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Review

Recent Advances in Diagnostics and Therapeutic Interventions for Drug-Resistant Malaria

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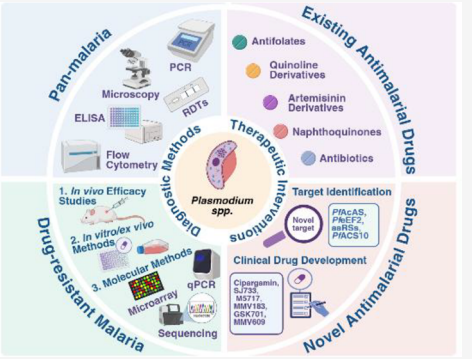
ABSTRACT: The emergence of drug-resistant malarial parasites has been a growing challenge to medical science to safeguard public health in the malaria-endemic regions of the globe. With time, the parasite develops newer resistance mechanisms to defunct the drug's action one after another. Genetic mutation is the prime weapon parasites rely upon to initiate the resistance mechanism in a case-specific manner, following various strategies such as structural changes in the target protein, metabolic alterations, and tweaking the drug-transported channels. In order to combat these resistances, different approaches have evolved among these developing inhibitors against critical parasite enzymes and metabolic pathways, combinatorial/hybrid drug therapies, exploring new drug targets and analogues of existing drugs, use of resistance-reversal agents, drug-repurposing, gene blocking/altering using RNA interference and CRISPR/Cas systems are prominent. However, the effectiveness of these approaches needs to be earnestly monitored for better management of the disease, which demands the development of a reliable diagnosis technique. Several methodologies have been investigated in search of a suitable diagnosis technique, such as *in vivo*, *in vitro*, *ex vivo* drug efficacy studies, and molecular techniques. A parallel effort to transform the efficient method into an inexpensive and portable diagnosis tool for rapid screening of drug resistance malaria among masses in the societal landscape is advocated. This review gives an insight into the historical perspectives of drug-resistant malaria and the recent developments in malaria diagnosis and antimalarial drug discovery. Efforts have been made to update recent strategies formulated to combat and diagnose drug-resistant malaria. Finally, a concluding remark with a future perspective on the subject has been forwarded.



KEYWORDS: *biosensors, CRISPR/Cas, genetic mutation, hybrid drug therapy, inhibitors, Plasmodium parasites*

INTRODUCTION

Malaria is a vector-borne life-threatening disease caused by *Plasmodium falciparum* (*Pf*), *P. vivax* (*Pv*), *P. malariae* (*Pm*), *P. ovale* (*Po*), and *P. knowlesi* (*Pk*). Among these, *Pf* is the deadliest one for humans, accounting for >90% of malaria-related deaths globally.[1] Symptoms of the disease include high fever, chills, and rigors that repeat every 48 h for *falciparum*, *ovale*, or *vivax* infections, 72 h for *malariae* infections, and 24 h for *knowlesi*. *Pv* hypnozoites can remain dormant in the liver and cause disease recurrence. The malaria burden is colossal in the WHO African Region. Outside of Africa, India is a main source of malaria-related incidence and deaths in the South-East Asia (SEA) region, accounting for 85.2% of all malaria in the region. North-East (NE) India contributes a high proportion of malaria cases, the vast majority (90%) of which are caused by *Pf*.[2]

The global vaccine development program for malaria prevention has been slow,[1] with only two vaccines, RTS,S (RTS, S/AS01), known as Mosquirix against *Pf* malaria for children, and R21/Matrix-M were approved by the WHO until date (World Malaria Report 2023). Commonly used medicines for malaria treatment are (a) Artemisinin (ART)-based combination therapy (ACT), the most efficient means for *Pf* malaria treatment; (b) chloroquine for the treatment of *Pv* infections, mostly in regions where the parasite is still responsive to the medication, and (c) primaquine to prevent relapses of *P. vivax* and *P. ovale* infections. ACTs, though, emerged as a suitable strategy to tackle malaria, their effectiveness waning over time due to the growing drug resistance of malarial parasites to most of the frontline antimalarial drugs (World Malaria Report 2023).

Antimalarial drug resistance refers to the ability of a parasite to survive and multiply even when the treatment is given at dosages that are equivalent to or higher than the recommended level but still within the patient's tolerance. The resistance

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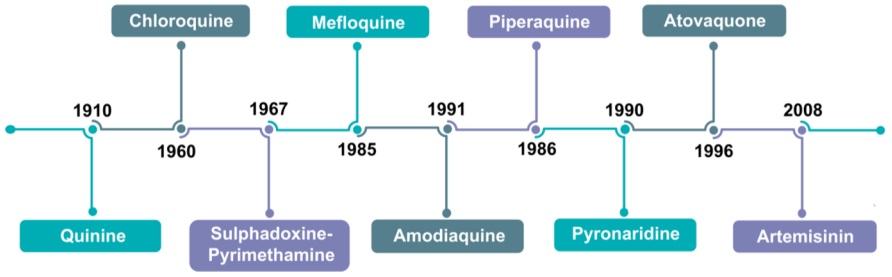

Figure 1. Emergence of antimalarial drug resistance over time.

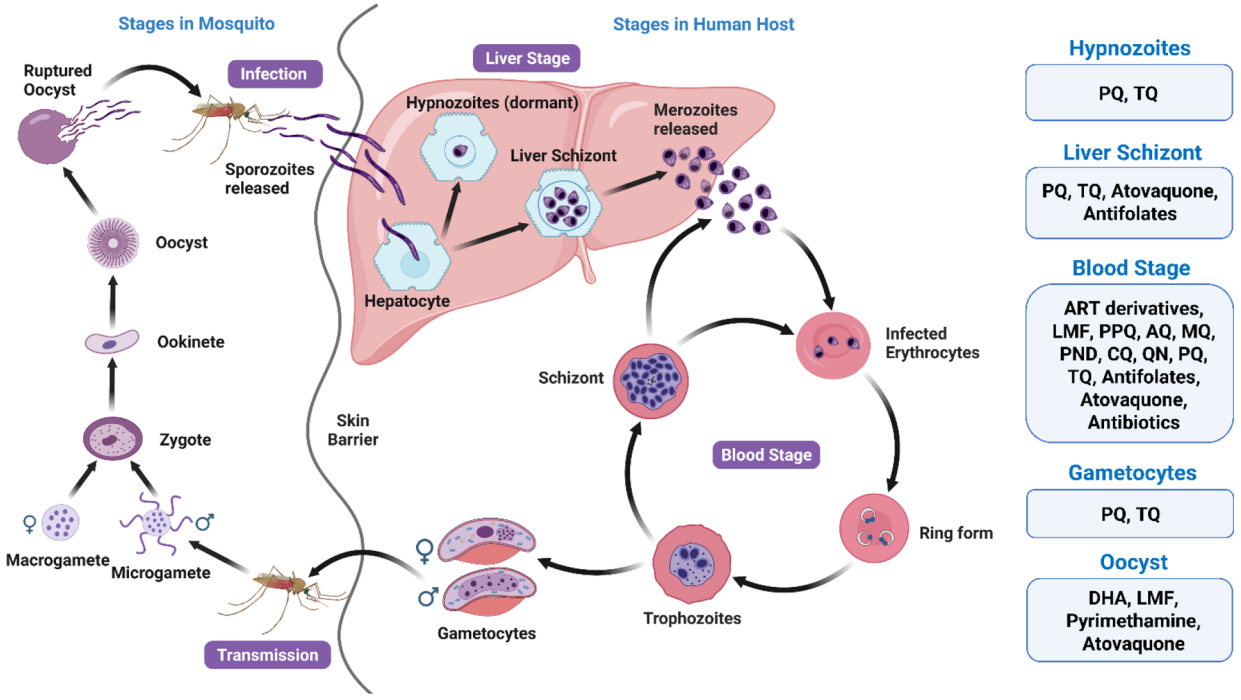

Figure 2. Antimalarial drugs targeting different stages of *Plasmodium spp.* life cycle (created in BioRender.com).

develops due to random genetic alterations in the parasite population. Under drug pressure, parasites may select genetic traits that aid their survival edge. Exposure to subtherapeutic amounts of antimalarial drugs increases the likelihood of parasites selecting a survival trait.[3]

The parasites withstand the effects of multiple antimalarial drugs that belong to different chemical classes. This phenomenon is commonly implicated in the resistance of *Pf* against chloroquine, sulfadoxine/pyrimethamine, mefloquine, halofantrine, etc. The chronological emergence of drug resistance has been depicted in Figure 1.[4] Lately, *Pf* has also developed 'artemisinin partial resistance', which implies delayed clearance of the parasite after treatment with ACT or Artemisinin-based monotherapy. This partial resistance of *Pf* is linked to mutations in *Pfkelch13*, the gene that has a vital role in the lifecycle of the malarial parasite.[3]

The extensive utilization of drugs contributes to the development of antimalarial drug resistance, resulting in increased chances of therapeutic failure. Approaches that can be implemented to minimize the risk of the spread and emergence of drug resistance include (i) transmission prevention from resistant cases, (ii) reducing drug pressure on parasites, (iii) monitoring drug therapeutic efficacy and tracking resistance, (iv) delivering high-quality interventions to ensure prevention, early diagnosis, and treatment, and (v) continuing research for future development of new drugs.[3] Drug resistance is a threat to the malaria control program.[5] Early and accurate diagnosis of resistance-related molecular markers in both clinical and laboratory settings is crucial to enhance the efficacy of existing antimalarial drugs.[6] Timely detection can also help to reduce mortality rates and impede the development of drug resistance in *Plasmodium spp.*[7]

Several review papers on different aspects of malaria have been published over the last five years.[1,6,8−15] However, this review strictly focuses on drug-resistant malaria, covering current resistance patterns, underlying processes, and advances in diagnostic and therapeutic strategies to highlight the need to develop novel solutions to combat this emerging risk for better management of global malaria control and elimination programs.

■ ANTIMALARIAL DRUG RESISTANCE

The emergence of molecular resistance to drugs can be delineated into two distinct events: the propagation of mutants due to drug selection pressure and the occurrence of genetic polymorphism randomly inside the pathogen population (*de novo* resistance), a process promoted by high pathogen density in patients. These genetic alterations may interrupt the mode of action of drugs.[15] Most of the antimalarial drugs focus on

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Table 1. Summary of Prominent Antimalarial Drugs and Their Mode of Action

Class of Antimalarial		Example	Inhibition Stage	Mode of inhibition	Site of action	Resistance-associated gene	Route of administration	Half-life	Toxicity	References
Quinoline derivatives	4-aminoquinolines	Chloroquine[a]	Blood stage	Interferes with heme detoxification, Interacts with double-stranded DNA and inhibits DNA replication	Food vacuole	*crt*, *mdr1*	Oral	20–60 days	Headache, drowsiness, visual disturbances, nausea, vomiting, etc	10, 13, 16–18
		Amodiaquine[a]		Interferes with heme detoxification		*crt*, *mdr1*		5.3–7.7 h	Headache, drowsiness, visual disturbances, vomiting, hypokalaemia, etc	
		Piperaquine[a]		Interferes with heme detoxification		*crt*, *mdr1*, *plasmepsin II* and *III*		20–30 days	Phospholipidosis, interference with cardiac conduction	
	8-aminoquinolines	Primaquine[b]	Hypnozoites, Liver schizonts, Blood stage, Gametocytes	Unclear, it may interfere with electron transport chain or generate reactive species	Mitochondria	_	Oral	4–10 h	Hemolysis in people with G6PD deficiency	13, 16, 19, 20
		Tafenoquine[b]						14 days		
	Aryl-amino alcohols	Quinine[a]	Blood stage	Interferes with heme detoxification	Food vacuole	*mdr1*	Oral	11–18 h	Thrombocytopenia	6, 9, 16, 17, 21–23
		Mefloquine[a]	Blood stage	It may interfere with hemoglobin uptake and heme detoxification, Targets *Pf*80S ribosomal subunit, Inhibits protein synthesis	Cytosol, Food vacuole	*mdr1*		2–4 weeks	Visual disturbances	
		Lumefantrine[a]	Blood stage, Oocyst	It may interfere with hemoglobin uptake and heme detoxification	Cytosol, Food vacuole	*mdr1*		3–4 days	Headache, anorexia, dizziness, vomiting	
Antifolates		Proguanil[a]	Liver schizonts, Blood stage	Interferes with folate biosynthesis pathway	Cytosol	*dhfr*	Oral	12–20 h	Gastrointestinal disorder	8, 16, 24
		Pyrimethamine[a]	Liver schizonts, Blood stage, Oocyst			*dhfr*		4 days	Gastrointestinal disorder, thrombocytopenia	
		Sulfadoxine[a]	Liver schizonts, Blood stage			*dhps*		4–9 days	Stevens-Johnson syndrome, erythema multiforme, erythroderma	
Artemisinin derivatives		Artesunate[a]	Blood stage	Generation of free radicals, DNA damage, alkylation of protein and lipids	Food vacuole, ER, vesicular structures	*kelch13*, ap-2 μ, ubp1, rad5	Intravenous, Intramuscular	0.1–1.8 h	Pancytopenia, melena, seizures, multiorgan failure	16, 25–30
		Artemether[a]	Blood stage				Oral	2–4 h	_	
		Dihydroartemisinin[a]	Blood stage, Oocyst				Oral	1 h	Phospholipidosis, decrease in reticulocytes	
Naphthoquinones		Atovaquone[a]	Liver schizont, Blood stage, Oocyst	Inhibition of electron transport chain	Mitochondria	*cytb*	Oral	2.2–3.2 days	_	10, 15, 16
Antibiotics		Clindamycin[a]	Blood stage	Inhibition of protein synthesis	Apicoplast	23S rRNA	Oral, Intravenous, Intramuscular	2.5–4 h	_	16, 31
		Tetracycline[a]				*mdt*, *tetQ*	Oral	6–12 h	Abdominal pain, nausea, vomiting, diarrhea	
		Doxycycline[a]					Oral, Intravenous	16–22 h		

[a]Resistance reported. [b]Resistance not reported; *crt*: chloroquine resistance transporter; *mdr1*: multidrug resistance protein 1; G6PD: glucose-6-phosphate dehydrogenase; *dhfr*: dihydrofolate reductase; *dhps*: dihydropteroate synthase; acid synthase; ER: Endoplasmic reticulum; *ap-2 μ*: adaptor protein complex 2 mu subunit; *ubp1*: upstream binding protein 1; *rad5*: DNA helicase Rad5; *cytb*: Cytochrome *b*; *mdt*: metabolite drug transporter; *tetQ*: Tetracycline resistance gene Q.

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targeting specific metabolic pathways in the parasite, while *de novo* mutations rapidly minimize their effectiveness leading to resistance. The antimalarial drugs targeting different stages of *Plasmodium* spp. lifecycle has been depicted in Figure 2.

Antimalarial Drug Classes and Resistance Patterns. Pharmacotherapy is the primary method of treating malaria, utilizing a range of drugs that target various intracellular targets of *Plasmodium spp*. Drugs currently available against malaria include quinoline derivatives (4-aminoquinolines, 8-aminoquinolines, and Aryl-Amino Alcohols), antifolates, artemisinin derivatives, Naphthoquinones, and antibiotics.[6] Prominent antimalarial drugs and their mode of action are described in Table 1.

Usually, a combination of drugs is administered simultaneously to combat malarial infection. Accurate selections of effective antimalarial drugs reduce the illness duration and decrease the probability of complications, mortality, and transmission risks. The best choice of medication, however, depends upon the patient's immunity condition and the drug's intracellular targets.

Quinoline Derivatives. This class of antimalarials consist of quinine (QN), chloroquine (CQ), mefloquine (MQ), amodiaquine (AQ), piperaquine (PPQ), primaquine (PQ), lumefantrine (LMF), and tafenoquine (TQ). These quinoline derivatives are widely categorized into three groups: 4-aminoquinolines, 8-aminoquinolines, and aryl-amino alcohols.[6] CQ, AQ, and PPQ are belonging to 4-aminoquinolines. CQ is primarily used as the first-line treatment for uncomplicated *Pf* malaria, whereas AQ and PPQ are utilized as partner drugs with artemisinin derivatives.

4-Aminoquinolines block the heme detoxification process of the parasites in its digestive vacuole (DV). Notably, heme, which is toxic to the parasite, is liberated during the hemoglobin breakdown, a process exploited by the parasite to acquire amino acids for its growth.[8] The released Fe^{2+}-heme moieties are oxidized into ferriprotoporphyrin IX (FPIX), which in turn are transformed into hemozoin as a part of the detoxification process. CQ has been a drug of choice for treating malaria due to its affordability, effectiveness, and safety. However, its rampant use led to the emergence of resistant *Pf* species against it.[19] Furthermore, CQ has been proposed to interact with the DNA double-strand, functioning as an intercalating agent that exerts cytotoxic effects on cells by inhibiting DNA replication.[17,18] It can form a stable DNA-CQ complex with the double-helical DNA of *Plasmodium*, and this complex has the ability to affect the process of DNA replication and RNA transcription, thereby inhibiting the growth and reproduction of the malarial parasite.[32] AQ (amodiaquine) was used mainly for the treatment of uncomplicated *Pf* infections in combination with artesunate (AS). However, clinical resistance against this drug surfaced after nearly four decades of its implementation. To overcome CQ resistance, PPQ was developed as a CQ derivative in the 1960s. Ironically, the parasite also developed resistance against PPQ.[33] PPQ was then used as a partner drug, with dihydroartemisinin (DHA) being the first-line treatment for *Pf* infection in Cambodia during the year 2010. Nevertheless, DHA + PPQ resistance emerged in Cambodia and Vietnam merely a few years after their implementation.[34]

Primaquine (PQ) and Tafenoquine (TQ) are examples of **8-aminoquinolines** used for malaria treatment. To prevent relapses in *Pv* and *P. ovale* malaria, PQ is administered in addition to CQ to treat the liver-stage parasites. PQ is used as a gametocytocidal treatment for the prevention of *P. falciparum* malaria transmission,[17] PQ functions as a prodrug that requires activation through the cytochrome P450 (CYP) enzyme system, involving two specific enzymes to generate its active metabolites.[35,36] The activation metabolism of PQ involves cytochrome P450 member 2D6 (CYP2D6) and cytochrome P450 nicotinamide adenine dinucleotide phosphate (NADPH): oxidoreductase (CPR), resulting in the formation of two hydroxylated active metabolites that can produce hydrogen peroxide. The active metabolites exert significant oxidative stress on both the malarial parasite and the host cells. Under normal homeostatic conditions, NADPH plays a crucial role in protecting cells from oxidative stress. The enzyme glucose-6-phosphate dehydrogenase (G6PD) is responsible for producing NADPH and is especially essential in red blood cells, where it serves as the sole source of NADPH.[37] PQ also interferes with the mitochondrial electron transport chain in the malarial parasite.[38] TQ is a long-acting analogue of PQ, specifically developed for the prophylaxis or radical cure of *P. vivax* malaria. Tafenoquine exhibits activity against all stages of malaria parasites, including the liver stage along with the hypnozoite (dormant stage) of *Pv*,[39] and the blood stage, and it is also capable of blocking parasite transmission.[20,40] CYP2D enzymes are also crucial for activating TQ into its active metabolite, which is responsible for its antimalarial activity. The proposed mechanism of action of TQ includes the generation of oxidative stress[41] or interfering with the parasite's DNA structure and compromising mitochondrial membrane integrity.[20,42] Thus, patients with G6PD deficiency are specifically prone to oxidative stress, and they can experience acute hemolytic anemia induced due to the administration of PQ and TQ.[17] There is no report of resistance against PQ and TQ.

