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Discovery and optimization of CRTH2 and DP dual antagonists

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ABSTRACT

A series of phenylacetic acid derivatives was discovered as CRTH2 antagonists. Modification of the series led to compounds that are also antagonists of DP. Since activation of CRTH2 and DP are believed to play key roles in mediating responses of asthma and other immune diseases, this series was optimized to increase the dual antagonistic activities and improve pharmacokinetic properties. These efforts led to selection of AMG 009 as a clinical candidate.

Prostaglandin D_2 (PGD_2) plays a key role in mediating allergic reactions such as those seen in asthma, allergic rhinitis, atopic dermatitis and allergic conjunctivitis.[1] PGD_2 is the major cyclooxygenase product formed and secreted by activated mast cells and its levels in bronchoalveolar lavage (BAL) fluid increase in response to antigen provocation.[2–6] In animals, including humans, PGD_2 stimulates several responses observed in asthma and other immune diseases such as airway constriction, mucus secretion, increased microvascular permeability and recruitment of eosinophils.[7–12] In addition, mice that overexpress PGD_2 synthase, resulting in overproduction of PGD_2, experience increased levels of Th2 cytokines and chemokines accompanied by enhanced accumulation of eosinophils and lymphocytes in the lung following an allergic response to ovalbumin.[11] Thus, PGD_2 is thought to be involved in the acute and late phases of allergic reactions. PGD_2 activates two receptors, DP (prostanoid D receptor, DP_1) and CRTH2 (chemoattractant receptor-homologous molecule expressed on Th2 cells, DP_2).

DP was the first PGD_2 receptor discovered.[13] It belongs to the prostanoid receptor family of GPCRs and is expressed on airway epithelium, smooth muscle and platelets. Upon stimulation, DP activates adenylate cyclase and increases the level of cAMP primarily via Gs-dependent pathways.[14] Genetic analysis of DP function using DP deficient mice has shown that mice lacking DP do not develop asthmatic responses in an ovalbumin-induced asthma model.[15] A selective DP antagonist in guinea pig allergic rhinitis models dramatically inhibited early nasal responses, as assessed by sneezing, mucosal plasma exudation, and nasal blockage, as well as late responses, such as mucosal plasma exudation and eosinophil infiltration.[16] Moreover, DP antagonism alleviated allergen-induced plasma exudation in the conjunctiva in a guinea pig allergic conjunctivitis model and antigen-induced eosinophil infiltration into the lung in a guinea pig asthma model.[16] In addition, human genetic data suggest that DP may play a role in asthma.[17] These results demonstrate the importance of DP signaling for allergic responses.

The second PGD_2 receptor, CRTH2, is related to the *N*-formyl peptide receptor (FPR) subfamily of chemoattractant receptors.[18] CRTH2 is selectively expressed on Th2 cells, T cytotoxic type 2 (Tc2) cells, eosinophils, and basophils.[19–21] CRTH2 activation induces an increase in intracellular Ca^{2+} mobilization via Gi dependent pathways, allowing CRTH2 to transmit promigratory signals in response to PGD_2.[17,22,23] In leukocytes, PGD_2 induces migration exclusively via CRTH2.[17,23–26] The ability of PGD_2 to stimulate the migration of inflammatory cells has led to the hypothesis that CRTH2 may play a role in the recruitment of cellular components of the allergic response into diseased tissues. Studies in rats using agonists and antagonists selective for CRTH2 over DP lend support to such a hypothesis. Selective activation of CRTH2 by intravenous injection of 13,14-dihydro-15-keto (DK)-PGD_2 into rats led to a dose- and time-dependent increase in the number of eosinophils in the peripheral blood. Pretreatment of the animals with Ramatroban (BAY u3405, 17), a CRTH2/thromboxane A2 receptor dual antagonist, completely abrogated DK-PGD_2-induced eosinophilia.[12] Furthermore, like DP, human genetic data also suggest that CRTH2 may play a role in asthma.[27]

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Several groups have reported their efforts of identifying selective DP and CRTH2 inhibitors. Figures 1 and 2 show a few examples of the DP and CRTH2 antagonists.[28–32] Comprehensive reports on DP and CRTH2 antagonists can be found in two review articles.[33,34] Because the collective observations suggest that DP and CRTH2 play important and complementary roles in the physiological responses to PGD_2, blockade of both receptors may prove more beneficial in alleviating allergic diseases triggered by PGD_2. In this article, we report our efforts towards the identification of dual DP and CRTH2 antagonists.

