Site-directed Mutagenesis of CC Chemokine Receptor 1 Reveals the Mechanism of Action of UCB 35625, a Small Molecule Chemokine Receptor Antagonist*

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The chemokine receptor CCR1 and its principal ligand, CCL3/MIP-1α, have been implicated in the pathology of several inflammatory diseases including rheumatoid arthritis, multiple sclerosis, and asthma. As such, these molecules are the focus of much research with the ultimate aim of developing novel therapies. We have described previously a non-competitive small molecule antagonist of CCR1 (UCB 35625), which we hypothesized interacted with amino acids located within the receptor transmembrane (TM) helices (Sabroe, I., Peck, M. J., Jan Van Keulen, B., Jorritsma, A., Simmons, G., Clapham, P. R., Williams, T. J., and Pease, J. E. (2000) J. Biol. Chem. 275, 25985–25992). Here we describe an approach to identifying the mechanism by which the molecule antagonizes CCR1. Thirty-three point mutants of CCR1 were expressed transiently in L1.2 cells, and the cells were assessed for their capacity to migrate in response to CCL3 in the presence or absence of UCB 35625. Cells expressing the mutant constructs Y41A (TM helix 1, or TM1), Y113A (TM3), and E287A (TM7) were responsive to CCL3 but resistant to the antagonist, consistent with a role for the TM helices in CCR1 interactions with UCB 35625. Subsequent molecular modeling successfully docked the compound with CCR1 and suggests that the antagonist ligates TM1, 2, and 7 of CCR1 and severely impedes access to TM2 and TMS, a region thought to be perturbed by the chemokine amino terminus during the process of receptor activation. Insights into the mechanism of action of these compounds may facilitate the development of more potent antagonists that show promise as future therapeutic agents in the treatment of inflammatory disease.

Chemokines represent a family of ∼50 proteins that are involved in the cellular migration of leukocytes under basal conditions and in situations of underlying inflammation (1, 2). To exert their functions, chemokines bind to and activate seven transmembrane (TM) domain G protein-coupled receptors found on the surface of leukocytes. Because the chemokine system is implicated in many disease processes involving chronic inflammation, autoimmunity, allergy, human immunodeficiency virus infection, and tumor metastasis, considerable efforts have been made to characterize and understand the chemokine/chemokine receptor interactions, with the ultimate aim of therapeutic intervention.

CC chemokine receptor 1 (CCR1) was the first CC chemokine receptor to be identified (3, 4). Initially this receptor was identified as the CCL3/MIP-1α and CCL5/RANTES (regulated on activation, normal T cell expressed and secreted) receptor, but later studies have shown it to bind signal in response to a variety of chemokines including CCL8/MCP-2 (monocyte chemotactrant protein), CCL7/MCP-3, CCL15/Lkn-1, CCL24/MIPF-1 (myeloid progenitor inhibitory factor), and CCL14/HCC-1 (hemofiltrate CC-chemokine) (5–7). Studies have shown that CCR1 is involved in the mobilization of myeloid progenitor cells (8) and the trafficking of monocytes and Th1/CD45RO(+) T cells (9) and that this receptor has an important role in host defense and inflammation (10, 11). In terms of disease processes, CCR1 has been hypothesized to play a role in an experimental acute encephalitis model of multiple sclerosis (12), rheumatoid arthritis (13), organ transplant rejection (14), Alzheimer's disease (15), and eosinophil infiltration of the asthmatic lung (16, 17). Thus, antagonism of CCR1 is an attractive therapeutic ambition for the treatment of a wide range of diseases.

Several studies have been published to date on the interactions of chemokine receptors with small molecule antagonists. Certain features, typical of antagonists, are apparent. In general, chemokine receptor antagonists possess an halogen-modified aromatic ring system and a basic region (18). Antagonism of the CCL2 receptor, CCR2, by spiropiperidine-derived compounds has been postulated to involve an interaction between a glutamic acid residue found in TM7 of the receptor and a basic nitrogen atom within the compound (19). Similarly, a glutamate residue in an analogous location in CCR5 and CXCR4 has been shown to be important for interactions with the antagonists TAK-779 and AMD3100 respectively (20, 21).

