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Identification of a Putative Intracellular Allosteric Antagonist Binding-Site in the CXC Chemokine Receptors 1 and 2[S]

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ABSTRACT

The chemokine receptors CXCR1 and CXCR2 are G-protein-coupled receptors (GPCRs) implicated in mediating cellular functions associated with the inflammatory response. Potent CXCR2 receptor antagonists have been discovered, some of which have recently entered clinical development. The aim of this study was to identify key amino acid residue differences between CXCR1 and CXCR2 that influence the relative antagonism by two compounds that have markedly different chemical structures. By investigating the effects of domain switching and point mutations, we found that the second extracellular loop, which contained significant amino acid sequence diversity, was not important for compound antagonism. We were surprised to find that switching the intracellular C-terminal 60 amino acid domains of CXCR1 and CXCR2 caused an apparent reversal of antagonism at these two receptors. Further investigation showed that a single amino acid residue, lysine 320 in CXCR2 and asparagine 311 in CXCR1, plays a predominant role in describing the relative antagonism of the two compounds. Homology modeling studies based on the structure of bovine rhodopsin indicated a potential intracellular antagonist binding pocket involving lysine 320. We conclude that residue 320 in CXCR2 forms part of a potential allosteric binding pocket on the intracellular side of the receptor, a site that is distal to the orthosteric site commonly assumed to be the location of antagonist binding to GPCRs. The existence of a common intracellular allosteric binding site at GPCRs related to CXCR2 may be of value in the design of novel antagonists for therapeutic intervention.

Chemokines are small, secreted proteins of 8 to 14 kDa that regulate a broad spectrum of cellular functions and typically induce cell movement along a concentration gradient. There are three groups of chemokines exhibiting characteristic cysteine sequence motifs: the C-X-C, C-C, and C-X3-C families (Horuk, 2001). The emergent role of chemokines in immune and inflammatory responses has identified chemokine receptors as attractive targets for therapeutic intervention in various diseases and disorders (D'Ambrosio et al., 2003). The two GPCRs CXCR1 and CXCR2 have been identified as important mediators of inflammation and display distinct ligand specificities. CXCL8 (interleukin-8) and CXCL6 (granulocyte chemotactic protein-2) interact with both CXCR1 and CXCR2; however, the chemokines CXCL5 (epithelial cell-derived neutrophil-activating protein 78), CXCL7 (neutrophil-activating peptide 2), and CXCL1 (growth-related oncogene-α) are efficacious for CXCR2 only (Wolf et al., 1998). CXCR2 is expressed on a variety of cells including neutrophils, keratinocytes, mast cells, eosinophils, macrophages, and endothelial and epithelial cells. In addition to chemotaxis, activation of CXCR2 is known to stimulate a variety of cellular responses including calcium mobilization, adhesion molecule up-regulation, and angiogenesis. These pleiotropic effects have implicated CXCR2 in the pathology of various diseases with inflammatory components such as chronic obstructive pulmonary disease, arthritis, and psoriasis. The diverse nature of CXCR2 in biology has stimulated much interest in the pharmaceutical industry, and the

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ABBREVIATIONS: GPCR, G-protein-coupled receptor; PCR, polymerase chain reaction; TM, transmembrane; CXCR1–2-1, CXCR1 receptor with amino acids 168 to 218 (CXCR2 numbering system, equivalent to 159 to 209 in the CXCR1 sequence) substituted from CXCR2; CXCR1–2long, CXCR1 receptor with C-terminal 60 amino acids substituted from CXCR2; CXCR2–1long, CXCR2 receptor with C-terminal 59 amino acids substituted from CXCR1; CXCR1–2short, CXCR1 receptor with C-terminal 34 amino acids substituted from CXCR2; CXCR2–1short, CXCR2 receptor with C-terminal 33 amino acids substituted from CXCR1; HEK, human embryonic kidney; KT5720, hexyl (5*R*,6*S*,8*S*)-6-hydroxy-5-methyl-13-oxo-5,6,7,8,14,15-hexahydro-13*H*-5,8-epoxy-4*b*,8*a*,14-triazadibenzo[*b*,*h*]cycloocta[1,2,3,4-*jkl*]cyclopenta[*e*]-*as*-indacene-6-carboxylate.

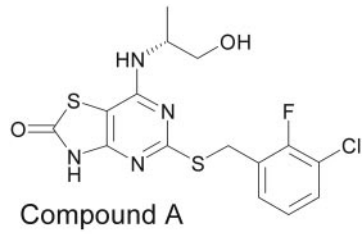
synthesis of several nonpeptide antagonists has been described previously (White et al., 1998; Catusse et al., 2003; Matzer et al., 2004; Souza et al., 2004; Widdowson et al., 2004; Baxter et al., 2006; Gonsiorek et al., 2007). These nonpeptide antagonists fall into various structural classes and display different receptor-selectivity profiles (Busch-Petersen, 2006). Understanding the nature of antagonist interactions with receptors and their selectivity is key for rational drug design. Because CXCR1 is the closest homolog to CXCR2 and compounds have been shown to bind to both receptors, it is important to understand the origin of the observed selectivity. For GPCRs, predictions for ligand-binding interactions have until recently been largely based on comparisons with rhodopsin. The retinal binding site is clearly defined in the exofacial core of rhodopsin and has been the focus of attention for mutagenesis and homology modeling studies to predict ligand-binding interactions (Klabunde and Hessler, 2002; Kristiansen, 2004; Schwartz et al., 2006). However, reports describing allosteric modulation of GPCRs suggest the existence of alternative interaction sites, which regulate receptor function and dimerization (Bertini et al., 2004; Soudijn et al., 2004; Birdsall and Lazareno, 2005; Gao and Jacobson, 2006). Although the term "allosteric" refers to a recognition domain on the receptor that is distinct from the primary (orthosteric) site (Neubig et al., 2003), the precise location of alternative interaction sites within GPCRs is not clearly understood. We describe here evidence to support the existence of a nonpeptide antagonist-binding site near the intracellular C-terminal domain of CXCR2, which is distal to the classic retinal-binding site in rhodopsin. We have used domain swap experiments and site-directed mutagenesis in conjunction with homology modeling to identify amino acids within the intracellular region of CXCR1 and CXCR2 that are important for conferring receptor selectivity by structurally distinct nonpeptide antagonists. In addition, we present evidence suggesting that access to the intracellular side of the receptor is required for inhibition by allosteric antagonists. The existence of a potential intracellular binding pocket in chemokine receptors and other GPCRs could influence the design of novel agents for therapeutic intervention.

Materials and Methods

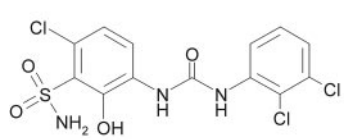
Reagents. Oligonucleotide primers were synthesized by Eurogentec (Southampton, UK) using standard methods. Standard tissue culture and molecular biology reagents including restriction endonucleases, alkaline phosphatase, T4 DNA ligase, and TOP 10 *Escherichia coli* were supplied by Invitrogen (Paisley, UK). QuikChange XL site-directed mutagenesis kits were purchased from Stratagene (Amsterdam, the Netherlands). QIAquick Gel Extraction kits were supplied by Qiagen (Crawley, UK). CXCL8 and CXCL1 were purchased from BioSource (Nivelles, Belgium). ^{125}I-Labeled CXCL8 (specific activity, 74 TBq/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Beaconsfield, Buckinghamshire, UK). CXCR2 antagonists (Fig. 1), compound A, (1*R*)-5-[[(3-chloro-2-fluorophenyl)methyl]thio]-7-[[2-hydroxy-1-methylethyl]amino]-thiazolo[4,5-*d*]pyrimidin-2(3*H*)-one (Walters et al., 2008), and compound B, *N*-(3-(aminosulfonyl)-4-chloro-2-hydroxyphenyl)-*N*′-(2,3-dichloro-phenyl) urea (Podolin et al., 2002), were synthesized by the Department of Medicinal Chemistry at AstraZeneca (Charnwood, UK). Compound A is protected by European Patent 1222195, U.S. Patent 6790850, and corresponding patents and patent applications.

