nature portfolio

Peer Review File

Structural insights into the agonist selectivity of the adenosine A3 receptor

Corresponding Author: Professor Osamu Nureki

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

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors reported high-resolution structures of sheep adenosine receptor A3R bound with nonselective and selective agonists, in which one selective agonist was screened out. Based on Cryo-EM structures, authors analyzed the selectivity mechanism of A3R, the hydrophobic pocket located at the extracellular side. The authors applied structure-guided engineering for m6A-insensitive and adenosine-sensitive A3R to facilitate further physiological and pathological studies of m6A. The article presented the importance of the structural study of A3R and facilitated further functional study and drug development. Most results and explanations are reasonable and arranged in a logical way, while some need to be checked and improved.

Major issues:
1. The thickness of helices in figures should be decreased to clarify domain comparison and interacting residues.
2. Fig. 4h: Figure 4h2 is nearly identical to 4d1. Figure 4h5 did not present E17445.53 well.
3. Line 152-155: P34.50 involves interaction with G protein in class A GPCRs. Please clarify whether the interaction has been changed, given that P34.50 has been replaced by T34.50. Furthermore, please explain how “slightly elongated TM3” influences “extensive interactions across ICL2”.
4. Line 174-176: Residue histidine adopts a bulking sidechain compared to serine, while both residues can form polar hydrogen interactions. Discussion of these two residues should probably emphasize the size difference, further resulting in loss of polar interaction.
5. Lines 180 and 191: The mutagenesis study is better done and presented in figures instead of references.
6. Line 176-182: Residue V16945.53 in human A3R is different both in sidechain size and polarity compared to other adenosine receptors of human (E45.53) and sheep A3R (R45.53). Since the article mentioned position 45.53 consists of part of the hydrophobic pocket, which is important for ligand selectivity, please clarify the confidence level that the selectivity mechanism between human adenosine receptor subtypes can be explained by sheep A3R structures. Also, please give a possible explanation for the decrease in potency through nonpolar to polar mutation in A3R, while decreased potency in other adenosine receptors is through polar to nonpolar mutation.
7. Line 196: Providing figures superposing hydrophobic pocket of A3R and other adenosine receptors could be better exhibiting narrower volume. Furthermore, please explain more clearly that tight packing could result in higher potency.
8. Line 204: Providing a figure superposing i6A and m6A is needed to exhibit distinct placement of N6 substitution.
9. Section “Structure-guided engineering of m6A-insensitive A3R”: Homology mutagenesis is a widely used method to evaluate selectivity-related residues. Please check this method to evaluate engineered residues and engineered results.
10. Line 260: “… exhibits more extensive hydrophobic interactions than m6A.” Readers would expect to see the comparison of hydrophobic interaction between m6A and namodenoson.

Minor issues:
1. Line 53-54: “… inhibition of the A2AR has been effective … the activation of A1R has helped prevent …”. Tense is not proper.
2. Line 66: “… effectively induced inflammation and type I allergy through A3R.” Reference is needed.
3. Line 95: “A 3R” should be “A3R”.

Reviewer #2

(Remarks to the Author)
In this present study, Oshima and coauthors identified a new Adenosine A3 receptor (A3R)-selective ligand via modified adenosine library screening. The authors also solved the structures of A3R-Gi in complex with several selective or nonselective ligands. Furthermore, the authors conducted structure-guided engineering of m6A-insensitive A3R by mutagenesis studies of the ligand binding pocket. Overall, I find the study to be well designed and well presented. The work is significant to the understanding the structure and signaling of A3R. I have some suggestions and comments, which can be addressed with some revisions.

Major points:
1. It is yet clear to me how the modified adenosine library was generated. The authors should provide more information on that. Since there are only 42 modified adenosine compounds in this library ('a limited set of modified nucleosides according to the authors' own account), I suggest refrain calling it a comprehensive screening.

2. How did author determine the receptor selectivity of the ligands? Based on Fig.1A, i6A appears to activate both A3R and A2bR, although with higher potency on A3R.

3. Since NECA and Namodenoson are used for structural studies, the authors should show their activation data, as a side-by-side comparison with the other ligands.

4. Fig. 2b, the authors should show the density of ligands in the EM maps.

5. In all the maps shown in Fig.2, the Gα densities appear to be rather poor, especially given that the overall resolution is close to 3Å. Can the authors offer some insights why?
The authors should also provide information about the local resolution estimate in each EM map, e.g., color the map with different colors to indicate different local resolution.

6. It is interesting to me that the A3R-Gi complex with two nonselective ligands had higher resolution than the three selective ligands. This is surprising because m6A and Namodenoson showed higher binding affinity towards A3R based on Fig. S1C. Can the authors offer some explanation?

7. A discussion of the differences of five EM maps and respective structural models of the A3R shown in Fig.2 would be helpful. The current way of describing the maps gave the impression that all the maps are the same thus only NECA is discussed.

