Review Article

Recent advances in identification of potential drug targets and development of novel drugs in parasitic diseases. Part IV. *Plasmodium* spp.

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ABSTRACT

In the last two decades, transporters attracted much attention in identification of potential drug targets against intracellular protozoa. Several membrane molecules exhibit essential roles in trafficking pathways for nutrients, essential enzymes and virulence factors. In this context, *P. falciparum* possesses a complex of genomic plasticity-encoding transporters with high potentiality for aneuploidy, and gene expression modulation in response to drug exposure. Therefore, it is able to undergo gene mutations in enzymes controlling drug uptake, and evade host immune response by antigenic variations as well. The genetic mechanism of antimalarial drug resistance arises early with monotherapy using fast-acting drugs or a single targeting drug. Accordingly, combined therapy acting on multiple targets would decrease the emergence of drug resistance. Evolutionary technology on genetic approach enabled researchers to identify and propose novel *Plasmodium* drug targets. The main objective of this part is to review potential drug targets for apicomplexans and discuss recent approaches in identifying *Plasmodium* targets, as well as advances in the design and development of novel antimalarial drugs.

Keywords: apicoplast; drug targets; egress; intracellular protozoa; mitochondria; novel drugs; *Plasmodium* spp.

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Abbreviations: AMA1: Apical membrane antigen 1; **ART:** Artemisinin; **CP:** Cysteine protease; **eIF2:** Eukaryote initiation factor 2; **ER:** Endoplasmic reticulum; **HAT:** Histone acetyltransferase; **HDAC:** Histone deacetyltransferase; **HSP:** Heat shock protein; **HTS:** high-throughput screening; **PAT:** Palmitoyl-acyltransferase; **PIK:** Phosphoinositide kinase; **PK:** Protein kinase; **PKA:** Cyclic AMP-dependent PK; **PTM:** Post-translational modification; **PV:** Parasitophorous vacuole; **MSP1:** Merozoite surface protein 1; **NMT:** N-myristoyl transferase; **RON2:** Rhoptry neck protein 2; **SERCA:** Sarco/endoplasmic reticulum calcium ATPase; **TK:** Tyrosine kinase; **Topo:** Topoisomerase; **Ub:** Ubiquitin; **Ubl:** Ubiquitin-like.

INTRODUCTION

Several approaches were utilized to identify potential drug targets aiming to design and develop novel drugs in treatment of protozoal diseases, e.g., bioinformatics analyses, functional genomic studies, and phenotypic screening. Several reviews were published to discuss the recent advances in essential molecules required for survival, growth, replication, and virulence of protozoa causing tropical diseases with high morbidity and mortality risks worldwide. Among them, heat shock protein 90 (HSP90) isoforms[1], plasma membrane ion channels and transporters[2], protein lipidation enzymes[3], Sarco/endoplasmic reticulum calcium ATPase (SERCA) transporter[4], ubiquitin (Ub) and ubiquitin-like (Ubl) modifiers[5], and host mechanistic target of rapamycin (mTOR)[6]. The latter is linked with phosphoinositide 3-kinase (PI3K) that controls cell signaling and functions employed in several cellular processes. Identification of a specific parasitic interaction and its essential role in pathogenicity and immunopathology, or survival and proliferation will lead to recognition of potential drug targets[6].

American scientists[7] discussed the apicoplast role in spite of absence of photosynthesis requirement. Isopentenyl pyrophosphate biosynthesis and its metabolic 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway was suggested. This was evidenced by inhibition of *Plasmodium* growth *in vitro*, and *in vivo* using Fosmidomycin, a specific DOXP inhibitor[7]. A Swiss review was published recently discussing the role of apicoplast transporters and their potentiality as drug targets in *Plasmodium* spp. The parasitophorous vacuole (PV) membrane works as a molecular sieve for proteins and metabolites transport and distribution *via* the apicoplast to the cytosol, endoplasmic reticulum (ER), Golgi apparatus, and mitochondrion. Metabolic pathways within the apicoplast, targeted by several antibiotics such as clindamycin and doxycycline, confirmed its drug target potentiality[8]. On the other hand, ferredoxin/ferredoxin-$NADP^+$ reductase (Fd/FNR) system, one of the redox systems within the apicoplast, was identified essential for *Plasmodium* survival. A recent study demonstrated that both molecules (Fd and FNR) are individually dispensable in erythrocytic stages,

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but the Fd/FNR system is essential for survival, i.e., simultaneous knocking down of both encoding genes was lethal. It was hypothesized that the Fd/FNR system provides reducing power to several iron-sulfur cluster-dependent proteins in the apicoplast. Due to Fd/FNR absence in human, the study proposed it an attractive drug target[9].

To persist longer in their host, apicomplexans developed different mechanisms to tolerate unfavorable stress conditions utilizing development of latent life cycle stages. In this context, *Plasmodium* spp. possess a well-developed capacity to sense environmental changes and initiate an integrated stress response (ISR). The latter provides a rapid adaptive change, i.e., progression to a latent state through gene regulation switching from proliferative to dormant states. In their review[10], the scientitsts defined ISR as a post-transcriptional mechanism of gene regulation widely conserved in eukaryotes. They claimed that ISR is mediated by stress-induced phosphorylation of α subunit of eukaryote initiation factor 2 (eIF2) to transiently repress protein synthesis. The reviewers discussed mechanisms involved in relief of translational repression and conversion from latency into proliferative states causing malaria relapse. In the latter, few hepatic merozoites of *P. vivax*, and *P. ovale* have the ability to differentiate into quiescent stages (hypnozoites) in the liver serving as reservoir for malaria for years[10].

Mitochondria, distinctive double-membrane organelles, involved in several metabolic pathways, e.g., energy conversion, respiration, thermogenesis, calcium homeostasis, cell signaling, and apoptosis. Except for *Cryptosporidium* spp., apicomplexan mitochondria are characterized by massive gene transcription and translation with sequence rearrangements and functional reductions. Mitochondrial genomes and mitogenomics revealed the loss of several subunits of conserved mitochondrial ribosomes, and genes encoding transfer RNAs. It was suggested that this functional reduction was saved for apicoplast proteins. After comparing mitochondrial functions in apicomplexans and the evolutionarily related alveolates, the reviewers proposed cyclooxygenases (Cox 1 and Cox 3), and cytochrome bc1 complex potential drug targets. While the former are enzymes for thromboxane formation, the latter is a subunit of ubiquinol-cytochrome c reductase complex. Except for cox 3 gene, all other genes are conserved among all apicomplexans[11].

Drug targets in *Plasmodium* spp.

The Malaria Drug Accelerator (MalDA) (https://www.malariada.org/), a public-private partnership formed in 2012, constitutes 15 leading scientific laboratories expertises. Its primary objective is to improve and accelerate development of novel antimalarial drugs by identifying essential druggable targets. In 2021, two reviews were published. The first[12] discussed advantages and limitations of the most common approaches, such as phenotypic screening (chemogenomics) and target-based screening (genome-wide associated studies). For validation and prior to clinical trials, both approaches require additional steps regarding cytotoxicity, structure-activity relations, mutations identification in the genes encoding drug uptake, and druggability, i.e. pharmacokinetics and pharmacodynamics. With the concept that gene mutations may arise during long term cultures, the reviewers validated using CRISPR/Cas9 technology to achieve drug validation. The reviewers also discussed other approches utilized in identification of novel drug targets such as metabolomics, chemical proteomics, and reverse genetic approach[12]. The second review[13] discussed process and criteria for target prioritization with the aim of identifying potential targets already processed in drug discovery programs or those possessing insufficient validation data for further progress. The reviewers classified plasmodial drug targets into four categories, high priority targets, those under considerations, new emerging targets, and deprioritized targets. They also updated progress carried out for the first two categories, and highlighted specific requirements to validate or invalidate the last two categories. It is worth mentioning that *Plasmodium* monoacylglycerol lipase was the only new emerging target that was discussed regarding its chemical, structural, genetic validation, cytotoxicity and druggability, as well as its resistance potential[13].

During the last years (2019-2022), several studies[14-21] were published discussing new approaches to identify novel drug targets for *Plasmodium* spp. Stanway *et al*.[14] hypothesized that identifying genes encoding the high metabolic activities behind rapid development of a single sporozoite (invading the liver) into thousands of daughter-merozoites (invading RBCs) should reveal several potential drug targets. Utilizing *in silico* tools, the investigators analyzed the screening in context of genomic, transcriptomic, and metabolomics data of *P. berghei* hepatic stages metabolism. Compared to erythrocytic stages, seven essential metabolic processes were identified: type II fatty acid synthesis, translational elongation factor, and metabolism of five components: heme, amino sugar, tricarboxylic acid, lipoate, and shikimate[14]. Tricarboxylic acid is an organic carboxylic acid, and the best-known example is citric acid, lipoate is an anti-oxidant, and shikimate is a metabolic pathway for biosynthesis of folates and aromatic amino acids.

Incorporation of platinum group metals such as iridium into bioactive ligands enhanced their antimalarial activity with neglected toxicity towards mammalian cells[15]. Nanotechnology was also utilized in development of a delivery system for drugs that exhibited satisfactory antimalarial activity. A series of azacarbazoles were synthesized and evaluated against *P. falciparum* chloroquine-sensitive and -resistant strains. The led compound (No. 3) was nanoemulsified, and analyzed for its pharmacokinetics properties, stability, and cytotoxicity to *Caco-2* cell lines.