DNA Constructs and Site-Directed Mutagenesis. The cDNAs encoding the human chemokine receptors CXCR1 and CXCR2 were cloned into pIRESneo2 using standard methods as described in Sambrook et al. (1989) and were confirmed by sequencing. These plasmids were used as a template to produce the CXCR1 and CXCR2 chimeras. An alignment of CXCR1 and CXCR2 was generated (Fig. 2), and throughout this article, the amino acid numbering of all mutant and hybrid proteins corresponds to that of CXCR2 in Fig. 2. The amino acid residues at positions 320 and 325 in the CXCR2 sequence were equivalent to residues 311 and 316 in CXCR1, respectively. All oligonucleotide primers used for genetic manipulations are listed in the supplementary data. The first chimera was CXCR1–2-1 and was generated by exchanging the cDNA sequence of CXCR1 encoding residues 159 to 209 with residues 168 to 218 of CXCR2 using an overlapping PCR reaction. Primers CXCR1 5′-start and CXCR1 5′-back were used to amplify the N-terminal sequence of CXCR1. Primers CXCR2 F1 and CXCR1 R1 were used to amplify the middle CXCR2 region, and primers CXCR1 3′-start and CXCR1-stop were used to amplify the C-terminal region of CXCR1. The resulting PCR reaction was cloned into pIRESneo using NheI and NotI restriction sites on the PCR product. The next chimera constructs were generated by exchanging the cDNA encoding C-terminal 60 amino acids of CXCR2 with the C-terminal 59 amino acids of CXCR1 using an internal Xcm I site. These hybrid receptors were designated CXCR1–2long and CXCR2–1long. A second set of chimera constructs designated CXCR1–2short and CXCR2–1short was generated by first introducing an AflII restriction enzyme site in the cDNAs of both CXCR1 and CXCR2 by site-directed mutagenesis followed by exchanging the cDNA encoding the C-terminal 34 amino acids from CXCR2 and the C-terminal 33 residues from CXCR1. Receptor mutants CXCR1 N311K, CXCR1 F316L, CXCR2 K320N, CXCR1 N311K/F316L, and CXCR2 K320N/L325F were generated using DNA primers with single- or double-base mismatches. Mutagenesis was performed using the QuikChange XL site-directed mutagenesis kit. The correct sequence of all DNA constructs was confirmed by di-deoxy-terminator sequencing using standard methods.

Cell Culture. HEK293 cells were grown in Dulbecco's modified Eagle's glutamax medium containing nonessential amino acids and 10% (v/v) fetal calf serum in a humidified incubator at 37°C with 5% CO_2/95% air. Cells were harvested at approximately 80% confluence from the flasks using 10× trypsin. The cells were transfected with plasmids for CXCR1 and CXCR2 receptor chimeras and mutants using the transfection reagent Fugene 6 (Roche, Burgess Hill, UK). Stable transfectants expressing CXCR1 and CXCR2 proteins were selected for and maintained by the addition of Geneticin G418 at 1 mg/ml (Invitrogen).



Compound A



Compound B

Fig. 1. Chemical structures of compound A, (1*R*)-5-[[(3-chloro-2-fluorophenyl)methyl]thio]-7-[[2-hydroxy-1-methylethyl]amino]thiazolo[4,5-*d*]-pyrimidin-2(3*H*)-one, and compound B, *N*-(3-(aminosulfonyl)-4-chloro-2-hydroxyphenyl)-*N*′-(2,3-dichloro-phenyl) urea.

Functional Calcium Assays for CXCR2. Intracellular calcium mobilization was determined in 96-well poly(D-lysine)-coated plates. Cells (100,000/well) were allowed to adhere to plates overnight and then were incubated with 10 μM final concentration of Fluo-3/AM for 60 min in media at 37°C. Plates were washed twice in 100 μl of HEPES-buffered phosphate-buffered saline solution (25 mM HEPES, 10 mM phosphate buffer, pH 7.4, containing 137 mM NaCl and 1.5 mM $CaCl_2$), and then 50 μl of buffer per well was added to the cells. Compounds in HEPES-buffered phosphate-buffered saline solution, containing 1.5% (v/v) dimethyl sulfoxide, were added in a volume of 50 μl/well and incubated at room temperature for 30 min. Cells were primed by the addition of 50 μl/well carbachol solution (1 mM final concentration). After 3 min, 50 μl/well of solutions containing CXCL8 at various concentrations was added. Calcium transients were measured using a fluorometric imaging plate reader (Molecular Devices, Wokingham, UK).

Membrane Preparation. Cells were resuspended and then disrupted by homogenization using a Polytron tissue homogenizer (Kinematica, Basel, Switzerland) in hypotonic buffer at 4°C (3:1 mix of water and HEPES-buffered Tyrode's solution). The membrane preparation was purified by centrifugation at 140,000*g* for 1 h at 4°C on 41% (w/v) sucrose. The membrane fraction at the interface was recovered, diluted, and centrifuged at 100,000*g* for 20 min at 4°C. The membrane pellet was resuspended at 1×10^8 cell equivalents/ml (typically 3 mg/ml protein) in HEPES-buffered Tyrode's solution [10 mM HEPES, pH 7.4, containing 2.7 mM KCl, 137 mM NaCl, 0.4 mM $KHPO_4$, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% (w/v) gelatin, and 100 μg/ml bacitracin] and subsequently stored in aliquots at −80°C.

Radioligand Binding Assays. Radioligand binding assays were performed in HEPES-buffered Tyrode's solution using 0.45-μm 96-well filter plates (Millipore, Watford, UK). Membranes (30 μg/ well) and ^{125}I-CXCL8 (60 pM) were incubated with compounds for 2 h at room temperature in the presence of 1% (v/v) dimethyl sulfoxide. Membrane-bound ^{125}I-CXCL8 was separated from ^{125}I-CXCL8 in solution by washing with 200 μl of HEPES-buffered Tyrode's solution at 4°C. Individual filters were transferred to polypropylene tubes, and the radioactivity was measured by direct γ counting using a Cobra II Gamma counter (PerkinElmer Life and Analytical Sciences, Beaconsfield, UK). Nonspecifically bound radioactivity was determined in the presence of 10 μM unlabeled CXCL8.

