Molecular insights into the inhibition of plasmepsins by HIV-1 protease inhibitors: Implications for antimalarial drug discovery

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1. Abstract

Malaria is a deadly disease, and the worldwide emergence of drug resistance in the *Plasmodium* parasites demands the development of novel and potent antimalarials. HIV-1 protease inhibitors (HIV-1 PIs) alleviate the *Plasmodium* pathogenesis during malaria/HIV-1 co-infection plausibly by inhibiting vacuolar plasmepsins (PMs), the hemoglobin degrading proteases from *P. falciparum*. All five FDA-approved HIV-1 PIs tested against PMII exhibit the K_i values in the low micromolar range of which RTV and LPV display the highest inhibition activity. Both inhibitors impede *in vitro* growth of *P. falciparum* at low micromolar IC_{50} values. We report the first crystal structures of PMII complexed with RTV and LPV that reveal the binding mode and interactions of the inhibitors in the active site as well as elucidate the mechanism underlying their differential potency. The conformational flexibility of the P4 group in RTV allows it to explore multiple regions of the S4 pocket. The present study establishes vacuolar PMs as potential drug targets of HIV-1 PIs. The molecular details explaining the inhibitory mechanism of HIV-1 PIs might be crucial in designing novel and potent antimalarial analogs. This work strengthens the prospect of drug repurposing as an alternative strategy towards antimalarial treatments and provides an opportunity to tackle malaria and HIV-1 co-infection.

Keywords: Plasmepsins, protease, parasite, malaria, hemoglobin, drug design, ritonavir, lopinavir, inhibitors, crystal structures, drug repurposing

Abbreviations: Plasmepsins, PMs; Hemoglobin, Hb; Plasmepsin II, PMII; Protease inhibitor, PI; Ritonavir, RTV; Lopinavir, LPV; Human Immunodeficiency Virus, HIV

2. Introduction

Malaria has emerged as one of the most severe infectious diseases globally and remains one of the primary causes of illness and mortality. Half of the world's population lives in malaria-endemic areas, primarily tropical and subtropical regions. An estimated 229 million cases of malaria and 409,000 deaths were recorded worldwide in 2019; currently, malaria is the priority tropical disease of the World Health Organization [1]. Among the five different species of *Plasmodium* causing human malaria, *P. falciparum* is the most virulent one and an obligate intracellular parasite, resulting in most malaria-related deaths [2]. The present treatment of the disease relies on a combination of multiple antimalarial drugs such as artemisinin combination therapy but recently has led to the emergence of drug-resistant parasites [3]. Malaria affects the poorest regions of the globe, where it poses enormous health and economic challenges; the geographical overlap of malaria and HIV-1 in several parts of the world further exacerbates the ferocity of the disease. Such challenges emphasize the urgent need to identify and validate novel drug targets, decipher the molecular mechanisms underlying the inhibitory action of potential compounds, provide structural guidance to improve the inhibitors' desired properties, and develop new affordable chemotherapeutic strategies. However, one of the major focus areas is to discover cost-effective therapeutics for economically weaker and developing countries. Therefore, to reduce cost and the timeline for de novo drug discovery, drug repurposing is an alternative strategy that includes the evaluation of approved drugs to identify their new therapeutic uses [4,5]. Such a strategy may also result in the discovery of lead chemical scaffolds for further optimization studies, thus can fill the gap between the starting hit selection and the clinical trials [5].

Interestingly, in the population co-infected with HIV-1 and malaria, treatment of the former condition has shown reduction in *Plasmodium* parasites [6]. Subsequent studies have confirmed the ability of HIV-1 PIs to reduce the *in vitro* growth of *P. falciparum* at clinically

relevant concentrations [7,8]. These HIV-1 PIs were also found to be effective against other *Plasmodium* species, such as *P. chabaudi* in a murine model of malaria [7], *P. yoelii* in the hepatic stages of rodent [9], and *P. berghei* parasites [10].

