Preclinical evaluation of an inhibitor of cytosolic phospholipase A$_2$α for the treatment of asthma

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Running Title: cPLA₂α for the treatment of asthma

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Number of Text Pages: 19
Number of Tables: 3
Number of Figures: 5
Number of References: 37
Number of Words in Abstract: 246
Number of Words in Introduction: 545
Number of Words in Discussion: 1742

List of Non-Standard Abbreviations: AHR, airway hyperresponsiveness; ANOVA, analysis of variance; AUC, area under the curve; COX, cyclooxygenase; cPLA₂α, cytosolic phospholipase A₂α; CRTH2, chemoattractant receptor expressed on T helper type 2 cells; DNP, dinitrophenyl; DPI, dry powder inhaler; ELISA, enzyme linked immunosorbent assay; FA, formic acid; FCeRI, high affinity IgE receptor; FCS, foetal calf serum; GLU, 7-hydroxycoumarinyl-γ-linolenate; HSA, human serum albumin; KH, Krebs-Henseleit; LT, leukotriene; PBS, phosphate buffered saline; PG, prostaglandin; TX, thromboxane.

Recommended Section Assignment: Inflammation, Immunopharmacology, and Asthma
Abstract

Asthma is a chronic inflammatory lung disease with considerable unmet medical requirement for new and effective therapies. Cytosolic phospholipase A$_2$α (cPLA$_2$α) is the rate-limiting enzyme ultimately responsible for production of eicosanoids implicated in the pathophysiology of asthma. We investigated a novel cPLA$_2$α inhibitor, PF-5212372, to establish the potential for this drug as a treatment for asthma. PF-5212372 was a potent inhibitor of cPLA$_2$α (7nM) and was able to inhibit prostaglandin D$_2$ (PGD$_2$) and cysteinyl leukotriene release from anti-IgE stimulated human lung mast-cells (0.29nM and 0.45nM, 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$_2$ (2.6nM, 2.6nM and 4.0nM, respectively), but was significantly less effective against prostaglandin E$_2$ (>301nM, $p<0.05$). In an in vitro cell retention assay, PF-5212372 retained its potency up to 24 h post wash-off. In a sheep model of allergic inflammation, inhalation of PF-5212372 significantly inhibited late-phase bronchoconstriction (78% inhibition, $p<0.001$) and airway hyper-responsiveness (94% inhibition, $p<0.001$) and isolated sheep lung mast cell assays confirmed species translation via effective inhibition of PGD$_2$ release (0.78nM). Finally, PF-5212372 was assessed for its ability to inhibit the contraction of human bronchus induced by adenosine 5’monophosphate (AMP). PF5212372 significantly inhibited AMP induced contraction of human bronchus (81% inhibition, $p<0.001$) and along with the ability of this drug to be effective in a wide-range of pre-clinical asthma models, suggests that inhibition of cPLA$_2$α using PF-5212372 may represent a new therapeutic option for the treatment of asthma.
**Introduction**

Asthma is a chronic inflammatory disorder of the airways causing recurrent episodes of wheezing, breathlessness, chest tightness, and coughing in susceptible individuals. The disease represents a significant global disease burden with major socio-economic consequences. Asthma prevalence in the developed world has been increasing in recent decades (particularly in children) and is estimated at ~5-10% of the population in developed countries (Global initiative in Asthma, 2002. NIH publication No 02-3659).

Although anti-inflammatory therapeutic options exist for the treatment of asthma, there remains unmet medical need for agents displaying high degrees of efficacy in the absence of significant adverse effects following chronic administration. Inhaled and oral steroids have been shown to be effective anti-inflammatories in the treatment of asthma, but chronic use of these agents can be associated with a range of side effects (e.g. dysphonia, oral candidiasis, suppression of hypothalamic pituitary axis) especially at high doses (Baptist and Reddy, 2009).

An asthma therapy inhibiting cytosolic phospholipase A$_2 \alpha$ (cPLA$_2 \alpha$) may address this unmet medical need. cPLA$_2 \alpha$ releases arachidonic acid from the phospholipid membrane and is the rate limiting enzyme in the biosynthesis of prostaglandins (PG), thromboxane (TBX) and leukotrienes (LT) (Ghosh et al., 2006) all implicated in airway inflammation, mucus production, bronchoconstriction, and airway hyperresponsiveness (AHR) associated with asthma (Drazen et al., 1999). LTB$_4$ is known to contribute to inflammation by both recruiting and activating leukocytes, while cysteinyl leukotrienes (LTC$_4$, D$_4$, and E$_4$) are powerful bronchoconstrictors that promote oedema by increasing vascular permeability and permitting leakage of plasma in the extravascular space (Boyce, 2008). Both 5-lipoxygenase inhibitors
(e.g. zileuton/ZYFLO) and leukotriene receptor antagonists (e.g. montelukast/Singulair) have been shown to have efficacy in the treatment of asthma (Price et al., 2011).

