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Pharmacological characterization of MK-7246, a potent and selective CRTH2 antagonist

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Running title: Pharmacological characterization of a novel CRTH2 antagonist

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Non-standard abbreviations:

CRTH2, chemoattractant receptor-homologous molecule expressed on T-helper cells; Th2, T-helper cells type 2; PGD_2, Prostaglandin D_2; DK-PGD_2, 13-14-dihydro-15-keto-PGD_2; HEK, human embryonic kidney cell; cAMP, cyclic adenosine monophosphate; i.v., intra-venously

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ABSTRACT

The chemoattractant receptor-homologous molecule expressed on T-helper type 2 cells (CRTH2) is a G protein-coupled receptor that has been reported to modulate inflammatory responses in various rodent models of asthma, allergic rhinitis and atopic dermatitis. In this study, we describe the biological and pharmacological properties of MK-7246, a novel synthetic CRTH2 antagonist. We show that MK-7246 1) has high affinity for the human, monkey, dog, rat and mouse CRTH2, 2) interacts with CRTH2 in a reversible manner, 3) exhibits high selectivity over all prostanoid receptors as well as 157 other receptors and enzymes, 4) acts as a full antagonist on recombinant and endogenously expressed CRTH2, 5) demonstrates good oral bioavailability and metabolic stability in various animal species, 6) yields ex vivo blockade of CRTH2 on eosinophils in monkeys and sheep and 7) significantly blocks antigen-induced late phase bronchoconstriction and airway hyperresponsiveness in sheep. MK-7246 represents a potent and selective tool to further investigate the in vivo function of CRTH2.

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INTRODUCTION

Prostaglandin D_2 (PGD_2) is primarily released by mast cells in the lungs of asthmatic subjects upon allergen/IgE-induced degranulation (Miadonna et al., 1990). DP (also known as DP1) was the first G-protein coupled receptor identified for PGD_2 and is recognized to have a role in vasodilation (Walch et al., 1999). A second PGD_2 receptor termed CRTH2 (Chemoattractant Receptor expressed on TH2 cells; also known as DP2) has also been identified (Hirai et al., 2001). The role of PGD_2 acting via DP1 and/or CRTH2 in human asthma is under clinical evaluation using selective antagonists to these receptors. Clinical evaluation with a specific DP1 antagonist demonstrated that blockade of DP1 had no effect on a variety of endpoints in asthma or seasonal allergic rhinitis (Phillip et al 2009). If PGD_2 does play a role in asthma, it is likely via CRTH2.

CRTH2 was initially identified as a GPCR expressed on human Th2 but not on Th1 cells (Nagata et al., 1999a). CRTH2 was also shown to be expressed on innate cells such as eosinophils and basophils (Nagata et al., 1999b). *In vitro*, PGD_2 and at least one of its metabolites, DK-PGD_2 (13,14-dihydro-15-keto-PGD_2), can recruit and activate these leukocytes via CRTH2. CRTH2 activation leads to the recruitment of leukocytes by 1) stimulating the expression of the surface protein CD11b which favors cell adhesion to the vascular wall and transmigration of cells from the blood circulation to the inflamed tissue and 2) stimulating cell movement to the site of inflammation (chemotaxis). CRTH2 activation also promotes the release of Th2 cytokines, such as IL-13, from Th2 cells and degranulation of basophils and eosinophils (Kostenis and Ulven 2006).

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Early preclinical studies indicate that genetic and pharmacological blockade of CRTH2 signaling have variable response in attenuating inflammation (Chevalier et al., 2005 and Merck unpublished data in rodent models). However, recent studies show that CRTH2 blockade can significantly reduce allergic inflammation in rodent models of antigen-induced airway inflammation, allergic rhinitis, and atopic dermatitis, as well as airway hyperresponsiveness in rodent models of asthma (Uller et al., 2007; Shiraishi et al., 2008; Lukacs et al., 2008; Takeshita et al., 2004; Satoh et al., 2006; Oiwa et al., 2008). In humans, a CRTH2 genetic polymorphism leading to increased CRTH2 mRNA stability is significantly associated with asthma in two independent populations (Huang et al., 2004). Pharmacologically, Ramatroban, a dual TP/CRTH2 antagonist commercialized in Japan is reported to exhibit some degree of efficacy in allergic rhinitis (Terada et al., 1998).

TM30089 is a Ramatroban-derivative that has been shown to be selective for CRTH2 (Ulven and Kostenis 2005). We have identified and extensively characterized MK-7246, the corresponding inverse indole analog of TM30089. In this paper, we show that MK-7246 is a potent and selective CRTH2 antagonist. We also show that MK-7246 can significantly reduce antigen-induced late phase bronchoconstriction and airway hyperresponsiveness in sheep demonstrating that MK-7246 is a useful tool which can be used for elucidating the role of CRTH2 in inflammatory responses both in vitro and in vivo.

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METHODS

Chemicals

MK-7246 ({(7R)-7-[[(4-fluorophenyl)sulfonyl](methyl)amino]-6,7,8,9-tetrahydropyrido[1,2-a]indol-10-yl}acetic acid) was synthesized at Merck Frosst's Department of Medicinal Chemistry (Kirkland, PQ, Canada; Fig.1).

