicit elevated anti-inflammatory effect in asthmatic rats.

As a continuation, we measured two specific pro-inflammatory enzyme levels, MPO and LDH, in the BAL fluid (Fig. 3b and c). MPO, an indicator of neutrophil infiltration, levels in BAL fluid provide information regarding the severity of lung inflammation caused by ovalbumin challenge (21,22). The MPO levels of untreated asthmatic animals were elevated by 6.3-fold ($p<0.05$). Plain montelukast administered *via* the oral and intratracheal routes reduced the MPO levels to the same extent ($p>0.05$). The enzyme levels in rats treated with montelukast loaded large porous particles were similar to those observed in sham animals but were remarkably lower than those observed in other three treatment groups ($p<0.05$). LDH, a cytoplasmic enzyme, releases in the BALF when cell damage occurs (23,24). The profiles of LDH levels in four treatment groups closely resemble the MPO profiles: a major elevation in untreated rats but reduction in montelukast treated rats (Fig. 3c). These data hold true to the previous published reports that montelukast reduces injury markers that are indicator for cell damage (25,26). Overall, inhaled large porous particles containing montelukast provide protection against cell damage associated with allergen-induced inflammation.

Evaluation of Infiltrated Inflammatory Cells

When the lung is exposed to inhaled allergen, systemic leukocytes deploy themselves onto the alveolar region and intensify the inflammatory response. The infiltrated leukocytes spit out inflammatory mediators and propagate the inflammation. Thus we counted three infiltrated cells—macrophages, eosinophils, and neutrophils—in the BALF (Fig. 3d). The total number of infiltrated cells in untreated asthmatic animals and blank particle-treated animals were several-fold greater than those observed with normal animals. In fact, ovalbumin challenge increased the total numbers of macrophages, eosinophils and neutrophils by 4.3-, 11-, and 2.2-fold ($p<0.01$). Compared with untreated asthmatic group, plain montelukast (oral and intratracheal) reduced the number of cells by ~30% ($p<0.01$). The total number of cells went down even further when rats were treated with montelukast particles, a >61% reduction compared with untreated rats. The differential cell count data show that oral montelukast reduced the eosinophil count by >48% without influencing much other cell counts. Compared with untreated rats, a 2-, 5.7-, and 1.54-fold reduction in the number of macrophages, eosinophils, and neutrophils were observed ($p<0.05$) with montelukast particles. The cell counts were further confirmed by photographing the Hema-3-stained lung sections (Fig. 4). Images of the lung section back up the cell count data presented in Fig. 3d—a higher number of infiltrated cells in untreated airways but little or no infiltration in airways of rats treated with montelukast particles. True to these observations, published report showed that montelukast provides protection against infiltration of inflammatory cells, especially eosinophils, in both rat and human airways (27,28). Further, sustained drug concentrations in the lungs constrained the cell extravasation and inflammatory triggers.

Histological Examination of the Lungs

In asthma, allergen-induced airway inflammation thickens the airway walls, increases goblet cell proliferation, and thus obstructs the airways. The disease worsens with the increase in structural changes in the airways (29). Also, chronic inflammation remodels the airways by increasing the deposition of extracellular matrix proteins—collagen, laminin, and fibronectin—under the airway basement membrane (30). To

