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REVIEWS

Opinion

Artemisinin Bioactivity and Resistance in Malaria Parasites

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Artemisinin is the most widely-used compound against malaria and plays a critical role in the treatment of malaria worldwide. Resistance to artemisinin emerged about a decade ago in Southeast Asia and it is paramount to prevent its spread or emergence in Africa. Artemisinin has a complex mode of action and can cause widespread injury to many components of the parasite. In this review, we outline the different metabolic pathways affected by artemisinin, including the unfolded protein response, protein polyubiquitination, proteasome, phosphatidylinositol-3-kinase, and the eukaryotic translation initiation factor 2α. Based on recently published data, we present a model of how these different pathways interplay and how mutations in K13, the main identified resistance marker, may help parasites survive under artemisinin pressure.

Artemisin, the Front-Line Compound against Malaria

The fight against malaria has been a resounding success in the past two decades, during which morbidity and mortality due to the disease have halved. Nonetheless, *Plasmodium falciparum* still accounts for nearly 500 000 deaths per year, and its prevalence may have started to (re)increase in recent years [1]. Progress has been due mostly to the combination of large-scale distribution of insecticide-treated bed nets and the massive use of **artemisinin (ART)-based combination therapies (ACTs)** (see Glossary). ART derivatives are currently the pillar of malaria treatment for both severe and uncomplicated malaria and are used in combination with other antimalarial drugs. Therefore, ongoing malaria control and possible elimination efforts rely heavily on the sustained efficacy of ART.

Clinical ART derivatives are semisynthetic sesquiterpene endoperoxides. They exert potent activity against the pathogenic *Plasmodium* blood stage through their common active metabolite, dihydroartemisinin (DHA). ART first needs to be activated by the cleavage of its endoperoxide bridge to yield free radicals which then alkylate a very large number of parasite proteins [2,3]. ART is extremely potent and fast-acting, reducing the parasite load up to 10 000 fold in a single ~48 h erythrocytic multiplication cycle [4]. All parasite blood stages are sensitive to ART; however, early blood stages – young **rings**, 0–6 hpi (hours postinvasion) – can be over 100-fold less sensitive than the **trophozoite** stage (24–40 hpi) [5] (Figure 1).

A major drawback of DHA is its very short plasmatic elimination half-life (typically ~1–2 h in humans). Monotherapies with an ART derivative for the standard 3-day regimen often do not completely clear the total parasite burden in patients, and are frequently (3–50%) followed by parasite recrudescence, that is, reappearance of blood-stage parasites that are still sensitive to ART. Indeed, even with a 10 000-fold reduction in parasite burden per cycle, it is not possible for a typical symptomatic infection – comprising several billion parasites – to be entirely cleared by exposure to artemisinin for only two asexual cycles. Therefore, ART is used only in combination with an antimalarial partner drug with a longer elimination half-life, such as mefloquine, piperaquine, amodiaquine, pyronaridine, or lumefantrine. A 3-day treatment with an ACT typically is fully curative and completely clears the parasite's asexual blood stage [6], and therefore has been recommended by the WHO since 2001 as a first-line treatment of uncomplicated malaria.

Emergence of ART Resistance: An Issue of Latency

Resistance to ART derivatives was demonstrated for the first time in 2007 [7]. At that time, a clinical trial showed that the efficacy of artesunate monotherapy in treating *P. falciparum*-infected patients had decreased compared with earlier studies. This trial, conducted in Western Cambodia, identified a series of patients who remained infected by the parasite for up to 7 days after treatment. A parallel

Highlights

Artemisinin is currently the most widely used and effective compound against malaria parasites and it plays a critical role in treatment and ongoing control efforts of malaria worldwide.

Unfortunately, emergence of resistance to artemisinin has occurred in Southeast Asia and was shown to be associated with mutations in the K13 gene.

Artemisinin kills parasites by increasing oxidative stress in the infected red blood cell, leading to an increased level of unfolded protein.

The unfolded protein response of the parasite is associated with a PK4/Elf2a response that can lead to parasite latency. K13 mutations are thought to alter ubiquitination patterns and help parasites to resist the accumulation of polyubiquinated proteins. How these two pathways interplay is not fully understood.

Recombinant parasite and single-cell technologies provide powerful avenues to decipher the mode of action of artemisinin and the function of K13.