Several small molecule antagonists of CCR1 have been described to date (for review see Refs. 18 and 22), including a compound named UCB 35625 (the trans-isomer J113863 published to date (for review see Refs. 18 and 22), including a compound named UCB 35625 (the trans-isomer J113863 published by Banyu Pharmaceutical Company, patent WO98/04554). We have previously characterized this compound as a potent inhibitor of CCR1 that is effective at low nanomolar

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‡ The abbreviations used are: TM, transmembrane; CCR, CC chemokine receptor.
FIG. 1. Use of a transient expression system to examine the modus operandi of UCB 35625. A, the structure of UCB 35625 1-cycloheptenylmethyl-1-ethyl-4(2,7-dichloroxanthene-9-carboxamido)-piperidinium iodine. B, the transient expression of CCR1 as determined by flow cytometry. CCR1-expressing L1.2 cells are shown by a gray line (open histogram), whereas naive cells are shown in black. Data are shown as a representative experiment of at least seven independent experiments. C, the chemotactic responses to CCL3 of L1.2 cells transiently expressing CCR1 24 h post-transfection in the presence or absence of fixed concentrations of UCB 35625. Data shown are the mean ± S.E. of four independent experiments, carried out in duplicate. Untransfected cells showed no detectable chemotaxis to CCL3 (data not shown).

FIG. 2. Residue Glu-287 is required for the interaction of UCB 35625 with CCR1. L1.2 cells were transiently transfected separately with the cDNAs for D80N, D92N, E120Q, or E287Q. 24 h post-transfection, cells were stained with the anti-CCR1 antibody to detect expression of these constructs on the surface of the cells. Expression of the mutant receptors is shown in gray and is compared with the staining profile of the untransfected L1.2 cells in black (A, C, E, and G). All four constructs were capable of migrating in response to CCL3 (B, D, F, and H). In the presence of 100 nM UCB, only cells expressing the E287Q mutant were capable of chemotaxis (H).
concentrations despite an apparent inability to displace CCL3 from the receptor (23). This characterization led us to hypothesize that the molecule exerts its antagonistic effects by interacting with amino acids within an intra-helical binding pocket of CCR1 and stabilizing the receptor in an inactive conformation that cannot induce signal transduction but can still bind ligand. We report here the findings from a large scale mutagenesis study directed at the TM domains of CCR1 to examine this hypothesis directly.

EXPERIMENTAL PROCEDURES

Materials—Reagents were purchased from Sigma-Aldrich and Invitrogen, unless stated otherwise. Recombinant CCL3 was purchased from PeproTech EC Ltd. (London, United Kingdom). The monoclonal anti-human CCR1 antibody was purchased from R&D Systems (Abingdon, United Kingdom). Fluorescein isothiocyanate-conjugated goat anti-mouse antibody was purchased from DAKO (Ely, United Kingdom).

Generation of a Model Structure of CCR1—A computer model of the human CCR1 receptor was built on the solved structure of bovine rhodopsin (24) (Protein Data Bank code 1F88) (25). The modeling software used was QUANTA (release 4.0; MSI) running on a Silicon Graphics INDIGO2 workstation under the UNIX operating system. The “Modeler” option (26) within QUANTA was used to build 20 models of the CCR1 receptor, including a conserved disulfide bridge. The different models were calculated by varying the initial model and optimizing the objective function using conjugate gradients and molecular dynamics with simulated annealing that employed the CHARMm force field (27). The model with the lowest objective function was chosen for further study and allowed the estimation of which TM residues of CCR1 were likely to project their side chains into the helix bundle of the receptor and therefore be available for interactions with the compound. This facilitated the design of relevant point mutations within CCR1.

A structural model of the TM regions of human CCR1 was subsequently built using the program DeepView/Swiss-Pdb Viewer (ca.expasy.org/spdbv), which was also used for the manual docking of UCB 35625 to the receptor. Energy minimization was performed using the Gromos96 implementation of the Swiss-Pdb Viewer. The final representations of the TM regions of CCR1 were obtained with the program PyMol (www.pymol.org).

