Chemistry & Biology
Article

Functional Characterization of a SUMO Deconjugating Protease of *Plasmodium falciparum* Using Newly Identified Small Molecule Inhibitors

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DOI 10.1016/j.chembiol.2011.04.010

SUMMARY

Small ubiquitin-related modifier (SUMO) is implicated in the regulation of numerous biological processes including transcription, protein localization, and cell cycle control. Protein modification by SUMO is found in *Plasmodium falciparum*; however, its role in the regulation of the parasite life cycle is poorly understood. Here we describe functional studies of a SUMO-specific protease (SENP) of *P. falciparum*, PfSENP1 (PFL1635w). Expression of the catalytic domain of PfSENP1 and biochemical profiling using a positional scanning substrate library demonstrated that this protease has unique cleavage sequence preference relative to the human SENPs. In addition, we describe a class of small molecule inhibitors of this protease. The most potent lead compound inhibited both recombinant PfSENP1 activity and *P. falciparum* replication in infected human blood. These studies provide valuable new tools for the study of SUMOylation in *P. falciparum*.

INTRODUCTION

Small ubiquitin-related modifier (SUMO) is a posttranslational modifier in eukaryotes that is structurally related to ubiquitin yet functionally distinct. SUMO modification has been implicated in the regulation of numerous biological processes including transcription (Desterro et al., 1998), protein localization (Matunis et al., 1996), and cell cycle control (Ulrich, 2009). Like other reversible posttranslational modifications (i.e., ubiquitination, acetylation, methylation and phosphorylation), SUMOylation is highly dynamic with diverse and complex functional implications. SUMOylated proteins were recently identified in *Plasmodium falciparum*, a unicellular eukaryotic parasite that causes human malaria (Issar et al., 2008), suggesting that SUMOylation may play similarly important roles in parasite growth and survival.

P. falciparum is the causative agent of human malaria, infecting 250 million people per year and causing nearly 800,000 deaths (Aregawi et al., 2008). The parasite has a complex and highly regulated life cycle in both the human and mosquito hosts. Numerous transcriptional and proteomic profiling studies have demonstrated that patterns of gene and protein expression are highly regulated during the blood stage life cycle (Bozdech et al., 2003; Khan et al., 2005; Le Roch et al., 2003). *P. falciparum* lacks canonical eukaryotic transcription factors, but novel transcription factors and epigenetic and posttranscriptional regulatory factors have recently been identified (Painter et al., 2011). Beyond regulation at the transcriptional level, multiple unexpected protein isoforms in proteomic analyses suggest that posttranscriptional and posttranslational modifications may play important and unique roles in the regulation of parasite survival inside the host (Coulson et al., 2004; Foth et al., 2008; Shock et al., 2007). Thus, SUMOylation may represent one of several key mechanisms that the parasite uses to control gene expression profiles. Proteomic analysis of SUMOylated proteins from both *P. falciparum* and *Toxoplasma gondii*, demonstrated that proteins from numerous essential biological pathways, including proteins that are unique to these parasites, are modified by SUMO (Braun et al., 2009; Issar et al., 2008). In *P. falciparum*, PfSir2, an epigenetic regulator of parasite antigenic variation, is modified by SUMO (Issar et al., 2008). Although a preliminary list of SUMO modified proteins has been generated, the importance of SUMO modification and its regulatory machinery in *P. falciparum* remains unclear.

SUMOylation of target proteins is regulated by dedicated enzymatic machinery, including a family of SUMO-specific proteases (SENPs) (Yeh, 2009). SENPs play two primary roles in SUMO regulation: they process SUMO precursors to reveal a C-terminal di-glycine before conjugation, and they cleave the isopeptide bond between the C-terminal glycine of SUMO and the lysine side chain of a target protein. Bioinformatic analysis has predicted two *P. falciparum* SENPs (Issar et al., 2008; Ponder and Bogyo, 2007; Wu et al., 2003). However, functional studies of the *P. falciparum* SENPs have posed many challenges. Traditional genetic disruption of *P. falciparum* SENPs will likely not be possible given the essential role of SENPs in

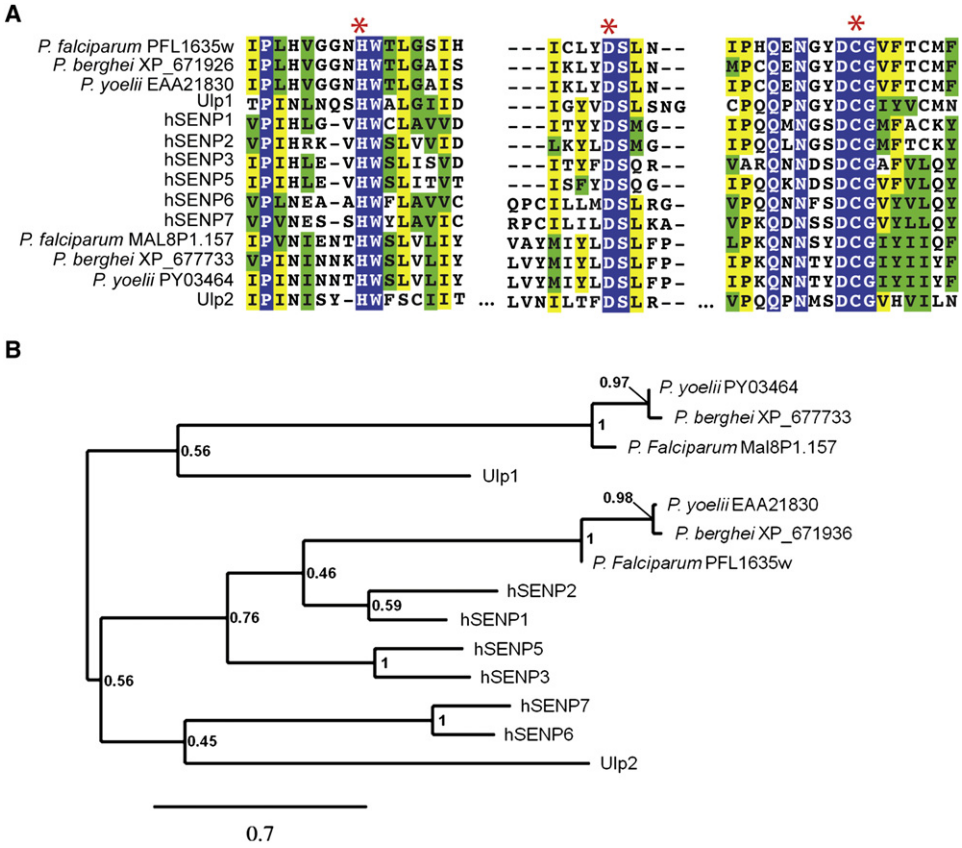

Figure 1. *Plasmodium falciparum* Has Two Predicted Sumo-Specific Proteases

(A) ClustalW alignment of the catalytic residues of six human SENPs, two yeast SENPs, and the two predicted SENPs from *P. falciparum*, *Plasmodium berghei*, and *Plasmodium yoelii*. Completely conserved identical residues are blocked in blue, conserved identical residues in green, and conserved similar residues in yellow. Aligned catalytic residues are denoted by the red (*).

(B) Phylogram depicting the relationship between the human, yeast, and *Plasmodium sp*. SENP catalytic domains. The bootstrap number is shown for each node.

yeast and mammals (Li and Hochstrasser, 2003; Yeh, 2009). Knockdown of gene expression by RNAi is not possible in *P. falciparum*, and, although two promising conditional gene knockout systems have been developed, the inherent leakiness of both systems limits their applications (Armstrong and Goldberg, 2007; Baum et al., 2009; Meissner et al., 2005). As an alternative to genetic disruption, small molecule inhibitors have been successfully used to characterize the function of several *P. falciparum* proteases (Arastu-Kapur et al., 2008; Yeoh et al., 2007). However, there are currently no small molecule inhibitors that target SENPs from any organism.

