This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

nature portfolio

Peer Review File

A small molecule enhances arrestin-3 binding to the β2-adrenergic receptor

Corresponding Author: Dr Ozge Sensoy

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript the authors explored “the role of small molecule to enhance arrestin-3 binding to Beta 2 adrenergic receptor” . Here, authors used various approach to confirmed the validity of the studies and potentially important for the regulation of G protein-coupled receptors in a pathological conditions scenario. NMR, Nano Bit and FRET approach to validate the results.
I have the following concern
1. It is not clear from the manuscript, what criteria authors employed to select these compounds from the Virtual screen ? What was the initial screen results?. How have they done?

2. Authors study the L SH-3 is the most interesting compound to evaluate the arrestin 3 recruitment. How specific these interactions? Do this compound interact with arrestin 1 and arrestin 2.?

3. Authors made a phosphorylation deficient mutant of B2 adrenergic receptor, but lacking the characterization of this construct, or its phosphorylation patten. It is better to show these details.
Minor Comments
1. Figure 1A, labeling the residues is helpful to understand the distance and position of the residues.

2. Figure 4- Authors studied the arrestin 3 interaction with PD- B-AR, but the figure showing as arrestin 2. Need to be corrected.

Reviewer #2

(Remarks to the Author)
The manuscript presents an interesting study on interactions between the β2-adrenergic receptor and arrestins. Kurt et al discovered a small molecule that increases in-cell arrestin-3 binding to phosphorylation-deficient and wild-type β2-adrenergic receptor, but not to muscarinic M2 receptor. Several issues should be addressed to improve the quality and impact of the manuscript.

1. Different functional states (inactive/pre-activated/active states) of protein conformations are mentioned. Why does a conformation belong to a specific state? The criteria for classifying the inactive and active conformations are unclear and not well explained, particularly for the intermediate state conformation. Please provide more information on the quantitative classification of functional states. Also, discuss the functional states of all related experimental structures.

2. The manuscript uses bovine (PDB ID: 3P2D) arrestin-3 as a template to model human arrestin-3, but the reasons for choosing this template are not discussed. More information should be provided for homology modeling and initial conformation selection.

3. In the virtual screening process, seven snapshots were used. It is not explained how these were selected from various conformational states. The manuscript mentions pharmacophore modeling based on binding pockets. How was the pharmacophore constructed from the geometric structure of the binding pocket? More information should be provided for virtual screening.

4. Under the Plasmid transfection method, the authors mentioned the plasmid transfection into H9C2 cells and cAMP detection. Please provide relevant experimental data.

5. Did the authors detect the expression differences between mutants and wildtype? Mutation may have a significant impact on the expression level and thus potentially affect the FRET/Donor value.

6. The manuscript and supplementary could benefit from further polishing.

Reviewer #3

(Remarks to the Author)
Precise regulation of signal initiation and termination is essential for cellular homeostasis. This study focuses on targeting pre-activated arrestin-3 conformations derived from molecular dynamics (MD) simulations, identifying a small molecule predicted to bind arrestin-3. The binding was verified using saturation-transfer difference (STD) NMR. These insights may interest drug discovery researchers. However, some issues should be addressed before publication.I recommend the manuscript for publication after substantial revisions.

1. The homology-based structural model requires thorough evaluation. Consider comparing it with relevant AI-predicted models to validate its accuracy, and other evaluation methods.
2. The methods section includes non-essential details but omits crucial information, such as simulation time and the number of independent simulations in aMD simulations. Details like the threshold energy for dihedral angles, dependent on the number of atoms and the cMD state, have limited reference value.
3. Is the use of several interatomic distances sufficient to distinguish conformational states (inactive, pre-active, active)? It might be beneficial to include assessments using secondary structure angles or residue-specific angles for a more robust evaluation.
4. The study employs SiteMap for predicting potential binding sites. To reinforce the accuracy of these predictions, consider using additional tools and comparing the results.
5. The study could further emphasize its relevance to the development or treatment of specific diseases, thereby highlighting its significance in a broader biomedical context.
6. The manuscript and supplementary information lack detailed MD simulation data, such as RMSD and changes in distances between key residues with the simulation. Providing this data would facilitate a comprehensive evaluation of the simulation's robustness.
7. The manuscript's clarity could be improved by reducing lengthy or complex sentences and adopting a more concise and direct writing style. Additionally, enhancing the quality of the figures is recommended.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Authors clearly answered all my quires. Additional information provided by the authors improved the manuscript and the readers are able to understand the logic behind the use of these small compounds.
I recommend the manuscript can be acceptable for publication.

