nature portfolio

Peer Review File

Structural insights into the mechanisms of urea permeation and distinct inhibition modes of urea transporters

Corresponding Author: Professor Jin-Peng Sun

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)
This is an interesting study that provides structural insights into the mechanisms of urea permeation and distinct inhibition modes of urea transporters. The authors use cryo-EM to provide a structural understanding of 4 different urea transports and how they interact with urea transport inhibitors.
The work will be of significance in designing urea transport inhibitors as salt-sparing diuretics.
The work supports the conclusions.
There are no flaws in the data analysis that require revision.
The methodology is sound and sufficient detail is provided.

Minor Comment: in line 73-74 and 643-644, the authors refer to the thin descending limb or the proximal tubule. It would be better to refer to it as the thin descending limb of the loop of Henle.

Reviewer #2

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Huang et al presented an impressive structural biology study of the UT family members. The manuscript performed a detailed analysis of UT's structural dynamics in resting states, urea transport states, or inactive states bound with different synthetic inhibitors. In particular, the study directly observed how urea passes through the UT channel, and further combined molecular dynamics simulations with biochemical validation to uncover the conserved urea recognition motif (Q-T-T-Q motif) among different UT members. Moreover, in the manuscript, distinct binding modes to hUT-A2 were observed for the competitive inhibitors C25a and ATB3, the uncompetitive inhibitor F11 and the noncompetitive inhibitor HQA2. The overall manuscript is of high quality and well written, which provide a structural view for understanding of urea transport via UTs, and afforded a structural landscape of UT-A2 inhibition by competitive, uncompetitive and noncompetitive inhibitors. These structures combined with mutagenesis data studies, provide new insights for developing selective human UT-A inhibitors as novel salt-sparing diuretics. Therefore, this study offers conceptual advance at UTs structural level as well as the development of selective UT antagonists. There are several questions need to be addressed to improve the manuscript. I would like to recommend for minor revision before publication in Nature Communications.
1. The structures presented by the authors are in high resolution. To enhance the clarity of the findings, it would be beneficial to include additional views of the antagonists' density in the Supporting Information, though Figure 2 already showcases the density, supplementing with more perspectives would undoubtedly improve the overall understanding.
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3. The electron microscopy-density of CF11 seems to exhibit little misalignment with its molecular counterpart. To ensure a more accurate representation, it is strongly suggested that the authors perform supplementary molecular dynamics simulations of CF11 within the hUTA2 structure for a better understanding of the molecular interactions.
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5. The methodology section on molecular dynamics lacks appropriate citations for the crucial software tools employed in the simulations.
6. In Figure 7i, the interaction between HQA2 and F282, compared to C25a, should also be a special interaction.
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The authors did some research about urea permeation mechanism of the urea transporter and early stage salt-sparing diuretics discovery by solving 11 cryo-EM structures of four UT members, wet-lab and in silico experiments. Please check the following:

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[1] Levin EJ, Cao Y, Enkavi G, et al. Structure and permeation mechanism of a mammalian urea transporter. Proc Natl Acad Sci U S A. 2012;109:11194–9.

Thus, please reconsider this: “Because our current cryo-EM structure of urea-hUT-A2 could not capture the stable interaction of urea molecules inside the Sm region, we performed MD simulation of the process of a urea molecule passing through the Sm region.”

Try to extract conformation carefully from biased molecular dynamics because it is not under equilibrium.

2. I am very interested in why authors chose these 4 specific small organic molecules to represent competitive, uncompetitive and noncompetitive inhibitors. Could authors give some reasons and the original reference papers where these molecules come from?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have responded satisfactory to the previous review.

Reviewer #3

(Remarks to the Author)
The authors have carefully answered my questions and tried different collective variables selection. It could be observed that the urea stays in the hydrophobic pocket of sm zone for a while in the cartoon supplement material. The free energy

calculation looks consistent with this cartoon, but the local minimal potential at the sm zone is till brought by the calculation. As shown in Fig. 5f, the sm zone is the narrowest part of this channel, that is also a hint of a high energy barrier because the urea has to get rid of waters to pass through, that takes energy. I suggest the authors to recheck the parameters, consult some metadynamics experts or express their doubt attitude and explain that it is still challenging to get a reasonable result for the free energy calculation for this channel.

Reviewer #4

(Remarks to the Author)
Overall Comments
The authors present impressive work on uncovering the molecular mechanisms of urea channels and inhibition mechanism with a series of high-resolution structures, molecular dynamics and biophysical assays. While I find the results of the manuscript publication-worthy, I recommend that Nature Communications sends it back to the authors with serious reservations due to the reason below.

The cryo-EM maps presented in this work and provided to the journal, which I assume were also deposited to PDB, appear to have been generated with DeepEMhancer (as stated in the methods). This is evident in the way the map looks at lower contours. For example, with the human UT-A2/C25a map, 1) at contour level of 0.1, the map looks mask-ish; 2) at 0.00001 contour level, computational artefacts such as regular noise distributions are clear; and 3) detergent belt is not visible at any contour. The use of DeepEMhancer is likely the reason why the inhibitor densities look poor and rounded despite the high nominal and local resolutions (each R-group should be clearly visible at stated resolutions but it is not), as it has tendency to make non-protein densities look worse.

This is not an accepted practice for cryo-EM structure refinement and deposition. Such maps are supposed to be used only for guidance during manual model building/refinement, and should not be used for automatic refinement (e.g. Phenix RSR), density assessment/analysis such as those used in the figures, or map/model deposition to PDB.

Version 2:

Reviewer comments:

Reviewer #4

(Remarks to the Author)
The authors have addressed the major concerns regarding the cryo-EM reconstructions and analysis. I have only minor comments (below), otherwise I am satisfied with the quality of the manuscript and the authors' response.

