Basis for drug selectivity of plasmepsin IX and X inhibition for *Plasmodium falciparum* and *vivax*

Anthony N. Hodder[1,2*], Janni Christensen[1,2*], Stephen Scally[1,2], Tony Triglia[1], Anna Ngo[1], Richard W. Birkinshaw[1,2], Brodie Bailey[1,2], Paola Favuzza[1,2], Melanie H. Dietrich[1,2], Wai-Hong Tham[1,2], Peter E. Czabotar[1,2], Kym Lowes[1], Zhuyan Guo[3], Nicholas Murgolo[3], Manuel de Lera Ruiz[3], John A. McCauley[3], Brad E. Sleebs[1,2], David Olsen[3], Alan F. Cowman[1,2,@]

[1] The Walter and Eliza Hall Institute of Medical Research, Parkville 3052, Australia

[2] University of Melbourne, Melbourne 3010, Australia

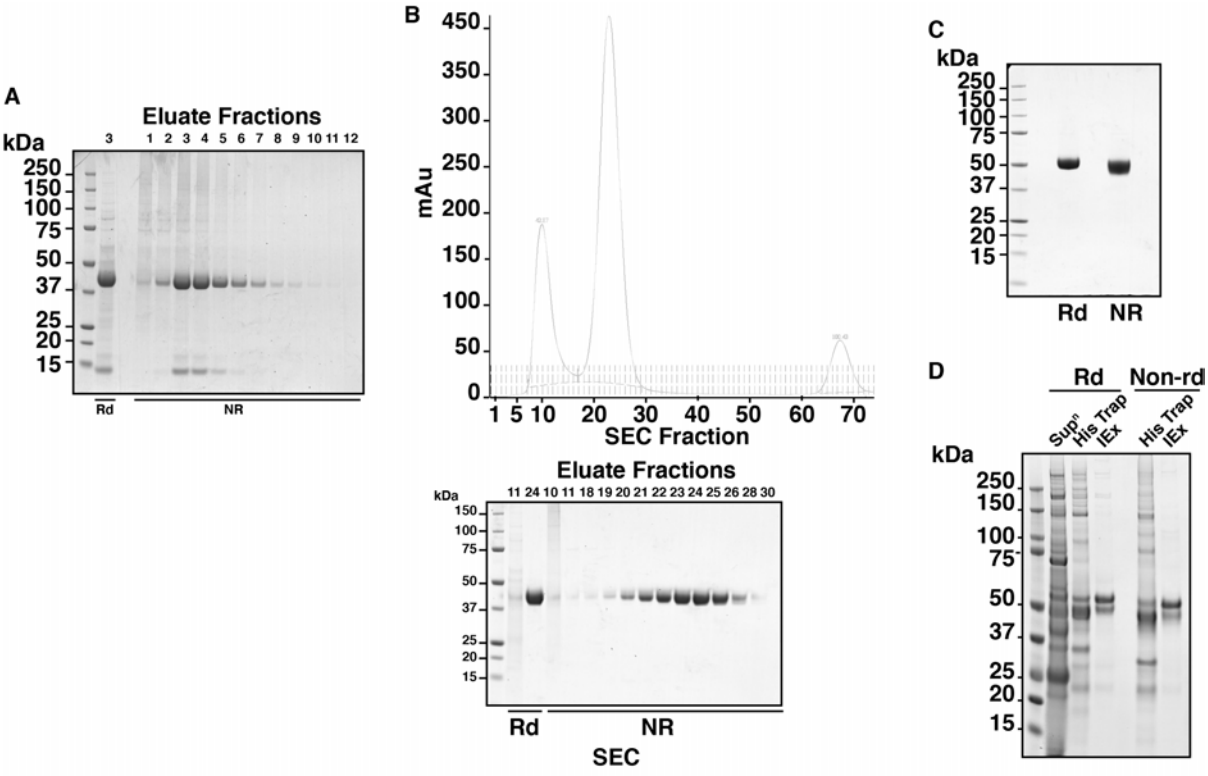
[3] Merck & Co., Inc., 770 Sumneytown Pike, West Point, PA 19486, USA

