Identification and Characterization of Small Molecule Functional Antagonists of the CCR1 Chemokine Receptor*

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Joseph Hesselgesser, Howard P. Ng, Meina Liang, Wei Zheng, Karen May, John G. Bauman, Sean Monahan, Imadul Islam, Guo Ping Wei, Ameen Ghannam, Dennis D. Taub, Mary Rosser, Richard Horuk, H. Daniel Perez, and Richard Horuk

From the Departments of Immunology and Pharmaceuticals Discovery, Berlex BioSciences, Richmond, California 94806 and the Laboratory of Immunology, NIA, National Institutes of Health, Baltimore, Maryland 21224

The CC chemokines macrophage inflammatory protein-1α (MIP-1α) and RANTES (regulated on activation normal T cell expressed) have been implicated in rheumatoid arthritis and multiple sclerosis. Since their effects are mediated through the CCR1 chemokine receptor, we set up a small molecule CCR1 antagonist program to search for inhibitors. Through high capacity screening we discovered a number of 4-hydroxypiperidine compounds with CCR1 antagonist activity and report their synthesis and in vitro pharmacology here. Scatchard analysis of the competition binding data revealed that the compounds had Ki values ranging from 40 to 4000 nM. The pharmacological profile of the most potent member of this series, compound 1 (2-2-diphenyl-5-(4-chlorophenyl)piperidin-yl)valeronitrite), was further evaluated. Compound 1 showed concentration-dependent inhibition of MIP-1α-induced extracellular acidification and Ca2+ mobilization demonstrating functional antagonism. When given alone, the compound did not elicit any responses, indicating the absence of intrinsic agonist activity. Compound 1 inhibited MIP-1α- and RANTES-induced migration in peripheral blood mononuclear cells in a dose-responsive manner. Selectivity testing against a panel of seven transmembrane domain receptors indicated that compound 1 is inactive on a number of receptors at concentrations up to 10 μM. This is the first description of CCR1 receptor antagonists that may be useful in the treatment of chronic inflammatory diseases involving MIP-1α, RANTES, and CCR1.

The directed migration of select populations of leukocytes from the circulation to sites of inflammation is an integral part of the immune response. The chemokines are a diverse group of proteins that play an important role in this process (1). They are classified into two major groups, CXC and CC, based on the position of the first two of their four invariant cysteines (2). Each of the chemokines recognizes and induces the chemotaxis of a particular subset of leukocytes. For example, the CXC chemokines, like IL-8 and melanoma growth stimulatory activity, mainly chemoattract and activate neutrophils, in contrast to the CC chemokines, like RANTES and monocyte chemoattractant protein, which preferentially attract T lymphocytes and monocytes and induce their activation by producing changes in cellular morphology, transient increases in cellular calcium concentration, and the up-regulation of surface adhesion proteins.

The chemokines produce their biologic effects by interacting with specific receptors on the cell surface of their target cells (3). To date, 14 different chemokine receptors including eight CC chemokine receptors have been identified by cloning (4, 5). All of these receptors are characterized by a heptahelical structure and belong to a superfamily of serpentine receptors that are coupled to guanine nucleotide-binding proteins (G-proteins) (6).

Occasionally the immune system can turn upon its host, giving rise to chronic inflammation and disease. Given their important role in this process, chemokines have been implicated in the pathophysiology of autoimmune diseases like multiple sclerosis and rheumatoid arthritis. For example a recent study by Karpus et al. (7) provides strong in vivo concept validation for a role of MIP-1α in a mouse experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis. These investigators were able to show 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. In addition Godiska et al. (8) have shown an up-regulation of mRNA for a number of chemokines, including MIP-1α, in EAE.

A number of reports have shown that both RANTES mRNA and protein appear to be up-regulated in rheumatoid arthritis (9, 10). For example, cultured synovial fibroblasts isolated from rheumatoid patients when treated with IL-1 were shown to up-regulate the expression of RANTES both at the mRNA and protein levels (9). Similarly Snowden et al. have used reverse transcriptase-polymerase chain reaction to detect RANTES mRNA in synovial tissue samples from patients with rheumatoid arthritis (10).

