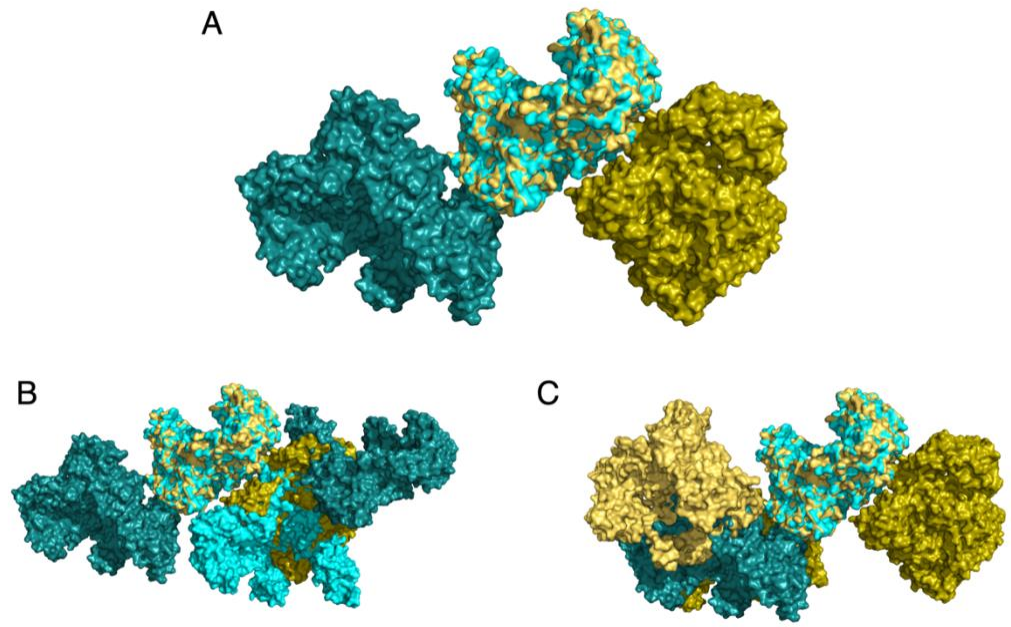
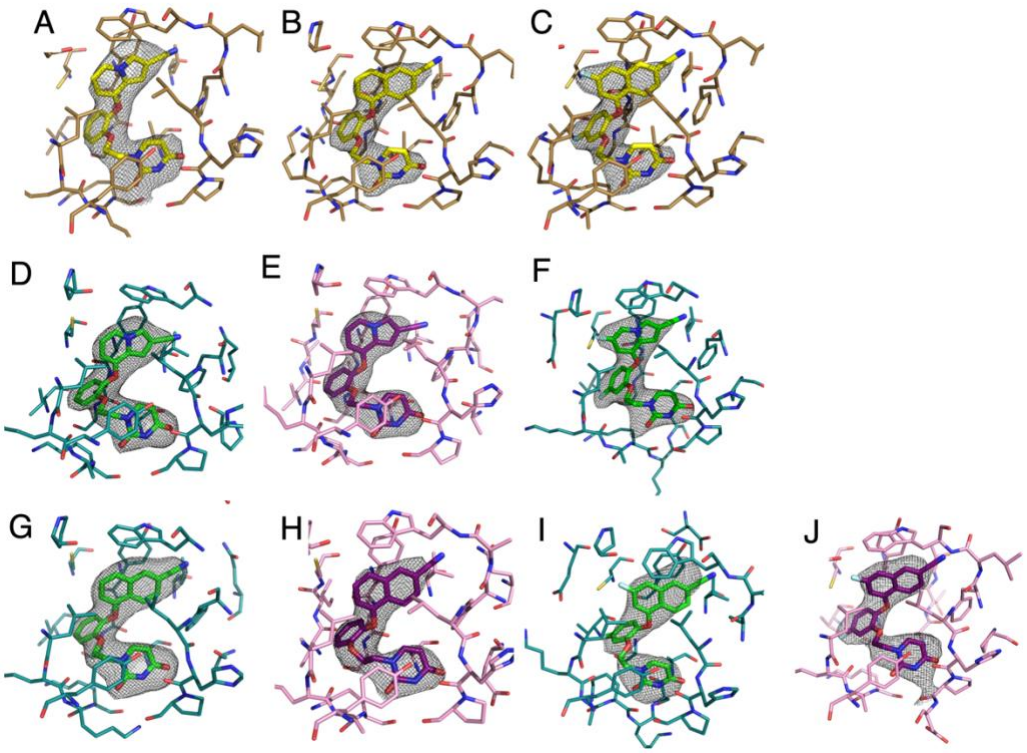


