Small Molecule Targeting of the STAT5/6 Src Homology 2 (SH2) Domains to Inhibit Allergic Airway Disease

J. Morgan Knight1,2*, Pijus Mandal3, Pietro Morlacchi3, Garbo Mak1, Evan Li1,7, Matthew Madison1, Cameron Landers4, Brandon Saxton6, Ed Felix3, Brian Gilbert5, Joel Sederstrom6, Atul Varadhachary9, Melissa M. Singh9, Dev Chatterjee9, David B. Corry1,2,4,7,8*, and John S. McMurray3

From the Departments of 1Medicine, 2Pathology & Immunology, 3Molecular Virology and Microbiology, the 4Translational Biology and Molecular Medicine Program and the 7Biology of Inflammation Center, Baylor College of Medicine, Houston, Texas, 77030
3Department of Experimental Therapeutics, MD Anderson Cancer Center, Houston, Texas, USA
6Flow cytometry Core Facility, Baylor College of Medicine, Houston, Texas, USA
8Michael E. Debakey VA Center for Translational Research in Inflammatory Diseases, Houston, Texas, 77030
9Fannin Innovation Studio and Atrapos Therapeutics, LLC, 3900 Essex Ln, Ste. 575, Houston Texas 77027

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To whom correspondence should be addressed: David B. Corry, M.D., or J. Morgan Knight, PhD, Department of Medicine, Baylor College of Medicine, One Baylor Plaza BCM285 Houston, Texas 77030, Telephone: (713) 798-8740; FAX: (713) 798-5780; E-mail: dcorry@bcm.edu or JMK@bcm.edu

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ABSTRACT

Asthma is a chronic inflammatory disease of the lungs and airways and one of the most burdensome of all chronic maladies. Previous studies have established that expression of experimental and human asthma requires the IL-4/IL-13/IL-4 receptor alpha (IL-4Rα) signaling pathway, which activates the transcription factor STAT6. However, no small molecules targeting this important pathway are currently in clinical development. To this end, using a preclinical asthma model, we sought to develop and test a small-molecule inhibitor of the Src homology 2 domains in mouse and human STAT6. We previously developed multiple peptidomimetic compounds on the basis of blocking the docking site of STAT6 to IL-4Rα and phosphorylation of Tyr-641 in STAT6. Here, we expanded the scope of our initial in vitro structure–activity relationship studies to include central and C-terminal analogs of these peptides to develop a lead compound, PM-43I. Conducting initial dose experiments with PM-43I, we found that it potently inhibits both STAT5- and STAT6-dependent allergic airway disease in mice. Moreover, PM-43I reversed preexisting allergic airway disease in mice with a minimum ED05 of 0.25 μg/kg. Of note, PM-43I was efficiently cleared through the kidneys with no long-term toxicity. We conclude that PM-43I represents the first of a class of small molecules that may be suitable for further clinical development against asthma.

Introduction

Asthma is a chronic inflammatory disease of the lungs and airways that is marked by exaggerated airway constrictive responses against diverse provocative challenges (airway hyperresponsiveness; AHR), accumulation of mucus and fibrin plugs in the airway that further compromise airflow (plastic bronchitis), airway and systemic eosinophilia, T-helper type 2 (Th2) cells and innate lymphoid cells type 2 (ILC2) that secrete the cytokines interleukin-4 (IL-4),
IL-5 and IL-13 and vigorous IgE antibody responses (1-3). Affecting up to 30 million Americans with 3 million experiencing severe, therapy-resistant disease, asthma is one of the most common and burdensome of all chronic afflictions (4, 5).

Current asthma therapy provides symptomatic relief for many patients, but the inability of these agents to cure disease ensures their long-term use and attendant risk of inducing serious, even life-threatening side effects. We reasoned that a safer and more effective means of treating asthma could be developed through an improved understanding of the critical immune effector pathways that drive disease expression. The development of asthma is complex and is initiated in part by airway epithelial cells (AEC) that recognize exogenous antigen or pathogens through pattern recognition receptors. AEC then secrete the inflammatory cytokines IL-25, IL-33, and TSLP (1, 2) to promote ILC2 recruitment and enhance dendritic cell luminal sampling, migration to lymph nodes, and antigen presentation to initiate B and T cell activation (1, 6-8).

Adaptive immunity is critical for maintaining the chronic inflammation associated with asthma that is mediated by T(H)2 and T(H)17 cells that migrate to the lungs (3). Once in the lung, T(H)2 cells work cooperatively with ILC2s to coordinate production of the cytokines IL-4 and IL-13 (8). These cytokines signal through distinct multimeric receptor complexes that share the common IL-4Rα subunit (9). Cytokine binding to IL-4Rα induces the recruitment of tyrosine kinases Jak1 and Jak3 (Jak1 and Tyk2 for IL-13) that then phosphorylate Tyr631 within the Tyr-Lys-Pro-Phe docking site for the latent cytoplasmic transcription factor signal transducer and activator of transcription 6 (STAT6) (9). Upon binding to this motif via its Src Homology 2 (SH2) domain, STAT6 is phosphorylated by Jak kinases, becoming competent to homodimerize, translocate to the nucleus and promote STAT6-dependent gene expression (10). The absence of IL-4/IL-13/STAT6 responses in STAT6(-/-) mice results in complete disease resistance in murine models of asthma, highlighting the central role of STAT6 in the development of asthma (11, 12).

In addition to STAT6, STAT5 also contributes to the development of allergic airway disease. STAT5 together with STAT6 is required for optimal T(H)2 development (13-15). STAT5 is also essential for ILC2 development and is the major transcription factor activated by IL-5, which promotes eosinophil development, recruitment and survival (5, 16). STAT5 and STAT6 are both activated by TSLP (17, 18), underscoring the fact that multiple, diverse ligands drive the activation of a limited number of STAT factors that are crucial to the expression of asthma. Thus, although targeting select cytokines is likely to be beneficial in asthma (19, 20), a more effective approach is likely to be targeting the key STAT factors that represent the final common allergic disease mediators (21).

Our laboratories have focused on the design of small molecule phospho-peptidomimetic inhibitors of the SH2 domain of STAT6 that prevent recruitment to the critical docking site on IL-4Rα and phosphorylation of Tyr641. Early generation compounds were shown to be potent ligands of STAT6 in vitro, blocking STAT6-dependent gene expression in a variety of human airway epithelial cells, breast cancer cells, and primary splenocytes (22, 23). Furthermore, the cell-permeable, phosphatase-stable prodrug PM-242H was effective at preventing and/or reversing allergic lung disease in a murine model of asthma (11). Here we expand the scope of our initial in vitro structure-activity relationship studies to include novel central and C-terminal analogs. The phosphopeptide PM-301 was previously shown to have a high affinity (IC_{50} = 117 nM) for STAT6 (23). The cell-permeable, phosphatase-stable PM-301 prodrg, PM-37I, displayed high cellular potency, completely blocking IL-4-stimulated STAT6 phosphorylation at approximately 100 nM. PM-37I also blocked EGF-stimulated pSTAT5 in MDA-MD-468 breast cancer cells and IL-4-dependent STAT6 activation in primary murine splenocytes (23, 24). Here we considered the effects of conformationally-constrained modifications of PM-37I on the central dipeptide region and a survey of cyclic amides on the C-terminus on STAT inhibition.
RESULTS

 Novel central scaffold and C-terminal analog affinities

The phosphopeptide PM-301 was previously shown to have a high affinity (IC_{50} = 117 nM) for STAT6 (32). The cell-permeable, phosphatase-stable PM-301I prodrug, PM-37I, displayed high cellular potency, completely blocking IL-4-stimulated STAT6 phosphorylation at approximately 100 nM. PM-37I also blocked EGF-stimulated pSTAT5 in MDA-MB-468 breast cancer cells and IL-4-dependent STAT6 activation in primary murine splenocytes (32, 33). Here we considered the effects of conformationally-constrained modifications of PM-37I on the central dipeptide region and a survey of cyclic amides on the C-terminus on STAT inhibition.

