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Orally bioavailable allosteric CCR8 antagonists inhibit dendritic cell, T cell and eosinophil migration

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ABSTRACT

The chemokine receptor CCR8 is associated with asthma. Herein, we describe that both mature and immature dendritic cells (DC) express CCR8, whereas only mature DC migrate towards CCL1. Moreover, transient LPS challenge significantly down-regulates CCR8 expression hence attenuating CCL1 chemotaxis.

To inhibit CCR8 pathophysiology, we recently developed a novel series of small molecule CCR8 antagonists containing a diazaspiroundecane scaffold, which had micromolar potency. However, these first generation antagonists had high lipophilicity that endowed the compounds with poor physicochemical properties, and were thus not suitable for further development. By introducing polar bicyclic groups on the N-benzyl substituent and building in further polar interactions on the amide group we now show second generation diazospiroundecane antagonists with significantly improved overall properties. Potency is substantially improved from micromolar to nanomolar potency in CCR8 binding and inhibition of chemotaxis in human primary T cells, DC and in an eosinophil cell line. In addition to high potency, the most attractive antagonist, AZ084 showed excellent selectivity, high metabolic stability in vitro and an attractive in vivo PK profile with a long half-life in rat.

Interestingly, in ligand saturation experiments and in wash-off experiments, CCL1 was shown to have two binding sites to CCR8 with K_d at 1.2/68 pM respectively, and on-off rates of 0.004 and 0.0035/0.02 pM min, respectively. The lead antagonist, AZ084, appears to act as an allosteric inhibitor with a K_i at 0.9 nM.

Taken together, we herein report a novel oral allosteric CCR8 antagonist with predicted low once-daily dosing capable of potent inhibition of both human T cell and DC functions.

1. Introduction

The prevalence of asthma and the associated cost burden for the health care system is high [1]. Despite current therapies and improved diagnosis, asthma related deaths still occur especially in severe asthma patients during an exacerbation of their disease. It is well recognized that airway inflammation is a fundamental driver of the chronic nature of uncontrolled asthma [2]. This inflammatory state is characterized by a massive infiltration of cells such as eosinophils and lymphocytes, and a concomitant release of various factors that contribute to a long term remodeling of the tissue [2].

The infiltration of cells is orchestrated by small peptides and proteins belonging to the chemokine family [3,4]. These proteins have multiple biological roles including recruitment of leukocytes upon binding to a seven transmembrane G-protein-coupled receptor (GPCR). A subfamily of chemokine receptors belong to the CC-family and including among others CCR3, CCR4 and CCR8, all of which have been suggested to play a role in allergic airways disease [5]. A number of studies have shown that lung T cells express both CCR4 and CCR8, and the latter has conclusively been shown to play a role in asthmatic patients where expression has been correlated with decline in FEV1 [6,7]. Likewise, the levels of the ligands of these receptors, such as CCL1, CCL17 and CCL4, are enhanced in asthmatic patients [6,8,9].

In contrast to CCR4, CCR8 is so far reported to have only one major mammalian ligand, namely CCL1 [10]. CCR8 positive cells respond to CCL1 stimulation with increased intracellular calcium,

Abbreviations: DC, dendritic cell; LPS, lipopolysaccharide; PK, pharmacokinetics; GPCR, G-protein-coupled receptor; hERG, human ether-à-go-go related gene; FEV1, forced expiratory volume 1; KO, knock-out; wt, wild-type; CYP, cytochrome P450; MEC, minimal effective concentration; DtM, dose-to-man; SPA, scintillation proximity assay; BSA, bovine serum albumin; HLM, human liver microsomes.

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chemotaxis and enhanced survival [11–14]. In addition to T cells, other inflammatory cells such as dendritic cells and eosinophils have been shown to express CCR8 during certain conditions [15,16]. The role of CCR8 in allergic airway inflammation has been evaluated in vivo using CCR8 KO mice and recombinant installation of CCL1 in the trachea, and these data suggest that CCR8 may play a pivotal role [6,17–19]. The current understanding is that mast cell activation in the lung tissue is essential to induce high concentrations of CCL1, which attracts peripheral T cells to the inflammatory loci [6].

Small molecule chemokine receptor antagonists most often share a common pharmacophore, which includes lipophilic groups and a centrally located protonated amine that forms an ionic bond with the conserved glutamate $E^{7.39}$ of chemokine receptors [20]. This pharmacophore is also inherent in hERG ion channel blocking agents [21]. Increased bulkiness and rigidity of the lipophilic periphery in CCR8 antagonists render the compounds unable to fit into the narrow part of the hERG ion channel and hence any potential hERG channel inhibition of the chemokine antagonists described herein is reduced [22].

In the present study we demonstrate novel, highly selective, hERG free, CCR8 antagonists that possess excellent in vivo pharmacokinetic properties, and are potent inhibitors of chemotaxis not only in primary human T cells, but also in human dendritic cells. Taken together, we believe that these new antagonists that also have a low predicted dose-to-man are excellent tools with which to evaluate and firmly establish CCR8 as an important non-redundant factor in allergic airway diseases.

2. Materials and methods

2.1. Differentiation of human DC

Monocyte derived dendritic cells were routinely derived from peripheral human blood. Briefly, mononuclear cells were enriched by Ficoll separation and monocytes were then obtained by magnetic sorting with monocyte isolation kit II (Miltenyi Biotec, Auburn, CA) to >95% purity. Monocyte derived DC were obtained after culture for 7 days at 37 °C in medium [RPMI 1640 supplemented with Glutamax, 1 mM essential amino acids, penicillin–streptomycin, 10% HIFCS (Gibco BRL Life Technologies Paisley, Scotland), 5×10^{-5} M β-mercaptoethanol (Sigma, St. Louis, MO), 10 ng/mL rhIL-4 and 10 ng/mL GM-CSF (R&D systems, Minneapolis, MN)]. Mature dendritic cells were obtained by 24 h incubation of the immature DC with 0.5 ng/mL LPS (L4516, Sigma–Aldrich, St. Louis, MO) at 37 °C.

2.2. Flow cytometric analysis

Expression of CCR8 on monocytes and DC cells was evaluated by flow cytometric analysis according to standard procedures. Briefly, cells were re-suspended in PBS/0.5% bovine serum albumin (BSA) and non-specific binding was blocked with CD16 & CD32 antibodies (BD Biosciences, San Jose, CA). The cells were then incubated with CCR8 antibodies (R&D systems, Minneapolis, MN) at 4 °C for 30 min. Viability was monitored and dead cells excluded by 7-AAD staining. Surface expression was monitored on a FACS Fortessa using standard settings and analyzed by Diva Software.

