

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^[S]

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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-ylmethylindo-1-yl)-acetic acid (OC000459) is an indole-acetic acid derivative that potently displaces [³H]PGD₂ from human recombinant DP₂ (K_i = 0.013 μM), rat recombinant DP₂ (K_i = 0.003 μM), and human native DP₂ (Th2 cell membranes; K_i = 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 (pK_B = 7.9) and human whole blood (pK_B = 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).

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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-EETE, 5-oxoeicosatetraenoic acid; PI, propidium iodide; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; EIA, enzyme immunoassay; IL, interleukin; OC000459, (5-fluoro-2-methyl-3-quinolin-2-ylmethylindo-1-yl)-acetic acid; C5a, complement component 5a; TP, thromboxane receptor; EP, prostaglandin E; ANOVA, analysis of variance; SQ29548, [1S-[1α,2α(Z),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-epoxymethano-prosta-5Z,13E-dien-1-oic acid; BW245C, (4S)-3-[(3R,S)-3-cyclohexyl-3-hydroxypropyl]-2,5-dioxo-4-imidazolidineheptanoic acid; NS398, N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide.

Prostaglandin D₂ (PGD₂) 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., 1986). PGD₂ 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 PGD₂ is mediated by the G protein-coupled receptor DP₂, which is also known as chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) (Hirai et al., 2001; Monneret et al., 2001). Furthermore, PGD₂ can stimulate the production of interleukins 4, 5, and 13 by Th2 cells in the absence of costimulation through a DP₂-dependent mechanism (Xue et al., 2005). These *in vitro* data are supported by findings that the effect of PGD₂ in exacerbating allergic inflammation in the lungs and skin of mice can be mimicked by the selective CRTH2 agonist DK-PGD₂ (Spik et al., 2005). Studies in mice genetically deficient in DP₂ have implicated this receptor in the development of allergic responses. In a mouse model of skin inflammation, genetic ablation of DP₂ 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 (Sato et al., 2006). Production of IgE was also reduced in DP₂-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 DP₂ (Oiwa et al., 2008). The effects of genetic ablation of DP₂ in mice are mimicked by selective small-molecule DP₂ antagonists that have been shown to be effective in reducing allergen-induced airway inflammation (Uller 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 DP₂ 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 DP₂ including tetrahydroquinolone derivatives, carbazole derivatives, indole acetic acids, azaindole-3-acetic acids, phenoxyacetic acids, phenylacetic acids, thiazoleacetic acids, 3-indoyl sultams, 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-ylmethylindol-1-yl)-acetic acid (OC000459), an indole-1-acetic acid derivative that is a potent and selective DP₂ 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]PGD₂, [³H][1S-[1α,2α(Z),3α,4α]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid (SQ29548), and [³H]PGE₂ were purchased from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). PGD₂, PGF_{2α}, iloprost, 5-oxo-eicosatetraenoic acid, and PGD₂-methoxime enzyme immunoassay were purchased from Cayman Chemical (Ann Arbor, MI). Eotaxin (CCL11), complement component C5a, 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 CD4⁺ isolation, anti-DP₂ 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). Cytofix buffer was purchased from BD Biosciences (Oxford, Oxon, UK). Phycocerythrin-annexin V, propidium iodide (PI), and annexin-binding buffer were obtained from Invitrogen (Carlsbad, CA). Pentobarbitone 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]PGD₂ Ligand Binding Assays. Membranes from CHO cells expressing human recombinant DP₁ or DP₂ were prepared as described previously (Gazi et al., 2005), and membranes from CHO cells expressing rat recombinant DP₂ 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]PGD₂ (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/well 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 by 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 PGD₂. 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 PGD₂ 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

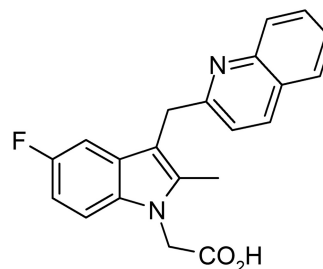


Fig. 1. Chemical structure of the CRTH2 antagonist OC000459.

binding is included in Supplemental Table 1, using 9,11-dideoxy-9 α ,11 α -epoxymethano-prosta-5Z,13E-dien-1-oic 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 EP₁-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 PGF_{2 α} -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 Sf9 cells was incubated with arachidonic acid (4 μ M) 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 μ M) for 15 min at 37°C, and PGE₂ was measured by EIA with diclofenac included as a reference standard. Human recombinant COX2 expressed in Sf9 cells was incubated with arachidonic acid (2 μ M) for 10 min at 25°C, and PGE₂ was measured by EIA with *N*-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide (NS398) included as a reference standard.

