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1 **Artemisinin Bioactivity and Resistance in Malaria Parasites**

2

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10

11 **Keywords**

12 *Plasmodium falciparum*, artemisinin resistance, dormancy, quiescence, latency, unfolded protein
13 response

14

15 **Abstract**

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

25

26 **Artemisin, the front-line compound against malaria**

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

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

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

54

55 **Emergence of ART resistance: an issue of latency**

56 Resistance to ART derivatives was demonstrated for the first time in 2007 [7]. At that time, a clinical
57 trial showed that the efficacy of artesunate monotherapy to treat *P. falciparum*-infected patients had
58 decreased compared to earlier studies. This trial, conducted in Western Cambodia, identified a series
59 of patients that remained infected by the parasite for up to 7 days after treatment. A parallel trial,
60 performed in the same endemic area, confirmed these results and suggested that the observed clinical

61 resistance was linked to a decrease in parasite clearance rate [8]. In these early *in vivo* studies, it was
62 not possible to study the molecular mechanism underlying this delayed clearance because one could
63 not distinguish contributions of the parasite or the host to this phenotype. Delayed clearance could
64 indeed be due to altered immune response against the parasite or to altered pharmacokinetics of the
65 drug, and not necessarily to the appearance of a *per se* “resistant” parasite.

66 In parallel, long-term ART pressure *in vitro* on initially sensitive parasites gave rise to resistance [9-
67 12]. Remarkably, the resistance phenotype of these *in vitro*-generated parasites was unusual. The *in*
68 *vitro*-pressured parasites did not display an increased 50% inhibitory concentration (IC₅₀) to DHA
69 using standard growth inhibition assays but contained a sub-population of parasites capable of
70 surviving a 6 to 48 h DHA pulse at clinical concentration. The outcomes measured differed in the two
71 studies, with either the time taken by surviving parasites to attain a parasitemia threshold or the
72 proportion of surviving parasites. In both cases, the surviving sub-population was restricted to young
73 blood stages of the parasites, i.e. early ring stages, that were found to be arrested in the cell cycle
74 under ART pressure and to resume cell cycle progression upon drug removal. Therefore, the
75 phenotype of these surviving parasites appeared to be one of tolerance to ART rather than *bona fide*
76 resistance, and both groups proposed that the tolerance phenotype was due to the ability of a parasite
77 sub-population to enter some form of dormancy [10] or quiescence [12], at least *in vitro*. Thus, the
78 phenotype described by the two groups, although not strictly equivalent in terms of experimental
79 design and outcome, appear to involve similar cell cycle regulation, hereafter referred to as latency
80 (Box 1, Figure 2). It is now clear that the parasite cell cycle can be abruptly arrested following
81 exposure of ring-stage to ART, with a proportion of exposed parasites (typically very small,
82 depending on the ART regimen and the parasite genetic background) resuming growth up to 25 days
83 after exposure [10,11]. Therefore, to resist the widespread injuries caused by ART, the ring stage has
84 the ability to shield itself into latency until drug exposure has ceased. Such latency is highly
85 reminiscent of other *Plasmodium* parasite stages which are known to arrest proliferation at various
86 steps of the life cycle: gametocytes, which are differentiated non-replicative forms transmissible to
87 mosquitoes; **sporozoites** inside mosquito salivary glands, awaiting transmission to the vertebrate host;
88 *P. vivax* and *P. ovale* hypnozoites inside host hepatocytes that can arrest cell division and
89 differentiation for up to several years before inducing a blood infection. Whether ART-induced
90 latency has hijacked some of the mechanisms used in these other arrested stages is not known.

91

92 **K13-dependent ART resistance**

93 These pioneering studies led to the development of an *in vitro* phenotypic resistance test that was
94 applicable to culture-adapted field parasites. This test, called the ring-stage survival assay (RSA, see
95 Figure 3), consisted in synchronizing cultured parasites at early stages, subjecting them to a clinical

96 concentration of DHA (700 nM) for 6 h, and evaluating the parasite survival rate after a complete
97 intra-erythrocytic cycle, i.e., 72 h [12].

