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*

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Monocyte chemoattractant-1 (MCP-1) stimulates leukocyte chemotaxis to inflammatory sites, such as rheumatoid arthritis, atherosclerosis, and asthma, by use of the MCP-1 receptor, CCR2, a member of the G-protein-coupled seven-transmembrane receptor superfamily. These studies identified a family of antagonists, spiropiperidines. One of the more potent compounds blocks MCP-1 binding to CCR2 with a \( K_d \) of 60 nM, but it is unable to block binding to CXCR1, CCR1, or CCR3. These compounds were effective inhibitors of chemotaxis toward MCP-1 but were very poor inhibitors of MCP-1-mediated chemotaxis. The compounds are effective blockers of MCP-1-driven inhibition of adenylate cyclase and MCP-1- and MCP-3-driven cytosolic calcium influx; the compounds are not agonists for these pathways. We showed that glutamate 291 (Glu291) of CCR2 is a critical residue for high affinity binding and that this residue contributes little to MCP-1 binding to CCR2. The basic nitrogen present in the spiropiperidine compounds may be the interaction partner for Glu291, because the basicity of this nitrogen was essential for affinity; furthermore, a different class of antagonists, a class that does not have a basic nitrogen (2-carboxypyrroles), were not affected by mutations of Glu291. In addition to the CCR2 receptor, spiropiperidine compounds have affinity for several biogenic amine receptors. Receptor models indicate that the acidic residue, Glu291, from transmembrane-7 of CCR2 is in a position similar to the acidic residue contributed from transmembrane-3 of biogenic amine receptors, which may account for the shared affinity of spiropiperidines for these two receptor classes. The models suggest that the acid-base pair, Glu291 to piperidine nitrogen, anchors the spiropiperidine compound within the transmembrane ovoid bundle. This binding site may overlap with the space required by MCP-1 during binding and signaling; thus the small molecule ligands act as antagonists. An acidic residue in transmembrane region 7 is found in most chemokine receptors and is rare in other serpine receptors. The model of the binding site may suggest ways to make new small molecule chemokine receptor antagonists, and it may rationalize the design of more potent and selective antagonists.

Chemokines are a large family of small proteins that mediate attraction of leukocytes to inflammatory sites (1–3). The chemokine family shares a common pattern of disulfide bonds and a common overall tertiary structure as shown in solution or crystallographically determined structures (4–6). The chemokine family is divided into four subfamilies based on the number of residues between the first and second cysteine. Among the chemokines, the CC chemokine monocyte chemotactant-1 (MCP-1)\(^1\) has received a great deal of attention because of its involvement in diseases. MCP-1 expression is elevated in the inflamed synovium of rheumatoid arthritis, and its expression is reduced by anti-arthritis drugs (7, 8). Other work has shown that MCP-1 is elevated in asthmatic patients; the amount of elevation correlates with symptoms. MCP-1 expression is suppressed by successful immunotherapy (9–12). MCP-1 is elevated in human atherosclerosis and is elevated by consumption of high fat diets in monkeys and rabbits (13, 14). Treatment with MCP-1 neutralizing antibodies or other biological antagonists can reduce inflammation in a number of animal models; these include lung granuloma (15, 16), lipopolysaccharide-induced death (17), glomerulonephritis (17), delay type hypersensitivity in the skin (18), and adjuvant arthritis in MRL mice (19). Transgenic over expression of MCP-1 induces monocyte and T-cell migration to the site of expression in skin (20), lung (21), brain (22), and pancreas (23). Mice genetically deleted for the MCP-1 or its receptor CCR2 are protected from inflammation and atherosclerosis driven by bacterial products and high fat diets (24–26). These findings suggest that MCP-1 functions \textit{in vivo} as a monocyte and T-cell attractant and that MCP-1 is present and elevated during disease. These findings also demonstrate that modulation of MCP-1 expression or activity will be beneficial in treating inflammatory diseases.

Because of the involvement of MCP-1 in pathophysiological inflammatory diseases, we initiated a program to discover small molecule antagonists of the MCP-1 receptor. These efforts lead to the identification of several classes of compounds that inhibited MCP-1 binding to its receptor with affinities between 1 and 15 \( \mu \)M; this report details the properties of one class of inhibitors. We examined several of these compounds in detail with regard to binding affinity and antagonist function at several receptor sites. One class, the spiropiperidines, has affinities that range from 60 nM to inactive and has distinct structure activity relationships; we wished to understand the mechanism of action for these compounds and model their

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\(^1\) The abbreviations used are: MCP-1, monocyte chemoattractant-1; GPC, G-protein-coupled; TM, transmembrane region; CHO, Chinese hamster ovary; RANTES, regulated on activation normal T cell expressed; SP, spiropiperidene; 2CP, 2-carboxy-pyrrole.
interaction with CCR2 receptor. Using several types of function studies, we examined the effect of the compounds on several post-receptor signaling pathways simulated by MCP-1 and another CCR2 ligand, MCP-3. We also examined the effect of certain site-directed receptor mutations on the binding affinity of MCP-1 and several compounds. These studies revealed an unappreciated similarity between binding of compounds to biogenic-amine receptors and their binding to MCP-1 receptor. We have built models of the CCR2 receptor incorporating the mutagenesis data, the spiropiperidine structure activity relationships, and ligand binding and signaling models that we have previously presented (27, 28). These observations provide a model to understand the interaction of the small molecule chemokine antagonists with chemokine receptors and provide a path to the design of better antagonists.

MATERIALS AND METHODS

Compound Synthesis—Spiropiperidines were prepared by adding the appropriate ortho-lithiated tert-butoxy carbonyl-protected aniline (prepared from the tert-butoxy carbonyl-aniline and 2.2 eq of tert-butyl lithium in tetrahydrofuran at −78 °C) to the N-tertbutoxy carbonyl-4-piperidone followed by a spontaneous cyclization under (29). Yields averaged 60% after chromatography. Subsequent deprotection of the tert-butoxy carbonyl-protected spirobenzoxazinones using standard procedure (20 eq of trifluoroacetic acid in CH₃Cl) followed by alkylation with the appropriate alkyl bromide yielded the desired compounds (5 eq of Hunig’s base and 1.2 eq of the bromide in acetonitrile at reflux, 5 h. The compounds were purified by column chromatography or by preparative thin layer chromatography using 5:1 hexanes/CH₂Cl₂/MeOH mixtures. Each sample was >98% pure.