The **aryl-amino alcohols** include QN, MQ, and LMF. The mechanism of action of aryl amino alcohols is not clearly understood as compared to 4 aminoquinolines. There is a secondary aspect for their mechanism of action, which states that they may partially interfere with hemoglobin uptake, hemozoin formation, and detoxification of hemoglobin degradation byproducts.[17,21,34] The first antimalarial drug, QN, also known as a cinchona alkaloid, inhibits the erythrocytic phase of the *Plasmodium* life cycle, leading to the rupture of the infected red blood cells.[6] Unfortunately, QN was challenged by a *Pf* parasite more than 110 years ago. MQ, developed initially to treat CQ-resistant malaria, was also resisted later on.[9] Currently, MQ is used as a partner drug with AS in the ACT. The mechanism of action of MQ has been primarily attributed to the intraerythrocytic asexual stages. One of the plausible mechanisms is its interaction with heme prevents the formation of β-hematin, resulting in the toxic accumulation of heme byproduct FPIX in the food vacuole of the parasite.[8] An *in vitro* study by Wong et al., have suggested that MQ inhibits protein synthesis in *P. falciparum* by targeting the GTPase catalytic site located on the cytosolic 80S ribosomal subunit of the malarial parasite. They have solved a 3.2 Å resolution cryo-electron microscopy (cryo-EM) structure of the *Pf* 80S ribosome with the (+) MQ enantiomer bound to the residues within the GTPase-associated center. The binding site of MQ to the 80S ribosome is on the highly conserved ribosomal protein uL13.[22] Nevertheless, this finding was countered by another research group, which stated that MQ had no effect on the *Pf* 80S ribosomal subunit *in vitro* and it has been mischaracterized as a ribosomal inhibitor.[17,43]

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LMF is suggested to inhibit the conversion of toxic heme into nontoxic hemozoin by the malarial parasite.[44] The primary site of action of LMF is the cytosol of the *Plasmodium* parasite, and as mentioned above, it can also inhibit hemozoin formation within the DV of the parasite. However, mutations in the *Pfmdr1* gene, which are believed to regulate the transport of arylamino drugs like LMF into the DV, can alter the parasite's susceptibility to the drug by taking it away from its primary target site.[21] *Pfmdr1* gene amplification increases protein expression and has been implicated in resistance to MQ, LMF, and QN.[34] While widespread resistance to LMF alone is not commonly reported, studies have shown evidence of decreased susceptibility to LMF associated with mutations in the *Pfmdr1* gene, suggesting the potential for developing resistance.[23,45−47] LMF is also used as a partner drug for an artemisinin derivative named artemether together known as Coartem.[8]

Antifolates. These drugs prevent the synthesis of folic acid, which is essential for the synthesis of amino acids and nucleotides. The antifolate drug includes inhibitors of dihydrofolate reductase (DHFR), i.e., proguanil, pyrimethamine, and inhibitors of dihydropteroate synthase (DHPS) i.e., sulfa drugs (sulfadoxine). Two essential enzymes in *Plasmodium's* folate synthesis pathway are DHPS and DHFR.[6] Malaria parasites cannot use folate directly in the host environment. Antifolates interfere with the activity of these enzymes, diminishing the growth of *Plasmodium* sporozoites along with the development of gametocytes. Proguanil was one of the earliest antifolate antimalarial drugs. It inhibits DHFR by interfering with the formation of deoxythymidylate through its metabolite, cycloguanil (CG). As the malarial parasites enter the bloodstream, proguanil kills them before they can invade the erythrocytes. Proguanil has been used in combination with atovaquone (Malarone). The combination of the two drugs has produced a synergistic effect that has made it a very effective antimalarial action.[9] Pyrimethamine inhibits DHFR, preventing the formation of tetrahydrofolate, thus affecting the biosynthesis of amino acids and nucleic acids.[8] The structures of sulfadoxine and *p*-aminobenzoic acid (PABA), a component of folic acid, are similar. They prevent the synthesis of dihydrofolate acid by inhibiting the DHPS enzyme.[19] A combination of sulfadoxine-pyrimethamine (SP) was approved as an antimalarial drug.[9] Due to rising resistance to quinoline derivatives, CQ was substituted with SP as the first-line treatment to combat malaria. However, resistance to folic acid synthesis inhibitors developed more quickly as compared to resistance against derivatives of quinolone; since around 1967, *Pf* developed resistance to SP and spread to various countries. The drug resistance developed as a result of mutations at crucial loci of the two important targets for SP, i.e., DHPS and DHFR. Studies have confirmed that mutations in the *pfdhps* at the S436A, A437G, K540E, and A581G positions and mutations in S108N, N51I, C59R, and I164L of *pfdhfr*. Among these, the A437G variation is an important factor contributing to sulfadoxine resistance,[24] and the substitution of S at position 108 by N is the significant factor of pyrimethamine resistance.

Artemisinin (ART) Derivatives. ART, isolated from the herb *Artemisia annua*, belongs to a sesquiterpene lactone peroxides class that contains unique peroxy-bridge groups within the molecules. These compounds became indispensable for efficient malaria treatment after CQ and antifolates were redundant due to the resistance. ART-based combination therapies (ACTs) are first-line treatments for uncomplicated malaria, combining a shorter-acting time and extremely potent ART-derivative (artesunate (AS), artemether (ATM), and dihydroartemisinin (DHA)) with a longer-acting time partner drug.[48] Presently, WHO recommends six first-line ACTs: artesunate-amodiaquine (AS-AQ), artesunate-pyronaridine (AS-PND), artemether-lumefantrine (AL), dihydroartemisinin-piperaquine (DHA-PPQ), artesunate-mefloquine (AS-MQ), and artesunate-sulfadoxine-pyrimethamine (AS-SP).[49]

ART derivatives need to be activated to exert their impact. The activation is initiated by the Fe^{2+} heme produced through the digestion of hemoglobin. Particularly, *Pf* invades the host red blood cells (RBCs) during the erythrocytic phase of the parasite life cycle, and hemoglobin endocytosis occurs. ARTs affect several metabolic machinery and functions, including unfolded protein response (UPR), eukaryotic translation initiation factor 2α (eIF2α), phosphatidylinositol 3-kinase (PI3K), and proteasome protein polyubiquitination.[25] ARTs kill parasites by damaging their proteins and preventing the functionality of the proteasome. Proteasome activity inhibition results in the accumulation of polyubiquitinated proteins, followed by the parasite death.[50] ARTs are also considered to aid in the formation of free radical species because of endoperoxide bridge cleavage following artemisinin activation. The ART peroxide bridge breaks when it interacts with hematin, a byproduct of hemoglobin degradation. The bridge cleavage generates reactive oxygen species (ROS) that attack the parasite's nucleophilic proteins and lipids and occurs inside the DV in the erythrocytic stage. It primarily kills the parasites in the ring and trophozoite forms.[26] The accumulation of ROS is also associated with increased DNA damage. Eventually, both ROS and DNA damage caused the death of the parasite. ART also blocks hematin crystallization and heme detoxification after the drug is activated.[51] Additionally, ARTs enhance *Pf* PI3K ubiquitination, resulting in a decrease in the lipid product phosphatidylinositol 3-phosphate (PI3P). PI3P localizes to the membrane of the parasite food vacuole and apicoplast, and it is an important lipid for vesicular transport, for instance, in the exportation of protein.[34]

Like many other antimalarial drugs, ART (artemisinin) and ACTs also succumb to resistance by the parasite.[49] Many cases of ART resistance, particularly resistance to the DHA-PPQ combination therapy, were detected in the SEA region.[52] A mutation in the *kelch13 (Pfk13)* gene's propeller region is one of the molecular markers identified as a key factor in ART resistance. The *k13* in *Pf* produces the 726-amino acid protein known as K13. The asexual intraerythrocytic stage of the *Plasmodium* lifecycle depends on the *k13* gene.[27] The propeller domain of the K13 protein presents various protein−protein interaction sites and aids various physiological processes, e.g., the degradation of ubiquitin-regulated proteins and oxidative stress responses. Parasites that are resistant to ARTs due to mutated K13 protein displayed a reduction in the process of hemoglobin endocytosis, leading to decreased hemoglobin digestion. This resulted in the reduction of drug activator Fe^{2+} heme and disrupted the activation of ARTs, thereby diminishing the effectiveness of ARTs.[53,54] C580Y, I543T, R539T, and Y493H, mutations in *k13* were most closely associated with the emergence of resistance. The *k13* C580Y mutation reduces interactions between the *Pf*PI3K and ART, resulting in a reduction in polyubiquitination by the *Pf*PI3K and subsequent reduction in PI3P, which is involved in phospholipid signaling.[55] Additionally, a protein, Kelch13

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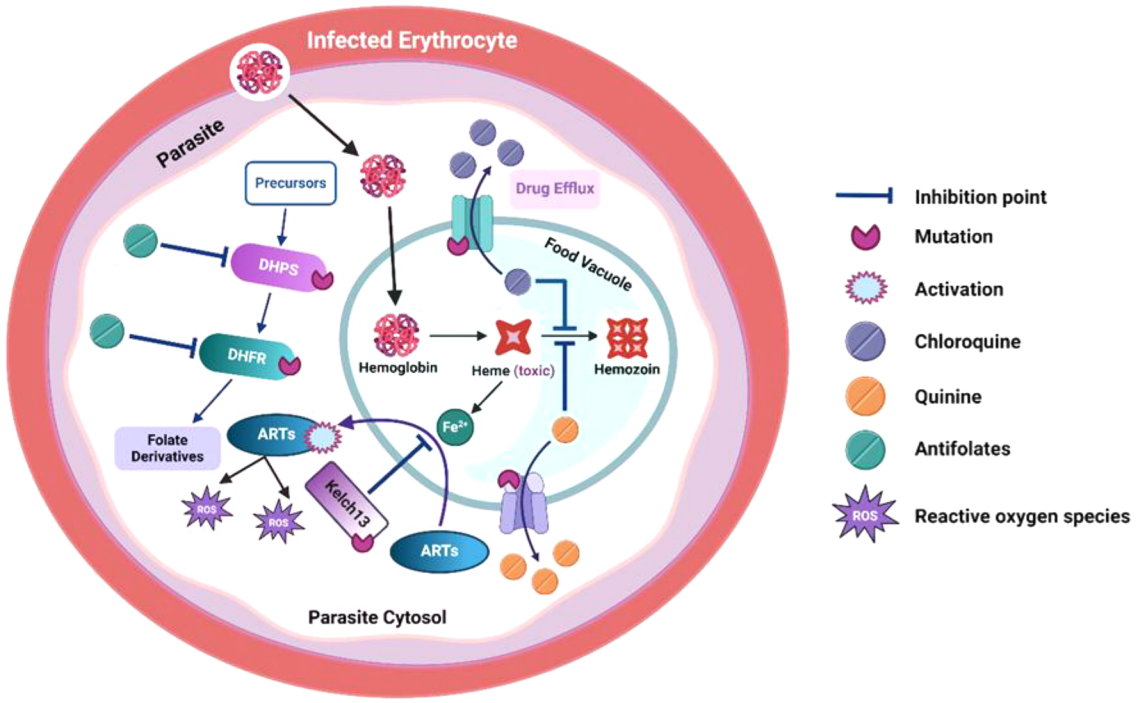

Figure 3. Mutation triggered drug resistance in *Plasmodium* spp. (created in BioRender.com).

interacting candidate 5 (KIC5), has been linked to playing a role in regulating the ART stress response in the malarial parasite.[56] A study reported an epitranscriptomic stress-response mechanism in ART-resistant parasites, in which thiouridine tRNA modifications regulate the response to ART exposure. Using mass spectrometry, genetic knockdowns, and proteomics, it was confirmed that these tRNA modifications contribute to ART resistance and other drug stressors. Furthermore, K13 could be modulated by codon bias translation when ART pressure is removed, revealing a novel function for epitranscriptomic mechanisms in regulating drug resistance of malarial parasites.[57]

Apart from *kelch13*, some other genes have also been identified to be associated with ART derivative resistance, such as, adaptor protein complex 2 mu subunit (*Pf*AP-2 μ), upstream binding protein 1 (*Pf*UBP1), and DNA helicase Rad5 (*Pf*Rad5). *Pf*AP-2 μ and *Pf*UBP1 proteins are associated with the endocytosis process of host hemoglobin along with *Pf*Kelch13.[58,59] *Pf*AP-2 μ is also important for schizont maturation and localization of key merozoite proteins during the intraerythrocytic stage of the parasite's lifecycle.[60] *Pf*Rad5 is one of the important factors of the DNA repair machinery which plays key roles in processes like postreplication repair and template switching.[30,61] Henrici et al. have modified both *pfap-2* μ and *pfubp1* genes using CRISPR/Cas9 editing, which showed a notable reduction in ring-stage susceptibility of *Pf* to Artemisinin. In *pfap-2* μ I592T mutation showed more than a 9-fold increase in ring-stage survival assay $(RSA)_{0-4h}$ survival%, and in *pfubp1* V3275F mutation showed more than a 5-fold increase in RSA_{0-4h} survival% as compared to wild-type (WT).[28,62] Mutations in *pfap-2* μ and *pfubp1* can interfere with the uptake of hemoglobin by the malarial parasite, which in turn may affect the activation of ART, causing a reduction in ROS production and ART resistance.[58] In the case of *pfrad5*, studies have found nonexclusive nonsynonymous SNPs in *pfrad5* (N1131I and N821K) which seemed to be linked with artemisinin resistance. Moreover, these mutations (N1131I and N821K) have persisted over time, spreading throughout the Greater Mekong Subregion (GMS) over the past two decades as covariates of *pfk13*.[30] The N821K and N1131I mutations have been associated with a prolonged parasite clearance half-life (PC1/2) in the GMS.[29] Moreover, the human homologue of Rad5 is known to prevent autophagy-induced cell death by repairing DNA damage caused by ROS.[30] Interestingly, artemisinin-resistant *P. falciparum* is thought to evade a programmed cell death-like process which is at least partially triggered by ROS-mediated stress as part of ART's mechanism of action.[63] The findings of these studies further support the role of *Pf*RAD5 in the mechanism of artemisinin resistance. Therefore, ART resistance appears to be governed by a multifaceted mechanism arising from the combined influence of various cellular and metabolic factors.

Naphthoquinones. Atovaquone (hydroxy-1,4-naphthoquinone, brand name Malarone), is the first antimalarial drug approved for targeting *Plasmodium* mitochondria. It is presently administered in conjunction with proguanil for malaria prevention. Atovaquone acts as a ubiquinone analogue to block Cytochrome *b* (*cytb*) parts of the cytochrome bc1 complex, inhibiting the electron transport chain.[19] This system is essential for asexual blood stage parasites, as it activates dihydroorotate dehydrogenase (DHODH), an enzyme accountable for *de novo* pyrimidine synthesis. Resistance to atovaquone monotherapy is linked to single-point mutations in the gene that encodes *cytb*.[8] However, many mutations (Y268S/C/N) of resistant isolates were nontransmissible[64] since the parasites harboring *cytb* mutants failed to form sporozoites in mosquitoes.

Antibiotics. The key enzymes in the apicoplast are fascinating targets for malaria treatment. The clinical treatment for malaria already employs translation inhibitors as antimalarial medications; clindamycin, tetracycline, and doxycycline are likely the most extensively studied that inhibit the

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protein synthesis in the parasite.[6] Clindamycin is a semisynthetic derivative of lincomycin. Synthetic antibiotics and tetracyclines are based on a cycline that is produced naturally by *Streptomyces*. Tetracycline is classified into three groups on the basis of pharmacological distinctions. The long-acting group, which includes doxycycline, is the most active against *Plasmodium in vitro*. The "delayed death" phenomenon is an inhibitory behavior that they display despite their potency and effectiveness.[13] While indirect evidence points to apicoplast as the focus of the "delayed death" phenomena, the exact mechanism is yet unknown. Tetracycline and doxycycline inhibit the apicoplast genome's expression, resulting in defects in the apicoplast. *Pf* resistance is mediated by mutations in apicoplast rRNA. Resistance field isolates that were cultured for clindamycin have an A1875C mutation in the gene encoding the apicoplast 23S rRNA.[8] Currently, there are no recognizable markers for doxycycline resistance. However, research has indicated that *pfmdt*, a multidrug transporter gene that shares a high sequence similarity with efflux pumps and copy numbers of *Pf* GTPase *TetQ* gene (*pftetQ*), which is involved in bacterial resistance to the cycline drugs, are the possible markers of reduced *in vitro* susceptibility to doxycycline.[31] These medications are frequently used as preventatives and in combination with fast-acting antimalarial drugs due to their slow onset of action. Identifying new antimalarials with novel targets is paramount due to the increasing prevalence of drug-resistant malaria.[65]

Molecular Mechanisms of Drug Resistance. A change in a protein's structure or function appears to be the primary cause of drug resistance. The alteration in the protein is regulated by genetic modifications such as single nucleotide polymorphisms (SNPs) or copy number variations (CNVs).[10] Different mutation-triggered drug resistance sites in *Plasmodium* spp. are depicted in Figure 3.

Structural Changes in the Target Protein. Mutations in the gene that encodes the target protein of an antimalarial drug are one of the factors that contribute to the formation of drug-resistant parasites. This subject was intensively studied in relation to parasite's resistance to pyrimethamine, which works by interfering with the folate synthesis pathway of *Plasmodium*. Resistance to pyrimethamine is developed by SNPs in the *pfdhfr* gene which reduces the enzyme's affinity toward the inhibitor.[4] Similar mutations in *pvdhfr* and *pvdhps* cause resistance to the antifolate drugs since the enzymes involved in the folate synthesis pathway are identical in *Pv*. Lately, a study identified the A383G mutation in the *pvdhps* gene, which has the ability to alter the affinity of drugs and proteins.[66] There have been reports of this mutation at various prevalence rates across the world, with the highest level found in western Thailand and western Cambodia.[67] Several resistance-conferring SNPs, sometimes occurring in combinations, were reported in many African and non-African countries. Using transgenic *S. cerevisiae* strains carrying *pfdhfr*, evolutionary trajectories were analyzed. The results indicate that different subsets of SNPs emerge subsequently and compensate for the loss of fitness generated by the reduced affinity for dihydrofolate, leading to nearly as strong an enzyme activity as the wild-type.[68] Mutations in *pfcytb*, the target of atovaquone, have led to changes in the catalytic activity of the protein, resulting in resistance to atovaquone.[45]

Drug-Tolerance Induced by Metabolic Alterations. *Pf* has developed mechanisms for drug resistance that involve a variety of metabolic and intracellular signaling pathways. Currently, with the help of antimalarial medicines, ART, and PPQ, the mechanism has been explored. As already mentioned, ARTs encounter free ferrous iron (Fe^{2+}) in the DV of the malarial parasite, resulting in the formation of ROS. This leads to severe damage to lipids and proteins and, thereafter, dysfunction of proteasomes because of the excessive level of polyubiquitinylated proteins and activation of antiproliferative stress response pathways, causing parasite growth arrest and eventually death.[50] The K13 protein is not a pump or an enzyme; instead, it is anticipated as a substrate adapter for the cullin E3 ligase, with a possible substrate of *Pf*PI3K and a redox sensor.[45] ART resistance is defined by WHO as ″partial″ resistance, which is phenotypically defined as a delayed parasite clearance following treatment with an ACT that may result in parasite reactivation.[3] In the SEA region, the majority of parasites were discovered to carry a C580Y mutation despite the identification of at least 20 mutations in *k13*, and this mutation accounts for 80% of resistant cases.[69] The specific physiological role of *Pf*k13 is yet to be elucidated. However, considering all the possible roles of *Pf*k13 mentioned in the previous section, it is assumed that a combination of these factors permits ring-stage parasites containing mutant *pfk13* to endure longer periods of ART exposure.[49] Zhu et al. reported a range of transcriptionally linked 156 genes that probably play a role in ART resistance through changed transcription levels. They named this Artemisinin Resistance-associated Transcriptional Profile (ARTP) and provided proof, suggesting that its constitutive expression evolved from the initial transcriptional responses of sensitive *Pf* parasites to the ARTs.[70] An orthologue of *Pfk13* exists in *Pv* (*Pvk13*), but there are no mentions of *Pv* resistance yet to ARTs.[45]

Metabolomic studies revealed that PPQ, which is nearly identical in structure to CQ, inhibits the parasite's nucleotide metabolism in addition to inhibiting hemozoin production.[71] High-level resistance to PPQ is associated genetically with an increase in the copy numbers of *Pf plasmepsin II* and *III*;[72] these genes encode aspartic proteases and contribute to the breakdown of host hemoglobin and particular SNPs in *pfcrt*.[73] This fact was supported by an investigation that found an epistatic interaction between these mutations, with the M343L mutation in *pfcrt* being crucial for resistance to high PPQ concentrations exceeding 200 nM.[74] In terms of mechanism, this signifies a synergistic effect of enhanced hemoglobin digestion and removal of PPQ out of the DV of the malarial parasite.[75]

SNPs and CNVs of Drug Transporter Proteins. Mutations in transporter proteins can also cause resistance to antimalarial drugs. The most notable example is CQ resistance, which is mostly caused by SNPs in the genes that encode the *Pf* chloroquine resistance transporter (*Pf*CRT) and, to a smaller extent, the multidrug resistance protein 1 (*Pf*MDR1). The protein *Pf*CRT is highly polymorphic in nature, existing more than 20 distinct point mutations.[8] This transporter protein is outward-directed and is situated on the DV membrane of the malarial parasite. When *Pf*CRT is in mutant form, it can effectively flush out CQ from the DV, thereby prohibiting it from attaching to its specific target. Almost all strains of *Pf* that are resistant to CQ contain the crucial K76T point mutation in the *pfcrt* gene, with some exceptions, such as instances where a K76A mutation has been noted instead of a K76T mutation.[4] *Pf*MDR1 is an inward-directed transporter protein situated on the membrane of the parasite's DV. Both *in vitro* investigations and field studies conducted in Africa, SEA, and Oceania

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demonstrated that the N86Y mutation in *pfmdr1* was associated with CQ resistance, although with a minimal effect on the intensity of resistance.[8] Mechanistic studies on transgenic *Xenopus laevis* oocytes indicated that *Pf*MDR1 may promote the transport of CQ (chloroquine) into the oocyst and QN (quinine) out of the oocytes, implying a contribution in resistance to both 4-aminoquinolines and aryl amino alcohols.[4] The multidrug resistance-associated protein (*Pf*MRP) belongs to the ABC protein family and the ABC transporter C subfamily. *Pf*MRP genetic disruption increases parasite susceptibility to antimalarial medicines such as CQ, QN, ART, PPQ, and PQ and accumulates more glutathione (GSH), CQ, and QN.[76] CQ-resistant *Pv* was reported first in Papua New Guinea. There is no definitive molecular marker of CQ resistance in *Pv*. Although *pvcrt-o* is orthologous to *pfcrt*, no direct link has been observed between CQ resistance and mutations in *pvcrt-o*. The most prevalent *pvcrt* polymorphism is a lysine insertion at position 10 (K10).[77] Also, no evident relationship has been detected between *pvmdr1*, the homologue of *pfmdr1*, and CQ (chloroquine) resistance. While some studies revealed point mutations of *pvmdr1* within resistant parasite populations, including a Y976F substitution in Indonesia and an F1076L mutation in SEA, these polymorphisms are not uniform between parasite populations. Additionally, CQ-resistant parasites with the wild-type *pvmdr1* have also been identified. A study examining recurrent *Pv* infections in the Brazilian Amazon reported that CQ resistance is linked to higher copies of *pvcrt-o*.[78] For increased tolerance to aryl amino alcohols, CNVs of *pfmdr1* may play an important role. Since aryl-amino alcohols are expected to function at least partially by disrupting hemozoin formation, increased synthesis of *Pf*MDR1 might facilitate effective efflux and sequestration of the compounds outside of the parasite's DV, thereby keeping the drugs apart from their respective targets. Several investigations have stated that increasing the copy number of *pfmdr1* is associated with enhanced sensitivity to both CQ and PPQ. After DHA-PPQ was introduced as a first-line treatment, a decrease in copy number of *pfmdr1* was seen in *Pf* strains that were circulating in Thailand. This decreased the sensitivity to PPQ while increasing the sensitivity to MQ.[72] As discussed, both *Pf* and *Pv* can quickly adapt to the current antimalarial drug interventions.