Compared to non-encapsulated formulation, intra-gastric administration of encapsulated nanoemulsion to experimentally infected mice exhibited 2.8, and 4.2 more activity against both strains, respectively[16].

A Pakistani study retrieved drug targets proposed in recent studies and utilized comparative proteomics analyses to scan them against human orthologues. Results revealed six *P. falciparum* novel drug targets that had not been indexed in the DrugBank. Using *in silico*, molecular docking studies, and pharmacophore modelling-based virtual screening, they proposed diadenosine tetraphosphate hydrolase (DDTPH) a potential drug target. Since DDTPH is a membranous enzyme in the infected RBCs, it regulates the levels of signaling molecules, i.e., hydrolyzes DDTPH to ATP and AMP. This hydrolysis mediates and enhances *P. falciparum* cellular communication and function[17]. Similarly, utilizing drug repositioning approach and *in silico* methods, a South African study screened ~800 DrugBank compounds against 36 *P. falciparum* drug targets. The top leading compounds were selected for further molecular docking, and phenotypic *in vitro* studies. Results revealed four active compounds (Fingolimod, Abiraterone, Prazosin, and Terazosin), and the first two drugs showed relevant IC_{50} values (2.21, and 3.37 μM)[18].

Virtual screening approach was also used to identify compounds with inhibitory activity against *Plasmodium* actin I since it is expressed in all erythrocytic stages and plays several functions including motility, transport, and division. The investigators succeeded to select five potential compounds, and recommended further *in vitro* and *in vivo* studies to validate proposing actin I a promising drug target[19]. In Thailand, a recent study evaluated a mitochondria-penetrating peptide (MPP) termed $(Fxr)_3$ as a novel antimalarial drug. The investigators observed $(Fxr)_3$ intensive localization in mitochondria of merozoites that entered the infected RBCs without membrane disruption with subsequent death of erythrocytic stages. Compared to atovaquone, $(Fxr)_3$ exhibited significant lethal effects on both freshly isolated, and laboratory cultured strains as well as a chloroquine-resistant strain, with minimum toxicity towards various mammalian cells[20].

Since glycolysis is the sole energy-yielding process in *Plasmodium* spp., an Indian study proposed phosphoglycerate mutase (PGM), a glycolytic enzyme, a potential drug target. By conditional knockdown, the investigators confirmed *Pf*PGM essential role in intra-erythrocytic stages development, and survival. The study characterized *Pf*PGM1 structure that revealed its existence in tetramer form, and in-depth oligomerization analysis showed that this tetramerization contributed to the optimal enzymatic function *in vitro*, and *in vivo*. Understanding PGM enzymatic mechanism of action might provided a promising strategy to design and develop a specific inhibitor as novel antimalarial drug[21].

Similarly, other reviews were recently published discussing usefulness of new scaffolds[22,23], proposals of new approaches[24], potential plasmodial drug targets[25,26], and novel antimalarial drugs in clinical trials[27]. It was reported that use of new chemical scaffolds, utilizing molecular hybridization, exhibited slightly better antimalarial activity than using the antimalarial drug without scaffolds[22]. On the other hand, multiple available drugs targeting hemozoin formation or ATP4 transporter were produced from natural products that have an indole scaffold. An Ethiopian report reviewed several indole scaffolds that showed potent anti-plasmodial properties. The reviewers recommended synthesis of novel indole analogues holding two pharmacophore units to bear both hemozoin, and ATP4 inhibitors. It was suggested that such a multi-target drug therapy would decrease emerging drug resistant strains[23].

In vitro evolution and whole genome analysis was proposed. It is a new approach with three steps: 1) *in vitro* exposure of *Plasmodium* strain to sub-lethal doses of a certain compound until reaching increased IC_{50} that indicates drug resistance; 2) whole genome sequencing of resistant parasites to be compared to the non-exposed parasites; 3) detected genetic changes would pinpoint genes encoding drug targets[24]. In addition, molecules involved in *Plasmodium* epigenetic processes and mitochondrial metabolic pathways were proposed promising drug targets. Koumpoura and her colleagues[25] reviewed a recent evolution conducted to synthesize selective efficient inhibitors targeting three essential molecules. They included DNA methyltransferases, one of post-translational modifications (PTMs) involved in epigenetic processes, and cytochrome bc1 complex, and dihydroorotate dehydrogenase (DHODH) involved in essential mitochondrial metabolic pathways. Another recent review proposed hematin-artemisinin (ART) adducts novel antimalarial drugs. Action of ART, an endoperoxide drug is activated by bio-reductive reaction by heme (FeII-PPIX) leading to radical oxygen species (ROS), and inhibition of heme detoxification. Besides, ROS cause radical-induced protein alkylation with subsequent inactivation of several biological processes essential for *Plasmodium* survival and growth. The reviewers reported that relationship between endoperoxide drugs and heme relied on two concepts, *Plasmodium* redox homeostasis and heme detoxification. Since heme alkylation alters the heme/hematin ratio of the iron porphyrin IX complex, it induces an imbalance in *Plasmodium* redox hemostasis of iron species. Therefore, metalloporphyrins, hematin-adducts, irreversibly bind with β-hematin to initiate heme detoxification inhibition. In ART resistance, the reviewers recommended combined treatment of hematin adducts with ART since the highly lipophilic lactone from ART contributes to overcome the poor lipophilicity of metalloporphyrins[26]. Shibeshi *et al.*[27] summarized the results of studies that proposed *Plasmodium* glucose transporter, protein kinases (PKs), and proteases potential drug targets. The reviewers also discussed several metabolic pathways involved

in the digestive vacuole, mitochondria and apicoplast that render them drug targets. Currently in clinical trials, they listed 13 drugs inhibiting the growth of the erythrocytic stages. Among them were Cipargamin, Fosmidomycin, Albitiazolium, DSM265, KAF156, MMV048, and M5717[27].

[I] Growth and replication

1. Nucleic acids synthesis (salvage pathways)

In a short communication, Brazilian investigators identified an essential nucleotide hydrolase; S-adenosyl-L-homocysteine hydrolase (SAHH) in *Plasmodium* spp. The enzyme is involved in purine salvage pathway, with no homolog in human RBCs, hence a potential drug target. They investigated the efficacy of a SAHH inhibitor *in vitro*, and observed significant reduction in development of erythrocytic stages, and number of reinvaded RBCs[28]. A recent interesting approach was utilized by a study conducted to evaluate using subversive substrates instead of selective inhibitors of enzymes and/or transmembrane transporters involved in purine salvage pathway. Since allopurinol, a nucleoside derivative, exhibited *in vitro* anti-*T. cruzi* activity[29], Spanish investigators synthesized 81 purine derivatives and pyrimidine analogs with benzoxy or aromatic or triazolylmethoxy groups at positions 6, 8, and 9 of the purine ring. The investigators conducted primary phenotypic screening at three fixed concentrations that kept *Plasmodium* growth <30%, followed by cytotoxicity assays. Compounds 33 and 76 with triazolylmethoxy group at C6 were the most potent against *Plasmodium* with no cytotoxicity to mammalian cell lines. An *in silico* docking study proved that *Plasmodium* hypoxanthine guanine phosphoribosyl-transferase (HGPRT) a potential target. It was concluded that purine-based chemotypes represented a new strategy for further optimization to be used as novel antimalarial drug[30].

As previously reviewed[31], adenosine deaminase (ADA), purine nucleoside phosphorylase (PNP), and HGXPRT are essential enzymes in purine salvage pathway. Besides, inosine monophosphate dehydrogenase (IMPDH) obtained from *Staphylococcus aureus*, an enzyme producing NADH over time to monitor the activity of purine pathway, gained much attention for treatment of multi-drug resistant bacterial and fungal infections, i.e., a valuable drug target. Since traditional high-throughput screening (HTS) approach focuses on a single target, American investigators performed *in vitro* assay for the simultaneous HTS of potential inhibitors against *Pf*ADA, *Pf*PNP, *Pf*HGXPRT, and *Sa*IMPDH. After expression in *Escherichia coli*, the recombinant enzymes were used to simulate purine pathway *in vitro* to be screened against online library. Results revealed identification of five compounds with inhibitory efficacy against all investigated enzymes. The investigators validated this approach to be effectively utilized for development of novel drugs with inhibitory activity against multiple targets[32].

On the other hand, dihydroorotate dehydrogenase (DHODH) is an essential enzyme for *de novo* pyrimidine synthesis. In his review, Belete[33] claimed that DHODH was inhibited by DSM265, and KAF156 that were evaluated in either phase I or II clinical trials[33]. After identification of the crystal structure of *Plasmodium* aspartate transcarbamoylase (ATCase), involved in pyrimidine *de novo* biosynthesis pathway, a review[34] summarized the studies conducted on *Pf*ATCase structure. The reviewers aimed to develop allosteric inhibitors with selective binding affinity with its pocket that supports its catalytic mechanisms. Since *Pf*ATCase exists in two distinct states with different substrate affinity, and activity, the reviewers hypothesized that further understanding of the mechanism of *Pf*ATCase inhibition would provide an opportunity for novel antimalarial drug development[34].

