JPET Fast Forward. Published on January 21, 2003 as DOI:10.1124/jpet.102.046748

JPET/2002/46748

An orally-bioavailable small molecule antagonist of CRTH2, ramatroban (BAY u3405), inhibits PGD_2-induced eosinophil migration in vitro

Hiromi Sugimoto*, Michitaka Shichijo*, Takashi Iino, Yoshihisa Manabe, Akihiko Watanabe, Makoto Shimazaki[1], Florian Gantner and Kevin B. Bacon.

Respiratory Diseases Research, and Medicinal Chemistry[1], Bayer Yakuhin, Ltd., 6-5-1-3 Kunimidai, Kizu-cho, Soraku-gun, Kyoto 619-0216, Japan.

*H.S. and M.S. contributed equally to this work.

JPET/2002/46748

Running Title

Ramatroban is an antagonist of CRTH2

Author for correspondence: Michitaka Shichijo, Ph.D.

Therapeutic Research Area Respiratory Diseases, Bayer Yakuhin, Ltd., 6-5-1-3 Kunimidai, Kizu-cho, Soraku-gun, Kyoto 619-0216, Japan.

Phone +81 774 75 2470 Fax +81 774 75 2506

e-mail : michitaka.shichijo.ms@bayer.co.jp

The number of

text pages: 25

tables: none

figures: 4

references: 32

words in the Abstract : 170

words in the Introduction : 647

words in the Discussion : 929

Abbreviations

CRTH2: chemoattractant receptor-homologous molecule expressed on Th2 cells

GPCR: G-protein coupled receptor

ICAM-1: intercellular adhesion molecule-1

VCAM-1: vascular cell adhesion molecule-1

ABSTRACT

Ramatroban (Baynas™, BAY u3405), a thromboxane A_2 (TxA_2) antagonist marketed for allergic rhinitis, has been shown to partially attenuate prostaglandin (PG)D_2-induced bronchial hyperresponsiveness in man, as well as reduce antigen-induced early and late-phase inflammatory responses in mice, guinea-pigs and rats. PGD_2 is known to induce eosinophilia following intra-nasal administration, and to induce eosinophil activation in vitro. In addition to the TxA_2 receptor, PGD_2 is known as a ligand for the PGD_2 (DP) receptor, and the newly-identified G-protein-coupled chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2). In order to fully characterize PGD_2-mediated inflammatory responses relevant to eosinophil activation, further analysis of the mechanism of action of ramatroban has now been performed. PGD_2-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^{TM}, Bay u3405), 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 A_2 (TxA_2)-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 LPS-induced shock (Atavilla et al., 1994), myocardial ischemia reperfusion injury (Squadrito et al., 1993), vagal neuro 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 PGD_2-induced 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 PGD_2-mediated bronchoconstriction (Magnussen et al., 1992; Johnston et al., 1992; Rajakulasingam et al., 1996). The broad efficacy 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), own unpublished observations).

JPET Fast Forward. Published on January 21, 2003 as DOI: 10.1124/jpet.102.046748
This article has not been copyedited and formatted. The final version may differ from this version.

One possible indirect mechanism to block eosinophil recruitment into tissues by ramatroban might be the inhibition of TxA_2-mediated expression of adhesion molecules on endothelial cells. TxA_2 has been reported to augment the expression of intercellular adhesion molecule-1 (ICAM-1) (Ishizuka et al., 1994; Ishizuka et al., 1998) and vascular cell adhesion molecule-1 (VCAM-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. Various chemoattractants are known for eosinophils (eotaxin, eotaxin-2, MCP-3, MCP-4, RANTES, LTD_4, C5a, PAF, and PGD_2, see (Jose et al., 1994; Hirai et al., 2001; Elsner et al., 1996; Fukuda et al., 1992) for details), 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. While no evidence exists for a direct interaction of ramatroban with chemokine receptors (own unpublished observations), the blockade of PGD_2-mediated eosinophilia and bronchoconstriction by ramatroban is well documented in animals and man (Nagai et al., 1995; Narita et al., 1996; Magnussen et al., Johnston et al., 1992; Rajakulasingam et al., 1996).

PGD_2 is an agonist for TP (Coleman et al., 1990). However, it also specifically binds to two other receptors, PGD_2 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

PGD_2 could induce degranulation of eosinophils via CRTH2 stimulation. These reports strongly suggest a critical role of PGD_2 and CRTH2 in allergic diseases.