2.3. Chemotaxis

Differentiated AML14.3D10 cells and CCR8 expressing T cells were obtained as described previously [16] and chemotaxis was evaluated in AML14.3D10 cells, T cells and DC as previously described [16]. Briefly, cells were re-suspended at 4×10^6 cells/mL in RPMI 1640/0.1% BSA (Gibco BRL Life Technologies Paisley, Scotland). 10 nM CCL1 (R&D systems Oxon, UK) ± CCR8 antagonist was added to the lower wells of a chemotaxis plate with 5 μm filter pores (Neuro Probe, Gaithersburg, MD) and the cells were added as drops on top of the filter. The cells migrated at 37 °C for 2 h and then the drops were wiped off the filter and the filter was removed. 3 μL of TOX-8 (Sigma, St. Louis, MO) was added to each well and incubated overnight at 37 °C. Fluorescence was measured by Spectramax Gemini at 550 nm.

Inhibition% was calculated by 100 × (1 − ((chemotaxis with antagonist − min)/(max − min))) where min = chemotaxis without CCL1 and max = cells added directly to bottom.

2.4. Primary screening assays; binding assays, toxicity assays and selectivity

A human recombinant CCR8 membrane preparation (0.05 μg/μL) (Euroscreen SA Brussels, Belgium) and 4 μg/μL SPA beads (Amersham Bioscience, Piscataway, NJ) were pre-incubated on ice for 2 h in binding buffer pH 7.4 [50 mM Hepes (Gibco BRL Life Technologies, Carlsbad, CA), 1 mM $CaCl_2$ (Merck Brookfield, WI), 5 mM $MgCl_2$ (Merck Brookfield, WI), 75 mM NaCl (Merck Brookfield, WI)]. Then an equal volume of the membrane/SPA mixture and 15 pM ^{125}I-CCL1 were added to a 96-well in the presence of increasing concentrations of the CCR8 antagonists and incubated for 1.5 h at RT with shaking. After centrifugation the plates were read in a Wallac 1459 microbeta counter Trilux. Binding affinity towards murine CCR8 was determined in BW5147 mouse CCR8 expressing cells, as described in [23]. Briefly, cells were incubated with 0.2 mg/well wheat germ agglutinin-coated SPA beads (GE Healthcare, Buckinghamshire, UK) for 30 min at RT in binding buffer (PBS/Ca/Mg, 0.1% (w/v) BSA), followed by addition of 100 nM unlabeled CCL1 ± compound to the cell/bead mixture. Finally, 100 pM ^{125}I-CCL1 (specific activity 2400 Ci/mmol) was added to the mixture and incubated at RT for 2 h. Bound ^{125}I-CCL1 was measured by scintillation counting (Wallac TriLux, PerkinElmer). Cellular toxicity was determined in THP-1 cells using WST as substrate. Briefly, cells were seeded at a density of 1×10^4 cells/well and incubated in DMEM supplemented with 10% FCS at 37 °C under 5% CO_2 for 24 h. This was followed by overnight culture in serum-free DMEM medium in presence of the CCR8 antagonist. Finally, 10 μL of WST-1 was added to each well and the plates were incubated for 1 h and then analyzed using a microplate reader (SpectraMax, Cambridge, UK) at 450 nm with reference wavelength of 655 nm. Selectivity in vitro was determined by MDS Pharma Services, Taiwan Ltd according to standard procedures (www.mdsps.com).

Inhibition% is calculated by 100 × (1 − ((cpm with antagonist − min)/(max − min))) where min = cpm with 3 nM CCL1 alone and max = cpm in buffer alone.

2.5. Ligand saturation experiment, wash-off experiments and antagonist competition experiments

The CCR8 membrane and SPA beads were prepared as in the binding assay above. In ligand saturation a serial dilution from 220 pM to 0.11 pM ^{125}I-CCL1 was prepared and non-specific binding was estimated by adding 300 nM cold CCL1 to control wells. The plate was incubated for 90 min at RT. Binding curves

were analyzed by best fit by Prism 5 for Windows (GraphPad Software Inc, La Jolla, CA) and $K_{d_{1/2}}$ and $B_{max_{1/2}}$ were generated.

In the wash-off experiments 5–500 pM ^{125}I-CCL1 were then added to the wells and the plate was read every 30 s until steady state occurred when an excess of cold CCL1 (500 nM) was added and plate was read every 30 s again until the signal was gone. $K_{on/off}$ were established through best fit using Prism 5 for Windows.

In the antagonist competition experiments, IC_{50} values for the antagonists were established in the binding assay at 5–100 pM ^{125}I-CCL1 concentration. The obtained IC_{50} value was then plotted against the corresponding antagonist concentration and best fit was obtained by Prism 5 for Windows.

2.6. In vivo PK, PPB, stability and allometric scaling

The pharmacokinetics of the antagonists was investigated by single intravenous (*i.v.*) doses to Balb/C female mice, male Wistar rats and female Beagle dogs and by oral administration (*p.o.*) in male Wistar rats and blood samples were taken from 10 min up to 24 h post-administration. Briefly, antagonists were administered *i.v.* (1–2 μmol/kg) or *p.o.* (5 μmol/kg) in 0.9% NaCl. Blood was drawn at indicated time-points and directly mixed with three parts of Milli-Q water containing Na-heparin to lyze the blood cells. The samples were then precipitated with MeCN/1% HAc, containing internal standard, and blood concentrations were determined by LC–MS/MS.

PK parameters (using the blood concentrations as inputs) were derived from a three-compartment model using Pharsight WinNon-lin version 5.2.1 (Pharsight, Mountain View, CA). The plasma protein binding was performed by ultra filtration and the intrinsic metabolic clearance was performed in hepatocytes as previously described [24]. Cyp inhibition was determined according to standard protocol. Briefly, all antagonists were diluted in 0.1% DMSO. HLM/substrate mixture (0.1 M PBS with 10 mM $MgCl_2$) and 3 μL of antagonist were added to each well and the reaction was started with addition of 1 mM NADPH. The reaction was quenched by CAN/levallorphan after 20 min and the supernatant analyzed by LC/MS/MS. Caco-2 was determined according to standard protocol. Briefly, Caco-2 cells from ATCC were cultured to a monolayer on Millipore Minicell plates for 20 days. Following this, medium was changed to HBSS/HEPES buffer at pH 6.0 above and HBSS/HEPES at pH 7.0 bellow the cells. CCR8 antagonists were then added either above (A > B permeation) or below (A < B permeation). Permeation was continued for 2 h before the concentration of drug in each well was quantified by reversed HPLC with MS detection. Log *D* was determined according to standard procedures. Briefly, CCR8 antagonists were incubated on an orbital shaker at 450 rpm overnight in 1-octanol in 0.1 mol/L phosphate buffer at pH 7.4. The clear supernatant was then transferred to a new vial and saturated 1-octanol was added. After incubation for 30 min at 20 °C the plate was centrifuged for 30 min at 160 × *g* and the antagonist concentration in the two phases was measured by HPLC and expressed on the log scale as concentration of antagonist in octanol/concentration of antagonist in water.

