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Artemisinin action and resistance in *Plasmodium falciparum*

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Abstract

The worldwide use of artemisinin-based combination therapies (ACTs) has contributed in recent years to a substantial reduction in deaths resulting from *Plasmodium falciparum* malaria. Resistance to artemisinins, however, has emerged in Southeast Asia. Clinically, resistance is defined as a slower rate of **parasite clearance** in patients treated with an **artemisinin derivative** or an ACT. These slow clearance rates associate with enhanced survival rates of ring-stage parasites briefly exposed *in vitro* to dihydroartemisinin. We describe recent progress made in defining the molecular basis of artemisinin resistance, which has identified a primary role for the *P. falciparum* K13 protein. Using K13 mutations as molecular markers, epidemiological studies are now tracking the emergence and spread of artemisinin resistance. Mechanistic studies suggest potential ways to overcome resistance.

Keywords

artemisinin resistance; Kelch 13; ubiquitination; proteasome; unfolded protein response; malaria

Artemisinins: Front-line Antimalarials under Threat

In 2015 the malaria parasite *Plasmodium falciparum* killed over 400 000 people, most of whom were children under the age of five [1]. Thus it is of acute concern that resistance to the artemisinin derivatives (**ARTs**, see **Glossary**), the first-line drug class used to treat malaria, emerged several years ago, and is now evident in six countries in South East Asia. If resistance spreads to India and Africa, a major health crisis is feared. The World Health Organization has warned: "There is a limited window of opportunity to avert a regional public health disaster, which could have severe global consequences." This review discusses

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recent insights into how ARTs kill malaria parasites, and how *P. falciparum* achieves resistance. We also examine potential ways to improve treatment outcomes in regions where resistance is established and to slow the spread of resistance.

Artemisinin (ART, also known as Qinghaosu; Figure 1) is a sesquiterpene lactone produced by the Chinese medicinal herb *Artemisia annua*. Activation of this drug's **endoperoxide bridge** is essential for antimalarial activity [2]. Semisynthetic **lactol derivatives** such as **dihydroartemisinin** (DHA), artesunate, and artemether (Figure 1) exhibit improved bioavailability and efficacy [3]. DHA is the active *in vivo* metabolite of all clinically used ARTs [4]. ARTs are exceptionally fast acting against intra-erythrocytic asexual blood-stage malaria parasites, effecting up to 10 000-fold reductions in parasite burden every 48 hr. This pharmacodynamic hallmark is of critical benefit in treating severe malaria and reversing its otherwise lethal course [5, 6]. An inherent disadvantage of ARTs is their very short *in vivo* half-lives (typically ~1 hr in humans [5, 7]). As a result, ARTs are co-administered with longer half-life partner drugs, such as lumefantrine, amodiaquine, piperaquine, mefloquine, sulphadoxine-pyrimethamine or pyronaridine in **ART-based combination therapies** (**ACTs**). These combinations help prevent recrudescence (which can occur even after five days of ART **monotherapy**), and are employed to slow the development of parasite resistance (Box 1) [8, 9]. An important goal in the malaria chemotherapy field is to develop compounds that benefit from longer plasma half-lives yet achieve the same rapid parasite killing as ART derivatives through a similar mode of action. These objectives appear to have been achieved with the ozonides (or 1,2,4-trioxalanes), a class of fully synthetic endoperoxide antimalarials (Figure 1). Two members of this class, namely OZ277 and OZ439, have been evaluated in humans [10–14]. The combination of OZ277 (arterolane) and piperaquine, known as Synriam™, is available in India. The usefulness of OZ277 may be limited however, as its half-life is only 2– to 3–fold longer than that of DHA [15], and it is reported to have lower plasma exposure in malaria patients than in uninfected volunteers [16]. OZ439 (artefenomel) is currently being assessed either alone or as a **combination therapy** in Phase II human clinical trials ([12, 17, 18]; ClinicalTrials.gov NCT02083380). Ongoing studies are also investigating whether OZ439 (whose terminal half-life is 46–62 hr, [17, 19]) can overcome resistance to ARTs (see below), and provide an effective drug for new antimalarial combinations [12, 14].

ARTs are Activated to Cytotoxic Species *In Situ*

ART activation is thought to involve iron-catalyzed reductive scission of the endoperoxide bond, generating carbon-centered radicals that react with susceptible groups in parasite proteins and other biomolecules [20, 21] (Figure 2). The major source of iron in parasites, which develop inside a parasitophorous vacuole inside infected erythrocytes, is thought to be in the form of heme species that are liberated as a result of plasmodial protease-mediated degradation of imported host hemoglobin. Most of this proteolysis occurs during the **trophozoite stage** when parasites endocytose large quantities of hemoglobin from the erythrocyte into an acidic digestive vacuole [22–24]. Although most of this pool of highly reactive heme is sequestered via incorporation of Fe^{3+}-heme dimers (known as β-hematin) into chemically inert hemozoin crystals [25], some heme is available in the reduced Fe^{2+} form to activate ARTs [2, 25, 26]; see below). ARTs are less active against mid-ring stage

parasites than against trophozoites [27], and are inactive against mature (stage V) gametocytes [28] and liver stages [29], consistent with decreased or absent hemoglobin digestion at these stages. However ARTs are active against very early asexual **ring-stage parasites**, just after erythrocyte invasion [27, 30]. This finding suggests that early rings can already import and digest host hemoglobin, even before formation of the digestive vacuole where most of the hemoglobin is degraded during the trophozoite stage. In support of this idea, ART action can be substantially mitigated in very early rings as well as trophozoites by inhibiting falcipains, which proteolytically cleave host hemoglobin and release heme-iron [30]. Recent evidence suggests that biosynthetic heme produced by the parasite might also contribute to ART activation in very early rings [31–33].

Another secondary activator of ARTs might be reduced (Fe^{2+}) iron that is not heme-bound. *P. falciparum* maintains a low steady-state labile iron pool [34]. Additional iron may be released via hydrogen peroxide-mediated degradation of heme in the digestive vacuole [35] or by reaction with reduced glutathione in the parasite cytoplasm [36]. Iron chelators weakly antagonize ART activity [30, 37, 38]. However, this antagonism is much less potent than that observed with hemoglobinase inhibitors, providing further evidence that heme-iron rather than free iron is the main activator. At present the heme-iron hypothesis seems more compelling than earlier reports suggesting alternative mechanisms for activation, such as cofactors involved in maintaining redox homeostasis [39, 40]. Nonetheless, it remains possible that factors in addition to heme-iron or other iron sources might also contribute to ART activation.

