CP-481,715, a Potent and Selective CCR1 Antagonist with Potential Therapeutic Implications for Inflammatory Diseases*

Received for publication, June 27, 2003, and in revised form, July 28, 2003
Published, JBC Papers in Press, August 7, 2003, DOI 10.1074/jbc.M306875200

Ronald P. Gladue‡, Laurie A. Tylaska, William H. Brissette, Paul D. Lira, John C. Kath, Christopher S. Poss, Matthew F. Brown, Timothy J. Paradis, Maryrose J. Conklyn, Kevin T. Ogborne, Molly A. McGlynn, Brett M. Lillie, Amy P. DiRico, Erin N. Mairs, Eric B. McElroy, William H. Martin, Ingrid A. Stock, Richard M. Shepard, Henry J. Showell, and Kuldeep Neote

From Pfizer Global Research and Development, Groton, Connecticut 06340

The Journal of Biological Chemistry Vol. 278, No. 42, Issue of October 17, pp. 40473–40480, 2003

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

‡ To whom correspondence should be addressed: Pfizer Global Research and Development, Dept. of Immunology, MS 8220-2410, Eastern Point Rd., Groton, CT 06340. Tel.: 860-441-5677; Fax: 860-868-1109; E-mail: Ronald_P.Gladue@Groton.Pfizer.com.

The abbreviations used are: TNF, tumor necrosis factor; PBS, Dul-becco’s phosphate-buffered saline; PBMC, peripheral blood mononuclear cells; MMP, matrix metalloproteinase.

The chemokines CCL3 and CCL5, as well as their shared receptor CCR1, are believed to play a role in the pathogenesis of several inflammatory diseases including rheumatoid arthritis, multiple sclerosis, and transplant rejection. In this study we describe the pharmacological properties of a novel small molecular weight CCR1 antagonist, CP-481,715 (quinoxaline-2-carboxylic acid [4(R)-carbamoyl-1(S)-(3-fluorobenzyl)-2(S),7-dihydroxy-7-methyloctyl]amide). Radiolabeled binding studies indicate that CP-481,715 binds to human CCR1 with a Kd of 9.2 nM and displaces 125I-labeled CCL3 from CCR1-transfected cells with an IC50 of 74 nM. CP-481,715 lacks intrinsic agonist activity but fully blocks the ability of CCL3 and CCL5 to stimulate receptor signaling (guanosine 5’-O-(thiotriphosphate) incorporation; IC50 = 210 nM), calcium mobilization (IC50 = 71 nM), monocyte chemotaxis (IC50 = 55 nM), and matrix metalloproteinase 9 release (IC50 = 54 nM). CP-481,715 retains activity in human whole blood, inhibiting CCL3-induced CD11b up-regulation and actin polymerization (IC50 = 165 and 57 nM, respectively) on monocytes. Furthermore, it behaves as a competitive and reversible antagonist. CP-481,715 is >100-fold selective for CCR1 as compared with a panel of G-protein-coupled receptors including related chemokine receptors. Evidence for its potential use in human disease is suggested by its ability to inhibit 90% of the monocyte chemotactic activity present in 11/15 rheumatoid arthritis synovial fluid samples. These data illustrate that CP-481,715 is a potent and selective antagonist for CCR1 with therapeutic potential for rheumatoid arthritis and other inflammatory diseases.

Rheumatoid arthritis is a chronic inflammatory disease affecting 0.5–2% of the population in the Western world, the majority of whom are female. Central to the pathogenesis of this disease is the infiltration of monocytes into synovial tissue. This is supported by the predominance of monocytes in the joint during flare (1, 2), the role of monocyte-derived proinflammatory mediators on disease progression (e.g. TNF), interleukin 1) (3, 4), and the ability of monocytes to secrete tissue-damaging proteolytic enzymes that participate in joint destruction (5). Consequently, an agent that inhibits monocyte infiltration into synovial tissue has the potential to decrease tissue damage and joint destruction, thus, providing a novel therapy for rheumatoid arthritis.

