Chapter 2

Mitochondria of Malaria Parasites as a Drug Target

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Additional information is available at the end of the chapter

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Abstract

Mitochondria are organelle, which is found in most eukaryotic cells, and play an important roll in production of many biosynthetic intermediates as well as energy transduction. Recently, it has been reported that mitochondria contribute to cellular stress responses such as apoptosis and autophagy. These functions of mitochondria are known to be essential for survival and maintenance of homeostasis. The mitochondria of malaria parasites are quite different from those of their vertebrate hosts. Because these differences markedly contribute to drug selectivity, we have focused on the *Plasmodium* mitochondrion to develop antimalarial drugs. Here we summarize recent advances in our knowledge of the mitochondria of malaria parasites and discuss future prospective antimalarial drugs targeting the parasite mitochondrion.

Keywords: malaria, *Plasmodium*, mitochondria, antimalarial drugs, atovaquone, 5-aminolevulinic acid

1. Introduction

Malaria is a major global health problem, shortening over 500,000 human lives annually, mainly children in tropical and subtropical regions [1]. Due to difficulties in developing antimalarial vaccine, chemotherapy is important for controlling malaria. Parasites causing malaria, however, can rapidly develop resistance against the available chemotherapies [2]. Thus, new drugs with different modes of action are urgently needed. Malaria parasites are disseminated by female *Anopheles* mosquitoes and belong to the *Plasmodium* genus. *Plasmodium* has a complicated life cycle, comprising two major cycles: asexual multiplication in humans and sexual multiplication in mosquitoes (Figure 1) [3]. The parasites invade the hepatocytes of their host and mature into merozoites. After release, the merozoites infect red blood cells (RBCs). In the RBCs, the parasites differentiate into the following stages: ring, trophozoite, and schizont. Subsequently, the infected RBCs burst and release merozoites, which invade

uninfected RBCs. These stages are called the erythrocytic stages, where the parasites multiply asexually. Following the establishment of infection, some parasites differentiate to gametocytes [4]. The gametocyte stage is essential for subsequent transmission because this is the only stage where the organism undergoes sexual development in the mosquito vector. Therefore, *Plasmodium* has a complex life cycle, which seems to be an adaptation to its host environment [5]. In addition to the complex life cycle, the malaria parasites have evolved sophisticated pathways of energy transduction to adapt to their hosts.

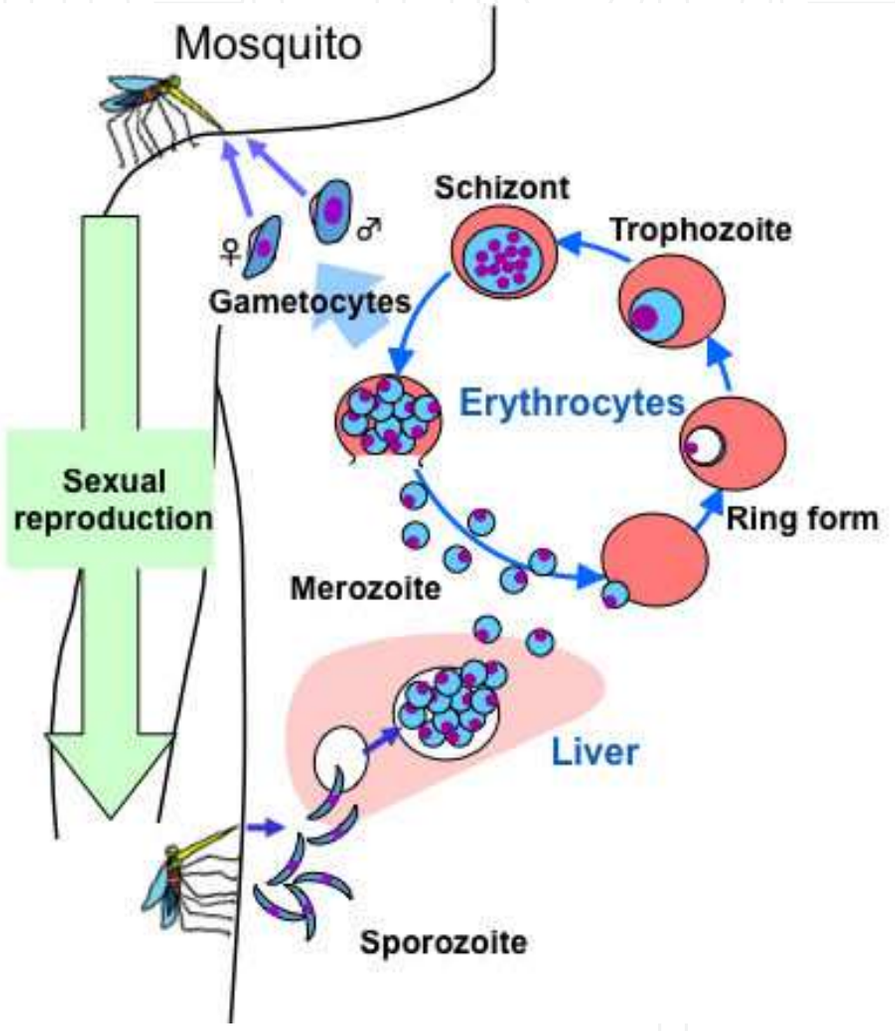

Figure 1. Life cycle of the human malaria parasite *Plasmodium falciparum*.

