From every angle: novel insights into CC chemokine receptor 2 pharmacology

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Chapter 6

Conclusions and future perspectives

1. Conclusions

Allosteric modulation of CCR2

Most prospective drugs targeting chemokine receptors including CCR2 have failed in various stages of clinical trials, mainly due to inefficacy, which shows the need for alternative targeting strategies. In **chapter 2**, the pharmacological characterization of intracellular allosteric modulators for chemokine receptors was discussed. Advantages of allosteric modulators include insurmountability, increased selectivity and a controlled manner of (partial) receptor activation or inhibition, including attenuation of receptor signalling through probe dependence and biased signalling. A number of published small molecules were included if they showed clear signs of allosteric modulation *in vitro*, as well as a confirmed topologically distinct binding site compared to chemokines. Ligands were discussed in terms of their (kinetic) binding profile or their effect on signalling events in functional assays to substantiate possible assay outcomes with hypothetical graphs as a starting point for the discovery and characterization of novel allosteric ligands for chemokine receptors. It became clear that multiple assays are necessary to confirm (non-)competitiveness compared to the chosen orthosteric or allosteric ligand. Furthermore, the nature of the allosteric ligand (e.g. negative vs positive allosteric modulator) should be confirmed in (multiple) biological assays. Finally, the binding site could be elucidated to be topographically distinct from the chemokine binding site with the help of computational methods, and confirmed through site-direct mutagenesis or structure elucidation of the receptor with the ligand.

An understudied allosteric binding site in chemokine receptors is the sodium ion binding site. This site is characterized by the highly conserved aspartic acid $D^{2.50}$ (Ballesteros-Weinstein numbering[1]) and allows modulation of class A GPCRs by sodium ions.[2] Raised local sodium ion concentrations compared to healthy tissues were found in a number of solid tumours.[3–6] CCR2 also plays a prominent role in cancer (see *Cancer-associated mutations and CCR2*). We found the CCR2 sodium binding site to be atypical compared to e.g. A_{2A}AR[7], with a diverging subset of residues in the binding pocket. Also sodium ion modulation was only seen for CCL2 binding or at high concentrations of [^{3}H]CCR2-RA-[*R*] (**chapter 5**), unlike A_{2A}AR. The amiloride derivate 6-substituted Hexamethylene Amiloride (HMA) which has generally been confirmed to bind the sodium ion binding site in other GPCRs, showed allosterism compared to both the orthosteric ligand [^{3}H]INCB3344 and the intracellular ligand [^{3}H]CCR2-RA-[*R*], confirming the existence of a third binding site. Molecular

dynamics simulations with HMA and CCR2 showed multiple conformations of the ligand, which insinuated a more spacious sodium ion binding site compared to other class A GPCRs. Introduction of mutations in the CCR2 sodium ion binding site resulted in the mutants being inadequately expressed or functional, indicating that the makeup of this site is crucial for receptor integrity. Only G123A$^{3.35}$ and G127K$^{3.39}$ were expressed to a similar extent as WT *and* were functionally intact. The residues in the sodium ion binding site are distinct compared to CXCR's and other class A GPCRs, and represent an opportunity for increased selectivity. This novel binding site could be explored as a novel druggable binding site by e.g. bitopic ligands.

Cancer-associated mutations and CCR2

CCR2, and its main cognate ligand CCL2, are highly involved in later stages of malignant cancer development, including metastasis formation, immune suppression, and angiogenesis.[8] The receptor would therefore be an excellent target to minimize tumour growth and spread. However, no efficacious drugs are yet on the market due to a plethora of reasons. In **chapter 3**, we examined the effect of cancer-associated mutations in CCR2 on receptor function and drugability using an extracellular orthosteric and an intracellular allosteric (see also **chapter 2**) radioligand as prototypical drugs. Mutations were selected based on their location in or near small molecule binding sites in the receptor, as well as their occurrence in highly conserved regions. Most mutations were disadvantageous for receptor expression or function, and mutations near either binding site severely impacted ligand binding. As CCR2 is an attractive drug target in cancer, the existence of cancer-associated mutations with impact on receptor function and targeting should be taken along in the drug discovery process.

