

MOL# 101832

Fevipirant (QAW039) a slowly dissociating CRTh2 antagonist with the potential for improved clinical efficacy

David A Sykes, Michelle E Bradley, Darren M Ridby, Elizabeth Willard, John Reilly, Asadh Miah, Carsten Bauer, Simon J Watson, David A Sandham, Gerald Dubois and Steven J Charlton

Novartis Institutes for Biomedical Research, Wimblehurst Road, Horsham, West Sussex, RH12 5AB, UK (DAS, MEB, DMR, EW, JR, AM, SW, DAS, GD, SJC)

Novartis Institutes for Biomedical Research, Cambridge, Massachusetts, USA (DAS, JR)

Novartis Institutes for Biomedical Research, Basel, Switzerland (CB)

School of Life Sciences, Queen's Medical Centre, University of Nottingham, Nottingham NG7 2UH (DAS and SJC)

MOL# 101832

Running title:

Fevipiprant a slowly dissociating CRTh2 antagonist

Addresses for correspondence:

David Sandham PhD
Global Discovery Chemistry
Novartis Institutes of Biomedical Research
100 Technology Square
Cambridge MA-02139
USA
E-mail: david.sandham@novartis.com

and

Prof Steven Charlton
School of Life Sciences
Queen's Medical Centre
University of Nottingham
University of Nottingham
E-mail: [Steven Charlton@nottingham.ac.uk](mailto:Steven.Charlton@nottingham.ac.uk)

Number of text pages: 43 (Supplemental 12)

Number of tables: 4 Tables (Supplemental Tables 3)

Number of figures: 8 (Supplemental Figures 6)

Number of references: 50

Number of words in Abstract: 262

Number of words in Introduction: 749

Number of words in Discussion: 1443

Abbreviations:

MOL# 101832

CRTh2 chemoattractant receptor-homologous molecule expressed on T-helper type 2;

PGD₂ prostaglandin D₂;

CHO, Chinese hamster ovary;

HBSS, Hanks Balanced Salt Solution;

QAW039, 2-(2-methyl-1-(4-(methylsulphonyl)-2-(trifluoromethyl)benzyl)-1H-pyrrolo[2,3-b]pyridine-3-yl)acetic acid (fevipiprant);

NSB, non-specific binding;

CPM, counts per minute;

WBSC, whole blood shape change;

ISC, isolated cellular shape change;

Abstract

Here we describe the pharmacological properties of a series of clinically relevant chemoattractant receptor-homologous molecule expressed on T-helper type 2 (CRTh2) receptor antagonists including fevipiprant (NVP-QAW039 or QAW039) which is currently in development for treatment of allergic diseases. [³H]-QAW039 displayed high affinity for the human CRTh2 receptor (1.14 ± 0.44 nM) expressed in CHO-cells, the binding being reversible and competitive with the native agonist prostaglandin D₂ (PGD₂). The binding kinetics of QAW039 determined directly using [³H]-QAW039 revealed mean kinetic k_{on} and k_{off} values for QAW039 of 4.5×10^7 M⁻¹min⁻¹ and 0.048 min⁻¹ respectively. Importantly, the kinetic off-rate (k_{off}) of QAW039 ($t_{1/2} = 14.4$ min) was >7 fold slower than the slowest reference compound tested AZD-1981. In functional studies QAW039 behaved as an insurmountable antagonist of PGD₂ stimulated [³⁵S]-GTPγS activation and its effects were not fully reversed by increasing concentrations of PGD₂ following an initial 15min incubation period. This behaviour is consistent with its relatively slow dissociation from the human CRTh2 receptor. In contrast the inhibitory effect of the other ligands tested was fully reversed by the 15min time point, whereas QAW039's effects persisted for >180min. All CRTh2 antagonists tested inhibited PGD₂ stimulated human eosinophil shape change but importantly QAW039 retained its potency in the whole blood shape change assay relative to the isolated shape change assay, potentially reflective of its relatively slower off-rate from the CRTh2 receptor. QAW039 was also a potent inhibitor of PGD₂ induced cytokine release in human Th2 cells. Slow CRTh2 antagonist dissociation could provide increased receptor coverage in the face of pathological PGD₂ concentrations, which may be clinically relevant.

Introduction

The chemoattractant receptor-homologous molecule expressed on T-helper type 2 cells (CRTh2) is a class A G protein-coupled receptor involved in the modulation of inflammatory responses. CRTh2 (or DP₂) is also expressed on innate immune cells such as eosinophils, basophils (Nagata et al., 1999a and 1999b) and group 2 lymphoid cells (ILC2) (Xue et al., 2014) and is known to play a role in the pathophysiology of airway disease.

Activation of CRTh2 receptors occurs via the natural ligand prostaglandin D₂ (PGD₂), a major product of the cyclooxygenase pathway released primarily by allergen activated mast cells (Miadonna et al., 1990) in asthmatic and allergic rhinitis patients. High levels of PGD₂ (Murray et al., 1986; Wenzel et al., 1989; Crea et al., 1992; Nowark et al., 1993; Horlak et al., 1998) lead to recruitment and activation of Th2-cells and stimulate eosinophil shape change and migration (Pettipher et al., 2007). PGD₂ also promotes increased CD11b expression, which facilitates cell adhesion on the vascular cell wall and movement of eosinophils from the circulation to the site of inflammation (Monneret et al., 2001; Gyles et al., 2006). More recently it has been reported that patients with severe asthma accumulate a particular subtype of PGD₂-producing mast cells in the airway submucosa and epithelium resulting in increased sputum levels of PGD₂ relative to other steroid treated asthma patients (Balzar et al., 2011).

Stimulation of CRTh2 receptors found in the bronchial epithelium has been shown to drive epithelial differentiation, suggesting that in addition to its well-characterized role in inflammatory cell migration, CRTh2 may contribute to airway remodeling in asthmatic patients (Stinson et al., 2015). Preclinical studies suggest that CRTh2 antagonism may also potentially accelerate apoptosis and clearance of Th2-cells from inflamed tissue, thereby promoting the resolution of the underlying inflammation in allergic asthma and other related conditions such as allergic rhinitis (Xue et al., 2009). In animal models of acute allergen

challenge PGD₂ mediated effects are blocked by CRTh2 antagonists acting on CRTh2 receptors on Th2-cells and inflammatory effector cells downstream of the allergic cascade (Uller et al., 2007). Multiple selective orally available antagonists have been since described in the literature with compounds such as setipiprant (Diamant et al., 2014), BI-671800 (Krug et al., 2014) and OC-459 (Barnes et al., 2012) providing clinical proof of concept in asthma and allergic rhinitis. A comprehensive review of the current status of these and other compounds including the dual CRTh2/thromboxane A₂ receptor (TP) antagonist ramatroban is available (Norman 2014).

Drug-receptor interactions are traditionally characterised in terms of potency and efficacy in preclinical screening programs. However increasing evidence suggests that the time molecules reside at their cellular target can provide an important indicator of their clinical performance (Swinney 2009). In functional assays these slowly dissociating compounds exhibit the well documented phenomenon of ‘hemi-equilibrium’ (Charlton & Vauquelin 2010), characterised functionally as a depressed agonist response and one which can potentially aid the selection of molecules for further pre-clinical development. For these reasons we employed the [³⁵S]-GTPγS assay format to assess the ability of CRTh2 antagonists to inhibit PGD₂ responses over time.

We recently reported the discovery of QAV680, a potent/selective CRTh2 receptor antagonist, suitable for clinical testing in allergic diseases (Sandham et al., 2013). QAV680 dosed twice or four times daily was shown to reduce nasal symptom scores following allergen challenge in patients with allergic rhinitis (Erpenbeck et al., 2014a, 2014b). A follow-up compound was consequently sought with improved duration of action (DOA) giving the potential for once daily dosing in order to increase patient compliance. This optimization resulted in the discovery of 2-(2-methyl-1-(4-(methylsulphonyl)-2-

(trifluoromethyl)benzyl)-1H-pyrrolo[2,3-b]pyridine-3-yl)acetic acid (QAW039, fevipiprant), which is currently in clinical development for asthma and atopic dermatitis.

Previous work has demonstrated different modes of action (MoA) of antagonists of CRTh2 receptors for example studies have suggested a non-competitive MoA for the small molecule antagonist AZD-1981 (Schmidt et al., 2013) whilst structurally similar compounds such as OC-459 and ramatroban have been shown to be competitive (Pettipher et al, 2012; Uller et al., 2007; Mathiesen et al., 2006). A complicating factor/explanation could be the phenomenon of hemi-equilibrium resulting from slow dissociation which leads to insurmountable antagonism under certain conditions and can be inadvertently mistaken for non-competitive inhibition. The aim of the present study was to determine the binding characteristics of QAW039 and a cohort of clinically relevant reference antagonists at the human CRTh2 receptor and to explore the influence of kinetics on compound MoA by investigating the effect of these ligands on PGD₂ stimulated CRTh2 responses in both recombinant and relevant inflammatory cell types.