Sub-threshold contractile concentrations of PGD2 have been demonstrated to increase AHR to inhaled histamine and methacholine (Fuller et al., 1986), increase acute bronchoconstriction (Johnston et al., 1995) and PGD2 is increased in the lung following allergen-challenge of asthmatic subjects (Murray et al., 1986). PGD2 has also been implicated in multiple aspects of allergic inflammation via the chemoattractant receptor expressed on T helper type 2 cells (CRTH2). This receptor is preferentially expressed in T helper type 2 cells, eosinophils, and basophils in humans, has been shown to mediate PGD2-dependent cell migration of blood eosinophils and basophils as well as intracellular calcium mobilization and chemotaxis in Th2 cells (Nagata and Hirai, 2003). Recent data in asthmatic subjects has also demonstrated positive effects with a CRTH2 antagonist able to reduce eosinophil numbers in sputum, reduce circulating IgE levels and partially improve forced expiratory volume in 1 second (Barnes et al., 2009). TXA2, another potent constrictor of smooth muscle, has been implicated in the late asthmatic response after inhaled allergen in humans (Shephard et al., 1985), and in AHR in asthmatic subjects (Fujimura et al., 1986).

Direct evidence of a role for cPLA2α in respiratory disease has been demonstrated using cPLA2α deficient mice. These animals are resistant to bronchial hyperreactivity in an anaphylaxis model (Uozumi et al., 1997) as well as 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 cPLA2α (McKew et al., 2008). The purpose of the present investigation was to establish the preclinical effects of PF-5212372 as a potential novel anti-inflammatory treatment for asthma.
Methods

cPLA₂α enzyme assays. The 7-hydroxycoumarinyl-γ-linolenate (GLU) micelle enzyme assay was carried out as previously described (McKew et al., 2005).

Compound and vehicle use for in vitro and ex vivo experimentation. PF-5212372 and all other compounds used in this investigation were prepared in DMSO (Sigma Aldrich, St Louis, MO) and assessed at a DMSO final assay concentration of 0.1% (v/v). All vehicle controls were also assessed using a DMSO final assay concentration of 0.1% (v/v).

Human lung mast cell assay. Mast cells were isolated by physical and enzymatic dispersion of lung tissue obtained from surgical resections according to methods that have been described elsewhere (Ali and Pearce, 1985). Ethical approval for the types of experiment to be performed was in place and all patients donating tissue gave their informed consent. Mast cells were incubated with a variety of concentrations of PF-5212372 for 15 min prior to being activated with 2 µg/mL human anti-IgE (clone HP6061; Hybridoma Reagent Laboratory, Baltimore, MD). Supernatants were harvested 25 minutes later and stored at -80°C until required for analysis.

Sheep lung mast cell assay. Lung tissue from Suffolk Crosses was obtained from Matrix Biologicals Limited (Hull, UK). Mast cells were isolated by physical and enzymatic dispersion as described for the human assay. Following dispersion, cells were rested for 24 h at 37°C and 5% CO₂ in RPMI medium (Invitrogen, Carlsbad, CA) supplemented with 10% foetal calf serum (FCS) (Sigma Aldrich), gentamycin (Sigma-Aldrich) and penicillin-streptomycin (Sigma Aldrich). Cells were then centrifuged for 10 min at 200g for 10 min. Contaminating red blood cells were removed by hypotonic lysis for 30 s in ice-cold distilled water before isotonicity was restored by addition of an equal volume of ice-cold 2X phosphate buffered saline (PBS). Cells were centrifuged as above and re-suspended 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 prior to being activated with 1 µM ionomycin (Tocris Bioscience, Bristol UK). Supernatants were harvested 1 h later and stored at -80°C until required for analysis.

**Human whole lung digest assay.** Human lung tissue was obtained from Papworth Hospital, Cambridge, UK. Approximately 3 g of lung parenchyma was placed into a single gentleMACSTM C tube (Miltenyi Biotec, Bergisch Gladbach, Germany) followed with 10 mL 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 on a gentleMACSTM 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 first through 100 µm then 40 µm cell strainers. The cleared cell preparation was then centrifuged at 200 g and 4°C for 10 min and re-suspended in ice-cold PBS containing 5% (v/v) FCS (Sigma Aldrich). Contaminating red blood cells were removed by hypotonic lysis for 30 s in ice-cold distilled water before isotonicity was restored by addition of an equal volume of ice-cold 2X PBS. Cells were centrifuged as above before being re-suspended in assay buffer containing 0.1% (w/v) bovine serum albumin (Sigma Aldrich) and 10mM HEPES in gassed Tyrode’s solution at 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 prior to being activated with 3 µM ionomycin (Tocris Bioscience). Supernatants were harvested 1 h later and stored at -80°C until required for analysis.

**RBL-2H3 cell retention assay.** The RBL-2H3 mast cell line was purchased from the American Type Culture Collection (ATCC number CRL-2256) 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 and 5% CO₂ in a humidified incubator.

