Molecular Pharmacology Fast Forward. Published on March 8, 2005 as DOI: 10.1124/mol.104.009068
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MOL 9068

Title Page

Identification of a potent and selective synthetic agonist at the CRTH2 receptor

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Running Title: Synthetic agonist of CRTH2

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Number of text pages: 21

Number of Tables: 2

Number of Figures: 4

Number of references: 20

Number of words in Abstract: 128

Number of words in Introduction: 490

Number of words in Discussion: 695

Non-standard abbreviations:

cAMP, Cyclic adenosine monophosphate; CRTH2, chemoattractant receptor-homologous molecule expressed on T_H2 cells; DK-PGD_2, 13-14-dihydro-15-keto-PGD_2; GPCR, G protein-coupled receptor.

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Abstract

The Chemoattractant Receptor-homologous molecule expressed on T-Helper type 2 cells (CRTH2) is a G-protein-coupled receptor whose function *in vivo* has been incompletely characterized. One of the reasons is that its current known ligands, prostaglandin D_2 (PGD_2) and some of its metabolites, have either poor selectivity for CRTH2 or are metabolically unstable *in vivo*. In this study, we describe the biological and pharmacological properties of L-888,607, the first synthetic potent and selective CRTH2 agonist. We show that L-888,607 exhibits: 1) sub-nanomolar affinity for the human CRTH2 receptor, 2) high selectivity over all other prostanoid receptors and other receptors tested, 3) agonistic activity on recombinant and endogenously expressed CRTH2 receptor, and 4) relative stability *in vivo*. L-888,607 thus represents a suitable tool to investigate the *in vivo* function of CRTH2.

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Introduction

CRTH2 (Chemoattractant Receptor-homologous molecule expressed on T-Helper type 2 cells) is a G-protein-coupled receptor closely related to chemoattractant receptors for the N-formyl peptide, the complement peptides C3a and C5a and the leukotriene LTB4. It was first reported to be selectively expressed on human T-helper type 2 cells (Nagata *et al.*, 1999b). It is also expressed on T-cytotoxic type 2 cells, eosinophils and basophils (Nagata *et al.*, 1999a;Cosmi *et al.*, 2000;Sawyer *et al.*, 2002). CRTH2 mRNA is found in various adult tissues in the digestive tract, heart, thymus, spleen and brain (Sawyer *et al.*, 2002). The roles played by this receptor throughout the body are currently unknown. However, it has been demonstrated that its activation by prostaglandin D_2 (PGD_2) can increase eosinophil, basophil and Th2 cell motility (Gervais *et al.*, 2001;Hirai *et al.*, 2001;Monneret *et al.*, 2001). Activation of CRTH2 has also been shown to modulate eosinophil morphology and degranulation (Gervais *et al.*, 2001), basophil degranulation (Gervais *et al.*, 2001;Yoshimura-Uchiyama *et al.*, 2004) and Th2 cell cytokines secretion (Tanaka *et al.*, 2004). The currently known high affinity ligands for CRTH2 are the agonists PGD_2, some PGD_2 metabolites (13-14-dihydro-15-keto PGD_2 ($DK\text{-}PGD_2$), 15-deoxy-$\Delta^{12,14}$-PGJ_2, PGJ_2, and Δ^{12}-PGJ_2) (Sawyer *et al.*, 2002), and indomethacin (Hirai *et al.*, 2002) and the antagonist ramatroban (Sugimoto *et al.*, 2003). PGD_2 is an arachidonic acid metabolite implicated in a wide range of physiological events including sleep induction, goblet cell depletion, vasodilation, increased microvascular permeability, eosinophil infiltration, smooth muscle relaxation, contraction of the myometrium, inhibition of platelet aggregation, cell survival, control of intraocular pressure and allergic responses (Nagata and Hirai, 2003). PGD_2 binds with equivalent

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affinity to DP and with lower affinities to FP and EP_3, all members of the prostanoid receptor family. Because of the non-selectivity of PGD_2 and its instability *in vivo*, it has been difficult to determine which receptor is responsible for each effect mediated by PGD_2 (Robinson *et al.*, 1989;Liston and Roberts, 1985). The discovery of BW245C, a selective and stable synthetic agonist of DP has allowed the identification of DP-specific effects mediated by PGD_2 such as vasodilation, relaxation of smooth muscle and platelet aggregation (Whittle *et al.*, 1983;Wright *et al.*, 1998). However, a number of PGD_2 functions could not be mimicked by BW245C including increased microvascular permeability, eosinophil infiltration, and goblet cell depletion (Woodward *et al.*, 1990;Fernandes and Crankshaw, 1995). To investigate whether these functions are mediated through the CRTH2, investigators have used the selective agonist DK-PGD_2, a metabolic product of PGD_2. Unfortunately, given the inherent metabolic instability of PGD_2 and some of its metabolic intermediates, one must interpret functional data with caution. Therefore, given that PGD_2 and potentially DK-PGD_2 can be metabolized and that some intermediates may serve as activators of receptors other than the CRTH2 (Bocher *et al.*, 2002), a synthetic, stable CRTH2 ligand is needed to investigate the role of this receptor *in vivo*.

Here, we describe the biological and pharmacological properties of L-888,607, the first synthetic, potent and selective CRTH2 agonist.

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Materials and methods

Chemicals.