Leukocyte infiltration into inflammatory sites is believed to be regulated by small molecular weight cytokines known as chemokines. Chemokines are 7–10-kDa proteins that can be divided into four groups (CC, CXC, CX3C, and C) depending on the spacing of their N-terminal cysteine residues. Approximately 40 different chemokines have been identified. Some of these are constitutively expressed and play a crucial role in development and lymph node architecture. However, most chemokines are specifically induced at inflammatory sites during disease (6). Chemokines are of interest as therapeutic targets because they exert their effects through seven-transmembrane G-protein-coupled receptors, well preceded drug targets. Although chemokines are best known for their ability to stimulate cell migration, they also have additional activities that can contribute to tissue damage and inflammation including enhancing T cell activation (7), regulating TH-1/TH-2 polarization (8–10), and stimulating macrophage function (11) and protease secretion (12, 13).

In rheumatoid arthritis two chemokines believed to play a major role in regulating monocyte infiltration into synovial tissues are CCL3 (macrophage inflammatory protein-1α) and CCL5 (RANTES, regulated on activation, normal T-cell expressed and secreted). CCL3 and CCL5 have potent monocyte chemotactic activity both in vitro (14) and in vivo, as demonstrated in human subjects injected intradermally with these agents (15, 16). Furthermore, several studies have demonstrated an elevation of CCL3 and CCL5 in the synovial tissue and fluid of rheumatoid arthritis patients (17–22) coincident with the infiltration of monocytes expressing their shared receptor, CCR1 (23). In these studies, the levels of CCL3 and monocytes in synovial tissue were directly proportional to the magnitude of joint pain (2). The role of these chemokines in the pathogenesis of arthritis is also supported by human genetic association studies (24) and by the beneficial effects observed in animal models of arthritis using neutralizing antibodies and receptor antagonists (25–27).

The potential importance of CCR1 in the pathogenesis of rheumatoid arthritis coupled with the well known success in identifying antagonists of G-protein-coupled receptors...
prompted us to initiate a search for small molecular weight antagonists of CCR1. These efforts led to the identification of the novel antagonist, CP-481,715. In this study we describe the in vitro biological properties of CP-481,715 and demonstrate its ability to dose-dependently inhibit CCL3- and CCL5-induced chemotaxis, integrin up-regulation, intracellular calcium mobilization, and matrix metalloprotease production. Furthermore, CP-481,715 retains activity in whole blood, and as evidence for its potential therapeutic utility, we demonstrated the ability to inhibit the monocyte chemoattractant activity produced by synovial fluid from rheumatoid arthritis patients. These studies suggest that blocking CCR1 may represent a novel therapeutic approach to prevent monocyte infiltration into inflammatory sites such as the synovial tissue.

EXPERIMENTAL PROCEDURES

Materials—The CCR1 antagonist CP-481,715 (quinoline-2-carboxylic acid (4R-carbamoyl-1-S)-(3-fluorobenzyll)-2(S),7-dihydroxy-7-methyloctylamide; see Fig. 1) and [3H]CP-481,715 were prepared by the Pfizer Medicinal chemistry group. 125I-Labeled CCL3 was purchased from PerkinElmer Life Sciences. All unlabeled chemokines were obtained from Peprotec (Rocky Hill, NJ) unless otherwise indicated and checked for purity by high performance liquid chromatography. Human recombinant interleukin-2 and TNFα were obtained from R&D Systems (Minneapolis, MN).

