BX471: A CCR1 Antagonist with Anti-Inflammatory Activity in Man

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Abstract: Chemokines belong to a large family of chemoattractant molecules involved in the directed migration of immune cells. They achieve their cellular effects by direct interaction with cell surface receptors. The chemokine receptor CCR1 appears to be involved in a variety of proinflammatory and autoimmune diseases and this makes it a very attractive therapeutic target. This review discusses the identification, chemistry, biology and therapeutic potential of BX 471 a potent CCR1 antagonist that is currently in the clinic for a variety of indications.

INTRODUCTION

Chemokine receptors belong to one of the most pharmacologically exploited family of proteins; the G-protein coupled receptors (GPCR's). Drugs that target these receptors make up greater than 45% of all known-marketed medicines. The first recorded uses of drugs directed at this important family of proteins can be traced back to ancient Chinese and Indian physicians who were using plant extracts to treat a variety of disorders [1, 2]. For example, the fumewort plant was first described for its tranquilizing effects as early as the fifth century [1], although its active principle tetrahydropalmitine, a potent dopamine receptor antagonist, was isolated only a few years ago. Extracts from the deadly nightshade family have been widely used as analgesics and anesthetics in medicine since ancient times [1]. The active principles that were identified in modern times were shown to be the potent muscarinic receptor antagonists, atropine and scopolamine.

From the ancient shaman who searched for medicinal plants to treat disease to the modern pharmaceutical industry with its sophisticated high-throughput mechanism-based screening programs; the quest to find drugs to help the sick and ailing is an important activity that has been around since the dawn of mankind. Today, the modern pharmas concentrate on increasing resources and money, in finding potent drugs that target both old diseases such as multiple sclerosis and rheumatoid arthritis, and modern diseases such as AIDS and organ transplant rejection. Collectively the chemokines, because of their important role in these and other diseases, have been the focus of much attention by drug companies, and almost all of the major pharmaceutical houses have screens to identify chemokine receptor antagonists.

CHEMOKINES AND THEIR RECEPTORS

Chemokines belong to a large family of small, chemotactic cytokines, characterized by a distinctive pattern of four conserved cysteine residues [3]. They are divided into two major (CXC and CC) and two minor (C and CX3C) groups, dependent on the number and spacing of the first two conserved cysteine residues. Although originally identified on the basis of their ability to regulate the trafficking of immune cells, the biological role of chemokines goes well beyond this simple description of their function as chemoattractants, and they have been shown to be involved in a number of biological processes, including growth regulation, hematopoiesis, embryological development, angiogenesis and HIV-1 [3, 4].

Chemokines mediate their biological effects by binding to cell surface receptors which belong to the GPCR superfamily [5]. Receptor binding initiates a cascade of intracellular events, mediated by the receptor associated heterotrimeric G proteins. These G-protein subunits trigger various effector enzymes which leads to the activation not only of chemotaxis, but also to a wide range of functions in different leukocytes such as an increase in the respiratory burst, degranulation, phagocytosis and lipid mediator synthesis [5].

Chemokines have been shown to be associated with a number of autoinflammatory diseases including multiple sclerosis, rheumatoid arthritis, atherosclerosis, dermatitis, organ transplant rejection, etc. [6]. Evidence, reviewed below, is mounting that chemokines may play a major role in the pathophysiology of these diseases and thus, chemokine receptor antagonists could prove to be useful therapeutics in treating these and other proinflammatory diseases.

DISCOVERY OF CCR1

Although numerous reports had described specific effects of the chemokines RANTES and MIP-1a on T lymphocytes, and monocytes the identity of the putative receptor for these ligands was unknown [7]. However, cloning of this receptor was aided by the fact that the primary sequences of the C5a, fMLP and IL-8 receptors revealed domains which were conserved in receptors associated with cell motility, but not in other seven-transmembrane-spanning receptors [8-11]. These similarities were exploited using PCR technology to obtain several orphan receptor cDNA clones, which were then expressed and screened by receptor binding and functional assays. Using this homology hybridization cloning approach, the molecular cloning and functional

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1389-5575/05 $50.00+.00

expression of CCR1 was reported by two separate groups [12, 13]. The open reading frame for human CCR1 is on a single exon, and predicts a protein of 355 amino acids (Fig. **1**). The gene, cmkbr1, is located on human chromosome 3p21. The expressed human CCR1 was able to bind MIP-1α and RANTES with high affinity and physiological concentrations of both ligands induced an increase in intracellular Ca^{2+}. CCR1 was specific for these ligands and showed a poor response to MIP-1ß and MCP-1. In addition to these ligands, CCR1 has been shown to respond with high affinity and to signal in response to a variety of other C-C chemokines including MCP-3, MPIF-1, leukotactin-1, and HCC-1.

ROLE OF CCR1 IN PATHOPHYSIOLOGY

Assigning biological activities and elucidating pathophysiological roles for CCR1 has been difficult for several reasons. First, as discussed above, several chemokines including MIP-1α, RANTES, MCP-3, leukotactin-1, and HCC-1 that bind with high affinity to CCR1 can also bind with high affinity and activate other chemokine receptors, RANTES for example, can also bind to CCR3 and CCR5 and MIP-1α can also bind to CCR5. Second, there are no known commercially available specific neutralizing antibodies to CCR1. Consequently, current ideas regarding physiological and pathological roles of CCR1 have come mainly from a consideration of the roles of its ligands in biology, recognizing that these ligands may also be also acting upon other chemokine receptors. Knockout studies of CCR1 (see later) have also provided some information of a rather limited nature, as have studies employing CCR1 transfected cell lines. For instance, MIP 1α and RANTES can induce predictable biological responses in CCR1 transfected cells, chemotaxis for example. While such information is useful, it does not however convey a very clear picture of the role that CCR1 plays in the intact animal, since depending upon the physiological circumstances, the receptor may or may not even be expressed in its normal target cell. Given these and other difficulties consideration of the potential pathophysiological roles of CCR1 were of necessity, originally based upon a consideration of the roles of its ligands. A clearer indication of the biological roles of CCR1 have been provided by studies with CCR1 specific antagonists, and these will be discussed later.

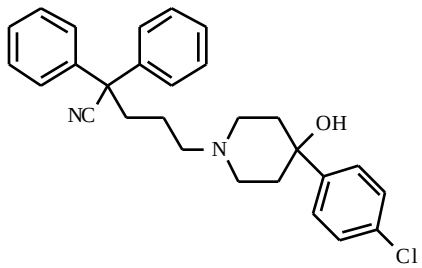
ROLE OF CCR1 IN MULTIPLE SCLEROSIS

Multiple sclerosis is an autoimmune disease mediated by T and B lymphocytes, and macrophages, which results in extensive inflammation and demyelination of the white matter [14]. Although the mechanisms responsible for causing this immunological damage in the CNS are still unknown, they are almost certainly mediated by infiltrating leukocytes. Initial interactions between invading T-cells and monocytes in the CNS, result in the production of cytokines such as TNF and IL-1. These cytokines induce a variety of effects that culminate in the recruitment of activated T-cells and macrophages. It is likely that a chemotactic gradient of immobilized chemokines, possibly bound to sulfated glycans [15] on the subendothelial matrix [16], guides the directed flow of these blood leukocytes across the endothelium into the CNS.

