Preclinical Evaluation of an Inhibitor of Cytosolic Phospholipase A₂α for the Treatment of Asthma

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Received July 27, 2011; accepted December 6, 2011

ABSTRACT

Asthma is a chronic inflammatory lung disease with considerable unmet medical needs for new and effective therapies. Cytosolic phospholipase A₂α (cPLA₂α) is the rate-limiting enzyme that is ultimately responsible for the production of eicosanoids implicated in the pathogenesis of asthma. We investigated a novel cPLA₂α inhibitor, PF-5212372, to establish the potential of this drug as a treatment for asthma. PF-5212372 was a potent inhibitor of cPLA₂α (7 nM) and was able to inhibit prostaglandin (PGD₂) and cysteinyl leukotriene release from anti-IgE-stimulated human lung mast cells (0.29 and 0.45 nM, respectively). In a mixed human lung cell population, PF-5212372 was able to inhibit ionomycin-stimulated release of leukotriene B₄, thromboxane A₂, and PGD₂ (2.6, 2.6, and 4.0 nM, respectively) but was significantly less effective against PGE₂ release (>301 nM; p < 0.05). In an invitro cell retention assay, PF-5212372 retained its potency up to 24 h after being washed off. In a sheep model of allergic inflammation, inhalation of PF-5212372 significantly inhibited late-phase bronchoconstriction (78% inhibition; p < 0.001) and airway hyperresponsiveness (94% inhibition; p < 0.001), and isolated sheep lung mast cell assays confirmed species translation via efficacious inhibition of PGD₂ release (0.78 nM). Finally, PF-5212372 was assessed for its ability to inhibit the contraction of human bronchi induced by AMP. PF5212372 significantly inhibited AMP-induced contraction of human bronchi (81% inhibition; p < 0.001); this finding, together with the ability of this drug to be effective in a wide range of preclinical asthma models, suggests that inhibition of cPLA₂α with PF-5212372 may represent a new therapeutic option for the treatment of asthma.

Introduction

Asthma is a chronic inflammatory disorder of the airways that causes recurrent episodes of wheezing, breathlessness, chest tightness, and coughing in susceptible individuals. The disease represents a significant global disease burden, with major socioeconomic consequences. Asthma prevalence has been increasing in recent decades in the developed world (particularly among children) and is estimated at ~5 to 10% of the population in developed countries (Global Initiative for Asthma, 2002).

Although anti-inflammatory therapeutic options exist for the treatment of asthma, there remain unmet medical needs for agents that display high degrees of efficacy in the absence of adverse side effects. Cytosolic phospholipase A₂α (cPLA₂α) is the rate-limiting enzyme that is ultimately responsible for the production of eicosanoids implicated in the pathogenesis of asthma. We investigated a novel cPLA₂α inhibitor, PF-5212372, to establish the potential of this drug as a treatment for asthma.
of significant adverse effects after chronic administration. Inhaled and orally administered steroids have been shown to be effective anti-inflammatory agents in the treatment of asthma, but chronic use of these agents may be associated with a range of side effects (e.g., dysphonia, oral candidiasis, and suppression of the hypothalamic–pituitary axis), especially at high doses (Baptist and Reddy, 2009).

An asthma therapy that inhibits cytosolic phospholipase A₂α (cPLA₂α) may address these unmet medical needs. cPLA₂α releases arachidonic acid from the phospholipid membrane and is the rate-limiting enzyme in the biosynthesis of prostaglandins (PGs), thromboxanes, and leukotrienes (LTs) (Barnes et al., 2009), all of which are implicated in airway inflammation, mucus production, bronchoconstriction, and airway hyper-responsiveness (AHR) associated with asthma (Drazen et al., 1999). LTs are known to contribute to inflammation by both recruiting and activating leukocytes, whereas cysteinyl leukotrienes (LTC₄, LTD₄, and LTE₄) are powerful bronchoconstrictors that promote edema and metachronal migration of blood eosinophils and basophils, as well as inflammation by both recruiting and activating leukocytes, whereas cysteinyl leukotrienes (LTC₄, LTD₄, and LTE₄) are powerful bronchoconstrictors that promote edema by increasing vascular permeability and permitting leakage of plasma into the extravascular space (Boyce, 2008). Both 5-lipoxygenase inhibitors (e.g., zileuton) and leukotriene receptor antagonists (e.g., montelukast) have been shown to reduce eosinophil numbers in sputum and then 40-100 𝑚 cell strainers. The cleared cell preparation was processed again before the lung homogenate was passed through a gentleMACS dissociator (Miltenyi Biotec) before incubation at 37°C with 5% CO₂ in a humidified incubator.

