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Cationic CaMKII Inhibiting Nanoparticles Prevent Allergic Asthma

Angie S Morris[‡], Sara C Sebag[‡], John D Paschke[‡], Amaraporn Wongrakpanich^α, Kareem Ebeid[‡], Mark E Anderson^β, Isabella M Grumbach^{‡,*,*}, and Aliasger K Salem^{‡,*}

[‡]Department of Pharmaceutical Sciences and Experimental Therapeutics, College of Pharmacy, University of Iowa, 115 S. Grand Avenue, S228 PHAR, Iowa City, IA 52242, USA [♦]Department of Internal Medicine, Carver College of Medicine, University of Iowa, 200 Hawkins Drive, Iowa City, IA 52242, USA ^αDepartment of Pharmacy, Faculty of Pharmacy, Mahidol University, Bangkok, 10400, Thailand ^βDepartment of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD ^{*}Iowa City Veterans Affairs Healthcare System, 601 US-6, Iowa City, IA 52246, USA

Abstract

Asthma is a common lung disease affecting over 300 million people worldwide and is associated with increased reactive oxygen species (ROS), eosinophilic airway inflammation, bronchoconstriction and mucus production. Targeting of novel therapeutic agents to the lungs of patients with asthma may improve efficacy of treatments and minimize side effects. We previously demonstrated that Ca²⁺/calmodulin-dependent protein kinase (CaMKII) is expressed and activated in the bronchial epithelium of asthmatic patients. CaMKII inhibition in murine models of allergic asthma reduces key disease phenotypes, providing the rationale for targeted CaMKII inhibition as a potential therapeutic approach for asthma. Herein we developed a novel cationic nanoparticle (NP)-based system for delivery of the potent and specific CaMKII inhibitor peptide, CaMKIIN, to airways. CaMKIIN-loaded NPs abrogated the severity of allergic asthma in a murine model. These findings provide the basis for development of innovative, site-specific drug delivery therapies, particularly for treatment of pulmonary diseases such as asthma.

Introduction

Asthma is a chronic, wide-spread disease that occurs in people of all ages. Exposure of the respiratory epithelium to allergens is the initiating event in allergic asthma which is

*CORRESPONDING AUTHORS. Aliasger Salem, aliasger-salem@uiowa.edu, Phone: (319)-335-8810, Isabella Grumbach, isabella-grumbach@uiowa.edu, Phone: 319-384-4610.

[‡]These authors contributed equally.

AUTHOR CONTRIBUTIONS

ASM, SCS, JDP, IMG and AKS designed the experiments and analyzed the data. ASM, SCS, JDP, AW, KL and KAE carried out the experiments. ASM, SCS, MEA, IMG and AKS wrote the manuscript.

COMPETING FINANCIAL INTERESTS

IMG and MEA are named inventors on an issued patent claiming to treat asthma by CaMKII inhibition. AW, MEA and AKS are named inventors on an issued patent on CAMKII inhibiting nanoparticles.

characterized by excessive pulmonary inflammation, airway hyperreactivity and mucus production. New studies have shown enhanced oxidative stress in patients with asthma.^{2–3} Specifically, our group has shown that Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) plays a pivotal role in ROS generation^{4–6} and contributes to asthma phenotypes in asthmatic patients and in vivo models of allergic asthma.^{7–8} Despite contemporary stepwise treatment approaches, 5–10% of the estimated 26 million Americans with asthma do not achieve adequate symptom control⁹, in part because molecular disease mediators are not specifically targeted.^{10–11}

Delivery of therapeutic agents, specifically to the respiratory epithelium, is likely to quell core asthmatic phenotypes and has the potential to improve drug efficacy.¹² Nanoparticle delivery systems allow for local delivery of drugs while offering additional advantages such as sustained release of therapeutic molecules over a desired amount of time, ability to deliver both water soluble and lipophilic drugs, the need for fewer administered doses and decreased enzymatic degradation of drug.^{13–14} Poly(lactic-co-glycolic acid) (PLGA) is a well-established biodegradable polymer that is FDA approved for use in a wide variety of biomedical applications and can be utilized to fabricate NPs in which therapeutically active molecules are entrapped.^{12, 15}

In the case of pulmonary drug delivery, it has been shown that drug-loaded PLGA NPs offer superior therapeutic effects over delivery of soluble drug alone.¹⁶ The increase in therapeutic effects for PLGA NP systems can be attributed to sustained release of the drug over time and a longer residence time of NPs in the lungs compared to drug alone.¹⁷ Furthermore, the surface chemistry of PLGA NPs can easily be manipulated to increase the bioavailability of the system.

