Inhibitory effect of the 4-aminotetrahydroquinoline derivatives, selective CRTH2 antagonists, on eosinophil migration induced by prostaglandin D2

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Running title: Effect of CRTH2 antagonists on eosinophil migration

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Abbreviations list:

K117, (2R*,4S*)-N-(1-Benzoyl-2-methyl-1,2,3,4-tetrahydroquinolin-4-yl)-N-phenylisobutyramide; K376, (2R*,4S*)-N-(2-Methyl-1-propionyl-1,2,3,4-tetrahydroquinolin-4-yl)-N-phenylacetamide; K604, (2R*,4S*)-N-(1-Benzoyl-2-methyl-1,2,3,4-tetrahydroquinolin-4-yl)-N-phenylcyclopropanecarboxamide; BW245c, (4S)-(3-[(3R,S)-3-cyclohexyl-3-hydroxypropyl]-2,5-dioxo)-4-imidazolidineheptanoic acid; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; CD, cluster of differentiation; IL-, interleukin-; PGD2, prostaglandin D2; PCR, polymerase chain reaction; SAS, statistical analysis system; ANOVA, analysis of variance.

Section assignment: Inflammation & Immunopharmacology
Abstract

Prostaglandin (PG) D$_2$, a major cyclooxygenase metabolite generated from immunologically stimulated mast cells, is known to induce activation and chemotaxis in eosinophils, basophils and Th2 lymphocytes via a newly identified PGD$_2$ receptor, chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2). CRTH2 is hypothesized to play an important role in the outcome of allergic responses. However, the absence of selective CRTH2 antagonists has prevented the elucidation of the role of CRTH2 in pathogenesis of allergic diseases. We now report compounds discovered as selective CRTH2 antagonists, (2$\text{R}^*,4\text{S}^*$)-N-(1-Benzoyl-2-methyl-1,2,3,4-tetrahydroquinolin-4-yl)-N-phenylisobutyramide (K117) and (2$\text{R}^*,4\text{S}^*$)-N-(1-Benzoyl-2-methyl-1,2,3,4-tetrahydroquinolin-4-yl)-N-phenylcyclopropanecarboxamide (K604). K117 and K604 have inhibitory effects on human CRTH2 with Ki values of 5.5 nM and 11 nM, respectively. The effect of these compounds is CRTH2-specific with no cross-reactivity against 15 other receptors and 4 arachidonic acid metabolizing enzymes. K117 and K604 has no effect on the basal Ca$^{2+}$ level and inhibited the Ca$^{2+}$ response induced by PGD$_2$ in 293EBNA cells expressing human CRTH2. Also, K117 and K604 inhibit PGD$_2$-induced human eosinophil chemotaxis with IC$_{50}$ values of 7.8 nM and 42.2 nM, respectively, but do not inhibit the CCR3 agonist, eotaxin-induced chemotaxis. These results indicate that K117 and K604 are highly potent and selective antagonists for human CRTH2. These compounds have possibilities to become useful tools to explore CRTH2 functions in allergic diseases.
Introduction

Prostaglandin D₂ (PGD₂) is a major cyclooxygenase metabolite produced by mast cells responding to IgE-dependent stimuli (Lewis et al., 1982). In patients with allergic rhinitis, bronchial asthma and atopic dermatitis, PGD₂ levels increase dramatically following allergen challenge in nasal and bronchial lavage fluids, and skin chamber fluids, respectively (Naclerio et al., 1983, 1985; Murray et al., 1986; Charlesworth et al., 1991).