A phenylacetic acid derivative **1** (Table 1), discovered in a high throughput screen for CRTH2, inhibited the binding of ^{3}H-PGD_2 to CRTH2 receptors on 293 cells with an IC_{50} of 0.010 μM, and inhibited the binding of ^{3}H-PGD_2 to DP receptors with an IC_{50} of only 8.3 μM (Table 1).[35] Compound **1** also inhibited CRTH2 mediated cell migrations in response to PGD_2 with an IC_{50} of 0.0047 μM using hCRTH2 stably transfected CEM cells.[36]

Compound **1** and many its derivatives (**2–12** and **20–37**) were synthesized according to Scheme 1. 4-Chloro-3-nitrobenzoyl chloride was reacted with amines to form the corresponding amides. Displacement of the chlorine adjacent to the nitro group with hydroxyphenylacetic acids gave the bis-aryl ethers in good yields. Reduction of the nitro group followed by treatment with sulfonyl chlorides in pyridine afforded the sulfonamides.

Compounds **13, 15** and **16** were synthesized in three steps (Scheme 2), as described in Scheme 1, steps b, c and d.

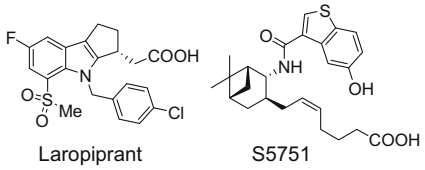


Figure 1. DP antagonists.

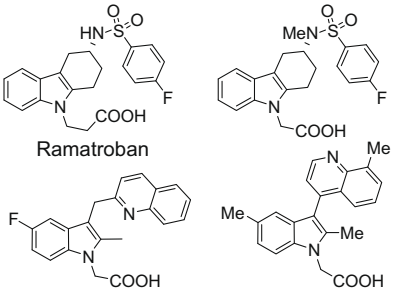
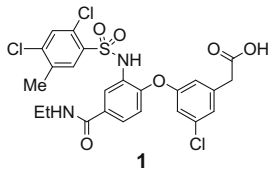


Figure 2. CRTH2 antagonists.

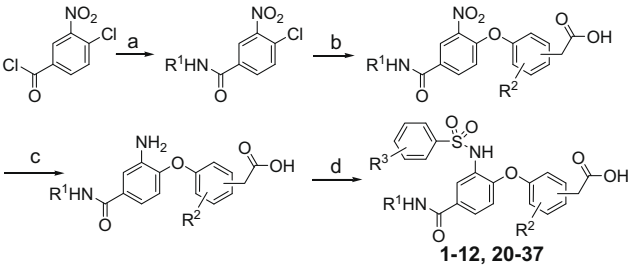
Table 1



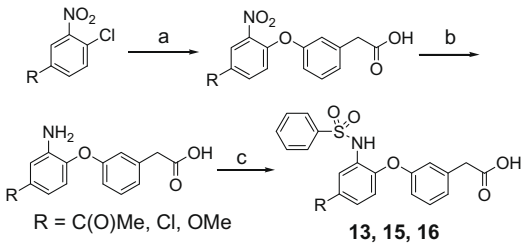
1

Compd	CRTH2 IC_{50}[a] in buffer (μM)	DP IC_{50}[a] in buffer (μM)
1	0.010	8.3

[a] Displacement of ^{3}H-PGD_2 from the CRTH2 or DP receptor expressed on 293 cells. Assay run in buffer containing 0.5% BSA. See Ref. 35 for assay protocol. Values are means of three experiments, standard deviation is ±30%.



