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

Ortiz Zacarías, N.V.

Chapter 8

Conclusions and Future Perspectives

CONCLUSIONS

Crystal structures to advance drug discovery

The first X-ray crystal structure of CC chemokine receptor 2 isoform b (CCR2) in complex with two antagonists, crystallized at 2.8 Å resolution, is described in **Chapter 3**. Engineering of CCR2 resulted in the more stable crystallization construct CCR2-T4L, in which the flexible intracellular loop 3 (ICL3) was replaced by the T4 lysozyme (T4L) fusion protein, among other modifications. However, sufficient stabilization and subsequent crystallization was only achieved after the simultaneous addition of two antagonists: the orthosteric BMS-681 and the allosteric CCR2-RA-[*R*]. The obtained crystal structure (PDB 5T1A, **Chapter 3**) provided insight on the binding mode and mechanism of inhibition of the two antagonists. The structure shows that BMS-681 binds in the minor subpocket of the canonical orthosteric binding site of CCR2, while CCR2-RA-[*R*] binds in a previously suggested intracellular binding site,[1, 2] located ~30 Å away from the chemokine binding site. By binding at this intracellular site, CCR2-RA-[*R*] inhibits the receptor in a noncompetitive manner with regard to chemokine binding, which results in its previously observed insurmountable behavior: CCR2-RA-[*R*] decreased the maximum receptor response even at the highest agonist concentration tested.[2] This is particularly relevant due to the high local concentration of chemokine ligands, such as CCL2, during inflammatory conditions.[3-5]

In addition, the structure suggests a cooperative mechanism of inhibition between the two antagonists: BMS-681 directly interferes with chemokine binding through competition in the orthosteric binding site, but is not associated with G protein coupling, while CCR2-RA-[*R*] directly disrupts G protein binding and allosterically intervenes with chemokine binding. Hence, both BMS and CCR2-RA-[*R*] help to stabilize the receptor in an inactive conformation (Figure 1). This cooperative binding between these antagonists was further supported by data from the stability and radioligand binding assays. For instance, the binding capacity of CCR2-RA-[*R*] was increased by the presence of BMS-681 in both equilibrium and kinetic radioligand binding assays, indicative of allosteric enhancement. Finally, this crystal structure may facilitate the rational design of novel antagonists for CCR2.

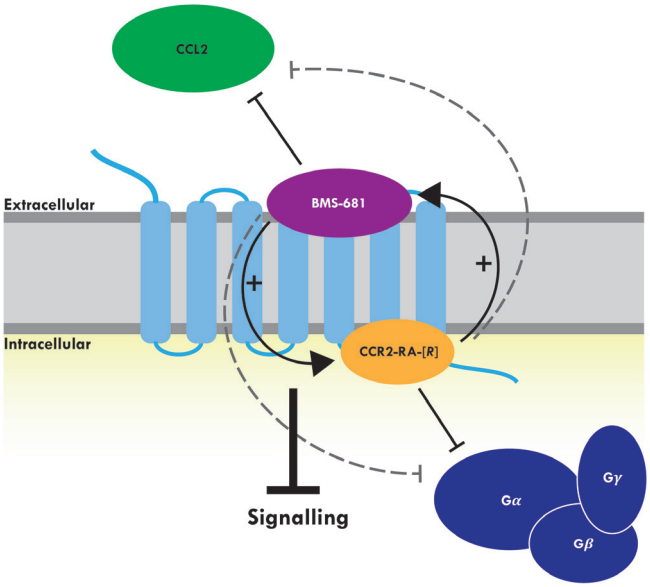

Figure 1. Binding and mechanism of inhibition of BMS-681 and CCR2-RA-[*R*] based on the crystal structure of CCR2. BMS-681 binds at the orthosteric binding site, where it directly interferes with CCL2 binding, and promotes an inactive, G protein-uncoupled CCR2 conformation. CCR2-RA-[*R*] binds in an allosteric binding site located at the intracellular region of the receptor, where it directly interferes with G protein-coupling and allosterically inhibits CCL2 binding. As a consequence, when the two inhibitors bind simultaneously, they act in a cooperative manner as shown by an enhancement of each other's binding, resulting in a highly inactive conformation of CCR2.

