Pharmacologic Profile of OC000459, a Potent, Selective, and Orally Active D Prostanoid Receptor 2 Antagonist That Inhibits Mast Cell-Dependent Activation of T Helper 2 Lymphocytes and Eosinophils


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ABSTRACT

D prostanoid receptor 2 (DP2) [also known as chemoattractant receptor-homologous molecule expressed on T helper 2 (Th2) cells (CRTH2)] is selectively expressed by Th2 lymphocytes, eosinophils, and basophils and mediates recruitment and activation of these cell types in response to prostaglandin D2 (PGD2). (5-Fluoro-2-methyl-3-quinolin-2-ylmethyl-1-yl)-acetic acid (OC000459) is an indole-acetic acid derivative that potently displaces [3H]PGD2, from human recombinant DP2 (Kᵢ = 0.013 μM), rat recombinant DP2 (Kᵢ = 0.003 μM), and human native DP2 (Th2 cell membranes; Kᵢ = 0.004 μM) but does not interfere with the ligand binding properties or functional activities of other prostanoid receptors (prostaglandin E₁–4 receptors, D prostanoid receptor 1, thromboxane receptor, prostacyclin receptor, and prostaglandin F receptor). OC000459 inhibited chemotaxis (IC₅₀ = 0.028 μM) of human Th2 lymphocytes and cytokine production (IC₅₀ = 0.019 μM) by human Th2 lymphocytes. OC000459 competitively antagonized eosinophil shape change responses induced by PGD₂ in both isolated human leukocytes (pKᵢ = 7.9) and human whole blood (pKᵢ = 7.5) but did not inhibit responses to eotaxin, 5-oxo-eicosatetraenoic acid, or complement component C5a. OC000459 also inhibited the activation of Th2 cells and eosinophils in response to supernatants from IgE/anti-IgE-activated human mast cells. OC000459 had no significant inhibitory activity on a battery of 69 receptors and 19 enzymes including cyclooxygenase 1 (COX1) and COX2. OC000459 was found to be orally bioavailable in rats and effective in inhibiting blood eosinophilia induced by 13,14-dihydro-15-keto-PGD₂ (DK-PGD₂) in this species (ED₅₀= 0.04 mg/kg p.o.) and airway eosinophilia in response to an aerosol of DK-PGD₂ in guinea pigs (ED₅₀ = 0.01 mg/kg p.o.). These data indicate that OC000459 is a potent, selective, and orally active DP₂ antagonist that retains activity in human whole blood and inhibits mast cell-dependent activation of both human Th2 lymphocytes and eosinophils.

Introduction

Mast cells are believed to play a central role in the pathophysiology of asthma and other allergic diseases. These cells are a rich source of mediators that may contribute to early-phase allergic responses such as bronchoconstriction and the characteristic pattern of Th2 lymphocyte and eosinophil infiltration seen during the late-phase airway response to allergen. Immunological, IgE-dependent activation of mast cells can promote the accumulation of CD4⁺ T cells and enhance Th2-mediated eosinophilic airway inflammation in experimental animals (Williams and Galli, 2000; Maezawa et al., 2004), whereas treatment with the anti-IgE antibody omalizumab reduces eosinophil and lymphocyte numbers in the airway mucosa of patients with asthma (Djukanović et al., 2004).

