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CRTH2-specific binding characteristics of [^{3}H]ramatroban and its effects on PGD_2-, 15-deoxy-$\Delta^{12,14}$-PGJ_2- and indomethacin-induced agonist responses

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Abstract

We previously showed that ramatroban (Baynas™), a thromboxane A_2 (TxA_2) antagonist, had inhibited prostaglandin D_2 (PGD_2)-stimulated human eosinophil migration mediated through activation of chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2). However, detailed pharmacological characterization of its inhibitory activity has not been described. In the present study, we showed that [^{3}H] ramatroban bound to a single receptor site on CRTH2 transfectants with a similar K_d value (7.2 nM) to a TxA_2 receptor (8.7 nM). We also demonstrated that ramatroban inhibited PGD_2-, 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2)- and indomethacin-induced calcium responses on CRTH2 transfectants in a competitive manner with similar pA_2 values (8.5, 8.5, and 8.6, respectively). This is the first report showing the evidence for direct binding of ramatroban to CRTH2, revealing its competitive inhibitory effects and another interesting finding that PGD_2, indomethacin and 15d-PGJ_2 share the same binding site with ramatroban on CRTH2.

Keywords: Ramatroban; CRTH2 (Chemoattractant receptor-homologous molecule expressed on Th2 cells); Indomethacin; 15d-PGJ_2 (15-deoxy-$\Delta^{12,14}$-PGJ_2); Competitive inhibitory effect

1. Introduction

Ramatroban (Baynas™, (+)-(3R)-3-(4-fluorobenzensulfonamido)-1,2,3,4-tetra-hydrocarbazole-9-propionic acid), is a potent antagonist of the thromboxane A_2 (TxA_2) receptor (prostanoid TP receptor), and has been used for the treatment of allergic rhinitis in Japan. It was reported that ramatroban antagonizes the contraction of human, guinea-pig, rat and ferret airway smooth muscle induced by the prostanoid TP receptor agonist, 9, 11-dideoxy-9α, 11α-methanoepoxy $PGF_{2\alpha}$ (U-46619) (McKenniff et al., 1991), and prostagrandin D_2 (PGD_2)-mediated human bronchoconstriction (Johnston et al., 1992) via prostanoid TP receptor antagonism (Johnston et al., 1992). However, the lack of evidence for functional prostanoid TP receptor expression on eosinophils (Monneret et al., 2001) suggests that the significant antagonism of eosinophil infiltration into the nasal space and nasal obstruction in allergen-challenged patients suffering from perennial rhinitis by ramatroban (Terada et al., 1998) was not caused solely by prostanoid TP receptor antagonism by ramatroban. We reported that ramatroban inhibited eosinophil migration by interacting with chemoattrantant receptor-homologous molecule expressed on Th2 cells (CRTH2) (Sugimoto et al., 2003). There are, therefore, potentially two mechanisms through which ramatroban can block eosinophil migration into the nasal space. One is that ramatroban inhibits thromboxane-induced adhesion molecule expression on endothelial cells via prostanoid TP receptor antagonism, and another is that ramatroban directly inhibits their migration by CRTH2 antagonism. However, there are no reports describing the comprehensive characterization of

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the interaction of ramatroban with the prostanoid TP receptor and the CRTH2 receptor.

PGD_2, a predominant prostanoid produced by activated mast cells has been implicated in the pathogenesis of allergic asthma and atopic dermatitis (Lewis et al., 1982). PGD_2 is generated by cyclooxygenase (COX)-1 and COX-2 from arachidonic acid and exerts its effects through two G-protein coupled receptors, the PGD_2 receptor (prostanoid DP receptor) (Boie et al., 1995) and CRTH2 (Nagata et al., 1999; Hirai et al., 2001). The prostanoid DP receptor is coupled to G_s-type G proteins (G_s), and increases intracellular cyclic AMP (cAMP) and calcium (Hirata et al., 1994), which mediate both inflammatory and anti-inflammatory events (Matsuoka et al., 2000), and inhibition of colonic granulocyte infiltration in the rat (Ajuebor et al., 2000). CRTH2 is coupled to G_i-type G proteins (G_i), and inhibits cAMP production and increases intracellular calcium (Hirai et al., 2001), which mediate pro-inflammatory effects including migration or degranulation of eosinophils (Hirai et al., 2001; Gervais et al., 2001). It is also known that PGD_2 induces the contraction of human isolated bronchial smooth muscle via a prostanoid TP receptor (Coleman and Sheldrick, 1998). The prostanoid TP receptor couples to G_q-type G proteins (G_q), activates phospholipase C (PLC) and subsequently increases inositol triphosphate (IP_3), diacylglycerol (DAG) and intracellular calcium concentrations (Hirata et al., 1991). Thus, PGD_2 induces both inflammatory and anti-inflammatory effects via three different G-protein coupled receptors; prostanoid DP receptors, CRTH2 and prostanoid TP receptors.

Recently, it was described that 15-deoxy $\Delta^{12,14}$-PGJ_2 (15d-PGJ_2), a metabolite of PGD_2, and indomethacin bound to CRTH2 and induced migration or degranulation of eosinophils (Hirai et al., 2002; Monneret et al., 2002). 15d-PGJ_2 is known as an agonist of the peroxisome proliferator-activated receptor (PPAR)γ (Forman et al., 1995), which plays a central role in the adipogenesis, enhances the sensitivity to insulin, and inhibits inflammatory responses (Murphy and Holder, 2000).