Receptor Modeling. Recently, two crystal structures for the β_2 adrenergic receptor have been described (Cherezov et al., 2007; Rasmussen et al., 2007), and these structures were compared with the rhodopsin structure to evaluate their suitability for modeling CXCR2. A multiple sequence alignment of all class A human GPCR receptors suggests the presence of a single residue gap in the β_2 receptor sequence at the bottom of helix 7, within three or four residues of the CXCR2 K320N mutation described in this study. This gap is not present in rhodopsin or CXCR1 or CXCR2, and when the rhodopsin and β_2-receptor structures are structurally aligned, this adversely affects any model of the intracellular region based on the β_2 receptor structure. More fundamentally, the β_2 receptor structures were solved using techniques likely to introduce artifacts into the intracellular portion of the receptor. The Rasmussen structure was obtained at low resolution as a complex with an antibody Fab bound to the intracellular site and the Cherezov structure as a chimera with T4-lysozyme. A structural alignment of the rhodopsin and the chimeric β_2 receptor structures shows a very strong correspondence between the intracellular portions of the two receptors. However, the greatest deviation is in helices 5 and 6, where the lysozyme molecule is fused in the chimeric β_2 receptor, and it is likely that this is an artifact introduced by engineering the chimera. For these reasons, the bovine rhodopsin structure was used as a starting point for modeling the intracellular site of CXCR2.

A sequence alignment of the CXCR2 coding sequence with other class A GPCRs and bovine rhodopsin was generated using ClustalW (Chenna et al., 2003) and then modified to reflect known TM-defining motifs. This alignment along with the structure of bovine rhodopsin (Okada et al., 2002) was used as input to Modeler version 5 (Sali and Blundell, 1993) run via the InsightII 2000 interface (Accelrys Software Inc., Cerius2 Modeling Environment, release 4.10; Accelrys Software Inc., San Diego, CA). A total of 20 models were

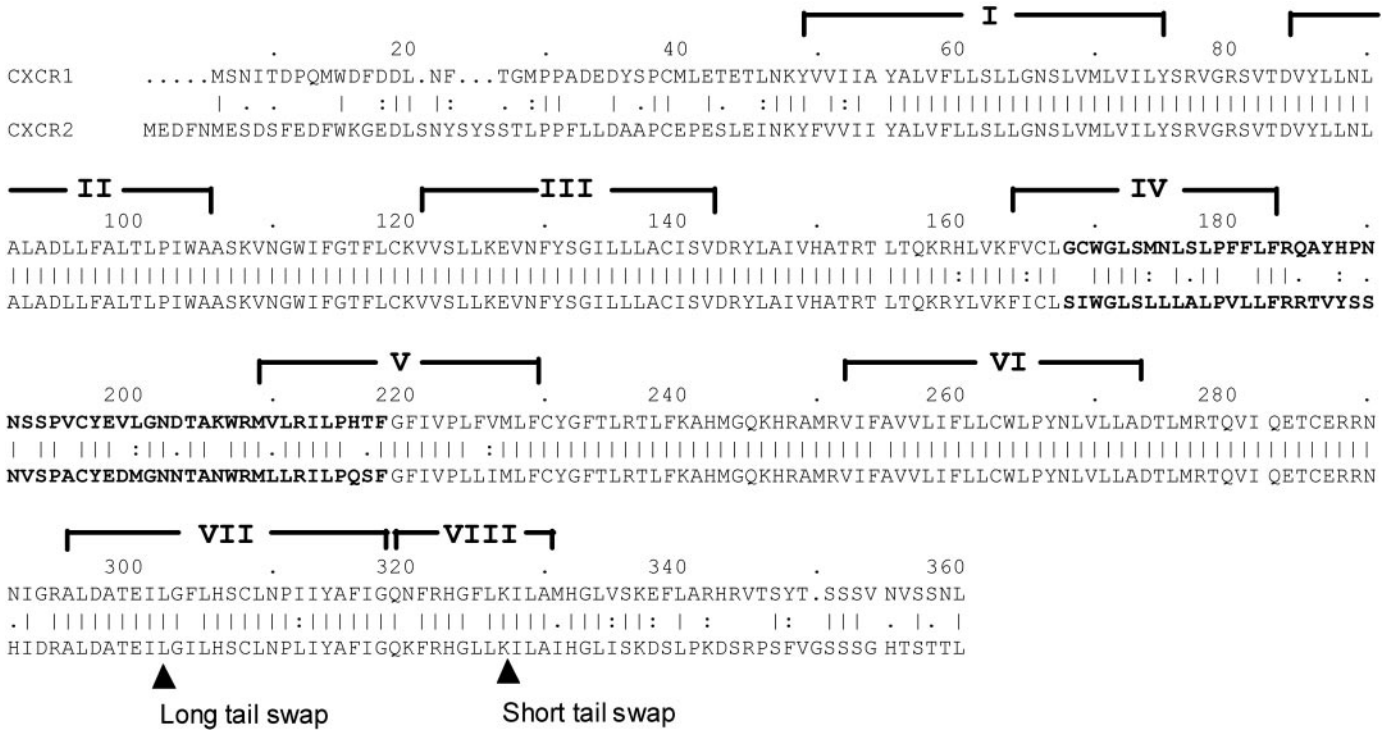


Fig. 2. Amino acid sequence alignment of CXCR1 and CXCR2. Amino acid positions are numbered according to the CXCR2 sequence. Boldface type denotes a region exchanged in CXCR1–2-1 chimera construct. ▲, substitution positions for the long and short tail swap constructs. Brackets denote the predicted seven TM spanning regions on the receptors.

produced, and the structure with the lowest penalty function used. Hydrogens and charges were subsequently added in Sybyl version 6.9 and siteID (Tripos Inc., St. Louis, MO) was used to visualize the intracellular binding site. The resulting model was manually refined and subjected to a series of constrained minimizations using CHARMm Version 31.1 (Accelrys Software). Dockings were performed manually and subjected to constrained minimization in the active site using the Tripos force field.

Data Analysis. Concentration-response data were fitted to a four-parameter logistic function using the Excel-based program XL-fit (I.D. Business Solutions Ltd., Guildford, UK). Estimates of pA_2 values for compounds were determined by analyzing the dose ratio relative to the vehicle control curve. The IC_{50} value was defined as molar concentration of compound required to give 50% inhibition of specifically bound ^{125}I-CXCL8.

Results

Agonist Potency and Compound Antagonism at CXCR1 and CXCR2. As expected, agonist potency differences at CXCR1 and CXCR2 were clearly evident in CXCL8- and CXCL1-mediated calcium responses (Fig. 3, A and D). Antagonism and affinity differences were evaluated by comparing the ability of compounds to inhibit both CXCL8-mediated calcium responses and the binding of radio-labeled CXCL8 to membranes from HEK293 cells expressing recombinant receptors. Consistent with findings from previous studies (Podolin et al., 2002; Walters et al., 2008), both compounds A and B were at least 100 times more potent antagonists of CXCR2 compared with CXCR1 (Table 1). Moreover, compound A is representative of a diverse chemical series that shows a consistent 100-fold decrease in apparent affinity against CXCR1 compared with CXCR2 (data not shown). The antagonism of CXCR2-mediated intracellular calcium mobilization by compound A produced parallel right-shifted concentration-response curves (Fig. 4). In this respect, compound A seemed to demonstrate an inhibition profile resembling that of a classic competitive antagonist with a pA_2 value of 8.7 ± 0.1 (n = 6), similar to the pIC_{50} for inhibition of radiolabeled CXCL8 binding to membranes in a cell-free system (Table 1).

Dependence of Lipophilicity. A large series of compounds of which A is a representative was generated to investigate the structure-activity relationship with CXCR2. Therefore, all the compounds were tested in the cellular CXCL8-mediated calcium-response assay and the CXCL8 cell-free radioligand displacement assay. The partition coefficient (logD) between octanol and aqueous solution at pH 7.4 of each of these compounds was also determined as a measure of lipophilicity. Compounds with lower lipophilicity showed a weak but significant trend toward reduced antagonism in the cellular calcium mobilization assay compared with the cell-free system (Fig. 5). Because permeability of a compound by passive diffusion through a biological membrane is dependent on lipophilicity, molecular size, and hydrogen bonding capacity, these data suggest that compounds in this series may require penetration of the biological membrane to interact with the receptor in the calcium-response assay. Compounds A and B demonstrate a comparable degree of antagonism in the cell and cell-free systems, and they both have high lipophilicity, each with a logD of approximately 3.5 (Fig. 5).

