The ins and outs of ligand binding to CCR2

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Chapter 1

General Introduction

As long as human beings have recognized and suffered from disease, there has been a quest for cures and therapies. Historically, natural product extracts served as the main source of drugs. In the second half of the nineteenth century, the first isolation of biologically active molecules from these extracts succeeded, and soon after that the first synthesis of the pharmaceutical drug aspirin took place [1]. Due to enormous progress in all fields related to pharmaceutical sciences, the art of drug discovery has evolved greatly in the 20th and 21st century. We are now able to synthesize large chemical libraries of up to millions of synthetic small molecules, which can be screened against the target of interest in order to identify potential drug candidates [2]. As a consequence, it is not a surprise that for one particular target many different drug-like molecules are discovered. This is also the case for the chemokine receptor CCR2, a receptor that is involved in a large variety of diseases ranging from autoimmune and metabolic diseases to atherosclerosis and pain. Despite major efforts of the pharmaceutical industry and synthesis of many inhibitors, there is at this moment no clinically effective drug available that targets this receptor. In order to improve current drug candidates, one would benefit from understanding their mechanism of action at a molecular level, which is often incomplete in the current process of drug discovery. In this thesis we therefore zoom in at the molecular level of CCR2, and reveal novel insights in mechanisms of action of existing as well as novel drug-like molecules. These findings serve as a fundament for future drug discovery programs, and will be equally relevant for understanding the outcomes of current drug candidates in later stages of development.

In order to grasp the relevance of the research and the concepts that will be discussed in this thesis, first of all the world of G protein-coupled receptors (GPCRs) will be introduced, the protein family to which CCR2 belongs. The activation and inhibition of GPCRs will be outlined, as well as their role in physiology and disease. This brings us to the receptor of interest, CCR2, a member of the GPCR subfamily of chemokine receptors. Finally the current status of drug discovery targeting CCR2 will be addressed, followed by the outline of the aim and contents of this thesis.

G protein-coupled receptors

Classification and structure

GPCRs comprise the largest family of membrane receptors in mammalian cells; the human genome has been estimated to encode approximately 800 GPCRs [3]. GPCRs are located at the cell surface and transduce an extracellular signal into an intracellular response. They are

expressed in nearly all organs and tissues of the human body, and therefore they regulate a broad range of physiological processes. The structure of a GPCR consists of an extracellular N-terminus and an intracellular C-terminus with seven transmembrane-spanning α-helices, resulting in three extra- and intracellular loops (Fig. 1) [4]. The understanding of the 3D-conformational structure has substantially increased since attempts to crystallize GPCRs became more successful; we now have snapshots of at least 23 different GPCRs available at the time of writing of this introduction, including those for the chemokine receptors CXCR4 and CCR5 [5, 6]. According to the IUPHAR Committee on Receptor Nomenclature and Drug Classification, the superfamily of GPCRs is divided in six classes based on their functional similarity and sequence homology [7]. Each family generally shares over 25% sequence identity in the transmembrane core region, with specific sets of highly conserved residues and motifs. The largest and most studied subfamily is formed by the class A rhodopsin-like receptors, to which the chemokine receptors belong. The remaining classes are the class B secretin receptor family, class C metabotropic glutamate/pheromone receptors, class D fungal mating pheromone receptors, class E cyclic AMP receptors and class F frizzled/smoothened like receptors. In addition, for ~15% of all GPCRs the endogenous ligand is at present unknown, and therefore these receptors are accordingly named orphan GPCRs [7].

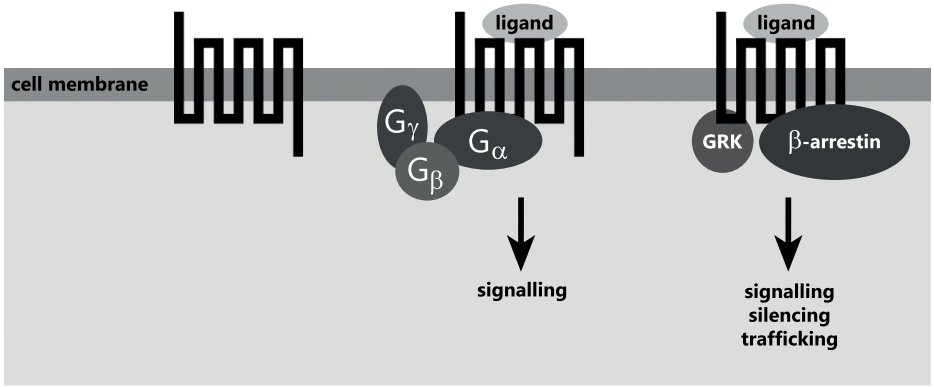


Fig. 1. Schematic representation of GPCRs embedded in the cell membrane. Upon ligand binding and receptor activation, signal transducing proteins like G proteins, GRKs and β-arrestins can bind at the intracellular side.

