Drugs and drug targets against malaria

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The development of resistance by the parasite against first line and second line antimalarial drugs, has underscored the importance to develop new drug targets and pharmacophores to treat the disease. The absence of a vaccine for protection and the availability of artemisinin and its derivatives as the only option has made the situation rather serious. With the availability of increased support for malaria research, a variety of drug targets and candidate molecules are now available for further development. However, the success rate of a candidate molecule to become a drug is very low and it does become necessary to start with a large basket, identified on a rational basis. This review focuses on the present efforts to identify a variety of drug targets in the malaria parasite and to develop candidate drug molecules.

Keywords: Artemisinin, drug molecules, drug targets, malaria parasite.

ALL intervention strategies do not seem to have controlled malaria, which still takes its toll of 1–2 million deaths every year. An effective vaccine is not in sight and the parasite has developed resistance to frontline antimalarials such as chloroquine and antifolates (sulfadoxine + pyrimethamine). Artemisinin and its derivatives (ART) seem to be the only effective antimalarials in the basket and the situation calls for major initiatives to discover new antimalarials. This review focuses on the efforts to discover and develop new antimalarials.

Cell cycle as a drug target

Cyclin-dependent kinases (CDKs) play an important role in cell cycle progression and are conserved in all eukaryotic species[1]. CDKs have been investigated as possible drug targets that include cancer, infectious diseases, cardiovascular and neurological disoders[2,3]. CDK7 is an upstream kinase that activates other CDKs in the cell cycle[4,5]. Among the CDKs identified in *Plasmodium falciparum*, Pfmrk shares the highest homology with CDK7 (ref. 6). Pfmrk associates with Pfcyc-1 and phosphorylates the carboxy terminal domain of RNA polymerase II, just as is the case with CDK7 (ref. 7). With the use of high throughput screening assays, chemical synthesis as well as virtual screening and computer-aided design, attempts are underway to develop molecules that could act as universal *Plasmodium* CDK inhibitors. Based on the three-dimensional structure of pfmrk obtained through homology modelling, the similarities and differences with known CDKs have been studied. Starting with known CDK inhibitors, indorubin-3′-monoxime was identified as a template compound and a starting point for iterative screening process. Most potent activity was obtained with quinolinone and oxindole compounds. Further iterative searching of chemical databases has identified chalcones and tryptanthrins as potent inhibitors with greater specificity for Pfmrk[3].

Metabolic pathways in the apicoplast as drug targets

The identification of the 35 kb extrachromosomal DNA established the plastid origin of the apicoplast in *P. falciparum* and the apicomplexan parasites[8]. It is proposed that a secondary endosymbiotic event, where a red algal cell engulfed by an ancestral alveolate protist was acquired by the parasite, gave rise to the plastid organelle. The 35 kb apicoplast DNA codes for a full set of tRNAs, a few ribosomal proteins, three genes for the subunits of an oligomeric RNA polymerase, a gene for the elongation factor PfTu and a gene contributing to the Fe–S pathway. The plastid organelle lost most of its other genes to the nucleus and is incapable of photosynthesis[9].

The origin of apicoplast proteins has attracted a significant investigation. There is consensus that the nuclear-coded apicoplast proteins carry a bipartite signal at the N-terminal end. After entering the secretory pathway, the signal peptide is cleaved of, exposing the transit peptide that has a specific recognition mechanism to transit the multimembrane structure of the apicoplast[10,11]. Although transit peptides do not have a unique primary or secondary structure to carry out specific targeting, it is proposed that a chaperone may impart a characteric structure with specificity[12]. It has been possible to develop an algorithm to define a set of rules predicting the apicoplast-targeted protein[13]. A total of 551 proteins have been predicted to be targeted to the apicoplast[14], although experimental proof is perhaps available with only about 10–15% of the predicted Open Reading Frames (ORFs). These include: enzymes of fatty acid, isoprenoid and haem biosynthesis, subunits of DNA gyrase, Ti protease and ferredoxin. The predictions need not be absolute and each case has to be validated experimentally.

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Fatty acid biosynthesis

The genome of *P. falciparum* contains the genes for fatty acid synthase II (FAS II) pathway[14]. Earlier, individual studies had identified and located the FAS II enzymes in the apicoplast[11,15]. FAS I pathway involves multifunctional enzymes catalysing the elongation steps and is found in the cytosol of fungi and eukaryotes. In the FAS II pathway, each reaction is catalysed by a discrete enzyme and is present in algae and plants[16]. Figure 1 depicts the fatty acid biosynthetic pathway. Malonyl-ACP is the starting point and is produced from malonyl CoA and ACP, catalysed by the enzyme Malonyl CoA : ACP transacetylase (FabD). Malonyl-ACP condenses with acetyl-CoA to give β-ketoacyl ACP, catalysed by the enzyme β-ketoacyl-ACP synthase III (FabH). Three enzymes, namely β-ketoacyl-ACP reductase (FabG), β-hydroxy acyl-ACP dehydratase (FabA or FabZ) and enoyl-ACP reductase (FabI) sequentially convert β-ketoacyl-ACP to Acyl-ACP. This product will condense with another molecule of malonyl-ACP and produce β-ketoacyl-ACP. This reaction is catalysed by β-ketoacyl–ACP synthase I (FabB) or β-ketoacyl-ACP synthase II (FabF) depending on chain length. This product will go through a second cycle of reactions catalysed by FabG, FabZ (or FabA) and Fab I, extending the acyl-ACP product by two carbons.