For preparation of 3-chlorobenzyl 2-pyrole carboxylic acid, 2-trifluoroacetylpyrrolo (25 g, 0.12 mol) was combined with sodium hydride (6.2 g, 0.15 mol) in dimethylformamide (300 ml) at 0 °C. 3-Chlorobenzyl bromide was added, and the resulting mixture was stirred for about 1 h at room temperature. The reaction was quenched with water followed by dilute sodium hydroxide (3 x 80 ml) and stirred for 2 h. Additional sodium hydroxide was added, and the water layer was washed with ether. Addition of 6 M HCl to the aqueous phase until the pH was 2 resulted in the precipitation of the desired carboxylic acid (75% pure). All compounds were characterized by NMR spectroscopy (¹H and ¹³C), IR spectroscopy, mass spectroscopy, melting point, and elemental analysis; the data were consistent with the desired structure.

Binding Assay—A detailed description of the binding assay is described in a previous manuscript (30). Briefly, binding was measured using membranes prepared from two cell types, THP-1 and CCR2-CHL cells. Each competition assay (Table I) was composed of cell membranes, 50 pmol ¹²⁵I-MCP, MCP buffer, protease inhibitors, and test compound. Equilibrium was achieved by incubation at 28 °C for 90 min. Membrane-bound ¹²⁵I-MCP was collected by filtration through GF/B filters pre-soaked in polyethylenimine and bovine serum albumin, followed by four rapid washes with approximately 0.5 ml of ice-cold buffer containing 0.5 M NaCl and 10 mM HEPES, pH 7.4. MCP buffer consists of 50 mM HEPES, pH 7.2, 1 mM CaCl₂, 5 mM MgCl₂, and 0.1% bovine serum albumin. Protease inhibitors include 0.1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, and 0.35 mg/ml pepstatin. THP-1 cells are a human monocytic cell line (ATCC TIB-202) that have been stably transformed with an expression vector bearing the human CCR2 receptor (30).

The other binding assays reported in Table I were performed as described above with appropriate changes in radioligand, buffer, temperature, and cell or tissue source. Thus, CCR1 and CXCR1 binding assays used 50 pmol ¹²⁵I-MIP1a or ¹²⁵I-interleukin-8, a buffer composed of 50 mM HEPES, 5 mM MgCl₂, 1 mM CaCl₂, 0.1% bovine serum albumin, and a pH of 7.4, a temperature of 28 °C and 100,000 CHO-CCR1 cells or CHO-CXCR1 cells. These are performed in CHO lasting comedy using the pSW104 vector containing the human CCR1 cDNA or the human CXCR1 cDNA. The α₅ or α₇ binding assay used 300 pmol [¹²⁵I]prazosin a buffer composed of 50 mM Tris-Cl, 0.5 mM EDTA, pH 7.4, a temperature of 28 °C, and 50,000 CHO-α₅ or CHO-α₇ membranes from transformed CHO cell lines containing the pSW104 vector containing the human α₅ or α₇ adrenergic receptor. The SHT₁a binding assay used 300 pmol [¹²⁵I]H-hydroxyaminopropionamidetetranil, a buffer composed of 50 mM Tris-Cl, 0.5 mM EDTA, pH 7.4, a temperature of 32 °C, and rat membranes containing 0.25 mg/ml protein. All the binding buffers also contained 100 μM phenylmethylsulfonyl fluoride and 1 μM leupeptin. The biogenic amine binding assays used 0.1 mM NaCl to wash the glass fiber filters, and the chemokine assay filters were washed as for the CCR2 assay. The cell lines bearing biogenic amine receptors were kindly gift from David Chang and Richard Panor (Roche BioScience).

Freeze-thaw lysed L1.2 cell clones bearing the wild type receptor and mutant receptors were used for saturation binding experiments with MCP-1 (Table I) and for competition experiments with MCP-1 and various compounds (Table II). Freeze-thaw lysed cells were prepared from washed centrifuge-packed cell pellets, which had been stored at −80 °C prior to binding analysis. These cells were thawed at room temperature and refrozen once. Between 1 x 10⁶ and 1 x 10⁷ freeze thawed cells were used for saturation binding experiments. These experiments used ¹²⁵I-MCP-1 at 440 Ci/mmol and varied the MCP-1 concentration between 3 and 0.023 nM. Data analysis for these saturation experiments was performed as described previously (31). Competition experiments using the L1.2 cell bearing mutant receptors also used freeze-thaw lysed cells; 1 x 10⁶ wild type bearing cells and 5 x 10⁵ mutation bearing cells were used for the competition experiments.

### Binding Site of Small Molecule MCP-1 Receptor Antagonists

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Kᵦ⁹⁰</th>
<th>Number of receptors/cell</th>
<th>Surface expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0.06 ± 0.01</td>
<td>21000 ± 8000</td>
<td>302 ± 147</td>
</tr>
<tr>
<td>D284A</td>
<td>0.27 ± 0.14</td>
<td>36000 ± 17000</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>D284N</td>
<td>1.58 ± 0.45</td>
<td>33000 ± 20000</td>
<td>246 ± 92</td>
</tr>
<tr>
<td>D284K</td>
<td>&gt;3</td>
<td>UTM</td>
<td>121 ± 45</td>
</tr>
<tr>
<td>D284E</td>
<td>0.89 ± 0.36</td>
<td>12000 ± 6000</td>
<td>81 ± 36</td>
</tr>
<tr>
<td>D284Q</td>
<td>0.73 ± 0.33</td>
<td>14000 ± 13000</td>
<td>102 ± 67</td>
</tr>
<tr>
<td>D284A/E291A</td>
<td>&gt;3</td>
<td>UTM</td>
<td>73 ± 31</td>
</tr>
<tr>
<td>D284N/E291Q</td>
<td>&gt;3</td>
<td>UTM</td>
<td>177 ± 96</td>
</tr>
<tr>
<td>D284K/E291K</td>
<td>&gt;3</td>
<td>UTM</td>
<td>54 ± 48</td>
</tr>
<tr>
<td>DOM4A</td>
<td>&gt;3</td>
<td>UTM</td>
<td>53 ± 36</td>
</tr>
</tbody>
</table>

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**a** Saturation binding experiments, as in Fig. 5, measured the binding affinity and receptor content of the various cell lines.

