British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2012.02076.x
www.brjpharmacol.org

RESEARCH PAPER

Molecular requirements for inhibition of the chemokine receptor CCR8 – probe-dependent allosteric interactions

PC Rummel[1], KN Arfelt[1], L Baumann[2], TJ Jenkins[3], S Thiele[1], HR Lüttichau[1], A Johnsen[4], J Pease[5], S Ghosh[3], R Kolbeck[3] and MM Rosenkilde[1]

[1]*Department of Neuroscience and Pharmacology, Faculty of Health Sciences, Copenhagen University, Copenhagen, Denmark,* [2]*Institute of Biochemistry, Faculty of Biosciences, Pharmacy and Psychology, Universität Leipzig, Leipzig, Germany,* [3]*Millennium Pharmaceuticals, Cambridge, MA, USA,* [4]*Department of Clinical Chemistry, Rigshospitalet, Copenhagen, Denmark, and* [5]*Leukocyte Biology Section, National Heart and Lung Institute Division, Faculty of Medicine, Sir Alexander Fleming Building, Imperial College, London, UK*

Correspondence
Mette M. Rosenkilde, Blegdamsvej 3, Building 18.6, Copenhagen DK-2200, Denmark.
E-mail: rosenkilde@sund.ku.dk.

Present address: Tempero Pharmaceuticals Inc., Cambridge, Massachusetts (SG), MedImmune Gaithersburg, Maryland (RK) and BiogenIdec Cambridge, Massachusetts (TJJ).

Keywords
chemokine receptor; small-molecule antagonist; allosteric interaction; receptor conformation

Received
23 December 2011
Revised
30 March 2012
Accepted
7 May 2012

BACKGROUND AND PURPOSE

Here we present a novel series of CCR8 antagonists based on a naphthalene-sulfonamide structure. This structure differs from the predominant pharmacophore for most small-molecule CC-chemokine receptor antagonists, which in fact activate CCR8, suggesting that CCR8 inhibition requires alternative structural probes.

EXPERIMENTAL APPROACH

The compounds were tested as inverse agonists and as antagonists against CCL1-induced activity in $G\alpha_i$ signalling and chemotaxis. Furthermore, they were assessed by heterologous competition binding against two radiolabelled receptor ligands: the endogenous agonist CCL1 and the virus-encoded antagonist MC148.

KEY RESULTS

All compounds were highly potent inverse agonists with EC_{50} values from 1.7 to 23 nM. Their potencies as antagonists were more widely spread (EC_{50} values from 5.9 to 1572 nM). Some compounds were balanced antagonists/inverse agonists whereas others were predominantly inverse agonists with >100-fold lower potency as antagonists. A correspondingly broad range of affinities, which followed the antagonist potencies, was disclosed by competition with [^{125}I]-CCL1 (K_i 3.4–842 nM), whereas the affinities measured against [^{125}I]-MC148 were less widely spread (K_i 0.37–27 nM), and matched the inverse agonist potencies.

CONCLUSION AND IMPLICATIONS

Despite highly potent and direct effects as inverse agonists, competition-binding experiments against radiolabelled agonist and tests for antagonism revealed a probe-dependent allosteric effect of these compounds. Thus, minor chemical changes affected the ability to modify chemokine binding and action, and divided the compounds into two groups: predominantly inverse agonists and balanced antagonists/inverse agonists. These studies have important implications for the design of new inverse agonists with or without antagonist properties.

Abbreviations

7TM, 7 transmembrane receptors; LH, luteinizing hormone; LMD-A, N-(5-[N-((3R,4R)-1-((S)-2-aminopropanoyl)-3-methyl-piperidine-4-yl)sulfamoyl)naphthalene-1-yl]-2-methylbenzamide; LMD-B, N-(4-[N-((3R,4R)-1-((S)-2-aminopropanoyl)-3-methylpiperidine-4-yl)sulfamoyl)naphthalene-1-yl]-2-methylbenzamide; LMD-C, N-(5-[N-((3R,4R)-1-butyryl-3-methylpiperidine-4-yl)sulfamoyl)naphthalene-1-yl]-2-methyl-benzamide; LMD-D,

2-methyl-N-(4-(N-(1-(4-methylpiperazine-1-carbonyl)piperidine-4-yl)sulfamoyl)-naphthalene-1-yl)-2-methylbenzamide; LMD-E, 2-methyl-N-(4-[N-(1-(pyrimidine-2-yl)piperidine-4-yl)-naphthalene-1-yl]benzamide; LMD-F, ethyl 4-[4-(2-methylbenzamide)-naphthalene-1-sulfonylamino]-piperidine-1-carboxylate; LMD-G, ethyl 4-[5-(thiophene-2-carboxamido)-naphthalene-1-sulfonamido]-piperidine-1-carboxylate; LMD-H, ethyl 4-(5-benzamidonaphthalene-1-sulfonamino)piperidine-1-carboxylate.NS, naphthalene-sulfonamide; TSH, thyrotropin

Introduction

With approximately 50 members, the chemokines constitute a special class of cytokines possessing chemoattractant properties. This enables them to induce migration of their target cells towards the site of their secretion as well as coordinating the homeostatic circulation of leukocytes. Chemokines exert their effect through chemokine receptors (approximately 20 members), which belong to the family of 7 transmembrane (7TM) GPCRs. As a consequence of its essential role in a number of biological processes, mostly related to the immune regulation, the chemokine system is also important in the pathogenesis of many human diseases (Viola and Luster, 2008). These considerations have increased interest in the development of potent, selective and clinically useful chemokine receptor antagonists.

However, the chemokine system is characterized by a large degree of promiscuity as many chemokines bind several receptors, and in this way result in a wide range of effects. Furthermore, several receptors bind many different chemokines, thereby adding a further degree of complexity to the system. Thus, a small-molecule antagonist (or allosteric modulator) that blocks (or modulates) the action of a certain chemokine might be ineffective towards the action of other chemokines on the same receptor. This was recently shown in CCR1, where a series of small-molecule compounds acted as positive allosteric modulators of CCL3 binding, whereas they competed with CCL5 binding (Jensen *et al.*, 2008; receptor and ligand nomenclature follows Alexander *et al.*, 2011). However, CCR8 is monogamous in its binding of CCL1 and this receptor is expressed by a variety of cells, but is mainly associated with T_H2 cells. As T_H2 recruitment is implicated in a number of autoimmune diseases (Miesher and Vogel, 2002; Pease, 2010), CCR8 and CCL1 have been tested for, and shown to be involved in these diseases (Panina-Bodignon *et al.*, 2001; Chung *et al.*, 2003; Gombert *et al.*, 2005; Soler *et al.*, 2006). Altogether, these studies have increased interest in CCR8 as a drug target.

Many recent attempts to discover CCR8-selective small-molecule antagonists however have resulted in the discovery of small-molecule agonists for CCR8, which surprisingly share an overall similar structure with other CC-chemokine receptor antagonists (Haskell *et al.*, 2006; Jensen *et al.*, 2007). Thus, these compounds also contain a centrally located positively charged nitrogen atom, which interacts with the chemokine receptor-conserved Glu in position VII:06/7.39 (nomenclature according to the Baldwin/Schwartz system (VII:06) (Baldwin, 1993; Schwartz, 1994) and according to the Ballesteros–Weinstein nomenclature (7.39) (Ballesteros and Weinstein, 1995)), and flanking aromatic moieties, which bind in the main binding pocket of CCR8 (Jensen *et al.*, 2007). Therefore, these molecules resemble the binding mode of structurally highly similar antagonists in CCR1, CCR2, CCR3 and CCR5 (Forbes *et al.*, 2000; Mirzadegan *et al.*, 2000; Berkhout *et al.*, 2003; Castonguay *et al.*, 2003; de Mendonca *et al.*, 2005; Nishikawa *et al.*, 2005; Maeda *et al.*, 2006; Vaidehi *et al.*, 2006).

Today, however, structurally different small-molecule antagonists have been described for CCR8, including oxazolidinone structures from Glaxo Smith Kline (Jin *et al.*, 2007) and naphthalene-sulfonamides (NS) from Millennium Pharmaceuticals (Jenkins *et al.*, 2007). Besides these, the viral chemokine MC148 shares the same phenotype. This poxvirus-encoded CC-chemokine was identified in 1997 and encompasses a shorter N-terminus than human chemokines, leading to the prediction that it would act as an antagonist (Krathwohl *et al.*, 1997). Indeed, a screening of all human chemokine receptors identified MC148 as a CCR8 antagonist (Luttichau *et al.*, 2000).