[*] These authors contributed equally to this work

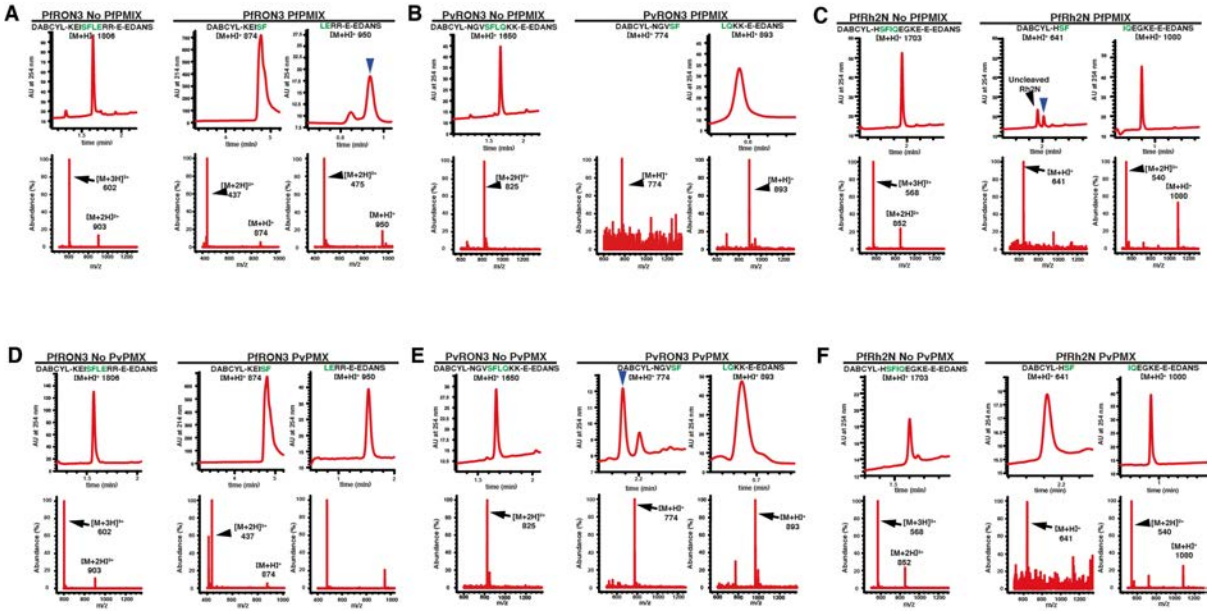
[@] Correspondence to cowman@wehi.edu.au

Running title: Molecular mechanism of antimalarial inhibition for plasmepsin IX and X

