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Title page

Title

Chemokine receptor crystal structures: what can be learnt from them?

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Running title page

a) Running title

Lessons from chemokine receptor x-ray structures

b) Corresponding authors

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(Q)SAR, (quantitative) structure-activity relationships; ACKR, Atypical Chemokine Receptor; ADBR2, β2 adrenergic receptor; BRET, bioluminescence resonance energy transfer; CCL5, C-C motif chemokine ligand 5; CCR2, C-C chemokine receptor 2; CCR4, C-C chemokine receptor 4; CCR5, C-C chemokine receptor 5; CCR9, C-C chemokine receptor 9; CD4, cluster of differentiation 4; CRS, Chemokine Recognition Site; CXCL12, C-X-C

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chemokine ligand 12; CXCR4, C-X-C chemokine receptor 4; ECL, extracellular loop; FDA, Food and Drug Administration, FRET, fluorescence resonance energy transfer; gp160, glycoprotein 160; GPCR, G-protein coupled receptor; GPCRdb, GPCR database; ICL, intracellular loop; SBDD, structure-based drug design; SDM, site-directed mutagenesis; SNP, single nucleotide polymorphism; T4L, T4 lysozyme; TM, transmembrane; vMIP-II, viral macrophage inflammatory protein 2; VS, virtual screening

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Abstract

Chemokine receptors belong to the class A of G-protein coupled receptors (GPCRs) and are implicated in a wide variety of physiological functions, mostly related to the homeostasis of the immune system. Chemokine receptors are also involved in multiple pathological processes, including immune and autoimmune diseases, as well as cancer. Hence, several members of this GPCR subfamily are considered to be very relevant therapeutic targets. Since drug discovery efforts can be significantly reinforced by the availability of crystal structures, substantial efforts in the area of chemokine receptor structural biology could dramatically increase the outcome of drug discovery campaigns. This short review summarizes the available data on chemokine receptor crystal structures, discusses the numerous applications from chemokine receptor structures that can enhance the daily work of molecular pharmacologists, as well as the challenges and pitfalls to consider when relying on crystal structures for further research applications.

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Significance statement

This short review summarizes the available data on chemokine receptor crystal structures, discusses the numerous applications from chemokine receptor structures that can enhance the daily work of molecular pharmacologists, as well as the challenges and pitfalls to consider when relying on crystal structures for further research applications.

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Introduction

G protein-coupled receptors (GPCRs) are one of the biggest families of transmembrane proteins in the human genome (Lander et al., 2001). Their relevance is reflected by the large proportion of marketed drugs targeting GPCRs (Hauser et al., 2018; Hopkins and Groom, 2002) and the dramatic increase in the number of GPCR structures deposited in the Protein Data Bank in the last decade (Hauser et al., 2018; Katritch et al., 2013). To illustrate, 337 GPCR-ligand complexes have been solved to date, including 63 unique receptor proteins according to data from the GPCR database (GPCRdb) (Pandy-Szekeres et al., 2018). These GPCR structures have been obtained binding a wide variety of ligands and show a range of conformational GPCR states. This wave of structural knowledge is stimulating virtual screening (VS) and structure-based drug design (SBDD) approaches, as well as the elucidation of the molecular mechanisms of receptor activation and functional selectivity (Erlandson et al., 2018; Venkatakrishnan et al., 2016).

Chemokine receptors are a subfamily of class A GPCRs with a number of key physiological roles. These roles include a variety of developmental functions, the homeostasis of the immune system by controlling the homing of hematopoietic stem cells, and regulating the activation, differentiation, migration, and survival of leukocytes (Koenen et al., 2019; Scholten et al., 2012). Chemokine receptors are therefore key in inflammatory processes and are also involved in a number of immune and autoimmune diseases, including psoriasis, atherosclerosis, and allergies, amongst others (Murdoch and Finn, 2000), and are also considered to be important in cancer (Neves et al., 2019). Some chemokine receptors are also used by the human immunodeficiency virus (HIV) to enter into target cells (Scarlatti et al., 1997). Consequently, several chemokine receptors are very interesting drug targets (Kufareva et al., 2017). So far, three chemokine receptor-based therapeutics have been approved by the

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Food and Drug Administration (FDA) to date: plerixafor (Mozobil®) targeting C-X-C chemokine receptor 4 (CXCR4), indicated for stem cell mobilization in non-Hodgkin lymphoma and multiple myeloma (Sancho et al., 2016); maraviroc (Celsentri®) targeting C-C chemokine receptor 5 (CCR5), indicated for HIV-1 infection (Meanwell and Kadow, 2007); and the monoclonal antibody mogamulizumab (Poteligeo®) targeting C-C chemokine receptor 4 (CCR4), indicated for cutaneous T cell lymphoma (Ishii et al., 2010).

As mentioned, structural knowledge on the family of GPCRs has substantially increased over the past years, and both NMR and x-ray crystallography approaches have been successfully applied for solving chemokine receptor structures as well (Apel et al., 2019; Burg et al., 2015; Miles et al., 2018; Oswald et al., 2016; Park et al., 2012; Peng et al., 2018; Qin et al., 2015; Tan et al., 2013; Wu et al., 2010; Zheng et al., 2017; Zheng et al., 2016). This review will only focus on x-ray structures; for a recent overview of the application of NMR to GPCR structure elucidation the reader is referred to Shimda et al. (2019). To date, crystal structures of 5 different chemokine receptor complexes are available (Figure 1): CXCR4 binding the small molecule isotiourea-1t or IT1t (Wu et al., 2010), the peptide-like ligand CVX15 (Wu et al., 2010), and the chemokine-like viral macrophage inflammatory protein 2 or vMIP-II (Qin et al., 2015); CCR5 binding the small molecule FDA-approved drug maraviroc (Tan et al., 2013), the C-C motif chemokine ligand 5 (CCL5) truncation 5P7 (Zheng et al., 2017), and the small molecule antagonist compounds 21 and 34 (Peng et al., 2018); US28, a viral chemokine-like receptor, binding the human chemokine agonist C-X3-C chemokine ligand 1 (CX3CL1) (Burg et al., 2015), an engineered version of CX3CL1 with randomized N-terminus residues, namely CX3CL1.35 (Miles et al., 2018), and an intracellular nanobody 7, that keeps the receptor in an active-like state (Burg et al., 2015; Miles et al., 2018); C-C chemokine receptor 2 (CCR2), binding small molecule ligands in two different binding sites, BMS-681 and CCR2-RA-[R] (Zheng et al., 2016), as well as the small molecule antagonist

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MK-0812 (Apel et al., 2019); and C-C chemokine receptor 9 (CCR9) binding the intracellular small molecule vercirnon (Oswald et al., 2016). Additionally, a CCR5 electron-microscopy structure has been solved bound to the HIV envelope glycoprotein gp160 and the CD4 (cluster of differentiation 4) receptor (Shaik et al., 2019).

The potential of using SBDD methods is reflected by the large amount of ligands found for various chemokine receptors (Arimont et al., 2017; Das et al., 2015; Davies et al., 2009; Kellenberger et al., 2007; Liu et al., 2008; Mishra et al., 2016; Mysinger et al., 2012; Perez-Nueno et al., 2009; Schmidt et al., 2015; Vitale et al., 2013; Wang et al., 2014; Yoshikawa et al., 2013). Moreover, SBDD methods prove to be more effective when a crystal structure is available over the use of de novo techniques or homology models (Arimont et al., 2017; Mysinger et al., 2012). This illustrates one of the numerous advantages of relying on structural knowledge over the protein sequence only, and the necessity for new structures of other chemokine receptors. However, chemokine receptors, as any other GPCR and transmembrane proteins, are inherently challenging to crystallize (Kobilka, 2013; Piscitelli et al., 2015). The first prerequisite for experimentally solving a protein structure is obtaining large amounts of stable, purified, homogeneous protein (Piscitelli et al., 2015). Protein purification has proven challenging with highly dynamic GPCRs (Milic and Veprintsev, 2015). The multiple advances on the use of protein engineering, including mutagenesis, truncations, and chimeric constructs, as well as the use of stabilizing interaction partners, such as antibodies and nanobodies (Bobkov et al., 2019, submitted), have helped to overcome the challenges of GPCR instability (Ayoub et al., 2017; Manglik et al., 2017). To illustrate, CXCR4 and CCR2 have been crystallized fused to the T4 lysozyme (T4L) (Qin et al., 2015; Wu et al., 2010; Zheng et al., 2016), CCR5 and CCR2 fused to rubredoxin (Apel et al., 2019; Peng et al., 2018; Tan et al., 2013), CCR9 with seven thermostabilizing mutations (Oswald et

al., 2016), and US28 binding the intracellular nanobody 7 (Burg et al., 2015; Miles et al., 2018).

