

Supplementary Information for

Molecular basis of antagonism of the dimeric human arginine vasopressin receptor 1A

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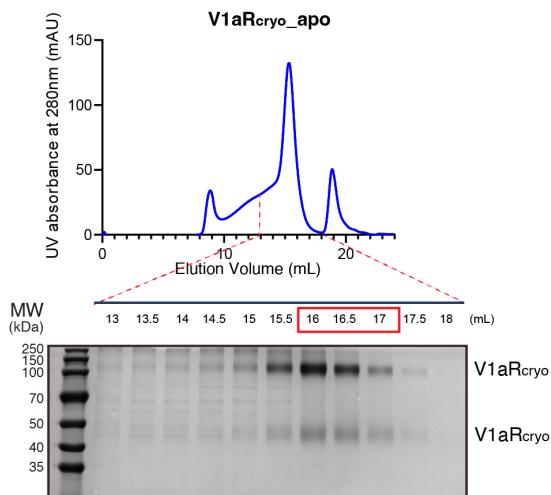
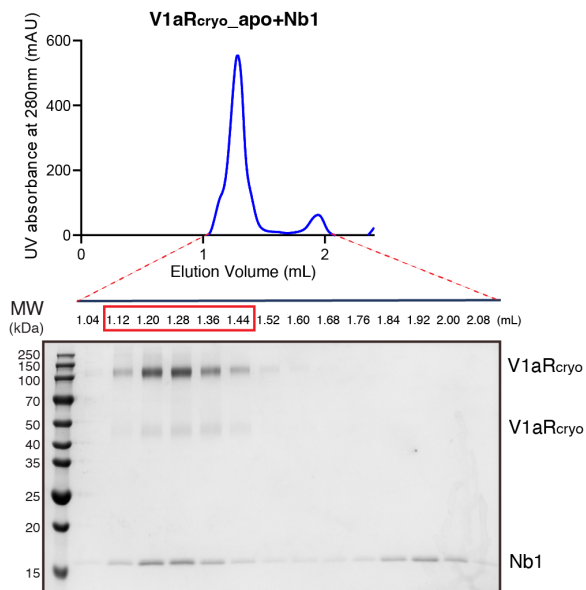
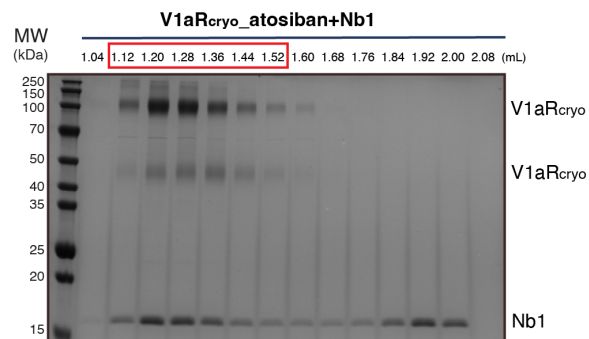
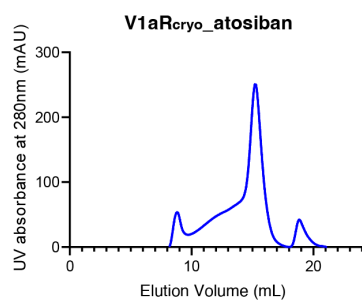
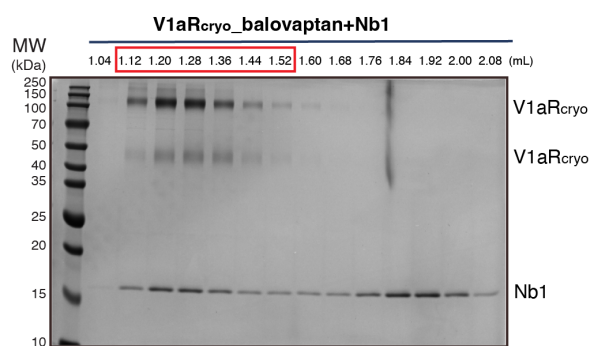
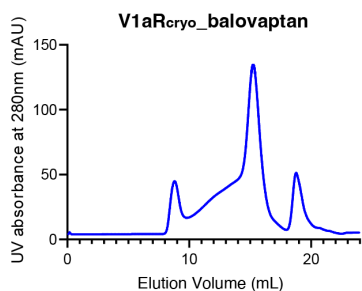
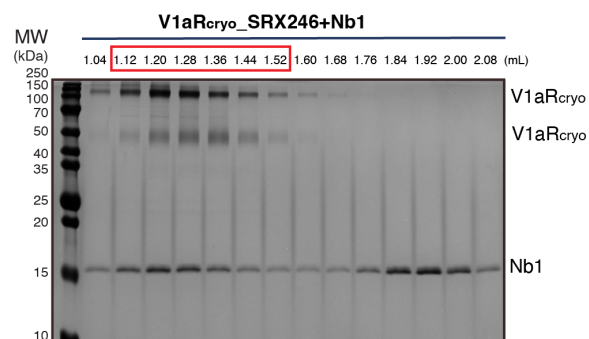
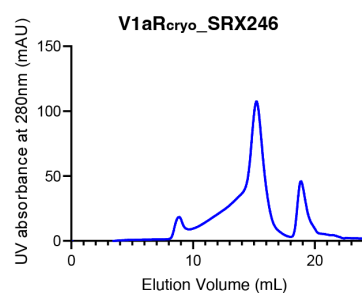
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This PDF file includes:

Supplementary Figures. 1-13

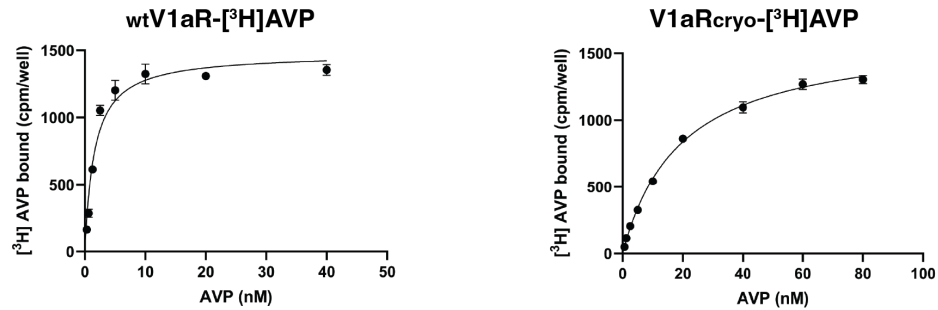
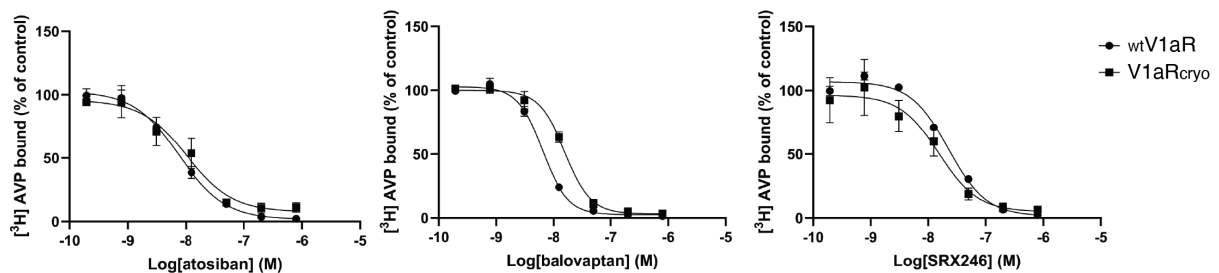
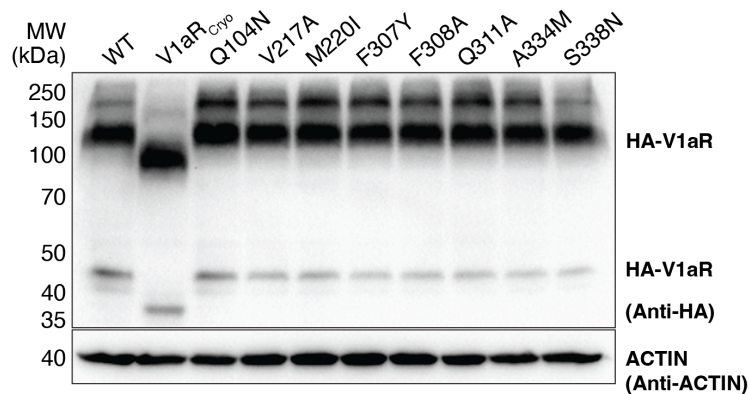
Supplementary Tables. 1-4

