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-tetra-hydrocarbazole-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 antagonize U-46619 [a thromboxane A₂ (TxA₂)-mimetic]-induced contraction of airway smooth muscle derived from human, guinea pig, rat, and ferret (McKenniff et al., 1991). Ramatroban has also shown antagonistic effects on U-46619-induced bronchoconstriction in the guinea pig in vivo when given intravenously, orally, or by aerosol (Francis et al., 1991). These results suggested that TP is associated with contraction of airway smooth muscle and that ramatroban inhibited these responses by TP antagonism.

In addition, ramatroban has been reported to suppress lipopolysaccharide-induced shock (Atavilla et al., 1994), myocardial ischemia reperfusion injury (Squadrito et al., 1993), vagal nerve effector transmission in tracheal smooth muscle (Aizawa et al., 1996), allergen- and IgE antibody-mediated skin and nasal reactions (Nagai et al., 1995; Narita et al., 1996), and eosinophilia in experimental animal models of asthma (Nagai et al., 1995). Likewise, ramatroban significantly blocked eosinophil infiltration into the nasal space of allergen-challenged patients suffering from perennial rhinitis (Terada et al., 1998) and PGD₂-mediated bronchoconstriction (Johnston et al., 1992; Magnussen et al., 1992; Rajaku-lasingam et al., 1996). The broad efficacy that ramatroban exerts in these pathological situations is unlikely to be explained solely by direct TP antagonism. This is especially true for the inhibition of eosinophilia—believed to be the reason for the improvement of nasal symptoms seen under ramatroban treatment—since no evidence for functional TP receptor expression on eosinophils exists (Monneret et al., 2001; our unpublished observations).

One possible indirect mechanism to block eosinophil recruitment into tissues by ramatroban might be the inhibition of TxA₂-mediated expression of adhesion molecules on endothelial cells. TxA₂ has been reported to augment the expression of intercellular adhesion molecule-1 (Ishizuka et al., 1994, 1998) and vascular cell adhesion molecule-1 (Ishizuka et al., 1998) by human vascular endothelial cells.

A further potential mechanism of ramatroban action might...
be the blockade of the chemotactic reaction itself. Although 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 and bronchoconstriction 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 in Ca2+ was 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-quik 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′-AATAGCTTCTCA-GAGCCCCACAGTGTCGCCC and 5′-AATGAATTCCTAACTCGAG-GTCTGCTTCAG. 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 clone-sequence 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).

**Generation of Human CRTH2 and DP Stable Transfectants.** CRTH2 transgene inserted into the pEAK10 expression vector was transfected into L1.2 cells (a kind gift from Prof. Eugene Butcher, Stanford, CA) by electroporation (250V/1000 μF, Gene Pulser II; Bio-Rad, Hercules, CA). Stable transfectants were selected in the presence of puromycin (1 μg/mL, P7255; Sigma-Aldrich). The DP cDNA cloned into the pcDNA3.1(−) expression vector (Invitrogen) was transfected into Chinese hamster ovary cells, which express Gα16 using LipofectAMINE Plus (Invitrogen). Stable transfectants were selected in the presence of G418 (0.5 mg/mL; Invitrogen).

**Receptor Binding Assay.** CRTH2 transfectants were resuspended in binding buffer (50 mM Tris-HCl, pH 7.4, 40 mM MgCl2, 0.1% BSA, 0.1% Na3VO4). Cell suspension (2 × 107 cells/mL) was incubated with various concentrations of ramatroban 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 at 60 min at room temperature. After incubation, the cell suspension was transferred to a filtration plate (MAPB; Millipore Corporation, Bedford, MA) and washed three times with binding buffer. Scintillant was added to the filtration plate, and radioactivity remaining on the filter was measured by a scintillation counter (TopCount; PerkinElmer Life Sciences). Nonspecific binding was determined by incubating the cell suspension and [3H]-labeled PGD2 in the presence of 1 μM unlabeled PGD2.

**Ca2+ Mobilization Assay.** Ca2+ loading buffer was prepared by mixing 1 μM Fluo-3/AM and pluronic F-127 in Ca2+ assay buffer (20 mM HEPES, pH 7.6, 0.1% BSA, 1 mM probenecid, Hanks’ solution) and the CRTH2 transfectants established were resuspended in Ca2+ loading 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-botted 96-well plates (3831; Costar, Corning, NY) at 2 × 104 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 FDS5000 fluorimeter (Hamamatsu Photonics, Hamamatsu, Japan). For inactivation of Gα, 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 FACSscan (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 × 10⁶ cells/ml. Fifty microliters of the cell suspension (5 × 10⁵ 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 [³H]-labeled PGD₂ to CRTH2 and Scatchard transformation is shown in Fig. 1A. [³H]-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 [³H]-labeled PGD₂ to CRTH2 transfectants in a concentration-dependent manner with an EC₅₀ value of 2.7 nM (Fig. 1B). Ramatroban showed significant inhibitory effects on the binding of [³H]-labeled PGD₂ to CRTH2, albeit with much lower potency (IC₅₀ = 100 nM, Fig. 1C).

