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IMMUNOLOGY AND HOST-PARASITE INTERACTIONS - REVIEW

Current scenario and future strategies to fight artemisinin resistance

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Abstract

Despite several setbacks in the fight against malaria such as insecticide and drug resistance as well as low efficacy of available vaccines, considerable success in reducing malaria burden has been achieved in the past decade. Artemisinins (ARTs and their combination therapies, ACTs), the current frontline drugs against uncomplicated malaria, rapidly kill plasmodial parasites and are non-toxic at short exposures. Though the exact mode of action remains unclear, the endoperoxide bridge, indispensable for ART activity, is thought to react with heme released from hemoglobin hydrolysis and generate free radicals that alkylate multiple protein targets, thereby disrupting proteostasis pathways. However, rapid development of ART resistance in recent years with no potential alternatives on the horizon threaten the elimination efforts. The Greater Mekong Subregion in South-East Asia continues to churn out mutants resistant to multiple ACTs and detected in increasingly expanding geographies. Extensive research on ART-resistant strains have identified a potential candidate Kelch13, crucial for mediating ART resistance. Parasites with mutations in the propeller domains of *Plasmodium falciparum* Kelch13 protein were shown to have enhanced phosphatidylinositol 3-kinase levels that were concomitant with delayed parasite clearance. Current research focused on understanding the mechanism of Kelch13-mediated ART resistance could provide better insights into *Plasmodium* resistome. This review covers the current proposed mechanisms of ART activity, resistance strategies adopted by the parasite in response to ACTs and possible future approaches to mitigate the spread of resistance from South-East Asia.

Keywords Plasmodium · Artemisinin · Artemisinin resistance · Artemisinin combination therapies · Kelch13 · Proteostatic dysregulation · Free radicals · Oxidative stress

Introduction

The global malaria effort seems to have plateaued with the recent WHO report indicating ~445,000 deaths occurred in 2016 as compared to ~446,000 deaths in 2015 (WHO 2016, 2017a). Effective methods for malaria prevention still lie in vector control through reducing contact between humans and malaria-propagating vectors. Successful prevention strategies include usage of insecticide-treated bed nets, indoor residual spraying (IRS), and malathion fogging (Bayoh et al. 2010; Mutuku et al. 2011; Singh et al. 2014). More recently, endosymbiotic *Wolbachia* bacteria have been employed in several geographic locations for controlling mosquito populations (Werren et al. 2008; Shaw et al. 2016). On the other hand, treatment of malaria infection includes administering either artemisinin (ART) combination therapies (ACTs) or vaccination with vaccines such as RTS, S. However, emerging drug resistance in parts of Africa and South-East Asia and further lack of potent long-term vaccine candidates threaten current malaria eradication efforts.

Malaria is caused by the apicomplexan group of protozoan parasites belonging to the genus *Plasmodium*. The species responsible for human malaria infections include *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* with *P. knowlesi* being the latest addition (Figtree et al. 2010; Ahmed and Cox-Singh 2015).

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Of the five species, *P. falciparum* is most fatal to humans especially in young children below 5 years and in pregnant women (Uneke 2007; Schantz-Dunn and Nour 2009; Schumacher and Spinelli 2012). *P. vivax*, the other deadly parasite, can stay dormant in its hosts for prolonged periods before reemergence (recrudescence) even after treatment (Campo et al. 2015). Published reports indicate that in India, *P. vivax* accounts for ~ 40% of malaria cases, a sixth of global infections, therefore demands equal attention as *P. falciparum* (Anvikar et al. 2016).

Earlier antiplasmodial drugs and development of resistance

Several efforts were undertaken to develop antimalarial drugs that can inhibit key pathways or enzymes and thus kill or arrest parasite growth. Majority of the drugs were initially isolated from natural sources and later semi-synthetic variants were developed to improve potency. Hemoglobin degradation, a key process within the parasite machinery, generates a toxic by-product heme that is converted to non-toxic hemozoin. Methanolquinoline class of compounds including mefloquine, quinine, and lumefantrine specifically target hemozoin formation (Nosten et al. 2012). Another class of compounds called aminoquinolines contain potent members such as chloroquine and amodiaquine which prevent heme crystallization, leading to accumulation of toxic heme, while primaquine was found to target the hypnozoite and gametocyte stage parasites (Foley and Tilley 1998; Egan 2006; Müller and Hyde 2010). Processes involving DHFR (dihydrofolate reductase) and DHPS (dihydropteroate synthetase) enzymes which play important roles in purine and pyrimidine biosynthesis have been targeted by DHFR inhibitors such as pyrimethamine and chloro or sulfonamide compounds such as sulfadoxine (Nzila 2006). The sesquiterpene lactone group of compounds include dihydroartemisinin (DHA), artesunate, and ART; function based on their alkylating capabilities, thus destabilizing and killing the parasite (Amorim et al. 2013). The parasite cytochrome bc1 complex was also targeted by the drug atovaquone which inhibits the electron transport chain (ETC) functioning in parasite mitochondria (Srivastava et al. 1999; Kessl et al. 2003) (Table 1).

