Biomolecular interactions between *Plasmodium* and human host: A basis of targeted antimalarial therapy

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1. INTRODUCTION

Malaria is a grievous disease, which is caused by the protozoan genus plasmodium parasite and spread in humans through the female anopheles. It remains one of the major health concerns especially in children and pregnant women. Five major Plasmodium species are responsible for malaria: *P. falciparum*, *P. vivax*, *P. ovale, P. malariae, and P. knowlesi* [1]. *P. falciparum* infection is the most virulent form. Extensive morphological variations and metabolic changes take place during the complex life cycle of plasmodium, which enables them to adapt to various host milieu. In 2020, there were approximately 241 million cases of clinical malaria worldwide, and 627000 estimated deaths occurred due to malaria. African countries share a high score of malaria burden globally. In 2020, 95% of malaria cases were found in the African region and 96% of malaria deaths occurred in this region only. In the African region children below 5 age accounted for 80% of death [2]. In the 21st century along with tuberculosis and HIV, malaria remains a major concern worldwide. Tropical areas are more prone to malaria due to the high humidity and warm temperature. Africa is the most affected continent, where improper treatment and approachability have become limiting factors. The world health assembly aims to decrease the malaria burden by 90% up to 2030 [3,4]. Malaria is an ancient disease, which exerts a strong evolutionary pressure on the human population to resist disease. Vice versa strong evolutionary pressure also has been exerted on plasmodium by the antimalarial drug and the human immune system. Transmissibility is written in the genome of each parasite, to coevolutionary exist between human and female Anopheles. Thus genomic tools and data are very important to understanding the fundamental genetic underpinning of the disease and developing novel strategies to eradicate malaria [5]. Although chemotherapy continues to be a clinical option that has been used for many decades, there are still some limitations that cause the failure of treatment. Chloroquine is being used as a first-line treatment; however, due to the development of resistance, the situation has changed drastically in endemic areas

[6]. Currently, combination therapy is recommended for the treatment and to prevent resistance; ACT (Artemisinin-based combination therapies) is nowadays most commonly used to overcome resistance. Moreover, recent reports suggest growing plasmodium resistance to artemisinin in Southeast Asia, which could result in an ineffective treatment thus further adding to the cases of malaria and death [7]. In the Greater Mekong Subregion (GMS), the emergence and spread of artemisinin and combination drug resistance in the *P. falciparum* occurred. When artemisinin is given along with the other drugs, all recommended ACT have been shown to decrease efficacy in some countries of GMS. Although novel drugs are not available in the near future, thus a Triple artemisinin-based combination therapies are recommended (combining artemisnin with two other partner drugs). It became the last safe and effective treatment option in the GMS to prevent the global emergence and spread of drug resistance [8]. Apart from the resistance, the cost of the ACT treatment also makes it less accessible, especially for patients in rural areas of developing countries. Development of resistance and emergence of drug-resistant parasites along with the absence of an effective vaccine[9] and the high cost of treatment further complicate the treatment therefore, there is an urgent need for newer antimalarial drugs based on novel molecular targets associated with *plasmodium*, which could enhance the efficacy of treatment [4]. This review focuses on various novel molecular targets exploitable for designing and developing efficacious and cost-effective drugs and drug delivery systems for the treatment and management of malaria.

2. LIFE CYCLE OF PLASMODIUM

The life cycle of the *plasmodium* is complex and is shared between two hosts. To infect multiple cell types, significantly different metabolic environments are adopted by the parasite for its survival and transmission. Within the erythrocytic phase alone, plasmodium encounters various key nutrients, including amino acids, lipids, and sugar due to significant differences in the host dietary nutrition,

pathogenesis, and cellular tropism [10]. The *P. falciparum* life cycle in humans includes the pre-erythrocytic stage, which initiates the infection; the asexual erythrocytic stage, which causes the disease; and the gametocyte stage, which infects mosquitoes and transmits the parasite. The malaria life cycle begins when the female *Anopheles* releases worm-like sporozoite into the human blood from its salivary gland. After invading the liver, each sporozoite multiplies over several days into tens of thousands of tiny merozoites packed into a single hepatocyte. No clinical signs or symptoms are seen during the pre-erythrocytic stages. After completion of the pre-erythrocytic stage, the hepatocyte is ruptured releasing merozoites into the circulation and initiating the "blood stage" of malaria infection. This stage is responsible for the disease. The intra-erythrocytic stage starts with the penetration of erythrocytes by invasive merozoites, which develop into trophozoites and further replicate into schizonts, which undergo segmentation to produce 16-20 merozoites. These merozoites start an additional round of invasion [11]. Merozoites quickly invade another erythrocyte where asexual multiplication takes place. Merozoites divide and grow within the erythrocyte, and then finally burst the erythrocyte. Malaria vaccines that target the blood stage are considered anti-disease vaccines and prevent or reduce the clinical illness without preventing subsequent infection[12–14]. Some blood-stage parasites develop into male and female gametocytes. These sexual forms of *Plasmodium* are taken up by the mosquito during a blood meal. These gametes form a diploid stage and develop through further haploid stages before migrating from the gut to the salivary glands of mosquitoes (**Figure 1**). Each mating pair of gametocytes yields up to 1,000 infectious sporozoites and further, these sporozoites are released into the host to complete the transmission cycle [13,15,16].

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3. PROPERTIES AND STRUCTURAL COMPONENTS OF RBC MEMBRANE

Unusual biconcave disc shape structure and exceptional membrane mechanical stability are important features of the RBC membrane, which are crucial for passing through the blood vessels for O_2-CO_2 exchange. These properties are furnished by the cytoskeleton of the RBCs[17]. A human red blood cell (RBC) membrane consists of 50% of protein, 20% of phospholipids, 20% of cholesterol molecules, and 10% of carbohydrates. Throughout the bilayer of lipids, there are multiple proteins are present, these proteins are vital for multiple functions. There are 20 major proteins and approx 850 minor proteins present in the erythrocyte membrane. These are transmembrane proteins, some are present on the outer surface, and others are located on the inner surface. Proteins are named according to their position occupied during electrophoresis. Like protein 4.1, it is located near band 4 and band 3, which is present in the electrophoresis band 3[18]. The most important protein of the RBC membrane is spectrin, which forms the membrane skeleton. It is a helical protein and is composed of 2 subunits α and β. Spectrin is attached to the transmembrane proteins at various locations. RBC is simply described as a "sack of hemoglobin", specifically adapted to carry out the gaseous exchange [19]. Erythrocytes have the unique feature of a flexible disk shape that helps to increase the surface area to volume ratio. Apart from that mature RBCs do not have a nucleus, ribosomes, and mitochondria in order to provide maximum space for hemoglobin (Hb). Thus, RBC is not competent to synthesize new proteins. The membrane of the erythrocyte is made up of three layers: the exterior part consists of the glycocalyx, which predominantly contains carbohydrates; the lipid bilayer, which possesses many transmembrane proteins, beneath its membrane skeleton is located. The membrane skeleton is a structural framework of proteins situated on the inner part of the lipid bilayer.