Radioligand Binding assays

Radioligand binding assays were as described elsewhere (Abramovitz et al., 2000 and Sawyer et al., 2002).

Binding Kinetic determination

The binding kinetics of [^{3}H]MK-7246 (specific activity of 41 Ci/mmol) at human CRTH2 was characterized using recombinant HEK293E cell membranes. The radioligand binding experimental condition for CRTH2 was as described previously (Sawyer et al., 2002) with the following exceptions: the incubation mixture contained 10 mM $MgCl_2$ instead of $MnCl_2$, 10 nM of [^{3}H]MK-7246 and 1.25 μg of membrane protein. Total binding represented 10 % of the radioligand added to the incubation media, and specific binding at equilibrium corresponded to 85% to 95% of the total binding. The membranes were first incubated with [^{3}H]MK-7246 for 120 min in the absence (total binding) or presence

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(non-specific binding) of 10 μM MK-7246. To one series of total binding incubation tubes, 10 μM MK-7246 or 100 μM PGD_2 was added to initiate dissociation of the radioligand from the receptor and the reaction was left to proceed for up to 300 min. The samples were then harvested and processed as detailed above. The association and dissociation kinetic data analysis was done by non-linear regression curve fitting using Prism software (GraphPad Software Inc., San Diego, California) to determine the observed on-rate (K_{obs}) and dissociation rate (K_{off}) constants, and $T_{1/2}$ of on- and off-rates. The association rate constant (K_{on}) was calculated as (K_{obs}-K_{off})/[radioligand] and the equilibrium dissociation constant (K_d) was calculated as the ratio of K_{off}/K_{on}.

Functional cAMP assay and DP/TP functional selectivity assays

Inhibition of Forskolin-induced increase in intracellular cAMP using HEK-hCRTH2 cells was evaluated as described elsewhere (Sawyer et al., 2002). Functional cell-based assays to measure activity on DP1 (inhibition of PGD_2-induced increases in cAMP in platelets) and the prostanoid receptor TP (inhibition of thromboxane-induced platelet aggregation) were performed as described elsewhere (Sturino et al., 2007).

Eosinophil shape change assay

Blood was collected in vacutainers containing EDTA. The antagonist was added to blood and incubated for 10 min at room temperature. DK-PGD_2 was then added to blood for 4 min at 37^{o}C in a running water bath. Blood cells were then fixed in presence of cold 0.25%(v/v) paraformaldehyde prepared in 75%(v/v) PBS for 1 min on ice. 175μL of

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fixed blood was transferred into 870µL of cold 155mM NH_4Cl lysis solution and incubated at 4°C for at least 40 min. The solution was then centrifuged at 430g for 5min and the supernatant was discarded. Centrifuged cells were analyzed with a FACs Calibur flow cytometer (Becton Dickinson). Flow cytometry raw data were analyzed with FlowJo software by isolating the eosinophils from the neutrophils based on their intrinsic autofluorescence and determining the percent of total eosinophils with increased forward scatter (FSC-H) value. Maximum (100%) and minimum (0%) shape change were determined in the presence of 10µM DK-PGD_2 and PBS, respectively. MK-7246 was tested in 10-point dose titration curves in the presence of 30nM DK-PGD_2 (~EC80) to determine the IC_{50}.

Eosinophil CD11b assay

Human blood was collected in vacutainers containing citrate. The antagonist was added to blood and incubated for 10 min at 37°C. DK-PGD_2 was then added to blood and again incubated for 10 min at 37°C. The samples were put on ice for 5min, the anti-human CD11b-APC (Becton Dickinson) antibody was added and the blood was incubated on ice for 20 min. in the dark. After gentle mixing, blood cells were fixed by adding cold BD FACs lysing solution (Becton Dickinson) for 15 min at room temperature in the dark. The samples were then centrifuged at 430g for 5min, the supernatant was discarded, and cells were resuspended in PBS. Cells were analyzed by flow cytometry as described above with the geometric mean fluorescence (APC fluorochrome intensity) of the eosinophil population. Maximum and minimum mean fluorescence intensity were determined in the presence of 10µM DK-PGD_2 and PBS, respectively. MK-7246 was

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tested in 10-point dose titration curves in the presence of 30 nM DK-PGD_2 (~EC_{80}) to determine the IC_{50}.

Basophil CD11b assay

The procedure was as described above for the eosinophil CD11b assay with the following exceptions: 1) the human blood was collected in vacutainers containing heparin, 2) an anti-IgE-FITC (from Abcam, Cambridge, MA, USA) was added with the anti-CD11b–APC antibody and 3) basophils were identified by their IgE positive, side scatter low profile.

Ex vivo CD11b assay using monkey or sheep whole blood

Male cynomolgus monkeys (*Macaca fascicularis*), weighing between 4 and 10 kg were from the Primate Import Corp., Fort Washington, NY (Charles River Research Primate Corporation and Covance Research Products Inc, Denver, PA.). Female sheep, weighing between 32 and 46 kg were housed at the Mount Sinai Medical Center Animal Research Facility (Miami Beach, Florida). All animals were selected on the basis of their naturally acquired sensitivity to the soil pathogen *Ascaris suum*. The animals were administered a single dose of MK-7246 orally in monkeys and by i.v. administration in sheep. Blood samples were taken at various time points after dosing for evaluation of eosinophil CD11b expression following ex vivo challenge with various concentrations of DK-PGD_2 as described above.

