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Structure Function Differences in Nonpeptide CCR1 Antagonists for Human and Mouse CCR1

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A useful strategy for identifying ligand binding domains of G protein-coupled receptors has been the exploitation of species differences in antagonist potencies. We have used this approach for the CCR1 chemokine receptor with a novel series of antagonists, the 4-hydroxypiperidines, which were discovered by high throughput screening of human CCR1 and subsequently optimized. The structure-activity relationships for a number of different 4-hydroxypiperidine antagonists for human and mouse CCR1 were examined by receptor binding and functional assays. These compounds exhibit major differences in their rank order of potency for the human and mouse chemokine receptor CCR1. For example, the initial lead template, BX 510, which was a highly potent functional antagonist for human CCR1 ($K_i = 21$ nM) was >400-fold less active on mouse CCR1 ($K_i = 9150$ nM). However, increasing the length of the linker between the piperidine and dibenzothiepine groups by one methylene group generated a compound, BX 511, which was equipotent for both human and mouse CCR1. These and other analogs of the lead template BX 510, which have major differences in potency for human and mouse CCR1, are described, and a model for their interaction with human CCR1 is presented. The Journal of Immunology, 2003, 170: 1910–1916.

Chemokines are small, mainly basic proteins involved in host defense that summon immune cells in response to invasion by pathogenic organisms. Well over 40 human chemokines have been described (1), and all of them transmit intracellular signals by binding to and activating specific G protein-coupled receptors on the cell surface of their target cells (2). Inadvertent activation of chemokine receptors has been shown to lead to autoimmunity by inappropriately targeting self Ags for destruction by cytotoxic T cells and macrophages (3). The role of chemokine receptors in diseases such as multiple sclerosis, rheumatoid arthritis, and organ transplant rejection has been well described (4). Given their role in disease and inflammation, chemokine receptors have received much attention from the pharmaceutical industry, and a number of chemokine receptor antagonists have been described (5).

Previously we have identified several novel small molecule antagonists for the human chemokine receptor CCR1 (6–8). The structure-activity relationships for one of these classes of compounds, the 4-hydroxypiperidines, has been well described for human CCR1 (9). During the optimization of these compounds we discovered that the lead candidate from this series, which was highly potent and had a binding affinity of 40 nM on human CCR1, was relatively ineffective in the mouse (10). These findings provided the first clear evidence of the impact of mouse/human species variability of CCR1 on nonpeptide receptor antagonist potency. Recently, researchers from Banyu have reported that CCR1 inhibitors based upon a xanthenecarboxamide template also exhibit differential potencies on human and mouse CCR1s (11). These results demonstrate that the CCR1 mouse/human species variability also affects compounds from different template classes, a finding that may result from these inhibitors using a common antagonist binding site on CCR1.

In this study we have further characterized the pharmacological basis for the species selectivity of these compounds by carrying out detailed structure function studies using our previous wide array of human CCR1 antagonists (9). These detailed studies have allowed us to identify a series of CCR1 antagonists displaying species selectivity for mouse and human CCR1 and to rationalize this selectivity based on compound structure. Since human and mouse CCR1 share a very high degree of homology, and sequence identity reaches 81% (12, 13), divergent residues may represent potential major determinants responsible for the binding of species-selective CCR1 antagonists. These results should be of broad interest to pharmacologists and immunologists studying the molecular mechanisms of receptor binding and signal transduction of CCR1 and other G protein-coupled chemokine receptors.

Materials and Methods

Materials

Unlabeled chemokines were from PeproTech (Rocky Hill, NJ). $^{125}$I-labeled chemokines were obtained from NEN (Boston, MA).

Chemistry

Compounds BX 510 through BX 523 (see Figs. 3 and 5–7) were synthesized as outlined in a prior publication (9).

Cell lines

The human embryonic kidney cell line HEK 293 was obtained from the American Type Culture Collection (Manassas, VA) and was maintained in RPMI or DMEM containing 10% heat-inactivated FBS, 2 mM l-glutamine, penicillin, and streptomycin in a 5% CO$_2$ atmosphere at 37°C. For binding assays the cells were harvested and washed once with PBS. Cell viability was assessed by trypan blue exclusion, and cell number was determined by counting the cells in a hemocytometer.
CCR1-expressing cells

HEK 293 cells stably expressing human or mouse CCR1 were grown to confluent monolayers in 225-cm² flasks as previously described (7). Cells were tested for their ability to bind ¹²⁵I-labeled macrophage inflammatory protein-1α (MIP-1α)⁵ and biological responses by changes in intracellular Ca²⁺ or microphysiometry.

