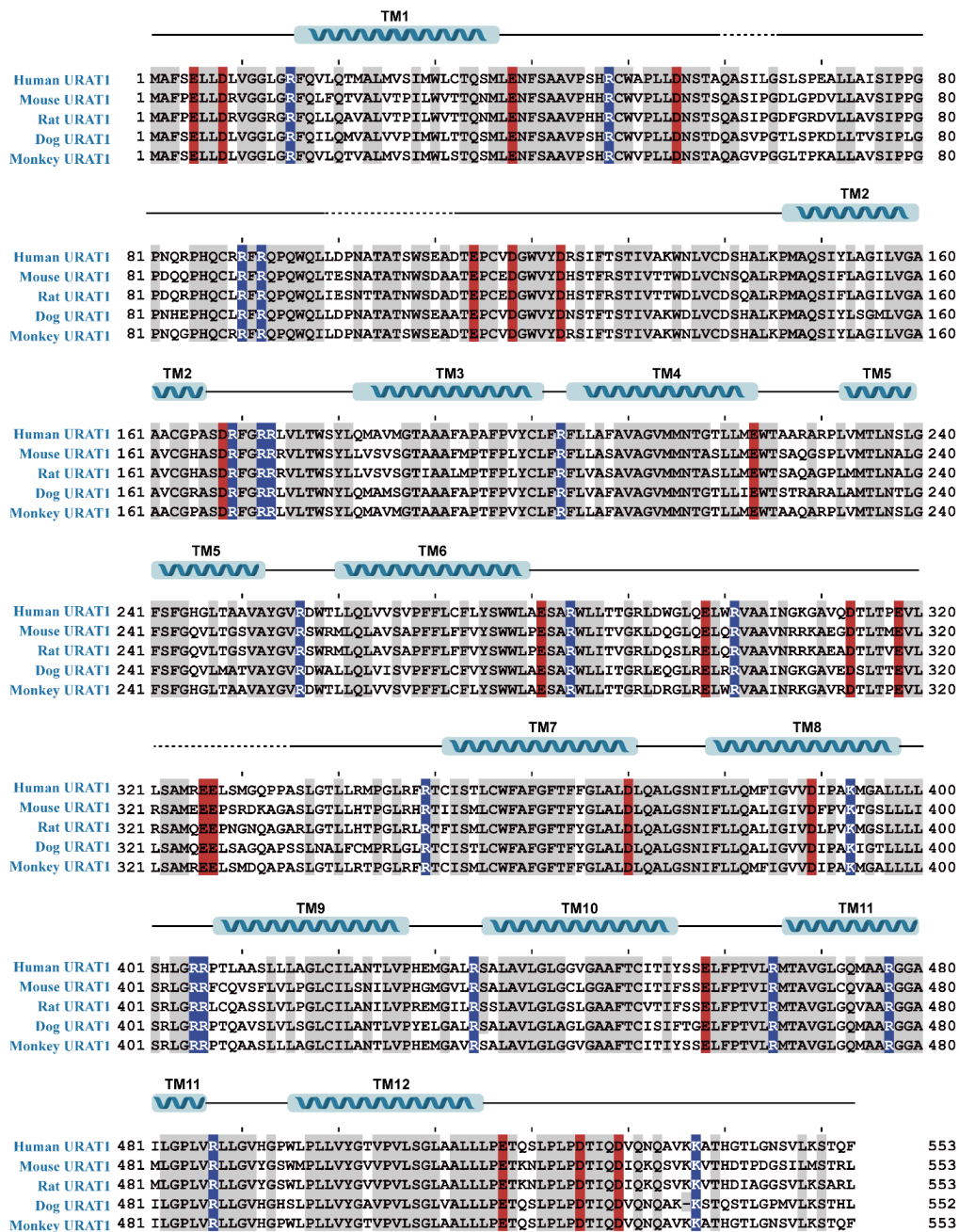


Supplementary Information for

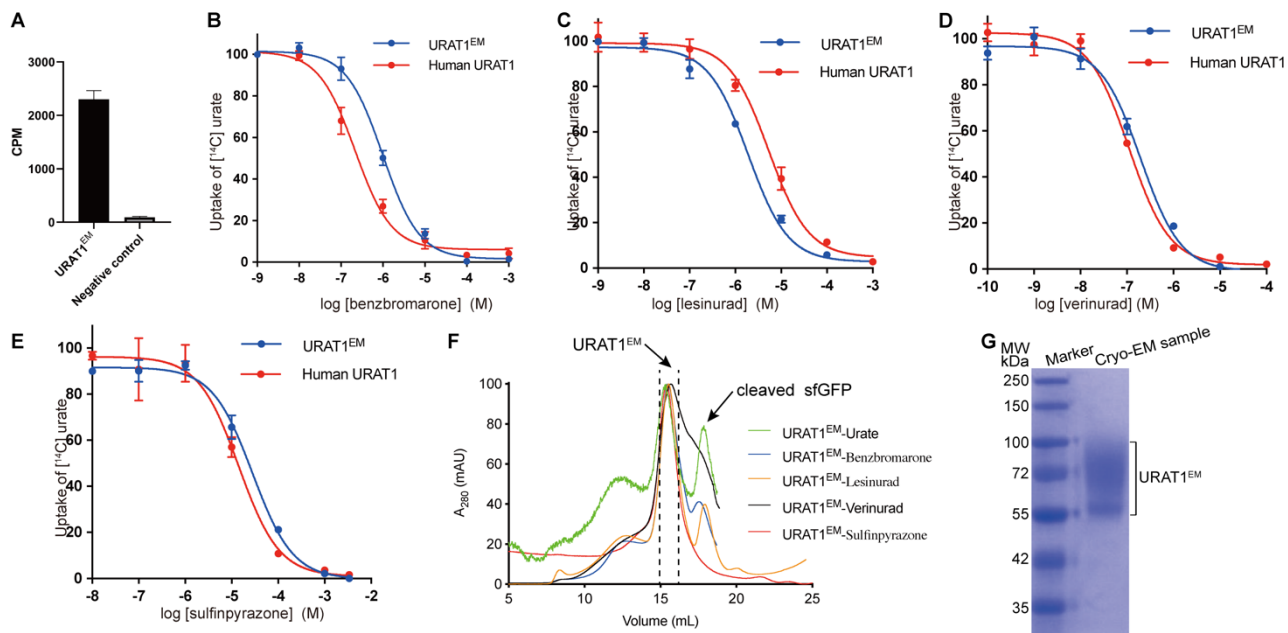
Molecular mechanism of drug inhibition of URAT1

This document contains Supplementary Figures 1-17 and Supplementary Tables 1-2.