1. Please check if figure 8 c and figure 9 c have been updated - they also have EM density representations but are not on the list of corrections in the authors' response.

2. Please add a row for the Q-scores of the structures in the data tables (supplementary tables 1 and 2).

3. It is not clear what the relevance of "contour level" in Supplementary table 3 is.

4. Line 395 - didn't => did not

5. Line 449, 499 - Flag => FLAG (the tag is styled as FLAG elsewhere)

6. Line 450 - Tobacco Etch cleavage site => Tobacco Etch Virus protease recognition site

7. Line 457/458/461 (there could be more) - centrifugation unit g is usually italicised

8. Line 477 - 488 - centrifugation is often in rpm instead of g

9. Methods generally lack vendor information. For example, Line 517 - 520 - manufacturers for the grids and Titan Krios need to be stated. Softwares such as Cryosparc are proprietary, as well.

10. References to supplementary tables 1 and 2 in either Image processing or Model building section are missing.

11. Line 537 has a sentence that is re-statement of line 525. Line 525 should state Supplementary Fig. 2 - 5 instead of 2- 4.

12. Line 522 - superscript for "2" in "A2"

13. Supplementary figures - Inconsistent labelling and terms ("supplemental" instead of "supplementary" in places, use of "calculations" instead of "data processing" or other more commonly used terms)

REVIEWER COMMENTS

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The work will be of significance in designing urea transport inhibitors as salt-sparing diuretics.

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Minor Comment: in line 73-74 and 643-644, the authors refer to the thin descending limb or the proximal tubule. It would be better to refer to it as the thin descending limb of the loop of Henle.

Reply: We thank you for your very helpful suggestions. We have revised the description of the thin descending limb of the loop of Henle in line 73-74 and 664-665 in the revision manuscript.

Reviewer #2 (Remarks to the Author):

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1. The structures presented by the authors are in high resolution. To enhance the clarity of the findings, it would be beneficial to include additional views of the antagonists' density in the Supporting Information, though Figure 2 already showcases the density, supplementing with more perspectives would undoubtedly improve the overall understanding.

Reply: We thank you for your very helpful suggestions. valuable suggestions. We have included the ligand's EM-density with multiple views in the Supplementary Fig. 2-5 in the revised manuscript.

2. The electron microscopy protocol, although comprehensive, would be more complete if it incorporated an orientation distribution histogram for the particles. This would provide essential information on the orientation of the particles within the homotrimer sample, enhancing the overall analysis and interpretation of the data.

Reply: We thank you for your valuable suggestions. We have included the orientation distribution histogram figures in the calculation processes of all structures in Supplementary Fig 2-5 in the revised manuscript.

3. The electron microscopy-density of CF11 seems to exhibit little misalignment with its molecular counterpart. To ensure a more accurate representation, it is strongly suggested that the authors perform supplementary molecular dynamics simulations of CF11 within the hUTA2 structure for a better understanding of the molecular interactions.

Reply: We thank you for your valuable suggestions. We have performed supplementary molecular dynamics simulations of CF11 within the hUTA2 structure for a better understanding of the molecular interactions (Figure R1), which indicated a stable binding pose of CF11 in hUTA2 We have included these data in Supplementary Fig. 10f in the revised manuscript.

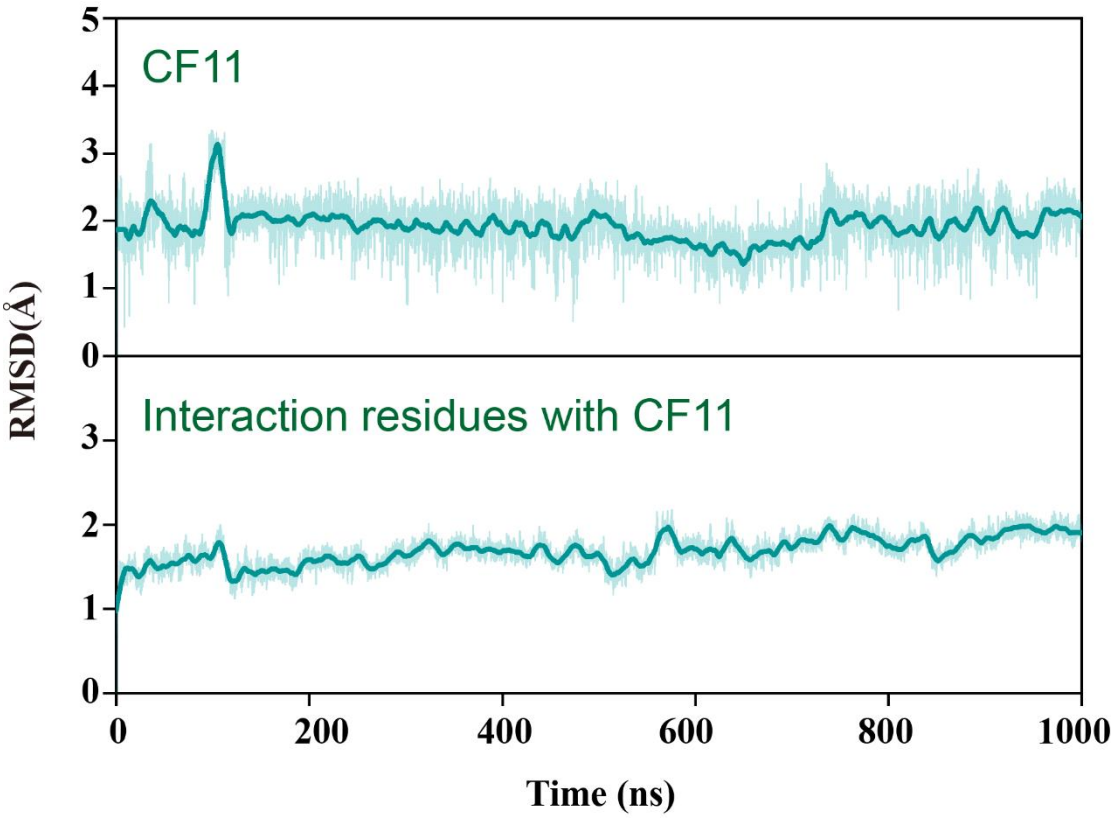

Figure R1. RMSD of CF11(upper panel) and the interacting residues with CF11 (lower panel) in CF11-hUTA2 during 1000 ns MD simulation. The shaded area represents the original, unsmoothed value of distance, with reduced transparency, and the thick line was the result of smoothing the original distance value every 200 points.

