SB 239063, a p38 MAPK inhibitor, reduces neutrophilia, inflammatory cytokines, MMP-9, and fibrosis in lung

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Underwood, David C., Ruth R. Osborn, Steven Bochnowicz, Edward F. Webb, David J. Rieman, John C. Lee, Anne M. Romanic, Jerry L. Adams, Douglas W. P. Hay, and Don E. Griswold. SB 239063, a p38 MAPK inhibitor, reduces neutrophilia, inflammatory cytokines, MMP-9, and fibrosis in lung. Am J Physiol Lung Cell Mol Physiol 279: L895–L902, 2000.—The effects of a second generation p38 mitogen-activated protein kinase (MAPK) inhibitor, SB 239063 [trans-1-(4-hydroxycyclohexyl)-4-(4-fluorophenyl)-5-(2-methoxypyrimidin-4-yl)imidazole; IC50 = 44 nM vs. p38α], were assessed in models that represent different pathological aspects of chronic obstructive pulmonary disease (COPD) [airway neutrophilia, enhanced cytokine formation and increased matrix metalloproteinase (MMP)-9 activity] and in a model of lung fibrosis. Airway neutrophil infiltration and interleukin (IL)-6 levels, assessed by bronchoalveolar lavage fluid 48 h after lipopolysaccharide (LPS) inhalation, were inhibited dose dependently by 3–30 mg/kg of SB 239063 given orally twice a day. In addition, SB 239063 (30 mg/kg orally) attenuated IL-6 bronchoalveolar lavage fluid concentrations (>90% inhibition) and MMP-9 activity (64% inhibition) assessed 6 h after LPS exposure. In guinea pig cultured alveolar macrophages, SB 239063 inhibited LPS-induced IL-6 production (IC50 of 362 nM). In a bleomycin-induced pulmonary fibrosis model in rats, treatment with SB 239063 (2.4 or 4.8 mg/day via osmotic pump) significantly inhibited bleomycin-induced right ventricular hypertrophy (indicative of secondary pulmonary hypertension) and increases in lung hydroxyproline synthesis (indicative of collagen synthesis and fibrosis). Therefore, SB 239063 demonstrates activity against a range of sequelae commonly associated with COPD and fibrosis, supporting the therapeutic potential of p38 MAPK inhibitors such as SB 239063 in chronic airway disease.

Chronic obstructive pulmonary disease; interleukin-6; bleomycin; alveolar macrophage; mitogen-activated protein kinase; matrix metalloproteinase-9

Chronic obstructive pulmonary disease (COPD) is characterized by a chronic inflammatory process in the lung that includes 1) increased number of inflammatory cells (neutrophils, macrophages, and CD8+ T cells) in the airways and parenchyma, 2) increased inflammatory cytokine and chemokine expression, and 3) increased number of proteases [elastases, cathepsins, and matrix metalloproteinases (MMPs)] (31). Trafficking and activation of the neutrophil, believed to play a central role in the pathophysiology of COPD, results in the release of a number of inflammatory mediators and proteases, most importantly, neutrophil elastase, which contributes to the progressive fibrosis, airway stenosis, and destruction of the lung parenchyma that leads to an accelerated decline in airway function and presentation of cor pulmonale (11, 22). Neutrophil elastase is also a powerful mucus secretagogue and thus may contribute to the characteristic mucus hypersecretion that characterizes lung inflammation (11, 13).

The mitogen-activated protein kinase (MAPK) p38 is a ubiquitous and highly conserved, proline-directed serine/threonine protein kinase considered important in many processes that are critical to the inflammatory response and tissue remodeling (9, 19) that are hallmarksof pulmonary diseases such as COPD (1). Although a role for p38 kinase inhibitors in the treatment of pulmonary disease has only recently been postulated (2, 20), it is well known that inflammatory cytokines and chemokines are capable of regulating or supporting chronic airway inflammation (3). The production and action of many of the potential mediators of airway inflammation have been shown to be dependent on the stress-induced MAPK or p38 kinase cascade (7). Through the use of specific inhibitors, the potential role of this stress-induced kinase in airway disease is beginning to be studied. Several reports support the association of p38 kinase activation with a plethora of pulmonary events: lipopolysaccharide (LPS)- and tumor necrosis factor-α (TNF-α)-induced intercellular adhesion molecule-1 expression on pulmonary microvascular endothelial cells (28), MMP-9 activation (26), hypoxia-induced stimulation of pulmonary arterial cells (24), hyperosmolarity-induced IL-8 expression in...
bronchial epithelial cells (10), and enhanced eosinophil trafficking and survival (17, 29).