Uncropped scans of all blots and gels

a**b****c****d****e**

Supplementary Figure. 1 | Protein preparations for cryo-EM analysis

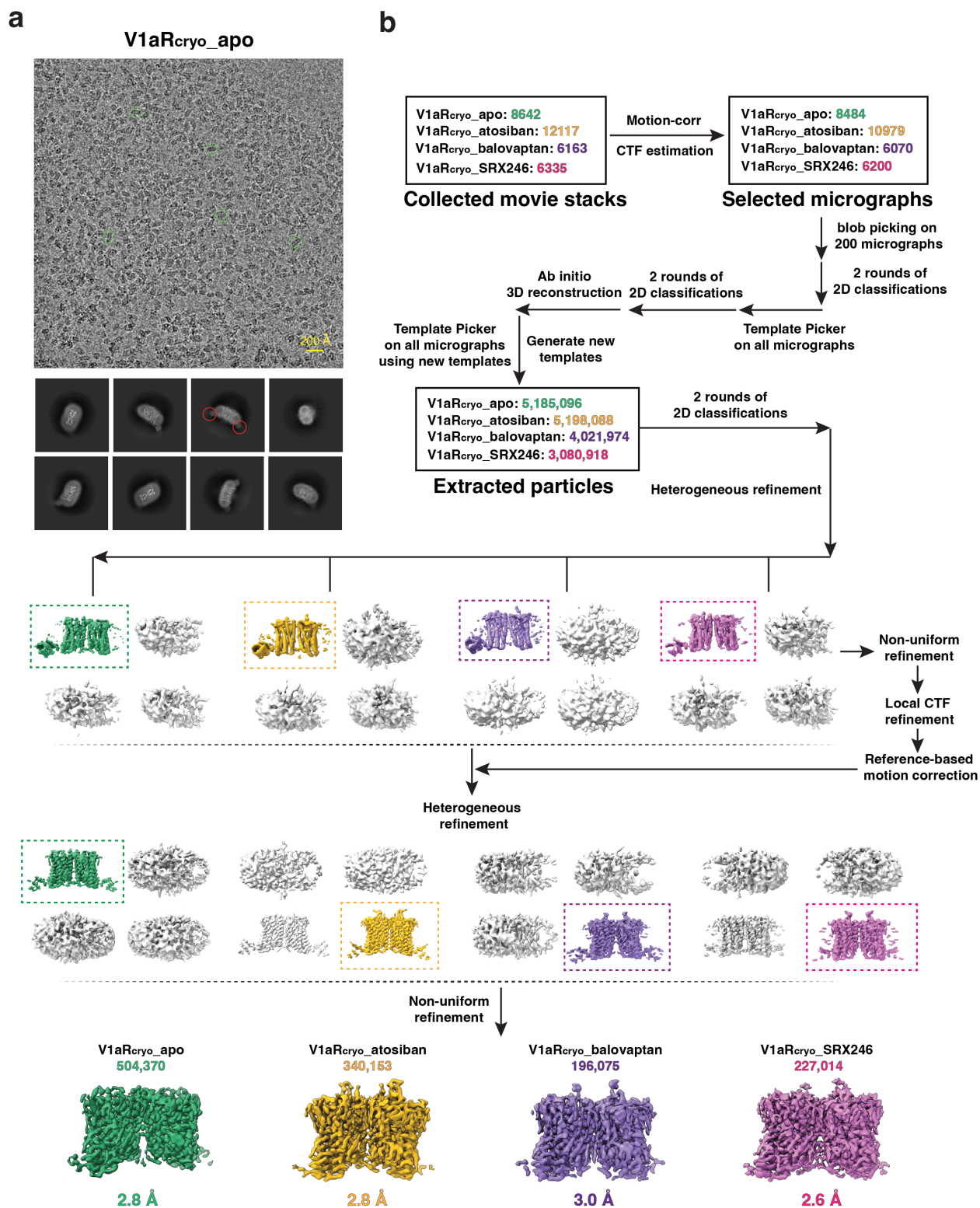
a. Human V1aR_{cryo} in its apo state was subjected to size-exclusion chromatography. The top panel illustrates the elution profile, and the bottom panel shows the analysis of fractions using SDS-PAGE and Coomassie-blue staining. The red circle indicates the fractions pooled for downstream experiments. **b.** The same as **a**, but for V1aR_{cryo} in its apo state in complex with the nanobody Nb1. **c.** The left panel shows the elution profile for the size-exclusion chromatography of V1aR_{cryo} with atosiban bound. The right panel shows the SDS-PAGE gel of fractions from the elution profile. The red circle indicates the fractions pooled for downstream experiments. **d, e.** The same as **c**, but for V1aR_{cryo} with balovaptan (**d**) and SRX246 (**e**) bound.

a**b****c**

Supplementary Figure. 2 | Binding properties of V1aR for different ligands

a. A cell-based binding assay was used to measure the saturation binding of $[^3\text{H}]\text{AVP}$. The presented data are background subtracted, and the means from three independent experiments $\pm$ S.E.M. (except that two independent experiments were used for the 80nM datapoint of V1aRcryo), and the data were fitted using the one-site-specific binding equation. The K_d values for the two constructs are summarized in **Supplementary Table 3**. wt stands for full-length wild-type sequence. **b.** Inhibition of $[^3\text{H}]\text{AVP}$ binding was done by increasing concentrations of atosiban, balovaptan, and SRX246, respectively. Data were normalized to the values of zero inhibitor concentration (control). The presented data are the means from three independent experiments $\pm$ S.E.M., and were fitted to a four-parameter logistic equation. The obtained IC_{50}

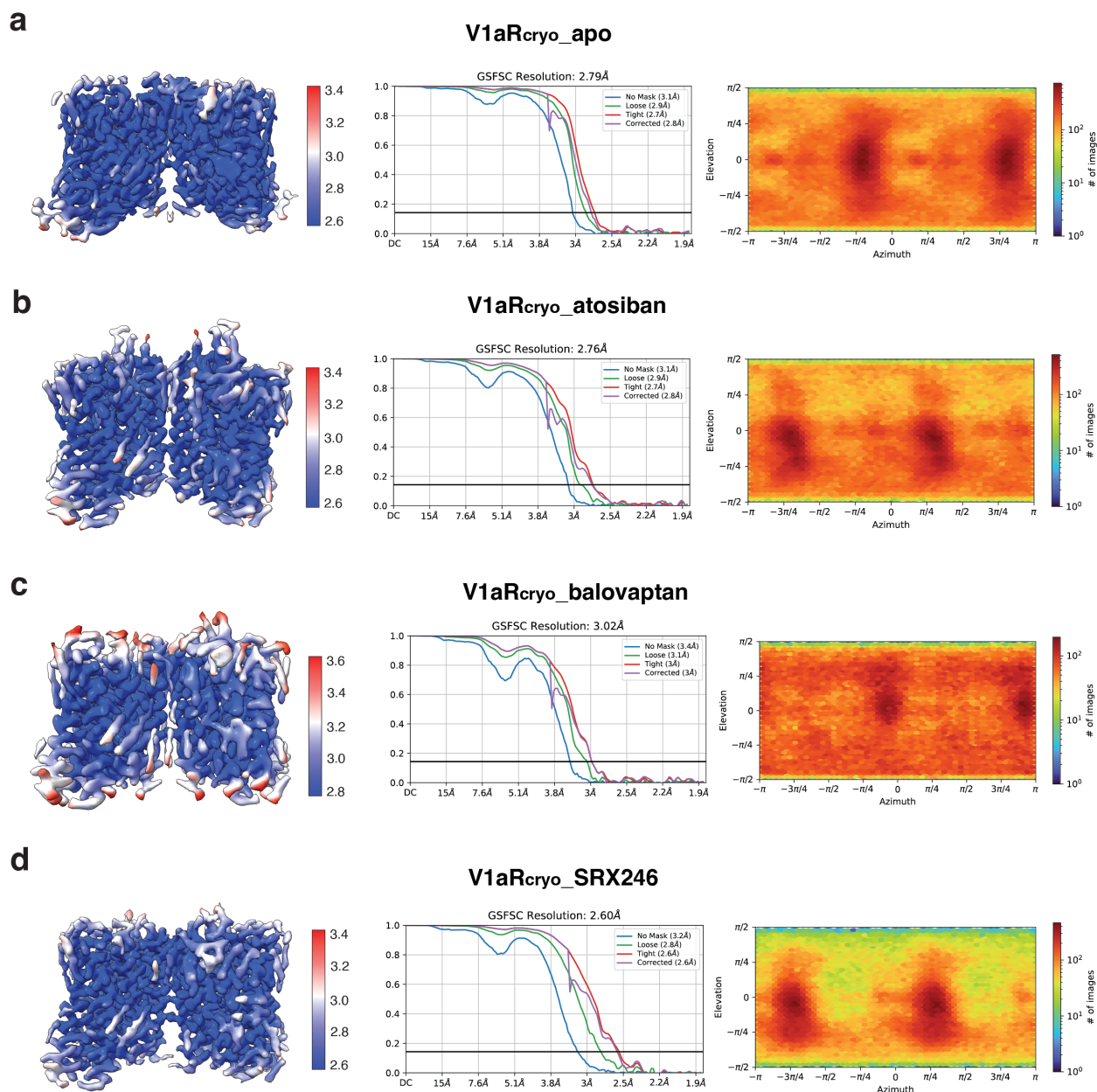
values were converted to K_i using the Cheng–Prusoff equation. The K_i values were summarized in **Supplementary Table 3. c**. The expression levels of different mutants used in the study were analyzed by SDS-PAGE and Western blot using anti-HA antibody (Sigma, #11867423001). The experiments were repeated twice.