Here, we present and characterize eight new small-molecule structures based on the NS core (LMD-A, -B, -C, -D, -E, -F, -G and -H) (Figure 1). As opposed to other CC-chemokine receptor antagonists, as well as previously described CCR8 agonists, these compounds do not contain the centrally located, positively charged, nitrogen atom. They all acted as highly potent inverse agonists for CCR8 with rather similar potencies, whereas their potencies as antagonists of CCL1-induced activation were far more diverse, in some cases >100-fold lower than the corresponding inverse agonist potency. A similar broad – but corresponding – range of affinities was observed in the competition binding against agonist ($[^{125}I]$-CCL1), whereas the affinities measured against a radiolabelled chemokine antagonist ($[^{125}I]$-MC148) were less widely spread. Thus, the binding studies revealed that the small-molecule ligands were able to displace CCL1 with affinities – that in some cases were rather low – similar to their antagonist potencies, and that affinities determined against $[^{125}I]$-MC148 – that were high and in the nanomolar range in all cases – were similar to their inverse agonist potencies. Consequently, these small-molecule antagonists variably modified CCL1 binding through probe-dependent allosteric effects, where structural traits of the compounds made it possible to compare how different chemical groups placed on the 'right side' or the 'left side' of the NS core influenced the antagonist (allosteric) properties relative to the inverse agonist (direct) counterparts.

Methods

Transfections and tissue culture

COS-7 cells (LGC/ATCC, Teddington, Middlesex, UK) were grown at 10% CO2 and 37°C in Dulbecco's modified Eagle's medium with glutamax (Gibco, Cat. No. 21885-025) adjusted

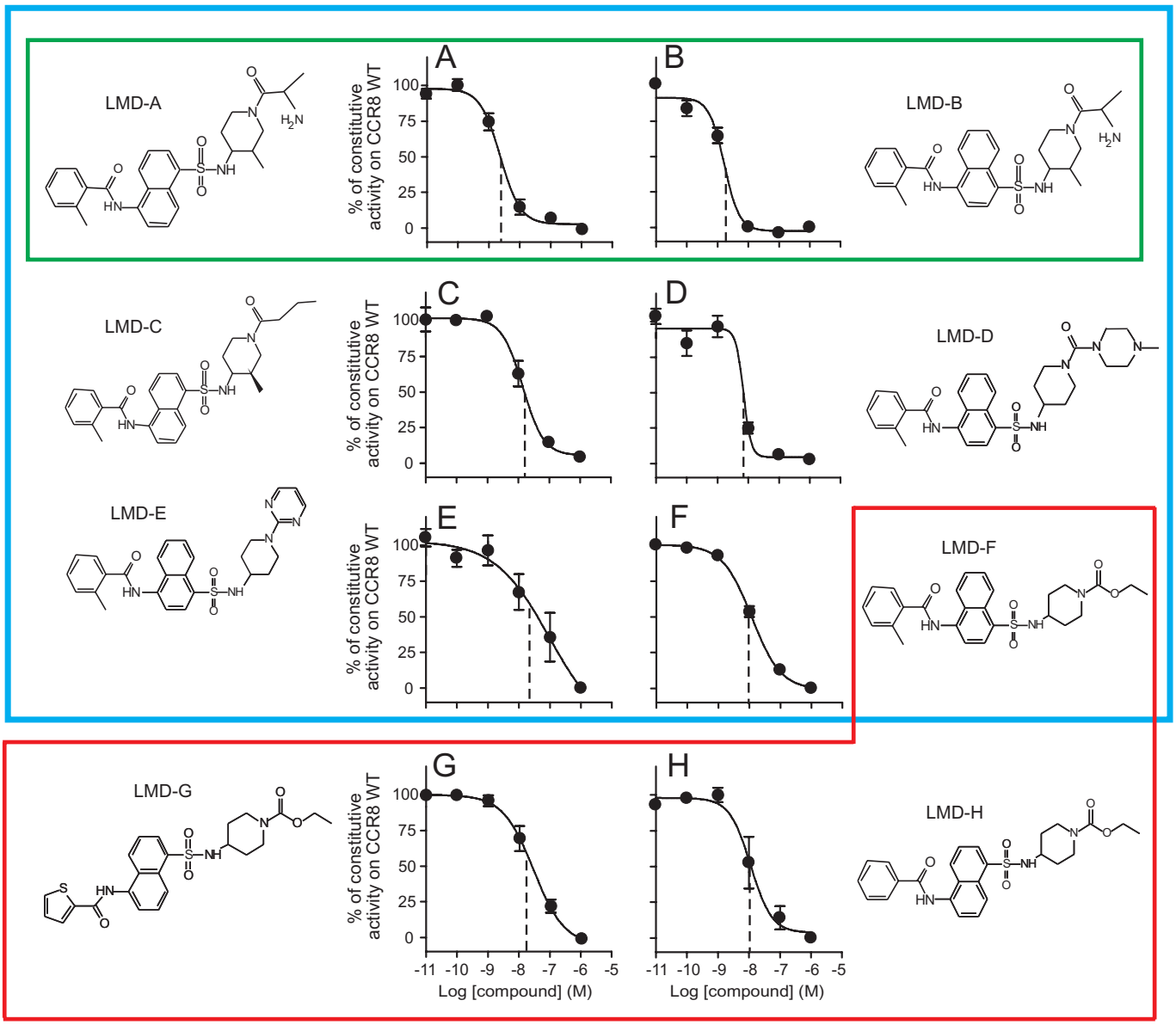

Figure 1

Compunds LMD-A through -H are highly potent inverse agonists. Structures of the compounds are shown together with results of inverse agonism on WT CCR8. All curves were normalized to the constitutive activity of CCR8 (100%) and untransfected cells (0%). Coloured boxes indicate similarities and differences in chemical structure among the small-molecule ligands. Molecules in the green box (LMD-A and –B) have similar chemical structure, but differ in the placement on the two benzyl rings on the naphthalene stucture. Molecules in the blue box indicate structures containing similar 'left side' moieties. In the red box are compounds with similar 'right side' moieties. (A–H) The IP accumulation experiments were performed in transiently transfected COS-7 cells (n = 3–4).

with 10% fetal bovine serum, 180 U·mL^{-1} penicillin and 45 ug·mL^{-1} streptomycin (PenStrep, Copenhagen University, Denmark). 4DE4 cells stably expressing CCR8 (see Tiffany *et al.*, 1997 fpr details of preparation) were grown in suspension at 5% CO_2, 37°C in HEPES-modified RPMI 1640 medium containing 10% fetal bovine serum, 100 U·mL^{-1} penicillin, 0.1 mg·mL^{-1} streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 1 × minimal essential medium non-essential amino acids and 1 mg·mL^{-1} Geneticin. Transfection of COS-7 cells was performed by the calcium phosphate precipitation method. For 75 cm^2 flasks, 10 μg receptor DNA and 15 μg GΔ6αqi4myr were mixed with 30 μL 2 mM $CaCl_2$ and TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH7.4) to a total volume of 240 μL. About 240 μL 2xHBS was added and the mixture was left for precipitation for 45 min before added to the cells, with chloroquine 2 mg·mL^{-1}). The flask was incubated for 5 h at 37° before replacing the media with 10 mL fresh COS-7 media.

MC148 purification

COS-7 cells were transiently transfected as described above. Serum-free medium was collected 24, 48 and 56 h after transfection. The medium was adjusted to pH 4.5 and centrifuged at 1500 *g* for 20 min at room temperature. The medium was filtered through a 0.22 μm Nalgene filter, a 1:1 volume of sterilized water added and then loaded on cation SP Sepharose fast flow columns equilibrated with 50 mM acetate buffer pH 4.5. MC148 was eluted by 50 mM acetate buffer pH 4.5 containing 2 M NaCl. The eluate was made 0.2% in trifluoroacetic acid (TFA), filtered and loaded on a Vydac C-8 column for reverse-phase HPLC. MC148 was eluted by 0.1% TFA in water using a gradient of CH_3CN. The position and amount of MC148 were identified by mass spectrometry and N-terminal sequencing on an ABI 494 protein sequencer.

