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# Preclinical Evaluation of an Inhibitor of Cytosolic Phospholipase $A_2\alpha$ for the Treatment of Asthma

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## ABSTRACT

Asthma is a chronic inflammatory lung disease with considerable unmet medical needs for new and effective therapies. Cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) is the rate-limiting enzyme that is ultimately responsible for the production of eicosanoids implicated in the pathogenesis of asthma. We investigated a novel $cPLA_2\alpha$ inhibitor, PF-5212372, to establish the potential of this drug as a treatment for asthma. PF-5212372 was a potent inhibitor of $cPLA_2\alpha$ (7 nM) and was able to inhibit prostaglandin (PG)$D_2$ 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 $B_4$, thromboxane $A_2$, and $PGD_2$ (2.6, 2.6, and 4.0 nM, respectively) but was significantly less effective against $PGE_2$ release (>301 nM; $p < 0.05$). In an in vitro 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 effective inhibition of $PGD_2$ 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 $cPLA_2\alpha$ 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

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**ABBREVIATIONS:** $cPLA_2\alpha$, cytosolic phospholipase $A_2\alpha$; AHR, airway hyper-responsiveness; AUC, area under the concentration-time curve; COX, cyclooxygenase; CRTH2, chemoattractant receptor expressed on T helper type 2 cells; DPI, dry powder inhaler; ELISA, enzyme-linked immunosorbent assay; FA, formic acid; FCS, fetal calf serum; GLU, 7-hydroxycoumarinyl-γ-linolenate; LT, leukotriene; PBS, phosphate-buffered saline; PG, prostaglandin; TX, thromboxane; HPLC, high-performance liquid chromatography; MOX, methoximated; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MK 571, (*E*)-3-[[[3-[2-(7-chloro-2-quinolinyl)ethenyl]phenyl][[3-(dimethylamino)-3-oxopropyl]thio]methyl]thio]-propanoic acid.



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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 A$_2\alpha$ (cPLA$_2\alpha$) may address these unmet medical needs. cPLA$_2\alpha$ releases arachidonic acid from the phospholipid membrane and is the rate-limiting enzyme in the biosynthesis of prostaglandins (PGs), thromboxanes, and leukotrienes (LTs) (Ghosh et al., 2006), all of which are implicated in airway inflammation, mucus production, bronchoconstriction, and airway hyper-responsiveness (AHR) associated with asthma (Drazen et al., 1999). LTB$_4$ is known to contribute to inflammation by both recruiting and activating leukocytes, whereas cysteinyl leukotrienes (LTC$_4$, LTD$_4$, and LTE$_4$) 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 have efficacy in the treatment of asthma (Price et al., 2011).

Subthreshold contractile concentrations of PGD$_2$ 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 PGD$_2$ levels in the lungs are increased after allergen challenge of subjects with asthma (Murray et al., 1986). PGD$_2$ 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 PGD$_2$-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., 2009). TXA$_2$, another potent constrictor of smooth muscle, has been implicated in the late asthmatic response to inhaled allergens among human subjects (Shephard et al., 1985) and in AHR among subjects with asthma (Fujimura et al., 1986).

Direct evidence of a role for cPLA$_2\alpha$ in respiratory disease has been demonstrated by using cPLA$_2\alpha$-deficient mice. These animals are resistant to bronchial hyper-reactivity in an anaphylaxis model (Uozumi et al., 1997) and models of acute inflammation and lung injury (Nagase et al., 2000).

PF-5212372 was identified as part of an effort to identify novel, high-potency inhibitors of cPLA$_2\alpha$ (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.

## Materials and Methods

**cPLA$_2\alpha$ Enzyme Assays.** The 7-hydroxycoumarinyl-γ-linolenate (GLU) micelle enzyme assay was used as described previously (McKew et al., 2005).