Reviewer #2

(Remarks to the Author)
No more comments.

Reviewer #3

(Remarks to the Author)
The authors have reasonably addressed my concerns, and now I suggest accepting it for publication in this journal.

Dear Editor,

We would thank you and the reviewers for careful reading of the manuscript and constructive suggestions. Please find enclosed the revised manuscript, where all changes are highlighted in yellow. In addition, below are point-by-point responses to all reviewers' comments. We hope that you find the revised manuscript suitable for publication.

Sincerely,

Reviewer #1 (Remarks to the Author):

In this manuscript the authors explored "the role of small molecule to enhance arrestin-3 binding to Beta 2 adrenergic receptor" . Here, authors used various approach to confirmed the validity of the studies and potentially important for the regulation of G protein-coupled receptors in a pathological conditions scenario. NMR, Nano Bit and FRET approach to validate the results.
I have the following concern:
1. It is not clear from the manuscript, what criteria authors employed to select these compounds from the Virtual screen ? What was the initial screen results? How have they done?

We are grateful to the reviewer for raising this point. We have revised the Methods sections entitled `reaction coordinates and structure classification`, `virtual screening on selected conformations`, and `docking` (Page 12: Lines 462-497), to explain what we did in more detail. Also, we have included additional tables and figures in the supplementary information (highlighted below) to address these comments.

Briefly, we performed three atomistic MD simulations of basal arrestin-3 in water. We clustered conformations based on two reaction coordinates reporting on the status of the gate loop and the back loop, based on values found in the basal and receptor-bound structures of arrestin (Supplementary Table 1 for the list of structures used). Then, we picked seven conformations (Supplementary Fig.2) representing various combinations of the two reaction coordinates (Supplementary Table 2). By means of pocket mapping we consistently identified the back loop and the gate loop as preferred binding sites on all structures (Supplementary Table 4), then proceeded to virtual screening using the three conformations in which these two pockets showed the highest druggability score, site score, and size (Supplementary Table 3 and Supplementary Table 5). Virtual screening returned 2055 compound hits. We then filtered them by means of a combined strategy. First, we preliminarily ranked the compounds considering their docking scores (Supplementary Table 6), ADME predictors (number of metabolites, human oral absorption value, molecular weight, QplogHERG, and QplogKhsa,) and binding poses (those that block interaction between the finger loop and the back loop, which stabilizes basal conformation, are prioritized). Then, the 22 highest ranking molecules were subjected to two 1 microsecond MD simulations in complex with arrestin-3. After filtering out the compounds that

dissociated within 50 ns of MD, we checked the impact of the remaining ones on the protein inter-domain rotation angle. We selected compounds inducing a rotation angle higher than or close to that adopted by basal arrestin-3 (Supplementary Table 6). The selected compounds L^{SH1}, L^{SH2}, L^{SH3} satisfy these criteria. The L^{GL} compound, although predicted to have a limited effect on the inter-domain rotation angle, was included in the experimental studies to compare the effect of a compound targeting the gate loop.

2. Authors study the L^{SH}-3 is the most interesting compound to evaluate the arrestin 3 recruitment. How specific these interactions? Do this compound interact with arrestin 1 and arrestin 2.?

We thank the reviewer for bringing up the selectivity issue. To address this question, we performed additional studies, where we tested the selectivity of the compound towards arrestin subtypes by means of both computational and experimental approaches. Specifically, we performed induced fit docking using arrestin-1 and arrestin-2 as targets in comparison to arrestin-3. For arrestin-3 and arrestin-2, we used as target the representative conformation of the most populated cluster of atomistic MD trajectories. For arrestin-1, we used as targets the pre-activated (PDB ID: 4ZRG) and the activated crystal structure (PDB ID: 5W0P). Our results suggested that compound L^{SH3} significantly binds to the predicted pocket only in arrestin-3, while the other subtypes show poorer score and mostly outside of the target site. To experimentally test the effect of L^{SH3} on arrestin-2, we performed the same NanoBIT experiment where the biological activity of L^{SH3} was tested on arrestin-3 (Fig. 6). No effect on arrestin-2 binding to the receptors was detected. We could not perform this experiment with arrestin-1, as its cognate receptor, rhodopsin, is light sensitive and decays within seconds of activation. We described new results and corresponding methods in the revised version of the manuscript (Fig. 6 ; Page 8; lines 312-321).

3. Authors made a phosphorylation deficient mutant of B2 adrenergic receptor, but lacking the characterization of this construct, or its phosphorylation patten. It is better to show these details.

