



Pharmacological characterization of CRTh2 antagonist LAS191859: Long receptor residence time translates into long-lasting *in vivo* efficacy[☆]

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ABSTRACT

The chemoattractant receptor-homologous molecule expressed on T-helper type 2 cells (CRTh2) is a G protein-coupled receptor expressed on the leukocytes most closely associated with asthma and allergy like eosinophils, mast cells, Th2-lymphocytes and basophils. At present it is clear that CRTh2 mediates most prostaglandin D₂ (PGD₂) pro-inflammatory effects and as a result antagonists for this receptor have reached asthma clinical studies showing a trend of lung function improvement. The challenge remains to identify compounds with improved clinical efficacy when administered once a day.

Herein we described the pharmacological profile of LAS191859, a novel, potent and selective CRTh2 antagonist. *In vitro* evidence in GTPγS binding studies indicate that LAS191859 is a CRTh2 antagonist with activity in the low nanomolar range. This potency is also maintained in cellular assays performed with human eosinophils and whole blood.

The main differentiation of LAS191859 vs other CRTh2 antagonists is in its receptor binding kinetics. LAS191859 has a residence time half-life of 21 h at CRTh2 that translates into a long-lasting *in vivo* efficacy that is independent of plasma levels. We believe that the strategy behind this compound will allow optimal efficacy and posology for chronic asthma treatment.

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1. Introduction

Prostaglandin D₂ (PGD₂) is a prostaglandin derived from the metabolism of arachidonic acid by cyclooxygenases and downstream PGD₂ synthases. PGD₂ is a pleiotropic molecule that is involved in many different processes such as sleep, regulation of nerve-cell function, reduction of intraocular pressure, modulation of nociception, hormone release, bronchoconstriction, vasodilation and inflammation [1]. In the immune system, PGD₂ is mainly produced by mast cells [2] but also, although at lower levels, by other cells like dendritic cells and Th2 lymphocytes [3,4]. PGD₂ is induced in response to local tissue damage and allergic inflammation in dis-

eases such as asthma [5–7], allergic rhinitis and atopic dermatitis among others [8–11].

PGD₂ binds to three different receptors: the prostaglandin D₂ receptor or DP (also known as DP1) [12], the chemoattractant receptor-homologous molecule expressed on Th2 cells or CRTh2 (also known as DP2) [13] and the thromboxane-type prostanoid receptor (TP), although at higher concentrations [14,15].

Activation of CRTh2 by PGD₂ induces a reduction of intracellular cAMP and the mobilization of calcium. It is involved in the chemotaxis of Th2 lymphocytes, eosinophils, mast cells and basophils [16–19], and inhibits the apoptosis of Th2 lymphocytes [20]. Moreover, CRTh2 activation has a role in the production of IL4, IL5, and IL13 [21] which are cytokines involved in important biological responses like eosinophil recruitment and survival, mucus secretion, airway hyper responsiveness and immunoglobulin E (IgE) production among others.

Therefore, molecules that antagonize the mentioned pro-inflammatory PGD₂ effects mediated by CRTh2 on key cell types associated with allergic inflammation including basophils, eosinophils, mast cells and Th2 lymphocytes (most relevant T cell population in mild-moderate asthma), would have a potential

Abbreviations: cAMP, cyclic adenosine monophosphate; CHO, chinese hamster ovary cell line; CRTh2, chemoattractant receptor-homologous molecule expressed on T-helper cells; DK-PGD₂, 13-14-dihydro-15-keto-PGD₂; GDP, guanosine diphosphate; GPCR, G protein-coupled receptor; GTP, guanosine triphosphate; PGD₂, prostaglandin D₂; PMN, polymorphonuclear cells; Th2, T-helper cells type 2.

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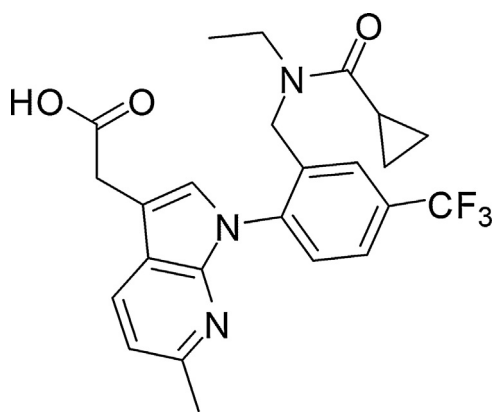


Fig. 1. Chemical structure of CRTh2 antagonist LAS191859.

benefit in asthma and related pathologies like allergic rhinitis and atopic dermatitis.

The roles of DP1 and CRTh2 in human asthma have been under debate for several years but recently preclinical and clinical evidences seem to clarify this situation. DP1 mediates both pro and anti-inflammatory effects of PGD₂ while CRTh2 mediates mostly proinflammatory effects of this prostaglandin and with the potential that responses mediated by both receptors could be antagonizing each other [22–24]. Asthma clinical studies of the DP1 antagonist Laropiprant and also of the dual DP1-CRTh2 compound AMG-853 showed no efficacy vs placebo or vs montelukast [25,26]. So, at present, preclinical and clinical studies are mainly focussed on pure CRTh2 antagonists.

Several compounds are currently in clinical trials for asthma, allergic rhinitis and atopic dermatitis. Positive results have been reported for OC-459 in allergic rhinitis and asthma clinical studies. After 28 days of 200 mg bid OC-459 administration to asthmatic patients, it was observed an improvement in pulmonary function of 7.4% in FEV1, a significant decrease in total IgE levels, a reduction of eosinophils in sputum, a reduction in night time symptoms and an improvement in quality of life. Even with high dosing, CRTh2 antagonism seems to be a very safe mechanism with no adverse effects associated to it [27–29]. Further OC-459 studies in eosinophilic asthma patients showed positive results at a dose of 25 mg once daily [30].

Herein, we present the preclinical profile of LAS191859, a potent, selective and long-lasting CRTh2 antagonist. Our strategy was the optimization by design of compounds with slow receptor dissociation kinetics, that translates into a long duration of action independent of plasma levels. This strategy would provide a compound with the potential to show clinically relevant asthma control with a once-a-day posology.