The allometric scaling approach is based on the power function, as the body weight from three species is plotted against the unbound pharmacokinetic parameter of interest [25]. Where *Y* is the parameter of interest, *W* is the body weight, log *a* is the *y*-intercept and *b* is the slope. The log transformation of the equation above is represented as follows: $\text{Log } Y = \log a + b \log W$.

2.7. hERG ion works, hERG trafficking assay, QTc in dog, 7-day tolerability study in the rat and the dog, AMES and MLA assay

hERG trafficking was monitored in stable hERG-WT transfected HEK293 cells stimulated with antagonist for 16 h at 37 °C followed by paraformaldehyde fixation and staining with fluorescently labeled antibody to detect surface hERG expression. Functional interference with the hERG ion channel was determined by monitoring voltage clamp according to standard procedures [26]. In vivo evaluation on cardiovascular effects was monitored in beagle dogs. Briefly, CCR8 antagonists were dissolved in 200 nM phosphate buffer containing 0.5% (w/v) hydroxypropyl methylcellulose and 0.1% (w/v) polysorbate 80 and administered orally by gavage (1 mL/kg). Animals were chronically instrumented through a DSI PhysioTel transmitter residing *i.p.* The telemetry assessments took place in a telemetry pen. Blood pressure, heart rate, body temperature and lead II ECG intervals PR, RR, QRS and QT were recorded via receivers placed inside the dogs pens in conscious beagle dogs for 1 h pre-dosing and 24 h post-dosing. Dose–response curve is derived in Graph Pad Prism using non-linear regression best fit.

In vivo evaluation of tolerability of the CCR8 compounds was evaluated in Wistar rats and beagle dogs. Briefly, CCR8 antagonists were dissolved in 200 nM phosphate buffer containing 0.5% (w/v) hydroxypropyl methylcellulose and 0.1% (w/v) polysorbate 80 and administered orally by gavage. The animals were necropsied on the day after the last dose. In all animals the following parameters were evaluated: clinical observations, ECG, body weight, food consumption, hematology, coagulation, plasma chemistry, gross and microscopic pathology.

Evaluation of mutagenic activity was evaluated in Salmonella typhimurium LT2 strain TA97, TA98, TA100, TA102, TA105 in presence and absence of S9 fraction from Aroclor 1254-induced rat liver. After 3 days culture on minimal glucose agar medium plates the number of colonies were counted as a measurement of mutagenicity.

The capacity to induce forward mutation at the thymidine kinase locus in mouse lymphoma L5178Y TK ± 3.7.2 C cells was determined in presence or absence of S9 fraction from Aroclor 1254-induced rat liver.

2.8. Statistical analyses

The significance of differences between groups was calculated using one-way ANOVA using Bonferroni's multiple comparison test with by GraphPadPrism. Statistical power is described as *p*-value >0.05 = NS, 0.05–0.01*, 0.01–0.001** and <0.001***.

3. Results

3.1. Human dendritic cells express CCR8 and respond to CCL1 by chemotaxis

To evaluate human dendritic cell responses to CCL1, an in vitro model was established.

Purified monocytes from peripheral human blood expressed low levels of CCR8, whereas 75% of the in vitro generated immature DC acquired CCR8 after 8 days in culture (Fig. 1A and B). Transient exposure (2 h) of LPS, highly significantly down-regulated CCR8 expression on the immature DC (Fig. 1A and B). When exposed to LPS for 24 h the CCR8 expression was partly regained reaching almost 50% in the now mature DC (Fig. 1A and B). Monocytes and transiently LPS stimulated immature DC did not migrate towards CCL1, although immature DC showed a marginal migration at high doses of CCL1 (Fig. 1C). In sharp contrast, significant chemotaxis was observed at 10 nM in fully mature DC cells (Fig. 1C).

These data demonstrate that human dendritic cells express CCR8 and migrate to CCL1 in a dose- and maturation dependant manner.

