Identification and Characterization of a Potent, Selective, and Orally Active Antagonist of the CC Chemokine Receptor-1*

Received for publication, February 11, 2000
Published, JBC Papers in Press, March 29, 2000, DOI 10.1074/jbc.M001222200

The CC chemokine receptor-1 (CCR1) is a prime therapeutic target for treating autoimmune diseases. Through high through screening followed by chemical optimization, we identified a novel non-peptide CCR1 antagonist, R-N-[5-chloro-2-[2-[4-[(4-fluorophenyl)methyl]-2-methyl-1-piperazinyl]-2-oxoethoxy]phenyl]urea hydrochloric acid salt (BX 471). Competition binding studies revealed that BX 471 was able to displace the CCR1 ligands macrophage inflammatory protein-1α (MIP-1α), RANTES, and monocyte chemotactic protein-3 (MCP-3) with high affinity (K_i ranged from 1 nM to 5.5 nM). BX 471 was a potent functional antagonist based on its ability to inhibit a number of CCR1-mediated effects including Ca2+ mobilization, increase in extracellular acidification rate, CD11b expression, and leukocyte migration. BX 471 demonstrated a greater than 10,000-fold selectivity for CCR1 compared with 28 G-protein-coupled receptors. Pharmacokinetic studies demonstrated that BX 471 was orally active with a bioavailability of 60% in dogs. Furthermore, BX 471 effectively reduced disease in a rat experimental allergic encephalomyelitis model of multiple sclerosis. This study is the first to demonstrate that a non-peptide chemokine receptor antagonist is efficacious in an animal model of an autoimmune disease. In summary, we have identified a potent, selective, and orally available CCR1 antagonist that may be useful in the treatment of chronic inflammatory diseases.

It is clear that the inappropriate interaction of immune cells, such as T lymphocytes and monocytes, can lead to extensive inflammation and tissue destruction, which is a hallmark of several autoimmune diseases such as rheumatoid arthritis and multiple sclerosis. Immune cells are sent on their destructive journey by chemoattractant molecules known as chemokines, which interact with and signal through specific cell surface chemokine receptors. Chemokine receptors belong to the GPCR superfamily and have been viewed as attractive therapeutic targets by the pharmaceutical industry mainly because of their central role in regulating leukocyte trafficking. The premise that drugs that can inhibit the directed migration and activation of immune cells could be useful therapeutically has prompted the search for specific and highly potent chemokine receptor antagonists.

Autoimmune diseases like multiple sclerosis and rheumatoid arthritis are characterized by interactions between invading T lymphocytes and tissue macrophages that result in extensive inflammation, tissue damage, and chronic disease pathologies. Numerous studies have demonstrated CCR1 expression in these cell types, and a variety of evidence provides strong in vivo concept validation for a role of this receptor in animal models of these diseases. For example, Karpus et al. (1, 2) were able to show in a mouse EAE model of multiple sclerosis that antibodies to MIP-1α prevented the development of both initial 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 disease. These results led the authors to conclude that MIP-1α plays an important role in this T-cell-mediated disease. Furthermore, in a recent study, Rottman et al. (3) were able to demonstrate in a mouse EAE model of multiple sclerosis that CCR1 knockout mice had a significantly reduced incidence of disease compared with wild type mice. The spinal cords of the wild type mice showed non-suppurative myelitis, whereas those from the CCR1 knockouts were minimally inflamed. Taken together these data strongly support the idea that CCR1 plays a role in the pathogenesis of EAE and furthermore suggest a role in the pathophysiology of the human disease, multiple sclerosis.

From the data discussed above it is apparent that CCR1 antagonists could potentially be very important therapeutically in multiple sclerosis and in other autoimmune and inflammatory diseases.

* 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 1744 solely to indicate this fact.

§ To whom correspondence should be addressed: Meina Liang, Berlex Biosciences, Dept. of Molecular Pharmacology, 15049 San Pablo Ave., Richmond, CA 94804. Tel.: 510-689-4091; Fax: 510-262-7844; E-mail: meina_liang@berlex.com.

**Current address: Coulter Pharmaceutical, South San Francisco, CA 94080.

§§ Current address: Mirus Corp., Madison, WI 53719.

††‡‡§§ Current address: Mirus Corp., Madison, WI 53779.

