nature portfolio

Peer Review File

Molecular basis of the urate transporter URAT1 inhibition by gout drugs

Corresponding Author: Professor Seok-Yong Lee

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Reviewer Comments:
The study by Yang et al. presents cryo-EM structures and MD simulations of URAT1 in both apo state and in complex with inhibitors, including benzbromarone, lesinurad, and the novel compound TD-3. The authors employed consensus mutagenesis to enhance yield and stability, achieving 91% sequence identity to human URAT1. They report the structures of URAT1 complexed with benzbromarone and TD-3 for the first time in the context of gout treatment. All three inhibitors are non-competitive and bind to the inward-open state of URAT1. The TM5 region of TD-3-URAT1 shows slight conformational changes compared to the structures bound to benzbromarone and lesinurad, indicating structural adjustments upon binding different inhibitors. MD simulations were conducted to validate ligand model building, and mutations in the central cavity were introduced to further support the binding poses. While the cryo-EM structures are generally well resolved and the data are presented adequately, I have several significant concerns.

Major Concerns:
1. Ligand Assignment: I disagree with the assertion that ligands were “modeled confidently into strong, unambiguous…”. Without access to the cryo-EM maps and PDB files, it is challenging to evaluate the accuracy of the model building. Based on previous studies of OAT or OCT structures, it is likely that the hydrophobic components of the inhibitors, such as the naphthalene ring, are stabilized by aromatic clamp residues. Supporting this, recent work by Chia-Hsueh Lee et al. (PMID: 39245778) showed URAT1 bound to lesinurad in a nearly flipped orientation compared to this study. Additionally, their study indicates that urate, a substrate of URAT1, is clamped by aromatic residues in a manner that differs from what is reported in Qian’s work (PMID: 39146184), which has relatively lower resolution. It appears the authors may have submitted their manuscript prior to the publication of Lee’s findings; I recommend a comparison of structures with their study. I have concerns that the ligands in this study may have been incorrectly modeled. While the RMSD suggests overall stability in the MD simulations, caution is warranted, especially with large, neutral ligands. For instance, the authors state that “the carboxylates of both LESU and TD-3 show considerable rotatability during MD simulations” (line 210). To improve model building of the ligands, I suggest performing ligand-free docking first, considering free energy assessments like the MM-GBSA method using Schrodinger software to exclude incorrect poses, followed by MD simulations based on the generated ligand configurations. I also strongly recommend uploading the density maps and PDB files for further review.
2. Consensus Mutagenesis: Firstly, while consensus mutagenesis greatly enhanced the yield and stability of URAT1, introducing too many mutations may not be the most effective approach. The use of consensus mutagenesis in URAT1 raises questions about how representative the engineered protein is of the wild-type human URAT1. The functional data indicate that while the expression level of the mutated protein is significantly higher than that of the wild type, the transport activity is notably lower. The IC50 assays for hURAT1 versus URAT1CS were conducted under different incubation conditions; thus, comparable data under uniform conditions are essential to evaluate the impact of mutations on activity. It is recommended to further assess the IC50 values of URAT1 and URAT1CS with respect to benzbromarone and lesinurad, as this may provide a clearer understanding of how URAT1CS retains most of the functions of URAT1.
3. Surface Expression Experiments: There appears to be a discrepancy between the surface expression levels determined by western blot and the microscopy images. The western blot results suggest that the reduced transport capacity in several mutants is primarily due to significantly lower surface expression rather than impaired transport functionality. Therefore, the authors should not conclude that reduced uptake capacity indicates an essential role of the mutated residues in transport. Instead, these residues may be more critical for folding and surface targeting. Quantification of the surface expression data is necessary to address this. When performing western blots, including an internal control, such as Na+-K+-ATPase, would

strengthen the assessment of surface expression levels across the mutants.

Minor Issues:
Line 153: F449 is from TM10, but only TM5 and TM7 are mentioned.
Line 178: Clarify whether F364 or F365 is the correct residue number, and verify this residue number in Fig. 3e.
Line 187: Should Fig. 3b be referred to as Fig. 3d?

Figure 3b and e shows T217A, while Extended Data Figure 6b displays N217A.

The term “No ligand added” in the manuscript and figures should be replaced with “apo.”

In Extended Data Figure 2b, the 2D classification indicates an orientation preference for the top view. Please provide detailed methodology on how to calculate the structure appropriately. Do the final resolved structures exhibit any orientation preference?

Reviewer #2

(Remarks to the Author)
Suo et al. report the structures of the URAT1 transporter in its apo form, as well as three structures resolved in complex with therapeutically relevant inhibitors. This is the second study to report URAT1 structures resolved using cryo-EM, but the first to resolve the structure with clinically used inhibitors. This is of particular interest, as inhibition of URAT1 is one of the strategies for treating gout, and this study may help improve the specificity and potency of URAT1 inhibitors.

1) My major concern is that, similar to their previous papers describing cryo-EM structures of the SLC22 family, the authors used a consensus sequence of URAT1 (URAT1cs). This amino acid sequences does not exist in nature, making the structural results difficult to interpret and highly prone to artifacts.

a. The URAT1cs is much more stable in the inward-open conformation. While this naturally facilitates the generation of high-resolution cryo-EM structures, it may be completely artificial. On one hand, the structural mechanisms stabilizing this conformation may be absent in hURAT1 (or URAT1 from any other species), leading to potential misinterpretation of the rest of the data.

b. As the protein is more stable in the inward-open conformation, it may be entirely artificial that the inhibitors bind in this conformation as well. This raises doubts about the major finding of this study. The arguments suggesting that in vitro experiments showed a non-competitive mode of inhibition, and that in vivo, the inhibitors approach the transporter intracellularly, are not convincing without confirming the cryo-EM findings. Non-competitive inhibition could also result from binding extracellularly, rather than in the urate binding pocket. Indeed, all the inhibitors are polar organic anions, and it is unclear how they could enter the cells in general (and particularly into the HEK293 cells used in this experiment) without a transport process. In humans, the inhibitors are likely to be excreted in the urine, acting extracellularly at URAT1. For example, "Urinary excretion of unchanged lesinurad was generally between 30% and 40% of the dose" (PMID: 26170627). The autors should deliver a cryo-EM data of the native hURAT1 structure with at least on of the inhibitors to address this consern.

2) The authors should clearly acknowledge that their study represents the second report on cryo-EM structures of URAT1. This should be stated in both the introduction and the abstract, with the additional knowledge provided by their study being clearly highlighted.