In the present study, we therefore examined the effect of ramatroban on PGD_2-induced CRTH2 activation, using CRTH2 transfectants and peripheral blood eosinophils. We demonstrate that ramatroban is an antagonist for CRTH2, and inhibits PGD_2–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 PGD_2-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 ((+)-(3R)-3-(4-fluorobenzenesulfonamido)-1,2,3,4-tetra-hydrocarbazole-9-propionic acid) was synthesized at Bayer Yakuhin Ltd., Shiga factory (Japan). BWA868C was synthesized by SOGO Pharmaceutical Co. Ltd. (http://www.sogo-pharma.co.jp/index.html). U-46619 was from BIOMOL Research Labs Inc. (Plymouth Meeting, PA), PGD_2 from Sigma-Aldrich (St. Louis. MO), and [^{3}H]PGD_2 was purchased from Amersham Pharmacia (Buckinghamshire, UK). Sodium butyrate was purchased from Wako Pure Chemicals (Osaka, Japan). Fluo-3AM and pluronic F-127 were purchased from Molecular Probes (Eugene, OR, USA). Anti-human CRTH2 monoclonal antibody (clone BM16, rat IgG2a) was provided by BML (Saitama, Japan), and FITC conjugated rat IgG2a (for isotype control) and anti-rat IgG2a antibodies were purchased from PharMingen (San Diego, CA). Ramatroban, BWA868C, U-46619 and PGD_2 were dissolved in dimethyl sulfoxide (DMSO, Nacalai Tesque, Inc., Kyoto, Japan). As confirmed in preliminary experiments, the concentrations of DMSO in working dilutions used in this study (< 0.1 %) had no effect on receptor binding, Ca^{2+} 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^R (ICN Biomedicals, Co. Ltd., 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® (Gibco BRL, Rockville, MD). First-strand cDNA was then synthesized with the SUPERSCRIPT™ First-Strand Synthesis System (Gibco BRL). loning 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'-AATAAGCTTCAGAGCCCCACGATGTCGGCC and 5'-AATGAATTCCTAACTCGAGGTGCTGCTCAG. PCR was carried out with KOD Plus polymerase (Toyobo, Osaka, Japan) under the following parameters: 15 sec at 94˚C, 30 sec at 60˚C and 90 sec at 68˚C for 35 cycles. PCR products obtained were cloned into pCRII®-TOPO® (Invitrogen, Carlsbad, CA) for sequencing and subcloned into pEAK vector (Edge Biosystems, Gaithburg, 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).

Generation of human CRTH2 and DP stable transfectants

The CRTH2 gene 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 CHO cells which express G . 16 using lipofectamine plus (Invitrogen). Stable transfectants were

selected in the presence of G418 (0.5 mg/ml, Gibco BRL).

Receptor binding assay

CRTH2 transfectants were resuspended in binding buffer (50 mM Tris-HCl pH 7.4, 40 mM $MgCl_2$, 0.1% BSA, 0.1% NaN_3). Cell suspension (2 x 10^5 cells), [^{3}H]-labeled PGD_2, and various concentrations of ramatroban were then mixed in a 96-well U-bottom polypropylene 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, Millipore, Bedford, MA) and washed 3 times with binding buffer. Scintillant was added to the filtration plate, and radioactivity remaining on the filter was measured by a scintillation counter (TopCount, Packard Bioscience, Meriden, CT). Non-specific binding was determined by incubating the cell suspension and [^{3}H]-labeled PGD_2 in the presence of 1 µM unlabeled PGD_2.

Ca^{2+} mobilization assay

Ca^{2+} loading buffer was prepared by mixing 1 µM of Fluo-3AM (Molecular Probes, Eugene, OR) and pluronic F-127 (Molecular Probes) in Ca^{2+} assay buffer (20 mM HEPES pH 7.6, 0.1% BSA, 1 mM probenecid, Hanks' solution). The CRTH2 transfectants established were resuspended in Ca^{2+} loading buffer at 1 x 10^7 cells/ml, and incubated for 60 minutes at room temperature. After the incubation, cells were washed and resuspended in Ca^{2+} assay buffer, then dispensed into transparent-bottom 96-well plates (#3631, Costar-Corning, NY) at 2 x 10^5 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 FDSS6000 fluorimeter (Hamamatsu Photonics,

Hamamatsu, Japan). For inactivation of Gαi proteins, cells were incubated with 1 μg/ml of pertussis toxin (Sigma) at 37 ^{o}C for 2 h before start of the experiment.