1 The abbreviations used are: GPCR, G-protein-coupled receptors; CCR1, CC chemokine receptor-1; MIP-1α, macrophage inflammatory protein-1α; MCP-3, monocyte chemoattractant protein-3; MCP-1, monocyte chemotactic protein-1; EAE, experimental allergic encephalomyelitis; HEK293, human embryonic kidney cells; FLIPR, fluorometric imaging plate reader; FACScan, fluorescence-activated cell scanner; HPCL-MS, high pressure liquid chromatography-mass spectrometry; BX 471, R-N-[5-chloro-2-[2-[4-[(4-fluorophenyl)methyl]-2-methyl-1-piperazinyl]-2-oxoethoxy]phenyl]urea hydrochloric acid salt.
flamatory diseases in which target cells expressing the receptor play a role. For this reason we have established a program to develop highly potent and specific CCR1 antagonists. Recently we described a functional CCR1 antagonist belonging to the 4-hydroxypiperidine class (4). This molecule was shown to have a $K_i$ of 40 nM for CCR1 and a 250-fold selectivity against other GPCR tested.

In this report, we describe the discovery and pharmacological characterization of a novel, potent, and selective functional CCR1 antagonist. The compound, BX 471, is able to displace the CCR1 ligands MIP-1α, RANTES, and MCP-3 with high affinity ($K_i$ ranged from 1 nM to 5.5 nM) and is a potent functional antagonist based on a number of in vitro biological assays including the inhibition of Ca$^{2+}$ mobilization, extracellular acidification rate, CD11b expression, and leukocyte migration. BX 471 demonstrated a greater than 10,000-fold selectivity for CCR1 versus other GPCR in both receptor binding assays and functional assays. Pharmacokinetic studies revealed that BX 471 had an oral bioavailability of 60% in dogs. Finally, even though BX 471 binds to rat CCR1 with a 100-fold reduced affinity ($K_i = 121 \pm 60$ nM), it is still able to effectively reduce disease in a rat EAE model of multiple sclerosis. The in vivo demonstration of efficacy for a CCR1-specific receptor antagonist could represent a major advance in the treatment of multiple sclerosis, where peripheral leukocyte recruitment and activation are critical to disease pathology.

**EXPERIMENTAL PROCEDURES**

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

**CCR1 Expression Vectors**—CCR1 cDNA was obtained as described (5) and inserted into a mammalian expression vector containing the SV40 replication origin, the human cytomegaloviral enhancer with the puromycin-N-acetyltransferase gene (puromycin resistance) and hygromycin B gene (hygromycin resistance) similar to that described previously (6).

**Cell Lines**—The human monocytic cell line THP-1, Jurkat cells, and the HEK293 cell line were obtained from the American Type Culture Collection and were cultured as described previously (4). For binding assays, cells were harvested and washed once with phosphate-buffered salt solution. Cell viability was assessed by trypan blue exclusion, and cell number was determined by counting the cells in a hemocytometer.

**CCR1 Expressing Cells**—The transfection and selection of HEK293 cells stably expressing human CCR1 was as described previously (4).

**Cytosolic Ca$^{2+}$ Measurements**—HEK293 cells expressing human CCR1 were plated on poly-l-lysine-coated black 96-well plates (Becton Dickinson, Franklin Lakes, NJ) at 8,000 or 300,000 cells per assay point were used as the receptor source. Nonspecific binding was determined in the presence of 100 nM unlabeled chemokine. The binding data were curve-fitted with the computer program IGOR (Wavemetrics) to determine the affinity and number of sites.

**Cytotoxic Ca$^{2+}$ Measurements**—HEK293 cells expressing human CCR1 were plated onto poly-lysine-coated black 96-well plates (Becton Dickinson, Franklin Lakes, NJ) at 80,000 cells/well and were cultured overnight. Cells were then loaded with 4 μM Fluo-3 (Molecular Probes, Eugene, OR), a calcium-sensitive fluorescence dye, for 60 min at 37°C in Hanks balanced salts solution (Life Technologies, Inc.) containing 20 mM Hepes, 3.2 mM calcium chloride, 1% fetal bovine serum, 37 °C in Hanks’ balanced salts solution (Life Technologies, Inc.). Probes, Eugene, OR), a calcium-sensitive fluorescence dye, for 60 min at 37 °C in Hanks’ balanced salts solution (Life Technologies, Inc.).

**Measurement of Extracellular Acidification in HEK Cells**—Extracellular acidification was measured in a microphysiometer as described previously (4).