To examine the importance of the central (Tle-Pro) scaffold on STAT6 affinity, we synthesized a series of conformationally constrained dipeptides (Fig. 1a). In PM-28I, linkage of the Cα of pY+1 to the Nα of pY+2 with a Freidinger lactam resulted in a five-fold decrease in affinity (IC_{50} of 630 nM) as assessed by fluorescence polarization. Lactamation of the pY+2 and the C-terminal amide had a detrimental effect on affinity as the IC_{50} of PM-60I was 2.33 μM, a 20-fold decrease in affinity. In contrast to the Freidinger lactams, replacing the central Tle-Pro of PM-301H with the tricyclic dipeptide mimic, Haic (PM-34I), was well tolerated with only a 2-fold loss in affinity compared to PM-301H. These data show a slight, but significant decrease in affinity with increasing rigidity in the central scaffold.

The C-terminal methylanilide of PM-301H was replaced with a series of cyclic amides (Fig. 1a). In the piperidinamide series (PM-67I-(25)), morpholine (PM-67I-B) was approximately 2-fold higher in affinity than piperidine (PM67I-A) and was equal to the starting methyl, phenyl amide (PM-301H). Interestingly, the positively charged 4-methylpiperazine analog (PM-67I-C) bound the STAT6 SH2 domain with a 20-fold lower affinity than the lead PM-301H. Fused alky-aryl amides with varying alky rings were also probed. Increasing the ring size from N=5 to N=7 had no effect on affinity, with all three tested compounds (N = 5: PM-59I, N = 6: PM-87I, N = 7, PM-71I-A) displaying IC_{50} values of 230-260 nM. Interestingly, the tetrahydroisoquinolinyl amide (PM-71I-B), which moves the benzene ring slightly farther from the main chain, was very avid, with an IC_{50} of 50 nM. Taken together, these data show that STAT6 affinity as defined by fluorescence polarization is only mildly impacted by changes in ring conformation.

Cellular Activity Screen

The phosphate-containing inhibitors in Figure 1a were converted to a series of cell-permeable, phosphatase-stable prodrugs by addition of phosphate-blocking POM groups (24), and screened for ability to inhibit IL-4 stimulated STAT6 inhibition (data not shown). Of this series, PM-43I, PM-63I, PM-74I, PM-80I, PM-81I, and PM-86I, were the most potent (STAT6 inhibition >90% at 5 μM) and selected for more detailed analyses. Titration of the inhibitors in Beas-2B cells indicated EC_{50} values of 100 – 500 nM, as judged by pSTAT6 inhibition (Fig. 1b). The exception was PM-43I, which required between 1 and 2 μM to completely inhibit STAT6 phosphorylation.

The six leads were also evaluated for their potential for inhibition against additional SH2 domain containing proteins, including other STAT factors, in MDA-MB-468 cells stimulated with epidermal growth factor (EGF) for STAT3, STAT5, AKT (via SH2 domain containing p85 subunit of PI3K) and FAK activation (via SH2 containing Src kinase activity), or IFN-γ for STAT1 activation (Fig. 1c) (26, 27).

Considerable variability in cross-reactivity was observed between the screened compounds, with PM-86I showing the highest specificity for STAT6 and no cross-reactivity to any of the additional targets at the highest dose tested. At 5 μM, PM-43I and PM-63I showed significant cross-reactivity to STAT5 and PM-43I showed slight inhibition of STAT3. PM-74I was cross-reactive with STAT1, STAT3, STAT5, and AKT (p85/PI3K). PM-80I was the only compound to show cross-reactivity to STAT5 below the 5 μM maximum dose. PM-81I did not
cross-react with STAT5, but did moderately inhibit FAK and STAT1.

Prodrug cytotoxicity after 72 h exposure was assessed in Beas-2B (Fig. 1d) and MDA-MB-486 (Fig. 1e) cells by the MTA oxidation assay. With near complete viability at ≤ 1 μM and IC₅₀ values between 8 μM and 10 μM, the apparent reduction in STAT6 phosphorylation cannot be attributed due to significant cytotoxicity of drug at the tested concentrations. A moderate reduction in cellular activity was consistently observed in cells treated with 5 μM of PM-63I, suggesting slight toxicity with this species.

Although this is not a complete survey of all of the SH2 domains that could be encountered in a cell, these results show that it is indeed possible to develop inhibitors selective for the conserved STAT family. Furthermore, the surveyed proteins allowed for the identification of potential inhibition of proteins associated with regulation of cell cycle (AKT) and non-allergic immune responses (STAT1). Because of their favorable combination of biochemical and toxicity features, PM-43I and PM-86I were selected for additional in vivo studies.

In vivo allergic lung disease screen
To determine the in vivo activity of these selected compounds, we assessed the impact of PM-43I and PM-86I on the expression of IL-13-STAT5/6-dependent allergic airway disease using a fungal infectious murine model (Fig. 2a)(28). C57BL/6 mice exposed to inhaled drug vehicle (DLPC) and challenged with Aspergillus niger (AN) developed robust airway hyperresponsiveness as induced by increasing doses of acetylcholine chloride (Ach). In contrast, fungal challenged mice treated with PM-43I or PM-86I had significantly reduced maximal increases in respiratory system resistance (Rₛₛ; a measure of AHR) (Fig. 2b, c). Although both drugs significantly inhibited development of AHR, the lowest dose of PM-43I (25 μg/kg) produced the most consistent inhibition (Fig. 2c).

Lung inflammatory responses were also quantified. Compared to fungal challenged mice treated with vehicle, drug treated mice had significantly fewer cells recovered from bronchoalveolar lavage fluid (Fig. 2d), as well as marked reductions in eosinophilia (Fig. 2e). Similarly, mice treated with high and low doses of PM-43I had significantly reduced numbers of total lung cells secreting IL-4 (Fig. 2f). IL-4-secreting cells were also significantly reduced in the lungs of mice treated with 250 μg/kg of PM-86I, but again the 25 μg/kg dose of PM-43I yielded remarkably greater and more consistent suppression of eosinophils and other indices of allergic inflammation.