ABBREVIATIONS: Th, T helper; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; DP₂, D prostanoid receptor 2; PGD₂, prostaglandin D₂; DK-PGD₂, 13,14-dihydro-15-keto-PGD₂; FCS, fetal calf serum; COX, cyclooxygenase; CHO, Chinese hamster ovary; 5-oxo-ETE, 5-oxo-eicosatetraenoic acid; PI, propidium iodide; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; EIA, enzyme immunoassay; IL, interleukin; OC000459, (5-fluoro-2-methyl-3-quinolin-2-ylmethyl-1-yl)-acetic acid; C5a, complement component 5a; TP, thromboxane receptor; EP, prostaglandin E; ANOVA, analysis of variance; SC29548, [1S-[1α,2α(2S,3α,4α)-7-[3-[(2-[([phenylamino]carbonyl)hydrazino)methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; U44069, 9,11-dideoxy-9α,11α-epoxy-methano-prosta-5Z,13E-dien-1-ol acid; BW245C, (4S)-3-[3(3R,5S)-3-cyclohexyl-3-hydroxy-propyl]-2,5-dioxo)-4-imidazolidinedione-hexanoic acid; NS398, N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide.
Prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) is produced in abundant quantities by mast cells in response to IgE-dependent activation (Lewis et al., 1982) and has been detected in the airways of allergic asthmatics challenged with antigen (Murray et al., 1989). PGD<sub>2</sub> can exert a number of biological effects relevant to the pathogenesis of allergic disease including the ability to promote eosinophil accumulation (Emery et al., 1989) and enhance Th2 cytokine production in the allergic airways of experimental animals (Fujitani et al., 2002). Chemotaxis of Th2 lymphocytes, eosinophils, and basophils in response to PGD<sub>2</sub> is mediated by the G protein-coupled receptor DP<sub>2</sub>, which is also known as chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) (Hirai et al., 2001; Monneret et al., 2001). Furthermore, PGD<sub>2</sub> can stimulate the production of interleukins 4, 5, and 13 by Th2 cells in the absence of costimulation through a DP<sub>2</sub>-dependent mechanism (Xue et al., 2005). These in vitro data are supported by findings that the effect of PGD<sub>2</sub> in exacerbating allergic inflammation in the lungs and skin of mice can be mimicked by the selective CRTH2 agonist DK-PGD<sub>2</sub> (Spik et al., 2005). Studies in mice genetically deficient in DP<sub>2</sub> have implicated this receptor in the development of allergic responses. In a mouse model of skin inflammation, genetic ablation of DP<sub>2</sub> was associated with diminished dermal infiltration of various leukocyte populations including lymphocytes and eosinophils, reduced tissue swelling, and a reduction in the levels of serum IgE (Satoh et al., 2006). Production of IgE was also reduced in DP<sub>2</sub>-deficient mice exposed to Japanese intranasal cedar pollen, an effect associated with reduced inflammation of the nasal mucosa and signs of rhinitis (Nomiya et al., 2008). In Japanese cedar pollen-induced dermatitis, skin inflammation was shown to depend on both mast cell activation and the presence of DP<sub>2</sub> (Oiwa et al., 2008). The effects of genetic ablation of DP<sub>2</sub> in mice are mimicked by selective small-molecule DP<sub>2</sub> antagonists that have been shown to be effective in reducing allergen-induced airway inflammation (Uluer et al., 2007; Lukacs et al., 2008; Stebbins et al., 2010; Bain et al., 2011) and allergic skin inflammation (Boehme et al., 2009b) in murine models.

The observations linking DP<sub>2</sub> to the development of allergic inflammation has spurred interest in identifying more potent, selective, and orally bioavailable antagonists of this receptor for treating asthma and related disorders. A number of chemical series have been described that antagonize DP<sub>2</sub> including tetrahydroquinolone derivatives, carbazole derivatives, indole acetic acids, azaindole-3-acetic acids, phenoxacycetic acids, phenylacycetic acids, thiazoleacetic acids, 3-indoyl sulfamates, and other series as reviewed in detail by Ulven and Kostenis (2010). Here, we describe the pharmacological profile of (5-fluoro-2-methyl-3-quinolin-2-ylmethylindolo-1-yl)-acetic acid (OC000459), an indole-1-acetic acid derivative that is a potent and selective DP<sub>2</sub> antagonist, which, in proof-of-concept phase IIa clinical trials, has been shown to reduce airway inflammation and improve symptoms and quality of life in subjects with moderate persistent asthma (Barnes et al., 2011). It is currently being evaluated in longer-term phase IIb trials.

Materials and Methods

Materials. [3H]PGD<sub>2</sub>, [3H][1S-](1a,2α[Z],3α,4α)-7-[3-[2-[[phenylamino]carbonyl]hydrazinomethyl]-7-oxa-bicycle[2.2.1]hept-2-yl]-5-heptenoic acid (SQ298548), and [3H]PGF<sub>2α</sub> were purchased from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). PGD<sub>2</sub>, PGE<sub>2</sub>, iloprost, 5-oxo-eicosatetraenoic acid, and PGD<sub>2</sub>-methoxime enzyme immunoassay were purchased from Cayman Chemical (Ann Arbor, MI). Eotaxin (CCL11), complement component C5α, and IL-13 immunoassay kits were purchased from R&D Systems Europe Ltd (Abingdon, Oxfordshire, UK). HitHunter cAMP II was purchased from DiscoveRx (Fremont, CA). Calcium-3-dye was purchased from Molecular Devices (Wokingham, Berkshire, UK). Mono poly resolving medium was purchased from Dainippon Pharmaceuticals (Osaka, Japan). Magnetic cell sorting CD<sub>4</sub> isolation, anti-DP<sub>2</sub> microbead, and T cell expansion kits were purchased from Miltenyi Biotec (Bisley, Surrey, UK). Iscove’s modified Dulbecco’s media and X-VIVO 15 medium were purchased from Cambrex BioScience (Wokingham, Berkshire, UK). Ficoll Hypaque was purchased from GE Healthcare, and 96-well Chemotx plates were purchased from Neuroprobe (Gaithersburg, MD). Cytok buffer was purchased from BD Biosciences (Oxford, Oxon, UK). Phycoerythrin-annexin V, propidium iodide (PI), and annexin-binding buffer were obtained from Invitrogen (Carlsbad, CA). Pentobarbital sodium (Euthetal) was purchased from Merial Ltd (Harlow Essex, UK). OC000459 was synthesized by Evotec OAI (Abingdon, Oxfordshire, UK) as described in patent WO-2005044260 (Middlemiss et al., 2005). The structure of OC000459 is shown in Fig. 1.