Taken together, these data suggest that there is considerable potential for a p38 kinase inhibitor in the treatment of inflammatory lung diseases. In this report, we describe the in vitro and in vivo pharmacology of a novel, potent, and selective inhibitor of p38 MAPK, SB 239063 [(trans-1-(4-hydroxycyclohexyl)-4-(4-fluorophenyl)-5-(2-methoxypyrimidin-4-yl)imidazole), including its effects in pulmonary disease models. The results support the contention that this class of compound may have promise in the treatment of chronic diseases of the airways.

METHODS

Inhibition of Cytokine Production in Human Whole Blood

Heparinized blood drawn from four normal volunteers was placed as 1-ml aliquots into Eppendorf tubes containing SB 239063 or vehicle and bacterial endotoxin (50 ng/ml) and tested in triplicate. The samples were incubated at 37°C for 4 h. Plasma was then collected by centrifugation in a microfuge at 10,000 rpm for 5 min at 4°C. Plasma content of various cytokines was determined by the appropriate ELISA assay for IL-6, IL-8, and IL-1 receptor agonist (IL-1ra; R&D Systems, Minneapolis, MN). ELISA assays for human IL-1 and TNF-α were developed in-house.

Animals

Male Lewis rats and male Hartley guinea pigs, obtained from Charles River Breeding Laboratories (Raleigh, MA), were maintained in a barrier facility. All experimental procedures conformed to Animal Care and Use Committee protocols filed at SmithKline Beecham Pharmaceuticals (King of Prussia, PA).

Guinea Pig Studies

Inhaled LPS-induced airway neutrophilia, IL-6 recovery, and MMP-9 activity. LPS exposure. Guinea pigs were placed four at a time into a 20-liter plastic box that had been modified with an intake and exhaust port; a small fan in the lid increased aerosol circulation. An aerosol of LPS (Sigma, St. Louis, MO) dissolved at 30 μg/ml in normal saline was generated by a modified DeVilbiss Pulmosonic nebulizer (DeVilbiss, Somerset, PA) and delivered into the box for 15 min via the intake port at a rate of 250 ml/min. For 6-h IL-6 and MMP-9 studies, LPS aerosol was generated by a DeVilbiss forced-air disposable nebulizer at a rate of 4 l/min, providing a more vigorous exposure to LPS.

SB 239063 (3, 10, or 30 mg/kg) or vehicle (acidified 0.5% tragacanth) was administered intragastrically via a size 8 French feeding tube 1 h before and 4 h after LPS challenge. For airway neutrophilia and IL-6 recovery experiments (48-h studies), SB 239063 or vehicle was administered twice, 6 h apart, on the day after LPS challenge. For MMP-9 and IL-6 recovery experiments (6-h studies), SB 239063 (30 mg/kg) or vehicle was administered 1 h before and 4 h after LPS challenge.

Bronchoalveolar lavage. For airway neutrophilia experiments, bronchoalveolar lavage (BAL) was performed 48 h after LPS exposure. Guinea pigs were euthanized with an overdose of pentobarbital sodium. The lungs were lavaged with 50 ml of Dulbecco’s PBS (5 × 10 ml), which was aspirated after a gentle chest massage. The BAL fluid was centrifuged, and the pellet was resuspended in 0.25% NaCl to lyse residual erythrocytes; after centrifugation, the pellet was resuspended again in 1 ml of 0.9% NaCl. After a total cell count, slides were prepared and stained, and the cells were differentiated as eosinophils, neutrophils, and mononuclear cells by counting a minimum of 200 cells and expressing the results as a percentage of total cells as well as expressing the actual number of each cell type. This measurement and expression technique has been previously validated by histological methodology as accurately reflecting endothelial and subendothelial airway leukocytosis (30).