Antigen-specific immunity
STAT6 signals in both immune (i.e., T cells) and non-immune (i.e., airway epithelial) cells to coordinate the expression of allergic airway disease. Induction of STAT6-dependent T½2 cells is further believed to occur in secondary lymphoid organs such as the spleen even when allergen challenge occurs remotely from the spleen (e.g., the airway). Thus, we questioned if the reduced allergic disease illustrated in Figure 2 was due to systemic or local suppression of STAT6. To address this, we assessed antigen-specific cytokine recall responses from splenocytes of mice exposed to inhibitors given through distinct routes. Mice were sensitized to ovalbumin through intraperitoneal sensitization while receiving either PM-43I or PM-86I systemically (i.p.; 5,000 μg/kg) or locally (i.n.; 250 μg/kg) (Fig. 2g). Antigen-specific cytokine recall responses of splenocytes were then determined using an ELISpot format. Mice receiving either drug i.p. had significantly reduced ovalbumin-specific IL-4-secreting cells compared to vehicle (DLPC) treated mice (Fig. 2h). No difference was observed in the total numbers of IFN-γ or IL-17 secreting cells between the systemically treated mice, showing that both PM-43I and PM-86I can specifically inhibit STAT6-dependent adaptive T½2 immune responses when given systemically. In contrast, local administration of PM-43I or PM-86I to the lungs did not inhibit the development of splenic cytokine responses (Fig. 2i). These observations indicate that the reduced allergic airway disease
observed could not be attributed to impaired Th2 responses, but rather was a result of local inhibition of STAT6 in airway cells.

Dose ranging analysis
Our data thus far suggested that, although similar in their anti-inflammatory activity, PM-43I was overall more consistently effective compared to PM-86I. We therefore next sought to identify the lowest efficacious in vivo dose of drug in the allergic airway disease model, focusing on PM-43I (Fig. 3a). To improve our ability to detect subtle differences, Balb/c mice were used in these studies given their exaggerated Th2 responses and AHR in this model (29). Relative to vehicle treated animals, mice treated with PM-43I over a wide dose range (0.25 – 25 µg/kg) showed progressive reductions in AHR. Paradoxically, the efficacy of PM-43I increased inversely with respect to dose given down to a maximally effective dose of 0.25 µg/kg; at lower doses (0.025 µg/kg), the drug began to again lose the ability to control AHR (Fig. 3a, b). Similarly, airway inflammatory cells and cytokine producing cells were also significantly reduced most effectively in the lungs of mice receiving 0.25 and 0.025 µg/kg doses (Fig. 3c, d). Thus, PM-43I demonstrates therapeutic control at very low doses (2.5 µg/kg).

Phosphatase-stable pro-drug cross-reactivity
Although the initial drug screen with the fluorescent polarization assay consisted of drugs containing a naturally occurring phosphotyrosine functional group (Fig 1a; PM-28I), PM-43I has a phosphatase-stable (-CF3PO2-) motif that may influence binding efficiency and specificity for the highly conserved SH2 domains of STAT family members. Initial in vitro functional screens showed potential cross reactivity to STAT5 and, to a much lesser extent, STAT3 (Fig 1c). Sequence analysis of STAT6 (P42226.1) and the STAT6 SH2 domain showed considerably more sequence similarity to STAT5B (P51692.2; 41.55% and 49.22%, respectively) than STAT3 (P40763.2; 28.04% and 36.15%, respectively) (Fig. S1a). Further analysis of the conserved protein SH2 domain family showed that 3 of the 4 amino acids that form the SH2 domain phosphotyrosine binding pocket of STAT6 are conserved in STAT5B, but only two are conserved in STAT3. The more conservation in STAT family sequences and binding pocket amino acids makes it more likely that an inhibitor would be cross reactive.

To better characterize the potential impact of the phosphostable PM-43I on binding to the phosphotyrosine binding pocket of STAT6, STAT5B, and STAT3, we sought to determine the cell-free affinity of PM-43I to STAT6, STAT5B, and STAT3 (Fig. S1b-d). PM-43I bound recombinant STAT6 with an IC₅₀ of 1.8 µM, which reflects the in-cell STAT6 inhibitory concentration of 1-2.5 µM (Fig. S1b, Fig 1b). PM-43I bound recombinant STAT5B with slightly lower affinity (IC₅₀ = 3.8 µM) similar to the observed cell-based inhibition (Fig. S1c, Fig. 1c). In contrast, the affinity for STAT3 was found to be much lower (IC₅₀ = 29.9 µM), consistent with the limited STAT3 inhibition observed at the maximum dose tested in the initial cross-reactivity screen (Fig. S1d, Fig. 1c). Taken together, the affinity analysis appears to reflect the degree of sequence similarity among the tested STAT proteins and supports the observed STAT5/6 in vitro selectivity and in vivo attenuation of STAT5/6 dependent immunity.

In vivo structure-function analysis
To identify the structural features that make PM-43I a more potent inhibitor than PM-86I in vivo, we tested PM-43I/PM-86I hybrid prodrugs in the allergic airway disease model. PM-43I and PM-86I differ structurally at their central scaffold regions and C-termini. We created two phospho-stable structural hybrids, PM-37I and PM-205I, where these regions have been swapped (Fig. 4a). Using the lowest efficacious dose identified in the dose ranging experiment (0.25 µg/kg), a direct comparison of the hybrid compounds was performed. As expected from previous experiments, mice treated with PM-43I, but not PM-86I, had significantly reduced airway reactivity when compared to vehicle treated, fungal challenged mice (Fig. 4b, c). PM-37I, the prodrg of the first high affinity peptide mimetic (PM-301; Figure 1a) containing...
the PM-43I C-terminus and PM-86I central scaffold, failed to inhibit AHR (Fig. 4b, c). In contrast, PM-205I, containing the PM-43I central scaffold and PM-86I C-terminus, significantly inhibited AHR.

As expected, PM-43I reduced total BALF inflammatory cells, but interestingly no other inhibitor affected this quantity (Fig. 4d). All inhibitors reduced BALF eosinophils, with PM-43I showing the greatest reduction. Lung IL-4 (Fig. 4e) and IL-17 (Fig. 4f) secreting cells were also significantly reduced by PM-43I treatment, consistent with a general reduction in lung inflammation by STAT6 blockade in the lungs. Similarly, PM-205I treated mice also had significantly fewer lung IL-4 secreting cells, but only moderately reduced IL-17 (Fig. 4f). PM-37I had no inhibitory effect on cytokine producing cells and in fact caused marked upregulation of lung IFN-γ-secreting cells (Fig. 4e-g). Taken together, these data show that the central scaffold Freidinger lactam of PM-43I is the critical structural feature contributing to the superior in vivo efficacy of PM-43I and PM-205I. As evident by the moderately enhanced potency of PM-43I over PM-205I (Fig. 4f), variation in the C-terminus did not appear to significantly impact drug efficacy beyond IL-17 production.