Intracellular ligands to better target CCR1, CCR2 and CCR5

As reviewed in **Chapter 2**, targeting the intracellular binding site of GPCRs offers several advantages over targeting the orthosteric binding site: allosteric modulation of the affinity/efficacy of orthosteric ligands, insurmountable mode of inhibition, possibility to design multitarget ligands and potential biased signaling (**Chapter 2**). Thus, throughout **Chapters 4** and **5**, we explored the possibility of targeting other chemokine receptors with intracellular allosteric modulators. For this, we focused on the highly homologous CC chemokine receptors 1 (CCR1) and 5 (CCR5), where intracellular pockets for small-molecules have been previously suggested.[6-9] The radiolabeled version of the co-crystallized intracellular ligand CCR2-RA-[*R*] in **Chapter 3**, [^{3}H]-CCR2-RA-[*R*], was first characterized in both CCR2 and CCR1 (**Chapter 4**). In addition to its high affinity in CCR2, [^{3}H]-CCR2-RA-[*R*] also displayed high affinity for CCR1, rendering it a suitable tool for studying CCR1. Moreover, in [^{3}H]-CCR2-RA-[*R*] displacement assays, the CCR1 orthosteric antagonist BX471 was not able to displace the radioligand, further supporting that CCR1 also possesses an intracellular binding pocket, which may be used for the design of both selective and 'multitarget' inhibitors. The fact

that the CCR1, CCR2 and CCR5 are involved in the pathogenesis of many inflammatory diseases, such as rheumatoid arthritis (RA) and multiple sclerosis (MS), makes a strong case for the development of multitarget ligands, i.e. dual/triple antagonists, as a promising therapeutic approach.[10, 11] Several dual antagonists have already been reported for CCR2/CCR5;[12] however, we are the first to undertake the design of CCR1/CCR2 dual antagonists (**Chapter 4**). Based on the pyrrolone scaffold of CCR2-RA-[*R*], ~50 pyrrolone derivatives were synthesized and evaluated in both CCR1 and CCR2 (Figure 2). This medicinal chemistry approach allowed us to find several compounds with improved selectivity towards CCR1, as well as potential dual CCR1/CCR2 antagonists. Functional characterization of selected compounds revealed that these intracellular ligands behave as inverse agonists in CCR1, which has previously been characterized as a constitutively active receptor.[13] To the best of our knowledge, these ligands represent the first intracellular inverse agonists for CCR1, providing a new pharmacological approach to modulate this receptor.

All previously reported CCR2/CCR5 dual antagonists[12] bind to the orthosteric binding site of the receptors, which results in a competitive and surmountable mode of inhibition. Thus, in **Chapter 5** we explored the potential of developing intracellular CCR2/CCR5 antagonists by synthesizing and evaluating the activity of a series of triazolo-pyrimidinone derivatives (Figure 2), which also bind to the intracellular binding site as confirmed in CCR2 radioligand binding assays. However, CCR2-RA-[*R*] binds with much lower affinity to CCR5 (~100 nM) compared with CCR2 and CCR1, preventing us from using this radioligand to determine the affinity of triazolo-pyrimidinone derivatives in CCR5. Thus, we relied on functional β-arrestin recruitment assays to evaluate the activity of the derivatives in CCR5. In contrast to **Chapter 4**, where we found several pyrrolone derivatives with high-affinity towards CCR1, most triazolo-pyrimidinone derivatives remained selective towards CCR2; however, few derivatives were able to inhibit CCR5 signaling with approximately 100 nM potency, indicating that the design of intracellular multitarget ligands is quite feasible for these receptors as well. Moreover, evaluation of two compounds in [^{35}S]GTPγS binding assays confirmed that these compounds behave as insurmountable antagonists in both CCR2 and CCR5, which might translate into higher *in vivo* efficacy in inflammatory diseases where these receptors are involved.

