A Prodomain Peptide of *Plasmodium falciparum* Cysteine Protease (Falcipain-2) Inhibits Malaria Parasite Development

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Falcipain-2 (FP-2), a papain family cysteine protease of *Plasmodium falciparum*, is a promising target for antimalarial chemotherapy. Designing inhibitors that are highly selective for falcipain-2 has been difficult because of broad specificity of different cysteine proteinases. Because propeptide regions of cysteine proteases have been shown to inhibit their cognate enzymes specifically and selectively, in the present study, we evaluated the inhibitory potential of few falcipain-2 proregion peptides. A 15 residue peptide (PP1) inhibited falcipain-2 enzyme activity in vitro. Studies on the uptake of PP1 into the parasitized erythrocytes showed access of peptide into the infected RBCs. PP1 fused with Antennapedia homeoprotein internalization domain blocked hemoglobin hydrolysis, merozoite release and markedly inhibited *Plasmodium falciparum* growth and maturation. Together, our results identify a peptide derived from the proregion of falcipain-2 that blocks late-stage malaria parasite development in RBCs, suggesting the development of peptide and peptidometric drugs against the human malaria parasite.

Introduction

Malaria proteases are attractive antimalarial targets due to their critical roles in parasite infection and development. In silico approaches on parasite genome have identified 92 putative proteases that are grouped into five major classes (aspartic, cysteine, metallo, serine, and threonine) in the *Plasmodium falciparum* genome.[1] Among these proteases, cysteine and aspartic proteases of *P. falciparum* known as falcipains and plasmepsins respectively, play an important role in parasite food assimilation by their ability to degrade hemoglobin. Because hemoglobin degradation is an essential step in the parasite life cycle, blocking of these proteases offers an attractive target to arrest development of malaria parasite.[2–4]

Studies with protease inhibitors have suggested that malaria cysteine proteases are important for the hydrolysis of hemoglobin, erythrocyte rupture, and erythrocyte invasion.[5,6] In *P. falciparum*, four falcipains (falcipain-1, falcipain-2 and -2′, and falcipain-3) have been described at intraerythrocytic stage of parasite life cycle and investigations are on to elucidate their physiological role(s).[7–10] These enzymes belong to the papain superfamily with a number of unique features. Genes encoding falcipain homologues have also been identified in other *plasmodium* species.[3,11]

One of the difficulties in developing chemotherapeutic inhibitors against cysteine proteases is the ubiquitous presence of these enzymes in viruses, prokaryotes, and higher organisms and the identity of their catalytic mechanism.[12] These enzymes also have a broad substrate specificity, which makes it difficult to develop inhibitors that specifically inhibit individual proteinases.[13] As a consequence of these limitations, propeptide regions of different cysteine proteases are being employed to specifically inhibit individual protease.[13–17] Prosegment of proteases, especially cysteine proteases, have been shown to play a role in protein folding, intracellular trafficking, secretion of mature protease, and in the control of proteolytic activity by masking the active site.[16,18–20] Like many proteases, parasite cysteine proteinases are also synthesized as inactive zymogens that are converted to the mature form upon proteolytic cleavage and the release of the proregion polypeptide.[21,22] Recent studies on the mapping of the proregion peptides have revealed that subpeptides derived from the proregions inhibit their cognate enzymes in a selective manner.[13] For example, propeptide derived from cathepsin L of *Fasciola hepatica* is a selective inhibitor of the parasite cathepsin L and does not inhibit human cathepsin L.[23] Similarly, peptides containing pentapeptide sequence (LCGTV) from the proregion of trypanosomal cysteine proteinase, congopain, were found to be competitive inhibitors of the corresponding trypanosomal cysteine proteinases but did not inhibit rat cathepsins B and L.[24,25]

Of the four identified cysteine proteases of *P. falciparum*, falcipain-2 is the most intensely studied enzyme, and recent functional and structural data suggest that it is an attractive target for therapeutic intervention.[26,27] In comparison to other papain family proteases, falcipain-2 possesses an unusually long prosequence that is 2−3 times larger than in related enzyme prosequences.[21] Unlike other related proteases, falcipain-2 does not require its prodomain for acquiring a catalytically competent conformation.[21,28] Nevertheless, recombinant proregion of falcipain-2 has been shown to inhibit falcipain-2 activity effectively.[29] Considering the uniqueness and sequence divergence of falcipain-2 proregion, we explored the possibility of using small prodomain peptides of falcipain-2 for evaluating their antimalarial activity. A series of peptides from the prosegment of falcipain-2 were designed, and their effects on falcipain-2 catalytic activity and parasite development in vitro were studied. This study establishes the inhibitory potential of a peptide within

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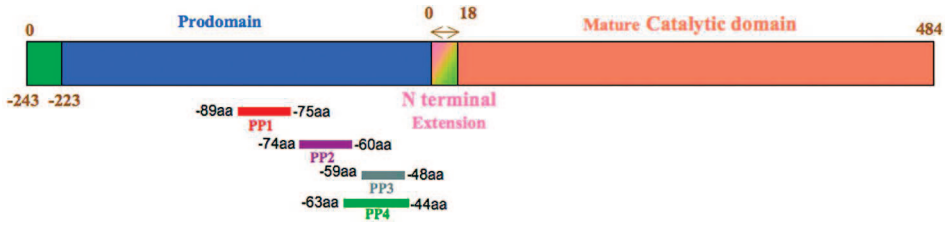

Figure 1. Schematic representation of falcipain-2 protein and its proregion peptides spanning the region −89 aa to −44 aa. Numbering is based on the position of first amino acid (Q) of falcipain-2 mature region.

