

Review

Protein Degradation Systems as Antimalarial Therapeutic Targets

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Artemisinin (ART)-based combination therapies are the most efficacious treatment of uncomplicated *Plasmodium falciparum* malaria. Alarming, *P. falciparum* strains have acquired resistance to ART across much of Southeast Asia. ART creates widespread protein and lipid damage inside intraerythrocytic parasites, necessitating macromolecule degradation. The proteasome is the main engine of *Plasmodium* protein degradation. Indeed, proteasome inhibition and ART have shown synergy in ART-resistant parasites. Moreover, ubiquitin modification is associated with altered parasite susceptibility to multiple antimalarials. Targeting the ubiquitin–proteasome system (UPS), therefore, is an attractive avenue to combat drug resistance. Here, we review recent advances leading to specific targeting of the *Plasmodium* proteasome. We also highlight the potential for targeting other nonproteasomal protein degradation systems as an additional strategy to disrupt protein homeostasis.

Combating Drug-Resistant Malaria: Upsetting the Proteostatic Balance

Malaria in humans is caused by five species of *Plasmodium* eukaryotic parasites (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*) and is transmitted by female *Anopheles* mosquitoes. Due to the range of the mosquito, half the world's population is at risk of contracting a malaria infection. In 2015, an estimated 212 million cases were reported worldwide, leading to ~430 000 deaths [1]. The WHO recommends artemisinin-based combination therapy (ACT) for first-line treatment of uncomplicated falciparum malaria. Alarming, resistance to ART has arisen in geographic pockets in multiple countries in Southeast Asia [2–5], and one case of a Chinese migrant worker was recently reported in equatorial Guinea [6]. ART-resistant parasites are defined as parasites that clinically display delayed parasite clearance times in patients treated with an ART derivative or ACT, or that, *in vitro*, can withstand short pulses of high ART concentrations (for recent reviews of artemisinin resistance see [7,8]).

To maintain the gains we have made against malaria, and to stem the spread of artemisinin resistance, we must discover novel targets and pathways that are not compromised by existing drug resistance. One such avenue is to target proteostatic pathways, as a dysregulation of these pathways leads to cellular death [9–18]. The major pathway for degradation in eukaryotes is undertaken by the 26S proteasome (for a comprehensive review of the proteasome see [19,20]). Other compartmentalized proteolytic complexes whose activity is regulated by AAA ATPase chaperones include the archaeal 20S proteasome, the prokaryotic proteasome HslV, and the ClpP proteases, all of which exhibit similar architecture in which the chaperone is docked at either end of the proteolytic core. These chaperones recognize substrates, remove degradation tags, unfold and thread the substrate into the proteolytic core in an ATP-dependent manner, and allosterically activate gate opening into the channel where substrates are

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Proteasome inhibitors are effective against *Plasmodium* spp., but past inhibitors had a low therapeutic index due to inhibition of the host enzyme complex.

Recent cryo-EM data have illuminated differences between the human and *P. falciparum* 20S proteasome core particles, allowing the generation of *P. falciparum*-specific proteasome inhibitors.

The recent characterization of *Plasmodium* proteins involved in the ubiquitin–proteasome system (UPS) has identified several enzymes, involved in attachment and removal of ubiquitin, which could be viable drug targets.

Inhibitors of the human proteasome, ubiquitin E1, E2, and E3 enzymes, as well as deubiquitinating enzymes (DUBs), have been FDA-approved or are in clinical trials, demonstrating the therapeutic potential of inhibitors against these enzymes.

Inhibitors to PfClp proteases are attractive since there is no human homolog to the mitochondrial-based PfClpY/Q, while PfClpC/P resides in the apicoplast, a cyanobacterial relic organelle that is not present in humans.

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proteolytically processed [21]. *Plasmodium* spp. are unique in that they possess the eukaryotic 26S proteasome in addition to a homolog of the prokaryotic proteasome HslV and a homolog of the cyanobacterial Clp protease. Maintaining multiple degradation systems points to the importance of this process. We discuss the potential of inhibiting degradation systems in *Plasmodium* parasites as a means to develop new antimalarial therapy agents that may overcome some of the current issues with resistance.

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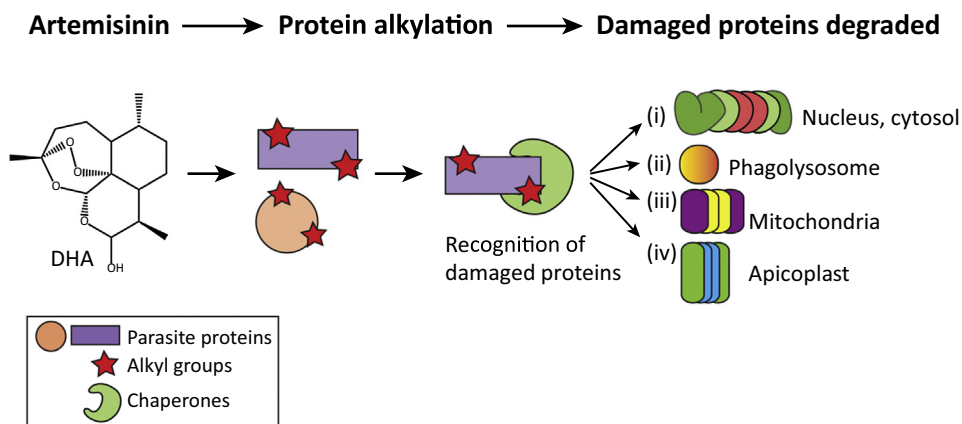
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Targeting the 26S Proteasome