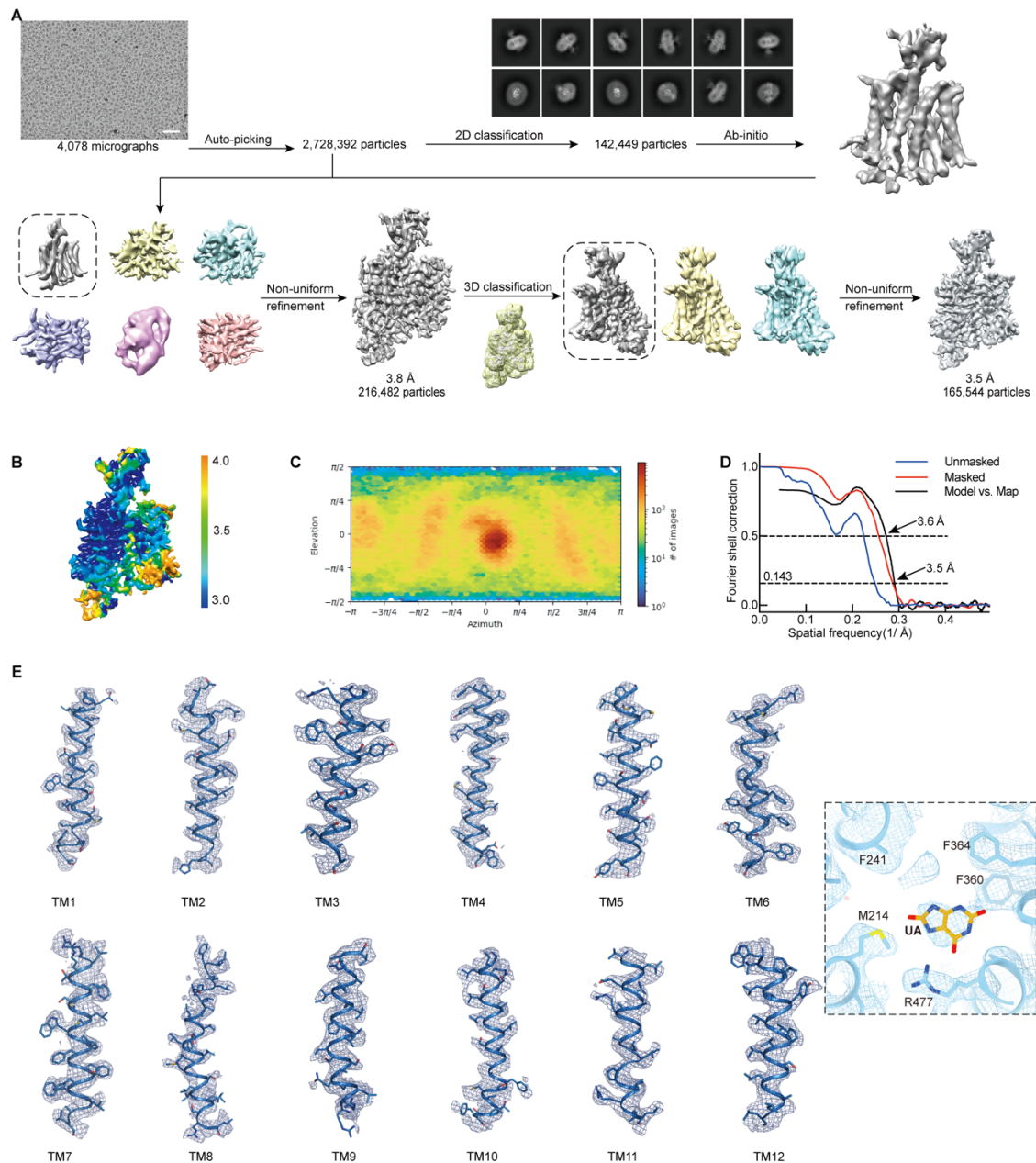
Supplementary Fig. 1 Sequence alignment of URAT1 from different species.

Secondary structural elements of URAT1 from different species are depicted above the sequence alignment. Highly conserved amino acids are highlighted with gray shadows, while positively and negatively charged amino acids shaded in blue and red, respectively. The sequences were retrieved from the UniProt database, with the following accession numbers: human (Q96S37), mouse (Q8CFZ5), rat (Q3ZAV1), dog (*Canis lupus familiaris*, I7HI71), and monkey (*Macaca mulatta*, I7HH13).



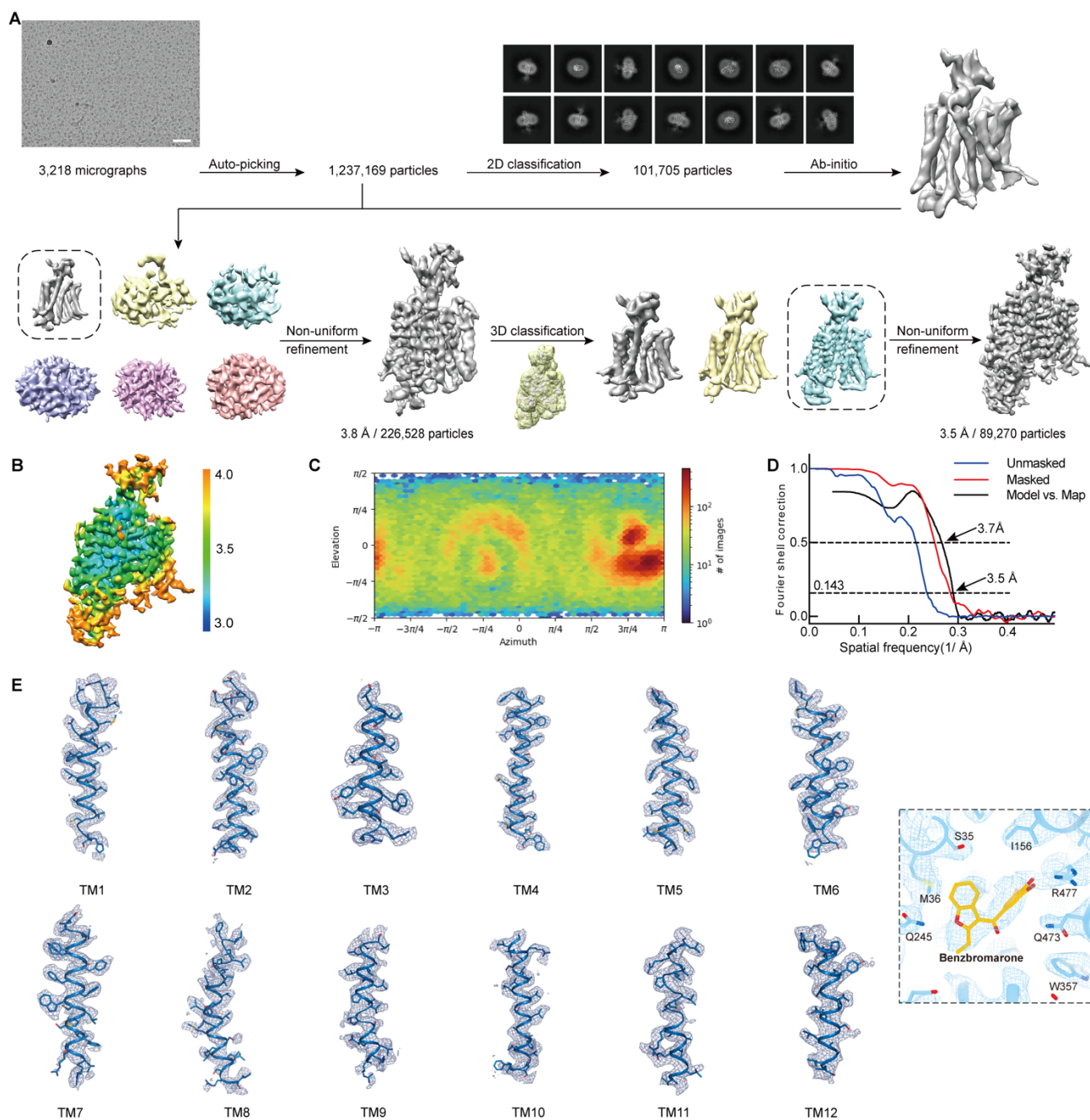
Supplementary Fig. 2. Functional characterization and purification of URAT1^{EM}.

(A) The transport activity of URAT1^{EM}, quantified by [¹⁴C] urate uptake assay. Negative control is untransfected HEK293 cells. Data are mean mean $\pm$ s.e.m. from three independent assays, each with one measurement ($n = 3$). (B-E) Inhibition of [¹⁴C] urate uptake double-mutant rat URAT1 (URAT1^{EM}) and human URAT1 (hURAT1) by benzbromarone (B), lesinurad (C), verinurad (D), and sulfinpyrazone (E). Data represent mean $\pm$ s.e.m. obtained from three biological experiments. The normalized data of inhibition assay were fitted using non-parametric nonlinear regression. (F) Gel-filtration profile of URAT1^{EM} in LMNG, showing the SEC spectra for each sample: URAT1^{UA} (green), URAT1^{BEN} (blue), URAT1^{LES} (orange), URAT1^{VER} (black), and URAT1^{SPZ} (red). (G) Coomassie-blue-stained SDS-PAGE gel of URAT1^{EM} in LMNG.