Some studies have speculated that vacuolar plasmepsins (PMs) from different *Plasmodium* species are putative targets of HIV-1 PIs in malaria/HIV-1 co-infection [11,12]. Vacuolar PMs (PMI, PMII, histo-aspartic protease (HAP), and PMIV [13]) are pepsin-like aspartic proteases that reside in the acidic food vacuole. They play a crucial role in hemoglobin (Hb) degradation, which is the source of essential amino acids required for the survival of the parasites [14]. Owing to the high sequence and structural similarity among these functionally redundant proteases [15], it is crucial to design effective inhibitors targeting multiple PMs. Notably, lopinavir, an FDA-approved HIV-1 PI, has been shown to mediate its antimalarial effect by blocking the glucose uptake pathway by targeting the glucose transporter of *P. falciparum* [16]. Such additional targets exploited by these inhibitors might be beneficial in delaying the development of drug resistance and contributing towards their antimalarial action. Despite the prominent roles of HIV-1 PIs in mitigating the *Plasmodium* pathogenesis, no reports are available corroborating the inhibitory mechanism of HIV-1 PIs against PMs. Therefore, structural details underlying the antimalarial activities of HIV-1 PIs with PMs are crucial for designing improved inhibitors that could be useful as antimalarial drugs.

This study investigates the inhibitory action of five FDA-approved HIV-1 PIs against one of the pivotal vacuolar PMs in Hb degradation, PMII from *P. falciparum*. These inhibitors are ritonavir (RTV), lopinavir (LPV), saquinavir (SQV), nelfinavir (NFV), and indinavir (IDV). Our biochemical experiments have demonstrated the highest inhibitory activity of RTV and LPV against recombinant PMII, and shown their ability to arrest the parasite growth *in vitro*. Here, we present the first report of the crystal structures of PMII complexed with RTV and LPV that revealed the molecular mechanism of inhibition of vacuolar PMs by HIV-1 PIs. Such

structural investigations elucidating the interactions between these inhibitors and their targets pave the way towards repurposing the FDA-approved HIV-1 PIs, and also providing a scaffold for designing a novel class of antimalarials to tackle malaria/HIV-1 co-infection.

3. Materials and Methods

3.1. Inhibition of PMII by HIV-1 PIs

The expression and purification of recombinant, soluble thioredoxin-tagged truncated PMII (Trx-tPMII) was performed as described previously [17], with a few modifications; the primary and secondary culture of bacteria (Rosetta-gami B(DE3)pLysS) were grown in terrific broth media at 28 °C overnight to achieve higher expression levels of soluble Trx-tPMII.

Five FDA-approved HIV-1 PIs used in this study (RTV, LPV, SQV, NFV, and IDV) were purchased from Sigma-Aldrich (St. Louis, MO). Except for IDV soluble in double distilled water, the other four inhibitors were dissolved in 100% dimethylsulfoxide (DMSO). Aliquots of stock solutions of inhibitors (10 mM) were stored at -20 °C. Inhibition assays were performed as reported [17]. The experiment was performed in triplicates at 25 °C, pH 5.0. The data were fit to sigmoidal dose-response curves with nonlinear regression to calculate IC₅₀ values using GraphPad Prism (version 8.0). IC₅₀ values were converted to K_i values using the Cheng-Prusoff equation for competitive inhibitors, $K_i = \text{IC}_{50} / (1 + S/K_m)$ [18].