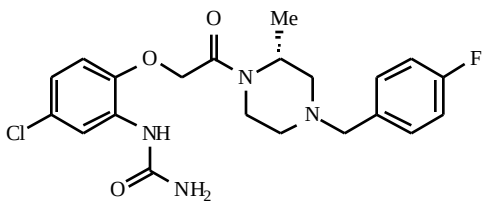
A variety of evidence implicates CCR1 in multiple sclerosis. For instance, Trebst *et al*. [17] analyzed the expression of the chemokine receptors CCR1 and CCR5 in human cerebrospinal fluid and in MS brain lesions. They found that the majority of infiltrating monocytes in the cerebrospinal fluid were CCR1+/CCR5+, compared to less than 20% of circulating monocytes. In active MS lesions, CCR1+/CCR5+ monocytes were found in perivascular cell cuffs and at the demyelinating edges of evolving lesions. Mononuclear phagocytes in early demyelinating stages comprised CCR1+/CCR5+ hematogenous monocytes and CCR1-/CCR5- resident microglial cells. These findings suggested that monocytes expressing CCR1, migrate into the CNS, where they could play a role in the pathophysiology of multiple sclerosis. A role for CCR1 in multiple sclerosis is strongly supported by studies from Rottman *et al*. [18], who demonstrated, in an EAE model of multiple sclerosis, that CCR (-/-) mice had a significantly reduced incidence of disease compared to wild type mice. The spinal cords of the wild type mice showed non-suppurative myelitis, while those from the CCR1 knockouts were minimally inflamed.

ROLE OF CCR1 IN RHEUMATOID ARTHRITIS

There is accumulating evidence from a number of studies, to implicate RANTES in the progression of rheumatoid arthritis. Rheumatoid arthritis is a chronic inflammatory disease, characterized in part by a memory T lymphocyte



1
Berlex
BX 513
K_i (MIP-1α) = 40 nM
K_i (RANTES) = 60 nM



2
Berlex
BX 471
K_i (MIP-1α) = 1 nM
K_i (RANTES) = 6 nM

Fig. (1). Structures of Berlex CCR1 antagonists.

and monocyte infiltrate [19, 20]. This process is thought to be mediated by chemotactic factors released by inflamed tissues. Rheumatoid synovial fibroblasts upregulate RANTES mRNA in response to IL-1ß, TNFα and γIFN. Rathanswami *et al*. demonstrated by Northern blot and ELISA, that cultured synovial fibroblasts isolated from rheumatoid patients were capable of expressing and producing RANTES and other chemokines in response to IL-1ß [19]. Snowden *et al*. have used reverse transcriptase-PCR to detect RANTES mRNA in four out of seven synovial tissue samples from rheumatoid arthritis patients [20]. By contrast, osteoarthritis tissue does not express RANTES mRNA [20].

In addition to these studies, we have recently obtained strong evidence implicating RANTES in the pathophysiology of rheumatoid arthritis [21]. We were able to show in an adjuvant-induced arthritis (AIA) model in the rat, that antibodies to RANTES greatly reduced the development of disease in animals induced for AIA. Polyclonal antibodies to either MIP-1α or KC were ineffective. Recently, a small phase Ib clinical trial with a CCR1 antagonist from Pfizer yielded preliminary data implicating CCR1 in the disease [22]. In this double-blind, placebo-controlled, phase–Ib clinical trial, a specific, oral CCR1-antagonist was tested in 16 patients with active rheumatoid arthritis. In patients treated with the antagonist, there was a significant reduction in the number of macrophages in the synovium compared to the placebo treated group. There were also significant decreases in overall cellularity, intimal lining layer cellularity, CD4+T-cells, and CD8+T-cells in treated patients. Cells lacking CCR1 were not affected. Trends towards clinical improvement were observed within the treated patients, but not in the placebo group. Severe side-effects were not reported. The latest information suggests that Pfizer is conducting multi center CCR1 trials in rheumatoid arthritis patients. These results provide the first evidence that specific chemokine-receptor blockade can result in potential beneficial effects in patients with active rheumatoid arthritis.

ROLE OF CCR1 IN ORGAN TRANSPLANT REJECTION

The classic signs of acute cellular rejection during organ transplantation includes the infiltration of mononuclear cells into the interstitium [23]. This cellular infiltrate consists mainly of T-cells and macrophages, cell types that express CCR1, and thus respond to RANTES. Several early studies provided evidence for a role of the CCR1 ligand RANTES in organ transplant rejection, particularly of the kidney. In a model of reperfusion injury in the rat, RANTES levels were increased over normal and remained high for more than a week, correlating with the peak of infiltrating macrophages [24]. RANTES protein was detected in infiltrating mononuclear cells, tubular epithelium, and vascular endothelium of renal allograft biopsy specimens from patients with cyclosporin nephrotoxicity, but not in normal kidney [23]. A recent study suggests that RANTES may play a role in graft atherosclerosis [25]. Increased levels of RANTES, both mRNA and protein, were detected in mononuclear cells, myofibroblasts, and endothelial cells of arteries undergoing accelerated atherosclerosis, compared with normal coronary arteries. Direct evidence for a role of CCR1 in transplant rejection was provided recently by Gao *et al*. [26], who demonstrated a significant prolongation of allograft survival in CCR1(-/-) mice in 4 separate models of cardiac allograft rejection. In one model, levels of cyclosporin that had marginal effects in CCR1(+/+) mice resulted in permanent allograft acceptance in CCR1(-/-) recipients. These studies strongly implicate a pathophysiological role for CCR1 in transplant rejection, and suggest that therapies to inhibit CCR1 may prove useful in preventing acute and chronic rejection clinically.

ROLE OF CCR1 IN ATHEROSCLEROSIS

Atherosclerosis and coronary artery disease result from intimal thickening of the blood vessels due to localized accumulation of lipids, known as atheromas. Although the exact mechanism of atherosclerotic plaque formation remains unclear, it can be viewed as an inflammatory process involving macrophages and T lymphocytes. The presence of substantial numbers of T-lymphocytes in the lesion, and local and circulating autoantibodies to plaque components suggests that a specific immune response is operating. Expression of adhesion molecules and local secretion of chemokines help to recruit inflammatory cells to the lesion, and CC chemokines in particular, have been postulated to play a role in this process. Investigation of RANTES expression in transplant-associated acceleratedatherosclerosis revealed an increased expression of the chemokine at both mRNA and protein levels in T-cells, macrophages, myofibroblasts, and endothelial cells of arteries undergoing accelerated atherosclerosis, but not in normal coronary arteries [25]. Human vascular smooth muscle cells treated with IL-1 or TNF produce a number of chemokines including RANTES. In contrast, very low amounts of RANTES (assessed by specific ELISA) are produced under basal conditions [27].