Inositol phosphate assay (IP turnover)

COS-7 cells were transfected as described above. The co-transfection with GαΔ6qi4myr turns the G_i signal into a G_q coupled signal, making it possible to measure the activation of PLC as IP turnover. One day after transfection, the cells were seeded in 24 well plates (1.5×10^5 cells per well) and incubated with 2 μCi of ^{3}H-*myo*-inositol in 0.3 mL growth medium for 24 h. Cells were washed twice with HBSS supplemented with $CaCl_2$ and $MgCl_2$ (Gibco14025) and afterwards incubated for 15 min in 0.3 mL buffer supplemented with 10 mM LiCl prior to ligand addition followed by 90 min incubation. For antagonist testing, the proposed antagonists were added 10 min before the agonist. Cells were extracted by addition of 1 mL 10 mM formic acid followed by incubation on ice for 30 min. The [^{3}H]-IP generated was purified on AG 1X8 anion-exchange resin. All determinations were made in duplicate.

Chemotaxis assay

Cell migration was assayed using 96-well ChemoTx® plates with 5 μm pores (Neuroprobe, Gaithersburg, MD, USA). Various concentrations of chemokine were applied in a final volume of 31 μL to the lower wells of a chemotaxis chamber. Following incubation with different concentrations of antagonist, 2×10^5 cells were applied to the filter, and the plates were incubated for 5 h in a humidified chamber at 37°C in the presence of 5% CO2. Migrating cells were detected by use of CellTiterGlo® dye (Promega, Southampton, UK) and resulting luminescence measured using a TopCount scintillation counter (PerkinElmer, Waltham, MA, USA). Determinations were made in duplicates.

Binding

COS-7 cells were transferred to culture plates 1 day after transfection. The number of cells seeded per well was determined by the apparent expression efficiency of the receptors and aimed at obtaining 5–10% specific binding of the added radioactive ligand (1×10^4–3×10^5 cells per well for the different CCR8 compounds). Two days after transfection, the cells were assayed by competition binding for 3 h at 4°C using 10–15 pM ^{125}I-CCL1 plus unlabelled CCL1 in 0.2 mL 50 mM HEPES buffer, pH 7.4, supplemented with 1 mM $CaCl_2$, 5 mM $MgCl_2$ and 0.5% (w/v) bovine serum albumin. After incubation, the cells were washed quickly twice in 4°C binding buffer supplemented with 0.5 M NaCl. Non-specific binding was determined as the binding in the presence of 0.1 μM unlabelled CCL1. Determinations were made in duplicate. IC_{50} values were determined and Kd and Ki were calculated according to the equations of Cheng and Prusoff (1973):

$$K_d = IC_{50}\text{-}[L] \text{ and } K_i = IC_{50}/(1+([L]/K_d)), \text{ where } [L] \text{ is the concentration of radioligand.}$$

Data analysis

All curves as well as calculations of EC_{50} and IC_{50} values were performed with Prism 4. Where the concentration-response graphs did not reach a plateau (complete displacement or inhibition), the values were estimated based on a Hill coefficient of –1 and the bottom values of similar curves. These estimated values are marked with '#' in Table 1.

Materials

Human CCL1 was purchased from Peprotech (Rocky Hill, NJ, USA) and the viral receptor ligand MC148 was prepared as described above. [^{125}I]-CCL1 (100T Bq mmol^{-1}) was supplied by Perkin Elmer, MA, USA. and [^{125}I]-MC148 (~ 100TBq mmol^{-1}) was prepared in house. The human CCR8 WT chemokine cDNA was kindly provided by Tim Wells (Serono Pharmaceutical Research Institute, Geneva, Switzerland). ^{3}H-*myo*-Inositol (PT6-271) was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). The promiscuous chimeric G-protein GαΔ6qi4myr (abbreviated Gqi4myr) was kindly provided by Evi Kostenis (University of Bonn, Germany). AG 1-X8 anion-exchange resin was purchased from Bio-Rad Laboratories (Hercules, CA, USA).

Results

The structures of the eight novel NS compounds are shown in Figure 1 where a colour code is used to clarify chemical differences. Highlighted in blue (Figure 1A–F) are compounds containing similar 'left sides' (consisting of a 2-methylbenzamide group) but different 'right sides' – either concerning chemical structure or placement on the naphthalene structure. Thus, LMD-A and -B are similar in chemical structure for both subgroups, but are placed on opposite benzene rings on the naphthalene – as highlighted in green (Figure 1A and B). Highlighted in red are compounds with similar 'right side' structure but different 'left side' structure (Figure 1F–H).

A screening on all endogenous chemokine receptors (CCR1-10, CXCR1-6, XCR1 and CX3CR1) for antagonist properties of these compounds revealed that they were specific for CCR8 (data not shown). The screening was performed by co-transfection with a promiscuous G-protein (Gqi4myr); a chimeric Gα-subunit recognized as a $G\alpha_i$ subunit by the receptor, but resulting in a $G\alpha_q$ response, upon activation (Kostenis, 2001). Thus, chemokine receptor activation was measured by IP accumulation, a signalling readout

Table 1

Results of IP3 accumulation and competition binding for the small molecule compounds (LMD-A to –H) and for the viral protein MC148

	IP3 accumulation Ligand Potency (inverse agonist)			Ligand + CCL1 (10 nM) Potency (antagonist)				Competition binding ^{125}I-MC148 Affinity			^{125}I-CCL1 Affinity		
	IC_{50} (log)	EC_{50} (nM)	(*n*)	IC_{50} (log)	EC_{50} (nM)	(*n*)	Fold difference	IC_{50} (log)	Ki (nM)	(*n*)	IC_{50} (log)	Ki (nM)	(*n*)
LMD-A	–8.60± 0.12	2.5	(4)	–7.93± 0.16	12	(4)	4.6	–9.39± 0.38	0.37	(3)	–8.46± 0.05	3.4	(3)
LMD-B	–8.76± 0.06	1.7	(3)	–8.23± 0.18	5.9	(3)	3.4	–8.56± 0.09	2.6	(3)	–8.09± 0.22	8.0	(3)
LMD-C	–7.63± 0.12	23	(3)	#–6.34± 0.13	455	(3)	19	–9.01± 0.63	0.86	(3)	–7.96± 0.09	11	(3)
LMD-D	–8.33± 0.06	4.7	(4)	–7.08± 0.22	83	(4)	18	–8.75± 0.07	1.6	(3)	–8.08± 0.19	8.1	(3)
LMD-E	–7.67± 0.37	22	(4)	#–6.03± 0.12	937	(3)	43	–7.52± 0.61	27	(3)	#–6.07± 0.49	842	(3)
LMD-F	–8.08± 0.16	8.2	(4)	#–6.03± 0.14	925	(4)	112	–8.30± 0.19	4.6	(3)	–6.91± 0.31	121	(3)
LMD-G	–7.86± 0.30	14	(4)	#–5.80± 0.50	1572	(3)	115	–7.66± 0.12	20	(3)	#–6.42± 0.07	371	(3)
LMD-H	–7.91± 0.28	12	(3)	#–6.26± 0.08	546	(3)	45	–8.00± 0.10	9.1	(3)	–7.27± 0.15	52	(3)
MC148	–9.92± 0.62	0.12	(5)	–8.39± 0.33	4.1	(7)	34	–9.86± 0.38	*0.13	(3)	ND		

Results of IP_3 accumulation experiments display the potency of inverse agonism and antagonism against CCL1 (10 nM) of LMD-A, -B, -C, -D, -E, -F, -G, -H and MC148 on WT CCR8 transiently transfected in COS-7 cells. The table shows the potency (as log EC_{50} and EC_{50}) and the number of experiments given as '*n*'. Ratios of EC_{50} values from assays for inverse agonism and antagonism are shown as 'Fold difference'. Binding results are given as log IC_{50} and K_i determined by competition binding against [^{125}I]-MC148 and [^{125}I]-CCL1. K_i and K_d were calculated as described. *, K_d values for these two ligands were 0.68 and 0.13 nM for CCL1 and MC148; #, refers to estimated IC_{50} values in experiments where the displacement of agonist (competition binding) or inhibition of CCL1 action (IP_3 accumulation) were incomplete.
ND, not determined.

that was previously shown to correlate with chemotaxis and calcium release following activation of CCR8 (Jensen *et al.*, 2007).