PGD₂ exerts a variety of actions in various tissues and cells via two G protein-coupled receptors, DP and CRTH2 (Chemoattractant Receptor-homologous molecule expressed on T-Helper type 2 cells) (Boie et al., 1995; Hirai et al., 2001). CRTH2 was cloned as a Th2-specific marker by differential display (Nagata et al., 1999). CRTH2-mediated signals enhance the IL-2, IL-4, IL-5, and IL-13 production by Th2 cells and cause up-regulation of CD11b and CD40L in resting Th2 cells (Tanaka et al., 2004). CRTH2 is expressed not only on Th2 cells, but also on eosinophils and basophils, and induces their migration (Hirai et al., 2001). Gervais et al., (2001) have demonstrated that PGD₂ induces degranulation of eosinophil via CRTH2 stimulation. Since PGD₂ has been shown to enhance the release of leukotriene C₄ in response to the calcium ionophore A23187 in eosinophils (Raible et al., 1992), this phenomenon may be through the activation of CRTH2. These reports have strongly suggested the critical role of PGD₂ and CRTH2 in allergic diseases. The pharmacologic blockade of PGD₂-mediated events at CRTH2 receptors may then reduce the allergic pathogenesis caused by activated Th2 cells, basophils and eosinophils. However, the importance of CRTH2 in allergic pathogenesis still remains to be clarified. To elucidate the implications of CRTH2 in these pathological conditions, one of the best
tools is a selective ligand. Recently, ramatroban (BAY u3405), a thromboxane-type prostanoid (TP) receptor antagonist, has been revealed to be a CRTH2 antagonist (Sugimoto et al., 2003) and indomethacin, a cyclooxygenase inhibitor, has been identified as a CRTH2 agonist (Hirai et al., 2002). The use of these compounds is, however, limited due to the other activities they have in addition to their effects on CRTH2. Therefore, we have explored molecules specific for CRTH2.

We now report a small molecule agonist, K376, and antagonists, K117 and K604, which are selective for CRTH2, and the effects of these compounds on eosinophil migration.

Materials and Methods

Chemicals.

The sources of materials used in this study were as follows:  
[5,6,8,9,12,14,15(n)-3H]-prostaglandin D$_2$ ([3H]PGD$_2$, 192 Ci/mmol) was from Amersham Pharmacia Biotech (Buckinghamshire, UK); PGD$_2$, BW245c and 13,14-dihydro-15-keto-PGD$_2$ (DK-PGD$_2$) were from Cayman Chemical (Arbor, MI); Indomethacin was from Sigma (St. Louis, MO); Fura 2-acetoxymethyl ester (Fura 2-AM) was from Dojindo (Kumamoto, Japan); Human recombinant eotaxin was from Strathmann Biotech (Hannover, Germany); Fetal bovine serum was from Intergen (Purchase, NY). K117, K376, and K604, were synthesized in Research Laboratories of Kyowa Hakko Kogyo. All other chemicals and solvents were used in their analytical pure forms.

Construction of pAMoh-hCRTH2 mammalian expression vector.
The human CRTH2 cDNA was generated by reverse-transcription (RT)-PCR using a human leukocyte total RNA as a template and the following primers:

5′-CCAAGCTTCCACCAGTGCGCAAGCAGCAGCTG-3′ (sense) and
5′-TAGGTACCTAATTGAGTGCTGCTCAGC-3′ (antisense). The amplified DNA fragment was cloned into pCR-Blunt vector using Zero Blunt PCR Cloning Kit (Invitrogen, Carlsbad, CA). The nucleotide sequence of cloned cDNA was confirmed to be identical to the reported sequence (Genbank accession number AY507142). The human CRTH2 cDNA was subsequently subcloned into HindIII-Asp718 sites in the mammalian episomal expression vector pAMoh (the Hygromycin B resistant-type of pAMo vector; Sasaki et al., 1993) for generation of a cell line stably expressing human CRTH2.

**Cells and culture.**

The cell line 293EBNA was purchased from Invitrogen and maintained in culture in a humidified atmosphere at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, 100 units/ml of penicillin, 100 µg/ml of streptomycin, and 250 µg/ml of G418 (for EBNA selection). Stable expression of human CRTH2 in 293EBNA cells was achieved by transfection of the pAMoh-hCRTH2 plasmid using Fugene 6 reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. Cells were maintained in culture for 48 h post transfection and then grown in the presence of 300 µg/ml hygromycin B (Wako Jun-yaku, Osaka, Japan) for 2 weeks to select for resistant cells expressing the human CRTH2 receptor (293EBNA/hCRTH2).
Receptor binding assay.

Cultured 293EBNA/hCRTH2 cells were homogenized in ice-cold assay buffer [10 mM HEPES-KOH (pH 7.4), 1 mM EDTA, 10 mM MnCl₂] using a Polytron homogenizer (Kinematica, Luzern, Switzerland). The homogenate was centrifuged at 40,000×g for 20 min at 4°C and then the pellet was suspended in assay buffer and stored frozen at -80 °C until use.