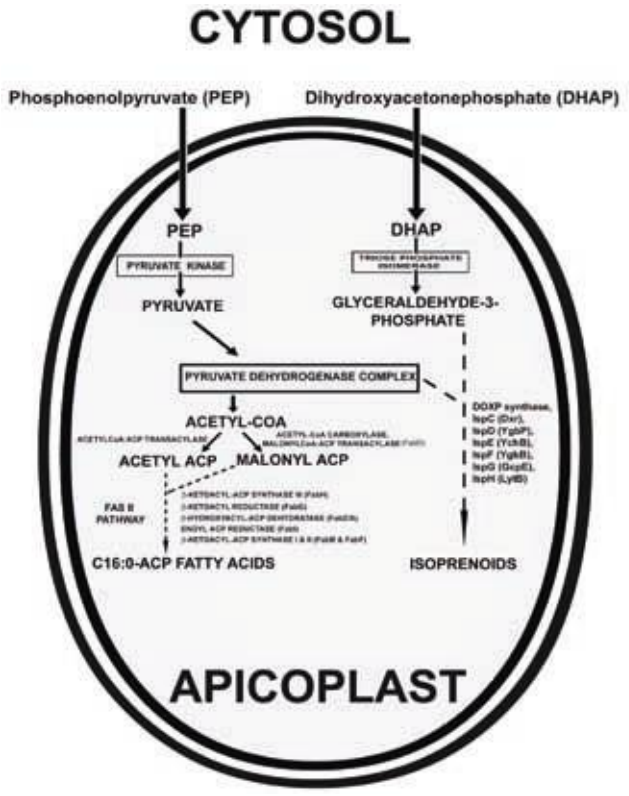

Figure 1. Hypothetical pathways for the formation of fatty acids (FAS II) and isoprenoids (non-mevalonate) in the apicoplast. Different components of the pathway have been identified at various levels: gene annotation, bioinformatics tools, enzyme activity, etc. Abbreviations: DOXP, 1-deoxy-D-xylulose-5-phosphate; IspC(Dxr), 1-deoxy-D-xylulose-5-phosphate reductase; IspD(Ygbp), 4-diphospho cytidyl-2C-methylerythritol synthase; IspE (YchB), 4-diphosphocytidyl-2C-methyl-D-erythritol kinase; IspF(YgbB), 2C-methyl-D-erythroitol 2,4 cyclodiphosphate synthase; IspG(GcpE), (E)-4-hydroxy-3-methylbut-2enyl diphosphate synthase; IspH(LytB), (E)-4-hydroxy-3-methylbut-2 enyldiphosphate reductase.

In the apicomplexan parasites including the *Plasmodium* species, the enzymes of the fatty acid biosynthetic pathway are predicted to be localized in the apicoplast, based on the N-terminal extensions suggesting such a localization[17]. Malonyl-CoA itself is formed from acetyl-CoA and biocarbonate and a single acetyl-CoA carboxylase (ACCase) is predicted to be localized to the apicoplast in the *Plasmodium* genome. *Toxoplasma gondii* has two ACCases, one in the cytosol and another in the apicoplast. It has been shown that only the plastidic ACCase is sensitive to acryloxyphenoxy propionate herbicides[18]. The inhibitors act on the carboxyl-transferase (CT) domain of ACCase as revealed by the crystal structure of the CT domain of yeast ACCase with the inhibitors[19]. Although the growth of *P. falciparum* is inhibited by a fairly high concentration of the inhibitors (100–200 μM), these could be the starting molecules for further modifications, since they are non-toxic to mammalian cells. ACP is the other component needed to generate malonyl-ACP. PfACP encodes a protein product of 137 amino acids with a 60 amino acid bipartite sequence at the N-terminus. The potential of PfACP as a drug target needs to be explored[20].

Among the enzymes of the FASII pathway in *P. falciparum*, FabI or enoyl-ACP reductase, catalysing the final step in the chain elongation cycle, has been investigated in great detail from the viewpoint of identifying potent inhibitors. The N-terminus of this gene product carries a bipartite sequence with potential to target the protein to the apicoplast. The enzyme catalyses the conversion of trans-2-acyl-ACP to acyl-ACP and requires NADH as the cofactor. Based on the potential of compounds to inhibit the bacterial enoyl-ACP reductase[21], the inhibitory effects of triclosan, diazoborine, isoniazid and ethionamide on the *Plasmodium* enzyme and growth in culture have been studied. Triclosan has been shown to inhibit the growth of *P. falciparum* in culture with an IC_{50} value around 1 μM[22,23]. It also inhibits radiolabelled acetate incorporation into fatty acids. The structural basis for the inhibition of the Pfenoyl-ACP reductase by triclosan has been investigated based on the resolution of the crystal structure[24] as well as kinetic studies using the recombinant enzyme[25]. It is inferred that triclosan traps the enzyme in the non-productive NAD^+ cofactor state, inhibiting the binding of NADH. The cofactor has a critical role in the binding of the inhibitors such as thienodiazaborine, imidazoles, aminopyridines and naphthyridinones[26]. Triclosan itself has not been found suitable for therapeutic use[22] with a very high potential for resistance development by a single A217V mutation in the enzyme[25]. A 6-hydroxy naphthalene derivative of triclosan with a K_i around 150 nM appears to have a potential for further development[27].