Supplementary Figure. 3 | Workflow for cryo-EM analysis

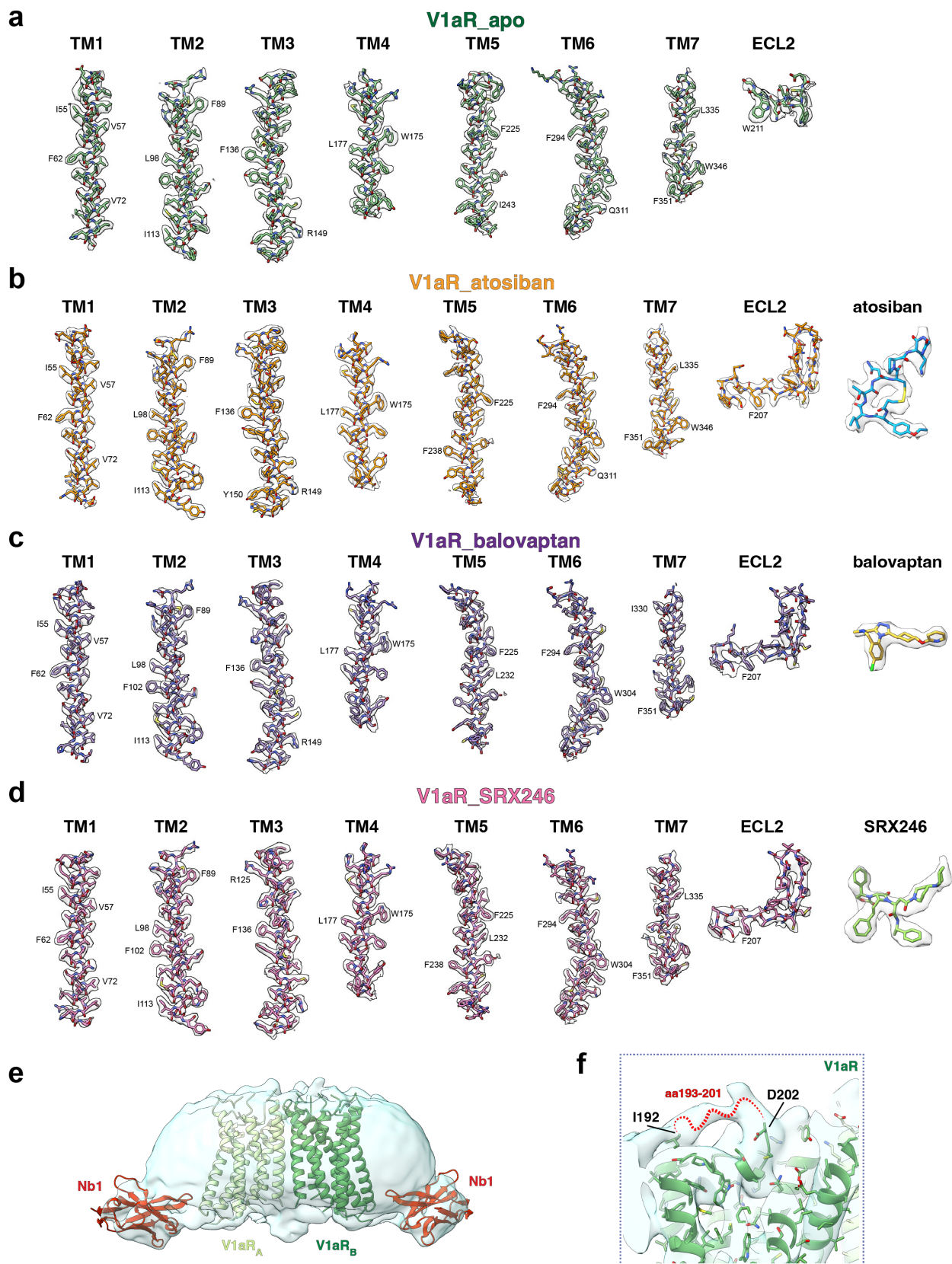
a. The upper panel shows the representative cryo-EM image (with a defocus at $\sim 2 \mu\text{m}$) of the V1aR_{cryo}_apo sample. The green circles, with a diameter of 160 Å, indicate representative

particles picked for downstream analysis. The bottom panel displays some representative 2D class averages during data analysis, with red circles indicating the position of two bound nanobodies. Cryo-EM images and 2D class averages of the other samples were not shown due to high similarity. **b.** Image processing workflow for the four samples. Views of 3D reconstructions parallel to the membrane were shown. The classes highlighted in color and boxed were selected for further analysis.



Supplementary Figure. 4 | Estimated resolution and direction distribution

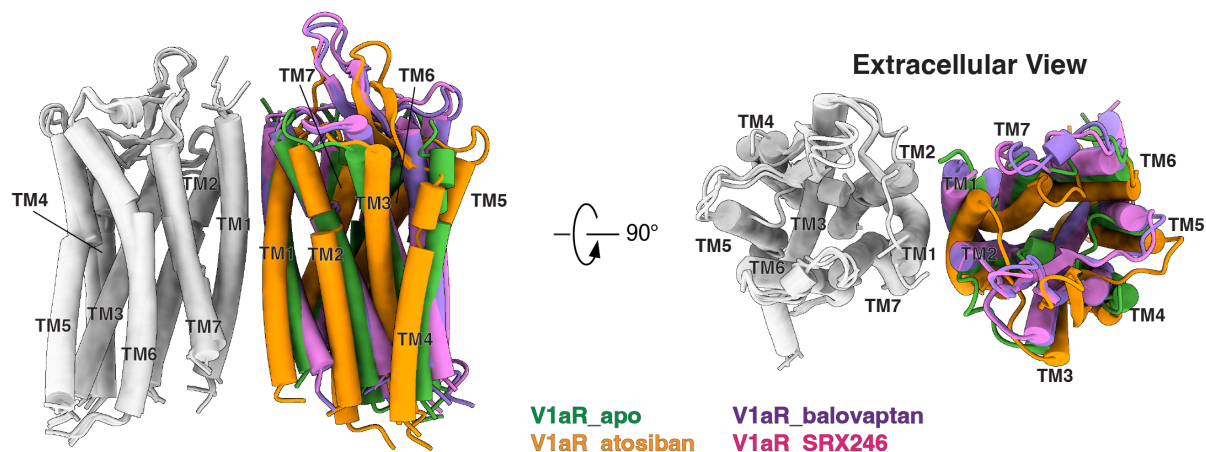
a. The left panel shows the local resolution estimation of the cryo-EM map of the V1aR_{cryo}_apo sample. The final unsharpened map was colored based on local resolution estimation, and the scale bar represents values in angstroms (Å). The middle panel shows the Golden Standard Fourier Shell Correlation (GSFSC) curve for the 3D reconstruction. The black line indicates the 0.143 criterion. The right panel displays the direction distribution of the 3D reconstruction. **b, c, d.** The same illustration as **a**, but for V1aR_{cryo}_atosiban, V1aR_{cryo}_balvopatan, and V1aR_{cryo}_SRX246, respectively.



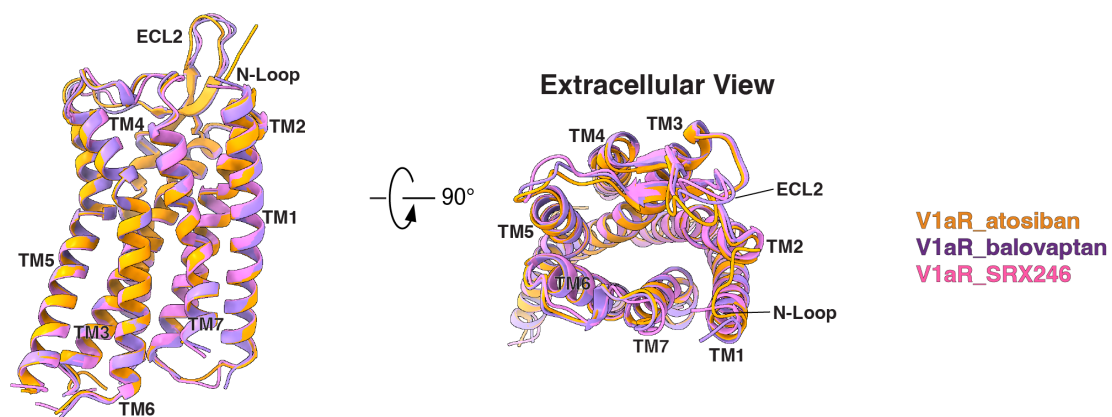
Supplementary Figure. 5 | Cryo-EM densities and fitted models for the apo state of V1aR_{cryo} and for its complex with different antagonists

a. Cryo-EM densities with the fitted model for the V1aR_{cryo_apo} sample. **b, c, d.** The same illustration as **a**, but for the V1aR_{cryo_atosiban}, V1aR_{cryo_balvopatan}, and V1aR_{cryo_SRX246} samples, respectively. **e.** The Cryo-EM density map (low-pass filtered to 6 Å, contoured at 0.056) for the V1aR_{cryo_apo} sample was shown together with the fitted model of V1aR and two docked models of Nb1 predicted by Alphafold. The positions of Nb1 in other maps are the same. **f.** The same map and fitted model were shown as in **e**. The shown region is the densities accounting for the flexible part of ECL2 (aa193-201) that was not modeled.