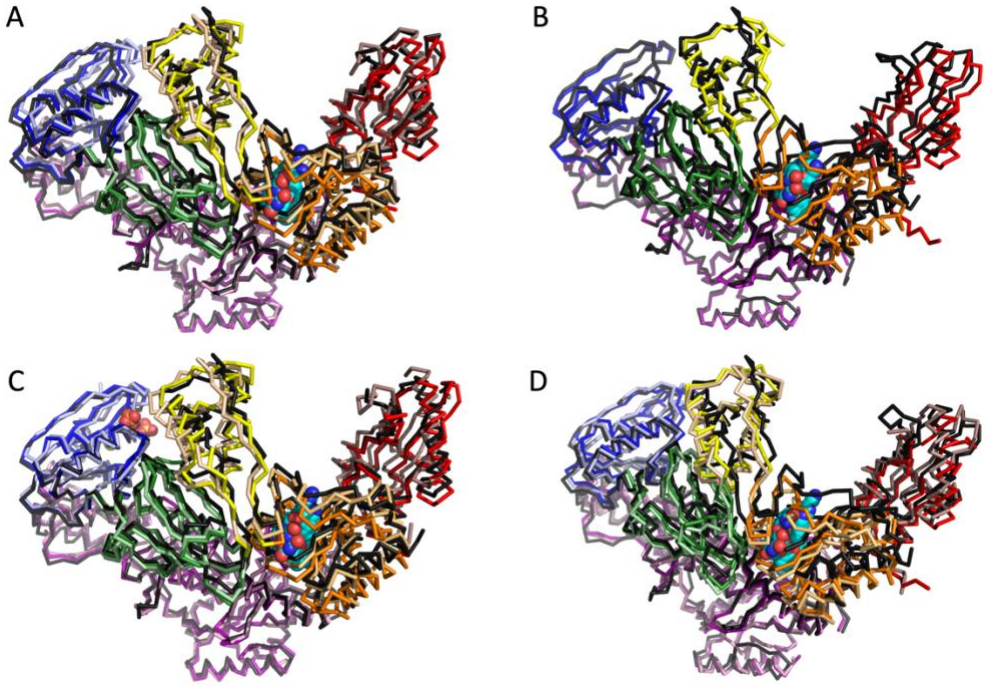
Supplementary Figure 1: Asymmetric units of V106A/Y181C structures form from different arrangements of p66/p51 heterodimers.

(A) V106A/Y181C:Compound 1a (Assembly 1: cyan, Assembly 2: deep teal) superimposed on V106A/Y181C:Compound 2b (Assembly 1: yellow, Assembly 2: gold). Assembly 1 of each structure aligned. (B) Alignment from A with one additional Compound 1a asymmetric unit. (C) Alignment from A with one additional Compound 1b asymmetric unit.