The proteasome is a multisubunit protein-degradation complex that is necessary for protein turnover and cell differentiation in all eukaryotes. Several features render the *Plasmodium* proteasome an attractive therapeutic target. First, *Plasmodium* parasites require the proteasome to progress through their complex lifecycle, and a number of different classes of inhibitors have been shown to block growth or kill the parasite at all lifecycle stages, including the transmissible gametocyte stages [9–18]. Second, proteins damaged by indiscriminate protein alkylation caused by the antimalarial action of endoperoxides, such as ART, or the newer synthetic ozonides (OZ277, OZ439) that mimic artemisinin action [22–24], must be degraded to maintain homeostasis. The proteasome carries the largest burden of protein degradation, and has been the best characterized of the various degradative systems in *Plasmodium*. Third, ART-resistant *P. falciparum* isolates demonstrate an increased cell stress response and reliance on the proteasome in the basal, non-drug-treated state [25,26]. Presumably, constitutive reliance on chaperones and degradative capacity allows these parasites to withstand short pulses of ART. However, this cellular rewiring also exposes vulnerabilities. An increased reliance on the proteasome pathway can result in increased potency of proteasome inhibitors in ART-resistant parasites [18]. Lastly, ART resistance is mediated primarily by mutations in K13 (also known as Kelch13, PF3D7_1343700). Based on homology, K13 is suspected to be an adaptor protein for E3 ubiquitin ligases [27,28]. Comparisons with a human kelch-like protein lead to the speculation that downstream effects on autophagy might rely on K13 and proteasome-mediated degradation [29].

ART and synthetic ozonides alkylate proteins that are involved in a wide variety of cellular processes, including hemoglobin degradation, antioxidant responses, and response stress pathways [22–24]. Recent evidence suggests that ART-treated parasites accumulate ubiquitinated polypeptides [25], leading to an increased load of proteins that require degradation. Parasite survival in the presence of ART might depend upon the ability of the proteasome to dispose of these damaged proteins (Figure 1). Indeed, transcriptomics studies of untreated ART-resistant parasites from Cambodia reported upregulation of the catalytic proteasome β -subunits as well as components of the *Plasmodium* reactive oxidative stress complex (PROSC) and T-complex protein-1 Ring Complex (TRiC) chaperone complexes [26]. Proteasome inhibitors were also observed to be more potent against ART-resistant parasites, and they synergized with dihydroartemisinin (DHA), the active metabolite of ART, to mediate parasite killing [18,25]. Together, the data suggest that increasing cellular damage, while decreasing the ability of the proteasome to process that workload, can create a homeostatic imbalance that is lethal to *P. falciparum*. Thus, specific targeting of the proteasome has the potential to overcome *P. falciparum* resistance to ART and mechanistically related drugs.

Aside from the synergy displayed between proteasome inhibitors and ART derivatives in asexual blood-stage parasites, proteasome inhibitors possess the additional benefit of being active throughout the parasite lifecycle: liver stages, asexual blood stages, sexual stages that are transmissible to *Anopheles* mosquitoes, and the mosquito stages. Proteasome inhibitor-treated *Plasmodium berghei* sporozoites fail to establish a liver stage infection and cannot develop into exoerythrocytic forms in hepatocytes both *in vitro* and *in vivo* [9]. Moreover, asexual blood-stage parasite growth is potently inhibited when exposed to proteasome

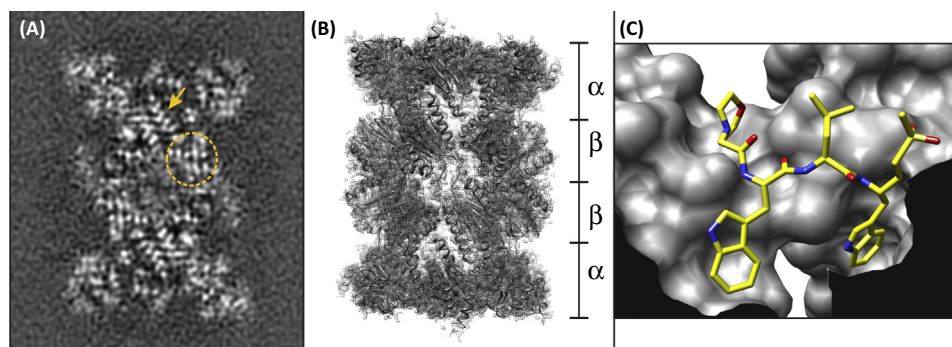


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Figure 1. Artemisinin, Its Derivatives, and the Synthetic Ozonides Cause Widespread Protein Damage That Induces Stress Response Pathways. Treatment with dihydroartemisinin (DHA), the active metabolite of artemisinin (ART), has been found to lead to nonspecific protein alkylation. Damaged proteins are recognized by chaperones, and targeted for degradation by one of four pathways: (i) the ubiquitin–proteasome system (UPS), (ii) autophagy, (iii) the PfClpY/Q protease, or (iv) the PfClpC/P protease. The subcellular locations of these pathways are denoted to the right of each proteolytic system.

inhibitors at the ring, trophozoite, or schizont stages [9–11,16,30]. Proteasome inhibition can also block parasite transmission by inhibiting stage V gametocyte development [13] and can prevent development in the mosquito by interfering with oocyst formation in the midgut [9]. These studies, performed with inhibitors that also target the human proteasome, demonstrate that the *Plasmodium* counterpart is indispensable for parasite survival.