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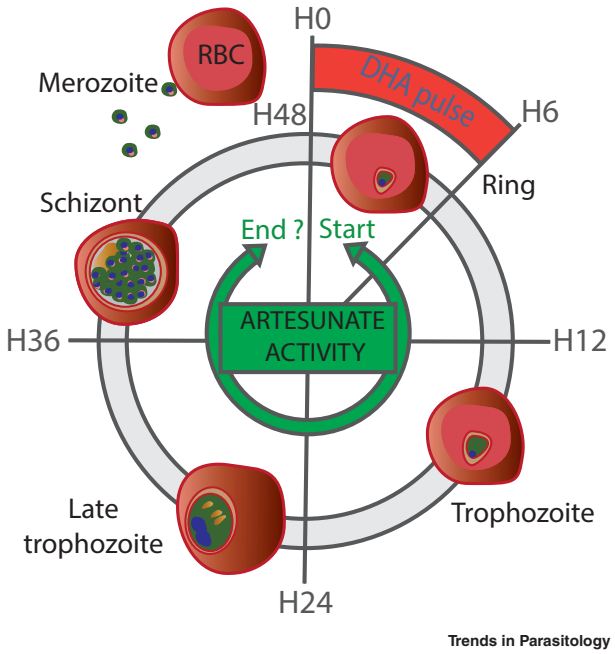

Figure 1. Schematic Representation of the Erythrocytic Cycle of *Plasmodium falciparum*.
In this 48 h cycle, the parasite undergoes multiplication via endomitosis, with different stages of the cycle having different drug sensitivities. Green arrows highlight the supposed highest activity window of artemisinin (ART) derivative drugs. The parts of the lifecycle that have the lowest sensitivity to ART in K13 wild-type (WT) parasites are ~6–16 hours postinvasion (hpi) and the last few hours of schizogony. The red bar corresponds to the standard dihydroartemisinin (DHA) pulse in a ring-stage survival assay (RSA) test (Figure 3). Abbreviation: RBC, red blood cell.

trial, performed in the same endemic area, confirmed these results and suggested that the observed clinical resistance was linked to a decrease in the parasite clearance rate [8]. In these early *in vivo* studies, it was not possible to study the molecular mechanism underlying this delayed clearance because one could not distinguish contributions of the parasite or the host to this phenotype. Delayed clearance could indeed be due to an altered immune response against the parasite or to altered pharmacokinetics of the drug, and not necessarily to the appearance of a per se 'resistant' parasite.

In parallel, long-term ART pressure *in vitro* on initially sensitive parasites gave rise to resistance [9–12]. Remarkably, the resistance phenotype of these *in vitro*-generated parasites was unusual. The *in vitro*-pressured parasites did not display an increased 50% inhibitory concentration (IC_{50}) to DHA using standard growth-inhibition assays but contained a subpopulation of parasites capable of surviving a DHA pulse of 6–48 h at clinical concentration. The outcomes measured differed in the two studies, with either the time taken by surviving parasites to attain a parasitemia threshold or the proportion of surviving parasites. In both cases, the surviving subpopulation was restricted to young blood stages of the parasites, that is, early ring stages, that were found to be arrested in the cell cycle under ART pressure and to resume cell-cycle progression upon drug removal. Therefore, the phenotype of these surviving parasites appeared to be one of tolerance to ART rather than bona fide resistance, and both groups proposed that the tolerance phenotype was due to the ability of a parasite subpopulation to enter some form of dormancy [10] or quiescence [12], at least *in vitro*. Thus, the phenotype described by the two groups, although not strictly equivalent in terms of experimental design and outcome, appears to involve similar cell-cycle regulation, hereafter referred to as latency (Box 1 and Figure 2). It is

Glossary

Alkylation: the transfer of an alkyl group from one molecule to another.
Artemisinin (ART)-based combination therapies (ACTs): therapies involving a combination of two types of molecule: a semi-synthetic molecule derived from artemisinin and a synthetic molecule whose role is to increase the effect of the first molecule, mostly due to the long half-life of the partner drug.
Autophagy: denotes a degradation of a part of the cytoplasm of a cell by its own lysosomes.
Cell-cycle checkpoint: a point in the eukaryotic cell cycle at which the progression of a cell to the next stage in the cycle can be halted until conditions are favorable.
Eukaryotic translation initiation factor 2α (eIF2α): a protein that catalyzes an early regulated step of protein synthesis initiation, promoting the binding of the initiator tRNA to 40S ribosomal subunits.
Kelch domain: this sequence motif is composed of about 50 amino acid residues which form a structure of a four-stranded β-sheet 'blade'.
K13: *Plasmodium falciparum* protein (PF3D7_1343700); the presence of nonsynonymous mutations in his propeller domain (kelch domain) has been associated with decreased efficacy of artemisinin derivatives in the young stages of parasites.
Metalloprotein: a generic term for a protein that contains a metal ion cofactor. A large number of proteins are in this category.
Proteasome: a protein complex that degrades unneeded or damaged proteins by proteolysis.
Pyknotic: irreversible condensation of chromatin in the nucleus of a dying cell.
Rings: red blood cell stages from 0 to 24 h postinvasion.
Sporozoites: parasite stages transmitted from mosquito to mammal.
Trophozoites: red blood cell stages from 24 to 40 h postinvasion.
Ubiquitination: an enzymatic process that involves bonding of a ubiquitin protein to a substrate protein that usually becomes