ROLE OF CCR1 IN PSORIASIS

Intraepidermal collections of neutrophils and T lymphocytes are unique features of the inflammatory reaction of psoriasis. Migration of leukocytes from dermis to the epidermis suggests a role for chemotactic agents in the pathophysiology of psoriasis. Studies have provided evidence for a role of the CCR1 chemokines RANTES and MIP-1α in psoriasis. In a recent study, the CC chemokine RANTES was detected, by immunostaining with specific antibodies, in keratinocytes of skin biopsies obtained from chronic psoriatic plaques [28]. The keratinocytes of patients with psoriasis showed almost a 40-fold increase in the expression of RANTES, compared to those from controls. Since RANTES is chemotactic for memory T-cells, and activated naive T cells then the increased amounts of RANTES reported in this study could provide an explanation for the increased migration of activated T-cells to the epidermis of the psoriatic lesions.

Another report demonstrates expression of RANTES in psoriatic lesions [29]. In this study, the authors showed by immunohistochemistry, that RANTES was present in the intercellular spaces between epidermal keratinocytes, in the fully developed lesions from the middle to the edge of

Table 1. K_i Values of CCR1 Antagonist BX 513 for Binding to CCR1 Receptors

Competing ligand	Human	Rabbit	Mouse	Marmoset	Rat
			K_i ± SEM (nM)		
human MIP-1α	2.1 ± 0.1	1.5 ± 0.08	32 ± 3.5	81 ± 23	61 ± 26
BX 513	40 ± 6	245 ± 60	>3500	451 ± 191	Not tested

psoriatic plaques, but not in the perilesional uninvolved and healthy control skin. Furthermore, they demonstrated by ELISA, that RANTES was produced by cultured normal human epidermal keratinocytes. Stimulation of these cells with TNF-α and IFN-γ synergistically increased the RANTES production in this system. Finally, Tacalcitol (an active vitamin D-3 analogue) inhibited RANTES production in cultured normal epidermal keratinocytes which may partly account for its action as an antipsoriatic drug. These results demonstrate the expression of RANTES in psoriatic lesions and suggest the involvement of this chemokine in the outcome of cutaneous inflammatory diseases.

ROLE OF CCR1 IN MULTIPLE MYELOMA

A variety of studies suggest a role for CCR1 in multiple myeloma. For example, Choi *et al*. [30] showed that that MIP-1α was an osteoclast stimulating factor in human marrow cultures and that it was overexpressed in patients with multiple myeloma, but not in controls. In addition, a neutralizing antibody to MIP-1α blocked the osteoclast stimulating factor activity present in bone marrow plasma from multiple myeloma patients. These data strongly support a role for MIP-1α in the bone destruction observed in patients with multiple myeloma.

Recently the role of MIP-1α was investigated in an *in vivo* model of mutiple myeloma [31]. A human mutiple myeloma–derived cell line ARH was stably transfected with an antisense construct to MIP-1α and tested for its capacity to induce a mutiple myeloma like bone disease in SCID mice. Human MIP-1α levels in marrow plasma from antisense treated mice were markedly decreased, compared with control ARH cells treated with empty vector. Mice treated with cells containing antisense construct to MIP-1α

Table 2. Specificity of CCR1 Antagonist BX 513 for GPCRs

Neurotransmitter Receptor	BX 513 % inhibition at 10 μM	Ratio of activity versus MIP-1α affinity
Adenosine	8.3	
α_1-Adrenergic	90.2	86
α_2-Adrenergic	24.7	
β–Adrenergic	17.7	
Dopamine	95.2	23
Histamine-1	24.4	
Histamine-2	58.8	257
Serotonin	65.2	229
Muscarinic (central)	85.8	114
Muscarinic (peripheral)	36.9	
Glutamate	0.9	
Opiate	62.6	229
Angiotensin II	49.6	
Arg-vasopressin	0	
CCK-peripheral	2.9	
Endothelin-A	3.2	
Substance P	0	
Neuropeptide Y	12.2	
Neurotensin	5.0	
VIP	0	
Galanin	9.3	
C5a	12.0	

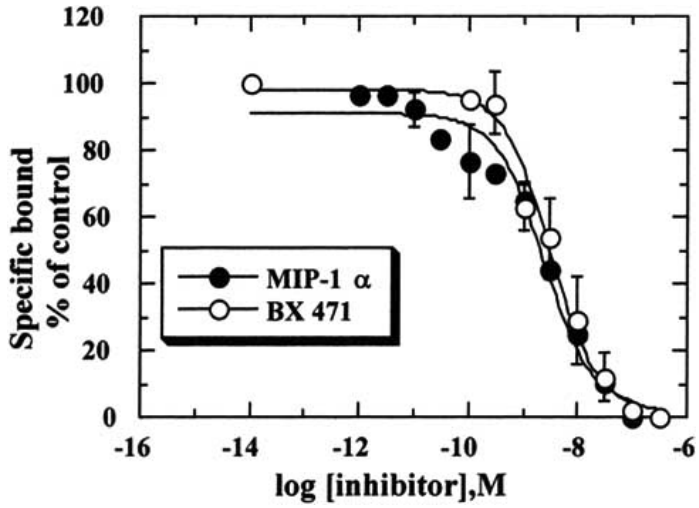


Fig. (2). BX 471 and MIP-1α displace ^{125}I-MIP-1α from the human CCR1 receptor expressed in HEK293 cells.

cells lived longer than controls and, unlike the controls, they showed no radiological identifiable lyric lesions. These and other data supported an important role for MIP-1α in cell homing, survival, and bone destruction in mutiple myeloma. Furthermore, these studies suggest that blocking MIP-1α activity may be useful in treating patients with myeloma to decrease both their tumor burden and bone destruction.

CCR1 ANTAGONISTS

The strong association of CCR1 with a wide variety of autoimmune and proinflammatory diseases have made the protein an attractive therapeutic target and for these reasons, we initiated a high throughput binding assay to identify CCR1 antagonists. A variety of compounds were identified, and the most interesting belonged to a family of 4-hydroxypiperidines (Fig. **1**). The most potent member of this class of compounds, 2-2-diphenyl-5-(4-chlorophenyl) piperidin-lyl)valeronitrite (BX 513), dose-responsively inhibited the ability of MIP-1α to induce a variety of biological responses in cells expressing human CCR1, including increases in extracellular acidification and intracellular Ca^{2+} mobilization [32]. The potency of BX 513 was 40 nM (Table **1**). Furthermore, it dose-responsively inhibited MIP-1α and RANTES induced migration in PBMC, but had no effects on the migration of cells stimulated with MIP-1β, MCP-1 or SDF-1α. These data demonstrated that BX 513 was a potent antagonist for CCR1, but had no effects on the related chemokine receptors CCR5, CXCR2 or CXCR4.

