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A Review on Cysteine Protease Inhibitors as Antimalarial Agents

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ABSTRACT

Malaria is a significant worldwide health challenge as evident from the latest WHO malaria report. The disease, predominantly caused by *Plasmodium falciparum*, has become progressively challenging to manage due to the establishment and dissemination of resistance to existing antimalarial medications, including chloroquine, sulfadoxine-pyrimethamine, and artemisinin-based therapies. The increasing resistance underscores the pressing necessity to identify and formulate new antimalarial agents with innovative modes of action. A promising research avenue focuses on targeting parasite-specific enzymes critical for survival, especially cysteine proteases. Falcipain-2, a papain-like cysteine protease, is crucial for hemoglobin breakdown throughout the intraerythrocytic phase of the parasite's life cycle. Recent research has revealed many falcipain-2 inhibitors, such as vinyl sulfones, artemisinin hybrids, quinoline-based compounds, and suramin analogues, many of which exhibit significant antiparasitic efficacy and the ability to surmount resistance. This review highlights the potential of falcipain-2 as a target for antimalarial drug development. Continued research in this domain may result in the identification of efficacious treatments for drug-resistant malaria.

Keywords: *Cysteine protease, Falcipain-2, Malaria, Resistance, Plasmodium falciparum*

1. INTRODUCTION

Malaria is a significant worldwide health threat, particularly impacting tropical and subtropical countries where warm weather facilitates the lifecycle of Plasmodium parasites and their mosquito vectors. The World Health Organization's World Malaria Report 2024 indicates that malaria continues to pose a substantial worldwide health burden, with an anticipated 263 million cases documented in 2023 which was an increase of almost 11 million over the preceding year. The global malaria incidence rate increased to 60.4 cases per 1,000 individuals at risk, up from 58.6 in 2022. Malaria-related fatalities were estimated at 597,000, a figure that has remained comparatively constant. The burden remains most severe in the WHO African Region, which constituted around 95% of all malaria fatalities and 94% of cases worldwide. The WHO South-East Asia Region has demonstrated significant advancement, with malaria occurrences decreasing from 22.8 million in 2000 to around 4 million in 2023. India, the predominant contributor in this region, documented a 9.6% reduction in cases from 2022 to 2023 [1].

Malaria is caused by five known species of *Plasmodium*, with *P. falciparum* and *P. vivax* being the most common [2]. *P. falciparum* is responsible for the most severe and fatal cases [3], especially in Africa, while *P. vivax* is more prevalent in Asia and Latin America and is capable of remaining dormant in the liver, leading to relapses [4]. The disease is transmitted through the bite of an infected female *Anopheles* mosquito. Once inside the human host, the parasite undergoes a complex lifecycle involving both asexual and sexual stages, making treatment and eradication especially challenging. The Plasmodium lifecycle involves several stages, namely, sporozoite, liver, blood, and gametocyte which play a crucial role in malaria transmission and pathogenesis [5]. Targeting these stages offers strategic points for therapeutic intervention. In the liver stage, sporozoites infect hepatocytes and mature into merozoites; drugs like primaquine act here to prevent progression to symptomatic blood stages and can help achieve radical cures by eliminating dormant forms in *P. vivax*. The blood stage is the most clinically significant, as merozoites infect red blood cells, multiply, and cause symptoms such as fever and

anaemia. Despite decades of control efforts, including the widespread use of insecticide-treated bed nets, indoor residual spraying, and antimalarial drugs, malaria remains a leading cause of death and illness worldwide.