The NS ligands are highly potent inverse agonists for CCR8

As CCR8 displays constitutive activity of approximately 12% (Jensen *et al.*, 2007), the ligands were tested for inverse agonist properties using IP_3 accumulation as readout. Thus, COS-7 cells were transiently transfected with human CCR8 together with Gqi4myr. Indeed, all compounds were high potency inverse agonists for CCR8 with rather similar potencies between 1.7 and 23 nM (Figure 1 and Table 1). The highest potencies were found for LMD-A and -B (2.5 and 1.7 nM respectively), which both contain a positively charged amino group in the exterior of the 'right side' group. In fact, the only structural differences between these two ligands reside on the subgroup placements on the naphthalene, indicating that this specific feature does not influence the potency of the ligands and that the positive charge contributes to the high potency interaction with the receptor.

Large differences in potencies as antagonists despite similar potencies as inverse agonists

All eight compounds were tested as antagonists against 10 nM CCL1-induced activation of CCR8. This concentration of CCL1 activates CCR8 to approximately 80% of full activation. In contrast to the rather similar potencies as inverse agonists (Figure 1), the compounds were found to inhibit CCL1-induced activation with potencies between 5.9 and 1572 nM (Figure 2 and Table 1). In general, the compounds were less potent as antagonists compared with the potencies observed as inverse agonists, as illustrated by dotted lines in Figure 2. However, again, we found the highest potencies of LMD-A and -B (Figure 2A and B) with EC_{50} values of 12 and 5.9 nM, respectively, which were comparable to the inverse agonist potencies, with only 4.6- and 3.4-fold difference respectively (Table 1). LMD-C, -D and -E, which share similar 'left side' structure with these two ligands, displayed between 18- and 43-fold lower potencies as antagonists, compared with the inverse agonist potencies (Figure 2C–E). Noticeable, the lowest antagonist potencies were observed for the four compounds LMD-E, F, -G and –H, of which the latter three share the same right side structure (piperidine-1-carboxylate). Thus, these four compounds inhibited CCL1-induced activation with EC_{50} values between 546 and 1572 nM. Compared with their high potencies as inverse agonists, the potencies as antagonists were thus 43- to 115-fold lower (Figure 2F–H). Thus, the chemical structure of the compounds was more critical for the inhibition of CCL1-induced activation, than for inhibition of basal CCR8 activity.

In order to determine the effect of the ligands in a more natural signalling pathway, all ligands were tested for their ability to antagonize the effect of CCL1- induced chemotaxis in 4DE4 cells stably expressing CCR8.

As shown in Figure 3A, CCL1 induced cell migration in these cells in a classical bell-shaped manner. Furthermore, all compounds antagonized cell migration induced by 3 nM CCL1 (Figure 3B–D). Although the compounds displayed higher potencies in these experiments, compared with those

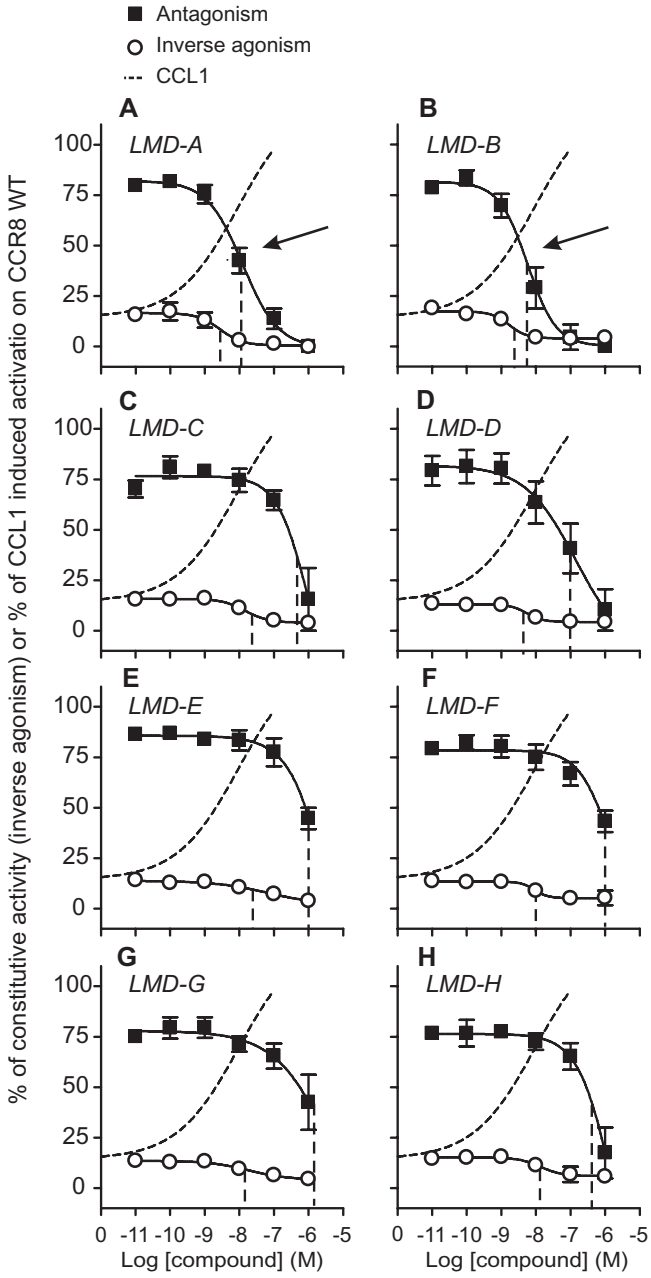

Figure 2

Balanced antagonists/inverse agonists versus predominant inverse agonists. Inverse agonist and antagonist properties of the compounds (LMD-A to -H) measured by IP accumulation experiments in transiently transfected COS-7 cells. CCL1 dose–response curve is shown in dotted lines. (A) LMD-A, (B) LMD-B, (C) LMD-C, (D) LMD-D, (E) LMD-E, (F) LMD-F, (G) LMD-G and (H) LMD-H. The EC_{50} values are indicated by dotted lines. (n = 3–4).

from the IP_3 accumulation experiments (Figure 2), we found them to be structure-dependent in a similar manner. Thus, LMD-A and -B again displayed the highest potencies with EC_{50} values below 1 nM (0.58 and 0.69 nM respectively), whereas LMD-E, -F, -G and -H displayed the lowest with EC_{50} values between 43 and 240 nM.

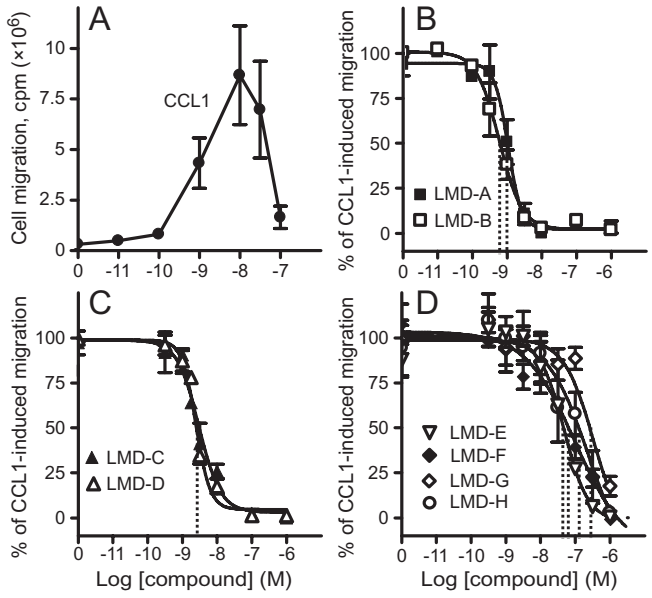

Figure 3

Inhibition of CCL1-induced chemotaxis by small-molecule antagonists. (A) CCL1-induced chemotaxis in a classical bell-shaped manner. Migrating cells were measured as luminescence by use of CellTiterGlo® dye (data shown as CPM). (B–D) Antagonism of 3 nM CCL1-induced migration. (B) LMD-A and -B. (C) LMD-C and -D. (D) LMD-E, -F, -G and -H. The curves were normalized to the CCL1-induced migration in the absence of antagonist and buffer. The EC_{50} values are indicated by dotted vertical lines. (n = 3–4).

