Pharmacologic Profile of OC000459, a Potent, Selective and Orally Active DP₂ Antagonist that Inhibits Mast Cell-Dependent Activation of Th2 Lymphocytes and Eosinophils


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Abbreviation list: DP₂, D prostanoid receptor 2, CRTH2, chemoattractant receptor homologous molecule expressed on Th2 cells; PGD₂, prostaglandin D₂; DK-PGD₂, 13,14 dihydro-15-keto-prostaglandin D₂; IgE, immunoglobulin E; FCS, foetal calf serum; BAL, bronchoalveolar lavage; COX, cyclo-oxygenase; CHO, Chinese Hamster Ovary; 5-oxo-ETE, 5-oxoicosatetraenoic acid.

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Abstract

DP2 (also known as chemoattractant receptor-homologous molecule expressed on Th2 cells, CRTH2) is selectively expressed by Th2 lymphocytes, eosinophils and basophils and mediates recruitment and activation of these cell types in response to PGD2. 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 (EP1-4, DP1, TP, IP, FP). OC000459 inhibited chemotaxis (IC50=0.028 µM) of, and cytokine production (IC50 =0.019 µM) by, human Th2 lymphocytes. OC000459 competitively antagonised eosinophil shape change responses induced by PGD2 in both isolated human leukocytes (pKb=7.9) and in human whole blood (pKb=7.5) but did not inhibit responses to eotaxin, 5-oxo-eicosatetraenoic acid or C5a. OC000459 also inhibited 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 COX1 and COX2. OC000459 was found to be orally bioavailable in rats and effective in inhibiting blood eosinophilia induced by 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 which 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 to 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 accumulation of CD4+ T cells and enhance Th2-mediated eosinophilic airway inflammation in experimental animals (Williams and Galli, 2000; Maezawa et al, 2004) while treatment with the anti-IgE antibody omalizumab reduces eosinophil and lymphocyte numbers in the airway mucosa of patients with asthma (Djukanovic et al, 2004).

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 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 co-stimulation 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). Recent 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 (Satoh 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 be dependent 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 which 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, 2008) in murine models.

The observations linking DP$_2$ to the development of allergic inflammation has spurred interest in identifying more potent, selective and orally bioavailable antagonists of this receptor to treat asthma and related disorders. A number of chemical series have been described that antagonise DP$_2$ 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 OC000459, an indole-1-acetic acid derivative which is a potent and selective DP$_2$ antagonist which, in proof-of-concept Phase IIa clinical trials, has been shown to reduce airway inflammation, improve symptoms and quality of life in subjects with moderate persistent asthma (Barnes et al, 2011) and is currently being evaluated in longer term Phase IIb trials.
Materials and Methods

Materials

[^3H]-PGD$_2$, [^3H]-SQ29548 and [^3H]-PGE$_2$ were purchased from Amersham Biosciences (Amersham, Buckinghamshire, UK).

PGD$_2$, PGF$_{2\alpha}$, iloprost, 5-oxo-eicosatetraenoic acid and PGD$_2$-MOX enzyme immunoassay were purchased from Cayman Chemical (Ann Arbor, Michigan, USA).

Eotaxin (CCL11), complement C5a and IL-13 immunoassay kits were purchased from R and D Systems (Abingdon, UK).

HitHunter™ cAMP II was purchased from DiscoveRx (Fremont, CA, USA).

Calcium-3 dye was purchased from Molecular Devices (Wokingham, Berkshire, UK).

Mono poly resolving medium was purchased from Dainippon Pharmaceuticals (Osaka, Japan). MACS CD4$^+$ isolation, anti-DP$_2$ microbead and T cell expansion kits were purchased from Miltenyi Biotec (Bisley, Surrey, UK).

Cove’s Modified Dulbecco’s Media and X-VIVO 15 medium were purchased from Cambrex BioScience (Wokingham, Berkshire, UK).

Ficoll Hypaque was purchased from Amersham Biosciences (Amersham, Buckinghamshire, UK).