RBL-2H3 cells were seeded into 96-well plates at 1x10⁵ cells per well in growth medium containing 0.5 μg/ml dinitrophenol (DNP) -specific murine IgE (clone SPE7; Sigma-Aldrich). Cells were cultured for 30 h before medium was replaced with Roswell Park Memorial Institute-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 (UW) were incubated for 1 h with compound before activation with 100 ng/mL DNP-human serum albumin (DNP-HSA) (Sigma-Aldrich). For “washed” cells, the compound containing medium was removed and the cells washed three times with fresh experimental medium. Cells were then either immediately activated as above (T=0), or incubated for a further 3 (T=3) or 24 h (T=24) in experimental medium before activation. Cells were centrifuged at 300g 1 h post stimulation and supernatants were harvested and stored at -80°C until required for analysis.

**PGD₂ and cysteinyl LT ELISAs.** Cysteinyl LT and PGD₂ levels were quantified using commercially available competition ELISAs following manufacturer’s instructions (Cayman Chemical, Ann Arbor, MI). Briefly, for PGD₂ quantification supernatants were treated with methoxyamine hydrochloride for 30 min at 60°C and diluted before being placed into pre-coated ELISA plates, together with acetylcholinesterase-conjugated PGD₂-MOX and PGD₂-MOX antiserum. For cysteinyl leukotriene quantification, the same process was followed, but samples did not require any treatment before being diluted and placed into pre-coated 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 PGD₂ and 18 h at room temperature for
cysteinyl LT) and absorbance measured at 412nM. Higher absorbance indicated less PGD2 or cysteinyl LT. Sensitivity was 3.1 pg/mL for PGD2 and 34 pg/mL for cysteinyl LT.

**Histamine quantification assay.** Histamine levels were quantified using the automated fluorometric assay developed by Siraganian (Siraganian, 1974). Histamine was determined as a percentage of the total histamine content by lysing control cells with perchloric acid and subtraction of the spontaneous histamine release.

**TXB2, PGE2, PGD2 and LTB4 measurement by LC-MS/MS.** Lung tissue or cell supernatants were added to a Whatman 2 mL 96-well plate (50 µL per well). 300 µL acetonitrile containing 500 pg of each of the stable isotope labelled analogues of the analytes, TXB2 (TXB2-d4), PGE2 (PGE2-d4), PGD2 (PGD2-d4) and LTB4 (LTB4-d4), was then added to each well and the plate agitated for 15 min to allow proteins precipitation. 1300 µL 0.1% formic acid (FA) (aq) was then added to each well, the plate sealed and centrifuged at 4000 rpm on an Eppendorf Centrifuge 5810R for 30 min at 4°C. The plate was transferred to a CTC PAL autosampler and 500 µL of the supernatant was injected into the LC-MS/MS system for measurement of TXB2, PGE2, PGD2 and LTB4. Sample analysis was performed using on-line solid phase extraction with liquid chromatography turbo-V ionspray ionisation tandem mass spectrometry (SPE-LC-MS/MS). The LC-MS/MS system consisted of an API4000 triple quadrupole mass spectrometer (Applied Biosystems, Warrington, UK) equipped with a Turbo-V ion source operating in negative ESI mode, two Agilent 1100 binary HPLC pumps (pump 1 as analytical pump and pump 2 as the loading pump) and degasser (Agilent, Winsorsh, UK) and CTC PAL Autosampler (Presearch Ltd, Milton Keynes, UK). A valco switching valve was used to perform the on-line SPE switching method (10-port, VICI Valco Instruments, Houston, TX). For the on-line SPE and chromatographic separation, a trapping cartridge, Thermo Biobasic 10 x 2.1 mm (Part No 72105-012101, Thermo) and a TARGA C18, 75 x 2.1 mm 3 µm analytical column (Higgins
Analytical, Mountain View, CA). Gradient elution was employed to clean-up, elute and separate the four analytes. The HPLC system comprised of two HPLC pumps, a CTC autosampler and an additional Valco valve driver unit. The system was set up such that following 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 using pump 1, continued separating and were eluted into the mass spectrometer source between 1.7 and 3.8 min from injection. The mobile phase for pump 1 (elution) started at 40% acetonitrile in 0.1% FA (aq) rising 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. Overall chromatography time was 4 min and total cycle time (injection to injection) was 4.75 min. The mass spectrometer was operated in the negative ion mode with an ionspray voltage of -4200 V at 650°C. Multiple reaction monitoring was used for quantification. All quadrupoles were working at “low” resolution (FWHM 1 Da). Quantitation was performed with Analyst Software V1.5 (Applied Biosystems) using the internal standard method (isotope-dilution mass spectrometry). Ratio of analyte peak area and internal standard peak area (y axis) 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 by retention time and elution order with PGE2 eluting first (2.2 min) followed by PGD2 (2.4 min). Mass transitions for these molecules were: PGE2/PGD2, m/z 351.2 to m/z 271.2 and for PGE2-d4/PGD2-d4, m/z 355.2 to m/z 275.2. A collision energy of -26 V, declustering potential of -48 V and a dwell time of 25 ms was used for all analytes and transitions. Results were reported as ratio of native
molecule to stable isotope labelled molecule; a ratio of “1” would indicate an approximate concentration of 10 ng/mL in the original cell culture supernatant.