**b** The surface expression was monitored by treating whole cells with epitope tag-specific antibodies and was quantified using flow cytometry.

**p < 0.001 (highly significant).**

**p < 0.05.**

**p < 0.01 (significant).**

Dom4A, a mutant receptor containing the quadruple mutation E270A, E278A, D284A, and E291A.
These experiments used $^{125}$I-MCP-1 at 440 Ci/mmol and MCP-1 concentrations of 50 pM for wild type bearing cells and 100 pM for cells bearing mutations. Data analysis of these type of competition was performed as described by Jarnagin et al. (31). The buffers used for these experiments as well as the equilibration times and temperatures were identical to those used with THP-1 cells.

**Intracellular Signaling and Chemotaxis**—The methods for measurement of intracellular signaling and chemotaxis have been described in detail in our previous paper (32). Briefly, cytosolic calcium influx was measured in CCR2-CHL cells loaded with the fluorescent dye Fura-2-AM. Quantitation of signal intensity used the integrated signal intensity for 82 s after the addition of chemokine and thus has units of ms$^{-1}$. Antagonism by various compounds of calcium influx was measured using an approximate ED$_{50}$ dose of MCP-1 (3 nM) and an approximate ED$_{95}$ attractant concentration for MCP-1 and for RANTES as agonists.

Adenylate cyclase inhibition was measured using a reporter linked with a correlation coefficient of 0.96 (32). The antagonist was present in both the cell clone, CHO-K1-CCR2b-cAMP-Luc-neo-22. The assay was performed as described by Jarnagin et al. (31). The methods for measurement of intracellular signaling and chemotaxis have been described in detail in our previous paper (32). Briefly, cytosolic calcium influx was measured in CCR2-CHL cells loaded with the fluorescent dye Fura-2-AM. Quantitation of signal intensity used the integrated signal intensity for 82 s after the addition of chemokine and thus has units of ms$^{-1}$. Antagonism by various compounds of calcium influx was measured using an approximate ED$_{50}$ dose of MCP-1 (3 nM) and an approximate ED$_{95}$ attractant concentration for MCP-1 and for RANTES as agonists.

The data were expressed by normalization to the uninhibited migration of $^{82}$ s after the addition of chemokine and thus has units of M product was further purified using Geneclean columns (Bio101, La Jolla, CA) and then sequenced. The mutations in the third extracellular domain of the pCDNA3.1 vector and a 3′ primer within pCDNA3.1 vector, 5′-TAGAAGGCACGTCGAGG. The polymerase chain reaction product was further purified using Geneaclean columns (Bio101, La Jolla, CA) and then sequenced. The mutations in the third extracellular loop and at the top of the seventh transmembrane domain were sequenced using a primer that included the receptor stop codon, 5′-CTA CGG TGC GAC TTA TAA ACC AGC CGA G. These sequences confirmed that all of the clones lines reported contained the desired mutant receptor.

**Preparation and Isolation of L1.2 Transfectants—**L1.2 cells were a gift from E. Butcher (Stanford University, CA). Untransfected L1.2 cells were grown at 37 °C in humidified 5% CO$_2$ and in RPMI 1640 medium (Life Technologies, Inc.) containing 10% (v/v) heat-inactivated fetal calf serum (Hyclone, Logan UT), 2 mM minimal essential medium sodium-pyruvate (Life Technologies, Inc.), 55 μM β-mercaptoethanol, 50 units/ml penicillin G, and 50 μg/ml streptomycin (Life Technologies, Inc.). Transfected stable L1.2 cells were grown under the same conditions with additional 400 μg/ml Geneticin® (G-418, Life Technologies, Inc.). L1.2 cells were transfected for 4 h at 37 °C using 30 μg of LipofectAMINE and 5 μg of plasmid DNA2× 10$^6$ cells in Opti-MEM serum-free medium. The cells were allowed to recover for 24 h in RPMI medium containing 15% fetal calf serum. After a washing, the cells were cultured for 6 days in RPMI medium containing 1000 μg/ml G-418. Cells were maintained for two passages in 400 μg/ml G-418 prior to fluorescence-activated cell sorter-based cell cloning.

**Preparation, Isolation, and Characterization of Mutant Receptor Encoding cDNA and Verification of Transfected Receptor Sequences—** Mutations were introduced into a pcDNA3.1 vector containing an amino-terminal FLAG-tagged hCCR2 receptor (33) using a polymerase chain reaction method similar to that of Jarnagin et al. (31). This method amplifies the region of receptor sequence immediately surrounding the target mutation site; thus it avoids amplification of the whole receptor. The region of the plasmid DNA amplified during the mutagenesis procedure was completely sequenced to verify the presence of the mutation and confirm the absence of unintended mutations.

Clones of L1.2 cells bearing a single mutant receptor were further checked after cloning to confirm that each isolated cell line contained DNA encoding the desired mutation. Genomic DNA from the clone was isolated using a QIAamp blood DNA isolation kit (Qiagen GmbH, Hilden Germany). The genomic DNA was used as the template in polymerase chain reaction reactions using primers encoded by the T7 region of the pCDNA3.1 vector and a 3′ primer within pCDNA3.1 vector, 5′-TAGAAGGCACGTCGAGG. The polymerase chain reaction product was further purified using Geneaclean columns (Bio101, La Jolla, CA) and then sequenced. The mutations in the third extracellular loop and at the top of the seventh transmembrane domain were sequenced using a primer that included the receptor stop codon, 5′-CTA CGG TGC GAC TTA TAA ACC AGC CGA G. These sequences confirmed that all of the clones lines reported contained the desired mutant receptor.