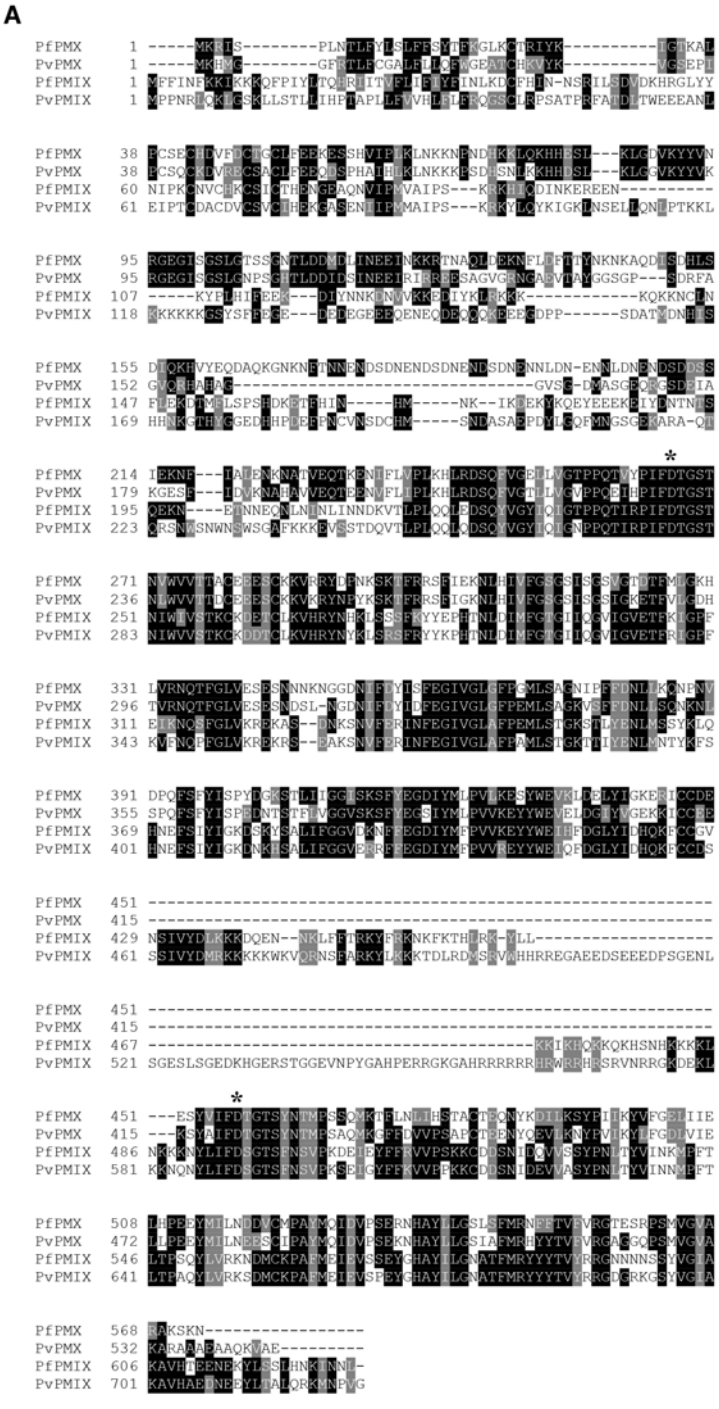
Keywords: malaria, aspartic protease, antimalarial, plasmepsin IX, plasmepsin X



Supplementary Figure 1. Expression and purification of active recombinant PvPMX and PfPMIX aspartic proteases. A. PvPMX was expressed in insect cells and protein purified from supernatants using anti-FLAG M2-agarose. Shown is a Coomassie stained gel of eluted fractions. **B.** Pooled fractions were further purified by Size Exclusion Chromatography (SEC). **C.** Purified recombinant PvPMX run under reduced (Rd) and non-reduced (NR) conditions and visualized using Coomassie staining. **D.** Expression and purification of PfPMIX in CHO cells. Shown are proteins from insect cell supernatant (Sup^n), protein purified by His-affinity chromatography (His Trap) and further purification using Ion Exchange Chromatography (IEX). Samples were run under reduced (Rd) and non-reduced (Non-rd) conditions and visualized by Coomassie staining.



Supplementary Figure 2. Mass spectrometry analysis of PfRON3 and PfRh2N peptides before and after cleavage with PvPMX or PfPMIX. A. PfRON3 peptide (100 µM) was incubated without and with recombinant PfPMIX (50 nM) and analysed by LC-ES/MS to identify the products. **B.** PvRON3 peptide (100 µM) was incubated without and with recombinant PfPMIX (50 nM) and analysed by LC-ES/MS to identify the molecular species present. The N-terminal fragment could not be detected by HPLC but was identified by mass spectrometry. **C.** PfRh2N peptide (100 µM) was incubated without and with recombinant PfPMIX (50 nM) and analysed by LC-ES/MS to identify the molecular species present. For all fluorogenic peptides (PfRON3, PvRON3 and Rh2N) cleavage by PfPMIX occurs on the C-terminal side of the phenylalanine (F). Blue arrows indicate the cleavage product. **D.** PfRON3 peptide (100 µM) was incubated without and with recombinant PvPMX (50 nM) and analysed by LC-ES/MS to identify the products. **E.** PvRON3 peptide (100 µM) was incubated without and with recombinant PvPMX (50 nM) and analysed by LC-ES/MS to identify the molecular species present. **F.** PfRh2N peptide (100 µM) was incubated without and with recombinant PvPMX (50 nM) and analysed by LC-ES/MS to identify the molecular species present. For all fluorogenic peptides (PfRON3, PvRON3 and Rh2N) cleavage by PvPMX occurs on the C-terminal side of the phenylalanine (F). Blue arrows indicate the cleavage product.