The 26S proteasome is a large, barrel-shaped multisubunit protease complex composed of a 19S regulatory particle (RP) and a 20S core particle (CP). All subunits of the *P. falciparum* 26S proteasome have been identified [31], and are expressed throughout the lifecycle [32–34]. Recent characterization of the *P. falciparum* 26S proteasome and associated proteins recapitulate many of the lessons learned from yeast and mammals, with minor differences that can be exploited for therapeutic purposes [31]. The *P. falciparum* 20S CP exists freely, or is capped singly or doubly with a 19S RP [31], similar to that of other eukaryotes [35]. Subunits of the 19S RP recognize, unfold, and deubiquitinate degradation substrates. The 20S CP is responsible for proteolytic cleavage [19]. A recent cryo-EM structure of the *P. falciparum* 20S CP reveals four stacked heptameric rings, with two outer rings consisting of α_1 – α_7 , and the catalytic activity located in the two inner beta rings consisting of β_1 – β_7 [18] (Figure 2A,B). The catalytically-active β_1 , β_2 , and β_5 subunits use an N-terminal threonine as the nucleophile [36] and have peptidylglutamyl-peptide (caspase)-like, trypsin-like, and chymotrypsin-like activities, cleaving after the carboxy-terminal side of acidic residues, tryptic residues, and hydrophobic residues respectively. These active sites face into the channel of the barrel to allow degradation of a substrate only after it has been actively inserted into the CP complex. The N-terminal tails of the α subunits prevent polypeptide access to the inner β core catalytic subunits. Proteasomal subunits in the 19S RP regulate gate opening of the 20S CP [37]. Major structural differences between human and plasmodial proteasomes include an unusually open β_2 active site in *P. falciparum* proteasomes [18]. To identify specific differences in the substrate specificities of the human and parasite enzymes, Li *et al.* [18] performed an unbiased screen using diverse peptide substrates whose cleavage could be biochemically defined using mass spectrometry. This approach produced a map of substrate sequences that were accepted by the parasite but not by the human proteasome. While this information provided general specificity data for the full proteasome complex, it did not allow direct monitoring of specificities of each of the three



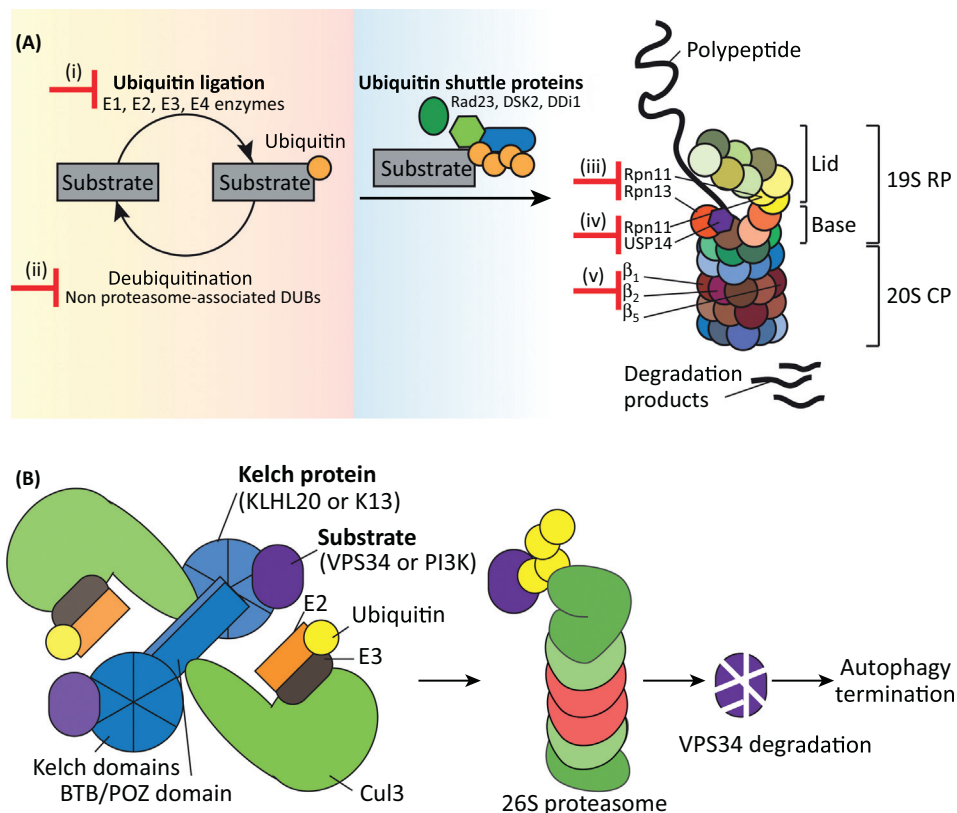
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Figure 2. Visualizing the *Plasmodium falciparum* 20S Core Particle. (A) Cryo-EM image of the *P. falciparum* 20S core particle (CP). Image shows regions where β -sheet structures (circle) and α -helices (arrow) can be visualized. (B) Fitted model of the 20S core particle based on the cryo-EM images, denoting the stacked α and β rings. (C) Ball-and-stick representation of the parasite-specific peptide vinyl sulfone inhibitor Mu-WLV-vs [18] bound in the β_2 active site of the 20S CP. The image shows the large S1 and S3 pockets that can accommodate the two tryptophan residues that do not fit in the human active site.