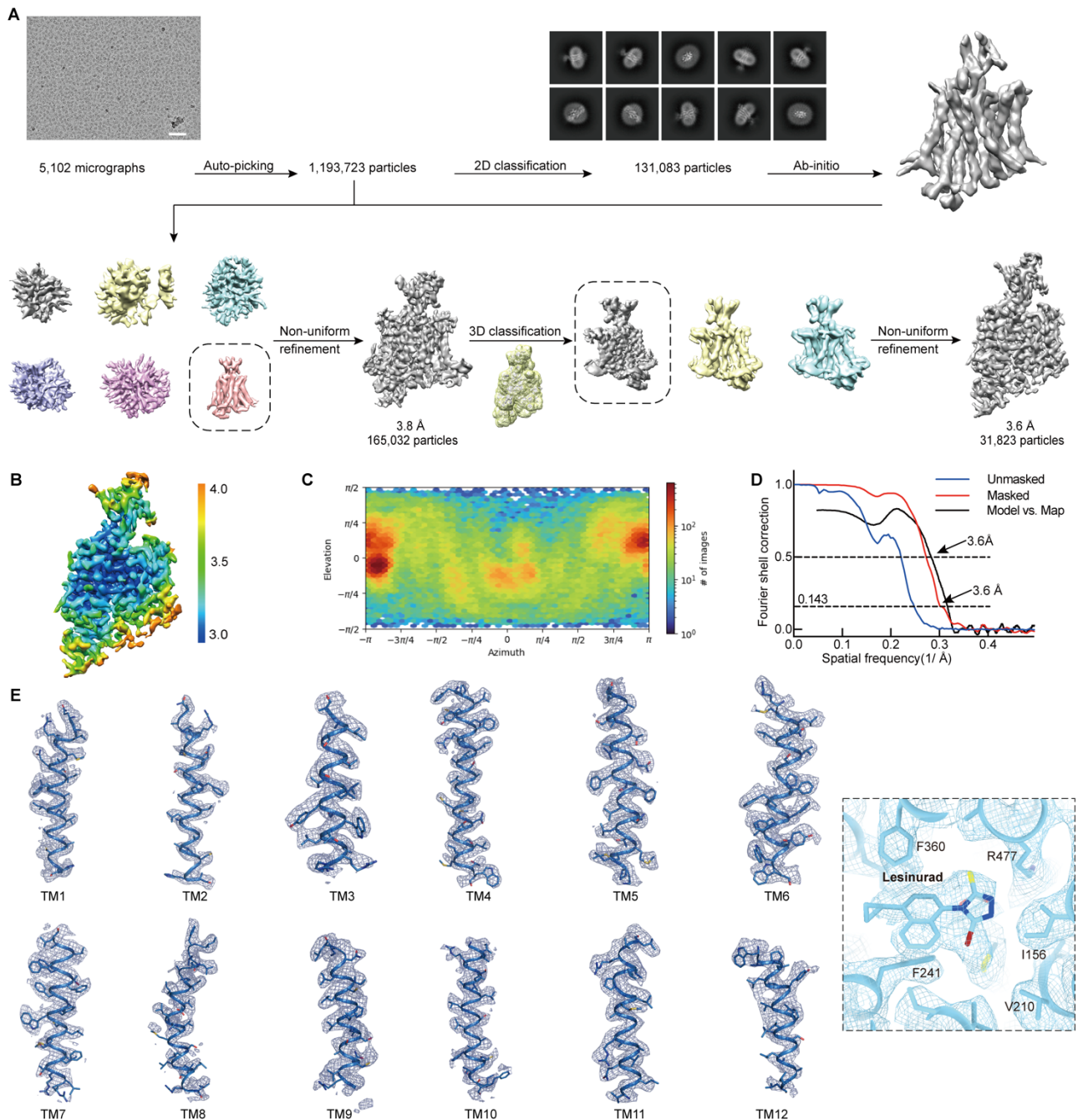
Supplementary Fig. 3. Cryo-EM data analysis of the URAT1^{UA}

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



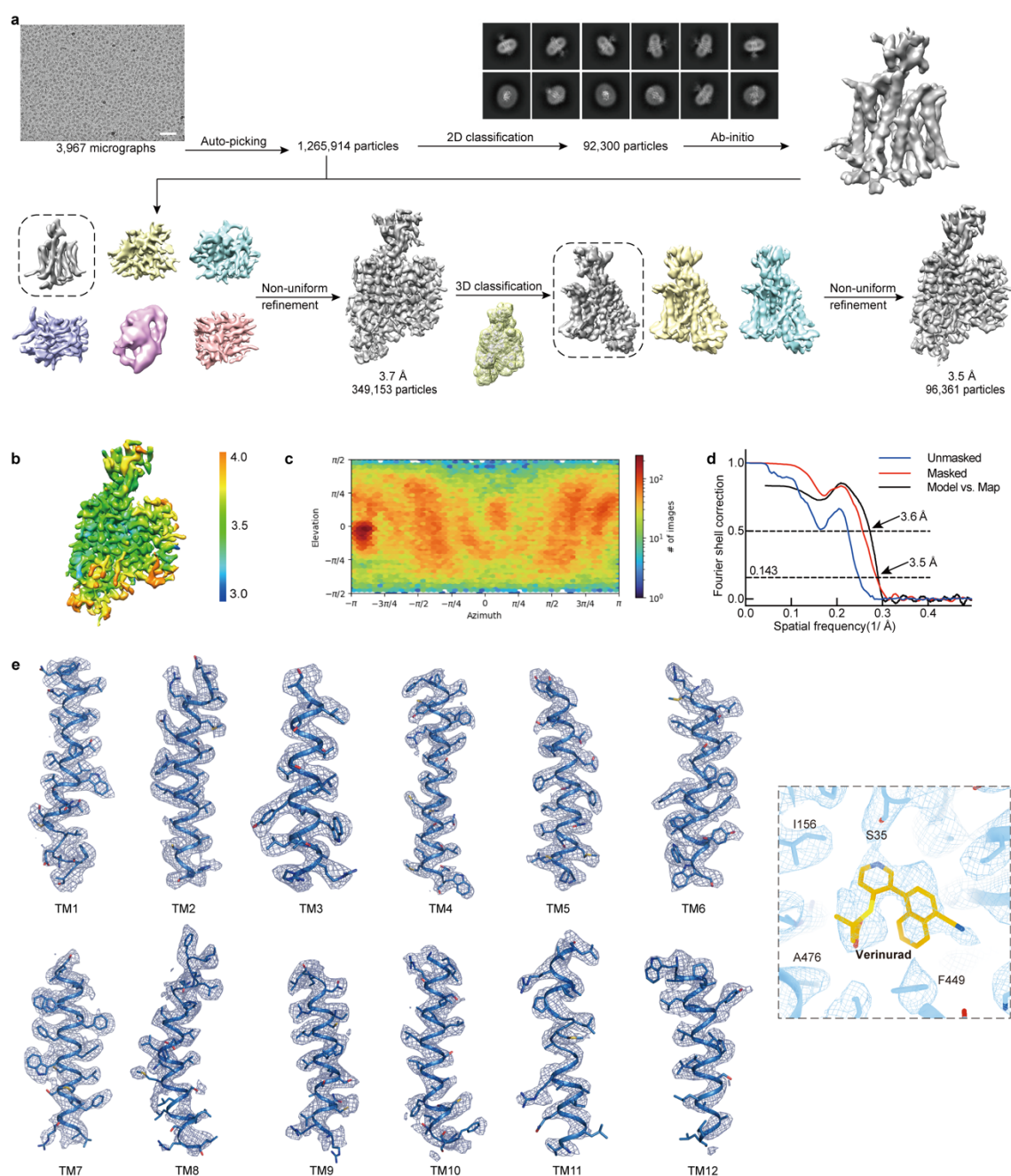
Supplementary Fig. 4. Cryo-EM data analysis of the URAT1^{BEN}.