**Sheep model of allergic inflammation.** Allergic sheep were used to assess the anti-asthmatic action of PF-5212372 as previously described (McKew et al., 2008). PF-5212372 was delivered as an aerosol via a Raindrop disposable medical nebuliser (Puritan Bennett, Lenexa, KS) or as a dry powder using a single-dose Spinhaler dry powder inhaler (DPI). For nebulisation studies, PF-5212372 was dissolved in 5 mL ethanol and nebulised until dry. For DPI delivery, PF-5212372 was loaded into a Size No. 2 gelatin capsule, placed into 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 all of the compound was inhaled. Aerosols were delivered with a mass median aerodynamic diameter of 3.2 µm, or <4.6µM for the DPI.

**Human bronchus contractility assay.** Regions of macroscopically normal lungs were taken from uninvolved areas of the resection from 3 patients (2 male and 1 female, 52.4 ± 3.7 years old) undergoing lobectomy surgery for lung cancer, but without a history of chronic airway disease. Airways were immediately placed into oxygenated Krebs-Henseleit (KH) buffer solution (119 mM NaCl; 5.4 mM KCl; 2.5 mM CaCl₂; 1.2 mM KH₂PO₄; 1.2 mM MgSO₄; 25 mM NaHCO₃ and 11.7 mM glucose at pH 7.4) containing the cyclooxygenase (COX) inhibitor indomethacin (5.0 µM), and transported at 4°C. None of the patients were chronically treated with theophylline, β₂-adrenoceptor agonists or glucocorticosteroids. Serum IgE levels determined on the day of surgery were within the normal range. Bronchial rings were transferred into a 4 chamber isolated organ bath system containing KH buffer solution (37°C), continuously bubbled with carboxygen mixture and connected to an isometric force displacement transducer. Airways were allowed to equilibrate for 90 min flushing with fresh KH buffer solution every 10 min. Passive tension was determined by gentle stretching of tissue (0.5 – 1.0g) during equilibration. The isometric change in tension...
was measured with a transducer Fort 10 WPI (Basile Instruments, Italy). The tissue responsiveness was assessed using 100 µM acetylcholine and when the response reached a plateau, rings were washed three times and allowed to equilibrate for 30-45 min. Bronchial rings were incubated for 30 min with 200 nM PF-5212372 or 1 µM MK-571 (cysteinyl LT receptor antagonist) before stimulation with an AMP concentration response curve (1 nM to 100 µM). Contractile responses were calculated as a percentage of the initial contraction observed with 100 µM acetylcholine.

**Statistical analysis.** Data are presented as mean ± the standard error of the mean or, for IC50s, as geometric mean ± the standard error of the geometric mean. Data were analysed using the 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 when 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 in McKew et al. (McKew et al., 2008) to identify next generation inhibitors of cPLA$_{2\alpha}$. This entire series was previously demonstrated to be exquisitely selective for cPLA$_{2\alpha}$, and when PF-5212372 was profiled in the GLU micelle cPLA$_{2\alpha}$ enzyme assay it demonstrated high potency with an IC$_{50}$ value of 7 nM. This made PF-5212372 one of the most potent inhibitors to date from this indole series. With the demonstration that PF-5212372 was a highly potent and selective (data not shown) inhibitor of cPLA$_{2\alpha}$, we have now interrogated this novel drug in a range of human cells and tissues in vitro and 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 as 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 pre-formed histamine and the rapidly metabolised cysteinyl LT and PGD$_2$ all implicated in asthma pathogenesis. To assess the ability of PF-5212372 to inhibit this activation, mast cells were isolated from human lung surgical resections and pre-incubated with a variety of concentrations of PF-5212372 for 15 min. The mast cells were then activated using an anti-IgE antibody, binding and cross-linking the bound IgE. Supernatants were harvested 30 min post-stimulation and cysteinyl LT and PGD$_2$ quantified separately by ELISA. Histamine was also quantified using a fluorometric assay. PF-5212372 significantly inhibited cysteinyl leukotriene and PGD$_2$ release with IC$_{50}$’s of 0.45 nM and 0.29 nM, respectively (Fig. 2; Table 3). Maximum inhibition was 99% and 98%, respectively,
compared to uninhibited control cells. In contrast, no inhibition of histamine 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 at 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 end-points generated from different cells in a single assay via a non-specific activation process (in this case a calcium ionophore). To this end, pieces of human lung parenchyma were homogenised to yield a viable mixed cell population. Various concentrations of PF-5212372 were pre-incubated for 1 h with the mixed cell population before stimulation with 3 µM ionomycin. Supernatants were harvested 1 h later LTB₄, TXB₂, PGE₂ and PGD₂ assessed concurrently by mass-spectrometry. Percentage inhibition was calculated in comparison to ionomycin-alone conditions. PF-5212372 was an effective inhibitor of LTB₄, TXB₂ and PGD₂ (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 to the other end-points (<11%; p <0.001 cf. LTB₄; p <0.01 cf. TXB₂ and PGD₂). When compared to the COX inhibitor indomethacin (Table 1), the COX inhibitor was effective in inhibition of PGE₂ with a comparable potency (173 nM; p >0.05) and <2-fold reduction in efficacy for this end-point compared to the others (63%; p <0.01 cf. PGD₂; p <0.05 cf. TXB₂). By way of control, it was observed that indomethacin had no effect on LTB₄ release confirming the expected inhibition of COX only with no effect on 5-lipoxygenase activity.

