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What do structures tell us about chemokine receptor function and antagonism?

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Abstract

Chemokines and their cell surface G protein-coupled receptors are critical for cell migration not only in many fundamental biological processes but also in inflammatory diseases and cancer. Recent X-ray structures of two chemokines complexed with full-length receptors provide unprecedented insight into the atomic details of chemokine recognition and receptor activation, and computational modeling informed by new experiments leverages these insights to gain understanding of many more receptor:chemokine pairs. In parallel, chemokine receptor structures with small molecules reveal complicated and diverse structural foundations of small molecule antagonism and allostery, highlight inherent physicochemical challenges of receptor:chemokine interfaces, and suggest novel epitopes that can be exploited to overcome these challenges. The structures and models promote unique understanding of chemokine receptor biology, including the interpretation of two decades of experimental studies, and will undoubtedly assist future drug discovery endeavors.

Keywords

G protein-coupled receptor; crystallography; molecular modeling; receptor activation; allostery; druggability

Introduction

As master regulators of cell migration, chemokines and chemokine receptors are critical to fundamental biological processes including embryonic development early in life, immune surveillance, host defense and wound repair throughout life, and disease that comes with age (3, 41, 46). The receptors belong to the Class A family of G protein-coupled receptors (GPCRs) and are expressed on the migrating cells. The ligands (chemokines) are small proteins that are secreted by most cell types either constitutively or inducibly, in response to a wide variety of stimuli and environmental cues (110). By forming gradients on cell surfaces and extracellular matrices in tissues, chemokines serve as the directional signals for cell migration (82, 111).

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Because they control leukocyte migration, chemokines and chemokine receptors were initially recognized as fundamental mediators of inflammation, which motivated many drug discovery campaigns. In 1996, two chemokine receptors were identified as the coreceptors that facilitate HIV entry into cells (38) and in 2001, a link between chemokines and cancer metastasis was made (87). Today they have been identified as important mediators in an unusually large number of diseases, many anticipated based on their roles in cell migration and inflammation, and many unexpected like AIDS (120). By analogy with other members of the class A GPCR family, chemokine receptors were initially considered highly "druggable" targets, which inspired more than two decades of drug discovery efforts. However, progress has often been described as slow (101, 114), and only two small molecules (a CCR5 antagonist Maraviroc (31) and a CXCR4 antagonist Plerixafor (26)) against the chemokine receptor system are currently approved for use. Additionally, two antibody-based drugs, an anti-CCR4 monoclonal antibody Mogamulizumab (33) and an anti-CXCL8 antibody Abcream (145), are approved in Japan and China, respectively.

Factors contributing to clinical failures have been attributed to both deficiencies of the therapeutic candidates and the complexities of receptor:chemokine biology (54, 102, 114, 120). As for other GPCRs, important properties of drug candidates include target residence time for ensuring adequate receptor occupancy and receptor selectivity for minimizing off-target effects (22, 139). However chemokine receptors pose additional challenges that may be less problematic for other GPCRs. For example, their sequences, expression patterns, and function can differ markedly between species, which complicates the design and interpretation of animal studies (54, 120). Other factors include the apparent redundancy of the chemokine system (which provides a rationale for developing multi-targeted antagonists), and the limited understanding of the complexities of diseases where multiple chemokine receptors and cell types are involved (114). Additionally, the overall "druggability" of Class A GPCRs may not translate as easily to chemokine receptors since their endogenous ligands are proteins and the interaction interfaces are extensive (15, 103). Finally, the difficulties of competing with abundant levels of endogenous chemokines compared to e.g. hormone receptors, calls for the development of insurmountable or allosteric antagonists. Fortunately, the recent plethora of GPCR structures is providing new insights into ligand binding modes, binding locations, binding kinetics and associated ligand pharmacology, all of which can address the above issues and contribute to the design of more efficacious drugs (21). In this review we describe recent breakthroughs in understanding receptor interactions with chemokines and small molecules, and receptor activation mechanisms, all based on findings from structural biology and experiment-guided molecular modeling. We also discuss the implications of this information for the development of drugs targeting chemokine receptors.

Chemokines: structure and biology

In humans, there are approximately 45 chemokines (115) that have been classified into four families (CC, CXC, CX3C and XC) based on the pattern of cysteine residues (Fig. 1a,b). Unlike most GPCR ligands, chemokines are proteins, ~8–12 kDa in size. They have a highly conserved tertiary structure defined by an N-terminal unstructured domain that is critical for receptor activation, an irregular "N-loop", a 3-stranded β-sheet connected by loops (e.g. the

30s and 40s loops) and a C-terminal helix (Fig. 1a,b) (68, 69). The 22 chemokine receptors share the canonical topology of most GPCRs. They have seven transmembrane (TM) helices, three extracellular (EC) and three intracellular (IC) loops, an unstructured extracellular N-terminus (NT) and an intracellular C-terminus. Chemokine binding at the extracellular side of the receptor triggers a conformational change that transmits the signal towards the intracellular side where direct coupling to G proteins and β-arrestins activate cell migration pathways. Receptors are classified as CC, CXC, CX3C, or XC according to the subfamily of their preferred ligands (115).

Most chemokines dimerize or form higher order oligomers (69). The elongated structure of CC chemokine dimers is stabilized by interactions between the CC motifs and preceding N-terminal residues of the monomers (Fig. 1c) while dimerization of CXC chemokines is driven by interactions between the β_1 strands (Fig. 1d). Some chemokines form higher order oligomers (Fig. 1e) (74), many of which utilize both CC and CXC dimerization interfaces (72, 89).

Despite the propensity of chemokines to oligomerize, studies with constitutively monomeric chemokine variants have demonstrated that monomers are sufficient and in some cases required for receptor activation (58, 96, 105, 128). By contrast, oligomerization is required for high affinity binding of chemokines to their “other receptors”, the glycosaminoglycans (GAGs), which are found on cell surfaces and the extracellular matrix (34). These GAG interactions are important for immobilizing chemokines and facilitating the formation of haptotactic chemokine gradients that effectively provide a path for chemokine receptor bearing cells to follow (49, 82, 111, 116). Thus in the multistep process of cell migration, chemokine oligomers bound to GAGs must reversibly dissociate to fully engage receptors as monomers.

Structural basis of chemokine receptor interactions with chemokines

Early studies: the two-site model of receptor activation

Prior to the recent crystallographic studies of intact receptor:chemokine complexes, structural information was derived from mutagenesis experiments, structures of chemokines alone, and from NMR studies of chemokines bound to N-terminal peptides from the receptors (reviewed in (68, 69)). Mutagenesis studies revealed that chemokine N-termini largely control receptor activation: even subtle modification of the N-termini can alter ligand pharmacology by converting agonists into partial agonists, antagonists or even super agonists. By contrast, mutation of the globular “core domain” of chemokines usually produce effects on potency proportional to effects on receptor-binding affinity. Because of the disproportionate control of signaling by the two chemokine domains, a “two-site model” became the paradigm for the interaction (85, 97). In this model, the receptor N-terminus binds to the chemokine core (an interaction often referred to as chemokine recognition site 1, CRS1) while the chemokine N-terminus binds in the pocket of the receptor transmembrane helical domain (chemokine recognition site 2, CRS2) (Fig. 2a) (115). The affinity of the CRS1 interaction is enhanced by post-translational sulfation of tyrosine residues in the receptor N-termini (37, 77, 83, 106, 136, 137, 149, 150) which may regulate selective chemokine binding (149).

