Pharmacophore anchor models of flaviviral NS3 proteases lead to drug repurposing for DENV infection

(Additional file 1)

Contents:

Additional text:

PA models of HCV, DENV, WNV and JEV NS3 proteases:

The PA model of the HCV NS3 protease contains 12 distinct anchors, five core (highlighted in pink dotted circles) and 7 specific anchors. The five core anchors have been discussed in the main manuscript, so the general features of seven HCV specific anchors are explained below (Fig. S2A). At the S2 subsite, a specific anchor HHV4 favors both H-bonding (by D1081, D1079 and V1078) and hydrophobic interactions (by H1057, D1081 and Y1056) with compound moieties. Between the S2 and S3 sites we find HH3 anchor (D1168, R1155, D1079) preferring polar groups (like -CO-NH-, -CO- and -COO-) for anchoring the flat region. Near the S1' site, adjacent to CEH1, the HH2 anchor with three residues (Q1041, T1042 and G1137) forms H-bonds with carbonyl and ketone moieties from compounds. Also the anchors HV1 (H1057, Q1041, F1043) and HV2 (Q1041, T1042, G1137) represented the consensus hydrophobic interactions of aromatic and heterocyclic moieties of the docked compounds with subsite residues. Anchors HV4 (K1136, I1132, A1157) and HV6 (R1123, A1156, A1157) at S3 site also favor van der Waals interactions as shown in Figure S2A.

The DENV NS3 protease PA model consists of 13 anchors including 8 specific anchors in addition to 5 core anchors (highlighted in pink) (Fig. S2B). A dual-type DHV4 anchor, an electrostatic DE2 anchor, three H-anchors (DH2, DH5, DH9) and three V-anchors (DV6, DV8 and DV9) constituted the specific anchors. Anchors DHV4 and DH2 at the S1 sub-pocket, accommodated the P1 Arg side chain of the substrate as seen in ligand-bound structure [1]. While the DHV4 anchor offers both H-bonds (by D129 and F130 side chains) and van der Waals bonds (by Y161 and P132), the adjacent DH2 anchor is found buried deep inside the S1 subsite favoring carbonyl and ketone groups to interacts with its polar residues S135, T134 etc. At the S1' subsite, we observe an DENV-exclusive DE2 electrostatic anchor formed by consensus electrostatic interactions of positively charged R54 residue with negatively charged compound moieties ($-PO4^-$ or $-COO^-$). This anchor could be exploited during drug design to achieve selectivity towards inhibiting DENV NS3 protease. Adjacently we find a hydrophobic DV6 anchor (H51, V52 and V36) which helps to stabilize substrate during catalysis. Anchors DH9 and DV9 at the S2 subsite as shown in Figure S2B, are supported by the residues H51, D75, N152 and N152, G82, G83 respectively (from both NS3 and NS2B cofactor chains). Lastly, the S3 site contains anchors DH5 (Y161, G151, N152 and G153) and DV8 (N152, G82, and T83) which prefer polar and hydrophobic groups respectively also interact with P3 group of the substrate [1].

The WNV PA model has 7 specific anchors (WHV4, WHV8, WH2, WH5, WH6, WV5 and WV6) as shown in the Figure 2C. In the WNV NS3 protease ligand-bound crystal structure: 2FP7 [2], at S2 sub-pocket the WHV4 anchor (analogous to HHV4 from HCV PA model) supports P2 Arg side chain of peptide-like inhibitor,

in accordance with its moiety preferences. Similarly, at S1 subsite we find a WHV8 anchor (D129, Y130, P131, Y161), which helps in anchorage of the 'Arg' side chain at P1 region of the inhibitor [2]. Adjacent to WHV8 and deeply located is a WH5 anchor (Y130, T134, S135) available for H-bonding (analogous to DH2). The specific anchor, WH2 (Y161, G151, N152, G153) engages the amino acid backbone carbonyl groups of the peptide substrate by H-bonding. The anchor WV5 forms interaction with compound hydrophobic moieties (like with phenyl group of inhibitor in 2FP7) by anchor residues Y151, G153, V154 and I155. The two anchors WH6 and WV6, engage the substrate/inhibitor functional groups at the S3 subsite of the WNV protease.

In the JEV NS3 protease PA model, we observed 13 anchors (5 core and 8 specific) (Fig. S2D). Among the specific anchors, JHV3, JHV4, JHV7 and JEH4 anchors belonged to the dual interaction type; JH2 and JH6 were H-type; JV1 and JV10 were V-type. In the vicinity of the S1 sub-pocket, we find the three mixed-type anchors and a JH2 anchor. The JHV3 and JHV7 anchors supported H-bonding (with polar amide, ketone, alcohol groups) and van der Waals interactions (with aromatic, alkyl and aliphatic moieties). The JEH4 anchor preferred electrostatic bonding of D129 residue with charged groups like –SO2- of compounds, while residues like Y130 helped in forming H-bond with polar carbonyl moieties. The JH2 anchor located deep in the S1 sub-pocket is analogous to DH2 and WH5 anchors sharing similar binding features. The JH6 (near the core anchor CV3) aids to stabilize the main chains of substrate or peptide inhibitor by H-bonding. At the S3 site, there exists the JHV4 anchor (A125, G151, N152 and G153) H-bonding with polar groups (amide, tertiary amine and alcohol moieties) and forming van der Waals interactions with hydrophobic groups (aromatic, alkyl and phenolic moieties). Finally, JV1 at S3 site and JV10 at S2 site, hold the substrate/inhibitor hydrophobic functional groups in position by van der Waals interactions.

Flaviviral NS3 protease sequence and structure analysis:

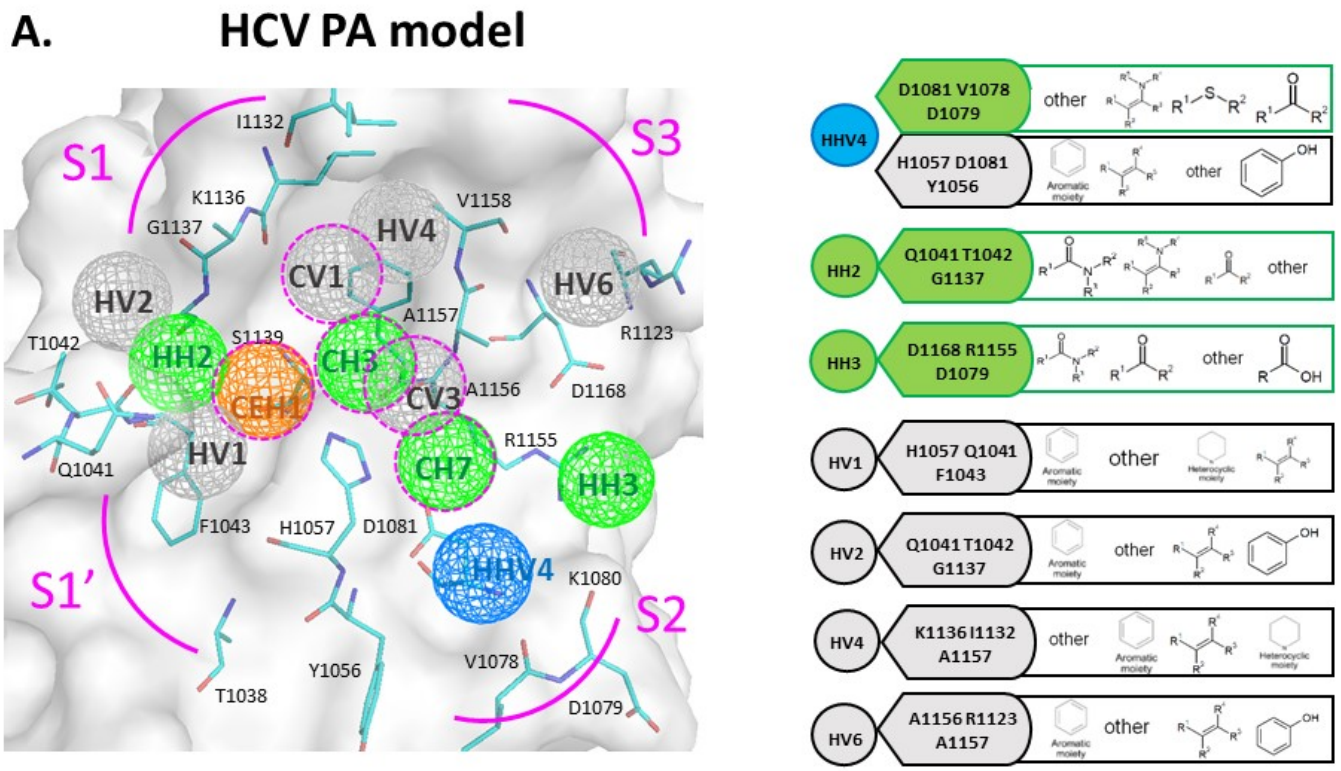
We analyzed the four flaviviral NS3 protease sequences by CLUSTALW, a multiple sequence alignment (MSA) tool [3] and structures by CEalign, a structure alignment tool [4]. We observed a significant sequence similarity in aligned NS3 protease (Fig. S1A), except for HCV NS3/4A protease which differs distinctly by the use of NS4A as a cofactor unlike others which used the cofactor NS2B. In addition, the phylogenetic evolutionary tree derived from MSA for NS3 and the co-factors showed that the HCV protease is branched farther away from other viruses denoting its distant evolution (Fig. S1B). The structural alignment revealed a conserved chymotrypsin-like fold with aligned catalytic triad residues *His-Ser-Asp* (Fig. S1C). In DENV, WNV and JEV NS3 proteases, the cofactor NS2B extends to the substrate binding site for substrate stabilization making a deeper active site, but the cofactor 4A in HCV NS3 protease does not extend to the substrate binding pockets resulting in a much flat and wider active site. These subtle similarities and differences in proteases are of great importance in inhibitor design and discovery.