ART-Mediated Killing is Stage- and Exposure Time-Dependent

The level of potency of short pulses of ARTs against *P. falciparum* asexual intra-erythrocytic parasites *in vitro* is notably impacted by the stage of parasite development [27, 41]. Interestingly, this stage-specificity appears to reflect altered temporal responses to ARTs rather than differences in intrinsic sensitivity. When exposed to short (physiologically relevant) pulses of ARTs, mid-ring stage 3D7 parasites are less susceptible to ARTs than do trophozoites, yet the former are rendered non-viable if ART exposure is prolonged. The complex dose response can be explained by invoking the concept of an effective dose of drug to which the parasite is exposed. This concept assumes that cytotoxicity occurs when parasites are exposed to activated drug for a sufficient period of time. Therefore, the extent of killing would therefore be determined by the rate of production of the ART activator (*e.g.* the rate of production of heme-iron resulting from hemoglobin proteolysis), as well as the duration of the drug pulse (which depends on drug degradation rates) and stage- and strain-dependent differences in the parasite's ability to defend itself (Figure 2). These parameters have been incorporated into a cumulative effective dose mathematical model [27] that predicts parasite responses to ARTs using K_m values (*i.e.*, the drug concentration resulting in half the maximum effective dose) and $\widehat{t_{50}}$ values (*i.e.*, the time required to render 50% of the parasites non-viable). Of note, the similarity between the half-lives of different ARTs and the exposure times needed to induce killing implies that even small stage- or strain-dependent differences in the K_m or $\widehat{t_{50}}$ values can yield large differences in drug efficacy [27]. Based on this model, the enhanced *in vivo* half-lives of synthetic ozonide

endoperoxides would be predicted to increase the effective dose and thus clear parasites more effectively. Pharmacokinetic/pharmacodynamic data from human clinical trials will provide valuable data to assess this prediction.

What are the Targets of Activated ARTs?

The parasite cytoplasm enables reduction of the Fe^{3+} form of heme (released from hemoglobin and oxidized in the acidic digestive vacuole) to Fe^{2+} heme, which appears to be the primary activator of ARTs [2]. Once activated, ART radicals will react rapidly with proteins that have accessible nucleophiles (*e.g.* in enzyme active sites), as well as with unsaturated membrane lipids and heme itself [42–44]. The mitochondrion [40], the endoplasmic reticulum (ER) [37, 45, 46], and the digestive vacuole [46–49] have all been suggested as sites of early damage, but convincing identification of specific vital targets is still lacking.

Two recent studies have investigated the mode of action of ARTs by identifying parasite proteins that were covalently modified by ARTs. These experiments used clickable alkyne or azide derivatives of ART as bait, followed by biotinylation and pull-down of tagged proteins [32, 50]. A broad spectrum of proteins (~70 to 125) was identified across many functional categories and cellular locations. There was a significant overlap in the proteins identified between these two studies (~25 proteins), but it is also noteworthy that there was a large degree of overlap with proteins that are reproducibly detected as the most abundant representatives in non-targeted proteomic analyses of trophozoite-stage parasites [51–53]. These abundant (housekeeping) proteins might include specific targets, one of which might be the membrane-bound glutathione-S-transferase Exp-1 [54], but it is also possible that death occurs following generalized damage to multiple functional pathways. It would be interesting to determine the labeling profiles of tagged ARTs in parasites with different levels of ART sensitivity and to determine whether overexpression (in transfected parasite lines) of one or more of the putative target proteins results in a decrease in ART sensitivity.

Studies with a fluorescently labeled ART derivative found evidence of probe accumulation in neutral lipid bodies [49]. Furthermore, ART treatment was shown to perturb parasite membrane components [55] and trigger oxidative damage in both lipid [49] and soluble [56] compartments. Similarly, the general level of protein ubiquitination increases following ART treatment [41] indicative of widespread protein damage. Taken together the available data support the idea that parasite death occurs when damage accumulates to a level that overwhelms the parasite's protein repair system, exacerbated by inactivation of multiple proteins with important housekeeping functions. This damage might also extend to lipids.

Recently the *P. falciparum* phosphatidylinositol-3-kinase (PfPI3K) has been proposed as a direct target of ARTs [57]. PfPI3K phosphorylates phosphatidylinositol (PI) to produce phosphatidylinositol 3-phosphate (PI3P) in ring stage parasites [58]. These authors showed that DHA quickly and reversibly alters the subcellular distribution of a PI3P-binding FYVE-domain reporter, consistent with DHA inhibiting the production of PI3P. Treatment of *P. falciparum* with a known PfPI3K inhibitor, wortmannin, inhibits delivery of hemoglobin to the digestive vacuole [58], providing evidence that this kinase plays a role in regulating

hemoglobin endocytosis. ART treatment produces a similar inhibitory effect on endocytosis [27, 59]. The reported data implicate PI3P in the mode of action of ART and suggest a role for PfPI3K in the ring stage of infection, although there are likely to be other critical targets (including heme, lipids and an array of proteins, see above), particularly in the trophozoite stage. A recent review provides a more detailed discussion of possible roles for PI3P/PI3K in ART resistance [60]. Interestingly, a recent study with ART-pressured, resistant rodent parasites also implicates an important role for altered hemoglobin endocytosis in ART resistance [61].

In addition to protein damage, it is also important to note that ART-alkylated reactive heme species might constitute a major cause of parasite death [2]. Interestingly, substantially lower levels of heme-ART adducts were observed in an ART-resistant *P. yoelii* rodent parasite line that had been selected by continuous ART pressure for more than five years in recipient mice [62]. Removal of drug pressure led this line to lose its resistance phenotype and heme-ART adducts were once again observed to similar degrees as with the parental drug-sensitive line. These data reinforce the importance of heme in ART action and the possibility that alterations in heme production represent one route towards reduced parasite susceptibility.

ART Resistance is Conferred Primarily by Mutations in the K13-Propeller Protein

Decreased sensitivity to ARTs, manifesting clinically as slower rates of parasite **clearance**, is now documented in multiple Southeast Asian countries [63, 64]. *In vitro* studies show that parasites isolated from patients with either fast- or slow-clearing infections exhibit similar sensitivity to DHA in standard growth inhibition assays (*i.e.*, where drug pressure is maintained throughout one or more generations of intra-erythrocytic parasite development) [65, 66]. By contrast, the response of parasites in a **ring-stage survival assay** ($RSA_{0–3hr}$) generally correlates well with parasite clearance times [67, 68]. The $RSA_{0–3hr}$ assay exposes parasites to ARTs for a 6 hr pulses starting at 0–3 hr post-invasion and then monitors viability in the next cycle. This mimics the short half-lives of ARTs and targets the stage (very early rings) that shows reduced sensitivity in resistant strains [67]. Additional methods to monitor parasite clearance are discussed in Box 2.

Initial investigations into the genetic basis of ART resistance included genome-wide studies performed using parasites isolated from regions with cases of delayed parasite clearance [69–71]. These studies identified regions on chromosome 13 that were associated with increased parasite clearance half-lives and that displayed genetic evidence of recent positive selection. One remarkable observation was the discovery of several distinct but apparently sympatric (geographically co-existing) parasite sub-populations that displayed exceptionally high levels of genetic differentiation, circulating in the drug resistance epicenter of western Cambodia. Three of these sub-populations (KH2–4) were associated with increased clearance times and displayed genetically distinct clonal structures with high levels of haplotype homozygosity, indicative of founder effects and recent sub-population expansions [71].