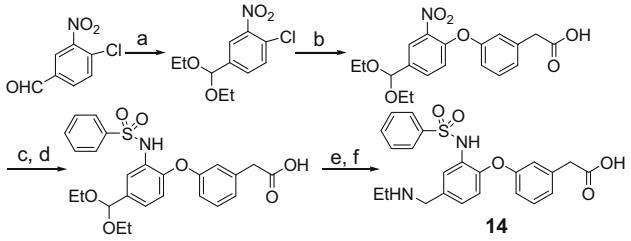
Scheme 1. Reagents and conditions: (a) amines, triethylamine, DCM, rt, 4 h, ~90%; (b) phenylacetic acids methyl ester, cesium carbonate, DMSO, 70 °C, 6 h, ~85%; (c) H_2, Pd/C, EtOH, rt, 1 h, 100%; or $SnCl_2$, EtOAc, 60 °C, 4 h, 80%; (d) sulfonyl chlorides, pyridine, rt, 24 h, 70%.



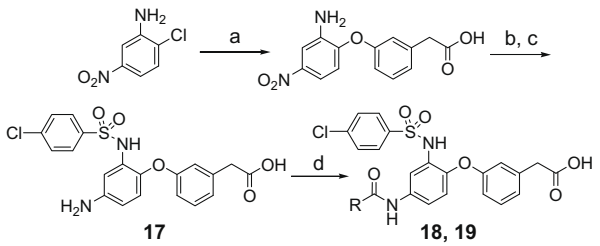
Scheme 2. Reagents and conditions: (a) 3-hydroxyphenylacetic acid, cesium carbonate, DMSO, 70 °C, 6 h, ~85%; (b) $SnCl_2$, EtOAc, 60 °C, 4 h, 80%; (c) benzenesulfonyl chlorides, pyridine, rt, 24 h, 70%.

The synthesis of compound **14** is shown in Scheme 3. 4-Chloro-3-nitrobenzaldehyde was protected as the diethyl acetal, which was carried into the same three steps as described in Scheme 2. Finally, the aldehyde was unmasked and converted into the ethyl benzyl amine via reductive amination.

Compound **17** (Scheme 4) was also synthesized similarly in three steps, as described in Scheme 2. Compounds **18** and **19** were synthesized from compound **17**. Reaction of propionyl chloride



Scheme 3. Reagents and conditions: (a) EtOH, HCl, rt, 14 h, 100%; (b) 3-hydroxyphenylacetic acid methyl ester, cesium carbonate, DMSO, 70 °C, 6 h, ~85%; (c) $SnCl_2$, EtOAc, 60 °C, 4 h, 80%; (d) benzenesulfonyl chlorides, pyridine, rt, 24 h, 70%; (e) TFA, DCM/H_2O, 0 °C, 2 h, 90%; (f) ethylamine, $NaBH(OAc)_3$, DCE, rt, 3 h, 80%.



Scheme 4. Reagents and conditions: (a) 3-hydroxyphenylacetic acid methyl ester, cesium carbonate, DMSO, 70 °C, 6 h, ~85%; (b) benzenesulfonyl chlorides, pyridine, rt, 24 h, 70%; (c) $SnCl_2$, EtOAc, 60 °C, 4 h, 80%; (d) for **18**, propionyl chloride, triethylamine, EtOAc, rt, 12 h, 80%; for **19**, ethyl isocyanate, triethylamine, EtOAc, rt, 12 h, 80%.

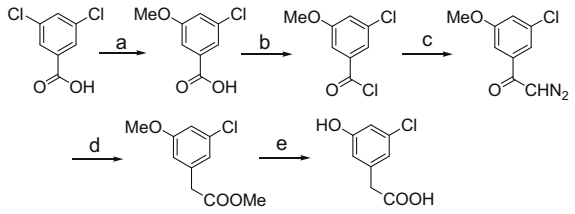
with **17** yielded amide **18** and reaction of ethyl isocyanate with **17** afforded urea **19**.

The non-commercially available 3-hydroxy-5-chloro phenylacetic acid was synthesized according to Scheme 5. Monochloro displacement of 3,5-dichlorobenzoic acid by methoxide gave 3-chloro-5-methoxybenzoic acid. The carboxylic acid was then converted into the acyl chloride, which was treated with trimethylsilyldiazomethane to afford the diazoketone. Wolff rearrangement of the diazoketone provided the homologated methyl ester. Finally, demethylation afforded 3-hydroxy-5-chloro phenylacetic acid.