In this study, we tested the hypothesis that, in response to allergen challenge, CaMKII contributes to the induction of hallmark features of allergic asthma. Utilizing a novel drug delivery system, we exposed mice to a CaMKII inhibitor peptide encapsulated in PLGA NPs. These NPs were directly delivered to the lung via oropharyngeal instillation (OP).¹⁸ Furthermore, we found chitosan coating of the CaMKIIIN-loaded PLGA-NPs increased uptake in lung cells compared to uncoated NPs and led to reduced core features of allergic asthma including inflammation, mucus production and airway hyperreactivity.

Methods and Materials

PLGA NP Fabrication

PLGA NPs were prepared using an established double emulsion solvent evaporation method. PLGA (50 mg, Resomer® RG503, viscosity 0.32–.044 dl/g, MW 24,000–38,000, Boehringer Ingelheim KG) and amine-end capped PLGA (50 mg, MW 10,000–20,000, PolyScitech) were dissolved in a mixture of 2.35 mL ethyl acetate (EA) and 0.250 mL of dimethyl sulfoxide (DMSO). Sixty milliliters of 2.5% (w/v) poly (vinyl alcohol) (PVA, Mowiol® 8–88, 87–89% hydrolyzed, MW 67,000, Sigma-Aldrich) in 10 mM phosphate buffered saline (PBS) was prepared and 9 mL was added to a 20 mL scintillation vial along with 1 mL EA. Fluorescently-labeled CaMKIIIN peptide (sequence: H - KRP PKL GQI GRA KRV VIE DDR K (HF488) - NH₂; HF488: HiLyte™ Fluor 488 acid) (AnaSpec Inc)

was dissolved in a solution of water containing 1% PVA (w/v) at a concentration of 5mg/mL. To prepare the nanoparticles, the organic and aqueous phases were emulsified using a probe sonicator (Fisher Scientific). First, 125 μ L of the CaMKIIN solution was sonicated into the polymer/EA/DMSO solution at 40% amplitude for 60 seconds. Next, this emulsion was sonicated into 9 mL 2.5% PVA solution containing 1 mL EA. Finally, the emulsion was poured into the remaining 51 mL 2.5% PVA solution. The particle suspension was stirred using a magnetic stir bar for 30 minutes, followed by centrifugation at $4,500 \times g$ for 5 minutes to pellet larger unwanted particles. The supernatant was removed and centrifuged at $10,000 \times g$ for 30 minutes to form a pellet of NPs. NPs were then washed by discarding the supernatant and replacing it with water, followed by centrifugation at $10,000 \times g$. The particles were washed twice to remove residual surfactant. After washing, the particles were frozen at -80°C overnight and lyophilized (Labconco).

NPs were loaded with a near infrared fluorescent dye (XenoLightTM DiR, Perkin Elmer) to evaluate biodistribution. Particles were prepared according to the method described above except only a single emulsion was used. Due to the poor aqueous solubility of the dye, it was added directly to 100 mg PLGA dissolved in 2.5 mL EA. This solution was sonicated into 9 mL 2.5% PVA solution containing 1 mL EA. Then the emulsion was poured into 51 mL 2.5% PVA and the particle suspension was stirred using a magnetic stir bar for 30 minutes. The same collection, washing, and storage procedures as described above were used.

Chitosan Purification

Chitosan (low molecular weight, deacetylation degree 96.1%, Sigma-Aldrich) was purified according to a previously established method.¹⁹ Chitosan (2 g) was dissolved in 200 mL 1% (v/v) acetic acid then filtered (Whatman 541 filter paper). The filtrate was titrated with 1 N NaOH until the pH was approximately 8.5 to precipitate the chitosan. The precipitate was removed via filtration and resuspended in 500 mL buffer (0.1 M sodium bicarbonate, pH 8.3). Next, 2.5 g sodium dodecyl sulfate (SDS) and 3.72 g ethylenediaminetetraacetic acid (EDTA) were added to the solution and stirred using a magnetic stir bar for 30 minutes. The insoluble chitosan was filtered, rinsed and dialyzed (Snakeskin) in nanopure water for 24 hours. During dialysis, the water was changed after 10 hours and every hour afterwards. The chitosan was collected from the dialysis tubing, frozen at -80°C overnight and lyophilized.

Chitosan Coating of NPs

Dry NPs were suspended in 0.5 mL of chitosan (3 mg/mL) dissolved in 1% (v/v) acetic acid. Subsequent to complete resuspension, NPs were centrifuged at $10,000 \times g$ for 20 minutes. The supernatant was removed and replaced with 1 mL water.

