

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

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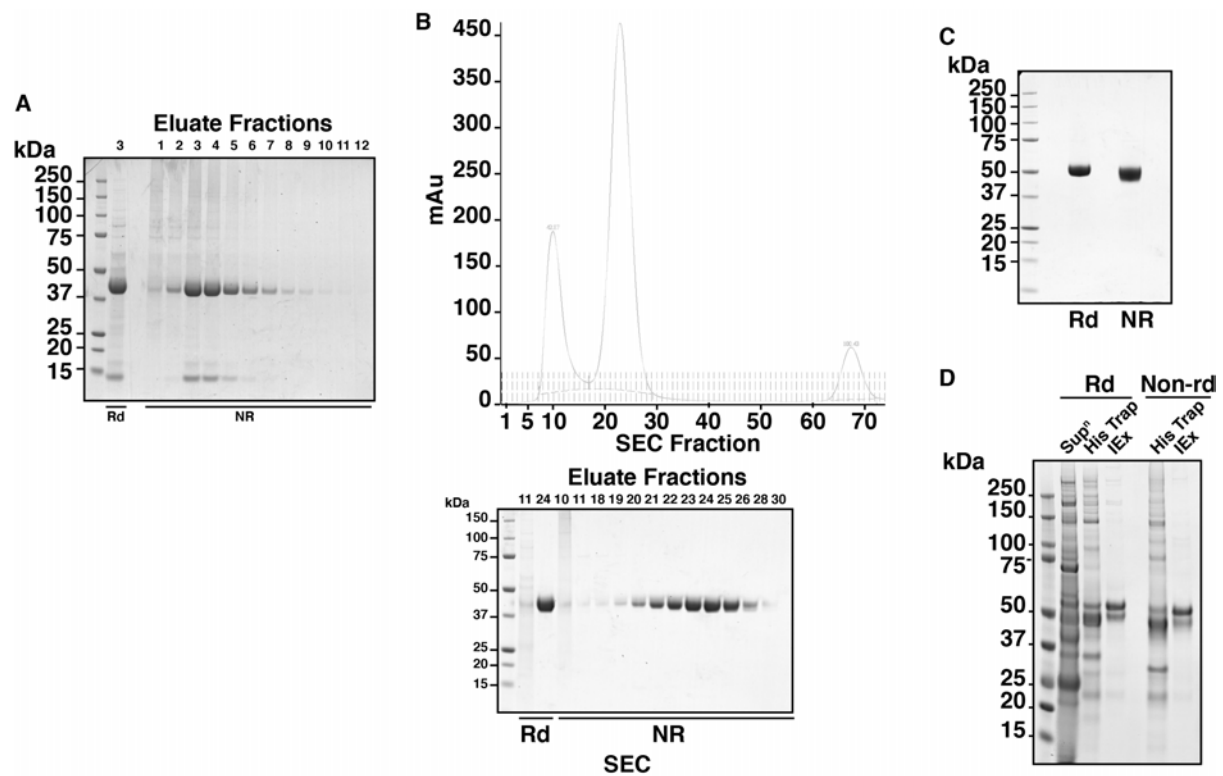
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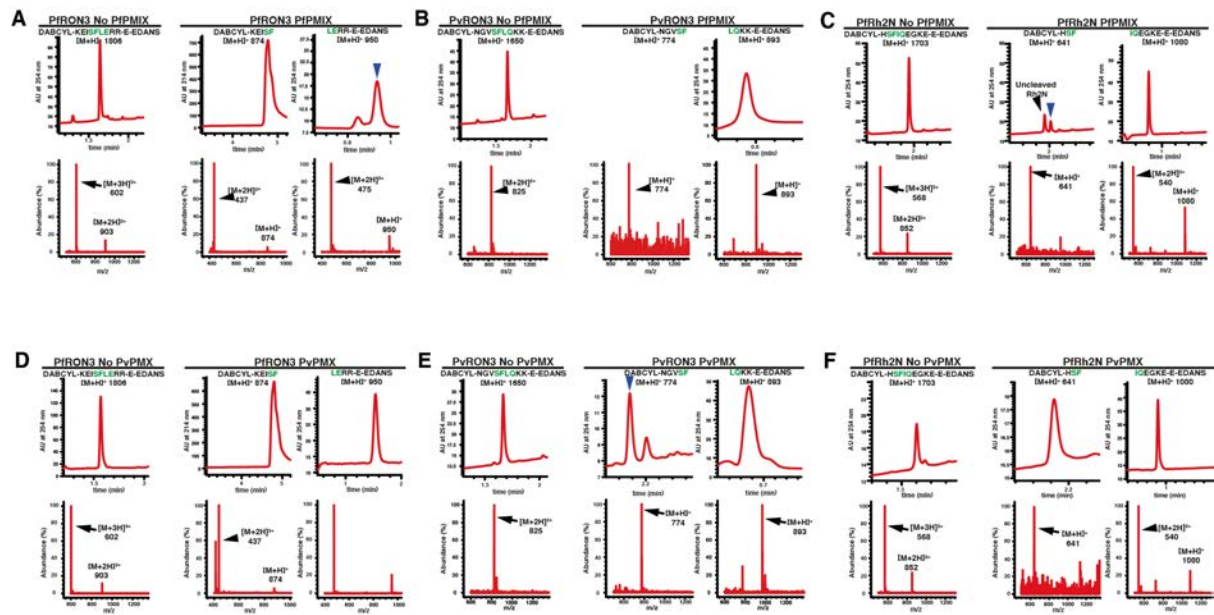
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Running title: Molecular mechanism of antimalarial inhibition for plasmepsin IX and X

