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European Journal of Pharmacology 389 (2000) 41–49

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Species selectivity of a small molecule antagonist for the CCR1 chemokine receptor

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Received 6 September 1999; received in revised form 22 November 1999; accepted 30 November 1999

Abstract

The species specificity of a small molecule antagonist for the human CCR1 chemokine receptor, 2-2-diphenyl-5-(4-chlorophenyl)piperidin-1-yl)valeronitrile (CCR1 antagonist 1), has been examined using cloned CCR1 receptors from various species. The compound was able to bind to rabbit, marmoset, and human CCR1, and was able to block the functional activation of these receptors. However, it failed to significantly displace radiolabeled macrophage inflammatory protein-1α (MIP-1α) binding to mouse CCR1 at concentrations up to 10 μM. These data suggested that the antagonist binding site is well-conserved in rabbit, marmoset and human CCR1, but not in mouse CCR1. The functional selectivity and mechanism of action for CCR1 antagonist 1 were further characterized. CCR1 antagonist 1 blocked the increase in intracellular Ca^{2+} stimulated by CCR1 agonists, but had no effect on *N*-formyl-Met–Leu–Phe (FMLP), monocyte chemotactic protein-1 (MCP-1) and stromal-derived factor 1α (SDF1α)-induced Ca^{2+} mobilization, demonstrating functional selectivity for CCR1. Since CCR1 antagonist 1 is a functional antagonist of marmoset and rabbit CCR1 receptors, it should be possible to test its efficacy in animal models of disease.

Keywords: Species selectivity; molecule antagonist; CCR1

1. Introduction

The chemokines are a group of small proteins that play an important role in leukocyte-trafficking during immune response (Schall, 1994; Baggiolini, 1998). They can be classified into four groups, CC, CXC, C, and CX_3C, according to the positions of the cysteines at the N-terminus of the molecule (Schall, 1994). The chemokines produce their biological effects by activating specific receptor proteins on their target cells (Horuk, 1994). To date, a total of 16 chemokine receptors, including nine CC chemokine receptors and five CXC chemokine receptors, have been identified (Mackay, 1996; Luster, 1998). All of these receptors belong to a G-protein-coupled receptor superfamily (Dohlman et al., 1991).

Chemokines have been implicated in the pathogenesis of chronic inflammatory diseases, such as multiple sclerosis and rheumatoid arthritis. For example, expression of RANTES and macrophage inflammatory protein-1α (MIP-1α) correlated with the disease onset in a mouse experimental autoimmune encephalomyelitis model of multiple sclerosis (Godiska et al., 1995). A recent study by Karpus et al. (1995) demonstrated that antibodies to MIP-1α prevented the development of both acute and relapsing paralytic disease as well as infiltration of mononuclear cells into the central nervous system. Treatment with MIP-1α antibody was also able to ameliorate the severity of ongoing clinical disease. These results led the authors to conclude that MIP-1α plays an important role in this T-cell-mediated disease.

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0014-2999/00/$ - see front matter
PII: S0014-2999(99)00863-8

A number of studies have also suggested a role for RANTES in rheumatoid arthritis. Both RANTES mRNA and protein appear to be upregulated in rheumatoid arthritis (Rathanaswami et al., 1993; Snowden et al., 1994). Antibodies against RANTES significantly decreased the severity of ongoing clinical disease in a rat adjuvant-induced rheumatoid arthritis model (Barnes et al., 1998).

These studies provided strong evidence for an important role of the chemokines, RANTES, and MIP-1α in chronic inflammatory diseases. Since MIP-1α and RANTES are ligands for CCR1, antagonists for this receptor may prove to be useful in treatment of these diseases. In our previous report, we identified a novel series of small molecule antagonists for human CCR1 receptor. In this study, we further characterize the pharmacological properties of one of these compounds, 2-2-diphenyl-5-(4-chlorophenyl)-piperidin-1-yl)valeronitrile (CCR1 antagonist 1), report the cloning of CCR1 receptor from marmoset and rabbit, and discuss the selectivity of CCR1 antagonist 1 for CCR1 receptors from a number of animal species. The study will provide useful information for testing the efficacy of CCR1 antagonists in animal disease models in vivo.

2. Materials and methods

2.1. Materials

Unlabeled chemokines were from Peprotech (Rocky Hill, NJ). ^{125}I-labeled chemokines were obtained from New England Nuclear (Boston, MA).

2.2. Synthesis of compounds

CCR1 antagonist 1 was synthesized as previously described (Hesselgesser et al., 1998).