In this review, we will summarize the available data on chemokine receptor structures, discuss the numerous applications of chemokine receptor structures for drug discovery, as well as the challenges and pitfalls that one can encounter when relying on crystal structures for research applications.

Chemokine receptor structures

To date, 5 of 28 chemokine receptor family members have been crystallized (Figure 1). Despite the low representation of co-crystallized chemokine GPCRs, the structures available offer rather representative information for this GPCR subfamily: co-crystallized ligands of different chemical nature, binding in different binding sites, and receptors in different conformational states have been crystallized. Additionally, a complex of CCR5-CD4-gp160 of HIV-1 has very recently been solved by cryo-electron microscopy (Shaik et al., 2019). Chemokine-bound crystal structures include the CXCR4-vMIP-II (Qin et al., 2015), US28-CX3CL1 (Burg et al., 2015; Miles et al., 2018), and CCR5-5P7-CCL5 (Zheng et al., 2017) and support the two-steps/two-sides mechanism of chemokine binding (Kufareva et al., 2014). The sequences of the co-crystallized chemokines can be found in Figure 2. According to the two-steps/two-sides model, the globular core of the chemokine binds first to the extracellular surface of the receptor (Chemokine Recognition Site 1, or CRS1), which allows for a subsequent interaction between the N-terminus of the chemokine and the orthosteric binding site within the 7TM domain (Chemokine Recognition Site 2, or CRS2) (Scholten et al., 2012). This model has been recently expanded in accordance to the most recent crystal structure of a chemokine-bound receptor, the CCR5-5P7-CCL5 complex (Zheng et al., 2017), and to extensive experimental evaluation of the contributions of amino acid residues in both C-C chemokine receptor 1 (CCR1) and its ligands to affinity and receptor activation (Sanchez

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et al., 2019). The CCR5-5P7-CCL5 complex shows a third site, that authors annotate as Chemokine Recognition Site 1.5, where the conserved receptor motif P19-C20 is packed against the chemokine disulphide bond (Zheng et al., 2017). This third site would act as a pivot point between CRS1 and CRS2, allowing a specific arrangement of interactions in between receptor and ligand (Zheng et al., 2017). Experimental evaluation of CCR1 and its ligands has shown that CRS1 and CRS2 contribute to ligand binding but that full receptor activation cannot be explained solely by high affinity ligand binding. This observation has led to the proposal of a third step for the model, now called three-step model, where a conformational change of the receptor-ligand complex that would results in receptor activation (Sanchez et al., 2019).

Despite the overall conserved geometry and stoichiometry of chemokine binding, the specific interactions that occur in CRS2 are chemokine-receptor specific, as illustrated by the reported chemokine-bound x-ray structures (Burg et al., 2015; Miles et al., 2018; Qin et al., 2015; Zheng et al., 2017). Despite the knowledge on the conserved mechanism of binding and the specific pattern of interactions between the chemokine and its receptors, the redundancy and functional selectivity of the chemokine system is still poorly understood. Multiple chemokines are able to bind the same GPCR, whereas some GPCRs are able to bind multiple chemokines (Scholten et al., 2012). Chemokine receptors are also able to bind small molecule and peptide ligands (Figure 2). The CVX15-bound CXCR4 structure shows the ability of peptide mimetics to mimic the binding of the large chemokines, interacting with multiple chemokine binding residues or hotspots (Arimont et al., 2017; Wu et al., 2010) (Figure 3A). Small molecules occupy a space in the binding site that can (partially) overlap with the chemokine binding site, as has been reported for IT1t (Wu et al., 2010), maraviroc (Tan et al., 2013), compounds 21 and 34 (Peng et al., 2018), BMS-681 (Zheng et al., 2016), and MK-0812 (Apel et al., 2019) (Figure 1, Figure 2, Figure 3A). The (partial) overlap explains the

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different levels of chemokine displacement that is observed in experiments using different small molecule (potentially allosteric) modulators (Adlere et al., 2019). Moreover, the orthosteric binding site is divided into a minor subpocket and a major subpocket (Figure 3A). Ligands can bind to either pocket exclusively (IT1t, minor pocket, CVX15, major pocket, Figure 1 in CXCR4, green and magenta, respectively) (Wu et al., 2010) or simultaneously (maraviroc and compounds 21 and 34, Figure 1 in CCR5, green) (Tan et al., 2013). BMS-681and MK-0812 have been crystallized protruding from the TM domain towards the membrane interface (Apel et al., 2019; Zheng et al., 2016). Intriguingly, the CCR2 and CCR9 crystal structures show that small molecule modulators CCR2-RA-[R] (Zheng et al., 2016) and vercirnon (Oswald et al., 2016) are also able to bind at the intracellular side of chemokine receptors, showing the targetability of an intracellular pocket (Figure 1, Figure 2, Figure 3C).

Most chemokine receptor crystal structures are co-crystallized with antagonists and resemble an inactive conformational state. However, the human cytomegalovirus-encoded viral receptor US28 is bound to a human chemokine CX3CL1 and a stabilizing nanobody Nb7, and resembles an active state (Burg et al., 2015; Miles et al., 2018). Yet, the rearrangements do not completely resemble the fully active conformation observed in the G-protein-bound β_2 adrenergic receptor (Rasmussen et al., 2011) (Figure 1, Figure 3D).

Applications of chemokine receptor crystal structures for molecular pharmacologists

The information that can be extracted from crystal structures goes far beyond the ligand interaction or protein structural analysis and is often neglected. In this section we review multiple aspects of chemokine receptor crystal structures that can be useful for molecular pharmacologists. We will focus on the distinct aspects of chemokine receptor structures that can guide the understanding of the mechanistic details of GPCR function. Moreover, we will

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provide insights on how these structures can be used for the design of tools and experiments to explore different pharmacological aspects of chemokine receptors.

Structural determinants of ligand binding

Understanding the mechanism of binding of small molecule ligands and chemokine mimics is key to rationally modulate their pharmacology (Arimont et al., 2017). Crystal structures are key to reveal interactions of ligand-receptor complexes (Figure 4, yellow), which can subsequently be used to: i) generate ligand analogues with improved interactions with key residues; ii) drive SBDD efforts, or iii) to design site-directed mutagenesis experiments and elucidate the interactions that contribute most to the free energy of binding. Medicinal chemistry efforts are often focused on modifying ligands to generate structure-activity-relationships, exploring the different chemical features of the ligand that enhance the desired activity parameters (e.g. affinity, potency, selectivity, kinetics). However, this exercise requires significant synthetic efforts, as identifying these key features implies designing modifications in all possible chemical substituents of the ligands. These efforts are exponentially bigger as the ligand complexity increases, and chemokine receptor ligands are often rather complex: from big and flexible small molecules, to peptides and chemokines. The challenges of such a ligand-based approach can often be overcome by combining it with structure-based methods. Analysis of key interactions in crystal structures offers a rational for designing ligand modifications. To illustrate, a recent study focused on the IT1t-bound CXCR4 crystal structure as a baseline to identify new fragment hits, and analysed the crystal structure to rationally design a fragment growing strategy (Adlere et al., 2019). In this study, the identification of a highly hydrophobic hotspot in the binding site of the co-crystallised ligand was used to generate analogues with increased lipophilicity to target the hotspot. Furthermore, experimentally-validated ligand binding modes provided by crystal structures can be used for the rational design of ligand modifications. Such modifications include the

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attachment of radioactive or fluorescent probes (Iliopoulos-Tsoutsouvas et al., 2018), which are key tools for a wide variety of molecular pharmacology experiments, from affinity determination (ligand displacement), kinetics profiling, to in vitro and in vivo imaging. To illustrate, the IT1t-bound CXCR4 crystal structure was successfully used to design fluorescent probes based on the chemical structure of IT1t and analogues (Dekkers et al., manuscript in preparation).