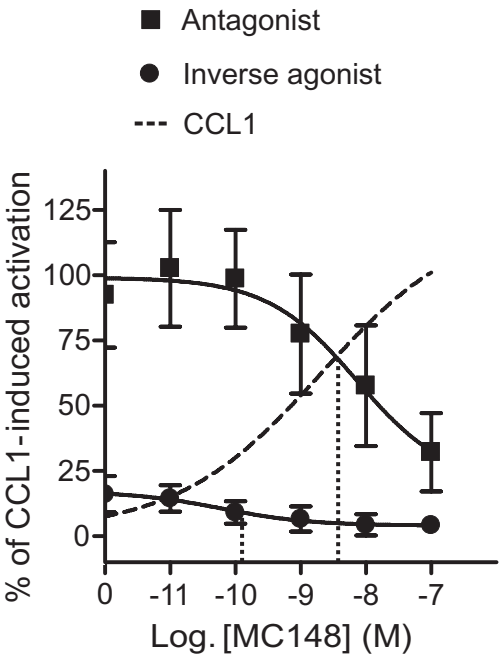

Figure 4

MC148 also displays predominant inverse agonism with 34-fold higher potency as an inverse agonist compared with its potency as an antagonist. Inverse agonism and antagonism against CCL1 (10 nM) activation on WT CCR8 The concentration-response curve for CCL1 is shown with a dotted line. The EC_{50} values for MC148 are indicated by dotted vertical lines. The experiments were performed in transiently transfected COS-7 cells. (n = 5–7).

The LMD compounds displace MC148 with affinities similar to their inverse agonist potencies

To investigate whether the difference in inverse agonist potencies, compared with antagonist potencies observed for the majority of the small-molecule ligands was also applicable to large protein ligands, MC148 was tested using IP_3 assays, as described above. Here, the potency as inverse agonist and antagonist was found to be 0.12 and 4.1 nM, respectively, resulting in a potency difference of 34-fold (Figure 4 and Table 1). In this way, MC148 resembles the majority of the small-molecule compounds (LMD-C to -H), which also showed higher potencies when acting directly on the ligand-free receptor instead of antagonizing CCL1. Furthermore, these data show that not only small molecules inhibited the basal activity.

The differences in potencies of the LMD compounds as inverse agonists and as antagonists inspired us to study the binding affinities. Therefore, we tested them in heterologous competition-binding experiments against radio-labelled chemokine agonist ([^{125}I]-CCL1) and chemokine antagonist ([^{125}I]-MC148) in transiently transfected COS-7 cells. The small-molecule ligands displaced [^{125}I]-MC148 with affinities (K_i) between 0.37 and 27 nM. Again, the highest affinities were found for LMD-A and -B with 0.37 and 2.6 nM, respectively, and as for the other compounds, the affinities correlated very well with their inverse agonist potencies (Figure 5 and Table 1).

The affinities measured against radiolabelled agonist (CCL1) correlate well with the antagonist potencies

Heterologous binding experiments were also performed using ^{125}I-CCL1 as radioligand. Compared with the affinities measured against ^{125}I-MC148, much lower affinities were identified in competition against ^{125}I-CCL1 with Ki values ranging between 3.4 and 842 nM. Thus, these results correlated with the antagonist potencies determined against 10 nM CCL1-induced activation. In general, however, the affinities were higher than the antagonist potencies. Especially, LMD-C had a markedly higher affinity as compared with its potency as antagonist (42-fold). All other compounds had higher resemblance between these values, ranging between 1.3- and 10-fold difference. Again, LMD-A and -B displayed the highest affinities with K_i values of 3.4 and 8.0 nM, respectively, as shown in Figure 6 and Table 1. The wide variation in binding affinities of the small molecules when measured against [^{125}I]-CCL1 and the correlating low antagonist potencies indicate a probe-dependent allosteric effect.

Finally, the affinity of MC148 was determined by homologous competition-binding experiments. Again, we found that the potency of MC148 as an inverse agonist correlated very well with the affinity, with almost identical K_d and EC_{50} values (0.13 and 0.12 nM respectively) (Figure 4 and Table 1). Furthermore, the potency of MC148 as an antagonist against 10 nM CCL1 (EC_{50} of 4.1 nM) was comparable to its affinity

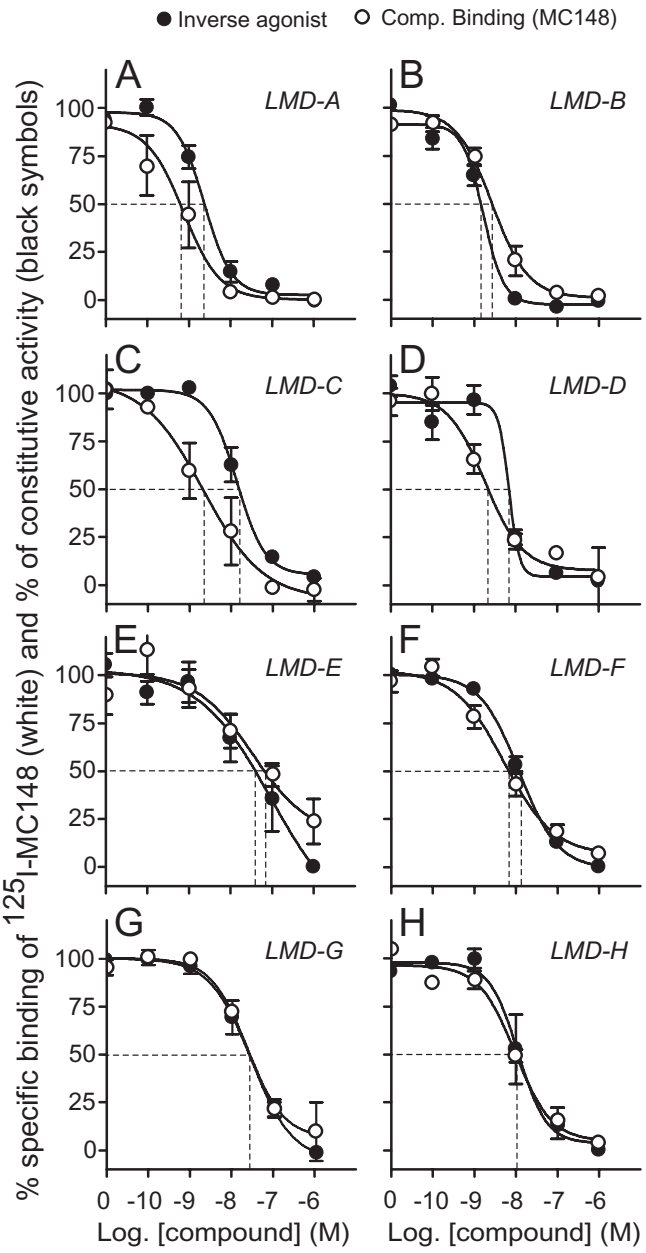

Figure 5

The small-molecule antagonists displace [^{125}I]-MC148 with affinities similar to their inverse agonist potencies. The heterologous competition binding (Comp.binding) against the inverse agonist/antagonist [^{125}I]-MC148 is shown together with the dose–response curves as inverse agonists of basal IP_3 accumulation for each ligand. Potencies and affinities are indicated by dotted vertical lines. All experiments were performed in transiently transfected COS-7 cells. (n = 3–4).