Culture and Activation of Human Mast Cells. Human mast cells were cultured from CD34⁺ progenitor cells as described previously (Gyles et al., 2006). In brief, CD34⁺ progenitor cells from human cord blood were cultured at a density of 1×10^5 cells/ml with Iscove's modified Dulbecco's medium containing 10% human serum, 0.55 μ M 2-mercaptoethanol, penicillin/streptomycin, human recombinant stem cell factor (100 ng/ml), and human recombinant IL-6 (50 ng/ml) in 5% CO₂ at 37°C for 8 to 10 weeks. Half of the culture medium was replaced twice weekly with fresh medium containing the same concentration of cytokines. The expression of tryptase and chymase of the cells was tested by immunostaining. The cytospin smears were first air-dried for 2 h at room temperature and then fixed with Carnoy's solution (ethanol/chloroform/glacial acetic acid, 6:3:1, v/v/v) for 1 min. The fixed smears were stained by using monoclonal antibodies against human mast cell tryptase and human mast cell chymase. The mast cells used in this study were tryptase-positive (> 80%) and chymase-negative (<1%). The cells were pre-treated with 5 μ g/ml purified human myeloma IgE and human recombinant IL-4 (10 ng/ml) for 4 days, washed, and then passively sensitized with fresh IgE (5 μ g/ml) for 2 h. The cells were washed with medium for 20 min and then incubated with medium or challenged with goat anti-human IgE (1 μ g/ml) in the presence or absence of diclofenac (10 μ M). The supernatants of the cells were collected 1 h after challenge. The supernatants were assayed for PGD₂ by using a PGD₂-methoxime enzyme immunoassay kit according to the manufacturer's instructions (Cayman Chemical).

Culture of Human CRTH2⁺CD4⁺ Th2 Cells. The study was approved by the NHS Oxfordshire Local Research Ethics Committee. Human CRTH2⁺CD4⁺ Th2 cells were prepared by using a modified method described previously (Xue et al., 2005). In brief, peripheral blood mononuclear cells were isolated from buffy coats (National Blood Service, Bristol, UK) by Ficoll Hypaque density gradient centrifugation, followed by CD4⁺ cell purification using a magnetic cell sorting CD4⁺ T cell isolation kit II (Miltenyi Biotec). After a 7-day culture in X-VIVO 15 medium containing 10% human serum, 50 U/ml IL-2, and 100 ng/ml IL-4, CRTH2-positive cells were isolated from the CD4⁺ cultures by positive selection using an anti-human CRTH2 MicroBead Kit (Miltenyi Biotec). The harvested CD4⁺ CRTH2⁺ cells were treated as Th2 cells, further amplified by stimulation with the T cell activation/expansion kit (Miltenyi Biotec), and grown in X-VIVO 15 medium containing 10% human serum and 50 U/ml IL-2 before use.

Chemotaxis Assays. For measurement of chemotaxis of Th2 cells, cells were resuspended in X-VIVO media at 2×10^6 cells/ml; 25 μ l of cell suspension and test samples (29 μ l) prepared in X-VIVO media were applied to the upper and lower chambers of a 5- μ m-pore-

sized, 96-well ChemoTx plate. After incubation at 37°C for 60 min, any cells remaining on top of the filter were wiped off, and the plates were centrifuged at 300g for 2 min to collect any cells on the underside of the filters. The upper membrane was carefully removed, and cell migration was quantified by fluorescence-activated cell sorting analysis. Background cell migration was determined by measuring the response to media alone.

Eosinophil Shape Change. The study was approved by the NHS Oxfordshire Local Research Ethics Committee. The shape change response of human eosinophils to chemoattractants in both isolated leukocyte preparations and whole blood by gated autofluorescence forward-scatter assay were measured by a modification of the methods described previously by Bryan et al. (2002). For the measurement of responses of isolated leukocytes, heparinized blood was collected from healthy volunteers and incubated with red blood cell lysis buffer for 5 min at room temperature (300 μ l of lysis buffer was used for every 100 μ l of blood). Samples were then centrifuged at 300g for 5 min. The supernatant (containing lysed red cells) was removed, and leukocytes were resuspended in 50 μ l 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 μ l 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 μ l 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 μ l, 200 $\times$ final concentration) was added directly to 200 μ l of heparinized whole blood, mixed well, and incubated for 15 min at 37°C in a 5% CO₂ incubator. Chemoattractant agonists (1 μ l, 200 $\times$ final concentration) were added to each sample and incubated for another 1 h at 37°C. After this time, cell shape was fixed by the addition of 300 μ l 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 μ l 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 or absence of PGD₂ or other compounds as indicated under *Results* at 37°C and 5% CO₂ for 5 h. The supernatants were collected and assayed 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 ultracentrifugation 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). Wistar rats, used in the blood eosinophilia studies, were bred at the University of Hertfordshire (Hatfield, Hertfordshire UK).

