Published in final edited form as:

Transl Res. 2018 August ; 198: 40–47. doi:10.1016/j.trsl.2018.04.007.

The proteasome as a target to combat malaria: Hits and Misses

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Abstract

The proteasome plays a vital role throughout the life cycle as Plasmodium parasites quickly adapt to a new host and undergo a series of morphologic changes during asexual replication and sexual differentiation. Plasmodium carries three different types of protease complexes: typical eukaryotic proteasome (26S) that resides in the cytoplasm and the nucleus, a prokaryotic proteasome homolog ClpQ that resides in the mitochondria, and a caseinolytic protease complex ClpP that resides in the apicoplast. In silico prediction in conjunction with immunoprecipitation analysis of ubiquitin conjugates have suggested that over half of the Plasmodium falciparum proteome during asexual reproduction are potential targets for ubiquitination. The marked potency of multiple classes of proteasome inhibitors against all stages of the life cycle, synergy with the current frontline antimalarial, artemisinin, and recent advances identifying differences between Plasmodium and human proteasomes strongly support further drug development efforts.

Proteasomes in *Plasmodium* biology

Plasmodium life cycle

Plasmodium parasites that cause malaria undergo a series of developmental transformations as they cycle between vertebrate and mosquito hosts. The vertebrate infection begins when an infected female *Anopheles* mosquito deposits parasites called sporozoites in the skin and vasculature during a blood meal (1). From the blood stream sporozoites enter liver cells and replicate to form schizonts containing thousands of merozoites. When the liver cell ruptures the merozoites are released and invade red blood cells (RBC) where they grow from a ring stage parasite to a trophozoite before again replicating to produce a schizont with 8-32 new merozoites that are released and invade RBCs. This cycle leads to clinical symptoms and continues until treated or suppressed by the immune response. A subpopulation of schizonts produce merozoites that are committed to sexual differentiation after RBC invasion generating a single male or female gametocyte, that when taken up in a blood meal by a female anopheles mosquito, undergo gametogenesis into microgametes (male) and macrogametes (female). Fertilization takes place in the mosquito midgut beginning a developmental cascade that leads to the production of tens of thousands of sporozoites that

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Disclosures: All authors have read the journal's policy on authorship agreement and disclosures of potential conflicts of interest and have none to declare. The authors have read the journal's authorship agreement and the manuscript has been reviewed and approved by all named authors. The manuscript was written and edited only by the named authors.

concentrate in the salivary glands ready to be transmitted to new vertebrate hosts during a blood meal.

Proteasome in erythrocytic asexual stages

The proteasome plays a vital role throughout the life cycle as the parasite quickly adapts to a new host and undergoes a series of morphologic changes during asexual replication and sexual differentiation. *Plasmodium* carries three different types of protease complexes: typical eukaryotic proteasome (26S) that resides in the cytoplasm and the nucleus, a prokaryotic proteasome homolog ClpQ that resides in the mitochondria, and a caseinolytic protease complex ClpP that resides in the apicoplast (2–5). In silico prediction and immunoprecipitate analysis of ubiquitin conjugates have suggested that during asexual reproduction over half of the *Plasmodium falciparum* proteome are potential targets for ubiquitination (6). This high turnover is consistent with the observation that *P. falciparum* protein sequences are rich in lysine, an anchor point for polyubiquitin chain. *P. falciparum* is responsible for the most virulent human malaria and is the only *Plasmodium* for which a continuous in vitro culture system has been developed facilitating drug discovery efforts. It will be the primary focus of the rest of the review.

Aminake et al. have reported the proteasome expression profile in blood-stage parasites, which shows that the α - and β - subunits are expressed in all blood stages and located in the nucleus and cytoplasm of trophozoites, schizonts, and gametocytes (7). Reports further show that the quantity of immuno-precipitated ubiquitin increases dramatically during the transition from the ring stage to the schizont stage (6). An elegant study by Witola and Mamoun showed that choline, a precursor for phospholipids, mediates proteasome-mediated degradation of phosphoethanolamine methyltransferase (PfPMT), which provides an alternate path to produce phosphatidylcholine (8). While the importance of PfPMT for parasite growth, multiplication, and viability was established by the same authors in a subsequent study (9), the physiological importance of proteasome-mediated PfPMT degradation was more difficult to establish directly, but is thought to be a result of negative feedback regulation mediated by the accumulation of choline from the host. Together these studies suggest a central role for the proteasome during parasite replication and the production of merozoites. The importance of the proteasome is also strongly supported by the data showing effective killing of these erythrocytic stages by a range of proteasome inhibitors, which will be the subject of this review.