Assessment of cell retention as a surrogate for in vitro duration of action. For an inhaled drug it is desirable to have retention at the site of pharmacology to allow for a good duration of action. A surrogate in vitro assay was therefore employed to make an assessment for 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 PGD\(_2\) release 1 h later by mass spectrometry. In unwashed cells, PF-5212372 was a potent inhibitor of PGD\(_2\) release (IC\(_{50}\) of 0.8 nM) and this level of inhibition was retained even when the compound was removed and the cells subsequently stimulated up to 24 h later (Figure 3A; Table 2). In contrast, Compound A, a cPLA\(_2\) inhibitor from a different series with reduced lipophilicity, protein binding and enzyme potency (Figure 1B), was effectively shown to be “washed-off” the cells with a progressive and significant reduction in potency observed over time (Figure 3B; Table 2).

**In vivo assessment of PF-5212372 in a sheep model of allergic inflammation.** With the demonstration that PF-5212372 was a potent and effective inhibitor of cPLA\(_2\)\(\alpha\) in primary human lung cell assays and with evidence for cell retention, it was 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 dosed with 3 mg PF-5212372 via inhalation as a dry powder or nebulisation within a liquid vehicle 16 h and 1 h prior to challenge. PF-5212372 was highly effective at inhibiting the late phase bronchoconstriction, with 78% inhibition of the area under the curve (AUC) in comparison to an inhaled vehicle (lactose or ethanol as appropriate) control (Figure 4A). When AHR was assessed using inhaled carbachol, PF-5212372 was again highly effective, with 94% inhibition of the response when compared to vehicle (Figure 4B). Identical data was generated when 1 mg PF-5212372 was chronically dosed once-daily for 7 days prior to challenge as a dry powder (data not shown). However, when 1 mg PF-5212372 was dosed acutely (16 h and 1 h prior to challenge) in the same manner as the 3 mg experiments described above, this did not inhibit bronchoconstriction or AHR (data not shown).
We then sought to follow up the in vivo results in allergic sheep with effects on sheep cells. A series of in vitro experiments were therefore run using primary mast cells isolated from the lungs of sheep. In an analogous manner to the human assay, isolated sheep mast cells were pre-incubated for 1 h with PF-5212372 before stimulation with 1 µM ionomycin. Supernatants were harvested 1 h later and PGD₂ levels quantified by mass spectrometry. Anti-IgE stimulation was not possible with sheep mast cells due to the lack of availability of sheep-specific antibodies. However, experiments were carried out in human mast cells to demonstrate that comparable data was produced when comparing anti-IgE and ionomycin stimulations (data not shown). PF-5212372 inhibited ionomycin-stimulated PGD₂ release from sheep mast cells with an IC₅₀ of 0.79 nM and a maximum inhibition of 95% (Figure 4C; Table 3). This compared well with the data generated in human mast cells confirming translation of primary pharmacology 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 prior to challenge with an AMP concentration response curve, a significant 81% inhibition of the AUC was observed ($p <0.01$; Figure 5). Additionally, analysis of the data at the AMP $E_{\text{max}}$ concentration (100 µM) indicated PF-5212372 produced a significant 69% inhibition compared to the control tissues ($p <0.05$). The level of inhibition was comparable to that observed with 1 µM MK571, a specific leukotriene receptor antagonist, confirming previous data with AMP-induced contraction of human bronchial tissue (Björck and Dahlén, 1992), and was not significantly different for either the AUC or AMP $E_{\text{max}}$ comparisons ($p >0.05$).
Discussion

