Pharmacological Blockade of the DP2 Receptor Inhibits Cigarette Smoke-Induced Inflammation, Mucus Cell Metaplasia, and Epithelial Hyperplasia in the Mouse Lung

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ABSTRACT

Prostaglandin D₂ (PGD₂) is one of a family of biologically active lipids derived from arachidonic acid via the action of COX-1 and COX-2. PGD₂ is released from mast cells and binds primarily to two G protein-coupled receptors, namely DP1 and DP2, the latter also known as chemoattractant receptor-homologous molecule expressed on Th2 cells. DP2 is predominantly expressed on eosinophils, Th2 cells, and basophils, but it is also expressed to a lesser extent on monocytes, mast cells, and epithelial cells. Interaction of PGD₂ and its active metabolites with DP2 results in cellular chemotaxis, degranulation, up-regulation of adhesion molecules, and cytokine production. Chronic obstructive pulmonary disease (COPD) is a chronic progressive inflammatory disease characterized by elevated lung neutrophils, macrophages, and CD8+ T lymphocytes and mucus hypersecretion. Cigarette smoke contributes to the etiology of COPD and was used here as a provoking agent in a murine model of COPD. In an acute model, (2'-[cyclopropanecarbonyl-ethyl-amino]-methyl)-6-methoxy-4'-trifluoro-methyl-biphenyl-3-yl)-acetic acid, sodium salt (AM156) and (5-{2-[((benzoyloxycarbonyl-ethyl-amino)-methyl]-4-trifluoromethyl-phenyl}-pyridin-3-yl)-acetic acid, sodium salt) (AM206), potent DP2 receptor antagonists, dose-dependently inhibited influx of neutrophils and lymphocytes to smoke-exposed airways. In a subchronic model, AM156 and AM206 inhibited neutrophil and lymphocyte trafficking to the airways. Furthermore, AM156 and AM206 treatment inhibited mucus cell metaplasia and prevented the thickening of the airway epithelial layer induced by cigarette smoke. These data suggest that DP2 receptor antagonism may represent a novel therapy for COPD or other conditions characterized by neutrophil influx, mucus hypersecretion, and airway remodeling.

Chronic obstructive pulmonary disease (COPD) is a complex disease in which airflow obstruction is not fully reversible. It is a chronic, progressive inflammatory condition of the small airways and lung parenchyma that is characterized by elevated pulmonary macrophages, neutrophils, and CD8+ T cells. Proinflammatory mediators, including IL-8, MCP-1, and IL-1β, are elevated in bronchoalveolar lavage fluid (BALF) and sputum samples obtained from patients with COPD (Keatings et al., 1996; Yamamoto et al., 1997; Hill et al., 1999; Soler et al., 1999). Chronic inflammation contributes to the decline in pulmonary function associated with chronic bronchitis, mucus hypersecretion, and emphysema via the release of proinflammatory mediators, reactive oxygen species, and tissue degradation enzymes (Corrigan and Kay, 1991; Saetta, 1999; Shapiro, 1999, 2002; Cosio and Majo, 2002; Stockley, 2002; Tetley, 2002). At present, there

ABBREVIATIONS: COPD, chronic obstructive pulmonary disease; AM156, (2'-[cyclopropanecarbonyl-ethyl-amino]-methyl)-6-methoxy-4'-trifluoro-methyl-biphenyl-3-yl)-acetic acid, sodium salt; AM206, (5-{2-[((benzoyloxycarbonyl-ethyl-amino)-methyl]-4-trifluoromethyl-phenyl}-pyridin-3-yl)-acetic acid, sodium salt); BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; COX, cyclooxygenase; PGD₂, prostaglandin D₂; DK-PGD₂, 13,14-dihydro-15-keto prostaglandin D₂; IL, interleukin; KG, keratinocyte-derived chemokine; PBS, phosphate-buffered saline; MCP-1, monocyte chemoattractant protein-1; MIP-2, macrophage inflammatory protein-2; DMSO, dimethyl sulfoxide; MC, methylcellulose; PAS, periodic acid Schiff; ANOVA, analysis of variance; AZ11805131, 2-[4-ethylsulfonyl-2-methylphenyl]-4-[trifluoromethyl]phenox)acetic acid.

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are no therapies that treat the underlying mechanisms of COPD. Corticosteroids, which are effective in other inflammatory conditions such as asthma are effective only against COPD exacerbations. Thus, a large unmet medical need exists for novel therapies aimed at ameliorating the progression of COPD.