Proteasome in erythrocytic sexual and mosquito stages

The proteasome is also expressed continuously as the intraerythrocytic parasite undergoes sexual differentiation to form mature male and female gametocytes that are required for malaria transmission via a mosquito (7,10). The biological relevance of the proteasome in these stages was demonstrated by showing that nanomolar levels of the specific proteasome inhibitor, epoxomicin, effectively killed all gametocytes, even mature stage-Vs which are resistant to all approved antimalarials, except the 8-aminoquinolines (11). Interestingly, thiostrepton, an anti-malarial compound that targets the proteasome as well as the large ribosomal subunit at the apicoplast, has a 15-fold higher selectivity for male gametocytes (7,12). The Ubiquitin/Proteasome System (UPS), which is required for cellular homeostasis

during shifts in temperature in many cell types, was thought to play a role as parasite is transmitted from vertebrate host to mosquito and vice versa (7). However, transcriptomic analysis during initial phase of transmission from the human to the mosquito show down-regulation of proteasome subunits, suggesting that the proteasome-mediated degradation may actually be reduced during fertilization and early zygotic development (13). Whether this difference persists through further development into infectious sporozoites within the mosquito has been difficult to study due to the technical challenges associated with in vivo mosquito experiments, but would be interesting to evaluate.

Proteasome in sporozoites

The proteasome requirement for sporozoites to transition from the mosquito to the vertebrate liver was the first stage evaluated using proteasome inhibitors (14). In 1998 Gantt et al. demonstrated that the transformation of sporozoites into liver stage in culture and in mice decreases over 10-fold when treated with the proteasome-inhibitor lactacystin at low concentration. Up-regulation of UPS components during sporozoite transformation was shown in a subsequent study (15). A report by Jayabalasingham et al. further shows that the metamorphosis of sporozoites into trophozoites in the liver is associated with loss of organelles deemed unnecessary for replication by a process termed “organelle clearance” (16). It is hypothesized that as in mammals, the UPS in *Plasmodium* could allow selective degradation of outlived organelles (17). These studies suggest major contributions from the proteasome in sporozoite remodeling.

Proteasome inhibitors as drug candidates

Proteasome inhibitors as antimalarials

Given the essential role of the proteasome throughout the *Plasmodium* life cycle in the human host, a number of inhibitors have been tested as potential drug candidates and will be reviewed in detail below. However, the original inhibitors tested had been identified using mammalian systems and in anti-cancer drug screens and therefore lacked specificity for the *Plasmodium* proteasome, leading to toxicity concerns. Coupled with this, many of the early proteasome inhibitors also inhibited other cellular proteases increasing the potential for toxicity and limiting direct application as antimalarials (18,19). Nevertheless, proteasomes remain attractive as anti-infective agents. See Bibo-Verdugo et. al. for a detailed review focused on targeting proteasomes to combat disease caused by a variety of infectious organisms (20).

The recent emergence of artemisinin (ART)-resistance in Southeast Asia (21–24), and the determination of the structure of the *P. falciparum* proteasome using cryo EM (25) has reinvigorated efforts to develop *P. falciparum* specific protease inhibitors. The demonstration by Mok et al. that ART-resistance is associated with up-regulation of genes involved in unfolded protein response (UPR) pathways, including UPS components, provided further impetus to identify safe and effective proteasome inhibitors as antimalarials (26). The UPR pathways are thought to be important for elimination of proteins damaged by ART, and this has led to the idea that proteasome inhibitors may reduce resistance and prolong the usefulness of ART (26). The involvement of UPS in ART-resistance is also supported by

finding that the mutations found to be the primary molecular markers of ART resistance localize to Kelch13 (PF3D7_1343700), which is thought to be a ubiquitin ligase that is involved in labeling proteins for degradation (27,28). This hypothesis has been tested by Dogovski et al., and the data shows that proteasome inhibitors strongly synergize with ART activity in killing both sensitive and resistant parasites (29). For a recent review related to combating drug resistance by targeting the *Plasmodium* UPS, please see Ng et. al. (30).