The elasticity of the plasma membrane is largely contributed by structural interaction between outer membranes with protein skeleton. Unusual deformability of the RBC membrane is associated with 3 factors; (i) The greater surface area to volume ratio (ii) Cytoplasmic viscosity of RBC, largely due to hemoglobin occupancy (iii) The

viscoelastic feature of RBC membrane, which is composed of 3 layers, outer surface covered by carbohydrate-rich glycocalyx, lipid bilayer at the middle and protein meshwork beneath lipid bilayer [11]. The deformability and durability of the erythrocyte membrane are largely due to the membrane skeleton. The membrane skeleton of the erythrocyte is composed of a pseudohexagonal array of spectrin, ankyrin, actin, actin-associated proteins, and protein 4.1R that laminate the cytosolic surface of the cell [20,21]. This network is attached to the erythrocyte cell membrane at two different locations: the first Ankyrin makes a bridge between spectrin and band-3 while the second protein 4.1R attaches junctional complex (JC) to glycophorin C [22]. Spectrin is the most plentiful protein of the membrane skeleton and is responsible for its extreme deformability as it forms heterodimers via lateral association. Spectrin acts like a coiled spring, which can stretch and snap back as the RBCs cross the capillary, distorted during the circulation due to shearing stress. An ordered arrangement of the above-mentioned proteins provides deformability during circulation in the blood throughout its lifespan. The deformable nature of the erythrocyte membrane is due to some helical linkers which are associated with spectrin and partly by "breathing action", in which association and dissociation of tetramer accommodate the deformity which is enforced by shear force in circulation [23,24]. Various membrane proteins are present on the surface of RBCs, some of them transport the molecules across the RBC membrane also called the transporter. For example, band 3 (anion transporter), aquaporin 1 (water transporter), Glut1 (glucose and L-dehydroascorbic acid transporter), RhAG (gas transporter, probably of carbon dioxide), Kidd antigen protein (urea transporter), Na^{+}-K^{+}-ATPase, Ca^{++} ATPase, Na^{+}-K^{+}-$2Cl^{-}$cotransporter, Na^{+}-Cl^{-}cotransporter, Na^{+}-K^{+} cotransporter, K^{+}-Cl^{-}cotransporter, and Gardos Channel. Apart from the transporter adhesive proteins are also present on membranes such as ICAM-4, which interacts with integrins and Leucine [25]. Figure 2 depicts the structural features of the RBC membrane.

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4. WHY PLASMODIUM HARBOR WITHIN THE CONFINEMENT OF RBC?

There are three main reasons why RBC is the primary choice for plasmodium to shelter in humans [26,27].

- RBC does not contain any organelle; thus, this unique feature provides maximum space for replication and growth of plasmodium.
- RBC predominantly contains Hb, which becomes a rich source of amino acids (after digestion) for the survival of plasmodium after the invasion.
- RBC, being devoid of any organelles cannot present antigen on their surface via MHC I (Major Histocompatibility Complex) thus providing immunogenic clearance to the parasite.

5. RENOVATION AND DECORATION OF IRBC BY PLASMODIUM

As the journey of merozoite begins in the erythrocyte it extensively modifies the host RBC, as required for its survival. Upon invasion, the parasite alters the permeability and adhesive property of the erythrocyte. Several unusual structures appear in the infected RBC (iRBC), which are usually absent in noninfected RBC. Plasmodium merozoite invades RBC through a cascade of mechanisms, which involve adhesion, reorientation, junction formation, and finally invagination. This whole process requires close interplay between merozoite-derived ligands and RBCs cell surface receptors [28]. Different subcellular localization of proteins, sub-compartmentalization inside various secretory organelles, and "Just in time" release of proteins play an important role during RBC invasion. Before the invasion, only Merozoite surface proteins (MSP) are expressed on the surface of RBC while maximum proteins are released after attachment of merozoite to the RBC membrane, which enables merozoite to remain undetected during circulation and hence not recognized by the immune system [29]. Invasion is initiated by the binding of merozoite surface protein to ligand receptors present on the RBC membrane. MSP-1 is a repertoire of surface protein, the most

plentiful and functionally conserved protein of merozoite. During the invasive phase apical structure of plasmodium rhoptries and micronemes, secrete various proteins such as erythrocyte binding antigen (EBA) and *P.falciparum* reticulocyte binding protein homolog (PfRh) that are thought to be involved in the formation of the tight junction [30]. Subsequently, two classes of proteins take charge to carry out the further strong attachment, which are the adhesion family and reticulocyte binding, protein family. The adhesion family includes erythrocyte-binding antigens, EBA-175, Eb11, and EBA-140, which bind to glycophorin A, B, and C respectively. Another family is reticulocyte binding like (RBL or Rh) protein, which provides an alternative pathway of invasion via neuraminidase-resistant and chymotrypsin-sensitive host receptors [31]. PfRh4also binds to the complement receptor 1 (CR-1). Further merozoite is irreversibly attached to the RBC membrane via a tight junction mediated through parasite-derived proteins, mainly RON complex and AMA1 [32,33]. After the formation of a tight junction, merozoite enters into RBC via parasite actin-myosin motor in the pellicle of invading parasite. The parasite is captured within the vacuole, which is formed in the erythrocyte after the initiation of the actin-myosin moving junction. In sporozoites, TRAP proteins interact with skeletal proteins, although a similar molecule in merozoite is not yet been recognized. After the invasion phase, to seal the merozoite inside the parasitophorous vacuole and RBC, the fusion of membranes takes place at the posterior part of the merozoite. Glideosome is conserved in apicomplexa genera and is thought to play an important role in generating force, which is a prerequisite for host cell invasion. Plasmodium set (s) up unique "extra parasite and intraerythrocytic" protein transportation pathway. To survive within the host cell, merozoite generates membranous compartments to carry out the transportation of synthesized protein within and to the surface of the cell membrane which is collectively known as exomembrane system. It includes tubovesicular network, parasitophorous vacuole, Maurer's cleft, J dots, caveola vesicle complex, and other small vesicles found in iRBC [34–36] (Figure 3).

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5.1 The Tubulovesicular network (TVN)

Within 33 hours of infection, merozoites start to synthesize TVN extending from the parasitophorous vacuole, up to the erythrocyte membrane [37,38]. Tubulovesicular membrane (TVM) arises from the parasitophorous vacuolar membrane, which extends up to the erythrocyte membrane. TVM is very close to NPPs, thus nutrients having a size below 80 nm, reach directly to the parasite [39]. The function of the TVN is to import nutrients, lipid, and raft proteins from the RBC membrane. Additionally, it also alters the permeation pathway of the membrane by creating non-selective ion channels [40,41].

5.2 Maurer's clefts (MC)

MC is the parasite-induced membranous compartment present in iRBCs. These are flat and disc-shaped structures that are joined to and by the erythrocyte membrane. Transportation of cargo proteins between the Plasmodium parasite and erythrocyte membrane is carried out via MC [42,43].

5.3 Knob

When plasmodium invades erythrocytes, various modifications occur on the cell surface. Out of them, one important modification is the nanoscale protrusions, also known as Knobs. Knobs act as a site for anchoring PfEMP1 protein. PfEMP1 imparts adhesion of iRBC to several host receptors and thus escapes splenic capture and clearance and is also responsible for clonal antigenic variation, iRBC avoids immune recognition. A Knob is composed of various additional plasmodium encoded proteins, i.e., Knob associated histidine-rich proteins (KAHRP), PfEMP3, mature parasite-infected erythrocyte surface antigen (MESA), plasmodium helical interspersed subtelomeric (PHIST) [43,44]. Figure 4 depicted the RBC membrane modification carried out by plasmodium.

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5.4 Placental malaria

Placental malaria is characterized by the seclusion of iRBCs and the infiltration of cells of the immune system within the placenta. The density of plasmodium is much higher in the placenta as compared to peripheral blood. It is reported that women who are pregnant for the first time are supposed to be more susceptible to placental malaria [42,45].

5.5 Cytoadherence

Cytoadherence is an important characteristic of *P. falciparum* infection whereby iRBCs adhere to endothelial cells. It can lead to life-threatening conditions when adhering to the brain and placenta. Cytoadherence causes a partial or complete blockage of blood flow, which is thought to have a major contribution to the fatal syndrome. Various receptors on endothelial cells may interact and sequester an iRBC. The receptors include intercellular adhesion molecules (ICAM-1), vascular cell adhesion molecules (VCAM), platelet endothelial cell adhesion molecules, thrombospondin, CD36, selectins (P-selectin and E-selectin), glycosaminoglycan (chondroitin sulfate A (CSA), heparin sulfate) [46–48]. **Figure 5** shows cytoplasmic modifications in infected RBC induced by plasmodium.

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6. CHARACTERISTICS OF AN IDEAL TARGET

An ideal molecular target for an antimalarial activity/targeting should have the following characteristics [39,49–51]:

- Target should be crucial in the parasitic life cycle.
- Target should differ from a similar process within the host.
- The target must be involved in the rate-limiting step of the biochemical pathway.
- Inhibition of the target biochemical pathway should have a significant effect on the inhibition of parasitic growth.
- There should not be any alternative pathway to the target.

- There should be the least probability of development of resistance.