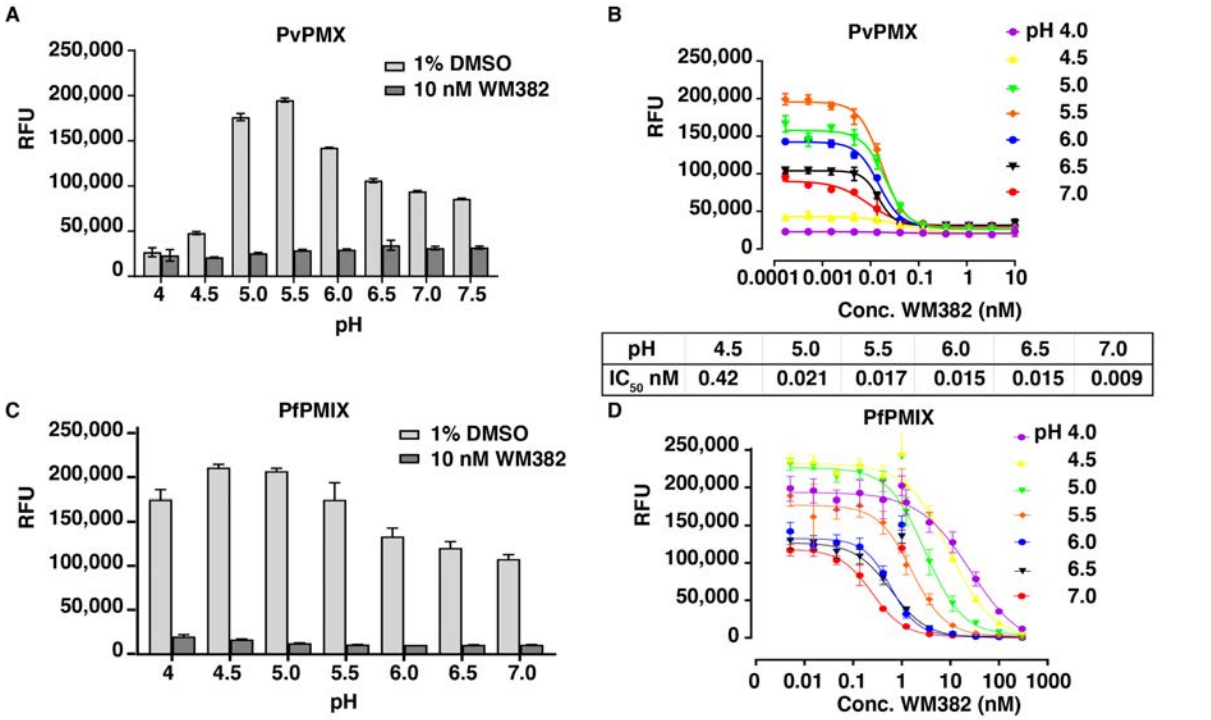


B

Percent Identity Matrix				
1: PfPMX	100.00	63.13	34.73	34.00
2: PvPMX	63.13	100.00	34.84	35.47
3: PfPMIX	34.73	34.84	100.00	56.87
4: PvPMIX	34.00	35.47	56.87	100.00

Supplementary Figure 3. Comparison of the protein sequence for PfPMX, PvPMX, PfPMIX and PvPMIX and determination of the percent identity. A. Comparison of the protein sequence for PfPMX, PvPMX, PfPMIX and PvPMIX with identical residues shaded in

black. Amino acid positions are numbered at the left. **B.** Percent identity matrix of for PfPMX, PvPMX, PfPMIX and PvPMIX. PlasmoDB and Genbank Database Sequence Accessions: PfPMX PF3D7_0808200, CAD51290.1; PvPMX PVP01_0112200, VUZ93138.1; PfPMIX PF3D7_1430200, CZT99999.1; PvPMIX PVP01_1319200, VUZ98253.1.