When considered in light of the two recent structures of chemokines complexed to intact receptors, the two-site model is clearly oversimplified (68). Nevertheless, it captured the essential roles of the chemokine domains and guided numerous efforts towards engineering chemokines with altered pharmacology, both for proof-of-concept studies of the efficacy of antagonizing specific receptors in various diseases (44, 50, 134), and as therapeutic candidates. One of the best examples of the latter is a series of N-terminally modified CCL5/RANTES variants that show a superior ability to inhibit HIV entry into cells compared to WT CCL5 (10, 39).

Architecture of receptor:chemokine complexes elucidated by crystallography and experimentally guided modeling

Apart from earlier structures of rhodopsin (98), breakthroughs in structural studies of GPCRs with intact transmembrane domains started with the 2007 structure of the β_2-adrenergic receptor (19, 109) and were followed by structures of other GPCRs including chemokine receptors CXCR4 and CCR5 (130, 142). In 2015, the first structure of a chemokine receptor in complex with a chemokine was solved, that of human CXCR4 in complex with a viral chemokine antagonist vMIP-II (103). Shortly thereafter the structure of the viral chemokine receptor, US28, was solved in complex with the human chemokine agonist CX3CL1 (15). Both the CXCR4:vMIP-II and US28:CX3CL1 structures demonstrate the CRS1/2 interactions of the two-site model; however, in both structures, density was missing for much of the receptor N-termini, including tyrosine residues known to be sulfated. The observed parts of receptor N-termini bound in pockets defined by the chemokine N- and 40s-loops (CRS1). Modeling of sulfotyrosines proximal to the observed N-termini of the receptors allowed their placement in favorable positions in the N-loop/40s-loop groove for interaction with basic residues of the chemokines (Fig. 2b,c). Although more structures will be needed for confirmation, it seems likely that the N-loop/40s-loop interaction with receptor N-termini will be a common CRS1 feature of most complexes. For example, the binding of a small inhibitor to this pocket in CXCL12 (118) validated it as a hotspot and closely paralleled the predicted interactions of the CXCR4 N-terminus in a model of the CXCR4:CXCL12 complex (103).

As expected, the N-termini of the chemokines were observed to bind in a large wide open polar pocket defined by the receptor 7TM bundle (CRS2) (Fig. 2b,c). In this pocket, they directly contact residues at key positions involved in signal initiation in many other GPCRs. Despite this trend, the features of CRS2 interactions appear to be receptor- and chemokine-specific, in line with the variation of the binding pockets for small molecule ligands in GPCRs (63).

A surprising observation in both receptor:chemokine structures was how extensive, in fact virtually contiguous, the interaction interface was, which prompted naming of an intermediate interaction site as CRS1.5 (103) (Fig. 2b,c). Located between CRS1 and CRS2, this region is centered on a conserved Pro-Cys motif of the receptor N-terminus that packs against the conserved disulfide of the chemokines. Interestingly, these interactions result in the formation of an antiparallel β-sheet similar to those observed in the respective chemokine dimer interfaces (Fig. 1c), and explain in part why CC chemokines cannot bind

receptors as dimers (58, 128). In contrast, the geometry of CXC chemokine dimers permits them to bind and activate receptors (103), although they tend to have lower affinity and altered pharmacology as compared to respective monomeric chemokines (32, 91, 105, 136). When the receptors from the CXCR4:vMIP-II and the US28:CX3CL1 structures are superimposed, the RMSD of the chemokines is as large as ~8.3 Å suggesting that CRS1.5 acts as a flexible pivot that allows chemokines to differentially engage extracellular loops (ECL2 and ECL3) of the receptor while maintaining the CRS1/2 interactions. This may be important for structural adaptability that enables promiscuous receptor:chemokine interactions, including binding of a single receptor to multiple ligands that share very low sequence homology.

Other experimental data and modeling studies suggest some unanticipated receptor:chemokine interaction epitopes (68). In addition to CRS1/1.5 interactions involving the N-terminus of the receptor proximal to the first conserved Cys, several reports suggest involvement of the distal N-terminus. Mutation or deletion of amino acids within the first 10 residues of the receptor have been shown to affect chemokine binding and/or receptor activation in the case of CXCR4:CXCL12 (12, 141) and CXCR2 with CXCL1, CXCL7 and CXCL8 (62). An NMR structure of CXCL12 in complex with an N-terminal peptide (residues 1–38) from CXCR4 suggests that the distal end pairs with the β_1-strand of CXCL12 (119), a region also implicated by cross-saturation NMR experiments (67). Mutagenesis and chemical shift perturbation studies of CXCR1:CXCL8 also support a role for the CXCL8 β_1-strand (59, 78), as well as a potential direct interaction of the chemokine β_1-strand with the receptor N-terminus (59). Finally, a recent radiolytic footprinting, disulfide crosslinking, and molecular modeling study of ACKR3:CXCL12 suggests interactions of the ACKR3 distal N-terminus and the CXCL12 β_1-strand (48). The constraints imposed by the TM domain of the receptor as well as disulfide crosslinks between ACKR3 and CXCL12, suggest that the interaction results in the formation of an anti-parallel β-sheet between receptor and chemokine (Fig. 2d, e) in contrast to the parallel β-sheet suggested by NMR studies (119). The compatibility of this antiparallel orientation with earlier models of CXCR4:CXCL12 (70, 103) also prompted the proposal of a similar extended model for CXCR4:CXCL12 (not shown). While structures of additional receptor:chemokine complexes will be required to obtain precise structural details, it is interesting to note that an antiparallel β-sheet would mimic the interface of CXC chemokine dimers (Fig. 2d), analogous to the CRS1.5 region of the CXCR4:vMIP-II and US28:CX3CL1 structures that mimic the dimer interfaces of CC and CX3C chemokines. Regardless of orientation, this interaction has sufficient support to deserve a name, which we refer to as CRS0.5, in line with the CRS1/1.5/2 nomenclature.

The extent and distributed nature of receptor:chemokine interfaces explain why mutation of individual receptor or chemokine residues, particularly involving CRS1, rarely has a dramatic impact on binding or signaling (141). Unless the mutated residue is an interaction hotspot (9), multiple mutations or truncations are typically required to produce a significant effect.

Repurposing of chemokine interfaces

Regions of chemokines corresponding to CRS1.5 and CRS0.5 represent interfaces that are "repurposed" for interactions with chemokine receptors versus other binding partners. These interfaces facilitate CC and CXC chemokine oligomerization, respectively, which is critical for their high affinity binding to GAGs. However, when chemokine oligomers dissociate, these oligomerization interfaces become available, and given their propensity to mediate protein:protein interactions (68), it is not surprising that they are repurposed for binding receptors and other proteins.