7. HOW INFECTED RBC IS SELECTIVELY TARGETED?

Plasmodium parasites are auxotrophic to most of the nutrients such as glucose, lipid, and amino acids. These metabolites are the primary nutritional requirement of the plasmodium, and their need should be fulfilled by the host [10]. After the invasion, the rapid growth of parasites does take place in the erythrocyte, which usually requires a high amount of lipid, glucose, choline, and various ions which are usually supplemented or derived from/by the host. Lipid uptake is required for the synthesis and maintenance of membranes and membranous compartments of cells such as plasma membrane, food vacuole (FV), apicoplast membrane, PVM membrane, and formation of various apical organelles i.e., rhoptries, micronemes during the growth of the parasite. Although parasites can synthesize phospholipids and neutrolipids (Diacylglycerol, triacylglycerol) through *de novo* pathway, precursors are needed and are derived from the serum of the host. Thus, by tagging or incorporating the essential nutrients into formulation the targeted delivery of antimalarials can be achieved. Various studies are reported in which RBC is targeted through lipid carriers with better results [52–54]. During the parasite growth apart from the lipid, glucose uptake is also increased by manifolds, and this demand is fulfilled by the host, thus it has become an attractive therapeutic target. Various strategies have been adapted to target over-expressed GLUT-1 of RBC by incorporating ligands for sugar transporters such as glucose, starch, and glucose-bearing polymers; the approach demonstrated promising results [55–57]. Another study carried out by Shafi et al. reported that dehydroascorbic acid as such competes with glucose in GLUT-1 binding [58]. Thus, formulation bearing the DHA as a ligand could preferentially be targeted to infect RBC to deliver the drug payload to the infected RBC. Plasmodium induces various new permeation pathways (NPPs) into the RBC membrane

for access to various ions required for their growth and survival. Various strategies are being explored to target NPPs and nowadays have become potential mechanisms of interest. The inhibition of NPPs affects the supply of nutrients to the parasite; the inhibitors include sugar, amino acids, vitamins, and organic cations. NPPs also allow the elimination of waste material such as lactate. Thus, inhibition of NPPs is fatal to the parasite and ultimately causes the death of plasmodium [59,60]. Thus, iRBCs could be targeted preferentially with the help of their nutrient uptake mechanism. Along with the nutrients, the drug would go to the iRBCs, and thus growth of RBCs may be inhibited (Figure 6).

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8. STRATEGIES INVOLVED IN MALARIA TARGETING

8.1 Lipid targeting

Lipids are ubiquitous and considered to be an essential constituent of the cell membrane playing an important role in the life cycle of the malaria parasite. The demand for lipids is dramatically increased in various biological processes including the synthesis of membranes, post-translational modification [61], haem crystallization, and as a source of energy. Lipid metabolism is insignificant in normal RBC [62], while its level is increased significantly in iRBCs [63] during the intraerythrocytic cycle of the parasite. The enormous demand for the lipid thus makes it a suitable and attractive target and thus has gained the attention of many researchers. Metabolic pathways that are used for phospholipids and fatty acids synthesis could be promising targets for designing novel antimalarial therapeutic strategies. Various important precursors are provided by the host such as fatty acids, serine, choline, and ethanolamine which are the key constituents of the parasite membrane. Phosphatidylcholine (PC) is the primary phospholipid of the cell membrane of plasmodium and represents approx. 45% of the total phospholipid, while normal RBC has cholesterol and phospholipid as major lipids at the ratio of approximately 1. After the Plasmodium infection, there is a six-fold increase in phospholipid [including fatty acid (FA), diacylglycerol

(DAG), triacylglycerol (TAG)] content during the trophozoite stage [64,65]. The increment of these neutral lipids is largely supported by *de novo* synthesis**.** However, the requirement of DAG, TAG is significantly increased during the last stage of schizogony, which depicts that neutral lipid plays a key role in the development and release of merozoite [66,67].

8.2 Choline analogues as a target

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) represent the major phospholipid content of the eukaryotic cell as well as plasmodium. Synthesis of the phospholipid is a crucial step for the membrane synthesis of plasmodium. Phosphatidylcholine is synthesized via two routes, the CDP-choline pathway (Kennedy pathway) and the Serine decarboxylation phosphoethanolamine methylation (SDPM) pathway. Kennedy's pathway of the PC synthesis requires the choline uptake from the host into the iRBCs, which occurs through the NPPs along with the normal choline transporter in erythrocytes [68,69]. CDP-choline pathway (Kennedy pathway) involves three enzymes: choline/ethanolamine-phosphotransferase, choline kinase, and Cytidine triphosphate (CTP) phosphocholinecytidyltransferase (PfCCT). PfCCT is a rate-limiting enzyme of PC synthesis and hence appears to be a potential target. As compared to mammalian orthologs, this enzyme contains a duplicate catalytic domain [70]. SDPM pathway utilizes serine as a precursor from the host for the synthesis of PtdCho. Serine is also obtained from the degradation of the hemoglobin in the digestive vacuoles. Five enzymes are used in the SDPM pathway, out of them serine decarboxylase (PfSD) and phosphoethanolaminemethyltransferase (PfPMT) are not present in mammalian cells [71] (**Figure 7**). Transmethylation reactions are present in plants, protozoans, and worms but are absent in mammals. Genetic studies revealed that in the case of plasmodium that lacks a functional SDPM pathway, its development is severely hampered. Thus these enzymes are critical in this pathway and should be an alluring target for drug development, due to the absence of such

processes in mammalian cells [72].

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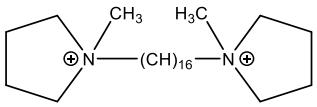
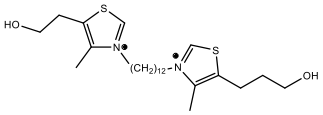
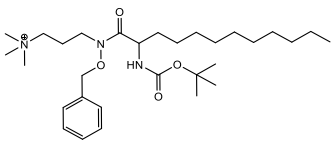
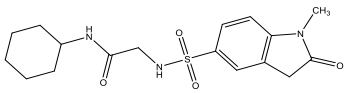
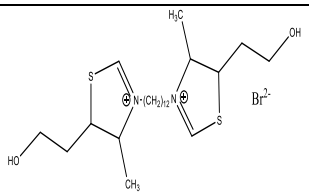
Choline analogs could be emerging drug candidates due to their broad-spectrum inhibition activity and effectiveness against multidrug resistance. Various choline analogs have been studied for their antimalarial activity. Analog of choline G25 [1,16-hexadecamethylenebis (N-methylpyrrolidium) di-bromide] could be an effective antimalarial drug that preferentially targets two major metabolic pathways of phospholipids, i.e. inhibition of *de novo* synthesis of phosphatidylethanolamine and phosphatidylcholine thus emerging antimalarial agent [73–75]. G25 inhibits the growth of *P.falciparumin-vitro* and suppresses the infection of *P. falciparum* and *P. cynomolgi* infected monkeys even at very low doses [76]. A study reveals that the tritium-labeled bis quaternary ammonium salt analog of G25 preferentially targets iRBCs several hundred times as compared to normal RBC with an IC_{50} value of 18nM. The antimalarial potency of G25 is comparable to chloroquine. It eradicates parasite load at a very low (nM) concentration [77]. Another study reveals that quaternary ammonium choline analogs are transported to the iRBCs and integrated into the membrane of the parasite, leading to altered lipid composition and physicochemical properties and hence resulting in the death of the parasite. The absence of recrudescence, complete parasite clearance, and the ability to cure severely infected animals has opened up a new area for antimalarial treatment in the future [78]. Albitiazolium, another inhibitor of PC synthesis is under phase 2 clinical development and could be a promising lead compound in the area of antimalarial research [79]. It is a choline analog consisting of two thiazolium cationic heads, which is a prerequisite for its high efficacy. Albitiazolium entered into the iRBCs via furosemide-sensitive NPPs and transferred to the plasmodium via a poly-specific cation carrier. Choline entry into the iRBCs through the plasmodium-derived cation transporter was competitively inhibited by Albitiazolium which is the primary mechanism for its antimalarial effect. Albitiazolium showed greater efficacy *in-vitro* and *in-vivo* in *P.falciparum* and mice