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Evaluation of the effect of MK-7246 on lung function in sheep

Three sheep were treated with 1 mg/kg MK-7246 (in 5% dextrose solution) delivered intra-venously over a period of 4 hours. This dose regimen was selected based on its ability to fully block the blood biomarker (ex vivo stimulation of eosinophil CD11b) over a period of 24 hours (data not shown). The i.v. infusion was initiated 1 hour prior to the challenge of the sheep airways with aerosolized extracts of the antigen (Ascaris suum extract). A detailed description of the animals used as well as the measurement of airway mechanics (lung resistance) and airway hyperresponsiveness following the antigen challenge are described elsewhere (Shichijo et al., 2009). The same three sheep were infused with the vehicle (5% dextrose) 3 weeks before and 3 weeks after the study with MK-7246 in order to establish the control lung function response to the antigen in the absence of drug.

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RESULTS

Determination of MK-7246 affinity and selectivity for CRTH2

The affinity and selectivity of MK-7246 for human CRTH2 and recombinant human prostanoid receptors was determined by equilibrium competition analysis using the relevant radioligands and cell membranes expressing the various receptors. MK-7246 competed for [^{3}H]PGD_2 specific binding to cell membranes expressing recombinant human CRTH2 with high affinity (K_i of 2.5 nM; Table 1). MK-7246 displayed a relatively high selectivity for CRTH2 with an affinity 149-fold lower for the DP receptor and ≥ 1500-fold lower for the other prostanoid receptors (Table 1). MK-7246 was also tested in a panel of 157 enzyme and receptor assays by MDS Pharma Services Taiwan, Ltd., at concentrations up to 100 μM and small but significant activity was detected only on Phosphodiesterase 1 (IC_{50} = 33.2 μM) and MAP kinase 3 (ERK1; IC_{50} = 49.4 μM). MK-7246 was also shown to bind to recombinant mouse, rat, dog and Cynomolgus monkey CRTH2 receptors with high affinity (Table 2).

Association and Dissociation Kinetics of [^{3}H]MK-7246 from Recombinant Human CRTH2 Receptor

In order to calculate an equilibrium dissociation constant and to determine whether MK-7246 associates to CRTH2 in a reversible manner, the association and dissociation rates of [^{3}H]MK-7246, from recombinant CRTH2 were determined. The on-rate of association

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of [^{3}H]MK-7246 at CRTH2 (K_{on}) was 0.0016 to 0.0017 min^{-1} . nM^{-1} and the $T_{1/2}$[on] was 19.8 to 20.9 min (Figure 2; Table 3). Dissociation of [^{3}H]MK-7246 from the recombinant CRTH2 receptor, initiated with 10 μM of MK-7246 or 100 μM of PGD_2, was comparatively slower than its on-rate with a K_{off} of 0.0212 to 0.0216 min^{-1} and a $T_{1/2}$[off] of 32.2 to 33.9 min (Figure 2; Table 3). As described in the methods section, the association and dissociation kinetic analysis was used to deduce an equilibrium dissociation constant (K_d) for [^{3}H]MK-7246 at CRTH2 of 13.6 to 18.6 nM. Another method of determining the K_d of a radiolabeled receptor ligand consists in performing saturation analysis. This method establishes the relationship between receptor bound and unbound ligand fraction seen at the equilibrium after adding various concentrations of [^{3}H]MK-7246. The K_d for [^{3}H]MK-7246 determined by saturation analysis was 2.3 ± 0.7 nM (n = 3; data not shown). This K_d value is similar to the affinity constant determined for MK-7246 in competition binding assays (K_i = 2.5; Table 1).

Determination of Antagonist Potency of MK-7246 in Blocking DK-PGD_2-Induced Inhibition of cAMP Accumulation in Recombinant Cells Overexpressing Human CRTH2

CRTH2 is a Gi-coupled receptor that signals through inhibition of adenylate cyclase leading to an inhibition of intracellular cAMP production (Sawyer et al., 2002). Accordingly, an assay that measures intracellular cAMP levels was used as a functional assay for receptor activation using recombinant cells overexpressing human CRTH2 (HEK293E/CRTH2). The CRTH2-selective metabolite of PGD_2, termed DK-PGD_2, was

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used to activate CRTH2 at the surface of these cells and thus to trigger a decrease in intracellular cAMP. MK-7246 blocked DK-PGD_2-induced inhibition of cAMP formation in HEK293E-CRTH2 cells with an IC_{50} value of 3.0 nM (Table 4). We have also performed Schild analysis using this cAMP assay. The Schild analysis consists in challenging HEK293E-CRTH2 cells with increasing concentrations of DK- PGD_2 after pre-incubation with different concentrations of MK-7246 (Figure 3). The fact that increasing concentrations of MK-7246 causes an attenuation of maximal response to DK-PGD_2 is suggestive of insurmountable antagonism.

Determination of Antagonist Potency of MK-7246 in Blocking DK-PGD2-Induced Eosinophil Shape Change and Up-Regulation of CD11b on Eosinophils and Basophils in Human Whole Blood.