**Binding Site of Small Molecule MCP-1 Receptor Antagonists**

<table>
<thead>
<tr>
<th>Mutants</th>
<th>hMCP-1</th>
<th>Wild type</th>
<th>0.06 ± 0.01 (4)</th>
<th>1.0</th>
<th>550 ± 385 (4)</th>
<th>1.0</th>
<th>105 ± 15 (4)</th>
<th>1.0</th>
<th>3.50 ± 0.72 (4)</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>D284A</td>
<td>0.29 ± 0.06 (4)$^a$</td>
<td>4.8</td>
<td>1892 ± 382 (3)</td>
<td>3.44</td>
<td>413 ± 77 (4)$^a$</td>
<td>3.9</td>
<td>2.27 ± 0.62 (4)</td>
<td>0.64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D284N</td>
<td>0.38 ± 0.09 (4)$^a$</td>
<td>6.3</td>
<td>568 ± 326 (4)</td>
<td>1.0</td>
<td>99 ± 34 (4)</td>
<td>0.94</td>
<td>1.25 ± 0.71 (4)</td>
<td>0.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E291Q</td>
<td>0.32 ± 0.07 (4)$^a$</td>
<td>&gt;100,000 (4)</td>
<td>&gt;180</td>
<td>64,000 ± 33,000 (4)$^a$</td>
<td>600</td>
<td>2.12 ± 0.35 (2)</td>
<td>1.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E291Q</td>
<td>0.38 ± 0.15 (4)$^a$</td>
<td>6.3</td>
<td>&gt;100,000 (4)</td>
<td>&gt;180</td>
<td>26,000 ± 11,000 (4)$^a$</td>
<td>250</td>
<td>1.65 ± 0.67 (4)</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D284A/E291A</td>
<td>2.05 ± 0.59 (4)$^a$</td>
<td>4</td>
<td>&gt;100,000 (4)</td>
<td>&gt;180</td>
<td>&gt;100,000 (4)</td>
<td>&gt;950</td>
<td>96.6 ± 16.1 (2)$^a$</td>
<td>28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D284N/E291Q</td>
<td>11.31 ± 3.97 (4) $^a$</td>
<td>190</td>
<td>&gt;100,000 (4)</td>
<td>&gt;180</td>
<td>&gt;100,000 (4)</td>
<td>&gt;950</td>
<td>9.43 ± 0.86 (2)$^a$</td>
<td>2.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ p < 0.001 (highly significant).

$^b$ >100,000 represents displacement potencies larger than 100 μM. At approximately 200–300 μM, many of the compounds become insoluble; thus, affinity estimates greater than 100 μM were felt to be inaccurate and are not reported.

$^*$ p < 0.01 (significant).
FIG. 1. Structure and affinities of several small molecule inhibitors of chemokine binding: their affinities for biogenic amine receptors and their potencies as chemotaxis inhibitors driven by MCP-1 or RANTES.

MCP-1 binding was measured in THP-1 cells using \(^{125}\text{I}-\text{MCP-1}.\) MIP1\(\alpha\) binding was measured in a transfected cell line CHO-CCR1 using \(^{125}\text{I}-\text{MIP1}\alpha.\) The biogenic amine binding receptors were measured using transfected cell lines bearing the human \(\alpha_2\) and \(\alpha_1\) receptors or a tissue preparation for the 5HT1a receptor, rat brain cortex. Characteristic radioligands appropriate for each receptor were used: prazosin for \(\alpha_2\) and \(\alpha_1\) receptors or 8-hydroxyaminopropylaminotetralin for 5HT1a receptors. Chemotaxis inhibition was measured using THP-1–5X strain of THP-1 cells and 3 \(\mu\)M MCP-1 or RANTES as the chemoattractant. All measurements are reported in \(\text{nM,}\) and the error statistic is the standard deviation for the number of replicate experiments shown in parentheses. ND, not determined.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC(_{50}) CCR2 Binding (nM)</th>
<th>IC(_{50}) CCR1 Binding (nM)</th>
<th>IC(_{50}) Alpha-1a Binding (nM)</th>
<th>IC(_{50}) Alpke-1d Binding (nM)</th>
<th>IC(_{50}) 5-HT1a Binding (nM)</th>
<th>IC(_{50}) Chemotaxis to MCP-1 (nM)</th>
<th>IC(_{50}) Chemotaxis to RANTES (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS-21825</td>
<td>11,400</td>
<td>&gt;100,000</td>
<td>5.6</td>
<td>43</td>
<td>62</td>
<td>28,300</td>
<td>32,000</td>
</tr>
<tr>
<td>RS-29634</td>
<td>940</td>
<td>17,800</td>
<td>35</td>
<td>150</td>
<td>97</td>
<td>1900</td>
<td>64,000</td>
</tr>
<tr>
<td>RS-102895</td>
<td>360</td>
<td>&gt;100,000</td>
<td>120</td>
<td>320</td>
<td>470</td>
<td>1700</td>
<td>37,000</td>
</tr>
<tr>
<td>RS-504393</td>
<td>89</td>
<td>&gt;100,000</td>
<td>72</td>
<td>460</td>
<td>1070</td>
<td>330</td>
<td>61,000</td>
</tr>
<tr>
<td>RS-136270</td>
<td>11,900</td>
<td>95,000</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>3500</td>
<td>&gt;50,000</td>
</tr>
</tbody>
</table>

FIG. 2. The spiropiperidine class and the carboxypyrrole class of inhibitors block MCP-1-driven chemotaxis and MCP-1-driven adenylate cyclase suppression. A, inhibition of chemotaxis of THP-1 cells to the attractants MCP-1 (3 \(\mu\)M) or RANTES (3 \(\mu\)M) by several spiropiperidine drugs. Each data point is the mean of quadruplicate data points; the standard deviations for the two curves shown are representative of all the curves. The data shown are representative of experiments repeated on from 2–6 separate days. B, the antagonistic effect of spiropiperidine and carboxypyrrole compounds on MCP-1 caused inhibition of cAMP-dependent luciferase expression. The left-most curve shows the inhibition of luciferase expression (cAMP-dependent reporter) caused by MCP-1. The three right-most curves use RS-102895, RS-136270, and RS-29634 as antagonists of MCP-1 (5 \(\mu\)M). In both the antagonism experiments and the MCP-1 control, 2.0 \(\mu\)M forskolin was used to stimulate cAMP production. The data are presented as a percentage of the luciferase expression caused by 2.0 \(\mu\)M forskolin (100\%) and the background luciferase expression in untreated cells (0\%). The data points shown are the means of triplicate determinations. The standard deviations are about 10\% and are omitted for clarity. The curves shown are representative of dose titrations repeated from two to four times.