3.2. Crystallization of PMII complexed with ritonavir (RTV) and lopinavir (LPV)

Mature PMII (0.1 mM) in the acidic buffer (0.1 M sodium acetate, pH 4.5) was mixed with a five-fold molar excess of each inhibitor and was incubated overnight at 4 °C to form a protein-inhibitor complex. The mixture was buffer-exchanged in 50 mM phosphate buffer containing 0.15 M NaCl, 0.2% CHAPS (pH 7.5), and concentrated using a 10 kDa cut-off ultra-

centrifugation device up to 8 mg/ml. Crystallization screens of the protein-inhibitor complexes were set up with the help of the Phoenix robotics system (Protein Crystallography Facility, IIT Bombay) using commercial screens from Hampton (Index, PEGIon, PEGRx), Molecular Dimensions (JCSG, JCSG+), and Qiagen (PEG suite). Sitting drops containing 0.3 μ l protein solution and 0.3 μ l reservoir solution were equilibrated against 50 μ l reservoir solution at 293 K. The initial hit for the cocrystal of PMII-RTV was observed after a few weeks in the crystallization condition comprising 2.5 M sodium formate, pH 7.0, while the crystals for PMII-LPV complex were observed after several months in solution containing 1.4 M ammonium sulfate and 0.1 M Bis-Tris pH 5.5. Upon rigorous optimization of the above crystallization conditions, good quality crystals were obtained for data collection.

3.3. Data collection, structure solution, and refinement

The PMII-inhibitor cocrystals were transferred to the cryoprotectant solutions (mother liquor containing 30% ethylene glycol) and then to a nitrogen gas stream at 100 K. The data set for the PMII-RTV complex were collected at the PX-BL21 beamline (BARC) at Indus-2, Raja Ramanna Centre for Advanced Technology (RRCAT), India, using an MX225 CCD detector. The data set for the PMII-LPV complex was collected by the rotation method using a home X-ray radiation source consisting of a Rigaku Micromax 007HF generator equipped with R-Axis IV++ detector at the Protein Crystallography Facility, IIT Bombay. Data sets were indexed and integrated using the program XDS [19]. Integrated intensities were converted to structure factors with the modules F2MTZ and CAD of CCP4 [20].

The coordinates of PMII without the inhibitor (extracted from PDB ID: 5YIC) were used as a search model to solve the RTV- and LPV-bound PMII structures by molecular replacement. After the first cycle of refinement of the model with REFMAC5 [21] in the CCP4 suite, the sigma-A weighted $F_o - F_c$ electron density maps indicated the presence of inhibitor in the active

site pocket of PMII. Multiple iterative cycles of refinements with REFMAC5 and interactive model building in the electron density map using COOT [22] were carried out. Local anisotropy was modeled with translation-libration-screw (TLS) parameters by dividing the protein molecule into distinct TLS groups [23]. The validation parameters of the structures were obtained with MolProbity [24]. The statistics of data collection and refinement are presented in Table 1. All structure-related figures were generated with PyMOL (<https://www.pymol.org/>).

3.4. Antiparasitic assay of HIV-1 PIs by flow cytometry

3D7 strain of *P. falciparum* was cultured on human O+ erythrocytes maintaining 2% hematocrit with 5% CO₂, 1% O₂, and 94% N₂ environment in RPMI (Roswell Park Memorial Institute, Gibco, Grand Island, NY, USA) medium supplemented with Albumax 5 g/l, glucose 2 g/l, sodium bicarbonate 2 g/l, hypoxanthine 55 mg/l, and gentamycin 40 µg/ml [25,26]. Synchronization of the parasite was done with 5% D-sorbitol [27]. The inhibitors were diluted in DMSO. For the assay, 2 ml of 0.2-0.5% parasitemia culture and different concentrations of RTV and LPV (0.1 µM-50 µM) were added in each well of a 24-well culture plate with growth control and DMSO control. The plates were incubated at 37 °C for 48 h.

The dye dihydroethidium (Sigma-Aldrich) having an excitation_{max}/emission_{max} = 535/610 nm was used to stain infected red blood cells (iRBCs). The stock solution of dihydroethidium (1mg/ml) was diluted in phosphate-buffered saline (PBS) (1:2500). 50 µL of the 2% hematocrit blood media mixture (BMM) was pelleted by centrifugation at 1500 rpm for 2 min. The iRBCs were stained by adding 50 µl dihydroethidium working solution (0.63 mM) to iRBCs pellet and incubated in the dark at 37 °C for 25 min. To remove the excess of staining solution, iRBCs were washed with 200 µL PBS, centrifuged at 1500 rpm for 2 min, and finally resuspended in 500 µL PBS [28].