We have described here for the first time a clear rationale for inhibition of cPLA$_2^\alpha$ as a potential treatment of asthma using a potent and selective inhibitor of the enzyme, PF-5212372. We have demonstrated that PF-5212372 is potent and broad-spectrum inhibitor of eicosanoid release in primary human mast cells, an acknowledged pivotal cell type in the pathogenesis of allergic asthma – importantly using a physiologically relevant stimulation (FcεRI cross-linking). We followed up this data by demonstrating a 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 using a “washed” whole cell potency assay. We were able to demonstrate that PF-5212372 was retained within the cells and able to inhibit PGD$_2$ release up to 24 h after the excess compound was removed from the cell medium. This demonstrated that PF-5212372 may have a suitable duration of action to be effective as once a day inhaled medicine. In line with this, we established the preclinical activity of PF-5212372 as an inhaled therapy using a sheep model of allergic lung inflammation. The sheep allergic inflammation model developed by Abraham et al. uses naturally allergic sheep and challenges them with *Ascaris suum* antigen via inhalation (Abraham, 2008). This results in development of both an early phase and late phase bronchoconstriction as well as AHR to inhaled carbachol. Therefore this model exhibits several features that are reminiscent of the response observed following allergen challenge in subjects with allergic asthma and allows preclinical assessment of potential asthma therapies. Using this model, we established that inhaled PF-5212372 was able to inhibit the late-phase allergic response as well as allergen-induced AHR assessed by carbachol inhalation – although as we did not specifically evaluate effects on cellular infiltration (for example eosinophils) into the lung, we can only infer that additional effects on cellular infiltration were produced by the inhibition of cPLA$_2^\alpha$-mediated
We then confirmed translation of this effect by demonstrating that PF-5212372 was equally potent and effective in a primary sheep lung mast cell assay when compared to the human assay. Finally, we demonstrated that PF-5212372 was effective at inhibiting AMP-induced contraction of human bronchus. 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 per sheep for 7 days) a trend for inhibition of the early-phase was observed (data not shown), but not complete inhibition. It remains a possibility that higher or more extended dosing may improve inhibition of the early-phase (although sufficient compound was available to completely inhibit both the late-phase and AHR) but it is also likely that the role of non-prostanoids, for example histamine, are more important in this phase. We clearly demonstrated in both human and sheep mast cells that PF-5212372 had no effect on release of histamine. It is therefore possible that inhibition of cPLA2α may not be clinically efficacious as a reliever-type medicine, but may be more suitable for maintenance therapy.

cPLA2α 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 implicated in the pathogenesis of asthma (Drazen et al., 1999), it seems logical that inhibition of the enzyme could be a useful therapeutic option for the treatment of this common disease. In addition, while unlikely to be a direct effect, downstream inhibition of cytokine release from a variety of immune cells may also be expected as a result of inhibition of cPLA2α mediated prostanoid release thereby further improving the rationale for effective treatment of asthma (Schuligoli et al., 2010). However, to our knowledge this publication is the first to clearly demonstrate a convincing rationale, including primary human lung cell and tissue data, that a potent and specific inhibitor of cPLA2α should be effective in the treatment of asthma. It is possible that the reason oral cPLA2α inhibitors have focused primarily on
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Arthritis and inflammatory pain indications is due to the relative success of COX-2 inhibitors, especially prior to 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 also limit the utility of an oral 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 minimise unwanted side-effects. One of the central themes for designing an effective inhaled medicine is to design in duration of pharmacological action. The improved patient convenience and compliance associated with either twice-daily or, ideally, once-daily administration is expected to yield a more effective therapy and is a central tenet for the latest generation of anti-inflammatory and bronchodilator therapies (Van Den Berge et al., 2010; Cazzola et al., 2011). Clearly, preclinical demonstration for an extended duration of pharmacology would be a desirable feature of a novel anti-inflammatory drug. As cPLA₂α inhibitors such as PF-5212372 must gain access to the enzyme via dissolution into a lipid membrane, any cellular retention of the compound may yield a suitable duration of pharmacology. As outlined in this publication, PF-5212372 is able to maintain inhibition of PGD₂ release for up to 24 h after the cells are washed to remove the compound from the aqueous phase (Figure 2). This is in contrast to a related cPLA₂α inhibitor (compound A) which can be “washed” from the cells progressively over time. Compound A has reduced lipophilicity and protein binding in comparison to PF-5212372 and it is hypothesised that it is this change in physical-chemical properties that drives the reduced retention. While this type of experiment does not provide a clear indication of whether PF-5212372 could be once- or twice-daily, encouragement for a suitable duration was obtained by the observation that once-daily chronic dosing of 1 mg in the sheep model (data not...
shown) was as effective as more acute dosing with 3 mg PF-5212372 (Figure 3). It was also be noted that PF-5212372 was not detected systemically following inhalation in the sheep, confirming the intended reduction in systemic exposure.