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

105 Interestingly, the ART-resistant strain selected *in vitro* by Witkowski *et al.* [11] displayed a survival
106 rate > 1% as assessed by the RSA. By comparing the genomes of the *in vitro*-selected ART-resistant
107 strain (F32-ART) and of the original parental strain (F32-TEM), which was cultured in the same
108 conditions but without ART pressure, the group identified seven genes in F32-ART that bore specific
109 non-synonymous mutations. Using whole genome data of Cambodian isolates displaying varying
110 survival rates assessed by RSA, it was found that ART resistance was associated to mutations in the
111 **Kelch**-type propeller domain of a single parasite protein called **K13** [13]. K13 shares homologous
112 BTB and Kelch domains with proteins that function as substrate adaptors facilitating protein
113 **ubiquitination** *via* cullin-3 ligases. Some mutations in the Kelch domain of other Kelch-containing
114 proteins decrease the binding of protein substrates, and their ubiquitination and degradation.

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

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

129 Several other parasite determinants were shown to associate with some ART resistance in strains
130 pressured by ART *in vitro*, including increased chaperone production and proteostasis protein

131 expression (see below), and amplification of *pfmdr1* [see 20, 21]. Notably, the *P. falciparum*
132 phosphatidylinositol-3-kinase (PI3K) has been proposed to play a central role in resistance. The
133 polyubiquitination level of PI3K is controlled by K13 [22], presumably through a direct interaction but
134 which still needs to be formally demonstrated. In a K13 mutant background, the K13-PI3K interaction
135 may be altered, leading to decreased ubiquitination and increased levels of PI3K, along with its
136 products, e.g. the lipid phosphatidylinositol-3-phosphate (PI3P), which was found to be sufficient to
137 confer modest ART resistance *in vitro* [22]. It was also hypothesized that a ‘proteostatic’ mechanism
138 dependent on PI3P⁺ vesicle formation from the ER could help remove misfolded or aggregated
139 proteins, a process similar to **autophagy** [21-25].

140

141 **A central response to ART-induced stress: the unfolded protein response**

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

153 Importantly, recent functional studies have shown that an accumulation of ubiquitinated proteins
154 (UbP) was the main toxic event associated with ART treatment. First, the levels of UbP increase upon
155 parasite exposure to ART, with resistant parasites exhibiting lower levels of UbP post-exposure [22,
156 28, 29]. Second, inhibition of deubiquitinases, which remove ubiquitin from substrates before
157 degradation or from the ubiquitin receptor, exacerbates the build-up of UbP and lead to parasite death
158 following ART treatment; conversely, inhibition of the ubiquitin machinery or depletion of
159 intracellular pools of activated ubiquitin (with compound C1, 5'-O-sulphamoyl-N(6)-[(1S)-2,3-
160 dihydro-1H-inden-1-yl]-adenosine), or inhibition of protein translation (with cycloheximide), all
161 reduced ART-mediated accumulation of UbP and strongly antagonized ART-mediated killing [29].
162 Third, proteasome inhibitors (e.g., epoxomicin) cause an accumulation of UbP [29], and strongly
163 enhance ART activity against both ART-sensitive and ART-resistant parasites [28]. Fourth, ART
164 depresses proteasome function, which may occur by clogging the proteasome with UbP or *via* direct
165 proteasome damage by ART [30]. In sum, the emerging picture of ART-induced injury is that of a

166 'double whammy' [29], i.e. misfolded/unfolded protein generation and proteasome inhibition, death
167 coming from an excess of UbP and an unresolved ER stress. Of note, mutations in deubiquitinase,
168 UBP1, were identified in rodent malaria parasites subjected to ART pressure [31] as well as in African
169 [32] and Southeast Asian [33] *P. falciparum* isolates with decreased ACT efficacy. Fittingly, adapted
170 and natural resistant parasites typically displayed a deceleration of the developmental cycle during
171 early stages following an ART pulse, which is reminiscent of the cell stress response observed in
172 many organisms [5, 12, 27, 28].

