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Pharmacological characterization of allosteric modulators: A case for chemokine receptors

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Abstract

Chemokine receptors are relevant targets for a multitude of immunological diseases, but drug attrition for these receptors is remarkably high. While many drug discovery programs have been pursued, most prospective drugs failed in the follow-up studies due to clinical inefficacy, and hence there is a clear need for alternative approaches. Allosteric modulators of receptor function represent an excellent opportunity for novel drugs, as they modulate receptor activation in a controlled manner and display increased selectivity, and their pharmacological profile can be insurmountable. Here, we discuss allosteric ligands and their pharmacological characterization for modulation of chemokine receptors. Ligands are included if (1) they show clear signs of allosteric modulation in vitro and (2) display evidence of binding in a topologically distinct manner compared to endogenous chemokines. We discuss how allosteric ligands affect binding of orthosteric (endogenous) ligands in terms of affinity as well as binding kinetics in radioligand binding assays. Moreover, their effects on signaling events in functional assays and how their binding site can be elucidated are specified. We substantiate this with examples of published allosteric ligands targeting chemokine receptors and hypothetical graphs of

pharmacological behavior. This review should serve as an effective starting point for setting up assays for characterizing allosteric ligands to develop safer and more efficacious drugs for chemokine receptors and, ultimately, other G protein-coupled receptors.

KEYWORDS
allosteric modulation, chemokine receptor, medicinal chemistry, molecular pharmacology

1 | INTRODUCTION: CHEMOKINE RECEPTORS AND THE IMPORTANCE OF ALLOSTERIC MODULATION

Chemokine receptors are class A G protein-coupled receptors (GPCRs) activated by small cytokines called chemokines.[1] There are four classes of chemokine receptors based on the number and spacing of cysteines in their corresponding chemokines (C, CC, CXC, and CX3C), as well as so-called atypical chemokine receptors. Chemokine receptors are mainly expressed on leukocytes and are crucial in regulation of the immune system in homeostasis and trafficking of leukocytes to sites of inflammation among others.[1]

Chemokine receptors are membrane proteins consisting of an extracellular N-terminus, followed by seven transmembrane (TM) helices and ending with the intracellular C-terminus. The chemokine ligands bind at the extracellular side of the protein, which is termed the orthosteric binding pocket. It is divided in a major (TMIII, -IV, -V, -VI, -VII) and minor (TMI, -II, -III) pocket (Figure 1A,B).[2] Orthosteric antagonists blocking the binding of the endogenous chemokines occupy either both or one of these pockets.[3] Allosteric modulators, usually negative allosteric modulators (NAMs) for chemokine receptors, represent another, less explored opportunity for drug design and development. Allosteric ligands are defined as small molecules that bind a spatially and topologically distinct site different from the orthosteric/endogenous binding site.[4] Of note, small molecules binding the orthosteric binding site of chemokine receptors have been reported to act in an allosteric manner as well, such as maraviroc.[5–7] However, crystal structures of CCR5 in complex with CC chemokine ligands 3 and 5 (CCL3 and CCL5) show considerable overlap with maraviroc's binding pocket.[8,9] Hence, this could be a case of so-called "orthosteric allosterism,"[10] where this (partial) overlap between small molecules and chemokines can be an explanation for observed allosterism based on the chemokine used. However, as these types of ligands are not spatially distinct from the overall chemokine binding site, we have not included such ligands in this review.

Targeting an allosteric binding site provides multiple advantages over orthosteric ligands including a lack of competition from endogenous ligands such as chemokines.[13] Allosteric inhibitors act in an insurmountable manner, meaning that they can alter orthosteric ligand affinity and/or potency even at high local concentrations of chemokines, eventually allowing dosages to be reduced while maintaining efficacy.[13,14] Furthermore, as the allosteric binding site is generally less evolutionarily conserved than the orthosteric site, there is opportunity to develop ligands selective for one or multiple receptors, which might be beneficial for diseases where multiple chemokine receptors are involved.[15] Finally, as allosteric ligands can show preference in modulating receptor signaling pathways (biased signaling) depending on the agonist (probe dependence),[16,17] signaling can be selectively adjusted to hinder disease progression but not completely incapacitate receptor function.

An intracellular allosteric ligand binding site has been discovered for multiple chemokine receptors (Figure 1).[11,18–21] This binding site seems to (partially) overlap with the G protein and β-arrestin binding sites, thereby avoiding competition with chemokines. For an excellent overview of the history of intracellular allosteric