Assays using human whole blood were performed to evaluate the potency of MK-7246 at the CRTH2 receptor endogenously expressed at the surface of human cells in a physiologically relevant environment. The potency of MK-7246 was evaluated on two different endpoints (shape change and CD11b expression) on two different types of leukocyte expressing CRTH2 (eosinophils and basophils) to verify consistency. Both shape change and up-regulation of CD11b have previously been shown to be a consequence of eosinophil activation (Gervais et al., 2001 and Monneret et al., 2001) and basophil activation (Bohm et al., 2004 and Yoshimura-Uchiyama et al., 2004) by a CRTH2 agonist. Shape change is a consequence of cytoskeleton reorganization predisposing the leukocyte for cell movement and transmigration and can be quantified

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by flow cytometry through changes in forward light scatter (FSC). CD11b is part of an integrin complex named MAC-1 which interacts with ICAM-1 at the surface of endothelial cells facilitating local entry of leukocytes in inflamed tissue. Up-regulation of CD11b at the surface of a cell is quantified by flow cytometry using a fluorescent antibody specific for CRTH2. MK-7246 inhibited DK-PGD_2-induced eosinophil shape change with an IC_{50} value of 2.2 nM (Table 4). MK-7246 also efficiently blocked DK-PGD_2-induced CD11b up-regulation on eosinophils and basophils with IC_{50} values of 6.2 and 5.4 nM, respectively (Table 4). Importantly, no agonistic activity of MK-7246 at concentrations up to 10 μM was observed in the whole blood assays mentioned above as well as in the cAMP functional assay (data not shown).

Pharmacokinetic profile of MK-7246 in animals

MK-7246 was administered to mice, rats, dogs, sheep, Rhesus and Cynomolgus monkeys orally (p.o) and/or intravenously (i.v) to determine its pharmacokinetic profile over a 24-h period. (Table 5 and 6). The pharmacokinetic properties of MK-7246 are overall good. The compound has excellent oral bioavailability in all species (F> 50%) except in Cynomolgus monkeys (F = 10%). The reason for the low bioavailability in Cynomolgus monkeys is unknown and is not aligned with the observed low plasma clearance. Low plasma clearance is also observed in rodents, Rhesus monkeys and sheep. Moderate clearance is observed in dogs. The plasma half life is overall longer in higher species compared to rodents. Higher doses or multiple dosing might be required if sustained coverage is required in rodents.

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Ex vivo effects of orally dosed MK-7246 on DK-PGD2-induced increases in CD11b expression on blood eosinophils in Cynomolgus monkeys and sheep.

In order to establish a relationship between MK-7246 plasma levels (pharmacokinetic; PK) and the ex vivo inhibition of DK-PGD_2-induced CD11b expression (pharmacodynamic; PD), blood samples collected at different time points from 8 monkeys dosed with different concentrations of MK-7246 were analyzed in three independent experiments and are summarized in Figure 4A. A plot of the MK-7246-mediated inhibition of CD11b expression versus the plasma levels of MK-7246 established that on average 15 nM of MK-7246 is necessary to block 50% of DK-PGD_2-induced CD11b expression. When MK-7246 was added directly to the monkey blood in vitro the IC_{50} value was 3.5 ± 1.5 nM (n = 4). The PK/PD relationship for MK-7246 was also evaluated in sheep. Nine sheep were dosed i.v. with different concentrations of MK-7246 and blood was challenged with DK-PGD_2 ex vivo at different time points. The data are summarized in Figure 4B. The IC_{50} value for MK-7246 inhibition of ex vivo expression of eosinophil CD11b from sheep blood was 107 nM. When MK-7246 was added directly to the blood in vitro the IC_{50} value was 22.5 nM (n = 2).

Effects of MK-7246 on antigen-induced changes in lung function in sheep

Three sheep were initially dosed i.v. with a vehicle control (5% dextrose solution) and then challenged with an antigen (aerosols of *Ascaris suum* extract) in order to establish their control lung function response. The antigen challenge in vehicle-treated animals

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triggered a rapid increase in airway resistance (early phase bronchoconstriction between 0-4 hr post challenge) as well as a delayed increase in airway resistance (late phase bronchoconstriction between 4-8 hr post-challenge) as shown in Figure 5A. The antigen challenge in vehicle-treated animals also caused an increase in airway responsiveness measured 24 hours post-challenge as reflected by a decrease in the amount of carbachol (muscarinic agonist) required to cause a 400% increase in lung resistance (Figure 5B). Three weeks later, the same three sheep were dosed i.v. with MK-7246 and challenged with the antigen. Although MK-7246 had no significant effect on the early phase bronchoconstriction, it decreased the late phase bronchoconstriction on average by 80% (Figure 5A; $p < 0.00001$). Moreover, MK-7246 completely prevented antigen-induced airway hyperresponsiveness (Figure 5B). 3 weeks after the dosing with MK-7246, the same three sheep were dosed again with the vehicle only and challenged with the antigen. All sheep had a normal response to the antigen in terms of early and late phase bronchoconstriction as well as airway hyperresponsiveness (data not shown).