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 (Supⁿ), 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 μ M) was incubated without and with recombinant PfPMIX (50 nM) and analysed by LC-ES/MS to identify the products. **B.** PvRON3 peptide (100 μ M) 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 μ M) 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 μ M) was incubated without and with recombinant PvPMX (50 nM) and analysed by LC-ES/MS to identify the products. **E.** PvRON3 peptide (100 μ M) 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 μ M) 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.

A

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PfPMX 1  ---MKKIS-----PLNLFYSLFFSTFRLKTRLYK-----IGKAL
PvPMX 1  ---MKHMG-----GFRFLCGALFLDFWGBATGKRYK-----VGEPI
PfPMIX 1  MFFINFKKIKKQFPPIYLQHRITITVELFHYFINLKDFFHN-NSRILSDWKHRLGY
PvPMIX 1  MPPNRKIKKISLLSTLIHFPAPLLEVHLFLRQSSCLFSAFPRFADITWEEENL

PfPMX 38  PCSSEIVFTQIGCLPEEKSSHVIFPKLNKKNNDKKLOKHHESI---KLGSVKYYYN
PvPMX 38  PCSQCHVRECSACLFEEQSPHAIHFKLNKKKSDSNKKHHESI---KLGSVKYYVK
PfPMIX 60  NIPKENVCHKCSITHENGSAQNVIPVAIPS---KRRKCDINKEREEN---
PvPMIX 61  EIPTDADCVCSVTHKKAASENLIIPMAIPS---KRRKCDYKIGKINSELDNPTKKL

PfPMX 95  RGEGISGSLGTSSNTLDDMLINEEINKKRTNAQLEKNFLFTTNKKNKAQDLSHLS
PvPMX 95  RGEGISGSLGNPSCNTLDDTISINEEIRIRIESAGVGNAPVYANGGSGP---DRFA
PfPMIX 107  ---KYFHLITFEK---DIYNNKINNVKKELIYKLKKK-----KOKKNCIN
PvPMIX 118  KKKKKKSYSPFEK---EEEGRESENEQDFKQFEESDPP-----SDATDNHLS

PfPMX 155  DQKHVVEQDAQKGNKNTNNENDSDNENDSDNENSDNENNLDN-ENLDNENDSDSS
PvPMX 152  GVCCHHAFAC-----GVSF-DMASGQSGSDIA
PfPMIX 147  FIERKDTGLSPSHDKRTHIN-----HM-----NK--IKDEKVKQYEEEEKIYNTNLS
PvPMIX 169  HNKRTTIVGSELHHPTEFNVCVNSDCIM-----SNBASAPVYLQFMNGSGEAKARA-CH

PfPMX 214  IEKNE---LAEKNNAIVEOTDENFLPLKHLRDSQVGTLLGTFPPQTIPIFDTGST
PvPMX 179  KGESF---LDKNNNAIVEOTDENFLPLKHLRDSQVGTLLGTFPPQTIPIFDTGST
PfPMIX 195  DEKN---EINNECNLNLINNDKVTLPLQQLDSDQVGYQLGTFPPQTIPIFDTGST
PvPMIX 223  KRSNLSNWNWSGAPKKKLVSTQVTLPLQQLDSDQVGYQLGTFPPQTIPIFDTGST