The uncontrolled use of antimalarial drugs in the last 50 years led to their prolonged exposure to the parasite, resulting in the development of drug resistance. Drug resistance w.r.t malaria is defined as delayed parasite clearance upon drug administration, thereby reducing the drug effectiveness and/or increase in dosage to achieve parasite clearance (Witkowski et al. 2013; White 2017). Severe malaria causing *P. falciparum* has developed resistance to almost all the

Table 1 Summary of different class of compounds used for the treatment of *P. falciparum* infections discussed in this study

Antimalarial drugs							
Hemozoin inhibitors			Antifolates		Sesquiterpene lactones	Miscellaneous compounds	Combinations (ACTs)
Methanolquinolines	Aminoquinolines	Others	DHFR inhibitors	Sulfonamides			
Melfoquine	Chloroquine	Lumefantrine	Pyrimethamine	Sulfadoxine	Artemisinin	Atovaquone	Artemether-lumefantrine (AMLF)
Quinine	Primaquine	Halofantrine	Chloroproguanil		Artesunate	Diaminodiphenyl sulfone (DDS)	Artesunate-melfoquine (ASMQ)
	Amodiaquine		Sulfadoxine-pyrimethamine (SP)		Artemether	Piperaquine	Artesunate-amodiaquine (ASAQ)
					Dihydroartemisinin (DHA)	Tetracycline	Dihydroartemisinin-piperaquine (DHA-PPQ)
							Artesunate-sulfadoxine-pyrimethamine (ASSP)

current drugs in use with resistance to chloroquine arising in 1950s, quinine and antifolates in 1980s, and more recently to ARTs in 2000s in certain parts of the world (White 2008; Dondorp et al. 2009; Petersen et al. 2011; Cui et al. 2015). Gene duplications of *pfmdr1* and single nucleotide polymorphism (SNP) N1042D were found to be responsible for reduced drug sensitivity of quinine (Sidhu et al. 2005; Pascual et al. 2013; Veiga et al. 2016). Resistance against chloroquine drug involved the SNP K76T in transporter *pfcrt* gene which enhanced the efflux of chloroquine out of the parasite and recent studies have associated the role of PfCRT in iron homeostasis (Pascual et al. 2013; Bakouh et al. 2017). Antifolate drug treatment such as sulfadoxine-pyrimethamine (SP) combination therapy which targeted purine and pyrimidine synthesis pathways were effective against chloroquine resistance parasites (Plowe et al. 1998; Gregson and Plowe 2005); however, mutations in DHFR and DHPS that decreased their binding affinity of to pyrimethamine were reported in sub-Saharan Africa and in South-East Asia, rendering the treatment futile (Alifrangis et al. 2003; Sharma et al. 2015). The ETC of the parasite was targeted by the drug atovaquone, but SNPs at codon 268 in the cytochrome b gene (*pfcytb*) made the drug ineffective upon parasite treatment (Sutherland et al. 2008). Other *Plasmodium* species though not widespread as *P. falciparum*, were also reported to develop resistance against commercially used antimalarial drugs. Chloroquine-resistant *P. vivax* strains were found in parts of South-East Asia and South America (Baird 2004; de Santana Filho et al. 2007), while SP resistance by *P. vivax* was associated with the presence of a single amino acid in DHPS enzyme (Korsinczky et al. 2004). *P. malariae* strains were discovered to have developed resistance to chloroquine in Southern Indonesia (Maguire et al. 2002).

Artemisinins: structure and mechanism of action

ART (Chinese: Qinghaosu), extracted from the Chinese plant *Artemisia annua*, has been used in traditional Chinese medicine to treat malaria since 1500s (Tu 2011). Since the development of resistance to most antimalarials by 1980s, ARTs and its derivatives have gained importance as a successful treatment model against uncomplicated malaria. Commonly used ART derivatives include artesunate, DHA, and artemether (Cui and Su 2009) (Fig. 1a). While pure ART has poor pharmacokinetic properties, its derivative artesunate is water soluble. DHA is the active metabolite in all ARTs while artemether is used against both uncomplicated and chloroquine-resistant *P. falciparum* and *P. vivax* parasites. Although piperaquine, a bisquinoline antimalarial, declined in usage owing to *P. falciparum* resistance in 1980s, it has recently been suggested to be administered in combination with ART derivatives owing to its longer half-life (Davis et al. 2005). Broad-spectrum antibiotics such as tetracycline were also used along with ARTs due to its potency in areas with chloroquine-resistant *P. falciparum* parasites (Ye and Van Dyke 1994; Gaillard et al. 2015).

