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## The ins and outs of ligand binding to CCR2

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# Chapter 7

**Conclusions and future perspectives**

In this thesis the molecular pharmacology of the chemokine receptor CCR2 has been extensively studied and discussed. Insights in the mechanism of action of novel as well as existing drug-like molecules for CCR2 were presented. This chapter concludes the findings of these studies, to then discuss its impact on drug discovery and to reflect on future directions of this research field.

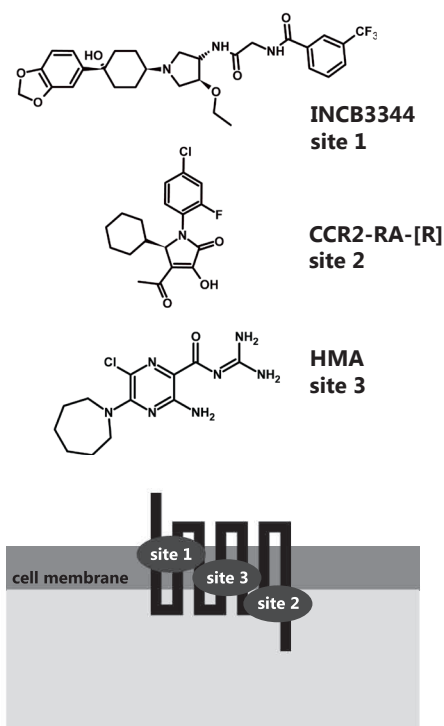
## Conclusions

### Multiple binding sites for small molecules at CCR2

CCR2 is a membrane-bound receptor protein that transduces signals into the cell due to direct interactions with a number of signalling molecules. It is activated by its endogenous chemokine ligands at the extracellular side, which each most likely bind in a slightly different manner to the extracellular and TM domains, given their distinct effects (Chapter 2). Receptor activation allows interactions with large proteins such as G proteins and  $\beta$ -arrestins at the intracellular side of the GPCR, which is often preceded or followed by additional GPCR-interacting proteins (GIPs) [1-3]. Therefore, CCR2, as well as all other GPCRs, naturally possesses multiple binding sites for ligands or proteins. In my thesis I conclude that such distinct binding sites also exist for chemically-derived small molecule ligands for the chemokine receptor CCR2 (Chapter 3). Not only do these ligands bind to the well-established binding sites at the extracellular or TM domain regions, but also to the core region of the receptor and at a hitherto unknown intracellular binding pocket (Chapters 4 and 5, Fig. 1).

### Novel routes towards insurmountable inhibition of CCR2

Throughout this thesis I have presented data on the mechanism of action of orthosteric antagonists and allosteric antagonists. In Chapter 3 it was described that the allosteric antagonists act in a noncompetitive manner with respect to the endogenous chemokine CCL2. This implies that the maximal response of the receptor is suppressed even when high concentrations of the agonist CCL2 are present; a phenomenon that has been described as insurmountable antagonism in *in vitro* functional assays [4, 5]. Due to this property, insurmountable antagonists are proposed to be highly clinically relevant. This situation is particularly relevant for chemokine receptors, since chemokine levels are highly increased (1-10 nM) during inflammatory conditions [6-8]. In addition, as discussed in Chapter 2, chemokines are sequestered on glycosaminoglycans (GAGs) near the chemokine receptors, thereby creating a depot in close proximity to the receptor that further increases the local concentration of these ligands. Leukocytes expressing CCR2 that pass the site of inflammation, will only be inhibited from chemotaxis towards these sites if a drug can bind irrespective of the high local chemokine concentrations; a requirement that is fulfilled by the allosteric insurmountable antagonists described in Chapters 3 and 4. The binding site of these antagonists was discovered to be located at the intracellular side of the receptor. Since the surrounding amino acid residues of these ligands have now been revealed (Chapter 4), this may facilitate the design of novel and improved allosteric insurmountable antagonists.



**Fig. 1.** Schematic representation of CCR2 with its multiple ligand binding sites throughout the entire transmembrane domain. A representative ligand for each of these binding sites is depicted at the top.

An allosteric mode of action is not the only possibility to obtain insurmountable antagonism, since orthosteric antagonists with a long residence time on the receptor can manifest a similar inhibition profile [5]. In this latter scenario, the orthosteric antagonists are bound to the receptor for such a long time that they prevent agonists from binding and thereby diminish their maximum effect. The structure-kinetics relationship (SKR) presented in Chapter 6 is among the first examples of a comprehensive medicinal chemistry approach that aims to increase ligand residence times. Small structural changes prolonged the residence time of these antagonists, revealing the first molecular determinants that can lead to insurmountable antagonism via this orthosteric binding pocket. Importantly, SKR-driven ligand optimization resulted in final compounds distinct from a situation in which the affinity would have driven the optimization process. This emphasizes that SKR should be incorporated in early stages of hit-to-lead optimization in order to identify long residence time antagonists.