Table 1. Amino Acid Sequences of Synthetic Falcipain-2 Proregion and Control Peptides Used in the Present Study[a]

peptide	amino acid sequence	position	molecular mass (Da)
PP1	LMNNAEHINQFYMFI	−89aa to −75aa	1885.19
PP2	KTNNKQYNSPNEMKE	−74aa to −60aa	1824.98
PP3	RFQVFLQNAHKV	−59aa to −48aa	1486.74
PP4	EMKERFQVFLQNAHKVNMHN	−63aa to −44aa	286.77
PP5	LMNNVEHINQFYTFI	−89aa to −75aa	1883.15
FITC PP1	FITC-LMNNAEHINQFYMFI	−89aa to −75aa	2274.57
Ant-PP1	RQIKIWFQNRRMKWKK-LMNNAEHINQFYMI	−89aa to −75aa	4113.92
Ant	RQIKIWFQNRRMKWKK		2246.75
OVA	ISQAVHAAHAEINEAGR		1773.93

[a] The position of each proregion peptide with respect to first amino acid (Q) of the falcipain-2 mature region is indicated. Molecular mass of each peptide in Daltons is presented.

the prosegment of falcipain-2 that has the potential of being used as a therapeutic molecule.

Results

Inhibition of Falcipain-2 Activity by Synthetic Proregion Derived Peptides. To delineate inhibitory segments in the falcipain-2 proregion sequence, we designed five peptides (PP1, PP2, PP3, PP4, and PP5)[a] corresponding to the −89aa to −44aa of the falcipain-2 proregion (Figure 1). This segment was chosen to design small peptides because it includes a conserved motif ER(F/W)N(I/V)N that is considered to be a functional unit involved in the inhibition of cathepsin L-like cysteine proteases.[30] Table 1 depicts the sequence of each peptide and their molecular masses. To analyze the effect of prodomain peptides on falcipain-2 activity, active recombinant falcipain-2 was refolded and purified to near homogeneity by affinity chromatography and ion-exchange chromatography using a protocol described previously by Shenai et al., 2000[21] (Figure 2A,B). The refolded protein was enzymatically active as it cleaved Z-FR-AMC, a substrate of falcipain-2, and enzymatic activity of the protein was significantly inhibited by a broad spectrum inhibitor of cysteine protease, E-64 (Figure 2C). The effect of different proregion peptides on enzyme activity was analyzed by preincubating the active falcipain-2 with each of these peptides before addition of the substrate. The proteolytic activity of falcipain-2 was significantly inhibited by peptide PP1, while peptides PP2 and PP3 inhibited the enzyme activity marginally (Figure 2C). Consequences of interaction of peptides with purified enzyme were monitored kinetically and biophysically. V_{max} of the substrate Z-Phe-Arg-AMC was estimated to be 120 pmol/min. Inhibition of enzyme activity by PP1 peptide was linear with time and concentration (Figure 2D). Inhibition constant (K_i) was calculated by double reciprocal plot and was 3.8 μM (Table 2). A peptide, PP5, corresponding to falcipain-2′, that contains two substitutions in the primary sequence (A-V, M-T), also inhibited the falcipain-2 activity with a similar K_i value. To know whether PP1 peptide specifically inhibits the cognate enzyme or it also affects the activities of other falcipains, PP1 peptide was incubated with active recombinant falcipain 1. As shown in Figure 2C (inset), PP1 peptide did not inhibit the falcipain-1 activity. An unrelated Ova peptide (OVA) also failed to inhibit the falcipain-2 activity (Figure 2C). Together, these results suggested that the peptide PP1, corresponding to the subpeptide of the proregion of falcipain-2, inhibited the enzyme activity specifically. Circular dichroism of peptide PP1 and purified enzyme produced a significant diminution in the elepticity of falcipain-2 protein, while an unrelated peptide, Ova peptide, did not produce any such change, suggesting an interaction between PP1 peptide and the falcipain-2 protein (Figure 3).

Uptake of Falcipain-2 Proregion Peptide by *P. falciparum* Infected RBC's and Inhibition of Falcipain-2 Activity. Peptides have been shown to get access into the parasitized erythrocytes selectively.[31] To investigate the translocation of PP1 peptide into the infected RBCs, PP1 peptide was labeled with FITC and FITC-PP1 peptide was incubated with RBC infected with *P. falciparum* 24 h postinvasion. The parasites treated with the FITC labeled peptides were washed and visualized by confocal microscopy at three different time intervals. Parasites inside RBC at trophozoite and schizont stages were observed to accumulate fluorescent PP1 peptide, whereas uninfected RBCs did not permit the accumulation of PP1 peptide (Figure 4). Because fluorescent PP1 peptide was able to translocate into the *P. falciparum* infected erythrocytes, we examined the ability of FITC-PP1 peptide to block the falcipain-2 mediated hydrolysis of Z-FR-AMC substrate by confocal microscopy. The hydrolysis of Z-FR-AMC was measured quantitatively by capturing the blue fluorescence generated as a result of release of free AMC by cysteine proteases. As shown in Figure 4A, PP1 treated parasites showed significant reduction in blue fluorescence in comparison to the untreated parasites. Quantitative measurements by confocal microscopy showed up to ∼50% block in substrate hydrolysis in PP1 peptide treated infected erythrocytes over untreated infected cells (Figure 4B). These results demonstrated that FITC-PP1 peptide enters into infected

