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Various Mechanisms of Drug Resistance in *Plasmodium falciparum* Malaria – A Comprehensive Review

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ABSTRACT
It is crucial to comprehend and stop the spread of antimalarial resistance, especially to artemisinin and its companion medications. Mutations in the *P. falciparum* chloroquine resistance transporter (PfCRT), a transporter located on the digestive vacuole membrane that, in its variant forms, can transport these weak-base 4-aminoquinoline drugs out of this acidic organelle, preventing these drugs from binding heme and inhibiting its detoxification, are present in *Plasmodium falciparum* parasites that are resistant to chloroquine, amodiaquine, or piperaquine. Through the use of cryogenic electron microscopy, the structure of PfCRT was solved. It reveals mutations surrounding an electronegative core drug-binding cavity, which is likely where medications and natural substrates interact to regulate transport. Overexpression or mutations in the digestive vacuole membrane-bound ABC transporter PfMDR1 (*P. falciparum* multidrug resistance 1 transporter) also affect *P. falciparum* sensitivity to heme-binding antimalarials. Mutations in *P. falciparum* Kelch13 protein (K13), a protein involved in several intracellular processes including haemoglobin endocytosis, which is necessary for parasite development and artemisinin activation, are the main cause of artemisinin resistance. The creation of innovative antimalarial medications with unique mechanisms of action is critically needed to combat drug-resistant malaria.

KEYWORDS: Antimalarial drug resistance, Artemisinin-based combination therapy, Piperaquine, endocytosis

I. INTRODUCTION
According to the World Health Organisation, the *Plasmodium falciparum* parasite continues to cause malaria worldwide, with over 200 million cases recorded in 2018 [157]. This is in spite of decades of clinical research and treatment procedures. An estimated 405,000 people died as a result of these infections, including 272,000 (67%) children under the age of five. Sub-Saharan Africa accounted for an astounding 94% of the fatalities. Antimalarial medications are largely used for prevention and the treatment of infected individuals because the use and efficacy of experimental vaccines have been extremely restricted [30]. Natural compounds found in the bark, roots, or leaves of plants have been used for thousands of years to cure malaria. However, it was not until the twentieth century that their active constituents were discovered and employed as separate pharmaceutical substances. Quinine, an alkaloid derived from the bark of cinchona trees that contains quinoline, was arguably the most effective of these medications [101]. The 4-aminoquinoline quinacrine and additional toxicological and pharmacological investigations, chloroquine were produced as a result of advancements in the chemical synthesis of drug analogues. This quick-acting antimalarial was the primary malaria therapy by the 1950s. Because of chloroquine's effectiveness, accessibility, minimal toxicity, and cost, it was used as a single-agent treatment for decades around the world [150]. Antimalarial drugs are among the most widely used drugs in tropical areas, where *P. falciparum* parasites are under intense pressure to develop resistance mechanisms due to the high treatment demand, which is further aggravated to some extent by poor patient adherence. Malaria resurfaced in the early 1960s with the emergence of chloroquine resistance, and it persisted for decades in the majority of nations [151]. The US Army discovered pyrimethamine, amodiaquine, an arylaminoalcohol called halofantrine, and mefloquine, an analogue of chloroquine, through high-throughput screening and drug discovery and development activities. Since the late 1980s, their effectiveness has also decreased due to the expansion of parasites that have developed resistance. More recently, Southeast Asian combination treatments have

started using piperaquine, another derivative of chloroquine, which was created under the Chinese National Malaria Elimination Program as a bis 4-aminoquinoline that may overcome chloroquine resistance [14]. The World Health Organisation advises treating malaria using two or more drugs that have distinct mechanisms of action in order to postpone the establishment of resistance and achieve the required cure rates. However, historically, frontline drug resistance has emerged first in places with low rates of mixed-strain transmission, particularly in Southeast Asia [86]. In fact, it was in that area that resistance to sulfadoxine-pyrimethamine, mefloquine, and chloroquine first developed [14, 112]. With the worldwide incidence of malaria declining by 37% between 2000 - 2015, artemisinin-based combination treatments (ACTs) have shown effective in managing the disease and saving many lives in more recent times [43]. Fast-acting substances with a distinct endoperoxide bridge are artemisinin, which was first isolated from the Chinese sweet wormwood *Artemisia annua*, and its derivatives artemether, artesunate, and dihydroartemisinin (DHA). For the treatment of simple *P. falciparum* malaria, they are usually used in combination with a companion medication that has a longer half-life, such as lumefantrine, piperaquine, mefloquine, amodiaquine, or more recently, pyronaridine, as advised by the World Health Organisation. Southeast Asia, especially the Greater Mekong Subregion (GMS), was the site of growing artemisinin resistance as early as 2008 [86, 153]. The most troubling issue at hand is the sharp rise in DHA + piperaquine failure rates, which has been the standard therapy and favoured ACT for the majority of Southeast Asia [106, 125, 142]. The emergence of these novel parasites that are resistant to piperaquine and DHA threatens the recent gains gained in the fight against malaria and emphasises the need for fresh approaches. Using the preventive combination medication atovaquone-proguanil was one first option [83]. A word of caution: changes in cytochrome b can easily provide atovaquone resistance, but the resulting mutant parasites may become non-transmissible [48]. Additionally, proguanil has a restricted dosage, and point mutations in the target dihydrofolate reductase can make its active metabolite, cycloguanil resistant [123]. Recent reviews of the antifolate combinations pyrimethamine-sulfadoxine and atovaquone-proguanil independently examined the mechanisms driving resistance to these drugs [53, 127]. Instead, the mechanisms of action and resistance to ACT medications are the main topics of this study.