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DISCUSSION

In this study, we describe a novel CRTH2 antagonist. We show that MK-7246 1) has high affinity for the human CRTH2 receptor, 2) interacts with CRTH2 in a reversible manner, 3) exhibits high selectivity over all prostanoid receptors as well as 157 other receptors and enzymes, 4) acts as a full antagonist on recombinant and endogenously expressed CRTH2, 5) is devoid of any agonistic activity in all functional assays described in this study, 6) demonstrates good oral bioavailability and metabolic stability in various animal species leading to ex vivo blockade of CRTH2 on eosinophils in monkeys and sheep and 7) significantly blocks antigen-induced late phase bronchoconstriction and airway hyperresponsiveness in sheep.

Ligand binding kinetics analysis with radiolabeled MK-7246 revealed that its dissociation from CRTH2 is slower than its association leading to a high affinity ligand with a K_d in the low nM range. The slow dissociation ($T_{1/2}$ = 33 minutes) most likely explain the insurmountability (reduction in maximal response/plateau against high concentrations of CRTH2 agonist) observed in the Schild analysis using the recombinant cell system. Similar insurmoutability was observed in the whole blood assays (data not shown). In these functional assays, the cells are pre-incubated for 10 minutes with the antagonist and the agonist stimulation lasts for 4 to 10 minutes. This ~20 minute time interval is shorter than the dissociation $T_{1/2}$ of MK-7246 and thus most likely leads to the sustained occupancy of the CRTH2 receptors by the antagonist for the duration of the assay.

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Administration of single oral doses of MK-7246 can lead to a complete blockade of DK-PGD_2-induced up-regulation of CD11b on blood eosinophils ex vivo. A relatively good correlation between MK-7246 blood exposure and the level of inhibition of CD11b expression is observed. Based on the PK/PD relationship curve, the MK-7246 blood exposure necessary to block 50% of CD11b expression (IC_{50}) in this ex vivo assay is 15 nM in monkeys. This IC_{50} value obtained ex vivo is about 4-fold higher than the IC_{50} value of 3.5 nM obtained in the CD11b in vitro whole blood assay. The reason for this discrepancy is unknown, however, a similar shift between the IC_{50}'s generated ex vivo and in vitro was observed using sheep blood.

MK-7246 is a high affinity ligand at recombinant mouse, rat, dog and Cynomolgus monkey CRTH2 and has favorable pharmacokinetic properties across these species. MK-7246 thus represents a useful tool to investigate the role of CRTH2 in various animal models. For example, we used this compound in a sheep model to demonstrate favorable effects on lung function following a lung antigen challenge. MK-7246 did not have an effect on the early phase bronchoconstriction which is associated with the immediate release of bronchoconstrictor agents and pro-inflammatory mediators such as PGD_2 (Abraham, 2008). However, MK-7246 significantly blocked the late phase bronchoconstriction and airway hyperresponsiveness which are thought to be the physiological indicators of a heightened and continued inflammatory response in part associated with the recruitment and activation of pro-inflammatory leukocytes to the lung (Abraham, 2008). It is thus hypothesized that MK-7246 has a significant impact on the late phase airway response because it interferes with the recruitment and activation of key CRTH2-positive cells involved in the allergic response (Th2 cells, eosinophils and

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basophils) following the release of the CRTH2 ligand PGD_2 during the early response. In support of this hypothesis, it has been shown that known blockers of cellular recruitment such as L-selectin inhibitors, when administered post antigen challenge, can completely block the late airway response and airway hyperresponsiveness without affecting the early airway response (Abraham et al., 1999). To our knowledge, this is the first demonstration of the modulation by a CRTH2 antagonist of the late phase airway response after an antigen challenge. These data also support previous findings in mice that a CRTH2 antagonist can block the development of airway hyperresponsiveness (Gonzalo et al., 2005; Lukacs et al., 2008 and Shiraishi et al., 2010).

An obvious advantage of MK-7246 over the commonly used Ramatroban for in vivo studies is the improved potency on CRTH2 and selectivity against other prostanoid receptors. Characterization of Ramatroban in our in vitro assays confirmed its weaker affinity for the human CRTH2 receptor (Ki = 137 ± 21 nM; n = 4), its comparatively reduced potency in the whole blood eosinophil shape change assay (IC_{50} = 520 ± 370 nM; n = 4) and its lack of selectivity for CRTH2 versus the prostanoid TP receptor. Specifically, in our binding assays Ramatroban has greater affinity at human TP (Ki = 0.6 ± 0.1 nM; n = 3) as compared to human CRTH2 (Ki = 137 ± 21 nM; n = 4). The comparison of MK-7246 with other potent and selective CRTH2 antagonists with reported in vivo activity is more difficult in the absence of side-by-side comparison in the same assays and lack of complete datasets from published data. Other CRTH2 antagonists with reported activity in animal models for allergic inflammation include TM30089, ARRY-063, AZ11805131, AZ11665362, Amira's Compound 23, AM156, AM206 and Actimis' Compound A (recently reviewed by Norman in 2010). TM30089, a

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close analog of MK-7246, was partially characterized in some of our assays and was confirmed to have a similar profile as MK-7246 in terms of affinity for CRTH2, selectivity versus other prostanoid receptors, potency in various functional assays and pharmacokinetic properties in rodents.

Herein, we have described MK-7246, a potent and selective CRTH2 antagonist which can be used as a tool to interrogate the physiological and pathophysiological roles of CRTH2 both in vitro and in vivo.