individual β -subunit activities and thus the exact specificity profiles for each subunit remain to be determined. However, prior results using active-site probes and subunit-selective inhibitors showed that parasites were most sensitive to inhibition of the β_5 subunit, and that effective killing by β_2 -specific inhibitors required inhibition of additional subunits [16]. Using the substrate specificity data, the authors generated peptide vinyl sulfone inhibitors containing the sequences preferred by the parasite enzyme. These inhibitors bound covalently to the β_2 alone or to both the β_2 and β_5 proteasome subunits (Figure 2C), resulting in potent killing of parasites (with IC_{50} values of 6 nM (Mu-WLL-vs) and 290 nM (Mu-WLV-vs) in 72 h assays beginning with *P. falciparum* ring stages). Furthermore, Mu-WLL-vs was able to clear *P. chaubaudi* from infected mice without evident toxicity to the host [18]. The identification of these potent *Plasmodium*-specific proteasome inhibitors validates the parasite proteasome as a viable drug target, and has identified important leads for ongoing antimalarial drug discovery and development efforts. Currently, there are a number of different efforts to identify potent and selective proteasome inhibitors from multiple different classes of inhibitor scaffolds. We anticipate that many more reports of *Plasmodium*-specific proteasome inhibitors will appear in the literature in the near future. Below, we discuss other attractive targets of the UPS, as well as other proteolytic systems in *Plasmodium* spp.

Targeting Enzymes Involved in the Ubiquitin Cascade

Considering that the vast majority of *P. falciparum* genes are tightly regulated so that transcripts are expressed according to the developmental lifecycle [38], post-translational modifications such as ubiquitination may provide a way for parasites to respond more dynamically to external stimuli such as temperature changes or antimalarial drugs. Indeed, the polyubiquitin gene was found to be upregulated in response to heat shock at 41°C [39], presumably to provide an abundance of ubiquitin molecules to tag damaged proteins for proteasome-mediated degradation. Thus, inhibiting ubiquitin conjugation or removal constitutes another potential strategy to kill parasites (Figure 3A). Ubiquitin is a 76-amino-acid protein that is highly conserved across prokaryotes and eukaryotes. The primary amino acid sequence of human and plasmodial ubiquitin differs by only one amino acid (E16 in *Homo sapiens*; D16 in *P. falciparum*) [39,40]. In *P. falciparum*, ubiquitin can be derived from two sources: a polyubiquitin gene that encodes five tandem repeats of ubiquitin (PfUpUb, PF3D7_1211800), or a gene fusion protein consisting of ubiquitin conjugated to the 60S ribosomal protein L40 (PF3D7_1365900) [39–41]. Both sources of ubiquitin are expressed in asexual blood stages, gametocytes, and sporozoites.



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Figure 3. The Ubiquitin-Proteasome System Facilitates Cellular Homeostasis. (A) There are multiple ways to target the ubiquitin-proteasome system. Inhibiting enzymes involved in (i) attachment or (ii) removal of ubiquitin will affect homeostasis of protein degradation. Targeting (iii) proteasomal regulation, (iv) proteasome-associated deubiquitinating enzymes, or (v) the catalytic subunits will impair the ability of the 26S proteasome to degrade proteins. (B) A hypothetical relationship between K13 and PI3K in regulating autophagy. Kelch-like proteins dimerize via their BTB/POZ domains and bind substrate via their Kelch domains. Theoretically, each Kelch-like protein dimer can bind two different substrates. Here, we show a model in which human KLHL20 binds Cul3 to mediate ubiquitination of the class III PI3K VPS34. VPS34 then undergoes proteasome-mediated degradation. This, and degradation of other proteins in the VPS34 complex, turns off autophagy. In a similar manner, K13 could mediate ubiquitination and proteasomal degradation of PI3K, leading to autophagy termination. Artemisinin-resistant parasites, harboring mutations in the propeller domain of K13, are unable to bind and target PI3K for proteasomal degradation. Prolonged autophagy may confer on these parasites an enhanced ability to dispose of damaged proteins upon artemisinin treatment. Abbreviations: CP, core particle (20S) of the 26S proteasome; DUBs, deubiquitinating enzymes; K13, Kelch 13; PI3K, phosphatidylinositol 3 kinase; RP, regulatory particle (19S) of the 26S proteasome.

Polyubiquitin expression peaks during the intraerythrocytic trophozoite and schizont stages [33,39,42], which are also the most susceptible to proteasome inhibitors [9,16]. These findings suggest that the highly coordinated UPS is essential for parasite multiplication.

Ubiquitin is attached in a controlled and step-wise manner. First, ubiquitin is proteolytically processed from its precursor form by ubiquitin-specific proteases (USPs) to reveal a diglycine motif. Then, in an ATP-dependent step, an E1 ubiquitin-activating enzyme forms a thioester bond between its active site cysteine and the C-terminus of ubiquitin. Ubiquitin is then transferred via a transthioesterification reaction to the active site cysteine of an E2 ubiquitin-conjugating enzyme. Finally, E3 ligases belonging to the Homologous to E6-associated protein C-terminus (HECT) family bind ubiquitin prior to transfer onto a substrate, while those in the Really Interesting New Gene (RING) and U-box families facilitate transfer of ubiquitin directly