In summary, it can be concluded that two distinct paths towards insurmountable antagonism for CCR2 have been paved.

## Future perspectives

A variety of pharmacological topics are covered in this thesis, ranging from allosteric modulation and insurmountability to ligand binding kinetics and biased signalling. In addition, the functioning and regulation of the intertwined chemokine receptor system was discussed, along with the challenges to target these receptors in the clinic. The following sections will discuss some future perspectives of all these findings for targeting CCR2, and GPCRs in general.

### CCR2 as a single drug target

This thesis is mainly focused on the molecular pharmacology of CCR2, without emphasis on any particular disease state to which this receptor is associated. However, the complexity and functioning of the chemokine receptor system was discussed in Chapter 2, and should also be taken into account while speculating on the future directions of disease management through CCR2 antagonism. For example in rheumatoid arthritis (RA), patients suffer from joint pain, stiffness, and swelling as a result of synovial inflammation. CCR2 is clearly involved in this disease, concluded from many *in vitro* studies as well as *in vivo* animal studies in which knock-out or pharmacological blockade of CCR2 decreased disease symptoms [9]. Nevertheless, no clinical trial targeting CCR2 in this disease has been proven successful so far [10]. There can be multiple reasons for this failure, including inappropriate drug-receptor kinetics or molecular mechanisms of action, i.e. surmountable vs. insurmountable antagonism, as discussed in Chapters 3, 4 and 6. Additionally, in case of these complex inflammatory diseases it must be remembered that multiple chemokines as well as multiple chemokine receptors are involved, which can in turn have both synergistic and counteracting modes of action (Chapter 2). In case of RA, monocytes in peripheral blood of RA patients expressed higher levels of CCR2 than CCR5 [11]. In contrast, substantially higher expression of CCR5 compared to CCR2 was observed on macrophages in synovial fluid [11]. Yet another study revealed that neither CCR2 nor CCR5 antagonists were able to block chemotaxis of monocytes towards synovial fluid of patients, but inhibition of CCR1 was proven to be effective [12]. In this particular case it should be identified which receptor(s) are critical for the migration of monocytes towards the synovial compartment in RA in order to know which receptor(s) to target [13].

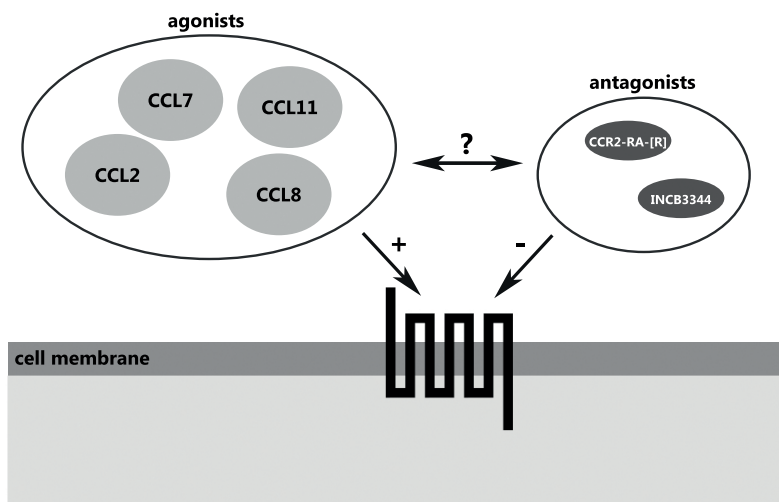
There are several other diseases for which multiple chemokine receptors have been found to be involved, which has stimulated the development of dual antagonists, mainly for CCR2 and CCR5 [14, 15]. The dual CCR2 and CCR5 antagonist cenicriviroc has made most progress so far, since it is has entered phase 3 clinical trials for the treatment of HIV-1 infection,

and is currently also in preclinical development for fibrosis, graft-versus-host disease, and other indications in which CCR5 and CCR2 play a role [14, 16]. Future results of these ongoing trials will demonstrate whether dual inhibition is the key to successful treatments for these and other diseases, like RA (see above). For this particular disease, one could think of dual/triple antagonists that additionally target CCR1, which does not seem impossible in view of the high sequence similarity of CCR1, CCR2 and CCR5 [17]. Regarding this polypharmacology as an approach [18], the development of dual intracellular antagonists could be another way to proceed, given the evidence that such a binding pocket is at least conserved among the highly homologous chemokine receptors CCR2 and CCR5 (Chapter 4).