Development of covalent ligands, either as tool compounds or pharmaceutical products, has gained increased interest due to their many potential applications and therapeutic advantages.[14, 15] Thus, while **Chapters 4** and **5** focused on the design of reversible intracellular ligands, **Chapter 6** describes the design, synthesis and characterization of the first irreversible intracellular ligand for CCR2. Using a variety of assays, including time-dependent affinity determination, radioligand wash-out assays, and functional (wash-out) assays, we validated compound **14** as a covalent negative allosteric modulator (NAM) for

CCR2 (Figure 2). The binding mode of compound **14** was studied using computational modeling followed by site-directed mutagenesis of CCR2. These studies identified Cys75^{2x37}, at the intracellular binding pocket, as the primary residue for covalent interaction, although secondary interaction sites remain possible. Altogether, compound **14** represents a potential tool compound to further study CCR2 pharmacology.

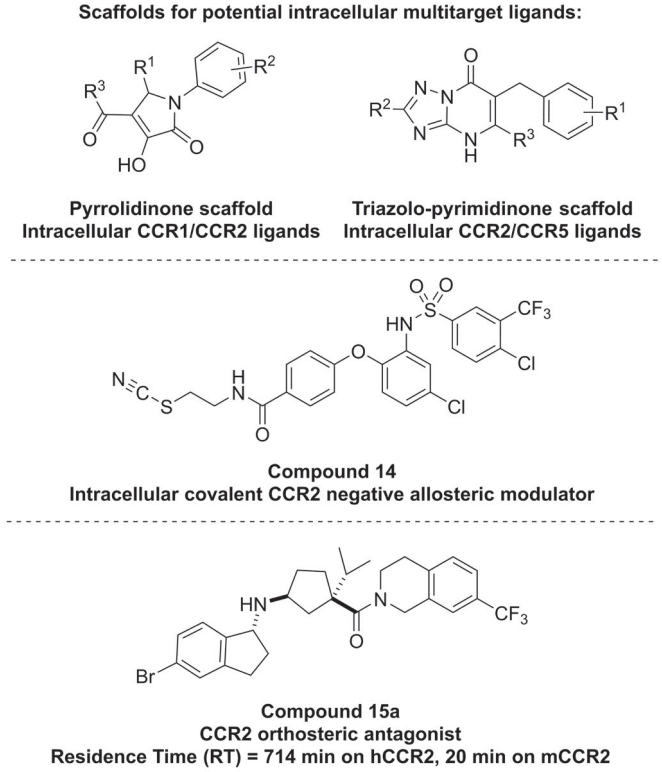

Figure 2. Scaffolds and chemical structures of compounds discussed in this thesis.

CCR2 antagonists for the treatment of atherosclerosis

CCR2 and its endogenous ligand CCL2 have been found to play a key role in the recruitment of monocytes to atherosclerotic lesions, representing potential targets for the treatment of atherosclerosis. Previous research in our group led to the discovery of the CCR2 orthosteric antagonist **15a** (Figure 2), which displays a prolonged residence time (RT) on its target (RT of 714 min).[16] Long RT antagonists can also inhibit the receptor in an insurmountable manner,[17] and they have been proposed to lead to enhanced *in vivo* efficacy.[18] Thus, we aimed to determine whether **15a** is effective in inhibiting atherogenesis in the apolipoprotein

E-deficient (apoE$^{-/-}$) mouse model of atherosclerosis. Compared to vehicle control, treatment of apoE$^{-/-}$ mice with **15a** resulted in significant inhibition of CCR2^{+} monocytes recruitment to the atherosclerotic plaques, as well as significant reduction of the plaques size at both the carotid artery and the aortic root. Assessment of **15a** binding kinetics in mouse CCR2 (mCCR2) revealed a poor translation of kinetic parameters between the human and mouse orthologues: **15a** displays a RT of less than 30 min in mCCR2 compared with 714 min in human CCR2 (Figure 2), while its affinity was comparable in both receptors. These findings emphasize the need to characterize equilibrium and kinetic parameters of drug candidates in all relevant species for preclinical studies, especially among chemokine receptors, where high species variation has been found.[19] Pharmacokinetic analysis and calculation of CCR2 occupancy levels indicated that a single dose of **15a** led to >90% CCR2 occupancy levels for over 24 hours. Such prolonged target occupancy resulted from the long elimination half-life of **15a** combined with the use of target-saturating concentrations. Overall, these data support high receptor occupancy as a key parameter for an effective anti-inflammatory response, and suggests **15a** as a promising candidate for further drug development studies.

