Artemisinin Resistance in *Plasmodium falciparum* Malaria

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Xiaoxing Wang, Bo Xiao, and Lubin Jiang

Abstract

The worldwide use of artemisinin-based combination therapies (ACTs) has contributed in recent years to a substantial reduction in deaths resulting from *Plasmodium falciparum* malaria. However, resistance to artemisinin and its derivatives (ARTs) has emerged worldwide, which is a threat to the global malaria elimination campaign. In this chapter, we will summarize the emergence of ARTs resistance, the molecular mechanisms of action of ARTs, and measures to slow down or prevent the emergence and spread of ARTs resistance. Combined efforts in strengthened surveillance, resistance detection, molecular mechanism elucidation of artemisinin resistance, and the development of novel targets and antimalarial drugs will help us to apply more effective antimalarial drug policies, putting us ahead of the curve in the fight against artemisinin resistance.

Keywords

Artemisinin resistance · ACT · kelch13 mutations · *Plasmodium falciparum*

11.1 Background

Malaria remains a major public health problem and is prevalent in tropical and subtropical regions worldwide. Globally, an estimated 1.7 billion malaria cases and 10.6 million malaria deaths were averted in the period 2000–2020. Most of the cases (82%) and deaths (95%) averted were in the World Health Organization (WHO) African Region, followed by the WHO Southeast Asia Region (cases 10% and deaths 2%) (WHO, 2021). In the World Malaria Report 2021, the WHO

X. Wang · B. Xiao · L. Jiang (✉)
Institut Pasteur of Shanghai, Chinese Academy of Sciences, Huangpu, Shanghai, China
e-mail: xxwang@ips.ac.cn; bxiao@ips.ac.cn; lbjiang@ips.ac.cn

H. Mehlhorn et al. (eds.), *Malaria Control and Elimination in China*, Parasitology Research Monographs 18, https://doi.org/10.1007/978-3-031-32902-9_11

recommends the broad use of the RTS,S/AS01 (RTS,S) malaria vaccine, which can save tens of thousands of children's lives every year (WHO, 2021). Before the WHO recommended the use of malaria vaccines, malaria control was mainly through antimalarial drugs (chloroquine, sulfadoxine-pyrimethamine, artemisinin, etc.), the spraying of insecticides, and insecticide-treated mosquito nets. With the prolonged use of antimalarial drugs, chloroquine resistance has emerged in the Greater Mekong Subregion (GMS). In 2006, the WHO mandated that artemisinin and its derivatives (ART) be used only in combination with other drugs to avoid the development of chloroquine and sulfadoxine-pyrimethamine resistance in GMS, which are applied in combination with partner drugs, so-called ART combination therapies (ACTs). Unfortunately, some ACTs are unable to rapidly clear *Plasmodium falciparum* parasites from the bloodstream and fail to cure malaria patients, which is a major concern. There is a fear of resistant *Plasmodium falciparum* emerging in the world. In this chapter, we will summarize the emergence of artemisinin resistance, the molecular mechanisms of artemisinin resistance, and measures to slow down or prevent the emergence and spread of ART resistance.

11.2 Emergence of Artemisinin Resistance

In 2006, Afonso et al. first found that *Plasmodium chabaudi* can develop stable resistance to artemisinin in the laboratory but lacks mutations in the candidate genes atp6 (encoding the sarcoplasmic and endoplasmic reticulum Ca^{2+} ATPase), tctp, mdr1, and cg10 (Afonso et al. 2006). In 2008, clinical artemisinin resistance was first reported in Cambodia, which manifested as delayed parasite clearance, with a higher parasitemic by microscopy on day 3 after artemisinin treatment (Noedl et al. 2008). Subsequently, artemisinin resistance is redefined by a delayed parasite clearance half-life ($PCt_{1/2} > 5$ h) in vivo after ART treatment compared to approximately 2 h for sensitive parasites or by an increased parasite survival rate following brief exposure to a high dose of dihydroartemisinin (DHA) in the ring-stage survival assay ($RSA^{0–3h}$) in vitro (Noedl et al. 2008; Witkowski et al. 2013). In this assay, parasite clinical isolates are adapted to culture for several weeks, synchronized at the early-ring stage (0–3 h after invasion of RBCs), treated with a pharmacologically relevant dose of DHA for 6 hours, and then cultured for 66 hours (Witkowski et al. 2013). The percentage of parasites surviving DHA exposure was then calculated as the ratio of parasites surviving exposure to DHA versus those surviving exposure to DMSO. $^{RSAD0–3h}$ discriminates two groups of parasites, one with $<1\%$ survival and another with $\geq 1\%$ survival, which are generally defined to be artemisinin-sensitive and artemisinin-resistant, respectively (Witkowski et al. 2013).