L-883,595 ({9-[(4-chlorophenyl)thio]-6-fluoro-2,3-dihydro-1*H*-pyrrolo[1,2-*a*]indol-1-yl}acetic acid, compound A ([9-(4-chlorobenzyl)-2,3-dihydro-1H-pyrrolo[1,2- *a*]indol-1-yl] acetic acid), compound B ([10-(4-chlorobenzyl)-6,7,8,9-tetrahydropyridol[1,2-*a*]indol-9-yl] acetic acid), compound C ([10-(4-chlorophenyl)thio]-6,7,8,9-tetrahydropyridol-[1,2- *a*]indol-9-yl] acetic acid), compound D ({8-bromo-9-[(4-chlorophenyl)thio]-6-fluoro-2,3-dihydro-1H-pyrrolo[1,2- *a*]indol-1-yl] acetic acid), compound E ({9-[(4-chlorophenyl)thio]-8-ethyl-6-fluoro-2,3-dihydro-1H-pyrrolo[1,2-*a*]indol-1-yl] acetic acid), compound F ({9-[(4-chlorophenyl)thio]-8-cyclopropyl-6-fluoro-2,3-dihydro-1H-pyrrolo[1,2- *a*]indol-1-yl] acetic acid), compound G ({9-[(4-chlorophenyl)thio]-8-cyclopentyl-6-fluoro-2,3-dihydro-1H-pyrrolo[1,2- *a*]indol-1-yl] acetic acid), and compound H ({9-[(4-chlorophenyl)thio]-6-cyano-2,3-dihydro-1H-pyrrolo[1,2- *a*]indol-1-yl] acetic acid) are racemic compounds that were synthesized at Merck-Frosst (Montreal, Canada). The two enantiomers L-888,607 and L-888,291 (S- and R-enantiomers, respectively) were isolated from L-883,595. PGD_2, 13-14-dihydro-15-keto-PGD_2 (DK-PGD_2), and BW-245C were purchased from Cayman Chemical.

Radioligand binding assay

Prostanoid receptor binding assays were performed at room temperature in a final volume of 0.2 ml in 10 mM HEPES/KOH pH 7.4 (CRTH2, DP and IP) or 10 mM MES/KOH pH 6.0 (EP subtypes, FP and TP), containing 1 mM EDTA and 10 mM $MnCl_2$ (CRTH2) or 10 mM $MnCl_2$ only (DP, FP, IP and TP) or 1 mM EDTA and 10 mM $MgCl_2$ (EP subtypes)

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and radioligand (0.4 nM [^{3}H]PGD_2, 172 Ci mmol^{-1} for CRTH2 and DP, 0.5 nM [^{3}H]PGE_2, 181 Ci mmol^{-1} for EP subtypes, 0.95 nM [^{3}H]$PGF_{2\alpha}$, 170 Ci mmol^{-1} for FP, 5 nM [^{3}H]iloprost, 16 Ci mmol^{-1} for IP and 1.8 nM [^{3}H]SQ-29548, 46 Ci mmol^{-1} for TP. EP_3 assays also contained 100 μM GTPγS. Competing ligands (from Biomol and Cayman) were diluted in dimethylsulphoxide (DMSO) that was kept constant at 1% (v v^{-1}) of the final incubation volume. Non-specific binding was determined in the presence of 1 μM of the corresponding non-radioactive prostanoid. The reaction was initiated by the addition of membrane proteins from HEK293 (EBNA) cells stably expressing the appropriate receptor (23 μg of membrane proteins for CRTH2, 30 μg for DP and EP1, 20 μg for EP2, 2 μg for EP3, 10 μg for EP4, TP and IP, 60 μg for FP). Incubations were conducted for 60 minutes at room temperature and terminated by rapid filtration through a 96-well Unifilter GF/C (Packard) using a Tomtec MachIII semi-automated harvester. The filters were then washed with 4 ml of the same buffer and residual radioligand bound to the filter was determined by liquid scintillation counting following equilibration in 50 μl Ultima Gold F^{TM} (Unifilter) (Packard) using a 1450 MicroBeta (Wallac).

i [cAMP] measurements

The intracellular concentration of cAMP was determined using the [^{125}I]-cAMP scintillation proximity assay (Amersham) as previously described (Sawyer *et al.*, 2002). Briefly, cells were in Hanks' balanced salt solution containing 25mM HEPES pH 7.4 (HBSS/HEPES). The assay was performed in 0.2 ml HBSS/HEPES containing 5 μM forskolin (Sigma), 100 μM RO 20-1724 (Biomol) and 2 μl of test compound. The reaction was initiated by the addition of 100 000 cells and left to proceed for 10 min at

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37°C. the reaction was stopped by a 3 min incubation in a boiling water bath. The samples were centrifuged for 10 min at 500g and the cAMP content in the supernatant was determined using a [^{125}I]-cAMP scintillation proximity assay (Amersham). All compounds were prepared in DMSO kept constant at 1% (v v^{-1}) of the final incubation volume.

Eosinophil purification

Circulating eosinophils were isolated from heparinized venous blood from healthy volunteers as previously described (Gervais *et al.*, 2001). Briefly, erythrocytes were removed by addition of Dextran-T500 (Pharmacia) and mononuclear cells were removed by means of centrifugation over Ficoll-Paque (Pharmacia). Remaining erythrocytes were lysed by brief incubation in water and the eosinophils were isolated from the granulocyte fraction by negative depletion using immunomagnetic beads directed against CD16 (Miltenyi Biotec). The purity of the eosinophil fraction was evaluated by flow cytometry on a CELL-DYN 3700 System (Abbott Laboratories) on the basis of size, granularity and lobularity. In general, the populations were composed of over 90-95% eosinophils with 5-10% contaminating neutrophils and lymphocytes.

Immunofluorescence Microscopy

Purified eosinophils were resuspended in RPMI-1640 media supplemented with 0.5% (v v^{-1}) FBS (RPMI-FBS). Poly-D-lysine coated culture slides (Becton Dickinson) were seeded at 150,000 cells/well and incubated with test compounds for 20 minutes at 37°C in a humidified atmosphere (6% CO_2). Cells were washed with phosphate-buffered saline

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(PBS) and fixed with ice-cold 70% ethanol for 30 min. The purified eosinophils were stained with anti-actin antibody (Sigma), washed with PBS, and stained with Alexa-594 goat anti-rabbit antibody (Molecular Probes) prior to visualization on an Axioplan2 Fluorescence Microscope (Zeiss).