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Wrote or contributed to the writing of the manuscript: Gervais, O'Neill

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LEGENDS FOR FIGURES

Figure 1. Structure of MK-7246

Figure 2 . Association and Dissociation of [^{3}H]MK-7246 at Human CRTH2. The binding kinetics of [^{3}H]MK-7246 at human CRTH2 was characterized using recombinant HEK293E cell membranes. The membranes were first incubated with [^{3}H]MK-7246 in the absence (total binding; blue curve) or presence (non-specific binding; black curve) of 10 μM of MK-7246. To one series of total binding incubation tubes, 10 μM of MK-7246 was added to initiate dissociation of the radioligand from the receptor (dissociation; red curve; panel A). To another series of total binding incubation tubes, 100 μM of PGD_2 was added to initiate dissociation (panel B). The association was determined 6 times and the dissociation was performed three times with MK-7246 and three times with PGD_2 as cold ligands yielding similar results. A representative experiment is shown.

Figure 3. Schild analysis of MK-7246. HEK cells overexpressing hCRTH2 were challenged with increasing concentrations of the CRTH2 agonist DK-PGD_2 (X-axis) and the extent of inhibition of cAMP production was monitored (Y-axis). The effects of different concentrations of the CRTH2 antagonist MK-7246 (color code; in nM) on the DK-PGD_2 dose response curve are shown.

Figure 4. Correlation between PK and PD for MK-7246 in Cynomolgus monkeys (A) and sheep (B). Blood samples were collected at different time points after dosing with MK-7246 orally in monkeys (1, 2 and 5 mpk) or intravenously in sheep (0.1, 0.3, 1 and 3 mpk). Blood was then challenged with increasing concentration of the CRTH2 agonist DK-PGD_2 and up-regulation of eosinophil CD11b expression was monitored. The percentage of inhibition of CD11b up-regulation in a given blood sample was calculated at an EC90 concentration of DK-PGD2 (Y-axis) and was correlated with the concentration of MK-7246 detected in that same blood sample (X-axis). A total of 8

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monkeys across 3 independent experiments and 9 sheep across 2 independent experiments were used.

Figure 5. Evaluation of MK-7246 in a sheep lung allergen challenge model. Sheep were infused for 4 hours with 1 mpk of MK-7246. The infusion was initiated 1 hour prior to an inhaled antigen challenge with an Ascaris extract. Effect on early (0-4hr) and late (4-8hr) phase bronchoconstriction were measured (A) as well as airway hyperresponsiveness to carbachol 24hr after the antigen challenge (B, a decrease in the carbachol level indicates the development of airway hyperesponsiveness). Results are expressed as the average of 3 sheep per group. Control responses with the vehicle were obtained with the same sheep 3 weeks before the drug treatment.

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TABLES

Table 1

Competitive Receptor Binding Activity of MK-7246 on Recombinant Human CRTH2 and Prostanoid Receptors

Receptor M	K-7246 K_i (nM)
CRTH2	2.5 ± 0.5 (8)
CRTH2 + HS†	8.1 ± 2.1 (6)
DP	373 ± 96 (6)
EP_1	>23330 (4)
EP_2	7668 ± 2169 (4)
$EP_{3\text{-III}}$	>20040 (4)
EP4	>15040 (4)
FP	>25100 (4)
IP	>23030 (4)
TP	3804 ± 1290 (6)

All values are mean ± SD, with n values indicated in parentheses.
†Affinity determined in the presence of 10% (w/v) human serum.

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Table 2

Competitive Receptor Binding Activity of MK-7246 on Recombinant CRTH2 Receptors from Different Species

Species MK	-7246 IC_{50} (nM)
Human	3.5 ± 0.8 (8)
Mouse	9.2 ± 0.3 (3)
Rat	4.6 ± 0.9 (3)
Dog	6.3 ± 2.2 (3)
Cynomolgus Monkey	6.9 ± 1.6 (3)

All values are mean ± SD, with n values indicated in parentheses.

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Table 3

Association and Dissociation Kinetics of [^{3}H]MK-7246 from Recombinant Human CRTH2

Asso	ciation Kinetics		Dissociation Kinetics		
Receptor	K_{on} ($min^{-1}.nM^{-1}$)	$T_{½}$ (on) (min)	Dissociating Ligand	K_{off} (min^{-1})	$T_{½}$ (off) (min)
CRTH2 (n=3)	0.0016 ± 0.0008	20.9 ± 6.0	PGD_2	0.0216 ± 0.0019	32.2 ± 2.7
CRTH2 (n=3)	0.0017 ± 0.0003	19.8 ± 1.9	MK-7246	0.0212 ± 0.0048	33.9 ± 7.6

All values are mean ± SD

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Table 4

Functional Antagonism of MK-7246 in human cell-based assays

	HEK-CRTH2	Whole blood			Platelet-rich plasma	
	cAMP	Eosinophil Shape Change	Eosinophil CD11b	Basophil CD11b	DP/cAMP	TP/aggregation
IC_{50} (nM)	3.0	2.2	6.2	5.4	8692	>30000
Std Dev	1.3	1.0	2.4	3.8	5289	
n	4	10	5	4	8	3