Physicochemical Characterization of PLGA NPs

Physical characterization of the NPs was performed using a scanning electron microscope (SEM, Hitachi S4800). Dry NPs were dispersed in water. A small drop of NP suspension was placed on a silicon wafer fixed to an aluminum stub. After all water had evaporated, the sample was sputter-coated (Emitech Sputter Coater K550, Quorum Technologies) with a mixture of gold and palladium before imaging. Hydrodynamic diameter, zeta potential and

polydispersity index (PDI) of the NPs were determined in water using dynamic light scattering (DLS, Zeta Sizer NanoZS, Malvern Instruments).

CaMKIIN Loading and Release

To assess CaMKIIN loading, PLGA NPs were dissolved in 0.3 N NaOH (1 mg/100 μ L). Once all NPs were degraded, the solution was neutralized to pH 7 using 1 N HCl. The concentration of fluorescently-labeled CaMKIIN in the sample was determined by linear regression using standard CaMKIIN solutions ranging from 0.4–50 μ g/mL (diluted in PBS). The standards and samples were analyzed simultaneously in a 96-well plate using a SpectraMax Plus 384 microplate reader (Molecular Devices) with an excitation wavelength of 500 nm and emission collected at 530 nm. The background signal was determined using PBS. The encapsulation efficiency (equation below) was calculated according to Joshi et al.

$$\text{Encapsulation Efficiency} = \frac{(\text{Total Mass of Nanoparticles} \times \text{Peptide Loading})}{\text{Initial Mass of Peptide Used}} \times 100$$

To measure CaMKIIN release, 29.4 mg of NPs were suspended in 0.5 mL PBS and agitated at 300 rpm and 37°C. At time points from 30 minutes to 48 hours, the sample was centrifuged at 15,000 $\times$ g to pellet NPs. The supernatant was collected and NPs resuspended in 0.5 mL PBS. Samples were stored at –20°C until the time of analysis. The amount of CaMKIIN released at each time point was determined using a SpectraMax Plus 384 microplate reader (Molecular Devices) with 500 nm excitation and 530 nm emission as described for analysis of CaMKIIN loading.

In Vitro Cellular Uptake

Human airway epithelial cells (HAECs) were cultured in keratinocyte serum-free medium supplemented with 1% penicillin/streptomycin (Gibco) on collagen (rat tail, type 1, Sigma-Aldrich) at 37 °C and 5% CO₂.²¹ Next, HAECs were plated in a 6-well plate at a density of 2×10^5 cells per well and incubated at 37 °C and 5% CO₂ for 24 hours. After changing the medium, chitosan-coated and uncoated CaMKIIN-loaded PLGA NPs (300 μ g) were added to the wells and incubated for 24 hours. The cells were collected from the wells by trypsinization and centrifuged at 230 $\times$ g for 5 minutes. The supernatant was removed and replaced with 0.5 mL fresh medium and cells were stored on ice until analysis. The amount of fluorescently-labeled CaMKIIN associated with the cells was assessed by flow cytometry (FACScan, Becton Dickinson Immunocytometry Systems). The excitation wavelength was 488 nm and the emission was collected at 530 nm using a 30 nm bandpass filter. The mean fluorescence intensity for 10,000 cells was determined for each sample.

Primary bronchial murine epithelial cell culture and treatment

Primary murine tracheal epithelial cells (MTBEC) were isolated from B62 mice (obtained from Charles River Laboratories) as previously described.²² For analysis of expression of Th2 cytokines, cells were plated onto collagen (BD Biosciences)-coated coverslips and maintained in MTEC Plus culture medium as described previously.²² Cells were grown until

confluent and then exposed to chitosan-coated empty (E) or CaMKIIN (CN) loaded NPs (25, 50 or 100 µg) with recombinant murine IL-13 (10 ng/ml, R&D Systems) for 14 days. RNA was isolated as stated below.

Animals

Six to ten week old C57Bl/6J female and male mice (equal proportions) were obtained from Charles Rivers Laboratories International, Inc. All animal studies complied with NIH guidelines and were approved by the University of Iowa Institutional Animal Care and Use Committee.

OVA Sensitization, Challenge and NP Delivery

Mice were sensitized by intraperitoneal injection (IP) of 10 µg OVA (Sigma) mixed with 1 mg alum (or saline alone for control) on days 0 and 7. Mice were subsequently challenged with by nebulization of OVA (1% solution in 0.9% saline, 40 minutes challenge) or saline on days 14–17. Prior to OVA challenge by inspiration of soluble OVA, on days 14 and 16, oropharyngeal (OP) delivery of chitosan-coated NPs was performed as described previously with some modifications.^{23–24} Briefly, mice were anesthetized with 2% isoflurane vapor in oxygen and then suspended by cranial incisors on a thin rubber band from a ring stand. To visualize the base of the tongue and the pharynx, the nares were pinched with curved forceps and the tongue gently extracted from the mouth using blunt forceps. NPs (25, 50 or 100 µL corresponding to 25, 50 or 100 µg NP) were placed in the posterior pharynx with a micropipettor. Respiration was monitored to ensure the suspension was fully delivered before the tongue and nares were released. Airway reactivity to methacholine was determined 24 hours after the last OVA challenge (day 18). In control experiments, 25 ng CaMKIIN peptide alone was administered. Based on a loading of 0.6 (± 0.02) µg CaMKIIN per mg of NPs, this dose corresponds to the delivery of 50 µg CaMKIIN-loaded NPs.