**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 Pearce, 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% CO$_2$ 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 200*g* for 10 min. Contaminating red blood cells were removed through hypotonic lysis for 30 s in ice-cold distilled water before isotonicity was restored with the addition of an equal volume of ice-cold 2× 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 Biochemical, Lakewood, NJ), 0.5 mM CaCl$_2$, and 0.6 mM MgCl$_2$ 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 homogenate was passed through 100-μm and then 40-μm cell strainers. The cleared cell preparation was centrifuged at 200*g* 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 isotonicity was restored with the addition of an equal volume of ice-cold 2× 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% CO$_2$ in a humidified incubator.

RBL-2H3 cells were seeded into 96-well plates, at $1 \times 10^5$ cells per well, in growth medium containing 0.5 μg/ml dinitrophenol-specific

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murine IgE (clone SPE7; Sigma-Aldrich). Cells were cultured for 30 h before the medium was replaced with RPMI 1640 medium (Sigma-Aldrich) containing 1% FCS and buffered with 0.15% sodium bicarbonate (Invitrogen). This experimental medium was then used throughout the experiment. Unwashed cells were incubated for 1 h with compound before activation with 100 ng/ml dinitrophenol-human serum albumin (Sigma-Aldrich). For washed cells, the compound-containing medium was removed and the cells were washed three times with fresh experimental medium. Cells were then either immediately activated as described above (time 0) or were incubated for an additional 3 or 24 h in experimental medium before activation. Cells were centrifuged at 300*g* 1 h after stimulation, and supernatants were harvested and stored at −80°C until analysis.

**$PGD_2$ and Cysteinyl LT ELISAs.** Cysteinyl LT and $PGD_2$ levels were quantified by using commercially available competition ELISAs, following the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI). For $PGD_2$ quantification, supernatants were treated with methoxyamine hydrochloride for 30 min at 60°C and were diluted before being placed in precoated ELISA plates, together with acetylcholinesterase-conjugated $PGD_2$-MOX and $PGD_2$-MOX antiserum. For cysteinyl leukotriene quantification, the same process was followed but samples did not require any treatment before being diluted and placed in precoated ELISA plates together with acetylcholinesterase-conjugated cysteinyl leukotriene-MOX and cysteinyl leukotriene-MOX antiserum. Plates were incubated overnight at 4°C, washed, and developed with Ellman's reagent (18 h at 4°C for $PGD_2$ and 18 h at room temperature for cysteinyl LT), and absorbance was measured at 412 nm. Higher absorbance indicated less $PGD_2$ or cysteinyl LT. The sensitivity was 3.1 pg/ml for $PGD_2$ and 34 pg/ml for cysteinyl LT.

**Histamine Quantification Assay.** Histamine levels were quantified by using the automated fluorometric assay developed by Siraganian (1974). Histamine levels were determined as proportions of the total histamine content through lysis of control cells with perchloric acid and subtraction of the spontaneous histamine release.