In the present study, we showed, using human CRTH2 transfectants, that ramatroban bound to CRTH2 with high affinity comparable to the prostanoid TP receptor, and that ramatroban antagonized CRTH2 in a competitive manner. Furthermore, we examined the effects of ramatroban on CRTH2 activities induced by 15d-PGJ_2 and indomethacin, also recently identified as CRTH2 agonists.

2. Materials and methods

2.1. Reagents

Ramatroban was synthesized at Bayer Yakuhin Ltd. (Shiga, Japan).[^{3}H]ramatroban and (*E*)-5-[[[(3-pyridinyl)[3-(trifluoromethyl)phenyl]-methylen]amino]oxy] pentanoic acid (ridogrel) were prepared at Bayer AG. (Wuppertal, Germany). PGD_2 was purchased from Sigma-Aldrich (St. Louis, MO). 13, 14-dihydro-15-keto-prostaglandin D_2 (13, 14-dihydro-15-keto-PGD_2), 15R-methyl-prostaglandin D_2 (15R-methyl-PGD_2), 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), 7-[3-[[2-[(phenylamino)carbonyl] hydrazino]methyl]7-oxabicyclo[2.2.1]hept-2-yl]-,[1*S*-[1α, 2α(*Z*), 3α, 4α]]-]5-heptenoic acid (SQ29548) and 5-(6-Carboxyhexyl)-1-(3-cyclohexyl-3-hydroxypropyl) hydantoin (BW245C) were purchased from Cayman (Ann Arbor, MI). 9, 11-dideoxy-9α, 11α-methanoepoxy $PGF_{2\alpha}$ (U46619) and 3-isobutyl-1-methylxanthin (IBMX) were purchased from BIOMOL Research Labs Inc. (Plymouth Meeting, PA). Fluo-3AM and pluronic F-127 were purchased from Molecular Probes (Eugene, OR).

2.2. Generation of human CRTH2 transfectants

The human CRTH2 stable transfectants were generated as described previously (Sugimoto et. al., 2003). Briefly, the pEAK10 expression vector containing human *CRTH2* gene 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) and maintained in RPMI-1640 medium (Gibco BRL, Scotland, U.K.) supplemented with 10% heat-inactivated fetal calf serum (JRH Biosciences, KS), 292 μg/ml L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, St. Louis, MO).

In preliminary experiments, the concentrations of dimethyl sulfoxide (DMSO) in working dilutions used in this study (<0.1%) were shown to have no effect on receptor binding, Ca^{2+} mobilization, cAMP production and cell migration assays. We also confirmed that there were no functional prostanoid DP or TP receptors on CRTH2-transfected L1.2 cells (Sugimoto et al., 2003).

2.3. Receptor binding assay

CRTH2 transfectants were suspended in binding buffer (50 mM Tris-HCl, pH 7.4, 40 mM $MgCl_2$, 0.1% bovine serum albumin, 0.1% NaN_3). Cell suspension (2×10^5 cells) and [^{3}H] ramatroban were mixed in a 96-well U-bottom polypropylene plate and incubated 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). For saturation binding experiments, non-specific binding was determined by incubating the cell suspension in the presence of 100 μM unlabeled ramatroban. Competitive binding experiments were performed in the presence of 2.5 nM [^{3}H]ramatroban and various concentrations of competitive ligands.

2.4. Ca^{2+} mobilization assay

Ca^{2+} loading buffer was prepared by mixing 1 μM of Fluo-3AM and pluronic F-127 in Ca^{2+} assay buffer (20 mM HEPES, pH 7.6, 0.1% bovine serum albumin, 1 mM probenecid, Hanks' solution). The CRTH2 transfectants were suspended in Ca^{2+}

loading buffer at 6×10^6 cells/ml, and incubated for 60 min 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, NY) at 2×10^5 cells/well. Cells were incubated with various concentrations of ramatroban for 5 min at room temperature. Fluorescence was measured with emission at 480 nm on a FDSS6000 fluorometer (Hamamatsu Photonics, Hamamatsu, Japan).

2.5. cAMP production assay

CRTH2 transfectants were suspended in cAMP assay buffer (20 mM HEPES, pH 7.4, 0.1% bovine serum albumin, 250 mM IBMX, Hanks' solution) at 5×10^5 cells/well and incubated with various concentrations of ramatroban for 5 min at room temperature. After stimulation with 10 μM of forskolin for 5 min, cells were incubated with various ligands for 30 min at 37 °C, 5% CO_2. The cAMP content was determined using cAMP-Screen™ System (Applied Biosysytems, Foster City, CA). Maximal inhibition of forskolin-stimulated cAMP production was determined in the presence of 1 μM PGD_2. In preliminary experiments, the production of cAMP was not observed after pre-incubation with ramatroban alone.

