nature portfolio

Peer Review File

Structural basis for CCR6 modulation by allosteric antagonists

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
Wu et al reported two cryo-EM structures of inactive CCR6 in complex with different allosteric antagonists. The electron density maps are with overall high quality. Here, I have some minor issues:
1. In the structure of CCR6/SQA1/OXM1, the distance between atom OD1 of D82 (chain A) and atom N12 of SQA1 (PFE in chain C) is only 1.3 Å, which seems to be a covalent bond between them. The distance for non-covalent interaction must >1.5 Å and the rational distance range of hydrogen bond should be 2-4 Å.
2. In the structure of CCR6/SQA1/OXM1, although a cholesterol (CLR) molecule is located at the sides of TM2 and TM4, there is no description of the CLR in the PDB validation.
3. The procedure of model building and structure refinement must be described in the part of 'Method', including initial models, softwares, etc.
4. The B factors of ligands should be showed in Table S1.
5. The outliers of Ramachandran plot of CCR6/SQA1/OXM1 is 0.11%, which is not consistent with the PDB validation report.
6. Please refine the bond angle outliers to zero in the two structures.
7. Please show the EM density maps for ligands in Fig. 2.
8. It's better to include the SEC profile and SDS-PAGE results in Figure S3 and S4.
9. The color for local resolution map is strange, please reverse the color scheme.

Reviewer #2:
Remarks to the Author:
I have carefully reviewed the manuscript titled "Structural basis for CCR6 modulation by allosteric antagonists" and focused specifically on the utilisation of NMR spectroscopy techniques within the study. As an expert in NMR spectroscopy, I offer the following assessment:

The authors present a compelling investigation into the structural dynamics of CCR6 utilizing a combination of cryo-EM and NMR spectroscopy. Specifically, they explore the binding of oxomorpholine (OXM) analogues within the extracellular allosteric pocket, highlighting the importance of the U-shaped conformation for binding activity.

The NMR spectroscopy experiments conducted by the authors meet expected standards and provide crucial insights into the conformational preferences of the OXM analogues in solution. The ROESY experiment yields surprising yet undeniable evidence supporting the predominantly U-shaped conformation of OXM1. However, the absence of ROESY results for the other analogues raises questions regarding the experimental approach, which I recommend the authors address to enhance clarity.

Subsequent RDC analysis of all four OXM analogues in DMSO-d6 swollen poly-HEMA gels yields

sound results, with very low Cornilescu Q-factors. This analysis confirms the predominantly U-shaped conformation of the active OXM1 and OXM2 analogues, while revealing an extended conformation for the other two inactive analogues in solution. The authors' meticulous approach and robust results commendably support their conclusions regarding the structural basis for CCR6 modulation by allosteric antagonists.

Overall, I find the NMR work presented in this manuscript to be of excellent quality, meeting expected standards, and I recommend its publication in Nature Communications. However, I suggest that the authors deposit the NMR data in F.A.I.R. format alongside other supporting data to enhance transparency and reproducibility.

Reviewer #3:
Remarks to the Author:
Manuscript#: NCOMMS-24-17415
Title: Structural basis for CCR6 modulation by allosteric antagonists
Chemokine receptors, a crucial class of G protein-coupled receptors (GPCRs), are significant targets for various conditions such as cancers, autoimmune disorders, and infectious diseases. CCR6 belongs to the subfamily of ~20 chemokine receptors. It is activated by its protein agonist CCL20 resulting in several physiological responses, but also pathophysiological effects involving inflammation, psoriasis, and inflammatory bowel disease. Small molecule antagonists are needed for testing against CCR6 and these diseases. The structure of the CCL20-CCR6-G protein has previously been determined. CCL20 interacts with extracellular loops and the N-terminus of CCR6, leaving a shallow orthosteric cavity with little interactions of 3-4 residues but interactions with other residues at the N-terminal region of the CCL20. In contrast to other known three-dimensional chemokines-receptor complexes, the entire N-terminal chemokine region interacts with a much deeper cavity composed of the receptors' transmembrane regions (TM).

According to this manuscript, single-particle cryogenic electron microscopy (cryo-EM) employed an engineered fiducial marker (BRIL/Fab/Nb module). The allosteric antagonists, oxomorpholine analogues (OXM1 and OXM2) and squaramide (SQA1), bind to the receptor simultaneously at the extracellular and the intracellular regions and result in CCR6/SQA1/OXM1 and CCR6/SQA1/OXM2. The highly selective OXM1 and OXM2 oxymorphone (OXM) analogues disrupt the molecular network crucial for receptor activation by binding to an extracellular allosteric while SQA1 binds to the intracellular G protein site. A variety of chemical moieties from the allosteric antagonists and their H-bond, ionic, and hydrophobic interactions with CCR6 for affinity are probed by mutagenesis and NMR, and validated.

