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DHFR: A promising drug target for the identification of antimalarial agents

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ABSTRACT
Malaria continues to be a significant worldwide health concern, characterized by a substantial illness burden and fatality rate. The escalating resistance of Plasmodium falciparum to standard antimalarial medications highlights the pressing need for novel therapeutic approaches. Dihydrofolate reductase (DHFR) is a prominent therapeutic target in malaria, serving as a crucial enzyme in the folate biosynthesis pathway, which is important for the replication and survival of the parasite's DNA. Nonetheless, extensive resistance to antifolates such as pyrimethamine has emerged due to point mutations in the dhfr gene. This has resulted in a revitalized focus on creating innovative DHFR inhibitors that are efficacious against resistant strains. Recent advancements in medicinal chemistry and structure-based drug design have enabled the identification of novel molecules with enhanced affinity for mutant DHFR variants. The present review examines malaria incidence, resistance mechanisms, and highlights DHFR as a crucial target for the development of next-generation antimalarials..

Keywords: *Plasmodium falciparum, malaria, Dihydrofolate reductase, resistance.*

1. INTRODUCTION

Malaria is a life-threatening vector-borne illness induced by protozoan parasites of the genus Plasmodium, with the five primary human-infective species being P. falciparum, P. vivax, P. malariae, P. ovale, and P. knowlesi [1]. Its manifestation extends from low/high fever to severe consequences such as anemia, neurological involvement, or organ failure. Notwithstanding extensive control initiatives, malaria continues to pose a significant worldwide health threat. The WHO's World Malaria Report 2024 indicates an expected 263 million malaria cases and 597,000 fatalities in 2023, reflecting an increase of 11 million cases from 2022, with almost 95% of deaths happening in the WHO African Region. Over fifty percent of global cases are concentrated in five nations: Nigeria, the Democratic Republic of Congo, Ethiopia, Mozambique, and Uganda. The incidence (60.4 per 1,000 individuals at risk) significantly exceeds the objectives established in WHO's 2025 and 2030 Global Technical Strategy. The primary factors contributing to this revival are funding deficits—projected at $4.3 billion each year—climatic disturbances, humanitarian emergencies, and increasing resistance in both vectors and parasites. The rise of artemisinin partial resistance in certain regions of East Africa, such as Eritrea, Rwanda, Uganda, and Tanzania, is particularly alarming [2]. In 2023, India accounted for roughly 50% of all malaria cases and deaths in the WHO South-East Asia region. Malaria is transmitted when infected female Anopheles mosquitoes bite humans and introduce sporozoites during blood feeding. These migrate to the liver, undergo schizogony, and release merozoites into the bloodstream, infecting red blood cells (RBCs). Numerous asexual replication cycles occur, resulting in symptoms, with a portion developing into gametocytes. Subsequently, these are ingested by mosquitoes, thereby concluding the cycle. In *P. vivax* and *P. ovale*, dormant hepatic forms known as hypnozoites can revive after weeks to months, resulting in relapses [3]. Chemotherapy for malaria is essential for illness management and elimination, particularly in areas with high transmission rates. The principal treatment for *Plasmodium falciparum* is Artemisinin-based Combination Therapies (ACTs), as endorsed by the World Health Organization (WHO). Artemisinin-based combination therapies (ACTs) integrate a rapid-acting artemisinin derivative with a prolonged-acting companion medication, such as lumefantrine or piperaquine, to swiftly diminish parasite burden and avert resistance. Commonly utilized ACTs comprise AL, ASAQ, DHA-PPQ, and ASMQ [4]. The emergence of resistance to artemisinin and its partner drugs, particularly in Southeast Asia and East Africa, is a significant danger to treatment efficacy [5]. To address this issue, Triple ACTs (TACTs),

which comprise artemisinin and two accompanying pharmaceuticals, are now being studied [6]. Chloroquine remains efficacious against *P. vivax*, *P. malariae*, and *P. ovale* in the absence of resistance. Artemisinin-based combination therapies (ACTs) are employed in resistant regions. Infections caused by *P. vivax* and *P. ovale* necessitate a 14-day primaquine treatment to eradicate dormant hepatic stages and avert relapse; however, screening for G6PD deficiency is essential prior to administration [7]. Severe malaria is treated with intravenous artesunate, which has predominantly supplanted quinine owing to its enhanced efficacy and safety. Fixed-dose combinations such as Coartem® and ASAQ Winthrop® [8] improve adherence and mitigate resistance. Current research is concentrated on next-generation pharmaceuticals such as ganaplacide [9] and cipargamin [10] to combat resistance and enhance therapeutic efficacy. Two malaria vaccines, RTS,S/AS01 [11], demonstrating 30–50% efficacy, and R21/Matrix-M [12], exhibiting ≥75% efficacy—have received WHO endorsement and are being deployed in endemic areas to improve malaria prevention.