Box 1. Dormancy, Quiescence, and Latency

Due to the ambiguity of the terms 'dormancy' and 'quiescence', we wanted to define these two concepts and explicitly state how they differ. Dormancy and quiescence are observed in many life forms. They correspond to the period when, in the life cycle of an organism, growth and/or development are temporarily halted. This is a bet-hedging strategy to mitigate risks; it is implemented in a wide range of taxa and is linked to environmental conditions.

Predictive dormancy or primary dormancy occurs when entrance into a dormant phase precedes unfavorable conditions. It is a genetically coded mechanism. For example, in *Plasmodium falciparum*, it corresponds to arrests observed in the mature gametocyte and sporozoite stages.

Consecutive dormancy (known as secondary dormancy), which for us is synonymous with quiescence, occurs when organisms enter a phase of metabolic slowdown as a result of adverse conditions. This is commonly found in areas with a random environment but is not *a priori* 'anticipated' in the life cycle of the organism.

In the context of what is observed for *P. falciparum* rings when submitted to DHA treatment, we are currently unable to establish which of the two terms is most appropriate; therefore, we here use the more generic neutral term 'latency'.

inactivated and tagged for degradation by the proteasome as a result.

Unfolded protein response (UPR): cellular stress response related to the endoplasmic reticulum (ER) stress by unfolded proteins.

now clear that the parasite's cell cycle can be abruptly arrested following exposure of the ring stage to ART, with a proportion of exposed parasites (typically very small, depending on the ART regimen and the parasite's genetic background) resuming growth up to 25 days after exposure [10,11]. Therefore, to resist the widespread injuries caused by ART, the ring stage has the ability to shield itself into latency until drug exposure has ceased. Such latency is highly reminiscent of other *Plasmodium* parasite stages which are known to arrest proliferation at various steps of the life cycle: gametocytes, which are differentiated nonreplicative forms transmissible to mosquitoes; **sporozoites** inside mosquito salivary glands, awaiting transmission to the vertebrate host; and *Plasmodium vivax* and *Plasmodium ovale* hypnozoites inside host hepatocytes that can arrest cell division and differentiation for up to several years before inducing a blood infection. Whether ART-induced latency has highjacked some of the mechanisms used in these other arrested stages is not known.

K13-Dependent ART Resistance

The studies discussed in the previous section led to the development of an *in vitro* phenotypic resistance test that was applicable to culture-adapted field parasites. This test, called the ring-stage survival assay (RSA; Figure 3), consisted of synchronizing cultured parasites at early stages, subjecting them to a clinical concentration of DHA (700 nM) for 6 h, and evaluating the parasite survival rate after a complete intraerythrocytic cycle, that is, 72 h [12].

When the RSA was applied to Cambodian isolates, parasites exhibiting an *in vitro* survival rate >1% were also found to exhibit delayed parasite clearance *in vivo* [12]. Not only do these results prove that the RSA provides a valid surrogate measure of delayed clearance, they also suggest that ART resistance is indeed attributable to the parasite. Importantly, it was further shown that the population of parasites surviving an RSA, when subjected to a subsequent RSA, displayed a survival rate identical to that obtained in the first RSA. The stability of the survival rate determined by the RSA further supported the idea of a peculiar mechanism of resistance to ART.

Interestingly, the ART-resistant strain selected *in vitro* by Witkowski *et al.* [11] displayed a survival rate >1% as assessed by the RSA. By comparing the genomes of the *in vitro*-selected ART-resistant strain (F32-ART) and of the original parental strain (F32-TEM), which was cultured in the same conditions but without ART pressure, the group identified seven genes in F32-ART that bore specific nonsynonymous mutations. Using whole-genome data of Cambodian isolates displaying varying survival rates assessed by RSA, it was found that ART resistance was associated with mutations in the **Kelch**-type propeller domain of a single parasite protein called **PfK13** [13]. K13

(A)

Organism	Stage	Latency type	Duration of latency
Plasmodium spp	Ring	? ART-induced	Days to weeks
Plasmodium spp	Mature gametocyte	Developmental, obligate	Several days to a week
Plasmodium spp	Sporozoite	Developmental, obligate	Several days to a week
Plasmodium vivax *Plasmodium ovale*	Hypnozoite	Developmental, facultative	Months–years
Toxoplasma gondii	Bradyzoite	Developmental, stress-induced, facultative	Months–years