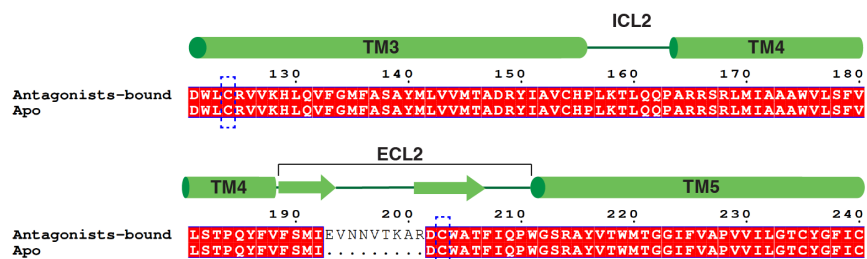
a



b



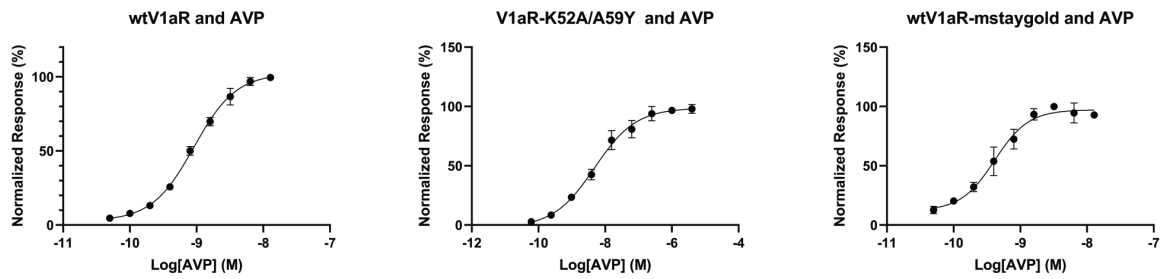
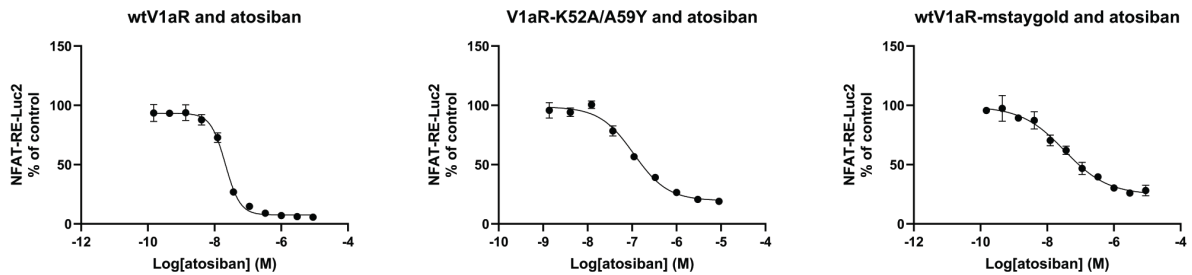
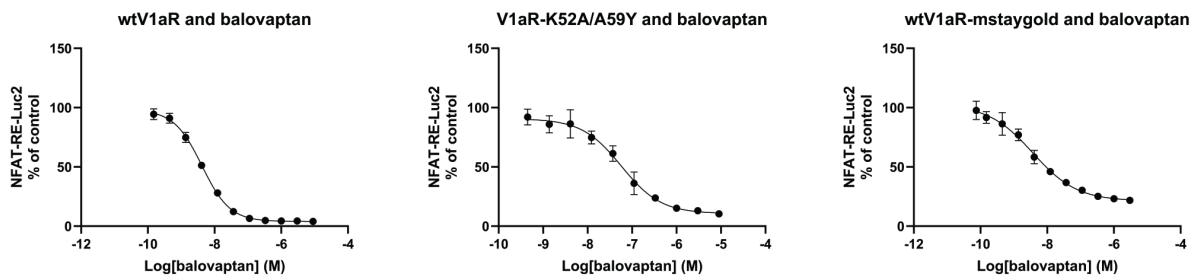
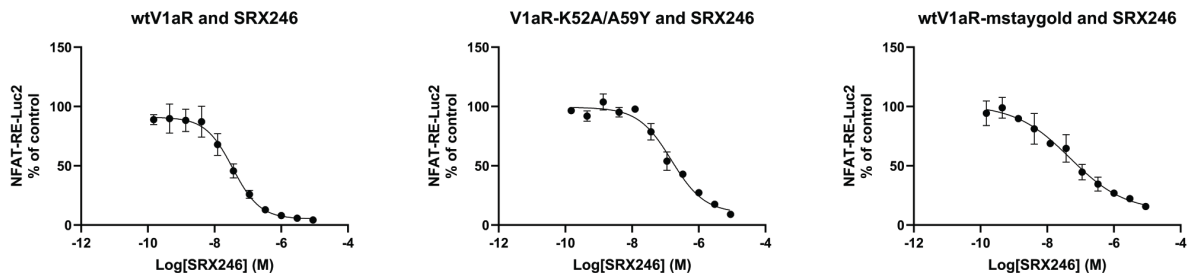
c



Supplementary Figure. 6 | Comparisons of different V1aR structures

a. The structures of V1aR (shown in cylinders) in its apo state or with different antagonists bound were superimposed based on one protomer of the dimer. The copy used for superimposition was shown in grey, and the other copy was colored accordingly. **b.** The structures of V1aR (shown as ribbons) bound by different antagonists were superimposed. **c.** Sequence alignment of Antagonists-bound and apo structures. Blue dashed boxes are two

cysteines that form a disulfide bond in antagonist-bound structures but remain unpaired in the apo structure. Secondary structures of the corresponding protein sequence are indicated above the alignment. The exact position of the two segments in ECL2 that form the antiparallel β -sheet fold is shown as arrowed lines.

a**b****c****d**

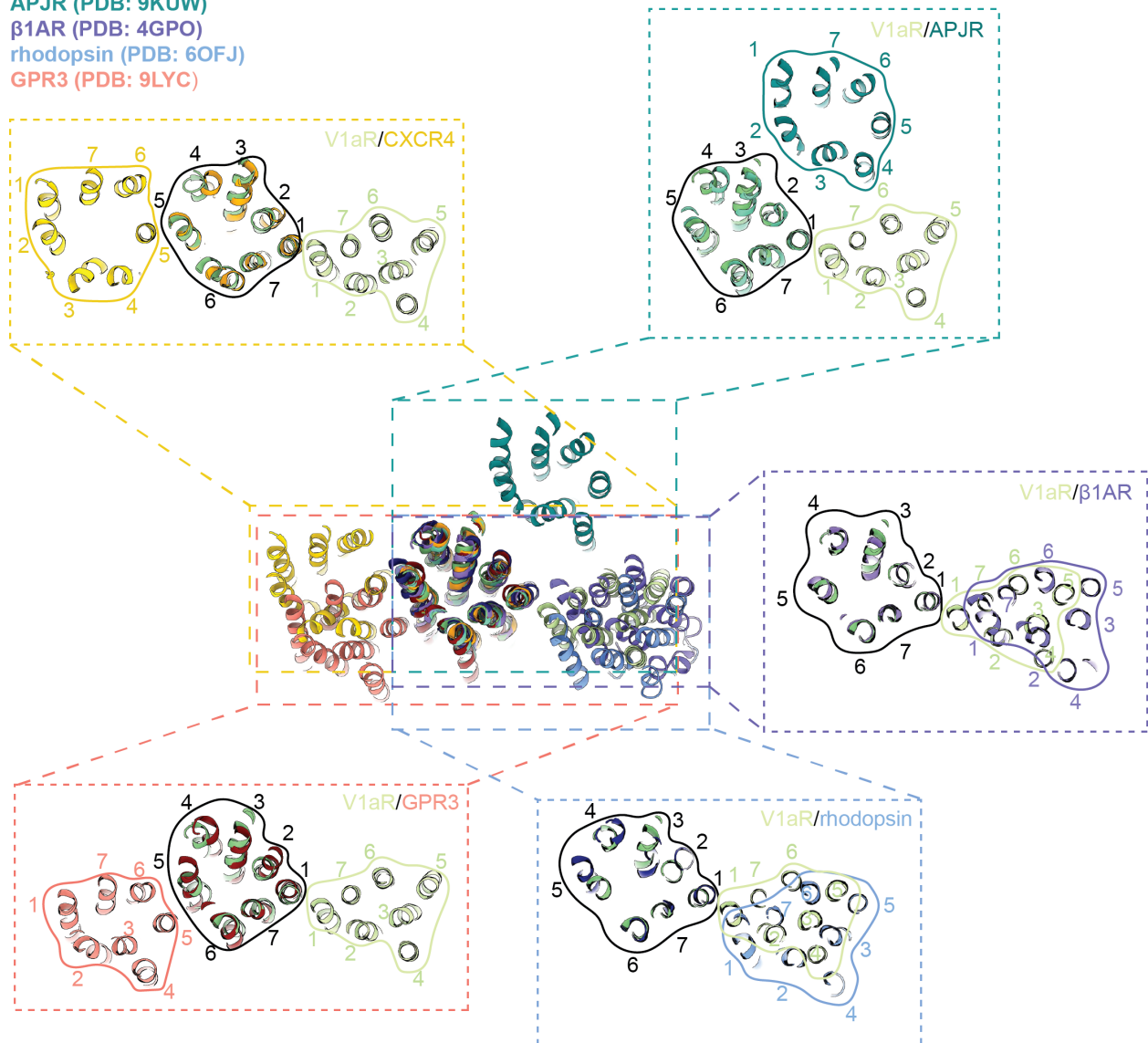
Supplementary Figure. 7 | Dose–response and antagonist inhibition of V1aR variants measured by NFAT luciferase assays

a. AVP-induced G-protein signaling was measured by NFAT-driven luciferase activity for wild-type V1aR and mutants. Data were normalized to the values of the maximal AVP response. The presented data are the means from three independent experiments $\pm$ S.E.M. and were fitted to a four-parameter logistic equation. The EC_{50} values were summarized in **Supplementary Table 4**.

b. Inhibition of AVP-induced signaling by atosiban was measured. Data were normalized to the

values of zero inhibitor concentration. The presented data are the means from three independent experiments $\pm$ S.E.M. and were fitted to a four-parameter logistic equation. The IC₅₀ values were summarized in **Supplementary Table 4. c. d.** The same as **b**, but for balovaptan and SRX246, respectively.