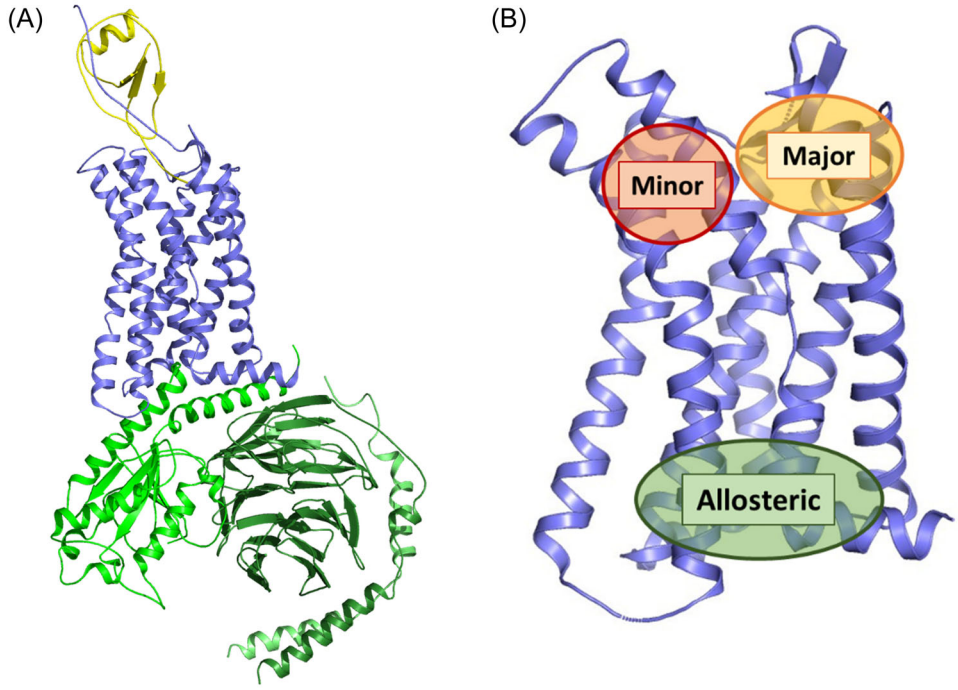

FIGURE 1 General structure of chemokine receptors. (A) A general structure of a chemokine receptor in complex with a chemokine (Yellow) and G protein (Orange/yellow). Note, the β-arrestin binding site overlaps with the G protein binding site. (B) General structure of a chemokine receptor annotated with multiple binding sites, with the major (Yellow, TMIII, -IV, -V, -VI, -VII) and minor (Orange, TMI, -II, -III) orthosteric binding pocket that binds (part of) the chemokines and small molecule antagonists, and the intracellular allosteric (green) binding site that binds small molecule antagonists and seems to (partially) overlap with the G protein and β-arrestin binding sites. Images are based on 5T1A and 6FLO, respectively.[11,12] [Color figure can be viewed at wileyonlinelibrary.com]

ligands for chemokine receptors, the authors refer to Billen et al.[22] In this review, we aim to provide a guide on how to pharmacologically characterize allosterically binding ligands (depicted in Figure 2) in in vitro binding and functional assays, as well as how to elucidate their binding site.

2 | QUANTIFICATION OF ALLOSTERIC MODULATION

Several descriptive models have been developed to understand and quantify allosteric modulation. The simplest receptor-ligand binding model is the one-step binding model, where the receptor is either unbound or bound to a ligand driven by a one-step association (k_1/k_{on}) or dissociation (k_{-1}/k_{off}) constant (Figure 3A).[23] This model describes the interaction of either an orthosteric or allosteric ligand in a single ligand environment. However, since allosteric ligands bind in a topologically distinct manner that may allow for the binding of an orthosteric/endogenous ligand, this model is not always applicable to explain certain in vitro or in vivo findings.

The allosteric ternary complex model (ATCM) describes the effect an allosteric modulator on the receptor and the orthosteric ligand-receptor complex in equilibrium (Figure 3B).[24] This model can be expanded to include G protein-binding and active/inactive receptor states,[25,26] however for the sake of simplicity we do not take these extensions into account. We can do this as most described compounds in this review are negative allosteric modulators, which have no preference for the active receptor. Affinity of orthosteric/endogenous

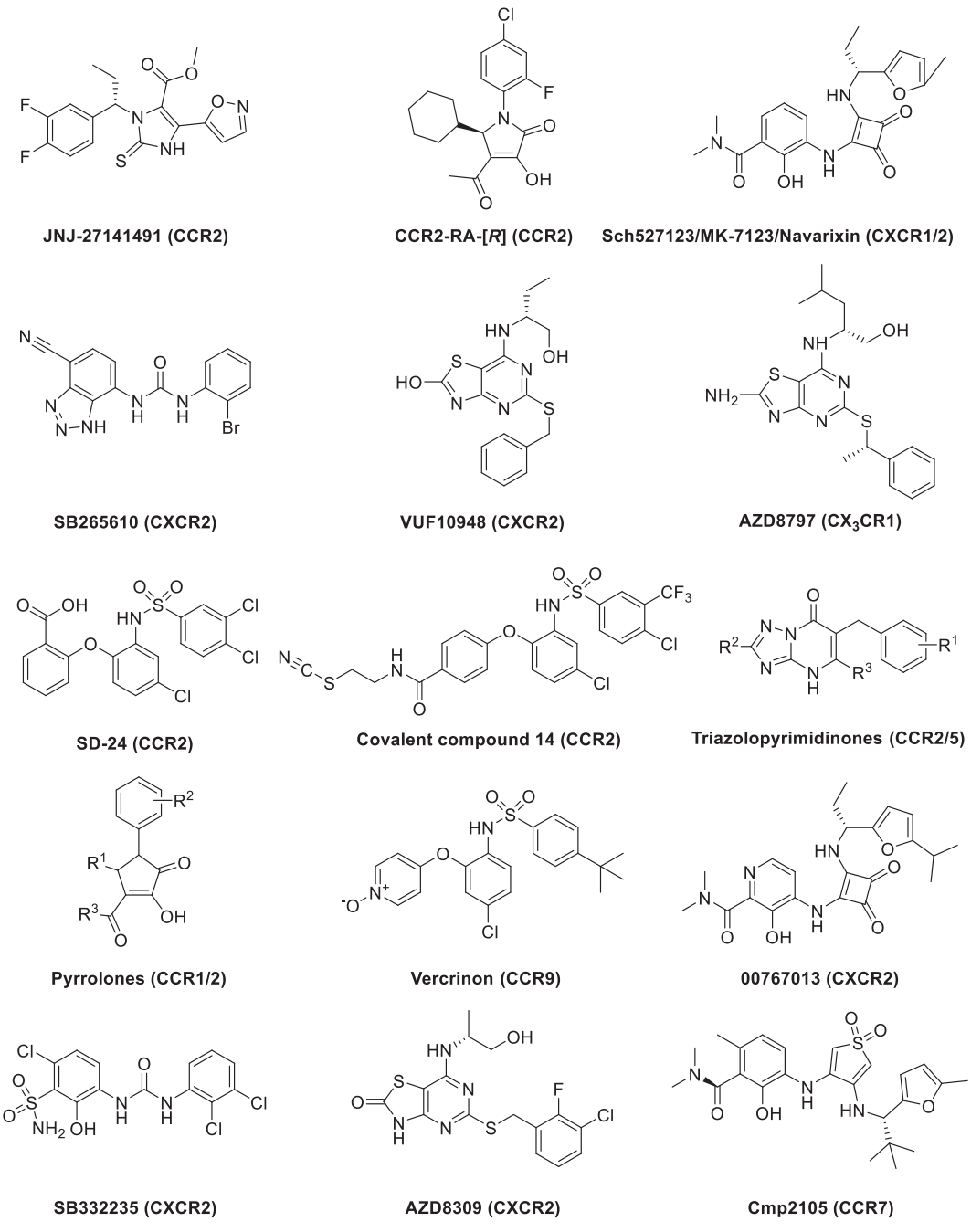

FIGURE 2 Chemical structures of intracellularly binding allosteric ligands for chemokine receptors discussed in this review. Depicted in order of appearance with target receptor(s) shown between brackets.