(B)

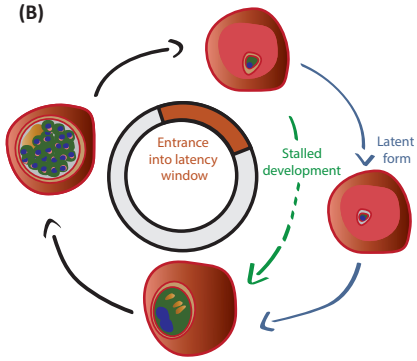

Figure 2. Latency in Apicomplexan Parasites.
(A) Several stages of apicomplexan parasites have the ability to enter developmental arrest; these phases can either be obligate whilst awaiting a suitable host/vector or facultative when parasites can bifurcate into latent stages for later re-emergence (e.g., hypnozoites, bradyzoites). (B) In the case of the artemisinin (ART)-induced ring arrest, it is not known whether the latent ring corresponds to a bona fide developmental bifurcation, or whether the parasite is simply stalled in development.

shares homologous BTB and Kelch domains with proteins that function as substrate adaptors, facilitating protein **ubiquitination** via cullin-3 ligases. Some mutations in the Kelch domain of other Kelch-containing proteins decrease the binding of protein substrates, and their ubiquitination and degradation.

Several site-directed mutagenesis studies have confirmed that ring-stage parasites bearing mutations in K13 had the capacity to better cope with a 6 h DHA pulse [14,15]. Further studies on the relationship between mutations in the K13-encoding gene and the RSA have yielded important information. First, the different mutations in K13 each associate with a specific and reproducible survival rate after 72 h. Second, the parasite geographic origin and genomic background are important modulators of the survival rate associated with a K13 mutation that was either selected by drug pressure or engineered by molecular biology. In particular, when bearing a K13 mutation, the Asian or South American backgrounds associate with higher survival rates compared with African ones [16,17].

A recent study has also found that K13 mutations conferred increased tolerance to ART in male gametogenesis [18,19], a process essential to transmission to the insect vector. Remarkably, highly differentiated ART-resistant parasites infect highly diverse *Anopheles* species. Competition experiments are needed to further dissect the impact of resistance-conferring mutations on the fitness of the *Plasmodium* sexual stage. This will be key to understanding how such mutations are selected for and spread in the population.

Several other parasite determinants were shown to associate with some ART resistance in strains pressured by ART *in vitro*, including increased chaperone production and proteostasis protein expression (see below), and amplification of *pfmdr1* (see [20,21]). Notably, the *P. falciparum* phosphatidylinositol-3-kinase (PI3K) has been proposed to play a central role in resistance. The polyubiquitination level of PI3K is controlled by K13 [22], presumably through a direct interaction, but which still needs to be formally demonstrated. In a K13 mutant background, the K13–PI3K interaction may be altered, leading to decreased ubiquitination and increased levels of PI3K, along with its products, for example, the lipid phosphatidylinositol-3-phosphate (PI3P), which was found to be sufficient to confer modest ART resistance *in vitro* [22]. It was also hypothesized that a 'proteostatic' mechanism dependent on $PI3P^{+}$ vesicle formation from the endoplasmic reticulum (ER) could help to remove misfolded or aggregated proteins, a process similar to **autophagy** [21–25].

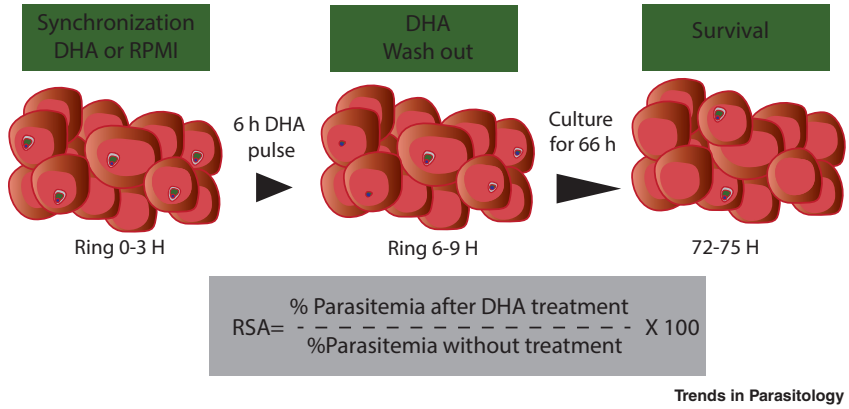

Figure 3. Schematic Representation of the Ring-Stage Survival Assay (RSA).
Parasites are exposed to a pulse of dihydroartemisinin (DHA), and the relative survival rate is measured after another erythrocytic cycle. Resistance to DHA is defined as an RSA value above 1%. Circular versus dotted parasites symbolize viable and pyknotic parasites, respectively.