The optimization began with the examination of the effect of substitutions on the sulfonamide benzene ring (Table 2). It was found that the substitutions had little effect on the compounds' affinity for CRTH2, as the unsubstituted compound **3** has similar potency to other compounds in Table 2. However, in vitro metabolic stability studies let us have a preference for the benzenesulfonamides with halogen substitutions, such as the 2,4-dichloro benzenesulfonamide (**2**), because the stability studies using liver microsomes indicated that the benzene ring of the benzenesulfonamides might be a vulnerable site of oxidative metabolism and benzenesulfonamides with alkyl groups or other electron-donating substitutions tend to have less optimal microsomal stability.

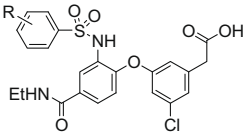
Next we investigated the role of the ethyl amide moiety. Analogs devoid of the hydrogen bond donor NH, such as tertiary amide **12** and methyl ketone **13**, has significant decreased activity. Likewise, analogs lacking the carbonyl group, such as compounds **14** (compared with **9**) and **17** (compared with **18**), also has significantly decreased CRTH2 activity. This data suggests that a hydrogen bond donor and a hydrogen bond acceptor together is likely to be beneficial for the activity on CRTH2, as demonstrated by the primary or secondary amides (**9** to **11**), the reversed amide (**18**) and the urea (**19**) derivatives.

Evaluation of the phenylacetic acid moiety revealed that modifications in this part of the molecule affect the selectivity of the antagonists to the CRTH2 and DP receptors (Table 4). Compound **20** (Table 4) and compounds in Tables 2 and 3 that feature the acetic acid moiety *meta* to the oxygen-linker are selective for CRTH2 over DP receptor. However, compounds that feature the acetic acid moiety *para* to the oxygen-linker, such as **21,** display CRTH2 and DP dual inhibitory activity. Since we were interested in identifying dual antagonists of CRTH2 and DP receptors, we decided to explore the analogs of **21**. Replacement of the acid moiety of **21** with a tetrazole group (**22**) maintained the affinity for the DP receptor, but the affinity for CRTH2 was greatly reduced. Likewise, addition of a methyl group at the methylene next to the carboxylic acid (**23**) resulted in a moderate enhancement of the affinity for DP, but a significant loss of the CRTH2 affinity.



Scheme 5. Reagents and conditions: (a) NaOMe, HMPA, 120 °C, 2 days, 95%; (b) $SOCl_2$, reflux, 4 h, 100%; (c) $TMSCHN_2$, triethylamine, ACN, THF, 0 °C, 3 h, 55%; (d) silver benzoate, triethylamine, MeOH, -25 to 25 °C, 6 h, 70%; (e) 48% HBr, HOAc, reflux, 22 h, 95%.

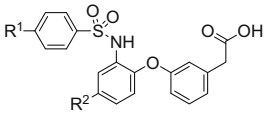
Table 2
Evaluation of the sulfonamide benzene ring



Compd	R	CRTH2 IC_{50}[a] in buffer (μM)
1	2,4-Cl-5-Me	0.010
2	2,4-Cl	0.012
3	H	0.003
4	4-Me	0.005
5	4-Cl	0.003
6	2-Cl	0.004
7	3-Cl-4-Me	0.004
8	2-Cl-4-CF_3	0.028

[a] Displacement of ^{3}H-PGD_2 from the CRTH2 receptor expressed on 293 cells. Assay run in buffer containing 0.5% BSA. See Ref. 35 for assay protocol. Values are means of three experiments, standard deviation is ±30%.

