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Plasmodial enzymes in metabolic pathways as therapeutic targets and contemporary strategies to discover new antimalarial drugs: a review

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Abstract. Malaria continues to pose imminent threat to the world population, as the mortality rate associated with this disease remains high. Current treatment relies on antimalarial drugs such as Artemisinin Combination Therapy (ACT) are still effective throughout the world except in some places, where ACT-resistance has been reported, thus necessitating novel approaches to develop new anti-malarial therapy. In the light of emerging translational research, several plasmodial targets, mostly proteins or enzymes located in the parasite's unique organelles, have been extensively explored as potential candidates for the development of novel antimalarial drugs. By targeting the metabolic pathways in mitochondrion, apicoplast or cytoplasm of *Plasmodium,* the possibility to discover new drugs is tremendous, as they have potentials as antimalarial therapeutic targets. This literature review summarizes pertinent information on plasmodial targets, especially enzymes involved in specific metabolic pathways, and the strategies used to discover new antimalarial drugs.

Keywords: *Plasmodium* enzymes, metabolic pathways, antimalarial target, drug discovery

INTRODUCTION

Despite the reduced numbers of malaria cases in the world, the morbidity and mortality rates remain disturbing. The World Health Organization (WHO) in 2017, reported that 219 million malaria cases have been registered worldwide. From this number, almost 92% of the cases occurred in WHO African Region, WHO South-East Asia Region (5%) and the Eastern Mediterranean (2%) regions (WHO, 2018). Malaria is caused by the parasites from genus *Plasmodium*, where infected *Anopheles* mosquitoes play a role as the transmission vector. Human infection with *Plasmodium* is normally associated with four species; specifically, *Plasmodium falciparum*, *P. vivax*, *P. malariae* and *P. ovale*. *P. ovale* that caused malaria in human can be divided into two distinct *ovale* malaria species, which are *P. ovale curtisi* and *P. ovale walkeri,* after a study conducted by researchers from Mahidol University, Bangkok and UK Malaria Reference Laboratory, where polymorphisms in six loci were observed in 55 isolates. It was found that two diverse major haplotypes of each locus were identified and these did not recombine in any of the parasites examined (Alemu *et al.*, 2013; Rowe *et al.*, 2006; Sutherland *et al.*, 2010)

The recent distribution of *Plasmodium* species shows that majority of *P. falciparum* infection

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occurred in tropical Africa, while *P. vivax* infection happens more in South America, compared to *P. falciparum*. Meanwhile, in South-eastern Asia and Western Pacific, both *P. falciparum* and *P. vivax* are prevalent. Even though *P. malariae* could arise in all malarious areas, the *P. malariae* prevalence is usually low. Meanwhile in tropical Africa, *P. ovale* is prevalent and sometimes *P. falciparum* and *P. malariae* co-infection is found (Autino *et al.,* 2012). However, in the recent years, the fifth human malaria parasite, known as *P. knowlesi* that was formerly infecting monkeys has been discovered, raising concerns on zoonotic transmission (White, 2008). *P. knowlesi* infection occurs only in forested area in certain countries in South-East Asia (Autino *et al.,* 2012). The zoonotic malaria species *P. knowlesi* has become the main cause of human malaria in Malaysian Borneo (Fornace *et al.*, 2016; William *et al.*, 2011; Yusof *et al.*, 2016).

The clinical features shown on malaria patients usually appear during parasite's maturation in human blood and the symptoms emergence coincides with the release of antigens during the host cell rupture (Trampuz *et al.,* 2003). According to its severity, malaria is usually classified as uncomplicated (or mild) or complicated (severe) (Phillips *et al.,* 2017). To circumvent severe illness or fatality, an appropriate management of malaria is vital. At present, malaria treatment according to the standard treatment recommended by the WHO, is Artemisinin Combination Therapy (ACT) as the first line of malaria treatment for countries that are prevalent with *P. falciparum* infection. In areas of multidrug resistance (South-East Asia), drugs presently used for malaria treatment including combination of artemether-lumefantrine or artesunate + mefloquine while in Africa artemether-lumefantrine, artesunate + amodiaquine, or artesunate + sulfadoxine-pyrimethamine is used. The second line treatment could be alternative of ACT, quinine + tetracycline or doxycycline or clindamycin. Chloroquine is generally used for *P. vivax, P.ovale* and *P. malariae.* (CDC, 2015; WHO, 2015).

The reduction of death due malaria nowadays is largely contributed by the collective efforts of adoption of new-artemisinin-based medicine, increased of investments for research and development that leads to improvements in antimalarial treatments. Jagoe mentioned in the Medicine for Malaria Ventures (MMV) that a single dose treatment has been introduced in 2018 for vivax malaria. The current treatment always lead to poor compliance because of the treatment period; this could replace the current 14 days treatment to stop relapse (Jagoe, 2018). The trial for the new vivax malaria treatment was performed in Brazil, Cambodia, Ethiopia Peru, Philippines and Thailand, where they have selected 522 malaria patients with confirmed infection with *P. vivax* and normal glucose-6-phosphate dehydrogenase (G6PD), resulted in significantly lower risk of recurrence of *P. vivax* as compared to placebo in patient with normal G6PD activity (Lacerda *et al.,* 2019).