Biodistribution

Near infrared dye-loaded nanoparticles were administered to mice via OP delivery. Particles were chitosan coated as described above or uncoated and instilled by OP delivery. For controls, mice were instilled with PBS alone. At 1, 24 and 48 hour time points the fluorescence intensity of the organs was measured using a Xenogen In Vivo Imaging System (IVIS-200).

Assessment of Airway Hyperreactivity (AHR)

AHR in response to methacholine was measured on a flexiVent small-animal ventilator (Scireq) using a single compartment model, which determines the dynamic resistance of the respiratory system (R), as described previously.⁸

Bronchoalveolar Lavage

After the assessment of AHR, mice were euthanized, the trachea was cannulated, and two PBS washings were collected for analysis of total and differential counts in the bronchoalveolar lavage fluid (BALF). BALF cellular differential was determined on 250 µL cytopins stained with Diff-Quik (Dade Behring).

Quantification of ROS in murine bronchial epithelial cells

ROS were measured from freshly isolated MTBEC from mice exposed to OVA in the presence of blank (E) or CaMKIIN (CN) loaded, chitosan-coated NP using dihydroethidium red (5 mM, Invitrogen).²² The cellular staining was confirmed by colocalizing with CellTracker Green (50 nM, Thermo Fisher Scientific). Cells were imaged using a LSM 510 confocal microscope (Carl Zeiss), and analyzed with ImageJ software (ImageJ64, version 1.48, National Institutes of Health). All images were taken at the same time and using the same imaging settings. Data are presented as fold change compared to blank (OVA+E) NPs.

Liver Toxicity and Weight Change in Mice

Commercially available kits were used to evaluate bilirubin concentration (Sigma) and AST activity (Sigma) in the blood serum of mice. For bilirubin analysis, all experimental groups were normalized to saline control. The weight of individual mice was recorded before they were given any treatments and at the end of the treatment regimen (i.e., immediately before assessment of AHR) to determine percent weight change.

Lung Histology

Lungs were fixed with 4% paraformaldehyde and then processed by paraffin embedding. Tissue sections (5 μ m) were cut and stained using hematoxylin and eosin (H&E) or Alcian Blue/periodic acid–Schiff (PAS) to determine mucin distribution. Images were acquired using a Leica light microscope. Eosinophilia from H&E sections was determined using the 40 $\times$ objective; 4–5 random digital images per group were taken within areas of overt peri-bronchiolar inflammation. Total eosinophil cell counts were determined using ImageJ software (ImageJ64, version 1.48, National Institutes of Health), and expressed as number of cells per 10 μ m². Severity of perivascular inflammation was quantified by a 4-point scoring system where 0=absence of cell cuffs, 1=rare to few scattered perivascular inflammatory cell cuffs, 2=multifocal to moderate numbers of perivascular inflammatory cell cuffs, 3=large number of diffuse perivascular inflammatory cell cuffs.²⁵ For mucin measurements, PAS-stained slides were imaged (20 $\times$ objective) and then ImageJ software was used to determine the percentage of positively stained area per total area.

IL-5 Cytokine Determination

IL-5 was analyzed in lung homogenates by cytokine-specific ELISA Duo Set kit (R&D Systems) and normalized to total protein content (DC Assay, Bio-Rad) according to the manufacturer's instructions.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated using the Qiagen RNeasy column-based kits. Complementary DNA was prepared using the SuperScript III reverse transcription system (Invitrogen) with random nanomer primers. Expression of mRNA was quantified with the iQ LightCycler (Bio-Rad) and SYBR Green dye system and normalized to acidic ribosomal phosphoprotein 1 (Arp) mRNA. Primer sequences (mouse): Arp forward – TCA TCC AGC TGT TTG ACA A, Arp reverse – ATT GCG GAC ACC CTG TAG GAA G. Muc5ac forward-GTG GTG GAA ACT GAC ATT GG, Muc5ac reverse - CAT CAA AGT TCC CAC ACA GG. Eotaxin

forward - CAC TTC CTT CAC CTC CCA GGT GC, Eotaxin reverse - CCC ACT TCT TCT TGG GGT CAG CA. IL-4 forward: TCT TTA GGC TTT CCA GGA AGT C, IL-4 reverse - GAG CTG CAG AGA CTC TTT CG.