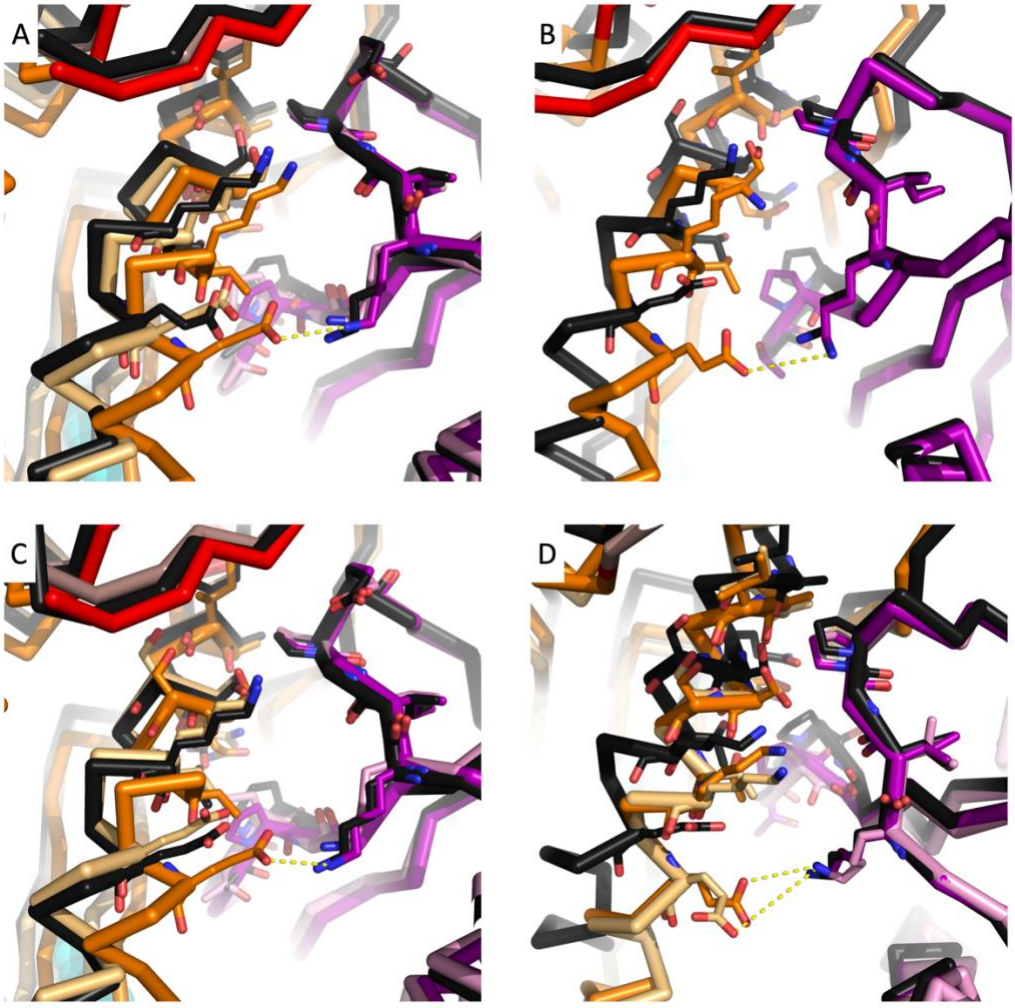
Supplementary Figure 2: There is good quality density for the ligand in all solved structures.

$2F_o$-F_c electron density omit maps of ligand for each RT-inhibitor complex. Maps contoured to 3.0σ. (A) Y181C:1a PDB: 8STP (protein in brown, Compound 1a in yellow) (B) Y181C:2a PDB: 8STQ (protein in brown, Compound 2a in yellow) (C) Y181C:2b PDB: 8STR (protein in brown, Compound 2b in yellow) (D) V106A/Y181C:1a Assembly 1 PDB: 8STT (protein in deep teal, Compound 1a in green) (E) V106A/Y181C:1a Assembly 2 PDB: 8STT (protein in pink, Compound 1a in purple) (F) V106A/Y181C:1b Assembly 1 PDB: 8STU (protein in deep teal, Compound 1b in green) (G) V106A/Y181C:2a Assembly 1 PDB: 8STV (protein in deep teal, Compound 2a in green) (H) V106A/Y181C: 2a Assembly 2 PDB: 8STV (protein in pink, Compound 2a in purple) (I) V106A/Y181C:2b Assembly 1 PDB: 8STR (protein in deep teal, Compound 2b in green) (J) V106A/Y181C:2b Assembly 2 PDB: 8STR (protein in pink, Compound 2b in purple)