Selectivity against other GPCRs was shown by screening against a panel of human GPCR's (Table **2**). Selectivity is important because of the vital roles that GPCR's play in regulating homeostasis. Interestingly, BX 513 showed significant crossreactivty with a number of biogenic amine neurotransmitter receptors including adrenergic, dopaminergic and serotinergic (Table **2**). This was not surprising, given that its structure is reminiscent of the typical neuroleptic or antidepressant structural motif [33, 34]. Lack of GPCR selectivity is a well recognized problem in the development of small molecules targeting GPCRs and even weak activity of a compound for a related receptor can give rise to side effects when used therapeutically in humans. These selectivity issues can limit the potential therapeutic use of receptor antagonists. Attempts to "dial out" these undesired crossreactivities with the biogenic amine receptors while maintaining potency for CCR1, proved to be difficult for this group of compounds.

In addition, these problems of specificity were coupled with problems of species selectivity, and this class of compounds had very poor affinity for non human receptors (for example the K_i for BX 513 on mouse CCR1 was 3500 nM) which precluded testing them for efficacy in appropriate animal models of disease (Table **1**). Based on these data and the rapid metabolism of these compounds, no further development was carried out and we concentrated our efforts on identifying other novel CCR1 leads.

To this end, a related series of CCR1 antagonists identified from screening and exemplified by BX 471 showed much more promise as potential drug candidates (Fig. **1**).

Table 3. K_i Values of CCR1 Antagonist BX 471 for Binding to CCR1 Receptors

Competing ligand	Human	Rabbit	Mouse	Marmoset	Rat
			K_i ± SEM (nM)		
human MIP-1α	2.1 ± 0.1	1.5 ± 0.08		81 ± 23	61 ± 26
BX 471	1.0 ± 0.03	0.8 ±0.02	215 ± 46	117 ± 28	121 ± 67

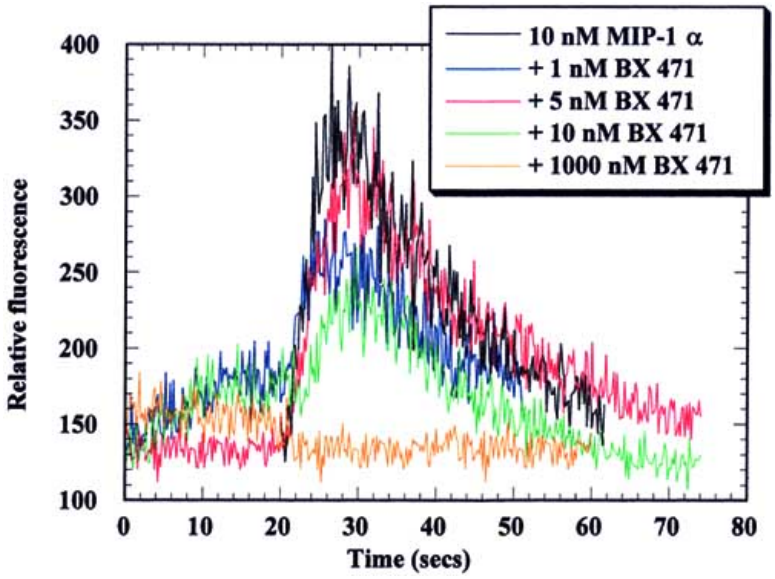


Fig. (3). BX 471 dose responsively inhibits Ca^{2+} flux induced by 10 nM MIP-1α in HEK293 cells expressing human CCR1.

For example, in competition binding experiments with HEK293 cells expressing human CCR1, BX 471 was able to displace ^{125}I-MIP-1α, (Fig. **2**) ^{125}I-RANTES and ^{125}I-MCP-3 binding in a concentration-dependent manner with K_i's of 2.8 nM, 1.0 and 5.5 nM, respectively, which are similar to the K_D's for ligand binding to CCR1. These data demonstrated that BX 471 was a potent inhibitor of human CCR1. In contrast to these data, the compound has much weaker affinity for rodent CCR1 with K_i's for inhibition of MIP-1α binding to rat and mouse CCR1 of 121 nM, and 215 nM respectively (Table **3**).

This poor affinity of potent small molecule inhibitors of human GPCR's for non human receptors represents one of the more challenging problems in drug development, and numerous examples abound in the literature. For example, the quinoxaline CCR1 antagonist CP-481,715 from Pfizer that has significant activity for the human receptor (K_D for displacement of ^{125}I-MIP-1α binding = 74 nM and K_D for displacement of ^{3}H-CP481,715 binding = 9.2 nM) does not inhibit the binding of CCR1 ligands to mouse, rat, guinea pig, dog, rabbit or monkey CCR1 receptors [35]. Drug substances that are limited in specificity to human target proteins can be problematic during drug development, since they are difficult to test in surrogate animal efficacy models. Without efficacy data, it can become very difficult to justify further development of the drug, given the considerable risks and costs involved. This example probably represents the tip of the iceberg, as more extensive pharmacological

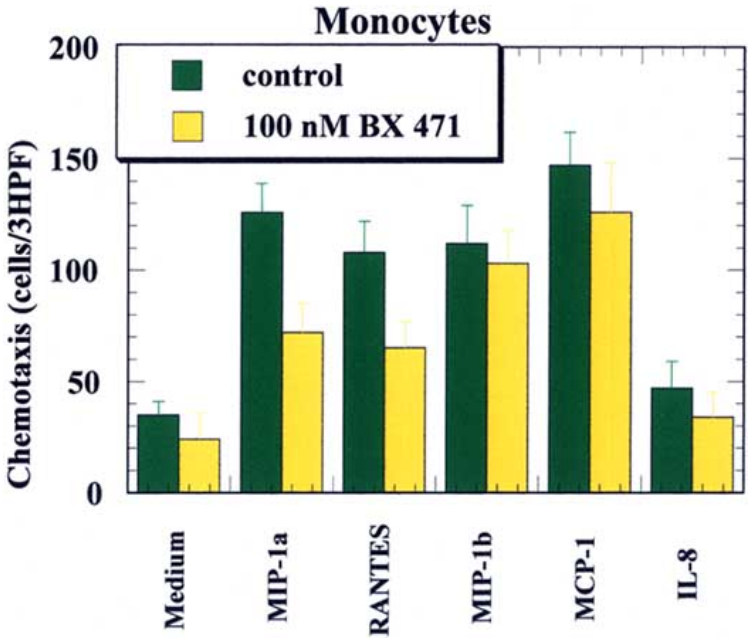


Fig. (4). BX 471 inhibits chemotaxis of monocytes induced by MIP-1α and RANTES.

characterization of other GPCR antagonists will most likely reveal.

The functional antagonism of BX 471 was demonstrated by its ability to inhibit agonist-induced Ca^{2+} mobilization in CCR1 expressing cells (Fig. **3**). BX 471 inhibited the Ca^{2+} transients induced by submaximal concentrations of the CCR1 ligands, MIP-1α, RANTES and MCP-3 in a concentration-dependent manner with IC_{50}'s of 5, 2 and 6 nM, respectively, demonstrating functional antagonism for CCR1. When given alone, the compound did not induce Ca^{2+} transients, indicating that it had no intrinsic agonistic activity.