Prostaglandin D₂ (PGD₂) is one of a family of biologically active lipids derived from arachidonic acid via the action of COX-1 and COX-2. Mast cells are the primary source of PGD₂, but antigen-presenting cells and Th2 cells also contribute to the production of PGD₂ (Urade et al., 1989, 1990; Tanaka et al., 2000). PGD₂ binds primarily to two distinct G protein-coupled receptors, namely DP1 and DP2; DP2 is also known as chemoattractant receptor-homologous molecule expressed on Th2 cells. PGD₂ is rapidly degraded after release and gives rise to several metabolites that are selective and potent agonists of the DP2 receptor (Hirai et al., 2001; Heineemann et al., 2003; Almishri et al., 2005). This suggests that PGD₂ release may cause sustained activation of DP2.

At least one preclinical study has demonstrated an increase in PGD₂ in the BALF after exposure to cigarette smoke (Hong and Lee, 1996). Furthermore, the concentrations of PGD₂ in BALF recovered from COPD patients inversely correlated with forced expiratory volume in 1 s and peak expiratory flow, suggesting that PGD₂ plays a role in the worsening of the disease (Csanky et al., 2009). However, the role for PGD₂ in COPD remains to be further defined.

DP2 is predominantly expressed on eosinophils, Th2 cells, and basophils and is thought to play a key role in Th2-driven conditions, including allergic rhinitis and asthma (Pettipher, 2008). Relatively little attention has been given to DP2 in non-Th2-driven pathophysiology, including, for example, neutrophilia associated with COPD. This may be because, under normal conditions, human neutrophils do not express DP2 (Sawyer et al., 2002). However, DP2 expression has been found to be up-regulated on pulmonary neutrophils in patients with cystic fibrosis (Tirouvanziam et al., 2008), suggesting that, in some pathological conditions, DP2 is expressed on neutrophils as well. In rodents, however, DP2 is expressed on neutrophils under basal conditions and does not require activation of expression (Takeshita et al., 2004). Experiments in the Brown Norway rat reveal a role for DP2 in neutrophil migration in vivo. Injection of the selective DP2 agonist 13,14-dihydro-15-keto prostaglandin D₂ (DK-PGD₂) resulted in mobilization of leukocytes, including neutrophils, from the bone marrow into the circulating blood (Shichijo et al., 2003). Furthermore, in a murine model of contact hypersensitivity, a selective antagonist of DP2 inhibited recruitment of neutrophils to the skin (Boehme, 2009). Thus, rodent models of neutrophil trafficking may be useful in examining the PGD₂-DP2 axis.

Exposure of animals to cigarette smoke induces pulmonary inflammation that is useful for the pharmacological evaluation of novel therapeutics for COPD. In acute models (<4 days) inflammation is predominantly neutrophilic (Leclerc et al., 2006; Morris et al., 2008; Doukas et al., 2009), whereas in subchronic exposure models (<30 days), the inflammatory phenotype is more complex and includes mucus hypersecretion and epithelial hyperplasia (Medicherla et al., 2008).

The goal of the studies described herein was to examine the efficacy of AM156 and AM206, novel DP2 receptor antagonists, in murine models of cigarette smoke exposure. First, we profiled our antagonists by use of in vitro radioligand membrane binding and whole-blood eosinophil shape-change assays. Next, we used a guinea pig pharmacodynamic assay to establish that these molecules could block in vivo effects of the selective DP2 agonist DK-PGD₂. Last, we profiled AM156 and AM206 in acute and subchronic models of cigarette smoke exposure to determine the effect of DP2 antagonism on cellular influx, COPD-relevant inflammatory mediators, mucus hypersecretion, and airway remodeling.

### Materials and Methods

**Drugs**

AM156 (Fig. 1A) and AM206 (Fig. 1B) are novel DP2 receptor antagonists developed by Amira Pharmaceuticals. Representative examples of reference DP2 antagonists

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**Fig. 1.** Chemical structures of AM156 (A), AM206 (B), and representative reference DP2 antagonists from Actimis (Ly et al., 2007) (C) and Oxagen (Lovell, 2007) (D).
[4-chloro-6-dimethylamino-2-[4-(4-trifluoromethyl-benzoyl-amino)-benzyl]-pyrimidin-5-yl]-acetic acid, mono trifluoracetate salt (Fig. 1C) and (5-fluoro-2-methyl-3-quino lin-2-ylmethyl-indol-1-yl)-acetic acid, sodium salt (Fig. 1D) outlined in patents from Actimis Pharmaceuticals, Inc. (San Diego, CA) (Ly et al., 2007) and Oxagen (Abingdon, Oxfordshire, UK) (Lovell, 2007), respectively, were synthesized in-house. DK-PGD₂ was purchased from Cayman Chemical (Ann Arbor, MI). Rolipram ([4S-(cyclopentolxy)-4-methoxyphenyl]-2-pyrrolidione) and dexamethasone ((11β,16α)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione) were purchased from Sigma-Aldrich (St. Louis, MO).