ligand L to allosteric ligand (A)-receptor (R) complex RA is influenced by cooperativity factor α, while A to LR is influenced by β. The ATCM model can also be adjusted to describe kinetic parameters, where α and β influence association and dissociation rate constants.[26,27] If α>1 A can be classified as a positive allosteric modulator (PAM), if α<1 A can be classified as a negative allosteric modulator (NAM) and if $\alpha = 1$ the ligand is a neutral or

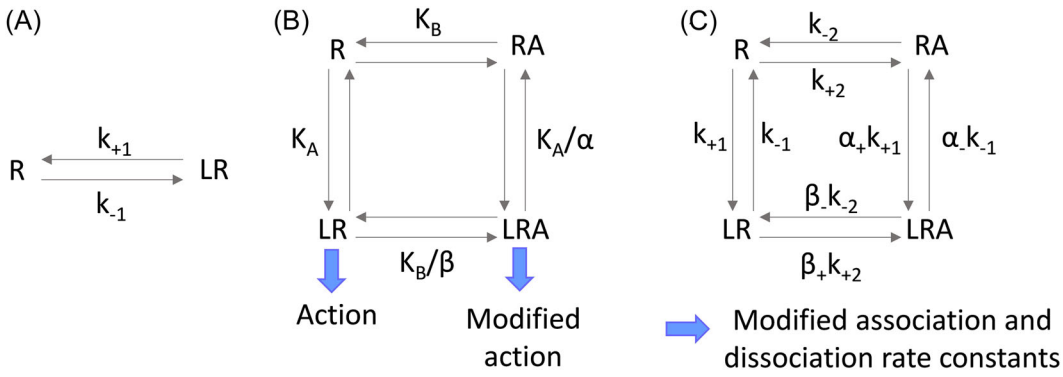

FIGURE 3 Ligand binding models (A) Classical model with one ligand (L) and receptor (R) which form a ligand-receptor complex (LR). Affinity of R to L is derived from association rate constant k_{+1} and dissociation rate constant k_{-1} (B) ACTM model supplemented with allosteric ligand (A) to form receptor-allosteric ligand complex (RA) and ligand-receptor-allosteric ligand complex (LRA). Affinities of A and L to R are expressed as KA and KB, respectively. When LR is formed, affinity of A to the LR complex is influenced by cooperativity factor β, or R to RA by cooperativity factor α. (C) ACTM model expressed in kinetic parameters with association rate constant of L to R being k_{+1} and dissociation rate constant k_{-1} and A to R being k_{+2} and k_{-2}. For the transition LR/LRA rate constants are influenced by cooperativity factor β_- and β_+, for the transition RA/LRA by cooperativity factor α_- and α_+. [Color figure can be viewed at wileyonlinelibrary.com]

"silent" allosteric modulator (NeAM or SAM). The same factors have a similar effect on potency and maximal activation in functional assays.[25]

3 | EFFECT ON LIGAND BINDING

One of the traditional assays in the GPCR field is the ligand binding assay, for which different types of endogenous, orthosteric, and allosteric ligands are labeled with a radioactive isotope or tagged with a fluorophore. Competition or modulation by an unlabeled ligand can be measured using these types of assays. The terms K_D and K_i both depict affinity and describe affinity of the labeled or competing ligand to the receptor, respectively. For more information on how to determine these constants, the authors refer to other reviews, such as Van der Velden et al.[23] Here, we focus on how to perform these assays to determine the effect of an allosteric modulator on affinity and binding kinetics of the labeled ligand, substantiated by examples from literature.

3.1 | Displacement assays

The displacement assays mentioned in this review are end-point assays, although continuous displacement assays have been reported. Here we will discuss the former, one concentration of labeled ligand is added together with increasing concentrations of unlabeled ligand to tissue expressing the target of interest, such as cell membranes or whole cells (Figure 4A). This assay can be performed with either labeled chemokines, orthosteric or allosteric ligands in combination with all types of unlabeled ligands.

Directly competing ligands culminate in a sigmoidal-shaped curve as they displace the labeled ligand, with complete displacement at the highest concentrations (Figure 4B). Noncompetitive ligands can result in a variety of curves. They can appear similarly as a competitive ligand showing full displacement, show partial or no displacement of the labeled ligand or binding of the labeled ligand can even be increased.

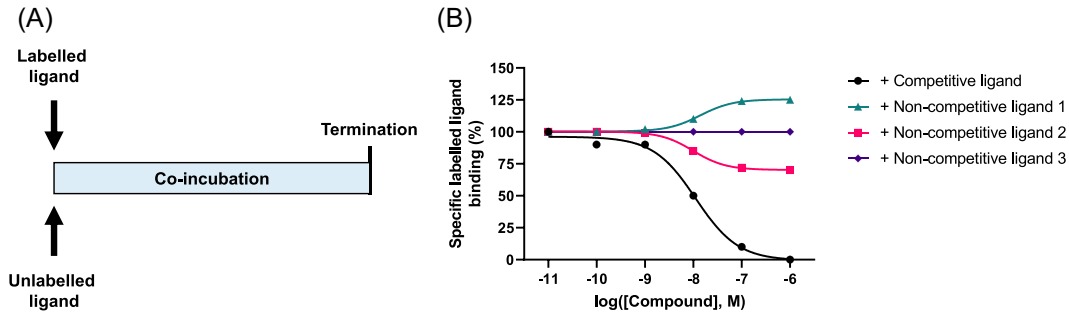

FIGURE 4 Displacement assays with a labeled ligand. (A) General set-up of displacement assays where a (fluorescently or radio-)labeled ligand and unlabeled ligand are added simultaneously to cells or cell membranes expressing the target of interest before co-incubation and termination of the experiment. This assay can be executed with either an orthosteric or allosteric radioligand with a variety of (non-)competitive cold ligands. (B) Hypothetical outcomes of displacement assays with a competitive (black circles), or different types of noncompetitive ligands (turquoise triangles, pink squares, purple diamonds). [Color figure can be viewed at wileyonlinelibrary.com]

Chemokines labeled with an iodine-125 isotope have been used for screening of novel ligands. At CCR2, JNJ-27141491 and CCR2-RA-[*R*] were able to fully displace ^{125}I-CCL2,[28] similarly as Sch527123/MK-7123/Navarixin, SB265610 and VUF10948 for ^{125}I-CXCL8 from CXCR1 and/or CXCR2,[29–31] and AZD8797 for ^{125}I-CX3CL1 from CX3CR1.[32] However, some ligands have been shown to only partially displace chemokines, as illustrated by SD-24 in a ^{125}I-CCL2 displacement assay at CCR2.[33]

When using radiolabelled orthosteric antagonists, it is possible that they are not displaced by allosteric ligands. Intriguingly, CCR2-RA-[*R*], JNJ-27141491, and SD-24 showed an increase in binding of the orthosteric (antagonist) radioligand [^{3}H]INCB3344 at CCR2,[28,33] thus displaying positive cooperative binding.