In addition, the functional antagonism of BX 471 for CCR1, was demonstrated in two further assays. First, by its ability to inhibit the MIP-1α induced expression of the integrin CD11b on monocytes in a whole blood FACScan assay . MIP-1α dose-responsively induced the expression of CD11b on monocytes with an EC_{50} of 110 nM. The CCR1 antagonist inhibited CD11b up-regulation by MIP-1α by 100% and 65%, respectively . Second, BX 471 was able to inhibit the directed migration of both human lymphocytes and monocytes (Fig. **4**) in response to the CCR1 ligands MIP-1α and RANTES, but had no effect on the CCR5 ligand MIP-1ß, the CCR2 ligand MCP-1, or the CXCR4 ligand SDF-1α. Thus, BX 471 is a potent inhibitor of

Table 4. Specificity of CCR1 Antagonist BX 471 for GPCRs

	Receptor Ligand Tissue		**% Inhibition of Binding**		
			10 μM	**1 μM**	**Selectivity**
Adenosine A_3	[^{125}I]AB-MECA	Human	38	-6	>10,000
Adrenergic α_{1a}	[^{3}H]Prazosin	Rat	-7	5	>10,000
Adrenergic α_{2a}	[^{3}H]MK912	Human	24	-8	>10,000
Adrenergic α_{2c}	[^{3}H]MK912	Human	22	21	>10,000
Adrenergic ß$_1$	[^{125}I]-cyanopindolol	Human	1	2	>10,000
Bradykinin B_1	[^{3}H]des Arg^{10} Kallidin	Human	-11	-9	>10,000
Cannabinoid	[^{3}H]WIN-55,212-2	Human	13	23	>10,000
Cholecystokinin	[^{3}H]-Me-N(±)L364,718	Human	16	-14	>10,000
Dopamine D_1	[^{3}H]SCH23390	Human	13	-2	>10,000
Dopamine D_2	[^{3}H]Spiperone	Human	9	6	>10,000
Dopamine D_3	[^{3}H]Spiperone	Human	17	21	>10,000
Dopamine $D_{4.2}$	[^{3}H]Spiperone	Human	21	2	>10,000
Dopamine D_5	[^{3}H]SCH23390	Human	23	29	>10,000
Endothelin B	[^{125}I]Endothelin-1	Human	8	-15	>10,000
Leukotriene B_4	[^{3}H]LTB$_4$	Human	14	-13	>10,000
Muscarinic M_1	[^{3}H]NMS	Human	-7	-9	>10,000
Muscarinic M_2	[^{3}H]NMS	Human	21	12	>10,000
Muscarinic M_3	[^{3}H]NMS	Human	-8	7	>10,000
Muscarinic M_4	[^{3}H]NMS	Human	15	15	>10,000
Muscarinic M_5	[^{3}H]NMS	Human	18	11	>10,000
Neurokinin	[^{3}H]SR-140333	Human	2	5	>10,000
NPY	[^{125}I]NPY	Human	19	12	>10,000
Serotonin 5-HT1$_A$	[^{3}H]8-OH-DPAT	Human	8	4	>10,000
Serotonin 5-HT1$_6$	[^{3}H]NMS	Human	19	19	>10,000
CXCR2	[^{125}I]IL-8	Human	-4	-2	>10,000
CXCR4	[^{125}I]SDF-1α	Human	5	1	>10,000
CCR5	[^{125}I]MIP-1α	Human	2	3	>10,000
DARC	[^{125}I]MGSA	Human	6	-3	>10,000

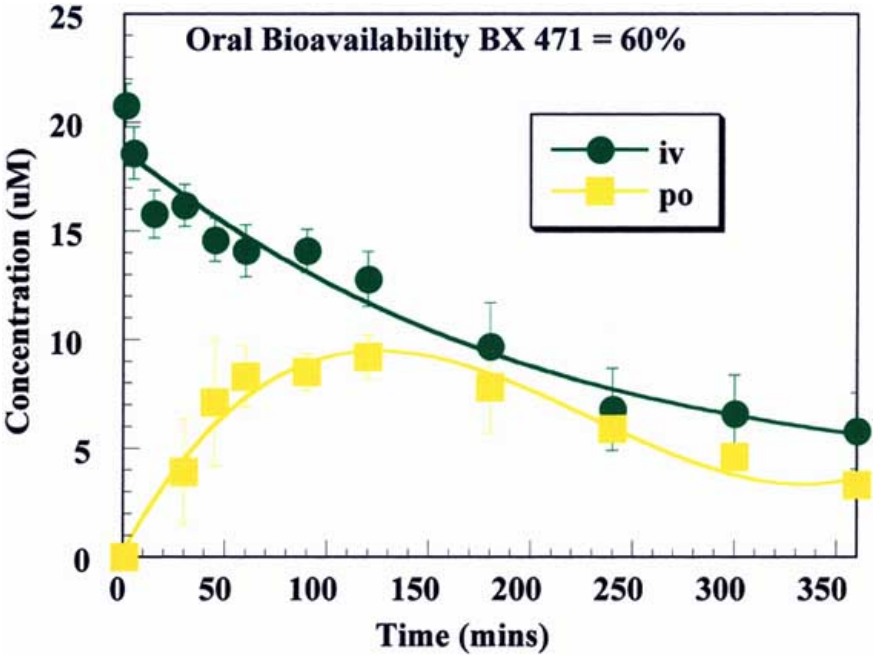


Fig. (5). Bioavailability of BX 471 in fasted male beagle dogs.

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.

The CCR1 antagonist BX 471 was shown to demonstrate reversible and competitive binding kinetics. The reversibility of receptor inhibition by an antagonist is a desirable property for a therapeutic agent, and we showed that cells initially inhibited by BX 471 could be made responsive to CCR1 ligands again, simply by washing out the compound. Competitive binding kinetics were demonstrated by Schild analysis of the concentration-response curves for Ca^{2+} transients induced by MIP-1α, in the presence of increasing concentrations of BX 471. CCR1 inhibition by BX 471 could be overcome by increasing concentrations of MIP-1α, suggesting surmountable antagonism . After transformation of the data, a linear Schild plot was generated with a slope of 1.29, which was not significantly different from unity.

Since CCR1 belongs to a large family of GPCR, which numbers well over 450 members, it was important to determine the specificity of the CCR1 antagonist to establish its therapeutic utility. The selectivity of BX 471 was thus