**Chemotaxis—Cell migration was examined using a 48-well microchemotaxis assay as described previously (8).**

**CD11b Expression on Peripheral Blood Mononuclear Cells**—CD11b expressed on peripheral blood mononuclear cells in a whole blood assay was measured as described (9). Briefly, human whole blood was collected by venipuncture into 2.5-mL Vacutainer tubes containing EDTA. The blood was kept at room temperature and used immediately after collection. The whole blood sample (200 μL) was mixed with 10 μL of agonist or without 1 μM BX 471 at 37 °C for 15 min followed by treatment with or without 100 nM MIP-1α for an additional 15 min. The reaction was terminated by the addition of 1 ml of cold phosphate-buffered salt solution wash. The tubes were centrifuged (200 × g for 7 min at 4 °C), and the supernatant was removed by aspiration. The cell pellet was resuspended in cold phosphate-buffered salt solution, 10 μl of 1 mg/ml heat-aggregated IgG was added, and the tubes were incubated for 10 min at 4 °C. Antibodies CD11b FITC (5 μl) and CD14 PE (20 μl) were added to each assay tube and incubated for 20 min at 4 °C. Finally, 1 ml of ice-cold phosphate-buffered salt solution was added, and the cells were pelleted as above and analyzed by FACSAn (Becton Dickinson, San Jose, CA).

**Determination of Pharmacokinetic Parameters in Dogs**—Fasted male beagle dogs (n = 3 per treatment group) were given BX 471 either by oral gavage or by intravenous injection via the cephalic vein at a dose of 4 mg/kg. The compound was dissolved in a vehicle of 40% aqueous cyclodextrin. Serial blood samples were collected utilizing an in-dwelling catheter in the jugular vein at the indicated time points up to 6 h post-dosing. EDTA was used as an anticoagulant. The samples were centrifuged (1000 × g for 10 min at 4 °C), and plasma was stored frozen until analyzed for drug levels by HPLC-MS (electrospray mode operated under a positive ion mode). Plasma samples were thawed and denatured by the addition of four parts of ice-cold methanol containing a fixed amount of an internal standard to one part of plasma. The resulting protein precipitate was removed by centrifugation at 5000 × g, and the supernatants were analyzed directly. Concurrently plasma calibration standards of BX 471 were prepared over the range of quantification, processed, and analyzed under identical conditions. A Fisons, VG Platform single quadrupole instrument was used in these analyses with an electrospray ion source. MRM was used in the positive ion mode and the transitions monitored were 402.2–142.2 for BX 471 and 420.2–142.2 for the internal standard. The limit of quantification of BX 471 was 1 ng/ml.

**WST-1 Staining**—THP-1 and HEK293 cells expressing human CCR1 were incubated with various concentrations of BX 471 for 24 and 72 h followed by the addition of 10 μl of WST-1 (Roche Molecular Biochemicals) into 100 μl of culture medium. After 1 h of incubation with the dye at 37°C, the optical density was measured by the SpectraMax plate reader at 440 nm.
data were analyzed using an analysis of variance and Fisher's least significant difference.

**RESULTS**

**BX 471 Displaced Ligand Binding to CCR1**—Chemical optimization of compounds identified from high capacity screening of our compound libraries, using a CCR1 binding assay as described (4), yielded the lead compound, designated BX 471 (Fig. 1). In competition binding experiments with HEK293 cells expressing human CCR1, BX 471 was able to displace 125I-RANTES binding in a concentration-dependent manner with a *Kd* of 2.8 nM, which is similar to the *Kd* for RANTES of 1 nM (Fig. 2, Table I). In addition to RANTES, other ligands that bind to CCR1 with high affinity include MIP-1α and MCP-3. Competition binding studies showed that BX 471 could displace both 125I-MIP-1α and 125I-MCP-3 binding from human CCR1 with *Kd* values of 1 and 5.5 nM, respectively (Table I). These data demonstrated that BX 471 was a potent inhibitor of CCR1.

**Functional Antagonism for CCR1**—To demonstrate that BX 471 is a functional antagonist for human CCR1, we measured the ability of the compound to inhibit agonist-induced Ca2+ mobilization in CCR1-expressing cells. As shown in Fig. 3, MIP-1α at 30 nM induced a rapid and transient increase in intracellular Ca2+ (Fig. 3, inset). BX 471 inhibited the Ca2+ transients induced by submaximal concentrations of CCR1 ligands, 30 nM MIP-1α, 300 nM RANTES, and 100 nM MCP-3 in a concentration-dependent manner with *IC50* values of 5.2, and 6 nM, respectively, demonstrating functional antagonism for CCR1 (Fig. 3). When given alone, the compound did not induce Ca2+ transients, indicating that the compound has no intrinsically agonistic activity (data not shown).

