An Orally Bioavailable Small Molecule Antagonist of CRTH2, Ramatroban (BAY u3405), Inhibits Prostaglandin D2-Induced Eosinophil Migration in Vitro

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ABSTRACT

Ramatroban (Baynas, BAY u3405), a thromboxane A2 (TxA2) antagonist marketed for allergic rhinitis, has been shown to partially attenuate prostaglandin (PG)D2-induced bronchial hyperresponsiveness in humans, as well as reduce antigen-induced early- and late-phase inflammatory responses in mice, guinea pigs, and rats. PGD2 is known to induce eosinophilia following intranasal administration, and to induce eosinophil activation in vitro. In addition to the TxA2 receptor, PGD2 is known as a ligand for the PGD2 receptor, and the newly identified G-protein-coupled chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2). To fully characterize PGD2-mediated inflammatory responses relevant to eosinophil activation, further analysis of the mechanism of action of ramatroban has now been performed. PGD2-stimulated human eosinophil migration was shown to be mediated exclusively through activation of CRTH2, and surprisingly, these effects were completely inhibited by ramatroban. This is also the first report detailing an orally bioavailable small molecule CRTH2 antagonist. Our findings suggest that clinical efficacy of ramatroban may be in part mediated through its action on this Th2-, eosinophil-, and basophil-specific chemoattractant receptor.

Ramatroban (Baynas, BAY u3405; (+)-(3R)-3-(4-fluorobenzensulfonamido)-1,2,3,4-tetrahydrocarbazole-9-propionic acid), marketed in Japan for allergic rhinitis, has been characterized as a selective thromboxane-type prostanoid (TP) receptor antagonist and has been reported to partially attenuate prostaglandin (PG)D2-induced bronchial hyperresponsiveness in humans, as well as reduce antigen-induced early- and late-phase inflammatory responses in mice, guinea pigs, and rats. PGD2 is known to induce eosinophilia following intranasal administration, and to induce eosinophil activation in vitro. In addition to the TxA2 receptor, PGD2 is known as a ligand for the PGD2 receptor, and the newly identified G-protein-coupled chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2). To fully characterize PGD2-mediated inflammatory responses relevant to eosinophil activation, further analysis of the mechanism of action of ramatroban has now been performed. PGD2-stimulated human eosinophil migration was shown to be mediated exclusively through activation of CRTH2, and surprisingly, these effects were completely inhibited by ramatroban. This is also the first report detailing an orally bioavailable small molecule CRTH2 antagonist. Our findings suggest that clinical efficacy of ramatroban may be in part mediated through its action on this Th2-, eosinophil-, and basophil-specific chemoattractant receptor.

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ABBREVIATIONS: BAY u3405, ramatroban; TP, thromboxane-type prostanoid; TxA2, thromboxane A2; PG, prostaglandin; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; Ig, immunoglobulin; DP, PGD2 receptor; PCR, polymerase chain reaction; BSA, bovine serum albumin; PTX, pertussis toxin; RANTES, regulated on activation normal T cell expressed and secreted; ANOVA, analysis of variance.
be the blockade of the chemotactic reaction itself. Various chemoattractants are known for eosinophils (eotaxin, eotaxin-2, MCP-3 and -4, RANTES, leukotriene D4, C5a, platelet-activating factor, and PGD2 (Fukuda et al., 1992; Jose et al., 1994; Elsner et al., 1996; Hirai et al., 2001), all of which are known to be produced or present in elevated amounts in allergen-challenged nasal areas of rhinitis patients and lungs of asthmatics. Although no evidence exists for a direct interaction of ramatroban with chemokine receptors (unpublished observations), the blockade of PGD2-mediated eosinophilia andbronchoconstriction by ramatroban is well documented in animals and humans (Johnston et al., 1992; Magnussen et al., 1992; Nagai et al., 1995; Narita et al., 1996; Rajakulasingam et al., 1996).