CXCR1–2-1 Chimera Construct. The antagonism of CXCR1 and CXCR2 by compounds A and B was clearly different as shown by right-shifted CXCL8-mediated calcium mobilization in the presence of antagonist (Fig. 6, A and B).

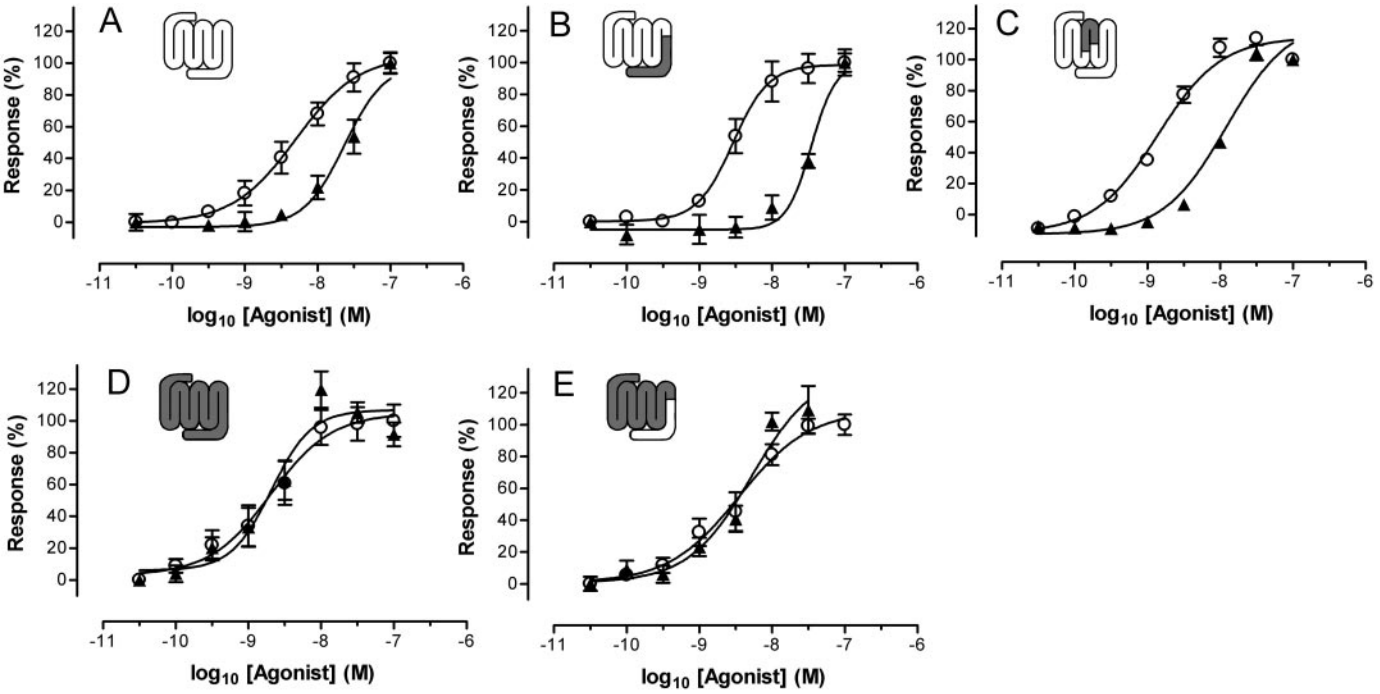


Fig. 3. Effect of receptor tail substitution on agonist-induced calcium mobilization. Calcium mobilization in HEK293 cells expressing recombinant receptor constructs was determined in response to stimulation with CXCL8 (○) or CXCL-1 (▲). Receptor constructs are illustrated schematically, where CXCR1 is represented in white fill and CXCR2 represented in gray fill. A, CXCR1; B, CXCR1–2long; C, CXCR1–2-1; D, CXCR2; E, CXCR2–1long. Data are the mean of three to eight individual experiments normalized to the maximum observed response determined in the absence of compound.

We wished to understand, at a molecular level, the relative differences in antagonism for compounds acting at CXCR1 and CXCR2. It is generally assumed that small-molecule binding sites in GPCRs are located in the retinal-binding pocket, described in the rhodopsin structure, which has extracellular access for agonists and antagonists. Therefore, the chimeric construct CXCR1–2-1 was generated to investigate the effect of exchanging the second extracellular loop and sections of the transmembrane regions of CXCR1 and CXCR2. This region has a high degree of sequence diversity (Fig. 2), is equivalent to the retinal binding pocket, and was considered likely to have amino acid residues involved in compound interactions. When expressed in HEK293 cells, the chimeric protein retained functional activity after stimulation with the agonists CXCL8 or CXCL1 showing the CXCR1–2-1 construct possesses a similar agonist profile to wild-type CXCR1 (compare Fig. 3, A and C). The CXCR1–2-1 chimera is similar to the ABA chimera generated previously (Ahuja et al., 1996), which also maintained agonist potency differences between CXCL8 and CXCL-1. We were surprised to find that when incubated with compounds A or B, the CXCR1–2-1 construct gave an antagonism profile similar to that of wild-type CXCR1 (compare Fig. 6, A and C). Thus, exchanging the second extracellular loop and sections of the transmembrane region of CXCR1 and CXCR2, comprising the equivalent of the retinal binding pocket in rhodopsin, had little effect on either agonist potency or antagonist activity.

Long C-Terminal Tail Chimera Constructs. Two chimeric constructs were generated to investigate the effect of exchanging the C-terminal region of CXCR1 and CXCR2 (CXCR1–2long and CXCR2–1long). Exchange of these C-terminal domains had little effect on agonist potency for either CXCL8 or CXCL1 (Fig. 3), suggesting that key amino acid residues describing differences in agonist effects between CXCR1 and CXCR2 were not located in the C-terminal domain. However, differences in compound antagonism were observed when the chimeras were compared with the wild-type CXCR1 and CXCR2 receptors. CXCR1–2long showed enhanced compound antagonism (Fig. 7A), which was absent previously in the CXCR1 wild-type receptor. On the other hand, the reciprocal mutation CXCR2–1long had a reduced compound antagonism compared with the CXCR2 wild-type receptor.

Short C-Terminal Tail Chimera Constructs. To further investigate key amino acid residues dictating the selectivity difference between CXCR1 and CXCR2, hybrid receptor constructs were created where the most divergent region, the last 34 C-terminal amino acid residues, was exchanged (CXCR1–2short and CXCR2–1short). We were surprised to find that there was no effect on the antagonist profile when this smaller domain was exchanged because both hybrid receptors (Fig. 7, C and D) were similar to wild-type CXCR1 and CXCR2, respectively.