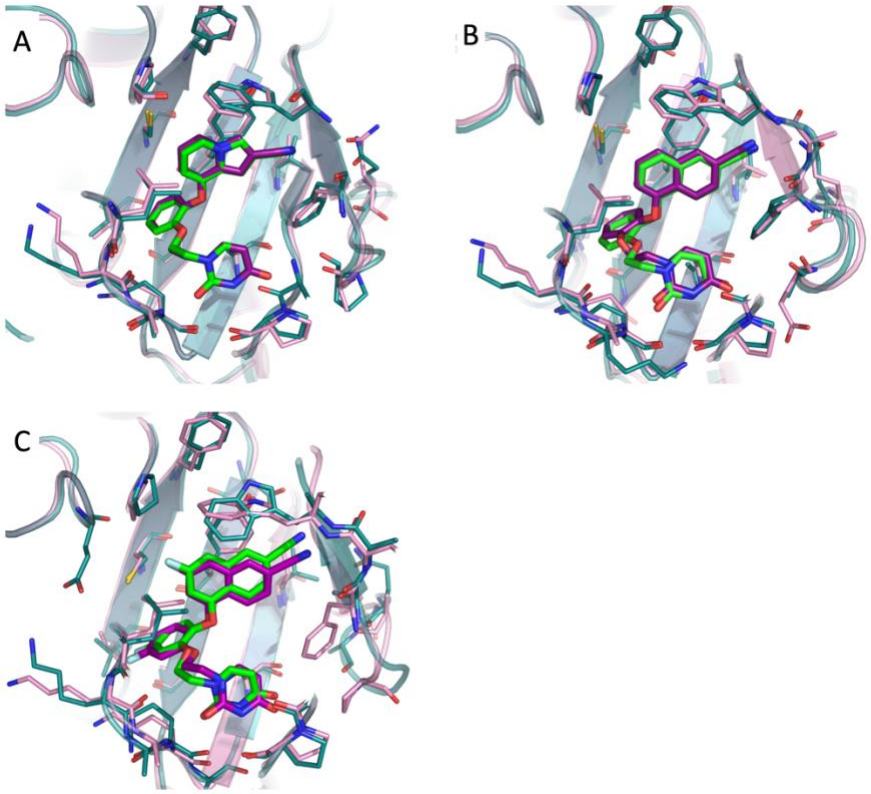
Supplementary Figure 3: Overall structure of V106A/Y181C RT is similar to that of WT RT.

Superposition of (A) Compound 1a, (B) Compound 1b, (C) Compound 2a and (D) Compound 2b in complex with wildtype (black), V106A/Y181C Assembly 1 (Bright colors), and V106A/Y181C Assembly 2 (pale colors). V106A/Y181C structures are colored based on subdomain: p66 thumb (red), palm (orange), fingers (yellow), connection (green), RNAse H (blue), p51 (pink). Bound inhibitors are shown as cyan spheres.



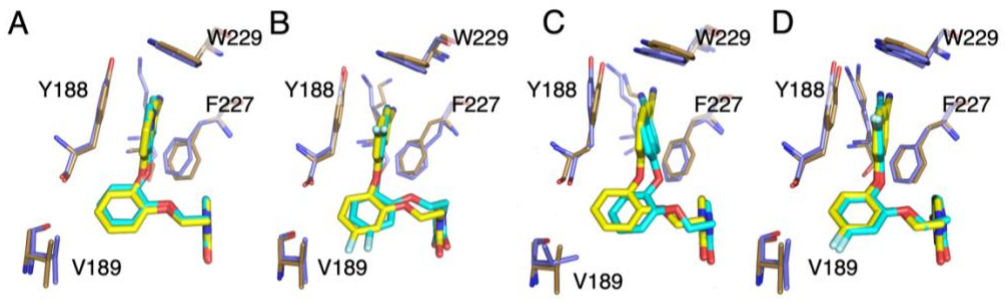
Supplementary Figure 4: Additional hydrogen bonding interactions between E169 and K49 in V106A/Y181C double mutant

Superposition of (A) Compound 1a, (B) Compound 1b, (C) Compound 2a and (D) Compound 2b in complex with wildtype (black), V106A/Y181C Assembly 1 (Bright colors), and V106A/Y181C Assembly 2 (pale colors). Closer view of the alpha helix in palm subdomain (orange) which shifts closer to the p51 subunit (pink). Residues within 6Å of the opposite domain are shown as sticks. The interaction between p66 E169 and p51 K49 is highlighted with yellow dashed lines.