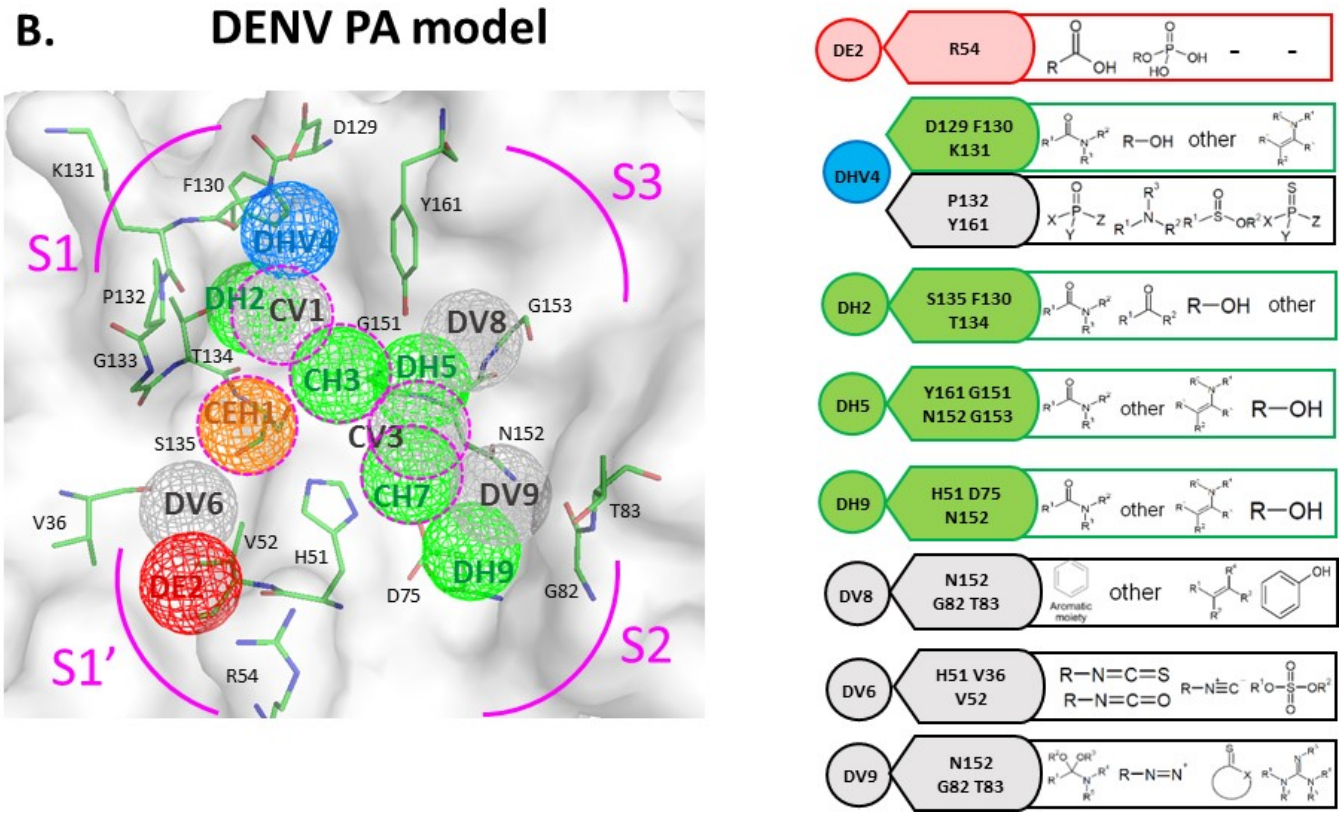
Evolutionary conservation and mutational analysis of anchor residues:

To verify our PA/CPA models, we primarily evaluated anchor-residue conservation by ConsurfDB residue conservation scores [5]. The NS3 protease residues were grouped into four categories as core anchor residues, specific anchor residues, binding site residues (active site residues (<8Å) but not anchor residues) and other residues (not in above groups). Based on the residue conservation scores for four proteases (Fig. S4A), we observed that the >60% of core anchor residues and >40% of specific anchor residues had the highest conservation score of 9. This high conservation of core anchor residues confirms their critical role in the protease structure and function. The specific anchor residues were less conserved compared to core anchors as they tend to have subtle differences among species. Moreover, when comparing the anchor conservation scores (an average of conservation scores of anchor residues) the highest scores 8-9 were attained by core (>60%) and specific (>40%) anchors in all the four proteases (Fig. S4B). This shows that core anchors are critically conserved across the protein family during evolution followed by specific anchors, and points out their key role.

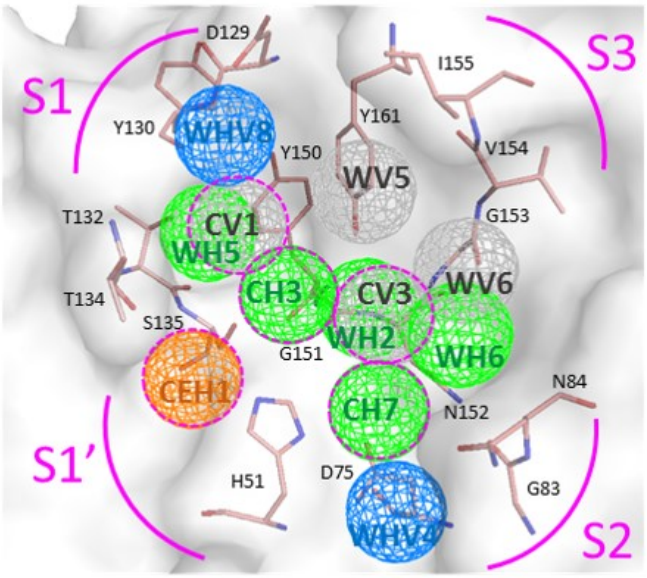
The PA/CPA models were further verified by analyzing the effect of the anchor residue mutations on the overall protease enzymatic activity. For this, we collected the mutation-activity effect data of HCV, DENV, and WNV NS3 protease residues from literature (Table. S2). In general, we observed that anchor residue led to abrogation of enzyme activity depicting their functional role pointing out their targeting for function inhibition. In the HCV NS3 protease (Table. S2A), when His1057 (a catalytic residue) involved in four core and two specific anchors was mutated to Ala, the positively charged His side chain was lost resulting in disruption of the protease activity evident by the inactive mutant. Also, another anchor residue D1081 of CH7 and HHV4 anchors when mutated to Gly, lost it negative charged aspartate side chain leaving the mutant enzyme inactive. But when residue R1123 of specific anchor HV6 mutates to threonine the enzyme retains its biological activity similar to wild type, as both Arg and Thr have similar hydrophobic interactions at the anchor. In the DENV NS3 protease (Table. 2B), the CEH1 anchor residue G133 forming the catalytic oxyanion hole, when mutated to Ala can no longer accommodate oxyanion during catalysis resulting in its undetectable protease activity. Similarly, mutations in residues S135, Y150, G151, N152 and G153 all involved in core and specific anchors resulted in mutant enzymes with undetectable or very low activities. We see similar observations in the residue mutations occurring in the WNV NS3 protease (Table. 2C). Catalytic D75 involved in CH7, WHV4 and WV6 anchors if mutated to Ala lost the ability to stabilize substrate, resulting in an enzymatically inactive enzyme. Interactions of WHV4 and WV6 anchor residues D82 and G83 with substrates are disrupted due to their mutations causing enzyme inactivity. From this analysis, we inferred that enzymatic activity is preserved in mutant when the anchor interaction is preserved in spite of the anchor residue change; while activity is lost when anchor interaction is lost due to residue mutation. Hence, the role of anchors (consensus interactions) in enzymatic activity as well as inhibition is justified.

