

Novel Tight-Binding Inhibitory Factor- κ B Kinase (IKK-2) Inhibitors Demonstrate Target-Specific Anti-Inflammatory Activities in Cellular Assays and following Oral and Local Delivery in an in Vivo Model of Airway Inflammation

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ABSTRACT

Nuclear factor- κ B (NF- κ B) is one of the major families of transcription factors activated during the inflammatory response in asthma and chronic obstructive pulmonary disease. Inhibitory factor- κ B kinase 2 (IKK-2) has been shown to play a pivotal role in cytokine-induced NF- κ B activation in airway epithelium and in disease-relevant cells. Nevertheless, the potential toxicity of specific IKK-2 inhibitors may be unacceptable for oral delivery in chronic obstructive pulmonary disease. Therefore, local delivery to the lungs is an attractive alternative that warrants further exploration. Here, we describe potent and selective small-molecule IKK-2 inhibitors [8-(5-chloro-2-(4-methylpiperazin-1-yl)isonicotinamido)-1-(4-fluorophenyl)-4,5-dihydro-1*H*-benzo[g]indazole-3-carboxamide (PHA-408) and 8-(2-(3,4-bis(hydroxymethyl)-3,4-dimethylpyrrolidin-1-yl)-5-chloroisonicotinamido)-1-(4-fluorophenyl)-4,5-dihydro-1*H*-benzo[g]indazole-3-carboxamide (PF-184)] that are competitive for ATP have slow off-rates from IKK-2 and display broad in vitro anti-inflammatory activities resulting from NF- κ B pathway

inhibition. Notably, PF-184 has been designed to have high systemic clearance, which limits systemic exposure and maximizes the effects locally in the airways. We used an inhaled lipopolysaccharide-induced rat model of neutrophilia to address whether inhibiting NF- κ B activation locally within the airways would show anti-inflammatory effects in the absence of systemic exposure. PHA-408, a low-clearance compound previously shown to be efficacious orally in a rodent model of arthritis, dose-dependently attenuated inhaled lipopolysaccharide-induced cell infiltration and cytokine production. Interestingly, PF-184 produced comparable dose-dependent anti-inflammatory activity by intratracheal administration and was as efficacious as intratracheally administered fluticasone propionate (fluticasone). Together, these results support the potential therapeutic utility of IKK-2 inhibition in inflammatory pulmonary diseases and demonstrate anti-inflammatory efficacy of an inhaled IKK-2 inhibitor in a rat airway model of neutrophilia.

Chronic obstructive pulmonary disease (COPD) is a multifactorial chronic inflammatory disease involving the activa-

tion of many different types of inflammatory cells, including macrophages, neutrophils, lymphocytes, eosinophils, and structural cells, in the airways (Barnes, 2004b). The disease is mainly characterized by an oxidative stress (e.g., cigarette smoking)-induced pulmonary inflammation, which results in

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ABBREVIATIONS: COPD, chronic obstructive pulmonary disease; PHA-408, 8-(5-chloro-2-(4-methylpiperazin-1-yl)isonicotinamido)-1-(4-fluorophenyl)-4,5-dihydro-1*H*-benzo[g]indazole-3-carboxamide; PF-184, 8-(2-(3,4-bis(hydroxymethyl)-3,4-dimethylpyrrolidin-1-yl)-5-chloroisonicotinamido)-1-(4-fluorophenyl)-4,5-dihydro-1*H*-benzo[g]indazole-3-carboxamide; NF- κ B, nuclear factor- κ B; I κ B, inhibitory factor- κ B; IKK, I κ B kinase; LPS, lipopolysaccharide; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; TNF- α , tumor necrosis factor- α ; MIP, macrophage inflammation protein; Gro- α , growth-related oncogene- α ; MCP-1, monocyte chemoattractant protein-1; PMN, polymorphonuclear; PBMC, peripheral blood mononuclear cell; DMEM, Dulbecco's modified Eagle's medium; BAL, bronchial alveolar lavage; IT, intratracheal; rh, recombinant human; RASF, rheumatoid arthritis synovial fibroblast; SC-514, 3-amino-5-(thiophen-3-yl)thiophene-2-carboxamide; CSC, cigarette smoke condensate; FBS, fetal bovine serum; GM-CSF, granulocyte-macrophage colony-stimulating factor; ANOVA, analysis of variance; PGE₂, prostaglandin E₂; PHA-357, 8-(5-chloro-2-(4-methylpiperazin-1-yl)isonicotinamido)-1-(pyridin-4-yl)-4,5-dihydro-1*H*-benzo[g]indazole-3-carboxamide; UK-436303, *N*-(5-methyl-1*H*-pyrazol-3-yl)-2-(2-methylpyrrolidin-1-yl)pyrido[2,3-*d*]pyrimidin-4-amine; BAY-117082, (E)-3-[(4-methylphenylsulfonyl)-2-propenenitrile].

a progressive decline in lung function. Standard corticosteroid treatments have limited efficacy and untoward effects (Pauwels et al., 2001; Barnes and Hansel, 2004) that substantiate the need for novel anti-inflammatory strategies.

The NF- κ B pathway is activated during the inflammatory response in asthma and COPD (Adcock et al., 2006). It is activated by many inflammatory stimuli and, in turn, regulates the expression of proinflammatory cytokines, chemokines, enzymes, and cell adhesion molecules that drive disease pathology (Balwin, 2001; Bonizzi and Karin, 2004). Increased NF- κ B expression and p65 nuclear localization have been demonstrated in bronchial biopsies from smokers and COPD patients and in sputum alveolar macrophages during COPD exacerbations (Di Stefano et al., 2002; Caramori et al., 2003). Furthermore, the indispensable role of NF- κ B in airway inflammation has been substantiated in several preclinical models using a variety of approaches. For example, I κ B super-repressor transgenic mice demonstrate an impaired cell infiltration and airway inflammation in response to LPS challenge (Poynter et al., 2003; Skerrett et al., 2004). NF- κ B "decoy" oligonucleotide treatment resulted in a significant reduction of LPS-induced lung injury compared with control animals (Matsuda et al., 2005). The proinflammatory consequence of IKK-2 activation has also been shown in preclinical models using small-molecule IKK-2 inhibitors and adenoviral delivery of dominant-negative IKK-2, IKK-1, and dominant I κ B α (I κ B α Δ N) (Birrell et al., 2005a, 2006; Catley et al., 2006; Everhart et al., 2006; Newton et al., 2007).

The hallmark of COPD is characterized by dramatically increased numbers of activated neutrophils in sputum and bronchial alveolar lavage (BAL) fluid (Lacoste et al., 1993; Keatings and Barnes, 1997). In fact, neutrophil numbers in bronchial biopsies and induced sputum are correlated with COPD disease severity (Di Stefano et al., 1998). Activated macrophages drive the inflammatory process by recruiting neutrophils via the release of neutrophil chemotactic factors, including interleukin-8 (IL-8), growth-related oncogene- α (Gro- α), IL-1 β , and tumor necrosis factor- α (TNF- α) (Barnes, 2004a). Administration of endotoxin to the airways leads to an NF- κ B-dependent macrophage activation and neutrophil recruitment (Corteling et al., 2002). This acute model has been used to evaluate clinical therapies such as salmeterol (Maris et al., 2005) and new targets, including p38 mitogen-activated protein kinase (Branger et al., 2002). Unlike COPD where corticosteroids are ineffective in a significant number of patients, fluticasone and budesonide are fully efficacious after inhalation delivery in human and rodent models of LPS-induced neutrophilia (Alexis et al., 2001; Birrell et al., 2005) and can be used to benchmark novel anti-inflammatory agents. To our knowledge, a comparison of oral versus IT delivery of potent small molecule IKK-2 inhibitors with IT administration of the gold standard, fluticasone, has not been described.

In the present study, we characterize two novel potent and selective IKK-2 inhibitors that bind tightly to IKK-2, show broad anti-inflammatory activities *in vitro*, and are fully efficacious in an *in vitro* model of oxidative stress. Furthermore, utilizing an aerosolized LPS model of neutrophilia in the rat, we demonstrate that airway inflammation is inhibited through an IKK-2 mechanism by local delivery to the lung and is comparable with orally active IKK-2 inhibitors.

Interestingly, the anti-inflammatory efficacy following IT administration of the IKK-2 inhibitor appeared to be driven by local activity in the lungs because there was no inhibition of LPS-induced TNF- α production in an *ex vivo* whole-blood assay. In contrast, inhaled fluticasone blocked *ex vivo* LPS challenges in whole blood and in lung-derived BAL cells equally. The combination of data from the reported *in vitro* and *in vivo* studies provides a strong rationale for using an IKK-2 anti-inflammatory inhibitor approach for airway diseases that may also be suitable for inhaled delivery.

Materials and Methods

Compounds. PHA-408 (D. F. Bonafoux, S. L. Bonar, M. Clare, A. M. Donnelly, J. L. Glaenger, J. A. Guzova, H. Huang, N. N. Kishore, F. J. Koszyk, P. J. Lennon, et al., manuscript in preparation describing the synthesis procedure), PF-184, and the reversible analog PHA-357 were synthesized at Pfizer, Inc. (St. Louis, MO). For *in vitro* assays, compounds were dissolved in Me₂SO and stored at -20°C in 50 mM aliquots. Fluticasone Propionate (Sequoia Research Products, Oxford, UK) was reconstituted in 100% Me₂SO. For all cell assays, compounds were preincubated with cells for 1 h before treatment in a final Me₂SO concentration of 0.2%.