Chemokine dimer interfaces are not the only repurposed regions. Many chemokines, such as CCL2 and CXCL1 show significant overlap between residues important for receptor binding and those important for GAG binding, where both sets map to the N-loop/40s-loop groove (Fig. 2f). For both CCL2 and CXCL1, the groove features a number of basic residues while the receptor N-termini feature acidic residues and validated (CCR2) or predicted (CXCR1) sulfated tyrosines (51, 75, 117, 129). Similarly to receptor N-termini, GAGs are rich in acidic moieties and they must be sulfated to bind virtually all chemokines with high affinity (34). Interface repurposing contributes to the ability of some (especially CC) chemokines to bind both receptors and GAGs but in a mutually exclusive manner.

Interface repurposing is also prevalent in complexes of chemokines with chemokine binding proteins (CBPs) from viruses and ticks (23, 76, 143) that target and neutralize chemokines to suppress the immune response. For example, a dimer of the γ-herpesvirus CBP, M3, features a cleft where chemokines bind (1). In the case of an M3:CCL2 complex, the C-terminal domain of M3 interacts with the chemokine by positioning a proline against the conserved chemokine disulfide (Fig. 2h), similar to the interaction observed in chemokine:chemokine dimers Fig. 2g) and receptor:chemokine complexes (Fig. 2b,c). The M3 N-terminal domain binds to the N-loop/40s loop groove: like receptor N-termini or GAGs, this domain is highly acidic and complementary to the basic surface of the chemokine (not shown). Thus the M3:chemokine interaction mimics both receptor:chemokine and GAG:chemokine interactions, and as such, effectively blocks chemokine from its two main functions. Moreover, a comparison of a panel of CBPs from multiple viruses and ticks, showed universal targeting of the variable chemokine N-loop and the invariant disulfide (76) (Fig. 2i,j). The repurposing of these interfaces and motifs in multiple unrelated complexes suggests their role as fundamental determinants of chemokine interactions that can serve as reliable guides for future computational docking studies.

Insights into receptor activation from experiments interpreted in the context of structures and models

GPCRs exist in equilibria between multiple conformational states that can be defined as active or inactive based on their ability to couple to downstream effectors (G proteins or β-arrestins) (29, 80). Ligand binding shifts these equilibria towards one of the states, which effectively defines ligand pharmacology. The chemokine receptor structures solved thus far represent a wide range of conformational states: the CX3CL1-complexed US28 is in an active state, the maraviroc-bound CCR5 and all structures of CXCR4 are inactive, and a recent double antagonist bound CCR2 structure has the conformational signature of a deep

inactive state (15, 103, 147). However, these static structures do not reveal the intricate and dynamic details of the residue network that couples agonist binding at the extracellular side to the intracellular conformational changes. Recent mutagenesis and radiolytic footprinting efforts, in combination with structural models, provided important residue-specific clues to the mechanism in the context of CXCR4 and ACKR3, respectively (48, 141).

Intramolecular signal transmission network in CXCR4

Using high throughput shotgun mutagenesis covering every amino acid of CXCR4, Wescott and coworkers identified residue positions where mutations disrupted CXCL12-induced receptor signaling (141). The mutations may exert their effect through several mechanisms such as biasing the receptor conformational equilibrium, disrupting chemokine or G protein binding, or disrupting the intramolecular communication network. Interestingly, when mapped onto active and inactive state models of CXCR4:CXCL12 (103), about one half of the identified residues formed a continuous chain connecting CXCL12-binding residues in the receptor extracellular pocket to the intracellular G protein coupling residues. These residues were clustered into five functional layers and their roles tentatively assigned as (i) chemokine engagement, (ii) signal initiation, (iii) signal propagation, (iv) activation microswitches and (v) G protein binding (Fig. 3a–f) (141).

The chemokine engagement residues form a solvent-accessibile “ring” in the receptor CRS2 (Fig. 3a) and are predicted to capture the proximal N-terminus of the chemokine while directing the distal N-terminus towards signal initiator residues in the base of the binding pocket (Fig. 3b). The initiator residue E288$^{7.39}$ (superscript denotes Ballesteros-Weinstein numbering of residues in GPCRs (4)) engages the side chain of CXCL12 K1, which orients CXCL12 P2 for interaction with other initiator residues W94$^{2.60}$ and Y116$^{3.32}$ (Fig. 3c). K1 and P2 in CXCL12 are critical activation determinants, as deletion or mutation of K1 or substitution of P2 by glycine results in complete loss of agonist activity (24). The models suggest that K1 deletion or substitution lead to loss of contact with the engagement residue D97$^{2.63}$ or the initiator residue E288$^{7.39}$, respectively. The antagonism of the P2G mutant may be due to loss of steric contact with initiator residues W94$^{2.60}$ and Y116$^{3.32}$ (Fig. 3c).

Along with binding to the distal N-terminus of the chemokine, the initiator residues contact the “signal propagation” residues in the TM7 part of the receptor core (Fig. 3d). These, in turn, make contact with W252$^{6.48}$ of the CWxP rotamer motif and could thereby communicate structural shifts to helix VI, which is well known to undergo the largest conformational changes upon activation (36, 42, 64, 108). Within the signal propagation group, a sequential cluster of residues (V242$^{6.38}$-ILILA-F248$^{6.44}$) in the intracellular half of helix VI appears to act as a bridge connecting W252$^{6.48}$ to the key intracellular GPCR signaling motifs, including the DRY box and NPxxY and Y(x)$_5$KL microswitch motifs (64) (Fig. 3d). The results of mutagenesis suggests that the bridge region must be hydrophobic and strictly helical, while evaluation in the context of active and inactive state models demonstrates that the bridge undergoes significant repacking with respect to the conserved signaling microswitches. Therefore, its helical hydrophobic nature may enable it to serve as a “lubricant” for helix and side chain repacking during the conformational transition.

To transition from an inactive to active state, the conserved microswitch residues Y219$^{5.58}$ and Y302$^{7.53}$ (parts of the Y(x)$_5$KL and NPxxY motifs, respectively, and both identified by mutagenesis) undergo major repositioning that brings them in close proximity inside the TM bundle to support a G protein compatible interface (43) (Fig. 3e). Finally, R134$^{3.50}$ and L226 were identified as critical by the screen and likely represent G protein interaction hotspots as their coupling role is conserved across several GPCRs (8, 108) (Fig. 3f).

The interconnected chain of propagation, microswitch and G protein coupling residues is likely conserved across all class A GPCRs. This is in agreement with the fact that in multiple GPCRs, activation has been shown to involve uniform conformational changes of evolutionarily conserved structural elements (63, 138). By contrast, the engagement and initiator residues are likely to be most relevant to the chemokine receptor family (63), and even within this family, may be chemokine- and receptor-specific.