The effectiveness of antimalarial therapy is increasingly impeded by the emergence of antimalarial drug resistance, and scientists are diligently pursuing research to address this challenge. Continual development of novel molecules, medications, and antimalarial drugs is necessary to prevent the emergence of resistant parasite strains,[79] since the malaria parasite, *P. falciparum*, has already developed resistance to most antimalarial drugs and their derivatives.

DIAGNOSIS OF DRUG-RESISTANT MALARIA

To eradicate malaria, proper and timely diagnosis in community settings is very important. There are various conventional and advanced techniques known for diagnosis. Along with this, several methods have been used for the detection of antimalarial drug resistance, which include *in vivo* drug efficacy studies, *in vitro/ex vivo* drug sensitivity studies, and molecular diagnosis.

Conventional Diagnostic Methods for Malaria. There exist numerous well-established and conventional methods for diagnosing malaria, such as nucleic acid amplification techniques (NAATs), rapid diagnostic test (RDT) kits, and gold-standard microscopic analysis. The light microscopy technique commonly utilizes thin and thick blood smears with Giemsa stain. Its sensitivity depends on the parasitemia level.[14] To increase its sensitivity, several techniques have been developed. For instance, fluorescent dyes like acridine orange have been employed to stain the *Plasmodium* DNA within infected blood cells, making it easier to detect parasites under a fluorescence microscope. NAATs include several PCR-based assays, including loop-mediated isothermal amplification (LAMP), which can detect parasites at much lower densities. PCR is a high-throughput method and has a limit of detection (LOD) of 1 to 5 parasites/μL of blood.[80] Various types of PCR can be employed to identify *Plasmodium* species, such as real-time quantitative PCR (qPCR), Nested PCR, and multiplex PCR.

All the above techniques, though offer sensitive diagnosis, they face some limitations, such as the requirement of multistep analysis and skilled labor, costly equipment, and thus are difficult to deploy in PoC settings. However, LAMP uses simpler, less expensive equipment and is relatively faster than PCR and hence has the potential to offer detection in PoC settings.

The RDTs are lateral flow Immunochromatography-based devices widely used for routine diagnosis of malaria by detecting malaria antigens in the blood. The RDT relies on dye-labeled antibodies interacting with the specific target antigen in the blood, which leads to a visible color band on the strip. The common target antigens are *Pf* histidine-rich protein-II (*Pf*HRP-II), *Pf* glutamate dehydrogenase (*Pf*GDH), *Pf* lactate dehydrogenase (*Pf*LDH), *Pv*LDH, common *Plasmodium* LDH (pan- *p*LDH), and aldolase.[81] The RDTs are simple, portable, less expensive, and can deliver results within 30 min. Nevertheless, humidity and extreme temperatures might impede their effectiveness. Moreover, in regards to HRP-II as the target antigen for specific detection of *Pf*, its growing gene deletion cases, and persistence in the blood even after ceasing the disease condition, the RDT exhibits false negative and false positive results, respectively, for *Pf* species.

The immunofluorescence antibody test (IFAT) identifies *Plasmodium* species indirectly using fluorescently tagged secondary antibodies to detect both IgG and IgM antibodies produced by the immune response of the body against the asexual blood stage of the parasite's lifecycle.[80] The indirect ELISA is a technique used to identify antibodies specific to *Plasmodium* species. In this method, a serum sample is incubated with malaria-specific antigens, and the primary antibodies present in the serum sample are detected by using an enzyme-conjugated secondary antibody. Conjugated enzymes then react with substrates to produce a color change, confirming the presence of *Plasmodium*-specific antibodies. This approach allows for the simultaneous analysis of *Pf* samples with high throughput; however, it is less sensitive compared to IFAT.[80,82] Malaria infection can be directly confirmed by detecting parasite-specific antigens with the Enzyme Immuno Assay (EIA) or Sandwich ELISA. In these methods, antibodies are immobilized in the wells of a plate. When the sample is added, primary antibodies capture the *Plasmodium* species-specific antigen. The reaction used for the detection is similar to the ELISA test.

The flow cytometry-based approach initially developed for detecting malarial parasites was later further developed into a rapid and simple flow cytometry, known as the tricolor method

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Table 2. Summary of the Methods Used for the Detection of Antimalarial Drug Resistance and Their Advantages and Disadvantages[a]

Type		Name of Method	Advantages	Disadvantages
In vivo studies		TES	Easy to standardize, No heavy equipment, Minimal supplies and training required,[91] Results have direct clinical relevance, Can confirm the association between parasite resistance markers and drug efficacy *in vivo*	Complexity, Logistical constraints,[90] Impact of drug resistance could be undetected if patient has a high degree of acquired immunity,[5] Unable to differentiate recrudescence from reinfection, Difficult to conduct in low transmission settings,[92] High-cost
In vitro/ex vivo studies		WHO Microtest (Microscopy)	Simple, Easy to operate, No heavy equipment, Small amount of capillary blood required,[91] Highly sensitive	Labour-intensive
		Isotopic test	Automated reading, Highly sensitive, Easy to perform	Expensive, High parasite density required,[91] Involves risk factors due to the use of radioactive reagents[12]
		Flow cytometry	Highly sensitive, Fast method, Automated and nonradioactive[91]	Expensive, Generally needs heavy equipment, Requires skilled personal
		ELISA	Cost-effective, simple procedure,[12] Highly sensitive	Labour-intensive, Antibody instability
		Fluorescent markers	Inexpensive, Fast assay	Heavy equipment, Underestimation of parasitemia, Interaction of drugs and dye instead of DNA[5]
		RSA	No heavy equipment	Labour-intensive, Difficult to standardize[5]
Molecular methods	PCR-based	PCR-RFLP	Relatively easy to operate, Multiplexed analysis, Low cost per test, simple instrumentations	Needs trained staff, Complex assay, Low throughput, Time-consuming, Unable to detect CNV
		Nested PCR combined with sequencing	High sensitivity and specificity	Needs trained staff, Possibility of false positive result, qualitative, Time-consuming
		SSOP-ELISA	Simple, Multiplexed analysis, Low cost	Needs trained staff, Low throughput, Unable to detect CNV
		qPCR	Can be multiplexed to identify many antimalarial drug resistance-associated SNPs in a single run, Rapid, Higher sensitivity and Specificity than conventional PCR,[93] Low risk of contamination	Needs trained staff, probes are expensive, need labeling, and sophisticated equipment
		AS-PCR	Low-cost, simple, High throughput, Rapid	Needs trained staff, More stringent requirements
		LDR-FM assay	High sensitivity and specificity; high throughput,[94] Rapid, Multiplexed analysis	Needs trained staff, Expensive reagents and equipment, Unable to detect CNV
	Isothermal amplification	LAMP	High specificity,[95,96] Simple, Low cost, single-step suitable for POCT[97]	Low-throughput, Complex primer designing, High risk of aerosol contamination increased the rate of false positive results[6]
	Sequencing-based	Sanger sequencing	High throughput, Can detect CNV	Time-consuming, Needs trained staff, Requires extensive data analysis
		Next-generation sequencing	High throughput, Can detect CNV, High accuracy, Multiplexed analysis	Needs trained staff, Short-read length, Needs high volume computing system for data analysis
		Nanopore sequencing	longer read length, High throughput, Real-time sequence reads	Error-prone, Needs trained staff, poor yield of high-quality data[12]
	CRISPR/Cas-based	_	Easy to operate, High sensitivity and specificity[88]	Longer detection time, Off-target activity
	Other techniques	HRM	Easy to operate, High sensitivity and specificity,[98] Low cost, High throughput, no risk of sample contamination	High sample quality needed, Possibility of false positive result
		NALFIA	High sensitivity and specificity,[99−101] Rapid	Low throughput, Unable to detect CNV, Needs trained staff
		Q-poc	Easy to operate, Portable	–
		DNA microarray	Can detect CNV, High throughput	Needs trained staff and computational facilities, and involves sample processing and data analysis steps

[a]TES: Therapeutic efficacy studies; ELISA: Enzyme-linked immunosorbent assay; Ring stage survival assay: RSA; CNV: Copy number variation, PCR-RFLP: PCR-restriction fragment length polymorphism; SSOP-ELISA: Sequence-specific oligonucleotide probe-based ELISA; qPCR: Real-time fluorescence quantitative PCR; AS-PCR: Allele-Specific PCR; LDR-FM: Ligase detection reaction-fluorescent microsphere; LAMP: Loop-mediated isothermal amplification; HRM: High-resolution melting, NALFIA: Nucleic acid lateral flow immunoassay.

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(TCM), which is for quantification and grading of different malaria parasites in red blood cells or *in vitro* cultured *Pf*. In this flow cytometry, the malaria pigment, hemozoin, is widely used as a marker. However, like other conventional methods, flow cytometry also suffers from several drawbacks that impede its application outside established laboratory settings.

Biosensors are emerging as a cutting-edge technology for PoC and point-of-need (PoN) deployment for diverse applications, including healthcare, biomedical, pharmaceuticals, and many more.[83] It combines the fine selectivity of biorecognition elements with the power of a small transducer that transduces the biochemical signals generated as a result of the interaction between the biorecognition element and the target of interest into a quantifiable electrical signal in a readable format. These devices should comply with the ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable to end user) criteria for their healthcare applications in developing and underdeveloped countries.[83] Several types of biosensors based on types of recognition element (e.g., aptamer, enzyme), transducer (e.g., electrochemical, optical, piezoelectrical), and platform substrate (e.g., paper) have been developed for the detection of malaria.[84−86] Some of the latest additions of prominent biosensors to the field of malaria diagnosis are smartphone-based optical fiber platforms for quantitative detection of *Pf*GDH,[87] and Specific High-Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK) method for detection of *Plasmodium* strain. The SHERLOCK is a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) based method utilizing *Lw*Cas13a protein activity to detect the genomic DNA of a *Plasmodium* strain, with a sensitivity level ranging from 2.5 to 18.8 parasites per reaction. In comparison to a real-time PCR-based assay, the SHERLOCK assay for *Pf* detection demonstrated better sensitivity and selectivity.[88] Using this molecular technology, a field-deployable ultrasensitive detection of *Plasmodium* species for both symptomatic and asymptomatic cases of malaria has been reported.[89] However, biosensor research on malaria diagnosis needs more attention for their miniaturization into a portable device for PoC applications.

Techniques for Detecting Antimalarial Drug-Resistant Parasites. Precise and sensitive molecular diagnostic technologies are essential for monitoring the geographical and temporal spread of drug-resistant *Plasmodium spp*. and identifying genetic markers connected to the resistance.[49,90] Identification of resistance-associated molecular markers in both clinical and laboratory settings can also increase the effectiveness of available antimalarial medications. The ability to monitor drug resistance is crucial for advancement in therapy, disease surveillance, and epidemiology, prompting the development of sophisticated diagnostic tools.[12] At present, the primary techniques for detecting drug resistance in *Plasmodium spp*. include *in vivo* drug efficacy studies, *in vitro/ex vivo* drug sensitivity studies, and molecular diagnostic studies that evaluate known markers of antimalarial drug resistance.[90] A summary of the methods used for the detection of Antimalarial drug resistance and their advantages/disadvantages are presented in Table 2.

In Vivo Drug Efficacy Studies. *In vivo* studies, particularly therapeutic efficacy studies (TES), are regarded as the 'gold standard' for evaluating antimalarial drug resistance and drug efficacy, as recommended by WHO.[92] Malaria patients are treated with known dosages of antimalarial medicines, and their parasitemia levels are recorded after treatment. TES are crucial for evaluating the impacts of medications and drug resistance inside the body, as host-parasite interactions can influence the efficacy of therapeutics and clinical outcomes.[12] *In vivo* studies, however, involve many uncontrollable factors. First, individual differences, especially in absorption and metabolism, can vary significantly. Additionally, patients receive a range of essential treatments, including fluid resuscitation, mechanical ventilation, antibiotics, nutritional support, and oxygen therapy.[6] Due to the complexity of the immune response and diversity among humans, associations seen in *in vitro* tests or molecular studies may not always convey the expected clinical result in patients during *in vivo* studies. Furthermore, the impact of drug resistance in the malarial parasite could remain undetected if the patient possesses a high degree of acquired immunity from repeated malaria exposure in an endemic region because the immune response will still be adequate to eliminate the infection even if the drug is ineffective. However, *in vivo* studies are most effective in correlating resistance impact on actual clinical outcomes in patients.[12] These studies can be carried out along with molecular assays to evaluate the relationships between resistance markers and *in vivo* drug efficacy; e.g., Tun et al. have studied SNPs in the *pfkelch13* and drug failure rates after ART treatment.[102] Animal models are used for *in vivo* testing of drugs to reduce the impact of host immunity despite their significant differences from humans. However, these models have several limitations, including the fact that the results may not always accurately reflect what occurs in human patients and may not eliminate all host factors that could affect the efficacy of antimalarial drugs. Furthermore, there are ethical concerns with utilizing animal models, especially nonhuman primates, for the study of human diseases. Gabriel et al. conducted a single-arm *in vivo* prospective TES to assess the parasitological and clinical response to the first-line treatment of *Pv* in Ethiopia. They found that coadministration of CQ with PQ was efficacious and well-tolerated, with fast fever resolution and a high parasite clearance rate but with a 7.4% failure rate.[103] This *in vivo* method is, however, difficult to deploy, especially in areas with low transmission where screening thousands of patients is required.[5]

In Vitro and Ex Vivo Techniques. Unlike *in vivo* models, the evaluation of the susceptibility of malarial parasites to antimalarial medicines can be done phenotypically. This can be achieved by using parasite samples obtained from patients (*ex vivo* method) or by utilizing culture-adapted *Plasmodium* isolates (*in vitro* method) in a controlled condition.[5] In these methods, *Plasmodium* parasite is cultured and exposed to different drug concentrations to assess the growth-inhibitory effects of drugs, or parasites are exposed to a specific high concentration for a brief period of time to assess their sensitivity to the drug. These methods have the advantage of classifying parasites as "resistant" or "sensitive" with minimal confounding variables. Drug resistance is measured through calculations like survival rate or IC_{50}, which is the half-maximal inhibitory concentration that slows the growth of the parasite by 50%.[12] At present, *in vitro/ex vivo* drug sensitivity assays employ various methods to detect parasite growth as described below.

WHO Microtest (Microscopy). The basic strategy of this method involves manual counting of *Plasmodium* parasite growth in response to varying drug exposure using a microscope. Here, parasites (whole capillary blood of patients)

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are cultured in a microtiter plate precoated with varying concentrations of drugs along with drug-free control wells and incubated for 24 to 30 h. After completion of the incubation, thick blood films are prepared from each well, followed by Giemsa staining. Using microscopy, the number of schizonts present in the stained sample film is counted and compared with respect to the count from drug-free control wells.[91] The count is then employed to determine the IC_{50} value. To interpret the results, one needs to consider the count of parasites at various developmental stages, such as the schizont or ring stage. However, parasite visibility may change during its developmental stages.[5]

Isotopic Test. This test measures the growth of the parasite by incorporating radioactive dye into the culture. The dye can be added to a DNA precursor, like hypoxanthine or ethanolamine tagged with radioactive tritium.[12] Parasites are cultured with various drug concentrations in 96-well plates for 48 h. After that, the culture is filtered using a filter paper, and then the paper is dried. In the next step, a scintillating liquid is added to the dried paper and tested for beta radiation, thereby measuring the DNA of the parasite.[5] The incorporation of hypoxanthine is directly proportional to the number of *Plasmodium*-infected red blood cells.[91] Further, the IC_{50} value is calculated by using the relative radioactivity corresponding to various drug concentrations. This approach is not widely adopted because it relies on radioactive reagents.

Flow Cytometry. This assay evaluates *Plasmodium* growth by quantifying the number of infected red blood cells in a blood sample. Here also, *Plasmodium* parasites are cultured for 48 h adding varying concentrations of drug in a microtiter plate. Along with this, intraerythrocytic parasite DNA is labeled with a fluorescent dye to detect infected red blood cells using this flow cytometry technique. Different permeable markers can be utilized to label the DNA.[5] The labeled cells of each well are then excited with laser or ultraviolet light, to measure the fluorescence intensity depending on the dye used. Measured fluorescence intensity is directly proportional to the development of trophozoites into schizonts.[91] The combination of noninfected and infected red blood cells is examined to determine the number of infected cells. This allows for the assessment of parasite growth in the absence or presence of the antimalarial drug, which is used to calculate the IC_{50}.[104] Portable flow cytometers can also be utilized in field settings to obtain comparable IC_{50} values. Flow cytometers can also be adapted for the detection of hemozoin, eliminating the need for expensive fluorescent dyes for field application.

ELISA. The ELISA is used to detect the growth of the parasite by quantifying the concentration of *Pf*-specific marker proteins such as *p*LDH and HRP-II. At first, malarial parasites are cultured for 24 to 72 h with varying drug concentrations in a microtiter plate, following which the culture plate is kept at −20 °C for freezing and then thawed 3 times to completely lyse the cells, ensuring the release of *Pf*-specific marker proteins. Meanwhile, another 96-well plate used in this technique is coated with monoclonal antibodies against the above-mentioned proteins at 4 °C overnight.[5] Then, cell lysates taken from thawed parasite culture plates are added to each well of the microtiter plates for incubation.[91] After 2 h of incubation, biotinylated antibodies and required reagents for colorimetric detection are added, and then the microtiter plate is read at $\lambda_{450\ nm}$. The absorbance results are utilized to determine the IC_{50}, parasite growth, and accumulation of related byproducts, which are inhibited by the drugs.[12] Parasitemia in infected patients has a positive correlation with the protein antigen.

Fluorescent Markers. This assay measures parasite growth employing a fluorescent marker that reacts with both DNA and RNA. Parasites are first cultured with varying doses of drugs over 48−72 h. After incubation, the culture plate is stored at −80 °C. Then, the SYBR Green I dye is added and incubated with the samples. The dye interacts with the nucleic acid present in erythrocytes infected with parasites. At the end, fluorescence intensities are recorded to determine the DNA amount in the culture, and the IC_{50} is calculated using the results obtained.[5]

Ring-Stage Survival Assay (RSA). Artemisinin primarily targets the parasite's ring stage, and after ART treatment, the parasite is exposed to extremely high concentrations of the drug *in vivo*. Several previously stated parasite quantification techniques become less reliable when applied for measuring cultured parasites exposed to minimal quantities of ART for 48 to 72 h, as these include several stages of the parasite lifecycle.[12] RSA was specifically developed to evaluate the resistance of *Pf* parasites against ART derivatives, which previously could not be effectively detected using conventional *in vitro/ex vivo* assays.[5] To perform this assay, malarial parasites are cultured in the absence of antimalarial drugs until the culture reaches a high density, following which they are synchronized to remove schizont-stage parasites. Then, the ring-stage parasites (0−3 h after erythrocyte invasion) are exposed to drug concentrations similar to those observed in the human body after ART treatment, allowing for comparisons with parasite populations that were cultured without the drug. Thereafter, microscopic identification of the fraction of the initial parasite population that develops into morphologically normal trophozoite-stage parasites or second-generation ring-stage is used to calculate survival rates.[12]

In vitro and *ex vivo* techniques give important information on parasite susceptibility but need extensive laboratory infrastructure for parasite culture and highly skilled personnel.[5] Thus, these assays are mainly limited to laboratories to confirm novel molecular markers of drug resistance or to associate a resistance phenotype with a genotype.

Molecular Methods for Diagnosis. Molecular techniques have made it possible to understand the occurrence and spread of drug resistance in malaria. The mechanisms of resistance to the available antimalarial drugs associated with CNVs and SNPs have been identified in part by employing molecular techniques.[5] Antimalarial drug resistance can be tracked by passive surveillance reports of treatment failure and suspected resistance; however, active monitoring needs more precise high throughput technologies for resistance detection that decreases clinical status variability in patients. Multiple molecular techniques have been developed to detect drug resistance in malaria, such as allele-specific PCR, real-time PCR, PCR-RFLP, next-generation sequencing (NGS), Sanger sequencing, microarray, etc.[90] Most of the resistance-detecting molecular methods depend on PCR amplification and sequencing of loci that contain resistance-associated SNPs.

