# Targeting the *Plasmodium falciparum* Proteome and Organelles for Potential Antimalarial Drug Candidates.

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## Abstract

There is an overarching need to find alternative treatment options for malaria and this quest is more pressing in current times due to the morbidity and mortality data arising from most endemic countries and partially owing to the fact that the SARS-Cov-2 pandemic has diverted much public health attention. Additionally, the therapeutic options available for malaria has been severely threatened with the emergence of resistance to almost all existing drugs by the human malaria parasite. The Artemisinin Combination Therapies (ACTs) which hitherto have been the mainstay

for malaria have encountered resistance in South East Asia, a notorious ground zero for the emergence of antimalarial drug resistance. This review analyses few key druggable targets of the parasite and the potential to leverage strategic inhibitors to mitigate the scourge of malaria by providing a concise assessment of the essential proteins of the malaria parasite that could serve as targets. Furthermore, this work provides a summary of the advances made in malaria parasite biology and the potential to leverage such findings for antimalarial drug production.

**Keywords**

Malaria, proteases, Plasmodium rhomboids, dipeptidyl aminopeptidases, apical membrane antigen, subtilisin-like proteins, glucose transporters, schizogony, plasmepsins

## Introduction

*P. falciparum* is a protozoan parasite responsible for the malaria disease in humans and transmitted by female Anopheles mosquitoes. The parasite on entry into the host invades and multiplies in the hepatocytes producing invasive merozoites (Deu, 2017), which invade and multiply in the host's erythrocytes. Other invasive forms of the parasite are ookinete and sporozoites. *P. falciparum* is classified as; Kingdom Protozoa, Subkingdom Biciliata, Phylum Myzozoa, Subphylum Apicomplexa, Class Aconoidasida, Order Haemosporina, Genus *Plasmodium*. Plasmodium has an alternating life cycle between human and mosquito hosts. Sporozoite injected into the human host during a blood-feed with anticoagulant saliva, circulates through the bloodstream to infect hepatocytes (Burda et al., 2017; Choudhary et al., 2019). They multiply and proliferate asymptomatically in the liver to form the invasive merozoites released into the bloodstream to invade erythrocytes. The parasite feeds on the host cell and multiplies to form more of the merozoites to invade new erythrocytes (the erythrocytic stage, repeated many times) (Shown in

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figure1). In the host, hemoglobin is degraded and used for parasite biosynthesis of polyamines (Becker et al., 2010), pyrimidine (Phillips & Rathod, 2010), fatty acids, while other compounds are obtained from the host directly.

During the merozoites entry into the RBC, parasitophorous vacuolar membrane (PVM) forms to separate the merozoites from the erythrocyte's cytosol. The ring stage parasite degrades the host hemoglobin for food to form the schizonts after several rounds of nuclear divisions (Perrin et al., 2021). At or before the merozoites phase, some parasites transform into sexually active gametocytes (micro- and macro gametocytes) (Liu, 2020) which when ingested by a mosquito combines to form zygotes. The zygote develops into invasive ookinete, escapes the mosquito's gut to form an oocyst, which grows into other invasive sporozoites to infect other human hosts (Fig. 1).

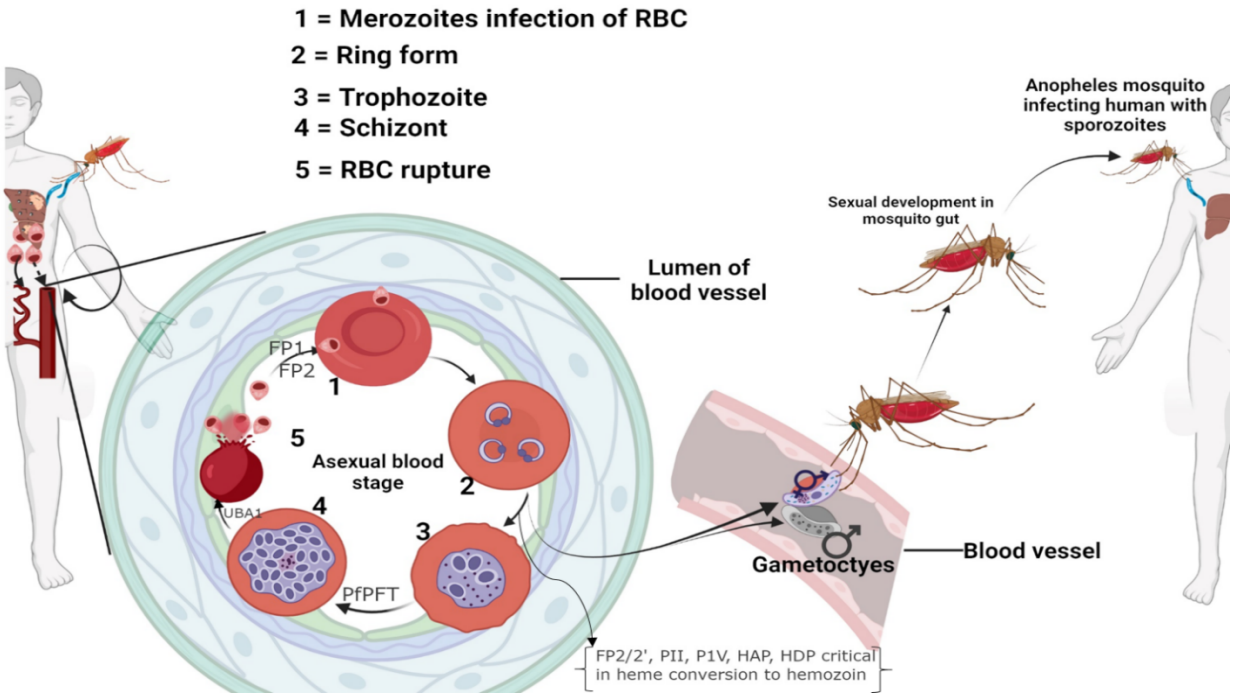

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**Figure 1. Life Cycle of *P. falciparum* Indicating Stage Specific Expression of Essential Parasite Proteins.** The malaria parasite expresses crucial stage specific proteins which facilitates its survival in the human host. Typical among these are Falcipain-1 (FP1), Falcipain-2 (FP2), Plasmepsin II and IV among others. The roles of these proteins are as indicated in (table 1**) (Fig.1. created using Biorender.com)**

**Table 1. Important Proteins of the Malaria Parasite**

| Abbrev. | Name | Activity | Reference |
|---|---|---|---|
| **FP1** | falcipain-1 | Host Cell Invasion | (Greenbaum et al., 2020) |
| **FP2** | falcipain-2 | Merozoite egression | (Dasaradhi et al., 2005) |
| **PII** | plasmepsin II | Heme conversion to hemozoin | (Chugh et al., 2013) |
| **PIV** | plasmepsin IV | Heme conversion to hemozoin | (Chugh et al., 2013) |
| **HAP** | histo aspartic protease | Heme conversion to hemozoin | (Chugh et al., 2013) |
| **HDP** | heme detoxification protein | Heme conversion to hemozoin | (Chugh et al., 2013) |
| **UBA1** | Ubiquitin Activating enzyme 1 | Ubiquitin activation is essential for schizont maturation in *Plasmodium falciparum* blood-stage development | (Green et al., 2020) |

| | | | |
|---|---|---|---|
| **PfPFT** | *Plasmodium falciparum* farnesyltransferase | Trophozoites differentiation to schizonts and schizonts to ring transitions | (Chakrabarti et al., 2002) |

***P. falciparum* essential parasite proteins and their roles in the survival of the malaria parasite in the human host.**

## The Key Stages of the Malaria Parasite in the Human Host

Upon entry into the human host, the malaria parasite goes through several stages that facilitates its survival and evasion of the human immune system as has been exemplified in the life cycle above. Briefly, sporozoites mature into schizonts in the hepatocytes which burst to yield merozoites. The merozoites invade other red blood cells which develop into the ring, trophozoite and schizont stage and the schizont ruptures and amplifying the merozoite repertoire in the human host. Some of the merozoites may develop into gametocytes via the ring stage. These various stages of the malaria parasite could be targeted in an attempt to cure the disease (Rono et. al., 2018)

## Merozoites

They are invasive forms that allow the entry of the parasite into the host erythrocytes. Their specialized organelles, rhoptries and micronemes enable a non-lytic entry into the host erythrocytes while their small, rounded vesicles and exonemes are for merozoites to exit from schizonts. The rhoptries, two in number and the micronemes, quite numerous, converge at the merozoites' apex where they secrete the proteins required for erythrocyte binding and entry. After the full development of the merozoites, they secrete small vesicles and elongated exonemes to dissolve the schizonts for exit (Nasamu et al., 2020). The pellicle (a three-membrane layer)

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underneath the thick bristly coat on the merozoites surface contains the actomyosin motor for propelling the merozoites during invasion. Other components of the merozoites are microtubules, a nucleus, a mitochondrion, an apicoplast, and some ribosomes.