IL-6 bioassay. IL-6 levels in BAL fluid or culture supernatants were determined with the IL-6-dependent B9 hybridoma cytotoxicity assay. Briefly, dilutions of test samples were incubated with B9 cells (5,000/well) in a 96-well microtiter plate for 72 h. A colorimetric readout (optical density (OD) at 630-nm frequency) of cell viability was generated after a 4-h incubation with PMS-XTT [phenazine methosulfate-2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; Sigma]. A human IL-6 standard curve was run on each plate to determine IL-6 levels in test samples. IL-6 results are measured as nanograms per milliliter for culture supernatants and expressed as total nanograms per lavage (i.e., value × dilution factor × BAL volume) for BAL fluid. Each sample was tested in triplicate.

MMP SDS-PAGE zymography. MMP enzyme expression was assayed by SDS-PAGE zymography with gelatin as the substrate. Equal volumes (2 μl) of BAL samples, without boiling or reduction, were subjected to electrophoresis through a 10% polyacrylamide gel copolymerized with gelatin (0.5 mg/ml) at 4°C. After electrophoresis was complete, the gel was incubated for 1 h at 25°C in a 2.5% Triton X-100 solution, washed two times (20 min each) with water, and then incubated overnight at 37°C in a 0.05 M Tris-HCl buffer, pH 8.0, containing 5 mM CaCl2. The gels were fixed with 40% methanol and 7% acetic acid, stained with 0.25% Coomassie brilliant blue R250, and then destained with 10% methanol and 7% acetic acid. Enzyme activity attributed to MMP-1, MMP-2, and MMP-9 could be visualized in the gelatin-containing zymograms as clear bands against a blue background. Standards for the recombinant active forms of MMP-2 and MMP-9 were included on the gels for comparison and identification. Recombinant MMP-2 and MMP-9 were purified from conditioned medium of HT-1080 human fibrosarcoma cells that were transfected with a cDNA for MMP-2 or MMP-9. Relative clearing of each sample was quantitated by determining the inverse OD units with the National Institutes of Health Image version 1.60 software package. Values are means ± SE expressed as inverse OD units relative to background.

Isolated alveolar macrophage experiments. Alveolar cells (>95% alveolar macrophages) were collected from two sets of four pentobarbital sodium-overdosed normal guinea pigs by BAL with 50 ml of Dulbecco’s PBS (5 × 10 ml), which was aspirated after a gentle chest massage. The pooled cells were centrifuged at low speed, washed twice in PBS, and resuspended in complete medium (MEM plus 10% FBS). The macrophages were plated in a 48-well multidish at a density of 5 × 104 cells/well and incubated overnight at 37°C. Cultures were washed and replenished with medium containing vehicle or various concentrations of SB 239063. The cultures were challenged with 0.001–10 ng/ml of LPS (for an initial LPS concentration-response determination), 1 ng/ml of LPS in the presence of vehicle, or 0.001–10 μM SB 239063 (for IC50 determination) and incubated for 24 h. Culture supernatants were collected and analyzed for IL-6 production as described in IL-6 bioassay.
**Rat Studies**

**LPS-induced TNF-α production.** To evaluate the ability of SB 239063 to function as a cytokine inhibitor in vivo, the ability of the compound to inhibit LPS-induced TNF-α in aged and weight-matched Lewis rats was examined. Various doses of SB 239063 were administered orally, followed 60 min later by a challenge with LPS (3.0 mg/kg ip; Sigma). For time-course studies, 15 mg/kg of SB 239063 were administered orally 1, 2, 3, or 4 h before LPS challenge. Heparinized whole blood was collected from each animal by cardiac puncture 90 min after the LPS injection. The blood samples were centrifuged, and the plasma was collected for analysis by ELISA for TNF-α levels.