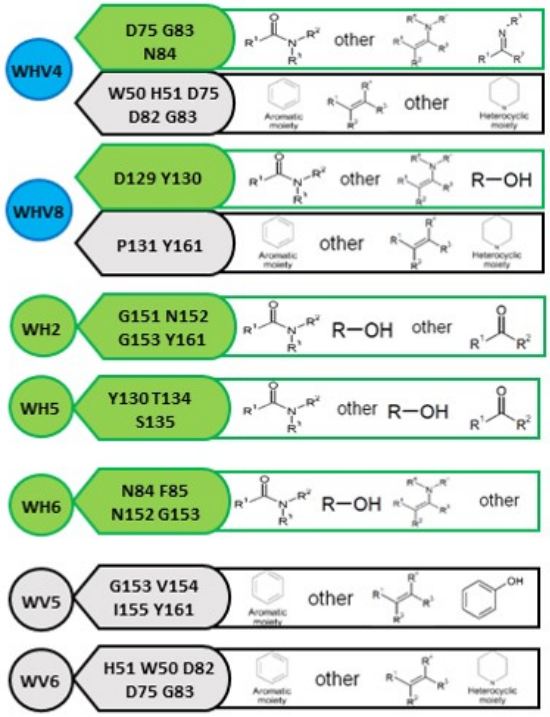
Additional figures:



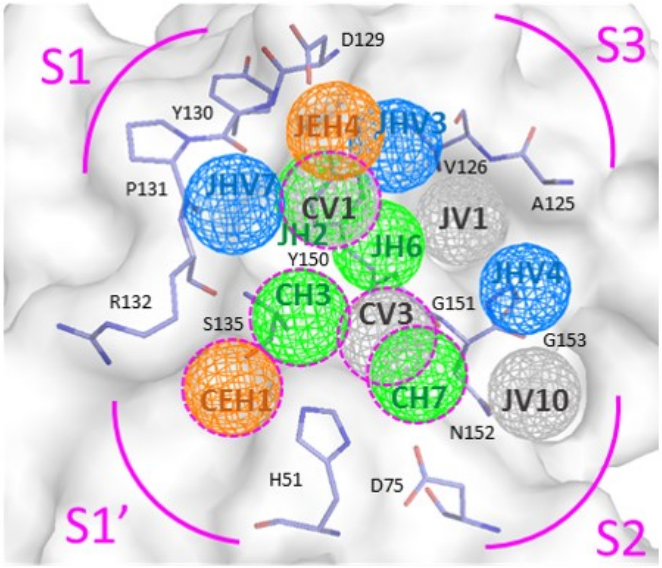


C. WNV PA model





D. JEV PA model



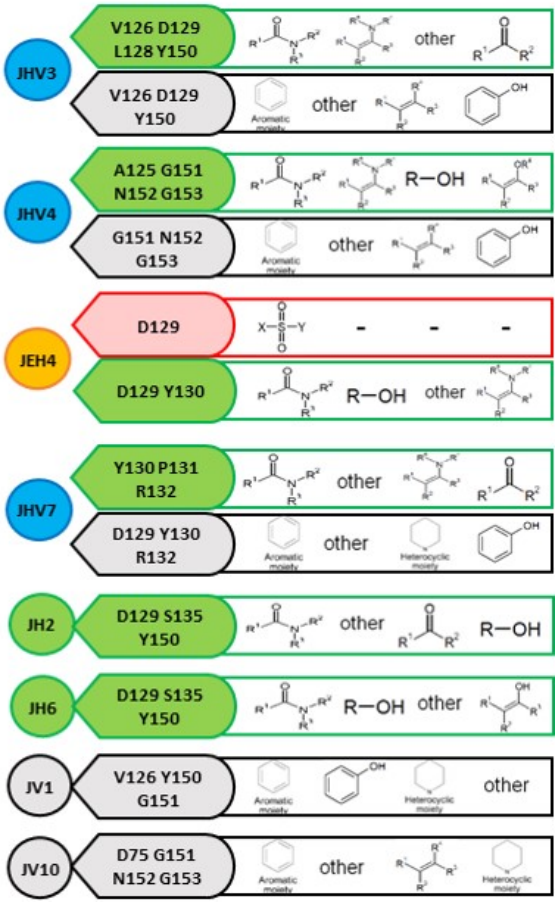


Figure S1. **Individual pharmacophore anchor (PA) models of four flaviviral NS3 proteases:** (A) HCV NS3 protease PA model; (B) DENV NS3 protease PA model; (C) WNV NS3 protease PA model; (D) JEV NS3 protease PA model. Each PA model shows core anchors (dotted pink circles), specific anchors and the protease subsites (pink arcs). The anchor features (type, residues and moiety preferences) for specific anchors are depicted.

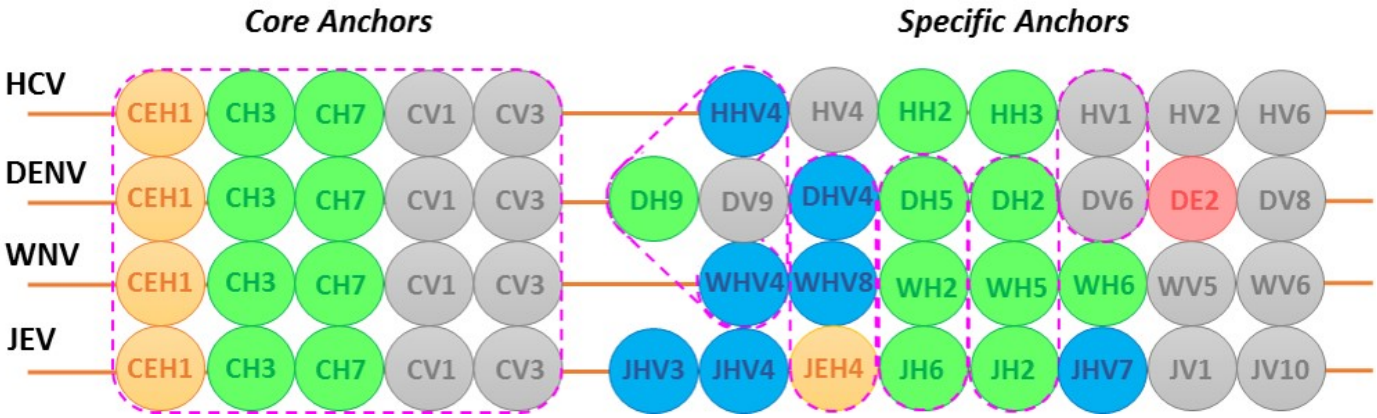


Figure S2. **Summary of the core and specific anchors.** The matched core and specific anchors between proteases are highlighted by dotted pink lines.

