

Aerosolized BIO-11006, a Novel MARCKS- Related Peptide, Improves Airway Obstruction in a Mouse Model of Mucus Hypersecretion

Anurag Agrawal^{1,*#}, Edwin C. Murphy III², Joungjoa Park³, Kenneth B. Adler^{3,*#} and Indu Parikh²

¹Baylor College of Medicine, Houston, Texas; ²BioMarck Pharmaceuticals, Durham, North Carolina; ³North Carolina State University, Raleigh, North Carolina, USA

Abstract: *Rationale:* Mucus hypersecretion has been shown to be an important cause of airway obstruction in COPD and asthma, and has been associated with fatal asthma. Previous *in-vitro* and *in-vivo* studies have provided direct evidence that the myristoylated alanine-rich C kinase substrate (MARCKS) is a central regulatory molecule linking secretagogue stimulation at the goblet cell surface to mucin granule release. BIO-11006 is a novel, highly soluble, aerosolized peptide that inhibits MARCKS function and results in reduced mucus secretion and improved airway obstruction. BIO-11006 is being tested in Phase 2 clinical studies of COPD.

Objective: To determine access of BIO-11006 to the intracellular compartment of normal human bronchial epithelial (NHBE) cells, and assess the effectiveness of BIO-11006 inhalation solution in inhibiting airway obstruction caused by mucus hypersecretion as a function of dose and time.

Methods: Biotin labeled BIO-11006 was incubated with NHBE cells and visualized with fluorescein-labeled streptavidin. Ovalbumin-sensitized and challenged mice with airway inflammation and hyperresponsiveness to methacholine (MCh) were treated with aerosolized BIO-11006 solution prior to MCh challenge, and effects on airway obstruction and mucus secretion were measured.

Results: A 30-minute aerosol exposure to a 10 mM BIO-11006 solution was maximally effective in inhibiting MCh-induced airway obstruction and mucus hypersecretion. There was a long-lasting effect on inhibition of airway obstruction after a single treatment with BIO-11006 ($t_{1/2}$ ~4 hrs). The duration of effect on inhibition of mucin secretion was shorter ($t_{1/2}$ ~2 hrs).

Conclusion: The pharmacodynamic properties of aerosolized BIO-11006 Inhalation Solution are suitable for therapeutic use. Inhaled BIO-11006 effectively inhibits mucin secretion and improves airway obstruction.

Keywords: BIO-11006, MARCKS, mucin, asthma, COPD.

INTRODUCTION

Mucus hypersecretion has been shown to be an important cause of airway obstruction in COPD and asthma, and has been associated with fatal asthma that fails to respond to conventional medical therapy [1-3]. Previous *in vitro* and *in vivo* studies have provided direct evidence that the myristoylated alanine-rich C kinase substrate (MARCKS) is a central regulatory molecule linking secretagogue stimulation at the goblet cell surface to mucin granule release [4-6]. These studies include experiments in which intranasal administration of a 24 amino acid myristoylated peptide identical to the N-terminus of the MARCKS protein (MANS peptide: Myristoylated N-terminal Sequence) was used to inhibit MARCKS function. Low solubility of the MANS peptide excluded a nebulizer based aerosol therapy, which would be

required for future human use. Therefore, more highly soluble derivatives of the MANS peptide were tested for their ability to inhibit mucus hypersecretion and airway obstruction. The effects of one such peptide, BIO-11006, are reported here. BIO-11006 Inhalation Solution is an investigational new drug product that is being developed as a new therapeutic agent indicated for chronic bronchitis associated with COPD. BIO-11006 is a 10-amino acid synthetic peptide that inhibits the function of the MARCKS protein, thereby inhibiting mucus secretion. The BIO-11006 peptide has also been shown to be an effective inhibitor of inflammatory mediator release from neutrophils [7] and ozone-induced airway inflammation in mice [8]. The studies presented in this communication were designed to assess the effectiveness of BIO-11006 in inhibiting airway obstruction caused by mucus hypersecretion as a function of dose and time in an established mouse model of mucus hypersecretion. The duration of action of any compound is an important determinant of its clinical utility. While it has been previously shown that BIO-11006 is an effective inhibitor of mucus secretion and related airway obstruction in mice, these results were exclusively reported for a single time point immediately following a 30 minute exposure to aerosolized BIO-11006. The current study further examines whether significant effects can be

*Address correspondence to these authors at the Baylor College of Medicine, Houston, Texas, USA; Tel: 713 798 4951;

E-mail: a.agrawal@igib.in

North Carolina State University, Raleigh, North Carolina, USA;

Tel: 919 513 1348; Fax: 919 515 4237;

E-mail: kenneth_adler@ncsu.edu

#Currently at Institute of Genomics & Integrative Biology, Delhi, India

seen for longer durations, when BIO-11006 is administered 1 to 4 hours prior to methacholine stimulated mucus hypersecretion.