FACS aalysis of CRTH2 expression

Cell surface expression of CRTH2 on transfected cells and eosinophils was determined according to standard protocols. CRTH2 tranfected L1.2 cells, wild type L1.2 cells and purified eosinophils were incubated with anti-human CRTH2 mAb for 20 min in the cold PBS containing 1% bovine serum albumin and 0.01% sodium azide. After washing cells were incubated with FITC-conjugated anti-rat IgG2a for 20 min before analysis by FACScan (Becton Dickinson, San Jose, CA). Rat IgG2a was used as a control.

Migration assays

Human eosinophils were purified as described above and re-suspended in migration buffer (20 mM HEPES pH 7.6, 0.1% BSA, Hanks' solution) at a density of 6 x 10^6 cells/ml. Fifty μl of the cell suspension (3 x 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, Gaithesburg, MD) and 30 μl of ligand solution was added to the lower chamber. Cells were pre-incubated with various concentrations of ramatroban or BWA868C at 37 ^{o}C for 10 minutes. The migration assays were performed in a humidified incubator at 37 ^{o}C, 5% CO_2 for 2 hours. 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

JPET Fast Forward. Published on January 21, 2003 as DOI: 10.1124/jpet.102.046748
This article has not been copyedited and formatted. The final version may differ from this version.

ligands (compared to controls without ligand) and Student's *t*-test for drug evaluations (compared to controls without drug). *P* values <0.05 were considered as statistically significant (**P* <0.05, ***P*< 0.01).

JPET Fast Forward. Published on January 21, 2003 as DOI: 10.1124/jpet.102.046748
This article has not been copyedited and formatted. The final version may differ from this version.

RESULTS

Ramatroban antagonizes PGD_2 binding to CRTH2 transfectants

Analysis of the binding of [^{3}H]-labeled PGD_2 to CRTH2 and Scatchard transformation is shown in Fig. 1A. [^{3}H]-labeled PGD_2 bound to a single site on CRTH2 transfectants with high affinity (K_D = 6.3 nM, Bmax = 450 pM). Non-labeled PGD_2 inhibited the binding of [^{3}H]-labeled PGD_2 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 PGD_2 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 PGD_2 stimulation was monitored. PGD_2 stimulated Ca^{2+} mobilization in CRTH2-L1.2 transfectants (Fig. 2A) and DP-CHO transfectants (Fig. 3A) in a concentration-dependent manner with EC_{50} values of 15 nM and 150 nM, respectively. U-46619 (TxA_2 mimetic) failed to induce Ca^{2+} mobilization in either transfectant (Fig. 2A, 3A). As expected for a Gαi-coupled receptor, PGD_2 (10nM)-induced Ca^{2+} mobilization in CRTH2 transfectants was completely suppressed by pretreatment of cells with the Gαi inhibitor pertussis toxin (PTX, Fig. 2A). Ramatroban and indomethacin also inhibited PGD_2-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

JPET Fast Forward. Published on January 21, 2003 as DOI: 10.1124/jpet.102.046748
This article has not been copyedited and formatted. The final version may differ from this version.

was confirmed as an agonist of Ca^{2+} mobilization at concentrations greater than 10 nM ((Hirai et al., 2002), Fig. 2C). As expected, PGD_2-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 PGD_2-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). PGD_2 but not U-46619 induced migration of human eosinophils (Fig. 4B) that peaked at 100 nM and was completely suppressed by 1 μg/ml of PTX pretreatment. As shown in Fig. 4C, ramatroban completely inhibited the PGD_2 -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 PGD_2-induced eosinophil migration, the inhibitory effect of a DP selective antagonist, BWA868C, was evaluated. BWA868C completely suppressed PGD_2–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 PGD_2–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 TxA_2 and PGD_2 are known ligands for TP (Seuter et al., 1989). PGD_2 has been shown to bind to DP and CRTH2 with relatively similar affinities (45 and 61 nM, respectively (Hirai et al., 2001)), whereas TxA_2 does not bind either receptor. Surprisingly, our study using GPCR-transfected cells has revealed a 10-fold higher affinity of the PGD_2/CRTH2 interaction as compared to the interaction of PGD_2 with DP. The reason for this is unclear and we are currently investigating the expression of CRTH2 in different host backgrounds. McKenniff et al. (1991) showed selective antagonism of ramatroban at TP but not at PGE_2 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 Cmax value (1.83 hr) 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 μM (MW=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) and Fig. 2C in the present study) while ramatroban did not even at 1,000 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 μM (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 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 Kd 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 FcεR stimulation (Georgitis et al., 1994). In allergic rhinitis patients, allergen challenge caused an increase in PGD_2 levels in nasal lavage fluid (Beppu et al., 1994). Several articles (Klimek and Rasp, 1996; Wang and Clement, 2000; Hamilos et al., 1996; Fan et al., 2000) demonstrate the importance of eosinophils in nasal obstruction in allergic rhinitis and sinusitis, and eosinophilia is a characteristic feature of allergen-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 ICAM-1 and VCAM-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 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.