Subthreshold contractile concentrations of PGD₂ have been demonstrated to increase AHR to inhaled histamine and methacholine (Fuller et al., 1986) and to increase acute bronchoconstriction (Johnston et al., 1995), and PGD₂ levels in the lungs are increased after allergen challenge of subjects with asthma (Murray et al., 1986). PGD₂ has been implicated in multiple aspects of allergic inflammation mediated through the chemoattractant receptor expressed on T helper type 2 cells (CRTH2). This receptor is preferentially expressed on T helper type 2 cells, eosinophils, and basophils in humans and has been shown to mediate PGD₂-dependent cell migration of blood eosinophils and basophils, as well as intracellular calcium mobilization and chemotaxis in T helper type 2 cells (Nagata and Hirai, 2003). Data from subjects with asthma demonstrated positive effects, with a CRTH2 antagonist being able to reduce eosinophil numbers in sputum, to reduce circulating IgE levels, and to improve forced expiratory volume in 1 s (Barnes et al., 2008). Both 5-lipoxygenase inhibitors (e.g., zileuton) and leukotriene receptor antagonists (e.g., montelukast) have been shown to have efficacy in the treatment of asthma (Price et al., 2011).

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Materials and Methods

cPLA₂α Enzyme Assays. The 7-hydroxycoumarinyl-γ-linolenate (GLU) micelle enzyme assay was used as described previously (McKew et al., 2008). The purpose of the present investigation was to establish the preclinical effects of PF-5212372 as a novel anti-inflammatory treatment for asthma.

Compound and Vehicle Used for In Vitro and Ex Vivo Experiments. PF-5212372 and all other compounds used in this investigation were prepared in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) and were assessed at a final assay concentration of dimethyl sulfoxide of 0.1% (v/v). All vehicle controls also were assessed by using a final assay concentration of dimethyl sulfoxide of 0.1% (v/v).

Human Lung Mast Cell Assay. Mast cells were isolated through physical and enzymatic dispersion of lung tissue obtained from surgical resections, according to methods described elsewhere (Ali and Pearse, 1985). Ethical approval for the types of experiments to be performed was in place, and all patients who donated tissue gave informed consent. Mast cells were incubated with a variety of concentrations of PF-5212372 for 15 min before being activated with 2 μg/ml human anti-IgE (clone HP6061; Hybridoma Reagent Laboratory, Baltimore, MD). Supernatants were harvested 25 min later and were stored at −80°C until analysis.

Sheep Lung Mast Cell Assay. Lung tissue from Suffolk Crosses was obtained from Matrix Biologicals (Hull, UK). Mast cells were isolated through physical and enzymatic dispersion as described for the human assay. After dispersion, cells were rested for 24 h at 37°C and 5% CO₂ in RPMI 1640 basal medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich), gentamicin (Sigma-Aldrich), and penicillin-streptomycin (Sigma-Aldrich). Cells were then centrifuged at 200g for 10 min. Contaminating red blood cells were removed through hypotonic lysis for 30 s in ice-cold distilled water before isotoncity was restored with the addition of an equal volume of ice-cold 2X phosphate-buffered saline (PBS). Cells were centrifuged as described above and were resuspended in PBS supplemented with 0.1% (w/v) D-glucose (Sigma-Aldrich) and 0.03% (w/v) human serum albumin (Sigma-Aldrich) (pH 7.0). This medium was then used throughout. Mast cells were incubated with a variety of concentrations of PF-5212372 for 1 h before being activated with 1 μM ionomycin (Tocris Bioscience, Bristol, UK). Supernatants were harvested 1 h later and were stored at −80°C until analysis.

Human Whole-Lung Digest Assay. Human lung tissue was obtained from Papworth Hospital (Cambridge, UK). Approximately 3 g of lung parenchyma was placed in a single gentleMACS C tube (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by 10 ml of collagenase buffer containing 15 μg/ml DNase (Sigma-Aldrich), 150 units/ml collagenase 3 (Worthington Biological, Lakewood, NJ), 0.5 mM CaCl₂, and 0.6 mM MgCl₂ in PBS. The tissue was processed with a gentleMACS dissociator (Miltenyi Biotec) before incubation at 37°C for 30 min, with gentle shaking (300–1000 rpm). The tissue was processed again before the lung homogenerate was passed through 100-μm and then 40-μm cell strainers. The cleared cell preparation was centrifuged at 200g at 4°C for 10 min and was resuspended in ice-cold PBS containing 5% (v/v) FCS (Sigma-Aldrich). Contaminating red blood cells were removed through hypotonic lysis for 30 s in ice-cold distilled water before isotoncity was restored with the addition of an equal volume of ice-cold 2X PBS. Cells were centrifuged as described above before being resuspended in assay buffer containing 0.1% (w/v) bovine serum albumin (Sigma-Aldrich) and 10 mM HEPES in gassed Tyrode’s solution (pH 7.4). This medium was then used throughout. The isolated human lung cells were incubated with a variety of concentrations of PF-5212372 or indomethacin (Sigma-Aldrich) for 1 h before being activated with 3 μM ionomycin (Tocris Bioscience). Supernatants were harvested 1 h later and stored at −80°C until analysis.