Lastly, allosteric ligands themselves can be labeled. In this way, binding affinities can be determined for ligands that compete for the same allosteric binding site. For example, the CCR2 allosteric radioligand [^{3}H]CCR2-RA-[*R*] was used to characterize the covalent compound 14 for CCR2,[34] triazolopyrimidinones for CCR2 and CCR5,[35] and pyrrolone derivatives for CCR1 and CCR2.[36] Furthermore, several carboxytetramethylrhodamine-tagged (TAMRA-tagged) allosteric ligands have been developed which were validated for cell membrane and whole cell displacement assays using NanoBRET technology. Displacement data was similar to that obtained using a radiolabeled ligand, and includes the CCR9 allosteric ligand vercirnon, CCR2 allosteric ligand SD-24 and CXCR2 allosteric ligand 00767013.[37–39] These ligands serve as a promising alternative for radioactive assays in drug optimization. With reversed setups, little to no displacement of the radioligand by a chemokine was seen for [^{3}H]CCR2-RA-[*R*] for CCL2 from CCR2,[28] and [^{3}H]Sch527123 and [^{3}H]SB264510 for CXCL8 from CXCR2.[29–31] As stated in Section 2, these radioligands are negative allosteric modulators with no inherent preference for receptor state, whereas chemokines prefer the G protein-bound (active) state. Thus, receptors bound by the radioligand are no longer available for binding of the chemokines.

3.2 | Saturation assays

With saturation assays the maximal amount of receptors in the tissue used (B_{max}) and affinity (K_D) of the radiolabelled ligand can be determined by incubating increasing concentrations of radiolabelled ligand with membranes expressing the target of interest (Figure 5A). By co-incubating an unlabeled allosteric ligand, the (non-) competitive nature compared to the radiolabelled ligand, and vice versa, can be established. Competitively binding ligands alter the K_D of the radioligand, while the B_{max} is unaffected. On the contrary, for fully noncompetitive or allosteric ligands the K_D of the radioligand remains unchanged, while the B_{max} is decreased (Figure 5B).[26]

The B_{max} of ^{125}I-CX_3CL1 was decreased by AZD8797 in saturation experiments at CXCR3 without affecting the K_D of the radioligand. Of note, it was shown that unlabeled CX_3CL1 resulted in a reduced K_D for the radioligand and an unchanged B_{max}.[32] For CXCR2, SB265610, Compound 1, and VUF10948 produced comparable effects in ^{125}I-CXCL8 saturation assays, as B_{max} was significantly reduced.[30,31] Thus, the noncompetitive nature of these small molecule antagonists at CXCR3 and CXCR2 compared to the chemokine was confirmed.

3.3 | Kinetic binding assays

Kinetic binding assays are used to determine the association (k_{on} or k_{+1}) or dissociation rate constant (k_{off} or k_{-1}) of a radioligand by initiating the experiment at multiple timepoints (Figure 6A,C). Addition of an allosteric ligand at the time of initiation can result in a positive or negative effect on either or both rate constants as determined by the previously mentioned cooperativity factors. By principle, PAMs enhance association and/or decrease dissociation rates, while NAMs decrease association and/or enhance dissociation rates (Figure 6B,D).[26]

At CX_3CR1, addition of the allosteric AZD8797 in ^{125}I-CX3CL1 binding experiments resulted in no effect on chemokine association, but the dissociation was significantly decreased and changed the fit of the curve from monophasic to biphasic.[32] For CCR2, addition of the orthosteric antagonist BMS-681 in [^{3}H]CCR2-RA-[*R*] kinetic assays increased k_{obs} and decreased $k_{off,fast}$ but not $k_{off,slow}$, confirming their cooperativity.[11]

However, radioligand dissociation assays are not always the optimal choice. For example, addition of SB265610 to ^{125}I-CXCL8 dissociation and vice versa resulted in no difference in $k_{off,}$ even though modulatory effects were present in other (functional) assays.[30] In this case, it appears that a change in association rate is leading and kinetic association assays should be performed.

4 | EFFECT ON RECEPTOR SIGNALING

Allosteric ligands cannot only alter endogenous agonist binding, but also its functional response. Most functional assays for GPCRs measure upstream (G protein and beta-arrestin assays) or downstream (e.g., calcium influx or cAMP production) signaling events. These assays can be used to determine if an allosteric modulator is a PAM or a NAM for the tested endogenous ligand. Insurmountability is an indication of an

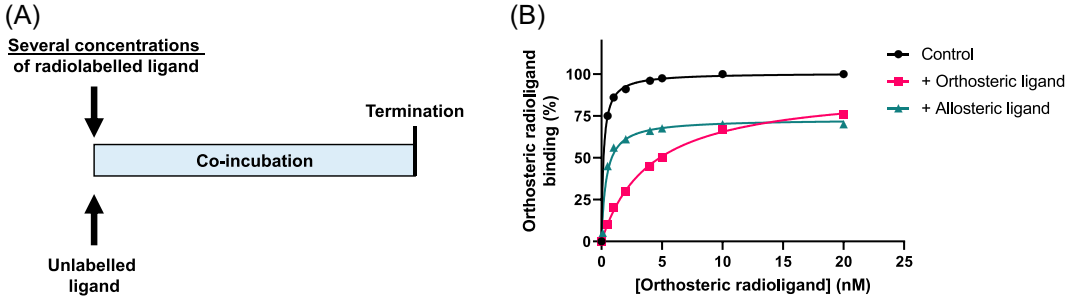

FIGURE 5 Radioligand saturation assays. (A) Increasing concentrations of radiolabelled ligands are incubated with membranes expressing the target of interest to determine B_{max} and K_D. When co-incubated with an unlabeled ligand, (non-)competitiveness with respect to the chosen radioactively labeled ligand can be determined. (B) Hypothetical outcomes of a saturation assay of only radiolabelled ligand (black circles), with the addition of a competitive unlabeled ligand (pink squares) or the addition of a noncompetitive unlabeled ligand (turquoise triangles). [Color figure can be viewed at wileyonlinelibrary.com]