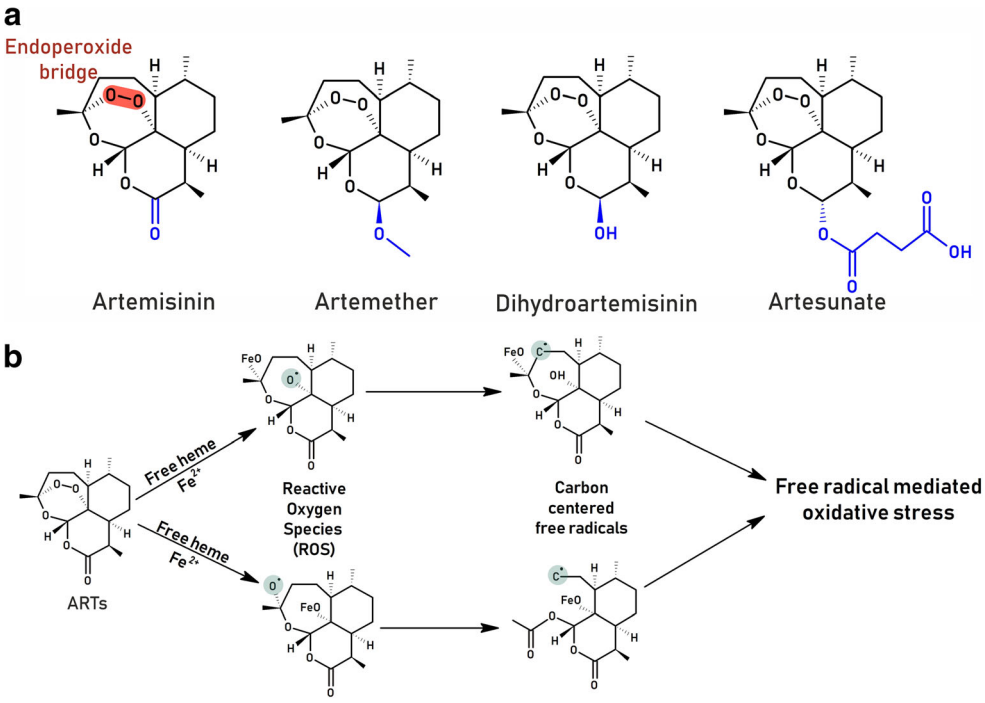

Fig. 1 Structural comparison of ART, its derivatives, and proposed mechanism of activation. **a** The endoperoxide bond is highlighted in red. The motifs differing in each derivative are highlighted in blue. **b** The prodrug ART is thought to undergo reductive activation in the presence of free heme/Fe^{2+} ions at either O1 or O2 positions and generate ROS which further rearranges to form carbon-centered free radicals (highlighted). Adapted and modified from Tonmunphean et al. (2001)

ART and its derivatives contain the signature endoperoxide bridge which upon reductive activation (Fig. 1a, highlighted in red) leads to the formation of reactive oxygen species (ROS, ex. suerpoxides (O_2^-), peroxides (O_2^{-2})) which further reorganize to form carbon-centered free radicals. ART derivatives devoid of the peroxide bond were found to have no antimalarial activity indicating its essentiality (Kaiser et al. 2007). The activation of these pro-drugs is mainly thought to occur upon interaction of ARTs with free heme and to a partial extent by ferrous ions, though the exact mechanism is still debated (Klonis et al. 2013a; Tilley et al. 2016) (Fig. 1b). As heme is inherently toxic to the parasite, heme monomers are polymerized into hemozoin crystals; however, two contrasting mechanisms have been proposed (Coronado et al. 2014). The heme detoxification protein (HDP) was validated to play a crucial role in heme polymerization (Jani et al. 2008), which along with proteases of multiple classes create a hemozoin formation complex that detoxifies free heme (Chugh et al. 2013). This theory also supports earlier findings that heme-ART adducts disrupt hemozoin formation (Pandey et al. 1999; Kannan et al. 2002). The other proposed mechanism states that hemozoin formation is autocatalytic and is promoted by a neutral lipid environment based on the evidence that *P. falciparum* initial hemozoin crystals are always directly observed enveloped in neutral lipid droplet-like structures (NLDs) (Hartwig et al. 2009; Hoang et al. 2010). It further argues hemozoin formation is apparent due to heme's inherent chemical properties with NLDs serving as nucleation centers (Kapishnikov et al. 2012) and more importantly that no protein involvement is required for this process (Egan 2008a, b). Since most of the heme is synthesized during hemoglobin degradation in the trophozoite stage, ARTs were shown to be active at trophozoite stage and inactive against mature gametocyte and liver stages (Meister et al. 2011). ARTs were also found to be effective against very early-ring stage parasites but were relatively ineffective against mid-ring stage parasites (Klonis et al. 2013a, b). After ART treatment, parasite remnants are removed from infected erythrocytes in a process called "pitting" where the previously infected erythrocytes are re-introduced back into the bloodstream (Chotivanich et al. 2000; Buffet et al. 2006).

The natural river basin of the Mekong River called the Greater Mekong Subregion (GMS), continues to be the driver of resistance against all the drugs used against malaria. While ART monotherapy was initially used to treat malaria, resistance was first reported in Cambodia in 2008 (Noedl et al. 2010). Delayed parasite clearance was observed and increased ART dosage was used to achieve parasite clearance similar to ART-sensitive strains in Thailand and Cambodia in 2009 (Dondorp et al. 2009). In Thailand-Myanmar border, parasite clearance half-lives upon artesunate treatment were found to increase from 2.6 h in 2001 to 3.7 h in 2010 (Phyo et al. 2012). Owing to the poor half-life and pharmacokinetic properties, and rapid emergence of resistance, WHO prohibited the use of pure ART or derivative monotherapies (WHO 2012; Elfawal et al. 2015) and recommended ACTs consisting of fast-acting ARTs combined with a slow-acting drug of different class, resulting in different modes of action and thus making it harder to develop resistance.

ACTs, the current frontline drug treatment against uncomplicated malaria infection, are administered selectively depending on the presence of resistance to any of the co-administered drugs. The current WHO-recommended therapies include artemether-lumefantrine (AMLF), artesunate-melfoquine (ASMQ), artesunate-amodiaquine (ASAQ), DHA-piperaquine (DHA-PPQ), and artesunate-sulfadoxine-pyrimethamine (ASSP) (WHO 2015) (Table 1), while in places where ACTs are not readily available, SP + amodiaquine (SPAQ) treatment is recommended for seasonal malaria chemoprevention (SMC) depending on the geography and severity of the ART resistance. While ACTs achieved considerable success in curbing malaria, the threat of resistance has forced regions in GMS to switch to different strategies (Alonso and Noor 2017). Cambodia currently has been identified to contain strains resistant against all the 5 ACTs while Thailand, Laos, and Vietnam were identified to have strains resistant to 2 ACTs while Myanmar, parts of China, and North-East India have been found to contain markers known to be resistant against one ACT (ASSP) (WHO 2017b, a) (Fig. 2).