One of the most interesting and unexpected pieces of data presented here is around the human lung homogenate experiments. We had expected that this assay would provide comparable data to the primary human lung mast cell experiments, but demonstrate additional evidence for broad spectrum eicosanoid inhibition in a complex cell mixture derived from the human lung. Indeed, our data does demonstrate that inhibition of cPLA2α with PF-5212372 produces a potent and effective inhibition of multiple arachidonic acid metabolites in this mixed cell population. However, the first observation is that, while the potency is comparable for LTB4, TXB2 and PGD2, the potency is some 10-fold reduced in comparison to the human lung mast cell assay. This can be explained again by the knowledge that cPLA2α cleaves its phospholipid substrate at the membrane/water interface and that inhibitors must therefore sequester into 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 very much higher cell numbers allowing 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 those compounds that would be effective when dosed systemically. Indeed, the human lung homogenate potency data is very comparable to the GLU micelle potency described for PF-5212372 (7 nM).

Which potency value is most relevant for inhaled delivery is debatable. While the compound is dosed topically and the level of systemic free concentration to drive pharmacology is moot from a lung efficacy perspective, the identification that potency is reduced in a complex cell system does potentially argue that this will 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 inhibition of PGE$_2$. Indeed, of the three separate experiments run with PF-5212372, in two of them no apparent inhibition was observed with up to 1 µM PF-5212372. This is of great interest due to 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 was 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 in susceptible asthmatics, likely due to the shunting of arachidonic acid through the 5-lipoxygenase pathway leading to enhanced production of leukotrienes (Szczeklik, 1990) and it is therefore possible that inhibition of cPLA$_2$ may cause shunting down the PGE$_2$ pathway – or that PGE$_2$ may be preferentially generated through non-cPLA$_2$ pathways. Indeed there is some historical evidence for different stimuli causing release of PGE$_2$ through non-cPLA$_2$ mechanisms (Berenbaum et al., 1996) as well as recent evidence for cross-talk between enzymes involved in arachidonic metabolism (Niknami et al., 2010). Finally, there is evidence for the role of the secreted PLA$_2$’s in asthma pathophysiology (Granata et al., 2010) and so it is possible that in the complex mixed cell population, cPLA$_2$ is not the most relevant enzyme responsible for ultimate generation of PGE$_2$. The mechanism is clearly not defined here, but it does raise an interesting question for further investigation.

In summary, we have presented a wide range of data on a novel inhibitor of cPLA$_2$, PF-5212372, from a preclinical perspective as a potential new inhaled therapy for the treatment of asthma. We have demonstrated that PF-5212372 is a potent and broad spectrum inhibitor of inflammatory eicosanoid release from human primary lungs cells, combining this with translatable efficacy in a preclinical animal model of allergic airways disease and
demonstration of a functional response in human lung tissue. In addition, we have identified for the first time a potential for sparing PGE$_2$ inhibition in the lung that may provide a distinct advantage for this mechanism in treating patients with asthma.
Authorship Contributions

Participated in research design: Hewson, Patel, Williams, Clark, N.P. Clarke, Yeadon, Page, Cazzola, Abrahams, Peachell, Matera, Liu

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

Performed data analysis: Hewson, Patel, Campwala, Williams, Clark, Luscombe

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

Contributed new reagents or analytical tools: Clark, P.A. Clarke, Luscombe
References


Footnotes

a) This work was funded by Pfizer Ltd.

b) N/A

c) Reprint requests to Christopher A. Hewson PhD, The Old House Cottage, Easole Street, Nonington, Dover, KENT CT 15 4HE, UK, c_hewson@sky.com

d) N/A
Legends for Figures

Figure 1.

Chemical structure of (A) PF-5212372, C_{42}H_{38}ClF_{3}N_{2}O_{6}S_{2} (molecular weight 823.35) and (B) compound A.

Figure 2. PF-5212372 inhibits anti-IgE mediated PGD$_2$ and cysteinyll LT release from isolated human lung mast cells.

Human lung mast cells were isolated and pre-incubated 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 (A) cysteinyll LT or (B) PGD$_2$ measured by ELISA. * and *** indicates data significantly different to control cells without PF-5212372 at $p < 0.05$ and $p < 0.001$, respectively (n=3).

Figure 3. PF-5212372 retains cellular potency following “wash-off” in contrast to an unrelated Compound A.

RBL-2H3 cells were sensitised with IgE and pre-incubated for 60 min with or without various concentrations of (A) PF-5212372 or (B) the unrelated compound A. Cells were then immediately stimulated with 100 ng/mL DNP-HSA (“UW”; closed circles), or were washed three times and left in fresh experimental medium for 0 hours (“T=0”, closed squares), 3 hours (“T=3”, open circles) or 24 hours (“T=24”, open squares) before stimulation with DNP-HSA. Supernatants were harvested 60 min later and PGD$_2$ measured by mass spectrometry (n=3).
Figure 4. Inhaled PF-5212372 inhibits allergen-induced late-phase bronchoconstriction and AHR in a sheep model of allergic asthma, and inhibits ionomycin-stimulated PGD₂ release from isolated sheep lung mast cells.