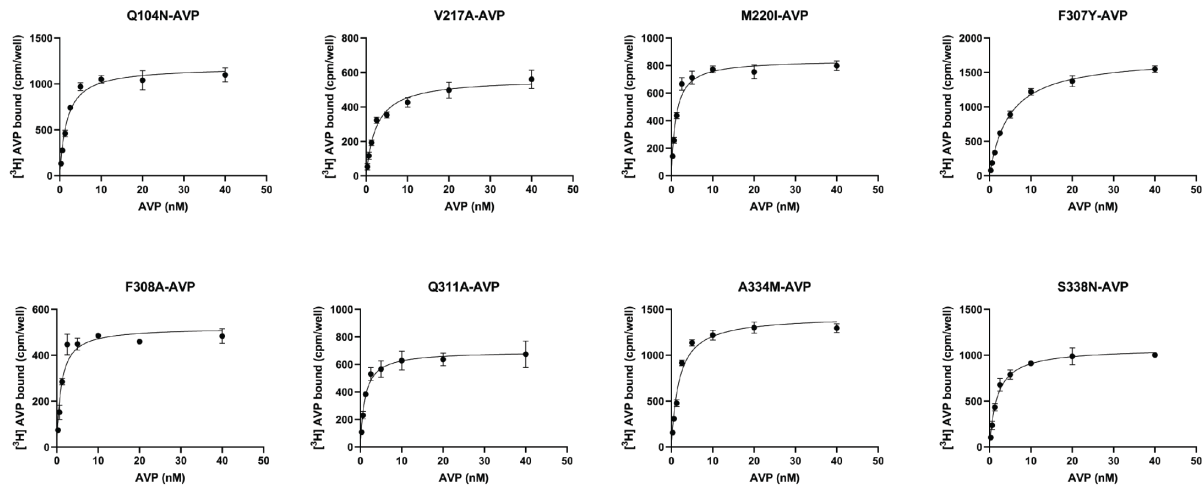
V1aR (This study)
 CXCR4 (PDB: 3ODU)
 APJR (PDB: 9KUW)
 β 1AR (PDB: 4GPO)
 rhodopsin (PDB: 6OFJ)
 GPR3 (PDB: 9LYC)



Supplementary Figure. 8 | Structural comparison of Class A GPCR dimer interfaces

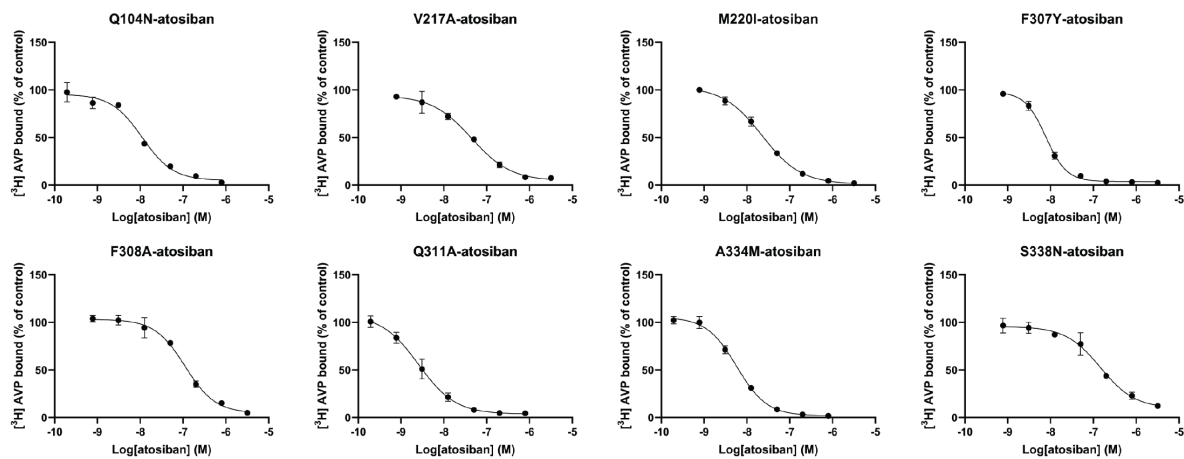
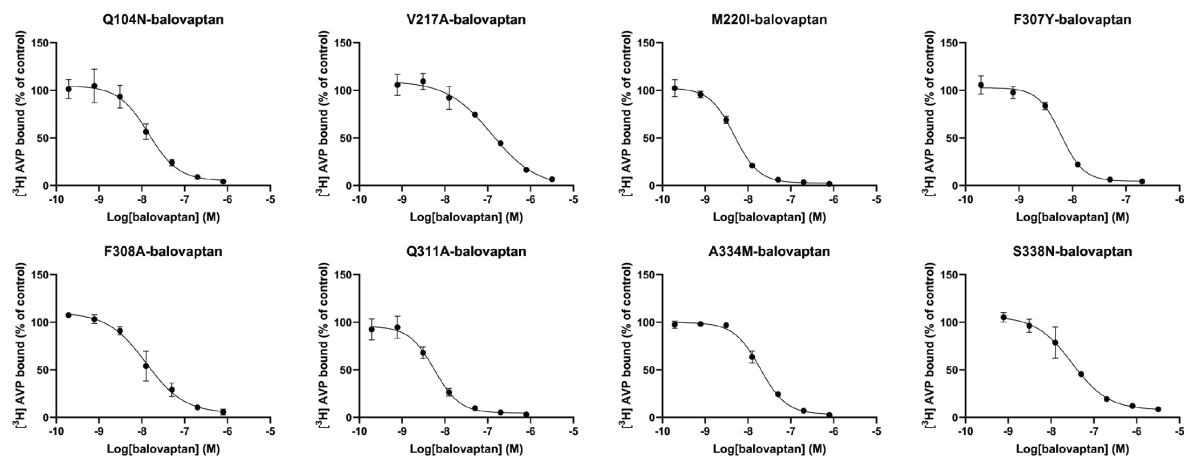
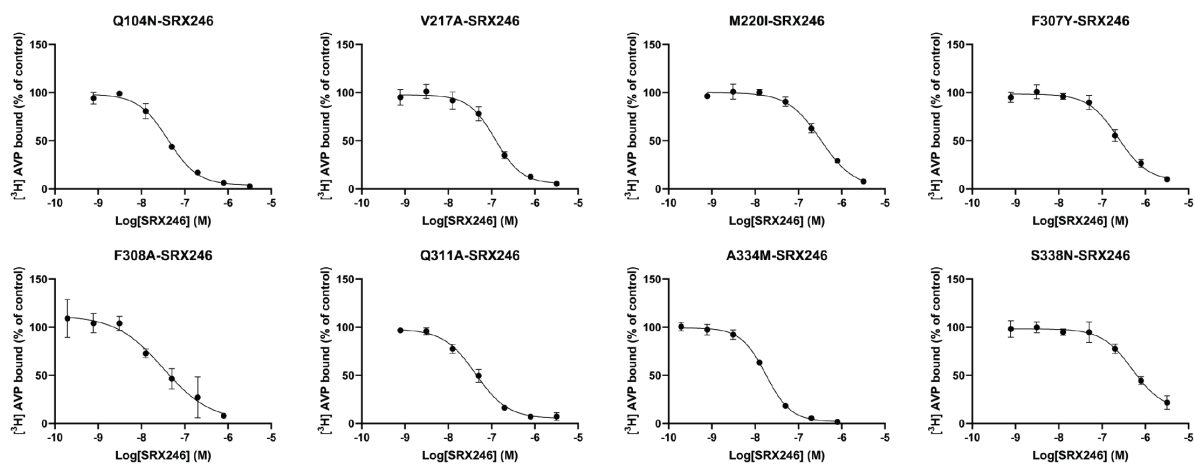
a. Top-down (extracellular) views in the middle of the figure show the comparison of V1aR with representative Class A GPCR dimers/oligomers: CXCR4 (PDB: 3ODU), APJR/apelin receptor (PDB: 9KUW), β 1AR (PDB: 4GPO), rhodopsin (PDB: 6OFJ), and GPR3 (PDB: 9LYC). In each separate panel, one protomer is aligned to V1aR; the aligned protomers are outlined in black, and the other protomers are outlined in their corresponding colors (V1aR: light green; CXCR4: yellow; APJR: teal; β 1AR: purple; rhodopsin: blue; GPR3: salmon). TM helices are labeled to enable direct comparison of interface geometry across receptors.