Female Dunkin Hartley guinea pigs (250–350 g) were purchased from Darley Oaks Farm (Newchurch, 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, pH4) and the internal standard ($^{13}\text{C}_4$ -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 (MDS SCIEX API365; Applied Biosystems, Warrington, UK) with TurboIonSpray 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 μl) was added to Turk's solution, and the total leukocyte count was determined with a hemocytometer.

A further aliquot of blood (500 μl) was mixed with an equal volume of 4% dextran (molecular weight 500,000), and the erythrocytes were allowed to settle. A cytospin 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, cytopins 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 (DeVilbiss 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 IC_{50} 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). IC_{50} values of competitors were derived from this analysis, and the K_i values (inhibition constants) were calculated by using the method of Cheng and Prusoff (1973). The pK_B values of antagonist [according to the formula $\text{pK}_B = \log(\text{CR}-1) - \log[B]$, where K_B is the equilibrium dissociation constant, $[B]$ is the concentration of the antagonist used, and CR (concentration ratio) is the ratio of agonist EC_{50} 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 $\pm$ 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

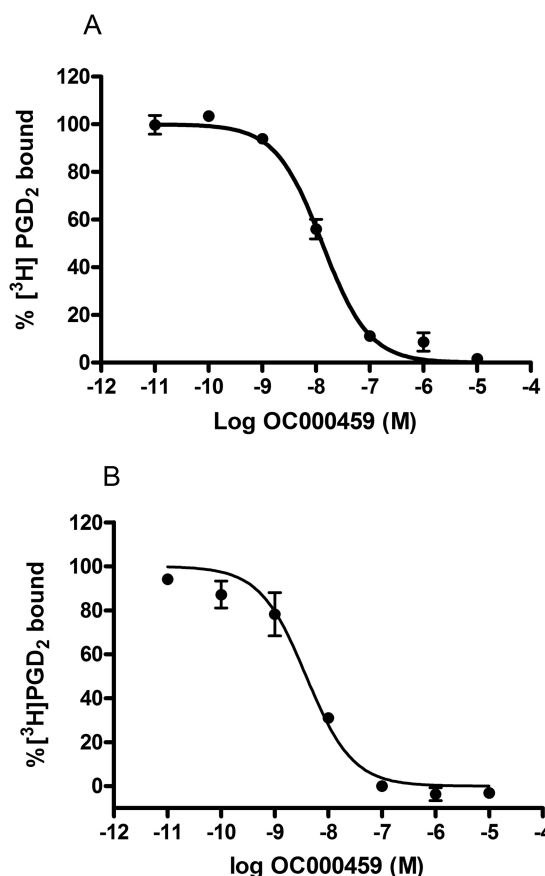


Fig. 2. Competition binding analysis of OC000459 to recombinant human DP₂ (A) and human Th2 cell membranes (B). Membranes isolated from CHO cells expressing human recombinant DP₂ or Th2 cell membranes were incubated with [^3H]PGD₂ in the presence of various concentrations of OC000459 as described under *Materials and Methods*. Data are presented as mean $\pm$ S.E.M. from a representative experiment performed in triplicate. The mean K_i derived from multiple independent experiments is shown in Table 1.

TABLE 1

 K_i values (μM) for OC000459 for DP₂ binding compared with other prostanoid receptors

	K_i or IC_{50}									
	Human DP ₂	Rat DP ₂	DP ₁	TP	EP ₁	EP ₂	EP ₃	EP ₄	FP	IP
Mean	0.013	0.003	>10	>10	>10	>10	>10	>10	>10	>10
S.E.M.	0.002	0.001								
<i>n</i>	13	5	7	4	3	5	5	4	3	3

FP, prostaglandin F receptor; IP, prostacyclin receptor.

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 $p < 0.05$. 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 [³H]PGD₂ to Human DP₂. OC000459 inhibited the binding of [³H]PGD₂ to membranes from CHO cells transfected with human DP₂ with K_i of $0.013 \pm 0.002 \mu\text{M}$ ($n = 13$ independent experiments) as shown in Fig. 2A. OC000459 also displaced [³H]PGD₂ from membranes from human Th2 lymphocytes ($K_i = 0.004 \pm 0.001 \mu\text{M}$; $n = 3$ independent experiments), indicating that the compound was active on the native receptor as shown in Fig. 2B.

OC000459 was active on rat recombinant DP₂ ($0.003 \pm 0.001 \mu\text{M}$; $n = 5$ independent experiments) but did not affect ligand binding or functional activity of the other prostanoid receptors (Table 1). Unlabeled PGD₂, PGE₂, U44069, and iloprost displaced [³H]PGD₂, [³H]PGE₂, [³H]SQ29548, and

[³H]iloprost from their respective receptors at concentrations in the nanomolar range.