from a E2 onto the substrate [43]. E4 ubiquitin chain elongation factor can further polyubiquitinate polypeptides [44]. Ubiquitin and its E1, E2, E3, and E4 enzymes are expressed throughout the asexual blood stages [32,38,41,45]. Molecular characterization of a set of E1, E2, and E3 enzymes revealed significant functional conservation in *Plasmodium*. The *P. falciparum* homologs of E1 UBA (PfUBA1, PF3D7_1225800), E2 UBC7 (PfUBC, PF3D7_1203900), and E3 RING ligase HRD1 (PfHRD1, PF3D7_1422500) were shown to have *in vitro* ubiquitinating activities. Furthermore, these proteins were found by immunofluorescence assays to reside in the expected subcellular localizations (PfUBA1 and PfUBC in the cytosol, PfHRD1 at the ER membrane), indicating that these enzymes likely function in endoplasmic reticulum-associated degradation (ERAD) [46]. Ubiquitin itself has seven lysine residues (K6, K11, K27, K29, K33, K48, and K63) that can be mono- or polyubiquitinated, either in single or multiple types of ubiquitin branches. In addition, ubiquitin can be attached to its first amino acid (M1), thereby forming linear chains [47]. Ubiquitin conjugation promotes a variety of cellular processes [43], including targeting proteins for proteasome-mediated degradation. Proteins destined for destruction are modified with K48-linked polyubiquitin chains, although the proteasome is also able to associate with and process all other non-K63-linked polyubiquitin chains, albeit less efficiently [48]. Proteomic analysis of *P. falciparum* parasites have revealed that K48-linkage is found in ~80% of ubiquitin conjugates [42]. Inhibiting ubiquitin conjugation will affect the homeostasis of the UPS and could negatively impact parasite growth. Inhibitors of human ubiquitin E1, E2, and E3 enzymes have been approved for hematological malignancies or are in clinical trials (ClinicalTrials.gov identifier: NCT02045095) [49,50], demonstrating the feasibility of targeting these enzymes.

Deubiquitinating enzymes (DUBs) are required for maturation of the C termini of ubiquitin precursors, ubiquitin chain editing, and removal of ubiquitin tags prior to polypeptide threading into the proteasome for protein degradation. Five classes are papain-type cysteine proteases: ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), otubains (OTUs), ataxin-3/Josephin ubiquitin proteases (MJDs), and the predicted family of permuted papain fold peptidases of dsRNA viruses and eukaryotes (PPPDE). A sixth class of Jab1/Mov34/Mpr1 Pad1 N-terminal (MPN+) (JAMM) isopeptidases is comprised of zinc-dependent metalloproteases [51]. Members of each class have been found in *Plasmodium* spp. [41]. Proteasome-associated DUBs and their regulators are potential drug targets. One such candidate is the DUB USP14, which removes polyubiquitin chains from degradation substrates [52] to recycle ubiquitin molecules, and regulates Rpn11-mediated unfolding of substrates targeted for proteasome-mediated degradation, thereby controlling the timing of protein degradation [53,54]. The *P. falciparum* homolog PfUSP14 (PF3D7_0527200) was shown to bind plasmodial proteasomes, and small-molecule (b-AP15) inhibition of this enzyme led to accumulation of proteasomal substrates and *P. falciparum* death [31]. Inhibitors of the human USP14 are currently in clinical trials to treat multiple myeloma [55,56]. Recently, multiple natural and synthetic compounds have been identified that inhibit the deubiquitinating activity of the 19S subunit Rpn11 [57–59].

In addition to targeting the catalytic activities of DUBs, targeting DUB regulators has been shown to be effective in inhibiting proteasomal activity. Inhibitors of human Rpn13 perturbed proteasome function without inhibiting 20S catalytic activity or 19S deubiquitinating activity [60]. Rpn13 is a proteasome subunit that resides in the base of the 19S RP, binds K48-linked diubiquitin polypeptides via a pleckstrin-like receptor for ubiquitin (Pru) domain [61], and activates the proteasome-associated DUB UCH37 [62–64]. This strategy could potentially work in *P. falciparum*. The Pru domain of PfRpn13 (PF3D7_1414000) expressed as a recombinant protein was unable to bind ubiquitin *in vitro* [31], but ubiquitin binding might depend upon conformational changes that were lacking without full protein expression. PfUCH54 (PF3D7_1117100), the *P. falciparum* homolog of UCH37, was found in a complex with the

P. falciparum 26S proteasome in three of four mass spectrometry runs in the presence of formaldehyde crosslinking [31], and was demonstrated to have *in vitro* deubiquitinating and deneddylating activities [65].

The Roles of Ubiquitin Ligases and Deubiquitinating Enzymes in *P. falciparum* Drug Resistance

Field-based studies have shown an association between delayed parasite clearance times in artesunate or ACT-treated patients and mutations in the propeller domain of *P. falciparum* K13 [3,6,27]. *In vitro* gene editing of several individual K13 mutations demonstrated that they mediate high levels of survival following exposure of early ring stages to short pulses of DHA [66,67]. K13 contains a BTB/POZ (Broad-Complex, Tramtrack and Bric a Brac/Poxvirus or Zinc-finger) domain as well as a six-bladed Kelch propeller domain [7,27]. BTB domains dimerize, and each can associate with Cullin3 substrate adaptors in the Cullin-Ring E3 ubiquitin ligase (CRL) complex, whereas Kelch domains associate with substrate to be ubiquitinated [28]. The function of K13 may include being an adaptor for an as-yet-unidentified E3 ubiquitin ligase.