2.3. Cloning of marmoset and rabbit CCR1 genes

2.3.1. Marmoset CCR1 gene cloning

Previous studies had shown that the mouse and human *CCR1* genes are highly conserved and contain no introns (Gao et al., 1993; Neote et al., 1993). A number of polymerase chain reaction (PCR) primers were synthesized based on the 5′ and 3′ ends of the human coding region. In addition, these primers contained mostly untranslated regions and only a few bases of the 5′ or 3′ coding regions. One pair of primers (5′-CCAGAGAGAAGCCGG-GATGGA-3′ and 5′-GGTCTGAGTCAGAACCCAGC-3′) resulted in a PCR fragment of ~1100 bp with marmoset genomic DNA as substrate. Cloning and sequencing of this fragment indicated an open reading frame of 355 amino acids with high homology to both human (91%) and mouse (75%) CCR1 proteins.

2.3.2. Rabbit CCR1 gene cloning

A PCR strategy similar to that used above for cloning of the marmoset gene was unsuccessful for the rabbit gene. Instead, degenerate primers were designed to the highly homologous transmembrane segments 2 and 5 of the human, mouse, and marmoset *CCR1* genes. Using these primers (5′-TTYCTNTTYACNCTNCCNTTYTGGAT-3′ and 5′-GGNGTNACRCARCARTGNGTRTANGC-3 j7 where Y = C or T; N = A, G, C or T; R = A, T, or G) in a PCR reaction with rabbit genomic DNA as template, we were able to amplify and clone a 650-bp fragment with high homology to the human, marmoset, and mouse *CCR1* genes. This fragment was used to screen 300,000 plaques from a rabbit genomic DNA library (Stratagene, La Jolla, CA) using standard methods. Two clones with homology to the 650-bp probe were purified and their DNA subjected to Southern analysis with a variety of restriction enzymes (*Bam*H1, *Kpn*1, *Eco*R1, and *Bgl*II). For each enzyme, both clones gave the same pattern of fragments homologous to the 650-bp CCR1 probe. From one-phage DNA, a 2800-bp *Bgl*II fragment was cloned into pBluescript SK(+) and sequenced to reveal a rabbit *CCR1* gene containing a 1053-bp open reading frame. The amino acid sequence of rabbit CCR1 showed 82, 79, and 76% homology to the human, marmoset, and mouse receptor sequences, respectively (Table 1). The nucleotide sequences reported in this manuscript have been submitted to the Genebank/EBI data bank with accession numbers AF 127527 (rabbit) and AF 127528 (marmoset).

2.4. CCR1 expression vectors

Human CCR1 cDNA was obtained as described (Neote et al., 1993) and ligated into a mammalian expression vector containing the myeloproliferative sarcoma virus promoter followed by the SV40 origin of replication, the human cytomegaloviral enhancer with the Puromycin-*N*-acetyl-transferase gene (puromycin resistance) and Hygromycin *B* gene (hygromycin resistance) similar to that described previously (Perez et al., 1994). The marmoset and rabbit *CCR1* receptor genes were cloned into the same mammalian expression vector, pBBS242, as described for human CCR1 (Hesselgesser et al., 1998).

2.5. Cell lines

The human monocytic cell line, THP-1, and the human embryonic kidney (HEK) 293 cell line were obtained from

Table 1
Sequence identity of human, marmoset, rabbit and mouse CCR1 receptors (% homology)

	Human	Marmoset	Rabbit	Mouse
Human	–	91	82	80
Marmoset	91	–	79	75
Rabbit	82	79	–	76
Mouse	80	75	76	–

the American Type Culture Collection and were cultured as previously described (Hesselgesser et al., 1998). For binding assays, the cells were harvested and washed once with phosphate-buffered saline solution. Cell viability was assessed by trypan blue exclusion and cell number was determined by counting the cells in a hemocytometer.

2.6. CCR1-expressing cells

The transfection and selection of HEK 293 cells stably expressing human CCR1 have been previously described (Hesselgesser et al., 1998). HEK 293 cells stably expressing marmoset and rabbit CCR1 were generated using a method similar to that described for human CCR1 cells (Hesselgesser et al., 1998). The population of clones was pooled and used in subsequent experiments. Stable transfectants were tested for their ability to bind ^{125}I-labeled MIP-1α and RANTES, and to respond biologically to these ligands by measuring changes in intracellular Ca^{2+} concentrations. HEK 293 cells expressing mouse CCR1 were a kind gift of Dr. Phillip Murphy and Dr. Ji-Liang Gao (Gao and Murphy, 1995).

2.7. Chemokine binding studies

The binding assays were performed by either filtration or centrifugation methods as previously described (Hesselgesser et al., 1998). Non-specific binding was determined in the presence of either 100 nM or 1 μM unlabeled ligand. The binding data were curve-fitted with the computer program, IGOR (Wavemetrics), to determine the affinity and number of sites.