1.1 Pathogenesis of malaria

The infection commences when sporozoites are introduced into the human bloodstream by a mosquito bite. The sporozoites migrate to the liver within minutes, enter hepatocytes, and undergo asexual reproduction, resulting in the formation of schizonts that contain thousands of merozoites. Upon maturation, the hepatocytes disintegrate, liberating merozoites into the circulatory system—a process that signifies the commencement of symptomatic infection. In *Plasmodium vivax* and *Plasmodium ovale*, certain sporozoites develop into dormant hypnozoites in the liver, which may reactivate after months or years, resulting in relapses. The merozoites subsequently infiltrate red blood cells (RBCs), commencing the erythrocytic phase [6]. Within red blood cells, they progress through a trophozoite stage, thereafter undergoing schizogony, resulting in the generation of new merozoites. Infected red blood cells burst, releasing merozoites that invade more red blood cells, leading to recurrent episodes of fever, chills, and other symptoms [7]. The coordinated destruction of red blood cells (RBCs) and the subsequent release of parasite poisons and cellular debris initiate a host immune response, marked by the secretion of pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6, which lead to fever and systemic inflammation [8]. *Plasmodium falciparum*, the most pathogenic species, can induce significant problems owing to its capacity to bind to vascular endothelium via PfEMP1 proteins expressed on infected red blood cells. Cytoadherence results in the sequestration of infected red blood cells inside the microvasculature, particularly in organs such as the brain, lungs, and placenta, leading to consequences including cerebral malaria [9], acute respiratory distress syndrome (ARDS), and placental malaria. Microvascular blockage and inflammation lead to hypoxia and metabolic abnormalities, such as lactic acidosis and hypoglycemia. Severe anemia arises from the hemolysis of both infected and uninfected red blood cells, as well as the inhibition of erythropoiesis. Moreover, the splenic clearance of parasitized cells adds to anemia and splenomegaly. In regions with high transmission rates, recurrent infections in children can result in chronic anemia and hindered development [10].

1.3 Resistance in malaria

Resistance in malaria, namely *Plasmodium falciparum*, constitutes a significant challenge to worldwide malaria control and eradication initiatives. The term "resistance" in malaria denotes the parasite's capacity to endure and proliferate despite the application of antimalarial medications at therapeutic levels. Resistance has developed against almost all categories of antimalarial medications, primarily due to genetic alterations, selective drug pressure, and incorrect usage of these treatments. Chloroquine was historically the foundation of malaria treatment. Chloroquine resistance arose in the 1950s in Southeast Asia and South America, subsequently disseminating to Africa. The resistance was mainly ascribed to changes in the Pfcrt gene, which modified drug accumulation in the parasite's digestive vacuole [11]. Resistance to sulfadoxine-pyrimethamine subsequently emerged, driven by point mutations in the DHFR (dihydrofolate reductase) and dihydropteroate synthase genes, which are essential enzymes in the folate production pathway of the parasite [12]. The primary treatment, artemisinin-based combination treatments (ACTs), is increasingly jeopardized by prolonged parasite clearance durations. Resistance to artemisinin has been associated with mutations in the Pfkelch13 gene, initially identified in the Greater Mekong Subregion of Southeast Asia [13]. While these mutations are not yet prevalent in Africa, increasing evidence suggests developing patterns of resistance [14], highlighting the necessity for ongoing genetic surveillance and therapeutic efficacy assessment. Resistance development is exacerbated by variables like inferior or counterfeit medications, inadequate treatment regimens, and the use of monotherapies. Moreover, resistance of mosquito vectors to insecticides—particularly pyrethroids utilized in insecticide-treated nets (ITNs)—complicates vector control initiatives, as it diminishes their efficacy [15]. To address resistance, the WHO underscores the importance of combination therapy, pesticide rotation, and the enhancement of pharmacovigilance and molecular surveillance initiatives. The advancement of novel antimalarial drugs, including next-generation endoperoxides (e.g., OZ439) [16] and transmission-blocking compounds, is essential for maintaining long-term control.

1.4 Drug targets of malaria

Malaria medication discovery emphasizes the identification of critical molecular targets within *Plasmodium* parasites, especially *Plasmodium falciparum*, the most lethal variety. The parasite's intricate life cycle, encompassing both hepatic and erythrocytic phases in humans, presents multiple susceptible targets for therapeutic intervention. Malaria drug targets are often categorized into those that influence metabolic pathways, organelles, and particular parasite proteins essential for survival and replication. Dihydrofolate reductase (DHFR) is a well-established target, an enzyme integral to folate biosynthesis, which is crucial for DNA synthesis. Antifolate agents, including pyrimethamine and proguanil, block dihydrofolate reductase (DHFR), hence obstructing nucleotide synthesis [17]. The emergence of resistance to these medications has prompted the creation of novel DHFR inhibitors that exhibit enhanced affinity for mutant variants. Heme detoxification is an essential step addressed by antimalarials. During hemoglobin catabolism in the food vacuole, toxic heme is liberated and subsequently detoxified into hemozoin. Drugs such as chloroquine function by obstructing this polymerization, resulting in the demise of the parasite [18]. Despite the extensive resistance that has curtailed the utilization of chloroquine, the food vacuole continues to be a crucial target. Inhibitors of the mitochondrial electron transport chain have