malaria models respectively [80] with PC50 value (amount of phosphatidylcholine prepared from choline by 50%) as 2.8, 9.3, and 26 mM at the ring, schizont, and trophozoite stage respectively [77] [56]. Another inhibitor of PC synthesis is PG12 which impedes the intraerythrocytic growth of parasite, however, has a limited effect on reinvasion or parasite rupture. PG12 shows low toxicity and outstanding antimalarial efficacy. Some studies suggest that only PC synthesis is completely inhibited by PG12, while phosphatidylethanolamine synthesis is partially inhibited [72]. *Plasmodium falciparum* CTP Phosphocholinecytidylyltransferase (PfCCT) is a rate-limiting enzyme in PC synthesis, thus has gained the attention of many researchers as an attractive target. PfCCT bears two catalytic active domains while eukaryotic CCTs contain only one catalytic domain and are expressed as a dimer. CDP-choline is a competitive inhibitor of CTP and P-choline. C12 is also a competitive inhibitor of both substrates. IC50 of C12 is 42 μM, which is approx. 15 times lower in comparison to the *in-vitro* inhibiting capacity of PfCCT. Contest and coworker studied the effect of C12 on *de novo* synthesis of PC and PC12 and found them to reduce the PC synthesis from choline with IC50 value of 187μM [70]. It was well established that choline transport into the infected erythrocyte is one of the limiting steps in de novo PC biosynthesis [11]. Interfering with this crucial step through the use of choline analogues could thus lead to a novel and promising pharmaceutical approach for malaria treatment. Some examples of choline analogous with their Mol. Structure and PC50/IC50 value are given in table 1.

Table 1: Choline analogues are represented with their molecular structure and PC50/IC50 value

SNo.	Drug/ Compound Name	Comments	Structures	PC_{50}/ IC_{50}	Ref
1.	G25	G25 is a potent inhibitor of parasite growth *both in vitro* and *in vivo*. G25 prevents choline uptake into Saccharomyces cerevisiae and thus inhibits its growth.		1 to 5.3 nM	[73]
2.	Albitiazolium	It inhibits the choline entry into the parasite. It also inhibits the enzymes involved in de novo synthesis of PC, thus acting as a potent antimalarial.		26 mM (Trophozoite stage)	[80]
3.	PG12	PG12 acts as a phospholipid mimetics, PG12 specifically prevents PC synthesis from both SDPM and CDP-choline pathways through inhibition of PfCCT.		0.42 µM	[72]
4.	C12	C12 competitively inhibits the PfCCT enzyme in vitro.	- 	42 µM	[70]
5.	T3/SAR97276 and T4	It exhibits greater antimalarial activity as caused retardation of the growth of the parasite.	 Bis-thiazolium drug R = H T3 R = CH3 T4	2.25 and 0.65 respectively	[75]

Abbreviations: Phosphatidylcholine (PC); cytidine diphosphate (CDP); serine-decarboxylase-phosphoethanolamine-methyltransferase(SDPM); phosphocholine cytidylyltransferase (PfCCT)

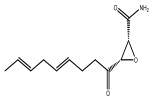
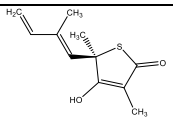
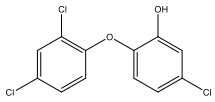
8.3 Apicoplast as a target

The apicoplast is a residual plastid found in apicomplexans, which is necessary for the survival of plasmodium. Apicoplast discovery offers an opportunity to think of a new target that is being explored in drug development against *plasmodium*. *Plasmodium* apicoplast does not have pigments, required for photosynthesis, while it has one operational anabolic pathway which is required for its growth and viability. Various metabolic pathways operate in apicoplast such as fatty-acid type II (FASII) biosynthesis, lipoic acid, isoprenoids (IPP), heme and iron-sulfur clusters synthesis, etc. These metabolic pathways are different from their human host; however, they are a prerequisite for the pathogenesis of plasmodium. Therefore, due to the bacterial origin and significant difference from mammalian pathways, the apicoplast could be an excellent and distinct target [81,82]. The synthesis of isopentenyl pyrophosphate is an important function of the apicoplast. A product of isoprenoid is essential for electron transport, apoptosis, membrane structure, and cell signaling. The precursor of isoprenoid is synthesized from MEP (2-C-methyl-D-erythritol 4-phosphate) pathway in plasmodium. It has previously been assumed that the *de novo* pathway for fatty acid synthesis is absent in plasmodium and it is supplemented by the serum of the host. However, the gene sequence of *P. falciparum,* has revealed that plasmodium has the ability to synthesize fatty acids and has the gene which encodes for the type II fatty acid synthase (FAS) located in its apicoplast [83–85]. Fungi and higher eukaryotes contain type I fatty acid synthase (FAS-I), which is a multifunctional enzyme that catalyzes the fatty acid chain elongation reactions. FAS-II is present in bacteria and plastids of plants and algae. In the type-II system, each reaction of initiation and elongation is catalyzed by different enzymes. This pathway is similar to the type II pathway of plant chloroplast and bacteria, however, it differs from the human wherein

type I pathway occurs; this may be seminal in the development of novel antimalarial drugs [86]. Several antibiotics such as triclosan, cerulenin, and thiolactomycin, are specific inhibitors of the type II pathway (**Table II**) and were found to decrease the parasite numbers in *in-vitro* culture [87,88].

<Insert table 2 here>

Table 2: Antibiotics that are specific inhibitors of type II fatty acid synthase (FAS) pathway

S.N	Drug	Inhibition	Remarks	Structure	Ref.
1.	CER	KAS I and II	CER inhibits parasitic protein and is thus a potent inhibitor of the parasite growth		[89]
2.	Thiolactomycin	ACP synthase & FabH	*In vitro* inhibition		[90]
3.	Triclosan	FabI	Inhibits purified *P. falciparum* enoyl-ACP reductase and parasite growth as well		[91]

Abbreviations : Cerulenin (CER); β-oxoacyl-ACP synthase (β-ketoacyl-ACP synthase) (KAS); Acyl carrier protein (ACP); β-Ketoacyl-Acyl Carrier Protein Synthase III (FabH); enoyl-ACP reductases I enzyme (FabI).

8.4 Post Translational Modifications (PTMs) based on targeting

PTMs involve covalent modification of proteins, catalyzed by enzymes that occur after protein translation. PTMs of protein enhance the functional diversification of the proteomics of the biological system, through a covalent conjoining of a variety of functional groups. These chemical modifications affect all basic cell functioning and pathogenesis, the modifications include involves glycosylation [92], acetylation [93] methylation, phosphorylation [94], ubiquitination [95] sumoylation [96], myristoylation [97], etc. Thus, identifying and understanding the PTMs target can open a new area for drug discovery for the treatment of malaria and could serve to be a potential drug target. One of the important PTMs in plasmodium is O-GlcNAcylation, which is involved in many biological processes such as glycolysis, protein folding, etc. involving O-GlcNAc transferase. It has a structural difference from the human O-GlcNAc transferase thus selective intervention of the same will have antimalarial activity [92]. Another potential drug target for *Plasmodium* is N-myristoyltransferase is involved in the transfer of myristate (fatty acid with C14 carbon) group from myristoyl coenzyme A to the α-amino group of glycine moiety of the protein substrate. Protein N-myristoylation is mainly a co-translational process followed by the removal of the methionine group. Myristoylation is not only the process of diversification of protein functions, but its most common function is in membrane association, cellular localization of modified proteins, ion channel regulation, and regulation of protein complex assembly [97]. Palmitoyl proteins in *P. falciparum* play a key role in the development of the asexual stage and virulence. In the asexual stage of the parasite, its expression is upregulated. One of the mechanisms involved in PTMs is palmitoylation of cysteine moiety, which occurs in reversible lipid modification and is bound to regulate target proteins. Palmitoylated proteins regulate many important activities of parasites for cytoadherence, invasion, development, signaling process, and drug resistance. Palmitoylated protein directly affects the invasion of the

parasite in the RBCs by influencing the stability of actin-myosin protein invasion motor components. Thus, it plays a central role in controlling the parasitic blood-stage and could be a potential therapeutic target. Another finding suggests that histone deacetylase inhibitors can be a potential target (blood-stage) to design novel antimalarial. Histone is abundant, has an antigenic nature, and plays an important role in the pathogenesis of malaria. These are nuclear core proteins, which regulate the transcriptional regulation through continuous acetylation and deacetylation of a specific lysine residue [98]. Multiple copies of histone genes per haploid genome are present in higher eukaryotes while the plasmodium haploid genome consists of only one copy of H3, H3A, H2B, and H4 histone proteins. Understanding the basic biology of malaria parasites may enable us to find new ways to treat malaria, which will prove to be of significance, especially to reduce drug resistance [8,99,100].