Mitochondria, an organelle arising from alpha-proteobacterium engulfed by a eukaryotic progenitor [6], play a key role in energy transduction of eukaryotic cells. In vertebrates, that can become a host for malaria parasites, mitochondria have been reported to contribute to cellular responses such as autophagy, apoptosis, and ATP production [7]. The vertebrate mitochondrion comprises two separate and functionally distinct outer and inner membranes that form cristae, and it also contains its own circular genome, the mitochondrial genome (mtDNA). With few exceptions, vertebrate mtDNA is approximately 16 kb in size, encoding

37 genes: two for ribosomal RNAs (rRNAs), 13 for proteins, and 22 for tRNAs [8]. In contrast to the vertebrate mitochondrion, the *Plasmodium* mitochondrion is a single tubular organelle structure [9] that possesses a 6-kb mtDNA, encoding only three genes for proteins and highly fragmented rRNA genes [10], and it is the smallest eukaryotic mtDNA. Furthermore, the erythrocytic stages of the parasite have been considered to mainly rely on glycolysis, with secretion of end products such as lactate and pyruvate [11, 12]. The mitochondria of malaria parasites are thus quite different from those of their vertebrate hosts. Because these differences markedly contribute to drug selectivity, we have focused on the *Plasmodium* mitochondrion to develop antimalarial drugs. Here we summarize recent advances in our knowledge of the mitochondria of malaria parasites and discuss future prospective antimalarial drugs targeting the parasite mitochondrion.

2. Biochemical functions of malaria parasite mitochondria

2.1. ATP production in canonical eukaryotes

Conventionally, a mitochondrion is the cell's powerhouse, in which energy stored in chemical bonds is turned into ATP via oxidative phosphorylation. ATP production can be divided into three pathways: glycolysis, mitochondrial tricarboxylic acid (TCA) cycle, and mitochondrial electron transport chain (mtETC). Glycolysis breaks down one molecule of glucose into two molecules of pyruvate, generating two molecules of ATP. Pyruvate then moves into the mitochondrion where it is converted to acetyl-CoA and carbon dioxide by pyruvate dehydrogenase complex (PDH). Subsequently, acetyl-CoA enters the TCA cycle. The mtETC involves the passage of electrons from TCA-cycle NADH or from succinate via mtETC complexes to oxygen, with concomitant translocation of protons into the mitochondrial intermembrane space. Generally, the mtETC comprises four integral membrane enzyme complexes in the mitochondrial inner membrane: NADH-ubiquinone oxidoreductase (complex I), succinate-ubiquinone oxidoreductase (SQR, complex II), ubiquinol-cytochrome *c* oxidoreductase (complex III or cytochrome bc_1), and cytochrome *c* oxidase (complex IV). Ubiquinone (Q) and cytochrome *c* (complex IV) function as electron carriers and the complexes I, III, and IV function as sites generating potential. The resultant potential across the mitochondrial inner membrane is used to drive ATP synthesis.

2.2. ATP production in malaria parasites

Similar to canonical eukaryotes, in the mosquito stages of malaria parasites, the organisms produce ATP in their mitochondria [13]. In the erythrocytic stages, however, the mitochondrial energy transduction system for oxidative phosphorylation is downregulated to adapt to host environments and produce ATP mainly via glycolysis using blood glucose [14, 15]. As a consequence, in malaria parasite-infected patients, plasma lactate levels tend to be high and highly variable, ranging from 2 to 26.7 mM [16, 17], compared with plasma lactate levels (0.3-1.3 mM) in normal individuals. Apart from the minor flux of carbon backbone derived from glucose, TCA metabolism of *Plasmodium* was believed to involve a branched architecture

bifurcating from 2-oxoglutarate until recently [18]; however, this report was subsequently retracted [19]. More recently, the malaria parasites have been reported to use the canonical oxidative mitochondrial TCA cycle to catabolize host glucose and glutamate (Figure 2) [20], even during asexual multiplication. The TCA cycle begins with malate generated by anaplerotic reactions and 2-oxoglutarate produced from glutamine as well as conversion of acetyl-CoA to citrate by citrate synthase [20-22]. In general, pyruvate, the end product of glycolysis, is transported via the monocarboxylate transporter (MCT) family [23]. *Plasmodium* possesses two MCT genes (PF3D7_0210300 and PF3D7_0926400) identified in its genome (PlasmoDB version 11.0, website: http://plasmadb.org/plasma/). Although further evidence is required, these MCTs are considered to be associated with the transport of pyruvate across the mitochondrial membrane [24]. To convert pyruvate into acetyl-CoA, *Plasmodium* retains branched chain ketoacid dehydrogenase (BCKDH), the only enzyme implicated in branched chain amino acid degradation [22]. PDH complex, linking cytoplasmic glycolysis to the TCA cycle in canonical eukaryotes, is not localized to the mitochondrion but to a plastid, apicoplast, in *Plasmodium* [25]. The function of the *Plasmodium* PDH complex seems to include the provision of acetyl-CoA for *de novo* fatty acid synthesis within the apicoplast.