GPCR signalling in health and disease

Upon activation due to ligand binding at the extracellular side of the GPCR, the receptor undergoes conformational changes that allow recruitment of intracellular signalling proteins (Fig. 1) [8]. These signalling proteins subsequently become activated and start a downstream

signal transduction cascade. Multiple types of signalling proteins have been associated with GPCRs, among which the family of G proteins is most ubiquitous and best characterised [9].

There are four members of the family of heterotrimeric G proteins, being G_s, G_i, G_q, $G_{12/13}$, which individually consist of a G_α, G_β and G_γ subunit (Fig. 1) [10]. Activation of G proteins results in an exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) in the alpha subunit, which is followed by dissociation of the activated G_α and $G_{\beta\gamma}$ subunits. These subunits can activate a wide variety of signalling molecules, of which adenylyl cyclase (AC), the MAP kinase pathways and phospholipase C (PLC) are most prominent [11]. Second messengers, including cyclic AMP, inositol triphosphate and calcium ions, then turn on a range of effector systems to change the behavior of the cell, ranging from morphological changes and secretion of molecules, to the regulation of gene transcription.

Besides G proteins, GPCRs can bind and activate other cytosolic proteins such as G protein-coupled receptor kinases (GRKs) and β-arrestins (Fig. 1) [9]. GRKs and β-arrestins orchestrate GPCR activities at three different levels [12]. First of all they induce *silencing*, which is the functional uncoupling of the receptor from its G protein by a mechanism known as desensitisation. In addition they mediate receptor *trafficking*, characterized by receptor internalization, resensitisation and/or degradation. Finally, they can induce *signalling*, via the activation or inhibition of intracellular signalling pathways independently of heterotrimeric G proteins.

Together, these signalling proteins determine the response of a cell to an extracellular stimulant. Due to the great divergence in GPCRs this can vary from the regulation of the heart rate to the perception of odors and flavors. All of these processes are carefully fine-tuned, and therefore malfunctioning of any GPCR can result in severe diseases. Since GPCRs comprise a large protein-family, and are involved in the most prevalent disease areas including cancer, obesity, diabetes and cardiac dysfunction, approximately one third of the pharmaceuticals on the market today target these proteins [13].

Ligands for G protein-coupled receptors

Activation and inhibition of GPCRs

GPCRs are very flexible membrane proteins and their conformation varies from an inactive state (R) to several active states (R*) [14, 15]. The ratio between active and inactive states is dependent on the type of GPCR and its cellular environment. Some are naturally present with high proportions in an active state; these GPCRs signal without any ligand binding, a

phenomenon that is named 'constitutive activity' or 'basal activity' [16, 17]. The ratio between active and inactive states is affected by binding of ligands at the extracellular face of the GPCR. Agonist ligands preferentially bind to and stabilize the active state R* of a receptor, resulting in an increase in receptor activity and signalling (Fig. 2A+B). Some ligands behave as partial agonists; these ligands activate the receptor, but cannot elicit the maximum possible response that is induced by a full agonist (Fig. 2B). These partial agonists have been reported to stabilize a distinct conformational state of the receptor compared to full agonists [18, 19]. Others propose that partial agonists are able to dynamically bind with multiple orientations to a receptor, which results in active and inactive populations of receptors of which the ratio determines the level of the response [20]. Inverse agonists preferentially bind to the inactive state R and reduce the receptor activity (Fig. 2A+B) [21]. Again, a distinction can be made between full and partial inverse agonists [22, 23]. Neutral antagonists prevent GPCR activation, but bind equally well to active as well as inactive conformations, and therefore these ligands do not affect the basal activity of the receptor (Fig. 2A+B) [21].

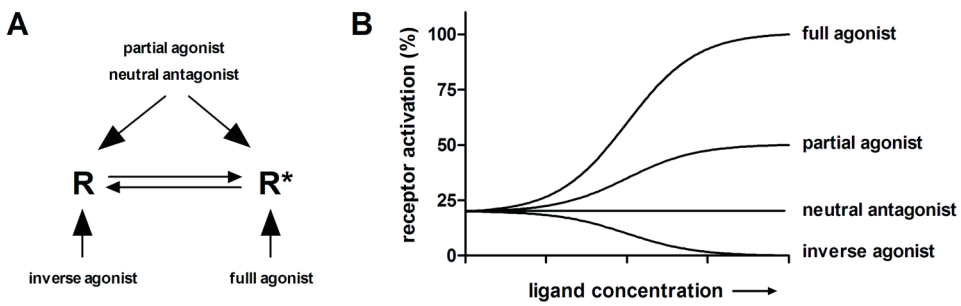


Fig. 2. (A) The preference of different ligands to bind to the inactive (R) and/or active (R*) receptor state. (B) Receptor activation upon binding of a full agonist, partial agonist, neutral antagonist or inverse agonist.

