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The ins and outs of ligand binding to CCR2

Zweemer, A.J.M.

Citation
Zweemer, A. J. M. (2014, November 20). *The ins and outs of ligand binding to CCR2*. Retrieved from https://hdl.handle.net/1887/29763

Version:	Corrected Publisher's Version
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Downloaded from:	https://hdl.handle.net/1887/29763

Note: To cite this publication please use the final published version (if applicable).

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Author: Zweemer, Annelien
Title: The ins and outs of ligand binding to CCR2
Issue Date: 2014-11-20

Summary

This thesis has provided novel insights in the molecular mechanism of action of antagonists for the chemokine receptor CCR2. CCR2 belongs to the protein family of G protein-coupled receptors (GPCRs). It is involved in several inflammatory diseases and therefore many small molecule antagonists targeting this receptor have been developed over the years. Unfortunately all clinical candidates tested so far appeared to lack efficacy in man, which stresses the need for a better understanding of their mechanism of action.

Chapter 1 introduces the main subjects that were discussed in this thesis. Prior to our study of CCR2, the entire family of chemokine receptors and its endogenous chemokines were introduced and discussed in **Chapter 2**. Chemokine receptors are widely expressed on a variety of immune cells and play a crucial role in normal physiology as well as in inflammatory and infectious diseases. The existence of 23 chemokine receptors and 48 chemokine ligands guarantees a tight control and fine-tuning of the immune system. In this chapter the multiple regulatory mechanisms of chemokine signalling at a systemic, cellular, and molecular level were discussed. In particular, this chapter was focused on the impact of biased signalling at the receptor level, an emerging concept in molecular pharmacology. An improved understanding of these mechanisms may provide a framework for more effective drug discovery and development at a target class that is so relevant for immune function.

A selection of reference CCR2 antagonists was studied in a variety of pharmacological binding and functional assays as described in **Chapter 3**. All these antagonists displaced the endogenous agonist ^{125}I-CCL2 from CCR2 with nanomolar affinity. Two antagonists, INCB3344 and CCR2-RA, were radiolabeled to study the binding site in greater detail. It was discovered that [^{3}H]-INCB3344 and [^{3}H]-CCR2-RA bind to distinct binding sites at CCR2, the latter being the first allosteric radioligand for CCR2. Besides the binding properties of the antagonists, CCR2 inhibition was examined in multiple functional assays, including a novel label-free whole-cell assay. INCB3344 competitively inhibited CCL2-induced G protein activation, whereas CCR2-RA showed a noncompetitive (insurmountable) or allosteric mode of inhibition. These findings demonstrated that the CCR2 antagonists described in this chapter can be classified into two groups with different binding sites and thereby different modes of inhibition.

In **Chapter 4** the binding site of the allosteric antagonist CCR2-RA-[R] was identified. A chimeric CCR2/CCR5 receptor approach was used to obtain insight into the binding site of the allosteric antagonists, and additionally eight single point mutations were introduced in CCR2 to further characterize the putative binding pocket. All constructs were studied in radioligand binding as well as functional IP turnover assays, providing evidence for an intracellular binding site for CCR2-RA-[R]. The most important residues for binding were found to be the highly conserved tyrosine $Y^{7.53}$ and phenylalanine $F^{8.50}$ of the $NPxxYX_{(5,6)}F$ motif, as well as $V^{6.36}$

at the bottom of TM-VI and $K^{8.49}$ in helix-VIII. In addition, the antagonists JNJ-27141491 and SD-24 were identified to bind at the same binding pocket as CCR2-RA-[R], albeit with distinct orientations. This chapter demonstrated for the first time the presence of an allosteric intracellular binding site for CCR2 antagonists.

In **Chapter 5**, yet another binding pocket was revealed via which amiloride analogues and sodium ions were discovered to modulate CCR2. In radioligand binding studies the amiloride analogue HMA allosterically inhibited binding of the agonist ^{125}I-CCL2, the orthosteric antagonist [^{3}H]-INCB3344 and the intracellular antagonist [^{3}H]-CCR2-RA-[R]. Differently, sodium ions only allosterically inhibited ^{125}I-CCL2 binding, while they enhanced binding of [^{3}H]-CCR2-RA-[R]. Three residues located in the core of the transmembrane domain, $D^{2.50}$, $W^{6.48}$ and $H^{7.45}$, turned out to be important for modulation of the antagonist radioligands, since mutation of these residues abolished or diminished the allosteric effects induced by HMA and sodium ions. Upon induced-fit docking of HMA in a homology model of CCR2, its interactions with $D^{2.50}$, $W^{6.48}$ and $H^{7.45}$ were visualized, and additional surrounding residues of this binding pocket were predicted.

Chapter 6 was focused on the design of high-affinity and long-residence-time orthosteric CCR2 antagonists, which share the same binding site as INCB3344. A new competition association assay was developed for CCR2, which allowed investigation of the relationship between the structure of the ligand and its receptor residence time (i.e., structure–kinetic relationship (SKR)) next to a traditional structure–affinity relationship (SAR). By applying combined knowledge of SAR and SKR, the hit-to-lead process of cyclopentylamines as CCR2 antagonists was re-evaluated. Affinity-based optimization yielded a compound with good binding (K_i = 6.8 nM) but very short residence time (2.4 min). However, when the optimization was also based on residence time, the hit-to-lead process yielded a new high-affinity CCR2 antagonist (3.6 nM), with a residence time of 135 min.

In summary this thesis revealed novel *ins* and *outs* of ligand binding to CCR2, presenting three separate binding pockets via which this receptor can be pharmacologically modulated. Different routes towards insurmountable antagonism of CCR2 were described, either via noncompetitive or via long residence time antagonists, as concluded in **Chapter 7**. These results may allow a more rational design of future antagonists, and are equally important to understand the outcomes of studies with existing CCR2 antagonists. 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.