8. In discussion of the binding site of NECA, the authors should also compare it with other known AR-NECA structures. Are the binding mode the same?

9. The illustration of ligand binding sites in Fig.4 d and h is unhelpful due to the transparency used. The authors might want to improve that.

10. line 205, the authors suggested that 'interaction between i6A and the receptor is looser compared to that of m6A'. How did the authors come to that conclusion?

11. Since sheep A3R is used for cryoEM analysis, the authors should also provide the sequence alignment with the sheep A3R sequence in Fig.3.

12. Line 187, R168 is a polar residue, it is misleading to state that its aliphatic size chain forms hydrophobic interaction with the ligand. Since position 168 is a valine in human A3R, would this suggest differed ligand binding mode between human and sheep A3R? It would be interesting to test that with R168V mutation.

13. Line 193, the authors stated that 'A3R exhibits much tighter packing in this pocket', however, the tightness in this pocket is not clearly observable from the illustration. The author should try quantitatively measuring the pocket size to strengthen this statement.

14. In general, I find the explanation of why the smaller residues such as V169A and M174 had unexpected results unsatisfactory and speculative. To me, the pocket size theory is not strongly supported by evidence and needs revision. Similarly, it is baffling why would I253A, I253F and I253N have quite different activation profile by i6A compared to other ligands. The authors might want to consider some MD simulation studies to investigate how these mutations would affect the ligand binding pockets.

Minor pointts:
1. The fonts used in table S1 and S2 are too small.

2. Fig1b is more suitable for a table instead of being part of the figure.

3. Fig.S1C, it is better to overlay the two plots to show direct comparisons of ligand binding properties of human and sheep A3R.

4. There are two panel d in Fig.2 and label for Fig.2C is missing.

5. Fig.S1d. please consider improve the constrast and the size of the gel image with better labeling. Is the first lane one of the fractions? Which fraction was used for cryo-EM grids preparations?

6. Citations are needed for the following statements:
Line 85, the previous study;
Line 95.

Reviewer #3

(Remarks to the Author)
see attachment

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I have carefully reviewed the rebuttal leter and revised manuscript. I feel the authors have fully addressed the reviewers' questions and suggestions. The original manuscript was greatly improved. I support the revised manuscript is in good shape to be published in Nature Communications.

Reviewer #2

(Remarks to the Author)
I appreciate the authors' hard work revising the manuscript. All my comments and concerns are addressed.

Reviewer #3

(Remarks to the Author)
The manuscript has been improved.

Point-by-point responses

The parts of the text that have been changed are highlighted in yellow. The major changes are as follows:

- During the review and revision, we re-analyzed the cryo-EM data of the A_3R-Gi complexes bound to m^6A, i^6A, and namodenoson, The improvement of the resolution for each complex is summarized in the following figure.

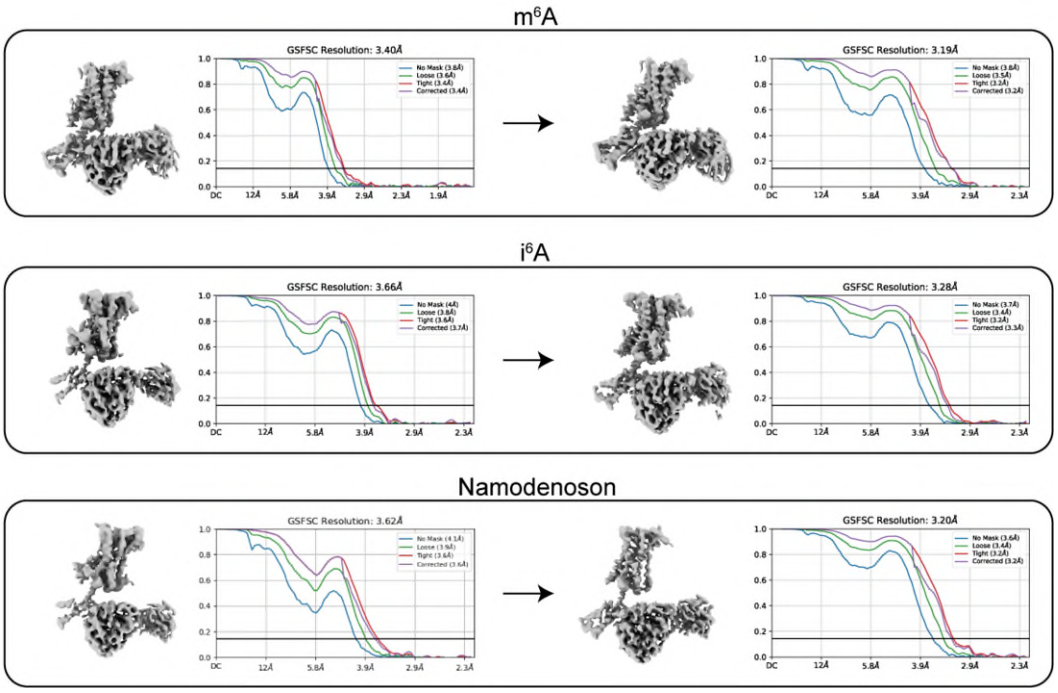

Fig. L1 | The improvement of the maps.
Previous maps (left) and improved maps (right) for each selective agonist are shown with FSC curves. Details are described in Supplementary Fig. 2.