become increasingly significant. Atovaquone, an element of the atovaquone-proguanil combination, inhibits the cytochrome bc1 complex in the parasite's mitochondrion, hence interrupting ATP and pyrimidine production [19]. The swift emergence of resistance during monotherapy underscores the need of combination medicines. The apicoplast, a non-photosynthetic plastid found in Plasmodium, is another distinctive target. Antibiotics such as doxycycline and clindamycin interfere with apicoplast protein synthesis, resulting in a "delayed death" effect [20]. The prokaryotic characteristics of the organelle facilitate selective targeting while preserving human cells' integrity. PfATP4, a P-type ATPase that regulates Na^+ efflux, has recently emerged as a viable target. Compounds like cipargamin (KAE609) interfere with ion homeostasis, resulting in parasite mortality [21]. Furthermore, research is focused on proteases that facilitate hemoglobin breakdown and calcium-dependent kinases (CDPKs) that govern invasion and egress. Pharmaceutical development is progressively concentrating on multi-stage inhibitors that are effective in both the blood and liver phases, as well as transmission-blocking medicines aimed at gametocytes [22,23].

2. CYSTEINE PROTEASES AS MALARIA TARGET

Cysteine proteases are critical enzymes in *Plasmodium falciparum* and have surfaced as potential targets for antimalarial pharmacotherapy. The falcipain family of enzymes is essential for hemoglobin breakdown, parasite proliferation, and erythrocyte lysis. Their essential role and unique structural characteristics, in contrast to human cysteine proteases, present options for selective inhibition.

2.1 Role of Cysteine Proteases

Cysteine proteases in P. falciparum are papain-like enzymes primarily engaged in hemoglobin breakdown, a vital metabolic step during the intraerythrocytic phase of the parasite. Hemoglobin is absorbed into the parasite's feeding vacuole, where it is systematically degraded by several proteases, including aspartic proteases (plasmepsins), metalloproteases, and cysteine proteases. Falcipain-2 and falcipain-3 are the primary cysteine proteases involved in this pathway [24]. The breakdown of hemoglobin supplies essential amino acids for parasite proliferation and mitigates osmotic lysis caused by the buildup of undigested globin. Interruption of this process consequently results in starvation and the accumulation of harmful intermediates, such as free heme, culminating in the demise of the parasite [25].

2.2 Cysteine Proteases as validated target

Numerous lines of evidence substantiate falcipains as confirmed therapeutic targets. Genetic knockdown studies have shown that the deletion of falcipain-2 markedly hinders hemoglobin breakdown and parasite proliferation. Likewise, falcipain-3, despite being somewhat redundant, plays a crucial role in the survival of the parasite within erythrocytes [26,27]. These enzymes are also produced during the early trophozoite stages, a crucial phase of active hemoglobin digestion, hence underscoring their therapeutic significance. Falcipains include a highly conserved catalytic dyad (Cys-His) [28] in their active site, rendering them vulnerable to covalent inhibitors. A diverse array of inhibitor classes has been examined, encompassing peptidyl vinyl sulfones, epoxides, aldehydes, and nitrile-based compounds [29,30]. These compounds generally operate by irreversibly attaching to the active site cysteine, hence inhibiting proteolytic activity. Numerous strong inhibitors, including E64 and vinyl sulfone compounds, exhibit nanomolar efficacy against falcipain-2 and falcipain-3, demonstrating effectiveness in vitro and in murine malaria models. A primary hurdle in the development of cysteine protease inhibitors has been attaining selectivity over human cathepsins to prevent off-target effects. Structure-based drug design, guided by high-resolution X-ray crystallography and computer modeling, has facilitated the identification of distinctive structural characteristics in falcipains that can be utilized for specificity [31].