Effect of OC000459 on Calcium Mobilization in Intact CHO Cells Expressing Human DP₂. In intact CHO cells expressing DP₂, OC000459 antagonized PGD₂-mediated calcium mobilization in a concentration-dependent manner ($\text{IC}_{50} = 0.028 \pm 0.005 \mu\text{M}$; $n = 4$ independent experiments) as shown in Fig. 3. OC000459 did not inhibit calcium mobilization in response to ATP at concentrations up to $10 \mu\text{M}$.

Effect of OC000459 on PGD₂-Mediated Activation of Th2 Lymphocytes. OC000459 inhibited chemotaxis of human Th2 cells in response to PGD₂ (10 nM) with an IC_{50} of $0.028 \pm 0.006 \mu\text{M}$ ($n = 3$ independent experiments) (Fig. 4A) and inhibited production of IL-13 production by Th2 cells in response to PGD₂ (100 nM) with an IC_{50} of $0.019 \pm 0.006 \mu\text{M}$ ($n = 3$ independent experiments) (Fig. 4B; Table 2). OC000459 also inhibited the antiapoptotic effect of PGD₂ on Th2 cells with an IC_{50} of $0.035 \pm 0.007 \mu\text{M}$ ($n = 3$ independent experiments) (Fig. 4C; Table 2).

Effect of OC000459 on Eosinophil Activation in Response to PGD₂, IL-5, C5a, Eotaxin, Leukotriene B₄, and 5-Oxo-Eicosatetraenoic Acid. The effect of OC000459

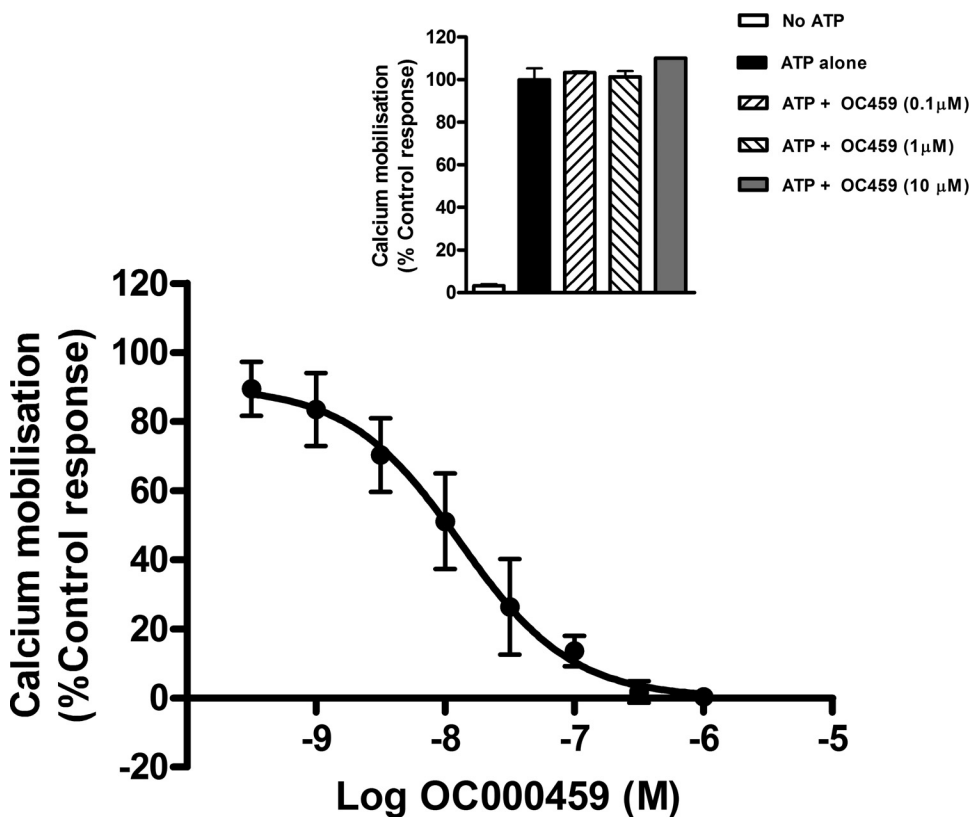


Fig. 3. Effect of OC000459 on PGD₂-mediated calcium mobilization in intact CHO cells transfected with human recombinant DP₂. Calcium mobilization in responses to PGD₂ in transfected CHO cells was measured in the presence and absence of various concentrations of OC000459. Data are presented as mean $\pm$ S.E.M from four independent experiments conducted in triplicate. Inset, the effects of OC000459 on calcium mobilization responses to ATP are shown.