TNF-α levels were measured with a sandwich ELISA (23) that used a hamster monoclonal anti-murine TNF-α (Genzyme, Cambridge, MA) as the capture antibody and a polyclonal rabbit anti-murine TNF-α (Genzyme) as the second antibody. For detection, a peroxidase-conjugated goat anti-rabbit antibody (Pierce, Rockford, IL) was added, followed by a substrate for peroxidase (1 mg/ml of orthophenylenediamine with 1% urea peroxide). TNF-α levels in the plasma samples from each animal were calculated from a standard curve generated with recombinant murine TNF-α (Genzyme).

**Bleomycin-induced pulmonary fibrosis.** **Bleomycin Administration.** Male Lewis rats (11–16 wk old, 250–350 g) were initially anesthetized in a 6-liter acrylic box with 4% isoflurane gas. The animals were removed from the chamber, shaved, and prepared with an ethanol and iodine solution, and connected to a nose cone delivering 4% isoflurane gas to maintain anesthesia level. The intact trachea was exposed with blunt dissection, and a 1-cm length of trachea was elevated and supported intact. Bleomycin (1 U; 300 µl of a 3.3 U/ml solution in sterile 9% saline) was administered with a 26-gauge needle and a 1-ml syringe and injected directly into the tracheal lumen. The incision in the skin of the neck was closed with two to three wound clips. On day 4, animals were euthanized with an intraperitoneal overdose of Fatal Plus and assessed for the development of pulmonary fibrosis.

**Osmotic Pump Implantation.** Immediately after the intratracheal instillation of bleomycin, osmotic pumps were implanted in the animals for continuous drug delivery over the 14-day period for development of pulmonary fibrosis. The intraperitoneal implantation of osmotic pumps (model 2ML2, Alzet, Palo Alto, CA) was performed after the surgical procedures outlined in the Alzet technical information manual. Sterile conditions were maintained to reduce the incidence of infection associated with surgery, and surgical instruments were sterilized with a hot bead sterilizer (Inotech Biosystems, Lansing, MI) between procedures on each animal. The implantation site was shaved and cleaned with alcohol and Betadine solution, and the animals were connected to a nose cone to maintain anesthesia. A 1- to 2-cm-long midline incision was made, and an osmotic pump was inserted into the intraperitoneal cavity. The musculoperitoneal layer was closed with a running 3-0 silk suture, and the cutaneous incision was closed with two to four wound clips. Animals were removed from the nose cone and returned to room air to recover for a minimum of 1 h; then they were returned to normal housing within the animal facility. SB 239063 or vehicle was released from the osmotic pumps immediately after implantation and began operating at a constant rate of 5.0 µl/h (0.12 ml/day) within 4–6 h for a maximum duration of 14 days.

**Drugs**

LPS from *Escherichia coli* serotype 055-85, bleomycin, DMSO, and PEG 400 were obtained from Sigma. TNF-α was

**Fig. 1.** Effects of SB 239063 on cytokine production from lipopoly saccharide (LPS)-stimulated human whole blood. IL, interleukin; IL-1ra, IL-1 receptor agonist; TNF, tumor necrosis factor. Heparinized whole blood (as described in METHODS) was incubated for 4 h with bacterial endotoxin in the presence and absence of SB 239063. Cytokine production was assayed by ELISA as described, and results are expressed as percent inhibition by SB 239063 (control response; without SE for clarity); n = 4 human subjects.
significant based on random probabilities of 5 in 100, 1 in
number and IL-6 recovered; stimulated with bacterial endotoxin, a concentration-

RESULTS

Statistical Analysis

As appropriate, Student’s t-test, ANOVA, or Fisher’s protected least significant difference test was used to determine significance, with \( P < 0.05 \), \( P < 0.01 \), or \( P < 0.001 \) considered significant based on random probabilities of 5 in 100, 1 in 100, and 1 in 1,000, respectively.