Reagents—Bovine serum albumin, fluorescein diacetate, and AccupinTM System-Histopaque-1077 tubes were purchased from Sigma. Heparin was purchased from American Pharmaceutical Partners Inc. (Los Angeles, CA). Dulbecco’s phosphate-buffered saline (PBS) without calcium chloride and magnesium chloride, Hanks’ balanced salt solution, and Geneticin were obtained from Invitrogen. Fetal bovine serum was purchased from HyClone, Logan, Utah. RPMI 1640, HEPES, glutamine, and penicillin/streptomycin were all obtained from BioWhittaker (Walkersville, MD). Tissue culture medium for cell cultures contained RPMI 1640 containing fetal bovine serum (10%), L-glutamine (2 mM), HEPES (10 mM), penicillin (100 units/ml), and streptomycin (50 μg/ml).

Cell Lines—CCR1-transfected HEK 293 cells (ATCC CRL-1573) were prepared as previously described (28). CCR1-, CCR2-, CCR5-, CXCR1-, CXCR2-, and CXC4-transfected 300-19 cells were obtained from Dr. Israel Chao at the Gladstone Institute. Cells were maintained in Geneticin for selection, split twice weekly, and discarded after passage 12. The human monocyte cell line, THP-1 (ATCC TIB 202), was maintained in RPMI medium and split 24 h before use.

Chemokine and CP-481,715 Binding Studies—Binding assays were conducted with CCR1-transfected HEK 293 cells as previously described (28). The number of CCR1 receptors on the cells was calculated to be 75,000/cell. Cells were typically grown to 80% confluency, removed by trypsinization and plated into flasks at least 48 h before the experiment. On the day of the experiment, cells were collected, washed in Hanks’ balanced salt solution, and resuspended in the assay buffer (50 mM HEPES, 1 mM CaCl2, 5 mM MgCl2, 0.5% bovine serum albumin, pH 7.4) at a concentration of 2.5 × 106 cells/ml. Cells were then plated into a 96-well plate (25,000 cells/well) and incubated with 50 pm 125I-labeled CCL3 or 125I-labeled CCL5 (specific activity 2200 Ci/mmol) in the presence or absence of varying concentrations of CP-481,715 at 4 °C for 60 min. The reaction was terminated by harvesting through a GF/B filter and the number of migrating cells was quantitated by reading the intensity of the color on a microtiter plate reader at 490 nm.

Calcium Measurements—Human CCR1-transfected 300-19 cells were harvested, centrifuged, and resuspended at 2 × 105 cells/ml in Hanks’ balanced salt solution containing 1.6 mM CaCl2. The cells were loaded with indo-1 AM (2 μM) and the number of migrating cells was quantitated by reading the intensity of the color on a microtiter plate reader at 490 nm.

Human blood collected in EDTA was incubated with various dilutions of CP-481,715 or diluent for 5 min at room temperature. CCL3 (10 ng/ml) was then added, and after 50 s the reaction was stopped by adding 2% cold EDTA. The cells were then pelleted at 200 × g for 10 min at 4 °C. CD11b expression on CD14+ monocytes was then determined by fluorescence-activated cell sorter analysis.

Identification of CP-481,715 and Characterization of Its Effects on CCR1 Using Binding Assays—A binding assay using CCR1-transfected HEK 293 cells was used to screen our compound library for small molecular weight compounds that inhibit CCL3 binding to CCR1. Chemical optimization of active compounds from this screen led to the identification of quinoxa-
line-2-carboxylic acid \([4(R)\text{-carbamoyl}-1(S)-(3\text{-fluorobenzyl})-2(S)\text{-dihydroxy-7-methyloctyl} amide]\) (CP-481,715) as the lead compound (Fig. 1). As shown in Fig. 2A, competition binding experiments demonstrated that CP-481,715 displaces \(^{125}\text{I}\)-labeled CCL3 from CCR1-transfected HEK 293 cells in a concentration-dependent manner (IC\(_{50}\) = 74 nM). Similar results were observed with \(^{125}\text{I}\)-labeled CCL5 (data not shown). To better define its interaction, competition binding experiments were also conducted with radiolabeled CP-481,715. As shown in Fig. 2B, \([3H]\)CP-481,715 binds to human CCR1-expressing HEK 293 cells with a \(K_d\) of 9.2 nM. These data demonstrate that CP-481,715 binds to human CCR1 and prevents its interaction with CCL3.