Structure and function based inhibitor design

The earliest attempt to understand the structural interaction of the parasite proteasome and an inhibitor involved homology modeling of the yeast proteasome in complex with an inhibitory compound (31). This homology modeling based virtual ligand interaction showed that the catalytic domain was extremely well-conserved except for one residue in the β -5 subunit (Y168G) that differed between human and plasmodium proteasome sequence. Another modeling study of the proteasome inhibitor bortezomib bound to *P. falciparum* 20S proteasome predicted low binding affinity when compared with eukaryotic 20S proteasome (32). These studies have further established the need to characterize the parasite proteasome at high resolution to directly compare the ligand binding preferences of human and parasite proteasome.

The Bogoy and Craik laboratories collaborated to identify the substrate preference of purified human and *Plasmodium* proteasomes for a diverse set of 228 synthetic peptides (25). Monitoring the degradation of synthetic peptides using liquid chromatography-tandem mass spectrometry generated a “frequency profile” showing a preference for cleavage at specific sites. The frequency profile showed that the parasite proteasome prefers to cleave 113 unique sites, with a strong preference for aromatic residues at P1 (first residue on the N-terminal side of the scissile bond) and P3 (third residue from the scissile bond on the N-terminal side) positions of a peptide. The authors then used Z-L3-vs [a benzyloxycarbonyl group (Z) coupled to a peptide containing leucines at positions P1, P2 and P3 (L3) and a vinyl sulfone side chain (vs)] as a scaffold to synthesize novel synthetic compound variants with tryptophan (W) at P1 and/or P3 positions to replace Leucine (L). The compounds WLL-vs and WLW-vs exhibit potent inhibition of the parasite proteasome, with high selectivity for the β -2 subunit. High-resolution cryo-EM and single-particle analysis revealed that the major difference between WLL-vs and WLW-vs was their ability to inhibit β -2 and β -5 subunits or just the β -2 subunit, respectively. WLL-vs displayed potent parasite killing, blocking erythrocytic development at low nanomolar concentrations. Specificity and potency of WLL-vs was remarkable, with a 675-fold selectivity for parasites over human fibroblasts in just 1 hr treatment. They further showed that a single administration of WLL-vs at 80 mg/kg decreased *P. chabaudi* parasitemia in mice by >95% without any sign of toxicity. They further established the high effectiveness of WLL-vs in killing ART-resistant parasites and synergy with DHA, suggesting potential use in artemisinin-based combination therapy (ACT). These findings have significantly enhanced the understanding of the unique characteristics of parasite proteasome’s active site architecture, highlighting the importance of structure-activity relationship for selective targeting and allowing directed compound optimization.

Candidates for future optimization

The new structural data for the *P. falciparum* proteasome has led to renewed efforts to design novel inhibitors as well as take a fresh look at the 6 well-established classes of proteasome inhibitors: β -lactones, α',β' -Epoxyketones, peptide aldehydes, dipeptidyl boronic acids, vinyl sulfones and cyclic peptides. Compounds representing all six proteasome inhibitor classes have been tested against *Plasmodium* and will be discussed in turn followed by a brief review of the use of related compounds in other parasitic infections.

β -lactones

Lactacystin was the first natural proteasome inhibitor identified and also one of the earliest inhibitors to be tested for its anti-*Plasmodium* activity (14,33). Lactacystin binds to all catalytic β -subunits irreversibly and inhibits trypsin-like, chymotrypsin-like, and peptidyl glutamyl peptide hydrolyzing activities (33). Lactacystin is effective against both pre-erythrocytic and erythrocytic stages in vitro and in vivo using a rodent malaria model (14,34). Lactacystin treatment of sporozoites in vitro appears to block conversion of major rRNA pool from C-type (sporozoites) to A-type (EEF stage) in HepG2 cells, a requirement for successful propagation in the mammalian host. Lactacystin also inhibits erythrocytic stages, blocking the trophozoite to schizont transformation with defects found in DNA synthesis initiation. Treatment of schizonts resulted in defects that prevent them from rupturing, suggesting effects on control of cell cycle progression or, possibly, inhibition of proteases required for egress. However, the inhibitory effects of lactacystin are variable, and the lack of specificity results in toxicity at doses required to achieve parasite clearance, precluding any use in clinical therapeutics.