Major drug-screening efforts involving drug companies and academic institutions using triclosan and isonicotinic hydrazide as the starting molecules are underway. Synthesis of additional compounds guided by structure-based molecular modelling and docking simulations are being employed. Two pyrazole compounds, Genz-8575 and Genz-10850, have been found to inhibit the drug-resistant and drug-sensitive enzyme at 32 μM and 18 μM, respectively. These are from a library screened against the *M. tuberculosis* enzyme and are structurally different from triclosan. Another interesting development is the creation of transgenic *P. berghei* parasites with the Pfenoyl-ACP reductase replacing the endogenous counterpart to serve as an *in vivo* mice model for studying drug efficacy[26].

Three condensing enzymes β-ketoacyl-ACP synthases (PfKAS I, II & III) essentially involved in chain initiation and FabB/F (I & II) involved in chain elongation are also being investigated as possible drug targets. *P. falciparum* appears to code for a single enzyme with 37% and 31% identity to the *E. coli* FabF and FabB, respectively and therefore the enzyme is designated as PfKAS I/II[26]. Thiolactomycin analogues such as 1,2-dithiole-3-one derivatives are inhibitors of PfKAS III (FabH), having an IC_{50} as low as 0.5 μM. Similarly cerulenin and its derivatives are also suggested for development as inhibitors for PfKAS I/II[26]. The availability of crystal structures of thiolactomycin, cerulenin and fatty acid bound bacterial KAS I/II enzymes can provide the basis for the development of inhibitors for the parasite enzyme[28].

Major drug discovery programmes have been initiated to screen for PfKAS III inhibitors from compound libraries using a pharmacophore-guided *in silico* screen. The pharmacophore template is then used as a query to search chemical databases for new inhibitors and the potential hit compounds further screened for ADME properties. This effort has led to around 40 compounds with IC_{50} values lower than 10 μM[26].

β-ketoacyl–ACP reductase (PfKAR, FabG) catalyses the reduction of β-ketoacylACP to β-hydroxyacylACP in presence of NADPH and the latter is converted to trans-2-acyl-ACP by the dehydratase enzyme (PfHAD, FabA/Z). PfKAR is considered as an ideal target, since it is found as a single isoenzyme. However, no specific inhibitor of PfKAR is known at present. The structure of PfHAD has been modelled using the *E. coli* enzyme as template and two compounds, NAS-21 and NAS-91, have been found to inhibit at around 1 μM. The IC_{50} values in culture were found[29] to be 100 μM for NAS-21 and 7.4 μM for NAS-91. Further, PfHAD has been crystallized[30], providing a basis for structure-based inhibitor screening.

Isoprenoid biosynthesis

Isoprenoids, consisting of isopentenyl pyrophosphate (IPP) repeat units, form prosthetic groups of some enzymes and are also used in the synthesis of ubiquinone and dolichol. The malaria parasite genome provides evidence for the presence of the non-mevalonate pathway for IPP biosynthesis (Figure 1). The presence of 1-deoxy-D-xylose-5-phosphate (DOXP) synthase in the parasite genome was first reported by Jomaa *et al.*[31]. The pathway has been characterized in more detail recently[9]. The DOXP pathway utilizes pyruvate and glyceraldehyde-3-phosphate to give DOXP, catalysed by the enzyme DOXP synthase. DOXP is also utilized for the biosynthesis of thiamine pyrophosphate and pyridoxal. Including an auxiliary protein, eight enzymes are required for the formation of isopentenyl pyrophosphate and dimethyl allylpyrophosphate (DMAPP) and the *Plasmodium* genome has homologues for all the genes with potential for apicoplast targeting of the proteins[32].

The mechanism of export of IPP and DMAPP is not clear, but the close association of the apicoplast with the mitochondrion is visualized to facilitate its import, facilitating ubiquinone biosynthesis in the mitochondrion. Similarly, IPP is also likely to be involved in the prenylation of proteins in the parasite cytoplasm, where specific prenyl transferases have been identified[9,38].

Fosmidomycin, an inhibitor of DOXP reductoisomerase, has antimalarial activity[31]. A clinical trial with fosmidomycin indicates that although the drug clears the parasite, it is prone to recrudescence[34,35] and a useful synergy has been found between fosmidomycin and clindamycin in preliminary studies[36].

Haem biosynthesis

Studies in this laboratory had shown that the malarial parasite synthesizes haem *de novo*, despite accumulating haem derived from red cell haemoglobin as haemozoin pigment. Inhibition of the pathway with a specific inhibitor such as succinylacetone leads to death of the parasite[37]. While animals use glycine and succinyl-CoA to make δ-aminolevulinic acid (ALA), the committed precursor for haem biosynthesis, plants utilize glutamate to make ALA in a 3-step reaction[38].

Studies in this laboratory had also shown that the parasite (*P. falciparum*) imports host ALA dehydratase from the red cell[39] and inhibition of this import process by the N- and C-terminal deleted fragment of host ALAD added to a culture of *P. falciparum* blocks this import, leading to inhibition of haem synthesis and death of the parasite[40]. More recent studies have shown that the parasite imports ferrochelatase, the terminal enzyme of the haem-biosynthetic pathway, from the host red cell and the parasite cytosol is capable of independent haem synthesis *in vitro* starting from ALA, suggesting the presence of all the down stream enzymes of the pathway of likely host origin[41].