The breakthrough in identifying the primary genetic determinant came from laboratory-based selection studies in which the ART-sensitive Tanzanian F32 isolate was pressured in a dose-escalating, 125-cycle regimen of exposure to ART over five years [72]. $RSA_{0–3hr}$ studies with the resulting F32-ART5 line showed increased parasite survival when exposed to DHA. Whole-genome sequence analysis of both F32-ART5 and F32-TEM ("témoin", or control), its sibling clone cultured without ART, revealed a mutation (M476I) in the propeller domain of the ***K13*** (*Kelch13,* PF3D7_1343700) gene [73]. Subsequent analysis of parasite isolates revealed the presence of several mutations in the K13 propeller domain in isolates from western Cambodia, which associated with delayed parasite clearance in patients [73]. Definitive evidence that asexual blood-stage parasites harboring mutant *K13* alleles are less susceptible to ART was provided in genome editing studies with the CRISPR/Cas9 system and with zinc-finger nucleases [74, 75]. Levels of parasite susceptibility to DHA were quantified using the $RSA_{0–3hr}$. The CRISPR/Cas9 experiments reported a gain of *in vitro* resistance upon introduction of the prevalent C580Y mutation into the *K13* gene of the reference parasite 3D7 (of likely African origin) [74]. Using zinc-finger nucleases, several K13 mutations were introduced or removed from recently derived clinical Cambodian isolates or older reference laboratory strains. Studies with isogenic lines generated in the reference strain Dd2 (Indochina) revealed a spectrum of resistance levels, with R539T and I543T conferring greater resistance than the more widespread C580Y mutation [75]. These data suggest that the prevalence of individual mutations might also be influenced by other factors, such as parasite fitness, or ability to enter a dormant state, or transmissibility to *Anopheles* mosquitoes. K13 mutations conferred *in vitro* resistance in all strains tested, with the highest levels observed in recent Cambodian isolates, evoking an important contribution of the parasite genetic background (see below for additional discussion of potential secondary loci).

The discovery of K13 mutations and their implication in conferring ART resistance led quickly to the identification of a substantial number of polymorphisms in parasites from malaria patients in different regions. In a comprehensive analysis conducted by the Tracking Resistance to Artemisinin Collaboration (**TRAC**), multiple mutations were identified throughout the entire *K13* gene, of which many in the β-propeller domain (beginning at amino acid 442) were associated with parasite clearance half-lives of greater than 5 hr [63]. Surveillance studies conducted in 59 countries by the K13 Artemisinin Resistance Multicenter Assessment (KARMA) consortium recently identified 108 non-synonymous K13 mutations, with an overall prevalence of 37% in South East Asia. These mutations showed strong regional differences. For example, in Cambodia-Vietnam-Laos the dominant mutation was C580Y (at ~50% prevalence), whereas in the samples from Thailand-Myanmar-China the dominant mutation was F446I (at 20% prevalence), with minimal C580Y [76]. Similar findings were reported in other studies [77–79]. These data are consistent with multiple *de novo* mutational events accompanied by gene flow between parasite populations. Among the rare mutations observed in Africa, A578S was found to be the most common, but gene-editing experiments showed that it does not mediate a resistance phenotype *in vitro* [76]. Independent genome sequence analysis of over 3,000 Asian and African isolates has also recently documented a large array of K13 mutations in Asia and Africa [80]. Importantly, Asian parasites showed a substantial excess of non-synonymous

mutations, consistent with strong selective pressure, in contrast with Africa where both non-synonymous and synonymous mutations were at similar levels [80]. Importantly, several studies in Africa have reported equivalent parasite clearance rates in patients harboring wild-type or mutant *K13* alleles [81–84]. These data suggest that in Africa K13 mutations are not under significant selection pressure and are not adversely impacting ACT efficacy [85]. One possibility is that the combined effects of high levels of immunity in African populations, relatively reduced drug pressure and the frequent occurrence of polyclonal infections (that select against resistance phenotypes that have reduced growth rates), are sufficient to prevent the relatively mild levels of K13-mediated resistance from altering clinical efficacy and beginning to spread under selective sweeps. This situation might be predicted to change if the partner drugs used in Africa (primarily lumefantrine and to a lesser extent amodiaquine) start to succumb to resistance.

An Enhanced Cell Stress Response is a Feature of ART-Resistant Parasites

*S*tudies with *in vitro* cultured parasites have begun to shed light on the molecular mechanism underlying K13-mediated ART resistance. Assays with very early ring stage parasites (average age 1.2 hr post-invasion with a 1 hr synchronization window) found that the 50% lethal dose of DHA, when given as a 3 hr pulse, was ~70–fold higher in K13 mutant compared to wild-type parasites [41]. This effect was most pronounced in these very early ring stages, although decreased sensitivity could be observed during about one-third of the cycle, *i.e.* from just after invasion to ~12 hr post-invasion and in the last 4 hr of the **schizont stage**. Modeling predicted that this resistance arose from an increase in the $\widehat{t_{50}}$ value (*i.e.*, the time required to render 50% of the parasites non-viable) rather than a change in the intrinsic sensitivity to killing (*i.e.*, the K_m value), evoking the suggestion that ART-resistant parasites exhibit an enhanced cell stress response [41].

The involvement of a cell stress response in the parasite's defense against ARTs is further indicated by the observation that parasites that survive a short pulse exposure to a sub-lethal concentration of ART exhibit a delay in their progress through the intraerythrocytic cycle [27, 41, 72]. This observation of quiescence and growth retardation is reminiscent of the cytostatic stress response observed in other organisms, in which stress events activate an unfolded protein response leading to shut-down of protein translation and other metabolic pathways [86, 87]. Consistent with this type of mechanism, *P. falciparum* can undergo eIF2α-mediated arrest of protein translation, leading to stalled parasite growth [88]. Separately, evidence of parasite entry into dormancy has been observed in parasites exposed to ART pressure in vitro [89, 90]. Further mechanistic studies are required to better delineate the contributions of quiescence and dormancy in ART-resistant and sensitive parasites and how this is impacted by the gain of mutation in K13.

Interestingly, a large-scale transcriptional profiling analysis of *P. falciparum* isolates collected from patients during the TRAC project recently found that ART resistance was associated with the upregulation of genes encoding proteins involved in the unfolded protein response [91]. In particular, increases in the levels of expression of two major chaperone complexes - the ER-located PROSC (*Plasmodium* Reactive Oxidative Stress Complex) and the cytoplasmic TRiC (T-complex protein-1 (TCP1) Ring Complex) - were strongly

correlated with a longer parasite clearance half-life [91]. Further support for a role for protein repair mechanisms in ART resistance came from the finding of higher ubiquitination levels in DHA-treated K13 wild-type parasites compared to K13 mutants [41]. Moreover, inhibitors of the proteasome - a proteinase complex that plays a critical role in degrading unfolded proteins - strongly synergize the action of ARTs against both sensitive and resistant strains of *P. falciparum in vitro* [41, 92]. These findings suggest that resistance arises from an enhanced cytoprotective capacity (Figure 2). Of note, altered protein ubiquitination has also been implicated in the mechanism of ART resistance in a rodent malaria model [93].