We thank the reviewer for this question. In fact, this phosphorylation-deficient mutant is widely used in the literature and cited in the manuscript (Krasel, C. *et al*. Dual role of the β2-adrenergic receptor C terminus for the binding of β-arrestin and receptor internalization. *J Biol Chem* **283**, 31840–31848 (2008)). Therefore, we believe that it did not need additional characterization.

Minor Comments

1. Figure 1A, labeling the residues is helpful to understand the distance and position of the residues.

Thanks! We have included the labels in the revised version of the manuscript.

2. Figure 4- Authors studied the arrestin 3 interaction with PD- B-AR, but the figure showing

as arrestin 2. Need to be corrected.

Thanks for your attention to detail. We have corrected this error.

Reviewer #2 (Remarks to the Author):

The manuscript presents an interesting study on interactions between the β2-adrenergic receptor and arrestins. Kurt et al discovered a small molecule that increases in-cell arrestin-3 binding to phosphorylation-deficient and wild-type β2-adrenergic receptor, but not to muscarinic M2 receptor. Several issues should be addressed to improve the quality and impact of the manuscript.

1. Different functional states (inactive/pre-activated/active states) of protein conformations are mentioned. Why does a conformation belong to a specific state? The criteria for classifying the inactive and active conformations are unclear and not well explained, particularly for the intermediate state conformation. Please provide more information on the quantitative classification of functional states. Also, discuss the functional states of all related experimental structures.

We thank the reviewer for his/her comment. Following the suggestion, we clarified our classification by rephrasing the relevant parts in the Results section (Page 11; lines 436-446). Briefly, for the classification of conformations sampled in MD trajectories as basal/pre-activated/active, we first determined relevant regions that undergo rearrangement upon receptor binding, focusing on the N-C interface including the gate loop and back loop. To this end, we measured distances between the two residue pairs located on the gate loop and back loop in structures of different functional forms of arrestin subtypes determined experimentally. Supplementary Table 1 has been added, listing all the experimental structures used in classification, along with the values measured for the coordinates, and the protein functional states. As shown in Figure 1B of the original manuscript, these two coordinates successfully discriminate between basal and receptor-bound forms of arrestin. To define the pre-activated state, we measured the same pair distances in crystal structures of pre-activated arrestin-1 (PDB ID: 4ZRG & 3UGU). The corresponding values are in between those in the basal and receptot-bound arrestins. MD snapshots falling within this interval, formerly labeled as intermediate, are therefore classified as pre-active.

2. The manuscript uses bovine (PDB ID: 3P2D) arrestin-3 as a template to model human arrestin-3, but the reasons for choosing this template are not discussed. More information should be provided for homology modeling and initial conformation selection.

Thanks! We provided this information in the Methods section of the revised manuscript. There is no crystal structure of human arrestin-3, so we used the crystal structure of bovine arrestin-3 (PDB ID: 3P2D) to model it since the sequence identity between the two organisms is 95.2%. Moreover, in this crystal structure, the number of C-tail residues resolved is higher than in the other arrestin crystal structures available. The missing C-tail residues were

modeled as coil, using Modeller. The lowest DOPE energy conformation was selected as the starting structure. The modeled C tail residues were rather mobile along the MD simulations, as should be expected, as they were not resolved in crystal structures (Page 10; lines 386-387 & lines 390-395).

3. In the virtual screening process, seven snapshots were used. It is not explained how these were selected from various conformational states. The manuscript mentions pharmacophore modeling based on binding pockets. How was the pharmacophore constructed from the geometric structure of the binding pocket? More information should be provided for virtual screening.

We thank the reviewer for pointing this out. To address this issue, we revised the Methods sections entitled `reaction coordinates and structure classification`, `virtual screening on selected conformations`, and `docking` in the Results (Page 12: Lines 462-497). Also, we included additional tables and figures in the supplementary information to provide details that were missing in the original manuscript.
Briefly, we performed three atomistic MD simulations of basal arrestin-3 in water. We clustered conformations based on two reaction coordinates, monitoring the status of the gate loop and the back loop through specific pair distances varying in basal, pre-activated and receptor-bound structures of arrestin. Accordingly, we picked seven conformations representing various combinations of coordinates (Supplementary Table 2). By pocket mapping we consistently identified the back loop and the gate loop pockets in all structures, which was then confirmed using different methods (Supplementary Table 3 & 4). We then proceeded to virtual screening using the three conformations in which the selected two pockets showed the highest druggability, site score, and size (Supplementary Table 5). The pharmacophore model based on receptor bound conformation was built of residues 123-128/306-308/316 and 168-172/291-299 for the back loop and the gate loop, respectively (Supplementary Table 5). Virtual screening returned 2055 compound hits.
We then filtered the hits by combined strategy. First, we preliminarily ranked the compounds considering their docking scores (Supplementary Table 6), ADME predictors (number of metabolites, human oral absorption value, molecular weight, QplogHERG, and QplogKhsa,) and binding poses (those that block interaction between the finger loop and the back loop, which stabilizes basal conformation, are prioritized). Then, the 22 highest ranking molecules were subjected to two one-microsecond MD simulations in complex with arrestin-3. After filtering out the compounds which dissociated within 50 ns of MD, we measured the impact of the remaining ones on the arrestin inter-domain rotation. We selected compounds inducing a rotation angle higher than or close to that adopted by basal arrestin-3 (Supplementary Table 6). The selected compounds L^{SH1}, L^{SH2}, L^{SH3} satisfy these criteria. The L^{GL} was added to the list to examine the effect of a compound predicted to target the gate loop.