Despite significant progress in malaria treatment, the emergence of resistance to current antimalarial drugs—including ACTs—underscores the urgent need to explore novel drug targets. Existing therapies largely focus on blood-stage parasites, leaving liver-stage and transmission-blocking targets underexplored. This review highlights the necessity of identifying and validating new molecular targets to enable the development of novel chemical entities (NCEs) with multi-stage efficacy and reduced risk of resistance.

2. Drugs targets of malaria

To facilitate the discovery of novel antimalarial compounds, it is essential to understand the key biological pathways and molecular targets within the Plasmodium parasite that can be exploited for therapeutic intervention. Some of the most important pathways and the associated drug targets have been discussed in the following sections.

2.1 Hemoglobin Digestion Pathway

The hemoglobin degradation route in *Plasmodium* is an essential metabolic mechanism that transpires during the intraerythrocytic phase of the parasite's life cycle. *Plasmodium* parasites within red blood cells internalize host hemoglobin into their acidic feeding vacuole by endocytosis. Hemoglobin is then destroyed by a series of proteases produced from the parasite, including aspartic proteases (plasmepsins), cysteine proteases (falcipains), and metalloproteases. This breakdown supplies the parasite with vital amino acids necessary for growth and protein synthesis. The digestive process, however, generates poisonous free heme (ferriprotoporphyrin IX), which the parasite detoxifies by crystallizing it into an inactive form known as hemozoin (malaria pigment). The interruption of this detoxification process results in the buildup of free heme, which is fatal to the parasite due to its oxidative and membrane-damaging properties. Literature suggests four unique mechanisms of hemoglobin acquisition in the malaria parasite *Plasmodium falciparum* [13]. Various antimalarials, including chloroquine, amodiaquine, and quinine, utilize this mechanism by disrupting heme detoxification, resulting in the demise of the parasite [14]. Resistance to chloroquine and analogous medications has arisen due to mutations in the *Plasmodium falciparum* chloroquine resistance transporter (PfCRT), underscoring the necessity for novel medicines that target this route. The hemoglobin digesting route is a recognized and appealing target for antimalarial drug development because of its essential nature and the presence of parasite-specific enzymes that are missing in humans. The important drug targets involved in this pathway include the *Plasmodium* falcipain-2, falcipain-3, and plasmepsins.

2.2 Mitochondrial Electron Transport Chain

The mitochondrial electron transport chain (ETC) in *Plasmodium* species is essential for the parasite's energy metabolism and pyrimidine biosynthesis. In contrast to humans, where mitochondria produce ATP via oxidative phosphorylation, *Plasmodium* predominantly utilizes anaerobic glycolysis for ATP synthesis. The mitochondrial ETC of the parasite is crucial for the regeneration of ubiquinone, necessary for the function of dihydroorotate dehydrogenase (DHODH), a vital enzyme in the de novo pyrimidine biosynthesis pathway [15]. Inhibiting DHODH or disrupting the mitochondrial electron transport chain effectively impedes parasite growth by depriving it of the nucleotides required for DNA and RNA synthesis [16]. Various antimalarial agents affect mitochondrial function. Atovaquone inhibits the cytochrome bc1 complex [17], obstructing electron transfer and resulting in the collapse of the mitochondrial membrane potential. Resistance to atovaquone develops rapidly due to point mutations in the cytochrome b gene, necessitating the creation of new mitochondrial ETC inhibitors [18]. DSM265 is a selective DHODH inhibitor that demonstrates efficacy against both the liver and blood stages of the parasite [19]. The mitochondrial ETC is a significant target for antimalarial drug discovery due to its divergence from the human equivalent and its essential function in nucleotide biosynthesis, particularly during the rapid replication of the parasite in the blood stage.