RESULTS

Inhibition of Cytokine Production in Human Whole Blood

As seen in Fig. 1, which shows results from the experiment in which human isolated whole blood was stimulated with bacterial endotoxin, a concentration-dependent inhibition of IL-1, TNF-\( \alpha \), IL-8, and IL-6 by SB 239063 was determined, with \( IC_{50} \) values from 0.02 to 0.09 \( \mu \)M, demonstrating the ability of SB 239063 to modify the production of a series of inflammatory cytokines. In contrast, weak inhibition of IL-1ra production was noted, with an \( IC_{50} \) of \( \sim 2 \) \( \mu \)M.

Guinea Pig Studies

Inhaled LPS-induced airway neutrophilia and IL-6 recovery. As seen in Fig. 2A and Table 1, LPS (30 \( \mu \)g/ml) administered as an aerosol produced a profound infiltration of leukocytes (10-fold compared with unexposed animals), especially neutrophils (>1,000-fold), 48 h after LPS exposure. A fivefold increase in mononuclear cells, predominantly alveolar macrophages, was also demonstrated (Table 1). The resultant airway neutrophilia was reduced in a dose-related fashion by administration of SB 239063 (\( P < 0.05 \) and \( P < 0.01 \) at oral doses of 10 and 30 mg/kg twice a day, respectively). Mononuclear cell numbers were also reduced (\( P < 0.05 \); Table 1). In addition, the concentration of IL-6 recovered in BAL fluid was strongly inhibited by SB 239063 administration (Fig. 2B); 3, 10, and 30 mg/kg twice a day produced significant inhibition (\( P < 0.05 \), \( P < 0.01 \), and \( P < 0.001 \), respectively), with almost complete normalization of IL-6 seen with oral administration of 30 mg/kg twice a day.

Inhaled LPS-induced enhancement of MMP-9 activity in airways. The activity of MMP-9 was clearly demonstrated by zymography of BAL fluid recovered 6 h after LPS challenge (Fig. 3A). Oral administration of SB 239063 (30 mg/kg) 1 h before and 4 h after LPS challenge produced a significant reduction in the protein expression and activity of MMP-9 (\( P < 0.01 \); Fig. 3B). In the same set of animals, slight but significant increases in MMP-2 activity were also demonstrated in the BAL fluid from LPS-exposed, vehicle-treated guinea pigs (1.05 \pm 0.003 OD units) relative to those in BAL fluid from unexposed animals (0.99 \pm 0.007 OD

Table 1. Effects of SB 239063 on leukocyte recovery in BAL fluid after LPS challenge

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total WBCs</th>
<th>Neutrophils</th>
<th>Mononuclear Cells</th>
<th>Eosinophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unexposed</td>
<td>10.0 \pm 2.2</td>
<td>0.04 \pm 0.03</td>
<td>9.51 \pm 1.97</td>
<td>0.50 \pm 0.26</td>
</tr>
<tr>
<td>LPS exposed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>110.5 \pm 8.5 ( \uparrow )</td>
<td>56.4 \pm 5.5 ( \uparrow )</td>
<td>49.9 \pm 4.5 ( \uparrow )</td>
<td>4.2 \pm 0.6</td>
</tr>
<tr>
<td>3mg/kg</td>
<td>112.9 \pm 7.3</td>
<td>60.0 \pm 5.3</td>
<td>49.9 \pm 2.4</td>
<td>2.9 \pm 1.0</td>
</tr>
<tr>
<td>10mg/kg</td>
<td>62.3 \pm 2.5 ( \uparrow )</td>
<td>29.1 \pm 6.4 ( \uparrow )</td>
<td>29.2 \pm 3.6 ( \uparrow )</td>
<td>4.0 \pm 1.2</td>
</tr>
<tr>
<td>30mg/kg</td>
<td>47.4 \pm 5.4 ( \uparrow )</td>
<td>19.8 \pm 3.2 ( \uparrow )</td>
<td>26.2 \pm 3.6 ( \uparrow )</td>
<td>1.4 \pm 0.4</td>
</tr>
</tbody>
</table>