RBL-2H3 Cell Retention Assay. The RBL-2H3 mast cell line was purchased from the American Type Culture Collection (ATCC number CRL-2256; American Type Culture Collection, Manassas, VA) and was cultured in minimal essential medium including Earle’s salts and Glutamax (Invitrogen) and supplemented with 10% FCS (Invitrogen). The cells were cultured at 37°C with 5% CO₂ in a humidified incubator.

RBL-2H3 cells were seeded into 96-well plates, at 1 × 10⁵ cells per well, in growth medium containing 0.5 μg/ml dinitrophenol-specific
Fisher Scientific, Waltham, MA) and a Targa analytical column.

PGD2 and 18 h at room temperature for cysteinyl LT), and absorbance was measured at 412 nm. Higher absorbance indicated less cholinesterase-conjugated cysteinyl leukotriene-MOX and cysteinyl dilution mass spectrometry. The analyte peak area/internal-standard peak area (y-axis) ratio was used as an index of response. The mass transitions used were as follows: TXB2, m/z 369.2 to m/z 169.2; TXB2-d4, m/z 373.2 to m/z 173.2; LTB4, m/z 335.2 to m/z 195.2; LTB4-d4, m/z 339.2 to m/z 197.2. PGE2 and PGD2 are isobaric and have common product ions; therefore, the same mass transitions were used for both molecules, which were separated chromatographically, and identity was defined on the basis of retention times and elution order, with PGE2 eluting first (2.2 min), followed by PGD2 (2.4 min). Mass transitions for these molecules were as follows: PGE2/PGD2, m/z 351.2 to m/z 271.2; PGE2-d4/PGD2-d4, m/z 355.2 to m/z 275.2. A collision energy of ~26 V, a declustering potential of ~48 V, and a dwell time of 25 ms were used for all analytes and transitions. Results were reported as native molecule/stable isotope-labeled molecule ratios; ratios of 1 would indicate concentrations in the original cell culture supernatant of approximately 10 ng/ml.

**Sheep Model of Allergic Inflammation.** Allergic sheep were used for assessment of the antiasthmatic action of PF-5212372, as described previously (McKew et al., 2008). PF-5212372 was delivered as an aerosol through a Raindrop dispensable medical nebulizer (Puritan Bennett, Lenexa, KS) or as a dry powder through a single-dose Spinhaler dry powder inhaler (DPI) (Fisons, Ipswich, UK). For nebulization studies, PF-5212372 was dissolved in 5 ml of ethanol and nebulized until dry. For DPI delivery, PF-5212372 was loaded into a size 2 gelatin capsule, placed in the DPI, and punctured. A slow stream of air (~<20 psi) was applied to the back of the DPI for approximately 5 min, to ensure that all of the compound was inhaled. Aerosols were delivered with a mass median aerodynamic diameter of 3.2 μm, or <4.6 μm for the DPI.

**Human Bronchial Contractility Assay.** Regions of macroscopically normal lungs were taken from uninvolved areas of resection from three patients (two male patients and one female patient; age, 52.4 ± 3.7 years) who were undergoing lobectomies for treatment of lung cancer but did not have a history of chronic airway disease. Airways were immediately placed in oxygenated Krebs-Henseleit buffer solution (119 mM NaCl, 5.4 mM KCl, 2.5 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 25 mM NaHCO3, 11.7 mM glucose, pH 7.4) containing the cyclooxygenase (COX) inhibitor indomethacin (5.0 μM) and were transported at 4°C. None of the patients was chronically treated with theophylline, β-adrenoceptor agonists, or glucocorticosteroids. Serum IgE levels determined on the day of surgery were within the normal range. Bronchial rings were transferred into a four-chamber, isolated, organ bath system containing Krebs-Henseleit buffer solution (37°C), continuously bubbled with carbon dioxide, and were connected to an isometric force-displacement transducer. Airways were allowed to equilibrate for 90 min, with flushing with fresh Krebs-Henseleit buffer solution every 10 min. Passive tension was determined through gentle stretching of the tissue (0.5–1.0 g) during equilibration. Isometric changes in tension were measured with a WPI Fort 10 transducer (Basile Instruments, Comerio, Italy). The tissue responsiveness was assessed by using 100 μM acetylcholine; when the response reached a plateau, the rings were washed three times and allowed to equilibrate for 30 to 45 min. Bronchial rings were incubated for 30 min with 200 nM PF-5212372 or 1 μM (E)-3-[2-(7-chloro-2-quinolinyl)ethyl]phenyl][3-dim-
ethylamino)-3-oxopropyl][thio][methyl][thio]-propanoic acid (MK 571) (cysteinyl LT receptor antagonist) before stimulation with various AMP concentrations (1 nM–100 μM). Contractile responses were calculated as percentages of the initial contraction observed with 100 μM acetylcholine.