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Table 5

Intravenous Pharmacokinetics of MK-7246 in Mouse, Rat, Dog, Sheep and Monkeys

Species	Gender	Dose (mg/kg)	$AUC_{0\text{-}24h}$ (µM.h)	Cl_p (ml/min/kg)	Vd_{ss} (L/kg)	$t_{1/2}$ (h)
Mouse	M	5	9.2	22	3.8	2.8
Rat	M	1	17	2.1	0.9	5.6
Dog	M	0.5	1.2	15	2.3	8.4
Cynomolgus Monkey	M	0.5	4.2	4.8	0.5	11
Rhesus Monkey	M	0.5	2.6	6.9	2.3	8.1
Sheep	F	1	42	0.9	0.5	6.5

MK-7246 was dosed intravenously as the in situ sodium salt in 5% dextrose (1 mL/kg) in fed animals, except in mice where 80:20 PEG200:5%Dextrose was used. Concentrations of MK-7246 were determined by a validated LC-MS/MS assay. Values represent average of at least two animals. Plasma samples were stabilized with 2% formic $acid_{(aq)}$ upon collection to prevent acyl-glucuronide migration and hydrolysis.

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Table 6

Oral Pharmacokinetics of MK-7246 in Mouse, Rat, Dog and Monkeys

Species	Gender	Dose (mg/kg)	$AUC_{0\text{-}24h}$ (µM.h)	C_{max} (µM)	T_{max} (h)	F (%)
Mouse	M	30	56	6.8	1	109
Rat	M	1	20	2.5	2	114
Dog	M	1	1.6	0.58	0.25	67
Cynomolgus Monkey	M	1	0.8	0.06	0.5	10
Rhesus Monkey	M	1	2.9	0.28	0.5	57

MK-7246 was dosed orally as a suspension of crystalline free acid in 0.5% methylcellulose (5 mL/kg) to fasted animals. Concentrations of MK-7246 were determined by a validated LC-MS/MS assay. Values represent average of at least two animals. Plasma samples were stabilized with 2% formic $acid_{(aq)}$ upon collection to prevent acyl-glucuronide (L-002245377) migration and hydrolysis.

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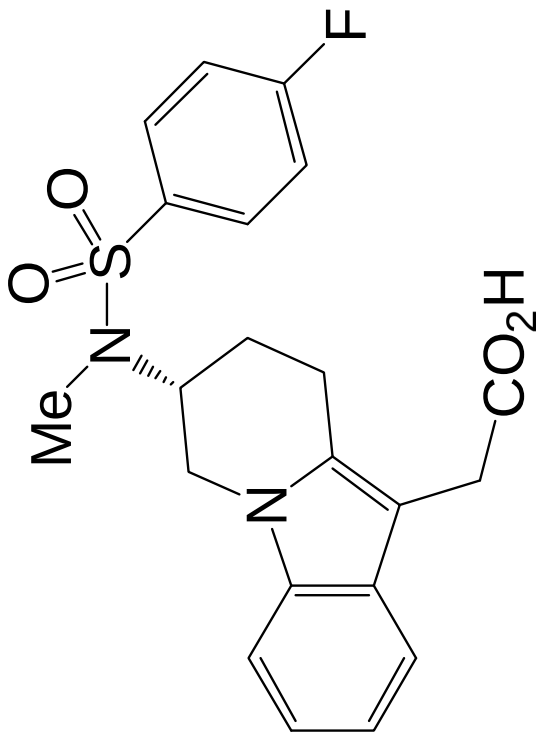