At the same time, the *P. falciparum* genome reveals the presence of all the genes of the haem-biosynthetic path-

way with the exception of uroporphyrinogen III synthase that is yet to be annotated[32]. Studies in this laboratory have shown that the parasite genome-coded ALA synthase (PfALAS), catalysing the formation of ALA from glycine and succinyl-CoA, is localized to the parasite mitochondrion[42]. Based on the localization of the GFP reporter in promoter–reporter fusion gene transfected parasites, Sato *et al.*[43] have also shown that PfALAS is localized to the parasite mitochondrion. In addition, they have also shown that PfALAD and PfPBGD (porphobilinogen deaminase) are targeted to the apicoplast. Studies in this laboratory have also shown by immunoelectron microscopy that the native PfALAD of the parasite is localized to the apicoplast[44]. The terminal enzymes of the pathway, PfCPO, PfPPO and PfFC do not manifest an N-terminal sequence targeting to the apicoplast and, therefore, Sato *et al.*[43] have suggested that these could be targeted to the parasite cytosol or mitochondrion. They have further speculated that while, PfPPO and PfFC could be mitochondrial, PfCPO could be cytosolic in terms of localization. Studies in this laboratory have, however, shown that the native PfFC is localized to the apicoplast as analysed using immunoelectron microscopy[41]. These results have been contested on the basis that in PfFC–GFP fusion gene transfected parasites, the reporter is targeted to the mitochondrion[45]. It is not clear at this stage whether there could be two PfFC species in the parasite. The present information regarding the localization of the parasite genome-coded and host-derived enzymes of the haem biosynthetic pathway in *P. falciparum* is summarized in Table 1.

Table 1. Localization of parasite genome-coded (Pf) and host-derived (h) haem biosynthetic enzymes in *P. falciparum*

Enzyme	Localization
PfALAS	Mitochondria
PfALAD	Apicoplast
hALAD	Cytosol
PfPBGD	Apicoplast
hPBGD	?*
PfUROS	Not annotated
hUROS	?*
PfUROD	Apicoplast (?)**
hUROD	?*
PfCPO	Cytoplasm (?)**, Mitochondria(?)**
HCPO	?*
PfPPO	Mitochondria (?)**
hPPO	?*
PfFC	Apicoplast/Mitochondria
hFC	Cytosolic

*Unknown; **Experimental validation required.

Abbreviations: ALAS, δ-aminolevulinate synthase; A; ALAD. δ-aminolevulinate dehydratase; PBGD, porphobilinogen deaminase; UROS, uroporphyrin III synthase; UDOD uroporphyrinogen III decarboxylase; CPO, coproporphyrinogen III oxidase; PPO, protophorphyrinogen IX oxidase; FC, ferrochelatase.

A hybrid pathway for haem biosynthesis involving the parasite genome coded enzymes localized in the mitochondrion, apicoplast and cytosol with the eventual formation of haem in the mitochondrion has been proposed[9,43,45]. The results obtained in this laboratory, however, show that the parasite cytosol and the organellar fractions are capable of independent haem synthesis[41]. The imported host ALAD and FC in the parasite account for more than 75–80% of the total respective enzyme activities in the parasite[41,44]. These results suggest the possibility that there could be two independent haem biosynthetic pathways in the parasite, one catalysed by the imported host enzymes and the other catalysed by the parasite genome-coded counterparts.

Further studies are needed to establish the need for two independent pathways of haem biosynthesis in the parasite. At the same time, experimental proof for the actual localization of native PfPBGD, PfUROD, PfCPO and PfPPO and their imported host counterparts needs to be obtained. Mechanisms for shuttling of the intermediates between compartments need to be understood to validate the proposed hybrid pathway.

Inhibition of the haem-biosynthetic pathway by succinyl acetone or the import of host-derived enzymes leads to death of the parasite[37]. Studies in this laboratory have shown that the biochemical properties of PfALAS[42] and PfALAD[44] are different from those of the corresponding host enzymes and that both components are needed for parasite survival. Therefore, the complex haem-biosynthetic pathway of the malaria parasite would constitute an ideal drug target.

DNA transactions

As already mentioned, the 35 kb apicoplast DNA has a marginal coding potential and undergoes replication, transcription and translation, essentially following a prokaryotic mechanism. A number of antibacterial compounds inhibiting this prokaryotic machinery act as slow acting antimalarials[46]. Tetracyclins, clindamycin, macrolids and chloramphenicol inhibit different steps in protein synthesis. Quinolone inhibits DNA gyrase and rifampicin inhibits RNA polymerase. All these compounds have been shown to cause delayed death, manifesting the effects in the cycle succeeding that in which the additions have been made[47].

At the level of protein synthesis, peptide deformylase from *P. falciparum*, that deformylates N-formylmethionine of the newly synthesized protein has been investigated as a possible drug target. The *Plasmodium* enzyme shows structural differences with that of the *E. coli* enzyme and many inhibitors (e.g. iron chelators) have been identified for further development[48,49]. Earlier studies from this laboratory had shown that chloroquine inhibits parasite protein synthesis by chelating haem and activating

eIF2α-kinase leading to enhanced phosphorylation of eIF-2α[50].