MOLECULAR MECHANISMS OF THE 4-AMINOQUINOLINES AND ARYLAMINOALCOHOLS

Mode of Action of the 4-Aminoquinolines and Arylaminoalcohols - Ever with the emergence of chloroquine resistance in Southeast Asia and South America, which subsequently expanded from Asia to Africa, researchers have been attempting to comprehend the molecular underpinnings of drug action and resistance [115]. The breakdown of host haemoglobin and the subsequent detoxification of heme products have become the focus of this effort. By endocytosing haemoglobin, a main cytosolic host erythrocyte protein, through cytostomes, the growing trophozoite consumes up to 75% of the available haemoglobin during the asexual blood stage of its life cycle. Hemoglobin-containing vesicles are sent to the digestive vacuole (DV), a pH of 5.2 acidic secondary lysosome [70]. The DV parasite undergoes a catabolic process facilitated by various proteases to breakdown haemoglobin in order to get the amino acids necessary for its development and maturation [47]. These consist of the zinc protease falcilysin; the aspartic proteases plasmepsins 1, 2, 4, and 3 (the latter also referred to as histo-aspartic protease); and the falcipain cysteine proteases. Iron protoporphyrin IX [Fe(II)PPIX], a heme byproduct, and denatured globin are the products of this process. O_2 has the ability to auto-oxidize Fe(II)PPIX into the cytotoxic Fe(III)PPIX, which may peroxide lipids. Fe(III)PPIX is biomineralized into hemozoin, an inert and very insoluble crystalline substance, to lessen its toxicity. Prior to the development of merozoites, this brown birefringent crystal is mostly generated during the trophozoite stage [132]. Chloroquine and its 4-aminoquinoline analogues have been shown in several investigations to block hemozoin production in the DV, which results in DV enlargement and pigment clumping. Haemoglobin proteases can be inhibited to reverse their activity [49, 131]. 4-Aminoquinolines extracellular biomimetic tests, it has also been demonstrated that aminoquinolines prevent the synthesis of β-hematin, or synthetic hemozoin [96]. Moreover, dose-dependent increases in intracellular free heme and corresponding decreases in hemozoin are seen in parasitised red blood cells treated with chloroquine, amodiaquine, or piperaquine [26, 32]. By attaching to the fastest-growing crystal face, maybe in the form of a chloroquine-hematin complex, chloroquine prevents the production of hemozoin [49, 100]. The DV may accumulate quinoline antimalarials over 1,000 times because to their potent binding to Fe(II)PPIX and pH trapping [16]. Antimalarial drugs including lumefantrine, mefloquine, and quinine are among the

arylaminoalcohols. As a side effect of their modes of action, they may also partially interfere with hemozoin synthesis and detoxification of haemoglobin breakdown by products. In general, their mechanism of action is not as well known as that of the 4-aminoquinolines. It has been demonstrated that, albeit having lower binding constants, the arylaminoalcohols form 1:2 drug:hematin μ-oxo dimer complexes similarly to the 4-aminoquinolines [37]. In a cell fractionation study, parasites treated with quinine, mefloquine, and lumefantrine at 2.5 times their 50% inhibitory concentration (IC50) values showed a statistically significant increase in free heme and a decrease in hemozoin. These effects, however, were not as noticeable as the diverging heme/hemozoin levels seen following chloroquine therapy [27].

PfCRT as a Primary Driver of Resistance to Chloroquine and Amodiaquine - Through an energy-dependent drug efflux mechanism, chloroquine buildup in the DV is reduced in parasites that are resistant to the medication [73]. The primary causative factor was discovered in 2000 when changes in the *P. falciparum* chloroquine resistance transporter gene, pfcrt, were found by genetic cross-analysis between chloroquine-sensitive and chloroquine-resistant strains [40]. In line with its function in facilitating chloroquine efflux out of the DV and away from its heme target, the 49-kDa drug/metabolite transporter protein PfCRT localises to the DV membrane [29, 40, 81, 115]. In order to overcome the size and analytical limitations of cryo-EM, PfCRT-specific antigen-binding fragments (Fabs) that formed complexes with PfCRT were successfully screened, and very recently, single-particle cryo-EM was used to determine the PfCRT structure to 3.2 Å resolutions [67]. The transporter is made up of 424 amino acids, 10 transmembrane helix domains organised in five helical pairs, two juxtamembrane helices, and two-helix hairpins with an inverted antiparallel topology, which are characteristic of drug/metabolite transporters. In the solved antibody-bound conformation, they combine to create a negatively charged central chamber of around 3300 $Å^3$, which is open to the DV side. Positively charged medications and other compounds/solutes that concentrate in the DV are thought to be accommodated in this cavity, enabling their transfer to the cytosol when PfCRT alternates conformations during its transport cycle. Experiments on trans-Stimulation efflux have shown that extracellular chloroquine accelerates the efflux of intracellular chloroquine that has been preloaded. This finding supports the transporter or carrier hypothesis for PfCRT-mediated drug efflux rather than the channel model [117]. The precise mutations that result in a given PfCRT isoform have a significant impact on the process's kinetics, efficiency, and mechanisms [16, 19, 67]. Chloroquine-resistant mutations in pfcrt-modified clones are sufficient to produce chloroquine-resistant phenotypes on diverse genetic backgrounds, as previously described in studies that revealed genetic transformation via pfcrt allelic exchange [122]. Lysine to threonine at position 76 is a significant and common amino acid alteration in chloroquine-resistant alleles, regardless of origin. There are always many other region-specific mutations present in addition to this K76T mutation. In contrast to the chloroquine-sensitive wild-type 3D7 isoform, the chloroquine-resistant South American 7G8, African GB4, and Southeast Asian Dd2 PfCRT isoforms, respectively, include five, six, and eight mutations. After the 7G8 isoform's cryo-EM structure was determined, it was discovered that the core drug-binding cavity of PfCRT is lined by all five of its mutations: C72S, K76T, A220S, N326D, and I356L. Chloroquine resistance can only be conferred by a minimum of four of these mutations, indicating a codependent function for these extra amino acid alterations [42]. While the 7G8, GB4, and Dd2 PfCRT isoforms are all thought to be resistant to chloroquine, the South American SVMNT PfCRT haplotypes are more closely linked to resistance to amodiaquine than the African/Asian CVIET haplotypes [116]. Consequently, 7G8 backgrounds are mostly where cross-resistance between chloroquine and amodiaquine is shown. Studies on transport have shown that isoforms of PfCRT, expressed in proteoliposomes [66], *Saccharomyces cerevisiae* yeast (8), or *Xenopus oocytes* [10, 81], only exhibit a dose-dependent uptake of quinine or chloroquine when these resistance-conferring mutations are present. K76T is a crucial requirement for transport. A pH gradient and a positive membrane potential are necessary for this proton-coupled transport, which is blocked by amodiaquine and piperaquine, among other 4-aminoquinolines. Verapamil, a drug resistance reversal medication that competes for the PfCRT drug-binding site, similarly inhibits transport [67]. Since PfCRT is vital to the parasite, 4-aminoquinolines may target it directly and function as a competitive inhibitor of natural PfCRT substrates. In fact, although exhibiting comparable levels of chloroquine accumulation, parasites expressing smaller amounts of mutant PfCRT are more susceptible to the drug [74]. Studies have indicated that PfCRT may have a role in facilitating the transfer of iron, glutathione, Cl^- ions, H^+ ions, hemoglobin-derived peptides, and amino acids out of the DV, even if its primary function is unknown [7, 73, 75, 81, 103, 162]. Basic amino acids including arginine, lysine,