Many approaches using the latest technology has been conducted to develop new drug candidates for the treatment of antimalarials, as alternative to the currently available treatments. Four years ago, a team at MVV lead by Burrows and colleagues has established an outline for malaria drug design by defining the minimum acceptable profile of target candidates and target product for developing a novel malaria therapy. Target candidate profile (TCP) with their mechanism of action has been listed out in their review. Each TCP has their own mechanism of actions, as for example TCP1 targeted molecules that would clear asexual plasmodial blood stages in patients, while TCP3 aim the molecule that aids in clearing the dormant hypnozoites as prevention of relapses due to *P. vivax* or *P. ovale* infections. Meanwhile, TCP4 and TCP5 have different target where TCP4 targeted molecules that can clear the liver stage infection. However, TCP5 and TCP6 action by blocking the transmission, TCP5 rendering the gametocyte non-functional and TCP6 kill the mosquito following a blood meal (Burrows *et al.,* 2017). MMV predicted that only less than two percent of project for new antimalarial will proceed to final stage of clinical trial.

Okombo and Chibale reviewed in 2018 on the latest progress of drug candidates as described in MMV and the NIH-hosted repository for clinical trials. A literature search was performed by Okombo and Chibale from articles in the life sciences journal archive and PubMED that was published in the last five to six years, where it was described that the drug candidate for antimalaria at present are under various phases of

consideration for development. Six drugs were at lead optimization stage, five drugs are at preclinical stage, three antimalarial drugs candidates are already for clinical evaluation and additional six drug candidates are in product development under patient exploratory stage. These current discoveries will require long term investments, scientific venture and political support to warrant the decrease of disease burden globally (Okombo & Chibale, 2018).

1. Antimalarial resistance

Over the years, the efficacy of most antimalarial drugs is declining, which is mainly due to the more widespread resistance. This issue is further exacerbated by the fact that the compounds currently used in malaria treatment share related mechanisms of action. This hinders the efforts to reduce the morbidity and mortality rates related to this disease (Olliaro & Mussano, 2003). Resistance of *P. falciparum* towards antimalarial starting from the first report of quinine resistance in 1910, followed by chloroquine in 1960 (Blount 1967; da Silva & Benchimol, 2014; Tan *et al.,* 2014). An analysis of molecular, genetic and biochemical approaches to *P. falciparum* gene resistance towards chloroquine has been conducted by Le Bras & Durand (2003), which the study aimed to elucidate *P. falciparum* multidrug resistance 1 gene (*pfmdr*1). The authors identified a gene on chromosome 5, encoding a P-glycoprotein homolog 1 and *P. falciparum* chloroquine resistance transporter (*pfcrt*) on chromosome 7 as the main determinants of chloroquine resistance. Their findings have also shown that mutations in *pfmdr1* and *pfcrt* genes eventually lead to chloroquine resistance. Mutations in these genes causes reduction in chloroquine uptake by the parasite's vacuole (Le Bras & Durand, 2003; Ibraheem *et al.,* 2014). Chloroquine resistance of *P. vivax* has already grasped a worrying prevalence in Indonesia, East Timor and Papua New Guinea. Recently, chloroquine resistance of *P. vivax* infections in Guyana, Peru and Brazil has been investigated in *in vivo* studies (Gonçalves *et al.,* 2014). In the same year, molecular evidence of increased resistance to chloroquine and sulphadoxine/pyrimethamine has been reported. Both *in vivo* treatment efficacy and molecular assays were used to identify the range of *P. falciparum* resistance towards both drugs in two of north-east Indian state. The study reported 81.5% treatment failure for chloroquine and 13.7% treatment failure to sulphadoxine / pyrimethamine. Moreover, 99% of the failure had mutant *pfcrt* genotype (76T), while 68% had mutant of *pfmdr-1* genotyoe (86Y). The study indicates high level of resistance was observed in North-east India and it triggered an alarm for malaria management in north-east India (Mohapatra *et al.,* 2014). Another study conducted during 2008−2013 in Kolkata and Purulia, India, resulted in a rise of *in vitro* chloroquine resistance to *P. falciparum*, even after five years following chloroquine withdrawal (Das *et al.,* 2017).

Since chloroquine is no longer used in treating *P. falciparum* infection, a standardized treatment using ACT has been employed to replaced chloroquine. Presently, ACT is still the most effective treatment for malaria since to date, no other alternative treatment can match its potency (WHO, 2015). However, in an earlier study, Rogers *et al.* (2009) found that artesunate-mefloquine, a type of ACT antimalarial, has started to fail in southern Cambodia (Rogers *et al.,* 2009). *P. falciparum* resistance to artemisinin has also been identified in the Greater Mekong Subregion including Cambodia, Myanmar, Laos, Thailand and Vietnam (WHO 2018). The mutations in the kelch propeller (K13-propeller) domain has been associated with the development of artemisinin resistance involving point mutations at Y493H, C580Y, M476I, R539T and I543T (Ariey *et al.,* 2014; Straimer *et al.,* 2015). The development of *P. falciparum* resistance towards artemisinin derivatives is causing limited efficacy of the current antimalarial drugs (Ringwald *et al.,* 2012). An investigation into the efficacy and safety of artemisinin-based antimalarials in the treatment of uncomplicated malaria for children in southern Tanzania indicated that artemether-lumefantrine (AL) is very efficacious in areas of high sulphadoxine–pyrimethamine (SP) resistance (Kabanywanyi *et al.,* 2007). More recently, Hastings *et al.* (2014) reported that artemisinin component typically shows insignificant contribution (<0.0001%) to the therapeutic capacity of the most widely used ACTs (Hastings & Hodel, 2014). On the other hand, a study to determine the efficacy of conventional antimalarials against *P. knowlesi*, the fifth human malaria parasite, revealed that it is uniformly

highly sensitive to artemisinin, variably and moderately sensitive to chloroquine, and less sensitive to mefloquine (Fatih *et al.,* 2013). Table 1 summarized the regions where antimalaria drug resistance have been reported with respective molecular markers.