Values are means \pm SE expressed as total cells recovered \( \times 10^6 \); \( n = 6-14 \) guinea pigs/treatment group. WBCs, white blood cells. Airway leukocytosis was assessed by bronchoalveolar lavage (BAL) performed 48 h after lipopolysaccharide (LPS; 30 \( \mu \)g/ml for 15-min challenge). Vehicle or SB 239063 was administered 1 h before and 4 h after LPS challenge. Significantly different from LPS-exposed, vehicle-treated guinea pigs: *\( P < 0.05 \); †\( P < 0.01 \); ‡Significantly different from unexposed, vehicle-treated guinea pigs, \( P < 0.05 \).
Rat Studies

LPS-induced TNF-α production. TNF-α production induced by LPS exposure in the peritoneal cavity of the rat was potently inhibited in a dose-related manner by oral administration of SB 239063 (1.875–15 mg/kg) with an ED₅₀ of 2.6 mg/kg (Table 2). In addition, in this model, SB 239063 produced significant inhibition of LPS-induced TNF-α production for >4 h, suggesting a long duration of action (Fig. 5).

Bleomycin-induced pulmonary fibrosis. The implantation of osmotic pumps delivering SB 239063 (2.4 and 4.8 mg/day) allowed for the continuous exposure of animals to SB 239063 over 14 days. As seen in Fig. 6A, bleomycin exposure (1 U; 300 μl of a 3.3 U/ml solution) induced an increase in right ventricular wall mass, which was significantly inhibited by 4.8 mg/day of SB 239063 (P < 0.05). Similarly, SB 239063 (4.8 mg/day)

units; P = 0.02; data not shown). The modest increase in MMP-2 activity was normalized in LPS-exposed animals that had been treated with SB 239063 (0.98 ± 0.007 OD units; P = 0.001 compared with the LPS-exposed, vehicle-treated group). In addition to increases in MMP-9 and MMP-2 activity, in the same animals, LPS exposure produced substantial increases in total lung lavage fluid levels of IL-6 (695.5 ± 191.6 ng) as well as increases in BAL fluid neutrophils (14.8 × 10⁶ ± 2.1 × 10⁶ cells in LPS-exposed vs. 0.09 × 10⁶ ± 0.08 × 10⁶ cells in unexposed animals). The levels of IL-6 (56.5 ± 8.6 ng) and neutrophils recovered by BAL (7.14 × 10⁶ ± 0.7 × 10⁶ cells) were significantly lower in the SB 239063-treated animals (P < 0.01; n = 4 guinea pigs; data not shown).

Inhibition of LPS-induced IL-6 production in isolated alveolar macrophages. As shown in Fig. 4A, LPS (0.001–10 ng/ml; EC₅₀ = 0.035 ng/ml) produced a concentration-dependent amount of IL-6 from alveolar macrophages. A maximal response to LPS was demonstrated at 1 ng/ml. The increase in IL-6 elicited by LPS (1 ng/ml) was potently inhibited by SB 239063 (IC₅₀ = 362 nM; n = 3–4 wells/treatment group; Fig. 4B).

![Fig. 3. Effects of SB 239063 on inhaled LPS-induced increases in matrix metalloproteinase (MMP)-9 activity in BAL fluid. Vehicle or SB 239063 (30 mg/kg) was administered orally 1 h before and 4 h post-LPS exposure (30 μg/ml for 30 min). A: zymogram of the gelatin gel of BAL fluid from unexposed and LPS-exposed (vehicle- or drug-treated) standards as well as from MMP-9 and MMP-2 standards. B: means ± SE of the scanned gels in LPS-exposed, vehicle- or SB 239063-treated guinea pigs relative to unexposed, vehicle-treated animals (n = 4/group). p.o., orally; OD, optical density. ** P < 0.01.](http://ajplung.physiology.org/)

![Fig. 4. Effects of SB 239063 on IL-6 production from LPS-stimulated guinea pig isolated alveolar macrophages. A: IL-6 production in response to LPS exposure for 24 h. Results are means ± SE. B: effects of SB 239063 on LPS (1 ng/ml)-induced IL-6 production. Values are means ± SE of triplicate samples for each concentration of SB 239063.](http://ajplung.physiology.org/)
significantly attenuated the increase in lung hydroxyproline content that was seen after exposure to bleomycin \( (P < 0.05; \text{Fig. 6B}) \).

