An Inhaled Inhibitor of Myristoylated Alanine-Rich C Kinase Substrate Reverses LPS-Induced Acute Lung Injury in Mice

Qi Yin1*, Shijing Fang1†, Joungjoa Park1, Anne L. Crews1, Indu Parikh2, and Kenneth B. Adler1

1Department of Molecular Biomedical Sciences, North Carolina State University, Raleigh, North Carolina; and 2Biomarck Pharmaceuticals, Incorporated, Durham, North Carolina

Abstract

Intratracheal instillation of bacterial LPS is a well-established model of acute lung injury (ALI) and/or acute respiratory distress syndrome (ARDS). Because the myristoylated alanine-rich C kinase substrate (MARCKS) protein is involved in neutrophil migration and proinflammatory cytokine production, we examined whether an aerosolized peptide that inhibits MARCKS function could attenuate LPS-induced lung injury in mice. The peptide, BIO-11006, was delivered at 50 μM via inhalation either just before intratracheal instillation of 5 μg of LPS into Balb/C mice, or 4, 12, 24, or 36 hours after LPS instillation. Effects of BIO-11006 were evaluated via analysis of mouse disease-related behavior, lung histology, bronchoalveolar lavage fluid total protein, neutrophil counts and percentages, cytokine (KC [CXCl1, mouse IL-8 equivalent] and TNF-α) expression, and activation of NF-κB in lung tissue. Treatment with aerosolized BIO-11006 at 0, 4, 12, 24, and even 36 hours after LPS instillation reversed the disease process: mouse behavior returned to normal after two treatments 12 hours apart with the inhaled peptide after LPS injury, whereas control LPS-instilled animals treated with PBS only remained moribund. Histological appearance of inflammation, bronchoalveolar lavage fluid protein levels, leukocyte and neutrophil numbers, KC and TNF-α gene and protein expression, and NF-κB activation were all significantly attenuated by inhaled BIO-11006 at all time points. These results implicate MARCKS protein in the pathogenesis of ALI/ARDS and suggest that MARCKS-inhibitory peptide(s), delivered by inhalation, could represent a new and potent therapeutic treatment for ALI/ARDS, even if administered well after the disease process has begun.

Keywords: myristoylated alanine-rich C kinase substrate; acute lung injury; LPS; neutrophils

Clinical Relevance

These studies show that treatment of mice with acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) with an inhaled inhibitor of myristoylated alanine-rich C kinase substrate (MARCKS) protein reverses disease progression, even when treatment is initiated well into the disease process, a time when the animals are moribund. These results implicate MARCKS protein in the pathogenesis of ALI/ARDS and suggest that MARCKS-inhibitory peptide(s), delivered by inhalation, could represent a new and potent therapeutic treatment for ALI/ARDS, even if administered well after the disease process has begun.

Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS), are major causes of respiratory failure; worldwide, over 1 million cases occur annually, with over 200,000 adult and 20,000 pediatric cases per year in the United States. This disorder is characterized by a large influx of leukocytes, especially neutrophils, to the lung as part of an acute inflammatory response, with injury to epithelial and endothelial cell barriers and pulmonary edema, all of which lead to respiratory failure. Treatment options remain limited to mechanical ventilation, a supportive therapy that still results in an in-hospital mortality rate of approximately 40% in the United States. There are currently no effective pharmacological...
therapies to counter disease progression in patients with ARDS (1, 2).

A major component of the initial phases of the pathophysiology of ARDS is recruitment of neutrophils into the lung (1, 3). In previous studies, we have shown that a key molecule regulating directed migration of neutrophils (4–7) and other inflammatory cells (8, 9) is myristoylated alanine-rich C kinase substrate (MARCKS) protein. These reports used a peptide that inhibits MARCKS function called the myristoylated N-terminal sequence (MANS) peptide (10). Additional studies demonstrated that a smaller analog of MANS, BIO-11006, has the same MARCKS-inhibitory properties as MANS (11), and its smaller size and high solubility make it more suitable for use as a drug: in fact, BIO-11006, administered as an inhaled aerosol, has already been tested in a phase 2a clinical trial in human patients with chronic obstructive pulmonary disease. Given that BIO-11006 attenuates neutrophil motility and migration, its effects as a potential therapy in ALI/ARDS were investigated in an animal model of the disease.