and histidine are transported by PfCRT variations produced in proteoliposomes. These amino acids would be positively charged when they entered PfCRT's cavity from the acidic DV [66]. Leucine and other neutral amino acids, such as lumefantrine and atovaquone, do not compete with each other for the drug-binding site, indicating that PfCRT is selective to cationic or protonatable 4-aminoquinoline compounds [67]. Metabolomics studies that assessed the impact of mutations on peptide levels in parasite extracts have provided additional proof of PfCRT's function in transferring vital nutrients from the DV. Elevated hemoglobin-derived peptides and the chloroquine-resistance-conferring pfcrt locus are correlated, according to metabolic quantitative trait locus analysis, which shows the relationship between the genome-wide contributions of individual alleles to metabolite concentration [75]. Due to impaired haemoglobin metabolism, chloroquine-resistant K76T parasites exhibit the greatest accumulation of these peptides, including PEEK, and a marked fitness disadvantage. Significant fitness costs are associated with other PfCRT mutations, such as C101F and L272F, which developed in vitro under amantadine and blasticidin selection, respectively. These mutations exhibit significantly expanded DVs in the trophozoite and schizont phases. According to findings on chloroquine uptake from Xenopus oocytes, the insertion of these mutations on a background of resistance to chloroquine induces reversal of resistance to chloroquine, likely by blocking PfCRT-mediated chloroquine transport [32, 72, 108]. Moreover, chloroquine-resistant Dd2 parasites that have the L272F mutation are unable to metabolise or get methionine, which is a required amino acid [72].

PfMDR1 as a Modulator of *P. falciparum* Susceptibility to 4-Aminoquinolines and Arylaminoalcohols - The P-glycoprotein homolog PfMDR1 (also called Pgh1), which is encoded by the *P. falciparum* multidrug resistance 1 transporter gene pfmdr1, is another component that contributes to resistance to heme-targeting antimalarials [109, 140]. PfMDR1 is located on the DV membrane, just as PfCRT, although transport is thought to be inwards directed towards the DV. PfMDR1 has two membrane-spanning homologous domains, each of which has six predicted helices followed by a hydrophilic nucleotide-binding pocket [118]. Topologically, PfMDR1 is similar to a normal P-glycoprotein-type ABC transporter. The cytosolic side of the DV appears to have this binding domain, which may be where it initially interacts with antimalarial drugs. Mefloquine, halofantrine, lumefantrine, quinine, and derivatives of artemisinin are more susceptible to parasites when the copy number of pfmdr1 is decreased [121]. Common in African strains, the amino acid alteration N86Y modifies medication sensitivity by making the parasite more resistant to amodiaquine and chloroquine while making it more susceptible to lumefantrine, mefloquine, and DHA [144, 145]. In parasites that reemerged following therapy, the use of artesunate + amodiaquine has been shown to select for PfMDR1 N86Y and D1246Y, with lower sensitivity to monodesethyl-amodiaquine, the active metabolite of amodiaquine [28, 98]. Significant isoform-specific interactions between PfCRT and PfMDR1 are suggested by the fact that PfMDR1 mutations in 7G8 South American parasites have a greater impact on chloroquine resistance than those in Asian Dd2 or African GB4 parasites. For medications to have a high level of action, they must have optimal access to their target. PfMDR1 mutations likely prevent antimalarials from being transported from the cytosol to the DV, which lowers the amount of heme-targeting medications like amodiaquine and chloroquine in the DV. However, when their trafficking via PfMDR1 out from the cytosol is blocked, antimalarials such mefloquine, lumefantrine, and halofantrine—which are expected to inhibit targets outside the DV—become more effective [111]. PfMDR1 transports chloroquine, quinine, and halofantrine, with single-nucleotide polymorphisms (SNPs) influencing substrate selectivity, according to studies using pfmdr1 complementary RNA-injected Xenopus oocytes [118]. For instance, the mutation N86Y causes the transporter Pgh-1Dd2 to acquire the capacity to transport halofantrine but lose the ability to transport quinine and chloroquine. It is probable that quinine and mefloquine, two antimalarial medications, not only disrupt Pgh-1's normal function by occupying a shared drug-binding site and preventing the transport of other solutes, but also because they are Pgh-1 substrates [111, 118]. In contrast to the scenario in Asia, where fitness appears to be less of a dominating influence, pfmdr1 gene amplification is extremely rare in Africa, most likely because of the more frequent mixed infections and less frequent medication pressure that reinforce the impact of its recognised fitness cost [113]. In Uganda, the wild-type D1246 variation and the N86Y mutant have the greatest selection benefit; nonetheless, copy number typically has a greater effect on fitness than SNPs in this gene [99].