[a] Abbreviations: PP1, propeptide 1; PP2, propeptide 2; PP3, propeptide 3; PP4, propeptide 4; PP5, propeptide 5; Ant PP1, propeptide 1 with Antennapedia homeoprotein internalization domain; FITC-PP1, fluorescein isothyocyanate lableled propeptide 1; OVA, ovalbumin peptide.

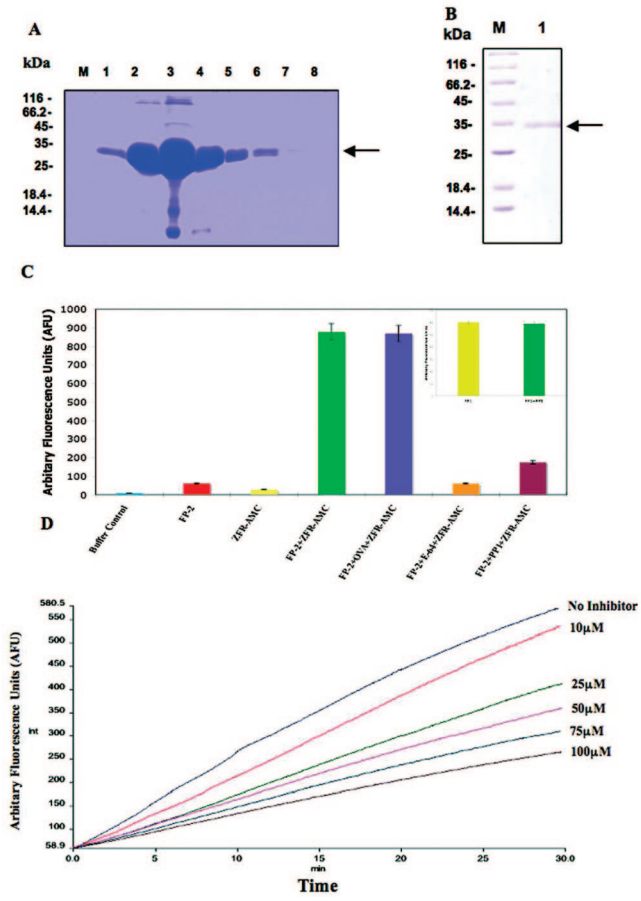

Figure 2. Expression and activity analysis of recombinant falcipain-2. (A) Coomassie blue stained 12% SDS-polyacrylamide gel of Ni^{2+}-NTA purified falcipain-2. Lanes 1−8, elutes from Ni^{2+}-NTA column. Lane M, molecular mass markers (kDa). Arrow indicates the position of the recombinant protein. (B) Coomassie stained 12% SDS-PAGE of refolded falcipain-2 protein. (C) Inhibition of recombinant falcipain-2 activity by falcipain-2 prodomain peptide (PP1). Proteolytic activity of falcipain-2 was measured at pH 5.5. Activity was analysed for the release of fluorescence units measured over a period of 30 min. Error bars represent SD of the results from three different sets of experiments. AFU, arbitary fluorescence units. The inset shows the effect of PP1 on falcipain-1 activity. (D) Dose-dependent activity profile of falcipain-2 incubated with varying concentrations of PP1 peptide over a period of 30 min.

Table 2. Kinetic Constants

V_{ma} picomol/min	K_m μM	K_i μM
120	10.11	3.8

erythrocyte and blocks the hydrolysis of Z-Phe-Arg-AMC, a substrate of falcipain-2.

Effect of Falcipain-2 Proregion Peptide on *P. falciparum* Development. We next investigated the effect of PP1 peptide on parasite growth and development in vitro by hypoxanthine uptake assay and by microscopic examination. Although PP1 peptide was able to access the intraerythrocytic parasites as shown previously, to enhance its uptake further, a 16aa sequence of Antennapedia (Ant) homeoprotein was coupled to the NH_2-terminus of PP1. The Antennapedia internalization sequence has been known to facilitate the uptake of peptides into *P. falciparum* and mammalian cells.[32,33] As shown in Figure 5, both PP1 and Ant-PP1 peptides inhibited parasite growth in vitro in a dose dependent manner. However, differences in the quantum of inhibition by these peptides were noteworthy. At 100 μM concentration, PP1 inhibited parasite growth up to 50%, whereas at the same concentration, Ant-PP1 inhibited the growth up to 80%. Peptides PP3, PP4, Ant, and OVA did not show any effect on parasite development. We also investigated the effects of these peptides on the total parasitemia by counting the Giemsa stained smears microscopically. Treatment of parasites with PP1 and Ant-PP1 considerably reduced the total parasitemia (∼50−70%) in comparison to the parasites treated with control OVA peptide. To visualize the effect of PP1 and Ant-PP1 peptides on parasite development, a transgenic parasite line expressing green fluorescent protein (GFP) fused with a region of knob associated histidine rich protein, KAHRP (KAHRP(-His)-GFP) was treated with the PP1 peptide. At 100 μM PP1 concentration, significant cessation in growth as indicated by loss of GFP fluorescence was observed in the treated parasites (Figure 5). These results were similar to that observed after the treatment of transgenic parasites with E-64 (Figure 5B,C). The cessation of growth observed by microscopic examination correlated with the [^{3}H] hypoxanthine uptake assay. These results suggested that PP1 inhibits falcipain-2 and consequently arrests the growth and development of the parasite.