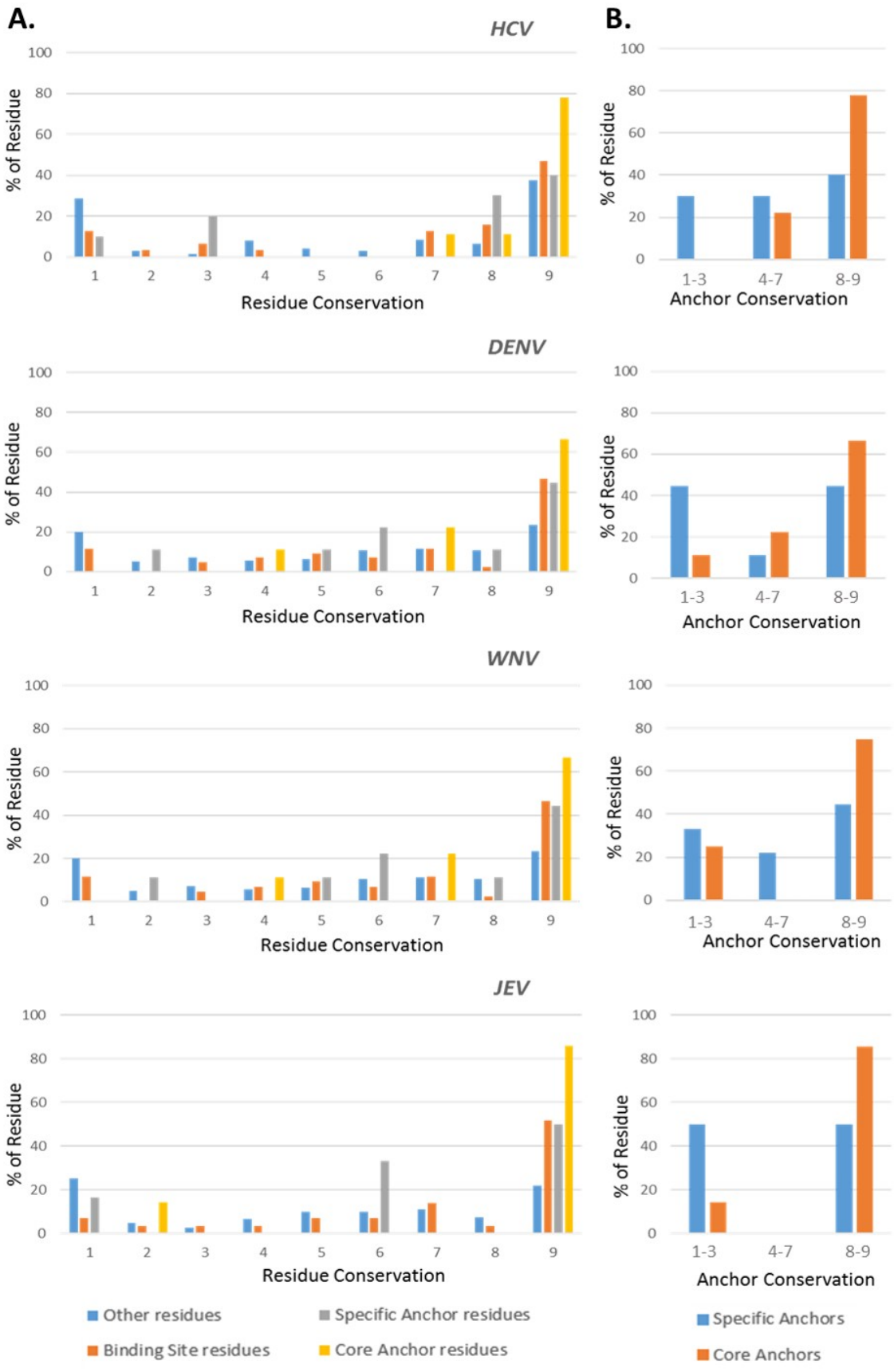

A.
B.
HCV
DENV
WNV
JEV
% of Residue
Residue Conservation
Anchor Conservation
1-3
4-7
8-9
Other residues
Specific Anchor residues
Binding Site residues
Core Anchor residues
Specific Anchors
Core Anchors

Figure S3. **Conservation analysis of core and specific anchor residues of PA/CPA models. (**A) Residue conservation scores and (B) anchor conservation scores. The % of residues with conservation scores (1-9, 1-least conserved, 9-most conserved) for four groups (core anchor residues, specific anchor residues, binding site residues and other residues) in HCV, DENV, WNV and JEV NS3 proteases are shown.

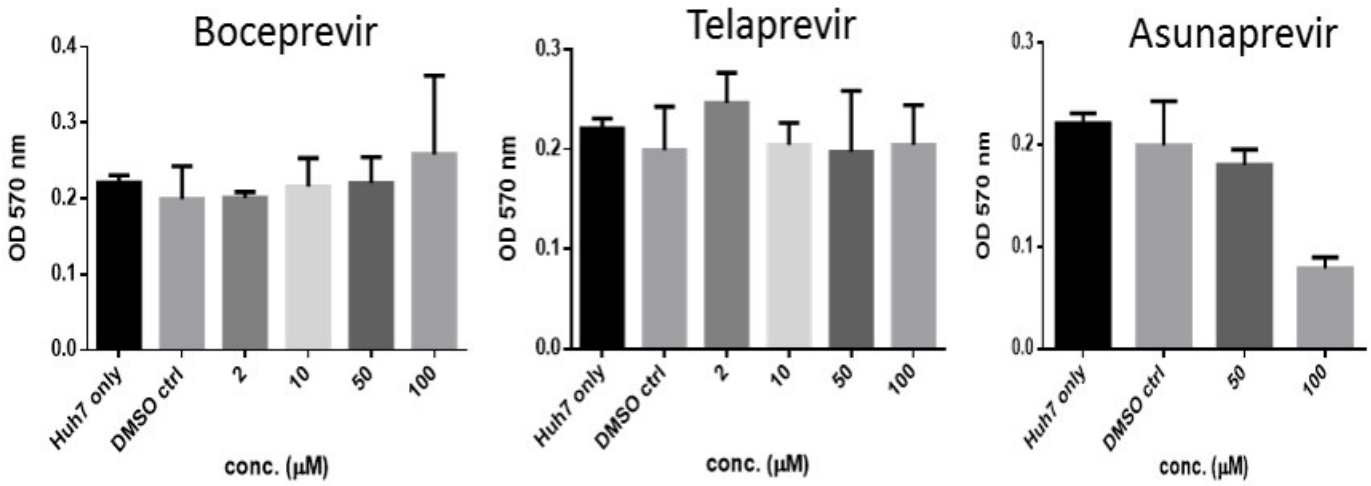


Figure S4. **MTT assays for cytotoxicity studies.** The non-cytotoxic concentrations of the inhibitor candidates are determined by testing increasing compound concentrations (μM) on Huh7 cells, and finding highest concentrations at which majority of cells are viable. OD values at 570 nm represents the cell viability.