Thus, there is a vital need to identify novel targets that can be employed in the improvement of the next generation of antimalarial drugs. Since antimalarial drugs are a vital component of disease control and elimination, potential failure of these drugs in the future would hinder the efforts dedicated to disease eradication and treatment. The aim of this review is to elucidate the applicability of enzymes found in *Plasmodium spp.* as possible therapeutic targets in combating malaria. In order to achieve this objective, pertinent information related to antimalarial targets, especially the enzymes involved in specific pathways, the mechanism of action of the enzymes and inhibitors, as well as the strategies used to discover new antimalarial drugs are discussed and presented in the following sections.

Table 1. Specific regions where antimalaria drug resistance have been reported with their respective molecular markers

Drugs	Species	Region	Molecular marker	Reference
Chloroquine	*P. falciparum*		Mutation on *pfmdr1* and *pfcrt* genes	(Le Bras & Durand, 2003)
Chloroquine	*P. vivax*	Indonesia, East Timor, Papua New Guinea Guyana, Peru and Brazil		(Gonçalves *et al.,* 2014)
Chloroquine and sulphadoxine /pyrimethamine	*P. vivax*	North-east India	Mutation on *pfmdr1* and *pfcrt* genes	(Mohapatra *et al.,* 2014)
Chloroquine	*P. falciparum*	Kolkata and Purulia, India		(Das *et al.,* 2017)
Artesunate-mefloquine	*P. falciparum*	Southern Cambodia		(Rogers *et al.,* 2009)
Artemisinin	*P. falciparum*	Cambodia, Myanmar, Laos, Thailand and Vietnam	Mutations in the kelch propeller (K13-propeller)	(WHO, 2018)
Sulphadoxine–pyrimethamine		Tanzania		(Kabanywanyi *et al*, 2007)

2. Antimalarial target

Over the years, most of the antimalarial drug discovery efforts tend to focus on parasite-specific processes, such as hemoglobin degradation, parasite's egress from the host cell, and host cell invasion by the parasite. As these approaches are becoming increasingly ineffective in combatting and treating malaria, it is essential to conduct further studies aiming to identify new targets for the development of antimalarial drugs. The findings are expected to facilitate construction of new compounds that can interact with the target receptor to diminish the malaria parasite. Targets such as mitochondrion, apicoplast and cytoplasm of *Plasmodium* have been studied extensively in extant studies and their potential as drug targets has been elucidated. Table 2 summarizes the Plasmodial enzymes in metabolic pathways that have been identified as drug targets.

Table 2. Plasmodial enzymes in metabolic pathways as therapeutic targets.

Metabolic pathway	Inhibitor	Therapeutic target	References
Iron and heme metabolism	Artemisinin and its derivatives	*Pf* ring stage activates ART	(Klonis, Creek, & Tilley, 2013)
Pyrimidine biosynthesis pathway	Small molecule species specific inhibitors of *Plasmodium falciparum* DHOD	Dihydroorotate dehydrogenase	(Patel *et al.,* 2008)
	Triazolopyrimidine-based	Dihydroorotate dehydrogenase	(Gujjar *et al.,* 2009)
	Aryl and aralkyl amine-based triazolopyrimidine	Dihydroorotate dehydrogenase	(Gujjar *et al.,* 2011)
	SPROUT-designed inhibitors (a software package for structure-based drug discovery and lead optimization).	Dihydroorotate dehydrogenase	(Davies *et al.,* 2009)
Mitochondrial inner membrane enzyme	Tetracyclic benzothiazepines	Cytochrome bc1	(Dong *et al.,* 2011)
	4(1H)-pyridones	Cytochrome bc1	(Bueno *et al.,* 2012)
Mitochondrion	Heterocyclic Quinolones	NADH: ubiquinone oxidoreductase	(Leung *et al.,* 2012)
Folate pathway	Antifolates	Dihydrofolate reductase and dihydropteroate synthase	(Hyde *et. al.,* 2005)
	Sulfur based drugs (analogs of sulphanilamide)	Dihydropteroate synthase	(Nzila *et. al.,* 2006)
	Quinazolinones	Dihydrofolate reductase	(Patel *et al.,* 2017)
	GTP analogue inhibitor (8-oxo-GTP)	GTP cyclohydrolase I	(Kümpornsin *et al.,* 2014)
Glycolytic pathway	Galloflavin	Lactate dehydrogenase	(Manerba *et al.,* 2012)
	Itraconazole, atorvastatin and posaconazole	Lactate dehydrogenase	(Penna-Coutinho *et al.,* 2011)
	Azole-based compound	Lactate dehydrogenase	(Cameron *et al.,* 2004)
	3-Br-isoxazoline	Glyceraldehyde-3-phosphate dehydrogenase	(Bruno *et al.,* 2016)
	Analogues of inorganic diphosphate	*T. cruzi* hexokinase	(Hudock *et al.,* 2005)
	*Pf*HK inhibitors with antiparasitic activity	hexokinase	(Davis *et al.,* 2016)

2.1 The mithochondrian target

According to Goodman *et al.*, 2017, *Plasmodium* mitochondrion is an effective drug target with safe effective drugs for medical use. The mitochondrion is an organelle that plays a key role in the eukaryotic cell energy production. *Plasmodium* mitochondrion, a single tubular organelle structure (6-kb mtDNA) is highly fragmented rRNA gen and only encodes three genes for proteins (Das *et al.,* 1997; Hikosaka *et al.,* 2011). Plasmodium and the host's mitochondrial are different in terms of molecular and their function. Plasmodial mitochondrial is essential in the parasite's life cycle (Lunev *et al.,* 2016), where mitochondrion dysfunctional can cause cell death. An overview of studies examining mitochondria