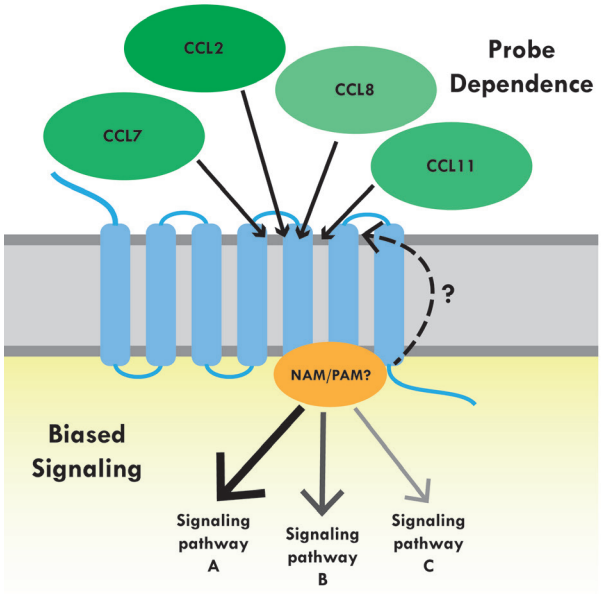

Figure 3. Intracellular modulation of G protein-coupled receptors (GPCRs). Targeting the intracellular binding site of GPCRs provides several opportunities and challenges. For example, this binding site can be used for the development of both negative allosteric modulators (NAM), which inhibit the receptor, and positive allosteric modulators (PAM), which activate or enhance the receptor activity. These intracellular ligands also have the potential to promote biased signaling, by preferentially activating or inhibiting one pathway over another. Finally, it is important to investigate a potential probe dependent behavior of intracellular ligands, as their effect might differ depending on the agonist bound. The latter is particularly important for chemokine receptors, where multiple chemokines can activate one receptor.

FUTURE PERSPECTIVES

Intracellular modulation of GPCRs

Although this thesis is mostly focused on intracellular modulation of CCR1, CCR2 and CCR5, this strategy should not be limited to chemokine receptors (**Chapter 2**). In fact, small-molecule ligands have been reported to bind to the intracellular region of β_2 adrenergic receptor (β_2AR),[20, 21] proteinase activated receptor 1 (PAR1)[22] and dopamine D1 receptor (D1R),[23] suggesting the presence of intracellular binding pockets among class A GPCRs in general. For instance, the crystal structure of β_2AR in complex with the negative allosteric modulator (NAM) 15PA (PDB 5X7D),[20] demonstrates that 15PA binds to a similar intracellular pocket compared to that of CCR2-RA-[*R*] in CCR2 (**Chapter 3**) and vercirnon in CCR9.[24] In addition, another crystal structure of β_2AR (PDB 6N48)[21] shows that the β_2AR positive allosteric modulator (PAM) 6FA also binds to the intracellular region, but at the interface between the cytoplasm and the lipid membrane, suggesting an additional intracellular pocket to modulate GPCR signaling.[21] In combination with screening campaigns, the increasing number of crystal structures with intracellular ligands can be used for *in silico* drug discovery studies, such as virtual screening, in order to identify novel intracellular ligands for chemokine receptors and GPCRs in general. The potential to activate the receptor via the intracellular site has also been demonstrated by the recently described intracellular PAMs for β_2AR[21] and dopamine D1 receptor (D1R).[23] Although no intracellular small-molecule PAMs have been reported for chemokine receptors, intracellular pepducins, i.e. lipidated peptides derived from the ICLs, with agonistic activity have been developed for CXCR4, such as ATI-2341.[25] Moreover, ATI-2341 displayed biased signaling towards $G\alpha_i$-coupling over $G\alpha_{13}$-coupling or β-arrestin recruitment,[25] suggesting that functional bias can also be achieved by targeting this intracellular pocket. In line with this, the suggested intracellular modulator AZD8797, targeting the CX_3CR1 receptor, has been found to act as a NAM of G protein-activation, and as a PAM for β-arrestin recruitment.[26] Indications of functional bias have also been found with CCR2 intracellular NAMs; for instance, the CCR2 intracellular ligand JNJ-27141491 displayed a higher potency in inhibiting G protein activation than β-arrestin recruitment,[2] while compounds **7** and **14** described in **Chapter 6** of this thesis appeared more potent in the β-arrestin recruitment assay than in the [^{35}S]GTPγS binding assay. Biased ligands for chemokine receptors have been found to differentially control physiological responses, such as leukocyte recruitment and inflammation,[27] as well as receptor endocytosis and the development of tolerance,[28] which highlights their potential therapeutic benefit. Thus, further studies are warranted to investigate the functional profile of intracellular allosteric modulators in multiple signaling pathways, in order to identify functional bias (Figure 3). Finally, evaluation of their functional effects in the presence of different chemokines, i.e. probe-dependence, is particularly relevant for the development