PfPMX 271  NWWVVTACEESCKKVRRYLNNKSKIFRRSIEKNLHIVFGSGSTSSVCHTFMCKKH
PvPMX 236  NWWVVTACEESCKKVRRYLNNKSKIFRRSIEKNLHIVFGSGSTSSVCHTFMCKKH
PfPMIX 251  NIWVVTCKQDETLKVHRYNKKLSSEFYYEFHNLDIMFGTGLIAGVIGVETFKIGGF
PvPMIX 283  NIWVVTCKQDETLKVHRYNKKLSSEFYYEFHNLDIMFGTGLIAGVIGVETFKIGGF

PfPMX 331  LVRNQTFLVSESSNNKNGGDNIFYISFEGIVGLFFPEMLSAENIPFDNLKKNPV
PvPMX 296  TVRNQTFLVSESSNDL-NGDNIFYISFEGIVGLFFPEMLSAENIPFDNLKKNPV
PfPMIX 311  EIKNCSFGLVKREKAS--DNKSNVFRINFEFIVGLAFPEMLSTGKSTLYBNLSSYKLG
PvPMIX 343  KVENCFEGLVKREKRS--DAKSNVFRINFEFIVGLAFPEMLSTGKSTLYBNLNTYKFS

PfPMX 391  DPQSFSPYISPYCKSTLIFGLSKSFEGDIYMFVVKESYWEKLFELYIGKEHCCDE
PvPMX 355  SPQSFSPYISPYNTSTLIFGVSKSFEGDIYMFVVKESYWEKLFELYIGKEHCCDE
PfPMIX 369  HNEFSIYIGKSKYSALIFGGVGRFEGDIYMFVVKESYWEKLFELYIGKEHCCGV
PvPMIX 401  HNEFSIYIGKSKYSALIFGGVGRFEGDIYMFVVKESYWEKLFELYIGKEHCCGS

PfPMX 451  -----
PvPMX 415  -----
PfPMIX 429  NSIYVDLKKKQDEN--NKLFTKKYFKNKFKTHIRN-ILL-----
PvPMIX 461  SSIYVDLKKKKKKWKVDENSEARKYLAKKTDLRDLSVQHHRREGAEEDSEEDPSGENL

PfPMX 451  -----
PvPMX 415  -----
PfPMIX 467  -----KQIKQKQKQKHSNHKKKKL
PvPMIX 521  SGESLSGEKDHGERSTGGEVNPYGAHPERRKGGAHRRRRRRRERERHRSRVNRRGDKKL

PfPMX 451  ---ESYLIIFDGTSTNNMPSQKQKFLNLHSTACTQNYKDIKSYFLIKVFGELIIE
PvPMX 415  ---ESYLIIFDGTSTNNMPSAQKQFENVVPSADTSENYCEVKNYFVKHFGDIVIE
PfPMIX 486  NKKKNYLIFDGTSTNSVPKDEIDFFENVVPSKKCDUSRIEIVSSYPNLYVINKNFT
PvPMIX 581  KKNQNYLIFDGTSTNSVPKSELGYFFENVVPSKKCDUSNIDEVVASYPNLYVINNFET

PfPMX 508  LIPPEYIMLNDCMPAYQIIVSEKHNHAYLGSLFMRNSETVVRGTESRPSVVGVA
PvPMX 472  LIPPEYIMLNESCLPAYQIIVSEKHNHAYLGSLAFMRHYTYTVRGASQPSVVGVA
PfPMIX 546  LTFQCYLVKKNDMCKPAEMELIVSSEYCHAYLGNAFMRYYYTVVRGNNNNSVVGVA
PvPMIX 641  LTFQCYLVKKSMDCKPAEMELIVSSEYCHAYLGNAFMRYYYTVVRGDRKCSVVGVA

PfPMX 568  KAKSKN-----
PvPMX 532  KARAAFAAAQKIAE-----
PfPMIX 606  KAVHDEENKYLESHNKNNNI-
PvPMIX 701  KAVHAEINBEYLIALQRKNNPVG