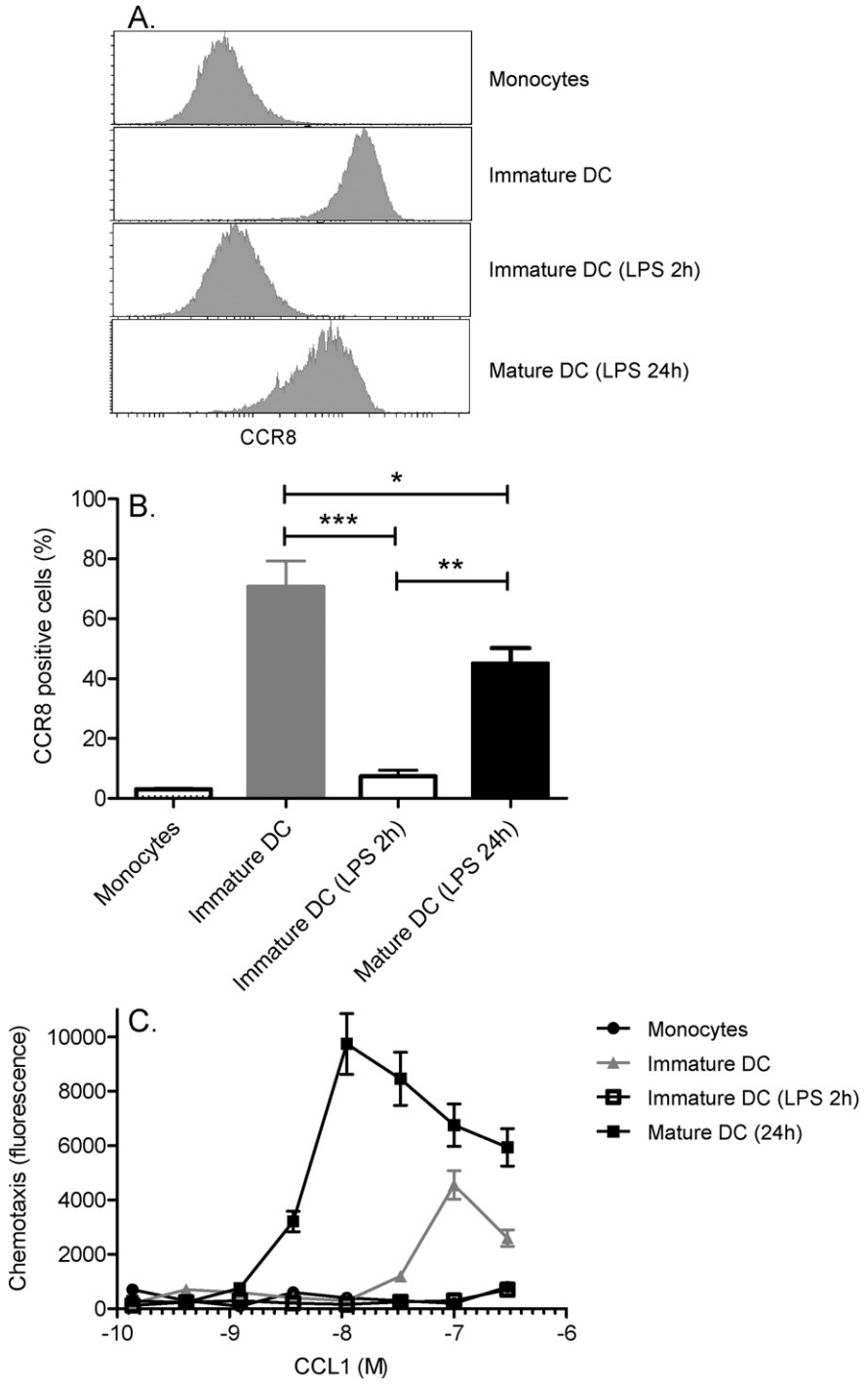


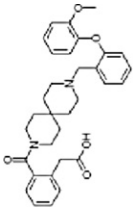
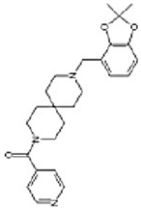
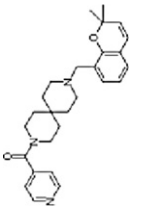
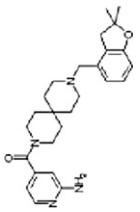
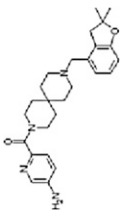
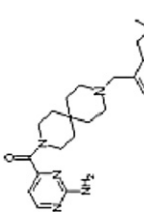
Fig. 1. Human mature DC expresses CCR8 and migrates towards CCL1. Expression of CCR8 on human peripheral blood monocytes and corresponding derived DC was evaluated by flow cytometric analysis, and shown from one representative donor (A). Mean values from three independent donors show a clear LPS dependent down-regulation of CCR8 expression on human DC (B). Only when DCs have been matured with LPS do they acquire chemotactic capacity to respond to CCL1 with chemotaxis (C). Data shows mean ± SD from three independent donors. Significance was calculated with one-way ANOVA using Bonferroni's multiple comparison test. Statistical power is described as p-value <0.05 = NS, 0.05–0.01*, 0.01–0.001** and >0.001***.

3.2. Biochemical and cellular potency and in vitro physicochemical properties of the CCR8 antagonists

We recently characterized novel CCR8 antagonists, based upon a diazaspiroundecane scaffold. These antagonists had micromolar activity, were generally metabolically unstable and were associated with hERG inhibition [16]. To improve the potency and overall properties further examples were synthesized and the structure–activity relationship of this series was explored.

It was found that polar substituents (e.g. pyridine or pyridimine) on the amide group reduced lipophilicity substantially, but retained potency. By synthesizing the corresponding pyridyl N-oxide amides (AZ760) or by inclusion of an acidic group to provide a zwitterionic series of amides (AZ491) it was discovered that extremely low lipophilicity could be achieved and yet acceptable potency could still be retained (Table 1). However, these antagonists although metabolically stable in vitro, still showed unacceptable hERG inhibition thus no further evaluation of these series were conducted.

Table 1
Primary parameters of CCR8 compounds in the diazaspiroundecanes series.

	AZ760	AZ491	AZ442	AZ463	AZ435	AZ084	AZ133
							
Molecule formula weight	488	529	422	432	435	435	436
CCR8 Hu bind IC50 (nM)	80	57.4	5.91	10.8	22.6	4.6	9.1
CCR8 Mu bind (nM)	907	115			4061	359	6638
Cell toxicity IC50 (nM)	>20,000	>20,000	>20,000	>20,000	>20,000	>20,000	>20,000
Lipophilicity (Log *D*) pH 7.4	0.5	−0.1	1.3	1.4	1.4	1.3	1.1
Hu mics metab K CLint (μL/min/mg)	10	10	41	25	12	18	10
CYP3A4 Hu BFC Enz CR IC50 (μM)	40	40		15	34	5.4	40
CYP2D6 Hu AMMC Enz CR IC50 (μM)	40	40	0.8	0.3	32	22	20
CYP1A2 Hu MROD Enz CR IC50 (μM)	40	40		40	40	40	40
CYP2C19 Hu CEC Enz CR IC50 (μM)	40	40		40	40	40	40
CYP2C9 Hu MFC Enz CR IC50 (μM)	40	40		40	40	40	40
hERG Hu EPhs IC50 (nM)	2800	10,000	9300	5100	40,000	35,000	93,000
hERG margin (fold)	35	174	1574	472	1770	4305	8017

Each assay was repeated at least three times and standard deviation is routinely less than 20%.

Firstly, by introducing specific bicyclic groups to replace the phenyl ring of the N-benzyl substituent, a new series was discovered with improved properties, for example AZ442 and AZ463. These new antagonists showed excellent potency, good lipophilicity and high free fraction in blood. These heterocyclic antagonists were however unstable in human microsomes, inhibited Cytochrome P450 2D6 and still showed low hERG margins (Table 1). Further improvement of these bicyclic diazaspiroundecanes was seen by extending the polar interactions on the amide group, exemplified by AZ084, AZ133 and AZ435. These three examples all show overall excellent primary properties such as high potency, good lipophilicity range, high free fraction in blood, acceptable stability in vitro, much reduced CYP 2D6 inhibition and importantly excellent hERG selectivity (Table 1).