The reversibility of receptor inhibition by an antagonist is a desirable property for a therapeutic agent. The Ca2+ mobilization assay was used to test the reversibility of the BX 471 effect. HEK293 cells expressing human CCR1 were treated with 300 nM BX 471 for 30 min at 37 °C, and under these conditions CCR1 was completely inhibited. The compound was then removed from cells by 8 washes with media and assay buffer. The Ca2+ transients in response to 30 nM MIP-1α was then measured. As shown in Fig. 4, MIP-1α at 30 nM increased intracellular Ca2+ concentration, and the response was completely inhibited by 300 nM BX 471. After removal of BX 471, the MIP-1α-induced Ca2+ transients recovered, indicating the reversibility of the CCR1 inhibition by BX 471.

To further examine the functional antagonism of BX 471, we measured its ability to inhibit the extracellular acidification rate in response to MIP-1α in THP-1 cells using the microphysiometer (10). As shown in Fig. 5, MIP-1α induced a rapid increase in the extracellular acidification rate, reaching a maximum after about 2 min and returning close to base-line levels within 8 min. The response elicited by MIP-1α was inhibited by BX 471 in a concentration-dependent manner with an *IC50* of approximately 1 nM (Fig. 5).

As an additional measurement of the functional antagonism of BX 471 for CCR1, the expression of the integrin CD11b on monocytes was measured in a whole blood assay using FACS-can analysis (Fig. 6). MIP-1α dose responsively induced the expression of CD11b on monocytes with an *EC50* of 110 nM (Fig. 6A). Blood from two separate donors was incubated with 1 μM BX 471 and then stimulated with 100 nM MIP-1α. The CCR1 antagonist inhibited CD11b up-regulation by MIP-1α by 100% and 65%, respectively (Fig. 6, B and C).

Chemokines were originally defined and classified as potent leukocyte chemoattractants mediating their effects through GPCR like CCR1 (11). Thus, characterization of a putative CCR1 antagonist would be incomplete without demonstrable inhibition of leukocyte migration elicited by a chemokine stimulus. Fig. 7 shows that BX 471 is able to inhibit the directed migration of both human lymphocytes and monocytes in response to the CCR1 ligands MIP-1α and RANTES but has no effect on the CCR5 ligand MIP-1β, the CCR2 ligand MCP-1, or the CXCR4 ligand stromal-derived factor 1α. Thus, BX 471 is a potent inhibitor of leukocyte migration and is specific for the CCR1 receptor since it is unable to affect the directed migration of cells in response to various chemokine ligands for CCR5, CCR2, CXCR1, and CXCR4. It thus shows functional selectivity as well as functional antagonism.

**Schild Analysis of BX 471**—The mechanism of the antagonistic effect of BX 471 was examined on CCR1-transfected cells by Schild analysis (12). The concentration-response curves for
Fluo-3-loaded cells were pretreated with or without 300 nM BX 471 for 15 min and then stimulated with increasing concentrations of BX 471 for 30 min and then stimulated with 1 nM MIP-1α. The increase in acidification rate was monitored using a microphysiometer. Data have been normalized as percentage biological response. Data shown are representative of at least three separate studies.

**Effect of BX 471 on General Toxicity**—To demonstrate that CCR1 antagonism by BX 471 was not due to the cellular toxicity of the compound, THP-1- or CCR1-transfected HEK293 cells were treated with BX 471 at concentrations up to 10 μM for 24 h, and cellular toxicity was monitored by measuring WST-1 staining. No significant toxicity was observed (data not shown). The toxicity for BX 471 was further examined in vivo by a battery of serum diagnostic tests including hepatic and renal function tests and blood electrolytes on rabbits that had been dosed with BX 471 at 20 mg/kg/day for 30 days. The test results all fell within the normal range (data not shown). The results suggest that the inhibition of BX 471 on CCR1 activation was not due to cellular toxicity and that chronic treatment with the drug had no adverse effects on the normal physiology of the animals.