Visualizing CCR2

The development of antibodies targeting GPCRs has been infamously difficult.[9] The use of affinity-based probes (AfBPs) could provide a valuable alternative.[10] In **chapter 4**, the design and validation of the first AfBP, probe **6c**, for CCR2 is described. This is also the first probe binding to an intracellular allosteric binding site on GPCRs to-date. Due to the incorporation of a click handle, we were able to use this probe in a variety of experiments based on the reporter tag attached *via* click chemistry. At low concentrations the probe

showed highly specific labelling of the receptor in SDS-PAGE with a fluorescent tag, and LC/MS-based proteomics experiments with a biotin tag. We also showed its application as a tool for ligand characterization and detection of post-translational modifications (PTMs) of CCR2. Finally, using cancer cell lines which endogenously express CCR2, we provided clear evidence that this probe could effectively label CCR2 in low-expression systems. All in all, probe **6c** was shown to be highly versatile and selective, and therefore a useful novel tool for CCR2 drug discovery.

2. Future perspectives

Precision medicine for chemokine receptors in cancer

Mutations in GPCRs have been found in ~20% of human solid tumours.[11] We have shown in **chapter 3** that these mutations can alter receptor functionality and drugability. It would be beneficial for patients to screen cancer biopsies for targets and possible mutations therein.

CCR2 represents a valuable drug target in cancer due to its involvement in various malignant cancer processes, such as metastasis formation and immune suppression.[8] Inhibiting CCR2 on tumour cells would hypothetically result in a tumour which is growing slower, and metastasis development is decreased. A few clinical trials are currently in progress to target CCR2 in cancer (NCT04123379, NCT03496662). The first is a study pertaining Programmed cell death protein 1 (PD-1)-receptor inhibitor nivolumab in combination with an undisclosed CCR2/CCR5 inhibitor against non-small cell lung cancer or hepatocellular carcinoma. The second is a combination of ribonucleotide reductase inhibitor gemcitabine/ nanoparticle albumin-bound paclitaxel in combination with nivolumab and CCR2/CCR5 antagonist BMS-813160 for pancreatic ductal adenocarcinoma (PDAC). However, mutations are not considered in these studies and could drastically alter patient outcome.

CCR2 expression has been correlated to poor patient health and survival.[12,13] Determination of (functional) CCR2 expression could therefore be beneficial to prevent patient suffering from side effects and ineffective treatments. Clinical trials are ongoing for screening of CCR2 expression using positron emission tomography (PET) in patients with PDAC (NCT03851237) and head and neck cancer (NCT04217057). The latter is a ^{64}Cu radiolabelled tracer based on the ECL1 of CCR2. An alternative for in patient screening of CCR2 could

be screening biopsy samples with probe **6c** (**chapter 4**, see also **Probes for CCR2**). As we showed in **chapter 3** that cancer-associated mutations could alter small molecule binding, it is possible that these molecules for screening could also be affected. No binding of CCR2 could therefore rule out the receptor as a potential target.

Probes for CCR2

The application of the probe described in **chapter 4** could provide an alternative for CCR2-targeting antibodies, which are generally difficult in their use and development[9], in *in vitro* or *ex vivo* settings. As such, the assays described in **chapter 4** could be further expanded to answer a variety of research questions. For example, SDS-PAGE experiments could be performed as an alternative for western blot or enzyme-linked immunosorbent assay (ELISA) experiments. For CCR2, these assays rely on antibodies directed towards a tag introduced into the protein for reliable protein expression detection. Probe **6c** could be used for native protein without possible interference by a tag. In addition, due to its high sensitivity, proteomics could be used to examine complex interactions and modifications that would go unnoticed in SDS-PAGE due to their small size in comparison to the protein. Expansion of the proteomics experiments can also be used to further our understanding of CCR2 biology. For example, exploring PTMs and protein-protein interactions, which has been done before with enzyme-targeting activity-based probes.[14]

These experiments could also be expanded towards fixed cell-based assays such as fluorescence-activated cell sorting (FACS) and microscopy experiments to determine receptor expression and distribution in a number of cell types. This has been demonstrated for example for the cannabinoid CB_2 receptor with a photoaffinity probe for FACS and the Adenosine A_3 Receptor for fluorescent microcopy.[15,16] It is important to note that the CuI used as a catalyst in the click reaction is toxic to cells. However, this can be circumvented by fixation of the cells or modification of the protocol for a reduction in CuII or a copper-free click reaction.[17,18]

Finally, probe **6c** could be used to confirm CCR2 expression in patient derived material, such as cancer biopsies.[19] This is caused by conformational variability and a small exposed target area of the receptors. Hence, the AfBP discussed in **chapter 4** is a suitable substitute as it is unaffected by these factors.