**CP-481,715 Inhibits GTP Hydrolysis**—Because interaction with the CCR1 receptor could impart both agonist and/or antagonist properties, we next sought to characterize this interaction from a functional perspective. During receptor activation, GDP bound to the \(\alpha\) subunit of G-protein receptors is released, leading to the rapid binding of GTP and the subsequent dissociation of \(\beta\gamma\) subunits. The release of these \(\beta\gamma\) subunits initiates a cascade of intracellular events (i.e. calcium mobilization, adenylyl cyclase activation, etc.) culminating in the hydrolysis of GTP to return the receptor to its resting state. The initial step in G-protein signaling can be quantitated by measuring the incorporation of the non-hydrolyzable form of GTP, \([35\text{S}]\)GTP\(_\gamma\). As shown in Fig. 3, THP-1 cells had a basal level of receptor activity (\([35\text{S}]\)GTP\(_\gamma\)) that was not significantly affected by CP-481,715, indicating that CP-481,715 was not acting as an inverse agonist (31). In contrast, CP-481,715 was able to block CCL3 (100 nM)-induced \([35\text{S}]\)GTP\(_\gamma\) incorporation in a dose-dependent manner (IC\(_{50}\) of 210 nM).

**CP-481,715 Inhibits Calcium Mobilization but Has No Agonist Properties**—We next characterized the effects of CP-481,715 on CCR1 function by measuring intracellular calcium mobilization in CCR1-transfected 300-19 cells. In these studies CP-481,715 did not directly induce calcium mobilization, supporting its lack of intrinsic agonist activity (data not shown). In contrast, as shown in Fig. 4, calcium mobilization induced by CCL3 was blocked by CP-481,715 in a concentration-dependent manner. The amount of CP-481,715 necessary to block this effect was directly proportional to the concentration of CCL3 utilized, demonstrating a 5–6-fold change in IC\(_{50}\) with each log change in CCL3 concentration. This data suggest that CP-481,715 is a competitive antagonist of CCR1.

To provide evidence that CP-481,715 was not acting nonspecifically (e.g. by inhibiting downstream signaling), we performed experiments on THP-1 monocytes that express both CCR1 and CCR2. As shown in Fig. 5A, calcium mobilization was induced by THP-1 cells using either CCL3 or CCL2. CP-481,715 was able to inhibit CCL3- but not CCL2-induced in-
tracellular calcium mobilization (Fig. 5). Again, CP-481,715 did not directly induce any calcium mobilization on THP-1 cells. To further establish that the inhibitory effects of CP-481,715 were mediated through direct interaction with CCR1 and not through effects on its ligands, we also measured calcium mobilization on CCR5-transfected cells because both CCL3 and CCL5 also interact with this receptor. CP-481,715 did not inhibit calcium mobilization in CCR5-transfected cells in response to either ligand (data not shown). Collectively, these studies indicate that CP-481,715 completely antagonizes the CCR1 receptor, lacks intrinsic agonist activity, does not directly interfere with its ligands, and does not antagonize CCR5 or CCR2.

**CP-481,715 Inhibits Leukocyte Chemotaxis**—A major property of chemokines is their ability to induce leukocyte migration and mediate the infiltration of these cells into sites of inflammation. As such, to better characterize the activity of CP-481,715 on CCR1 in a way that may be more relevant for disease, we performed a series of experiments examining the effects of CP-481,715 on chemotaxis. As shown in Fig. 6, CP-481,715 inhibited the chemotaxis of THP-1 cells and human peripheral blood monocytes to an optimal concentration of CCL3 (1.0 nM) with an IC$_{50}$ of 110 and 55 nM, respectively. CP-481,715 also inhibited the chemotaxis of monocytes to other CCR1 ligands, including CCL5 and CCL14, and was able to partially inhibit the response to the shared ligand CCL7, which interacts with both CCR1 and CCR2 on monocytes (data not shown). Concentrations of CP-481,715 up to 25 nM did not inhibit the chemotaxis of THP-1 or primary human monocytes in response to the CCR2 ligand, CCL2, indicating that CP-481,715 did not exert nonspecific effects on cells that would generally interfere with migration.