4. Literature indicates that urea channels are capable of bidirectional urea transport. The manuscript reveals that UT channel antagonists can bind at either the intracellular, extracellular, or both ends, blocking urea transport. The discovery, implies that certain antagonists, like CF11, require membrane crossing to exert their impact. This intriguing finding holds significant implications for the design and modification of UT antagonists, and it merits extensive discussion in the paper.

Reply: We thank you for your very helpful suggestions. We have calculated the membrane permeability of CF11 (UTA_{inh}-F11) using the schrödinger (Science. 2022 Sep 30;377(6614):eabn7065; Chem Rev. 2019 Sep 11;119(17):10241-10287.; J Chem Inf Model. 2012 Jun 25;52(6):1621-36.). The results indicated CF11 is membrane permeable, with the values of Log Perm RRCK (cm/s) calculated as -4.963. We have included corresponding discussion regarding to the antagonists which binds solely on the intracellular side. They are described as "Our structures reveal that certain UT inhibitors, such as CF11, exclusively bind to the intracellular pocket of the urea transporter. These inhibitors require membrane crossing to exert their inhibitory effect on urea transport, suggesting that it is crucial to take into account the transmembrane efficiency for modifying the UTA_{inh}-F type urea channel blockers as intracellular binders." in the discussion of

the revised manuscript at line 402-405. The data of Table R1 is also included in the Supplementary table 9 in the revised manuscript.

Membrane Permeability Prediction of CF11 (UTA_{inh}-F11)			
[1]Log Perm RRCK (cm/s)	[2]Membrane HDLD	[3]Membrane GB	[4]Membrane *dG Insert
-4.963	10.851	−8.788	11.020

Table R1. Membrane Permeability Prediction of CF11 (UTA_{inh}-F11).
[1]Log Perm RRCK: logarithm of the RRCK permeability in cm/s. This property is optimized to reproduce RRCK permeability assay results, with fitted energy; [2]Membrane HDLD: the free energy penalty for the neutral form of the ligand in its conformation inside the membrane to enter the membrane (i.e., move from the high dielectric region to the low dielectric region, hence HDLD). [3]Membrane GB: an implicit membrane generalized born theory model closely reproduces the Poisson–Boltzmann (PB) electrostatic solvation energy profile across the membrane. [4]*Partition energy "dG" Insert prediction; Membrane dG Insert: the total free energy penalty for the ligand to change state and enter the membrane. This is the net of the energy of Membrane HDLD and Membrane State Penalty.

5. The methodology section on molecular dynamics lacks appropriate citations for the crucial software tools employed in the simulations.

Reply: We thank you for your very helpful suggestions. We have included the reference citations for the crucial software tools employed in the simulations in the revised manuscript.

6. In Figure 7i, the interaction between HQA2 and F282, compared to C25a, should also be a special interaction.

Reply: We thank you for your very helpful suggestions. We have remarked the residue F282 as a special interaction of HQA2 (Figure R2) in Figure 7i in the revised manuscript.

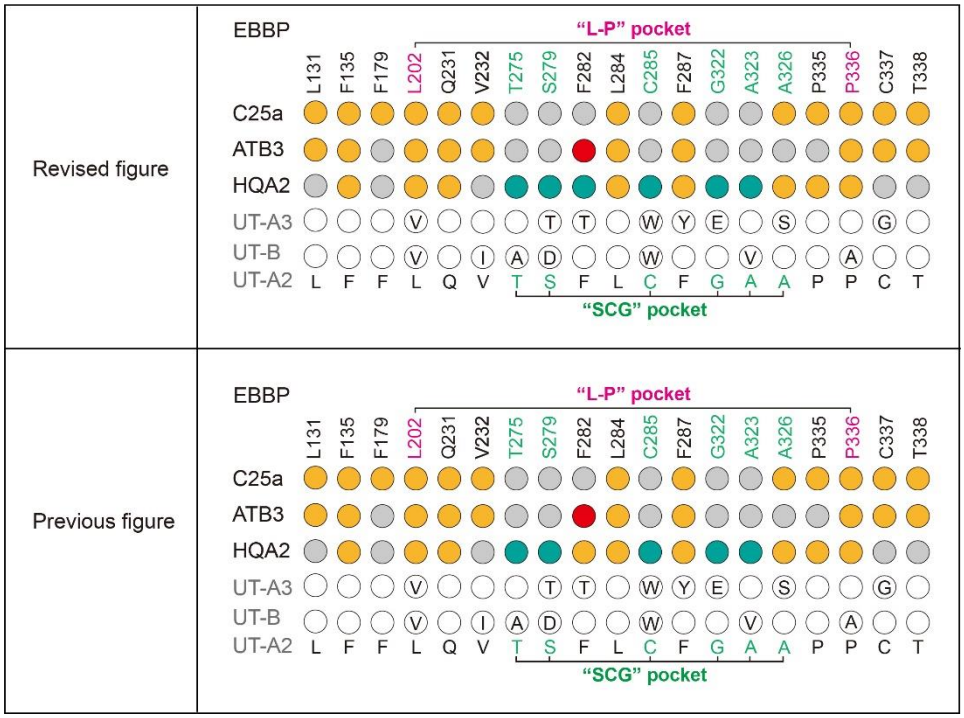

Figure R2. The residue F282R is remarked as a special interaction of HQA2

7. In Figure 8b, a schematic representation, similar to Figure 9b, depicting the urea's entry into the channel would significantly enhance the clarity of the CF11's uncompetitive inhibition behavior.