In 2003 a novel β -lactone Salinosporamide A (Marizomib) was discovered from marine actinomycete *Salinispora tropica* (35). It possesses significantly higher potency and has been found to have an IC_{50} of 11.4 nM against the erythrocytic stages of *P. falciparum* (31). The study by Prudhomme et al. shows that the compound blocks progression of all the erythrocytic stages, including blocking schizont rupture, with a concomitant increase in ubiquitinated proteins in parasite extracts after treatment. In vivo treatment of infected mice showed that a dose of 130 μ g/kg of Salinosporamide A decreased *P. yoelii* parasitemia significantly. At these levels toxicity is a major issue, necessitating further optimization studies to develop synthetic derivatives with specificity for the parasite.

α',β' -Epoxyketones

These peptide derivatives are generally very potent and highly specific inhibitors that bind the proteasome irreversibly and inhibit the chymotrypsin activity of the β -5 subunit. The first naturally occurring epoxyketones: eponemycin and epoxomicin, were discovered in early 1990s from screens targeting melanoma cells (36–38). Since their discovery, this actinomycete-derived family has expanded to include many peptides with different modifications and lengths.

Effective killing of erythrocytic stages by epoxomicin at low concentrations was first reported by Kreidenweiss et al. (34). Subsequent study by Czesny et al. established the gametocytocidal activity of epoxomicin at concentrations as low as 0.1 μ M (11). In a recent

study, Dogovski et al. further show that the anti-plasmodial activity of epoxomicin was independent of Kelch13 genotype in several field isolates, suggesting potential use in ACTs (29). However, 1 μ M epoxomicin treatment results in a 20% reduction in viability of A549 and NIH 3T3 cells, suggesting that toxicity is still an issue and establishing a need to develop analogs that preferentially bind the *Plasmodium* proteasome.

Parallel studies involving synthetic analogs of epoxomicin have made some progress. One such analog, YU101, was shown to inhibit erythrocytic stages at nanomolar range, and was used as a parent lead compound by Proteolix (now Onyx) in a screen that yielded carfilzomib (PR171, Kyprolis) (39). Carfilzomib went on to become the second FDA approved proteasome inhibitor for the treatment of multiple myeloma in 2012. While carfilzomib showed no toxicity at 1.5 mg/kg in the *P. berghei* mouse model, it had only weak activity against asexual stages (40). Importantly, carfilzomib worked synergistically when used along with dihydroartemisinin (DHA) even at a lower dose (29). Li et al. screened a library of 670 carfilzomib-analogs (curated at Onyx pharmaceuticals) in a *P. falciparum* replication assay (40). The screen yielded a parasite-selective compound PR3, which kills both asexual stages and gametocytes at nanomolar concentrations. The exciting data from this report was the lack of toxicity to human foreskin fibroblast cells treated with PR3 at its solubility limit (50 μ M), due to its inability to bind and block the activity of the human proteasome β -2 subunit. As a result, PR3 administration moderately delayed the onset of *P. berghei* parasitemia in infected mice with no toxicity. More recently, a screen of 20 synthetic analogs of carmaphycin B, a natural tripeptide containing an α,β' -epoxyketone group at its C-terminus yielded analog-18 with potent antimalarial efficacy against asexual stages as well as gametocytes. Analog-18 had low IC₅₀ against asexual stages (~3nM), and its therapeutic window was 100-fold wider when compared with that of carmaphycin B, demonstrating that subtle structural differences between the parasite and human proteasome can be exploited to reduce toxicity and increase selectivity of proteasome inhibitors (41). These studies indicate that the epoxyketone derivatives have high potential in the search for compounds with high selectivity and potency.

Peptido sulfonyl fluorides (PSF)

Sulfonyl fluorides were first synthesized and evaluated in 1995 as potent irreversible inhibitors of cysteine proteinases and calpain, and their activity against the proteasome was reported in 1997 (42–44). Tschan et al. showed that PSF targets the plasmodium proteasome by binding and inactivating the β -1 and β -5 subunits (45). They further showed that the lead PSF compound PW28 blocks development of asexual stages irrespective of the time of treatment initiation, and that gametocyte development was arrested at all stages, without affecting commitment. Most compounds tested in this study also showed no in vitro toxicity at 500 μ M. However, *P. berghei* infected mice showed signs of toxicity when treated with 10 mg/kg of PW28 despite showing significant decrease in parasitemia. The recent synthesis of WLL-vs based on the parasite-specific peptide cleavage analysis described above in **Structure and function based inhibitor design** clearly demonstrates the potential to improve the therapeutic window of PSF compounds.