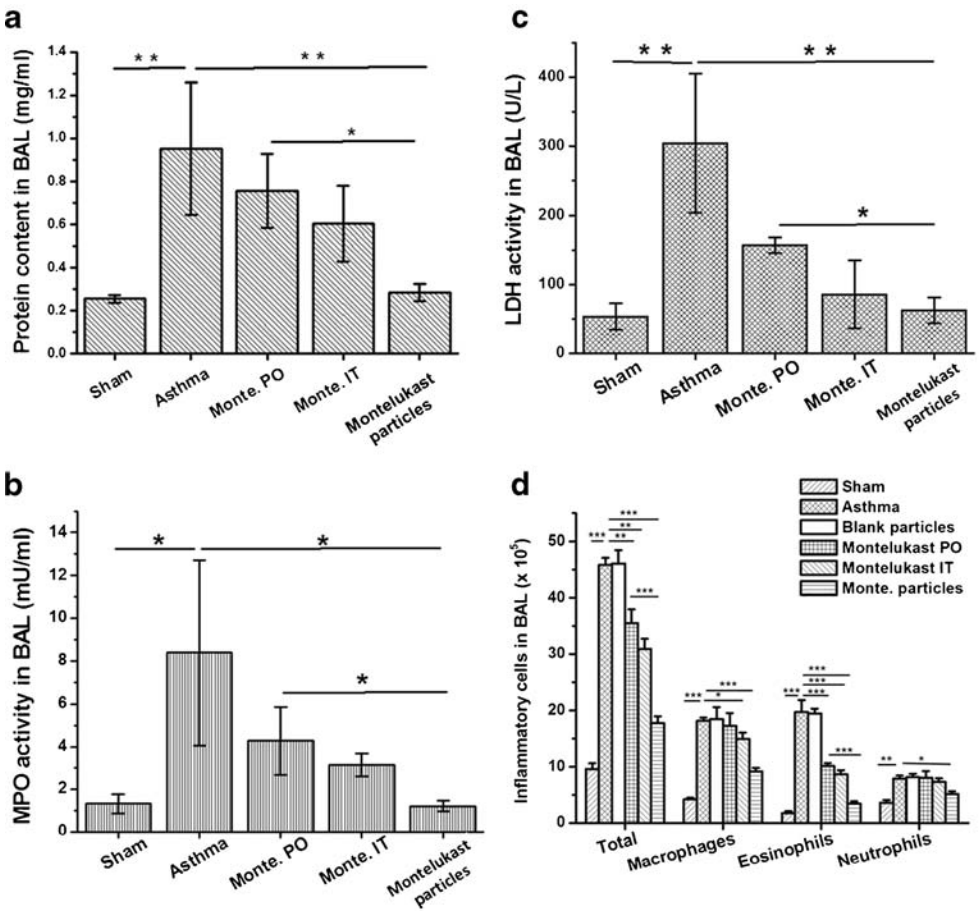

Fig. 3 **(a)** Total protein content, **(b)** myeloperoxidase, **(c)** lactate dehydrogenase activity levels, and **(d)** total and differential leukocyte cell counts in the bronchoalveolar lavage (BAL) fluid collected 24 h after last ovalbumin challenge. Data represent mean ± SD ($n = 3$).

evaluate these pathological features, we have measured the airway wall thickness and goblet cell hyperplasia and assessed whether inhaled montelukast particles prevent airway remodeling. Similar to inflammatory marker data, the airways of sham lungs showed little or no increase in wall thickness although the airways of asthmatic lungs underwent major remodeling and lumen narrowing (Fig. 5a). Plain montelukast administered either *via* the oral or pulmonary route reduced

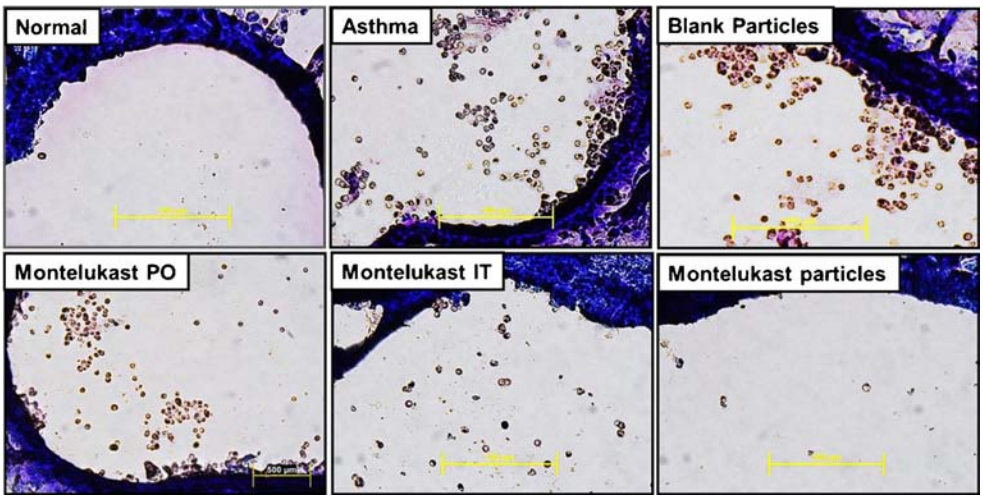

Fig. 4 Histological evaluation of lung sections for infiltrated cells after Hema-3 staining. For this experiment, rat lungs from each treatment group were fixed without collecting BAL fluid.