2.8. Measurement of extracellular acidification with the microphysiometer

Extracellular acidification was measured in a microphysiometer as previously described (Hesselgesser et al., 1998). HEK 293 cells transfected with marmoset CCR1 were cultured in Alpha Minimum Essential Medium supplemented with 10% heat-inactivated fetal bovine serum, 150 μg/ml hygromycin, and 2 mM glutamine. HEK 293 cells transfected with rabbit CCR1 were cultured in 50:50 Dulbecco's Modified Eagle Medium/F12 medium supplemented with 10% heat-inactivated fetal bovine serum and 150 μg/ml hygromycin. HEK 293 cells transfected with human CCR1 were cultured in 50:50 Dulbecco's Modified Eagle Medium/F12 medium supplemented with 10% heat-inactivated fetal bovine serum, 150 μg/ml hygromycin and 5 μg/ml puromycin. The transfected cells were harvested by aspirating off the medium, rinsing the cells with 25 ml of phosphate-buffered saline solution, and adding 1 ml of versene to the flask. Cells were dislodged from the flask and rinsed with 10 ml of complete medium. The cell suspension was collected into a 15-ml centrifuge tube and centrifuged at 800 rpm for 5 min. Prior to centrifugation, an aliquot of cell suspension was removed for a trypan blue dye exclusion cell count. The cells were resuspended to 2×10^5 cells ml^{-1} and inoculated into FNC COATING MIX® (BRFF, Jamsville, MD)-coated polycarbonate cell capsules. The cell capsules were incubated overnight at 37°C. To measure the rate of acidification, the cell capsules were assembled and loaded into the chambers of the microphysiometer.

2.9. Cytosolic Ca^{2+} measurements in HEK 293 cells

HEK 293 cells expressing CCR1 were plated on poly-D-lysine-coated black-wall 96-well plates (Becton Dickinson, Franklin Lakes, NJ) at 80,000 cells/well and were cultured overnight. Cells were then loaded with 4 μM Fluo-3 (Molecular Probes, Eugene, OR), a Ca^{2+}-sensitive fluorescence dye, for 60 min at 37°C in Hanks balanced salts solution (Gibco, Grand Island, NY) containing 20 mM HEPES, 3.2 mM Ca^{2+} chloride, 1% fetal bovine serum, 2.5 mM probenecid, and 0.04% pluronic acid. The excess dye was removed by gently washing four times with Hanks balanced salts solution containing 20 mM HEPES, 2.5 mM probenecid, and 0.1% bovine serum albumin, using a Denley washer (Labsystems, Franklin, MA). THP-1 cells were loaded in tubes by incubation for 60 min at 37°C with Hanks balanced salts solution containing 20 mM HEPES, 1% fetal bovine serum and 4 μM Fluo-3. The excess dye was removed by gently washing the cells three times. Cells were plated at a density of 200,000 cells/well into black-wall 96-well plates (Corning/Costar, Acton, MA) and the plates were spun for 5 min at $800 \times g$. Changes in intracellular free-Ca^{2+} concentration were measured with Fluorometric Imaging Plate Reader (FLIPR) (Molecular Devices, Sunnyvale, CA) immediately after addition of agonist at 37°C or room temperature for HEK 293 or THP-1 cells, respectively. To examine the antagonistic activity of CCR1 antagonist 1, the cells were pretreated with the compound for 15 min before addition of agonist.

2.10. Lactate dehydrogenase release

HEK 293 cells expressing CCR1 were plated at a density of 5×10^6 cells/well in six- well tissue culture plates. The cells were grown to 70% confluency and treated with CCR1 antagonists up to a concentration of 10 μM for 24 h. The cell-free supernatants were measured for lactate dehydrogenase activity using a kit from Sigma (St. Louis, MO) as per manufacturer's instructions.

2.11. WST-1 staining

Cells were incubated with compound for 24 and 72 h followed by addition of 10 μl of WST-1 (Boehringer

Mannheim) into 100 μl of culture medium. After 1 h of incubation with the dye at 37°C, the OD was measured by the SpectraMax plate reader at 440 nm.

3. Results

3.1. Cloning of CCR1 receptors from rabbit and marmoset

We have previously identified and described a novel CCR1 antagonist, (2-2-diphenyl-5-(4-chlorophenyl)piperidin-1-yl)valeronitrile), designated CCR1 antagonist 1. In order to examine the species selectivity of this antagonist, CCR1 receptors were cloned from rabbit and marmoset. As expected, these receptors were highly homologous (Table 1 and Fig. 1). However, as shown in Fig. 1, the rabbit receptor is missing 12 nucleotides corresponding to four amino acids in the cytoplasmic tail region of the protein. In order to determine whether this ''deletion'' existed in the true CCR1 receptor, a number of PCR and sequencing experiments, utilizing additional genomic clones, rabbit genomic DNA, and rabbit lymphocyte polyA RNA, were carried out (PK and HP, unpublished results). All of these experiments indicated the existence of this deletion in the rabbit CCR1 receptor.