Genetic knockdown of one of the two copies of *pfmdr1* gene resulted in increased susceptibility to almost all ART partner drugs including mefloquine, lumefantrine, halofantrine, quinine, and artemisinin, while the N-terminal N86Y mutation confers resistance to chloroquine and amodiaquine (Sidhu et al. 2006; Veiga et al. 2016). Whole genome sequence analysis of SE Asian (Cambodian) *P. falciparum* field isolates where DHA-PPQ was the preferred treatment model identified unique mutations in the *pfcrt* gene (F145I, M343L, or G353V) which resulted in increased survival rates. Given that PPQ is structurally similar to chloroquine containing two chloroquine moieties, it seems plausible that a similar strategy of PfCRT mediated chloroquine resistance is being employed (Ross et al. 2018). A single PfCRT C101F mutation generated through zinc-finger nuclease (ZFN) technology conferred PPQ resistance in Dd2 strains (Dhingra et al. 2017). Other genes such as plasmepsin II/III were found to have elevated copy numbers upon DHA-PPQ treatment and also correlated with DHA-PPQ treatment failure (Witkowski et al. 2017; Bopp et al. 2018) implying the involvement of hemoglobin degradome proteases in mitigating resistance. These studies collectively show the gradual rise in failures of ACT partner drugs and the threat of emerging ACT resistance.

Fig. 2 Spread of ACT resistance across GMS. The current WHO-recommended ACTs are quickly failing across South-East Asia. The Mekong River basin area in Cambodia has high failure rates against all 5 ACTs and these resistant strains are quickly spreading into new surrounding territories. Adapted and modified from WHO status report (WHO 2017b)

Kelch13 and its role in ART resistance

Since the discovery of the ART resistance in 2009 (Dondorp et al. 2009), concerted efforts to identify the molecular markers have found mutations in the gene *P. falciparum* Kelch propeller protein 13 (*pfk13*) in chromosome 13 that are correlated with ART resistance (Ariey et al. 2014). The Kelch group of proteins are highly conserved across different species with the closest human counterpart being KEAP1 that has a role in antioxidant stress response (Nguyen et al. 2003; Li et al. 2004; Wang et al. 2015b). The sequence of PfK13 indicates the presence of an N-terminal *P. falciparum*-specific region, a BTB/POZ (*B*road-Complex, *T*ramtrack, and *B*ric à brac; *Po*xvirus and *z*inc finger respectively) domain, followed by six Kelch repeat motifs (Fig. 3a). PfK13 was shown to localize near the food vacuole and the parasite membrane (Birnbaum et al. 2017). While the BTB/POZ domain was suggested to help in dimerization, solved crystal structure of PfK13 (PDB ID: 4YY8) (Jiang et al. 2015) indicated that the six Kelch motifs form a symmetric propeller structure with BTB/POZ domain protruding away in accordance with the crystal structure of human KEAP1 (Li et al. 2004) (Fig. 3b). Thus, PfK13 could play similar roles to that of human KEAP1 in mitigating antioxidant stress in *Plasmodium* parasites through protein ubiquitination. Bioinformatic analysis have identified interacting partners such as PF3D7_1353900 and PF3D7_1474800, while putative candidates such as PF3D7_1104400 and PF3D7_1461900 have been identified through pull-down/co-immunoprecipitation (Co-IP) assays in our lab that could help further advance our understanding of PfK13 interactions with the plasmodial proteasome (Atul et al., 2018 unpublished).

The landmark study by Ariey et al. identified mutations within the propeller domains in ART-resistant parasite populations in GMS. Four non-synonymous single nucleotide polymorphisms (SNPs) Y493H, R539T, I543T, and C580Y were shown to confer resistance against ART in Cambodia. The SNP N458Y was identified along the Thai-Myanmar border (Ashley et al. 2014; Talundzic et al. 2015). Another study indicated that the K13 mutations had the effect of shortening the duration of trophozoite stage, where the parasite is particularly susceptible to ARTs, and increasing the duration of the ring stage that is relatively ART insensitive due to low heme content (Hott et al. 2015). Presence of any of the above 5 mutations validated by both in vivo and in vitro data (Ashley et al. 2014; Straimer et al. 2015) (Fig. 3a, red highlights) indicates ACT resistance, while other candidate mutations are correlated with delayed parasite clearance and are yet to be verified in vitro (Fig. 3a, yellow highlights) (WHO 2017b). While less prominent than South-East Asian countries, regions in North-East India were documented for the first time to contain K13 candidate mutations (F446I and A578S) associated with parasite resistance (Mishra et al. 2015, 2016). Recently, another candidate mutation marker (A675V) has been observed in Northern Uganda in Africa (Ikeda et al. 2018). A total of ~ 130 PfK13 SNPs were identified until now in various parts of South-East Asia and Africa, though not all have been associated with ART resistance (Fairhurst 2015; Ménard et al. 2016).