Although the antibiotics mentioned are slow acting, combinations such as clindamycin and quinine[51] and azithromycin and artesunate[52] are effective in clinical trials.

Other apicoplast targets

Fe–S clusters play an important role as prosthetic groups of enzymes and proteins involved in the electron transfer of redox and non-redox catalysed reactions and anabolic pathways. These clusters are found in mitochondria as well as chloroplasts in plants. An orthologue of sufB has been annotated in the plastid genome of *P. falciparum*[53]. Candidate genes for sufC, sufA, sufD, sufE and sufS have all been located in the *P. falciparum* database. Evidence has been obtained that sufC is targeted to the apicoplast and it would interact with sufB. SufS is a desulphurase generating sulphur to be incorporated into the Fe–S cluster and it has been shown that in the presence of sufE, sufBCD complex can stimulate the desulphurase activity by 30-fold. SufD is implicated in iron mobilization[9]. In addition to the suf operon, two other operons, nif and isc, have been implicated in Fe–S cluster assembly[54].

Fe–S cluster is also involved in the maturation of apoferredoxin and a *Plasmodium* orthologue of a NIF protein from *A. thaliana*, involved in the transfer of Fe–S cluster to apoferredoxin has been identified among the lypothetical proteins of the parasite. Fe–S clusters are also needed for the two terminal enzymes of the isoprenoid pathway[9], as well as lipoic acid synthase[55]. The extensive involvement of the suf system in the apicoplast in providing the reducing capacity and repairing oxidative damage to facilitate fatty acid and isoprenoid synthesis is visualized to make it an attractive drug target[9].

Lipoic acid is an essential cofactor of pyruvate dehydrogenase and other dehydrogenases. Two isoforms of lipoic acid synthase, LipA and LipB, are predicted to be plastidic in nature in *P. falciparum*[55]. A biosynthetic pathway involving lipoic acid synthase and lipoyl (octonoyl)–ACP : protein N6-lipoyl transferase B has been identified in the apicoplast, which is visualized to provide exogenous lipoic acid to the mitochondrial α-keto acid dehydrogenase complex involving lipoate protein ligaseA. These pathways are also considered as excellent drug targets[56].

Mitochondrial targets

The *P. falciparum* mitochondrion is atypical. In the asexual stage, it lacks the cristae, but perhaps, develops cristae in the gametocyte stage[57]. The mitochondrion is closely associated with the apicoplast at all stages of parasite development. It has a small genome of 6 kb, encoding the genes for cytochrome b, cytochrome oxidase I and III, besides fragmented tRNA[58,59]. It acquires several nuclear gene-coded proteins through import mechanisms involving Tim–Tom complexes.

The organization and role of the parasite mitochondrion has been envisaged through a bioinformatics approach based on data mining from the *P. falciparum* genome, with experimental evidence being available in a limited number of cases[45]. An assessment of the main functions of a typical mitochondrion would indicate its roles in energy generation, electron transport and intermediary metabolism. In all these cases, the role of the parasite mitochondrion remains unclear.

The *Plasmodium* derives its energy through glycolysis and there is no evidence that it leads to the formation of acetyl-CoA. The citric acid cycle itself appears dysfunctional, although the genes for most of the component enzymes can be identified in the database. The electron transport chain appears to function via the FAD-linked tricarboxylic acid cycle enzymes, malate-quinone oxidoreductase and succinate dehydrogenase. Coenzyme Q (uniquinone) occupies a pivotal position and at least five enzymes in *P. falciparum* can transfer electrons to this molecule. Clear cut evidence is available in the case of dihydroorotate dehydrogenase localized to the inner membrane of the mitochondrion. The dihydroorate synthesized in the cytosol is converted to orotate in the mitochondrion by dihydroorotate dehydrogenase transferring the electrons to coenzyme Q[45]. *Plasmodium* does not have a salvage pathway for pyrimidine biosynthesis and *de novo* pyrimidine biosynthesis is an ideal drug target. Recently, it has been shown that the malarial parasite maintains an active electron transport chain just to serve one metabolic function, namely to regenerate ubiquinone required as the electron acceptor for dihydroorotate dehydrogenase[60].

This leaves the role of FAD-linked tricarboxylic acid cycle enzymes, malate : quinone oxidoreductase and succinate dehydrogenase in electron transport to be clarified. The contribution of electrons donated from cytosolic and mitochondrial NADH and NAD(P)H to the electron transport chain also needs to be understood and in this context an understanding of the localization and function of NAD(P)H dehydrogenase and glycerol-3-phosphate dehydrogenase is important. The NAD(P)H dehydrogenase in *P. falciparum* does not have a human counterpart and, therefore, is a unique drug target[45]. A recent study has shown that the over expression of yeast NAD(P)H dehydrogenase enhances the sensitivity to artemisinin and ablation lowers the sensitivity to the drug. Expression of the PfNAD(P)H dehydrogenase in such an yeast restored the sensitivity, attesting to the role of the parasite enzyme in mediating the effects of aretemisinin[61].

Atovaquone was developed as an antimalarial, targeting against ubiquinol-cytochrome C oxidoreductase and collapsing the membrane potential as well. However, resistance to atavaquone developed very quickly and the

combination drug atovaquone–proguanil has been found to be effective, but expensive[62]. Orotidine decarboxylase catalysing the conversion of orotidine monophosphate to uridine monophosphate has also been investigated as a unique drug target and novel pyrimidine derivatives are also under investigation as potential antimalarials[63].