Piperaquine Resistance Mechanisms - In vitro and in vivo parasite sensitivities to chloroquine have often been restored for a number of countries, most notably China, Vietnam, and Malawi, which have successfully avoided the use of chloroquine

monotherapy. Additionally, certain resistance-associated pfcrt and pfmdr1 mutations have decreased [41, 52]. While most countries of the world no longer employ quinoline antimalarials as monotherapies, the widely used ACTs frequently use them as companion medications. Regional acceptance of DHA + piperaquine occurred as a result of the earlier use of artesunate + mefloquine as the first-line treatment in various Southeast Asian nations, which led to selection for multicopy pfmdr1, associated with lower effectiveness [14, 28, 145]. But the companion medications are under more strain now that the artemisinin derivatives in the GMS have lost some of their efficacy. In Cambodia, piperaquine resistance was noted by 2015 [2, 21, 38]. Piperaquine-resistant parasites sometimes display biphasic or partial growth inhibition curves in typical dose-response experiments, which makes calculating the IC50 or IC90 values more difficult. The piperaquine survival assay has shown to be a reliable substitute. In this method, drug-free culture is maintained for a further 24 hours after synchronised ring-stage parasites are exposed to a pharmacologically appropriate dosage of 200 nM piperaquine for 48 hours. The proportion of the ratio of drug-treated vs mock-treated living parasites is used to compute survival ratios, which are determined after 72 hours [38]. A criterion for identifying piperaquine resistance in vitro is provided by survival rates ≥10%, which are linked to an increased probability of piperaquine treatment failure [155]. It has been of great importance to define the genetic basis of piperaquine resistance, which has led to the discovery of various putative molecular markers by genome-wide association studies (GWASs). Among these is the amplification of the plasmepsin 2 and 3 (pm2–3) genes, which are implicated in the breakdown of haemoglobin [3, 15, 93, 155]. It was suggested that the DV's heme detoxifying mechanisms may be inhibited by piperaquine if these plasmepsins are produced in excess [155]. Nevertheless, it was unable to determine if plasmepsins were compensating mechanisms for the fitness disadvantages of the real resistance determinant(s) or if they were actively engaged in inducing piperaquine resistance [78]. A further investigation using 78 isolates from western Cambodia collected between 2011 and 2014 clearly showed that piperaquine resistance must be caused by other loci [102]. After treating patients with DHA + piperaquine, Duru et al. [38] isolated artemisinin-resistant parasites that recrudesced. They also observed piperaquine survival rates ≥10%, which were correlated with single-copy pfmdr1 and several novel mutations in PfCRT, such as H97Y, M343L, or G353V, which originated on the Dd2 allele. Separately, 183 Cambodian isolates with genome-wide SNPs identified another PfCRT variant, F145I, which was linked to lower piperaquine sensitivity in isolates with amplified pm2-4 [3]. When a PfCRT mutation, C350R, from field isolates from French Guiana was discovered to be linked to lower piperaquine sensitivity, it originally offered evidence that PfCRT mutations may lead to piperaquine resistance. Genetic editing in the 7G8 line was used to validate this phenotype, and the outcome was a slight but noteworthy shift in the piperaquine IC50 value of about 1.5 fold [105]. Furthermore, when an SNP in pfcrt that results in the C101F substitution was genetically edited in Dd2 parasites, it produced a biphasic dose-response curve, a 3-fold increase in the 50% lethal dose (LD50), and an IC90 increase of about 140 fold compared to the Dd2 edited isogenic control [32]. This SNP was discovered through an in vitro piperaquine selection experiment. Another research demonstrated that even in the absence of pm2–3 amplification, the PfCRT mutations F145I, M343L, and G353V resulted in ≥10% piperaquine survival rates and substantial IC90 changes [114]. This finding was validated using pfcrt-modified parasites generated in Dd2. The mutations that led to a partial or complete reversal of chloroquine resistance were intriguing since they all happened on lines that were resistant to the drug. This suggests that minor physicochemical alterations within the PfCRT transporter have a significant role in determining drug associations and phenotypes [32, 105, 114]. Together with the fitness cost of the specific mutation, the frequency of these various point mutations in the field appears to be primarily influenced by the relative parasite survival they confer under a certain medication pressure within the area [51, 142]. T93S and I218F, two PfCRT mutations that also border the drug-binding cavity of PfCRT, have grown the fastest in the field over the past five years. Recently, it was demonstrated that these point mutations individually result in ~10% parasite survival rates under high piperaquine concentrations using pfcrt-edited parasites, while maintaining reasonable fitness levels in comparison to the more strongly piperaquine-resistant PfCRT mutation F145I [33]. The investigation of PfCRT's interactions with piperaquine and chloroquine has involved subjecting variant isoforms of PfCRT carrying mutations linked to piperaquine resistance to radiolabeled piperaquine or chloroquine, either in proteoliposomes to measure drug uptake or in nanodiscs to evaluate drug binding [67]. No matter the isoform, both piperaquine and chloroquine bind within the range of 0.1–0.2 μM K_d at pH 5.5; however, drug absorption is much higher in resistant isoforms. As an illustration, the

piperaquine-sensitive, chloroquine-resistant 7G8 and Dd2 isoforms exhibit the least piperaquine uptake and the biggest chloroquine uptake, whereas the F145I or C350R mutation-carrying isoforms exhibit the reverse effect, resulting in insignificant absorption of both drugs relative to the wild type. These results indicate that PfCRT point mutations result in various drug phenotypes and reaffirm the specificity of PfCRT for variably mediating resistance to structurally unique drugs. Using antimalarials like piperaquine and chloroquine together might be used to take advantage of these phenomena by applying opposing selection pressure to parasites [67]. Reversing resistance through the identification of certain PfCRT substrate-binding cavity inhibitors that stop the transport of natural substrates and medications is a more long-term strategy [124].