8.5 Purine transporter-based strategies

Plasmodium has a huge demand for purines for DNA replication and RNA synthesis and metabolism at all stages of its life cycle. However, *de novo* synthesis of purine does not occur in the plasmodium as well as in RBC, this is entirely dependent on host plasma. Plasmodium species are purine auxotrophs, hence, to fulfill metabolic needs it is obtained from the host via equilibrative nucleoside transporters (ENTs). Purine obtained from the host is modified by the purine salvage pathway enzymes. Thus, the plasmodium nucleoside import and salvage pathway could be another attractive target for the antimalarial drug development pipeline [101]. Human ENT1 (hENT1) is present on the surface of the erythrocyte, which is essential for the uptake of adenosine. 17-18% of the amino acid sequence of hENT1 is similar to Plasmodium falciparum ENT1 (PfENT1) and plasmodium berghei ENT1 (PbENT1). hENT1 is inhibited by FDA-approved drugs, dipyridamole, and nitrobenzylmercaptopurineriboside (NBMPR) at nM concentration, while PfENT1 and PbENT1 are not affected even up to mM concentration. Thus, specificity and selectivity for parasitic ENT1 can be achieved by designing a novel molecule that could selectively

inhibit parasitic ENT1. Arora *et al.* expressed PbENT1 in purine auxotrophic yeast and reported that guanosine (IC_{50}=21.3 μM) and adenosine (IC_{50}=3.7 μM) have exceedingly high affinity for PbENT1. They also reported that PfENT1 inhibitors are 10 times more potent for PfENT1 as compared to hENT1. They identified 9 inhibitors that could retard parasite proliferation in culture and also in the *in-vivo* mouse malaria model [102]. Kagami et al., have used *Plasmodium falciparum* purine nucleoside phosphorylase (PfPNP) for drug design because its inhibition was found lethal to parasites both *in-vivo* and *in-vitro*. Using a pharmacophore-based virtual screening model and molecular docking, they reported 59 PfPNP inhibitors, which seem to be absorbed orally in Caco-2 cell model. Out of 59 compounds, four compounds continued as leads due to their plausible interplay and intervention with the unique hydrophobic pocket of PfPNP, which is more selective towards PfPNP as compared to human PNP [103]. Synthesis of nucleic acid takes place by the hypoxanthine salvage pathway in *P. falciparum* but does not occur in humans. The synthesis of hypoxanthine can be arrested by DADMe-Immucillin-G, which binds to the transition state of purine nucleoside phosphorylase (PfPNP). Catalytic functions of enzymes are targeted by the transition state analogs. The mutation that mitigates the affinity of transition state analogs is believed to diminish the catalytic action of the target enzyme. DADMe-Immucillin-G is lethal to plasmodium both *in-vitro* and *in-vivo*. Possibilities of developing resistance for DADMe-Immucillin-G are least in *P.falciparum,* which shows its potential as antimalarial [[104].

8.6 Sugar Transporter based strategies

Intraerythrocytic plasmodium relies entirely on energy from glycolysis (metabolism of glucose). Anaerobic glycolysis takes place in plasmodium and lactate is produced as an end product. iRBCs require 100 times more glucose as compared to normal RBCs [5,105]. Sugar transporters present on the host and parasitic plasma membrane provide glucose to parasites from human blood. It requires a huge amount of host-derived glucose to meet the need of growing and

dividing parasites. The expression of glucose transporter is increased on the iRBC to cope with the need for rapid uptake of glucose [106]. Thus, the hexose transporter (PfHT) of *P. falciparum* could be a promising drug target. The increased glucose usage by iRBC may also divert this essential substrate from humans to plasmodium sequestered in small blood vessels, thereby increasing pathophysiological processes in cerebral malaria and have thus offered another reason for discovering PfHT inhibitors. Various approaches have been adapted to target GLUT-1 transporter by exploring ligands of hexose transporters such as polymer-bearing glucose and glucosamine. Infected RBC can be distinguished from uninfected RBC by starch, which has a terminal glucose moiety [55]. The malaria parasite is surrounded by an additional parasitophorous vacuole (PV) membrane inside the RBC. So, glucose must cross this membrane before reaching the parasite. This membrane allows the molecules up to 1400 Da molecular weight and these molecules can easily permeate between the cytoplasm of RBC and vacuolar space. Blood glucose is transported into the cytosol of RBC by GLUT1 transporter and reaches to PV membrane via facilitative transport. Finally, glucose reaches the plasmodium through a hexose transporter (PfHT) on the parasitic membrane [107]. The formulation in which hexose moiety is attached has the ability to bind the human RBC glucose transporter (GLUT1) along with the hexose transporter PfHT1 of the parasite, which would preferentially be targeted to infected RBC due to the overexpression of these receptors on iRBC. PfHT is considered as a potential antimalarial target due to the following reasons [108]:

- It is a single copy gene, not having any close orthologous.
- Plasmodium can't survive without glucose as they mature to trophozoite and no energy source as storage (glycogen) is available.
- The structural and functional difference between host and plasmodium transporter, thus enables selective inhibition of *plasmodium* transporter is possible.

Jiang et.al. crystallized the structure of PfHT1, hexose transporter within the plasmodium species at the 2.6 Aº resolution in complex with

D-glucose and 3.7Aº with a C3361, moderately selective inhibitor of PfHT1. Binding of the C3361 to PfHT1 brings out significant rearrangement, which results in a subsidiary pocket. Thus, on the basis of inhibitor binding induced pocket, PfHT1-specific inhibitors can be designed. Hence designed C3361 derivatives showed increased inhibition of PfHT1 and cellular potency against plasmodium, with a better selectivity profile to human GLUT1. Thus next-generation antimalarials can be designed by selectively targeting allosteric and orthosteric sites of PfHT1 [109].

8.7 Plasmodium Glycolytic enzyme

Survival of malaria parasite largely depends on glycolysis because of the absence of mitochondria in the parasite and RBC, thus oxidative phosphorylation does not occur. Glycolysis is pivotal for the synthesis of ATP in the plasmodium. Anaerobic conversion of glucose into lactate takes place in parasites due to the absence of Kreb's cycle and the rate of this conversion is 20-100 times higher as compared to normal RBC. Parasitic enzymes involved in glycolysis have a structural difference with respect to host enzymes, which could be used for selective targeting. These enzymes are involved in several specific functions of the parasite such as development and transmission. Compounds that target parasite energy machinery could be potential antimalarial because inhibition of glycolysis is lethal to the parasite. A glycolytic enzyme does not only take part in glycolysis but also exhibits some unrelated functions within or outside the cell. It is a Single protein that carries out multiple functions; this dual functionality is referred to as protein "moonlighting". These diverse functions of a protein or moonlighting arise through post-translational modification or differential binding patterns. This phenomenon plays an important role in the pathogenesis of many infectious diseases hence the knowledge of moonlighting protein may be seminal in antimalarial therapeutics. In the case of glycolytic enzymes, 7 out of 10 proteins show moon lightning activity. The major function of the moon-lightening enzymes of glycolysis is apoptosis, cell motility, and transcriptional regulation [110]. Normally, inhibitors target the active

site of the enzyme but the active site of the host and parasitic enzymes are highly conserved thus it is not feasible to target this active site [111,112]. Moreover, subunit interfaces and moon-lightening enzymes could be targeted due to their unique structural differences and mutation at these sites. Various attempts have been made to target parasitic glycolytic enzymes. Isobenzothiazolinone is one of the lead compounds that inhibits plasmodium hexokinase thus inhibiting glycolysis [113]. Another study reveals that plasmodium lactate dehydrogenase (PfLDH) inhibition could be a promising target that declines the growth of plasmodium *in-vitro*. In the last step of glycolysis, LDH catalyzes the final interconversion of pyruvate to lactate, which is necessary for the production of energy in the living cell [114].