The parasite mitochondrion has also possible roles to play in folate, Fe–S cluster and haem biosynthesis and with experimental validation many steps in these pathways could become drug targets. While apicoplast Fe–S clusters are involved in isoprenoid biosynthesis and other pathways mentioned earlier, the mitochondrion, perhaps, contributes to Fe–S complexes in the cytosol and some enzymes functioning in the mitochondrion. It is also of importance to assess the role of the parasite mitochondrion in stages other than that of the red cell stage. Reports have indicated that the parasite mitochondrial functions are upregulated in the mosquito stage[64,65].

Cytosolic targets

The folate pathway has been a major drug target with the combination of pyrimethamine, inhibiting dihydrofolate reductase (dhfr), and sulfadoxine, inhibiting dihydropteroate synthase (dhps), proving to be an effective and cheap antimalarial combination. Unfortunately, resistance to this combination has become widespread[66]. A new combination, lapdap™, consisting of proguanil (a triazine metabolite) inhibiting dhfr and dapsone inhibiting dhps has been found to be effective in Africa[67], although its efficacy in South East Asia, where the parasite has developed four mutations in the *dhfr* gene, is open to question. P-15, a biguanidine precursor of diaminotriazine derivative in combination with dhps inhibitors is being developed as a combination therapy against folate-resistant *P. falciparum* cases. There is also an effort to make methotrexate and aminopterin, potent inhibitors of dhfr, *in situ* from non-toxic precursors, in view of their toxicity to the host[68].

Since *Plasmodium* derives most of its energy through glycolysis, inhibitors of this pathway have been investigated for antimalarial activity. One of the well-investigated targets is *Pf* lactate dehydrogenase (PfLDH). Structural information on the enzyme co-crystallized with inhibitors including oxamic acid and chloroquine is available. A large effort is focused on developing PfLDH inhibitors such as gossypol derivatives and naphthoic acid-based compounds[69]. Although a clinically relevant candidate molecule is not yet available, this target needs to be further investigated.

Another glycolytic enzyme that has attracted attention is the triose phosphate isomerase (TPI). Structural differences between the human and PfTPI have been observed, including the mutation of a conserved mammalian serine residue near the active site to a phenylalanine in the parasite enzyme. This can have profound effects on the interplay between the active site catalytic loop and inhibitors that could have therapeutic possibilities[70].

Plasmodium does not manifest the *de novo* pathway for purine nucleotide biosynthesis and depends on the salvage pathway for its supply of purines. Some of the enzymes of the salvage pathway have been investigated as potential drug targets. Purine nucleoside phosphorylase (PfPNP) converts inosine or 5′-methylinosine to hypoxanthine. Immucillin H, a potent inhibitor of PNP, kills the parasite in culture, but would also inhibit the host red cell enzyme. 5′-Methylthio-imucillin H has been developed as a potent and selective inhibitor of PfPNP based on the crystal structure of the inhibitor-bound enzyme[71].

Hypoxanthine-guanine-xanthine phosphoribosyl transferase (PfHGXPRT) catalyses the transfer of a phosphoribosyl group to hypoxanthine, xanthine or guanine to give the corresponding nucleotide. The structure of the enzyme is very similar to that of the host enzyme, although only the parasite enzyme is capable of utilizing xanthine as substrate. NMR and kinetic studies have indicated possibilities for selectivity in drug design based on structural differences[72,73]. Adenylosuccinate synthetase (PfAdSS) catalyses the first committed step in the purine salvage pathway giving rise to adenosine monophosphate. The crystal structure of PfAdSS containing Hadacidin, an aspartate analogue, is available[74]. Threonine 307 appears unique to the parasite enzyme forming a hydrogen bond with the inhibitor. Unlike purine biosynthesis, the malaria parasite synthesizes pyrimidines *de novo*. The drug targets in this pathway have been discussed in the earlier section.

Thiols play an important role in maintaining the redox state of the cell and the malarial parasite is very sensitive to an oxidative environment. Therefore, inhibition of thiol metabolism is a potential strategy to develop antimalarials. *Plasmodium* has a single isoform of glutathione-s-transferase (PfGST, m or p class) and its crystal structure is available[75]. An extended hydrophobic pocket in the enzyme is considered suitable for drug targeting. Haemin has a stronger inhibition on PfGST than on the placental p class enzyme. Inhibition of PfGST can potentiate the inhibitory effect of chloroquine. Glutathione reductase is also a potential drug target. There is substantial difference in the geometry and internal surface chemistry of the inter subunit cavity of the homodimer between the parasite and mammalian enzyme. Methylene blue, a phenothiazine drug, which inhibits the *P. falciparum* enzyme more than the human enzyme binds to this cavity. Thioredoxin reductase and glutathione reductase may have a competing physiological function to reduce glutathione disulfide and the essentiality of either enzyme for parasite survival needs to be proven[76].