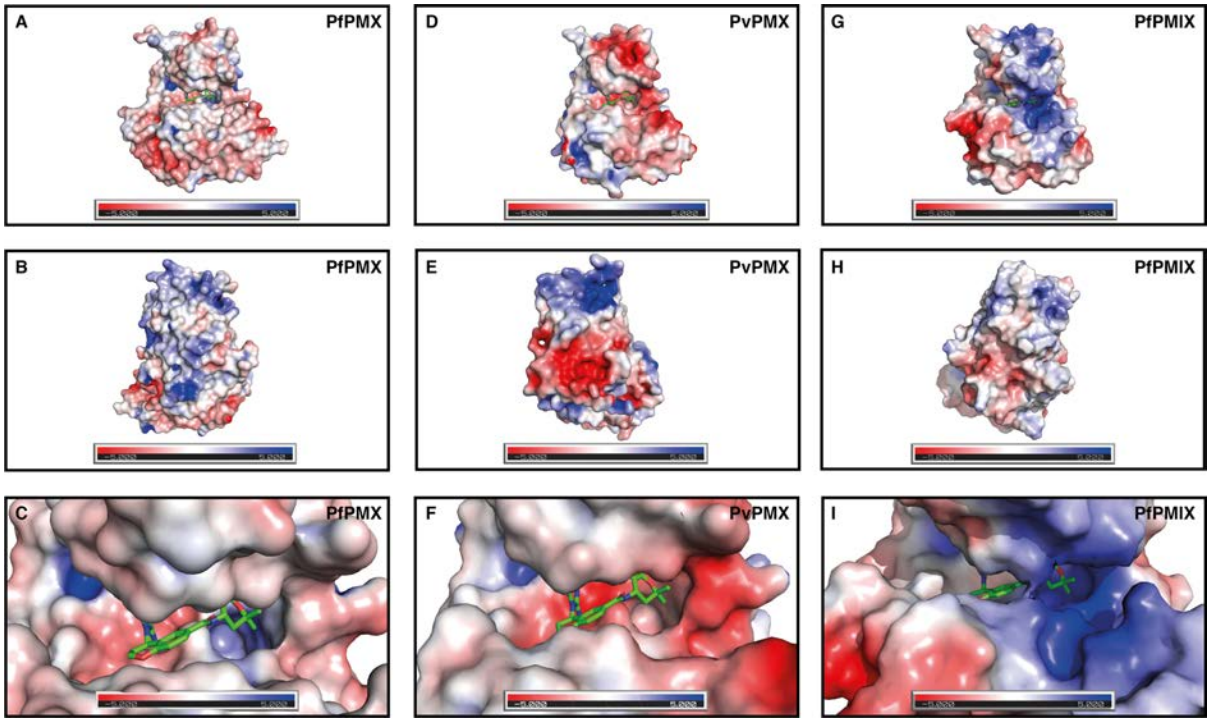
pH	4.5	5.0	5.5	6.0	6.5	7.0
IC_{50} nM	0.42	0.021	0.017	0.015	0.015	0.009