Activation of an atypical chemokine receptor

The findings on CXCR4 are especially interesting when compared to the activation mechanism of the atypical chemokine receptor ACKR3. ACKR3 shares its ligands CXCL11 and CXCL12 with CXCR3 and CXCR4, respectively (16) but, unlike these receptors, does not couple to G proteins and signals exclusively through β-arrestin mediated pathways (92, 104). Thus, ACKR3 provides a unique opportunity to determine the contribution of receptor structure to activation of one specific intracellular signaling pathway. A recent study of ACKR3 (48) collected over 100 structural restraints from radiolytic footprinting, disulfide trapping and mutagenesis, and utilized molecular modeling to produce experimentally driven models of ACKR3:ligand complexes (Fig. 3g). By comparing ACKR3 complexes with a small molecule partial agonist CCX777 and with chemokine full agonist CXCL12, radiolytic footprinting identified two classes of residues: those directly involved in chemokine binding, and those undergoing structural transitions in the course of ACKR3 activation. The conformational change upon full agonist binding led to increased exposure of intracellular residues, reconfiguration of the highly conserved W265$^{6.48}$ (of the CWxP rotamer motif) and increased solvent accessibility of the transmembrane region. A number of similarities are apparent between this study involving an atypical receptor and previous studies of GPCRs; e.g. the reorientation of W265$^{6.48}$ is consistent with a conformational transition, and the increased solvent accessibility of the intracellular region with opening of a binding cleft to accommodate intracellular effectors (60, 108). Furthermore, the results corroborate the hypothesis of activation being mediated by an extensive water-mediated intramolecular polar network in GPCRs (8, 55). Taken together, these results suggest that although ACKR3 exclusively activates β-arrestin dependent pathways, its active conformation is similar to that of canonical GPCRs.

On allostery of chemokine receptors

Upside-down and downside-up allostery

As detailed above, GPCRs are fundamentally allosteric machines, because their intracellular action (G protein coupling) is controlled by binding of ligands on the extracellular side (132) (Fig. 4a). For the most comprehensively studied receptors, it has been demonstrated that the

allosteric communication works both “upside down” (i.e. from the extracellular ligand binding site to the intracellular G protein-coupling interface) and “downside up”. For example, addition of an extracellular agonist to β_2AR results in conformational changes at the intracellular ends of receptor TM helices (45, 71, 107), and enhances the stability of receptor:G protein complexes (144) (“upside-down”). On the other hand, addition of either G protein or a G protein-mimicking nanobody Nb80 (124), both of which bind intracellularly, affects affinity and binding kinetics of extracellular agonists (30, 107) (“downside-up”). The bi-directional communication works not only in the context of receptor activation, but also in inactivation: inactive-state-specific intracellularly-binding nanobodies and antibodies reduce receptor affinity for extracellular agonists and increase affinity for inverse agonists (52, 123).

Obtained for β_2AR and A_{2A} adenosine receptor, these insights likely apply to most other GPCRs, including chemokine receptors. For CCR5 and CXCR4, high affinity chemokine agonist binding cannot be achieved without receptor coupling to a nucleotide-free G protein α-subunit (20, 94, 122) (Fig. 4a). On the other hand, for CCR2, intracellularly binding synthetic antagonists inhibit binding of the chemokine agonist CCL2 while increasing binding of extracellularly acting antagonists (151, 152). All of these observations are consistent with the idea of chemokine receptors being allosterically regulated “upside-down” and “downside-up” similar to β_2AR and A_{2A}AR (Fig. 4a).

“Orthosteric allostery” of chemokine receptor antagonists

Small molecule antagonists of chemokine receptors interfere with chemokine binding, G protein coupling, or both, and it is now clear that they can do so by binding intracellularly or extracellularly (Fig. 4b-e). Intracellular antagonists are by definition allosteric with respect to chemokine (Fig. 4c). However, even extracellularly binding small molecules that (presumably) spatially overlap with the bound chemokine can also demonstrate apparent allosteric behavior (Fig. 4d,e). Many small molecule antagonists of CCR5 (e.g. TAK-779, SCH-C (125), SCH-D/vicriviroc, or aplaviroc) inhibit chemokine (e.g. CCL3/MIP-1α and CCL4/MIP-1β) binding and chemokine-induced Ca^{2+} mobilization in an insurmountable manner (88, 140). By contrast, chemokine inhibition of antagonist binding is surmountable with increasing concentration of antagonist. For other chemokines (e.g. CCL5/RANTES), antagonists may allow residual binding (e.g. maraviroc and Sch-D/vicriviroc) or may not inhibit chemokine binding at all (aplaviroc) (140). A small molecule CCR1 antagonist, UCB-35625, has been shown to effectively disrupt receptor function with minimal effects on chemokine binding (112). An allosteric, non-competitive mechanism has also been demonstrated for DF1681Y/repertaxin/reparixin (5), a CXCR1/2 antagonist currently in clinical trials (ClinicalTrials.gov Identifiers: NCT01817959 and NCT01967888, NCT02370238).

Such complicated, probe-dependent inhibition mechanisms often lead to classification of small-molecule antagonists (including maraviroc for CCR5, UCB-35625 for CCR1, and DF1681Y/repertaxin/reparixin for CXCR1/2) as allosteric (40). Nevertheless, the X-ray structure of the CCR5:maraviroc complex (130) and subsequent structures of chemokine-bound complexes of homologous receptors (15, 103) suggest that the binding site of

maraviroc overlaps with CRS2, i.e. the TM domain pocket of the receptor that also accommodates the N-terminus of the chemokines (Fig. 5a,b). Similarly, mutational studies suggested that UCB-35625 binds in the orthosteric pocket of CCR1 (28) and that DF1681Y/repertaxin/reparixin bind in the orthosteric pocket of CXCR1/2 (6).

Based on these findings, the non-competitive behavior of such antagonists must have a different explanation than simply binding at an alternative, non-overlapping site of the receptor. One possibility is related to distributed nature of receptor:chemokine interfaces: receptors that mostly depend on CRS1 for chemokine binding can conceivably allow simultaneous binding of chemokine at CRS1 and small molecules at CRS2, resulting in the formation of ternary complexes (79) (Fig. 4d). This phenomenon has been recently described in great detail for heterobivalent ligands and is also sometimes referred to as “partial competition” (133). The large volume and flexibility of the receptor pocket (CRS2) may also make it permissive to ternary complex formation. Alternatively, these effects can be explained by the presence of a mixed population of receptors in cells: receptors that are coupled to G protein have different ligand preferences compared to uncoupled receptors (20), allowing for the apparent simultaneous binding of both types of ligands (Fig. 4e). Apart from a structural explanation, unusually slow dissociation kinetics of the chemokine probe or the test compound can translate into apparently insurmountable inhibition on the time scale of a typical experiment.