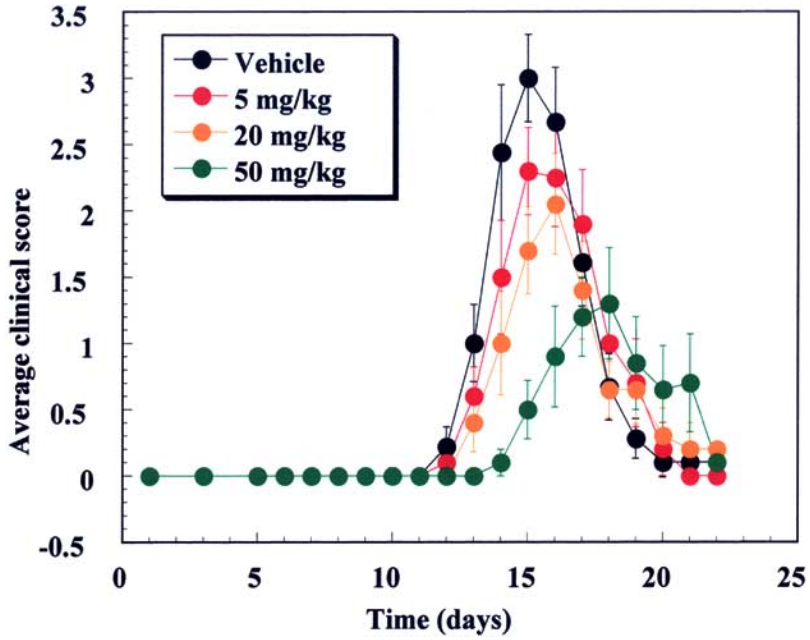


Fig. (6). Average clinical scores in EAE model of multiple sclerosis in Lewis rats treated with BX 471.

tested for inhibition of radioligand binding against a panel of 28 GPCR, including related chemokine receptors. Although BX 471 had a K_i of inhibition for CCR1 ranging from 1 to 5.5 nM, it had less than 50% inhibitory activity for all receptors tested at a concentration of 10 uM (Table **4**). These data indicated that BX 471 had a greater than 10,000-fold selectivity for CCR1, compared to all other GPCR's tested.

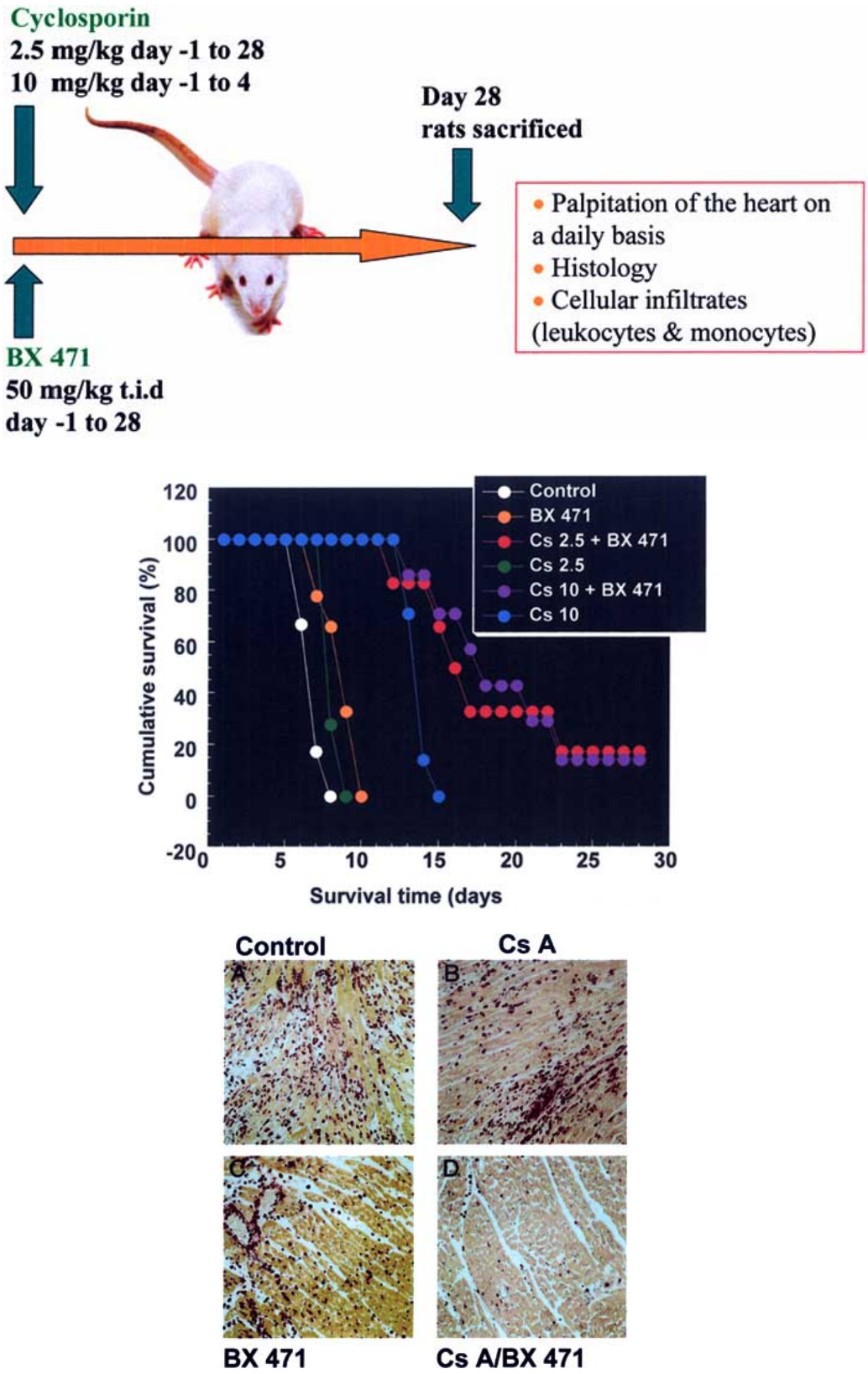


Fig. (7). Efficacy of BX 471 in a rat heart transplant model.