Eosinophil chemotaxis

Purified eosinophils were resuspended at $3.0x10^6$ cells/ml of RPMI-FBS and 0.1 ml was deposited in the top half of a transwell chamber (6.5-mm Transwell, 3.0 μm polycarbonate membrane from Costar). Test compounds (100 nM of either DK-PGD_2 or L-888,607) or DMSO vehicle were added to 0.6 ml of RPMI-FBS to the bottom chamber to a final vehicle concentration of 0.1% v v^{-1}. After 30 minute in a CO_2 chamber, the upper chamber was removed and the eosinophils that had migrated to the lower chamber were photographed with a 35 mm SLR camera (Contax) mounted on an Axiovert25 microscope (Zeiss). Individual cells were counted and the mean of two chambers was determined for each test condition. Chemotaxis efficiency is expressed as the number of transmigrating cells with the agent, divided by the number of transmigrating cells in presence of vehicle only (fold-increase over background).

Pharmacokinetic profile of L-888607

Male (ICR)BR mice with an average weight of 42 grams were obtained from Charles River Laboratories. All procedures were approved by the Animal Care Committee at Merck-Frosst Canada.

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A single dose (5 mg/kg in 60% (v v^{-1}) PEG 200) of L-888607 was given intravenously via the saphenous vein or a single dose (20 mg/kg in 60% (v v^{-1}) PEG 400) was given orally by gavage. No obvious side effects were observed. n of four in each case. Blood (10 μL) was taken from the jugular vein at each time point indicated and added to to 30 μL of 0.1M aqueous trisodium citrate. The mixture was kept at -20^{o}C until analysis. To the mixture was added 60 μL of acetonitrile and the samples were mixed for 20 sec. prior to centrifugation at 9000 rpm for 20 min. The supernatant was removed and analyzed by LC/MS on na APCI 2000 instrument equipped with a Luna 50X2 mm column 5 μ and using a 10-90% gradient of CH_3CN/20MM NH_4OAc. The LC/MS analysis was done by SIM in negative mode.

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Results

Identification of a synthetic human CRTH2 receptor ligand

L-883,595 a racemic compound with some affinity for the human DP receptor (K_i = 211 nM) was identified in a screen to have a higher affinity for the human CRTH2 (K_i = 4 nM) (see structure in figure 1). The affinity values were determined by equilibrium competition analysis using [^{3}H]-PGD_2 and cell membranes expressing recombinant human DP or CRTH2 receptors. Separation of the two enantiomers yielded: L-888,291 with affinities (K_i) for DP and CRTH2 of 40 and 48 nM, respectively and L-888,607 with affinities of 2331 and 0.8 nM, respectively.

Other analogs of L-883,595 shown in figure 1 were tested for their affinity for the human CRTH2 receptor. Results obtained from these racemic mixtures provided useful information about the structure-activity relationship around the L-883,595 structure. The first evidence was related to the carbocycle fused with the indole ring at position 1 and 2 (see the annotated indole structure at the bottom of figure 1) . The cyclopentyl analog compound A exhibited a 3-fold better affinity for the human CRTH2 receptor (K_i = 80 nM) than the cyclohexyl analog compound B (K_i = 269 nM). The sulfur atom linking the 4-Cl phenyl and the indole groups on the L-883,595 scaffold appeared to be also very important for potency. An example is the replacement of the sulfur atom of compound C (K_i = 6 nM) by a carbon atom as for compound B which led to a compound with an affinity for the human CRTH2 receptor decreased by 45-fold (K_i = 269 nM). Substitution at position 4 of the indole ring did not yield increased affinity compared to the hydrogen atom which confered maximal affinity for CRTH2 (L-883,595 K_i = 3 nM).

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Examples are the substitution by a bromine atom (Compound D, K_i = 21 nM) or by a small alkyl group such as ethyl (Compound E, K_i = 12 nM) or cyclopropyl (Compound F, K_i = 20 nM). These substitutions resulted in compounds with 4 to7-fold lower affinity for the human CRTH2 receptor. The loss of affinity was amplified with larger sustituents such as the cyclopentyl group (Compound G, K_i = 94 nM). Possibilities of substitution were also limited at position 6 of the indole ring. Replacement of the fluorine atom of L-883,595 at this position by larger atoms or groups such as a nitrile group in Compound H led to a less potent compound (K_i = 104 nM) compared to L-883,595.

L-888,607 is a highly selective ligand for CRTH2

The affinity of L-888,607 for all the other recombinant human prostanoid receptors was evaluated by equilibrium competition analysis (Table 1). This analysis revealed that L-888,607 displays a relatively high selectivity for CRTH2 with an affinity 363-fold lower for the TP receptor and more than 1000-fold lower for all the other prostanoid receptors. The rank order of affinity is CRTH2 >> TP > EP3 > DP > EP4 > EP2 > FP > IP > EP1. No significant binding was observed at a concentration up to 10 μM on various chemokine receptors (CCR1, CCR2, CCR4, CCR5, CXCR1, CXCR2 and CXCR3) on the anaphylatoxin receptors (C3aR and C5aR) and on the cyclooxygenases (Cox-1 and Cox-2; data not shown).

L-888,607 is a potent agonist at the human recombinant CRTH2 receptor

In a previous study, we reported that recombinant human CRTH2 is coupled to adenylate cyclase via the PTX-sensitive inhibitory trimeric Gαi/o protein in HEK293

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cells. Activation of CRTH2 on these cells with PGD_2 or its metabolite DK-PGD_2 can reduce forskolin-stimulated cAMP accumulation with a measured potency (EC_{50}) of 1.6±0.3 nM and 4.9±1.1 nM respectively (Sawyer *et al.*, 2002). Substitution of PGD_2 for L-888,607 in this functional assay revealed that this synthetic compound is a full agonist of the hCRTH2 by inhibiting cAMP production with an EC_{50} of 0.5 ± 0.3 nM (Figure 2). BW245C, a selective DP agonist, did not inhibit cAMP production in this assay at concentrations up to 1 μM (data not shown).