Materials and Methods

Cell culture and membrane preparation

CHO-cells expressing the human CRTh2 receptor were grown to 80-90% confluence in 500 cm² cell-culture plates at 37°C in 5% CO₂. All subsequent steps were conducted at 4°C to avoid receptor degradation. The cell-culture media was removed and ice cold HBS-EDTA (1x 10 mL; 10 mM HEPES, 0.9% w/v NaCl, 0.2% w/v EDTA pH 7.4) was added to the cells which were then scraped from the plates into a 50 mL Corning tube and subsequently centrifuged at 250 xg for 5 min to allow a pellet to form. The supernatant fraction was aspirated and 10 mL per 500 cm² tray of wash buffer (10 mM HEPES, 10 mM EDTA, pH 7.4) was added to the pellet. This was homogenized using an electrical homogenizer 'ultra-turrax' (position 6, 4x 5 second bursts) and subsequently centrifuged at 48,000 xg at 4°C (Beckman Avanti J-251 Ultracentrifuge) for 30 min. The supernatant was discarded and the pellet re-homogenized and centrifuged as described above, in wash buffer. The final pellet was suspended in ice cold 10 mM HEPES, 0.1 mM EDTA, pH 7.4 at a concentration of 5-10 mg mL⁻¹. Protein concentration was determined by the Bio-Rad Protein Assay based on the method of Bradford (1976), using BSA as a standard. Membrane aliquots were maintained at -80°C until required.

Compounds

QAW039 was prepared using procedures reported for QAV680 (Sandham et al., 2013) and all reference CRTh2 antagonists were prepared according to patent procedures referenced in Lamers et al., (2013). The preparation of [³H]-QAW039 has been previously described, see Luu et al., (2015). [³H]-OC-459 was prepared by direct hydrogen isotope exchange on solid support, further details plus the structures, specific activity and chemical purity of both radioligands are provided in the Supplemental Methods.

Common procedures applying to [³H]-QAW039 and [³H]-OC-459 radioligand binding experiments

All radioligand experiments were conducted in 96 deep-well plates in assay binding buffer (HBSS containing 10 mM HEPES, 1% DMSO and 0.02% pluronic acid pH 7.4) at 37°C. In all cases non-specific binding (NSB) was determined in the presence of 3 µM AZD-1981 a selective CRTh2 antagonist. After the indicated incubation period, bound and free radioligand were separated by rapid vacuum filtration using a FilterMate™ Cell Harvester (Perkin Elmer, Beaconsfield, U.K.) onto 96-well GF/B filter plates pre-treated with assay buffer and rapidly washed three times with ice cold wash buffer 20 mM HEPES containing 1 mM MgCl₂ pH 7.4. After drying (> 4 h) 40 µL of Microscint™ 20 (Perkin Elmer, Beaconsfield, U.K.) was added to each well and radioactivity quantified using single photon counting on a TopCount™ microplate scintillation counter (Perkin Elmer, Beaconsfield, U.K.) Aliquots of [³H]-QAW039 and [³H]-OC-459 were also quantified accurately to determine how much radioactivity was added to each well using liquid scintillation spectrometry on a Hidex 300SL scintillation counter (LabLogic, Sheffield, U.K.). In all experiments total binding never exceeded more than 10% of that added and therefore limiting significant depletion of the free radioligand concentration (Carter et al., 2007). To ensure the experiment was at the described temperature all components were pre-warmed for ≥ 2 hours.

[³H]-QAW039 and [³H]-OC-459 saturation binding

Binding was performed with a range of concentrations of [³H]-QAW039 and [³H]-OC-459 to construct saturation binding curves, as described by Sykes et al., (2010). CHO-CRTh2 cell-membranes (0.5 µg well⁻¹ respectively) were incubated in 96-deep well plates at 37°C in assay binding buffer with gentle agitation for 3 hour, to ensure equilibrium was reached. Due

to the low concentrations of [³H]-QAW039 employed, the assay volume was increased to 1.5 mL to avoid significant depletion of the radioligand and unlabelled competitors.

[³H]-QAW039 competition binding

To obtain affinity estimates for unlabeled QAW039 and the other unlabeled CRTh2 reference compounds, [³H]-QAW039 displacement experiments were performed in a total reaction volume of 0.5 ml. [³H]-QAW039 was used at a concentration of approximately 1 nM such that the calculated total binding never exceeded more than 10% of that added and therefore avoided ligand depletion. [³H]-QAW039 was incubated in the presence of the indicated concentration of unlabeled CRTh2 antagonist and CHO-cell membranes expressing the CRTh2 receptor (0.5 µg well⁻¹ respectively) at 37°C in assay binding buffer containing GTPγS (30µM) in 96-deep well plates with gentle agitation for 2.5 hour to ensure equilibrium was reached. In a separate study designed to assess the mode of action (MoA) of QAW039 increasing concentrations of [³H]-QAW039 (0.5, 1, 3, 10, 30 x K_d) were incubated against increasing concentrations of unlabeled QAW039 (homologous binding) and increasing concentrations of the native agonist PGD₂ plus selected CRTh2 antagonists all in the presence of GTPγS (30µM).

Determination of the binding kinetics of [³H]-QAW039 at human CRTh2 receptors

To accurately determine the k_{on} and k_{off} , and the observed rate of association (k_{ob}) was calculated with at least three different concentrations of [³H]-QAW039 (0.3, 1 and 3 x K_i). The appropriate concentration of [³H]-QAW039 was incubated with CRTh2 CHO-cell membranes (0.5 µg well⁻¹) in assay binding buffer with gentle agitation (final assay volume 500 µL). The binding of [³H]-QAW039 to the CRTh2 receptor was initiated with the addition of CHO-cell membranes and bound and free [³H]-QAW039 were separated at the indicated

time-points to construct association kinetic curves. Care was taken to ensure that equilibrium was reached for each concentration, before the experiment was terminated. After incubation, bound was separated from free by rapid filtration, plates were left to dry and radioactivity quantified (all as previously described).

Determination of the dissociation rate (k_{off}) of [3 H]-QAW039 and [3 H]-OC-459 at the human CRTh2 receptor

A single concentration of [3 H]-QAW039 (2.5 nM) and [3 H]-OC-459 (5 nM) was allowed to fully associate with human CRTh2 CHO-cell membranes (0.5 μ g well⁻¹) in a final reaction volume of 500 μ L. To construct dissociation kinetic curves an excess of AZD1981 (3 μ M) was added at various time intervals to the reaction mixture to initiate dissociation of the radioligands. Dissociation was monitored for up to 150 min until [3 H]-QAW039 was fully dissociated from the human CRTh2 receptor. Bound and free [3 H]-QAW039 and [3 H]-OC-459 were separated at the indicated time-points by rapid filtration, plates were left to dry and radioactivity quantified (all as previously described).

Competition kinetics at human CRTh2 receptors

The kinetic parameters of unlabeled QAW039 and the other CRTh2 antagonists were assessed using the methodology of Sykes et al., (2010). An experimentally calculated concentration of [3 H]-QAW039 (~2.5 nM, a concentration which avoided ligand depletion in this assay volume) was incubated with the indicated concentration(s) of unlabeled competitor in a final volume of assay binding buffer of 500 μ L. Each time-point was conducted on the same 96 deep-well plate incubated with constant gentle agitation. The experiment was initiated by the addition of CRTh2 CHO-cell membranes (0.5 μ g well⁻¹) containing GTP γ S (30 μ M) and the plates harvested (as previously described) at the indicated time-points.

[³⁵S]-GTP γ S functional binding assay

A range of concentrations of test compound were prepared in 100% DMSO and 2.5 μ L added to a 96-well white OptiPlate. To each well 200 μ L of assay buffer (20 mM HEPES, 10 mM MgCl₂, 100 mM NaCl and 1 mM EDTA) containing 0.1% HSA, 30 μ g mL⁻¹ saponin (added fresh on the day of experimentation), 50 μ g mL⁻¹ CHO-CRTh2 membranes, 3.7 μ M guanosine 5'-diphosphate sodium salt (GDP), 2.5 mg mL⁻¹ WGA PVT SPA beads and 50 μ L of [³⁵S]-Guanosine 5'-(γ -thio)triphosphate ([³⁵S]-GTP γ S) at a concentration of 300 pM was added and incubated at room temperature with gentle agitation for 2 hr. Following this incubation 2.5 μ L of increasing concentrations of PGD₂ prepared at 100x concentration in 100% DMSO was added and incubated at room temperature with gentle agitation for 15, 60 and 180 min. Eleven concentrations of PGD₂ were studied in total. Following this the plates were centrifuged for 3 min at 1000 g and bound [³⁵S]-GTP γ S quantified using single photon counting on a TopCountTM microplate scintillation counter (Perkin Elmer, Beaconsfield, UK).

Isolated eosinophil shape change assay

Granulocytes were isolated from the blood of healthy normal volunteers. The granulocytes were incubated with PGD₂ for 5 min at 37 °C. The reaction was terminated by addition of CellFixTM and data was acquired on FACSCalibur flow cytometer, first with FSC/SSC plots and then FSC/FL-2 to distinguish eosinophils from neutrophils. Data were fitted to a sigmoidal curve (Origin 7 or XL fit) to give a typical EC₅₀ for PGD₂ of 0.72 \pm 0.11 nM (n = 3, data not shown). Antagonist curves were determined as follows: aliquots of granulocyte in assay buffer containing 1% DMSO were treated with aliquots of 10 increasing test compound concentrations and the mixture incubated for 5 min at 37 °C. Agonist (EC₇₀ concentration) was added and the mixture was incubated for a further 5 min. The reaction was terminated

and activity measured as above. Data were fitted to a sigmoidal curve (Origin 7 or XL Fit) to give an IC₅₀ value.