Generation of Hemagglutinin-tagged CCR1—CCR1 cDNA was excised from CCR1.pREP9 (4), a kind gift of Dr. Philip Murphy, Laboratory of Host Defenses, National Institutes of Health, Bethesda, MD. The amino terminus of CCR1 was tagged with the hemagglutinin epitope by PCR using the sense primer 5′-GCCCATAGCTGCCACCATGATCATGATGTCCCAGATTATGCCAAAGAATTCGAAACTCCAAACACCACAGAGGA-3′ and the antisense primer 5′-ATTAGGTGACTTTAGATC-3′. The PCR product was then inserted back into pcDNA3.1 at the HindIII and XhoI sites, and its authenticity was verified by fluorescent DNA sequencing of both strands (MWG-Biotech, Milton Keynes, UK).

Generation of Point Mutants—Point mutants of CCR1 were generated by PCR using the QuikChange™ site-directed mutagenesis kit (Stratagene, Amsterdam, Netherlands) according to the manufacturer’s instructions. The fidelity of each construct was verified by DNA sequencing on both strands.

Tissue Culture—L1.2 cells were maintained as described previously (29) in suspension at 37 °C with 5% CO2 at a density of no more than 1 × 106 cells/ml. Cells were transiently transfected by electroporation as described previously (29). In brief, 1 × 106 cells per microgram of DNA were electroporated and incubated overnight with medium supplemented with 10 mM sodium butyrate. Cells were harvested and assayed the following day.

Flow Cytometry—Approximately 5 × 105 cells were harvested, washed once with fluorescence-activated cell sorting buffer (0.25% bovine serum albumin and 0.01% NaN3 in phosphate-buffered saline) and then incubated with the primary antibody or the corresponding IgG isotype control for 15 min in a final volume of 100 μl. Cells were then

**Fig. 3.** The amino acid sequence of CCR1. Shown is a schematic diagram of CCR1 illustrating the location of the 33 transmembrane residues that were mutated during the course of this study (filled circles).
Site-directed Mutagenesis of CCR1

FIG. 4. Cell surface expression of CCR1 point mutants. Each of the CCR1 point mutant constructs were transiently transfected into L1.2 cells separately. Twenty-four hours after transfection, cells were incubated with anti-CCR1 antibody, and cell surface expression was analyzed by flow cytometry. Surface expression is shown as a percentage of the expression observed for cells simultaneously transfected with the wild-type CCR1 construct. Data shown are the mean ± S.E. of four independent experiments and were analyzed by non-parametric analysis using the Mann-Whitney test. *, p < 0.05.

washed with fluorescence-activated cell sorting buffer and incubated with the fluorescein isothiocyanate-conjugated secondary antibody for 15 min in a final volume of 50 μl. Subsequently, cells were washed with fluorescence-activated cell sorting buffer and resuspended in a final volume of 400 μl before being analyzed by flow cytometry as described previously (30). The anti-CCR1 antibody was used at 10 μg/ml, the anti-hemagglutinin antibody was used at 5 μg/ml, and the fluorescein isothiocyanate-conjugated secondary antibody was used at a 1:50 dilution. All incubations were carried out on ice.

Chemotaxis Assay—Assays of chemotactic responsiveness were performed using Chemotx™ plates (Receptor Technologies Limited, Oxon, UK) as described previously (29). Briefly, different concentrations of CCL3 in the presence or absence of a fixed concentration of UCB 35625 were loaded onto the bottom wells in a final volume of 31 μl. The 5-μm pore filter was placed on top of the wells, and 2 × 10⁵ cells in a volume of 20 μl were loaded onto the filter. Following incubation for 5 h in a humidified chamber at 37 °C with 5% CO₂, the numbers of cells migrating into the well were counted using a hemocytometer. In a primary screen, all 33 point mutants were assessed at least three times in triplicate using concentrations of 0, 0.1, 1, and 10 nM CCL3 in the presence or absence of a fixed 100 nM concentration of UCB 35625. The percentage of inhibition was calculated using an equation in which the percentage of inhibition = 100 − (the number of cells migrating in the presence of the agonist ÷ the number of cells migrating in the absence of the antagonist × 100).

RESULTS

Previously, we have described the compound UCB 35625 (Fig. 1A) as a potent inhibitor of CCR1 at nanomolar concentrations, concentrations at which it is unable to displace the ligand from the receptor (29). This led us to hypothesize that the compound interacts with the TM helices of the receptor, thereby inhibiting the conformational changes necessary to initiate intracellular signaling (29). To examine this hypothesis, site-directed mutagenesis was employed to generate a large panel of TM mutants. These constructs were expressed in a leukocyte cell line, and the effects of mutagenesis upon the migratory response to ligand and sensitivity to UCB 35625 were determined.