**Efficacy of aerosolized drug**

To more accurately simulate the delivery of a peptidomimetic therapeutic clinically, we next sought to assess the efficacy of a PM-43I aerosol. PM-43I packaged in DLPC was aerosolized under standard conditions, quantified by HPLC-MS (Figure S2) and found to deliver PM-43I pro-drug at sufficient quantities for in vivo studies, without significant degradation (data not shown). Particle size analysis showed that >70% of aerosol particles containing PM-43I were of the appropriate size (0.5-3 micron) to deposit in the lower airways (Fig. 5a). These data show that PM-43I pro-drug can be stably delivered to the lower airways by aerosolization.

We next delivered PM-43I in a reversal model of pre-existing allergic airway disease, comparing aerosol versus intranasal delivery of the same dose (0.25 µg/kg; Fig. 5b). Both PM-43I-treated groups showed significantly reduced AHR after one week of therapy when compared to DLPC control (Fig. 5c). However, mice that received aerosolized PM-43I had dramatically lower, and effectively abrogated, AHR compared to i.n. treated mice, suggesting that aerosolization is a more efficient method of drug delivery to the lungs. Total lung cellularity was also significantly reduced in drug treated mice, but no significant differences were observed in lung eosinophilia (Fig. 5d).

Lung cytokine production was also assessed, with both PM-43I treated groups showing significantly reduced lung IL-4 secreting cells (Fig. 5e), but with no difference found in the number of IL-17 (Fig. 5f) or IFN-γ (Fig. 5g) secreting cells. Importantly, the total number of fungal colony forming units recovered from the lungs of challenged mice was not enhanced by drug therapy, showing that PM-43I did not impair anti-fungal immunity. These data show that PM-43I can reverse and can potentially serve as an effective therapy for established allergic airway disease, without compromising immunity against Th2-polarizing fungi that are likely causes of asthma and related allergic airway diseases of humans (30).

**Anti-viral immunity is maintained**

Given the chronic nature of asthma, affected subjects will experience intercurrent infections with a variety of pathogens, including viruses. We therefore further assessed the effect of PM-43I on lung immunity against the pulmonary viral pathogen influenza, clearance of which requires IFN-γ responses (31,32). Mice receiving a daily inhaled dose of PM-43I (75 µg/kg), were infected with influenza and monitored for changes in weight and survival over the course of the infection (Fig. S2a). Infected mice as expected showed significant weight loss and mortality, but mice treated with a relatively high dose of PM-43I were not adversely affected in either regard and in fact showed improved survival when compared to vehicle control (Fig. S2b,c). This experiment indicates that lung anti-viral immunity is maintained in the context of STAT inhibition by PM-43I.
PM-43I is renally excreted and eliminated from the lung within 48 hours

We next evaluated the pharmacokinetics of PM-43I by assessing its distribution and clearance in naïve mice using a highly sensitive HPLC-MS method (Fig. S3a-d) for identification of prodrug containing two phosphate blocking groups (Fig. S3a), one blocking group (Fig. S3b), or active drug (Fig. S3c). Mice i.n. treated with 250 µg/kg of PM-43I were assessed for drug distribution in the lungs (Fig. 6a), liver (Fig. 6b), kidney (Fig. 6c), and urine (Fig. 6d) over 48 hours. All three drug forms were identified at time 0 in the lungs, which likely reflects the time laps between treatment is tissue stabilization/freezing (Fig. 6a). The Bis-POM species was the least abundant species at time 0 and was not detectable beyond 5 minutes, showing rapid in vivo removal of at least one phosphate protecting group. The mono-POM species was the most abundant at time 0 in the lungs, but was also quickly converted to the bioactive non-POM form or cleared within 30 minutes. In contrast, bioactive non-POM drug was detectable in the lungs beyond 24 hours after i.n. treatment and was not fully cleared until 48 hours.

In the liver, only the non-POM species was identified with peak abundance at 5 minutes (Fig. 6b). Trace quantities of the mono-POM species were identified in the kidneys at time 0, but non-POM was the predominant species identified and cleared by 30 minutes (Fig. 6c). Only the non-POM species was detected in the urine, with levels peaking at one hour (Fig. 6d). Together, these data indicate that PM-43I prodrug is rapidly taken up intracellularly at the site of delivery, converted to active drug rapidly and then retained within the lung for more than 24 hours. Systemic distribution of drug appears to be limited to the cell-impermeable, non-POM species and is likely due to the high blood volume of these tissues. Active non-POM drug is excreted primarily in the urine and not retained in either the kidney or liver.

Impact of PM-43I on long-term immunity and toxicity

To determine if PM-43I elicits any significant toxicity, we evaluated body weight, blood chemistry, CBC, lung function, splenic immune cellularity, and humoral immunity in naïve mice that received PM-43I 3 times a week at doses 1000-fold more than the effective dose for up to 8 months, while receiving antigen once a week (Fig. 7a). Long-term exposure to PM-43I had no significant impact on airway reactivity when compared to vehicle control (Fig. 7b). Further, PM-43I did not impair weight gain (Fig. 7c). Similarly, blood cellularity (Fig. S4a), blood chemistry (Fig. S4b) and splenic cellularity (Fig. S4c, d) were not different between vehicle and PM-43I treated mice after 8 months of therapy. Serum ova-specific antibody responses revealed that the ratio of antigen-specific IgG2a (Tn1- associated) to IgE (Tn2-associated) antibody isotypes was significantly increased after 8 months of PM-43I treatment in mice that has simultaneously immunized with ovalbumin, suggesting that long-term therapy may suppress previously established allergic memory responses (Fig. 7d). Overall, these data suggest that long-term treatment of mice with PM-43I is non-toxic, but does influence antibody isotype profiles, favoring type 1 over type 2 antibodies.

DISCUSSION

Based on seminal work conducted in the mouse (33,34) and verified through human clinical trials (19,20), the IL-4/IL-13-IL-4Rα-STAT5/6 signaling pathway is now recognized as a critical to determining the expression of asthma and related allergic diseases. However, because of redundant and alternative pathways for activation of STAT6 (e.g., through TSLP (17), LABAs (25), and IL-4 (35)), therapeutics that target the extracellular limb of this pathway (including cytokines and receptors) will likely fail to completely suppress STAT6 activation. Additional redundant cytokine pathways support STAT5 activation, underscoring this concern (36). Thus, existing and future asthma therapeutics that target STAT5/6 ligands and receptors are destined to be incompletely effective in blocking unwanted allergic responses. Moreover, the agents that have been developed in this regard are all monoclonal antibodies, which are prohibitively expensive especially for patients with mild to moderate disease. Additionally, monoclonal antibodies must be delivered systemically, increasing the
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The high specificity of monoclonal antibodies can be achieved through the intelligent design of mimetic peptides around known protein-protein interactions. Several groups have embraced this strategy and developed large peptides (> 10 amino acids) to modulate BH3-mediated apoptosis, SOCS modulation of inflammation, and HDL-driven cardiovascular disease (37,38,40). We have previously reported our work developing small peptidomimetics (4-mers) targeting the SH2-domain of STAT3 and the suppression of tumor growth (24,26,39). Here we report the development of a novel phosho-peptidomimetic small molecule that is based on the cytoplasmic docking site on IL-4Rα for STAT6, PM-43I.