Based on these studies there is strong evidence in support of the concept that the chemokines RANTES and MIP-1α play an important role in the pathogenesis of multiple sclerosis and rheumatoid arthritis. Since MIP-1α and RANTES are ligands for CCR1, this receptor, located on circulating mononuclear cells, is a prime therapeutic target for these debilitating diseases. These studies then provided the rationale for establishing a program to inhibit MIP-1α- and RANTES-mediated biological activities by developing highly potent and specific non-peptide CCR1 receptor antagonists. We developed an empirical screening program to discover potential inhibitors of 125I-MIP-1α binding to the CCR1 receptor. We describe here a
series of 4-hydroxypiperidines that possess functional antagonist activity at the CCR1 receptor. The compounds in this series have $K_i$ values of inhibition of chemokine binding ranging from 40 to 4000 nM and are functional antagonists.

**EXPERIMENTAL PROCEDURES**

**Materials—**Unlabeled chemokines were from Peprotech (Rocky Hill, NJ). 125I-Labeled chemokines were obtained from NEN Life Science Products.

**Synthesis of Compounds—**Compounds 1–7 (see Fig. 1) were synthesized in a manner similar to that outlined as follows. To a solution of diphenylacetonitrile (2.0 g, 10.3 mmol) in 100 ml of N,N-dimethylformamide was carefully added sodium hydride (0.5 g, 12.4 mmol, 60% in oil) in small portions. The mixture was stirred at ambient temperature for 15 min, and then 1-chloro-3-isopropane (3.1 g, 15 mmol) was added in one portion. The mixture was stirred at ambient temperature. After 20 h, the reaction was quenched with 200 ml of water and extracted with two portions of ethyl acetate (200 ml). The combined organic extracts were washed with water (2 × 400 ml) and then brine (400 ml), dried over MgSO4, filtered, and concentrated in vacuo to a yellow oil. Purification by flash column chromatography on silica gel afforded 2.0 g from 40 to 4000 nM and are functional antagonists.

**Chemokine Binding Studies—**High capacity screening assays were performed in 96-well V-bottom microtiter plates (Nunc, polysurface) in a total volume of 100 µl. 125I-Labeled chemokines (1.25, 12.5, and 125 nM) were incubated with various concentrations of compounds at room temperature for 30–40 min. The reactions were terminated by harvesting through a GF/B filter (Whatman, Maidstone, UK). PCl (Packard) contained fitted filtration and cell-agarose mixture was spotted into the center of a disposable polycarbonate cell capsules (Molecular Devices) (16). The cell numbers in each well were about 300,000–600,000. To measure the rate of acidification, the assembled cell capsules with the agarose-entrapped cells were loaded into the chambers of the microphysiometer (Molecular Devices). The chambers were perfused with the low buffering media at a rate of 100 µl/min. For each cycle of operation, the cells were perfused with the media for 50 s, and then the flow was interrupted for 40 s, during which the rate of acidification of the media was measured and recorded. The rate of flow was reversed, and the chamber temperature was 37 °C. After stabilization, the cells were perfused with the indicated concentrations of chemokine and/or compound for about 100 µl during which the rate of acidification was measured.