3) The authors claim that the previous study failed to resolve the structure with inhibitors because the binding pocket of URAT1 was much smaller and less accessible from the outside. However, Suo et al. used a much higher concentration of inhibitors in this study, and this could be the reason for their success.

4) The apo structure indicates the potential binding of a small molecule inside the binding pocket. The authors suggested that this may be an endogenous molecule (line 110). Please specify which molecule it could be. If the molecule is not resolved structurally, it should be discussed in the context of its possible physiological role and previous data on the transport mechanism.

5) Reference 20 does not claim that the substrate of OAT1, PAH, interacts directly with a negative charge in the binding pocket. They only claim this for the inhibitor probenecid. Regarding OCTs, only the group that authored this manuscript claims a direct interaction between the substrate and E386. Several papers that resolved OCT structures after this study do not directly confirm this. Please revise the statement in lines 234-236 accordingly.

Minor comments:
Abstract:
Please remove the references form the abstract.

Line 112:
Include PMID: 36344565, the first cryo-EM structure of the SLC22 transporter, in the references.

Figure 4f:
There are substantial differences in the activity of the F241A mutant, ranging from a complete lack of activity to activity identical to or even stronger than the wild-type. Averaging these results does not make sense in this case, and the experiments should be repeated. To a lesser extent, the same issue applies to the S238A mutant.

Reviewer #3

(Remarks to the Author)
The paper presents apo and holo cryo-EM structures with three clinical drugs proposing an inward-facing conformation for molecular recognition. My concern is that the authors make several extrapolations not strongly supported by results. Further, I suggest they end their discussion with a summary of the amino acids they deem important to support their notion that this paper will contribute to better drug design/development.

Recommendation: Reject, but further work might justify a resubmission.

Lack of clarity:
1. Lines 117-12. The statement is not clear. The authors suggest inward-facing referring to Fig 1e, which does not indicate that, yet the clinical transporters stabilize outward-facing.
2. Lines 128-130. Rationalization of the hypothesis (their stated prediction) is needed. Explain or justify the correlation of central binding pocket occupancy to the state and/or kinetics (competitive, non-competitive).
3. Line 136. How and in what manner does it compromise transport activity?
4. Lines 138-140. Explain “restrictive.” Is it sterics? Are there amino acids blocking the entrance and if so, which ones?
5. Lines 157-160. The statement “distinct mechanism in substrate/inhibitor recognition and function” needs clarification.
6. Line 178. In one sentence they talk about interactions with henylalanines 241 and 364, yet in the next they speak of the mutation F365A (not F364) to justify it. Figure 3e shows small effect of F365, and not F364.
7. Lines 185-189. (i) Benzbromarone’s acidic (ionized hydroxyl) form is very minor, especially in kidney’s pH. The whole MD run with the negative phenolic does not make sense from a chemical perspective. (ii) K393 seems to be far from the ligand in Figure 3c. (iii) Figure 3g needs to be labelled as such. (iv) Even though in my opinion the charged MD was not necessary, the authors do not explain or even mention the new configuration at 650 ns in Figure 3b. (v) Did their MD runs converge?
8. Lines 201-202. What is the distance between Met214 for S- to occur? Citation 29 refers to a totally different receptor.
9. Lines 209-216. (i) Binding affinity does not relate to mobility in the binding cavity. (ii) Salt bridge formation between a weak (and mostly minor) phenolic hydroxyl and protonated nitrogen is not feasible, as noted in 7i above.
10. Lines 217-22. What is the significance of the urate being perpendicular to the inhibitors’ naphthalene? Also, the sentence “The additional heterocycle….for high affinity binding” does not make sense. What interactions does the carboxylate make with? Is there a salt bridge? When I look at Extended Figure 8, I see the carboxylate being close to a water or floating with no interactions for LESU and TD-3.
11. Line 242. What is the solvent accessible surface area?
12. Lines 283-288. A measure of hydrophobicity of the cavity would be helpful.

Figures:
Fig 1. Perhaps a more specific explanation of 1c and 1d can be informative. As it stands, 1d shows the three ligands binding similarly and in the same locus with no major changes by surrounding amino acids.
Fig 2e. Steric restriction by what? There is an X in the schematic, but further elaboration would be helpful in terms of amino acids blocking the outward cavity.
Fig 3c versus Extended Fig 8. Shouldn’t F449 and F365 appear in the latter since they seem perpendicular to the phenol for interactions? Please label g.
Fig 4d. Labels are missing. Once again what is the distance between Met and the ring?
Fig 4g. The RMSD is about 2 angstroms. The investigators may want to explore the different conformations here, which in turn can support either induced or conformational selection for molecular recognition.
Extended Fig 5. Which amino acids are conserved? What is the functional implication if any?

Extrapolations:
1. Lines 212-214. S238 in Figure 4 has a rather moderate effect. The effect is not the same among the inhibitors.
2. Lines 219-22. The sentence starting with “Therefore” does not follow or support the previous one.
3. Lines 251-253 and 265-268. The results do not support the proposed mechanism. There is no MD run with urate, no discussion on the inward or outward cavities in terms of receptor conformations observed in the MD simulations. No results have been presented regarding the various conformations. Perhaps the investigators could consider the most populated conformations in order to suggest conformational selection. As it stands right now, the mechanism is mostly induced fit (Figures 3 and 4).
4. Line 306. I realize this is a typical ending statement, but I would like to see exactly which data in their paper will help with better drug designs. Notably, it is the tightness of the bound complex that leads to better potency.

Reviewer #4

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

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
According to the higher-resolution maps, the PDBs fit the maps well, suggesting that the authors have accurately modeled the ligands. The authors have effectively addressed my questions, and I recommend acceptance.

In Extended Data Figure 8, PDB 9B1L is a better choice as a comparative structure, and it is recommended to replace PDB 8WJQ.

Reviewer #2

(Remarks to the Author)
We agree with the authors that the structure they resolved does not differ substantially from the previously published URAT1 structures, which have now been reported by three independent groups. These studies have also resolved URAT1 in the presence and absence of inhibitors.

However, this was not our primary concern. Our primary concern is that the structure resolved here is based on a non-existing consensus sequence. While this does not affect the spatial resolution, it may impact the stability of the protein in an inward-open conformation. The authors themselves acknowledge that their structure might represent an over-stabilized conformation. Therefore, we see this work as yet another confirmation of the potential binding of inhibitors in the inward-open state—an observation that has already been reported by others. However, this confirmation is based on a more artificial structure.

The rest of our comments have been addressed correctly.