Aldehydes and boronates

The ability of peptide aldehydes to block proteasome activity was first reported in the early 1990s (46,47). In a subsequent study, Goldberg's group reported potent proteasome inhibition by peptide aldehyde derivatives MG115 and MG132 which bind (reversibly) and inhibit the chymotrypsin activity in the β -5 subunit of the proteasome (48,49). MG132 inhibitory activity extends to calpains and serine/cysteine proteases to a lesser extent (50). Most peptide aldehydes are synthetic, with a few exceptions, such as the tyropeptin A, produced by *Kitasatospora sp.* (51).

MG132 shows anti-plasmodium activity at low nanomolar concentrations, with complete block of ring and trophozoite development and ~90% block of late trophozoite development (34,52). However, enlargement of food vacuoles also occurs during treatment regardless of treatment time point suggesting the compound is also inhibiting the cysteine proteases, falcipain 2a & 2b (52). The inhibitory effect of MG132 extends to many field isolates, suggesting possible incorporation in ACT regimens. MG132 could serve as a dual-target inhibitor of malaria parasites. However, the lack of selectivity remains a concern.

Another member of the boronic acid class, bortezomib (Velcade, PS-341), reversibly binds and blocks the β -5 subunit of the human proteasome (53). It was the first proteasome inhibitor approved by the FDA and went on to become the first therapeutic proteasome inhibitor for treatment of multiple myeloma in 2003 (54). However, MLN-273, an analog of bortezomib with a longer half-life was the first dipeptidyl boronic acid derivative to be tested against *Plasmodium* (55). The authors show that MLN-273 (100 nM) blocks *P. falciparum* erythrocytic development at the ring stage in multiple field isolates. In addition, MLN-273 (100 nM) also reduced *P. berghei* exo-erythrocytic progression to schizonts in vitro, with nuclear condensation and loss of multinucleation occurring at very high doses (1000 nM). While 100 nM MLN-273 showed minimal effects on uninfected RBCs and HepG2 cells, 1000 nM MLN-273 induced apoptosis to HepG2 cells.

Later, Reynolds et al. tested the anti-parasite effects of bortezomib and its analog ZL3B [analog of Z-L3-vs with boronate side chain (B) replacing the vinyl sulfone side chain], showing inhibition of intraerythrocytic development (56). They reported that the block occurred prior to DNA synthesis without any effect on parasite egress. The IC₅₀ of bortezomib and ZL3B were shown to be in the range of 30-40 nM. There is a divergence of views with regards to differences in the sensitivity of drug-sensitive and -resistant parasites to bortezomib (34,56). Nevertheless, Dogovski et al. showed a strong synergism between DHA and bortezomib during co-treatment of *P. falciparum* (29). Despite the progress made, toxicity of boronic acid derivatives are yet to be thoroughly evaluated. The advent of new boronic acid derivatives, some of which are in clinical trials (ixazomib) make this class of proteasome inhibitors good candidates in the search for potent and selective anti-malarial compounds (20,57,58).

Cyclic peptides

Cyclic peptides are a relatively new class of natural proteasome inhibitors from the fungus *Apiospora montagnei* (59). A cyclic peptide, TMC-95A, is the only known proteasome

inhibitor that inhibits all three β subunits via noncovalent bonding. Non-covalent inhibitors hold an advantage in the treatment of malaria because of their higher availability due to lack of permanent sequestration in the red blood cell proteasome. Using a 1600 non-covalent proteasome inhibitor library, Li et al. discovered an analog of TMC-95A called cyclic peptide-1 with >1000-fold specificity of *Plasmodium* proteasome over human proteasome in vitro (60). Like WLL-vs, the selectivity of cyclic peptide-1 was due to co-inhibition of the β -2 and β -5 subunits. They further showed that a pulse-treatment (1h) with cyclic peptide-1 blocked progression of the erythrocytic cycle of *P. falciparum* independent of the stage of the parasite. A subsequent study by Wilson et al. shows that a macrocycle added to peptide aldehydes significantly increases selectivity for the proteasome over intracellular cysteine proteases, suggesting the possibility of further improvements to potency and selectivity to cyclic peptide compounds (61).