PCR-Based Techniques. PCR technique is widely used in various configurations such as PCR-restriction fragment length polymorphism (PCR-RFLP), nested PCR combined with sequencing technology, Sequence-specific oligonucleotide probe-based ELISA (SSOP-ELISA), real-time fluorescence quantitative PCR (qPCR), allele-specific PCR (AS-PCR), and Ligase detection reaction fluorescent microsphere (LDR-FM)

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assay for detecting drug-resistant parasite strains. The detection specificity, sensitivity, and efficiency of different types of methods, however, vary depending on their underlying principles.[6]

PCR-RFLP. This technique was used to distinguish nucleotide sequence variations within a DNA fragment.[105] It uses PCR to amplify the target DNA, which is then cut into fragments of varying sizes using sequence-specific restriction enzymes. The size of the digested DNA products is ascertained by gel electrophoresis.[106] Then the outcome can be classified as 'wild-type' (i.e., no mutation linked to drug sensitivity), 'mutant' (i.e., a mutation linked to drug resistance), or mixed (i.e., a combination of parasite strains with both wild-type and mutant alleles). PCR-RFLP was utilized to identify mutations in the resistance loci of *Plasmodium*, such as *dhps, dhfr, kelch13, crt, mdr-1*.[12] In one study examining the reasons for SP treatment failure, *pfdhfr*, and *pfdhps* were assessed in 176 samples using PCR-RFLP. A new isoleucine mutation was identified at codon 108 of *pfdhfr*, showing a strong correlation with *in vitro* resistance to SP (sulfadoxine-pyrimethamine) along with early treatment failure.[107] The technique was successfully employed for determining the genetic signatures of *Pv* isolates collected from four regions with varying malaria transmission risks. RFLP analysis showed that a single clonal type was almost exclusively present in epidemic samples from three indigenous villages. This study showed the utility of PCR-RFLP for the evaluation of *Pv* genetic diversity and the detection of imported cases in a low-transmission setting.[108,105] However, not all SNP sites can be discovered with adequate restriction endonucleases, making them unsuitable for all SNP locus typing.

Nested PCR Combined with Sequencing Technology. This PCR method uses two primer pairs to amplify the entire DNA fragment. Like a standard PCR, the first set of primers amplifies a fragment. To increase the sensitivity of the reaction, the second set, known as nested primers, binds to the initial PCR product. Recently, this technique has been extensively used for rapidly identifying drug resistance and genotyping of *Pf*. One study employed nested PCR combined with sequencing technology to determine the prevalence of *Pfdhfr* and *Pfdhps* polymorphisms.[109] Using the technique, a study evaluated mutations and analyzed the SNPs and haplotypes of *Pfdhfr* and *Pfdhps* and found that the rate of mutation of *Pfdhfr* 108 and *Pfdhps* 437 codons were 94.7%, and 79%, respectively.[110] To evaluate the effectiveness of the existing treatment regimen used for uncomplicated *P. falciparum*, Singh et al. examined the mutations in four distinct genes (*Pfdhps, Pfdhfr, Pfmdr1, and Pfkelch13*) that result in resistance to artesunate and sulfadoxine-pyrimethamine. Analysis showed that there was a 53.3% occurrence of the double mutation in *Pfdhfr*, 89.3% single mutation in *Pfdhps*, 13.5% single mutants, and 51.1% synonymous mutations in *Pfmdr1* in the study area. Two synonymous and five different nonsynonymous point mutations in *Pfkelch13* were discovered, neither linked to ART resistance.[111] The advantage of this technique is that if an incorrect fragment is amplified during the initial amplification, the likelihood of the primers pairing with and amplifying this incorrect fragment in the subsequent amplification is significantly reduced. As a result, the nested PCR exhibits high selectivity and can confirm the diagnosis in cases of mixed *Plasmodium* infections and in patients with low malaria density that are not detectable under the microscope.

Sequence-Specific Oligonucleotide Probe-Based ELISA (SSOP-ELISA). This technique involves coating PCR products onto microtitration plates and incubating them with oligonucleotide probes specific to drug-resistance loci.[112] High-stringency washes eliminate unbound probes, leaving only those attached to SNPs, which are identified when the optical density reaches a minimal positive threshold. This approach was used to investigate the frequencies of the *Pfcrt* resistance allele in Tanzania.[12] This method, however, is inefficient at detecting uncommon SNPs among mixed-genotype infections.

Real-Time Fluorescence Quantitative PCR (qPCR). qPCR can identify both antimalarial drug resistance SNPs and gene copy number variations linked to resistance. It involves the addition of fluorophores to the PCR system, continuously monitoring the PCR process in real-time via the accumulation of fluorescence signals, and constructing a standard curve to quantify the specific DNA sequence present in the sample. Two primary types of fluorescent markers, specific (TaqMan probe) and nonspecific markers (SYBR Green I), are used.[6] Oligonucleotide probes containing a 5′ fluorophore and a 3′ quencher are added into the real-time PCR reaction to detect SNPs in the target resistance loci. If the polymorphism is present in the resistance locus, the probe will anneal and subsequently be cleaved by a polymerase during the extension step, releasing the fluorophore from the quencher and producing a detectable signal with intensity proportional to the amount of amplified product.[12] This enables high-throughput automation and quick determination of the SNP copy number, but it needs reagents like fluorescently labeled oligonucleotides. In a quantitative assay for gene CNV detection, the PCR mix is supplemented with fluorescent or intercalating dyes to monitor the real-time accumulation of the PCR product throughout the PCR cycles. In a study that analyzed 406 *Pfcrt* mutations, a sensitivity of 96.1% was achieved by qPCR.[6] An increased copy number of *Pfplasmepsin II* has been linked to PPQ resistance, which can be measured utilizing qPCR-based assays.[72] An onsite experimental package was developed to detect *kelch13* mutations promptly and evaluated partner drug susceptibility with minimum load in resource-limited settings. They have used qPCR to find mutations in the *kelch13* gene; it showed 100% sensitivity and specificity in the detection of the two common ART resistance mutations, C469Y and A675V, in Uganda.[93]

Allele-Specific PCR (AS-PCR). It is a simple technique for the detection of SNPs in genes based on the conventional PCR technique to enhance the resolution of base mismatches at the 3′ ends. Efforts have been made to utilize artificially modified bases as 3′ terminal bases to minimize the mismatch extension rate and to integrate mismatched bases to improve the specific detection of hotspot mutations in target genes.[6] A study employed AS-PCR along with a lateral flow test to identify *Pfcrt* and compared it to Sanger sequencing. Both selectivity and sensitivity produced by the method were 100% and 95.83%, respectively.[113] SNPs can be identified using the AS-PCR technique combined with lateral flow assays, eliminating the need for complex procedures and specific equipment.[100,101] For detecting *Plasmodium* resistance mutations, qPCR, AS-PCR, PCR-RFLP, and nested PCR combined with sequencing technology offer remarkable sensitivity and specificity. Nevertheless, the gold standard for detection is still nested PCR combined with sequencing technology.

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Ligase Detection Reaction-Fluorescent Microsphere (LDR-FM) Assay. The remarkable specificity of the ligase enzyme for specific target antimalarial drug resistance-associated SNPs signifies that ligase assays can also be employed to detect SNPs. The LDR-FM assay amplifies PCR products using a multiplex ligase detection reaction that includes specific primers for every resistance loci/mutation and common primers. Each primer has a fluorescent microsphere bead attached to a 5′ tag. If corresponding resistance loci are detected, this bead hybridizes with the PCR product at the 3′ end. The fluorescence intensity is measured automatically by a liquid fluorescence array reader.[5] To measure the abundance of distinct alleles, labeled products are examined on a fluorescence array reader. The technique was used for SNP-genotyping in *Pf* to study drug resistance markers. By accurately identifying *Pf* SNPs in *Pfcrt* and *Pfmdr1* in Uganda, this technique has proven helpful in the field.[94] Additionally, the scalability and effectiveness of the LDR-FM multiplex platform were validated for the identification of a wide range of antimalarial drug resistance markers, even in cases of low parasitemia.

Loop-Mediated Isothermal Amplification (LAMP). LAMP is an innovative amplification approach that eliminates the use of thermocycler since the amplification of the target sequence takes place isothermally by using displacement reaction. It employs four primers, two outer and two inner primers that specifically recognize six specific regions on the target sequence, and a *Bst* DNA polymerase, which has strand-displacement activity. It amplifies nucleic acid up to 10^9−10^{10} times in around 15−60 min under isothermal conditions (60−65 °C).[114] SNP genotyping can be performed by incubating genomic DNA alongside a fluorescent detection reagent, e.g., calcein, for a set duration. LAMP-amplified products exhibit a shift in color from light orange to bright yellow when using calcein. LAMP-based SNP genotyping is scalable for antimalarial drug resistance. LAMP and PCR-RFLP assays on CQ-resistant *Pf* specimens from India were carried out, and it was observed that the specificity, sensitivity, and accuracy of the LAMP assay was 100% for *Pfcrt* K76T, whereas, for the PCR-RFLP assay, it was 94%, 97%, and 96%, respectively.[95] Later on, a study employed the LAMP assay to identify 20 Cambodian and 74 Bangladeshi *Pfk13* C580Y mutant *Pf* malaria samples. Their results demonstrated 90% sensitivity, 91.9% specificity, and 2.5 h of detection time.[96] Another LAMP-based method was reported for rapid and specific detection of *Pf* malaria targeting the gene *kelch13*.[115] Further, on the line, they developed a Lab-on-Chip (LoC) device using an Ion-Sensitive Field-Effect Transistors (ISFET) that is coupled with LAMP for detecting C580Y SNP, which is linked to ART-resistant malaria.[115] Another study used LAMP and SNP analysis visualization along with a lateral flow assay (LAMP-SNP-LFA) to detect ART resistance in clinical samples. They observed that the LAMP-SNP-LFA approach is easier and faster than the conventional PCR/DNA sequencing approach, offering the potential to help malaria control.[116] The LAMP approach offers good efficacy in identifying the ART resistance-associated mutant *Pfk13*.

Sequencing-Based Technique. Recently, sequencing methods have been used to identify antimalarial resistance mutations by producing the sequences of specific regions associated with resistance.

Sanger Sequencing. This DNA sequencing technique relies on selectively incorporating chain-terminating dideoxynucleotides (ddNTPs) during the *in vitro* process of DNA replication.[12] It uses fluorescently labeled ddNTPs to terminate DNA replication as soon as a sequence randomly incorporates ddNTPs. After being separated by capillary electrophoresis, the sequence fragments are passed through a laser and a light sensor that can distinguish between fluorescent labels that correspond to the base present at the terminated end. Computer software turns this into a nucleotide sequence, which is then matched to a sequence taken from a reference strain (usually *Plasmodium* 3D7) to search for any resistance SNPs.[5] This technique has improved our understanding of the genetics of antimalarial drug resistance and supports molecular surveillance programs for malaria resistance markers. A study employed Sanger sequencing to investigate the mutation rate of three resistance markers i.e., *Pfdhps* (codons 436, 437, 540, 581, 613), *Pfdhfr* (codons 50, 51, 59, 108, 164), and *Pfcrt* (codons 72, 74, 75, 76) in 778 samples collected from Haiti. The prevalence of the *Pfdhfr* S108N single mutation was found to be 47%, and a triple mutant *Pfdhfr* haplotype (108N, 51I, and 59R) was also detected in a single isolate.[117] In a latter study, a computational method to deconvolute chromatograms generated through standard Sanger sequencing of PCR amplicons was developed to assess molecular marker variations of antimalarial drug resistance genes *Pfdhps* and *Pfdhfr*. The study identified two novel point mutations in the *Pfdhps* gene (codons D484T, D545N). This deconvolution approach can distinguish between variable amounts of alleles that are sensitive versus resistant to antimalarial drugs. Furthermore, this affordable and quantitative variant-sequencing method will be valuable for population-based surveys that can characterize mixed infections at the individual level, allowing for the evaluation of known and unknown mutations in *Pf* drug-resistance genes.[118] For Sanger sequencing, the average read length of the target DNA that can be sequenced with high confidence is 400−700 bases. However, Sanger sequencing requires the construction of clones for unknown sequences prior to sequencing, making genome-wide sequencing challenging.

Next-Generation Sequencing (NGS). NGS can be utilized to sequence either specific genes or loci of interest or the complete genome to identify potential novel mutations or drug resistance mechanisms. It can also be used to monitor the origin and spread of resistant parasites through microsatellite data analysis.[5,69] Various methods are used to extract target genes from the pooled samples, after which amplicons are sequenced. Next, a DNA library is created from small DNA fragments that range in size from ∼ 100 base pairs.[105] Deep sequencing is performed that involves repeatedly sequencing a region of the genome. Additionally, this NGS method enables the identification of rare clonal types as compared to the traditional capillary electrophoresis sequencing.[119] Bioinformatics tools are used for analyzing sequencing data to reconstruct the desired gene or the entire genome and then it is compared to a reference strain. In general, NGS operates similarly to Sanger sequencing, employing a computer to identify the fluorescent signal produced when DNA polymerase facilitates the binding of fluorescently tagged dNTPs onto the DNA template throughout each sequencing cycle. Nonetheless, NGS greatly increases efficiency over Sanger by simultaneously detecting signals from thousands of pores. The broad applicability of the NGS platform has been validated by studying drug resistance-associated genes in *P. falciparum*.[119,120] A study was performed using NGS to evaluate

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important resistance genetic markers such as *Pfk13*, *Pfdhps*, *Pfcrt*, and *Pfdhfr*, and discovered mixed genotypes with varying drug sensitivities, confirming the ability of NGS to assess resistance in *Pf* clinical isolates. The study used deep amplicon sequencing (AmpSeq) to distinguish between single and multiclonal infections.[121] Another study used the NGS and molecular inversion probes (MIPs) to test five *Pf* drug resistance-associated molecular markers (*Pfk13*, *Pfdhps*, *Pfcrt*, *Pfdhfr, and Pfmdr1*). The study discovered that the *Pfdhfr/Pfdhps* quadruple genotype linked to SP (sulfadoxine-pyrimethamine) resistance was nearly saturated. However, it also detected increased SP resistance, and a high mutation rate of *Pfmdr1* (Y184F) mutant parasites in the region.[122] Single-stranded oligonucleotides, known as MIPs, comprise unique linker sequences and two target-specific probes that complement the target in the genome. The target sequences present in the genome are captured by these hybridizing probes. The concurrent hybridization of probes to the same fragment of DNA results in the capture of the target sequence between them, and it goes through an inversion in configuration, therefore called molecular inversion.[105] One study employed NGS technology to assess the CNVs of numerous *Plasmodium* genes. To rationalize this approach, they developed the PlasmoCNVScan algorithm.[123] Additionally, the Illumina MiSeq NGS technique generated a vast amount of information on *Plasmodium* resistance mutations. This method was used to find resistance markers in the *Pfcrt, k13, dhfr, mdr1*, and *dhps* loci in samples from Guinea-Bissau,[120] and the *Pvdhfr* locus in samples from Pakistan.[124] Maximum depth sequencing (MDS) is a technology that relies on high-throughput sequencing to enhance the accuracy of DNA sequencing. It achieves this by eliminating some methods used in existing high-speed DNA sequencing machines.[6] MDS is particularly effective in identifying extremely rare mutations that may be mistaken as errors by machines. MDS can reveal how *Pf* employs high-speed evolution to overcome antimalarial drugs. Thus, the scope of NGS has been rapidly broadening.

Nanopore Sequencing. This third-generation sequencing technique utilizes electrophoresis to pass individual molecules one after another through nanopores to facilitate the sequencing. Because each ATCG base has a distinct electrical property, the passing base classes can be identified in real-time by comparing the electrical signals of the various bases.[6] Using nanopore sequencing, Girgis et al. carried out an end-to-end approach to identify *Pf* antimalarial drug resistance-associated markers. They found that *Pf* parasites in Ghana are primarily susceptible to CQ, with persisting SP resistance and little evidence of ART resistance.[125] Portable devices like the MinION sequencer, which employs nanopore technology, enable real-time sequencing in field settings. Runtuwene et al. employed the MinION sequencer to genotype *Pf* and discovered an average base identification accuracy of 74.3%.[126] A fast and highly sensitive detection of the *Pfk13* C580Y mutation in *Pf* was developed by integrating LAMP with MinION. They have clinically evaluated 34 blood samples of humans for LAMP testing, which yielded a 100% positive detection rate. Using MinION, all LAMP amplicons from as many as 12 samples were sequenced at the same time. The sequencing results aligned with traditional PCR and Sanger sequencing methods.[127] Nanopore technology enables users to make decisions based on real-time information throughout the sequencing process, which is crucial for POCT (point-of-care testing). A versatile and affordable nanopore sequencing technique to detect drug resistance and diagnostic escape for *Pf* malaria has also been developed that can be utilized in low-resource settings.[128] However, this technique employs a hydrolysis process that cannot be duplicated, resulting in insufficient sequencing accuracy.

CRISPR/Cas System-Based Methods. CRISPR sequences are observed in many bacterial organisms. The CRISPR/Cas system in bacteria, employing CRISPR-related (Cas) effector proteins, binds specifically to target nucleic acids and functions as an adaptive immune system to protect against viral and other invading foreign genetic material.[88] The CRISPR system, discovered in 1987, has been extensively used for gene editing. Owing to its high sensitivity and specificity, this system has also been employed for developing nucleic acid biosensors, more frequently, for the diagnosis of infectious and chronic diseases.[89] Researchers developed an innovative malaria detection method based on the SHERLOCK principle. This method, which combines Recombinase polymerase amplification (RPA) and CRISPR-based technique, has the potential to detect all known *Plasmodium* species and drug-resistance gene types. Compared to AmpSeq, the SHERLOCK assay showed 73% sensitivity and 100% selectivity when tested on a panel of 43 clinical samples in studies on *dhps* (A581G) related to sulfadoxine resistance, implying its possibility for detecting malaria drug resistance alleles.[88] Thus, SHERLOCK assays illustrate the flexibility of CRISPR-based detection methods and their promise as a new class of molecular tools for diagnosing and monitoring malaria. An equipment-free microfluidic (FREM) platform for multiplex diagnosis has been reported for simultaneously testing malaria infection and genotyping species. This platform achieved a 90.91% accuracy in genotyping the five human *Plasmodium* species.[129] Recent advances in CRISPR technology produced a plethora of innovative methods for identifying certain nucleic acid sequences, which could prove to be highly advantageous for biological research endeavors. However, more thorough research with a larger sample size is required before considering its application in the field setting.

Other Techniques. High-Resolution Melting (HRM) Technique. It is a novel method for genotyping and identifying mutations in fragments of DNA that can find single-base mutations in these fragments rapidly. The underlying concept behind the HRM technique is to distinguish samples based on their different melting curves.[6] In one study, six molecular markers (*Pfdhps*, *Pfdhfr*, *Pfmdr1*, *Pfk13*, *Pfcrt*, and *PfATPase6*) linked to drug resistance were genotyped using HRM. Finally, sequencing confirmed that the result aligned perfectly with 100% of the stated sequence. This study also stated that the HRM technique was capable of identifying and distinguishing alleles from low-concentration template material regardless of the presence of abundant contaminating genetic material, indicating the sensitivity and specificity of this technique.[130] Ndiaye et al. used the HRM technique to compare common population signatures and drug resistance marker (*Pfdhps* and *Pfdhfr*) frequencies between Tanzania and Senegal, two populations with varying levels of malaria endemicity and antimalarial drug use. They identified a high degree of resistance polymorphisms in both *dhfr* and *dhps* in samples from these regions.[98]

Nucleic Acid Lateral Flow Immunoassay (NALFIA). It is a quick immunochromatographic assay, which can be utilized to identify resistant SNPs. This approach eliminates the requirement for DNA extraction steps by directly adding blood

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containing *Plasmodium* to a 1 h PCR reaction. The tagged PCR product forms a compound with carbon nanoparticles, detected by a line of antibodies linked to the nitrocellulose test strip.[12] The amplicons are tagged with specific primers containing a hapten and a biotin molecule. This complex is identified through direct interaction with a colloidal carbon nanoparticle labeled neutravidin. The strip functions like an RDT, with results displayed as the corresponding test line appears. This strip also includes a positive control line that captures the PCR product of a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). NALFIA has demonstrated good concordance with real-time PCR and sequencing techniques.[5] A new study developed a NALFIA using lateral flow chromatography to identify amplified nucleic acids instead of antigens. SNPs in *Pfdhfr* S108N, C59R, and N51I were identified in 98 clinical isolates. Compared to PCR, the sensitivities of S108N, C59R, and N51I were all determined to be 97.96%. The selectivity of S108N, C59R, and N51I, were found to be 100%, 95.92%, and 100%, respectively.[101] Cheng et al. developed a scalable platform using AS-PCR and a gold nanoparticle-based LFA. The platform was optimized and employed to find the SNPs in the *pfmdr1* drug-resistance gene. The AS-PCR-LFA platform exhibited up to 100% selectivity and 99.43% sensitivity.[100] Guirou et al. established a molecular method for nucleic acid amplification that uses RDTs as a source of nucleic acids. This technique can be employed to identify SNPs in the *kelch13* gene of *Pf* in order to acquire data on the transmission of ART-resistant *Pf* strains.[99] The NALFIA is a promising technique for POCT of resistant *Plasmodium* infections and can offer therapeutic guidance on an individual level.

Q-poc. This technique uses nanowires coated with specific probes to detect genetic variations and includes cloud-based connectivity for sharing and analyzing epidemiological data.[131] The tool comprises a cassette for sample collection and preparation, DNA amplification, and detection through microarray. The Q-poc device can distinguish *Plasmodium* parasites and identify many molecular markers related to antimalarial drug resistance.[5] It can identify resistance SNPs in just 20 min.

