The road to Insurmountability: Novel avenues to better target CC Chemokine Receptors

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Chapter 1

General Introduction

The history of drug discovery and medicine can be traced back to the early human civilizations, which used natural products obtained from plants, animal materials and minerals for treating a variety of ailments and diseases.[1] Records of such prescriptions and medicinal recipes have been found in ancient Egyptian papyri, such as the Ebers papyrus written around 3000 BCE,[2] as well as in ancient Chinese texts and Aztec codices among others.[1, 3] However, drug research as we know it, only began in the late 19th century with the rise of synthetic chemistry and pharmacology.[4] It was until the 1860s that the relationship between chemical structure and pharmacological activity started to be systematically studied,[5] and until the early 20th century that the receptor theory started to emerge, including the concepts of drug affinity and efficacy.[6] In the course of the 20th century, the advent of new technologies and the development of numerous disciplines led to unprecedented progress in drug discovery and development.[4] Today, more than 1500 drugs have been approved by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA), and more than 30% of them target one single protein family: the superfamily of G protein-coupled receptors (GPCRs).[7]

G protein-coupled receptors (GPCRs)

With ~800 members identified, G protein-coupled receptors (GPCRs) comprise the largest family of membrane-bound proteins in the human genome.[8] Based on sequence homology and phylogenetic analysis, human GPCRs can be divided in five families or classes: glutamate family (class C), rhodopsin family (class A), adhesion family, frizzled/taste2 and secretin family (class B).[9, 10] Of these, the class A or rhodopsin family is the largest and most studied class of receptors, which includes aminergic receptors, protein receptors and nucleotide receptors, among others. Structurally, class A GPCRs are characterized by a bundle of seven transmembrane α-helices (TM1-TM7) connected by three extracellular loops (ECL1-3) and three intracellular loops (ICL1-3), an extracellular N-terminus, an intracellular helix 8 (H8) and an intracellular C-terminus (Figure 1).[11, 12] GPCRs transduce extracellular signals—such as photons, odorants, small molecules or proteins—into intracellular responses by interacting with different signal transducers, including heterotrimeric G proteins, GPCR kinases (GRKs) and arrestins.[13, 14] In general, after binding of an endogenous agonist to its cognate GPCR, the receptor undergoes a series of conformational changes that facilitate the activation of a G protein or recruitment of other signaling effectors, such as β-arrestin.[14] Signaling via GPCRs is linked to many physiological, but also pathological processes, making them potential drug targets for many disease indications. In fact, more than 100 unique non-olfactory GPCRs are currently targets for approved drugs, with many more potential targets in clinical trials.[7, 15]

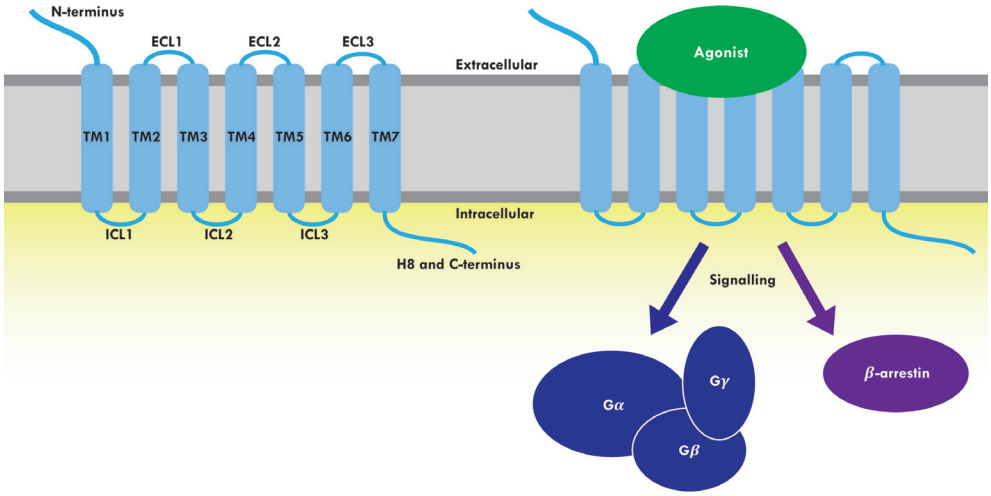

Figure 1. Schematic representation of a class A G protein-coupled receptor (GPCR) embedded in the cell membrane. Class A GPCRs share a general architecture of seven transmembrane alpha-helical domains (TM1-TM7) connected by three extracellular loops (ECL1-ECL3) and three intracellular loops (ICL1-ICL3), an N-terminus at the extracellular side, and Helix 8 (H8) and C-terminus at the intracellular side. After binding of an agonist from the extracellular side, the receptor undergoes conformational changes that allow the recruitment of different signaling effectors, such as the heterotrimeric G proteins or β-arrestins.