4. Under the Plasmid transfection method, the authors mentioned the plasmid transfection into H9C2 cells and cAMP detection. Please provide relevant experimental data.

We thank the reviewer for careful reading. We removed that section in the revised version of the manuscript, as this is still ongoing work.

5. Did the authors detect the expression differences between mutants and wildtype? Mutation may have a significant impact on the expression level and thus potentially affect the FRET/Donor value.

We thank the reviewer for bringing up this important issue. The wild type and the mutant proteins are fluorescently tagged, and transfected cells emitted similar amounts of fluorescence. We did ratio-metric fluorescence measurements in the cells where these constructs were expressed at comparable levels.

6. The manuscript and supplementary could benefit from further polishing.

Thanks! We revised the figures and the text both in the manuscript and the supplementary information.

Reviewer #3 (Remarks to the Author):

Precise regulation of signal initiation and termination is essential for cellular homeostasis. This study focuses on targeting pre-activated arrestin-3 conformations derived from molecular dynamics (MD) simulations, identifying a small molecule predicted to bind arrestin-3. The binding was verified using saturation-transfer difference (STD) NMR. These insights may interest drug discovery researchers. However, some issues should be addressed before publication.I recommend the manuscript for publication after substantial revisions.

1. The homology-based structural model requires thorough evaluation. Consider comparing it with relevant AI-predicted models to validate its accuracy, and other evaluation methods.

Thanks! We have provided additional information in the Methods section of the revised manuscript regarding the template selection used for homology modeling and the protocol. The crystal structure of human arrestin-3 is not solved, so we used the crystal structure of bovine arrestin-3 (PDB ID: 3P2D) as a template, since the sequence identity between the two proteins is 95.2%. Also, in this crystal structure, the number of C-tail residues resolved is higher than in the other crystal structures available. The missing C-tail residues were modeled as coil, using Modeller. The lowest Modeller energy conformation was selected as the starting structure. The modeled C tail residues were rather mobile along the MD simulations, as should be expected, as this element is likely disordered and therefore not resolved in available structure (Page 10; lines 386-387 & lines 390-395). Given very high sequence identity with bovine homolog, an AI-based structure prediction method like Alpha Fold, which relies on sequence alignment and structural databases, is likely to give consistent results with the one we obtained via homology modelling. Nonetheless, to

address the question raised by the reviewer, we generated a model for human arrestin-3 using Alpha Fold 3 server. The average RMSD between the 5 best AF3 models and our homology-modelled starting structure ranges from 2.1 Å and 2.2 Å, considering backbone atoms in the residue set 8-170, 190 to 348, i.e. the high confidence parts of the AF model. We believe that this result is consistent with our prediction and validates the homology model.

2. The methods section includes non-essential details but omits crucial information, such as simulation time and the number of independent simulations in aMD simulations. Details like the threshold energy for dihedral angles, dependent on the number of atoms and the cMD state, have limited reference value.

We thank the reviewer for pointing this out. In the Methods section `accelerated molecular dynamics` of the original manuscript we inadvertently omitted the number/length of aMD simulations. aMD was used as an independent validation to check whether the system might be exploring more conformational states than the ones observed in standard MD. Overall, a single aMD trajectory of length 1μs yielded a distribution of coordinates that was comparable with the classical one. We added this information to the revised version of the manuscript (Page 11; lines 420).

3. Is the use of several interatomic distances sufficient to distinguish conformational states (inactive, pre-active, active)? It might be beneficial to include assessments using secondary structure angles or residue-specific angles for a more robust evaluation.