**Cytosolic Ca2+ Measurements in Hek Cells—**HEK 293 cells expressing the CCR1 receptor were loaded by incubation with 10 nM MIP-1α for 30–40 min at room temperature. Prior to transfection, the cells were detached by trypsinization and plated into flasks at least 48 h prior to the experiment. HEK 293 cells expressing the CCR1 receptor were detached by shaking, washed once in PBS, and resuspended in the assay buffer (130 mM NaCl, 5 mM KCl, 1 mM MnCl2, 50 mM Tris, 30 µM β-lactamase, 0.1% bovine serum albumin, pH 7.4) to about 1.1 × 106 cells/ml. Cells (about 8000 cells/well) were incubated with 125I-MIP-1α (specific activity 2200 Ci/mmol; about 15,000–20,000 cpm/well) in the presence and absence of varying concentrations of compounds at room temperature for 30–40 min. The reactions were terminated by harvesting through a GF/B filter (Whatman, Maidstone, UK). PCl (Packard) contained fitted filtration. The dose curves of each compound were fitted to the log-logit equation (linear) with an EXCEL spread-sheet. The $K_i$ values were then calculated by dividing the IC50 by 1.025. For other binding assays, cells (5 × 105 cells/ml) were incubated in PBS with 125I-labeled ligands (0.2 nM) and varying concentrations of unlabeled ligands or compounds at 4 °C for 1 h. The incubation was terminated by removing aliquots from the cell suspension and separating cells from buffer by centrifugation through a silicone/paraffin oil mixture as described previously. (15) Nonspecific binding was determined in the presence of 1 µM unlabeled ligand. The data were curve fit with the computer program IGOR (WaveMetrics) to determine the affinity ($K_i$), number of receptor sites.

**Measurement of External Acidification with the Microphysiometer—**THP-1 cells were washed once with a modified RPMI 1640 medium containing 10% FBS, 1% L-glutamine, 1% sodium pyruvate, 1% nonessential amino acids (Life Technologies, Inc.), 2.2% bovine serum albumin and resuspended in the same media at a density of 1 × 106 cells/0.1 ml. Cell suspension of 150 µl was mixed with 50 µl of agarose cell entrapment medium (Molecular Devices), and 7 µl of the cell-agarose mixture was spotted into the center of a disposable polycarbonate cell capsules (Molecular Devices) (16). The cell numbers in each well were about 300,000–600,000. To measure the rate of acidification, the assembled cell capsules with the agarose-entrapped cells were loaded into the chambers of the microphysiometer (Molecular Devices). The chambers were perfused with the low buffering media at a rate of 100 µl/min. For each cycle of operation, the cells were perfused with the media for 50 s, and then the flow was interrupted for 40 s, during which the rate of acidification of the media was measured and recorded. The rate of flow was reversed, and the chamber temperature was 37 °C. After stabilization, the cells were perfused with the indicated concentrations of chemokine and/or compound for about 100 µl during which the rate of acidification was measured.

**Chemotaxis—**Cell migration was examined using a 48-well microchemotaxis assay as described previously (17). Briefly, various concentrations of chemokines (1.25, 12.5, and 125 nM) were placed in the lower wells of a 48-well microchemotaxis chamber. PMBCs (2 × 106 cells/ml) derived from a normal healthy leukopheresis donor (CIS0003, age 27, male, Caucasian) treated with or without compound 1 at concentrations of 35, 12.5, and 125 nM were placed in the lower wells of the upper chambers. The chambers were incubated for 4 h at 37 °C (a time period over which chemokin equilibrium between the upper and lower chambers is optimally achieved) after which the filters were scraped, washed, fixed with methanol, and stained with Diff-Quik. Cell migration was measured by counting the number of cells attached to the lower surface of the filter in six high power fields, and each concentration of chemokine was tested in either triplicate or sets of six wells. The results were expressed as the number of migrating cells per six high power fields (± S.D.).
sclerosis and rheumatoid arthritis, this receptor is a prime therapeutic target. Although peptide antagonists of chemokine receptors have been described, they suffer from poor metabolic stability and oral bioavailability, thereby limiting their therapeutic utility. For this reason, we set out to discover potent, selective, small molecule antagonists of the CCR1 receptor for use as potential therapeutics in autoimmune diseases like multiple sclerosis and rheumatoid arthritis.