Reagents. The following agents were purchased from Sigma-Aldrich (St. Louis, MO): LPS, EDTA, methylcellulose, Tween 20, bovine serum albumin, sodium azide, Ficoll histopaque-1077, and phosphatase inhibitor cocktail. Tris-buffered saline, DMEM, Dulbecco's phosphate-buffered saline, penicillin-streptomycin, nitrocellulose, and Tris-glycine acrylamide gels were obtained from Invitrogen (Carlsbad, CA). Electroplated 96-well plates for pI κ B and cytokine ELISAs, blocking buffer, sulfo-tagged antibodies, read buffer, and the Sector Imager 6000 were obtained from Meso Scale Discovery (Gaithersburg, MD). IL-1 β from R&D Systems (Minneapolis, MN). All other reagents used were of the highest grade commercially available.

Endogenous IKK-2 Kinase and rhIKK-2 Activities. Phospho-I κ B production from rhIKK-2 and from cell and lung lysates was quantitated by electrochemiluminescence. Electroplated 96-well plates were coated with anti-phospho-I κ B α (Santa Cruz Biochemicals, Santa Cruz, CA) at a concentration of 5 pmol/well. All kinase assays were performed in a 30- μ l total volume for 90 min in kinase buffer containing 25 mM HEPES, 10 mM NaF, 0.1% bovine serum albumin, 0.0005% Triton X-100, 5 mM MnCl₂, 5 mM MgCl₂, 1 mM dithiothreitol, and 2% Me₂SO, pH 7.5. For rhIKK-2 activity, inhibitor and rhIKK-2 (0.1 nmol/well) were added simultaneously to plates with substrate (1 μ M ATP, 2 μ M biotinylated I κ B α Ser32-Ala36 peptide). For endogenous IKK complex activity determination, 80 μ g of tissue lysates or 10 μ g of cell lysates were incubated with ATP (1 μ M) and biotin-labeled I κ B peptide (5 μ M). For all assays, the reaction was stopped by washing the plates twice with Tris-buffered saline/Tween 20. Electrochemiluminescence was generated by adding sulfo-tagged streptavidin (25 μ l/well) for 1 h and following the manufacturer's (Meso Scale Discovery) instructions. Phospho-I κ B α levels were quantitated with use of a Sector Imager 6000 (Meso Scale Discovery). Specific IKK kinase was determined by subtracting values from a no-peptide control.

The dissociation rate constants of PHA-408 and PF-184 from rhIKK-2 were determined by monitoring the recovery of IKK-2 kinase activity over time as described in Mbalaviele et al. (2009). In brief, the kinase activity of rhIKK-2 was determined by monitoring the phosphorylation of 5FAM-GRHDSGLDSMK (American Peptide Co., Inc., Sunnyvale, CA) with use of the LabChip 3000 (Caliper Life Sciences, Hopkinton, MA). PHA-408 or PF-184 was preincubated (200 nM) with 100 nM rhIKK-2 for 2 h at room temperature. The preincubation mixture was diluted 100-fold into an assay mixture containing 100 μ M MgATP (100 $\times$ K_m) and 2.0 μ M 5FAM-GRHDS-

GLDSMK. The production of the phosphorylated 5FAM-GRHDS-GLDSMK was monitored for 6 h.

Human Liver Microsomal Stability. PF-184 and PHA-408 (1 μ M) were incubated in human liver microsomes (0.8 mg/ml) with the addition of a NADPH-regenerating system (1 mM NADP⁺, 5 mM isocitric acid, 1 unit/ml isocitric acid dehydrogenase) in 100 mM potassium phosphate buffer (pH 7.4) for 30 min. Acetonitrile with internal standard was added to stop the reaction. The reaction mixture was centrifuged, and supernatant was analyzed for test compound by liquid chromatography coupled to tandem mass spectrometry.

Rat Intravenous and Oral PK. PF-184 and PHA-408 in the formulation of ethanol/polyethylene glycol 400/phosphate-buffered saline (8:76:76) were dosed to male Sprague-Dawley rats intravenously at 2 mg/kg and orally at 5 mg/kg. Blood was drawn at 5, 15, and 30 min and 1, 2, 4, 6, 8, 12, 16, 20, and 24 h. Acetonitrile with internal standard was added to precipitate plasma protein, and samples were centrifuged. Supernatants were analyzed by liquid chromatography coupled to tandem mass spectrometry for test compound.

Primary Cell Culture. Alveolar macrophages from BAL cells were plated in 96-well plates at a density of 3×10^5 /well in DMEM containing 5% FBS (Hyclone Laboratories, Logan, UT) and were either stimulated with 10 μ g/ml LPS or untreated for 16 h. Cells were pretreated with increasing concentrations of PHA-408 or fluticasone for 1 h before 16-h stimulation with LPS for cytokine analysis or 1-h stimulation for p65 translocation. Human whole blood was collected from healthy donors in sodium-heparinized tubes (BD, Franklin Lakes, NJ). Neutrophils or PBMC were isolated by Ficoll separation. Cells were resuspended in RPMI 1640 medium with penicillin-streptomycin (10 U/ml) and 5% FBS and plated into 96-well culture plates at 2.5×10^5 cells/well. Compounds were added to the cells and incubated for 1 h before a 16-h LPS (20 ng/ml) stimulation. Human A549 lung epithelial cells and human endothelial cells were grown following the manufacturer's instructions (American Type Culture Collection, Manassas, VA) and rheumatoid arthritic synovial fibroblasts (RASf) were grown as described previously (Mbalaviele et al., 2009). Cells were treated with compounds in fresh medium containing 1% FBS 1 h before stimulation with 5 ng/ml IL-1 β for 15 min or 18 h for native kinase assessment from lysates or mediator production, respectively. Conditioned medium was collected from in vitro assays and stored at -80°C before cytokine determinations.

Cell Duration of Action Assay. A549 cells or PBMCs were plated and pretreated with inhibitors as described above. After a 1-h compound incubation, plates were either immediately stimulated with human IL-1 β (A549) or LPS (PBMC) or were washed twice with phosphate-buffered saline and placed in a CO₂ incubator in the absence of inhibitor for the respective times before stimulation and cell lysis. For IL-8 production, RASf were either washed or not washed and incubated in the absence of inhibitor for 4 h before an 18-h IL-1 β stimulation.

In Vitro Oxidative Stress Assay. Cigarette smoke condensate (CSC) (Murty Pharmaceuticals, Lexington, KY) was prepared by smoking University of Kentucky's 1R3F standard research cigarettes on an Federal Trade Commission smoke machine and calculating the total particulate matter as weight gained on the filter. Me₂SO was used to extract the particulate matter by sonication at an estimated volume to yield a 4% (40 mg/ml) mass/volume solution. Aliquots were stored in dark vials at -80°C . PBMC were plated in 96-well plates as described above and treated with 20 μ g/ml CSC or 0.8% Me₂SO for 4 h at 37°C with 5% CO₂. After 4 h, both plates of cells were incubated for 1 h with PHA-408, PF-184, or fluticasone, without removing CSC. Cells in both plates were then stimulated with 1 ng/ml IL-1 β for 16 h.

Cytokine Assays. A rat 24-plex cytokine/chemokine immunoassay panel was used to profile BAL fluid cytokines (Millipore Corporation, Billerica, MA) following the manufacturer's instructions. The cytokine levels were quantitated by use of a Luminex¹⁰⁰ System

(Luminex Corporation, Austin, TX). Human cytokine, chemokine, and adhesion molecule levels were determined by use of electroplated 96-well plates that had been custom coated with multiple anti-human antibodies. The 100% control value was defined in the presence of LPS or IL-1 β stimulation and the 0% control reflected basal release. EC₅₀ values were generated with GraFit data analysis software (Erihtacus Software, Horley, Surrey, UK).

Animal Model. The Pfizer Institutional Animal Care and Use Committee reviewed and approved the animal use in these studies. The animal care and use program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. Fasted male Sprague-Dawley rats (350 g) were placed into a chamber connected to a Devilbiss UltraNebBlue 100 large volume nebulizer (Sunrise Medical, Longmont, CO) filled with 20 ml of 1 mg/ml solution of LPS (*Escherichia coli* serotype 0111:B4). At the indicated times after LPS aerosol challenge, rats were terminally anesthetized and bled to collect plasma. A total of 10 ml [4×2.5 ml] of Ca²⁺/Mg²⁺-free Dulbecco's phosphate-buffered saline containing 2.6 mM EDTA was instilled into the lung and recovered. After lavage, lung tissue was removed, immediately frozen in liquid nitrogen, and stored at -80°C . For oral delivery, PHA-408 was prepared as a suspension in a vehicle consisting of 0.5% methylcellulose and 0.025% Tween 20 and administered by oral gavage in a volume of 1 ml. For IT delivery, PF-184 or fluticasone was prepared as a nano-suspension and administered intratracheally in a volume of 100 μ l. To make a nanosuspension formulation, a bench scale wet milling (micronization) device was used with an appropriate amount of glass beads and Tween 80 0.5% (w/w) in phosphate-buffered saline (pH 7.4). Particle size distribution was determined on a LS 230 particle size analyzer using the small volume accessory (Beckman Coulter, Fullerton, CA). BAL cells were analyzed, following red cell lysis, with use of a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) equipped with a 96-well sampler that also determined the absolute cell count (cells per microliter; Cytex Development, Fremont, CA). The cytometry-based cell count was validated against a Coulter Z2 cell counter (Beckman Coulter). For in vitro experiments, alveolar macrophages were plated at 5×10^5 /well in 96-well plates for LPS 10 μ g/ml challenge. For ex vivo LPS challenge studies, whole blood from each animal was plated in triplicate in 96-well plates (175 μ l/well) and stimulated with LPS (10 μ g/ml) for 16 h. The plasma was collected after centrifugation to measure TNF- α production.