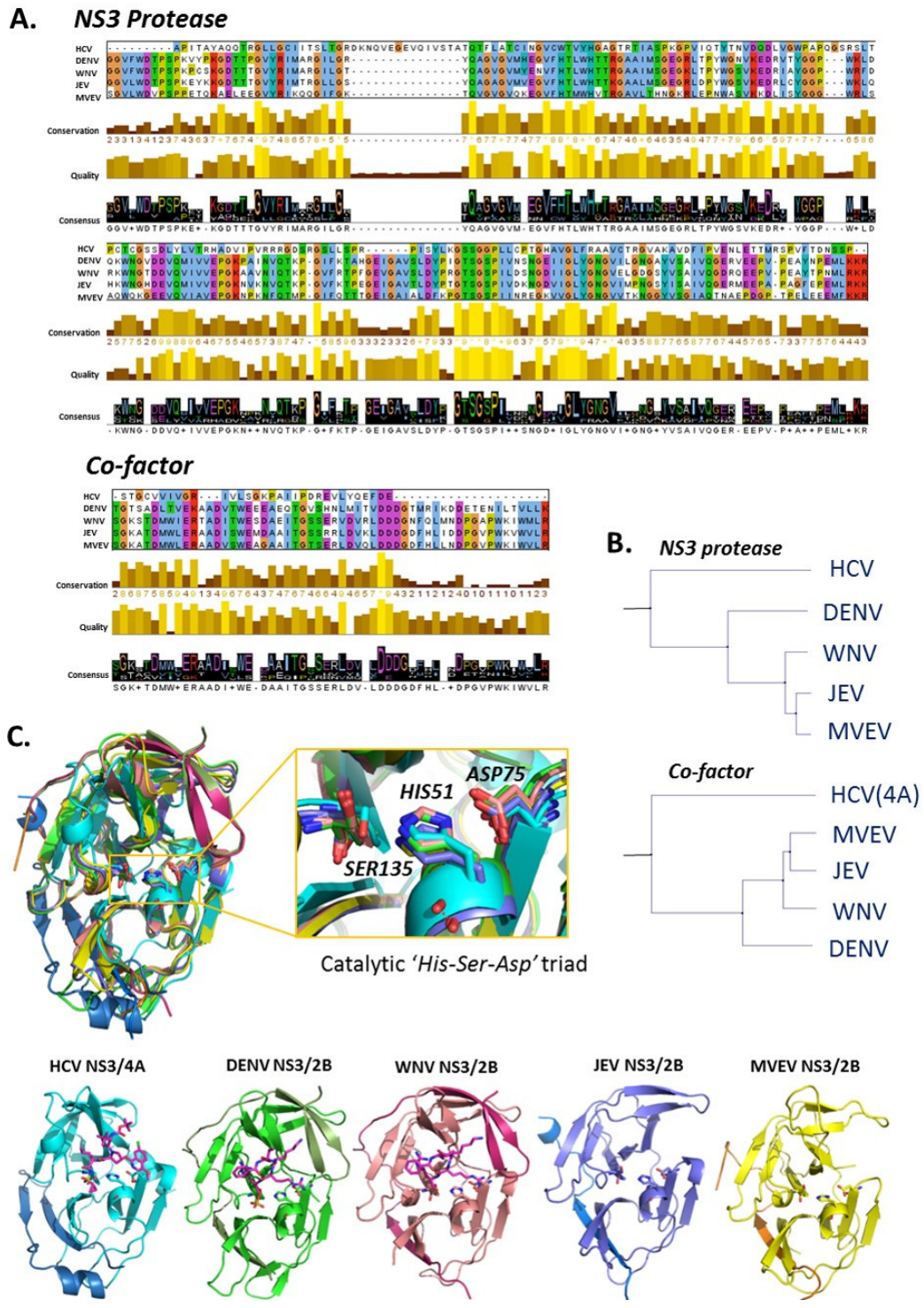


Figure S5. Flaviviral NS3 proteases: sequence and structure comparison. (A) Multiple sequence alignment (MSA) of flaviviral NS3 protease and cofactor (NS4A/NS2B) sequences. (B) Phylogenetic trees based on MSA (by nearest neighborhood method). (C) Aligned flaviviral protease structures with conserved catalytic triad '*His-Ser-Asp*' (in insight). Individual NS3 proteases from HCV (4WF8), DENV (3U1I), WNV (2FP7), JEV (4R8T) and MVEV (2WV9) with conserved protease fold (NS3 and cofactor chains colored differently).

Additional tables:

Table S1. Anchor residue mutation-activity data analysis. The change in residue mutant enzyme activity compared to wild-type (WT) are shown for anchor residues of (A) HCV PA model, (B) DENV PA model and (C) WNV PA model. Mutation types (represented by different fonts): Site directed mutagenesis - normal, Natural variants – italic, Resistance mutations – underlined. Activities (compared to WT): WT-like(WTL): 60%-100%; Decreased(D): 30%-60%; Very less(VL): 5%-30%; Undetectable(UD): 0-5%.

(A) Anchor residue	**Mutated to**	**Activity (compared to WT)**	**Anchor**	**Reference**
F1043	C S Y	- - -	HV1	[6] [6] [7]
H1057	A	UD	CEH1, CH3, CH7, CV3, HHV4, HV1	[7, 8]
D1079	E H	- -	HHV4, HH3	[7] [7]
D1081	G	UD	CH7, HHV4	[9]
R1123	*T* S	WTL -	HV6	[10] [7]
I1132	N	-	HV4	[7]
L1135	F	-	CV1	[7]
K1136	M R E	D WTL -	CV1, HV4	[11] [11] [7]
S1139	A P	UD UD	CEH1, CH3	[7, 8] [9]
F1154	S G	- -	CV1	[7]
R1155	*Q* T M K S	- - - - D	CH3, CH7, CV3, HH3	[6, 12] [7] [7] [7] [11]
A1156	S T D	- - -	CV3, HV6	[6] [7] [7]

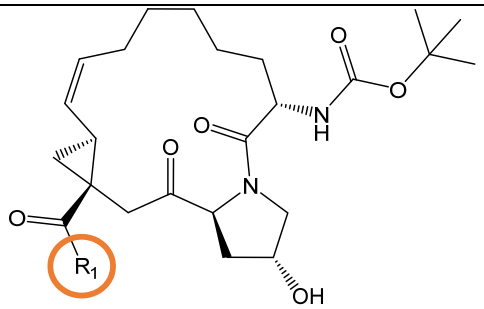
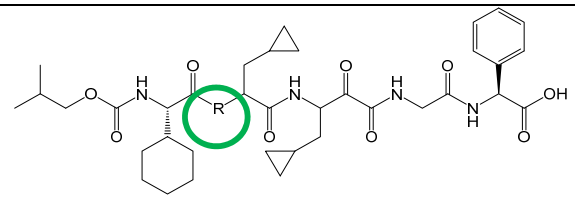
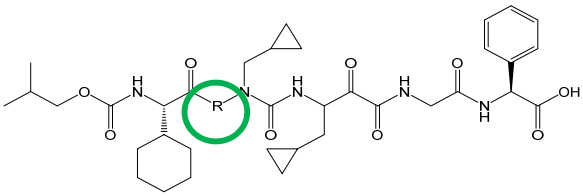
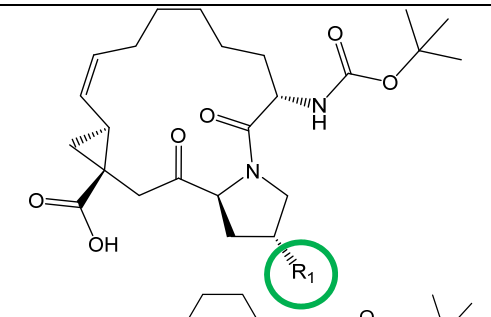
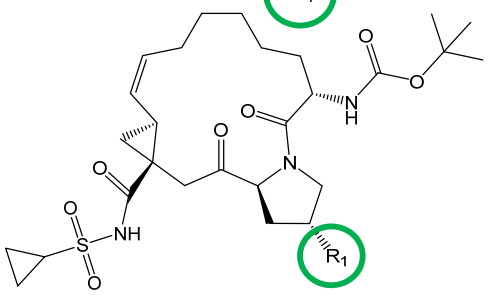
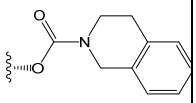
D1168	Q E *V* *Y* *A* *G*	WTL WTL - - - -	HH3	[10] [9] [12, 13] [13] [13] [14]