Reply: We thank you for your very helpful suggestions. We have presented a schematic representation for depicting the urea's entry into the channel (Figure R3) in Figure 8b in the revised manuscript.

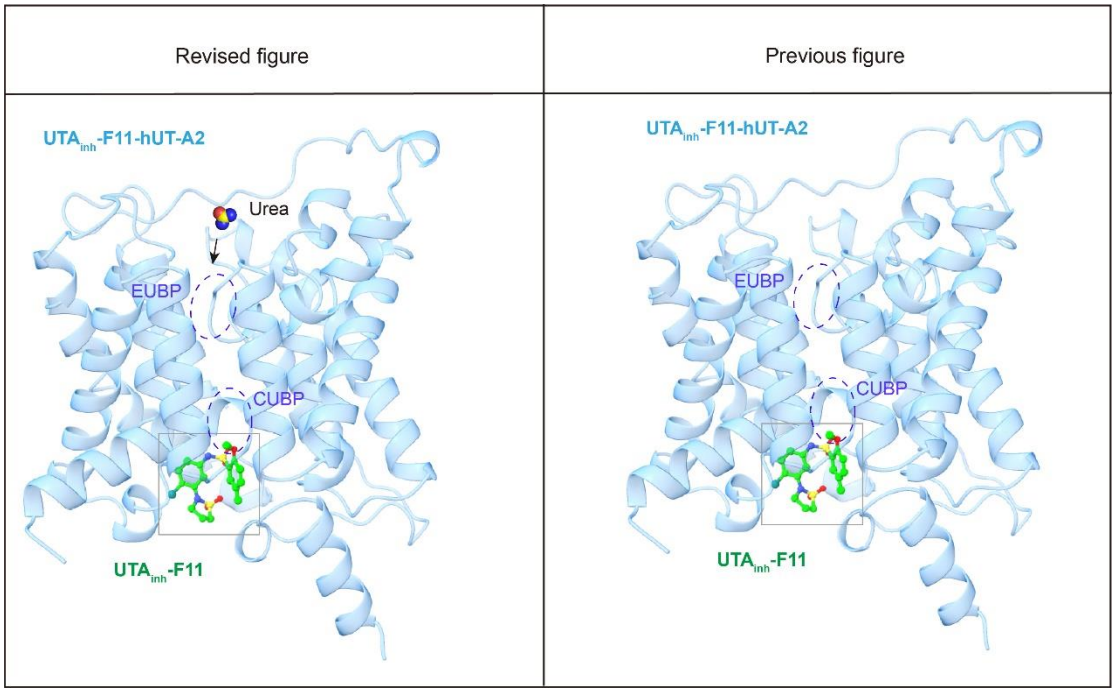

Figure R3. The schematic representation for depicting the urea's entry into the channel bound with CF11.

8. Discrepancies were observed in the data presentation for the map resolution range of structure ATB3-hUT-A2 and the MolProbity score of structure C25a-hUT-A2 in Supplementary Table 1.

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Reply: We thank you for your very helpful suggestions. We have conducted a thorough review and standardized all abbreviations throughout the revised manuscript。

Reviewer #3 (Remarks to the Author):

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Thus, please reconsider this: "Because our current cryo-EM structure of urea-hUT-A2 could not capture the stable interaction of urea molecules inside the Sm region, we performed MD simulation of the process of a urea molecule passing through the Sm region."

Try to extract conformation carefully from biased molecular dynamics because it is not under equilibrium.

Reply: We thank you for your very helpful comments and suggestions. Since the results of molecular dynamics simulations are directly associated with the original structural model used in each manuscript, we have compared the urea-hUT-A2 structure with the previously reported selenourea-bound UT-B structure (PDB: 4EZD) (*Proc Natl Acad Sci U S A. 2012;109:11194–9*). We found that our urea molecules are closer to the Sm region compared to selenourea molecules in the So and Si regions (Figure R4a), most likely because the radius of selenium atom is approximately 2-fold larger than that of oxygen, preventing selenourea from getting closer to the narrow Sm region (Figure R4a). In the urea-hUT-A2 structure, we found that urea molecules form hydrogen bonds with the hydroxyl groups of $T338^{5b}$/$T176^{5a}$ in the So/Si region (Figure R4b), whereas in the selenourea-bUT-B structure, there is no similar interaction between selenourea and bovine UT-B (Figure R4c). Therefore, the MD simulations based on the urea-hUT-A2 structure have demonstrated the conformational changes in $T338^{5b}$/$T176^{5a}$, which may be related to the initial structure model used in the simulations.