NF- κ B p65 Immunocytochemistry/Immunohistochemistry. Alveolar macrophages were plated in 96-well plates and stained for p65 with use of an NF- κ B activation kit (Cellomics; Thermo Fisher Scientific, Waltham, MA) following the manufacturer's instructions. p65 nuclear translocation was quantified with use of an ArrayScan imager. In brief, cells were fixed in 5% buffered formalin and permeabilized with 1% Triton X-100. A 4,6-diamidino-2-phenylindole-2-HCl stain was used to define the nucleus, and a fluorescein isothiocyanate was labeled secondary for p65 localization. For in vivo experiments, rat BAL cells were fixed with 10% buffered formalin overnight at a concentration of 10^6 cells/ml formalin. Cytospins slides were exposed to 3% hydrogen peroxide for 15 min, blocked 10 min with Innovex FC blocker, and treated 30 min with 1:100 anti-p65 (Santa Cruz Biochemicals) or 1:50 Dako IgG1 isotype control antibody. The p65 staining was developed with Vector Elite ABC (Vector Laboratories, Burlingame, CA) followed by treatment with a 3,3'-diaminobenzidine-positive chromogen substrate (Vector Laboratories). Hematoxylin was used as a counterstain. All slides were evaluated under 600 $\times$ magnification. Three randomly selected fields of each slide were counted, and the total number of cells was used for final percentage calculations (positive versus total cell counts). The intensity of nuclear or cytoplasmic staining was scored with the following criteria: 0 = no stain; 1 = minimum to mild stain; 2 = mild to moderate stain; 3 = moderate to marked stain; and 4 = marked stain. To ensure the accuracy, the cells with equivocal staining were excluded.

Western Blotting and p65 DNA Binding Assay. Tissues lysates and the detection of proteins by Western blot analysis was performed by use of anti-rabbit IKK-2, anti-rabbit p65, or anti-mouse total I κ B α antibodies (Santa Cruz Biochemicals) as described previously (Mbalaviele et al., 2009). The generation of nuclear lysates from frozen powdered lung and specific p65 DNA binding was performed with a kit available from Active Motif Inc. (Carlsbad, CA). In brief, 10 μ g of nuclear lysate was added to the oligo-coated plates for 1 h after subsequent additions of anti-p65 and horseradish peroxidase-labeled secondary antibodies. A wild-type oligonucleotide was added to a set of the nuclear lysates to determine specific p65 DNA binding.

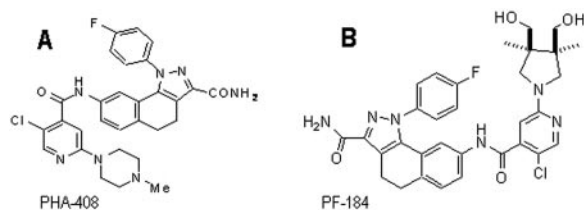
Statistical Analysis. All values are expressed as means plus or minus the standard deviation (S.D.) or standard error of the mean (S.E.M.). Statistical significance was assessed by use of one-way ANOVA (*, $p < 0.01$; **, $p < 0.001$) for comparison with the control group.

Results

PHA-408 and PF-184 Are Potent Tight-Binding Inhibitors of IKK-2 with Distinct Metabolism. PHA-408 (Fig. 1A) and PF-184 (Fig. 1B) were identified from a tricyclic pyrazole chemical series with potent activity against rhIKK-2. PHA-408 was described recently as a highly selective IKK-2 inhibitor with 350-fold selectivity over rhIKK-1 and no inhibitory activity against its closely related members, Tank-binding kinase 1, IKKi, and a panel of more than

30 tyrosine and serine/threonine kinases (Mbalaviele et al., 2009). On further enzyme characterization, PHA-408 showed competitive inhibition with respect to the ATP site ($K_i = 6$ nM) and was described as a tight-binding inhibitor with relatively slow dissociation kinetics with off rate (k_{off}) of approximately 0.36 ± 0.002 h $^{-1}$ (Mbalaviele et al., 2009). In arthritis-relevant cells, PHA-408 blocked agonist-induced I κ B- α phosphorylation and degradation, p65 phosphorylation, and DNA binding activity, while sparing p38 and *c-jun*-N-terminal kinase, mitogen-activated protein kinase pathways, demonstrating a selective IKK-2 biochemical mechanism of action (Mbalaviele et al., 2009). Oral administration of PHA-408 in the rat demonstrated a reasonably good bioavailability (50%), intravenous clearance rate (11.5 ml/min/kg), and low P450 metabolism (66% remaining after incubation with human liver microsomes) suitable for use as an oral tool inhibitor (Fig. 1). PF-184 was identified subsequent to PHA-408 and shared similar enzyme potency (37 nM) and slow dissociation kinetics with a $T_{1/2}$ of 6.7 h from rhIKK-2. Like PHA-408, PF-184 (up to 30 μ M) was very selective against more than 85 other kinases having $<50\%$ inhibition of all of the targets tested. In contrast to PHA-408, PF-184 had very low oral bioavailability (5%), high intravenous clearance (59 ml/min/kg), and high P450 metabolism in human liver microsomes (Fig. 1). The in vitro biochemical properties of PF-184 combined with poor oral bioavailability make it a potential candidate for local delivery to the lung.

PHA-408 and PF-184 Bind Tightly to Endogenous IKK-2 and Show Extended Inhibition of Kinase Activity and Cytokine Production. Based on the relatively slow K_{off} values (0.36 – 0.011) h $^{-1}$ and long $T_{1/2}$ of PHA-408 and PF-184 (2–6.7 h) from rhIKK-2 in Fig. 1, we reasoned that these inhibitors might also bind endogenous kinase for extended periods of time. To test this idea, lung epithelial cells (A549 cells) were pretreated for 1 h with varying concentrations of PHA-408 or a structurally similar reversible analog and subjected to multiple wash steps. After a 4-h incubation without inhibitor, cells were stimulated with IL-1 β for kinase activity measurement. The data show that washing had no effect on the potency of PHA-408 to inhibit endogenous IKK-2 activity (Fig. 2A) and demonstrate that it is a tight-binding inhibitor of IKK-2 in the cell. In contrast, this washing procedure completely abolished the potency of a reversible analog in inhibiting IKK-2 activity (Fig. 2A). PF-184 also demonstrated a similar inhibitory profile after successive washes of LPS-stimulated PBMC kinase activation (Fig. 2B), thus validating the tight-binding nature of both compounds. To further understand the duration of activity we repeated the experiment in A549 cells with much longer intervals between washing and IL-1 β stimulation. When IC_{50} values for the inhibition of kinase activity were compared, there was only a gradual increase from 17 to 100 nM when the washing interval increased from 0 to 12 h (Fig. 2C). These data support a long-lasting, tight-binding property of these inhibitors to resting and activated forms of the endogenous kinase. Finally, we wanted to assess a functional readout such as IL-8 production to validate the downstream consequence of extended kinase inhibition after compound washout. Synovial fibroblast cells were used for these experiments due to the rapid growth cycle of A549 cells to avoid complicating the results of these longer (22 h) experiments. Fibroblasts were pretreated with PHA-408 for 1 h and washed twice with



In Vitro Biochemistry	PHA-408	PF-184
IC ₅₀ (nM)	40 $\pm$ 2	37 $\pm$ 3
K _{off} (h ⁻¹)	0.36 $\pm$ 0.002	0.011 $\pm$ 0.002
T _{1/2} (h)	2.2	6.7
Microsomal Clearance (% remaining)	66 $\pm$ 32	1 $\pm$ 1
Rat i.v. CL (ml/min/kg)	11 $\pm$ 2	59 $\pm$ 6
Rat i.v. T _{1/2} (h)	2.9 $\pm$ 0.5	1.0 $\pm$ 6
C _{max} (μ g/ml)	1.0 $\pm$ 0.4 (10 mg/kg)	0.08 $\pm$ 0.02 (5 mg/kg)
Oral Bioavailability (%)	60 $\pm$ 7	10 $\pm$ 6

Fig. 1. PHA-408 and PF-184 are potent tight-binding inhibitors of IKK-2 with distinct metabolism. A and B, chemical structures of PHA-408 and PF-184. In vitro biochemistry parameters. rhIKK-2 (0.1 nmol/well) was incubated with varying concentrations of PHA-408 or PF-184 in the presence of ATP (1 μ M) and biotinylated I κ B α (2 μ M). Inhibition of kinase activity was assessed as described under *Materials and Methods*. IC_{50} data are expressed as a mean $\pm$ S.D. of more than three experiments. Dissociation kinetics of PHA-408 and PF-184 from rhIKK-2 are described under *Materials and Methods*. Human liver microsomal clearance, rat intravenous clearance, and rat oral bioavailability were determined as described under *Materials and Methods*. Data are expressed as a mean $\pm$ S.D. from three rats (PK) or three independent experiments (microsomal clearance, $T_{1/2}$) or mean $\pm$ range from two rats (BA) or a representative from three independent experiments (K_{off}).

