

Molecular Mechanism of Drug Inhibition of URAT1

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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)

Yu et al. present cryo-EM structures of the rat URAT1 transporter (with two mutations) bound to its substrate, urate, and four distinct small molecule drugs. The transporter is involved in the renal reabsorption of urate and is pharmacologically relevant as a target in developing therapies for hyperuricemia.

The authors obtained cryo-EM density maps with resolutions ranging between 3.5 and 3.7 Å. The text focuses on describing the interactions between the ligands and surrounding residues. Structural findings are supported by cell-based radioactive transport assays, a discussion of disease-causing mutations, and the transition between inward- and outward-open conformations. The outward-open conformation was constructed by generating a homology model of URAT1 based on the published OCT3 structure.

After analyzing the cryo-EM maps and models provided by the authors, I have concerns about the resolvability of the ligands in the structures. I have included a PDF file with my screenshots of the figures for better clarity on my points. Additionally, the manuscript could be structured in a more comprehensive way.

Major points:

1. The EM density map assigned to the urate molecule is not shown in any figure. Urate is a small and flat molecule, which makes its unambiguous assignment in the 3.5 Å cryo-EM density map particularly challenging. Additionally, there is another strong density feature adjacent to the assigned urate molecule (figure attached). Moreover, the nearby Phe241 residue is fully unresolved, regardless of the applied threshold. Both of these features should be presented and discussed in detail. Could this indicate distinct modes of urate binding?

2. In the benzbromarone-bound rat URAT1 structure, the EM density feature poorly covers at least half of the molecule (figure attached). A similar issue applies to the verinurad-bound structure, where the molecule is ambiguously positioned. This requires either improving the resolution of the EM density maps, particularly in the binding pocket, or presenting and discussing these ambiguous features.

3. The sulfinpyrazone-bound structure (figure attached) shows very low occupancy level in the binding pocket at higher threshold levels of the EM density maps. This also requires further discussion in the text. Are drug-free and sulfinpyrazone-bound URAT1 particles mixed in the final EM reconstruction?

4. The abstract should explicitly state that the authors studied the rat homolog of URAT1. Despite the 74% sequence identity and 84% similarity, the residues lining the binding pocket should be analyzed and compared between the rat and human homologs. Two mutations were introduced into the rat URAT1 construct to "enhance substrate affinity and drug sensitivity to levels similar to those of human URAT1." Please elaborate on these affinities and sensitivities. It is important to convince readers that the binding modes of the drugs are expected to be the same in human and double-mutant rat URAT1.

5. The authors claim to provide detailed insight into the inhibition mechanism of URAT1, stating that the studied drugs prevent the transporter from undergoing conformational transitions. Which results support this statement? Do the authors expect to observe different conformations in the apo sample of their URAT1 protein?

6. The manuscript text requires improvement. The title should be revised, as neither urate reabsorption nor (multiple) inhibitory mechanisms of URAT1 are directly studied in the manuscript. All literature-based fragments (e.g., l. 104–107) should be moved to the introduction. Additionally, avoid repetition between the introduction and discussion sections.

7. The manuscript does not mention the counter-ion or driving force of the transporter, which is a key element in any mechanistic discussion of transporters, including inhibition mechanisms.

8. The authors should provide SEC spectra showing the expression levels of URAT1 mutants used for normalization in the transport assays.

Minor points:

9. Please do not use the phrase “electron density” when referring to cryo-EM density maps

10. L. 78-80 - to comment on homogeneity, please specify the detergent-specific condition and avoid the phrase “ideal”

11. L. 88 - if the map covers 515 residues, why does Ext Table 1 indicate 472 protein residues in the model? Please clarify this discrepancy

12. L.125 - did the authors mean 1d?

13. L. 137 – please avoid the phrase “wild-type” if you are referring to the construct containing two mutations

14. Does the transporter adopt exactly the same conformation in all structures? Are there any conformational differences? Is the degree of the opening between the halves different?

15. Please provide a detailed cryo-EM analysis for each structure separately. Include the processing pipeline, local resolution estimation, 2D class representatives, angular distribution, etc. Please provide the final SEC spectra for each cryo-EM sample.

I would like to share an additional comment that is not critical to the decision on the manuscript. The authors prepared the cryo-EM samples by supplementing the buffers with each drug prior to SEC. However, the EM density maps show several unassigned, strong features in the binding pocket, which appear to resemble elements of the other drugs. Is it possible that the same column was used for all samples and was not properly cleaned before running subsequent samples? The comparison of the EM density maps suggests there may be traces of different molecules in the binding pocket. Please consider checking this.

Reviewer #2

(Remarks to the Author)

The submitted manuscript, "Urate reabsorption and inhibitory mechanisms of URAT1," provides data on high resolution structures of rat URAT1 (rURAT1), with human residues at positions 35 and 365 to increase substrate and inhibitor binding affinity, complexed to its substrate urate as well as URAT1 inhibitors benzbromarone, lesinurad, verinurad, and sulfinpyrazone. This work is relevant as it is important to know the structure of this pathologically- and pharmacologically-relevant target for hyperuricemia and gout. It is commendable that the authors successfully obtained the structure of rURAT1 and performed some validation studies on the structure. However, the manuscript requires revisions to be acceptable for publication, namely further validation studies, as outlined below.

1) The residues identified as interacting with urate and the inhibitors need to be verified using mutagenesis and activity assays, as was done in the recently published report on the structure of human URAT1 (DOI:10.1038/s41422-024-01023-1). For the inhibitors this was performed for residues 35 and 477, but this needs to be done for other interacting residues. Since the binding sites for urate and the inhibitors overlap, mutations in the binding site will affect substrate transport activity which can preclude reliably measurement of these assays. However, conservative mutations can be performed in many of these residues to preserve activity, as has been shown previously (DOI:10.1038/s41422-024-01023-1 and DOI:10.1038/s41598-017-00706-7):

1a) For urate, F365A mutation should be analyzed (all other interacting residues were analyzed in this way);

1b) For benzbromarone, the phenylalanine residues at 241, 360, 364, 365, and 449 should all be individually converted to tyrosine, Q245 should be converted to the human residue histidine and/or alanine, Q473 should be converted to histidine or alanine, M214 should be converted to glutamine to disrupt hydrophobic interactions, and T32 should be converted to alanine to disrupt hydrogen bonding;

1c) For lesinurad and verinurad, the phenylalanine residues at 241, 360, 364, 365, and 449 should all be individually converted to tyrosine, M214 should be converted to glutamine to disrupt hydrophobic interactions, and importantly D389 should be converted to glutamate or alanine to verify that this residue enhances the affinity to verinurad relative to lesinurad;

1d) For sulfinpyrazone, the phenylalanine residues at 241, 360, 364, 365, and 449 should all be individually converted to tyrosine, W357 should be converted to tyrosine (or alanine if the transporter is still active for substrate transport), N237 should be converted to alanine to disrupt the hydrogen bond, and methionines at 215, 222, 234, and 466 should all be individually converted to glutamine to disrupt hydrophobic interactions;

1e) For the URAT1 conformational transition, Q149 or R487 should be mutated to disrupt the hydrogen bond.

The statement in lines 213-216 that verinurad...exhibits approximately 10-20 times higher affinity for URAT1 needs to be referenced, and also checked for accuracy. If the authors are referring to human URAT1 (hURAT1), then the affinity is over 1000 times higher for verinurad based on DOI:10.1038/s41598-017-00706-7 for verinurad and DOI:10.1038/srep34995 for lesinurad. For rat URAT1 (rURAT1) which is less relevant, the affinity is only two times higher for verinurad based on the same references.

Here are other minor comments that should be addressed:

- 1) The sentence in lines 260-262 needs supportive reference(s). What were the previous studies?
- 2) Extended Data Figure 7f should include some features for clarification. G470 should be added since it is mentioned in the results section in line 266, and the entire sequence of motif A should be shown (it is currently shown up to residue 171, while the text in line 265 also mentions residues 172 and 173). Also, the location of motif A should be indicated, as well as the locations of the salt bridges (lines connecting the salt bridging residues should be shown).
- 3) There are some typographical errors that should be corrected:
 - a) Figure 3 legend - add the "a" in verinurad which is missing,
 - b) In line 245, M466TM1 is incorrect and should be M466TM11,
 - c) In line 266, G146TM5 is incorrect and should be G146TM2,
 - d) In line 267, "while" should be removed.
- 4) The sentence from lines 286-287 is supported by results that should be referenced, doi:10.1093/molbev/msw116.
- 5) The recently published report on human URAT1, DOI:10.1038/s41422-024-01023-1, should be referenced in some way, indicating that the results in that report are in high agreement with the results in this study, with differences that can be attributed to the use of human URAT1 in that study.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors addressed the majority of my concerns in a comprehensive and scientifically relevant manner. The issue of the below-average quality of ligand resolvability has also been tackled through the single point mutations and the MD simulations. I want to emphasize that I am not an expert in MD and therefore will not comment on the quality or methodology of these results.

While the additional results improved the overall quality of the research, the manuscript text still does not meet the expected standard. For instance, the authors confuse readers regarding the R477 residue. They speculate that R477 may play a role in coordinating counter-ions. This is a bold hypothesis that must either be elaborated or, ideally, supported by further experimental evidence. This hypothesis appears inconsistent with subsequent statements such as: "Neutralizing R477 by mutating it to asparagine (R477N) significantly reduces the affinity for benzbromarone (Fig. 2d)" and "The R477N mutation disrupted a critical hydrogen bond with sulfinpyrazone, significantly reducing binding affinity." The R477 residue interacts with the moiety of the benzbromarone molecule, which I previously described as having doubtful resolvability. Additionally, the EM density map in SI Fig. 14B and C does not clearly demonstrate the critical hydrogen bond between R477 and sulfinpyrazone. A mutation that abolishes transport should not be mistaken for one that disrupts binding.

I.210–215: These statements should appear at the beginning of the paragraph as descriptions of experimental results, rather than as the paragraphs final conclusions.

SI Fig. 1 and 12: The captions do not specify the source (or codes) of the sequences.

I.113. The text mentions residue E118, whereas SI Fig. 1 shows D118.

I still believe that readers should be directly introduced to the quality of the EM density maps covering the ligands and surrounding residues. These panels should be included in the main figures rather than in the SI figures only.

Reviewer #2

(Remarks to the Author)

The revised manuscript, "Molecular Mechanism of Drug Inhibition of URAT1" by Yu et al, contains many improvements from the original manuscript to offer insights in the molecular mechanisms of urate substrate transport and inhibition by small molecule compounds/drugs of the uric acid transporter URAT1. The authors submitted a rebuttal that addresses all concerns by the reviewers to the original manuscript. Furthermore, the authors now correctly cite two other recent publications on the structure of human URAT1 and its interactions with urate and URAT1 inhibitors (doi:10.1038/s41422-024-01023-1 and doi:10.1016/j.celrep.2024.114628) and provide important comparisons of their findings to the findings of these other publications. From this reviewers point of view, this manuscript is now acceptable for publication but with a minor revision:

In the rebuttal, the authors state that they analyzed Q245H and Q245A mutations and found them to be inactive (lines 479-480 of rebuttal), and show this data as Figure 13*A in the rebuttal. However, this figure, and any mention that these mutants were made and analyzed, is absent in the revised manuscript. Therefore, Figure 13*A needs to be added within Figure 2 of

the manuscript, along with the text from lines 479-480 of the rebuttal stating that these mutants were inactive. Furthermore, it is important to note the discrepancy of this result, that these mutants are inactive, with findings from doi:10.1038/s41422-024-01023-1. That research article shows that residue 245 in human URAT1 which is histidine (as shown in Supplementary Figure 1) is involved in urate transport, so it is surprising that conversion of this residue to human histidine abolishes activity in rat URAT1. Furthermore, mutation of human URAT1 histidine 245 to alanine (H245A) in the other report reduces transport, but does not eliminate it as found in this study using rat URAT1. Hence, the authors should at least mention these discrepant findings with the other research article, and perhaps speculate on the differences in the results.

Two other minor points:

- 1) in the paragraphs for lines 324-349, the referred Supplemental Figure needs to be changed from Supplemental Figure 13 to Supplemental Figure 15,
- 2) For the legend to Supplemental Figure 15, panel G needs to be mentioned and explained.

To summarize, according to this reviewer, this manuscript is acceptable for publication with the minor revisions outlined above. The authors have addressed all of concerns of this reviewer, which were mainly validations of the structural biology results of URAT1EM using mutagenesis and functional assays. If it is acceptable with the other reviewer who has focused on the structural biology aspects of the work, then it is publishable.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Upon revision, the manuscript "Molecular Mechanism of Drug Inhibition of URAT1" by Yu et al. has improved and the authors have addressed my concerns. Similar to the other reviewer, I consider the manuscript acceptable for publication.

Reviewer #2

(Remarks to the Author)

The authors have presented another revision to the manuscript, and the prior comments of this reviewer have been adequately addressed. However, the manuscript still needs revision because it is unclear in many areas, specified below. Also, two more constructs must be analyzed to complete the validation of the structural findings.

For the results section that discusses Figure 1, it should be mentioned that F365 is one of the residues that was changed to from the rat residue tyrosine to human phenylalanine in the construct. And it also should be mentioned that this human phenylalanine residue confers a higher affinity to urate, with reference to #40 in the list of references. This should be stated after the sentence that ends in line 135.

Also, for Figure 1, F241A should be analyzed for measuring urate affinity, since F241 is mentioned in line 142.

For the results section that discusses Figure 2, similar to the comment above, it should be mentioned that S35 was the other residue that was changed from rat residue asparagine (N) to serine in this construct, and that this change increases the inhibitory potency of benzbromarone with reference to #39. This should be stated after the sentence that ends in line 181. For Figure 2, lines 168-170 state, "Benzbromarone is primarily coordinated by a relatively conserved aromatic cluster consisting of F241, F360, F364, F365, and F449." All of these residues were converted to tyrosine and the effects on benzbromarone inhibitory potency were measured in Figure 2f. However, only the results of F365Y are discussed in lines 184-187, while the findings of the other constructs are not discussed in this paragraph, probably because they had no effect. These results need to be discussed here with speculation on why they had no effect. I think that it is reasonable to speculate that while, "introduction of the hydroxyl group in F365Y directly clashes (clashing) with benzofuran group of benzbromarone," (lines 185-186) introduction of the hydroxyl group to the other residues does not sterically hinder binding of URAT1 to benzbromarone. In addition, M214Q is analyzed (panel e) but not discussed in the text, so this residue and the findings of this mutation need to be discussed and summarized.

Suppl. Fig 4E – key residues involved in the binding of benzbromarone should be labeled, as they are for the corresponding figures for urate and the other inhibitors.

Suppl. Fig 9. To clarify, the legend should list the other structures as human URAT1 and also include the source of those structures. Specifically, they should be labeled as such: human URAT1 (PDB:8WJQ, ref. 44), and human URAT1 (PDB:9B1J, ref. 44).

Suppl. Fig 11. A reference for the structure of human URAT1 PDB:9B1G should be provided, either in panel A or in the legend. I think that this is reference 44, from Suppl. Fig 9. This reference should also be displayed in lines 216 or 217 of the text.

Suppl. Fig 12. Residues in URAT1 (35, 364, 365, 241) that are distinct from the other OATs and are involved in

benzbromarone binding, as described in the manuscript in lines 200-210, should be highlighted, suggesting yellow highlights.

Suppl. Fig 13. A reference for the structure of human URAT1 PDB:9B1I should be provided (ref 44, I think), either in panel A or the figure legend. This reference should also be displayed in line 255 of the text.

For Figure 3, the role of 9 residues in the affinity of lesinurad and verinurad is analyzed by mutagenesis (panels e and h). However, there is no discussion for some of these mutations in the text for this figure in lines 259-278. Specifically, these are F241Y, F364Y, and F449Y – the authors need to discuss the findings for these residues and speculate on their role on lesinurad and verinurad binding. Confusingly, there is discussion of findings for a M466Q mutation (line 263) but no results shown and also no indication of residue 466 in panels d, e, f, or g. The authors need to show the results in the figure, or omit M466Q from the text.

The finding that D389 is important for verinurad binding affinity, and not important for lesinurad binding affinity, is important and noteworthy. If this residue has not been identified in other studies, then this should be further highlighted.

For Figure 4, M215 is shown (panel e) and mentioned as forming hydrophobic interactions with the benzenesulfinyl group of sulfinpyrazone (line 306). However, no data is presented on the role of this residue. The authors should analyze an M215Q mutation and report the findings. The rest of the discussion of Figure 4 and all the mutations, however, is more complete than the discussions of Figures 2 and 3.

Suppl. Fig 15. In line 151, “22” appears to be a typographical error and should be removed.

Another observation by this reviewer is that the results show that URAT1 interacts with its substrate urate and inhibitors through a number of common residues. There are electrostatic interactions (or by hydrogen bonding) with R477, with a binding pocket that involves hydrophobic interactions with M214 (or M215 for sulfinpyrazone), F241, F360, F364 (except for urate), F365, and F449. Conversion of these residues individually to alanine perturbs the transport activity of urate, showing that they are involved in the urate binding pocket. And conversion of many of these phenylalanines to tyrosine generally does not dramatically affect inhibitor affinity, showing the hydrophobic nature of the interaction, except for F365Y which is known to reduce affinity to urate and the inhibitors from prior studies. This could be added to the discussion to enhance the impact of the findings.

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Urate reabsorption and inhibitory mechanisms of URAT1

Referees' comments:

Referee #1 (Remarks to the Author):

Yu et al. present cryo-EM structures of the rat URAT1 transporter (with two mutations) bound to its substrate, urate, and four distinct small molecule drugs. The transporter is involved in the renal reabsorption of urate and is pharmacologically relevant as a target in developing therapies for hyperuricemia.

The authors obtained cryo-EM density maps with resolutions ranging between 3.5 and 3.7 Å. The text focuses on describing the interactions between the ligands and surrounding residues. Structural findings are supported by cell-based radioactive transport assays, a discussion of disease-causing mutations, and the transition between inward- and outward-open conformations. The outward-open conformation was constructed by generating a homology model of URAT1 based on the published OCT3 structure.

After analyzing the cryo-EM maps and models provided by the authors, I have concerns about the resolvability of the ligands in the structures. I have included a PDF file with my screenshots of the figures for better clarity on my points. Additionally, the manuscript could be structured in a more comprehensive way.

Reply: We are grateful to the reviewer for his/her positive comments and specific suggestions to improve our manuscript.

Major points:

1. The EM density map assigned to the urate molecule is not shown in any figure. Urate is a small and flat molecule, which makes its unambiguous assignment in the 3.5 Å cryo-EM density map particularly challenging. Additionally, there is another strong density feature adjacent to the assigned urate molecule (figure attached). Moreover, the nearby Phe241 residue is fully unresolved, regardless of the applied threshold. Both of these features should be presented and discussed in detail. Could this indicate distinct modes of urate binding?

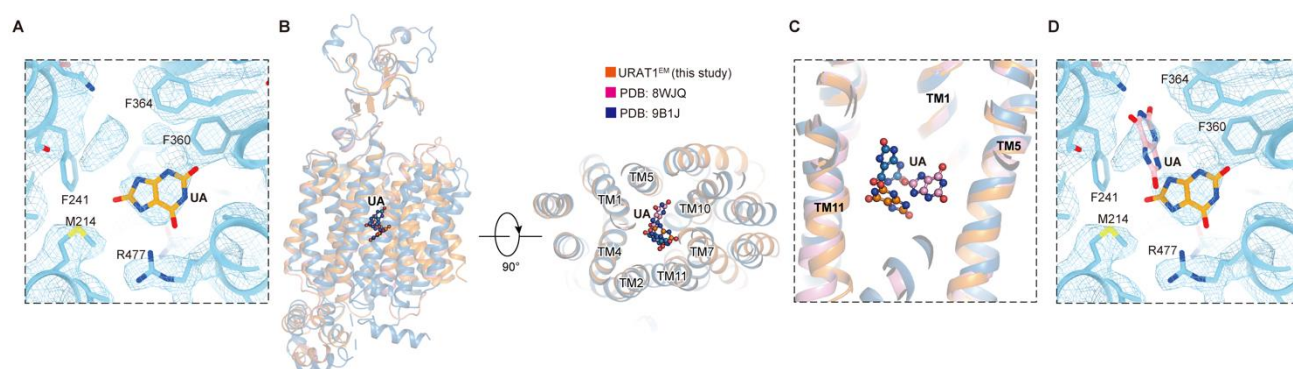
Reply: We appreciate the insightful feedback from the reviewer. We have now included a dedicated panel (Fig. 1*A) highlighting the cryo-EM density corresponding to the urate molecule within the binding pocket. We acknowledge the reviewer's point about the presence of an additional density adjacent to the assigned urate and agree with reviewer that it may also represent an alternative urate binding site. Considering the transporter is trapped in an inward-facing conformation—a state that releases urate to the cytosol and represents a low-affinity binding site—we cannot rule out the possibility of multiple binding sites in the intracellular pocket. A similar scenario has been reported with four taurine molecules bound within the cavity of the taurine transporter (TauT) facing the cytosol^[1].

During the manuscript submission, we were aware of two studies presenting URAT1 structures in complex with urate^[2-3]. We compared the binding modes of urate in these structures and found that the binding poses of urate are distinct (Fig. 1*B-C), further supporting the possibility that urate may bind at multiple

37 sites within the intracellular cavity, likely due to low substrate binding affinity at this conformation. Notably,
38 one of the structures showed a urate-binding site that closely partially overlaps with the additional density
39 observed in our study (Fig. 1*D), albeit not entirely identical.

40 Phe241 is located near the additional density (Fig. 1*A and D). As the reviewer pointed out, the density of
41 the side chain is not well resolved. We speculate that this may be due to the side chain undergoing a local
42 conformational change to accommodate urate at the distinct position.