Because calcium mobilization studies demonstrated a proportional relationship between the amount of CP-481,715 necessary to inhibit activity and the concentration of CCL3, we next determined whether the same effects were observed on chemotaxis. As shown in Fig. 7, the concentration of CP-481,715 necessary to inhibit 50, 75, or 90% of the chemotactic response to CCL3 was directly dependent on the amount of agonist used to stimulate the response. For example, the IC$_{50}$ for CP-481,715 was 5, 16, and 66 nM using CCL3 concentrations of 0.25, 0.5, and 1.0 nM, respectively. Similar results were also observed when CCL5 was used as the agonist (data not shown). These studies further suggest that CP-481,715 is a competitive antagonist of CCR1.

**Selectivity of CP-481,715 for CCR1**—CP-481,715 was evaluated against a panel of G-protein-coupled receptors using binding assays (Table I). In all cases, <10% inhibitory activity was observed at a concentration of 3 $\mu$M. Furthermore, drug concentrations as high as 25 $\mu$M did not inhibit receptor binding of the appropriate ligand to cells expressing CCR2, CCR3, CCR5,
IC75, and IC90 concentrations for CP-481,715 were determined for each assessment of whole blood activity that may be more indicative of at least three separate studies.

The data are representative of at least three experiments done with different human donors.

**Table I**

**Panel of G-protein-coupled receptors evaluated for activity with CP-481,715**

| Class                      | Receptor evaluated |
|----------------------------|--------------------|
| Adenosine                  | A1, A2A, A3        |
| Adrenergic II              | α1, non-selective, α2, non-selective, β1, β2 |
| Bradykinin                 | B1, B2             |
| Dopamine                   | D1, D2, D3, D4.4   |
| GABA                       | Non-selective      |
| Glutamate                  | AMPA, KAinste, NMDA|
| Histamine                  | H1, central, H2, H3|
| Melanocortin               | MC4                |
| Muscarinic                 | M1, M2, M3, M4     |
| Neurokinin                 | NK1                |
| Nicotinic                  | Neuronal, muscle-type|
| Opiate                     | δ, κ, μ            |
| Platelet-activating factor | PAF                |
| Serotonin                  | 5-HT1A, 5-HT2A, 5-HT2C, 5-HT3, 5-HT4, 5-HT7 |

CXCR1, and CXCR2, and did not inhibit the chemotaxis of human cells to the chemokines CCL2, CCL27, CCL20, CCL19, CXCL8, CXCL11, and CXCL12 (data not shown). These results indicate that CP-481,715 is >100-fold selective for CXCR1. In addition to its selectivity as compared with other receptors, CP-481,715 is also selective for the human receptor and does not inhibit the effects of CCL3 on mouse, rat, guinea pig, dog, rabbit, or monkey leukocytes as demonstrated with binding, chemotaxis, and whole blood studies at concentrations up to 25 μM (data not shown).

**Effects of CP-481,715 on CCL3-mediated CD11b Up-regulation in Human Whole Blood**—A crucial attribute for therapeutic agents is their ability to retain activity in the presence of high protein concentrations. This may be especially true for chemokine receptor antagonists since the cells migrating into inflammatory sites in response to a chemotactic gradient would come from the peripheral blood. To address this, we measured two components of CXCR1 activity, integrin up-regulation and cytoskeletal rearrangement in whole blood. The up-regulation of integrins, such as CD11b, mediate the firm adherence of leukocytes to the endothelium, a prerequisite to cell infiltration. As shown in Fig. 8, CP-481,715 dose-dependently inhibited CD11b up-regulation on monocytes in human blood (average IC50 from 10 different donors = 160 nM). As an alternative assessment of whole blood activity that may be more indicative of cell migration, we also measured actin polymerization on monocytes in response to CCL3. CP-481,715 also inhibited CCL3-induced actin polymerization in human blood with an IC50 of 58 nM (Fig. 8).