Effect of Falcipain-2 Proregion Peptide on *P. falciparum* Morphology. Cysteine protease inhibitors have been shown to cause morphological abnormalities, especially in the food vacuole of malaria parasites.[9,34] Treatment of parasites at late ring or early trophozoite stage with PP1 resulted in a phenotype similar to that seen in cysteine protease inhibitor-treated parasites, whereby the food vacuole of early trophozoites was swollen and darkly stained. The abnormal food vacuole also showed tight clumps of malaria pigment that appeared to be different from the normal hemozoin (Figure 6). In PP1 treated parasites, we also observed several incompletely ruptured parasites after 56 h postinvasion of the first cycle of treatment. These schizonts contained spherical clusters of merozoites, enclosed in a delicate membrane covering (Figure 6). Similar clusters have been previously reported in parasite cultures wherein the parasite cysteine proteases especially falcipain-2 were inhibited by either cysteine protease inhibitors or by cognate siRNAs.[6,35] These results indicate that, in addition to its role in hemoglobin degradation, falcipain-2 also plays a role in merozoite release. Falcipain-2 has been previously shown to cleave the two erythrocyte skeletal proteins, ankyrin and band 4.1.[32] It is likely that cleavage of cytoskeletal elements by falcipain-2 destabilizes the erythrocyte membrane that in turn leads to its rupture and release of merozoites.

Inhibition of Hemoglobin Degradation by Falcipain-2 Proregion Peptide. To know whether the above-mentioned food vacuole abnormalities seen in PP1 treated parasites were due to a block in globin hydrolysis, parasites were incubated with PP1 peptide for 24 h (beginning at the ring stage) and their proteins were analyzed by SDS-PAGE. Compared to the wild type (WT) parasites, PP1-treated and E-64-treated parasites showed accumulation of undigested hemoglobin. Taken together, our data indicates that a proregion peptide (PP1) inhibits the falcipain-2 activity, which in turn blocks the hydrolysis of hemoglobin in *P. falciparum* trophozoite.

Discussion

Parasite encoded cysteine proteinases from the genus *Trypanosoma* and *Plasmodium* have been reported as potential targets for chemotherapeutic inhibitors.[11,36] In *P. falciparum*, the use of cysteine protease inhibitors such as peptidyl fluoromethyl ketone, vinyl sulfone, aldehyde, and nonpeptide inhibitors has supported the development of cysteine protease

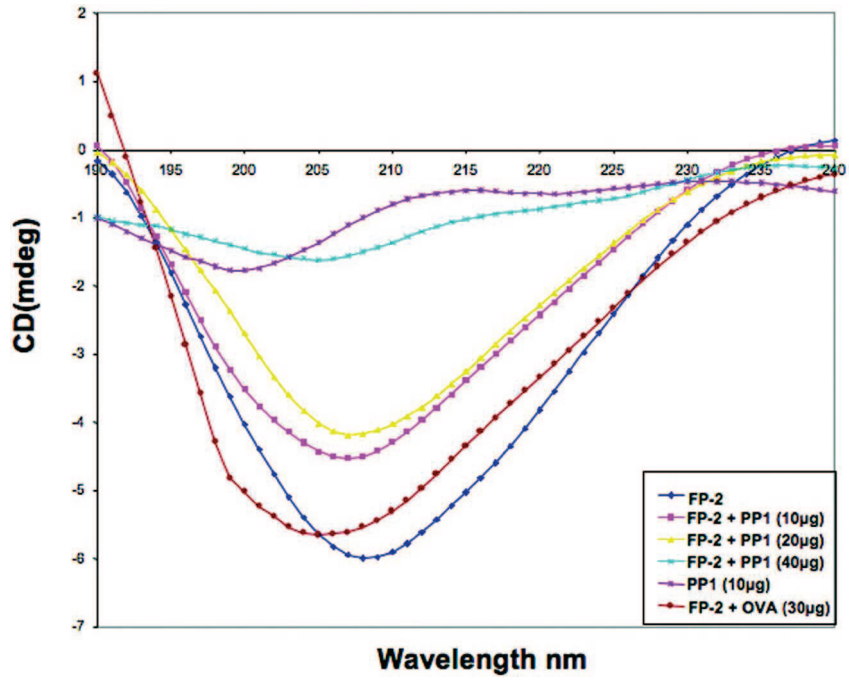

Figure 3. Analysis of interaction of PP1 with falcipain-2 by circular dichorism (CD). Far-ultraviolet CD spectrum of falcipain-2 in presence of different concentrations of PP1 peptide.