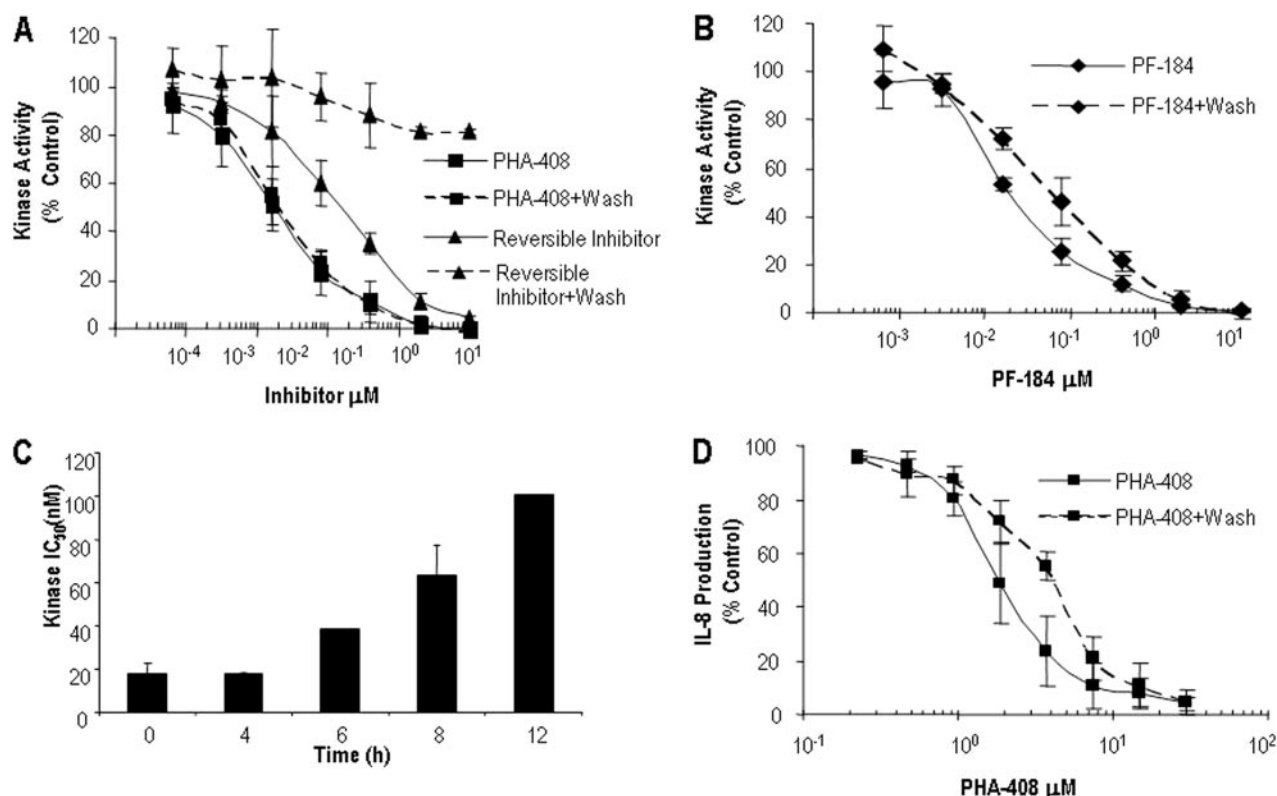


Fig. 2. PHA-408 and PF-184 bind tightly to endogenous IKK-2 and show extended inhibition of kinase activity and cytokine production. A and B, concentration response curves for the inhibition of endogenous IKK-2 activity. A, A549 cells were preincubated with PHA-408 (10^{-4} – 10^1 μM) (squares) or a reversible analog from the PHA-408 series (10^{-4} – 10^1 μM) (triangles) for 1 h and untreated (solid line) or washed with media (dashed line) and further incubated in the absence of compounds for 4 h prior to 15 min of IL-1 β stimulation. B, PBMC were pretreated with PF-184 (0.7×10^{-3} – 10^1 μM) for 1 h and untreated (diamonds; solid line) or washed (diamond; dashed line) with media 4 h prior to 15 min of LPS stimulation. Kinase activity was measured as described under *Materials and Methods*. C, duration of tight-binding assessed by IC_{50} for inhibition of endogenous kinase activity. A549 cells were pretreated with varying concentrations of PHA-408 (solid bars) for 1 h and washed with media for the indicated times before IL-1 β stimulation. IC_{50} values for PHA-408 were determined at each time of prewashing or untreated controls from separate assay plates and plotted as shown. D, concentration-response curves for the inhibition of IL-8 production. Synovial fibroblasts were preincubated with PHA-408 (0.1 – 30 μM) for 1 h and untreated (square; solid line) or washed with media (square; dashed line) 4 h prior to 18 h of IL-1 β stimulation. IL-8 levels in the supernatant were determined by ELISA. Results from A, B, and D are expressed as the mean $\pm$ S.D. from 3 independent experiments. Results from C are expressed as the mean $\pm$ S.D. from three independent experiments (0, 4, and 8 h) and mean from two independent experiments (6 and 12 h).

media and incubated without inhibitor for 4 h before an 18-h IL-1 β stimulation. PHA-408 displayed similar EC_{50} for IL-8 inhibition as shown in Fig. 2D.

PHA-408 and PF-184 Broadly Inhibit IKK-2-Dependent Inflammatory Products in a Variety of Human Disease-Relevant Cells. To demonstrate the wide-ranging anti-inflammatory activity of IKK-2 inhibition in human *in vitro* models of airway disease, the effects of PHA-408 and PF-184 on LPS- and IL-1 β -induced production of inflammatory mediators were evaluated in PBMC, neutrophils, airway epithelial cells, and airway endothelial cells. PHA-408 and PF-184 demonstrated a concentration-dependent inhibition of multiple inflammatory cytokines, chemokines, and adhesion molecules produced by these cells (Table 1) with maximal inhibition of at least 85% in each system (data not shown). Whereas the PHA-408 and PF-184 EC_{50} for inhibition of TNF- α production was approximately 5- to 10-fold lower than the EC_{50} for inhibiting IL-8, the EC_{50} for inhibiting IL-6, MCP-1, MIP- β , and p65 nuclear translocation were in between TNF- α and IL-8. Note that the EC_{50} values for TNF- α inhibition were closely correlated with the IC_{50} values on rhIKK-2 (Fig. 1B). Likewise, the similar potencies of PHA-408-mediated inhibition of p65 translocation and that of other LPS- and IL-1 β -induced gene products shown suggest that they are also inhibited through an

IKK-2 mechanism of action (Table 1; Fig. 4A). Fluticasone, a potent glucocorticoid with well documented cellular anti-inflammatory properties, was used as a benchmark and, as expected, potently inhibited most of the IL-1 β - and LPS-induced cytokines and chemokines, with the exception of MCP-1 and adhesion molecule production from airway endothelial cells (Table 1). Also as expected, fluticasone did not inhibit p65 nuclear translocation (Table 1; Fig. 4B). Similar profiles were obtained with other selective IKK-2 inhibitors in this series and with other corticosteroids (data not shown). These data demonstrate a broad anti-inflammatory profile for IKK-2 inhibition in multiple cell types, including LPS-challenged human neutrophils, which to our knowledge has not been reported.

IKK-2 Inhibitors Are Efficacious in a Steroid-Insensitive *In Vitro* Model of Oxidative Stress. Because cigarette smoking is the major risk factor for the development of steroid-insensitive types of asthma and COPD, CSC or smoke-bubbled medium has been used to develop *in vitro* models of steroid insensitivity (Culpitt et al., 2003; Walters et al., 2005; Yang et al., 2006). We recently developed an *in vitro* model in PBMC in which multiple corticosteroids, including fluticasone, beclamethasone, and dexamethasone, were significantly less efficacious in the presence of CSC or H_2O_2 treatment before IL-1 β compared with IL-1 β alone (C. D. Sommers, unpublished

TABLE 1
Disease-relevant in vitro inflammatory assays

Stimuli	Cell Assays	Inhibitors (IC ₅₀)		
		PHA-408	PF-184	Fluticasone
			<i>nM</i>	
LPS ^a	PBMC TNF	25 ± 4	8 ± 2	0.10 ± 0.02
	PBMC IL-8	223 ± 12	147 ± 70	0.26 ± 0.05
LPS ^a	Neutrophil TNF	44 ± 6	130 ± 46	0.14 ± 0.01
	Neutrophil IL-8	281 ± 50	183 ± 60	0.09 ± 0.01
	Neutrophil MCP-1	193 ± 30	290 ± 148	>1 μM
	Neutrophil MIP-1β	144 ± 8	201 ± 58	Partial curve
IL-1β ^a	Epithelial cell TNF	101 ± 66	190 ± 58	0.08 ± 0.02
	Epithelial cell IL-8	138 ± 68	343 ± 38	0.05 ± 0.02
	Epithelial cell p65 translocation	100 ± 50	ND	>1 μM
IL-1β ^b	Endothelial ICAM	134	ND	>10 nM
	Endothelial VCAM	50	ND	>10 nM
	Endothelial E-selectin	149	ND	>10 nM

ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule.