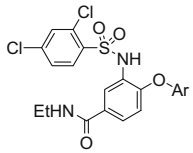
Table 3
Evaluation of the amide moiety

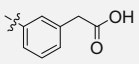
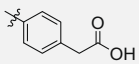
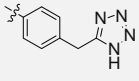
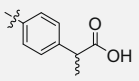


Compd	R^1	R^2	CRTH2 IC_{50}[a] in buffer (μM)
9	H	CONHEt	0.013
10	H	$CONH_2$	0.047
11	Me	CONHEt	0.007
12	Me	$CONEt_2$	1.10
13	H	COMe	0.38
14	H	CH_2NHEt	>10
15	H	Cl	0.68
16	H	OMe	1.40
17	Cl	NH_2	0.13
18	Cl	NHCOEt	0.007
19	Cl	NHCONHEt	0.016

[a] Displacement of ^{3}H-PGD_2 from the CRTH2 receptor expressed on 293 cells. Assay run in buffer containing 0.5% BSA. See Ref. 35 for assay protocol. Values are means of three experiments, standard deviation is ±30%.

Table 4
Exploration of the phenyl acetic acid moiety



Compd	Ar	CRTH2 IC_{50}[a] in buffer (μM)	DP IC_{50}[a] in buffer (μM)
20		0.016	>10
21		0.004	0.12
22		2.37	0.132
23		0.107	0.043

[a] Displacement of ^{3}H-PGD_2 from the CRTH2 or DP receptor expressed on 293 cells. Assay run in buffer containing 0.5% BSA. See Ref. 35 for assay protocol. Values are means of three experiments, standard deviation is ±30%.

Substitutions on the benzene ring of the phenyl acetic acid moiety were evaluated to study their effect on DP and CRTH2 selectivity (Table 5). Compound **21** has strong affinity for CRTH2, but it only has moderate affinity for DP. Small substitutions such as methoxy (**24**) and fluorine (**25**) next to the aryloxy group improve the compounds' affinity for the DP receptor (Table 5). However, ethoxy derivative **26** has decreased activity on DP compared to methoxy analog **24**, which suggests that the increased bulk may be detrimental. Introduction of methoxy groups on both sides next to the aryloxy group afforded a compound (**27**) with high affinity for CRTH2 and dramatically decreased activity on DP.

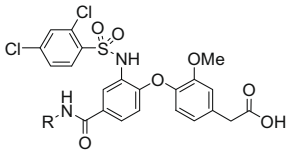
The linker between the carboxylic acid and the phenyl ring was also studied (Table 5). A methylene linker (**24**) is preferred over an ethylene linker (**29**) or direct attachment of carboxylic acid to the phenyl ring (**28**).

Further study of the amide moiety (Table 6) provided additional improvement on DP potency. Longer *n*-alkyl groups, such as *n*-propyl (**30**), *n*-butyl (**31**) and *n*-pentyl (**32**), improved the affinity for DP, while maintaining the affinity for the CRTH2 receptor. Investigation of branched alkyl groups, such as isopropyl (**33**) or isobutyl (**34**), and cyclic alkyl groups, such as cyclopropyl (**35**) or cyclopentyl (**37**), showed that these groups were well tolerated. However, they did not lead to improvements on the affinity towards either receptor. The cyclobutyl analog (**36**), on the other hand, displayed increased affinity for the DP receptor while maintaining the affinity for the CRTH2. All comparisons here are made to the parental ethyl amide (**24**).

Because of its high affinity towards both receptors, compound **31** (AMG 009) was further investigated in DP and CRTH2 functional assays. AMG 009 inhibited PGD_2-induced down-modulation of CRTH2 on CD16 negative granulocytes (eosinophils) in human whole blood with a K_i of 1 nM.[37] In addition, AMG 009 also inhibited PGD_2-induced cAMP response mediated by DP in platelets in 80% human whole blood with a K_i of 148 nM.[38] The selectivity of AMG 009 was studied in a commercially available GPCR panel and AMG 009 demonstrated excellent selectivity, including its selectivity against prostanoid receptors TP, EP2 and EP4.[39] The pharmacokinetic properties of AMG 009 were evaluated in several species (Table 7). AMG 009 has low to moderate clearance across species and good oral absorption.