Targeting the SH2 domain of STAT6, PM-43I is also a potent inhibitor of STAT5. As STAT5/6 require activation through their SH2 domains in all contexts and share a significant degree of sequence similarity, PM-43I is theoretically capable of completely inactivating STAT5 and STAT6 regardless of the activating stimulus. Indeed, the highly effective nature of PM-43I in our experimental asthma model suggests that we are achieving inhibition of most of the latent STAT5/6 within mouse airway cells at the observed therapeutic doses. As a small molecule, PM-43I is relatively easily synthesized in large quantities and to high purity. Due to the low dose required (0.25 – 25 µg/kg) and the need only for topical delivery to the airway to be highly effective, PM-43I should compare economically favorably to monoclonal antibody-based asthma therapies. Moreover, PM-43I appears in our preliminary studies to be well tolerated while at the same time highly effective. Together, our findings indicate that PM-43I is suitable for entry into clinical development in asthma and related afflictions.

Phospho-peptidomimetics represent a new class of pharmacologic agents that have been developed successfully to target the SH2 domains of a variety of signaling proteins (33,26,39), but have not previously been evaluated for the blockade of multiple STAT factors or for the treatment of asthma. Previously, we developed the phospho-peptidomimetic prodrug PM-242H to target STAT6 selectively and demonstrated that this novel agent was effective at both preventing and reversing asthma-like allergic airway disease in mice (25). Although PM-242H proved the validity of this therapeutic strategy, this agent has an unfavorably high minimum effective dose of 2.5 mg/kg. The iterative redesign of PM-242H as presented here was undertaken largely to improve compound potency, but also to determine the efficacy of more broadly cross-reactive STAT inhibitors.

Our in vitro observations were critically important in informing our choice of compounds selected for subsequent in vivo testing. Specifically, based on in vitro testing, we selected PM-43I as one compound that inhibited STAT5 and STAT6, and PM-86I as a compound that was highly selective for inhibiting STAT6. Although PM-43I inhibited STAT6 less effectively than PM-86I in vitro, PM-43I performed significantly better in vivo regarding inhibition of multiple parameters of allergic airway disease (Fig. 2). We presume that PM-43I was more effective because of its ability to inhibit STAT5 in addition to STAT6. The superior in vivo response may be due to the inhibition of the Stat5-dependant ILC2-driven innate immune responses that promote the Stat6-dependant Th2 adaptive immunity, both of which contribute to the expression of this disease phenotype (12-18,41-44). Another compound, PM-63I, also inhibited STAT5 and was at least 10-times more effective than PM-43I at inhibiting STAT6 (Fig. 1a-c). However, we did not pursue in vivo studies with this compound due to the enhanced cellular toxicity exhibited against two cell lines at the lower tested doses (Fig. 1d,e). Nonetheless, the in vivo potency of PM-43I, which potentially extends to the entire family of molecules that we have synthesized, suggests that such in vitro toxicity might be irrelevant in vivo. Therefore, further studies with PM-63I using the same mouse model to determine the therapeutic potential of this compound may be warranted.
The chronic, incurable nature of asthma mandates that approved treatments and pharmaceuticals be selected such that they minimize long-term side effects. However, standard asthma care includes the use of pharmaceuticals with relatively poor long-term safety records. The original LABAs approved for use in the United States, formoterol and salmeterol, lead to loss of disease control and excess asthma-related mortality in a small subset of patients that is, in part, but not completely (45), alleviated by the co-formulation with inhaled corticosteroids as now mandated by the Food and Drug Administration. However, inhaled and especially oral corticosteroids have their own notorious, long-term safety concerns that include laryngitis, diabetes, weight gain, hypertension, cataracts, etc. Serious fungal infectious complications of chronic corticosteroid use (46-49) are particularly concerning because asthma and related conditions such as chronic rhinosinusitis are increasingly recognized as manifestations of airway mycosis (airway fungal infection) (30). A primary benefit of agents such as PM-43I that are highly selective in their inhibition of immune pathways is that they are less likely to compromise protective immunity compared to broad-spectrum immunosuppressants like corticosteroids. Indeed, our preliminary toxicity studies suggest that PM-43I did not impact airway antifungal or antiviral immunity even when given in excessive doses (Fig. 5h; Sup. Fig. 2).

An important component of the drug delivery strategy presented here is the direct delivery of pro-drug to the lungs and deposition on the target cells of the airway, where it is then converted, extracellularly and intracellularly, to active drug by removal of the POM groups by reactive oxygen species, ions and/or esterases. Delivery of the drug by aerosol bypasses the caustic gastric and first pass metabolism to allow the immediate cellular absorption and intracellular activation of PM-43I in the lungs where the drug persists for more than 24 hours (Fig. 6). It is important to note that extracellular activation, by removal of the protective POM groups, abrogates membrane permeability and systemic drug activity (Fig. 2h, i), allowing efficient renal clearance of nonabsorbed drug within hours of airway delivery (Fig. 6). We cannot be certain which airway cells primarily uptake PM-43I, but presume that airway epithelial and smooth muscle cells are main PM-43I targets given their proximity to the airway lumen. Other airway cells are likely to be targeted also and the more that are affected by PM-43I, the stronger the suppressive effect on allergic disease expression should be.

The efficient renal clearance of non-POM drug is a critical observation and supports the apparent lack of toxicity observed in the 8-month toxicity study (Fig. 7). The potential toxicity of the inactive POM group byproducts – formaldehyde and pivalic acid – could be a concerning factor, but it is well known that both formaldehyde and pivalic acid are readily metabolized and excreted at the therapeutic range of PM-43I (50-54). Additionally, numerous drugs containing POM-groups (Adefovir, Pivampicillin, Cefditoren, Valproate, etc.) are now FDA approved and regularly used in the clinic.

Numerous drugs targeting immune molecules have been evaluated in asthma, many of which have proven successful (e.g., drugs targeting IL-5 and IgE), but not all. PM-43I offers several distinct advantages over all of these agents. First, as a small, easily synthesized molecule, it avoids the complexities and expense of biological agents such as monoclonal antibodies. Second, whereas biologics that individually target STAT6-activating cytokines have been developed, none individually blocks STAT6 completely and none block both STAT5 and STAT6 as does PM-43I. PM-43I is therefore predicted to be both more effective and less expensive than existing agents.

The direct delivery by aerosolization and activation of PM-43I in the lungs is an additional novel aspect of this drug that minimizes possible systemic toxic effects that are unavoidable with parenterally-administered biologics. Assuming immediate and complete POM-group breakdown and pro-drug molar equivalence for the observed PM-43I therapeutic range (0.25 – 25 µg/kg), a 15-minute aerosol treatment would produce a formaldehyde concentration (0.006 – 0.6 ppm) below the OSHA defined 15-minute permissible exposure limit (PEL) of 2 ppm and the 8-hr PEL of 0.75
ppm (53). Furthermore, the EPA estimated average indoor formaldehyde concentration of 0.03 ppm is well above the optimal PM-43I therapeutic dose (2.5 µg/kg; 0.006 ppm). Together, these data suggest that concerns regarding PM-43I-related toxicities related to the POM groups are negligible.