8.8 Anion channels-based targeting

Plasmodium utilizes various approaches to adapt the RBCs environment for a continuous supply of nutrients, thus the ability to adapt for its replication and survival. Therefore, the parasite must alter membrane permeability either by upregulation of anion channels or creating a new permeation pathway. To achieve this goal and to escape the host immune system, the parasite modulates the structure of RBC i.e., increases the permeability of the RBCs membrane. After a few hours of invasion in RBC, plasmodium induces the formation of some unusual small conductance ion channels, which are referred to as plasmodium surface anion channels (PSAC), responsible for its increased permeability such channels are absent in the normal RBCs [115]. Various organic and inorganic solutes i.e., sugar, vitamins, amino acid, purine, and organic cations enter into the RBCs via these channels due to the altered permeability of the membrane. The requirement of the majority of amino acids is thus fulfilled by the digestion of hemoglobin, although isoleucine is absent in hemoglobin and must be supplied from the host (plasma). Apart from the amino acid, plasmodium also requires vitamins (pantothenate) and purine precursor from the host. There is no native transport mechanism for a few essential nutrients, which are required for the growth of the

parasite. Some of the nutrients are either not transported or transported to a rate, which is not sufficient for the rapid growth of the parasite. NPPs allow the entry of a large number of nutrients such as anionic and nonpolar molecules, required for its rapid growth. Apart from nutrient acquisition NPPs also help in the elimination of metabolic waste such as lactate [60]. The number of these plasmodia induced PSAC eventually increased from the normal functional copy of 1000/cell and 2000/cell. This anion channel (Figure 8) plays an important role in nutrient acquisition in parasites but may be fatal because water-soluble drugs can easily enter through this channel. Thus, it can become an attractive target. These anion channels differ from the human counterpart in order of its selectivity of ions (SCN^-> I^-> Br^->Cl^-> acetate> lactate > glutamate).

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Various recent studies i.e., genetic mapping with site-directed mutagenesis, gene silencing along with the isolate-specific inhibitors suggest that PSAC activity is determined by a family of gene CLAGs protein and two conserved RhopH2 and RhopH3 proteins. CLAGs genes are a multigene family, which is specific to the Plasmodium parasite. Some of the genes of the family are associated with cytoadherence [116], while the other family members on the 3rd chromosome are associated with PSAC activity [117]. CLAGs proteins are encoded by parasitic chromosome 3 and become an integral part of the RBCs membrane. CLAGs proteins are directly involved in the formation of channels and transportation of various solutes [118]. Recent studies reveal that CLAGs proteins do not possess the transmembrane domain and thus cannot form an ion channel pore, however associated proteins RhopH2 and RhopH3 assist them in the formation of the pore [119]. CLAGs proteins are synthesized on the ribosome, initially packaged into organelles which is known as rhoptries. Then this protein is trafficked to the RBCs membrane, the exact mechanism is still not clear. Thus, there is a limitation in our understanding as to how it contributes to the enhanced permeability of the erythrocyte membrane. Because NPPs are of paramount significance for

plasmodium, thus NPPs could be exploited as an attractive therapeutic target owing to their surface occupancy which removes the risk of resistance development by the eviction of drugs from the site of action. PSAC is inhibited by Specific Sarcoplasmic reticulum Ca^{2+} channel antagonists such as dantrolene and non-specific channel blockers namely furosemide, glibenclamide, phloridzin, and 5-nitro-2-(3-phenylpropyl-amino) benzoic acid (NPPB). Most of these compounds inhibit parasite growth at the nanomolar range. However, Dantrolene seems to be a better antimalarial as compared to NPPB, which impedes cyclooxygenase and additional mammalian anion channel [49,51].

Discussion

Recent advancements in parasite biology and genomic techniques have expanded the pool of potential targets for developing new antimalarial therapies. This review aims to explore the latest scientific and technological progress in discovering and developing novel antimalarial agents. Several target proteins have emerged as promising antimalarial medicines in the last few decades. Choline transport inhibitors are among the most intriguing antimalarial target proteins that are being researched. Such insights hold promise for the development of more effective and resilient therapies against malaria. Due to the importance for the growth, proliferation, and pathogenesis of the malaria parasites, the metabolic machineries for the phospholipid and fatty acid synthesis have revealed great interest as a potential target for the development of novel antimalarial drugs. Phosphatidylcholine is synthesized de novo by the parasite, using choline as a precursor. This pathway is vital for parasite growth and survival. Inhibiting choline transport into the parasite blocks phosphatidylcholine biosynthesis, leading to parasite death. Albitiazolium, a drug in Phase II trials, achieves this by inhibiting choline transport, accumulating in Plasmodium up to 1000-fold, and effectively halting parasite growth, even in severe cases. Remarkably, a single injection can cure high parasitemia levels [120]. Cholinephosphate cytidylyl transferase is a critical step in phosphatidylcholine de novo biosynthesis. Compounds like G25 inhibit

this process in the parasite. G25, a bisquaternary ammonium compound, is significantly less toxic to mammalian cell lines, making it a promising lead compound for antimalarial drug discovery and development [121]. For the designing of antimalarial drugs, targeting the genetic machinery of apicoplast offers some advantages; however, challenges associated with the development of resistance impede its uses as antimalarials. Mefloquine-resistant strains of *P. falciparum* were resistant to triclosan (IC50 of 2 μg/ml), raising concerns that triclosan could share mechanisms of drug resistance [122]. From the extensive literature review, we concluded that choline targets are more desirable than apicoplasts; nonetheless, much work is currently being done on choline analogues as promising new therapeutic targets.

CONCLUSION

Treatment of malaria becomes difficult due to the lack of suitable vaccines and the development of resistance to antimalarial drugs. Thus, there is an urgent need for novel antimalarial drug targets. Various aspects should be kept in mind before developing an antimalarial. The drug should be safe, effective, and affordable to poor men and has the least susceptibility to develop resistance.

Targeted drug delivery may be based on variations in infected cells compared to normal versions. These variations are mainly at the molecular level, which helps them to survive in the host and also to meet our requirements of nutrients from the host. Various modifications are carried out by plasmodium which includes Maurer's clefts, Knob, tubulovesicular network, and cytoadherence phenomenon, which enable plasmodium to survive within the RBCs. Malaria parasite synthesized the tubulovesicular network, which not only exports synthesized protein to the host but also clears off the burned proteins into the extracellular milieu. The variant critical in pathogen metabolism could be an ideal target for drug discovery and design, while the variant associated with the host surface could be an ideal target for nanocarrier-loaded drug delivery for infected RBCs.

Competing Interest

The authors declare that they have no conflict of interest.

Author Contributions

AJ, LKG and RS contributed equally to this article. SPV and KK made critical revisions and approved the final version of the paper. AJ, LKG, and PS contributed to the writing of the preliminary draft of the manuscript. SPV, KK and SV editing the manuscript. All authors reviewed and approved the final manuscript.