^a EC₅₀ values (mean ± S.E.M.; *n* = 3) for inhibition of LPS- and IL-1β-induced cytokines from human PBMC, neutrophils, and A549 epithelial cells.

^b EC₅₀ values (mean; *n* = 2) for inhibition of IL-1β-induced adhesion proteins from primary endothelial cells.

data). CSC treatment alone had no effect on TNF-α production or cell viability over a 24-h period (C. D. Sommers, unpublished data). PF-408 and PF-184 inhibited IL-1β-induced TNF-α in a concentration-dependent manner with maximal efficacies of 98 and 94% and relative potencies of 176 and 163 nM, respectively (Fig. 3, A and B). On pretreatment with CSC, maximal efficacy and potency were maintained for both PF-408 and PF-184. Maximal efficacy has also been shown with other IKK inhibitors (C. D. Sommers, unpublished data). In contrast, fluticasone inhibited IL-1β-induced TNF-α, with maximal efficacy of 71% and a relative potency of 0.16 nM, which was significantly decreased to 49% maximal efficacy and lower potency in CSC pretreatment conditions (Fig. 3C). This suggests that IKK-2 inhibitors may be efficacious in cells less sensitive to steroid action.

Correlated Inhibition of NF-κB Nuclear Translocation and Inflammatory Mediator Production Links an IKK-2 Mechanism of Action in Primary Airway Cells. To further substantiate an IKK-2-dependent anti-inflammatory profile and NF-κB inhibition in relevant cells from the

rat neutrophilia model, the effects of PHA-408 and fluticasone were evaluated in vitro in LPS-challenged rat alveolar macrophages. PHA-408 demonstrated a concentration-dependent inhibition of LPS-induced Gro-α, TNF-α, IL-6, and GM-CSF production in rat alveolar macrophages with EC₅₀ values in the range of 0.2 to 2.1 μM (Fig. 4A). Fluticasone completely inhibited LPS-induced rat alveolar macrophage TNF-α, IL-6, and GM-CSF levels with picomolar potency but only partially blocked (60% inhibition) the production of Gro-α (Fig. 4B). PHA-408 demonstrated a concentration-dependent inhibition of LPS-induced p65 nuclear localization in alveolar macrophages with an EC₅₀ consistent with blockade of NF-κB-dependent cytokine production (Fig. 4A). Translocation of p65 in cells treated with the maximally efficacious concentration of PHA-408 (5 μM) were indistinguishable from untreated cells (Fig. 4C). In contrast, fluticasone, at doses that completely inhibited TNF-α, IL-6, and GM-CSF production, did not inhibit LPS-induced p65 translocation (Fig. 4, B and C). PF-184 and SC-514 (Kishore et al., 2003) also inhibited LPS-induced cytokine production from rat al-

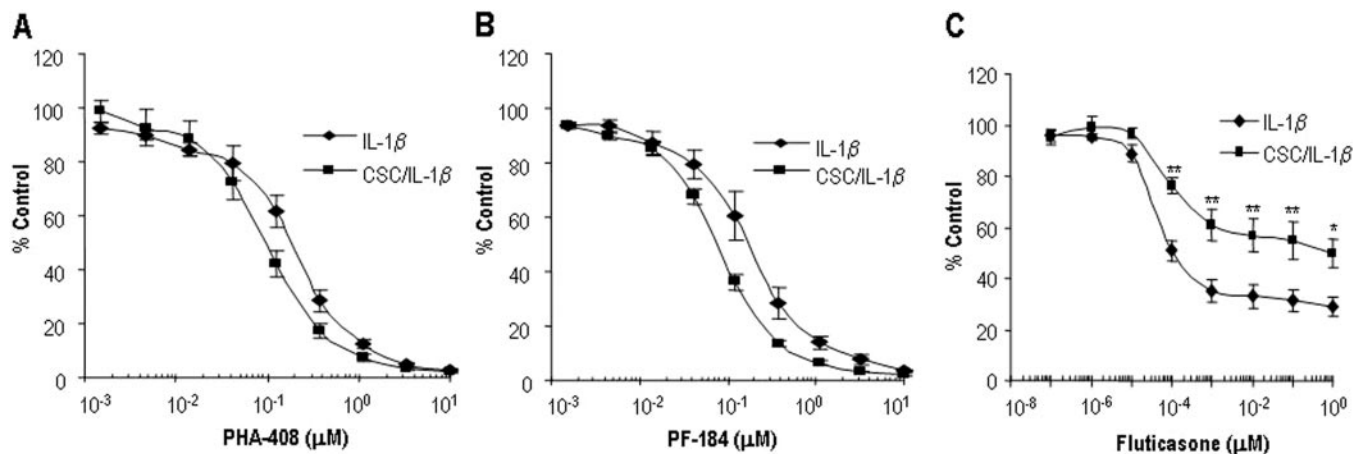
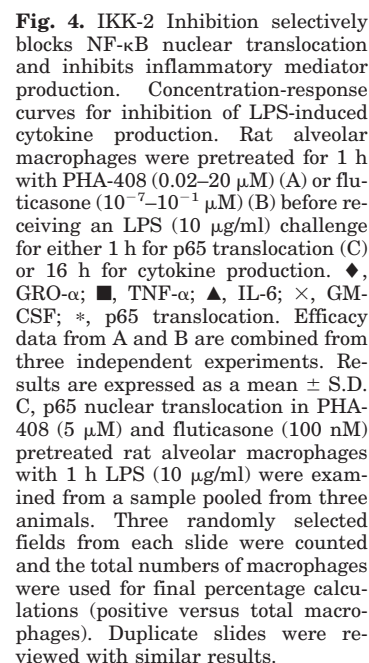


Fig. 3. IKK-2 Inhibitors are efficacious in a steroid-insensitive in vitro model of oxidative stress. A—C, concentration-response curves for the inhibition of TNF-α production. PBMC were treated with 20 μg/ml CSC or media containing 0.8% Me₂SO. After 4 h, cells were preincubated for 1 h with PHA-408 (A) (0.002–10 μM), PF-184 (0.002–10 μM) (B), or fluticasone (C) (10⁻⁷–10⁰ μM), without removing CSC. Cells were then stimulated with 1 ng/ml IL-1β for 16 h and TNF-α production in the supernatant measured by ELISA. The mean ± S.E.M TNF-α levels for 100% control from three donors were 567 ± 153 pg/ml for IL-1β alone and 867 ± 115 pg/ml for IL-1β plus CSC. The mean ± S.E.M TNF-α levels for CSC alone and basal conditions were 19 ± 9 and 21 ± 10 pg/ml, respectively. Data are expressed as a mean ± S.E.M from three independent experiments of triplicate samples. Statistical analysis was assessed with use of one-way ANOVA (*, *p* < 0.01; **, *p* < 0.001) denotes significance differences to the IL-1β-treated cells.



Inhaled LPS Activates the IKK-2 and NF- κ B Pathway and Causes a Time-Dependent Sequela of Cell Infiltration and Mediator Production. As part of the effort to characterize the IKK-2/NF- κ B pathway in the acute rat LPS model and to optimize in vivo studies with our selective IKK-2 inhibitors, we characterized the time course of inflammatory and signaling events. Inhalation of LPS resulted in a time-dependent increase in total cells and neutrophils (Fig. 5A). The earliest increase for both was detected at 2 h, peaked at 8 h, and declined to baseline by 48 h. As shown in Fig. 4, LPS induces a number of cytokines and chemokines in vitro. Distinct cytokine time course profiles were detected after aerosolized LPS challenge (Fig. 5B). TNF- α , MIP-1 α , and Gro- α were detected as early as 30 to 60 min and peaked between 2 and 4 h. IL-6 and IL-1 β peaked at 4 to 8 h and returned to baseline by 16 and 40 h, respectively. MCP-1 increased gradually, peaking at approximately 16 h and declined to baseline by 48 h. IKK-2 kinase activity from whole-lung homogenates was detected as early as 15 min after LPS exposure, peaked at 60 min, and returned to baseline by 4 h (Fig. 5C). This activity was paralleled by a sustained p65 DNA binding profile that was consistent with the broad activation time course profiles of the cytokines (Fig. 5B). I κ B α degradation and the presence of nuclear p65 coincided with p65 DNA binding activity as demonstrated by Western blot analysis in lung lysates (Fig. 5D). In alveolar macrophages, nuclear translocation of p65 peaked 30 to 60 min after LPS challenge with >80% of the macrophages displaying intense nuclear p65 staining then returning to baseline by 8 h (Fig. 5E).

philia. Oral administration of PHA-408 demonstrated a dose-dependent inhibition of total cell and neutrophil infiltration when predosed 1 h before a 4-h aerosolized LPS challenge (Fig. 6A). The calculated EC_{50} for total cell and neutrophil inhibition were 27.3 ± 4 and 29.4 ± 3 mg/kg, respectively. Inhibition of BAL fluid MCP-1, TNF- α , and Gro- α levels correlated with the inhibition of neutrophils (Fig. 6B). We evaluated the potential of using alveolar macrophages obtained from lavage samples after LPS treatment to assess the effect of orally administered PHA-408 (100 mg/kg) on p65 nuclear translocation. PHA-408 reduced p65 translocation measured 4 h following LPS by 54% (Fig. 6C).