Seven-transmembrane domain G-protein-coupled receptors have been an extremely fertile source of biological targets in the pharmaceutical industry. The actual drugs that interact with this receptor superfamily are too numerous to detail but include antihistamines, antipsychotics, antidepressants, antimuscarinics, \(\alpha\)- and \(\beta\)-adrenoreceptor agonist and antagonists, opiates, prostanoids, etc. More recently, compound library

\[\text{Table I}\]

| Compound | \(K_i\) of MIP-1\(\alpha\) binding\(^a\) | \(K_i\) of RANTES binding\(^a\) |
|----------|----------------------------------|----------------------------------|
| MIP-1\(\alpha\) | 2 ± 0.2 | 0.3 ± 0.1 |
| RANTES | 7 ± 0.5 | 21 ± 0.4 |
| 1 | 40 ± 6 | 60 ± 3 |
| 2 | 347 ± 65 | 268 ± 17 |
| 3 | 651 ± 27 | ND\(^b\) |
| 4 | 153 ± 8 | 618 ± 84 |
| 5 | 715 ± 27 | ND\(^b\) |
| 6 | 4000 ± 600 | ND\(^b\) |
| 7 | 5000 \(^c\) | 6 \(^c\) |

\(^a\) S.E. for \(n \geq 2\).
\(^b\) ND, not determined.
\(^c\) Data from Ref. 22.
screening has proven successful in the discovery of antagonists of neuropeptide receptors, i.e. Pfizer’s substance P antagonist (18), Merck’s CCK antagonists (19), Sterling’s bradykinin antagonist (20), and Sanofi’s neurotensin antagonist (21). Thus, based on these and numerous other additional (endothelin, vasopressin, angiotensin II, etc.) past successes using an empirical file screening approach, it seemed reasonable to pursue leads for the CCR1 receptor in this manner.

The initial approach involved empirical screening of our available compound libraries to discover potential CCR1 receptor antagonists. Initial screening of 125I-MIP-1α binding to the CCR1 receptor transfected into the HEK 293 cell line yielded 258 compounds that demonstrated 50% inhibition of binding at a concentration of 5 μM. Several of these active compounds belonged to the same structural family, the 4-hydroxypiperidines (Fig. 1) and were investigated in greater detail.

The Kᵢ values of these compounds were determined from concentration-response curves based on their ability to displace the specific binding of 125I-labeled MIP-1α in HEK 293 cells stably expressing the CCR1 receptor. Competition binding studies for the 4-hydroxypiperidine compounds 1 and 2 (Fig. 1) are shown in Fig. 2A. Compounds 1 and 2 were able to competitively displace radiolabeled MIP-1α with an IC₅₀ of 40 and 347 nM, respectively. In addition, the 4-hydroxypiperidines were shown to displace radiolabeled RANTES binding from the same transfected cells with similar IC₅₀ values (Fig. 2B and Table I). These data demonstrate that templates possessing the 4-hydroxypiperidine moiety are potent inhibitors of RANTES and MIP-1α binding to the CCR1 receptor.

The 4-hydroxypiperidine moiety was optimized by synthesizing a number of analogs (Fig. 1) and examining their ability to inhibit MIP-1α binding to CCR1 (Table I). Several interesting points are worth noting from this study. First, substitution of the piperidinol phenyl segment by a halogen (compound 1) improves activity over compound 3, which does not have a halogen. Other groups such as OMe or CF₃ (compounds 2 and 4) appeared to be less favorable. In addition, the phenyl ring appears to be important for activity, since replacement by a naphthyl group (compound 5) reduced potency almost 20-fold, while replacement by a butyl group (compound 6) lead to an almost total loss of activity.

Interestingly, we were recently made aware of a patent filed by Takeda Chemical Industries, Ltd. in which they describe a...
urea piperidine derivative of the nitrile group (compound 7), which had a reported IC$_{50}$ for MIP-1$\alpha$ binding of 5000 nM and a reported IC$_{50}$ for RANTES binding of 6 nM (22).

![Figure 6](image)

**FIG. 6.** 4-Hydroxypiperidine analogs inhibit the ability of MIP-1$\alpha$ and RANTES to induce chemotaxis in PBMCs. **A**, PBMCs were examined for their ability to migrate in response to various CC and CXC chemokines (1.25 nM) in the presence or absence of the CCR1 antagonist compound 1 (20 nM) as described under “Experimental Procedures.” B, PBMCs were stimulated with increasing concentrations of MIP-1$\alpha$ (1.25, 12.5, and 125 nM) in the presence of 16-fold excess concentrations of compound 1 (20, 200, and 2000 nM). C, PBMCs were stimulated with increasing concentrations of RANTES (1.25, 12.5, and 125 nM) in the presence of 16-fold excess concentrations of compound 1 (20, 200, and 2000 nM).