[³H]PGD₂ binding to the membrane fraction of 293EBNA/hCRTH2 was measured by a modification of the method described by Wright et al., 1998. Briefly, the reaction mixture containing an assay buffer, the membrane suspension (20 µg 293EBNA/hCRTH2), 0.5% bovine serum albumin (BSA) and 1 nM [³H]PGD₂ was incubated for 120 min at 25°C, in a volume of 0.2 ml. Nonspecific binding was determined in the presence of 1 µM PGD₂. Under these conditions, specific binding (total minus non-specific) of the radioligand to the receptor reached equilibrium. The binding reactions were terminated by filtration through GF/B filters (Whatman, Maidstone, UK) under reduced pressure using a MT-24 cell harvester (Brandel, Gaithersburg, MD). The filters were washed three times with ice-cold assay buffer and placed in scintillation vials, and the bound radioactivity was determined using a liquid scintillation counter, LS6500 (Beckman Coulter, Fullerton, CA). The concentration-response curves were obtained from assays performed in duplicate at four concentrations of each drug.

Selectivity Panel.

The binding assays for 15 receptors and the enzymatic assays for 4 arachidonic acid metabolizing
enzymes were run by MDS Pharma Services (Bothell, WA). The target run were: adrenergic receptors $\alpha_1$ (non-selective), $\alpha_2$ (non-selective), and $\beta$ (non-selective); dopamine D$_1$ and D$_2$ receptors; histamine H$_1$ and H$_2$ receptors; muscarinic receptor M$_1$; serotonin receptors 5-HT$_{1A}$ and 5-HT$_2$ (non-selective); leukotriene D$_4$ receptor (Cys-LT$_1$); leukotriene B$_4$ receptor (BLT$_1$); platelet activating factor (PAF) receptors; thromboxane A$_2$ receptor (TP); prostaglandin D$_2$ receptor (DP); cyclooxygenase 1 and 2 (COX-1, COX-2); 5-lipoxygenase (5-LO); and thromboxane (TX) synthase. All assays were run using recombinant human receptors and enzymes, except where noted. The full methods and references can be found on the MDS Pharma Services Website (http://www.mdsps.com/). The assays were run at 1 $\mu$mol/L of all test compounds, and the percentage of inhibition is given as the average of two experiments performed in duplicate. The abbreviation definitions for used ligands are as follows: SCH23390, $R(\pm)$-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1$H$-3-benzazepine hydrochloride; NMS, N-methylscopolamine; 8-OH-DPAT, 8-hydroxy-2-di-n-propylamino-tetralin; SQ29548, [1S-[1$\alpha$,2$\alpha$(Z),3$\alpha$,4$\alpha$]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid.

**Ca$^{2+}$ mobilization assay.**

Cells (2 $\times$ 10$^6$ cells/ml) were preincubated with 3 $\mu$M Fura 2-AM and 0.003% pluronic F-127 (Asahi-denka Kogyo, Tokyo, Japan) in Hanks' balanced salt solution (HBSS) buffer for 1 h at 37°C. After washing three times with HBSS buffer to remove any extracellular dye, the cells were suspended in HBSS buffer at 2 $\times$ 10$^6$ cells/ml and kept in the dark at room temperature. The cell suspension was...
transferred to each cuvette, stirred with a magnetic stirrer, and the fluorescence intensity of Fura 2 was quantified using a CAF-100 Ca\(^{2+}\) analyzer (Japan Spectroscopic, Tokyo, Japan). For measuring the antagonistic activity of compounds, antagonists were added to each cuvette 1.5 min before the addition of PGD\(_2\). The ratio of the fluorescence intensities at excitation wavelengths of 340 nm and 380 nm was recorded.

**Preparation of human eosinophils.**

All donors fully understood and consented to the procedure. Human eosinophils were purified according to the procedure described by Hansel et al. (1991). In brief, granulocytes were isolated from the peripheral blood of non-allergic, normal volunteers by discontinuous Ficoll gradient centrifugation. The granulocytes were then incubated for 30 min at 4\(^{\circ}\)C with magnetic beads coated with anti-CD16 (Miltenyi Biotech, Bergish Gladbach, Germany). Eosinophils and neutrophils were thereafter separated in a steel matrix column. Neutrophils attached to anti-CD16 beads stayed in the column, whereas eosinophils were eluted from the column. The purity of the isolated eosinophils was over 90%.