- We corrected the atomic models with the new maps. While the models of the m^6A-bound and namodenoson-bound A_3R complexes showed minor changes, in the i^6A-bound model, i^6A was revealed to interact with the hydrophobic pocket of A_3R, as in the cases with m^6A and namodenoson.
- Thus, we divided the section “Binding modes of m^6A and i^6A” into 2 sections to provide a detailed discussion for each agonist. Also, the section “Structure-guided engineering of m^6A-insensitive A_3R” was divided and merged into these sections.
- To investigate the artificiality of the functional studies, which was pointed out by reviewer #3, we measured the expression levels of WT and each mutant A_3R, as in our previous work. The results of the TGFα-shedding assay were minimally affected by the expression level within the tested range (Supplementary Fig. 5e, f).
- Moreover, we performed the mutagenesis studies additionally required by the reviewers (Supplementary Fig. 5a-d).

- We moved the discussion on adenosine receptor-selective drugs to the results, so that it would be more appealing.
- Finally, we corrected grammatical errors and improved the structure of the main text.

The point-by-point responses for each reviewer follow.

Reviewer #1

The authors reported high-resolution structures of sheep adenosine receptor A3R bound with nonselective and selective agonists, in which one selective agonist was screened out. Based on Cryo-EM structures, authors analyzed the selectivity mechanism of A3R, the hydrophobic pocket located at the extracellular side. The authors applied structure-guided engineering for m6A-insensitive and adenosine-sensitive A3R to facilitate further physiological and pathological studies of m6A. The article presented the importance of the structural study of A3R and facilitated further functional study and drug development. Most results and explanations are reasonable and arranged in a logical way, while some need to be checked and improved.

We appreciate your contribution and positive feedback to this work. We corrected and improved the manuscript following your advice.

Major issues:

1. The thickness of helices in figures should be decreased to clarify domain comparison and interacting residues.

Thank you for your advice. We reduced the thickness of helices in figures (e.g. Fig. 2f, g).

2. Fig. 4h: Figure 4h2 is nearly identical to 4d1. Figure 4h5 did not present E17445.53 well.

We removed the corresponding figures from the manuscript since we changed the discussion on the binding mode of i^6A. However, we appreciate your comment.

3. Line 152-155: P34.50 involves interaction with G protein in class A GPCRs. Please clarify whether the interaction has been changed, given that P34.50 has been replaced by T34.50. Furthermore, please explain how “slightly elongated TM3" influences “extensive interactions across ICL2”.

We appreciate your insightful comment. $T^{34.50}$ in A_3R interacts with the same residue of G_i as $P^{34.50}$ in A_1R (Supplementary Fig. 4). However, upon careful consideration, we have decided to remove the statement regarding 'slightly elongated TM3' influencing 'extensive interactions across ICL2' from our manuscript. This decision was made to ensure that our claims are fully supported by the available evidence and to maintain the focus of our discussion. We thank you for bringing this to our attention.

4. Line 174-176: Residue histidine adopts a bulking sidechain compared to serine, while both residues can form polar hydrogen interactions. Discussion of these two residues should probably emphasize the size difference, further resulting in loss of polar interaction.

We appreciate your suggestion. We added an explanation that is focused on the size difference, as you recommended (lines 183-187).

5. Lines 180 and 191: The mutagenesis study is better done and presented in figures instead of references.

Thank you for your recommendation. Since the mutagenesis studies were done with human A_3R in the reference[1], we conducted the same mutagenesis studies with both human and sheep A_3R. Both mutations reduced the potencies of agonists, so we mentioned them in the manuscript (lines 191-193, Supplementary Fig. 5c, d).

6. Line 176-182: Residue V16945.53 in human A3R is different both in sidechain size and polarity compared to other adenosine receptors of human (E45.53) and sheep A3R (R45.53). Since the article mentioned position 45.53 consists of part of the hydrophobic pocket, which is important for ligand selectivity, please clarify the confidence level that the selectivity mechanism between human adenosine receptor subtypes can be explained by sheep A3R structures. Also, please give a possible explanation for the decrease in potency through nonpolar to polar mutation in A3R, while decreased potency in other adenosine receptors is through polar to nonpolar mutation.