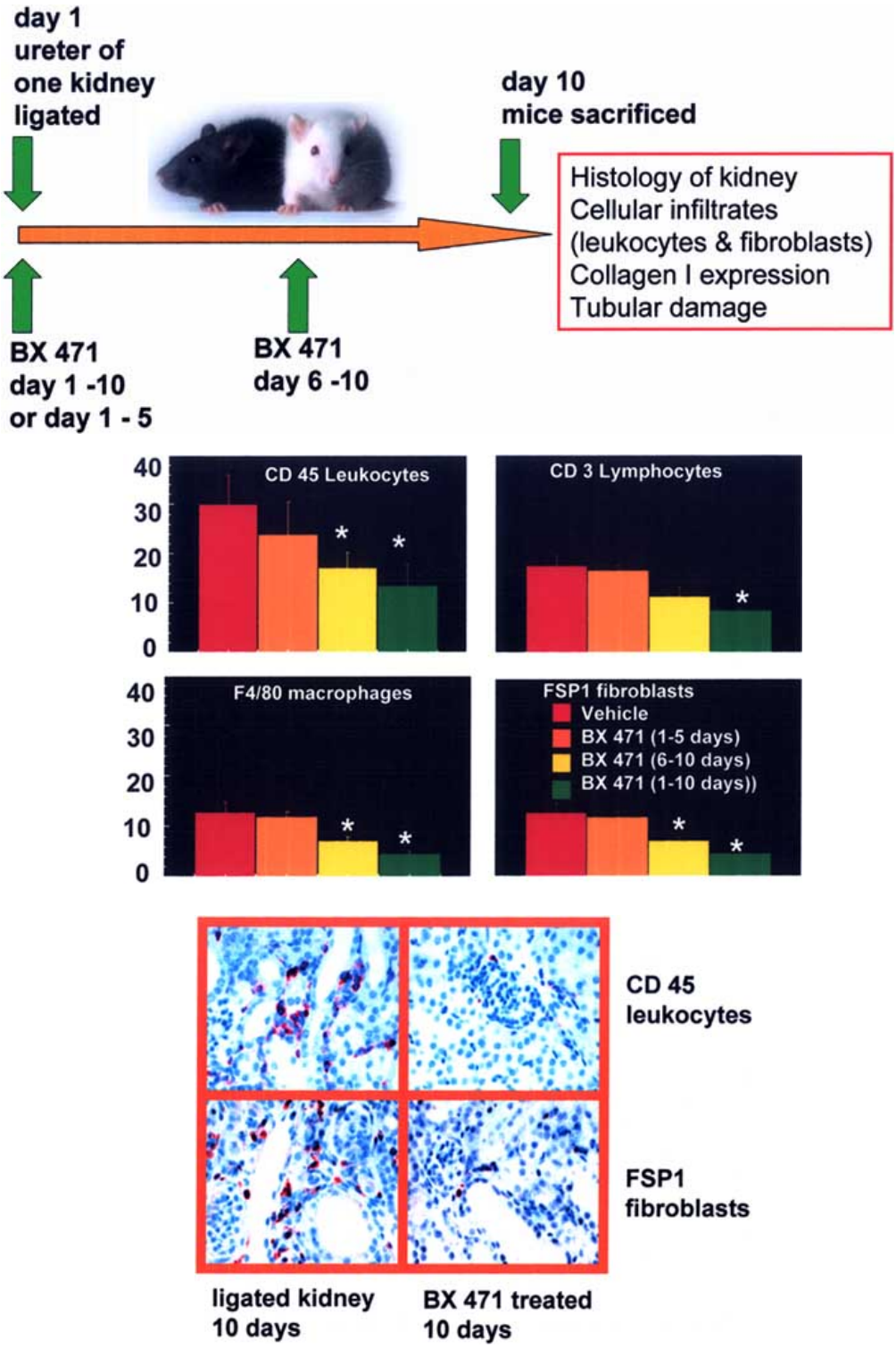


Fig. (8). Efficacy of BX 471 in a rat UUO model of renal fibrosis.

The oral bioavailability of BX 471 was examined in conscious dogs. It was administered to fasted male beagle dogs at 4 mg/kg in a vehicle of 40% cyclodextrin/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. 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 indicated that BX 471 is an orally absorbed drug in fasted dogs, with an oral bioavailability of approximately 60% (Fig. **5**).

Given the potentially important pathophysiological role of CCR1 discussed above, it is obvious that CCR1 antagonists could be therapeutically beneficial in treating these and other human diseases. Indeed, four separate studies with potent CCR1 receptor antagonists have illuminated the

role of CCR1 in the pathophysiology of multiple sclerosis, organ transplant rejection and renal fibrosis [36-39].

In a rat EAE model of multiple sclerosis, BX 471 dose-responsively decreased the clinical score (Fig. **6**). At the highest dose of 50 mg/kg, BX 471 reduced the clinical score by around 50% [36]. The much higher doses of BX 471 that are required to be effective in rat EAE, are due to the fact that the compound has an IC_{50} of 121 nM for inhibition of MIP-1α binding to rat CCR1, compared with an IC_{50} of 1-2 nM for human CCR1. Based on these considerations, it is likely that much lower doses of BX 471 (500 ug/kg or less) would be required to be therapeutically effective in treating multiple sclerosis in humans.

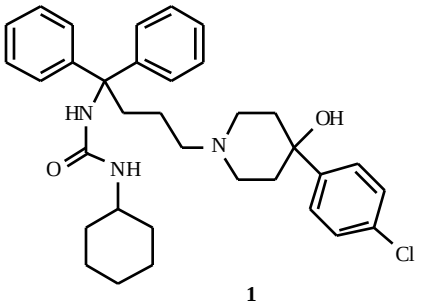
The CCR1 receptor antagonist BX 471 is alsoefficacious in a rat heterotopic heart transplant rejection model [38]. Animals treated with BX 471 and a sub-therapeutic dose of cyclosporin, 2.5 mg/kg, which is by itself ineffective in prolonging transplant rejection, was much more efficacious in prolonging transplantation rejection than animals treated with either cyclosporin or BX 471 alone (Fig. **7**). Immunohistology of the rat hearts for infiltrating monocytes confirmed these data. Three days after transplantation, the extent of monocytic graft infiltration was significantly reduced by the combined therapy of BX 471, and cyclosporin. Thus, BX 471 given in combination with cyclosporin resulted in a clear increase in efficacy in heart transplantation, compared to cyclosporin alone. These data were in line with the observed effects of BX 471 in dose-responsively blocking the firm adhesion of monocytes triggered by RANTES on inflamed endothelium. Together, these data demonstrate a significant role for CCR1 in allograft rejection.

A recent model of renal fibrosis in the mouse showed that inhibition of CCR1 with BX 471 reduced leukocyte infiltration and renal fibrosis (Fig. **8**). BX 471-treated mice (day 0-10 and day 6-10) revealed a 40-60% reduction of interstitial macrophage and lymphocyte infiltrate compared with controls [39]. Treated mice also showed a marked reduction of CCR1 and CCR5 mRNA levels, and FACS analysis showed a comparable reduction of CD8+/CCR5+ T cells. Markers of renal fibrosis, such as interstitial fibroblasts, interstitial volume, mRNA and protein expression for collagen I, were all significantly reduced by BX 471- treatment compared with vehicle controls. In summary, blockade of CCR1 substantially reduced cell accumulation and renal fibrosis after UUO. Most interestingly, late onset of treatment was also effective, and this is the first published example showing that a CCR1 antagonist is effective when given therapeutically, as compared to prophylactically in an animal model of disease.

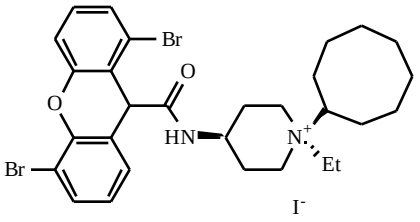
The studies demonstrating the effectiveness of BX 471 in animal models of multiple sclerosis and transplantation have also been confirmed by targeted gene disruption studies [18, 26, 40, 41]. Collectively, these data suggest that CCR1 antagonists are likely to be effective in treating human disease, and BX 471 is currently being evaluated in a phase II human clinical trial as an oral therapeutic treatment for multiple sclerosis.

A number of other pharmaceutical companies have also disclosed CCR1 antagonists (Fig. **9**). Most of these approaches with the exception of Pfizer are at the preclinical level and are described below.