SBDD requires detailed knowledge on the ligand-receptor interactions. Molecular docking predicts the conformation and interactions of the ligand in the binding site based on their shape and physicochemical properties. However, despite the rather successful application of ligand docking in SBDD for some GPCRs, relatively small conformational differences in protein binding sites (Coudrat et al., 2017a) and the availability of experimentally validated structural information, including site-directed mutagenesis (SDM) or ligand quantitative structure-activity relationships (QSAR), can be critical for the success of virtual screening (Yoshikawa et al., 2013). The use of GPCR crystal structures has long proven to enhance hit identification and lead optimization (Coudrat et al., 2017a; Coudrat et al., 2017b; Kooistra et al., 2014; Kooistra et al., 2015; Kuhne et al., 2016; Lee et al., 2018). In the case of chemokine receptors, multiple crystal structure-based virtual screening campaigns have led to the discovery of new chemokine receptor ligands (Arimont et al., 2017). Hence, the use of crystal structures is key for the discovery of new tool compounds and potential drug candidates targeting chemokine receptors. A major contribution from chemokine receptor crystal structures to the field of SBDD in GPCRs was the revelation of a novel druggable pocket in the intracellular side of the receptor (Oswald et al., 2016; Zheng et al., 2016), exposed to the cytosol, which partially overlaps with the binding sites for both G-proteins (Rasmussen et al., 2011) and β-arrestins (Kang et al., 2015; Zhou et al., 2017). This allosteric binding site was explored before in chemokine receptors (de Kruijf et al., 2011; Zweemer et al., 2014), but the

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ligand-bound crystal structures now clearly reveal a conserved pharmacophore that opens new doors to SBDD for chemokine receptors (Figure 3C).

Crystal structures can also be used to guide SDM experiments of a receptor to identify the key residues that drive the ligand pharmacology of interest. To illustrate, multiple models of peptide ligand binding (e.g. T140 (Trent et al., 2003) and FC131 (Vabeno et al., 2006)) were proposed before the release of the CVX15-bound CXCR4 structure. However, the release of the crystal structure revealed a different binding mode from the previously suggested. New peptide-bound CXCR4 models have been proposed after the release of the crystal structure that consider the observed interaction pattern in the x-ray structure, and it has been used to guide SDM experiments and validate the proposed new models (Thiele et al., 2014). The structural insights gained from this experimental validation allowed rational design strategies towards the discovery of new CXCR4 ligands (Di Maro et al., 2017; Vabeno et al., 2015), and even to translate this information to other chemokine receptor ligands (Oishi et al., 2015).

Structural determinants of protein function

All GPCRs share a common seven-transmembrane (TM) domain topology, despite the low sequence identity between class A subfamilies (~20-25%) (Katritch et al., 2013) and the different classes A-F (<20%) (Fredriksson et al., 2003). Furthermore, GPCRs possess highly-conserved sequence motifs that have been shown to contribute to their structural integrity and folding, including a highly conserved disulphide bridge between TM3 and the extracellular loop (ECL) 2 or ECL2, which upon mutation induces protein misfolding (Rader et al., 2004). This disulphide bridge is also present in chemokine receptors, as well as an additional disulphide bridge between the N-terminus and ECL3 uniquely conserved amongst chemokine receptors (Szpakowska et al., 2014) (Figure 3B). Disruption of this covalent interaction through mutagenesis destabilizes the tertiary structure of CXCR4, and as a consequence also potentially decreases ligand-binding affinity (Zhou and Tai, 2000). The crystal structures of

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chemokine receptors show the impact of the N-terminus-ECL3 disulphide bridge on the protein conformation and, specifically, on the orthosteric ligand binding site between the 7TM domains (Figure 3B): TM7 is two alpha-helical turns longer than in other GPCRs, and the N-terminus is repositioned towards TM7 (Arimont et al., 2017). The effects of these structural motifs must therefore be considered when designing chemokine or peptide ligands, small molecule fluorescent probes, and even protein constructs such as N-terminal probes. Another characteristic structural motif in chemokine receptors with a big impact regarding ligand binding and mutant design is the so-called S/T$^{2.56}$xP$^{2.58}$ motif in TM2. Mutation of residue Thr$^{2.56}$ in CCR5 has a significant impact on binding affinity and functional response of CCR5 to CCL5 (Govaerts et al., 2001). The available crystal structures of chemokine receptors show that this motif induces a unique helical kink in TM2 that places the residues 2.60 and 2.63 toward the ligand-binding site instead of toward the membrane interface as in other GPCRs (Arimont et al., 2017). Consequently, it is necessary to introduce two gaps in sequence alignments of chemokine receptors in order to translate this structural effect into the sequence space (Gonzalez et al., 2012).

Moreover, GPCRs share a conserved mechanism of signal transmission and activation (Venkatakrishnan et al., 2013), even though they bind a wide variety of ligands of different chemical natures. Identification of this conserved mechanism has been possible through the study of the multiple crystal structures released not only in the inactive state, but also in the active and even intermediate states (Venkatakrishnan et al., 2016). This mechanism seems to extend to chemokine receptors as well, as proven by the conserved active-like conformation in the CX3CL1-bound US28 structures (Burg et al., 2015; Miles et al., 2018). The most notorious structural changes associated to the active state include a significant outward shift of the intracellular half of TM6, accompanied by an inward movement of the bottom of TM7, and a subtler lateral displacement of the bottom of TM5 (Figure 3D).

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GPCRs are intrinsically allosteric by nature, as signal transduction involves changes occurring at spatially distinct protein sites (Thal et al., 2018). Many sequence motifs conserved throughout class A GPCRs are long known to be key for GPCR signalling (Figure 4, cyan, Figure 5), including e.g. the DRY motif (Rovati et al., 2007), the NPxxY motif (Audet and Bouvier, 2012), the sodium binding site (Katritch et al., 2014), the PIF motif or transmission switch (Venkatakrishnan et al., 2013), and the CWxP motif (Kobilka and Deupi, 2007). A study reporting on point mutations at all residues of CXCR4, proved that at least one of the key residues in each of the aforementioned structural motifs, upon mutation, significantly decreased CXCR4-mediated signalling (Wescott et al., 2016). However, only the analysis and comparison of inactive and active crystal structures has elucidated the molecular mechanism that triggers the aforementioned effects on signalling (Venkatakrishnan et al., 2016). With crystal structures at hand, the environment of these conserved motifs has been identified, supporting the rational design of specific mutant points to disrupt or enhance their structural or pharmacological effect. By these means, the role of the motifs has been thoroughly studied in CXCR4, where mutation in a residue on any of these motifs, with the exception of the sodium pocket, significantly impairs G-protein signalling without significantly affecting CXCL12 binding (Wescott et al., 2016) (Figure 4).

Rational evaluation of chemokine receptor orthologue variants and polymorphisms

The basis of most molecular pharmacology projects ultimately includes the evaluation of the effect of drugs or other chemical entities on a human protein target, often expressed in human-derived cell lines. Yet, for proper translating of the pharmacological effects of a drug candidate from *in vitro* to *in vivo* settings, knowledge on the effect of drug candidates on a number of animal orthologues is crucial, as protein sequence divergence between species can be substantial (Figure 4 – orange). Despite the high similarity between orthologues of