PGD2 is an agonist for TP (Coleman et al., 1989). However, it also specifically binds to two other receptors, PGD2 receptor (DP) and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) (Hirai et al., 2001), the latter two of which, in contrast to TP, have been identified on human eosinophils. CRTH2 was cloned as a Th2-specific marker by differential display (Nagata et al., 1999). It was also clarified that CRTH2 was expressed not only on Th2 cells, but also on eosinophils and basophils, and induced their migration (Hirai et al., 2001). Gervais et al. (2001) also demonstrated that PGD2 could induce degranulation of eosinophils via CRTH2 stimulation. These reports strongly suggest a critical role of PGD2 and CRTH2 in allergic diseases.

In the present study, we therefore examined the effect of ramatroban on PGD2-induced CRTH2 activation, using CRTH2 transfectants and peripheral blood eosinophils. We demonstrate that ramatroban is an antagonist for CRTH2, and inhibits PGD2-induced migration of eosinophils via CRTH2 blockade. In addition and in accordance with data published recently by others (Hirai et al., 2001), we show that PGD2-mediated eosinophil migration is solely dependent on CRTH2 agonism as evidenced by the lack of efficacy of a DP-selective antagonist (BWA868C).

**Materials and Methods**

**Reagents.** Ramatroban was synthesized at Bayer Yakuhin Ltd. (Shiga, Japan). BWA868C was synthesized by Sogo Pharmaceutical Co. Ltd. (Tokyo, Japan; http://www.sogo-pharma.co.jp/index.html). U-46619 was obtained from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA), PGD2 from Sigma-Aldrich (St. Louis, MO), and [3H]PGD2 was purchased from Amersham Biosciences Ltd. UK (Little Chalfont, Buckinghamshire, UK). Sodium butyrate was purchased from Wako Pure Chemicals (Osaka, Japan). Fluo-3/AM and pluronic F-127 were purchased from Molecular Probes (Eugene, OR). Anti-human CRTH2 monoclonal antibody (clone BM16, rat IgG2a) was provided by BML, Inc. (Saitama, Japan); and fluorescein isothiocyanate-conjugated rat IgG2a (for isotype control) and anti-rat IgG2a antibodies were purchased from PharMingen (San Diego, CA). Ramatroban, BWA868C, U-46619, and PGD2 were dissolved in dimethyl sulfoxide (Nacalai Tesque, Inc., Kyoto, Japan). As confirmed in preliminary experiments, the concentrations of dimethyl sulfoxide in working dilutions used in this study (<0.1%) had no effect on receptor binding, Ca2+ mobilization, and eosinophil migration assays.

**Cloning of Human CRTH2.** Peripheral blood was collected from healthy volunteers and the polymorphonuclear fraction purified on a Mono-Poly Resolving Medium (ICN Biomedicals Inc., Costa Mesa, CA). Under standard conditions according to manufacturer’s instruction, eosinophils were isolated by negative selection following removal of neutrophils using anti-CD16 MACS beads (Miltenyi GmbH, Bergisch-Gladbach, Germany). The purity of isolated eosinophils was more than 95% as assessed by Diff-quick staining (International Reagents, Kobe, Japan). Messenger RNA from eosinophils was isolated by extraction in Trizol (Invitrogen, Carlsbad, CA). First-strand cDNA was then synthesized with the SUPERSCRIPTTM first-strand synthesis system (Invitrogen). Cloning of the coding region of CRTH2 was performed by PCR using two primer pairs, designed from the reported CRTH2 sequence (GenBank accession no. AB008535). The primer sequences used were 5′-AATTAGCTTCA-GAGGCCCAGACGATGCGGCC and 5′-AATGAAATCTAATCGAGTGTGCTGTCACG. PCR was carried out with KOD Plus polymerase (Toyobo, Osaka, Japan) under the following parameters: 15 s at 94°C, 30 s at 60°C, and 90 s at 68°C for 35 cycles. PCR products obtained were cloned into pCRII-TOPO (Invitrogen) for sequencing and subcloned into pEAK vector (Edge Biosystems, Gaithersburg, MD) for expression. Clones were cycle-sequenced using the ABI Prism dye terminator cycle sequencing reaction kit (Applied Biosystems, Foster City, CA), and the sequence was analyzed on an ABI Prism 377 sequencing system (Applied Biosystems).