IKK-2 Inhibition by Inhaled Drug Delivery Blocks Neutrophil Infiltration and BAL Cell Cytokine Production. We were interested in demonstrating the potential of using a local delivery approach to determine whether similar anti-inflammatory efficacy would be observed. Given the low oral bioavailability (5%), rapid clearance (59 ml/min/kg), and prolonged inhibition of IKK-2 in cells because of slow off-rate (Figs. 1 and 2), PF-184 represented an ideal candidate to compare oral (PHA-408) versus IT delivery. PF-184 (0.3–2.5 mg) or fluticasone (0.01–0.1 mg) given as a nanosuspension 60 min before aerosolized LPS resulted in a comparable attenuation of total cell and PMN cell infiltration 4 h after LPS exposure (Fig. 7A). Both inhibitors dose-dependently inhibited cell infiltration with EC₅₀ values of 0.025 and 1 mg for fluticasone and PF-184, respectively. Likewise, IT delivery of PF-184 dose-dependently suppressed BAL fluid TNF- α and PGE₂ levels comparable with cell infiltration (Fig. 7B). To compare the inhibition of nuclear p65 translocation after oral delivery shown in Fig. 6C versus IT delivery, BAL cells were evaluated 4 h following LPS exposure. PF-184 (2.5 mg) treatment inhibited p65 translocation to a similar degree as PHA-408 (100 mg/kg) (Fig. 6C) (51 versus 54%) in alveolar macrophages, whereas inhaled fluticasone (0.1 mg) had no effect (Fig. 7C), demonstrating the specificity of this endpoint in vivo. To evaluate the effect of inhaled compound delivery on the signaling of resident airway cells, BAL cells

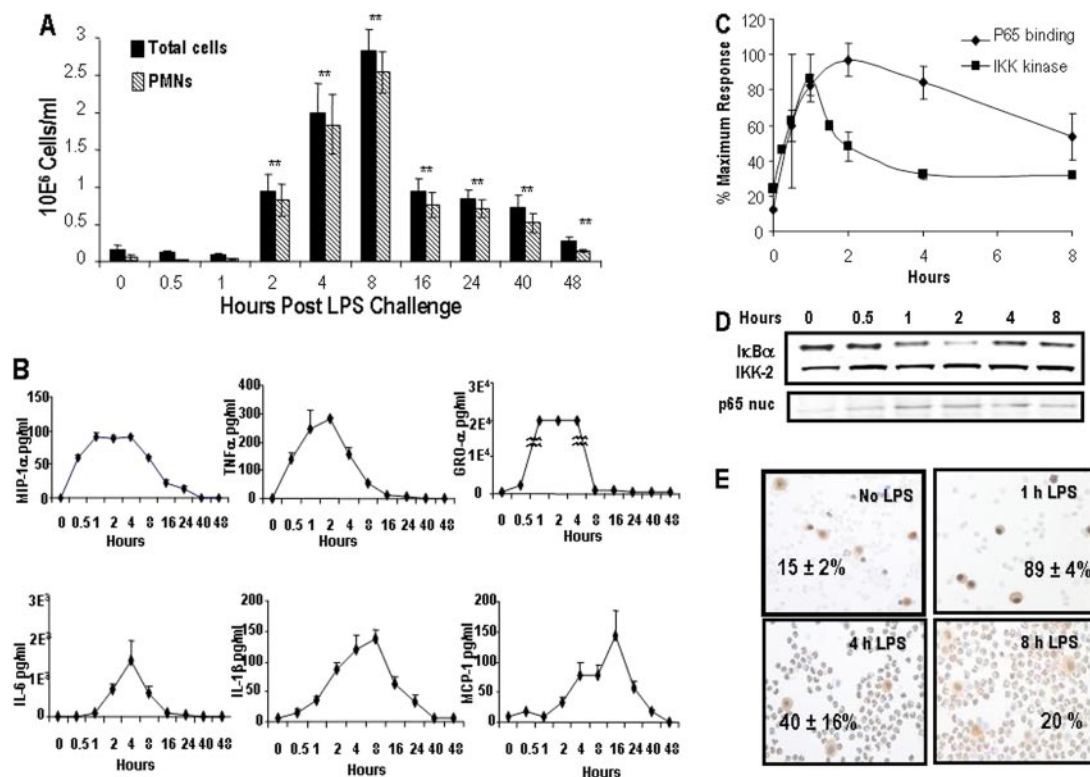


Fig. 5. Inhaled LPS activates the IKK and NF- κ B pathway and results in a time-dependent sequela of cell infiltration and mediator production. A, rats were exposed to 1 mg/ml aerosolized LPS. At times ranging from 0.5 to 48 h, lung lavages were collected, and total cell and cell differential counts were determined. Results are expressed as a mean $\pm$ S.E.M. of four animals. Data were representative from two independent studies. Statistical analysis was assessed by use of one-way ANOVA (**, $p < 0.001$) denotes significance differences from 0-h baseline for both PMN and total cells. B, MIP-1 α , TNF- α , GRO- α , IL-6, IL-1 β , and MCP-1 levels were detected in lavage fluid collected from animals at various times (0.5–48 h) after receiving aerosolized LPS (1 mg/ml). Cytokines were quantified by use of Luminex technology with a custom rat cytokine/chemokine 24-plex kit. Results are expressed as a mean $\pm$ S.E.M. of four animals. The data were from a representative experiment of two independent studies. C, four rats were exposed to 1 mg/ml aerosolized LPS. At various times ranging from 0.5 to 8 h, lung tissue samples were collected and frozen. IKK-2 kinase activity was assessed from whole-lung lysates with use of a capture plate coated with phospho-I κ B polyclonal antibody. NF- κ B p65 DNA binding activity was measured in nuclear lung lysate fractions by use of a p65 oligonucleotide-coated 96-well plate. Results are expressed as a mean $\pm$ S.E.M. The data were from a representative experiment of two independent studies. D, Western blot analysis of total I κ B α and IKK-2 from whole-lung lysates and p65 from nuclear lung lysates prepared from pooled samples described in C. E, p65 immunohistochemistry was performed in formalin-fixed cells from lung lavages collected 1 to 8 h after aerosolized LPS. Percentage of nuclear positive p65 staining in BAL macrophages from lung lavages was collected and pooled from four animals at the indicated times. Three randomly selected fields of each slide were counted and the total number of macrophages was used for final percentage calculations (positive versus total macrophages). Duplicate slides were evaluated with similar results. Results are expressed as a mean $\pm$ S.D. of three independent experiments (0, 1, and 4 h) or a mean of two experiments for 8 h.

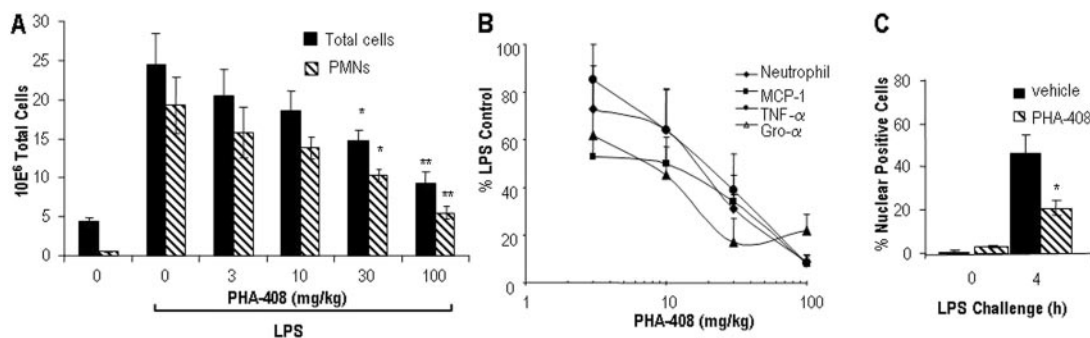


Fig. 6. IKK-2 inhibition dose-dependently inhibits LPS-induced neutrophil infiltration, cytokine production, and NF- κ B translocation in vivo. A, rats ($n = 12$) were dosed with vehicle (methylcellulose) or PHA-408 at doses ranging from 3 to 100 mg/kg 1 h before 1 mg/ml aerosolized LPS. After 4 h, lung lavages were collected for cell counts and cytokine determination. The PHA-408 efficacy data were combined from two independent experiments. Results are expressed as a mean $\pm$ S.E.M. B, cytokine production in BAL fluid was examined 4 h after LPS aerosol challenge. C, p65 nuclear localization in alveolar macrophages. Rats ($n = 3$) were dosed with vehicle or PHA-408 (100 mg/kg) 1 h before 1 mg/ml aerosolized LPS. Four hours after exposure, lung lavages were collected for cell cytopins and p65 staining. Three randomly selected fields of each slide from three animals were counted and the total numbers of macrophages were used for final percentage calculations (positive versus total macrophages). Results are expressed as a mean $\pm$ S.E.M. Statistical analysis for A and C was assessed by use of one-way ANOVA ($p < 0.01$; **, $p < 0.001$) denotes significance differences to the LPS-challenged vehicle group.