43 We fully agree with the reviewer that we should discuss these detailed observations, and we thank you for
44 helping make our manuscript more rigorous. Therefore, we have revised urate binding related content in
45 the revised manuscript, starting from line 138, which reads “We also observed weaker density near the
46 position discussed above, which partially overlaps with the urate-binding position observed in human
47 URAT1 (ref.¹) (Figure S10B-D). Notably, the density of the nearby residue F241 is not well-defined, likely
48 because its side chain adopts distinct conformations when stabilizing urate in various binding sites,
49 supporting the notion of heterogeneous urate binding in the inward-facing conformation. Moreover, the
50 complex structure of human URAT1(R477S) bound with urate revealed that urate binds at a position
51 distinct from the two locations discussed above². These observations suggest that urate may adopt
52 multiple binding modes and be highly dynamic and flexible within the intracellular cavity of URAT1. Similar
53 observations have been reported³. This variability is likely due to the low affinity of the substrate for the
54 intracellular cavity, a state that is ready to release the substrate.”.



55

56 Figure 1*. The cryo-EM density of urate binding pocket and distinct urate binding modes.

57 (A) Cryo-EM density of urate (UA) binding pocket. The cryo-EM densities are shown as mesh, with urate and

58 surrounding residues shown as sticks. (B-C) Superposition of urate-bound URAT1 structures based on

59 alignment of the N-domains. The structures are colored orange (URAT^{EM}), pink (PDB:8WJQ), and blue (PDB:

60 9B1J), respectively. The urate molecules are shown as sticks and spheres. (D) Cryo-EM density of urate (UA)

61 binding pocket. The urate molecule in the URAT1 structure determined in this study (URAT^{UA}) is colored orange,

62 while the urate molecule in the 8WJQ structure is colored pink.

63 Reference:

64 [1] Zhang, H., Cui, N., Ma, X. & Xu, H. E. Structural basis of augment taurine uptake by taurine transporter alleviating
65 cellular senescence. *bioRxiv*, 2024.2011.2013.623519 (2024). <https://doi.org/10.1101/2024.11.13.623519>

66 [2] He, Jingjing et al. Structural basis for the transport and substrate selection of human urate transporter 1. *Cell Reports*,
67 Volume 43, Issue 8, 114628. <https://doi.org/10.1016/j.celrep.2024.114628>

68 [3] Dai, Y., Lee, CH. Transport mechanism and structural pharmacology of human urate transporter URAT1. *Cell Res* 34,
69 776–787 (2024). <https://doi.org/10.1038/s41422-024-01023-1>

2. In the benzbromarone-bound rat URAT1 structure, the EM density feature poorly covers at least half of the molecule (figure attached). A similar issue applies to the verinurad-bound structure, where the molecule is ambiguously positioned. This requires either improving the resolution of the EM density maps, particularly in the binding pocket, or presenting and discussing these ambiguous features.

Reply: We appreciate the reviewer for this comment. For benzbromarone, we analyzed the EM density of the molecule and its surrounding residues from multiple angles, revealing that the benzofuran group is well-defined in the density map, while the bromophenol group is less clearly resolved (Figure 2*A). When comparing our structure to the recently reported human URAT1 structure bound to the benzbromarone analog dotinurad, we observed that the benzofuran group exhibits a good alignment, with significant overlap in binding position, including the side chains surrounding the benzofuran group (Figure 2*B). In contrast, the bromophenol group shows poor positional overlap, accompanied by notable side chain rearrangements, particularly involving residue Q473. Based on this comparison, we propose that the bromophenol group exhibits greater positional flexibility within the binding pocket compared to the benzofuran group, likely reflecting a higher degree of binding heterogeneity. Furthermore, following the suggestion of Reviewer-2, we have designed more mutations and conducted additional functional experiments, which have further validated the drug binding pocket (Figure 4*C-D). To further validate the binding pose of these drugs with URAT1, we carried out molecular dynamics simulations to evaluate the stability of the drug binding. The molecular dynamics simulations revealed that both the protein and ligand reached a stable state over the course of the simulation. The average Root-mean-square-deviations (RMSD) values for the protein and ligand remained below 4.0 Å and 2.0 Å, respectively, indicating minimal conformational fluctuations and high stability of the system (Figure 4*E). These findings further support the binding mode of benzbromarone.

Following the reviewer's suggestion, we have included a brief discussion in the revised manuscript. Starting from line 204, it reads: "We compared the structure of benzbromarone-bound URAT1 with the recent dotinurad-bound URAT1 structure, revealing that both benzbromarone and dotinurad bind within the same pocket. The benzofuran group aligns well, while the bromophenol group exhibits conformational diversity, and the side chain of nearby Q473 is in a different rotamer (Figure S11B). Notably, when we further analyzed the EM density of the benzbromarone, we found the benzofuran group is well-defined in the cryo-EM map, while the bromophenol group is less clearly resolved (Figure S11A). Based on this comparison, we propose that the bromophenol group exhibits greater positional flexibility within the binding pocket compared to the benzofuran group, likely reflecting a higher degree of binding heterogeneity."

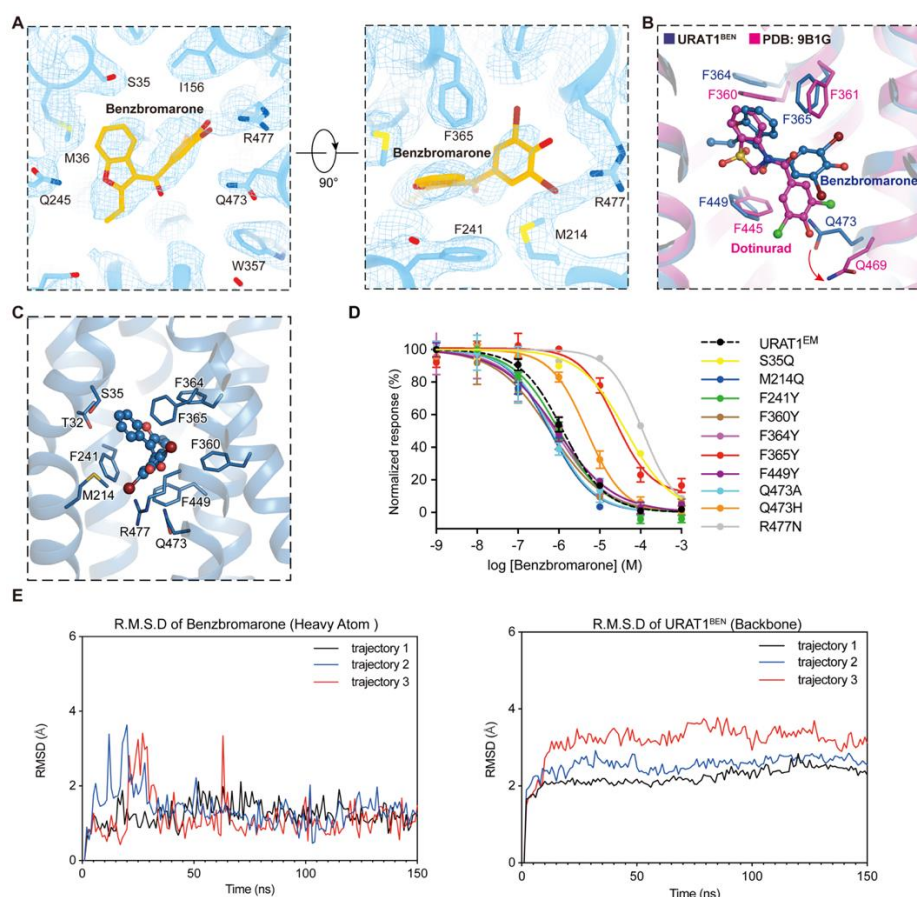


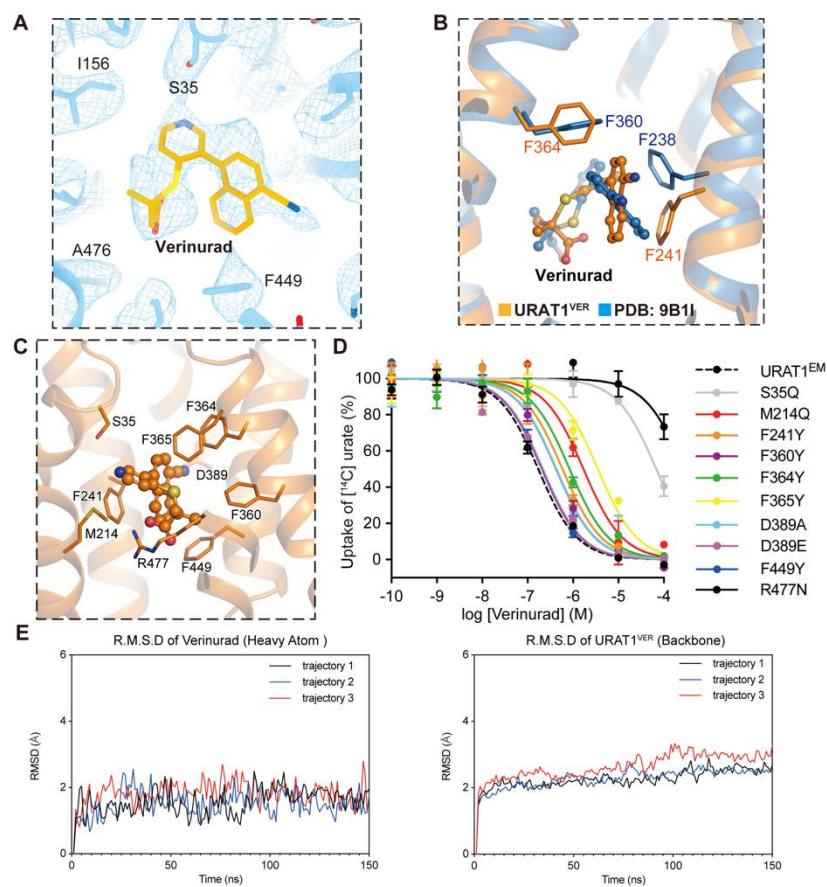
Figure 2*. (A) The cryo-EM density of benzbromarone and surrounding residues is shown as mesh while the structure of which are presented as sticks. (B) The structure alignment of URAT1^{BEN} and dotinurad-bound human URAT1(PDB: 9B1G). (C) Detailed view of the benzbromarone binding pocket of URAT1^{BEN}. Surrounding residues are represented as sticks. (D) Inhibition of ¹⁴C-urate transport by benzbromarone in URAT1^{EM} and its mutants. Data are presented as normalized mean $\pm$ s.e.m. from three biologically independent assays ($n = 3$). IC₅₀ values are calculated by fitting into a nonlinear regression model. (E) R.M.S.D. plots for ligand and protein backbone during MD simulation. R.M.S.D. plots were generated for both benzbromarone and the protein backbone. The calculation of backbone R.M.S.D. for URAT1^{BEN} using residues 2 to 42, 132 to 324, and 347 to 515. The calculation of benzbromarone R.M.S.D. involved the heavy atoms of benzbromarone, using its initial structure as the reference. Results from three independent trajectories were distinguished by different colors.

For verinurad, we present the cryo-EM density of the verinurad molecule and its surrounding residues (Fig. 3*A). While most regions show good density matching, the acene group's density does not perfectly align, likely due to its flexibility within the binding pocket (Fig. 3*A). To further understand these observations, we compared our structure with the verinurad-bound hURAT (PDB:9B1J) structure. This comparison revealed minor differences in the angle of the acene group, which correlate with changes in the side chains of surrounding residues (Fig. 3*B). To confirm the current orientation of verinurad, we performed more functional experiments, which supported our proposed binding pose (Fig. 3*C-D). Additionally, molecular dynamics simulations demonstrated that verinurad can dynamically bind during the simulation, further validating the binding pose of verinurad (Fig. 3*E).

Following reviewer's suggestion, we have included a brief discussion in the revised manuscript. Starting

123 from the line 240, it reads “Molecular dynamics simulations were conducted to further validate the binding
 124 pose of the ligand. The results demonstrated a stable RMSD value for the ligand throughout the simulation
 125 trajectories (Figure S13), indicating consistent behavior of the ligand during the simulations. These findings
 126 suggest that verinurad maintains a stable binding state with URAT1^{EM}. Comparison with the verinurad-
 127 bound hURAT1 structure (PDB: 9B1J) highlights minor differences in the orientation of the acene group
 128 and surrounding residue side chains (Figure S13B). The observed variation in the orientation of the acene
 129 group suggests greater positional flexibility and binding heterogeneity for this group, which is also
 130 evidenced by its weaker cryo-EM map density (Figure S13A).”.

131



132

133 Figure 3*. (A) The cryo-EM density of verinurad and surrounding residues is shown as mesh while the structure
 134 of which are visualized as sticks. (B) The structure alignment of URAT1^{VER} and verinurad-bound human
 135 URAT1(PDB: 9B1J). (C) Detailed view of the verinurad binding pocket of URAT1^{VER}. Surrounding residues are
 136 represented as sticks. (D) Inhibition of ¹⁴C-urate transport by verinurad for URAT1^{EM} and its mutants. Data are
 137 presented as normalized mean ± s.e.m. from three biologically independent assays (n = 3). IC₅₀ values are
 138 calculated by fitting into a nonlinear regression model. (E) R.M.S.D. plots for ligand and protein backbone
 139 during MD simulation. R.M.S.D. plots were generated for both verinurad and the protein backbone. The
 140 calculation of backbone R.M.S.D. for URAT1^{VER} using residues 2 to 42, 132 to 324, and 347 to 515. The
 141 calculation of verinurad R.M.S.D. involved the heavy atoms of benzbromarone, using its initial structure as the
 142 reference. Results from three independent trajectories were distinguished by different colors.

143 3. The sulfinpyrazone-bound structure (figure attached) shows very low occupancy level in the binding

pocket at higher threshold levels of the EM density maps. This also requires further discussion in the text. Are drug-free and sulfinpyrazone-bound URAT1 particles mixed in the final EM reconstruction?

Reply: We appreciate the reviewer for highlighting this issue. We examined the cryo-EM map and found that the sulfinpyrazone groups deep within the pocket exhibit stronger density compared to the benzenesulfinyl group closer to the intracellular side (Fig. 4*A-C). Structural analysis has shown that the diphenylpyrazolidine touching the base forms extensive interactions with surrounding residues, while the benzenesulfinyl group near the intracellular side has fewer interactions with surrounding residues (Fig. 4*D). Therefore, the benzenesulfinyl group may exhibit high flexibility, leading to its density not being well-resolved. Our functional assay indicates that mutations around the diphenylpyrazolidine group result in a greater reduction in affinity than mutations around the benzenesulfinyl group, suggesting that the residue around diphenylpyrazolidine group plays a more important role in stabilizing sulfinpyrazone. Furthermore, molecular dynamics simulations reveal that while the overall binding of sulfinpyrazone to the protein is relatively stable (Fig. 4F), the benzenesulfinyl group exhibits significant variation across different clusters, displaying more dynamic states compared to the deeply embedded diphenylpyrazolidine group (Fig. 4G-H). However, we cannot rule out the possibility of low occupancy of the sulfinpyrazone bound within the binding pocket, as pointed out by the reviewer. In particular, we were unable to differentiate between drug-free and sulfinpyrazone-bound particles because the drug molecule is too small relative to the protein, and the protein and micelles predominate in the particle alignment.

Following reviewer's suggestion, we have included a brief discussion in the revised manuscript. Starting from the line 277, it reads "The benzenesulfinyl group of sulfinpyrazone reaches the bottom of the intracellular cavity (18 Å deep), while the dione group extends towards the cytosol. Molecular dynamics simulations indicate that the overall binding of sulfinpyrazone to the protein remains relatively stable (Figure S14D). However, the clustering data mining technique generates seven main clusters and reveals that the benzenesulfinyl group shows significant variation across different clusters (Figure 14E-F), whereas the diphenylpyrazolidine group remains highly stable. These observations suggest that the benzenesulfinyl group exhibits more dynamic states compared to the deeply embedded diphenylpyrazolidine group. These findings are consistent with observations that the deeper sulfinpyrazone groups show stronger density than that of the benzenesulfinyl group near the intracellular side (Figure 14A-C).".

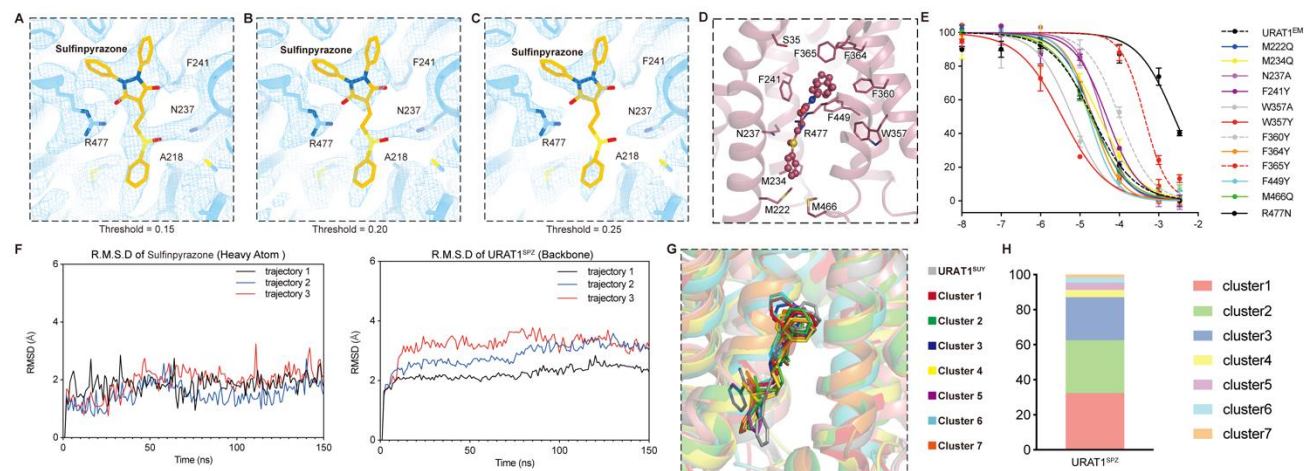


Figure 4*. (A-C) The cryo-EM densities of sulfinpyrazone and surrounding residues, in different thresholds, are shown as mesh while the structure of which are visualized as sticks. (D) Detailed view of the sulfinpyrazone

binding pocket of URAT1^{SPZ}. Surrounding residues are represented as sticks. (E) Inhibition of ¹⁴C-urate transport by sulfinpyrazone for URAT1^{EM} and its mutants. Data are presented as normalized mean $\pm$ s.e.m. from three biologically independent assays (n = 3). IC₅₀ values are calculated by fitting into a nonlinear regression model. (F) R.M.S.D. plots for ligand and protein backbone during MD simulation. R.M.S.D. plots were generated for both sulfinpyrazone and the protein backbone. The calculation of backbone R.M.S.D. for URAT1^{SPZ} using residues 2 to 42, 132 to 324, and 347 to 515. The calculation of sulfinpyrazone R.M.S.D. involved the heavy atoms of benzbromarone, using its initial structure as the reference. Results from three independent trajectories were distinguished by different colors. (G) Structural comparisons of initial poses of sulfinpyrazone with representative poses derived from clustering analysis. (H) Cluster populations obtained from clustering analysis. The clustering analysis was conducted for URAT1^{SPZ}, which involving three independent 150 ns trajectories.

4. The abstract should explicitly state that the authors studied the rat homolog of URAT1. Despite the 74% sequence identity and 84% similarity, the residues lining the binding pocket should be analyzed and compared between the rat and human homologs. Two mutations were introduced into the rat URAT1 construct to "enhance substrate affinity and drug sensitivity to levels similar to those of human URAT1." Please elaborate on these affinities and sensitivities. It is important to convince readers that the binding modes of the drugs are expected to be the same in human and double-mutant rat URAT1.

Reply: We thank the reviewer for this insightful comment. following reviewer's suggestion, we have revised the abstract to explicitly state the focus of our study on the rat homolog of URAT1. The updated sentence now reads: "Here, we present structures of the double-mutant rat homolog of URAT1 in complex with its substrate urate, and the clinical drugs benzbromarone, lesinurad, verinurad, and sulfinpyrazone."

We also agree with the reviewer that it is important to compare the binding affinities of these drugs to both double-mutant rat URAT1 (URAT1^{EM}) and human URAT1. Upon structural comparison between URAT1^{EM} and human URAT1, we observed some variations within the central binding pocket (Figure 5*). To confirm that the binding poses determined using URAT1^{EM} are relevant to those of human URAT1, we compared the inhibitory potency of each drug on both human URAT1 and URAT1^{EM}. We found that the IC₅₀ values of drugs in inhibiting the URAT1^{EM} closely match those of the human URAT1 (Figure 6*). These findings support that the drug-binding modes obtained using URAT1^{EM} in this study highly relevant to those in human URAT1.

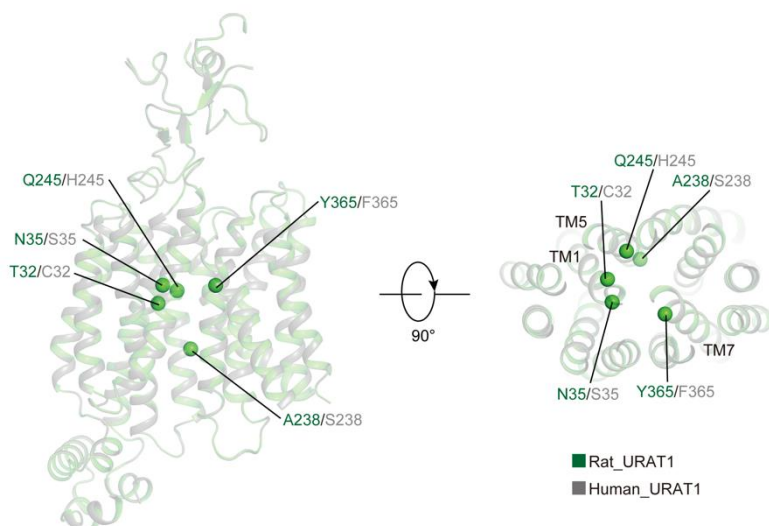


Figure 5*. Structural alignment of rat URAT1 (green) and human URAT1 (gray). Residues exhibiting differences within the binding pocket are shown as spheres and labeled for clarity.