Whole blood eosinophil shape change assay

Blood was collected into sterile 3.8% (w/v) trisodium citrate from healthy atopic volunteers and used immediately with minimum disturbance. Agonist curves were determined as follows: aliquots of whole blood (80 µL) were incubated in assay buffer containing 1% DMSO for 5 min at 37 °C. Increasing concentrations of PGD₂ were added and the mixture was incubated with gentle shaking at 37 °C for 5 min. The reaction was terminated by addition of ice cold CellFix™ (250 µL), followed by ammonium chloride lysis solution (1.5 ml). After incubation on ice for 40–60 min, data was acquired on FACSCalibur flow cytometer, first with FSC/SSC plots and then FSC/FL-2 to distinguish eosinophils from neutrophils. Data were fitted to a sigmoidal curve (Origin 7 or XL fit) to give a typical EC₅₀ for PGD₂ of 0.94 ± 0.18 nM (n = 3, data not shown). Antagonist curves were determined as follows: aliquots of whole blood (80 µL) and test compound (10 concentrations) were incubated in assay buffer containing 1% DMSO for 5 min at 37 °C. Agonist (EC₇₀ concentration in 10 µL in assay buffer) was added and the mixture incubated with gentle shaking at 37 °C for 5 min. The reaction was terminated and samples measured as described above.

Th2 cell cytokine release assay

CRTh₂⁺ Th2 cells isolated by either MACS or MoFlo (see Supplemental methods) were counted and seeded in flat-bottom 96 well plates at a concentration of 100,000 cells per well. Cells were seeded in X-VIVO 15 medium plus 10 % human AB serum and 1 % Penicillin/Streptomycin. QAW039 (10 mM stock solution in 100 % DMSO) was applied to

the cells (10 μ L of a 10x final concentration) at a range of concentrations between 1000 nM – 0.3 nM and incubated with the cells (37°C, 5% CO₂) for 30 minutes prior to PGD₂ being added. PGD₂ was added to the cells at a final concentration of 200 nM (10 μ L of a 10x solution – stock made up in 100% ethanol). Unstimulated cells were included in the assay – cells treated with medium alone; and PGD₂ at 200 nM \pm vehicle (0.01 % DMSO) was also included as a control. Once all additions had been made, the plate was incubated for a further 6 hours at 37°C, 5 % CO₂ after which the plate was centrifuged at 400 xg for 10 minutes and the supernatant was harvested into 96-well plates and stored at -20°C prior to cytokine analysis was carried out. The supernatant samples were analysed for levels of the Th2 cytokines IL-13, IL-5 and IL-4, and also IL-12 (as a negative control) using the Mesoscale Discovery (MSD) human IL-13 issue culture assay kit plus detection antibodies for human IL-4, IL-5 and IL-12 (total). Briefly, 25 μ L of each Calibrator (standard) at a range of 10,000 – 2.4 pg/mL was applied to the plate in duplicate and 25 μ L of neat test sample in triplicate. The plate was then incubated at RT for 2 hours with shaking at 800 rpm. 25 μ L of the Detection Antibody solution mix (each antibody diluted 1 in 50 in antibody dilution buffer) was then added to each well and incubated for a further 2 hours at RT with shaking at 800 rpm. The plate was then washed 3 times in PBS + 0.05 % Tween-20 and 150 μ L of 2x Read Buffer T was added to each well. The plate was analysed on the SECTOR™ Imager and the results for each cytokine were analysed using Discovery Workbench software. IC₅₀ values were calculated using GraphPad Prism v4.0 software.

Interpretation of data

Data analysis

As the amount of radioactivity varied slightly for each experiment (< 5%), data are shown graphically as the mean \pm s.d. for individual representative experiments, whereas all values

reported in the text and tables are mean \pm s.e. mean for the indicated number of experiments. All experiments were analyzed by non-regression using Prism 6.0 (GraphPad Software, San Diego, U.S.A.). Competition displacement binding data were fitted to sigmoidal (variable slope) curves using a “four parameter logistic equation”:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / \left(1 + 10^{(\log EC_{50} - X) \cdot \text{Hillcoefficient}} \right)$$

IC₅₀ values obtained from the inhibition curves were converted to K_i values using the method of Cheng and Prusoff (1973). [³H]-QAW039 association data was fitted as follows to a global fitting model using GraphPad Prism 6.0 to simultaneously calculate k_{on} and k_{off} using the following equation where k_{ob} equals the observed rate of association:

$$k_{ob} = [\text{Radioligand}] \cdot k_{on} + k_{off}$$

Association and dissociation rates for unlabeled antagonists were calculated using the equations described by Motulsky and Mahan (1984):

$$K_A = k_1[L] + k_2$$

$$K_B = k_3[I] + k_4$$

$$S = \sqrt{\left((K_A - K_B)^2 + 4 \cdot k_1 \cdot k_3 \cdot L \cdot 10^{-18} \right)}$$

$$K_F = 0.5 \cdot (K_A + K_B + S)$$

$$K_S = 0.5 \cdot (K_A + K_B - S)$$

$$DIFF = K_F - K_S$$

$$Q = \frac{B_{\max} \cdot K_1 \cdot L \cdot 10^{-9}}{DIFF}$$

$$Y = Q \cdot \left(\frac{k_4 \cdot DIFF}{K_F \cdot K_S} + \frac{k_4 - K_F}{K_F} \cdot \exp(-K_F \cdot X) - \frac{k_4 - K_S}{K_S} \cdot \exp(-K_S \cdot X) \right)$$

Where: X = Time (min), Y = Specific binding (CPM), $k_1 = k_{on}$ [^3H]-QAW039, $k_2 = k_{off}$ [^3H]-QAW039, L = Concentration of [^3H]-QAW039 used (nM), B_{max} = Total binding (CPM), I = Concentration unlabeled antagonist (nM).

Fixing the above parameters allowed the following to be calculated: k_3 = Association rate of unlabeled ligand ($\text{M}^{-1} \text{min}^{-1}$), k_4 = Dissociation rate of unlabeled ligand (min^{-1}).

Dissociation of [^3H]-QAW039 and [^3H]-OC-459 was fitted to a one phase mono-exponential decay function to estimate the dissociation rate of QAW039 directly.

PGD₂ stimulated [^{35}S]-GTP γ S binding data were fitted to sigmoidal (variable slope) curves using the four-parameter logistic equation described above. pA₂ values were estimated directly from Schild analysis following normalisation of data to the control maximal response (see supplemental Figure 3 and supplemental Table 1). Specifically data were fitted to a using a four-parameter logistic equation in Prism 6.0 as described above (each curve was fit to its own value of n, EC₅₀, bottom (E_{min}) and top (E_{max})) and to a simpler model (where a common E_{min} , E_{max} and n values were used). When the F-statistic indicated that a significantly better fit was obtained with the simple model, dose ratios (DR) were calculated for construction of Schild plots. Data were then fitted to the Gaddum/Schild equation in Prism 6.0 to obtain an estimation of the Schild slope. If the Schild slope did not differ significantly from unity (F-test) the data was refit to a slope of 1.0 to derive pA₂ values. Corrected pA₂ estimates were derived by correcting for % bound to HSA (0.1%) using the method of Toutain and Bousquet-Melou (2002). The validity of this correction is borne out of the fact that plasma protein binding (see Table 3) and HSA binding are directly correlated for the CRTh2 antagonists tested in this study ($P < 0.0001$, $r^2 = 0.97$ data not shown). For a full explanation of this conversion and the effects of protein on apparent receptor affinity see Blakeley et al., 2015. PGD₂ did not reach equilibrium with QAW039 in the time frame of the GTP γ S assay so EC₃₀ estimates were taken from which we were able to estimate log DR-1

values to derive a Schild plot and obtain a pA2 value. Estimated pA2 values along with corrected estimated pA2 estimates are shown in supplemental Table 1.

Functional GTP γ S data for QAW039 were fitted according to a combined operational and hemi-equilibrium model for competitive antagonism under non-equilibrium conditions (Kenakin, 2009; Mould et al., 2014; Riddy et al., 2015) in order to derive kinetic parameters for QAW039.

$$\alpha = \frac{[B]/K_B}{([B]/K_B + [A]/K_A + 1)}$$

$$\beta = \frac{[B]/K_B}{([B]/K_B + 1)}$$

$$\gamma = \frac{([B]/K_B + [A]/K_A + 1)}{([A]/K_A + 1)}$$

$$Y = \frac{[A]/K_A (1 - (\alpha \cdot (1 - e^{-k_{\text{off}} \cdot \gamma \cdot t}) + \beta \cdot e^{-k_{\text{off}} \cdot \gamma \cdot t})) \cdot \tau \cdot E_m}{[A]/K_A ((1 - (\alpha \cdot (1 - e^{-k_{\text{off}} \cdot \gamma \cdot t}) + \beta \cdot e^{-k_{\text{off}} \cdot \gamma \cdot t})) \cdot \tau + 1) + 1}$$

where [A] and [B] represent the concentrations of PGD₂ and QAW039, respectively, K_A and K_B represent the respective equilibrium dissociation constants, k_{off} is the dissociation rate constant for the antagonist (min⁻¹), t is the assay incubation time (min), τ is the operational efficacy of PGD₂ and E_m is the maximal system response. All parameters were shared across all data sets except t which were fixed to the assay incubation time. In all cases, potency and affinity values were estimated as logarithms (Christopoulos, 1998). Data shown are the mean \pm SEM (see supplemental Figure 4).