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chemokine receptors (90-100% within mammals (data retrieved from GPCRdb, (Pandy-Szekeres et al., 2018))), there are receptor regions with lower conservation, especially in the loops. To illustrate, the mouse CXCR4 sequence presents a 5 amino acid insertion in ECL2, proven to be key for the binding of multiple ligands (Arimont et al., 2017). Crystal structures are useful to map sequence differences and to predict, based on their location, if the orthologue variant may affect the ligand binding and signalling. By means of *in silico* methods, crystal structures can be used to predict the effect of such orthologue variants, for example by a combination of homology modelling (if needed) and molecular docking. Differences exist not only in between species, but within the human population (Figure 4, blue). Natural genetic variations can cause differences in the individual response to drugs, including therapeutic efficacy and safety. To illustrate, 8 of the 9 residues in the binding site of maraviroc in CCR5 are polymorphic, which suggests that HIV patients that carry one of these variants may show an altered response to the antiretroviral drug (Hauser et al., 2018). These naturally occurring genetic variations may therefore represent a challenge to the health care system, and may be taken into consideration in early stage drug development. This is key in the field of chemokine receptors as illustrated by the wide distribution of single nucleotide polymorphisms or SNPs that have been reported for these GPCRs, covering residues from the binding site, to key signalling structural motifs (Supplementary Table 1). To illustrate, GPCRdb reports ~2500 SNPs for chemokine receptors, from which ~43% corresponds to the C-C chemokine motif subfamily, 23,5% to the C-X-C chemokine motif subfamily, and ~17,7% to the Atypical Chemokine Receptor (ACKR) subfamily. Within these subfamilies, the receptors with most reported SNPs are CCR5 (175), C-X-C chemokine receptor 1 (160), and atypical chemokine receptor 2 (165), respectively. The average amount of SNPs observed per each transmembrane domain within the entire chemokine receptor family is rather conserved, with an average of 2 SNPs reported per residue (maximum TM5 =

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2.21 SNPs/residue, minimum TM3 = 1.6 SNPs/residue) (Hauser et al., 2018; Pandy-Szekeres et al., 2018). Also, in this context, crystal structures can assist to map these genetic variations in order to predict potential pharmacological effects. Additionally, crystal structures can guide drug design to prevent a negative impact of these polymorphisms, for example, avoiding key ligand interactions with the polymorphic amino acid when possible.

Analysis of oligomerization interfaces

Many GPCRs, including chemokine receptors (Figure 4, green) (Stephens and Handel, 2013), are known to form functional oligomers in the cell membrane (Bulenger et al., 2005; Cottet et al., 2012),. Chemokine receptor oligomers cause negative cooperativity in the binding of their chemokine ligands and, in the case of some receptors, oligomerization can either enhance or inhibit receptor activation through allosteric communication (Armando et al., 2014; Percherancier et al., 2005; Stephens and Handel, 2013). The current methodologies that are able to probe dimerization, are mainly based on fluorescence and bioluminescence resonance energy transfer approaches (FRET and BRET) (Fumagalli et al., 2019; Goddard and Watts, 2012; Heuninck et al., 2019; Percherancier et al., 2005), fluorescence fluctuation spectroscopy (Briddon et al., 2018; Isbilir et al., 2017), and spatial intensity distribution analysis (Pediani et al., 2018). Such biophysical approaches are very sensitive to detect oligomers, but are not able to identify the oligomerization interface between protomers, which is key for the molecular understanding of the quaternary GPCR structures. Multiple chemokine receptors have been crystallised in an oligomeric state, including the IT1t-bound CXCR4 structures (Wu et al., 2010), the maraviroc-bound CCR5 structure (Tan et al., 2013), the compound 21-bound CCR5 structure (Peng et al., 2018), and the vercirnon-bound CCR9 structure (Oswald et al., 2016). Although the biological relevance of GPCR oligomers cannot

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be deduced from crystal structures and the oligomeric states could originate from crystallographic artefacts, they provide a relevant basis for the design of validation experiments. The most commonly observed dimerization interface derived from GPCR crystal structures is the one involving TM5-TM6, also observed in the IT1t-bound CXCR4 structures (Ferre et al., 2014). This interface was suggested by use of synthetic peptides mimicking the sequence of the transmembrane domains (Baneres and Parello, 2003; Hebert et al., 1996) and providing evidence that GPCR dimers may be biologically relevant. Another common method to probe oligomerization interfaces is the use of site-directed mutagenesis experiments to disrupt the interactions needed for the formation of the complex. In this case, the use of crystal structures to guide the experimental design has been key. Formation of oligomers requires interactions between the membrane-oriented faces of transmembrane domains, which, by nature, are highly hydrophobic (Deber and Li, 1995; Li and Deber, 1994). The hydrophobic nature of the oligomeric interfaces suggests that the stabilization of the complexes may require significant hydrophobic packing. Consequently, the impact of a single mutation along the interface may not be enough to disrupt oligomer formation, but multiple mutations may be required. Alternatively, identification of the few key polar contacts (if present) only found in the top or bottom of the helices is key (Ferre et al., 2014). Crystal structures have provided a relevant guide towards the rational design of such mutations in strategic positions. This strategy has proven effective to map the CCR5 dimeric interface, where disulphide cross-linking experiments based on the dimeric interfaces observed in the CXCR4 and μ opioid receptor crystal structures (Jin et al., 2018). Another technique that relies on the knowledge of interaction interfaces is disulphide crosslinking or disulphide trapping. Disulphide trapping is an experimental approach in which, the introduction of two cysteine residues in different protein partners at strategical positions of the interface, generates (irreversible) covalently bound protein complexes

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(Kufareva et al., 2016). Consequently, disulphide trapping allows protein complexes to be stabilised in specific conformations preventing spontaneous dissociation, hence facilitating structural studies. To illustrate, crystallising chemokine receptors in complex with endogenous chemokines has often proven very challenging potentially due to lower affinity of chemokines for detergent-solubilized receptors yielding lower stability complexes, and/or the high selectivity of chemokines for specific conformational states of their receptors in order to bind with high affinity (e.g. active state) (Kufareva et al., 2016; Rasmussen et al., 2011). Such high affinity conformational states (e.g. an active state) are so far quite challenging to achieve under crystallisation conditions and have so far only succeeded for US28 with the aid of a stabilizing nanobody (Burg et al., 2015; Miles et al., 2018). Despite these challenges, the use of disulphide trapping has yielded the crystallisation of CXCR4 in complex with the viral chemokine v-MIP-II (Qin et al., 2015). Disulphide crosslinking also facilitates the evaluation of the proximity between residues, providing insights into complex interactions when combined with molecular modelling. Using these methods, the interaction geometry of ACKR3 and its endogenous ligand C-X-C chemokine ligand 12 or CXCL12 has been mapped, and proven to be consistent with the hypothesis of two sites of chemokine binding (Gustavsson et al., 2017).

Challenges and pitfalls

Despite the great value of GPCR crystal structures, there are some challenges and pitfalls to be considered when interpreting them. X-ray structures as visualized are molecular models built to fit an electron density map as good as possible. This fit is done by means of advanced computational methods, but sometimes assumptions (e.g. about missing data) are necessary (Piscitelli et al., 2015). One of the most used quality metrics is resolution, measured in angstroms, which reports the highest angle reflections recorded in the x-ray diffraction

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pattern. Resolution is a measure of the data quality that is used to build the models, and not a measure of the model quality itself (Piscitelli et al., 2015). The lower the value for the resolution, the better the quality of the data is. The resolution of GPCR crystal structures ranges from 1.7 up to 7.7Å, with an average of 2.89Å at the day of writing. Structures with a resolution lower than 2Å are considered of very high quality, and at this resolution it is possible to accurately position water molecules and ions in the protein structures. The resolution of the solved chemokine receptor crystal structures ranges from 2.2Å in the 5P7-CCL5-bound CCR5 structure, to 3.8Å in one of the US28 structures (PDB ID 4XT3), with an average of 3.03Å. Despite the comparable average resolution values, no high-quality structures have been reported for any chemokine receptor so far and experimental finetuning is needed to improve the quality of chemokine receptor structures. A summary of the current chemokine receptor structure quality metrics can be found in Supplementary Table 2.

Next to differences in the overall resolution of protein structures, the quality of the electron density of a GPCR structure can significantly vary between the transmembrane domains, the solvent-exposed loops, and ligand binding sites. The N- and C-terminal, and sometimes the intra- and extracellular loops, are often truncated or shortened for optimized protein crystallization and/or are not solved due to their flexibility. So often only a few residues of such protein regions can be modelled (Piscitelli et al., 2015). In the case of chemokine receptors, the N-terminus is key for binding chemokines, but only 1 (CCR2, PDB ID: 6GPX) to 10 (US28, PDB ID: 4XT1) residues can be modelled in the solved crystal structures (Figure 6, dark blue). For e.g. CCR2 (PDB ID: 5T1A) and CCR9, no residues of the N-terminus have been solved, limiting the value of the structure for e.g. designing probes or engineering ligands that interact with the N-terminus (e.g. chemokines, antibodies, nanobodies) (Figure 6, cyan).