of intracellular ligands for chemokine receptors, as many chemokines are known to activate a single receptor (Figure 3).[29]

Multitarget ligands for GPCRs

The development of multitarget drugs, which act on multiple receptors or enzymes, has been proposed as a more effective approach to treat complex, multifactorial diseases such as multiple sclerosis (MS), rheumatoid arthritis (RA) and cancer.[10, 11, 30, 31] Thus, in **Chapters 4** and **5** from this thesis, we explored the possibility to design multitarget intracellular ligands for CCR1, CCR2 and CCR5, which resulted in the identification of potential CCR1/CCR2 (**Chapter 4**) and CCR2/CCR5 (**Chapter 5**) multitarget ligands. Multitarget ligands have been reported for many chemokine receptor pairs, including CCR1/CCR3,[32] CCR2/CCR5,[12] CCR2/CXCR2,[33] CCR5/CXCR4,[34] CXCR1/CXCR2,[35] and CXCR3/CXCR4.[36] Multitarget ligands can also be developed to target different receptor classes, such as CCR3 and histamine receptor H_1 (H_1R), which are both involved in the pathogenesis of asthma and atopic dermatitis.[37] Although CCR3 and H_1R have limited homology, high-affinity dual-target antagonists have been developed for this pair,[37] opening up the possibility to design multitarget ligands against highly dissimilar proteins. Although there is ample evidence on the beneficial effects of CCR2/CCR5 combined inhibition in several (pre)clinical studies,[38-44] more studies are still needed to investigate whether combined inhibition of other receptor pairs is in fact more efficacious than selective inhibition. However, one of the main challenges in the development of multitarget drugs is choosing the right targets. For instance, several chemokine receptors seem to play a role in RA, including CCR1, CCR2, CCR5, CXCR2 and CXCR3,[45] which complicates the selection of relevant combinations of drug targets. In this regard, the generation of *in silico* biological network models may be used in combination with *in vitro*/*in vivo* studies to identify successful combinations of drug targets to achieve the highest efficacy.[46] These biological network models have also shown that inhibition of several network components, even if partially, is more effective than inhibition of a single component in modulating complex and robust disease models.[46] This implies that the use of low-affinity ligands might be sufficient to achieve the desired effect; thus, selection of multitarget ligands should not be based purely on affinity but on the desired activity profile.[47] Finally, optimization of selectivity for the desired targets remains a challenge in the rational design of multitarget ligands, warranting more structure-affinity/activity relationships studies as well as target structure-based studies.[47] Overall, the described multitarget inhibitors represent potential tool compounds to study the *in vitro* and *in vivo* effects of combined inhibition.