a



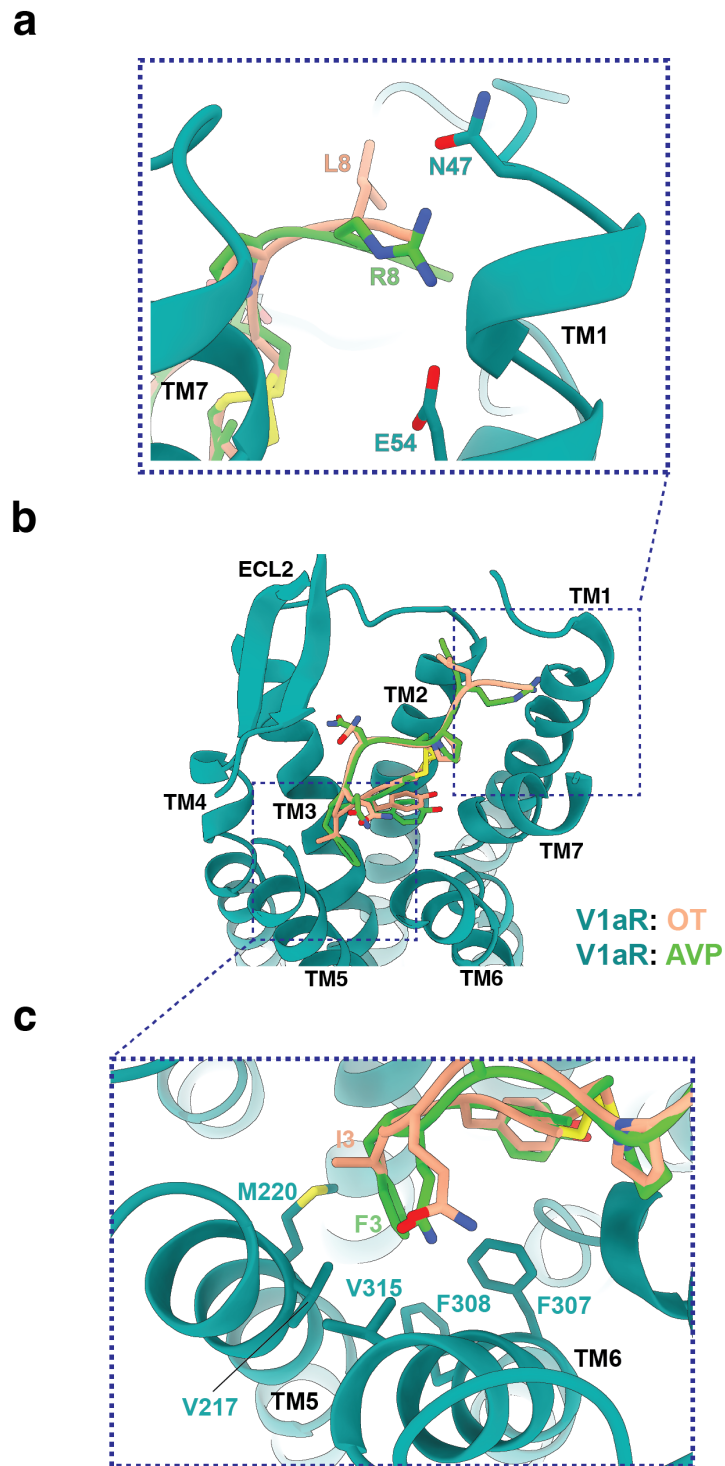
Supplementary Figure. 9 | AVP binding properties for V1aR mutants

a. A cell-based binding assay was used to measure the saturation binding of $[^3\text{H}]$ AVP. The presented data are background-subtracted, and the means from three independent experiments $\pm$ S.E.M., and the data were fitted using the one-site-specific binding equation. The K_d values for different mutants are summarized in **Supplementary Table 3**.

a**b****c**

Supplementary Figure. 10 | Competition assays using different antagonists for V1aR mutants

a. Inhibition of [³H]AVP binding for different mutants was done by increasing concentrations of atosiban. Data were normalized to the values of zero inhibitor concentration (control). The presented data are the means from three independent experiments $\pm$ S.E.M. and were fitted to a four-parameter logistic equation. The obtained IC₅₀ values were converted to K_i using the Cheng–Prusoff equation. The K_i values were summarized in **Supplementary Table 3. b, c**. The same as **a**, but for balovaptan and SRX246, respectively.

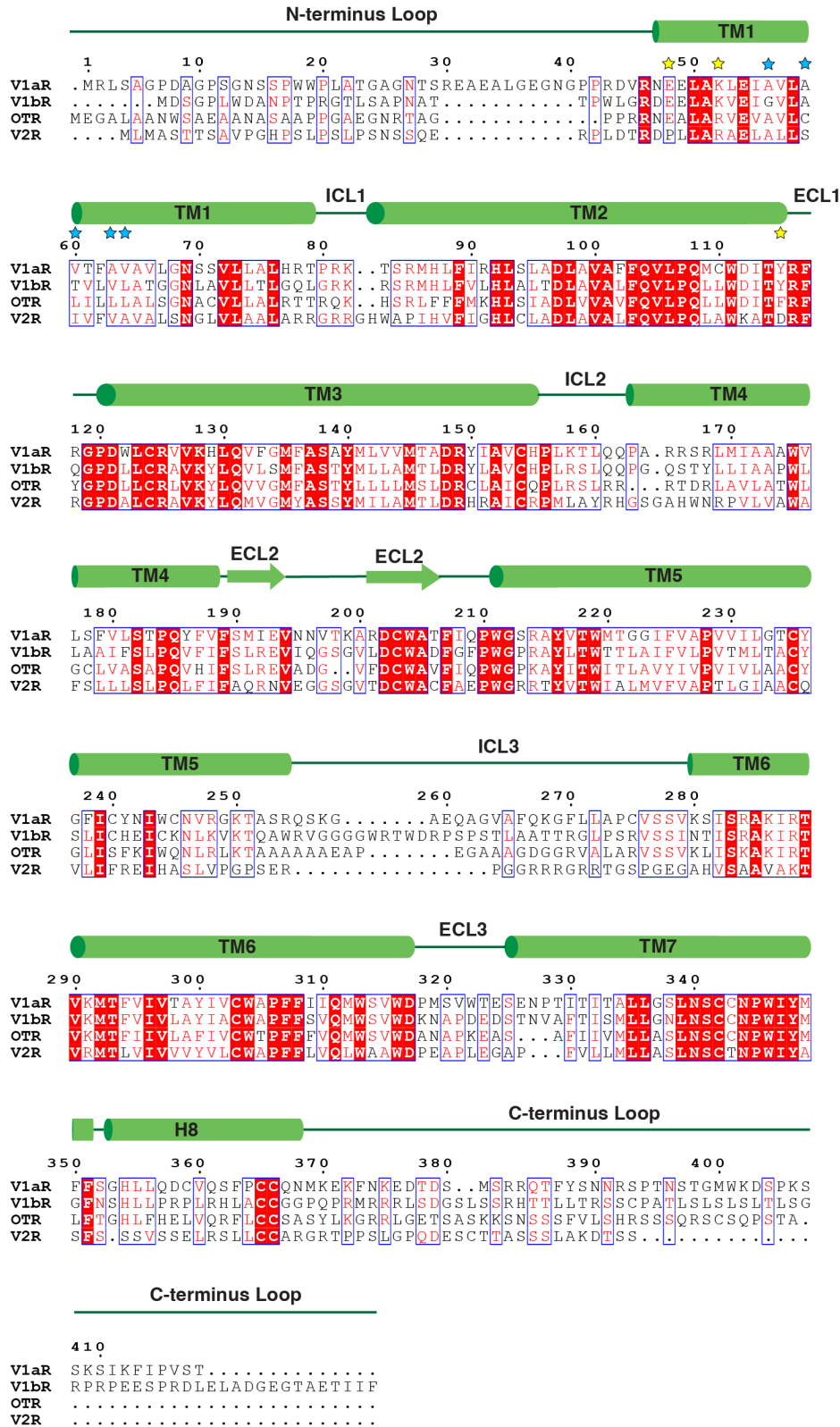


Supplementary Figure. 11 | Structural comparison of V1aR-oxytocin with V1aR-AVP models

V1aR-AVP and V1aR-oxytocin models were generated using MODELLER based on the OTR-oxytocin (PDB: 7RYC) and V2R-AVP (PDB: 7DW9) structures. **a.** Close-up view of the sub-

binding pocket for AVP/oxytocin position 8. **b.** Overview of the binding pocket. **c.** Close-up view of the sub-binding pocket for AVP/oxytocin position 3.