**Sporozoites**

Sporozoites are the invasive forms transmitted to a human host by Anopheles mosquitoes and the migrate to the liver upon injection (Yang et al., 2017). They possess organelles containing a number of invasive proteins such as the circumsporozoite protein (CSP) which currently is a component of the RTSS and R21 vaccines, and thrombospondin-related antigen protein (TRAP) that enable attachment to the glycosaminoglycans of the host cells. Plasmepsin VI is an important protein required for the development of sporozoites in the midgut of an Anopheles mosquito.

**Trophozoites**

These are asexual forms of the parasite occurring in the human host. They are vacuolated, amoeboid and uninucleated. The application of PfSpdSyn inhibitor cyclohexylamine confirmed the arrest of parasite development at trophozoite stage in *P. falciparum* 3D7 (Becker et al., 2010).

**Target sites for antimalarial drugs**

Malaria drugs over the years have changed with the development of resistance (Colón-Lorenzo et al., 2020). *S*everal sites and biosynthetic pathways are used as antimalarial targets: merozoite invasion of the erythrocytes, trophozoite growth, sporozoite development, hemoglobin metabolism and biosynthesis of essential compounds, sporozoites invasion of the hepatocytes, nuclear division

in the erythrocytic stage, merozoite and sporozoite egress, gametocytogenesis, and the parasite's other proteins produced by genome translation. Selective inhibition of one of these proliferative processes provides therapeutic intervention strategies (Morahan et al., 2020).

**Proteases**

Proteases are catalytic and regulatory molecules involved in protein turnover to their constituent amino acids for generating building blocks for new proteins (Teixeira, Gomes & Gomes, 2011;Alam, 2014). These enzymes catalyze the degradation of proteins into smaller peptides and amino acids in a process called proteolysis. Most proteins are activated or deactivated through proteolysis for the survival of the organism and for effective mounting of disease. The plasmodium genome encodes approximately 170-plasmodium protease, as indicated in the MEROPS database of proteases (Rawlings, Obrien & Barrett, 2002), which presumably play both regulatory, and effector roles in the various developmental stages of the plasmodium parasite e.g., protein homeostasis, invasion, immune evasion, trafficking, cell signaling, inflammation and catabolism (Li, Child & Bogyo, 2012; Roy, 2017). Falcipains and plasmepsins are specific proteases involved in hemoglobin degradation (Goldberg, 1992; Sijwali & Rosenthal, 2004; Qidwai, 2015). Deu (2017) contends that some protease family members have their genomic homologues in the human host making it more important to identify which ones to target: those with human homologues or those with no human homologues. Similarly, the aforementioned review intimates that proteases with no human homologues might play specific roles in the parasite (ability to enter RBCs, degrade hemoglobin for nutrients and escape host defensive mechanisms) and their inhibition providing a major drug discovery step . The review however, argues strongly that similar amino acid sequences do not always produce a specific target site sequence, and therefore, inhibition of human host proteases does not always cause adverse side effects, hence, targeting host proteases might provide some advantage. There are about five families of *Plasmodium* proteases, which can serve as targets for malarial drug or vaccine discovery; few members of these families have been studied (Verma, Dixit & Pandey, 2016). The families of proteases are aspartate (Plasmepsins, SPP), cysteine (serine repeat antigen (SERA), Dipeptidyl aminopeptidases **(**DPAP)), metallo (falcilysin, peptidase), serine (subtilisin-like protease (SUB), Rhomboid proteases **(**ROM), Proline aminopeptidase (PAP)) and threonine (proteasome) proteases (Mckerrow et al., 1993; Gluzman et al., 1994;

Blackman, 2000; Rosenthal, 2004, 2011; Klemba and Goldberg, 2012 ; Deu, 2017). Proteases have effectively served as drug targets for diseases such as hepatitis C virus, Human Immunodeficiency virus, and hypertension (Alam, 2014). Protease inhibitors currently considered include among others leupeptin, anti-pain, E-64 and chymostatin (Shibeshi, Kifle & Atnafie, 2020)

**Falcipain**

These are *P. falciparum* cysteine proteases that are involved in the degradation of hemoglobin (Machin et al., 2019), erythrocyte invasion and erythrocyte rupture (Marco and Miguel, 2012). Lee et al., (2003) investigated the antimalarial activities of protease inhibitors and showed that the cysteine proteases inhibitors block the hydrolysis of hemoglobin and thus hinder the development of cultured *P. falciparum*. FP1 plays a crucial role in oocyst production in the developmental stages occurring in the midgut of the mosquito and could aid in host cell invasion. Falcipain 1, 2 and 3 (FP2, FP2' and FP3) are the key hemoglobinases in the food vacuole (Marco & Miguel, 2012;Nair et al., 2013). Another suggested function played by these Falcipains is the transformation of pro-plasmepsins into mature active enzymes (Teixeira et al., 2011). Their inhibition could affect the degradation of hemoglobin, killing the parasite. Falcipain 2 has close interacting partners that may depict functional relationship (Fig. 2). (E)-chalcone 2 inhibitors have been shown to bind FP2 effectively (Machin et al., 2019).

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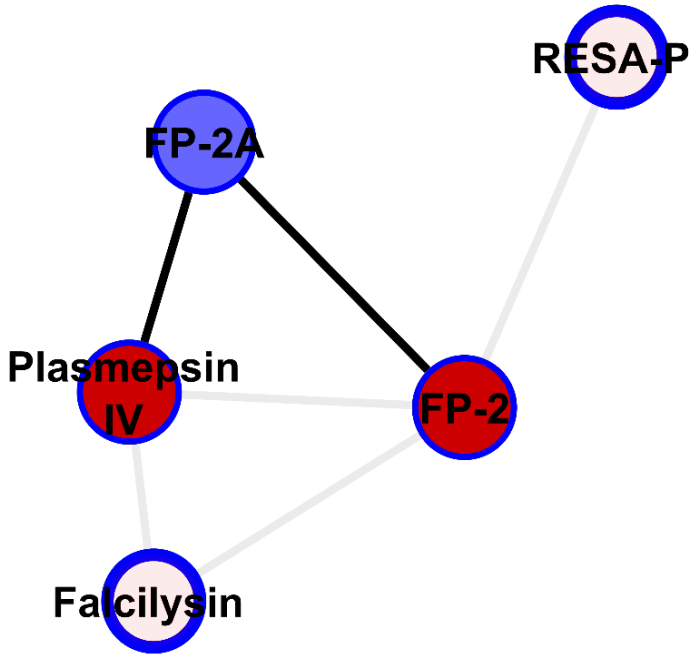


**Figure. 2. Protein network interaction diagram for falcipain 2.** This protein network shows the closest proteins that associate with it and may imply a functional relationship with interacting partners such as Plasmepsin, falcilysin, RESA-like protein and falcipain 2A. created using cytoscape 3.8.1.