The specificity of OXM1 towards CCR6 was analyzed using the GPCR β-arrestin assay panel. Additionally, an NMR-RDC study confirmed the low-energy U-shaped conformation of OXM in the solution state. The study also highlights the non-specificity of SQA1, as the binding pockets are similar in CCR6 and CXCR2. The quality of data for this study is excellent for chemotaxis, ΔTM (C), cryo-EM, radiolabeled binding studies, SPR, and NMR. The analysis of the results is well supported,

although some minor observations are noted below. Overall, this study contributes novel insights into the diverse mechanisms of CCR6 allosteric antagonism due to their different binding locations. Furthermore, it suggests potential avenues for drug discovery targeting CCR6 (and CXCR2) in autoimmune disorders. The study represents a significant contribution to the field of CCR6 specifically and GPCR in general. The manuscript is well-structured, and the authors have provided ample details to support the conclusions drawn.

Significance: CCR6-CCL20 signaling axis is a well-known target for psoriasis and inflammatory bowel disease. Based on previous structural analyses, the molecular size of the extracellular pocket in CCR6 presents obstacles to the development of small molecule antagonists that target the orthosteric site. The current study has demonstrated that manipulating chemokine receptors through allosteric ligands, whether at extracellular or intracellular, represents an appealing and promising strategy in drug discovery.

I strongly recommend accepting the manuscript after minor changes.

Observations and changes to seriously consider:
1. In Fig. 1b, the IC50s of SQA1, OXM1, and OXM2 are shown individually as a response to CCL20 for chemotaxis. What is the response if SQA1 and the OXM (1 or 2) are used concurrently? Fig. 1c, d show thermostability for SQA + OXM1 and SQA + OXM2 but that is a different parameter than cellular migration.
2. Binding of an agonist at the orthosteric site and the intracellular G-protein in the intracellular region affect their affinity when used together versus alone. SQA and the OXMs affect the conformation at the extracellular and intracellular regions (Fig. 5c, d). Is there any effect on Ki's of SQA1 and the OXMs used simultaneously versus alone?
3. Both small molecules stabilize the complexes. It is important to know if the binding order affects this stability (another panel for Fig. 1 or supplementary figure).
4. The authors explored the binding pocket of SQA1 by mutating the interacting residues and assessing their saturation binding using radioligand in HEK293 cells. Similar experiments could be conducted to investigate the OXM binding pocket.
5. The authors should include labels for secondary structures and residue names (in sticks) in all figures to assist the reader.
6. Reference 17 describes the structural biology of CCR2 binding two antagonists simultaneously: one site is the orthosteric agonist and the other is an allosteric site at the G protein binding site. I think it deserves more explanation in the text, rather than part of a set of citations because that manuscript is a prelude to two allosteric antagonists for CCR6.

Reviewer #4:
Remarks to the Author:
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

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1. In the structure of CCR6/SQA1/OXM1, the distance between atom OD1 of D82 (chain A) and atom N12 of SQA1 (PFE in chain C) is only 1.3 Å, which seems to be a covalent bond between them. The distance for non-covalent interaction must >1.5 Å and the rational distance range of hydrogen bond should be 2-4 Å.

We first would like to thank the reviewer for the favorable comment to the quality of the electron density maps. We would also like to thank the reviewer for their thorough evaluations of the two models and for suggestions to improvement. We have refined the models based on the suggestions and are happy to report that the clashes between OD1 of D82 (chain A) and N12 of SQA1 (PFE in chain C) in the structure of CCR6/SQA1/OXM1 have been resolved in the updated model. The distance between the two abovementioned atoms in the updated model of CCR6/SQA1/OXM1 is 3.0 Å.

2. In the structure of CCR6/SQA1/OXM1, although a cholesterol (CLR) molecule is located at the sides of TM2 and TM4, there is no description of the CLR in the PDB validation.

We thank the reviewer for pointing out the absence of the cholesterol (CLR) molecule in our previous PDB validation. We identified a formatting issue with the previous PDB which resulted in exclusion of the CLR molecule in the validation. The issue has been fixed in the updated PDB files and the CLR molecule is now included in the updated validation reports.