Since guinea pigs have been shown to respond to aerosolized PGD_2 in a similar manner as humans,[40] AMG 009 was investigated in an acute guinea pig model of PGD_2-induced airflow obstruction. In this model, airway resistance is measured in response to increased concentrations of PGD_2 using a whole body plethysmography.[41] Groups of four Dunkin-Hartley guinea pigs were dosed once with vehicle or AMG 009 at 3, 10 or 30 mg/kg by subcutaneous administration 15 min prior to PGD_2 challenges. The enhanced pause (Penh), a measure of airway resistance, to inhaled PGD_2 was measured over a 10-min span after the guinea pigs were subjected to 1-min challenges with aerosolized saline or PGD_2 in saline of ascending concentrations at 0.063, 0.125, 0.25, 0.5 and 1.0 mg/

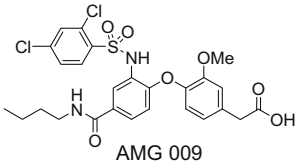
Table 6
Evaluation of the amide moiety



Compd	R	CRTH2 IC_{50}[a] in buffer (μM)	DP IC_{50}[a] in buffer (μM)
24	Et	0.003	0.055
30	*n*-Pr	0.002	0.031
31	*n*-Bu	0.003	0.012
32	*n*-Pentyl	0.007	0.009
33	*i*-Pr	0.002	0.036
34	*i*-Bu	0.003	0.059
35	*c*-Pr	0.004	0.041
36	*c*-Bu	0.003	0.011
37	*c*-Pentyl	0.006	0.179

[a] Displacement of ^{3}H-PGD_2 from the CRTH2 or DP receptor expressed on 293 cells. Assay run in buffer containing 0.5% BSA. See Ref. 35 for assay protocol. Values are means of three experiments, standard deviation is ±30%.

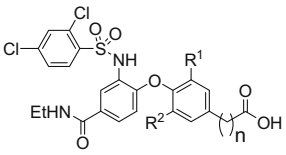
Table 7
Pharmacokinetic properties of AMG 009



Species	Clearance[a] (L/h/kg)	MRT (h)	V_{dss} (L/kg)	Oral bioavailability[b] (%)
Rat	1.2	0.9	1.0	28
Dog	0.77	1.4	1.0	60
Cyno	0.27	1.3	0.37	39

[a] IV dose at 0.5 mg/kg.
[b] Oral dose at 2 mg/kg.

Table 5
Substitutions on the benzene ring of the phenyl acetic acid moiety



Compd	R^1	R^2	*n*	CRTH2 IC_{50}[a] in buffer (μM)	DP IC_{50}[a] in buffer (μM)
21	H	H	1	0.004	0.12
24	OMe	H	1	0.003	0.055
25	F	H	1	0.002	0.036
26	OEt	H	1	0.002	1.91
27	OMe	OMe	1	0.002	16.0
28[b]	OMe	H	0	>50	>10
29[b]	OMe	H	2	0.74	2.07

[a] Displacement of ^{3}H-PGD_2 from the CRTH2 or DP receptor expressed on 293 cells. Assay run in buffer containing 0.5% BSA. See Ref. 35 for assay protocol. Values are means of three experiments, standard deviation is ±30%.
[b] Compounds are benzene sulfonamides instead of 2,4-dichlorobenzene sulfoamides.

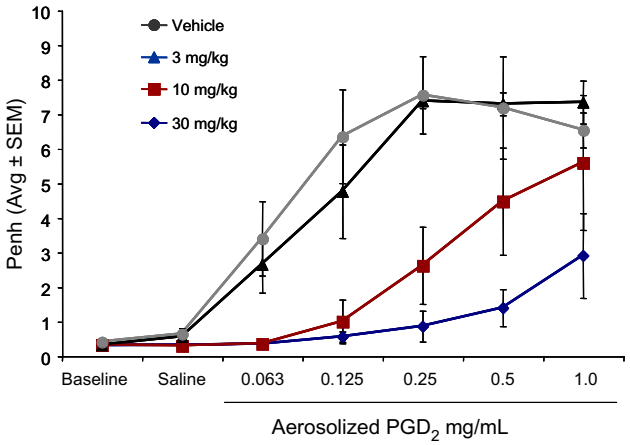


Figure 3. Dose–response of AMG 009 in the guinea pig model of PGD_2-induced airway constriction. AMG 009 was dosed SC 15 min before the challenges with aerosolized PGD_2; $n = 4$ guinea pigs/group. Data reported as mean ± standard error of the mean (sem).