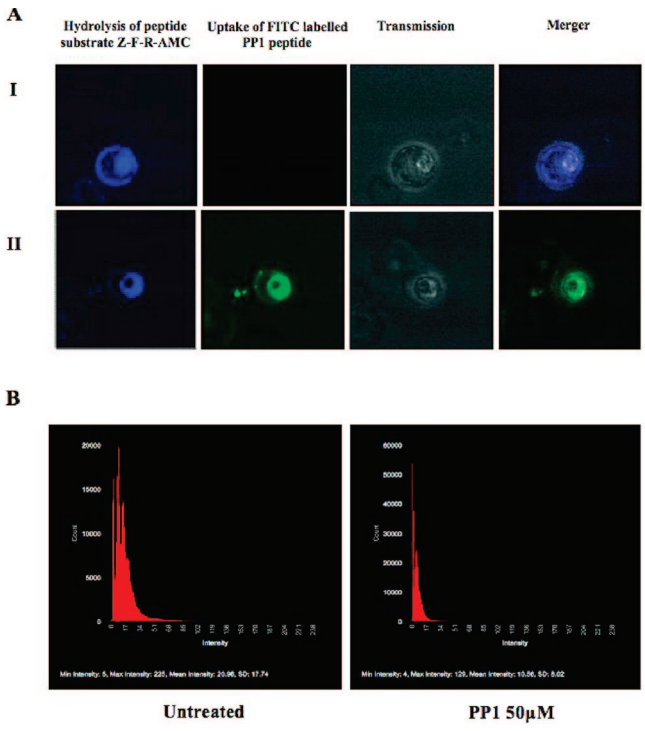

Figure 4. Inhibitory effect of PP1 peptide on substrate hydrolysis in the intracellular malaria parasite. (A) Confocal fluorescence imaging of *P. falciparum* trophozoite to show uptake and block in hydrolysis by PP1 peptide in the parasitized RBC. Panel 1, uptake and hydrolysis of fluoregenic substrate, Z-F-R-AMC. Note the presence of blue fluorescence in parasitophorous membrane and food vacuole, indicating the hydrolysis of substrate by native falcipain-2. Panel II, uptake of FITC PP1 peptide and its inhibitory effect on subtrate hydrolysis. (B) Quantitative analysis of fluorescence activity in the parasitzed RBCs to show inhibition in hydrolysis of Z-F-R-AMC by PP1 peptide.

inhibitors as antimalarials[11,37,38] Recent analysis has shown that the antiparasitic effects of cysteine protease inhibitors correlate with potent inhibition of falcipain-2 and falcipain-3 in *P. falciparum* and their homologous enzymes in *P. vivax* and *P. vinckei*.[39,40] Extensive studies are now underway to develop specific inhibitors corresponding to these enzymes for antimalarial therapy.[3,11] One of the limitations of developing specific inhibitors to a particular class of proteases is the conservation of active site residues in each class of proteins and broad substrate specificity.[25] On the other hand, prodomain of proteinases have been shown to be unique for each enzyme and inhibition of proteases by their corresponding prosequence is highly specific.[13,24] This specificity of prodomains inhibition of cognate enzyme is being exploited to develop a new generation of specific peptide inhibitors.

A large number of recent reports have shown that the parasitic proteases can be inhibited by their cognate prodomains. Several of these reports have employed *E. coli* expressed and purified protein of vector or parasite origin.[15,17,23,25,29,41] In the present study, we evaluated the potential of synthetic peptides corresponding to falcipain-2 proregion (−89aa to −44aa) for the antiparasitic activity. This region of prodomain was selected based on previous observations, suggesting that an evolutionary conserved α-helical motif in the proregion of cathepsin L-like proteases is likely to be involved in the inhibition of enzyme activity.[42] Of these peptides, only PP1 (−89aa to −75aa) significantly inhibited the falcipain-2 activity in a dose dependent manner. A corresponding peptide from the proregion of falcipain-2′ that has two amino acid substitutions (A-V and M-T) also showed similar inhibitory activity. The peptide, PP1, was highly selective as it failed to inhibit falcipain-1 activity. Surprisingly, PP4 peptide, which included the conserved ER(F/W)N(I/V)N motif, did not inhibit falcipain-2 activity in the present study as previously reported by Karrer et al.[30,42]

Import of proteins and macromolecules into malaria infected erythrocytes has been the subject of contention among different groups.[43] Recently, the access of fluorescent peptides with molecular mass ranging from 653 to 3146 Da into the parasitized erythrocyte has been investigated in detail, and the results