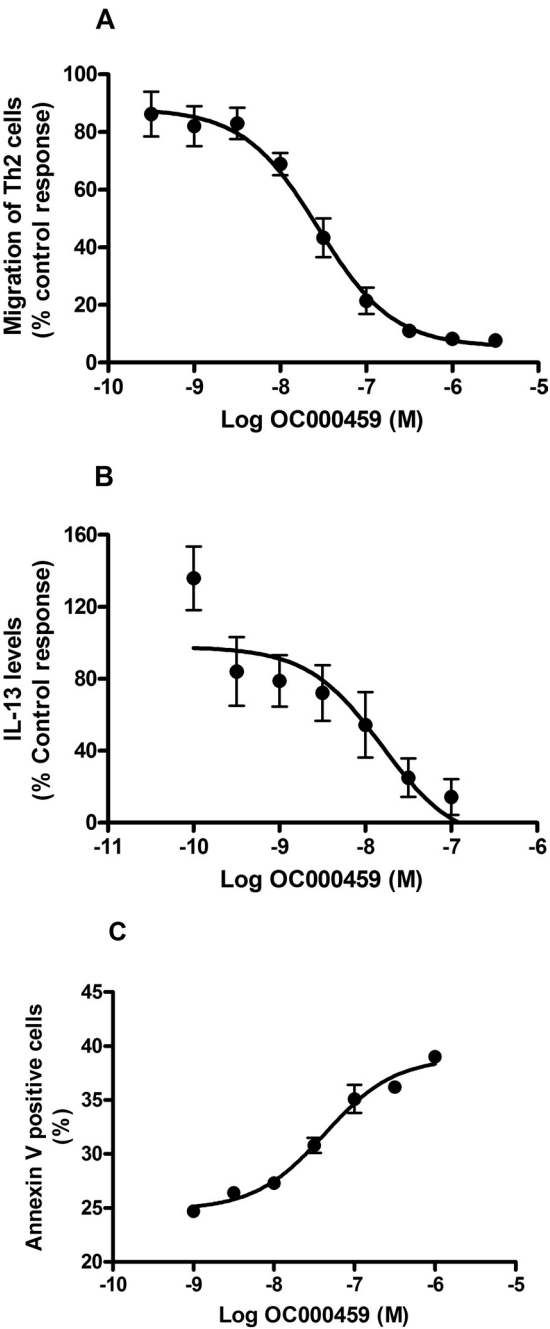


Fig. 4. Effect of OC000459 on chemotaxis of Th2 lymphocytes (A), IL-13 production by Th2 lymphocytes (B), and prevention of apoptosis of Th2 cells (C) in response to PGD₂. Chemotactic responses to PGD₂ (10 nM), IL-13 production, and prevention of apoptosis in response to PGD₂ (100 nM) were measured in the presence and absence of various concentrations of OC000459. Data are presented as mean $\pm$ S.E.M from a representative experiment performed in triplicate. Mean IC₅₀ values derived from multiple experiments are shown in Table 2.

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 PGD₂ competitively in both the isolated leukocyte preparation (Fig. 5A) and whole blood (Fig. 5B). In isolated leukocytes pK_B was calculated as 7.9 $\pm$ 0.2 (*n* = 3 independent experiments), and in human whole blood potency decreased by 2.5-fold (pK_B = 7.5 $\pm$ 0.1; *n* = 3 independent experiments). In the mixed leukocyte preparation,

TABLE 2
Potency of OC000459 for inhibition of PGD₂-mediated activation of Th2 lymphocytes
For all experiments *n* = 3.

	IC ₅₀ or EC ₅₀		
	Th2 Cell Chemotaxis	IL-13 Production by Th2 Cells	Promotion of Apoptosis of Th2 Cells
	μ M		
Mean	0.028	0.019	0.035
S.E.M.	0.006	0.008	0.007

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 PGD₂ (Fig. 6). OC000459 also inhibited eosinophil shape change responses to DK-PGD₂ (IC₅₀ = 0.011 $\pm$ 0.006 μM; *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 μM, 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 μM) (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 $\pm$ 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 $\sim$ 200 and $\sim$ 190 times the in vitro IC₅₀ for inhibition of Th2 cell and eosinophil function and 44.1 and 41.2 times higher than the whole blood IC₅₀, 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-PGD₂ in Rats. Intracardiac injection of DK-PGD₂ (10 μg) 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-PGD₂ led to a dose-dependent reduction in blood eosinophilia (Fig. 9; ED₅₀ = 0.04 mg/kg).