MECHANISM OF ARTEMISININ ACTION AND RESISTANCE IN *P. FALCIPARUM*

Mode of Action of Artemisinins - Acts have been the first-line therapy for malaria since the early 2000s and have been widely embraced globally [151]. Artesunate + mefloquine, DHA + piperaquine, which are used in Southeast Asia, artemether + lumefantrine (Coartem), and artesunate + amodiaquine, which are used in Africa, are the four primary medication combinations that include artemisinin derivatives. Dr. Youyou Tu won the Nobel Prize in Medicine in the 1970s for her team's definition of the therapeutic properties of artemisinin and their successful resolution of its chemical structure [139]. Despite having a short half-life of less than an hour, the compound's extraordinary properties include fast drug-activated death of both the asexual blood stage and early sexual gametocyte forms of *P. falciparum* parasites within hours of exposure at low nanomolar concentrations. Most antimalarials such as sulfadoxine-pyrimethamine, atovaquone, and chloroquine inhibit either a single target or a single pathway, e.g., DHFR-mediated folate synthesis by sulfadoxine-pyrimethamine, atovaquone inhibition of cytochrome bc_1, and heme detoxification by chloroquine. On the other hand, artemisinins have been reported to bind to a very broad array of parasite proteins and appear to affect a multitude of organellar and cellular processes including hemoglobin endocytosis, glycolysis, protein synthesis and degradation, and cell cycle regulation [17, 64, 119, 148]. This unique property is due to the cleavage of its endoperoxide bridge by free Fe(II)PPIX liberated from digested hemoglobin. After becoming active, the carbon-centered radical found in heme drugs alkylates heme, proteins, and lipids. This process increases the production of additional harmful reactive oxygen species, ultimately resulting in cell death [64, 148]. According to unaffiliated research, artemisinins can potentially affect mitochondrial function by depolarising the membrane potential of this organelle [77, 147].

Origins, Spread, and Prevalence of Artemisinin Resistance - In western Cambodia, the epicentre of growing antimalarial multidrug resistance, delayed parasite clearance after artemisinin therapy was initially noted [36, 97]. The World Health Organisation classified clinical locations as regions of resistance if more than 30% of patients had microscopically positive parasites that were visible 72 hours after starting artemisinin or ACT therapy. Consequently, parasite clearance half-life ($PCT_{1/2}$) > 5 h was characterised as clinical artemisinin resistance. The PCT is the amount of time on the log-linear part of the normalised parasite clearance curve [152] needed to lower the parasite density by 50%. This was the first time that antimalarial resistance was identified as a longer period to remove the parasite rather than a higher dosage of medication needed to do so. As of right now, resistance has been reported in several GMS nations, including Laos, Vietnam, Myanmar, Thailand, and Cambodia [86]. Due to permissive infections in native *Anopheles* mosquito species, artemisinin-resistant parasites were thought to have spread by population movement and de novo emergence in other locations, contributing to this regional diversity [5, 126, 136]. Outside of Southeast Asia, clinical evidence of artemisinin resistance has not yet been thoroughly reported [28]. When artemisinin loses its effectiveness, resistance to it increases the strain on other antimalarial medications to act swiftly and efficiently, which might be disastrous for them. Two malaria control techniques, mass drug administration (MDA) and triple ACTs (TACTs) have been developed to counteract artemisinin resistance [34, 146]. MDA seeks to eradicate pockets of asymptomatic malaria that act as reservoirs for the transmission and persistence of resistant parasites, whereas TACTs use combinations of drugs with different mechanisms of action to prevent the emergence of multidrug resistance or to eradicate infections resistant to one of the ACT partner drugs.

In the second phase of the multi-site project Tracking Resistance to Artemisinin Collaboration II (TRAC II), clinical trials are being conducted to evaluate the effectiveness of TACT. According to preliminary data, the combinations of DHA + piperaquine + mefloquine and artemether + lumefantrine + amodiaquine show promise in delaying the emergence of artemisinin resistance and perhaps restoring antimalarial sensitivity in previously artemisinin-resistant regions [143].