MATERIALS AND METHODS

This study was conducted in conformity with the American Physiological Society's Guiding Principles in the Care and Use of Animals. Experimental protocols and study design were approved by the institutional review boards at the Institute of Genomics and Integrative Biology, New Delhi, India.

Uptake of BIO-11006 by Normal Human Bronchial Epithelial (NHBE) Cells

NHBE cells were cultured on air/liquid interface in 12-well Transwell[®] microtiter plates (Costar, Corning, NY) by previously published procedures [6]. Biotin labeled BIO-11006 peptide was synthesized by standard solid phase synthesis and dissolved in PBS to provide a 1.0 mM solution. Biotin labeled BIO-11006 (100 uM final concentration) was incubated for various time periods from 0 to 60 minutes with fully differentiated NHBE cells that were on air/liquid interface, at 37°C. The cells were washed with PBS, fixed with 4% formaldehyde in PBS (pH 7.4) at room temperature for 20 minutes, and further incubated for 1 hour with 10% normal donkey serum dissolved in 100 mM PBS containing 1.0% Triton X-100. The cells were then incubated overnight at 4°C with mouse anti-mucin monoclonal antibody 17Q2 (Covance, Berkeley, CA). The cells were then incubated with a rhodamine-coupled donkey anti-mouse secondary antibody TRITC (red color) (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour at room temperature. The cells were washed with PBS and further incubated with FIFC labeled streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 minutes at room temperature (green color). Following this, the cells were washed again with PBS and then incubated with Alexa 647-phalloidin (blue) (Molecular probe, Eugene, OR) for 3 minutes at room temperature to visualize actin. Cells were washed and mounted on glass slides with fluorescent mounting media (Dako Cytomation, Carpinteria, CA) and examined with a Nikon Eclipse 2000E inverted microscope equipped with a Nikon C1 confocal laser scanning system. Standard excitation and emission wavelengths for rhodamine (red) (554, 573 nm), FIFC (green) (488, 520 nm), and Alexa 647 (blue) (650, 668 nm) were used. The pictures of each slide were taken at 1 μm thickness (Z-stack) and the data were rendered into 2D figures. Finally, the intensity of each fluorescence label was measured and documented using EZ-C1 software.

Induction of Airway Hyperresponsiveness (AHR) and Experimental Allergic Asthma

The study was conducted using eight to twelve week old (16-20 g) male, inbred, BALB/c mice obtained from V.P. Chest Institute (New Delhi, India). Mucus metaplasia and AHR were induced by weekly intraperitoneal injection of 20 μg ovalbumin (Ova, Sigma life science, USA) in 2.25 mg alum for four weeks followed by airway challenge with 1% Ova aerosol (Ova/Ova) within 30 days of the last sensitization [9]. Reference mice were sensitized but, instead of challenge with Ova, saline was administered (Ova/Sal).

Quantification of Airflow Obstruction

Specific airway conductance (sGaw) was measured as the time lag between flows recorded from the head and thoracoabdominal chambers in consciously breathing, restrained mice, using the two-chambered body plethysmograph system devised by Buxco (PLY-3351, Biosystem XA software) as previously described [9]. sGaw was converted to Raw by inverting and dividing by estimated normal mouse lung volume of 0.5 ml. For repeated measurements, changes in Raw were estimated by comparing any given Raw to baseline Raw for that mouse.

Experimental Protocol for Dose Response

For studying the dose response characteristics of BIO-11006, three days after the ovalbumin challenge, solutions of BIO-11006 (obtained from BioMarck Pharmaceuticals, Durham, NC), saline, or a negative randomized control sequence peptide (either BIO-11006R or RNS, as described previously [9]) were administered for either 30 minutes (3, 10, 30 mM solutions) or shorter durations of 5 and 15 minutes (10, 30 mM) by nebulizer to mice (n=3 per group). Approximately 30 minutes after start of peptide treatment (i.e. immediately after a 30 minute treatment and 25 minutes after a 5 minute treatment) mice were challenged with an aerosolized 60 mM solution of the secretagogue methacholine (MCh) for 5 minutes. Airway obstruction was measured by determining SGaw (specific airway conductance) five minutes after MCh challenge.

