Supplemental Information

Pan-3C Protease Inhibitor Rupintrivir Binds SARS-CoV-2 Main Protease in a Unique Binding Mode

*Gordon J. Lockbaum, Mina Henes, Jeong Min Lee, Jennifer Timm, Ellen A. Nalivaika, Paul R. Thompson, Nese Kurt Yilmaz, Celia A. Schiffer**

Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, Massachusetts 01605, United States

*Corresponding author. Email: Celia.Schiffer@umassmed.edu

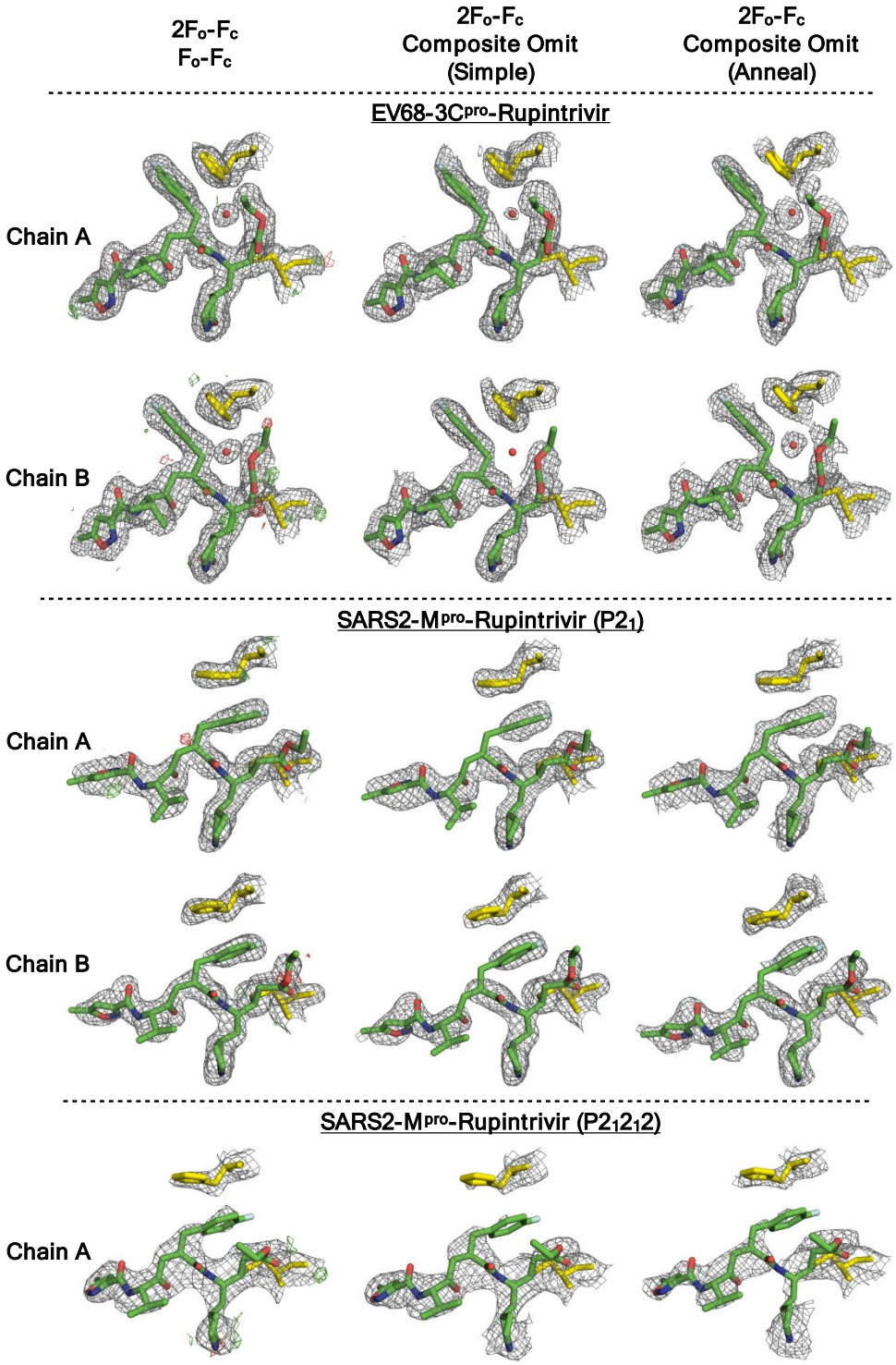

Figure S1. Evidence that the inhibitor and side chains were modeled correctly. Electron density maps around rupintrivir and the catalytic residues, shown as green and yellow sticks, respectively. The $2F_o$-F_c direct maps are depicted as grey mesh contoured at 1.0σ while the F_o-F_c difference maps have positive density depicted as green mesh contoured at 3.0σ and negative density as red mesh contoured at -3.0σ. PDB: L78H (top), 7L8I (middle), 7L8J (bottom).