In addition to the currently described probe, further efforts could be made to develop orthosterically binding AfBP for probes for CCR2. This would take away the need for cell penetration and thus potentially increasing its signal. Well known orthosteric CCR2 antagonists include BMS-681 and INCB3344[20,21], which could be starting points for future optimizations. Another interesting option would be to develop an AfBP based on the synthetic CCR2 agonist J113863.[22] Although J113863 is a low potency ligand for which pharmacology on CCR2 is still unfamiliar, docking of the enantiomer UCB35625 into CCR1 reveals its potential binding mode.[23] A cycloheptyl exposed on the extracellular side could be a potential location for modification of this ligand into an AfBP, which could allow to monitor (mutant) receptor internalization in real-time using confocal microscopy.

To study native CCR2 in a cellular environment, probe **6c** may not be sufficient. This probe is based on a negative allosteric modulator[24], which could interfere with receptor signalling (see **chapter 2**). Other receptor tags such as green fluorescent protein (GFP)[25], are bulky and might hinder receptor functionality. Hence, ligand-directed probes (LDPs) could be designed targeting CCR2.[26] LDPs are non-covalent ligands decorated with a fluorescent moiety attached to a cleavable electrophile.[27] A nucleophilic residue near the binding pocket reacts with the electrophilic group, after which the fluorescent moiety is transferred to the protein of interest. For GPCRs, CB_2R and $A_{2A}R$ were visualized using an LDP using fluorescence microscopy.[26,28] In addition, tagging of interacting proteins such as the G protein or β-arrestin could allow us to perform Förster resonance energy transfer (FRET) assays.[29] In these assays the receptor's C terminus and the interacting protein are tagged with fluorescent proteins between which an energy transfer is measurable. Tagging the receptor with a small fluorescent chemical moiety using an LDP would allow us to follow receptor fate, and signalling events and kinetics with minimal interference to the receptor.

Challenges in CCR2 drug discovery

The chemokine receptor system is tightly regulated[30] and as yet not fully understood. Factors such as post-translational modifications (PTMs)[31], homo- and heterodimerization[30], and receptor fate[32] should be considered in drug design. Confirmed CCR2 PTMs are N-glycosylation of Asn14 and sulfation of Tyr26, and phosphorylation of Tyr139 by Janus Kinase 2 (JAK2).[33,34] CCR2 has been established as a homodimer, or a heterodimer with CCR5

or CXCR4.[30] N-glycosylation and a CCR2 homodimer have been visualized using the AfBP probe **6c** described in **chapter 4**, which could aid in understanding these phenomena.

Furthermore, additional consideration should be given to translational research between animal models and humans to decrease attrition rates.[35] Great care should be taken to select the animal model with high homology in receptor sequence to human CCR2. The long RT ligand 15a successfully inhibited atherogenesis in apoE deficient mice, while CCR2-RA-[*R*] inhibited macrophage recruitment in zebrafish.[36,37] Hence, both represent good options for screening *in vivo* potency of CCR2 inhibitors.

Additionally, inhibition of CCR2 with orthosteric inhibitors has resulted in an increase of its cognate ligand, CCL2.[38] For example, treatment with long RT CCR2 antagonist 15a resulted in an increase in circulatory CCL2.[36] This could cause a vicious cycle of increasing levels of chemokine while targeting of the receptor becomes increasingly difficult due to heightened competition.

Finally, tolerance of chemokine receptor drugs (drug resistance) is an upcoming issue for the limited number of antagonists that have made it to the market.[39] Although mechanisms are still unclear, both maraviroc and plerixafor have shown signs of tolerance. These mechanisms should be further examined to avoid tolerance in future chemokine receptor-targeting drugs.

Opportunities of allosterism in CCR2 drug discovery

It is clear that classic strategies are not suitable for CCR2 drug development. Development of intracellular allosteric ligands for CCR2 has been ongoing, as exemplified by the emergence and optimization of novel scaffolds in the last ten years.[40–42] However, additional characteristics of these ligands could be further explored.