**$TXB_2$, $PGE_2$, $PGD_2$, and $LTB_4$ Measurements with LC-MS/MS.** 50 μl of lung tissue or cell supernatants were added to a Whatman 2-ml, 96-well plate (Whatman, Maidstone, UK); 300 μl of acetonitrile containing 500 pg of each of the stable isotope-labeled analogs of the analytes ($TXB_2$-$d_4$, $PGE_2$-$d_4$, $PGD_2$-$d_4$, and $LTB_4$-$d_4$) was then added to each well, and the plate was agitated for 15 min, to allow protein precipitation. A total of 1300 μl of 0.1% formic acid (FA) (aq) was then added to each well, and the plate was sealed and centrifuged at 4000 rpm for 30 min at 4°C with an Eppendorf 5810R centrifuge (Eppendorf AG, Hamburg, Germany). The plate was transferred to a CTC PAL autosampler (Presearch, Milton Keynes, UK), and 500 μl of the supernatant was injected into the LC-MS/MS system for measurement of $TXB_2$, $PGE_2$, $PGD_2$, and $LTB_4$. Sample analysis was performed by using on-line solid-phase extraction with liquid chromatography-ion spray-tandem mass spectrometry. The LC-MS/MS system consisted of an API 4000 triple-quadrupole mass spectrometer (Applied Biosystems, Warrington, UK) equipped with a Turbo-V ion source operating in negative electrospray ionization mode, two Agilent 1100 binary HPLC pumps (with pump 1 as the analytical pump and pump 2 as the loading pump) and degasser (Agilent, Winnersh, UK), and a CTC PAL autosampler (Presearch). A Valco switching valve (10-port; VICI Valco Instruments, Houston, TX) was used to perform the on-line solid-phase extraction switching. For the on-line solid-phase extraction and chromatographic separation, a Thermo Biobasic trapping cartridge (10 × 2.1 mm; Thermo Fisher Scientific, Waltham, MA) and a Targa analytical column (C18, 75 × 2.1 mm, 3 μm; Higgins Analytical, Mountain View, CA) were used. Gradient elution was used to clean, elute, and separate the four analytes. The HPLC system consisted of two HPLC pumps, a CTC autosampler, and an additional Valco valve driver unit. The system was configured such that, after loading of the injection loop, pump 2 loaded the sample onto the trapping cartridge. The analytes were eluted from the trapping cartridge onto the analytical column with pump 1, continued separating, and were eluted into the mass spectrometer source between 1.7 and 3.8 min after injection. The mobile phase for pump 1 (elution) started at 40% acetonitrile in 0.1% FA (aq) and increased to 70% acetonitrile in 0.1% FA, delivered at a flow rate of 400 μl/min. The mobile phase for pump 2 (loading) was 2% acetonitrile in 0.1% FA (aq) at 1500 μl/min. The overall chromatography time was 4 min, and the total cycle time (injection to injection) was 4.75 min. The mass spectrometer was operated in the negative ion mode, with an ion-spray voltage of −4200 V at 650°C. Multiple-reaction monitoring was used for quantification. All quadrupoles were operated at low resolution (full width at half-maximum, 1 Da). Quantitation was performed with Analyst 1.5 software (Applied Biosystems), by using the internal-standard method (isotope-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: $TXB_2$, *m/z* 369.2 to *m/z* 169.2; $TXB_2$-$d_4$, *m/z* 373.2 to *m/z* 173.2; $LTB_4$, *m/z* 335.2 to *m/z* 195.2; $LTB_4$-$d_4$, *m/z* 339.2 to *m/z* 197.2. $PGE_2$ and $PGD_2$ 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 $PGE_2$ eluting first (2.2 min), followed by $PGD_2$ (2.4 min). Mass transitions for these molecules were as follows: $PGE_2$/$PGD_2$, *m/z* 351.2 to *m/z* 271.2; $PGE_2$-$d_4$/$PGD_2$-$d_4$, *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 disposable 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 $CaCl_2$, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 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, $\beta_2$-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 carboxygen mixture, 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-[[[3-[2-(7-chloro-2-quinolinyl)ethenyl]phenyl][[3-(dim-

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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 $IC_{50}$ 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 cPLA$_2\alpha$. The entire series was demonstrated previously to be exquisitely selective for cPLA$_2\alpha$, and PF-5212372 demonstrated high potency in the GLU micelle cPLA$_2\alpha$ enzyme assay, with an $IC_{50}$ 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 cPLA$_2\alpha$, 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.