Chemokine Receptors

Chemokine receptors encompass a large subfamily of class A GPCRs, which are activated by highly conserved proteins called chemokines (**chemo**tactic cyto**kines**). So far, 23 different chemokine receptors and more than 40 different chemokines have been identified, which form a complex and seemingly redundant system: one chemokine receptor can respond to multiple chemokines, and one chemokine can act on multiple receptors (Figure 2).[16, 17] Most chemokine receptors are classified in four different families based on the pattern of N-terminal cysteine residues of their endogenous chemokines: XC, with only one cysteine residue; CC, with two adjacent cysteines; CXC and CX3C, with one or three residues separating the cysteine residues, respectively. In addition, there are five atypical chemokine receptors, which do not (seem to) signal via the heterotrimeric G proteins.[16, 18] Chemokine receptors are widely expressed in leukocytes, and upon activation by chemokine ligands they control a variety of leukocyte functions including migration, differentiation, and survival. According to their main function, chemokine receptors can be divided in inflammatory or homoeostatic, depending on whether they regulate functions required during an inflammatory response or under homeostatic conditions.[19]

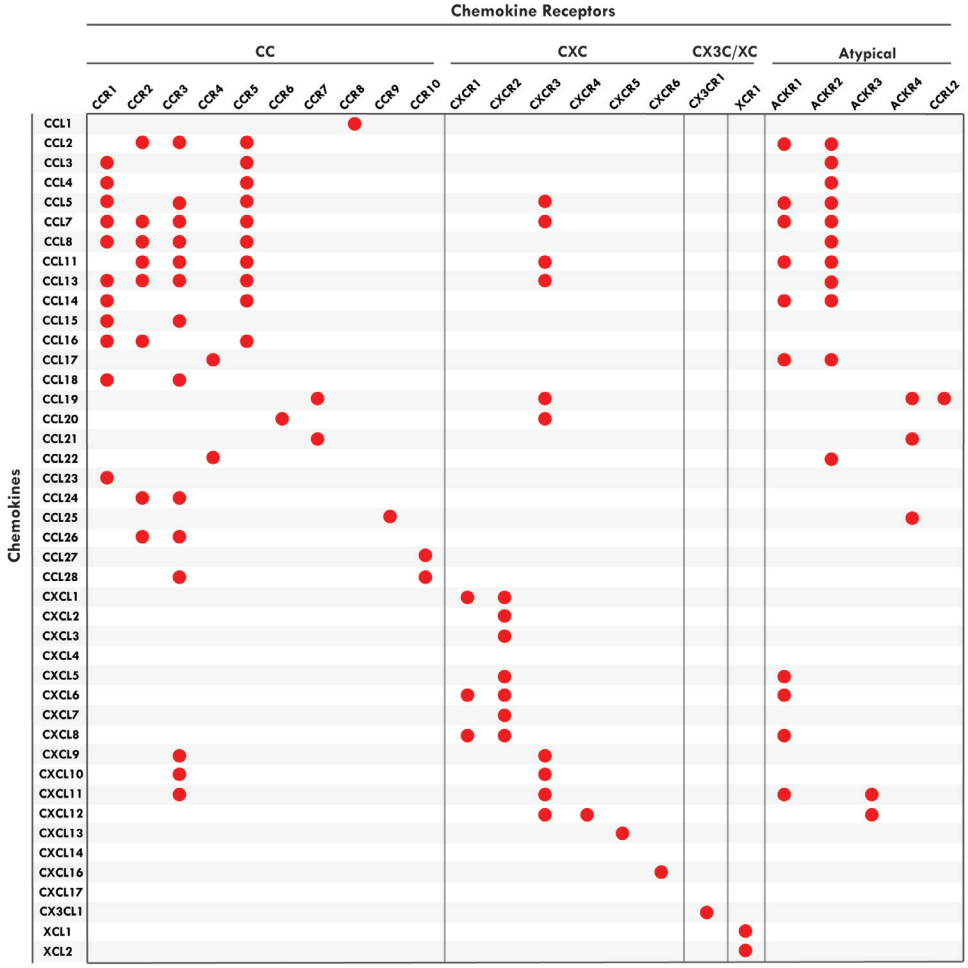

Figure 2. The human chemokine system. Chemokine receptors can be activated by multiple chemokines and several chemokines can act on multiple receptors.