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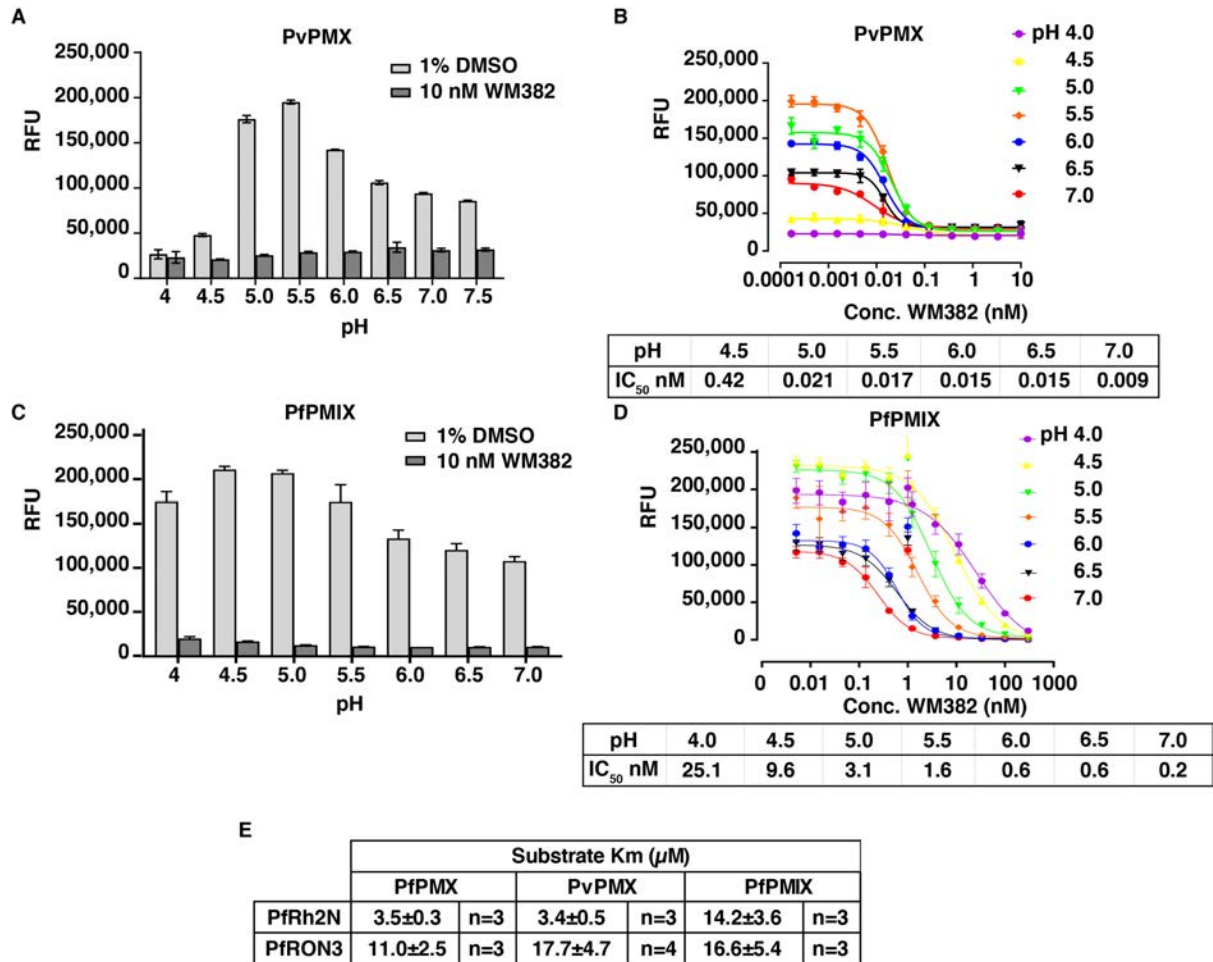
B