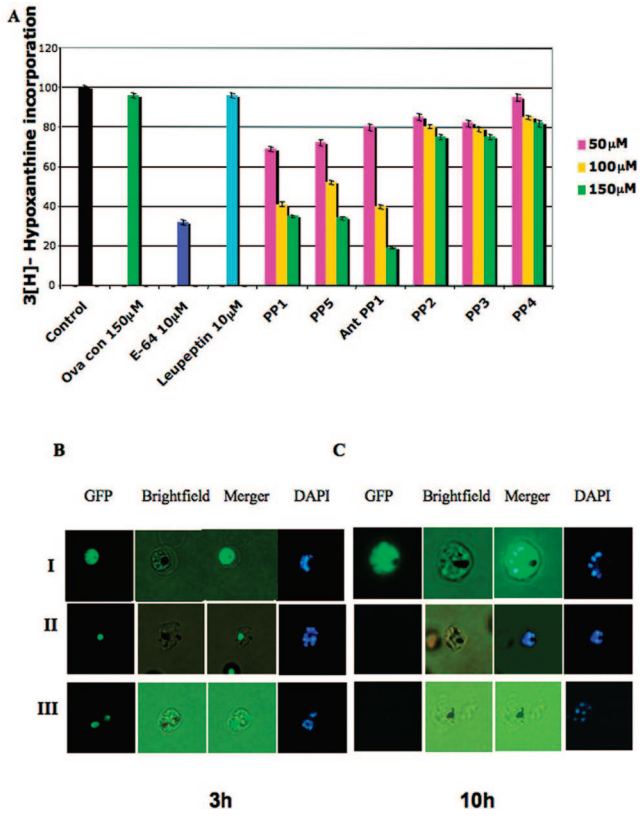

Figure 5. Effect of PP1 peptide on the growth and development of *P. falciparum.* (A) Inhibition of ^{3}[H] hypoxanthine uptake in cultured *P. falciparum* parasites by falcipain-2 proregion peptide, PP1. Synchronized cultures of parasite infected RBCs at ring stage were incubated with different concentrations of PP1 peptide for 24 h in a 96-well microtiter plate. Approximately 1 μCi of ^{3}[H] hypoxanthine was added in each well, and cultures were maintained for an additional 24 h. The cells were harvested, and ^{3}[H] hypoxanthine was quantified using a scintillation counter. OVA peptide was used as a control. (B) Fluorescence image analysis after 3 h of treatment and (C) after 10 h of treatment, to show the effect of PP1 peptide on the development of KAHRP (-His) transgenic parasite line. Note the loss of fluorescence in E-64 and PP1 peptide treated parasites. In each panel, the first image (left to right) represents fluorescence signal from GFP chimeric protein, second is the bright field image, third is an overlay of these two images, fourth is the DAPI stained image. Panel I, untreated parasites; panel II, E-64 treated parasites; panel III, PP1 peptide treated parasites.

revealed that peptides up to a molecular mass of 2365 Da are transported into the parasite.[31] We studied the uptake of substrate, Z-Phe-Arg-AMC and FITC labeled PP1 peptide by *P. falciparum* parasite and subsequently analyzed the effect of PP1 peptide on substrate hydolysis within the parasite using confocal microscopy. Both the substrate and FITC PP1 peptide were able to access the intracellular parasites. It appears that the substrate and labeled peptide are transported through the membrane channels from the outside directly to the parasites as no such uptake was seen inside the uninfected erythrocytes. The substrate hydrolysis was observed inside the parasite in PV as well in the food vacuole in the wild type parasite, thereby indicating the presence of falcipain-2 or other cysteine proteases in these sites. The fluorescent peptide PP1 was also detected in the intracellular compartments of *P. falciparum* trophozoites and schizonts. Treatment of parasites with PP1 peptide resulted in a block in Z-Phe-Arg-AMC hydrolysis inside the parasites. These results suggest that the small peptides can enter into the parasitized RBCs and can produce their biological affects effectively.

Recently, small peptides have been successfully used to inhibit merozoite invasion and block the parasite growth and development. Haldar and co-workers showed that peptides designed to inhibit Gαs protein function, considerably reduced parasitemia in *P. falciparum* cultures in vitro and in *P. berghei* infection in vivo.[44,45] Likewise, Hanspal and co-workers showed that an ankyrin peptide that blocks the cleavage of ankyrin mediated by falcipain-2 markedly inhibits the parasite growth and maturation.[32] Because PP1 peptide inhibited the native falcipain-2 activity in the intracellular parasites, we further examined the effect of PP1 peptide on the growth and development of *P. falciparum*. To do so, a modified PP1 peptide (Ant-PP1) that contains an Antennapedia internalization sequence at its NH_2 terminus was synthesized. This design has been previously used to deliver peptides into HeLa cells and into infected RBCs.[32] Ant-PP1 inhibited the falcipain-2 catalytic activity in a similar way as that of PP1 peptide. Both the peptides showed almost same potency (K_i for PP1 and Ant-PP1 were ~3 μM). Addition of each of these peptides to *P. falciparum* culture resulted in marked inhibition of parasite growth and development as determined by ^{3}[H] hypoxanthine assay, microscopic examination, and the counting of Giemsa stained smears. Less than 20% new ring stage parasites were observed in Ant-PP1 peptide treated culture in comparison to the control culture containing the same final concentration of OVA peptide. Interestingly, Ant-PP1 peptide was 1.5 fold more potent in inhibiting parasite growth than the PP1 peptide which may be a consequence of increased uptake of Ant-PP1 peptide within the parasite as a result of presence of the Antennapedia internalization sequence.