Table 2
The K_i of inhibition of radiolabeled MIP-1α binding to HEK 293 cells expressing CCR1 receptors by MIP-1α and CCR1 antagonist 1
HEK 293 cells expressing CCR1 receptors from various species were incubated for 30 min at room temperature with ^{125}I-MIP-1α in the presence of increasing concentrations of unlabeled MIP-1α or CCR1 antagonist 1. The binding reactions were terminated by centrifugation of cells through a paraffin/oil mixture. The K_d or K_i were determined from dose curves with computer program IGOR. Data are given as ±SEM.

Species	K_d of MIP-1α binding (nM)	K_i of inhibition of MIP-1α binding by compound (nM)
Human	2±0.2	40±6
Rabbit	43±17.8	245±60
Marmoset	81±23	451±191
Mouse	32±3.5	>10,000

3.2. Species selectivities of the CCR1 antagonist 1

The selectivities of the CCR1 antagonist 1 for human, mouse, rabbit and marmoset CCR1 were examined using stably transfected HEK 293 cells expressing these CCR1 receptors (Table 2 and Fig. 2). In competition binding experiments, the CCR1 antagonist 1 was able to displace human ^{125}I-MIP-1α from human, rabbit, and marmoset CCR1 with K_i of 40, 245, and 451 nM, respectively. However, the CCR1 antagonist 1 was unable to displace

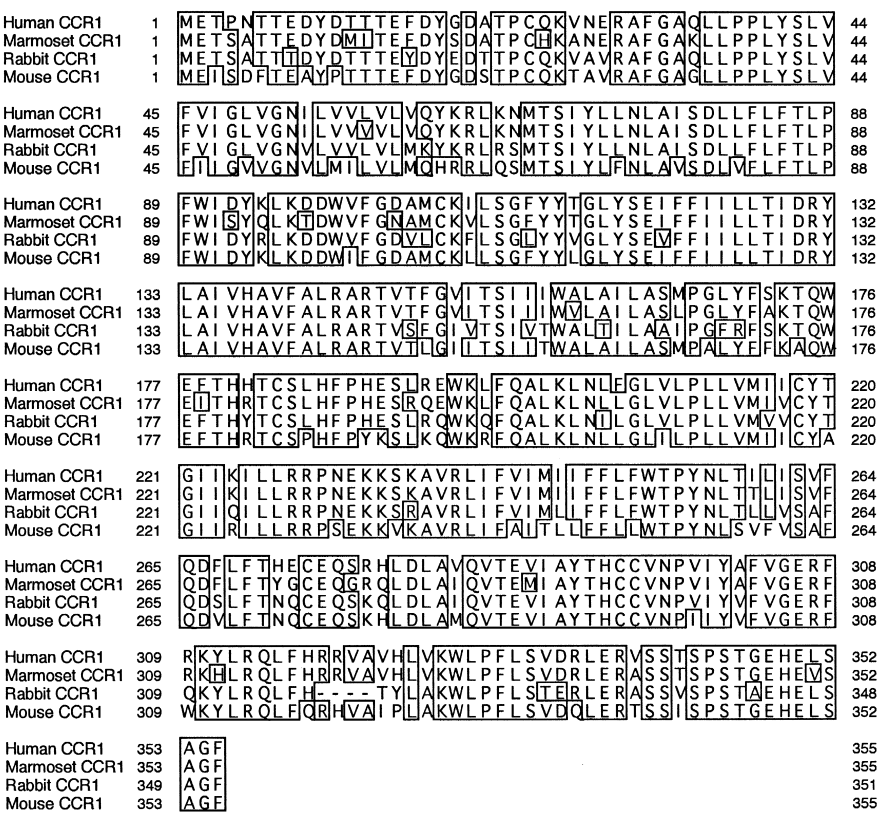

Fig. 1. Structure alignment of CCR1 from human, marmoset, rabbit and mouse.

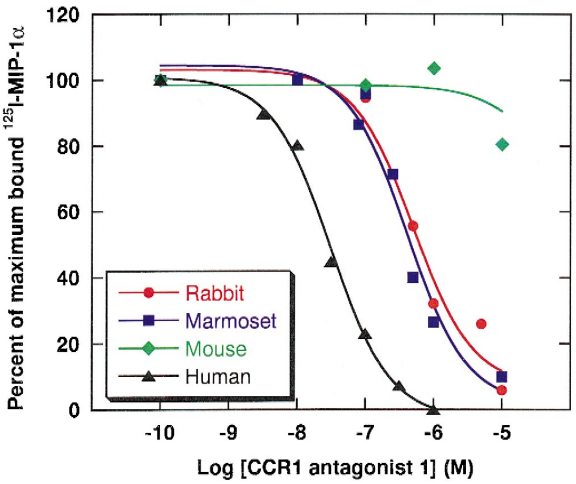

Fig. 2. CCR1 antagonist 1 displaced radiolabeled human MIP-1α binding from human, rabbit, marmoset, and mouse CCR1 receptors. HEK 293 cells expressing CCR1 receptors from various species were incubated for 30 min at room temperature with ^{125}I-MIP-1α in the presence of increasing concentrations of CCR1 antagonist 1. The binding reactions were terminated by centrifugation of cells through a paraffin/oil mixture. Binding shown represented specific binding as percent of total binding. Specific binding was around 10% of total ^{125}I-MIP-1α added.