2.3 Apicoplast Pathway

The apicoplast is a non-photosynthetic organelle resembling a plastid, present in *Plasmodium* species and other apicomplexan parasites. Originating from a secondary endosymbiotic event, it is crucial for parasite survival and represents a distinctive and compelling target for antimalarial drug development, given its non-existence in humans [20-22]. The apicoplast participates in essential biosynthetic pathways, such as fatty acid synthesis (FAS-II) [23], isoprenoid precursor synthesis through the methylerythritol phosphate (MEP) pathway [24], and heme synthesis. These pathways supply critical metabolites

necessary for parasite development, especially during the liver and blood stages. Inhibition of apicoplast function disrupts the metabolism and replication of the parasite. Fosmidomycin targets the DOXP reductoisomerase enzyme in the MEP pathway, effectively inhibiting isoprenoid biosynthesis and parasite growth [25,26]. Antibiotics like clindamycin[27] and doxycycline[28] inhibit apicoplast protein translation and demonstrate a "delayed death" effect, resulting in the demise of treated parasites in the subsequent replication cycle. This delayed action indicates the apicoplast's function in synthesizing metabolites essential for the viability of daughter cells. The apicoplast, owing to its prokaryotic origins and vital metabolic roles, presents distinct molecular targets for the development of new antimalarial therapeutics. Utilizing these pathways may result in multi-stage antimalarials that exhibit a diminished likelihood of cross-resistance with current treatments.

2.4 Protein Synthesis

Protein synthesis in *Plasmodium* constitutes a crucial cellular mechanism that engages both cytoplasmic and organelle-specific ribosomes, thereby presenting a significant target for the development of antimalarial drugs. The parasite depends on the efficient translation of its proteins to facilitate rapid growth and replication during the blood stage. In contrast to human ribosomes, *Plasmodium* possesses unique ribosomal structures within its cytoplasm, mitochondrion, and apicoplast, presenting potential avenues for selective inhibition. Elongation factor 2 (eEF2) represents a significant target [29], as it is crucial for peptide chain elongation in the translation process. M5717 (formerly DDD107498) is a novel antimalarial compound that inhibits eEF2, resulting in a rapid cessation of protein synthesis and subsequent parasite mortality [30]. M5717 has shown efficacy against various life stages, including liver and transmission stages, and is currently in clinical development. Furthermore, antibiotics like doxycycline and clindamycin utilize variations in apicoplast ribosomes to interfere with protein translation [31], leading to a “delayed death” phenotype, in which parasites perish in the subsequent replication cycle. Protein synthesis is essential for parasite survival and displays notable structural differences from the human system, making it a validated and appealing target for antimalarial drug development. Inhibitors of this pathway possess the potential to provide both curative and transmission-blocking effects, rendering them significant assets in malaria elimination strategies.

2.5 Ion homeostasis and membrane transport

Ion homeostasis and membrane transport are critical physiological processes in *Plasmodium* parasites, vital for sustaining intracellular ionic balance, nutrient acquisition, and waste elimination during their development within host red blood cells. The parasite significantly alters the host erythrocyte and develops its own ion transport systems to facilitate growth and replication. PfATP4, a P-type ATPase, represents a significant drug target as it regulates sodium (Na^+) efflux from the cytosol of the parasite [32-34]. Disruption of PfATP4 function results in intracellular sodium accumulation, cellular swelling, and ultimately leads to parasite death. Compounds like cipargamin (KAE609) [10,35] and SJ733 [36,37] selectively inhibit PfATP4, exhibiting strong and rapid efficacy against asexual blood stages and early gametocytes. These agents exhibit a minimal propensity for cross-resistance with current therapies, rendering them appealing options in the context of increasing antimalarial resistance. Alongside PfATP4, other notable targets are PfNCR1 [38,39], which is implicated in lipid homeostasis and membrane integrity, and PfCRT, known for its involvement in drug resistance and ion exchange within the digestive vacuole. Ion regulation and membrane transport are crucial for parasite survival, making these systems significant targets for developing next-generation antimalarials with innovative mechanisms of action.

2.6 Gametocyte and Liver Stage Targets

Targeting the gametocyte and liver stages of *Plasmodium* is essential for malaria elimination, as these stages are responsible for transmission and early infection, respectively. Gametocytes, particularly the mature stages of *P. falciparum*, represent the sexual stages that are transmitted to mosquitoes during a blood meal. Pharmaceuticals targeting gametocytes can inhibit transmission and mitigate disease propagation. Primaquine and tafenoquine are the only approved drugs exhibiting transmission-blocking activity [40,41], specifically targeting mature gametocytes and the dormant liver forms (hypnozoites) of *P. vivax* and *P. ovale*. However, their use necessitates screening for G6PD deficiency because of the associated risk of hemolytic anemia [42]. The liver stage, especially the dormant hypnozoite form in *P. vivax*, presents a significant challenge in malaria control. Hypnozoites may remain in a dormant state for several weeks to months prior to reactivation, leading to relapses. Targeting liver-stage parasites may inhibit the establishment of blood-stage infections and prevent relapses. Tafenoquine's extended half-life provides a single-dose radical cure alternative; however, issues of resistance and safety persist. New drug candidates seek to inhibit parasite development at both stages, with certain compounds focusing on mitochondrial or apicoplast functions essential for early liver-stage growth. The development of multi-stage drugs capable of treating, preventing, and blocking transmission is essential for the eradication of malaria [43].