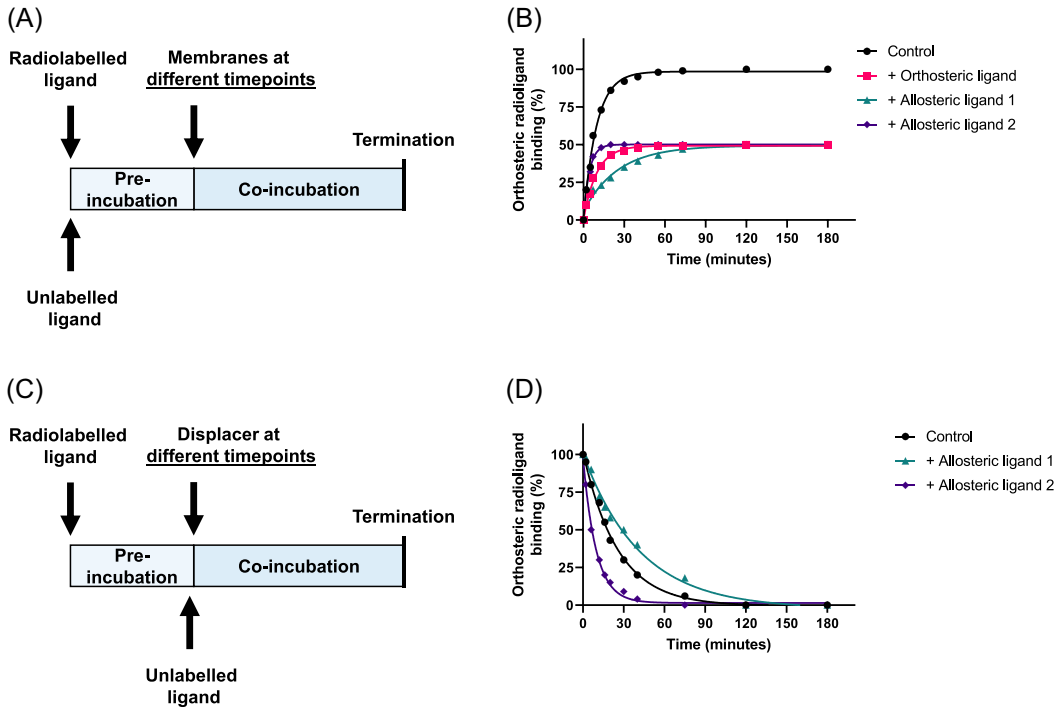

FIGURE 6 Kinetic radioligand binding assays. (A) Starting the incubation of a radiolabelled ligand with membranes that express the target of interest in the absence or presence of an allosteric modulator allows for the determination of the effect of the allosteric modulator on the association rate constant of the radiolabelled ligand. (B) Hypothetical graph of the association of a radioligand towards a receptor (black circles) with the addition of an orthosteric ligand (pink squares) or influenced by allosteric modulators which can decelerate (turquoise triangles) or accelerate (purple diamonds) the association rate of a radiolabelled ligand. The concentration of unlabeled ligand should correspond to 50% radioligand displacement. (C) Preincubation of the radiolabelled ligand with membranes that express the target of interest followed by initiation of the dissociation by an excess of the unlabeled version of the ligand in the absence or presence of an allosteric modulator allows for the determination of the effect of the allosteric modulator on the dissociation rate constant of the radiolabelled ligand. (D) Hypothetical graphs of a dissociation of a radioligand (black circles) in the presence of allosteric modulators which can decelerate (turquoise triangles) or accelerate (purple diamonds) the dissociation rate of a radiolabelled ligand. [Color figure can be viewed at wileyonlinelibrary.com]

allosterically binding antagonist, however this effect can also be caused by for example, compounds with a long target-residence time or covalent mode of action, so care must be taken to further characterize the ligand if allosterism is expected.[40]

4.1 | Insurmountability

To assess (in)surmountability in functional assays, typically three different concentrations of an (allosteric) ligand are co-incubated with increasing concentrations of agonist (chemokine) (Figure 7A). Surmountable antagonists can be recognized in functional experiments by their ability to cause a rightward shift in the dose–response curve of the agonist without affecting its maximal response (E_{max}) (Figure 7B).[41] In contrast, insurmountable antagonists will alter the maximal response, and may or may not have an effect on potency (Figure 7C).

At CXCR2, SB265610, compound 1, and VUF10948 showed insurmountability in β-arrestin2 recruitment with a rightward shift in the concentration-effect curve and a decrease in maximal effect.[31] In CCL2-mediated activation of CCR2 measured by Ca^{2+} mobilization or [^{35}S]GTPγS recruitment, JNJ2714191, CCR2-RA-[*R*] and triazolopyrimidinone derivatives 39 and 43 all showed insurmountability by reducing E_{max} and reducing potency.[28,35,42] At CCR5, compounds 39 and 43 also reduced the maximal CCL3-mediated binding of [^{35}S]GTPγS, but only the highest concentration of 43 affected the potency for this receptor.[35] At CX_3CR1, modulation by AZD8797 showed the reverse for maximal activation in β-arrestin2 recruitment assays, as E_{max} was increased while potency was unaffected,[32] and this is thus one of the few reported PAMs for chemokine receptors.

4.2 | Change in Hill slope

The Hill–Langmuir equation describes the degree of cooperativity of ligands binding to the receptors, while the Hill coefficient is used to determine the degree of interaction between ligand binding sites, similarly as the cooperation factors derived from the ACTM model.[43] For determining the effect of allosteric ligands, the Hill coefficient is more applicable compared to the Hill-Langmuir equation. A Hill coefficient different from 1 (unity) indicates a system with multiple different binding sites.[44] Similarly as the cooperativity factors derived from the ACTM models, n_H>1 shows positive cooperative binding, n_H=1 noncooperative binding, and n_H<1 negative cooperative binding.[43,45] Increasing concentrations of (allosteric) ligand are co-incubated with a submaximal concentration of agonist (chemokines) to obtain a sigmoidal concentration-effect curve where the highest concentration of ligand fully inhibits the receptor