2. DNA replication

(A) Topoisomerases (Topos): *Plasmodium* spp. genomic analyses revealed seven genes encoding Topos, one for each I, II and III, while two genes encode Topo VI (α, and β) and DNA gyrase (A and B). Chalapareddy *et al*.[35] showed existence of two putative Topos (VIA and VIB) belonging to type IIB Topo. Therefore, a suggestive Topos nomenclature was introduced; subfamily IA has two putative Topos (IIIα, and IIIβ), IB (IVα, and IVβ), IIA (Vα, and Vβ), and IIB (VIα, and VIβ), and Arabic numbers were used; i.e., 3 through 6. Indian investigators observed that Topo VIA possessed the Topo primase domain present in type IIA, involved in DNA cleavage. Besides, it showed significant similarity with Spo11, a Topo-like enzyme with an essential role in solving topological problems during meiotic recombination. Knocking out gene encoding Topo VIB revealed inhibition of mitochondrial replication leading to schizont death. Accordingly, the investigators proposed *Pf*Topo VIβ a promising drug target[35].

Six synthetic isoflavonoid derivatives (LQBs) were investigated against Topo I of both *P. falciparum* and human (*Hs*Topo I). All investigated compounds were previously used in cancer therapy beside their anti-parasitic activity *in vitro* and *in vivo*. Because the study had no access to *Pf*Topo I, the investigators conducted computational molecular docking studies validating LQBs fitting into the binding sites of known Topo I inhibitors (Camptothecin and Topotecan). Although all compounds fitted at the DNA cleavage site, LQB223 was selected because it showed similar docking energy to anti-cancer inhibitors. Utilizing experimental assays using chloroquine-resistant and –sensitive isolates, LQB223 exhibited the highest therapeutic index against both isolates[36].

The potential antimalarial activity of acriflavine (ACF) that exhibited potential anti-cancer and antibacterial activities was investigated. It was found that ACF significantly cleared parasitemia in experimentally infected mice and suppressed

parasite growth of chloroquine sensitive and resistant isolates *in vitro*. The potential ACF inhibitory activity against *P. falciparum* gyrase B was demonstrated[37]. Although ciprofloxacin and GSK299423 (gyrase inhibitor) exhibited inhibitory activity against *P. falciparum* Topo II and gyrase B, respectively, the investigators recommended further studies for crystal structures identification to characterize their binding sites[38]. Structure activity studies demonstrated that fluoroquinolones, bacterial poisons, possess potential toxic activity against *P. falciparum* gyrase. However, further studies with more structural modifications improving their efficacy as novel antimalarial drugs were recommended[39].

(B) Helicases: They are a highly conserved group of enzymes that play an essential role in all aspects of DNA and RNA metabolism; i.e., replication, repair and transcription. They are motor proteins with powerful ability to resolve DNA or dsRNA into single strands deriving energy from ATP hydrolysis. It was reported that the identified RNA helicase (DOZI) in *Plasmodium* spp. binds with RNA proteins complex of macrogametocytes to suppress translational control of protein synthesis. These RNA-binding proteins (Pufs) are responsible for storage of untranslated mRNAs as stress granules or germ granules described in erythrocytic gametocytes (Puf1 and Puf2), and salivary gland sporozoites (Puf2). Both Bufs were suggested to maintain sporozoites' infectivity for days in the salivary glands[40]. Gene sequencing of *P. falciparum* genome showed three ATP-dependent DNA helicases, and *Pf*RuvB was proved an essential factor for intra-erythrocytic schizogony. A study was conducted in 2016, the investigators demonstrated the inhibitory activity of the commonly used DNA helicase inhibitors on *Pf*RuvB3 activity. The investigators recommended subjecting recombinant *Pf*RuvB3 for further studies to design a novel specific inhibitor as novel antimalarial drug[41].

Recently, two Indian studies[42,43] were conducted on *Plasmodium* helicases. In the first, the investigators reported existence of two proteins crucially involved in *P. falciparum* mitochondrial genome repair, *Pf*Rad51, and Bloom helicase (*Pf*Blm). Loss of both proteins resulted in blockage of the repair of the chromosomal and nuclear DNA double stranded breaks, respectively in *P. falciparum* mitochondrial genome. They were recruited to schizonts mitochondria for DNA repair due to exposure to endogenous and physiologic DNA-damaging agents. The investigators observed that both recombinant proteins were expressed in the mitochondrial DNA once chromosomal and nuclear genomes were repaired. To achieve its role, *Pf*Blm should interacte with *Pf*Rad51, and *Pf*TopoIII. Overexpression or selective inhibition, using Rad51 and Blm inhibitors, kinetically increased or blocked mitochondrial DNA repair, respectively. Therefore, *Pf*Rad51, and *Pf*Blm were suggested promising drug targets[42]. Utilizing *in silico* analyses, the second study identified and biochemically characterized *Pf*DDX17, an active RNA helicase. It was highly expressed in trophozoites and mainly localized in infected RBCs membrane. Knocking down of *Pf*DDX17 demonstrated significant impairment in the progression of intra-erythrocytic stages differentiation[43].

(C) Microtubules (MTs) of the *Plasmodium* cytoskeleton play an essential role in the mitotic and meiotic replication required for proliferation. Since evidence from genetic *Plasmodium* studies revealed tubulin essential role in microtubules nucleation, assembly, and polarity establishment, Indian investigators performed molecular dynamics simulations to explore tubulin structure stability. Results revealed high potentiality of proposing *Plasmodium* tubulin promising drug target[44]. On the other hand, kinesin (K) superfamily includes several families of motor proteins with specialized MTs functions such as translocation, and organization of mitotic and meiotic spindles. A British study identified *Pf*K5 structural and biochemical characters, and observed three issues. First *Pf*K5 had highly conserved α- and β-tubulin that played a MT-organizing role within mitotic spindles. Second, loop5 in the drug-binding site of *Pf*K5 motor domain represented a good opportunity for development of a selective allosteric inhibitor. Third, knockout studies of *P. berghei* K5 significantly reduced sporozoites in mosquito salivary glands. Accordingly, it was suggested using K5 allosteric inhibitors as novel antimalarial drug, and to decrease malaria transmission as well[45].

3. Gene expression and regulation

Plasmodium life cycle constitutes sexual forms in mosquitos and specialized asexual stages in vertebrate hosts with distinct cellular environments throughout stages differentiation. Therefore, it requires a specialized elaborated adaptation using several PTMs that provide spatiotemporal control of cellular activity including acylation/deacetylation, phosphorylation, methylation, glycosylation, protein lipidation, and sumoylation (ubiquitination). Notably, SUMOs (small ubiquitin-like modifiers, Ubl modifiers), are required to fine tune their target protein in *Plasmodium* spp. *via* covalent attachment, altering its stability, conformation, localization, or affinity to binding partners. They include Ub-related modifier 1 (URM1), Ub-fold modifier 1 (UFM1), and autophagy-related proteins 8 and 12 (ATG8 and ATG12). British reviewers assigned regulation of endosomal processes, mitochondrial functions, and autophagy for URMI, UFM1, ATGs, respectively. Since Ubl modifiers are essential across all *Plasmodium* life cycle stages, development of specific inhibitors might yield an effective drug against multiple stages[5].

(A) Histone modifying enzymes: Genomic analysis of *P. falciparum* showed 25 histone modifying enzymes; 10 histone acetyltransferases (HATs), 7 histone deacetyltransferases (HDACs), and 8 bromodomain-

containing proteins (BDPs)[46]. In a recent report, a review[47] claimed three features for *Plasmodium* histone modifying enzymes. First, among *Plasmodium* HATs, only MYST and GCN5 attracted much attention due to their essential roles in gene activation and DNA repair controlling life cycle stages, and upregulation of virulence gene expression upon stress induction, respectively. Therefore, MYST and GCN5 play crucial roles in antigenic variation, and emergence of antimalarial drug resistance, respectively. Second, *in vitro* studies showed the efficacy of HDAC1 inhibitors on *Plasmodium* growth however, they exhibited unsatisfactory results *in vivo*. Therefore, the reviewers proposed using HDAC1 inhibitors as a combined therapy. Third, further characterization of *Plasmodium* BDPs was recommended to develop selective inhibitors particularly against those involved *in vivo* pathogenesis[47]. Later, a network-based comprehensive computational approach was utilized to conduct an interactome study, i.e. analysis of combined networks for host-*P. falciparum* relationship and protein–protein interactions. Results revealed identification of a potential drug target (C6KTD2) for which the knocking down of its encoding gene showed its essentiality in chromatin structure, histone lysine methylation, and gene expression[48].

(i) HATs: Sequence analysis of *Pf*GCN5 allowed Indian investigators to identify the difference in the catalytic pocket between *P. falciparum* and its human homologue. Utilizing virtual screening, ten compounds were identified with high affinity towards *Pf*GCN5 than its human orthologe. *In vitro* studies revealed that compounds of C14 complex exhibited inhibitory potency against growth with no cytotoxic effects[49]. *In silico* docking studies conducted on the crystal structures of *Pf*GCN5 using specific BDPs inhibitors showed growth inhibition of *P. falciparum* erythrocytic stages[50]. In addition, *in silico* analysis demonstrated its homology to human HAT (TIP60). Indian investigators succeeded to show its successful acetylation of its substrate, i.e. histone H4. Significant inhibition of *P. falciparum* growth *in vitro* was achieved using NU9056, a selective TIP60 inhibitor. The investigators recommended future studies to identify further compounds, utilizing HTS and *in vitro* assays, capable of inhibition of *Pf*MYST catalytic activity. Accordingly, *Pf*MYST was proposed a potential drug target[51]. In a recent study, it was demonstrated that *Pf*GCN5 regulated gene expression of *P. falciparum* stress responsive genes during intra-erythrocytic stages. Utilizing transcriptomics, the investigators showed increased *Pf*GCN5 genome-binding sites upon ART exposure. In addition, inhibition of *Pf*GCN5 in ART-resistant strains increased their ART sensitivity. Accordingly, CGN was proposed promising drug targets in ART-resistant strains. An interesting diagram was drawn illustrating mechanisms proposed for ART resistance in *P. falciparum*, and the role played by *Pf*GCN5 to increase ART-resistance generation[52].