“Allosteric allostery” of chemokine receptor antagonists

By contrast with the apparently allosteric antagonists that bind in the extracellular, orthosteric pocket of the receptors, a number of chemokine receptor antagonists have been unambiguously demonstrated to bind elsewhere. For CCR4, antagonists of the pyrazinyl sulfonamide series require intracellular access for activity and lose efficacy when the intracellular part of helix VII and the C-terminus of CCR4 are replaced with homologous regions from CCR5 (2). For CXCR2, several compounds (Pteridone-1, Navarixin/Sch527123/MK-7123, and diarylurea SB265610) have been demonstrated to be dependent on intracellular residues via site-directed mutagenesis (113). For diarylurea SB265610, intracellular binding site mapping followed the discovery of its ability to inhibit binding of [^{125}I]-CXCL8, as well as functional response to multiple chemokines, in a non-competitive, insurmountable manner (11).

For CCR2, numerous chemotypes of “acidic” antagonists have been described (13, 14, 25, 66, 84, 99). The potency and efficacy of these antagonists are not affected by mutations in the orthosteric pocket (84), their presence does not interfere with the binding of canonical orthosteric antagonists (151, 152) but their ability to inhibit function is insurmountable (152). For several compounds, the binding site was directly mapped to an intracellular pocket contacting helices VI, VII, and VIII (151). Finally, a recent crystallographic study of CCR2 confirmed that one of these antagonists, CCR2-RA-[*R*] (25), does indeed bind intracellularly (147) (Fig. 4c, 5d).

The binding of small molecules to the intracellular side of CCR2 is possible because this receptor has a sufficiently large cavity between the intracellular ends of helices I, II, VI and VII. Crystallography suggests that CCR2-RA-[R] binds through a balanced combination of

nonpolar and polar interactions within the cavity, the latter through hydrogen bonding with the exposed backbone amides on CCR2 helix VIII. Both the volume and the hydrogen bonding capacity of this cavity depend on the glycine residue at position 8.47 (G309$^{8.47}$ in CCR2). The residues lining the cavity are characterized by moderate to high conservation within the chemokine receptor family, and G$^{8.47}$ is conserved in most of them. The fact that some of the intracellularly binding CCR2 antagonists have only moderately weaker potencies against CCR1 (99) and CCR5 (151) supports the existence of the homologous cavity in these receptors. The binding site of several CXCR1 and CXCR2 antagonists was also mapped to a homologous region through mutagenesis (93, 113) and competition binding studies (27). Moreover, an X-ray structure has recently been solved for CCR9 with an allosteric antagonist Vercirnon bound at a homologous site (153)

The numerous structural mechanisms for apparent allostery of chemokine receptor antagonists (Fig. 4c–e) have important implications for the design of receptor-targeting therapeutics, as does deciphering these mechanisms by means of structural biology and molecular modeling.

On druggability of chemokine receptors

As described in the introduction, given the prominent roles of chemokine receptors in disease and the extensive drug discovery efforts that have been deployed to develop drugs targeting the chemokine system, the success stories of approved therapeutics has been disproportionately low. The biological challenges in targeting these receptors have been described in many reviews as summarized in the introduction (54, 102, 114, 120). A challenge that has received less consideration is related to the nuances of the chemokine receptors as targets where recent structures have been particularly revealing.

Physicochemical challenges of receptor:chemokine interfaces

Even though chemokine receptors belong to the superfamily of GPCRs (targeted by a huge fraction of approved small molecule drugs), they have evolved to bind *proteins* such as chemokines (Fig. 5a). As for many other protein:protein interfaces, receptor interfaces with chemokines are extensive, comparatively flat, flexible, and excessively polar; they lack hydrophobicity and enclosure – the two features associated with the concept of *druggability* (21). As such, they are conceptually challenging targets for small molecules.

Only three orthosteric small molecule antagonists have been crystallized with chemokine receptors so far: a CXCR4 antagonist isothiourea IT1t (131, 142), the above-mentioned HIV entry CCR5 inhibitor Maraviroc (130), and BMS-681, a potent dual affinity CCR2/CCR5 antagonist (17, 147) (Fig. 5b-d). Owing to the conformational plasticity of the respective receptor pockets, each crystallized antagonist finds and utilizes a unique enclosed non-polar subpocket. Nevertheless, the high degree of solvent exposure for all three crystallized antagonists, as well as their scarce hydrophobic anchoring to the pocket surface (Fig. 5b-e), are in stark contrast with other GPCR antagonists, for example Naltrindole (an opioid receptor antagonist) and Aprenolol (a β_2AR antagonist) (Fig. 5f). It surely is not by chance that most disclosed chemokine receptor antagonist series consist of large, polar, flexible

molecules, which may negatively impact their oral bioavailability, metabolic stability, and other pharmacokinetic properties (100).

The ultimate PD/PK conflict in small molecule antagonists of chemokine receptors

To aggravate the challenges even further, several studies suggest that achieving therapeutic endpoints in inflammatory and autoimmune diseases requires that an unusually large fraction of the target receptor (90–95%) is occupied (and inhibited) at all times in the course of treatment (114). This imposes constraints on potency, residence time (139), selectivity, and toxicity parameters of chemokine receptor drug candidates that by far exceed typical ranges for other targets. In combination with the inherently poor druggability of the receptor:chemokine interfaces, it creates a conflict between pharmacodynamics (PD) and pharmacokinetics (PK) requirements and makes discovery and development of successful competitive small molecule chemokine receptor antagonists a daunting task.

Biologics and biomimetics

Because of the small molecule challenge, biologics and biomimetics have attracted attention as alternative chemotypes for inhibition of receptor:chemokine interactions. For example, in the case of CXCR4, a series of cyclized peptides originating from a horseshoe crab antimicrobial peptide polyphemusin-II (T22 and T140 series, (126, 127)) has been well-characterized (73, 86, 146). CVX15, a member of this series, was crystallized with CXCR4 in 2010 (142) demonstrating a much better fit with the binding pocket than can ever be achieved with a small molecule. Other biologic scaffolds used for antagonist development include engineered chemokines (10, 50, 121) as well as nanobodies and antibodies (47, 56, 57, 61, 81, 135). Mogamulizumab, a monoclonal antibody targeting CCR4, recently became the first biologic to be approved for cutaneous T-cell lymphoma (33). In a complementary effort, antibodies (7, 148) and therapeutic nucleotides (35, 53, 95) are pursued as agents targeting chemokines. With all of these agents, oral availability is out of question; however, various approaches to improving metabolic stability have been successful (18, 95). In combination with the ample potential for optimization of receptor inhibition properties, this suggests that biologics and biomimetics may become a promising next generation class of therapeutics targeting the chemokine receptor system.