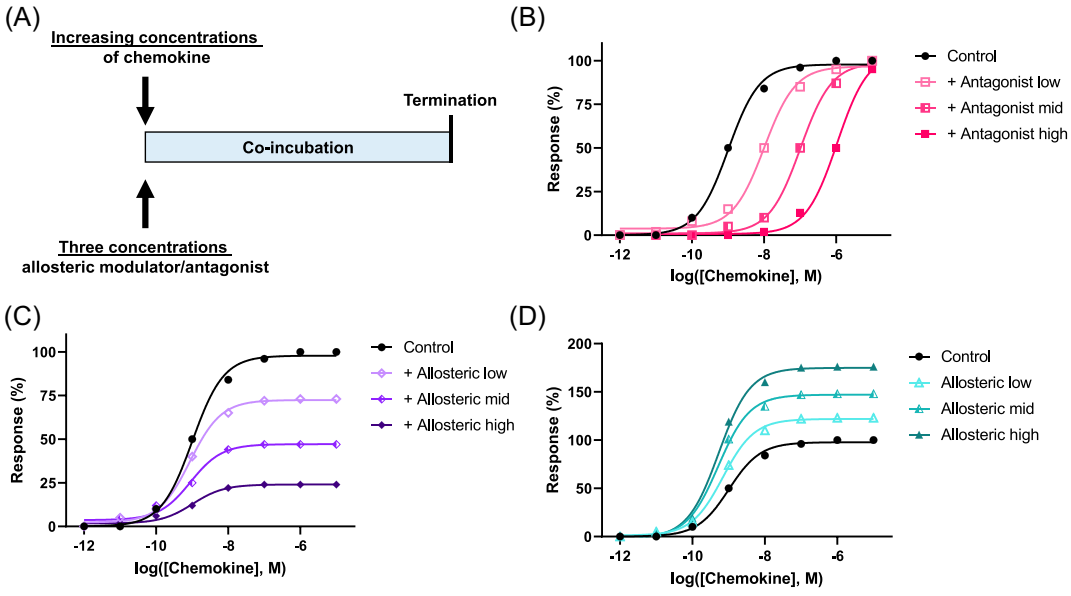

FIGURE 7 Insurmountability in functional assays. (A) Insurmountability can be assessed by co-incubating increasing concentrations of agonist (chemokine) and three different concentrations of antagonist or allosteric modulator. (B) Hypothetical graph of increasing concentrations of chemokine in the presence of an surmountable antagonist with high, mid, and low ligand concentrations (squares). (C) Hypothetical graph of increasing concentrations of chemokine in the presence of an insurmountable (allosteric) NAM with high, mid, and low ligand concentrations (diamonds), or (D) an insurmountable (allosteric) PAM with high, mid, and low ligand concentrations (triangles). [Color figure can be viewed at wileyonlinelibrary.com]

(Figure 8A). This coefficient can be determined from the steepness of the curve expressed in terms of potency, based on 10% and 90% of the maximal response (Figure 8B).[46]

At CCR1, pyrrolone derivatives were characterized as inhibitors of CCL3-mediated [^{35}S]GTPγS binding by Ortiz-Zacarías et al.[36] These compounds were based on the known allosteric modulator CCR2-RA-[*R*] for CCR2, and were presumed to bind at a similar allosteric site to CCR1. Indeed, three of the derivatives, compounds 39, 41, and 43, showed n_H values significantly lower than unity with −0.62 ± 0.0, −0.72 ± 0.08, and −0.73 ± 0.02, respectively, indicative of allosteric inhibition.

Other CCR2 targeting compounds, namely triazolopyrimidinone derivatives, were tested as novel antagonists at CCR2 and CCR5.[35] For CCR5, reference compounds TAK779 (orthosteric) and CCR2-RA-[*R*] (allosteric) were tested in CCL3-induced β-arrestin recruitment assays and exhibited n_H values of −1.1 and −2.4, respectively, confirming differential binding modes. Moreover, it was shown that compound 8 acted similarly to CCR2-RA-[*R*], with a n_H value of −2.2 ± 0.3. Triazolopyrimidinone derivatives 39 and 43 showed similar n_H values between CCR2 and CCR5. Derivative 39 showed a slope of n_H = −3.7 for CCR2 and n_H = −2.5 for CCR5, while derivative 43 showed a slope of n_H = −4.4 for CCR2 and n_H = −3.4 for CCR5.

5 | ELUCIDATION OF ALLOSTERIC BINDING SITE

Per definition, allosteric ligands should bind in a topologically different location compared to the endogenous ligands at their target. The location of this allosteric binding site can be determined by several methods that are discussed below.

5.1 | Mutagenesis

For mutational studies, any of the previously discussed assays can be used to determine an impact by the amino acid change, which is often a decrease or loss in affinity or potency of the compound under investigation for the receptor. This outcome is an indication of the residue's involvement in compound interactions, which can be supported by computational docking studies. However, an altered receptor state or expression can also cause different pharmacology.

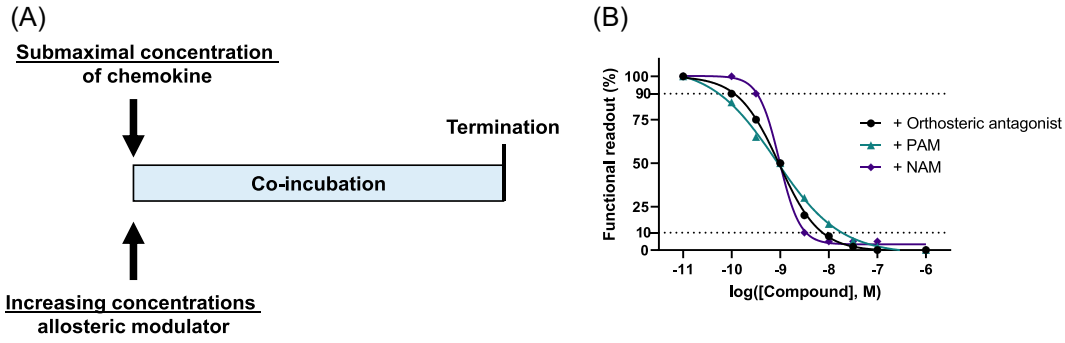

FIGURE 8 The Hill coefficient can be determined by calculating the steepness of the slope. (A) A submaximal concentration of agonist (i.e., chemokine) is co-incubated with increasing concentrations of ligand to obtain (B) concentration-inhibition curves of an orthosteric ligand (black circles), a PAM (pink triangles), and NAM (purple diamonds). [Color figure can be viewed at wileyonlinelibrary.com]