Results

QAW039 and related CRTh2 compounds

The structure of QAW039 and related CRTh2 antagonists and the agonist indomethacin are shown in Figure 1. QAW039 is a trifluoromethyl derivative of the previously described 7-azaindole-3-acetic acid QAV680 (Sandham et al., 2013). The measured lipophilicity ($\log D_{7.4}$) of these compounds is lower than other described CRTh2 antagonists (see Table 3) which likely contributes to their lower plasma protein binding.

Characterisation of CHO-CRTh2 expressing cell line.

Specific [^3H]-QAW039 and [^3H]-OC-459 binding to human CRTh2 receptors in CHO membranes was saturable and best described by the interaction of the radioligand with a single population of high affinity sites. A representative saturation binding curve for [^3H]-QAW039 and [^3H]-OC-459 binding to the human CHO-CRTh2 receptor is shown in Figure 2A and B. The expression level of the CHO-CRTh2 in the CHO-cell line, and the corresponding equilibrium dissociation constant (K_d) of [^3H]-QAW039 and [^3H]-OC-459 determined from [^3H]-QAW039 saturation binding studies are shown in Table 1.

Determination of the dissociation rate (k_{off}) of [^3H]-QAW039 and [^3H]-OC-459 at the human CRTh2 receptor

A single concentration of [^3H]-QAW039 (2.5 nM) and [^3H]-OC-459 (5 nM) was allowed to fully associate with the human CRTh2 receptor. An excess of AZD-1981 (3 μM) was then added to the reaction mixture to initiate dissociation of [^3H]-QAW039 and [^3H]-OC-459. Dissociation was monitored until [^3H]-QAW039 and [^3H]-OC-459 was fully dissociated from the human CRTh2 receptor (see Figure 1C and D). The data was fitted to a mono exponential

decay function to estimate a k_{off} value for QAW039 and OC-459 directly. A k_{off} value of $0.050 \pm 0.006 \text{ min}^{-1}$ ($t_{1/2} = 14.2 \text{ min}$) was determined for the slowly dissociating [^3H]-QAW039 and a value of $2.80 \pm 0.53 \text{ min}^{-1}$ ($t_{1/2} = 0.28 \text{ min}$) for the more rapidly dissociating [^3H]-OC-459 binding to human CRTh2 receptors.

Determination of the equilibrium binding affinity constant of CRTh2 antagonists and agonists at CRTh2 receptors.

IC₅₀ values determined from competition binding experiments were converted to equilibrium binding constants (pK_i) by using the equation of Cheng and Prusoff (1973) and using the K_d values of [^3H]-QAW039 as described in the methods section. The competition binding curves showing displacement of [^3H]-QAW039 by QAW039 and the other CRTh2 compounds tested is shown in Figure 3A and B, affinity values (pK_i) for CRTh2 antagonist and agonist binding to human CRTh2 receptors are summarized in Table 2.

Determination of the kinetic parameters of [^3H]-QAW039 at the human CRTh2 receptor

In order to calculate the kinetic on (k_{on}) and off-rates (k_{off}) of the unlabeled QAW039 and the other CRTh2 antagonists the kinetic parameters of the radioligand, [^3H]-QAW039 were first determined. A family of association kinetic curves was constructed using a range of [^3H]-QAW039 concentrations. Each association curve was monitored until equilibrium so that Y_{max} was reached (see Figure 4A). From this type of analysis it is possible to estimate both k_{on} and k_{off} values for QAW039 by fitting the data to a global kinetic model. A k_{on} value for [^3H]-QAW039 of $4.52 \pm 0.45 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$ and a k_{off} value of $0.048 \pm 0.005 \text{ min}^{-1}$ was determined. From the k_{off} value a $t_{1/2}$ of dissociation ($0.693/k_{\text{off}}$) of 14.4 min was calculated for [^3H]-QAW039. K_{obs} increased in a linear manner with [^3H]-QAW039 concentration (see Figure 4B) and the mean kinetic parameters estimated from these plots were in good

agreement with the values estimated from the global fit described above (linear fit mean $k_{\text{off}} = 0.044 \pm 0.004$ and $k_{\text{on}} = 4.86 \pm 0.53 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$).

Determination of the kinetic parameters of QAW039 and competitor compounds at human CRTh2 receptors.

Using the equations described in methods section and the kinetic values obtained for [³H]-QAW039 as detailed above, the association and dissociation rates for unlabeled QAW039, OC-459 and the other CRTh2 antagonists for the human CRTh2 (see Table 2 and Figures 4C-D and supplemental Figure 1A-E respectively) receptor were calculated. The kinetic on (k_{on}) and off-rates (k_{off}) determined for QAW039 using the Motulsky-Mahan methodology were not significantly different from the values calculated from the global kinetic analysis suggesting that [³H]-QAW039 is suitable for the determination of kinetic parameters of unlabeled CRTh2 antagonists. To validate the rate constants, the kinetically derived dissociation constant (K_d) values ($k_{\text{off}}/k_{\text{on}}$) were compared with the dissociation constant (K_i) obtained from equilibrium competition binding experiments. There was a very good correlation ($r^2 = 0.95$, $P < 0.001$, data not shown) between these two values, indicating the kinetic parameters were accurate.

QAW039 is a competitive antagonist of the native CRTh2 receptor agonist PGD₂.

In order to explore the nature of the interaction of QAW039 with the CRTh2 receptor we employed the technique of competitive binding. Using this technique we have explored the inhibitory potency of unlabelled QAW039, AZD-1981, OC-459 and the native agonist PGD₂ over a range of concentrations of [³H]-QAW039 designed to saturate the receptor. At the lowest radioligand concentration employed, ~0.3 nM, the pIC₅₀ value for displacement by QAW039 itself was 9.05 ± 0.10 (see Figure 5A). As expected a linear increase in the pIC₅₀

value was obtained as the radioligand concentration was increased to the maximum concentration employed in the study, ~30nM (~30-fold greater than K_d from saturation binding) (see Figure 5E). This same pattern of behaviour was mimicked by the other structurally related CRTh2 antagonists, and PGD₂ which were investigated in parallel. The difference in pIC₅₀ values for unlabelled PGD₂ were precisely in line with those predicted by the Cheng–Prusoff relationship (Cheng and Prusoff, 1973), using the K_d value for QAW039 obtained from saturation binding, see Table 1), suggesting that unlabelled PGD₂ displaced the radioligand competitively.

Determination of the effect CRTh2 antagonists on PGD₂ stimulated [³⁵S]-GTPγS binding to human CRTh2 receptors.

To investigate the potential for QAW039 and the other CRTh2 antagonists to display insurmountable behavior in a [³⁵S]-GTPγS binding assay, PGD₂ concentration response curves were constructed in the presence of increasing concentrations of pre-equilibrated antagonist, see Figure 6. [³⁵S]-GTPγS binding to CHO-hCRTh2 membranes was measured at different time intervals following incubation with the endogenous CRTh2 agonist PGD₂. QAW039 produced a clear reduction in the E_{max} of PGD₂ stimulated GTPγS binding following a 15 min incubation period with PGD₂ (Figure 6A) demonstrating its ability to produce insurmountable antagonism in a functionally relevant second messenger reporter assay. The incubation was extended to 180 min to allow the PGD₂ to reach equilibrium with the receptor and fully activate a second messenger response. The inhibitory effect of QAW039 was readily reversible with time, however the inhibitory effects of QAW039 were not fully reversed even following a 180 min incubation period with PGD₂ (see supplemental Figure 3). Also apparent was a reduction in the basal accumulation of GTPγS binding in the presence of increasing concentrations of QAW039 and the other CRTh2 antagonists (see

Figure 6A-G). This demonstrates that these compounds behave as inverse agonists of CRTh2 mediated GTP γ S accumulation. In contrast to QAW039, the more rapidly dissociating CRTh2 antagonists tested did not produce any noticeable depression of the PGD₂ maximal response following a 15 min incubation (see Figure 6B-G). PGD₂ stimulated [³⁵S]-GTP γ S binding in the presence of these more rapidly dissociating CRTh2 compounds following a 180 min incubation period are shown for comparison with QAW039, see supplemental Figure 3B-G respectively.