**Schild Analysis of the Interaction of CP-481,715 in Whole Blood**—To further characterize the antagonist properties of CP-481,715, we assessed the effect of multiple concentrations of CP-481,715 on the amount of CCL3 necessary to up-regulate CD11b in human whole blood. As shown in Fig. 9A, increasing concentrations of CP-481,715 produced a parallel rightward shift of the CCL3 dose response curve, suggestive of competitive antagonism. The amount of CP-481,715 necessary to shift the CCL3 EC50 10-fold was determined to be 211 ng/ml (436 nM). An analysis of these data by Schild regression (Fig. 9B) gave a slope of 1.23, which is not significantly different from unity (32), and a pA2 value of 7.068.

**Effects of CP-481,715 on Monocyte Chemotactic Activity in Synovial Fluid**—To better understand its therapeutic potential and its ability to antagonize CXCR1 ligands in a physiologically relevant medium, we assessed the ability of CP-481,715 to inhibit monocyte chemotaxis induced by synovial fluid. A total of 15 synovial fluid samples were obtained from rheumatoid arthritis patients. All subjects had active disease of >4 years duration and were receiving non-steroidal anti-inflammatory agents and methotrexate. All 15 synovial fluid samples caused significant in vitro monocyte chemotactic activity as demonstrated using standard Boyden chamber analysis, which could be partially inhibited with neutralizing antibodies to macrophage inflammatory protein 1α and RANTES. CP-481,715 neutralized 90% of this monocyte chemotactic activity in 11/15 (73%) of the samples (Table II). The average amount of CP-481,715 necessary to inhibit chemotaxis was 1.3 μM and ranged from 0.05 to 6.5 μM, depending on the sample. These studies illustrate the ability of CP-481,715 to inhibit monocyte chemotaxis induced by complex biological fluids, demonstrate its ability to inhibit the effects of CXCR1 ligands in their natural forms, and further suggest the therapeutic potential of CP-481,715 in rheumatoid arthritis.

**Effects of CP-481,715 on CCL3 and TNF-stimulated MMP9 Production by Monocytes**—MMPs have been implicated in the pathogenesis of rheumatoid arthritis. In synovial fluid samples, MMP9 levels were detected in the range of 10–500 ng/ml. Because CCL5 has been shown to induce monocyte MMP9 production, we next assessed the ability of CP-481,715 to inhibit this response. Human mononuclear cells cultured with 20 nM CCL5 induced 8 ng/ml MMP9, which was totally inhibited...
by CP-481,715 (IC₅₀ = 52 nM), confirming previous reports that the ability of CCL5 to induce MMP9 was CCR1-dependent (12) (Fig. 10). TNFα (10 ng/ml) induced 150 ng/ml MMP9 by mononuclear cells, which was partially inhibited (47%) by CP-481,715, indicating that TNFα exerts its effects in part by stimulating CCR1 ligands. Although it is difficult to assess due to the high level of MMP9 already present, CP-481,715 also inhibited 30% of the MMP9 induced by synovial fluid on human cells (data not shown). These studies suggest that CCR1 may play a role in MMP9 production in rheumatoid arthritis and suggest that a CCR1 antagonist may dampen this production.