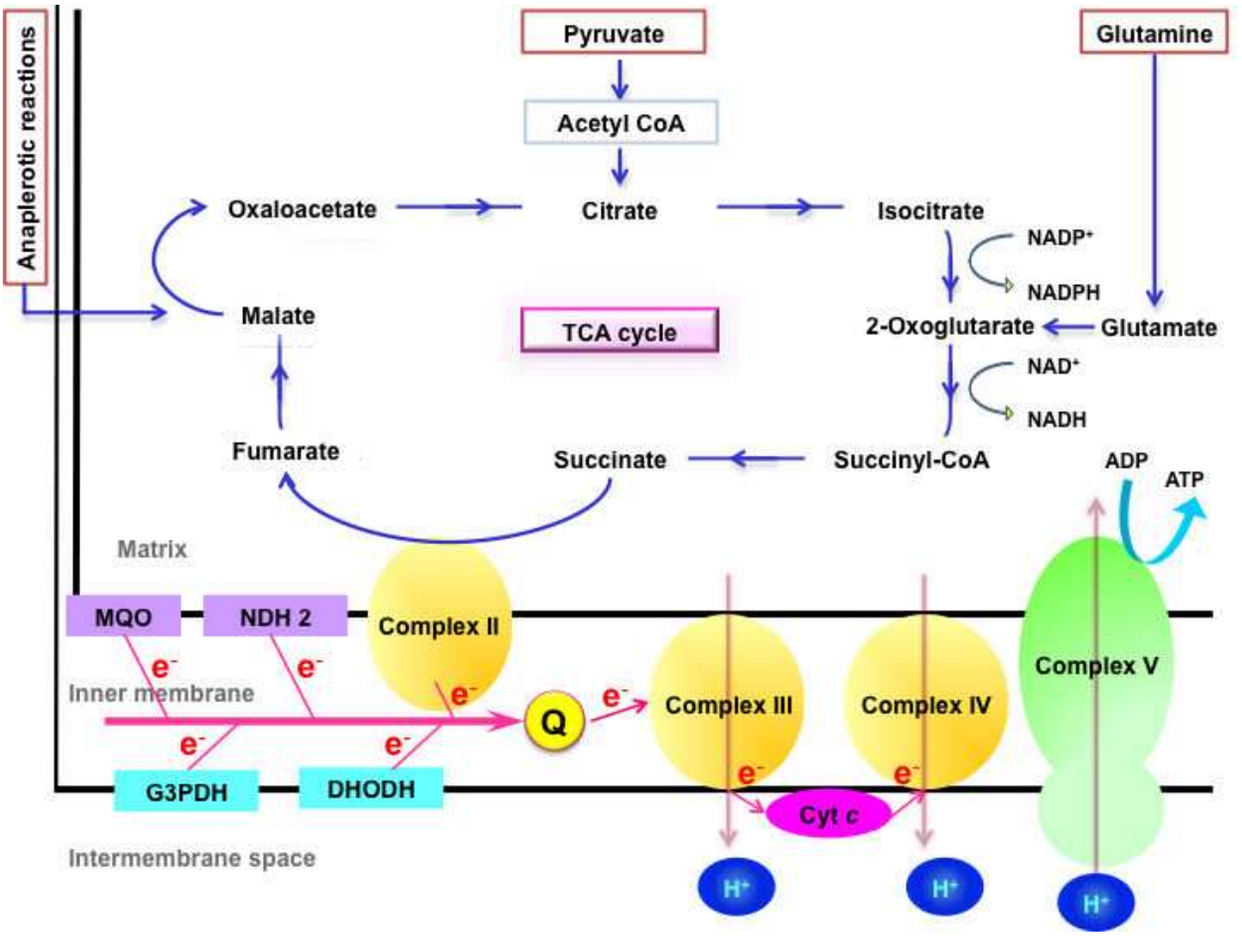

Figure 2. TCA cycle and oxidative phosphorylation of malaria parasites. The TCA cycle of malaria parasites begins with malate, 2-oxoglutarate, and citrate [20-22].

In the *Plasmodium* TCA cycle, succinate and malate are oxidized by SQR and malate-ubiquinone oxidoreductase (MQO), respectively, with transporting electrons to the matrix [26] (Figure 2). Similar to SQR of most eukaryotes, the *Plasmodium* SQR comprises four polypeptides: a flavoprotein (Fp) subunit, iron-sulfur (Ip) subunit [27], and two cytochrome *b* (cyt*b*) subunits (CybL and CybS) [28]. Fp and Ip form the catalytic portion of the complex. This portion acts as a succinate dehydrogenase (SDH), catalyzing the oxidation of succinate by water-soluble electron acceptors such as phenazine methosulfate in SQR, and is bound to the matrix side of the mitochondrial inner membrane via the membrane-anchoring proteins CybL and CybS. Because the mitochondria of erythrocytic stage parasites show both SQR and SDH activities [27, 29, 30], complex II has been considered to have some role in parasite survival. These activities, however, are very low, compared with those of the other eukaryotes (Table 1) [31-35]. Furthermore, our previous studies have demonstrated that disruption of the Fp subunit genes *pfsdha* and *Pbsdha* does not affect growth in the erythrocytic stages *in vitro* [36] and *in vivo* [37], respectively. These findings reveal that complex II is not essential for survival of the erythrocytic stage parasites, and this appears to be associated with relatively low activities of SQR and SDH in these developmental stages.

Organism	SQR specific activities* (nmol/min/mg)
Plasmodium falciparum	1.75
Trypanosoma cruzi	85
Ascaris suum	136
Rat liver	298
Bovine heart	111
Human cell	79.7

*Activity values of *P. falciparum*, *T. cruzi*, *A. suum*, rat liver, bovine heart, and human cell are obtained from references [29, 31, 32, 33, 34], and [35], respectively.