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



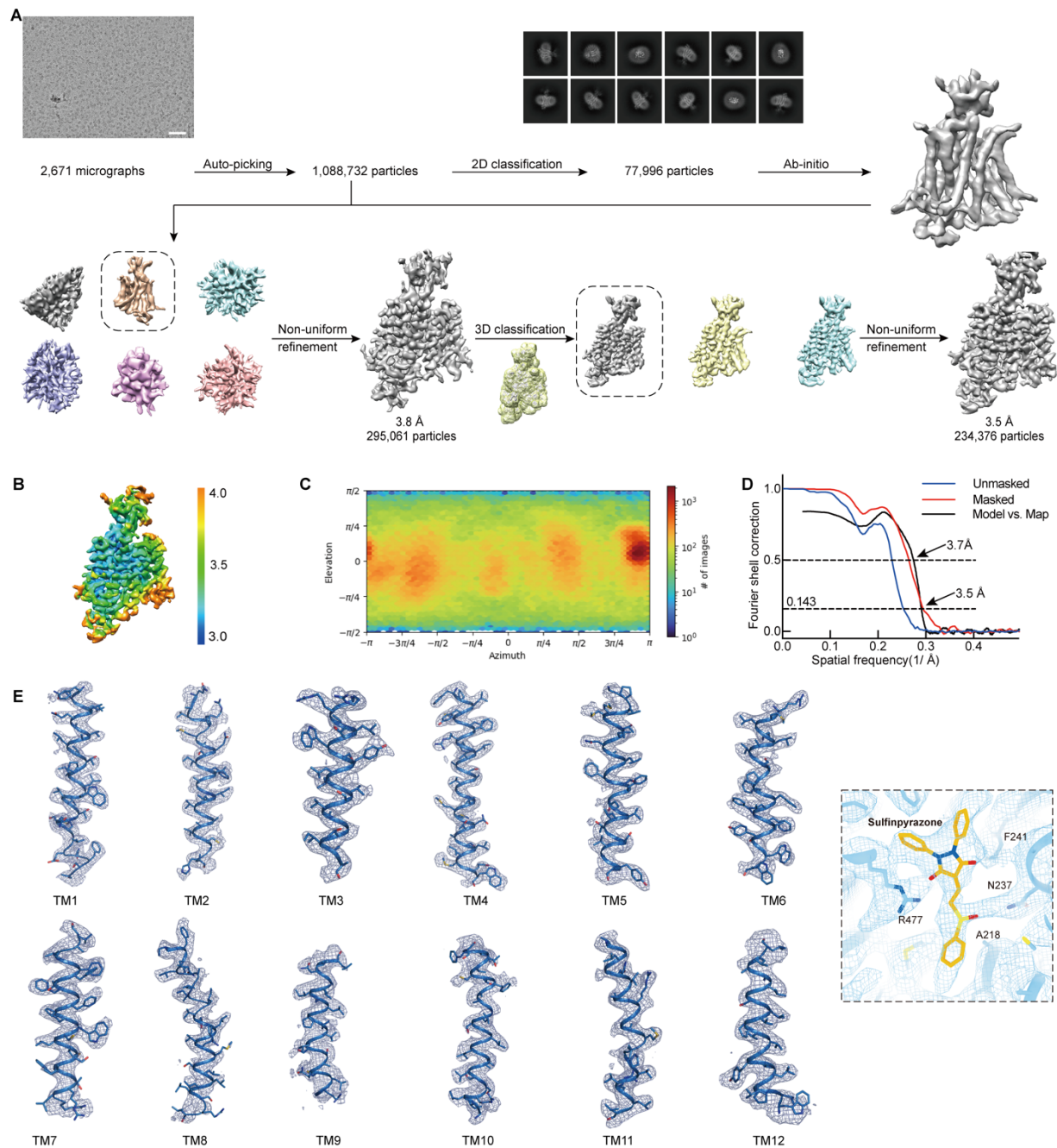
Supplementary Fig. 5. Cryo-EM data analysis of the URAT1^{LES}.

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



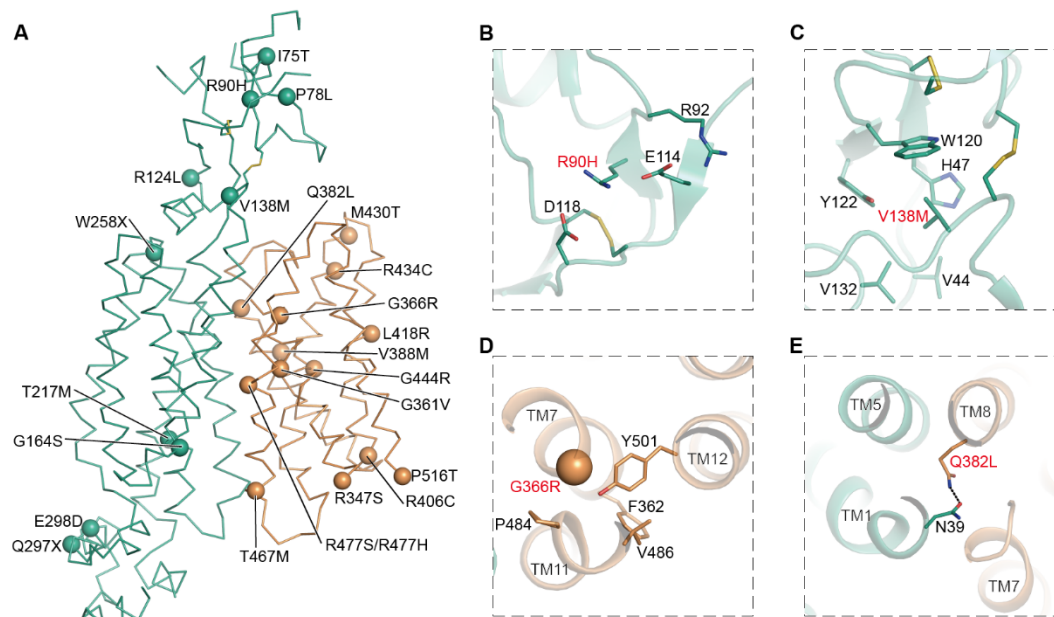
Supplementary Fig. 6. Cryo-EM data analysis of the URAT1^{VER}.