2. Material and methods

2.1. Chemicals

LAS191859 ({1-[2-{{[(Cyclopropylcarbonyl)(ethyl)amino]methyl}-4-(trifluoromethyl)phenyl] -6-methyl-1H-pyrrolo[2,3-*b*]pyridin-3-yl}acetic acid) was synthesized at Almirall S.A.'s Department of Medicinal Chemistry (Sant Feliu de Llobregat, Spain; Fig. 1). This compound was obtained as the result of an exhaustive optimization exercise of bicyclic heretoaromatic acetic acid series, fully published [40] and included in patent WO2013/010880A1.

2.2. CRTh2 overexpressing cell lines

CHO-K1 cell line stably overexpressing human CRTh2 (Genbank accession: AB008535) was obtained from Perkin Elmer.

Murine CRTh2 coding sequence (GenBank accession: AB109092) was obtained by RT-PCR from mouse brain total RNA and inserted into pORF-LHN4 expression vector. This vector was transfected by electroporation into CHO-K1 cell line and a suitable stable clone selected based on response to PGD₂ in cAMP, pERK and GTPγS assays.

Rat CRTh2 coding sequence (GenBank accession: NM.022241) and guinea pig CRTh2 coding sequence (GenBank: AY964064.1) were synthesized at Gene Oracle (Sigma) and Biomedal respectively and inserted into pORF-LHN4 expression vector. Both expression vectors were transfected by electroporation into CHO-K1 cell line and suitable stable clones selected based on response to PGD₂ in cAMP, pERK and GTPγS assays.

2.3. GTPγS antagonism binding assay

The assay was performed by pre-incubating 4–8 μg of membranes (obtained from CHO.K1 cells stably over expressing the CRTh2 receptor from different species: human, guinea pig, rat and mouse) per well with the test compound for 1 h, followed by incubation with 50 nM PGD₂ and 0.1 nM [³⁵S]-GTPγS in incubation buffer (20 mM HEPES, 10 mM MgCl₂, 100 mM NaCl, 10 μM GDP, 10 μg/ml Saponine and 0.2% BSA in order to avoid non-specific binding) for 2 h at room temperature. Compound was initially solubilized in dimethylsulfoxide (DMSO) at 10 mM stock concentration and several dilutions were performed to finally reach 1% DMSO in the GTPγS incubation buffer. The reaction was terminated by filtering with Whatman GF/C glass fibre filter plates pre-treated for 30 min with 20 mM NaH₂PO₄, 20 mM Na₂HPO₄ (wash buffer) using a MultiScreen® HTS vacuum manifold from Millipore and washing 4 times with the same buffer. After washing, the plates were dried and Optiphase scintillation cocktail was added. The radioactivity retained in the filter was counted using a Microbeta liquid scintillation counter. Percentage of inhibition for antagonist compounds was calculated by normalizing activity to total binding and basal binding. Assays were performed in duplicate. Compounds IC₅₀s were determined using Excel XL-fit software for calculations.

2.4. Radioligand binding dissociation assay (Residence time)

GTPγS binding assay was also performed to assess residence time in the receptor. The assay was performed as described in “GTPγS antagonism binding assay” with some modifications. Membranes were pre-incubated with an excess (5 × K_i) of the test compound for 2 h to ensure at least 90% of receptor occupancy. After the pre-incubation period, PGD₂ was added at a high concentration of 100 μM to occupy unbound receptor followed by addition of [³⁵S]-GTPγS (0.1 nM). At various time points, ranging from 2 min to 28 h the reactions were terminated as described in “GTPγS antagonism binding assay”.

2.5. Mechanism of action studies. Antagonist behavior

In order to better understand the behavior of LAS191859, the dose-effect curve

for the agonist (PGD₂) was determined in the absence and presence of various

concentrations of the antagonist using the [³⁵S]-GTPγS binding assay. The assay was performed as described in “GTPγS antagonism binding assay” but in the presence of increasing PGD₂ concentrations. In brief, membranes were pre-incubated with the antagonist at different concentrations and then PGD₂ (dose-curve) and 0.1 nM [³⁵S]-GTPγS were added, and the mixture was incubated at two different times (2 h and 22 h). After this period, the reaction was terminated by filtration and assay continued as described in previous sections.

2.6. Selectivity assays

Off-target selectivity of LAS191859 was tested at CEREP in a panel including 63 targets representing major classes of biogenic amine receptors, prostanoid receptors, neuropeptide receptors, ion channel binding sites, neurotransmitter transporters through *in vitro* receptor binding assays and COX1-2 selectivity in cellular assays. Compounds were tested at 10 μ M and results were expressed as percentage of inhibition of specific binding measured in the presence of the specific ligand.

2.7. Cytotoxicity assay

CHO-K1 cells (American Type Culture Collection) were seeded at a concentration of 10^4 cells/well in 96-well plate. Compound was initially solubilized in DMSO at 10 mM stock concentration and dilutions were performed to finally reach 1% DMSO in F12 culture medium with 0.5% FBS for 24 h incubation with cells. The reduction of the ATP-status of the cells was studied with ATPLite, a luminescence ATP detection assay system (PerkinElmer) according to the manufacturer instructions and luminescence was measured using a Luminoskan luminometer (ThermoElectron). To create a standard curve, luminescence values vs cell number were plotted and the data adjusted to a lineal equation. Luminescence values of the samples were interpolated to obtain number of cells. The inhibition of the ATP status for LAS191859 was determined by using six different concentrations ran in duplicate. IC₅₀ values were obtained by non-linear regression using XL-fit software.

2.8. CRTh2 isolated cell eosinophil cell shape assay

Citrated whole blood was obtained from consenting human volunteers (according to Spanish biomedical research law 14/2007, which complies with Helsinki declaration) and polymorphonuclear leukocytes (PMN) were separated by gradient centrifugation on Polymorphprep for 40 min at 500g brake off. The PMN fraction was resuspended in calcium free buffer (10 mM HEPES, 10 mM glucose, 0.1% BSA in calcium-free PBS) and centrifuged for 30 min at 400g at room temperature. Lysis of red blood cells was performed by adding 20 ml of 0.2% saline buffer (0.2% NaCl) for 40 s and stopped by adding 20 ml of 1.6% saline buffer (1.6% NaCl). For compound treatment, 90 μ l of PMNs cells were resuspended at a density of 5×10^6 cells/ml in 10 mM HEPES, 10 mM glucose, 0.1% BSA in calcium containing PBS and were incubated for 10 min with 10 μ l of compound, at the defined concentration to be tested, followed by incubation with 10 μ l of 500 nM PGD₂ for exactly 4 min at 37 °C. LAS191859 was initially solubilized in DMSO at 10 mM stock concentration and dilutions were performed to finally reach 0.1% DMSO when incubated with cells. Fixation was performed by addition of 0.2 ml of ice-cold 1:5 diluted CellFix (BD Biosciences) and immediately analysed on a FACSCalibur (BD Biosciences). Eosinophils were selected in front of other cells on the basis of their intrinsic autofluorescence in the FL1/FL2 channels and shape change of 600 cells was assayed by forward scatter and side scatter analysis. Compound IC₅₀ was determined using Excell XL-fit software for calculations.