Reviewer #3

(Remarks to the Author)

Reviewer #4

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

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

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The study by Yang et al. presents cryo-EM structures and MD simulations of URAT1 in both apo state and in complex with inhibitors, including benzbromarone, lesinurad, and the novel compound TD-3. The authors employed consensus mutagenesis to enhance yield and stability, achieving 91% sequence identity to human URAT1. They report the structures of URAT1 complexed with benzbromarone and TD-3 for the first time in the context of gout treatment. All three inhibitors are non-competitive and bind to the inward-open state of URAT1. The TM5 region of TD-3-URAT1 shows slight conformational changes compared to the structures bound to benzbromarone and lesinurad, indicating structural adjustments upon binding different inhibitors. MD simulations were conducted to validate ligand model building, and mutations in the central cavity were introduced to further support the binding poses. While the cryo-EM structures are generally well resolved and the data are presented adequately, I have several significant concerns.

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We thank the reviewer for this point. We have performed careful analyses and concluded that our lesinurad binding pose is the most accurate pose.

(1) Our maps (2.74 Å) have a higher nominal resolution compared to those reported by CH Lee (2.9 Å). Furthermore, we built our lesinurad pose with a pose based on that of TD-3, which we could model with high confidence (2.55 Å map) and is structurally very similar to lesinurad, so both are expected to adopt a very similar pose (see Figure 1 below).

(2) To further validate our model, we compared the reported poses for Lesinurad from our work (pose 1) and that reported by CH Lee (pose 2), into our respective maps/models and into each other's maps/models (see Figure 2, below). We also show the map and model fit for lesinurad from Wu, C., et al, which is clearly of poor quality (see Figure 2, below), so was not subjected to further comparison and analysis. We show that (1) our pose has the highest ligand map-to-model correlation when using either our map or CH Lee's map, (2) the ligand in pose 1 visually fits better in either map, and (3) MD simulations show that our ligand pose has superior stability (lower mean RMSD and narrower distribution) when docked into either model. This is represented by the RMSD histograms for the ligand based on five replicates of 1 μs MD simulations of each combination.

(3) Following the reviewer's suggestion, we also performed MMGBSA binding energy calculations using our structure (9DKB) and that from CH Lee's group (9B1H), testing our pose for lesinurad (pose 1) and theirs (pose 2). The results are summarized in Table 1 below and generally agree with the RMSD data from the MD simulations in Figure 2. Basically, the strongest interaction was for our pose (pose 1) to our structure (9DKB) (-42.93 kcal/mol) and the weakest was CH Lee's pose to their structure (-35.78 kcal/mol).

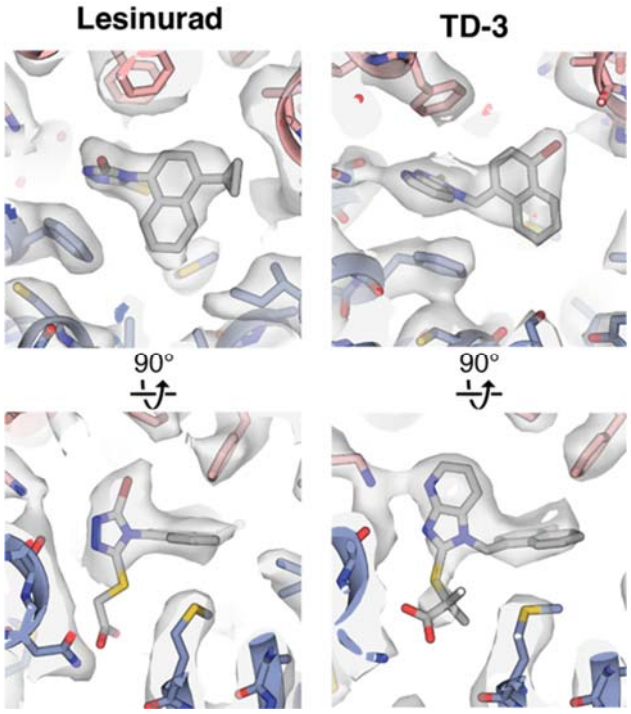

Figure 1 Comparison of lesinurad and TD3 poses from our manuscript.