2.3 Resistance for Cysteine Proteases

In contrast to the well-studied resistance mechanisms for medications such as chloroquine or artemisinin, resistance to cysteine protease inhibitors is comparatively under investigated. Nonetheless, *P. falciparum* has exhibited the ability to acquire resistance via gene mutations, overexpression, or compensating mechanisms. Resistance to falcipain inhibitors generated in the laboratory has been documented in prolonged drug pressure experiments. Inhibition of falcipain-2 using vinyl sulfone-based inhibitors resulted in the emergence of parasite strains that overexpressed alternative proteases, such as falcipain-3 or plasmepsins, thus partially compensating for the reduced enzymatic activity [25]. A further method of possible resistance entails point mutations in the active site or substrate-binding cleft of falcipains, which may diminish inhibitor binding without substantially impacting enzyme activity. Molecular modeling studies have indicated that these mutations may modify the conformational flexibility or steric characteristics of the active site, resulting in diminished drug affinity while maintaining catalytic activity [32]. These alterations, while not yet clinically validated, indicate the parasite's ability to adapt. Gene amplification of falcipain genes under pharmacological pressure has been documented, resulting in heightened expression and diminished effectiveness of reversible inhibitors [33]. To alleviate resistance, combination therapy utilizing cysteine protease inhibitors alongside other antimalarial agents—such as artemisinin or apicoplast-targeting drugs—have demonstrated synergistic effects and diminished selection pressure [34]. Furthermore, the creation of inhibitors that concurrently target numerous proteases or critical pathways is being investigated to mitigate the possibility of resistance evolution. Computer-aided drug design including docking-based studies offer a promising approach to overcoming these

resistance mechanisms by identifying new compounds that can either avoid or bypass the mutations responsible for drug resistance. For example, computational docking can simulate how specific drugs interact with mutant forms of *Plasmodium* proteins, predicting whether the drug can still bind effectively despite the presence of mutations. This allows researchers to identify potential compounds that could bind with high affinity to resistant targets, offering an alternative treatment strategy. Furthermore, docking studies can help identify compounds that target new proteins or metabolic pathways that are less likely to develop resistance. Another promising strategy is the design of combination therapies, where two or more compounds with different mechanisms of action are used together to reduce the likelihood of resistance development. By leveraging computational drug discovery methods, it is possible to design novel drugs or combinations that can circumvent existing resistance mechanisms, improving the efficacy of malaria treatment in the long term.

2.4 Falcipain-2

Falcipain-2 is a principal cysteine protease of *Plasmodium falciparum*, essential for the parasite's intraerythrocytic phase. It primarily facilitates the decomposition of host hemoglobin within the feeding vacuole, supplying critical amino acids for the growth and development of the parasite. Falcipain-2 commences hemoglobin degradation by cleaving globin into smaller peptides, which are then processed by other proteases [35]. This function is essential for the survival and growth of parasites within red blood cells. Gene disruption experiments have validated the necessity of falcipain-2 [36]. Parasites devoid of falcipain-2 demonstrate markedly diminished hemoglobin hydrolysis and compromised development, underscoring its essential role. Furthermore, falcipain-2 plays a role in the lysis of the host erythrocyte membrane, enabling parasite escape and the invasion of new red blood cells. Falcipain-2's distinctive structural and functional characteristics, unlike those of human proteases, render it a compelling target for pharmacological intervention [37]. Selective inhibitors of falcipain-2 exhibit significant antimalarial efficacy both in vitro and in vivo, establishing it as a viable target for the development of new antimalarial therapeutics. Consequently, falcipain-2 becomes a crucial enzymatic target in the pursuit of next-generation antimalarial treatments. Resistance to FP-2 inhibitors may arise due to mutations in the FP-2 enzyme that reduce drug binding. However, the **low likelihood of cross-resistance** with other antimalarial drugs (e.g., artemisinin or chloroquine) makes FP-2 inhibitors an attractive addition to existing antimalarial regimens. To prevent resistance, FP-2 inhibitors can be used in **combination therapies,** which lower the likelihood of the parasite developing resistance to multiple drug classes simultaneously. Molecules identified through different techniques including CADD studies play a pivotal role in targeting cysteine proteases in *Plasmodium* parasites that are involved in the parasite's growth and replication cycle.

3. CYSTEINE PROTEASE (FALCIPAIN-2) INHIBITORS

Aratikatla et al. developed artemisinin–peptidyl vinyl phosphonate hybrid molecules to address drug-resistant malaria, demonstrating superior efficacy compared to artemisinin alone against chloroquine- and multidrug-resistant strains of *Plasmodium falciparum*, achieving picomolar EC_{50} values. These hybrids demonstrated enhanced efficacy against artemisinin-resistant ring-stage parasites, resulting in complete parasite clearance in a *P. berghei* mouse model. Mechanistic studies demonstrated a dual action: the inhibition of falcipain-2, a cysteine protease critical for hemoglobin degradation, and the obstruction of hemozoin formation. These compounds demonstrate synergistic efficacy by targeting multiple steps in the parasite's metabolic pathway, suggesting their potential as effective antimalarials to mitigate resistance development [38].