It is not possible to accurately derive pK_B values directly from the GTP γ S binding experiments since the compounds are bound to plasma proteins (eg HSA, see Table 3) resulting in irregular shifts of the PGD₂ concentration response curves at concentrations of compound which come close to saturating the protein (0.1% HSA or 15 μ M) present in the assay buffer. Once HSA gradually becomes saturated by the increasing concentrations of antagonist the Schild slopes become gradually steeper as more antagonist becomes free in solution and available to bind the CRTh2 receptor (i.e. deviation from constant % bound, see Blakeley et al., 2015). As would be predicted this effect is most pronounced for the low CRTh2 affinity, highly protein bound compound setipiprant (see supplemental Figure 3G). However Schild plots of the lower concentrations of QAW039, QAV680, AZD-1981, OC-459, BI-671800 and ramatroban (<10 μ M, i.e. below the saturating protein concentration) tested resulted in Schild slopes not significantly different from 1.0 and pA₂ values and in line with expected pK_i estimates following correction for HSA binding further suggesting that these compounds are behaving competitively with PGD₂ (see supplemental Figure 3, Table 2 and supplemental Table 1). Although PGD₂ did not fully reach equilibrium with QAW039 in the 180 min time frame of the GTP γ S assay, we previously determined it to be competitive with PGD₂ based on competitive binding data, hence we have estimated DR-1 values to

derive a Schild plot and obtain an estimated PA_2 value. Thus the [^{35}S]-GTP γ S competition assay data is entirely consistent with the radioligand competition binding analysis and suggest that these molecules interact with the same orthosteric binding pocket as PGD $_2$.

When a comparison of CRTh2 antagonist inhibitory potency was made at the 15min time point in the [^{35}S]-GTP γ S assay, following addition of a maximally effective concentration of PGD $_2$ (1 μ M), the superior potency of QAW039 compared to the other CRTh2 compounds tested in this study was clearly visible (see Figure 7). This suggests that kinetic off-rate could potentially play a role in driving the superior potency of this compound.

The effect of time on the potency of CRTh2 antagonists to inhibit PGD $_2$ (1 μ M) stimulated GTP γ S accumulation is shown in Figure 8. QAW039 is >10-fold more effective at inhibiting the PGD $_2$ response following a 15min incubation compared to the full 180min incubation period (Figure 8A). This reflects its very slow dissociation from the CRTh2 receptor in the face of high concentrations of PGD $_2$. The other CRTh2 antagonist examined are equi-effective at all-time points demonstrating that kinetics does not play any role in determining their potency under these experimental conditions (Figure 8B-G).

An estimate of the affinity and dissociation rate of the CRTh2 antagonist QAW039 from functional [^{35}S]-GTP γ S data following a 60 min incubation was obtained by fitting data according to an operational hemi-equilibrium model for competitive antagonism first described by Kenakin (2009) and recently employed practically to estimate the dissociation rates of orexin-2 antagonists and muscarinic M $_1$ antagonists in functional assays (Mould et al., 2014; Riddey et al., 2015), see supplemental Figure 4 and supplemental Table 2. A pA_2 value for QAW039 of 3.2 ± 0.7 nM was estimated which is very much in line with affinity estimates obtained from kinetic association experiments performed at room temperature (K_d 1.39 ± 0.23 nM, see supplemental Figure 5 and supplemental Table 3) and the pA_2 obtained

from the Schild analysis (see supplemental Table 1). Importantly the k_{off} value for QAW039 of 0.005min^{-1} was also within 3-fold of that estimated in kinetic association binding experiments performed at room temperature (QAW039 $k_{\text{off}} = 0.012 \pm 0.001$ see supplemental Figure 5 and supplemental Table 3) demonstrating the potential for the GTP γ S assay to identify very slowly dissociating compounds. In contrast it was not possible to fit other ligands to the operational hemi-equilibrium model as their effects were fully reversible within the 15min incubation period. Confirmation that [^3H]-OC-459 was relatively rapidly dissociating when profiled at room temperature comes from kinetic association binding experiments see supplemental Figure 5 and supplemental Table 3.

Determination of the effect of QAW039 on PGD₂ stimulated whole blood eosinophil shape change.

Migration of eosinophils from the microcirculation into sites of inflammation occurs as a consequence of cytoskeletal rearrangements in a process known as eosinophil shape change. Purified human eosinophils undergo shape change upon exposure to PGD₂ in response to activation of CRTh2 and a similar response is seen in whole blood. This study confirms the inhibitory effect of the tested CRTh2 antagonists in both the isolated and whole blood shape change assays. QAW039 was the most potent compound tested at inhibiting PGD₂ stimulated eosinophil shape change, with comparable potencies in both the isolated and whole shape change assays (see Table 3).

Determination of the effect of QAW039 on cytokine release from PGD₂ stimulated CD4+ Th2 cells

The chronic inflammatory response characteristically observed in allergic asthma occurs by the selective accumulation of Th2 lymphocytes which further potentiate the inflammatory

response by releasing Th2 cytokines such as IL-5 and IL-13. The localized increase in the release of Th2 cytokines at the primary site of inflammation causes activation of Th2 cells, eosinophils and basophils. Here we show that QAW039 is a potent inhibitor of PGD₂ induced release of IL-5 and IL-13 from human CD4⁺ Th2 lymphocytes (see Table 4). The potency of QAW039 in this cellular functional assay system is 15-20 fold greater than the previous lead compound QAV680.

Discussion

It is potentially advantageous when developing antagonists of G protein coupled receptors to identify compounds which exhibit slow dissociation from the receptor (Tummino and Copeland 2008). CRTh2 antagonists exhibiting such behavior may display greater clinical efficacy as a consequence of prolonged receptor blockade, which will effectively reduce the recruitment of proinflammatory eosinophils into existing areas of inflammation. Such a profile could be particularly important in the asthmatic lung where local concentrations of PGD₂ are likely to vary dependent on the numbers of activated mast cells, especially given that PGD₂ release occurs in a matter of minutes after IgE activation (Kawata et al., 1995). Importantly, the transit time of ‘mixed leukocytes’ across the pulmonary circulation has very recently been quantified and is thought to be similar to that of neutrophils measured precisely at 14.2 seconds, only marginally slower than that reported for RBCs (Summers et al., 2014). This implies that sustained CRTh2 blockade will protect against eosinophil recruitment as they transit through areas of high PGD₂ concentration in the lung. Clinical data generated with QAW039 in eosinophilic asthmatics shows a reduction in both sputum and tissue bronchial biopsy eosinophils supporting this hypothesis (Berair et al., 2015). Prolonged CRTh2 receptor blockade therefore represents a new and potentially powerful approach for the treatment of allergic asthma.

The binding properties of several clinically relevant CRTh2 antagonists including the recently developed QAW039 were determined at the human CRTh2 receptor in assay buffer containing physiological sodium ion concentration at 37°C. Previous studies designed to assess the kinetics of unlabelled CRTh2 antagonists have employed non-physiological assay conditions, experiments being conducted at room temperature in the absence of salt (Gervais et al., 2011). Findings from these early kinetic studies are therefore likely to overestimate the receptor target coverage since it has been well documented that compound kinetics is highly

dependent on temperature and sodium ion concentration (Sykes et al., 2012). This phenomenon was also apparent in the current study with the off-rates of QAW039 and OC-459 being up to 5-fold slower when measured at room temperature compared to 37°C.

Other researchers have attempted to estimate dissociation $t_{1/2}$ s indirectly from functional [35 S]-GTP γ S assays performed at room temperature by measuring responses to PGD $_2$ (100 μ M) at fixed time periods following a 60 min pre-incubation period with test compounds, allowing antagonist $t_{1/2}$ s to be estimated by fitting an exponential decay curve to the PGD $_2$ responses observed over time (Andrés et al., 2014). Although these results enabled a rank order of residence time to be established, the use of an accumulation assay mean the dissociation half-lives are again likely to be overestimated.

In saturation binding experiments [3 H]-QAW039 and [3 H]-OC-459 display nM affinity for the human CRTh2 receptor expressed in CHO-cells. The mean kinetic k_{on} and k_{off} values determined for [3 H]-QAW039 were $4.5 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$ and 0.048 min^{-1} respectively, producing a $t_{1/2}$ value of 12 min and a kinetic K_d value of $1.06 \pm 0.06 \text{ nM}$ which is in excellent agreement with the equilibrium K_d value obtained in the saturation binding experiments described above. In contrast, despite displaying comparable nM affinity, OC-459 was ~30x faster at dissociating from the CRTh2 receptor ($t_{1/2} = 0.41 \text{ min}$). To put this in context > 50% of OC-459 could potentially be displaced from the CRTh2 receptor in the time it takes for eosinophils to pass through the asthmatic lung, whereas in direct contrast <1.2% of QAW039 will likely become displaced in the same time frame.

The binding kinetics of unlabelled QAW039 and the other related CRTh2 antagonists including OC-459 were also determined indirectly using [3 H]-QAW039 as the tracer through application of a competition binding model first described by Motulsky and Mahan (1984). The kinetic K_d values generated indirectly were in agreement with the K_i values generated by equilibrium competition kinetic binding validating the use of the current methodology. The

pharmacological properties of QAW039 are a significant improvement on those of our original lead compound QAV680 and the other CRTh2 antagonists examined in this study. In particular QAW039 is significantly slower to dissociate from the CRTh2 receptor (>9 fold compared to QAV680) which potentially affords this compound increased efficacy and an improved duration of action at the CRTh2 receptor due to sustained blockade of the receptor in the face of increasing concentrations of PGD₂. This phenomenon of insurmountability is highlighted in the GTP γ S assay, where QAW039 which exhibits the slowest dissociation rate constant, also produced the most pronounced suppression of the maximal response to PGD₂ in the GTP γ S binding assay. The slower the compound k_{off} the greater the % inhibition achieved in the GTP γ S binding assay for any given multiple of the compound K_i added. QAW039 displays a time-dependent inhibitory effect in the GTP γ S binding assay its potency being highest at early time points (see Figure 8A) characteristic of its slow dissociation from the CRTh2 receptor. This may contribute to QAW039's increased potency in the eosinophil whole blood shape change assay relative to the more rapidly dissociating reference compounds.