**Legend**: FP-2 = Falcipain 2, FP-2A = Falcipain 2A, RESA-P = RESA-like protein.

**Subtilisin-like proteins**

Sub1, a calcium-dependent serine protease that aids in the final maturation step of MSP1 is important for erythrocyte egress and invasion (Boonyalai et al., 2018; Nava et al., 2019). Sub2 synthesized during asexual stage (Withers-Martinez et al., 2004) schizogony and expressed during merozoite differentiation encodes an integral protein located in the merozoite dense granules (Barale et al., 1999), and possibly participates in the latter stages of erythrocyte invasion. SUB2 is involved in the shedding of the surface proteins of the parasite (Collins et al., 2020). This aids in

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the erythrocytic enveloping of the parasite during the internalization in parasitophorus membrane. This provides a suitable target for malaria drug development. Molecular docking and dynamic studies have leveraged the SUBI inhibitors as drug targets (Fulle et al., 2013). It has also been shown that the action of PfSUB1 is under the control of cGMP-dependent Protein Kinase. SUB2 and its interaction partners in an elaborate protein network is shown in (Fig. 3)

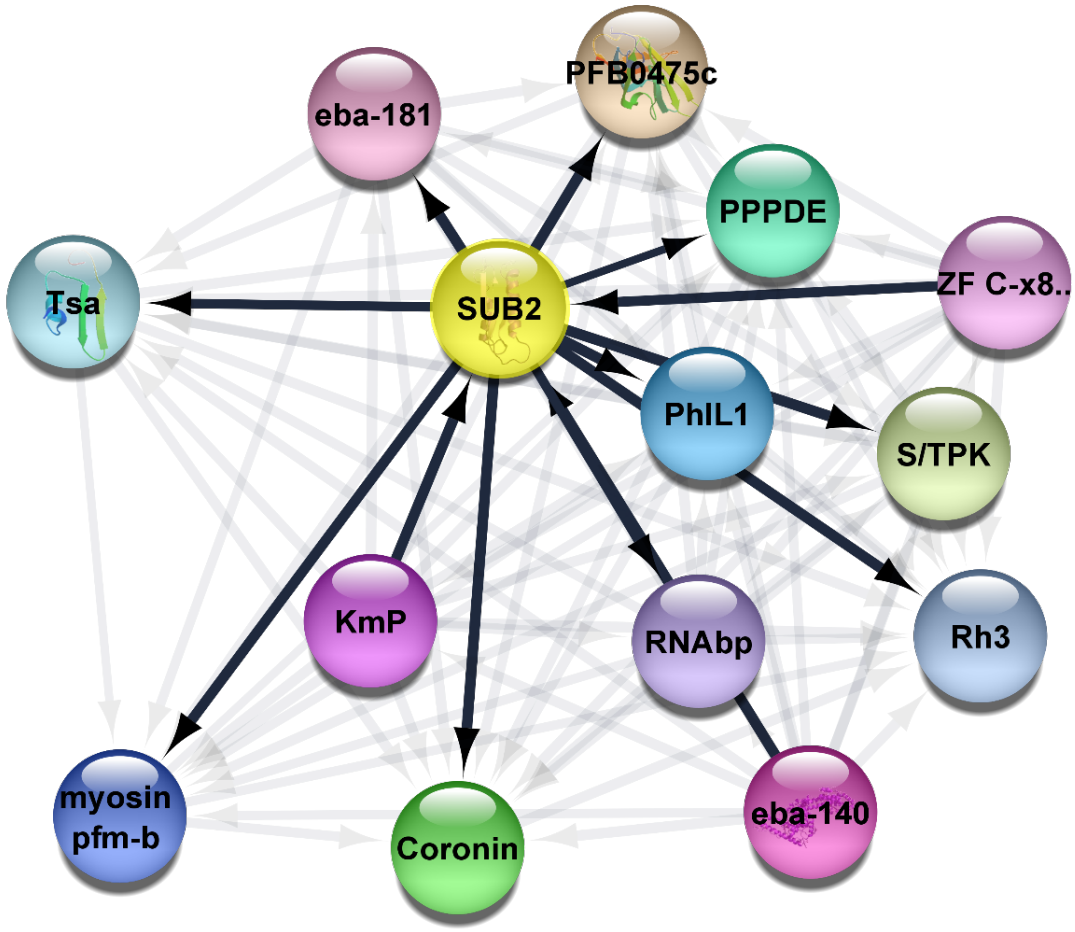


**Figure. 3. Protein network interaction diagram for SUB2.** Subtilisin-like protein 2 and its closest interacting partners. This network is suggestive of and predictive of Subtilisin-like protease 2 close association with putative photosensitized INA-labeled protein 1, putative RNA binding

protein, putative Kelc motif containing protein, putative coronin, Myosin motor domain-containing myosin pfm-b domain that belongs to the TRAFAC class myosin-kinesin ATPase superfamily, trophozoite stage antigen, erythrocyte binding antigen -181, Zinc finger C-x8-C-x5-C-x3-H type, putative serine/threonine protein kinase, putative reticulocyte-binding protein 3 and erythrocyte binding antigen -140. Network created using cytoscape 3.8.1

**Legend:** SUB2 = Subtilisin-like protease 2, PFB0475c = Conserved Uncharacterized protein, PPPDE = PPPDE Peptidase, PhIL1 = Photosensitized INA-Labeled Protein 1, Putative, RNAbp= RNA binding protein, putative, KmP = Kelc motif containing protein, putative, Coronin = coronin, Myosin pfm-b = Myosin motor domain-containing protein, an unconventional myosin pfm-b;, belonging to the TRAFAC class myosin-kinesin ATPase superfamily.,Tsa = Trophozoite stage antigen, eba-181 = erythrocyte binding antigen -181, ZF C-x8.. = Zinc finger C-x8-C-x5-C-x3-H type, putative, S/TPK = Serine/threonine protein kinase, putative, Rh3 = Reticulocyte-binding protein 3, eba-140 = erythrocyte binding antigen -140

**Merozoites invasion and egress of erythrocytes and inhibition.**

The discharge of merozoite from schizont exposes it to the host immune system a few minutes after, hence has to invade new erythrocytes before being noticed. Proteolysis plays important regulatory and effector roles in rapid and highly regulated egress (Collins et al., 2017) and invasion. Proteolysis involving cysteine and serine proteases is required in the rupture of the parasitophorous vacuole (PV) and erythrocyte membranes for egress. Cell signaling through cGMP activates downstream processes which lead to the secretion of proteins from exonemes and micronemes into the PV then on the merozoites surface, which cause the exonemal subtilisin-like protease 1 (SUB1) (Boonyalai et al., 2018) to process egress-invasion specific proteins. Examples

are members of the serine repeat antigen (SERA) family with three active proteins (SERA 6-8) and six inactive proteins (SERA 1-5, 9) due to the presence of ser instead of catalytic cys. Cysteine and serine inhibitors block egress and removes the protein coat around the merozoite. Deletion of the *Plasmodium berghei* homologue prevents the expression of SERA8 and therefore, prevents sporozoites egress and invasion. In addition, the knockout of some SERA5 leads to incomplete egress phenotype decreasing invasion efficiency.