So far, several crystal structures of chemokine receptors have been solved, which include the inactive-state structures of CCR2,[20, 21] CCR5,[22-25] CCR9[26] and CXCR4[27, 28], as well as the active-state structure of the viral chemokine receptor US28 in complex with the chemokine ligand CX3CL1 or derivatives.[29, 30] These structures provide structural insight into receptor activation by chemokines, as well as inhibition by small-molecule or peptide antagonists (Figure 3). As all class A GPCRs, chemokine receptors present a similar architecture of seven TM domains connected by three ECLs and three ICLs (Figure 1). In addition, these structures reveal a broad, open and very polar binding pocket for chemokines, located within the

extracellular region—the so-called orthosteric binding site[31] (Figure 3). This orthosteric pocket can be divided into a major and a minor subpocket, formed by TM3-6 or TM1-3 and 7, respectively.[32] Small-molecule antagonists can inhibit chemokine receptor function by binding to only one or both subpockets.[32, 33] For example, the CCR2 antagonists BMS-681 and MK-0812 bind exclusively to the minor pocket of the receptor,[20, 21] while the CCR5 antagonist Maraviroc appears to extend to both subpockets.[22] Furthermore, the crystal structures of CCR2 (**Chapter 3**) and CCR9 show that small-molecule ligands can also inhibit the receptors by binding to an intracellular binding site[20, 26] (Figure 3).

The different structures of chemokine receptors in complex with chemokine ligands[23, 28, 29] have also shed light on several epitopes necessary for chemokine recognition and activation: i) chemokine recognition site 1 (CRS1), where the chemokine first interacts with the N-terminus of the receptor; ii) CRS2, where the N-terminus of the chemokine extends into the TM domain of the receptor; and iii) CRS1.5 between CRS1 and CRS2, where conserved chemokine cysteine motifs and the N-terminus of the receptor are brought in close proximity to allow proper interaction.[31] As such, these structures have extended the so-called "two-site/two-step model" of chemokine-receptor activation, which only considered CRS1 and CRS2.[32] In addition, recent studies on CCR1 have led to the proposal of a three-step model, in which a conformational change of the receptor is also required for receptor activation.[34]

Chemokine receptors as drug targets: Focus on CCR1, CCR2 and CCR5.

CC chemokine receptors 1 (CCR1), 2 (CCR2) and 5 (CCR5) are expressed on many leukocyte cells, including antigen-presenting cells (dendritic cells and macrophages), basophils, neutrophils, natural killer cells and different types of T cells.[19] As inflammatory receptors, they play a key role in the recruitment of leukocytes to sites of inflammation—a process called chemotaxis.[19] Although this inflammatory response is an essential mechanism of defense, an aberrant response can lead to leukocyte accumulation and tissue damage, resulting in many inflammatory or immune diseases.[35]

In this regard, (pre)clinical studies have suggested a critical role of CCR1, CCR2 and CCR5 and their ligands in the pathogenesis of multiple sclerosis (MS)[36, 37] and rheumatoid arthritis (RA).[38, 39] Several studies have also shown that CCR1, CCR2 and CCR5 are necessary for monocyte recruitment and accumulation into the atherosclerotic plaques, suggesting a role of these receptors in atherosclerosis.[40-42] These chemokine receptors might also represent potential targets for the treatment of neuropathic pain, diabetes, psoriasis, and transplant

rejection, among others.[35, 43-45] In addition, a recent phase II clinical trial has successfully demonstrated that combined inhibition of CCR2 and CCR5 is beneficial for patients with nonalcoholic steatohepatitis (NASH).[46] Besides its role in inflammatory and immune diseases, CCR5 also acts as a co-receptor for the entry of the CCR5-tropic human immunodeficiency virus-1 (R5-HIV-1) into the host cells.[47] In addition, the chemokine system seems to be involved in tumor growth, tumor progression and metastasis.[48] For example, CCR1 has been implicated in colorectal cancer progression and metastasis to liver and lung,[49-51] while several preclinical studies have suggested a role for CCR2 and CCR5 in breast cancer progression and metastasis,[52-54] pancreatic cancer,[55, 56] and prostate cancer[57, 58] among others.