Effect of OC000459 on Airway Eosinophilia Induced by Aerosolization of DK-PGD₂. Exposure of guinea pigs to

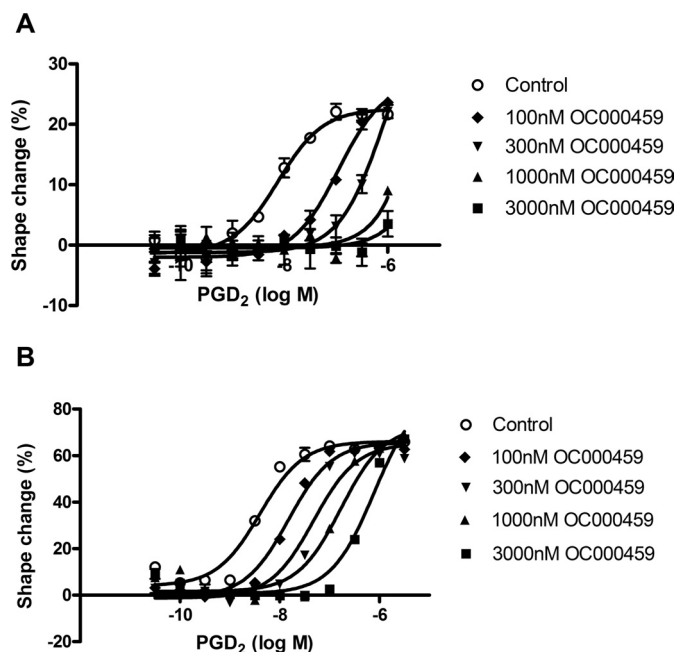


Fig. 5. Effect of OC000459 on PGD₂-mediated eosinophil shape change in a preparation of mixed human leukocytes containing 10% FCS (A) and in heparinized human whole blood (B). Eosinophil shape change responses were measured in response to increasing concentrations of PGD₂ in the presence and absence of various concentrations of OC000459 as described under *Materials and Methods*. Data are presented as mean $\pm$ S.E.M from a representative experiment performed in triplicate. The effect of OC000459 was competitive; the pK_B was 7.9 ± 0.2 in mixed leukocytes and 7.5 ± 0.1 in human whole blood (mean $\pm$ S.E.M from three independent experiments).

an aerosol of DK-PGD₂ (10 μ g/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).

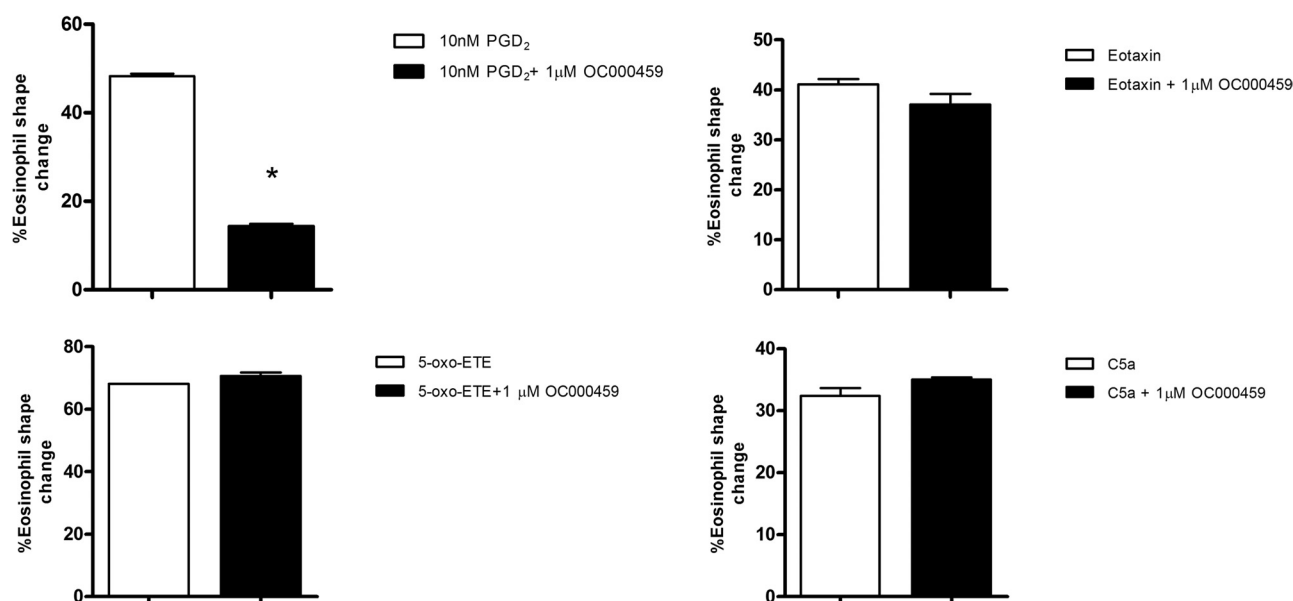


Fig. 6. Effect of OC000459 on eosinophil shape change induced by PGD₂, eotaxin 1, 5-oxo-ETE, and C5a in a human mixed leukocyte preparation. OC000459 at a concentration of 1 μ M caused significant inhibition of eosinophil responses to PGD₂ (*, $p < 0.01$) but did not affect responses to eotaxin 1, 5-oxo-ETE, or C5a. Results are presented as the mean $\pm$ S.E.M from three independent experiments.