Eosinophils were washed with 0.2% BSA-phosphate-buffered saline (PBS) and resuspended in a chemotaxis assay buffer at the concentration of \(1 \times 10^6\) cells /ml.

**Human eosinophil chemotaxis.**

For the chemotaxis assay, the test compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted in RPMI1640 medium with 10% heat-inactivated fetal bovine serum (chemotaxis assay buffer).
The final concentration of DMSO in a test compound solution was 0.1%. PGD$_2$ and BW245c were dissolved in ethanol. DK-PGD$_2$ dissolved in methyl acetate was evaporated with a nitrogen stream and dissolved in the chemotaxis assay buffer before use. Human recombinant eotaxin was dissolved in distilled water. All chemoattractants were diluted with chemotaxis assay buffer before use. The chemotaxis assays were performed using 24-well transwell plates with 3-µm pore-size polycarbonate filters (Corning Costar), as already described (Gervais et al. 2001). The chemoattractants in chemotaxis assay buffer (0.5 ml) were placed in the lower chamber. In the upper chamber, purified eosinophils (1 × 10$^5$ cells) were loaded in a volume of 0.1 ml. The transwells were then incubated for 3 h at 37°C in a 5% CO$_2$ humidified atmosphere. The migrated cells into the lower chamber were recovered and counted by a flow cytometry (Beckton Deckinson). For evaluation of agonistic activity on human CRTH2, the test compounds in chemotaxis assay buffer were placed in the lower chamber without any chemoattractants and the cells were loaded into the upper chamber. For evaluation of antagonistic activity on human CRTH2, the test compounds were placed in the lower chamber with chemoattractants and the cells incubated with the test compound for 15 min at 37°C were loaded into the upper chamber. For the control wells, chemotaxis assay buffer with 0.1% DMSO was used. As the chemoattractants, 100 nM of PGD$_2$ and 10 ng/ml of human recombinant eotaxin were used as a CRTH2 agonist and a CCR3 agonist, respectively. All experiments were performed in triplicate.

**Statistical Analysis.**

Data are expressed as mean ± SEM. The statistical significance was determined by one-way
Results

Competition for $[^3H]PGD_2$ specific binding to membrane of 293EBNA/hCRTH2 cells.

The analysis of $[^3H]PGD_2$ binding to human CRTH2 and the Scatchard transformation were shown in Fig. 1A. As previously reported (Sawyer et al., 2002), $[^3H]PGD_2$ bound with high affinity ($K_d = 1.4 \pm 0.035 \text{nM}$, $B_{max} = 1758 \pm 19 \text{fmol/mg membrane protein}$) to the membranes prepared from 293EBNA/hCRTH2. We identified three low molecular weight compounds, K376, K117 and K604, (Fig. 1B) having potent inhibitory effects in this equilibrium competition assay. Then, further evaluation was performed to reveal their ability to compete with $[^3H]PGD_2$ specific binding. These compounds inhibited the binding of $[^3H]PGD_2$ to human CRTH2 in a concentration-dependent manner (Fig1C). The $K_i$ values of K376, K117 and K604 for hCRTH2 were 91, 5.5 and 11 nM, respectively.

In addition, these compounds showed a significant selectivity for human CRTH2. Even at a concentration of 1 $\mu$M, these compounds have little effect on the specific binding of radioligands for $\alpha_1$- and $\alpha_2$-adrenoceptors, $\beta$-adrenoceptors, dopamine $D_1$ and $D_{2L}$ receptors, histamine $H_1$ and $H_2$ receptors, muscarinic $M_1$ receptors, serotonin 5-HT$_{1A}$ and 5-HT$_2$ receptors, Cys-LT$_1$ receptors, BLT$_1$ receptors, PAF receptors, thromboxane A$_2$ receptors, and prostanoid DP receptors, and on the enzymatic activities of arachidonic acids metabolizing enzymes such as COX-1, COX-2, 5-LO, and TX synthase (Table 1).
Effects of K376, K117 and K604 on Ca\textsuperscript{2+} mobilization in 293EBNA/hCRTH2 cells.