96-well ChemoTx plates were purchased from Neuroprobe (Gaithersburg, MD, USA).

Cytofix buffer was purchased from BD Biosciences (Oxford, Oxon, UK).

PE-annexin V, propidium iodide (PI) and annexin-binding buffer were obtained from Invitrogen.

Pentobarbitone Na (Euthetal) was purchased from Merial Ltd, Harlow Essex, UK.

OC000459 ((5-Fluoro-2-methyl-3-quinolin-2-ylmethylindo-1-yl)-acetic acid) was synthesised by Evotec OAI (Abingdon, Oxfordshire, UK) as described in patent WO-2005044260. The structure of OC000459 is shown in Fig.1.

[^3H]-PGD$_2$ ligand binding assays. Membranes from CHO cells expressing human recombinant DP$_1$ or DP$_2$ were prepared as previously described (Gazi et al, 2005) and membranes from CHO cells expressing rat recombinant DP$_2$ were prepared in a similar manner. Cell membranes (15 µg) were pre-incubated at room temperature with various concentrations of competing ligand in 80 µl HBSS supplemented with 10 mM HEPES, pH 7.3. 20 µl of [^3H]-PGD$_2$ (160 Ci/mmol) was then added to a final concentration of 5 nM and incubated for a further 60 min at room temperature. Reactions were terminated by the addition of 100 µl ice cold assay buffer to each well, followed by rapid filtration through Whatman GF/B glass fibre filters using a Unifilter Cell Harvester (Perkin-Elmer Life Sciences). The filters were washed six times with 300 µl/well of ice cold buffer and the plates dried at room temperature for
at least 60 min. The level of radioactivity retained on the filters was measured using a Beta Trilux counter (Perkin-Elmer Life Sciences) after addition of scintillant. Non-specific binding was defined in the presence of 10 µM unlabelled PGD$_2$. Assays were performed in duplicate.

**Calcium mobilisation in CHO cells expressing human recombinant CRTH2.**
Culture of CHO cells expressing CRTH2 and measurement of calcium mobilisation in response to PGD$_2$ 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 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 the supplementary information using U 44069 as a reference compound (Supplemental Table 1). IP antagonist activity was assessed by measuring inhibition of iloprost-induced cAMP production in intact human platelets and by [³H]-iloprost binding using unlabelled iloprost as a reference compound (Supplemental Table 1). Activity on EP$_1$-EP$_4$ was assessed by measuring displacement of [³H]-PGE$_2$ from CHO cells expressing human recombinant EP receptors. FP antagonist activity was assessed by measuring inhibition of PGF$_{2\alpha}$-induced calcium mobilisation in mouse 3T3 fibroblasts.
Effects of OC000459 on COX activity (Supplemental Table 1) was determined as follows:
Human recombinant COX1 expressed in Sf9 cells was incubated with arachidonic acid (4 µM) for 10 min at 25°C and PGE$_2$ 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 PGE$_2$ measured by EIA with diclofenac included as a reference standard.
Human recombinant COX2 expressed in Sf9 cells was incubated with arachidonic acid (2 µM) for 10 min at 25°C and PGE$_2$ measured by EIA with 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). Briefly, CD34+
progenitor cells from human cord blood were cultured at a density of 1 x 10^5 cells/ml with Iscove’s modified Dulbecco’s medium containing 10% human serum, 0.55 µM 2-mercaptoethanol, penicillin/streptomycin, human recombinant stem cell factor (100 ng/ml) and human recombinant IL-6 (50 ng/ml) in 5% CO_2 at 37°C for 8–10 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 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 pretreated with 5 µg/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 µg/ml) for 2 hr. The cells were washed with medium for 20 min and then incubated with medium or challenged with goat anti-human IgE (1 µg/ml) in the presence or absence of diclofenac (10 µM). The supernatants of the cells were collected 1 h after challenge. The supernatants were assayed for PGD_2 using a PGD_2-MOX enzyme immunoassay kit according to the manufacturer's instructions.