**Statistical Analysis.** Data are presented as mean ± S.E.M. or, for IC50 values, as geometric mean ± S.E.M. Data were analyzed by using Student’s t test or one-way analysis of variance and Bonferroni’s multiple-comparison post hoc test, as described. Data were accepted as significantly different at p < 0.05.

### Results

**Chemical Structure of PF-5212372.** The chemical structure of PF-5212372 is shown in Fig. 1.

**Enzyme Data.** PF-5212372 was designed as part of the research process described by McKew et al. (2008), to identify next-generation inhibitors of cPLA2α. The entire series was demonstrated previously to be exquisitely selective for cPLA2α, and PF-5212372 demonstrated high potency in the GLU micelle cPLA2α enzyme assay, with an IC50 value of 7 nM. This made PF-5212372 one of the most potent inhibitors from this indole series identified to date. With the demonstration that PF-5212372 was a highly potent and selective (data not shown) inhibitor of cPLA2α, we assessed this novel drug in a range of human cells and tissues in vitro and in other models relevant to our understanding of asthma.

**Inhibition of Anti-IgE-Activated Human Lung Mast Cells.** Mast cells are well established as important cells in the mediation of allergic asthma, because they express and are activated through the high-affinity IgE receptor, FcεRI (Brightling et al., 2003). Activation of mast cells with an anti-IgE antibody therefore replicates the physiological activation of these cells in the disease state, leading to release of preformed histamine and the rapidly metabolized cysteinyl LT and PGD2, all of which are implicated in asthma pathogenesis. For assessment of the ability of PF-5212372 to inhibit this activation, mast cells were isolated from human lung surgical resections and were preincubated with various concentrations of PF-5212372 for 15 min. The mast cells were then activated by using an anti-IgE antibody, with binding and cross-linking of the bound IgE. Supernatants were harvested 30 min after stimulation, and cysteinyl LT and PGD2 were quantified separately with ELISAs. Histamine was also quantified, with a fluorometric assay. PF-5212372 significantly inhibited cysteinyl LT and PGD2 release, with IC50 values of 0.45 and 0.29 nM, respectively (Fig. 2). Maximal inhibition values were 99% and 98%, respectively, compared with uninhibited control cells. In contrast, no inhibition of histamine release was observed at any concentration (data not shown).

**Inhibition of Calcium Ionophore-Stimulated Human Lung Homogenate.** In addition to the demonstration that PF-5212372 was effective in inhibiting human lung mast cells, we were keen to investigate effects in a mixed cell population representative of lung tissue. This would allow effective assessment of multiple endpoints generated from different cells in a single assay with a nonspecific activation process (in this case, a calcium ionophore). To this end, pieces of human lung parenchyma were homogenized to yield a viable mixed cell population. Various concentrations of PF-5212372 were preincubated for 1 h with the mixed cell population before stimulation with 3 μM ionomycin. Supernatants were harvested 1 h later, and LTD4, TXB2, PGE2, and PGD2 were assessed concurrently through mass spectrometry. Percentage inhibition was calculated in comparison with ionomycin-alone conditions. PF-5212372 was an effective inhibitor of LTD4, TXB2, and PGD2 release (Table 1). In contrast, PF-5212372 was largely ineffective in inhibiting PGE2 release, with a >75-fold reduction in potency (>301 nM; p < 0.05) and >6-fold reduction in efficacy, compared with the other endpoints (<11%; p < 0.001 versus LTD4; p < 0.01 versus TXB2 and PGD2). In comparisons with the COX inhibitor indomethacin (Table 1), the COX inhibitor was effective in inhibiting PGE2 release, with a comparable potency (173 nM; p > 0.05) and a >2-fold reduction in efficacy for this endpoint, compared with the others (63%; p < 0.01 versus PGD2; p < 0.05 versus TXB2). It was observed that indomethacin had no effect on LTD4 release, which confirmed the expected inhibition of COX only, with no effect on 5-lipoxygenase activity.