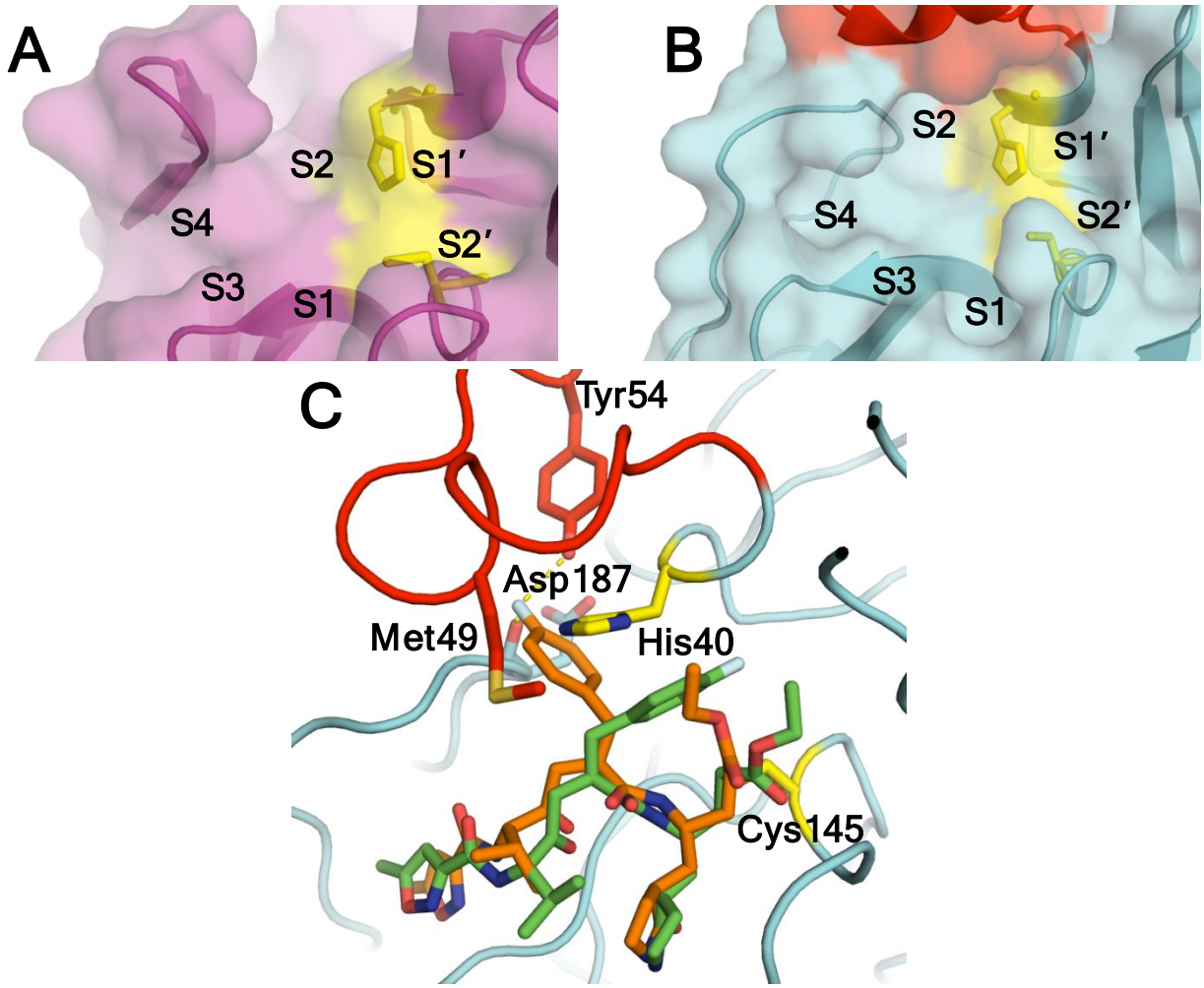

Figure S2. A) Human rhinovirus 3C^{pro} active site with subsites labeled and catalytic residues shown as sticks and colored yellow (PDB 1CQQ). **B)** SARS-CoV-2 M^{pro} active site with subsites labeled and catalytic residues shown as sticks and colored yellow, and residues 43-63 colored red (PDB: 3L0D). **C)** Alignment of rupintrivir (orange sticks) from the EV68 3C^{pro} bound structure onto SARS-CoV-2 M^{pro} active site with bound rupintrivir (green sticks) shows that although there are some polar atoms in the S2 subsite of SARS-CoV-2 M^{pro} such as Tyr54 and the backbone carbonyl oxygen of Asp187, there is a lack of space and flexibility to optimize those interactions to accommodate the canonical 3C^{pro} binding mode.