Statistical Analysis

Data are shown as means $\pm$ SEM. Analysis of experiments was performed using 2-tailed students t-test, two-way ANOVA or one-way ANOVA and post hoc comparisons tested using Tukey correction. The GraphPad Prism statistical software program was used for the analyses. *P < 0.05; ** P < 0.01; ***P < 0.001 was regarded as statistically significant. Results of experiments are compared between OVA-challenged mice or OVA-challenge in the presence of empty NPs.

Results and Discussion

Our group originally discovered that CaMKII is activated by ROS (oxidized-CaMKII or ox-CaMKII).²⁶ Ox-CaMKII is increased in airway epithelium from asthmatic patients after allergen exposure and correlates with asthma severity.⁸ Inhibition of CaMKII in the lungs of mice protected against allergen-induced phenotypes.⁸ Inhibitory peptides such as CaMKIIN are notable for lacking activity against other calmodulin kinases or protein kinase C¹ and provide a potential approach for highly specific CaMKII inhibition.

Herein, we examined CaMKIIN-loaded PLGA NPs as an inhalable therapeutic tool for allergic airway disease. PLGA NPs were loaded with the 21-amino acid peptide CaMKIIN which was conjugated with a HiLyte™ Fluor moiety for ease of detection. Afterwards, NPs were coated with chitosan and imaged using scanning electron microscopy (SEM). We established that the NPs were smooth in morphology and spherical in shape (Figure 1a). The loading of CaMKIIN in the PLGA NPs was 0.5 ($\pm$ 0.02) μ g CaMKIIN per mg of NPs. Because of the high water solubility of the peptide, the encapsulation efficiency was around 3%. An in vitro release study demonstrated that ~50% of CaMKIIN was released within the first 30 minutes, with an additional 15% released by 48 hours (Figure 1b).

Chitosan is a natural, cationic polymer with known mucoadhesive properties and has been shown to promote adsorption, uptake and retention of therapeutic compounds into lung epithelial cells^{27–28} in a mechanism that may include glycoprotein-mediated endocytosis.²⁹ To optimize the formulation of CaMKIIN delivery to the lungs, a chitosan layer was self-assembled onto the surface of the PLGA NPs via electrostatic interactions. To confirm the adsorption of chitosan on the surface of the NPs, DLS was used to measure size and zeta potential. In the absence of chitosan, the average hydrodynamic diameter of the PLGA NPs was 160 nm and the average zeta potential was 4 mV (Figure 1c). After coating of the NPs with the chitosan layer, the average hydrodynamic diameter increased to 230 nm and the zeta potential to approximately 40 mV (Figure 1c). This confirmed that the surface of the NPs was modified by chitosan.³⁰ Furthermore, the polydispersity index (PDI) of uncoated and chitosan-coated NPs were 0.2 ($\pm$ 0.1) and 0.22 ($\pm$ 0.09) respectively, indicating that there was no aggregation after coating the NPs with chitosan.

To determine the functional properties of chitosan-coated CaMKIIN-loaded PLGA NPs, we assessed cellular uptake in primary human airway epithelial cells (HAECs) by flow cytometry. There was a significant (4-fold) increase in uptake of chitosan-coated NPs in HAECs compared to uncoated NPs and the control (Figure 1d). Because of the drastic enhancement of uptake in HAECs, chitosan-coated NPs were utilized for subsequent in vivo testing of allergic asthma.

To assess specific delivery of NPs to the lungs, we performed a biodistribution study in male and female C57Bl/6J mice using chitosan-coated and uncoated PLGA NPs loaded with a near-infrared dye (thought to be retained within the PLGA matrix) administered by oropharyngeal instillation (OP) which would directly deliver NPs to the lung.^{31–32} After 1 hour, both coated and uncoated NPs were robustly detected in the lungs. Other organs had no appreciable increase in signal over PBS controls (Figure 2a, b). The fluorescent signal in the lungs of mice treated with chitosan-coated NPs was significantly lower at 1 hour post instillation compared to the lungs of mice treated with uncoated NPs (Figure 2b). However, there was higher reproducibility across the replicate measurements in the chitosan-coated group compared to non-coated NPs in which there was a large variability (1 hour, Figure 2b). In addition, at 24 and 48 hours after nanoparticle instillation, the near-IR dye signal was significantly reduced in mice treated with uncoated NP compared to 1 hour. In contrast, mice exposed to chitosan-coated NPs showed steady-state lung retention between 1, 24 and 48 hours (Figure 2a, b).