Table 1. Specific activities of succinate-ubiquinone oxidoreductase of various organisms

MQO is an FAD-dependent membrane-associated protein that catalyzes the oxidation of malate to oxaloacetate [38]. The electrons are donated to quinones of the mtETC, and NAD is accepted as an electron donor. The MQO has been not observed in mammals but has been found in *Plasmodium* [39] and some bacteria [40]. This implies that the *Plasmodium* MQO could be a target for drug design. In addition to SQR and MQO in the TCA cycle, *Plasmodium* possesses three oxidoreductases in its mitochondrial inner membrane: type II NADH:ubiquinone oxidoreductase (NDH2) [41], dihydroorotate dehydrogenase (DHODH) [42, 43], and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) [44, 45], all of which can reduce ubiquinone (Figure 2). Unlike the large multisubunit complex I in most mitochondria, the *Plasmodium* NDH2 is a single subunit enzyme, not involved in the direct pumping of protons across the membrane [46]. The absence of NDH2 in mammalian mitochondria shows that this enzyme would be a promising target of a novel antimalarial drug. Some antimalarial activities of NDH2 inhibitors, such as HQNO [47] and 1-hydroxy-2-dodecyl-4(1H) quinolone [48], have

been reported. However, a recent *in vivo* study on *Plasmodium berghei* revealed that the *Plasmodium* NDH2 could be deleted by targeted gene disruption, indicating that it is dispensable in the erythrocytic stages [49]. This disproves that NDH2 is a candidate drug target. Thus, the potential of targeting NDH2 as an antimalarial drug remains controversial.

The other dehydrogenases (DHODH and G3PDH) transfer electrons from reduced compounds in the cytosol (Figure 2). In the erythrocytic stages of the parasite, DHODH plays two roles—a generator of reduced ubiquinone and the fourth enzyme in the pyrimidine biosynthetic pathway. Since *Plasmodium* cannot salvage pyrimidine [50], DHODH is essential for its survival [42]. Therefore, in the erythrocytic stages, the mtETC appears to be essential for the pyrimidine biosynthetic pathway rather than for contributing to the ATP pool [11].

As presented above, in *Plasmodium* mitochondria, five mitochondrial dehydrogenases (SQR, MQO, NDH2, DHODH, and G3PDH) can generate reduced ubiquinone, which in turn is reoxidized by cytochrome bc_1 complex (complex III). Complex III is inhibited by atovaquone [51], which collapses the mitochondrial membrane potential [52]. As an antimalarial, atovaquone is very effective; however, atovaquone-resistant parasites develop easily. Mechanisms of atovaquone resistance are described in Section 4. Similar to canonical eukaryotes, *Plasmodium* utilizes cytochrome *c* (cyt*c*) as electron carriers and complexes III and IV as sites generating potential. The resultant potential across the mitochondrial inner membrane is used to drive ATP synthesis. *Plasmodium* ATP synthase is markedly different from that of its host [53]—it is assembled as a large dimeric complex in the erythrocytic stages. In the ciliates *Tetrahymena thermophila* and *Paramecium*, the structure and arrangement of dimeric ATP synthase have been suggested to determine the tubular morphology of the mitochondrial cristae [54, 55]. This could explain how the tubular cristae found in the mitochondria of erythrocytic stages are generated.

2.3. Mitochondrial energy metabolism: a target of antimalarial drugs

Recently, in addition to the genetic disruptions of SDH and NDH2 described above, it has been reported that six TCA cycle enzymes can be genetically disrupted in the erythrocytic stage or sexual development stage [45]. These reports suggest that the TCA cycle would not be essential for survival in these developmental stages. Hence, to develop an antimalarial drug, promising mitochondrial targets would be DHODH, which is associated with the pyrimidine biosynthesis pathway and mtETC, and the mitochondrial complexes III, IV, and V that generate electron gradients on the mitochondrial inner membrane.

On the other hand, it has been recently demonstrated that parasites derived directly from infected patients show three distinct gene expression states. One of these states demonstrates that the expression levels of the TCA cycle- or mtETC-related genes are increased [56]. Furthermore, mice infected with *P. berghei* or *Plasmodium yoelii* perform active oxidative phosphorylation [57], suggesting that, in some physiological conditions, malaria parasites may produce ATP via the mitochondrial TCA cycle and mtETC. Thus, we cannot exclude the possibility that all the mitochondrial enzymes are potential targets for antimalarial drugs.

3. The mitochondrial genome of malaria parasites

Malaria parasites possess a mitochondrial genome in the form of circular and/or tandemly repeated linear elements of 6 kb, the smallest in size among eukaryotic cells [58]. Copy numbers for this element are approximately 20-fold and 150-fold of the nuclear genomes in the human malaria parasite *Plasmodium falciparum* [58] and the rodent malaria parasite *P. yoelii* [59], respectively. These differences in the copy number may reflect differences in oxidative phosphorylation activities as noted previously (see Section 2.3). The 6-kb element contains only three mitochondrial protein-coding genes in addition to the large subunit (LSU) and small subunit (SSU) rRNA genes [60, 61, 62] (Figure 3). The three protein-coding genes are cyt*c* oxidase subunit 1 (*cox1*) and subunit 3 (*cox3*), members of the cyt*c* oxidase complex (complex IV), and cyt*b* (*cob*), a member of cytochrome bc_1 complex. In all eukaryotic cells possessing mitochondria, *cox1* and *cob* are encoded by the mitochondrial genome. Because the organisms possessing mitochondrion-like organelles without its own DNA (e.g., hydrogenosome and mitosome) do not have *cox1* and *cob*, these two genes appear to be essential for maintenance of the mtETC.