The detailed pharmacological analysis presented in this study confirms the competitive nature of QAW039 and structurally related ligands binding to the human CRTh2 receptor. In particular the observation that the pIC₅₀ for PGD₂ displacement of [³H]-QAW039 binding (to the CRTh2 receptor) increased in a linear fashion with equivalent pA₂ values at increasing radioligand concentration demonstrates that QAW039 behaves as a simple competitive antagonist. Similarly PGD₂ itself and the two other reference CRTh2 antagonists examined OC-459 and AZD-1981 appeared fully competitive with increasing concentrations of QAW039 demonstrating that all these molecules occupy the same CRTh2 receptor binding pocket.

Limited information exists on the MoA of other CRTh2 antagonists, but there are previous publications using functional assays that highlight potential insurmountable antagonist profiles (Mathiesen et al., 2006; Gervais et al., 2011). Both publications attribute this compound profile to slow receptor dissociation kinetics whereby equilibrium between PGD₂ and receptor is not achieved in the time frame of their experiments. In contrast, AZD-1981 has been shown to exhibit a non-competitive binding profile despite possessing rapid dissociation kinetics (Schmidt et al., 2013). This was demonstrated using an agonist ([³H]-PGD₂) binding assay and could potentially result from stabilisation of the uncoupled form of the CRTh2 receptor.

In functional studies performed at room temperature and in salt containing buffer QAW039 behaves as an insurmountable antagonist of PGD₂ stimulated GTPγS activation following a 15min agonist equilibration time. This insurmountable behaviour is entirely consistent with QAW039 relatively slow dissociation ($t_{1/2} = 14.4\text{min}$) from the human CRTh2 receptor but does not result from a non-competitive interaction. Thus the time-dependant concentration dependent rightward shifts observed with QAW039 in the human GTPγS assay coupled with the Schild analysis (see Figure 8A and supplemental Figure 3) supports the findings of homologous binding studies (see Figure 5) and demonstrates that QAW039 behaves as a simple competitive antagonist of PGD₂ functional responses.

To investigate the profile QAW039 at the cellular level, we chose two relevant inflammatory cell types. CRTh2 mediated shape change in eosinophils was used to profile QAW039 in whole blood and represents a physiologically relevant environment. The comparable IC₅₀ values for QAW039 in the whole blood and isolated shape change assays are consistent with its lower plasma protein binding and its relatively slow dissociation kinetics that drive its increased potency (see Figure 8A). Other compounds showed large discrepancies between these two assay formats which are likely the result of higher plasma protein binding coupled

with rapid dissociation from the CRTh2 receptor. In the case of QAV680, the reduced protein binding does not compensate for the faster dissociation kinetics. These data demonstrate that QAW039 is highly potent in whole blood systems with the IC₅₀ value obtained being consistent with the affinity values calculated from radioligand experiments. In a further disease-relevant cellular context the potency of QAW039 in the isolated Th2 cell cytokine inhibition assay, is consistent with its CRTh2 receptor affinity, and as with eosinophil assay readouts this represents an improved potency compared to QAV680.

In summary, in these human functional studies across diverse cellular systems, we have determined that the potency of QAW039 is cell type and cell function independent. Importantly, the pharmacological profile determined for QAW039 suggests it should competitively inhibit other disease relevant CRTh2-mediated responses in human cells including Th2 cell cytokine production, PGD₂-mediated Th2 cell apoptosis, and basophil chemotaxis and eosinophil activation. To our knowledge, this is the first study to demonstrate in both binding and functional studies the simple competitive antagonist nature of clinically relevant CRTh2 antagonists including the slowly dissociating compound QAW039 and represents an important step forward in our understanding of the pharmacology of low molecular weight CRTh2 antagonists in competition with their native agonist PGD₂. QAW039 (fevipiprant) is under ongoing clinical study as a novel oral therapy for allergic diseases.

Author Contributions

Participated in research design: Sykes, Dubois and Charlton.

Conducted experiments: Sykes, Riddy, Bradley, Willard, Reilly and Miah.

Contributed new reagents or analytic tools: Bauer, Watson

Performed data analysis: Sykes, Riddy, Bradley, Willard, Reilly and Miah.

Wrote or contributed to writing on manuscript: Sykes, Sandham and Charlton.

Competing Financial Interests

The authors declare no competing financial interests.

References

Andrés M, Buil MA, Calbet M, Casado O, Castro J, Eastwood PR, Eichhorn P, Ferrer M, Forns P, Moreno I, Petit S, Roberts RS (2014). Structure-activity relationships (SAR) and structure-kinetic relationships (SKR) of pyrrolpiperidinone acetic acids as CRTh2 antagonists. *Bioorganic & Medicinal Chemistry Letters* 24(21), 5111–5117.

Balzar S, Fajt ML, Comhair SA, Erzurum SC, Bleecker E, Busse WW, Castro M, Gaston B, Israel E, Schwartz LB, Curran-Everett D, Moore CG, Wenzel SE (2011). Mast cell phenotype, location, and activation in severe asthma. Data from the Severe Asthma Research Program. *Am J Respir Crit Care Med.* 183(3):299-309.

Banker MJ, Clark TH, Williams JA (2003). Development and validation of a 96-well equilibrium dialysis apparatus for measuring plasma protein binding. *J Pharm Sci.* 92(5):967-74.

Barnes N, Pavord I, Chuchalin A, Bell J, Hunter M, Lewis T, Parker D, Payton M, Collins LP, Pettipher R, Steiner J, Perkins CM (2012). A randomized, double-blind, placebo-controlled study of the CRTH2 antagonist OC000459 in moderate persistent asthma. *Clin Exp Allergy.* 42(1):38-48.

Berair R, Gonen S, Singapuri A, Hartley R, Laurencin M, Bacher G, Holzhauser B, Bourne M, Mistry V, Pavord I, Mansur AH, Wardlaw J, Siddiqui S, Kay R, Brightling CE (2015). Effect of QAW039, an Oral Prostaglandin D₂ Receptor (DP₂/CRTh2) Antagonist, Upon Sputum and Bronchial Eosinophilic Inflammation and Clinical Outcomes in Treatment-Resistant Asthma: A Phase 2a Randomized Placebo-Controlled Trial. *ATS poster abstract* 69051.

Blakeley D, Sykes DA, Ensor P, Bertran E, Aston PJ, Charlton SJ (2015). Simulating the influence of plasma protein on measured receptor affinity in biochemical assays reveals the utility of Schild analysis for estimating compound affinity for plasma proteins. *Br J Pharmacol.* 172(21):5037-49.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 72:248-54.

Carter CM, Leighton-Davies JR, Charlton SJ (2007). Miniaturized receptor binding assays: complications arising from ligand depletion. *J Biomol Screen.* 12(2):255-66.

Charlton SJ, Vauquelin G (2010). Elusive equilibrium: the challenge of interpreting receptor pharmacology using calcium assays. *Br J Pharmacol.* 161(6):1250-65.

Cheng Y, Prusoff WH (1973). Relationship between the inhibition constant (K_1) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol.* 22(23):3099-108.

Christopoulos A (1998). Assessing the distribution of parameters in models of ligand-receptor interaction: to log or not to log. *Trends Pharmacol Sci* 19: 351–357.

Crea AE, Nakhosteen JA, Lee TH (1992). Mediator concentrations in bronchoalveolar lavage fluid of patients with mild asymptomatic bronchial asthma. *Eur Respir J.* 5(2):190-5.

Diamant Z, Sidharta PN, Singh D, O'Connor BJ, Zuiker R, Leaker BR, Silkey M, Dingemans J (2014). Setipiprant, a selective CRTH2 antagonist, reduces allergen-induced airway responses in allergic asthmatics. *Clin Exp Allergy.* 44(8):1044-52.

Erpenbeck VJ, Salapatek AM, Patel P, Sanni J, Dubois G, Zhou P, Perry S, Larbig M (2014a) Efficacy and safety of 10 day treatment with the CRTh2 antagonist QAV680 evaluated in patients with allergic rhinitis in an environmental exposure chamber. European Respiratory Society poster abstract P4075.

Erpenbeck VJ, Salapatek AM, Patel P, Sanni J, Carew D, Dubois G, Perry S, Larbig M (2014b) QAV680, cetirizine and their combination reduce nasal symptoms in patients with intermittent allergic rhinitis in an environmental exposure chamber. European Respiratory Society poster abstract P4069.