To understand the dynamics of SUMOylation in *P. falciparum*, we aimed to characterize the predicted SENPs and to identify small molecule inhibitors that perturb their function. In this study, we identified two putative SENPs of *P. falciparum* using bioinformatics analyses: PfSENP1 (PFL1635w) and PfSENP2 (MAL8P1.157). We were able to recombinantly express PfSENP1 and determine that it has robust SUMO processing activity and a unique cleavage site preference relative to the human SENPs. Using a SUMO processing assay, we screened a library of irreversible cysteine protease inhibitors and identified a single compound, JCP-666, that inhibits PfSUMO processing both by parasite lysates and by recombinant PfSENP1 and also blocked parasite replication in human blood. We subsequently synthesized a more potent analog, VEA-260, with improved stability. Together, our data suggest that PfSENP1 activity is essential for parasite growth, and that the novel inhibitor scaffold identified here may be useful for the development of inhibitors of PfSENP1 that do not inhibit SENPs of the human host.

RESULTS

P. falciparum Has Two Putative SENPs

A search of the *P. falciparum* genome using sequence homology identified two genes encoding putative SENPs: PFL1635w and MAL8P1.157 (Ponder and Bogyo, 2007; Wu et al., 2003). Alignment of the catalytic domain of these proteins with the human and yeast SENPs suggested that the essential catalytic residues are conserved (Figure 1A). The catalytic domain of PFL1635w, which we refer to as PfSENP1, is more closely related to the human SENPs whereas MAL8P1.157, referred to as PfSENP2, is divergent and found within a branch that is only loosely related to the yeast Ulp1 and highly related to a similar sequence in other *Plasmodium* species (Figure 1B). Analysis of microarray data

from the *P. falciparum* transcriptome indicates that both genes are expressed during the intraerythrocytic life cycle with peak expression in the late trophozoite phase (~25 hr postinvasion), and lowest expression in the early ring stage, immediately after invasion (Bozdech et al., 2003; Le Roch et al., 2003).

Lysates of *P. falciparum* Have SUMO Processing Activity

Although the predicted SENP mRNAs appear to be transcribed in *P. falciparum*, it is unclear if the resulting proteins are expressed or catalytically active. One function of the previously characterized SENPs in higher eukaryotes is to prepare SUMO for conjugation by proteolytically removing the C-terminal amino acids from the precursor SUMO protein (SUMO-pro) to reveal a C-terminal di-glycine motif. By adding a His_6-tag after the C-terminal extension of SUMO-pro, processing can be monitored by gel shift assay (Mikolajczyk et al., 2007). Using this assay we found that *P. falciparum* lysates efficiently processed SUMO-pro (PfSUMO-pro) (Figure 2A). Furthermore, this processing activity could be blocked by preincubation of parasite lysates with either *N*-ethylmaleimide (NEM) or human SUMO protein modified with a vinyl sulfone reactive group after the C-terminal di-glycine (hSUMO-VS). NEM blocks SENP activity by acting as a general alkylating agent that modifies the active site cysteine, whereas hSUMO-VS is an irreversible inhibitor of SENP proteases that contains the full length SUMO protein fused to a reactive vinyl sulfone group (Borodovsky et al., 2001). Inhibition by these agents suggests that the PfSUMO-pro processing observed in parasite lysates is the result of active PfSENPs.

P. falciparum development in the red blood cell is highly regulated, with many genes and proteins varying in expression and activity over the course of replication (Bozdech et al., 2003; Le Roch et al., 2003). To determine when PfSENPs are active during the parasite blood stage life cycle, we harvested synchronous cultures of *P. falciparum* every 8 hr for 48 hr starting at ~14 hr postinvasion. Incubation of lysates with PfSUMO-pro resulted in a peak in protease activity at 30 hr postinvasion, corresponding to the late trophozoite stage. Processing activity was lowest during the ring stage (14 and 48 hr postinvasion) (Figure 2B; see Figure S1 available online), in agreement with the gene expression patterns of both of the predicted SENPs (Bozdech et al., 2003; Le Roch et al., 2003).

To assess the specificity of the SUMO processing activity in parasite lysates, we monitored processing of multiple related human SUMO sequences (hSUMO-1-pro, hSUMO-2-pro, or hSUMO-3-pro). Recombinantly expressed human SENP1 protease (hSENP1) processed both PfSUMO-pro and all three human SUMOs. *P. falciparum* lysates, on the other hand, processed PfSUMO-pro and hSUMO-1-pro but not hSUMO-2-pro or hSUMO-3-pro (Figure 2C). Thus, the parasite enzyme differs from the human enzyme in its specificity toward its substrate. Because recent studies have demonstrated that processing of SUMO-pro by SENPs is sensitive to the sequence of the C-terminal amino acid extension after the di-glycine (Mikolajczyk et al., 2007), we wanted to determine if specific sequence differences in the C-terminal extensions of the SUMOs could explain the substrate specificity observed in parasite lysates. We generated chimeric SUMOs by swapping the C-terminal amino acid tails to create PfSUMO-h2pro, PfSUMO-h3pro, and hSUMO2-Pfpro. Whereas *P. falciparum* lysates failed to cleave chimeric PfSUMO-h2pro and -h3pro, chimeric hSUMO2-Pfpro was cleaved by parasite lysates (Figure 2D). Therefore, differences in C-terminal amino acid sequences rather than differences in the core SUMO sequence impact substrate recognition by parasite SENPs.

Expression and Characterization of PfSENP1 and 2

Although these results confirmed SENP activity in *P. falciparum* extracts, it was not clear if PfSENP1 and/or PfSENP2 were responsible for this activity. Therefore, we attempted to express the catalytic domains of both PfSENP1 and PfSENP2 in *Escherichia coli*. Despite multiple attempts, we could only express and purify the catalytic domain of PfSENP1. The resulting recombinant PfSENP1 efficiently cleaved PfSUMO-pro, and cleavage was inhibited by both NEM pretreatment and by mutation of the predicted catalytic cysteine to alanine (Figure 3A). In addition, we found that recombinant wild-type but not the catalytically dead mutant PfSENP1 cleaved hSUMO-1 from the lysine side chain of RanGAP1, a well-characterized SUMO-modified protein (Figure 3B).

PfSENP2 homologs (yeast Ulp2 and hSENP6 and 7) primarily function in the processing of poly-SUMO chains (Kroetz et al., 2009; Lima and Reverter, 2008), on hSUMO-2, -3, and yeast SUMO (SMT3) at canonical SUMOylation motif sites. However, *P. falciparum* SUMO, like hSUMO-1, lacks canonical SUMOylation sites (Figure S2), suggesting that the parasite does not poly-SUMOylate substrates. To determine if PfSUMO can itself be SUMOylated, we used a previously described system (Uchimura et al., 2004) in which *E. coli* is cotransformed with a tricistronic plasmid for the overexpression of the SUMOylation machinery. Expression of the human SUMOylation proteins resulted in the formation of poly-SUMO chains that could be enriched using the C-terminal His_6 tag on the unprocessed hSUMO-2 (Figure 3C). In contrast, cotransformation with PfSUMO-pro resulted in the recovery of only unprocessed PfSUMO (Figure 3C). These data suggest that PfSUMO cannot be SUMOylated and therefore cannot form poly-SUMO chains in the parasite. Given the lack of poly-SUMOylation and our inability to express recombinant PfSENP2, it is unclear what role, if any, PfSENP2 plays in the regulation of SUMO in *P. falciparum*.