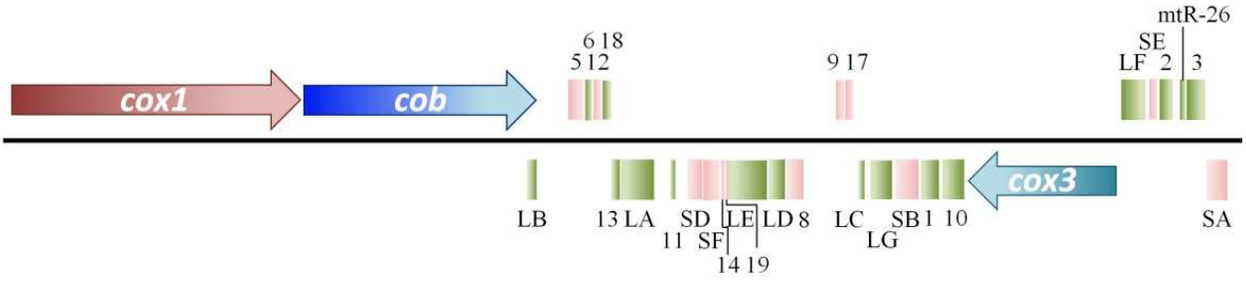

Figure 3. Mitochondrial (mt) genome structure of malaria parasites. Mt-genome organization is perfectly conserved among 23 *Plasmodium* species [63]. Elements within the mt genome of *Plasmodium* are tandemly repeated, so the designation of both termini is arbitrary. Light green and light magenta boxes indicate fragments of LSU and SSU rRNA genes, respectively.

The two rRNA genes of the *Plasmodium* mitochondrial genome are highly fragmented [63], and the fragmentation is the most extreme example of any described rRNA fragmentation. Recently, transcription of almost all intergenic regions of the *Plasmodium* mitochondrial genome has been demonstrated [63]. The results show that 27 small rRNA fragments (12 SSU rRNAs and 15 LSU rRNAs), ranging from 23 to 190 nt, are present in its mitochondrial genome (Figure 4). All the rRNAs are predicted to pair with at least one of the other rRNA, creating interactions that would help maintain the appropriate location and orientation of each rRNA. Notably, among the *Plasmodium* genera, the nucleotide sequences of noncoding regions, as well as fragmented rRNA gene regions, are more conserved when compared with those of the protein-coding gene regions [10]. It thus appears that these highly conserved sequence regions code for functional RNAs, including additional fragmented rRNAs.

In addition to the highly fragmented rRNAs, the mitochondria of malaria parasites have a unique property—transfer RNA (tRNA) is absent; therefore, protein translation in the

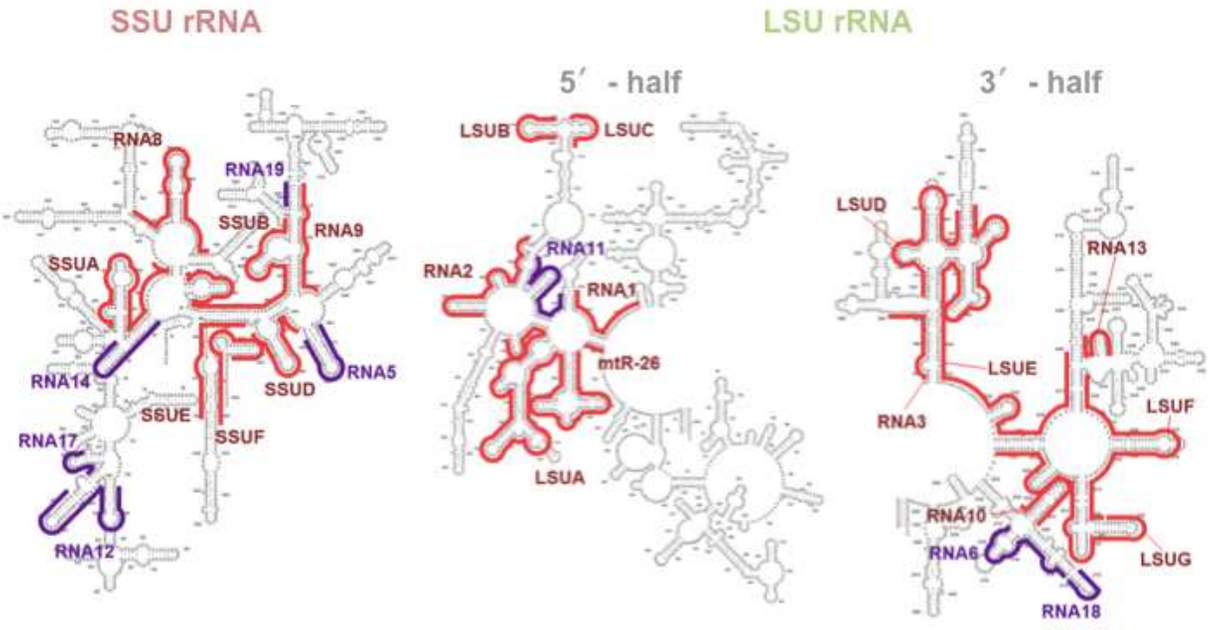

Figure 4. Fragmentation of the mitochondrial LSU and SSU rRNA genes of *Plasmodium falciparum*. Red line indicates rRNA regions. Purple lines indicate recently identified additional fragmented rRNA candidates[63].

mitochondrion was to date considered as being impossible. However, recently, extramitochondrial phenylalanyl-tRNA synthesis has been found in mitochondria of the erythrocytic stages, suggesting that the parasite mitochondrion can import tRNAs from the cytoplasmic tRNA pool [64]. These findings referring to the parasite rRNAs and tRNAs would make the parasite mitochondrial protein translation a desirable organelle to target as an antimalarial drug.