**Receptor Binding Assay.** CRTH2 transfectants were resuspended in HEPES buffered saline containing 1% BSA, 0.1% NaN3. Cell suspension (2 × 10⁷ cells/ml), [3H]-labeled PGD2, and various concentrations of ramatroban were then mixed in a 96-well U-bottomed polystyrene plate and incubated in a final volume of 100 μl for 60 min at room temperature. After incubation, the cell suspension was transferred to a filtration plate (MAFB; MPM, Co. Ltd. Tokyo, Japan) at 37°C for 2 h before start of the experiment.

**Receptor Binding Assay.** Ca2+ mobilization was measured by mixing 1 μM Fluo-3/AM and Fluronic F-127 in Ca2+ assay buffer (20 mM HEPES, pH 7.6, 0.1% BSA, 1 mM probenecid, Hanks’ solution). The CRTH2 transfectants established were resuspended in Ca2+ assay buffer at 1 × 10⁶ cells/ml and incubated for 60 min at room temperature. After the incubation, cells were washed and resuspended in Ca2+ assay buffer, then dispensed into transparent-bottomed 96-well plates (3631; Costar, Corning, NY) at 2 × 10⁶ cells/well. Cells were incubated with various concentrations of ramatroban for 5 min at room temperature. The emitted 480-nm fluorescence was measured on a FDS8600 fluorimeter (Hamamatsu Photonics, Hamamatsu, Japan). For inactivation of Gαs proteins, cells were incubated with 1 μg/ml pertussis toxin (Sigma-Aldrich) at 37°C for 2 h before start of the experiment.

**FACS Analysis of CRTH2 Expression.** Cell surface expression of CRTH2 on transfected cells and eosinophils was determined according to standard protocols. CRTH2-transfected L1.2 cells, wild-type L1.2 cells, and purified eosinophils were incubated with anti-human CRTH2 monoclonal antibody for 20 min in the cold phosphate-buffered saline containing 1% bovine serum albumin and 0.01% sodium azide. After washing, cells were incubated with fluorescein isothiocyanate-conjugated anti-rat IgG2a for 20 min before analysis by FACS (BD Biosciences, San Jose, CA). Rat IgG2a was used as a control.
Migration Assays. Human eosinophils were purified as described above and resuspended in migration buffer (20 mM HEPES, pH 7.6, 0.1% BSA, Hanks’ solution) at a density of $6 \times 10^6$ cells/ml. Fifty microliters of the cell suspension ($3 \times 10^5$ cells/well) was then dispensed into the upper chamber of a 96-well type chemotaxis chamber (pore diameter = 5 μm, 106-5; Neuro Probe, Gaithersburg, MD), and 30 μl of ligand solution was added to the lower chamber. Cells were preincubated with various concentrations of ramatroban or BWA868C at 37°C for 10 min. The migration assays were performed in a humidified incubator at 37°C, 5% CO2 for 2 h. The number of cells migrating into the lower chamber was counted by FACScan, as described previously (Palframan et al., 1998).

Statistics. Statistical analysis was performed using ANOVA for concentration-response studies of ligands (compared with controls without ligand) and Student’s t test for drug evaluations (compared with controls without drug); p values < 0.05 were considered as statistically significant (*p < 0.05, **p < 0.01).