Difficulties in targeting Chemokine Receptors

Despite the wealth of evidence regarding the involvement of chemokine receptors in many diseases, only three drugs targeting chemokine receptors have successfully reached market approval: the CCR5 small-molecule antagonist Maraviroc, the CXCR4 small-molecule antagonist Plerixafor, and the CCR4 monoclonal antibody Mogamulizumab. In most cases, preclinical findings have failed to translate into successful chemokine inhibitors, mainly due to lack of efficacy in clinical trials.[59, 60] Overall, difficulties with targeting the chemokine system can be grouped into three main categories: drug-related problems, relevance of the model, and complexity of the system. Drug-related problems include poor drug-like properties, insufficient target occupancy, and off-target effects, among others. For example, it has been predicted that > 90% receptor occupancy is required at all times for a sufficient anti-inflammatory effect, which is not always achieved in clinical trials.[17, 61] Relevance of the model refers to differences between the immune and chemokine systems of humans and animal species such as rodents, which renders these models poorly predictive in immune and inflammatory diseases.[62] For example, some chemokines have different functions in different species, while some others only exist in one species.[62] In addition, the potency of many chemokine receptor inhibitors can differ greatly between species, such as the CCR1 antagonist CP-481,715 that only inhibits the human receptor.[59, 63] Finally, the complexity of the system refers to the "redundancy" of the chemokine system, characterized by multiple cross-interactions between chemokines and chemokine receptors (Figure 2). The latter implies that targeting one single receptor might be insufficient in complex diseases where many chemokines and chemokine receptors are involved.[59, 60, 64] Added to the complexity is the suggested spatiotemporal regulation of the chemokine system, implicating that different biological responses are expected depending on the expression level, site of expression, or interaction with certain chemokine ligands, among others.[17, 65]

Modulating Chemokine Receptors and GPCRs

Chemokine receptors, and GPCRs in general, are modulated by **orthosteric** or **allosteric** ligands which activate or block the receptor response in different ways. Orthosteric ligands bind to the same site as the endogenous ligand, i.e. at the chemokine binding site. Allosteric ligands, on the other hand, modulate the receptor by binding to a site spatially distinct from the orthosteric site, a so-called allosteric binding site[66] (Figure 3). Such allosteric binding sites have been identified across all GPCR regions, including extracellular, intracellular, and even extrahelical regions.[67] Depending on their functional effect, orthosteric ligands can be classified as **agonists**, **inverse agonists** or **antagonists**. Agonists can fully activate (full agonists) or partially activate (partial agonists) the receptor by inducing or stabilizing an active receptor conformation. Inverse agonists inhibit the constitutive or basal activity of the receptor, while (neutral) antagonists inhibit the agonist response without decreasing the constitutive activity.[68] Similarly, allosteric modulators can be classified as **positive allosteric modulators** (PAMs), which potentiate the affinity and/or efficacy of the orthosteric ligand; **negative allosteric modulators** (NAMs), which decrease the affinity and/or efficacy of the orthosteric ligand; or **neutral allosteric ligand** (NAL), with no effect on the orthosteric ligand.[66]

Ligands are usually designed to bind to their target in a reversible manner: the ligand can freely associate and dissociate from the receptor. Optimization of a ligand's binding kinetics—association (k_{on}) and dissociation (k_{off}) rate constants—can result in improved *in vivo* efficacy and safety.[69] By calculating the reciprocal of the k_{off} ($1/(k_{off})$, the **drug-target residence time** (RT) of a ligand can be determined, which measures the lifetime of the drug-target complex. In addition, ligands that bind irreversibly to their target, i.e. **covalent ligands**, have been developed and used in the clinic.[70] These ligands bind in a two-step process, in which the ligand first binds to the receptor in a reversible manner, followed by the formation of the covalent or irreversible bond between the target protein and the reactive group of the ligand.[71] Inhibition via allosteric or covalent binding results in **insurmountable antagonism**, in which the ligand is able to inhibit receptor signalling despite high local concentration of the endogenous agonist, such as the presence of high chemokine levels during inflammatory conditions.[72]

Finally, although ligands have been traditionally designed to selectively act on a single target, recent evidence suggests that targeting one single protein might be insufficient in complex diseases where more than one protein is involved. Thus, inhibition of multiple drug targets (i.e. **polypharmacology**) may be more effective in disrupting complex biological systems than selective inhibition.[73] In this regard, three different approaches to polypharmacology have been proposed: i) drug cocktail, which refers to the administration of two different drugs,

each formulated differently; ii) multicomponent drugs, which refers to a single formulation containing two drugs; and iii) **multitarget ligands**, which refers to the design of one single ligand interacting with multiple targets.[74]