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



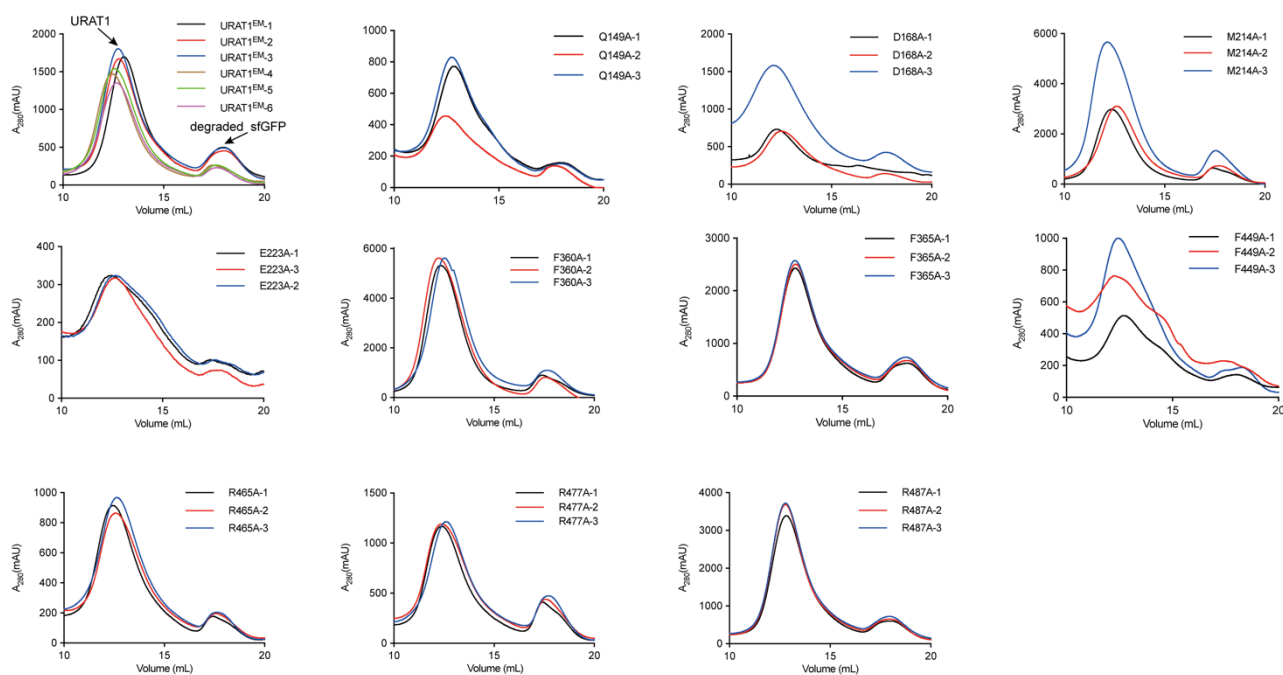
Supplementary Fig. 7. Cryo-EM data analysis of the URAT1^{SPZ}.

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



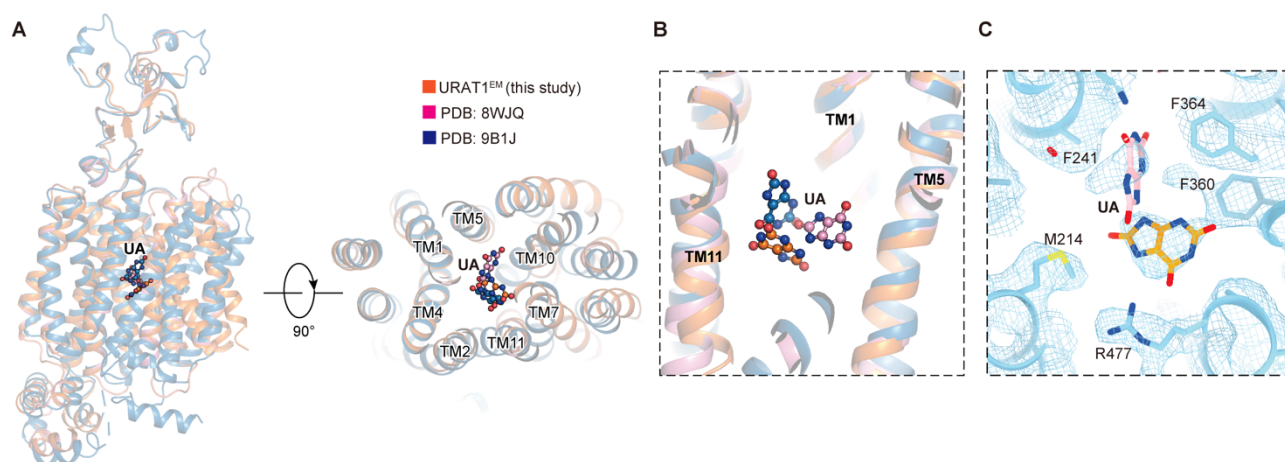
Supplementary Fig. 8. Distribution and interaction of the pathogenic mutations in URAT1

(A) Side view of the structure of URAT1^{UA}. The N- and C-terminal domains of URAT1^{UA} are shown in green and orange, respectively. The C α atoms of the pathogenic mutations are shown in spheres. (B-E). The interaction modes of four pathogenic residues, including R90 (B), V138 (C), G366 (D) and Q382 (E). The key residues for interactions are shown in sticks.



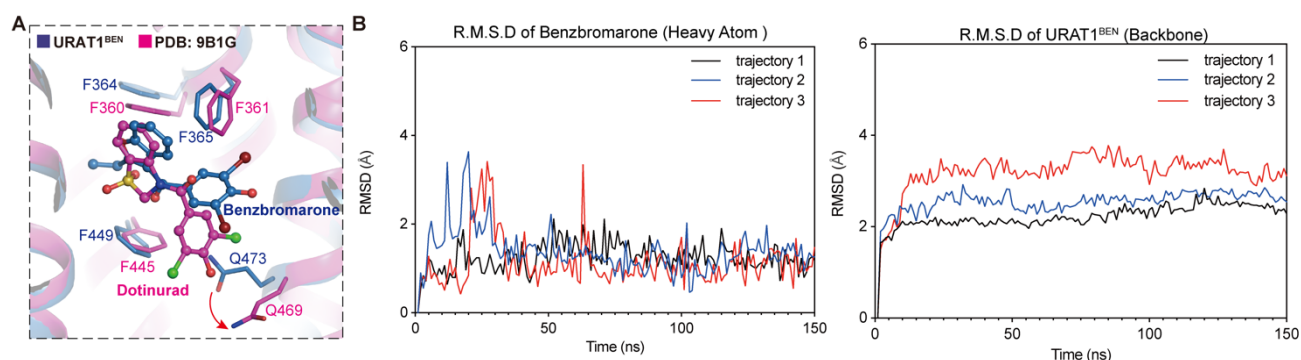
Supplementary Fig. 9. The SEC spectra of URAT1^{EM} and mutants.

The peak values within the 10-15 mL range of these spectra were used for normalization of expression levels in the transport assays.