Figure 1

Figure 2

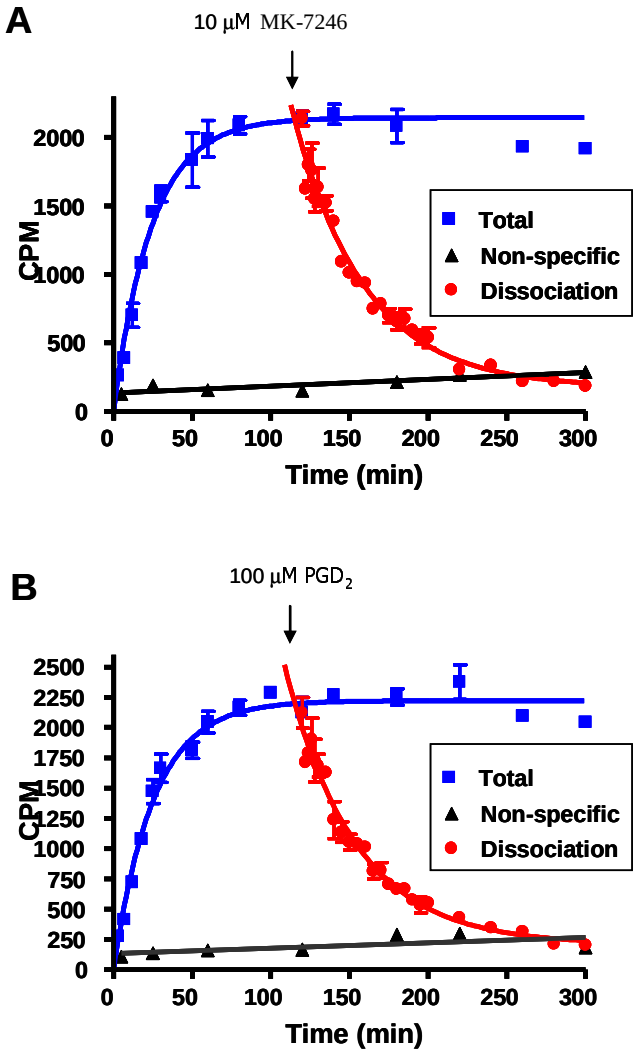

Figure 3

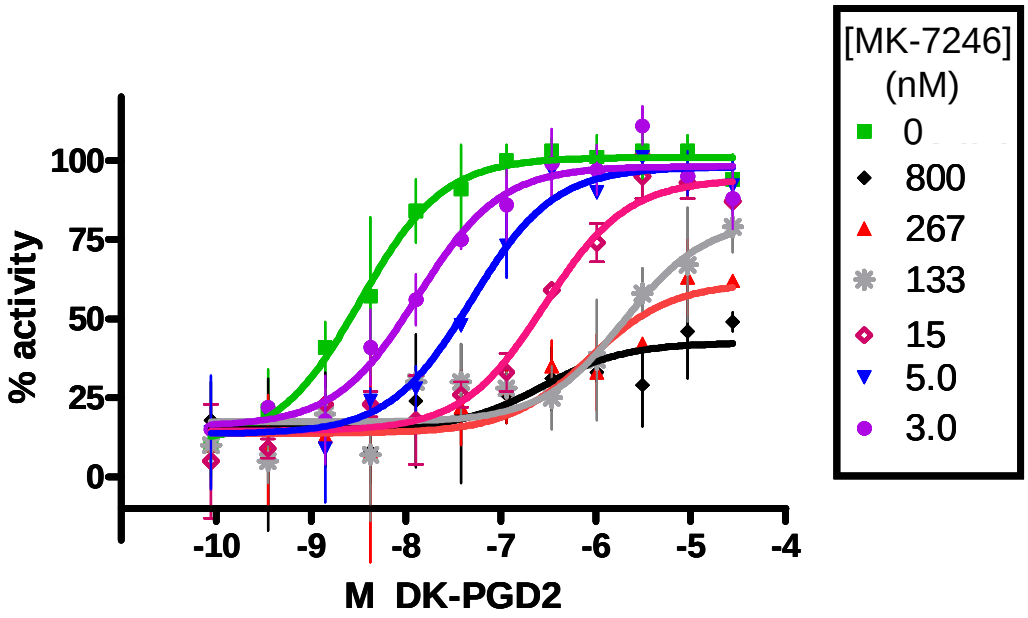

Figure 4

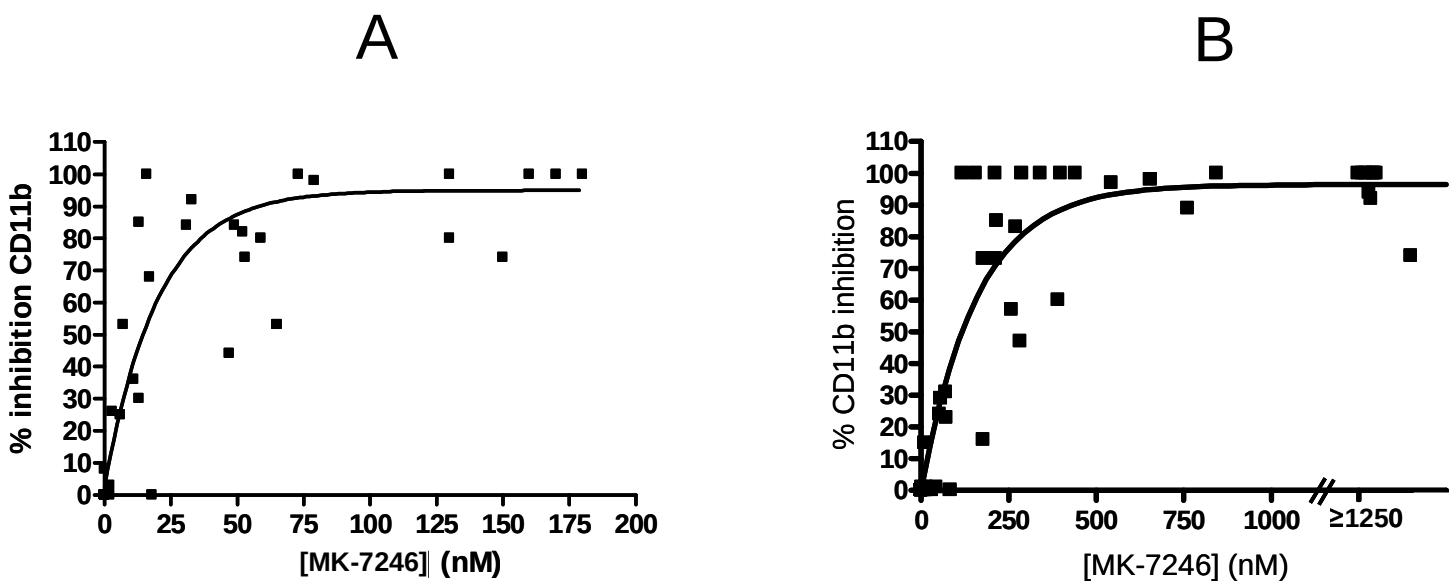

Figure 5