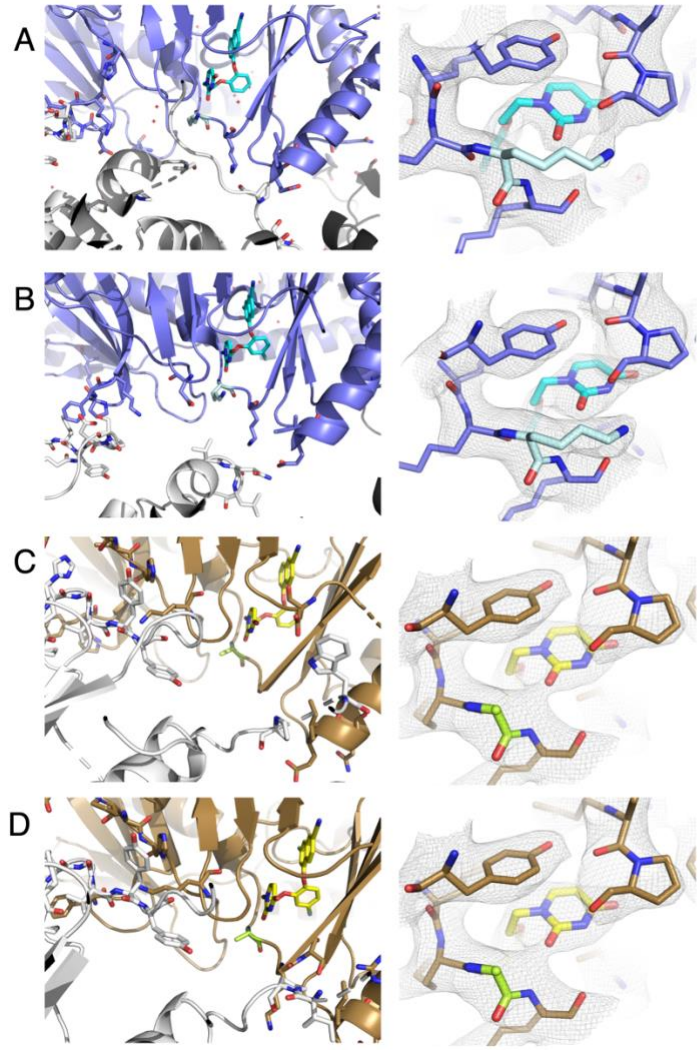
Supplementary Figure 5: Ligand placement is similar in both RT heterodimers in each asymmetric unit.

(A) Superposition of Compound 1a bound to V106A/Y181C RT Assembly 1 (protein in deep teal, Compound 1a in green) and Assembly 2 (protein in pink, Compound 1a in purple). (B) Superposition of Compound 2a bound to V106A/Y181C RT Assembly 1 (protein in deep teal, Compound 2a in green) and Assembly 2 (protein in pink, Compound 2a in purple). (C) Superposition of Compound 2b bound to V106A/Y181C RT Assembly 1 (protein in deep teal, Compound 2b in green) and Assembly 2 (protein in pink, Compound 2b in purple).