In Vitro Potency Screening and Counterscreening

Membrane-Binding Assays for DP2, DP1, IP, and TP. Membranes were isolated from human embryo kidney-293 cells stably transfected with human, mouse, or guinea pig DP2 receptor or human IP receptor. For the TP receptor and DP1 receptor counterscreens, membranes were prepared from enriched human platelets (Biological Specialty Corporation, Colmar, PA). For all experiments, DMSO or DP2 antagonists (diluted in DMSO) were added to membranes (4–17.5 µg of DP2/293, 10 µg of IP/293, 20 µg of human platelets), and titrated ligand was added (1 nM [3H]PGD₂ (PerkinElmer Life and Analytical Sciences, Waltham, MA); 5 nM [3H]Iloprost (GE Healthcare, Little Chalfont, Buckinghamshire, UK); 10 nM [3H]SQ 29,548 (PerkinElmer Life and Analytical Sciences); 2 nM [3H]BW A868C (American Radiolabeled Chemicals, Inc., St. Louis, MO)). DP2 binding experiments were performed in the presence or absence of 0.2% human, mouse, or guinea pig serum albumin (Sigma-Aldrich). Reactions were incubated in 96-well deep-well plates for 60 min at room temperature. Membranes were harvested onto glass filter binding plates (UniFilter GPC; PerkinElmer Life and Analytical Sciences) and washed three times with cold buffer containing 50 mM HEPES, pH 7.4, 0.5 M NaCl with use of a 96-tip cell harvester (Brandel, Gaithersburg, MD). Plates were dried for 60 min, and 50 µl of Microscint 20 (PerkinElmer Life and Analytical Sciences) was added to each well. Plates were sealed with TopSeal (PerkinElmer Life and Analytical Sciences), and the counts per minute were evaluated by use of a Packard TopCount NXT microplate scintillation counter (PerkinElmer Life and Analytical Sciences). The percentage inhibition of [3H]ligand binding relative to the vehicle control was graphed and the inhibitory concentration for 50% inhibition (IC₅₀) was generated by nonlinear regression analysis of the data by use of MDL Assay Explorer (Elsevier MDL).

In Vivo Animal Experiments

Animals. Female BALB/c mice (Harlan, 8–10 weeks at study initiation) were used for pharmacokinetic and smoke studies. Male Hartley guinea pigs (200–300 g at study initiation; Charles River Laboratories, Inc., Wilmington, MA) were used for pharmacodynamic studies. Animals were housed in accordance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals. Animal protocols were approved by the Amira Pharmaceuticals Institutional Animal Care and Use Committee.

Pharmacokinetics. The oral exposure of AM156 and AM206 was determined in fasted mice. Animals received an oral dose of AM156 or AM206 (10 mg/kg) in 0.5% methylcellulose (MC) vehicle and were then euthanized by CO₂ inhalation at 1, 2, 4, 6, or 24 h after dose for AM156 and 1, 2, 4, 8, and 24 h after dose for AM206 (n = 2 animals per time point for each test compound). Blood (approximately 300 µl) was collected via cardiac puncture into EDTA-containing tubes and centrifuged at 1450g for 10 min. The plasma was removed and analyzed for AM156 or AM206 content by liquid chromatography-mass spectrometry.

Cigarette Smoke Exposure

General Exposure Procedures. Mice were placed into a Plexiglas chamber (12 mice maximum per exposure group) and exposed to the smoke of seven cigarettes per day (3R4F filters removed, University of Kentucky, Lexington, KY). Smoke was generated by use of a peristaltic pump (Cede Parmer Masterflex L/S, Vernon Hills, IL) set to a delivery rate of 45 ml/min. Before entrance into the exposure chamber, smoke was diluted with air from an in-house air source. Mice were exposed to one cigarette every 15 min, for a total exposure time of 1.75 h. Ideally, air controls should be sham-treated by use of an identical procedure as used for the smoke-exposed mice minus the attachment of a cigarette to the apparatus. In our laboratory, however, we modified this procedure to accommodate space and equipment constraints. In this modified procedure, the filter tops were removed from the cages of air control groups and mice were exposed to room air for 1.75 h. In our experience, this modified procedure does not result in baseline values that differ from sham-exposed mice.