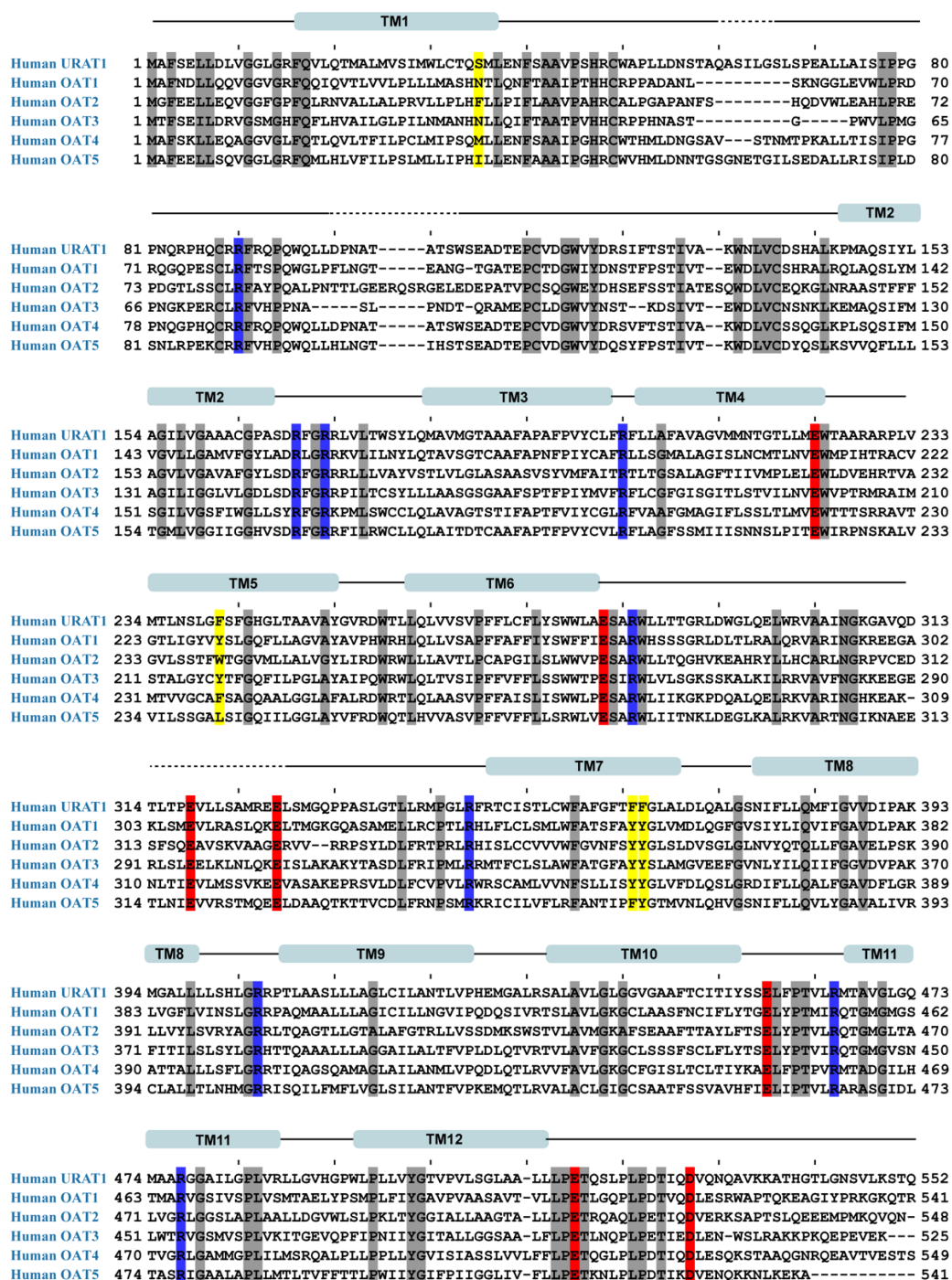
Supplementary Fig. 10. EM density of urate binding pocket and distinct urate binding modes.

(A-B) Superposition of urate-bound URAT1 structures based on alignment of the N-domains. The structures are colored orange (URAT1^{EM}, this study), pink (hURAT1, PDB:8WJQ, ref. 45), and blue (hURAT1, PDB:9B1J, ref. 44), respectively. The urate molecules are shown as sticks and spheres. (C) Cryo-EM density of urate (UA) binding pocket. The urate molecule in the URAT1 structure determined in this study (URAT1^{UA}) is colored orange, while the urate molecule in the 8WJQ structure is colored pink.



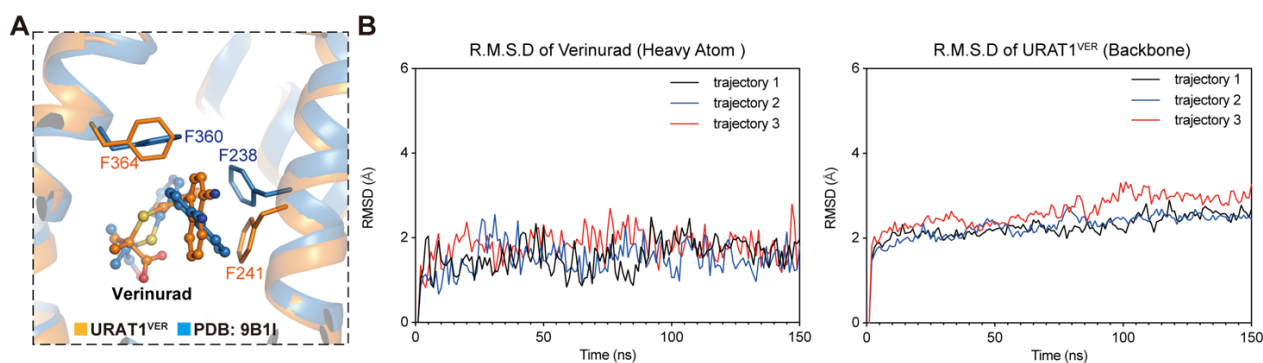
Supplementary Fig. 11. Dynamic stability analysis of benzbromarone binding to URAT1^{EM}

(A) The structure alignment of URAT1^{BEN} and dotinurad-bound human URAT1(PDB: 9B1G, ref. 44). (B) R.M.S.D. plots for ligand and protein backbone during MD simulation. R.M.S.D. plots were generated for both benzbromarone and the protein backbone. The calculation of backbone R.M.S.D. for URAT1^{BEN} using residues 2 to 42, 132 to 324, and 347 to 515. The calculation of benzbromarone R.M.S.D. involved the heavy atoms of benzbromarone, using its initial structure as the reference. Results from three independent trajectories were distinguished by different colors.



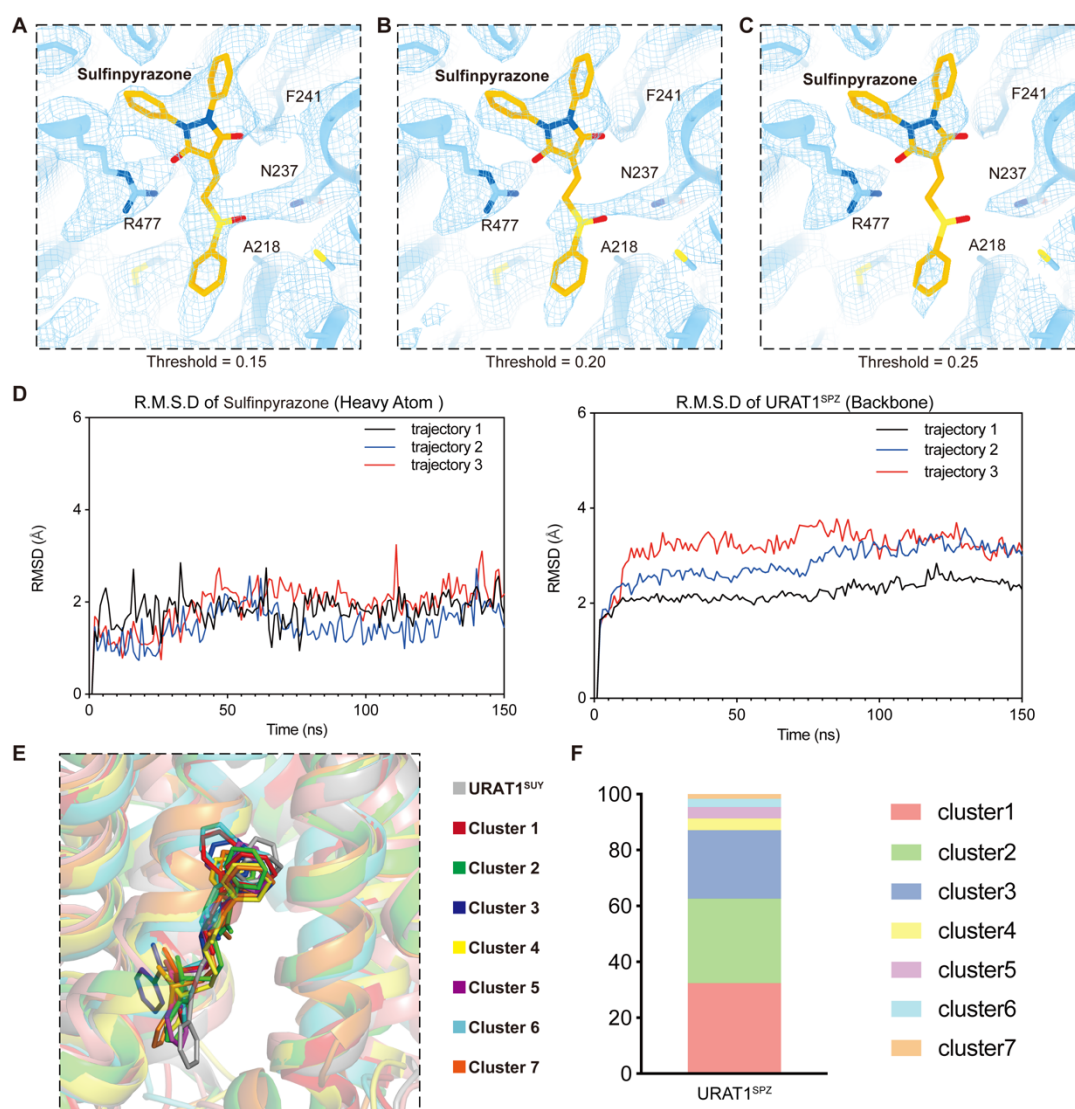
Supplementary Fig. 12. Amino acid sequence alignment of human URAT1 and human OAT1-4