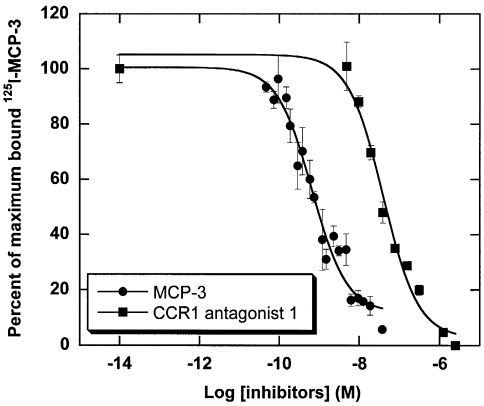

Fig. 3. CCR1 antagonist 1 displaced radiolabeled human MCP-3 binding to the human CCR1 receptor. HEK 293 cells expressing human CCR1 receptor were incubated for 30 min at room temperature with ^{125}I-MCP-3 in the presence of increasing concentrations of unlabelled MCP-3 or CCR1 antagonist 1. The binding reactions were terminated by filtration of cells through a GFB glass fiber filter soaked in 3% polyethyleneimine. The data shown are specific binding as a percentage of total binding ± SD. Specific binding was around 10% of total ^{125}I-MCP-3 added. The results shown in each case were from two separate studies.

^{125}I-MIP-1α from mouse CCR1. Even at a concentration of 10 μM, it only displaced 16% of total ^{125}I-MIP-1α binding to mouse CCR1. In contrast to these data, MIP-1α was able to bind to all four CCR1 receptors, including mouse, with high affinity (Table 2). These data demonstrated that the CCR1 antagonist 1 binding site is relatively well-conserved in human, marmoset, and rabbit receptors, but not in mouse receptors.

In addition to MIP-1α and RANTES, other ligands that bind to CCR1 with high affinity include monocyte chemotactic protein-3 (MCP-3). Thus, we carried out competition binding assays to determine whether the CCR1 antagonist 1 could displace ^{125}I-MCP-3 from cells expressing human CCR1. Competition binding studies showed that the CCR1 antagonist 1 could displace ^{125}I-MCP-3 binding from human CCR1 in a concentration-dependent manner with a K_i of 40 nM compared to a K_d for MCP-3 of 0.8 nM (Fig. 3). These data suggested that the CCR1 receptor antagonist 1 was able to displace all of the known CCR1 ligands that we tested.

To show that CCR1 antagonist 1 is a functional antagonist for human, rabbit, and marmoset CCR1, the effect of the compound on MIP-1α-induced extracellular acidification rate was measured by microphysiometry on THP-1 cells and HEK 293 cells expressing rabbit and marmoset CCR1 receptors (Fig. 4). Pretreatment of cells with 10 μM CCR1 antagonist 1 for 30 min completely inhibited the extracellular acidification rate induced by MIP-1α in all cases (Fig. 4) with no agonistic activities observed (data not shown). In addition, CCR1 antagonist 1, at doses of 1 and 10 μM, was able to inhibit MIP-1α induced Ca^{2+} mobilization in cells expressing human and rabbit CCR1 (data not shown). These data were consistent with that obtained with the microphysiometer. In contrast, even at 10 μM, CCR1 antagonist 1 did not inhibit MIP-1α-induced Ca^{2+} transients in cells transfected with mouse CCR1 (data not shown), consistent with the data obtained in the receptor binding assay.

3.3. Functional selectivities of the CCR1 antagonist 1

The functional selectivity of the CCR1 antagonist 1 was examined in THP-1 cells. These cells were known to express a number of chemokine receptors as well as other G-protein-coupled receptors. THP-1 cells were treated with

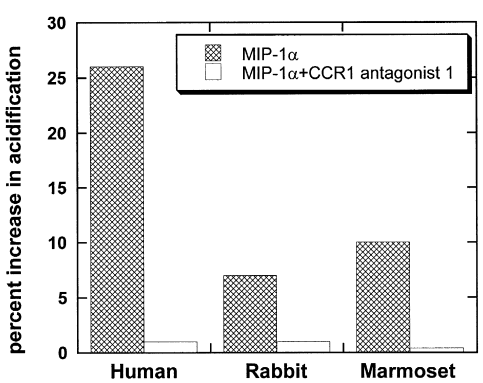

Fig. 4. CCR1 antagonist 1 inhibited the ability of MIP-1α to increase the acidification rate of THP-1 cells expressing human CCR1 and HEK 293 cells expressing rabbit and marmoset CCR1. Cells were pretreated with CCR1 antagonist 1 at 1, 10, and 10 μM followed by 1, 10, and 30 nM of MIP-1α for human, rabbit, and marmoset CCR1 cells, respectively. The increase in acidification rate was monitored using a microphysiometer. Data have been normalized as percentage biological response. Data shown are representative from at least two separate studies.