Crucial targets of ARTs

ARTs interact with the plasmodial proteome in a ROS-dependent manner in which heme-activated ART generates ROS that proceeds to react with susceptible proteins and disrupt key pathways such as carboxylic acid and cellular amine metabolic pathways, and nucleoside and ribonucleoside synthesis pathways (Wang et al. 2015a). As described earlier, ARTs disrupt hemozoin formation leading to free heme accumulation which further exacerbates parasite stress response mechanisms. ARTs were shown to interact with the translationally controlled tumor protein homolog (PfTCTP) both in food vacuole (FV) and cytosol (Bhisutthibhan et al. 1998, 1999) and crucial pockets were identified within PfTCTP responsible for ART binding (Eichhorn et al. 2013). Since ARTs were structurally similar to thapsigargin, an inhibitor of human sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), an investigation of ART interactions with its *Plasmodium* ortholog PfATP6 indicated that ARTs

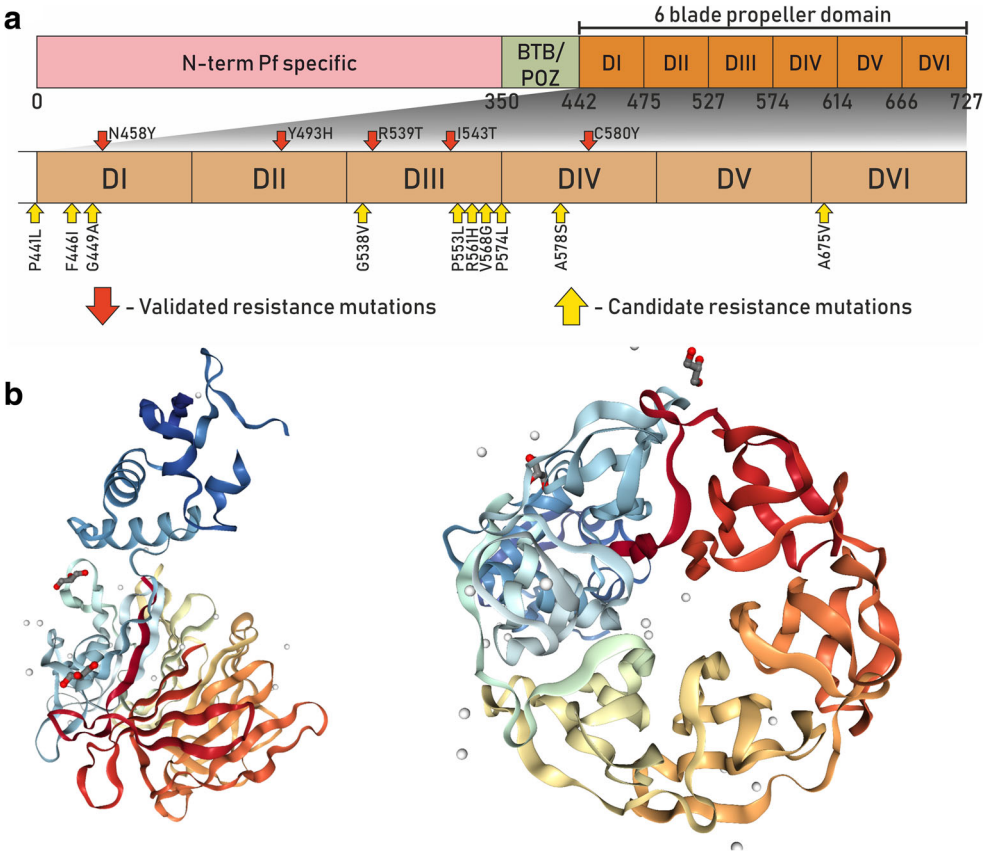

Fig. 3 Schematic of various domains of PfK13 and solved crystal structure. **a** Mutations that were validated to cause resistance and future candidate mutations yet to be verified in vitro are indicated in red and yellow, respectively. Validated and candidate resistance mutations are identified as per WHO definitions (WHO 2017b). **b** The front and bottom views of PfK13 indicate the orientation of six propeller domains. Images of the crystal structure were taken from PDB structure (PDB ID: 4YY8) and visualized using NGL Viewer (Jiang et al. 2015; Rose and Hildebrand 2015)

specifically inhibited PfATP6 (Ludwig 2003) and a single residue Leu^{263} was crucial for modulating ART activity (Uhlemann et al. 2005; Nagasundaram et al. 2016). The genes *pfmdr1* and *pfcrt* associated with chloroquine resistance were also shown to indirectly interact with ART, with SNPs in both genes observed in ART-exposed strains (Chavchich et al. 2010; Beez et al. 2011; Ferreira et al. 2011; Ehlgen et al. 2012; Eastman et al. 2016). However, not all mutations observed in these markers were associated with ART resistance (Afonso et al. 2006; Wang et al. 2015a). ARTs were also shown to interact with parasite mitochondria, with the ETC possibly activating ARTs (Wang et al. 2010) that rapidly depolarize mitochondrial membrane as well as the parasite plasma membrane (PM) (Antoine et al. 2014). ROS produced by ARTs were also shown to damage DNA by causing dsDNA breaks (Gopalakrishnan and Kumar 2015). Incidentally, SNPs were also identified in falcipain-2 (FP2), principal hemoglobinase of *P. falciparum* which interacts with HDP in heme detoxification process (Pandey et al. 2005; Chugh et al. 2013). Knockout of FP2 led to decrease in ART activity, indicating the importance of hemoglobin degradation in ART activation and subsequent ROS generation (Klonis et al. 2011; Conrad et al. 2014) (Fig. 4a).