Samples were examined using a blue laser (488 nm) BD FACS (Fluorescence-activated cell sorting) AriaIII cytometer system (BD SINGAPORE SORP_Fusion). Gating was done according to their FSC-H/SSC-H profile to separate debris from the red blood cell population. Differentiation of cell doublets from single cells was done by gating based on the FSC-A/FSC-W profile. Further, the SSC-A/FITC-A profile was used to analyze stained and unstained singlet RBCs. For data acquisition, 50,000 events were analyzed. The experiments were performed in triplicates, and the graph was plotted using GraphPad Prism with inhibitor concentration versus percent response.

4. Results

4.1. Inhibition studies of PMII with HIV-1 PIs

The inhibition activity of HIV-1 PIs (RTV, LPV, SQV, NFV, and IDV) (Figure 1) was assessed by the rate of hydrolysis of the Hb-based fluorogenic peptide substrate by PMII. All five HIV-1 PIs used in this study were found to inhibit PMII with the K_i values in the low micromolar range of 0.3-2.4 μM (Figure 2). RTV and LPV exhibit the highest inhibitory activity with the K_i values of 0.3 and 0.7 μM , respectively, followed by SQV, NFV, and IDV.

Figure 1. Chemical structures of HIV-1 PIs, used in this study.

Schematic diagrams showing the chemical structures of five FDA-approved HIV-1 PIs: Ritonavir (RTV), Lopinavir (LPV), Saquinavir (SQV), Nelfinavir (NFV), and Indinavir (IDV).

Figure 2. Inhibition assay of HIV-1 PIs against PMII.

(A) Plots showing the IC_{50} values at different concentrations (in μM) of HIV-1 PIs inhibiting PMII. (B) Bar graph representing the K_i values of HIV-1 PIs against PMII. The experiments were performed in triplicate, and the graphs were plotted using GraphPad Prism (version 8.0).

4.2. Crystal structures of PMII bound to ritonavir (RTV) and lopinavir (LPV)

To further evaluate the binding mode of the two potent HIV-1 PIs with PMII, the protein was co-crystallized in complexes with either RTV or LPV. The structures of the PMII-RTV and PMII-LPV complexes were solved at 1.9 Å and 3.2 Å resolution, respectively. The unambiguous omit $F_o - F_c$ electron density in the active sites of the complexes indicate the presence and orientation of the inhibitors (Figure 3A, E). The Matthews coefficient [29] suggest the presence of two molecules (subunits A and B) and one molecule of protein in the asymmetric unit for the PMII-RTV and PMII-LPV structures, respectively. The inhibitor-bound protein structures were refined satisfactorily as indicated by appropriate R -factor, acceptable stereochemical properties, and residues in the suitable Ramachandran region for their respective resolutions range. The quality of the final $2F_o - F_c$ electron density maps in the active site is also acceptable, unambiguously defining the conformations of inhibitors. Both inhibitors bind in the extended conformation in the active sites of PMII with their central hydroxyl group located between the two catalytic aspartates; the flap is closed and interacts with the inhibitors (Figure S1). Interestingly, the binding orientation of inhibitors in the PMII

active site is similar to the binding of the pepstatin A (a potent non-specific aspartic protease inhibitor) and opposite to that of KNI inhibitors observed in our previously reported structures [17]. Seven and eight CHAPS molecules are present in A and B subunits of the PMII-RTV complex, respectively, enclosing the active site and masking the hydrophobic surface of the protein (Figure S2), while the LPV-bound PMII structure has two CHAPS molecules close to the active site.

Table 1. Data collection and refinement statistics of PMII complexed with ritonavir (RTV) and lopinavir (LPV).