The discovery of shikimate pathway in the malaria parasite has opened up a new drug target, although chorismate synthase is the only enzyme identified conclusively[77]. Direct immunological evidence is now available

for the localization of this enzyme in the parasite cytosol[78], although partitioning of the pathway between the parasite compartments is not ruled out. The herbicide glyphosate, a selective inhibitor of 5-enolpyruvylshikimate-3-phosphate synthase, and analogues of shikimate inhibit the growth of *P. falciparum*. Computational methods have been used to provide evidence for the identification of shikimate pathway enzymes that were not detected in the *P. falciparum* genome annotation[79].

Food vacuole targets

A major function of the food vacuole is to degrade the host red cell haemoglobin sequestered through the cytostome machinery and provide amino acids to the parasite. This is brought about by a variety of proteases and inhibition of this process is well investigated as a drug target[80,81]. Massive degradation of haemoglobin also leads to generation of a large quantity of haem that is toxic to the parasite, promoting membrane damage due to its peroxidative properties. The most important pathway of haem detoxication in *P. falciparum* is the formation of haemozoin pigment. Detailed studies have revealed that haemozoin is a crystalline polymer and identical to β-haematin with the haem units linked to each other through a coordinate bond between ferric iron of one unit and the propionate carboxyl group of the adjacent unit, the different cyclic dimers being linked through hydrogen bonds between the remaining carboxylate groups[82].

The mechanism of haemozoin formation is a matter of debate. While it is essentially considered to be a non-enzymatic process, various factors such as parasite lipids, hrpII protein, etc., have been shown to promote its formation. There are strategies to form haemozoin *in vitro*, providing a high throughput screening method for identifying inhibitory molecules[83]. Chloroquine and many quinolines have been shown to interact with haem, chloroquine non-covalently interacting with the growing face of the haemozoin crystal preventing further growth[84]. Blood schizonticidal antimalarials (e.g. quinine, quinidine, halofantrine, mefloquine and 8-aminoquiniolines including tafequine as well as bisquinoline analogues) in general are visualized to act through this mechanism. Although the sequiterpene endoperoxide artemisinin was also shown to interfere with haemozoin formation, its action through the process of alkylation of proteins by the reactive intermediates generated through iron/haem-mediated activation of the endoperoxide bridge, has also been implicated[85]. Specific inactivation of PfATP6, a calcium ATPase, has been projected as the main target[86]. Many other compounds such as hydroxamates, clotrimazole, pentamidine, cyprohepatidine, a variety of metalloporphyrins, natural compounds such as ellagic acid from the bark of *Tristaniopsis calobuxus* and terpene isonitriles from marine sponges have all been implicated to act as antimalarials through inhibiting haemozoin formation[83]. High throughput screening of molecules to inhibit β-haematin formation *in vitro* has led to the identification of new candidate antimalarials such as triacylcarbinol and the related benzophenone[87].

As already mentioned, parasite proteases involved in haemoglobin degradation have been investigated as potential drug targets. Detailed studies have shown that plasmepsins I and II (along with IV) are aspartic proteases that initiate the degradative process. This is followed by falcipains (I, II & III) that are cysteine proteases and the metalloprotease falcilysin[88]. At least ten plasmepsin genes have been identified and therefore, identifying a universal inhibitor may be a challenging task. While these events occur in the food vacuole, the peptides have to be translocated to the cytoplasm, where specific aminopeptidases will cleave them to amino acids. Many groups of inhibitors for plasmepsins such as statins and hydroethylamines and for falcipains such as semicarbazones, vinylsulfones and isoquinolines have been investigated for antimalarial activity[89].

A library of 1,4-bis(3-aminopropyl)piperazine has been investigated for inhibition of PfA-M1, a zinc aminopeptidase in the parasite. This also has a quinoline structural component that can inhibit β-haematin formation. Hydroxamate derivatives have metalloprotease inhibitor activity. It appears that a variety of derivatives can be designed to inhibit the peptidase activity as well as β-haematin formation[90]. Members of the methionine aminopeptidase family have been examined as potential antimalarial targets. From a library of 175,000 compounds, using one of the four cloned methionine peptidase 1a as target, a potent inhibitor containing a 2-(2-pyridinyl)-pyrimidine core has been identified. The compound is active against chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* in culture as well as *in vivo* in the mouse model[91].

Membrane targets – Biosynthesis and transport

During the intraerythrocytic stage, *P. falciparum* synthesizes considerable amounts of membrane components involving phospholipid metabolism[92]. An approach to inhibit *de novo* phosphatidyl choline biogenesis through the use of mono- and bis-quaternary ammonium salts mimicking choline structure has been evolved. These compounds were shown to inhibit choline entry into the infected erythrocytes and maximal activity was demonstrated with the derivative E13 (N,N,N-tripropyl dodecan-1-ammonium bromide, IC_{50} of 33 nM)[106]. The bis-quaternary ammonium compound G25 [1,6-hexamethylene-bis-(N-methylpyrrolidinium) bromide] was significantly more active with an IC_{50} of 0.64 nM[93].

Although the bis-quaternary ammonium salts are potent in parenteral administration, their bioavailability through

oral delivery is low. Therefore, bioisosteric analogues (bisamidimes) were tested, but without much success. This led to the synthesis of bis-thiazolium salts with appropriate substituents as prodrugs. The prodrug TE3, while having an IC_{50} in the range 1–2 nM was found to have an ED_{50} of 5 mg/kg with an absolute bioavailability of around 16% in Sprague-Dawley rats and a good therapeutic index. These compounds have a dual mode of action on the intracellular parasite, inhibiting phosphotidyl choline biosynthesis as well as forming complexes with protoporphyrin IX to inhibit haemozoin formation in the cell-free system. These compounds are indicated to be more active than chloroquine/artemisinins and most potent in the rodent model[94].