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Other receptor areas that are often not solved are the intra- and extracellular loops (Figure 6, green). When considering ECLs, ECL2 is usually the longest and most flexible loop in GPCRs, and therefore its resolution is often lower. However, the ECL2 can exhibit different secondary structural elements, including alpha-helical or beta-sheet elements (Woolley and Conner, 2017). In chemokine receptors, the ECL2 loop has an organised secondary structure of two antiparallel β-sheets (Arimont et al., 2017) and is solved in all structures, except CCR9. In the CCR9 x-ray only ECL3 is resolved, and a residual signal is observed for ECL2 C187$^{45.50}$, involved in the highly conserved disulphide bridge with TM3 (Arimont et al., 2017). In the US28 structures, ECL1 is not fully solved (PDB IDs 4XT1 and 4XT3), and in one CCR2 structure (PDB ID: 6GPS), ECL3 is not fully solved. Regarding intracellular loops (ICLs), all structures fused to stabilising proteins, including T4 lysozyme and rubredoxin, lack ICL3, as the fusions were engineered in ICL3. Furthermore, the CVX15-bound and vMIP-II-bound CXCR4 structures also lack ICL1, whereas a CCR2 structure misses ICL2 (PDB ID: 6GPX).

A challenging aspect when analysing electron density maps is the interpretation of ligand densities (Figure 6, orange). As mentioned, the average resolution of the solved GPCR structures is around 3Å, which allows the visualization of electron density basic contours of amino acid side chains and ligands (Piscitelli et al., 2015). To illustrate, the symmetric thiourea group of IT1t is solved in the crystal structure in a tautomeric state in which the nitrogen N4 forms a salt bridge with Asp97$^{2.63}$ (Wu et al., 2010). However, the electron density does not exclude the existence of a very similar conformation with the thiourea group flipped, in which the nitrogen N3 forms the salt bridge with Asp97$^{2.63}$. These ambiguities in the interpretation of ligand electron density should be considered when designing new ligands, side-directed mutagenesis experiments, or attachment points for fluorescent probes or bivalent ligands.

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The role of water molecules in GPCRs is of great interest as waters play a critical role in the binding of drugs and the function of many proteins (Ball, 2008; Chaplin, 2006; Mason et al., 2012) (Figure 6, yellow). Information extracted from crystal structures is key to identify the biological functions of conserved structural waters and to exploit them in drug discovery. For example, modulating the presence of waters in a binding site by means of ligand scaffold modifications has proven successful to modulate ligand affinity (Mason et al., 2013) or predict ligand kinetics (Bortolato et al., 2013). On a structural level, water molecules are postulated to contribute to the functional plasticity needed for the GPCR conformational changes related with signal transmission and activation (Angel et al., 2009a; Angel et al., 2009b). Importantly, structures with resolutions >2.5 Å do not provide clear electron density maps for waters and hence these can often not be modelled. This explains the scarce amount of water molecules in chemokine receptor crystal structures. Additionally, in order to consider a water molecule as conserved and structurally relevant, it has to be visible in a number of crystal structures, and crystallised chemokine receptor-ligand complexes are so far mostly unique. The receptor-ligand complexes crystallised multiple times include CXCR4-IT1t, but only the PDB ID: 3ODU structure contains water molecules, and US28-CX3CL1, for which only the PDB ID: 4XT1 contains water molecules. Therefore, at this stage no conclusions on relevant water-mediated interactions can be drawn based only on chemokine receptor crystal structures and the role of water molecules in chemokine receptor function will have to be further explored experimentally.

Ions, and specifically monovalent cations, are long known to be key for GPCR structure and function (Childers et al., 1979; Vickery et al., 2018). The most representative example is the Na^+ ion, which in the inactive GPCR state binds in a pocket surrounding the most conserved aspartic acid in TM2 ($Asp^{2.50}$), in the centre of the TM domain (Liu et al., 2012). This sodium ion is coordinated directly by interactions with $Asp^{2.50}$ and $Ser^{3.39}$, and indirectly through a

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water network by Trp$^{6.48}$, Asn$^{7.49}$, Asn$^{7.45}$, and Ser$^{7.46}$. These coordinating residues are highly conserved throughout the class A of GPCRs, and numerous high-resolution crystal structures of different class A GPCRs evidence the presence of a sodium ion in this conserved site. Therefore, sodium binding in this pocket is assumed to be a conserved feature shared by the majority of class A GPCRs (Katritch et al., 2014; Vickery et al., 2018). Multiple studies reveal that mutation of the residues in the sodium binding pocket modulates agonist binding, signalling, and biased signalling (Fenalti et al., 2014; Liu et al., 2012; Massink et al., 2015). It has been proposed that, driven by electrochemical gradients, the sodium ion can traverse the receptor to the cytosol, coupled to the protonation of Asp$^{2.50}$, and that this is a key step in GPCR activation (Vickery et al., 2018; White et al., 2018). No chemokine receptor crystal structure has been solved with a sodium ion in the sodium binding pocket, which may be explained by the lack of high-resolution structures. However, it is important to consider that the sodium binding site is not fully conserved in chemokine receptors according to the GPCRdb sequence alignments (Pandy-Szekeres et al., 2018): Asp$^{2.50}$, Trp$^{6.48}$, and Asn$^{7.49}$ are highly conserved, but residue 3.39 is a glycine in 48% of chemokine receptors, residue 7.45 is a histidine in 74%, and residue 7.46 is a cysteine in 74%. Therefore, the role of the sodium ion in chemokine receptors has to be addressed experimentally and a study with mutagenesis of these residues in CXCR4 has failed to severely affect agonist mediated calcium flux (Wescott et al., 2016).

Often other co-crystallised ions, and especially cations, are solved in GPCR structures, including chemokine receptor structures. To illustrate, clear electron density of a metal ion is observed in the CCR2 structure (PDB ID 5T1A), which was identified as zinc through X-ray fluorescence scans. This zinc is positioned between the bottom of TM3, ICL3, and the T4L. However, the biological relevance of such ions has to be further studied, as divalent cations such as zinc or magnesium are common components of the buffers used for crystallisation

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and can induce to artefacts in the crystal environment (Piscitelli et al., 2015; Salom et al., 2013).

Concluding remarks

Members of the family of chemokine receptors are important therapeutic targets due to their involvement in numerous inflammatory and immune diseases, as well as in cancer. Despite the lack of crystal structure for the majority of chemokine receptors (only 5 out of 28 GPCRs of this subfamily have been crystallised to date), the available structures highlight several relevant characteristics of chemokine receptors and their structure: the ability to bind ligands of different chemical natures in different druggable binding sites, and the ability of the GPCRs to adopt multiple conformational states. Analysis of the crystal structures of chemokine receptors provide detailed understanding of chemokine receptor ligand binding and insights in the molecular mechanisms that drive receptor function. Moreover, it allows the rational design of tools and experiments for a wide variety of applications. These applications may include the design of site directed mutagenesis experiments for the identification of key determinants of receptor pharmacology, receptor oligomerization interfaces, or the functional effect of conserved structural motifs, orthologue variants and polymorphisms. However, despite the great impact of GPCR x-ray structures, there are some pitfalls to be considered as well. The quality of the electron density maps used to model receptor structures is a key determinant to confidently interpret them and to understand e.g. highly dynamic regions like loops, ambiguous ligand moieties and side chain rotamers, as well as conserved water molecules and structurally relevant ions. Awareness of the strengths and pitfalls of crystal structure analysis is crucial in order to fully exploit the value of GPCR x-ray structures by medicinal and computational chemists and molecular pharmacologists.

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Prospectively, acquisition of new chemokine receptor crystal structures would boost the field of chemokine receptor drug discovery. Chemokine receptor structures that have not been solved yet would be of great added value to drive drug discovery campaigns, but especially interesting would be the resolution of the structure of an atypical chemokine receptor, which could shed some light on the still puzzling biased signalling of these chemokine receptors. New ligand-bound complexes, including different modulator types such as small molecules, peptides, or biologicals, would deepen the understanding of the different binding patterns and mechanism of action of the different ligand types, potentially enhancing the design of new ligands and tools. Furthermore, future small molecule agonist-bound structures may allow for the identification of activation-driving interactions that could be targeted in order to achieve a pharmacological effect of interest. Similarly, solving a G-protein-bound complex or an β-arrestin-bound chemokine receptor complex would enhance the analysis of the geometry and interaction patterns of a fully active receptor. The field of cryo-EM has made a great progress on the resolution of big protein complexes including multiple GPCR:G-protein complexes. In the field of chemokine receptors, the cryo-EM structure of CCR5 bound to gp160 has provided very valuable insights on the binding mechanism of HIV (Shaik et al., 2019). However, a G-protein-bound chemokine receptor structure is still missing. Understanding the mechanism of binding of G-proteins or β-arrestins to chemokine receptors would provide valuable information that could potentially drive the exploration of new mechanisms of modulation of these intracellular partners, including for example the design of modulators targeting the intracellular side of the receptors.