2.6. Migration assay

CRTH2 transfectants were suspended in migration buffer (20 mM HEPES, pH 7.6, 0.1% bovine serum albumin, Hanks' solution) at 4×10^6 cells/ml. Fifty micro liters of the cell suspension (2×10^5 cells/well) was then dispensed into the upper chamber and 30 μl of ligand solution was added to the lower chamber of a 96-well type migration chamber (diameter=5 μm, #106-5, Neuro Probe, Gaithesburg, MD). Cells were pre-incubated with various concentrations of ramatroban for 10 min at 37 °C. The migration assay was performed in a humidified incubator at 37 °C, 5% CO_2 for 4 h. The number of cells migrated into the lower chamber was counted by a fluorescence activated cell sorter (FACS), as described previously (Palframan et al., 1998).

3. Results

3.1. Binding profile of [^{3}H]ramatroban to CRTH2

Receptor binding assays were performed to investigate the binding profile of [^{3}H]ramatroban to CRTH2. [^{3}H]ramatroban bound to CRTH2 transfectants in a concentration-dependent and saturable manner but not to non-transfected parental cells (Fig. 1A). From a Scatchard plot analysis, the K_d and B_{max} values were calculated as 7.2 nM and 92.5 pM (a number of 27,800 binding sites/transfectant), respectively (Fig. 1B). Hill plot analysis showed a slope of 1.00, signifying a non-cooperative bimolecular interaction between ramatroban and CRTH2 (Fig. 1C). In competitive binding assays, non-labeled ramatroban inhibited the binding of [^{3}H]ramatroban to CRTH2 in a concentration-dependent manner with a K_i value of 41 nM (Fig. 2B). The binding of [^{3}H]ramatroban to CRTH2 was inhibited by CRTH2 agonists such as PGD_2, 13, 14-dihydro-15-keto-PGD_2 or 15R-methyl-PGD_2 with K_i values of 23, 40, and 1.2 nM, respectively, but not by the prostanoid TP receptor agonist, U46619, or prostanoid DP receptor agonist, BW245C, up to 10 μM (Fig. 2A). Indomethacin also inhibited the binding of [^{3}H]ramatroban to CRTH2 in a concentration-dependent manner with a K_i value of 890 nM, which was 20-fold lower affinity than that of ramatroban (Fig. 2B). Prostanoid TP receptor antagonists, SQ29548 and ridogrel did not show any effects up to 10 μM (Fig. 2B).

3.2. Effects of ramatroban on various CRTH2 agonists-induced Ca^{2+} mobilization in CRTH2 transfectants

At first, we confirmed that neither PGD_2 nor U46619 induced Ca^{2+} mobilization in empty vector-transfected or in non-transfected parental cells. This suggests that there are no functional CRTH2, prostanoid DP or TP receptors on parental cells. We also confirmed that U46619 did not induce Ca^{2+} mobilization in CRTH2-transfectants and BWA868C did not inhibit PGD_2-induced Ca^{2+} mobilization in CRTH2 transfectants. These results suggest that there are no functional prostanoid TP or DP receptors on CRTH2-transfectants. Then,

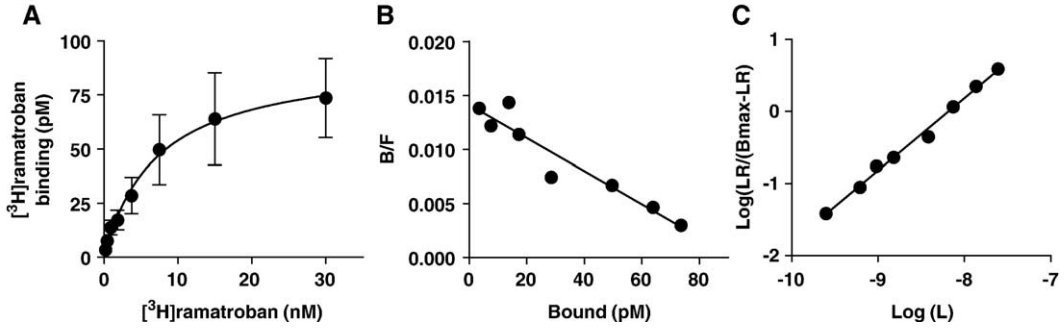

Fig. 1. The binding of [^{3}H]ramatroban to CRTH2 transfectants. (A) Saturation binding of [^{3}H]ramatroban to CRTH2 transfectants. (B) Scatchard plot of [^{3}H]ramatroban binding to CRTH2 transfectants. (C) Hill plot of [^{3}H]ramatroban binding to CRTH2 transfectants. Various concentrations of [^{3}H]ramatroban were incubated with CRTH2 transfectants as described in the Methods. Non specific binding was obtained by incubating with 100 μM unlabeled ramatroban. Data represent mean values±S.E.M. of 5 independent experiments.