K13 might potentially be involved in regulating autophagy by modulating levels of phosphatidylinositol (3)-phosphate (PI3P), which is essential for autophagosome formation [68,69]. *P. falciparum* phosphatidylinositol 3 kinase (PI3K, PF3D7_0515300), most homologous to the class III PI3K VPS34 (first identified in vesicle-mediated vacuolar protein sorting in yeast), phosphorylates phosphatidylinositol to generate PI3P. Recent evidence suggests that PI3K might form a complex with wild-type K13, but not with mutant K13 that confers ART resistance [70]. ART-resistant parasites had slightly increased levels of PI3K, leading the authors to speculate that PI3K escapes K13-mediated proteasomal degradation in ART-resistant lines. A molecular mechanism demonstrating how increased levels of PI3P could lead to ART resistance has not been reported. In human cells, a Cul3-KLHL20 ubiquitin ligase complex promotes ubiquitination and proteasome-mediated degradation of VPS34, leading to termination of autophagy [29]. K13 is homologous to KLHL20 in the propeller domains, with 29% identity. This level of identity is similar to that of KLHL12 (30%), KLHL2 (27%), and Keap1 (28%), three proteins reported to be homologous to K13 [27]. K13 could similarly associate with PI3K and promote its degradation. In ART-resistant parasites harboring mutations in the K13 propeller domains, K13 cannot bind and target PI3K for proteasome-mediated degradation. One possibility is that prolonged autophagy might help in removing proteins damaged by ART treatment, thereby allowing survival of short pulses of ART (Figure 3B). Details of autophagy in *Plasmodium* are still being elucidated. Using the ubiquitin-like protein ATG8 (PF3D7_1019900) as a marker, researchers have demonstrated punctate staining and partial colocalization with the apicoplast marker acyl carrier protein (ACP, PF3D7_0208500). In these studies, ART treatment decreased PfATG8 levels as evidenced by a decrease in intensity in immunofluorescence assays and Western blot, while the number of vesicles remained the same [71,72]. However, treatment with the lysosomal Na⁺/H⁺ pump inhibitor Bafilomycin A1, or the lysosomal cysteine protease inhibitor E64d, did not lead to accumulation of ATG8. Accumulation of ATG8 in the presence of these inhibitors would indicate efficient autophagic efflux [73]. Therefore, these drugs may have affected other cellular processes.

Variants of E3 ligases and DUBs have been associated with parasite susceptibility to a range of antimalarial drugs, presumably via altered ubiquitin modifications of particular substrates. Multiple studies have shown an association between mutations in the DUB ubiquitin carboxy-terminal hydrolase 1 (UBP1) and reduced parasite susceptibility to a number of antimalarials. Resistance selection and genetic mapping experiments followed by whole-genome sequencing of the rodent parasite *P. chabaudi* identified two distinct mutations (V2697F and V2728F) in PcUBP1 (PCHAS_0207200) that were associated with ART resistance, as

measured by the speed of parasite recrudescence after ART treatment. The UBP1 V2728F mutation appears to contribute to a multidrug resistance phenotype: this mutation was also identified in *P. chabaudi* parasites subjected to chloroquine or mefloquine drug pressure (but were not exposed to ART pressure) [74,75]. The contribution of UBP1 mutations to reduced susceptibility to ART derivatives was also seen in *ex vivo* studies. Culture-adapted *P. falciparum* Kenyan isolates subjected to whole-genome sequencing and determination of half-maximal inhibitory concentrations (IC₅₀) of standard antimalarial drugs associated the K873R mutation in PfUBP1 (PF3D7_0104300) with reduced DHA susceptibility [76]. In yeast, Ubp1 plays a role in the endocytic pathway [77], consistent with the known role of ubiquitin in the internalization step of endocytosis at the plasma membrane [78]. Variants of UBP1 might interfere with endocytosis of host products such as hemoglobin, thus allowing reduced susceptibilities to drugs that interact with host heme moieties.

Mutations in other components of the endocytosis pathway have also been proposed to modulate parasite susceptibility to ART. Whole-genome sequencing of ART-pressured *P. chabaudi* parasites referred to above [74] identified a single mutation: I568T in the μ -chain of the AP2 adaptor complex (PCHAS_143590) [79]. In humans, the AP2 complex is involved in clathrin-mediated endocytosis [80]. Homology modeling studies predict that the I568T mutation alters binding affinity of the PcAP2 μ -chain to cargo protein undergoing endocytosis [79]. Analysis of parasite isolates from ACT-treated Kenyan children suggested an increased prevalence of S160N/T mutations in PfAP2- μ (PF3D7_1218300) or a E1528D mutation in PfUBP1 in parasites surviving ACT treatment [81]. Expression of an extra copy of *pfap2-mu* harboring a S160N mutation increased *P. falciparum in vitro* sensitivity to chloroquine and quinine [82]. A separate study associated the HECT E3 ligase *P. falciparum* ubiquitin transferase (PfUT) (PF3D7_0704600) variant harboring a Y1387F mutation, in the presence of the K76T mutation in the *P. falciparum* chloroquine resistance transporter (PfCRT), with quinine resistance [83]. The molecular mechanisms underlying how mutations in PfUT could contribute to quinine resistance remain to be identified. Since ART can be activated by Fe²⁺-heme released by hemoglobin digestion, one possibility is that a decrease in hemoglobin endocytosis caused by mutations in UBP1 or AP2- μ might allow parasites to escape ART action due to a lower amount of activated drug. Alternatively, a decrease in endocytosis might be a general mechanism of survival by reducing the uptake of antimalarial drugs. These data suggest that inhibition of endocytic trafficking might be one strategy to allow parasites to escape drug-mediated cell death.