To assess if CaMKIIN-loaded, chitosan-coated PLGA NPs could be a potential therapeutic agent for preventing exacerbations in asthmatic patients, we used an established murine model of allergic asthma⁸ by sensitization to ovalbumin (OVA, Figure 2c). It is known that after delivery to the lungs, NPs can be detected in other organs such as the liver, heart, spleen, gastrointestinal tract and brain.^{33–36} Therefore, we examined toxicity after euthanizing animals on day 18, using serum biomarkers including bilirubin and aspartate transaminase (AST).³⁷ Administration of chitosan-coated CaMKIIN-loaded PLGA nanoparticles, as diagrammed in Figure 2c, had no significant effect on total bilirubin (Figure 2d), serum AST activity (Figure 2e), or animal weight (Figure 2f).

NPs have proven themselves to be good candidates for targeted drug delivery; they are widely available, easily functionalized, biocompatible, and stable.³⁸ We hypothesized that chitosan-coated, CaMKIIN-loaded NPs delivered directly to the lungs would be able to ablate OVA-mediated, CaMKII-induced asthma phenotypes better than soluble CaMKIIN delivered to the lungs. We tested this hypothesis by instilling mice with CaMKIIN peptide without NP encapsulation prior to assessing OVA-mediated airway hyperreactivity (AHR). After challenge with methacholine, both groups (OVA alone or OVA + CaMKIIN peptide) had significantly higher AHR compared to the saline control (Figure 3a). These results suggest that encapsulating the peptide into PLGA-NPs followed adding a chitosan-coating to the surface of NPs may be necessary for sufficient cellular uptake (as discussed previously and shown in Figure 1d) as well as for modification of asthma phenotypes in an experimental murine model of allergic asthma.

We next evaluated the effect of three doses (25, 50 or 100 μ g) of blank NPs on OVA-mediated, methacholine induced AHR. Our results showed that exposure of mice to blank NP at any concentration had a similar outcome as OVA alone (Figure 3b), suggesting there were no dose-dependent effects. Next, mice were given the same doses of NPs used in Figure 3b but this time loaded with a set concentration of CaMKIIN peptide (0.5 μ g CaMKIIN/mg NP, see materials and methods). The low dose of CaMKIIN-loaded NPs (25 μ g) did not alleviate OVA-mediated AHR, while the high dose (100 μ g) caused a significant increase in OVA-mediated AHR compared to OVA alone (Figure 3c) at the highest methacholine concentration. This suggested there was a no observable effect level (NOEL) at the low dose and an adverse effect at the highest dose. However, the intermediate dose (50 μ g) was effective at reducing OVA-induced AHR (Figure 3c). These data also emphasize that NP-based approaches can be adapted to deliver to other peptide-based therapies in asthma and other lung diseases.

Exposure of mice to 50 μ g of CaMKIIN-loaded NPs (OVA+CN) significantly reduced resistance, whereas empty NPs (OVA+E) did not protect against OVA-mediated AHR (Figure 3d). Administration of CaMKIIN-loaded NPs to non-asthmatic control mice (Saline-CN) did not alter airway resistance (Figure 3d). As the 50 μ g dose was effective at preventing increased airway resistance in OVA challenged mice after methacholine exposure and did not induce notable toxicity (Figure 2g), this dose was utilized for subsequent experiments.

In vivo studies investigating reactive oxygen species (ROS) production in models of cardiovascular disease have shown a role for CaMKII.^{39–40} Previous studies from our lab have demonstrated inhibition of CaMKII in transgenic mice reduces cytoplasmic ROS in airway epithelium, mucus production and inhibits allergen-mediated AHR.⁸ Recently, in a murine model of asthma using oxidant-resistant CaMKII MMVV6 where the protein could not be activated, compared to wild type mice, MMVV6 mice also had reduced asthmatic phenotypes as well as reduced levels of ROS.⁴¹ Therefore, CaMKII is thought to be a key mediator of ROS production. To test whether inhibition of CaMKII in bronchial epithelial cells decreases ROS production, we isolated primary murine tracheal bronchial epithelial cells²² (MTBEC) from OVA-challenged mice treated with empty NP (50 μ g, OVA-E) or CaMKIIN-loaded NP (50 μ g, OVA-CN). Staining for cytoplasmic superoxide production (DHE) showed significantly higher signal in mice treated with blank-NPs compared to mice treated with CaMKIIN-NPs (Supplemental Figure 1). These results further support a role for CaMKII in ROS production and suggest CaMKII activity is indeed inhibited by CaMKIIN-loaded, chitosan-coated NPs.