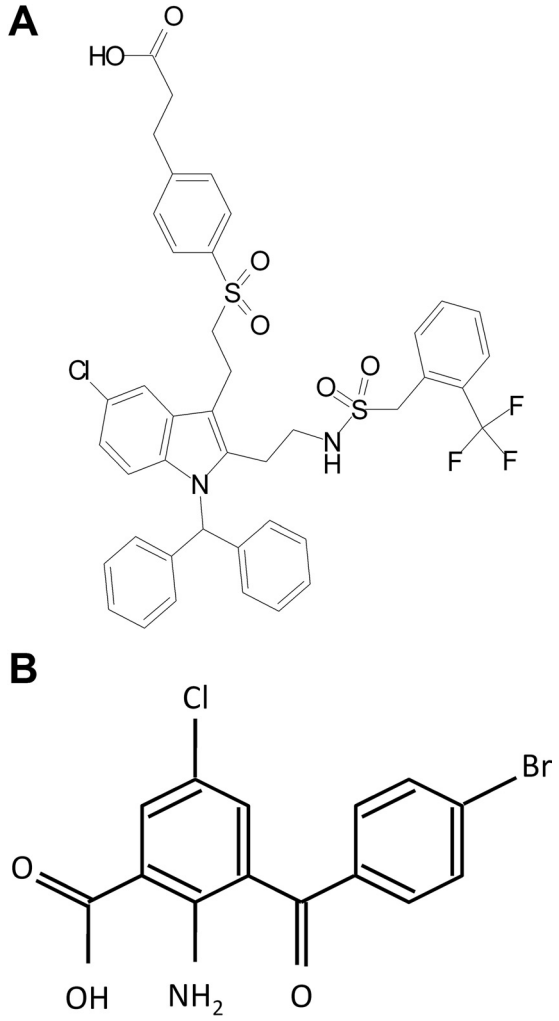


**Fig. 1.** Chemical structures of PF-5212372 ($C_{42}H_{38}ClF_3N_2O_6S_2$; molecular weight, 823.35) (A) and compound A (B).

**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 $PGD_2$, 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 $PGD_2$ were quantified separately with ELISAs. Histamine was also quantified, with a fluorometric assay. PF-5212372 significantly inhibited cysteinyl LT and $PGD_2$ release, with $IC_{50}$ 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 $LTB_4$, $TXB_2$, $PGE_2$, and $PGD_2$ were assessed concurrently through mass spectrometry. Percentage inhibition was calculated in comparison with ionomycin-alone conditions. PF-5212372 was an effective inhibitor of $LTB_4$, $TXB_2$, and $PGD_2$ release (Table 1). In contrast, PF-5212372 was largely ineffective in inhibiting $PGE_2$ release, with a >75-fold reduction in potency (>301 nM; $p < 0.05$) and a >6-fold reduction in efficacy, compared with the other endpoints (<11%; $p < 0.001$ versus $LTB_4$; $p < 0.01$ versus $TXB_2$ and $PGD_2$). In comparisons with the COX inhibitor indomethacin (Table 1), the COX inhibitor was effective in inhibiting $PGE_2$ 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 $PGD_2$; $p < 0.05$ versus $TXB_2$). It was observed that indomethacin had no effect on $LTB_4$ 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

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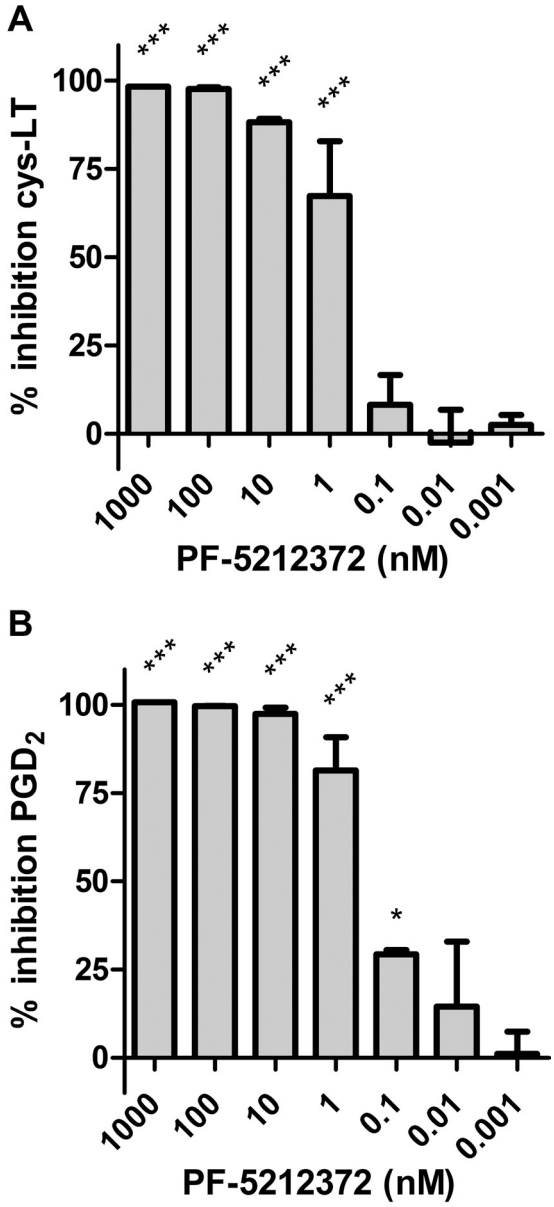