**Plasmodium Rhomboids (PROMs)**

PROMs are serine proteases involved in erythrocytic invasion of *P. falciparum* (Srinivassan et al., 2009). They cleave their substrates within transmembrane domains (Mataradchakul et al., 2018; Singh et al., 2007). Mataradchakul and colleague (2018) identified PvROM-like 1 protein in all the eukaryotic stages of P. vivax and suggested the possibility of its involvement in the invasion of erythrocytes due to the similarity of their amino acid sequence to PfROM1. Similarly, Gallenti et al., (2021) suggested the roles of ROM4, 6-8 in erythrocyte invasion. Survival through escape from the host defensive mechanisms is inhibited with downgrading of the activities of the rhomboids. The identification of the *Plasmodium* rhomboids, PfROMs (recently found to be secreted by mononemes (Singh et al., 2007) for *falciparum* and PvROM for *P. vivax* would be essential for malaria treatment and vaccine development as inhibitors would block host-cell invasion(Gandhi et al., 2020). The ROMs mostly likely have other interacting partners that possibly interact with them spatiotemporally to potentiate their roles the cleavage of adhesins (Fig. 4)

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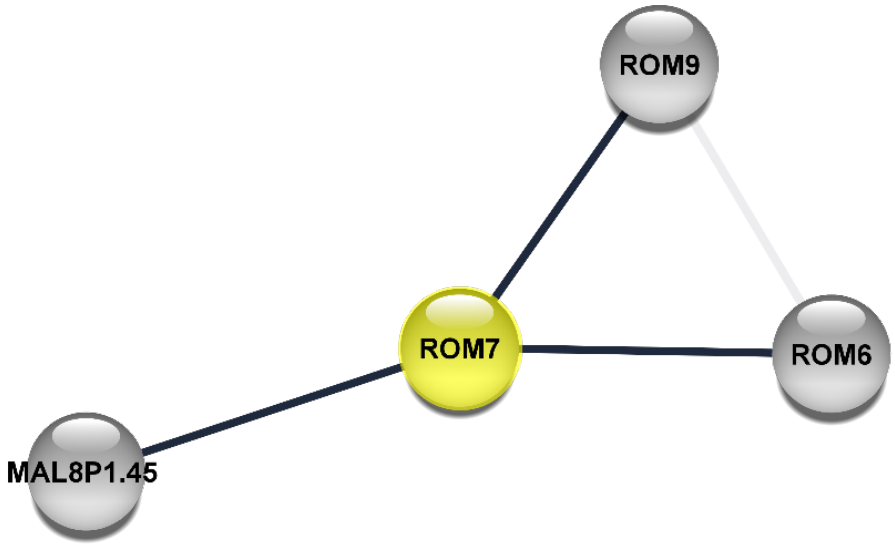


**Figure. 4. Protein network interaction diagram for ROM7.** The Rhomboid protease (ROM7) (Neafsey et al., 2013) possesses a serine-type endopeptidase activity, its serine nucleophile when activated by a proton it carries out hydrolysis of the internal, alpha-peptide bonds in a polypeptide chain. This protein has shown putative interactions with ROM8 and ROM9. The *Plasmodium* rhomboid proteases are involved in most enzymatic events during the invasive stages of the malaria lifecycle. The invasion of *Plasmodium* depends on the parasite transmembrane adhesins and these adhesins have to be processed by cleavage to be activated by PfROMs such as PfROM1, PfROM4 PfROM7, etc. (Baker et al., 2006). Interaction network created using cytoscape 3.8.1.

**Legend:** ROM = Rhomboid protease, MAL8P1.45 = Uncharacterized protein

**Dipeptidyl aminopeptidases (DPAPs)**

DPAPs are cysteine proteases that play important roles in various stages of parasite development and other microbes causing malaria, toxoplasmosis, and Chagas's disease (Dalal & Klemba, 2007;

Klemba, Gluzman & Goldberg, 2004). They cleave protein substrates from the N-terminus producing dipeptides (M. Tan et al., 2020). In the inhibition assay performed by Sanchez and colleagues (2019), it was found that some selected compounds from both UCSF Small Molecule Discovery Center's Diversity collection and Celera Protease Inhibitor Collection inhibited the activity of DPAPs. DPAP1 localizes in the digestive vacuole and plays roles in the hemoglobin degradation pathway (Deu et al., 2010). DPAP2 is expressed in gametocytes and its knockout (KO) reduces gamete egress from iRBCs. DPAP3 is involved in erythrocyte invasion and egress (Deu et al., 2010; Lehmann et al., 2018). DPAP1 and -3 are essential in asexual growth (Yamada et al., 2017) and their knockout results in stagnation of the erythrocyte cycle. The selective inhibition of these enzymes provides a new approach for vaccine and drug development. The role played by DPAPs in growth and transmission of the parasite provides possible targets for eliminating the parasite at those stages (Tanaka et al., 2013). Development of DPAPs inhibitors are a great approach to combating the disease.

**Apical membrane antigen**

AMA complexes with plasmodium rhoptry neck (RON) as part of the moving junction is formed between the host cell and invading parasite. AMA1 plays an important role in blood-stage replication and antibodies against AMA1 inhibit erythrocyte invasion (Drew et al., 2012). AMA1 could be involved in the reorientation or initiate junctional contact (Mitchell et al., 2004). This cleft is conserved in both the P. falciparum and P. vivax (MacRaild et al., 2011). AMA inhibition interferes host-cell infection by sporozoite (Silvie et al., 2004). This makes AMA1 an essential tool during zoites invasions of host cells and as their inhibition, an excellent antimalarial target.

A subset of serine protease inhibitors block the processing and shedding of both AMA-I and TRAP and inhibits sporozoite infectivity, suggesting that interference with sporozoite proteolytic processing may constitute a valuable strategy to prevent hepatocyte infection.

**Cyclic GMP-Dependent Protein Kinase**

cGMP and PfPKG play roles in the asexual replication invasion and egress (Vanaerschot et al., 2020) of the plasmodium parasite as cGMP regulate the activation of PKG (Kim & Sharma, 2021). Taylor et al., (2010) used 4-[2-(4-Fluorophenyl)-5-(1-methylpiperidine-4-yl)-1H pyrrol-3-yl] pyridine as inhibitor to identify its inhibitory effect on ring stage formation and reported the damaging effect on schizont. They further stated that the inhibitor had no other effect on the host red cell kinases. PKGs could serve as plausible antimalarial targets for drug or vaccine development as it is vital in the replicative cycle occurring in erythrocytes. The amidated analogue 3,6-diphenylated imidazopyridazines has moderate *in vitro* inhibitory effect on cyclic guanidine monophosphate (cGMP)-dependent protein kinase (PKG) activity (Cheuka et al., 2021). Baker et al. (2017) carried out study using imidasoyridine and reported a similar result. Further study could lead to the synthesis of more potent drugs or vaccines with definite inhibitory effect on the protein and possibly end the replicative cycle.

**Merozoite surface protein**

MSP, a ligand, coats the merozoite surface in all species of the parasite (Baldwin et al., 2015) and is essential in erythrocyte invasion (Beeson et al., 2016; Chandramohanadas et al., 2014; S.-H. Lee et al., 2020). MSP forms a complex with RBC glycophorin A (GPA) and RBC band 3 during the adhesion of merozoite to the RBCs. Antibody prevalence to Pf antigen provides promise as a key to gaining immunity (Ondigo et al., 2020). MSP1 (Jäschke et al., 2017; Thái et al., 2018), MSP2

(Das et al., 2017; Eacret et al., 2019; Lu et al., 2019; MacRaild et al., 2011), MSP3 (Lê et al., 2020),and MSP4 (Deshmukh et al., 2018; Perraut et al., 2017), and MSP10 (Bendezu et al., 2019; Nagaoka et al., 2019) blood-stage protein providing a promising vaccine candidate as they have their-specific antibodies have shown protection against malaria.

**Transfer RNA binding proteins (tRNAbp)**

In malarial infection, the parasite does switch hosts (mosquito to human and back to mosquito). The switch from one host to the other require specialized factors and gene regulatory mechanisms. Research works pointing to the role of tRNAbp in translational repression required by the parasite to establish and colonize the mosquito guts has been demonstrated (Braks et al., 2008; Zhang et al., 2010; Quenault, Lithgow & Traven, 2010)

For instance, the sexual parasite development is controlled by one tRNAbp known as the DEAD-box RNA helicase of the DDX6 family (Braks et al., 2008). Other factors such as the eIF2alpha kinase IK2 controls sporozoites latency, it carries out this function by phosphorylating eIF2alpha and down-regulating protein synthesis ( Zhang et al., 2010). The RNA-binding proteins of the Puf-family, *Plasmodium* Puf1 and Puf2 has been demonstrated to be critical during sporozoite stage transformation ( Quenault, Lithgow & Traven, 2010). Usually, Puf proteins bind to the 3′ UTR of target mRNAs and repress their translation or activate their degradation (Wickens et al., 2002; Quenault, Lithgow & Traven, 2010). For instance, the *P. berghei*, *Puf2* play a part in the regulation of *IK2* and inhibits premature sporozoite conversion (Müller, Matuschewski, & Silvie, 2011). Inside mosquito salivary glands *puf2(-)* mutants are unable to initiate malaria infection. In a gene disruption study in *P. falciparum Pf*Puf2 has been demonstrated to hold critical role in repressing

gametocytogenesis and male gametocyte differentiation in the human malaria parasite (Miao et al., 2010).