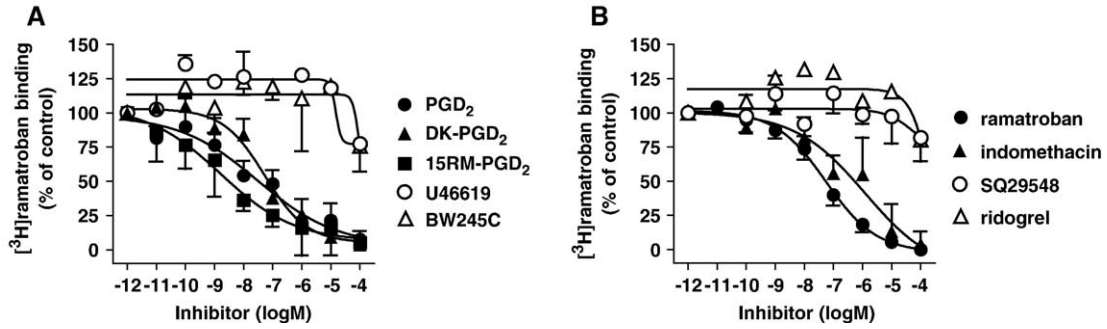

Fig. 2. Effects of ramatroban on [3H]ramatroban binding to CRTH2 transfectants. (A) CRTH2 transfectants were incubated with 2.5 nM [^{3}H]ramatroban together with various agonists such as PGD_2 (n=5), 13, 14-dihydro-15-keto-PGD_2 (DK-PGD_2, n=9), 15R-methyl-PGD_2 (15RM-PGD_2, n=3), U46619 (n=4) or BW245C (n=4). (B) CRTH2 transfectants were incubated with 2.5 nM [^{3}H]ramatroban together with various antagonists such as ramatroban (n=9), indomethacin (n=3), SQ29548 (n=5) or ridogrel (n=2). Data represent mean values±S.E.M.

we evaluated the effects of various CRTH2 agonists on CRTH2 by using CRTH2-transfectants.

Various CRTH2 agonists, such as PGD_2, 13, 14-dihydro-15-keto-PGD_2 and 15R-methyl-PGD_2, induced Ca^{2+} mobilization in CRTH2 transfectants with EC_{50} values of 1.2, 3.1, and 1.6 nM, respectively (Fig. 3A). These agonist-induced Ca^{2+} responses were inhibited by ramatroban in a concentration-dependent manner with IC_{50} values of 160, 110, and 760 nM, respectively (Fig. 3B).

The PPARγ agonist, 15d-PGJ_2 and a COX inhibitor, indomethacin also induced Ca^{2+} responses in CRTH2 transfectants and this was confirmed in our study with EC_{50} values of 110 and 49 nM, respectively (Fig. 3A). However, 15d-PGJ_2 induced a greater Ca^{2+} response at higher concentrations compared to other CRTH2 agonists (Fig. 3A). Ramatroban inhibited 15d-PGJ_2- and indomethacin-induced Ca^{2+} mobilization in a concentration-dependent manner with IC_{50} values of 46 and 37 nM, respectively (Fig. 3B).

3.3. Competitive inhibitory effects of ramatroban on CRTH2 activation

To further examine the inhibitory effects of ramatroban on CRTH2 activation, we performed PGD_2-induced Ca^{2+} mobilization assays in CRTH2 transfectants in the presence of various concentrations of ramatroban. Ramatroban caused a concentration-related rightward shift of the PGD_2 concentration–response curves with a pA_2 value of 8.5 and slope value of 0.81 as assessed by Schild plot (Fig. 4A), suggesting that ramatroban is a competitive antagonist for human CRTH2. Ramatroban also shifted the concentration–response curves of 13, 14-dihydro-15-keto-PGD_2 and 15R-methyl-PGD_2 to the right with pA_2 values of 8.1 and 7.8, with slope values of 0.81 and 0.83, respectively (data not shown). Furthermore, ramatroban caused a concentration-related rightward shift of the 15d-PGJ_2 and indomethacin concentration–effect curves with pA_2 values of 8.5 and 8.6, and slope values of 0.81 or 0.76, respectively (Fig. 4B, C).

3.4. Effects of ramatroban on cAMP production stimulated by indomethacin and 15d-PGJ_2 in human CRTH2 transfectants

Another functional assay, cAMP production, was measured to investigate the effects of ramatroban on 15d-PGJ_2-or indomethacin-treated CRTH2 transfectants. Various CRTH2 agonists such as PGD_2, 13, 14-dihydro-15-keto-PGD_2 and 15R-methyl-PGD_2 reduced forskolin-induced cAMP production in CRTH2 transfectants with EC_{50} values of 0.24, 2.8, and 0.43 nM, respectively (Fig. 5A). These effects by PGD_2, 13, 14-dihydro-15-keto-PGD_2 and 15R-methyl-PGD_2 were reversed by ramatroban in a concentration-dependent manner (Fig. 5B). 15d-PGJ_2 and indomethacin also reduced