Acute Model. Mice were exposed to smoke on days 0 and 1 and bronchoalveolar lavage (BAL) was performed on day 3, approximately 46 h after the final smoke exposure (Fig. 2A). AM156 (1 and 10 mg/kg) was prepared in 0.5% MC and dosed once daily. Rolipram (3 mg/kg q.d.) served as a positive control, whereas dexamethasone (0.3 mg/kg b.i.d.) served as a negative control. This dose of dexamethasone has been shown to inhibit lipopolysaccharide-induced pulmonary neutrophilia (Medicherla et al., 2008; Doukas et al., 2009). The vehicle for rolipram was 0.5% MC, whereas that for dexamethasone was 25% 2-hydroxypropyl-β-cyclodextrin (Sigma-Aldrich). AM156...
A

**Acute Smoke Exposure Model Study Design**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vehicle (PO, q.d.)</th>
<th>AM156 (1 or 10 mg/kg PO, q.d.)</th>
<th>Rolipram (3 mg/kg PO, q.d.)</th>
<th>Dexamethasone (0.3 mg/kg PO, b.i.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAL</td>
<td>d0</td>
<td>d1</td>
<td>d2</td>
<td>d3</td>
</tr>
</tbody>
</table>

7 cigarettes/day or air (1.75 h/day)

**Subchronic Model.** Mice were exposed to cigarettes on days 0 to 2, beginning at time 0 h. On these days, vehicle, AM156, and rolipram were dosed at −1 h. On day 2, vehicle and drug were dosed in the morning. Rolipram was dosed once daily. Dexamethasone was dosed twice a day. Bronchoalveolar lavage was performed on day 3. Subchronic model was performed on day 14.

B

**Acute Smoke Exposure Model Variation**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vehicle (PO, q.d.)</th>
<th>AM206 (1 or 10 mg/kg PO, q.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAL</td>
<td>d0</td>
<td>d1</td>
</tr>
</tbody>
</table>

7 cigarettes/day or air (1.75 h/day)

**DP2 Antagonists in Murine Cigarette Smoke Model of COPD**

**Efficacy of AM206 was explored in a variation of the acute smoke exposure model. BALB/c females were exposed to the smoke of seven cigarettes on days 0 and 1, beginning at time 0 h. On these days, vehicle, AM156, and rolipram were dosed at −1 h. On day 2, vehicle and drug were dosed in the morning. Rolipram was dosed once daily. Dexamethasone was dosed twice a day. Bronchoalveolar lavage was performed on day 3.**

**Subchronic Model.** Mice were exposed to cigarettes on days 0 to 2, beginning at time 0 h. On these days, vehicle, AM156, and rolipram were dosed at −1 h. On day 2, vehicle and drug were dosed in the morning. Rolipram was dosed once daily. Dexamethasone was dosed twice a day. Bronchoalveolar lavage was performed on day 3.

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TABLE 1
Species-specific DP2 binding and human whole blood eosinophil shape change potencies of AM156, AM206, and reference DP2 antagonists

<table>
<thead>
<tr>
<th>Compound</th>
<th>hDP2 Binding IC₅₀</th>
<th>hDP2 Binding + 0.2% HSA IC₅₀</th>
<th>mDP2 Binding + 0.2% MSA IC₅₀</th>
<th>gpDP2 Binding + 0.2% gpSA IC₅₀</th>
<th>hESC IC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM156</td>
<td>13.5</td>
<td>24.2</td>
<td>61</td>
<td>203</td>
<td>6.8</td>
</tr>
<tr>
<td>AM206</td>
<td>2.9</td>
<td>9.8</td>
<td>296</td>
<td>625</td>
<td>15.5</td>
</tr>
<tr>
<td>Oxagen a</td>
<td>6.2</td>
<td>50.8</td>
<td>761</td>
<td>712</td>
<td>359</td>
</tr>
<tr>
<td>Actimis b</td>
<td>0.7</td>
<td>8.4</td>
<td>N.D.</td>
<td>2.5</td>
<td>11.6</td>
</tr>
</tbody>
</table>

h, human; m, mouse; gp, guinea pig; HSA, human serum albumin; MSA, mouse serum albumin; gpSA, guinea pig serum albumin; hESC, human eosinophil shape change; N.D., not determined.

In Vivo
Pharmacokinetics. After oral administration of 10 mg/kg AM156, the peak plasma concentration of AM156 was 7.3 μM (1-h time point). AM156 concentrations then decreased to a trough value of 160 nM at 24 h (Fig. 4A). Oral administration of AM206 resulted in peak plasma concentrations of 2.9 μM (1-h time point) (Fig. 4B). At 8 h, AM206 concentrations were 70 nM. By 24 h, AM206 concentrations were below the limit of detection of the instrument (<10 nM).

Pharmacodynamics. Intravenous DK-PGD2 resulted in an increase in total leukocytes (Fig. 5). This increase was dose-dependently inhibited by AM156 and AM206 and by the reference DP2 antagonists from the patent literature (Lovell, 2007; Ly et al., 2007).