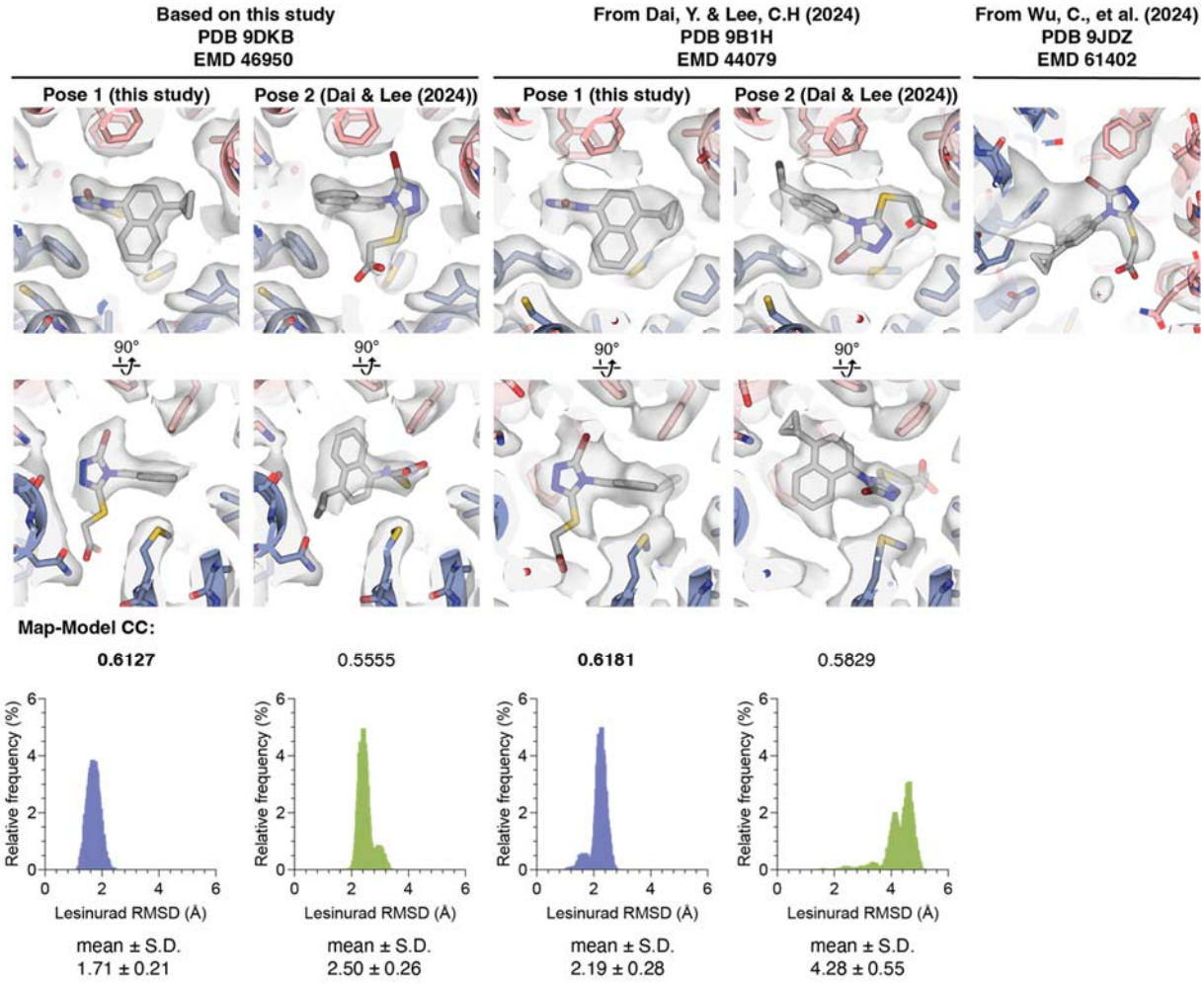

Figure 2, Comparison among proposed lesinurad poses, ligand map-model CC values, and MD plots for ligand RMSD from 5 replicates of 1 μs simulations, each.

MMGBSA – the first 10 ns simulations

	pose1	pose2
9dkb	−42.93 ± 1.08 kcal/mol	−42.89 ± 0.86 kcal/mol
9b1h	−40.28 ± 1.48 kcal/mol	−35.78 ± 1.46 kcal/mol

Table 1, MMGBSA results of comparison among proposed lesinurad poses. Calculations were done using the first 10 ns of simulation runs.

Therefore, after our careful analyses, we concluded that our lesinurad binding pose is the most accurate pose. We included our additional analysis in the revised text (Extended Data Fig. 10). We thank the reviewer for this point so that we can include our analysis to provide the most accurate binding pose to the field of gout drug development, thereby increasing the impact of our study.

2. Consensus Mutagenesis: Firstly, while consensus mutagenesis greatly enhanced the yield and stability of URAT1, introducing too many mutations may not be the most effective approach. The use of consensus mutagenesis in URAT1 raises questions about how representative the engineered protein is of the wild-type human URAT1. The functional data indicate that while the expression level of the mutated protein is significantly higher than that of the wild type, the transport activity is notably lower. The IC50 assays for hURAT1 versus URAT1CS were conducted under different incubation conditions; thus, comparable data under uniform conditions are essential to evaluate the impact of mutations on activity. It is recommended to further assess the IC50 values of URAT1 and URAT1CS with respect to benzbromarone and lesinurad, as this may provide a clearer understanding of how URAT1CS retains most of the functions of URAT1.

We understand the reviewer's concern. It is very typical to have to engineer constructs for greater conformational stability to facilitate structural elucidation, either by introducing mutations, truncating loops, engineering in domains, or through binding of stabilizing nanobodies, etc. Our structural construct is derived from the consensus sequence with consideration to preserve the residues that line the central cavity, and contact the substrate/inhibitors, as human URAT1 (hURAT1). Uptake rates for the consensus constructs are much slower than hURAT1, indicating an over-stabilized conformation. Importantly, our structure is nearly identical (≤1A RMSD, see Table 2) to the other reported inward-facing URAT1 structures. IC_{50} determinations using this construct demonstrate high binding affinities for benzbromarone, lesinurad and TD-3. The similar conformation of URAT1 to others' constructs and high inhibitor affinities indicate that our structures are relevant and not artefactual.

As a note on the functional assays, to ensure robust signal:noise while maintaining uptake in the temporally-linear range, hURAT1 and URAT1cs uptake was conducted for different durations (10 min vs 30 min). Provided uptake is linear, this difference in duration should not affect the apparent IC_{50} measurement.

Finally, we included the following paragraph in the discussion to acknowledge the potential limitation of our study. "We acknowledge that a limitation of our study is the use of a consensus construct (Extended Data Fig. 1a), which greatly facilitated our structural studies by being highly stabilized in the inward-open conformation albeit with substantially compromised activity (Extended Data Fig. 1c). …."

3. Surface Expression Experiments: There appears to be a discrepancy between the surface expression levels determined by western blot and the microscopy images. The western blot results suggest that the reduced transport capacity in several mutants is primarily due to significantly lower surface expression rather than impaired transport functionality. Therefore, the authors should not conclude that reduced uptake capacity indicates an essential role of the mutated residues in transport. Instead, these residues may be more critical for folding and surface targeting. Quantification of the surface expression data is necessary to address this. When performing western blots, including an internal control, such as Na+-K+-ATPase, would strengthen the assessment of surface expression levels across the mutants.

We agree that quantification of surface expression would help assess whether reduced activity of a given mutant is due to poor surface expression/folding, or genuine activity reduction. However, we found that, despite trying to optimize extraction conditions, some variants were prone to crashing out of solution after extraction. This results in lower apparent surface expression for some mutants. We therefore decided to exclude surface expression western blot data and instead rely on fluorescence confocal imaging of intact cells.

Minor Issues:
Line 153: F449 is from TM10, but only TM5 and TM7 are mentioned.
Thank you for catching this error. We now include mention of TMs 5, 7, and 10.

Line 178: Clarify whether F364 or F365 is the correct residue number, and verify this residue number in Fig. 3e.
Thank you for pointing this out. F364 is correct, but we have modified the text for clarity. "Interestingly, the brominated phenolic group interacts with F364 as well as F241 and F449 of the aromatic clamp via π-π interactions. F241 and F364 are both required for uric acid transport as their mutation to alanine eliminates activity, therefore the effect of these mutations on inhibitor binding could not be probed by activity assays (Fig. 2g). The F365A mutant also significantly reduces activity (~25% WT; Fig. 2g), but has a rather minimal impact on BBR binding, interacting only slightly with the benzofuran group (Fig. 3a-c)."

Line 187: Should Fig. 3b be referred to as Fig. 3d?
We mean to show that K393A/R has very low activity. We have corrected this by referencing Fig. 3a,b to show the proximity of K393 to the BBR hydroxyl, and also refer to Fig. 2g when discussing the low activity of K393 mutants.