Although PM-43I demonstrated dose-dependent efficacy in vivo, the inverse in vivo relationship between dose and efficacy is unusual, such that the drug begins to lose efficacy at doses higher than the maximally effective dose of 0.25µg/kg. This issue highlights the complexities of targeting the intracellular compartment where many distinct potential targets exist, including more than 140 SH2 domain-containing proteins that are potentially cross-reactive targets. Although PM-43I did not cross react at highly effective pharmacologic doses with other STAT proteins of considerable sequence homology (Fig. S1a) and other critical immune signaling proteins such as AKT and FAK (Fig. 1C), potential cross reactivity with the SH2 domains of suppressors of cytokine signaling 1 and 3 (SOCS1,3) could block their ability to inhibit STAT6 activity, in effect extending the activity of STAT6 that was not inactivated by drug (55-57). Similarly, protein tyrosine phosphatase non-receptor type 6 (PTPN6; SHP-1) is an SH2 domain-containing phosphatase that dephosphorylates active STAT factors, especially STAT5 and STAT6 (58,59). Inhibition of this protein via its SH2 domain with PM-43I could potentially produce paradoxically enhanced STAT5/6 activity at supra-therapeutic doses of PM-43I. If additional testing suggests that PM-43I possesses an excessively narrow therapeutic window related to such potential cross-reactivity, PM-63I could be explored further as a therapeutic alternative or PM-43I could be further chemically modified to reduce such cross reactivity while preserving specificity for STAT5/6.

Concluding remarks

We have developed a potent inhibitor of STAT5 and STAT6 that can be stably delivered to the lungs and inhibit the induction of or reverse pre-existing allergic lung disease in a murine model of allergic lung disease. The lead compound does not appear to impair immunity or present with any apparent toxicity, but can skew circulating humoral immunity towards a less dominant Th2 profile. Our studies confirm the feasibility to target the SH2 domain therapeutically and indicate that PM-43I is suitable for entry into clinical development.
**EXPERIMENTAL PROCEDURES**

**Drug/Inhibitor**

1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC; 770335) was acquired from Avanti Polar Lipids (Alabaster, AL) and used as a vehicle at a 1:5 (drug:DLPC) ratio. In brief, drug and DLPC were solubilized in t-butanol at the identified mass ratio, frozen and lyophilized. Individual treatments were suspended in PBS and sonicated for 30 seconds before use.

**Fluorescence Polarization Assays.**

Serial dilutions of peptides (200 µM) in “fluorescence polarization buffer” (FPB, 50 mM NaCl, 10 mM HEPES, 1 mM Na4EDTA, 2 mM DTT, and 1% NP-40) were prepared. Aliquots of STAT6 (480 nM) and FAM-Ala-pTyr-Lys-Pro-Phe-Gln-Asp-Leu-Ile-NH2 (20 nM) were plated in a 96-well plate and peptide dilutions were added. Fluorescence polarization was then read on a Tecan Infinite F200Pro plate reader and IC$_{50}$ values were obtained from the non-linear regression analysis.

**Aerosol Characterization**

Drug (PM-43I) or DLPC was aerosolized using an Aerotech II nebulizer with a flow rate of 10 L/min. Aerosols were captured using an all-glass Impinger under a vacuum. Aerosol particle size was determined with an Andersen cascade impactor. Captured drug was quantified by HPLC-MS and used to calculate drug dose for animal studies as previously reported (60-63).

**Cell Culture**

A549, BEAS-2B, and MD468 cells were acquired from American Type Culture Collection (Manassas, VA). Cells were maintained in 50% DMEM and 50% F-12 complete media until sufficient confluency and switched to 2% FBS 24 hour prior to stimulation. Cells were pretreated up to 2 hours with identified drugs at various concentrations, stimulated with IL-4 (2ng/mL; RnD), EGF (100ng/mL; RnD), or IFN-γ (25ng/mL; R&D) for identified times, and harvested for protein analysis.

**Western Blot**

Total protein was harvested from cell with RIPA buffer (9806S; Cell Signaling, Danvers, MA), quantified with BCA Protein Assay Reagent (23227; Thermo Fisher Scientific, Rockford, IL), and denatured with Laemmli sample buffer (161-0737; Bio-Rad, Hercules, CA) according to manufacturer’s protocol. Proteins were analyzed by separation in hand-poured SDS-PAGE gels with standard electrophoretic units (Bio-Rad, Hercules, CA) and transferred to PVDF membranes with iBlot Gel Transfer Device (IB1001; Invitrogen, Grand Island, NY). After blocking with 2% FBS PBST, membranes were probed for pSTAT6, STAT6, pSTAT5, STAT5, pSTAT3, STAT3, pAKT, AKT, pFAK, FAK, pSTAT1, STAT1, and β-actin. Protein signals were detected using the ChemiDoc XRS+ system (BioRad; Hercules, CA).

**Mice**

4-8 week old female Balb/c, C57BL/6, and CR1 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in the American Association for Accreditation of Laboratory Animal Care-accredited Transgenic Mouse Facility at Baylor College of Medicine under specific pathogen free conditions. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine and followed federal guidelines.

**Infectious Allergic Lung Disease/Reversal Models**

Mice were challenged intranasally every other day with 4 × 10$^5$ A. niger conidia for a minimum of 6 challenges and treated with the identified STAT6-inhibitors at identified doses and/or vehicle (DLPC) as described (Infectious allergic lung disease model, Figure 3a; Reversal model, Figure 7a) for each experiment. Following the final challenge, mice rested for a day and allergic airway disease was assessed.

**Immunosuppression Model**

Mice were concurrently sensitized to Ova-alum by i.p. and immunosuppressed with 5 µg PM-43I, PM-86I or vehicle control (DLPC) as detailed in Figure 4. Briefly, mice were vaccinated with alum-bound ovalbumin once a week for 2 weeks, and PM-43I (5 µg), PM-86I (5 µg), or DLPC (25 µg) by i.p. (systemic immunosuppression) or i.n. (local...
imunosuppression). Splenocytes were harvested and assessed for ova-specific responses.

**Allergic Airway Disease Analysis**

Allergic airway disease was assessed in accordance to previous reports (64). Increases in airway resistance ($R_{RS}$) due to intravenous acetylcholine, bronchoalveolar lavage (BAL) fluid differential counts, and lung IL-4, IL-17A and IFN-$\gamma$ secreting cells were quantified as previously described (64,65).

**Ovalbumin Restimulation**

Splenocytes from Ova-sensitized mice were assessed for antigen-specific recall by ELISpot for relevant cytokine. In brief, cells were cultured in the presence of media or whole ovalbumin (1 mg/mL) overnight and plates were developed for IL-4, INF-$\gamma$ and IL-17 as previously described.

**Histology**

Lungs were perfused with 4% paraformaldehyde and embedded in paraffin. Lung sections were cut and stained for mucus with the Periodic Acid-Schiff (PAS) kit (395B) from Sigma-Aldrich (St. Louis, MO) or cells with H&E. Lung sections were immunohistologically stained for p S6 and pSTAT5.