	PMII-RTV	PMII-LPV
Data collection statistics^a		
Space group	C121	I4
Unit cell parameters <i>a</i> , <i>b</i> , <i>c</i> (Å) <i>α</i> , <i>β</i> , <i>γ</i> (°)	150.29, 70.41, 106.67 90, 133.61, 90	108.94, 108.94, 70.20 90, 90, 90
Temperature (K)	100	100
Wavelength (Å)	0.9795	1.5419
Resolution (Å)	39.89-1.9 (2.0-1.9)	38.52-3.2 (3.3-3.2)
<i>R</i> _{merge} (%)	4.9 (75.7)	21.0 (231.4)
Completeness (%)	95.9 (96.6)	99.9 (100)
Mean <i>I</i> / <i>σ</i> (<i>I</i>)	16.16 (1.84)	9.95 (1.04)
Total reflections	177775 (23868)	51868 (4560)
Unique reflections	61329 (8741)	6877 (602)
Redundancy	2.89 (2.73)	7.54 (7.57)
CC _{1/2}	99.9 (65.1)	99.6 (37.5)
Wilson B factor (Å ²)	41.24	87.22
No. of molecules in ASU	2	1
Refinement statistics		
Resolution (Å)	39.89-1.9	38.52-3.20
Working set: no. of reflections	58262	6531
<i>R</i> _{factor} (%)	22.26	22.46
Test set: no. of reflections	3067	344
<i>R</i> _{free} (%)	26.13	26.62
Protein atoms	5257	2606
Water molecule	365	0
Inhibitor molecule	Ritonavir (2)	Lopinavir (1)
CHAPS molecule	15	2
Ethylene glycol molecule	2	0
Geometry statistics		
r.m.s.d. bond distance (Å)	0.004	0.004
r.m.s.d. bond angle (°)	1.31	1.44
Isotropic average B-factor (Å²)		
Protein	16.64	50.54
Solvent	53.3	-
Ligand	43.62 (RTV)	98.24 (LPV)
CHAPS	63.96	140.55
Ethylene glycol	47.19	-

<i>Ramachandran plot</i>		
Most favoured region (%)	96.3	82.6
Allowed regions (%)	3.7	15.3
Outlier (%)	0	2.1
PDB ID	7VE0	7VE2

^a Values in parentheses correspond to the highest-resolution shell. PMII-RTV: PMII complexed with ritonavir (RTV), PMII-LPV: PMII complexed with lopinavir (LPV).

4.3. Interactions of ritonavir (RTV) and lopinavir (LPV) with PMII

RTV is anchored in the active site of PMII via various hydrogen bonds and hydrophobic interactions (Figure 3C, D). The 5-thiazo ring at P2' position forms a hydrogen bond with Met77 and makes a hydrophobic contact with Leu133. The P1' of RTV is hydrogen-bonded to the carbonyl oxygen of Gly38. The carbonyl of the inhibitor interacts with the main chain amide of Val80 *via* a hydrogen bond. At the P1' position, the phenyl ring is packed in the pocket formed by residues Tyr79, Ile214, Tyr194, and Phe296 of the S1' pocket. The S1 pocket contains several hydrophobic residues (Ile34, Phe113, Thr116, Phe122, and Ile125) that tightly hold the other phenyl ring at the P1 position. The P1 amide of the inhibitor makes a hydrogen bond with the carbonyl oxygen of Gly218. The central hydroxyl group forms hydrogen bonds with the active site aspartates, Asp36 and Asp216. The N-dimethyl P2 is further stabilized by multiple hydrophobic residues (Val80, Thr223, Ile292, Leu294, and Ile302) in the S2 pocket. The P2 carbonyl and the amide of the inhibitor engage in hydrogen bond formation with the main chain amide of Ser220 and the main chain carbonyl of Ser81, respectively. At P3, RTV makes a hydrogen bond with Ser220. The P4 2-isopropyl-4-thiazolyl moiety is packed in the S4 hydrophobic pocket consisting of residues Ile16, Ala221, Phe246, Met288, and Ile292. The superposition of main chain atoms of subunits A and B (r.m.s.d. 0.3 Å) in the crystal structure indicates that most of the protein-inhibitor interactions are well conserved. Notably, at the P4 position, the inhibitor exhibits conformational variability A and B subunits; RTV is also observed to exist in a double conformation in subunit B (Figure 3A, B, S1B).