DNA Microarray. This technique, also known as DNA chip, facilitates high-throughput analysis of DNA sequences, which makes comprehensive CNV (copy number variation) analysis in malaria research feasible. Microarrays detect DNA from SNPs to vast CNVs.[14] Here, usually, a special glass sheet coated with 10^3−10^4 nucleic acid probes in arrays constitutes the chip allowed to hybridize with fluorophore-conjugated complementary DNAs in the sample. The fluorescence signals generated from the fluorophores upon excitation with lights of the corresponding wavelength for the fluorophores are then analyzed with the help of a computer-based spectral analyzer to retrieve information about the regulation of the genes.[6] A microarray-based technique allowed attributing *in vitro* derived fosmidomycin resistance to a CNV in the *Pf* 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*pfdxr*) gene, which allows the parasite to evade fosmidomycin-mediated inhibition of isoprenoid biosynthesis. The authors utilized the match-only integral distribution technique to detect gene deletions systematically, eliminating noise.[132] Similarly, Cheeseman et al. employed microarrays to show that 134 high-confidence CNVs are present across the parasite exome, comprising 102 amplifications and 33 deletions.[133] Another study used microarrays to identify 94 CNV sites in 183 freshly isolated field cases from three populations in Eastern Africa with varying malaria transmission intensity.[134]

Molecular technologies have significantly improved our understanding of malaria, facilitating the diagnosis and detecting drug resistance. These techniques for identifying and validating resistance SNPs have simplified resistance surveillance by lowering the requirement for additional *in vivo* studies after a resistance-associated marker has been confirmed.[12] The information obtained utilizing molecular techniques for resistance identification has given us in-depth knowledge of the genetic dynamics of *Plasmodium* parasites in the context of *in vitro* and *in vivo* research experiments. However, specialized equipment and highly skilled staff are also required in these techniques. Thus, there is a need to develop user-friendly and inexpensive technologies that could provide early warning signs before the spreading of significant levels of drug resistance parasites.

THERAPEUTIC INTERVENTIONS FOR DRUG-RESISTANT MALARIA

Several studies have indicated that the emergence of drug-resistant *Plasmodium* species minimizes the efficacy of a broad array of antimalarial drugs. Thus, there is a dire need to develop new antimalarial drugs that can target both established and new antimalarial targets.

Novel Antimalarial Targets for Drug Discovery. *Plasmodium Proteases.* *Plasmodium* proteases are a widespread and regulatory enzyme essential for the *Plasmodium* parasite's survival. Proteases are mostly utilized by merozoite-stage parasites to rupture and subsequently reinvade erythrocytes, as well as for the breakdown of hemoglobin by intraerythrocytic trophozoites.[10] Ten distinct aspartic proteases, or plasmepsin enzymes, are encoded by the *Pf* genome, four of which plasmepsins I−IV reside in the parasite DV and contribute to hemoglobin degradation. Hemoglobin degradation is assumed to be initiated by plasmepsins I and II hydrolyzing the peptide bond in the protein's hinge region.[135] This leads to protein unfolding, which releases heme in the process.[136] Plasmepsin-V plays a crucial role in the survival of parasites, as it is involved in the exportation of proteins of *Plasmodium* into the host erythrocyte.[79] These exported *Plasmodium* proteins are prepared by plasmepsin-V, cleaving five amino acid residues in their N-terminal sequence, which is known as the *Plasmodium* Export Element (PEXEL). Additionally, plasmepsins IX and X have therapeutic potential as they have a crucial role in the invasion, replication, and egress of merozoites throughout the blood stage of malaria infection. Plasmepsin X causes egress by cleaving the serine protease subtilisin 1 (SUB1) and promotes erythrocyte invasion by cleaving the apical membrane antigen 1 (AMA1) present on the surface of the parasite. Plasmepsin IX also promotes invasion by cleaving merozoite-invasion-related proteins, including apical sushi protein (ASP) and rhoptry-associated protein 1 (RAP1).[137]

Proteasome. Proteasomes, which are responsible for the proteolytic degradation of proteins through ubiquitination, are a part of biological mechanisms that cells use to regulate the concentration of specific proteins. This process of protein degradation plays a crucial role in several cellular processes, such as the cell cycle, gene expression regeneration, and responses to oxidative stress.[79] *Pf* proteasome subunit β5 is a component of the 26S proteasome, an enzymatic complex in the form of a barrel that consists of multiple subunits, such as

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the 19S regulatory factors and the 20S catalytic core.[138] Inhibition of the β5 subunit of *Pf* proteasome leads to the death of malaria parasites in all asexual stages.

Transporters. Pf Glucose Transporter 1 (PfHT1). *P. falciparum* is highly dependent on glucose uptake through glycolytic metabolism to extract energy. *Pf*HT1, a hexose transporter (HT), plays an important role in facilitating the uptake of glucose from the blood of the mammalian host. Erythrocytes infected with *Plasmodium* parasites raise their glucose intake multiple-fold higher, particularly because they lack alternate energy-generating pathways.[135] Without a transporter, an inadequate supply of glucose makes it very difficult for the parasite to survive, hence validating *Pf*HT1 as a possible target for antimalarial drugs.[139]

Pf Formate-Nitrite Transporter (PfFNT). *Pf* relies on glycolysis to promote fast growth and proliferation. The parasite consumes glucose from the host to produce ATP through glycolysis, which results in the formation of lactates and protons as byproducts.[140] The parasites instantly use the *Pf*FNT lactate transporter to excrete out lactate from the cytosol to prevent acidification.[141] *Pf*FNT is a high-capacity lactate-proton symporter, and it belongs to the family of microbial formate-nitrite transporter (FNT). It efficiently performs the lactate excretion function, preventing the lactic acid buildup, thus saving the cells from the lethal acidic pH effects.[142] *Pf*FNTs are a promising target for antimalarial drugs because they are dissimilar in terms of mechanistic and sequence perspective from human monocarboxylate transporters (e.g., MCT1) present in the membranes of red blood cells.

Pf p-Type Na^+ ATPase Transporter 4 (PfATP4). P-type ATPases, also referred to as E1-E2 ATPases, are a superfamily of cation transporters that facilitate the selective transportation of different ions, such as H^+, Na^+, and K^+, across a variety of biological membrane systems. This process is crucial for maintaining cell homeostasis and steep electrochemical gradients.[79] Erythrocytes, like other normal cells, typically maintain a lower concentration of cytoplasmic Na^+. To maintain the low cytosolic concentration of Na^+ ions, *Pf*ATP4, an ATP-consuming transporter, imports H^+ ions, and transfers Na^+ ions out of the cell. The cytosolic Na^+ level of the parasite increases due to the inhibition of this target, which induces osmotic water uptake and eventually leads to the death of the parasite.[11] *Pf*ATP4 uses the energy released by ATP hydrolysis to pump out Na^+ ions from the cytosol to the extracellular environment of the parasite. *Pf*ATP4 represents a potential target for antimalarial drug discovery, considering the absence of this transporter in humans, and inhibitors of *Pf*ATP4 are expected to exhibit a broad safety margin for the human host.[135]

Pf Niemann-Pick-Type C1-Related Protein 1(PfNCR1). *Pf*NCR1 is a lipid/sterol: H^+ symporter located on the plasma membrane of the parasite. *Pf*NCR1 is a crucial protein in the *Plasmodium* parasite, encoded by the gene PF3D7_0107500, and has been identified as a potential antimalarial target.[135] *Pf*NCR1 belongs to a family of multipass transmembrane proteins with various essential biological functions, such as maintaining the normal functioning of the food vacuole and plasma membrane of the parasite. These functions are especially important for the survival of the asexual intraerythrocytic stages of *Pf* lifecycle. Notably, cholesterol seems to be the predominant sterol involved, as the parasite does not synthesize it and primarily obtains it from erythrocytes, where a gradient of cholesterol level exists in the parasitic cytoplasm.[143] *Pf*NCR1 is indeed associated with human NPC1 (hNPC1), which plays an essential role in the egress of cholesterol from late endosomes.[144] Unlike hNPC1, *Pf*NCR1 is not an essential part of internal organelle membranes.

Plasmodium Kinases. In the *Plasmodium* parasite's life cycle, kinases play roles in phosphorylation, protein degradation, transcriptional regulation, and post-transcriptional control.[10] Protein kinase G (PKG) is a potential target for antimalarial drug development. PKG is a cGMP-dependent protein kinase that phosphorylates serine and threonine amino acid residues in proteins. *Pf*PKG is required for gametogenesis (in the human host), blood-stage replication, and ookinete motility in the mosquito. *Pf*PKG is also essential for schizont rupture, merozoite egress, along with merozoite invasion of erythrocytes.[145] It facilitates merozoite egress and invasion through its essential role in calcium mobilization.[135] The type III beta *Pf* phosphatidylinositol 4-kinase (*Pf*PI4K) has been identified as an essential antimalarial therapeutic target.[146] *Pf*PI4K is a widely distributed enzyme that catalyzes the synthesis of phosphatidylinositol 4-phosphate (PI4P) by phosphorylating lipids at the fourth position of the inositol ring. The PI4P subsequently determines the Golgi and trans-Golgi network membranes and modulates the trafficking of vesicles to and from the Golgi apparatus.[11] In *Plasmodium*, during merozoite formation to facilitate later-stage membrane invasion, Rab11A, a small GTPase of the Rab family, uses the lipid binding effector proteins recruited by PI4P.[147] Therefore, it is known that PI4K modulates intracellular signaling and trafficking through the synthesis of PI4P. *Pf* cyclin-dependent protein kinase 4 (*Pf*CDPK4) is another example of kinase that is involved in the exflagellation of male gametocytes, necessary for the formation of infectious sporozoites during the sexual phase of the *Plasmodium* parasite lifecycle.[79]

Target Enzymes from Plasmodium DNA and RNA Metabolic Pathway. Pf Purine Nucleoside Phosphorylase (PfPNP). *Pf*PNP, a purine salvage enzyme involved in a salvage process for the synthesis of purines, is necessary for the survival of parasites and is thus considered a potential target for new antimalarial drug discovery.[148] Malaria parasites cannot synthesize purines *de novo* and must rely on salvage processes to obtain these essential DNA synthesis precursors. *Pf*PNP converts inosine, its natural substrate, into hypoxanthine base and ribose-1-phosphate.[135] *Pf*PNP has been identified as a target of immucillins, which have been reported to induce purine-free death of *Pf* because of the lack of DNA precursors.[148]

Serine Hydroxymethyltransferase (SHMT). It is a ubiquitous enzyme involved in both the thymidylate and methionine synthesis pathways and strategically located within the folate pathway.[79] Two isoforms of SHMT, the cytosolic and mitochondrial enzymes, are known to be encoded by *Plasmodium* parasites. The enzyme facilitates the conversion of tetrahydrofolate (THF) into 5,10-methylene-tetrahydrofolate, which is an intermediary in the biosynthesis of purines and deoxythymidine monophosphate (dTMP).[135] dTMP is essential for the synthesis of DNA and RNA. Since nucleic acid synthesis is crucial for parasite survival, SHMT serves as a promising target for antimalarial therapeutics.

Deoxyuridine 5′-Triphosphate Nucleotido-hydrolase (dUTPase). Deoxyuridine triphosphate (dUTP) is hydrolyzed by the enzyme dUTPase into deoxyuridine monophosphate

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(dUMP) and inorganic pyrophosphate. Hence, it is essential for pyrimidine metabolism. dUMP serves as an intermediary compound in the synthesis of dTMP.[135] dUTPase also reduces cellular concentrations of dUTP to prevent the incorrect incorporation of uracil into DNA. *Pf*dUTPase is a potential antimalarial target because it is required for parasite survival during the intraerythrocytic stages of the *Pf* lifecycle.

Pf Cleavage and Polyadenylation Specificity Factor Subunit 3 (PfCPSF3). The gene PF3D7_1438500 encodes CPSF, a eukaryotic enzyme complex that is a member of the zinc-dependent metallo-β lactamase (MBL) family. *Pf*CPSF3 contains endonuclease activity that is responsible for cleaving and polyadenylating the 3′-end of pre-mRNA during the process of converting it into mRNA.[149] Thus, *Pf*CPSF3 has been recognized as a promising candidate for developing drugs to combat malaria.

Pf Dihydroorotate Dehydrogenase (PfDHODH). Pyrimidines are necessary metabolites that facilitate the biosynthesis of phospholipids, glycoproteins, as well as DNA and RNA. In contrast to its human host, *Pf* is unable to salvage pyrimidines and hence relies on their *de novo* biosynthesis.[79] DHODH is located in the inner membrane of mitochondria. The fourth step of the *de novo* pyrimidine synthesis pathway is catalyzed by *Pf*DHODH, and it oxidizes dihydroorotate to orotate in the presence of the cofactor flavin mononucleotide (FMN).[150] Therefore, *Pf*DHODH is considered a potential target for the discovery of novel antimalarial drugs.

Aspartate Transcarbamoylase (ATCase). As mentioned earlier, the *Plasmodium* parasite does not have the salvage enzymes and depends on the *de novo* synthesis of pyrimidines for survival.[151] ATCase catalyzes the second step of the pyrimidine biosynthesis pathway, and it is encoded by the gene PF3D7_1344880. This step involves the condensation of L-aspartate and carbamoyl phosphate to produce N-carbamoyl aspartate and inorganic phosphate.[135] Thus, *Pf*ATCase also represents a potential target for antimalarial drug discovery.

Targets from Protein Synthesis Pathway. Aminoacyl tRNA Synthetases (aaRSs). aaRSs are known to represent a diverse class of antimalarial targets. The discovery that cladosporin, a compound having antimalarial activity, can inhibit lysyl tRNA synthetase (*Pf*LysRS) highlighted the significance of aaRSs as promising targets for antimalarial drug discovery. The aaRSs play a crucial role in protein synthesis by facilitating the formation of aminoacyl tRNA. This involves the covalent attachment of an amino acid to a tRNA molecule. The resulting complex then binds to the ribosome, and the amino acid carried by the tRNA is afterward transferred to the developing peptide chain.[135] Some examples of aaRS antimalarial targets are Prolyl-tRNA synthetase (*Pf*ProRS), Phenylalanyl tRNA-synthetase (*Pf*PheRS),[152] Lysyl-tRNA synthetase (*Pf*LysRS) and Tyrosyl tRNA synthetase (*Pf*TyrRS).[153] Recently, cytoplasmic Isoleucyl tRNA synthetase (*Pf*IleRS) has been identified as a multistage antimalarial target for drug development.[154]

Pf Eukaryotic Elongation Factor 2 (PfeEF2). *Pf*eEF2 is an essential component of the ribosome and facilitates its translocation along the mRNA strand, moving from one codon to the next in a manner that depends on guanosine triphosphate (GTP). Therefore, it plays an important role in protein synthesis in eukaryotic organisms. When *Pf*eEF2 binds and hydrolyzes GTP into guanosine diphosphate (GDP), the released energy is used to propel the ribosome from one codon to the next during translation. *Pf*eEF2 is thus considered an important target in the prevention of malaria.[155]

Target Enzymes from Isoprenoid Biosynthesis Pathway. Pf Protein Farnesyltransferase (PfPFTase). While normal erythrocytes lack membrane-bound organelles, infected erythrocytes have a significant presence of diverse endomembrane systems formed by *Plasmodium* parasites.[141] Protein prenylation, the process of attaching a hydrocarbon to a protein, has an important impact on the formation of this membrane. Protein prenylation consists of three enzymatic steps, with farnesylation being the initial step. Farnesyltransferase transfers the farnesyl group, a 15-carbon isoprenoid lipid unit, from farnesyl pyrophosphate to the C-terminus of proteins that contain the 4-amino acid Cysteine-aliphatic-aliphatic-terminal residue (CaaX) motif.[135] *Pf*PFTase is a possible novel candidate for developing antimalarial drugs since its inhibition kills the parasite.

Plasmodium 2-C-Methyl-D-erythritol-4-Phosphate Cytidyltransferase (IspD). *Plasmodium* IspD is the third enzyme within the seven-step methylerythritol phosphate (MEP) pathway that results in the production of dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP), the precursor molecules of isoprenoid molecules.[156] Since Isoprenoid molecules are essential for all living cells and the MEP pathway is unique to *Plasmodium* parasites, inhibiting IspD has been identified to be a promising strategy for developing antimalarial drugs that are safe for humans.[135] Furthermore, the MEP pathway is crucial for developing the asexual blood stage and gametocyte parasites. *Plasmodium* IspD facilitates the conversion of MEP to cytidine diphosphate-methylerythritol (CDP-ME) through cytidylation. Attempts to genetically knockout the *pfispd* gene throughout intraerythrocytic stages of *Pf* parasites were unsuccessful, underscoring the important role of this gene in parasite development.[156] Therefore, *Plasmodium* IspD could be a potential antimalarial target.

Pf Farnesyl/Geranylgeranyl Diphosphate Synthetase (PfFPPS/GGPPS). *Pf*FPPS/GGPPS is a bifunctional enzyme that catalyzes a critical branch-point reaction during isoprenoid biosynthesis. Initial studies performed on the bifunctional enzyme isolated from *Methanobacterium thermoautotrophicum* revealed that it employs DMAPP, farnesyl diphosphate (FPP), and geranyl diphosphate (GPP) as allylic substrates.[135] FPPS/GGPPS synthesizes C15 and C20 prenyl chains, respectively, which are essential for many downstream enzymes, and for chain lengths greater than C20, its activity significantly decreases. FPPS catalyzes the sequential head-to-tail condensation of IPP with DMAPP to generate GPP, generated GPP is then further condensed with IPP to form FPP. The further condensation of FPP with an additional IPP result in the synthesis of geranylgeranyl diphosphate (GGPP).[157]

Other Targets. Plasmodium Dihydrofolate Reductase-Thymidylate Synthase (DHFR-TS). In malaria parasites, DHFR is the enzyme necessary for the NADPH-dependent conversion of dihydrofolate to tetrahydrofolate. This reduction reaction is particularly important for the biosynthesis of purines and some amino acids and the *de novo* synthesis of pyrimidines.[141] Therefore, inhibiting DHFR imbalances the pathways responsible for synthesizing thymidylate, leading to the disruption of DNA replication and linked cell death. Thymidylate synthase (TS) and DHFR coexist as a single-chain bifunctional enzyme in *P. falciparum*. DHFR catalytically reduces dihydrofolic acid to tetrahydrofolic acid. Subsequently,

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tetrahydrofolic acid is also converted to N5,N10-methylenetetrahydrofolate. Then, TS catalyzes the production of 2-deoxythymidine-5-monophosphate from 2-deoxyuridine-5-monophosphate by using N5,N10-methylenetetrahydrofolate as both a methyl donor and reducing agent.[158] Therefore, a highly promising way to prevent the growth of the *Plasmodium* parasite is to inhibit DHFR or the junctional region between DHFR and thymidylate synthase.[147]

Pf Acetyl-Coenzyme A Synthetase (PfAcAS) and Pf Acyl-CoA Synthetase (PfACS) 10. AcASs catalyze the ATP-dependent condensation of acetate and coenzyme A (CoA) to produce acetyl-CoA. Acetyl CoA is produced in *Pf* by two independent biosynthetic pathways: the *Pf*AcAS pathway and the branched-chain keto acid dehydrogenase (BCKDH) pathway. It has been established that *Pf*AcAS is necessary for supplying the acetyl moieties required for the parasite's chromatin modification and intraerythrocytic development. Additionally, it plays a role in the regulation of chromatin.[135] The proliferation of the parasite gets hindered when *Pf*AcAS is inhibited through compounds or gene knockdown. In this context, decreased levels of active *Pf*AcAS cause disruption in acetyl-CoA levels, changes in histone acetylation patterns, variable gene expression, and a decrease in the cytoadherence of infected erythrocytes.[159] This crucial enzyme can be utilized as an antimalarial target since inhibition of *Pf*AcAS leads to rapid cell death and disruption of acetyl-CoA levels.

*Pf*ACS 10 belongs to the acyl-CoA synthetase (*Pf*ACS) enzyme family. Enzymes of this family share more similarities to long-chain fatty acid synthetases (ACSLs). ACSLs activate free fatty acids (FA) with a preferred acyl chain length of 12−20 by attaching them to coenzyme A in a two-step, ATP-dependent reaction, producing acyl-CoA. *Pf*ACS10 is crucial in the asexual blood stage of the parasite lifecycle. Parasites obtain fatty acids (FAs) from the host, and activation of these FAs by *Pf*ACSs enables their incorporation into different lipid species crucial for the growth of the parasite.[160]

Pf N-Myristoyl Transferase (PfNMT). It facilitates the covalent attachment of myristoyl (tetradecanoyl) from myristoyl-CoA to the glycine residue at the N-terminal of target proteins. N-myristoylation has been shown to modulate protein−protein interactions, stabilize protein structures, and enhance protein association into membranes, playing a crucial role in protein trafficking processes.[161] *Plasmodium spp.* has a single NMT enzyme. In malaria parasite biology, several key parasitic proteins have been demonstrated to require myristoylation for precise localization and to perform their biological functions effectively. Myristoylation promotes a protein−membrane interaction that can be improved by additional lipidation; inhibition of NMT causes erroneous protein localization and subsequent disruption in function.[162] Various metabolic pathways, such as protein homeostasis, organelle biogenesis, ion channel regulation, parasite development, and parasite motility, are mediated by various myristoylated proteins in *Plasmodium* parasites.[135] The multitude of essential metabolic pathways that rely on N-myristoyl location indicates the importance of NMT in parasite survival, making it a promising antimalarial drug target.