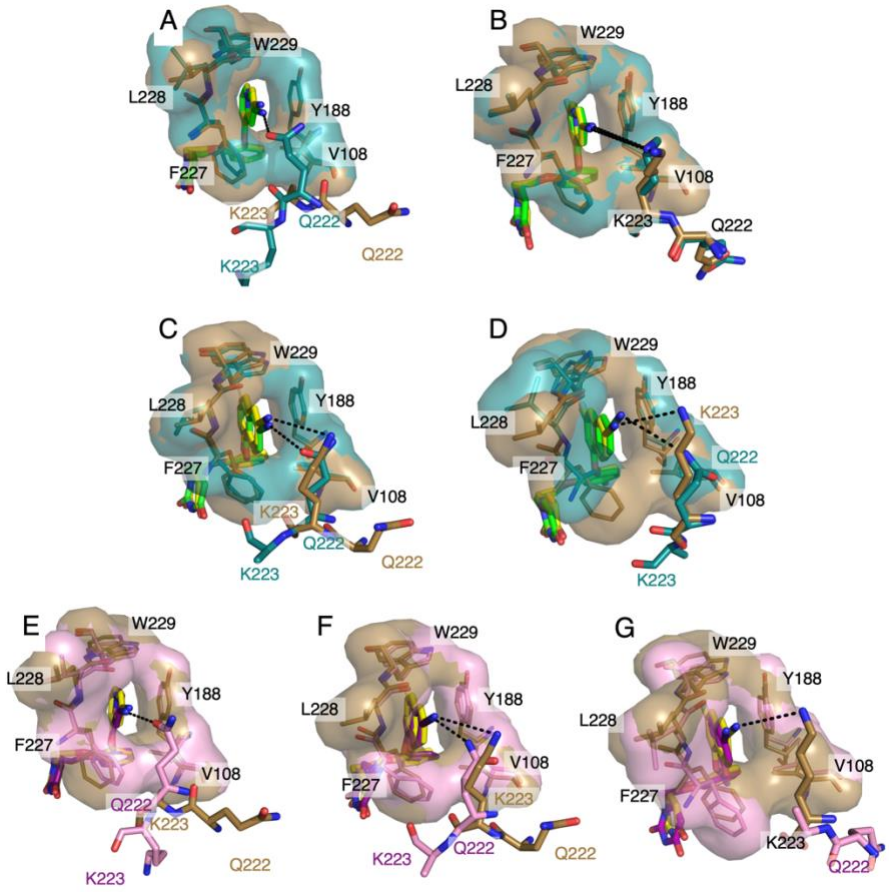
Supplementary Figure 6: Slight shifts within the binding pocket accommodate for the Y181C single mutation.

(A) Superposition of Compound 1a bound to WT RT (protein in lilac, Compound 1a in cyan; PDB: 4MFB) and Y181C RT (protein in brown, Compound 1a in yellow). (B) Superposition of Compound 1b bound to WT RT (protein in lilac, Compound 1b in cyan; PDB: 6DTX) and Y181C RT (protein in brown, Compound 1b in yellow; PDB: 6DTW). (C) Superposition of Compound 2a bound to WT RT (protein in lilac, Compound 2a in cyan; PDB: 4WE1) and Y181C RT (protein in brown, Compound 2a in yellow). (D) Superposition of Compound 2b bound to WT RT (protein in lilac, Compound 2b in cyan; PDB: 5TW3) and Y181C RT (protein in brown, Compound 2b in yellow).