Figure 3b and e shows T217A, while Extended Data Figure 6b displays N217A.
Extended Data Fig. 6b has a typo where it should be T217A. However, we have removed Extended Data Fig. 6b according to comment #3.

The term "No ligand added" in the manuscript and figures should be replaced with "apo." We respectfully disagree since this is not "true" apo as there is some unmodelled density in the cavity (Extended Data Fig. 4). Therefore, "No ligand added" is more accurate as apo implies an empty binding site (only water bound).

In Extended Data Figure 2b, the 2D classification indicates an orientation preference for the top view. Please provide detailed methodology on how to calculate the structure appropriately. Do the final resolved structures exhibit any orientation preference?
There is indeed some level of orientation preference in the raw data as shown in the 2D classes. Our iterative *ab initio* reconstruction method (described in this manuscript and Suo., *et al.* (2023) Nat. Struct. Mol. Biol. **30**: 1001-1011) successfully enabled us to obtain high-resolution reconstructions in the presence of orientation preference by iteratively selecting a subset of particles with improved orientation sampling, resulting in a better 3D reconstruction. Extended Data Fig. 3c shows that there is still some residual

orientation bias in the final reconstruction, but it does not significantly affect data quality.

Reviewer #2 (Remarks to the Author):

Suo et al. report the structures of the URAT1 transporter in its apo form, as well as three structures resolved in complex with therapeutically relevant inhibitors. This is the second study to report URAT1 structures resolved using cryo-EM, but the first to resolve the structure with clinically used inhibitors. This is of particular interest, as inhibition of URAT1 is one of the strategies for treating gout, and this study may help improve the specificity and potency of URAT1 inhibitors.

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a. The URAT1cs is much more stable in the inward-open conformation. While this naturally facilitates the generation of high-resolution cryo-EM structures, it may be completely artificial. On one hand, the structural mechanisms stabilizing this conformation may be absent in hURAT1 (or URAT1 from any other species), leading to potential misinterpretation of the rest of the data.

Similar to our findings, several inhibitor-bound URAT1 structures reported by CH Lee[1] and Eric Xu[2] also adopt inward-facing conformations, despite the use of very different structural constructs. When overlaying our LESU-$URAT1_{CS}$ with other inward-facing URAT1 structures, the models are virtually identical with Cα R.M.S.D values ≤ 1 Å (Table 2).

Table 2 Cα R.M.S.D values for aligned LESU- $URAT1_{CS}$ with other currently available inward facing URAT1 structures in the PDB.

LESU-URAT1cs vs.	R.M.S.D (Å)
9JDV	0.906
9JDY	1.012
9JDZ	0.957
9JE0	0.977
9JE1	0.982
9B1F	0.685
9B1G	0.907
9B1H	0.702
9B1I	0.877
9B1J	0.984

We would also argue that a functionally less active construct does not mean structurally incorrect, just that the energy landscape for the transporter has been modulated such that a single state is highly stabilized so that it becomes the predominant conformation in the sample. This facilitates structural characterization of that state, while hampering interconversion between states (activity). Our construct allowed us to determine the highest resolution structures and most accurate ligand binding poses (Extended Data Fig. 10a).

b. As the protein is more stable in the inward-open conformation, it may be entirely artificial that the inhibitors bind in this conformation as well. This raises doubts about the major finding of this study. The arguments suggesting that in vitro experiments showed a non-competitive mode of inhibition, and that in vivo, the inhibitors approach the transporter intracellularly, are not convincing without confirming the cryo-EM findings. Non-competitive inhibition could also result from binding extracellularly, rather than in the urate binding pocket. Indeed, all the inhibitors are polar organic anions, and it is unclear how they could enter the cells in general (and particularly into the HEK293 cells used in this experiment) without a transport process. In humans, the inhibitors are likely to be excreted in the urine, acting extracellularly at URAT1. For example, "Urinary excretion of unchanged lesinurad was generally between 30% and 40% of the dose" (PMID: 26170627).
The autors should deliver a cryo-EM data of the native hURAT1 structure with at least on of the inhibitors to address this consern.
As detailed above, we have validated our binding poses and all other reported structures of URAT1 with inhibitors bound are also in the inward-facing open state with nearly identical conformations (RMSD ≤1 A). So we are confident that despite being an engineered construct with a strong preference for the inward-open state, our structure is valid and interpretable in the context of URAT1 function.
One could envision a mechanism where drugs bind from the extracellular environment and fix the conformation of the protein in the inward open state, but it is more likely that they bind to and directly stabilize the transporter in the inward-open state. This is congruent with the mode of inhibition being non-competitive. A recent set of structures with URAT1 bound to uric acid[1] show uric acid binding partially overlaps with inhibitor binding. This means that inhibitor binding from the extracellular milieu would be

expected to manifest as competitive with uric acid and manifest as such in the uptake assays. Our kinetic model fitting clearly shows that a competitive mode of inhibition (where inhibitors bind from the extracellular side of the membrane) is the least well-fitting model. We have included a comparison of the inward-facing uric acid bound structure (PDB 8WJQ)[1] to demonstrate the overlap of interactions uric acid and the inhibitors make with URAT1 (Extended Data Fig. 8).

2) The authors should clearly acknowledge that their study represents the second report on cryo-EM structures of URAT1. This should be stated in both the introduction and the abstract, with the additional knowledge provided by their study being clearly highlighted.
We have modified our text to acknowledge the other work has been published and so conducted comparative analyses against these structures, with respective modifications to the text, including addition of Fig. 2f and Extended Data Fig. 10.

3) The authors claim that the previous study failed to resolve the structure with inhibitors because the binding pocket of URAT1 was much smaller and less accessible from the outside. However, Suo et al. used a much higher concentration of inhibitors in this study, and this could be the reason for their success.
While submitting this work, other structures have since been published, which all show inhibitor binding only to the inward-open states. Uric acid, however, was shown to bind to multiple different conformations, including the outward-open. This agrees with our model that the outward-open conformation is too restrictive for inhibitor binding, but inhibitors are readily accommodated by the more expansive pocket of the inward-open state.

4) The apo structure indicates the potential binding of a small molecule inside the binding pocket. The authors suggested that this may be an endogenous molecule (line 110). Please specify which molecule it could be. If the molecule is not resolved structurally, it should be discussed in the context of its possible physiological role and previous data on the transport mechanism.
Based on the shape of the unknown ligand and previous literature[3], it is possible that this unknown ligand is fatty acid. However, without further characterization by other methods (mass spec, etc.) supporting what it might be, we refrain from discussing the nature of the unknown endogenous ligand density. URAT1 is known to bind a wide variety of molecules, making it likely this density does not correspond to a single species. We would rather call this the “no ligand added” conformation, rather than “apo” and not over-interpret the density in the cavity.