A Central Response to ART-Induced Stress: The Unfolded Protein Response

The most likely scenario of ART-induced death is through widespread **alkylation** and irreversible damage to proteins, resulting in the production of misfolded proteins associated with the disruption of key metabolic pathways in the parasite. Transcriptomic analysis of a variety of resistant clinical isolates or laboratory strains concurred in showing that the core adaptive response to ART in ring-stage parasites resembled a typical **unfolded protein response (UPR)** [26,27], including the following: (i) an increase in expression levels of heat-shock proteins and two major chaperone complexes, the ER-located reactive oxidative stress complex (ROSC) and the cytoplasmic T-complex protein-1 ring complex (TRiC); (ii) an upregulation of genes involved in protein folding, unfolded protein binding, protein transport (particularly vesicular trafficking between the ER and Golgi apparatus), and **proteasome** function/proteolysis; and (iii) the formation of P-bodies involved in the repression of translation initiation.

Importantly, recent functional studies have shown that an accumulation of ubiquitinated proteins (UbPs) was the main toxic event associated with ART treatment. First, the levels of UbPs increase upon parasite exposure to ART, with resistant parasites exhibiting lower levels of UbPs postexposure [22,28,29]. Second, inhibition of deubiquitinases, which remove ubiquitin from substrates before degradation, or from the ubiquitin receptor, exacerbates the build-up of UbPs and leads to parasite death following ART treatment; conversely, inhibition of the ubiquitin machinery or depletion of intracellular pools of activated ubiquitin (with compound C1, 5′-O-sulphamoyl-N(6)-[(1S)–2,3-dihydro-1H-inden-1-yl]-adenosine), or inhibition of protein translation (with cycloheximide), all reduced ART-mediated accumulation of UbPs and strongly antagonized ART-mediated killing [29]. Third, proteasome inhibitors (e.g., epoxomicin) cause an accumulation of UbPs [29], and strongly enhance ART activity against both ART-sensitive and ART-resistant parasites [28]. Fourth, ART depresses proteasome function, which may occur by clogging the proteasome with UbPs or via direct proteasome damage by ART [30]. In sum, the emerging picture of ART-induced injury is that of a ‘double whammy’ [29], that is, misfolded/unfolded protein generation and proteasome inhibition, death coming from an excess of UbPs and an unresolved ER stress. Of note, mutations in deubiquitinase, UBP1, were identified in rodent malaria parasites subjected to ART pressure [31] as well as in African [32] and Southeast Asian [33] *P. falciparum* isolates with decreased ACT efficacy. Fittingly, adapted and naturally resistant parasites typically displayed a deceleration of the developmental cycle during early stages following an ART pulse, which is reminiscent of the cell stress response observed in many organisms [5,12,27,28].

The Terminal Sensor/Effector of ER Stress Response: The PK4-eIF2α Connection

In mammalian cells, UPR is a cellular stress response that modulates phosphorylation of the **eukaryotic translation initiation factor 2α (eIF2α)**, which delivers tRNA initiators to ribosomes [34]. In turn, phosphorylated eIF2α, which forms an inactive eIF2–eIF2B complex, leads to a general reduction in protein synthesis, accompanied by preferential translation of mRNAs encoding products that are important for recovery from the stress. Only three kinases of eIF2α are expressed in *Plasmodium*: IK1, IK2, and PK4 [35]. IK1 is dispensable for blood stages and, like its mammalian homolog GCN2, regulates eIF2α phosphorylation in response to amino acid starvation [36,37]. IK2 ensures the latency of sporozoites in mosquito salivary glands [38]. PK4 is a homolog of mammalian PERK, an integral ER membrane protein with an N terminus that protrudes in the ER lumen and senses unfolded proteins and a cytoplasmic C terminal catalytic domain that, upon activation, phosphorylates eIF2α. PK4 was shown to be essential for blood-stage growth using Flp/FRT conditional mutagenesis in *Plasmodium berghei* [39] and inducible knockdown by riboswitch in *P. falciparum* [29].