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 μ M. 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-

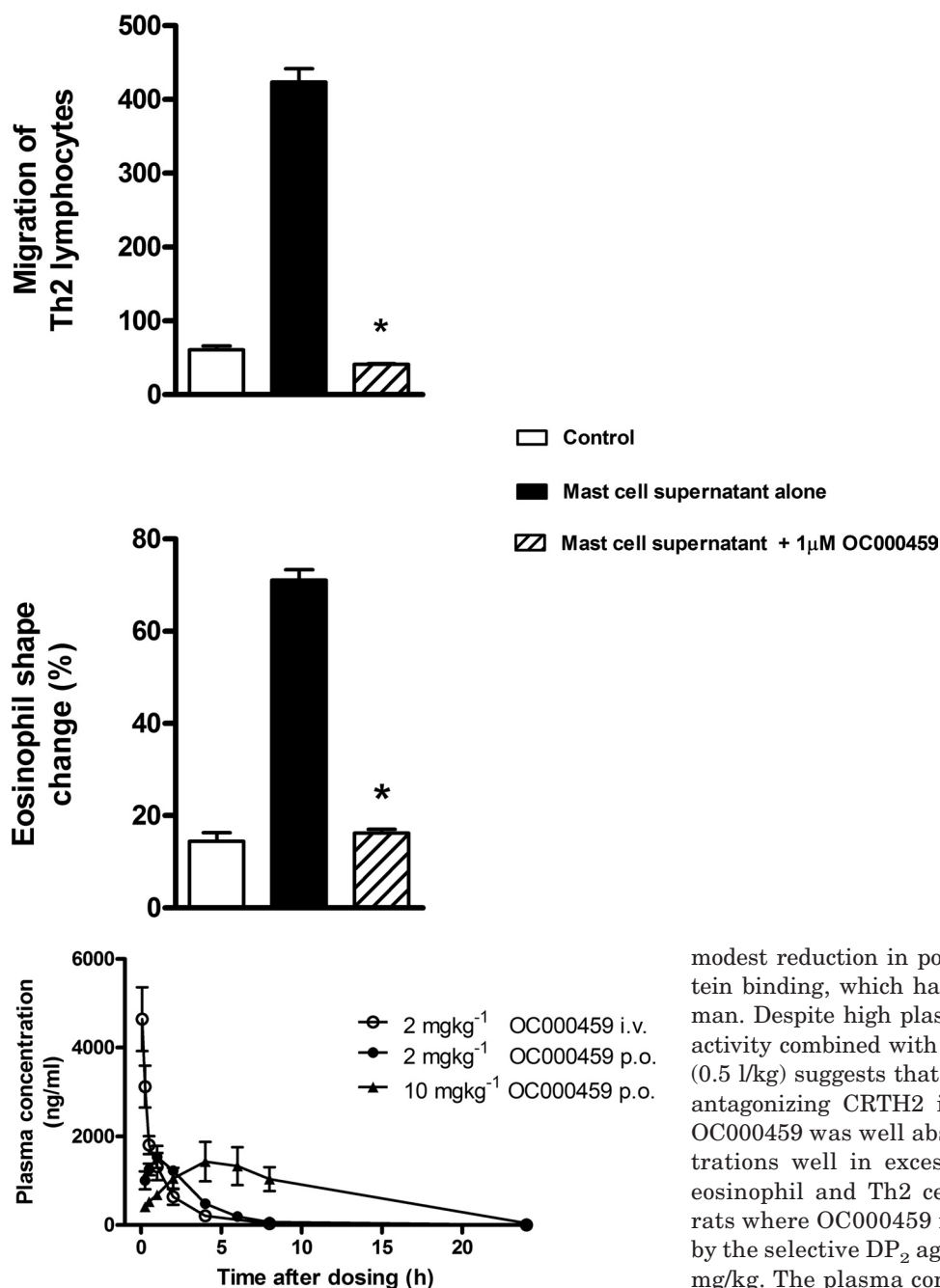


Fig. 8. Plasma concentrations of OC000459 at various times after administration of 2 mg/kg i.v. and 2 or 10 mg/kg p.o. to groups of Sprague-Dawley rats. Results are presented as the mean $\pm$ S.E.M ($n = 3$ per group).

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 pK_B 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 pK_B of 7.5. This

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 $\pm$ S.E.M ($n = 3$).

modest reduction in potency is likely to reflect plasma protein binding, which has been estimated to be $\sim 99\%$ 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

TABLE 3

Pharmacokinetic parameters for OC000459 in male Sprague-Dawley rats

Data are presented as the mean from three rats.