**Fig. 2.** PF-5212372 inhibition of anti-IgE-mediated $PGD_2$ and cysteinyl LT release from isolated human lung mast cells. Human lung mast cells were isolated and preincubated for 15 min, with or without various concentrations of PF-5212372, before stimulation with 2 μg/ml anti-human IgE. Supernatants were harvested 30 min later, and cysteinyl LT (A) or $PGD_2$ (B) levels were measured with ELISAs. *, $p < 0.05$; ***, $p < 0.001$, significantly different from control cells without PF-5212372 ($n = 3$).

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 $PGD_2$ release 1 h later, with mass spectrometry. PF-5212372 was a potent inhibitor of $PGD_2$ release in unwashed cells ($IC_{50}$ = 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 $cPLA_2$ 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 $cPLA_2\alpha$ 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 $PGD_2$ 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 $PGD_2$ release from sheep mast cells with an $IC_{50}$ 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 $E_{max}$ 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 ($p > 0.05$).

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TABLE 1

IC$_{50}$ and maximal inhibition values for PF-5212372 and indomethacin inhibition of ionomycin-induced eicosanoid release from human lung homogenate cells

Indomethacin did not inhibit LTB$_4$ release.

| | LTB$_4$ | | TXB$_2$ | | PGD$_2$ | | PGE$_2$ | |
|---|---|---|---|---|---|---|---|---|
| | IC$_{50}$ | Maximal Inhibition | IC$_{50}$ | Maximal Inhibition | IC$_{50}$ | Maximal Inhibition | IC$_{50}$ | Maximal Inhibition |
| | *nM* | % | *nM* | % | *nM* | % | *nM* | % |
| PF-5212372 | 2.6 ± 2.3* | 92 ± 1.2$^{\dagger\dagger\dagger}$ | 2.6 ± 3.3* | 68 ± 10.5$^{\dagger\dagger}$ | 4.0 ± 4.8* | 72 ± 15.7$^{\dagger\dagger}$ | >301 ± 361 | <11 ± 11.3 |
| Indomethacin | N.T. | N.T. | 60 ± 30 | 91 ± 3.3$^{\dagger}$ | 37 ± 22 | 97 ± 1.5$^{\dagger\dagger}$ | 173 ± 151 | 63 ± 9.7 |

N.T., not tested.

* $p < 0.05$, IC$_{50}$ data significantly different from PGE$_2$ ($n = 3$).

$^{\dagger}$ $p < 0.05$, $^{\dagger\dagger}$ $p < 0.01$, $^{\dagger\dagger\dagger}$ $p < 0.001$, maximal inhibition data significantly different from PGE$_2$ ($n = 3$).