UCB 35625 Inhibits the Chemotaxis of L1.2 Cells Transiently Expressing CCR1—To examine such a large panel of mutants, means of transiently expressing the constructs was desirable. Previously, we have described the transient expression of the receptors CCR3 and CXCR3 in the L1.2 murine pre-B cell background (30–32). Using this system, CCR1 was transiently expressed at high levels on the surface of L1.2 cells as detected by flow cytometry after incubation with the anti-CCR1 antibody (Fig. 1B). CCR1-transfected cells were then assayed in a chemotaxis assay to determine their ability to migrate in re-
response to increasing concentrations of CCL3 in the presence or absence of UCB 35625 (Fig. 1C). CCR1-transfected L1.2 cells migrated in response to low nanomolar concentrations of CCL3, and this response was inhibited by the presence of 100 nM UCB 35625. Naïve cells were unresponsive to similar concentrations of CCL3, and no detectable cell surface expression of CCR1 was evident (Fig. 1B).

Residue Glu-287 Is Important for the Interaction of UCB 35625 with CCR1—An initial screen targeted four acidic amino acids (Asp-80, Asp-92, Glu-120, and Glu-287) in the TMs of CCR1, because we hypothesized that one of these residues was likely to act as the counterion for the positively charged quaternary nitrogen of UCB 35625 (Fig. 1A) (19, 20). Residue Asp-80 is highly conserved among G protein-coupled receptors, and mutation of this residue in CCR5 has been reported previously to lead to the loss of certain intracellular responses without affecting ligand binding (33). Each residue was mutated in turn to its amide counterpart to minimize undue conformation changes within the receptor.

All four mutant receptors were expressed at the cell surface at levels comparable with those observed for wild-type CCR1, as deduced by flow cytometry (Fig. 2A, C, E, and G). Migration in response to CCL3 was also observed for all four mutant receptors, albeit with some reduction in efficacy compared with that of wild-type CCR1 (Fig. 2A, B, D, F, and H). UCB 35625 inhibited CCL3-induced migration of these mutants in all but one case. Cells expressing the mutant receptor E287Q migrated in response to CCL3, but this response was not inhibited in the presence of 100 nM UCB 35625 (Fig. 2H), suggesting that E287 in TM7 interacts with the compound. Interestingly, the highly conserved Asp-80 residue does not seem to be essential for chemotaxis mediated by CCR1, as cells expressing the mutant D80N still migrated in a dose-dependent manner to CCL3 (Fig. 2B).

Mapping the Site of Interaction for CCR1-UCB 35625—The structure of CCR1 was initially modeled upon the published crystal structure of rhodopsin (24). The resulting model allowed us to estimate which TM residues of CCR1 were likely to project their side chains into the helix bundle of the receptor and be available for interactions with the compound (Fig. 3). Candidate residues within each TM domain were individually mutated to alanine. These mutants were generated and transfected into L1.2 cells as detailed above. Twenty-four hours post-transfection, expression of the constructs was visualized by flow cytometry. With the exception of the construct Y118A, all of the mutants examined were expressed on the surface of L1.2 cells at respectable levels approaching those of wild-type CCR1 (Fig. 4). Permeabilization of Y118A-transfected cells showed that this mutant receptor was expressed intracellularly (data not shown), suggesting that its trafficking to the membrane is compromised as a result of mutation.

L1.2 cells expressing the mutant CCR1 constructs were subsequently assayed for their ability to migrate in response to CCL3 over a limited concentration range of 0, 0.1, 1, and 10 nM CCL3 in the presence or absence of a fixed 100 nM concentration of UCB 35625. For practical reasons, only the responses of the constructs to 10 nM CCL3 are shown (Fig. 5). For the vast majority of the constructs assayed, chemotaxis to CCL3 was abolished in the presence of UCB 35625 with the exception of cells expressing the mutants Y41A, Y113A, and E287Q, which...
were significantly resistant to the compound. The majority of
constructs assayed presented with a chemotactic profile similar
to that of wild-type (data not shown), with the exception of the
L87A construct. Cells expressing this mutant were completely
unable to migrate in response to CCL3 (Fig. 6A), despite flow
cytometry studies suggesting that it was expressed on the
surface of L1.2 cells at levels comparable of those observed for
CCR1 (Fig. 4). Subsequent experiments employing 125I-CCL3
in a competitive binding assay confirmed that the L87A mutant
was expressed at the cell surface and bound CCL3 with an
affinity very similar to that of the wild-type receptor (Fig. 6B).