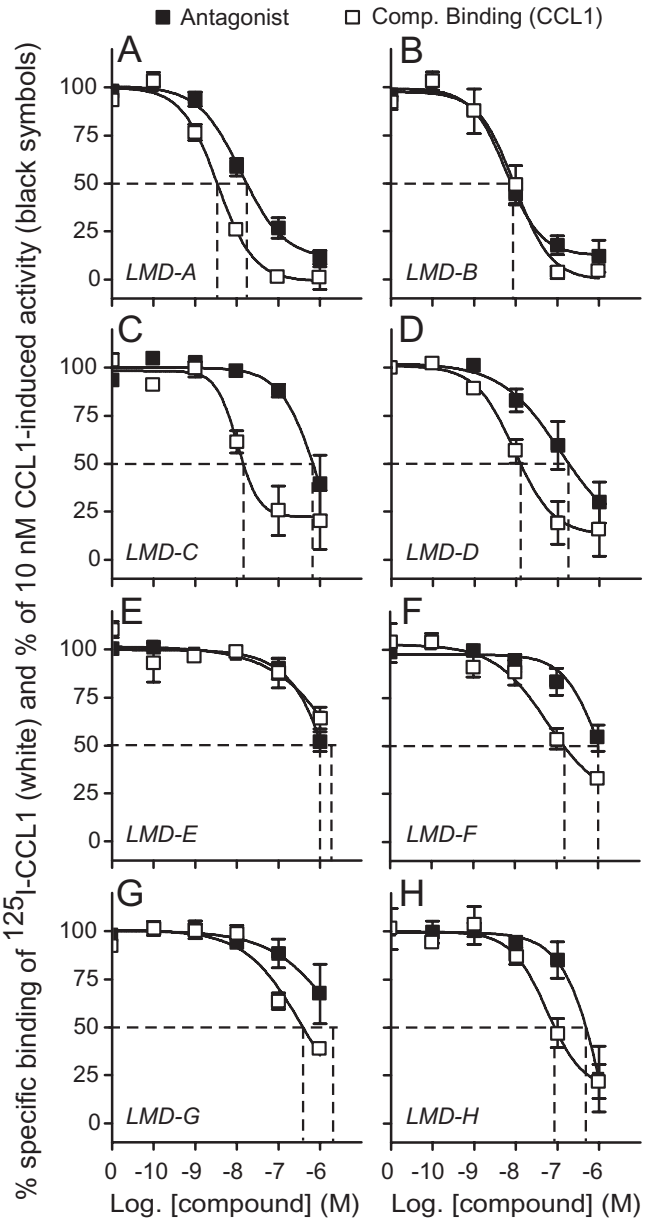

Figure 6

The small-molecule antagonists displace [^{125}I]-CCL1 with affinities similar to their antagonist potencies. The heterologous competition binding (Comp.binding) against the agonist [^{125}I]-CCL1 is shown together with the dose–response curves as antagonists of 10 nM CCL1-induced IP_3 accumulation for each ligand. Potencies and affinities are indicated by dotted vertical lines. All experiments were performed in transiently transfected COS-7 cells. (n = 3–4).

previously determined in heterologous binding competition against [^{125}I]-CCL1 (0.47 nM) (Luttichau *et al.*, 2000).

Discussion

Here we characterize the receptor interaction of a series of structurally related small-molecule ligands for CCR8. All compounds acted as highly potent inverse agonists whereas their action as antagonists was more diverse. Some compounds were 'balanced' antagonists/inverse agonists (LMD-A and -B), others 'predominant' inverse agonists with >100-fold lower potency as antagonists (LMD-C through -H). A correspondingly broad range of affinities was observed in competition with ([^{125}I]-CCL1 (agonist), whereas the affinities measured against ^{125}I-MC148 (antagonist) were closer and

matched the inverse agonist potencies. Thus, small chemical alterations in the compounds resulted in the loss of (allosteric) action as antagonist, but maintained (direct) action as inverse agonists.

General structural traits and common action of small-molecule CC-chemokine receptor ligands

The NS structure described here is uncommon for CC-chemokine receptor antagonists, as these usually are relatively elongated structures containing one or two centrally located positively charged nitrogens and flanking aromatic motifs (Rosenkilde and Schwartz, 2006). For several receptors, the positively charged nitrogen(s) anchor to the negatively charged (chemokine receptor-conserved) Glu residue in the top of TM-VII (in position VII:06/7.39) and that the aromatic moieties interact with aromatic residues confining the shallow part of the binding crevice (Forbes *et al.*, 2000; Mirzadegan *et al.*, 2000; Berkhout *et al.*, 2003; Castonguay *et al.*, 2003; de Mendonca *et al.*, 2005; Maeda *et al.*, 2006). Glu VII:06 is also found in CCR8 together with conserved aromatic residues in the main ligand-binding pocket. Importantly, however, small molecules displaying the common pharmacophore act as *agonists* in CCR8, as exemplified by LMD-009 (and related compounds), all of which activated CCR8 with nanomolar potencies by interacting with these conserved residues in the main binding crevice (Jensen *et al.*, 2007; 2012; Jensen and Rosenkilde, 2009). Thus, as these *agonists* contain the general CC-chemokine receptor *antagonist* pharmacophore, and interact with the same residues as other non-peptide *antagonists*, it is evident that CCR8 antagonism requires a different pharmacophore, as exemplified by the oxazolidinones (Jin *et al.*, 2007) and by the NS structure presented here (Figure 1) and previously (Jenkins *et al.*, 2007). The NS compounds do not contain the centrally located positive charge, confirming the completely different structural requirements for CCR8 inhibition. Interestingly, other antagonists containing the NS structure (although not as central as in those for CCR8) have been identified for CCR4 (Burdi *et al.*, 2007), the closest homologue to CCR8 (Lió and Vannucci, 2003).

Binding mode of chemokines in chemokine receptors – an orthosteric interaction

Chemokines share a similar tertiary structure with two conserved disulfide bonds. Thus, despite low sequence homology, NMR and X-ray crystallography have shown an overall structure consisting of a distorted N-terminus of approximately 6–10 residues, followed by a long loop (the N-loop), a 3_{10} helix, a three-stranded β-sheet and a C-terminal α-helix (Allen *et al.*, 2007). The interactions of chemokines with their cognate receptors are mainly driven by electrostatic interactions between the positively charged chemokines and the correspondingly negatively charged extracellular parts of the receptor (and of glycosaminoglycans) on the cell surface. The receptor N-terminus and most extracellular loops are essential for ligand recognition, as the initial interaction triggers a docking of the chemokine N-terminus into the binding pocket, thereby stabilizing an active receptor conformation (Samson *et al.*, 1997; Wu *et al.*, 1997; Rosenkilde *et al.*, 2000; Schwarz and Wells, 2002; Jensen *et al.*, 2012; Thiele *et al.*, 2011). The central role of the chemokine N-terminus has been confirmed, as modifications in this region alter the biological properties of the chemokine. For instance, CCL5 turns into a high-affinity antagonist for CCR1 and CCR5, upon retention of the N-terminal Met residue (Proudfoot *et al.*, 1996), and CCL1 displays 10-fold lower potency when the N-terminal is extended by a Ser residue (Fox *et al.*, 2006). Furthermore, N-terminal truncations of CCL3 and CCL7 result in broad-spectrum antagonists (Gong *et al.*, 1996; Struyf *et al.*, 1998). MC148 has the same tertiary structure as endogenous CC-chemokines (thus also CCL1) suggesting an overall similar binding mode, and that the antagonist properties are likely to be explained by the shorter N-terminus of MC148. Thus, in agreement with the overall chemokine/receptor binding mode (Schwarz and Wells, 2002; Allen *et al.*, 2007), we suggest that the opposite action of CCL1 and MC148 is caused by unique interactions in the main binding crevice exerted by the N-termini of these chemokines.

Small-molecule ligands for chemokine receptors act via an allosteric interaction

Given the predominant extracellular receptor recognition of chemokines, most small-molecule ligands act allosterically by targeting the main binding pocket independently of extracellular receptor domains, as shown in CCR5 and CXCR4 (Rosenkilde *et al.*, 2004; 2007; Watson *et al.*, 2005; Maeda *et al.*, 2006). However, some molecules also interact with extracellular loops (predominantly ECL2), as shown for the CCR5 antagonist aplaviroc and for a series of dual active CCR1-CCR8 compounds (Maeda *et al.*, 2006; Jensen *et al.*, 2012; Thiele *et al.*, 2011). All compounds described here interact well with CCR8 as determined by the high inverse agonist potencies (the direct interaction in the absence of other ligands) and the high affinities determined in competition with the antagonist MC148. However, at the same time, the majority showed highly impaired competition with CCL1 *binding* and CCL1 *action* suggesting a probe-dependent allosteric interaction, where minor chemical changes influenced the degree of antagonism, and divided the compounds into the so-called 'balanced antagonists/inverse agonists' (LMD-A and -B) and the 'predominant inverse agonists' (LMD-C-H).