Gervais FG, Sawyer N, Stocco R, Hamel M, Krawczyk C, Sillaots S, Denis D, Wong E, Wang Z, Gallant M, Abraham WM, Slipetz D, Crackower MA, O'Neill GP (2011). Pharmacological characterization of MK-7246, a potent and selective CRTH2 (chemoattractant receptor-homologous molecule expressed on T-helper type 2 cells) antagonist. *Mol Pharmacol.* 79(1):69-76.

Gyles SL, Xue L, Townsend ER, Wetley F, Pettipher R (2006) A dominant role for chemoattractant receptor-homologous molecule expressed on T helper type 2 (Th2) cells (CRTH2) in mediating chemotaxis of CRTH2+ CD4+ Th2 lymphocytes in response to mast cell supernatants. *Immunology*. 119(3):362-8.

Kawata R, Reddy ST, Wolner B, Herschman HR (1995). Prostaglandin synthase 1 and prostaglandin synthase 2 both participate in activation-induced prostaglandin D₂ production in mast cells. *J Immunol*. 155(2):818-25.

Kerns EH, Di L, Petusky S, Kleintop T, Huryn D, McConnell O, Carter G (2003). Pharmaceutical profiling method for lipophilicity and integrity using liquid chromatography-mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 791(1-2):381-8.

Kenakin TP (2009). Orthosteric drug antagonism. In: Kenakin T(ed.). *A Pharmacology Primer*, 3rd edn. Elsevier Academic Press:London, pp. 105–127.

Krug N, Gupta A, Badorrek P, Koenen R, Mueller M, Pivovarova A, Hilbert J, Wetzel K, Hohlfeld JM, Wood C (2014). Efficacy of the oral chemoattractant receptor homologous molecule on TH2 cells antagonist BI 671800 in patients with seasonal allergic rhinitis. *J Allergy Clin Immunol*. 133(2):414-9.

Lamers C, Flesch D, Schubert-Zsilavec M, Merk D (2013). Novel prostaglandin receptor modulators: a patent review (2002 - 2012) - part I: non-EP receptor modulators. *Expert Opin Ther Pat*. 23(1):47-77.

Luu VT, Goujon JY, Meisterhans C, Frommherz M, Bauer C (2015). Synthesis of a high specific activity methyl sulfone tritium isotopologue of fevipiprant (NVP-QAW039). *J Labelled Comp Radiopharm*. 58(5):188-95.

Mathiesen JM, Christopoulos A, Ulven T, Royer JF, Campillo M, Heinemann A, Pardo L, Kostenis E (2006). On the mechanism of interaction of potent surmountable and insurmountable antagonists with the prostaglandin D₂ receptor CRTH2. *Mol Pharmacol*. 69(4):1441-53.

Miadonna A, Tedeschi A, Brasca C, Folco G, Sala A, Murphy RC (1990). Mediator release after endobronchial antigen challenge in patients with respiratory allergy. *J Allergy Clin Immunol.* 85(5):906-13.

Monneret G, Gravel S, Diamond M, Rokach J, Powell WS (2001). Prostaglandin D2 is a potent chemoattractant for human eosinophils that acts via a novel DP receptor. *Blood.* 98(6):1942-8.

Motulsky HJ, Mahan LC (1984). The kinetics of competitive radioligand binding predicted by the law of mass action. *Mol Pharmacol.* 25(1):1-9.

Mould R, Brown J, Marshall FH, Langmead CJ (2014). Binding kinetics differentiates functional antagonism of orexin-2 receptor ligands. *Br J Pharmacol.* 171(2):351-63.

Murray JJ, Tonnel AB, Brash AR, Roberts LJ 2nd, Gosset P, Workman R, Capron A, Oates JA (1986). Release of prostaglandin D2 into human airways during acute antigen challenge. *N Engl J Med.* 315(13):800-4.

Nagata K, Tanaka K, Ogawa K, Kemmotsu K, Imai T, Yoshie O, Abe H, Tada K, Nakamura M, Sugamura K, Takano S (1999a). Selective expression of a novel surface molecule by human Th2 cells in vivo. *J Immunol.* 162(3):1278-86.

Nagata K, Hirai H, Tanaka K, Ogawa K, Aso T, Sugamura K, Nakamura M, Takano S (1999b). CRTH2, an orphan receptor of T-helper-2-cells, is expressed on basophils and eosinophils and responds to mast cell-derived factor(s). *FEBS Lett.* 459(2):195-9.

Norman P (2014). Update on the status of DP2 receptor antagonists; from proof of concept through clinical failures to promising new drugs. *Expert Opin Investig Drugs.* 23, 55–66.

Pettipher R, Hansel TT, Armer R (2007). Antagonism of the prostaglandin D2 receptors DP1 and CRTH2 as an approach to treat allergic diseases. *Nat Rev Drug Discov.* 6(4):313-25.

Pettipher R, Hansel TT (2008). Antagonists of the prostaglandin D2 receptor CRTH2. *Drug News Perspect.* 21(6):317-22.

Riddy DM, Valant C, Rueda P, Charman WN, Sexton PM, Summers RJ, Christopoulos A, Langmead CJ (2015). Label-Free Kinetics: Exploiting Functional Hemi-Equilibrium to Derive Rate Constants for Muscarinic Receptor Antagonists. *Mol Pharmacol.* 88(4):779-90.

Sandham DA, Arnold N, Aschauer H, Bala K, Barker L, Brown L, Brown Z, Budd D, Cox B, Docx C, Dubois G, Duggan N, England K, Everatt B, Furegati M, Hall E, Kalthoff F, King A, Leblanc CJ, Manini J, Meingassner J, Profit R, Schmidt A, Simmons J, Sohal B, Stringer R, Thomas M, Turner KL, Walker C, Watson SJ, Westwick J, Willis J, Williams G, Wilson C (2013). Discovery and characterization of NVP-QAV680, a potent and selective CRTh2 receptor antagonist suitable for clinical testing in allergic diseases. *Bioorg Med Chem.* 21(21):6582-91.

Schmidt JA, Bell FM, Akam E, Marshall C, Dainty IA, Heinemann A, Dougall IG, Bonnert RV, Sargent CA (2013). Biochemical and pharmacological characterization of AZD-1981, an orally available selective DP2 antagonist in clinical development for asthma. *Br J Pharmacol.* 168(7):1626-38.

Stinson SE, Amrani Y, Brightling CE (2015). D prostanoid receptor 2 (chemoattractant receptor-homologous molecule expressed on TH2 cells) protein expression in asthmatic patients and its effects on bronchial epithelial cells. *J Allergy Clin Immunol.* 135(2):395-406.

Sugimoto H, Shichijo M, Iino T, Manabe Y, Watanabe A, Shimazaki M, Gantner F, Bacon KB (2003). An orally bioavailable small molecule antagonist of CRTH2, ramatroban (BAY u3405), inhibits prostaglandin D2-induced eosinophil migration in vitro. *J Pharmacol Exp Ther.* 305(1):347-52.

Summers C, Singh NR, White JF, Mackenzie IM, Johnston A, Solanki C, Balan KK, Peters AM, Chilvers ER (2014). Pulmonary retention of primed neutrophils: a novel protective host response, which is impaired in the acute respiratory distress syndrome. *Thorax.* 69(7):623-9.

Sykes DA, Dowling MR, Charlton SJ (2010). Measuring receptor target coverage: a radioligand competition binding protocol for assessing the association and dissociation rates of unlabeled compounds. *Curr Protoc Pharmacol.* Chapter 9:Unit 9.14.

Sykes DA, Dowling MR, Leighton-Davies J, Kent TC, Fawcett L, Renard E, Trifilieff A, Charlton SJ (2012). The Influence of receptor kinetics on the onset and duration of action and the therapeutic index of NVA237 and tiotropium. *J Pharmacol Exp Ther.* 343(2):520-8.

Swinney DC (2009). The role of binding kinetics in therapeutically useful drug action. *Curr Opin Drug Discov Devel.* 12(1):31-9.

Toutain PL, Bousquet-Melou A (2002). Free drug fraction vs free drug concentration: a matter of frequent confusion. *J Vet Pharmacol Ther.* 25(6):460-3.

Tummino PJ, Copeland RA (2008). Residence time of receptor-ligand complexes and its effect on biological function. *Biochemistry.* 47(20):5481-92.

Uller L, Mathiesen JM, Alenmyr L, Korsgren M, Ulven T, Högberg T, Andersson G, Persson CG, Kostenis E (2007). Antagonism of the prostaglandin D2 receptor CRTH2 attenuates asthma pathology in mouse eosinophilic airway inflammation. *Respir Res.* 8:16.

Valko K, Du CM, Bevan CD, Reynolds DP, Abraham MH. (2000) Rapid-gradient HPLC method for measuring drug interactions with immobilized artificial membrane: comparison with other lipophilicity measures. *J Pharm Sci.* 89(8):1085-1096.

Wenzel SE, Westcott JY, Smith HR, Larsen GL (1989). Spectrum of prostanoid release after bronchoalveolar allergen challenge in atopic asthmatics and in control groups. An alteration in the ratio of bronchoconstrictive to bronchoprotective mediators. *Am Rev Respir Dis.* 139(2):450-7.

Xue L, Barrow A, Pettipher R (2009). Interaction between prostaglandin D and chemoattractant receptor-homologous molecule expressed on Th2 cells mediates cytokine production by Th2 lymphocytes in response to activated mast cells. *Clin Exp Immunol.* 156(1):126-33.