DISCUSSION

The results of the present study clearly demonstrate the ability of the potent and selective p38 MAPK inhibitor SB 239063 to reduce proinflammatory cytokine production that leads to diminished neutrophil trafficking and activation in the lung. The major specific findings of this study include 1) inhibition of inflammatory cytokine production, including IL-1, TNF-\( \alpha \), IL-6, and IL-8, in human whole blood; 2) inhibition of LPS-induced airway neutrophil infiltration and IL-6 recovery from BAL fluid in guinea pig airways; 3) inhibition of MMP-9 activity recovered from inflamed guinea pig airways; 4) inhibition of IL-6 production by guinea pig isolated alveolar macrophages; 5) inhibition of peritoneal LPS-induced TNF-\( \alpha \) production in the rat; and 6) inhibition of bleomycin-induced pulmonary fibrosis in the rat.

Previous work in this laboratory (29) has shown the presence of phosphorylated p38 in guinea pig and rodent lung tissue. This observation, coupled with the implication of inflammatory cytokines such as TNF-\( \alpha \), IL-8, and IL-6 in airway inflammation, suggests that p38 MAPK inhibition would have an impact in models that reflect chronic lung diseases (3, 20). The data generated with human whole blood strongly support the contention that SB 239063, a potent p38 kinase inhibitor (17), can modify inflammatory cytokine production including TNF-\( \alpha \), IL-8, and IL-6. Of interest is the observation of the markedly reduced inhibitory activity of SB 239063 versus IL-1ra. This indicates the selectivity of SB 239063 and also suggests that a major

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**Table 2. Effect of SB 239063 on LPS-induced TNF-\( \alpha \) production in rats**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose, mg/kg</th>
<th>TNF-( \alpha ), pg/ml</th>
<th>%Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td></td>
<td>53,189 ± 1,375</td>
<td></td>
</tr>
<tr>
<td>SB 239063</td>
<td>1.875</td>
<td>30,573 ± 2,762</td>
<td>-43*</td>
</tr>
<tr>
<td></td>
<td>3.75</td>
<td>21,478 ± 1,544</td>
<td>-60*</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>15,271 ± 887</td>
<td>-71*</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>5,012 ± 839</td>
<td>-91*</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( n = 5–6 \) male Lewis rats pretreated orally with various doses of SB 239063 or vehicle (0.5% tragacanth). Ninety minutes later, heparinized blood was collected, and the plasma was analyzed for tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)) levels by ELISA. *Significant difference from vehicle, \( P < 0.001 \).
impact on IL-1-associated events might be anticipated because both inhibition of IL-1 production and a sparing of its natural antagonist appear to occur.

That cytokine inhibition is important in an in vivo setting was suggested by the ability of SB 239063 to inhibit LPS-induced airway neutrophil infiltration in the guinea pig. It is tempting to speculate that the cascade of events involves the inhibition of a chemotactic cytokine (e.g., IL-8) and that this accounts for the reduced number of neutrophils in the BAL fluid. In addition, there is evidence in recovered BAL fluid of an inhibition of IL-6; it is possible that a reduction of IL-6 concentrations in the lung removes a potential stimulus for neutrophil activation, including enhancement of adhesion (12) and elastase release (15). That this results from a direct inhibition by SB 239063 of IL-6 production by alveolar macrophages is supported by the in vitro results of experiments in these cells. IL-6 appears to play a major role as judged by recent data in IL-6 knockout mice (5), which suggest an important role for IL-6 in mediation of the inflammatory response. In addition, the results showing the ability of IL-6 to activate and degranulate neutrophils (25) provides an explanation for the recovery of the proteolytic enzyme MMP-9 in BAL fluid in the present experiments and its subsequent inhibition by SB 239063.