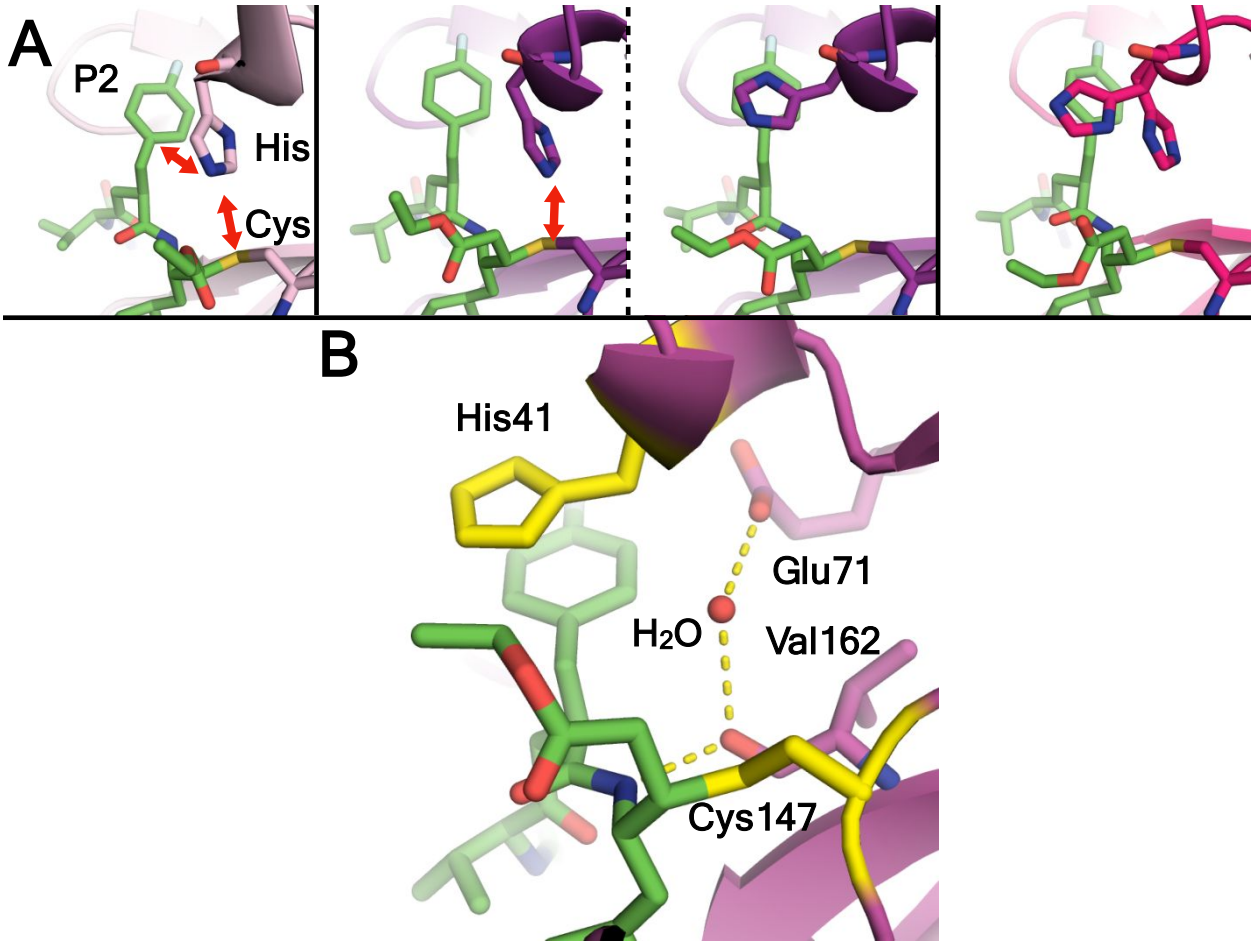

Figure S3. A) Previously published 3C proteases show the catalytic histidine modeled either: near the catalytic cysteine, in an orientation that creates pi-pi stacking, or in both conformations (PDB: (PDB: 1CQQ, 3SJO [chain B and chain A], and 3ROU). **B)** Our EV68 $3C^{pro}$-rupintrivir complex includes a crystallographic water molecule that creates a water-mediated salt bridge between the side chain of Glu75 and the main chain carbonyl oxygen of Val162.