**Pharmacokinetics of BX 471 in Dogs**—The oral bioavailability of BX 471 was examined in conscious dogs. BX 471 was administered to fasted male beagle dogs at 4 mg/kg in a vehicle of 40% cyclodextrin in saline by bolus intravenous injection via the cephalic vein or by oral gavage. The plasma samples were prepared, and compound concentrations in the plasma were determined by HPLC-MS. As shown in Fig. 9, BX 471 reached peak plasma levels approximately 2 h after oral dosing and maintained measurable concentrations for up to 6 h. BX 471 exhibits a volume of distribution (0.5 l/kg) close to the volume of body water (0.6 l/kg), suggesting that the compound is confined primarily to the aqueous volume (Table III). Low clearance, 2 ml/min/kg (which represents less than 10% of the total liver blood flow) in the dog resulted in a moderate terminal half-life of 3 h (Fig. 9 and Table III). For dogs that were orally dosed, the half-life for BX 471 was approximately 3 h. Calculations of percent oral availability using area under curve measurements obtained from analysis using TOPFIT software indicated that BX 471 is an orally absorbed drug in fasted dogs with an oral bioavailability of approximately 60% (Fig. 9 and Table III).

**Efficacy of BX 471 in a Rat EAE Model of Multiple Sclerosis**—Before determining the efficacy of BX 471 in a rat EAE model of multiple sclerosis, we tested its ability to inhibit MIP-1α binding to rat CCR1 receptors. Scatchard analysis of
competition binding studies with BX 471 demonstrated that the compound was able to inhibit chemokine binding to rat CCR1 with a $K_i$ of 121 ± 60 nM (data not shown), which was approximately 100 times less effective for rat CCR1 than for human CCR1. In addition, BX 471 does not inhibit chemokine binding to rat CCR5 (data not shown) and is thus specific for rat CCR1.

From pharmacokinetic studies in rats, we determined that the subcutaneous administration of BX 471 three times a day would give blood drug levels of 1 to 5 μM (data not shown), which we calculated would be about 10–50 times the $K_i$ on rat CCR1 receptors and should be sufficient for inhibition of MIP-1α binding. Unfortunately in contrast to the pharmacokinetic data obtained in the dog, BX 471 was poorly orally available in the rat (<20%, data not shown). Based on these studies, a rat EAE model of multiple sclerosis was set up.

Animals were dosed subcutaneously three times a day with vehicle or with BX 471 at 5, 20, and 50 mg/kg. The CCR1 antagonist, BX 471, dose-dependently decreased the severity of the disease (Fig. 10A). At the highest dose, 50 mg/kg, there was a marked reduction in the clinical score that was statistically significant at $p = 0.05$ (analyzed by analysis of variance) compared with the vehicle control. However, even at the lower two doses of BX 471, 20 mg/kg and 5 mg/kg, there were still noticeable decreases in the clinical score. This is more readily observed by expressing the data as the average accumulated clinical score per treatment group (Fig. 10B). Statistical analysis of the average accumulated clinical score data by $t$ test revealed that the 50 and 20 mg/kg doses were statistically significant compared with the vehicle control $p = 0.003$ and $p = 0.014$, respectively.

**DISCUSSION**

Increasing evidence suggests a role for the chemokines MIP-1α and RANTES in autoimmune diseases like multiple sclerosis and rheumatoid arthritis (1, 2, 14, 15) and in organ transplant rejection (16–18). A receptor for these chemokines, CCR1, could therefore be a major therapeutic target. Indeed, recent studies with CCR1 knockout mice in animal models of multiple sclerosis (3) and organ transplant rejection (19) have implicated this receptor in the pathogenesis of these diseases. Based on these data, we established a program to discover potent, selective, small molecule antagonists of CCR1 for the treatment of autoimmune diseases and organ transplant rejection.

The approach to identify CCR1 antagonists was to screen our compound library with a high throughput 125I-MIP-1α binding assay (4). The compounds identified from screening were chemically optimized, and this led to the discovery of BX 471 (Fig. 1). BX 471 was a potent inhibitor of chemokine binding to CCR1 (Fig. 2 and Table I) and inhibited the activation of CCR1...
The direct mechanism by which BX 471 inhibits the activation of human CCR1 has not yet been established. However, based on our studies, we favor the idea that the compound directly binds to CCR1, leading to the blockade of receptor activation. MIP-1α was able to bind to both CCR1 and CCR5 with high affinity. However, BX 471 was able to inhibit the binding and activation of CCR1 but not CCR5. Therefore, it is unlikely that BX 471 inhibits CCR1 by direct binding to the CCR1 ligand, MIP-1α. In addition, BX 471 did not demonstrate toxicity either in vitro or in vivo. Furthermore, BX 471 showed greater than a 10,000-fold selectivity for CCR1 when tested in 28 other GPCR binding assays and in cell migration assays in response to MIP-1α, MCP-1, interleukin-8, and stromal-derived factor 1α. These data suggest that CCR1 inhibition by BX 471 is not due to either cellular toxicity or nonspecific interaction with other cell surface receptors. Furthermore, a radiolabeled active analogue of BX 471 was able to bind to CCR1 with high affinity (data not shown).