Chemokine binding studies

The binding assays were performed in transfected cells as previously described (7). Nonspecific binding was determined in the presence of either 100 nM or 1 μM unlabeled ligand. The binding data were curve-fitted with the computer program IGOR (WaveMetrics, Lake Oswego, OR) to determine the affinity and number of sites.

Cytosolic Ca²⁺ measurements.

Cytosolic Ca²⁺ was measured in HEK 293 cells expressing human CCR1 (293 MR) or mouse CCR1 (293 M3X) as previously described (14).

Modeling of CCR1

A model of human CCR1 was built based on bovine rhodopsin (PDB1F88, resolution 2.8 Å) (15), using the program HOMOLOGY (Accelrys, San Diego, CA). The alignment shown in Fig. 1 was used to build the model, allowing for most insertions to occur away from the core. The sequence identity for this alignment is 20%. Coordinates for insertions and variable regions were extracted from the Protein Data Bank (http://www.rcsb.org). The model was refined by energy minimization using DISCOVER (Accelrys) with constraints on the backbone, except where deletions and insertions occurred. The model coordinates are available upon request from the corresponding author.

In the CCR1 homology model the pocket corresponding to the retinal binding site of rhodopsin was chosen as a potential ligand binding site, although in our model the pocket was slightly too small to accommodate our ligands without any steric clashes. There were no other internal pockets that were as large. Binding of these ligands to the extracellular surface of CCR1 cannot be ruled out. However, there were no obvious binding pockets present in the extracellular region of the model that were complementary in shape to the ligands. The predicted structure of the extracellular loop region of CCR1 is probably less accurate than that of the helical regions, because loop structures are more difficult to predict using homology modeling.

Because the proposed binding site of BX 510 was a bit too small to accommodate the ligand without strain, we used molecular dynamics (MD) calculations instead of simple docking and minimization. The advantage of using MD calculations was that the protein residues very close to the inhibitor were allowed to shift their positions slightly to enlarge the binding pocket. Attempts to use automated protein-ligand docking to place the ligand in the binding site failed, perhaps due to the constricted nature of the pocket. Therefore, manual docking was used with the ligand oriented according to the shape and lipophilic nature of the pocket.

The initial position of BX 510 was docked into the protein similarly to the bound position of retinal in 1F88. A few water molecules were placed in the central channel of CCR1 near the BX 510 binding site wherever there was enough space, according to the standard solvation protocol of the LeaP module in AMBER (University of California, San Francisco, CA). For simplicity, no attempt was made to simulate the actual membrane

FIGURE 1. Alignment of human and mouse CCR1 with bovine rhodopsin (PDB1F88). Known (PDB1F88) or predicted helixes (based on Psi-Pred) are highlighted in yellow. Known (PDB1F88) or predicted β strands are highlighted in turquoise. Conserved cysteine residues are highlighted in green. The letter “s” indicates a disulfide bond.

FIGURE 2. Displacement of radiolabeled MIP-1α from human and mouse CCR1 by CCR1 antagonists. Transfected HEK cells expressing human (A) and mouse (B) CCR1 were incubated with ¹²⁵I-labeled MIP-1α in the presence of increasing concentrations of the CCR1 antagonists BX 510, BX 511, and BX 512. The binding reactions were terminated as described previously (7). The binding shown represents specific binding. Nonspecific binding was <10% of the total ¹²⁵I-labeled MIP-1α added. The results shown are from a typical experiment (n = 2 or more).

⁵ Abbreviations used in this paper: MIP-1α, macrophage inflammatory protein-1α; MD, molecular dynamics.
environment surrounding CCR1. A distance-dependent dielectric of 2r was used. The receptor-antagonist complex was minimized and subjected to 2 ns of MD using the AMBER 6.0 program package. Harmonic restraints were applied to keep the structure close to the original structure. Harmonic position restraints of 2.0 kcal/mol Å² were applied to backbone atoms of the intracellular side of the receptor away from the binding site: residues 53–75, 125–154, 215–245, and 298–345. Restraints of 0.2 kcal/mol Å² were applied to the rest of the receptor backbone. After 2 ns of dynamics at 298 K, the structure was minimized for 3000 steps with the same restraints.