Site-directed mutagenesis of CXCR1 and CXCR2 resulted in the first evidence of an intracellular allosteric binding site in 2008.[47] SB332235, described as compound B, and AZD8309, described as compound A, both showed a preference in inhibition of CXCR2 over CXCR1 in CXCL8-induced calcium mobilization assays. This was reversed after switching the last 60 residues of the receptors' C-terminal tail, indicating the presence of an intracellular binding site. Site-directed mutagenesis and docking of the ligands showed that K320$^{8.49}$ (Ballesteros–Weinstein numbering[48]) in CXCR2 and N311$^{8.49}$ in CXCR1 were crucial for maintaining antagonist potency. Other CXCR2 ligands include [^{3}H]Navarixin, for which binding to CXCR2 was significantly decreased by mutations T83L$^{2.39}$, D84N$^{2.40}$, A249L$^{6.33}$, and [^{3}H]SB265610, while T83A$^{2.39}$, D84N$^{2.40}$, D143R$^{3.49}$, K320A$^{8.49}$, and Y314A$^{7.53}$ resulted in significantly affected affinity or SB265610 potency in response to CXCL8-induced G protein activation.[49]

For CCR2-RA-[*R*] at CCR2, site-directed mutagenesis showed that its binding site was indeed located intracellularly.[33] Affinity of [^{3}H]CCR2-RA was abolished at receptors containing V244A$^{6.36}$, Y305A$^{7.53}$, and F312A$^{8.50}$ mutations, while affinity of SD-24 and JNJ-27141491 was significantly decreased by D78N$^{2.40}$/K311A$^{8.49}$ and D78N$^{2.40}$/K72A$^{2.34}$. Induced fit docking of CCR2-RA-[*R*] into a CCR2 homology model indeed confirmed the involvement of V244$^{6.36}$, K311$^{8.49}$, Y305$^{7.53}$, and F312$^{8.50}$ in its binding.

5.2 | Structure elucidation

Receptor structures accommodating allosteric ligands are the most unambiguous proof of a ligand binding site distinct from the endogenous ligand binding site and involvement of specific residues. The currently available chemokine receptor structures with allosteric ligands are discussed below.

Crystal structures of CCR2 and CCR9 were published at the same time in 2016 (PDB identifier 5T1A and 5LWE, Figure 9A–D).[11,18] Both structures confirmed the binding location and poses of CCR2-RA-[*R*] and vercirnon for CCR2 and CCR9, respectively, to be (partially) overlapping with the G protein binding site.

CCR2-RA-[*R*]'s binding site in CCR2 was presented as a hydrophobic cage consisting of V63$^{1.53}$, L67$^{1.57}$, L81$^{2.43}$, L134$^{3.46}$, A241$^{6.33}$, V244$^{6.36}$, I245$^{6.37}$, Y305$^{7.53}$, and F312$^{8.50}$, while the bottom, cytosolic region consisted of the polar residues T77$^{2.39}$, R138$^{3.50}$, G309$^{8.47}$, K311$^{8.49}$, and Y315$^{8.53}$.[11] Hydrogen bonds between CCR2-RA-[*R*] and the exposed backbone amides of E310$^{8.48}$, K311$^{8.49}$, and F312$^{8.50}$ affixed the ligand in place. Interestingly, this structure confirmed the involvement of the residues found earlier in mutagenesis studies.[33] Notably, CCR2 was co-crystallized with the orthosteric BMS-681 and the allosteric CCR2-RA-[*R*] and their positive binding cooperativity resulted in the most inactive conformation of a chemokine receptor to date.[11]

For CCR9, the sulfone group of vercirnon forms hydrogen bonds with the backbone of amino acids E322$^{8.48}$, R323$^{8.49}$ and F324$^{8.50}$, and hydrophobic interactions with Y317$^{7.53}$.[18] The pyridine-*N*-oxide is surrounded by T83$^{2.39}$, D84$^{2.40}$, R144$^{3.50}$, R323$^{8.49}$ and T81^{ICL1}, and the ketone forms a hydrogen bond with the side chain of T256$^{6.37}$. The *tert*-butylphenyl group forms hydrophobic interactions with V69$^{1.53}$, V72$^{1.56}$, Y73$^{1.57}$, and L87$^{2.43}$, while Y317$^{7.53}$ shows edge-to-face π–π stacking with its aromatic core. The chlorophenyl is engaged with L87$^{2.43}$ in an apolar cavity consisting of L87$^{2.43}$, I140$^{3.46}$, A255$^{6.36}$, and V259$^{6.40}$.

Later, the crystal structure of CCR7 in complex with Cmp2105 was solved (PDB: 6QZH, Figure 9E,F).[20] The sulfonyl group interacts with Y326$^{7.53}$, G330$^{8.47}$, V331$^{8.48}$, K332$^{8.49}$, and F333$^{8.50}$ while the two central amino groups in the core form stabilizing hydrogen bonds with D94$^{2.40}$. Other substituents of Cmp2105 interact with TMI-III, forming hydrogen bonds with T91$^{2.37}$ and T93$^{2.39}$, hydrophobic interactions with V79$^{1.53}$, T82$^{1.56}$ and F86$^{1.60}$, and interacts with the highly conserved R154$^{3.50}$.

Most recently, multiple crystal structures of CXCR2 were determined of which one is an inactive conformation with the intracellular allosteric ligand 00767013 (PDB: 6LFL, Figure 9D).[12] This ligand forms hydrogen bonds with D84$^{2.40}$ and the conserved residues K320$^{8.49}$ and F321$^{8.50}$ via two amino groups and the ketone oxygens from the