PfSENP1 Has Unique Substrate Sequence Specificity

The SUMO regulatory machinery of *P. falciparum* differs from its human host in that *P. falciparum* has only two predicted SENP proteases whereas humans have six SENPs. Differences in the ability of parasite lysates to cleave a panel of human SUMOs suggested that PfSENP1 might be biochemically distinct from the SENPs of its human host. We therefore analyzed the cleavage site specificity of PfSENP1 using a positional scanning substrate library. This library was previously used to characterize the preferred amino acid cleavage sequences of five human SENP proteases, hSENP8, and three human de-ubiquitinating proteases (DUBs), a distinct class of proteases that can cleave after the C-terminal diglycine of ubiquitin (Drag et al., 2008a, 2008b). Specifically, we used the P2, P3, and P4 positional scanning libraries to compare the specificity profiles of PfSENP1 to the human SENPs (Figure 3D). Not surprisingly, PfSENP1 only

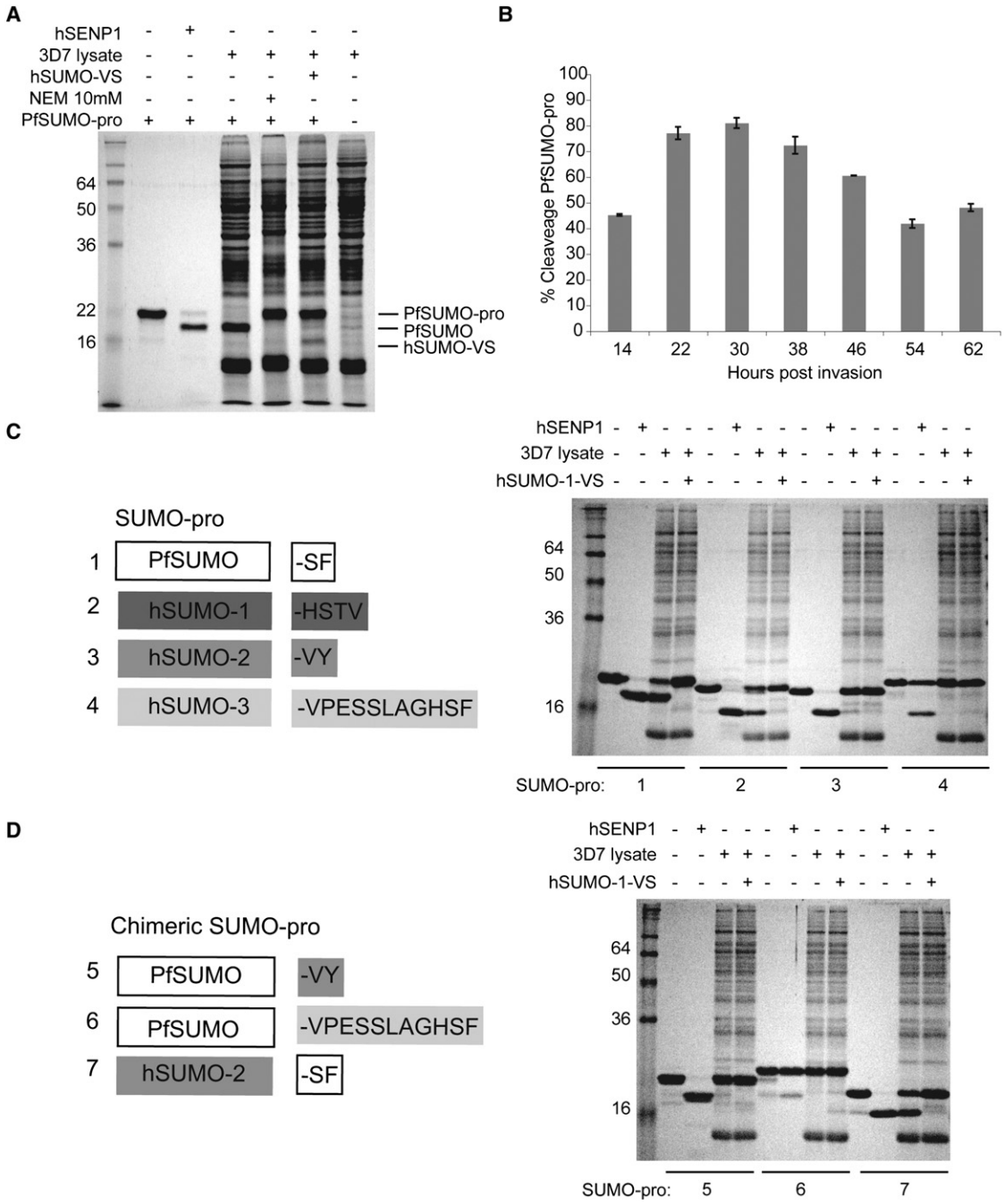

Figure 2. *Plasmodium falciparum* Lysates Have SENP activity

(A) SENP activity in *P. falciparum* lysates. The soluble fraction of mixed stage parasite lysates (3D7 lysate) was incubated with or without *N*-ethylmaleimide (NEM; 10 mM) or hSUMO-VS (10 μM) followed by PfSUMO-pro. Cleavage reactions were resolved by SDS-PAGE and visualized with Gelcode blue protein stain. PfSUMO-pro alone and recombinant human SENP1 (hSENP1)-cleaved PfSUMO-pro were included as negative and positive controls for cleavage, respectively.

(B) SENP activity throughout intra-erythrocytic life cycle. Synchronous blood stage cultures of *P. falciparum* were harvested every 8 hr for 48 hr, lysed by hypotonic lysis, and normalized for protein content (excluding hemoglobin). Equal protein concentrations from each time point were incubated with PfSUMO-pro, resolved by SDS-PAGE, visualized by Gelcode Blue, and the percent cleavage was quantified by ImageJ analysis. Bars represent average percent cleavage for each time point and error bars denote standard deviation (n = 3).

(C) Cleavage of SUMO-pro panel. Mixed stage parasite lysates, with or without hSUMO-VS pretreatment, or hSENP1 were incubated with SUMO-pro from *P. falciparum* (PfSUMO) or humans (hSUMO-1, -2, -3). Cleavage reactions were resolved and visualized as described above. The C-terminal amino acid sequence that is processed after the diglycine by SENPs is denoted in the detached boxes.

(D) Cleavage of chimeric SUMO-pro. Chimeric SUMO-pro proteins, as depicted on the left, were incubated with mixed stage parasite lysates, with or without hSUMO-VS preincubation, or hSENP1 and visualized as described above.

See also Figure S1.