The model of ALI/ARDS used in this study was intratracheal instillation of endotoxin (LPS from Escherichia coli) into mice, a widely used and well characterized model of inflammation and lung injury that involves massive neutrophil infiltration, enhanced production of proinflammatory cytokines and chemokines, edema, and tissue damage (12–15). BIO-11006 was administered to LPS-instilled mice as an inhaled aerosol (similar to its use in the clinical trial mentioned previously here on patients with chronic obstructive pulmonary disease). Several agents and treatments can prevent ALI in LPS-instilled mice when administered previous to, simultaneously with, or within a few hours after LPS instillation (12–15). However, human patients with ALI/ARDS clearly do not present until the disease has progressed to a point where there are clear symptoms, well into the course of the disease. To mimic this in the mouse model, we started aerosolized treatments (two treatments 12 h apart) at various times after LPS instillation: 0, 4, 12, 24, and as long as 36 hours. The results showed that administration of aerosolized BIO-11006, even as long as 36 hours after LPS instillation, a time when the mice were quite moribund, resulted in reversal of the disease process and full recovery of the animals. Parameters related to ALI, including mouse disease-related behavior, neutrophil influx, chemokine/cytokine production, and NF-κB activation, were all significantly ameliorated by treatment with BIO-11006, regardless of the time after LPS instillation that peptide treatment was initiated.

Materials and Methods

**LPS Lung Injury Model**

Animals were handled in accordance with the policies of the North Carolina State University (Raleigh, NC) Institutional Animal Care and Use Committee. Briefly, 7- to 10-week-old female BALB/c mice (Charles River Laboratories, Wilmington, MA) were kept quarantined for 7 days. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) intraperitoneally, and then instilled intratracheally with 5 μg of LPS (E. coli endotoxin no. L2630; Sigma-Aldrich, St. Louis, MO) in 50 μl PBS.

**BIO-11006**

BIO-11006 is an analog of the MANS peptide, which is identical to the first 24 amino acids of the MARCKS molecule (10). BIO-11006 contains the active site of MANS (the first 10 amino acids) and is more suitable as a drug because of its smaller size and enhanced solubility; it is identical to MANS in its MARCKS-inhibitory actions (11). BIO-11006 was synthesized by PolyPeptide (Torrence, CA). A concentration of 50 μM for N-terminal MARCKS inhibitors was shown to have optimal effects on attenuating neutrophil motility in vitro (4, 5), and showed efficacy in preliminary in vivo studies in LPS-injured mice, so this concentration of BIO-11006 (equivalent to ~0.75 mg/kg) was chosen for time-related studies detailed subsequently here.

**Treatment Groups**

Mice were divided into seven treatment groups containing six mice each;

1. Group 1: No LPS or peptide (negative control).
2. Group 2: LPS; treated only with inhaled PBS (positive control for each group).
3. Group 3: LPS; treatment started with aerosolized BIO-11006 just before LPS instillation (0 time); treated again at 12 hours.
4. Group 4: LPS; treatment started with aerosolized BIO-11006 4 hours after LPS instillation; treated again at 16 hours.
5. Group 5: LPS; treatment started with aerosolized BIO-11006 12 hours after LPS instillation; treated again at 24 hours.
6. Group 6: LPS; treatment started with aerosolized BIO-11006 24 hours after LPS instillation, treated again at 36 hours.
7. Group 7: LPS; treatment started with aerosolized BIO-11006 36 hours after LPS instillation, treated again at 48 hours.

The experimental groups were treated at the indicated times using 50 μM BIO-11006 aerosolized in PBS and administered via inhalation. The nebulizer (TREK S portable aerosol system; PARI Respiratory Equipment, Inc., Midlothian, VA) and the exposure chamber were placed inside a biological safety cabinet. Mice (three in the exposure chamber at a time) were exposed to aerosolized PBS or BIO-11006 for 30 minutes, with treatments repeated 12 hours later such that each group was exposed to the aerosolized peptide or PBS control twice over the course of the experiment. All animals were killed at 72 hours after exposure and lung injury and inflammation measured as described subsequently here.

**Disease-Related Behavior**

Mice were recorded by video for at least 30 seconds directly after they had received two treatments of BIO-11006. Videos of mice were analyzed blindly by a laboratory animal veterinarian for parameters related to disease severity and behavior using a scoring system similar to those described previously (12, 13). Each disease-related parameter was quantified via a scoring of 0 (no disease apparent, normal behavior) to 3+ (extremely sick). The disease-related parameters were: (1) activity in cage; (2) “hunching”; (3) ptosis; (4) piloerection; and (5) labored/rapid breathing.

**Analysis of Lung Injury**

At 72 hours after LPS instillation, mice were killed with an overdose of ketamine/xylazine. One lobe of the left lung was tied off and fixed, sectioned, and stained using standard hematoxylin and eosin for assessment of inflammation.