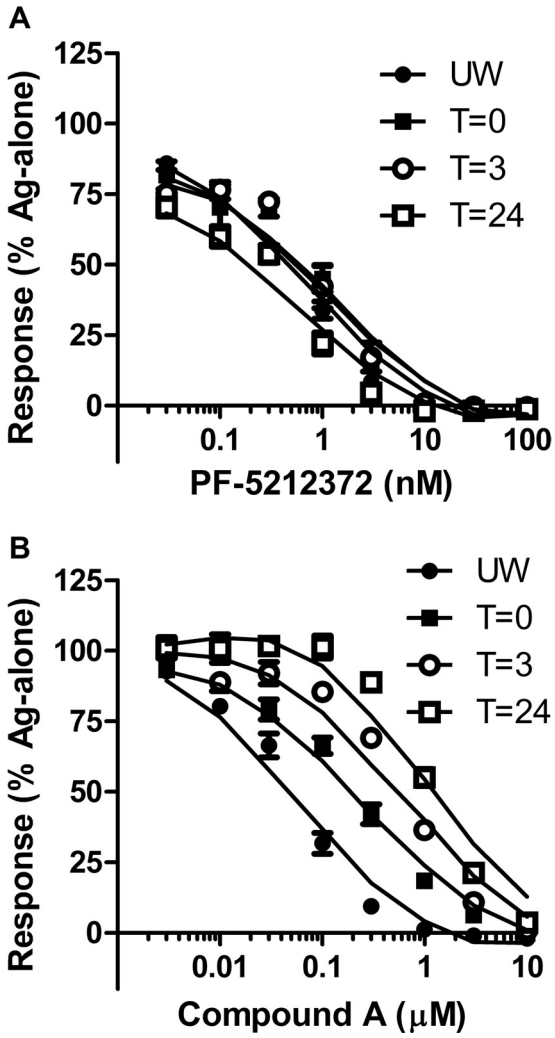


**Fig. 3.** Retention of PF-5212372 cellular potency after wash-off, in contrast to unrelated compound A. RBL-2H3 cells were sensitized with IgE and preincubated for 60 min, with or without various concentrations of PF-5212372 (A) or the unrelated compound A (B). Cells were then immediately stimulated with 100 ng/ml dinitrophenol-human serum albumin [unwashed (UW), ●] or were washed three times and left in fresh experimental medium for 0 h (T=0, ■), 3 h (T=3, ●), or 24 h (T=24, □) before stimulation with dinitrophenol-human serum albumin. Supernatants were harvested 60 min later, and PGD$_2$ levels were measured with mass spectrometry ($n = 3$). Ag, antigen.

## Discussion

We have described for the first time a clear rationale for the inhibition of cPLA$_2\alpha$, with the potent selective enzyme inhibitor PF-5212372, as a potential treatment for asthma. 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 PGE$_2$ inhibition with this mechanism. We then investigated the potential of PF-5212372 to have a long 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 PGD$_2$ 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 cPLA$_2\alpha$-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)

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TABLE 2

$IC_{50}$ values for PF-5212372 and unrelated compound A in RBL-2H3 cell retention assay

$IC_{50}$ values at each time point after washing are presented in relation to the unwashed time values.

| | Unwashed | 0 h | 3 h | 24 h |
|---|---|---|---|---|
| PF-5212372 | | | | |
| $IC_{50}$, nM | 0.82 ± 0.11 | 1.25 ± 0.10 | 1.25 ± 0.48 | 0.74 ± 0.19 |
| Fold of unwashed value | 1.0 | 1.5 ± 0.09 | 1.6 ± 0.41 | 0.9 ± 0.11 |
| Compound A | | | | |
| $IC_{50}$, nM | 60.0 ± 13.8 | 257 ± 63.2** | 721 ± 68.6*** | 1177 ± 198*** |
| Fold of unwashed value | 1.0 | 4.3 ± 0.50 | 13 ± 3.7 | 20 ± 3.3 |

** $p < 0.01$, *** $p < 0.001$, $IC_{50}$ values significantly different from the unwashed $IC_{50}$ value for the same compound.