3. The procedure of model building and structure refinement must be described in the part of 'Method', including initial models, softwares, etc.

We thank the reviewer for this suggestion. The procedures of model building and structure refinement including initial model and software used are now included as part of 'Methods' on page 30.

4. The B factors of ligands should be showed in Table S1.

We thank the reviewer for pointing this out. The B-factors of ligands are now included in the revised Table S1.

5. The outliers of Ramachandran plot of CCR6/SQA1/OXM1 is 0.11%, which is not consistent with the PDB validation report.

We thank the reviewer for catching this inconsistency. We have updated the table to reflect the Ramachandran outliers to be consistent with the PDB validation reports of the two updated models submitted with the revision.

6. Please refine the bond angle outliers to zero in the two structures.

We thank the reviewer for the comment. We have refined the bond angle outliers to zero in both structures.

7. Please show the EM density maps for ligands in Fig. 2.

We thank the reviewer for this suggestion. The EM density maps for the ligands have been moved from Figures S3 and S4 to Figure 2.

8. It's better to include the SEC profile and SDS-PAGE results in Figure S3 and S4.

We have included the SEC profile and SDS-PAGE of the complex purification in Figures S3 and S4.

9. The color for local resolution map is strange, please reverse the color scheme.

We thank the reviewer for pointing out the discrepancy in the coloring of the figure. The color scheme of the local resolution map has been reversed.

Reviewer #2 (Remarks to the Author):

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The NMR spectroscopy experiments conducted by the authors meet expected standards and provide crucial insights into the conformational preferences of the OXM analogues in solution. The ROESY experiment yields surprising yet undeniable evidence supporting the predominantly U-shaped conformation of OXM1. However, the absence of ROESY results for the other analogues raises questions regarding the experimental approach, which I recommend the authors address to enhance clarity.

We thank the reviewer for this excellent suggestion. We have added NOESY results for OXM2 which clearly show a long-range NOE from H-21/24/25 to H-17/H-19 and from H-21/24/25 to H20/H-16, supporting the U-shaped conformation (Fig. S35). OXM3 and OXM4 did not exhibit long range ROE/NOE correlations, consistent with linear conformations in solution for these two compounds. We have also modified the corresponding text on page 8, lines 160-169 to describe the new NMR data and have updated the NMR methods on pages 31-32 to reflect the changes.

Subsequent RDC analysis of all four OXM analogues in DMSO-d6 swollen poly-HEMA gels yields sound results, with very low Cornilescu Q-factors. This analysis confirms the predominantly U-shaped conformation of the active OXM1 and OXM2 analogues, while revealing an extended conformation for the other two inactive analogues in solution. The authors' meticulous approach and robust results commendably support their conclusions regarding the structural basis for CCR6 modulation by allosteric antagonists.

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We would like to thank the reviewer for their compliment regarding our NMR work. We also thank the reviewer for the suggestion of submitting data in the F.A.I.R. format to support transparency and reproducibility. We are in agreement with this suggestion and have supported this initiative in our previous publications: *J. Org. Chem.* 2019, **84**, 8, 4803–4813 (https://pubs.acs.org/doi/10.1021/acs.joc.8b02811) and *Angew. Chem. Int. Ed*. 2021, **60**, 26314 – 26319 (https://doi.org/10.1002/anie.202106794). As we did in the past, we will publish the RDC solution conformer files for OXM1, OXM2, OXM3, and OXM4, as well as the MSpin/Stereofitter input files for each compound with this manuscript. These data have been provided alongside the revision. With all these data, the lowest energy solution conformer for each compound can be reproduced using Mestre MSpin/ Stereofitter software.

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OXM1 and OXM2 oxymorphone (OXM) analogues disrupt the molecular network crucial for receptor activation by binding to an extracellular allosteric while SQA1 binds to the intracellular G protein site. A variety of chemical moieties from the allosteric antagonists and their H-bond, ionic, and hydrophobic interactions with CCR6 for affinity are probed by mutagenesis and NMR, and validated.

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We first would like to thank the reviewer for their strong recommendation and endorsement of our work. We also thank the reviewer for the question about CCR6 inhibition by SQA1 and OXM co-treatment. We have demonstrated that SQA and OXM analogues antagonize CCR6 via binding to different pockets and co-treating the receptor by both ligands, SQA1+OXM1 or SQA1+OXM2 led to additive stabilization of the receptor. Given that both SQA and OXM analogues are antagonists which fully antagonize CCR6 with comparable IC_{50}s, we anticipate full inhibition of T-cell migration mediated by CCR6 when the cells are co-treated by both ligands, like that was observed with single ligand.