This classical view of receptor signalling has been refined during the past couple of years, since we began to appreciate that one GPCR is able to activate multiple signalling proteins, via different active states [15]. It is now evident that certain ligands are able to stabilize a specific active state of a GPCR, and the first structural basis for this phenomenon was recently reported for the serotonin 5-$HT_{1B/2B}$ and the β_2-adrenergic receptors [24, 25]. This can result in ligand-directed signalling via one specific pathway, named 'functional selectivity' or 'biased signalling' [26]. In extreme cases it might occur that a certain ligand for one GPCR is an agonist for signalling pathway A, while it behaves as an antagonist for signalling pathway B. This concept is also applicable to chemokine receptors and is therefore important to take into account during drug discovery, as will be further discussed in Chapter 2.

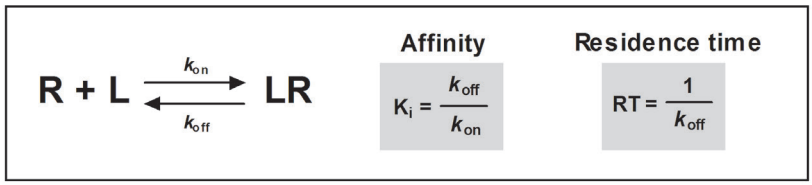
Orthosteric and allosteric ligands

The endogenous ligands for the GPCR superfamily are very diverse, ranging from peptide hormones, lipids and nucleotides to odorants and ions [7]. The binding site of these endogenous ligands is called the "orthosteric" binding site. Especially since the introduction of small molecule and peptide drugs, it was discovered that multiple ligand binding sites are present on GPCRs. If a ligand binds to the same binding site as the endogenous ligand, it is named an orthosteric ligand. In contrast, when it binds to a topographically distinct site from where the endogenous ligand binds, it is named an 'allosteric' ligand [27]. This term has been derived from the Greek word 'allo', which means 'other'. Allosteric ligands can exert a variety of effects at a functional level [28]. Allosteric agonists can activate GPCRs by themselves without the presence of any orthosteric ligand. In addition, since the allosteric site is different from the orthosteric site, a GPCR is in some cases able to simultaneously bind both orthosteric and allosteric ligands. Allosteric ligands are thereby able to alter the binding and/or signalling properties induced by the ligand at the orthosteric site and are accordingly named allosteric modulators, which can be further classified as allosteric enhancers (or positive allosteric modulators- PAMs) and allosteric inhibitors (or negative allosteric modulators – NAMs). Since the binding pocket of chemokine receptors is quite large and the size of synthetic drugs very small compared to the endogenous chemokine protein ligand, allosteric modes of action are often observed for this family of GPCRs [5, 29]. In this thesis two novel allosteric binding pockets were discovered, located within the core domain and at the intracellular side of CCR2, as described in Chapters 4 and 5.

Ligand-receptor binding kinetics

In order to activate or inhibit signalling events via a GPCR, a ligand first needs to bind to the receptor [30]. Both agonists and antagonists bind to the receptor with a certain association rate (k_{on}), followed by their release of binding from the receptor with dissociation rate k_{off} (Scheme 1). The strength of binding is represented by the parameter K_i, which stands for the affinity of a ligand for its receptor, and is determined by the ratio k_{off}/k_{on} (Scheme 1). This affinity can be easily measured in pharmacological assays, and drug discovery programs classically optimize this equilibrium parameter to end with high affinity ligands that are put forward in the drug development cycle. The affinity of a ligand is a very important measure, but next to that the concept of individually optimizing k_{on} and k_{off} has gained more and more attention during the last decade [31, 32]. Importantly, affinity is measured in a closed system (*in vitro*) at equilibrium, whereas in open systems like the human body (*in vivo*) the drug and target can have fluctuating concentrations [33]. This discrepancy may make equilibrium

measurements less appropriate to predict the effect of a drug *in vivo*. It would be better suited to additionally measure the lifetime of the drug-target complex, represented by the term 'residence time' that can be calculated as the reciprocal of k_{off} (Scheme 1) [34].