The strong correlation between PfK13 SNPs and ART resistance stimulated further research to understand unknown molecular aspects of K13 interactions. Mutations in six blade regions of K13 could potentially alter the propeller structure, thus affecting downstream protein-protein interactions (Ariey et al. 2014). A previously characterized but an unlikely target of such interactions, *P. falciparum* phosphatidylinositol-3-kinase (PfPI3K), was identified when DHA specifically blocked production of phosphatidylinositol 3-phosphate (PI3P) (Mbengue et al. 2015), which is the resultant product of phosphatidylinositols (PIs) phosphorylation by PfPI3K in early-ring stages (Vaid et al. 2010). Wild PfK13 was shown to bind to PfPI3K which led to ubiquitination and subsequent degradation of PfPI3K, while mutant PfK13 (C580Y) failed to bind PfPI3K, observed with a diminished detection of degraded PfPI3K fragments (Fig. 4b). This was also observed in clinical isolates where ~1.5- to 2-fold increase of PfPI3K levels was detected in resistant strains (Mbengue et al. 2015). ART-treated wild PfK13 parasites showed suspended/slow growth and higher levels of protein ubiquitination as compared to resistant strains, indicating the involvement of cell stress response mechanisms and was established with certainty when proteasome inhibitors were found to synergistically enhance ART-mediated killing even in ART-resistant parasites (Dogovski et al. 2015). Overall, the elevated PI3P levels are suggested to trigger AKT signaling cascades which promote oxidative stress response pathways (Mok et al. 2015) and K13 mutants which promote unfolded protein response, thus increasing parasite survivability (Fairhurst 2015; Paloque

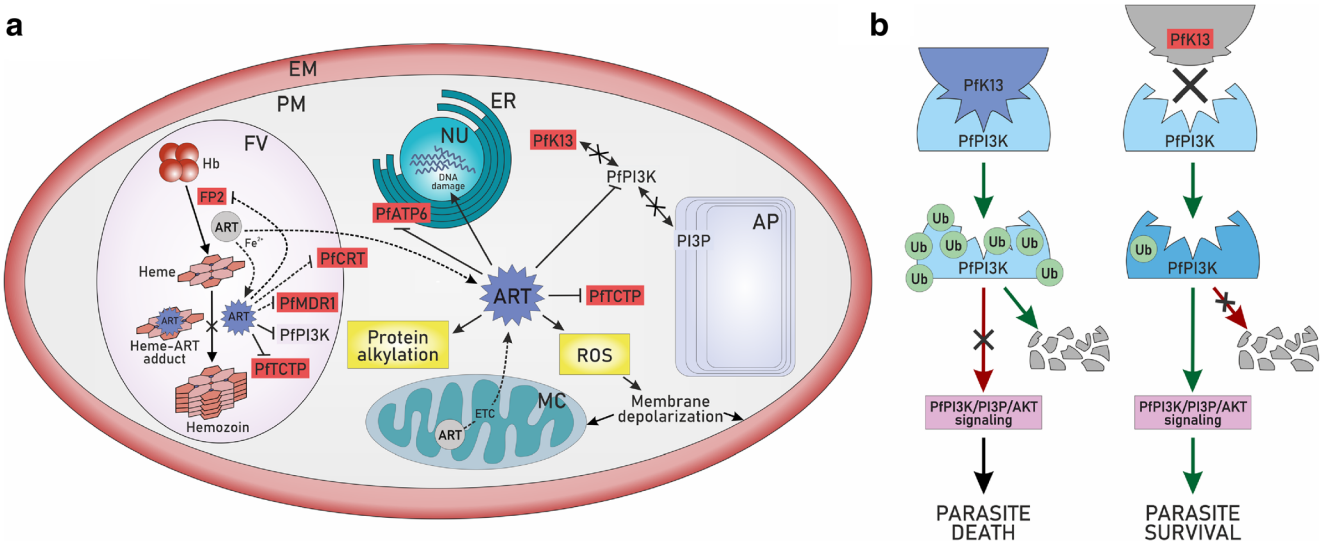

Fig. 4 Schematic view of ART interactions within malarial parasite and K13 mediated ART resistance. **a** Free heme or ferrous ions in FV or components in ETC are suggested to activate ARTs, which further interacts with and disrupts key proteins including FP2, PfCRT, PfMDR1, PfTCTP in FV, PfATP6 in ER, PfPI3K in the cytoplasm, and PI3P at AP membrane. ARTs further are responsible for protein alkylation of multiple targets and ROS-mediated membrane depolarization of PM and MC membranes. Proteins highlighted in red indicate those in which mutations have been observed. **b** In presence of ART, wild PfK13 binds with PfPI3K, which results in PfPI3K ubiquitination and degradation, thus dysregulation of phosphoinositide signaling pathways ultimately leading to parasite death, while in ART-resistant strains, mutated PfK13 does not bind to PfPI3K initiating PI3P signaling, thus promoting parasite survival. Adapted and modified from Ding et al. 2011 and Mbengue et al. 2015 (Ding et al. 2011; Mbengue et al. 2015). FV, food vacuole; PM, parasite plasma membrane; EM, erythrocyte membrane; NU, nucleus; ER, endoplasmic reticulum; MC, mitochondria; AP, apicoplast

et al. 2016), although more research is clearly needed to understand K13-mediated ART resistance.