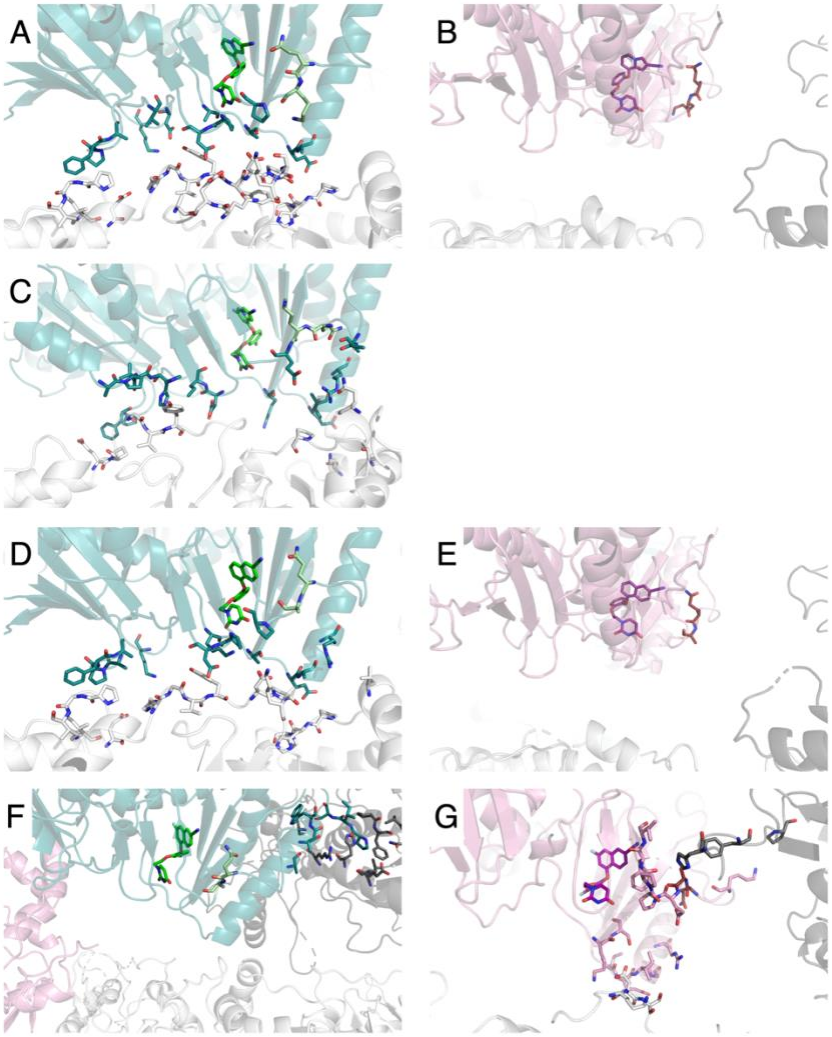
Supplementary Figure 7: Crystal contacts influence the positioning of the side chain of K102.

Crystal contacts around K102 in WT structures with (A) Compound 2a, (B) Compound 2b, or Y181C structures with (C) Compound 2a, or (D) Compound 2b. WT and Y181C structures are colored (ligand: cyan, protein: slate, K102: pale cyan) or (ligand: yellow, protein: brown, K102: green) respectively. Symmetry mates shown in white. Residues within 6Å of a symmetry mate are shown as sticks. $2F_o$-F_c electron density maps for the region surrounding K102 are shown to the right for each structure. Maps are contoured to 1 sigma.



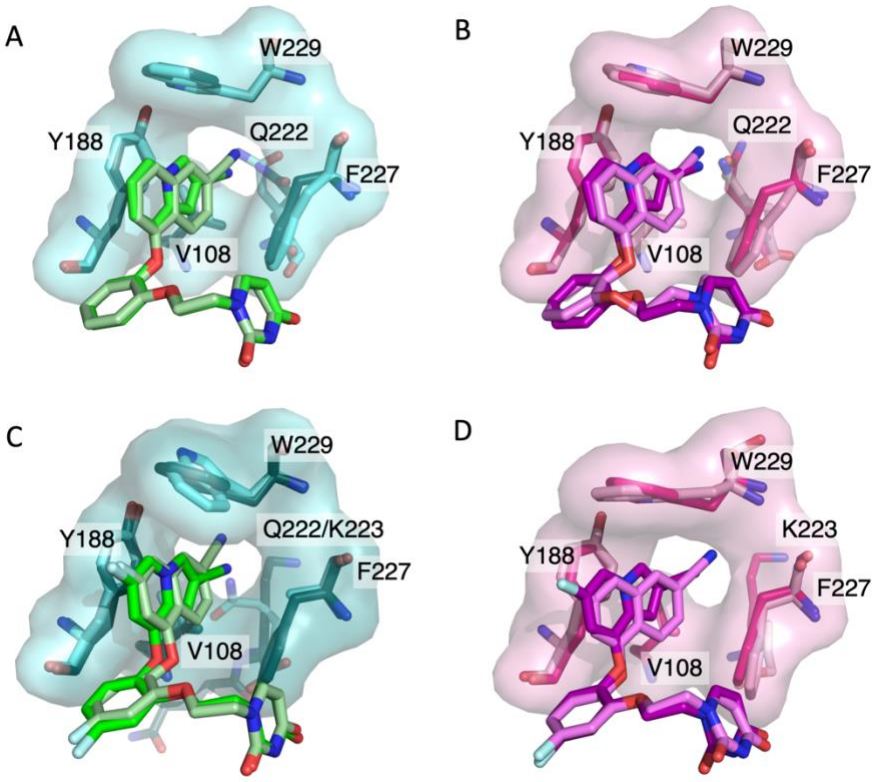
Supplementary Figure 8: Q222 commonly replaces K223 at the end of the tunnel in V106A/Y181C double mutant structures.