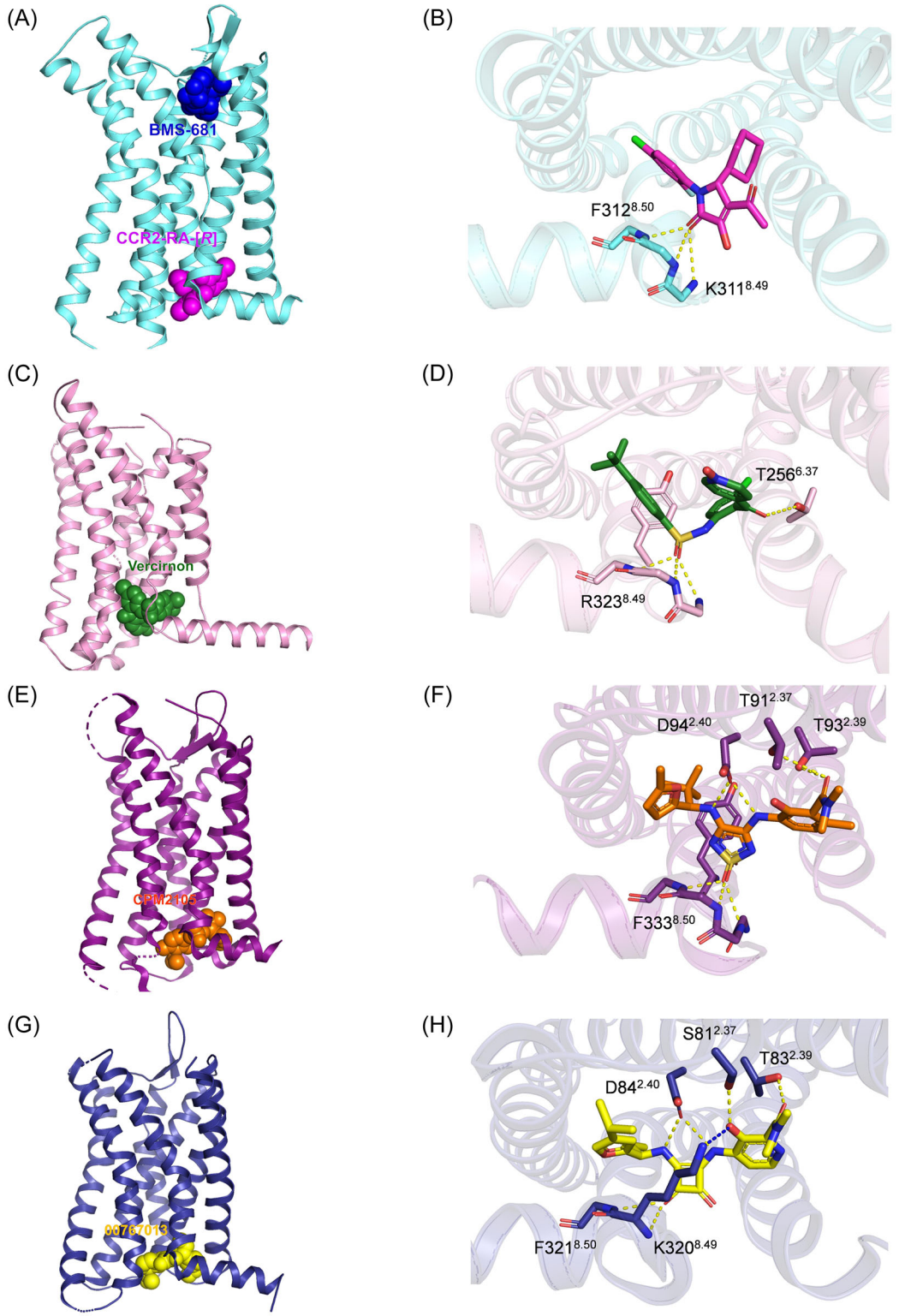

FIGURE 9 (See caption on next page).

core. The hydroxyl group in the pyridin-3-ol moiety and ketone oxygen on the left side of the molecule form further hydrogen bonds with S81$^{2.37}$ and T83$^{2.39}$ and a salt bridge with K320$^{7.49}$. Extensive hydrophobic interactions are formed with V69$^{1.53}$, V72$^{1.56}$, and I73$^{1.57}$ by the other side of the molecule.

6 | OUTLOOK

Chemokine receptors represent attractive drug targets, but the drug attrition rate for these receptors is particularly high. Allosteric modulators, and especially intracellularly binding antagonists, represent a valuable alternative to the classical orthosteric antagonists. Here, we have discussed the characterization of allosteric ligands for chemokine receptors in binding and functional assays and how to interpret the results. Furthermore, we have listed current knowledge of the binding site of intracellular allosteric ligands through published mutagenesis studies and elucidated structures.

First, allosteric modulation cannot be confirmed by using the discussed assays individually. Each provides an indication of (non-)competitiveness, while multiple assays should be performed to affirm both the noncompetitiveness as well as the nature of the allosteric ligand, that is, PAM or NAM. Although computational methods are an adequate step in obtaining insight into a ligand's binding site, mutational analysis or structure elucidation should be performed to substantiate the in silico data.

Allosteric modulators represent great opportunities as chemokine receptor drugs due to their relatively high selectivity compared to orthosteric ligands. Single- or multitarget approaches, such as triazolopyrimidinone derivatives for CCR2 and CCR5,[15,35] can be prioritized based on disease at hand. In addition, dual therapy through positive cooperativity between orthosteric and allosteric ligands such as shown by BMS-681 and CCR2-RA-[*R*][11] is an interesting avenue to explore for possible future treatments. Furthermore, allosteric ligands act in an insurmountable manner, as in the examples described above, which allows these ligands to be efficacious even with high local concentrations of chemokines present during disease.[13]

Other benefits of allosteric modulators which are currently under-investigated for intracellular ligands for chemokine receptors, are biased signaling and probe dependence, for which the authors recommend the reviews by Keov and colleagues and Slosky and colleagues.[16,17] In a few studies, it has been shown that orthosterically binding allosteric antagonists can selectively inhibit a signaling pathway (biased signaling) or block/modulate the effect of a specific chemokine (probe dependence).[50,51] With increasing knowledge of chemokine receptor-related diseases, specific allosteric ligands can therefore be selected to minimize disease-associated symptoms, while preventing adverse effects due to blockade of the immune system.

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FIGURE 9 Structures of chemokine receptors with intracellular allosteric ligands. (A) CCR2 (PDB: 5T1A) with BMS-681 (dark blue) and CCR2-RA-[*R*] (magenta) with (B) bottom view with CCR2-RA-[*R*] interactions. (C) CCR9 (PDB: 5LWE) with vercirnon (green) and (D) bottom view with vercirnon interactions. (E) CCR7 (PDB: 6QZH) with Cmp2105 and (F) bottom view with Cmp2105 interactions. (G) CXCR2 (PDB: 6LFL) with 00767013 and (H) bottom view with 00767013 interactions. Specific amino acids are represented as sticks, hydrogen bonding is shown as yellow dashed lines and ionic interactions in a blue dashed line. [Color figure can be viewed at wileyonlinelibrary.com]

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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