Results

Ramatroban Antagonizes PGD2 Binding to CRTH2 Transfectants. Analysis of the binding of $^3$H-labeled PGD2 to CRTH2 and Scatchard transformation is shown in Fig. 1A. $^3$H-labeled PGD2 bound to a single site on CRTH2 transfectants with high affinity ($K_D = 6.3$ nM, $B_{max} = 450$ pM). Nonlabeled PGD2 inhibited the binding of $^3$H-labeled PGD2 to CRTH2 transfectants in a concentration-dependent manner with an $EC_{50}$ value of 2.7 nM (Fig. 1B). Ramatroban showed significant inhibitory effects on the binding of $^3$H-labeled PGD2 to CRTH2, albeit with much lower potency ($IC_{50} = 100$ nM, Fig. 1C).

Effects of Ramatroban on Ca$^{2+}$ Mobilization in CRTH2 and DP Transfectants. To determine the functional expression of CRTH2 and DP on each transfectant, calcium mobilization after PGD2 stimulation was monitored. PGD2 stimulated Ca$^{2+}$ mobilization in CRTH2-L1.2 transfectants (Fig. 2A) and DP-Chinese hamster ovary transfectants (Fig. 3A) in a concentration-dependent manner with $EC_{50}$ values of 15 and 150 nM, respectively. U-46619 (TxA2 mimetic) failed to induce Ca$^{2+}$ mobilization in either transfectant (Figs. 2A and 3A). As expected for a Gs-coupled receptor, PGD2 (10 nM)-induced Ca$^{2+}$ mobilization in CRTH2 transfectants was completely suppressed by pretreatment of cells with the Gs inhibitor pertussis toxin (PTX; Fig. 2A). Ramatroban and indomethacin also inhibited PGD2-induced Ca$^{2+}$ mobilization in CRTH2 transfectants to almost the same extent with an $IC_{50}$ value of 30 nM (Fig. 2B). However, indomethacin but not ramatroban was confirmed as an agonist of Ca$^{2+}$ mobilization at concentrations greater than 10 nM (Hirai et al., 2002; Fig. 2C). As expected, PGD2-induced Ca$^{2+}$ mobilization in DP transfectants was not inhibited by PTX since DP is coupled directly to Gs-mediated adenylate cyclase activation (Hirata et al., 1994; Fig. 3A). In addition, ramatroban was ineffective at concentrations up to 10 μM, suggesting that it is not a direct antagonist of DP (Fig. 3B).

Effects of Ramatroban on PGD2-Mediated Migration of Human Eosinophils. It is known that eosinophils express DP and CRTH2 receptors. Analysis of receptor expression on human eosinophils in this study revealed high expression of cell surface CRTH2, comparable with CRTH2 levels found on transfected L1.2 cells (Fig. 4A). PGD2, but not U-46619, induced migration of human eosinophils (Fig. 4B) that peaked at 100 nM and was completely suppressed by 1 μg/ml PTX pretreatment. As shown in Fig. 4C, ramatroban completely inhibited the PGD2-induced migration of eosinophils in a concentration-dependent manner with an $IC_{50}$ value of 170 nM. To determine the relative contributions of DP and CRTH2 on PGD2-induced eosinophil migration, the inhibitory effect of a DP-selective antagonist, BWA868C, was evaluated. BWA868C completely suppressed PGD2-induced calcium mobilization in DP transfectants with an $IC_{50}$ value of 32 nM (Fig. 3B), whereas it only partially affected Ca$^{2+}$ mobilization in CRTH2 transfectants at 10 μM (Fig. 2B). BWA868C also slightly inhibited PGD2-induced migration of eosinophils at 10 μM (39% inhibition), but this effect did not reach statistical significance (Fig. 4C). Since only partial inhibition at the highest concentration of BWA868C was seen in CRTH2 transfectants, the effect on eosinophil migration might be nonspecific.