Molecular Modeling—The primary sequence of more than 100 G-protein-coupled receptors including CCR2 were aligned using the Needleman-Wunsch multi-sequence alignment as implemented in the PILEUP command in GCG (receptors that bind peptides and other ligands were included). The sequences were further aligned manually with special attention to transmembrane regions. The recently published bacteriorhodopsin structure and low resolution structures of rhodopsin were used as a template for the modeling of human CCR2 receptor (27, 28, 34). The methods used for building these first models are described elsewhere (35). Briefly, the appropriate residues of the helices of the rhodopsin template were changed to the corresponding amino acid of the CCR2. The amino acid side chains were energy minimized and placed in a reasonable conformation. For this study the small molecule antagonist binding site analysis. The final structure was minimized with a limited cycle using the Discover software (Bio-sym) and CVFF force field. Only a few cycles are used to improve the stereochemistry of the model and to remove the unfavorable clashes.

RESULTS

Identification and Modification of Small Molecule MCP-1 Receptor Antagonists—Because of the involvement of MCP-1 in several inflammatory pathologies, we began a search to find small molecule antagonists of the MCP-1 receptor. A large chemical library was screened using a high throughput radioligand binding and a secondary chemotaxis inhibition assay. This screen identified about 10 structurally diverse classes of small molecule inhibitor with affinities between 5 and 30 \(\mu\)M for receptor binding and chemotaxis inhibition.

Inhibitor Structure Activity Relationships—Two of these classes were judged to be drug-like and amenable to medicinal chemistry optimization. The spiropiperidine (SP) class, represented by RS-21825, RS-29634, RS-102895, and RS-504393 (Fig. 1), had a distinct structure activity relationship and affinities that spanned IC\(_{50}\) values from 90 \(\text{pM}\) to completely inactive compounds. The central features of this class includes a pharmacophore defined by a basic nitrogen in a piperidine ring; another important feature is the orthogonal relationship between a phenyl urethane system and a piperidine moiety, imposed by a spiro-carbon atom. The hydrogen bonding potential provided by a urethane moiety is very important. Another important feature is the restriction to small moieties for substituents on the phenyl-urethane heterocycle. Finally, a large range of hydrophobicities and sizes are acceptable for substituents in the phenethyl portion of the inhibitors, RS-504393 for example.
The 2-carboxy-pyrrrole (2CP) class, represented by RS-136270 in Fig. 1, had a less distinct and weak structure activity relationship. The best compounds had affinities near 5 μM. Two-carboxypyroles and 2-carboxyindoles were acceptable. Substituents at position three or four (pyroles) were acceptable if small; however, large substituents of any kind were not well tolerated. Benzyl substitution of the nitrogen was required for activity, and halogen substituents on the ring were well tolerated. Large benzyl substituents were not well tolerated. The requirement for a carboxylic acid at position 2 was absolute.

Inhibition of Chemotaxis by SP and 2CP Class—All of the compounds shown in Fig. 1 inhibited chemotaxis driven by MCP-1. The chemotaxis inhibitor concentrations of the spiropiperidine class are well correlated with the binding affinities and thus with the binding-derived structure activity relationship. Similar close correlation between binding affinities, structure and chemotaxis were observed for the carboxypyrrrole and carboxyindole class (data not shown). Fig. 2A shows representative dose response relationships for chemotaxis inhibition. These curves are single phase curves with Hill slopes close to 1, suggesting that the compounds interact with a single population of receptors. Figs. 1 and 2A also illustrate that although these compounds are potent inhibitors of MCP-1-driven chemotaxis mediated by CCR2, none of the compounds are potent inhibitors of RANTES-driven chemotaxis mediated by CCR1. The best spiropiperidine, RS-504393, was 8000-fold better as a MCP-1-driven chemotaxis inhibitor than as a RANTES-driven chemotaxis inhibitor (Fig. 1). The highest affinity CCR2 binder, RS-504393, was a 700-fold better binder on CCR2 than CCR1. Neither class of compound, SP or 2CP, displayed any affinity for CCR1 or CCR3 (not shown). Thus, both classes of compounds are highly selective among chemokine receptors.

Inhibition of MCP-1-stimulated cAMP Inhibition—Fig. 2B illustrates that both SP compounds and 2CP compounds inhibit MCP-1-stimulated cAMP inhibition, measured here using a pentameric cAMP responsive promoter element, CREB binding element, linked to a reporter cDNA, luciferase. Previous studies (30, 32) have demonstrated that this reporter assay accurately reflects cAMP changes in these cells. The rank order of potencies observed for these two SP compounds, RS-29634 and RS-102895, and one 2CP compound are very similar to their rank order receptor binding and chemotaxis inhibition potency.

Inhibition of MCP-1- and MCP-3-stimulated Calcium Influx—Fig. 3 demonstrates that both SP compounds and 2CP compounds inhibit MCP-1- and MCP-3-stimulated calcium influx into CCR2-CHL cells. The IC50 values for RS-504393, RS102895, and RS-136270 as MCP-1 inhibitors are, respectively, 35 ± 9, 32 ± 7, and 260 ± 45 nm, and as MCP-3 inhibitors they are 160 ± 26, 130 ± 27, and 500 ± 170 nm. We note that as antagonists of calcium flux, RS-504393 and RS-102895 do not show the 3-fold separation in potency that is exhibited by these two compounds in CCR2 binding assays and chemotaxis antagonist assays. This finding may reflect an equal ability of these two inhibitors to block formation of a conformation of the receptor that is able to stimulate calcium influx. Thus both classes of compounds inhibit calcium influx caused by either MCP-1 or MCP-3. None of these compounds were able to stimulate calcium influx at up to 50 μM for RS-504393 and RS-102895 or 20 μM for RS-136270 (data not shown). They are not agonists for calcium influx, adenylyl cyclase inhibition, or chemotaxis. These small molecular weight ligands block all post receptor events and do not distinguish between calcium stimulatory receptor conformations caused by either CCR2 agonist, MCP-1, or MCP-3.