REFERENCES

Aizawa H, Shigyo M, Nogami H, Hirose T and Hara N (1996) BAY u3405, a thromboxane A_2 antagonist, reduces bronchial hyperresponsiveness in asthmatics. *Chest* **109**:338-342.

Atavilla D, Canale P, Squadrito F, Sardella A, Ammendolia L, Urna G, Ioculano M, Squadrito G and Caputi AP (1994) Protective effects of BAY u3405, a thromboxane A_2 receptor antagonist, in endotoxin shock. *Pharmacol Res* **30**:137-151.

Beppu T, Ohta N, Gon S, Sakata K, Inamura K, Fukase S, Kimura Y and Koike Y (1994) Eosinophil and eosinophil cationic protein allergic rhinitis. *Acta OTO-Laryngologica* Suppl. **511**:221-223.

Coleman RA, Kennedy I, Humphrey PPA, Bunce K and Lumley P (1990) Prostanoids and their receptors. *In* the Comprehensive Medicinal Chemistry. Emmett JC, editor. Pergamon Press, U.K. **3**:644-706.

Elsner J, Oppermann M and Kapp A (1996) Detection of C5a receptors on human eosinophils and inhibition of eosinophil effector functions by anti-C5a receptor (CD88) antibodies. *Eur J Immunol* **26**:1560-1564.

Fan G-K., Itoh T, Imanaka M, Fujieda S and Takenaka H (2000) Eosinophilic apoptosis in sinusitis mucosa: relationship to tissue eosinophilia and its resolution in allergic sinusitis.

J Allergy Clin Immunol. **106**:551-558.

Francis HP, Greemham SJ, Patel UP, Thompson AM and Gardiner PJ (1991) BAYu3405 an antagonist of thromboxane A_2- and prostaglandin D_2-induced bronchoconstriction in the guinea-pig. *Br J Pharmacol* **104**:596-602.

Fukuda T, Numao T, Akutsu I and Makino S (1992) Platelet-activating factor (PAF)-induced chemotaxis and PAF binding to human eosinophis. *J Lipid Mediators* **5**:145-149.

Georgitis JW, Stone BD and Gottschlich G (1994) Inflammatory cells and mediators in nasal secretions during early response to ragweed challenge-Correlation between type of cellular influx and mediator release. *Am J Rhinol* **8**:217-224.

Gervais FG., Cruz RPG, Chateauneuf A, Gale S, Sawyer N, Nantel F, Metters KM and O'Neill GP (2001) Selective modulation of chemokinesis, degranulation, and apoptosis in eosinophils through the PGD_2 receptors CRTH2 and DP. *J Allergy Clin Immunol* **108**:982-988.

Hamilos DL, Leung DYM, Wood R, Bean DK, Song YL, Schotman E and Hamid Q (1996) Eosinophil infiltration in nonallergic chronic hyperplastic sinusitis with nasal polyposis (CHS/NP) is associated with endothelial VCAM-1 upregulation and expression of TNF-α. *Am J Respir Cell Mol Biol* **15:**443-450.

Hirai H, Tanaka K, Yoshie O, Ogawa K, Kenmotsu K, Takamori Y, Ichimasa M, Sugamura K, Nakamura M, Takano S and Nagata K (2001) Prostaglandin D_2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2. *J Exp Med* **193**:255-261.

Hirai H, Tanaka K, Takano S, Ichimasa M, Nakamura M and Nagata K (2002) Agonistic effect of indomethacin on a prostaglandin D-2 receptor, CRTH2. *J Immunol* **168**:981-985.

Hirata M, Kakizawa A, Aizawa M, Ushikubi F and Narumiya S (1994) Molecular characterization of a mouse prostaglandin D receptor and functional expression of the cloned gene. *Proc Natl Acad Sci USA.* **91**:11192-11196.

Ishizuka T, Suzuki K, Kawakami M, Kawaguchi Y, Hidaka T, Matsuki Y and Nakamura H (1994) DP-1904, a specific inhibitor of thromboxane A_2 synthesizing enzyme, suppresses ICAM-1 expression by stimulated vascular endothelial cells. *Eur J Pharmacol* **262**:113-123.