Percent Identity Matrix

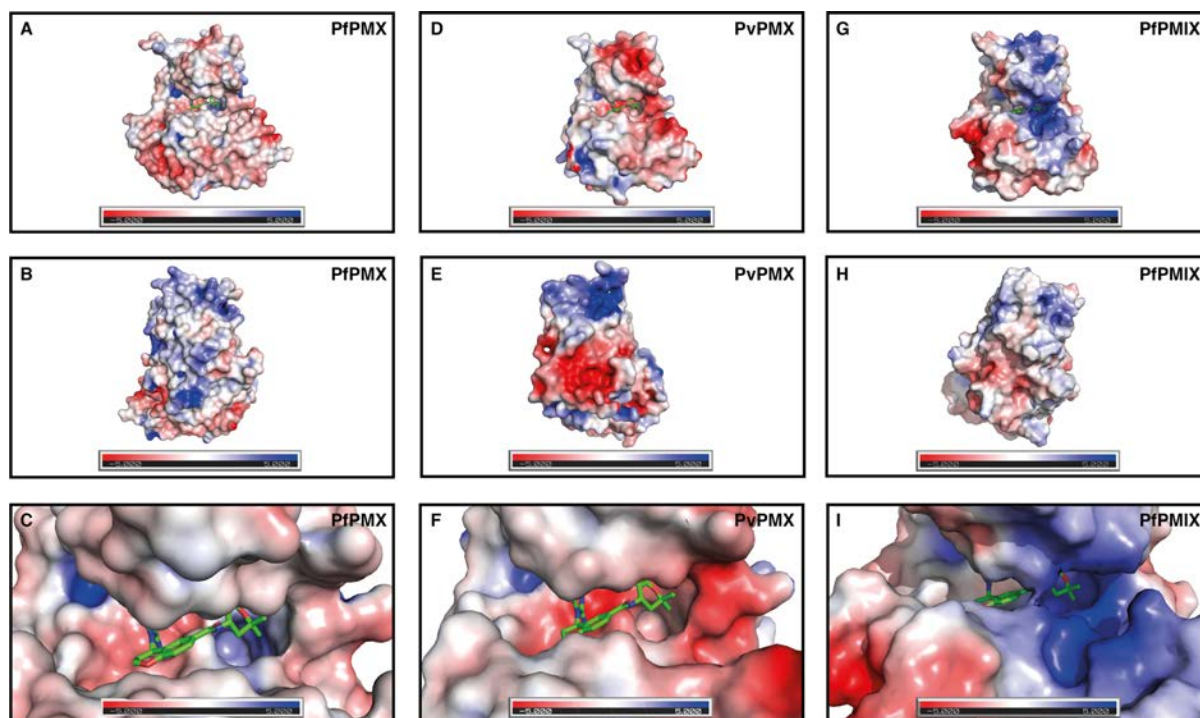
1: PfPMX	100.00	63.13	34.73	34.00
2: PvPMX	63.13	100.00	34.84	35.47
3: PfPMIX	34.73	34.84	100.00	56.87
4: PvPMIX	34.00	35.47	56.87	100.00

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

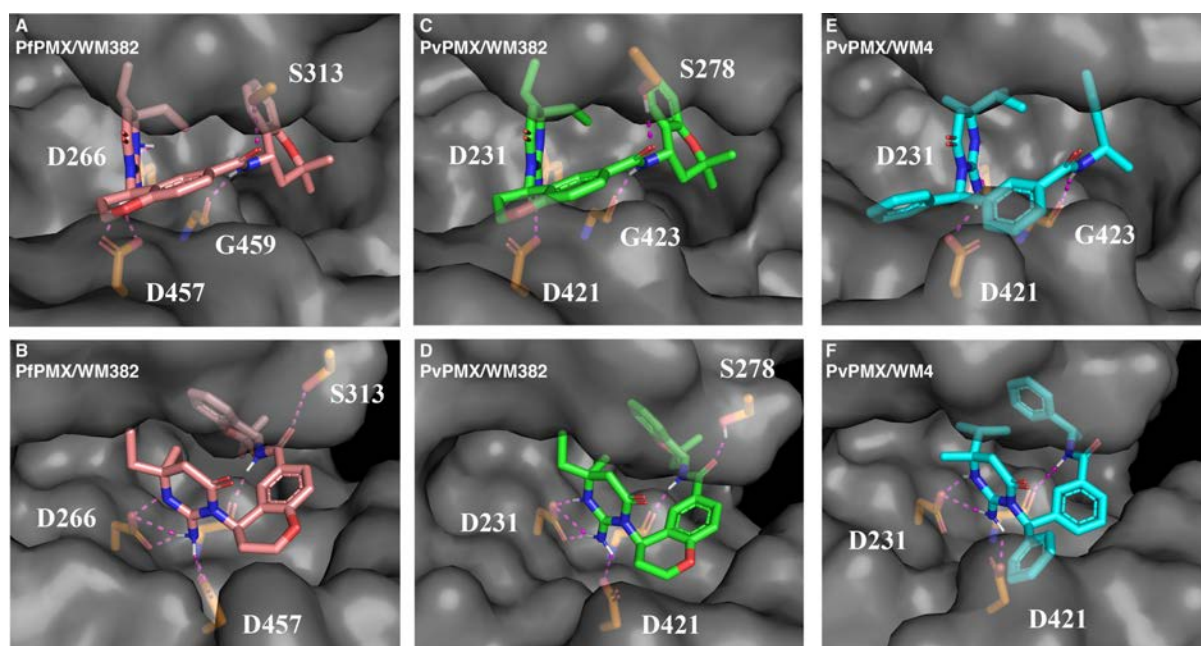
black. Amino acid positions are numbered at the left. **B.** Percent identity matrix of for PfPMX, PvPMX, PfPMIX and PvPMIX. PlasmoDB and Genbank Database Sequence Accessions: PfPMX PF3D7_0808200, CAD51290.1; PvPMX PVP01_0112200, VUZ93138.1; PfPMIX PF3D7_1430200, CZT99999.1; PvPMIX PVP01_1319200, VUZ98253.1.