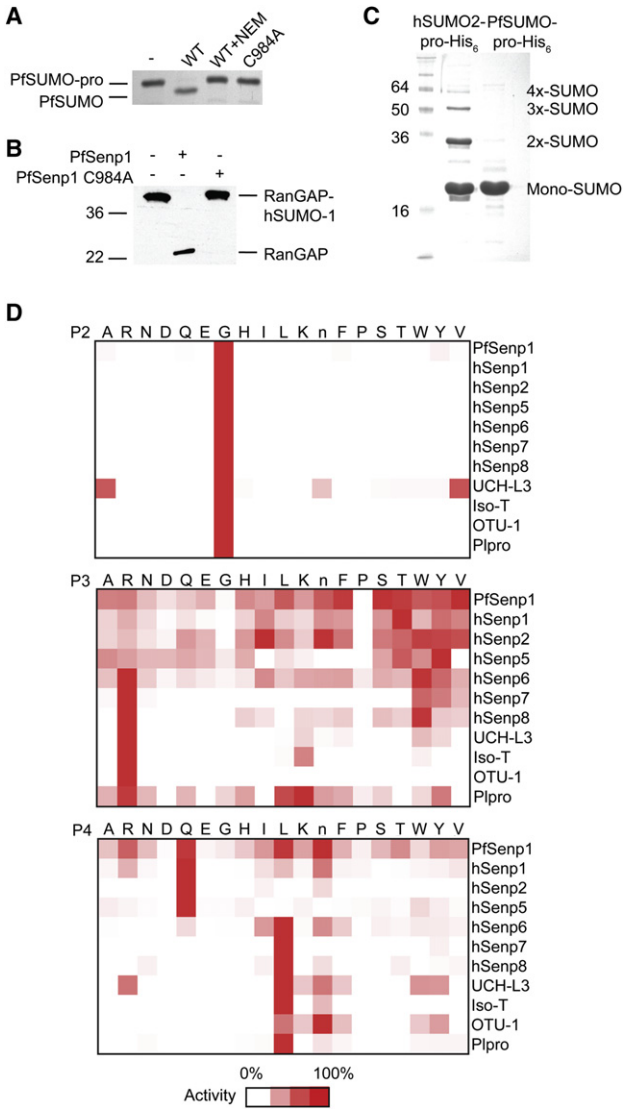

Figure 3. In Vitro Biochemical Characterization of PfSENP1 and Poly-SUMOylation

(A) PfSUMO-pro cleavage by PfSENP1. PfSUMO-pro was incubated with no enzyme, PfSENP1 (WT), PfSENP1 pretreated with 10 mM N-ethylmaleimide (WT+NEM), or PfSENP1 catalytic mutant (C984A). Cleavage reactions were resolved by SDS-PAGE and visualized with Gelcode Blue protein stain reagent.

(B) Cleavage of SUMO from a target protein by PfSENP1. hSUMO-1 modified Flag-RanGAP was incubated with PfSENP1 or PfSENP1 C984A. Cleavage reactions were resolved by SDS-PAGE and visualized by western blot using an anti-Flag antibody.

(C) *E. coli* were cotransformed with pT-E1/E2S2 and either pET28a-hSUMO2-pro-His_6 or pET29a-PfSUMO-pro-His_6. After induction, proteins were enriched with Ni^{2+}-NTA, and eluted with imidazole. Eluted proteins were resolved by SDS-PAGE and visualized by Gelcode Blue protein stain reagent.

(D) PfSENP1 (0.5 uM) was incubated with the indicated positional scanning substrate library, and ACC production was measured in triplicate using a fluorescence plate reader. Activity relative to the best amino acid was determined. A heat map of these data as well as published data on hSENPs 1-2, 5-8, and four human deubiquitinating enzymes, UCH-L3, Iso-T, OTU-1 and Plpro, is shown. Red indicates 100% cleavage activity and white indicates 0% cleavage activity.

See also Figure S2 and Figure S3.

tolerated glycine in the P2 position. In the P3 position, PfSENP1, like hSENP1 and hSENP2, tolerated a wide range of amino acids. In the P4 position, PfSENP1 showed the greatest preference for glutamine, leucine, and norleucine. Glutamine is the native P4 amino acid of *P. falciparum* SUMO and all three human SUMOs; it also is the preferred P4 amino acid of hSENP1, 2, and 5. Leucine is the native P4 position of ubiquitin and is therefore strongly preferred in this position for DUBs. Among the human SENPs, only hSENP6, 7, and 8 preferentially cleave leucine over glutamine in the P4 position. Our findings also demonstrate that PfSENP1 shares P4 specificity with both groups of human SENPs but also accepts several additional amino acids in the P4 position that are not well tolerated by the hSENPs, including arginine, isoleucine, and threonine.

To determine if differences observed in peptide substrate specificity translate into differences in specificity in the context of the native SUMO substrate, we mutated glutamine 95 (the equivalent of the substrate P4 position) of PfSUMO-pro to threonine (PfSUMOQ95T-pro). The PfSUMOQ95T-pro mutation resulted in a 2-fold decrease in substrate processing by PfSENP1 relative to the wild-type PfSUMO-pro substrate (Figure S3A). In contrast, hSENP1 processing of PfSUMOQ95T-pro was decreased by >25-fold relative to processing of the wild-type PfSUMO-pro (Figure S3B). These data suggest that the P4 position could be used to drive selectivity away from the closest human homologs of PfSENP1, namely hSENP1 and 2.

Identification of PfSENP1 Inhibitors

The only reported SENP inhibitors are general cysteine alkylating agents and the full-length SUMO protein modified with a reactive group, such as a vinyl sulfone (Borodovsky et al., 2002). Although effective for some biochemical assays, these inhibitors cannot be used to study SENPs in live cells. In addition, biotinylated probes consisting of 5–13 amino acids from the C terminus of SUMO modified with a vinyl sulfone reactive group have been reported as probes of SENP proteases (Borodovsky et al., 2005). However, these probes have not been shown to have SENP inhibitory activity. Therefore, we decided to screen a highly focused library of 508 irreversible cysteine proteases inhibitors (Arastu-Kapur et al., 2008) using the parasite lysate PfSUMO-pro processing assay to identify novel small molecule SENP inhibitors (Figure S4). This library was used previously in *P. falciparum* to identify novel inhibitors of host cell rupture and includes general scaffolds of covalent irreversible cysteine protease inhibitors (Arastu-Kapur et al., 2008). Because we were unable to express PfSENP2, we screened the inhibitor library against the native SUMO processing activity in parasite lysates rather than recombinant PfSENP1 alone. Interestingly, of the 508 compounds screened, only one inhibitor, JCP-666, reproducibly blocked SUMO-pro processing (Figure 4A). JCP-666 contains an aza-epoxide reactive group with a P1 aspartic acid side chain. Unlike the majority of aza-epoxide-based inhibitors in the library, JCP-666 lacks a P2 amino acid side chain. Chemical characterization of the compound indicated the primary species in the library was a mixture of chlorohydrin forms of the ring opened epoxide that formed during synthesis as a result of the removal of a BOC group with HCl (Figure 4B; Supplemental Compound Synthesis). Because we could not isolate the epoxide form of JCP-666, we used the mixture of