The antagonism of CCR1 by BX 471 appeared to be reversible and surmountable (Figs. 4 and 8). The data is consistent with competitive antagonism. Mechanistically, competitive antagonism is observed when an antagonist binds to the same site on a receptor as the agonist but is incapable of activating the receptor, therefore preventing receptor binding and subsequent activation of the receptor by its agonist. In addition, similar Schild data could also be obtained if the antagonist were to bind to a separate but interacting site on the receptor from the agonist binding site.

Studies with a number of GPCR have revealed that the receptor binding sites for antagonists can be distinct from the agonist binding sites (20–22). For example, Gether et al. (21) demonstrate that non-conserved residues in transmembrane segments V and VI were essential for the binding of the neu- rokinin-1 receptor antagonist, CP-96345, to its receptor but were not important for the binding of the natural peptide ligand, substance P. Since the chemical structure of BX 471 does not resemble the natural ligands of CCR1, it is tempting to speculate that BX 471 binds to separate or overlapping sites on CCR1 rather than to those involved in chemokine binding.

Although peptide antagonists of chemokine receptors have been described (23, 24), their sub-optimal metabolic stability and oral bioavailability have limited their therapeutic utility. Non-peptide antagonists could potentially overcome these disadvantages. Studies show that BX 471 was 60% orally available when tested in conscious dogs. It appeared to be relatively stable in plasma with a clearance rate of 2 ml/min/kg and maintained a measurable level for at least 6 h (Fig. 9 and Table III). Therefore, if these profiles of BX 471 are confirmed in humans, this compound would provide a better alternative as a therapeutic candidate than the peptide antagonists reported.

We have previously identified a small molecule CCR1 antagonist, 2–2-diphenyl-5-(4-chlorophenyl)piperidin-1-ylvaleronitrile (4, 25). In addition, a patent filed by Takeda Chemical Industries, Ltd. reported a CCR1 antagonist, a urea piperidine derivative of the nitrile group, that is structurally similar to our 4-hydroxyxypiperidine series (26). This compound, however, demonstrated potential cross-reactivity with several biogenic amine neurotransmitter receptors. Furthermore, Banyu Pharmaceutical Co. Ltd. has recently reported small molecule CCR1 antagonists (27). This patent disclosure reports a group of tricyclic amides that inhibited receptor binding with an IC₅₀ of 1.8 nm. These compounds were also reported to inhibit binding to CCR3 with a similar IC₅₀ (1.7 nM), making them less specific than the Berlex compound. The fact that these compounds are quaternary ammonium salts may further limit their therapeutic use due to potential pharmacokinetic problems such as poor oral absorption and rapid elimination. Thus, based on these studies, BX 471 appears to be more highly specific and superior on pharmacological and pharmacokinetic grounds to the other reported CCR1 antagonists.

Multiple sclerosis is an autoimmune disease mediated by extensive infiltration of T lymphocytes and monocytes into the central nervous system followed by resident macrophage and microglia activation. This results in an extensive inflammation and subsequent demyelination of the white matter in the central nervous system (28). Although the mechanisms responsible for causing this immunologic damage in the central nervous system are still unknown, regulation of the recruitment of activated immune cells and the extravasation of these cells into the central nervous system has been readily studied. Chemokine-induced recruitment of leukocyte subsets clearly plays roles in chronic inflammatory diseases and has been implicated in the pathogenesis of multiple sclerosis (29–31). Based on...
TABLE II
Specificity of BX 471 binding to GPCR