Asthma is characterized by excessive airway inflammation and accumulation of eosinophils.⁴² We determined whether chitosan-coated NPs loaded with CaMKIIN could attenuate lung eosinophilic inflammation induced by allergen challenge. Sensitization to OVA significantly increased total cell counts (Figure 4a) and eosinophils in bronchoalveolar lavage (BAL) fluid (Figure 4b). Although empty-NPs alone (OVA+E) significantly reduced OVA-mediated eosinophilic inflammation in the BAL, OVA-challenged mice exposed to CaMKIIN-loaded NPs had a further reduction in cell count and BAL-eosinophils (Figure 4a,b). This is interesting, and shows similar results to a previous study in which chitosan displayed

immunosuppressive properties in OVA and prostate specific antigen (PSA) tumor models.⁴³ Similarly, histologic analysis of lung sections demonstrated that, following allergen challenge, eosinophil infiltration into the airway was significantly abrogated by CaMKIIN-loaded NPs compared to OVA-alone or OVA-exposure with empty NPs (Figure 4c, d). Further assessment of allergen-mediated inflammation was determined by quantification of perivascular cuffs present in lung tissue sections. There was a reduction in perivascular cuffing in the lungs of mice exposed to CaMKIIN-loaded NPs compared to OVA-alone or mice challenged with OVA and exposed to empty NPs (Figure 4e).

Eotaxin, an eosinophil chemoattractant, is induced in different allergy models.^{42, 44} CaMKIIN-loaded NPs eliminated eotaxin mRNA expression following OVA challenge, in contrast to control or empty NP-treated mice where eotaxin mRNA was significantly increased (Figure 4f). Eotaxin cooperates with other interleukins, including IL-5, to promote tissue eosinophilia.²³ Compared to saline-treated mice, animals exposed to saline in conjunction with CaMKIIN-loaded NPs (N.S.+CN) showed a trend towards an increase in lung-derived IL-5 protein, however OVA alone or in the presence of empty NPs had significantly increased cytokine levels while CaMKIIN-loaded NPs reduced IL-5 protein (Figure 4g). Finally, analysis of another prominent type II cytokine involved in asthma phenotypes⁴⁵, IL-4, in OVA-challenged mice treated with CaMKIIN-NPs had significantly lower mRNA expression compared to mice treated with empty NPs (Supplemental Figure 2). These results provide compelling evidence that delivery of chitosan-coated CaMKIIN-loaded PLGA NPs reduce key features of allergic asthma, including AHR, eosinophilic airway inflammation and production of inflammatory cytokines.

Another key feature of allergic asthma is increased mucus production in the lungs²⁴, and previous work from our group has implicated CaMKII in this process.⁸ Consistently, mucin expression and MUC5AC mRNA gene expression were significantly reduced in CaMKIIN NP-treated mice compared to empty NP treated mice or untreated mice following OVA challenge (Figure 4h,i,j). Although mice instilled with empty NPs also had a significant reduction in MUC5AC mRNA, mice instilled with CaMKIIN-loaded NPs had a significantly greater MUC5AC mRNA reduction relative to untreated mice challenged with OVA (Figure 4j).

Although these data provide a proof-of-concept study investigating the potential benefit of CaMKIIN-loaded NPs as a treatment option for preventing asthmatic exacerbations in patients, we further assessed whether the dose dependent phenomenon in CaMKIIN-NP effects seen in Figure 3c could be recapitulated ex vivo in bronchial epithelial cells. MTBEC isolated from wild type mice and treated with increasing concentrations of NPs in the presence of a potent type II cytokine⁴⁶, IL-13, was similar to our in vivo assessment of AHR (Figure 3c), that compared to blank-NPs, the intermediate dose of CaMKIIN-loaded NPs (50 µg) significantly reduced IL-13-mediated effects on mRNA expression of eotaxin, IL-5 and MUC5AC (50 µg, Supplemental Figure 3). Interestingly, the low (25 µg) dose had no effect while the high (100 µg) dose chitosan-coated CaMKIIN-NPs increased IL-13-induced effects on mRNA expression (Supplemental Figure 3), similar to our AHR assessment in Figure 3c. This study demonstrates that while CaMKII acts as a key mediator of the asthma disease phenotypes, more in depth analysis of the pharmacological and toxicological profile

of CaMKIIN is needed to further investigate chitosan-coated, CaMKIIN-NPs as a viable treatment option for patients with asthma.

Conclusion

In summary, we have demonstrated that cationic NPs are effective vehicles for drug delivery to the lung. Surface modification of PLGA NPs with chitosan enhanced the uptake of the encapsulated therapeutic agent in primary airway epithelial cells compared to uncoated NPs, with a favorable in vivo safety profile. In addition, we provide evidence for a novel peptide-based formulation for CaMKII inhibition in the lungs. The translational potential of our findings is high given that CaMKII inhibitors and use of nanotechnology to improve retention of therapeutic agents are currently under development.^{25, 47} As such, utilization of NPs for drug delivery to the lungs could offer a novel, more efficacious and safer treatment option for asthmatic patients. In future studies, we plan to optimize the peptide loading in the PLGA NPs by varying the formulation used during fabrication and to test the NPs in a therapeutic setting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AHR	airway hyperreactivity
AST	aspartate transaminase
BALF	bronchoalveolar lavage fluid
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase
CaMKIIN	Ca ²⁺ /calmodulin-dependent protein kinase inhibitor
DLS	dynamic light scattering
HAECs	human airway epithelial cells
INH	inhalation
IP	intraperitoneal injection
NOEL	no observable effect level
NP	nanoparticle

NS	normal saline
OP	oropharyngeal
OVA	ovalbumin
ox-CaMKII	oxidized-CaMKII
PLGA	poly(lactic-co-glycolic acid)
ROS	reactive oxygen species.