173

174 **The terminal sensor/effector of ER stress response: the PK4-eIF2 α connection**

175 In mammalian cells, UPR is a cellular stress response that modulates phosphorylation of the
176 **eukaryotic translation initiation factor 2 α** (eIF2 α), which delivers tRNA initiators to ribosomes
177 [34]. In turn, phosphorylated eIF2 α which forms an inactive eIF2-eIF2B complex, leads to a general
178 reduction in protein synthesis, accompanied by preferential translation of mRNAs encoding products
179 that are important for recovery from the stress. Only three kinases of eIF2 α are expressed in
180 *Plasmodium*: IK1, IK2 and PK4 [35]. IK1 is dispensable for blood stages and, like its mammalian
181 homologue GCN2, regulates eIF2 α phosphorylation in response to amino acid starvation [36, 37]. IK2
182 ensures the latency of sporozoites in mosquito salivary glands [38]. PK4 is a homologue of
183 mammalian PERK, an integral ER membrane protein with an N-terminus that protrudes in the ER
184 lumen and senses unfolded proteins and a cytoplasmic C-terminal catalytic domain that, upon
185 activation, phosphorylates eIF2 α . PK4 was shown to be essential for blood stage growth using
186 Flp/FRT conditional mutagenesis in *P. berghei* [39] and inducible knockdown by riboswitch in *P.*
187 *falciparum* [29].

188 Recently, two studies linked ART-induced latency and recrudescence to PK4 and phosphorylation of
189 eIF2 α in these two *Plasmodium* species. The first [40] showed that: (i) ART treatment resulted in
190 eIF2 α phosphorylation; (ii) ART activated PK4, which is ER-resident; (iii) overexpression of a PK4
191 dominant-negative or pharmacological inhibition of PK4/PERK (ER and PERK being absent from
192 erythrocytes) blocked parasites from entering latency and abolished recrudescence after treatment in
193 *P. berghei*-infected mice; (iv) augmentation of eIF2 α phosphorylation by salubrinal increased
194 recrudescence rates to 100% ; and (v) eIF2 α was phosphorylated in the young ring stage of an ART-
195 resistant K13-mutated parasite but not in the young ring stage of its ART-sensitive K13 wild-type
196 parent strain. The second study [29] largely confirmed these data, while adding that: (i) eIF2 α
197 phosphorylation in rings occurred upon exposure to ART in a concentration-dependent manner; and
198 (ii) inhibition of the ubiquitin machinery (with compound C1) or of protein translation (with
199 cycloheximide) both strongly antagonized ART-mediated killing, and also strongly inhibited DHA-
200 mediated eIF2 α phosphorylation. It is therefore tempting to speculate that eIF2 α phosphorylation

201 ultimately mediates the various accounts of latency as well as cell cycle elongation/growth retardation,
202 particularly during early stages of parasite development (see Figure 4 for a quick overview of 10 years
203 of *falciparum* artemisinin resistance research).

204

205 **A model for ART activity and resistance**

206 As schematized in Figure 5 (Key Figure), following activation by free heme, DHA induces protein
207 alkylation and misfolding. The parasite then triggers an UPR. In the cytosol, the response will tend to
208 ubiquitinate unfolded proteins and degrade them in the proteasome. However, since DHA also inhibits
209 the proteasome, ubiquitinated proteins will accumulate and eventually lead to parasite death. The
210 parasite may also activate a PI3P⁺-dependent vesicle formation process, possibly linked to autophagy,
211 to help remove UbP. In the ER, UbP induce an ER-stress response, which activates/oligomerizes the
212 ER membrane-bound PK4. Activated PK4 in turn phosphorylates eIF2 α , which induces parasite
213 translational arrest/slow-down to limit protein neosynthesis. In this context, a mutated K13 may help
214 the parasite resist ART injury in several ways: by decreasing polyubiquitination of UbP, and thus
215 accumulation of lethal UbP, and by decreasing the release of free heme, and thus decreasing ART
216 activation. Also, mutations in K13 may be involved in the control of eIF2 α phosphorylation thereby
217 enhancing delay of the process of ring maturation into trophozoites. This continued slowdown effect
218 of cell metabolism and translation would allow the parasite to withstand DHA induced stress, and
219 potentially lead to a complete cessation of parasite development, mimicking a dormant parasite.