Acute Cigarette Smoke Exposure Model. In the AM156 experiment, acute cigarette smoke exposure caused an increase in total BALF leukocytes, which comprised macrophages, neutrophils, and lymphocytes (Fig. 6, A–D). Both total cells and macrophages were similarly affected by all treatment groups; dexamethasone produced a significant effect on macrophage influx (Fig. 6D). AM156 dose-dependently inhibited pulmonary neutrophilia (Fig. 6B). BALF neutrophils were also inhibited by rolipram but not by dexamethasone. There was a small but statistically significant increase in the number of lymphocytes recovered in the BALF (Fig. 6C), which was inhibited dose-dependently by AM156. Rolipram and dexamethasone were both effective against the lymphocyte influx. Based on these results, 10 mg/kg was selected for profiling in the subchronic smoke model.

In the variation of the acute smoke model, AM206 dose-dependently reduced the smoke-induced increase in BALF neutrophils (Fig. 6F). Lymphocytes were modestly increased by smoke and dose-dependently reduced by AM206 (Fig. 6G). Total BALF cells and macrophages were not affected by smoke in this model variation (Fig. 6, A and H).

Subchronic Cigarette Smoke Exposure Model. Analysis of lung tissue homogenates revealed that lung PGD2 concentrations increased from 11.2 ± 0.7 ng/mg protein in air-exposed lungs to 34.2 ± 4.3 ng/mg protein in smoke-
exposed lungs. Subchronic smoke exposure increased total BALF leukocytes (Fig. 7, A and E). Prophylactic treatment with AM156 significantly inhibited this increase in total cells. Therapeutic treatment with AM156 also reduced total cell numbers, but this did not reach statistical significance. AM206 caused a trend toward a reduction in total cells (Fig. 7E); however, this was not significant. Differential analysis of subtypes revealed that subchronic smoke exposure in-
increased the numbers of neutrophils and lymphocytes (Fig. 7, B, C, F, and G). Only a modest increase in macrophages was observed (Fig. 7, D and H). AM156 reversed the smoke-induced effects on both neutrophils and lymphocytes after either prophylactic or therapeutic dosing regimens. No effect was seen on BALF macrophages. Likewise, AM206 reduced neutrophils and lymphocytes (Fig. 7, F and G). Trough plasma concentrations of AM156 were 114 and 169 nM, respectively, for prophylactic and therapeutic groups. Trough plasma concentrations of AM206 were below detection limits (≤8 nM). These concentrations are consistent with those seen in the separate pharmacokinetic studies described above. These data suggest that it is not necessary to maintain plasma concentrations above the IC_{50} at trough.

BALF mucin was elevated after 10 days of smoke exposure (Fig. 8). Although not statistically significant, there was a trend toward reduction after treatment with AM156 and AM206. In the case of AM156, therapeutic dosing was as effective as prophylactic dosing. Histological analysis revealed PAS-positive cells in the airway epithelium, predominantly in the large airways, of smoke-exposed mice (Fig. 9, A and C). A reduction in PAS staining occurred after treatment with either AM156 (Fig. 9, A and C) or AM206 (Fig. 10A). Furthermore, epithelial thickening occurred in response to cigarette smoke exposure (Fig. 9, B and D). This was attenuated by both treatment with AM156 (Fig. 9, B and D) or AM206 (Fig. 10B). Moreover, therapeutic treatment with AM156 was nearly as effective as prophylactic treatment.

The AM156 experiment was further profiled via analysis of both BALF and lung homogenates. Cigarette smoke elevated KC, MIP-2, and MCP-1 in the BALF (Table 3). Neither KC nor MCP-1 was reduced by AM156. MIP-2 was modestly reduced with prophylactic treatment and unaltered by therapeutic with AM156. IL-1β and IL-17 were below the limits of quantitation for the assays (3 and 5 pg/ml, respectively).

Cigarette smoke exposure resulted in elevated KC, MIP-2, MCP-1, and IL-1β in lung homogenates (Table 3). These concentrations were not altered by AM156. The lung concentration of IL-17 was not altered by smoke exposure; however, the basal concentration of IL-17 was reduced by DP2 antagonism.

**Discussion**

The results presented here are the first to demonstrate an effect of antagonism of the DP2 receptor on pulmonary inflammation, mucus cell metaplasia, and airway epithelial hyperplasia in a mouse model of COPD. DP2 antagonism inhibited cigarette smoke-induced influx of neutrophils and lymphocytes into the lungs. In the acute model, the degree of inhibition achieved was comparable with that of the well characterized phosphodiesterase 4 inhibitor rolipram. Dexam
methasone failed to inhibit neutrophil influx, which is consistent with previous literature reports showing that rodent smoke models are steroid-resistant (Leclerc et al., 2006; Doukas et al., 2009).