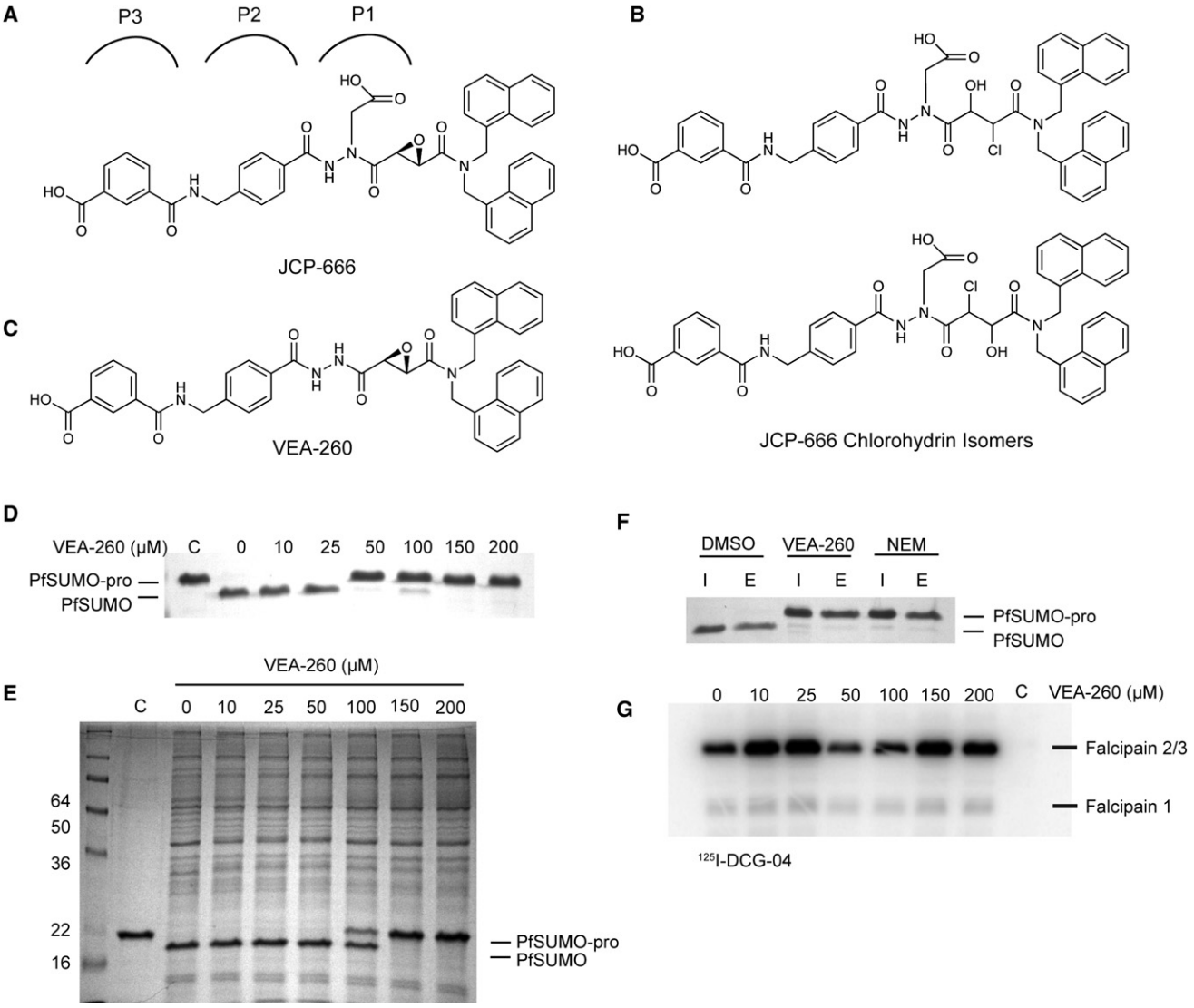

Figure 4. Screening and Identification of a Novel PfSENP Inhibitor

(A) Structure of JCP-666.

(B) Structure of chlorohydrin isomers of JCP-666.

(C) Structure of VEA-260.

(D) VEA-260 inhibits PfSENP1. Purified recombinant PfSENP1 (100 nM) was incubated with VEA-260 (0–200 μM) followed by cleavage of PfSUMO-pro and assessed as described above.

(E) Soluble mixed stage *P. falciparum* lysates were pretreated with VEA-260 (0–200 μM) followed by cleavage of PfSUMO-pro. PfSUMO-pro without lysate added was included as a control in lane C. Samples were separated by SDS-PAGE and visualized as described above.

(F) VEA-260 irreversibly inhibits PfSENP1. PfSENP1 was treated with VEA-260 (200 μM), NEM (10 mM), or DMSO. Input samples were taken. The inhibitor treated protease was diluted, enriched with Ni-NTA, and eluted with imidazole. Input (I) and elution (E) samples were added to PfSUMO-pro and cleavage visualized as described above.

(G) Total mixed stage *P. falciparum* lysates were pretreated with VEA-260 (0–250 μM) or DCG-04 (100 μM; lane C), a falcipain inhibitor, followed by labeling with ^{125}I labeled DCG-04. Proteins were separated by SDS-PAGE and visualized by autoradiography.

See also Figure S4 and Figure S5.

the chlorohydrin isomers in all our subsequent studies and refer to these simply at JCP-666. We did not find any evidence of similar chlorohydrin species for the related analogs JCP-665, JCP-667, JCP-668 even though these compounds were prepared using the same synthesis methods (Scheme S1). Interestingly, both the epoxide and chlorohydrin forms of JCP-666 were able to inhibit PfSENP1 activity (Figures S5). This suggests that either the chlorohydrin isomers interconvert to the epoxide in reaction buffer or the chlorohydrin forms are also active as inhibitors (Scheme S2). Thus, the chlorohydrin form of JCP-666 may inhibit the target SENP by SN2-like displacement of the chloride by the active site cysteine. This is consistent

Table 1. Inhibition of SENP and DUB Fluorogenic Substrate Cleavage by JCP-666 and VEA-260

Protease	Substrate	JCP-666 (**1b**) IC_{50} (μM)	VEA-260 IC_{50} (μM)
PfSENP1	QTGG-AFC	17.9 ± 1.0	16.2 ± 1.5
hSENP1	QTGG-AFC	9.0 ± 0.5	7.1 ± 0.6
hSENP2	QTGG-AFC	4.7 ± 0.5	3.7 ± 0.2
hSENP6	LRGG-AFC	>100	>100
hSENP8 (deNEDDylase)	LRGG-AFC	>100	9.1 ± 1.2
IsoT (USP5)	LRGG-AFC	>100	ND
UCH-L3	LRGG-AFC	>100	~100

ND, not determined.

with the fact that the chlorohydrin form of JCP-666 irreversibly inhibited PfSENP1. In addition, the chlorohydrins also inhibited human SENP1 activity against SUMO-pro and also the SUMOylated RanGAP substrate (Figure S5). Regardless, the overall instability of the JCP-666 epoxide and the presence of multiple isomers of the chlorohydrin prompted us to develop an alternative inhibitor for use in all subsequent studies.

For our first analog of JCP-666, we removed the aspartic acid side chain on the main aza-epoxide scaffold. We reasoned that changes in this region would likely alter the stability of the epoxide group. Furthermore, removal of this group would simplify the synthesis by reducing steric hindrance for the key coupling reaction between the peptide scaffold and the epoxide of the molecule. We therefore synthesized VEA-260, which is identical to JCP-666 but lacks the P1 aspartic acid side chain (Figure 4C). This new analog was as potent as the original JCP-666 against both recombinant PfSENP1 (Figure 4D; Figure S5A) and PfSENP activity in parasite lysates (Figure 4E and Figure S5B) suggesting that this aspartic acid side chain is not essential for binding to the target enzyme. Furthermore, VEA-260, like JCP-666, acted as an irreversible inhibitor of PfSENP (Figure 4F; Figure S5C). Importantly, VEA-260 was not susceptible to epoxide ring opening during synthesis and could be isolated in the epoxide form.

To assess the selectivity of VEA-260 for *P. falciparum* SENPs, we examined whether it inhibited other *P. falciparum* cysteine proteases. Specifically, we incubated mixed-stage *P. falciparum* lysates with various concentrations of VEA-260 and measured the residual activity of falcipains 1/2/3 using radiolabeled probes as described previously (Arastu-Kapur et al., 2008; Greenbaum et al., 2002). Importantly, VEA-260, like JCP-666 (Figures S5D and S5E**)** did not inhibit any of the falcipains (Figure 4G), suggesting that it is likely to be sufficiently selective to allow its use for studies in live parasite cultures.

To assess whether VEA-260 was selective for parasite SENP activity over other SENP proteases and the related deubiquitinating proteases (DUBs), we monitored inhibition using the QTGG-AFC and LRGG-AFC fluorogenic substrates previously optimized for these proteases (Table 1) (Drag et al., 2008a, 2008b). PfSENP1 and its closest human homologs (hSENP1 and 2) were all potently inhibited by VEA-260 and JCP-666. In contrast, hSENP6 (a homolog of PfSENP2) and UCH-L3 (a DUB) were not inhibited by VEA-260. Human SENP8 (a deNEDDylase) was inhibited by VEA-260 (Albrow et al., 2011) but not JCP-666, suggesting that removal of the aspartic acid P1 side chain may reduce selectivity to some extent. However, neither *P. falciparum* nor human red blood cells express hSENP8.