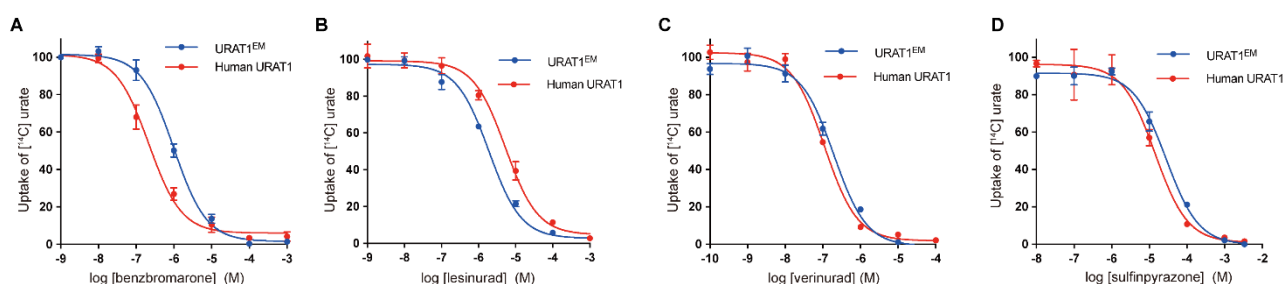


Figure 6*. Inhibition of [^{14}C] urate uptake double-mutant rat URAT1 (URAT1^{EM}) and human URAT1 (hURAT1) by benzbromarone (A), lesinurad (B), verinurad (C), and sulfinpyrazone (D). Data represent mean $\pm$ s.e.m. obtained from three biological experiments. The normalized data of inhibition assay were fitted using non-parametric nonlinear regression, yielding IC_{50} values of $0.99 \pm 0.14 \mu\text{M}$ (URAT1^{EM}) and $0.22 \pm 0.01 \mu\text{M}$ (hURAT1) for benzbromarone, $1.91 \pm 0.12 \mu\text{M}$ (URAT1^{EM}) and $5.32 \pm 0.59 \mu\text{M}$ (hURAT1) for lesinurad, $0.19 \pm 0.02 \mu\text{M}$ (URAT1^{EM}) and $0.11 \pm 0.002 \mu\text{M}$ (hURAT1) for verinurad, and $28.0 \pm 5.7 \mu\text{M}$ (URAT1^{EM}) and $14.9 \pm 1.1 \mu\text{M}$ (hURAT1) for sulfinpyrazone, respectively.

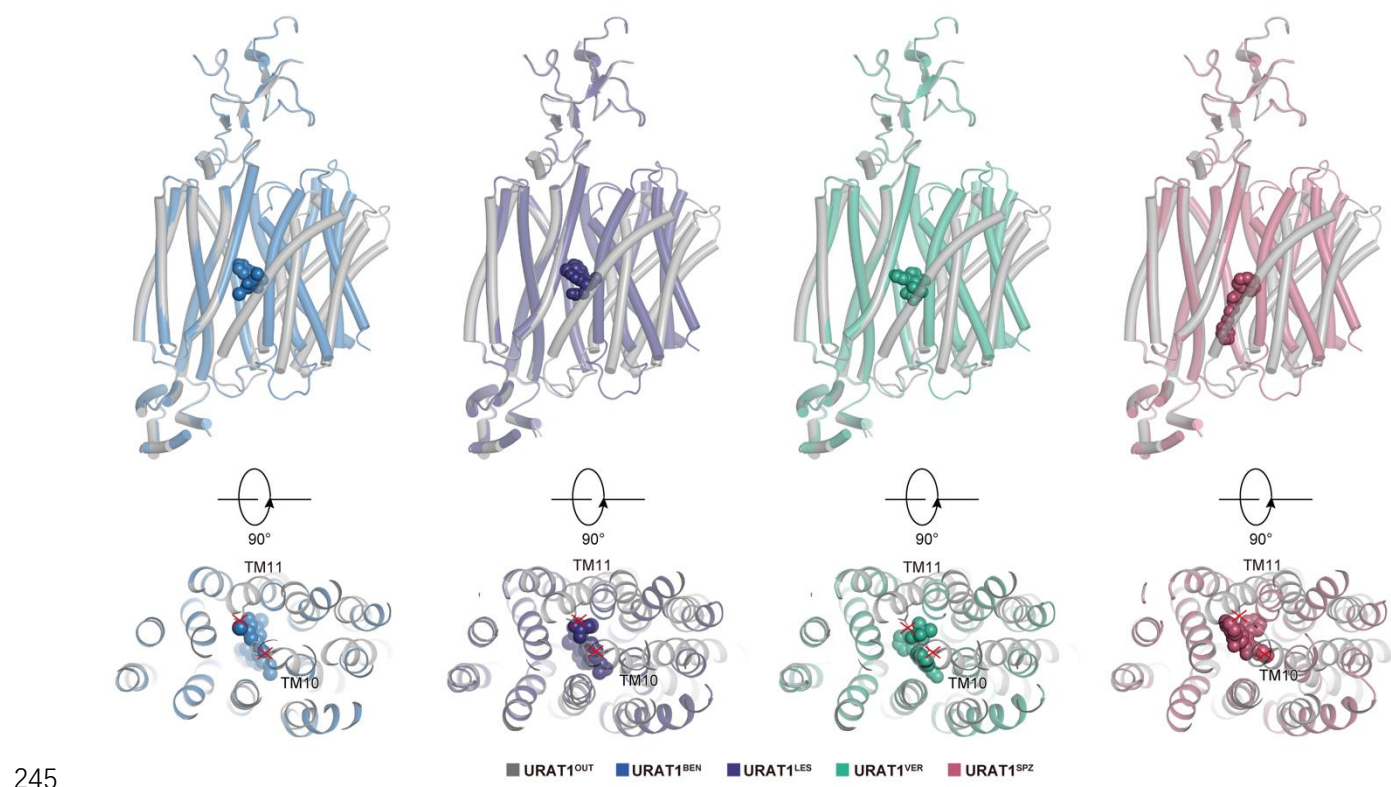
Figure 6* is shown in Supplementary Figure S2, and a description has been added in the manuscript at line 80 as follows: “Functional assays using [^{14}C]-urate confirmed the activity of this construct, which also exhibited similar drug sensitivity to compounds such as benzbromarone, lesinurad, verinurad, and sulfinpyrazone, compared to human URAT1(Figure S2A-E), consistent with earlier findings⁴.”

5. The authors claim to provide detailed insight into the inhibition mechanism of URAT1, stating that the studied drugs prevent the transporter from undergoing conformational transitions. Which results support this statement? Do the authors expect to observe different conformations in the apo sample of their URAT1 protein?

Reply: We appreciate the reviewer for raising this question. To address this, we constructed a homology model of URAT1 (URAT1^{OUT}) in the outward-facing conformation and aligned it with the drug-bound inward-facing structures of URAT1 using the N-domain as a reference (Figure 7*). Our analysis revealed

226 significant steric clashes between the studied drugs and the URAT1^{OUT} structure, particularly involving
 227 transmembrane helices TM10 and TM11. These clashes suggest that the drugs effectively stabilize
 228 URAT1 in the inward-facing conformation and obstruct its conformational transition to the outward-facing
 229 state, thereby preventing the transporter from completing its functional cycle. This mechanism is
 230 universally adopted by inhibitors to block transporters from the same MFS family and other families [1-2].
 231 Moreover, based on the structural alignment with chloride ions-bound OAT1 and pyrazinoate-bound
 232 hURAT1 (Figure 9*), we found that the inhibitor within the intracellular cavity block the counter-ion binding
 233 site, thereby inhibiting its activity.

234 In addition, we have added a new figure (Supplementary Figure 17) and provided the discussion in the
 235 revised manuscript. The revised text in the discussion section now reads: “We aligned the outward-facing
 236 homology model of URAT1 (URAT1^{OUT}) with the drug-bound inward-facing structures of URAT1
 237 individually using their N-domains as a reference (Figure S17A). This analysis revealed significant steric
 238 clashes between the drugs and the URAT1^{OUT} structure, suggesting a potential inhibitory mechanism of
 239 drugs by obstructing its conformational transition from the inward-facing to the outward-facing state,
 240 thereby preventing the transporter from completing its functional cycle. Furthermore, URAT1 utilizes
 241 intracellular anions or organic anions to exchange extracellular urate into the cytosol^{4,5}. The binding site
 242 for these drugs is located proximal to the potential counter-ion or organic anion pyrazinoic acid binding
 243 site^{44,45,47}, which likely obstructs anion binding and therefore effectively impairs the transport activity of
 244 URAT1 (Figure S17B).”.



246 Figure 7*. Structural alignment of URAT1^{OUT} and the drug-bound structures of URAT1. The structures of
 247 URAT1^{OUT}, URAT1^{BEN}, URAT1^{LES}, URAT1^{VER}, and URAT1^{SPZ} are superposed by aligning their N-domains and
 248 are shown in gray, sky blue, purple, green, and red, respectively. The bound drugs are represented as spheres.
 249 The TM10/11 of URAT1^{OUT}, which have steric clashes with the bound drugs, are labeled.

We have attempted to resolve the structure of URAT1 in its apo state. Unfortunately, we were unable to obtain a high-resolution map and only achieved a low-resolution map. The limited resolution is likely due to the sample in the apo state not being homogeneous and consisting of multiple conformations, rather than a specific conformation stabilized by inhibitors.

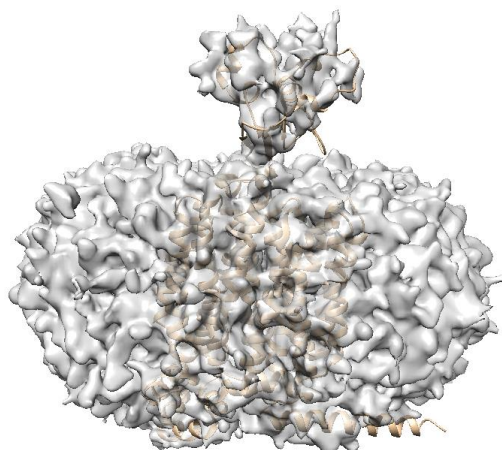


Figure 8*. The superpose of the low-resolution cryo-EM map and structure of URAT1 in the apo state.

[1] Wu, D., Chen, Q., Yu, Z. *et al.* Transport and inhibition mechanisms of human VMAT2. *Nature* **626**, 427–434 (2024). <https://doi.org/10.1038/s41586-023-06926-4>

[2] Drew, D., North, R. A., Nagarathinam, K. & Tanabe, M. Structures and General Transport Mechanisms by the Major Facilitator Superfamily (MFS). *Chemical Reviews* 121, 5289–5335 (2021). <https://doi.org/10.1021/acs.chemrev.0c00983>
6. The manuscript text requires improvement. The title should be revised, as neither urate reabsorption nor (multiple) inhibitory mechanisms of URAT1 are directly studied in the manuscript. All literature-based fragments (e.g., l. 104–107) should be moved to the introduction. Additionally, avoid repetition between the introduction and discussion sections.

Reply: We thank the reviewer for this valuable feedback. In response to your comment, we have revised the title to 'Molecular mechanism of drug inhibition of URAT1' to more accurately reflect the focus of our manuscript.

We have also moved the literature-based fragments (e.g., lines 104–107) to the introduction as you suggested. We believe these changes enhance the clarity of the manuscript.

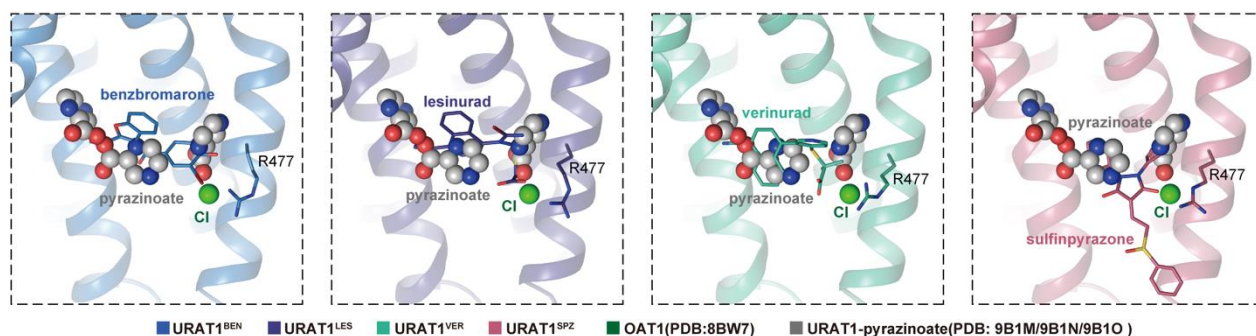
7. The manuscript does not mention the counter-ion or driving force of the transporter, which is a key element in any mechanistic discussion of transporters, including inhibition mechanisms.

Reply: We sincerely thank the reviewer for highlighting this important aspect. We fully agree that the counter-ion and driving force of the transporter are critical for understanding its mechanistic function, including inhibition mechanisms. URAT1, as an antiporter, mediates the exchange of urate with intracellular anions (such as chloride or organic anions). This transport is driven by the concentration gradients of urate and anions across the membrane. Our structural analysis of URAT1 reveals a positively charged intracellular binding pocket, which likely facilitates anion binding.

During our protein purification, chloride ions were present in the buffer. However, due to the resolution limitations of our cryo-EM map, we have not been able to identify the specific chloride ion binding sites on URAT1. Nevertheless, previous studies on OAT1 and OAT4 have identified a chloride ion coordination site

280 at the R466/R473 residue which is conserved as R477 in URAT1 [1-2]. We speculate that R477 may serve
281 an important role in coordinating counter-ions.

282 Moreover, the binding site for these drugs is located proximal to the potential counter-ion or organic anion
283 pyrazinoic acid binding site [1-3], which likely obstructs anion binding and therefore effectively impairs the
284 transport activity of URAT1 (Figure 9*).



285
286 Figure 9*. Structural alignment of chloride ions-bound OAT1(PDB: 8BW7), pyrazinoate-bound hURAT1(PDB:
287 9B1M/9B1N/9B1O) and the drug-bound structures of URAT1. The structures of URAT1^{BEN}, URAT1^{LES},
288 URAT1^{VER}, and URAT1^{SPZ} are superposed by aligning their N-domains and are shown in sky blue, purple,
289 green, and red, respectively. The bound drugs are represented as sticks while the chloride ions and pyrazinoate
290 are shown as deep green and grey sphere, respectively.

291 In response to the reviewer's suggestion, we have included the following statement in line 149: "URAT1
292 mediates urate transport through the exchange with counter-ions such as chloride or organic anions^{5,6}.
293 Although chloride ions were supplied in the buffer during protein purification. However, due to the moderate
294 resolution of the cryo-EM map, the chloride binding site was not identified. Nevertheless, previous studies
295 on OAT1 and OAT4 have identified a chloride ion coordination site at the R466/R473 residue which is
296 conserved as R477 in URAT1(ref.^{2,7}). We speculate that R477 may serve an important role in coordinating
297 counter-ions." and line373:"Furthermore, URAT1 utilizes intracellular anions or organic anions to exchange
298 extracellular urate into the cytosol^{5,6}. The binding site for these drugs is located proximal to the potential
299 counter-ion or organic anion pyrazinoic acid binding site^{1,2,7}, which likely obstructs anion binding and
300 therefore effectively impairs the transport activity of URAT1 (Figure S17B)."

301 [1] He, J. et al. Structural basis for the transport and substrate selection of human urate transporter 1. Cell Rep 43, 114628
302 (2024). <https://doi.org/10.1016/j.celrep.2024.114628>

303 [2] Parker, J. L., Kato, T., Kuteyi, G., Sitsel, O. & Newstead, S. Molecular basis for selective uptake and elimination of organic
304 anions in the kidney by OAT1. Nat Struct Mol Biol 30, 1786-1793 (2023). <https://doi.org/10.1038/s41594-023-01039-y>

305 [3] Dai, Y. & Lee, C. H. Transport mechanism and structural pharmacology of human urate transporter URAT1. Cell Res 34,
306 776-787 (2024). <https://doi.org/10.1038/s41422-024-01023-1>

307 8. The authors should provide SEC spectra showing the expression levels of URAT1 mutants used for
308 normalization in the transport assays.

309 **Reply:** Thank you for bringing attention to this matter. We have included the SEC spectra of URAT1^{EM} and
310 its mutants used in the transport assays as a supplementary Figure 9. The peak values within the 10-15
311 mL range of these spectra were utilized to normalize the expression levels across different samples.

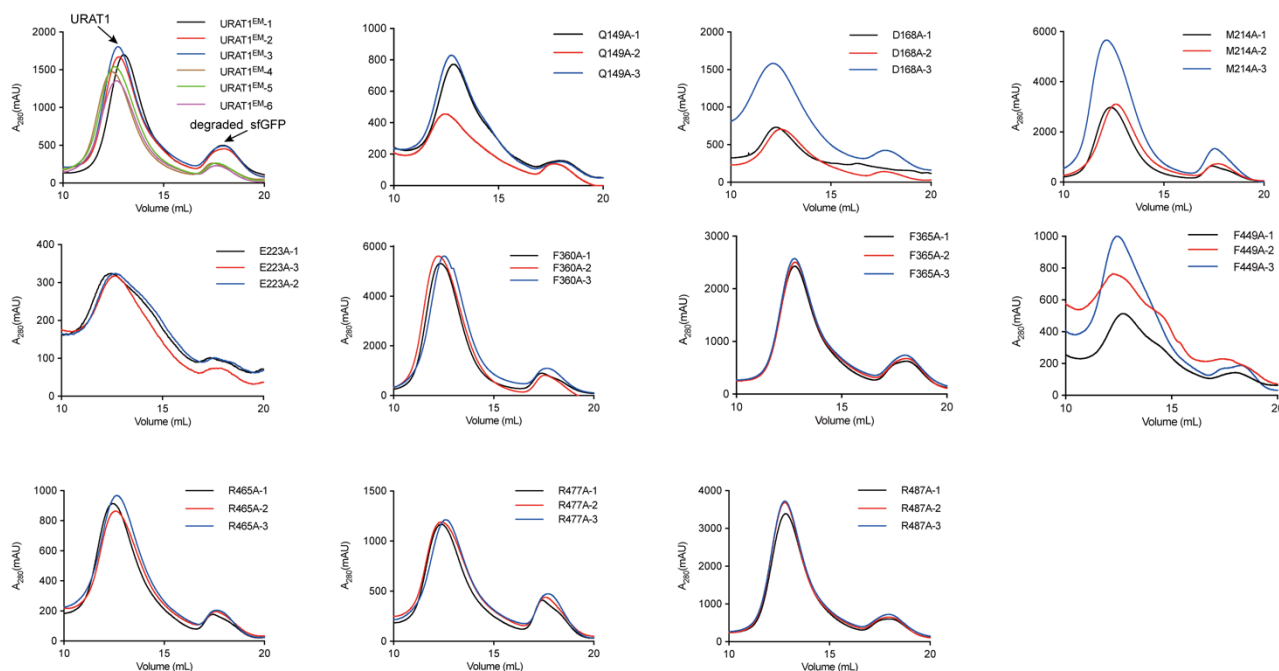


Figure 10*. The SEC spectra of URAT1^{EM} and mutants. the peak values within the 10-15 mL range of these spectra were used for normalization of expression levels in the transport assays.

Minor issues:

9. Please do not use the phrase “electron density” when referring to cryo-EM density maps.

Reply: Thank you for pointing this out. We have replaced “electron density” with “cryo-EM density maps” in the revised manuscript.

10. L. 78-80 - to comment on homogeneity, please specify the detergent-specific condition and avoid the phrase “ideal”

Reply: Thank you for this suggestion. We have revised this sentence. The revised sentence now reads: “The purified protein exhibited a symmetric peak in size exclusion chromatography under LMNG detergent conditions, indicating that the sample is homogeneous for the following cryo-EM study.”.

11. L. 88 - if the map covers 515 residues, why does Ext Table 1 indicate 472 protein residues in the model? Please clarify this discrepancy.

Reply: Thank you for pointing out this discrepancy. In our structure, some extracellular and intracellular loops connecting the TM helices are not determined, likely due to their conformational heterogeneity. We have clarified this in the revised manuscript. In line 91, it reads:” The URAT1^{EM} protein consists of 553 amino acids, with the structural model determined from a cryo-EM map covering 472 residues. Some flexible loop regions (residues 58-67, 100-110, and 325-346) are not clearly resolved, likely due to their flexibility.”.

12. L.125 - did the authors mean 1d?

Reply: Thank you for pointing this typo. We have corrected it in the text accordingly.

13. L. 137 – please avoid the phrase “wild-type” if you are referring to the construct containing two

mutations

Reply: Thank you for this suggestion. We have replaced 'wild-type' with 'URAT1^{EM}' construct throughout the manuscript.

14. Does the transporter adopt exactly the same conformation in all structures? Are there any conformational differences? Is the degree of the opening between the halves different?

Reply: Thank you for your insightful question. We conducted a structural comparison of URAT1^{UA} with inhibitor-bound structures. The calculated RMSD values for 472 C α -pairs are approximately 0.28 Å, 0.50 Å, 0.39 Å, and 0.31 Å, respectively, indicating that the transporter adopts a similar overall conformation. However, upon closer examination, the side chains involved in drug binding exhibit significant local conformational changes, which are likely important to accommodate inhibitors with distinct chemical structures (Figure 11*).