220

221 **What kind of latency?**

222 Conflicting accounts were made on morphological features of parasite latent forms associated with
223 ART pressure *in vitro*. Some displayed a round shape retaining a small amount of visible cytoplasm
224 and condensed chromatin (as opposed to **pyknotic** parasites) [11,41-43], whilst another study
225 described dormant forms as indistinguishable from ring stage parasites [9]. Dormant forms were also
226 found as retaining a mitochondrial membrane potential and associated with a compact mitochondrion
227 [42, 44], although only a subset of FACS-sorted parasites with a positive membrane potential were
228 capable of resuming growth. The duration of latency was also shown to be highly variable, lasting
229 from only 24 hours to 25 days post exposure [11]. Given the diversity of these phenotypes, are we
230 facing parasites in a formal latency stage, or is the observed latency phenotype just the result of a
231 probability of survival of the parasites population depending on its size, its genetic background and the
232 dose of DHA used?

233 Indeed, a major unresolved question is whether early blood stage developmental arrest and/or slow-
234 down results from an existing, qualitative control mechanism, i.e., a **cell cycle checkpoint** controlling

235 progression along the erythrocytic cycle, possibly by sensing ROS, or from a progressive decrease in
236 translation, irrespective of any control mechanism, leading to a gradual slowdown in parasite
237 development possibly culminating in a complete arrest. In other words, is it a real developmental
238 process into a *bona fide* latent stage (able to be identified by a “biological signature”) or is it only a
239 quantitative slowdown of the translation without any specificity? (Figure 2)

240 In favor of the first scenario, it is known that many *P. falciparum* strains [11] and rodent-infecting
241 parasites [45] are capable of entering a latency state without the necessity to preselect them by ART
242 pressure. In addition, as already mentioned, other *Plasmodium* stages, i.e., gametocytes, sporozoites
243 and liver stages, are known to be able to arrest translation at specific developmental steps as a means
244 to halt their cycle progression awaiting vector-to-host transmission. Nevertheless, it is important to
245 note that IK2-mediated latency as observed in salivary gland of sporozoites and PK4-mediated ER
246 stress response mediated arrest of translation would be expected to have different molecular
247 mechanisms, and that IK2 knock-out has no effect on ART sensitivity. Furthermore, blood stages are
248 known to be able to adapt their development in response to specific environmental conditions, such as
249 amino acid starvation that was shown to induce parasite ‘hibernation’ [46]. Latency may therefore be a
250 natural trait, or at least a natural option, of blood stages that allows them to escape certain types of
251 stress not limited to drug exposure. ART resistance may thus be linked to an increased parasite
252 propensity to engage into latency. There is one report of persistent low-density parasite DNA (using
253 ultra-sensitive PCR) following ACT treatment in Myanmar. Authors presume this signature originates
254 from dormant parasites [47]. However, despite persistence observed for up to 3 weeks, no
255 recrudescence was observed raising questions as to the viability of these parasites [47]. To date,
256 experimental proof of the existence of such naturally occurring, dormant blood stages is still lacking –
257 which may be due to their small proportion and the lack of adequate tools to isolate them.

258 In favor of the second scenario, there appears to be a quantitative correlation between parasite
259 survival, measured by the RSA, and the dose of DHA for a given parasite genetic background. This
260 could also explain the efficacy of longer courses of artemisinin derivatives in ACT clinical studies in
261 Southeast Asia [49]. As mentioned above, DHA must be activated and many studies agree that its
262 main bioactivator is heme – which breaks its endoperoxide bond. Free heme is released by the
263 degradation of **metalloproteins** and in particular hemoglobin in the case of late blood stages
264 (hemoglobin degradation starts around 6-10 hours after red blood cell invasion). Since DHA is also
265 active in young blood stages, the latter must contain free heme, and it has been shown that hemoglobin
266 digestion has already started in young rings even though the digestive vacuole is still not formed [49].
267 Alternative sources of free heme may be from heme biosynthesis or heme-containing proteins such as
268 the ones present in the mitochondrion and apicoplasts and/or the degradation of metalloproteins other
269 than hemoglobin [50]. K13 may be involved in the latter process by controlling the
270 ubiquitination/degradation of metalloproteins, thereby decreasing the release of free heme and thus

271 lowering ART activation and activity in young resistant stages. This quasi-linear relationship between
272 the amount of activated DHA and the probability of survival of the parasite is rather in favor of a
273 continuous process.