Site-Directed Mutagenesis. Taken together, switching of the short and long C-terminal domains demonstrated that key amino acid residues which influence compound antagonism were located in the intervening 25 amino acid region between residues 302 and 327 (as shown in Fig. 2). The most prominent difference between CXCR1 and CXCR2 in the amino acid sequence alignment between residues 302 and 327 occurs at position 320 (Fig. 2), where asparagine in CXCR1 is substituted for lysine in CXCR2. Other differences are relatively conservative changes with differences in leucine, isoleucine, and phenylalanine residues. These residues are similar in terms of bulk and lipophilicity, whereas the physicochemical properties of the asparagine and lysine

TABLE 1

Inhibition of CXCL8 binding

Compound inhibition at CXCR receptors and mutated receptor constructs was determined by measuring the displacement of ^{125}I-CXCL8 binding to membranes. Compound, CXCL8, and membranes were incubated for 2 h at 22°C. Inhibition of specifically bound CXCL8 was determined and analyzed by nonlinear regression. pIC_{50} values were derived from experiments presented in Fig. 9 and are the mean ± S.E.M. of three to six individual determinations.

Construct	pIC_{50}	
	Compound A	Compound B
CXCR1	6.3 ± 0.17	5.9 ± 0.13
CXCR2	8.9 ± 0.13	8.7 ± 0.11
CXCR1 N311K	8.1 ± 0.13	8.3 ± 0.11
CXCR2 K320N	7.9 ± 0.15	7.5 ± 0.22

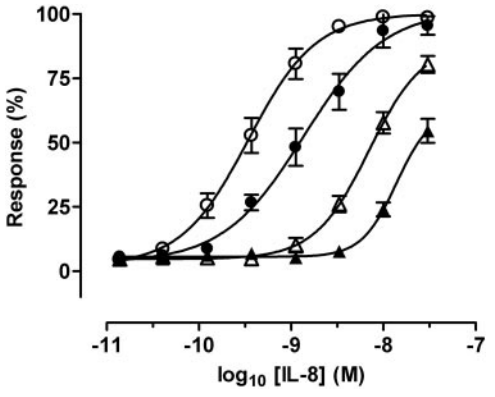


Fig. 4. Antagonism of CXCL8-induced calcium mobilization by recombinant CXCR2. Calcium mobilization was determined in HEK293 cells expressing CXCR2 in the absence (○) and presence of 10 (●), 32 (△), and 100 nM (▲) compound A. Data are the mean of six individual experiments normalized to the maximum observed response determined in the absence of compound.

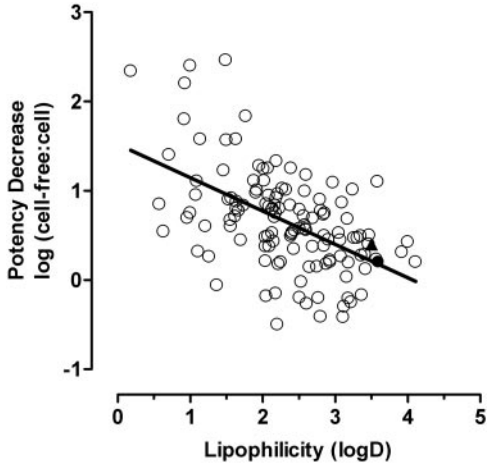


Fig. 5. Relationship of lipophilicity to potency loss for inhibition of cellular calcium response. Potency loss was determined by subtracting the pIC_{50} value for inhibition of ^{125}I-CXCL8 to cell membranes expressing CXCR2 (cell-free assay) from pA_2 for antagonism of CXCL8-mediated calcium mobilization in HEK293 cells expressing CXCR2 (cell assay). Lipophilicity was determined by measuring logD, defined as the partition coefficient between aqueous solution and octanol at pH 7.4. Compounds similar to those described by Walters et al. (2008) (○); ●, compound A; ▲, compound B.

amino acid side chains have markedly different ionization states at physiological pH.

Mutant receptor constructs of CXCR1 and CXCR2 were generated to investigate the effects of the N311K mutation on compound antagonism. A mutation at F316L was also included because it is in close proximity to N311K and could be included in the same mutagenic oligonucleotide. Simultaneous introduction of the two mutations N311K and F316L into CXCR1 (Fig. 8A) resulted in a mutant receptor that displayed an antagonist profile similar to that observed with native CXCR2 and the CXCR1–2long hybrid receptor. The reciprocal mutation in CXCR2 resulted in diminished compound antagonism at the concentrations tested (Fig. 8B). The single amino acid substitution of F316L into CXCR1 had no effect on receptor antagonism because this mutant receptor had an antagonist profile identical with wild-type CXCR1 (Fig. 8E). It is remarkable that the single amino acid substitution of N311K into CXCR1 (Fig. 8C) resulted in antagonism of the mutant receptor similar to the antagonist profile observed with wild-type CXCR2, CXCR1–2long, and the CXCR1 N311K/F316L double mutation constructs. The reciprocal mutation K320N in CXCR2 reduced antagonism at the concentration tested such that the antagonist profile was similar to wild-type CXCR1 (Fig. 8D). Taken together, these data suggest that the amino acid residue at position 320 (Fig. 2) has a profound effect on antagonism by both compounds A and B.

The contribution of lysine or asparagine at position 320 to compound potency in CXCR1 and CXCR2 was explored further by investigating the effect of mutations on inhibition of radiolabeled CXCL8 binding to membrane preparations expressing recombinant receptors (Table 1 and Fig. 9). The mutations N311K in CXCR1 and K320N in CXCR2 produced an increase and decrease in affinity, respectively, for both compounds tested. Mutation of the residue at position 320 does not result in a complete reciprocal change in affinity for compounds A and B between CXCR1 and CXCR2 (Table 1).

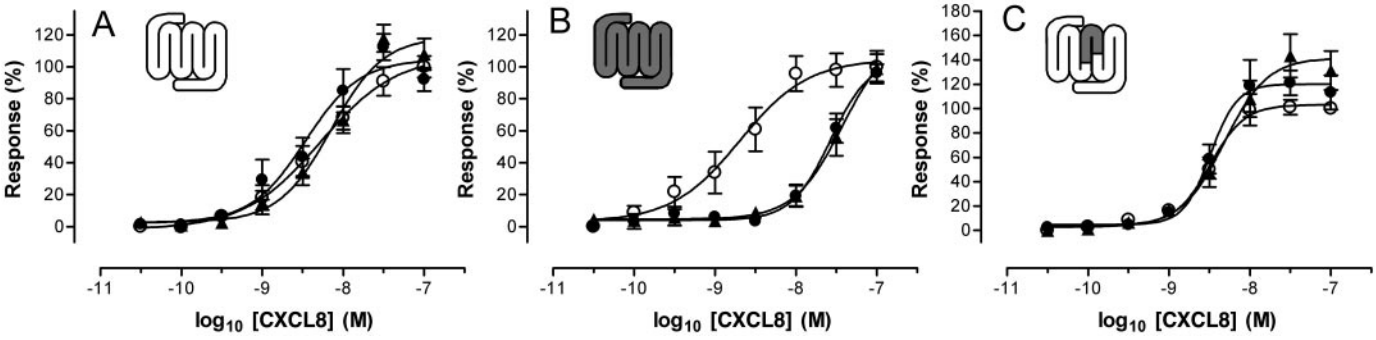


Fig. 6. Effect of second extracellular domain substitution on antagonism of CXCL8-induced calcium mobilization. Calcium mobilization was determined in response to stimulation with CXCL8 in the absence of compound (○) or in the presence of 32 nM compound A (●) or 100 nM compound B (▲). Receptor constructs are illustrated schematically, where CXCR1 is represented in white fill and CXCR2 is represented in gray fill. A, CXCR1; B, CXCR2; C, CXCR1–2-1. Data are the mean of three to four individual experiments normalized to the maximum observed response determined in the absence of compound.