PGD\textsubscript{2} induced Ca\textsuperscript{2+} mobilization in 293EBNA/hCRTH2 cells at nanomolar concentrations, but did not in mock-transfected 293EBNA/Vec cells even at 10 \( \mu \)M (data not shown). K376 induced Ca\textsuperscript{2+} mobilization in 293EBNA/hCRTH2 cells at submicromolar concentrations with around one order of magnitude lower potency than that of PGD\textsubscript{2} (Fig. 2B); however, K376 induced no significant Ca\textsuperscript{2+} mobilization in the mock-transfected 293EBNA/Vec cells even at 10 \( \mu \)M (Fig. 2A). Having once responded to PGD\textsubscript{2}, 293EBNA/hCRTH2 cells did not show any responses to K376 in the Ca\textsuperscript{2+} mobilization assay, and vice versa (data not shown).

On the other hand, although K117 and K604 showed no effect on the Ca\textsuperscript{2+} mobilization in 293EBNA/hCRTH2 cells, K117 and K604 inhibited the Ca\textsuperscript{2+} mobilization induced by 3 nM PGD\textsubscript{2} in a concentration-dependent manner (Figs. 3A and 3B, respectively).

Agonistic effect of K376 on human eosinophil chemotaxis.

Consistent with the previous observation (Hirai et al., 2001), human eosinophils exhibited chemotactic responses to either CRTH2 agonists PGD\textsubscript{2} or DK-PGD\textsubscript{2}, but not to a DP agonist BW245c (Fig. 4A). Also, a cyclooxygenase inhibitor, indomethacin, which has been reported to have an agonistic activity on human CRTH2 (Hirai et al., 2002), induced a chemotactic activity of human eosinophils (Fig. 4B). K376, a compound showing agonistic activity on human CRTH2 in the Ca\textsuperscript{2+} mobilization assay, induced the weak human eosinophil chemotaxis at the concentration of 10 \( \mu \)M (Fig. 4C).
Antagonistic effects of K117 and K604 on PGD$_2$-induced human eosinophil chemotaxis.

The optimal PGD$_2$ concentration of 100 nM and eotaxin concentration of 10 ng/ml were used to evaluate the antagonistic activity of the compounds. Both K117 and K604, which showed antagonistic activity on human CRTH2 in the Ca$^{2+}$ mobilization assay, inhibited the PGD$_2$-induced human eosinophil chemotaxis in a concentration-dependent manner (Figs. 5A and 5B). The inhibitory concentration 50% (IC$_{50}$) values were 7.8 and 42.2 nM, respectively. On the other hand, neither K117 nor K604 influenced eosinophil chemotaxis induced by eotaxin, the CCR3 agonist, at 1 µM (Fig 5C).

Discussion

CRTH2, identified as one of the two PGD$_2$ receptors, has been speculated to have a critical role in allergic diseases (Hirai et al., 2001). However, the precise role of CRTH2 in allergic pathogenesis still remains to be clarified. One of the best tools for probing the function of this receptor is a CRTH2-specific ligand. Ramatroban (BAY u3405), a thromboxane-type prostanoid (TP) receptor antagonist, has recently been revealed to be a CRTH2 antagonist (Sugimoto et al., 2003). However, the use of ramatroban as a CRTH2 antagonist is limited due to its TP antagonistic activity, although its CRTH2 antagonistic activity is more potent than its TP antagonistic activity. The object of this study is to identify a potent and selective CRTH2 antagonist.

In order to explore the CRTH2 antagonists, we employed a PGD$_2$ binding assay using 293EBNA cell membranes expressing human CRTH2. Saturation analysis of [$^3$H]PGD$_2$ specific binding to human
CRTH2 revealed the presence of a specific binding site with an equilibrium dissociation constant (Kd) of 1.4 nM. This value is one order of magnitude lower than the one (31.3 nM) previously reported by Hirai et al. (2001), in which a whole cell binding assay was performed. This dissociation may reflect the differences in experimental settings such as buffer constitution, incubation time, reaction temperature, and human CRTH2 expression system. It has also been reported that saturation analysis of [3H]PGD₂ specific binding to HEK293 (EBNA) cell membranes expressing CRTH2 reveals the presence of high affinity binding sites with a Kd of 2.5 nM, although there also exists low affinity binding sites (Sawyer et al., 2002).