In another research, Singh et al. identified ten quinoline-triazole compounds that bind to FP-2 and inhibit its activity at micromolar concentrations, thereby suppressing parasite growth. Two compounds exhibited IC_{50} values of 16.16 µM and 25.64 µM, respectively, and inhibited parasite development at the trophozoite stage with EC_{50} values of 21.89 µM and 49.88 µM. Both induced food vacuole abnormalities analogous to those caused by the established FP2 inhibitor E-64. These compounds serve as promising candidates for the development of FP2-targeted antimalarial agents [39].

Shenai et al. investigated 39 compounds, including vinyl sulfone, sulfonate ester, and sulfonamide, for their inhibitory effects on falcipain-2, falcipain-3, and the growth of *Plasmodium falciparum*. Numerous compounds demonstrated significant dual inhibition of both proteases, with optimal antimalarial activity identified in phenyl vinyl derivatives featuring P2 leucine moieties. These compounds demonstrated effective inhibition of parasite development at low nanomolar concentrations. The research identified specific structure-activity relationships related to enzyme inhibition and antiparasitic effects, underscoring the potential of peptidyl vinyl sulfones as effective antimalarial agents aimed at falcipains that play a role in hemoglobin degradation during the blood stage of the parasite [40].

Marques et al. showed that suramin, a polysulfonated naphthylurea, inhibited falcipain-2 at nanomolar concentrations via a noncompetitive allosteric mechanism. It successfully inhibited both synthetic and natural substrates, including hemoglobin. Modified suramin analogues, such as smaller derivatives and methyl-substituted forms, demonstrated significant inhibition. The findings underscore the suramin family as potential candidates for the development of falcipain-2-targeted antimalarials with a novel mechanism of action, presenting a new approach to address drug-resistant *Plasmodium falciparum* infections [41].

Huang et al. have designed and synthesized a series of first-generation small molecular dual inhibitors of FP-2 and DHFR, derived from a lead chemical that was fortuitously found during screening for FP-2 inhibitors in our laboratory. Six

compounds exhibited enhanced dual inhibitory activity against FP-2 (IC_{50}=2.7-13.2μM) and DHFR (IC_{50}=1.8-19.8μM), with one of the compounds demonstrating about 8-fold and 6-fold increases in inhibitory efficacy against FP-2 and DHFR, respectively, compared to the lead drug. The same compound demonstrated moderate *in vivo* antimalarial activity in a dose-dependent manner, with its safety and survival rate marginally superior to that of the positive control medication. The preliminary SAR was acquired, while molecular modeling results supplied essential structural information to preserve dual inhibitory activity, aiding in the design of future dual inhibitors.

4. CONCLUSIONS

Malaria continues to be a significant worldwide health concern, chiefly because of the ongoing evolution of drug-resistant forms of *Plasmodium falciparum*. Despite considerable progress in comprehending the parasite's biology and treatment weaknesses, resistance to traditional antimalarials like chloroquine, sulfadoxine-pyrimethamine, and artemisinin highlights the pressing necessity for innovative drug targets and modes of action. Cysteine proteases, especially the falcipain family, have emerged as interesting molecular targets due to their essential role in hemoglobin breakdown throughout the erythrocytic stage of the parasite. Falcipain-2 (FP-2) has been thoroughly verified as an essential enzyme for the parasite's survival. The distinctive structure, lacking in human host enzymes, facilitates the creation of selective inhibitors with reduced off-target toxicity. Recent advancements in medicinal chemistry have resulted in the development of new FP-2 inhibitors, including as vinyl sulfones, artemisinin hybrids, quinoline-triazoles, and suramin analogues, many of which have nanomolar potency and multi-stage antimalarial efficacy. Dual inhibitors that target FP-2 and additional enzymes, such as DHFR, augment therapeutic efficacy by diminishing the probability of resistance. In conclusion, targeting falcipain-2 constitutes a feasible and novel approach for next-generation antimalarial treatment. Ongoing investigation into its inhibitors offers potential for surmounting existing therapy constraints and addressing drug-resistant malaria.

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