The effects observed in the acute smoke model were further characterized in a subchronic smoke exposure model. In this setting, smoke exposure induced mucus cell metaplasia and airway epithelial thickening. The mucus hypersecretion, seen primarily in the large airways, could be detected both as released mucin in the BAL and histologically as a mucin-specific stain. Because lungs were lavaged before formalin fixation, histological analyses of small-airway inflammation, which would have been interesting, could not be performed accurately. Our results clearly demonstrate a beneficial effect of AM156 and AM206 in the subchronic cigarette smoke exposure model. As seen in the acute experimental model, the main anti-inflammatory effects were on inhibition of neutrophils and lymphocytes. In addition, there was a striking inhibition of both mucus cell metaplasia and airway epithelial hyperplasia. The longer duration of this model also allowed us to evaluate AM156 in both a prophylactic and therapeutic setting, the latter of which is more clinically relevant. Efficacy was achieved with both treatment paradigms. Because cigarette smoking is implicated in the etiology of COPD, DP2 antagonism may prove to be beneficial in the clinical setting, via prevention of airway inflammation, mucus hypersecretion, and airway remodeling.

The effects on neutrophil migration observed here are consistent with earlier reports in other murine models. In mice, DP2 is expressed on neutrophils, and intradermal injection of DK-PGD₂, a selective DP2 agonist, increased staining of Ly-6G-positive neutrophils in the skin, demonstrating that activation of the receptor can have a direct effect on neutrophil recruitment (Takeshita et al., 2004). Furthermore, this was inhibited by ramatroban, a dual DP2/TP antagonist, but not by ridogrel, a selective TP antagonist. In contrast, the same authors failed to demonstrate in vitro chemotaxis of murine neutrophils in response to DK-PGD₂, which perhaps suggests a role of the inflammatory milieu in the phenotypic response to DP2 agonists. In a murine model of contact hypersensitivity a selective antagonist of DP2 inhibited recruitment of neutrophils to the skin (Boehme et al., 2009). In vitro, cul-

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**Fig. 7.** Inhibition of BALF total cells (A, E), neutrophils (B, F), lymphocytes (C, G), and macrophages (D, H) by AM156 (A–D) and AM206 (E–H) in the subchronic smoke exposure model. Values represent means ± S.E.M. *, P < 0.05; **, P < 0.01 versus smoke by ANOVA. #, P < 0.05 versus air by Student’s t test; ##, P < 0.001 versus air by ANOVA.

**Fig. 8.** Inhibition of BALF mucin by AM156 (A) and AM206 (B). Values represent means ± S.E.M. ##, P < 0.05 versus air by ANOVA; ###, P < 0.01 versus air by ANOVA. Thera, therapeutic; Proph, prophylactic.
tured human neutrophils can up-regulate expression of DP2 (Carroll, 2006). Clinically, DP2 expression has been found to be up-regulated on sputum neutrophils in patients with cystic fibrosis (Tirouvanziam et al., 2008). These data suggest that in at least some pathological conditions, DP2 is expressed on human neutrophils. Therefore, it can be hypothesized that DP2 antagonism may provide a benefit in diseases such as COPD where neutrophils play a role in pathogenesis.

Chronic bronchitis is characterized by mucus hypersecretion.
smoke-exposed lungs may be via direct action on circulating lymphocytes, which DP2 antagonists prevent lymphocyte trafficking to the periphery (Shichijo et al., 2003). In vitro, Th2 lymphocytes chemotax in response to indomethacin, a DP2 agonist, and this was inhibited by an anti-DP2 antibody (Hirai et al., 2002). Thus, our results confirm that DP2 antagonism may be acting to down-regulate adhesion molecules on neutrophils, preventing adherence and subsequent chemotaxis of cells to the airways. Monneret et al. (2001) demonstrated that PGD2, probably acting via DP2, up-regulates expression of adhesion molecules on human eosinophils. Although this

Our results demonstrate that DP2 antagonism can inhibit the small, but significant, increase in BALF lymphocytes observed after cigarette smoke exposure. One mechanism by which DP2 antagonists prevent lymphocyte trafficking to the smoke-exposed lungs may be via direct action on circulating lymphocytes. In rats, the DP2 agonist DK-PGD2 stimulates the recruitment of lymphocytes from the bone marrow to the peripheral circulation, demonstrating a role for DP2 in lymphocyte trafficking (Shichijo et al., 2003). In vitro, Th2 lymphocytes chemotax in response to indomethacin, a DP2 agonist, and this was inhibited by an anti-DP2 antibody (Hirai et al., 2002). Thus, our results confirm that DP2 antagonism can inhibit lymphocyte trafficking in vivo.