L-888,607 triggers eosinophil morphological changes

In order to validate L-888,607 as an agonist on endogenous levels of CRTH2, we evaluated its effects on isolated human eosinophils. We have previously shown that eosinophils undergo rapid morphological changes when incubated with CRTH2-receptor agonists like PGD_2 and DK-PGD_2 (Gervais *et al.*, 2001). Using antibodies directed against actin to better visualize morphological changes of the cells we demonstrated that like PGD_2 and DK-PGD_2, L-888,607 is capable of inducing a morphological response in freshly isolated and purified human eosinophils (Figure 3).

L-888,607 stimulates eosinophil chemotaxis

In order to further confirm that L-888,607 is an agonist on endogenously expressed CRTH2, we evaluated its capacity to stimulate human eosinophil chemotaxis. It was previously reported that PGD_2 and DK-PGD_2 can optimally stimulate eosinophil chemotaxis at a concentration ≥ 100 nM (Hirai *et al.*, 2001;Monneret *et al.*, 2001). We thus placed freshly isolated and purified human eosinophils in the upper compartment of

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a transwell chamber and added either DK-PGD_2 or L-888,607 at 100 nM to the bottom chamber. DK-PGD_2 and L-888,607 significantly stimulated the migration of eosinophils to the bottom chamber when compared to the vehicle control, DMSO (Table 2). The concentration of 100 nM was determined to be optimal in this assay for both DK-PGD_2 and L-888,607 (data not shown). This result thus further confirms the agonistic nature of L-888,607.

Pharmacokinetic profile of L-888,607 in mice

L-888,607 was administered to mice either intravenously (i.v.) or orally (p.o.) to determine its pharmacokinetic profile over an 8 hour period (Figure 4). Following an intravenous administration of 5 mg/kg of the compound, blood analysis revealed a peak level (C_{max}) of 36.1 μM, a half-life (t½) of 2.9 hrs, trough level at 8 hours (C_{8h}) of 3.5 μM and an area under the curve ($AUC_{0\text{-}8h}$) of 87.1 μM. Following oral administration of 20 mg/kg of the compound, blood analysis revealed a peak level (C_{max}) of 31.6 μM, a half-life (t½) of 4 hrs, trough level at 8 hours (C_{8h}) of 15.4 μM, an area under the curve ($AUC_{0\text{-}8h}$) of 166 μM and a bioavailability (F) of 48%.

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Discussion

In this study, we describe the first synthetic compound to selectively bind and activate the PGD_2 receptor, CRTH2. We showed that L-888,607 exhibits: 1) sub-nanomolar affinity for the human CRTH2 receptor, 2) high selectivity over all other prostanoid receptors and some other chemokine and anaphylatoxin receptors, 3) agonistic activity on recombinant and endogenously expressed CRTH2 receptor, and 4) relative stability *in vivo*. L-888,607 thus represents a suitable tool to investigate the *in vivo* function of CRTH2.

The fact that the synthetic ligands L-888,607 and BW245C can selectively bind to CRTH2 and DP respectively indicates that while they share the same natural ligand (PGD_2), their binding pockets are sufficiently different to enable the design of selective ligands. This is consistent with the fact that CRTH2 shares less sequence similarity to DP and other prostanoid receptors than to receptors for leukotrienes and anaphylatoxins.

Another synthetic compound, indomethacin, has previously been reported to be a potent CRTH2 agonist (Hirai *et al.*, 2002). However, the use of indomethacin to study the endogenous function of CRTH2 would be complicated by its well established anti-inflammatory properties through cyclooxygenases inhibition (Barnett *et al.*, 1994). Importantly, we have demonstrated that L-888,607 does not inhibit cyclooxygenases at a concentration up to 10 μM (data not shown). Nevertheless, comparing the chemical structure of L-888,607 with indomethacin (see figure 1) reveals interesting chemical properties of synthetic agonists to CRTH2. Both compounds are indole core molecules having an acetic acid side chain. In addition, both the benzoyl group (from indomethacin) and the phenyl sulfide group (from L-888,607) are para-substituted by a chlorine atom.

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Of particular interest is the relative position of the acetic acid side chain compared to the benzoyl group on indomethacin which occupies the same arrangement in space as the acetic acid side chain and the phenyl sulfide group on L-888,607. This later similarity could explain, in part, a shared affinity of both compounds for the CRTH2 receptor. Interestingly, the CRTH2 antagonist ramatroban is also an indole core molecule which bears an aliphatic carboxylic acid side chain (see figure 1; Sugimoto *et al.*, 2003). In addition, the relative position of the acetic acid side chain could be considered as similar to the side chain position of L-888,607 and indomethacin.

When comparing the L-888,291 and L-888,607 stereoisomers, the stereochemistry at the chiral center bearing the acetic acid group appears to play a central role in determining selectivity toward the CRTH2 and DP receptors. The presence of a carboxylic acid is necessary for potency in both cases. Based on L-888,607's affinity for the CRTH2 receptor, it seems that an R configuration at the chiral center allows the compound to acquire a better conformation to fit the CRTH2 receptor rather than the S configuration, contrary to the DP receptor which prefers the S configuration.

Structure-activity relationship based on close analogs of L-888,607 shows the superiority of compounds with a 5-membered ring carbocyle fused with the indole ring over the six-membered ring analogs. The different orientations provided to the carboxylic acid group by these two class of compounds might explain their different ability to bind with the CRTH2 receptor. The distance between the 4-Cl phenyl group and the indole ring appears to be important. Replacement of the sulphur atom by a smaller spacer as a carbon atom results in significantly less potent compounds. We also showed that position

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4 and 6 on the indole ring are sensitive to steric effects. Steric hindrance induced by large atom or alkyl groups at these positions results in compounds with weaker affinity for the CRTH2 receptor.