**Fungal Burden**

Fungal burden in fungal challenged mice was determined with serial dilutions on Sabouraud agar plates (84088; Sigma Aldrich, St. Louis, MO) of lung homogenates, in the presence of 100 mg/mL chloramphenicol (C0378; Sigma Aldrich, St. Louis, MO). After an overnight incubation at 37$^\circ$C, plates were assessed for fungal colony forming units/lung from dilution.

**Influenza Infection**

Mice were placed on PM-43I (1.5$\mu$g) or DLPC aerosol therapy prior to challenge with an LD$_{30}$ of aerosolized influenza (H3N2), as detailed in Figure 6a. Changes in weight and survival were monitored throughout the experiment.

**Toxicity**

Mice were sensitized to Ova-alum by $i.p.$ (1/wk, x2) and treated with PM-43I (5$\mu$g) or DLPC for 8 months, as described in Figure 10a. In brief, sensitized mice were intranasally treated every other day with ovalbumin plus PM-43I or DLPC and monitored for changes in weight. Airway hyper-reactivity and ova-specific antibody were assessed as described. Blood and spleens were harvested at the end of the experiment for further analysis. Blood samples were submitted to the pathology core at The University of Texas MD Anderson Cancer Center for complete blood counts and blood chemistry. Detailed cellular analysis of splenocytes was performed by the Flow cytometry core at Baylor College of Medicine.

**Flow Cytometric analysis**

Spleens were disassociated using an Octo Dissociator (Miltenyi Biotech) in RPMI 1640 containing (Gibco) 10% heat inactivated FBS (HyClone), 2mM EDTA (Gibco), 0.2 mg/ml DNase I (Sigma), 1 mg/ml Collagenase II (Gibco). The suspension was incubated at room temperature for 20 minutes. Digestion was stopped by the addition of EDTA to a 12 mM concentration. Cells were filtered through a 40 $\mu$m mesh. Cells were collected and red blood cells lysed in RBC Lysis Buffer (eBioscience) for 5 minutes at room temperature. Cells were washed and re-suspended in Flow Buffer (DPBS without Ca++/Mg++ (Gibco) supplemented with 2% FCS, 25mM HEPES, 2mM EDTA). A portion of the cells were used for enumeration and trypan blue viability assessment using a ViCell (Beckman Coulter). Fc receptors were blocked on ice for 10 minutes using Flow Buffer containing 1:250 dilution of anti-mouse CD16/32 (BD Biosciences). Cells were incubated on ice for 30 minutes with one of two panels of antibodies, Panel 1 and Panel 2. Panel 1 includes anti-mouse CD5, CD4, CD44, CD8, CD25, CD161 and CD62L (BD Biosciences). Panel 2 includes anti-mouse CD5, Ly6G CD19, Ly6C, CD21/35, CD11b, CD11c, CD161, CD23 (BD Biosciences) and Anti-Mouse MHCII (Biolegend). Both groups were washed and resuspended in Flow Buffer with 1 drop/ml Nuc Blue Fixed DAPI Stain (Life Technologies). Single stained DAPI control and FMOs were prepared from remaining pooled spleen cells. Cells were run on a LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo.
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(Treestar) according to immunophenotyping gating strategies outlined by the International Mouse Phenotyping Consortium.

Ova-specific ELISA
Serum samples were serially diluted and incubated in 96-well plates coated with ovalbumin. Captured antibody were detected with biotinylated anti-IgE and anti-IgG2a, and developed with streptavidin-alkaline phosphatase. Substrate was added and signal was detected by optical density at 405 nm.

Pharmacokinetic Analysis

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CONFLICT OF INTEREST
JMK, PMandal, PMorlacchi, DBC and Atrapos Therapeutics, LLC hold intellectual property rights in compound PM-43I.

AUTHOR CONTRIBUTIONS
JMK, PMandal, PMorlacchi, GM, EL, MM, CL, BS, EF, BG, and JS conducted experiments, contributed essential data, and critically reviewed the manuscript; AV, MS, and DC analyzed and critically interpreted the data and revised the initial manuscript draft. JSM and DBC conceived the project. JMK and DBC designed the experimental flow and wrote the initial and revised manuscripts. All authors gave final approval of the manuscript version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Figure Legends

Figure 1. **In vitro** STAT6 inhibitor screen. (a) Structural variations assessed for binding affinity in fluorescence polarization analysis. (b) Structure of pro-drugs PM-43i, PM-74i, PM-63i, PM-86i, PM-80i, and PM-81I tested in BEAS-2B cells, pretreated (2hr) with drug or vehicle control (DMSO). Treated cells were stimulated with IL-4 (2ng/mL; 1hr) and assessed for phospho- and total STAT6 by western blot. (c) Cross-reactivity of STAT6 inhibitors to additional SH2-domain containing proteins. MDA-488-MB cells were pre-treated with identified inhibitors. Treated cells were then stimulated with EGF (100ng/mL) or INF-γ (25ng/mL) for 30 min and assessed for STAT5, STAT4, AKT, FAK, and STAT1 activation. Pro-drug toxicity was assessed in BEAS-2B (d) and MDA-468-MB (e) cells by MTA oxidation assay.

Figure 2. **In vivo** comparison of PM-43I and PM-86I in the allergic lung disease model. (a) C36BL/6 mice were challenged every other day with *A. niger* (AN), treated with drugs PM-43I, PM-86I, or vehicle (PBS/DLPC) for 16 days, and airway hyperresponsiveness (AHR) to increasing (b) and peak (5th dose only; c) doses of acetylcholine. Total bronchial airway lavage (BAL) cells (d), BAL differential counts (e), and IL-4 secreting cells (f) in the lungs of challenged mice were quantified. Immune suppression model (g) where C57BL/6 mice were sensitized to Ovalbumin with i.p. injections of Ova-alum, treated with PM-43I, PM-86I, or DLPC (vehicle control) by weekly systemic i.p. injection (b) or daily local i.n. delivery (c) and splenocytes were restimulated and assessed for Ova-specific responses. * P < 0.05 (n ≥ 3 mice). Data are representative of two or more independent experiments.

Figure 3. Titration of PM-43I in allergic lung disease model. Balb/c mice were intranasally treated daily with PM-43I (0.025 – 25 µg/kg) or vehicle (DLPC) and challenged every other day with *A. niger* (AN). The effect on airway hyper-responsiveness (AHR) to increasing (a) and peak (b) doses of acetylcholine, BAL cellularity (c), and lung cytokine production (d). *: P < 0.05, N ≥ 3/treatment group. Ns = not significant. Data are representative of two or more independent experiments.

Figure 4. **In vivo** hybrid structure analysis. (a) Matrix showing the cross comparison (central scaffold vs. C-terminus) of PM-43I and PM-86I structures evaluated in the allergic lung disease model. Balb/c mice were treated daily with 0.25 µg/kg of PM-37I, PM-43I, PM-86I, PM-205I, or vehicle control (DLPC) and challenged every other day with *A. niger* (AN) or PBS (DLPC, grey). Airway hyper-reactivity was measured in response to increasing (b) and peak (c) doses of acetylcholine. Bronchial airway lavage (BAL) differential counts (d), IL-4 (e), IL-17 (f), and IFN-γ secreting cells (g). *: P < 0.05, n ≥ 3. Data are representative of two or more independent experiments.