(ii) HDACs: Among the identified HDACs, only *Pf*HDAC1 showed significant homologous level with high potentiality for development of selective inhibitor against apicomplexan diseases. The inhibitory activity of four HDAC inhibitors, clinically approved for cancer therapy, against *Plasmodium* spp. was investigated. Although Vorinostat (SAHA), Panobinostat, Belinostat, and Romidepsin exhibited dose-dependent activity, the investigators did not support their use as novel drugs. The study hypothesized that HDAC differentially inhibited stage specific HDAC isoforms. The investigators recommended further studies to develop selective or pan HDAC inhibitors[53]. The previous anti-cancer drugs were investigated against *P. knowlesi in vitro* and *in vivo*. *In vitro* results revealed potent inhibitory activity of all drugs except Romidepsin with 8–45 fold selectivity for *P. knowlesi* over mammalian cell lines. *In vivo* results showed significant reduction in parasitemia on administration of oral dose (25 mg/kg twice daily for four days) of either SAHA or Panobinostat. Since *P. falciparum* HDAC1 showed high identity with that of *P. knowlesi*, the investigators generated a 3-dimensional structure of *Pk*HDAC1 and they observed high selective binding affinity towards *Pk*HDAC1 versus mammalian cell lines[54].

In treatment of *falciparum* malaria, two other studies utilized SAHA in a combined therapy regimen, either with primaquine[55], or with BIX-01294, another anti-cancer therapy[56]. Both exhibited low micromolar potency against chloroquine-resistant and –sensitive strains of *P. falciparum* and *P. berghei* with low cytotoxicity *in vitro*. Recently, Australian investigators hypothesized that poor *in vivo* efficacy of anti-cancer drugs such as SAHA and Panobinostat was attributed, in part, to their suboptimal pharmacokinetic profiles. To minimize their metabolism, a series of achiral analogues of AR-42, an anticancer HDAC inhibitor, were developed and investigated against *P. falciparum* intra-erythrocytic stages *in vitro*, *P. berghei* intra-erythrocytic stages *ex vivo*, and *P. berghei* exoerythrocytic forms in hepatocytes *in vivo*. Results reveled inhibitory potency of 13 analogues against *P. falciparum* (3D7 strain) intra-erythrocytic stages. Among them, only four were with more than 50-fold selectivity for *P. falciparum* versus mammalian cells. Oral administration (50 mg/kg/d single dose for four days) cured parasitemia in *P. berghei* infected mice[57].

In 2018, novel series of *Pf*HDACs inhibitors were investigated *in vitro* against erythrocytic stages of *P. falciparum* and *P. berghei*. Two inhibitors showed sub-micromolar efficacy against chloroquine-resistant and -sensitive strains, and one of them (MC1742 12) displayed nano-molar activity against *P. falciparum* isolates, with extremely low toxicity against murine and human cell lines *in vitro*[58]. An Indian study utilized molecular docking and virtual screening to identify novel potential inhibitors against *Pf*HDAC1. Out of 20 compounds, 14 exhibited inhibitory activity against various *P. falciparum* drug resistant and sensitive strains at nanoscale concentrations with minimum cytotoxic effects against mammalian cell lines[59]. Later, virtual screening of 51 inhibitors revealed that

MC1742-12 displayed *in vitro* satisfactory results against *P. falciparum*, but it failed to reduce parasitemia in *P. berghei*-infected mice[60].

(iii) BDPs: As previously explained[31], BDPs are conserved acetyl-lysine-specific protein-interaction modules that play important roles in regulating gene expression and mutations. In other words, they are proteins with a domain known to recognize acetyl-lysine residues on histones. Change in BDP expressions was linked to several human diseases, e.g., cancer, diabetes, and neurological disorders. Therefore, BDP serve as potential drug targets, i.e., interfering with lysine acetylation mediated signaling. Although seven BDP encoding genes were identified in *P. falciparum*, only two were partially characterized, the HAT *Pf*GCN5 that proved to have a bromodomain with a lysine acetyltransferase activity, and *Pf*BDP1. Using virtual screening, a study was conducted to investigate 42 compounds against *P. falciparum* growth. *In silico* docking studies showed that all compounds interacted with the conserved bromodomain of *Pf*GCN5. Unfortunately, when tested against *P. falciparum* (Dd2 strain) intra-erythrocytic stage *in vitro*, they exhibited ~50% growth inhibitory activity. Moreover, *in vitro* cytotoxicity assays showed that only one compound (SGC-CBP30) had ~7-fold better selectivity for *Pf*GCN5 versus mammalian cell lines. The investigators recommended future studies investigating further compounds to achieve better *in vitro* results accompanied with *in vivo* studies[61].

(B) Protein lipidation enzymes: Commonly observed in all apicomplexans cell membrane, protein lipidation enzymes [N-myristoltransferase (NMT), and palmitoyl acyltransferase (PAT)] play an essential role in trafficking of virulence factors between membrane compartments. In *P. falciparum*, ~30 NMTs were identified, e.g. glideosome-associated protein 45 (GAP45) that was functionally assigned to motility[62]. A British review[63] discussed NMTs as potential drug targets in intracellular parasites. *Plasmodium* spp. possess a single NMT isoform for N-myristoylation of several proteins involved in PTMs. These include glutamate receptor associated protein-1 (GRASP1) for Golgi functions, ADP-ribosylation factor-1 (ARF1) for trafficking, glideosome associated protein (GAP45) for host cell invasion, calcium-dependent protein kinase-1 (CDPK1), calpain for membrane localization during life cycle stages regulation and progression, and adenosine kinase (AK) for energy metabolism[63].

In a recent study, British investigators proposed other functions for NMTs that comprised schizonts development, merozoites egress and RBCs *de novo* invasion. Treated intra-erythrocytic stages during schizogony with IMP-1002, NMT inhibitor, resulted in a significant disruption of rhoptry function. The investigators identified 16 NMT substrates that significantly reduced myristoylation on NMT inhibitor treatment. Among them, GAP45, and loss of its myristoylation led to significant impairment of motor complex function that prevented RBC's *de novo* invasion. It was concluded that inhibition of myristoylation in *Plasmodium* spp. resulted in the formation of pseudoschizonts with impairment of merozoites development and egress, and loss of RBCs *de novo* invasion[64].

On the other hand, Brown *et al*.[65] reviewed PATs and claimed that they were mainly localized in the Golgi apparatus and ER. However, several PATs in *P. falciparum* spp. were localized in rhoptries involved in secretion of factors necessary for RBCs invasion[65]. In addition to their role in PTMs, evidences confirmed additional stage-specific functions of PATs. In contrast to NMTs, investigating PATs functions are challenged by absence of specific inhibitors, hence it was suggested that compounds with PATs inhibitory activity should be a high priority for further studies to develop novel therapeutic agents[3].

Lipolyation: Lipoic acid (LA), an organosulfur compound with antioxidant effects, is a cofactor for the action of two lipoate ligases (LipL1 and LipL2). Both enzymes are involved in protein lipoylation, another form of protein lipidation. While LipL1 lipolyates the H protein of glycine cleavage complex (GcvH), LipL2 lipolyates the E2 subunits of pyruvate dehydrogenase, and α-ketoglutarate dehydrogenase. Both enzymes are involved in lipoate biosynthesis in the apicoplast. Besides, LipL1 is a mitochondrial enzyme, while LipL2 exists in mitochondrion and apicoplast. In addition to their role in PTMs, LA proved its essential role in redox regulation. Since LipL1 and LipL2 inhibitors are used as anti-cancer drugs, the reviewers recommended further studies investigating the efficacy of these inhibitors against *Plasmodium in vitro* and *in vivo*[66].

(C) Sumoylation: In eukaryotes, sumoylation is an evolutionarily conserved PTM in which a small Ubl modifier (SUMO) modulates several biological and molecular processes, i.e., protein-protein interactions. Sumoylation is formed by the covalent linkage of SUMO (12 kDa) to the lysine residues on the protein substrate. In *P. falciparum*, three molecules trigger the current PTM, E1-activating enzyme (*Pf*SUMO-E1), E2-conjugating enzyme (*Pf*SUMO-E2), and *Pf*SUMO-E3 ligase. Through sumoylation, fine-tuning of cellular functions are maintained by modulations of protein stability, enzymatic activity, and interactions affinity. In addition, its role in regulating gene expression, oxidative stress response, and proteasome pathways renders *Pf*SUMO, and the involved enzymes potential drug targets. Besides, SUMO removal is catalyzed by SUMO-specific isopeptidases/proteases, and *P. falciparum* genome revealed two genes encoding SUMO-specific protease (*Pf*SENPs 1 and 2). In a recent report[67], the reviewers drew a schematic overview of *Pf*SUMO machinery roles throughout the intra-erythrocytic stages. They also discussed the functional conserved role played by ubiquitination in merogony development of erythrocytic schizonts to extracellular merozoites. They concluded that synergism between

ubiquitination and SUMOylation cascade following proteasome pathway activation might contribute to *Plasmodium* virulence by regulating the protein turnover[67]. In the same year, the investigators who utilized host-*P. falciparum* interactome approach suggested another potential drug target (C6KTB7). They claimed its involvement in the ubiquitination pathway required for regulation of several cellular signaling crucial for virulence of malignant malaria[48].