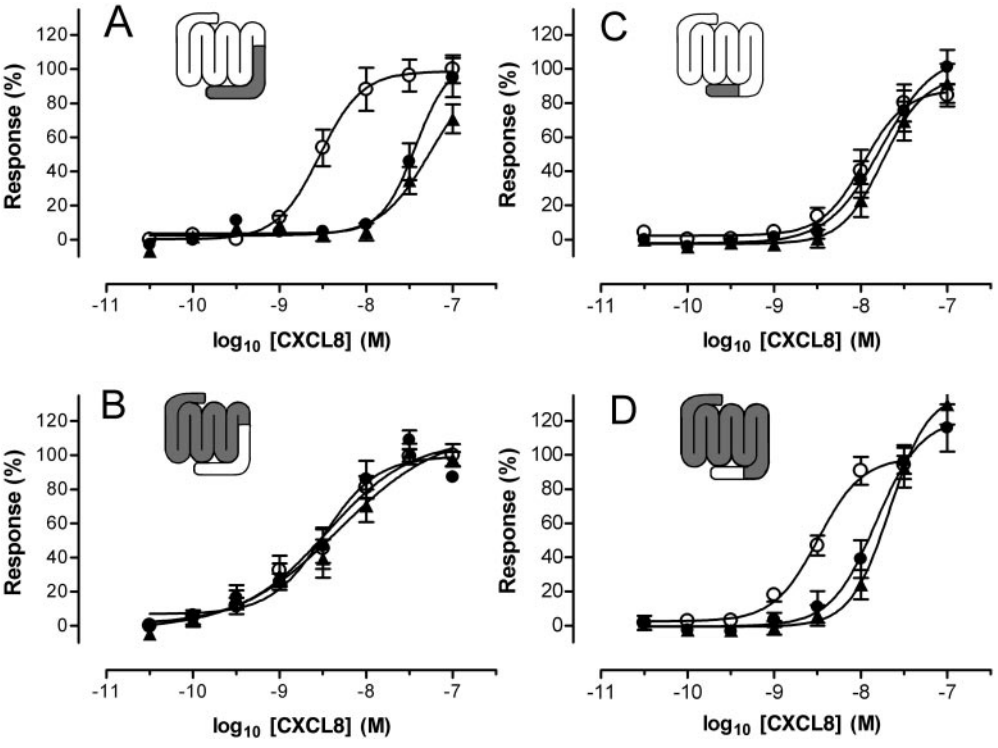


Fig. 7. Effect of receptor C-terminal domain substitutions on antagonism of CXCL8-induced calcium mobilization. Calcium mobilization was determined in response to stimulation with CXCL8 in the absence of compound (○) or in the presence of 32 nM compound A (●) or 100 nM compound B (▲). Receptor constructs are illustrated schematically, where CXCR1 is represented in white fill and CXCR2 is represented in gray fill. A, CXCR1–2long; B, CXCR2–1long; C, CXCR1–2short; D, CXCR2–1short. Data are the mean of three to four individual experiments normalized to the maximum observed response determined in the absence of compound.

The magnitude of the changes in affinity seemed to be larger for compound B than for compound A (Table 1).

Receptor Modeling. A homology model of CXCR2 based on the rhodopsin structure (Okada et al., 2002) was generated (Fig. 10). The rhodopsin model clearly shows the presence of a hydrophobic cavity in CXCR2 on the intracellular side of the TM bundle with apparent access from the cytosol. The surface of this cavity comprises residues of transmembrane helices 2, 3, 6, and 7, and the opening of the binding site is adjacent to lysine 320, which is highlighted in green in Fig. 10. The cavity is able to accommodate compounds of the size of A and B, with minimal adjustment either of individual residues or helices. The binding mode was modeled by introducing compound A into the cavity by a process of manual docking followed by minimization (Fig. 11). In this pose, the acidic nitrogen of the thiazole ring and the adjacent nitrogen of the pyrimidine ring interact directly with Lys320. The phenyl ring sits in a hydrophobic cavity within the transmembrane region, whereas the allaninol group is located in the interface between the membrane region and the cytosol, adjacent to the arginine and aspartate of the DRY motif on helix 3. A model of chemokine bound to receptor has recently been proposed in which the N terminus of the chemokine interacts with the receptor helical bundle and the core domain of the ligand interacts with the extracellular loops of the receptor (Allen et al., 2007). It is possible that with a degree of flexibility in the receptor, the cavity containing the chemokine N terminus could potentially extend through the receptor. However, the chemokine-binding model suggests that the N terminus does not extend far enough down into the receptor to interact at the intracellular site proposed here.

Discussion

The nonpeptide compounds A and B inhibit CXCL8 binding to both CXCR1 and CXCR2 (Table 1), although the compounds have approximately 100-fold lower affinity at

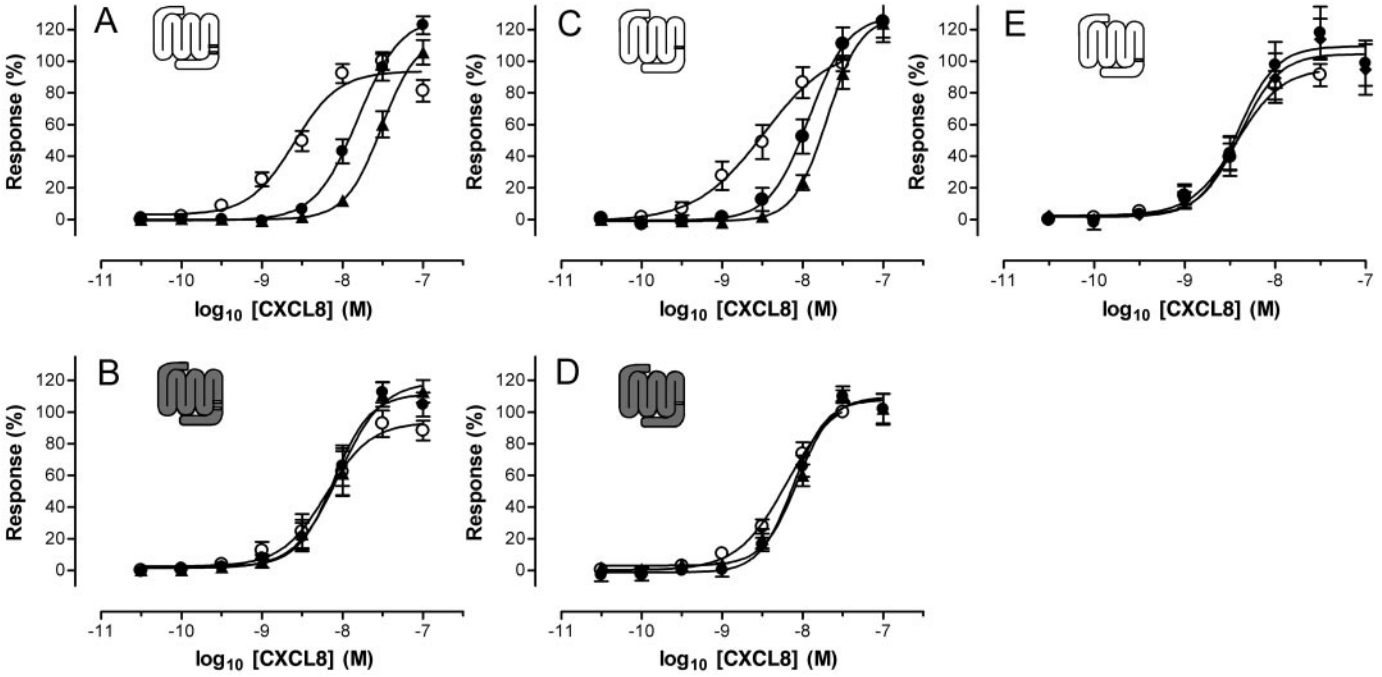


Fig. 8. Effect of receptor point mutations on antagonism of CXCL8-induced calcium mobilization. Calcium mobilization was determined in response to stimulation with CXCL8 in the absence of compound (○) or in the presence of 32 nM compound A (●) or 100 nM compound B (▲). Receptor constructs are illustrated schematically, where CXCR1 is represented in white fill and CXCR2 is represented in gray fill. A, CXCR1 N311K/F316L; B, CXCR2 K320N/L325F; C, CXCR1 N311K; D, CXCR2 K320N; E, CXCR1 F316L. Data are the mean of three to four individual experiments normalized to the maximum observed response determined in the absence of compound.