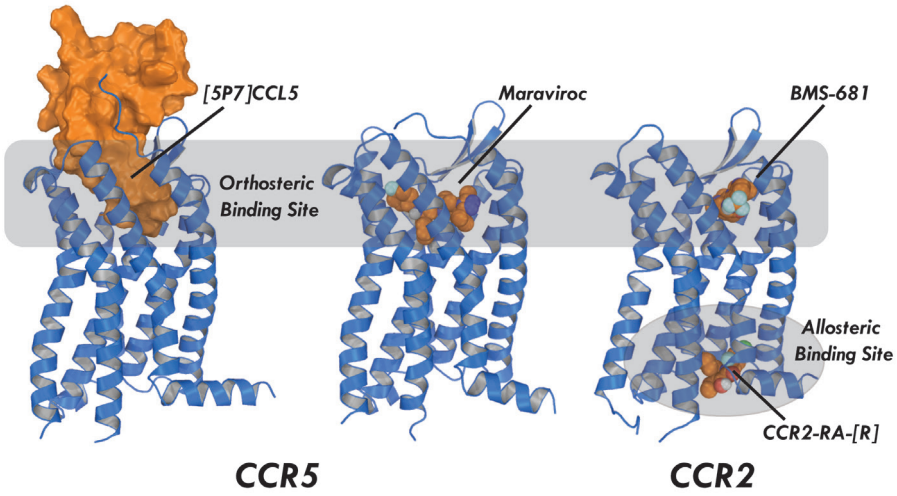

Figure 3. Representative crystal structures of chemokine receptors. Figure shows the crystal structure of CCR5 in complex with [5P7]CCL5, an engineered CCL5 variant; the crystal structure of CCR5 in complex with the small-molecule antagonist Maraviroc; and the crystal structure of CCR2 in complex with the small-molecule antagonists BMS-681 and CCR2-RA-[R]. Both Maraviroc and BMS-681 bind to the orthosteric binding site where the chemokines also bind, while CCR2-RA-[R] binds to an allosteric site located in the intracellular region.

AIM AND OUTLINE OF THIS THESIS

Despite the major advances in drug discovery and development, the attrition rate of drug candidates in clinical trials continues to be high: only ~10% of all drug candidates entering Phase I clinical trials is expected to reach final marketing approval.[75, 76] An analysis of the causes of drug failure has reported lack of efficacy as the main reason of Phase II and Phase III failures[77] and this is no different in the case of chemokine receptors.[59, 60] In this regard, a thorough understanding of the mechanism of action at a molecular level is key for the development of drug candidates with better safety and efficacy profiles. This requires the inclusion of novel concepts and novel tools in early phases of drug discovery, some of which we aimed to explore in this thesis.

Chapter 2 provides an overview on the available evidence of a common intracellular binding site among chemokine receptors and other class A GPCRs. Furthermore, the different

strategies to target such binding sites are discussed, with special focus on small molecules, as well as the potential advantages of intracellular ligands versus the traditionally designed orthosteric ligands. As crystal structures are paramount in drug discovery programs, **Chapter 3** focuses on the determination of the X-ray structure of human CCR2 in complex with two small-molecule antagonists: BMS-681, binding in the orthosteric binding site, and CCR2-RA-[*R*], binding in an intracellular binding pocket. The high conservation of this intracellular pocket among chemokine receptors can be exploited for the design of multitarget ligands, such as dual-targeting CCR1/CCR2 (**Chapter 4**) or CCR2/CCR5 (**Chapter 5**) intracellular ligands. Thus, **Chapter 4** explores whether the highly homologous CCR1 can also be targeted with intracellular small molecules. For this purpose, a series of CCR2-RA-[*R*] derivatives were synthesized and evaluated in both CCR1 and CCR2 using biochemical assays, allowing us to develop structure-affinity relationships for both receptors. A similar medicinal chemistry approach was used in **Chapter 5**, which describes the synthesis and biological evaluation of a series of triazolo-pyrimidinone derivatives in both CCR2 and CCR5, with the aim of gaining insight in the compounds' structural requirements to achieve selectivity and dual activity in the two receptors. With the aim of obtaining the first covalent probe for CCR2, **Chapter 6** describes the design, synthesis, pharmacological characterization and suggested binding mode of a covalent, intracellular NAM for this receptor. As *in vivo* drug efficacy is the ultimate goal of drug discovery efforts, **Chapter 7** investigates whether compound **15a**, an orthosteric antagonist with a long residence time on human CCR2, is efficacious in a mouse model of atherosclerosis. Finally, **Chapter 8** summarizes the results of the work presented in this thesis, as well as the future prospects and challenges in the field. Hopefully, this thesis will contribute to the development of better insurmountable antagonists and improved *in vivo* outcomes.

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