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



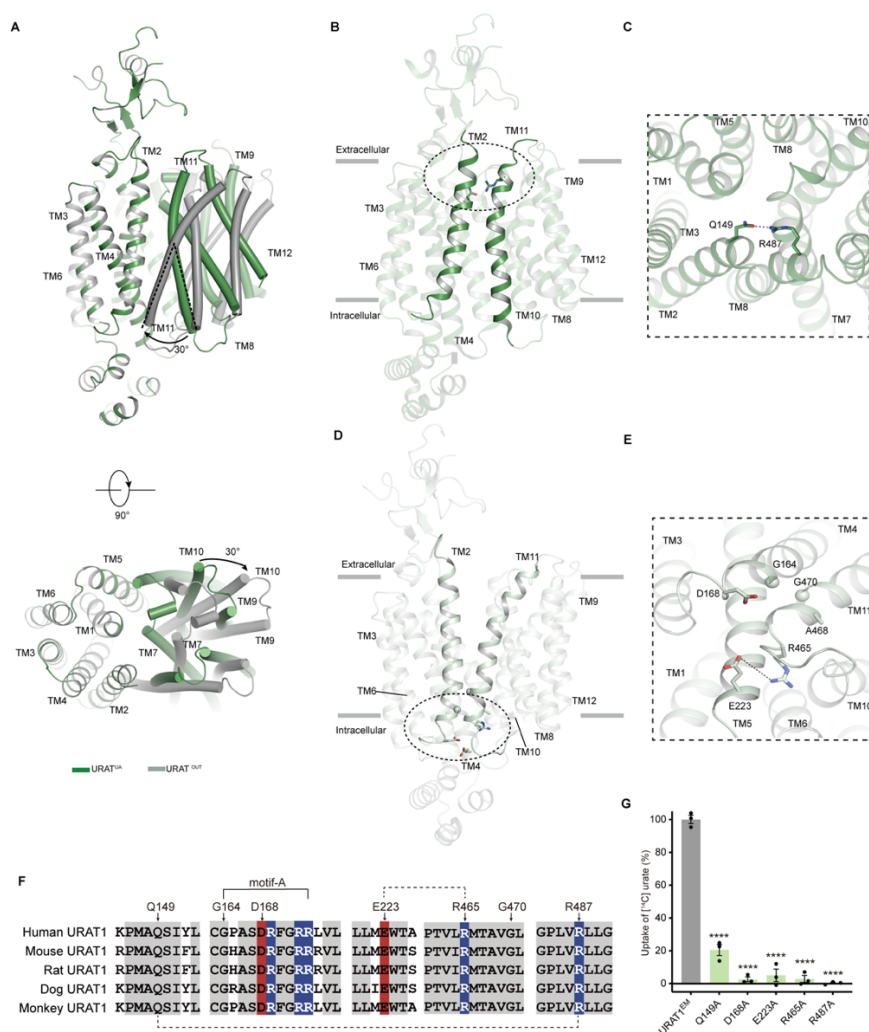
Supplementary Fig. 13. Cryo-EM density and dynamic stability analysis of verinurad binding to URAT1^{EM}

(A) The structure alignment of URAT1^{VER} and verinurad-bound human URAT1 (PDB: 9B1I, ref. 44). (B) R.M.S.D. plots for ligand and protein backbone during MD simulation. R.M.S.D. plots were generated for both benzbromarone and the protein backbone. The calculation of backbone R.M.S.D. for URAT1^{BEN} using residues 2 to 42, 132 to 324, and 347 to 515. The calculation of benzbromarone R.M.S.D. involved the heavy atoms of benzbromarone, using its initial structure as the reference. Results from three independent trajectories were distinguished by different colors.



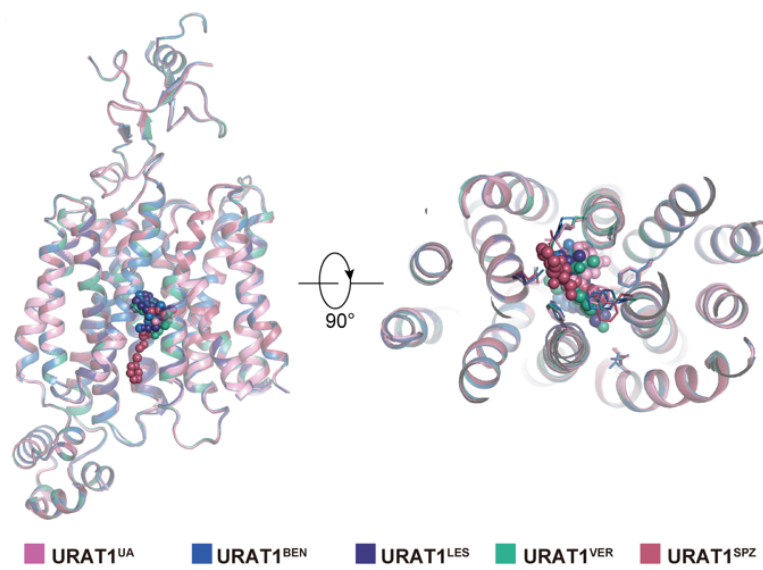
Supplementary Fig. 14. Cryo-EM density and dynamic stability analysis of sulfinpyrazone binding to URAT1^{EM}

(A-C) The cryo-EM densities of sulfinpyrazone and surrounding residues, in different thresholds, are shown as mesh while the structure of which are visualized as sticks. (D) R.M.S.D. plots for ligand and protein backbone during MD simulation. R.M.S.D. plots were generated for both sulfinpyrazone and the protein backbone. The calculation of backbone R.M.S.D. for URAT1^{SPZ} using residues 2 to 42, 132 to 324, and 347 to 515. The calculation of sulfinpyrazone R.M.S.D. involved the heavy atoms of benzobromarone, using its initial structure as the reference. Results from three independent trajectories were distinguished by different colors. (E) Structural comparisons of initial poses of sulfinpyrazone with representative poses derived from clustering analysis of production-phase trajectories (20-150 ns). (F) Cluster populations obtained from analysis of the combined 390 ns production data (3×130 ns from independent 150 ns trajectories; first 20 ns of each trajectory excluded as equilibration).