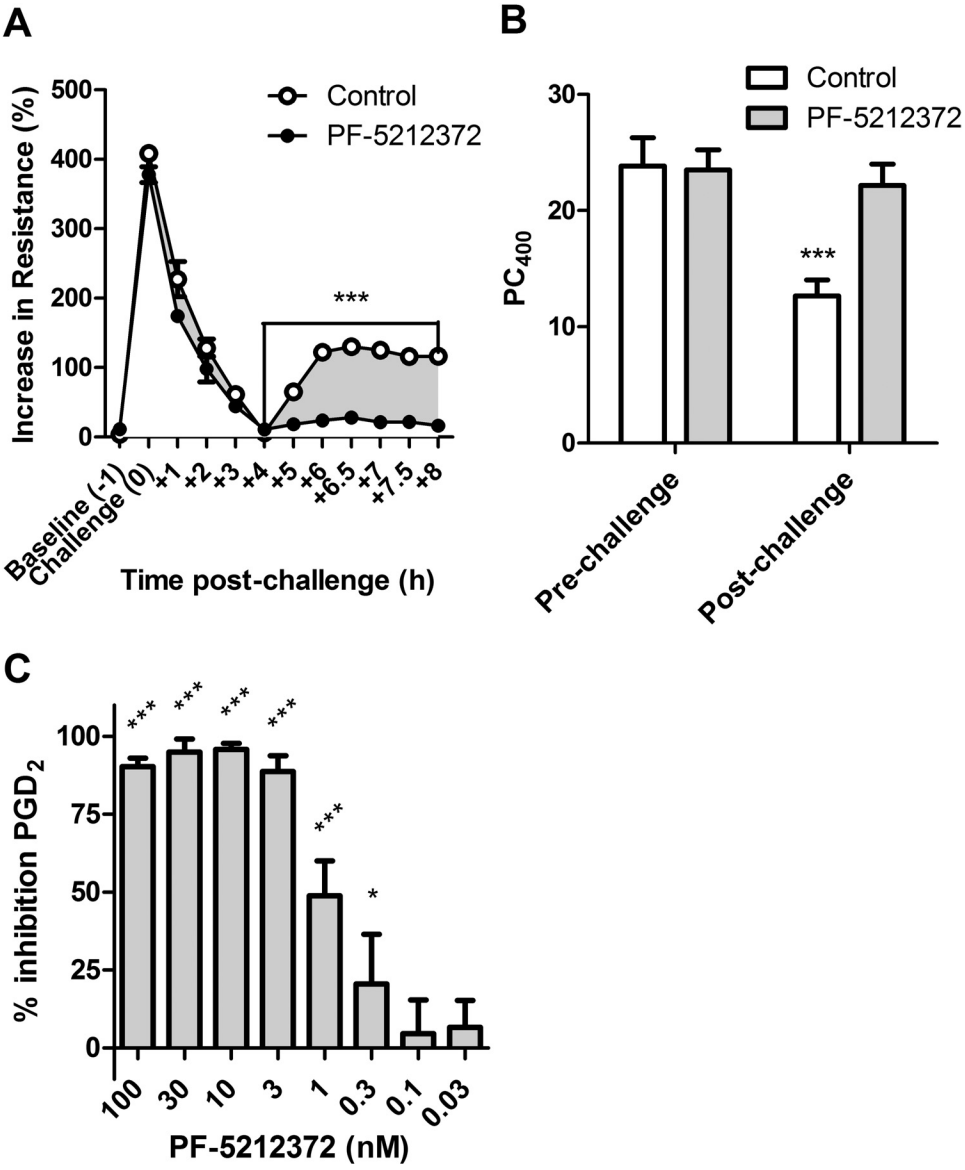


**Fig. 4.** Inhaled PF-5212372 inhibition of allergen-induced late-phase bronchoconstriction and AHR in a sheep model of allergic asthma and inhibition of ionomycin-stimulated $PGD_2$ release from isolated sheep lung mast cells. PF-5212372 (3 mg) or vehicle was delivered via inhalation to naturally allergic sheep, 16 h and 1 h before allergen challenge (*Ascaris suum*). A, airway resistance was measured through the subsequent 8 h. ***, $p < 0.001$, AUC data significantly different from vehicle control (4–8 h; $n = 3$). B, additional dosing of PF-5212372 or vehicle was performed 8 h after the allergen challenge, and carbachol-induced AHR was measured the following day. ***, $p < 0.001$, carbachol challenge data significantly different from vehicle control ($n = 3$). C, sheep lung mast cells were isolated and preincubated for 1 h, with or without various concentrations of PF-5212372, before stimulation with 1 μM ionomycin. Supernatants were harvested 1 h later, and $PGD_2$ levels were measured with mass spectrometry. *, $p < 0.05$; ***, $p < 0.001$, data significantly different from control cells without PF-5212372 ($n = 3$).

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 $cPLA_2\alpha$ may not be clinically efficacious as reliever-type medicines but may be more suitable for maintenance therapy.