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Figure 1. Chitosan coating of PLGA NPs increases size, zeta potential and cellular uptake by human airway epithelial cells

a, Representative SEM image of chitosan-coated PLGA NPs. b, Cumulative CaMKIIN release from PLGA NPs incubated at 37°C and agitated at 300 rpm (n=3). c, Size and zeta potential of PLGA NPs with and without chitosan-coating determined by DLS. d, Cellular uptake of CaMKIIN loaded PLGA NPs with and without chitosan in HAECs as measured by flow cytometry. Control: cells incubated with culture medium alone. (*P 0.05, **P < 0.01, ****P < 0.0001)

Figure 2. Chitosan-coated NPs localize in the lungs of mice and cause no significant toxicity in vivo

a, Representative images of control (PBS), uncoated and coated NPs (50 μ g NPs) loaded with fluorescent dye in various organ systems 1, 24 and 48 hours after oropharyngeal administration. b, Graphical analysis of NPs measured in lungs of mice at 1, 24 and 48 hours ($n = 3-5$ mice/group). c, Timeline of OVA sensitization (IP) and challenge and NP treatment (oropharyngeal (OP), inhalation (INH) of ovalbumin and alum (OVA/ALUM)). Animals were euthanized and samples taken on day 18. d,e Normalized (to saline control group) bilirubin content (d) and AST activity (e) in serum of OVA sensitized mice after treatment with normal saline (N.S.), saline + CaMKIIN-loaded NPs (N.S.+CN), OVA alone (OVA), OVA + empty NPs (OVA+E), and OVA + CaMKIIN NPs (OVA+CN) on day 18. f, Percent weight change during sensitization and NP treatment protocol. Data were calculated relative to body weight on day 0. For toxicity studies, all NPs were coated with chitosan.

Figure 3. Cationic CaMKIIN-loaded nanoparticles (NPs) reduce airway hyperreactivity (AHR) Mice were sensitized to OVA alone or in the presence of 25 ng of soluble CaMKIIN peptide. The dose of soluble CaMKIIN peptide was calculated based on the total amount of CaMKIIN peptide present in 50 µg of NPs (50 µg of NPs loaded with 0.5 µg CaMKIIN/mg NP = 25 ng total CaMKIIN). Control mice were sensitized to saline. b, AHR of OVA alone or OVA in the presence of empty chitosan-coated NPs (25, 50 and 100 µg-blank-NPs). NS= not significant. c, AHR of saline, OVA alone or OVA with chitosan-coated NPs (25, 50 and 100µg-CN) loaded with CaMKIIN. d, AHR in OVA-sensitized mice exposed to 50 µg of chitosan-coated empty NPs (OVA-E) or 50 µg of chitosan-coated NPs loaded with 0.5 µg/mg CaMKIIN (OVA-CN). Additional controls were not sensitized to OVA and given chitosan-coated NPs loaded with CaMKIIN (N.S.-CN). Data are means ± SEM; *P < 0.05 vs. saline; \$P < 0.05 100µg-CN vs. OVA; # P < 0.05 versus OVA+NP or OVA alone (n = 5–9 mice).

Figure 4. Airway inflammation, cytokine expression and mucus production are decreased by cationic CaMKIIN-loaded NPs

a, Total cell counts in bronchoalveolar lavage fluid (BALF) and b, Eosinophil counts in BALF (n=7–12 mice). c, Quantification of eosinophils/10 μ m² area (40 $\times$ magnification). d, H&E staining of lung sections. e, Perivascular cuffing score (10 $\times$ magnification). f, qRT-PCR for eotaxin in lung homogenates. g, IL-5 protein levels in lung homogenates by ELISA. h, i, Representative images of PAS staining (h, 20 $\times$ magnification) and (i) quantification (3–5 sections per mouse). j, qRT-PCR for MUC5AC in lungs of allergen challenged mice. Data

shown are mean $\pm$ SEM (n = 3 for saline groups and n= 6–8 for all OVA-challenged groups).

* P < 0.05 OVA alone vs. saline or as indicated by brackets. For g, p < 0.09 N.S.

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