Xue L, Salimi M, Panse I, Mjösberg JM, McKenzie AN, Spits H, Klenerman P, Ogg G (2014). Prostaglandin D2 activates group 2 innate lymphoid cells through chemoattractant receptor-homologous molecule expressed on Th2 cells. *J Allergy Clin Immunol.* 133(4):1184-94.

Figure legends

Figure 1. Structures of the selective CRTh2 antagonist QAW039 and reference CRTh2 ligands.

Figure 2. Characterization of the CRTh2 radioligands [³H]-QAW039 and [³H]-OC-459.

Saturation binding of [³H]-QAW039 (A) and [³H]-OC-459 (B) to human CRTh2 receptors expressed in membranes from CHO cells. Dissociation of [³H]-QAW039 (C) and [³H]-OC-459 (D) from human CRTh2 receptors expressed in membranes from CHO cells. Data are shown as Mean ± SEM and are representative of three separate experiments performed in triplicate.

Figure 3. Competition binding between [³H]-QAW039 and CRTh2 receptor ligands for

human CRTh2 receptors expressed in membranes from CHO cells. Displacement of [³H]-QAW039 (1 nM) by increasing concentrations of CRTh2 antagonist (A) QAW039, QAV680, AZD-1981, OC-459 and BI-671800 and (B) ramatroban and setipiprant plus the CRTh2 agonists PGD₂ and indomethacin. Data are presented as the mean range from a representative of three experiments performed in duplicate.

Figure 4. Direct determination of [³H]-QAW039 kinetic binding parameters plus competition kinetic binding to determine the kinetic rate constants of unlabeled

compounds. (A) Association of [³H]-QAW039 to the human CRTh2 receptor expressed in membranes from CHO cells. A family of association kinetic curves was constructed using a range of [³H]-QAW039 concentrations. k_{on} and k_{off} values for QAW039 were determined by fitting the data to a global kinetic association model. (B) Plot of ligand concentration verses k_{ob} . Binding followed a simple law of mass action model, k_{ob} increasing in a linear manner with radioligand concentration. [³H]-QAW039 competition kinetic curves in the presence of

unlabeled QAW039 (A) and OC-459. CHO-CRTh2 membranes were incubated with ~2.5 nM [³H]-QAW039 and the increasing concentrations of unlabeled competitor. Plates were incubated at 37°C for the indicated time points and nonspecific binding levels were determined in the presence of 3 μM AZD-1981. Data were fitted to the equations described in Materials and Methods to calculate k_{on} and k_{off} values for the unlabeled ligands; these are summarized in Table 2. Data are presented as mean ± range from representative three or more experiments performed in duplicate.

Figure 5. Determination of the competitive mode of action of QAW039 with PGD₂ and the selected other CRTh2 antagonists. Effect of increasing concentrations of [³H]-QAW039 on the degree of inhibition observed with increasing concentrations of (A) QAW039, (B) PGD₂, (C) AZD-1981 and (D) OC-459 at human CRTh2 receptors expressed in membranes from CHO cells. (E) Linear increase in the compound pIC₅₀ values with increasing [³H]-QAW039 concentrations. Data are shown as mean ± range and are representative of 3 separate experiments performed in duplicate.

Figure 6. Effect of CRTh2 antagonists on PGD₂ stimulated GTPγS accumulation. Insurmountable behavior of (A) QAW039 at human CRTh2 receptors expressed in membranes from CHO cells following a 15 min incubation period with the agonist PGD₂. Surmountable behavior of (B) QAV680, (C) AZD-1981, (D) OC-459, (E) BI-671800, (F) ramatroban and (G) setipirant following a 15 min incubation period. Data are shown as mean ± SEM and are representative of 3 separate experiments performed in singlet.

Figure 7. Functional consequences of hemi-equilibrium. Effect of CRTh2 antagonists acting at human CRTh2 receptors expressed in membranes from CHO cells on % depression of a maximally effective concentration of PGD₂ (1 μM) in the [³⁵S]-GTPγS assay at the 15min time point. Antagonist potency is expressed as a ratio of concentration added over compound

affinity corrected for % HSA bound. Data are shown as mean \pm SEM and are representative of 3 separate experiments performed in singlet.

Figure 8. Effects of hemi-equilibrium on CRTh2 antagonist potency. The effect of time on the inhibitory potency of CRTh2 antagonists (A) QAW039, (B) QAV680, (C) AZD-1981, (D) OC-459, (E) BI-671800, (F) ramatroban and (G) setipiprant in the [³⁵S]-GTP γ S binding assay following stimulation of the CRTh2 receptor expressed in membranes from CHO cells with a maximal effective concentration of PGD₂ (1 μ M). Data are shown as mean \pm SEM and of 3 separate experiments performed in singlet.

Tables

Table 1. Saturation binding and dissociation kinetic rate constant (k_{off}) data for [^3H]-QAW039 and [^3H]-OC-459 binding to human CRTh2 receptors expressed in membranes from CHO-cells. Data are mean \pm s.e. mean from at least 3 separate experiments.

Radioligand	Receptor expression level (pmol mg ⁻¹)	Dissociation kinetic rate constant k_{off} (min ⁻¹)	Equilibrium dissociation constant K_d (nM)
[^3H]-QAW039	6.26 \pm 0.51	0.050 \pm 0.006	1.13 \pm 0.44
[^3H]-OC-459	6.44 \pm 0.06	2.79 \pm 0.53	2.44 \pm 0.58

Table 2. Kinetic and equilibrium binding parameters of unlabeled QAW039 and other CRTh2 ligands interacting with the human CRTh2 receptor expressed in membranes from CHO-cells. Data are mean \pm s.e. mean from at least 3 separate experiments. Nd = not determined.

CRTh2 ligand	k_{on} ($M^{-1}min^{-1}$)	k_{off} (min^{-1})	$t_{1/2}$ (min)	Kinetic pK_d	Equilibrium pK_i
QAW039	$6.27 \pm 0.93 \times 10^7$	0.061 ± 0.006	12.04	8.99 ± 0.06	8.98 ± 0.03
QAV680	$4.80 \pm 2.14 \times 10^7$	0.66 ± 0.21	1.29	7.78 ± 0.04	7.54 ± 0.09
AZD-1981	$3.01 \pm 0.76 \times 10^8$	0.77 ± 0.23	1.26	8.57 ± 0.02	8.49 ± 0.03
OC-459	$9.50 \pm 5.14 \times 10^8$	1.83 ± 0.32	0.41	8.48 ± 0.04	8.35 ± 0.13
BI-671800	$2.47 \pm 0.36 \times 10^8$	1.22 ± 0.11	0.58	8.28 ± 0.01	8.14 ± 0.20
Ramatroban	$1.89 \pm 0.66 \times 10^7$	0.80 ± 0.22	0.80	7.35 ± 0.05	7.45 ± 0.02
Setipirant	$1.87 \pm 0.45 \times 10^8$	0.95 ± 0.15	0.76	8.28 ± 0.04	8.35 ± 0.06
PGD ₂	nd	nd	nd	nd	6.41 ± 0.08
Indomethacin	nd	nd	nd	nd	5.74 ± 0.12

Table 3. CRTh2 antagonist human whole blood (WBSC) and isolated cellular (ISC) shape change potency values plus protein binding and physicochemical descriptors clogP, logD_{7.4} and logK_{IAM}. Data are mean ± SEM for 3 or more experiments. ^a determined by HPLC column method (Kerns et al., 2003) ^b determined by HPLC column method (Valko et al., 2000) ^c determined by equilibrium dialysis (Banker et al., 2003)

CRTh2 antagonist	clogP	log D _{7.4} ^a	log K _{IAM7.4} ^b	% human plasma protein unbound ^c	WBSC IC ₅₀ (nM)	ISC IC ₅₀ (nM)
QAW039	2.26	0.65	1.12	7.8	0.44 ± 0.19	0.40 ± 0.04
QAV680	1.15	-0.34	0.55	21.4	31.0 ± 1.0	5.0 ± 1.0
AZD-1981	4.79	1.19	1.77	1.8	13.10 ± 4.03	2.21 ± 0.87
OC-459	4.57	1.32	1.91	0.5	422.5 ± 155.9	6.4 ± 1.4
BI-671800	4.58	1.65	1.95	0.4	104.9 ± 35.3	1.87 ± 0.88
Ramatroban	3.97	1.34	1.89	2.7	195 ± 75.4	11.4 ± 1.9
Setipiprant	4.28	1.35	1.88	0.4	>500	10.7 ± 1.7

Table 4. Comparison of potencies of CRTh2 antagonists QAV680 and QAW039 for inhibition of PGD₂ induced cytokine release in human CD4⁺ Th2 lymphocytes. Methods described in the [supplemental files](#). Data are mean \pm s.e. mean from at least 3 separate experiments.

CRTh2 antagonist	Inhibition of IL-5 release IC ₅₀ (nM)	Inhibition of IL-13 release IC ₅₀ (nM)
QAW039	2.56 \pm 1.24	1.40 \pm 0.30
QAV680	57 \pm 28	29 \pm 5