Recently, two studies linked ART-induced latency and recrudescence to PK4 and phosphorylation of eIF2α in these two *Plasmodium* species. The first [40] showed that: (i) ART treatment resulted in eIF2α phosphorylation; (ii) ART activated PK4, which is ER-resident; (iii) overexpression of a PK4 dominant-negative or pharmacologic inhibition of PK4/PERK (ER and PERK being absent from erythrocytes) blocked parasites from entering latency and abolished recrudescence after treatment in *P. berghei*-infected mice; (iv) augmentation of eIF2α phosphorylation by salubrinal increased recrudescence rates to 100%; and (v) eIF2α was phosphorylated in the young ring stage of an ART-resistant K13-mutated parasite but not in the young ring stage of its ART-sensitive K13 wild-type parent strain. The second study [29] largely confirmed these data, while adding that: (i) eIF2α phosphorylation in rings occurred upon exposure to ART in a concentration-dependent manner, and (ii) inhibition of the ubiquitin machinery (with compound C1) or of protein translation (with cycloheximide) both strongly antagonized ART-mediated killing, and also strongly inhibited DHA-mediated eIF2α phosphorylation. It is therefore tempting to speculate that eIF2α phosphorylation ultimately mediates the various accounts of latency as well as cell-cycle elongation/growth retardation, particularly during early stages of parasite development (see Figure 4 for a quick overview of 10 years of *P. falciparum* artemisinin resistance research).

A Model for ART Activity and Resistance

As schematized in Figure 5 (Key Figure), following activation by free heme, DHA induces protein alkylation and misfolding. The parasite then triggers a UPR. In the cytosol, the response will tend to ubiquitinate unfolded proteins and degrade them in the proteasome. However, since DHA also inhibits the proteasome, ubiquitinated proteins will accumulate and eventually lead to parasite death. The parasite may also activate a $PI3P^{+}$-dependent vesicle formation process, possibly linked to autophagy, to help remove UbPs. In the ER, UbPs induce an ER-stress response, which activates/oligomerizes the ER membrane-bound PK4. Activated PK4 in turn phosphorylates eIF2α, which induces parasite translational arrest/slow-down to limit protein neosynthesis. In this context, a mutated K13 may help the parasite to resist ART injury in several ways: by decreasing polyubiquitination of UbP, and thus accumulation of lethal UbP, and by decreasing the release of free heme, and thus decreasing ART activation. Also, mutations in K13 may be involved in the control of eIF2α phosphorylation, thereby enhancing delay of the process of ring maturation into trophozoites. This continued slowdown effect of cell metabolism and translation would allow the parasite to withstand DHA induced stress, and potentially lead to a complete cessation of parasite development, mimicking a dormant parasite.

What Kind of Latency?

Conflicting accounts were made on morphologic features of parasite latent forms associated with ART pressure *in vitro*. Some displayed a round shape retaining a small amount of visible cytoplasm and condensed chromatin (as opposed to **pyknotic** parasites) [11,41–43], whilst another study described dormant forms as indistinguishable from ring-stage parasites [9]. Dormant forms were