Covalent ligands as tools for GPCRs

Covalent probes are increasingly being developed for GPCRs as they can be used to further elucidate receptor function in both *in vitro* and *in vivo* systems, as well as to facilitate target crystallization.[14, 48] Such covalent probes have been reported for several class A GPCRs;[14, 49] however, no covalent probes have been reported for chemokine receptors, with the exception of the covalent reversible boronic acid-based probes for CXCR3, which were used to study CXCR3 allosteric modulation.[50] Thus, compound **14** described in **Chapter 6** of this thesis represents the first irreversible covalent ligand for chemokine receptors, in this case CCR2. Using a similar approach, allosteric or orthosteric covalent ligands can be designed for other chemokine receptors, including the constitutively active CCR1. These covalent ligands can be particularly useful in receptor stabilization for X-ray structure determination, as demonstrated by the crystal structures of β_2AR,[51] cannabinoid receptor CB_1,[52] and adenosine A_1 receptor,[53] all co-crystallized with covalent ligands. A covalent inverse agonist targeting CCR1 could represent an important step to stabilize the receptor, achieve crystallization and thus provide insight on the binding mode and mechanism of inhibition of CCR1 intracellular modulators. In addition, covalent ligands can be further functionalized as affinity-based probes by addition of a ligation or click handle (alkyne or azide group) to their chemical structure, which allows the introduction through "click chemistry" of a fluorophore or a biotin tag after covalently binding to a protein.[54] Although this is a relatively new field for membrane proteins, (photo)affinity-based protein profiling has been used in GPCRs to study target expression profiles and drug-target engagement, identification of off-targets, and target visualization in biological systems.[55-58] Thus, the development of affinity-based probes represents a novel and promising approach to advance drug discovery in the field of chemokine receptors (Figure 4).

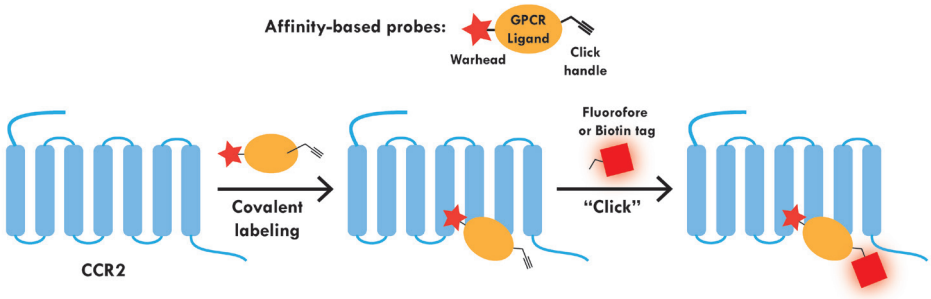

Figure 4. Covalent ligands as affinity-based probes. Covalent ligands such as compound **14** described in this thesis (**Chapter 6**), can be further functionalized as affinity-based probes by adding a ligation or "click" handle to the molecule, such as an alkyne group. In this way, a fluorophore or a biotin tag can be introduced to the receptor via "click-chemistry", allowing GPCR visualization or isolation from a complex cellular mixture.