(D) Coenzyme A (CoA) synthesis: Pantothenate (Pan, vitamin B5) is the precursor of CoA synthesis, an essential cofactor required for oxidation and acetylation of pyruvate, a fatty acid in the citric acid cycle. Since CoA synthesis is essentially required for gene regulation, and PTMs, de Vries and her colleagues[68] reviewed the biological and pharmacological aspects related to Pan synthesis and its metabolization to CoA in apicomplexans focusing on *Plasmodium* spp. Notably, CoA synthesis is a five-step reaction that requires Pan, four ATP molecules, and cysteine, and several enzymes. Among them are pantothenate kinase (PANK), dephospho-CoA kinase (DPCK), phosphopantetheine adenylyl-transferase (PPAT), phosphopantothenoyl-cysteine decarboxylase (PPCDC), and synthetase (PPCS). The reviewers discussed genomic analyses and *in vivo* studies conducted in *P. falciparum*, *P. yoelii*, and *P. berghei*. *In vitro* studies revealed potential dispensability of genes encoding PANK only in *P. falciparum*, and those encoding PPCDC and PPCS in all species. They observed that PANK was essential for the viability of intra-erythrocytic stages in *P. yoelii*, and *P. berghei*. Since DPCK showed higher homology to the human orthologues, the reviewers proposed PPAT a potential drug target. Due to its weak homology to the human bifunctional PPAT/DPCK enzyme, previous studies investigating the efficacy of pantothenamides (PnAms) as inhibitory compounds targeting CoA synthesis were reviewed. Further studies were recommended to investigate PnAms (Amb180780, KuWei173, and MMV689258) against *Pf*PPT activity[68]. One year later, an *in silico* study utilizing genetic, metabolomics, and chemogenomics analyses validated two compounds (MMV019721 and MMV084978) targeting acetyl-CoA synthetase (*Pf*AcAS). Both compounds exhibited significant growth inhibitory activity against exoerythrocytic and erythrocytic *P. falciparum* stages[69].

4. Translational control of protein synthesis

Diverse *Plasmodium* transmission stages rely on dynamic translational control to rapidly adapt on their host and vector. The asexual replication stages are closely timed and mediated by internal triggers, while the sexual stages are highly dependent on external signals. German reviewers tabulated updated molecules involved in machinery of *P. falciparum* translational regulation. They discussed the essential role of 1) IF2α kinases (eIK1, eIK2, and PK4), 2) the interaction between eIF4E and eIF4G, 3) DOZI, a RNA helicase, 4) the interaction of poly(A)-binding protein (PABP) to facilitate mRNA pseudo-circularization that activates transcript stabilization, and 5) the deadenylation process to induce degradation of mRNA decay using two complexes, CNOT and RNA exosome. The former is a highly conserved complex comprising two deadenylases, a nuclear one to control gene expression, while the cytoplasmic enzyme degrades mRNA decay. On the other hand, RNA exosome, a multi-protein complex, acts after mRNA decay deadenylation degrading various types of RNA molecules. The expression of erythrocyte membrane protein 1 (EMP1), the major *P. falciparum* virulence factor, is also under strict control of both transcriptional and translational regulation mechanisms. Therefore, molecules involved in the molecular machinery of *Plasmodium* translational control significantly represent attractive new avenues for development of novel antimalarial drugs[70].

Translational eIF2 is a ribosomal component working as a translational machine for protein synthesis between three *Plasmodium* genomes; nuclear, mitochondrial, and apicoplast. The essential role of RNA helicase (DOZI) in repression of translational control of protein synthesis was previously described (Helicases). When salivary gland sporozoites are injected into the host, they develop into clinically silent hepatic (exoerthrocytic) stages. After a few days, the intra-erythrocytic cycle develops with active processing of translational protein synthesis in rings and trophozoites that is repressed later in schizonts. This translational suppression was associated with increased levels of eIF2α kinase 4 (PK4) in *P. berghei* schizonts to phosphorylate eIF2α. Accordingly, it was hypothesized that this translation arrest, a conserved mechanism in *Plasmodium* spp. that was mediated by eIF2α phosphorylation, might be responsible for the generation of hypnozoites in *P. vivax*, and *P. ovale* causing malaria relapse[40]. *Plasmodium* eIF kinases provide an essential role not only in sensing stressful conditions, but also in initiating a specific translational mechanism to reprogram gene expression involved in disease latency (quiescence). *Plasmodium* genomics revealed three eIF2α kinases (eIK1, eIK2, and PK4), and each possesses specific regulatory regions triggerd by different stress conditions. It was observed that PK4 was activated in response to ER stress leading to eIF2α phosphorylation, i.e., essential enzyme for development and differentiation of intra-erythrocytic stages, and gametocytogenesis as well. American reviewers[10] described existance of germ granules, stored as untranslated mRNAs, in the transmitted sporozoites. They recommended further studies exploring the specific role of eIF2α phosphorylation in formation of *P. vivax*, and *P. ovale* hypnozoites. Future studies were also encouraged to discover mechanisms underlining conversion of hypnozoites to proliferative stages causing malaria relapse[10].

The first report describing the use of M5717 that targets eukaryote elongation factor (eEF2) in *Plasmodium* spp., was published in 2021. A clinical trial

was conducted in two parts to characterize its safety, tolerability, antimalarial activity, and pharmacokinetics in healthy volunteers. Previous *in vitro* and *in vivo* studies showed high selective inhibitory activity against hepatic and erythrocytic stages. A single dose of M5717 was well tolerated and showed initial clearance of asexual blood stages that lasted for 35-55 h. Its pharmacokinetic profile confirmed high potentiality for the development of novel antimalarial drug. The study recommended using M5717 in a combined therapy with other antimalarial drugs with different modes of action and rapid onset to avoid recrudescence acquired from mutations in the gene encoding eEF2[71]. Surprisingly, combined M5717 and pyronaridine treatment was investigated against *P. falciparum* erythrocytic stages *in vitro*, and in well-established *P. falciparum* infection in severe combined immunodeficient mice (SCID) *in vivo*. Since the former is a slow acting drug targeting eEF2, while the latter exhibits rapid action through inhibition of hemozoin formation, no pharmacokinetic interactions were observed[72].

As previously explained[31], the genetic code is a set of three letters (AUG) of nucleotides; adenosine (A), uracil (U) and guanosine (G) called codons. The rate of translational protein synthesis machinery is maintained by upstream AUGs (uAUGs). Therefore, both uAUGs position relative to the protein coding region, and their ability to form an upstream open reading frame (uORF) determine translation machinery. In *Plasmodium* spp., mRNAs translational efficiency is a complex process fine-tuned by cis- and trans-acting factors. It was reported that *Plasmodium* mRNAs showed unusual 5' untranslated regions suggesting cis-acting sequence complexity that exhibited an essential role on translational efficiency *via* fine-tuning protein synthesis levels. *In vitro* recent American study compared the role of cis-acting regulatory sequences in *P. falciparum* and human. The study characterized *P. falciparum* mRNAs with high or low translational efficiency, their termination status of upstream AUGs, and 5' untranslated regions (5′ UTR) composition. Results revealed remarkable conservation between *P. falciparum* and human since the investigators characterized unusual features of *Plasmodium* 5′ UTR. Features included its length, base content, and high upstream AUGs prevalence, suggesting its significant role in tuning translational efficiencies. Therefore, cis-acting sequence complexity was proposed as a promising drug target for development of novel antimalarial drug[73].

Utilizing a computational metabolic network for estimation of pathogen essential genes in *P. falciparum*, a recent study predicted five potential drug targets with no homology to human orthologues. From these, the investigators selected RNA pseudouridylate synthase putative (RPuSP) because no molecular docking studies were yet recorded. Notably, RPuSP is a member of RsuA family of enzymes essentially involved in translational protein synthesis and cell growth. The investigators succeeded to predict *Pf*RPuSP 3-dimensional structure, and its virtual screening revealed seven compounds with remarkable interactions with its active binding sites. Further studies to validate their efficacy *in vitro* and *in vivo* were recommended[74].

[II] Survival

1. Protein kinases (PKs)

After discussing several PKs inhibitors, Arendse *et al*.[75] considered two issues prior to selecting a kinase as a promising drug target, absence of a human homologue, and conserved phosphorylated site in the selected PK target. Therefore, a kinase-specific proteomic approach accompanied by *in vitro* kinase assays, and advanced gene editing to identify and validate the potential PK drug target, were recommended. Two main differences were observed between *Plasmodium* and human kinomes: 1) absence of tyrosine kinase (TK) and tyrosine-like kinase (TLK), and 2) presence of unique conservative several atypical PKs (aPKs) in *P. falciparum*[75].

To update PKs, and identify antimalarial drugs, kinomics and chemogenomics studies complemented with bioinformatics pipeline analyses of eight *Plasmodium* spp. were conducted. The study investigated a list of drugs targeting a selected subset of the top-ranked PKs on the *in vitro* growth of two *P. falciparum* isolates, sensitive and multidrug-resistant strains. Kinomics revealed that *P. falciparum* expressed 21 kinases belonging to the FIKK group (aPK), while other *Plasmodium* spp. expressed only one. The investigators suggested 33 eukaryote PKs (ePKs), and two additional aPKs (RIO, and PI4K) in the investigated species, beside FIKKs in *P. falciparum*. Chemogenomics revealed only three compounds with inhibitory activity against 30% of erythrocytic stages growth, with neglected cytotoxicity on mammalian cells[76].