Thus, further characterization of biological and pharmacokinetic properties were focused on these three antagonists.

3.3. Antagonism in primary human cells

To evaluate if these new compounds were functional antagonists in physiological relevant cellular systems, their potential to inhibit chemotaxis in three different human cells was evaluated.

In the endogenously CCR8 expressing eosinophilic cell line AML, all three antagonists showed high potency with pronounced dose–response dependent inhibition of chemotaxis with IC_{50} between 1.2 and 13 nM (Fig. 2A). All three antagonists were active in primary human T cells and inhibited chemotaxis at 5–50 nM (Fig. 2B). In the human LPS matured monocytic derived DC, a significant inhibition of chemotaxis was seen with all three antagonists with IC_{50} at 5–75 nM (Fig. 2C). Importantly, none of the antagonists acted as an agonist, measured as intrinsic capacity to drive chemotaxis (data not shown).

Taken together, all three antagonists are active in the low nanomolar range (Table 2) in three different CCR8 expressing human cells i.e. eosinophils, T cells and DC with AZ084 being the most active in all cell systems.

3.4. Pharmacokinetic evaluation of CCR8 antagonists in vitro and in vivo

To evaluate if the antagonists could be suitable for further development, in vitro and in vivo pharmacokinetic analysis of the three CCR8 antagonists was conducted.

The in vitro permeability profile for both antagonists suggests a rapid and complete absorption (Table 3). In vivo PK studies in rats supported the in vitro observation as all three antagonists were shown to have a bioavailability >70% in rat. The AUC was 5.4, 5.2 and 4.4 μM h (AZ133, AZ435 and AZ084, respectively) with a T_{max} less then 2 h (Fig. 3). Furthermore, a large free fraction was observed in human, dog, rat and mouse plasma (Table 3). The predicted CL and Vss in man were estimated by allometric scaling based on the *i.v.* pharmacokinetic data from mouse, rat and dog. The allometric scaling suggested that AZ084 would have the lowest CL in man. This is supported by the in vitro CL in human hepatocytes (Table 3). The major CL route is metabolic for all the three antagonists.

Taken together, based on the in vitro potency in combination with the in vivo PK AZ084 and AZ133 displayed the most attractive profile and were chosen for further development.

3.5. A two site binding mode to CCR8 for CCL1—allosteric and orthosteric antagonist inhibition

In order to establish apparent K_i-values for the antagonists the binding mode of CCL1–CCR8 was first established.

The binding profile in ligand saturation experiments suggested the best fit of CCL1 to the CCR8 receptor with two binding sites,

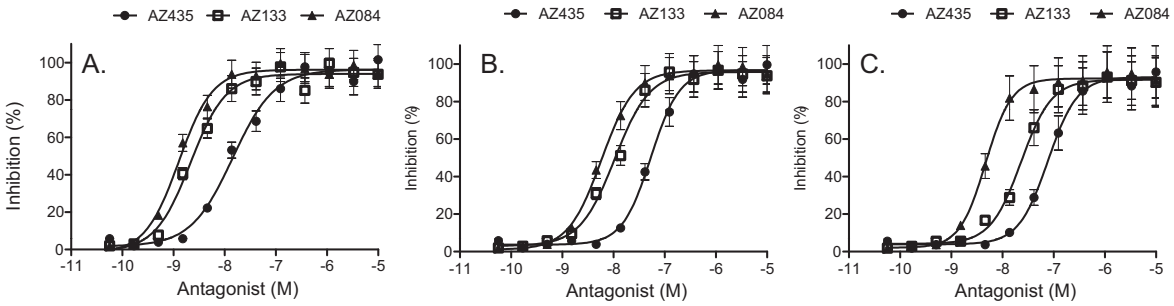


Fig. 2. CCR8 antagonist effect on chemotaxis in AML, T cell and DC. To determine the potency of the CCR8 antagonists in cellular systems, the percentage inhibition of CCL1 induced chemotaxis was evaluated with three CCR8 antagonists in AML (A), T cells (B) and DC (C). Data shows mean inhibition ± SD from three experiments (independent donors for T cells and DC).

Table 2
Inhibition of chemotaxis in AML14.3D10, human T cells and human monocyte derived LPS maturated DC (nM).

	AZ435	AZ133	AZ084
AML	14.0	2.3	1.3
T cells	50.9	10.2	5.7
Dendritic cells	73.1	22.1	4.6

Each assay was repeated at least three times and standard deviation is routinely less than 20%.

rater than one, interaction (Fig. 4A). The high affinity binding site has a K_d of 1.2 pM, whereas the low affinity site has a K_d of 67.9 pM (Table 4). In wash-off experiments of labeled CCL1 with cold CCL1 the on/off$_{(high/low)}$ rates were established to 0.004/pM min and 0.0035/0.020/pM min which generated $K_{d(high/low)}$ 0.88/5.1 pM, which are in agreement with the binding affinities obtained in the saturation experiment (Fig. 4C and D).

In competition experiments, the affinity of AZ084 and AZ133 for CCR8 was measured by competition with radiolabeled CCL1. Most interestingly, the two antagonists show strikingly different binding mode characteristics. AZ133 showed a classical orthosteric competitive binding to CCL1 (Fig. 4B). In contrast, the profile for AZ084 appears like a hyperbolic inhibition curve with a competitive inhibition profile up to 50 nM and thereafter the slope of the curve is shallower suggesting a potential allosteric antagonism mechanism (Fig. 4B). This results in an IC_{50} value of 9.1 nM for AZ133 and 4.6 nM for AZ084 at 5 pM CCL1. This translates to apparent K_i of 1.73 nM and 0.88 nM, respectively. With the competitive binding characteristics for AZ133 the IC_{50} and thereby K_i increase proportionally, whereas AZ084 appears to reach a maximum at 75 pM CCL1 with an IC_{50} 22.5 nM.

Table 3
In vitro and in vivo DMPK parameters for the three leading CCR8 compounds.