5) Reference 20 does not claim that the substrate of OAT1, PAH, interacts directly with a negative charge in the binding pocket. They only claim this for the inhibitor probenecid. Regarding OCTs, only the group that authored this manuscript claims a direct interaction between the substrate and E386. Several papers that resolved OCT structures after this study do not directly confirm this. Please revise the statement in lines 234-236 accordingly.
Thank you for raising this point. Reference 20 discusses how PAH binding is flexible and the carboxyl group of PAH can turn towards Arg466 or Lys382. Probenecid binding

is much more rigid with its carboxyl stabilized by Lys382. As for OCT1, Zheng et al (2023) does mention a direct interaction of the diltiazem amine with E386, although the other compounds were not found to specifically interact with either acidic residue directly. OCT3, however, just has a very electronegative cavity. We have modified our text as such:

“It also does not seem that there is a critical basic residue responsible for stabilization of the anionic inhibitors, but that these compounds bind to a generally electropositive cavity, where residues like K393 and R477 contribute. This appears to be the general case for the SLC22 family of transporters, which tend to lack a single defined critical charged residue that stabilizes the complementarily charged ligand.”

Minor comments:
Abstract:
Please remove the references form the abstract.
Thank you for this point. We have removed the references from the abstract.

Line 112:
Include PMID: 36344565, the first cryo-EM structure of the SLC22 transporter, in the references.
Thank you for noticing this omission, we have now included this reference.

Figure 4f:
There are substantial differences in the activity of the F241A mutant, ranging from a complete lack of activity to activity identical to or even stronger than the wild-type. Averaging these results does not make sense in this case, and the experiments should be repeated. To a lesser extent, the same issue applies to the S238A mutant.
We thank the reviewer for this point. We have re-validated the plasmids and conducted uptake characterizations of these mutants again, significantly improving the data quality. Figures 3 and 4 have been updated, as well as the text to reflect the revised data, which agrees with the structural data.

Reviewer #3 (Remarks to the Author):

The paper presents apo and holo cryo-EM structures with three clinical drugs proposing an inward-facing conformation for molecular recognition. My concern is that the authors make several extrapolations not strongly supported by results. Further, I suggest they end their discussion with a summary of the amino acids they deem important to support their notion that this paper will contribute to better drug design/development.

Thank you for the suggestion, we have modified the final paragraph of our discussion to more thoroughly discuss this point:
"Tailoring carboxylate positioning to perhaps better engage K393 and/or R477 could also be considered for future therapeutic development. This in addition to ensuring drug binding engages both the N- and C-domains of the transporter, and utilizes the aromatic clamp and hydrophobic region to rigidly stabilize the inward-open conformation of the transporter. Optimizing interactions with S238 and M214 also represent good targets for improving inhibitor binding."

Recommendation: Reject, but further work might justify a resubmission.

Lack of clarity:
1. Lines 117-12. The statement is not clear. The authors suggest inward-facing referring to Fig 1e, which does not indicate that, yet the clinical transporters stabilize outward-facing.
Fig. 1e is meant to show the inhibitors within the central binding pocket, showcasing the extent of interactions. This statement is meant to convey the contrary-to-expected finding that URAT1 binds clinically relevant inhibitors in the inward-facing conformation, as opposed to the more commonly observed outward-facing conformation for other drug-transporter complexes. We have clarified this statement by referencing Fig. 1d (which better demonstrates the inward-open conformation of the transporter) and 1e. We have also modified our wording to say that our URAT1 construct is stabilized in the inward-open conformation, to which the inhibitors are all bound, which is rather unique for clinical drugs that target transporters.

2. Lines 128-130. Rationalization of the hypothesis (their stated prediction) is needed. Explain or justify the correlation of central binding pocket occupancy to the state and/or kinetics (competitive, non-competitive).
If we frame the uptake of uric acid by URAT1 in terms of Michaelis-Menten kinetics, then competitive binding would mean that uric acid and inhibitors bind to the same conformation of the protein (outward-open) and to the same/overlapping binding sites. Non-competitive inhibition would be when inhibitors bind to the protein such that conformation and activity are altered to prevent substrate binding. For transporters, like URAT1, this could be achieved by binding of inhibitor to a remote binding (allosteric) site or an alternate conformation. Inhibitor binding to the inward-open conformation prevents turnover to the outward-facing conformation, and thus preventing uric acid binding, hence this inhibition manifesting non-competitive. We have modified our text in this section to better explain this mechanism of inhibition.

3. Line 136. How and in what manner does it compromise transport activity?
According to He et al[4], the R477S variant has a significantly elevated K_T for uric acid uptake (actual fit values were not reported). We have removed this sentence from the text.

4. Lines 138-140. Explain “restrictive.” Is it sterics? Are there amino acids blocking the entrance and if so, which ones?
We have modified our text to better explain what we mean. Namely, the binding site in the outward-open conformation is sterically restrictive and the enclosed space is smaller due to the alternate conformation of the transporter. With an experimental structure now available for the outward-open conformation, we have compared the binding sites to show that inhibitor binding (in the conformations we report) is not possible to the cavity of the outward-open conformation. Primarily, the aromatic clamp formed by F241 and F449 closes, restricting binding of the imidazo-pyridine ring of TD-3, the dichlorophenol ring of benzbromarone, and the triazole ring of lesinurad.

5. Lines 157-160. The statement “distinct mechanism in substrate/inhibitor recognition and function” needs clarification.
We simply mean that inhibitor binding involves residues from more TMs that is typical for MFS transporters, which mostly engage substrates/inhibitors with residues from TMs 1, 4, 7, and 10. URAT1 also employs residues on TMs 2, 5 and 8 to bind inhibitors, indicating a broader engagement of the inhibitors with structural elements of the transporter. We have clarified the text by explicitly stating that URAT1 engages inhibitors with residues from more TMs than is typical for MFS transporters and that this may be necessary for locking the transporter in the inward-open conformation.

6. Line 178. In one sentence they talk about p-p interactions with henylalanines 241 and 364, yet in the next they speak of the mutation F365A (not F364) to justify it. Figure 3e shows small effect of F365, and not F364.
We thank the reviewer for catching this error. F364 is correct, and we have modified the text for clarity: “Interestingly, the brominated phenolic group interacts with F364 as well as F241 and F449 of the aromatic clamp via π-π interactions. F241 and F364 are both required for uric acid transport as their mutation to alanine eliminates activity, therefore the effect of these mutations on inhibitor binding could not be probed by activity assays (Fig. 2g). The F365A mutant also significantly reduces activity (~25% WT; Fig. 2g), but has a rather minimal impact on BBR binding, interacting only slightly with the benzofuran group (Fig. 3a-c).”