(A) The ePKs: Several ePKs were proposed promising drug targets. According to *in vitro* and *in vivo* potent efficacy of calcium channel blockers, a Brazilian study conducted *in silico* studies to identify PK5 and glycogen synthase kinase 3β (GSK3 β) as molecular targets[77]. Recently, a study demonstrated several evidences proposing GSK3β a promising drug target in *P. falciparum* because 1) it is involved in apical membrane antigen 1 (AMA1) activation, 2) its inhibitors possess *in vitro* potent and selective inhibitory activity, in low micro-molar concentrations, 3) several auto-phosphorylation sites with regulatory activity were identified, and 4) inactivation of its N-terminal was mediated by cyclic AMP-dependent PKs, e.g., PKA and PKB[78]. Later, Lasonder *et al*.[79] claimed that ePKs involved in transmembrane signaling pathways to facilitate communication within *Plasmodium* merozoites were potential drug targets. Extracellular signals transduction leads to increased levels of intracellular messengers [calcium and cyclic nucleotides (cAMP and cGMP)] with subsequent activation of calcium-dependent PK (CMPK), and PKA, and PKG, respectively[79]. In this context, PKA signaling was established an essential kinase for the proliferation

of *P. falciparum* erythrocytic stages. The mechanisms involved in the tight regulation of PKA catalytic subunit (*Pf*PKAc) activity showed its essential role in RBCs invasion by egressed merozoites and subsequent proliferation. Using reverse genetics approach, the investigators showed that *Pf*PKA decreased or augmented expression that essentially contributes in regulating gametocyte-infected erythrocyte deformability, i.e,. with decreased transmission. Moreover, 3-phosphoinositide-dependent protein kinase-1 (*Pf*PDK1) was observed to activate *Pf*PKAc acting as a crucial upstream modifier in the tight regulation of *Pf*PKA signaling pathway. Therefore, both ePKs were proposed potential drug targets[80].

(B) The aPKs: In addition to FIKKs group, two aPKs were characterized in *Plasmodium* spp., PI3K and PI4K, and both proved essential in several cellular processes including proliferation, survival, trafficking, and intracellular signaling[81]. Later, it was claimed that all research focused on PI3K and PI4K in phase II clinical trials, with less attention on phosphatidylinositol phosphate kinases (PIPKs)[75].

Utilizing gene knocking out techniques, eight aPKs belonging to FIKK family were identified as potential drug targets. Among them, six FIKKs were exported into infected RBCs including three (9.1, 10.1, and 10.2) involved in growth and survival, and three (4.2, 7.1 and 12) involved in merozoites egress through altering infected RBC membrane rigidity. The role of FIKK4.2 in RBC adhesive properties and knob morphology was asserted. Two non-exported FIKKs (3 and 9.5) were involved in *de novo* RBC invasion and mitotic nuclear division, respectively[82].

(C) Novel drugs: In 2018, a study investigated the antimalarial potential efficacy of synthesized dihydro-pyrimidinones, calcium channel blockers against *P. falciparum* chloroquine-resistant strains *in vitro*, and *P. berghei*–infected mice *in vivo*. Only three compounds with potent *in vitro* activity and *in vivo* lower cytotoxicity were selected for *in silico* study to identify *P. falciparum* molecular target[77]. Recently, German investigators demonstrated that divalent heavy metal ions and metal chelating proteins inhibited *Pf*GSK3 activity[78].

In his review, an Ethiopian scientist[33] claimed that inhibition of the identified *Plasmodium* aPKs using MMV048 was processed in phase II, and IIa clinical trials in Ethiopia[83]. Recently, Lasonder *et al*.[79] described in their review two obstacles in developing new drugs with satisfactory inhibitory potency to cyclic nucleotides PKs (cAMP and cGMP). They were poor membrane permeability of cyclic nucleotides, and the structural similarity between *P. falciparum* kinase domains and those of host because they may be conserved across all eukaryotes. Accordingly, the reviewers proposed three alternatives. First, some PK domains are surrounded by additional domains essential for their signaling pathways that could serve as specific targets for selective inhibition of the PK of interest, without targeting host kinases. Second is utilizing allosteric inhibitors that bind at a site other than the enzyme catalytic active site. Third is using genetic manipulation (CRIPR-Cas9) that allows researchers to alter gene DNA sequences modifying its function[79].

2. Transporters

Molecules expressed by *P. falciparum* to create new permeation pathways (NPPs) and transporters involved in nutrients acquirement were discussed[84]. The reviewers discussed nutrient trafficking through three barriers. First, NPPs modifications to allow nutrients transfer through RBCs plasma membrane were created by CLAG3 proteins, and RhopH complex. Second, two proteins were identified responsible for nutrients trafficking through PV membrane, EXP2, and RON3 secreted by dense granules and rhoptry bulb, respectively. Notably, EXP2 is the pore-forming component of the *Plasmodium* translocon of exported proteins. Third, transport through parasite plasma membrane requires several transporters, among them ion channels, aquaglyceroporins, equilibrative nucleoside transporters, ABC transporters, and a variety of P-ATPases[84].

(A) Nucleoside transporters (NTs): Similar to *H. sapiens*, there are four equilibrative NTs (eNTs) in *Plasmodium* spp. importing nucleosides and nucleobases through plasma membrane[85]. Since *Plasmodium* spp. synthesize pyrimidine *de novo*, the reviewers claimed that *Plasmodium* eNT1 was the major transporter employed in importing purine nucleosides and nucleobases for purine slavage pathway. It was identified in all life-cycle stages with a small peak in early trophozoites. Knocking down of its encoding gene is conditionally lethal depending on purine concentrations in host blood. Comparing with *Pf*eNT1, tow differences were observed in *Pf*eNT2; absence in sporozoites, and localization in internal membranes, predominantly ER. Membranes localization of *Pf*eNT3 is unknown but is in constant expression levels in intra-erythrocytic stages without clear evidence to import purine. Lastly, *Pf*eNT4 is detected in all erythrocytic stages and sporozoites, and displays a low substrate affinity for most transported purine nucleosides and nucleobases. However, it possessed marked affinity to adenine derivatives, and its functional role is not known. Because erythrocytic purines concentration is not sufficient to build *Plasmodium* DNA genome, other purine-transport pathways were identified[85].

(B) Metabolite transporters: Australian investigators reported that *Plasmodium* spp. extruded L-lactate, a metabolite of glycolysis, through a formate nitrite transporter (FNT)[86]. Two years later, German researchers investigated the efficacy of a compound (MMV007839), identified as a new class of fluoroalkyl vinylogous acids. Results revealed its significant inhibitory potency against *Pf*FNT and killed intra-erythrocytic stages *in vitro* with sub-lethal concentrations. The investigators demonstrated that MMV007839 produced a single nucleotide exchange in the gene encoding *Pf*FNT[87]. In 2020, a review

reported three plasma membrane transporters, hexose, lactate:H^+, and choline, for glucose/fructose, lactate and choline transport, respectively, proposing them potential drug targets since they were essential transporters to maintain neutral intracellular pH[33].

(C) Primary and secondary active transporters: In their review, Meier *et al*.[2] tabulated all inhibitors investigated in the last two decades that showed potent inhibitory activity against *Plasmodium* transporters. Among them, several inhibitors are currently in clinical trials such as aquaporin (AQP) transporters, and SERCA that is also termed ATP4. *Plasmodium* SERCA, a P type ATPase transporter responsible for maintaining low cytosolic sodium concentration, is absent in mammals, i.e., potential drug targets[4]. Cipargamin, a *Pf*ATP4 inhibitor, was reported a novel antimalarial drug in clinical trials II for parenteral administration[88]. However, the study demonstrated variable inter-species differences in drug susceptibility[88].

The essential role of ATP2, a P4-ATPase, in the maintenance of erythrocytic stages membrane lipid asymmetry was demonstrated[89]. *Plasmodium* P4-ATPases are involved in the asymmetric phospholipid distribution through plasma membranes through their trafficking to the cytosol. Using recombinant *P. chabaudi* ATP2, the study predicted its mechanism of action, and demonstrated that its activity was upregulated by phosphatidylinositol 4-phosphate (PI4P)[89].

3. Mitochondrial respiratory chain

Although several drugs are used targeting *Plasmodium* mitochondrial respiratory chain machinery, none escapes drug resistance due to gene mutations. This is because almost all exhibit a mode of action targeting single active site of cytochrome bc1 complex. A study[90] designed a selective inhibitor of *P. falciparum* nicotinamide adenine dinucleotide (*Pf*NADH) with simultaneous allosteric inhibitory potency on both active sites of *Pf*NDH2. The investigators claimed that RYL-581 was the first report of developing multi-targeting allosteric inhibitory drug as novel antimalarial drug[90].

[III] Virulence

1. Proteases

In fact, no protease was established as a virulence factor in *Plasmodium* spp., however 12 proteases were proposed potential drug targets in a review published in 2019[91]. The reviewers proposed five cysteine [falcipains/vivapains 1 and 2, serine repeat antigens 5 and 6, and dipeptidyl aminopeptidase I (DPAPI)], three metallo (DPAP III, alanyl, and leucyl aminopeptidases), two aspartyl (plasmepsins II and V), a threonine (HsIV), and a subtilisin-like protease. Three crucial biological processes are assigned to proteases; 1) hemoglobin (Hb) degradation, 2) synthesis of protein required for growth, survival, and differentiation, and 3) RBCs invasion and egress[91]. Several protease inhibitors were investigated as novel antimalarial drugs, but with only *in vitro* significant results. In fact, the main obstacle in development of a specific selective CP inhibitor is the inefficiency of activity-based probes (ABPs) that determine the specificity of the tested inhibitors. Notably, ABPs are small molecules that use the enzymatic mechanism of an enzyme to covalently modify its active site. Unfortunately, the developed ABPs used nowadays for CPs target either endopeptidases (falcipians) or dipeptidyl aminopeptidases, but not both. In 2020, a study designed a new series of fluorescent broad-spectrum ABPs able to label the enzymatic mechanism of both CPs. The investigators claimed that it becomes feasible to design selective CP inhibitors using dipeptidic vinyl sulfone probes[92].