Dose	$C_{\max}$	$T_{\max}$	$t_{1/2}$	CL	AUC _{24h}	V_d	F
	ng/ml		h	ml/kg/min	ng/ml · h	l/kg	%
2 mg/kg i.v.	5660.4	0.08	1.3	8.4	4486.5	0.5	N.A.
2 mg/kg p.o.	1543.5	1.3	2.9	6.4	5262.5	N.A.	111.0
10 mg/kg p.o.	1443.5	3.3	3.5	9.6	13,062.1	N.A.	68.2

$C_{\max}$, maximal plasma concentration achieved; $T_{\max}$, time that maximal plasma concentration is achieved; $t_{1/2}$, plasma half-life; CL, clearance; AUC_{24h}, plasma concentration under the curve to 24 h; V_d , volume of distribution; F , percentage of drug orally compared with intravenous dosing; N.A., not available.

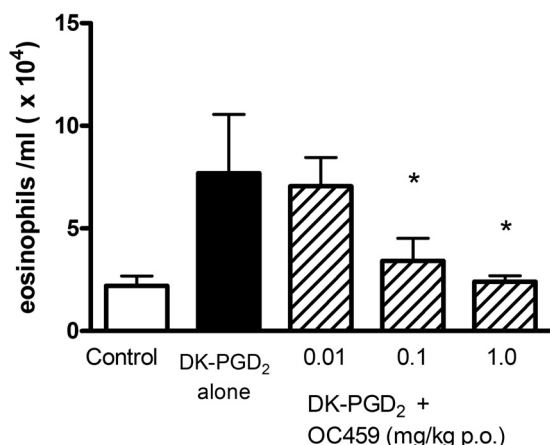


Fig. 9. Effect of OC000459 on blood eosinophilia induced by systemic treatment with DK-PGD₂ in rats. Blood eosinophilia was measured 60 min after intracardiac injection of DK-PGD₂ (10 µg) as described under *Materials and Methods*. Animals were dosed by oral gavage 30 min before injection of DK-PGD₂. OC000459 caused a dose-dependent reduction in DK-PGD₂-mediated blood eosinophilia (ED₅₀ = 0.04 mg/kg p.o.). Results are presented as the mean ± S.E.M. (5–6 animals per group). *, $p < 0.05$ compared with DK-PGD₂ + vehicle (analyzed by ANOVA followed by Dunnett's post hoc test).

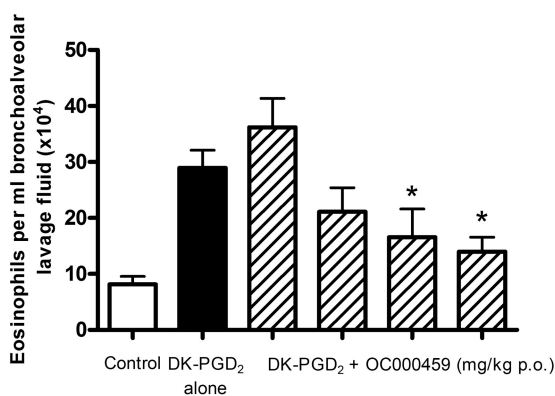


Fig. 10. Effect of OC000459 on airway eosinophilia induced by aerosolized DK-PGD₂. The numbers of eosinophils in bronchoalveolar lavage fluids from guinea pigs 24 h after exposure to an aerosol of DK-PGD₂ (10 µg/ml) were measured as described under *Materials and Methods*. Guinea pigs were dosed by oral gavage with vehicle or OC000459 30 min before exposure to aerosolized DK-PGD₂. OC000459 caused a dose-dependent inhibition of DK-PGD₂-mediated airway eosinophilia (ED₅₀ = 0.01 mg/kg p.o.). Results are presented as the mean ± S.E.M. (6–13 animals per group). *, $p < 0.05$ compared with DK-PGD₂ + vehicle (analyzed by ANOVA followed by Dunnett's post hoc test).

Th2 cells bind [³H]PGD₂ and this binding is inhibited by ramatroban and the selective DP₂ agonist DK-PGD₂ but not by the TP antagonist SQ29548 or the DP₁ agonist (4*S*)-(3-[(3*R,S*)-3-cyclohexyl-3-hydroxypropyl]-2,5-dioxo)-4-imidazolidineheptanoic acid (BW245C) (Xue et al., 2005), suggesting that human Th2 cells express DP₂ but not DP₁ or TP.

OC000459 displaced [³H]PGD₂ with high potency ($K_i = 0.004$ 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 K_B , 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. OC000459 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

Participated in research design: Pettipher, Vinall, Xue, Gazi, Whelan, Armer, Payton, and Hunter.

Contributed new reagents or analytic tools: Speight, Townsend, and Armer.

Wrote or contributed to the writing of the manuscript: Pettipher, Armer, Payton, and Hunter.

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