Together with the data from Fig. 2, this work suggests that
residues Tyr-41, Tyr-113, and Glu-287 found within TM2, TM3,
and TM7 respectively, interact with UCB 35625. To further
characterize these constructs, a full chemotactic dose response
to CCL3 was obtained for cells expressing each of the three
mutant receptors (Fig. 7). Transfection with all three con-
structs conferred upon the cells an antagonist-resistant capac-
ty to migrate in response to CCL3, confirming the previous
data. Of interest was the fact that in the presence of 1
μM compound, chemotaxis of cells expressing the E287A mutant
was markedly reduced, although not blocked completely (Fig.
7A). This observation suggests that Glu-287 is important for
facilitating binding of the antagonist and that in its absence a
lower affinity interaction occurs that is able to impede receptor
function when sufficient antagonist is employed.

Structural Modeling of the Interaction between CCR1 and
UCB 35625—A structural model of the TM regions of human
CCR1 was built based on its amino acid sequence homology
with bovine rhodopsin. Following the sequence alignment pre-
sented in Fig. 8, the structure of bovine rhodopsin, calculated
by x-ray crystallography (Protein Data Bank accession number
1L9H), was used as a template for building a model of the TM
regions of CCR1. Molecular dynamics simulations of the TM
regions of CCR5 have predicted that a TTP motif located in
TM2 and conserved in CCR1 (Fig. 8) causes a kink in TM2 that
is not observed in rhodopsin (34, 35). In addition to this bend-
ing in TM2, a rearrangement of TM3 was also postulated (35).
Because of the high degree of homology between CCR1 and
CCR5 (67% in the predicted transmembrane helices), such
rearrangements are likely to also occur in CCR1. Therefore,
following the building of a preliminary structural model of the
TM regions of CCR1 (based exclusively on the rhodopsin struc-
ture), TM2 and TM3 were manually adjusted to mimic the
results from molecular dynamics simulations performed on
CCR5. The adjustments were performed such as to preserve ϕ
and Ψ angles compatible with α-helical segments, prevent ste-
reochemical clashes, and minimize the energy of the confor-
mation (Fig. 9A). Within this model, Tyr-41, Tyr-113, and Glu-287,
shown by mutagenesis to be important for conferring resistance to UCB 35625 are highlighted by an asterisk. Residues in the rhodopsin sequence within 7 Å of the retinal binding site, as deduced by the crystal structure (24), are highlighted in boldface.

DISCUSSION

We report here the results of a study carried out to investi-
gate the mode of interaction of the receptor CCR1 with an
antagonist, UCB 35625, which was previously postulated to
interface with residues found within the TM domains (23). We
generated 33 point mutants of CCR1 in total, targeting residues in all seven TM helices that are postulated to project their side chain into a central binding pocket. Of all the mutants studied, only one was not expressed on the surface of the transfected cells, namely Y118A (located in TM2), suggesting that this tyrosine residue is important for the overall conformation of CCR1 and that its replacement severely affects trafficking to the plasma membrane. Similarly, the L87A mutant was expressed on the cell surface and bound CCL3 with an affinity similar to that of wild-type CCR1 but, importantly, did not impart migration in response to CCL3. As with Tyr-118, Leu-87 is conserved among the majority of other chemokine receptors, which suggests that these two residues may be important for the overall conformation and function of the receptors. Indeed, Leu-87 of CCR1 is part of the TXP motif (X here being leucine) that has been shown to play a key structural role in chemokine receptors by conferring the backbone flexibility necessary for the conformation change associated with the activation step induced by ligand binding (34, 36). Mutations of the threonine and proline residues of the TXP motif in CCR5 have previously demonstrated that this motif is mainly involved in receptor activation and contributes very little to ligand binding (34). Our data here suggest that the leucine side chain may also play an important role in this process. Inter-