Strategies to overcome ART resistance

ACTs, though facing the threat of resistance, can still be put to short- to medium-term use until viable drug candidates are established. Areas with high malaria occurrence and thus more prone to re-exposure can be administered with longer half-life antimalarials which can last long enough in the event of re-exposure. While AMLF remains the primary ACT of choice in several African countries, the piperaquine component of DHA-PPQ, which has a longer half-life than lumefantrine, indicated a modest 10–15% decrease in cases in children administered with DHA-PPQ as compared to AMLF (Okell et al. 2014; Pfeil et al. 2014). Alternatively, triple drug combinations along with ARTs (such as ASSP) could provide greater efficacy against multiple strains and/or have a synergistic action between different medications. However, this could also lead to increases in the cost of medication and unintended drug-drug interactions which may lead to depleted levels of active metabolites (Dennis Shanks et al. 2014). A detailed mechanism of drug resistance can lead to the identification of optimal rotational regimen strategies, where two or more ACTs can be administered that exert opposing drug pressures, thus either avoiding or delaying the onset of development of resistance (Taylor et al. 2016). Discontinuation of drugs can also lead to loss of resistance augmenting mutations due to lack of drug pressure, as has been observed in Malawi, Africa, where once chloroquine-resistant parasites were found to be susceptible to chloroquine treatment, 12 years after treatment withdrawal. Overall, locally optimized treatment policies with better surveillance, prevention, and treatment guidelines need to be developed with tailored dosing regimens to populations such as pregnant women and small children, who are most susceptible to the infection.

Despite the considerable amount of research which has undergone in understanding and mitigating ART resistance, it seems inadequate when compared to the rapid pace of evolution of resistant strains spreading across South-East Asia. Without the hope of alternative candidates as efficacious as ACTs in the foreseeable future, the fight against malaria may come to a standstill. Therefore, new strategies and tools are urgently required to mitigate ART resistance and novel targets involved in host-parasite interactions need to be characterized.

Synthetic compounds containing 1,2,4-trioxane-, 1,2,4-trioxolane-, and 1,2,4,5-teraoxane- scaffolds comprising the potent endoperoxide bridge are currently being considered as potential replacements or as partner drugs. OZ439 (artefenomel), one such compound that was found to be well tolerated and had a considerably longer half-life (McCarthy et al. 2016; Phyo et al. 2016). These compounds can be substituted with ARTs in areas of high resistance; however, this can only be an intermediate measure as these compounds

may be equally susceptible to resistance and/or, worse, have cross-resistance with ARTs (Siriwardana et al. 2016; Straimer et al. 2017).

Another strategy to mitigate resistance is to combine multiple pharmacophores into a single compound through a linker, a relatively unexplored concept in malaria. These hybrid molecules depending on the linked active moieties may have the advantage of targeting multiple life stages or exhibit synergistic and improved pharmacokinetic properties over combination of individual compounds. A primaquine-ART (PQ-ART) hybrid evaluated for in vivo efficacy showed potent activity against both liver and blood stage parasites (Capela et al. 2011). While chemosensitizing compounds, which can re-sensitize parasites were described earlier for chloroquine (Ch'Ng et al. 2013), a hybrid molecule with a synthetically linked chloroquine and a chemo reversal agent (CQ-CRA) was recently shown to potently affect chloroquine-resistant as well as ART-resistant strains (Boudhar et al. 2016). This study is significant, as it not only exemplifies the use of a hybrid compound strategy but also demonstrates a mechanism to re-sensitize previously resistant parasite strains, a potential game-changing approach. Genetic manipulation tools such as peptide-conjugated phosphorodiamidate morpholino oligomers (PPMOs) that specifically target drug efflux pumps and induce hypersensitivity in antibiotic-resistant bacteria (Ayhan et al. 2016) can be modified and adapted for protozoan parasites such as *Plasmodium* spp. Similarly, periodic cycling between alternate drugs has been shown to deter resistance in bacteria; thus, this strategy may be applied in malaria endemic regions to delayed the spread of ART-resistant strains (Kim et al. 2014).

Identification of proteasome inhibitors that synergistically act along with ARTs indicates a definite role of cellular homeostasis pathways in abating ART resistance (Dogovski et al. 2015; Rathore et al. 2015). This implies a potential scope for determining key players from a vast pool of candidate molecules within these signaling cascades, which may provide alternate targets for future drug development. Genetic modification tools such as CRISPR/Cas9, ZFN can be employed to identify such target enzymes or crucial residues within such enzymes. Two recent studies involving transcriptome analysis of laboratory generated ART-resistant parasites (Rocamora et al. 2018) and NGS analysis of all *P. falciparum* resistome strains (excluding ARTs) (Cowell et al. 2018) identified novel genes other than PfK13 that could impart drug resistance against almost all the currently used drug candidates, thus impacting future drug development efforts (Carlton 2018). Many of the SNPs identified in PfK13 and other drug-resistant protein variants were found to lie near or within the active site cleft which directly interacts with target substrates and more susceptible to resistance (Fairhurst 2015; Ménard et al. 2016); thus, it would be advantageous to target allosteric sites or exosites in these enzymes as they indirectly modulate protein activity and since they are under low drug pressure; these sites would likely be less susceptible to mutations. Such exosite-mediated interactions have been shown to either modulate protease auto-processing or capture substrate for proteolytic digestion (Pandey et al. 2005; Sundararaj et al. 2012). In the case of cysteine proteases of malaria parasite, it has been shown that allosteric sites can be targeted to inhibit the processing of enzyme and block the activity of these crucial hemoglobinases (Akansha et al., 2018, unpublished).

Malaria parasites upon ART interaction enter a quiescent phase wherein the parasites become dormant with minimal energy requirements and an arrested developmental cycle at very young ring stage (Witkowski et al. 2010). This phase was shown to be temporary with parasites efficiently resuming cellular functioning after removal of ARTs, indicating possible epigenetic control. This phenotype is similar to the hepatic *P. vivax* hypnozoites that can lie dormant from months to years, with a recent study identifying transcriptional differences between hypnozoites and dividing liver schizonts (Cubi et al. 2017). Another study has indicated the role of histone methyltransferases during hypnozoite to schizont stage maturation (Dembélé et al. 2014), supporting the claim of epigenetic control during crucial parasite life events or when under high drug pressure. Understanding and identifying drug targets in *P. vivax* hypnozoite stages have been unsuccessful due to difficulties in co-culturing hepatic and parasite cells, and due to lack of hypnozoite stage diagnostic tools; however, recent techniques such as micro-patterned primary human hepatocyte co-cultures (MPCCs) may help gain insights into hypnozoite biology (March et al. 2013; Gural et al. 2018). Identifying key players behind such modifications could be beneficial to fully destroy all stages of parasites and their further proliferation.