Intracellular ligands as PROTACs

In addition to GPCR modulation by small-molecule inhibitors, other strategies are now emerging to inhibit receptor function, including pepducins and nanobodies described in **Chapter 2**. Induced protein degradation has recently emerged as a novel strategy to inhibit protein function by using, for instance, proteolysis-targeting chimeras (PROTACs) that exploit the ubiquitin-proteasome system (UPS).[59] PROTACs are bifunctional molecules composed of a ligand that targets the protein of interest connected via a linker group to another ligand that recruits an E3 ubiquitin ligase. By forming a ternary complex with both the E3 ligase and the protein of interest, PROTACs induce poly-ubiquitination of the protein target and subsequent degradation of the protein of interest by the 26S proteasome (Figure 5).[60, 61] Recent advances in this technology have led to the development of the first PROTAC clinical candidate, which targets the nuclear androgen receptor for the treatment of prostate cancer.[62] Due to their mechanism of action, PROTACs need to engage their targets within the intracellular space, and thus, they have been mostly developed for cytosolic targets such as enzymes, nuclear receptors, transcription factors and kinases, among others.[61] Intracellular ligands for GPCRs, such as those described in **Chapters 2, 3, 4** and **5**, represent valuable starting points for PROTACs design, as they engage their target from the intracellular site. By linking these intracellular ligands to E3 ligase ligands, we can investigate whether the ubiquitination machinery can be hijacked to induce GPCR degradation (Figure 5).[63] In this regard, poly-ubiquitination of GPCRs has been found to play an important role not only on lysosomal or proteasomal degradation, but also on receptor signaling. For example, the E3 ligase VHL has been found to ubiquitinate the β_2-adrenergic receptor (β_2AR) to promote proteasomal degradation via 26S,[64] indicating that this strategy might be feasible for some, if not all, GPCRs. Moreover, Burslem *et al*. recently developed PROTACs for three different transmembrane receptor tyrosine kinases (RTK), suggesting that targeting transmembrane proteins is indeed possible.[65] Of note, lysosome targeting chimeras (LYTACs)[66] and endosome targeting chimeras (ENDTACs)[67] have been recently developed to induce lysosomal degradation of extracellular targets; providing another strategy to induce degradation of GPCRs. Due to their catalytic mode of action—in contrast to the occupancy-based mode of action of small-molecule inhibitors—PROTACs may offer several advantages for GPCRs, including potent target degradation at low concentrations, enhanced target selectivity, and prolonged inhibition of receptor signaling.[68, 69] Thus, the development of novel small-molecule intracellular ligands for GPCRs may facilitate the design of GPCR-targeting PROTACs as a novel strategy to modulate receptor pharmacology (Figure 5).

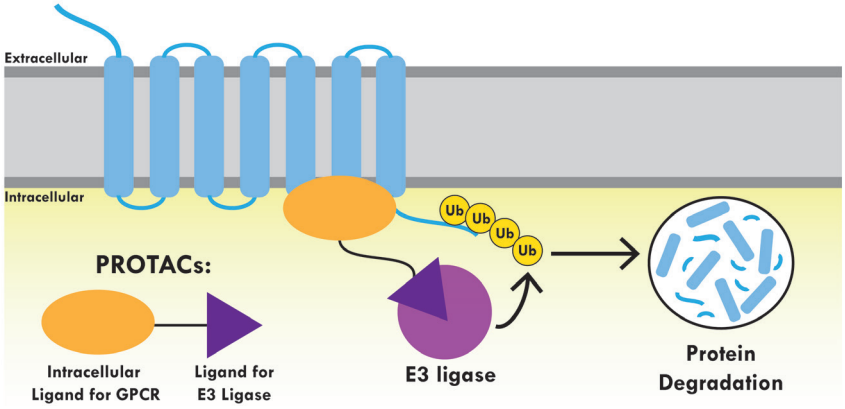

Figure 5. Intracellular ligands as PROTACs. Induced-protein degradation represents an alternative to inhibit protein function. Proteolysis-targeting chimeras (PROTACs) for GPCRs can be designed by linking an intracellular binding GPCR ligand to an E3 ligase ligand. By binding to both the GPCR of interest and the E3 ligase, PROTACs induce poly-ubiquitination (Ub) and subsequent degradation of the protein of interest.

Final notes

All in all, in this thesis we have explored different mechanisms to achieve insurmountable inhibition for chemokine receptors, including intracellular allosteric inhibition, covalent inhibition and long RT inhibitors, as this may lead to improved *in vivo* efficacy of chemokine receptors' inhibitors. In addition, with the crystallization of CCR2 and the identification of several intracellular ligands for CCR1, CCR2 and CCR5—including selective, multitarget and covalent ligands—we are expanding the toolbox to further study and modulate chemokine receptors. Finally, we hope that the data presented in this thesis contributes to advance drug discovery in the field of chemokine receptors and GPCRs in general.

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