**Culture of human CRTH2+CD4+ Th2 cells.** The study was approved by NHS Oxfordshire Local Research Ethics Committee. Human CRTH2+CD4+ Th2 cells were prepared using a modified method described previously [29]. Briefly, 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 MACS CD4+ T cell isolation kit II. After 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. The harvested CD4+ CRTH2+ cells were treated as Th2 cells and were further amplified by stimulation with the T cell Activation/Expansion Kit 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 2x10^6 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 µm-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 plates were centrifuged at 300 g 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 (FACS) analysis. Background cell migration was determined by measuring the response to media alone.

**Eosinophil shape change.** The study was approved by NHS Oxfordshire Local Research Ethics Committee. The shape change response of human eosinophils to chemoattractants in both isolated leukocyte preparations and in whole blood by gated autofluorescence forward scatter assay measured by a modification of the methods described by Bryan et al (2002). For measurement of responses of isolated leukocytes, heparinised blood was collected from healthy volunteers and incubated with red blood cell lysis buffer for 5 min at room temperature (300 µl of lysis buffer was used for every 100 µl of blood). Samples were then centrifuged at 300×g for 5 min. The supernatant (containing lysed red cells) was removed and leukocytes were resuspended in 50 µl PBS/2mM EDTA. Cells were washed twice by centrifugation at 300×g for 5 min. Leukocytes were resuspended in RPMI/10% FCS. Fifty microliters of cells were added to a 96-well microtitre plate containing 50 µl buffer containing test compounds. The plate was then incubated for 1 h, at 37°C, 5% CO₂. Following that incubation period, the plate was transferred on ice and the cell shape was fixed by addition of 150 µl cytofix buffer. Cell morphology was analysed using FACSCalibur. Eosinophils were gated based on their autofluorescence and 2000 events were counted per sample. Assays were performed in duplicate and were repeated at least three times.

For measurement of shape change responses in whole blood, OC000459 (1µl, 200x final concentration) was added directly to 200µl heparinised whole blood, mixed well and incubated for 15 min at 37°C in 5% CO₂ incubator. Chemoattractant agonists (1µl, 200x final concentration) were added to each sample and incubated for a further 1h at 37°C. After this time, cell shape was fixed by addition of 300µl cytofix buffer (BD biosciences) and incubated for 15 min on ice. 10ml red blood cell lysis buffer was added to the fixed cells, incubated for 5 min at room temperature and centrifuged 300xg, 5 min. Supernatant (containing lysed red blood cells) was removed and the lysis step was repeated. Leukocytes were resuspended in 250µl RPMI/10% FCS and shape change analysed by FACS. Eosinophils were gated based on their autofluorescence, and 2000 eosinophil events were counted per sample. Data were analysed in triplicate.
Cytokine release assays. Th2 cells were treated with X-VIVO 15 culture medium in the presence or the absence of PGD2 or other compounds as indicated in the results at 37°C and 5% CO2 for 5 hr. The supernatants were collected and assayed for IL-13 content by immunoassay according to the manufacturer’s instructions. The results were measured in a Victor2 V-1420 multilabel HTS Counter (PerkinElmer Life Sciences).

Measurement of apoptosis in Th2 cells. Apoptosis of Th2 cells was measured after 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, transferred to annexin-binding buffer, followed by incubation with PE-annexin V/PI at room temperature for 15 min according to the manufacturer’s instructions. The stained cells were analysed by FACSArray flow cytometer. 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. OC00459 was added to plasma samples to final concentrations of 25, 100 and 500 ng/ml and loaded onto duplicate ultracentrifugation devices (Amicon, Centrifree YM-30) and subjected to centrifugation (1,500 x g for 1 hour). The concentration of OC000459 in the plasma and ultrafiltrate were determined by LC-MS/MS as described below.