(A) Cysteine proteases (CPs): Falcipains and vivapains are the major CP in *P. falciparum*, and *P. vivax*, respectively. Both CPs have a conserved structural-functional relationship, C and N terminal domains for Hb degradation, four small subdomains for food vacuolar, ER trafficking, an inhibitory subdomain, and a hot spot interaction at the interface of pro- and mature domains for auto-processing[91]. In 2018, an Indian study presented the first trial to use azapeptides, a new generation of allosteric inhibitors, against falcipains 2 and 3. Without cytotoxic effects, two compounds arrested *in vitro* growth on a dose-dependent manner *via* blocking falcipains auto-processing. However, due to falcipains processing in a cellular compartment with high lipid cell wall content, only partial inhibition was occasionally observed. Further studies were recommended to synthesize other azapeptides compounds with high lipophilicity to access such cellular compartments with higher potency[93]. In his review, Abaza[94] reported that investigated inhibitors against the major CPs included peptide, non-peptide, peptidomimetic, and allosteric site inhibitors. Both peptide and non-peptide inhibitors exhibited unsatisfactory *in vivo* results due to their poor pharmacological profiles and their susceptibility to degradation by host enzymes. However, no efficient CP inhibitor was validated until then for the treatment of malaria[94].

(B) Aspartylproteases: *Plasmodium* genome showed 10 plasmepsins (PLMs), however, only four (II, V, IX and X) were suggested promising drug targets. While II and V contribute in Hb degradation, IX and X are involved in RBC's invasion and egress cascade[95]. The crystal structure of PLMs II, and V showed conserved features with relative evolutionary divergence from host' aspartyl proteases that increased their potentiality as drug targets[91]. Their inhibition by statine and allophenylnorstatin-based inhibitors that block Hb degradation was suggested[33]. Utilizing a HTS approach, an oral bioavailable lead compound (WM382) was identified with selective *in vitro* and *in vivo* inhibitory effects on PLMs IX and X, with complete cure in experimentally *P. berghei*-infected mice. The investigators demonstrated both PLMs essential role in the processing, maturation, and activation of SUBs 1 and 2, with subsequent maturation of AMA1, merozoite surface protein-1, and serine repeat antigen-5, essential

molecules in invasion and egress cascade. Further studies were recommended to validate WM382 in clinical trials[95].

(C) Metalloproteases (MPs): Alanyl and leucine aminopeptidases (M1 and M17, respectively) were identified contributing in Hb digestion. All *Pf*M17 and most of *Pf*M1 were strictly localized in the cytosol. Besides, *Pf*M1 was expressed in three isoforms, one of which was minimally expressed in the PV[96]. Because both MPs possess tightly bound zinc ions in their active site, the reviewers recommended investigating zinc ion chelating agents that inactivate MPs proteolytic functions[91]. In an attempt to develop inhibitors with dual activity against both MPs, a report studied their binding sites and substrates preferences in three species, *P. falciparum*, *P. vivax* and *P. berghei*. Althoug the results revealed largely conserved substrate specificity profiles., the investigators succeeded to recognize an approach that changed the substrate length and its intra-peptide sequence, i.e. high possibility to develop selective inhibitors[97]. In the same year, the same Australian investigators identified the metal active site of *Pf*M17 and *Pv*M17 that played a structural role regulating M17 catalytic activity. The investigators demonstrated that binding of metal ions to M17 active site regulated the dynamic equilibrium between inactive oligomers to active hexamers. This means that conversion of inactive oligomers (mono, di, and tetra) to active hexamer is a dynamic process directionally controlled by the metal active site. Accordingly, the study suggested M17 oligomerization a novel approach for developing new antimalarial drug instead of using MP inhibitors that block M17 active site[98].

(D) Threonine proteases: *Plasmodium* HsIV showed high similarity and conservancy in all species with highly different similarity to human orthologues. In addition to PLMs II and V, PLM III previously known as histoaspartyl proteases and expressed in merozoites' digestive vacuole, was recently proposed a potential drug target[99].

(E) Subtilisin-like protease 1 (SUB1): It has an additional role in processing proteases required for RBCs invasion and egress cascade. The crystal structure of *Plasmodium* SUB1 showed scissile bond allowing unusual interaction of its active site with substrate residues on prime and non-prime sides. Mature SUB1 was strongly suggested a novel drug target[91].

2. Heat Shock proteins (HSPs)

Since it belongs to HSP70 family, glucose-regulated protein (GRP78), also known as BiP, was investigated as novel drug target[100]. It is a luminal molecule in ER to maintain hemostasis and has an essential role in protein folding and membrane modification. *Plasmodium* GRP78 proved its essentiality for schizonts and gametocytes survival and growth. During stress conditions, accumulation of misfolded proteins triggers the unfolded protein response (UPR) to restore normal ER hemostasis. American investigators[100] evaluated the binding affinity of several GRP78 inhibitors against recombinant *Plasmodium* and human GRP78. In comparison to chloroquine, they evaluated their efficacy against drug-sensitive and -resistant *P. falciparum* strains *in vitro*. Only Apoptozole showed statistically significant activity between sensitive and resistant *P. falciparum* strains. Unfortunately, the drug exhibited limited binding activity towards *Pf*GRP78 due to its lower possession of phosphonucleotides required for ATP hydrolysis. To increase its affinity toward *Pf*GRP78, Apoptozole required further studies to identify and characterize *Pf*GRP78-Apoptozole structure interaction[100]. Later, the efficacy of allosteric modulators (SANC190 and SANC651) against *P. falciparum* HSP70 isoforms were investigated. Both compounds exhibited significant allosteric modulatory activity that changed *Pf*HSP70 conformation leading to its failure to respond to ATP binding affinity essentially required for its expression[101].

Recently, an American study identified *P. falciparum* HSP40 chaperone with a C-terminal thioredoxin (Trx) domain, termed *Pf*J2 localized in ER. Besides, four protein disulfide isomerases (PDIs), members of the Trx superfamily, with a redox regulatory role on *Pf*J2 were identified. Knocking down of the genes encoding PDIs revealed that *Pf*PDI8 was essentially required for growth and survival of asexual erythrocytic stages. The investigators suggested that *Pf*J2-*Pf*PDI8 interaction acted with GRP78 (HSP70-BiP) to accelerate ER protein folding. The investigators demonstrated that interaction between ER-*Pf*J2 and *Pf*PDI8 could be blocked by covalent inhibitors. Therefore, oxidative folding process in *P. falciparum* EC was proposed a drug target and using these inhibitors in a combined multi-target therapy[102].

[IV] Other targets

1. Molecules for invasion and egress cascade

As previously mentioned, molecules contributing in invasion and egress cascade are potential drug targets, e.g., ePKs involved in transmembrane signaling pathways[79], FIKKs[82], SUBs 1 and 2[91], and PLMs IX and X[95]. In an editorial[103], a diagram was drawn demonstrating essential molecules involved in egress of hepatic and erythrocytic merozoites, and gametocytes in the host, as well as sporozoites in mosquitoes. The diagram also showed molecules involved in RBCs invasion and cell traversal, and all these molecules were tabulated[103].

The cytoadherence linked asexual genes (CLAG), members of multigene family, are *Plasmodium* spp. conserved genes. They encode proteins localized in the rhoptries of newly formed merozoites that are termed RhopH proteins. American reviewers drew a schematic diagram describing their synthesis and trafficking from egressed merozoites to new RBCs. In trophozoites, RhopH proteins, in particular RhopH2 and RhopH3, are trafficked to the RBC membrane, contributing either in nutrient uptake or cytoadherence to receptors on blood vessel endothelium. Except for *P. falciparum* that possess five *clag* genes, other *Plasmodium* spp.

possess at least two per species. In addition to nutrient uptake, cytoadherence, and *de novo* RBCs invasion, RhopH proteins contribute to PV formation, liposomes synthesis, and increased RBCs permeability *via* increasing anion channels formation[104]. Three years later, the same group of investigators identified two *Pf*RhopH3 isoforms, termed CLAG3.1 and CLAG3.2 that were directly involved in the formation of plasmodial surface anion channels (PSACs). The study demonstrated the mechanism involved by CLAG3 isoforms to form PSACs, and recommended future studies validating CLAG3 potential drug target[105].

Investigated compounds inhibiting RBCs invasion, either in the developing schizont or during merozoite invasion were reviewed. This therapeutic approach is a promising strategy because the majority of molecules required for RBCs invasion has no equivalents in human, i.e., developing selective inhibitors is highly considered. The reviewers claimed that although several compounds with invasion-inhibitory activity were investigated, none proved to possess activity against RBC invasion to date. Accordingly, future studies were recommended to develop inhibitors targeting merozoite surface protein 1 (MSP1), AMA1, and rhoptry neck protein 2 (RON2)[106]. More recently, an Australian study conducted *in silico* HTS and identified several drugs that inhibited RBCs invasion and egress cascade. Among them, only three compounds were suggested as specific inhibitors, two of them blocked RBCs invasion through either blocking merozoite invasion or inhibiting ring development. The third direct inhibitor blocked egress *via* inhibition of the RBCs membrane breakdown. The investigators suggested that these inhibitors could complement current antimalarial drugs[107].