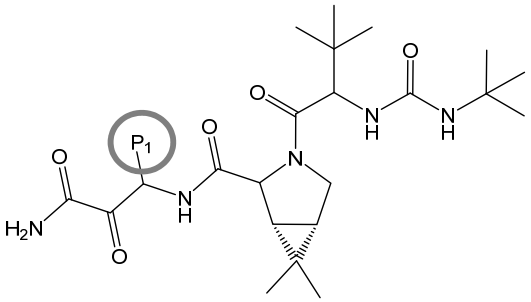
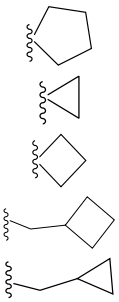
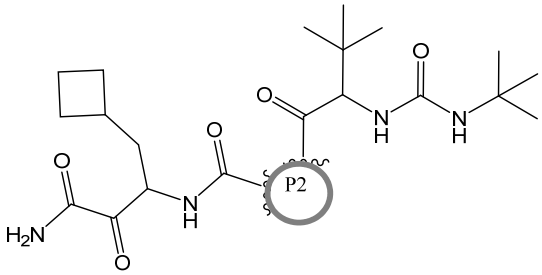
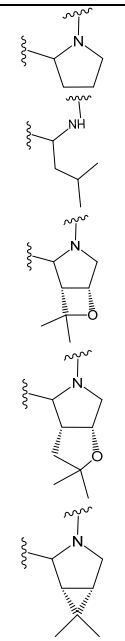
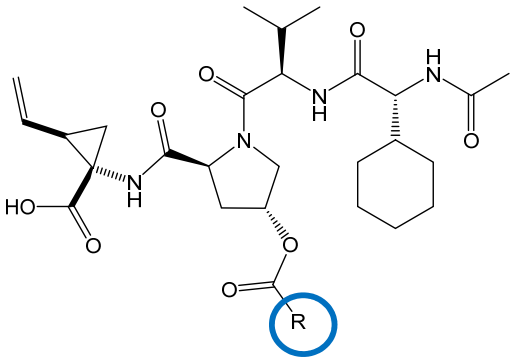
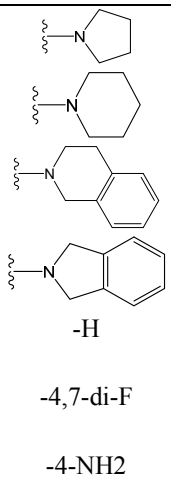
(B) **Anchor Residue**	**Mutated to**	**Activity (compared to WT)**	**Anchor**	**Reference**
D129	E S A K R L	D D D VL VL VL	DHV4	[15, 16]
F130	Y A S	D VL VL	DHV4, DH2	[15]
K131	S T	WT WT	CV1, DHV4	[15]
G133	A	UD	CEH1	[15, 16]
T134	A D	D UD	DH2	[15, 16]
S135	A C	UD UD	CEH1, CH3, DH2, DH9	[15, 16]
Y150	F A V H	D UD UD UD	CV1, DH9	[15, 16]
G151	A	UD	CH3, CH7, CV3, DH5	[15, 16]
N152	A Q	VL VL	CH7, CV3, DH5, DV8, DV9	[15, 16]
G153	A V	UD UD	CH7, CV3, DH5	[15, 16]

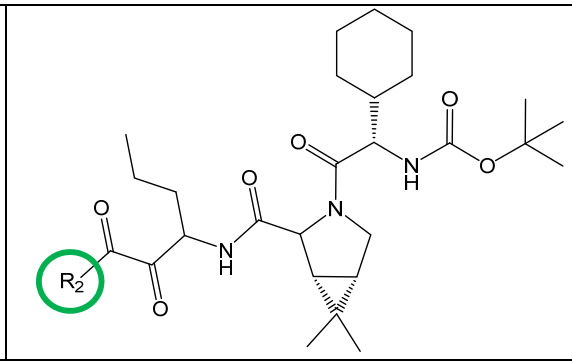
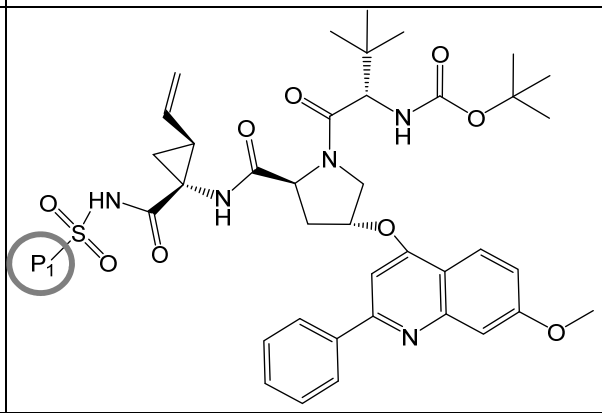
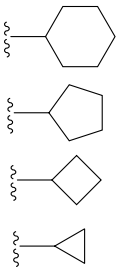
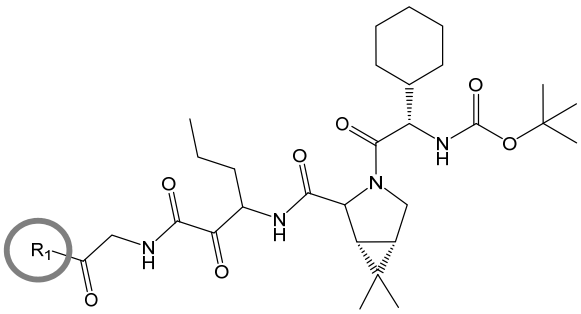
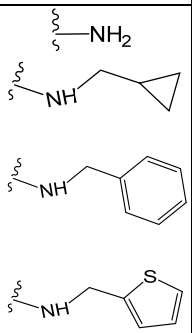
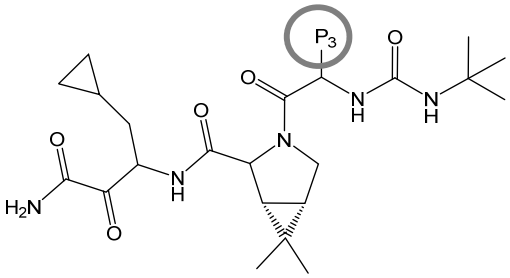
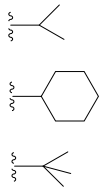
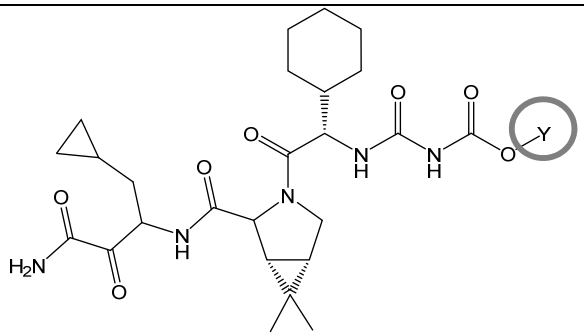
(C) **Anchor Residue**	**Mutated to**	**Activity (compared to WT)**	**Anchor**	**Reference**
D75	A	UD	CH7, WHV4, WV6	[17, 18]

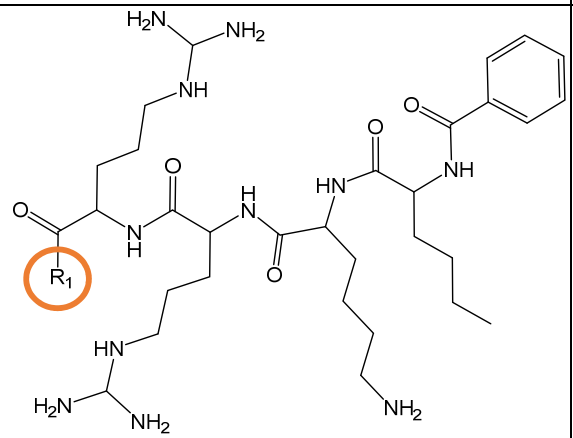
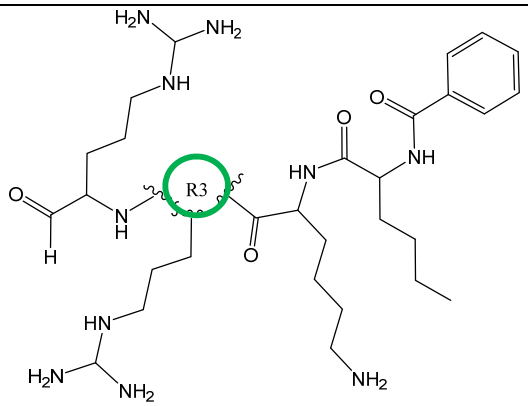
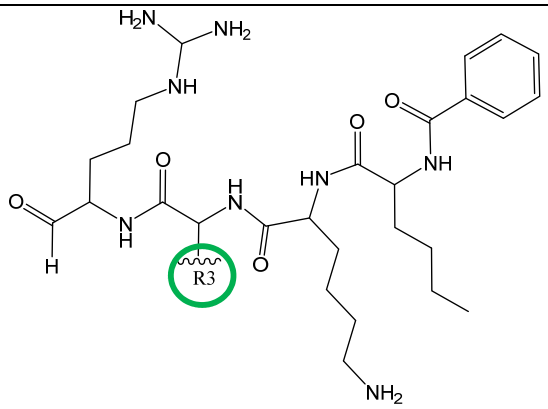
D82	A	VL	WHV4, WV6	[18]
G83	F S D K A	VL VL VL VL UD	WHV4, WV6	[17, 18]
N84	A D E L S	VL WT WT D D	WHV4, WH6	[18, 19]
F85	A	UD	WH6	[18]
D129	A E N	UD VL VL	WHV8	[17]
S135	A	UD	CEH1, CH3, WH5	[17]
Y150	F A	D UD	CV1	[17]
V154	F L	VL D	WV5	[19]
I155	F	D	WV5	[19]