To clarify these findings, we have included a supplementary figure (Supplementary Figure 16) and brief discussion in the revised manuscript. Starting from the line 364, it reads “These structures adopt the same inward-open conformation, with the overall structures being superimposable. However, to accommodate the distinct drug molecules, slight displacements occur in the residue side chains around the binding site (Figure S16).”.

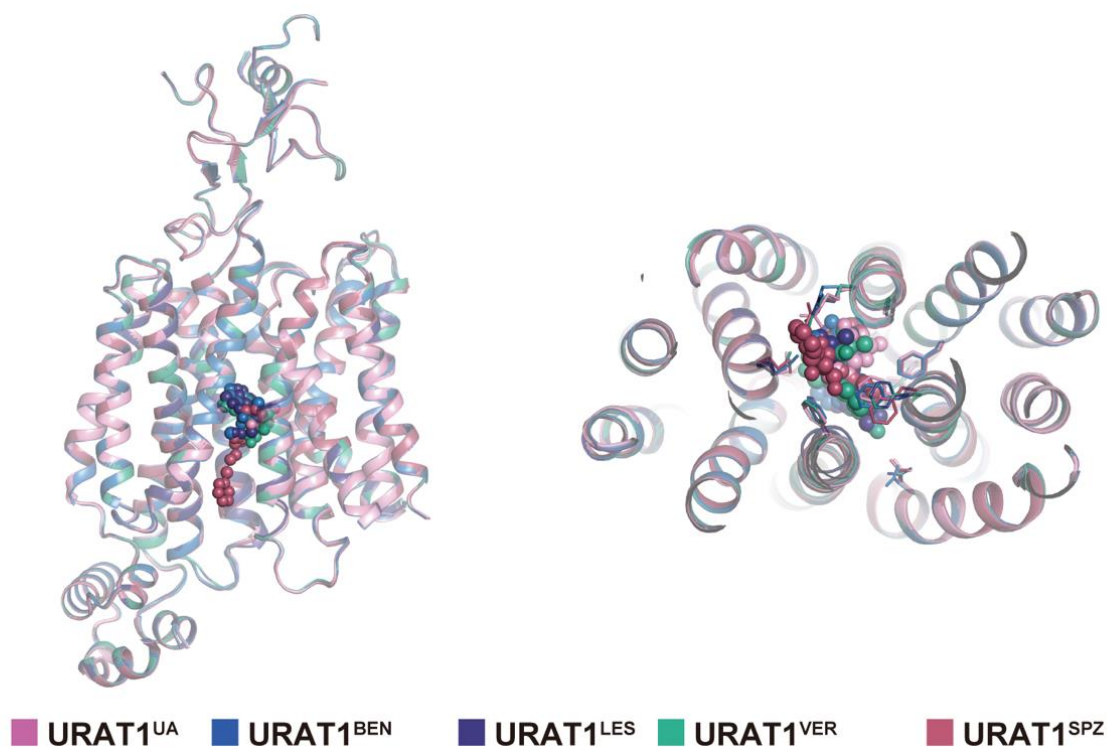


Figure 11*. The structural alignment of all structures which aligned by N-domain. The structures of URAT1^{UA}, URAT1^{BEN}, URAT1^{LES}, URAT1^{VER}, and URAT1^{SPZ} are colored in pink, sky-blue, purple, green, red, respectively. The surrounding residues are shown as sticks.

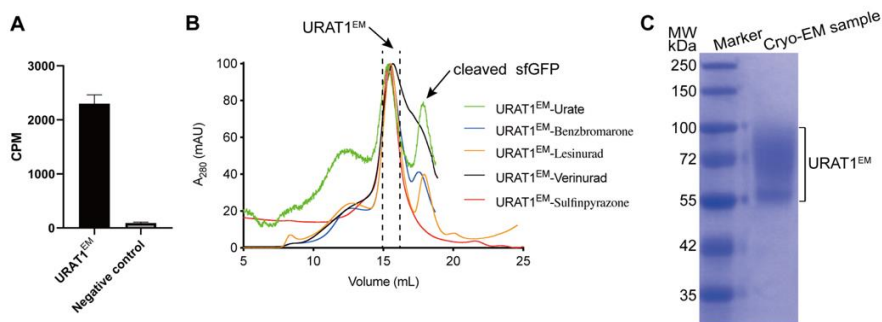
15. Please provide a detailed cryo-EM analysis for each structure separately. Include the processing

357 pipeline, local resolution estimation, 2D class representatives, angular distribution, etc. Please provide the
358 final SEC spectra for each cryo-EM sample.

359 **Reply:** Thank you for this suggestion. Following the reviewer's suggestions, we have now provided a
360 detailed cryo-EM analysis for all URAT1 complexes, including URAT1^{UA}, URAT1^{BEN}, URAT1^{LES}, URAT1^{VER},
361 and URAT1^{SPZ}. For each complex, we have included the processing pipeline, local resolution estimations,
362 2D class averages, and angular distributions, all presented in Supplementary Figures 3-7.

363 Additionally, the final SEC spectra for each cryo-EM sample are shown in Supplementary Figure 2B, which
364 are similar to each other.

365 For your convenience, we have included all relevant figures below.



366
367 Figure S2. Functional characterization and purification of URAT1^{EM}.

368 (A) The transport activity of URAT1^{EM}, quantified by [¹⁴C] urate uptake assay. Negative control is
369 untransfected HEK293 cells. Data are mean mean $\pm$ s.e.m. from three independent assays, each with one
370 measurement (n = 3). (B) Gel-filtration profile of URAT1^{EM} in LMNG, showing the normalized SEC spectra
371 for each sample: URAT1^{UA} (green), URAT1^{BEN} (blue), URAT1^{LES} (orange), URAT1^{VER} (black), and
372 URAT1^{SPZ} (red). (C) Coomassie-blue-stained SDS-PAGE gel of URAT1^{EM} in LMNG.

373

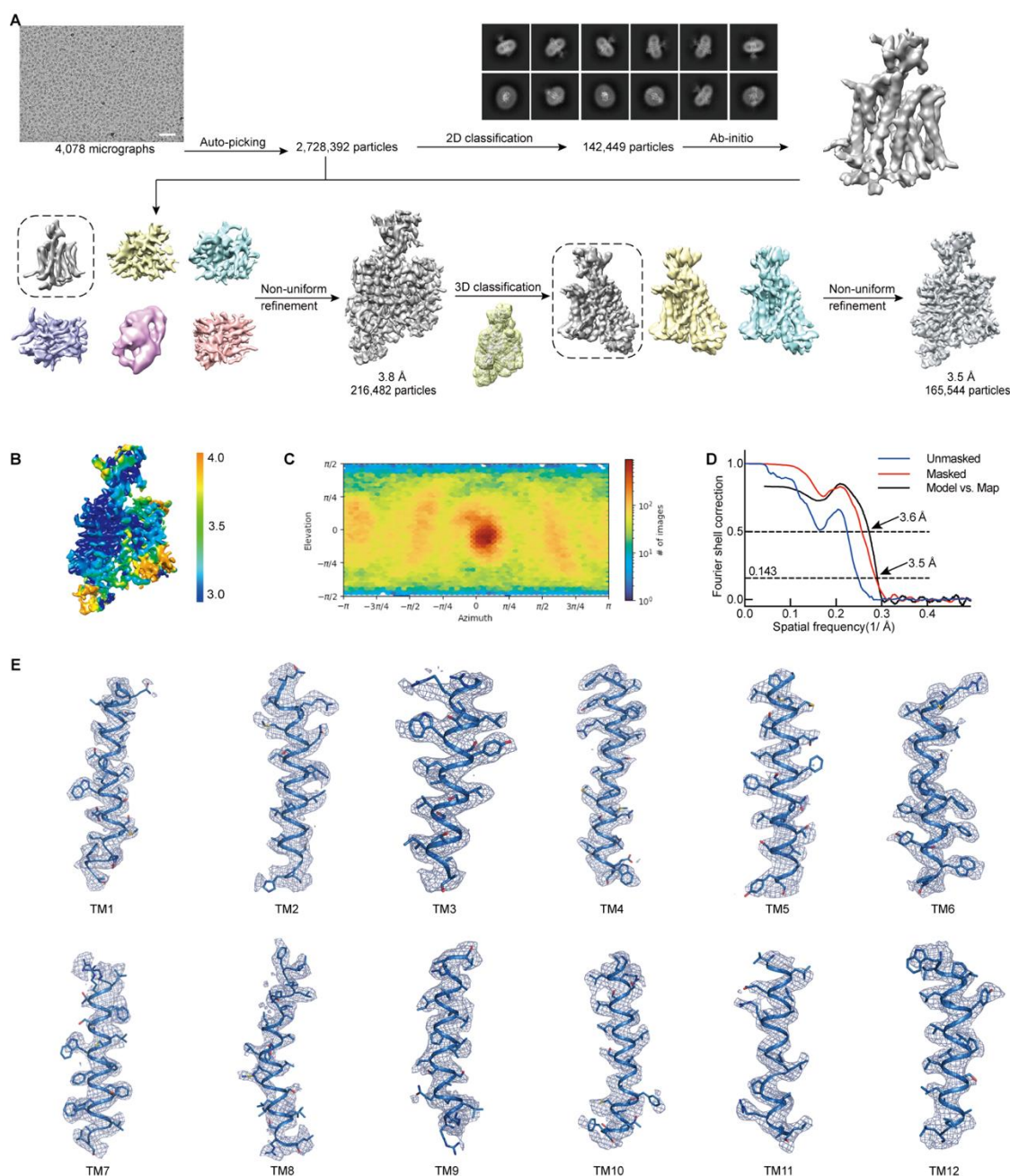
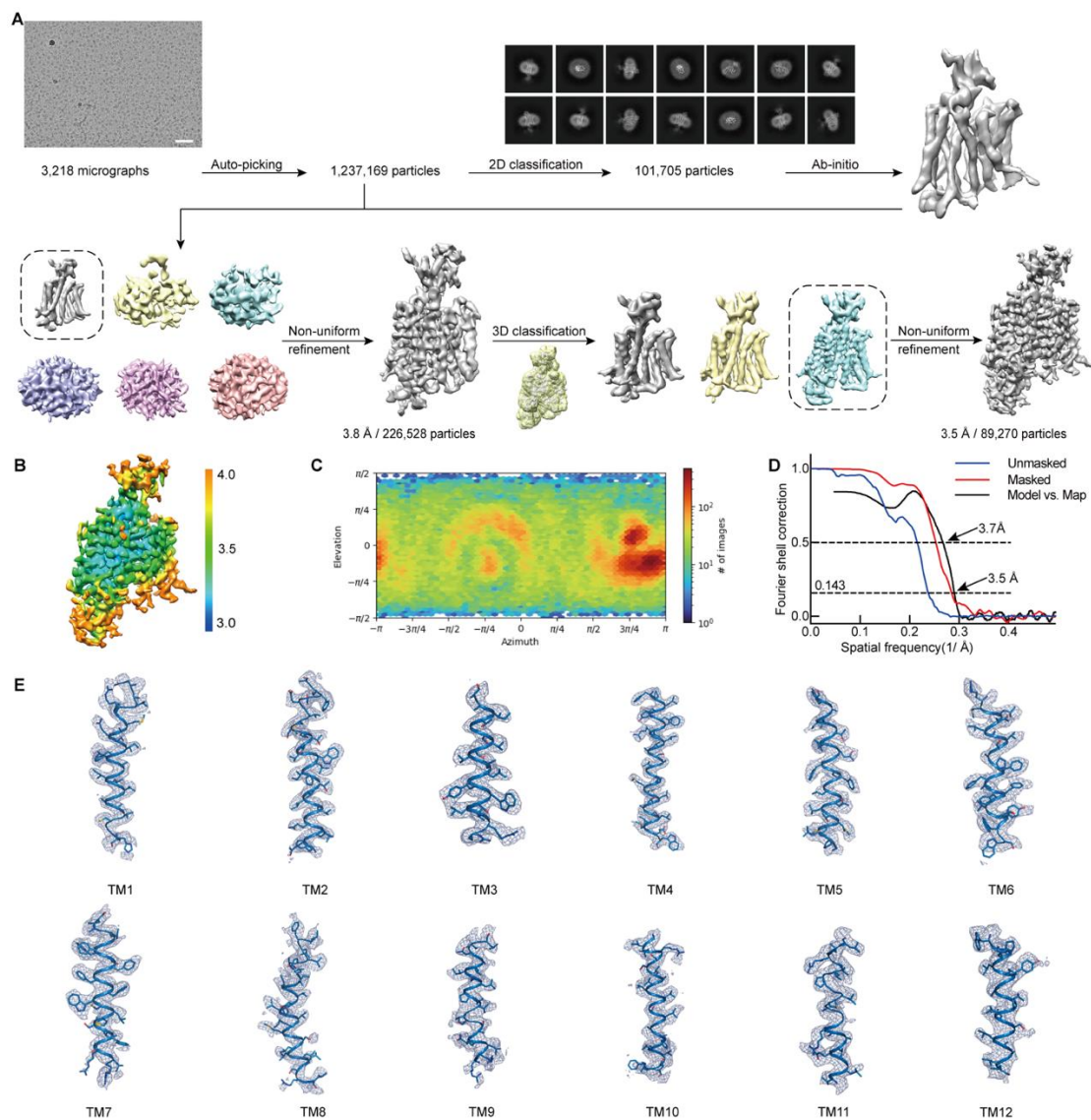


Figure S3. Cryo-EM data analysis of the URAT1^{UA}

(A) Flowchart for cryo-EM data processing of URAT bound with urate. A representative raw cryo-EM micrograph is shown here (Bar=100 nm). Particles were cleaned using multiple rounds of classifications. The final map was reported at 3.5 Å according to GSFSC criterion. (B) The sharpened map of URAT1^{UA} colored according to the estimated value of local resolution. (C) Angular sampling of the final reconstruction, illustrating the orientation distribution of particles in the final reconstruction of URAT1^{UA}. (D) *Fourier* shell correlations (FSC) curves of the URAT1^{UA} and its atomic model. The unmasked (blue) and masked (red) FSC curves were calculated between two independently refined half-maps before and after map sharpening. The model-vs-map FSC (black) was calculated between the full map and the atomic model. (E) The cryo-EM densities and atomic models of TM1-12 of URAT1^{UA}.



386

387 Figure S4. Cryo-EM data analysis of the URAT1^{BEN}.

388 (A) Flowcharts for cryo-EM data processing of URAT bound with benzbromarone. A representative raw
 389 cryo-EM micrograph is shown here (Bar=100 nm). (B) The sharpened map of URAT1^{BEN} colored according
 390 to the estimated value of local resolution. (C) Angular sampling of the final reconstruction, illustrating the
 391 orientation distribution of particles in the final reconstruction of URAT1^{BEN}. (D) Fourier shell correlations
 392 (FSC) curves of the URAT1^{BEN} and its atomic model. The unmasked (blue) and masked (red) FSC curves
 393 were calculated between two independently refined half-maps before and after map sharpening. The
 394 model-vs-map FSC (black) was calculated between the full map and the atomic model. (E) The cryo-EM
 395 densities and atomic models of TM1-12 of URAT1^{BEN}.

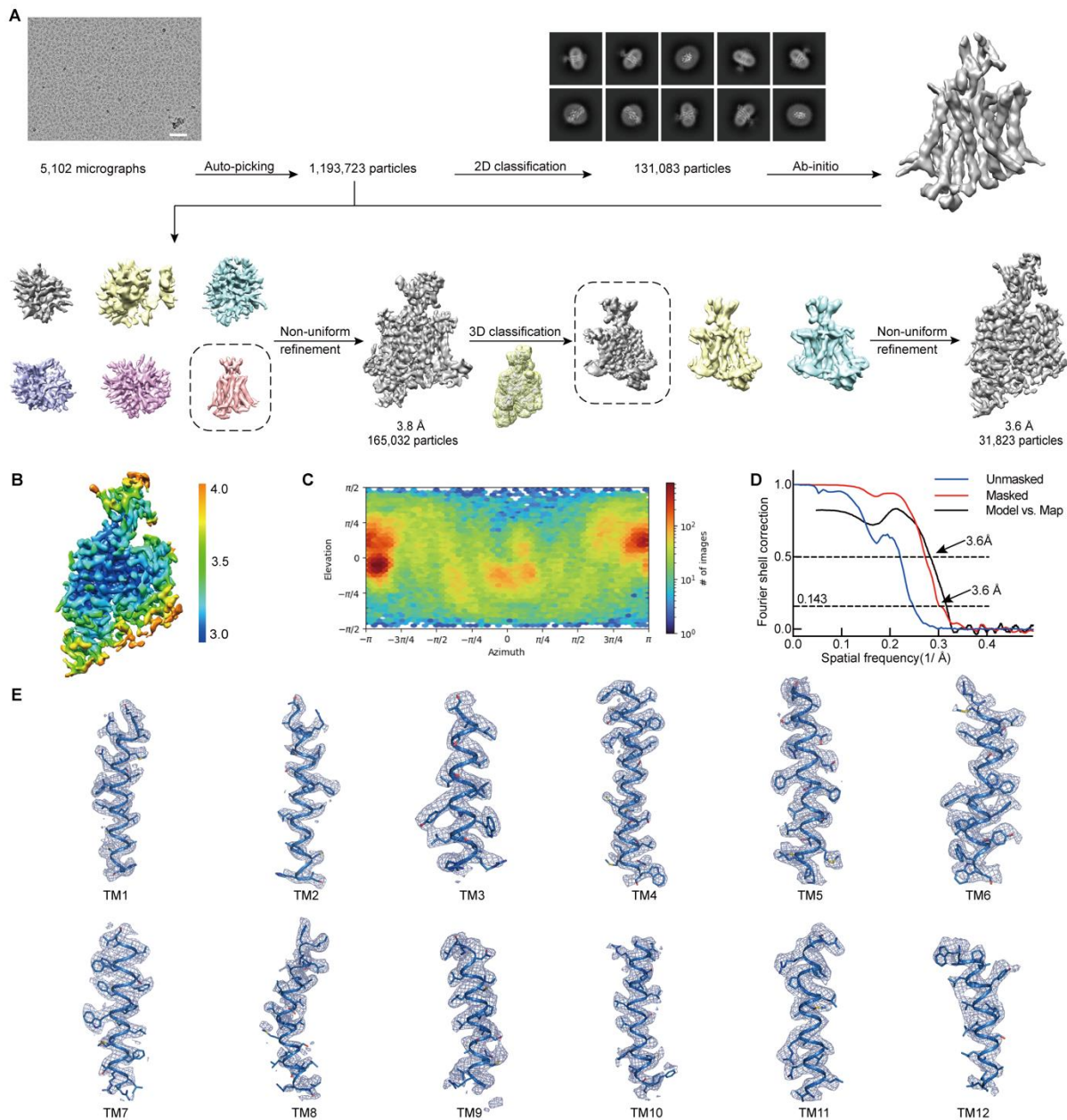


Figure S5. Cryo-EM data analysis of the URAT1^{LES}.

(A) Flowcharts for cryo-EM data processing of URAT bound with lesinurad. A representative raw cryo-EM micrograph is shown here (Bar=100 nm). (B) The sharpened map of URAT1^{LES} colored according to the estimated value of local resolution. (C) Angular sampling of the final reconstruction, illustrating the orientation distribution of particles in the final reconstruction of URAT1^{LES}. (D) Fourier shell correlations (FSC) curves of the URAT1^{LES} and its atomic model. The unmasked (blue) and masked (red) FSC curves were calculated between two independently refined half-maps before and after map sharpening. The model-vs-map FSC (black) was calculated between the full map and the atomic model. (E) The cryo-EM densities and atomic models of TM1-12 of URAT1^{LES}.

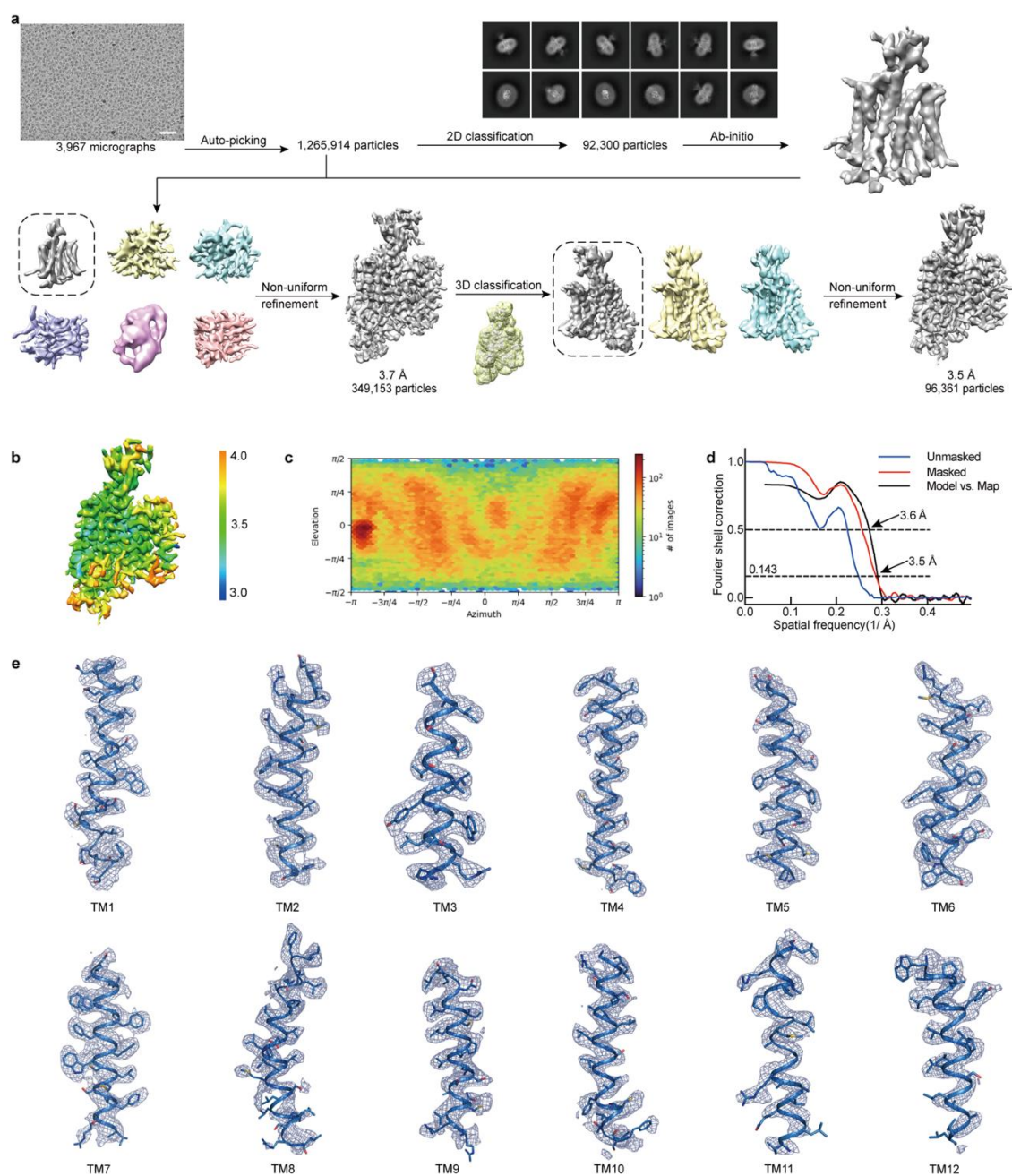


Figure S6. Cryo-EM data analysis of the URAT1^{VER}.