Bronchoalveolar lavage (BAL) was performed by cannulating the trachea with a
23-gauge catheter and lavaging the lungs with 850 μl sterile PBS solution. The collected lavage fluid was centrifuged (300 x g for 15 min), and the supernatant stored at −80°C. Protein concentrations were measured with a Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL) according to the manufacturer’s instructions. Protein levels of two prominent proinflammatory cytokines related to ALI (KC [CXCL1; the murine IL-8 equivalent] and TNF-α) were measured by ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. RNA was isolated from the right lung using a Qiagen RNAeasy kit (Qiagen Sciences, Germantown, MD), per the manufacturer’s instructions. Gene expression levels of KC and TNF-α were determined by RT-PCR. LPS-induced NF-κB activation was measured in cytoplasmic extracts from right lung tissue using a Western blotting technique to visualize phospho-NF-κB p65 levels, which were quantified with ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical Analysis
Data were analyzed for significance using one-way ANOVA with Bonferroni posttest corrections. Differences between treatments were considered significant at \( P < 0.05 \).

Results

Disease-Related Behavior
As shown in Table 1 and in Figure E1 (see Figure E1 in the online supplement), the effects of BIO-11006 on the mice were dramatic, regardless of time after LPS instillation that the peptide was administered. Treatment of mice with aerosolized BIO-11006 at 0 time prevented the onset of injury. Administration of BIO-11006 at 4, 12, 24, or 36 hours after LPS instillation actually reversed the disease process: mice treated with LPS plus PBS only at these times appeared sick and moribund, but mice treated with BIO-11006 showed behavior and disease parameters returning to normal after postinjury treatment initiated at each of these time points (see Table 1). The dramatic effect on the mice was apparent when their behavior after treatment with BIO-11006 is visualized (see Figure E1).

Histological Assessment
As illustrated in Figure 1, lungs of LPS-instilled mice treated only with PBS showed extensive infiltration of neutrophils and edema around airways and into the parenchyma when analyzed 72 hours after LPS instillation. Treatment with BIO-11006 appeared to ameliorate this response, with no apparent inflammation at 0 time, and increasing levels of neutrophilia as the time from LPS administration to initial treatment increased. Even with treatment starting at 36 hours, there clearly was less inflammation and edema apparent than in the LPS-instilled mice treated with PBS only.

Table 1. Blinded Analysis of Mouse Disease-Related Behavior Parameters by a Veterinarian after Two Treatments with BIO-11006 or PBS 12 Hours Apart at Indicated Times

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Lack of Activity</th>
<th>Hunched Posture</th>
<th>Ptosis</th>
<th>Piloerection</th>
<th>Labored Breathing</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS + PBS</td>
<td>+++++</td>
<td>+++++</td>
<td>+++++</td>
<td>+++++</td>
<td>+++++</td>
</tr>
<tr>
<td>LPS + BIO-11006; 0, 12 h</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LPS + BIO-11006; 4, 16 h</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LPS + BIO-11006; 12, 24 h</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>LPS + BIO-11006; 24, 36 h</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>LPS + BIO-11006; 36, 48 h</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>0</td>
</tr>
</tbody>
</table>

BIO-11006 ameliorates each of these acute lung injury–associated effects. Key: 0, normal; +, mild; ++, moderate; ++++, severe.

Leukocyte Influx
As illustrated in Figure 2, numbers of total leukocytes, and especially neutrophils, measured in BAL fluid (BALF) at 72 hours after LPS were decreased inversely proportional to the time after LPS instillation when treatment was initiated (i.e., the earlier the time after LPS that the peptide was given, the fewer leukocytes and neutrophils in the lung were observed).

Proinflammatory Cytokine Expression
As illustrated in Figures 3A and 3B, BALF levels of the proinflammatory cytokines, TNF-α and KC, were significantly attenuated at 72 hours after LPS instillation, inversely proportional to the time after LPS instillation when treatment was initiated. Message levels for both cytokines at 72 hours after LPS were also significantly attenuated by treatment with BIO-11006 (Figure 3C), shown here with treatment initiated at 4 hours after LPS instillation.

NF-κB Activation
As illustrated in Figure 4, BIO-11006 attenuated LPS-induced NF-κB activation, as assessed by measurement of phosphorylated p65 (p-p65) in the lung, when administered at each of the time points (4, 12, 24, or 36 h after LPS). Total p65 in cytoplasmic extracts increased over time (data not shown).

Discussion
The results of this study indicate that inhaled, aerosolized BIO-11006, a peptide that inhibits the function of MARCKS protein, attenuates LPS-induced neutrophil
influx into the lung, activation of NF-κB, and expression of the proinflammatory cytokines, KC and TNF-α, and thus reverses the development of lung injury in mice instilled intratracheally with LPS. Although it is known that several agents can affect the onset of ALI when given to mice before or even a few hours after LPS instillation (14–16), this is the first time, to our knowledge, that a treatment demonstrates the ability to stop, and even reverse, ALI, even when administered well into the course of the disease process. This reversal took place as long as 36 hours after LPS instillation, a time when the mice appeared quite moribund before treatment. These findings could potentially be extrapolated to the human situation, where patients usually present with ALI/ARDS that is already well established. There currently are no effective pharmacological treatments for ALI/ARDS, but targeting MARCKS protein with BIO-11006 or similar peptides could represent a novel pharmacological approach.