2.9. Eosinophil shape change assay in human whole blood

Whole blood was obtained from consenting human volunteers (according to Spanish biomedical research law 14/2007, which complies with Helsinki declaration) mixed with heparine and kept in rotation until use. 90 μ l of blood were mixed with 10 μ l of test compound and incubated for 30 min at room temperature followed by addition of 10 μ l of 500 nM PGD₂ for 4 min at 37 °C. LAS191859 was initially solubilized in DMSO at 10 mM stock concentration and dilutions were performed to finally reach 1% DMSO when incubated with cells. The reaction was stopped by placing the tubes on ice and

adding of 0.25 ml of ice-cold 1:5 diluted CellFix (BD Biosciences). Red blood cells were lysed in two steps by adding 2 ml of lysis solution (150 mM ammonium chloride, 10 mM potassium hydrogencarbonate), incubating for 20 min for the first step of lysis and 10 min for the second, centrifuging at 300g for 5 min at 6 °C. The pellet was resuspended in 0.2 ml of fixative solution and immediately analysed on a FACSCalibur (BD Biosciences). Eosinophils were selected in front of other cells on the basis of their intrinsic autofluorescence in the FL1/FL2 channels and the shape change of 600 cells was assayed by forward scatter and side scatter analysis. Compound IC₅₀ was calculated using Excel XL-fit software.

2.10. Eosinophil chemotaxis

Citrated whole blood was obtained from consenting human volunteers (according to Spanish biomedical research law 14/2007, which complies with Helsinki declaration) and polymorphonuclear leukocytes obtained by gradient centrifugation on Polymorphprep for 40 min at 500g brake off. Eosinophils were further purified from the granulocyte populations by negative magnetic selection using the human eosinophil isolation kit (MACS Miltenyi Biotec). LAS191859 was initially solubilized in DMSO at 10 mM stock concentration and dilutions were performed to finally reach 0.1% DMSO in assay buffer (PBS with Ca²⁺ and Mg²⁺, containing 0.1% BSA, 10 mM HEPES and 10 mM glucose). 225 μ l of Eosinophils at a concentration of 1.33×10^6 cells/ml were pre-incubated with 25 μ l of vehicle or each concentration of compound in assay buffer for 60 min in a round bottom 96-well plate at RT. 225 μ l of PGD₂ 110 nM (in assay buffer) and 25 μ l of each compound concentration were added to the bottom transwell chamber with 5- μ m pore size polycarbonate filter (Corning). 75 μ l of cells from 96-well pre-incubation plate were transferred to the upper transwell chamber. The plates were incubated at 37 °C in a humidified CO₂ incubator for 2 h, and the membrane was carefully removed. Cells in the lower chamber were counted by flow cytometry (60" high speed). Compound IC₅₀ was calculated using Excel XL-fit.

2.11. Pharmacokinetic profiles in rat and dog

For intravenous dosing, LAS191859 was formulated in 40% polyethylene glycol-400 while the suspension for oral gavage dosing was prepared in 0.5% aqueous methylcellulose/0.1% Tween-80. Blood samples were collected into heparinized tubes and centrifuged at 3300g for 10 min to obtain blood plasma. All animals were fasted overnight before compound administration on the next morning and regained access to food after collection of the 4-h blood sample. Male Wistar rats (n=2) cannulated at the carotid artery were administered a single intravenous dose (1 mg/kg) into the tail vein or by oral gavage (1 mg/kg). Blood was sampled at 0.1 (i.v. only), 0.25, 0.5, 1, 2, 3 (p.o. only), 4, 6, and 24 h post-dose. In case of male Beagle dogs, intravenous bolus dosing was done through a cephalic vein (1 mg/kg), the dose level of the oral gavage was 1 mg/kg. Blood samples were collected by venipuncture of a jugular vein at 0.1 (i.v. only), 0.25, 0.5, 1, 2, 3, 4, 6, 8, and 24 h post-dose.

For analysis plasma proteins were precipitated and supernatants injected onto the LC column. Quantitative analysis was achieved on an Acquity UPLC system coupled to a Quattro Premier triple quadrupole-mass spectrometer (Waters, Milford, MA). Samples were separated on a Acquity C¹⁸ BEH column using gradient elution.

2.12. Pharmacokinetic data analysis

Based on the plasma concentration-time profiles, the pharmacokinetic parameters were estimated by standard noncompartmental analysis using WinNonlin 5.0 (Pharsight, Mountainview,

CA). For the determination of the area-under-the curve (AUC), the linear-log trapezoidal method was used in case of intravenous dosing while the linear up-logarithmic down rule was applied to determine the AUC corresponding to oral administration. Terminal half-lives were calculated based on at least three time points. Absolute bioavailabilities were determined by comparing AUC_{po} with AUC_{iv} according to $F = (AUC_{po} \times Dose_{iv}) / (AUC_{iv} \times Dose_{po}) \times 100$ (in%).

2.13. Evaluation of the potency and duration of action of LAS191859 on DK-PGD₂-induced eosinophilia in guinea pig

Care of the animals was undertaken in compliance with European Community Directive 2010/63/UE for the use of laboratory animals and local regulations. Experimental procedures were approved by the institutional Animal Care and Use Committee of Almirall S.A.

Dunkin Harley Guinea pigs weighing between 400 and 450 g were obtained from Charles River (Cerdanyola del Vallès, Spain). During the study animals were housed in maintenance standard cages and kept under controlled light cycle, air cycle, temperature and humidity. Standard guinea pig chow and tap water were available ad libitum.

LAS191859 was suspended in oral standard vehicle in 0.5% methylcellulose and 0.1% Tween80 in distilled water (5 ml/kg).