of *Plasmodium* as a drug target conducted by Hikosaka *et al.* (2015) indicated that the enzymes of mitochondrial tricarboxylic acid (TCA) cycle and mitochondrial electron transport chain (mtETC) are attractive drug targets. TCA cycle, also known as Krebs cycle, is one of the important stages in aerobic respiration. It is well-established that the substrate for TCA cycle is acetyl-CoA from pyruvate oxidation or fatty acid/protein degradation. Lunev *et al.* (2016) also has discussed in detail regarding the potential pathways of the mitochondrial and carbon metabolisms, such as pyrimidine biosynthesis, aspartate metabolism and mitochondrial tricarboxylic acid (TCA) cycle as drug targets in *P. falciparum* (Lunev *et al.,* 2016). Although *P. falciparum* possesses all enzymes of the TCA cycle, it has been suggested that at least the asexual stages do not require TCA cycle for energy generation (van Dooren, Stimmler and McFadden 2006) and rely on glycolysis and fermentation for energy (Vander Jagt *et al.,* 1990). Moreover, Hikosaka *et al.* (2015) cautioned that drug targeting TCA cycle and mtRTC was a difficult approach, as it required rigorous biochemical analysis due to the complexity associated with obtaining intact and pure mitochondria from the parasites. Furthermore, studies mentioned that during erythrocytic cycle of plasmodium, oxidative phosphorylation is not crucial for the *Plasmodium* as the parasites depend mainly on glycolysis for the source of energy to survive during the blood stage (Ke *et al.,* 2015; MacRae *et al.,* 2015; Bryant *et al.,* 1964; Roth *et al.,* 1988). Thus, Lunev *et al.,* (2016). generally believed that the role of mitochondria in the parasite during blood stage is mainly for the maintenance of inner mitochondrial potential Hence, designing antimalarial drugs by targeting the mitochondrion, specifically by failing the inner mitochondrial potential and inhibit *Plasmodium* growth is still relevant, as it may potentially deliver new drugs for malaria treatment.

Nevertheless, mitochondrial electron transport remains as target for the existing and new antimalarials. An analysis has suggested that a mixture of chloroquine or its analogue, together with another drug, inhibits carbon fixation and/or increases oxidative stress, where this should increase the clearance of *P. falciparum* from the host system (Tewari *et al.,* 2017). Compounds such as atovaquone are known to target the mitochondrial electron transport chain, since it blocks the flux of metabolites through the TCA cycle (Ke *et al.,* 2015).The details of the studies of enzymes in these pathways are tabulated in Table 2.

Even though malaria parasite's mitochondria metabolic processes are limited, and less pathways appear during the intraerythrocytic cycle for the parasite survival, it is encouraging that various number of compounds selectively aiming Plasmodium mitochondrion. Inhibition of mitochondria resulting in accumulation of free fatty acid and triglycerides in the cytoplasm and their derivatives, which have toxic effects potential on mitochondrial respiration, ketone body production , gluconeogenesis and ATP synthesis (Olszewska & Szewczyk, 2013).

2.2 Apicoplast target

Apicoplast (vestigial plastid) is a chloroplast-like organelle characterized by a unique non-photosynthetic plastid. Apicoplast can be found in most parasites of Phylum Apicomplexa, a name derived from apical complex, except in *Cryptosporidium spp.* (Köhler *et al.,* 1997; McFadden *et al.,* 1996). Apical complex is an anterior structure that allows the parasite to invade the host cells and establish themselves in the host. Apicoplast is a specialized organ located below apical complex that performs multiple functions but its capacity to photosynthesize is limited. In addition, it holds a wide range of metabolic pathways that can be used by the parasite. Some of the product components are critical for the host cell invasion (Ralph *et al.,* 2001; Mukherjee & Sadhukhan, 2016). However, apicoplast retains the typical plastid functions, such as isoprenoid, fatty acid, and haem syntheses (Table 1), while also carrying cellular processes, such as deoxyribonucleic acid (DNA) replication, transcription, translation, and post-translational modification (Mukherjee & Sadhukhan, 2016). Nonetheless, the reason why apicoplast is essential to the parasite's survival is still unknown (Lim & McFadden, 2010).

Apicoplast as an antimalarial drug target has been examined by several researchers (Botté *et al.,* 2012; Mukherjee and Sadhukhan 2016; Ralph, D'Ombrain, & McFadden, 2001). According to their findings, the apicoplast target for antimalarial is based on the metabolic activity in

the *Plasmodium* itself. As reviewed by Mukherjee & Sadhukhan (2016), in apicoplast DNA replication, gyrase A (GyrA) and gyrase B (GyrB) play an important role in *Plasmodium*, where DNA cleavage and wrapping domains were located in GyrA subunit, whereas adenosine triphosphase (ATPase) and DNA binding/GyrA interaction domains are present in the GyrB subunit, where this structure exhibit strong homology to *Escherichia coli*. In *E. coli,* aminocoumarin drug novobiocin inhibits the ATPase activity of GyrB, while in *P. falciparum* the drug was shown to competitively inhibit the apicoplast's ATPase activity and DNA replication, which causing a lag in conversion of the parasite's stages from trophozoite to schizont stage. Novobiocin also disrupt the activity of *P. falciparum* GyrB ATPase that causes the parasite's death in cultures. On the other hand, the review also mentioned ciprofloxacin, which shown to inhibit apicoplast DNA replication in *P. falciparum* (Mukherjee & Sadhukhan, 2016).