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Footnotes

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Figure legends

Figure 1 – Overview of chemokines and chemokine receptor structures. All co-crystallized chemokine receptors are shown in gray cartoons and depicted according to their phylogenetic relationship within the chemokine receptors subfamily. The crystallized receptors include CCR5 (PDB IDs: 4MBS, 5UIW, 6AKX, 6AKY), CCR2 (PDB IDs: 5T1A, 6GPS, 6GPX), CCR9 (PDB ID: 5LWE), US28 (PDB IDs: 4XT1, 4XT3, 5WB1, 5WB2), and CXCR4 (PDB IDs: 3ODU, 3OE0, 3OE6, 3OE8, 3OE9, 4RWS). Ligands are colored based on chemical nature: small molecules (green), peptides (magenta), chemokines (cyan), nanobody (purple). Examples of soluble chemokine structures are shown in the central panel including: CCL5 co-crystallized in complex with a heparin-derived disaccharide (PDB ID: 5DNF) (Liang et al., 2016), CXCL12 dimer co-crystallized with a small molecule modulator (PDB ID: 4UAI) (Smith et al., 2014), and a C-X-C chemokine ligand 11 NMR model (PDB ID: 1RJT) (Booth et al., 2004). For a more complete overview of standalone chemokine structures the reader is referred to (Kufareva et al., 2017) and (Ziarek et al., 2017). The phylogenetic tree is based on the structure-based alignment in GPCRdb (Pandy-Szekeres et al., 2018) and calculated in JalView (Waterhouse et al., 2009) (average distance using % identity).

Figure 2 – Overview of co-crystallized chemokine receptor ligands, including small molecules, peptide, and chemokines.

Figure 3 – Details of unique features observed in chemokine receptor structures. A) CVX15 (magenta) and IT1t-bound (green) CXCR4 representation (in grey cartoon). CVX15 binds exclusively to the so-called major pocket between TMs 3-7, able to mimic to a greater extent the binding of a chemokine, while IT1t binds exclusively to the so-called minor pocket

between TMs 1-3 and 7. B) Comparison of the positioning of TM1 in chemokine receptors (CXCR4, pale yellow cartoon) and other class A GPCRs (exemplified by ADBR2, in grey cartoon). A disulfide bridge between the chemokine receptors N-terminus and the top of TM7 (2 helix turns longer than in ADBR2) positions TM1 towards TM7 and the binding site. C)The intracellular binding site of chemokine receptors (represented by CCR2, in grey cartoon) illustrates a conserved pharmacophore between intracellular ligand binders, where a key double backbone hydrogen bond occurs between residues 8.49, 8.50, and the ligands. D) Common structural rearrangements upon receptor activation in GPCRs. An outward movement of TM6 and an inward movement of TM7 allow for the accommodation of the stabilizing nanobody (purple spheres).

Figure 4 – Overview of potential applications of chemokine receptor crystal structures. Chemokine receptor crystal structures may allow the pharmacologist to: map orthologue variants that might potentially affect the pharmacological profile of ligands (orange), visualize and interpret clinically relevant single nucleotide polymorphisms (dark blue), understand the key features of ligand binding (yellow), critically analyze the potential dimerization interfaces of receptors (green), and understand the molecular mechanisms of receptor function based on key structural motifs (cyan).

Figure 5 - Key sequence motifs conserved throughout class A GPCRs known to be key for GPCR signalling, including the DRY motif (Rovati et al., 2007) (blue), the NPxxY motif (Audet and Bouvier, 2012) (orange), the PIF motif or transmission switch (Venkatakrishnan et al., 2013) (green), the CWXP motif (Kobilka and Deupi, 2007) (red), and the sodium binding site (Katritch et al., 2014) (purple, sodium ion is shown as a reference, but it has not been crystallised in any available chemokine receptor crystal structure). Residues mutated in

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CXCR4 that significantly reduce its signalling are highlighted in red boxes (Wescott et al., 2016).

Figure 6 –Electron density challenges and pitfalls for crystal structures of chemokine receptors. ED maps of: chemokine binding (cyan), receptors N-terminus (dark blue), co-crystallized ligands (orange), intracellular loops (green), and water molecules and ions (yellow).