	AZ435	AZ084	AZ133
Hu Caco-2 Perm pH 7.4 A–B Papp (1E−6 cm/s)	24.2	30.4	22.0
Hu Caco-2 Perm pH 7.4 A–B recovery (%)	95.0	101.0	101.0
Hu Hep Clint (μL/min/1E6)	<2.7	<2.7	<2.6
Dog plasma protein binding (% free)	54.9	45.7	52.7
Mu plasma protein binding (% free)	61.5	55.6	57.2
Hu plasma protein binding (% free)	44.0	31.0	43.0
Rat plasma protein binding (% free)	52.0	47.0	64.0
Rat HW plasma PK CL (mL/min/kg)	21.0	15.0	32.0
Rat HW plasma PK Vss (L/kg)	15.0	6.0	5.5
Rat HW plasma PK $t_{1/2}$ (h)	9.4	5.4	3.1
Rat HW plasma PK bioavailability (%)	92.0	68.0	200.0
Rat HW plasma PK C_{max} (μM)	0.2	0.5	1.0

Each assay was repeated at least two times and standard deviation was routinely less than 20%.

Thus, CCL1 showed a two site binding mode to CCR8. AZ133 apparently acts as a competitive antagonist, whereas AZ084 seems to be an allosteric inhibitor. Due to the apparent allosteric binding mode AZ084 was selected as the lead candidate to be progressed further.

3.6. Dose-to-man predictions

Considering its excellent PK profile, in vitro activity and binding mode AZ084 was chosen to progress into final development and thus prediction of human dose was carried out for this antagonist.

Human systemic concentrations of AZ084 were simulated in an oral one-compartment model assuming rapid absorption and with the predicted human PK estimates as inputs (CL = 5.8 mL/min/kg, Vss = 5.4 L/kg, F = 70% based on allometric scaling). The human minimal effective dose (MEC) was estimated to be 10 nM derived from the free concentration required to cover 3 times human chemotaxis EC_{50} (1.2 nM) for 24 h. This results in a predicted dose-to-man for AZ084 of 0.16 mg/kg u.i.d. giving a predicted free C_{max} to be 14 nM and a C_{max} to C_{min} ratio of 3.5-fold (Fig. 5).

3.7. Safety evaluation of AZ084

An extensive evaluation with respect to safety and selectivity was conducted with AZ084 to establish safety margins.

Initially, the antagonist was shown to have an excellent selectivity profile in vitro. In a broad screen evaluating 141

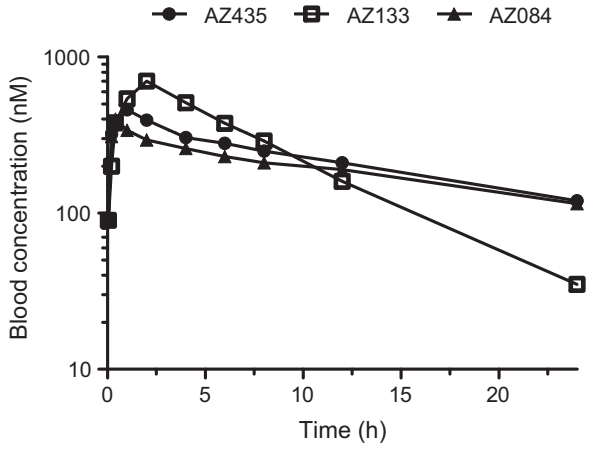


Fig. 3. Oral PK profile in male Wistar rats. To determine if the compounds had appropriate in vivo characteristics suitable for drug development, the three CCR8 antagonists were administered with a dose of 5 μmol/kg *p.o.* to rats and blood samples were taken from 10 min up to 24 h post-administration. Data shows mean ± SD from three experiments.