We thank the reviewer for this comment. We used two distances, within the gate and the back loop, to classify conformations sampled in arrestin-3 trajectories. The latter is also associated with the folding state of the short alpha helix, which unravels upon activation. The position of the gate loop has been previously used as an activation marker. We show that these two parameters are effectively separated between receptor-bound and basal structures (Figure 1). We also coupled them to the inter-domain rotation angle as a global measure of the activation state.

4. The study employs SiteMap for predicting potential binding sites. To reinforce the accuracy of these predictions, consider using additional tools and comparing the results.

We thank the reviewer for the suggestion which we followed. Our additional analysis further validates the pocket mapping we proposed. In detail, we have applied two further pocket detection tools in addition to the SiteMap. One of them is *fpocket*, which identifies a high number of pockets (~30) in each of the 7 arrestin snapshots (Supplementary Fig.2 & Supplementary Table 2). The back loop pocket appears in the top 10 pockets, whereas the gate loop pocket emerges as a relevant binding site in several snapshots. The convolutional neural network-based detection tool *DeepSite* yields only 3 major binding sites on arrestin; among them, the back loop and gate loop pockets are always present and often top ranking. We have expanded Supplementary Table 4 to include these results. The cross-validation is

now mentioned in the Methods and in the Results section of the revised manuscript (Page 13; lines 492-498).

5. The study could further emphasize its relevance to the development or treatment of specific diseases, thereby highlighting its significance in a broader biomedical context.

We thank the reviewer for this suggestion. We expanded the discussion and added relevant references. E.g., the following paragraph was included in the revised version of the manuscript (page 9: lines 344-353).

“Mutations in GPCRs are at the root of numerous congenital disorders (reviewed in (1-7)). Excessive signaling by GOF mutants of various GPCRs was shown to cause retinal degeneration (rhodopsin (8, 9)), hyperthyroidism or toxic thyroid adenomas (thyrotropin receptor (10-12)), male precocious puberty (LH receptor (13)), obesity (melanocortin-4 receptor (14)), and various forms of cancer (15), to name just a few disorders. Therefore, enhanced arrestins stabilized by small molecules, thus having binding capacity to hyperactive GPCRs might be considered as an alternative therapeutic approach to treat such diseases.”

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2. Schoneberg, T., Schulz, A., Biebermann, H., Hermsdorf, T., Rompler, H., and Sangkuhl, K. (2004) Mutant G-protein-coupled receptors as a cause of human diseases Pharmacology & therapeutics 104, 173-206
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4. Parnot, C., Miserey-Lenkei, S., Bardin, S., Corvol, P., and Clauser, E. (2002) Lessons from constitutively active mutants of G protein-coupled receptors Trends in endocrinology and metabolism: TEM 13, 336-343
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mutation altering the carboxyl terminal sequence of rhodopsin Br J Ophthalmol 77, 495-501
10. Kosugi, S., Hai, N., Okamoto, H., Sugawa, H., and Mori, T. (2000) A novel activating mutation in the thyrotropin receptor gene in an autonomously functioning thyroid nodule developed by a Japanese patient European journal of endocrinology / European Federation of Endocrine Societies 143, 471-477
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and Gs alpha genes as a cause of toxic thyroid adenomas The Journal of clinical endocrinology and metabolism 82, 2695-2701
13. Laue, L., Chan, W. Y., Hsueh, A. J., Kudo, M., Hsu, S. Y., Wu, S. M. et al. (1995) Genetic heterogeneity of constitutively activating mutations of the human luteinizing hormone receptor in familial male-limited precocious puberty Proceedings of the National Academy of Sciences of the United States of America 92, 1906-1910
14. Tao, Y. X. (2014) Constitutive activity in melanocortin-4 receptor: biased signaling of inverse agonists Advances in pharmacology 70, 135-154
15. Arang, N., and Gutkind, J. S. (2020) G Protein-Coupled receptors and heterotrimeric G proteins as cancer drivers FEBS Lett 594, 4201-4232

6. The manuscript and supplementary information lack detailed MD simulation data, such as RMSD and changes in distances between key residues with the simulation. Providing this data would facilitate a comprehensive evaluation of the simulation's robustness.

We thank the reviewer for this suggestion. We have provided backbone RMSDs of basal arrestin-3 calculated from classical atomistic simulations (Supplementary Fig.1). We also added timeline plots that show the distance between the center-of-mass of the compound and the center-of-mass of the back loop of arrestin-3 (Supplementary Fig. 3).

7. The manuscript's clarity could be improved by reducing lengthy or complex sentences and adopting a more concise and direct writing style. Additionally, enhancing the quality of the figures is recommended.

We have revised figures and text both in the manuscript and in the supplementary information and we hope that the revised version is better in this regard.