Human alveolar macrophages produce several MMPs including gelatinase B (MMP-9), macrophage elastase (MMP-12), and collagenase 1 (MMP-1), as well as elastolytic cysteine proteinases including cathepsins K, L, and S (24). Because the neutrophil also provides a readily releasable source of MMP-9 from its tertiary granules (21), there may be an important role for this proteinase in the elastolytic degradation of the lung in response to inhaled pathogens. Reduction of the activity of MMP-9 by SB 239063 may occur via the attenuation of cell-related inflammation through inhibition of chemotaxis and activation in the airways after LPS inhalation. The recent demonstration (16) of the importance of MMP-2 activity in airway smooth muscle proliferation suggests that inhibition of this protease may be beneficial in airway disease. Although the increases in MMP-2 activity in this acute model were modest compared with the enhanced MMP-9 activity, it is interesting to speculate whether MMP-2 inhibition coupled with the recently demonstrated airway eosinophilia-reducing capacity of SB 239063 may work in tandem to indicate a strong rationale for the use of p38 MAPK inhibitors in chronic airway disease, including asthma (29).

SB 239063 also had a strong inhibitory effect on inflammatory cytokine production in the rat. LPS-induced TNF-α production was inhibited in a dose-related fashion and served to establish the activity of this p38 kinase inhibitor in the rat. In addition, the responses associated with chronic fibrosis induced by bleomycin were also altered by SB 239063 administered via an osmotic pump. As a biochemical readout for the architectural changes occurring in this model, hydroxyproline levels in the tissue were measured. The striking increase in hydroxyproline levels after bleomycin was significantly normalized by SB 239063. Perhaps more importantly, one of the measures of the physiological consequences of the fibrosis, i.e., right ventricular hypertrophy, was attenuated by SB 239063. It is very encouraging that this complex pulmonary pathology (27) was modified by treatment with SB 239063. Among the mediators that have been linked to airway fibrosis because of their ability to regulate fibroblast proliferation and matrix production (6), IL-1, TNF-α, and IL-6 were potently inhibited by SB 239063. Because stress-activated protein kinases have been implicated in hypertrophic responses in the heart based on earlier generation kinase inhibitors, the need for more selective tools has been recognized (8). SB 239063 is a potent and selective inhibitor that displays specific and high-affinity binding to p38 MAPK, resulting in potent inhibition of its catalytic activity (ID₅₀ = 44 nM). Because p38 MAPK exists as four distinct isoforms (α, β, γ, and δ), SB 239063 exhibits equipotent inhibitory activity against the α- and β-isoforms and no activity (up to 100 μM) against the γ- and δ-kinase isoforms. A panel of closely related kinases, including lipid kinases, tyrosine kinases, Erk, and c-Jun NH₂-terminal protein kinase (JNK)-1, were not inhibited by SB 239063 in concentrations up to 10 μM (29). In addition, SB 239063 appears to be more selective than a previous generation of p38 MAPK inhibitors such as SB 203580, which also inhibited c-Raf (IC₅₀ = 360 nM) (20). It is not known whether the attenuation of right ventricular hypertrophy occurs directly or as a consequence of a reduction in pulmonary vascular resistance compared with that in vehicle-treated, bleomycin-exposed animals. From transgenic overexpression modeling experiments, a relationship linking the IL-6-type cytokines with airway fibrosis, including myocyte and myofibroblast abnormalities, has become evident (6). Although airway hyperresponsiveness was not demonstrated in a transgenic murine model of increased expression of IL-6 (6), further demonstration of an emphysematous pathophysiology in these animals makes it interesting to speculate on the existence of a multifactorial relationship of this cytokine to COPD (18). In addition, fibrosis has not been considered a typical finding in the pathology associated with COPD per se, and a recent review (14) addresses a controversy regarding their relationship.

Taken together, these results suggest the possible therapeutic value of p38 MAPK inhibitors such as SB 239063 in the treatment of pulmonary inflammatory diseases.

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