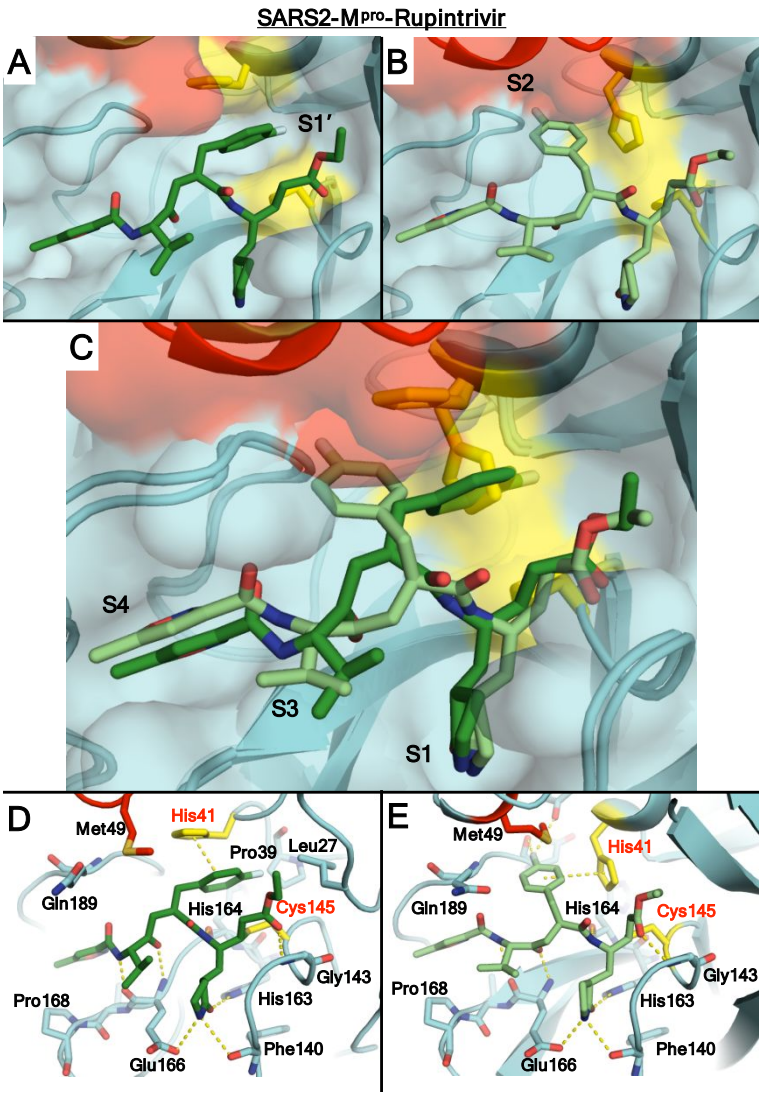

Figure S4. Comparison of rupintrivir binding mode in **A)** the crystal structure presented here (PDB ID: 7L8I), and **B)** another recent structure (PDB ID: 7P35). **C)** Overlay of rupintrivir in the two structures. **D, E)** Residues involved in inhibitor interactions are indicated.

Table S1. X-ray data collection and crystallographic refinement statistics. Values in parentheses are for the highest resolution shell.

Protein	**EV68-3C^{pro}**	**SARS2-3CL^{pro}**	**SARS2-3CL^{pro}**
Inhibitor	rupintrivir	rupintrivir	rupintrivir
PDB ID	7L8H	7L8I	7L8J
Data Collection			
Wavelength (Å)	0.9200	1.54178	0.9200
Space group	R3	$P12_11$	$P2_12_12$
a, b, c (Å)	80.9, 80.9, 197.2	47.8, 106.4, 54.1	44.9, 62.1, 107.2
α, β, *γ* (°)	90, 90, 120	90, 103.1, 90	90, 90, 90
Resolution (Å)	40.3 - 1.95 (2.02 - 1.95)	23.6 - 2.10 (2.18 - 2.10)	41.4 - 2.45 (2.54 - 2.45)
Total Reflections	161095 (16113)	97350 (7445)	66115 (6597)
Unique Reflections	34962 (3510)	28161 (2360)	11556 (1145)
Redundancy	4.6 (4.6)	3.5 (3.2)	5.7 (5.8)
Completeness (%)	99.7 (98.7)	92.2 (78.1)	99.7 (99.7)