**Assessment of Cell Retention as Surrogate for In Vitro Duration of Action.** For an inhaled drug, it is desirable to have retention at the site of pharmacological action, to allow for good duration of action. A surrogate in vitro assay was used for assessment of the potential duration of action of PF-5212372. PF-5212372 was incubated with the adherent
rat mast cell-like cell line RBL-2H3 for 1 h before the compound was washed off with an excess of culture medium. The cells were then left for a variety of times before stimulation and assessment of the resulting PGD2 release 1 h later, with mass spectrometry. PF-5212372 was a potent inhibitor of PGD2 release in unwashed cells (IC50 = 0.8 nM), and this level of inhibition was retained even when the compound was removed and the cells were stimulated up to 24 h later (Fig. 3A; Table 2). In contrast, compound A, a cPLA2 inhibitor from a different series with reduced lipophilicity, protein binding, and enzyme potency (Fig. 1B), was shown to be washed off the cells effectively, with progressive significant reductions in potency being observed over time (Fig. 3B; Table 2).

In Vivo Assessment of PF-5212372 in Sheep Model of Allergic Inflammation. With the demonstration that PF-5212372 was a potent effective inhibitor of cPLA2 in primary human lung cell assays and with evidence for cell retention, we decided to assess the ability of this drug to inhibit asthma-like symptoms in an in vivo model of allergic lung inflammation (Abraham, 2008). Sheep were treated with 3 mg of PF-5212372 through inhalation as a dry powder or nebulization with a liquid vehicle 16 h and 1 h before challenge. PF-5212372 was highly effective in inhibiting late-phase bronchoconstriction, with 78% inhibition of the area under the concentration-time curve (AUC), in comparison with an inhaled vehicle control (lactose or ethanol, as appropriate) (Fig. 4A). When AHR was assessed by using inhaled carbachol, PF-5212372 was highly effective, with 94% inhibition of the response, compared with vehicle (Fig. 4B). Identical data were generated when 1 mg of PF-5212372 was administered once daily for 7 days before challenge, as a dry powder (data not shown). When 1 mg of PF-5212372 was administered 16 h and 1 h before challenge, however, in the same manner as in the 3-mg experiments described above, it did not inhibit bronchoconstriction or AHR (data not shown).

We sought to follow up the in vivo results from allergic sheep with studies of effects on sheep cells. A series of in vitro experiments were performed by using primary mast cells isolated from the lungs of sheep. In a manner analogous to that for the human assay, isolated sheep mast cells were preincubated for 1 h with PF-5212372 before stimulation with 1 µM ionomycin. Supernatants were harvested 1 h later and PGD2 levels were quantified with mass spectrometry. Anti-IgE stimulation was not possible with sheep mast cells because of the lack of availability of sheep-specific antibodies. However, experiments were performed with human mast cells to demonstrate that comparable data were produced when anti-IgE and ionomycin stimulations were compared (data not shown). PF-5212372 inhibited ionomycin-stimulated PGD2 release from sheep mast cells with an IC50 of 0.79 nM and maximal inhibition of 95% (Fig. 4C; Table 3). This compared well with the data generated in human mast cells, confirming translation of primary pharmacological features between the species.