[3H]PGD<sub>2</sub>, Ligand Binding Assays. Membranes from CHO cells expressing human recombinant DP<sub>2</sub> or DP<sub>2</sub> were prepared as described previously (Gazi et al., 2005), and membranes from CHO cells expressing rat recombinant DP<sub>2</sub> were prepared in a similar manner. Cell membranes (15 μg) were preincubated at room temperature with various concentrations of competing ligand in 80 μl of Hanks’ balanced salt solution supplemented with 10 mM HEPES, pH 7.3. Twenty microliters of [3H]PGD<sub>2</sub> (160 Ci/mmol) was then added to a final concentration of 5 nM and incubated for an additional 60 min at room temperature. Reactions were terminated by the addition of 100 μl of ice-cold assay buffer to each well, followed by rapid filtration through Whatman GF/B glass fiber filters using a Unifilter Cell Harvester (PerkinElmer Life and Analytical Sciences, Waltham, MA). The filters were washed six times with 300 μl of ice-cold buffer, and the plates were dried at room temperature for at least 60 min. The level of radioactivity retained on the filters was measured using a Beta Trilux counter (PerkinElmer Life and Analytical Sciences) after the addition of scintillant. Nonspecific binding was defined in the presence of 10 μM unlabeled PGD<sub>2</sub>. Assays were performed in duplicate.

Calcium Mobilization in CHO Cells Expressing Human Recombinant CRTH2. Culture of CHO cells expressing CRTH2 and measurement of calcium mobilization in response to PGD<sub>2</sub> or ATP were conducted as described previously (Gazi et al., 2005).

Prostanoid Receptor and COX Selectivity Assays. Selectivity of OC000459 against the other prostanoid receptors was assessed by using the following methods: TP binding activity was assessed by measuring displacement of [3H]SQ29548 from human platelet membranes. In addition, a further assessment of effects on [3H]SQ29548
binding is included in Supplemental Table 1, using 9,11-dideoxy-9α,11α-epoxymethano-prosta-5,13E-dien-1-oxic acid (U44069) as a reference compound. Prostacyclin receptor antagonist activity was assessed by measuring inhibition of iloprost-induced cAMP production in intact human platelets and by [3H]iloprost binding using unlabeled iloprost as a reference compound (Supplemental Table 1). Activity on EP2/EP4 was assessed by measuring displacement of [3H]PGE2 from CHO cells expressing human recombinant EP receptors. Prostaglandin F antagonist activity was assessed by measuring the inhibition of PGE2-induced calcium mobilization in mouse 3T3 fibroblasts.

Effects of OC000459 on COX activity (Supplemental Table 1) were determined as follows: human recombinant COX1 expressed in S9 cells was incubated with arachidonic acid (4 μM) for 10 min at 25°C, and PGE2 was measured by EIA with diclofenac included as a reference standard. COX1 activity was also measured in human platelets incubated with arachidonic acid (0.3 μM) for 15 min at 37°C, and PGE2 was measured by EIA with diclofenac included as a reference standard. Human recombinant COX2 expressed in S9 cells was incubated with arachidonic acid (2 μM) for 10 min at 25°C, and PGE2 was measured by EIA with diclofenac included as a reference standard. Human recombinant COX2 expressed in S9 cells was incubated with arachidonic acid (4 μM) for 10 min at 37°C.

Chemotaxis Assays. For measurement of chemotaxis of Th2 cells, cells were resuspended in X-VIVO media at 2 × 10^6 cells/ml; 25 μl of cell suspension and test samples (29 μl) prepared in X-VIVO media were applied to the upper and lower chambers of a 5-m孔 filter. Effects of OC000459 and PGE2 on COX activity (Supplemental Table 1) were determined as follows: human recombinant COX1 expressed in S9 cells was incubated with arachidonic acid (4 μM) for 10 min at 25°C, and PGE2 was measured by EIA with diclofenac included as a reference standard. COX1 activity was also measured in human platelets incubated with arachidonic acid (0.3 μM) for 15 min at 37°C, and PGE2 was measured by EIA with diclofenac included as a reference standard. Human recombinant COX2 expressed in S9 cells was incubated with arachidonic acid (2 μM) for 10 min at 25°C, and PGE2 was measured by EIA with diclofenac included as a reference standard. Human recombinant COX2 expressed in S9 cells was incubated with arachidonic acid (4 μM) for 10 min at 37°C. The supernatant (containing lysed red cells) was removed, and leukocytes were resuspended in 50 μl of PBS/2 mM EDTA. Cells were washed twice by centrifugation at 300g for 5 min. Leukocytes were resuspended in RPMI/10% FCS. Fifty microliters of cells were added to a 96-well microtiter plate containing 50 μl of buffer containing test compounds. The plate was then incubated for 1 h at 37°C with 5% CO2. After that incubation period, the plate was transferred on ice, and the cell shape was fixed by the addition of 150 μl of cytofix buffer. Cell morphology was analyzed by using a FACSCalibur (BD Biosciences). Eosinophils were gated based on their autofluorescence, and 2000 events were counted per sample. Assays were performed in duplicate and repeated at least three times.