Guinea pigs were fasted for 18 h with free access to water. On the day of the experiment, guinea pigs were weighted, randomly distributed into various treatment groups (N=6) and administered orally with either vehicle or LAS191859. At 1 and 17 h post-administration of compound, the CRTh2 agonist, 13,14-dihydro-15-keto-prostaglandin D₂ (DK-PGD₂) (Cayman Chemical Company) was administered intravenously at 300 µg per animal in 0.5 ml of 2% ethanol to increase systemic eosinophilia. One hour after administration of the inducer, animals were anesthetized with isoflurane and blood samples were collected by cardiac puncture (1 ml for hematological counts and 1 ml for analytical determination of plasma levels). Total and differential blood cell counts were performed with a hemocytometer Sysmex XT-2000i (Roche Diagnostics, Barcelona, Spain). Animals were then sacrificed by cervical dislocation prior to anesthesia recovery.

Blood samples were collected into heparinized tubes and centrifuged at 3300g for 10 min. Plasma samples were stored at –20 °C for subsequent analysis. For plasma levels analysis, plasma proteins were precipitated and supernatants injected onto the LC column. Quantitative analysis was achieved on an Acquity UPLC system coupled to a Quattro Premier triple quadrupole-mass spectrometer (Waters, Milford, MA). Samples were separated on a Acquity C¹⁸ BEH column using gradient elution.

Table 1

Potencies and dissociation half-life ($t_{1/2}$) of LAS191859 in recombinant CRTh2 receptors from different species.

	IC ₅₀ (nM)	Dissociation $t_{1/2}$ (h)
Human	9.58 ± 2.85	21.89 ± 2.59
Guinea Pig	7.6 ± 2.35	16.26 ± 1.06
Rat	14 ± 6.65	ND
Mouse	15.5 ± 3.10	ND

Potency and residence time for CRTh2 antagonist LAS191859 were determined from [³⁵S]GTPγS binding assay. All values are mean at least three experiments performed in duplicate ± SD; ND: not determined.

Results are expressed as the mean percentage of the reduction of the number of circulating eosinophils with respect to the window between vehicle control group (basal number of eosinophils) and DK-PGD₂ induced control group (maximal increase of eosinophils). Compound ID₅₀ was calculated using GraphPad PRISM V5.0 software.

3. Results

3.1. Potency of LAS191859 in [³⁵S]-GTPγ binding studies in human, guinea pig, rat and mouse CRTh2 receptor

The binding of an agonist to a GPCR induces a conformational change in the receptor that promotes the exchange of GDP for GTP. This is the basis of the [³⁵S]-GTPγ binding functional assay that was used to measure the potency of the compounds to antagonize the effect induced by 50 nM PGD₂ in the CRTh2 receptor.

The potency of LAS191859 (see structure in Fig. 1) in the human, rat, mouse and guinea pig CRTh2 were determined using CHO cell membrane preparations expressing each species recombinant CRTh2. LAS191859 was a potent compound with an IC₅₀ of 9.58 ± 2.85 nM in the human CRTh2 and maintained potency in the different species receptor tested: 14 nM ± 6.65 in rat; 15.5 ± 3.10 nM in mouse and 7.6 ± 2.35 nM in guinea pig (Table 1).

3.2. Evaluation of the antagonist behaviour of LAS191859. Dissociation studies

In order to better understand the behavior of LAS191859, its mechanism of action was studied using the [³⁵S]-GTPγ binding assay and CHO cell membrane preparations expressing either the human or guinea pig CRTh2 receptor. Dose-response curves of agonist (PGD₂) in the absence and presence of different antagonist concentrations, showed that LAS191859 behaved as an unsurmountable compound after 2 h of incubation at concentrations higher than 4 nM (Fig. 2A). A clear reduction in the binding capacity (B_{max}) was observed and this effect was largely maintained even

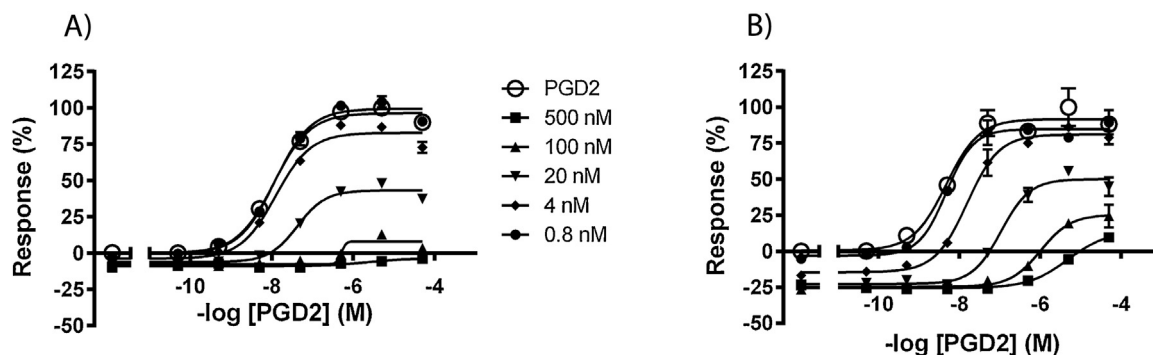


Fig. 2. Dose response curves for the agonist PGD₂ in presence of various concentrations of antagonist LAS191859 and at two different incubation times 2 h (A) and 22 h (B). Three independent experiments were performed in duplicate and the figure shows a representative experiment. (2 h studies basal: 612 ccpm, top: 2561 ccpm; 22 h studies: basal:1928 ccpm, top: 4188 ccpm).

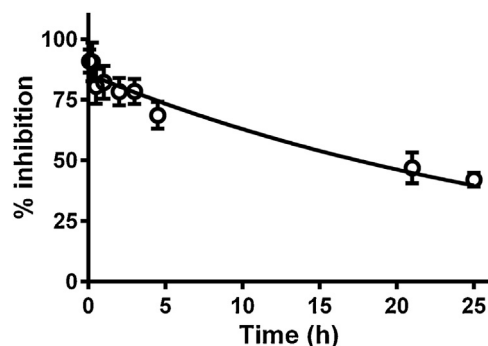


Fig. 3. Dissociation kinetics profile of LAS191859 from human CRTh2 from 0 to 24 h. Data shown correspond to a representative experiment. Two additional experiments performed in duplicate gave similar results.

after 22 h of incubation time (Fig. 2B). Although after 22 h incubation the experimental window in the [^{35} S]-GTP γ binding assay was much reduced vs 2 h time point and resulted in a less optimal quantification, the unsurmountable behavior of LAS191859 was still evident at concentrations of 20 nM and higher.