Natural products and whole plant extracts provide a diverse source of potent pharmacological agents such as ARTs from *A. annua* (Tu 2011), avermectins from *Streptomyces avermitilis* (Ōmura and Shiomi 2007), maytansinoids from *Maytenus ovatus* (Lopus et al. 2010), erythromycin, tetracycline, and doxocycline (Ginsburg and Deharo 2011). Though popular in various ancient cultures, traditional medicines derived from plant extracts are being replaced by a synthetic pharmaceutical industry driven by high throughput screening (HTS) approach. Before ARTs, one of the first widely used antimalarial was quinine isolated from the bark of *Cinchona* trees. The natural phenol compound curcumin of the curcuminoid family was found to have a cytotoxic effect on both chloroquine-resistant and sensitive strains through ROS-mediated DNA damage (Cui et al. 2007). A novel set of compounds, spiroindolones, based on a central spirocenter scaffold, discovered from screening of ~ 12,000 pure natural products and compounds with structural features found in natural products, rapidly inhibit protein synthesis in *P. falciparum* and

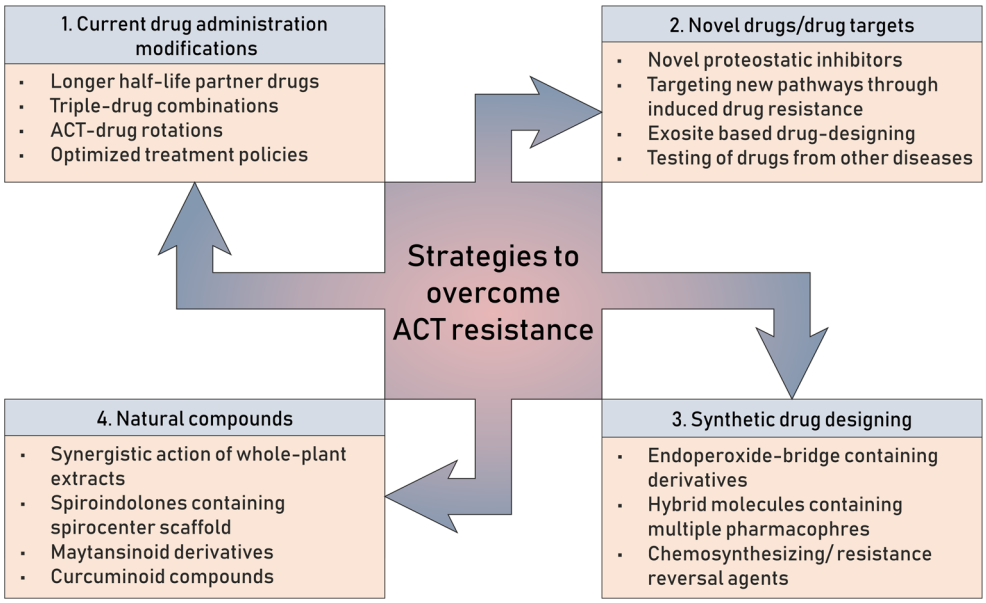

Fig. 5 A summary of possible future strategies to combat ART resistance. The major strategies can be divided into four main categories: Current drug administration modifications, novel drugs/drug targets, synthetic drug designing, and natural compounds

the derivative NITD609 was approved for preclinical trials (Rottmann et al. 2010). Along with monotherapies, whole plant extracts could provide potent remediation strategies with studies on dried whole plant (WP) extracts of *A. annua*, showing the effectiveness of WP extracts even against ART-resistant parasites. WP extracts were more resilient to develop resistance as compared to pure ART administration (Elfawal et al. 2015). While some flavonoids in these WP extracts had no antiplasmodial activity, the concomitant mixtures were highly effective indicating synergistic action between individual components (Ferreira et al. 2010). These findings suggest that future pursuit of novel antimalarials lie prominently in discovery of natural products.

Conclusion

ARTs, the endoperoxide bridge containing sesquiterpene lactones, are highly reactive in the presence of heme/Fe^{2+} ions and generate ROS that rearranges to form carbon-centered free radicals. Though the comprehensive mechanism of activation is debated, these free radicals were shown to disrupt several pathways in the parasite proteome essential for survival, making ARTs and its derivatives highly significant in the global fight against malaria. However, despite ~2.7 billion USD funding in 2016, WHO estimates it is less than half the total amount required as part of the global malaria strategy. Emerging resistance and rise in number of deaths to pre-2012 levels signify an insufficient global effort to eradicate malaria. The identification of PfK13-mediated ART resistance gives valuable insights into the *Plasmodium* resistome and indicates that current drug regimens including ACTs, though robust, may inevitably fail in future due to the rapid and unstoppable pace of plasmodial evolution. Therefore, it is essential to uncover new targets and develop prospective chemotherapies (Fig. 5). The development of next-generation drugs should potentially aim to target multiple plasmodial stages with either a combination of fast-acting and longer half-life counterparts or a hybrid molecule fused with multiple pharmacophores.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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