We have shown that the pharmacokinetic parameters of L-888,607 are suitable for *in vivo* investigations. In mice, exposure levels of L-888,607 when administered once intraveinously at 5 mg/kg or orally at 20 mg/kg are well above its affinity at the murine CRTH2 receptor (IC_{50} = 18.8 ± 1.7 nM) for a period of at least 8 hours.

In conclusion, we identified L-888,607, a potent, selective and stable CRTH2 agonist that will prove useful in identifying the role of CRTH2 *in vivo* and together with the selective DP agonist BW245C, can be used to distinguish whether some biological activities associated with PGD_2 are mediated through CRTH2 or DP.

Acknowledgements
The authors would like to thank Marc Ouellet and Rino Stocco for the assessment of L-888,607 affinity for the cyclooxygenases and C5aR, respectively.

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Reference List

Barnett J, Chow J, Ives D, Chiou M, Mackenzie R, Osen E, Nguyen B, Tsing S, Bach C, Freire J and . (1994) Purification, Characterization and Selective Inhibition of Human Prostaglandin G/H Synthase 1 and 2 Expressed in the Baculovirus System. *Biochim Biophys Acta* **1209**:130-139.

Bocher V, Pineda-Torra I, Fruchart J C and Staels B (2002) PPARs: Transcription Factors Controlling Lipid and Lipoprotein Metabolism. *Ann N Y Acad Sci* **967**:7-18.

Cosmi L, Annunziato F, Galli M I G, Maggi R M E, Nagata K and Romagnani S (2000) CRTH2 Is the Most Reliable Marker for the Detection of Circulating Human Type 2 Th and Type 2 T Cytotoxic Cells in Health and Disease. *Eur J Immunol* **30**:2972-2979.

Fernandes B and Crankshaw D (1995) Functional Characterization of the Prostanoid DP Receptor in Human Myometrium. *Eur J Pharmacol* **283**:73-81.

Gervais FG, Cruz R P, Chateauneuf A, Gale S, Sawyer N, Nantel F, Metters K M and O'Neill G P (2001) Selective Modulation of Chemokinesis, Degranulation, and Apoptosis in Eosinophils Through the PGD2 Receptors CRTH2 and DP. *J Allergy Clin Immunol* **108**:982-8.

Hirai H, Tanaka K, Takano S, Ichimasa M, Nakamura M and Nagata K (2002) Cutting Edge: Agonistic Effect of Indomethacin on a Prostaglandin D2 Receptor, CRTH2. *J Immunol* **168**:981-985.

Hirai H, Tanaka K, Yoshie O, Ogawa K, Kenmotsu K, Takamori Y, Ichimasa M, Sugamura K, M N, Takano S and Nagata K (2001) Prostaglandin D2 Selectively Induces Chemotaxis in T Helper Type 2 Cells, Eosinophils, and Basophils Via Seven-Transmembrane Receptor CRTH2. *J Exp Med* **193**:255-61.

Liston TE and Roberts L J (1985) Metabolic Fate of Radiolabeled Prostaglandin D2 in a Normal Human Male Volunteer. *J Biol Chem* **260**:13172-13180.

Monneret G, Gravel S, Diamond M, Rokach J and Powell W S (2001) Prostaglandin D2 Is a Potent Chemoattractant for Human Eosinophils That Acts Via a Novel DP Receptor. *Blood* **98**:1942-8.

Nagata K and Hirai H (2003) The Second PGD(2) Receptor CRTH2: Structure, Properties, and Functions in Leukocytes. *Prostaglandins Leukot Essent Fatty Acids* **69**: 169-177.

Nagata K, Hirai H, Tanaka K, Ogawa K, Aso T, Sugamura K, Nakamura M and Takano S (1999a) CRTH2, an Orphan Receptor of T-Helper-2-Cells, Is Expressed on Basophils and Eosinophils and Responds to Mast Cell-Derived Factor(s). *FEBS Lett* **459**:195-9.

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Nagata K, Tanaka K, Ogawa K, Kemmotsu K, Imai T, Yoshie O, Abe H, Tada K, Nakamura M, Sugamura K and Takano S (1999b) Selective Expression of a Novel Surface Molecule by Human Th2 Cells in Vivo. *J Immunol* **162**:1278-86.

Robinson C, Herbert C A, Bedwell S, Shell D J and Holgate S T (1989) The Metabolism of Prostaglandin D2. Evidence for the Sequential Conversion by NADPH and NAD+ Dependent Pathways. *Biochem Pharmacol* **38**:3267-3271.

Sawyer N, Cauchon E, Chateauneuf A, Cruz R P, Nicholson D W, Metters K M, O'Neill G P and Gervais F G (2002) Molecular Pharmacology of the Human Prostaglandin D(2) Receptor, CRTH2. *Br J Pharmacol* **137**:1163-1172.

Sugimoto H, Shichijo M, Iino T, Manabe Y, Watanabe A, Shimazaki M, Gantner F and Bacon KB (2003) An orally bioavailable small molecule antagonist of CRTH2, ramatroban (BAY u3405), inhibits prostaglandin D2-induced eosinophil migration in-vitro. *J Pharmacol Exp Ther* **305**:347-352

Tanaka K, Hirai H, Takano S, Nakamura M and Nagata K (2004) Effects of Prostaglandin D2 on Helper T Cell Functions. *Biochem Biophys Res Comm* **316**:1009-1014.

Whittle BJ, Moncada S, Mullane K and Vane J R (1983) Platelet and Cardiovascular Activity of the Hydantoin BW245C, a Potent Prostaglandin Analogue. *Prostaglandins* **25**:205-223.