Biogenic Amine Binding Properties of the SP and 2CP Class—Despite the high chemokine receptor specificity exhibited by the spiropiperidine compounds, these compounds are potent α1-adrenergic receptor blockers (Fig. 1); lower affinities were observed for other types of adrenergic receptors, α2 and β (not shown). In addition several other GPC-7TM receptors, the serotonin 5HT1a and μ-opioid receptors for example, also bound SP compounds. In the case of the serotonergic receptors, structures with improved CCR2 affinity had decreased 5HT1a affinity (Fig. 1). A remarkable property of the SP class is its high affinity for α-adrenergic receptors, particularly α1a receptors and to a lesser extent α1d receptors. Affinity at α1b receptors was much lower (not shown). Affinity at α1a sites became less because the compounds had improved affinity on CCR2 (Fig. 1). Affinity at α1a receptors also decreased as CCR2 affinity increased; however, because the α1a affinity of the initial lead compound, RS-21825, was 5.6 nm, the decrease in α1a affinity was insufficient to eliminate α1a affinity of the SP compounds. The 2CP class of compounds had no significant affinity for biogenic amine receptors (Fig. 1).

Selection of Residues Glu291 and Asp784 for Mutagenesis Studies—The findings of a strong requirement for a basic nitrogen in the SP class of blockers suggests that an acidic residue on CCR2 might provide part of the interaction site. The alignment of several chemokine receptors (Fig. 4) highlights an unusual feature of chemokine receptors; chemokine receptors...
contain an acidic glutamic acid, Glu\textsuperscript{291}, 1.5–2 turns within TM-7. Acidic residues are rare in this position in GPC-7TM receptors (36, 37). We also noted in our sequence alignment Asp\textsuperscript{284}. This residue is located two helical turns above and on the same helical face as Glu\textsuperscript{291} and is also conserved between chemokine receptors. Thus, we chose to make mutations of CCR2 at Glu\textsuperscript{291} and Asp\textsuperscript{284}. We made mutations to alanine to eliminate the residues side chain or made changes to asparagine or glutamine to remove the charge but maintain the hydrogen bonding potential of the side chain.

Creation and Characterization of Glu\textsuperscript{291} and Asp\textsuperscript{284} Mutants—The mutant receptors were made in an amino-terminal FLAG epitope-tagged receptor (38) and then transfected into L1.2 cells. Stable clones expressing moderate receptor densities (12–36 thousand receptors/cell) were isolated using flow cytometry. Fig. 5 shows representative cell population distributions of three important cell lines, epitope-tagged wild type receptors and E291A and E291Q mutant receptors. This figure demonstrates that the various stable mutations have similar population distributions and surface staining with anti-epitope antibody to mean levels 50–250 times the staining observed in untransfected L1.2 cells. However, we note with interest (Table I) that the only rough correlation between the surface receptor staining measured by flow cytometry and the binding assay measured receptor density, particularly for the D284A receptor mutant (Table I). The crudeness of the correlation may reflect a difference in intracellular membrane system distribution for the various mutants and the difference in sampling inherent in the two assay methods.

The use of amino-terminal FLAG epitope-tagged MCP-1 receptor does not affect the MCP-1 binding affinity of wild type receptors. The wild type receptor, naturally expressed in the human monocyte cell line THP-1, binds MCP-1 with a $K_d$ of 0.035 ± 0.02 nM (32), whereas the epitope-tagged receptors bind MCP-1 with a $K_d$ of 0.06 ± 0.01 (Table I and Ref. 38).
lines as well as lines bearing D284N, E291Q, and D284A/E291A (Table I). For these mutant receptors affinities ranged between 0.06 nM, for wild type to 1.53 nM for D284N. The double receptor mutations, such as D284A/E291A, have higher affinities than are measurable in saturation binding experiments using available radioactive ligands.

However, four mutant receptors have substantial surface expression (Table I) but bind with an affinity of less than 3 nM, the lowest affinity detectable in these saturation binding assays. These mutants, D284K, D284A/E291A, D284N/E291Q, D284K/E291K, and DOM4A, are well expressed on the cell surface with staining of 53–177-fold over untransfected control cells (Table I). Their population distributions were all quite similar to wild type receptors and the other mutants shown in Fig. 5. These findings indicate that these mutant receptors are cell surface localized but have very low MCP-1 affinities.

**Binding Affinities of Receptor Mutants Measured Using Competition**—To more fully characterize the mutants and to measure the effect of the mutations on drug binding, competition binding experiments were performed. Table II and the example graphs of Fig. 6 show that mutations at positions 284 and 291 significantly reduced MCP-1 binding affinity; however, none of these changes completely eliminated the ability of MCP-1 to bind to the receptor. The most detrimental combination of mutations, the double mutant D284N/E291Q, reduced the binding affinity by 190-fold to 11.3 nM. Other single amino acid mutants had reduced binding affinity by 4.8–8.7-fold. This observation, along with the ligand mutagenesis data showing the critical importance of certain basic residues on the MCP-1 surface (30), suggests that Asp284 or Glu291 may be a site for receptor ligand interaction with some of the MCP-1 basic residues. A model of this interaction has been presented (30).

**Binding Affinities of Mutants for Small Molecular Weight Antagonists**—The ability of these mutants to bind MCP-1 allowed competition measurements of binding affinity of several of the small molecule antagonists. Fig. 6 and the summary data presented in Table II show that the spiropiperidine compounds, RS-102895 and RS-504393, bind extremely poorly when Glu291 is changed to alanine (affinity reduced by 610-fold) or to glutamine (affinity reduced by 250-fold). These findings suggest that the negative charge of glutamic acid is important because glutamine has hydrogen bonding potential similar to that of glutamic acid, yet E291Q has significantly reduced binding affinity with RS-504393, 250-fold. In contrast to the spiropiperidine compounds, the 2-carboxypyrroles, represented by RS-136270, bind to all of the single acidic amino acid mutant receptors with affinities similar to wild type receptors (Fig. 6). Together these observations suggest that Glu291 interacts with the basic nitrogen of SP molecules.