Effects of PF-5212372 on Isolated Human Bronchial Tissue Contraction. Isolated rings of human bronchus were contracted with AMP as described elsewhere (Calzetta et al., 2011). When 100 nM PF-5212372 was preincubated for 30 min before challenge with various AMP concentrations, significant 81% inhibition of the AUC was observed (p < 0.01) (Fig. 5). Analysis of the data at the maximally effective AMP Emax concentration (100 µM) indicated that PF-5212372 produced significant 69% inhibition, compared with the control tissues (p < 0.05). The level of inhibition was comparable to that observed with 1 µM MK 571 (a specific leukotriene receptor antagonist), confirming previous data on AMP-induced contraction of human bronchial tissue (Björck et al., 1992), and results were not significantly different for either the AUC or maximally effective AMP concentration comparisons (p > 0.05).
We demonstrated that PF-5212372 is a potent, broad-spectrum inhibitor of eicosanoid release in primary human mast cells, a cell type acknowledged to be pivotal in the pathogenesis of allergic asthma, by using physiologically relevant stimulation (FcεRI cross-linking). We followed up these data by demonstrating comparable efficacy in a mixed lung cell population and established the first known evidence for potential sparing of PGD₂ inhibition with this mechanism. We then investigated the potential of PF-5212372 to have a longer duration of action by using a washed, whole-cell, potency assay. We were able to demonstrate that PF-5212372 was retained within the cells and was able to inhibit PGD₂ release up to 24 h after the removal of excess compound from the cell medium. This demonstrated that PF-5212372 might have a suitable duration of action for effectiveness as a once-daily inhaled medicine. In line with this, we established the preclinical activity of PF-5212372 as an inhaled treatment in a sheep model of allergic lung inflammation. The sheep allergic inflammation model developed by Abraham (2008) uses naturally allergic sheep and challenges them with *Ascaris suum* antigen through inhalation. This results in development of both early-phase and late-phase bronchoconstriction, as well as AHR to inhaled carbachol. This model exhibits several features that are reminiscent of the responses observed after allergen challenge in subjects with allergic asthma and allows preclinical assessment of potential asthma treatments. With this model, we established that inhaled PF-5212372 was able to inhibit the late-phase allergic response as well as allergen-induced AHR assessed with carbachol inhalation, although, because we did not specifically evaluate effects on the infiltration of cells (e.g., eosinophils) into the lung, we can only infer that additional effects on cellular infiltration were produced through inhibition of cPLA₂α-mediated eicosanoid release. We confirmed translation of this effect by demonstrating that PF-5212372 was equally potent and effective in a primary sheep lung mast cell assay, compared with the human assay. Finally, we demonstrated that PF-5212372 was effective in inhibiting AMP-induced contraction of human bronchi. We were not able to demonstrate clear effects on the early-phase allergic response in the sheep model. We did observe that, with chronic dosing of PF-5212372 (1 mg once daily for 7 days for each sheep), a trend for inhibition of the early-phase response was observed (data not shown), although not complete inhibition. It remains possible that higher or more-extended dosages might improve inhibition of the early-phase response (although sufficient compound was available to inhibit completely both the late-phase response and AHR), but it is also likely that the role of nonprostanoids (e.g., histamine)

**Table 1**

IC₅₀ and maximal inhibition values for PF-5212372 and indomethacin inhibition of ionomycin-induced eicosanoid release from human lung homogenate cells

Indomethacin did not inhibit LTB₄ release.

<table>
<thead>
<tr>
<th></th>
<th>LTB₄</th>
<th>TXB₂</th>
<th>PGD₂</th>
<th>PGE₂</th>
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<tr>
<td>IC₅₀</td>
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<tr>
<td>IC₅₀</td>
<td>2.6 ± 3.3*</td>
<td>68 ± 10.5††</td>
<td>72 ± 15.7††</td>
<td>&gt;301 ± 361</td>
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<tr>
<td>Indomethacin</td>
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<td>N.T.</td>
<td>37 ± 22</td>
<td>&lt;11 ± 11.3</td>
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<tr>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
<td>97 ± 1.5††</td>
<td>173 ± 151</td>
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</table>

N.T., not tested.

* p < 0.05, IC₅₀ data significantly different from PGE₂ (n = 3).
† p < 0.05, †† p < 0.01, ††† p < 0.001, maximal inhibition data significantly different from PGD₂ (n = 3).

**Discussion**

We have described for the first time a clear rationale for the inhibition of cPLA₂α, with the potent selective enzyme inhibitor PF-5212372, as a potential treatment for asthma.
is more important in this phase. We clearly demonstrated with both human and sheep mast cells that PF-5212372 had no effect on the release of histamine. It is possible that inhibitors of cPLA₂/H₁₁₅₆₉/H₁₁₅₆₉/H₁₁₅₆₉ may not be clinically efficacious as reliever-type medicines but may be more suitable for maintenance therapy.

cPLA₂/H₁₁₅₆₉ is now well accepted as the major enzyme involved in arachidonic acid metabolism leading to PG and LT production and release (Uozumi et al., 1997). With these mediators also being implicated in the pathogenesis of asthma (Drazen et al., 1999), it seems logical that inhibition of the enzyme might be a useful therapeutic option for the treatment of this common disease. In addition, although it is unlikely to be a direct effect, downstream inhibition of cytokine release from a variety of immune cells might be expected as a result of inhibition of cPLA₂/H₁₁₅₆₉-mediated prostanoid release, which strengthens the rationale for effective treatment of asthma (Schuligoi et al., 2010). To our knowledge, however, this publication is the first to demonstrate clearly a convincing rationale, with primary human lung cell and tis-
assays be effective in the treatment of asthma. It is possible that the then monitored.