Woodward DF, Hawley S B, Williams L S, Ralston T R, Protzman C E, Spada C S and Nieves A L (1990) Studies on the Ocular Pharmacology of Prostaglandin D2. *Invest Ophthalmol Vis Sci* **31**:138-146.

Wright DH, Metters K M, Abramovitz M and Ford-Hutchinson A W (1998) Characterization of the Recombinant Human Prostanoid DP Receptor and Identification of L-644,698, a Novel Selective DP Agonist. *Br J Pharmacol* **123**:1317-24.

Yoshimura-Uchiyama C, Iikura M, Yamaguchi M, Nagase H, Ishii A, Matsushima K, Yamamoto K, Shichijo M, Bacon K B and Hirai K (2004) Differential Modulation of Human Basophil Functions Through Prostaglandin D2 Receptors DP and CRTH2/DP2. *Clin Exp Allergy* **34**:1283-1290.

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Footnote

J.P.M. is supported by an Industrial Research Fellowship from the National Research Council of Canada

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Legends for figures

Figure 1. Chemical structure of indomethacin, ramatroban, the racemic compound L-888,595 and its two enantiomers, L-888,607 and L-888,291 as well as racemic analogs of L-883,595.

Figure 2. L-888,607 is a full agonist at the recombinant hCRTH2. L-888,607, PGD_2 and DK-PGD_2 dose-response curve of the inhibition of forskolin-stimulated i[cAMP] levels in HEK293 cells stably expressing the hCRTH2. The potency (EC_{50}) of L-888,607, PGD_2 and DK-PGD_2 in the experiment shown were 0.4, 1.3 and 1.8 nM, respectively. The curves shown are representative of at least four independent experiments where each data point was done in duplicate.

Figure 3. L-888,607 induces changes in eosinophil morphology. Purified human eosinophils were incubated with vehicle (A), or 100 nM of the CRTH2 agonists PGD_2 (B), DK-PGD_2 (C), and L-888,607 (D). Cells are stained with an anti-actin antibody coupled to an Alexa-594 fluorescent antibody and visualized using a Fluorescence microscope. A representative experiment from 3 donors tested is shown. Original magnification, x200.

Figure 4. Pharmacokinetic profile of L-888,607 in mice receiving 5 mg/kg intravenously or 20 mg/kg orally. Each point corresponds to the average obtained from four mice.

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Table I. Affinities of L-888607, PGD_2 and DK-PGD2 for the recombinant human prostanoids receptors

	K_i (nM)								
Compound	*CRTH2*	*DP*	*TP*	*EP_1*	*EP_2*	*$EP_{3\text{-}III}$*	*EP_4*	*FP*	*IP*
L-888,607	0.8±0.4 (5)	2331±947 (7)	283±52 (7)	>28671 (7)	8748±2571 (5)	1260±211 (5)	4634±1903 (5)	10018±2907 (5)	14434±5405 (3)
PGD_2	1.7±0.8 (30)	1.7±0.8 (47)	2024±1091 (19)	5302±2768 (7)	2390±1175 (4)	421±60 (3)	1116±782 (4)	11.5±6.5 (4)	>100000 (8)
DK-PGD_2	2.5±0.9 (15)	18522 (2)	30882±2747 (3)	>95150 (2)	9059.5 (2)	1268 (2)	3915 (2)	3915 (2)	72000 (2)
Indomethacin	25.0±3.6 (4)*	10539±4329 (3)*							

Radioligand binding assays were conducted as described in Methods. Ki values were determined as previously described [Sawyer et al., 2002].
The results are expressed as Mean± Standard Deviation (SD) with the number of determinations in parenthesis. * From Sawyer et al., 2002.

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Table 2. Chemotactic potential of DK-PGD_2 and L-888,607 on human eosinophils.

	donor 1		donor 2		average of 6 donors
	No. migrating cells	fold-increase vs DMSO ctrl	No. migrating cells	fold-increase vs DMSO ctrl	fold-increase vs DMSO ctrl
DMSO	890	-	1168	-	-
DK-PGD_2[†]	5676	6.4	5371	4.6	**5.1 ± 0.7**[*]
L-888,607[†]	4274	4.8	5969	5.1	**4.0 ± 0.4**[*]

[†] = the final concentration of L-888,607 and DK-PGD_2 was 100 nM
[*] = standard deviation

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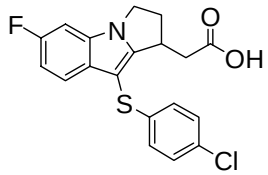
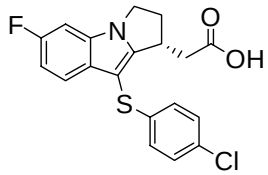
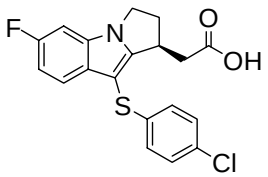
Figure 1.



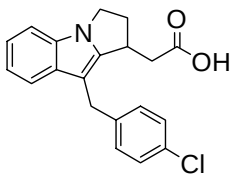
L-883,595
(4 ± 1 nM)



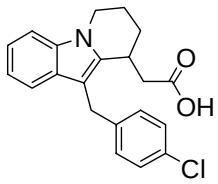
L-888,291
(48 ± 6 nM)



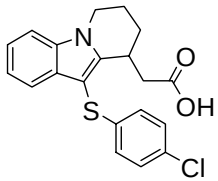
L-888,607
(0.8 ± 0.4 nM)



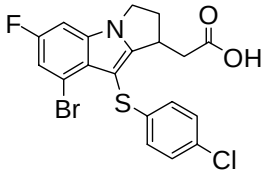
Compound A
(80 ± 25 nM)



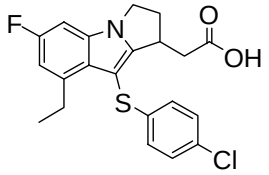
Compound B
(269 ± 69 nM)