pH	4.0	4.5	5.0	5.5	6.0	6.5	7.0
IC_{50} nM	25.1	9.6	3.1	1.6	0.6	0.6	0.2

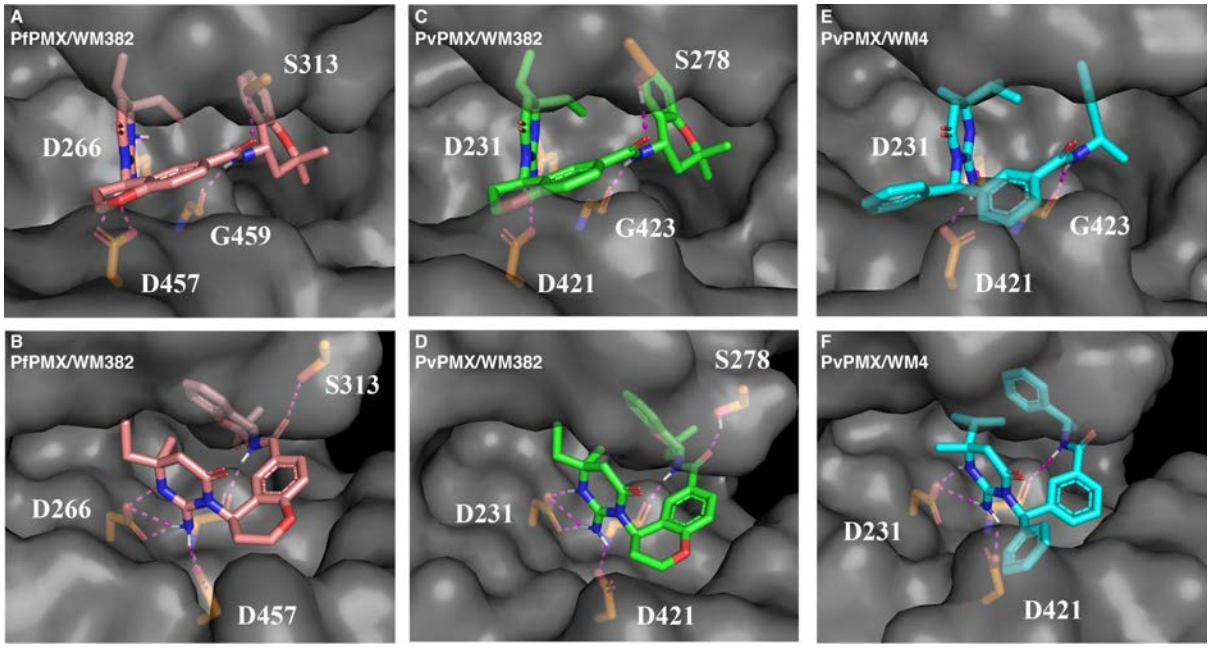
E

	Substrate Km (μM)					
	PfPMX		PvPMX		PfPMIX	
PfRh2N	3.5±0.3	n=3	3.4±0.5	n=3	14.2±3.6	n=3
PfRON3	11.0±2.5	n=3	17.7±4.7	n=4	16.6±5.4	n=3

Supplementary Figure 4. Recombinant PvPMX and PfPMIX aspartic proteases are enzymatically active. A. Cleavage activity of PvPMX with the PfRh2N fluorogenic peptide at different pH and inhibition with WM382. **B.** Determination of the IC_{50} for inhibition of PvPMX with WM382 and cleavage of the PfRh2N peptide. **C.** Cleavage activity of PfPMIX with the PfRON3 fluorogenic peptide at different pH and inhibition with WM382. **D.** Determination of the IC_{50} for inhibition of PfPMIX with WM382 and cleavage of the PfRON3 peptide. **E.** K_m for PfPMX, PvPMX and PfPMIX using peptide substrates PfRh2N and PfRON3 as indicated.



Supplementary Figure 5. Surface charge distribution for PfPMX, PvPMX and PfPMIX and binding of WM382 to the active site (red positive and blue negative charge). A. Surface charge distribution of a front view of PfPMX. **B.** Surface charge distribution for the back view of PfPMX. **C.** Surface charge distribution of the active site of PfPMX with WM382 bound. **D.** Surface charge distribution of a front view of PvPMX. **E.** Surface charge distribution for the back view of PvPMX. **F.** Surface charge distribution of the active site of PvPMX with WM382 bound. **G.** Surface charge distribution of a front view of PfPMIX. **H.** Surface charge distribution for the back view of PfPMIX. **I.** Surface charge distribution of the active site of PfPMIX with WM382 bound. For all panels electrostatic calculations were performed using Adaptive Poisson-Boltzmann Solver [30] and rendered in PyMOL (The PyMOL Molecular Graphics System, Version 2.0; Schrödinger, LLC); scale: −5 kT/e (red) to +5 kT/e (blue).