Average I/σ	12.8 (3.1)	9.1 (3.1)	10.0 (3.9)
R_{merge} [a]	0.077 (0.650)	0.086 (0.385)	0.125 (0.576)
R_{pim}	0.041 (0.340)	0.054 (0.248)	0.058 (0.237)
CC 1/2	0.996 (0.689)	0.994 (0.790)	0.992 (0.926)
Refinement			
Molecules in A.U.	2	2	1
Twin Law Twin Fraction	h, -h-k,-l 0.390	-	-
R_{factor} (%)[c]	15.8	20.6	22.4
R_{free} (%)[d]	18.6	26.3	28.1
RMSD[b] in:			
Bond lengths (Å)	0.004	0.003	0.010
Bond angles (°)	0.640	0.540	0.870
Ramachandrans:			
Favored (%)	94.55	96.86	96.37
Allowed (%)	4.90	2.64	3.30
Outliers (%)	0.54	0.50	0.33
B-factors:			
Average	35.91	36.22	60.67
Macromolecules	35.57	35.97	60.68
Ligands	30.07	40.24	69.58
Solvent	41.12	38.74	51.36

[a] $R_{sym} = \Sigma \mid I - \langle I \rangle \mid / \Sigma I$, where I = observed intensity, $\langle I \rangle$ = average intensity over symmetry equivalent

[b] RMSD, root mean square deviation.

[c] $R_{factor} = \Sigma \mid\mid F_o \mid - \mid F_c \mid\mid / \Sigma \mid F_o \mid$.

[d] R_{free} was calculated from 5% of reflections, chosen randomly, which were omitted from the refinement process.