K13 may Function as a Ubiquitin E3 Ligase Substrate Adaptor

Multiple efforts are underway (including subcellular localization, proteomics, metabolomics and transcriptomics) to determine the molecular function of K13. This 726 amino acid protein comprises three domains (Figure 3): *i)* a conserved *Plasmodium*-specific N-terminal domain; *ii)* a putative BTB/ POZ domain (Broad complex_Tramtrack_Bric-a-brac / Pox virus_Zinc finger); and *iii)* a C-terminal domain containing six Kelch motifs. The Kelch domain is predicted to form a six-bladed β-propeller. The Kelch motif is a repeat element that folds into a four-stranded antiparallel β-sheet or "blade". These blades are arranged radially around a central axis, forming the β-propeller, with the upper and lower surfaces available for protein-protein interactions [94]. The structure of the BTB/POZ and β-propeller domains of K13 has been solved and the coordinates made available through the Structural Genomics Consortium, Toronto (PDB ID: 4YY8). The crystallized protein is present as a homodimer. The publication of a manuscript describing the structural features is eagerly anticipated.

The superfamily of Kelch repeat proteins includes a subclass of KLHL (Kelch-like) proteins with an N-terminal dimerization domain and a C-terminal propeller domain. The KLHL proteins are widely distributed throughout eukaryotes, and have diversified into many family members, with humans encoding 42 members [95]. The Kelch domain sequence is often preceded by sequence encoding BTB/POZ and BACK (BTB-And-C-terminal-Kelch) domains. The BTB/POZ motif mediates protein binding and homo-dimerization [96, 97], and can be involved in transcriptional repression [98]. BTB/POZ/BACK domains can be involved in binding to cullin 3, the largest family of E3 ubiquitin ligases [99], with the downstream Kelch domain providing a substrate adaptor [100]. That is, these Kelch-like proteins partner with an E3 ligase to bind and orient specific substrates ready for polyubiquitination by an E2 ubiquitin-conjugating enzyme, which in turn leads to their degradation by the ubiquitin-proteasome system (Figure 4).

The Kelch domain of K13 shares some (25–30%) sequence identity with equivalent domains in human Kelch-like proteins such as KLHL8 (Kelch-like protein 8 isoform 3; NP_001278936.1) and Keap1 (Kelch-like ECH (erythroid cell-derived protein with CNC homology)-associated protein 1 or KLHL19; NP_987096.1). The BTB/POZ domain in K13, however, shares little sequence similarity with other Kelch proteins and K13 lacks the adjacent BACK domain, which contains the cullin 3 binding site in other KLHL gene family proteins [95, 100]. Despite this, particular interest has focused on Keap1 [73, 101]. In animals, Keap1 is a negative regulator of nuclear erythroid 2-related factor 2 (Nrf2), a

transcription factor that regulates the cellular response to oxidative stress [102]. Under unstressed conditions, Nrf2 is a substrate for the Cul3 ubiquitin E3 ligase/ Keap1 complex. That is, under unstressed conditions, Nrf2 is constantly ubiquitinated and rapidly degraded in proteasomes. Upon exposure to electrophilic and oxidative stresses, reactive cysteine residues of Keap1 become modified, leading to decreased presentation of Nrf2 for ubiquitination. Nrf2 accumulates and relocates to the nucleus where it binds (as a heterodimer with the Maf transcription factor) to the antioxidant response elements present in the promoters of genes involved in responding to oxidative stress. Nrf2 is a member of the cap 'n' collar (CNC) family of transcription factors. Other CNC homologues are also found in invertebrates, where they interact with Keap1 to fulfill comparable signaling responses to oxidative stress [103].

It has been suggested that K13 may perform a Keap1-like function in *P. falciparum* [73]. Mutations in K13 might impair its interactions with a transcription regulator (substrate), resulting in an enhanced anti-oxidant/cytoprotective response (Figure 4). While this is an attractive hypothesis, no clear Nrf2 or other CNC ortholog has been identified outside the metazoan lineage [104], let alone in in the *Plasmodium* genome [73]. Several fungal proteins have recently been reported to share small regions of similarity to Nrf2 [104], but the conservation of the Keap1-Nrf2 pathway is likely restricted to animals. While *Plasmodium* shares several chromatin remodeling factors and general transcription factors with animals, there is a paucity of specific DNA-binding transcription factors (and their matching target genes) that are conserved between Apicomplexa and the animal/fungal lineage [105]. While an analogous scenario to the Keap1/Nrf2 mechanism, whereby a transcription factor regulated by K13-interactions regulates proteins involved in proteostasis is plausible, further work is needed to experimentally confirm or refute a Keap1-like role for K13.

Another role that has been proposed for K13 is that of a regulator of poly-ubiquitination (and thus degradation) of PfPI3K. Evidence of PfPI3K binding to wild-type K13 was provided by immunoprecipitation – an interaction that was reduced in parasites harboring a mutant C580Y K13 [57]. The consequent increase in PfPI3K levels was proposed to enable ring stage parasites to survive ART-mediated inhibition of this kinase [57]. The increase in PfPI3K levels, however, was modest (2–4 fold), whereas ring stage parasites with mutant K13 can exhibit more than 70–fold lower sensitivity to ART [41]. Thus, while K13-dependent alterations in PfPI3K may contribute to ART resistance, it seems likely that additional factors are involved. It will be of particular interest to identify other K13 interacting partners.

Roles of other Proteins in Conferring High-Level ART Resistance

Sequencing of parasite strains collected as part of the TRAC study revealed that particular non-synonymous polymorphisms in apicoplast ribosomal protein S10, multidrug resistance protein 2, ferredoxin, and chloroquine resistance transporter (*pfcrt*) provide a genetic background on which *K13* mutations are likely to arise [106]. Moreover, *in vitro* drug-pressured lines have been reported that carry genetic changes other than at the K13 locus, notably copy number changes in *pfmdr1*, which might associate with ART resistance [90]. Genetic down-modulation of the falcipain 2 and 3 cysteine protease hemoglobinases

conferred a degree of ART resistance to early rings, although this is presumably because of the role of these hemoglobinases in producing heme as the ART activator as opposed to these being resistance modulators *per se* [30]. A central question underlying the role of secondary determinants is whether these are involved in reducing parasite susceptibility to ARTs during the trophozoite stage, when hemoglobin metabolism is at its peak. This is likely in the case of PfCRT and PfMDR1, which both reside on the digestive vacuole membrane and for which point mutations and (in the case of PfMDR1) changes in expression levels can modulate ART potency against trophozoites stages *in vitro* [107–111]. Mutations or expression level changes in these two transporters are also known to impact parasite susceptibility to several ACT partner drugs including lumefantrine, mefloquine and amodiaquine [109, 111–113]. Further experiments are required to dissect the roles of these associated secondary loci *in vitro* and their clinical contributions to reduced ART potency in patients.