Using an equilibrium competition binding assay, we have identified 4-aminotetrahydroquinoline derivatives having potent inhibitory activity on human CRTH2. The Ki values of K376, K117 and K604 are 91, 5.5 and 11 nM, respectively. K117 and K604 are more potent than ramatroban, because, calculated from the reported IC₅₀ value (Sugimoto et al., 2003), Ki value of ramatroban is 86 nM. The selectivity of our compounds for CRTH2 is demonstrated by the lack of binding to other representative GPCRs including DP receptors (Table 1).

We have further evaluated these compounds by Ca²⁺ mobilization assay in order to identify whether or not these compounds are antagonists. When PGD₂ is added, 293EBNA/CRTH2 cells show intracellular Ca²⁺ mobilization, but the vector-transfected 293EBNA cells do not show any responses to PGD₂ even at a concentration of 10 µM. Therefore, intracellular Ca²⁺ mobilization in 293EBNA/CRTH2 induced by PGD₂ can be the specific response via CRTH2. This result is consistent with a previous report, which have revealed that CRTH2 can induce Ca²⁺ influx (Hirai et al., 2001). In our assay system,
K117 and K604 do not influence the basal Ca\(^{2+}\) level in 293EBNA/CRTH2 cells by themselves, but inhibit intracellular Ca\(^{2+}\) mobilization induced by 3 nM of PGD\(_2\) in a concentration-dependent manner (Fig. 3). The concentration of 1 µM is sufficient to completely ablate the response. Therefore, these results suggest that K117 and K604 have an antagonistic activity on human CRTH2 without any agonistic activity. On the other hand, K376 induces intracellular Ca\(^{2+}\) mobilization in a concentration-dependent manner in 293EBNA/CRTH2 cells, even though the potency is weaker than PGD\(_2\) and DK-PGD\(_2\), CRTH2 full agonists. Since the potency of K376 in inducing eosinophil chemotaxis is also comparatively weak in spite of its potent activity in the binding assay (Fig. 1C and Fig. 4C), K376 may be a partial agonist, not a full agonist of human CRTH2. The structures of K376, K117 and K604 are quite similar among them. Therefore, it can be possible to manipulate the structure to convert an agonist to an antagonist and vice versa. However, more information about the structure-activity relationship is required, which we are now investigating.

CRTH2 is expressed on human eosinophils, and once activated by PGD\(_2\), a chemotactic response is induced (Hirai et al., 2001). On the other hand, DP is also expressed on human eosinophils, and DP has been considered to negatively regulate the action of other mediators (Monneret et al., 2002). According to previous studies, PGD\(_2\) induces the chemotactic response of eosinophils in our assay system. DK-PGD\(_2\), a metabolite of PGD\(_2\) and selective CRTH2 agonist, also induces eosinophil migration, but BW245c, a selective DP agonist, does not. Therefore, it has also been confirmed that eosinophils shows a chemotactic response via CRTH2. Since both K117 and K604 inhibit eosinophil migration induced by 100 nM of PGD\(_2\), but not by eotaxin, an agonist of CCR3 chemokine receptor,
these compounds selectively inhibit the response via CRTH2 (Figs. 5A and 5B). K117 is a more potent antagonist than K604, because an IC\textsubscript{50} value of K117 (7.8 nM) is lower than that of K604 (42.2 nM). This result is consistent with the results from the competitive binding assay and Ca\textsuperscript{2+} mobilization assay (Figs. 1C and 3). Furthermore, K117 and K604 are more potent compounds than ramatroban, because it has been reported that ramatroban has an IC\textsubscript{50} value of 170 nM in the eosinophil migration assay in which 1 nM of PGD\textsubscript{2} is used as a chemoattractant (Sugimoto et al., 2003).