(A) Flowcharts for cryo-EM data processing of URAT bound with verinurad. A representative raw cryo-EM micrograph is shown here (Bar=100 nm). (B) The sharpened map of URAT1^{VER} colored according to the estimated value of local resolution. (C) Angular sampling of the final reconstruction, illustrating the orientation distribution of particles in the final reconstruction of URAT1^{VER}. (D) Fourier shell correlations (FSC) curves of the URAT1^{VER} and its atomic model. The unmasked (blue) and masked (red) FSC curves were calculated between two independently refined half-maps before and after map sharpening. The model-vs-map FSC (black) was calculated between the full map and the atomic model. (E) The cryo-EM densities and atomic models of TM1-12 of URAT1^{VER}.

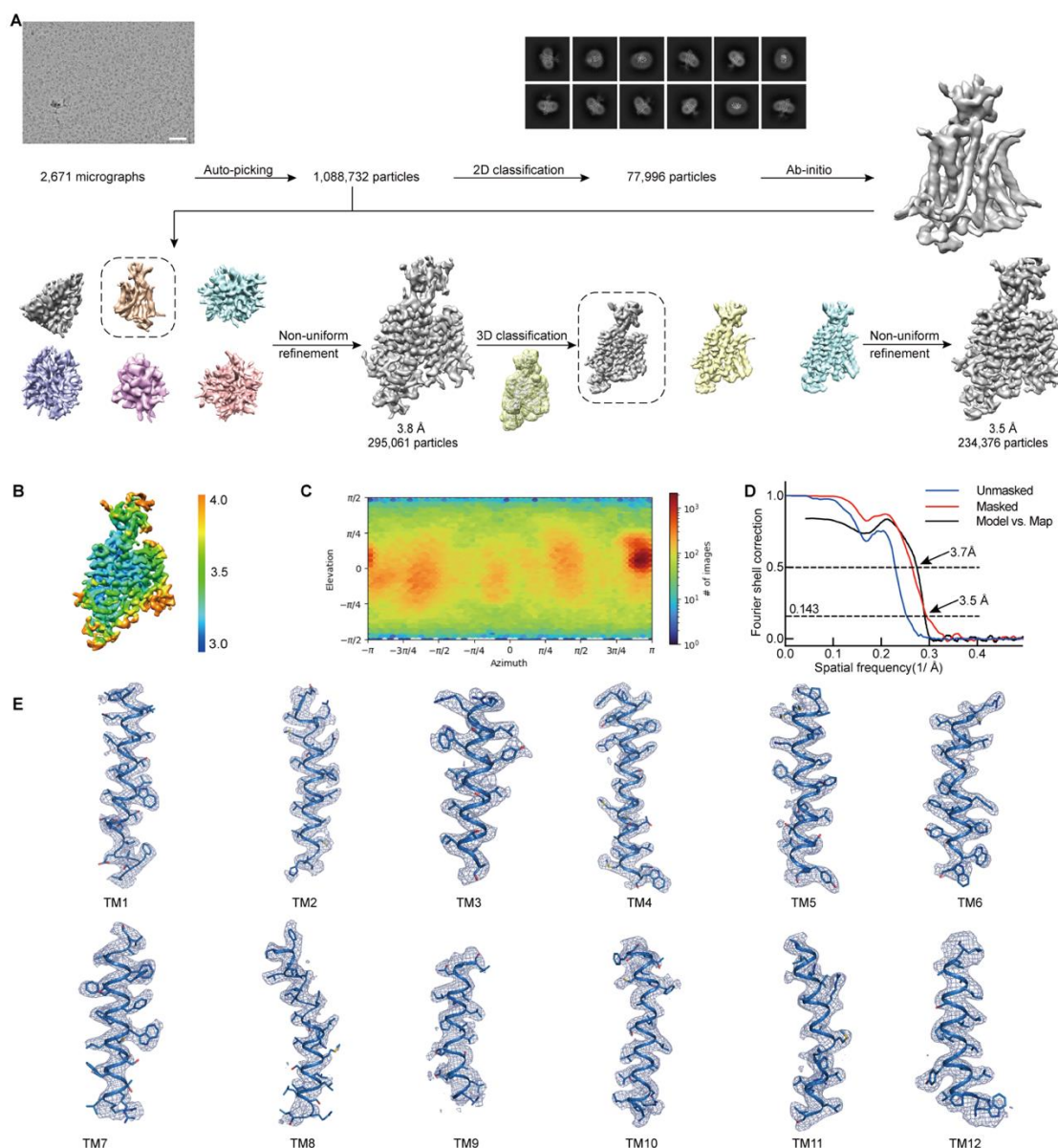


Figure S7. Cryo-EM data analysis of the URAT1^{SPZ}.

(A) Flowcharts for cryo-EM data processing of URAT bound with sulfipyrazone. A representative raw cryo-EM micrograph is shown here (Bar=100 nm). (B) The sharpened map of URAT1^{SPZ} colored according to the estimated value of local resolution. (C) Angular sampling of the final reconstruction, illustrating the orientation distribution of particles in the final reconstruction of URAT1^{SPZ}. (D) Fourier shell correlations (FSC) curves of the URAT1^{SPZ} and its atomic model. The unmasked (blue) and masked (red) FSC curves were calculated between two independently refined half-maps before and after map sharpening. The model-vs-map FSC (black) was calculated between the full map and the atomic model. (E) The cryo-EM densities and atomic models of TM1-12 of URAT1^{SPZ}.

I would like to share an additional comment that is not critical to the decision on the manuscript. The authors prepared the cryo-EM samples by supplementing the buffers with each drug prior to SEC. However, the EM density maps show several unassigned, strong features in the binding pocket, which appear to resemble elements of the other drugs. Is it possible that the same column was used for all samples and was not properly cleaned before running subsequent samples? The comparison of the EM density maps

suggests there may be traces of different molecules in the binding pocket. Please consider checking this.

Reply: Thank you for your comment. In fact, these complexes are prepared using a single column. However, we rinse the column with 2-5 column volumes of drug-free basic buffer before and after each use. This procedure is generally effective in removing any residual material. Additionally, the binding affinities of these drugs to URAT1 are in the micromolar (μM) range. During cryo-EM sample preparation, we typically incubate URAT1 with a specific inhibitor at millimolar (mM) concentrations to ensure full occupancy of this inhibitor bound with URAT1. In this scenario, we do not believe that a low concentration of residual inhibitor from the previous preparation would significantly affect the binding mode of URAT1 in the presence of a high concentration of specific inhibitor. Instead, we speculated that the unassigned feature in the binding pocket were derived from noise.

Referee #2 (Remarks to the Author):

The submitted manuscript, "Urate reabsorption and inhibitory mechanisms of URAT1," provides data on high resolution structures of rat URAT1 (rURAT1), with human residues at positions 35 and 365 to increase substrate and inhibitor binding affinity, complexed to its substrate urate as well as URAT1 inhibitors benzbromarone, lesinurad, verinurad, and sulfapyrazone. This work is relevant as it is important to know the structure of this pathologically- and pharmacologically-relevant target for hyperuricemia and gout. It is commendable that the authors successfully obtained the structure of rURAT1 and performed some validation studies on the structure. However, the manuscript requires revisions to be acceptable for publication, namely further validation studies, as outlined below.

Reply: We are grateful to the reviewer for acknowledging our work and providing specific suggestions to improve our manuscript.

1) The residues identified as interacting with urate and the inhibitors need to be verified using mutagenesis and activity assays, as was done in the recently published report on the structure of human URAT1 (DOI:10.1038/s41422-024-01023-1). For the inhibitors this was performed for residues 35 and 477, but this needs to be done for other interacting residues. Since the binding sites for urate and the inhibitors overlap, mutations in the binding site will affect substrate transport activity which can preclude reliably measurement of these assays. However, conservative mutations can be performed in many of these residues to preserve activity, as has been shown previously (DOI:10.1038/s41422-024-01023-1 and DOI:10.1038/s41598-017-00706-7):

1a) For urate, F365A mutation should be analyzed (all other interacting residues were analyzed in this way);

Reply: We appreciate the reviewer's suggestion. We constructed the F365A mutant and analyzed its transport activity. The results indicate that the activity of F365A is significantly reduced, suggesting its crucial role in the binding and transport of urate (Fig.12*).

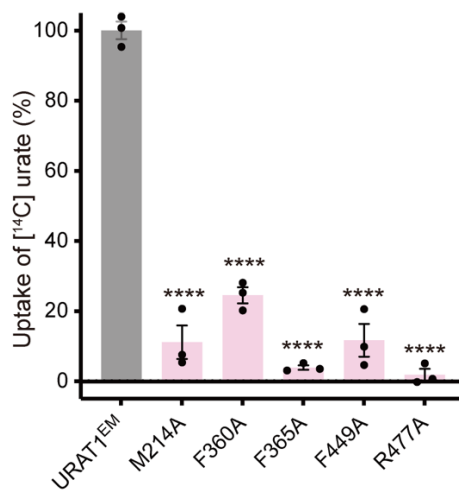


Figure 12*. Comparative evaluation of the [¹⁴C] urate transportation activity of URAT1 mutants in relation to the URAT1^{EM}. Data are mean $\pm$ s.e.m. from three independent assays, each with one measurement (n = 3).

1b) For benzbromarone, the phenylalanine residues at 241, 360, 364, 365, and 449 should all be individually converted to tyrosine, Q245 should be converted to the human residue histidine and/or alanine, Q473 should be converted to histidine or alanine, M214 should be converted to glutamine to disrupt hydrophobic interactions, and T32 should be converted to alanine to disrupt hydrogen bonding;

Reply: We appreciate the reviewer's comment. Following your suggestion, we constructed the mutations based on rURAT1^{EM}, including M214Q, F241Y, Q245H, Q245A, F360Y, F364Y, F365Y, F449Y, Q473A, and Q473H. T32 not directly interact with benzbromarone; therefore, we did not design a mutation at this site. The Q245H and Q245A mutants exhibited no transport activity, and we could not test if these mutations influence benzbromarone binding (Figure 13*A).

The residue S35 is located close to the benzofuran group of benzbromarone. Substituting it with glutamine (S35Q), which has a larger side chain, may create steric hindrance with benzbromarone, resulting in a significant decrease in affinity. The side chain of F365 points towards the benzofuran group. Moreover, the F365Y mutation significantly reduces the inhibitory potency of benzbromarone, likely due to the introduction of the hydroxyl group of tyrosine directly clashing with benzofuran group of benzbromarone. Although the residue Q473 is not directly involved in benzbromarone binding, mutating Q473 to histidine (Q473H) exhibits a slight reduction in its binding affinity, presumably due to introduced steric hindrance resulting from larger volume of histidine side chain. Furthermore, the R477 residue forms electrostatic interactions with benzbromarone, which are crucial for its binding. Neutralizing R477 by mutating it to asparagine (R477N) significantly reduces the affinity for benzbromarone.

We also tested the effect of mutations M214Q, F241Y, F360Y, F364Y, and F449Y on the inhibition by benzbromarone. It turns out that the IC₅₀ values for these mutants show no significant difference compared to URAT1^{EM}, likely because these residues may not be crucial for benzbromarone binding, or the alterations caused by these mutations do not significantly affect benzbromarone binding.

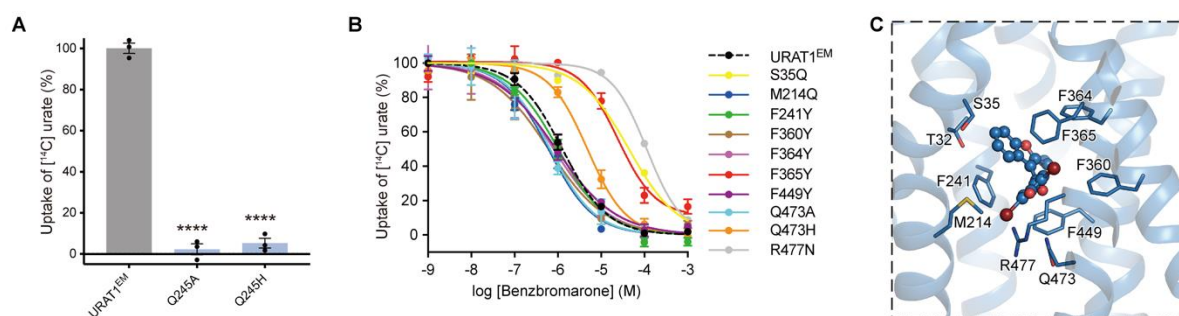


Figure 13*. (A) Comparative evaluation of the $[^{14}\text{C}]$ urate transportation activity of URAT1 mutants in relation to the URAT1^{EM}. Data are mean $\pm$ s.e.m. from three independent assays, each with one measurement ($n = 3$). (B) Inhibition of $[^{14}\text{C}]$ -urate transport by benzbromarone in URAT1^{EM} and its mutants. Data are presented as normalized mean $\pm$ s.e.m. from three biologically independent assays ($n = 3$). IC₅₀ values are calculated by fitting into a nonlinear regression model. (C) Detailed view of the benzbromarone binding pocket. Surrounding residues are represented as sticks while benzbromarone were shown as sphere and sticks.

These findings are now incorporated into Figure 2 of the manuscript, along with additional discussion to contextualize their relevance. Specifically, we have added the following in line 171: “The residue S35 is located close to the benzofuran group of benzbromarone. Substituting it with glutamine (S35Q), which has a larger side chain, may create steric hindrance with benzbromarone, resulting in a significant decrease in affinity (Figure 2D). The side chain of F365 points towards the benzofuran group. Moreover, the F365Y mutation significantly reduces the inhibitory potency of benzbromarone, likely due to the introduction of the hydroxyl group of tyrosine directly clashing with benzofuran group of benzbromarone. These observations are consistent with previous findings⁸. Although the residue Q473 is not directly involved in benzbromarone binding, mutating Q473 to histidine (Q473H) exhibits a slight reduction in its binding affinity, presumably due to introduced steric hindrance resulting from larger volume of histidine side chain. Furthermore, the R477 residue forms electrostatic interactions with benzbromarone, which are crucial for its binding. Neutralizing R477 by mutating it to asparagine (R477N) significantly reduces the affinity for benzbromarone (Figure 2D). The molecular dynamics simulations revealed that both the protein and ligand reached a stable state over the course of the simulation. The average Root-mean-square-deviations (R.M.S.D.) values for the protein and ligand remained below 4.0 Å and 2.0 Å, respectively, indicating minimal conformational fluctuations and high stability of the system (Figure S11C). These findings further support the binding mode of benzbromarone.”.

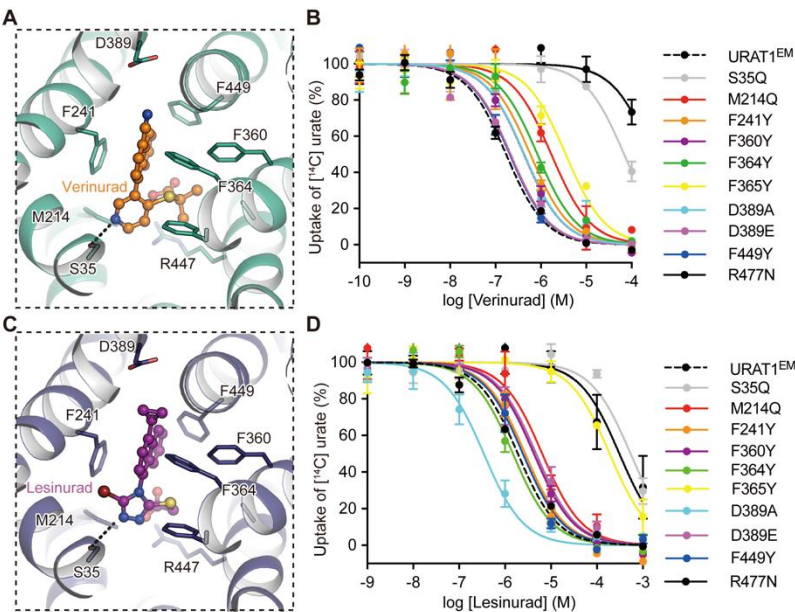
1c) For lesinurad and verinurad, the phenylalanine residues at 241, 360, 364, 365, and 449 should all be individually converted to tyrosine, M214 should be converted to glutamine to disrupt hydrophobic interactions, and importantly D389 should be converted to glutamate or alanine to verify that this residue enhances the affinity to verinurad relative to lesinurad;

Reply: We appreciate your suggestion and have constructed a series of point mutants, including S35Q, M214Q, F241Y, F360Y, F364Y, F365Y, D389A, D389E, F449Y, and R477N. We measured their IC₅₀ values to evaluate the impact of these mutations on the binding of lesinurad and verinurad.

According to our structure, the S35 residue forms a hydrogen bond with lesinurad and verinurad. Substituting S35 with glutamine (S35Q), which has a bulkier side chain, disrupted the hydrogen bond and introduced steric hindrance with benzbromarone, resulting in a significant reduction in binding affinity. The R477 residue stabilizes inhibitor binding through electrostatic interactions. The R477N mutation disrupted

these interactions, leading to a pronounced decrease in affinity and supporting the important role of R477 in stabilizing inhibitor binding. Several hydrophobic residues, including M214, F241, F360, F364, F365, and F449, contribute to establishing a hydrophobic environment for lesinurad and verinurad binding. Mutations such as M214Q, F241Y, F360Y, F364Y, F365Y, and F449Y lead to varying degrees of reduced affinity. Some reductions were modest, likely because the alterations caused by these mutations are not significant enough to affect drug binding.

The D389 residue may play nonequivalent roles in lesinurad and verinurad binding. It stabilizes verinurad binding through electrostatic interaction with the positively charged cyano group. Substituting aspartate with the negatively charged glutamate (D389E) does not affect the binding affinity of verinurad. In contrast, neutralizing D389 (D389A) causes a substantial reduction in verinurad binding affinity. These observations suggest that the electrostatic interaction between verinurad and D389 is important for its binding. Unlike the positively charged cyano group in verinurad, the cyclopropyl group at the equivalent position in lesinurad is hydrophobic. The hydrophilic D389 residue may not be conducive to lesinurad binding due to an energetically unfavorable hydrophobic mismatch. The D389E mutation results in a significant decrease in lesinurad binding affinity, likely because the hydrophobic mismatch is strengthened as the cyclopropyl group and the side chain of E389 come closer. Moreover, substituting D389 with alanine (D389A) reduces this hydrophobic mismatch, thus improving lesinurad binding affinity.



547

Figure 14*. (A and C) Detailed view of the verinurad (A) and lesinurad (C) binding pocket. Surrounding residues are represented as sticks while benzbromarone were shown as sphere and sticks. (B and D) Inhibition of ¹⁴C-urate transport by verinurad (B) and lesinurad (D) in URAT1^{EM} and its mutants. Data are presented as normalized mean ± s.e.m. from three biologically independent assays (n = 3). IC₅₀ values are calculated by fitting into a nonlinear regression model.

We have incorporated the data in the revised Figure 3 and discussed these observations in the manuscript. In line 250, it reads “To validate the binding pocket of lesinurad and verinurad, we designed mutations to assess their effects on binding of these two drugs. We found that the S35Q and R477N mutations significantly reduced binding affinity, supporting the important role of these residues in lesinurad and verinurad binding. Mutations such as M214Q, F360Y, F365Y, and M466Q disrupted hydrophobic

interactions, resulting in varying degrees of reduced binding affinity. These observations highlight the importance of these residue in establishing a hydrophobic environment to accommodate inhibitors (Figure 3E). Mutations at position D389 exhibit different effects on lesinurad and verinurad, as the D389 residue may play nonequivalent roles in their binding. It stabilizes verinurad binding through electrostatic interaction with the positively charged cyano group. Substituting aspartate with the negatively charged glutamate (D389E) does not affect the binding affinity of verinurad. In contrast, neutralizing D389 (D389A) causes a substantial reduction in verinurad binding affinity. These observations suggest that the electrostatic interaction between verinurad and D389 is important for its binding. Unlike the positively charged cyano group in verinurad, the cyclopropyl group at the equivalent position in lesinurad is hydrophobic. The hydrophilic D389 residue may not be conducive to lesinurad binding due to an energetically unfavorable hydrophobic mismatch. The D389E mutation results in a significant decrease in lesinurad binding affinity, likely because the hydrophobic mismatch is strengthened as the cyclopropyl group and the side chain of D389 come closer. Moreover, substituting D389 with alanine (D389A) reduces this hydrophobic mismatch, thus improving lesinurad binding affinity.”.