Residue Asp284 mutants bind SP compounds with affinities that are only very modestly changed from wild type affinities, which is in contrast to the large changes in affinity caused by mutations at residue Glu291 (Table II). Of the single Asp284 mutations only the affinity of RS-504393 was significantly affected by the change to alanine; however, the magnitude of reduction in affinity of this mutant (3.9-fold) is small when compared with the magnitude of Glu291 changes (610- and 250-fold). Thus it would seem that Asp284 contributes only very slightly to a SP compound binding pocket.

The 2CP compound, RS-136270 was unaffected by any of the single mutations at residues 284 or 291 (Table II). Thus these acidic residues are not required for 2CP binding, and the contrast in binding contact requirements compared with SP compounds suggests very different binding sites for these two classes of antagonists.

The double mutations to alanine and the amides, D284A/E291A
E291A and D284N/E291Q, respectively, have undetectable affinities for the SP class of compounds and are the most reduced in binding affinity for MCP-1. Thus, this combination of residues is important for both the SP and the MCP-1 binding interaction. In contrast, the double mutations to alanine and to the amisdes, D284A/E291A and D284N/E291Q, affect the 2CP compounds binding only modestly, 28- and 2.7-fold, respectively (Table II); these findings highlight the clear distinction between the binding mode of SP compounds and 2CP compounds.

**DISCUSSION**

**Structure-Activity Relationship of SP Compounds**—After identification of the spiropiperidines as CCR2 ligands we were able to define several central features of the structure-activity relationship of these compounds. The spiro arrangement between a phenyl urethane moiety and a piperidine is one of the striking features. The piperidine ring was di-substituted at the 4 position with a spiro-phenyl urethane system. Ring systems other than piperidine and straight chain substitutions of the piperidine system were inactive or quite reduced in affinity. In all the more potent analogs, the nitrogen was connected via an aliphatic chain to an aromatic system. For the SP class, the aromatic system-binding pocket was tolerant of a wide range of substituents of a variety of sizes, provided that they were nonpolar. Polar substituents were not well tolerated; this implied a binding pocket with substantial hydrophobicity. This phenyl urethane system was mostly intolerant of substitution, particularly large moieties. The urethane moiety could not be replaced or altered without significant losses in activity. The strength of this structure activity relationship and its defined preference for particular sizes and polarities in different parts of the molecule implies several different steric constraints around the phenyl-urethane portion. The absolute requirement for the urethane system, with the nitrogen bearing a hydrogen, implies the presence of a hydrogen bond partner for the urethane nitrogen in the binding pocket. Possibly, the most significant feature of this structure activity relationship is the absolute requirement for a tertiary nitrogen, preferably contained within a piperidine ring. Alterations of the nitrogen pKa to lead to complete loss of binding affinity. These features suggest that the register of the compound within its binding site was controlled by an acid-base interaction.

Our starting point of the optimization of these compounds was \( \text{RS-21825 (IC}_{50} 11.4 \, \mu \text{M}) \), and we were able to make steady improvements by increasing the affinity to \( \text{RS-504393, IC}_{50} 90 \, \text{nm} \). The discovery of small molecular weight CCR2 antagonists of several different classes was gratifying because, when we started this effort, no drug-like chemokine receptor antagonist had been revealed. In addition, we and others had suggested that the ligand was too big (molecular weight of 8,600) to allow small molecular weight compounds to inhibit its binding. This view held that the receptor ligand complex involved several thousand square angstroms of surface and was spread over a large flat interaction interface similar in nature to the surfaces defined for growth hormone and its receptor (39) or antibody-protein interactions surfaces (40). Clearly these results demonstrate the feasibility of development of MCP-1 antagonists, and they suggest that some part of the essential interaction space from MCP-1 contains a crevice or pocket with features necessary to bind small molecular weight inhibitors.

**Structure-Activity Relationship of 2-CP Compounds**—In contrast to the substantial improvements in affinity we obtained in the SP inhibitor class, affinities of the 2-CP inhibitor class improved by less than 4-fold from the initial screening hit (20 \( \mu \text{M} \)). This class of compounds had no requirement for a basic nitrogen, nor did it possess distinct geometric requirements imposed by a spiro-center or the molecular length and size requirements displayed by the SP inhibitor class. The absolute requirement for an acidic residue further distinguished the 2CP compounds from the SP compounds. These contrasting features clearly hint that the binding sites on CCR2 for SP compounds and 2CP compounds are distinct. Given the extraordinary contrast, we speculate that the sites were spatially distinct and certainly controlled by opposite acid-base interactions.

**Binding of SP and 2-CP Compounds to Other Chemokine Receptors**—These groups of compounds are uniquely able to interact with a binding pocket on CCR2 and not other chemokine receptors. Because both classes of inhibitors are extremely weak binders to other chemokine receptors, including CXCR1, CCR1, and CCR3. Small molecular weight ligands for CCR1 and CXCR4 have been described (41–43); these compounds have no affinity for CCR2, findings that we have corroborated. None of the CCR1 or CXCR4 antagonists similar structurally to either the SP or CP classes. Thus, insight of the sequence similarities between the chemokines (30–90\%), their receptors (30–70\%), and their conserved overall three-dimensional structures, small molecular weight inhibitors that distinguish the various family members can be found.

**Blockade of Receptor Signaling by SP Compounds**—In addition to inhibiting MCP-1 binding and MCP-1-driven chemotaxis, the SP compounds are potent inhibitors of MCP-1-driven cAMP-mediated gene transcription as well as MCP-1- or MCP-3-driven calcium influx. Furthermore, these compounds are not agonists of MCP-1-mediated events, even at concentrations 500 times their apparent affinities. These findings differentiate small molecular weight antagonists from MCP-1 mutants that are chemotaxis antagonists (32). These ligand-derived antagonists were of two types; one type \((1+9–76)\text{hMCP}) stimulates cAMP signaling through CCR2 but is not able to stimulate chemotaxis or calcium signaling. Another chemotaxis antagonistic ligand mutant (Y13A) was able to stimulate cAMP signaling and calcium signaling through CCR2 but was not able to stimulate chemotaxis. These findings suggest that ligand-derived antagonists can promote some receptor conformations that are necessary to drive signaling of post receptor pathways but are not able to promote the conformation necessary to drive chemotaxis. Unlike the ligand-derived antagonists, the small molecular weight inhibitors are not able to separate these functions; they block all measured post receptor events and are not agonists. Thus they have no ability to promote CCR2 conformations that can couple to post-receptor pathways. These findings indicate that the small molecular weight antagonists interact with the receptor quite differently than ligand-derived antagonists.