According to your suggestion, we have changed the collective variables in the molecular dynamics simulations. In the updated system, we have set CV1 as the distance between the center of mass of urea and residue $Q67^{Pa}$, whereas CV2 has been modified to the dihedral angle of residue $T338^{5b}$ located in the upper half of the channel (Figure R5a-c). We observed that as urea molecules pass through the Sm region, there is a conformational rotation of the side chains of $T338^{5b}$/$T176^{5a}$, which involves the formation of a hydrogen bond between the hydroxyl groups of $T338^{5b}$ and $T176^{5b}$, leading to a change in the orientation of their side-chain methyl groups, pointing towards the Sm cavity (Figure R5d-e). This is accompanied by the formation of a hydrophobic cavity in the Sm region, along with the hydrophobic side chains of

V232^{Pb}/V68^{Pa} and L127^{3a}/L291^{3b} (Figure R5f). Regarding to the conformational changes of T338^{5b}/T176^{5a}, the urea molecules move into the Sm region and engage polar interactions and hydrogen bonds with the hydroxyl groups of T338/T176, as well as with the carbonyl groups of V232^{Pb}/V68^{Pa} (Figure R5e). Moreover, the polar urea molecules interact with the hydrophobic side chains of T338^{5b}/T176^{5a}, V232^{Pb}/V68^{Pa} and L127^{3a}/L291^{3b} through van der Waals forces in the Sm region (Figure R5f). Consequently, the energy for the urea molecules in the Sm region is approximately 2.7-3.4 kcal/mol higher than that of Si or So sites (with an energy barrier with a maximum ΔG of approximately 8.0 kcal/mol) (Figure R5b-c). Similar results in energy calculations also observed in the simulation of urea-zf-UT (Figure R6a-b). Although there are different conformational rearrangements of T338^{5b}/T176^{5a} in the MD simulation of urea-hUT-A2 structure compared to selenourea-bUT-B structure, the thermal stability of urea molecular in the Sm region is lower than that in the Si and So regions (Figure R5b-c, R6a-b), which is consistent with the energy distribution observed in the MD system based on the selenourea-bUT-B structure. These results indicate that the urea molecules cannot keep stable states in the Sm region, and instead, they quickly pass through the Sm region from the So region and enter the Si region, facilitating the transmembrane transportation.

In summary, our data and calculation indicated that the Sm region is a critical structural domain for UT to regulate urea transport, and the movement of urea through the Sm site is the rate limiting step in urea transportation. We have integrated the above data and discussion into the revised manuscript, and have modified the description at line 183-185 to "Because our current cryo-EM data of urea-hUT-A2 could not capture clear EM density inside the Sm region, we performed MD simulation to explore the process of urea molecule passing through the Sm region".

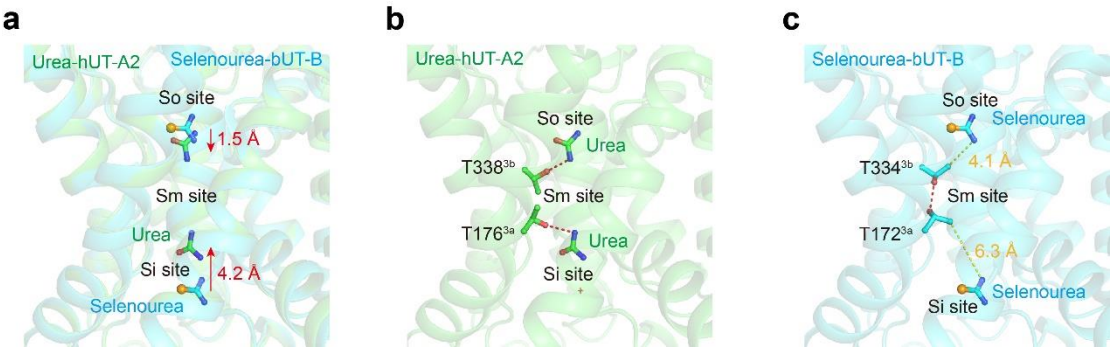

Figure R4. Structural comparison of the binding position of urea/selenourea in urea-hUT-A2 structure and selenourea-bUT-B structure.

a. Different binding position of urea/selenourea in urea-hUT-A2 structure (green) and selenourea-bUT-B structure (blue). The urea molecules in So site and Si site respectively shift by 1.5 Å and 4.2 Å closer to Sm site relative to the selenourea molecule.

b. The urea molecules form H-Bond interaction (red dashed line) with T338^{5b}-T176^{5a} in the urea-hUT-A2 structure.

c. The hydroxyl groups of T334[5b]-T172[5a] form stable H-Bond interaction but engage no interaction with the selenourea molecules in selenourea-bUT-B structure.