k13 as the Primary Determinant of Artemisinin Resistance and Impact on Fitness - The ring-stage survival test (RSA), which exposes immature, 0–3 h postinvasion rings to a pharmacologically relevant 700-nM concentration of the active artemisinin metabolite DHA for 6 h, is used to evaluate in vitro artemisinin resistance. A survival rate greater than 1% at the mature asexual blood stages without a change in sensitivity has been identified as the hallmark of artemisinin resistance's lack of effectiveness in early rings of resistant isolates [154]. The k13 gene (kelch13) of the parasite was initially discovered in a lab-based in vitro evolution research as a genetic predictor of artemisinin resistance. Ariey et al. identified the M476I mutation in K13 and the D56V mutation in the DNA-directed RNA polymerase II subunit RPB9 as early genetic changes that emerged after ~30 cycles of artemisinin pressure by gradually increasing artemisinin pressure on the Tanzanian strain F32 over 125 repeated drug cycles spanning 5 years [4]. During additional in vitro drug selection, mutations in six additional genes in addition to k13 also surfaced. These included two genes encoding unknown proteins, protein kinase PK7, gamete antigen 27/25 Pfg27, a stop codon in falcipain 2, and SNPs in a cysteine protease implicated in haemoglobin breakdown. A number of organisations' GWAS also identified k13 as a potential indicator of clinical artemisinin resistance [22, 89, 135]. A delayed clearance, defined as a $PCT_{1/2} > 5$ h, was linked to around 26 mutations in the K13 protein in the GWAS of approximately 1,500 clinical samples from the multiple-site TRAC investigation [5]. The M476I mutation derived from in vitro cultures has only been observed at a very low frequency of 0.3% in one field study [158], despite the fact that the k13 gene was the only gene that converged using the two powerful approaches. This suggests that in vitro selections can identify the correct gene, but that the mutations may differ from those that succeed in the field. The WorldWide Antimalarial Resistance Network's most recent extended pooled analyses of 3,250 isolates from a compilation of literature and new data revealed that 20 β-propeller mutations were linked to a prolonged PCT1/2 in Asia; however, this was not the case for the S522C, A578S, and Q613L mutations, which were found in Africa [158]. The apicomplexan-specific domain's only nonpropeller mutation, E252Q, has been linked to a 1.5-fold longer half-life, confirming the propeller mutations' predominant function in regulating artemisinin responses [158]. In the same way, not all propeller mutations result in the development of artemisinin resistance. For instance, A578S was shown to be rare in Africa but did not result in the development of resistance in vitro, at least not in Dd2 parasites [87]. Therefore, it is not possible to predict the resistance phenotype based on the existence of propeller mutations. Using CRISPR/Cas9 site-directed gene editing or gene-specific zinc-finger nucleases, reverse genetics has proven effective in establishing a causal relationship between the most prevalent K13 propeller mutations (C580Y, R539T, I543T, and Y493H) and in vitro artemisinin resistance (RSA >1%) in Asian parasites [44, 130]. Even on the same parasite background, the degree of resistance varies between K13 mutations, indicating that these point mutations can function differently [130]. In fact, the six-bladed β-propeller domain has these four stated point mutations dispersed among its second, third, and fourth blades. The identical K13 variant codon altered in different parasite settings also produced varied responses to artemisinin [130]. This highlighted the role that secondary factors play in varying the degree of resistance to artemisinin and the significant influence that genetic background plays in this process. Numerous GWASs revealed candidate secondary loci that may affect the artemisinin response [22, 89, 135]. In Southeast Asian isolates, Miotto et al. [89] identified the following mutations as parts of a genetic founder background: MDR2 (multidrug resistance protein 2) T484I, ferredoxin D193Y, PIB7 (phosphoinositide-binding protein 7) C1484F, ARPS10 (apicoplast ribosomal protein S10) V127M, and PfCRT I356T and N326S. Their role in artemisinin resistance hasn't been scientifically proven yet, though. Through epistatic interactions with the K13 C580Y or E252Q isoforms, Kelch10 P623T was found to be a possible regulator of artemisinin resistance in another GWAS of 192 samples from northeastern Thailand [20]. Independently emerging K13 mutations have been linked to certain geographic origins [87, 136, 166]. While E252Q is found along the western Thai border and in Myanmar, F446I and, more recently, G533S prevail in Yunnan, southern China, near the China-Myanmar border, with 56% and 44% frequency respectively [59, 107, 163]. While not seen in western Thailand, R539T and Y493H mutations have been found in Cambodia [87]. In Western Cambodia, Thailand, Vietnam, and across the GMS, the most common mutation is C580Y [87, 158]. Every mutation has a very dynamic prevalence; some change over time, while others, as C580Y in Cambodia, tend to fix [107]. The DHA- and piperaquine-resistant Pailin lineage's clonal proliferation is the primary cause of the C580Y mutation's high frequency. This lineage originated in western Cambodia and then extended to Thailand, Vietnam, southern Laos, and northeastern Thailand [51, 62]. This lineage has the PLA1/KEL1 (K13 C580Y and pm2–3 copy > 1)

haplotype, which was discovered by microsatellite typing of an area surrounding the k13 gene ranging from −31 kb to +50 kb [63]. Significant variations in K13 mutations have been seen among in vitro comparative growth rate investigations; modern Cambodian parasite strains have a lower fitness cost than earlier strains, which is in line with the advantageous effects of secondary determinants [94, 129]. K13 mutations have been reported to have emerged in 2 isolates from Rwanda (P574L and A675V), 14 from Guyana (1.6%; C580Y), and 3 from Papua New Guinea (1.3%; C580Y) according to recent publications. These seem to be different from the haplotype PLA1/KEL1 [24, 82, 90, 134]. There has been no independent confirmation of a previous study [79] that associated an M579I mutation with delayed clearance and low-level RSA survival (2.3%). Furthermore, the percentage of nonsynonymous to synonymous SNPs in k13 is much lower in Southeast Asia than it is in Africa, indicating that k13 is not subject to the same level of selection in African nations as it is in Southeast Asia [80]. This is presumably due in part to the fitness cost of the K13-mutant isolates being more deleterious to their survival in higher-transmission African environments where mixed infections are more common and untreated illness cases are higher. Adapted from the field or gene-edited to induce the C580Y mutation, K13-mutant isolates from Papua New Guinea and French Guiana have demonstrated higher RSA survival rates of 6.8% and 27.6%, respectively [82, 90], indicating that mutant K13 can confer resistance in those strains. On the other hand, there is no data about the clinical $PCT_{1/2}$ result that would point to the establishment of K13-mediated artemisinin resistance that is clinically characterised in these areas.