Supplementary Figure 6. Surface view of the active site of PfPMX, PvPMX showing the hydrogen bonds to WM382 or WM4 that have bound to the active site. A and B. Different views for the interaction of WM382 with the active site of PfPMX. **C and D.** Different views for the interaction of WM382 with the active site of PvPMX. **E and F.** Different views for the interaction of WM4 with the active site of PvPMX.

Supplementary Table 1. SPR kinetic parameters for WM4 against PvPMX, PfPMX and PfPMIX.

Ligand	Analyte	Values	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (M)	R_{max} (RU)	χ^2 (RU^2)
PvPMX	WM4	independent repeat 1	$3.88x10^6$	$2.19x10^{-3}$	$5.64x10^{-10}$	8.5	0.014
PvPMX	WM4	independent repeat 2	$1.38x10^7$	$5.67x10^{-3}$	$4.11x10^{-10}$	36.2	0.050
PvPMX	WM4	independent repeat 3	$5.01x10^6$	$2.27x10^{-3}$	$4.52x10^{-10}$	15.5	0.024
PvPMX	WM4	mean ± SD	$7.6x10^6 \pm 5.4x10^6$	$3.4x10^{-3} \pm 2x10^{-3}$	$4.8x10^{-10} \pm 8x10^{-11}$	N/A	N/A
PfPMX	WM4	independent repeat 1	$7.81x10^6$	$2.36x10^{-3}$	$3.03x10^{-10}$	4.0	0.036
PfPMX	WM4	independent repeat 2	$7.44x10^6$	$3.25x10^{-3}$	$4.10x10^{-10}$	4.6	0.021
PfPMX	WM4	independent repeat 3	$3.63x10^6$	$1.62x10^{-3}$	$4.45x10^{-10}$	2.3	0.023
PfPMX	WM4	mean ± SD	$6.3x10^6 \pm 2.31x10^6$	$2.4x10^{-3} \pm 8.2x10^{-4}$	$3.9x10^{-10} \pm 7.4x10^{-11}$	N/A	N/A
PfPMIX	WM4	independent repeat 1	$9.62x10^5$	$8.12x10^{-2}$	$8.45x10^{-7}$	79.4	1.98
PfPMIX	WM4	independent repeat 2	$4.23x10^5$	$2.61x10^{-2}$	$6.17x10^{-8}$	74.9	0.30
PfPMIX	WM4	independent repeat 3	$4.16x10^5$	$3.49x10^{-2}$	$8.38x10^{-8}$	76.6	0.07
PfPMIX	WM4	mean ± SD	$6.0x10^5 \pm 3.1x10^5$	$4.7x10^{-2} \pm 3x10^{-2}$	$7.7x10^{-8} \pm 1.3x10^{-8}$	N/A	N/A

Supplementary Table 2. Data collection and refinement statistics for 3-D crystal structures for PfPMX-apo, PfPMX-WM382, PvPMX-WM382 and PvPMX-WM4.