The mechanism of action of tRNAbp has been elucidated partially to include roles in 2-thiolation of mcm5S2U at tRNA wobble positions of tRNA(Lys), tRNA(Glu) and tRNA(Gln) (Duechler et.al., 2016). It directly binds tRNAs and probably acts by catalyzing adenylation of tRNAs, an intermediate required for 2-thiolation. It is unclear whether it acts as a sulfurtransferase that transfers sulfur from thiocarboxylated URM1 onto the uridine of tRNAs at wobble position. This protein is involved in the pathway 5-methoxycarbonylmethyl-2-thiouridine-tRNA biosynthesis, which is part of tRNA modification. tRNAbp and its interaction partners are illustrated in the protein network (Fig.5)

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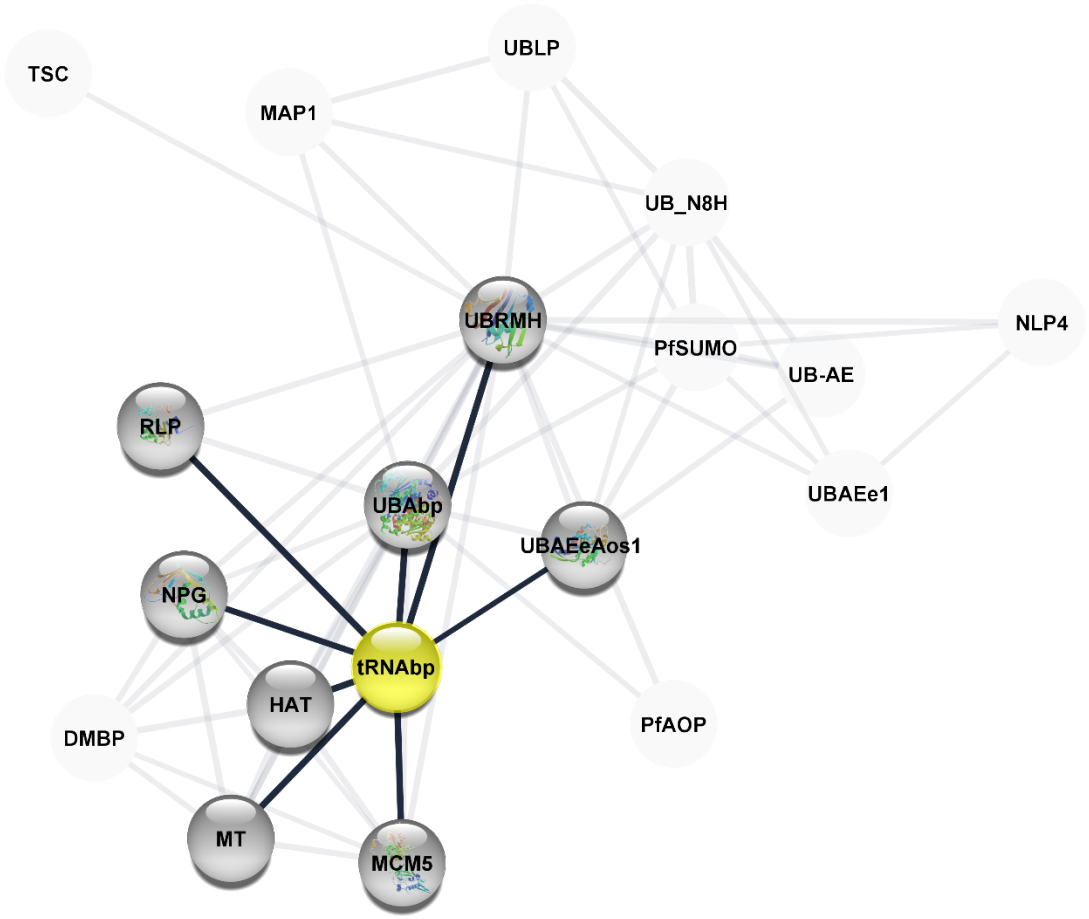


**Figure 5. Protein network interaction diagram for tRNA binding protein (tRNAbp).** tRNAbp interacts closely with Histone acetyltransferase, DNA replication licenses factor MCM5, methyl transferase, Nucleolar Peribosomal GTPase, Rhodanese like protein, Ubiquitin related modifier homologue, Ubiquitin binding protein and Ubiquitin activating enzyme E1. Interaction network created using cytoscape 3.8.1

**Legend:** TSC = Tubulin-specific chaperone, putative, MAP1 = Microtubule associated protein 1, putative, UBLP = Ubiquitin-like protein, UB_N8H = Ubiquitin-like protein nedd8 homologue, putative, UBRMH = Ubiquitin related modifier homologue, pfSUMO = Small ubiquitin related modifier, putative , UB_AE = Ubiquitin activating enzyme , NLP4 = Nuclear pore associated protein 4, putative, UBAEe1 = Ubiquitin activating enzyme E1, putative UBAEeAos1 = Ubiquitin

activating enzyme (E1), subunit Aos1, PfAOP = 1 cys peroxidoxinPeroxidoxin, UBAbp = UBA/THIF type NAD/FAD binding protein, putative, tRNAbp = cytoplasmic tRNA 2- thiolation protein 1, HAT = Histone acetyltransferase, putative, MCM5 = DNA replication licenses factor MCM5, putative, MT = methyl transferase, putative, DMBP = DPH-type MB domain-containing protein, NPG = Nucleolar Peribosomal GTPase, putative, RLP = Rhodanese like protein, putative, **pfSUMO**

**Protein Kinases**

The essentiality of protein kinases in the biological roles of the parasite is not fully understood. Imidazopyridazines were reported by Green et al., (2016) to be an inhibitor of the kinase PfCDPK1, an essential protein in asexual blood stages. The inhibition of Serine/Arginine-rich proteins, Protein-Phosphorylating Cyclin-dependent kinase-line kinases inhibit the erythrocytic-stage replication (Kern et al., 2014). Mustière et al., (2020) reviewed a number of inhibitors against plasmodial kinases.

**Organelles and plastids**

Several Organelles have been the target sites for antimalarial drugs. The food vacuole of the parasite serves as target sites for antimalarial drugs such as chloroquine-prevents the bio-crystallization of heme (Teixeira et al., 2011). The apicoplast is involved in the synthesis of isoprenoids (Tewari et al., 2021) from isopentenyl pyrophosphate (IPP) in the blood stage of the parasite (Miller et al., 2014; Rai et al., 2017). Prokaryotic DNA replication inhibitor (ciprofloxacin), prokaryotic translation inhibitors (chloramphenicol, doxycycline (Okada et al., 2020), tetracycline, clindamycin, azithromycin, erythromycin, and clarithromycin), a tRNA synthase inhibitor (mupirocin), and two IPP synthesis pathway inhibitors (fosmidomycin and

FR900098) are apicoplast targets (Uddin et al., 2018). The research conducted by Kumarihamy and colleagues (2020) showed the *in vitro* antimalarial activity of Botryosphaeria dothidea due to the common metabolic pathway shared by the apicoplast in Plasmodium parasites and plastids in plants. Apicoplast has no human homolog and would therefore provide a more promising antimalarial drug target (Tan et al., 2021).