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Figures

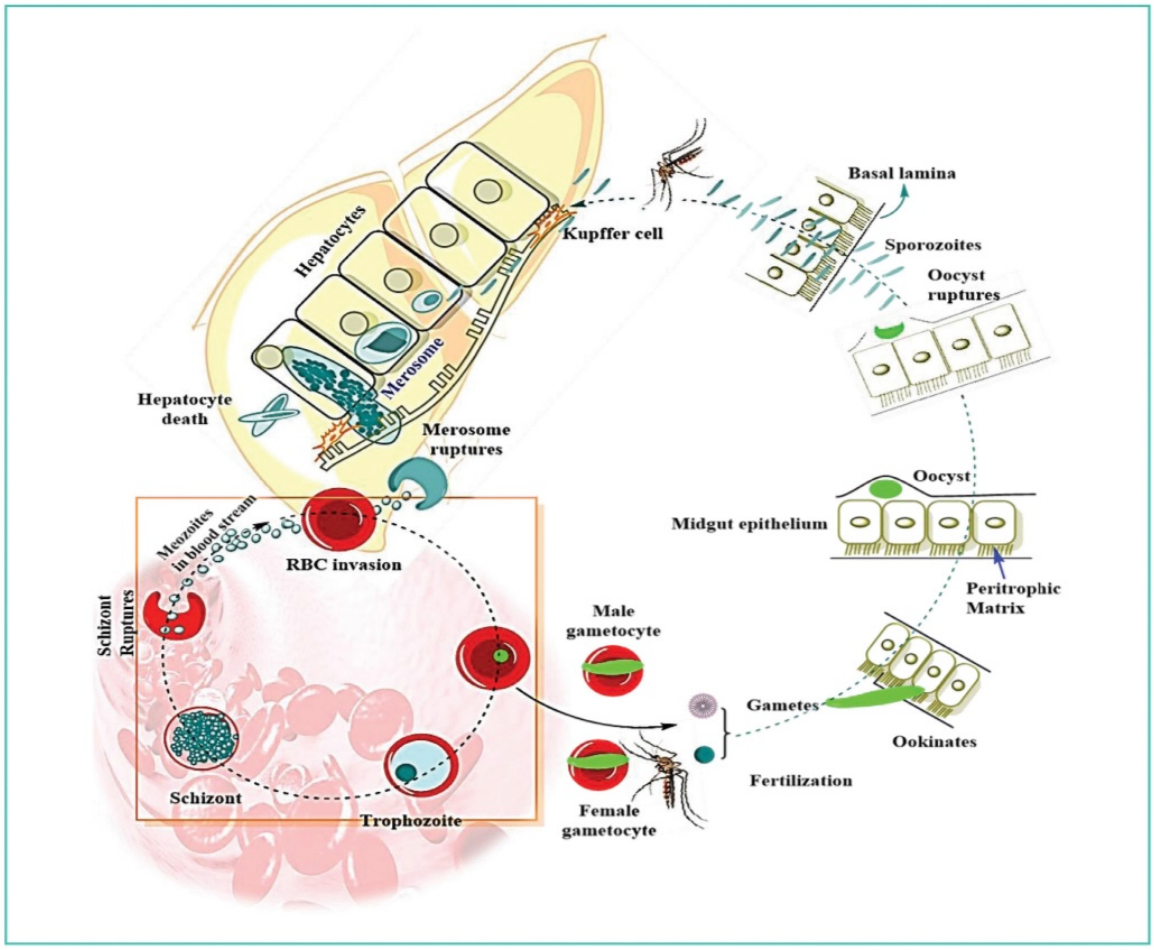

Figure 1: Life cycle of *Plasmodium* parasite.

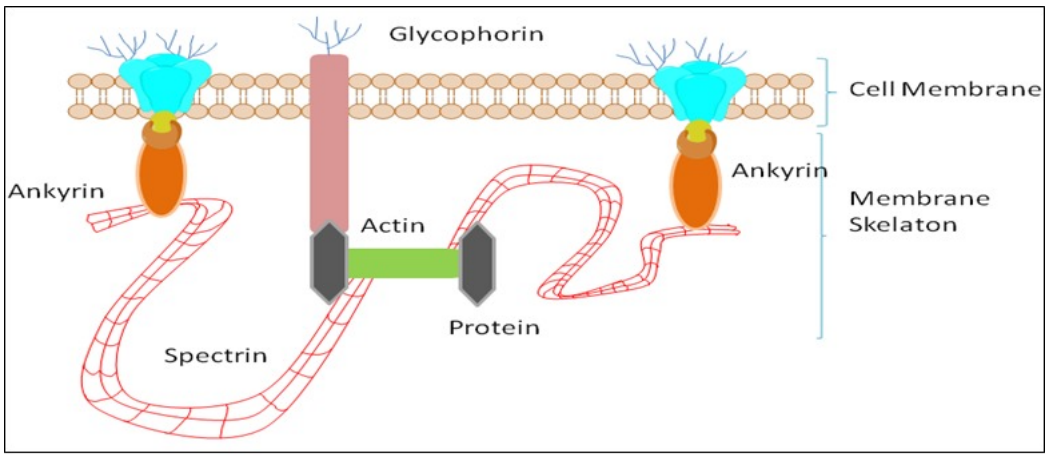

Figure 2: Structural features of normal RBC membrane spectrin protein is present underlying the cell membrane.

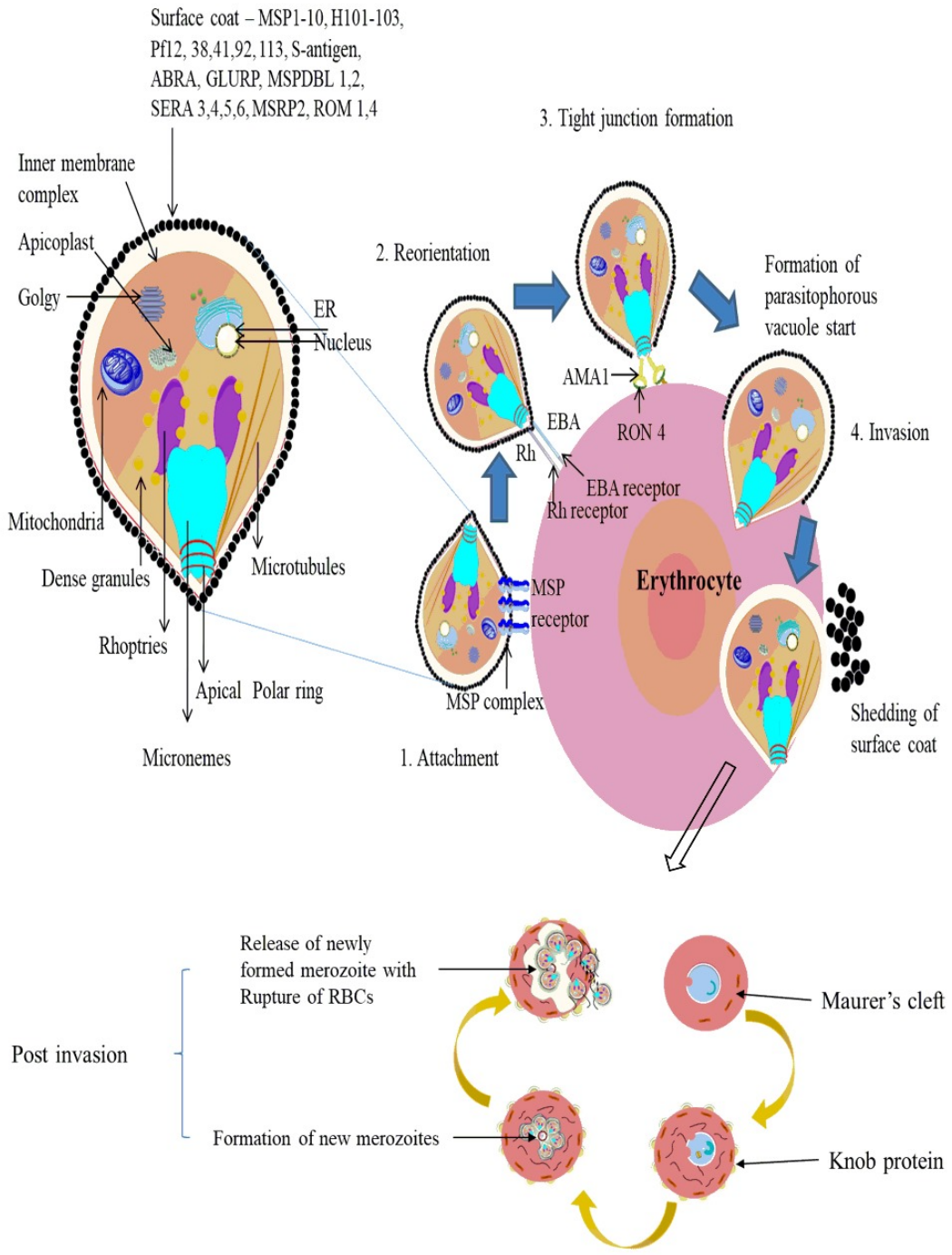

Figure 3: Different Stages of invasion of merozoite in RBC.