Conformational constraining may contribute to the allosteric appearance

Being membrane-expressed proteins, 7TM receptors interchange between different conformations – from complete inactivity to fully active receptors. Spontaneous equilibrium towards active states results in constitutive activity, whereas agonists shift the equilibrium towards activated receptors in contrast to inverse agonists that do the opposite (Schwartz and Rosenkilde, 1996). In some cases, the conformational interchange may be impaired, resulting in apparently low affinities as shown in the tachykinin NK_1 receptor system, where a series of mutations in the edge of TM-II facing into the main binding crevice prevented competition between the endogenous agonist (substance P) and radiolabelled non-peptide antagonists. Importantly, homologous binding with ([^{125}I]-substance P uncovered high affinity of this ligand,

indicating that the mutations blocked the conformational interchange (Rosenkilde *et al.*, 1994). This phenomenon has also been observed in the chemokine system, for instance by the endogenous chemokines targeting CCR1 and CCR5 or the herpes virus-encoded ORF74 and US28 (Simmons *et al.*, 1997; Kledal *et al.*, 1998; Rosenkilde and Schwartz, 2000). Thus, conformational constraining may contribute to the lack of competition of agonist (CCL1) *binding* and *action* in our system, although the overall structural similarities within the group of small-molecule ligands suggest similar conformational constraining and thus point towards differential allosteric interactions as a reason for the observed binding phenomena. A final level of complexity can be added to this system by the discoveries that small molecules may act differently in receptors with more than one endogenous ligand – which is the case for promiscuous chemokine receptors like CCR1. Thus, a series of small-molecule agonists for CCR1 acted as allosteric enhancers of CCL3 binding, but at the same time – and with the same high affinities – competed with CCL5 binding due to an identified partial overlap in the binding sites of CCL5 and of the small-molecule agonists centred around the conserved GluVII:06 (Jensen *et al.*, 2008). Partially overlapping binding sites of chemokines and small-molecule ligands could contribute to the phenomena observed here in CCR8, especially in the case of MC148.

The agonist-prone nature of CCR8 – a challenge in terms of medicinal chemistry

In contrast to the majority of endogenous chemokine receptors, CCR8 appears to be agonist prone. Not only does CCR8 display constitutive activity of 12%, its basal activity is also further enhanced by several mutations in the main binding crevice (Jensen *et al.*, 2007). Furthermore, high-throughput screening for small-molecule *antagonists* using the general CC-chemokine receptor antagonist pharmacophore resulted in the discovery of *agonists* for CCR8 (Jensen *et al.*, 2007). Constitutive activity was described for the first time in 1982 (Koski *et al.*, 1982) and today, it is generally accepted that receptors shift between different conformations and are seldom completely silent. Naturally occurring mutations that result in constitutive activity are well known in human disease, for example, the thyrotropin (TSH) receptor involved in thyroid adenomas (Seifert and Wenzel-Seifert, 2002). Furthermore, the constitutive activity of virus-encoded receptors has been associated with oncogenesis (Arvanitakis *et al.*, 1997; Rosenkilde *et al.*, 1999; 2001).

The availability of compounds which are selective inverse agonists and/or concomitant antagonists has obvious advantages as inverse agonists act independently of the endogenous agonist, whereas antagonists rely on the presence of agonists. We present here probe-dependent allosteric interactions, where minor chemical changes had major effects on the antagonist properties, without major alterations in the inverse agonist properties – an observation with important consequences for drug development. Another important lesson is that the screening method determines the outcome of the screen. Thus, screening for antagonism may result in false negative compounds with low ability to compete for, or inhibit the agonists, but which may still interact with high affinity with the receptors. Likewise, screening libraries on constitutive active receptors may result in false positive hits – compounds with inverse agonist properties, but with poor or no antagonism of activation by agonists. Finally, it could be advantageous to selectively target constitutive activity, and not antagonism, or the opposite, and thereby fine-tune the pharmacological properties of new compounds.

Acknowledgement

The studies were supported by The Danish Council for Independent Research | Medical Sciences, the NovoNordisk Foundation, the Lundbeck Foundation, the European Community's Sixth Framework program (INNOCHEM: LSHB-CT-2005-518167), the AP-Moller Foundation, and the Aase and Einar Danielsen Foundation.

Conflicts of interest

None.

References

Alexander SPH, Mathie A, Peters JA (2011). Guide to receptors and channels (GRAC), 5th edn. Br J Pharmacol 164 (Suppl. 1): S1–S324.

Allen SJ, Crown SE, Handel TM (2007). Chemokine: receptor structure, interactions, and antagonism. Annu Rev Immunol 25: 787–820.

Arvanitakis L, Geras-Raaka E, Varma A, Gershengorn MC, Cesarman E (1997). Human herpesvirus KSHV encodes a constitutively active G-protein-coupled receptor linked to cell proliferation. Nature 385: 347–350.

Baldwin JM (1993). The probable arrangement of the helices in G protein-coupled receptors. EMBO J 12: 1693–1703.

Ballesteros JA, Weinstein H (1995). Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors. In: Sealfon SC (ed.). *Receptor Molecular Biology*. Academic Press: New York, pp. 366–428.

Berkhout TA, Blaney FE, Bridges AM, Cooper DG, Forbes IT, Gribble AD *et al.* (2003). CCR2: characterization of the antagonist binding site from a combined receptor modeling/mutagenesis approach. J Med Chem 46: 4070–4086.

Burdi DF, Chi S, Mattia K, Harrington C, Shi Z, Chen S *et al.* (2007). Small molecule antagonists of the CC chemokine receptor 4 (CCR4). Bioorg Med Chem Lett 17: 3141–3145.

Castonguay LA, Weng Y, Adolfsen W, Di Salvo J, Kilburn R, Caldwell CG *et al.* (2003). Binding of 2-aryl-4-(piperidin-1-yl)butanamines and 1,3,4-trisubstituted pyrrolidines to human CCR5: a molecular modeling-guided mutagenesis study of the binding pocket. Biochemistry 42: 1544–1550.

Cheng Y, Prusoff WH (1973). Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem Pharmacol 22: 3099–3108.

Chung CD, Kuo F, Kumer J, Motani AS, Lawrence CE, Henderson WR Jr *et al*. (2003). CCR8 is not essential for the development of inflammation in a mouse model of allergic airway disease. J Immunol 170: 581–587.

Forbes IT, Cooper DG, Dodds EK, Hickey DM, Ife RJ, Meeson M *et al*. (2000). CCR2B receptor antagonists: conversion of a weak HTS hit to a potent lead compound. Bioorg Med Chem Lett 10: 1803–1806.

Fox JM, Najarro P, Smith GL, Struyf S, Proost P, Pease JE (2006). Structure/function relationships of CCR8 agonists and antagonists. Amino-terminal extension of CCL1 by a single amino acid generates a partial agonist. J Biol Chem 281: 36652–36661.

Gombert M, eu-Nosjean MC, Winterberg F, Bunemann E, Kubitza RC, Da Cunha L *et al*. (2005). CCL1-CCR8 interactions: an axis mediating the recruitment of T cells and Langerhans-type dendritic cells to sites of atopic skin inflammation. J Immunol 174: 5082–5091.

Gong J-H, Uguccioni M, Dewald B, Baggiolini M, Clark-Lewis I (1996). RANTES and MCP-3 antagonists bind multiple chemokine receptors. J Biol Chem 271: 10521–10527.

Haskell CA, Horuk R, Liang M, Rosser M, Dunning L, Islam I *et al*. (2006). Identification and characterization of a potent, selective nonpeptide agonist of the CC chemokine receptor CCR8. Mol Pharmacol 69: 309–316.

Jenkins TJ, Guan B, Dai M, Li G, Lightburn TE, Huang S *et al*. (2007). Design, synthesis, and evaluation of naphthalene-sulfonamide antagonists of human CCR8. J Med Chem 50: 566–584.

Jensen PC, Rosenkilde M (2009). Chapter 8. Activation mechanisms of chemokine receptors. Methods Enzymol 461: 171–190.

Jensen PC, Nygaard R, Thiele S, Elder A, Zhu G, Kolbeck R *et al*. (2007). Molecular interaction of a potent nonpeptide agonist with the chemokine receptor CCR8. Mol Pharmacol 72: 327–340.

Jensen PC, Thiele S, Ulven T, Schwartz TW, Rosenkilde MM (2008). Positive versus negative modulation of different endogenous chemokines for CC-chemokine receptor 1 by small molecule agonists through allosteric versus orthosteric binding. J Biol Chem 283: 23121–23128.

Jensen PC, Thiele S, Steen A, Elder A, Kolbeck R, Ghosh S *et al*. (2012). Reversed binding of a small molecule ligand in homologous chemokine receptors. Br J Pharmacol 166: 258–275.

Jin J, Wang Y, Wang F, Kerns JK, Vinader VM, Hancock AP *et al*. (2007). Oxazolidinones as novel human CCR8 antagonists. Bioorg Med Chem Lett 17: 1722–1725.