We appreciate your insightful comment. Although the sidechain size and polarity of the residues in 45.53 differ, both feature a carbon chain in the side chain. As seen in our sheep A_3R structure, the important part of R168 in the ligand-receptor interaction is the carbon chain. We also swapped the residues in 45.53 between human and sheep A_3R, and found only a small difference (Supplementary Fig. 5a, b). Taken together, we believe the selectivity mechanisms of sheep A_3R and human A_3R are quite similar. Also, nonpolar-to-polar mutations in A_3R may have resulted in repulsion between the residues in the hydrophobic pocket, thereby leading to a loss of tight packing and decreased potency. By contrast, polar-to-nonpolar point mutations in other adenosine receptors may have resulted in loss of polar interactions with a ligand.

7. Line 196: Providing figures superposing hydrophobic pocket of A3R and other adenosine receptors could be better exhibiting narrower volume. Furthermore, please explain more clearly that tight packing could result in higher potency.

We appreciate your suggestion. Instead of superposing the pocket, we changed the magnification of the figure so that it looks clearer. In addition, we measured the distance between the ligand and the residues and added them to Supplementary Table 4. We assume that the tightly packed hydrophobic pocket enables close and strong interactions with agonists, and endows agonists with high potencies (lines 204-210).

8. Line 204: Providing a figure superposing i6A and m6A is needed to exhibit distinct placement of N6 substitution.

The corresponding part has been removed from the manuscript. However, we appreciate your suggestion.

9. Section “Structure-guided engineering of m6A-insensitive A3R”: Homology mutagenesis is a widely used method to evaluate selectivity-related residues. Please check this method to evaluate engineered residues and engineered results.

We appreciate your insightful comment. We previously reported residue swapping experiments between adenosine receptors[1]. Furthermore, that paper also discussed the interspecies differences between the residues constituting the hydrophobic pocket of A_3R[1]. Based on these insights, in this study, we did not focus on homology but instead attempted to validate the function of the hydrophobic pocket by creating comprehensive mutants.

10. Line 260: “… exhibits more extensive hydrophobic interactions than m6A.” Readers would expect to see the comparison of hydrophobic interaction between m6A and namodenoson.

Thank you for this comment. After consideration, we changed the tone of the corresponding part, since adding the comparison of the interactions would be redundant (lines 262-264).

Minor issues:

1. Line 53-54: “… inhibition of the A2AR has been effective … the activation of A1R has helped prevent …”. Tense is not proper.

We corrected the tense (lines 52-54).

2. Line 66: “… effectively induced inflammation and type I allergy through A3R.” Reference is needed.

We added a reference (line 77).

3. Line 95: “A 3R” should be “A3R”.

We corrected it (line 103). We appreciate your kind corrections.

Reviewer #2

In this present study, Oshima and coauthors identified a new Adenosine A3 receptor (A3R)-selective ligand via modified adenosine library screening. The authors also solved the structures of A3R-Gi in complex with several selective or nonselective ligands. Furthermore, the authors conducted structure-guided engineering of m6A-insensitive A3R by mutagenesis studies of the ligand binding pocket. Overall, I find the study to be well designed and well presented. The work is significant to the understanding the structure and signaling of A3R. I have some suggestions and comments, which can be addressed with some revisions.

We appreciate your contribution and positive feedback to this work. We carefully corrected and improved the manuscript following your suggestions.

Major points:

1. It is yet clear to me how the modified adenosine library was generated. The authors should provide more information on that. Since there are only 42 modified adenosine compounds in this library ('a limited set of modified nucleosides according to the authors' own account), I suggest refrain calling it a comprehensive screening.

We thank you for this constructive comment. More than 170 types of post-transcriptional RNA modifications have been identified across the three domains of life[2]. Among them, approximately 50 types of modifications have been identified in humans[3]. Of these, 34 types could be either purchased or synthesized. Additionally, we purchased six types found in non-eukaryotic domain organisms (4-thiouridine, s^4U; 2,8-dimethyladenosine, $m^{2,8}A$; 2-methyladenosine, m^2A; N^4,2'-O-dimethylcytidine, m^4Cm; 2-thiocytidine, s^2C; and 2-methylthioadenosine, ms^2A), and three secondary metabolites (8-hydroxyguanosine, 8OHG; N^6-succinyl adenosine, N^6sucA; and 5-hydroxyuridine, ho5U), resulting in a total collection of 43 modified nucleosides. As noted in the initial draft, 1-methyladenosine (m^1A) was excluded from this collection as synthetic m^1A contains a significant amount of m^6A as a by-product of chemical synthesis. Therefore, the 43 (42) types of modified nucleosides tested in this study are the most mammalian-related modified nucleosides currently available. Nevertheless, we agree with the reviewer that the modified nucleosides used in this study are not complete. We thus have removed the term "comprehensive" from the subtitle.