3. Dihydrofolate reductase as drug target in malaria

Dihydrofolate reductase (DHFR) facilitates the conversion of dihydrofolate (DHF) to tetrahydrofolate (THF), which serves as an essential cofactor in the synthesis of purines, thymidylate, and specific amino acids through the folate pathway [44]. In Plasmodium falciparum, dihydrofolate reductase (DHFR) is a component of a bifunctional enzyme known as PfDHFR-TS [45], which includes both dihydrofolate reductase and thymidylate synthase domains. The DHFR domain exhibits significant

differences from the human variant in critical active-site residues, facilitating selective inhibition by antifolate agents [46]. Inhibition of DHFR halts parasite DNA replication and cell division, which is crucial during the asexual blood stages characterized by rapid proliferation of the parasite. The available drugs and clinical pipeline drugs have been given in Figure 1 [47-50].

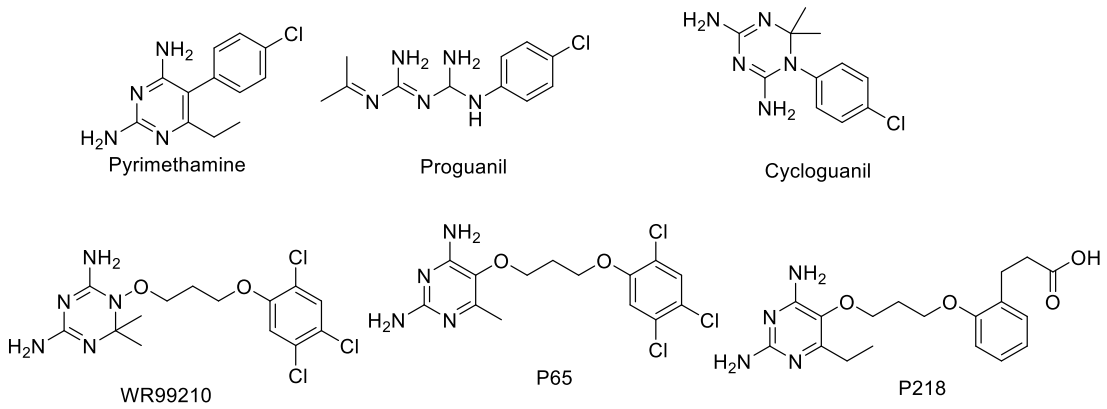

Figure 1. Marketed and clinical pipeline drugs as DHRF inhibitors.

X-ray structures of PvDHFR and mutant PfDHFR highlight the enzyme's adaptability; mutations alter loop conformations (e.g., Met20 and residues 51, 59, 108, 164), resulting in distortion of inhibitor binding [44]. The findings underscore two strategies for inhibitor design: first, adapting to resistance by creating compounds such as WR99210 that conform to the enzyme's "substrate envelope" to preserve affinity despite mutations; second, employing combination pressure modulation by co-administering inhibitors with varying residue interaction requirements to mitigate resistance evolution [51]. There have been several studies to inhibit the DHFR, and the most recent involved biphenyl-based inhibitors [52]. Biphenyl-based inhibitors constitute a significant category of compounds aimed at addressing resistance in Plasmodium falciparum dihydrofolate reductase (PfDHFR), especially concerning quadruple-mutant variants (N51I, C59R, S108N, I164L) that exhibit substantial resistance to traditional antifolates such as pyrimethamine. The molecules are organized around a biphenyl core connected to a 2,4-diaminopyrimidine moiety, facilitating robust binding within the PfDHFR active site, including in resistant variants. In a recent structure–activity relationship (SAR) study, compounds 4 and 8 were identified as potent leads. The compounds demonstrated Ki values of 0.29 nM and 0.25 nM, respectively, for mutant PfDHFR, signifying a strong enzyme-binding affinity. The in vitro antimalarial activity against the pyrimethamine-resistant *P. falciparum* strain TM4/8.2 was significant, with IC_{50} values of 0.77 µM and 0.72 µM, respectively. The observed submicromolar activities indicate significant inhibition of parasite growth at concentrations that are pharmacologically attainable. This class of inhibitors offered several advantages: they exhibit synthetic versatility, demonstrate strong selectivity for PfDHFR compared to human DHFR, and maintain potency against resistant strains. Current optimization efforts emphasize enhancing bioavailability, metabolic stability, and the potential for combination with other antimalarials. In other works, phthalide derivatives have been reported as DHFR inhibitors through the use of Computer-aided drug design studies [53]. Hybrid molecules (Figure 2) have also been reported as DHFR inhibitors which can tackle the issue of resistance [54].