In malaria parasites, mtDNA is replicated via rolling circle replication to generate the linear concatemers, similar to the replication mechanism used by some bacteriophages and plasmids [58]. This replication manner is remarkably different from that of the vertebrate mtDNA, which is replicated by a theta mechanism. Furthermore, mitochondrial DNA polymerase, which has been characterized as a γ-like DNA polymerase, is strongly resistant to 2,3-dideoxythymidine-5-triphosphate and, in this aspect, differs from its vertebrate homolog [65], suggesting structural differences between the *Plasmodium* and vertebrate DNA polymerase. Further research on translation and replication mechanisms of the parasite mtDNA may help identify potential targets for drug candidates.

4. Atovaquone resistance in malaria parasites

4.1. Predicted mode of action for atovaquone

Atovaquone (a hydroxy-1,4-naphthoquinone derivative) is a broad-spectrum antiparasitic agent active against malaria, *Pneumocystis carinii* pneumonia, toxoplasmosis, and babesiosis [66]. The mode of action for atovaquone involves selective inhibition of parasite mtETC

without affecting the host mitochondrial functions at effective doses, making it the first member of an entirely new class of antimalarial agents [52]. This drug shares structural similarity with ubiquinone, a coenzyme involved in mtETC and serves as a point of contact between energy metabolism and pyrimidine metabolism. Therefore, a potential molecular target of atovaquone can be the ubiquinol oxidation pocket, Qo site, of the cytochrome bc_1 complex [51, 67] because it may have a specific inhibitory effect on the parasite cytochrome bc_1 complex. Generally, the cytochrome bc_1 complex is a structural and functional homodimer. The catalytic core comprises three redox active subunits, cyt *b* with two *b*-type hemes, cytochrome c_1 (cytc_1) with a *c*-type heme, and Rieske protein with a [2Fe-2S] cluster [68]. Cyt*b* catalyzes the transfer of electron from ubiquinol to cytc_1, coupled to the transmembrane proton translocation across the mitochondrial membrane [69]. There are two distinct catalytic sites on the cyt*b* protein, which are involved in the proton motive Q cycle and are proposed to account for the electron transfer and proton translocating activity through the cytochrome bc_1 complex: center o (also designated as Qo or center P) on the cytoplasmic site of the mitochondrial inner membrane, where ubiquinol (QH_2) oxidation occurs, and center i (Qi or center N) on the matrix site, where ubiquinone (Q) reduction occurs. Because of its structural similarity with ubiquinone, atovaquone appears to inhibit the cytochrome bc_1 complex by competitive binding with coenzyme Q for one of these sites.

4.2. Emergence of atovaquone-resistant malaria parasites

Atovaquone is majorly used for treatment and chemoprophylaxis of falciparum malaria for international travelers [70], but the major problem is rapidity of emergence of drug resistance when it is used as a single agent. Thus far, proguanil, which inhibits the parasite dihydrofolate reductase, is combined with atovaquone to prevent the emergence. The combination drug, registered as Malarone® (GlaxoSmithKline group of companies), is approved for treating malaria in more than 30 countries and is used for chemoprophylaxis for international travelers. However, atovaquone-resistant parasites isolated from malaria patients have also been highly reported [71-73]. These studies demonstrate that atovaquone resistance is associated with point mutations of the amino acid residue at codon 268 of cyt*b* (*Pfcob*) constructing the cytochrome bc_1 complex. The mutations Y268S, Y268N, and Y268C have been found in atovaquone-resistant parasites.

To mimic the situation of emergence of atovaquone-resistant parasites in a clinical setting, we chose a mouse malaria model using BALB/c mice and the *P. berghei* ANKA strain. In the first trial, we administered atovaquone intraperitoneally on seven consecutive days at doses ranging from 0.4 μg/kg/day to 4.8 mg/kg/day and obtained *P. berghei* isolates with four genetic resistance variations in cyt*b* [74] (Table 2). We did not observe the mutation of the amino acid residue at codon 268, which is observed in *P. falciparum*. The two mutations, M133I and L144S, are located in Qo_1, and these code amino acids are critical for inhibitor resistance in yeast and mice [75, 76]. Moreover, in *Plasmodium*, the M133I and L144S amino acid changes appear to be structurally significant, altering the conformational structure of the ubiquinone-binding site and thus lowering the affinity of atovaquone to the Qo_1 site. The mutation V284F is located in the sixth transmembrane domain adjacent to the Qo_2 site, and the amino acid change by itself

confers only an approximately 10-fold resistance to atovaquone. Notably, the mutation V284F has been found in all atovaquone-resistant clones [74].

Isolate	Mutation	Reference
PbSK2A1Tb	M133I, L271V	[74]
PbSK2A1T	M133I, V284F	[74]
PbSK1A2	V284F	[74]
PbSR-1	L144S, V284F	[74]
PbLSJ1.1	Y268N	[77]
PbLSJ2.1	Y268C	[77]
PbLSJ3.1	L271V, K272R	[77]

Table 2. Mutations in the cytochrome *b* of *Plasmodium berghei* with atovaquone resistance

To obtain a better model for the biochemical and genetic studies of mutations found in *P. falciparum*, we performed further experiments to obtain *P. berghei* strains, resistant to atovaquone, with mutations in the Qo_2 region conferring high degrees of resistance [77]. The parasite-infected mice were treated intraperitoneally for 3 consecutive days at a dose of 14.4 mg/kg/day, a higher dose than in the previous experiment. The results showed three variations of the atovaquone-resistant mutation, including mutations at codon 268 (Y268N, Y268C, and L271/K272R; Table 2). All the mutations were located in the Qo_2 region, and these resistance levels were more than 500 times higher than those of the wild type, although the resistance levels of the previous isolates were more than 50 times higher. Administered doses of atovaquone affected the site of mutation in cyt*b* and the level of drug resistance.