In 2014, Ariey et al. found mutations in the PF3D7_1343700 kelch propeller domain ("K13-propeller") associated with artemisinin resistance both the longer $PC_{1/2}$ in vivo and higher RSA values in vitro by whole-genome sequencing of an artemisinin-resistant parasite line from Africa and clinical parasite isolates from Cambodia (Ariey et al. 2014). The K13 gene comprises a Plasmodium-specific N-terminal domain followed by a CCC (coiled coil-containing) domain, a BTB/POZ

(Broad complex Tramtrack Bric-a-brack/Pox virus Zinc finger) domain, and a Kelch propeller domain. The six-bladed β-propeller domain consists of six Kelch motifs, each folding into a four-stranded antiparallel β-sheet (Coppée et al. 2019). Single nucleotide polymorphisms (SNPs) have been identified throughout the K13 gene, but only nonsynonymous mutations in the propeller domain (K13PDmut, K13 propeller domain mutant) are associated with delayed parasite clearance (Ashley et al. 2014). In 2017, a Chinese researcher found that a 43-year-old man was diagnosed with malaria (*P. falciparum*) in Jiangsu Province and had worked in Equatorial Guinea for approximately 2 years. Importantly, parasites of this patient were still detected on day three with dihydroartemisinin and piperaquine treatment, and RSA resulted in a 2.29% survival rate and the mutation M579I in K13, which indicated that artemisinin-resistant strains have emerged in Africa (Lu et al. 2017). Mutations in the K13 propeller domain are recommended for conducting molecular surveillance as an assistant tool for monitoring resistance to artemisinin. To date, the mutations P441L, F446I, S449A, N458Y, M476I, P553L, V568G, P574L, M579I, D584V, and L675V in K13 have been reported to be associated with delayed parasite clearance from global parasite populations. In addition, a list of validated K13 mutations associated with decreased sensitivity to artemisinin has been issued, including F446I, N458Y, Y493H, I543T, R539T, R561H, P574 L, and C580Y (WHO, 2020). By 2022, some K13 mutations were found in Thailand, Vietnam, Myanmar, southern Pakistan, China, Laos, Nigeria, India, Papua New Guinea, South Africa, and Rwanda, which are also markers of slow parasite clearance (Ajogbasile et al. 2022; Ashley et al. 2014; Lautu-Gumal et al. 2021; Lu et al. 2017; Mishra et al. 2016; Na-Bangchang and Karbwang 2013; Thanh et al. 2017; Tun et al. 2015; Wang et al. 2015b). ART resistance was first reported in the GMS, and there are different geographical distributions of K13 mutations. C580Y is most prevalent in the Cambodia-Thailand and Thailand-Myanmar borders (eastern Thailand, Laos, Cambodia, and Vietnam) (Boullé et al. 2016), whereas F446I predominates along the China-Myanmar and Myanmar-India borders (Myanmar, China, and western Thailand) (Wang et al. 2015a, b; Zhang et al. 2019). This difference may result from different drug uses, demographic histories, vectors, genetic backgrounds, and antimalarial policies in these regions.