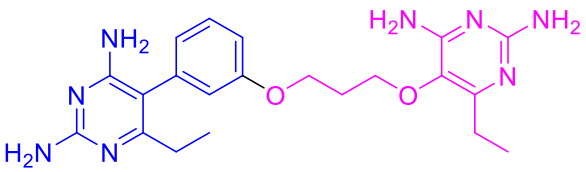

Figure 2. Hybrid molecule as DHFR inhibitor

Resistance to dihydrofolate reductase (DHFR) inhibitors in malaria, especially in *Plasmodium falciparum*, poses a substantial problem that has undermined the effectiveness of traditional antifolate treatments like pyrimethamine and proguanil. These pharmaceuticals operate by inhibiting the *Plasmodium* DHFR enzyme, essential for folate metabolism and DNA synthesis. Point mutations in the dhfr gene of *P. falciparum* diminish drug binding affinity while preserving the enzyme's catalytic function, resulting in treatment failure. The most thoroughly documented variants imparting resistance include S108N [55], the original mutation frequently observed, followed by N51I [56], C59R [57], and I164L [58]. The aggregation of these mutations leads to the emergence of the "quadruple mutant," which exhibits significant resistance to pyrimethamine, rendering it nearly ineffective in numerous endemic areas [59]. Analogous resistance pathways are identified in *P. vivax*, where mutations such as S117N/T and S58R diminish drug susceptibility [60]. These mutations modify the binding pocket of DHFR, obstructing effective contact with the inhibitor while permitting the enzyme to metabolize its normal substrate.

Structural analyses indicate that these mutations disrupt essential hydrogen bonds and introduce steric hindrance, resulting in diminished inhibitor potency. The swift proliferation of resistant DHFR alleles, especially in Southeast Asia and Africa, has considerably restricted the effectiveness of antifolate medications in the treatment and prevention of malaria. In response, novel inhibitors such as P218 and WR99210 have been formulated to maintain efficacy against resistant DHFR variations. These compounds are formulated utilizing structure-guided drug design methodologies to circumvent resistance mutations and preserve elevated binding affinity.

2. 4. CONCLUSIONS

The battle against malaria remains dependent on our comprehension of the parasite's biology and the tactical targeting of its critical pathways. This article examines various validated and emerging drug targets in *Plasmodium* species, including the hemoglobin digestion pathway, mitochondrial electron transport chain, apicoplast metabolism, protein synthesis, ion homeostasis, and the essential liver and gametocyte stages, emphasizing their therapeutic significance and roles in parasite survival. Dihydrofolate reductase (DHFR) is one of the most crucial and historically significant targets for antimalarial intervention. DHFR is pivotal in folate metabolism and nucleotide synthesis, both crucial for parasite DNA replication and survival throughout the erythrocytic phase. Pyrimethamine and cycloguanil have constituted the foundation of antimalarial treatment for decades. Nonetheless, extensive resistance resulting from point mutations in the *dhfr* gene has significantly undermined the efficacy of these medications. The rise of triple and quadruple DHFR mutations, particularly in Africa and Southeast Asia, highlights the parasite's ability for swift adaptation and the pressing necessity for novel remedies. Promisingly, next-generation DHFR inhibitors like P218 and WR99210 have demonstrated potential in surmounting resistance, attributed to advancements in structure-based drug design. These agents exhibit significant efficacy against mutant DHFR enzymes and present promise for application in both therapy and chemoprophylaxis. The ongoing advancement of selective, resistance-resilient DHFR inhibitors—particularly in conjunction with reasonable medication combinations—may reinstate the efficacy of this well-established target. Future tactics must emphasize combination therapy that integrate these novel DHFR inhibitors to postpone the onset of resistance. Combining DHFR inhibitors with medicines that target other critical pathways (such as mitochondrial, apicoplast, or transmission-blocking stages) can improve effectiveness and diminish the selection pressure on any singular target. Moreover, creating pan-species DHFR inhibitors that are effective against both *P. falciparum* and *P. vivax* will enhance therapeutic applicability.

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