Animals. Sprague-Dawley rats were supplied by Charles River Ltd (Margate, Kent, United Kingdom). Wistar rats, used in the blood eosinophilia studies, were bred at the University of Hertfordshire (Hatfield, Hertfordshire UK). Female Dunkin Hartley guinea pigs (250-350g) were purchased from Darley Oaks Farm (Newchurch, UK). All animals were housed and all experiments conducted under a project licence granted under the Animals (Scientific Procedures) Act 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. Heparinised blood was collected at various times after dosing and plasma was extracted with methyl tertiary butyl ether (MTBE) after addition of ammonium formate buffer (100 mM, pH4) and
the internal standard ($^{13}$C$_4$-OC000459). Liquid chromatography was carried out with a Prodigy C8, 5 μm, 30 x 4.6 mm Phenomenex analytical column and a Phenomenex C18 4 x 2 guard column. The mobile phase was a 0.1% formic acid in methanol gradient: (80:20>10:90>80:20 over a 5.5 minute run time). The injection volume was 10 μl and flow rate was 1.0 ml/min.

Samples were analysed by LC-MS/MS (Applied Biosystems MDS SCIEX API365, Warrington, UK) with TurboIonspray in the positive ion mode.

**Measurement of DK-PGD$_2$-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. 30 min after oral administration of OC000459 (0.01, 0.1 and 1.0 mg/kg) or vehicle, animals were anaesthetised with isoflurane and received an intracardiac injection of 10mg DK-PGD$_2$ in 0.3ml heparinised (10U/ml) saline. Control animals received an injection of 0.3ml heparinised saline.

60 min after the intracardiac injection, animals were injected with an overdose of pentobarbitone sodium and a blood sample taken (into heparin) by cardiac puncture while the rat was anaesthetised, but not dead.

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

A further aliquot of blood (500ml) was mixed with an equal volume of 4% Dextran (mw 500,000) and the erythrocytes 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 (5min) and Giemsa (15 min) stains. Finally cytospins were washed in phosphate buffer (pH6.8) 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 eosinophils (differential count).

**Measurement of DK-PGD$_2$-induced airway eosinophilia in guinea pigs.** DK-PGD$_2$-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-PGD$_2$, generated from a nebuliser driven by a compressor (DeVilbiss Pulmostar, Sunrise Medical Ltd, W Midlands, UK), for 10 min. At 24h after exposure to aerosols
animals were sacrificed by an overdose of pentobarbitone Na, the trachea was cannulated and the lungs lavaged twice with 5 ml heparinised (10 U/ml) PBS. A cytocentrifuge preparation (Thermo Shandon, cytospin 2) was prepared from an aliquot of the pooled bronchoalveolar lavage (BAL) fluid recovered and stained with May Grunwald Giemsa stain. A total leukocyte count was also made from the BAL fluid using a haemocytometer. The percentage of eosinophils was determined from the cytocentrifuge preparation under oil immersion microscopy and the number of eosinophils/ml blood 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 prior to aerosol exposure.

Data analysis. Data were analysed using the computer program GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA). Concentration–response curves for antagonists in functional assays were analysed by non linear 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 using an F-test). IC50 values of competitors were derived from this analysis and the Ki values (inhibition constants) were calculated using the method of Cheng and Prusoff (1973). The pKb values of antagonist (according to the formula pKb = log(CR−1)− log[B], where Kb 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 analysed 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 3 experiments were pooled and analysed by ANOVA followed by Dunnett’s post-hoc test (GraphPad Prism). A difference between groups was considered significant when p<0.05. In order to calculate ED50 values for OC000459, a non-linear, sigmoidal regression was fitted to the data obtained from animals treated with OC000459 and the ED50 value obtained using GraphPad Prism.
Results

Effect of OC000459 on binding of [³H]-PGD₂ to human DP₂. OC000459 inhibited binding of [³H]-PGD₂ to membranes from CHO cells transfected with human DP₂ with Ki of 0.013±0.002 µM (n=13 independent experiments) as shown in Fig. 2A. OC000459 also displaced [³H]-PGD₂ from membranes from human Th2 lymphocytes (Ki=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 DP₂ (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). Unlabelled 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 mobilisation in intact CHO cells expressing human DP₂. In intact CHO cells expressing DP₂, OC000459 antagonised PGD₂-mediated calcium mobilisation in a concentration dependent manner (IC₅₀=0.028±0.005 µM, n=4 independent experiments) as shown in Fig. 3. OC000459 did not inhibit calcium mobilisation in response to ATP at concentrations up to 10 µM.