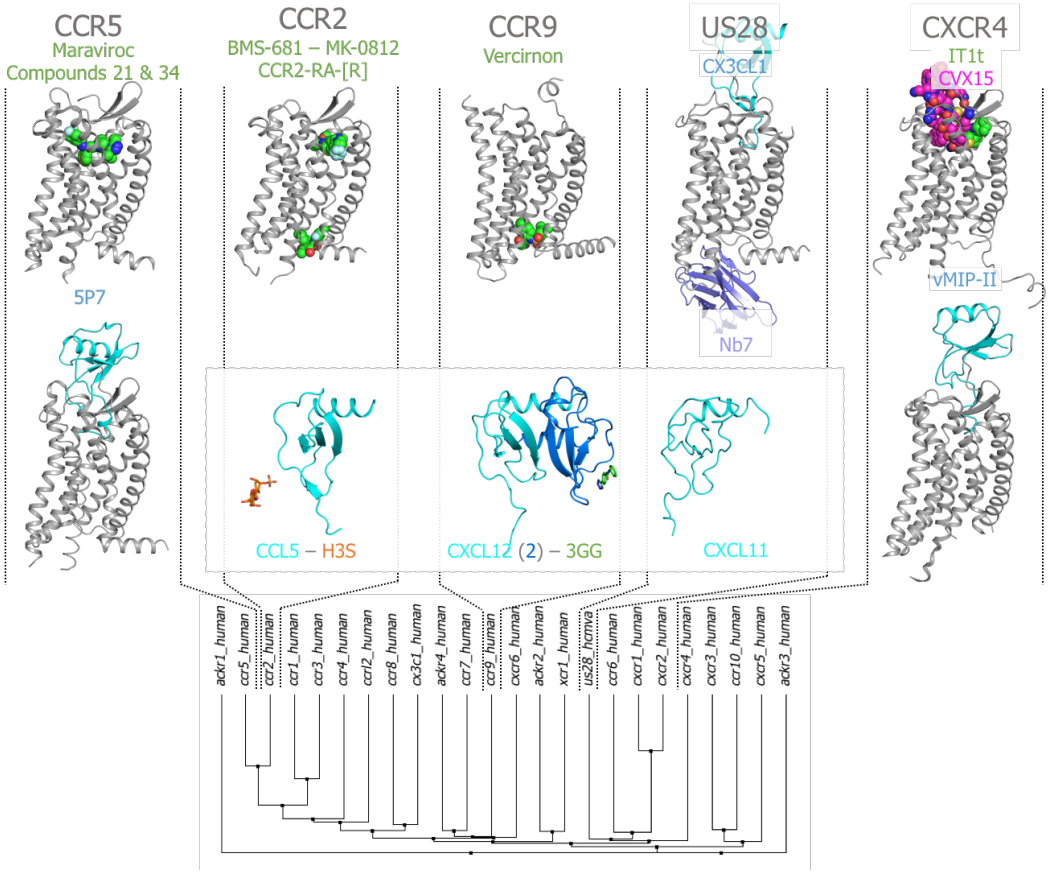

Figure 1

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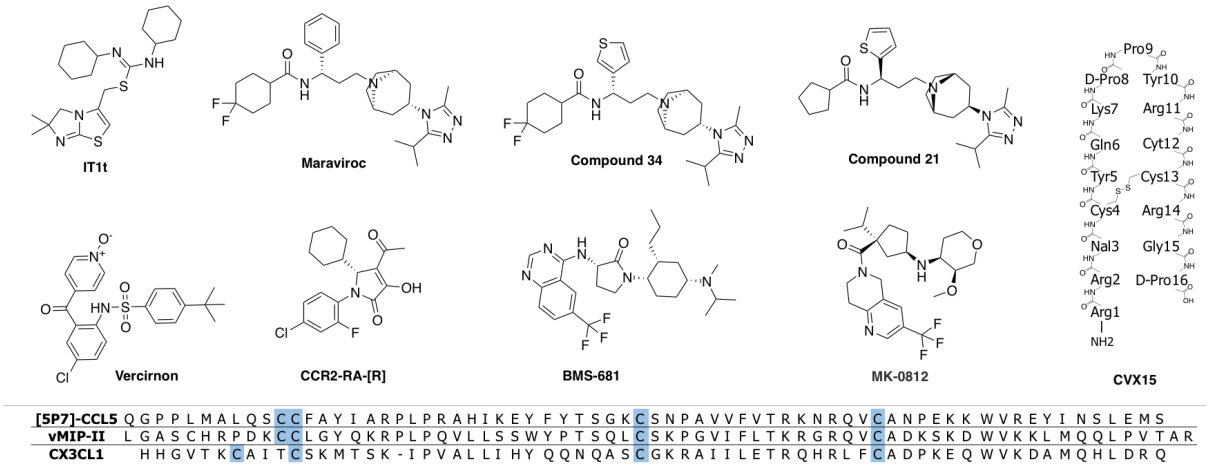

Figure 2

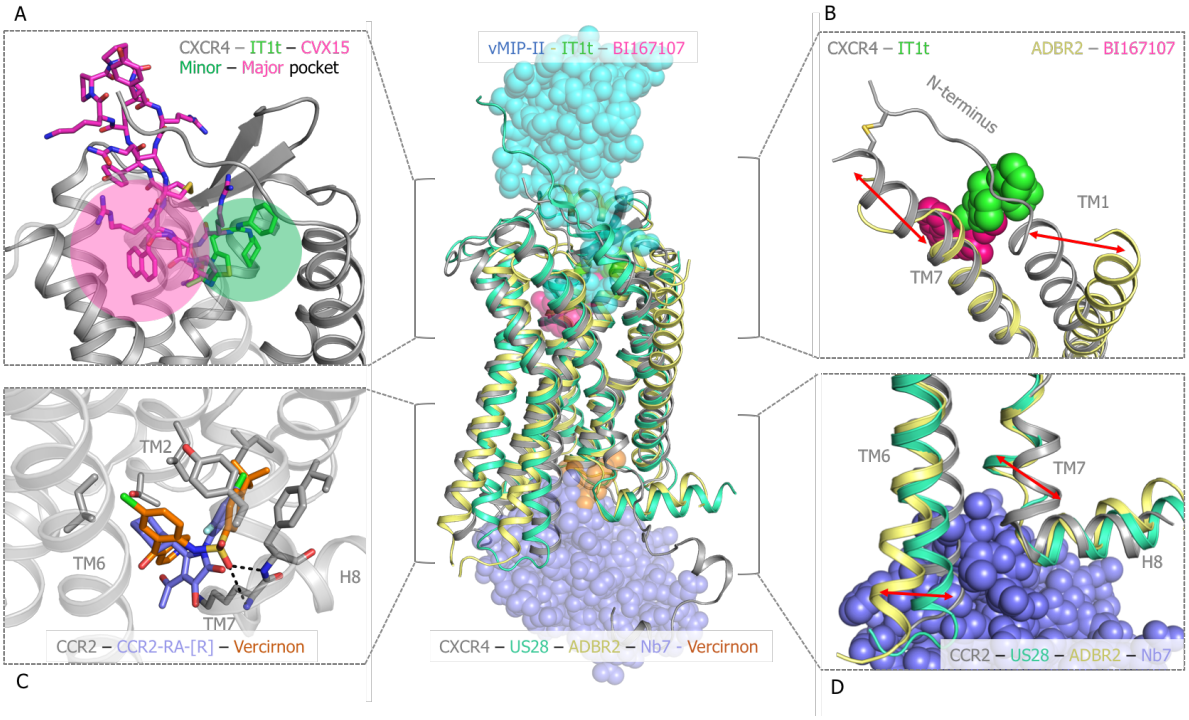

Figure 3

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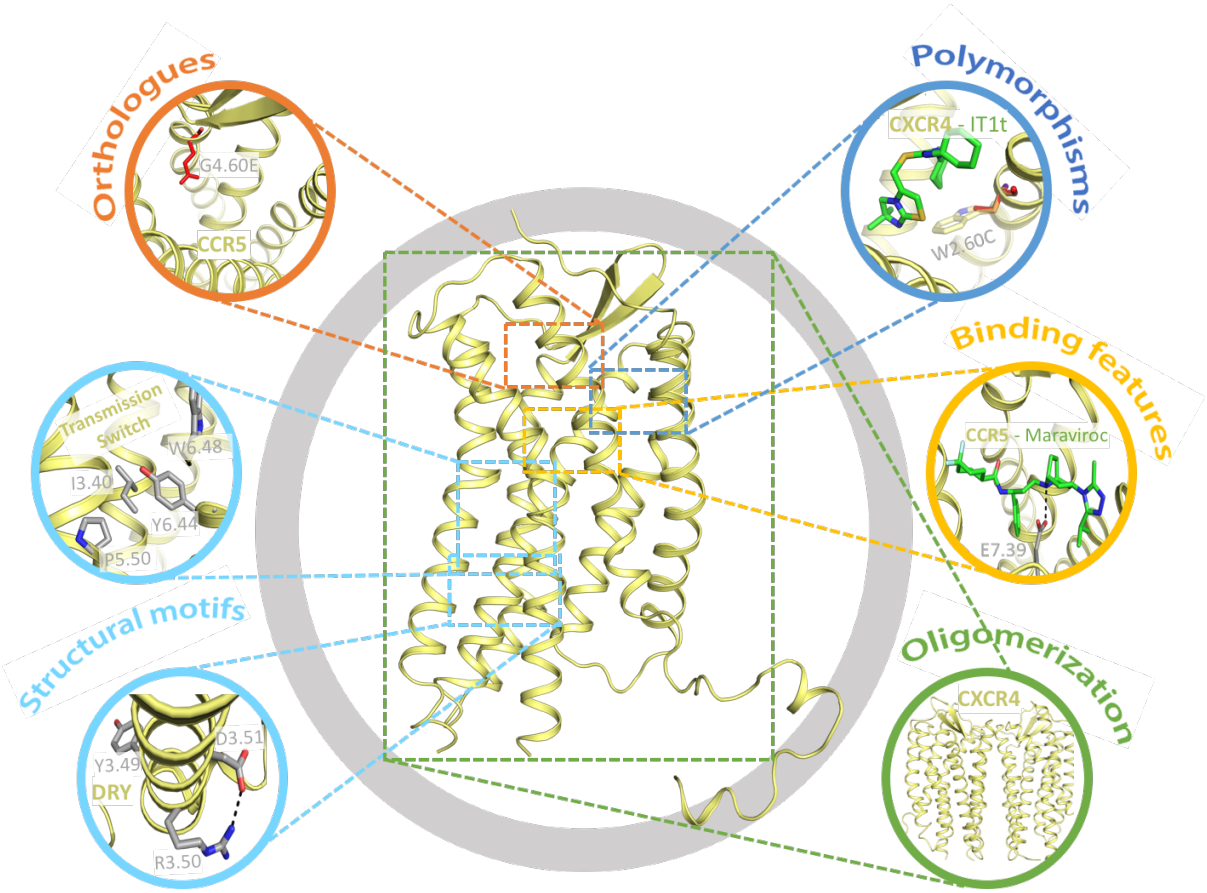