$cPLA_2\alpha$ 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 $cPLA_2\alpha$-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-

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TABLE 3
Human, rat, and sheep translation of $IC_{50}$ and maximal inhibition values for PF-5212372 inhibition of eicosanoid release in in vitro whole-cell assays

| | Human Lung Mast Cells | Rat RBL-2H3 Cells | Sheep Lung Mast Cells |
|---|---|---|---|
| Cysteinyl LT | | | |
| $IC_{50}$, nM | 0.45 ± 0.33 | N.T. | N.T. |
| Maximal inhibition, % | 95 ± 1.08 | N.T. | N.T. |
| $PGD_2$ | | | |
| $IC_{50}$, nM | 0.29 ± 0.12 | 0.82 ± 0.11 | 0.78 ± 0.12 |
| Maximal inhibition, % | 101 ± 1.44 | 101 ± 0.56 | 95 ± 2.73 |

N.T., not tested.

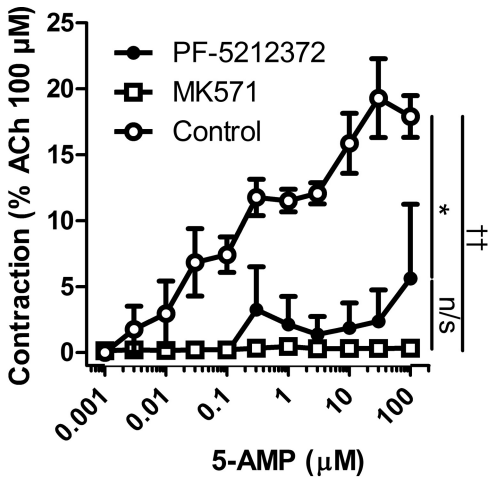


**Fig. 5.** PF-5212372 inhibition of AMP-induced contraction in isolated human bronchial sections. Isolated human bronchial rings were pretensioned and then preincubated for 30 min, with or without 200 nM PF-5212372 or 1 µM MK 571 (leukotriene receptor antagonist), before stimulation with various AMP 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 ($n = 3$).

sue data, for why a potent specific inhibitor of cPLA$_2\alpha$ should be effective in the treatment of asthma. It is possible that the reason why orally administered cPLA$_2\alpha$ 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 cPLA$_2\alpha$ 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 cPLA$_2\alpha$ 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 cPLA$_2\alpha$ 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 $PGD_2$ 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 cPLA$_2\alpha$ 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 cPLA$_2\alpha$ 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 $LTB_4$, $TXB_2$, and $PGD_2$, the potency is ~10-fold reduced in comparison with the human lung mast cell assay. This can be explained by the knowledge that cPLA$_2\alpha$ 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-

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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 $PGE_2$. 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 bronchoprotective effects of $PGE_2$ 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 $PGE_2$ in a clinical setting, this may be of great benefit (Mathison and Koziol, 2002). The reason for this reduced potency and efficacy versus $PGE_2$ 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 $cPLA_2\alpha$ causes shunting down the $PGE_2$ pathway or that $PGE_2$ is preferentially generated through non-$cPLA_2\alpha$ pathways. There is some historical evidence for different stimuli causing release of $PGE_2$ through non-$cPLA_2\alpha$ 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 $A_2$ in asthma pathophysiological conditions (Granata et al., 2010), and it is possible that, in the complex mixed cell population, $cPLA_2\alpha$ is not the most relevant enzyme responsible for the ultimate generation of $PGE_2$. 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 $cPLA_2\alpha$, 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 translatable efficacy 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 $PGE_2$ 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, Peachell, Matera, Cazzola, Page, Abraham, Williams, Clark, Liu, N.P. Clarke, and Yeadon.

*Conducted experiments:* Hewson, Patel, Calzetta, Campwala, Havard, Luscombe, P.A. Clarke, and Abraham.

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

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

*Wrote or contributed to the writing of the manuscript:* Hewson, Campwala, P.A. Clarke, Peachell, Cazzola, and Page.

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