Discussion

Ramatroban is known as a TP antagonist (McKenniff et al., 1991), and TxA2 and PGD2 are known ligands for TP (Seuter et al., 1989). PGD2 has been shown to bind to DP and CRTH2 with relatively similar affinities (45 and 61 nM, respectively) (Hirai et al., 2001), whereas TxA2 does not bind either recep-

![Fig. 1](image-url)
tor. Surprisingly, our study using G-protein-coupled receptor-transfected cells has revealed a 10-fold higher affinity of the PGD₂/CRTH2 interaction compared with the interaction of PGD₂ with DP. The reason for this is unclear, and we are currently investigating the expression of CRTH2 and DP in different host backgrounds. McKenniff et al. (1991) showed

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**Fig. 2.** PGD₂-induced Ca²⁺ mobilization in CRTH2 transfectants, and the effect of ramatroban, indomethacin, and BWA868C on this response. A, dose-response of Ca²⁺ mobilization in CRTH2 transfectants induced by PGD₂ and U-46619 (n = 3), and the effect of PTX on PGD₂-induced responses (n = 3). B, effects of ramatroban (n = 6), indomethacin (n = 3), and BWA868C (n = 3) on PGD₂ (10 nM)-induced Ca²⁺ mobilization in CRTH2 transfectants. C, agonistic effect of ramatroban (n = 6), indomethacin (n = 3), and PGD₂ (n = 3) on Ca²⁺ flux in CRTH2 transfectants. Significant difference between ligand-treated and untreated Ca²⁺ mobilization (A, C) was analyzed by ANOVA, *, p < 0.05, **, p < 0.01, and between drug-treated and -untreated Ca²⁺ mobilization (B) was analyzed by Student’s t test: ***, p < 0.01.

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**Fig. 3.** PGD₂-induced Ca²⁺ mobilization in DP transfectants, and the effect of ramatroban and BWA868C on this response. A, dose-response of Ca²⁺ mobilization in DP transfectants induced by PGD₂ (n = 3) and U-46619 (n = 1), and the effect of PTX on PGD₂-induced responses (n = 3). B, effects of ramatroban (n = 6) and BWA868C (n = 3) on PGD₂ (10 nM)-induced Ca²⁺ mobilization in DP transfectants. Significant difference between ligand-treated and untreated Ca²⁺ mobilization (A) was analyzed by ANOVA, *, p < 0.05, and between drug-treated and -untreated Ca²⁺ mobilization (B) was analyzed by Student’s t test: *, p < 0.05, **, p < 0.01.
selective antagonism of ramatroban at TP but not at PGE2 receptors (EP1 and EP2), PGF2 receptor (FP), and PGI2 receptor (IP). In the present study, we clearly demonstrated that ramatroban also antagonizes CRTH2 by inhibiting PGD2 binding and PGD2-mediated functions. The potency of CRTH2 blockade (the IC50 values for the inhibition of receptor binding, Ca2+ mobilization and migration of eosinophils were 100, 30, and 170 nM, respectively) was better than that for TP antagonism reported previously (the IC50 values for the inhibition of platelet aggregation induced by collagen, arachidonic acid and U-46619 in human plasma are 65, 160, and 700 nM, respectively) (Lewis et al., 1982). The Cmax value (1.83 h) of ramatroban in blood when a 75-mg tablet was administered to healthy adults was 418.8 ng/ml and is comparable to approximately 1/2 mol.wt. (416.5). The average drug concentration in blood was approximately 100 ng/ml and is comparable to approximately 240 nM. Therefore, the concentrations at which ramatroban acts on TP and CRTH2 in vitro are thought to be physiologically relevant. These results suggest that ramatroban is a dual antagonist for TP and CRTH2 in physiological concentrations, but it would appear that it is a stronger CRTH2 antagonist.

Indomethacin, a cyclooxygenase inhibitor, also inhibited CRTH2-mediated Ca2+ mobilization, confirming the results of Hirai et al. (2002). Ramatroban and indomethacin display a similar chemical structure, possibly satisfying a common requirement for the binding of CRTH2. It is interesting, however, that indomethacin showed agonistic activity in the Ca2+ flux assay (Hirai et al., 2002; Fig. 2C), whereas ramatroban did not, even at 1000 nM. The reasons for this are unclear but may be related to subtle differences at the molecular level of the respective structure and further in-depth chemical analyses may clarify this.