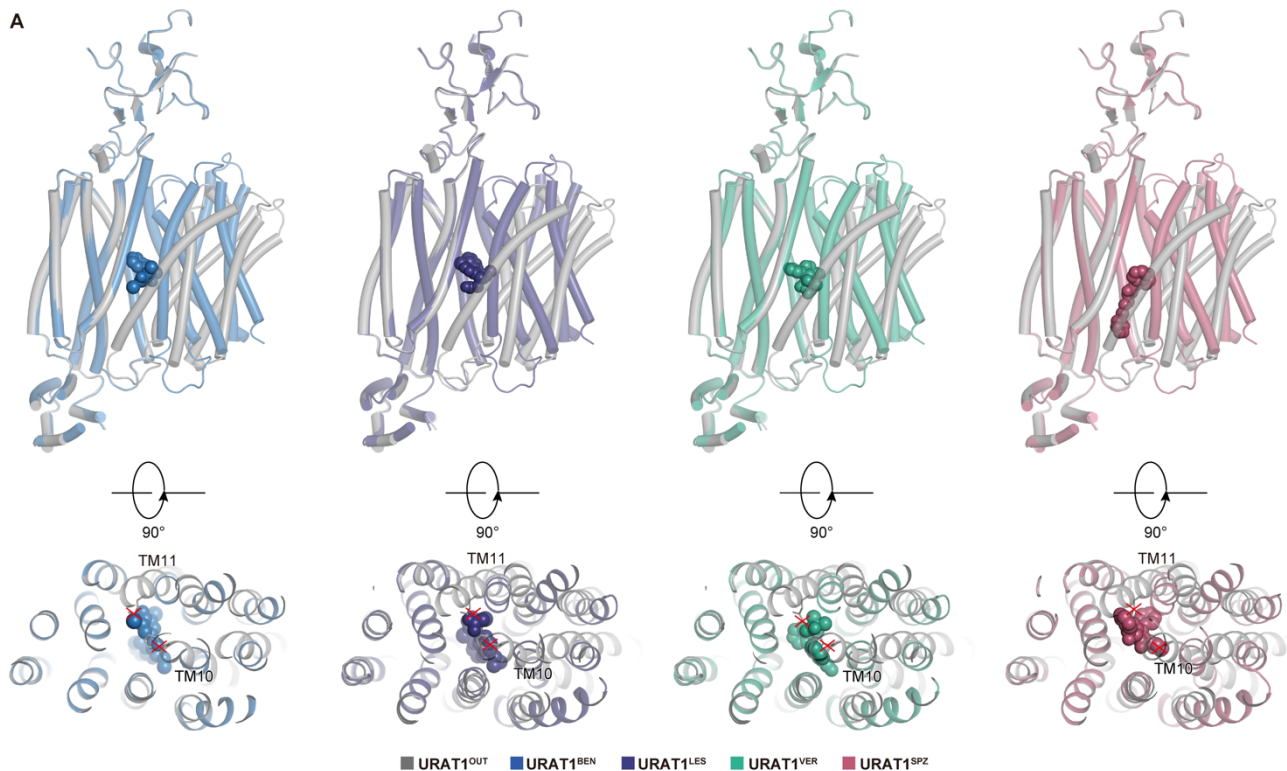
Supplementary Fig. 15. Conformational changes between cytoplasm- and extracellular-facing states.

(A) Side view (top) and extracellular view (bottom) of cytoplasm-facing URAT1^{UA} and extracellular-facing URAT1^{OUT}. The N-domains of URAT1 are shown as cartoon, while the C-domains of URAT1 are shown as cylinder. The extracellular-facing URAT1 model was generated according to the structure of extracellular-facing OCT3 (PDB: 7ZH0, ref. 78). (B) The region of motif-A and salt bridge on the extracellular side of URAT1 in the cytoplasm-facing state is outlined by a dark dashed circle. (C) A close-up view of residues involving in stabilizing URAT1 in the cytoplasm-facing state. The key residues are shown as sticks. (D) The region of motif-A and salt bridge on the cytoplasmic side of URAT1 in the extracellular-facing is outlined by a dark dashed circle. (E) A close-up view of residues involving in stabilizing URAT1 in the extracellular-facing state. The key residues are shown as sticks. (F) Sequence alignment of URAT1 from different species. The positions of the key residues involved in conformational change are highlighted, including motif-A and conserved salt bridges. Lines indicate the connections between residues forming salt bridges. (G) Comparative evaluation of the [¹⁴C] urate transportation activity of URAT1 mutants in relation to the URAT1^{EM}. Data presented are mean ± s.e.m. from three independent assays, each with one measurement (n = 3). Comparison of URAT1^{EM} with mutant constructs using an unpaired two-sided t-test showed significant differences (all P < 0.0001, denoted as ****).



Supplementary Fig. 16. The structural alignment of all structures which aligned by N-domian

The structures of URAT1^{UA}, URAT1^{BEN}, URAT1^{LES}, URAT1^{VER}, and URAT1^{SPZ} are colored in pink, sky-blue, purple, green, red, respectively. The surrounding residues are shown as sticks.



Supplementary Fig. 17. Structural alignment of URAT1^{OUT} and the drug-bound structures of URAT1.

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

Supplementary Table 1. Cryo-EM data collection, refinement and validation statistics.

	URAT1 ^{UA} (EMD-61191) (PDB-9J72)	URAT1 ^{BEN} (EMD-61192) (PDB-9J73)	URAT1 ^{LES} (EMD-61193) (PDB-9J74)	URAT1 ^{VER} (EMD-61194) (PDB-9J75)	URAT1 ^{SPZ} (EMD-61195) (PDB-9J76)
Data collection and processing					
Magnification	×105,000	×105,000	×105,000	×105,000	×105,000
Voltage (kV)	300	300	300	300	300
Electron exposure (e ⁻ /Å ²)	60	60	60	60	60
Defocus range (μm)	-1.2 – -2.2	-1.2 – -2.2	-1.2 – -2.2	-1.2 – -2.2	-1.2 – -2.2
Pixel size (Å)	0.85	0.85	0.85	0.85	0.85
Symmetry imposed	C1	C1	C1	C1	C1
Initial particle images (no.)	2,728,392	1,237,169	1,193,723	1,654,709	1,088,732
Final particle images (no.)	165,544	89,270	31,823	96,361	295,061
Map resolution (Å)	3.5 Å	3.5 Å	3.6 Å	3.5 Å	3.5 Å
FSC threshold	0.143	0.143	0.143	0.143	0.143
Refinement					
Model resolution (Å)	3.6	3.7	3.6	3.6	3.7
FSC threshold	0.5	0.5	0.5	0.5	0.5
Map sharpening <i>B</i> factor (Å ²)	-160.6	-159.2	-135.6	-150.0	-169.9
Model composition					
Non-hydrogen atoms	3636	3652	3654	3655	3659
Protein residues	472	472	472	472	472
Ligands	1	1	1	1	1
<i>B</i> factors (Å ²)					
Protein	86.71	78.60	69.07	85.32	93.79
Ligand	91.10	89.83	56.81	82.34	90.76
R.m.s. deviations					
Bond lengths (Å)	0.004	0.004	0.009	0.003	0.003
Bond angles (°)	0.744	0.704	1.105	0.647	0.714
Validation					
MolProbity score	1.89	1.76	1.79	1.73	1.78
Clashscore	8.39	5.95	6.22	5.67	6.08
Poor rotamers (%)	0.77	0.26	0.26	0.77	0.52
Ramachandran plot					
Favored (%)	93.32	93.32	93.10	93.53	93.10
Allowed (%)	6.68	6.68	6.90	6.47	6.90
Disallowed (%)	0.00	0.00	0.00	0.00	0.00

Supplementary Table 2. System setup of MD simulation

System	Total number of atoms	Total number of water molecules	Salt concentration	Lipid composition
URAT ^{BEN}	96,964	22,379	0.15 M NaCl	166 POPC
URAT ^{VER}	83,380	18,972	0.15 M NaCl	141 POPC
URAT ^{SPZ}	95,200	21,836	0.15 M NaCl	165 POPC