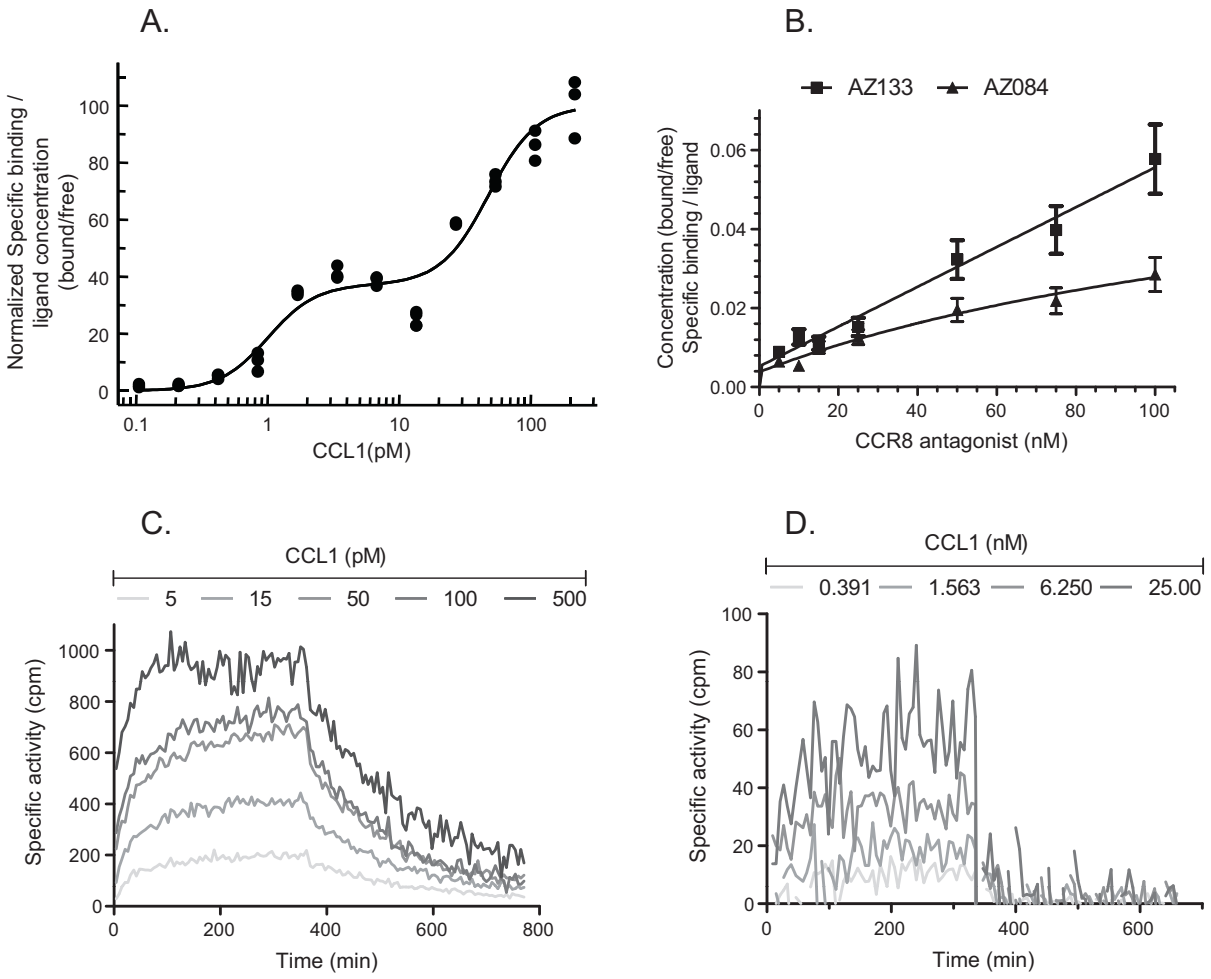


Fig. 4. Binding mode of CCL1 and CCR8 antagonists to human CCR8. To demonstrate the number of binding sites for CCL1–CCR8, a ligand saturation experiment with increasing concentrations of ^{125}I-CCL1 to CCR8 membrane was conducted. The graph shows total and specific binding at a constant concentration of unlabeled CCL1 and the corresponding obtained best fit of each point after normalization (A). To determine the binding mode of the CCR8 antagonists, competition experiments with AZ133 and AZ084 with ^{125}I-CCL1 were performed. Best fit shows AZ133 to be an oligosteric inhibitor whereas AZ084 is an allosteric inhibitor (B), data shown is mean ± SD from four experiments. The specific activity in wash-off experiments of ^{125}I-CCL1 with unlabeled CCL1 was determined for 700 min which provided K_{on}/K_{off} rates of CCL1–CCR8 (C and D), one out of four similar experiments.

various kinases and receptors only three activities with IC_{50}s <10 μM activity were recorded, with sigma σ1 with an K_i of 3 μM (3750-fold margin to CCR8 K_i) as the most active (Table 5). AZ084 showed no binding activity at 10 μM towards any of the other chemokine receptors evaluated (Table 5). The antagonist also showed very good selectivity (>40,000-fold) towards a panel of ion channels known to be involved in QT prolongation (Table 5). Moreover, the antagonist did not show any effect on trafficking of the hERG channel measured as surface expression in wild type hERG expressing HEK292 cells. AZ084 has no activity towards 5-strain AMES and is negative in the mouse lymphoma assay.

The antagonist was well tolerated in rats and dogs exposed for 7 consecutive days at doses up to 650 mg/kg/day and 8.7 mg/kg/day, respectively. In dog telemetry studies a small effect was observed

Table 4
Binding affinity and enzyme kinetic for CCL1–CCR8.

Saturation exp	B_{max} (high) (cpm)	278.1
	K_d (high) (pM)	1.17
	B_{max} (low)	643.9
	K_d (low)	67.87
Wash off experiment (5 pM)	On rate (1/pM min)	0.0040
	Off rate (high) (/min)	0.0035
	Off rate (low) (/min)	0.0201
	K_d (high) (pM)	0.88
	K_d (low)	5.07
	$T_{1/2}$ (min)	30

Each assay was repeated at least three times and standard deviation is routinely less than 20%.

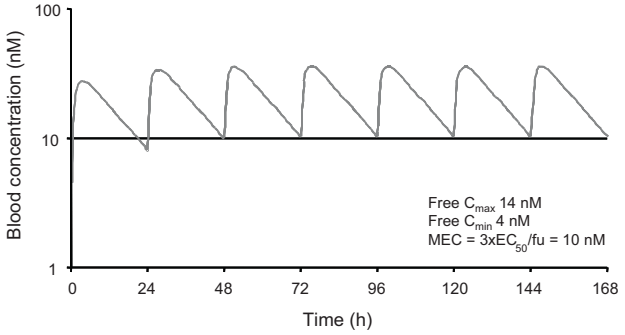


Fig. 5. Dose prediction to man for AZ084. To obtain an estimated dose to man for AZ084 and determine if AZ084 would be a once-daily drug, simulations of AZ084 in human were performed. The calculations were based on information from preclinical species and using minimal effective concentration (MEC) as 3-times the concentration required to inhibit 50% of chemotaxis. These data generated a predicted dose to man for AZ084 of 0.16 mg/kg u.i.d.

Table 5
Selectivity profile for AZ084 towards kinases, receptors and the hERG family.

	K_i (mM)	Fold to K_i CCR8
Sigma 1	3.3	3750
Muscarinic M2	7.4	8409
Non-selective muscarinic (OxoM)	9	10,227
CCR (1,2,3,4,5,6,7,9)	>10	>11,364
CXCR (1,2,3,4,5)	>10	>11,364
hERG	35.2	40,000
hNav	>100	>113,640
hKv	>100	>113,640
hKv-hKChIP	>100	>113,640
hCav	62.5	71,023
hIKs	>100	>113,640
hHCN	>100	>113,640
hERG trafficking	>100	>113,640

Each assay was repeated at least three times and standard deviation is routinely less than 20%.

with moderate QTc prolongation (~5%) at doses corresponding to 100× the predicted dose-to-man (Fig. 6).

4. Discussion

Asthma comprises a range of heterogeneous phenotypes that differ in presentation, etiology and pathophysiology and has more than doubled in prevalence and severity mainly in developed countries during the past 60 years [1,27]. Inflammatory factors such as cytokines and chemokines have been implicated in playing pivotal roles in the initiation and progression of the disease. In this report we describe novel small molecule antagonists of CCR8 that are potent in inhibiting CCL1 driven functions such as chemotaxis of T cells, dendritic cells and eosinophils.