For the measurement of shape change responses in whole blood, OC000459 (1 μl, 200× final concentration) was added directly to 200 μl of heparinized whole blood, mixed well, and incubated for 15 min at 37°C in a 5% CO2 incubator. Chemotactrant agonists (1 μl, 200× final concentration) were added to each sample and incubated for another 1 h at 37°C. At this time, cell shape was fixed by the addition of 300 μl of cytofix buffer (BD Biosciences) and incubated for 15 min on ice. Ten milliliters of red blood cell lysis buffer were added to the fixed cells, incubated for 5 min at room temperature, and centrifuged at 300g for 5 min. Supernatant (containing lysed red blood cells) was removed, and the lysis step was repeated. Leukocytes were resuspended in 250 μl of RPMI/10% FCS, and shape change was analyzed by fluorescence-activated cell sorting. Eosinophils were gated based on their autofluorescence, and 2000 eosinophil events were counted per sample. Data were analyzed in triplicate.

Cytokine Release Assays. Th2 cells were treated with X-VIVO 15 culture medium in the presence of absence of PGD2 or other compounds as indicated under Results at 37°C and 5% CO2 for 5 h. Supernatants were collected and assessed for IL-13 content by immunoassay according to the manufacturer’s (R&D Systems) instructions. The results were measured in a Victor2 V-1420 multilabel HTS Counter (PerkinElmer Life and Analytical Sciences).

Measurement of Apoptosis in Th2 Cells. Apoptosis of Th2 cells was measured after the withdrawal of IL-2 treatment as described previously (Xue et al., 2009). The cells were treated with PGD2 (100 nM) in the presence and absence of various concentrations of OC000459 (1–1000 nM) for 16 h. Cells were then harvested and transferred to annexin-binding buffer, followed by incubation with phycoerythrin-annexin V/PI at room temperature for 15 min according to the manufacturer’s (Invitrogen) instructions. The stained cells were analyzed by using a FACSArray flow cytometer (BD Biosciences). The cells with annexin V-positive and PI-negative staining were counted as apoptotic cells.

Plasma Protein Binding. Plasma protein binding in human and rat plasma was measured by ultrafiltration. OC000459 was added to plasma samples to final concentrations of 25, 100, and 500 ng/ml and loaded onto duplicate ultra centrifugation devices (Centrifree YM-30; Millipore Corporation, Billerica, MA) and subjected to centrifugation (1500g for 1 h). The concentration of OC000459 in the plasma and
ultrafiltrate was determined by liquid chromatography/tandem mass spectrometry as described below.

**Animals.** Sprague-Dawley rats were supplied by Charles River (Margate, Kent, UK). All animals were housed and all experiments were conducted under a project license granted under the Animals (Scientific Procedures) Act of 1986.

**Measurement of Pharmacokinetic Profile in Sprague-Dawley Rats.** Sprague-Dawley rats (n = 3 rats per group) were administered a single oral dose of 2 or 10 mg/kg OC000459 by gavage in 10% DMSO/saline solution or a single intravenous dose of 2 mg/kg OC000459 in 10% DMSO/saline solution. Heparinized blood was collected at various times after dosing and plasma was extracted with methyl tertiary butyl ether after the addition of ammonium formate buffer (100 mM, pH 4.4) and the internal standard ([4,4-Chloro-OC000459]. Liquid chromatography was carried out with a Prodigy C8, 5 μm, 30 × 4.6 mm Phenomenex analytical column and a Phenomenex C18 4 × 2 guard column (Phenomenex, Torrance, CA). The mobile phase was a 0.1% formic acid in methanol gradient (80:20 > 10:90 > 80:20 over a 5.5-min run time). The injection volume was 10 μl, and flow rate was 1.0 ml/min.

Samples were analyzed by liquid chromatography/tandem mass spectrometry (MDSCIEX API365; Applied Biosystems, Warrington, UK) with TurboLonspray in the positive ion mode.