1d) For sulfapyrazone, the phenylalanine residues at 241, 360, 364, 365, and 449 should all be individually converted to tyrosine, W357 should be converted to tyrosine (or alanine is the transporter is still active for substrate transport), N237 should be converted to alanine to disrupt the hydrogen bond, and methionines at 215, 222, 234, and 466 should all be individually converted to glutamine to disrupt hydrophobic interactions;

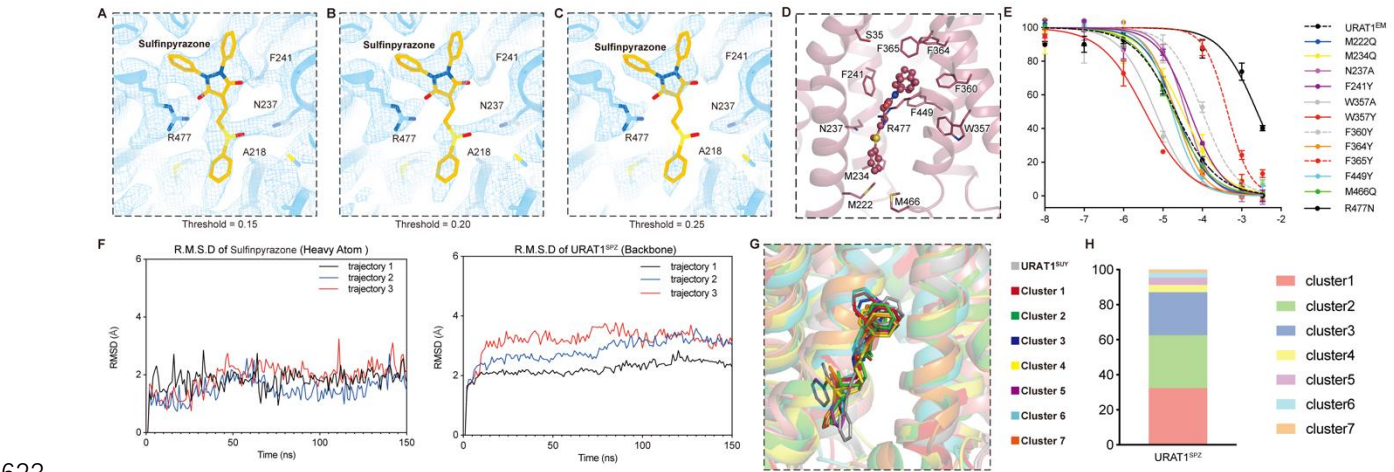
Reply: We have generated a series of point mutants, including M222Q, M234Q, N237A, F241Y, W357A, W357Y, F360Y, F364Y, F365Y, F449Y, M466Q, and R477N, to assess their impact on sulfapyrazone binding by measuring their IC50 values.

Residues F241, F360, F364, F365, and F449 constitute the binding pocket of sulfapyrazone and may stabilize the diphenylpyrazolidine group of sulfapyrazone deep within the pocket. Mutations at F360 and F365, where phenylalanine is replaced by tyrosine (F360Y and F365Y), led to a significant reduction in inhibitory potency (Figure 15*E). This decrease is likely due to the altered hydrophobicity of the binding pocket or introduced steric hindrance, disrupting the optimal binding conformation. Similarly, the F241Y mutation also reduced sulfapyrazone's binding affinity. Furthermore, the R477N mutation, which disrupts the hydrogen bond with sulfapyrazone, also leads to a significant reduction in binding affinity. However, mutations at F364Y and F449Y exhibited minimal impact on sulfapyrazone's affinity, likely because the introduced hydroxyl group in these mutants does not significantly alter the binding pocket's hydrophobic character or the overall binding interaction with sulfapyrazone.

Residues M222, M234, and M466, positioned near the intracellular side and close to the benzenesulfinyl group, exhibited minimal effects on the inhibitory effect of sulfapyrazone when mutated to glutamine, suggesting that interactions between these residues and the benzenesulfinyl group are not critical. This finding is consistent with cryo-EM density (Figure 15*A-C) and MD results (Figure 15*F-H), which all indicate that the benzenesulfinyl group is less tightly bound. Residue N237, which forms a hydrogen bond with sulfapyrazone, exhibited a slight reduction in binding affinity when mutated to alanine, highlighting the significance of this interaction. Additionally, residue W357, located farther from the sulfapyrazone molecule, provides a hydrophobic environment that contributes to the stability of the binding pocket. When W357 was mutated to tyrosine or alanine, an enhancement in the binding affinity of sulfapyrazone was observed. This could be attributed to alterations in the protein backbone or side-chain interactions, which

600 may have modified the overall structure of the binding pocket, thus facilitating stronger interactions with
601 sulfinpyrazone.

602 We have incorporated the data in the revised Figure 4 and discussed these observations in the manuscript.
603 In line xxx, it reads: “To confirm the sulfinpyrazone binding pocket, we designed several mutations and
604 conducted [¹⁴C]-urate transport assays to determine their effects on IC₅₀ values. Mutations of F360 and
605 F365 to tyrosine (F360Y, F365Y) significantly reduced the inhibitory potency, likely due to changes in
606 hydrophobicity and steric hindrance introduced by the tyrosine hydroxyl group. Similarly, the F241Y
607 mutation also decreased the binding affinity of sulfinpyrazone. In contrast, mutations F364Y and F449Y
608 had minimal impact, likely because the introduced hydroxyl group in these mutants does not significantly
609 alter the binding pocket's hydrophobic character or the overall binding interaction with sulfinpyrazone. The
610 R477N mutation disrupted a critical hydrogen bond with sulfinpyrazone, significantly reducing binding
611 affinity. Residues M222, M234, and M466, located near the intracellular side close to the benzenesulfinyl
612 group, exhibited minimal effects on sulfinpyrazone's inhibitory effect when mutated to glutamine,
613 suggesting that interactions between these residues and the benzenesulfinyl group may not be critical,
614 which is consistent with observations that the benzenesulfinyl group is less tightly bound, as evidenced by
615 the cryo-EM map and MD simulations (Figure S14). Residue N237, which forms a hydrogen bond with
616 sulfinpyrazone, exhibited a slight reduction in binding affinity when mutated to alanine, highlighting the
617 significance of this interaction. Additionally, residue W357, located farther from the sulfinpyrazone molecule,
618 provides a hydrophobic environment that contributes to the stability and integrity of the binding pocket.
619 When W357 was mutated to tyrosine or alanine, the binding affinity of sulfinpyrazone was slightly
620 enhanced (Figure 4E). This could be attributed to alterations in the side-chain interactions and possibly in
621 the protein backbone, which may have modified the local environment of the binding pocket, leading to
622 stronger interactions with sulfinpyrazone.”.



624 Figure 15*. (A-C) The cryo-EM density of sulfinpyrazone in different threshold and surrounding residues is
625 shown as mesh while the structure of which are visualized as sticks. (D) Detailed view of the sulfinpyrazone
626 binding pocket of URAT1^{SPZ}. Surrounding residues are represented as sticks. (E) Inhibition of ¹⁴C-urate
627 transport by sulfinpyrazone for URAT1^{EM} and its mutants. Data are presented as normalized mean ± s.e.m.
628 from three biologically independent assays (n = 3). IC₅₀ values are calculated by fitting into a nonlinear
629 regression model. (F) R.M.S.D. plots for ligand and protein backbone during MD simulation. R.M.S.D. plots
630 were generated for both sulfinpyrazone and the protein backbone. The calculation of backbone R.M.S.D. for
631 URAT1^{SPZ} using residues 2 to 42, 132 to 324, and 347 to 515. The calculation of sulfinpyrazone R.M.S.D.

involved the heavy atoms of benzbromarone, using its initial structure as the reference. Results from three independent trajectories were distinguished by different colors. (G) Structural comparisons of initial poses of sulfinpyrazone with representative poses derived from clustering analysis. (H) Cluster populations obtained from clustering analysis. The clustering analysis was conducted for URAT1^{SPZ}, which involving three independent 150 ns trajectories.

1e) For the URAT1 conformational transition, Q149 or R487 should be mutated to disrupt the hydrogen bond.

Reply: We appreciate the reviewer's comment. Based on your suggestion, we constructed Q149A and R487A mutants to disrupt the hydrogen bond and found that both mutations effectively reduced the transport activity (Figure 16*). We have also included these results in the Supplementary Figure 15.

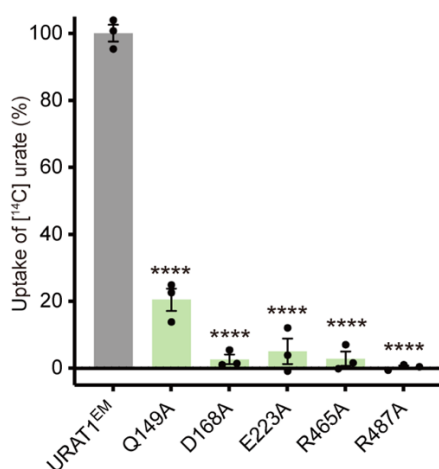


Figure 16*. Comparative evaluation of the [¹⁴C] urate transportation activity of URAT1 mutants in relation to the URAT1^{EM}. Data presented are mean $\pm$ s.e.m. from three independent assays, each with one measurement (n = 3).

The statement in lines 213-216 that verinurad...exhibits approximately 10-20 times higher affinity for URAT1 needs to be referenced, and also checked for accuracy. If the authors are referring to human URAT1 (hURAT1), then the affinity is over 1000 times higher for verinurad based on DOI:10.1038/s41598-017-00706-7 for verinurad and DOI:10.1038/srep34995 for lesinurad. For rat URAT1 (rURAT1) which is less relevant, the affinity is only two times higher for verinurad based on the same references.

Reply: Thank you for your insightful comment. We appreciate your attention to the accuracy of the affinity comparison between verinurad and lesinurad. As per the referenced studies ^{[1][2]}, the IC₅₀ of lesinurad for hURAT1 is 3.5 μ M, while the IC₅₀ of verinurad for hURAT1 is 0.025 μ M, indicating that verinurad has an affinity approximately 140 times higher than lesinurad. Based on this, we have revised the statement in lines 213-216 for clarity and accuracy. The updated statement now reads: "Verinurad, developed by modifying the molecular structure of lesinurad – replacing the cyclopropyl group with a cyano group and the acetic acid group with a methylpropanoic acid group – exhibits approximately 140 times higher affinity for hURAT1 and a longer half-life than lesinurad."

[1] Tan, P.K., Liu, S., Gunic, E. *et al.* Discovery and characterization of verinurad, a potent and specific inhibitor of URAT1 for the treatment of hyperuricemia and gout. *Sci Rep* 7, 665 (2017). <https://doi.org/10.1038/s41598-017-00706-7>

661 [2] Tan, P., Ostertag, T. & Miner, J. Mechanism of high affinity inhibition of the human urate transporter URAT1. *Sci Rep* 6,
662 34995 (2016). <https://doi.org/10.1038/srep34995>

663

664 Here are other minor comments that should be addressed:

665 1) The sentence in lines 260-262 needs supportive reference(s). What were the previous studies?

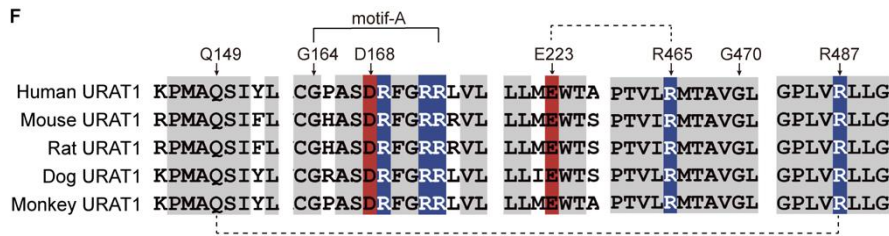
666 **Reply:** Thank you for this comment. A previous study has shown that the R487A mutation reduces the
667 maximum transport rate of human URAT1^[1], supporting its role in the conformational transition. We have
668 added this reference in the revised manuscript.

669 **Reference:**

670 [1] Zhao, Z. et al. Structural Insights into the Atomistic Mechanisms of Uric Acid Recognition and Translocation of Human
671 Urate Anion Transporter 1. *ACS Omega* 5, 33421-33432 (2020). <https://doi.org/10.1021/acsomega.0c05360>

672 2) Extended Data Figure 7f should include some features for clarification. G470 should be added since it
673 is mentioned in the results section in line 266, and the entire sequence of motif A should be shown (it is
674 currently shown up to residue 171, while the text in line 265 also mentions residues 172 and 173). Also,
675 the location of motif A should be indicated, as well as the locations of the salt bridges (lines connecting the
676 salt bridging residues should be shown).

677 **Reply:** We thank reviewer for the suggestion. We have updated Extended Data Figure 7f (now refer to
678 Supplementary Figure 15) to include all the requested features, including labeling G470 and the entire
679 sequence of motif A. The salt bridges are represented with dash lines connecting the relevant residues.
680 For your convenience, we have attached the revised Extended Data Figure 7f below.



681

682 Figure 17*. Extended Data Figure 7f. Sequence alignment of URAT1 from different species. The positions of
683 key residues involved in conformational changes are highlighted, including motif A and conserved salt bridges.
684 Lines indicate the connections between residues forming salt bridges

685 3) There are some typographical errors that should be corrected:

686 **Reply:** Thank you for your careful review of our manuscript. All typos have been corrected.

687 a) Figure 3 legend - add the "a" in verinurad which is missing,

688 **Reply:** Thank you for pointing out this typo. We have corrected it and checked throughout the manuscript
689 to ensure there are no similar errors.

690 b) In line 245, M466TM1 is incorrect and should be M466TM11,

691 **Reply:** Thank you for pointing out this typo. We have corrected it.

692 c) In line 266, G146TM5 is incorrect and should be G146TM2,

693 **Reply:** Thank you for pointing out this typo. We have corrected it.

694 d) In line 267, "while" should be removed.

695 **Reply:** Thank you for your suggestion. We have removed "while" as you suggested.

696 4) The sentence from lines 286-287 is supported by results that should be referenced,
697 doi:10.1093/molbev/msw116.

698 **Reply:** Thank you for this suggestion. We have added your suggested reference to the revised
699 manuscript.

700 5) The recently published report on human URAT1, DOI:10.1038/s41422-024-01023-1, should be
701 referenced in some way, indicating that the results in that report are in high agreement with the results in
702 this study, with differences that can be attributed to the use of human URAT1 in that study.

703 **Reply:** We appreciate the reviewer's comment. We have already cited the recently published report on
704 human URAT1 in the revised manuscript. In addition, we have compared substrates and inhibitors binding
705 with those in the report. We believe these comparisons provide further insights into the mechanistic
706 understanding of URAT1. Thank you again for your insightful comments.

707

Molecular Mechanism of Drug Inhibition of URAT1

Referees' comments:

Referee #1 (Remarks to the Author):

The manuscript by Hu et al. has improved considerably in the revised version. The additions and clarifications are extensive and largely performed to great satisfaction.

Reviewer #1 (Remarks to the Author):

The authors addressed the majority of my concerns in a comprehensive and scientifically relevant manner. The issue of the below-average quality of ligand resolvability has also been tackled through the single point mutations and the MD simulations. I want to emphasize that I am not an expert in MD and therefore will not comment on the quality or methodology of these results.

Reply: We sincerely thank the reviewer for their thorough evaluation and constructive feedback. We appreciate the reviewer's careful consideration and valuable insights, which have greatly contributed to improving our manuscript.

While the additional results improved the overall quality of the research, the manuscript text still does not meet the expected standard. For instance, the authors confuse readers regarding the R477 residue. They speculate that R477 may play a role in coordinating counter-ions. This is a bold hypothesis that must either be elaborated or, ideally, supported by further experimental evidence. This hypothesis appears inconsistent with subsequent statements such as: "Neutralizing R477 by mutating it to asparagine (R477N) significantly reduces the affinity for benzbromarone (Fig. 2d)" and "The R477N mutation disrupted a critical hydrogen bond with sulfinpyrazone, significantly reducing binding affinity." The R477 residue interacts with the moiety of the benzbromarone molecule, which I previously described as having doubtful resolvability. Additionally, the EM density map in SI Fig. 14B and C does not clearly demonstrate the critical hydrogen bond between R477 and sulfinpyrazone. A mutation that abolishes transport should not be mistaken for one that disrupts binding.

Reply: We are deeply grateful to the reviewer for thoroughly examining our manuscript and providing numerous detailed corrections. Our hypothesis that R477 might play a role in coordinating counter-ions was based on previous observations on OAT1 and OAT4, where the R466/R473 residues were found to be involved in chloride ion coordination^{[1][2]}. These residues are conserved as R477 in URAT1, leading us to speculate that R477 in URAT1 may serve a similar function in counter-ion coordination. However, we acknowledge that we currently lack additional evidence to support this hypothesis. To avoid potential confusion, we have removed this statement from the revised manuscript, in line with the editor's suggestion to focus on conclusion what can be demonstrated with data presented in the manuscript.

In our experiment, the R477N mutant retained significant and sufficient activity to reliably assess drug inhibition/binding on this mutant. In this context, we would like to clarify that the effect of the mutation on drug binding is not influenced by the reduced transport activity caused by the R477N mutation. Given that the Michaelis constant (K_m) of URAT1 is in the hundreds of micromolar range ^[3], while the urate concentration used in functional assays is only 5 μ M, the Cheng-Prusoff equation ^[4] predicts that IC_{50}

38 closely approximates K_i under these conditions. Therefore, changes in substrate binding should not
39 significantly impact IC_{50} measurements, and the observed decrease in inhibitor affinity upon R477N
40 mutation provides evidence that this residue is directly involved in inhibitor binding. For benzbromarone,
41 R477 forms an electrostatic interaction with the negatively charged bromophenol group. For sulfinpyrazone,
42 the side chain of R477 is positioned close to the oxygen atom on the diphenylpyrazolidine group, with a
43 distance of approximately 2.5 Å, suggesting the potential formation of a hydrogen bond. The R477N
44 mutation would disrupt these interactions critical for drug binding, and our functional assay further supports
45 these structural observations.

46 To enhance the accuracy and clarity of our descriptions regarding the interactions between R477 and the
47 inhibitors, we have carefully revised the relevant sections as follows: (i) In line 191, we now state:
48 “Furthermore, R477 forms electrostatic interactions with the bromophenol group of benzbromarone.
49 Mutation of R477 to asparagine (R477N), which disrupts this interaction, significantly reduces
50 benzbromarone binding affinity (Fig. 2f), aligning with structural observations and underscoring the critical
51 role of R477 in stabilizing benzbromarone binding.” (ii) In line 315, we clarify: “The R477N mutation disrupts
52 a potential hydrogen bond with the oxygen atom of the diphenylpyrazolidine group in sulfinpyrazone (Fig.
53 4d), resulting in a marked reduction in binding affinity and supporting R477 is important in sulfinpyrazone
54 binding.” We sincerely thank the reviewer for the insightful feedback, which has greatly improved the rigor
55 and coherence of our study.

56 [1] He, J. *et al.* Structural basis for the transport and substrate selection of human urate transporter 1. *Cell Rep* **43**, 114628
57 (2024). <https://doi.org/10.1016/j.celrep.2024.114628>

58 [2] Parker, J. L., Kato, T., Kuteyi, G., Sitsel, O. & Newstead, S. Molecular basis for selective uptake and elimination of organic
59 anions in the kidney by OAT1. *Nat Struct Mol Biol* **30**, 1786-1793 (2023). <https://doi.org/10.1038/s41594-023-01039-y>

60 [3] Tan PK, Farrar JE, Gaucher EA, Miner JN. Coevolution of URAT1 and Uricase during Primate Evolution: Implications
61 for Serum Urate Homeostasis and Gout. *Mol Biol Evol*. 2016;33(9):2193-2200. doi:10.1093/molbev/msw116

62 [4] Cheng Y, Prusoff WH. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes
63 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol*. 1973;22(23):3099-3108. doi:10.1016/0006-
64 2952(73)90196-2

65 **I.210–215:** These statements should appear at the beginning of the paragraph as descriptions of
66 experimental results, rather than as the paragraphs final conclusions.

67 **Reply:** Thank you for your suggestion. We have revised the manuscript accordingly. The description of
68 the density has been moved to the beginning of the paragraph, specifically to lines 167–169, to present
69 the experimental results first before drawing conclusions.

70 **SI Fig. 1 and 12:** The captions do not specify the source (or codes) of the sequences.

71 **Reply:** Thank you for your valuable suggestion. We have ensured that the source and accession numbers
72 of the sequences are explicitly stated in the figure legends. Specifically, in Figure S1, we have included
73 the statement: “The sequences were retrieved from the UniProt database, with the following accession
74 numbers: human (Q96S37), mouse (Q8CFZ5), rat (Q3ZAV1), dog (Canis lupus familiaris, I7HI71), and
75 monkey (Macaca mulatta, I7HH13).” Similarly, in Figure S12, we have specified: “The sequences were
76 retrieved from the UniProt database with the following accession numbers: URAT1 (Q96S37), OAT1
77 (Q4U2R8), OAT2 (Q9Y694), OAT3 (Q8TCC7), and OAT4 (Q9NSA0).”

I.113. The text mentions residue E118, whereas SI Fig. 1 shows D118.

Reply: Thank you for your careful review. This was a typographical error, and the correct residue is D118, as shown in SI Figure 1. We have now corrected it in the text. We truly appreciate your insightful comments and attention to detail.