CCR1 antagonist 1 for 15 min at 37°C and then maximal or submaximal concentrations of various receptor agonists were added. The increase in intracellular free-Ca^{2+} concentration was measured using FLIPR. No intrinsic agonist activity was observed when the CCR1 antagonist 1 was added at a concentration of 1 μM (data not shown). In contrast, CCR1 antagonist 1 pretreatment completely blocked MIP-1α and RANTES-induced Ca^{2+} mobilization with an IC_{50} of approximately 150 nM well in line with its predicted K_i from receptor binding studies. However, the compound at a concentration of up to 10 μM had no effect on intracellular free Ca^{2+} concentration induced by *N*-formyl-Met–Leu–Phe (FMLP), monocyte chemotactic protein-1 (MCP-1), and stromal-derived factor 1α (SDF1α) (Fig. 5). The Ca^{2+} signal induced by MCP-3, another CCR1 agonist, was only partially inhibited by the CCR1 antagonist 1 in THP-1 cells (Fig. 5). However, in HEK 293 cells expressing human CCR1, the CCR1 antagonist 1 was able to completely block the MCP-3-induced Ca^{2+} transients (Fig. 6). The IC_{50} for the compound to block a CCR1 response on CCR1 transfected cells was approximately 600 nM.

3.4. Schild analysis of the CCR1 antagonist 1

The mechanism of the antagonistic effect of CCR1 antagonist 1 was examined on CCR1 transfected cells by Schild analysis (Arunlakshana and Schild, 1959). The concentration–response curves for Ca^{2+} transients induced by MIP-1α in the presence of increasing concentrations of CCR1 antagonist 1 were determined (Fig. 7). CCR1 antagonist 1 shifted the concentration–response curves to the right and also decreased the maximal responses, suggesting insurmountable antagonism.

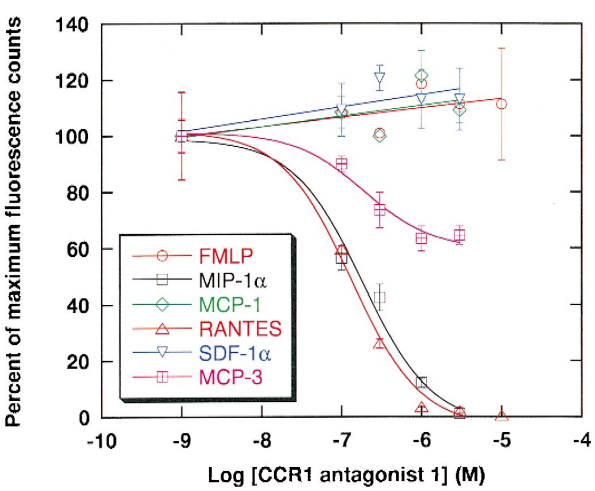

Fig. 5. CCR1 antagonist 1 inhibited the ability of MIP-1α and a number of G-protein-coupled receptor agonists to increase Ca^{2+} transients in THP-1 cells. Fluo-3-loaded cells were pretreated with increasing concentrations of CCR1 antagonist 1 for 15 min followed by a number of agonists at maximal or submaximal concentration (10 nM FMLP, 3 nM MIP-1α, 1 nM MCP-1, 30 nM RANTES, 30 nM SDF1α and 30 nM MCP-3). The changes in fluorescence representing the change in Ca^{2+} concentration were measured by FLIPR at room temperature. Data shown were average fluorescence counts (percent of the counts in the absence of CCR1 antagonist 1) ± SD. They are representative from at least two separate studies.

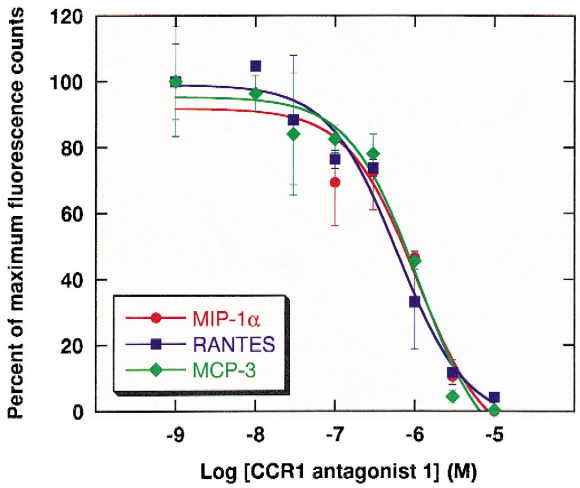

Fig. 6. CCR1 antagonist 1 inhibited the ability of CCR1 agonists, MIP-1α, RANTES and MCP-3, to increase Ca^{2+} transients in HEK 293 cells expressing human CCR1. Fluo-3-loaded cells were pretreated with increasing concentrations of CCR1 antagonist 1 for 15 min and then stimulated with CCR1 agonists, MIP-1α (30 nM), RANTES (300 nM) and MCP-3 (100 nM). The changes in fluorescence representing the changes in Ca^{2+} concentration were measured by FLIPR at 37°C. Data shown were average fluorescence counts (percent of the counts in the absence of CCR1 antagonist 1) ± SD.