Can Resistance to ARTs be Overcome?

While the very early ring (0–8 hr post-invasion) and late schizont stages (mean ~4 hr prior to egress) of K13 mutant parasites show markedly decreased sensitivity to short pulse exposure to DHA, the parasites remain susceptible to DHA for most of the intra-erythrocytic developmental cycle. A careful analysis of the kinetics of the responses of K13 wild-type and mutant parasites to DHA *in vitro* was used to inform predictions of *in vivo* parasite clearance profiles [41]. This analysis estimated that, for a patient treated with a typical 3-day regimen of DHA, the reduction in parasite burden would be 50–fold lower for a K13 mutant than for a wild-type parasite. This analysis also suggested that extending the dosing regimen to 4 days would decrease the parasite load of a K13 mutant to the level observed with a 3-day treatment of a K13 wild-type infection. This is consistent with data from a clinical trial in an area with a high prevalence of K13 mutants that showed 98% efficacy of a 6–day ACT treatment course [63]. These data speak to the appeal of extending treatments as a strategy to reduce the risk of **treatment failure** in areas with ART resistance, although this may face challenges in terms of ensuring compliance.

Similarly K13 mutants succumb to ART treatment *in vitro* if the duration of drug exposure is extended. That is, while K13 mutant early rings are able to withstand ART exposure for a longer period of time [41], prolonged exposure to drug is expected to overcome these parasites' enhanced defense mechanisms. The new synthetic ozonide, OZ439, exhibits a much longer half-life in the bloodstream [17, 19]. All other factors being equivalent, OZ439 would be expected to be much more effective at clearing K13-mutant parasites.

Another possibility is to combine ARTs with drugs that can reverse resistance. Clinically used proteasome inhibitors, such as Carfilzomib and Bortezomib, exhibit antimalarial activity and strongly synergize ART activity against both sensitive and resistant parasites [41]. This synergy has been demonstrated in Cambodian clinical isolates as well as isogenic lines expressing mutant and wild-type K13. Synergy was also observed in a *P. berghei* rodent malaria model [41]. These data suggest that supplementing an ACT with a proteasome inhibitor could provide therapeutic efficacy against ART-resistant parasites. Toxicity and regulatory issue may make it difficult to reposition a clinically used proteasome inhibitor.

Earlier efforts to develop *Plasmodium*-specific proteasome inhibitors [114–116] now benefit from a recent *tour de force*, in which a collaboration led by Dr. Matt Bogyo (Stanford University) applied cryo-electron microscopy and single particle analysis to solve the structure of the *P. falciparum* 20S proteasome to a resolution of 3.6 Å. Combined with substrate profiling, this provides valuable information regarding active site architecture that can be used to drive optimal inhibitor design [92].

Alternatively it is possible that K13 itself or its putative transcription regulator partner might be targeted. Given the increasing evidence that human Nrf2 plays a role in promoting oncogenesis and chemotherapeutic drug resistance, the Keap1/ Nrf2 pathway is considered a potential target [117]. If an Nrf2 equivalent in *P. falciparum* could be identified, and specific inhibitors generated, these compounds could be used to prevent the parasite from mounting a protective defense response. Ongoing studies into PI3K will also be valuable to determine whether inhibitors of this kinase phenocopy ART action and provide a novel strategy to overcome ART resistance.

It should be noted that even with the rising prevalence of K13 mutant genotypes, ACTs fail only when the partner drug efficacy also declines. One important consequence of the spread of K13 mutants is the increased number of parasites that remain following treatment of patients harboring ART-resistant infections, placing additional selection pressure on the partner drugs. In Cambodia, resistance to the partner drug piperaquine has now recently emerged, resulting in lower cure rates [118–122]. Resistance to artesunate-mefloquine has also been detected in different regions of South East Asia [123, 124]. This rapidly evolving situation raises the worrying specter that malaria in that region, particularly Cambodia, might be untreatable within a few years. An important short-term strategy to slow the emergence of resistance would be to rotate between different ACTs in an effort slow the development of resistance to the partner drugs.

Concluding Remarks

Artemisinin resistance is now well established in the Mekong region. Preventing ART resistance from gaining a foothold in Africa and other endemic regions including India is therefore essential. The identification of K13 as a molecular marker of ART resistance provides a critically important tool for the detection of new foci of resistance, enabling rapid mobilization of intervention strategies. Encouragingly, recent insights into the molecular basis of ART resistance provide hope that K13-mediated resistance can be combatted. The enhanced cellular defense response that underlies ART resistance enables very early ring stages to withstand drug exposure for longer but the intrinsic sensitivity to ARTs is retained. Extending the treatment regimen, rotating between ACTs with different partner drugs, or introducing new synthetic endoperoxides with longer half-lives might be effective strategies to overcome ART resistance at its current levels. This will be critical to have sufficient time to develop and introduce new antimalarial strategies.

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Glossary

Artemisinin
also known as Qinghaosu; a constituent of the Chinese medicinal herb *Artemisia annua*. Its semi-synthetic derivatives generally possess improved pharmacokinetic characteristics.

Artemisinin-based combination therapies (ACTs)
These drugs pair a short-lived artemisinin derivative with a second drug with a longer *in vivo* half-life in order to maximize treatment efficacy. Appropriate combinations should also delay the acquisition of parasite resistance compared to isolated use of either drug. Triple combination ACTs are currently in development.

Combination therapy
employing multiple drugs (normally two for malaria) to maximize treatment efficacy, delay emergence of drug-resistant parasites, and in some mixtures, kill dormant or transmissible forms of parasites.

Dihydroartemisinin (DHA)
the active *in vivo* metabolite of clinically used artemisinins.

Endoperoxide bridge
A peroxide (-O-O-) group that bridges two carbons that are both part of the ART molecule.

K13
P. falciparum Kelch protein K13 (gene ID PF3D7_1343700), the primary determinant of *P. falciparum* ART resistance in Southeast Asian parasites.

Lactol derivatives
DHA is the reduced lactol derivative of artemisinin, formed when artemisinin's cyclic carbonyl group is reduced to a hydroxyl group. The semisynthetic derivatives (artemether, arteether, artesunate and artelinate) are ethers or esters of the lactol.

Monotherapy
employing a single drug as a disease treatment.

Parasite clearance
the disappearance of parasites from blood circulation, as measured by a given assay (e.g. PCR, blood smear). Clearance is often reported in terms of its half time. The clearance half time is the number of hours required for the parasitemia to decrease by half during the log-linear phase of parasite reduction.

Pyknotic morphology
characterized by chromatin condensation in the nucleus.

Ring-stage parasites
the early phase of the intraerythrocytic asexual life cycle, defined as the period after invasion of red blood cells, but prior to the microscopic appearance of hemozoin (a sequestered form of heme released during hemoglobin breakdown).