FIG. 9. Modeling of the UCB 35625-CCR1 interaction. A, a model of the helical regions of CCR1 (cyan) superimposed on those of rhodopsin (orange) as viewed from the extracellular face. The seven TM helices are labeled. B, the same view of CCR1 with the side chains of Tyr-41 (Y41), Tyr-113 (Y113), and Glu-287 (E287) shown as yellow stick representations. C, the same three side chains in more detail, with the compound UCB 35625 docked to the receptor. Hydrogen bonds are indicated by dotted lines. D, a schematic representation highlighting the interactions between Tyr-41, Tyr-113, and Glu-287 with UCB 35625. E and F, the compound depicted as a space-filling representation, with TM helices shown as ribbons, as viewed from the extracellular face (E) and the side (F).
receptor antagonists and their receptors as postulated previously of common interactions between small molecule chemokine CXCR4 and CCR2 have been shown to be important for antagonists (37). Analogous glutamate residues within retinal binding site of rhodopsin, as has been described for CCR5. Moreover, these residues also map to the corresponding amino acid sequences of human CCR1 and human CCR5 for the interaction of TAK-779 with CCR5 (20). Alignment of residues Tyr-37, Thr-82, Tyr-108, and Glu-287 are important for the compound. It may be that this residue plays some minor role in maintaining the conformation of the antagonist binding site while not participating directly in either antagonist or ligand binding.

We have successfully docked the antagonist with our model of CCR1 helices (Fig. 9). Fig. 9B shows the location of Tyr-41, Tyr-113, and Glu-287 of CCR1 projecting into a binding pocket contained within the helix bundle. In Fig. 9C, the compound is docked with these residues, illustrating the proposed interactions between the small molecule and the receptor. Glu-287 is postulated to form a salt bridge with the quaternary nitrogen of the compound, whereas Tyr-41 and Tyr-113 are postulated to hydrogen bond with the oxygen of the xanthenyl group and the carboxyl group of the small molecule, respectively (Fig. 9D). Modeling of the interaction of CCR1 with the hydroxypropyridine BX510 also implicates Tyr-113 and Glu-287 as forming part of the antagonist binding site (39), although in that study there is no role for the Tyr-41 of CCR1 in docking with the compound.

Despite apparent similarities in docking sites, the cross-reactivity of small molecule antagonists with other chemokine receptors is limited. For example, although TAK-779 has some activity at CCR2, it is not effective at CCR1 (40). On the contrary, although UCB 35625 has activity at the related receptor CCR3, it has no effect on chemotaxis to CCL3 mediated by CCR5 (data not shown). It is plausible that the selectivity of the compounds may result from non-conserved residues within the receptor helix bundle that sterically hinder access of the compound to the binding pocket as suggested previously (41). Alternatively, the spatial separation of the conserved amino acid side chains may be subtly different, facilitating interactions with one compound but not another.

In our model, the antagonist is embedded within the transmembrane helices with a limited projection of the cycloheptyl ring out if the plasma membrane (Fig. 9F), as has been predicted for TAK-779 interactions with CCR5 (20). Such an interaction is envisaged to ligate TM helices 1, 3, and 7 of the receptor. Because movement of the latter two helices is thought to occur during the activation of rhodopsin class GPCRs (42, 43), this may explain how the compound exerts its antagonistic effects. The activation of CCR5 by CCL3 has been described by Govaerts and colleagues as a two-step process in which the hub of the chemokine interacts with the extracellular regions of the receptor, and the N terminus of the chemokine interacts with the intra-helical core of the receptor (28). This latter step is postulated to disturb interhelical interactions between side chains of TM2 and TM3, leading to the induction of an active receptor conformation (35). If we apply the same model to the activation of CCR1, then access to both TM2 and TM3 of CCR1 is likely to be severely restricted by the presence of the compound (Fig. 9, E and F). Thus, in addition to ligating the helices of CCR1, UCB 35625 may also be envisaged as physically impeding an interaction of the amino terminus of the chemokine with the receptor.

Thus, in accordance with previously published data from other laboratories, the TM domains of chemokine receptors represent an attractive target for small molecule antagonist molecules. Insights into the modus operandi of these compounds may facilitate the development of more potent antagonists that show promise as future therapeutic agents in the treatment of inflammatory disease.

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Site-directed Mutagenesis of CC Chemokine Receptor 1 Reveals the Mechanism of Action of UCB 35625, a Small Molecule Chemokine Receptor Antagonist
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