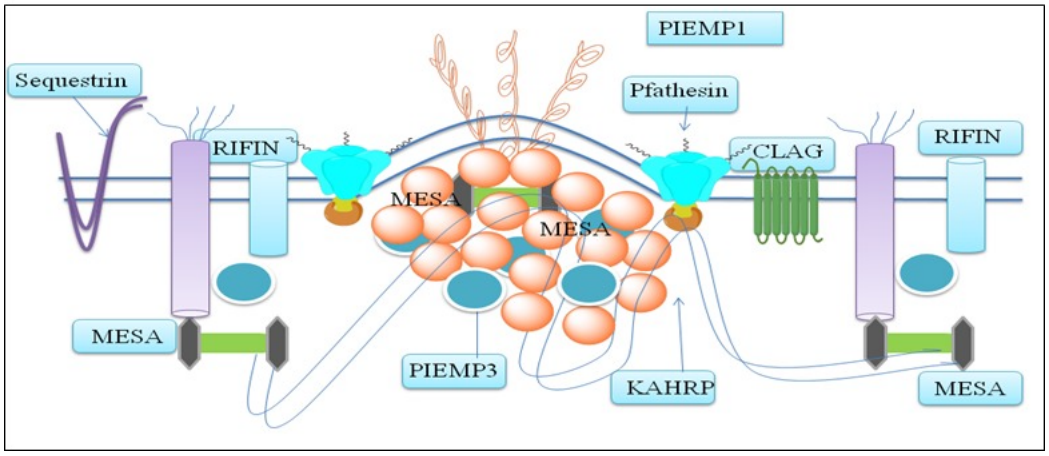

Figure 4: RBC cell membrane modifications carried out by *Plasmodium.*

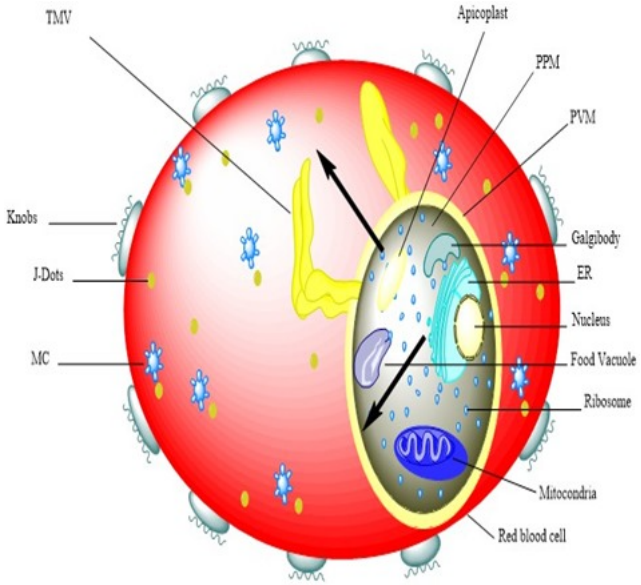

Figure 5: Cytoplasmic modifications carried out in Plasmodium in infected RBC.

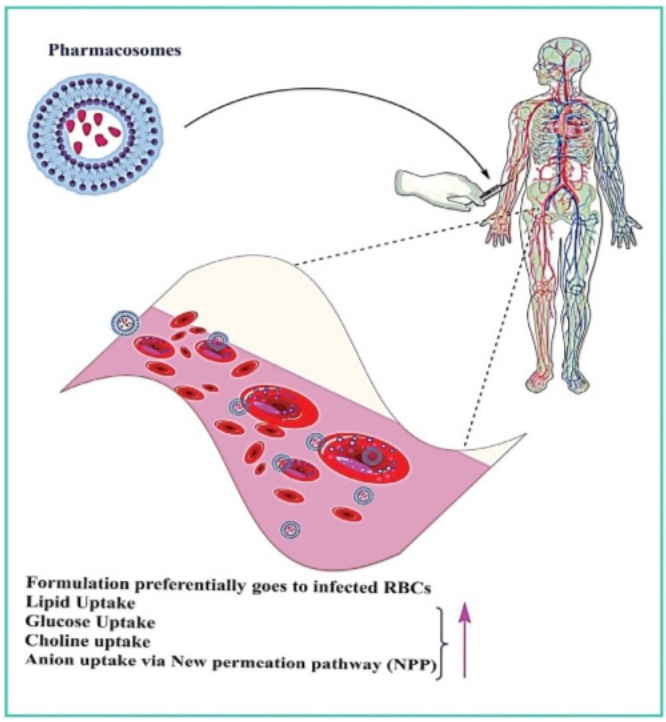

Figure 6: Diagrammatic representation of how infected RBC can be selectively targeted.

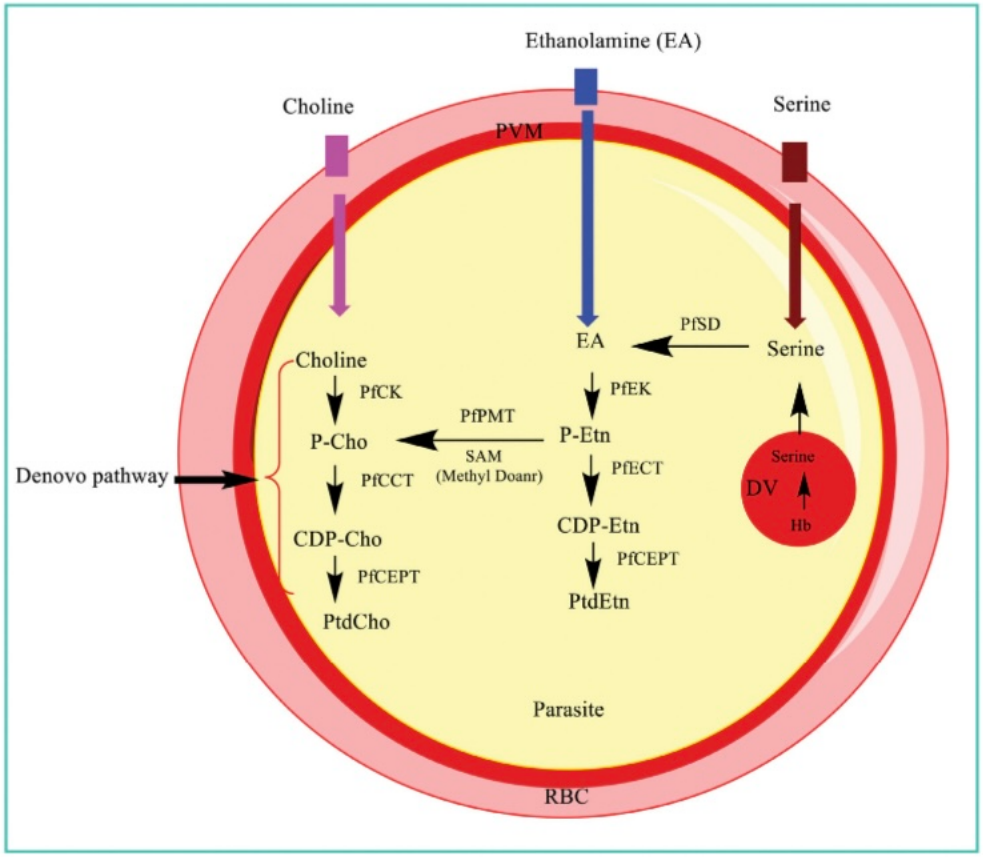

Figure 7: Phospholipid biosynthesis in *Plasmodium falciparum.*

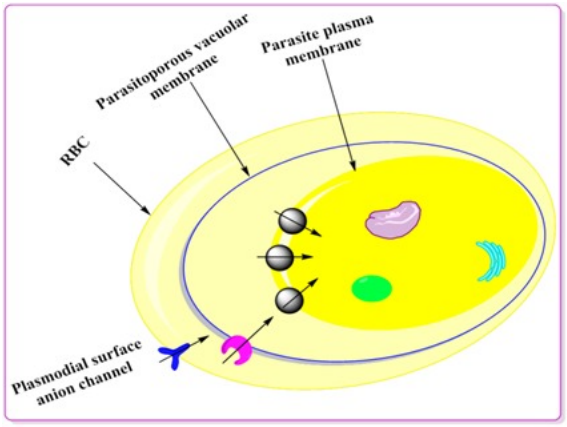

Figure 8: Schematic representation of Plasmodium surface anion channel