The structures from 1 and 2 ns were compared and were found to be very similar except in the tilt angle of the piperidine ring. After dynamics, the root-mean-squared deviation of the α carbons from the initial model was 1.0 Å after 1 or 2 ns. This represents a fairly small deviation in structure from the homology model. A small portion of extracellular loop 2 (ECL2), residues 177–180, deviated more significantly (2.9 Å carbon root-mean-square deviation) during dynamics. This larger deviation may be due to interactions with the antagonist or sequence differences with respect to the rhodopsin structure that was the source of the homology model. The portion of ECL2 that is close to BX 510, residues 182–185, did not deviate significantly from its initial position during 2 ns of restrained MD. The only residues showing significant shifts in their side chain positions during the MD were Tyr113 and Trp252, which opened up a binding pocket to accommodate the phenylpiperidine moiety. The binding pocket of the dibenzothiepine ring remained very similar to its initial coordinates. The fused ring system itself flexed into several different conformations during the dynamics, but the cyano group remained in a fairly constant position.

**Results and Discussion**

Previously we have shown that compounds of the 4-hydroxypiperidine class were potent antagonists of human CCR1 (6, 9). The lead candidate from this series, BX 513, was highly potent on human CCR1, but relatively ineffective in the mouse (10). Here we have further explored the differences in potency of these compounds for human compared with mouse CCR1. Compounds were initially analyzed for their ability to inhibit the binding of radio-labeled human MIP-1α to cells expressing human or mouse CCR1.

Fig. 2 shows the displacement of radiolabeled MIP-1α from human and murine CCR1 in the presence of increasing concentrations of the CCR1 antagonists BX 510, BX 511, and BX 512. As observed from the binding curves, changing the length of the alkyl linker between the piperidine and dibenzothiepine groups profoundly affected the ability of the compounds to inhibit chemokine binding. For example, BX 510, which has a three-atom linker (Fig. 3), had a low potency for mouse CCR1 (Kᵢ = 9150 nM) compared with human CCR1 (Kᵢ = 21 nM). In contrast, insertion of a four-atom linker, as in BX 511, increased the potency almost 30-fold in the mouse, but decreased the potency 20-fold in the human CCR1.

These data indicate that at certain chain lengths, a specific functional group in this series of 4-hydroxypiperidines interacts adversely with groups on CCR1.

We have previously shown that the CCR1 compounds are biologically active on mouse compared with human leukocytes. The compounds have similar specificity on these cells measured by migration assays, and they can block leukocyte migration in vitro and in vivo (7, 8, 15). For example, the CCR1 compounds ameliorate disease in three different rodent models, i.e., multiple sclerosis, transplant rejection, and renal fibrosis, albeit at concentrations much higher than would be required to inhibit in human

![FIGURE 3](image-url)

**FIGURE 3.** BX 510, which has a three-atom linker, had a low potency for mouse CCR1 (Kᵢ = 9150 nM) compared with human CCR1 (Kᵢ = 21 nM). In contrast, insertion of a four-atom linker, as in BX 511, increased the potency almost 30-fold in the mouse, but decreased the potency 20-fold in the human CCR1.

![FIGURE 4](image-url)

**FIGURE 4.** CCR1 antagonists inhibit the ability of MIP-1α to increase Ca²⁺ transients in HEK 293 cells expressing murine CCR1. The two panels show HEK 293 cells expressing murine CCR1 that were loaded with Fluo-3/AM and stimulated with 30 nM human MIP-1α in the presence of increasing concentrations of the CCR1 antagonists BX 511 and BX 523. The data shown are representative of at least two separate studies.
disease given their much reduced potency on rodent CCR1 (7, 8, 15). In a recent publication we showed that CCR1 antagonists were able to inhibit calcium flux in mouse cells transfected with CCR1 in line with their ability to inhibit the binding of CCR1 ligands such as MIP-1α (15). Furthermore, in this publication we also demonstrated that the CCR1 antagonist inhibited the infiltration of macrophages and lymphocytes in mouse kidney, demonstrating their in vivo ability to block the migration of leukocytes (15).

There are three sequence differences between the human and mouse CCR1 models that occur near the proposed antagonist binding site: Leu183 is Pro in mouse, Leu260 is Phe, and Ile259 is Val (Fig. 1). These differences in hydrophobic residues alter the shape of the pocket where the cyanodibenzothiepine or diphenylacetoni-trile groups are proposed to bind. This could subtly change the binding mode for that portion of the antagonists and thereby alter the preferred linker distance between these groups and the phenylhydroxypiperidine group.