Neutrophil infiltration and inflammation are major contributors to tissue damage in ALI (3, 17). Neutrophils are recruited to the injured lung within minutes after the initial insult, and neutrophil influx and the resultant cytokine “storm” are considered hallmarks of ALI. As shown in Figure 2, treatment of LPS-instilled mice with aerosolized BIO-11006 appears to rapidly inhibit further influx of neutrophils into the lung. The mechanisms through which this might occur were then investigated. One possibility is that BIO-11006 directly inhibits neutrophil motility, thereby blocking neutrophil migration to the injured lung. Indeed, a number of published studies from our laboratory and others show that treatment of isolated neutrophils and other cell types with N-terminal MARCKS-inhibitory peptides attenuates their motility/migration (4–9), probably through effects on the
actin-binding function of MARCKS (4). Thus, an obvious explanation for the effect of inhaled BIO-11006 is that it directly inhibits movement of neutrophils, not allowing them to migrate to the lung after injury. However, based on other published work describing a role for MARCKS in release of cytokines and chemokines (18), we looked at other potential mechanisms that might be involved in attenuating influx of neutrophils into the lung and cytokine/chemokine expression in response to LPS. We focused this study on the potent neutrophil chemoattractant, KC, or CXCL1, the mouse IL-8 equivalent, and the pleiotropic proinflammatory cytokine, TNF-α. Indeed, analysis of BALF at 72 hours after LPS showed that treatment with inhaled BIO-11006 significantly decreased levels of both cytokines (Figures 3A and 3B). We also investigated, using RT-PCR, whether this effect could be on the expression of KC and TNF-α at the mRNA level. As illustrated in Figure 3C, treatment with BIO-11006 provoked a significant decrease in mRNA expression of these cytokines in the lung, suggesting effects at the message level.

MARCKS also has been shown to be involved in activation of NF-κB in different cells (19–21), perhaps through a protein kinase C-δ-related mechanism (22), and it is known that KC and TNF-α contain NF-κB–binding sites in their promoter regions, so a logical next step was to look at whether or not BIO-11006 could affect NF-κB. Using phosphorylation of the p65 subunit of NF-κB in lung cytoplasmic extracts as an indicator of NF-κB activation, inhalation of BIO-11006 at any of the time points in this study inhibited LPS-induced NF-κB activation (Figure 4).

Given that BIO-11006 can inhibit the above proinflammatory parameters, a possible explanation is that each of these effects can contribute to attenuation of neutrophil influx into the lung after LPS injury. For example, if the initial event is blocking NF-κB activation, this would, in turn, attenuate production of the potent neutrophil chemoattractant, KC, which would result in decreased neutrophil migration to the lung. This could occur on top of the direct effect of the
MARCKS-inhibitory peptide on neutrophil motility, further decreasing neutrophil influx. At this point, the exact contribution of each of these potential inhibitory events is not known, nor is there a known temporal sequence, but it could be that decreased chemotaxis/inflammation and direct inhibition of neutrophil motility both play a role in the ameliorating effects of BIO-11006 on LPS-induced inflammation in the lung. The exact contribution of each of these parameters is a topic of ongoing study.

To ascertain that the effects of aerosolized BIO-11006 were not restricted to LPS-induced lung injury, we also tested the effects of inhaled BIO-11006 in two other well characterized murine models of ALI: (1) intratracheal instillation of bacterial lipotetrahexide acid (LTA) (23); and (2) the bacterial pneumonia model with intranasal instillation of Streptococcus pneumoniae (24). Inhaled BIO-11006 inhibited neutrophil influx in both of these ALI models as efficiently as in the LPS-instilled mice (data not shown).

The results of this study clearly indicate that MARCKS protein is involved integrally in the pathogenesis of LPS-induced ALI/ARDS. Inhibition of MARCKS in ALI/ARDS with an inhaled N-terminal inhibitor could represent a potential pharmacological approach to treat patients with ALI/ARDS and other forms of ALI (e.g., smoke inhalation, trauma) characterized by neutrophil influx into the lung. The finding that the drug was effective even when administered fairly deeply into disease progression indicates that it may be useful for treating patients who present with the disease process already well underway. Clinical trials of BIO-11006 in patients with ARDS are being planned.

**Author disclosures** are available with the text of this article at www.atsjournals.org.

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**References**