Figure 3. Mode of binding of ritonavir (RTV) and lopinavir (LPV) to PMII.

(A) Omit $F_o - F_c$ map (contoured at 3σ level) of RTV-bound in the active site of PMII. The $F_o - F_c$ map of two molecules of RTV from the subunits 'A' and 'B', represented in magenta and yellow, respectively; RTV in the 'B' subunit assumes a double conformation. (B) Superposition of RTV from the two subunits 'A' (magenta) and 'B' (yellow) of the PMII-RTV complex; inset depicts the zoomed-in-view of the region where the conformational variability exists. The binding pocket of PMII representing the (C) hydrogen bonding interactions in black dotted lines and (D) hydrophobic interactions between the protein and the inhibitor in the PMII-RTV complex shown as cyan spheres. (E) Omit $F_o - F_c$ map (contoured at 2σ level) of LPV(blue) bound in the active site of PMII. The binding pocket representing the (F) hydrogen bonding interactions in black dotted lines and (G) hydrophobic interactions as cyan spheres between the protein and the inhibitor in the PMII-LPV complex. The amino acid residues in PMII are depicted as green sticks, and the inhibitors are shown in ball and stick.

In the PMII-LPV complex, most of the interactions between the inhibitor and PMII are analogous to those observed in the PMII-RTV structure. The central hydroxyl group has a hydrogen bonding with two active site aspartates (Figure 3F), whereas hydrophobic interactions are more prominent than the polar contacts between the protein and the inhibitor,

similar to the PMII-RTV complex (Figure 3F, G). Notably, RTV, a longer molecule comprising an additional P4 group than LPV, creates more interactions in the S4 pocket (Figure 4A).

Figure 4. A comparison of the binding modes of HIV-1 PIs in the active site of aspartic proteases.

(A) Surface representation of the superposed structures of ritonavir (RTV, magenta) and lopinavir (LPV, blue) bound to PMII. Due to the P4 extension in RTV compared to LPV, the former creates additional hydrophobic contacts in the S4 pocket. The catalytic aspartates are marked with red stars and labeled. (B) Superposition of RTV-bound PMII complex (magenta) with the available crystal structures of RTV complexed with other aspartic proteases: HIV-1 protease (PDB ID: 1HXW) in cyan, endothiapepsin (PDB ID: 3PRS) in salmon, secreted aspartic protease (PDB ID: 3Q70) in grey and Sapp1 (PDB ID: 3TNE) in green; all represented as ball and stick models. The superposed structures demonstrate the conformational flexibility at the P4 position of the inhibitor (region marked in black dashed lines) in the RTV-bound aspartic protease crystal structures. The two active site aspartates are represented in the sticks and are labeled.

4.4. Structural analysis of RTV complexes of aspartic proteases

Comparison of the PMII-RTV structure with the previously solved crystal structures of RTV bound to other aspartic proteases, such as HIV-1 protease (PDB ID: 1HXW), endothiapepsin (PDB ID: 3PRS), secreted aspartic proteases from *Candida albicans* (PDB ID: 3Q70) and *Candida parapsilosis* (PDB ID: 3TNE), reveals that a substantial portion of the inhibitor including the central core is well superposed. However, significant conformational variability is observed at the P4 position of the inhibitor (Figure 4B) that occupies the S4 pocket in these RTV-bound protease structures. Interestingly, this pocket can accommodate molecules, such as ethylene glycol, DMSO, and CHAPS, derived either from purification buffer, mother liquor, or cryoprotectant, further highlighting the larger volume of the S4 pocket. This pocket is surface exposed, and the inhibitor is found to interact with these additional molecules. The conformation of the P4 group is plausibly governed by steric hindrance due to combination of

residues in the S4 pocket and the small molecules wedged into or close to the S4 pocket. Notably, the P4 group of RTV in the structure solved in this study occupies a different site than RTV conformations in all the reported structures.