ulation with various AMP concentrations. Contractile responses were then monitored. *p < 0.05, significantly different from control tissue when stimulated with 100 μM AMP (n = 3). n/s, not significantly different (p > 0.05) between PF-5212372 and MK 571 when stimulated with 100 μM AMP (n = 3). ††, p < 0.01, AUC data significantly different from control tissue (n = 3).

sue data, for why a potent specific inhibitor of cPLA₂α should be effective in the treatment of asthma. It is possible that the reason why orally administered cPLA₂α inhibitors have been focused primarily on arthritis and inflammatory pain indications is because of the relative success of COX-2 inhibitors, especially before the Vioxx withdrawal (Bresalier et al., 2005). In addition to potential cardiovascular issues, other systemically driven side effects associated with prostaglandin inhibition, such as intestinal bleeding (Goldstein et al., 2010), may limit the utility of an orally administered cPLA₂α inhibitor for the treatment of other inflammatory diseases, such as asthma. With this in mind, we chose to investigate whether the topical inhaled delivery of a potent cPLA₂α inhibitor would be effective, with the anticipation that this route of administration might limit systemic exposure and minimize unwanted side effects. One of the central themes for designing an effective inhaled medicine is to design the duration of pharmacological action. The improved patient convenience and compliance associated with twice-daily or, ideally, once-daily administration are expected to yield a more-effective therapy, which is a central tenet for the latest generation of anti-inflammatory and bronchodilator therapies (van den Berge et al., 2010; Cazzola et al., 2011). Preclinical demonstration of an extended duration of pharmacological action would be a desirable feature for a novel anti-inflammatory drug. Because cPLA₂α inhibitors such as PF-5212372 must gain access to the enzyme through dissolution into a lipid membrane, any cellular retention of the compound may yield a suitable duration of pharmacological action. As outlined in this article, PF-5212372 is able to maintain inhibition of PGD₂ release for up to 24 h after washing of the cells to remove the compound from the aqueous phase (Fig. 2). This is in contrast to an unrelated cPLA₂α inhibitor (compound A) that can be washed from the cells progressively over time. Compound A has reduced lipophilicity and protein binding, in comparison with PF-5212372, and it is hypothesized that it is this change in physicochemical properties that drives the reduced retention. Although this type of experiment does not provide a clear indication of whether PF-5212372 could be dosed once or twice daily, support for a suitable duration of action was obtained from the observation that once-daily chronic dosing with 1 mg in the sheep model (data not shown) was as effective as more-acute dosing with 3 mg of PF-5212372 (Fig. 3). It should be noted that PF-5212372 was not detected systemically after inhalation in the sheep, which confirmed the intended reduction in systemic exposure.

One of the most interesting and unexpected pieces of data presented here involves the human lung homogenate experiments. We had expected that this assay would provide data comparable to those from the primary human lung mast cell experiments but would provide additional evidence for broad-spectrum eicosanoid inhibition in a complex cell mixture derived from the human lung. Our data do demonstrate that inhibition of cPLA₂α with PF-5212372 produces potent effective inhibition of multiple arachidonic acid metabolites in this mixed cell population. However, the first observation is that, although the potency is comparable for LTB₄, TXB₂, and PGD₂, the potency is ~10-fold reduced in comparison with the human lung mast cell assay. This can be explained by the knowledge that cPLA₂α cleaves its phospholipid substrate at the membrane/water interface and therefore inhibitors must be sequestered in the lipid membrane to gain access to the enzyme. In contrast to the standard, low-cell number, human lung mast cell assay, the whole-lung homogenate contains much higher cell numbers, which allows PF-5212372 to be sequestered into multiple lipid membrane reservoirs, effectively reducing the free concentration of compound. This paradigm was highlighted previously (McKew et al., 2005) when the utility of whole-blood assays was demonstrated in combination with the GLU micelle enzyme to identify compounds that would be effective when administered systemically. Indeed, the human lung homogenate potency data were very comparable to the GLU micelle potency described for PF-5212372 (7 nM). Which potency value is most relevant for inhaled delivery is debatable. Although the compound is administered topically and the systemic free con-

### Table 3

<table>
<thead>
<tr>
<th>Cysteinylt LT</th>
<th>Human Lung Mast Cells</th>
<th>Rat RBL-2H3 Cells</th>
<th>Sheep Lung Mast Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC₅₀, nM</td>
<td>0.45 ± 0.33</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
<tr>
<td>Maximal inhibition, %</td>
<td>95 ± 1.08</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PGD₂</th>
<th>Human Lung Mast Cells</th>
<th>Rat RBL-2H3 Cells</th>
<th>Sheep Lung Mast Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC₅₀, nM</td>
<td>0.29 ± 0.12</td>
<td>0.82 ± 0.11</td>
<td>0.78 ± 0.12</td>
</tr>
<tr>
<td>Maximal inhibition, %</td>
<td>101 ± 1.44</td>
<td>101 ± 0.56</td>
<td>95 ± 2.73</td>
</tr>
</tbody>
</table>

N.T., not tested.