### **The INS of ligand binding to GPCRs**

**Intracellular CCR2 antagonists.** In Chapter 4 the discovery of an intracellular binding pocket for small molecule antagonists of CCR2 was presented. This is the first time that such a binding pocket was extensively mapped on a GPCR by means of mutations, and visualized in a homology model of CCR2 upon induced-fit docking of the antagonist CCR2-RA-[R]. The most recent review on CCR2 small molecule antagonists includes approximately 30 different structural scaffolds, of which only 10% is likely to bind to the intracellular pocket based on structural similarity with the antagonists presented in this thesis [19]. Therefore, the identification of this binding pocket in Chapter 4 will allow chemists to further explore this allosteric site. In addition, the radioligand binding studies in Chapter 3 revealed that the intracellular and orthosteric antagonists enhanced binding of each other to CCR2. Hence, it would be interesting to further study the nature of this enhancement, since a similar level of inhibition might be achieved with lower doses of both antagonists due to a synergistic mode of action.

**Allosteric modulation of CCR2 signalling by intracellular ligands.** Now that the binding properties of the intracellular CCR2 antagonists have been elucidated, several future research questions arise with respect to the effect of these allosteric ligands on a functional level. As mentioned in Chapter 2, CCR2 has been found to function as both a monomer and a (hetero)dimer. The allosteric intracellular antagonists might differentially inhibit these multimeric complexes compared to the orthosteric antagonists. In addition, a total of eight chemokine ligands can bind to CCR2, all exerting distinct actions as described in Chapter 2. The intracellular antagonists might differentially inhibit these endogenous chemokines compared to the competitive orthosteric antagonists (Fig. 2), and depending on their role in disease states this could have an impact on their efficacy *in vivo*.



**Fig. 2.** CCR2 can be activated by multiple endogenous chemokines, including CCL2, CCL7, CCL8 and CCL11. In this thesis multiple antagonists with a distinct binding site and mechanism of action were discovered, among which the orthosteric antagonist INCB3344 and the allosteric antagonist CCR2-RA-[R]. It remains a question for further research how these two distinct antagonists affect the receptor activation that is induced by the multiple endogenous chemokines.

Besides a distinct action in the presence of the different chemokine ligands for CCR2, one could also hypothesize that a small molecule ligand might exert biased antagonism itself, in which it inhibits certain signalling pathways better than others [20, 21]. There is one study that reported such behavior for a set of 24 CCR2 antagonists, in which inhibition of CCL2 stimulation was compared in five different functional assays [22]. Although the structures of these antagonists were not reported, and the effect in a functional assay is dependent on the off-rate of the antagonists and the incubation time of the assay, it is at least a first indication of biased antagonism for CCR2. Now that the receptor's distinct antagonist binding sites have been revealed, this phenomenon should be further investigated.

**Intracellular CCR2 antagonists *in vivo*.** Speculations on distinct *in vivo* effects of allosteric antagonists compared to orthosteric antagonists can be made based on several clinical studies with CCR2 antagonists. Most of the trials involve orthosteric antagonists, and in many of these studies enhanced CCL2 plasma levels were previously reported upon administration of the antagonists, including for INCB3344 [10, 23, 24]. These enhanced CCL2 levels could in turn diminish the inhibitory action of the administered antagonist due to direct competition. In contrast, no elevation of systemic CCL2 levels was observed in a recent phase II study in type 2 diabetics with the antagonist CCX140-B [25, 26]. Although the chemical identity

of CCX140-B has not been revealed, it is likely to be an allosteric (intracellular) antagonist since the associated patent describes a chemical scaffold that closely resembles SD-24 from Chapter 4 [27]. The allosteric nature of this ligand might be the cause of different modulation of CCL2 plasma levels compared to orthosteric antagonists. As described in Chapter 2, CCL2 is scavenged by CCR2 *in vivo* [28]. This implies that the CCR2-CCL2 complex can be internalized to regulate the level of CCL2 in the extracellular environment. So far, the effect of small molecule antagonists on this internalization pathway has not been studied for CCR2. However, a very recent *in vitro* study that compared orthosteric and intracellular antagonists of CCR4 revealed that orthosteric antagonists were able to induce internalization of CCR4, whereas intracellular antagonists left CCR4 surface expression unaffected [29]. Whether this phenomenon is also applicable to CCR2, linking the unaffected plasma levels of CCL2 to a lack of CCR2 internalization by an allosteric mode of inhibition, remains to be determined. However, these studies indicate that orthosteric and allosteric antagonists may differentially inhibit the CCR2-CCL2 axis *in vivo*.