Ring-stage survival assay
a measurement of the *in vitro* or *ex vivo* response of early blood-stage parasites to a short exposure to drug. This assay is performed with synchronized ring-stage parasites 0–3 hr post invasion. Parasites are pulsed for several hours (typically with DHA), then the drug is removed and culture continued a further 2–3 days. At that time survival of drug-treated parasites is measured relative to untreated parasites. This assay is particularly relevant for determining levels of *in vitro* ART resistance as well as for modeling inhibitors with modes of action similar to ARTs.

Schizont stage
the last phase of the intraerythrocytic asexual life cycle, after the onset of nuclear division, up until the rupture and release of daughter parasites (merozoites).

Tracking Resistance to Artemisinin Collaboration (TRAC)
a large-scale cooperative effort to study and document treatment failure to ARTs, and to characterize the phenotypes and genotypes of the implicated *Plasmodium* parasites. This effort initially focused on parasites in Southeast Asia, as well as a single site in Africa. A second phase of the project (TRAC II, begun in 2015) aims to extend this monitoring and to test the efficacy of novel triple-combination ACTs.

Treatment failure
lack of parasite clearance and/or lack of resolution of symptoms following administration of a drug regimen. Treatment failure may be due to parasite resistance to one or more drugs in a combination therapy, or other non-parasite factors (such as a suboptimal treatment regimen or an immuno-compromised state of the patient).

Trophozoite stage
in its general form, trophozoite refers to the entire growing phase of the parasite during the intraerythrocytic asexual life cycle, but by convention, this specifically refers to the period of the *Plasmodium* life cycle after the appearance of parasite hemozoin, and prior to the appearance of nuclear division.

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Box 1

Defining Artemisinin Resistance Clinically and *in vitro*

Clinically, the term “artemisinin resistance” is employed to describe delayed clearance of *P. falciparum* parasites from the peripheral blood of a patient treated with artesunate monotherapy or an artemisinin-based combination therapy (ACT). Treatment failure is not a necessary outcome of delayed parasite clearance and so far has been observed only in combination with existing or emerging resistance to the respective partner drug within the Greater Mekong Subregion (notably with piperaquine; see main text). In light of emerging artemisinin (ART) resistance, it is important to regularly monitor the therapeutic efficacy of current first and second line treatments, as recommended by the World Health Organization (WHO). These studies should determine the proportion of patients who are parasitemic on day 3 after starting treatment with an ACT, a routine indicator for suspected artemisinin resistance, and assess treatment failure rates in clinical trials with 28- or 42-day follow ups (depending on the ACT being employed). Should this proportion exceed 10% then the current WHO recommendation is to change national treatment policy. So far the working definition for partial ART resistance by the WHO has two subcategories. First, “suspected partial artemisinin resistance” includes three scenarios: either ≥5% of patients carry ART resistance mutations, or ≥10% of patients show persistent parasitemia on day 3, or ≥10% of patients show a clearance half-life ≥5 hr post treatment with an ACT or artesunate monotherapy. Second, “confirmed partial artemisinin resistance” is defined as ≥5% of patients carrying clinically or genetically validated mutations, with these patients showing parasites in the peripheral blood on day 3 following the start of artesunate monotherapy or ACT treatment or a prolonged clearance half-life ≥5 hours.

The potency of antimalarials against asexual blood stage parasites is mostly assessed in standard 72-hr growth assays in which the parasites are exposed to a range of concentrations to infer the amount of drug that inhibits parasite growth by 50% (IC_{50}). Delayed parasite clearance, however, does not correlate with changes in IC_{50_72h} values. The parasite’s susceptibility to ARTs is measured as the percentage of early ring-stage parasites that survive a pharmacologically relevant exposure to dihydroartemisinin (DHA), the active metabolite of all clinically used ARTs [125]. This ring-stage survival assay ($RSA_{0–3hr}$) correlates with long parasite clearance half lives (>5 hr) in *P. falciparum*-infected patients receiving an ART or an ACT. This assay can also be performed on *ex vivo*-cultured patient isolates to distinguish fast from slow clearing infections [67].

Box 2

Additional Methods to Monitor Parasite Clearance Profiles

Following *in vitro* treatment of ring stage *P. falciparum* with ARTs, non-viable rings (*i.e.*, rings that are have lost the ability to progress to the next cycle) can retain their ring-like morphologies for many hours [41]. In that study, the half-time to adopt a **pyknotic morphology** was reported to be significantly longer (up to 32 hr) for a K13 mutant than for a K13 wild-type parasite (which averaged 23 hr). This has implications for the interpretation of parasite clearance profiles *in vivo*. Adoption of a pyknotic morphology might trigger splenic clearance, which could be an important mechanism for clearing ring-stage parasites rendered non-viable by ARTs [126, 127]. New direct measures of parasite viability (*e.g.* based on loss of mitochondrial potential or DNA integrity) would complement existing methods for evaluating the effectiveness of new endoperoxide antimalarials and alternative treatment regimens. These methods could complement recent protocols that investigated ART resistance via a modified [^{3}H]-hypoxanthine incorporation assay as well as a delayed clearance assay that measured parasite growth upon repeated exposure to short pulses of DHA [90].

Outstanding Questions

- What is the impact of K13 mutations outside of Asia?
- Is there evidence of artemisinin resistance emerging in Africa?
- What is the contribution of secondary genetic determinants and how does this influence the geographic emergence and spread of resistance?
- What are the cellular components involved in resistance, including the role of the unfolded protein response, protein poly-ubiquitination and the parasite proteasome, phosphatidylinositol-3-kinase, and endocytosis of parasite hemoglobin?
- How can we contain ART resistance?
- Will ozonides or other longer-lasting agents with similar modes of action be able to overcome K13-mediated resistance in the field?

Trends Box

- Heme-activated artemisinin indiscriminately targets parasite biomolecules
- Stage- and exposure time-dependence of artemisinin killing underpins differential parasite response rates
- Mutations in the propeller domain of the *P. falciparum* Kelch-like protein K13 are central to artemisinin resistance
- K13 mutations appear to be associated with an enhanced cell stress response
- Targeting the *P. falciparum* proteasome might thwart K13-mediated artemisinin resistance and presents a strategy to block its spread