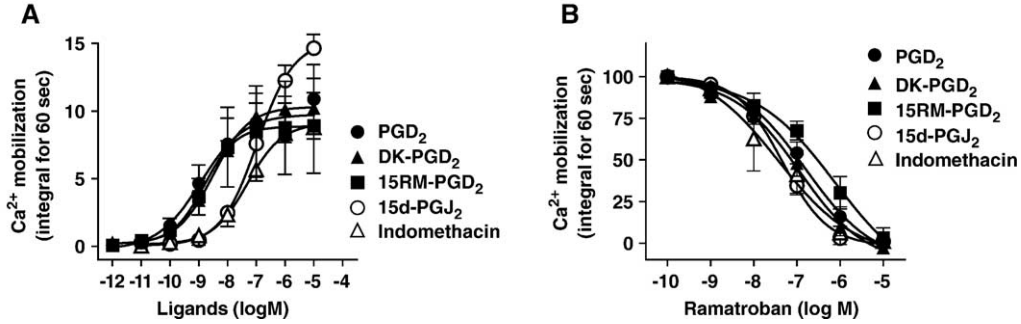

Fig. 3. Effects of ramatroban on Ca^{2+} mobilization in CRTH2 transfectants. (A) Concentration-response of Ca^{2+} mobilization in CRTH2 transfectants induced by PGD_2 (n=4), 13, 14-dihydro-15-keto-PGD_2 (DK-PGD_2, n=4), 15R-methyl-PGD_2 (15RM-PGD_2, n=3), 15d-PGJ_2 (n=3) or indomethacin (n=3). (B) Effects of ramatroban on Ca^{2+} mobilization in CRTH2 transfectants induced by 10 nM PGD_2 (n=4), 13, 14-dihydro-15-keto-PGD_2 (DK-PGD_2, n=4), 15R-methyl-PGD_2 (15RM-PGD_2, n=3) or 100 nM 15d-PGJ_2 (n=3), indomethacin (n=3). Data represent mean values±S.E.M.

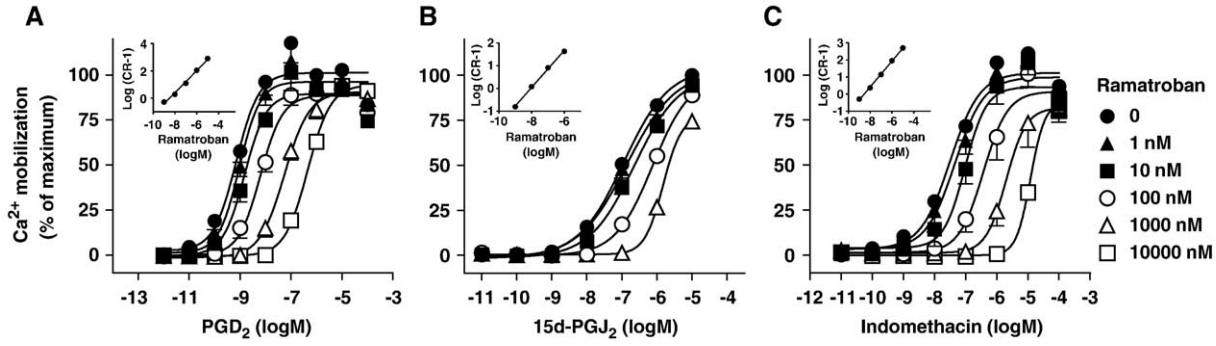

Fig. 4. Competitive inhibitory effects of ramatroban on CRTH2. CRTH2 transfectants were incubated with various concentrations of ramatroban and Ca^{2+} mobilization induced by various concentrations of PGD_2 (A. $n=4$), 13, 14-dihydro-15-keto-PGD_2 (DK-PGD_2, $n=4$), 15R-methyl-PGD_2 (15RM-PGD_2, $n=3$), 15d-PGJ_2 (B. $n=3$) or indomethacin (C. $n=3$) were monitored. pA_2 values were calculated by Schild plots.

forskolin-induced cAMP production in CRTH2 transfectants with EC_{50} values of 49 and 4.5 nM, respectively (Fig. 5A). These effects by 15d-PGJ_2 and indomethacin were reversed by ramatroban in a concentration-dependent manner (Fig. 5B).

3.5. Effects of ramatroban on indomethacin- and 15d-PGJ_2-mediated migration of CRTH2 transfectants

It is well known that cells such as eosinophils expressing CRTH2 migrate in response to PGD_2. We investigated the effects of ramatroban on CRTH2 ligand-induced migration using human CRTH2 transfectants. PGD_2, 14-dihydro-15-keto-PGD_2 and 15R-methyl-PGD_2-stimulated transfectants demonstrated characteristic bell-shaped concentration-dependent migration responses. The half maximal responses (EC_{50} values) were achieved at 0.5, 1.3, and 0.2 nM, respectively (Fig. 6A). 15R-methyl-PGD_2 produced three-fold greater response in the number of cells migrated when compared with PGD_2 and 14-dihydro-15-keto-PGD_2. Ramatroban completely blocked the migration of CRTH2 transfectants induced by sub-optimal concentrations of PGD_2 (1 nM), 14-dihydro-15-keto-PGD_2 (3 nM), or 15R-methyl-PGD_2 (1 nM), in a concentration-dependent manner with IC_{50} values of 140, 140, or 43 nM, respectively (Fig. 6C). Indomethacin and 15d-PGJ_2 similarly demonstrated bell-shaped dose–response curves in migration assays. Compared with 15R-methyl-PGD_2, both indomethacin and 15d-PGJ_2 were less potent, with EC_{50} values of 40 and 32 nM, respectively (Fig. 6B). The maximal response of indomethacin was similar to that of 15R-methyl-PGD_2 but the response of 15d-PGJ_2 was far greater than that of 15R-methyl-PGD_2. Ramatroban completely blocked the migration of CRTH2 transfectants induced by sub-optimal concentrations of indomethacin (100 nM) and 15d-PGJ_2 (100 nM) in a concentration-dependent manner with IC_{50} values of 120 and 60 nM, respectively (Fig. 6D).