To investigate whether unsurmountability of the compound was related to slow dissociation from the receptor, dissociation studies in both human and guinea pig CRTh2 receptors were performed. CHO-K1 cell membrane preparations expressing each species' recombinant CRTh2 receptor were tested in the [^{35} S]-GTP γ S assay. Antagonists were incubated at 5-fold the IC_{50} with the membranes during 2 h and then antagonist dissociation was initiated by displacement with 100 μ M PGD $_2$. LAS191859 showed a dissociation half-life in the human CRTh2 receptor of 21.89 ± 2.59 h, therefore demonstrating a long residence time in the receptor (Fig. 3) which is consistent with its sustained unsurmountable behavior. Dissociation half-life was also found to be long in the guinea pig CRTh2 with a value of 16.26 ± 1.06 h showing that, as in the human receptor, potency but also residence time is maintained in the guinea pig receptor (Table 1). The fact that potency and residence time was maintained in both species allowed the evaluation of *in vivo* potency and PK/PD disconnection in a guinea pig model.

3.3. Selectivity of LAS191859

LAS191859 was tested for its ability to inhibit the binding affinity or functional activity of a range of receptors, ion channels, transporters and enzymes at CEREP: A1, A2A, A3, Alpha 1, Alpha 2, Beta 1, Beta 2, AT1, BZD (rat), B2, CB1, CCK1, D1, D2S, ETA, GABA (rat), GAL2, CXCR2, CCR1, H1, H2, MC4, MT1, M1, M2, M3, NK2, NK3, Y1, Y2, NTS1, Delta2 (DOP) Dappa (KOP) (rat), Mu (MOP), NOP (ORL1) DP1, EP1, EP2, EP3, EP4, FP, IP (PGI2), 5-HT1A, 5-HT1B, 5-HT2A, 5-HT2B, 5-HT3, 5-HT5a, 5-HT6, 5-HT7, sst, VPAC, V1a, Ca $^{2+}$ channel (L.verapamil site) (rat), Kv channel (rat), SKCa channel (rat), Na $^{+}$ channel (site 2) (rat), Cl $^{-}$ channel (GABA-gated) (rat), NE transporter, DA transporter, 5-HT transporter, COX-1 and COX-2. All human targets unless rat is specified. Selectivity of LAS191859 tested at 10 μ M was very high with percentages of inhibition below 25% for all targets tested. Table 2 shows selectivity for all prostanoid receptor as more closely related GPCR targets.

3.4. Potency of LAS191859 in human cellular assays

The binding of PGD $_2$ to CRTh2 induces a change in the shape of eosinophils (ESC), that can be measured easily by flow cytometry based on eosinophil autofluorescence [31]. Fig. 4A shows the strategy followed to identify eosinophils from other leukocytes according to their intrinsic autofluorescence in FL-1/FL-2 channel compared to the neutrophils' lack of fluorescence, including

Table 2

Selectivity of LAS191859 tested at 10 μ M in binding assays for the different human prostanoid receptors.

Prostanoid receptor	% inhibition at 10 μ M
DP1	19 ± 2.4
TP	11 ± 10.6
EP1	-1 ± 1.2
EP2	-5 ± 0.4
EP3	15 ± 2.8
EP4	-5 ± 0.8
FP	16 ± 1.3
IP	-17 ± 11

Values represent percentage inhibition at 10 μ M (mean of two replicates $\pm$ SD).

Table 3

Potency of LAS191859 in human cellular assays.

Cellular Assays	IC_{50} (nM)
ESC isolated granulocytes	1.88 ± 0.9
ESC whole blood	9.2 ± 5.4
Eosinophil chemotaxis	1.3 ± 0.8

All values are mean of 3 independent experiments from different donors performed in duplicate $\pm$ SD. ESC: eosinophil shape change.

FSC/SSC plot, as well as back-gating of the identified population. Although the magnitude of the response in terms of shape change was not big, the assay turn out to be very robust. Compound potency in antagonizing the ESC induced by PGD $_2$ was measured in isolated human blood granulocytes as well as in whole blood in order to assess potency in a more physiologically relevant environment (Table 3). LAS191859 was a potent compound in both ESC assays with IC_{50} of 2 ± 0.9 nM in the isolated cell and IC_{50} of 8.2 ± 5.4 nM in the whole blood assay and in both cases in a dose-dependent manner (Fig. 4B, C). Shape change is the result of cytoskeleton reorganization that is required for cell migration. PGD $_2$ induces a dose-dependent increase of eosinophils migration in a transwell chemotaxis assay with an EC_{80} of 100 nM (Fig. 5A). Compound LAS191859 treatment led to a clear dose-response reduction in PGD $_2$ mediated eosinophil chemotaxis (Fig. 5B) with an IC_{50} of 1.3 ± 0.9 nM (Fig. 5C; Table 3) similar to the potency obtained in the ESC assay. To ensure that the reduction in migration was not due to a cytotoxic effect, cytotoxicity of LAS191859 was measured in CHO-K1 cells and ATP-lite assay to determine the cell viability (measured as a reduction of intracellular ATP levels). Results obtained after incubation of 24 h showed that LAS191859 was not cytotoxic with just 7% inhibition at 100 μ M (highest concentration tested)

3.5. Pharmacokinetic profile of LAS191859 in rat and dog

LAS191859 was administered to Wistar rats and Beagle dogs both orally (p.o) and intravenously (i.v) to determine its pharmacokinetic profile over a 24-h period with calculated parameters shown in Table 4. The compound has good oral bioavailability: F 27% in rat and 57% in dog. In both species the observed plasma clearances were moderate and the plasma half-lives long due to higher than expected volumes of distribution for an acidic compound. The pharmacokinetic profile in both species is favorable with potential for once a day administration.