274

275 **Concluding remarks**

276 Few topics in malaria research carry as much importance for the future of malaria control as
277 understanding ART resistance. ART injury and resistance are not only of a non-canonical nature but
278 also involve numerous cellular and metabolic pathways, making its study a massive challenge. In this
279 review, we have outlined how different molecular and cellular mechanisms participate to ART
280 resistance. Specifically we have tried to reconcile how different components of the stress response and
281 translation dynamics may affect cellular survival to ART, the exact molecular linkers and the dynamic
282 interplay between those cellular processes will still have to be addressed experimentally. We have also
283 stressed the importance of understanding the natural history of latency (see Outstanding Questions).
284 The nature of latency is still not understood, specifically if it is a physiological regulated process or an
285 adaptive arrest, what are the cellular and molecular players involved and what the dynamic of the
286 progression through latency is. Solving such questions may lead to more potent approaches to target
287 drug-resistant parasites. Some technical issues have to be resolved in order to address these questions:
288 1) to identify and isolate *P. falciparum* parasite at specific stages of their intracellular erythrocytic
289 development (perhaps through single cells technology); 2) to identify the K13-containing complex(s)
290 during the erythrocytic cycle, and its alteration(s) associated with K13 mutations.

291

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295

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422 retrospective genetic study. *Lancet Infect Dis* 18:337–345
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426 **Glossary**

- 427 • **Alkylation:** alkylation is the transfer of an alkyl group from one molecule to another.
- 428 • **Artemisinin based combination therapies (ACT):** a combination of two molecules: a
- 429 semisynthetic molecule derived from artemisinin and a synthetic molecule whose role is to
- 430 increase the effect of the first molecule mostly due to their long half-life.
- 431 • **Autophagy:** denotes a degradation of a part of the cytoplasm of the cell by its own lysosomes.
- 432 • **Cell cycle checkpoint:** A checkpoint is a point in the eukaryotic cell cycle at which the
- 433 progression of a cell to the next stage in the cycle can be halted until conditions are favorable.
- 434 • **Eukaryotic translation initiation factor 2 α (eIF2 α):** the protein eIF2 α catalyzes an early
- 435 regulated step of protein synthesis initiation, promoting the binding of the initiator tRNA to
- 436 40S ribosomal subunits.
- 437 • **Kelch domain:** this sequence motif is composed of about 50 amino acid residues which form
- 438 a structure of a four stranded beta-sheet "blade".
- 439 • **Metalloprotein:** generic term for a protein that contains a metal ion cofactor. A large number
- 440 of all proteins are part of this category.
- 441 • **PfK13:** *Plasmodium falciparum* protein (PF3D7_1343700), the presence of non-synonymous
- 442 mutations in his propeller domain (kelch domain) has been associated with decreased efficacy
- 443 of artemisinin derivatives in the young stages of parasites.
- 444 • **Proteasome:** protein complexes which degrade unneeded or damaged proteins by proteolysis.
- 445 • **Pyknotic:** irreversible condensation of chromatin in the nucleus of a cell undergoing death.
- 446 • **Rings:** red blood cell stages from 0 to 24 hours post invasion.
- 447 • **Sporozoites:** parasite stages transmitted from mosquito to mammal.
- 448 • **Trophozoites:** Red blood cell stages from 24 to 40 hours post invasion.
- 449 • **Ubiquitination:** enzymatic process that involves bonding of a ubiquitin protein to a substrate
- 450 protein that usually becomes inactivated and tagged for degradation by the proteasome as a
- 451 result.
- 452 • **Unfolded protein response (UPR):** cellular stress response related to the endoplasmic
- 453 reticulum (ER) stress by unfolded proteins.

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460 **Box 1. Dormancy, quiescence and latency.**

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

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

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

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

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477 **Legend to Figures**

478 **Figure 1. Schematic representation of the erythrocytic cycle of *Plasmodium falciparum*.** In this 48
479 h cycle, the parasite undergoes multiplication via endomitosis, with different stages of the cycle
480 having different drug sensitivities. Green arrows highlight the supposed highest activity window of
481 artemisinin derivative drugs. The parts of the lifecycle that have the lowest sensitivity to ART in K13
482 WT parasites are ~6-16 hpi and the last few hours of schizogony. The red bar corresponds to the
483 standard DHA pulse in an RSA test (see Figure 3).