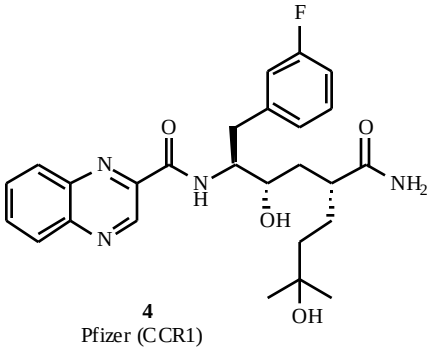
A group of structures similar to the original Berlex dihydrobenzothiepine template (Fig. **1**) was disclosed by



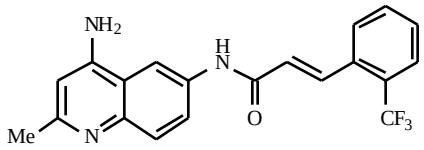
1
Takeda
K_i (MIP-1α) = 8 nM
K_i (RANTES) = 5 nM



2
Banyu
K_i (CCR1) = 1.9 nM
IK_i (CCR3) = 2.7 nM



4
Pfizer (CCR1)
K_i (MIP-1α) = 74 nM



3
Merck
CCR1, CCR2, CCR3, CCR4
CCR5, CXCR3, CXCR4
no activity reported

Fig. (9). Structures of other CCR1 antagonists.

Takeda Chemical Industries, Ltd. as CCR1 receptor antagonists [42]. These compounds share many structural features with the Berlex compound BX 513 and were reported to be potent for inhibition of binding of RANTES to CCR1. However, these researchers reported that the compounds inhibited MIP-1α binding with lower potency.

Banyu Pharmaceutical Co. Ltd. disclosed several CCR1 antagonists in a patent application [43]. In this disclosure, they reported a group of tricyclic amides which inhibited receptor binding in the low nanomolar range (IC_{50} =1.9 nM). These compounds were also reported to inhibit binding to CCR3 with a similar IC_{50} (2.7 nM), making them less specific than the Berlex compounds. In addition, 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. Recently, the same group published that their lead structure, a xanthene-9-carboxamide, had inhibitory activity against both human and murine CCR1 receptor, IC_{50} values of 0.9 and 5.8 nM, respectively [44].

Merck and Co. Inc. has also disclosed a number of different chemokine receptor antagonists in several recent published patents [45-47]. These patents claimed a number of compounds that shared a central 4-amino-2-methyl-quinoline with an acylated amine in the 6 position. All of these compounds were claimed to be modulators of the activity of several chemokine receptors including CCR1, CCR2, CCR3, CCR4, CCR5, CXCR3, and CXCR4 but no data were presented.

Most recently, Pfizer Inc. has disclosed a family of novel compounds which inhibit MIP-1α binding to CCR1 [48]. The compounds claimed in this patent are generally bicyclic aromatic herteoatomic systems, bound by an amide linkage to a substituted 5-aminovaleric acid derivative, whose carboxy terminus is generally derivatized as a primary amide. The level to which these compounds antagonize CCR1 binding has not been disclosed. Recently, Pfizer disclosed a number of novel heterocyclic amide derivatives as inhibitors of MIP-1α binding to CCR1 in the treatment of autoimmune diseases. The compounds were stated to have an IC_{50} value for inhibition of MIP-1α-induced migration of less than 25 nM [49]. Pfizer also recently published a patent application [50] claiming the synthesis of Berlex compounds such as BX 471 (Fig. **1**), that are covered by the issued Berlex CCR1 patent [51].

Millennium, in collaboration with Aventis, is investigating small molecule antagonists of CCR1 for the potential treatment of rheumatoid arthritis and multiple sclerosis. Compounds under investigation include MLX-010, MLX-025, MLX-011 and MLX-031. In January 2002, Millennium hoped to progress this program into clinical studies in 2002. In December 2001, data on CCR1 antagonists were presented at the BPS winter meeting in London [52]. MLX-010 was shown to be a potent and selective inhibitor of CCR1 (IC_{50} = 1.7 nM). The compounds tested were potent in humans on THP-1, but not in other typical study species; several species had been screened; potency was comparable in guinea pigs. In a guinea pig model, MLX-010 was shown to have good bioavailability and dose-dependently inhibit neutrophil infiltration to the skin.

AZD-8309 is a CCR1 antagonist under development by Astra Zeneca for the potential treatment of rheumatoid arthritis. In November 2002, clinical trials with AZD-8309 had not been initiated. The structure of AZD-8309 has not been revealed; however, in a separate patent application, the company disclosed a number of structures, including N- [1-(3,4-dichlorobenzyl)piperidin-4-yl]-4-methylbenzylamine, as CCR1 inhibitors [53].

CONCLUSION

In the late 1980's, scientists isolated the signaling molecules, chemokines that allowed leukocytes to communicate with one another and seek out and destroy invading pathogens. However, the immune response is a double edged sword and can under certain circumstances be inappropriately activated and targeted towards normal healthy tissue, leading to autoimmunity and disease. It was soon realized that an understanding of the mechanisms involved in these processes could provide a key for the identification of successful therapeutic approaches to treat these diseases. The identification of chemokine receptors as a subfamily of GPCR's has paved the way towards the realization of these early goals. CCR1 was first cloned in 1993, and in the ten years following this discovery, CCR1

Table 5. Chemokine Receptor Antagonists in Clinical Trials

Receptor	Company	Clinical phase	Compound	Indication
CCR1	Berlex	II	BX 471	MS
CCR1	Pfizer	II	????	RA
CCR3	GSK	I	766994	Asthma & allergic rhinitis
CCR3	BMS	I	DPC-168	Asthma
CCR5	Pfizer	II/III	UK-427857	HIV
CCR5	Schering Plough	I	SCH-C SCH-D	HIV
CXCR3	Tularik	I	T487	RA, MS transplant
CXCR4	Anormed	II	AMD3100	Stem cell transplant & multiple myeloma

antagonists are undergoing phase II human trials in a number of indications including multiple sclerosis, psoriasis and rheumatoid arthritis (Table **5**). The promise of highly specific therapies for a number of devastating diseases is on the horizon; thanks to the identification of chemokine receptor antagonists, and we can look forward with anticipation to the day when these drugs are finally marketed as potent therapeutics.

NOTE ADDED IN PROOF

As recently reported in a phase Ib clinical trial (22), patients with active rheumatoid arthritis responded to the antagonist as demonstrated by a significant reduction in the number of macrophages and CCR1 positive cells in the synovium compared with those from patients in the placebo group. A trend but no significant clinical improvements were seen in treated patients (22). Based on these data CP-481715 entered phase II studies for rheumatoid arthritis in February 2004, however the trial was stopped after 6 weeks since although the compound was well tolerated it did not demonstrate any efficacy (investigative drugs database IdDb).

Tularik initiated a Phase IIa clinical trial for psoriasis in late 2003 with Chemocentryx CXCR3 antagonist and the results were presented at the 12th Biennial International Inflammation Research Association meeting in Bolton Landing, NY. The study was performed in Europe and 40 patients with moderate-to-severe psoriasis received 50 or 200 mg T-487 or placebo once daily for 28 days. There was no significant difference in the psoriasis area and severity index scores between any of the patient groups. The lack of efficacy could have been due to the high variability in drug exposure as determined by the pharmacodynamic data. Although in general well tolerated, T-487 was discontinued in one patient due to exacerbation of symptoms.

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