In allergic responses, there are two phases of reactions. One is the immediate type reaction induced by mast cell-oriented mediators, including histamine and leukotrienes (Naclerio, 1988). The other is late reaction induced by migrated inflammatory cells, such as eosinophils and lymphocytes especially the Th2 phenotype, several hours after an immediate reaction (Frew and Kay, 1990). PGD\textsubscript{2} is well known as an important mediator of the immediate reaction in addition to histamine and leukotrienes, because plenty of PGD\textsubscript{2} is immediately released after the antigen-antibody reaction from mast cells (Schleimer et al., 1985; Murray et al., 1986). It has also been indicated that PGD\textsubscript{2} is continuously released several hours after the immediate reaction (Reddy and Herschman, 1997). Therefore, PGD\textsubscript{2} may work as a mediator in the late allergic reaction through activation of CRTH2 expressed on eosinophils and Th2 cells. To reveal the roles of CRTH2 in the allergic pathology, the evaluation with the CRTH2 antagonists in the animal allergic models is a beneficial approach. K117 and K604, potent and selective CRTH2 antagonists, can be suitable tools in this approach.

In conclusion, we have identified low molecular weight compounds K117 and K604 as selective and potent CRTH2 antagonists. These compounds can be useful tools to explore the CRTH2 functions in
allergic diseases in the future.

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References


Sasaki K, Watanabe E, Kawashima K, Sekine S, Dohi T, Oshima M, Hanai N, Nishi T and


Legends for figures

Fig. 1. Chemical structures of 4-aminotetrahydroquinoline derivatives and effect of compounds on \[^{3}H\]PGD\(_2\) binding to the membranes prepared from CRTH2 expressing cells.

A, Scatchard plot of \[^{3}H\]PGD\(_2\) binding to human CRTH2. B, chemical structures of a, K376, \((2R^*, 4S^*)-N-(2-Methyl-1-propionyl-1,2,3,4-tetrahydroquinolin-4-yl)-N-phenylacetamide; b, K117, \((2R^*, 4S^*)-N-(1-Benzoyl-2-methyl-1,2,3,4-tetrahydroquinolin-4-yl)-N-phenylisobutyramide; c, K604, \((2R^*, 4S^*)-N-(1-Benzoyl-2-methyl-1,2,3,4-tetrahydroquinolin-4-yl)-N-phenylcyclopropanecarboxamide.

C, Inhibitory effect of compounds on the \[^{3}H\]PGD\(_2\) binding to the membranes prepared from CRTH2 expressing cells. The membranes were incubated with various concentrations of compounds (K376: closed circle, K117: closed square, K604: closed triangle) and 1 nM \[^{3}H\]PGD\(_2\). The concentration-response curves were obtained from assays performed in duplicate at four concentrations of each drug.

Fig. 2. Responses induced by K376 in 293EBNA cells stably expressing CRTH2.

K376 was added at 0.1, 1, and 10 \(\mu\)M to Fura-2/AM loaded cells and intracellular Ca\(^{2+}\)-dependent fluorescence changes were recorded. A, Vector-transfected 293EBNA cells did not show any responsiveness to K376. B, Dose response of 293EBNA/CRTH2 cells to K376. K376 showed CRTH2 mediated agonistic activity. Arrows indicate the time points of compound addition. The vertical axis shows the 340/380 ratios. Studies are representatives of three separate experiments.
Fig. 3. Effects of K117 and K604 on PGD₂-induced Ca²⁺ mobilization in 293EBNA cells stably expressing CRTH2.

K117 (A) and K604 (B) were added at 0.1, 0.3, and 1 µM to Fura-2/AM loaded cells, and then PGD₂ was added at 3 nM. Intracellular Ca²⁺-dependent fluorescence changes were recorded. Intracellular Ca²⁺ mobilizations induced by PGD₂ were antagonized by K117 and K604. Arrows indicate the time points of compound addition. The vertical axis shows the 340/380 ratios. Studies are representatives of three separate experiments.

Fig. 4. Eosinophil chemotactic activities of indomethacin and K376.

Chemotaxis was induced by incubation of human eosinophils with PGD₂, DK-PGD₂ (a selective CRTH2 agonist) or BW-245C (a selective DP agonist) for 3 h (A). Chemotaxis was induced by incubation of human eosinophils with indomethacin (B) or K376 (C) for 3 h. Data are shown as means ± SEM of 3 experiments. #P<0.01 vs blank (Dunnett test).