A number of previous studies have demonstrated that treatment of malaria parasites with cysteine protease inhibitors results in morphological abnormalties at trophozoite stage; the food vacuole appears to be enlarged and filled with undigested globin.[34,46] Recently, knock out or RNAi studies suggest that these food vacuole abnormalities arise due to inhibition or complete loss of falcipain-2 activity.[9,35] In addition to its role in hemoglobin degradation, falcipain-2 has also been suggested to be involved in erythrocyte rupture.[32,35] In the present study, treatment of parasites with either PP1 or Ant-PP1 peptides produced distinct food vacuole abnormalities similar to that observed with other cysteine protease inhibitors. We also observed few parasites stuck at the schizont stage 48 h after either PP1 or Ant-PP1 treatment. A control peptide OVA did not produce these abnormalities even at 4-fold higher concentration. Taken together, our study identifies a falcipain-2 proregion peptide that inhibits parasite growth and development by blocking the hemoglobin hydrolysis.

In summary, our results validate falcipain-2 as a potential new chemotherapeutic target. In addition, this study also establishes the antimalarial potential of a prosegment of falcipain-2 that may contribute to the design of proteinase-directed antiparasitic drug(s) of therapeutic interest.

Although proregions of parasitic proteases have been previously shown to inhibit their cognate enzyme activites,[15,23,25,29] here we show for the first time the use of a synthetic prodomain peptide as an antimalarial effector. Even though PP1 peptide inhibited the *P. falciparum* growth and development with a K_i value in the micromolar range, this study has potential for the development of peptide and peptidometric drugs. It has been earlier shown that interplay of multiple falcipains and plasmepsins (aspartic proteases) is responsible for the hemoglobin degradation inside the malaria parasite. It will be useful to design

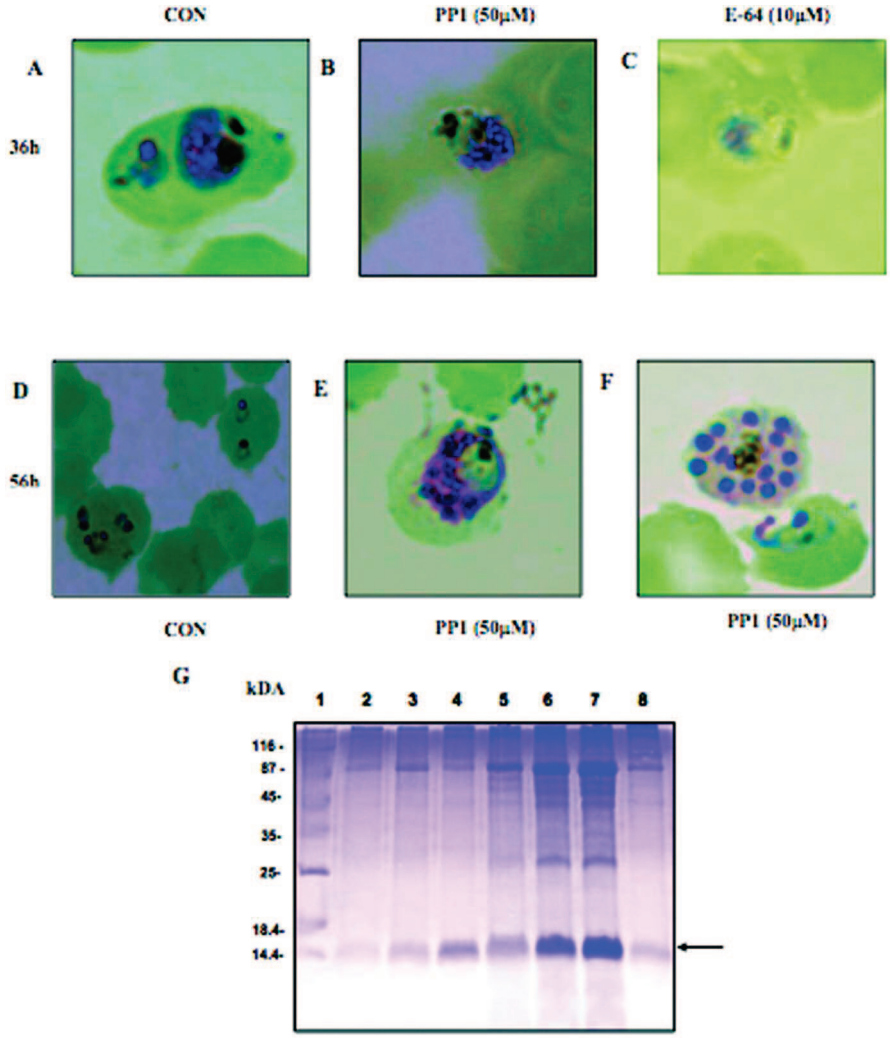

Figure 6. Prodomain peptide, PP1 blocks hemoglobin degradation in *P. falciparum.* (A) Effect of PP1 peptide on parasite morphology. Light microscope image of parasitized RBCs after 36 h of treatment. Note the presence of food vacuole abnormalities in E-64 and PP1 treated parasites. (B) Effect on globin hydrolysis by PP1 peptide treatment. Lane 1, molecular mass markers; lane 2, wild type parasite (control); lanes 3 and 4, E-64 (1 and 10 μM) treated parasites; lanes 5, 6, and 7, PP1 (50, 100, and 150 μM) treated parasites; lane 8, pepstatin (1 μM) treated parasites.

synthetic prodomain peptides against multiple food vacuole enzymes and then explore their potential to control malaria parasite.