3.6. *In vivo* potency and duration of action (DoA) of LAS191859 in a guinea pig model of DK-PGD $_2$ induced systemic eosinophilia

The effect of oral LAS191859 on the DK-PGD $_2$ induced systemic eosinophilia model in guinea pigs was assessed. The percentage of reduction of eosinophils in peripheral blood versus the vehicle group was measured at time points of 2 h and 18 h after administration in order to assess efficacy and duration of action of the

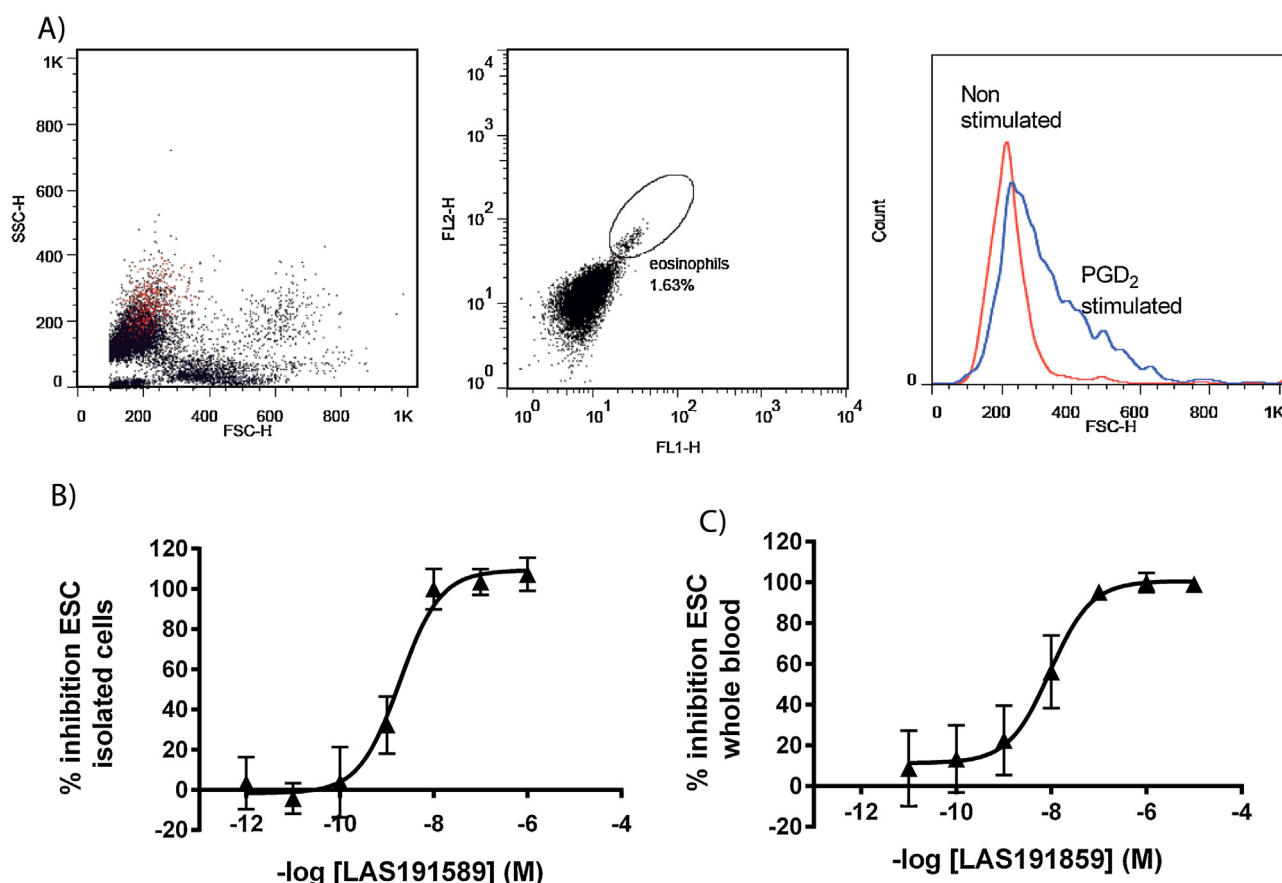


Fig. 4. Effect of LAS191859 on PGD₂ mediated eosinophil shape change response. A) Eosinophils were identified and plotted separately from the rest of leukocytes by flow cytometric measurement of autofluorescence, which is more pronounced in eosinophils. FSC/SSC plot shows also back-gating of the identified population. Changes in cell shape upon stimulation were reflected by increased forward scatter median and are illustrated by plots of nonstimulated vs stimulated eosinophils. B) Effects of LAS191859 on eosinophil shape change in human isolated polymorphonuclear cells. C) Effects of LAS191859 on eosinophil shape change in human whole blood. Three independent experiments were performed in duplicate from different donors.

Table 4

Pharmacokinetic parameters of LAS191859 in male Wistar rats and male Beagle dogs (N = 2).

Dose	C _{max} ng/ml	t _{max} h	AUC ng h/ml	Cl ml/min/kg	V _{ss} l/kg	t _{1/2} h	F %
RAT							
1 mg/kg i.v.	–	–	1230	14	1.8	4.9	–
1 mg/kg p.o.	136	0.5	328	–	–	3.6	27
DOG							
1 mg/kg i.v.	–	–	1580	11	1.8	6.3	–
1 mg/kg p.o.	1060	0.38	906	–	–	8.4	57

C_{max}: maximum plasma concentration, t_{max}: time to reach maximum plasma concentration, AUC: area under the plasma concentration–time curve (0–∞); V_{ss}: volume of distribution at steady-state; t_{1/2}: terminal plasma half-life; F: oral bioavailability.

Table 5

LAS191859 in vivo potency at 2 and 18 h after oral compound administration and duration of action (expressed as the ratio ID₅₀–18 h/ID₅₀–2 h) in the DK-PGD₂ induced systemic eosinophilia model in guinea pig.

Time after compound administration	ID ₅₀ (μg/kg)
2 h	13.27 (6.1–28.8)
18 h	131.4 (73.1–236.2)
Ratio 18 h/2 h	10

Values are mean values (95% CI) obtained from a mean of 2–3 experiments (n = 6 animals per dose).

compound. LAS191859 was a very potent compound after 2 h and 18 h of administration (Table 5 and Fig. 6) with an ID₅₀ of 13 and 131 μg/kg, respectively, indicating that only a 10 fold loss of activity was observed after 18 h of compound administration.