In a more recent study, Rai *et al.* (2017) noted that understanding of the function of apicoplasts will benefit in designing better therapeutics through calcium signaling because it plays a vital role in the development and growth of *Plasmodium*, specifically in apicoplast transcriptional regulation (Cheemadan *et al.*, 2014; M. Rai *et al.,* 2017). Cheemadan *et al.* (2014) studied the role of calcium signaling in the transcriptional regulation of the apicoplast genome of *P. falciparum*. In order to assess the effect of changing calcium distribution within the cell, the transcriptional responses of *P. falciparum* to two calcium ionophores was analysed. It was found that both inhibitors induced overlapping but not identical changes of the *P. falciparum* transcriptome. However, both inhibitors caused strong inhibition of transcriptional activity of all the important genes in the apicoplast genome. Due to this situation, the authors identified MAL13P1.156, which is a nuclear encoded apicoplast targeted protein that transports a calcium binding domain. Overexpression of the protein causing resistance of *P. falciparum* towards ionomycin and it was proposed that this protein possibly play an important role in calcium dependent signaling pathways in apicoplast (Cheemadan *et al.*, 2014).

In addition, apical membrane antigen 1 (AMA1) is one of the components in the invasion machinery of *Plasmodium*. AMA1 was found to be an important candidate for malaria vaccine. A review beheld at both structural details and functional significance of interactions at the hydrophobic cleft of AMA1 have been elucidated and it is suggested as an ideal target for the development of drugs that can prevent host cell invasion by malaria parasites (Macraild *et al.,* 2011).

2.3 Cytoplasmic target

An apicomplexan including *Plasmodium spp*., *Toxoplasma gondii*, and *Cryptosporidium parvum*, as well as opportunistic pathogens of immunocompromised individuals *Eimeria spp.* and *Theileria spp*., share distinct morphological features and cytoskeletal organization. Cytoskeletal system is multifaceted, comprising of interlinking filaments and tubules that spread throughout the cytoplasm. In *Plasmodium*, various metabolic pathways can be found in the cytoplasm, such as, folate metabolism and glycolysis. These metabolic pathways consist of hundreds of enzymes that may be potential drug targets (Gardner *et al.,* 2002). A general overview adapted and modified from Gardner *et al.,* 2002 on these pathways is shown in Figure 1.

2.4 Enzymes involved in folate pathway

Empirical evidence also indicates that folate pathway is vital for synthesizing amino acids, as it is important for building of proteins for the malaria parasites survival. By referring to previous literatures regarding folate pathway, several drugs were developed to disrupt folate pathway with the aim of developing new treatment and prophylaxis for malaria. Folate pathway is also critical in providing cofactors for the metabolic events. The enzymes in this pathway serve as molecular targets, including dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS). DHFR aids in the oxidation of tetrahydrofolate (THF) molecule to DHF and its reconversion to the THF form (Hyde, 2005). DHPS is an enzyme with two binding pockets, one of which binds to dihydropterin pyrophosphate (DHPP), while the other binds with p-amino benzoic acid (pABA). DHPS catalyzes the reaction that produces 7,8-dihydropteroate from these two substrates.

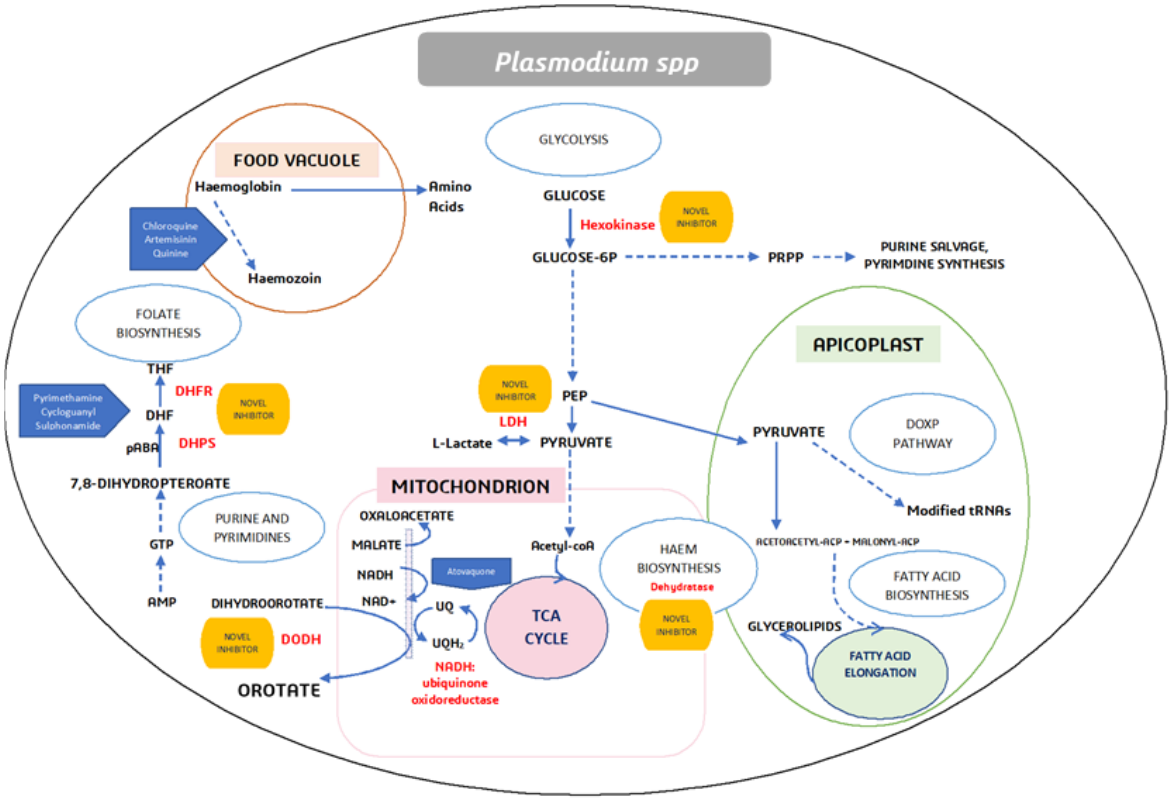

Figure 1. General overview of metabolism pathways and potential target for novel inhibitor in *Plasmodium spp*. Figures adapted and modified from Gardner *et al.,* (2002).