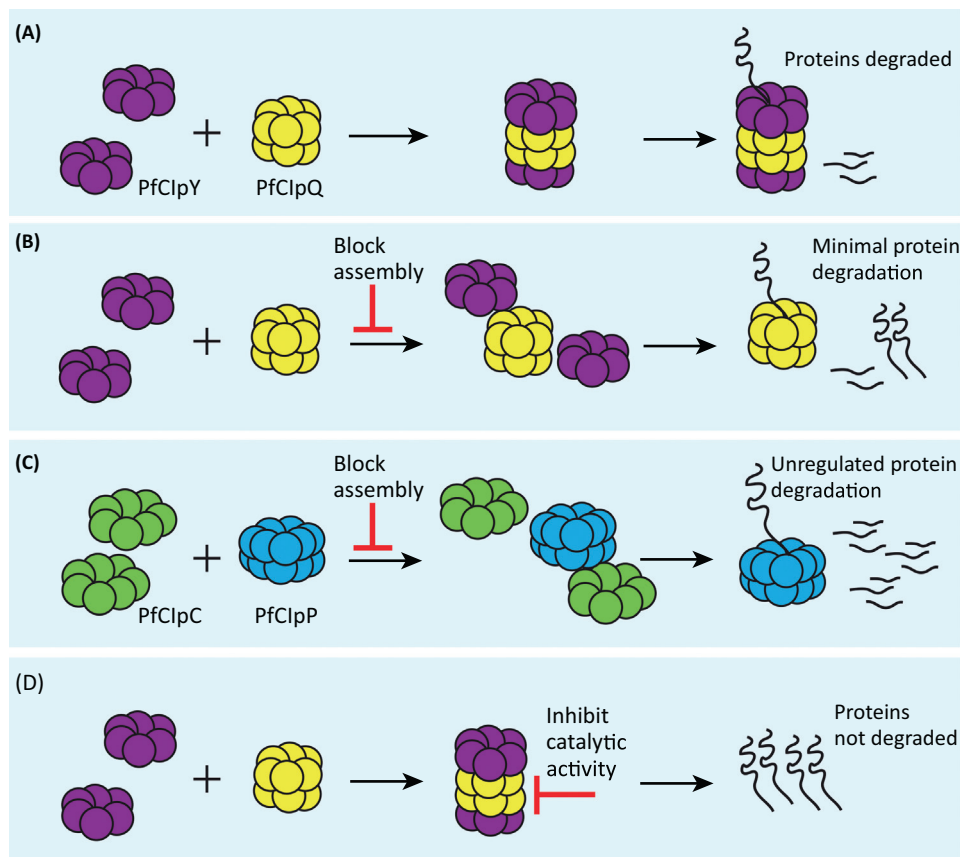
Targeting PfClpY/Q, the *Plasmodium* Homolog of the ClpY/ClpQ Prokaryotic Proteasome

In addition to the eukaryotic proteasome, *Plasmodium* homologs of the catalytic subunit of the prokaryotic proteasome caseinolytic protease Q (ClpQ), also known as heat shock locus V (HslV), and its regulatory chaperone ClpY/HslU, have been identified in *P. falciparum* (PfClpQ, PF3D7_1230400, and PfClpY, PF3D7_0907400) [84,85]. PfClpY and PfClpQ proteins are expressed at the trophozoite, schizont, and merozoite stages [84–86]. Bioinformatics analysis predicted mitochondrial localization of PfClpQ, which was confirmed by immunofluorescence assays and immunoelectron microscopy of an enhanced yellow fluorescent protein (EYFP)-tagged PfClpQ [87]. Thus, this protease may be responsible for degrading proteins specifically in the mitochondria. By homology to HslV, PfClpQ is predicted to consist of two stacked homohexameric rings [88]. *In vitro* fluorometric assays of recombinant PfClpQ demonstrated that this enzyme displays threonine protease-like, caspase-like and chymotrypsin-like activities, which were inhibited by lactacystin, chymostatin, and MG132, respectively [86]. The caspase-like and chymotrypsin-like activities are reminiscent of the proteolytic activity seen in the β_1 and β_5 subunits of the 26S proteasome respectively. Minimal PfClpQ activity is greatly enhanced upon activation by the AAA-ATPase chaperone PfClpY, which forms a complex with

PfClpQ via its C-terminal domain [85,89]. Like the 19S RP of the 26S proteasome, PfClpY recognizes, unfolds, and translocates substrates into PfClpQ. A peptide-based inhibitor that disrupts PfClpY/Q complex formation caused a loss of mitochondria membrane potential, activation of caspase-like cysteine proteases, and DNA fragmentation characteristic of apoptosis, leading to inhibition of parasite growth and an inability to form mature schizonts [85]. The PfClpY/Q protease resembles the 26S proteasome structurally and functionally. It would be interesting to test whether the recently identified *Plasmodium*-specific 20S proteasome inhibitors can also inhibit the PfClpY/Q system. Indeed, the absence of a homolog in humans makes this a particularly attractive target (Figure 4).