3.7. Pharmacokinetics/Pharmacodynamics (PK/PD) studies in guinea pig

The purpose of these studies was to understand the link between plasma levels of LAS191859 (PK) and the reduction of eosinophil counts in peripheral blood (PD) with time. These studies were used to assess either there was a direct PK/PD relationship, so that the observed pharmacological response correlates with systemic compound levels at that moment in time, or there was a disconnection between PK and PD by showing functional activity in the absence of significant compound plasma levels that could be attributed to compound residence time in the receptor.

Analysis of compound plasma concentrations of the 2 h guinea pig systemic eosinophilia study showed that response at this time point is dependent on plasma levels for all doses tested with an estimated IC₅₀ of approx 40 ng/ml (70 nM) (Fig. 7). This value was

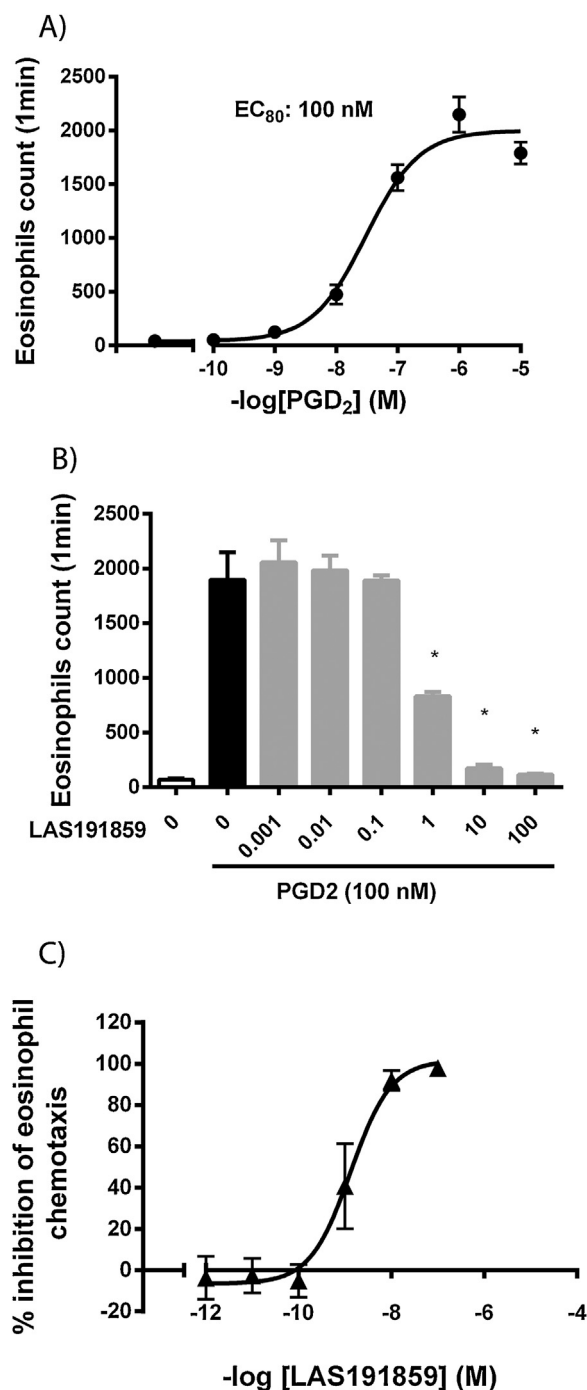


Fig. 5. Effects of LAS191859 on PGD₂ mediated human isolated eosinophils chemotaxis. A) Titration of PGD₂ effects on human eosinophil chemotaxis and EC₈₀ determination. Data shown are a representative of two repeated experiments. B) LAS191859 caused a dose-response reduction in PGD₂-induced eosinophil chemotaxis. Data presented as mean $\pm$ SD from a representative experiment performed in triplicate. * $p < 0.001$ compared with PGD₂ alone (analysed by ANOVA followed by Dunnett's post hoc test). C) Concentration response curve of LAS191859. Three independent experiments were performed in triplicate from different donors.

covering the 80–90% of the guinea pig GTP binding, activity that was considered optimal to translate in pharmacologic effect.

After 18 h of dosing most animals that received 0.3 mg/kg and some animals dosed with 0.1 mg/kg were still maintaining efficacy although compound levels detected in plasma were significantly reduced (Fig. 7). Analysing all doses and time points, a shift of the *in vivo* dose response curve to the left was observed after 18 h of compound administration. A functional activity of 60% could be

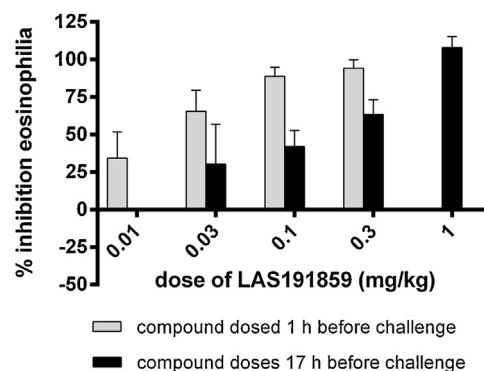


Fig. 6. Inhibition of systemic eosinophilia induced by DK-PGD₂ in a guinea pig model after 2 h and 18 h oral administration of LAS191859. Two-three independent experiments were performed with six animals per dose. (2 h studies basal: 0.3×10^5 eosinophils/ml top: 1.6×10^5 eosinophils/ml; 17 h studies basal: 1.0×10^5 eosinophils/ml top: 3.6×10^5 eosinophils/ml).

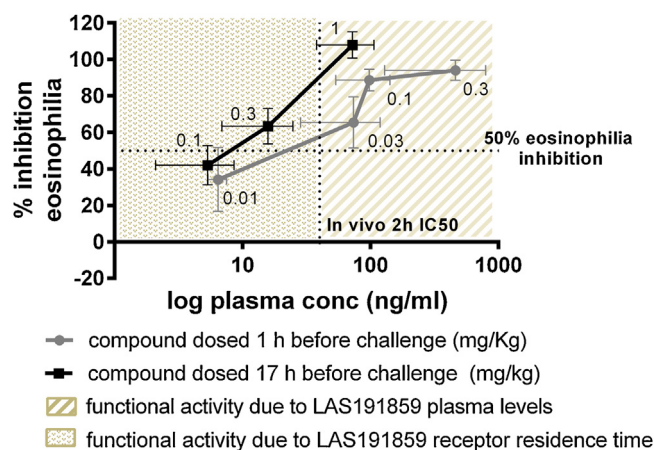


Fig. 7. Pharmacokinetics/Pharmacodynamics profile of LAS191859 in the eosinophilia induced by DK-PGD₂ model after 1 h and 17 h oral administration of several doses of LAS191859 in guinea pig. In the graph it is represented the plasma levels measured for each dose and time after administration of LAS191859 and the corresponding percentage of eosinophilia inhibition. In the symbols of the figure it is indicated the dose (mg/kg) administered. Dotted lines corresponds to the *in vivo* 2 h eosinophilia study IC₅₀ that was estimated from compound plasma concentrations and to 50% eosinophilia inhibition.

achieved with levels of 74 ± 45 ng/ml that can be explained with receptor occupancy but also with plasma levels of 15 ± 9 ng/ml, well below the measured *in vivo* IC₅₀ at 2 h and so insufficient to cover receptor occupancy. This result suggests that *in vivo* efficacy is maintained in time due to compound residence time in the receptor.