**Measurement of DK-PGD2-Induced Blood Eosinophilia in Rats.** Blood eosinophilia in response to systemic treatment with DK-PGD2 was determined by a method similar to that described by Shichijo et al. (2003). OC000459 was dissolved in DMSO and diluted with water to give a final dosing volume of 2 ml/kg. Thirty minutes after oral administration of OC000459 (0.01, 0.1, and 1.0 mg/kg) or vehicle, animals were anesthetized with isoflurane and received an intracardiac injection of 10 micrograms of DK-PGD2 in 0.3 ml of heparinized (10U/ml) saline. Control animals received an injection of 0.3 ml of heparinized saline. Sixty minutes after the intracardiac injection, animals were injected with an overdose of pentobarbitone sodium, and a blood sample was taken (into heparin) by cardiac puncture while the rat was anesthetized, but not yet dead.

An aliquot of blood (100 ml) was added to Turk’s solution, and the total leukocyte count was determined with a hemocytometer.

A further aliquot of blood (500 ml) was mixed with an equal volume of 4% dextran (molecular weight 500,000), and the erythrocytes were allowed to settle. A cytocentrifuge preparation was made from the resulting leukocyte-rich fraction.

Cytospin preparations were fixed with methanol (5 min) and stained with May-Grunwald (5 min) and Giemsa (15 min) stains. Finally, cytospins were washed in phosphate buffer, pH 6.8 and air-dried.

Differential leukocyte counts were obtained from the cytospin preparations. Blood eosinophil numbers were determined from the total leukocyte count and the percentage of eosinophils (differential count).

**Measurement of DK-PGD2-Induced Airway Eosinophilia in Guinea Pigs.** DK-PGD2-induced airway eosinophilia was measured as described by Whelan (2009). Groups of guinea pigs were placed in a plastic chamber and exposed to aerosols of DK-PGD2, generated from a nebulizer driven by a compressor (DeVilbis PulmoStar; Sunrise Medical Ltd, West Midlands, UK) for 10 min. At 24 h after exposure to aerosols animals were sacrificed by an overdose of pentobarbitone, the trachea was cannulated, and the lungs were lavaged twice with 5 ml of heparinized (10 U/ml) PBS.

A cytocentrifuge preparation (cytospin 2; Thermo Fisher Scientific, Waltham, MA) was prepared from an aliquot of the pooled bronchoalveolar lavage fluid, recovered, and stained with May Grunwald Giemsa stain. A total leukocyte count was also made from the bronchoalveolar lavage fluid using a hemocytometer. The percentage of eosinophils was determined from the cytocentrifuge preparation under oil immersion microscopy, and the number of eosinophils per milliliter of blood was determined from this percentage and the total leukocyte count.

Guinea pigs were dosed with OC000459 or vehicle (10% DMSO in water) by oral gavage 30 min before aerosol exposure.

**Data Analysis.** Data were analyzed by using the computer program Prism (GraphPad Software Inc., San Diego, CA). Concentration-response curves for antagonists in functional assays were analyzed by nonlinear least-squares regression using a sigmoidal concentration-response relationship, and IC50 values were derived from this analysis. Data from [3H]PGD2 competition experiments were fitted to one-binding site models (best fit as determined by using an F-test). IC50 values of competitors were derived from this analysis, and the K values (inhibition constants) were calculated by using the method of Cheng and Prusoff (1973). The pK values of antagonist [according to the formula pK = log(CR-1) ~ log[B], where K is the equilibrium dissociation constant, B is the concentration of the antagonist used, and CR (concentration ratio) is the ratio of agonist EC50 measured in the presence of antagonist over that measured in the absence of antagonist] were derived from a Schild plot where the slope was constrained to unity. Results are given as mean ± S.E.M. of the indicated number of experiments.

To assess statistical significance, data were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls test. Values of p < 0.05 were considered statistically significant.

For the rat blood eosinophilia and guinea pig airway eosinophilia...
experiments, data from at least three experiments were pooled and analyzed by ANOVA followed by Dunnett’s post hoc test (GraphPad Prism). A difference between groups was considered significant when \( p \leq 0.05 \). To calculate ED$_{50}$ values for OC000459, a nonlinear, sigmoidal regression was fitted to the data obtained from animals treated with OC000459, and the ED$_{50}$ value was obtained by using GraphPad Prism.

### Results

**Effect of OC000459 on Binding of [$^3$H]PGD$_2$ to Human DP$_2$.** OC000459 inhibited the binding of [$^3$H]PGD$_2$ to membranes from CHO cells transfected with human DP$_2$ with \( K_i \) of 0.013 ± 0.002 μM (\( n = 13 \) independent experiments) as shown in Fig. 2A. OC000459 also displaced [$^3$H]PGD$_2$ from membranes from human Th2 lymphocytes (\( K_i \) = 0.004 ± 0.001 μM; \( n = 3 \) independent experiments), indicating that the compound was active on the native receptor as shown in Fig. 2B.