VEA-260 Inhibits *P. falciparum* Replication

To determine the effects of inhibition of PfSENP activity on *P. falciparum* growth, we used VEA-260 for phenotypic studies in parasites cultured in human red blood cells. Although only JCP-666 was identified from the library screen, a further search of the library identified three additional compounds (JCP-665, 667, and 668) that are highly related in structure to VEA-260 and JCP-666 (Figure 5A). All three analogs have the aspartic acid P1 side chain like JCP-666. JCP-665 and JCP-667 further differ from VEA-260 only in the size of the aromatic groups on the terminal amide, whereas JCP-668 differs from VEA-260 in the reactive electrophile group. Interestingly, none of these analogs were obtained as the chlorohydrin form even though they were originally synthesized using the same chemistries. This suggests that the combination of the large di-naphthyl groups and the P1 aspartic acid side chain cause the reduced stability of the epoxide of JCP-666 to HCl. All three analogs in the library did not inhibit PfSUMO-pro processing in *P. falciparum* lysates at the concentration originally used in our screen. However, we evaluated their ability to inhibit PfSENP1 at higher concentrations and found that JCP-668 also has weak activity (Figure 5A). The lower potency of JCP-668 suggests that the reactive electrophile affects potency, whereas the reduced potency of JCP-665 and JCP-667 suggest that bulky aromatics are required for efficient binding to PfSENP1. Treatment of synchronous ring stage cultures with VEA-260, JCP-665, JCP-667, and JCP-668 demonstrated that VEA-260 most potently inhibits parasite replication (EC_{50} 70 ± 20 μM) followed by JCP-668 (EC_{50} 220 ± 30 μM; Figure 5B). JCP-667 had no effect that could be measured within the solubility range of the compound. Interestingly, JCP-665 showed a shallow cell killing curve but was unable to affect complete clearance of the parasites (EC_{50} could not be determined). This non-dose-dependent activity for JCP-665 may result from general toxicity of the compound. Regardless, these data confirm that potency against PfSENP1 correlates with parasite killing, suggesting that PfSENP1 is likely to be an essential protease.

Finally, to more carefully monitor the effects of VEA-260 on parasite growth, we treated synchronously grown cultures of infected RBCs with 100 μM concentrations of the drug at various points in the blood stage life cycle. We then analyzed thin blood

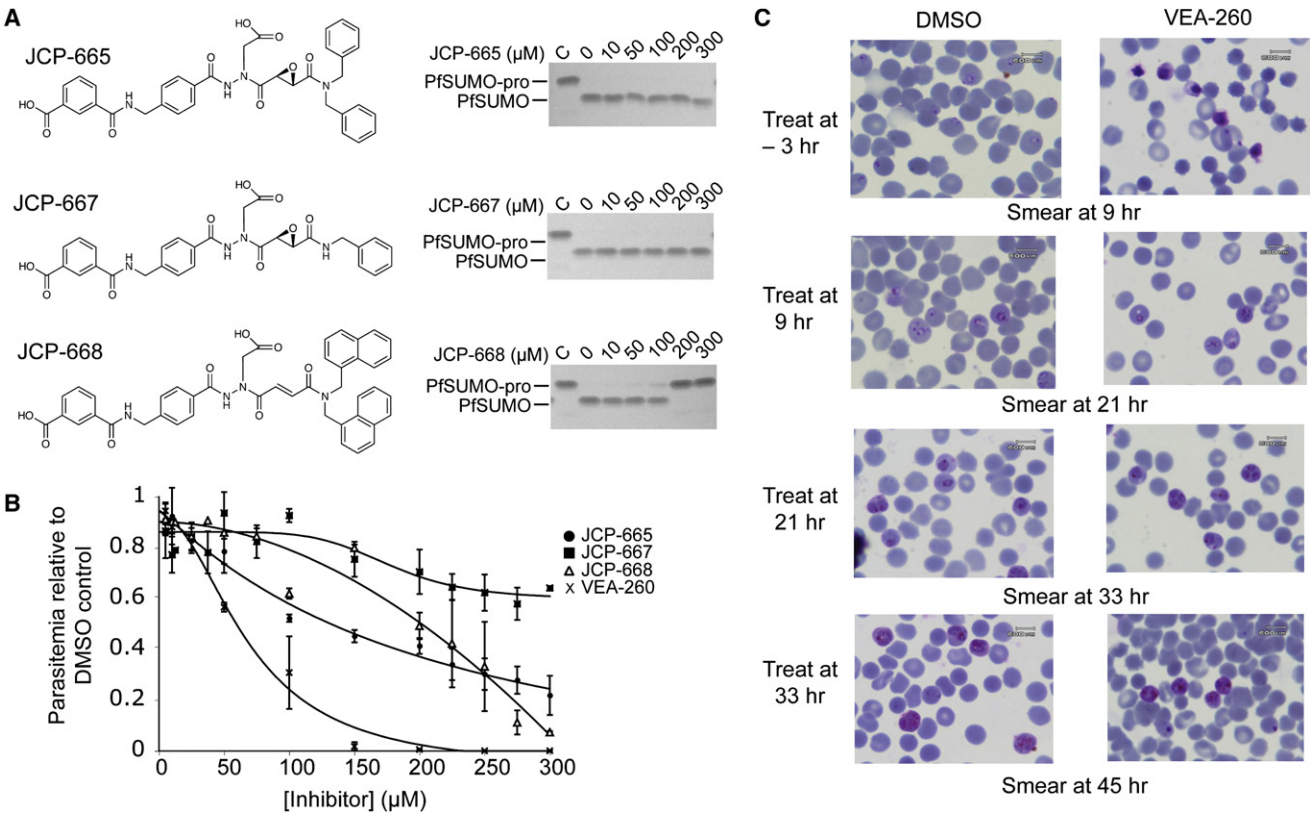

Figure 5. VEA-260 Inhibits Parasite Replication and Blocks Rupture When Added at Schizont Stage

(A) PfSENP1 was pretreated with JCP-665, -667, or -668 (0–300 μM) followed by addition of PfSUMO-pro. Cleavage reactions were resolved by SDS-PAGE and visualized by Gelcode Blue protein stain reagent. PfSUMO-pro without protease was included as a control (lane C).

(B) Synchronous ring-stage *P. falciparum* cultures were treated with indicated inhibitor (0–300 μM) and incubated for 75 hr. Cultures were then fixed, permeabilized, and stained with propidium iodide. Parasitemia was quantified by flow cytometry, and the parasitemia relative to DMSO treated controls was determined. The parasitemia relative to DMSO ± SD is shown for each concentration.

(C) Images of Giemsa-stained thin blood smears from synchronously grown parasite cultures treated with 100 μM VEA-260 at the indicated life cycle stage compared to a DMSO control. Smears were taken at the indicated times after addition of the compound.

smears of the parasites 12 hr later (Figure 5C). These results indicate that only parasites treated at the late schizont stage (i.e., 3 hr before rupture from the host [−3 hr]) looked morphologically altered and unable to release from the host RBC. The fact that parasites treated at earlier life cycle stages did not show any significant change in growth progression or cell morphology confirms that the compound is not generally toxic to the parasites. It also suggests that blocking deSUMOylation of proteins may be most detrimental for the parasite as it prepares to exit the host cell. Further studies into the reason for this stage specific effect of the inhibitor are currently ongoing.