Allosterics

Fortunately, competitive inhibition of receptor:chemokine interactions is not the only way to counteract receptor signaling. As described above, mechanisms of chemokine receptor activation suggest possibilities for allosteric regulation and indeed, numerous allosterically acting small molecules have been reported. The recent structure of CCR2 simultaneously bound to two antagonists (147) provided for the first time the opportunity to directly compare the physicochemical/druggability properties of the orthosteric pocket with those of an allosteric site in a chemokine receptor. The comparison is clearly in favor of the allosteric pocket! Unlike the orthosteric site, it is of a favorable size (not too large and not too small), well enclosed, and possesses a balanced combination of hydrophobic and polar features. The degree to which the co-crystallized allosteric antagonist CCR2-RA-[*R*] is buried from solvent (Fig. 5d,g) is more reminiscent of the close-to-ideal case of β_2AR antagonists (Fig. 5f) than the orthosteric antagonists of chemokine receptors (Fig. 5e). A similar trend is

observed with Vercirnon, the crystallized allosterically acting antagonist of CCR9 (not shown) (153)

Of course, an additional challenge posed by the allosteric inhibition approach is that intracellularly acting antagonists must be cell-permeable. Nevertheless, the favorable druggability properties of the allosteric pocket, and the fact that it appears to be present in other receptors, may open a new avenue for chemokine receptor inhibition. The structures of CCR2:BMS-681:CCR2-RA-[R] and CCR9:Vercirnon complexes (147, 153) will undoubtedly facilitate rational drug discovery efforts for these and possibly for other chemokine receptors, through ligand-informed homology modeling (65, 90).

Conclusions

Promising targets for inflammatory conditions, autoimmune diseases, and cancer, chemokine receptors have so far delivered only a small number of successful therapeutics. Drug discovery failures are caused in part by complex and poorly understood biology of receptors and chemokines, and in part by the inherently challenging nature of receptor:chemokine interactions as targets for small molecule development. Recent years brought several breakthroughs in understanding the structural basis of receptor interactions with chemokines and small molecules, as well as receptor activation, antagonism, and allosteric regulation. Structures and models may help address the major drug discovery hurdles and thus accelerate the discovery of drugs targeting the chemokine receptor axis.

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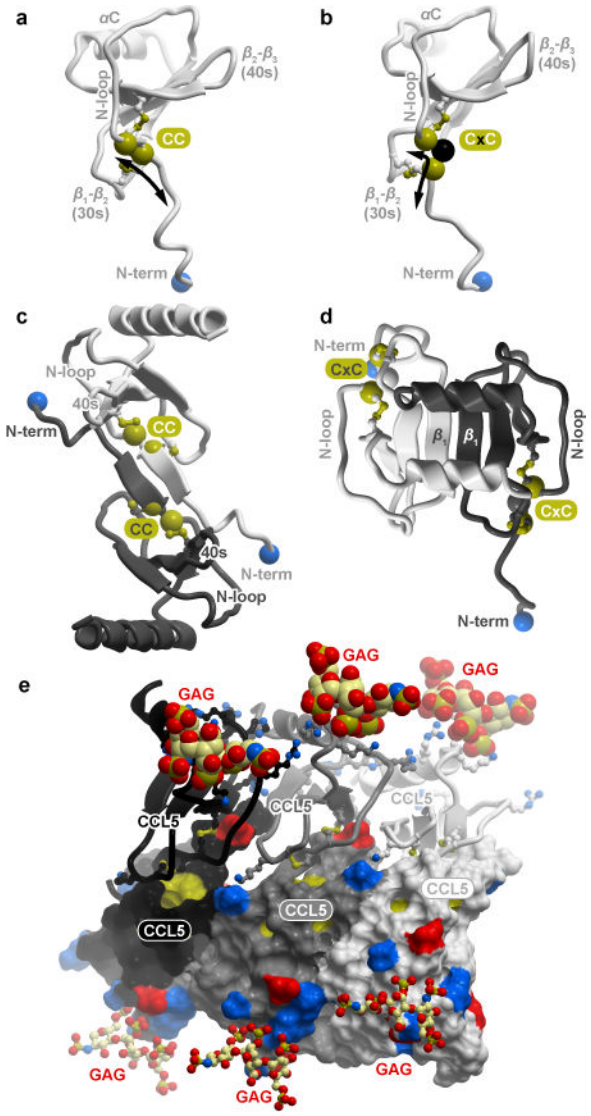

Figure 1. Topology and oligomerization behavior of chemokines

(*a–b*) Typical structures of a CC (*a*) and CXC (*b*) chemokines. (*c–d*) Typical dimerization geometry of CC (*c*) and CXC (*d*) chemokines. (*e*) CC chemokines bind GAGs through extensive positively charged interfaces formed through chemokine oligomerization. The oligomers are formed by polymerization of dimers that have a geometry consistent with (*c*).