Our data on mucus hypersecretion are consistent with previous findings with the dual DP2/TP antagonist ramatroban (Uller et al., 2007) and selective DP2 antagonists (Uller et al., 2007; Lukacs et al., 2008) in murine allergic settings. Although outside the scope of this manuscript, a possible mechanism by which DP2 antagonists affect mucus production may be via direct action on neutrophils to reduce elastase and other proteases.

The role of DP2 in epithelial cell proliferation and airway remodeling is an emerging concept indicated by this present report of DP2 antagonism inhibiting airway epithelial cell hyperplasia. While this manuscript was in preparation, similar results were reported with the selective DP2 antagonist AZ11805131 (Sargent et al., 2009). The epithelial cell acts as a first line of defense against harmful components in cigarette smoke, and DP2 is expressed on human bronchial epithelial cells (Chiba et al., 2006; Sargent et al., 2009). Intracellular expression of DP2 was demonstrated in NCI-H292 and normal human bronchial epithelial cells by both reverse transcription-polymerase chain reaction and flow cytometry (Chiba et al., 2007). The functional implications of DP2 expression on this cell type have yet to be fully elucidated; however, our data suggest that the efficacy of DP2 antagonists on cigarette smoke-induced pulmonary inflammation and mucus hypersecretion may be due to the direct effect of the DP2 antagonist on the epithelial cells, although, as stated above, a role for the DP2-neutrophil-epithelial cell axis cannot be ruled out.

We failed to demonstrate inhibition of KC, MIP-2, or IL-1β measured in either the BALF or lung homogenates, in agreement with those recently presented by Sargent et al. (2009). These results are in contrast to the inhibition of MIP-2, KC, and IL-1β levels in tissue homogenates by DP2 antagonism observed in an atopic dermatitis model (Boehme et al., 2009). We observed significant inhibition of neutrophil migration, even though the concentrations of the neutrophil chemoattractants KC and MIP-2 remained high in both BALF and lung tissue. This suggests that, in our model, the reduced number of neutrophils is not related to a DP2-dependent decrease in chemoattractants. Whereas the contrasting effects on cytokines and chemokines may simply reflect differences in the in vivo models used (smoke versus antigen or lung versus skin), we speculate that DP2 antagonists may be acting to down-regulate adhesion molecules on neutrophils, endothelial cells, or epithelial cells, thereby preventing adherence and subsequent chemotaxis of cells to the airways. Monneret et al. (2001) demonstrated that PGD2, probably acting via DP2, up-regulates expression of adhesion molecules on human eosinophils. Although this

<table>
<thead>
<tr>
<th>Cytokines and chemokines in the BALF and lung homogenates</th>
<th>Air</th>
<th>Smoke</th>
<th>AM156 Prophylactic</th>
<th>AM156 Therapeutic</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALF, pg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KC</td>
<td>54.9 ± 3.1</td>
<td>115.2 ± 6.0</td>
<td>120.9 ± 7.3</td>
<td>118.4 ± 5.8</td>
</tr>
<tr>
<td>MIP-2</td>
<td>2.2 ± 0.9</td>
<td>3.5 ± 0.6</td>
<td>2.1 ± 0.7</td>
<td>3.0 ± 1.3</td>
</tr>
<tr>
<td>MCP-1</td>
<td>&lt;2</td>
<td>19.0 ± 2.1</td>
<td>19.1 ± 3.3</td>
<td>18.1 ± 3.0</td>
</tr>
<tr>
<td>IL-8 KB</td>
<td>&lt;3</td>
<td>&lt;3</td>
<td>&lt;3</td>
<td>&lt;3</td>
</tr>
<tr>
<td>IL-17</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Lung, pg/mg protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCβ</td>
<td>5.0 ± 0.5</td>
<td>27.2 ± 3.2</td>
<td>29.5 ± 1.3</td>
<td>27.6 ± 2.8</td>
</tr>
<tr>
<td>MIP-2</td>
<td>0.6 ± 0.1</td>
<td>2.8 ± 0.5</td>
<td>2.8 ± 0.1</td>
<td>3.0 ± 0.4</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.9 ± 0.6</td>
<td>14.5 ± 0.5</td>
<td>11.9 ± 2.2</td>
<td>12.6 ± 2.0</td>
</tr>
<tr>
<td>IL-1</td>
<td>10.9 ± 0.4</td>
<td>21.1 ± 1.2</td>
<td>22.3 ± 2.5</td>
<td>20.8 ± 3.9</td>
</tr>
<tr>
<td>IL-17</td>
<td>0.80 ± 0.3</td>
<td>0.60 ± 0.1</td>
<td>0.32 ± 0.1*</td>
<td>0.19 ± 0.1*</td>
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</table>