3 mg PF-5212372 or vehicle was delivered via inhalation to naturally allergic sheep 16 h and 1 h prior to allergen challenge (Ascaris suum). (A) Airway resistance was measured through the following 8 h. *** indicates AUC data significantly different to vehicle control (+4 to +8 h) at $p < 0.001$ (n=3). (B) A further dosing of PF-5212372 or vehicle was performed 8 h after the allergen challenge carbachol-induced AHR measured the following day. *** indicates post-carbachol challenge data significantly different to vehicle control at $p < 0.001$ (n=3). (C) Sheep lung mast cells were isolated and pre-incubated for 1 h with or without various concentrations of PF-5212372 before stimulation with 1 µM ionomycin. Supernatants were harvested 1 h later and PGD₂ measured by mass spectrometry. * and *** indicates data significantly different to control cells without PF-5212372 at $p < 0.05$ and $p < 0.001$, respectively (n=3).

Figure 5. PF-5212372 inhibits AMP-induced contraction in isolated human bronchus sections.

Isolated human bronchial rings were pre-tensioned and then pre-incubated for 30 min with or without 200 nM PF-5212372 or 1 µM MK571 (leukotriene receptor antagonist) before stimulation with an AMP concentration response curve. Contractile responses were then monitored. * indicates data significantly different to the control tissue when stimulated with 100 µM AMP at $p < 0.05$ (n=3). n/s indicates data that was not significantly different between PF-5212372 and MK571 when stimulated with 100 µM AMP at $p > 0.05$ (n=3). †† indicates AUC data significantly different to control tissue at $p < 0.01$ (n=3).
Table 1: IC$_{50}$ and maximum 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. * indicates IC$_{50}$ data significantly different to PGE$_2$ at $p < 0.05$ (n=3). †, †† and ††† indicate maximum inhibition data significantly different to PGE$_2$ at $p < 0.05$, 0.01 and 0.001, respectively (n=3). – indicates “not tested”.

<table>
<thead>
<tr>
<th></th>
<th>LTB$_4$</th>
<th>TXB$_2$</th>
<th>PGD$_2$</th>
<th>PGE$_2$</th>
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<tr>
<td></td>
<td>IC$_{50}$ (nM)</td>
<td>Max inhib.</td>
<td>IC$_{50}$ (nM)</td>
<td>Max inhib.</td>
</tr>
<tr>
<td>PF-5212372</td>
<td>2.6* ±2.3</td>
<td>92%†††</td>
<td>2.6* ±3.3</td>
<td>68%†††</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>- -</td>
<td>60 ±30</td>
<td>91%†</td>
<td>37 ±3.3</td>
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</tbody>
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Table 2: IC₅₀ values for PF-5212372 and the unrelated compound A in the RBL-2H3 cell retention assay.

IC₅₀’s at each wash time-point presented in relation to the “unwashed” values. ** and *** indicate IC₅₀ values significantly different to the unwashed IC₅₀ for the same compound at \( p < 0.01 \) and \( p < 0.001 \), respectively.

<table>
<thead>
<tr>
<th></th>
<th>Unwashed</th>
<th>0 h</th>
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<th>24 h</th>
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<tr>
<td><strong>PF-5212372</strong></td>
<td></td>
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<tr>
<td>IC₅₀ (nM)</td>
<td>0.82</td>
<td>1.25</td>
<td>1.25</td>
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<tr>
<td>±0.11</td>
<td>±0.10</td>
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<td>1.6</td>
<td>0.9</td>
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<tr>
<td>±0.09</td>
<td>±0.41</td>
<td>±0.11</td>
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<tr>
<td><strong>Compound A</strong></td>
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</tr>
<tr>
<td>IC₅₀ (nM)</td>
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<td>257**</td>
<td>721***</td>
<td>1177***</td>
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<td>±13.8</td>
<td>±63.2</td>
<td>±68.6</td>
<td>±198</td>
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<tr>
<td>Fold “unwashed”</td>
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<td>13</td>
<td>20</td>
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<tr>
<td>±0.50</td>
<td>±3.7</td>
<td>±3.3</td>
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Table 3: Human, Rat and Sheep translation of IC₅₀ and maximum inhibition values for PF-5212372 inhibition of eicosanoid release in in vitro whole cell assays.

– indicates “not tested”.

<table>
<thead>
<tr>
<th></th>
<th>Human Lung Mast-Cell</th>
<th>Rat RBL-2H3</th>
<th>Sheep Lung Mast-Cell</th>
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<tr>
<td>Cys-LT</td>
<td>IC₅₀ (nM)</td>
<td>0.45 ±0.33</td>
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<tr>
<td></td>
<td>Max inhib.</td>
<td>95% ±1.08</td>
<td>-</td>
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<tr>
<td>PGD₂</td>
<td>IC₅₀ (nM)</td>
<td>0.29 ±0.12</td>
<td>0.82 ±0.11</td>
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<tr>
<td></td>
<td>Max inhib.</td>
<td>101% ±1.44</td>
<td>101% ±0.56</td>
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