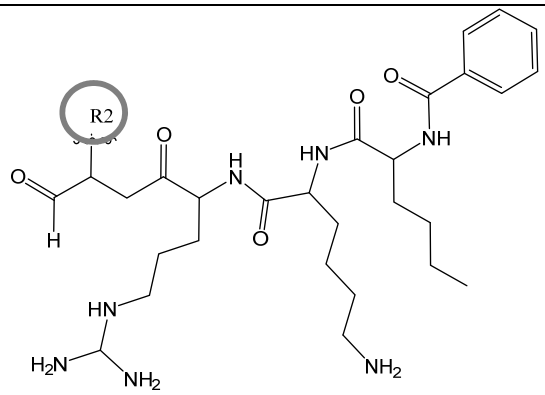
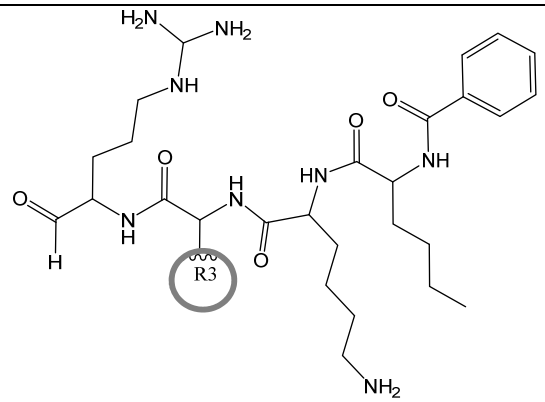
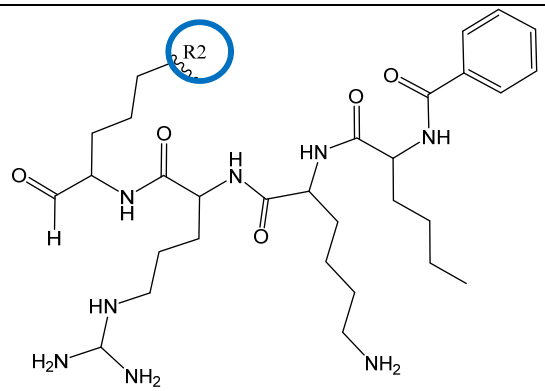
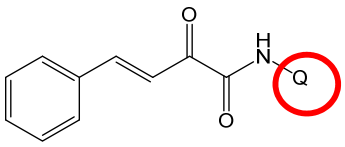
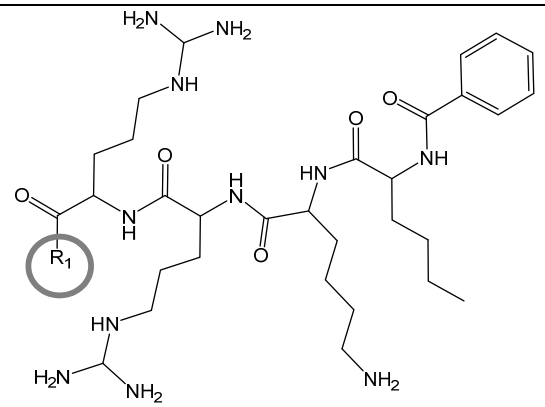
Table S2. PA/CPA model anchor analysis by known inhibitors. (A) HCV protease inhibitors, (B) DENV protease inhibitors and (C) WNV protease inhibitors. For each anchor, there are a group of inhibitors with a common scaffold but with different moieties at the anchor (colored circle). Their activities and efficacies are shown and explained in relation to the anchor.

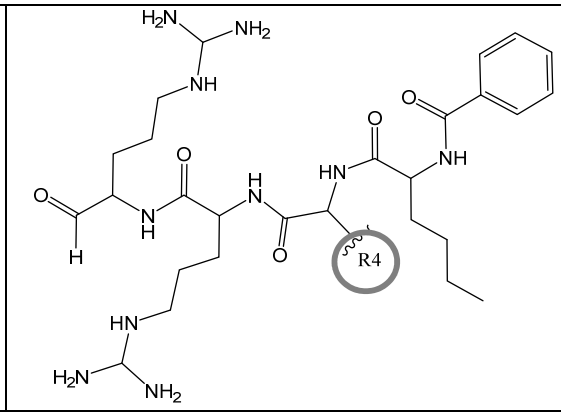
(A) Anchor	Inhibitor scaffold	Compound	Moiety at anchor	IC_{50}	Ki	Ref.
CEH1		130 131	-OH -NHSO2-()	>50000 nM 75 nM	-	[20]
CH3	 	30 33 1 31 32	-CH2- -N(CH3)- -NH- -CH2- -N(CH3)-	-	10 µM 0.12 µM 0.015 µM 2.1 µM 0.23 µM	[21]
CH7	 	130 4 132 131 133 134	-OH  -O-CH3 -OH -O-CO-CH3 -O-C4H9	>50000 nM 177 nM 220 nM 75 nM 12 nM 3 nM	-	[20]

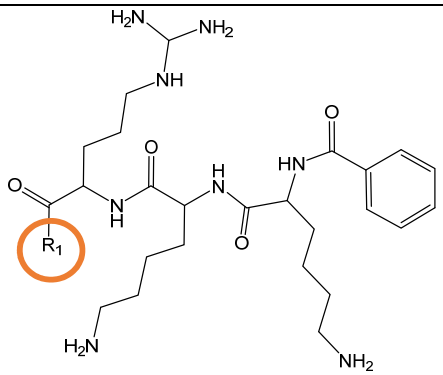
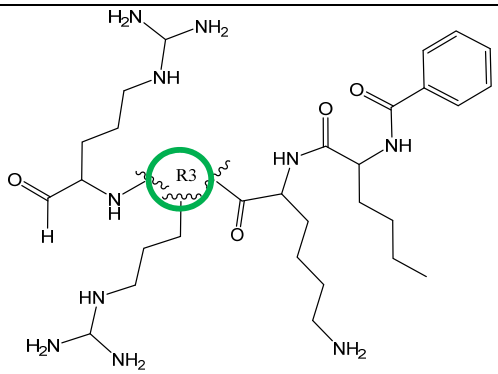
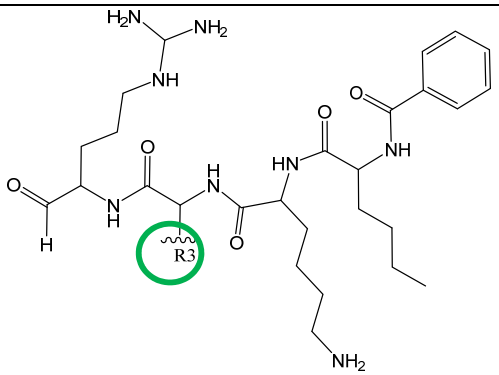
CV1		13 11 12 19 18		-	1800 nM 1600 nM 100 nM 14 nM 13 nM	[22]
CV3		26 27b 37 25 19		-	5000 nM 1000 nM 960 nM 500 nM 14 nM	[22]
HHV4		24 23 3 27 48 60 53	 -H -4,7-di-F -4-NH2	14877 nM 4286 nM 210 nM 186 nM 1.2 nM 0.7 nM 0.2 nM	-	[20]