Recently, Tang et al. found that only the C580Y mutation in K13 with low frequency was associated with artemisinin resistance in the China-Myanmar border region and detected some other high mutation sites of K13 (Tang et al. 2021). Additionally, it has been reported that the S522C mutation in K13 from Kenya was associated with delayed parasite clearance (Schmedes et al. 2021). A low frequency of M476I and C469Y mutations in K13 was found in Pakistan, which is significantly associated with artemisinin resistance (Ghanchi et al. 2021). One study reported that 13 patients from Northern Uganda were infected with *P. falciparum* parasites with mutations in the A675V or C469Y allele in the K13 gene, which were associated with prolonged parasite clearance half-lives, and the RSA also showed a higher frequency of parasite survival among organisms with the A675V allele than among those with the wild-type allele (Balikagala et al. 2021). K13 resistance mutations (including P574L and A675V) in southern Rwanda have been found,

which are common in Southeast Asia and associated with delayed parasite clearance (Tacoli et al. 2016). In 2022, Nima et al. reported that there was no association with artemisinin resistance and K13 mutation in Bangladesh but with a long parasite clearance half-life (8 h) and 2.01% survival in $RSA^{0–3\ h}$ (Nima et al. 2022). An interesting discovery has been reported that the P413A mutation in the BTB/POZ domain of K13 also conferred artemisinin resistance in vitro, which suggested the importance of monitoring for K13 mutations (Paloque et al. 2022). The emergence and spread of artemisinin-resistant strains have aroused widespread concern. Once these resistant strains spread and become popular, they will pose a serious threat to global malaria control.

11.3 Molecular Mechanisms of Artemisinin Resistance

Artemisinin resistance is primarily associated with point mutations in the parasite's K13 protein. At present, the mechanism of ART resistance has been connected to increased cellular stress, altered DNA replication, an activated unfolded protein response, reduced protein translation, and increased levels of phosphatidylinositol 3-phosphate (Gibbons et al. 2018; Mbengue et al. 2015; Mok et al. 2015; Rocamora et al. 2018).

The location and interactomes of K13 are involved in multiple cellular processes. Some studies have identified the location and interactomes of K13 to understand the functions of K13 using immunoprecipitation (IP) or dimerization-induced quantitative BioID (DiQ-BioID) (Birnbaum et al. 2020; Gnädig et al. 2020; Siddiqui et al. 2020). These results showed that K13 localized to the endoplasmic reticulum, food vacuole markers, Rab-positive vesicles, and the adaptor protein complex 2 μ subunit (AP-2 μ) (Birnbaum et al. 2020; Gnädig et al. 2020; Siddiqui et al. 2020). Birnbaum et al. found that the resulting list of associated proteins of K13 contained 10 uncharacterized proteins (designated K13 interaction candidates, KIC1–10), PFK9, MCA2, epidermal growth factor receptor substrate 15 (Eps15), ubiquitin-binding protein 1 (UBP1), CHDP, FKBP35, HSP90, GTPase-activating protein, MyosinC (MyoC), IMC protein and VPS52. Eps15 is involved in endocytosis in other organisms, which suggests that K13 may play a role in endocytosis (Birnbaum et al. 2020). The study also showed that K13 colocalizes with AP-2 μ but is distinct from clathrin. Disruption of the K13-compartment proteins AP-2 μ, KIC4, UBP1, KIC7, KIC5, Eps15, and MCA2 also resulted in ART resistance in RSAs. Other researchers also identified that Eps15, UBP1, KIC7 and AP-2 μ contribute to the endocytic transport of hemoglobin to the food vacuole in trophozoites using a food vacuole bloating assay (Birnbaum et al. 2020; Jonscher et al. 2019). These findings indicated that K13-compartment proteins influence endocytosis in all asexual life cycle stages, whereas K13 is only required for endocytosis in ring stages. Siddiqui et al. also identified some proteins associated with K13 by IP and liquid chromatography-tandem mass spectrometry analysis, and these proteins are involved in translation, transcription, and the unfolded protein response pathway. Among these proteins, putative endoplasmin (GRP94), heat shock protein 70 (BiP),