Figure 5. Aerosol characterization and allergic lung disease reversal. (a) Aerosol particle size and predicted lung distribution. (b) Balb/c mice were intranasally challenged with *A. niger* (AN) for two weeks and airway hyper-responsiveness was determined. Daily therapy of 0.25 µg/kg PM-43I was then given intranasally or by aerosol and fungal challenges were continued for an additional two weeks. Airway hyper-responsiveness was assessed weekly (c). Total BALF inflammatory cells (d), total lung IL-4 (e), IL-17A (f) and IFN-γ (g) secreting cells, and total recovered lung fungal CFU (h) were quantified at the end of the experiment. *: P < 0.05, n ≥ 6 mice/treatment group. Data are representative of two or more independent experiments.

Figure 6. Pharmacokinetics of PM-43I. CR1 mice were i.n. treated with 250 µg/kg of PM-43I, harvested over 48 hours, and lungs (a), liver (b), kidney (c), and urine (d) were analyzed for pro-drug state (Bis-, Mono-, and Non-POM). n ≥ 3. Data are representative of two independent experiments.

Figure 7. Long-term PM-43I treatment promotes antigen-specific Th1 antibody. (a) C57BL/6 mice were Intraperitoneally sensitized to Ovalbumin (1/wk x2), rested (2wk), and treated intranasally with Ova and PM-43I (250 µg/kg) every other day for 8 months. Changes in weights were monitored (b) and final airway hyperresponsiveness to acetylcholine (c) was assessed. Serum Ova-specific antibody was assessed (d). *: p < 0.05 one-tailed student t-test; n = 7. Data are representative of two independent experiments.
Figure 1

| Compound | Structure | IC₅₀ (µM) |
|----------|-----------|-----------|
| PM-63I   | [Image of molecule] | 0.13 ± 0.03 |
| PM-301H  | [Image of molecule] | 0.63 ± 0.013 |
| PM-80I   | [Image of molecule] | 2.33 ± 0.5  |
| PM-34I   | [Image of molecule] | 0.28 ± 0.06 |

**Central Scaffold**

| Compound | Structure | IC₅₀ (µM) |
|----------|-----------|-----------|
| PM-301H  | [Image of molecule] | 0.12 ± 0.03 |
| PM-67I-A | [Image of molecule] | 0.37 ± 0.09 |
| PM-67I-B | [Image of molecule] | 0.10 ± 0.05 |
| PM-59I   | [Image of molecule] | 1.9 ± 0.84 |
| PM-87I   | [Image of molecule] | 0.26 ± 0.09 |
| PM-71I-A | [Image of molecule] | 0.24 ± 0.13 |
| PM-71I-B | [Image of molecule] | 0.23 ± 0.12 |

**C-terminus**

| Compound | Structure | IC₅₀ (µM) |
|----------|-----------|-----------|
| PM-63I   | [Image of molecule] | 0.05 ± 0.04 |

**Prodrug IC₅₀**

| Prodrug | IC₅₀ (µM) |
|---------|-----------|
| PM-63I  | 0.13 ± 0.03 |
| PM-301H | 0.63 ± 0.013 |
| PM-80I  | 2.33 ± 0.5  |
| PM-34I  | 0.28 ± 0.06 |

**Compound Structure**

**Prodrug Conc. [µM]**

- PM-63I: 0.05 ± 0.04
- PM-301H: 0.10 ± 0.05
- PM-80I: 2.33 ± 0.5
- PM-34I: 0.28 ± 0.06
Figure 3

(a) R_{RS} (cm H_2O • s • ml^{-1})

- DLPC
- PBS

(b) R_{RS} (cm H_2O • s • ml^{-1})

- DLPC
- 25 µg/kg
- 2.5 µg/kg
- 0.25 µg/kg
- 0.025 µg/kg

(c) Cells/Lung (x10^5)

- Total
- Eos
- Mono
- Neut
- Lym

- DLPC
- 25 µg/kg
- 2.5 µg/kg
- 0.25 µg/kg
- 0.025 µg/kg

(d) Cytokine Cells/Lung (x10^4)

- IL-4
- IFN-γ

- DLPC
- 25 µg/kg
- 2.5 µg/kg
- 0.25 µg/kg
- 0.025 µg/kg

* indicates statistical significance.
Figure 4

**a.** C-terminus

**b.**

**c.**

**d.**

**e.**

**f.**

**g.**
Figure 5

a. Distribution of the aerosol particles through the preseparator and stages of the inhaler system.

b. Timeline of challenges and therapy weeks for assessing airway hyperreactivity.

Challenges:
- A. niger

Therapy Week (AHR):
- 0
- 1
- 2

Assess Inflammation

Day:

Challenge:
- DLPC/PM-43I (0.25 µg/kg)

PM-43I (Aerosol)
PM-43I (Intra Nasal)
DLPC (Aerosol)

Assess airway hyperreactivity:

Therapy (wk): 0 2 4

Dose Ach (mg g⁻¹)

c. Graph showing the relationship between dose of Ach and Rrs (cm H₂O • s • mL⁻¹).

d. Graph showing BAL Fluid cell counts across different categories.

e. Graph showing IL-4+ cells per lung.

f. Graph showing IL-17+ cells per lung.

g. Graph showing IFN-γ+ cells per lung.

h. Graph showing CFU/Lung.

Legend:
- DLPC (Aerosol)
- PM-43I (Intra Nasal)
- PM-43I (Aerosol)
Figure 6

a. Lung
b. Liver
c. Kidney
d. Urine

PM-43I Pro-drug State

- Bis-POM
- Mono-POM
- Non-POM
Figure 7

a. Ova-Alum Sensitization

b. 

PM-43I
DLPC

Dose Ach (mg g⁻¹)

Base 0.03 0.1 0.3 1.0 3.2

Ova (q.wk.) +/- PM-43I
(250 µg/kg Q.O.D. for 6 months)

Rest (2wk)
Assess Antigen Specific Antibody and Toxicity

Weight (g)

30
25
20
15
0 4 6 8

Month

PM-43I
DLPC

PM-43I
DLPC

IgG2a/IgE [Abs]

0 4 6 8
15
20
25
30

Figure 7

Assess Antigen Specific Antibody and Toxicity

Rest (2wk)

PM-43I
DLPC

PM-43I
DLPC

PM-43I
DLPC

PM-43I
DLPC

PM-43I
DLPC

PM-43I
DLPC

PM-43I
DLPC

PM-43I
DLPC
Small Molecule Targeting of the STAT5/6 Src Homology 2 (SH2) Domains to Inhibit Allergic Airway Disease
J. Morgan Knight, Pijus Mandal, Pietro Morlacchi, Garbo Mak, Evan Li, Matthew Madison, Cameron Landers, Brandon Saxton, Ed Felix, Brian Gilbert, Joel Sederstrom, Atul Varadhachary, Melissa M. Singh, Dev Chatterjee, David B. Corry and John S McMurray

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