The medicinal chemistry outlined above identified a number of small molecules as potent inhibitors of chemokine binding to the CCR1 receptor. To determine whether these molecules were also functional antagonists of the CCR1 receptor, we measured their ability to inhibit MIP-1$\alpha$- and RANTES-induced biological responses by microphysiometry (16). The biological activity of MIP-1$\alpha$ was initially assessed by stimulating THP-1 cells, which have been shown to express CCR1 (23), with the chemokine and measuring their increase in extracellular acidification rate. As shown in Fig. 3, MIP-1$\alpha$ induced a rapid increase in the extracellular acidification rate, reaching a maximum after about 2 min and returning close to baseline levels within 8–10 min. The MIP-1$\alpha$ effect on THP-1 cells was dose-dependent, and at 1 nM it produced an approximately 50–60% increase in extracellular acidification. These kinetics are quite similar to those reported for chemokines in human monocytes and in transfected cell lines (24, 25). Pretreatment of THP-1 cells with increasing concentrations of compound 1 dose-responsively inhibited the ability of MIP-1$\alpha$ to induce changes in the metabolic activity of the cells (Fig. 3). The concentration of compound 1 required to half-maximally inhibit 1 nM MIP-1$\alpha$ was 124 nM, about 6-fold higher than that predicted from the binding constants for compound 1 and MIP-1$\alpha$, which were 40 and 2 nM, respectively (Fig. 2). Compound 1 was also able to inhibit RANTES-induced changes in THP-1 cells, similar to the inhibition demonstrated for MIP-1$\alpha$ (data not shown).

**TABLE II**
Ancillary pharmacology of compound 1

| Receptor    | $K_i$ (nM) | Ratio $^a$ |
|-------------|------------|------------|
| CCR1        | 0.04       | 1          |
| CCR5        | >10        | >250       |
| CXCR2       | >10        | >250       |
| CXCR4       | >10        | >250       |
| Adenosine A3| 7          | 175        |
| Adrenergic $\alpha$-2A | 8          | 250        |
| Adrenergic $\beta$-2 | >10        | >250       |
| Bradykinin  | >10        | >250       |
| Cannabinoid | >10        | >250       |
| Dopamine D1 | >10        | >250       |
| Dopamine D2L| 2          | 50         |
| Endothelin  | >10        | >250       |
| Mucarinc M1 | 7          | 175        |
| Mucarinc M2 | 9          | 225        |
| Neuropeptide Y | >10        | >250       |
| Serotonin 5-HT$_6$ | 8          | 200        |

$^a$ $K_i$ compound 1 on test receptor/$K_i$ compound 1 on CCR1.
chemokine (Fig. 4). The concentration of compound 1 required to half-maximally inhibit 10 nM MIP-1α was around 200 nM, well in line with that predicted from the binding constants for compound 1 and MIP-1α (Fig. 2). Compound 1 was also able to effectively inhibit the RANTES-induced increase in Ca\(^{2+}\) (data not shown). To determine the reversibility of this effect, cells incubated with 1000 nM compound 1 were washed twice in PBS to remove the inhibitor and then stimulated with 10 nM MIP-1α. The washed cells were able to respond to the chemokine in a manner similar to that of cells untreated with the antagonist (Fig. 4).