Recent Progress in Antimalarial Drug Development.

The drug resistance to the existing first-line antimalarials is gradually spreading, emphasizing the need for the rapid development of drugs with mechanisms of action that can effectively and safely combat the resistance.[13] A list of novel antimalarial targets and their inhibitors, which are not yet halted under clinical trials, are discussed here and presented in Table 3.

Inhibitors of Plasmodium Proteases. **WEHI-842** or benzyl *N*-[(2*S*)-4-(diaminomethylideneamino)oxy-1-[[(2*S*)-1-[[(3*S*,4*S*)-3-hydroxy-6-methyl-1-oxo-1-(2-phenylethylamino)-heptan-4-yl]amino]-3-methyl-1-oxobutan-2-yl]amino]-1-oxobutan-2-yl]carbamate can inhibit the Plasmepsin-V of the parasites, thereby preventing the parasite's protein exportation and proliferation.[141] It mimics the transition state, causing proteolysis of amide bonds for the substrate of the *Plasmodium* export element (PEXEL).[208] At sublethal doses, WEHI-842 exhibited transmission-blocking efficacy.[164] Pino et al. reported the hydroxyl-ethylamine-based scaffold **Compound 49c** inhibits aspartic protease and exhibits multistage antiplasmodial activity. It is lethal for all parasite life cycle stages. Compound 49c prevents the SUB1-dependent egress of gametocytes, which are the sexual forms of *Plasmodium* that are transmitted to mosquitoes. Compound 49c also limits egress from hepatocytes during the liver stage, leading to fewer blood-stage infections *in vivo*.[137] Another example of a dual inhibitor of plasmepsins IX and X, is **WM382** or (4R)-4-(2-amino-4,4-diethyl-6-oxo-5H-pyrimidin-1-yl)-N-[(4S)-2,2-dimethyl-3,4-dihydrochromen-4-yl]-3,4-dihydro-2H-chromene-6-carboxamide. It was effective against *Pf* asexual infection in a humanized mouse model and prevented transmission of gametocytes to mosquitos.[166] WM382 is extremely potent with exceptional antimalarial activity *in vitro* and *in vivo* against the mosquito, blood, and liver stages.[165] **UCB7362** or N-{2-chloro-3-[(4*S*)-2-imino-4-methyl-1-[(2*S*,4*S*)-2-methyloxan-4-yl]-6-oxo-1,3-diazinan-4-yl]phenyl}-3-cyanobenzamide is another plasmepsin X inhibitor. It functions as an orally active and effective inhibitor that restricts parasite proliferation.[167] A macrocyclic amide inhibitor **7k** can eradicate blood-stage *Pf* in infected humanized mice and exhibited subnanomolar efficacy against plasmepsin X and the whole malarial parasite. In an *in vivo* model of *Pf* infection, 7k showed proof of principle for an orally administered medication,[168] and requires further development as an antimalarial drug.

Inhibitors of Plasmodium Proteasome. A novel and potentially effective antimalarial compound, **MPI-5** targets the β5 subunit of the *Pf* 20S catalytic core of proteasome. It is an amino-amide boronate possessing strong and quick antimalarial activity in a severe combined immunodeficiency (SCID) mouse model, resulting from specific inhibition of the *Pf* 20S β5 active site. Additionally, MPI-5 is also well tolerated following oral dosing in SCID mice.[169] Stokes et al. reported two peptide vinyl sulfones, **WLL-vs** (WLL) and **WLW-vs** (WLW), that specifically inhibit *Plasmodium* proteasome and have low nanomolar efficacy against genetically diverse parasites. Both have a distinct stage-specificity profile and are equally potent against parasites expressing either wild-type (WT) or mutant variants of the ART resistance determinant K13. Stage-specificity assays identified that WLL and WLW are more effective against schizonts and early ring stages of the parasite life cycle.[170] Furthermore, WLL shows a lower risk of resistance selection and maintains efficacy against a panel of proteasome mutants.[138] **TDI8304**, a macrocyclic peptide, binds to its target noncovalently and is effective, species-specific, and metabolically stable.[171] It inhibits the proliferation of the *Plasmodium* parasite in the erythrocytic stage and has shown antimalarial efficacy in a humanized mouse model of *Pf* infection.[209] Recently, a series of epoxyketone-based proteasome inhibitors were identified that have minimal cytotoxicity

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Table 3. List of Novel Antimalarial Targets along with Their Inhibitors[a]

Novel Target (Gene ID)[163]	Function of target	Inhibitors (IC_{50} values)	Reference
Plasmepsin V (PF3D7_1323500)	Exportation of parasite proteins into host erythrocyte	WEHI-842 (0.2 nM)	79, 164
Plasmepsin IX and X (PF3D7_1430200, PF3D7_0808200)	Both have role in invasion, replication, and egress of merozoites	Compound 49c (0.6 nM), WM382 (0.06 nM), 7k (0.4 nM), UCB7362 (7 nM *in vitro*)	137, 165−168
Pf proteasome subunit β2 and β5 (PF3D7_1470900, PF3D7_1011400)	Maintains cellular homeostasis of parasite	MPI-5 (5 ± 2 nM), TDI8304 (0.081 μM), WLL (16.8 ± 1.3 nM), WLW (54.3 ± 13.3 nM), J-18 (3.3 ± 0.23 nM)	138, 169−172
*Pf*HT1 (PF3D7_0204700)	Facilitates uptake of glucose from host blood	C3361 (33.1 ± 2.0 μM), TH-PF01 (0.615 ± 0.046 μM), TH-PF02 (0.329 ± 0.028 μM), TH-PF03 (1.22 ± 0.09 μM)	139, 173
*Pf*FNT (PF3D7_0316600)	Excretes out lactate from parasite cytosol	MMV007839 (0.14 μM), MMV000972 (1.8 μM), BH267.meta (0.11 μM)	140, 174, 175
*Pf*ATP4 (PF3D7_1211900)	Excretes out Na^+ from parasite cytosol and maintains Na^+ homeostasis	Cipargamin (0.5−1.4 nM *in vitro*), SJ733 (10−60 nM), MB14 (52 ± 16 nM), MB10 (62 ± 9 nM), MMV609, WJM-921	176−182
*Pf*NCR1 (PF3D7_0107500)	Maintains membrane lipid composition of blood-stage parasites	MMV009108, MMV019662, MMV028038	135, 144
*Pf*PKG (PF3D7_1436600)	Essential for parasite invasion, blood-stage replication, schizont rupture, and merozoite egress	ML10 (160 pM), MMV030084 (0.4 nM)	183, 184
*Pf*PI4K (PF3D7_0509800)	Phosphorylate lipids to regulate intracellular signaling and membrane trafficking during merozoite development	KDU691 (*Pf* ∼118 nM, and *Pv*: ∼69 nM), CHMFL-PI4K-127 (0.9 nM)	11, 141, 185
*Pf*PNP (PF3D7_0513300)	Essential enzyme for salvage pathway of purine synthesis	BCX4945 (164 ± 20 nM)	135, 148, 186
SHMT (PF3D7_1456100)	Facilitates conversion of tetrahydrofolate into 5,10-methylene-tetrahydrofolate, Essential for DNA synthesis	(±)-86 (97 ± 1 nM)	187
*Pf*CPSF3 (PF3D7_1438500)	Regulates cleavage and polyadenylation of 3′-end of pre-mRNA	AN3661 (32 nM)	149
*Pf*DHODH (PF3D7_0603300)	Catalyzes the rate-limiting step in *de novo* pyrimidine synthesis pathway	DSM421 (*Pf* 0.053 μM and *Pv* 0.094 μM), Z1481646084, Z24317941, Z951873618	141, 150, 188
ATCase (PF3D7_1344800)	Catalyzes the second step of the pyrimidine biosynthesis pathway	Torin 2 (8 nM *in vitro*)	189
aaRSs *Pf*ProRS (PF3D7_1213800) *Pf*PheRS (PF3D7_1232000) *Pf*LysRS (PF3D7_1350100) *Pf*TyrRS (PF3D7_1117500) *Pf*IleRS (PF3D7_1225100)	Facilitates the formation of aminoacyl tRNA, Essential for protein synthesis.	1-S (9 nM), Compound 5 (0.015 μM), BRD1389 (*Pf* 13 nM and *Pv* 22 nM), BRD7929 (*Pf* 23 nM and *Pv* 30 nM), ML901 (2.0 ± 0.1 nM), ASP3026 (5.61 ± 0.26 μM)	152−154, 190−193
*Pf*eEF2 (PF3D7_1451100)	Facilitates translocation of ribosome along mRNA strand during translation	M5717 (60.5 nM)	155, 194−196
*Pf*PFTase (PF3D7_1147500)	Facilitates transfer of farnesyl group from farnesyl pyrophosphate to C-terminus of proteins containing CaaX motif	BMS-388891 (10 nM), R115777 (11.48 nM *in vitro*)	135, 197
IspD (PF3D7_0106900)	Acts as a key enzyme methylerythritol phosphate isoprenoid biosynthesis pathway	MMV008138 (0.35 μM), 1R,3S-MMV008138 (0.47 μM)	156, 198
*Pf*FPPS/GGPPS (PF3D7_1128400)	Synthesizes C15 and C20 prenyl chains essential for many downstream enzymes of isoprenoid biosynthesis pathway	MMV019313 (0.82 μM), AIM-1290 (1.94 μM)	157, 199
DHFR-TS (PF3D7_0417200)	Essential in folate and thymidylate production required for DNA synthesis	Triclosan (0.775 μM), BT1 (0.29 ± 0.05 μM)	200, 201
*Pf*AcAS (PF3D7_0627800)	Catalyzes ATP-dependent condensation of acetate and CoA to produce acetyl-CoA	MMV019721 (0.073 ± 0.004 μM), MMV084978 (0.37 ± 0.04 μM), MMV689258 (5 nM), MMV183 (0.3 μM)	159, 202−204
*Pf*ACS10 (PF3D7_0525100)	Activates free fatty acids and enables their incorporation into lipid species essential for parasite growth	GSK701, MMV665924, MMV019719, MMV897615	13, 160, 180

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Table 3. continued

Novel Target (Gene ID)[163]	Function of target	Inhibitors (IC_{50} values)	Reference
*Pf*NMT (PF3D7_1412800)	Facilitates covalent attachment of myristoyl from myristoyl-CoA to glycine residue at the N-terminal of target proteins	IMP-1002 (3 nM), EXP90, ZBC205, ZDD968	161, 162, 205−207

[a]*Pf*: *Plasmodium falciparum*; *Pf*HT1: Hexose transporter; *Pf*FNT: *Pf* formate-nitrite transporter; *Pf*ATP4: *Pf* p-type Na^+ ATPase transporter 4; *Pf*NCR1: *Pf* Niemann-Pick-Type C1-Related Protein 1; *Pf*PKG: *Pf* Protein kinase G; *Pf*PI4K: *Pf* phosphatidylinositol 4-kinase; *Pf*PNP: *Pf* Purine Nucleoside Phosphorylase; SHMT: Serine Hydroxymethyltransferase; *Pf*CPSF3: *Pf* Cleavage and Polyadenylation Specificity Factor Subunit 3; *Pf*DHODH: *Pf* Dihydroorotate Dehydrogenase; ATCase: Aspartate Transcarbamoylase; aaRSs: Aminoacyl tRNA Synthetases; *Pf*ProRS: Prolyl-tRNA synthetase; *Pf*PheRS: Phenylalanyl tRNA-synthetase; *Pf*LysRS: Lysyl-tRNA synthetase; *Pf*TyrRS: Tyrosyl tRNA synthetase;*Pf*IleRS: Isoleucyl tRNA synthetase; *Pf*eEF2: *Pf* Eukaryotic Elongation Factor 2; *Pf* PFTase: *Pf* protein farnesyltransferase; CaaX: Cysteine-aliphatic-aliphatic-terminal residue, IspD: *Plasmodium* 2-C-Methyl-D-erythritol-4-Phosphate Cytidyltransferase; *Pf* FPPS/GGPPS: *Pf* Farnesyl/Geranylgeranyl Diphosphate Synthetase; DHFR-TS: *Plasmodium* Dihydrofolate reductase-thymidylate synthase; *Pf*AcAS: *Pf* Acetyl-Coenzyme A Synthetase; CoA: Coenzyme A; *Pf*ACS10: *Pf* acyl-CoA synthetase 10; *Pf* NMT: N-Myristoyl transferase.

for human cells and low nanomolar *in vitro* potency against blood-stage *P. falciparum*. **J-18**, the most effective inhibitor in this series, has a hexanoyl group at P4, a d-amino acid at P3, and a fluorinated aromatic l-amino acid at P1. These analogues are selective antimalarial drug candidates with promising pharmacokinetic features and effectiveness in animal studies.[172]

Inhibitors of Plasmodium Transporters. **C3361**, a glucose analogue, prevents the proliferation of blood-stage *Plasmodium* parasites *in vitro*. However, its low potency restricted its further development.[139,173] **TH-PF01**, **TH-PF02**, and **TH-PF03** are small molecule inhibitors that concurrently target both the orthosteric and allosteric binding sites of *Pf*HT1. These are structurally similar to glucose and are effective against the blood-stage development of various *P. falciparum* strains (3D7, Dd2).[173]

MMV007839 and **MMV000972**, two fluoroalkyl vinylogous compounds, are inhibitors of *Plasmodium* lactate transporter (*Pf*FNT).[140] Both inhibitors disrupt pH inside the parasite and kill the asexual blood-stage *P. falciparum* parasites at nanomolar concentrations.[142] MMV007839 was initially demonstrated to induce a fatal decrease in pH within trophozoite-stage *Pf* parasites. Treatment with sublethal dosages of MMV007839 caused a resistance mutation in the *Pf*FNT-encoding gene, leading to a conversion of Gly107 to serine on the protein level, *Pf*FNT G107S, and decreased sensitivity to the inhibitor. However, a serine hydroxy group has the potential to be utilized by exploiting the hydrogen bond as an interacting site with the inhibitor.[210] **BH267.meta**, a new class of pyridine-substituted pentafluoro-3-hydroxy-pent-2-en-1-ones with a single nitrogen atom serving as an H-bond acceptor site and lacking hydroxyl at the aromatic ring, exhibited nanomolar *Pf*FNT inhibition regardless of the presence of the G107S mutation.[174] In addition to the G107S mutation, G21E, and V196L, two new point mutations that impact inhibitor binding have been identified recently. An *in vivo* efficacy study of BH267.meta in malaria mouse models identified that *Pf*FNT is necessary for the trophozoite stage and that BH267.meta had >99% activity in mice. This supports the development of *Pf*FNT inhibitors as antimalarial drugs with a new mechanism of action.[175] **MMV000972** shares the identical structural scaffold as MMV007839, differing only by the presence of an aromatic methoxy group. It functions in the same way as MMV007839, inhibiting the lactate transport pathway.[140]

Cipargamin (KAE609) is a spiroindolone derivative that is effective against all intraerythrocytic stages of *Pf*, including gametocytes, and is currently in the phase-II development stage.[176,177] It causes swelling of intact infected erythrocytes and the isolated blood stage of *Pf* while increasing parasite cytosolic pH, resulting in the death of parasites.[211] Also, it directly disrupts the P-type Na^+ ATPase pump present in parasites and disrupts the homeostasis of their internal environment by preventing the excretion of Na^+ ions from the parasite's cytosol. Cipargamin is equally potent as ART derivative AS against drug-resistant strains of *Pv* and *Pf*. Nevertheless, a mutation G358S in *Pf*ATP4 was associated with 65% of treatment failures.[176] One of the most effective *Pf*ATP4 inhibitors, **SJ733**, a dihydroisoquinolone molecule, is a clinical lead that has completed first-in-human testing.[178] SJ733 is orally bioavailable, fast acting, efficacious against all drug-resistant *Pf*, and potent against all intraerythrocytic stages, leading to transmission blockage. The introduction of this inhibitor continuously inhibits *Pf*ATP4, leading to an increase in the parasite's cytoplasmic Na^+ concentration and elevating

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the pH of the cytoplasm. Therapy with SJ733 quickly induces the exposure of phosphatidylserine on the outer side of the parasite's plasma membrane, giving the signal for erythrophagocytosis.[141] Mutations in the gene coding *Pf*ATP4 conferred resistance to SJ733.[182] Furthermore, cross-resistance was identified between SJ733 and cipargamin. Gilson et al. produced a novel class of antimalarial compounds based on 4-cyano-3-methylisoquinoline. Two compounds present in this series, **MB14** and **MB10**, act in a similar way as spiroindolone because both directly target the Na^+ ATPase and inhibit the Na^+ efflux pathway. It induces swelling of infected red blood cells due to the accumulation of excess Na^+ and subsequent lysis.[179] **MMV609**, a small molecule drug, has been reported to be efficacious against the asexual blood stages, liver stage, and sexual stages.[180] Preclinical models indicate that MMV609 has similar parasite-killing kinetics to cipargamin.[179] **WJM-921** is a dihydroquinazolinone analogue that shows significant efficacy against asexual parasites. It alters parasite Na^+ homeostasis and pH, exhibits a fast-to-moderate asexual death rate, and inhibits gametogenesis. WJM-921 additionally exhibited oral effectiveness in a mouse model of malaria.[181]

*Pf*NCR1 has been discovered as a target for **MMV009108** (4-[2-(3,4-dimethylanilino)-1,3-thiazol-4-yl]-N,N-diethylbenzenesulfonamide), **MMV019662** (N-(2-(benzo[d][1,3]dioxol-5-yl)-2-(4-(4-methoxyphenyl)piperazin-1-yl)ethyl)-cyclohexanecarboxamide), and **MMV028038** (N-[cyclopentyl-(3-propan-2-ylphenyl)methyl]-2-(3-morpholin-4-ylbenzoyl)-3,4-dihydro-1*H*-isoquinoline-3-carboxamide), that have showed submicromolar potencies against the WT (wild-type) malaria parasites.[144] Treatment with these compounds led to slower replication of blood-stage parasites, very small and deformed food vacuoles, decreased growth rate, and parasite death.[135] Additionally, the parasites developed a hypersensitivity to saponin, a glycoside that forms membrane pores, suggesting that these inhibitors had an impact on the lipid composition of blood-stage parasite membranes.[144]

Inhibitors of Plasmodium Kinases. An example of a *Pf*PKG (*Pf* Protein kinase G) inhibitor is **ML10**, which showed nanomolar potency against the whole blood-stage *Pf* parasite, despite having picomolar potency on the kinase enzyme. **ML10** is an imidazopyridine, eradicates blood-stage parasite growth *in vitro* and in a *Pf* SCID mouse model, and inhibits the transmission of mature *Pf* gametocytes to mosquitoes.[183] Another *Pf*PKG inhibitor, **MMV030084**, a trisubstituted imidazole, was identified by phenotypic screening employing an assay for asexual stage growth and sporozoite invasion. It effectively prevents male gamete exflagellation, merozoite egress from asexual blood-stage schizonts, and *Plasmodium* sporozoites from invading hepatocytes.[184] Eck et al., have developed a series of isoxazole-based compounds that are effective, specific, and ATP-competitive inhibitors of *Pf*PKG.[212]

Imidazopyridine **KDU691**, a *Plasmodium* PI4K-specific inhibitor, has significant antiparasitic effects on blood-stage schizonts, gametocytes, and liver stages. KDU691 has transmission-blocking, preventative, and therapeutic functions in malaria by altering PI4P levels, which also includes membrane trafficking.[141] Dihydroartemisinin (DHA) pretreatment dormant rings (DP-rings) of *Pf* ART-resistant parasites harboring mutant K13 are also killed by KDU691.[213] Liang et al. reported bipyridine-sulfonamide chemotype *Pf*PI4K inhibitor **CHMFL-PI4K-127** (60-chloro-*N*-methyl-5′-(phenylsulfonamido)-[3,3′-bipyridine]-5-carboxamide). It showed antimalarial efficacy against the blood and liver stages of the *Plasmodium* parasite lifecycle. Furthermore, it showed strong selectivity across human lipid and protein kinases, and efficacy across a panel of clinical drug-resistant isolates.[185]

Inhibitors of Enzymes from Plasmodium DNA and RNA Metabolic Pathway. **BCX4945** (DADMe-Immucillin-G) is a potent inhibitor of *Pf*PNP, and it acts as a transition state analogue, effectively blocking the activity of *Pf*PNP. It functions as an oral antimalarial medication for *Pf* infections in *Aotus* primates and inhibits the PNPs from human and malaria parasites. BCX4945 kills parasites through purine deprivation, as the addition of exogenous hypoxanthine was able to rescue cultured parasites.[135] Furthermore, increasing drug pressure developed mutants resistant to BCX4945, resulting in point mutations in the *Pf*PNP gene at the catalytic site associated with drug binding. Resistance emerged gradually over 136 generations (2^{136} clonal selection). Therefore, slow resistance developing, transition-state analogue inhibitors against *Pf*PNP hold promise for application in the treatment of malaria.[186]

(±)-86, a pyrazolopyran-based ligand, is the first *Pf*SHMT inhibitor that has been shown to be efficacious *in vivo* in the *Pf* SCID mouse model, with a 73% reduction in parasitemia after 4 days of oral administration.[187]