**Glucose Transporters**

The transport of glucose across and within cells is essential for the growth and development of organisms. The transport of glucose for energy is employed by the parasite in the food vacuole and is carried out by glucose transporters (Slavic et al., 2011). The *Plasmodium* glucose transporter (PfHT) is known to be essential for parasite growth and survival (Kraft et al., 2015). Novel antimalarial drugs could be made to target these promising *Plasmodium* metabolic pathways (Jiang et al., 2020). Heitmeier et al., (2019) showed strong evidence that selective inhibition *of P. falciparum* hexose transporter (PfHT1) will lead to the shortage of glucose supply to the parasite. They investigated the role of O-3-hexose derivative and observed that it selectively inhibited PfHT1 and not Human Glucose Transporter 1(GLUT1). Notably, the O-3-hexose derivative has been shown to inhibit glucose uptake by *P. vivax* (Meireless, et al., 2017).

**Serine Repeat Antigen (SERA)**

These are serine-type (SERA 1-5 and SERA 9) and cysteine-type proteases (SERA 6-8) expressed in the parasitophorus vacuole of the blood-stage parasite (McCoubrie et al., 2007). *Plasmodium* SERA is one of the SERA multigene family of eight clustered homologues (Arisue et al., 2020) on chromosome 2 and one on chromosome 9. Putrianti et al., (2020) identified *Plasmodium bergei* SERA4 to an important requirement in blood-stage infection and a disruption of this protein

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consequently inhibiting egress from infected cells. PfSERA 5 is an asexual blood-stage antigen (Palacpac et al., 2011) that plays roles in parasite growth (Smith et al., 2020), egress (Iyer et al., 2018), and invasion (Kanodia et al., 2014) by regulating egress timing to coincide with merozoite maturation and disrupt red blood cell membranes. Table 2 provides a list of the essential parasite proteins, their location and known inhibitors.

**Table 2: Important proteins, site of localization or release, function, and modes of inhibition**

| Proteins | Sub | Site | Function | Inhibition/Inhibitor | Ref. |
|---|---|---|---|---|---|
| Serine Repeat Antigen (SERA) | SERA 5 | Erythrocytes (within the parasitophorous vacuole) | Regulate egress timing to coincide with maturation of merozoites | Conditional knockout (KO) produces immature egress phenotype leading to decrease in invasion efficiency | (Arisue et al., 2020) |
| | SERA 6 | | Proteolytically activated by SUB1 to disrupt the Red blood cell membranes | | (Thomas, et al., 2018; Ruecker, et al., 2012) |
| | SERA 8 | Expressed in sporozoites | | Inhibitors prevent sporozoite egress from oocysts | (Arisue et al., 2020) |
| Subtilisin-like Protease (SUB) | SUB1 | Erythrocytes, hepatocytes | Involved in the cleavage of parasitophorous vacuole membrane, rhoptry, and erythrocyte membrane proteins | | (Boonyalai et al., 2018; Nava et al., 2019) |

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| | | | | | |
|---|---|---|---|---|---|
| | SUB2 | Secreted by micronemes onto the surface of the merozoite | Sheddase involved in shedding the protein coat of the merozoite | | (Boonyalai et al., 2018; Nava et al., 2019) |
| Plasmodium falciparum Rhomboids (PfROM) | PfROM4 | Located on the surface of the parasite | Cleaves adhesin proteins involved in parasite attachment to erythrocyte surface | | (koussis, 2018; O'Donnell, et al., 2006) |
| | PfROM1 | Found at the apical end of merozoites in liver stages | Cleaves apical membrane antigen 1 | | (Baker, et al., 2006) |
| Merozoite Surface Protein (MSP) | MSP1 | | Destabilizes the erythrocyte cytoskeleton | Cleavage reduces parasite egress | (Das et al., 2015) |
| Dipeptidyl aminopeptidase (DPAP) | DPAP2 | Expressed in gametocytes | Regulates parasite egress, and efficient erythrocyte invasion by parasites | Knockout (KO) decreases gamete egress | (Lehmann et al., 2018) |
| | DPAP3 | Erythrocytes | erythrocyte invasion | DPAP3 inhibitors block egress upstream of SUB1 activation | (Lehmann et al., 2018) |
| Aspartyl protease (Plasmepsins, PM) | PM I | Erythrocytic stage | Essential in partial degradation of hemoglobin | Knockout has minimal effect on parasite replication | (Coombs et al., 2001) |

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| | | | | | |
|---|---|---|---|---|---|
| | PM II | Erythrocytic stage | Essential in partial degradation of hemoglobin | Knockout has minimal effect on parasite replication<br><br>Extra copies removal sensitizes the parasite to piperaquine | (Coombs et al., 2001) |
| | PM III | Erythrocytic stage | Essential in hemoglobin degradation into smaller oligopeptides | Knockout has minimal effect on parasite replication<br><br>Extra copies removal sensitizes the parasite to piperaquine | (Mohapatra et al., 2010; Moura, Dame & Fidock, 2009) |
| | PM IV | Erythrocytic stage | Essential in hemoglobin degradation into smaller oligopeptides | Knockout has minimal effect on parasite replication | (Liu et al., 2006) |
| | PM V | An endoplasmic-resident integral membrane protein | For protein export | Inhibition blocks biological functions such as protein trafficking on invaded host cell surface | (Blackman & Bannister, 2001) |
| | PM VI | Expressed in transmission-stage parasites | Important in midgut sporozoite development and function | Knockouts prevent sporozoite formation and thus blocks transmission | (Mastan et al., 2017) |

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| | | | | | |
|---|---|---|---|---|---|
| | PM VII | Located in cytoplasm of parasite's sporozoites and ookinetes | Function is not fully known but might play role in midgut transversal | | (Deu, 2017) |
| | PM VIII | Expressed in transmission -stage parasites | Function is not known | Knockout prevents sporozoite motility<br><br>Absence reduces the number of salivary gland and hemolymph sporozoites | (Cheuka et al., 2020; Myung, Marshall & Sinnis, 2004) |
| | PM IX | Localized to rhoptries | For RBC invasion | | Cheuka et al., 2020; Myung, Marshall & Sinnis, 2004) |
| | PM X | Localized to the exonemes | PM X is involved in both egress and invasion | Inhibitors prevent parasite progression from the liver to erythrocytes | (Nasamu et al., 2017) |
| Apical Membrane Antigen (AMA) | AMA1 | Secreted onto the merozoite surface | bridges interactions between components of the motor and rhoptry-derived proteins that are inserted into the RBCM after reorientation | | (Deu, 2017) |

## Gametocytogenesis

An essential part of the life cycle of malaria parasites is the sexual development from asexual blood stage into gametocytes. Burrows et al., (2017) indicate that the switch from asexual to sexual is essential as gametocytes are the form of malaria parasite taken up in blood by mosquitoes. Gametocytogenesis is therefore an important aspect in the transmission of the malaria parasite to the mosquito; making it an attractive target for the eradication of malaria (Rabinovich et al., 2017;Burrows et al., 2017; Sinden 2017).

*P. falciparum* gametocytes differentiate through five morphologically distinct stages into male (micro-) and female (macro-) gametocytes which are transmissible to mosquitoes for fertilization and formation of ookinetes (Hawking et al., 1971). Molecular mechanisms crucial during the differentiation stages of gametocytogenesis have been targeted to block the maturation of gametocytes and attenuate it transmission (Burrows et al., 2017). An aspartyl protease, Plasmepsin V (PMV) is active in both the asexual and sexual form of the malaria parasites. In gametocytes, PMV primes gametocyte exported proteins (GEXP) for export (Boddey et al., 2020; Russo et al., 2010). When PMV is inhibited, processing and export of GEXP is blocked and gametocyte's differentiation stages II-V inhibited (Jennison et al., 2019). *P. falciparum* casein kinase 2 (PfCK2) is another attractive potential antimalarial drug target. It is vital in both the proliferation of asexual forms of the malaria parasites and the stages of gametocytes development. Hitz et al., (2021) claim that the catalytic subunit PfCK2α is essential in the maturation of gametocytes. They opine that an experimental conditional knockout and knockdown of PfCK2α inhibit maturation of the gametocytes at early and late stages of gametocytogenesis respectively. Drugs such as 4-Aminoquinolines, 8-aminoquinolines, endoperoxides and antifolates have also been reported to show inhibition against one or two of the gametocytes' stages (early or late) (Duffy & Avery,

2013). Alsinol an arylacohol has exhibited inhibition against late-stage gametocytogenesis and this led to the formation of sterile immature gametocytes (Arias et al., 2020).