2. How did author determine the receptor selectivity of the ligands? Based on Fig.1A, i6A appears to activate both A3R and A2bR, although with higher potency on A3R.

We appreciate your comment. We determined the receptor selectivity of the ligands by calculating the intrinsic activity values relative to adenosine (RAi). The RAi value of i^6A toward the $A_{2B}R$ receptor is 12%, which is much lower than the value of 59% towards A_3R, indicating its specificity for A_3R.

3. Since NECA and Namodenoson are used for structural studies, the authors should show their activation data, as a side-by-side comparison with the other ligands.

Their activation data on A_3R are shown in Supplementary Fig. 1c.

4. Fig. 2b, the authors should show the density of ligands in the EM maps.

Thank you for the comment. The density of the agonists is now clearly shown in Fig. 2b.

5. In all the maps shown in Fig.2, the Gα densities appear to be rather poor, especially given that the overall resolution is close to 3Å. Can the authors offer some insights why?
The authors should also provide information about the local resolution estimate in each EM map, e.g., color the map with different colors to indicate different local resolution.

Thank you for this constructive comment. In our previous analyses, we excessively focused on the receptor, resulting in the poor Gα densities. In the new maps, the Gα densities as well as the receptor densities were improved. Also, we added figures showing the local resolution for each map (Supplementary Fig. 2a-e).

6. It is interesting to me that the A3R-Gi complex with two nonselective ligands had higher resolution than the three selective ligands. This is surprising because m6A and Namodenoson showed higher binding affinity towards A3R based on Fig. S1C. Can the authors offer some explanation?

We appreciate your comment. We were also surprised that NECA showed the highest resolution. Many factors can affect the resolution: potency of agonists, instability of the expression system, our technique, data collection conditions, and so on. However, here is a possible explanation. NECA has a long 5' tail, which closely interacts with the toggle switch $W^{6.48}$. We assume that this close interaction especially stabilizes the active form of the receptor, thus reducing the heterogeneity of the particles and thereby providing the highest resolution. However, since the other agonists do not interact as closely with the toggle switch, the overall resolutions of those complexes did not appreciably differ.

7. A discussion of the differences of five EM maps and respective structural models of the A3R shown in Fig.2 would be helpful. The current way of describing the maps gave the impression that all the maps are the same thus only NECA is discussed.

Thank you for this opinion. The receptor part of each model superposed well, so the NECA-bound model is representatively shown. However, to show the differences between the models, we added the superposition of the receptor part of each model in Fig. 2c.

8. In discussion of the binding site of NECA, the authors should also compare it with other known AR-NECA structures. Are the binding mode the same?

We appreciate your insightful suggestion. We added the comparison with the other NECA-bound adenosine receptors (lines 172-176, Fig. 2e). The binding modes are quite similar.

9. The illustration of ligand binding sites in Fig.4 d and h is unhelpful due to the transparency used. The authors might want to improve that.

Thank you for your comment. We changed the corresponding figures to show only the CPK models (Fig. 4d).

10. line 205, the authors suggested that 'interaction between i6A and the receptor is looser compared to that of m6A'. How did the authors come to that conclusion?

The corresponding part has been removed, since the model for the i^6A-bound complex changed. However, we appreciate your feedback.

11. Since sheep A3R is used for cryoEM analysis, the authors should also provide the sequence alignment with the sheep A3R sequence in Fig.3.

We appreciate your suggestion. We added the sequence alignment with sheep A_3R in Fig. 3g.

12. Line 187, R168 is a polar residue, it is misleading to state that its aliphatic size chain forms hydrophobic interaction with the ligand. Since position 168 is a valine in human A3R, would this suggest differed ligand binding mode between human and sheep A3R? It would be interesting to test that with R168V mutation.

We appreciate your insightful comment. As seen in the structure, the interacting portion of $R168^{45.53}$ is the carbon chain, which is supposed to be the same as the valine in human A_3R. Additionally, we conducted mutagenesis studies to swap the residues in 45.53 between human and sheep A_3Rs (Supplementary Fig. 5a, b). The potency of the agonists did not change much in the mutants, suggesting that the residues in 45.53 play quite similar roles.

13. Line 193, the authors stated that 'A3R exhibits much tighter packing in this pocket', however, the tightness in this pocket is not clearly observable from the illustration. The author should try quantitatively measuring the pocket size to strengthen this statement.

We greatly appreciate your suggestion. We changed the magnification of the figure, so that it looks clearer. Also, we measured the distances between the agonists and the residues in the hydrophobic pocket, revealing that m^6A-A_3R exhibits the closest contacts (line 204-208). The corresponding data are shown in Supplementary Table 4.

14. In general, I find the explanation of why the smaller residues such as V169A and M174 had unexpected results unsatisfactory and speculative. To me, the pocket size theory is not strongly supported by evidence and needs revision. Similarly, it is baffling why would I253A, I253F and I253N have quite different activation profile by i6A compared to other ligands. The authors might want to consider some MD simulation studies to investigate how these mutations would affect the ligand binding pockets.