	PfPMX-apo	PfPMX-WM382	PvPMX-WM382	PvPMX-WM4
Beamline	MX2	MX2	MX2	MX2
Wavelength (Å)	0.953649	0.95373	0.953732	0.953651
Space group	P 21 21 21	P 31 2 1	I 2 2 2	C 2 2 21
Cell dimensions				
***a,b,c* (Å)**	62.3, 63.2, 79.3	109.3, 109.3, 118.3	82.0, 88.4, 230.2	80.5, 253.3, 171.8
α, β, γ (°)	90, 90, 90	90, 90, 120	90, 90, 90	90, 90, 90
Resolution (Å)[a]	33.46-1.85(1.92-1.85)	49.63-2.76 (2.91-2.76)	38.63-2.22(2.29-2.22)	39.12-3.35(3.58-3.35)
No. molecules in ASU	1	1	2	4
No. observations	363,720 (35,939)	481,905 (18,533)	575,629 (54,084)	175,235 (31,403)
No. unique observations	27,357 (2,688)	21,289 (2,860)	41,807 (3,792)	25,719 (4587)
Multiplicity	13.3 (13.4)	22.6 (6.5)	13.8 (14.3)	6.8 (6.8)
R_{merge} (%)[b]	10.2 (144.1)	14.8 (120.9)	17.0 (174.0)	40.9 (120.8)
R_{pim} (%)[c]	2.9 (40.6)	3.0 (49.6)	4.7 (47.3)	16.8 (49.6)
$\langle I/\sigma I \rangle$	15.71 (1.43)	12.5 (1.2)	11.4 (1.7)	5.0 (1.9)
$CC_{½}$	100.0 (78.8)	99.9 (60.8)	99.8 (80.7)	97.5 (72.1)
Completeness (%)	99.9 (99.9)	99.0 (93.6)	100 (100)	99.9 (99.9)
Refinement Statistics				
Reflections (work)	27,333	21,253	41,778	25,684
Reflections (test)	1,995	1,061	4,099	1,286
Non-hydrogen atoms	2,831	2,790	5,770	11,048
Macromolecule	2,614	2,649	5,386	10,721
Water	165	9	231	83
Heteroatom	52	132	153	244
R_{work}[d] / R_{free}[e]	20.5/25.8	19.7/22.3	20.2/25.9	23.3/28.9
Rms deviations from ideality				
Bond lengths (Å)	0.007	0.009	0.008	0.003
Bond angle (°)	0.84	1.00	0.92	0.63
Ramachandran plot				
Favoured regions (%)	95.7	94.3	96.0	94.2
Allowed regions (%)	4.0	4.8	0.6	0.6
B-factors (Å^2)				
Wilson B-value	30.9	96.2	41.0	58.5
Average B-factors	40.2	92.2	45.6	52.1
Average macromolecule	39.6	91.9	45.2	52.1
Average heteroatom	59.1	99.8	54.7	55.3
Average water molecule	44.3	88.6	48.7	33.4

a Values in parentheses refer to the highest resolution bin.

b $R_{merge} = \Sigma_{hkl} \Sigma_i \mid I_{hkl,\,i} - \langle I_{hkl} \rangle \mid / \Sigma_{hkl} \langle I_{hkl} \rangle$

c $R_{pim} = \Sigma_{hkl} [1/(N-1)]_{1/2} \Sigma_i \mid I_{hkl,\,i} - \langle I_{hkl} \rangle \mid / \Sigma_{hkl} \langle I_{hkl} \rangle$

d $R_{work} = (\Sigma \mid\mid F_o \mid - \mid F_c \mid\mid) / (\Sigma \mid\mid F_o \mid)$ - for all data except as indicated in footnote e.

e 5% of data were used for the R_{free} calculation

Supplementary Table 3. Hydrogen bonding interactions between residues of the flap located within the S3 subpocket of PMX.

Protease/drug	#1	#2	#3	#4	#5	#6	#7	#8
PfPMX	Q247 D245 MC	Q247 MA D245 MC	Q247 MA L243 MA	Nil	Nil	Nil	Nil	Nil
PfPMX/WM382	Q247 S359 MC	Q247 D245	Q247 MA D245	Q247 MC L243 MA	D245 S359 MA	D245 D245 MA	D245 MC S246	S246 MA L243 MC
PvPMX/WM382	Q212 D323 MC	Q212 D210	Q212 D210	Q212 MA D210	Q212 MC L208 MA	S211 D210 MC	S211 MA L208 MC	D210 D323 MA
PvPMX/WM4	Q212 D323 MC	Q212 D210	Q212 MA D210	Q212 MC L208 MA	S211 D210 MC	S211 MA L208 MC	Nil	Nil

Amino Acid interaction with side chain function
MA interaction with main chain amide
MC interaction with main chain carbonyl