I still believe that readers should be directly introduced to the quality of the EM density maps covering the ligands and surrounding residues. These panels should be included in the main figures rather than in the SI figures only.

Reply: Thank you for your insightful suggestion. We have now incorporated the EM density map panels into the main figures, specifically in Figure 1d, Figure 2a, Figure 3a, and Figure 4a, to provide a more direct visualization of ligand binding and surrounding residues. Furthermore, in accordance with the editor's recommendation, we have included the map-to-ligand fit alongside the cryo-EM workflow for each protein preparation, which is presented in Supplementary Figures 3-7. We greatly appreciate your thoughtful feedback, which has helped us improve the clarity and completeness of our manuscript.

Reviewer #2 (Remarks to the Author):

The revised manuscript, "Molecular Mechanism of Drug Inhibition of URAT1" by Yu et al, contains many improvements from the original manuscript to offer insights in the molecular mechanisms of urate substrate transport and inhibition by small molecule compounds/drugs of the uric acid transporter URAT1. The authors submitted a rebuttal that addresses all concerns by the reviewers to the original manuscript. Furthermore, the authors now correctly cite two other recent publications on the structure of human URAT1 and its interactions with urate and URAT1 inhibitors (doi:10.1038/s41422-024-01023-1 and doi:10.1016/j.celrep.2024.114628) and provide important comparisons of their findings to the findings of these other publications. From this reviewers point of view, this manuscript is now acceptable for publication but with a minor revision:

Reply: We sincerely thank the reviewer for the positive feedback and recommendation for publication. We appreciate your valuable comments and will address the minor revision accordingly.

In the rebuttal, the authors state that they analyzed Q245H and Q245A mutations and found them to be inactive (lines 479-480 of rebuttal), and show this data as Figure 13*A in the rebuttal. However, this figure, and any mention that these mutants were made and analyzed, is absent in the revised manuscript. Therefore, Figure 13*A needs to be added within Figure 2 of the manuscript, along with the text from lines 479-480 of the rebuttal stating that these mutants were inactive. Furthermore, it is important to note the discrepancy of this result, that these mutants are inactive, with findings from doi:10.1038/s41422-024-01023-1. That research article shows that residue 245 in human URAT1 which is histidine (as shown in Supplementary Figure 1) is involved in urate transport, so it is surprising that conversion of this residue to human histidine abolishes activity in rat URAT1. Furthermore, mutation of human URAT1 histidine 245 to alanine (H245A) in the other report reduces transport, but does not eliminate it as found in this study using rat URAT1. Hence, the authors should at least mention these discrepant findings with the other research article, and perhaps speculate on the differences in the results.

Reply: Thank you for the reviewer's insightful comments. In response to this concern, we have incorporated the data for the Q245H and Q245A mutants into Figure 2e of the revised manuscript and added a corresponding statement in the text, explicitly noting that these mutations resulted in virtually

abolished activity.

Regarding the observed discrepancies between human and rat URAT1, we note that residue 245 in human URAT1 is naturally histidine (H), and the H245A mutation retains partial transport activity, as reported in the referenced study^[1]. In contrast, residue 245 in rat URAT1 is glutamine (Q), and our data showed that mutations to histidine (Q245H) or alanine (Q245A) resulted in virtually abolished activity. Although the underlying reasons for these differences remain unclear, we propose several possible explanations. First, the local structural microenvironment around residue 245 may differ between the two species. For example, position 32, which is spatially adjacent to residue 245, is a cysteine (C) in human URAT1 but a threonine (T) in rat URAT1, potentially altering local conformational dynamics of surrounding residues that are important for urea binding. Moreover, residues 246, 249, and 250, located on the same helix and in close proximity to residue 245, also differ between human and rat URAT1. These variations may impact the interactions and cooperative movements of helices within this region, contributing to differences in transport activity across species. Second, the transport efficiency of human URAT1 is significantly lower than that of rat URAT1, with the maximal transport rate of human URAT1 being approximately one-ninth that of rat URAT1^[ref.2]. This difference suggests that the functional consequences of mutations may be more pronounced in rat URAT1, where the higher transport efficiency amplifies the impact of mutations, whereas the lower transport rate in human URAT1 may result in minimal changes.

We have incorporated this discussion into the revised manuscript at line 173: "To validate the interaction between Q245 and benzbromarone, we designed the Q245H and Q245A mutations, but found that their transport activity was virtually abolished (Fig. 2e). However, in human URAT1, histidine occupies position 245, and the H245A mutant retained transport activity⁴⁴. We speculate that this discrepancy in functional activity may arise from non-conserved neighboring residues altering local conformational dynamics, including perturbations in residue-residue interactions and cooperative helix motions within this structural domain (Supplementary Fig. 1). Additionally, the significantly higher transport rate of rat URAT1 may amplify the functional impact of these mutations⁴⁰. However, these hypotheses remain speculative and warrant further investigation. "

[1] Dai Y, Lee CH. Transport mechanism and structural pharmacology of human urate transporter URAT1. *Cell Res.* 2024;34(11):776-787. doi:10.1038/s41422-024-01023-1

[2] Tan PK, Farrar JE, Gaucher EA, Miner JN. Coevolution of URAT1 and Uricase during Primate Evolution: Implications for Serum Urate Homeostasis and Gout. *Mol Biol Evol.* 2016;33(9):2193-2200. doi:10.1093/molbev/msw116

Two other minor points:

1) in the paragraphs for lines 324-349, the referred Supplemental Figure needs to be changed from Supplemental Figure 13 to Supplemental Figure 15,

2) For the legend to Supplemental Figure 15, panel G needs to be mentioned and explained.

Reply: We thank the reviewer for identifying these minor issues. The reference to Supplemental Figure 13 in lines 324–349 has been corrected to Supplemental Figure 15, and the legend for Supplemental Figure 15 has been updated to include and explain panel G. We appreciate your careful review and valuable suggestions.

To summarize, according to this reviewer, this manuscript is acceptable for publication with the minor revisions outlined above. The authors have addressed all of concerns of this reviewer, which were mainly

158 validations of the structural biology results of URAT1EM using mutagenesis and functional assays. If it is
159 acceptable with the other reviewer who has focused on the structural biology aspects of the work, then it
160 is publishable.

161 **Reply:** We sincerely thank the reviewer for the positive evaluation and constructive feedback. We are glad
162 that the manuscript is considered acceptable for publication after minor revisions. We look forward to the
163 feedback from the other reviewer. Thank you again for your valuable insights.

164

Point-by-point response for

Molecular Mechanism of Drug Inhibition of URAT1

Referees' comments:

Reviewer #1 (Remarks to the Author):

Upon revision, the manuscript "Molecular Mechanism of Drug Inhibition of URAT1" by Yu et al. has improved and the authors have addressed my concerns. Similar to the other reviewer, I consider the manuscript acceptable for publication.

Reply: We sincerely thank Reviewer #1 for their positive feedback and are pleased that our revisions addressed their concerns. We appreciate their time and constructive input on our manuscript.

Reviewer #2 (Remarks to the Author):

Upon revision, the manuscript "Molecular Mechanism of Drug Inhibition of URAT1" by Yu et al. has improved and the authors have addressed my concerns. Similar to the other reviewer, I consider the manuscript acceptable for publication.

Reply: We sincerely appreciate your time and constructive feedback, which has greatly improved our manuscript. We are pleased that the revisions addressed your concerns, and we thank you for your positive evaluation.

The authors have presented another revision to the manuscript, and the prior comments of this reviewer have been adequately addressed. However, the manuscript still needs revision because it is unclear in many areas, specified below. Also, two more constructs must be analyzed to complete the validation of the structural findings.

Reply: We appreciate the reviewer's feedback and acknowledge the remaining concerns regarding clarity in certain sections. We will carefully revise the manuscript to address these issues and ensure all points are presented clearly and comprehensively. Regarding the two additional constructs (F241A and M215Q), we will provide discussions below separately.

For the results section that discusses Figure 1, it should be mentioned that F365 is one of the residues that was changed to from the rat residue tyrosine to human phenylalanine in the construct. And it also should be mentioned that this human phenylalanine residue confers a higher affinity to urate, with reference to #40 in the list of references. This should be stated after the sentence that ends in line 135.

Reply: Thank you for your valuable comment. As suggested, we have now added the following sentence in the Results section (after the sentence ending in line 136): " Notably, F365^{TM7} was changed from rat tyrosine to human phenylalanine in the construct, and this human phenylalanine residue confers higher affinity for urate⁴⁰."

Also, for Figure 1, F241A should be analyzed for measuring urate affinity, since F241 is mentioned in line 142.

Reply: We sincerely appreciate the reviewer's valuable suggestion regarding the analysis of the F241A mutant. We completely agree that measuring urate affinity for this mutant would provide important

functional insights to complement our structural findings. While we were unable to include these experiments in the current study due to time constraints, we would like to note that previous work on the human ortholog has shown the F241A mutant retains approximately 50% of wild-type transport activity[1]. This observation suggests that while F241 contributes to urate binding or transport, it may not be absolutely essential. To address this important point, we have added the following statement in the revised manuscript (Line 146): "This conformational flexibility of F241 suggests its involvement in urate binding, though detailed characterization through mutagenesis studies and quantitative affinity measurements will be needed to fully elucidate its functional role."

[1] Dai, Y., Lee, CH. Transport mechanism and structural pharmacology of human urate transporter URAT1. Cell Res 34, 776–787 (2024). <https://doi.org/10.1038/s41422-024-01023-1>

For the results section that discusses Figure 2, similar to the comment above, it should be mentioned that S35 was the other residue that was changed from rat residue asparagine (N) to serine in this construct, and that this change increases the inhibitory potency of benzbromarone with reference to #39. This should be stated after the sentence that ends in line 181.

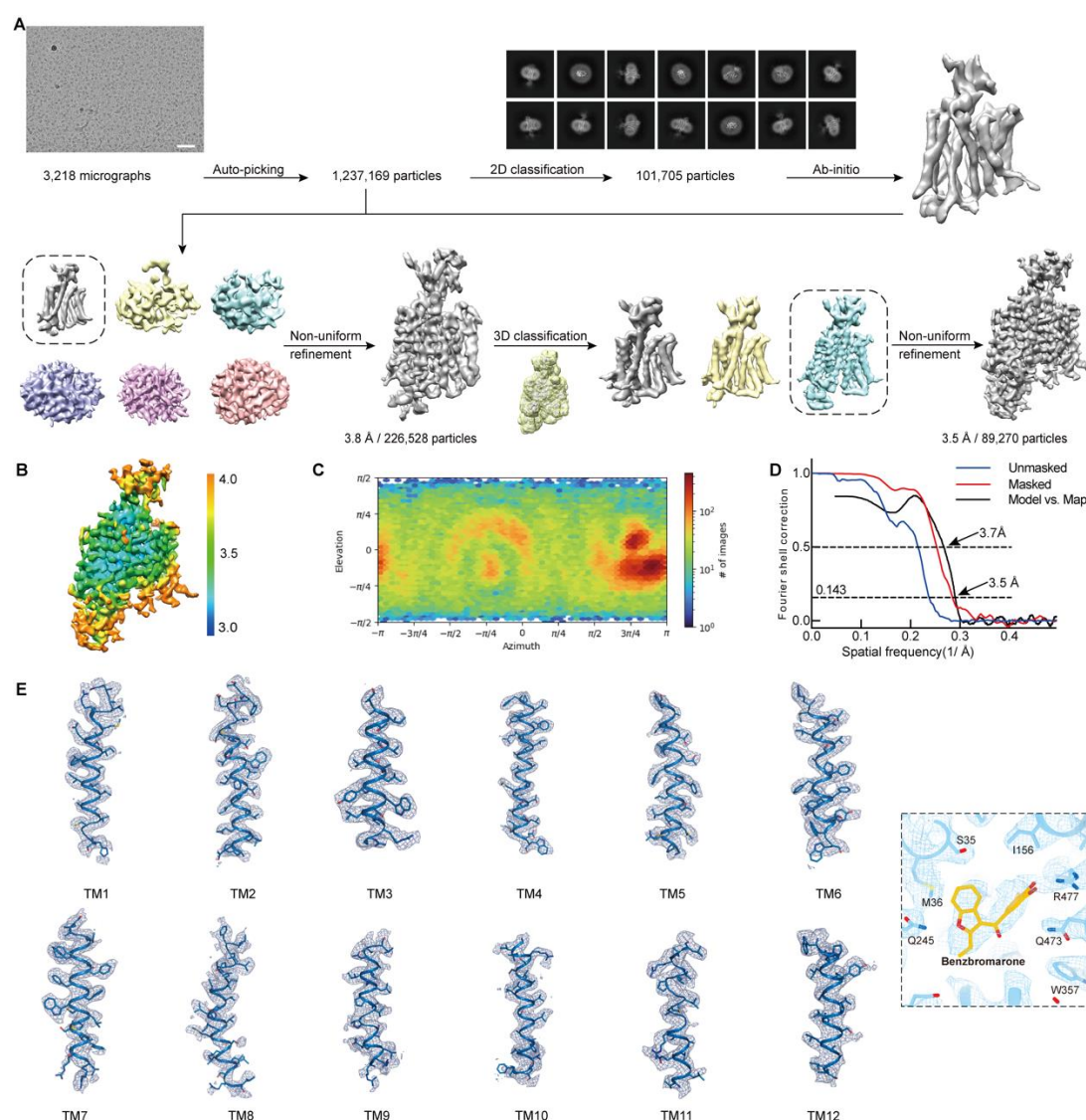
Reply: We sincerely appreciate the reviewer's insightful suggestion. As recommended, we have now explicitly stated in the Results section (in the discussion of Figure 2): "The residue S35 is located close to the benzofuran group of benzbromarone. Notably, S35 was changed from rat asparagine to human serine in the construct, and this human serine residue leads to increased inhibitory potency of benzbromarone³⁹".

For Figure 2, lines 168-170 state, "Benzbromarone is primarily coordinated by a relatively conserved aromatic cluster consisting of F241, F360, F364, F365, and F449." All of these residues were converted to tyrosine and the effects on benzbromarone inhibitory potency were measured in Figure 2f. However, only the results of F365Y are discussed in lines 184-187, while the findings of the other constructs are not discussed in this paragraph, probably because they had no effect. These results need to be discussed here with speculation on why they had no effect. I think that it is reasonable to speculate that while, "introduction of the hydroxyl group in F365Y directly clashes (clashing) with benzofuran group of benzbromarone," (lines 185-186) introduction of the hydroxyl group to the other residues does not sterically hinder binding of URAT1 to benzbromarone. In addition, M214Q is analyzed (panel e) but not discussed in the text, so this residue and the findings of this mutation need to be discussed and summarized.

Reply: Thank you for your valuable comment. Indeed, we initially did not discuss these residues in detail because their mutations had no observable effect on benzbromarone inhibition. Following your suggestion, we have now included a discussion of these results in the revised manuscript. The added text reads (line 194): "In contrast to F365Y, the introduction of a hydroxyl group at other aromatic residues (F241Y, F360Y, F364Y, and F449Y) did not affect benzbromarone's inhibitory potency, likely because these positions do not cause steric clashes with benzbromarone. M241 forms hydrophobic interactions with benzbromarone, and its mutation to glutamine does not significantly affect inhibitory potency, likely because this single substitution is not sufficient to effectively disrupt inhibitor binding."

Suppl. Fig 4E – key residues involved in the binding of benzbromarone should be labeled, as they are for the corresponding figures for urate and the other inhibitors.

Reply: Thank you for your helpful suggestion. We have now labeled the key benzbromarone-binding residues in Suppl. Fig. 4E. For your convenience, we have attached the revised Suppl. Fig. 4 below.



Supplementary Fig. 4. Cryo-EM data analysis of the URAT1^{BEN}.

(A) Flowcharts for cryo-EM data processing of URAT bound with benzbromarone. A representative raw cryo-EM micrograph is shown here (Bar=100 nm). (B) The sharpened map of URAT1^{BEN} colored according to the estimated value of local resolution. (C) Angular sampling of the final reconstruction, illustrating the orientation distribution of particles in the final reconstruction of URAT1^{BEN}. (D) Fourier shell correlations (FSC) curves of the URAT1^{BEN} and its atomic model. The unmasked (blue) and masked (red) FSC curves were calculated between two independently refined half-maps before and after map sharpening. The model-vs-map FSC (black) was calculated between the full map and the atomic model. (E) The cryo-EM densities and atomic models of TM1-12 and bound benzbromarone of URAT1^{BEN}.

Suppl. Fig 9. To clarify, the legend should list the other structures as human URAT1 and also include the source of those structures. Specifically, they should be labeled as such: human URAT1 (PDB:8WJQ, ref. 44), and human URAT1 (PDB:9B1J, ref. 44).

Reply: Thank you for your valuable suggestion. We have revised the legend for Supplementary Figure 10 as follows: “(A-B) Superposition of urate-bound URAT1 structures based on alignment of the N-domains. The structures are colored orange (URAT^{EM}, this study), pink (hURAT1, PDB:8WJQ, ref. 45), and blue (hURAT1, PDB:9B1J, ref. 44), respectively. The urate molecules are shown as sticks and spheres.”

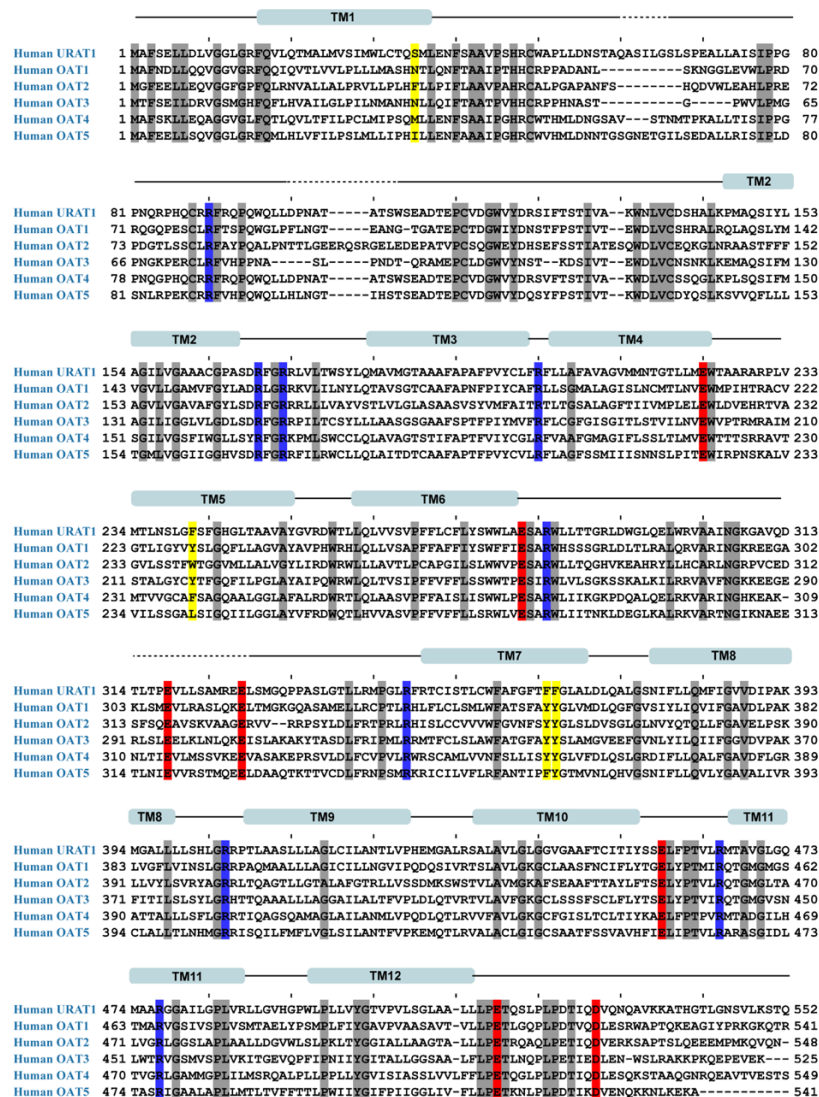
Suppl. Fig 11. A reference for the structure of human URAT1 PDB:9B1G should be provided, either in

92 panel A or in the legend. I think that this is reference 44, from Suppl. Fig 9. This reference should also be
93 displayed in lines 216 or 217 of the text.

94 **Reply:** We sincerely appreciate the reviewer's careful reading and constructive suggestions. We have
95 made the following modifications as recommended. For Supplementary Figure 11A, we have revised the
96 legend to: "The structure alignment of URAT1^{BEN} and dotinurad-bound human URAT1(PDB: 9B1G, ref.
97 44)". In the main text (line 227), we have updated the corresponding description to: "We compared the
98 structure of benzbromarone-bound URAT1 with the recent dotinurad-bound URAT1 structure⁴⁴, revealing
99 that both benzbromarone and dotinurad bind within the same pocket."

100 Suppl. Fig 12. Residues in URAT1 (35, 364, 365, 241) that are distinct from the other OATs and are involved
101 in benzbromarone binding, as described in the manuscript in lines 200-210, should be highlighted,
102 suggesting yellow highlights.

103 **Reply:** Thank you for the suggestion. We have now highlighted the URAT1-specific residues (Phe35,
104 Leu364, Val365, and Phe241) in yellow in Supplementary Figure 12 and the revised figure is provided
105 below for your reference.



107 Supplementary Fig. 12. Amino acid sequence alignment of human URAT1 and human OAT1-4

Sequence alignment of human URAT1, human OAT1, human OAT2, human OAT3 and human OAT4. Transmembrane helices are indicated above the alignment. Highly conserved amino acids are highlighted with gray shadows, while positively and negatively charged amino acids shaded in blue and red, respectively. Key URAT1-specific residues involved in benzbromarone binding are highlighted in yellow. The sequences were retrieved from the UniProt database, with the following accession numbers: human URAT1 (Q96S37), human OAT1(Q4U2R8), human OAT2(Q9Y694), human OAT3(Q8TCC7) and human OAT4 (Q9NSA0).