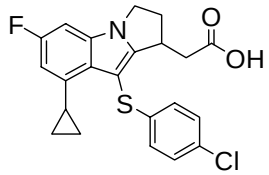
Compound C
(6 ± 2 nM)



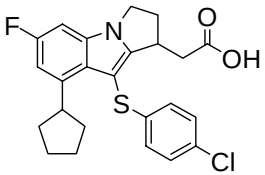
Compound D
(21 ± 6 nM)



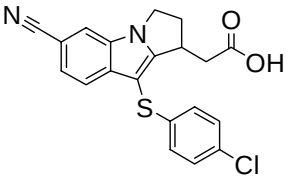
Compound E
(12 ± 8 nM)



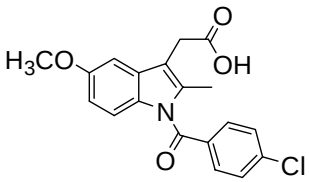
Compound F
(20 ± 12 nM)



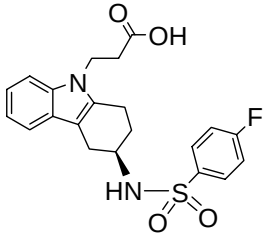
Compound G
(94 ± 32 nM)



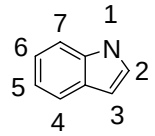
Compound H
(104 ± 10 nM)



Indomethacin
(25 ± 4 nM)



Ramatroban



Indole structure

* values in parenthesis = K_i value at CRTH2 ± standard deviation; n ≥ 3

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Figure 2.

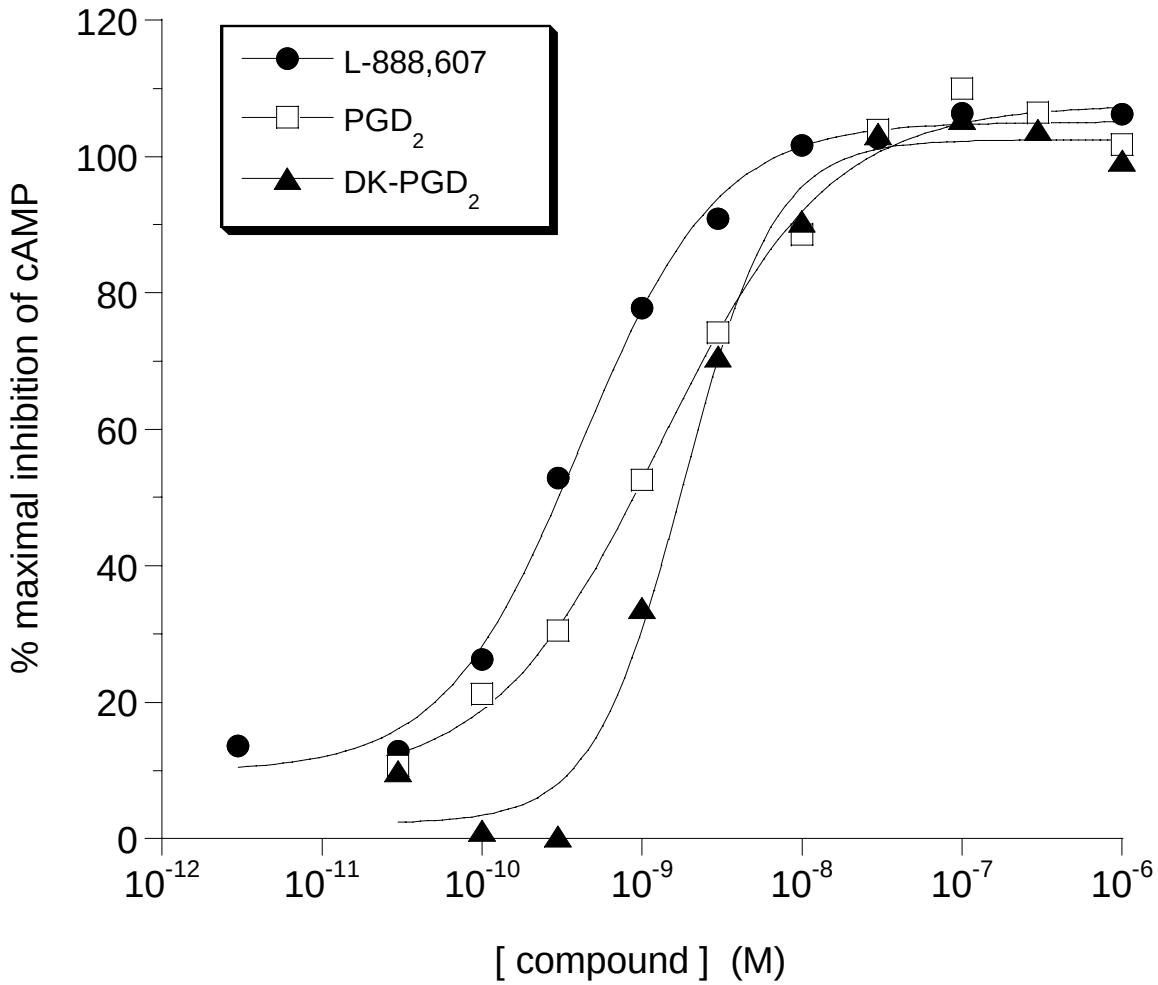

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Figure 3.