Ishizuka T, Kawakami M, Hidaka T, Matsuki Y, Takamizawa M, Suzuki K, Kurita A and Nakamura H (1998) Stimulation with thromboxane A_2 (TxA_2) receptor agonist enhances ICAM-1, VCAM-1 or ELAM-1 expression by human vascular endothelial cells. *Clin Exp Immunol* **112**:464-470.

Johnston SL, Bardin PG, Harrison J, Ritter W, Joubert JR and Holgate ST (1992) The effects of an oral thromboxane TP receptor antagonist BAY u3405, on prostaglandin D_2-

and histamine-induced bronchoconstriction in asthma, and relationship to plasms drug concentrations. *Br J Clin Pharmacol* **34**:402-408.

Jose PJ, Adcock IM, Griffiths-Johnson DA, Berkman N, Wells TNC, Williams TJ and Power CA (1994) Eotaxin - Cloning of an eosinophil chemoattractant cytokine and increased messenger RNA expression in allergen-challenged guinea-pig lungs. *Biochem Biophys Research Comm* **205**:788-794.

Klimek L and Rasp G (1996) Cell activation markers in rhinitis and rhinosinusitis. 1. Eosinophil cationic protein. *Laryngo-Rhyno-Otologie* **75**:665-670.

Lewis RA, Soter NA, Diamond PT, Austen KF, Oates JA and Roberts LJ (1982) Prostaglandin D_2 generation after activation of rat and human mast cells with anti-IgE. *J Immunol* **129**:1627-1631

Magnussen H, Boerger S, Templin K and Baunack AR (1992) Effects of thromboxane-receptor antagonist, BAY u3405, on prostaglandin D_2-and exercise-induced bronchoconstriction. *J Allergy Clin Immunol* **89**:1119-1126.

McKenniff MG., Norman P, Cuthbert NJ and Gardiner PJ (1991) Bay u3405, a potent selective thromboxane A_2 receptor antagonist on airway smooth muscle in vitro. *Br J Pharmacol* **104:**585-590.

Monneret G, Gravel S, Diamond M, Rokach J and Powell WS (2001) Prostaglandin D_2 is

a potent chemoattractant for human eosinophils that acts via a novel DP receptor. *Blood* **98**:1942-1948.

Nagai H, Takeda H, Yamaguchi S, Tanaka H, Matsuo A and Inagaki N (1995) The effect of a thromboxane A_2 receptor antagonist Bay u3405 on experimental allergic reactions. *Prostaglandins* **50**:75-87.

Nagata K, Hirai H, Tanaka K, Ogawa K, Aso T, Sugamura K, Nakamura M and Takano S (1999) CRTH2, an orphan receptor of T-helper-2-cells, is expressed on basophils and eosinophils and responds to mast cell-derived factor(s). *FEBS LETTERS* **459**:195-199.

Narita S, Asakura K and Kataura A (1996) Effects of thromboxane A(2) receptor antagonist (Bay u3405) on nasal symptoms after antigen challenge in sensitized guinea pigs. *Int Arch Allergy Immunol* **109**:161-166.

Palframan RT, Collins PD, Williams TJ and Rankin SM (1998) Eotaxin induces a rapid release of eosinophils and their progenitors from the bone marrow. *Blood* **91**:2240-2248.

Rajakulasingam K, Johnston SL, Ducey J, Ritter W, Howarth PH and Holgate ST (1996) Effect of thromboxane A_2-receptor antagonist on bradykinin-induced bronchoconstriction in asthma. *J Appl Physiol* **80**:1973-1977.

Seuter F, Perzborn E, Rosentreter U, Böshagen H and Fiedler VB (1989) Inhibition of platelet aggregation in vitro and ex vivo by the new thromboxane antagonist

(3R)-3-(4-fluorophenylsulfonamido)-1,2,3,4-tetrahydro-9-carbozolepropanic acid. *Arzneimittelforsch* **39**:1525-1527.

Squadrito F, Ioculano M, Altavilla D, Zingarelli B, Canale P, Campo GM, Saitta A, Oriti S, Faggioto A and Caputi AP (1993) Reduction of myocardial infarct size following adminstration on BAY u3405, a thromboxane A_2 receptor antagonist, in myocardial ischeamia-reperfusion injury. *Agents and Actions* **39**:143-149.