Effect of OC000459 on PGD₂-mediated activation of Th2 lymphocytes. OC000459 inhibited chemotaxis of human Th2 cells in response to PGD₂ (10nM) with an IC₅₀ of 0.028±0.006 µM (n=3 independent experiments) (Figure 4A) and inhibited production of interleukin 13 production by Th2 cells in response to PGD₂ (100nM) with an IC₅₀ of 0.019±0.006 µM (n=3 independent experiments) (Fig. 4B, Table 2). OC000459 also inhibited the anti-apoptotic effect of PGD₂ on Th2 cells with an IC₅₀ of 0.035±0.007 µM (n=3 independent experiments) (Fig. 4C, Table 2).

Effect of OC000459 on eosinophil activation in response to PGD₂, interleukin 5, C5a, eotaxin, leukotriene B₄ and 5-oxo-eicosatetraenoic acid. The effect of OC000459 on eosinophil shape change responses was measured by gated autofluorescence forward scatter in both a mixed leukocyte preparation and in whole blood. OC000459 antagonised the effect of PGD₂ competitively both in the isolated leukocyte preparation (Fig. 5A) and in whole blood (Fig. 5B). In isolated leukocytes pK₈ was calculated as 7.9±0.2 (n=3 independent experiments) and in human whole blood potency decreased by 2.5-fold (pK₈ =7. 5±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 PGD2 (Fig. 6). OC000459 also inhibited eosinophil shape change responses to DK-PGD2 (IC50 = 0.011±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 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 % eosinophil shape change in mixed leukocyte preparation compared to 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±0.4 % and 99.8±0.0% bound in human and rat plasma respectively, representing the mean ± SD of 6 measurements.

**Pharmacokinetic profile of OC000459 in the Sprague-Dawley rats.** Plasma concentrations of OC000459 at various times after iv and oral administration of OC000459 in rat are shown in Figure 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 t1/2 of 2.9-3.5h. 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 ~200 and ~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. At steady state the volume of distribution (Vd) was estimated to be 0.5l/kg which is similar to montelukast (0.7l/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) lead to a rapid increase
in blood eosinophil numbers detectable 60 min after injection. Oral administration of OC000459 0.5 h prior to 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 aerosolisation of DK-PGD$_2$.** Exposure of guinea pigs to an aerosol of DK-PGD$_2$ (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 prior to aerosolisation of DK-PGD$_2$ led to a dose-dependent inhibition of eosinophil accumulation (Fig. 10, ED$_{50}$ = 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). Recent studies in DP₂ knockout mice are consistent with 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; Pettipher, 2008; Oiwa et al, 2008; Lukacs et al, 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 indole-acetic 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 to native DP₂ expressed by Th2 cell membranes with high potency but 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 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 mobilisation in intact CHO cells expressing recombinant DP₂ without affecting responses to ATP which stimulates calcium mobilisation 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 (0.5L/kg) suggests that OC000459 is likely to be effective in antagonising 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 selective DP2 agonist DK-PGD2 with an ED50 of 0.04 mg/kg. The plasma concentrations at this ED50 level are 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 nebulisation of the DK-PGD2, illustrating OC00459 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 to blood eosinophilia may reflect lower protein concentration in lung tissue compared to 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 which enable it to inhibit inflammation of the target tissue when dosed by the intended therapeutic route.