DISCUSSION

SENPs play an essential role in the regulation of SUMOylation, a posttranslational modification that modulates numerous biological processes. The essential role of SENPs in other eukaryotes suggests these proteases may also be essential for *P. falciparum*. In this study, we demonstrated that *P. falciparum* has at least one active SENP protease, PfSENP1. Using a positional scanning substrate library, we demonstrated that PfSENP1 has distinct cleavage specificity relative to its human homologs. By screening a library of cysteine protease inhibitors we identified one compound, JCP-666, that inhibited SUMO processing by parasite lysates and recombinant PfSENP1. Because of the instability in the epoxide electrophile of JCP-666, we focused our efforts on a novel analog VEA-260 that showed increased potency and overall stability compared to JCP-666. This compound inhibited parasite replication in a dose-dependent manner, suggesting that PfSENP activity is essential for parasite survival.

The ability of VEA-260 to inhibit PfSUMO processing by both recombinant PfSENP1 and soluble parasite lysates, the selectivity of VEA-260 for hSENP1/2-like proteases over PfSENP2 human homologs, and the ability of VEA-260 to block parasite replication all suggest that PfSENP1 is the major SENP protease in *P. falciparum* lysates. Although the precise effect of VEA-260 on PfSENP2 is unknown, these data suggest one of three possible scenarios: VEA-260 inhibits both PfSENP1 and 2, inhibition of PfSENP1 alone is sufficient to block parasite replication, or PfSENP2 is not an active protease. *P. falciparum*, like yeast, has only two predicted SENP proteases. PfSENP1 is more closely related to yeast Ulp1 (hSENP1/2-like) and PfSENP2 is more closely related to yeast Ulp2 (hSENP6/7-like). Ulp1 is

cytosolic, lethal on deletion, and more highly related to the hSENP1/2 that are susceptible to VEA-260 inhibition. In contrast, Ulp2 is localized to the nucleus and can be genetically disrupted (Kroetz et al., 2009; Li and Hochstrasser, 2003). Defects resulting from Ulp2 disruption can be partially overcome by overexpression of the catalytic domain of Ulp1. Ulp2 is more closely related to the subset of human SENPs that includes hSENP6 and 7. As demonstrated here, VEA-260 is not an effective inhibitor of hSENP6, suggesting PfSENP2 is a less likely to be a target of this inhibitor. Therefore, we propose that PfSENP1 is the primary protease responsible for the SENP activity in *P. falciparum* soluble lysates and that inhibition of this protease alone is sufficient to block parasite replication.

Although we cannot rule out the possibility that VEA-260 inhibits PfSENP2, we speculate that PfSENP2 may not be an active protease. The catalytic domain of PfSENP2 has undergone a significant expansion relative to human SENPs, with nearly 200 additional amino acids relative to hSENP6 and 7, which themselves contain a 200-amino acid insertion relative to hSENP1 and 2 (Table S1) (Lima and Reverter, 2008). Furthermore, the expansion within PfSENP2 contains long stretches of amino acid repeat sequences that may have disrupted protease function and may have also prevented expression of PfSENP2 in *E. coli*. In addition, PfSENP2 homologs (yeast Ulp2 and hSENP6 and 7) primarily function in the processing of poly-SUMO chains (Kroetz et al., 2009; Lima and Reverter, 2008). We demonstrated here that PfSUMO cannot form poly-SUMO chains; therefore, it is unclear what role, if any, PfSENP2 plays in SUMO regulation in the parasite.

Regardless of whether or not PfSENP2 is a functional protease, VEA-260 is an inhibitor of PfSENP1. Most classes of protease inhibitors that target cysteine proteases in *P. falciparum* show some level of cross-reactivity with the falcipains (Arastu-Kapur et al., 2008; Greenbaum et al., 2002). Our lead SENP inhibitor, VEA-260, did not cross-react with the other major parasite cysteine proteases, including the falcipains, at nearly double its EC_{50} value in the replication assay. Furthermore, JCP-668 showed dose-dependent inhibition of parasite growth proportional to the decreased potency of this compound for PfSENP1, and JCP-667 showed no toxicity to parasite growth correlating with its inability to inhibit PfSENP1. Although JCP-665 showed toxic effects not related to PfSENP1 inhibition, these effects were not sufficient to fully block parasite replication. These data suggest that the complete block in parasite replication by VEA-260 and JCP-668 was due to inhibition of the target SENP. Modulation of PfSENP1 expression would be useful to validate that VEA-260 susceptibility is proportional to the level of PfSENP1. However, previous studies in yeast have demonstrated that Ulp1 overexpression exerts a dominant-negative effect and is therefore likely to result in lethality in *P. falciparum* (Mossessova and Lima, 2000). Although VEA-260 also inhibits hSENP1 and 2, we demonstrated that it does not inhibit all SENPs nor does it inhibit highly related cysteine proteases, such as the DUB UCH-L3. These data indicate that VEA-260 may be a promising lead compound with a high degree of selectivity for PfSENP1 over other cysteine proteases.

Identification of a small molecule SENP-selective inhibitor also provides a new tool for the analysis of the SUMOylation pathway in *P. falciparum*. Recent proteomic analysis of SUMO modified proteins in *P. falciparum* identified a preliminary list of 23 proteins (Issar et al., 2008). This is a surprisingly small number compared to proteomic studies in other organisms including yeast (>200 proteins identified) and the highly related parasite *Toxoplasma gondii* (>100 putative modified protein identified) (Braun et al., 2009; Denison et al., 2005). For example, SUMOylation of PfSir2 was not observed by mass spectrometry in *P. falciparum*, despite western blot and coimmunoprecipitation evidence that this protein is in fact modified by SUMO. This is likely due to the low levels of SUMO modified protein at any given time and the high level of activity of SENPs in *P. falciparum* lysates. With the discovery of small, cell permeable inhibitors of PfSENPs it should be possible to generate more complete profiles of SUMOylated proteins by mass spectrometry analysis. Furthermore, these inhibitors can be used to specifically block removal of SUMO at specific stages of the parasite life cycle to provide a more complete picture of the dynamics of SUMOylation.

SIGNIFICANCE

SUMOylation is a dynamic process that is involved in the regulation of a number of key biological processes. In particular, SUMO modification is required for correct transcriptional regulation. In the human parasite pathogen *Plasmodium falciparum*, the causative agent of malaria, a large percentage of genes are tightly regulated at the transcriptional level during its 48 hr blood stage life cycle inside the human host. However, very little is known about the molecular mechanisms used by the parasite to control this transcriptional program. Therefore, tools that will allow a more detailed biochemical analysis of posttranslational processes, such as SUMOylation, are likely to help shed light on how the parasite regulates its blood stage processes. In this study, we find that only one of two *P. falciparum* SENP genes produce a functional enzyme. Biochemical studies of this protease reveal that it has specificity for the parasite SUMO-pro and some degree of unique specificity for primary substrates compared to the human SENPs. To identify new classes of small molecule inhibitors of SENPs, we screened a small focused library of protease inhibitors in a newly developed assay in crude parasite extracts. This screen identified a class of nonpeptidic aza-epoxides that covalently inhibit the PfSENP1 protease. Using the lead compound and various analogs with reduced potency for PfSENP1, we were able to demonstrate that inhibition of PfSENP1 correlates with parasite killing. Together, these studies suggest that PfSENP1 may be a viable drug target and provide valuable new tools for studying SUMOylation in both parasites and other eukaryotic systems. These studies should also enable future studies of the molecular mechanisms of SUMOylation as it relates to the regulation of the blood stage life cycle of this important human pathogen.