Terada N, Yamakoshi T, Hasegawa M, Tanikawa H, Nagata H, Maesako K and Konno A (1998) Effect of a thromboxane A_2 receptor antagonist, ramatroban (BAY u3405), on inflammatory cells, chemical mediators, and non-specific nasal hyperreactivity after allergen challenge in patients with perennial allergic rhinitis. *Allergology International* **47**:59-67.

Wang DY and Clement P (2000) Pathogenic mechanisms underlying the clinical symptoms of allergic rhinitis. *Am J Rhinol.* **14**:325-333.

JPET Fast Forward. Published on January 21, 2003 as DOI: 10.1124/jpet.102.046748
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE LEGENDS

Fig.1 The binding of [^{3}H]-labeled PGD_2 to CRTH2 transfectants and the effect of ramatroban on their binding. A. Scatchard plot of [^{3}H]-labeled PGD_2 binding to CRTH2 transfectants. B. Homologous competitive binding in [^{3}H]-labeled PGD_2 and CRTH2 transfectants (n = 6). Various concentrations of non-labeled PGD_2 were added in the reaction mixture of 1 nM [^{3}H]-labeled PGD_2 and CRTH2 transfectants. C. Effect of ramatroban on [^{3}H]-labeled PGD_2 binding to CRTH2 transfectants. CRTH2 transfectants were incubated with various concentrations of ramatroban and 1nM of [^{3}H]-labeled PGD_2. Data represent mean ± SD of 7 independent experiments. Significant difference between ramatroban-treated and –untreated binding was analyzed by Student's *t*-test : ** $p < 0.01$.

Fig.2 PGD_2-induced Ca^{2+} mobilization in CRTH2 transfectants, and the effect of ramatroban, indomethacin and BWA868C on this response. A. Dose-response of Ca^{2+} mobilization in CRTH2 transfectants induced by PGD_2 and U-46619 (n = 3), and the effect of PTX on PGD_2-induced responses (n = 3). B. Effects of ramatroban (n = 6), indomethacin (n = 3) and BWA868C (n = 3) on PGD_2 (10 nM)-induced Ca^{2+} mobilization in CRTH2 transfectants. C. Agonistic effect of ramatroban (n = 6), indomethacin (n = 3) and PGD_2 (n = 3) on Ca^{2+} flux in CRTH2 transfectants.

Significant difference between ligand–treated and -untreated Ca^{2+} mobilization (A, C) was analyzed by ANOVA : * $p < 0.05$, $p < 0.01$, and between drug-treated and –untreated Ca^{2+} mobilization (B) was analyzed by Student's *t*-test : ** $p < 0.01$.

Fig.3 PGD_2-induced Ca^{2+} mobilization in DP transfectants, and the effect of ramatroban

and BWA868C on this response. A. Dose-response of Ca^{2+} mobilization in DP transfectants induced by PGD_2 (n = 3) and U-46619 (n = 1), and the effect of PTX on PGD_2-induced responses (n = 3). B. Effects of ramatroban (n = 6) and BWA868C (n = 3) on PGD_2 (10 nM)-induced Ca^{2+} mobilization in DP transfectants.

Significant difference between ligand–treated and -untreated Ca^{2+} mobilization (A) was analyzed by ANOVA : * $p < 0.05$, and between drug-treated and –untreated Ca^{2+} mobilization (B) was analyzed by Student's *t*-test : * $p < 0.05$, ** $p < 0.01$.

Fig.4 A. Surface expression of CRTH2 on L1.2 –CRTH2 transfectants, and circulating eosinophils derived from 2 donors. B. Dose-response of PGD_2- (n = 6) and U-46619- (n = 3) induced migration of human eosinophils. C. Effects of ramatroban (n = 5), BWA868C (n = 3) and 1 μg/ml of 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 : * $p < 0.05$, ** $p < 0.01$.