DISCUSSION

We describe the pharmacological characteristics of a novel CCR1 antagonist that has potential utility for the treatment of inflammatory diseases such as rheumatoid arthritis. CP-481,715 binds to human CCR1, prevents ligand interaction, and blocks the ability of CCR1 ligands to cause G-protein signaling, calcium mobilization, integrin up-regulation, cell migration, and monocyte protease secretion. CP-481,715 is structurally unique as compared with other small molecular weight CC chemokine receptor antagonists (33–36) in that it does not contain a positively charged group (i.e. a basic nitrogen or quaternary amine) thought to bind to acidic residues in the receptor (e.g. Glu²⁹¹). In support of its therapeutic potential, CP-481,715 retains activity under high protein concentrations (e.g. whole blood) and is able to block chemotaxis induced by CCR1 ligands present in a complex biological fluid (e.g. rheumatoid arthritis synovial fluid).

Agents that block receptor-ligand interactions and function can be classified by their relative level of agonist and antagonist activities, e.g. full antagonist, partial agonist, inverse agonist. Our studies provide evidence that CP-481,715 behaves as a full and competitive antagonist for human CCR1. CP-481,715 lacks any intrinsic agonist activity as determined by its lack of direct effects on intracellular calcium mobilization. Furthermore, CP-481,715 is not an inverse agonist, as assessed by the lack of direct effects on GTP₆₅ₛ incorporation. CP-481,715 totally blocked the ability of several CCR1 ligands to stimulate multiple functions on CCR1-transfected cells and on cells endogenously expressing CCR1 (THP-1 cells, primary human monocytes). Furthermore, the amount of CP-481,715 necessary to inhibit functional activity was directly proportional to the concentration of agonist utilized. A Schild analysis of whole blood dose-response curves indicated a slope of 1.23, close (within 95% confidence) to a value of 1.0, necessary to classify it as a competitive antagonist (32).

The ability of CP-481,715 to inhibit cell activity stimulated by CCR1 ligands appears to be solely due to its receptor antagonism and not through nonspecific effects on cell function or through direct effects on CCR1 ligands. CP-481,715 inhibited GTP₆₅ₛ incorporation, the first step after receptor ligation, at concentrations similar to that necessary for inhibition of calcium mobilization, chemotaxis, and CD11b up-regulation. CP-481,715 also had no effect on calcium mobilization and chemo-

![FIG. 9](image-url)

**TABLE II**

| Patient number | IC₅₀ CP-481,715 |
|---------------|-----------------|
| 1             | >25             |
| 2             | 0.050           |
| 3             | 0.025           |
| 4             | >25             |
| 5             | 0.250           |
| 6             | 0.090           |
| 7             | >25             |
| 8             | 1.2             |
| 9             | 0.090           |
| 10            | >25             |
| 11            | 6.5             |
| 12            | 0.250           |
| 13            | 5.2             |
| 14            | 0.290           |
| 15            | 0.290           |
taxis induced by non-CCR1 ligands in the same cell lines. In addition, CP-481,715 did not act directly on CCR1 ligands, as supported by its lack of effect on CCL5- and CCL5-induced intracellular calcium mobilization on CCR5-transfected cells. As such, we conclude that CP-481,715 did not act non-specifically on cells and exerted its effects solely as a CCR1 antagonist and not through modulation of ligand activity or intracellular signaling cascades.

A CCR1 antagonist has the potential to block several steps in the inflammatory response. First, it may prevent the firm adherence of cells to the endothelium during an inflammatory process by inhibiting CD11b (Mac-1) up-regulation. This integrin-mediated adherence is a necessary prerequisite to cell migration under shear flow (37). Second, CCR1 blockade should prevent the movement of cells into tissue as illustrated by the ability of CP-481,715 to block the chemotaxis of monocytes in response to purified CCR1 ligands as well as those in inflammatory fluid. Third, a CCR1 antagonist may also directly decrease tissue damage by leukocytes at inflammatory sites as illustrated by the ability of CP-481,715 to block monocyte MMP9 secretion. This inhibition of MMP9 production was observed not only in response to CCL5 but also TNFα (shown to induce CCL5 in culture), indicating that TNFα-induced MMP9 production is partially mediated through the generation of CCR1 ligands. Given the important role of TNF in the pathogenesis of inflammatory diseases such as rheumatoid arthritis (3, 4), this observation adds further support to the potential therapeutic utility of CP-481,715.