OC000459 was active on rat recombinant DP$_2$ (0.003 ± 0.001 μM; \( n = 5 \) independent experiments) but did not affect ligand binding or functional activity of the other prostanoid receptors (Table 1). Unlabeled PGD$_2$, PGE$_2$, U44069, and iloprost displaced [$^3$H]PGD$_2$, [$^3$H]PGE$_2$, [$^3$H]SQ29548, and [$^3$H]iloprost from their respective receptors at concentrations in the nanomolar range.

**Effect of OC000459 on Calcium Mobilization in Intact CHO Cells Expressing Human DP$_2$.** In intact CHO cells expressing DP$_2$, OC000459 antagonized PGD$_2$-mediated calcium mobilization in a concentration-dependent manner (IC$_{50}$ = 0.028 ± 0.005 μM; \( n = 4 \) independent experiments) as shown in Fig. 3. OC000459 did not inhibit calcium mobilization in response to ATP at concentrations up to 10 μM.

**Effect of OC000459 on PGD$_2$-Mediated Activation of Th2 Lymphocytes.** OC000459 inhibited chemotaxis of human Th2 cells in response to PGD$_2$ (10 nM) with an IC$_{50}$ of 0.028 ± 0.006 μM (\( n = 3 \) independent experiments) (Fig. 4A) and inhibited production of IL-13 production by Th2 cells in response to PGD$_2$ (100 nM) with an IC$_{50}$ of 0.019 ± 0.006 μM (\( n = 3 \) independent experiments) (Fig. 4B; Table 2). OC000459 also inhibited the antiapoptotic effect of PGD$_2$ on Th2 cells with an IC$_{50}$ of 0.035 ± 0.007 μM (\( n = 3 \) independent experiments) (Fig. 4C; Table 2).

**Effect of OC000459 on Eosinophil Activation in Response to PGD$_2$, IL-5, C5a, Eotaxin, Leukotriene B$_4$, and 5-Oxo-Eicosatetraenoic Acid.** The effect of OC000459

![Fig. 3. Effect of OC000459 on PGD$_2$-mediated calcium mobilization in intact CHO cells transfected with human recombinant DP$_2$. Calcium mobilization in responses to PGD$_2$ in transfected CHO cells was measured in the presence and absence of various concentrations of OC000459. Data are presented as mean ± S.E.M from four independent experiments conducted in triplicate. Inset, the effects of OC000459 on calcium mobilization responses to ATP are shown.](image-url)
on eosinophil shape change responses was measured by gated autofluorescence forward scatter in both a mixed leukocyte preparation and whole blood. OC000459 antagonized the effect of PGD$_2$ competitively in both the isolated leukocyte preparation (Fig. 5A) and whole blood (Fig. 5B). In isolated leukocytes p$_{K_B}$ was calculated as $7.9 \pm 0.2$ (n = 3 independent experiments), and in human whole blood potency decreased by 2.5-fold (p$_{K_B} = 7.5 \pm 0.1$; n = 3 independent experiments). In the mixed leukocyte preparation, OC000459 did not affect eosinophil shape change responses to C5a, eotaxin, or 5-oxo-eicosatetraenoic acid at concentrations that caused substantial inhibition of responses to PGD$_2$ (Fig. 6). OC000459 also inhibited eosinophil shape change responses to DK-PGD$_2$ (IC$_{50} = 0.011 \pm 0.006 \mu M$; n = 3 independent experiments).

**Selectivity of OC000459.** OC000459 was tested for its ability to inhibit the binding activity or functional activity of a range of receptors, ion channels, transporters, and enzymes (see Supplemental Tables 1 and 2). At a concentration of 10 $\mu M$, no significant inhibitory or stimulatory activity was detected on the 69 receptors, ion channels, and transporters or 17 enzymes tested, which included recombinant COX1 and COX2 and native COX1 expressed in platelets.

**Effect of OC000459 on the Activation of Th2 Cells and Eosinophils in Response to Mast Cell Supernatants.** Supernatants from human mast cells activated with IgE/anti-IgE stimulated increased migration of Th2 cells and increased the percentage of eosinophil shape change in mixed leukocyte preparation compared with supernatants from unactivated mast cells. The responses of Th2 lymphocytes or eosinophils were significantly suppressed in the presence of OC000459 (1 $\mu M$) (Fig. 7).

**Plasma Protein Binding.** OC000459 was 99.1 $\pm$ 0.4 and 99.8 $\pm$ 0.0% bound in human and rat plasma, respectively, representing the mean ± S.D. of six measurements.