Fig.1

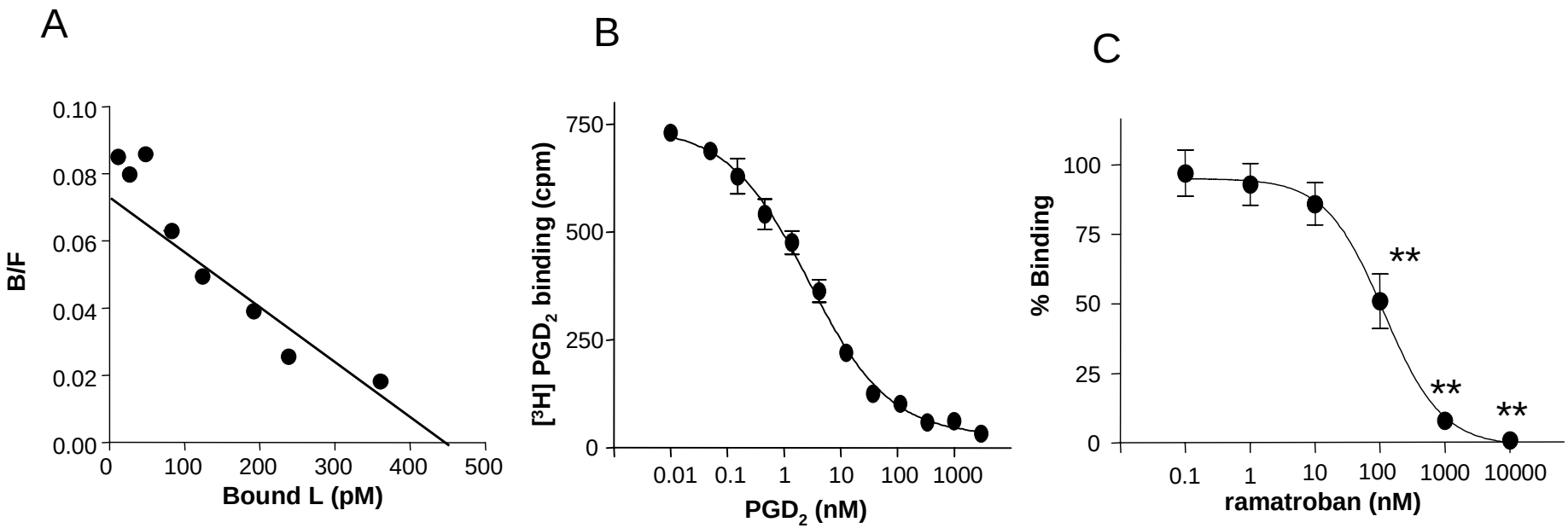

Fig.2