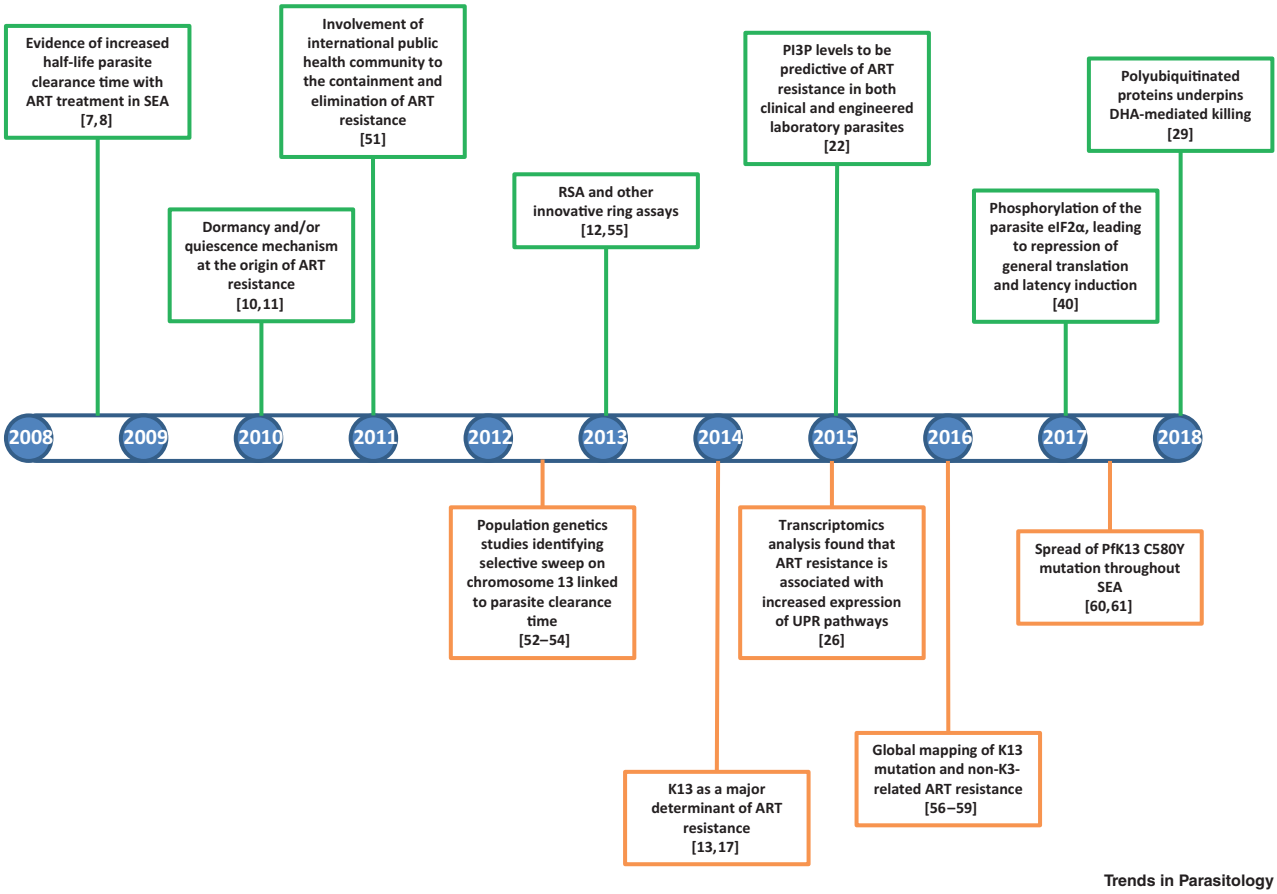

Figure 4. A Quick Overview of 10 Years of *Plasmodium falciparum* Artemisinin-Resistance Research.
In green (top) are major publications related to clinical and *in vitro* studies, and in orange (bottom) are genomics and transcriptomics studies (See [7,8,10–13,17,22,26,29,40,51–61]). Abbreviations: ART, artemisinin; DHA, dihydroartemisinin; eIF2α, eukaryotic translation initiation factor 2α; PI3P, phosphatidylinositol-3-phosphate; RSA, ring-stage survival assay; SEA, Southeast Asia; UPR, unfolded protein response.

also found as retaining a mitochondrial membrane potential and associated with a compact mitochondrion [42,44], although only a subset of flow cytometry sorted parasites with a positive membrane potential were capable of resuming growth. The duration of latency was also shown to be highly variable, lasting from only 24 h to 25 days postexposure [11]. Given the diversity of these phenotypes, are we facing parasites in a formal latency stage, or is the observed latency phenotype just the result of a probability of survival of the parasites' population depending on its size, its genetic background, and the dose of DHA used?

Indeed, a major unresolved question is whether early blood-stage developmental arrest and/or slowdown results from an existing, qualitative control mechanism, that is, a **cell-cycle checkpoint** controlling progression along the erythrocytic cycle, possibly by sensing ROS, or from a progressive decrease in translation, irrespective of any control mechanism, leading to a gradual slowdown in parasite development possibly culminating in a complete arrest. In other words, is it a real developmental process into a bona fide latent stage (able to be identified by a 'biological signature') or is it only a quantitative slowdown of the translation without any specificity? (Figure 2).

In favor of the first scenario, it is known that many *P. falciparum* strains [11] and rodent-infecting parasites [45] are capable of entering a latency state without the necessity to preselect them by ART pressure. In addition, as already mentioned, other *Plasmodium* stages, that is, gametocytes, sporozoites,

Key Figure

Proposed Model of the Induction by the Unfolded Protein Response (UPR) of Different Pathways in Artemisinin (ART) Resistance