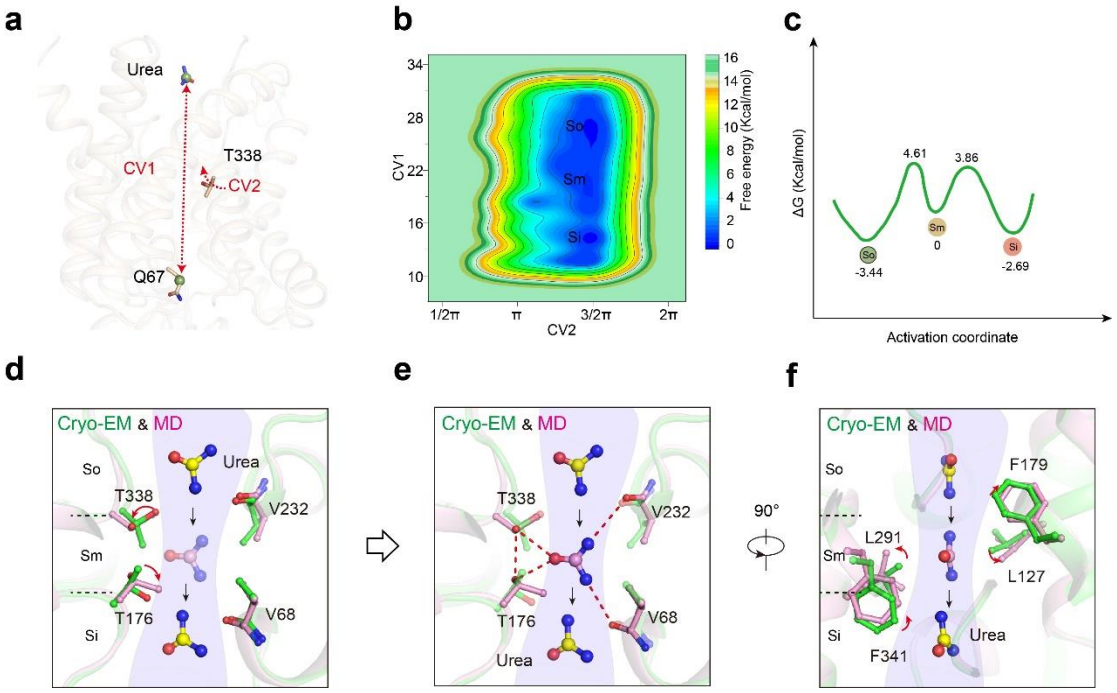

Figure R5. Metadynamics analysis of urea permeation mechanism of hUT-A2.

a. The collective variables in the molecular dynamics simulations. The CV1 have set as the distance between the center of mass of urea and residue Q67[Pa], while CV2 has been modified to the dihedral angle of residue T338[5b] located in the upper half of the channel.

b. Free energy surface (FES) of urea within the hUT-A2 channel. The simulation reveals three distinct low-energy states, which are characterized by prolonged urea residence times across defined regions within the channel and corresponds to the So, Sm, and Si regions respectively.

c. Simplified one-dimensional energy landscape highlighting the So, Sm, and Si states of urea within the hUT-A2 channel.

d-f. Metadynamics simulations elucidate the passage of urea through the Sm region. This process involves a rotation of approximately 40° in the side chains of T176[5a] and T338[5b], facilitating the rearrangement of T176[5a]-T338[5b] side chains (d). Notably, hydrogen bond formations between urea and specific residues, including T338[5b] and T176[5a], are depicted (e). The rotation of side chains of T338/T176 and structural rearrangement of L127/L291form a hydrophobic cavity in the Sm region, guiding urea transportation across the channel (f). The H-bond are shown as red dashed line. The black arrows indicate the direction of movement of the urea molecule.

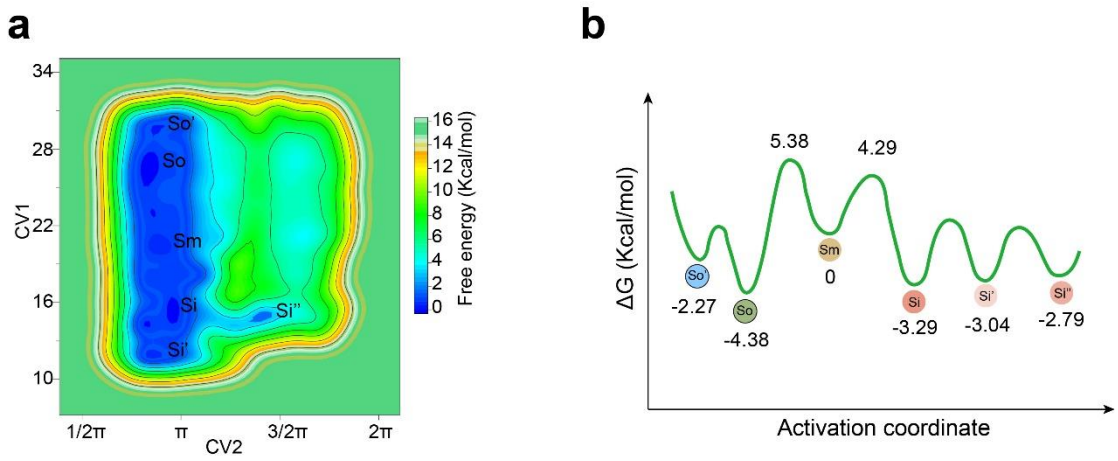

Figure R6. Free energy surface (FES) of urea within the zf-UT channel.

a-b. Simplified free energy landscape highlighting the So', So, Sm, Si, Si' and Si'' states of urea within the zf-UT channel (a). The free energy surface of urea's passage through zf-UT shows the conformational states of So', So, Sm, Si, Si' and Si'', as well as a simplified qualitative one-dimensional energy landscape (b).

2. I am very interested in why authors chose these 4 specific small organic molecules to represent competitive, uncompetitive and noncompetitive inhibitors. Could authors give some reasons and the original reference papers where these molecules come from?

Reply: We thank you for your very helpful comments and suggestions. For decades, urea transporter inhibitors hold promise as prospective novel diuretics. Notably, professor Yang (one of our correspondence authors, a world-famous urea transporter research expert) has push his efforts in developing urea transporter inhibitors for more than 30 years and maintained a long-standing international collaboration with the other laboratories. Several inhibitors used in this study are originally developed by Yang's team, and several are gifts from the collaborative laboratories years ago or synthesized by Yang's team. These inhibitors have been demonstrated with diuretic effects in vivo (*Eur J Med Chem. 226:113859*), or may exhibit selectivity towards UTA (*Chem Biol. 20(10):1235-44; FASEB J. 28(9):3878-90*) (Table R2, also see Supplementary table 4). However, the molecular mechanism by which they interact with the UT transporters is yet to be fully understood. After solving their high-resolution structures binding to UTA, we found that they exert their inhibitory effects through the competitive, uncompetitive or noncompetitive binding modes. We have cited the original reference papers of these inhibitor in the revision manuscript.

Inhibitor	IC50 (μM)			inhibition mode	Reference
	UT-A2	UT-A1	UT-B		
C25a	0.58 ± 0.01	＜8.0	0.63 ± 0.01	competitive	*Eur J Med Chem. 226:113859*
ATB3	6.38 ± 0.54	~ 7.0	~ 43	competitive	*Chem Biol. 20(10):1235-44*
CF11	2.78 ± 0.32	~ 1.0	~ 10.0	un-competitive	*FASEB J. 28(9):3878-90*
HQA2	3.66 ± 0.09	~ 5.3	＞50	non-competitive	*Chem Biol. 20(10):1235-44*

Table R2. The IC_{50} and inhibition modes of the different UT inhibitors.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have responded satisfactory to the previous review.