Further functional characterization of the antagonism by compound 1 was investigated in cells transfected with the chemokine receptors CCR5 and CXCR2. CCR5 is a related chemokine receptor that responds to MIP-1α, MIP-1β, and RANTES, while CXCR2 responds to IL-8 and melanoma growth stimulatory activity as agonists. Importantly, while compound 1 antagonized the effects of RANTES or MIP-1α on the Ca\(^{2+}\) response in cells expressing CCR1, no such attenuation of MIP-1β- or IL-8-stimulated Ca\(^{2+}\) flux was evident in cells expressing CCR5 and CXCR2, respectively (Fig. 5). This finding suggests selectivity of antagonism, consistent with the selectivity of this compound in binding studies (see below) and that the blockade of the Ca\(^{2+}\) signal is not due to a nonspecific effect.

Chemokines were originally defined and classified as potent leukocyte chemoattractants, mediating their effects through G-protein-coupled receptors (26). Thus, we assessed the ability of the CCR1 antagonist compound 1 to attenuate the directed migration of PBMCs stimulated with a number of CC and CXC chemokines. As shown in Fig. 6, a 16-fold excess of compound 1 dose-responsively inhibited the MIP-1α- and RANTES-mediated migration of PBMCs but had no effect on the migration of PBMCs stimulated with MIP-1β, monocyte chemoattractant protein, or stromal cell-derived factor-1α. These data demonstrate that compound 1 is a potent antagonist for CCR1 but has no effects on the related chemokine receptors CCR5, CXCR2, and CXCR4.

The specificity of antagonism of the CCR1 receptor antagonists versus other receptors or biological targets is a critical component to establishing a quality lead compound. This is particularly important, since the CCR1 receptor belongs to a superfamily of seven-transmembrane domain G-protein-coupled receptors that at the present time numbers well over 450 members and responds to a diverse array of ligands (27). Thus, the selectivity of the potential CCR1 antagonist compound 1 was tested in a panel of radioligand binding assays for activity with members of this superfamily of receptors, including related chemokine receptors and unrelated neurotransmitter receptors. It is quite encouraging that while the activity of this compound is under 40 nM at the CCR1 receptor, it is more than 250-fold less active for most of these other receptors, including the related chemokine receptor CCR5, which binds RANTES and MIP-1α with high affinity (Table I). The only potential cross-reactivity of compound 1 was with several biogenic amine neurotransmitter receptors, a result not surprising for a structure reminiscent of the typical neuroleptic/antidepressant structural motif (28, 29).

Although we have not established the direct mechanism by which these compounds are able to act as functional antagonists of the CCR1 receptor, we can rule out at least two formal possibilities. Since MIP-1α and RANTES are able to bind with high affinity to CCR1 and CCR5 but the CCR1 antagonist compound 1 only inhibited binding of the ligands to CCR1, these data would tend to rule out inhibition of ligand binding by direct interaction between the ligand and the compound. Compound 1 was also unable to inhibit MIP-1β binding to CCR5, IL-8, or melanoma growth stimulatory factor binding to CXCR2 and stromal cell-derived factor-1α binding to CXCR4, thus further demonstrating CCR1 receptor specificity. We can also rule out inhibition due to toxicity of the compounds, since, in Ca\(^{2+}\) flux experiments, the addition of the compound alone does not lead to an increase in intracellular Ca\(^{2+}\) by increasing uptake from the extracellular milieu as might be observed in cells that were semipermeable. We favor the idea that our compounds are directly competing for binding to specific sites on the CCR1 and thus antagonize the ability of the chemokine ligands to blockade a biologic effect by receptor blockade. Further studies will be carried out to determine the nature and site of interaction of the receptor-antagonist complex.

In summary, we have described here a class of highly potent nonpeptide antagonists of the CCR1 receptor. These compounds appear to be highly specific for the CCR1 receptor and are to our knowledge the first known examples of nonpeptide antagonists of a CC chemokine receptor. Classically, knowledge of the functional role of seven-transmembrane domain receptors has come from pharmacological studies using specific high affinity receptor antagonists. Thus, these compounds should also prove to be useful tools with which to evaluate the role of CCR1 receptors in normal physiology and in disease. Finally, this class of nonpeptide antagonists of the CCR1 receptor should hold promise for the future design of more potent, highly selective agents that could be useful therapeutically in treating autoimmune diseases like rheumatoid arthritis and multiple sclerosis.

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