We have previously shown that membranes from human 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 BW245C (Xue et al, 2005) suggesting that human Th2 cells express DP2 but not DP1 or TP. OC000459 displaced [3H]-PGD2 with high potency (Ki = 0.004 μM) indicating that it is active on native DP2. OC000459 also inhibited 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 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 to 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) and 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 (FEV₁ 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 Kᵦ, 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 which 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 to treat allergic disorders.
Acknowledgments

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Authorship Contributions

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

*Conducted experiments:* Vinall, Xue, Speight, Townsend, Gazi, and Whelan

*Contributed new reagents or analytical tools:* Speight, Townsend, and Armer

*Performed data analysis:* Pettipher, Vinall, Xue, Speight, Townsend and Whelan

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


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Vinall SL, Townsend ER, and Pettipher R (2007) A paracrine role for chemoattractant receptor-homologous molecule expressed on T helper type 2 cells (CRTH2) in mediating chemotactic activation of CRTH2⁺CD4⁺ T helper type 2 lymphocytes. *Immunology.*


Legends for Figures

**Fig. 1.** Chemical structure of the CRTH2 antagonist OC000459 (((5-Fluoro-2-methyl-3-quinolin-2-ylmethylindo-1-yl)-acetic acid)).

**Fig. 2.** Competition binding analysis of OC000459 to recombinant human DP2 (A) and to 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 in *Materials and Methods*. Data are presented as mean ± S.E.M from a representative experiment performed in triplicate. The mean Ki derived from multiple independent experiments is shown in Table 1.

**Fig. 3.** Effect of OC000459 on PGD₂-mediated calcium mobilisation in intact CHO cells transfected with human recombinant DP₂. Calcium mobilisation 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 ± S.E.M from 4 independent experiments conducted in triplicate. The effect of OC000459 on calcium mobilisation responses to ATP are shown (inserted).

**Fig. 4.** Effect of OC000459 on chemotaxis of Th2 lymphocytes (A), interleukin 13 production by Th2 lymphocytes (B) prevention of apoptosis of Th2 cells (C) in response to PGD₂. Chemotactic responses to PGD₂ (10 nM), interleukin 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 ± S.E.M from a representative experiment performed in triplicate. Mean IC₅₀’s derived from multiple experiments are shown in Table 2.

**Fig. 5.** Effect of OC000459 on PGD₂-mediated eosinophil shape change in a preparation of mixed human leukocytes containing 10% FCS (A) and in heparinised 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 in *Materials and Methods*. Data are presented as mean ± S.E.M from a representative experiment performed in triplicate. The effect of OC000459 was competitive; the pK₉ was 7.9 ± 0.2 in mixed leukocytes and the pK₉ was 7.5± 0.1in human whole blood (mean ± S.E.M from 3 independent experiments).
Fig. 6. Effect of OC000459 on eosinophil shape change induced by PGD$_2$, eotaxin 1, 5-oxo-ETE and C5a in a human mixed leukocyte preparation. OC000459 at a concentration of 1 µM caused significant inhibition of eosinophil responses to PGD$_2$ (*p<0.01) but did not affect responses to eotaxin 1, 5-oxo-ETE or C5a. Results are presented as the mean ± S.E.M from 3 independent experiments.

Fig. 7. Effect of OC000459 on migration of Th2 lymphocytes (A) and eosinophil shape change (B) 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 to 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 supernants compared to mast cell supernatants alone. Results are expressed as mean ± S.E.M (n=3).

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).

Fig. 9. Effect of OC000459 on blood eosinophilia induced by systemic treatment with DK-PGD$_2$ in rats. Blood eosinophilia was measured 60 min after intracardiac injection of DK-PGD$_2$ (10 µg) as described in Materials and Methods. Animals were dosed by oral gavage 30 min prior to injection of DK-PGD$_2$. OC000459 caused a dose-dependent reduction in DK-PGD$_2$-mediated blood eosinophilia (ED$_{50}$ =0.04 mg/kg p.o.). Results are presented as the mean ± S.E.M. (5-6 animals per group). *p<0.05 compared to DK-PGD$_2$ + vehicle (analysed by ANOVA followed by Dunnett’s post-hoc test).