Reply: We thank you very much for your positive comments.

Reviewer #3 (Remarks to the Author):

The authors have carefully answered my questions and tried different collective variables selection. It could be observed that the urea stays in the hydrophobic pocket of sm zone for a while in the cartoon supplement material. The free energy calculation looks consistent with this cartoon, but the local minimal potential at the sm zone is till brought by the calculation. As shown in Fig. 5f, the sm zone is the narrowest part of this channel, that is also a hint of a high energy barrier because the urea has to get rid of waters to pass through, that takes energy. I suggest the authors to recheck the parameters, consult some metadynamics experts **or** express their doubt attitude and explain that it is still challenging to get a reasonable result for the free energy calculation for this channel.

Reply: We thank you very much for your valuable comments and suggestions. In the previous round of revisions, we have rechecked the parameters and tried to simulate the process of urea molecules passing through the urea transport channel by employing different collective variables selection. The two common features of these simulations are that (I) significant energy barriers are found for urea molecules in the Sm region compared to the Si and So regions, though there are numerical differences in the size of the energy barriers under different simulation conditions; (II) the valley values of the free energy in the Sm region are higher than those in the Si and So regions, suggesting that urea molecules cannot stably reside in the Sm region. These results are consistent with the structural information of the urea-bound UTs shown in this manuscript, and the MD simulation of urea permeation processes described by Ming Han et al. on the other UT structures (Table R1, corresponding to Supplementary Table 9) *(Molecular Simulation, 47(12), 1022–1028.; Proc Natl Acad Sci U S A. 2012;109:11194–9; Nat Commun. 2013; 4:2900.)*. According to your suggestions, it is worth to note that the calculation of the free energy changes in urea molecules passing through the transport channel is still challenging and variable, because urea needs to get rid of water to pass through and the

role of waters in the urea permeation process is not yet clear *(J Phys Chem B. 2015 Apr 23;119(16):5124-31.; J Biol Chem. 1998 Apr 17;273(16):9369-72.)*. There is also a lack of structural information for urea molecule interacting with the Sm region. Therefore, in addition to the results of free energy simulations, we also combined the structural information and biochemical experimental data to propose a potential urea permeation mechanism of UT in this paper. We have discussed the above concerns on the MD stimulation in the revised manuscript at line 378-384 and line 395-396.

Table R1. The statistic of the free energy in MD stimulation on different UTs.

Structural models	Valley values of the free energy ΔG(kcal/mol)			Energy barriers $\Delta\Delta$G(kcal/mol)		Reference
	So	Sm	Si	So-Sm	Si-Sm	
hUT-A2-Urea	-3.4	0	-2.7	8.0	6.6	This paper
zf-UT-Urea	-4.4	0	-3.3	9.8	7.6	This paper
hUT-B (PDB: 6QD5)	-2.4	3.0	-2.5	6.0	6.5	*Molecular Simulation, 47(12), 1022–1028.*
Bos-UT-B (PDB: 4EZC)	0	3.0	0	4.0	4.8	*Proc Natl Acad Sci U S A 109: 11194-11199*
*Hp*UreI (PDB: 3UX4)	0	1.3	0.3	2.1	3.1	*Nat Commun. 2013;4:2900.*

Reviewer #4 (Remarks to the Author):

Overall Comments

The authors present impressive work on uncovering the molecular mechanisms of urea channels and inhibition mechanism with a series of high-resolution structures, molecular dynamics and biophysical assays. While I find the results of the manuscript publication-worthy, I recommend that Nature Communications sends it back to the authors with serious reservations due to the reason below.

The cryo-EM maps presented in this work and provided to the journal, which I assume were also deposited to PDB, appear to have been generated with DeepEMhancer (as stated in the methods). This is evident in the way the map looks at lower contours. For example, with the human UT-A2/C25a map, 1) at contour level of 0.1, the map looks mask-ish; 2) at 0.00001 contour level, computational artefacts such as regular noise distributions are clear; and 3) detergent belt is not visible at any contour. The use of DeepEMhancer is likely the reason why the inhibitor densities look poor and rounded despite the high nominal and local resolutions (each R-group should be clearly visible at stated resolutions but it is not), as it has tendency to make non-protein densities look worse.

This is not an accepted practice for cryo-EM structure refinement and deposition. Such maps are supposed to be used only for guidance during manual model building/refinement, and should not be used for automatic refinement (e.g. Phenix RSR), density assessment/analysis such as those used in the figures, or map/model deposition to PDB.

Reply: We thank you for your valuable comments and suggestions. To refine and deposit structures in line with community expectations and standards in the revised manuscript, we have replaced all the previously submitted maps which were modified with DeepEMhancer with their corresponding unprocessed maps for model building, automatic refinement and PDB deposition, including those of apo-hUT-A2, C25a-hUT-A2, apo-hUT-A3, apo-hUT-B, apo-zf-UT, urea-zf-UT, and C25a-zf-UT complexes. The newly refined models of these complexes are almost the same as the previously submitted models, except for the C25a-hUT-A2 complex. However, although the C25a-hUT-A2 structure revealed some subtle changes in the ligand binding pose (Figure R1), these changes did not alter the interactional patterns between C25a and hUTA2 (Table R2, corresponding to Supplementary table 5). Therefore, these maps and models still support the original conclusions in the manuscript. We have updated the relevant density assessment/analysis based on these currently submitted unprocessed maps and their corresponding models, including Fig. 2; Fig. 5a-b; Fig. 6b-f; Supplementary Fig. 2a-e, l-q; Supplementary Fig. 4-5; Supplementary Fig. 7a; Supplementary Fig. 8 and Supplementary Fig. 9c-d. The data collection and refinement statistics for these complexes have been updated in Supplementary table 1-3, and a newly produced PDB validation reports of all the structures are

provided with the resubmission.