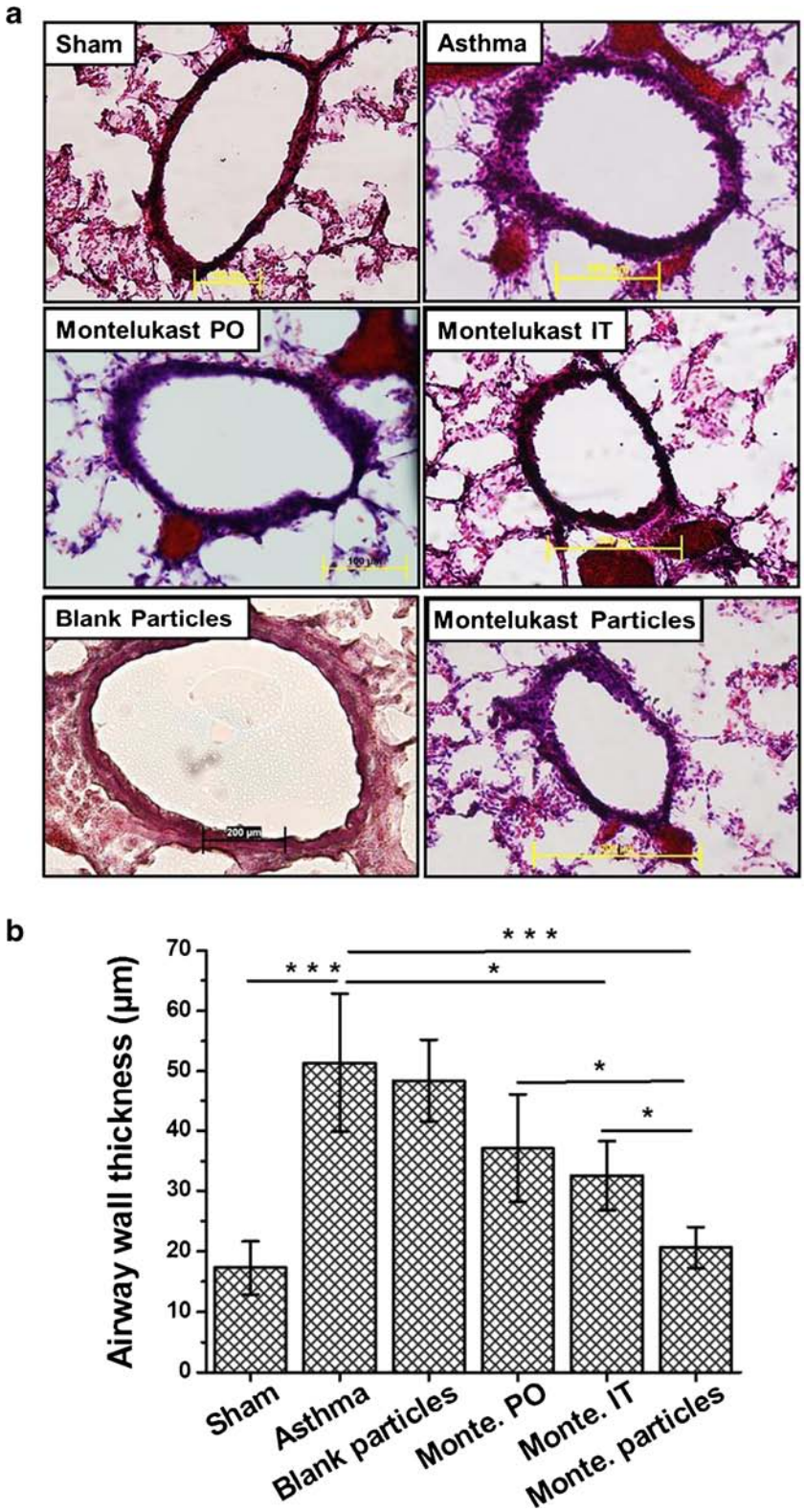

Fig. 5 **(a)** Photomicrographs of rat lung sections following haematoxylin and eosin staining for airway wall thickening. **(b)** Airway wall thickness measured from the haematoxylin and eosin stained rat lung sections by using ImageJ software. Data represent mean ± SD ($n = 8–10$ airway measurements).

the extent of remodeling to some degree. But montelukast-loaded particles substantially improved the structural alterations of airways—in this case, the extent of alterations were similar to those of sham animals. In agreement with the

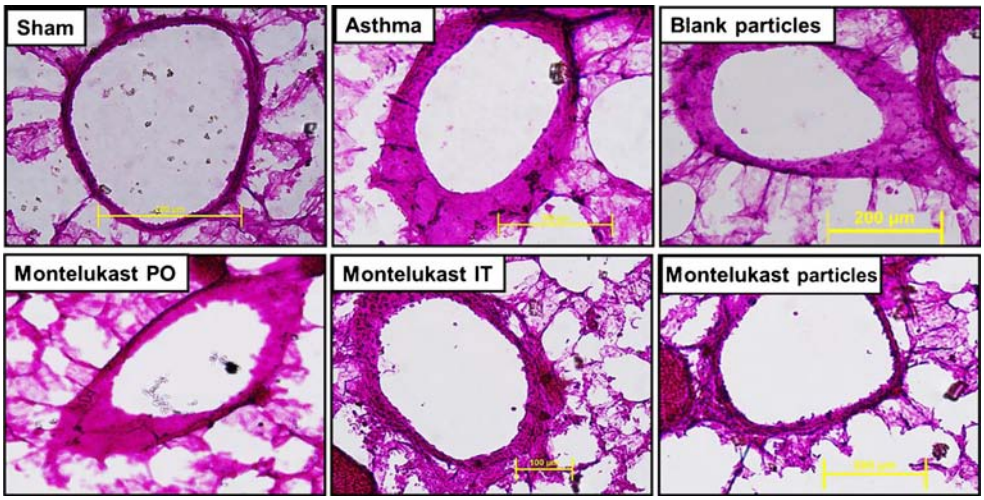

Fig. 6 Photomicrographs of rat lung sections following periodic acid-Schiff staining of goblet cells and mucus.

qualitative data in Fig. 5b, airway wall thickness increased in asthmatic animals and blank particle treated animals ($p<0.001$). Plain oral montelukast did not produce statistically significant reduction in wall thickness ($p>0.05$); but inhaled plain montelukast appreciably reduced the airway wall thickness ($p<0.05$). Importantly, montelukast particles reduced the wall thickness by 50% ($p<0.001$) when compared with untreated asthmatic rats. Thus inhalable particles of CysLTs inhibitor can attenuate airway remodeling by reducing collagen and extracellular matrix deposition. Further, montelukast, a $CysLT_1$ antagonist, reverses airway remodeling more efficiently when administered as inhaled polymeric particles (31,32).

Increased goblet cell proliferation in asthma is associated with abnormal mucin gene expression (33) that exacerbate mucus secretion. We have qualitatively evaluated the influence of the formulation on goblet cell hyperplasia (Fig. 6). The lung sections of untreated asthmatic and blank particles-treated animals showed intense pink stain, suggesting goblet cell proliferation and mucus buildup. Plain montelukast, oral and intratracheal, reduced the stain intensity to some extent. Maximal reduction in stain intensity was observed in the lung sections of rats that received large porous particles of montelukast. The stain intensity of these lung sections was very similar to that of sham animals. This data echoes the published report that montelukast reduces goblet cell hyperplasia by blocking $CysLT_1$ receptors (31).