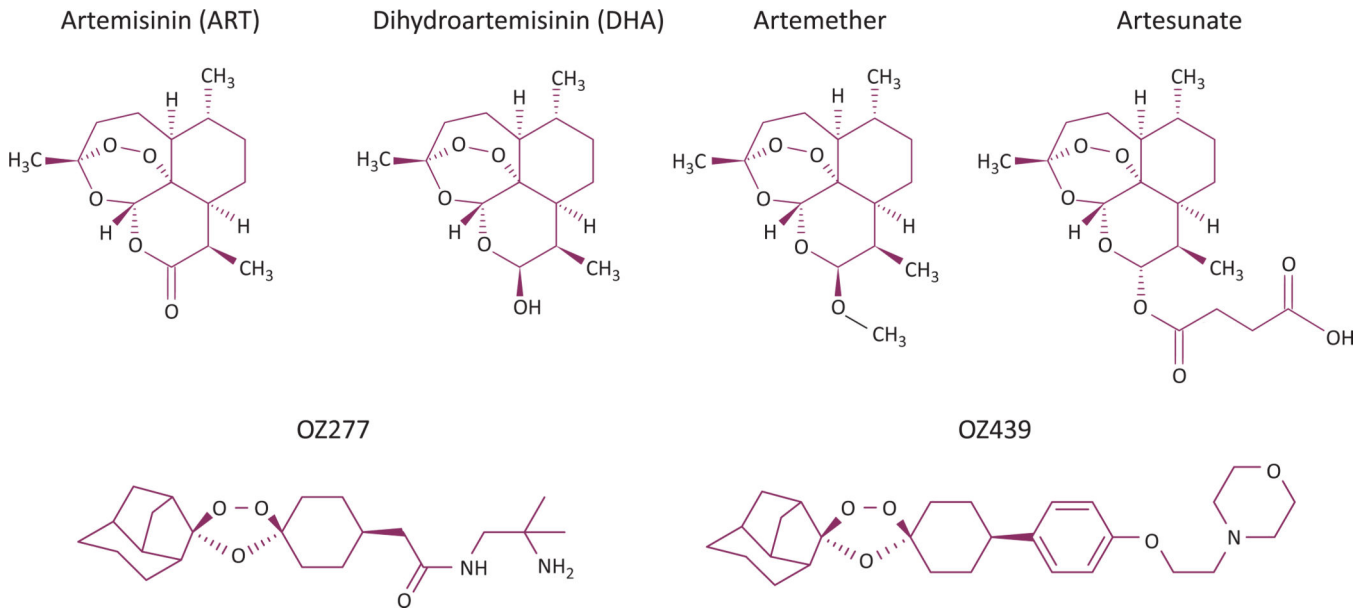

Figure 1. Structures of endoperoxide antimalarials

These are shown for the lactone artemisinin, its lactol derivatives dihydroartemisinin, artemether and artesunate, and the fully synthetic ozonides OZ277 and OZ439.

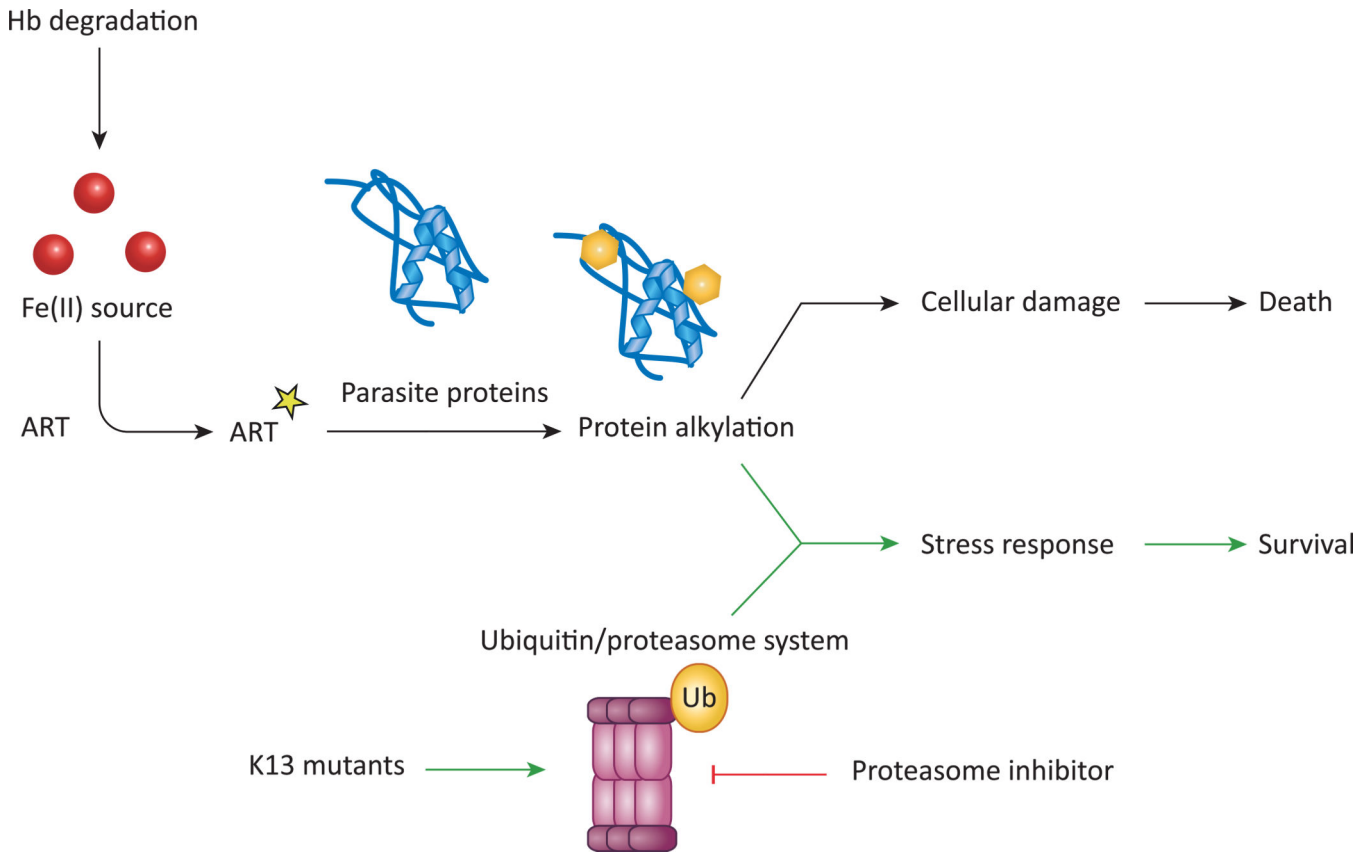

Figure 2. Diagram of putative cell death- and survival-promoting events following treatment with artemisinin (ART)

ART and its derivatives are activated by a reduced iron source (probably mainly heme released from hemoglobin digestion) to produce activated ART (ART*). Activated ART* reacts promiscuously with nucleophile-harboring cellular components, leading to damage and ultimately parasite death (black arrows). Parasites are thought to mount a stress response that engages the unfolded protein response (UPR), including the ubiquitin-proteasome system (green arrows). Recent evidence suggests that the stress response in K13 mutants is enhanced. Proteasome inhibitors are proposed to decrease the stress response (red), thus promoting parasite death.