New permeability pathways (NPPs) in the intraerythrocytic stage to supply nutrients to the parasite across the red cell membrane are also under investigation as drug targets. The NPPs are visualized to function to enhance supply of nutrients such as glucose, pantothenate, Na^+, etc. or to deliver drugs as well as to efflux excess metabolites, such as lactic acid or amino acids[95]. Techniques based on radiotracer uptake, haemolysis, fluorescence and electrophysiological methods have been used to screen compounds inhibiting the NPPs, which are essentially anion-selective[96]. The most potent blockers of NPP activity are based on furosemide and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) and the most effective derivatives have an IC_{50} around 100 nM[97]. The main issues are a lack of correlation between the inhibitory activity on NPP and parasite growth and non-selectivity due to inhibition of the RBC pathway component.

Another important strategy is to use the NPP for selective targeting of antimalarials[98], including the bis-quaternary ammonium compounds mentioned earlier. NPPs can also be used to deliver monovalent cationic moieties and non-physiological stero-isomers such as the toxic-L-nucleosides that are not accessible to normal biological membranes[95].

Natural compounds as antimalarials

Manzamine A, α-β-carboline alkaloid present in several marine sponges provides 90% clearance of *P. berghei* in mice after a single intraperitoneal injection and 40% of the mice survive after 40 days[99]. Cyclosporin A, an antifungal immunosuppressive agent and clotrimazole, another antifungal compound, have all been found to have antimalarial properties. Recently, studies in this laboratory have shown that curcumin from turmeric has antimalarial activity[100] and a combination of α-β-arteether–curcumin therapy is able to completely prevent recrudescence due to arteether monotherapy and provide 100% protection against mortality in *P. berghei*-infected mice[101]. In the context of artemisinin-based combination therapies being looked upon as the strategy to prevent development of resistance and recrudescence, the curcumin–artemisinin derivative combination therapy appears superior to many other combinations being tested or used.

A study of plant species in rain forests of Madagascar for possible antimalarial compounds has led to the isolation of a novel morphine compound named tazopsine from *Strychnopsis thouarsii* having a specific inhibitory action against the liver stage but not the blood stage parasite. A simple modification of tazopsine to give N-cyclopentenyl-tazopsine has improved the toxicity profile, providing an alternative to primaquine, the only licensed drug available against hepatic stage malaria[102]. A variety of herbal medicine and traditional practices to treat malaria in different countries and societies is available and some have undergone clinical trials as well. But, an internationally acceptable, safe and efficacious regimen is yet to emerge, although there are many more herbal treatments used throughout the world than the validated antimalarial drugs[103]. The natural compound artemisinin from the Chinese herb continues to inspire the synthesis of newer antimalarials. Vennerstrom *et al.*[104] utilized the endoperoxide bridge as the war head and synthesized synthetic trioxalane derivatives with high oral bioavailability and optimal half-life, performing superior to the native compound. The derivative OZ277 is under clinical trial. More recently, Posner *et al.*[105] have synthesized new generation trioxane dimers from artemisinin and four of these cure malaria in infected mice with a single subcutaneous injection.

Concluding remarks

A difficult situation to treat malaria has arisen in view of the development of resistance of the parasite to front line

Table 2. Summary of drug targets and candidate molecules against the malaria parasite (typical examples)

Target	Enzyme/ receptor	Candidate molecule
Cell Cycle	MRK	Quinolinones, oxindoles
Apicoplast	Fab I	Triclosan derivatives, pyrazoles
Apicoplast	Accase	Acryloxy phenoxy propionate
Apicoplast	Fab A/Z	NAS-21, NAS-91
Apicoplast	Fab H	Thiolactomycin analogues
Apicoplast	DOXR	Fosidomycin
Cytoplasm	LDH	Gossypol -, naphthoic acid derivatives
Apicoplast	PNP	5-Methylthio-iimucilin H
Apicoplast	EPPS	Shikimate derivatives
Food vacuole	Haemozoin	Triacyl carbinol and several other compounds
Food vacuole	Proteases, Peptidases	1,4-Bis(3-amino propyl) piperazine derivatives
Membrane	Phospholipid. … Haemozoin	Bis quaternary ammonium and Bbisthiazolium salts
Natural compounds	?	Curcumin, Tazopsine derivatives

antimalarials and the unavailability of a vaccine. At present the main drug available in the basket is artemisinin (ART) and its derivatives and serious efforts are underway to develop ART-based combination therapies to extend the life of the drug. Therefore, all the targets and molecules discussed in this review and elsewhere are important with the realization that only a few would eventually make the grade. Typical examples of potential drug targets and candidate antimalarials are given in Table 2. In particular, new pharmacophores attacking different targets in the parasite can bring in new strategies to combat malaria. Apart from efficacy, toxic side effects, pharmacokinetic compatibility and potential to develop resistance would all be the major parameters in the eventual development of a successful drug. Affected children and pregnant women would pose additional challenges. Above all, malaria is a poor man's disease and therapies to be developed have to be affordable to the poor communities of the world that form the prime targets for the disease.

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