Targeting PfClpC/P, the *Plasmodium* Homolog of the Cyanobacterial ClpP

P. falciparum homologs to the cyanobacterial ClpP (PfClpP, PF3D7_0307400) and its associated chaperone ClpC (PfClpC, PF3D7_1406600) have been identified and characterized [90,91]. PfClpC/P proteins are expressed during the late trophozoite and early schizont stages of the asexual blood stage cycle [90,91]. The PfClpC/P complex is structurally and functionally



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Figure 4. Inhibition of PfClpY/Q and PfClpC/P in the Mitochondria and Apicoplast, Respectively. (A) Assembly of the chaperone (PfClpY or PfClpC) and the protease (PfClpQ or PfClpP) allow regulated proteolytic cleavage of degradation substrates. Shown here is a representation for the PfClpY/Q complex. (B) Inhibition of PfClpY assembly with a short peptide prevents complex formation. Unassembled PfClpQ has shown minimal degradation activities. Through an unknown mechanism, incubation with the short inhibitory peptide causes loss of mitochondrial membrane potential and eventual parasite death. (C) An antibiotic that prevented closure of the *Staphylococcus aureus* ClpP proteolytic chamber led to nonspecific protein degradation and bacterial death. Similar strategies can be envisioned for *Plasmodium*. (D) Inhibiting the catalytic sites in PfClpQ (depicted here) or PfClpP will prevent substrate cleavage, leading to dysregulation of protein homeostasis.

analogous to the 26S proteasome and the ClpY/ClpQ systems. Complementing the protein degradation machinery in the cytosol and mitochondria, PfClpC/P functions in the apicoplast [91]. This organelle is responsible for biosynthesis of fatty acid metabolism, heme, and isoprenoids, and is essential for asexual blood stages [92,93]. Bioinformatics identified apicoplast-targeting sequences in PfClpP and PfClpC [90,91]. Immunofluorescence assays examining a streptavidin-3xhemagglutinin-tagged PfClpC [91] and a construct expressing the amino-terminus of PfClpP harboring the apicoplast-targeting sequences fused to GFP [90] confirmed colocalization with the apicoplast marker acyl carrier protein (ACP). Immunoelectron microscopy further confirmed that the PfClpP–GFP fusion construct resided in the apicoplast [90]. Size-exclusion chromatography, analytical ultracentrifugation, and electron microscopy revealed that amino-terminally truncated PfClpP primarily formed homoheptameric rings. Under the conditions studied, only a small percentage of PfClpP existed as an oligomeric complex consisting of two stacked heptamers [90,91].

PfClpP is thought to complex with and be activated by PfClpC via a ClpP binding loop [91]. Presumably, the protease subunits are docked at either end of the cylinder by the AAA ATPase PfClpC chaperone that recognizes, unfolds, and threads substrates into the proteolytic core. The amino-truncated PfClpP demonstrated chymotrypsin-like serine protease activity as measured by Suc-LLVY-AMC. Accordingly, PfClpP protease activity was inhibited by serine protease inhibitors [chymostatin and phenylmethylsulfonyl fluoride (PMSF)], but not cysteine protease inhibitors (E-64 and leupeptin), or an at the P1 position that are distinct from those of *Escherichia coli* and *Homo sapiens* [94]. These preferences could be exploited for drug discovery purposes. The β -lactone compound U1 bound PfClpP and affected apicoplast growth and segregation. U1 treatment also resulted in a delayed death phenotype [90], which is defined as parasites dying in the second generation following drug exposure and which is typically associated with inhibition of apicoplast protein translation [93,95–97]. Antibacterial research has identified an antibiotic (acyldepsipeptide, ADEP4) that mediates overactivation of *Staphylococcus aureus* ClpP. In these antibiotic-treated cultures, ADEP4 bound ClpP and kept the proteolytic chamber open. This prevented the chaperone from regulating substrate access to the ClpP protease, thus allowing the protease to nonspecifically degrade proteins. Dysregulation of this proteolytic system resulted in the death of bacteria that were actively growing or were in the stationary phase [98,99] (Figure 4C). Similar methods of proteolytic perturbations could be explored as a novel antimalarial approach in targeting nonreplicating forms of *Plasmodium*.

Concluding Remarks

The recent identification of *Plasmodium*-specific inhibitors of the catalytic subunits of the 26S proteasome provides a promising new avenue for antimalarial drug discovery. Small-molecule inhibitors of the proteasome have demonstrated that this catalytic multi-protease subunit is essential for *Plasmodium* parasite viability. Identification of multiple candidate drug targets within this system could lead to antimalarial combination therapies that are not compromised by existing mechanisms of multidrug resistance. Activators and regulatory subunits of the proteasome also constitute attractive drug targets, in addition to the proteasome catalytic subunits. Enzymes involved in the attachment and removal of ubiquitin have been shown to be viable targets in humans, and should be explored in *Plasmodium*. Additionally, the PfClp proteases remain attractive targets, especially since the mitochondria and apicoplast organelles are vital to *Plasmodium* survival and cell stress responses. The necessity of these degradation pathways and the abundance of targets within the pathways suggest that this is a good general strategy for the development of novel antimalarial drugs (see Outstanding Questions).

Outstanding Questions

Will it be possible to develop inhibitors against substrate ubiquitination and deubiquitination to block specific key events during the parasite lifecycle?

Are other nonproteasome targets in the UPS viable drug targets in *P. falciparum*?

Will parasites treated with proteasome inhibitors develop resistance?

Will parasite-specific proteasome inhibitors work equally well against parasites resistant to multiple drugs?

Can combining proteasome inhibitors with ART treatment suppress ART resistance?

Will it be possible to generate safe and orally available malaria proteasome inhibitors?

Is it possible to develop inhibitors against PfClp proteases based on inhibitors active against other organisms?

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