Experimental Protocol for Time Course of Drug Response

To determine the time course of the drug response, serial determinations of SGaw were made prior to MCh challenge (60 mM for 90 s), and at 3, 6, 9, 12, and 15 minutes post MCh challenge for each mouse. Mice were treated for 30 minutes with either aerosolized BIO-11006 or a negative randomized control sequence peptide (either BIO-11006R or RNS, as described previously) at 0, 30, 60, 120, or 240 minutes prior to the MCh challenge (n=5 per group).

Histological Analysis of Mucus Secretion

In selected animals, intracellular mucin remaining in the airways was assessed by periodic acid Schiff (PAS) staining to assess BIO-11006 inhibition of mucin secretion. Serial sections of the main axial bronchus along with minor daughter branches that we refer to as penetrating bronchi were used for quantitative morphometry. Quantitative morphometry was performed on comparable stained sections from the proximal airways as described previously [9].

RESULTS

BIO-11006 is Efficiently Taken Up by Normal Human Bronchial Epithelial Cells in a Time Dependent Manner

To determine whether BIO-11006 peptide is likely to penetrate into secretory cells, we applied biotin labeled BIO-11006 (100 uM final concentration) to fully differentiated NHBE cells for 0, 15, 30, and 60 minutes and studied its uptake by confocal microscopy. Fig. (1). shows that the peptide (green) is found intracellularly in abundance and is taken up by both mucus secreting cells (red granules) and non-mucus secreting cells. The uptake of biotinylated BIO-11006

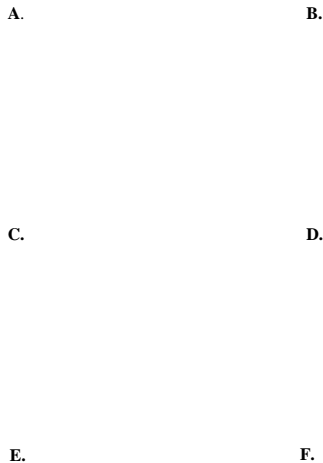


Fig. (6). Inhibition of mucin secretion after BIO-11006 aerosol administration. PAS stained airway section from mice treated with control peptide / BIO-11006 followed by MCh: A, control mice treated with control peptide followed by immediate MCh challenge (note lack of mucin); B, MCh challenge immediately after BIO-11006; C, 30 minutes after BIO-11006; D, 1 hr after BIO-11006; E, 2 hr after BIO-11006; F, 4 hr after BIO-11006. Note that even at 4 hrs, some degree of mucin retention compared to control can be seen.

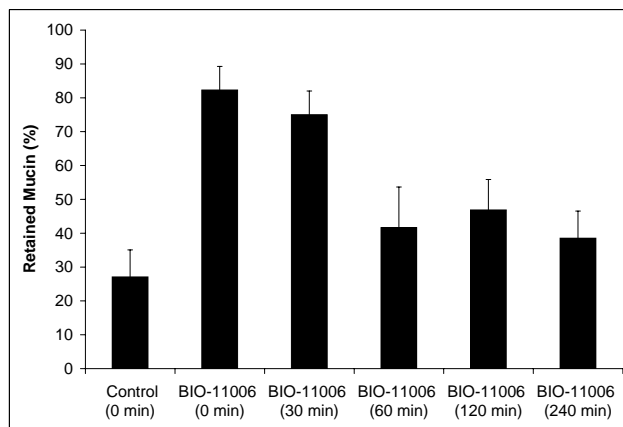


Fig. (7). Duration of inhibition of MCh-induced inhibition of mucin secretion after BIO-11006 treatment. Retained mucin in airways of mice treated with either control peptide or BIO-11006 followed by MCh at varying intervals is shown as percentage of mucin prior to MCh challenge. Time duration between treatment and MCh challenge is shown in parentheses. Asterisk (*) denotes statistically significant difference.

colytics such as inhaled N-acetyl-cysteine (NAC), which act to thin mucus, have limited utility in COPD, but have modest benefits in cystic fibrosis patients. DNase (Pulmozyme) is indicated for cystic fibrosis patients, and acts to cleave extracellular DNA in mucus, thus reducing adhesiveness and viscoelasticity; Pulmozyme is not indicated for treatment of