Importantly, the analogs were true antagonists for both the human and mouse receptors even though there were major differences in the ability of these CCR1 analogs to displace chemokine binding to human and mouse CCR1. Fig. 4 shows the ability of the CCR1 antagonists BX 511 and BX 523 to inhibit MIP-1α-mediated Ca^{2+} transients in cells expressing murine CCR1. HEK 293 cells expressing murine CCR1 were loaded with Fluo-3 and stimulated with 30 nM human MIP-1α in the presence of increasing concentrations of the CCR1 antagonists. The antagonists were able to inhibit Ca^{2+} transients with IC_{50} values of inhibition that were in line with their ability to inhibit MIP-1α binding (Figs. 3 and 5).

Interestingly, when the methylsulfide bridgehead was removed, varying the length of the alkyl linker between the piperidine and diphenyl groups did not produce as drastic an effect as previously for both human and mouse CCR1 (Fig. 6). For example, insertion of a three-atom linker, BX 513, resulted in a 90-fold lower potency for mouse compared with human CCR1. The removal of the bridging atoms improved binding to the mouse receptor almost 3-fold, but reduced the potency for human CCR1 2-fold (compare with Fig. 3). These data indicate that without the methylsulfide bridgehead, the diphenyl group interacts more productively with the mouse receptor.

As shown in Fig. 7, the activity of this class of compounds against human CCR1 was selectively sensitive to substitution at the diphenylacetoni-trile. Removal of one phenyl group from the diphenyl BX 513 resulted in a 6-fold loss in activity (BX 516) for human CCR1 and a slight gain in activity for mouse CCR1. However, substitution on a phenyl group with a basic moiety (dimethylamine; BX 519) resulted in a significant loss of activity against human CCR1, while a noticeable increase in activity was observed against mouse CCR1.
indicates that the basic group interacts with a complementary functional group in mouse CCR1 (e.g., an acidic group or a hydrogen bond donor), while the reverse is true against human CCR1 (perhaps by interrupting a hydrophobic interaction). The most dramatic change in activity was observed when both phenyl groups were removed (BX 520). No significant loss of activity was observed against mouse CCR1, while complete loss of activity was observed against human CCR1. This lends further evidence to an important hydrophobic interaction of the diphenyl system of the 4-hydroxypiperidines with human CCR1 that is not significant in mouse CCR1.

The high human/mouse ratio of A = CN vs A = H in Fig. 5 indicates that human CCR1 benefits more from the presence of the cyano group than does mouse CCR1. This lends further evidence to a hydrophobic interaction by this region of the 4-hydroxypiperidines with human CCR1, but not with mouse CCR1. The increased potency of BX 521 and BX 523 relative to BX 513 and BX 522 shows that both human and mouse CCR1 benefit from the increased basicity of a quaternary ammonium salt on the piperidine. This result is similar to that reported for the Banyu xanthene-carboxamide series following replacement of the piperidine nitrogen with a quaternary ammonium group (11).

Recently, the structure of bovine rhodopsin was solved to a resolution of 2.8 Å (16). Using these data we built a homology model of human CCR1. We attempted to increase our understanding of the structure function differences of the 4-hydroxypiperidines by

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**FIGURE 8.** Backbone structure of CCR1 model showing BX 510 as a stick representation with a dotted solvent accessible surface. CCR1 is colored from red (N terminus) to blue (C terminus). Midas-Plus (Computer Graphics Laboratory, University of California) was used to create Figs. 8 and 10.

**FIGURE 9.** Detailed view of the interactions of CCR1 with BX 510. Cerius2 (version 4.5, Accelrys) was used to create Fig. 5. Residues that are different in the mouse receptor are shown in magenta.
modeling the binding of a CCR1 antagonist that exhibits a strong species difference in binding and antagonism, BX510. After examining the protein model for potential small molecule binding pockets, we chose the pocket corresponding to the retinal binding site of rhodopsin. This pocket has a lipophilic region and an adjacent polar region, which complements the characteristics of our inhibitors. The pocket was initially a bit too small to accommodate BX510, but the ligands could be easily accommodated after MD simulations without major distortions in the protein structure. Fig. 8 shows the general orientation of BX 510 with respect to the helices of CCR1. The molecule is in a binding site just below and in contact with ECL2, in an analogous position to retinal in the rhodopsin crystal structure. Its long axis is perpendicular to the overall axis of the transmembrane helixes. In this model the antagonist makes a large number of favorable lipophilic and aromatic contacts with CCR1, but no direct polar interactions. The chlorophenyl ring of BX 510 is in contact with Pro88, Leu87, and Ile91 of helix 2, Tyr113 of helix 3, and Tyr294 and His295 of helix 7 (Fig. 9). Tyr113 shifted its side chain during the MD simulation to form a binding pocket for the chlorophenyl group. The dibenzothiepine ring occupies a pocket that contacts residues Tyr113 of helix 3, Leu203 of helix 5, Phe187 of extracellular loop 2, and Ile259, Asn256, and Phe248 of helix 6. The dibenzothiepine ring system was flexible during the dynamics, which would explain why this moiety can be replaced by a nonfused diphenyl acetonitrile group (BX 514) without losing potency.