Mechanisms Underpinning K13-Mediated Artemisinin Resistance - The K13 protein is necessary for the parasite's intraerythrocytic growth, much like proteins produced by other drug resistance genes. However, its level may be decreased by up to 50% in transgenic parasites by controlling its mislocalization by a knock-sideways method [12]. K13 has been found inside intracellular vesicles that link to endocytosis or vesicular trafficking of antigens, such as PfEMP1 or Rab-mediated protein transport, as well as cytostomes at the periphery of the parasite [11, 13, 46, 160]. It has been proposed that K13 mutations mostly facilitate artemisinin resistance in rings by decreasing the activation of artemisinin medications and increasing the parasite's ability to eliminate damaged proteins. Mutations causing decreased K13 levels have been suggested to cause decreased haemoglobin endocytosis and catabolism in developing rings, which in turn causes decreased amounts of free Fe(II)PPIX that can activate artemisinin [13, 160]. Additionally, DHA-induced reduction of proteasomal activity was more resistive to in vitro edited K13-mutant 3D7 parasites, which maintained protein turnover. Because rings can withstand artemisinin concentrations up to 100 times greater than those of the later trophozoite stages, when peak activity is reached, haemoglobin endocytosis and digestion play a critical role in artemisinin activation and consequent cytotoxicity [68, 69]. Reduced Fe(II)PPIX-mediated drug activation can decrease parasite sensitivity to artemisinin, as further evidenced by pathway perturbation caused by falcipain 2a disruption or the addition of the cysteine protease inhibitor E64 [159]. Further evidence that K13 mutations affect haemoglobin catabolism and intracellular liberation of globin-derived amino acids was also reported when artemisinin-resistant K13 mutant parasites were denied adequate external amino acid sources. These mutants were found to be less fit and to have not developed as far as K13 wild-type parasites [18]. When compared to their isogenic K13 wild-type counterparts, K13-mutant field isolates similarly had decreased heme levels during the trophozoite stage and produced less heme adducts upon DHA treatment [54, 55]. Notably, K13 mutant clinical isolates did not exhibit a change in the quantity of the K13 transcript during expression [91]. Furthermore, in contrast to the anticipated increased tolerance in the event of lower K13 steady-state protein levels, reduced expression of K13 in rings was reported to cause hypersensitivity to artemisinin as assessed by 72-hour dose-response assays (rather than RSAs) in piggyBac transposon-generated mutant parasite lines [45, 165]. When compared to the isogenic wild-type line, the Cam3.II R539T mutant showed lower levels of K13 protein in early rings, but not the Cam3.II C580Y mutant [11, 120]. It is evident that more research is needed to determine whether K13 protein functions or levels are impacted in all clinical isolates with mutations linked to clinical resistance to artemisinin. Reduced Fe(II)PPIX-mediated drug activation may possibly be explained by K13's longer duration of ring-to-trophozoite stage development in K13 C580Y clinical isolates, which similarly modifies the cell cycle [58, 91]. It has been suggested that K13 mutations destabilise the protein and deregulate PfPK4 phosphorylation, which activates transcriptional stress response pathways downstream and modifies growth by phosphorylating eIF2α (the parasite's eukaryotic initiation factor 2α) [160, 164]. It has also been noted that phosphorylation of eIF2α functions as a

stress-sensing regulatory mechanism responsible for lower growth rates in cells lacking the amino acid isoleucine [6]. When cells are treated with artemisinin, which is known to produce the buildup of unfolded, damaged and polyubiquitinated proteins as well as to impair proteasome action, maintaining protein turnover is essential to the cells' ability to survive [17]. It has been suggested that K13 mutations eliminate damaged proteins by decreasing the amounts of ubiquitinated proteins as part of their improved cell stress response and upregulating the unfolded protein response (UPR), as seen with field isolates [35, 91]. Protease inhibitors and artemisinin have been shown to work in concert by a number of organisations, offering a strong prospective means of overcoming artemisinin resistance [35, 76, 128, 161]. However, this synergy is found in parasites with K13 mutations as well as those with wild-type genetics [128], suggesting that K13 mutations do not mediate resistance via altering proteasomal activity. As an alternative, it has been suggested that K13 mutations protect against the proteostatic activity of artemisinin by decreasing drug binding to and polyubiquitination of PI3K. This increases the amount of PI3P-positive vesicles involved in cellular processes such as vesicle-mediated protein export, UPR, and protein folding [11, 84]. Additionally, recent studies point to a connection between parasite mitochondria and K13 function. Increased colocalization of K13 with mitochondria was seen by Gnadig et al. [46] after a DHA pulse, particularly in K13 mutants. Additionally, we saw that the artemisinin resistance conferred by mutant K13 was reversed when atovaquone, an inhibitor of mitochondrial cytochrome bc1, was additionally cultured with the cells.