*Pf*CPSF3 inhibitor **AN3661**, a benzoxaborole, is effective against *P. falciparum* strains that are susceptible (3D7) or resistant (W2, K1, Dd2, FCR3, TM90C2B, and HB3) to commonly available antimalarials.[149] Nevertheless, resistance to AN3661 emerged due to point mutations identified in the cluster around the active site of *Pf*CPSF3.[135]

DSM421, a triazolopyrimidine, inhibits various laboratory strains of *Pf* and has efficacy against both the blood stage and liver stage with a long half-life and significant oral exposure.[141] Recently, Alzain et al. reported three novel and potential *Pf*DHODH inhibitors, **Z1481646084**, **Z24317941**, and **Z951873618**, utilizing *in silico* studies.[188]

Torin 2 has been effective against both the asexual blood stage and gametocyte stages of *P. falciparum* 3D7 parasites.[135] It inhibited the formation of mosquito oocysts in a mouse model of transmission. A further study developed a double mutant of *Pf*ATC, which, when transfected into parasites, led to decreased parasite growth in a nutrient-deficient media, confirming the importance of *Pf*ATC.[189] Moreover, a 10-fold increase in tolerance to Torin 2 was seen when the compound was examined on transgenic strains that overexpressed *Pf*ATC. Wang et al. discovered a number of small-molecule allosteric inhibitors of *Pf*ATCase with low nanomolar binding affinities that specifically bind to an allosteric pocket, hindering *Pf*ATC activation. These compounds showed activity against the blood stage of the *Plasmodium* parasite lifecycle, inhibiting parasite proliferation.[214]

Inhibitors of Plasmodium Protein Synthesis Pathway. **1-S**, a 1-(pyridin-4-yl)pyrrolidin-2-one derivative that competes for the ATP binding site of cytoplasmic *Pf*ProRS, was shown to have *in vitro* nanomolar efficacy against resistant *Pf* laboratory strains while inhibiting hepatic schizont formation. Oral efficacy was noted in a humanized mouse model of *Pf* malaria despite the high tendency of 1-S to develop resistance *in vitro*.[190] Baragana et al. identified a series of inhibitors, among which **Compound 5** showed specific inhibition of *Pf*LysRS over the human counterpart (*h*LysRS), with *in vivo* efficacy shown in a SCID mouse model of malaria.[191] **BRD1389** and **BRD7929** are bicyclic azetidine-based compounds that target

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*Pf*PheRS. Both inhibitors kill blood-stage, liver-stage, and transmission-stage *Plasmodium* parasite.[193] In animal models of malaria, BRD1389 has been reported to prevent transmission, provide prophylaxis, and facilitate a single-dose cure.[152] Another inhibitor, **ML901**, is a sulfamate compound that suppresses the growth of *P. falciparum* parasite both *in vitro* and *in vivo*. ML901 targets *Pf*TyrRS by a reaction-hijacking mechanism, binding it to the tyrosine of *Pf*TyrRS, generating a dead-end product that inhibits the active site and restricts downstream protein synthesis in the parasite. It is safe for human cells, and in a mouse model of malaria, it showed potent antimalarial activity against all stages of the *Plasmodium* parasite lifecycle with a single dose that has low nanomolar potency.[153] **ASP3026** (2-N-[2-methoxy-4-[4-(4-methylpiperazin-1-yl)piperidin-1-yl]phenyl]-4-N-(2-propan-2-ylsulfonylphenyl)-1,3,5-triazine-2,4-diamine) an inhibitor of anaplastic lymphoma kinase, has been found as a potential *Pf*LysRS inhibitor. It inhibits the enzymatic activity of *Pf*LysRS at nanomolar concentrations, which is >380-fold more potent than its human counterpart. Additionally, this highlighted the promising possibility of repurposing kinase-inhibitory drugs as aaRS inhibitors for the treatment of other diseases.[192]

M5717 (formerly DDD107498) or 6-fluoro-2-(4-(morpholinomethyl)phenyl)-N-(2-(pyrrolidin-1-yl)ethyl)-quinoline-4-carboxamide possess nanomolar potency against *P. falciparum*. It has a multistage activity spectrum, including liver, blood, and transmission-blocking activity, and it is effective against parasites that have exhibited resistance to commonly used antimalarial drugs.[194] Rottmann et al. evaluated M5717 in combination with PND, a hemozoin formation inhibitor. No pharmacokinetic interactions have been seen with the combination of M5717 and PND (pyronaridine). M5717 did not adversely affect the rate of activity of the rapid-acting PND. Instead, PND successfully blocked the selection of M5717-resistant mutants while also considerably delaying parasite recrudescence under both suboptimal and optimal dosing regimens.[195] McCarthy et al. conducted the first-in-human clinical study to evaluate the safety, pharmacokinetics, tolerability, and antimalarial efficacy of M5717 in healthy individuals. They found that M5717 is well-tolerated in healthy individuals at doses effective in eradicating blood-stage *P. falciparum*. The longer half-life of M5717 offers the possibility of single-dose administration with extended duration of activity.[196]

Inhibitors of Plasmodium Isoprenoid Biosynthesis Pathway. **BMS-388891** is a tetrahydroquinoline (THQ) that potently suppresses parasite proliferation. At dosages of 5 and 10 nM, BMS-388891 caused severe abnormalities in *Pf* maturation, halting parasite growth at the trophozoite stage. Further, resistant mutants produced by exposure of *Pf* Dd2 strains to the compound exhibited a 12-fold rise in EC_{50} value, with a single mutation noted in the active site of *Pf*PFTase.[135] Another *Pf*PFTase inhibitor, **R115777**, a nonpeptidomimetic quinolinone, has been studied for its effect on the mitochondria of the malaria parasite. The farnesyltransferase inhibitor (FTI) R115777 was found to effectively suppress the proliferation of malaria parasites. Additionally, it had an impact on ROS levels and mitochondrial depolarization. FTI R115777 significantly lowered the copy number of cytochrome c oxidase III, which is encoded by the mtDNA. This study found that FTI R115777 has a significant impact on the mitochondria of *P. falciparum*, disrupting their function.[197]

MMV008138 or (1*R*,3*S*)-1-(2,4-dichlorophenyl)-2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]indole-3-carboxylic acid is a compound that inhibits *Pf*IspD and identified as a promising antimalarial lead following a phenotypic screening method. MMV008138 was later revealed to exhibit compromised antiparasitic activity in assays enriched with an exogenous supply of isopentenyl pyrophosphate (IPP), a key isoprenoid building block.[198] The possibility of therapeutic intervention against *Pf* infection by inhibiting *Pf*IspD was evaluated and it found that *Pf*IspD serves as the enzymatic target of 1*R*,3*S* diastereomer of MMV008138. It was suggested that **1*R*,3*S*-MMV008138** inhibits *Pf*IspD competitively with its cytidine 5′-triphosphate (CTP) substrate by targeting the CTP binding site of *Pf*IspD.[156]

MMV019313 is the very first nonbisphosphonate inhibitor of bifunctional *Pf*FPPS/GGPPS. It effectively reduced *Pf* growth throughout the absence of IPP, but its EC_{50} (half maximal effective concentration) was compromised in the presence of IPP. MMV019313 exhibited no potency against human FPPS or GGPPS. MMV019313 resistance was conferred by moderate overexpression of a single nucleotide variant of Ser228Thr in the *Pf*FPPS/GGPPS protein.[157] Kabeche et al. showed a new series of thiazole-containing amides that are effective and specific inhibitors of *Pf*FPPS/GGPPS. Out of this series, the *in vivo* mouse pharmacokinetic profile of **AIM-1290** is a high-quality tool compound that should be further evaluated as an ideal candidate for an antimalarial drug.[199] **MMV026468** or [4-[5-(3-Chloroanilino)-pyridin-3-yl]phenyl]methanol is an effective picomolar inhibitor of *Pf* IPP synthesis. However, its target enzyme remains unknown. Further medicinal chemistry optimization, could result in a novel category of antimalarial isoprenoid inhibitors to prevent the spread of drug-resistant parasites.[215]

Inhibitors of Plasmodium DHFR-TS, PfAcAS, PfACS10, and NMT. **Triclosan** is an antimicrobial and antifungal agent that can precisely target the DHFR enzymes of both *Pv* and *Pf*; it can specifically inhibit both wild-type and pyrimethamine-resistant enzymes. Triclosan inhibits the blood stage of the malarial parasite.[200] **BT1** is a hybrid compound that combines rigid and flexible pharmacophores in a single molecule. It exhibits excellent affinity for binding and inhibiting both quadruple mutant (QM)/108N series and wild type (WT)/S108 series *Pf*DHFR. BT1 exhibited significant antimalarial efficacy and the ability to prevent resistance mutations, making it an excellent template for future design and synthesis of antifolates that are less susceptible to resistance. This series of rigid-flexible hybrids can add to the growing list of novel antifolates with good efficacy against pyrimethamine-resistant *Pf*.[201]

MMV019721 (2-chloro-5-(diethylsulfamoyl)-*N*-(2-methyl-1,3-benzothiazol-6-yl)benzamide) and **MMV084978** (N′-(4-chlorobenzoyl)-1,2,3-thiadiazole-4-carbohydrazide) are two small molecules that can effectively inhibit *Pf*AcAS by competing with substrates. Both these compounds compete for CoA and acetate binding, leading to acetyl-CoA depletion and disruption of histone acetylation in the malarial parasite. Mechanistic investigations in compound-treated WT parasites showed inhibition of histone acetylation, with both compounds functioning as potent inhibitors of asexual and liver-stage growth in the parasite lifecycle.[202] Schalkwijk et al. reported another *Pf*AcAS inhibitor, a pantothenamide bioisostere **MMV689258**, effective against the blood stage of the *P. falciparum* parasite lifecycle. It can also inhibit the transmission

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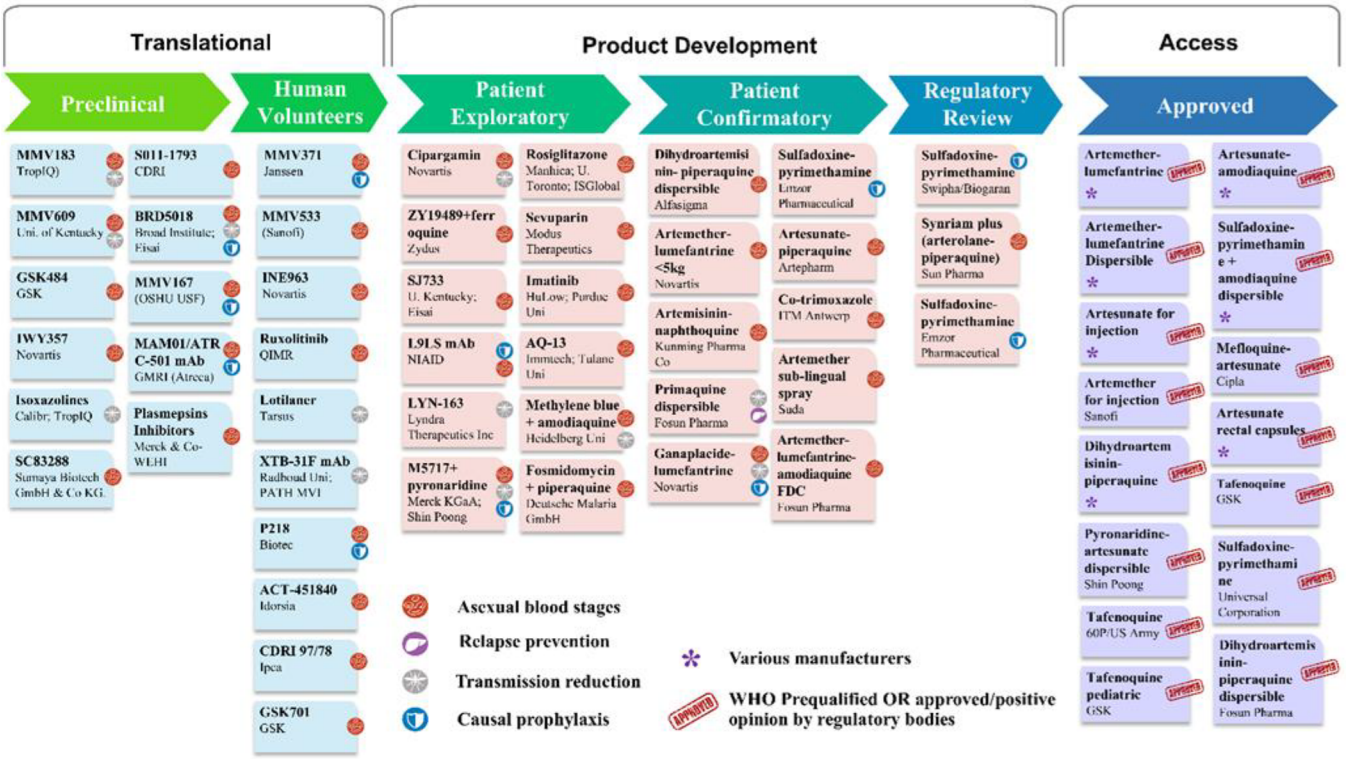

Figure 4. Antimalarial drug discovery and development pipeline (adapted from MMV Website[222]).

of sexual stages to the *Anopheles* mosquito. MMV689258 exhibited antimalarial efficacy, good pharmacokinetic characteristics, and parasite clearance in a humanized mouse model of *Pf* infection. The parasite converts this novel pantothenamide class into coenzyme A analogues that are very effective against malarial parasites at different stages of the malarial parasite lifecycle.[203] **MMV183** or N-[(2*S*)-1-[[(2*R*)-2,4-dihydroxy-3,3-dimethylbutanoyl]amino]propan-2-yl]-2,4,5-trifluorobenzamide is an antimetabolite exhibiting significant transmission-blocking and asexual blood-stage inhibiting activity that targets *Pf*AcAS. MMV183 is derived from the natural product pantothenic acid, also known as vitamin B5, and it is currently in the preclinical exploratory stage of drug development.[204]

GSK701 or [(3*R*)-3-(4-fluorophenyl)pyrrolidin-1-yl]-[4-[(2*R*)-2-hydroxy-3-(tetrazol-2-yl)propoxy]phenyl]methanone is a novel inhibitor of the acyl-CoA synthetases *Pf*ACS10 and *Pf*ACS11. The precursor of GSK701 was discovered in a phenotypic screening. It is currently in phase I of clinical drug development for the treatment of uncomplicated malaria. GSK701 has good potency and efficacy against both susceptible and resistant *Plasmodium* strains. It can eradicate asexual blood-stage parasitemia,[13] and is a fast-acting pyrrolidinamide derivative that is effective against *P. falciparum*.[180] Three chemical scaffolds, **MMV665924**, **MMV019719**, and **MMV897615**, target *Pf*ACS10, resulting in a reduction in triacylglycerols, which are necessary for parasite proliferation and the accumulation of its lipid precursors.[160]

IMP-1002 or 1-(5-{4-fluoro-2-[2-(1,3,5-trimethyl-1*H*-pyrazol-4-yl)ethoxy]phenyl}-1-methyl-1*H*-indazol-3-yl)-*N*,*N*-dimethylmethanamine is an NMT inhibitor (NMTi) that targets *Pf*NMT and *Pv*NMT by interacting with the protein substrate binding site of NMT.[206] When IMP-1002 is given to a synchronous population of ring-stage parasites, it irreversibly disrupts parasite growth during early schizont development (4 to 6 nuclei) prior to the formation of the inner membrane complex (IMC), producing a parasite form known as 'pseudoschizont'. This 'pseudoschizont' form is most likely the result of the suppression of *Pf*NMT during the trophozoite or early schizont stages.[205] A later study used the computer-aided drug design technique to identify three possible *Pv*NMT-targeting compounds: **EXP90**, **ZBC205**, and **ZDD968**. These compounds can be further investigated for inhibition of *Pv*NMT *in vitro* and *in vivo*.[207] Rodríguez-Hernandez et al. developed the next generation of *Pv*NMT inhibitors using structure-guided hybridization, combining chemical moieties from previously identified NMT inhibitors. The hybridized compounds effectively decrease *Pf* blood-stage parasite load while additionally inhibiting *Pv* liver-stage schizonts and hypnozoites in a dose-dependent manner. They showed that hybridized NMT inhibitors can act as multistage antimalarials, targeting the blood stage and both liver dormant and developing forms of the parasite.[161]

Transmission Blocking Agents. Antimalarial drug development is primarily driven by the critical necessity to develop effective therapeutic agents for reducing infections and mortality, particularly amid the ongoing emergence of drug-resistant strains.[216] Transmission-blocking agents are the most effective choice in this context, as they not only disrupt the parasite's life cycle but also help prevent the development of drug resistance.[217] To achieve transmission-blocking activity, eliminating or inactivating individual mature gametocytes would be necessary.[216] PQ (Primaquine) is the only drug approved by WHO for the malaria transmission-blocking purpose. While PQ is effective in removing mature gametocytes from the blood, its use is limited due to safety concerns in G6PD-deficient patients.[218] Late-stage gameto-

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cytes (IV−V) are the primary targets for transmission-blocking drugs, as they are more susceptible to therapeutic intervention, and can be readily targeted within the human blood compartment.[217] Examples of transmission-blocking antimalarial drugs currently under clinical trials are (+)-SJ733, M5717, Cipargamin, Artefenomel (OZ439), and Ganaplacide.[216] Over the past decade, the search for novel transmission-blocking agents has significantly increased, resulting in several promising candidates' identification. Some examples of potential lead compounds are DDD01035881, ML324, GSK-J4, ML901, MPI11, MPI13, Oxamflatin, Scriptaid, WM382, MMV183, MMV030084, Torin 2, Sapanisertib, etc. Appetecchia et al. have recently discussed these lead compounds.[217] Apart from transmission-blocking drugs, transmission-blocking vaccines (TBVs) are another way to prevent malaria transmission. In addition to eliminating gametocytes, malaria transmission could be halted by targeting other parasitic stages that occur in mosquitoes, including gametes, zygotes, oocytes, and oocysts. Thus, antigens from these stages are being thoroughly explored as potential targets for TBVs.[218] TBVs mainly focus on proteins linked to the developmental stages that occur after the gametocyte stage. TBVs do not directly prevent infection in humans. Instead, they promote the production of antibodies that target sexual-stage or sporogonic-stage parasites within mosquitoes, or antigens expressed by the mosquito itself.[219] When a blood-feeding mosquito ingests vaccine antibodies, these antibodies target parasites in the same bloodmeal, preventing the mosquito from being infectious to the next person.[220] Further, TBVs can help slow the spread of mutant parasites, thereby extending the effectiveness of antimalarial drugs and vaccines.[218] TBVs can be paired with vaccines targeting the sporozoite, liver, or blood stages to form a multistage vaccine, which may be more effective in controlling infection and preventing transmission. Prominent TBV candidate antigens include *P. falciparum* sexual-stage surface protein (*Pfs*25), *P. falciparum* gametocyte surface antigen (*Pfs*230), and *P. falciparum* gametocyte surface protein (*Pfs*48/45).[221] Vaccines targeting gamete surface antigens (*Pfs*230 and *Pfs*48/45) or zygote surface antigens (*Pfs*25) have progressed to clinical trials. *Pfs*25 was the first transmission-blocking candidate, followed by *Pfs*230 and *Pfs*48/45.[220] Recently, an mRNA-based vaccine strategy successfully elicited functional immune responses against the *Pfs*25 and *Pfs*230 domain 1 (*Pfs*230D1) candidates in animal models.[221] However, vaccine development is inherently a lengthy process that involves overcoming numerous challenges and technical hurdles.