Among the antimalarial drugs presently in use, the antifolates have emerged as the most promising owing to their well-defined molecular targets—dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS)—that are also involved in the folate metabolic pathway. Antifolates are inhibitors of folate synthesis or folate conversions and are currently used for malaria treatment. These are first-line drugs in some of the African countries, as they are a cost-effective choice for fighting chloroquine-resistant malaria (Hyde, 2005). The antifolates have also been developed as synergistic combinations of DHFR and DHPS inhibitors. Antifolates, such as sulfur-based drugs, which are analogs of sulfanilamide, can inhibit DHPS. These compounds compete with para-aminobenzoic acid in the DHPS active site. However, due to their inherent toxicity, there is paucity of studies on the sulfur-based drugs, which limits their use in antimalarial treatment (Nzila, 2006). Another evaluation of antimalarial screening of quinazolinones was carried out by Patel *et al.,* (2017) both computationally as well as *in vitro* and its findings revealed their DHFR inhibitory potency. The main contribution of this study stems from identifying five out of nineteen potent antimalarial molecules (Patel *et al.,* 2017).

Other enzymes in folate pathways were defined in an earlier work by Lee *et al.* (2001), which described the gene that encodes GTP cyclohydrolase I (*gtp-ch*), catalyzing the conversion of GTP to dihydroneopterin *via* triphosphate. Polymerase chain reactions and DNA sequencing were performed to isolate the *gtp-ch* gene of *P. falciparum*. The gene was identified by using six primers and direct sequencing of this fragment discovered an open reading frame that are comparable with the predicted protein sequence and those of known *gtp-ch* molecules. This gene is attractive for novel antimalarial therapeutic target because it is expressed in blood-stage parasites and *gtp-ch* provides the rate-limiting steps in folate pathway, as shown in another microorganism (Lee *et al.,* 2001). Years later, Kümpornsin *et al.* (2014) studied the enzymatic activity and genetic

complementation for *P. falciparum* GTP cyclohydrolase I (*PfGCH1*). Its findings indicated that this could be a new approach to antimalarial drug development, since the assay of this enzyme showed an inhibitory effect by 8-oxo-GTP, a known GTP analogue inhibitor (Kümpornsin *et al.,* 2014).

2.5 Enzymes involved in glycotic pathway

Finding the alternative molecular targets for antimalarial drug design, specifically on the energy-generating pathway of carbohydrate metabolism is deemed essential in most drug design studies. Glycolysis, the metabolic pathway for carbohydrate metabolism, is an ancient, conserved metabolic energy-producing machinery that converts glucose to pyruvate and lactate under aerobic and anaerobic conditions, respectively. The enzymes in this pathway are crucial for parasite survival and growth. It is well-established that *P. falciparum* solely depends on glycolysis for energy generation and meets its energy needs by anaerobic metabolism of glucose to lactate (Sabbatani, Fiorino, & Manfredi, 2010). Glycolysis occurs in two distinct phases—the preparatory and the payoff phase. The preparatory phase is the glucose activation phase, where phosphorylation of glucose and its conversion to glyceraldehyde-3-phosphate takes place, while the payoff phase is the extraction stage, during which conversion of glyceraldehyde-3-phosphate to pyruvate, as well as the coupled formation of ATP, occurs (Giri, 2016). Ten enzymes are involved in glycolysis, namely hexokinase, phosphohexose isomerase, phosphofructokinase-1, aldolase, triose-phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase. During anaerobic condition, an additional enzyme—lactate dehydrogenase—is required. Genome sequence analysis by Gardner *et al.* (2002) has provided a comprehensive understanding of the metabolic potential of *P. falciparum*.

Glycolysis is the key metabolic pathway for ATP production in malaria parasite and is pivotal for its survival during the *Plasmodium* intra-erythrocytic cycle. The glycolysis rate in *P. falciparum*-infected erythrocytes is 20–100 times higher than in uninfected erythrocytes, which renders them valuable indicators of current infections (Iqbal *et al.,* 2004; Singh & Daneshvar, 2013). In a much earlier study, Roth Jr. *et al.* (1988) demonstrated that nearly all glycolytic enzymes were upregulated in *P. falciparum*-infected red blood cells (RBC), which was proportional to the parasitemia level. More recently, Kantele & Jokiranta (2011) revealed that hexokinase, aldolase, enolase, pyruvate kinase, and adenosine deaminase were the most markedly upregulated enzymes. The functions of each enzyme in the glycolytic pathway of malaria parasites have been reviewed extensively by Alam *et al.* (2014). The authors also investigated the functions of *P. falciparum* glycolytic enzymes as a part of their review. They noted its exceptional structural differences and functional features, suggesting that these could be exploited as therapeutic targets.

Research on *Plasmodium* lactate dehydrogenase has revealed that *p*LDH could be a diagnostic biomarker, as well as antimalarial inhibitory target (Jain *et al.,* 2014; van Niekerk *et al.,* 2016). Manerba *et al.* (2012) conducted their study aiming to verify galloflavin (CAS 568-80-9) as a novel inhibitor of lactate dehydrogenase. The authors reported that, in cultured tumor cells, galloflavin blocked the aerobic glycolysis at micromolar concentrations, but did not affect the cell respiration or induced cell death by triggering apoptosis. Additionally, 50 commercially-available compounds that have been selected through molecular docking approach were tested by Penna-Coutinho *et al.* (2011) against *P. falciparum* lactate dehydrogenase (*Pf*-LDH) and three compounds (itraconazole, atorvastatin and posaconazole) have been identified with the closest binding energy values to NADH. These were subsequently proven active in immunoenzymatic assays. The study findings further demonstrated that molecular docking research can be a reliable approach for discovering new antimalarial drugs (Penna-Coutinho *et al.,* 2011). Following an earlier study on azole-based compound, which is also an inhibitor of *Pf*-LDH, Cameron *et al.* (2004) suggested that these compounds have limited chances for additional derivatization due to the close contacts made within the active site of the enzyme. As the authors also noted that they appeared to have limited cellular uptake in the current form, they called for further development

of extended azole-like compounds, which may result a beneficial route for the improvement of novel antimalarials (Cameron *et al.*, 2004).