COPD or asthma. In summary, a therapy that can effectively modulate mucus hypersecretion would represent a significant advance in treatment of several major airway diseases. In this study, the time dependent uptake of BIO-11006 into normal human bronchial epithelial cells, the dose response of BIO-11006 in mice, and the duration of effectiveness of BIO-11006 in a mouse model were explored. This expanded upon our previous work that showed BIO-11006 was an effective inhibitor of mucus hypersecretion [9]. In the previous studies, we demonstrated that inhibition of mucus hypersecretion resulted in increased airway conductance, and that the effects of BIO-11006 on airway conductance were a result of an attenuation of the pulmonary rather than the nasal component of airway resistance [9].

In the current studies, we found that BIO-11006 is efficiently taken up by human bronchial epithelial cells suggesting that it has potential use as therapy in the human setting. At the magnification limitations of the method (LCM) used to observe cellular uptake of the labeled peptides (Fig. 1) it was not possible to observe the specific intracellular localization of the peptides within the epithelial cells. Since BIO-11006 has an amino acid sequence identical to a region of the MARCKS N-terminus, any antibodies raised against the peptide or any of its sequences would not be able to differentiate between the peptide and the N-terminal region of endogenous MARCKS within the cell. It would be extremely interesting to observe this kind of subcellular localization of the peptide after it is taken up by cells, and studies to tag the peptide prior to treatment and identification of the tag ultrastructurally are presently under consideration, even with the obvious difficulties associated with these kinds of approaches.

Since this *in vitro* assay was not designed to study the effects on mucin secretion, the pharmacodynamic effects of BIO-11006 were studied in an established mouse model. While the data may not extrapolate directly to humans, the data appear to be promising. Administration of 10 mM BIO-11006 aerosol to mice for 30 minutes (estimated pulmonary dose of approximately 0.32 mg/kg) had persisting effects on MCh induced mucin secretion and airway obstruction. This effect was seen as late as 4 hrs, histologically and physiologically, based on PAS staining and measurement of airflow obstruction, respectively. The observed half-life was ~ 2 hours for inhibition of mucin secretion, and ~ 4 hours for inhibition of airway obstruction. The observed difference is not surprising because the two measurements are of very different natures. First, the relationship between airway obstruction and mucin secretion is certain to be non-linear. Even small reductions in mucin secretion may have significant effects on airway lumen size, which is related to airway resistance by a fourth power exponential function. Second, the linearity of quantitative morphometry of mucins is assumed but not definitive. Finally, BIO-11006, by simultaneously inhibiting secretion of inflammatory mediators and mucus secretion, may result in a longer duration of effect on airway obstruction.

The exact mucin gene product affected by treatment with BIO-11006 is not known, but previous studies in mice with goblet cell hyperplasia induced by either ovalbumin sensitization [4, 5] or instillation of human neutrophil elastase [10] have implicated the Muc5ac protein as the major mucin type

that is secreted in the mouse lung and affected by peptides directed against the MARCKS N-terminus. In the present study, we did not measure mucin release directly, but rather used histochemical staining with PAS to measure mucin retention in the airway goblet cells, which is the inverse of released mucin. Since this staining does not differentiate between the different mucin gene products, it is possible that other mucins that can be secreted by epithelial cells (i.e., Muc2, Muc5b or Muc19) also could be released in addition to Muc5ac.

With a half-life of 2-4 hours, the pharmacodynamics of BIO-11006 appear to be well suited for clinical use as a mucus and pulmonary inflammation inhibitor. While the efficacious clinical dose remains to be determined, it is critical to consider that both too high and too low a dose of BIO-11006 could be problematic. The effects of too low a dose would likely be ineffective inhibition of mucus secretion and inflammation and no improvement in lung function, and the effects of too high a dose might be to reduce levels of mucus below what is required for its physiological functions. In addition, too high a dose could generate a non-specific irritant effect that could provoke additional mucin secretion and/or inflammation.

CONFLICT OF INTEREST

Indu Parikh and Edwin Murphy are employees of BioMarck Pharmaceuticals. This work was supported by a grant from BioMarck Pharmaceuticals to Anurag Agrawal. Kenneth Adler holds founder's shares of stock in BioMarck, Inc. and serves in a scientific advisory capacity to the company without pay.

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