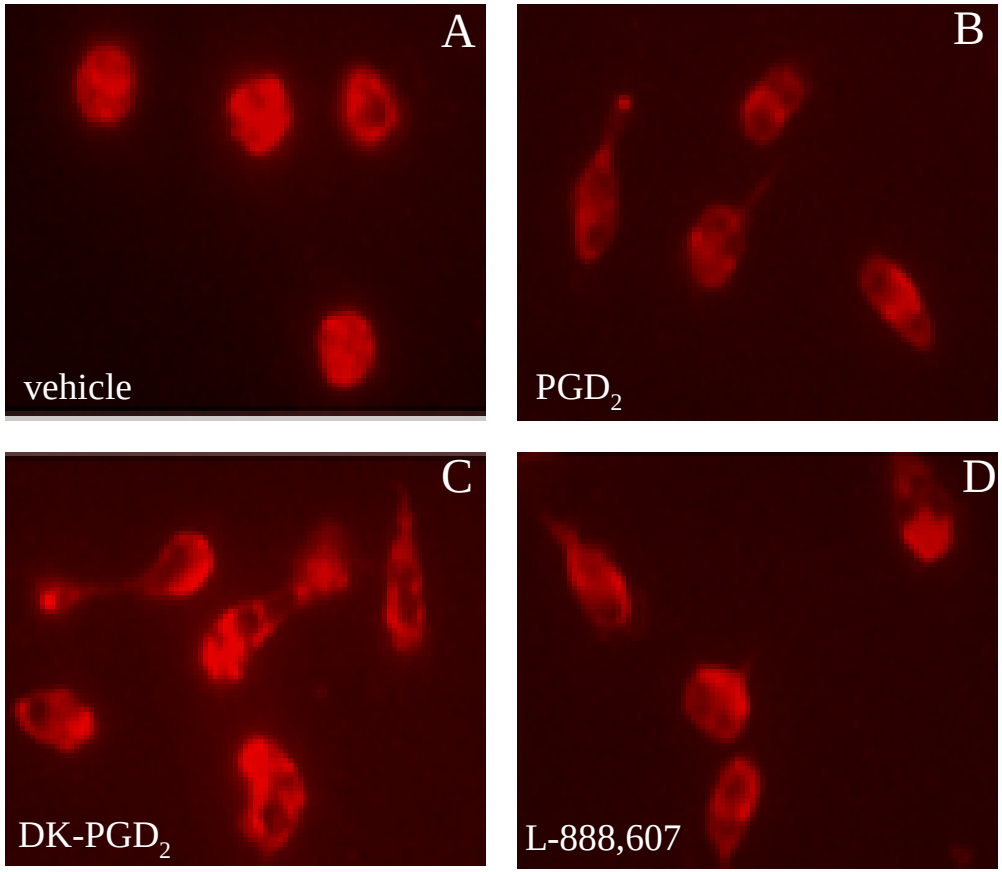

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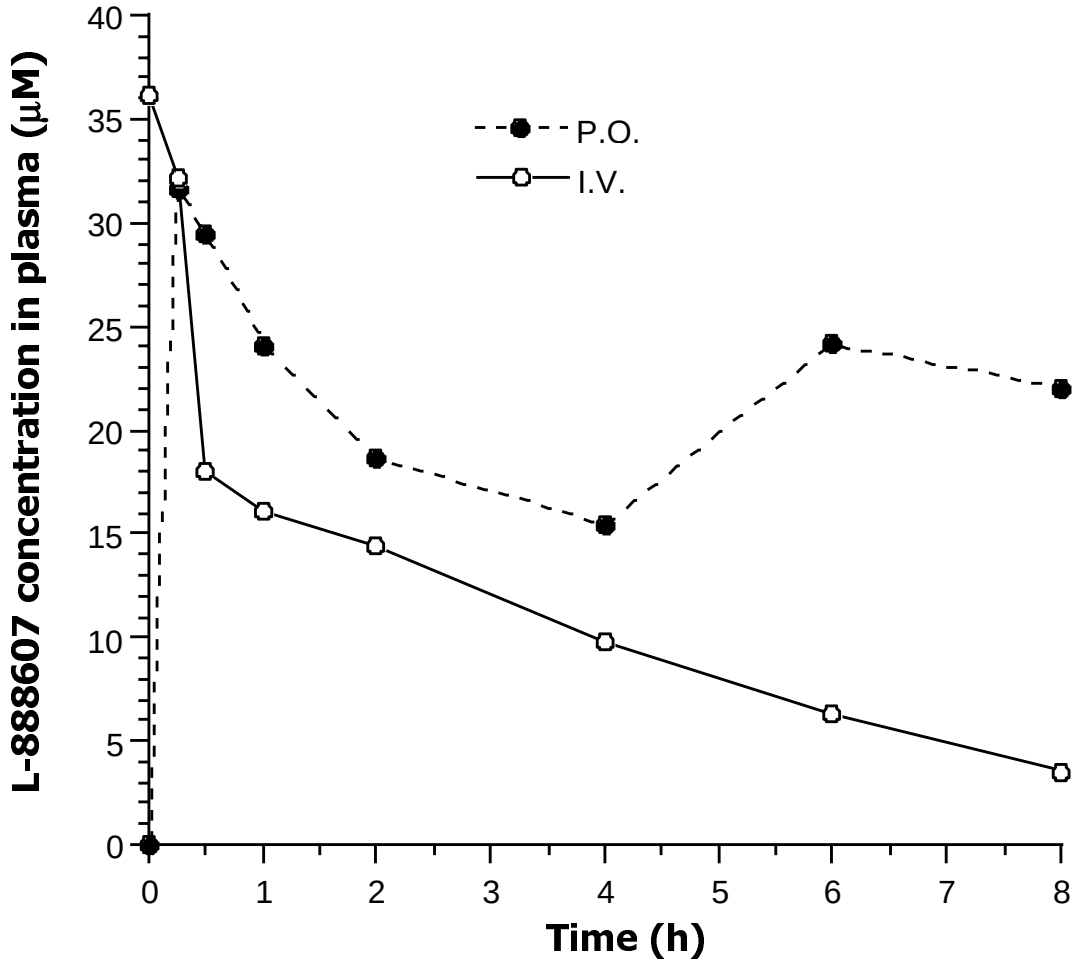
Figure 4.



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