**Fig. 5.** PF-5212372 inhibition of AMP-induced contraction in isolated human bronchial sections. Isolated human bronchial rings were pretensioned and then preincubated for 30 min, with or without 200 nM PF-5212372 or 1 μM MK 571 (leukotriene receptor antagonist), before stimulation with various AMP concentrations. Contractile responses were then monitored. *p < 0.05, significantly different from control tissue when stimulated with 100 μM AMP (n = 3). n/s, not significantly different (p > 0.05) between PF-5212372 and MK 571 when stimulated with 100 μM AMP (n = 3). ††, p < 0.01, AUC data significantly different from control tissue (n = 3).
centration to drive pharmacological behavior is moot from a lung efficacy perspective, the identification that potency is reduced in a complex cell system does suggest that this might be most relevant.

The second aspect of the human lung homogenate data that is intriguing is the apparent relative lack of potency and efficacy in the inhibition of PGE2. In two of the three separate experiments performed with PF-5212372, no apparent inhibition was observed with up to 1 μM PF-5212372. This is of great interest because of the apparent branchoptroective effects of PGE2 in the lung (Pavord et al., 1991; Sestini et al., 1996; Szczeklik et al., 1996; Vancheri et al., 2004). If PF-5212372 is able to spare lung inhibition of PGE2 in a clinical setting, this may be of great benefit (Mathison and Koziol, 2002). The reason for this reduced potency and efficacy versus PGE2 is not clear. It is known that inhibition of COX enzymes with aspirin has a deleterious effect for susceptible patients with asthma, likely because of the shunting of arachidonic acid through the 5-lipoxygenase pathway, leading to enhanced production of leukotrienes (Szczeklik, 1990). It is possible that inhibition of cPLA2α causes shunting down the PGE2 pathway or that PGE2 is preferentially generated in the setting, this may be of great benefit (Mathison and Koziol, 2002). The reason for this reduced potency and efficacy versus PGE2 is not clear. It is known that inhibition of COX enzymes with aspirin has a deleterious effect for susceptible patients with asthma, likely because of the shunting of arachidonic acid through the 5-lipoxygenase pathway, leading to enhanced production of leukotrienes (Szczeklik, 1990). It is possible that inhibition of cPLA2α causes shunting down the PGE2 pathway or that PGE2 is preferentially generated through non-cPLA2α pathways. There is some historical evidence for different stimuli causing release of PGE2 through non-cPLA2α mechanisms (Berenbaum et al., 1996), as well as evidence for cross-talk between enzymes involved in arachidonic acid metabolism (Niknami et al., 2010). Finally, there is evidence for a role of the secreted phospholipase A2 in asthma pathophysiological conditions (Granata et al., 2010), and it is possible that, in the complex mixed cell population, cPLA2α is not the most relevant enzyme responsible for the ultimate generation of PGE2. The mechanism is clearly not defined here, but this study does raise an interesting question for further investigation.

In summary, we have presented a wide range of data on a novel inhibitor of cPLA2α, PF-5212372, from a preclinical perspective, as a potential new inhaled therapy for the treatment of asthma. We demonstrated that PF-5212372 is a potent, broad-spectrum inhibitor of inflammatory eicosanoid release from human primary lung cells, in addition to demonstrating translatably efficacious in a preclinical animal model of allergic airway disease and a functional response in human lung tissue. We identified for the first time a potential for sparing PGE2 inhibition in the lung, which may provide a distinct advantage for this mechanism in treating patients with asthma.

Authorship Contributions

Participated in research design: Hewson, Patel, Peaknell, Matera, Cazzola, Page, Abraham, Williams, Clark, Liu, N.P. Clarke, and Yeandon.

Conducted experiments: Hewson, Patel, Calzetta, Campwalla, Ha vard, Luscombe, P.A. Clarke, and Abraham.

Contributed new reagents or analytic tools: Luscombe, P.A. Clarke, and Clark.

Performed data analysis: Hewson, Patel, Campwalla, Luscombe, Williams, and Clark.

Wrote or contributed to the writing of the manuscript: Hewson, Campwalla, P.A. Clarke, Peaknell, Cazzola, and Page.

References


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