**Receptor Distribution within Cells**—The point mutations we made in CCR2 were designed to investigate the role of specific residues in MCP-1 and small molecule antagonist binding; they also revealed interesting findings regarding GPC-7TM receptor intracellular sorting. As part of our efforts to characterize each mutant thoroughly, we measured receptor density using two techniques. The correlation between the two measurement methods was weak. We believe that the correlation is affected by the difference in receptor populations measured by the two techniques; the receptor binding assay is performed in crude cell membranes and thus measures binding competent receptors on the cell surface or on intracellular membranes. In contrast, the cell staining estimation of receptor density measures only surface receptors because it is performed on unper-
meabllized cells. These differences could account for the weak correlation between the methods. Several other workers have noted that various mutant GPC-7TM receptors do not transport well to the cell membrane (44–47). CCR2a, an alternative splice variant of CCR2b studied here, does not efficiently localize to the plasma membrane (48). These observations indicate that for D284A and possibly other receptors, some part of the receptor population is localized on intracellular membranes. These findings and the aberrant transport of several other GPC-7TM receptors to the cell surface are interesting and could indicate the existence of cellular membrane sorting systems that detects individual proteins and their conformations. Further investigation into the effect of these and similar point mutations on the distribution of GPC-7TM receptors may reveal novel details of the protein sorting process. Nevertheless, even in the most dramatic example of intracellular retention, D284A, adequate surface receptor was present to allow high quality saturation binding experiments.

**Binding of SP and 2-CP Compounds to Biogenic Amine Receptors**—The first identified member of the SP family, RS21825, had been previously synthesized as an α-adrenergic receptor antagonist (29) to be used as a treatment for hypertension. In addition to α₁-adrenergic receptor affinity, some members of this compound class also had significant affinity to 5-HT₁, α₂-adrenergic, and opioid receptors (not shown). Thus we sought to remove the α-adrenergic and biogenic amine receptor binding properties from this class while preserving CCR2 affinity. We were successful at eliminating or significantly reducing the affinities of these compounds for α₁, 5-HT₁, α₂, and opioid receptors while improving the affinities for CCR2. These results show that differences exist among the binding pockets on these G-protein coupled-seven transmembrane receptors; these differences can be exploited to convert molecules that bind to one class into molecules that bind to another class. However, the fact that these receptors were all from families that bind ligands that are basic amines was very provocative. This finding of a shared basic nitrogen motif and other results (below) formed the basis of our hypothesis of similar ligand binding modes between SP compounds and biogenic amines.

**Significance of Acidic Residues Glu 291 and Asp 284**—The results of our mutagenesis experiments establish that the SP compounds interact with Glu²⁹¹ and to a lesser extent with Asp²⁸⁴. The SP class of antagonists requires a basic nitrogen for high affinity. In addition the SP class has significant affinity for biogenic-amine receptors. These findings suggest that the way in which SP molecules bind to CCR2 may be similar to well characterized binding modes of biogenic amine receptor antagonists (49–51). The distribution of acidic amino acids in CCR2 further stimulated our interest (Fig. 4); the extracellular loops and transmembrane regions contain only 13 acidic residues. Five of the 13 residues are in an acidic cluster located in the amino terminus of the receptor. (This cluster was examined in a previous paper (30).) Parts of this cluster are important for MCP binding, but none of it is important for small molecular weight ligand binding. The alignment of several chemokine receptors (Fig. 4) highlights an unusual feature of chemokine receptors; chemokine receptors contain an acidic glutamic acid 1.5–2 turns within TM-7. Acidic residues are very rare in this position in GPC-7TM receptors (36, 37).

**Model of SP Compounds Binding to Receptor**—Models of GPC-7TM receptors were constructed based on the low resolution electron cryomicroscopic data available for rhodopsin (28, 34, 52) and the higher resolution structures of bacteriorhodopsin (27). These models predict that Glu²⁹¹ of TM-7 is inside the ovoid-helical bundle in a mirror image position to the critical binding residue of the biogenic amine receptor, an aspartic acid from TM-3.

This binding interaction is illustrated in Fig. 7. The model depicts Glu²⁹¹ interacting with the basic nitrogen of piperidine ring and the phenylurethane moiety extending the length of the ovoid bundle to interact with TM-3 and TM-6. The phenethyl moiety extends in the opposite direction toward TM-1 and TM-2, forming a hydrophobic pocket. The binding pocket is 1.5 to 2 helical turns within the transmembrane helices; and thus, no more than one-third of the extracellular portion of the transmembrane width is occupied by the SP compound.

**How Does the Binding of a SP Compound Prevent MCP-1 Interactions with CCR2?**—Comparison of the model presented in Fig. 7 with our model of MCP-1 binding to CCR2 (27) indicates that SP compounds prevent MCP-1 binding by occupying the same space, the inter-helical bundle region on the extracellular side of the receptor. Our models of the MCP-1/CCR2 complex place the amino terminus of MCP-1 in close proximity to Asp²⁸⁴ and Glu²⁹¹, crossing through the same volume of space as would be occupied by SP compounds.

In conclusion, we have discovered and optimized a class of small organic molecule antagonists of the CCR2 receptor, spiropiperidines. These compounds are not chemotaxis agonists and do not stimulate post receptor signaling of any kind. The compounds block MCP-1 and MCP-3 signaling through CCR2; however, they are not antagonists of CXCR1, CCR1, or CCR3. These antagonists block the receptor by occupation of a binding site that includes acidic residue Glu²⁹¹. We believe that the acidic moiety interacts with the piperidine basic nitrogen. The spiropiperidine blockade of MCP binding occurs by occupation of some of the space that CCR2-bound MCP-1 occupies. These results provide an excellent starting point for the design of new experiments directed toward refining theses models and may provide insights on how to improve the affinity and specificity of small organic ligands. Most of the chemokine receptors contain acidic residues in TM-7 in an analogous location to CCR2. Many of the early lead antagonists disclosed in the patent literature contain basic sites similar to the piperidine nitrogen; thus our models may provide a general framework to explain small molecule binding to chemokine receptors.

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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

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