Thank you for your comment. When the pocket size is reduced, a ligand cannot be accommodated since it will cause direct steric occlusion. In contrast, when the pocket size is enlarged, a ligand can still be accommodated, but may not form tight interactions. These differences may have affected the clear interpretation of the mutagenesis studies. However, considering the structural differences among the adenosine receptors, we believe that the pocket size theory is reasonable. The interactions between i^6A and the hydrophobic pocket have now been revealed, and we believe that our explanation of the mutagenesis studies on i^6A is sufficient. Taken together, we did not conduct MD simulation studies since we thought they were not necessary. However, we appreciate your suggestion.

Minor points:

1. The fonts used in table S1 and S2 are too small.

We enlarge the font size of the tables (Supplementary Tables 1, 2).

2. Fig1b is more suitable for a table instead of being part of the figure.

We moved the corresponding table to Supplementary Table 1.

3. Fig.S1C, it is better to overlay the two plots to show direct comparisons of ligand binding properties of human and sheep A3R.

We have revised the figure as follows to clarify the reactivity differences between humans and sheep for each ligand, based on the reviewers' comments (Supplementary Fig. 1c).

4. There are two panel d in Fig.2 and label for Fig.2C is missing.

We corrected the labelings.

5. Fig.S1d. please consider improve the constrast and the size of the gel image with better labeling. Is the first lane one of the fractions? Which fraction was used for cryo-EM grids preparations?

We changed the contrast of the gel image, and the corresponding fractions for the grid preparation are now shown in Supplementary Fig. 1d.

6. Citations are needed for the following statements:
Line 85, the previous study;
Line 95.

We added the references (lines 93, 103). We appreciate your kind corrections.

Reviewer #3

This manuscript reports several cryo-EM structures of non-selective and A3-selective adenosine receptor agonists in complex with the sheep A3 adenosine receptor. The authors could not obtain structures of the human A3 receptor and therefore used the sheep protein. These are the first A3 adenosine receptor structures (aside from those shown in oral presentations or posters at meetings/conferences). The authors additionally claim to have identified a "novel A3-selective ligand" (N6-Isopentenyladenosine). Docking studies led to an A3AR mutant, which the authors claim to be insensitive to N6-methyladenosine. The manuscript is not well written. Structure and language would require extensive improvement.

We appreciate your constructive feedback. We fully understand the problems with our manuscript. Following your kind advice, we tackled the problems carefully and improved the structure and language of the manuscript.

Major issues

- The authors do not appear to be experts in adenosine receptor pharmacology. The Introduction has to be carefully revised. The text is inconsistent. For example, allergies and inflammation induced by A3 are mentioned in line 209 of Results, but not in the Introduction. Moreover, this effect may be species-dependent, since mast cells in rodents express A3, while mast cells in humans express A2B receptors.

We appreciate your comment. We carefully corrected the introduction (e.g. lines 52-54, 56-59). Also, considering the species dependence of A_3R pharmacology and function, we emphasized which phenomenon is related with which species (in humans, in mice, and so on). For your information, we found that A_3R is also expressed in human mast cells, according to the Immune Cell Atlas (Fig. L2).

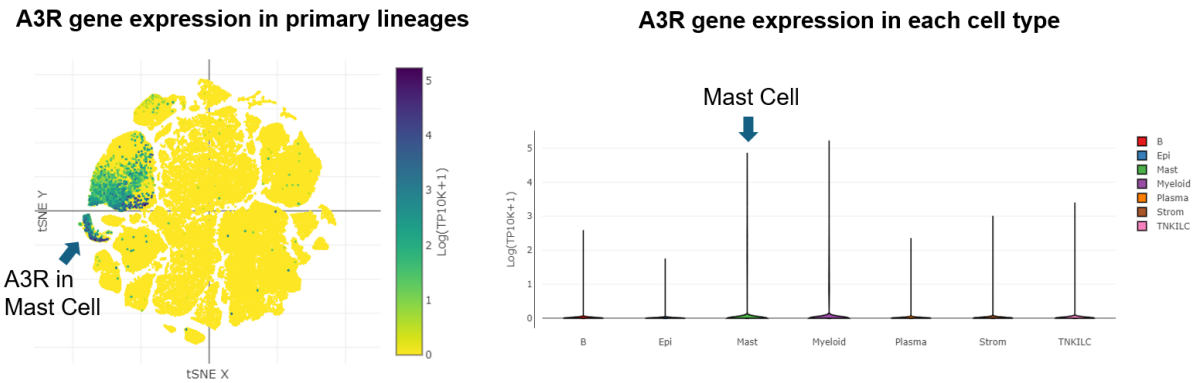

Fig. L2 | A_3R gene expression in human cells at single cell resolution.
Note that A_3R gene expression is enriched in mast cells (Upper left). The data are derived from human colon cancer patients (ID: Human Colon Cancer Atlas c295). Data are available at the Immune Cell Atlas (https://singlecell.broadinstitute.org).