mL. Treatment with AMG 009 resulted in a dose dependent decrease in airway resistance provoked by PGD_2 aerosol (Fig. 3). The in vivo effect of AMG 009 was most likely due to its DP antagonistic activity, because CRTH2 selective antagonist (**27**) did not reduce airway resistance in this guinea pig model.[42]

The ability of AMG 009 to block the guinea pig CRTH2 and DP receptors was also evaluated in vitro using a ^{3}H-PGD_2 displacement assay with cells transfected with the guinea pig CRTH2 receptors (IC_{50} = 3 nM)[43] and a PGD_2-induced cAMP response assay with cells expressing the guinea pig DP receptors (K_i = 131 nM).[44]

In summary, we have discovered a novel series of CRTH2 antagonists and optimized their properties to also inhibit the DP receptor. Based in part on the evaluation of CRTH2 and DP activities and the outcomes of pharmacokinetic studies, we selected AMG 009 (**31**) as a candidate compound for evaluation in clinical studies. This compound is a potent inhibitor of PGD_2-induced CRTH2 receptor down-modulation and DP mediated cAMP response in human whole blood. This compound was also efficacious in a guinea pig model of airway resistance.

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35. The CRTH2 or DP radioligand binding assay was performed on 293 cells stably expressing human CRTH2 or DP. To measure binding, [^{3}H]-PGD_2 was incubated together with 293(hCRTH2 or hDP) cells in the presence of increasing concentrations of compounds. After washing, the amount of [^{3}H]-PGD_2 that remained bound to the cells was measured by scintillation counting and the concentration of compounds required to achieve a 50% inhibition of [^{3}H]-PGD2 binding (the IC_{50}) was determined. The binding buffer contains either 0.5% BSA (buffer binding) or 50% human plasma (plasma binding).
36. CRTH2 mediated cell migration was analyzed in a transwell migration assay using hCRTH2 stably transfected CEM cells (a T lymphoblast cell line). The cells were incubated with increasing concentrations of compounds for 3 h in a 96-well migration chamber on top of a transwell filter and the number of cells migrating through the filter in response to 5 nM PGD_2 in the lower chamber was counted and the IC_{50} of the compounds determined. The migration buffer contains 0.5% BSA.
37. Human whole blood was drawn into ACD anti-coagulated tubes, treated compounds or DMSO and then stimulated with a dose response of PGD_2. Fluorochrome conjugated antibodies were used to label CRTH2 positive granulocytes and CRTH2 receptor internalization was monitored by flow cytometry. K_i was determined using Schild equation.
38. Human whole blood was drawn into ACD vacutainer tubes, treated compounds or DMSO and then stimulated with a dose response of PGD_2. Cells were lysed and cAMP was measured using a competitive ELISA. Comparison of the dose response to PGD_2 in samples containing DMSO only and samples containing compounds were used in determining K_i using Schild equation.
39. AMG 009 has an IC_{50} of 30 μM in a binding assay against ^{3}H-labeled TP agonist SQ29548 using membrane made from HEK-293 EBNA cells stably transfected with human TP receptors. The EP2 and EP4 activity of AMG 009 was evaluated in a PGE_2 induced cAMP response assay using HEK293 cells expressing EP2 and EP4. The K_i was 44 μM.
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42. Compound **27** was dosed SC at 30 mg/kg 15 min before challenge with aerosolized PGD_2.
43. Guinea pig ^{3}H-PGD_2 displacement assay was done in the same way as in Ref. 35 using 293 cells stably expressing guinea pig CRTH2.
44. 293 cells stably-transfected with guinea pig DP receptors were treated compounds or DMSO and then stimulated with a dose response of PGD_2. Cells were lysed and cAMP was measured using a competitive ELISA. Comparison of the dose response to PGD_2 in samples containing DMSO only and samples containing compounds were used in determining K_i using Schild equation.