Fig. 10. Effect of OC000459 on airway eosinophilia induced by aerosolised DK-PGD$_2$. The numbers of eosinophils in bronchoalveolar lavages fluids from guinea pigs 24 h after exposure to an aerosol of DK-PGD$_2$ (10 µg/ml) were measured as described in Materials and Methods. Guinea pigs were dosed by oral gavage with vehicle or OC000459 30 min prior to exposure to aerosolised DK-PGD$_2$. OC000459 caused a dose-dependent inhibition of DK-PGD$_2$-mediated airway eosinophilia (ED$_{50}$=0.01 mg/kg po.). Results are presented as the mean ± S.E.M. (6-13 animals per group).
*p<0.05 compared to DK-PGD\(_2\) + vehicle (analysed by ANOVA followed by Dunnett’s post-hoc test).
Table 1:  
Kᵢ Values for OC000459 for DP₂ Binding Compared With Other Prostanoid Receptors

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<td>mean</td>
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<tr>
<td>SEM</td>
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<td>0.001</td>
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Table 2: Potency of OC000459 for inhibition of PGD<sub>2</sub>-mediated activation of Th2 lymphocytes and eosinophils

<table>
<thead>
<tr>
<th></th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; or EC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
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<tbody>
<tr>
<td></td>
<td>Th2 cell chemotaxis</td>
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<td>Mean</td>
<td>0.028</td>
</tr>
<tr>
<td>SEM</td>
<td>0.006</td>
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<td>n</td>
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Table 3. Pharmacokinetic parameters for OC000459 in male Sprague-Dawley rats. Data are presented as the mean from 3 rats.

<table>
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<tr>
<th>Dose</th>
<th>Cmax (ng/ml)</th>
<th>Tmax (h)</th>
<th>t1/2 (h)</th>
<th>CL (ml/kg/min)</th>
<th>AUC_{24h} (ng/ml.h)</th>
<th>Vd (l/kg)</th>
<th>%F</th>
</tr>
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<tbody>
<tr>
<td>2 mg/kg i.v.</td>
<td>5660.4</td>
<td>0.08</td>
<td>1.3</td>
<td>8.4</td>
<td>4486.5</td>
<td>0.5</td>
<td>N/A</td>
</tr>
<tr>
<td>2 mg/kg p.o.</td>
<td>1543.5</td>
<td>1.3</td>
<td>2.9</td>
<td>6.4</td>
<td>5262.5</td>
<td>N/A</td>
<td>111.0</td>
</tr>
<tr>
<td>10 mg/kg p.o.</td>
<td>1443.5</td>
<td>3.3</td>
<td>3.5</td>
<td>9.6</td>
<td>13062.1</td>
<td>N/A</td>
<td>68.2</td>
</tr>
</tbody>
</table>

Cmax: Maximal plasma concentration achieved
Tmax: Time that maximal plasma concentration is achieved
\(t_{1/2}\): Plasma half-life
CL: Clearance
AUC_{24h}: Plasma concentration under the curve to 24h
Vd: Volume of distribution
\%F: % drug orally compared to i.v. dosing
Figure 1
Figure 4

A

Migration of Th2 cells (% control response)

B

IL-13 levels (% Control response)

C

Annexin V positive cells (%)

Log OC000459 (M)
Figure 8

Plasma concentration (ng/ml) vs. Time after dosing (h) for different dosing regimens:
- 2 mg/kg⁻¹ OC000459 i.v.
- 2 mg/kg⁻¹ OC000459 p.o.
- 10 mg/kg⁻¹ OC000459 p.o.
Figure 10

Eosinophils per ml bronchoalveolar lavage fluid ($\times 10^4$)

Control  | DK-PGD$_2$ alone | DK-PGD$_2$ + OC000459 (mg/kg p.o.)
---       |------------------|-----------------------------------
          |                  | *                                |
          |                  | *                                |