2. Binding of an agonist at the orthosteric site and the intracellular G-protein in the intracellular region affect their affinity when used together versus alone. SQA and the OXMs affect the conformation at the extracellular and intracellular regions (Fig. 5c, d). Is there any effect on Ki's of SQA1 and the OXMs used simultaneously versus alone?

We thank the reviewer for raising this important question to understand if there is any crosstalk between the two allosteric pockets of CCR6 discovered by our work. We have thereby conducted additional experiments to investigate the pocket communication, which include saturation binding of [^{3}H]-SQA1 on WT CCR6 with and without OXM1, as well as SPR experiments to measure OXM1 binding on a stabilized CCR6 with and without SQA1. To our surprise, we observed minimal communication occurred between the two allosteric pockets of SQA and OXM, differing from the cooperativity observed between orthosteric agonists and intracellular signaling transducers.

Findings from our crosstalk studies were summarized on page 12 before "Discussion". The experimental data were presented by two new supplementary figures, Figures S37 and S38 in the revised supplementary information. Please note that the crosstalk SPR was conducted with a further stabilized construct CCR6_Nβ10.2. We therefore modified the "CCR6 thermostabilization and characterization" method on pages 26-27 and Figures S1-S2 to include information of this new construct. We also updated the [^{3}H]-SQA1 saturation binding method on page 27 to include methods used to evaluate the crosstalk. Lastly, we added the method used to study OXM1 crosstalk with SQA1 on page 34, paragraph "Crosstalk study by SPR".

3. Both small molecules stabilize the complexes. It is important to know if the binding order affects this stability (another panel for Fig. 1 or supplementary figure).

We thank the reviewer for pointing out the question regarding the order of binding. In our thermostability assay, we have tried different orders of ligand additions (OXM first, then SQA, as well as SQA first, then OXM) in the cotreating conditions. We have not observed any differences in receptor stabilization because of different ligand binding orders. In fact, the data points provided in Figs. 1c and 1d contain data from both ligand binding orders. We agree it is important to address potential questions around the binding orders. Therefore, we included further clarification on page 5, lines 87 -91: "Such additive stabilization was not dependent on the order of ligand addition between SQA1 and OXM analogues but was not observed when CCR6 was co-incubated with the two different OXM molecules. These results suggest that … the two series … can bind independently and simultaneously to the receptor.".

4. The authors explored the binding pocket of SQA1 by mutating the interacting residues and assessing their saturation binding using radioligand in HEK293 cells. Similar experiments could be conducted to investigate the OXM binding pocket.

We thank the reviewer for this suggestion. We agree that the saturation binding of OXM analogue and mutagenesis of the OXM binding pocket using a radiolabeled OXM analogue like what we did for SQA1 could also be conducted. However, due to the absence of a radiolabeled OXM analogue, we did not carry out these experiments. But with the extensive biochemical, biophysical, and structural characterization we have conducted for the OXM analogues, we hope we have provided an ample amount of data to investigate the molecular mode of action of these molecules in this manuscript.

5. The authors should include labels for secondary structures and residue names (in sticks) in all figures to assist the reader.

We thank the reviewer for pointing this out. We have now added labels for all TMs and residues shown in sticks in Figures 3a, 3c, 4a, 4e-f, and 5e-h for clarity.

6. Reference 17 describes the structural biology of CCR2 binding two antagonists simultaneously: one site is the orthosteric agonist and the other is an allosteric site at the G protein binding site. I think it deserves more explanation in the text, rather than part of a set of citations because that manuscript is a prelude to two allosteric antagonists for CCR6.

We thank the reviewer for making this suggestion. We fully agree that the structure of CCR2 with orthosteric and allosteric antagonists is a highly relevant reference to discuss crosstalk between two non-overlapping pockets. Based on this suggestion, we used the reported work of CCR2 by Zheng, Y. *et al* as the reference when we discussed the crosstalk study between the OXM and SQA pockets of CCR6. The discussion was added to page 12.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We thank the reviewer for their time to co-review the manuscript. We would also like to thank all the reviewers again for their favorable comments and the very helpful feedback to further improve our work.

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
The authors have addressed all my concerns.

Reviewer #2:
Remarks to the Author:
I can report that in the revised manuscript the authors have addressed all my previous comments and thus I would thoroughly recommend its publication in Nature Communications.

Reviewer #3:
None

Reviewer #4:
Remarks to the Author:
Comments to the Authors' section of the review: "I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.