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

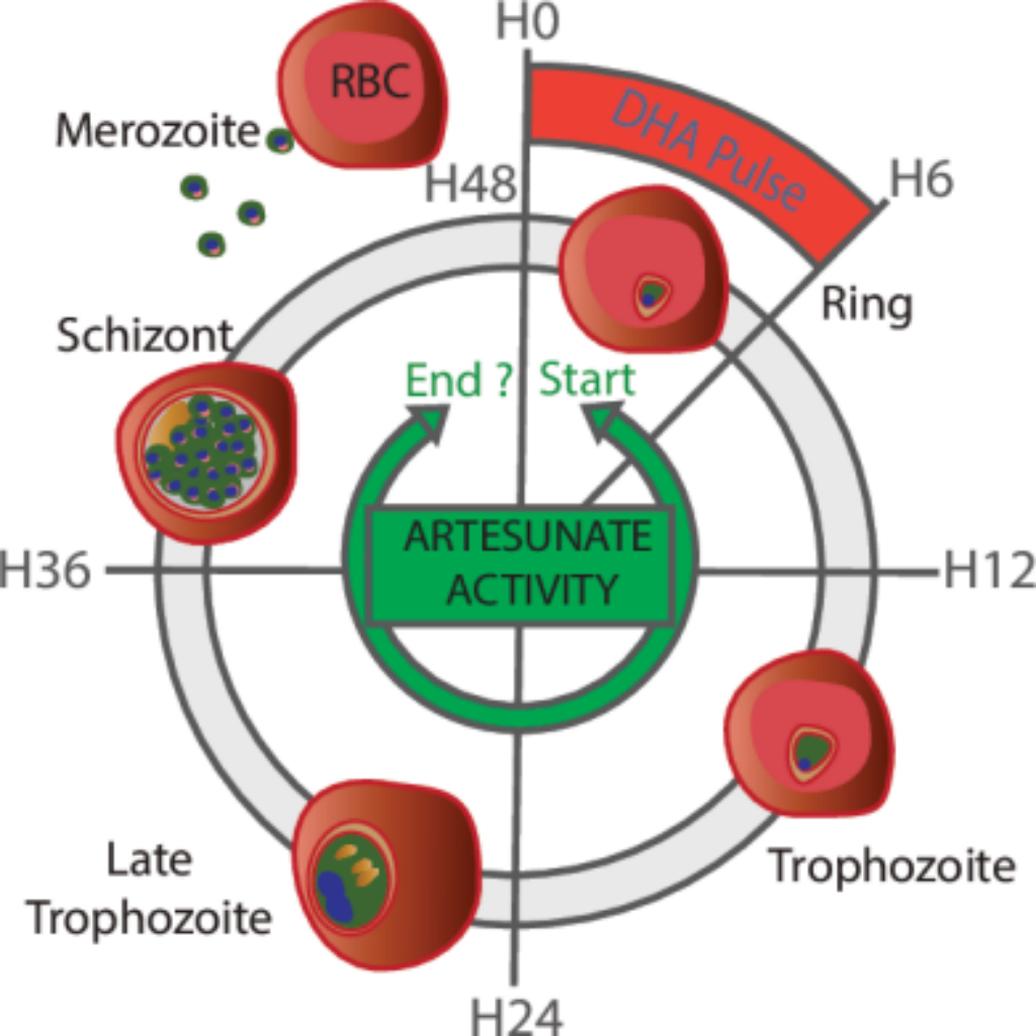
490 **Figure 3. Schematic representation of the ring-stage survival assay (RSA).** Parasites are exposed
491 to a pulse of DHA, and the relative survival rate is measured after another erythrocytic cycle.
492 Resistance to DHA is defined as an RSA value above 1%. Circular versus dotted parasites symbolize
493 viable and pyknotic parasites, respectively.

494 **Figure 4. A quick overview of 10 years of *P. falciparum* artemisinin resistance research.** In green
495 (Top) are major publications related to clinical and *in vitro* studies, and in orange (Bottom) are
496 genomics and transcriptomics studies. ART, artemisinin; DHA, dihydroartemisinin; eIF2 α , eukaryotic
497 translation initiation factor 2 α ; PI3P, phosphatidylinositol-3-phosphate; RSA, ring-stage survival
498 assay; SEA, Southeast Asia; UPR, unfolded protein response.