Other enzymes in the glycolytic pathway of *Plasmodium* were also widely studied, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in particular. GAPDH is a important glycolytic enzyme that catalyzes the oxidation of glyceraldehyde-3-phosphate (G3P) to 1,3-biphosphoglycerate (1,3-BPG), coupled to the reduction of nicotinamide adenine dinucleotide (NAD+) to NADH (Bruno *et al.,* 2016). Recent investigations have revealed that this enzyme is promising as a new malaria diagnostic biomarker because native *Pf*GAPDH protein levels were 4–6 times higher than the current target *Pf*LDH (Krause *et al.,* 2017). However, GAPDH has also been explored as potential antimalarial drug target. Compounds based on the 3-Br-isoxazoline scaffold completely inhibit GAPDH from *P. falciparum* by selectively alkylating all four catalytic cysteines of the tetramer, but only inhibit 25% of human orthologue (hGAPDH) (Bruno *et al.,* 2016).

Hexokinase has also been the subject of extensive research, as it is the first enzyme in the glycolytic pathway. An inhibition study on hexokinase of *Trypanosoma cruzi,* another species of blood protozoa, by bisphosphonates, showed that these non-hydrolyzable analogues of inorganic diphosphate are potent inhibitors of *T. cruzi* hexokinase (Hudock *et al.,* 2005). More recently, Davis *et al.* (2016) attempted to identify the inhibitor for *P. falciparum* hexokinase, where 57,654 molecules were screened from multiple small-molecule collections. Their findings revealed that the most potent inhibitors (GSK-650394) have 50% inhibitory concentrations as low as ~1 μM, and some were found to have low-micromolar concentrations (NCGC 00099116 and NCGC00099209) that were 50% effective against asexual blood stage of *P. falciparum* parasites.

3. Approaches employed in the discovery of new antimalarial drugs

3.1 Computational approach

Drug design and discovery pipeline comprises all the drug development stages, from identifying a target to clinical trials for testing the drug on human subjects. Hence, drug discovery is typically considered a time-consuming and costly process. However, computer aided drug design (CADD), a computational technology aimed at assisting the antimalarial drug discovery process, has become indispensable in recent years. It makes use of the structural knowledge of either the target (structure-based) or known ligands with bioactivity (ligand-based) to facilitate the identification of likely candidate drugs (Macalino *et al.,* 2015).

A study benefiting from this approach was performed by Granchi *et al.* (2015) with the aim of finding new human lactate dehydrogenase 5 (LDH5) inhibitors. As a part of this investigation, an automated docking-based virtual screening platform was developed by considering different protein conformations and the consensus docking strategy. The authors discovered that two of the ten of the selected compounds efficiently inhibit the enzyme activity *via* enzymatic assays (Granchi *et al.,* 2015). Given that it avoids carrying out enzymatic assays of thousands of compounds, CADD is highly cost- and time-efficient, as it helps optimizing the work in the laboratory. Several studies have been performed using both structure-based drug design (SBDD) and ligand-based drug design (LBDD) approaches in finding new inhibitors for the enzymes in *Plasmodium spp.* as the target, as summarized in Table 3.

Huthmacher *et al.* (2010) conducted their study with the goal of elucidating the capability of computational methods to foresee the metabolic activities during different stages of *P. falciparum* life cycle, which later led to the identification of new drug targets. Their findings suggested that the set of significant enzymes projected by flux balance approach signifies a reliable beginning for further drug development (Huthmacher *et al.,* 2010).

3.2 Natural products

It well-elucidated that countless compounds for drug discovery can be found in natural products. In the past two decades, their use has declined due to the technical difficulties associated with screening natural products in high-throughput assays compared to molecular targets (Harvey *et al.,* 2015). Azas *et al.,* (2002) evaluated potent *in vitro* synergistic antimalarial interactions between the extracts *of Mitragyna enermis* (Willd) O. Kuntze, Rubiaceae, *Nauclea latifolia* (Sm.) *Guera senegalensis* (GMel), Combretaceae and *Feretia apodhantera*

(Del) by conducting isobologram analysis. The findings yielded revealed that tetrahydroharman isolated from *G. senegalensis* exhibits antimalarial activity with neither cytotoxicity nor genotoxicity found in Salmonella Ames test, with and without metabolic activation (Azas *et al.,* 2002).