**Intracellular ligands for GPCRs.** During the last couple of years the intracellular region of GPCRs has become of increasing interest, either for therapeutic interventions or to study receptor functioning in general [30, 31]. Indications of intracellular GPCR binding pockets have been reported previously for CCR4, CCR5, CXCR1, CXCR2 and PAR1 [32-35]. In addition, the intracellular CCR2 antagonists CCR2-RA-[R], JNJ-27141491 and SD-24 from Chapters 3 and 4 have been reported to also bind to CCR1 [36-38]. Together these data suggest that an intracellular binding pocket for small molecule antagonists could be a shared feature among chemokine receptors, or even GPCRs in general. Such an intracellular antagonist binding pocket could be highly interesting for certain GPCRs, for example for those that have their endogenous ligand tethered at the extracellular side, like the PAR1 receptor [39]. These tethered ligands directly compete with orthosteric antagonists, preventing them to sufficiently block the receptor. Besides receptor blockade with long residence time orthosteric antagonists, inhibition via an intracellular allosteric site would be beneficial for targeting such receptors. For PAR1 this is likely to be possible, since indications of an intracellular binding pocket have been reported for this receptor [34].

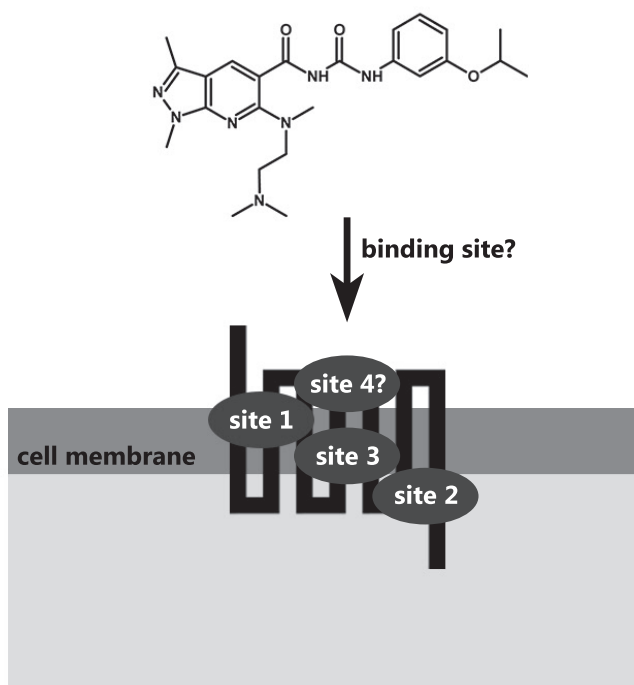
Intracellular antagonists act at the interface of the receptor and its signalling molecules, and therefore they most likely act as inverse agonists, inhibiting the basal activity or constitutive signalling of the receptor. This is indeed the case for the intracellular CXCR2 antagonist Sch527123 [32, 40], which has been found efficacious in a Phase II clinical trial for asthma [41]. Although the extent of constitutive activity in disease states remains largely

unknown, many GPCR antagonists are in fact inverse agonists [42]. Some of these drugs have been presented to display higher clinical efficacy than neutral antagonists targeting the same receptor [43, 44].

Last but not least, it is tempting to speculate on possibilities to enhance or activate GPCR signalling via the intracellular side. Small lipidated peptide sequences named pepducins have been proposed to act via the intracellular side of the receptor, and can thereby activate, inhibit or modulate various GPCRs, including the chemokine receptor CXCR4 [31, 45, 46]. In Chapter 4 the intracellular binding pocket for CCR2 small molecule antagonists was identified, which is surrounded by TM-I, II, VI and VII. One of the possible mechanisms of inhibition by these antagonists is the fixation of TM-VI, since relatively large outward movements of TM-VI are necessary for activation of the receptor [47, 48]. It would be interesting to study whether small molecule agonists can be designed that favor this outward movement of TM-VI, thereby creating a binding site for signalling proteins such as the G protein [49].

### **The OUTS of ligand binding to CCR2**

Several orthosteric CCR2 antagonists were described in Chapter 3, of which INCB3344 was radiolabeled and further studied. The binding site of other orthosteric antagonists was previously reported to reside in the upper half of the TM domain [50]. A similar location for binding of INCB3344 was confirmed in Chapter 4, where the conserved glutamate residue E<sup>7.39</sup> was identified to be important for binding. In close proximity to this binding site, an additional binding pocket for small molecule ligands and sodium ions was discovered (Chapter 5). This pocket is located in the core domain of the receptor, and allows amiloride analogues and sodium ions to modulate CCR2. Besides targeting one particular antagonist binding site, inhibition via multiple pockets simultaneously might offer additional opportunities to antagonize CCR2. This could for example be established via so-called bitopic ligands that would be able to bind both the binding pocket in the core domain as well as the orthosteric binding pocket [51].