Compound LAS191859 is a very potent compound with long-lasting efficacy in the DK-PGD₂ induced systemic eosinophilia model in guinea pig, showing a disconnection between the pharmacokinetic (plasma levels) and the pharmacodynamic (inhibition of systemic eosinophilia) profiles due to slow receptor dissociation.

4. Discussion

The aim of this study was to establish the preclinical profile of LAS191859, a novel oral long-acting CRTh2 antagonist for treatment of asthma and other eosinophilic conditions.

In vitro evidence indicates that LAS191859 is a potent CRTh2 antagonist as demonstrated in GTP γ S binding studies with potency in the low nanomolar range and in addition it demonstrates a long receptor residence time. This potency is also maintained in cellular assays performed with human eosinophils and whole blood.

In the functional assay measuring inhibition of PGD₂-mediated GTPγS binding, LAS191589 depressed the agonist binding capacity producing a progressive reduction of maximal response at 2 h of incubation that was observed even after 22 h of incubation time therefore showing a clear functional insurmountability.

This behavior can be related to several possible mechanisms of action including: slow dissociation of antagonist from the receptor, interaction of the antagonist with allosteric binding sites that induces changes in conformation in the receptor preventing proper agonist binding, irreversible covalent binding or even antagonist-mediated receptor internalization [35,36]. We and others have described that CRTh2 antagonists can have slow dissociation kinetics from the receptor [37–39] and this was our first hypothesis to explain the observed unsurmountable behavior of our compound.

We confirmed this hypothesis for LAS191589 in the dissociation studies for human and guinea pig CRTh2 receptors using the [³⁵S]-GTPγS functional assay, demonstrating long receptor residence time for the human and guinea pig receptors, with half lives of 21 h and 16 h, respectively. Also, Schild plots discarded the allosteric effect as the unsurmountability profile was overcome with time. In the process of these studies it was intriguing to note that related compounds whose only structural difference was presence or absence of a single methyl group could make a huge difference in terms of receptor kinetics. Changes from around 2 h up to 21 h could be observed adding a methyl group improving dissociation half-life by an order of magnitude while maintaining same potency [40].

Considering reported CRTh2 antagonists, receptor kinetics makes LAS191589 unique, as reported dissociation half-lives are typically in the range of minutes compared with the 21 h of LAS191589 [37,38,32].

Mechanistic *in vivo* studies of inhibition of the systemic eosinophilia in guinea pig showed that the compound is also very potent *in vivo* with an ID₅₀ of 13.25 μg/kg after 2 h of compound administration. Administration of a single 0.3 mg/kg oral dose of LAS191589 leads to a complete inhibition of DK-PGD₂-induced systemic eosinophilia. Potencies reported for other CRTh2 antagonists in similar models showed lower *in vivo* potency: ID₅₀ for AM211 was calculated to be 850 μg/kg in a guinea pig model measuring blood leukocytosis [33] and for OC000459 the effect on systemic eosinophilia induced by DK-PGD₂ in a rat model, ID₅₀ was reported to be 40 μg/kg [34].

Duration of action studies by dosing LAS191589 18 h before the administration of the agonist showed a shift in potency but still an inhibition (approx. 60%) that was maintained at a time point where plasma levels were no longer relevant to block the receptor. These are relevant findings proving that a long receptor residence time in the CRTh2 receptor translates into a long *in vivo* duration of action without pharmacological active plasma levels. This finding indicates that a disconnection between the pharmacokinetic (PK) and the pharmacodynamic (PD) profiles has been observed for LAS191589 in guinea pig.

On top of residence time in the CRTh2 receptor, LAS191589 pharmacokinetic profile in rat and dog by oral and intravenous route, demonstrated that the compound has an overall favorable profile with an appropriate oral bioavailability and long plasma half-life that suggest a once a day administration.

Toxicological and safety studies performed to complete the profile of LAS191589 showed that so far, it is a safe compound. No issues have been detected in any of the studies performed in terms of cytotoxicity, cardiovascular safety, central nervous system safety and genotoxicity. No relevant adverse effects have been observed in 1, 4, and 14 days toxicological studies in different preclinical species.

As a whole, these results validated our internal strategy designed to maximize the possibility of the discovery of a safe acidic

CRTh2 antagonist compatible with once-daily dosology combining both properties in LAS191589: favorable PK and long residence time in the CRTh2 receptor

Although it is premature to postulate that a slowly dissociating CRTh2 antagonist with the potential to produce long lasting receptor blockade will be an advantage for asthma treatment, other compounds with similar characteristics exist in the market. A number of drugs have slow dissociation rate, including among others, angiotensin receptor antagonists like candesartan (dissociation half-life: 11.5 h), muscarinic M3 receptor antagonists like tiotropium and aclidinium (dissociation half-life: 62 h and 29 h, respectively) or histamine H1R antagonist such as desloratadine (dissociation half-life: >6 h), that contribute to their therapeutic effect and duration of action [41–44].

5. Conclusion

The preclinical data reported in this study shows that LAS191589 is a potent and safe CRTh2 antagonist, with a long receptor residence time that provides a sustained *in vivo* efficacy. This property is reflected in the prolonged duration of action of LAS191589 in *in vitro* and *in vivo* functional models. The results presented here are the first to demonstrate that long receptor residence time in the CRTh2 receptor translates into a long-lasting efficacy *in vivo* and this efficacy is achieved in the absence of pharmacologically relevant systemic plasma levels.

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