Proteasome inhibition in other parasites

Proteasome-targeting has also been shown to be a good strategy to combat other parasitic diseases, and studies have yielded further insights into parasite-specific compounds as described below. As with *Plasmodium* the challenge is to identify new potent specific compounds with reduced toxicity and good pharmacokinetics. The β -lactone, Lactacystin appears to have wide-ranging effect (62). Lactacystin has been shown to block the growth of *Entamoeba histolytica* and *invadens* as well as *Leishmania Mexicana* (63,64). It also blocks transformation of *Trypanosoma cruzi* from trypomastigotes into amastigotes by inhibiting cell cycle progression (65), and with *Toxoplasma gondii*, lactacystin blocks DNA replication (66). MG-132, a peptide aldehyde derivative, causes significant cell death in *Leishmania donovani* promastigotes (67,68), and the α' , β' -Epoxyketone epoxomicin was shown to inhibit in vitro growth of *Babesia sp.* at nanomolar concentrations (69). A novel compound, GNF5343 (an azabenzoxazole) was identified as a proteasome inhibitor in a screen involving *L. donovani* and *T. cruzi* and *brucei* (70). GNF6702, an optimized analog of GNF5343, was shown to inhibit the β -5 subunit in an unusual manner: the compound binds β -4 subunit adjacent to the subunit interface of β 4 and β 5. More importantly, GNF6702 does not inhibit the human proteasome (70). Treatment with GNF6702 reduces parasite burden in blood, heart, colon and brain in a mouse model of Chagas disease caused by *T. cruzi*. Despite the universality of proteasomes for parasite survival, structural studies aimed at identifying and exploiting differences between human and parasitic proteasomes are lacking. Techniques developed for *Plasmodium* may be effectively transferred to additional protozoan parasites to facilitate the design and optimization of species specific inhibitors.

Future scope

It is evident that the proteasome, the central component of the ubiquitin-proteasome system, is vital to the survival of *Plasmodium sp.* throughout the life cycle, making it an attractive drug target. The studies highlighted in this review also show that sufficient structural differences exist between the *Plasmodium* and human proteasomes to support specific targeting. Promising compounds (WLL-vs and carmaphycin B-analog 18) have been identified and serve as scaffolds for continued development. Continuing to augmenting high throughput screens of novel synthetic and natural compounds with careful structural analysis and focused medicinal chemistry should yield additional candidates. Recent successes

identifying unique structural features and substrate preferences of the *Mycobacterium tuberculosis* proteasome demonstrate the utility of these approaches (71–73). Additionally, K777, a vinyl sulfone that inhibits cysteine proteases required for the survival of *T. cruzi* (cruzain), also progressed to safety pharmacology studies but unfortunately failed to achieve low dose tolerability requirements in primates. GNF6702, the selective inhibitor of the kinetoplastid proteasome, is currently undergoing preclinical toxicity studies and could provide additional support for proteasome inhibition as a viable strategy to treat parasitic diseases (70). Expanding the focus to proteasome-like complexes containing ClpP, ClpQ or ClpR proteases is another promising area (3,74,75). The disruption of mitochondrial ClpQY protease complex leads to loss of mitochondrial membrane potential, mitochondrial dysfunction and apoptosis-like cell death in *Plasmodium* (3,74). Similarly, inhibitors aimed at the ClpP protease complex hamper schizont development and apicoplast segregation, resulting in cell death (75). Further work aimed at compounds specifically targeting these unique proteasome-like complexes, as well as other UBC components remain attractive, especially with increasing concerns about artemisinin resistance. The ability of sub-therapeutic concentrations of proteasome inhibitors to synergize with artemisinin suggests they have potential to augment therapy at drug levels that would have limited toxicity during a typical three day ACT treatment regimen. Oral availability is another important requirement for an anti-malarial. The recent development of two orally available second generation proteasome inhibitors (ex. ixazomib, oprozomib) could serve as a resource for further optimization studies. Designing compounds that are taken up or activated specifically by *Plasmodium*-infected RBC is another strategy exploited by chloroquine and artemisinin, respectively to enhance efficacy. Despite the challenges associated with an essential, but conserved drug target like the ubiquitin proteasome system significant progress has been made, providing optimism for the development of potent parasite-specific drugs.

Acknowledgments

Funding: This work was supported by Public Health Service grant AI114761 (KW) from the National Institute of Allergy and Infectious Diseases.

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