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
<th>Tissue</th>
<th>10 μM % Inhibition of Binding</th>
<th>1 μM % Inhibition of Binding</th>
<th>Selectivity (K i)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine A 1</td>
<td>[125I]IB-MECA</td>
<td>Human</td>
<td>38</td>
<td>6</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Adrenergic α 1a</td>
<td>[3H]prazosin</td>
<td>Rat</td>
<td>-7</td>
<td>5</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Adrenergic α 2a</td>
<td>[3H]MK912</td>
<td>Human</td>
<td>24</td>
<td>-8</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Adrenergic α 2c</td>
<td>[3H]MK912</td>
<td>Human</td>
<td>22</td>
<td>21</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Adrenergic β 1</td>
<td>[125I]-cyanoepinodol</td>
<td>Human</td>
<td>1</td>
<td>2</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Bradykinin B 1</td>
<td>[125I]-Hides-Arg 11-kallidin</td>
<td>Human</td>
<td>-11</td>
<td>-9</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Cannabinoid</td>
<td>[3H]WIN-55,212-2</td>
<td>Human</td>
<td>18</td>
<td>23</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Cholecystokinin</td>
<td>[3H]Me-N(3)364,718</td>
<td>Human</td>
<td>16</td>
<td>-14</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Dopamine D 1</td>
<td>[3H]SCH23390</td>
<td>Human</td>
<td>13</td>
<td>-14</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Dopamine D 1</td>
<td>[3H]Sperone</td>
<td>Human</td>
<td>9</td>
<td>6</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Dopamine D 2</td>
<td>[3H]Sperone</td>
<td>Human</td>
<td>17</td>
<td>21</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Dopamine D 2</td>
<td>[3H]Sperone</td>
<td>Human</td>
<td>21</td>
<td>2</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Dopamine D 2</td>
<td>[3H]SCH23390</td>
<td>Human</td>
<td>23</td>
<td>29</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Endothelin B 1</td>
<td>[125I]Endothelin-1</td>
<td>Human</td>
<td>8</td>
<td>-15</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Leukotriene B 4</td>
<td>[125I]LTB 4</td>
<td>Human</td>
<td>14</td>
<td>-13</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Mucarinic M 1</td>
<td>[3H]NMS</td>
<td>Human</td>
<td>-7</td>
<td>-9</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Mucarinic M 1</td>
<td>[3H]NMS</td>
<td>Human</td>
<td>21</td>
<td>12</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Mucarinic M 1</td>
<td>[3H]NMS</td>
<td>Human</td>
<td>-8</td>
<td>7</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Mucarinic M 1</td>
<td>[3H]NMS</td>
<td>Human</td>
<td>15</td>
<td>15</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Mucarinic M 1</td>
<td>[3H]NMS</td>
<td>Human</td>
<td>18</td>
<td>11</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Neurokinin</td>
<td>[125I]SR-140333</td>
<td>Human</td>
<td>2</td>
<td>5</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Neuropeptide Y</td>
<td>[3H]neuropeptide Y</td>
<td>Human</td>
<td>19</td>
<td>12</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Serotonin 5-HT 1A</td>
<td>[3H]8-OH-DPAT</td>
<td>Human</td>
<td>8</td>
<td>4</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Serotonin 5-HT 1A</td>
<td>[3H]NMS</td>
<td>Human</td>
<td>19</td>
<td>19</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>CXCR2</td>
<td>[125I]interleukin-8</td>
<td>Human</td>
<td>-4</td>
<td>2</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>CXCR4</td>
<td>[125I]SDF-1α</td>
<td>Human</td>
<td>5</td>
<td>1</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>CCR5</td>
<td>[125I]SDF-1β</td>
<td>Human</td>
<td>2</td>
<td>3</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>DARC</td>
<td>[125I]MGSA</td>
<td>Human</td>
<td>6</td>
<td>3</td>
<td>&gt;10,000</td>
</tr>
</tbody>
</table>

* Represents K i, BX 471 on test receptor/K i BX 471 on CCR1.

Absolute bioavailability (%F) was estimated to be 60%. Pharmacokinetic parameters were determined using WinNonLin version 3.0 software. AUC, area under curve.

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>Intravenous</th>
<th>Oral</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terminal plasma half-life</td>
<td>3 h</td>
<td>3 h</td>
</tr>
<tr>
<td>AUC (0–6 h)</td>
<td>27 μg × h/ml</td>
<td>16 μg × h/ml</td>
</tr>
<tr>
<td>AUC (0–8 extrapolated)</td>
<td>39 μg × h/ml</td>
<td>21 μg × h/ml</td>
</tr>
<tr>
<td>Volume of distribution (V f)</td>
<td>0.5 l/kg</td>
<td>1.0 l/kg</td>
</tr>
<tr>
<td>Clearance rate (CL)</td>
<td>2 ml/min/kg</td>
<td>2 ml/min/kg</td>
</tr>
</tbody>
</table>

a possible therapy in the treatment of multiple sclerosis (36).