*, P = 0.07; +, P = 0.05 vs. smoke, by Student’s t test.
phenomenon has not been examined in murine granulocytes, it is possible that in the inflammatory milieu of cigarette smoke, DP2-dependent up-regulation of adhesion molecules on neutrophils or epithelial cells may occur. Thus, one mechanism by which DP2 antagonism could be inhibiting cell migration to the murine airways may be the down-regulation of cell surface adhesion molecules. Further exploration is needed to confirm this hypothesis.

In the naive lung, the resident alveolar macrophages are the predominant cell type. Mouse peritoneal macrophages can migrate toward the DP2 agonist, DK-PGD2, (Tajima et al., 2008). Our results in the acute smoke model support a role for DP2 in macrophage influx. However, neither macrophage influx nor local concentrations of MCP-1 were affected by AM156 in the subchronic model. Migration of peritoneal macrophages toward MCP-1 was unaltered in DP2-deficient mice (Tajima et al., 2008). Thus, the inability to reduce BAL macrophages with DP2 antagonism may be due to the role that MCP-1 may play in the recruitment of, and subsequent differentiation of, monocytes to macrophages in this subchronic smoke model.

It is noteworthy that we observed a reduction in basal lung IL-17 concentrations after treatment with a DP2 antagonist. In mice, Th17 cells are differentiated from Th0 cells in response to the production of IL-6 and transforming growth factor-β by dendritic cells (Romagnani, 2008). Elevations of IL-6 and transforming growth factor-β have been demonstrated in murine smoke models of COPD (Obot et al., 2004; Churg et al., 2006). The effect of DP2 antagonism on IL-17 confirms the modest reduction in IL-17 gene expression that Lukacs et al. (2008) observed in lungs from DP2 antagonist-treated mice relative to those from antigen-treated mice. IL-17 is implicated in promoting and sustaining neutrophilic inflammation; thus, the inhibitory effects of DP2 antagonism on pulmonary neutrophilia may, in part, be due to the reduction in basal lung IL-17 concentrations.

In summary, we report that DP2 antagonism with AM156 and AM206, potent and selective DP2 antagonists with favorable pharmacokinetic properties, inhibit cigarette smoke-induced pulmonary inflammation, mucus cell metaplasia, and airway epithelial hyperplasia. Furthermore, this is the first report that demonstrates the effectiveness of DP2 antagonism in the chronic smoke model. Migration of peritoneal neutrophilia may, in part, be due to the role for DP2 in macrophage influx. However, neither macrophage influx nor local concentrations of MCP-1 were affected by AM156 in the subchronic model. Migration of peritoneal macrophages toward MCP-1 was unaltered in DP2-deficient mice (Tajima et al., 2008). Thus, the inability to reduce BAL macrophages with DP2 antagonism may be due to the role that MCP-1 may play in the recruitment of, and subsequent differentiation of, monocytes to macrophages in this subchronic smoke model.

It is noteworthy that we observed a reduction in basal lung IL-17 concentrations after treatment with a DP2 antagonist. In mice, Th17 cells are differentiated from Th0 cells in response to the production of IL-6 and transforming growth factor-β by dendritic cells (Romagnani, 2008). Elevations of IL-6 and transforming growth factor-β have been demonstrated in murine smoke models of COPD (Obot et al., 2004; Churg et al., 2006). The effect of DP2 antagonism on IL-17 confirms the modest reduction in IL-17 gene expression that Lukacs et al. (2008) observed in lungs from DP2 antagonist-treated mice relative to those from antigen-treated mice. IL-17 is implicated in promoting and sustaining neutrophilic inflammation; thus, the inhibitory effects of DP2 antagonism on pulmonary neutrophilia may, in part, be due to the reduction in basal lung IL-17 concentrations.

In summary, we report that DP2 antagonism with AM156 and AM206, potent and selective DP2 antagonists with favorable pharmacokinetic properties, inhibit cigarette smoke-induced pulmonary inflammation, mucus cell metaplasia, and airway epithelial hyperplasia. Furthermore, this is the first report that demonstrates the effectiveness of DP2 antagonism in this setting. Efficacy after therapeutic dosing of AM156 suggests that DP2 antagonists, such as AM156 or AM206, may provide novel and beneficial therapy for diseases such as COPD in which neutrophils and epithelial cells mediate disease progression.

References


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