7. Lines 185-189. (i) Benzbromarone’s acidic (ionized hydroxyl) form is very minor, especially in kidney’s pH. The whole MD run with the negative phenolic does not make sense from a chemical perspective. (ii) K393 seems to be far from the ligand in Figure 3c. (iii) Figure 3g needs to be labelled as such. (iv) Even though in my opinion the charged MD was not necessary, the authors do not explain or even mention the new configuration at 650 ns in Figure 3b. (v) Did their MD runs converge?

i. Functional group pKa is modulated by its environment so while in solution benzbromarone may be largely neutral, binding to the protein may perturb the pKa and render it more anionic in character. We tested anionic and neutral forms of all the ligands to address how the charge state of the ligand affects it's binding.
ii. The K393 amine is 3.5 Å from the phenolic hydroxyl of benzbromarone. This is near enough for electrostatic interaction between K393 and benzbromarone, which will alter the pKa of Benzbromarone, depressing it's pKa and imparting a more partial negative charge.
iii. We have added the label and changed figure references, respectively.
iv. This increase in RMSD is a 1-2 Å drop in BENZ binding pose, but overall orientation is conserved.
v. We have performed five replicates of MD simulations for all liganded structures and the ligand R. M. S. D trajectories are similar (Extended Data Fig. 10), indicating their convergence.

8. Lines 201-202. What is the distance between Met214 for S-p to occur? Citation 29 refers to a totally different receptor.
According to the paper we cite and this work[5] the interaction distance for S-pi interaction is expected to be on the order of 4-5 Å. This is the distance of the S of M214 to the ring of LESIN and TD-3 (~4.5-4.7Å).

9. Lines 209-216. (i) Binding affinity does not relate to mobility in the binding cavity. (ii) Salt bridge formation between a weak (and mostly minor) phenolic hydroxyl and protonated nitrogen is not feasible, as noted in 7i above.
If the binding of a small molecule to a cavity is stable, the dynamics of that small molecule are lower than compared to a weaker binding molecule. This arises from thermodynamic consideration of the binding energy (binding equilibrium), where binding entropy terms include the protein-ligand conformational dynamics and the solvent. This is reflected in MD time course simulations as having a higher RMSD and broader RMSD distribution, which is what our analysis utilizes. We have modified the text to soften the statement:
"…but TD-3 shows less mobility within the cavity compared to LESU, which may correlate with its higher affinity to hURAT1."
As we discuss in point 7i, the pKa of BENZ within the protein cavity is likely to be perturbed relative to in the bulk solution, which will affect the charge state and distribution over the molecule. We have also modified the text to refer to the general electropositivity of the binding pocket (Fig. 5b), rather than there being any key electrostatic interactions.

10. Lines 217-22. What is the significance of the urate being perpendicular to the inhibitors' naphthalene? Also, the sentence "The additional heterocycle….for high affinity binding" does not make sense. What interactions does the carboxylate make with? Is there a salt bridge? When I look at Extended Figure 8, I see the carboxylate being close to a water or floating with no interactions for LESU and TD-3.
Thank you for pointing out this admittedly confusing statement. We have updated the manuscript to better compare uric acid and inhibitor binding (Fig. 2f and Extended Data

Figure 9). The main point is that uric acid binds uniquely but in a site that overlaps with the inhibitors. This is important as it makes inhibitor binding mutually exclusive with uric acid binding.
We have modified the text to clarify the point that the more extensive interaction of the inhibitors ensures they bind with higher affinity and lock the transporter in the inward-open conformation. Indeed, the carboxylate does not seem to make any specific interactions, but rather is electrostatically complementary to the rather electropositive nature of the cavity.

11. Line 242. What is the solvent accessible surface area?
The calculated solvent accessible surface areas are 2198 and 2745 $Å^2$ for LESU-URAT1$_{CS}$ and TD-3-URAT1$_{CS}$, respectively.

12. Lines 283-288. A measure of hydrophobicity of the cavity would be helpful.
We agree but did not find a satisfactory way of plotting hydrophobicity within the cavity. Rather, we believe it is evident by the nature of the residues lining the cavity.

Figures:
Fig 1. Perhaps a more specific explanation of 1c and 1d can be informative. As it stands, 1d shows the three ligands binding similarly and in the same locus with no major changes by surrounding amino acids.
This is correct and what the image is meant to show. All our URAT1 structures exhibit an inward-open conformation, which is what Fig. 1c and 1d are meant to convey.

Fig 2e. Steric restriction by what? There is an X in the schematic, but further elaboration would be helpful in terms of amino acids blocking the outward cavity.
As we explain in point 4, we have modified our figure and text to more explicitly demonstrate the steric restriction for inhibitor binding to the outward-open conformation of URAT1.

Fig 3c versus Extended Fig 8. Shouldn't F449 and F365 appear in the latter since they seem perpendicular to the phenol for p-p interactions? Please label g.
Thank you for pointing that out. We have included the panel label and F449 and F365.

Fig 4d. Labels are missing. Once again what is the distance between Met and the ring?
We had added the missing labels in Fig. 4d. The distance between Met214 and the naphthalene ring is about 4.5 Å.

Fig 4g. The RMSD is about 2 angstroms. The investigators may want to explore the different conformations here, which in turn can support either induced or conformational selection for molecular recognition.
The RMSD reported in Fig 4g is the ligand RMSD relative to the reference starting pose after aligning protein TMs, and the protein remains quite rigid throughout all the MD simulations. Since the protein conformation doesn't appreciably change we do not believe that this would allow us to use the MD simulations alone to propose an induced fit versus conformational selection mechanism of inhibitor binding.

Extended Fig 5. Which amino acids are conserved? What is the functional implication if any?
Sequence alignment in Supplemental Figure 1 and sequence conservation plot in Extended Data Fig. 5 are meant to demonstrate sequence conservation throughout the protein. As we mention at the start of the section introducing the central cavity of URAT1, there is mild sequence conservation of the cavity. The most highly conserved residues are residues like K393, D389, and R474, which are important for gating and do not appear to directly contact the ligands.

