Biochemical Pharmacology 83 (2012) 778–787

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

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

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ARTICLE INFO

Article history:
Received 20 October 2011
Accepted 15 December 2011
Available online 24 December 2011

Keywords:
Chemokine
Dendritic cell
Asthma
Receptor inhibitor
Mode of binding

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.

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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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0006-2952/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2011.12.021

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.

$$\text{Inhibition\% was calculated by } 100 \times (1 - ((\text{chemotaxis with antagonist} - \text{min})/(\text{max} - \text{min}))) \text{ where min} = \text{chemotaxis without CCL1 and max} = \text{cells added directly to bottom}.$$

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).

$$\text{Inhibition\% is calculated by } 100 \times (1 - ((\text{cpm with antagonist} - \text{min})/(\text{max} - \text{min}))) \text{ where min} = \text{cpm with 3 nM CCL1 alone and max} = \text{cpm in buffer alone}.$$

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

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