Although a number of chemokines have been reported to be expressed in the synovial fluid of rheumatoid arthritis patients, determining the relevant chemokines that mediate inflammation is difficult. Simply measuring their levels can give misleading results due to the complexity of synovial fluid, which often requires extraction and/or precipitation before analysis (19–21). Furthermore, regardless of the chemotactic factors present, those responsible for cell infiltration are dependent on their relative levels and the expression of the appropriate receptors on peripheral cells. Previous studies have demonstrated that the in vitro monocyte chemotactic activity of synovial fluid could be partially decreased (up to 49%) using CCR1 ligands in synovial fluid. For example, some CCR1 ligands have been reported to have increased agonist activity after cleavage by peptidases such as CD26, which could have conceivably affected the amount of CP-481,715 necessary to inhibit activity (39). Further studies will be needed to investigate these possibilities. Nonetheless, the ability of CP-481,715 to inhibit chemotaxis induced by complex inflammatory fluid may more realistically demonstrate its anti-inflammatory potential in clinic and the concentration of the compound that may be necessary to inhibit cell infiltration.

Our studies highlight a structurally novel and selective CCR1 antagonist. The ability of CP-481,715 to block several functions mediated by CCR1 ligands and to retain potency in whole blood underscores its clinical potential. Furthermore, our studies uncover effects of CCR1 antagonism not previously reported, including its predominant role on the monocyte chemotactic activity in synovial fluid and its effects on TNFα-mediated matrix metalloproteinase production. These studies support the possibility that CP-481,715 may be beneficial for the treatment of rheumatoid arthritis and other inflammatory diseases in which CCR1 has been implicated (40, 41).
33. Luttichau, H. R., and Schwartz, T. W. (2000) Curr. Opin. Drug Discov. Devel. 3, 610–623
34. Horuk, R., and Ng, H. P. (2000) Med. Res. Rev. 20, 155–168
35. Liang, M., Mallari, C., Rosser, M., Ng, H. P., May, K., Monahan, S., Busman, J. G., Islam, I., Ghannam, A., Buckman, R., Shaw, K., Wei, G. P., Xu, W., Zhao, Z., Ho, E., Shen, J., Oanh, H., Subramanyam, B., Vergona, R., Taub, D., Dunning, L., Harvey, S., Snider, R. M., Hesselgesser, J., Morrissey, M. M., and Perez, H. D. (2000) J. Biol. Chem. 275, 19000–19008
36. Mirzadegan, T., Diehl, F., Ebi, B., Bhakta, S., Polsky, I., McCarley, D., Mulkins, M., Weatherhead, G. S., Lapierre, J. M., Dankwardt, J., Morgans, D., Jr., Wilhelm, R., and Jarnagin, K. (2000) J. Biol. Chem. 275, 25562–25571
37. Dunne, J. L., Ballantyne, C. M., Beaudet, A. L., and Ley, K. (2002) Blood 99, 336–341
38. Nanki, T., Hayashida, K., El-Gabalawy, H. S., Suson, S., Shi, K., Girschick, H. J., Yavuz, S., and Lipsky, P. E. (2000) J. Immunol. 165, 6590–6598
39. Proost, P., Menten, P., Struyf, S., Schutyser, E., DeMeester, I., and Van Damme, J. (2000) Blood 96, 1674–1680
40. Rottman, J. B., Slavin, A. J., Silva, R., Weiner, H. L., Gerard, C. G., and Hancock, W. W. (2000) Eur. J. Immunol. 30, 2372–2377
41. Gao, W., Topham, P. S., King, J. A., Smiley, S. T., Csizmadia, V., Lu, B., Gerard, C. J., and Hancock, W. W. (2000) J. Clin. Invest. 105, 35–44