Table 3. Implementation of SBDD and LBDD approaches to find new inhibitors in malaria drug discovery

Methods	Species	Enzyme target	Inhibitor	References
Molecular modeling	*Plasmodium falciparum*	Enoyl reductase	VRC-007 and VRC-009 (analogues of N-benzylidene-4-phenyl-1,3-thiazol2-amine)	(Morde *et al.,* 2009)
Molecular modeling	*Plasmodium falciparum*	Dihydroorotate dehydrogenase	KMC-3 and KMC-15 (derivatives of N-phenylbenzamide)	(Desai, Shaikh, & Coutinho, 2011)
Molecular docking and 3D-QSAR	*Plasmodium falciparum*	Cysteine proteases	Derivatives of peptidyl vinyl sulfone	(Teixeira *et al.,* 2011)
3D-QSAR, molecular dynamics, docking, and genetic algorithm-based methods	*Plasmodium falciparum*	Dihydroorotate dehydrogenase	Derivatives of triazolopyrimidine	(Shah *et al.,* 2012)
Modelling the evolution of drug resistance in malaria.	*Plasmodium*	Dihydrofolate reductase	-	(Hecht & Fogel, 2012)
Docking and in silico ADMET	*Plasmodium falciparum*	S-adenosyl-L-homocysteine hydrolase	Noraristeromycin, curcumin and its derivatives	(Singh *et al.,* 2013)
Ligand-based 3D-QSAR analysis and virtual screening	*Plasmodium falciparum*	Glutathione reductase	N-(2,3-dihydrobenzo[b] [1,4]dioxin-6-yl)-2-(5-isopropyl4-(2-methoxyethyl)-4H-1,2,4-triazol-3-ylthio)acetamide	(Kamaria & Kawathekar, 2014)
Molecular docking	*Plasmodium falciparum*	Spermidine synthase	(1R,4R)-(N1-(3-aminopropyl) -trans-cyclohexane-1,4-diamine and analogue N-(3-aminopropyl)-cyclohexylamine	(Burger *et al.,* 2015)
Molecular docking	*Plasmodium falciparum*	Transketolase	6'-Methyl-Thiamin Diphosphate	(Hasan *et al.,* 2015)
In silico screening	*Plasmodium falciparum*	Enoyl-ACP reductase	Pyrimidine diones	(Lindert *et al.,* 2015)
3D QSAR, pharmacophore and molecular docking	*Plasmodium falciparum*	M18 aspartyl aminopeptidase	Derivative of quinine, chloroquine, 8-aminoquinoline and known antimalarial M18AAP inhibitors (AID743024)	(Kumari *et al.,* 2016)

More recently, Upadhyay *et al.* (2014) reported antimalarial potential of extracts and compounds from the chloroform and n-butanol fractions of *Ammannia baccifera* roots and methanol extract of *Ammannia coccinea* (AC). These extracts are commonly used in Chinese and Indian medicine as a therapy for diseases. The compounds were isolated from the extracted and fractionated dried and powdered samples, followed by repeated chromatographic separations of the fractions. The compounds effectiveness was demonstrated *in vitro* through antiplasmodial activity against *P. falciparum* NF-5. Extracts from AC showed potency in antiplasmodial activity and nontoxic to Vero cells. The authors thus concluded that the selective antiplasmodial activity from these plants will be advantageous in antimalarial drug development and discovery of safer therapeutics (Upadhyay *et al.,* 2014).

Thiengsusuk & Chaijaroenkul (2013) performed an evaluation of antimalarial activities of Thai traditional medicine which consist of 27 medicinal plants and 5 herbal formulations used against chloroquine-resistant (K1) and chloroquine-sensitive (3D7) *P. falciparum* clones. Their results indicate that that ethanolic extracts from 19 investigated plants/herbal formulations exhibited promising activity against both K1 and 3D7 clones of *P. falciparum*, with < 50% survival at the concentration of 50 μg/ml. In addition, eight medicinal plants and two herbal formulations that were included in this investigation showed potent antimalarial activity with median range IC50 values < 10 μg/ml against K1 or 3D7 *P. falciparum* clone or both (Thiengsusuk & Chaijaroenkul 2013).

Sethiya *et al.* (2014) screened methanol extract of *Evolvulus alsinoides* for *P. falciparum* lactate dehydrogenase enzyme inhibitory activity, reporting that *E. alsinoides* possesses a compound known as scopoletin that potentially inhibits the *Pf*LDH. *E. alsinoides* is generally recognized as *Shankhpushpi* and is used in traditional remedy for malarial treatment by some ethnic populations of India (Sethiya *et al.,* 2014).

3.3 High throughput screening

High Throughput Screening (HTS) is a drug-discovery method that is widely used in the pharmaceutical industry. It is an automated process that rapidly assays the biological or biochemical activity of many drug-like compounds. During a typical HTS assay, a collection of chemical composites is carefully chosen and tested against a biological target to evaluate the strength of the related inhibition or activation signal (Malo *et al.,* 2006). In order to successfully identify LDH inhibitors from library of a small molecule compound, two label-free high-throughput assays were planned using a kinetic high-throughput screen (VanderPorten *et al.,* 2013). In addition, examination on antimalarial elements from *Berberis thunbergii* and *Eugenia rigida* by automated HTS, using UPLC-MS-ELSD-PDA yielded two triterpenoids, namely α-betulinic acid and β-betulinic acid, that were acknowledged as antimalarial active elements from HTS hits of *E. rigida* (Zhang *et al.,* 2014)

High throughput screening is not only beneficial in the search for antimalarial drugs but is also indispensable in the discovery of small molecules that can interfere malaria transmission. Plouffe *et al.* (2016) developed a serum-free one-step assay for malaria transmission-blocking activity, which allowed them to analyze 13,983 known and new compounds. The authors noted that more than 90% recognized antimalarial drugs do not show activity to the late-stage gametocytes. Another high-throughput matrix screening approach was tested by Mott *et al.* (2015) on 13,910 approved and investigational drugs, allowing the researchers to identify several synergistic and antagonistic antimalarial drug combinations.

CONCLUSION

Malaria is a global issue, with the ongoing malaria transmission, there is a possibility for drug resistance to occur through increasing introduction of parasites to suboptimal drug levels. The growing resistance of *P. falciparum* to the established antimalarial drugs highlights the need for developing alternative drug regimens. Thus, identifying compounds that may disrupt the enzymes activities that have high control over the pathway's flux may lead to the discovery of novel and effective antimalarial drugs.

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