Radioligand binding kinetics for intracellular allosteric ligands

In **chapter 3**, we introduced [^{3}H]LUF7482 as a novel radioligand for the intracellular binding site of CCR2. So far the radioligand [^{3}H]CCR2-RA-[*R*] has been used for this binding site, while it has previously been shown to have biphasic binding kinetics.[20] This makes the radioligand unsuitable for determination of structure-kinetic relationship (SKR) studies, due to the inability

of the mathematical models to fit, i.e. the Motulsky-Mahan equation requires a monophasic fit.[43] Initial data on this [^{3}H]LUF7482 is hopeful, showing a monophasic association to CCR2. This will allow us screen for affinity and binding kinetics of newly synthesized allosteric antagonists at CCR2. For example, optimization of binding kinetics previously led to the long RT orthosteric ligand 15a.[44] Importantly, ligands with a long RT are a way to increase efficacy by extending their functional time on the receptor.[45] Furthermore, the dynamic drug-target interactions could provide a better prediction for *in vivo* efficacy in combination with traditional pharmacological parameters such as affinity or potency.[46] The ultimate long RT ligand binds in a covalent manner, as was previously published for CCR2.[47] However, concerns about adverse effects by covalent ligands have been raised.[48] Therefore, more balanced ligands in terms of association and dissociation rate should be developed. A long RT, intracellular allosteric ligand would lead to prolonged efficacy combined with all the benefits of allosteric ligands (see **chapter 2**).

Exploiting molecular pharmacology of intracellular modulators

Allosteric binding sites of chemokine receptors and GPCRs in general have been gaining attention in recent years. Allosteric ligands represent multiple advantages over orthosteric ligands, including lack of competition with the endogenous ligand(s), insurmountable inhibition and increased specificity, and attenuation of signalling pathways through biased signalling and probe dependence (**chapter 2**).[49,50] Especially the latter are currently understudied in chemokine receptors for intracellular allosteric ligands. With their recent popularity, more attention should be directed to bias introduced by these intracellular allosteric modulators. This includes probe dependence, the affinity or potency of orthosteric ligands can be altered in a pleiotropic system[51], and biased antagonism, where a ligand selectively inhibits one or multiple signalling pathways, yet allows activation of others.[52] Exploitation of these characteristics may lead to safer and more efficacious drugs in the future.

Exploration of further allosteric binding sites

In this thesis, two distinct allosteric binding sites were explored. Namely the intracellular allosteric binding site (**chapter 2**) and the sodium ion binding site

(**chapter 5**). However, additional (possible) binding sites in the extra-helical regions, such as the cholesterol binding site, have not yet been explored for CCR2.[53] These binding sites are relatively small compared to the orthosteric and allosteric binding sites and targeting them may prove difficult. However, bitopic ligands spanning both the allosteric and orthosteric binding site could provide increased stabilization of the receptor and receptor subtype selectivity.[54]

Target elimination

An up-and-coming strategy which could be applied are proteolysis targeting chimeras (PROTACs).[55] These bifunctional molecules consist of a ligand for the target protein connected by a linker to a ligand which interacts with an E3 ubiquitin ligase. This recruits the E3 ubiquitin ligase towards the target protein, which is tagged with ubiquitins for protein degradation. The protein will subsequently be internalized and either recycled or destroyed. This concept can be applied to chemokine receptors and CCR2 in particular, as a range of intracellularly binding ligands (**chapter 2**) are already available which can be modified into PROTACs.

3. Final notes

The development of drugs targeting chemokine receptors including CCR2 has been turbulent. In this thesis, I explored novel avenues on aiding drug development for this receptor, with an emphasis on cancer. We discussed the pharmacological characterization of intracellular allosteric ligands for chemokine receptors, the effect of cancer-associated mutations on CCR2 function and druggability, explored the sodium ion binding site as a third binding site in CCR2 and visualized CCR2 using a novel AfBP. However, many avenues are currently under-investigated and could lead to novel targeting strategies. As CCR2 is involved in so many autoimmune and inflammatory diseases, it is imperative that efforts continue to be made, as a single successful drug could alleviate symptoms or cure innumerable patients.

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