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 IC₅₀ 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 IC₅₀ for inhibition of PfPMIX with WM382 and cleavage of the PfRON3 peptide. **E.** K_m for PfPMIX, 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	3.88×10^6	2.19×10^{-3}	5.64×10^{-10}	8.5	0.014
PvPMX	WM4	independent repeat 2	1.38×10^7	5.67×10^{-3}	4.11×10^{-10}	36.2	0.050
PvPMX	WM4	independent repeat 3	5.01×10^6	2.27×10^{-3}	4.52×10^{-10}	15.5	0.024
PvPMX	WM4	mean \pm SD	$7.6 \times 10^6 \pm 5.4 \times 10^6$	$3.4 \times 10^{-3} \pm 2 \times 10^{-3}$	$4.8 \times 10^{-10} \pm 8 \times 10^{-11}$	N/A	N/A
PfPMX	WM4	independent repeat 1	7.81×10^6	2.36×10^{-3}	3.03×10^{-10}	4.0	0.036
PfPMX	WM4	independent repeat 2	7.44×10^6	3.25×10^{-3}	4.10×10^{-10}	4.6	0.021
PfPMX	WM4	independent repeat 3	3.63×10^6	1.62×10^{-3}	4.45×10^{-10}	2.3	0.023
PfPMX	WM4	mean \pm SD	$6.3 \times 10^6 \pm 2.31 \times 10^6$	$2.4 \times 10^{-3} \pm 8.2 \times 10^{-4}$	$3.9 \times 10^{-10} \pm 7.4 \times 10^{-11}$	N/A	N/A
PfPMIX	WM4	independent repeat 1	9.62×10^5	8.12×10^{-2}	8.45×10^{-7}	79.4	1.98
PfPMIX	WM4	independent repeat 2	4.23×10^5	2.61×10^{-2}	6.17×10^{-8}	74.9	0.30
PfPMIX	WM4	independent repeat 3	4.16×10^5	3.49×10^{-2}	8.38×10^{-8}	76.6	0.07
PfPMIX	WM4	mean \pm SD	$6.0 \times 10^5 \pm 3.1 \times 10^5$	$4.7 \times 10^{-2} \pm 3 \times 10^{-2}$	$7.7 \times 10^{-8} \pm 1.3 \times 10^{-8}$	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				
<i>a, b, c</i> (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
<i>α, β, γ</i> (°)	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 (4,587)
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)
<I/σ I>	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 (Å²)				
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 $R_{\text{merge}} = \sum_{\text{hkl}} \sum_i |I_{\text{hkl},i} - \langle I_{\text{hkl}} \rangle| / \sum_{\text{hkl}} \langle I_{\text{hkl}} \rangle$

^c $R_{\text{pim}} = \sum_{\text{hkl}} [1/(N-1)]^{1/2} \sum_i |I_{\text{hkl},i} - \langle I_{\text{hkl}} \rangle| / \sum_{\text{hkl}} \langle I_{\text{hkl}} \rangle$

^d $R_{\text{work}} = (\sum | |F_o| - |F_c| |) / (\sum |F_o|)$ - 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 ^{MC} S359	Q247 D245	Q247 ^{MA} D245	Q247 ^{MC} L243 ^{MA}	D245 ^{MA} S359	D245 ^{MA} D245	D245 ^{MC} S246	S246 ^{MC} 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 ^{MC}
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