Exposure of released merozoites to low blood potassium concentration is followed by their stimulation by increased cytosolic calcium levels that inducs AMA1 secretion from micronemes. A study[108] demonstrated the role of *Pf*PKA in simultaneous AMA1 phosphorylation, an essential molecule in RBCs invasion. The investigators showed several differences between *P. falciparum* and *P. yoelii*. In the former, merozoites invaded new RBCs immediately after egress, while it required several minutes after merozoites egress in the latter. Second, deleting gene encoding *Pf*PKA had no significant role in new RBCs invasion since *Pf*AMA1 was phosphorylated by another PK (*Pf*GS3K). In contrast, AMA1 impaired expression with altered growth and RBCs invasion was observed in deletion of the gene encoding *Py*PKA. Third, RON2 expression was not affected by *pka* gene deletion in *P. yoelii*. Accordingly, it was suggested that *P. falciparum* AMA1-RON2 interaction played the major role in RBC *de novo* invasion[108].

A recent study[109] characterized one of the essential proteins expressed by *P. falciparum* to localize on the surface of erythrocytic stages by glycosylphosphatidylinositol (GPI) anchors. Confirmed by a knocking down study, the characterized putative phosphomannomutase (PMM), also termed HAD5, was essential for RBCs *de novo* invasion and egress cascade. Notably, GPI anchored proteins are essential components for egress of merozoites (MSP1 and rhoptry-associated membrane antigen), gametocytes (*Pf*s25 and *Pf*s230), and sporozoites (circumsporozoite protein). The investigators succeeded to identify the 3D X-ray crystal structure of *Pf*HAD5 and developed a substrate analog that specifically inhibited HAD5 compared to human PMMs[109].

2. The Hb trafficking pathway: Phosphoinositide lipids (PPIs) play key roles in cell motility, and cytoskeletal reorganization. Hence, they are distributed throughout cellular membranes, and concentrated in Golgi apparatus, and endosomes. In *Plasmodium* spp., they have a crucial role in Hb trafficking pathway from infected RBCs cytosol to the digestive vacuoles of intra-erythrocytic stages. Knocking down the gene encoding the specific putative phosphoinositide-binding protein (*Pf*PX1) led to significant growth alteration. The investigators demonstrated its binding with phosphatidylinositol-3-phosphate (PI3P) localized in the digestive vacuole' membrane to facilitate Hb trafficking. Therefore, *Plasmodium* PX1 was proposed a potential drug target, and inhibitors of phosphoinositide metabolism through inhibition of PI3K, and PI4K are currently in development as novel antimalarial drugs[110].

3. Vitamins: Possibile use of the enzymatic routes of vitamins biosynthesis for the discovery of new generation of antibiotics as novel antimalarial drugs was investigated. Since vitamins B1 (thiamin) and B6 (pyridoxal) are involved in crucial metabolic process of carbohydrates and amino acids, molecules involved in their biosynthesis were proposed potential drug targets. Using proteomic and genomic data available for bacteria, a study[111] demonstrated that thiamin required three enzymes (ThiM, ThiM, and ThiE), while pyridoxal required only two (Pdx1 and Pdx2). Absence of these enzymes in humans renders them potential drug targets[111]. One year later, the same Brazilian investigators published a review and claimed the inability to knock down genes encoding Pdx1 and Pdx2, i.e. crucial enzymes for *Plasmodium* survival. They discussed pyridoxal involvement in several metabolic pathways protecting *Plasmodium* against oxidative stress, i.e., increased survival rate in stress environment. Both enzymes structure and dynamics, their crystal structures reported for *P. berghei* and other eukaryotes, and the mode of their interaction to synthesize pyridoxal were summarized. Two questions were raised regarding possibility of targeting Pdx1-Pdx2 interaction site, and availability of Pdx1, and Pdx2 inhibitors to disrupt pyridoxal de novo biosynthesis[112].

4. Lactate dehydrogenase: Since three calcium channel blockers exhibited satisfactory efficacy *in vitro* and *in vivo*, Brazilian investigators conducted *in silico* studies to identify new *P. falciparum* molecular target. They proposed L-lactate dehydrogenase potential drug target[77]. Since hybrid molecules might overcome re-emergence of drug resistance, Malaysian investigators

synthesized a new series of 4-aminoquinoline hybrids to be screened against chloroquine-resistant and -sensitive *P. falciparum* strains *in vitro*. With low cytotoxic effect against *Vero* cultured cells, hybrid (4b) displayed significant antimalarial activity against both strains with low IC_{50}. A molecular docking study confirmed the potentiality of lactate dehydrogenase as promising drug target[113].

5. Host-directed anti-malarial drugs

(A) Eryptosis enhancement: Similar to all intracellular protozoa, *Plasmodium* spp. manipulate host cell signaling pathways, e.g. programmed cell death pathway. Since RBCs life span is ~115 days, they undergo apoptotic features such as nuclear apoptosis, cell shrinkage and membrane ruffling, i.e. gradual degradation (eryptosis). For rapid establishment of *P. falciparum* in host RBCs, erythrocytic stages induce significant stress on host cell signaling to delay eryptosis. To avoid drug resistance for anti-malarial drugs, a review[114] was published discussing host apoptotic signaling pathways for future perspectives using host-directed therapies to enhance eryptosis. In addition to eryptosis enhancement, the reviewers discussed *Plasmodium* molecules expressed to manipulate host apoptotic signaling pathway through buffering intracellular calcium levels, exporting amino acids, and blocking host cellular kinases[114].

(B) Host TK: Interestingly, a new strategy was proposed utilizing the absence of TK in *Plasmodium* spp. Human RBCs are known to express TK involved in phosphorylation of the cytoplasmic domain of band 3 (cdb3), a RBC' transmembrane protein. Phosphorylated cdb3 within RBC membrane cytoskeleton accelerates separation of Ankyrin and spectin-based membrane cytoskeleton from the lipid bilayer, resulting in membrane breakdown. A recent study observed significant increase of cdb3 phosphorylation on maturation of *Plasmodium* merozoites. Therefore, the investigators hypothesized that merozoites might trigger host TK to increase cdb3 phosphorylation facilitating their egress, i.e. inhibition of host TK would prevent merozoites egress. Library online search for kinase inhibitors with potential antimalarial activity revealed only one subset, Syk inhibitors. Although the investigators observed insignificant results, they suggested that selective Syk inhibitors might be potential novel anti-malarial drugs without future drug resistance. In 2020, the hypothesis utilizing host target for development of novel antimalarial drug was achieved in a clinical trial in Vietnam and Laos[115].

CONCLUDING REMARKS

1. The online website (MalDA) was constructed to accelerate development of novel antimalarial drugs by identifying essential druggable targets. Several approaches were utilized, however two were commonly reported, phenotypic screening, and target-based screening.
2. Although *Plasmodium* PKs, proteases, and transporters attracted much attention, metabolic pathways involved in apicoplast, and mitochondrion remain attractive drug targets. The PV membrane works as molecular sieve for proteins, nutrients, and metabolites transport to be distributed *via* the apicoplast to several organelles including the mitochondrion. Therefore, among the proposed drug targets are apicoplast DOXP, and Fd/FNR system, and mitochondrial DHODH, Cox 1, Cox 3, and cytochrome bc1 complex.
3. Drugs currently in clinical trials in either phase include DSM265 and KAF156 (targeting DHODH), Cipargamin (targeting ATP4), Fosmidomycin (targeting DOXP), MMV048 (targeting PIKs), M5717 (targeting eEF2), and pantothenamides (targeting PPAT in acetyl-CoA synthetase).
4. Since PTMs provide crucial regulatory control of cellular activity during distinct life cycle stages, molecules involved in histone modifying enzymes (HATs, HDACs, and BDPs), phosphorylation (PKs), protein lipidation (NMTs, and PATs), lipolyation (LipL1, and LipL2), and SUMOylation (SUMO-Es) are proposed potential drug targets.
5. Vitamin biosynthesis pathways are also proposed potential drugs targets. Vitamin B5 (pantothenate) is the precursor of CoA synthesis, an essential cofactor required for gene regulation, and PTMs. Since they are absent in human, involvement of ThiM, ThiM, and ThiE in B1 biosynthesi, and Pdx1 and Pdx2 in B6 biosynthesis renders them potential drug targets.
6. Although *Plasmodium* proteases arsenal includes 12 proteases proposed potential drug targets, and plays crusial functional roles, no single protease was established as a virulence factor.
7. Among *Plasmodium* potential drug targets are several molecules involved in invasion and egress cascade, e.g. proteases (PLMs IX and X, SUBs 1 and 2), aPKs (FIKKs), and ePKs (calcium-dependent PK, and cyclic nucleotides PKs),
8. Cytoadherence linked asexual genes (CLAG) encode rhoptries proteins (RhopH2 and RhopH3). To facilitate *de novo* RBCs invasion, trafficking of both proteins to a new RBC membrane is the initial step for cytoadherence that increased RBCs permeability, and PV formation.
9. Two approaches were suggested to develop host-directed antimalarial drugs; eryptosis enhancement, and TK absence in *Plasmodium* spp.

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