Fig. 5. Effects of K117 and K604 on eosinophil chemotaxis mediated via CRTH2 or via CCR3. Human eosinophils pretreated with indicated concentrations of K117 or K604 for 15 min were incubated with 100 nM PGD₂ (A and B) or 10 ng/ml CCR3-agonist human eotaxin (C) for 3 h. The blank group was incubated without PGD₂. The percentages of inhibition were indicated above the columns. Data are shown as means ± SEM of 5 experiments. #P<0.05 vs blank (Aspin-Welch test). *p<0.05, **p<0.01, ***p<0.001 vs 0.1% DMSO (Williams test).
Table 1  Effect of K376, K117 and K604 on specific binding of radioligand to various receptors and on enzymes involved in arachidonic acid metabolism.

<table>
<thead>
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<th>Target</th>
<th>Percent of inhibition (at 1 µM)</th>
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<tr>
<td>Dopamine D$_{2L}$</td>
<td>0, -4</td>
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<tr>
<td>Histamine H$_1$</td>
<td>10, 0</td>
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</table>

<table>
<thead>
<tr>
<th>K376</th>
<th>K117</th>
<th>K604</th>
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<tbody>
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</table>

Rat brain     0.25 nM $[^3]$H|Prazosin
Rat cerebral cortex 0.7 nM $[^3]$H|Rauwolscine
Rat brain 0.25 nM $[^3]$H|Dihydroalprenol
Human recombinant 1.4 nM $[^3]$H|SCH23390
Human recombinant 0.16 nM $[^3]$H|Spiperone
Human recombinant 1.2 nM $[^3]$H|Pyrilamine
<table>
<thead>
<tr>
<th>Receptor</th>
<th>pIC50 Range</th>
<th>Affinity</th>
<th>Source</th>
<th>Ligand/Concentration</th>
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<tr>
<td>Histamine H&lt;sub&gt;2&lt;/sub&gt;</td>
<td>-11, 1</td>
<td>26, 12</td>
<td>-1, 11</td>
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<tr>
<td>Muscarine M&lt;sub&gt;1&lt;/sub&gt;</td>
<td>8, -6</td>
<td>0, 6</td>
<td>-2, 0</td>
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<td>5-HT&lt;sub&gt;1A&lt;/sub&gt;</td>
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<td>10, 2</td>
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<tr>
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<td>-1, -1</td>
<td>-8, 2</td>
<td>Rat brain</td>
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<tr>
<td>Cys-LT&lt;sub&gt;1&lt;/sub&gt;</td>
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<td>10, -8</td>
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<td>BLT&lt;sub&gt;1&lt;/sub&gt;</td>
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<td>11, 7</td>
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<td>TP</td>
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<td>5, 5</td>
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<td>DP</td>
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<td>COX-1</td>
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<td>TX synthase</td>
<td>-2, 10</td>
<td>-4, 18</td>
<td>4, 18</td>
<td>Human platelets</td>
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These data were obtained at MDS Pharma Services (Bothell, WA). The full methods and references can be found on the MDS Pharma Services website (http://www.mdsps.com/). Values in the table represent the percent inhibition of specific binding of radioligand to target receptors and the percent inhibition of arachidonic acid metabolizing enzymes. The average values for each of the two individual experiments performed in duplicate are shown. In the enzymatic assays, IC50 values of the reference standard compounds are as follows: COX-1; 44 nM Indomethacin, COX-2; 170 nM Rofecoxib, 5-LO; 130 nM Nordihydroguaiaretic Acid, TX synthase; 17 nM 1-(7-Carboxyheptyl)-Imidazole. Experimental details and abbreviation definitions are described in *Materials and Methods.*
Fig. 2

A  293EBNA/Vec

B  293EBNA/hCRTH2

Fluorescence ratio

0 150 Time (s)
Fig. 3

Fluorescence ratio

0 180 Time (s)
Fig. 4

A

Migrated Eosinophils

Concentration (nM)

PGD2
DK-PGD2
BW-245C

B

Migrated Eosinophils

Indomethacin (µM)

0 0.1 0.3 1

C

Migrated Eosinophils

K376 (µM)

0 1 10