499 **Figure 5, Key Figure. Proposed model of the induction by the unfolded protein (UP) response of**
500 **different pathways in ART resistance.** Different pathways include protein polyubiquitination (Ub),
501 proteasome, phosphatidylinositol-3-kinase (PI3K) and Protein Kinase 4 (PK4) / eukaryotic translation
502 initiation factor 2 α (eIF2 α) and their potential interactions with mutations in K13, the main identified
503 artemisinin resistance marker. Inhibition of the proteasome by the activated DHA (blue), associated
504 with a reduced ubiquitination due to the K13 mutations (green), reduces the harmful effects of protein
505 polyubiquitination and accumulation (red) and lead to a decrease in the degradation of metalloproteins
506 and subsequent reduced release of DHA activator heme. This virtuous circle contributes to the reduced
507 effectiveness of DHA in mutant K13 parasites without decreasing direct activation of PI3P and PK4
508 related protective pathways.

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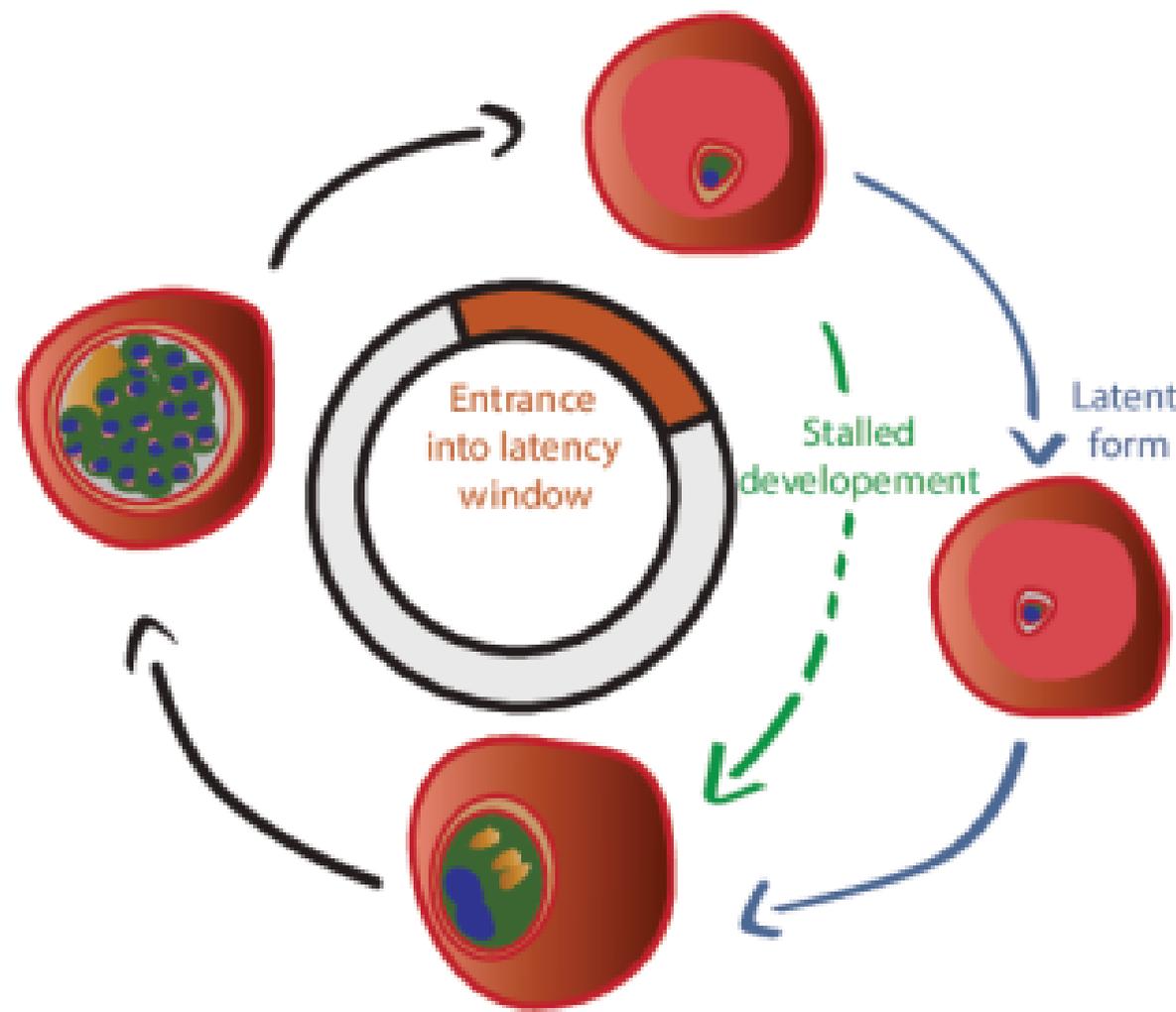
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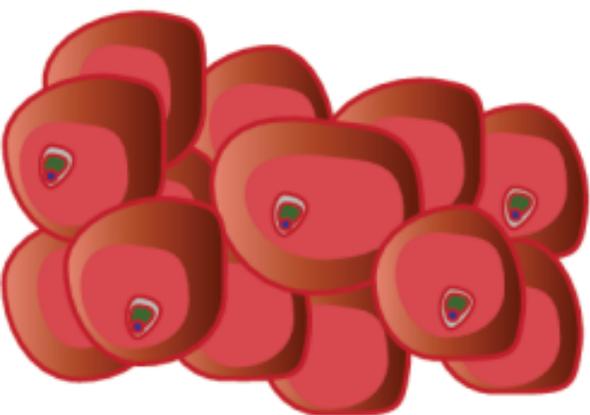
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Organism	Stage	Latency type	Duration of latency
<i>Plasmodium</i> spp	Ring	? ART-induced	days to weeks
<i>Plasmodium</i> spp	Mature Gametocyte	Developmental, obligate	several days to a week
<i>Plasmodium</i> spp	Sporozoite	Developmental, obligate	several days to a week
<i>Plasmodium vivax</i> <i>Plasmodium ovale</i>	Hypnozoite	Developmental, facultative	months-years
<i>Toxoplasma gondii</i>	Bradyzoite	Developmental, stress-induced, facultative	months-years