and protein disulfide isomerase (ERp72) may be involved in the reactive oxidative stress pathway in *Plasmodium* (Siddiqui et al. 2020). Gnadig et al. also identified many putative K13-interacting proteins, including S-adenosylmethionine synthetase, adenosylhomocysteinase, phosphoglycerate mutase 1 (PGM1), phosphoglucomutase 2 (PGM2), the Rab family of GTPases, the Rieske protein, a putative dynamin (involved in mitochondrial fission), ATP synthase F1 alpha subunit (involved in mitochondrial energy metabolism), mitochondrial matrix protein 33, and several proteins involved in the mitochondrial antioxidant system. These proteins are involved in the methionine metabolism pathway, parasite glycolysis, vesicular trafficking and endocytosis, proteasome-mediated degradation, mitochondrial fission, energy, and the antioxidant system (Gnädig et al. 2020). The resulting data sets of interacting proteins of K13 suggest that it is involved in multiple cellular processes (Gnädig et al. 2020; Mok et al. 2021; Siddiqui et al. 2020).

One study demonstrated that the resistance of *Plasmodium* to artemisinin-based compounds depends on alterations of heme metabolism and on a loss of hemozoin formation linked to the downregulation of the recently identified heme detoxification protein (HDP). These artemisinin-resistant strains could detoxify free heme by an alternative catabolism pathway involving glutathione (GSH) mediation (Witkowski et al. 2012). Henriques et al. found that an increased artemisinin-resistant phenotype of *Plasmodium chabaudi* is accompanied by a mutation in a functional element of the AP2 adaptor protein complex, which also suggests that artemisinin resistance may be associated with endocytosis and trafficking of membrane proteins (Henriques et al. 2013). An additional study was reported by Mbengue et al. (Mbengue et al. 2015), who suggested that artemisinin targets *P. falciparum* phosphatidylinositol-3-kinase (PI3K) and that PI3K is the binding partner of K13. Since these parasites have low basal levels of PI3-phosphate (PI3P), the product of PI3K activity, they are highly sensitive to artemisinin-induced inhibition of PI3K. Without a functional PI3K, parasites cannot generate the high PI3P levels they need for growth (PI3P is involved in membrane biogenesis and fusion events and increases in amount as parasites develop from rings to schizonts). This study speculates that K13 mutations fail to bind PI3K, and PI3K accumulates and produces high basal levels of PI3P. When subsequently exposed to artemisinin, high basal levels of PI3P enable the continuous PI3P-dependent growth of artemisinin-resistant parasites while they recover from the effects of PI3K inhibition. PI3P is located in the FV membrane and RBC cytoplasm vesicles. A membrane-bound glutathione S-transferase (PfEXP1, PF3D7_1121600), found on the parasitophorous vacuole membrane, was upregulated twofold in the DHA-tolerant 3b1 parasite strain compared to the parental Dd2 strain and was further upregulated twofold in 3b1 upon DHA exposure (Lisewski et al. 2014). In cell-free systems, PfEXP1 can conjugate reduced GSH to hematin, accelerating the degradation of the toxic hemoglobin digestion byproduct (Lisewski et al. 2014). Artesunate inhibits PfEXP1 glutathione transferase activity, likely by alkylation, as endoperoxide-containing antimalarials alkylate PfEXP1 (Ismail et al. 2016), which could functionally inactivate this protein. PfEXP1 was also among the antioxidant response genes upregulated in 3D7 parasites pressured with artemisinin in a separate study (Rocamora et al. 2018).

Some studies have found that K13 can act as an adaptor to bring E3 ligase(s) and their substrate(s) into proximity. Ubiquitin moieties are attached to substrates via an ATP-dependent cascade of E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases (Varshavsky 2017). K13 is hypothesized to bind a Cullin 3 RING E3 ligase complex (CRL3) via the BTB domain and bind a yet-to-be-identified substrate(s) via the kelch propeller domains (Coppée et al. 2019; Tilley et al. 2016). In this proposed scenario, wild-type K13 binds substrate(s) and allows for CRL3-mediated ubiquitination. In contrast, the K13 propeller domain mutant would be unable to bind substrate(s), preventing CRL3-mediated ubiquitination. Depending on the nature of ubiquitin chain linkages, ubiquitination of substrate(s) can act as a signal for localization, downstream signaling, or proteasome-mediated degradation (Varshavsky 2017).