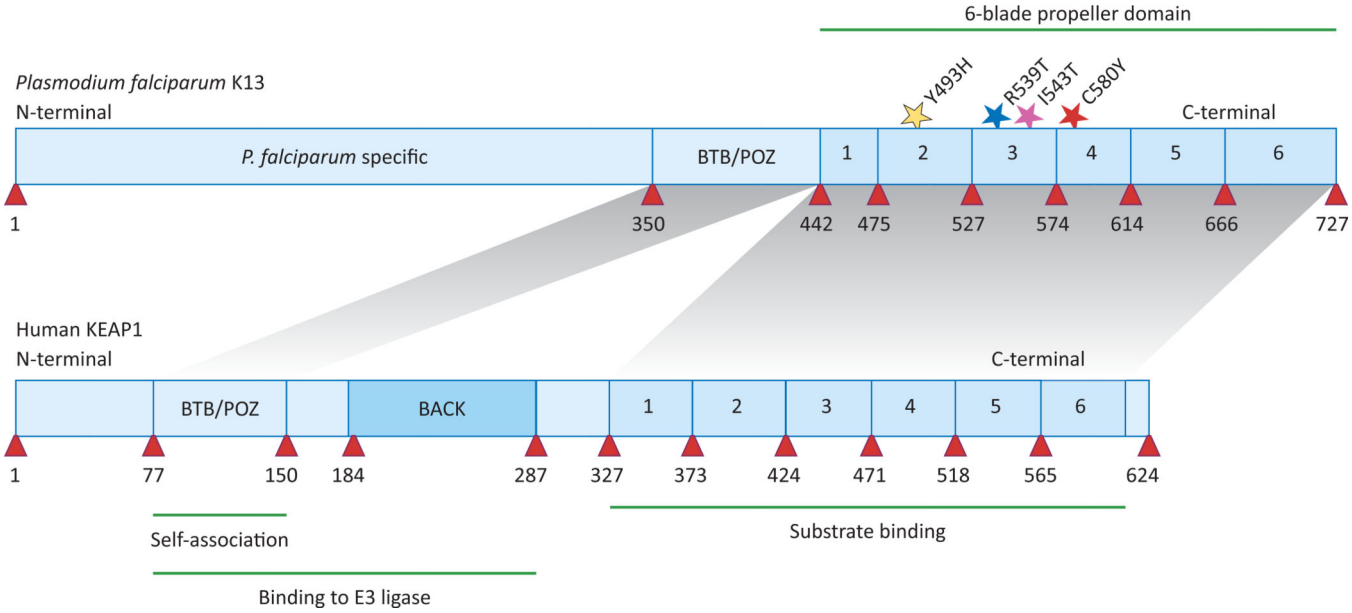

Figure 3. K13 and KEAP1 domains

Diagram of K13 domain structure illustrating the conserved *Plasmodium*-specific N-terminal domain, a BTB/POZ domain and a C-terminal domain containing six Kelch motifs, compared with human KEAP1, which has an E3 ligase-binding BACK domain adjacent to the BTB/POZ domain.

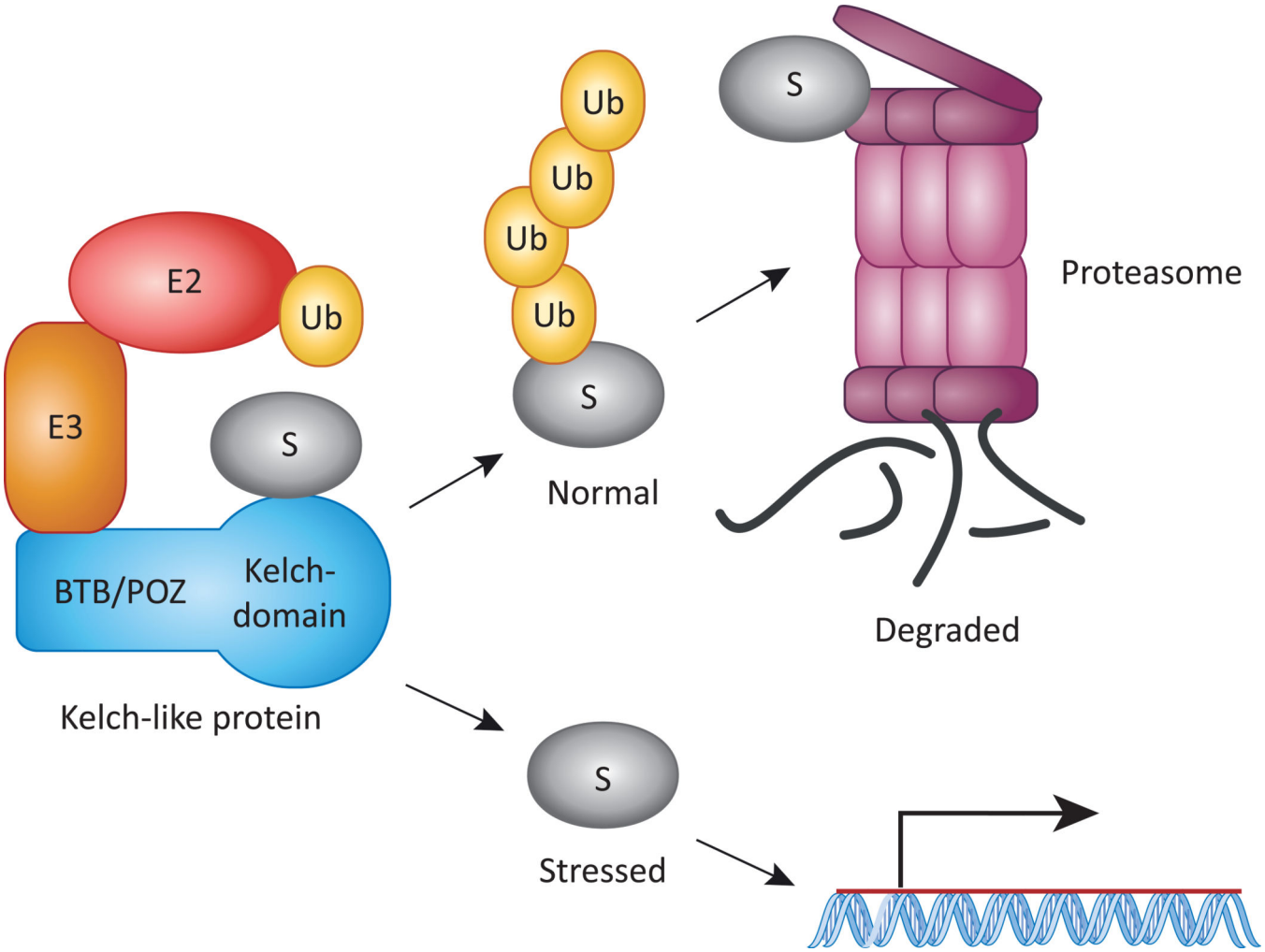

Figure 4. Proposed role of Kelch proteins as an E3 ligase substrate adaptor

The BTB/POZ domain of Kelch proteins contains a binding site for an E3 ligase, while the Kelch domain acts as an adaptor for a putative transcription factor substrate (S) that can be polyubiquitinated by an E2 enzyme. This would result in proteasomal degradation of the transcription factor. In stressed conditions the transcription factor is not degraded and can promote transcription of cellular defense proteins.