Extrapolations:
1. Lines 212-214. S238 in Figure 4 has a rather moderate effect. The effect is not the same among the inhibitors.
We thank the reviewer for this point. As mentioned above, the activity of S238A was re-evaluated and the resulting data is much cleaner. While there is not a substantial effect of S238A on lesinurad inhibition, it does substantially impact TD-3 binding. This is rather interesting and suggests that interaction with S238 is stronger with TD-3 than lesinurad, or simply that the interactions with TD-3 have greater weight on binding affinity, which is hard to tease out from our data and would require more in-depth measurements. It is an interesting phenomenon none-the-less. In light of the revised data we have updated the text accordingly.

2. Lines 219-22. The sentence starting with “Therefore” does not follow or support the previous one.
We have modified the text to provide improved logical flow:
“The larger size, electronegativity and hydrophobicity of the inhibitors are critical for their high affinity binding and ability to lock the transporter in the inward-open conformation. Therefore, the interactions mediated by the aromatic clamp and the polar group (both involving TM5) are important for activity and inhibitor binding.”

3. Lines 251-253 and 265-268. The results do not support the proposed mechanism. There is no MD run with urate, no discussion on the inward or outward cavities in terms of receptor conformations observed in the MD simulations. No results have been presented regarding the various conformations. Perhaps the investigators could consider the most populated conformations in order to suggest conformational selection. As it stands right now, the mechanism is mostly induced fit (Figures 3 and 4).
In light of recently published data by other groups, the urate-inhibitor exchange mechanism is well supported by the data and is in line with the general mechanism of MFS transporters.
We do not run MD simulations of urate or of transporter in the outward-facing conformation because we did not generate structural models to base such MD runs on. What does seem to suggest a conformation selection mechanism is the leftward shift in IC50s for the inhibitors to our consensus construct, which is highly stabilized in the inward-open conformation. Furthermore, if the mechanism of binding was induced fit, then it should be possible for the R477S construct used by He et al.[4] to bind the inhibitors. However, the authors noted that despite their efforts they were unable to get

an inhibitor-bound structure. Whereas our construct is essentially fixed in an inward-open state and we were able to obtain the inhibitor-bound structures. Therefore, we believe it is fair to suggest a conformational selection mechanism, which we do so in the discussion and toned down by saying “we speculate that inhibitors bind to URAT1 via a conformation selection mechanism”

4. Line 306. I realize this is a typical ending statement, but I would like to see exactly which data in their paper will help with better drug designs. Notably, it is the tightness of the bound complex that leads to better potency.
We end our discussion by highlighting some key interactions of the inhibitors with URAT1 and have expanded this slightly. Ultimately our functional and structural features add valuable data that can be used to guide drug design. As is always the case, any rationally applied modifications will need to be refined using screening approaches, but focusing on compounds that retain a degree of hydrophobicity (for lipid partitioning and cavity binding), are electronegative, and better engage with particular residues (K393, R477, S238, M214) may help find improved drug candidates.

Reviewer #4 (Remarks to the Author):

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

1 Dai, Y. & Lee, C.-H. Transport mechanism and structural pharmacology of human urate transporter URAT1. *Cell Research* **34**, 776-787 (2024).
2 Wu, C. *et al.* Molecular mechanisms of uric acid transport by the native human URAT1 and its inhibition by anti-gout drugs. *bioRxiv*, 2024.2009. 2011.612394 (2024).
3 Saito, H. *et al.* Omega-3 polyunsaturated fatty acids inhibit the function of human URAT1, a renal urate re-absorber. *Nutrients* **12**, 1601 (2020).
4 He, J. *et al.* Structural basis for the transport and substrate selection of human urate transporter 1. *Cell Reports* **43** (2024).
5 Ringer, A. L., Senenko, A. & Sherrill, C. D. Models of S/π interactions in protein structures: Comparison of the H2S–benzene complex with PDB data. *Protein Science* **16**, 2216-2223 (2007).

Response to Reviewer's comments - Revision

Reviewer #1 (Remarks to the Author):

According to the higher-resolution maps, the PDBs fit the maps well, suggesting that the authors have accurately modeled the ligands. The authors have effectively addressed my questions, and I recommend acceptance.

Thank you for your review and recommendations. We appreciate your work.

In Extended Data Figure 8, PDB 9B1L is a better choice as a comparative structure, and it is recommended to replace PDB 8WJQ.

Thank you for the suggestion, we have replaced the urate pose figure from PDB 8WJQ to PDB 9B1J. We chose 9B1J over 9B1L because 9B1L represents a urate-bound outward facing URAT1 conformation, whereas 9B1J represents urate-bound inward facing URAT1 conformation which is consistent with the conformations we reported in this manuscript.

Reviewer #2 (Remarks to the Author):

We agree with the authors that the structure they resolved does not differ substantially from the previously published URAT1 structures, which have now been reported by three independent groups. These studies have also resolved URAT1 in the presence and absence of inhibitors.

However, this was not our primary concern. Our primary concern is that the structure resolved here is based on a non-existing consensus sequence. While this does not affect the spatial resolution, it may impact the stability of the protein in an inward-open conformation. The authors themselves acknowledge that their structure might represent an over-stabilized conformation. Therefore, we see this work as yet another confirmation of the potential binding of inhibitors in the inward-open state—an observation that has already been reported by others. However, this confirmation is based on a more artificial structure.

The rest of our comments have been addressed correctly.

We appreciate the reviewer's concerns regarding the consensus sequence construct used in our structure determination. We have acknowledged that our construct is an engineered variant optimized for structural studies and the modifications overstabilized its inward-facing open conformation. Most importantly, we performed functional characterization of wild-type URAT1 to show that these inhibitors' mode of inhibition is non-competitive, consistent with this inhibitor binding to the inward-facing open state. To our knowledge, our study is the only one to provide functional data directly supporting the inhibitor binding mode amongst all the recent URAT1 inhibitor studies. Therefore, we respectfully disagree with the reviewer that our study is merely another confirmation of the potential binding of inhibitors in the inward state. Furthermore, we also believe the reviewer's term "artificial structure" is inappropriate in this context. "Artificial structure" implies incorrect structure. Our modification biased the conformation to the inward-facing open state, but the conformation/structure is not artificial, as our

resolved structure aligns closely with recently reported URAT1 structures, both in terms of inhibitor binding and overall architecture. Furthermore, our high-resolution structure enabled us to provide these inhibitors' most accurate binding pose, which we believe is an important contribution. We have clarified this point and explicitly discussed the implications of using a consensus-based construct in the revision. Last, we note that most URAT1 studies do not use native constructs. For example, Dai & Lee (2024) utilized a truncated chimeric construct where intracellular and extracellular loops of URAT1 were replaced with those from OAT4, which is also not a native sequence.

Reviewer #4 (Remarks to the Author):

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