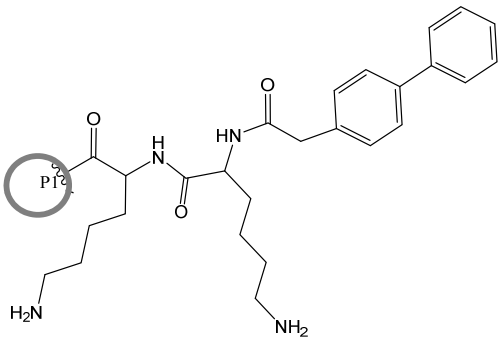
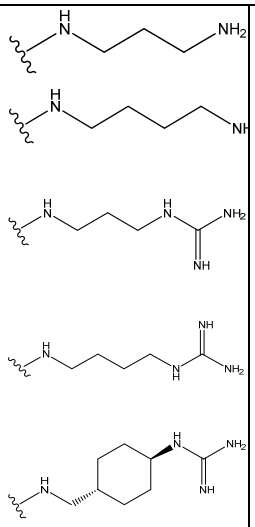
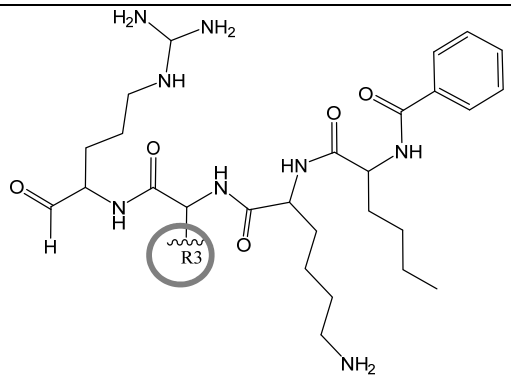
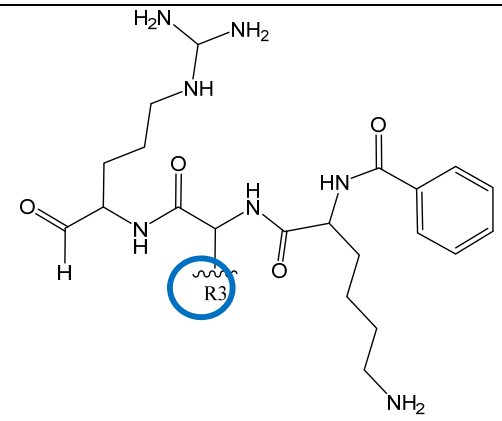
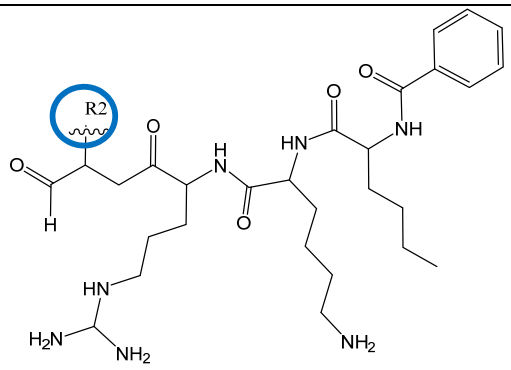
HH2		38 36 37 39	-N(CH3)2 -NH(CH3) -NH2 -OH	-	>13 µM 1.5 µM 0.1 µM 0.11 µM	[23]
HV1		9 8 7 6		149 nM 71 nM 7 nM 1 nM	-	[24]
HV2		30 29 26 27		-	0.790 µM 0.130 µM 0.056 µM 0.060 µM	[23]
HV4		29 30 18		-	100 nM 50 nM 13 nM	[22]
HV6		31 32 33 14	-CH3 -C2H5 -iPro -tBu	-	800 nM 230 nM 60 nM 25 nM	[22]

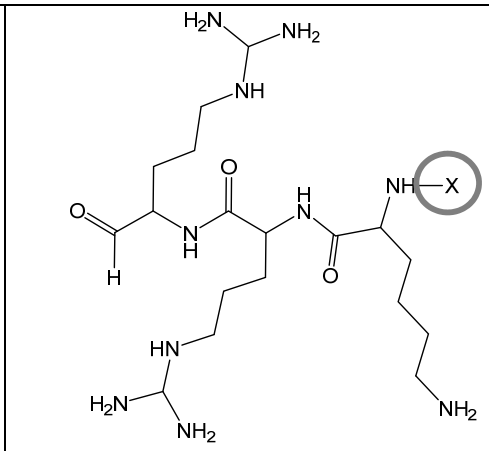
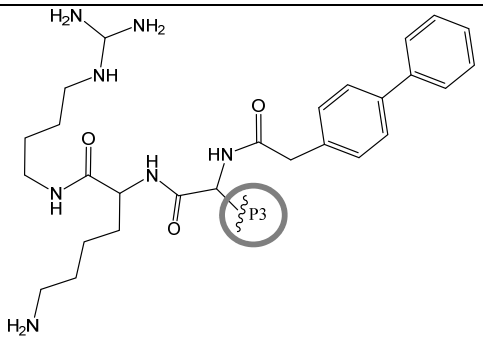
(B) Anchor	Inhibitor scaffold	Compo -und	Moiety at anchor	% Inhibition	Ki	Ref.
CEH1		2 4 18 21	-OH -NH2 -CF3 -B(OH)2	-	>500 µM 127.5 µM 0.85 µM 0.043 µM	[25]
CH3		12 18 1	-Pro- -DArg- -Arg-	-	109 µM 115 µM 5.8 µM	[25]
CH7		3 4 6 7	-Thr -Arg -Thr -Arg	-	>500 µM 127.5 µM >500 µM 5.8 µM	[25]

CV1		2 6	-Ala -Phe	-	193 µM 15.9 µM	[25]
CV3		3 7	-Ala -Phe	-	>500 µM 40.7 µM	[25]
DHV4		10 1 25 6 23	-Lys -Arg -(homo)Phe -Phe -(p-guanidinyl)Phe	-	20.5 µM 5.8 µM >500 µM 15.9 µM 2.8 µM	[25]
DE2		46 51 48	-C2H4(OCOCH3) -CH(OH)(COOCH3) -CH(CH3)(Phenyl)	35.8% 35.1% 0%	-	[26]
DV6		2 4 13a 13b	-OH -NH2 -Benzoxazole -Thiazole	-	>500 µM 127.5 µM 82.9 µM 42.8 µM	[25]

DV8		15 13 4 8	-NH(CH3) -Pro -Ala -Phe	-	113.3 µM 61.4 µM 22.1 µM 15.8 µM	[25]

(C) Anchor	Inhibitor scaffold	Compo -und	Moiety at anchor	IC_{50}	Ki	Ref.
CEH1		16 25	-H -CHO	0.42 µM 0.271 µM	-	[27]
CH3		7 3 1	-DArg- -N(CH3)-Arg- -Arg-	128.6 mM 57.8 mM 4.1 mM	-	[28]
CH7		15 1 28	-Phe -Arg -Lys	108 mM 4.1 mM 1.9 mM	-	[28]

CV1		1 3 2 4 17		-	134 µM 31 µM 16 µM 3.9 µM 1.2 µM	[29]
CV3		11 15	-Ala -Phe	262 mM 108 mM	-	[28]
WHV4		19 20 21 16 22	-Acetyl -Benzoyl -pAnisoyl -H -benzyl	112 µM 33 µM 8.3 µM 0.42 µM 24.2 µM	-	[27]
WHV8		14 21 20 19 25	-Phe -Tyr -(pCN)Phe -(pPh)Phe -p(Guanidinyl)Phe	109.8 mM 41.5 mM 62 mM 22.7 mM 11.8 mM	-	[28]

WV5		15	-(Phenyl)propionyl	19.7 μM	-	[27]
		8	-Propionyl	8.5 μM		
		1	-nBenzoyl	2.6 μM		
		6	-Acetyl	2.4 μM		
		13	-2-Napthoyl	1.8 μM		
		10	-4(Phenyl)phenylacetyl	0.99 μM		
		2	-Phenylacetyl	0.39 μM		
WV6		6	-Val	>100 μM	-	[30]
		7	-Leu	>100 μM		
		5	-Ala	53.7 μM		
		8	-Nle	47.9 μM		

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