- Results: The SARs of A3 agonists are already well known since they have been extensively investigated. The identified "new results" on the identification of agonists are not surprising based on

the previous data. This part is therefore not really exciting. Moreover, the new agonists are only weakly active.

We thank you for this reasonable opinion. The aim of the section "Screening of Modified Nucleosides Against Human A_3R" is to deepen the understanding of endogenous A_3R agonists. In this study, we utilized a library of modified nucleosides that exist in mammals. We believe that our findings provide valuable insights into the relationships between modified adenosines and adenosine receptors, a topic that remains largely unexplored. Furthermore, the three modified nucleosides discussed in this paper—m^6A, $m^{6,6}A$, and i^6A—are among the most toxic endogenous modified nucleosides derived from RNA molecules and are linked to human diseases[4]. Investigating their roles in the extracellular context, particularly their ligandability for receptors, is crucial given their potential role *in vivo*.

- Only (highly artificial) functional assays are performed to assess agonist potency on the receptor and its mutant. No binding studies have been performed. However, functional G protein-dependent potencies and efficacies are dependent on receptor expression levels, which likely differ depending on the expressed sequence. Therefore, binding studies need to be performed (or at least beta-arrestin recruitment assays, which appear to be less dependent on expression levels). This is particularly important for supporting the claim that a mutant has been developed that is insensitive to N6-methyladenosine.

We really appreciate your critical feedback. Using fluorescence-activated cell sorting, we investigated the surface expression levels of mutant A_3Rs that reduced the potency of m^6A, as representatives of the mutants. The results revealed that the potency studies are minimally affected by the expression level, at least within the tested range (Supplementary Figure 5e, f). Thus, we believe that the original discussion on the mutant remains valid, and only made minimal changes.

- Chemical interactions are insufficiently explained, e.g. lines 276-280.

We are grateful for this comment. In lines 279-283, we summarized the interaction between A_3R and each modification of namodenoson based on the detailed explanation written above. We believe we made sufficient explanations for the other portions.

- Structures of chemical compounds mentioned in the text (lines 316 ff) should be depicted, at least in SI.

We appreciate your suggestion. We have added the structures of the mentioned compounds in Supplementary Figure 6.

- Message in line 343-344: But very potent and selective A3 agonists already exist.

We thank you for this comment. We understand that very potent and selective A_3R agonists already exist. However, since all of them were developed by ligand-based modifications, there is yet room for further structure-based improvement. We believe that higher potency and selectivity are worthy goals.

- References: Some of the references are very old. E.g. Ref. 1: Two updates have appeared since that 2001 publication (in 2011 and in 2021).

We thank you for bringing it to our attention. We updated the references.

- Table in Fig. 1: Emax: what refers to 100%? The data in the table is difficult to understand.

Thank you for your comment. The principles of the TGFα shedding assay used to measure GPCR activity in this study are GPCR-induced ectodomain shedding of membrane-bound alkaline

phosphatase (AP)-fused-TGFα and subsequent ligand-induced release into conditioned media[5]. The GPCR activity is quantified by measuring the proportion of membrane-expressed AP-TGFα that is released into the supernatant. A release rate of 100% signifies that all of the membrane-bound AP-TGFα has been transferred into the supernatant. The detailed calculation method is as follows: (i) relative percentage AP activity in conditioned medium = ΔOD405 CM / (ΔOD405 CM + ΔOD405 Cell), where ΔOD405 CM and ΔOD405 Cell denote changes in OD405 in the conditioned medium and on the cell surface, respectively, before and after a 1-h incubation in the presence of para-nitrophenylphosphate (p-NPP), a substrate for AP, and (ii) percentage AP-TGFα release = (relative percentage AP activity in the conditioned medium in the ligand-stimulated condition) – (relative percentage AP activity in the conditioned medium in the unstimulated condition with vehicle treatment). Finally, we fit the data to the four-parameter sigmoid model (dose-response curve), from which we obtained values of maximal effect (Emax) and half-maximal effective concentration (EC50). Also, we have added an explanation in the figure legend and in the Materials and Methods section.

- Figure 2: Chemical structures of agonists on top are incorrect (double bonds missing) and they lack stereochemistry. The figures f and g could be improved.

We again thank you for your kind feedback. We corrected the structures of the agonists. Also, we improved Fig. 2g, h.

Minor points

- A3 adenosine receptor, A3 ligand etc.: A3 subscripted according to IUPHAR rules.

We corrected the words.

- Resolution of structures should be mentioned in the Abstract.

The resolution of each structure is now described in the Abstract.