Medicines for Malaria Venture (MMV). The Medicines for Malaria Venture (MMV), established in 1999, has been at the forefront of the discovery and development of novel malaria therapeutics. MMV is collaborating with universities and pharmaceutical companies globally to facilitate the development and commercialization of novel antimalarial drugs, thereby contributing to the battle against malaria.[9] The list of antimalarial compounds in the current development pipeline, as of 2024, is shown in Figure 4; this list is also regularly updated on the MMV Website.[222] Umumararungu et al. have recently discussed the drugs in the MMV drug development pipeline.[20]

Strategies to Combat Antimalarial Drug Resistance. *Use of Triple Artemisinin-Based Combination Therapies (TACT).* TACT emerged as an innovative approach for combating ACT resistance. It follows the biological principles of combining two distinct, slowly eliminated antimalarial drugs with an ART derivative. In this combination, one drug is expected to provide protection against partner-drug resistance and, thus, maintain high therapeutic efficacy. It is based on the assumption that parasites develop mutations conferring resistance to both partner drugs within a short period of time, which is extremely rare.[223] Ongoing research attempts to unravel the rationale and evaluate the pharmacodynamics and pharmacogenetics of triple artemisinin-based combinations.[224] Randomized clinical trials carried out in Asia have demonstrated that the combinations of dihydroartemisinin-piperaquine-mefloquine (DHA-PPQ-MQ) and artemether-lumefantrine-amodiaquine (ALAQ) are both well-tolerated, safe, and successful. These combinations have proven to be effective even in regions with a high prevalence of multidrug-resistant *Pf* malaria.[225] DHA-PPQ combines the rapid effect of DHA with the delayed action of PPQ for mass drug administration. DHA generates ROS after heme activation, which causes parasite cell damage, whereas PPQ interferes with metabolic processes by preventing hemozoin formation. Adding MQ improves the efficacy of DHA-PPQ and lowers resistance to PPQ. In the ALAQ combination, LMF binds to hemin, inhibiting detoxification into hemozoin, whereas ATM releases harmful free radicals. AQ complemented this activity by inhibiting protozoan nucleic acid and protein synthesis.[226] Each drug has the same pharmacokinetics as its companion drugs. In a small phase II clinical study, the combination of ACT and imatinib, a tyrosine kinase inhibitor used to treat leukemia, was studied. It was based on the rationale that interfering with tyrosine phosphorylation of erythrocyte membrane protein 3 could inhibit merozoite release, potentially having an antimalarial activity and enhancing the antimalarial activity of ART *in vitro*. Furthermore, imatinib partnered with DHA-PPQ generated faster parasite clearance than DHA-PPQ alone.[227] The effectiveness of TACT in overcoming resistance, providing a broader spectrum of action, minimizing the likelihood of treatment failure, and its adaptability to regional requirements contribute to the global effort to eradicate malaria.[226] However, the clinical trials are unable to evaluate the long-term evolutionary advantages of TACT implementation. A consensus mathematical modeling method was employed to predict the possible long-term evolutionary dynamics and clinical treatment results of TACT implementation in various malaria epidemiological settings. The result demonstrated that the use of TACTs could remarkably delay the emergence and spread of ART resistance and treatment failure, thereby extending the therapeutic lifespan of current antimalarial medicines and increasing the possibility of malaria elimination. Therefore, the immediate implementation of TACTs in areas of emerging ART resistance was advocated.[223]

Identification of New Drug Targets. Currently, numerous essential and unrelated metabolic processes or enzymes present in *Plasmodium* parasites have been considered as the targets for antimalarial drugs. The identification of novel therapeutic targets for antimalarial drug development is a major hurdle in the context of multidrug-resistant malaria. Drug resistance in malaria results in the ineffectiveness of closely related drugs, a phenomenon known as "cross-resistance". This reduces the available alternatives for managing the disease.[65] The primary reasons for seeking such novel targets include the development of chemically diversified antimalarial therapeutics that are less susceptible to cross-resistance, as well as the production of safer and more potent compounds utilizing these targets.

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Whole-genome sequencing of malarial parasites opened new possibilities for the discovery of a novel therapeutic target.[228] Several novel antimalarial drug target and their potential inhibitors are already discussed in the previous section. High-throughput *in vitro* or virtual screening of chemical libraries from various resources is a promising approach for identification and lead optimization. This method is not only important for the development of a novel antimalarial drug or combination, but its distinctive mode of action will also be advantageous in mitigating the likelihood of drug resistance, both with current antimalarial drugs and potential future candidates.

Analogues of Existing Drugs. The identification and lead optimization of a novel chemical series as an effective antimalarial has several economic, societal, and scientific hurdles. These hurdles include toxicity, expense, and ethical issues. On the contrary, developing analogues of currently available antimalarial drugs is a viable approach for developing novel therapeutic interventions, potentially addressing the above-mentioned concerns to a greater extent.[19] Developing analogues for further screening requires an understanding of the chemistry of existing antimalarial drugs and their active moieties. This strategy is an inexpensive approach and has proven to be a profitable endeavor. Several drugs have been produced using this strategy, including CQ and QN analogues such as PPQ/PND and AQ/MQ, respectively. These analogues are either components of currently used ACTs or are in the advanced stages of clinical trials.[229] Additional products of this strategy include PQ analogues, TQ, intended for a radical cure for *Pv*, and ART analogues, specifically ozonide OZ439 (artefenomel), synthetic endoperoxides developed to be used in combination with a partner drug.[222] The best examples in this category are the development of trioxanes and analogues of 4-aminoquinolines. CQ analogues are developed by linking 4,7-dichloro quinoline with monoalkynes; ferroquine, a methanogenic CQ analogue, and 1,2,4-trioxane, that share a similar core structure to ART. Both of these analogues are in clinical studies for the treatment of uncomplicated malaria.[230] Moreover, the effects of sulphonamides, AQ, and pyrimethamine on pyrimidine metabolism have prompted the creation of novel drugs that target *Pf*DHFR, P218 and *Pf*DHODH, DSM265, hence addressing pyrimethamine resistance issues.[11]

Use of Resistance-Reversal Agents. Antimalarial drug resistance is spreading rapidly due to a variety of factors, including drug overuse, mutations in transporter genes, and mutations in known target genes. The drug development process is time-consuming, and it is mostly constrained by the time required for lead optimization and clinical trials, along with high costs, and stringent regulatory guidelines.[229] An effective strategy for developing antimalarial drugs is preventing the development of resistance to existing treatments. This can potentially be achieved by utilizing resistance-reversal agents.[11] Nevertheless, the identification of resistance reversal agents is a difficult task, constrained by the limited knowledge regarding their mechanisms of action and potential toxicity. Numerous resistance reversal agents have been identified for the majority of the traditional medications, such as CQ (chlorpheniramine, chlorpromazine, verapamil, promethazine, etc.), MQ, and QN. Drug metabolizing enzymes may also reduce the efficacy. Specific inhibitors that block these drug-metabolizing enzymes can reverse the condition, allowing the drug to continue being effective. However, toxicity may be a significant concern for certain combinations, therefore it should be considered. This resistance reversal strategy has been employed successfully in research. The utilization of ketoconazole to reverse resistance to MQ, in combination with clarithromycin and MQ/QN/quinidine, has been shown to be beneficial in combating multidrug-resistant malaria.[229]

Drug Repurposing. Identifying new uses for existing treatments has appeared to be more beneficial than conventional *de novo* drug discovery processes, facilitating the rapid development of a safe drug at a lower cost.[231] Several anticancer, antibacterial, antiviral, and antiparasitic medicines have already exhibited tremendous antimalarial potential and offered numerous prospects for drug repurposing.[231,232] Currently, the majority of the clinical, pharmacological, pharmacodynamic, and pharmacokinetic information regarding these drugs is available, making it safer, inexpensive, as well as faster to proceed to the clinical stage. When selecting drugs for repurposing, it is important to take into consideration the drugs that showed efficacy against the target disease during the clinical development process. This strategy lowers the likelihood of failure since repurposed drugs are already proven to be safe for humans to use.[65] This approach has identified several compounds, especially those used for treating malaria, following multiple successful endeavors in this field. Some notable examples of these repurposed drugs include lopinavir and tipranavir (HIV protease inhibitors), imidazolopiperazine compound (which has antifungal and antihelminthic properties), atorvastatin (often used to lower cholesterol levels), and itraconazole (an antifungal compound).[229] These compounds with potent antimalarial activity specifically target multiple stages and particular targets of the malarial parasite. Furthermore, some of the antibiotics, such as doxycycline, tetracycline, clindamycin, etc., are clinically used in combination with partner drugs for the treatment of malaria. The antimalarial efficacy of nitrofurantoin (NTF), a therapeutically employed antibacterial drug, against intraerythrocytic stages of ART-sensitive (*Pf*3D7) and resistant (*Pf*Kelch13^{R539T}) *P. falciparum* strains has been evaluated. NTF showed an inhibitory effect on parasite growth at a submicromolar concentration by halting their growth at the trophozoite stage of the malaria lifecycle.[233]

Therapeutic Use of RNA Interference (RNAi) and CRISPR/Cas9. RNA interference (RNAi) is a prevalent biological mechanism seen in the majority of eukaryotes. It modulates gene expression by inhibiting translation through the degradation of mRNA. This is accomplished through the binding of small RNA molecules, particularly double-stranded RNA, with the target mRNA, resulting in the formation of a double-stranded structure. The RNA-induced silencing complex (RISC) then identifies this structure. The RISC directs the RNAi process toward the complementary mRNA, resulting in its degradation and the inhibition of mRNA translation.[234] Although the implementation of RNAi as a therapeutic approach was previously seen as challenging, mainly due to the specific and efficient delivery of RNAi to the target cell, its stability, and the limited duration of the silencing effect, recent advancements seem to be addressing these limitations.[235] Presently, these gene-silencing mechanisms are effectively employed. McRobert and McConkey used RNAi to inhibit the process of translating the mRNA of *Pf*DHODH, which is an essential enzyme involved in pyrimidine synthesis required for the parasite. This study focused on a specific region of this

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gene in the *Plasmodium* stage that infects erythrocytes, resulting in a parasite growth suppression of over 60%. Additionally, researchers evaluated the suppression of other important genes, such as Chorismate Synthase (CS), which plays a key role in the synthesis of folate. They noted a 44% reduction in parasite growth.[236] Notably, these authors introduced dsRNA into *Pf* cells through the electroporation method. However, advanced RNAi delivery techniques are now available, such as encapsulating RNA in lipid nanoparticles. These approaches successfully address past challenges associated with dsRNA stability and delivery. In a later work, Hentzschel et al. explained the invention of a strategy that uses AgoshRNAs (a modified short hairpin RNA) to silence specific genes in the *P. berghei* parasite that lacks Dicer ribonuclease, a crucial enzyme in the RNAi pathway. The authors successfully integrated the Argonaute protein into the *P. berghei* genome, producing a transgenic strain known as *Pb*Ago2. This line expresses the Argonaute protein constitutively without impacting the growth of the parasite. AgoshRNAs exhibited excellent specificity in silencing target genes without showing off-target effects. Finally, scientists have reported the specific expression of Ago2 in the hepatic stages of the malarial parasite's life cycle, temporally regulating gene silencing.[237] While this technology shows promise as a therapeutic strategy, more research is required to determine its viability in the treatment of malaria in humans. Even though there is evidence indicating the utilization of RNAi in *Pf*, few authors deny the presence of this machinery in the parasite. Therefore, the application of this therapeutic approach remains to be a topic of debate.[235,236] RNAi therapy includes drugs such as Patisiran and Inotersen. Considering the notable genomic flexibility of *Pf*, which confers its ability resistance to all currently available drugs, it may be appropriate to reevaluate this therapeutic approach based on the provided examples. Likewise, the CRISPR-Cas9 system can be used to silence the genes that have acquired drug resistance since they previously showed their efficacy as therapeutic targets prior to the parasite's development of drug resistance. CRISPR/Cas9 is a genetic editing technology that utilizes the Cas9 protein in conjunction with a guide RNA molecule to accurately modify genes. The guide RNA guides Cas9 to specific sites in the DNA, where it generates cuts. Controlled genetic modifications can be accomplished through the natural repair of these cuts.[235] A new study has shown that an integration approach called Cas9i, which utilizes the CRISPR/Cas9 system, can accelerate the production of transgenic strains in *Pf*.[238] However, comparable to previous technologies like RNAi, the main challenge in the CRISPR/Cas9 system is in the delivery mechanism. Even though the therapeutic targets stated above have been shown to be potent, genetic resistance remains a persistent concern. Instead of completely altering our therapeutic target, it might be advantageous to explore novel strategies or combinations of treatments to combat the ongoing emergence of genetic resistance.

Nanocarrier (NC)-Based Antimalarial Drug Delivery. Research in nanotechnology and nanomedicine promises to overcome the limitations of antimalarial treatments, such as multidrug resistance or lack of selectivity in targeting parasites directly. Nanotechnology-enabled nanomedicines provide benefits, including targeted drug delivery, enhanced therapeutic effectiveness, decreased drug toxicity, and improvement in drug resistance.[239] An important advantage of utilizing nanotechnology is its ability to develop small carriers capable of encapsulating drugs while transporting them to specific cells or tissues. The application of these NCs is critical for modifying the drug's pharmacokinetic profile, increasing solubility and bioavailability, decreasing drug-related toxicity, enhancing pharmacological properties, and overcoming multidrug resistance. As a result, NCs contribute to a more effective treatment of malaria compared to conventional therapy.[240] The development of NCs that can encapsulate drugs for targeted delivery offers an opportunity to enhance the effectiveness of antimalarial drugs while limiting potential adverse effects. Employing NCs for targeted drug delivery can effectively achieve the desired drug concentration at the specific site, hence minimizing the likelihood of multidrug resistance emergence in *Plasmodium*. It also offers drug targeting by surpassing many barriers to reaching the intracellular parasites, minimizing drug resistance, and improving the therapeutic efficacy of commonly administered antimalarial therapeutics through selective drug distribution. Various NCs, such as liposomes, dendrimers, micelles, solid lipid nanoparticles (SLNs), metallic nanoparticles, nanostructured lipid carriers (NLCs), and others, have been extensively studied and proven to be highly beneficial for drug delivery purposes.[38] Therefore, the utilization of nanostructured drug delivery systems has the potential to tackle the major issue of *Plasmodium spp*. multidrug resistance to long-running drugs in endemic regions. This offers a novel opportunity to administer the same drugs at precise dosages, in turn decreasing side effects.[241,242] However, the NC-based drug delivery system is associated with some limitations, such as expensive preparation, interaction with biological elements, inability to select the optimal route of administration, etc.

Development of Hybrid Drugs. In recent times, the hybrid concept has developed as a highly effective method for the synthesis of novel and effective antimalarial drugs. Hybrid drugs are more beneficial due to their lower incidence of drug−drug side effects. The hybrid molecule offers significant advantages, such as reduced likelihood of drug−drug interactions, less expensive, and enhanced patient compliance.[243] The hybrid molecule exhibits a dual mechanism of action that enables it to simultaneously affect several targets with improved effectiveness and reduced vulnerability to drug resistance. Thus, synthesizing hybrid molecules employing new chemical entities with two (or more) distinct heterocyclic skeletons (pharmacophores) is a rational approach to developing new antimalarial drugs. These hybrid drugs with a dual mode of action can prevent the rapid emergence of multidrug resistance by minimizing the likelihood of drug−drug interactions. Despite the development of resistance, the efficacy of conventional drugs such as CQ has been restored by this approach. Additionally, these drugs offer advantages like low toxicity to the host and shorter course duration. Currently, researchers are developing fully synthetic antimalarials and hybrid molecules that are showing promising results in laboratory experiments. Also, some of these compounds have progressed to clinical trials.[65] Recently, a group of researchers identified novel PPQ hybrid analogues linked by triazolopyrimidine and pyrazolopyrimidine scaffolds, which exhibit an inhibitory effect on gametocyte and transmission-blocking activities in the malarial parasite.[244] Herrmann et al. successfully synthesized novel ART-triazole antimalarial hybrids through facile organo-click reaction, achieving good yields of up to 83% from readily available substrates. Then, these novel compounds were tested for the first time against *Pf*

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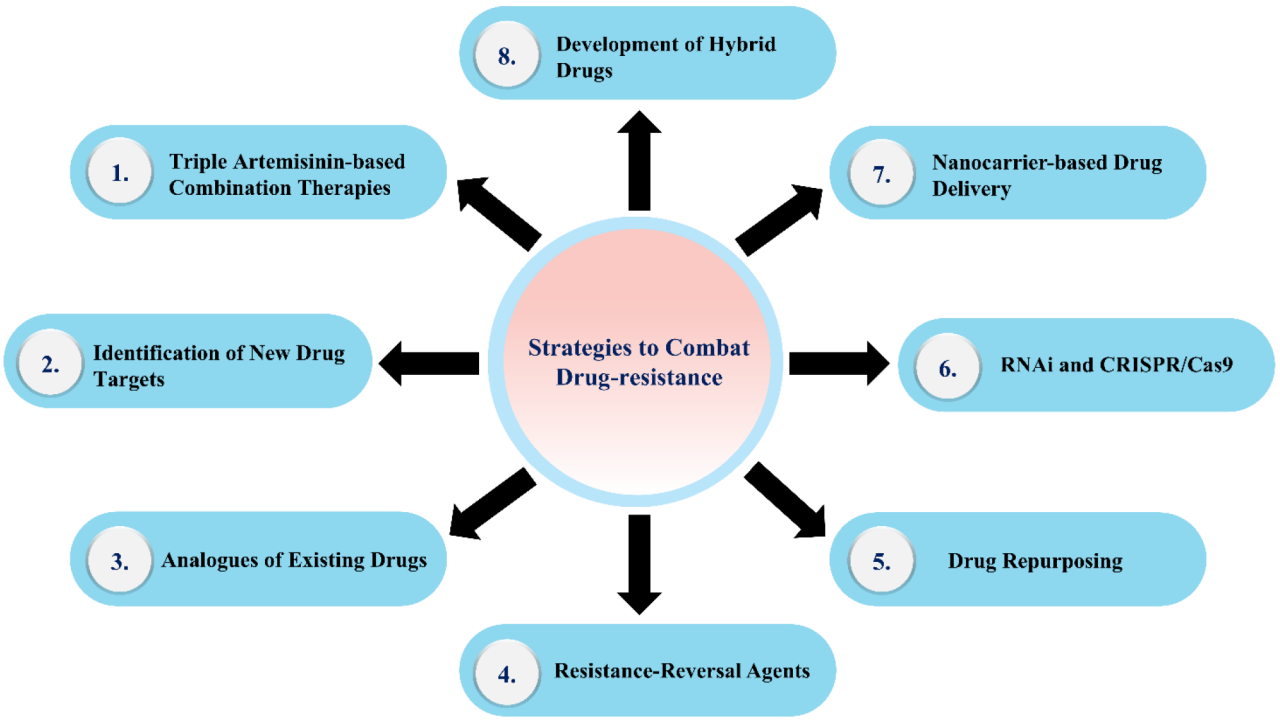

Figure 5. Different strategies to combat drug resistance

strains. Notably, all hybrid compounds showed excellent antimalarial efficacy *in vitro* against CQ-resistant and multi-drug-resistant *Pf* strains (IC_{50} of Dd2 strain decreased to 2.1 nM; IC_{50} of K1 strain decreased to 1.8 nM) compared to the reference drug CQ (IC_{50} of Dd2 = 165.3 nM; IC_{50} of K1 = 302.8 nM).[245] Raiguru et al. have discussed the recent advances in hybrid antimalarial drugs, including ART-based, 1,2,3-triazole-based, 4-amino-7-chloroquinoline, quinoline/quinolone/quinazolinone, and other hybrid molecules.[243] Figure 5 depicts different strategies to combat drug resistance.

CONCLUSION AND FUTURE PERSPECTIVE

Antimalarial drugs target different stages of *Plasmodium spp.* life cycles, such as hypnozoites, liver schizonts, blood stage, gametocytes, and oocyst. Most of these drugs become ineffective or partially effective against drug-resistant malarial parasites. Among the different parasites, *P. falciparum*-led drug-resistant cases are the highest, followed by *P. vivax*. Following the first report in 1910, several cases of antimalarial drug resistance have emerged, including the resistance against the most potent antimalarial drug, artemisinin. The parasites withstand the effects of antimalarial drugs of different chemical classes, such as aryl amino alcohols, Antifolate, and Artemisinin compounds. They acquire drug resistance primarily by changing the structure and function of the target proteins involved in various metabolic/signaling/ion-channel pathways through genetic modifications such as SNPs or copy number variations. One of the relatively better-known strategies adopted by the parasites to avert the effect of many drugs (e.g., quinine, chloroquine, ARTs) is the expulsion of these drugs from the food vacuole to maintain the heme detoxification functions for their survival inside the erythrocytes. These increased drug resistances have propelled intensive research to identify new targets for malaria and drug-resistant *Plasmodium* strains. These efforts identified various targets, including proteases, proteasomes, kinases, enzymes from the *Plasmodium* DNA and RNA metabolic pathway, protein synthesis pathway, isoprenoid biosynthesis pathway, and transporters proteins. Additionally, different inhibitors were also identified for these targets in malaria parasites. Various approaches were proposed to combat antimalarial drug resistance, such as the use of triple artemisinin-based combination therapies, analogues of existing drugs, resistance-reversal agents, therapeutic use of RNA interference and CRISPR/Cas9, drug repurposing, nanocarrier-based antimalarial drug delivery, development of hybrid drugs along with identification of new drug targets. These efforts have invented many drugs against drug-resistant strains, some of which are in the clinical pipelines, and few are approved by regulatory bodies. In order to offer precise and timely therapy to the patients infected by drug-resistant malaria, a reliable and rapid diagnosis is essential. Such diagnosis activities are also critical to monitor the patients during the treatment period for prognosis, evaluate the drug efficacy, and identify the linked drug-resistant strains. Currently, the prominent techniques employed for detecting the drug resistance in *Plasmodium* spp. include *in vivo* drug efficacy studies, *in vitro/ex vivo* drug sensitivity studies, and molecular diagnostics that evaluate known markers of antimalarial drug resistance. Among these techniques, *in vivo* studies are considered the gold standard due to their greater accuracy for detection. However, these methods involve many uncontrollable factors due to the complexity of the immune response and diversity among human subjects. Thus, they do not always provide real clinical results for patients.

Although significant progress has been made in developing both therapy and diagnosis of drug-resistant malaria, further research is warranted in these sectors for better management of these acute infectious diseases, particularly in the resource-limited regions. The efficacy of the drugs should be prolonged to protect the patient's health; the diagnostic methods/techniques should be simple, provide results rapidly and

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inexpensively, and be deployable to point-of-care settings for better surveillance in masses and remote environments. Parallel research for developing an efficient vaccine against malaria should also be encouraged. Notably, the global vaccine development program for malaria prevention is slow, with only limited progress visible from the recent past, where the first malaria vaccine (Mosquirix) for *Pf* malaria was approved in 2021, following which another malaria vaccine (R21/Matrix-M) was approved in October 2023 by the WHO.

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Kangkana Barman reviewed the literature and prepared the draft manuscript. Pranab Goswami edited the manuscript and administered the project.

Notes

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