**Pharmacokinetic Profile of OC000459 in the Sprague-Dawley Rats.** Plasma concentrations of OC000459 at various times after intravenous and oral administration of OC000459 in rat are shown in Fig. 8, and calculated pharmacokinetic parameters are shown in Table 3. After oral administration in 10% DMSO/PBS, OC000459 was well absorbed and had a plasma t$_{1/2}$ of 2.9 to 3.5 h. At doses of 2 and 10 mg/kg p.o. the plasma levels achieved were 1543.5 and 1443.5 ng/ml, respectively, which were $\approx 200$ and $\approx 190$ times the in vitro IC$_{50}$ for inhibition of Th2 cell and eosinophil function and 44.1 and 41.2 times higher than the whole blood IC$_{50}$ respectively. At steady state the volume of distribution (V$_d$) was estimated to be 0.5 l/kg, which is similar to montelukast (0.7 l/kg) and significantly greater than indomethacin (0.1 l/kg).

**Effect of OC000459 on Blood Eosinophilia Induced by Systemic Treatment with DK-PGD$_2$ in Rats.** Intracardiac injection of DK-PGD$_2$ (10 $\mu g$) led to a rapid increase in blood eosinophil numbers detectable 60 min after injection. Oral administration of OC000459 0.5 h before injection of DK-PGD$_2$ led to a dose-dependent reduction in blood eosinophilia (Fig. 9; ED$_{50}$ = 0.04 mg/kg).

**Effect of OC000459 on Airway Eosinophilia Induced by Aerosolization of DK-PGD$_2$.** Exposure of guinea pigs to
an aerosol of DK-PGD₂ (10 μg/ml) caused lung eosinophilia measured by increased numbers of eosinophils in bronchoalveolar lavage 24 h after challenge. Oral administration of OC000459 0.5 h before aerosolization of DK-PGD₂ led to a dose-dependent inhibition of eosinophil accumulation (Fig. 10; ED₅₀ = 0.01 mg/kg).

Discussion

There is emerging evidence that DP₂ plays a central role in the initiation and maintenance of allergic responses (Pettipher et al., 2007). Studies in DP₂ knockout mice are consistent with the effects of small-molecule antagonists and support the view that DP₂ plays a central role in leukocyte recruitment to allergic tissue (Satoh et al., 2006; Uller et al., 2007; Lukacs et al., 2008; Oiwa et al., 2008; Pettipher, 2008; Boehme et al., 2009a), airway hyper-responsiveness (Lukacs et al., 2008), and production of cytokines (Nomiya et al., 2008; Boehme et al., 2009a), mucus (Uller et al., 2007), and IgE (Satoh et al., 2006; Nomiya et al., 2008; Boehme et al., 2009b). Studies with isolated human cells have highlighted a dominant role for DP₂ in both mast cell-dependent and paracrine activation of Th2 cells (Gyles et al., 2006; Vinall et al., 2007).

An effort to discover potent and selective DP₂ antagonists resulted in the identification of OC000459, an N-1 indoleacetic acid derivative. The chemical starting point for this program was indomethacin, which possesses DP₂ partial agonist activity (Hirai et al., 2002; Sawyer et al., 2002). OC000459 inhibited binding of [³H]PGD₂ to human recombinant DP₂ expressed in CHO cell membranes and native DP₂ expressed by Th2 cell membranes with high potency, but it did not affect the ligand binding properties of the other prostanoid receptors at concentrations up to 10 μM. OC000459 had no activity on a diversity panel of 69 receptors and ion channels and 17 enzymes (Supplemental Tables 1 and 2). Of particular note is the finding that OC000459, unlike indomethacin, did not inhibit the activity of human recombinant COX1 or COX2 and did not inhibit COX activity in intact human platelets. In further contrast to indomethacin, OC000459 is a DP₂ antagonist rather than agonist as demonstrated by its ability to inhibit PGD₂-mediated calcium mobilization in intact CHO cells expressing recombinant DP₂ without affecting responses to ATP, which stimu-
lates calcium mobilization in CHO cells by activation of an endogenous purinergic receptor.

OC000459 also inhibited PGD₂-mediated activation of eosinophils in a competitive manner. In a mixed human leukocyte preparation, OC000459 inhibited PGD₂-induced eosinophil shape change with a pKᵦ of 7.9. The specificity of OC000459 was demonstrated by the finding that eosinophil shape change responses to eotaxin, 5-oxo-eicosatetraenoic acid, and C5a were unaffected at concentrations that caused profound inhibition of responses to PGD₂. OC000459 retained good activity in whole blood where PGD₂-mediated activation of eosinophils was inhibited with a pKᵦ of 7.5. This modest reduction in potency is likely to reflect plasma protein binding, which has been estimated to be ∼99% in human. Despite high plasma protein binding, the whole blood activity combined with an acceptable volume of distribution (0.5 l/kg) suggests that OC000459 is likely to be effective in antagonizing CRTH2 in vivo. When dosed orally to rats, OC000459 was well absorbed and achieved plasma concentrations well in excess of the levels required to inhibit eosinophil and Th2 cell function. This was confirmed in rats where OC000459 inhibited blood eosinophilia induced by the selective DP₂ agonist DK-PGD₂ with an ED₅₀ of 0.04 mg/kg. The plasma concentrations at this ED₅₀ level were estimated to be ∼30 ng/ml, which is sufficient to substantially inhibit PGD₂-mediated eosinophil activation in whole blood. OC000459 also inhibited airway eosinophilia in guinea pigs in response to nebulization of DK-PGD₂, illustrating OC000459 can inhibit inflammatory responses in the lung after oral delivery, a key property of a drug targeted to the treatment of asthma and related disorders. The slighter higher potency of OC000459 inhibiting airway eosinophilia compared with blood eosinophilia may reflect lower protein concentration in lung tissue compared with blood, which would lead to higher concentrations of free drug to interact with the receptor. Consequently, it is considered that OC000459 has “drug-like” properties that enable it to inhibit inflammation of the target tissue when dosed by the intended therapeutic route.