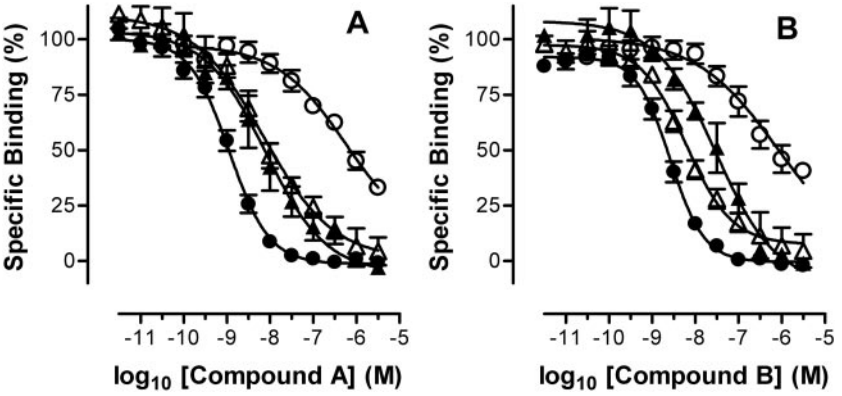


Fig. 9. Effect of receptor point mutations on compound inhibition of ^{125}I-CXCL8 binding to recombinant membranes. Compound inhibition of ^{125}I-CXCL8 binding was determined in membranes prepared from HEK293 cells expressing recombinant CXCR1 (○), CXCR2 (●), CXCR1 N311K (△), or CXCR2 K320N (▲). A, compound A; B, compound B. Data are the mean of three to seven individual experiments.

CXCR1. We investigated whether amino acid sequence variation between CXCR1 and CXCR2 could explain the differential antagonism of compounds A and B at these receptors. The second extracellular loop region between transmembrane domains IV and V was initially expected to be involved in compound binding, because this region (shaded red, Fig. 10) forms part of a potential pocket analogous to the retinal binding site in rhodopsin. However, it seems that residues in this region contribute little to agonist or compound interactions, because there were no apparent differences in either agonist potency or compound antagonism when the second extracellular loop was substituted in CXCR1 with that of CXCR2 to generate the chimera CXCR1–2-1.

The observation of a loss in potency for compounds in the cell assay compared with the cell-free system related to decreasing lipophilicity (Fig. 5) is consistent with the cell membrane acting as a barrier to passive diffusion of the compounds, with lipophilicity being a key determinant for permeability through a biological membrane. These data suggest that the compounds require intracellular access to the receptor antagonist binding site as described recently (Andrews et al., 2008) and may not be directly competing with CXCL8 at the extracellular site. This would imply a binding site involving residues in the intracellular region, and the region of highest sequence dissimilarity between the two receptors, the C terminus, was chosen as the next target for mutagenesis.

When the long C-terminal domains of CXCR1 and CXCR2 were exchanged, there was little effect on agonist signaling, consistent with previous reports in which the agonist binding location was attributed to segments of the receptor other than the C terminus (LaRosa et al., 1992; Gayle et al., 1993; Ahuja et al., 1996; Katancik et al., 2000; Rajagopalan and Rajarathnam, 2004; Andrews et al., 2008). In contrast, the chimera receptor constructs showed that exchanging the C termini clearly affected the ability of small-molecule antagonists to inhibit calcium responses. Although a decrease in potency can be attributed to trivial nonspecific effects, the increased compound antagonism demonstrated at the CXCR1–2long hybrid was compelling evidence to support involvement of the C-terminal region in compound-receptor interactions, as described for CCR4 and CCR5 (Andrews et al., 2008). We were surprised to find that when the short C-terminal regions containing the greatest sequence diversity between CXCR1 and CXCR2 were exchanged, there was little change in compound antagonism of the chimeric receptor constructs (Fig. 7, C and D). The striking difference in compound antagonism observed between the short and long amino acid C-terminal domains suggested that the intervening region (amino acid residues 302–327) plays a prominent role in describing compound interactions. Amino acid sequence differences between CXCR2 and CXCR1 in this region were limited to positions 304, 312, 320, and 325 (Fig. 2). Compound antagonism profiles analogous to wild-type CXCR2 and CXCR1–2long were generated in CXCR1 by introduction of the double point mutations CXCR1 N311K/F316L or the single point mutation CXCR1 N311K (Fig. 8).

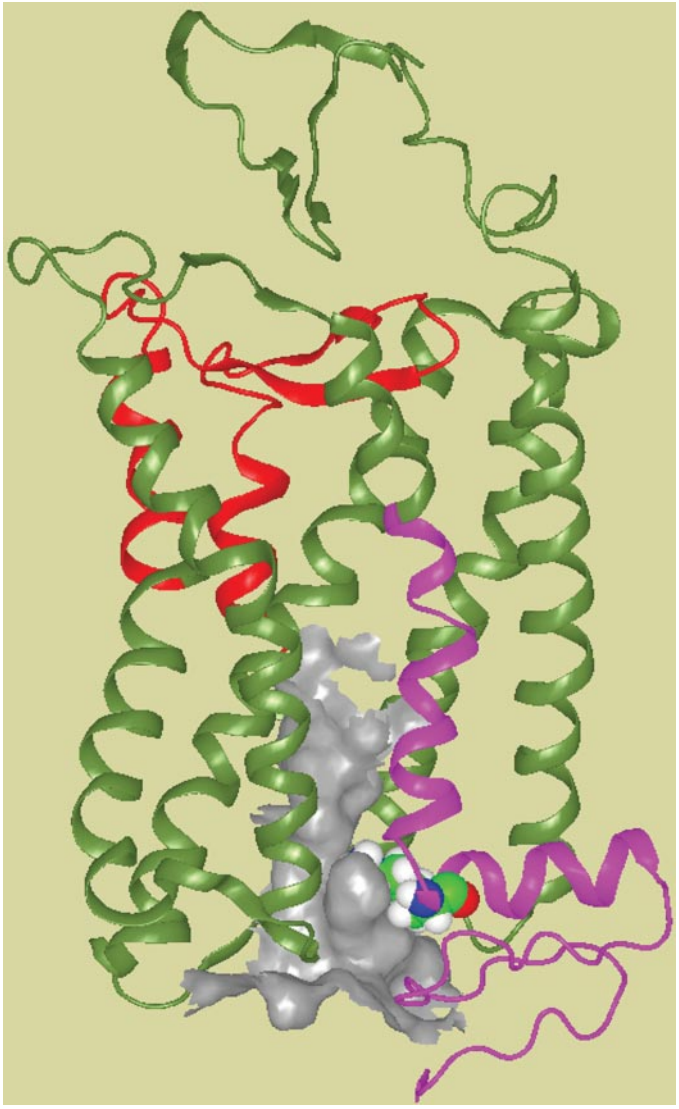


Fig. 10. Ribbon representation of the homology model of CXCR2. The ribbon is colored to show the second extracellular domain substitution (red) and the long C-terminal substitution (purple). The intracellular half of the interhelical cavity is surfaced in gray. Lysine 320 is shown in a space-filling representation.