As described above, our group has reported various mutations in the quinone-binding sites of the cyt*b* gene of *P. berghei*, such as M133I, L144S, L271V, K272R, Y268C, Y268S, Y268N, and V284F, using the mouse model with continuous atovaquone pressure. However, no direct evidence of a relationship between the mutations and resistance has been observed using intact mitochondria isolated from the malarial parasite, although biochemical analysis of the mutant has been reported using cell-free extract [78]. To address this point, we have further investigated the activity of dihydroorotate-cyt*c* reductase (regarding this mitochondrial pathway, see Section 2) in both atovaquone-resistant and atovaquone-sensitive *P. berghei* isolates [79]. The results showed that mutations in the quinone-binding site of the cyt*b* gene resulted in variable sensitivity to atovaquone and provided direct evidence for the atovaquone inhibitory mechanism in the parasite cytochrome bc_1 complex.

4.3. Cytochrome bc_1 complex as an antimalarial drug target

Recently, the X-ray crystallographic structure of the mitochondrial cytochrome bc_1 complex from *Saccharomyces cerevisiae* with atovaquone has been resolved, and it demonstrates atovaquone bound in the Qo site [80]. It can therefore explain the molecular basis for the broad spectrum of the antimalarial drug as well as for the species-specific differences in its effects. This would allow us to develop a drug targeting cytochrome bc_1 that would control the emergence of resistant parasites. Furthermore, the other group has reported cocrystallization

of a bovine cytochrome bc_1 complex with the 4(1H)-pyridone class of inhibitors [81], which are potent antimalarial agents *in vivo* [82, 83]. The X-ray structure demonstrates that these inhibitors do not bind at the Qo site but rather at the Qi site. Differences in the inhibitor-binding site to cytochrome bc_1 complex would aid the rational drug designing for reducing the emergence of inhibitor-resistant parasites and increasing selectivity against malaria parasites toward novel treatments. In the future, in addition to binding site analysis using modalities such as X-ray crystallography, we need to elucidate the molecular mechanisms explaining how atovaquone resistance mutation is generated in the parasite mt genome.

5. 5-Aminolevulinic Acid (ALA): A new antimalarial candidate targeting the mitochondrion

ALA is a precursor used in the biosynthesis of tetrapyrroles such as chlorophyll and heme. The heme is an iron-containing complex macrocycle that plays a fundamental role in several cellular processes, including oxygen transport and storage, mitochondrial respiratory chain, and detoxification [84]. Generally, in mammalian cells, heme biosynthesis begins with ALA formation by ALA synthase in the mitochondria from glycine and succinyl-CoA [85]. The next four steps and three final steps occur in the cytosol and mitochondria, respectively. In cancer cells, the uptake of a high concentration of ALA results in elevated levels of its metabolites, particularly protoporphyrin IX (PPIX), due to insufficient activity of ferrochelatase [86]. The PPIX accumulates in the mitochondria and consequently acts as a photosensitizer releasing singlet oxygen and other reactive oxygen species (ROS), resulting in induction of cell death in cancer. ALA therefore has been applied to the development of photodynamic diagnosis and photodynamic therapy (PDT) of various cancers [87, 88].

Recently, all the enzymes of *de novo* heme-biosynthetic pathway have been characterized in the human malaria parasite, *P. falciparum* [89-91]. In contrast to the mammalian enzymes of heme biosynthesis, the parasite enzymes have unique localizations (Figure 5). The first enzyme, ALA synthase, and the final two enzymes, protoporphyrinogen IX oxidase and ferrochelatase (FC), localize to the mitochondrion. The enzymes that catalyze the intermediate three steps—ALA dehydratase, porphobilinogen deaminase, and uroporphyrinogen decarboxylase (UROD)—localize to the apicoplast, a nonphotosynthetic plastid. The next enzyme, coproporphyrinogen III oxidase, is cytosolic. In addition, the catalytic efficiency of these enzymes of the erythrocytic stages differs from that of mammalian enzymes: the enzymes localizing to the apicoplast have very low catalytic efficacy [92]. Altogether, the properties of the heme-biosynthetic pathway are remarkably different between malaria parasites and their hosts. Hence, the heme-biosynthetic pathway has been recognized as a novel chemotherapeutic target in *Plasmodium*. Smith and Kain attempted PDT for human malaria parasites by adding ALA to an *in vitro* culture [93]. The growth of *Plasmodium* was completely inhibited by 0.2 mM ALA, followed by exposure to white light or by a higher concentration (2 mM) of ALA alone without light exposure. This use of PDT is, unfortunately, clinically unrealistic because white light cannot illuminate the inside of a malaria-infected patient's body and the concentration of 2 mM is extremely high to apply clinically.