We have previously shown that membranes from human

![Fig. 7. Effect of OC000459 on migration of Th2 lymphocytes (top) and eosinophil shape change (bottom) in response to supernatants from human mast cells activated with IgE/anti-IgE. Mast cell supernatants caused increased activation of both Th2 lymphocytes and eosinophils compared with control media, and this effect was completely inhibited by OC000459. *, p < 0.01 by ANOVA and Neuman-Keuls test for activated mast cell supernatants versus control media and p > 0.05 for OC000459 + mast cell supernatants compared with mast cell supernatants alone. Results are expressed as mean ± S.E.M (n = 3).](image)

![Fig. 8. Plasma concentrations of OC000459 at various times after administration of 2 mg/kg i.v. and 2 or 10 mg/kg p.o. to groups of Sprague-Dawley rats. Results are presented as the mean ± S.E.M (n = 3 per group).](image)
OC000459 displaced [3H]PGD2 with high potency (Kᵢ = 0.004 μM), indicating it is active on native DP2. OC000459 also inhibited the functional responses of Th2 cells. Both PGD2-mediated chemotaxis and cytokine production were potently inhibited by OC000459. OC000459 also inhibited the anti-apoptotic effect of PGD2 on cytokine-deprived Th2 cells, suggesting that not only will this drug prevent the accumulation of Th2 lymphocytes in allergic tissue but also stimulate their clearance from such tissue by promoting apoptosis.

The highly potent and selective properties of OC000459 make it an ideal tool to define the role of DP2 in mediating biological responses in vitro and in vivo. In this study we have used OC000459 to investigate the role of DP2 in mediating activation of Th2 cells and eosinophils in response to mast cell supernatants. OC000459 caused complete inhibition of chemotaxis of Th2 cells in response to supernatants collected from immunologically activated mast cells. This confirms our previous observations with ramatroban (Gyles et al., 2006) and suggests that mast cell-dependent recruitment of Th2 cells is mediated by DP2, most likely through the action of PGD2. OC000459 is also effective in inhibiting mast cell-dependent activation of eosinophils, which is consistent with the ability of DP2 antagonists to inhibit eosinophil accumulation at sites of allergic inflammation (Uller et al., 2007; Pettipher, 2008).

The ability of DP2 antagonists such as OC000459 to inhibit Th2 cytokine production and block mast cell-dependent recruitment of Th2 cells and eosinophils may have profound implications for the treatment of asthma and other allergic diseases. The late-phase airway response to inhaled allergen is T cell-dependent (Sihra et al., 1997; Khan et al., 2000) and is associated with the accumulation of eosinophils (Rossi et al., 1991; Gauvreau et al., 1999). Therefore inhibition of DP2-mediated recruitment and activation of Th2 cells and eosinophils may reduce late-phase airway obstruction.

The clinical effects of OC000459 have also been studied in subjects with moderate persistent asthma. Treatment with OC000459 was well tolerated and associated with a reduction in sputum eosinophilia, reduction in circulating levels of IgE, and improvements in lung function (Forced expiratory volume in one second and peak flow) and symptoms (Barnes et al., 2011). Plasma concentrations of 600 ng/ml were detected in patients taking OC000459, which is 50 times higher than the whole blood Kᵢ, suggesting that substantial receptor blockade had been achieved in this study. These clinical findings show remarkable concordance with the findings from the preclinical models of allergic disease discussed earlier.

In summary, OC000459 is a highly potent, selective, and orally active DP2 antagonist that inhibits mast cell-dependent activation of Th2 cells and eosinophils. This compound...
is proving to be an excellent tool in defining the role of DP2, in asthma and related allergic disorders and is currently being evaluated in phase IIb trials. OCO00459 has the potential to be one of a new class of oral anti-inflammatory agents used to treat allergic disorders.

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Authorship Contributions

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References


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