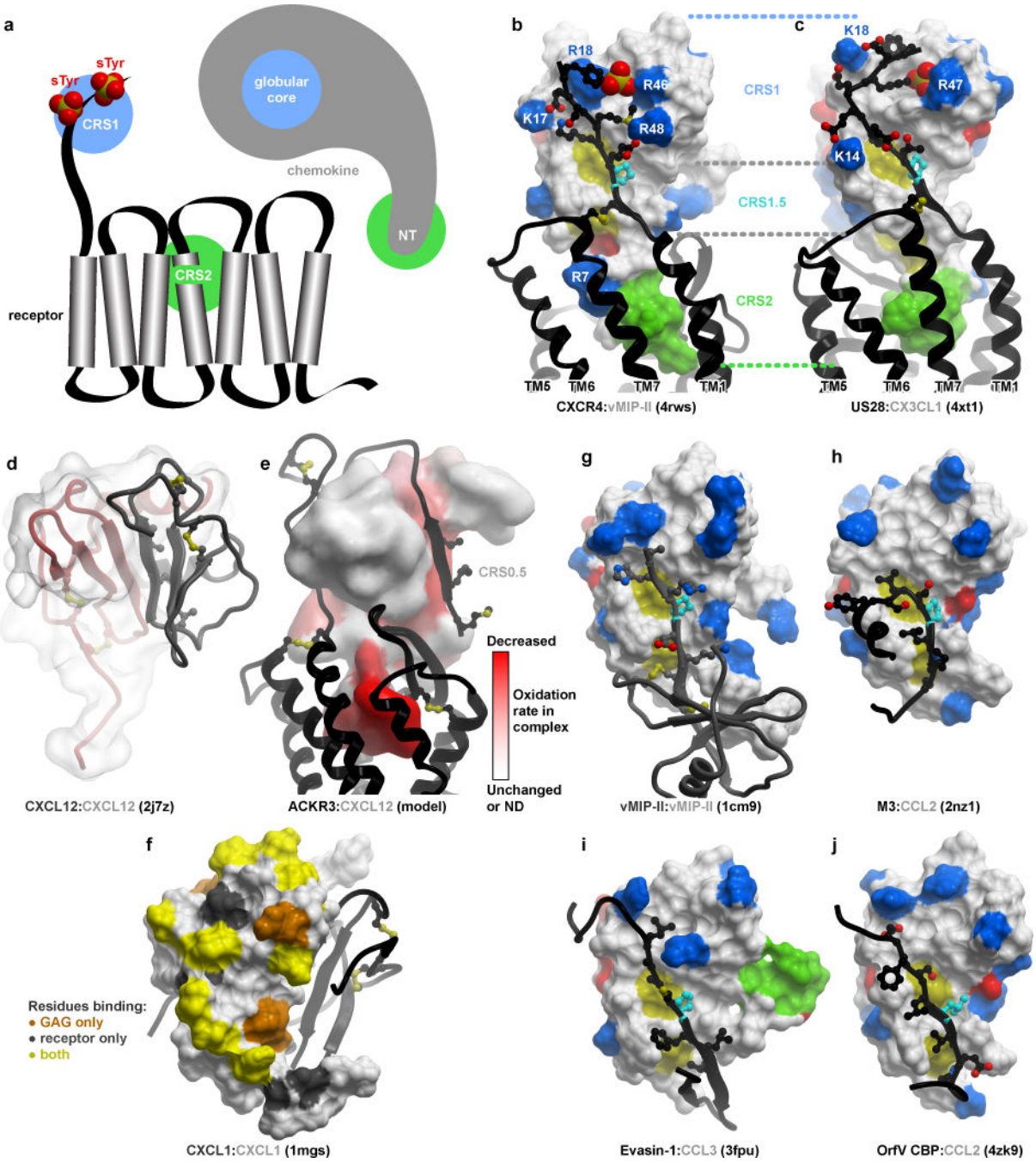

Figure 2. Chemokine interaction with receptors and other binding partners

(*a*) Canonical two-site model of receptor:chemokine recognition. (*b-c*) Structures of receptor:chemokines complexes solved in 2015 (CXCR4:vMIP-II and US28:CX3CL1) are consistent with the two-site model. Important basic residues on the chemokines are highlighted in blue. The chemokine N-terminus is highlighted in green. The Pro of the receptor Pro-Cys motif is colored cyan and packs up against the conserved disulfide of the chemokine (yellow surface). Proximal sulfotyrosine residues from the receptor N-termini were modeled and are shown with the sulfate colored red and yellow. (*d-e*) The CXC

chemokine dimerization geometry (*d*) is closely mimicked by the hypothetical interaction of distal receptor N-terminus with the β_1-strand of the chemokine in the modeled ACKR3:CXCL12 complex (*e*). The prediction is supported by the radiolytic footprinting data. (*f*) The binding interface of CXCL1 consisting of the proximal N-terminus of the chemokine and its N-loop/40s loop groove is shared by GAGs and the receptor. (*g-j*) CC chemokine dimers and pathogen chemokine-binding proteins share receptor binding interfaces and geometry.

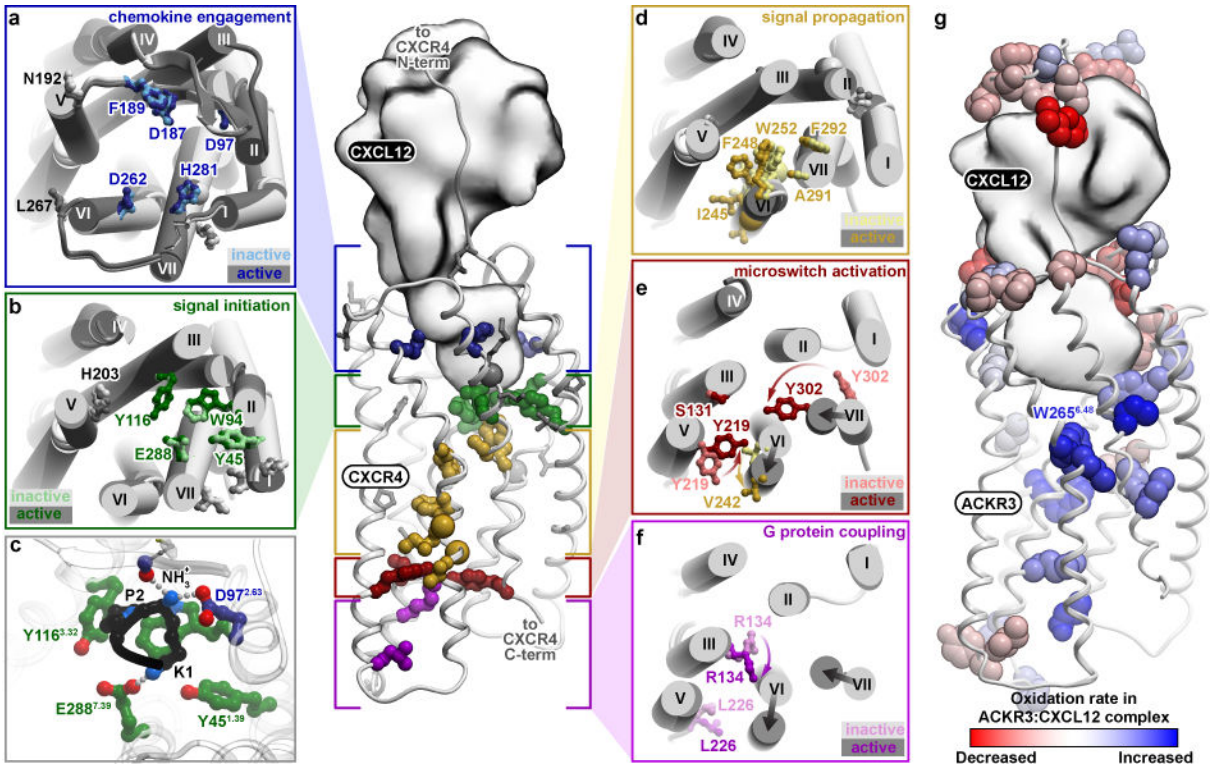

Figure 3. Insights into chemokine receptor activation

(*a-f*) Residues critical for CXCL12-induced activation of CXCR4 were identified and mapped onto an inactive and active state model of CXCR4:CXCL12 complex. (*a-b*) and (*d-f*) Five structural and functional layers are shown in the context of the full structure (center) and enlargements: (*a*) chemokine engagement (blue), (*b*) signal initiation (green), (*d*) signal propagation (yellow), (*e*) microswitch activation (red) and (*f*) G protein coupling (purple). Predicted residue conformations in the inactive and active state are shown in lighter and darker colors, respectively. (*c*) The proposed geometry of the interaction between the CXCL12 distal N-terminus (a critical signaling domain, shown in black) and chemokine engagement (blue)/signal initiation (green) residues of CXCR4. (*g*) Radiolytic footprinting mapping of residue solvent exposure in the atypical chemokine receptor ACKR3. Residues protected by the chemokine (as compared to a small-molecule bound state) are shown in shades of red: these residues are mostly located in the N-terminus and the extracellular loops of the receptor. On the contrary, numerous residues within the transmembrane domain become less protected in the chemokine-bound state; these residues report on increased solvent exposure of the TM core due to conformational changes upon chemokine-induced full activation of ACKR3.