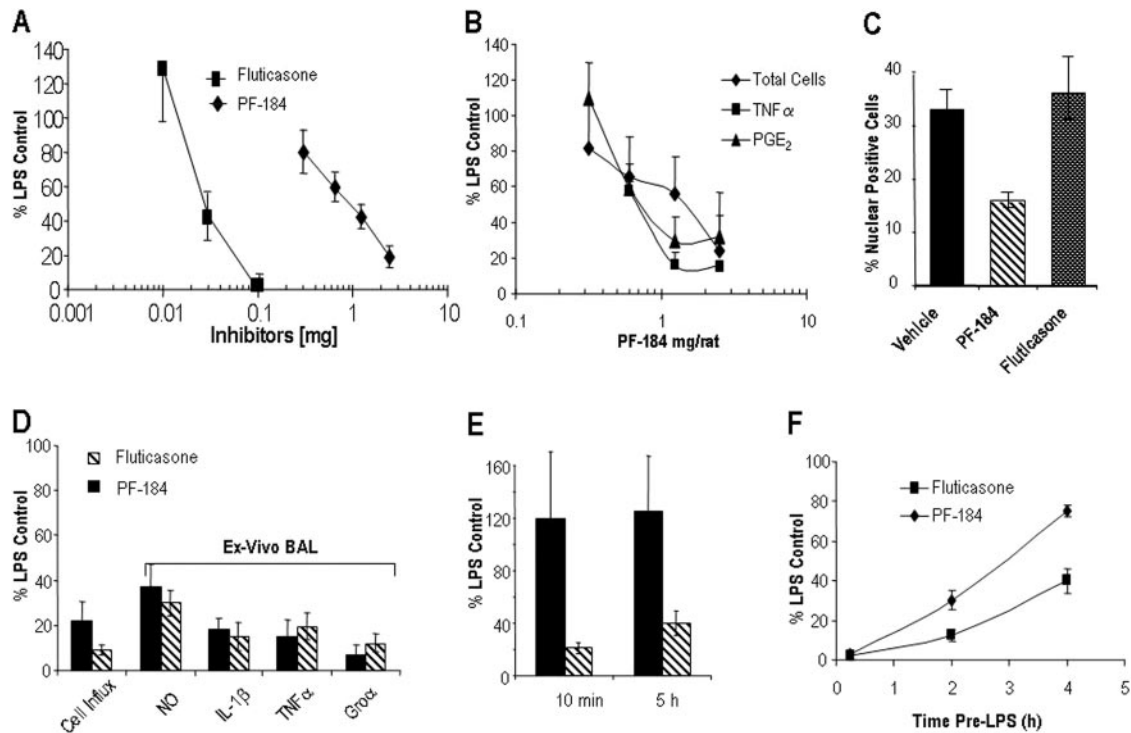


Fig. 7. IKK-2 inhibition by inhaled drug delivery blocks neutrophil infiltration and BAL cell cytokine production. A, rats ($n = 6$ /study) were dosed IT with vehicle (nanosuspension), PF-184 (diamonds) (0.3–2.5 mg), or fluticasone (squares) (0.01–0.1 mg) 1 h before 1 mg/ml aerosolized LPS. After 4 h, lung lavages were collected for cell counts and cytokine determination. The data are combined from two (PF-184) and three (fluticasone) independent experiments. Results are expressed as a mean $\pm$ S.E.M. B, dose-response curves of BAL fluid total cell counts (diamonds), TNF- α (squares), and PGE₂ (triangle) production from rats treated IT with PF-184 (0.3–2.5 mg) 1 h before aerosolized LPS as in A. The data are from a representative experiment that has been repeated with similar results. C, p65 nuclear localization in alveolar macrophages obtained from vehicle, PF-184 (2.5 mg) and fluticasone (0.1 mg) BAL samples in A. Four hours after LPS exposure, lung lavages were collected and pooled for cell cytopins and p65 staining. Three randomly selected fields of each slide from two experiments were counted and the total numbers of macrophages were used for final percentage calculations (positive versus total macrophages). Results are expressed as a mean $\pm$ range of values. D, inhibition of aerosolized LPS-induced cell influx and BAL cell mediator production following an ex vivo 10 μ g/ml LPS challenge. Rats ($n = 6$) were dosed IT with vehicle (nanosuspension), PF-184 (solid bars) (2.5 mg), or fluticasone (dashed bars) (0.1 mg) 1 h before 1 mg/ml aerosolized LPS or vehicle control. After 4 h, lung lavages were collected for cell counts and cell isolation from the vehicle control for ex vivo studies. BAL cells resuspended in DMEM media containing 10% FCS were plated at 5×10^5 /well for an 18-h 10 μ g/ml LPS challenge. Nitric oxide, IL-1 β , TNF- α , and Gro- α levels were determined in the culture supernatants by the Greiss assay and ELISA. The data are expressed as mean $\pm$ S.E.M from six animals. The percentage of LPS control values were determined from absolute levels for the LPS control groups, which were 17 μ M and 0.1, 1, and 2 ng/ml for nitric oxide, IL-1 β , TNF- α , and Gro- α , respectively. E, inhibition of TNF- α production from rat whole-blood following an ex vivo 10 μ g/ml LPS challenge. Rats ($n = 3$) were dosed IT with vehicle (nanosuspension), PF-184 (solid bars) (2.5 mg), or fluticasone (dashed bars) (0.1 mg) and bled after 10 min or 5 h compound treatment; 175 μ l of blood in duplicate from each animal was placed in 96-well plates for ex vivo LPS stimulation for 18 h. TNF- α production in plasma was determined by ELISA. The percentage of LPS control was determined from absolute levels of 1 ng/ml (10 min) and 0.5 ng/ml (5 h) in the control LPS groups. F, rats ($n = 6$) were dosed IT with vehicle (nanosuspension), PF-184 (diamonds) (2.5 mg), or fluticasone (squares) (0.1 mg) 20 min, 2 h, or 4 h before 1 mg/ml aerosolized LPS. Four hours after LPS challenge, lung lavages were collected for cell counts. The data are expressed as a mean $\pm$ S.E.M from a representative experiment that has been repeated with similar results.

were obtained 4 h after fluticasone (0.1 mg) or PF-184 (2.5 mg) administration and plated in 96-well plates for ex vivo LPS challenge studies. Treatment of control BAL cells with 10 μ g/ml LPS for 18 h, resulted in the production of nitric oxide, IL-1 β , TNF- α , and Gro- α in the media. Cells from fluticasone- and PF-184-treated rats showed significantly reduced ex vivo LPS-induced mediator production, which was similar to the inhibition of in vivo LPS-induced cell infiltration for both inhibitors (Fig. 7D). To determine whether IT delivery caused significant inhibition of LPS action in the systemic compartment, we evaluated whole blood from the vehicle-, PF-184-, or fluticasone-treated animals for TNF- α production in response to ex vivo LPS challenge. TNF- α produced from an ex vivo whole-blood LPS challenge was not inhibited in animals treated with PF-184, whereas it was inhibited in the fluticasone-treated animals when blood was drawn 10 min or 5 h after dosing (Fig. 7E). Consistent with these results are the PF-184 plasma concentrations, cor-

rected for protein binding, at 10 min (0.17 μ M) and 5 h (0.37 μ M), which, based on the human data in Table 1, may be below levels needed to inhibit LPS-induced TNF- α production. Plasma concentrations of fluticasone were in the high nanomolar range at 10 min and 5 h, which may have been sufficient to inhibit TNF- α production from ex vivo challenges in the BAL cells and in the whole blood. Finally, because PF-184 demonstrated prolonged inhibition of IKK-2 in cells, we wanted to explore the duration of inhibitory activity of inhaled PF-184 in vivo. IT administration of PF-184 or fluticasone at 20 min, 2 h, or 4 h before inhaled LPS challenge resulted in a time-dependent decrease in efficacy for both inhibitors. PF-184 was 100, 70, and 25% efficacious when administered 20 min, 2 h, and 4 h before LPS, respectively, demonstrating long-lasting activity in vivo, albeit shorter than observed in vitro (Fig. 7F). Collectively, for the first time, these data demonstrate that selective IKK-2 inhibitors are efficacious in a model of lung inflammation by

local delivery to the lung and that airway inflammation is inhibited by an IKK-2 mechanism by both oral and IT delivery.