Materials and Methods

Peptide Synthesis. The peptides, PP1, PP5, Ant-PP1, PP2, PP3, and Ova peptide were synthesized by the solid-phase synthesis method using the Fmoc procedure. PP4, Ant-PP1, and FITC PP1 peptides were obtained from Peptron, South Korea, and Genscript Corporation, NJ. All the peptides obtained were purified by semipreparative HPLC using a Bondapak C18 column. The molecular mass and purity of the synthesized peptides was checked by amino acid analysis and mass spectroscopy with MALDI-TOF. The stock solutions of the peptides were prepared in DMSO/water (1:1), and concentrations were measured spectrophotometrically.

Parasite Strains, Culture, and Peptide Treatment. *Plasmodium falciparum* strains, 3D7 and KAHRP (-His)-GFP strains were cultured with human erythrocytes (4% hematocrit) in RPMI media (Invitrogen, USA) containing gentamycin sulfate, $NaHCO_3$, and L-glutamine in vitro using protocol described previously.[47] The strain KAHRP (-His)-GFP is a transgenic cell line that is transfected with a construct in which a region (of knob-associated histidine-rich protein (KAHRP) gene encoding the first 60 amino acids was joined upstream of GFP coding sequence.[48] Trangenic strain is maintained in media containing 0.25 nM WR99210 drug. Both the wild type and transgenic parasites were synchronized using sorbitol treatment.[49] To assay the inhibitory effect of prodomain peptide(s), synchronized parasites cultures were adjusted to 4% hematocrit with 1% infected erythrocytes, and 0.2 mL of these cultures at the early ring stage were treated with prodomain peptides in a 24-well culture plate in triplicate. Parasites were maintained further for 24 or 48 h. For the microscopic analysis, smears were made from each well, stained with Giemsa, and examined for abnormalities as well as the number of new rings formed. The number of ring-stage parasites per 2000 RBCs was determined for each well. Parasites with enlarged food vacuoles and very little hemozoin accumulation were scored as abnormal. ^{3}[H] hypoxanthine uptake assay was performed as described previously by Malhotra et al.[50]

Preparation of Recombinant Falcipain-2 Protein. Recombinant falcipain-2 protein was prepared by a protocol described by Shenai et al. and Kumar et al.[21,28]

Enzyme Assay and Kinetic Analysis. Fluorimetric assay for falcipain-2 activity was carried out as described previously by Kumar et al..[28] Briefly, in 3 mL of assay buffer (100 mM sodium acetate pH 5.5 10 mMDTT) containing 200 nM enzyme, fluoregenic substrate Z-F-R-AMC was added at 7 μM concentration and the release of 7-amino-4-methyl coumarin (AMC) was monitored (excitation 355 nm; emission 460 nm) over 30 min at RT using a LS50B Perkin-Elmer fluorimeter. To analyze the effect of peptide(s) on enzyme activity, recombinant falcipain-2 was preincubated with

each peptide for 10 min at room temperature. Remaining activity was determined using the fluorogenic substrate.

Rate of hydrolysis at varied concentration of substrate Z-F-R-AMC was determined at constant enzyme concentration (200 nM) in 3 mL reaction volume. Kinetic constants k_m, V_{max}, and K_i values were determined using PRISM software (Graph Pad, San Diego).

CD Spectroscopy. The CD spectra of the PP1 peptide, falcipain-2 protein, and falcipain-2 protein + PP1 peptide were measured at room temperature (25 °C) between 190 and 300 nm using a Jasco J-715 spectrapolarimeter (Jasco, Easton, MD). The path length of the cell was 1 cm. Experiments were performed in 10 mM phosphate buffer, pH 7.0. All the spectra are averages of multiple scans.

Confocal Microscopy. To study the uptake of peptides into the infected erythrocytes, 3D7 parasites at 4−5% parasitemia were incubated with the FITC PP1 peptide at 24−28 h post invasion for 10 h. After the incubation, the cultures were washed with complete medium and incubated with the substrate Z-F-R-AMC for 10 min. The treated parasitized cells were immediately mounted on a glass slide with a coverslip and visualized using a Nikon TE 2000-U microscope. To capture FITC peptide, an excitation of 488 nm was used. Emitted light was collected through a band-pass filter at 505−530 nm. The release of AMC was captured using the UV band-pass filter. Transmitted light observations were performed during the experiments in order to follow the integrity of the cells. Fluorescence arbitary units were acquired from an average of selected whole parasites.

Analysis of Globin Hydrolysis in *P. falciparum* Parasites. The effects of PP1 peptide or E-64 on hemoglobin hydrolysis in malaria parasites were determined as previously described.[35] Briefly *P. falciparum* cultures were treated with these inhibitors for 24 h at ring stage. After the treatment, the infected erythrocytes were collected by centrifugation and lysed using 0.15% saponin in PBS on ice for 10 min. The lysed samples were centrifuged and washed three times with ice-cold PBS to remove erythrocyte cytoplasmic contents. Parasite pellets were solubilized in Laemmli buffer and separated on 15% SDS-PAGE. The gels were stained with Coomassie Brilliant Blue R250. Approximately 1×10^6 parasites were loaded in each well.

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Supporting Information Available: Table of purity of all target compounds; reverse phase HPLC profile of PP1; mass spectrometry data of PP1; reverse phase HPLC profile of Ant PP1; mass spectrometry data of Ant PP1. This information is available free of charge via the Internet at http://pubs.acs.org.

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