Scheme 1. Binding of a ligand (L) to the receptor (R) with association rate k_{on}, and dissociation of L from R with dissociation rate k_{off}. The affinity (K_i) and residence time (RT) can be derived from these rate constants.

Several studies have indicated that long residence time ligands can contribute to improved efficacy, reduced side effects and a longer duration of action. The latter would enable once-daily dosage forms as opposed to multiple doses and thus increases patient compliance [35-39]. Examples are the angiotensin II subtype-1 (AT_1) receptor antagonist olmesartan for treatment of hypertension [40, 41], and the neurokinin-1 (NK_1) receptor antagonist aprepitant for prevention of acute and delayed chemotherapy-induced nausea and vomiting [42]. In addition, the rate at which a drug binds to a target receptor is crucial to the onset of the drug effect, therefore quick binding of a drug to its target is preferred [43].

The residence time of a ligand can be measured in kinetic binding assays upon radiolabelling the compound of interest [44]. However, this is labor intensive and cost-inefficient, and therefore a method to determine the residence time of unlabelled ligands was invented in 1984 by Motulsky and Mahan [45]. It took twenty years before this competition association assay was picked up by a larger audience, but nowadays it is applied to assess ligand binding kinetics at many GPCRs [32, 36, 39, 46]. Based on this method a higher throughput screening assay was developed in our laboratory, named the 'dual point kinetic assay', which facilitates the screening for long residence time ligands [47]. In Chapter 6 we applied both of these assays to study the residence time of antagonists for CCR2, to stimulate drug discovery based on kinetic profiles next to affinity in order to eventually improve clinical efficacy.

Chemokine receptors and their ligands

The chemokine receptor family

The chemokine receptor CCR2 belongs to the GPCR subfamily of chemokine receptors. They are predominantly expressed on immune cells and serve an important role in the host immune response against invading pathogens [48, 49]. Approximately 23 different chemokine receptors are known to date, and these can be activated by one or several of the 48 different chemokine ligands. Chemokines are small peptides of 70 to 120 amino acid residues, which are classified into four families according to the interaction pattern of the cysteine residues in their N-terminus: XC, CC, CXC and CX3C, where C represents a cysteine bridge and X/X3 stands for one or three non-cysteine residues (Fig. 4) [50].

Chemokine receptor binding and activation are generally thought to occur via a two-step process in which the first step is governed by binding of the large peptide ligand to the N-terminus and extracellular loops of the GPCR protein [51]. Subsequently, the N-terminus of the chemokine is well-positioned to interact with the transmembrane (TM) domains, leading to activation of the receptor [52].

Chemokines can be divided into two functional groups: homeostatic chemokines that are involved in leukocyte homing, and inflammatory chemokines that are produced in inflamed tissue by resident and infiltrating cells [53]. Several chemokines have both a homeostatic and inflammatory function. The secretion of chemokines evokes a chemokine gradient that results in chemotaxis: direct migration of cells expressing the appropriate chemokine receptor towards the chemokine ligand [54]. More details of the functions and the regulation of the chemokine receptor system are described in Chapter 2.

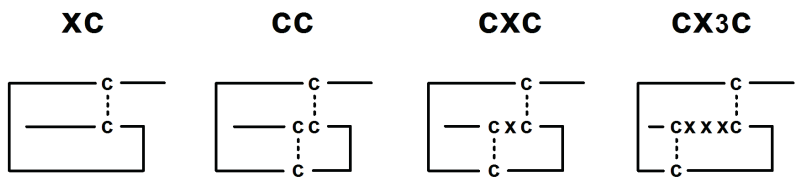


Fig. 3. The structure of chemokine families XC, CC, CXC and CX3C. Disulfide bridges are represented by the dotted lines.

The chemokine receptor CCR2

In 1994 CCR2 was fully cloned and characterized by Charo and co-workers [55]. It exists in two alternatively spliced forms: CCR2a and CCR2b [56]. CCR2b is the predominantly expressed variant on which the current study was focussed, therefore I refer to this variant as “CCR2” in

this thesis. CCR2 is abundantly expressed on immune cells such as monocytes, natural killer cells and T-lymphocytes and can be bound by eight different inflammatory chemokines, being CCL2, CCL7, CCL8, CCL11, CCL13, CCL16, CCL24 and CCL26 [57-60]. CCL2 is the most studied chemokine for CCR2, and is unique among the seven CCR2 chemokines since it is the only ligand that binds exclusively to CCR2. Intracellular signalling pathways that are activated by CCR2 are mainly driven by G_i proteins and β-arrestins [61]. Further downstream the activation of kinase cascades including extracellular signal-regulated kinase (ERK) 1/2 and Akt, as well as calcium signalling have been reported. Notably, the different chemokines have been reported to preferentially activate specific signalling pathways over others, implying that the concept of biased-signalling is applicable to this receptor, as discussed in Chapter 2 [61-63].