Evidence of Non-k13 Genetic Mediators of Artemisinin Resistance - Recent research has demonstrated that genes other than k13 can induce artemisinin resistance in *P. falciparum*. Only a small number of clinical isolates from Southeast Asia and Africa have occasionally shown delayed parasite clearance after ACT, indicating that non-K13-mediated resistance has not been extensively studied in clinical settings [92, 133]. Conversely, in vitro selection experiments using the Senegalese Pikine and Thies strains produced parasites with coronin mutations that were somewhat resistant to artemisinin. This protein has a WD40 seven-bladed β-propeller domain that resembles K13 in structure [31]. The elevated RSA was replicated to comparable levels (5–10%) in the wild-type parental lines using CRISPR-Cas9 editing of the coronin R100K, E107V, or G50E mutation. Different selection experiments using the African line 3D7 produced mutations in several genes, including a halt mutation in hemoglobinase falcipain 2A; nevertheless, that study lacked genetic confirmation [110]. Based on linkage group selection study of a genetic cross between a chosen artemisinin-resistant parent and an artemisinin-sensitive parent in the rodent parasite *Plasmodium chabaudi*, UBP-1 has also been proposed as a potential mediator of artemisinin resistance [60]. Research involving *P. falciparum* identified a pfubp1 mutation, V3275F, which was linked to resistance in vitro [56]; in contrast, same gene was not linked to in vivo resistance in clinical isolates from Southeast Asia [61]. Additionally, there was no correlation seen between higher RSA values in vitro and the S160N mutation in the AP-2 adaptin μ subunit found in African isolates treated with ACT [56]. However, no gene has been linked to a patient's delayed clearance rate of *P. falciparum* infections after receiving artemisinin treatment. More convincing is the GWAS of isolates from the border region between China and Myanmar, which discovered a correlation between the T38I mutation in the PI3P-binding autophagy-related protein ATG18 and reduced sensitivity of *P. falciparum* to artemisinin derivatives [149]. It will be fascinating to see if these genes, or any other genes, work along the same route as k13 and result in resistance levels that are comparable even in the absence of K13 mutations.

Features of Artemisinin-Induced Dormancy and Recrudescence - Dormancy, or the process by which cells assume a low-energy resting state, has been used as a bet-hedging tactic in bacterial systems to get around unfavourable environmental circumstances [65]. When cells perceive that growth circumstances have improved, growth resumes [39]. This phenomenon has been documented in Plasmodium parasites after short-term (6 - 144 h) treatment with antimalarial drugs such as derivatives of artemisinin, atovaquone, proguanil, pyrimethamine, and mefloquine, or after exposure to stressors such as starvation or cold shock [57, 71, 88, 95, 137, 138]. Condensed chromatin and decreased cytoplasm are features of dormant parasites, which are generally robust to disturbances, indicating that dormancy is a cell's adaptive reaction to external stress [9]. For instance, *P. falciparum* parasites treated with DHA had a brief developmental stop, which was followed by the recrudescence of a tiny percentage (0.04%-0.3%) of parasites nine to twenty days later [137]. It is interesting to note that latent forms were only reached by asexual rings. Artemisinin-induced dormancy may play a role in the recrudescence of artemisinin-resistant parasites and treatment failure, according to modelling studies (25). Dormant *P. falciparum* parasites treated with artemisinin were

shown to have lower metabolic levels but to still have functioning mitochondria and apicoplasts [23, 104]. Remarkably, atovaquone prevented DHA-induced inactive parasites from recovering too quickly [104], indicating that mitochondrial function is essential to the survival and regeneration of dormant parasites. The mechanics behind these processes have been suggested by a number of researches. According to one research, the prereplicative complex and the controlled expression of mitotic Ca^{2+}dependent kinases (cdpk4, pk2, nima, and ark2) are associated to the advancement of the *P. falciparum* asexual blood stage cell cycle [141]. A different research found that cyclins (pfcrk1, pfcrk4) and cyclin-dependent kinases had different expression levels when the cells recovered from DHA-induced dormancy [50]. Increased pre-tRNA expression to sustain protein translation, greater transcript levels of the RNA polymerase III repressor PfMaf1, and stress-response GCN2-mediated eIF2α phosphorylation were also linked to recovery after isoleucine deprivation [6, 85]. As suggested in a two-step process of artemisinin resistance acquisition [25], repeated drug pressures and induction of dormancy in wild-type parasite populations may eventually select for faster-recovering rings and result in artemisinin-resistant parasites that no longer require dormancy. This would result in increased rates of recrudescence and longer timeframes needed for the parasites to be cleared by artemisinin therapy. The emergence of an artemisinin-resistant K13 M476I mutant after several cycles of brief artemisinin pressure and subsequent parasite recovery might be explained by an in vitro illustration of this situation [4, 156]. According to observations made on this mutant line, quiescent, nonpyknotic rings could quickly begin intraerythrocytic growth when artemisinin pressure was removed. This suggests that mutant K13 eliminated the necessity for parasites to go into dormancy as a means of survival [156].

II. DISCUSSION AND CONCLUSION

Mutations in the transmembrane proteins PfCRT and PfMDR1 are mostly responsible for the mechanisms of resistance to substances that target parasite DV activities, including as haemoglobin breakdown and hemozoin production. These proteins transfer medications either towards or away from their main site of action. Mutations in K13 have been linked to resistance to endoperoxide artemisinins, which has been linked to decreased drug activation and increased UPR. Which haplotypes predominate in field isolates is influenced by a combination of fitness costs conferred by particular point mutations or sets of mutations, as well as the degree of drug sensitivity under the applicable pharmacological pressure. Important chances to mitigate drug resistance are presented by combining appropriate combinations of medications in combination therapy that apply opposing selection forces. Only by having a thorough grasp of the ways in which parasites resist different pharmacological pressures will this aim be accomplished.

ABBREVIATIONS

ACT	artemisinin-based combination therapy
DHA	dihydroartemisinin
GMS	Greater Mekong Subregion
DV	digestive vacuole
Fe(II)PPIX	iron protoporphyrin IX
PfCRT	*Plasmodium falciparum* chloroquine resistance transporter
Cryo-EM	cryogenic electron microscopy
PfMDR1	*Plasmodium falciparum* multidrug resistance 1 transporter
SNP	single-nucleotide polymorphism
GWAS	genome-wide association study
pm2–3	plasmepsin 2–3
PCT1/2 (parasite clearance half-life)	the time in hours that it takes for parasitemia to decrease by 50%
RSA	ring stage survival assay
UPR	unfolded protein response

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