Mok et al. have provided some evidence to suggest that they were able to link artemisinin resistance to an upregulated "unfolded protein response" pathway involving two major chaperone complexes: *Plasmodium* reactive oxidative stress complex (PROSC) and TCP-1 ring complex (TRiC) (Mok et al. 2015). Recently, mutation of ferredoxin (D97Y) was reported to be strongly associated with artemisinin resistance. Research has shown that ferredoxin is not a direct target of artemisinin, but its mutation may be involved in the protective response against the oxidative stress caused by artemisinin (Kimata-Ariga and Morihisa 2022). It has been shown that nutrient-permeable channels are important for nutrient and drug access and reveal amino acid deprivation as a critical constraint in artemisinin-resistant parasites (Mesén-Ramírez et al. 2021). Some findings elucidate the role of PfGCN5 as a global chromatin regulator of stress responses with a potential role in modulating artemisinin drug resistance and identify PfGCN5 as an important target against artemisinin-resistant parasites (Rawat et al. 2021). Additionally, it has been shown that the autophagy-related gene 18 (ATG18) T38I polymorphism may provide additional resistance against artemisinin derivatives but not partner drugs, even in the absence of kelch13 mutations, and may also be important in parasite survival during nutrient deprivation (Breglio et al. 2018).

11.4 Responding to Artemisinin Resistance

To monitor the occurrence and spread of artemisinin resistance and drug or treatment efficacy, surveillance for artemisinin resistance should combine parasite clearance half-life in vivo, RSA in vitro, and molecular monitoring of resistance gene markers. K13 mutations have been demonstrated to be a useful marker gene for surveillance of resistant parasites in Cambodia by Ariet et al. Artemisinin partial resistance is monitored using an established list of validated and candidate K13 markers associated with decreased sensitivity to artemisinin. Recently, the detection of K13 mutations in Africa, South America, and Oceania suggests the importance of further monitoring K13 mutations to understand artemisinin resistance globally. The proteins that interact with K13 may also be candidate marker genes for new resistance-conferring mutations (Birnbaum et al. 2020; Xie et al. 2016). Several

reports have found that parasites with delayed parasite clearance in Africa were not associated with K13 mutations, which may be due to mutations in other K13 pathway genes (Henriques et al. 2014; Kone et al. 2020; Li et al. 2019; Madamet et al. 2017; Sutherland et al. 2017). Specifically, it would be important to monitor the occurrence of SNPs in EPS15, UBP1, coronin, AP-2 μ, MCA2, KIC7, KIC4, KIC5, ATG18, and possibly PI3K in isolates from patients who present with delayed parasite clearance. Therefore, molecular monitoring of artemisinin resistance could play an important role in providing early warning of the emergence of resistance and could also help us map the spread of ART resistance.