For a long time, the key drivers of gametocytogenesis were unknown and inhibition of the various stages of the gametocytogenesis has been the focus in attenuating the transmission of the malaria parasites. With the advent of forward genetics, concomitant methodologies have been leveraged to exemplify extensively how gametocytogenesis is regulated (Josling et al., 2018). In vitro studies have identified two genes as essential for the production of gametocytes; apetala-2 transcription factor (ap2-g) and gametocyte development protein 1 (gdv1) (Filarsky et al., 2018; Kafsack et al., 2014). GDV1 is present in the nucleus and its overexpression results in an increase in the production of gametocytes (reviewed in Josling et al., 2018). It was suspected to be a possible transcriptional regulator of the sexual commitment of malaria parasite and this notion has been corroborated by studies that have elucidated GDV1 as a probable epigenetic regulator (Filarsky et al., 2018). C-terminal truncation of GDV1 has led to failure of malaria parasites to develop into gametocytes (Tibúrcio et al., 2021). Kafsack et al., (2014) showed that AP2-G is a crucial regulator of gametocytogenesis, employing reverse genetics; malaria parasites do not produce gametocytes in its absence. They further noted that AP2-G is a transcription factor that has the ability to change the fate of the malaria parasite through its overexpression, a hallmark of master regulator. They also posit with experimental evidence that its expression induces the asexual-sexual conversion thus it regulates the sexual commitment phase.

GDVI in combination with other epigenetic factors such as histone deacetylase 2 (HDA2) and heterochromatin protein 1 (HP1) regulate the expression of ap2-g gene (Sinha et al., 2014; Kafsack et al., 2014;Josling et al., 2018). AP2-G as a master regulator of gametocytogenesis is another probable route to block malaria transmission and regulators in both upstream and downstream

pathways of AP2-G can be potential targets in blocking malaria transmission (Josling et al., 2020). The gametocyte proteome and exportome could serve as essential targets for the development of transmission blocking drugs and treatment interventions towards the global malaria elimination efforts. Leveraging modern day genomics, gametocytes could be targeted for treatment interventions that certainly geared towards malaria eradication ( Chawla et al., 2021)

**Schizogony**

The parasite on invasion of the host's erythrocytes proliferates through a mitotic cell division mechanism where new cells assemble around daughter nuclei after a number of asynchronous, closed nuclear divisions, a process called schizogony (Morahan et al., 2020). The mitotic process is controlled through the interplay between a number of protein kinases and phosphatases (Morahan et al., 2020). Microtubule organizing center (MTOC) within the nuclear envelope signals the onset of mitosis and meiosis in the plasmodium parasite. Aurora kinases have been implicated in the control control of the mitotic pathway in schizogony and are crucial targets for malaria drug discovery for effective eradication (Reininger et al., 2011). Morahan et al., (2020) identified Hesperadin as a potent inhibitor of Aurora kinase, an essential instrument in controlling centromere and kinetochores, thus blocking nuclear division in the intraerythrocytic stage of the parasite. Gantt and colleagues (1998) reported the inhibitory effect of lactacystin and lactacystin analogs on erythrocytic schizogony through proteasome and nucleic acid synthesis inhibition.

**Plasmepsins**

Different classes of enzymes are involved in the catalytic and developmental stages of the parasite. Plasmodium pepsins (plasmepsins, PM) are aspartic proteases involved in the various developmental stages of the parasite (Banerjee, et al., 2002). They are involved in biological processes such as hemoglobin degradation, protein export, sporozoite formation, midgut transversal, egress, and invasion (Deu, 2017). Plasmepsins I-IV with falcipain (papain-like proteases) and falcilysin (metalloprotease) are involved in the degradation of hemoglobin (Hb) for food in the food vacuole of the parasite (Moura et al., 2009). Nonetheless, PMs are the key enzymes in the degradation process, PMs I-II degrade hemoglobin partially and it is further degraded by PM IV and a histo-aspartic protease (HAP) into smaller peptides (Gowda et al., n.d.). Inhibition of these plasmepsins have minimal effects on parasite replication but the removal of extra copies of PM II-III resensitizes the parasite to piperaquine. PM V is responsible for protein export leading to differing morphological changes in the parasite, conversion of asexual parasite to sexual parasite and escaping host defensive mechanisms. Inhibitors like WEHI-842 and WEHI-916 (Boonyalai et al., 2018) block protein trafficking on invaded host cell surfaces. PM IX-X are involved in egress and invasion of erythrocytes and hepatocytes and in mosquito guts as well. Favuzza et al., (2020) demonstrated the modulating activity of PMX in merozoite invasion and maturation of proteins involved in invasion, parasite development, and egress. They further showed the inhibitory effect of WM856, WM4, WM5, and WM382 on PMX with WM382 having a dual effect as it inhibits PMIX. Li et al., (2016) reported the expression of PfPM VII and PfPM X in ookinetes and their contribution to the parasite transmission to Anopheles mosquitoes. Inhibiting these enzymes reduces the invasion of the hepatocytes and erythrocytes (Deu, 2017). Jiang et al., (2001) identified the noneptidyl compound WR268961 as a potent inhibitor of P.

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falciparum plasmepsins and reported pepstatin, SC-50083 and Ro40-4388 to display inhibition against plasmepsins.

As early as the 1970s, the depletion of spectrin and protein 4.1 which are erythrocyte membrane cytoskeletal proteins has been well-known for Plasmodium parasite infection (Homewood & Neame, 1974).

Plasmepsin II is an aspartic protease which has been characterized from *P. falciparum* and is noted for its degradation of the host cell hemoglobin within the acidic food vacuole of the parasite (Banerjee et al., 2002). Works by Le Bonniec et al. demonstrated an *in vitro* hydrolysis of erythrocyte spectrin by plasmepsin II (Le Bonniec et al., 1999), to involved aspartic protease(s) cleaving mainly within the SH3 motif of the spectrin a-subunit. They further showed that a recombinant plasmepsin II could digest spectrin and actin at higher pH of 6.8 suggesting that plasmepsin not only degrades hemoglobin, but also involved in cytoskeleton digestion of infected erythrocytes. Gluzman et al. (1994) showed that in the slightly acidic food vacuole of the parasite plasmepsin I and plasmepsin II commences the digestion of hemoglobin tetramers, which is followed with the cysteine protease falcipain that digests the fragmented substrate (Gluzman et al., 1994). The digestion of spectrin, actin and hemoglobin inevitable leads to the erythrocytes plasma membrane destruction, burst and release of parasites from the infected cell (Goldberg et al., 1991). Below is a diagrammatical illustration of the mechanism of action of plasmepsin I and II (Fig.6.).