Discussion

Inhibition of the NF- κ B activation pathway has gained increasing interest as an effective target for anti-inflammatory therapies in diseases such as asthma and COPD (Bonizzi and Karin, 2004; Adcock et al., 2006; Barnes, 2006b). IKK-2, the proximal kinase leading to NF- κ B activation, is an exemplary target for inhibition in this pathway. This is supported by several preclinical studies using selective IKK-2 inhibitors and molecular approaches in disease-relevant airway cells and in vivo models (Adcock et al., 2006). In this report, we characterize the anti-inflammatory activities of two highly selective, potent, and long-acting IKK-2 inhibitors (PHA-408 and PF-184). We took advantage of the compounds' distinct drug metabolism, bioavailability, and clearance properties to elucidate the role of IKK-2 and to compare local and oral compound delivery in a model of airway inflammation.

Extended duration of action on the desired target significantly enhances the ability of a drug to demonstrate efficacy after inhaled delivery (Rohatagi et al., 2004). Slow dissociation kinetics of PHA-408 and PF-184 were demonstrated on rhIKK-2 and in several cell-based formats by use of extensive washing, gel filtration, dialysis, and immunoprecipitation procedures (Fig. 2 and Mbalaviele et al., 2009). Both compounds maintained efficacy when washed from cells several hours before cytokine or LPS stimulation. In fact, in the adherent A549 cell line, PHA-408 was still bound to the native IKK complex, albeit decreased, 12 h after the washout as shown by the nanomolar IC_{50} for kinase inhibition. This unique tight-binding property of these potent IKK-2 inhibitors is in contrast to the ATP-competitive, reversible inhibitors of IKK-2 reported previously (Kishore et al., 2003; Ziegelbauer et al., 2005; Wen et al., 2006) and provides valuable tools to directly link efficacy readouts with kinase inhibition and to evaluate dosing paradigms in preclinical models of airway disease. Our next objectives were to validate suitable anti-inflammatory profiles of these novel inhibitors and the requisite role of NF- κ B in the rat model of neutrophilia to characterize the pharmacological properties of PHA-408 and PF-184 in vivo.

Corticosteroids are thought to derive their unprecedented efficacy on a molecular level by recruiting histone deacetylase-2 to NF- κ B-activated gene complexes resulting in a broad anti-inflammatory profile in multiple disease-relevant cell types (Barnes, 2006a). As expected, fluticasone demonstrated picomolar potency against the activation and transcription of several NF- κ B-dependent gene products but did not inhibit human MCP-1 or adhesion molecules, whereas the IKK-2 inhibitors completely inhibited all the gene readouts with low micromolar potency concomitant with inhibition of p65 nuclear localization. Interestingly, a differential inhibition of gene expression by the IKK-2 inhibitors was observed. Whereas the shift in EC_{50} values for inhibition of TNF- α and IL-8 may be through alternative targets, it more likely reflects the complex nature of the interactions between NF- κ B and the regulatory elements of its targets in the context of chromatin structures. In support of this hypothesis, similar observations have been made with the use of

other reported IKK-2 inhibitors (Ziegelbauer et al., 2005; Wen et al., 2006) and with PHA-408 in RASF cells where EC_{50} for inhibition of IL-8 and NF- κ B SEAP (secreted alkaline phosphatase) reporter activity were right-shifted from TNF- α and rhIKK-2 inhibition (Mbalaviele et al., 2009). Furthermore, we have demonstrated that, with use of dominant negative approaches, a similar shift in inhibition of TNF- α versus IL-8 is observed (data not shown). Taken together, these data suggest that off-target effects of the inhibitors are unlikely.

In patients with COPD, histone deacetylase-2 levels are significantly decreased, which reduces the therapeutic benefit obtained from corticosteroids (Barnes, 2005). In fact, proinflammatory cytokines produced from alveolar macrophages from COPD patients are recalcitrant to dexamethasone treatment compared with alveolar macrophages from healthy subjects (Ito et al., 2001; Culpitt et al., 2003). IKK-2 inhibitors, however, act upstream by blocking NF- κ B activation. In the present study, we demonstrate that the efficacy and potency for an IKK-2 inhibitor is not reduced in a cigarette smoke-induced in vitro model of oxidative stress, unlike that of fluticasone. Other investigators have also shown that inhibition of IKK-2 activity by UK-436303, BAY-117082, or the antioxidant, *N*-acetylcysteine, reduced cigarette smoke extract-induced IL-8 or the combined cigarette smoke extract + IL-1 β -induced IL-8 production (Walters et al., 2005; Yang et al., 2006). The results herein demonstrating that IKK-2 mediated anti-inflammatory activities in several cell types, including neutrophils and rat alveolar macrophages, and full efficacy in a model of oxidative stress support published opinions (Barnes, 2006b), suggesting that an IKK-2 inhibitor may be efficacious in COPD and in steroid-resistant asthma and would be particularly useful if it could be delivered by the inhaled route.

Acute administration of endotoxin to the airways results in an NF- κ B-dependent sequela of inflammation that can be used as a model to characterize potential therapeutic agents for airway diseases. On oral administration, PHA-408 demonstrated dose-dependent inhibition of neutrophil infiltration and TNF- α , Gro- α , and MCP-1 production in BAL fluid. Likewise, other small-molecule IKK-2 inhibitors have also shown efficacy through oral administration in rodent models of asthma and COPD (Birrell et al., 2005b, 2006; Everhart et al., 2006). Glucocorticoids, however, are mainly administered by inhalation for treatment of chronic airway diseases. This increases the likelihood of achieving high local concentrations at the target in the lung and potentially minimizes the risk of adverse systemic effects (Rohatagi et al., 2004). By the same token, an IKK-2 mechanism by virtue of inhibiting systemic NF- κ B activity may also exert undesired systemic side effects on chronic oral administration. We demonstrate the unprecedented finding that IT administration of a small-molecule IKK-2 inhibitor, PF-184, dose-dependently inhibited LPS-induced cellular infiltration and mediator production into the BAL fluid to a magnitude similar to oral administration of PHA-408 and to that achieved with the inhaled positive standard, fluticasone. Ultimately it would be of interest to demonstrate IKK-mediated efficacy and improvement of lung function in a steroid-insensitive in vivo smoke model. These types of studies are being pursued and will help elucidate the role of IKK-2 in chronic airway disease.

Nuclear localization of p65 has been demonstrated by Hart et al. (1998) in alveolar macrophages obtained from COPD and asthmatic patients. Therefore, we were interested in understanding the potential feasibility of using this endpoint to track NF- κ B pathway modulation concomitant with efficacy after IKK-2 inhibitor treatment. Of interest is the inhibition of p65 nuclear translocation after in vivo treatment with PHA-408 and PF-184, but not fluticasone. Nevertheless, there seems to be a discord between the in vivo data showing a 50% reduction in p65 translocation and almost full repression of mediator release. These apparent discrepancies may result from limitations of the assays, time dependence, and/or additional regulatory mechanisms of NF- κ B activities. We have observed a time-dependent inhibition of I κ B phosphorylation and degradation as well as p65 nuclear localization in IL-1 β -stimulated synovial fibroblasts, whereas NF- κ B genes were completely inhibited (Kishore et al., 2003; Mbalaviele et al., 2009). The matter is complicated even more because p65 can be phosphorylated by other enzymes, including TBK1 and RSK1, which induce its nuclear export and turnover, respectively. Collectively, these potential regulatory events suggest that complete or extended inhibition of p65 nuclear translocation may not be required for blockade of gene induction. Further studies, including time courses and additional NF- κ B activation endpoints, are needed to elucidate which markers most closely correspond with IKK-2 activity.

Intratracheal administration of fluticasone blocked neutrophil infiltration as did PF-184. Interestingly, although PF-184 inhibited BAL fluid and alveolar macrophage TNF- α , there was no inhibition of TNF- α produced in whole blood after an ex vivo LPS challenge from PF-184-treated animals, which is consistent with the low plasma concentrations of inhibitor. These results suggest that local concentrations of PF-184 in the airways are sufficient for efficacy. Conversely, fluticasone inhibited TNF- α production in both the ex vivo LPS-challenged BAL cell and whole blood to a comparable magnitude, suggesting that part of the anti-inflammatory effects in this model may be derived from systemic exposure. It would be interesting, but beyond the scope of this article, to understand the relationship between the potencies and exposures needed after local and oral fluticasone dosing to elicit anti-inflammatory activities versus untoward immune and bone-related effects. These types of studies would require a more chronic model with a repeated dosing paradigm. In clinical studies, Sorkness and co-workers (2004) demonstrated that steroid effects on body and thymus weights were less after metered dose inhaler treatment versus oral treatment, which suggests that local delivery may reduce but not eliminate the systemic liabilities of steroids.

In conclusion, we have demonstrated several novel findings using two selective IKK-2 inhibitors including: 1) tight-binding properties; 2) efficacy in an in vitro model of oxidative stress and disease-relevant cells; 3) IKK-2-specific inhibition of p65 translocation; and finally 4) similar anti-inflammatory effects upon oral and local administration to the lung. Together these data support an essential role for IKK-2 in airway disease models and demonstrate the feasibility and therapeutic utility of inhaled IKK-2 inhibitor delivery.

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