- Line 70: “Numerous structures of the other adenosine receptors” is exaggerated. The A2AAR is an exception.

We corrected the words to avoid exaggeration (lines 79-80).

- Line 83: which species were screened?

The list of the modified nucleosides are shown in Fig. 1 and Supplementary Tables 1, 5.

- N has to be italic in 5’-N-ethylcarboxamidoadenosine.

We corrected the words.

-Line 88-90: intrinsic activity should be presented in percent (only full numbers).

We corrected the issues (lines 95, 96, and 98).

- Pi-Stacking should be π-stacking

We corrected the words (lines 170, 179, 198, 239, 250, and 261).

- Line 255: nomenclature/description of structure is wrong (“methylamino in the 5’-position”), also in line 275.

The structure of namodenoson is now correctly described (lines 259, 277).

- Line 265: Incomplete sentence.

We corrected the sentence (lines 268-269).

- Ref. 52 is incomplete.

We corrected the reference. We deeply appreciate your numerous kind corrections.

References

1. Ogawa, A. *et al.* N6-methyladenosine (m6A) is an endogenous A3 adenosine receptor ligand. *Mol. Cell* **81**, 659-674.e7 (2021).
2. Boccaletto, P. *et al.* MODOMICS: a database of RNA modification pathways. 2021 update. *Nucleic Acids Res.* **50**, D231–D235 (2022).
3. Chujo, T. & Tomizawa, K. Human transfer RNA modopathies: diseases caused by aberrations in transfer RNA modifications. *FEBS J.* **288**, 7096–7122 (2021).
4. Ogawa, A. *et al.* A sequential, RNA-derived, modified adenosine pathway safeguards cellular metabolism. 2024.05.23.595515 Preprint at https://doi.org/10.1101/2024.05.23.595515 (2024).
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Oshima et al.

Structural insight into agonist selectivity and structure-based engineering of adenosine A3 receptor

This manuscript reports several cryo-EM structures of non-selective and A3-selective adenosine receptor agonists in complex with the **sheep A3 adenosine receptor**. The authors could not obtain structures of the human A3 receptor and therefore used the sheep protein. These are the first A3 adenosine receptor structures (aside from those shown in oral presentations or posters at meetings/conferences).

The authors additionally claim to have identified a "novel A_3-selective ligand" (N^6-Isopentenyladenosine). Docking studies led to an A_3AR mutant, which the authors claim to be insensitive to N^6-methyladenosine.

The manuscript is not well written. Structure and language would require extensive improvement.

Major issues

- The authors do not appear to be experts in adenosine receptor pharmacology. The Introduction has to be carefully revised. The text is inconsistent. For example, allergies and inflammation induced by A3 are mentioned in line 209 of Results, but not in the Introduction. Moreover, this effect may be species-dependent, since mast cells in rodents express A3, while mast cells in humans express A2B receptors.
- Results: The SARs of A3 agonists are already well known since they have been extensively investigated. The identified "new results" on the identification of agonists are not surprising based on the previous data. This part is therefore not really exciting. Moreover, the new agonists are only weakly active.
- Only (highly artificial) functional assays are performed to assess agonist potency on the receptor and its mutant. No binding studies have been performed. However, functional G protein-dependent potencies and efficacies are dependent on receptor expression levels, which likely differ depending on the expressed sequence. Therefore, binding studies need to be performed (or at least beta-arrestin recruitment assays, which appear to be less dependent on expression levels). This is particularly important for supporting the claim that a mutant has been developed that is insensitive to N^6-methyladenosine.
- Chemical interactions are insufficiently explained, e.g. lines 276-280.
- Structures of chemical compounds mentioned in the text (lines 316 ff) should be depicted, at least in SI.
- Message in line 343-344: But very potent and selective A3 agonists already exist.
- References: Some of the references are very old. E.g. Ref. 1: Two updates have appeared since that 2001 publication (in 2011 and in 2021).
- Table in Fig. 1: Emax: what refers to 100%? The data in the table is difficult to understand.
- Figure 2: Chemical structures of agonists on top are incorrect (double bonds missing) and they lack stereochemistry. The figures f and g could be improved.

Minor points

- A3 adenosine receptor, A3 ligand etc.: A_3 subscripted according to IUPHAR rules.
- Resolution of structures should be mentioned in the Abstract.

- Line 70: “Numerous structures of the other adenosine receptors” is exaggerated. The A_{2A}AR is an exception.
- Line 83: which species were screened?
- N has to be italic in 5’-N-ethylcarboxamidoadenosineLine 88-90: intrinsic activity should be presented in percent (only full numbers).
- Pi-Stacking should be π-stacking
- Line 255: nomenclature/description of structure is wrong (“methylamino in the 5’-position”), also in line 275.
- Line 265: Incomplete sentence.
- Ref. 52 is incomplete.