4. Discussion

In this report, we showed that [^{3}H]ramatroban bound to CRTH2 with a K_d value of 7.2 nM (Fig. 1). We also demonstrated that ramatroban caused a concentration-related rightward shift of the PGD_2 concentration–response curves with a pA_2 value of 8.5 in the PGD_2-induced Ca^{2+}mobilization assay (Fig. 4A). The K_d value of [^{3}H]ramatroban binding to the prostanoid TP receptor on human platelets was reported as 8.7 nM (Theis et al., 1992) and the pA_2 value for ramatroban antogonism of U46619 (prostanoid TP receptor agonist)-induced contractions of human pulmonary vein smooth muscle was reported as 8.9 (Walch et al., 2001). These results suggest that ramatroban bound to CRTH2 and the prostanoid TP

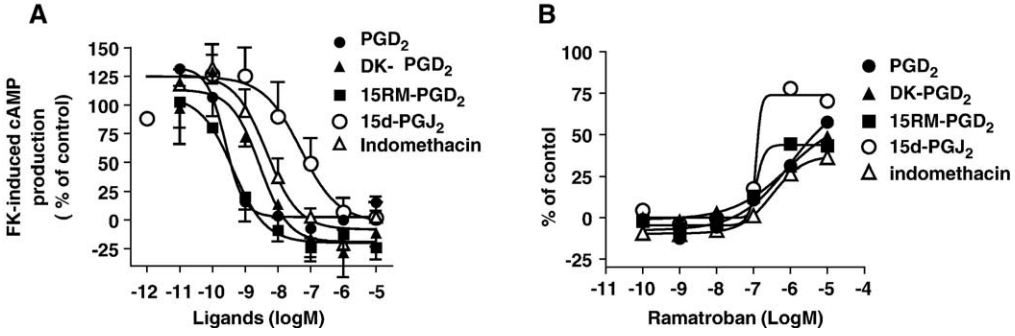

Fig. 5. Effects of ramatroban on cAMP production in CRTH2 transfectants. (A) Concentration-response of 10 μM forskolin (FK)-induced cAMP production in CRTH2 transfectants induced by PGD_2 ($n=6$), 13, 14-dihydro-15-keto-PGD_2 (DK-PGD_2, $n=4$), 15R-methyl-PGD_2 (15RM-PGD_2, $n=4$), 15d-PGJ_2 ($n=6$) or indomethacin ($n=6$). Data represent mean values±S.E.M. (B) Effects of ramatroban on 10 μM forskolin (FK)-induced cAMP production in CRTH2 transfectants induced by 10 nM PGD_2 ($n=2$), 100 nM 13, 14-dihydro-15-keto-PGD_2 (DK-PGD_2, $n=2$), 10 nM 15R-methyl-PGD_2 (15RM-PGD_2, $n=2$), 1000 nM 15d-PGJ_2 ($n=2$) or 100 nM indomethacin ($n=2$).