B



Synchronisation
DHA or RPMI

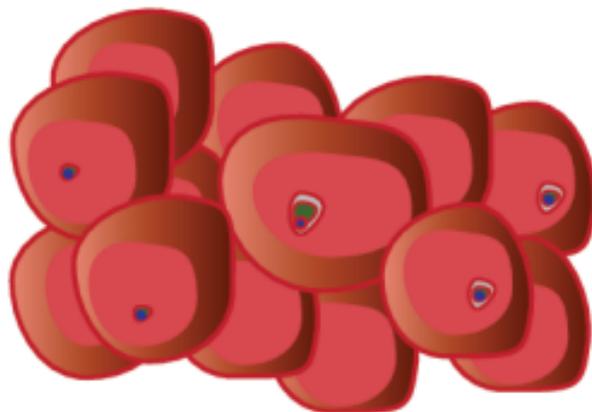


Ring 0-3 H

6 h DHA
pulse



DHA
Wash out

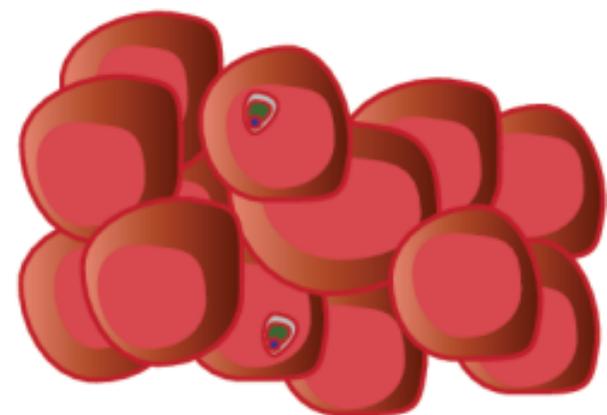


Ring 6-9 H

Culture
for 66 h



Survival



72-75 H

$$\text{RSA} = \frac{\% \text{ Parasitemia after DHA treatment}}{\% \text{ Parasitemia without treatment}} \times 100$$