**Fig. 3.** Chemical structure of the most potent heteroaroylphenylurea CCR2 antagonist described by Laborde *et al.* [56]. This antagonist inhibits CCR2 in a different manner than the antagonists that bind to sites 1, 2 and 3, and was previously suggested to interact with extracellular domains of the receptor. This raises the question if a fourth small molecule binding site exists in CCR2.

Binding and activation of CCR2 by its endogenous chemokines is highly dependent on the extracellular loops (ECLs) as well as the N-terminus [52]. The role of the ECLs in antagonist binding to CCR2 has not yet been deciphered, but the results on chimeric CCR2-CCR5 receptors in Chapter 4 suggest that these loops may also be involved in the binding of the orthosteric antagonist INCB3344. A general analysis of the extracellular domains for class A GPCRs has previously revealed that these parts of the receptor can host allosteric binding sites [53]. For CCR2, there is one particular antagonist described in literature that has been suggested to mediate its inhibitory action via extracellular domains of the receptor [54, 55]. This heteroaroylphenylurea CCR2 antagonist is structurally different from all of the antagonists reported in this thesis [56], and is also unique compared to all other CCR2 antagonist chemotypes [19]. Interestingly, this antagonist does not inhibit binding of CCL2 to the receptor, while it inhibits CCL2-induced chemotaxis *in vitro* as well as *in vivo* [56]. A lack of chemokine displacement was previously also reported for the CCR5 antagonist aplaviroc

[57] as well as the CXCR1 antagonist repertaxin [58], and is indicative of an allosteric mode of action. Whether this heteroaroylphenylurea antagonist indeed binds to a pocket within the extracellular domains remains to be determined (Fig. 3), but it does inhibit the receptor in a very distinct manner compared to the antagonists described in this thesis.

### Keeping up with kinetics

In Chapter 6 the kinetics of antagonist binding to CCR2 were explored. Although this study was solely focused on orthosteric antagonists, a similar approach can be applied to the intracellular antagonists in the future. In fact, it has already been demonstrated that intracellular antagonists have potential to result in prolonged receptor blockade, since a residence time of 29 hours at room temperature was previously measured for the intracellular CXCR2 antagonist Sch527123 [59].

Long residence time antagonists are proposed to lead to enhanced clinical efficacy and patient compliance, due to a prolonged receptor blockade, increased selectivity and a decreased dosing frequency [60]. Recently a short residence time antagonist for CCR2 was found to enhance vaccine immunity only after a multi-dose treatment regime, due to its poor pharmacokinetic properties [61]. Increasing the residence time of such ligands could lead to longer-lasting *in vivo* blockade, as was previously also reported for antagonists of the NK1 receptor [62]. To identify whether administration of long residence time antagonists results in more efficacious CCR2 blockade *in vivo*, comparative studies with short residence time antagonists should be performed. Additionally, long residence time antagonists can be used for structure elucidation of CCR2, since attempts to crystallize GPCRs have been proven to be more successful in the presence of long residence time ligands that stabilize the receptor complex [63].

Although several marketed and highly efficacious drugs were determined to slowly dissociate from their target receptor in retrospect [64], it is not solely the receptor residence time that is related to the clinical efficacy and duration of action [65]. Factors such as the association rate of a drug to its receptor, plasma protein binding, pharmacokinetics and rebinding of a drug to its target all contribute to the level of receptor occupancy and the duration of the effect [66-70]. Hence, chemists, pharmacologists and computational biologists should join forces and study the influence of drug binding kinetics on drug action at a molecular as well as a systems level in order to unravel the determinants for clinical success per target and disease [69, 71].

## **Final notes**

Altogether the molecular mechanisms of action of orthosteric and allosteric antagonists for CCR2 have been explored and detailed throughout this thesis. In addition, these ligands, with their distinct binding sites, were effective tools to study receptor modulation by physiologically relevant sodium ion concentrations and small molecule amiloride analogues. The discovery of the three different ligand binding sites throughout the entire transmembrane domain of the receptor illustrates that a GPCR behaves as an allosteric machine, rather than as the classically described receptor with a ligand binding site at the extracellular side and a signalling domain at the inside of the cell. In concert with the currently expanding insight in the structure and signalling capacities of GPCRs, the data presented in this thesis allow to better fine-tune the pharmacological modulation of the chemokine receptor CCR2, and GPCRs in general.

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