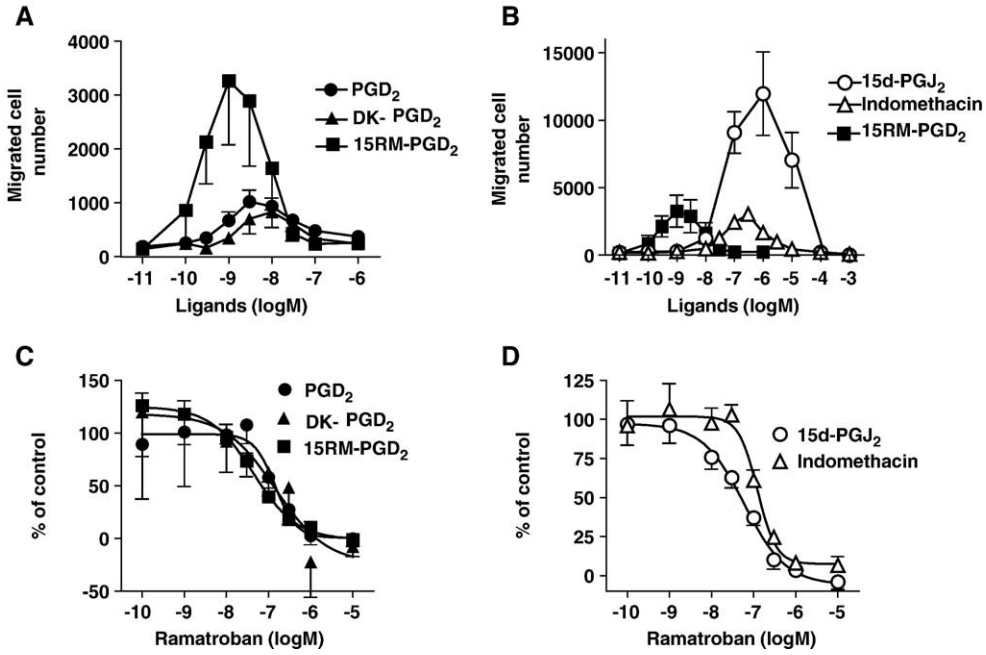

Fig. 6. Effects of ramatroban on migration of CRTH2 transfectants. (A, B) Concentration-response of migration of CRTH2 transfectants induced by PGD_2 ($n=5$), 13, 14-dihydro-15-keto-PGD_2 (DK-PGD_2, $n=4$), 15R-methyl-PGD_2 (15RM-PGD_2, $n=3$), 15d-PGJ_2 ($n=5$) or indomethacin ($n=4$). (C, D) Effects of ramatroban on migration of CRTH2 transfectants induced by 1 nM PGD_2 ($n=7$), 3 nM 13, 14-dihydro-15-keto-PGD_2 (DK-PGD_2, $n=3$), 1 nM 15R-methyl-PGD_2 (15RM-PGD_2, $n=4$), 100 nM 15d-PGJ_2 ($n=4$) or 100 nM indomethacin ($n=4$). Data represent mean values±S.E.M.

receptor with a similar affinity and its inhibitory effects on CRTH2 and the prostanoid TP receptor are competitive in manner. These findings will be critical reference points in experimental settings using ramatroban as a research tool, and in the clinical setting. These results also strongly support our previous report (Sugimoto et al., 2003). In our previous report, we showed that ramatroban, which had been thought of as a specific prostanoid TP receptor antagonist, antagonized CRTH2 activities with IC_{50} values of 100, 30 and 170 nM in [^{3}H]PGD_2 binding to CRTH2, PGD_2-induced Ca^{2+}mobilization in CRTH2 transfectants and PGD_2-induced migration of human eosinophils, respectively. Based on these results, we suggested that ramatroban antagonizes eosinophil recruitment into tissue by at least two different mechanisms; via the prostanoid TP receptor and CRTH2. Through prostanoid TP receptor antagonism on endothelial cells, ramatroban inhibits eosinophil adhesion to endothelial cells by inhibiting the TxA_2-mediated expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on human vascular endothelial cells (Ishizuka et al., 1998, our unpublished data). Through CRTH2 antagonism, ramatroban inhibits migration of eosinophils directly.

Our present study reveals another interesting results using indomethacin. Indomethacin is known as an anti-inflammatory agent for its inhibitory effects on COXs and recently it was reported that indomethacin was also an activator of CRTH2. In our experiment, indomethacin completely inhibited the binding of [^{3}H]ramatroban to CRTH2, although the affinity was 20-fold weaker (K_i=890 nM) than that of ramatroban (K_i=41 nM) (Fig. 2). We further studied the signaling mechanism of indomethacin and ramatroban through CRTH2 to clarify these mechanisms. Indomethacin induced Ca^{2+} mobilization in CRTH2 transfectants with the same efficacy as PGD_2 and other CRTH2 ligands such as 13, 14-dihydro-15-keto-PGD_2 or 15R-methyl-PGD_2, although the potency was 30 to 40-fold weaker than those of CRTH2 ligands. Ramatroban inhibited indomethacin-induced Ca^{2+} mobilization completely and caused a concentration-related rightward shift of the indomethacin concentration–response. These results suggest that ramatroban inhibits indomethacin-induced Ca^{2+} mobilization in a competitive inhibitory manner, suggesting that indomethacin shares the same binding site with ramatroban on CRTH2.

15d-PGJ_2 is known to have anti-inflammatory actions based on its agonistic effects on PPARγ and it was reported recently that 15d-PGJ_2 was also an activator for CRTH2. Interestingly, 15d-PGJ_2 induced Ca^{2+} mobilization in CRTH2 transfectants with greater efficacy than those of other CRTH2 ligands. This response was inhibited completely by ramatroban, suggesting that it was mediated via CRTH2. Furthermore, ramatroban also caused a concentration-related rightward shift of the 15d-PGJ_2 concentration–response curves. This result suggests that ramatorban inhibits 15d-PGJ_2-induced Ca^{2+} mobilization in a competitive manner, suggesting that 15d-PGJ_2 also shares a similar binding site with ramatroban on CRTH2.

Furthermore, indomethacin and 15d-PGJ_2 reduced forskolin-induced cAMP production, and induced cell migration of CRTH2 transfectants. Ramatroban inhibited all of these responses. Thus, ramatroban demonstrated antagonistic effects on responses induced by indomethacin and 15d-PGJ_2 via CRTH2, which further compounds the mechanisms by which they exert their anti-inflammatory action—through inhibition of COXs or activation of PPARγ. While this is counterintuitive it may be one

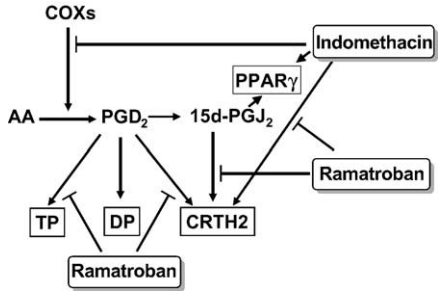

Fig. 7. Effects of ramatroban on migration of CRTH2 transfectants. Ramatroban inhibits PGD_2 activities via prostanoid TP receptor (TP) antagonism. Furthermore, ramatroban inhibits CRTH2 activities induced by PGD_2, 15d-PGJ_2 and indomethacin originally identified as three different ligands for the PGD_2 receptor (DP), peroxisome proliferator-activated receptor (PPARγ) and cyclooxygenases (COXs), respectively.

of many mechanisms dependent on the physiological or pathological processes in question. It is interesting to find that PGD_2, indomethacin and 15d-PGJ_2, originally identified as ligands for three different systems, the prostanoid DP receptor, COXs and PPARγ and stimulating different physiological effects, share a similar binding site with ramatroban on CRTH2 (Fig. 7).