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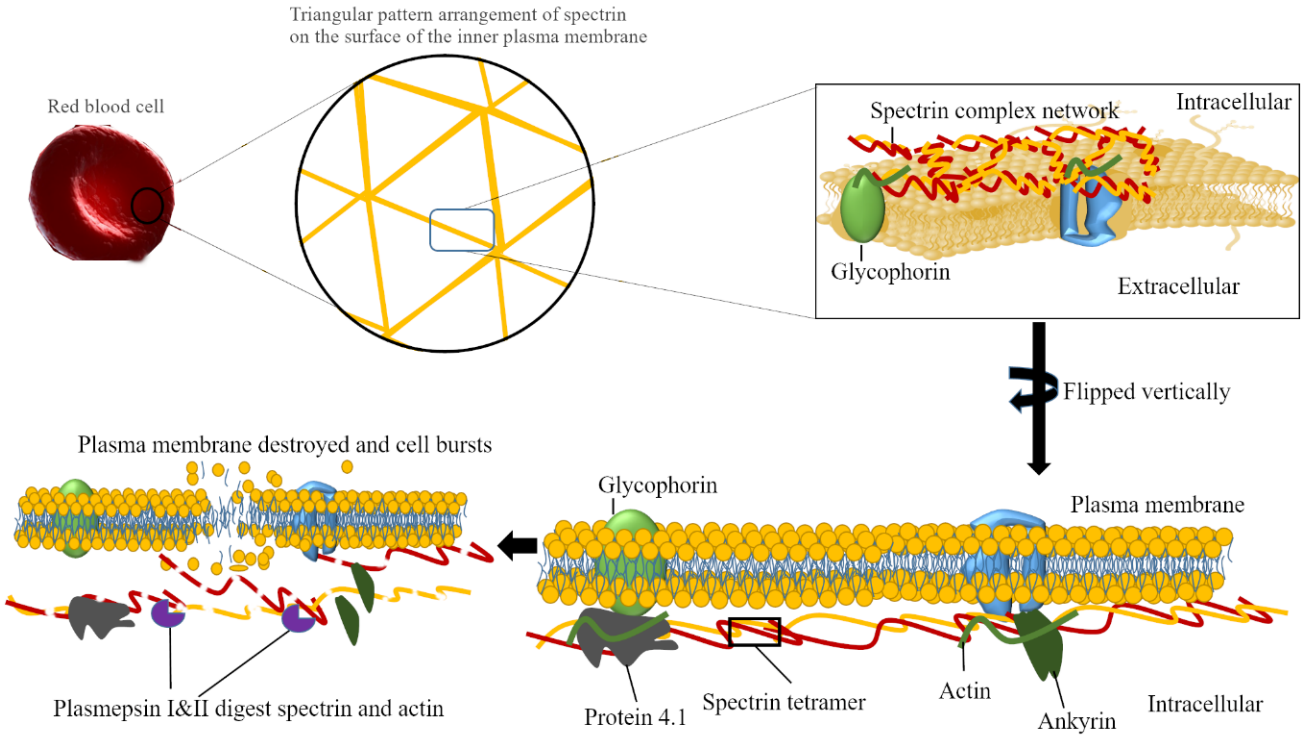


**Figure. 6. A Schematic showing the mechanism of action of plasmepsin I and II in *P. falciparum* destruction of erythrocytes.** This illustrates the role of Plasmepsin I & II in the destruction of spectrin and actin.

The two homologous aspartic proteases, plasmepsins I and II initiate the degradation of hemoglobin (Gluzman et al., 1994; Goldberg et al., 1991) and this is followed by cysteine protease, falcipain-2, and a metalloprotease, falcilysin, to degrade hemoglobin to small peptides (Eggleson et al., 1999). Known inhibitors of these proteases have been demonstrated to exert destructive effect on the malarial parasites, and hence the inhibition of the hemoglobin-degradation pathway has been employed heavily in drug development, for instance, polyhydroxyphenyl and hydroxamic acid derivatives has been shown to possess effective antimalarial activities (Holland et al., 1998). Similarly, mechanisms have been observed in the parasites' ability to digest and destroy the plasma membrane of the erythrocytes via the cleavage and degradation of the spectrin, actin and protein

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4.1 using the same proteases (plasmepsin I, II and falcipains). The "spectrinase activity" of plasmepsin II (Fig. 7) acts via cleaving the alpha-spectrin at a site located within the SH3 motif of the molecule (Le Bonniec et al., 1999). Plasmepsin II has a broad substrate selectivity and is known to localize extracellularly in the parasites in their late schizonts, presenting a strong evidence that plasmepsin II has a larger role in the parasites' life cycle than the digestion of hemoglobin alone (Le Bonniec et al., 1999).

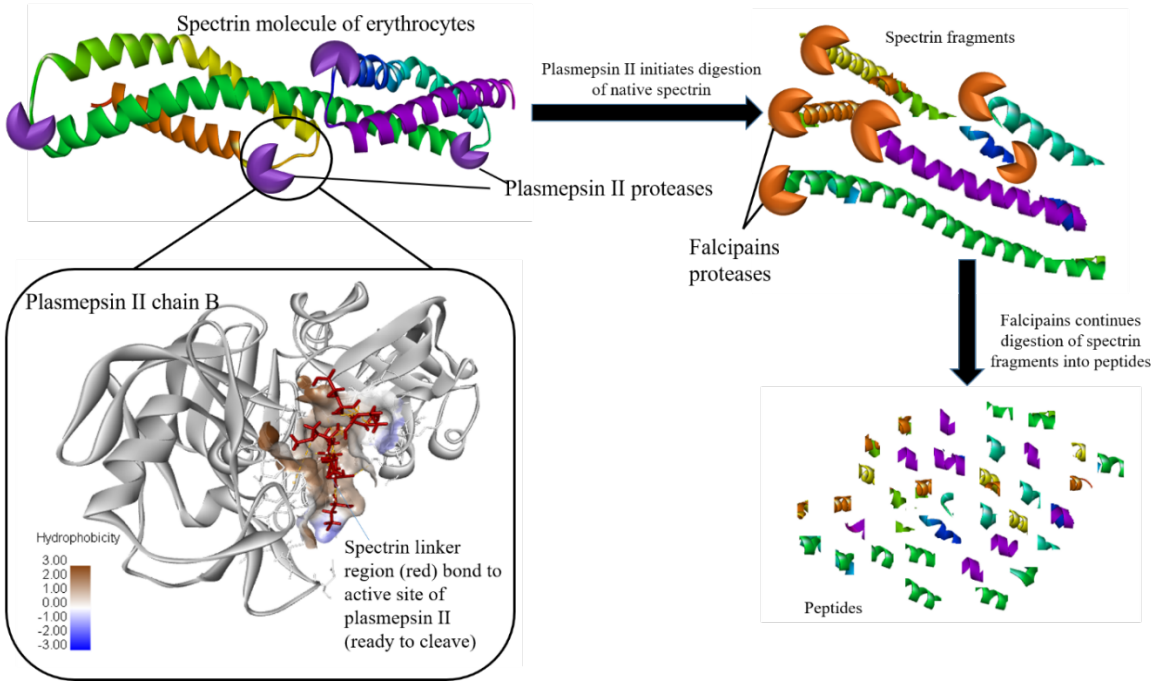

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**Figure 7. Illustration of "spectrinase activity" of plasmepsin II (PDB ID: 1SME) and falcipain of the malarial parasite on the human Erythroid Spectrin molecule (PDB ID:1S35).** Spectrin linker region was extracted using PyMol (Schrödinger, Inc.) and prepared as a ligand and docked onto the active site of Plasmepsin II chain B using PyRx software. Relative free binding energies of the interaction between Plasmepsin II and spectrin indicated a positive interaction between the two molecules indicative of the role of plasmepsin II in spectrin cleavages into short peptides.

## Conclusion

The need for surveys for new antimalarial drugs to augment the existing antimalarial therapeutics are made much more imperative at recent times since the malaria parasite has already developed resistance to some of these drugs. Several Proteases, organelles, and metabolic enzymes exceptionally peculiar to the malaria parasite are druggable targets for antimalarial agents. In this major review, we explored a number of these targets such as falcipain, *Plasmodium* rhomboids, dipeptidyl aminopeptidases, apical membrane antigen, cyclic GMP-Dependent Protein Kinases, merozoite surface protein, subtilisin-like proteins, Protein-Phosphorylating Cyclin-dependent kinase-like kinases, organelles and plastids, glucose transporters, serine repeat antigen (SERA), plasmepsins etc., elucidating the importance of these targets in the parasites' life cycle as well as demonstrating how such targets have or could influence drug discovery against malaria.

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