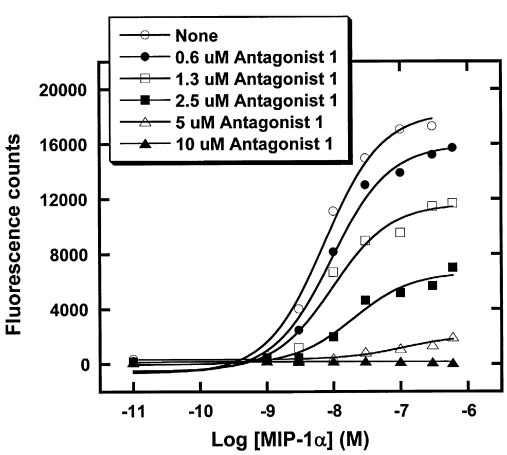

Fig. 7. Concentration–response curves for the ability of MIP-1α to increase Ca^{2+} transients in HEK 293 cells expressing human CCR1 in the presence of increasing concentrations of CCR1 antagonist 1. Fluo-3-loaded cells were pretreated with increasing concentrations of CCR1 antagonist 1 for 15 min followed by increasing concentrations of MIP-1α. The changes in fluorescence representing the changes in Ca^{2+} concentration were measured by FLIPR at 37°C. Data shown were average fluorescence counts. The results shown are representative from three separate studies.

3.5. Effect of the CCR1 antagonist 1 on general cell toxicity

To demonstrate that CCR1 antagonism by the CCR1 antagonist 1 was not due to the cellular toxicity of the compound, CCR1 transfected cells were treated with the antagonist at concentrations up to 10 μM for 24 and 72 h, and cellular toxicity was monitored by measuring both lactate dehydrogenase release and WST-1 staining. No toxicity was observed under any conditions tested (data not shown), suggesting that the inhibition of CCR1 antagonist 1 on CCR1 activation was not due to cellular toxicity.

4. Discussion

CCR1 could be a therapeutic target for autoimmune diseases like multiple sclerosis and rheumatoid arthritis since its ligands, MIP-1α and RANTES, have been implicated in these diseases (Karpus and Kennedy, 1997; Karpus et al., 1995; Plater-Zyberk et al., 1997; Barnes et al., 1998). Thus, a potent and selective CCR1 antagonist could be useful as a potential therapy. We have previously identified and described a novel CCR1 antagonist, CCR1 antagonist 1 (Hesselgesser et al., 1998). This compound was able to displace the binding of the CCR1 ligands, MIP-1α, and RANTES, to human CCR1 receptors to a similar degree with a K_i of approximately 40 nM. Further, it showed concentration-dependent inhibition of MIP-1α and RANTES-induced extracellular acidification, Ca^{2+} mobilization, and cellular migration demonstrating functional antagonism. Finally, the CCR1 antagonist 1 showed selectivity for human CCR1 when tested against a large panel of seven transmembrane domain receptors.

Although the studies described above identified potential CCR1 antagonists, the further utility of these compounds will depend on being able to demonstrate their effectiveness in animal models of disease. Several small mammals including mice, rabbits and marmosets have been used in animal models of multiple sclerosis and rheumatoid arthritis. Mouse CCR1 has been cloned but rabbit and marmoset CCR1 receptors are unknown. Thus, we set out to clone rabbit and marmoset CCR1, and to examine the species selectivity of CCR1 antagonist 1 for these CCR1 receptors compared to human CCR1. The results suggested that CCR1 antagonist 1 is a functional antagonist for human, rabbit, and marmoset CCR1, but not for mouse CCR1 (Figs. 2 and 4).

Although CCR1 were highly conserved among various species, comparison of the sequences for human, marmoset, and rabbit, with mouse CCR1, revealed several radical substitutions in mouse CCR1, compared to the other receptors (Fig. 1). It is interesting to speculate that perhaps, substitutions that are in the N-terminal region of mouse, CCR1 including Thr^{6} to Phe, Asp^{9} to Pro, Asp^{11} to Ala, could be involved in antagonist binding. This region of CCR1 has been shown to contain some determinants for ligand binding (Pease et al., 1998) and it is possible that the acidic Asp residues could, e.g., be involved in ionic interactions with the basic nitrogen in the piperadine ring of the CCR1 antagonist 1. In addition, there are several substitutions in the second extracellular loop of mouse CCR1 including two substitutions of charged residues His^{190} to Thr, and Glu^{191} to Lys, together with a Leu/Gln^{198} to Arg, that might also be candidates for antagonist binding. However, elucidation of the molecular basis for the species selectivity of CCR1 antagonist 1 will require the generation and testing of human/mouse chimeric CCR1 receptors.