Effects of Ramatroban on Ca²⁺ Mobilization in CRTH2 and DP Transfectants. To determine the functional expression of CRTH2 and DP on each transfected, calcium mobilization after PGD₂ stimulation was monitored. PGD₂ stimulated Ca²⁺ mobilization in CRTH2-L1.2 transfectants (Fig. 2A) and DP-Chinese hamster ovary transfectants (Fig. 3A) in a concentration-dependent manner with EC₅₀ values of 15 and 150 nM, respectively. U-46619 (TxA₂ mimetic) failed to induce Ca²⁺ mobilization in either transfec tant (Figs. 2A and 3A). As expected for a Gα₄-coupled receptor, PGD₂ (10 nM)-induced Ca²⁺ mobilization in CRTH2 transfectants was completely suppressed by pretreatment of cells with the Gα₄ inhibitor pertussis toxin (PTX; Fig. 2A). Ramatroban and indomethacin also inhibited PGD₂-induced Ca²⁺ mobilization in CRTH2 transfectants to almost the same extent with an IC₅₀ value of 30 nM (Fig. 2B). However, indomethacin but not ramatroban was confirmed as an agonist of Ca²⁺ mobilization at concentrations greater than 10 nM (Hirai et al., 2002; Fig. 2C). As expected, PGD₂-induced Ca²⁺ mobilization in DP transfectants was not inhibited by PTX since DP is coupled directly to Gα₃-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 IC₅₀ 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 IC₅₀ value of 32 nM (Fig. 3B), whereas it only partially affected Ca²⁺ 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. The binding of [³H]-labeled PGD₂ to CRTH2 transfectants and the effect of ramatroban on their binding. A, Scatchard plot of [³H]-labeled PGD₂ binding to CRTH2 transfectants. B, homologous competitive binding in [³H]-labeled PGD₂ and CRTH2 transfectants (n = 6). Various concentrations of nonlabeled PGD₂ were added in the reaction mixture of 1 nM [³H]-labeled PGD₂ and CRTH2 transfectants. C, effect of ramatroban on [³H]-labeled PGD₂ binding to CRTH2 transfectants. CRTH2 transfectants were incubated with various concentrations of ramatroban and 1 nM of [³H]-labeled PGD₂. Data represent mean ± S.D. of seven independent experiments. Significant difference between ramatroban-treated and untreated binding was analyzed by Student’s t test: **, p < 0.01.
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...
selective antagonism of ramatroban at TP but not at PGF_2\_alpha receptors (EP1 and EP2), PGF_2 receptor (FP), and PGI_2 receptor (IP). In the present study, we clearly demonstrated that ramatroban also antagonizes CRTH2 by inhibiting PGD_2 binding and PGD_2-mediated functions. The potency of CRTH2 blockade (the IC_{50} values for the inhibition of receptor binding, Ca^{2+} mobilization and migration of eosinophils were 100, 30, and 170 nM, respectively) was better than that for TP antagonism reported previously (the IC_{50} 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 C_{max} 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 \mu\text{M (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 Ca^{2+} 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 Ca^{2+} 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 have confirmed the surface expression of CRTH2 in the present study (Fig. 4A). PGD_2 binds to DP, TP, and CRTH2. Therefore, we checked the contribution of DP and TP in PGD_2-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 PGD_2-induced migration of eosinophils at concentrations below 5 \mu\text{M (Fig. 4C)}. Earlier speculation that effects of PGD_2 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

**Fig. 4.** A, Surface expression of CRTH2 on L1.2-CRTH2 transfectants and circulating eosinophils derived from two donors. B, dose-response of PGD_2-induced (n = 6) and U-46619-induced (n = 3) migration of human eosinophils. C, effects of ramatroban (n = 5), BWA868C (n = 3), and 1 \mu\text{g/ml PTX (n = 2)} on PGD_2 (1 nM)-induced eosinophil migration. Significant difference between ligand-treated and untreated eosinophil migration (B) was analyzed by ANOVA: *, p < 0.05, **, p < 0.01, and between drug-treated and untreated eosinophil migration (C) was analyzed by Student’s t test: *, p < 0.05, **, p < 0.01.
antagonize the PGD$_2$-induced response in a Ca$^{2+}$ 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 PGD$_2$-mediated migration responses in eosinophils. It has been suggested by Monneret et al. (2001) that stimulation via DP with PGD$_2$ 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 EC$_{50}$ value for PGD$_2$-mediated migration of eosinophils very closely approximates the K$_d$ for PGD$_2$ binding to CRTH2, suggesting that there is limited DP-CRTH2 signal cross-talk in the eosinophil.

PGD$_2$ 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 PGD$_2$ levels in nasal lavage fluid (Beppu et al., 1994). Several articles (Hamilos et al., 1996; Klimek and Rasp, 1996; Fan et al., 2000; Wang and Clement, 2000) demonstrate the importance of eosinophils in nasal obstruction in allergic rhinitis and sinusitis, and eosinophilia is a characteristic feature of allergic-induced airway inflammation. Increased PGD$_2$ 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 IC$_{50}$ 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 considered as a useful tool for clarifying the role of CRTH2 in diseases characterized by elevated levels of PGD$_2$ (if this is the sole CRTH2 ligand), eosinophils, basophils, and Th2 cells.