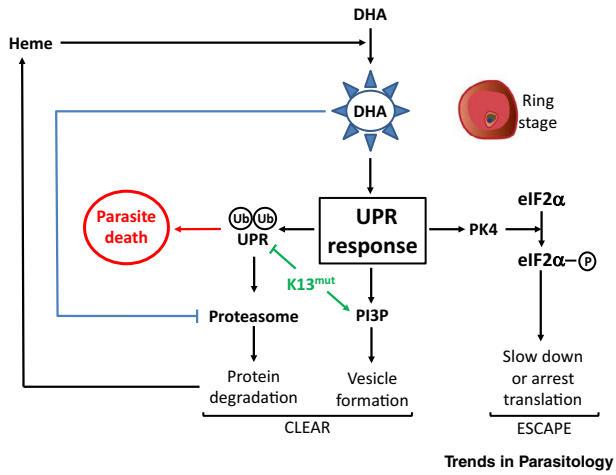

Figure 5. Different pathways include protein polyubiquitination (Ub), proteasome, phosphatidylinositol-3-kinase (PI3K) and protein kinase 4 (PK4)/eukaryotic translation initiation factor 2α (eiF2a) and their potential interactions with mutations in K13, the main identified artemisinin-resistance marker. Inhibition of the proteasome by the activated dihydroartemisinin (DHA) (blue), associated with a reduced ubiquitination due to the K13 mutations (green), reduces the harmful effects of protein polyubiquitination and accumulation (red) and leads to a decrease in the degradation of metalloproteins and subsequent reduced release of DHA activator heme. This 'virtuous circle' contributes to the reduced effectiveness of DHA in mutant K13 parasites without decreasing direct activation of PI3P- and PK4-related protective pathways.

Outstanding Questions

Does latency correspond to a bona fide developmental stage or a stalling of the cell cycle?

What precise window in the erythrocytic cycle of the parasite is capable of entering latency?

How does latency contribute to our understanding of ART resistance *in vivo*?

What are the determinants of entry and exit from latency?

What is the role of the genetic background associated with K13 polymorphisms in the ability to enter latency?

What is the contribution of K13-independent factors in ART resistance?

and liver stages, are known to be able to arrest translation at specific developmental steps as a means to halt their cycle progression awaiting vector-to-host transmission. Nevertheless, it is important to note that IK2-mediated latency as observed in salivary gland of sporozoites and PK4-mediated ER stress response mediated arrest of translation would be expected to have different molecular mechanisms, and that IK2 knock-out has no effect on ART sensitivity. Furthermore, blood stages are known to be able to adapt their development in response to specific environmental conditions, such as amino acid starvation that was shown to induce parasite 'hibernation' [46]. Latency may therefore be a natural trait, or at least a natural option, of blood stages that allows them to escape certain types of stress not limited to drug exposure. ART resistance may thus be linked to an increased parasite propensity to engage in latency. There is one report of persistent low-density parasite DNA (using ultrasensitive PCR) following ACT treatment in Myanmar. The authors presumed that this signature originates from dormant parasites [47]. However, despite persistence, observed for up to 3 weeks, no recrudescence was observed, raising questions as to the viability of these parasites [47]. To date, experimental proof of the existence of such naturally occurring, dormant blood stages is still lacking – which may be due to their small proportion and the lack of adequate tools to isolate them.

In favor of the second scenario, there appears to be a quantitative correlation between parasite survival, measured by the RSA, and the dose of DHA for a given parasite genetic background. This could also explain the efficacy of longer courses of artemisinin derivatives in ACT clinical studies in Southeast Asia [48]. As mentioned above, DHA must be activated, and many studies agree that its main bioactivator is heme – which breaks its endoperoxide bond. Free heme is released by the degradation of **metalloproteins** and, in particular, hemoglobin in the case of late blood stages (hemoglobin

degradation starts around 6–10 h after red blood cell invasion). Since DHA is also active in young blood stages, the latter must contain free heme, and it has been shown that hemoglobin digestion has already started in young rings even though the digestive vacuole is still not formed [49]. Alternative sources of free heme may be from heme biosynthesis or heme-containing proteins such as the ones present in the mitochondrion and apicoplasts and/or the degradation of metalloproteins other than hemoglobin [50]. K13 may be involved in the latter process by controlling the ubiquitination/degradation of metalloproteins, thereby decreasing the release of free heme and thus lowering ART activation and activity in young resistant stages. This quasi-linear relationship between the amount of activated DHA and the probability of survival of the parasite is rather in favor of a continuous process.

Concluding Remarks

Few topics in malaria research carry as much importance for the future of malaria control as understanding ART resistance. ART injury and resistance are not only of a noncanonical nature but also involve numerous cellular and metabolic pathways, making its study a massive challenge. In this review, we have outlined how different molecular and cellular mechanisms participate in ART resistance. Specifically, we have tried to reconcile how different components of the stress response and translation dynamics may affect cellular survival to ART, and the exact molecular linkers, and the dynamic interplay between those cellular processes will still have to be addressed experimentally. We have also stressed the importance of understanding the natural history of latency (see Outstanding Questions). The nature of latency is still not understood; specifically, if it is a physiologically regulated process or an adaptive arrest, what are the cellular and molecular players involved, and what are the dynamics of the progression through latency? Solving such questions may lead to more potent approaches to target drug-resistant parasites. Some technical issues have to be resolved in order to address these questions: (i) to identify and isolate the *P. falciparum* parasite at specific stages of its intracellular erythrocytic development (perhaps through single-cell technology); (ii) to identify the K13-containing complex(s) during the erythrocytic cycle, and its alteration(s) associated with K13 mutations.

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