Suppl. Fig 13. A reference for the structure of human URAT1 PDB:9B1I should be provided (ref 44, I think), either in panel A or the figure legend. This reference should also be displayed in line 255 of the text.

Reply: Thank you for your valuable comment. We have updated the Suppl. Fig. 13SA legend: "The structure alignment of URAT1^{VER} and verinurad-bound human URAT1 (PDB: 9B1I, ref. 44)." and cited ref. 44 in the main text (line 266): "Comparison with the verinurad-bound hURAT1 structure (PDB: 9B1I) highlights minor differences in the orientation of the acene group and surrounding residue side chains⁴⁴ (Supplementary Fig. 13B)."

For Figure 3, the role of 9 residues in the affinity of lesinurad and verinurad is analyzed by mutagenesis (panels e and h). However, there is no discussion for some of these mutations in the text for this figure in lines 259-278. Specifically, these are F241Y, F364Y, and F449Y – the authors need to discuss the findings for these residues and speculate on their role on lesinurad and verinurad binding. Confusingly, there is discussion of findings for a M466Q mutation (line 263) but no results shown and also no indication of residue 466 in panels d, e, f, or g. The authors need to show the results in the figure, or omit M466Q from the text.

Reply: We sincerely appreciate the reviewer's insightful comments. As suggested, we have now included a discussion of the F241Y, F364Y, and F449Y mutations in the revised manuscript (line 277): "The F241Y, F364Y, and F449Y mutations do not significantly impair binding affinity, likely because the structural perturbations induced by these individual mutations are not substantial enough to disrupt ligand recognition." Regarding M466Q, we confirm this residue does not participate in direct inhibitor interactions and have consequently removed all related discussion to ensure consistency between figures and text.

The finding that D389 is important for verinurad binding affinity, and not important for lesinurad binding affinity, is important and noteworthy. If this residue has not been identified in other studies, then this should be further highlighted.

Reply: We sincerely thank the reviewer for highlighting the significance of D389's differential roles in verinurad versus lesinurad binding. As suggested, we have expanded the discussion (lines 405-410) to emphasize this finding: "Additionally, certain residues exhibit differential recognition of specific inhibitors. For instance, D389 shows dual functionality: it stabilizes verinurad binding through electrostatic interactions with its cyano group, while creating unfavorable hydrophobic clashes with lesinurad's cyclopropyl moiety. These findings provide a detailed interaction map of URAT1's binding pocket, offering clear molecular guidance for the design of targeted drugs against this transporter."

For Figure 4, M215 is shown (panel e) and mentioned as forming hydrophobic interactions with the benzenesulfinyl group of sulfapyrazone (line 306). However, no data is presented on the role of this residue. The authors should analyze an M215Q mutation and report the findings. The rest of the discussion of Figure 4 and all the mutations, however, is more complete than the discussions of Figures 2 and 3.

Reply: We thank the reviewer for the insightful suggestion. We fully agree that experimental validation of M215's role would strengthen our conclusions. As suggested in the previous round of revision, we did attempt to generate and test the M215Q mutant. However, the mutant protein showed very low expression levels, making it impossible to obtain reliable binding or functional data. To ensure accuracy, we have now modified the text in line 306 to clarify that the proposed interaction is based on structural modeling rather than experimental mutational data. Starting from the line 320, it reads "The benzenesulfinyl group forms hydrophobic interactions with M215^{TM4}, M222^{TM4}, M234^{TM5} and M466^{TM11} based on structural analysis (Fig. 4d)." .

Suppl. Fig 15. In line 151, "22" appears to be a typographical error and should be removed.

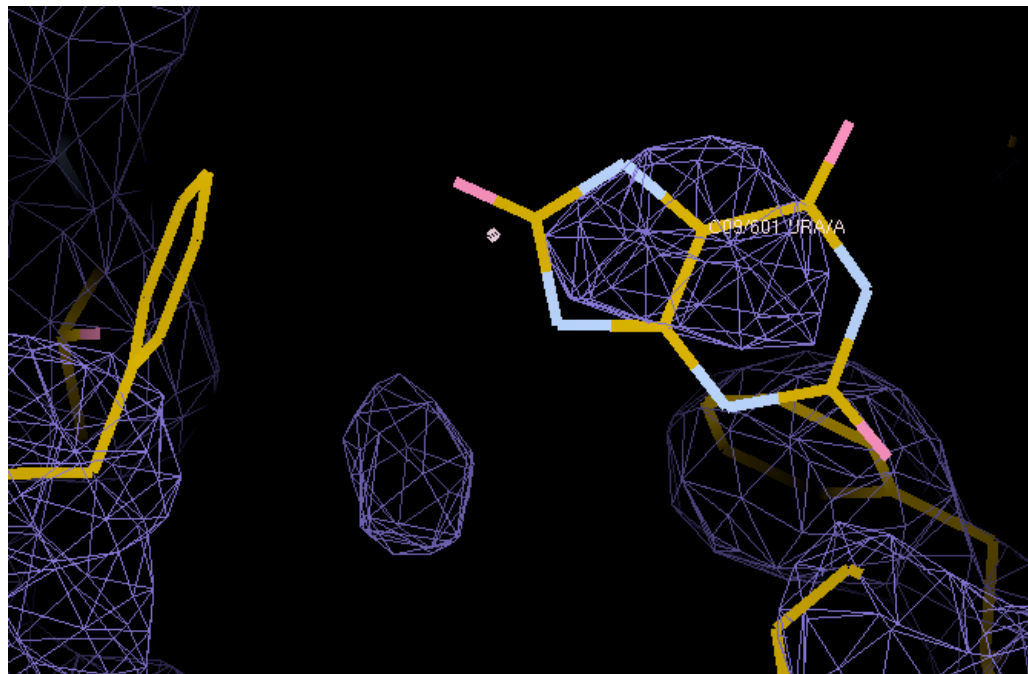
Reply: Thank you for catching this. We have removed "22" from as suggested.

Another observation by this reviewer is that the results show that URAT1 interacts with its substrate urate and inhibitors through a number of common residues. There are electrostatic interactions (or by hydrogen bonding) with R477, with a binding pocket that involves hydrophobic interactions with M214 (or M215 for sulfapyrazone), F241, F360, F364 (except for urate), F365, and F449. Conversion of these residues individually to alanine perturbs the transport activity of urate, showing that they are involved in the urate binding pocket. And conversion of many of these phenylalanines to tyrosine generally does not dramatically affect inhibitor affinity, showing the hydrophobic nature of the interaction, except for F365Y which is known to reduce affinity to urate and the inhibitors from prior studies. This could be added to the discussion to enhance the impact of the findings.

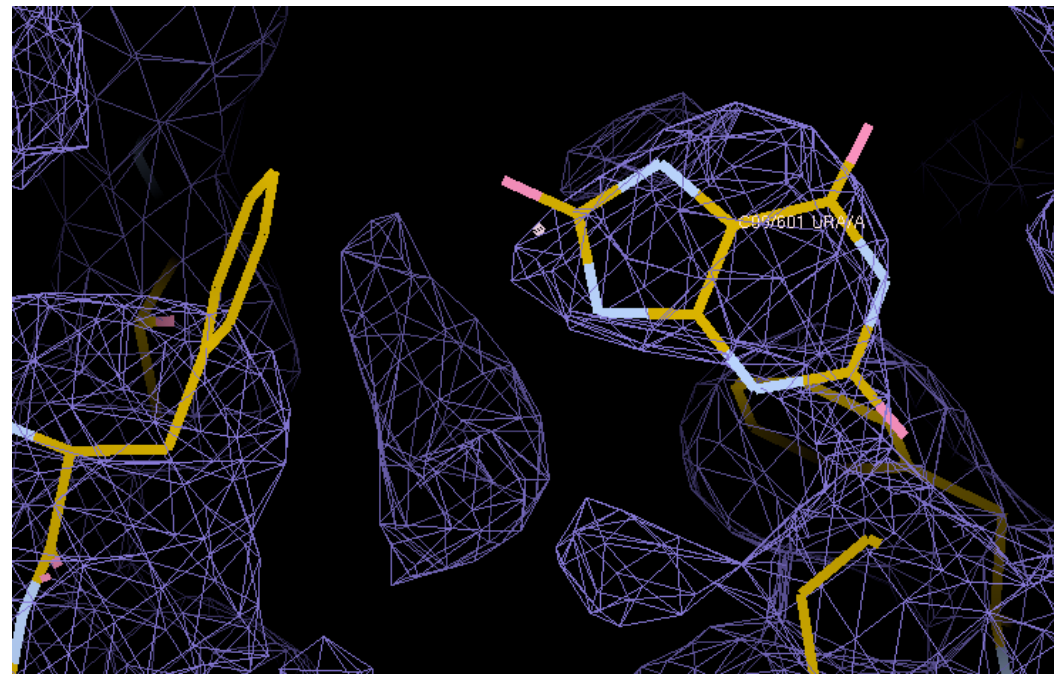
Reply: We sincerely appreciate the reviewer's insightful observation regarding the shared binding residues between urate and inhibitors. As suggested, we have added a dedicated discussion paragraph (lines 394-410) to highlight these findings: "Through systematic functional studies of these surrounding residues, we have elucidated the sophisticated recognition mechanism of URAT1's binding pocket. URAT1 recognizes its endogenous substrate urate and various inhibitors through a highly conserved binding site, where two major types of interactions work synergistically: R477 provides polar binding forces through hydrogen bonding and electrostatic interactions, while the hydrophobic cluster (M214, F241, F360, F364, F365, and F449) establishes a stable hydrophobic binding environment. Notably, alanine mutations of these residues significantly affect urate transport activity, confirming their collective role in maintaining the structural integrity of the binding pocket. Tyrosine mutation studies further revealed that most positions (F241/F360/F364/F449) maintain inhibitor binding, demonstrating the dominant role of hydrophobic interactions, while F365Y mutation uniquely reduces affinity for both urate and inhibitors. Additionally, certain residues exhibit differential recognition of specific inhibitors. For instance, D389 shows dual functionality: it stabilizes verinurad binding through electrostatic interactions with its cyano group, while creating unfavorable hydrophobic clashes with lesinurad's cyclopropyl moiety. These findings provide a detailed interaction map of URAT1's binding pocket, offering clear molecular guidance for the design of targeted drugs against this transporter."

Urate-bound

Treshhold 1

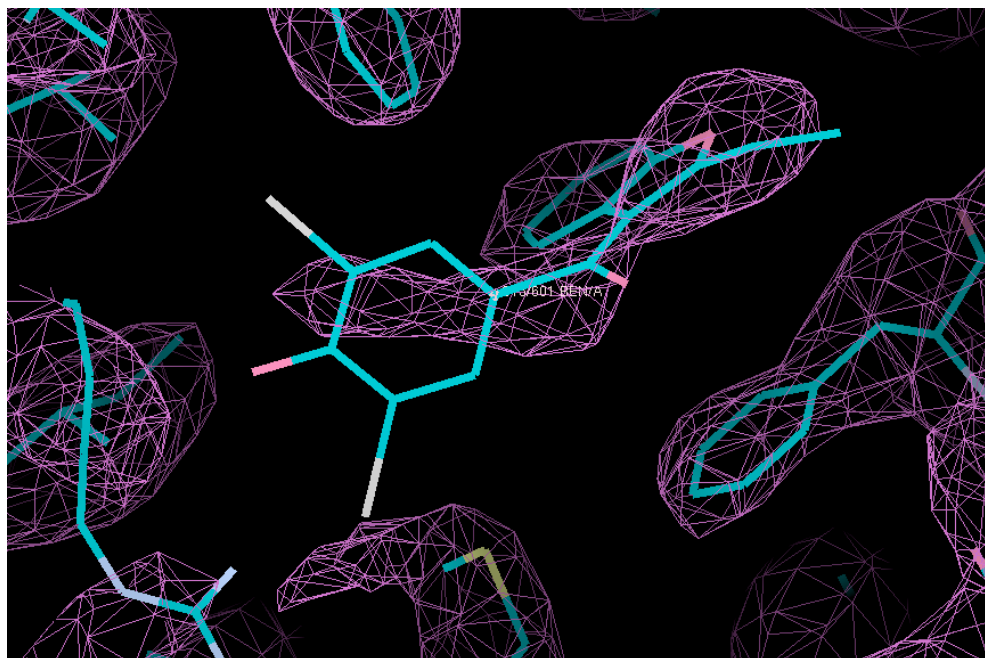


Treshhold 2

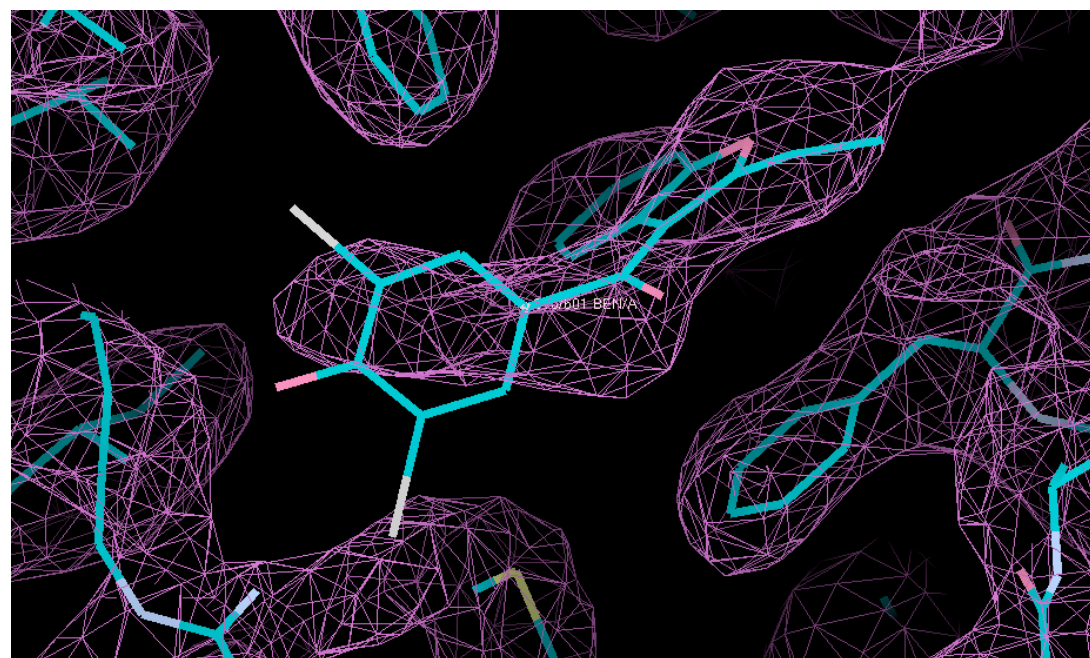


Benzbromarone-bound

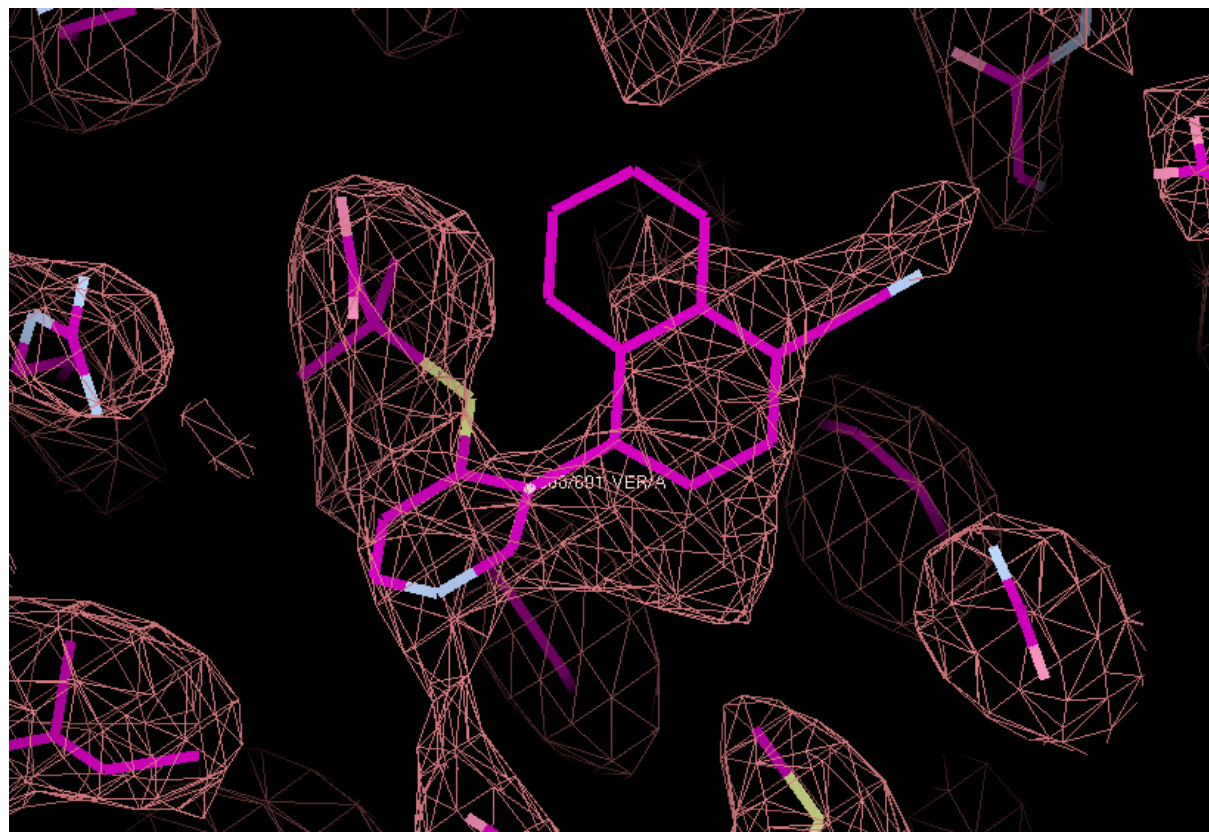
Treshhold 1

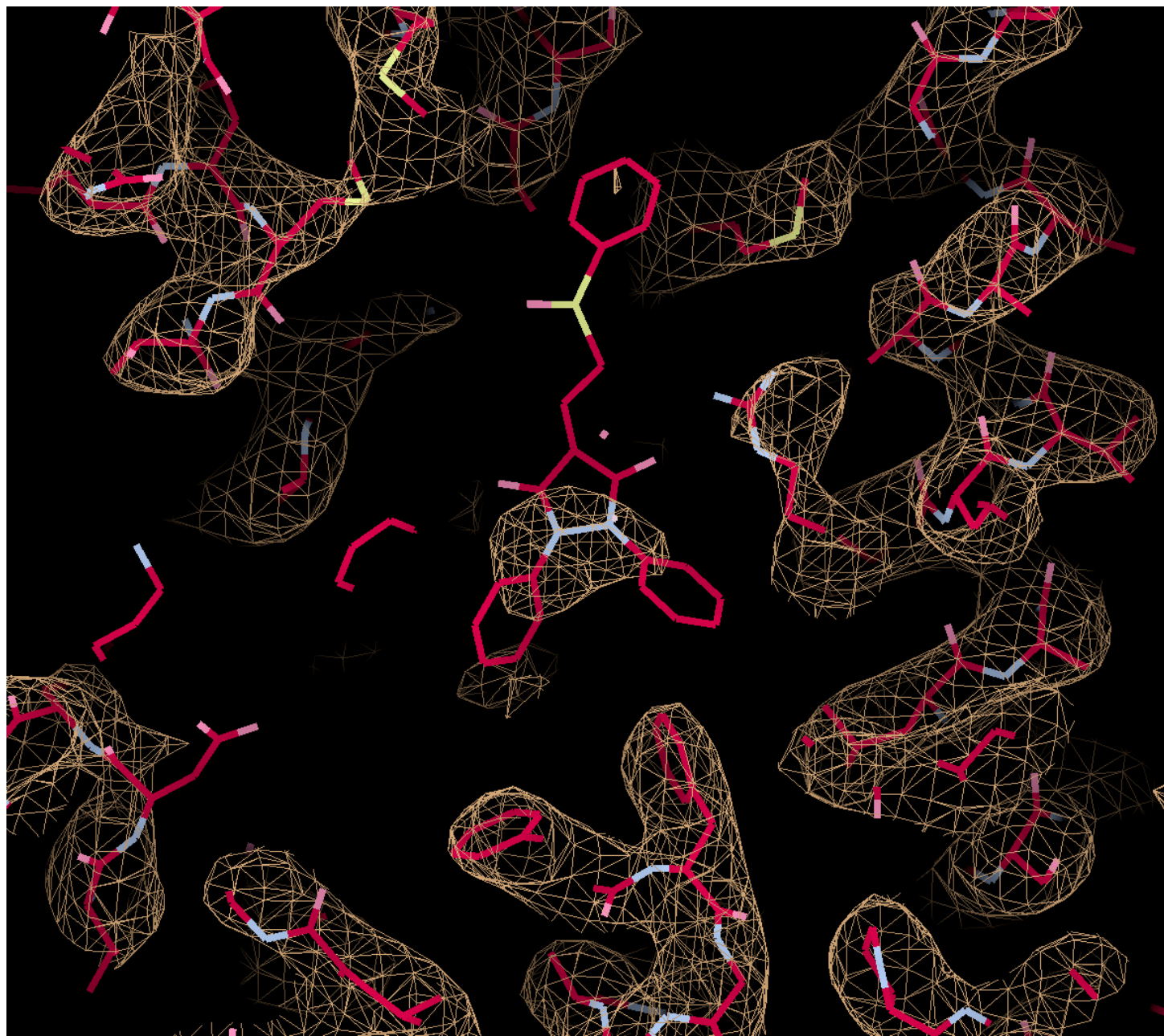


Treshhold 2



Verinurad-bound





Sulfinpyrazone-bound