Interventions that decrease the selective advantage of parasites with reduced sensitivity to a drug will slow the spread of drug resistance. Consequently, interventions can be done by using combinations of different drugs, which ensure that drug pressure on a parasite population is not from one drug only and that minimizes the risk that parasites from recrudescent cases are transmitted. It is critical that the right partner drugs were selected, which gives the relatively short window of action of artemisinin. The first-line treatments for *P. falciparum* include artemether lumefantrine (AL), artesunate-pyronaridine (AS-PY), artesunate-amodiaquine (AS-AQ), and dihydroartemisinin-piperaquine (DHA-PPQ) in the WHO African Region. The first-line treatment drugs were AL, artesunate mefloquine (AS-MQ), and chloroquine (CQ) in the WHO Region of the Americas. In the Southeast Asia Region, the first-line treatments were AL, AS-PY, AS-MQ, DHA-PPQ, and artesunate plus sulfadoxine-pyrimethamine (AS+SP). In 2015–2020, AL found high efficacy with all treatments in India, Bangladesh, Brazil, Myanmar, and Columbia. In India, although treatment failure rates with AS+SP remained low, one study from Chhattisgarh State detected a high prevalence of dhfr and dhps, which indicated decreased sensitivity to SP. DHA-PPQ conducted in Indonesia and Myanmar showed high rates of efficacy, with failure rates of less than 5%. In Thailand, where drug efficacy is assessed with integrated drug efficacy surveillance, treatment failure rates with DHA-PPQ plus primaquine in 2018, 2019 and 2020 were all less than 10%, whereas failure rates were high in Sisaket Province, which led the province to change its first-line therapy to AS-PY in 2020. In the Eastern Mediterranean Region, AL and AS+SP remain more efficacious and are the first-line treatments. In the WHO Western Pacific Region, AL, AS-MQ, AS-PY, and DHA-PPQ were the first-line treatments. The first-line treatment of DHA-PPQ was replaced with AS-PY in provinces where high treatment failure rates were observed in Vietnam (WHO, 2021). In addition, optimizing the course of therapy (for example, from 3 to 7 days) may also help us maintain the effect of the drugs (Wang et al. 2019). Triple artemisinin-based combination therapies (TACTs), combining artemisinin with two partner drugs, could be an effective therapy for treating multidrug-resistant malaria, which can prevent the global emergence and spread of artemisinin resistance (van der Pluijm et al. 2021). Furthermore, the use of the gametocytocidal drug (primaquine) might interrupt the transmission of artemisinin-resistant parasites.

Since the number of antimalarial drugs is limited and resistance is emerging, seeking to lower transmission and stop the resistance spread with interventions is

important. Malaria preventive measures must first manage the source of infection, improve epidemic reporting, and eradicate malaria patients and those with malaria parasites. In terms of cutting off the route of transmission, it is mainly to eliminate Anopheles mosquitoes, including preventing being bitten by *Anopheles* mosquitoes, using insecticides to remove places where Anopheles mosquito larvae breed, using mosquito nets or applying mosquito repellents to avoid being bitten. Currently, new intervention strategies are urgently needed to fight against malaria. Some *Plasmodium*-blocking symbiotic bacteria and their secretion products were identified from *Anopheles sinensis* and could confer mosquito resistance to malarial infection, which might provide a novel vector control tool for blocking the transmission of malaria (Gao et al. 2021; Wang et al. 2017). Drug resistance is a continuing challenge to control and eradicate malaria worldwide, and this scientific question remains a hot research topic. Studies on artemisinin resistance have contributed to a comprehensive understanding of the molecular mechanisms of ART resistance, which are involved in endocytosis, the cellular stress response, and multiple cellular processes (Ross and Fidock 2019). The identification of artemisinin targets and resistance markers offers convenient molecular surveillance tools to detect the emergence and forestall the spread of ART resistance. With the rapid emergence and spread of artemisinin resistance, there is also an urgent need to discover antimalarial drugs with new modes of action and activity against the malaria parasite. Recently, the innovative antimalarial drugs JX21108, compound 11 and JX_{35} were reported, which are histone deacetylase inhibitors developed from the clinical anticancer drug candidate quisinostat and could cure multiple life-stage and multidrug-resistant *Plasmodium*, indicating that these novel drugs could be used to cure artemisinin-resistant parasites and block malaria transmission in the future (Huang et al. 2020; Li et al. 2021; Wang et al. 2022). Additionally, a new antimalarial drug ASP3026 was developed based on the lysyl-tRNA synthetase (LysRS) novel drug targets of *P. falciparum* (Zhou et al. 2020). Combined efforts in strengthened surveillance, resistance detection, molecular mechanism elucidation of artemisinin resistance, and the development of novel targets and antimalarial drugs will help us to apply more effective antimalarial drug policies, putting us ahead of the curve in the fight against artemisinin resistance.

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