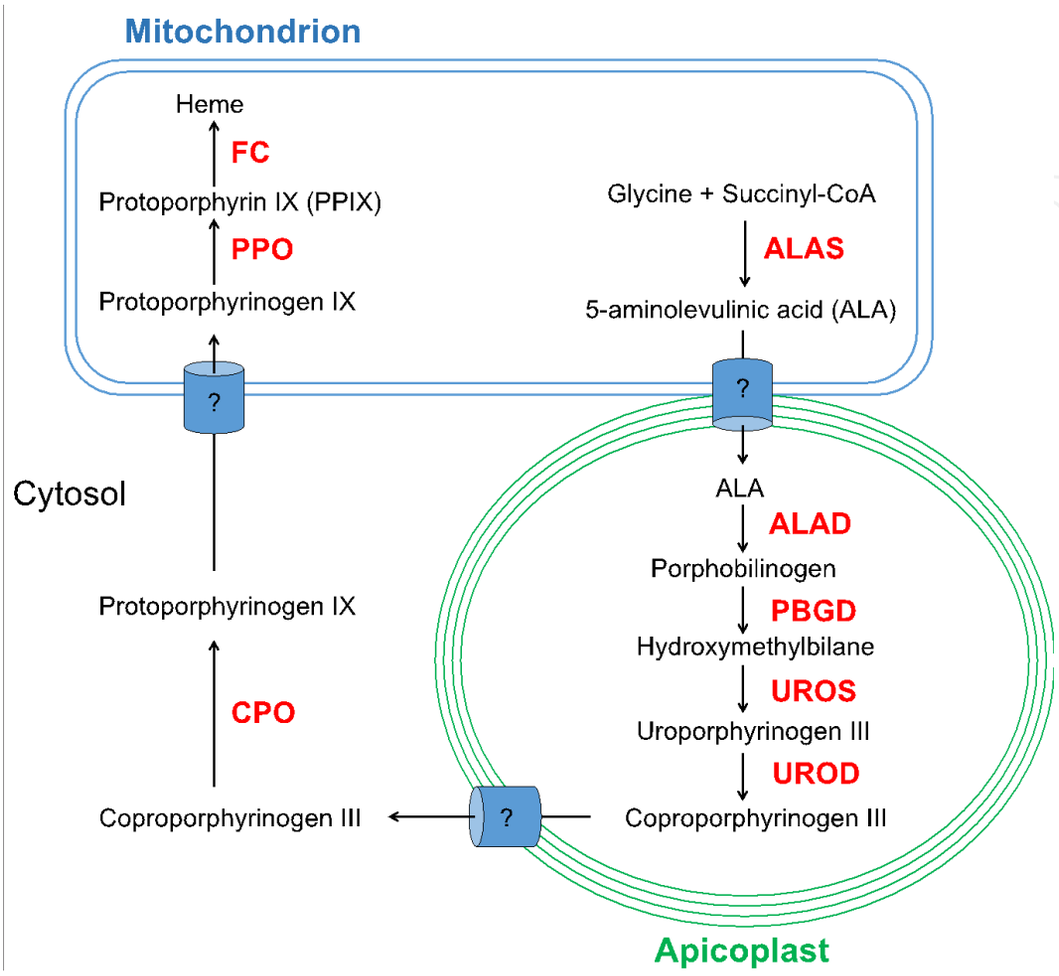

Figure 5. Heme biosynthesis of malaria parasites. The enzymes in the pathway are localized in the mitochondrion, apicoplast, and cytosol [89-91].

Our recent study resolved this issue: in the presence of ferrous ion, ALA efficiently inhibited the *in vitro* growth of *Plasmodium* even without light exposure [94]. Because there was a previous report on protection from malaria by elevated zinc protoporphyrin, which binds to heme crystals to inhibit further crystallization to form hemozoin [95], we first investigated effects of metal ions on growth inhibition by ALA using an *in vitro* culture system of *P. falciparum*. Our results showed that treatment with 10 μM sodium ferrous citrate (SFC) and 0.2 mM ALA increased the growth inhibition to more than 50% when compared with that of 0.2 mM ALA alone. Notably, no other metal ions (e.g., zinc, lead, and copper) had such a synergistic effect, indicating that only ferrous compounds are synergistic with ALA.

Next, to determine heme intermediate, we analyzed the cell extract of the parasite using HPLC. The extract contained three major intermediates: coproporphyrin I, coproporphyrin III (CPIII), and PPIX. Unlike in cancer cells, CPIII was majorly accumulated in the apicoplast. Although

its contribution to the parasite growth inhibition remains unknown, we believe that these differences are due to the complicated heme-biosynthetic pathway (Figure 5) and life cycle of *Plasmodium* (Figure 1). Moreover, PPIX, as is the case with cancer cells, accumulated mainly in the mitochondrion. In PDT of cancer cells, PPIX acts as a photosensitizer releasing ROS, resulting in extensive cellular damage and cell death [96]. This suggests that PPIX accumulated in the parasite mitochondria is a factor contributing to the inhibition of parasite growth. Thus, the parasite heme-biosynthetic pathway in the mitochondrion and apicoplast may be a potential target of an antimalarial drug.

Recently, to confirm the efficacy of the combination of ALA and SFC (ALA/SFC) in treating malaria using an animal model, we performed a preclinical drug evaluation of orally administered ALA/SFC for the treatment of mice infected with the malaria parasite. ALA/SFC cured 50% of the Py17XL-infected mice, and the cured mice showed long-lasting humoral immune responses to the same parasite strain and protection from homologous malarial infections [97]. ALA can be safe compound because a phase I clinical study has been successfully completed. Considering the safety and mild antimalarial activities of ALA/SFC, a combination with an available antimalarial drug, such as artemisinin or chloroquine, would be applicable for the treatment of malaria.

6. Concluding remarks

The energy metabolism of malaria parasites has been considerably elucidated with accumulating data from several "omics" analyses. These data suggest that enzymes of the mitochondrial TCA cycle and mtETC could be attractive targets for development of antimalarial drugs. However, activity of these energy transduction pathways in the mitochondrion is considered to be very low in the erythrocytic stages of the parasite. To address these possibilities, biochemical assay data are required. However, rigorous biochemical analysis of the parasite mitochondrion, in which the TCA cycle and mtETC are present, is highly difficult because intact and pure mitochondria cannot be obtained from the parasites thus far. As a consequence, the malaria parasite mitochondrion needs to be purified to perform these future biochemical studies. Biochemical data regarding the *Plasmodium* mitochondrion would shed light on the details of mitochondrial enzyme behavior and help in the management of malaria.

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