CCR2 as drug target

CCR2 is involved in a variety of diseases characterized by inflammation. Increased levels of CCL2 have been found associated to atherosclerosis, and CCR2 knock-out mice show a reduction in lesion size in the arterial wall [64, 65]. Several studies have shown that CCR2 on monocytes and macrophages mediates their recruitment to the atherosclerotic lesion and thereby contributes to plaque formation [66]. In addition, CCL2 and CCR2 are both highly expressed in the dorsal root ganglion (DRG) and on astrocytes and microglial cells in the peripheral and central nervous system during chronic pain states [67]. Knock-out of CCR2 in mice was found to diminish development of chronic pain states like neuropathic pain, and therefore many companies search for CCR2 antagonists as pain-reducing agents since no therapies are currently available for this disease [68]. Rheumatoid arthritis is characterized by inflammation in the joints, and again increased expression of CCL2 and CCR2-expressing macrophages has been found at these sites [69]. From these examples it seems that both CCR2 and its ligands are associated to different disease states through a common mechanism of action, which is a combination of direct activation of CCR2 in the cells of the target tissue and recruitment of circulating inflammatory cells into the tissue. Other diseases for which an important role of CCR2 and its chemokines has been reported include cancer [70], asthma [71], fibrosis [72], diabetes [73] and multiple sclerosis [74].

CCR2 inhibition by small molecule antagonists or monoclonal antibodies has been evaluated in a number of clinical trials targeting all the diseases mentioned above [75]. Unfortunately the majority of these trials failed, with the predominant reason being a lack of efficacy in Phase II. On-going trials include studies with the antagonists CCX140 and PF-04634817 for diabetic nephropathy [76, 77], and PF-04136309 for pancreatic adenocarcinoma [78]. The only marketed drugs for chemokine receptors at this moment are the CCR5 antagonist maraviroc and the CXCR4 antagonist AMD3100 [79, 80]. Maraviroc

inhibits entry of human immunodeficiency virus (HIV) into CCR5-positive cells, and AMD3100 is used to mobilize human hematopoietic stem cells from the bone marrow. Notably, both these conditions are not related to inflammatory diseases, highlighting the difficulty to intervene in those pathologies.

This thesis

Aim

The aim of the study was to provide a detailed insight in the molecular mechanism of action of CCR2 antagonists in order to improve drug discovery targeting this receptor. Three separate binding pockets via which CCR2 can be modulated were discovered, and different routes that lead to insurmountable antagonism of this receptor were revealed. In this thesis these results will be discussed in view of the complexity of the chemokine system. They should provide the reader with insights that will hopefully lead to the development of clinically effective drugs in the long term.

Outline

The family of chemokine receptors and their endogenous chemokines will be more extensively introduced and discussed in **Chapter 2**. This chapter is particularly devoted to the so-called biased-signalling of chemokines and its implications for drug discovery.

Chapter 3 is focused on small molecule antagonists for CCR2, for which multiple binding sites were discovered. This chapter presents four orthosteric antagonists, including INCB3344, and two allosteric antagonists, including CCR2-RA-[R].

The binding site of the allosteric antagonist CCR2-RA-[R] was discovered to be located at the intracellular side of the receptor. **Chapter 4** presents the amino acid residues in the receptor involved in binding of CCR2-RA-[R], which were revealed by means of an experimental and computational approach.

Besides the orthosteric and allosteric binding sites of the antagonists in Chapters 3 and 4, yet another binding site for CCR2 small molecule inhibitors was discovered. Modulation of CCR2 via this site by amiloride analogues as well as sodium ions is described in **Chapter 5**.

Chapter 6 is focused on the discovery of novel orthosteric antagonists. This chapter describes how the residence time of CCR2 antagonists was increased by specific and small structural changes. This type of structure-kinetics relationships (SKR) should be incorporated in hit-to-lead optimization in order to increase the discovery of clinically effective CCR2 antagonists.

The research presented in these chapters reveals that binding sites for small molecule ligands are present throughout the entire transmembrane domain of CCR2. Therefore this thesis literally presents the *ins* and *outs* of ligand binding to CCR2. These results and its forthcoming opportunities for drug discovery are concluded and discussed in **Chapter 7**.

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