There have been numerous reports in the literature of peptide and non-peptide G-protein-coupled receptor antagonists that demonstrate species selectivity. In many cases, the molecular basis for this selectivity turns out to be one or two amino acid substitutions. For example, two substance P antagonists exhibit opposite rank orders of potency for the human and rat tachykinin NK_1 receptors (Fong et al., 1992). Analysis of mutant receptors revealed that substitution of two residues Val^{116} to Leu and Ile^{290} to Ser in the transmembrane domain of the human tachykinin NK_1 receptor is necessary and sufficient to reproduce the antagonist binding affinities of the rat receptor. Molecular cloning and ligand binding studies have shown that the differences in binding affinity of the classical α_{2A}-adrenoceptor antagonist, yohimbine, to mouse and human α_{2A}-adrenoceptor is due to a single conservative Cys^{201} to Ser change in the fifth transmembrane domain (Link et al., 1992). Pharmacological analysis of mutant V2 vasopressin receptors revealed that residues 202 and 304 fully control the species selectivity of the rat vs. the human receptor for peptide vasopressin antagonists (Cotte et al., 1998).

In previous studies, we had demonstrated that the CCR1 receptor antagonist 1 had greater than 200-fold selectivity for CCR1 compared with 25 other G-protein-coupled receptors tested in receptor binding assays (Hesselgesser et al., 1998). Here, we further examined the functional selectivity of the CCR1 antagonist 1 in THP-1 cells (Fig. 5). These data suggested that the CCR1 antagonist 1 demonstrated greater than 100-fold selectivity for CCR1 over FMLP receptor, CCR2 and CXCR4 in a receptor functional study, in agreement with the results obtained from receptor binding studies (Hesselgesser et al., 1998).

Interestingly, the Ca^{2+} signal induced by MCP-3, another CCR1 agonist, was only partially inhibited by the CCR1 antagonist 1 in THP-1 cells (Fig. 5). This can be explained by the presence of CCR2 receptors on THP-1 cells. The CCR2 can be activated by MCP-3, but CCR1 antagonist 1 does not block CCR2 activation. This hypothesis was supported by the data presented in Fig. 6. In cells which express only the human CCR1 receptor but not CCR2, the CCR1 antagonist 1 was able to completely block the MCP-3-induced Ca^{2+} transients (Fig. 6). The IC_{50} for the compound to block a CCR1 response on

CCR1-transfected cells was approximately 600 nM, which was different from that obtained from THP-1 cells (IC_{50} = 150 nM). The discrepancy in IC_{50} values was not completely understood. However, it is likely due to the fact that the stimulating concentration of agonists used in the assay was higher in CCR1-transfected cells compared to that used in THP-1 cells, leading to an increase in the IC_{50} value obtained for CCR1 antagonist 1 in transfected cells.

Schild analysis demonstrated that CCR1 antagonist 1 decreased both affinity and maximal response of Ca^{2+} transients induced by MIP-1α (Fig. 7), the antagonism was very likely to be heterotropic-cooperative non-competitive inhibition, implying that antagonist and agonist bound to separate, but interacting, sites (Horn et al., 1998). The results are not surprising since the chemical structure of CCR1 antagonist 1 does not resemble the natural ligands of CCR1. It has been described for a number of G-protein coupled receptors that the binding sites on the receptor for agonist are distinct from that for antagonist (Beinborn et al., 1993; Gether et al., 1993; Kong et al., 1994). For example, Gether et al. (1993) demonstrated that non-conserved residues in transmembrane segment V and VI were essential for the binding of tachykinin NK_1 receptor antagonist, CP96345, on the tachykinin NK_1 receptor, but were not important for the binding of the natural peptide ligand, substance P.

In summary, we have identified CCR1 receptor genes from rabbit and marmoset. The sequence information has provided us with the advantage to study the species selectivity of CCR1 antagonist 1. Using the stably transfected cells expressing CCR1 from various species, we have demonstrated that CCR1 antagonist 1 is a potent, functional, selective, insurmountable CCR1 antagonist that binds with high affinity to human, rabbit, and marmoset CCR1. In contrast, CCR1 antagonist 1 has only very low affinity for the mouse CCR1. The species specificity of this compound has provided the basis for choosing an animal disease model to test the efficacy of this compound in vivo. This class of potent and selective CCR1 antagonists should provide insight into developing small molecule therapy for autoimmune diseases such as multiple sclerosis and rheumatoid arthritis.

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