Methacholine Provocation Test—Measurement of Airway Hyperreactivity

Airway hyper-responsiveness, a transient and inducible phenomenon, occurs upon exposure to allergen and is considered as one of the key pathological features of asthma (34,35). We have measured the midexpiratory flow (EF_{50}), the respiratory flow rate at 50% of tidal volume during expiration, to evaluate the airway hyper-reactivity (Fig. 7). A concentration-dependent reduction in initial EF_{50} was observed in all treatment groups. Compared to sham animals, untreated and blank particles treated animals showed significantly reduced EF_{50} when rats were exposed to 40 mg/mL methacholine. EF_{50} for plain montelukast (oral or intratracheal) treated rats underwent little or no changes compared with untreated rats. But montelukast-loaded large porous particles treated animals exhibited maximum resistance to methacholine provocation with a highest EF_{50} of $68.14 \pm 2.38\%$. Elevated EF_{50} suggest that particulate formulation inhibits allergen induced inflammation and reduce hyperreactivity. Similar to the data presented in Fig. 7, subcutaneous and inhaled montelukast was reported to reduce airway hyperreactivity and inhibit bronchoconstriction in ovalbumin sensitized asthma model (8,31). Unlike plain

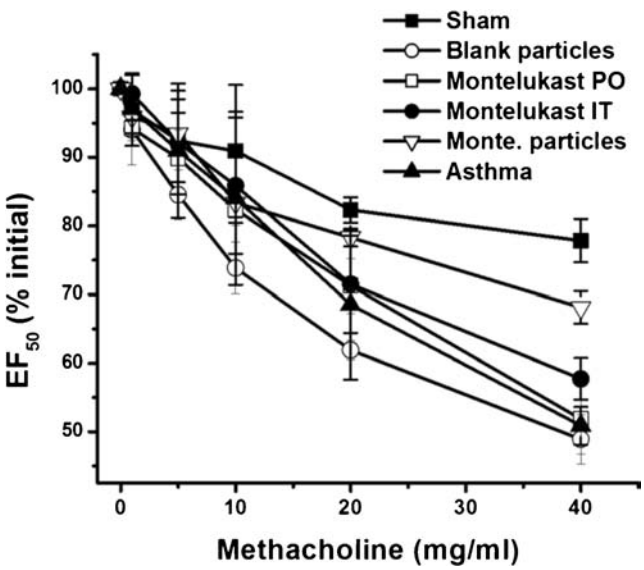

Fig. 7 Tidal midexpiratory flow (EF_{50}) was measured in response to increasing concentrations of inhaled methacholine exposure to rats to assess airway hyperreactivity. Data represent mean ± SD ($n=3$).

drug, montelukast loaded particles released the drug in a controlled manner for prolonged period and produced sustained drug concentrations at the site of disease.

The use of montelukast in asthma therapy has been limited to the patients unresponsive to inhaled corticosteroids therapy and who has mild to moderate asthma. Montelukast is a very hydrophobic drug. A fraction of the drug reaches the lungs after oral administration because of its high hepatic clearance and extensive distribution to adipose tissue. But the anti-inflammatory effect of the drug can be amplified by formulating it in PLGA based polymeric carrier systems (36,37). The heightened anti-inflammatory activity of montelukast-loaded particles observed in this study stemmed from the prolonged release of the drug and localized administration of the formulations into the lung. Although the data concerning the enhanced anti-inflammatory and protective effects of montelukast-loaded large porous particles are compelling, future studies will determine the clinical feasibility of this approach.

CONCLUSIONS

Once-a-day pulmonary administration of montelukast-loaded large porous particulate formulation improved the therapeutic efficacy by elevating and sustaining the anti-inflammatory effects compared with the plain drug given orally. The histological and *in vivo* studies revealed the power of particulate formulations in lessening allergen induced inflammation, morphological changes, and airway hyperreactivity in the lungs. All in all, this investigation establishes that montelukast-loaded large porous PLA particles as an efficacious treatment option for asthma.

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