The placement of the chlorophenyl and dibenzothiepine rings seems reasonable in terms of steric fit to the binding site. The hydroxyl group of BX 510 is close enough to form water-mediated contacts with the amide carbonyl of Ser110 and Tyr113 of helix 3. The piperidine nitrogen is near Glu287 (helix 7), but there is no direct hydrogen bond. The piperidine nitrogen is in direct contact with the indole ring of Trp252 (helix 6). The polar interactions of the central hydroxypiperidine ring in this model are relatively weak, although we believe it is an important pharmacophore for binding to the receptor. There may be an alternative binding mode that places this ring in a better position for interactions, or there may be bridging waters present in the bound complex that are not present in the model. Some effort was made to create a model with a direct salt bridge between the piperidine nitrogen and an acidic group, but no suitable binding mode with this feature was found with manual docking. The current model places Glu287 only 5.1 Å away from the charged amino group, which is close enough for a significant electrostatic interaction between the opposite charges. This electrostatic interaction would be expected to be strengthened following replacement of the piperidine nitrogen by a quaternary ammonium salt containing a formal positive charge. It is of interest that the acidic group, Glu287, is conserved in most of the chemokine receptors except for CCR7, CCR9, and CCR10 (2). A central conserved role for this residue has also been proposed in models of CCR2 and CCR5 chemokine receptor/small molecule antagonist interactions (17, 18).

Fig. 10 shows the solvent accessible surfaces of CCR1 and BX 510 showing surface complementarity. This view is from the extracellular side looking down the axis of the helixes. BX510, the yellow dot surface, has a good fit to the proposed binding site in
cyan, especially in the region of the chlorophenyl group and the hydroxypiperidine. The binding site for the dibenzothiepine can easily accommodate this large group, and the cyan group makes a close contact with Leu$^{185}$ that may explain why its removal is detrimental for binding in human, but not in mouse, CCR1.

Examination of the model of human CCR1 binding to BX 510 (Figs. 8–10) reveals that there are three sequence differences in mouse CCR1 that are within 6 Å of the antagonist atoms. These are Leu$^{185}$ to Pro, Leu$^{260}$ to Phe, and Ile$^{259}$ to Val. All three of these changes are near the cyano-dibenzothiepine portion of the molecule and may provide an explanation for the major differences observed in binding to human vs mouse CCR1. For example, if one assumes that BX 513 binds in the same manner in human CCR1 as BX 510, the sequence differences in the mouse receptor are within the contact area of the diphenylacetoniitrite. Removal of the phenyl groups has very little effect on mouse CCR1, but these groups are very important for human CCR1 affinity. Likewise, the removal of the cyano group actually improves affinity for the mouse receptor, while it lowers affinity for the human receptor, and the proposed position of the cyano group is near a key sequence difference. The model of the binding mode for this series of inhibitors is consistent with the structure-activity differences exhibited for human and mouse CCR1 and helps to explain these differences.

In summary, this study demonstrates the importance of the piperidine group for antagonism of 4-hydroxypiperidines for both human and mouse CCR1. The central conserved role for Glu$^{287}$ in the recognition of a basic moiety on chemokine antagonists further supports previous modeling efforts on CCR2 and CCR5 (17, 18). Based on the binding data and from examination of the molecular model of the human CCR1-BX 510 complex, it would appear that human CCR1 is also more dependent on a beneficial hydrophobic interaction that gives rise to an increase in binding affinity. However, in the diphenyl series of compounds, both human and mouse CCR1 were sensitive to the size of the 4-hydroxypiperidines. The similarities of the species selectivity of both the 4-hydroxypiperidines and the Banyu xanthenecarboxamide series with human and mouse CCR1 receptors suggests that the molecules interact with the receptor at a common antagonist binding site.

**References**