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

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SUMMARY

Chemokine receptors, including CCR1, CCR2 and CCR5, have been implicated in many inflammatory and immune diseases, such as atherosclerosis, rheumatoid arthritis and cancer. Thus, numerous drug discovery efforts have been made to develop drugs for these receptors. Yet, only one—maraviroc, targeting CCR5—has reached the market, while all other drug candidates have failed in clinical trials mostly due to lack of efficacy. Therefore, it is crucial to develop novel tools and concepts that allow us to better study and target these receptors in early phases of drug discovery, in order to develop safer and more efficacious drug candidates. **Chapter 1** introduces chemokine receptors, and the chemokine system in general, as potential drug targets, as well as the difficulties encountered when targeting such a complex system. In addition, this chapter introduces the main concepts studied throughout this thesis, including allosteric modulation, insurmountability, target residence time, multitarget and covalent inhibition.

While orthosteric ligands bind to the same binding site as the endogenous ligands, allosteric ligands bind to a nonoverlapping, spatially distinct site. All allosteric ligands described in this thesis bind to a highly conserved intracellular pocket, which (partially) overlaps with the binding site of intracellular signaling effectors, such as G proteins or β-arrestins. Thus, **Chapter 2** reviews the evidence for such an intracellular pocket among chemokine receptors and other class A G protein-coupled receptors (GPCRs). In addition, the structural features of this binding site, the different strategies to target it, and the potential advantages of intracellular ligands are discussed. One of the first crystal structures to provide direct evidence of an intracellular binding site is that of CCR2, described in **Chapter 3**. We solved the X-ray crystal structure of CCR2 in complex with two antagonists, BMS-681 and CCR2-RA-[*R*]. The structure showed that BMS-681 binds at the so-called orthosteric binding site, where the endogenous chemokines also bind. In this way, BMS-681 appears to inhibit chemokine binding by directly competing for the same binding site. In contrast, CCR2-RA-[*R*] binds to an intracellular pocket located more than 30 Å away from the orthosteric site. Thus, CCR2-RA-[*R*] inhibits chemokine binding in a noncompetitive, insurmountable manner, while it directly interferes with the binding of signaling effectors. Simultaneous binding of both ligands was not only crucial to achieve crystallization of CCR2, but it resulted in a highly inactive conformation of this receptor. Overall, this structure provides novel insights on how to better target CCR2, and chemokine receptors in general.

As this intracellular binding site is highly conserved among chemokine receptors, it was used for the design and development of 'multitarget' ligands, i.e. ligands that inhibit multiple

receptors. Thus, in **Chapter 4** we aimed to determine if CCR1 could also be targeted with intracellular antagonists. Using [^{3}H]-CCR2-RA-[*R*] as intracellular tool, we found that this compound also binds to CCR1 with high affinity. We synthesized around 40 different CCR2-RA-[*R*] analogues, which were further characterized in both CCR1 and CCR2 in order to perform a structure-activity relationships (SAR) analysis for both receptors. This strategy allowed us to identify ligands with higher selectivity towards CCR1 or CCR2, but also ligands with potential multitarget activity. Moreover, these intracellular ligands behaved as inverse agonists in CCR1, as they were able to decrease the basal activity of this receptor. Due to the high constitutive activity of CCR1, the development of inverse agonists for CCR1 is highly relevant. In addition to CCR1/CCR2 ligands, in **Chapter 5** we focused on the development of intracellular CCR2/CCR5 ligands. For this, we focused on a triazolo-pyrimidinone scaffold, which displayed the best activity in both receptors in comparison with other intracellular ligands. Next, we performed an SAR study in both receptors by synthesizing and characterizing a series of triazolo-pyrimidinone derivatives. Although most derivatives displayed much higher selectivity towards CCR2, we also found some compounds with improved activity towards CCR5, and thus, improved dual activity. Their mechanism of inhibition was also investigated, providing evidence that these ligands display insurmountable antagonism, as they led to a significant decrease in receptor activity even at the highest concentration of agonist tested.

Chapter 6 focuses on the design and pharmacological characterization of the first intracellular covalent ligand for CCR2. Based on the structure of a known intracellular ligand for CCR2, we designed a potential covalent ligand containing a reactive thiocyanate group. To validate its covalent binding mode, we used a variety of radioligand binding and functional assays, which showed that, even after extensive washing, this ligand remains bound and functional in CCR2. Furthermore, we used computational modelling followed by a mutagenesis study in order to identify the amino acids responsible for such covalent interaction. The results from this study point to one cysteine residue as the main interaction point, although other potential secondary interaction sites are also suggested.

In **Chapter 7** we investigated whether a previously characterized 'long residence-time' orthosteric CCR2 antagonist was efficacious in atherosclerosis. For this, the affinity and binding kinetics of this antagonist were characterized in murine CCR2, while its efficacy was determined in an apolipoprotein E-deficient mouse model of atherosclerosis. Treatment with the antagonist resulted in a significant reduction of monocytes recruitment and plaque size at both the carotid artery and the aortic root. Furthermore, pharmacokinetic data and calculation of occupancy levels showed that this compound achieved full receptor occupancy for a prolonged time. Lastly, **Chapter 8** summarizes the main conclusions and future perspectives that have been derived from the research presented in this thesis.