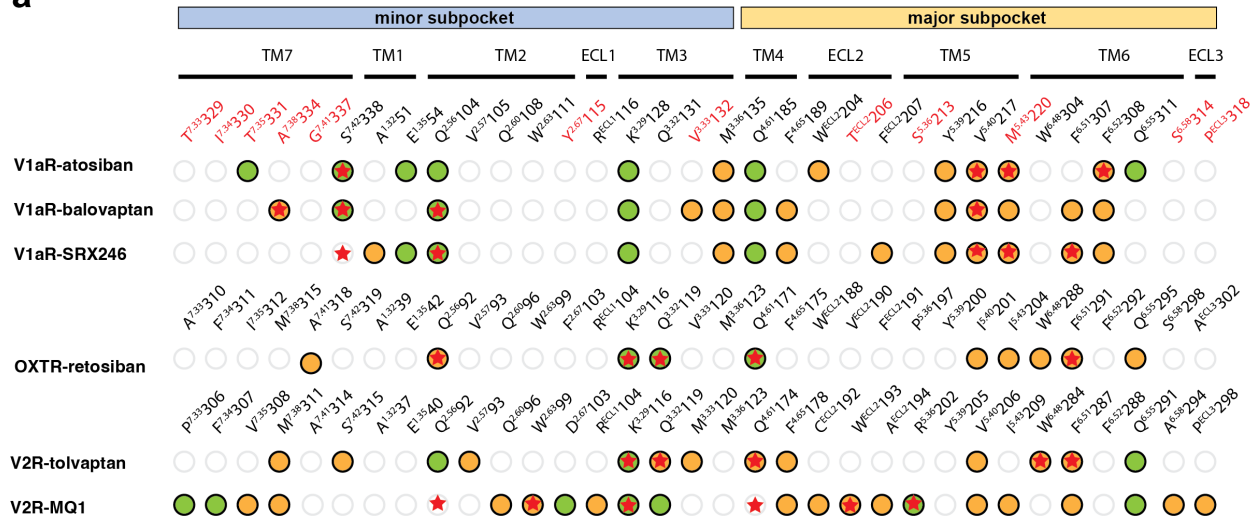
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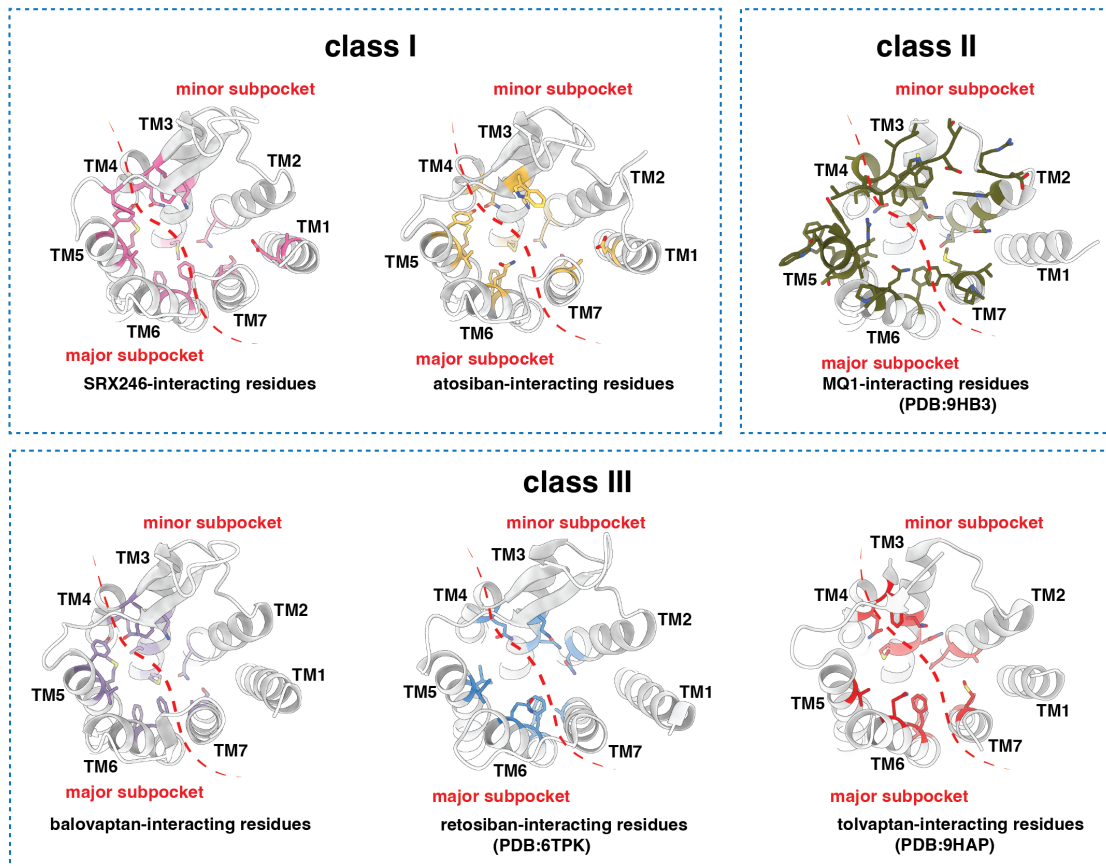
Supplementary Figure. 12 | Sequence alignment of the OT-AVP receptor family

a. Alignment of protein sequences from members of the OT-AVP receptor family. Residues involved in polar interactions at the V1aR dimer interface are marked with yellow stars, while residues with small hydrophobic side chains at the V1aR dimer interface are highlighted with blue stars. Secondary structures of the corresponding sequence for V1aR are indicated above the alignment.

a



b



Supplementary Figure. 13 | Analysis of antagonist binding modes across the AVP/OT receptor family.

a. Comparison of the binding sites of V1aR-atosiban, V1aR-balovaptan, V1aR-SRX246, OXTR-retosiban (PDB: 6TPK), V2R-tolvaptan (PDB: 9HAP), V2R-MQ1 (PDB: PHB3). The region

enclosed by TM7, TM1, TM2, and TM3 is defined as the minor subpocket, whereas the region bounded by TM4–TM6 is defined as the major subpocket. Residue labels correspond to the aligned positions in each receptor; superscript numbers denote the Ballesteros-Weinstein numbering scheme. Residues conserved across the AVP/OT receptor family are shown in black, while non-conserved positions are shown in red. Residues engaged in hydrophobic stacking are indicated by orange circles, and those involved in polar interactions are indicated by green circles. Residues whose mutations were experimentally verified to affect ligand binding are marked by a red five-pointed star within the circle. **b.** Spatial distribution of ligand-contacting residues. Receptors are shown as cartoons in white, and interacting residues are shown as sticks and color-coded by ligand: pink (SRX246), yellow (atosiban), olive (MQ1), purple (balovaptan), blue (retosiban), and red (tolvaptan). The major and minor subpockets are approximately separated by a dotted red line.

Supplementary Table 1. Statistics for data collection and refinement

Structure	V1aR_apo	V1aR_atosiban	V1aR_balovaptan	V1aR_SRX246
Data Accession				
PDB	9XB1	9UWI	9UWJ	9UWL
EMDB	EMD-66695	EMD-64555	EMD-64556	EMD-64559
Data Collection				
Microscope	Titan Krios G4			
Detector	Falcon 4i			
Voltage (kV)	300			
Automation software	EPU			
Energy filter	10 eV			
Nominal magnification	130k			
Pixel Size (Å)	0.92			
Electron exposure (e ⁻ /Å ²)	50			
Defocus range (μm)	-1.2~-2.2			
Movies collected	8642	12117	6163	6335
Reconstruction				
Software	cryoSPARC v4.1			
Micrographs used	8484	10979	6070	6200
Particles extracted	5,185,096	5,198,088	4,021, 974	3,080,918
Particles used in refinement	504,370	340,153	196,075	227,014
Symmetry	C1	C1	C1	C1
Overall resolution (Å) FSC=0.143 (masked)	2.8	2.8	3.0	2.6
Map sharpening B-factor (Å ²)	-65	-65	-64	-96
Model Refinement				
Software	AlphaFold2, Coot and Phenix			
Initial model used	From AlphaFold2 (AF-P37288-F1)			
Model Composition				
Protein residues	512	538	530	530
Water	0	0	0	8
Ligands	0	atosiban: 2	balovaptan: 2	SRX246: 2
Lipids	CLR: 2	CLR: 4	CLR: 4	CLR: 4
B factors (Å ²)				
Protein	52.0	68.4	82.2	55.5
Ligand		67.4	83.0	54.7
Water				35.7
R.M.S. deviations				
Bond length (Å)	0.002	0.004	0.004	0.004
Bond angle (°)	0.491	0.575	0.652	0.669
Ramachandran statistics (%)				
Outliers	0.0	0.0	0.0	0.0
Allowed	2.5	2.1	1.2	0.8
Favored	97.5	97.9	98.8	99.2
MolProbity score	1.31	1.27	1.43	1.08

All-atom clashscore	4.38	4.7	6.3	2.9
Poor rotamers (%)	0.45	0.00	1.30	0.43
Model resolution (Å) FSC threshold=0.5	2.8	2.9	3.1	2.7

Supplementary Table 2. Protein sequence of Nb1

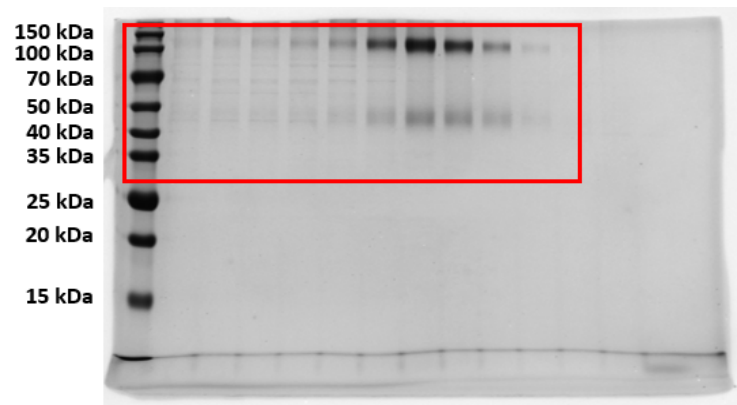
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Supplementary Table 3. Binding properties of AVP and different antagonists for various V1aR constructs