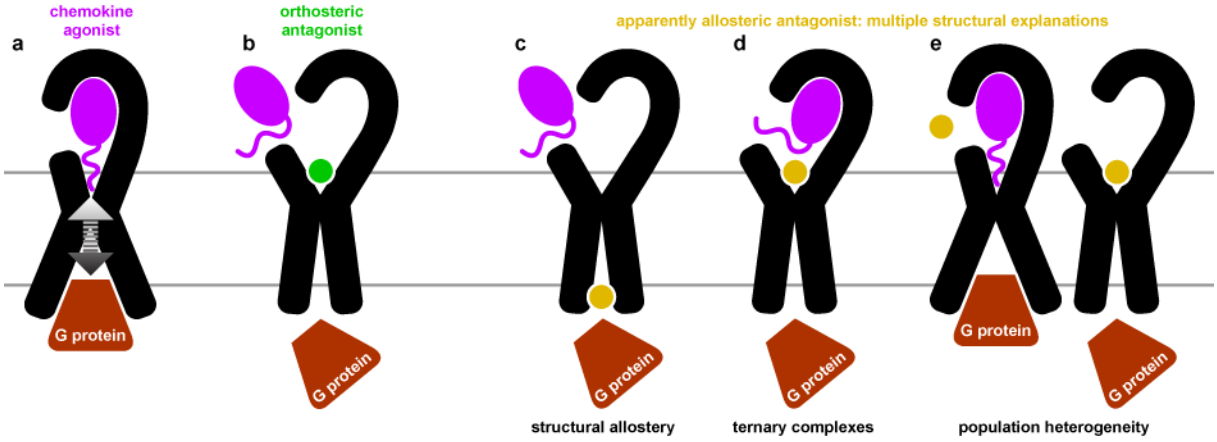

Figure 4. The allosteric nature of chemokine receptor activation and inhibition

(*a*) Extracellular chemokine binding translates into intracellular G protein coupling, and communication also works in reverse with G protein binding enhancing the affinity of chemokines. (*b*) Orthosteric antagonists directly block binding of chemokines in the CRS2 ligand binding pocket. (*c*-*e*) Apparently allosteric behavior of chemokine receptor antagonists can have multiple explanations including binding at a site distinct from the chemokine (*c*), occupying a part of the extensive chemokine interaction interface while allowing for a ternary complex formation (*d*), or binding to a distinct sub-population of receptors (*e*). The latter can happen in heterogeneous receptor populations where the G protein-coupled sub-population preferentially binds chemokine agonist while the uncoupled sub-population binds small molecule antagonists.

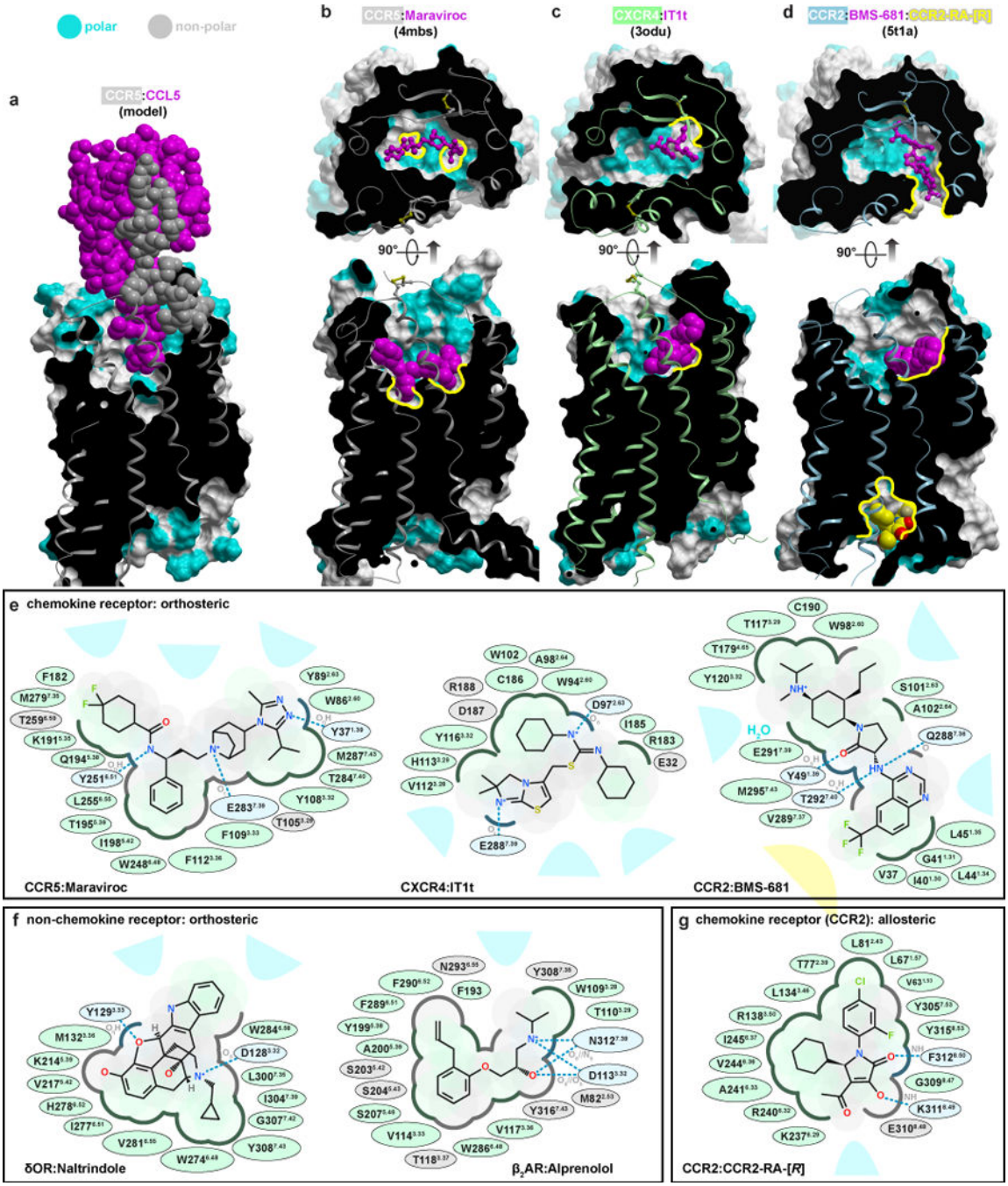

Figure 5. Structural basis of small molecule antagonism of chemokine receptors

(*a*) Receptors (grey) interact with chemokines (magenta) via extensive interfaces with numerous polar contacts (cyan); the interaction is additionally reinforced with the flexible N-terminus of the receptor (CRS1) essentially wrapping around the chemokine. Like most protein:protein interactions, chemokine:receptor interactions are conceptually difficult to inhibit with small molecules. (*b-d*) Structures of chemokine receptors with small molecule antagonists: (*b*) CCR5:maraviroc (*c*) CXCR4:IT1t, and (*d*) CCR2 in a ternary complex with BMS-681 and CCR2-RA-[*R*]. Each molecule explores a unique non-polar subpocket

(highlighted in yellow) within the overall large and polar (i.e. poorly druggable) binding pocket. (*e*) All three crystallized orthosteric small molecule antagonists of chemokine receptors demonstrate low degree of enclosure and high degree of solvent exposure. (*f*) This is in stark contrast with small molecule antagonists of non-chemokine GPCRs. (*g*) The crystallized allosteric antagonist of CCR2 binds in an intracellular pocket with favorable druggability properties and demonstrates a high degree of enclosure.