Superposition of (A) Compound 1a, (B) Compound 1b, (C) Compound 2a, and (D) Compound 2b bound to Y181C RT (protein in brown, ligand in yellow) and V106A/Y181C Assembly 1 (protein in deep teal, ligand in green). Superposition of (E) Compound 1a, (F) Compound 2a, and (G) Compound 2b bound to Y181C RT (protein in brown, ligand in yellow) and V106A/Y181C Assembly 2 (protein in pink, ligand in purple). The surface of residues which form the end of the tunnel are shown. Residues Q222 and K223 are shown as sticks. The interaction between the cyano group of each compound and either Q222 or K223 are indicated with black dashed lines. Labels which refer to residues in both structures are shown in black. Labels which refer to a residue in only one structure are shown in the color of the respective structure's backbone.



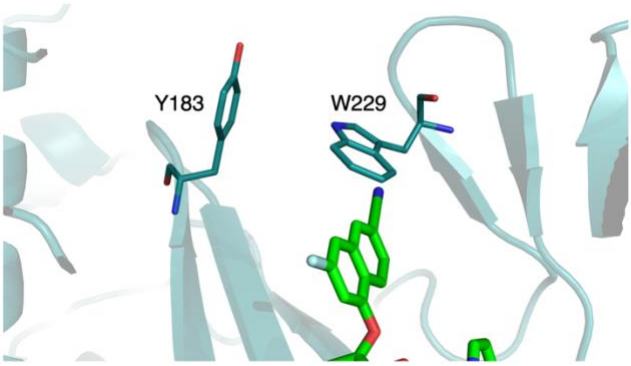
Supplementary Figure 9: Crystal contacts influence whether Q222 or K223 point into the tunnel region of the binding pocket.

Crystal contacts around Q222 and K223 in V106A/Y181C structures with (A, B) Compound 1a, (C) Compound 1b, (D, E) Compound 2a, or (F, G) Compound 2b. Assemblies 1 and 2 are colored (ligand: green, protein: deep teal, Q222 and K223: pale green) or (ligand: purple, protein: pink, Q222 and K223: brown) respectively. Symmetry mates shown in white. If interactions with multiple symmetry mates were identified, the second symmetry mate is shown in dark grey. Residues within 6Å of a symmetry mate are shown as sticks. For B, E, and F, no residues were within 6Å of a symmetry mate.



Supplementary Figure 10: The cyano group of naphthyl compounds extends farther into the tunnel than those of indolizine compounds.

Superposition of (A) V106A/Y181C:1a Assembly 1 (protein in deep teal, ligand in green) and V106A/Y181C:2a Assembly 1 (protein in cyan, ligand in pale green), (B) V106A/Y181C:1a Assembly 2 (protein in dark pink, ligand in dark purple) and V106A/Y181C:2a Assembly 2 (protein in light pink, ligand in light purple), (C) V106A/Y181C:1b (protein in deep teal, ligand in green) and V106A/Y181C:2b Assembly 1 (protein in cyan, ligand in pale green), (D) V106A/Y181C:1b (protein in dark pink, ligand in dark purple) and V106A/Y181C:2b Assembly 2 (protein in light pink, ligand in light purple). The surface of residues which form the end of the tunnel are shown. Residues Q222 and K223 are shown as sticks.



Supplementary Figure 11: Close up view of edge-to-face interaction between Y183 and W229

Compound 2b bound to V106A/Y181C Assembly 1 (protein in deep teal, Compound 2b in green). Y183 and W229 shown as sticks.