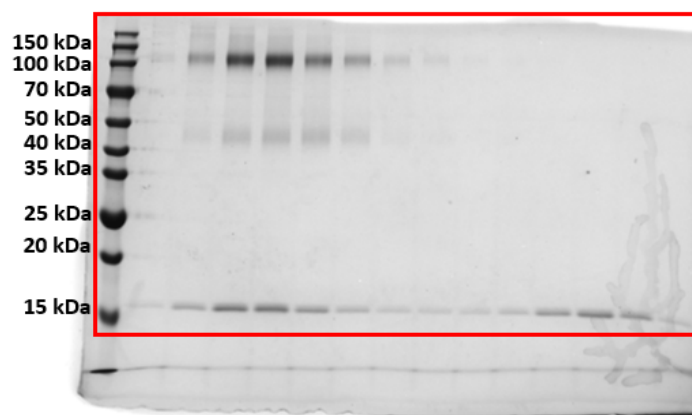
	AVP	atosiban		balovaptan		SRX246	
	Kd [nM]	Ki [nM]	95% CI [nM]	Ki [nM]	95% CI [nM]	Ki[nM]	95% CI [nM]
WT	1.57±0.19	2.15	1.64 - 2.67	1.83	1.48 - 2.19	6.35	4.82 - 7.89
cryoEM	19.30±0.89	8.67	4.56 - 12.79	13.16	11.66 - 14.66	12.74	5.84 - 19.64
Q104N	1.71±0.18	3.31	2.33 - 4.30	4.25	2.57 - 5.93	12.26	9.41 - 15.11
V217A	2.46±0.28	16.95	11.61 - 22.28	46.88	24.18 - 69.58	46.13	32.91 - 59.36
M220I	1.11±0.12	4.90	3.82 - 5.99	1.04	0.82 - 1.26	71.21	48.66 - 93.75
F307Y	4.70±0.26	4.18	3.67 - 4.70	3.29	2.70 - 3.87	126.08	89.12 - 163.15
F308A	1.04±0.16	23.05	15.65 - 30.45	2.59	1.51 - 3.67	6.62	0.59 - 12.65
Q311A	1.10±0.14	0.55	0.35 - 0.76	1.22	0.82 - 1.62	9.24	6.67 - 11.81
A334M	1.85±0.18	1.82	1.48 - 2.18	6.09	4.94 - 7.24	5.59	4.59 - 6.59
S338N	1.83±0.16	45.65	28.28 - 63.02	9.67	6.20 - 13.14	156.84	74.49 - 234.19

Supplementary Table 4. IC₅₀ and EC₅₀ of AVP and different antagonists for various V1aR constructs

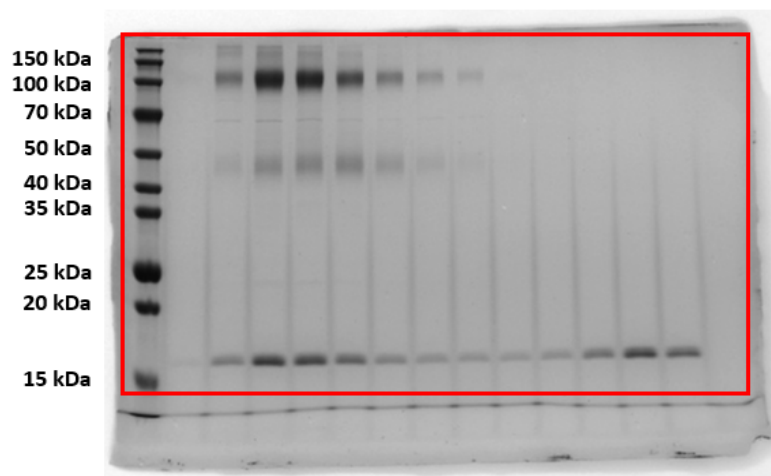
Construct	wtV1aR	wtV1aR-mstaygold	V1aR-K52A/A59Y
AVP			
EC50 (nM)	0.91	0.41	4.84
95% CI (nM)	0.83 - 1.00	0.33 - 0.52	3.39 - 6.92
atosiban			
IC50 (nM)	21.11	33.06	108.9
95% CI (nM)	18.91 - 23.55	21.32 - 51.26	85.67 - 138.40
Balovaptan			
IC50 (nM)	4.18	3.67	57.45
95% CI (nM)	3.71 - 4.71	2.34 - 5.74	41.06 - 80.83
SRX246			
IC50 (nM)	34.9	48.58	151.5
95% CI (nM)	25.82 - 47.28	23.13 - 102.0	104.80 - 218.90



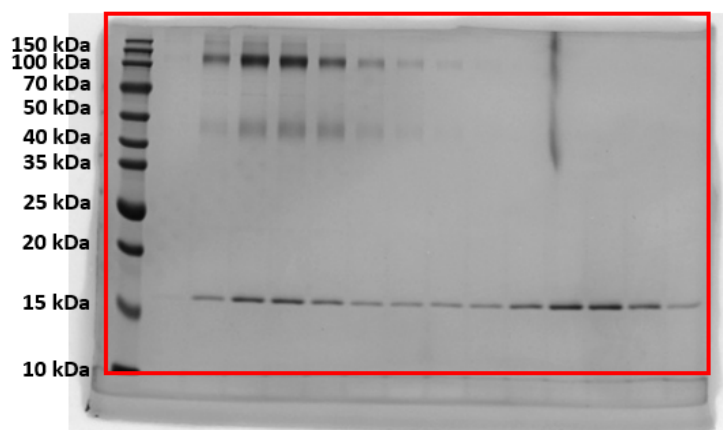
Uncropped scan of gel for Supplementary Figure. 1a



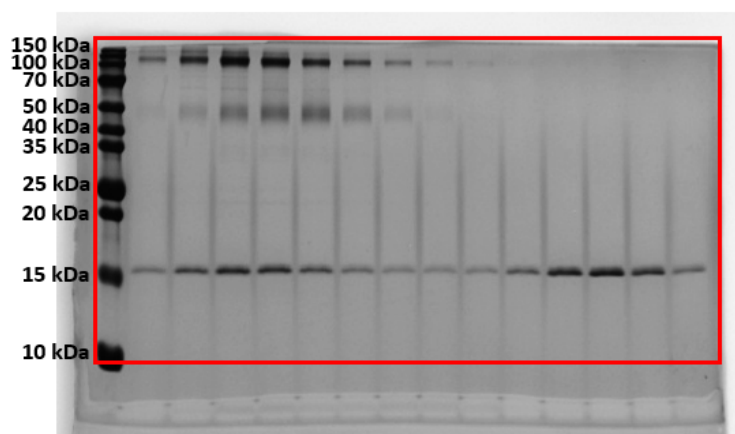
Uncropped scan of gel for Supplementary Figure. 1b



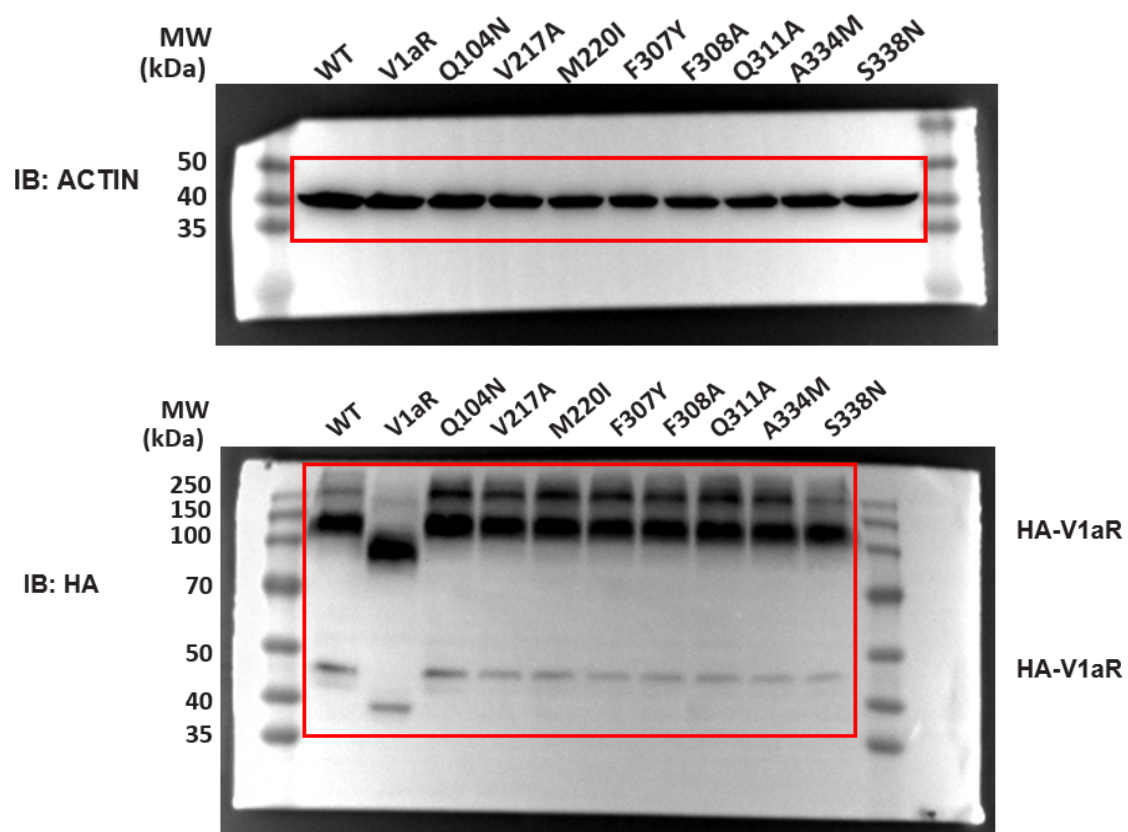
Uncropped scan of gel for Supplementary Figure. 1c



Uncropped scan of gel for Supplementary Figure. 1d



Uncropped scan of gel for Supplementary Figure. 1e



Uncropped scan of blot for Supplementary Figure. 2c