4.5. Antiparasitic activity of ritonavir (RTV) and lopinavir (LPV)

The ability of HIV-1 PIs (RTV and LPV) to arrest the growth of *P. falciparum* was further investigated. The *in vitro* growth of the malaria parasite in the infected RBCs pretreated with different concentrations of inhibitors (0.1 μ M-50 μ M) was assessed using flow cytometry. Both inhibitors are indicated to hamper the asexual life cycle of the malaria parasite with IC₅₀ values of 1.3 μ M and 3.0 μ M for RTV and LPV, respectively (Figure 5). The solvent control shows no inhibitory effect on the parasite. Notably, the higher antiparasitic activity of RTV as compared to LPV is also in agreement with the previously discussed inhibitory activities of these two compounds against PMIL.

Figure 5. Antiparasitic activity of HIV-1 PIs.

Dose-response curve of (A) RTV and (B) LPV, showing the log concentration of inhibitor versus percentage parasitemia with respect to control. The experiments were performed in triplicates, and the graphs were plotted using GraphPad Prism.

5. Discussion

Treatment of malaria with the currently available drugs is becoming more challenging due to the emerging drug resistance of *Plasmodium* parasites. Among various strategies implemented

to combat malaria, drug repurposing may be a promising approach to promptly provide cheaper drugs and potential leads. Owing to the observed efficacy of FDA-approved HIV-1 PIs in mitigating the pathogenesis of parasites during malaria/HIV-1 co-infection, vacuolar PMs were identified to be among the plausible targets of HIV-1 PIs [8,12,30]. However, there are no reports that corroborate and depict the molecular details of inhibition of PMs by HIV-1 PIs. In this study, we demonstrated that five FDA-approved HIV-1 PIs inhibit PMII with low micromolar inhibition activity; RTV has the highest activity showing two-fold higher inhibition than that of LPV. Both inhibitors suppress the *in vitro* growth of parasites with IC₅₀ in the low micromolar range. We obtained the first-ever crystal structures of any plasmepsin in complex with two potent HIV-1 PIs, RTV and LPV. These structures highlight the crucial interactions responsible for the inhibitory effects of RTV and LPV, and unveiled the mechanism underlying their differential potency. Unlike LPV, RTV possesses an additional P4 group creating more interactions that might be responsible for its higher inhibition activity. Our structural analysis shows that RTV exhibits conformational flexibility at the P4 position, implying that substitutions at this position could be well accommodated in the large S4 pocket. Thus, modification at the P4 position of RTV might be imperative in modulating its binding and inhibitory properties. These structural insights could enable the design of broad-spectrum inhibitors targeting the structurally similar aspartic proteases of therapeutic importance. One of our previous reports [31] has also elucidated the ability of SQV in inhibiting PMV, a non-vacuolar plasmepsin, as well as revealed the molecular basis of its inhibition. Such studies emphasize the plausibility of utilizing HIV-1 PIs for drug repurposing or as leads for expediting the development of effective inhibitors against a range of PMs. The latter strategy could also circumvent the need for the arduous drug discovery process, thus reducing the cost and time required for getting a lead molecule to design novel antimalarial inhibitors and reducing the burden of malaria/HIV-1 co-infection.

Author contributions

V.M, I.R., and P.B. conceived the idea and coordinated the study. V.M. prepared the proteins and undertook crystallographic and kinetics experiments under the supervision of P.B. The structure solution and analysis were done by V.M. and I.R. with inputs from P.B. The antiparasitic assay was performed by A.D. with an advice from S.P. The manuscript was jointly written by V.M., I.R., and P.B. with inputs from the remaining authors.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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