EXPERIMENTAL PROCEDURES

Sequence Alignment and Phylogram Generation of Predicted *P. falciparum* SENPs

The catalytic domains of hSENPs 1, 2, 3, 5, 6, and 7, yeast Ulp1 and Ulp2, and the predicted SENPs of *P. falciparum*, *P. berghei*, and *P. yoelii* were aligned

using ClustalW (MacVector 10.0.2). A phylogram was created based on this alignment using the PhyML program with a bootstrapping procedure (number of bootstraps = 100) available on Laboratoire d'Informatique, de Robotique et de Microélectronique (LIRM) at Montpellier (France) (http://www.phylogeny.fr).

Parasite Culture, Harvesting of Life Cycle Stages, and Lysate Preparation

For details, see Supplemental Experimental Procedures.

Cloning and Expression of PfSUMO, Chimeric SUMOs, PfSENP1, and PfSENP2

Strains, constructs, and primers for PfSUMO, chimeric SUMOs, PfSENP1, and PfSENP2 are described in the Supplemental Experimental Procedures. All constructs were expressed in BL21(DE3) *E. coli* (OD_{600} = 0.6; 0.1 mM IPTG) for 3 hr at 30°C, lysed in ML1B, and immobilized on Ni^{2+}-NTA agarose. His_6-tagged PfSENP1 was eluted with imidazole whereas untagged PfSENP1 was eluted from the CPD fusion with 100 μM $InsP_6$ (Lupardus et al., 2008). PfSENP2 was not successfully expressed in either system.

SUMO-pro and SUMO-RanGAP1 Cleavage Assays

SUMO-pro and SUMO-RanGAP cleavage assays were performed as previously described (Mikolajczyk et al., 2007). For details see Supplemental Experimental Procedures.

The SUMO-pro cleavage assay was modified to assess the effects of the PfSUMOQ95T-pro mutation. PfSUMOQ95T-pro or PfSUMO-pro were incubated with 2-fold serial dilutions of PfSENP1 or hSENP1 (400 nM–3.1 nM) for 1 hr at 37°C. Proteins were separated by SDS-PAGE and visualized by Gelcode Blue protein stain reagent (Pierce), and the protease concentration required for 50% cleavage activity of the substrate (AC_{50}) was estimated.

The SUMO-pro cleavage assay was also used to evaluate the reversibility of VEA-260 and JCP-666. PfSENP1 (200 nM) was treated for 2 hr at 37°C with VEA-260 or JCP-666 (200 μM), NEM (10 mM), or DMSO in a final reaction volume of 200 μL. An input sample was taken and then the inhibitor treated protease was diluted, enriched with Ni^{2+}-NTA, and eluted with 300 mM imidazole. The input and elution samples were subjected to the SUMO-pro processing assay described above.

Positional Scanning Fluorogenic Substrate Library Screen

The fluorogenic substrate library screen was performed as described previously (Drag et al., 2008a, 2008b). Method details are provided in the Supplemental Experimental Procedures.

Cysteine Protease Inhibitor Library Screen

A library of 508 cysteine protease inhibitors was screened for inhibition of PfSUMO-pro processing. For the screen, 1 μl of a 10 mM DMSO stock solution of each inhibitor (100 μM final concentration) was added to 10 μg of soluble *P. falciparum* lysate in reaction buffer (50 μL reaction) and incubated for 30 min at room temperature in a 96-well plate format. The PfSUMO-pro substrate was then added to the reaction (10 μM final concentration) and incubated for 1 hr at 37°C. PfSUMO-pro without lysate and lysate pretreated with DMSO alone were used as positive and negative controls for inhibition on each assay plate. The addition of 10 μL SDS loading dye quenched the reaction, and cleavage was analyzed as described for the SUMO processing assays.

The lead compound JCP-666 was initially assessed by liquid chromatography (Agilent 1100 Series)-mass spectrometry (API 150EX, Applied Biosystems) (LC-MS) (see Supplemental Compound Synthesis and Characterization). However, the expected mass was not found. Subsequent chemical analyses and re-synthesis of JCP-666 suggested that the epoxide of the reactive warhead was unstable and modified by hydrochloric acid (see Supplemental Compound Synthesis and Characterization, Supplemental Results, Scheme S1, and Scheme S2). VEA-260 was subsequently synthesized as described in the Supplemental Compound Synthesis and Characterization. The general scaffold of JCP-666 was used to perform a structure search of the library to identify related analogs. The purity and identity of the analogs JCP-665, JCP-667, and JCP-668 was assessed by LC-MS, HRMS, and proton NMR (see Supplemental Compound Synthesis and Characterization). Characterization of all compounds is described in detail in the Supplemental Compound Characterization.

Labeling and Competition for Protease Activity with Activity-Based Probes

The ability of VEA-260 to compete for labeling of common cysteine proteases of *P. falciparum* was measured using two general activity-based probes as described previously (Arastu-Kapur et al., 2008; Greenbaum et al., 2002) and in more detail in the Supplemental Experimental Procedures.

SENP and DUB Fluorogenic Substrate Activity Assay

The ability of VEA-260 and JCP-666 to inhibit a panel of ubiquitin-like protein deconjugating proteases, including PfSENP1, hSENP1, hSENP2, hSENP6, hSENP8 (a deNEDDylase), UCHL3 (DUB), and IsoT (USP5; DUB), was tested using a previously described fluorogenic substrate assay (Drag et al., 2008a, 2008b). See Supplemental Experimental Procedures for additional details.

P. falciparum Replication Assay

Synchronous *P. falciparum* ring-stage parasites (~9 hr postinvasion; ~1% parasitemia; 0.5% hematocrit) were treated with VEA-260, JCP-666, JCP-668, -665, or -667 at (0–300 μM) for 75 hr at 37°C in a 96-well plate format. Cells were fixed with 0.05% glutaraldehyde (Sigma) in phosphate-buffered saline (PBS) overnight at 4°C, permeabilized with 0.25% Triton X-100 for 5 min at room temperature (25°C), and stained with a 1:100 dilution of a 5 mg/ml working solution of propidium iodide (Sigma) in 200 μl PBS. Parasite replication was monitored by observation of a propidium iodide-stained population using a BD FACScan flow cytometer (Becton Dickinson) located in the Stanford Shared FACS Facility at Stanford University. The data obtained were analyzed to quantify the number of infected and uninfected red blood cells using Flowjo 8.7.3. Parasitemia was determined as the ratio of infected cells to total cells. Parasitemia was normalized to DMSO for each run. The assay was repeated three to six times per drug per dose, and the EC_{50} was calculated by nonlinear regression using GraphPad Prizm software.

Susceptibility of the Different Life Cycle Stages to VEA260 Treatment

Synchronous parasites were treated for 12 hr with either DMSO or 100 μM of VEA260 at late schizont (3 hr before rupture), ring (9 hr postinfection [p.i.]), late ring (21 hr p.i.), or trophozoite (33 hr p.i.) stages. The morphology of the VEA260-treated parasites was compared to that of the controls Giemsa-stained thin blood smears.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Supplemental Compound Synthesis and Characterization, Supplemental Results, five figures, one table, two schemes and can be found with this article online at doi:10.1016/j.chembiol.2011.04.010.

ACKNOWLEDGMENTS

We would like to thank K. Wilkinson for providing the IsoT and UCH-L3 proteases and M. Verdoes and A. Guzetta for assistance with the compound characterization. E.L.P. was supported by the US National Science Foundation Graduate Research Fellowship 2005021299. This work was funded by the Burroughs Wellcome Trust New Investigators in Pathogenesis Award and by the NIH grants R21-AI088541 and R01-AI078947 (to M.B.) and R01-CA69381 (to G.S.).

Received: December 7, 2010
Revised: March 24, 2011
Accepted: April 4, 2011
Published: June 23, 2011

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