To investigate the signaling efficiency among various CRTH2 agonists, we compared the efficacy and potency of various CRTH2 agonists in receptor binding, Ca^{2+} mobilization, cAMP production and migration assays. 15R-methyl-PGD_2 showed 3-fold higher migrated cell numbers with 2.5-fold higher potency than PGD_2 in inducing migration of CRTH2 transfectants (Fig. 6A), although they showed similar efficacy in inducing Ca^{2+} mobilization and reducing forskolin-induced cAMP production. These results show correlation with the results of human eosinophils. In human eosinophils, 15R-methyl-PGD_2 showed 5-fold higher potency than PGD_2 in upregulating CD11b expression (EC_{50} values of 1.4 and 7 nM, respectively), actin polymerization (EC_{50} values of 3.8 and 13 nM, respectively) and cell migration (EC_{50} values of 1.7 and 10 nM, respectively) (Monneret et al., 2003). Almost all of the maximally efficacious response seen in 15R-methyl-PGD_2-induced cell migration was inhibited by ramatroban in the present study (Fig. 6C), suggesting that its effect was mediated via CRTH2.

In contrast, indomethacin induced 3-fold higher migrated cell numbers but with 65-fold lower potency than PGD_2 in migration of CRTH2 transfectants. The potency in Ca^{2+} mobilization and suppression of cAMP production was 40-fold lower and 200-fold lower than PGD_2, respectively. These results are in close agreement with the study reported by Hirai et al. (Hirai et al., 2002). Indomethacin showed 50-fold lower potency than PGD_2 in Ca^{2+} mobilization using CRTH2 transfectants, and showed similar migrated cell numbers as PGD_2 with 15 to 50-fold lower potency than PGD_2 in human eosinophils, basophils and Th2 cells (Hirai et al., 2002). While it is difficult to speculate on the relevance of a 2 to 3-fold increase in cell number in vitro migration assays of transfectants, it is possible that the efficacy in response is derived from CRTH2-associated signals because ramatroban also inhibited this response.

For 15d-PGJ_2, there are some contradictory reports. Hirai et al. (2001) reported that 15d-PGJ_2 showed 40-fold lower affinity than PGD_2 in [^{3}H]PGD_2 binding to CRTH2-transfected K562 cells (K_i values of 2,300 and 61 nM, respectively). In contrast, Sawyer et al. (2002) described that 15d-PGJ_2 and PGD_2 showed similar affinities in [^{3}H]PGD_2 binding to CRTH2-transfected HEK293 cell membranes (K_i values of 3.2 and 2.4 nM, respectively). Monneret et al. (2002) reported that 15d-PGJ_2 and PGD_2 showed similar potencies in Ca^{2+} mobilization (EC_{50} values of 29 and 60 nM, respectively), actin polymerization (EC_{50} values of 11 and 7 nM, respectively) and CD11b expression (EC_{50} values of 9.4 and 11.7 nM, respectively) in human eosinophils. We obtained interesting results using CRTH2-transfected L1.2 cells. 15d-PGJ_2 showed 90-fold lower potency but showed 1.5-fold greater increase in Ca^{2+} level than PGD_2 in Ca^{2+} mobilization assays, and showed 80-fold lower potency but 12-times greater migrated cell numbers than that of PGD_2 in migration assay using CRTH2 transfectants. Only 15d-PGJ_2 showed such increases in Ca^{2+} level and migrated number of cells among the other CRTH2 agonists tested here. These results suggest that 15d-PGJ_2 has lower potency to CRTH2, but its function could be amplified through different signaling pathways especially at the higher concentration.

Finally, it is known that indomethacin has unwanted side effects causing various complications such as gastrointestinal injury. Shortening of the villi, epithelial stratification, basal lamina degeneration, eosinophil degranulation and infiltration of the epithelium prior to infiltration of the mucosa by neutrophils are earliest histological features of indomethacin-induced intestinal injury in rats (Anthony et al., 1993). It is also known that CRTH2 is expressed on infiltrating cells of the gut mucosa and has been found in acute manifestations of ulcerative colitis (Matsuzaki et al., 2003), thus, indomethacin may also act on these cells via CRTH2. Ramatroban, or specific CRTH2 antagonists, may therefore have potential as therapeutic agents for use in combination with these known anti-inflammatory principles, or as a counterbalance to toxicity associated with similar drugs.

This is the first report showing the evidence for direct ramatroban binding to CRTH2, revealing its competitive inhibitory effects and other interesting findings that PGD_2, indomethacin and 15d-PGJ_2 share the same binding site with ramatroban on CRTH2.

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