Human eosinophils have been reported to express both CRTH2 and DP at the mRNA level (Gervais et al., 2001). Using a specific antibody, we confirmed the surface expression of CRTH2 in the present study (Fig. 4A). PGD2 binds to DP, TP, and CRTH2. Therefore, we checked the contribution of DP and TP in PGD2-mediated eosinophil migration. U-46619 did not induce eosinophil migration (Fig. 4A) as there are no TP receptors on eosinophils, and a DP-selective antagonist, BWA868C, did not significantly inhibit the PGD2-induced migration of eosinophils at concentrations below 5 μM (Fig. 4C). Earlier speculation that effects of PGD2 on eosinophil migration were independent of DP activation (Monneret et al., 2001) and the likely effect of a DP-selective agonist, BW245C, on human eosinophil migration (Hirai et al., 2001) support our present findings. Ramatroban did not
antagonize the PGD₂-induced response in a Ca²⁺ mobilization assay using DP transfectants (Fig. 3B). Therefore, taking these findings together, it is clear that the inhibition by ramatroban can be solely attributed to its effects on CRTH2 in selectively antagonizing PGD₂-mediated migration responses in eosinophils. It has been suggested by Monneret et al. (2001) that stimulation via DP with PGD₂ might be inhibitory to CRTH2-mediated migration since DP is linked to Gs and would lead to elevation of intracellular cAMP levels. However, in our hands, the EC₅₀ value for PGD₂-mediated migration of eosinophils very closely approximates the Kᵦ₅₀ for PGD₂ binding to CRTH2, suggesting that there is limited DP-CRTH2 signal cross-talk in the eosinophil.

PGD₂ is a major prostanoid released from mast cells via FceR stimulation (Georgitis et al., 1994). In allergic rhinitis patients, allergic challenge caused an increase in PGD₂ levels in nasal lavage fluid (Beppu et al., 1994). Several articles (Hamilos et al., 1996; Klimek and Rasp, 1996; Fan et al., 1999; Wang and Clement, 2000) demonstrate the importance of eosinophils in nasal obstruction in allergic rhinitis and sinusitis, and eosinophils is a characteristic feature of allergic-induced airway inflammation. Increased PGD₂ in nasal inflammatory sites after antigen challenge may induce eosinophil chemotaxis via CRTH2 and induce nasal obstruction. The present study showed that ramatroban might inhibit these clinical phenomena by the antagonism of CRTH2. However, the inhibitory mechanism of ramatroban on nasal symptoms might not be caused only by CRTH2 antagonism on eosinophils. In our preliminary studies, ramatroban inhibited U-46619-induced expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 on human endothelial cells with IC₅₀ values of 60 nM and 50 nM, respectively (unpublished data). Therefore, ramatroban might affect eosinophil migration by at least two different mechanisms: 1) inhibition of chemotaxis by CRTH2 antagonism, and 2) inhibition of adhesion to endothelial cells by TP antagonism, assuming that eosinophils would selectively use only these adhesion molecules. Furthermore, CRTH2 is expressed on Th2 lymphocytes and basophils, suggesting additional targets involved in the chronic phase of the allergic response.

In the present study, we have detailed the first evidence for a small molecule CRTH2 antagonist, and a new mode of action of ramatroban. Ramatroban should therefore be a useful tool for clarifying the role of CRTH2 in diseases characterized by elevated levels of PGD₂ (if this is the sole CRTH2 ligand), eosinophils, basophils, and Th2 cells. References


Seuter F, Perzborn E, Rosenreuter U, Boshagen H, and Fiedler VB (1989) Inhibition of platelet aggregation in vitro and ex vivo by the new thromboxane antagonist (3-[(4-hydroxyphenyl)butylamino]-1,2,3,4-tetrahydro-9-carbolepropanoic acid. Arzneim Forsch 39:1525–1527.


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