Kledal TN, Rosenkilde MM, Schwartz TW (1998). Selective recognition of the membrane-bound CX3C chemokine, fractalkine, by the human cytomegalovirus-encoded broad-spectrum receptor US28. FEBS Lett 441: 209–214.

Koski G, Streaty RA, Klee WA (1982). Modulation of sodium-sensitive GTPase by partial opiate agonists. An explanation for the dual requirement for Na+ and GTP in inhibitory regulation of adenylate cyclase. J Biol Chem 257: 14035–14040.

Kostenis E (2001). Is Galpha16 the optimal tool for fishing ligands of orphan G-protein-coupled receptors? Trends Pharmacol Sci 22: 560–564.

Krathwohl MD, Hromas R, Brown DR, Broxmeyer HE, Fife KH (1997). Functional characterization of the C—C chemokine-like molecules encoded by *Molluscum contagiosum* virus types 1 and 2. Proc Natl Acad Sci USA 94: 9875–9880.

Lió P, Vannucci M (2003). Investigating the evolution and structure of chemokine receptors. Gene 317: 29–37.

Luttichau HR, Stine J, Boesen TP, Johnsen AH, Chantry D, Gerstoft J *et al*. (2000). A highly selective CC chemokine receptor (CCR)8 antagonist encoded by the poxvirus *Molluscum contagiosum*. J Exp Med 191: 171–180.

Maeda K, Das D, Ogata-Aoki H, Nakata H, Miyakawa T, Tojo Y *et al*. (2006). Structural and molecular interactions of CCR5 inhibitors with CCR5. J Biol Chem 281: 12688–12698.

de Mendonca FL, da Fonseca PC, Phillips RM, Saldanha JW, Williams TJ, Pease JE (2005). Site-directed mutagenesis of CC chemokine receptor 1 reveals the mechanism of action of UCB 35625, a small molecule chemokine receptor antagonist. J Biol Chem 280: 4808–4816.

Miesher SM, Vogel M (2002). Molecular aspects of allergy. Mol Aspects Med 23: 413–462.

Mirzadegan T, Diehl F, Ebi B, Bhakta S, Polsky I, McCarley D *et al*. (2000). Identification of the binding site for a novel class of CCR2b chemokine receptor antagonists: binding to a common chemokine receptor motif within the helical bundle. J Biol Chem 275: 25562–25571.

Nishikawa M, Takashima K, Nishi T, Furuta RA, Kanzaki N, Yamamoto Y *et al*. (2005). Analysis of binding sites for the new small-molecule CCR5 antagonist TAK-220 on human CCR5. Antimicrob Agents Chemother 49: 4708–4715.

Panina-Bodignon P, Papi A, Mariani M, Di Lucia P, Casoni G, Bellettato C *et al*. (2001). The C-C chemokine receptors CCR4 and CCR8 identify airway T cells of allergen-challenged atopic asthmatics. J Clin Invest 107: 1357–1364.

Pease JE (2010). Is there a role for CCR8 in the pathogenesis of asthma? Clin Exp Allergy 40: 1110–1112.

Proudfoot AEI, Power CA, Hoogewerf AJ, Montjovent M-O, Borlat F, Offord RE *et al*. (1996). Extension of recombinant human RANTES by the retention of the initiating methionine produces a potent antagonist. J Biol Chem 271: 2599–2603.

Rosenkilde MM, Schwartz TW (2000). Potency of ligands correlates with affinity measured against agonist and inverse agonists but not against neutral ligand in constitutively active chemokine receptor. Mol Pharmacol 57: 602–609.

Rosenkilde MM, Schwartz TW (2006). GluVII:06 – a highly conserved and selective anchor point for non-peptide ligands in chemokine receptors. Curr Top Med Chem 6: 1319–1333.

Rosenkilde MM, Cahir M, Gether U, Hjorth SA, Schwartz TW (1994). Mutations along transmembrane segment II of the NK-1 receptor affect substance P competition with non-peptide antagonists but not substance P binding. J Biol Chem 269: 28160–28164.

Rosenkilde MM, Kledal TN, Brauner-Osborne H, Schwartz TW (1999). Agonists and inverse agonists for the herpesvirus 8-encoded constitutively active seven-transmembrane oncogene product, ORF-74. J Biol Chem 274: 956–961.

Rosenkilde MM, Kledal TN, Holst PJ, Schwartz TW (2000). Selective elimination of high constitutive activity or chemokine binding in the human herpesvirus 8 encoded seven transmembrane oncogene ORF74. J Biol Chem 275: 26309–26315.

Rosenkilde MM, Waldhoer M, Luttichau HR, Schwartz TW (2001). Virally encoded 7TM receptors. Oncogene 20: 1582–1593.

Rosenkilde MM, Gerlach LO, Jakobsen JS, Skerlj RT, Bridger GJ, Schwartz TW (2004). Molecular mechanism of AMD3100 antagonism in the CXCR4 receptor: transfer of binding site to the CXCR3 receptor. J Biol Chem 279: 3033–3041.

Rosenkilde MM, Gerlach LO, Hatse S, Skerlj RT, Schols D, Bridger GJ *et al*. (2007). Molecular mechanism of action of monocyclam versus bicyclam non-peptide antagonists in the CXCR4 chemokine receptor. J Biol Chem 282: 27354–27365.

Samson M, LaRosa G, Libert F, Paindavoine P, Detheux M, Vassart G *et al*. (1997). The second extracellular loop of CCR5 is the major determinant of ligand specificity. J Biol Chem 272: 24934–24941.

Schwartz TW (1994). Locating ligand-binding sites in 7TM receptors by protein engineering. Curr Opin Biotechnol 5: 434–444.

Schwartz TW, Rosenkilde MM (1996). Is there a 'lock' for all 'keys' in 7TM receptors. Trends Pharmacol Sci 17: 213–216.

Schwarz MK, Wells TN (2002). New therapeutics that modulate chemokine networks. Nat Rev Drug Discov 1: 347–358.

Seifert R, Wenzel-Seifert K (2002). Constitutive activity of G-protein-coupled receptors: cause of disease and common property of wild-type receptors. Naunyn Schmiedebergs Arch Pharmacol 366: 381–416.

Simmons G, Clapham PR, Picard L, Offord RE, Rosenkilde MM *et al*. (1997). Potent inhibition of HIV-1 infectivity in macrophages and lymphocytes by a novel CCR5 antagonist. Science 276: 276–279.

Soler D, Chapman TR, Poisson LR, Wang L, Cote-Sierra J, Ryan M *et al*. (2006). CCR8 expression identifies CD4 memory T cells enriched for FOXP3+ regulatory and Th2 effector lymphocytes. J Immunol 177: 6940–6951.

Struyf S, De MI, Scharpe S, Lenaerts JP, Menten P, Wang JM *et al*. (1998). Natural truncation of RANTES abolishes signaling through the CC chemokine receptors CCR1 and CCR3, impairs its chemotactic potency and generates a CC chemokine inhibitor. Eur J Immunol 28: 1262–1271.

Thiele S, Steen A, Jensen PC, Mokrosinski J, Frimurer TM, Rosenkilde MM (2011). Allosteric and orthosteric sites in CC chemokine receptor (CCR5), a chimeric receptor approach. J Biol Chem 286: 37543–37554.

Tiffany HL, Lautens LL, Gao J-L, Pease J, Locati M, Combadiere C *et al*. (1997). Identification of CCR8: a human monocyte and thymus receptor for the CC chemokine I-309. J Exp Med 186: 165–170.

Vaidehi N, Schlyer S, Trabanino RJ, Floriano WB, Abrol R, Sharma S *et al*. (2006). Predictions of CCR1 chemokine receptor structure and BX 471 antagonist binding followed by experimental validation. J Biol Chem 281: 27613–27620.

Viola A, Luster AD (2008). Chemokines and their receptors: drug targets in immunity and inflammation. Annu Rev Pharmacol Toxicol 48: 171–197.

Watson C, Jenkinson S, Kazmierski W, Kenakin T (2005). The CCR5 receptor-based mechanism of action of 873140, a potent allosteric noncompetitive HIV entry inhibitor. Mol Pharmacol 67: 1268–1282.

Wu L, LaRosa G, Kassam N, Gordon CJ, Heath H, Ruffing N *et al*. (1997). Interaction of chemokine receptor CCR5 with its ligands: multiple domains for HIV-1 gp120 binding and a single domain for chemokine binding. J Exp Med 186: 1373–1381.