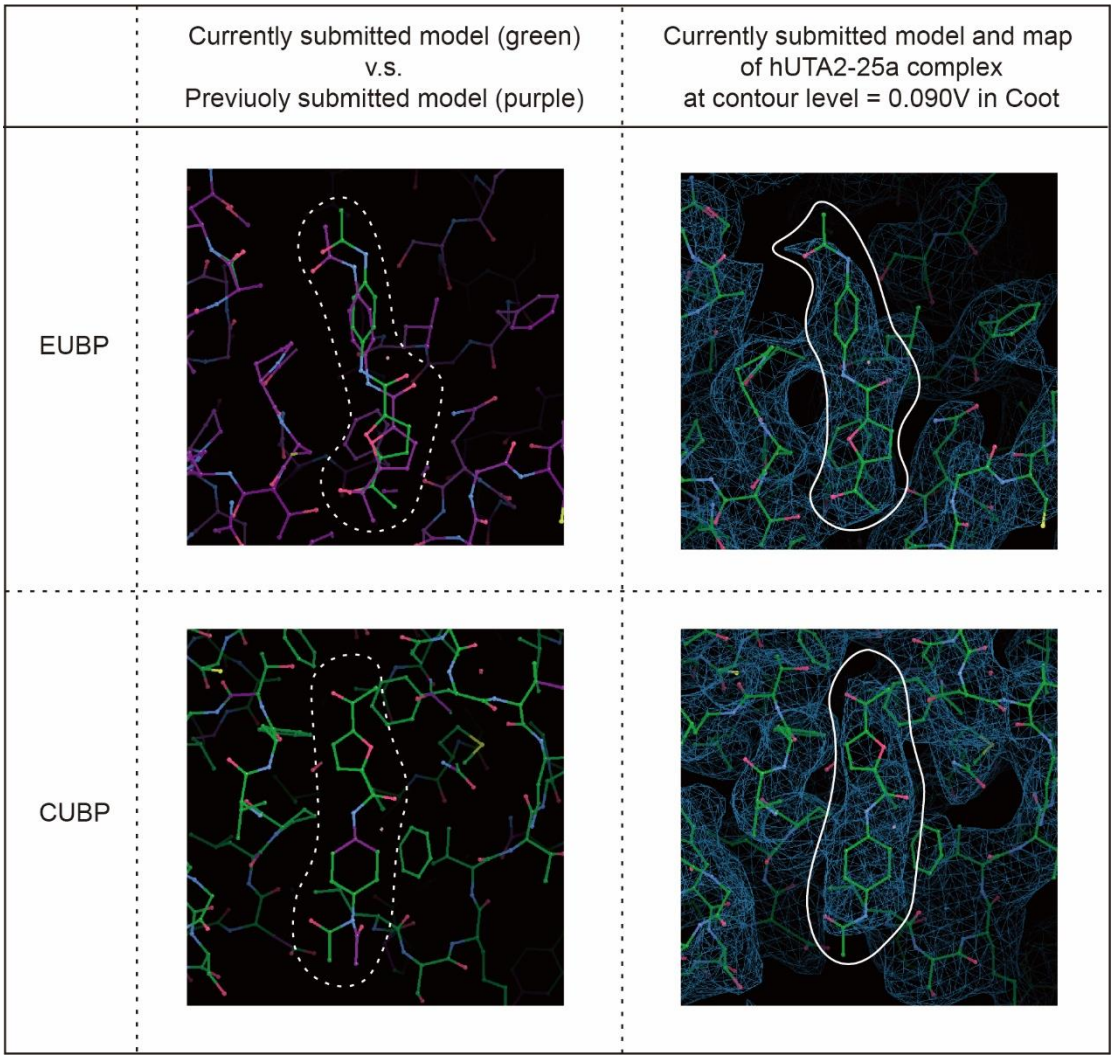

Figure R1. The left panel showed the structural comparison of C25a (surrounded by white dash line) in the extracellular blocker binding pocket (EBBP) and cytoplasmic blocker binding pocket (CBBP) between the currently submitted model (green) and the previously submitted model (purple) of hUTA2-C25a complex. The density of C25a of the currently submitted map of hUTA-C25a complex are shown on the right pane, which are highlight with the white line circle in Coot at the contour level of 0.090 V.

Table R2. The interactional patterns between C25a and hUT-A2

Residues	Interaction	Distance (Å)
Extracellular blocker binding pocket		
L131	Hydrophobic or van der Waals forces≤4.0 Å	3.3
F135		3.9
F179		4.0

L202		3.7
Q231	Polar interaction ⩽4.0 Å	3.9
V232	Hydrophobic or van der Waals forces⩽4.0 Å	2.9
L284		3.8
F287		3.5
A326		4.0
P335		3.6
P336		3.6
C337		3.6
T338	H-Bond ⩽3.5 Å	2.5
Cytoplasmic blocker binding pocket		
Q67	Polar interaction ⩽4.0 Å	3.9
V68	Hydrophobic or van der Waals forces⩽4.0 Å	3.2
F70		3.0
F120		3.3
Y123		3.3
P173		3.8
F175		3.3
T176	H-Bond ⩽3.5 Å	3.1
F300	π-π stacking ⩽6.0 Å	4.5
F341	Hydrophobic or van der Waals forces⩽4.0 Å	3.6
L363		3.8