The CCR1 antagonist, BX 471, at 50 mg/kg gives a 50% reduction of clinical score in the rat EAE model (Fig. 10). The much higher doses of BX 471 that are required to be effective in rat EAE could be due to the fact that the compound has a K i for inhibition of MIP-1α binding for rat CCR1 of 121 nM, compared with a compound K i of 1–2 nM for human CCR1. Based on these considerations, it is likely that much lower doses of BX 471 (500 μg/kg or less) would be required to be therapeutically effective in treating multiple sclerosis in humans.

The complex pathology of the development of multiple sclerosis demonstrates the involvement of several factors that are both peripheral and central nervous system-specific. Clearly, inhibition of peripheral leukocyte activation and recruitment can effect the development of central nervous system pathologies. Inhibition of central nervous system-specific glial activa-

chemokine antibody neutralization experiments, there is evidence for a role of MIP-1α in animal models of multiple sclerosis (1, 2). Although recruitment into the vasculature of the brain is likely to be directed by these chemoattractant proteins, leukocyte activation also takes place and results in the up-regulation of adhesion molecules. Neutralization of leukocyte integrins has demonstrated an ability to inhibit or prevent EAE in rodent models (32–35) and has even been proposed as
tion can also ameliorate the effects of neuronal and oligodendrocyte dysfunction and destruction. CCR1 expression within demyelinating plaques of multiple sclerosis brains on T-lymphocytes, macrophage/microglia, or vascular expression on T lymphocytes implicate this receptor in the pathology of this disease. It is highly likely that a stepwise process of leukocyte accumulation in the central nervous system occurs in multiple sclerosis. Lymphocyte expression of CCR1 in the periphery leads to accumulation of activated cells in blood vessels of multiple sclerosis brains; after transendothelial migration to the perivascular regions, the receptor would likely be down-modulated. Here the expression of other chemokine receptors such as CCR5 and CXCR3, markers of TH1 type T lymphocytes, would be necessary for disease progression. Although a number of chemokines, MIP-1α, RANTES, MCP-1, interferon inducible protein of 10KD, monokine induced by interferon γ (reviewed in Refs. 37–39) and various receptors CCR1, CCR5, CXCR3 (38, 39) have been visualized in EAE or multiple sclerosis brains, the consequential versus causative ramifications for the development of multiple sclerosis pathology remain to be proven. Only by the use of specific chemokine receptor inhibitors to treat multiple sclerosis in human clinical trials will the significance of these molecules be elucidated.

In summary, we have identified a potent, selective, small molecule functional antagonist for CCR1, BX 471. The inhibition of CCR1 by the compound appears to be reversible and surmountable. Studies in dogs demonstrated that the compound is orally available and relatively stable in plasma, and the compound appears to be effective in an EAE model of multiple sclerosis in the rat. We believe that BX 471 represents a CCR1 antagonist possessing high potential as a therapy among all the CCR1 inhibitors reported so far. Multiple sclerosis is a particularly devastating disease of the central nervous system. Other than immunosuppressive drugs like steroids, which have a myriad of side effects, or the use of interferons, no orally active drugs are available for its treatment. Safety studies with BX 471 in a variety of animal species have revealed no toxicity, hemodynamic, or central nervous system effects with the drug at doses way in excess of the blood plasma levels observed in this animal study (data not shown). Thus, a specific treatment with an orally available small molecule like BX 471 may represent a potential therapeutic alternative to the current drugs of choice in treating patients with multiple sclerosis.

**REFERENCES**

25. Ng, H. P., Mak, J. Y., Bauman, J. G., Grannam, A., Islam, I., Liang, M., Horuk, R.,
Identification and Characterization of a Potent, Selective, and Orally Active Antagonist of the CC Chemokine Receptor-1
Meina Liang, Cornell Mallari, Mary Rosser, Howard P. Ng, Karen May, Sean Monahan, John G. Bauman, Imadul Islam, Ameen Ghannam, Brad Buckman, Ken Shaw, Guo-Ping Wei, Wei Xu, Zuchun Zhao, Elena Ho, Jun Shen, Huynh Oanh, Babu Subramanyam, Ron Vergona, Dennis Taub, Laura Dunning, Susan Harvey, R. Michael Snider, Joseph Hesselgesser, Michael M. Morrissey, H. Daniel Perez and Richard Horuk

doi: 10.1074/jbc.M001222200 originally published online March 29, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M001222200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 36 references, 11 of which can be accessed free at http://www.jbc.org/content/275/25/19000.full.html#ref-list-1