The importance of CCR8 in diseases like asthma and atopic dermatitis has been much discussed over the last 10 years [5]. It has been established that T cells respond to CCL1 by Ca mobilization and chemotaxis in vitro upon binding to CCR8 and play a determinative role in clinical asthma [7,11–14]. The source for the gradient of CCL1 has been shown to be antigen activated cells such as mast cells, endothelial cells and Langerhans cells [6,28,29]. In addition, to create the CCL1 gradient, Langerhans cells have been associated with high CCR8 surface expression [28] in allergic conditions such as atopic dermatitis. Moreover, mouse lung DCs express CCR8 and migration to draining lymph nodes is

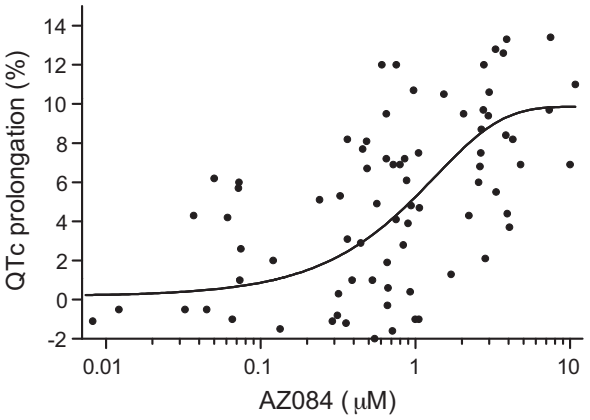


Fig. 6. QTc effect in dog after AZ084 administration. To determine if the AZ084 had a QTc liability beagle dogs dosed with AZ084 were monitored for a 24 h time period. The observed effect of QTc prolongation in dogs after *p.o.* administration of AZ084 is plotted individually based on the received dose of AZ084. The dose–response is then derived and the dotted line represents the dose required to induce 5% prolongation of QTc.

dependent on CCR7 and CCR8, also suggesting a role for CCR8 reactive DC's in asthma [15]. In this report we extend these observations and demonstrate that human monocytic derived dendritic cells express surface CCR8 and respond to CCL1. Interestingly, both the expression of and chemotactic function through CCR8 is influenced by the presence of bacterial products such as LPS, as previously demonstrated in macrophages [19]. While immature DCs are homogenously CCR8 positive, LPS down-regulates CCR8. Short exposure to LPS completely eliminates surface CCR8 expression and thereby renders the cells unresponsive to CCL1 stimulation. In contrast, although fully matured DC cells show lower CCR8 expression compared to immature DC, it is only the mature DC that respond robustly to CCL1 by chemotaxis. The down-regulation of CCR8 is in line with previous reports that long exposure to bacterial products only moderately regulate CCR8 expression on DCs [15]. However, the novel observation that a transient down-regulation at the first contact with LPS suggests that CCR8 may be internalized as a response to contact with bacterial endotoxins. Indeed, preliminary results in our lab demonstrates that the intracellular expression of CCR8 is in fact enhanced in DC exposed to short burst of LPS supporting this hypothesis (data not shown). In the context of the recurrent bacterial exposure in the lung and knowledge that many severe exacerbations of asthma are actually caused by invading bacteria, the altered expression pattern of CCR8 may significantly influence the cellular dynamics and thereby the progression of the disease [30]. Our observation that suggests mature DCs to be more prone to chemotaxis, compared to the immature DCs, may indicate that interference in the migratory capacity of these antigen presenting cells might constitute a novel means to treat disease.

Development of small molecule antagonists of CCR8 has been difficult and only a few examples have shown with potent (nanomolar) activity combined with good drug-like properties [31–33]. Based on this information it is encouraging that we herein demonstrate a series of novel antagonists containing a diazaspiroundecane scaffold that show low nanomolar activity in three different CCR8 endogenously expressing human cell types. As demonstrated the selected antagonist AZ084 also has good stability in vitro and good PK properties in vivo. The species sequence homology for chemokine receptors is generally low and CCR8 is no exception with only 71% homology between human and mouse CCR8 [34]. Therefore it is not surprising that the discovered antagonists have reduced potency in crossover to mouse, so despite the good PK profile, in vivo testing of the CCR8 antagonist concept in experimental models remains problematical. However, the support from in vivo CCR8 KO experiments and observations in human asthmatic patients with the close correlation of progression of disease to CCR8 expression is overwhelming [6,7,17–19]. This taken together with the solid in vitro data for AZ084 in three human CCR8 endogenously expressing primary cells warrants further studies to demonstrate efficacy in man for this antagonist.

CCR8 was originally cloned in 1997 and then demonstrated to have a high affinity interaction with the ligand CCL1 with a K_d at 1.2 nM [10]. In follow-up experiments the affinity has been confirmed both in CCR8 transfected 1D-21 cells and T cells to be 1.8 and 2.1 nM, respectively [13]. Both in these reports and in the mouse, CCL1 has been shown to interact with CCR8 with one binding site [10,13,34]. Therefore our demonstration that CCL1 in fact interacts with CCR8 with two distinct binding affinities is notable. The discrepancy between the previous studies and ours is intriguing and may relate to the observation that chemokine ligands form dimers or oligomers spontaneously. Since it is only the monomeric form that is the active form in vitro it can point towards higher presence of oligomeric forms in the previous studies which thus could have caused the higher apparent K_d [35,36]. The actual structure of CCR8 is not known and therefore all

modeling work is based on bovine rhodopsin [37]. Based on the rhodopsin structure, the hypothesis is that a chemokine ligand should interact with a chemokine receptor at two distinct sites. Site I is between the receptor N-domain and the ligand N-terminal loop and site II is between the receptor extracellular loop and the ligand N-terminal residue and cooperates to mediate binding affinity, ligand selectivity and activation of the receptor [38]. Importantly, the sites are coupled in function and thus do not operate independently [39]. This means that binding at site I/II increases the binding affinity at site II/I, although binding at one site only actually decreases the binding affinity at the other site [38]. With this in mind it is intriguing to consider the different inhibition profiles for the two CCR8 antagonist antagonists described. AZ133 acts as a classical orthosteric competitive inhibitor to CCL1 on CCR8. In contrast, AZ084 appears to act as an allosteric inhibitor with a hyperbolic curve. To our knowledge this is the first example of a CCR8 allosteric antagonist although examples of allosteric inhibitors have been shown towards CCR1, CCR5 and CXCR1 [40–42]. The biological consequence of the binding mode for AZ084 is that at pathophysiological concentrations of CCL1 less antagonist will be required to have a therapeutic efficacy, meaning the safety margin will be larger than initially expected and making AZ084 a very attractive candidate drug.

Chemokine and hERG blocking agents share a common pharmacophore which includes lipophilic groups and centrally located protonated amines [22,31]. In the course to discover AZ084 this was considered, and a profile with very large margins to hERG channels was developed to avoid this activity from the beginning. This is in sharp contrast to many other chemokine programs that have been stopped in the pre-clinical phase due to insufficient margins to hERG. The safe profile of AZ084 with respect to QTc was further demonstrated when no effect on hERG trafficking and only minor QTc prolongation in dogs at acceptably high margins were observed in vivo.

So, taken together, AZ084 is a novel, highly selective and metabolically stable small molecule CCR8 antagonist with high potency in human cells that has low QTc risk and a low predicted dose-to-man.

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