Figure 4

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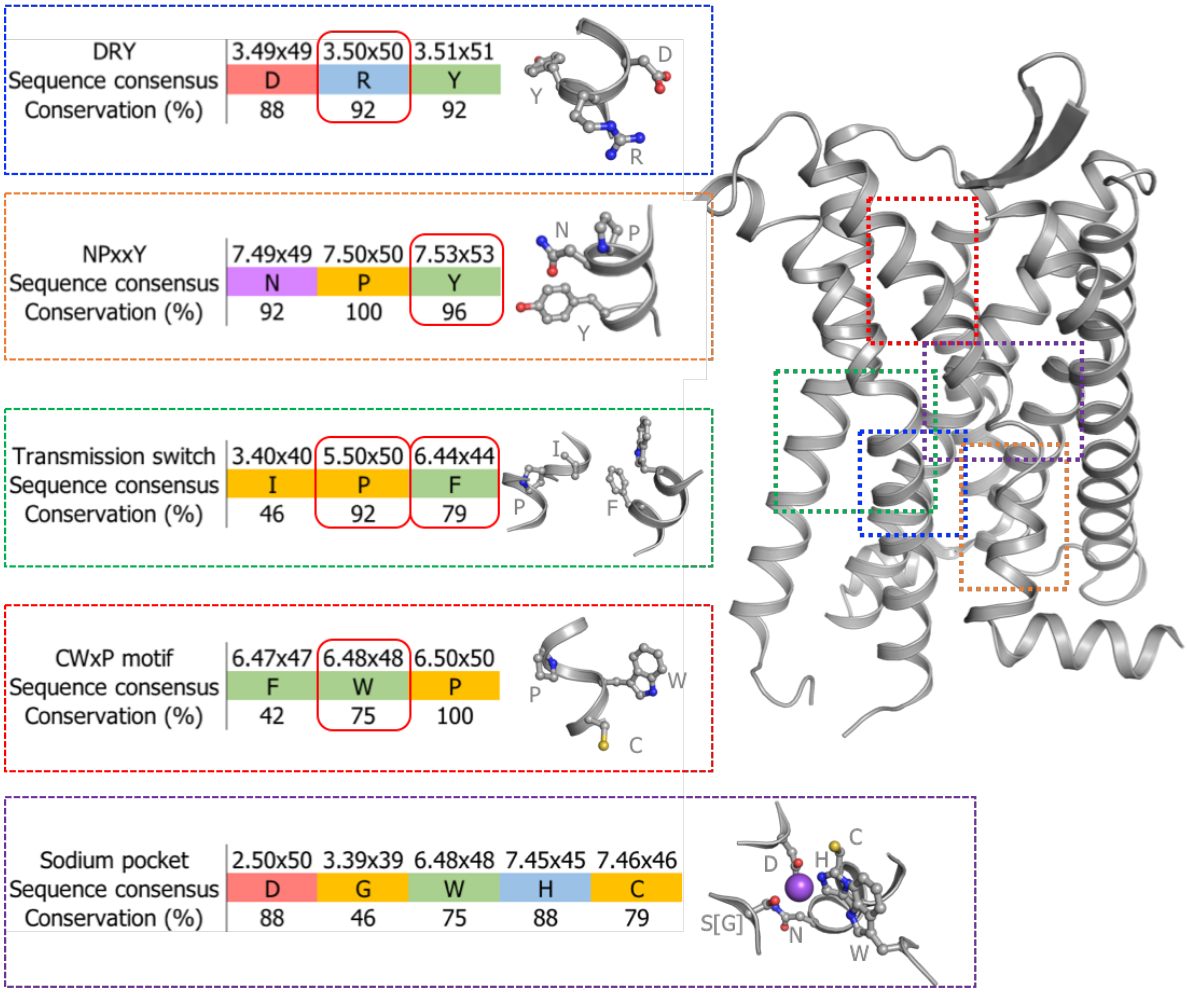

Figure 5

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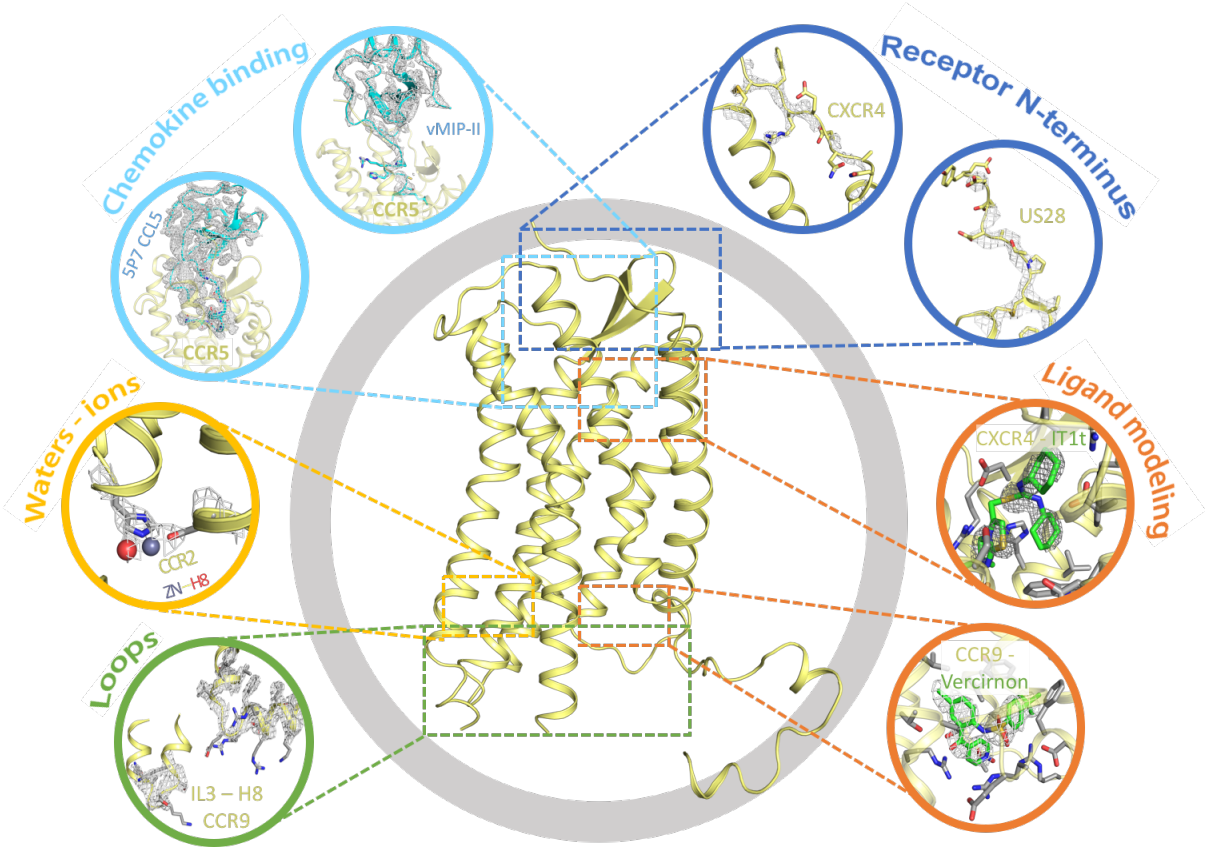

Figure 6

Chemokine receptor crystal structures: what can be learnt from them?

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Molecular Pharmacology

Supplementary Table 1 – Chemokine receptor genetic variants from GPCRdb (http://www.gpcrdb.org/) (Hauser et al., 2018; Pandy-Szekeres et al., 2018). Missense and loss of function mutations are reported for each chemokine receptor. The position, original amino acid, amino acid change, allele counts and frequencies, number of homozygotes, two predictions of the functional impact of the genetic variation (SIFT and PolyPhen), and a functional annotation of the position when available.

See excel file Supp_table_1

Supplementary Table 2 – List of all available chemokine receptor structures, including x-ray, cryo-EM and NMR structures. For each structure, the following information regarding the quality metrics of the structure is reported: the receptor, species, PDB identifier (PDB ID), method of resolution, resolution, R-value free, R-value work, clash score, Ramachandran outliers, side-chain outliers, and RSRZ outliers. Also general information from GPCRdb (http://www.gpcrdb.org/) (Pandy-Szekeres et al., 2018) is reported including: the conformational state (inactive, active, intermediate), the delta distance, the fusion protein, the endogenous ligands of the receptor, the endogenous ligand type, the co-crystallised ligand, the co-crystallised ligand function, the co-crystallised ligand type, the crystallisation method, the presence of the conserved sodium site, the senior author of the publication, the reference of the publication, and the release date of the PDB.

See excel file Supp_table_2

References

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