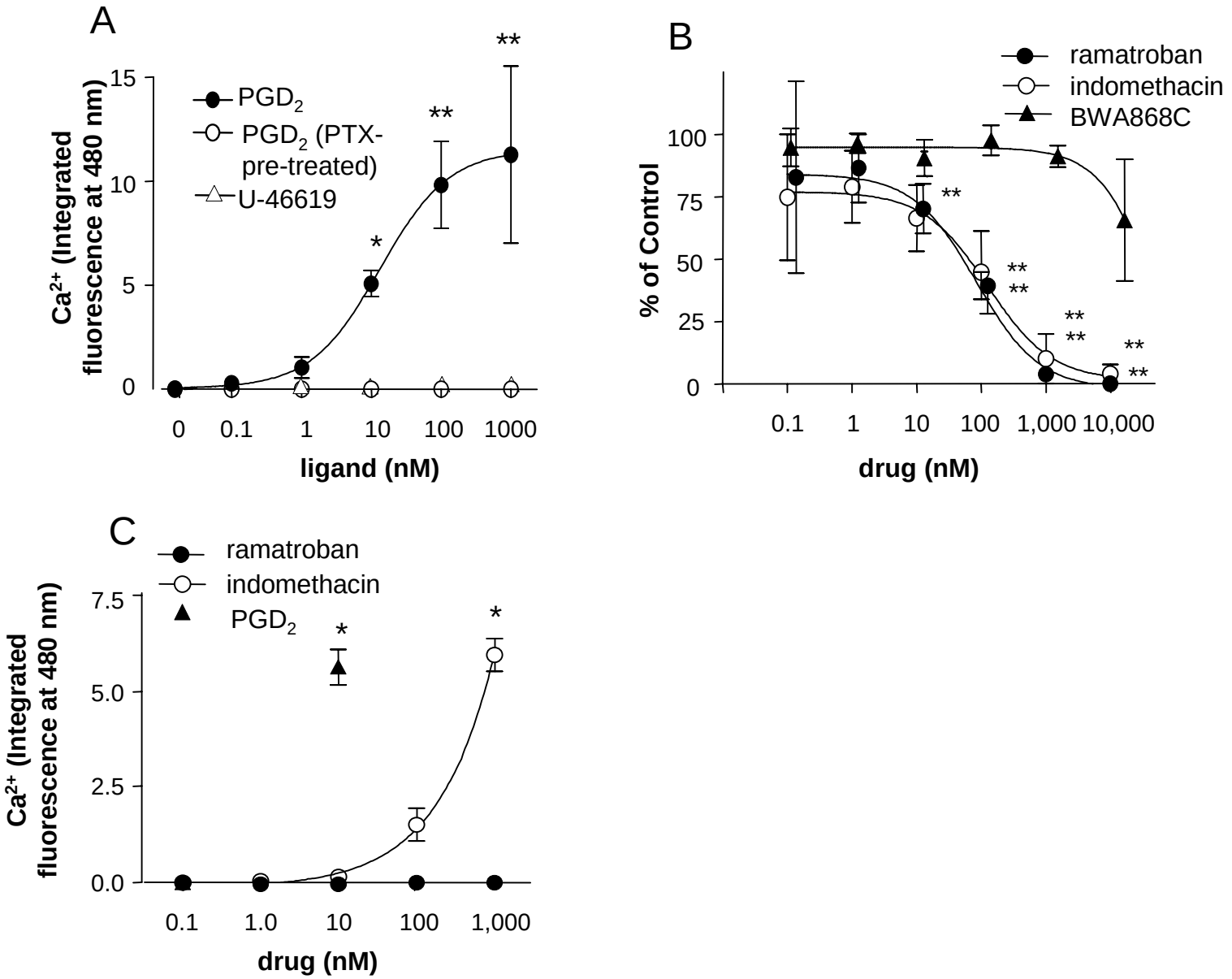

Fig.3

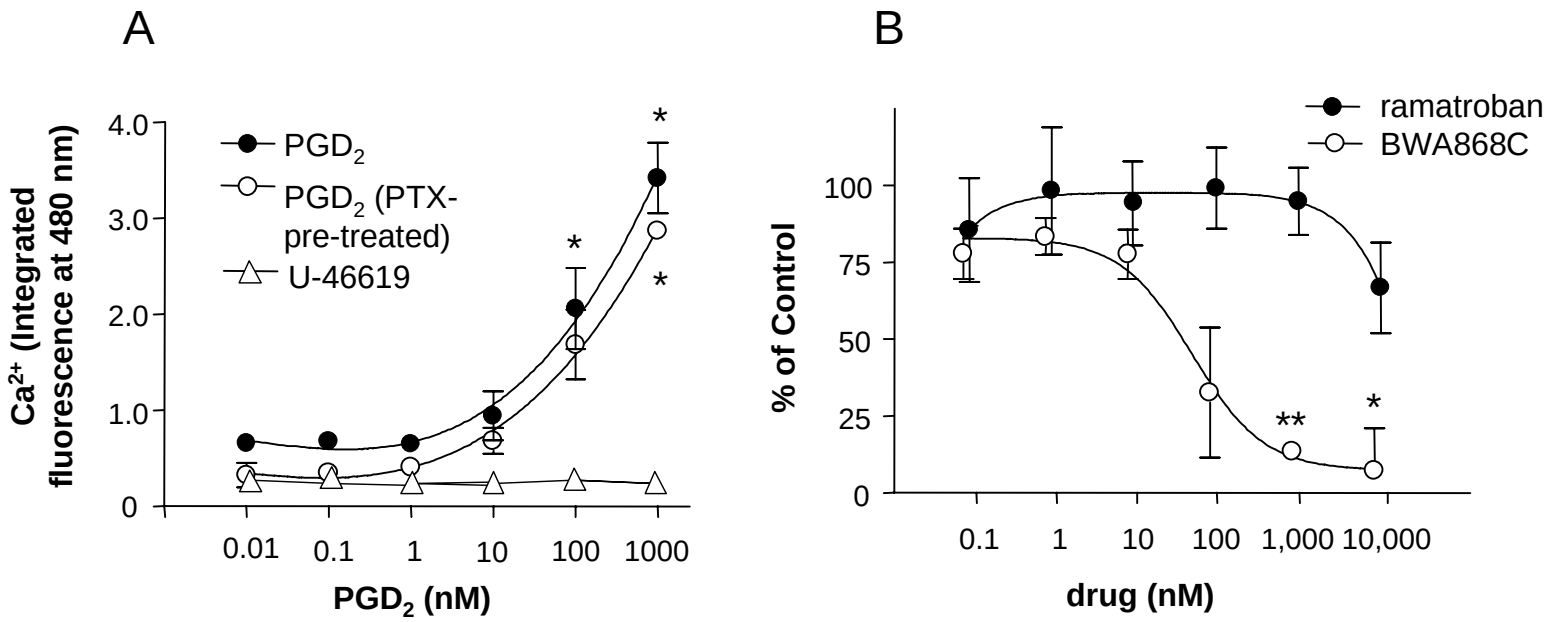

Fig.4