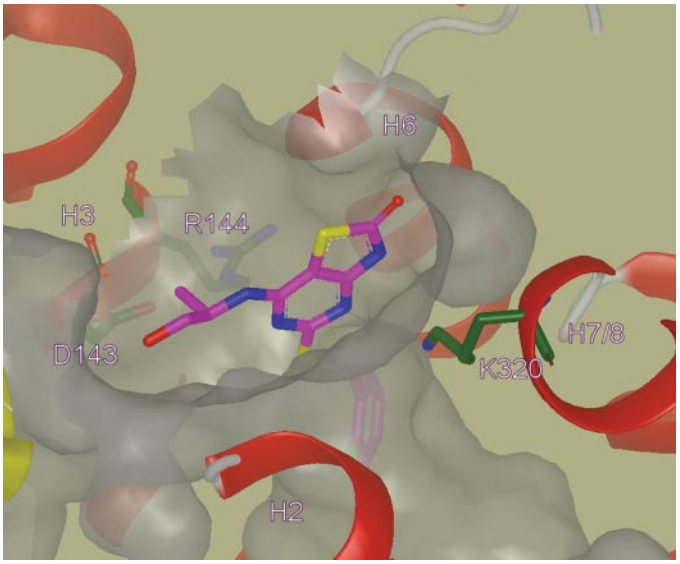


Fig. 11. Intracellular view of CXCR2 with compound A (magenta) docked. The interhelical cavity is surfaced in gray and helices—denoted H2, H3, H6, and H7/H8—are in red. The acidic heterocycle nitrogen of compound A is in contact with lysine 320 (green).

In contrast, the reciprocal double and single mutations introduced into the CXCR2 receptor, CXCR2 K320N/L325F and K320N (Fig. 8), diminished compound antagonism, which was similar to the profile observed with wild-type CXCR1 and the CXCR2–1long receptor construct. In addition, the single point mutation F316L was unable to confer compound antagonism in CXCR1. Taken together, these observations of mutually consistent reciprocal mutations suggest that the amino acid residue at position 320 (Fig. 2) plays a key role in describing compound antagonism differences between CXCR1 and CXCR2.

The contribution of the amino acid residue at position 320 was further investigated by determining the degree of inhibition of radiolabeled CXCL8 binding to membranes expressing recombinant receptors (Fig. 9). The mutation at position 320 did not produce a complete reciprocal switch in compound affinity between CXCR1 and CXCR2 (Table 1), suggesting that the residue at position 320 was not the sole factor in defining the relative compound affinity against these receptors. The increase in affinity afforded by the Lys311 mutation in CXCR1 was more pronounced for compound B (250-fold) than for compound A (60-fold), suggesting that residue 320 has a more profound effect on the relative affinity of compound B for CXCR1 and CXCR2. This may reflect differences in the structural and physicochemical nature of the acid isosteres.

The antagonist profile of compound A is consistent with competitive antagonism of the calcium response with a pA_2 value similar to the pIC_{50} value for the inhibition of radiolabeled CXCL8 binding to membranes (Fig. 4 and Table 1). In the absence of other information, it might seem that compound A competes directly with CXCL8 at the orthosteric binding site. However, the weaker antagonism of hydrophilic compounds in cells coupled with the mutational data suggest a compound binding site in CXCR2 and CXCR1 that is intracellular and therefore most likely to be allosteric rather than orthosteric to the chemokine binding site. Typically, allostery induces a conformation of the receptor that has either a very weak functional response or weak agonist binding. Antagonists that exert a strong negative allosteric effect can produce concentration-dependent shifts in agonist dose-response curves indistinguishable from competitive interactions, particularly at low concentrations (Ehlert, 1988). Thus, the observation that agonist concentration-response curves shifted in a parallel fashion with different concentrations of compound A (Fig. 4) is consistent with either an orthosteric or allosteric mode of action (Lazareno et al., 1998; Avlani et al., 2004).

Although it is possible that the agonist and antagonist binding sites overlap, it is apparent that amino acid changes in the intracellular portion of the receptor can independently modify the activity of antagonists without affecting agonist activity, suggesting that the chemokine does not extend as far as Lys320 into the receptor. This is consistent with the hypothesis presented here that the agonists and antagonists used exert their activity through independent sites. The hypothesis that an allosteric compound binding site at the C terminus can induce receptor conformation changes that are propagated to the agonist binding site is supported by studies on a related receptor, open reading frame-74 (Verzijl et al., 2006), in which perturbations in the region of helix 8 resulted in a reduction or loss of agonist binding.

The presence of a hydrophobic cavity in the homology model leading from the cytosol into the central TM region is highly suggestive of a binding mode in which the hydrophobic group of compound A binds within the lower half of the TM region and the acidic feature interacts directly with Lys320. The discovery here that the N311K mutant can confer CXCR2-like activity on CXCR1 and vice versa is supportive of this proposed mechanism of antagonism. Nevertheless, the existence of many basic residues in the intracellular region (including, among others, arginine 144 of the DRY motif on helix 3) introduces uncertainty, and the proposed binding mode is speculative in the absence of additional mutagenesis or structural data. This region of the protein has been shown previously to be critical in the function of GPCRs and alternative explanations for the mutagenesis data shown here may include indirect conformational effects as described previously (Verzijl et al., 2006; Li et al., 2007); however, the apparent requirement for access to the intracellular compartment alongside the mutagenesis data adds weight to the more straightforward explanation of the existence of a binding pocket in proximity to Lys320. Definitive location of the binding site by X-ray crystallography or NMR could provide the focus for future structural studies.

With muscarinic acetylcholine receptors, subtype selectivity has been achieved through ligand binding at an allosteric site (Lazareno et al., 1998). Binding to the intracellular face of the muscarinic M1 receptor has been proposed in a docking study of an allosteric modulator, KT5720 (Espinoza-Fonseca and Trujillo-Ferrara, 2006). Although no supporting experimental evidence was put forward, the study highlighted the same region proposed here as the binding site for compounds A and B.

The observation of a suitable binding site for a CXCR2 antagonist in a model based on the dark state of rhodopsin suggests that any compound binding to this site and stabilizing an inactive state of the receptor should act as an antagonist or inverse agonist. Because the surface of the proposed intracellular cavity comprises residues that are reasonably conserved throughout all GPCRs, it is tempting to speculate that other GPCRs can be modulated in this way. Evidence has been put forward recently for a common intracellular antagonist binding site in CCR4 and CCR5 (Andrews et al., 2008). It is also noteworthy that compound A used in the present study is an antagonist at the CCR2 receptor (Walters et al., 2008), which has a lysine residue at the equivalent position. The identification of this intracellular binding pocket should be of value in the design of new drugs, and our data for CXCR1 and CXCR2 suggest that subtle amino acid changes in this region markedly modify compound activity. Thus, even in closely related GPCRs, selective antagonism can be obtained through binding to a common intracellular site.

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