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# Isoprenoid Biosynthesis in *Plasmodium falciparum*

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**Malaria kills nearly 1 million people each year, and the protozoan parasite *Plasmodium falciparum* has become increasingly resistant to current therapies. Isoprenoid synthesis via the methylerythritol phosphate (MEP) pathway represents an attractive target for the development of new antimalarials. The phosphonic acid antibiotic fosmidomycin is a specific inhibitor of isoprenoid synthesis and has been a helpful tool to outline the essential functions of isoprenoid biosynthesis in *P. falciparum*. Isoprenoids are a large, diverse class of hydrocarbons that function in a variety of essential cellular processes in eukaryotes. In *P. falciparum*, isoprenoids are used for tRNA isopentenylation and protein prenylation, as well as the synthesis of vitamin E, carotenoids, ubiquinone, and dolichols. Recently, isoprenoid synthesis in *P. falciparum* has been shown to be regulated by a sugar phosphatase. We outline what is known about isoprenoid function and the regulation of isoprenoid synthesis in *P. falciparum*, in order to identify valuable directions for future research.**

Severe malaria remains a threat to human health worldwide, with over 250 million cases per year. Malaria is a leading cause of death in children, with almost one million deaths each year (1, 2). Despite ongoing and intensive control efforts, malaria remains endemic on five continents. Widespread resistance to former first-line agents, most notably chloroquine, has severely limited malaria control efforts (2). Currently, the recommended standard of care for malaria infection is combination therapy using artemisinin-based therapeutics. However, decreased sensitivity to artemisinin has been recognized in the field, particularly in Southeast Asia. The spread of artemisinin resistance threatens the progress that has been made in control of malaria, particularly in sub-Saharan Africa (3–5). New antimalarial agents, particularly agents with novel mechanisms of action, are urgently needed.

Malaria is caused by infection with protozoan parasites in the genus *Plasmodium*. Most cases of life-threatening malaria are attributable to infection with a single species, *Plasmodium falciparum*, although *P. vivax* and *P. knowlesi* have also been associated with severe disease (6–9). *Plasmodium* infection is transmitted through the bite of anopheline mosquitoes (Fig. 1 depicts their life cycle). Expelled from mosquito salivary glands, malaria sporozoites first traffic to the liver, where 10 to 100,000 daughter parasites are generated from a single invading cell. Upon egress from the liver, the parasite enters the host bloodstream. There, the malaria parasite begins an asexual cycle of growth and development within erythrocytes. This intraerythrocytic cycle leads to the signs and symptoms associated with malaria infection, including fever, anemia, and multiorgan dysfunction due to vascular adherence of parasitized red blood cells. New antimalarials must therefore target this pathogenic stage of parasite development. A small proportion of asexual-stage parasites leave the asexual cycle and commit to the production of sexual forms, known as gametocytes. Upon a new blood meal, gametocytes return to the mosquito midgut, where they complete sexual development and begin the life cycle anew.

One cellular peculiarity of *Plasmodium* species, as well as other apicomplexan parasites, such as *Toxoplasma* and *Babesia* species, is the presence of an unusual plastid organelle, the apicoplast (Fig. 2A and B). The apicoplast is surrounded by four membranes, suggesting an ancient secondary endosymbiotic event between a

protozoan parasite ancestor and red algae, similar to that of the chloroplast (10–12). While the apicoplast was previously believed to be of green algal origin, the recent discovery and genome sequencing of the alveolate *Chromera velia* has revealed *C. velia* as an evolutionary link between apicomplexans and their red algal ancestors (11, 12). *C. velia* can potentially serve as a useful tool to study the evolution of plastid pathways in apicomplexan parasites. While photosynthetic capabilities have been lost over time, the malaria parasite has retained some plantlike metabolic pathways that hold particular value as targets for antimalarial drug development, since these pathogen-specific processes are not present in humans.

Key among apicoplast metabolic pathways is that of isoprenoid biosynthesis. Isoprenoids comprise a very large and diverse group of biomolecules derived from the sequential assembly of two 5-carbon isomers, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Chains of isoprene units are subsequently modified through cyclizations, oxidations, reductions, and additions to generate the array of over 25,000 isoprenoids found in nature (13). In humans (as well as fungi, archeobacteria, cytoplasm of plants, and other metazoans), the isoprenoid building blocks IPP and DMAPP are produced through a mevalonate-dependent pathway from acetyl-coenzyme A (CoA). The rate-limiting step in the mevalonate pathway is the conversion of 3-hydroxy-3-methyl-glutaryl (HMG)-CoA to mevalonic acid by the enzyme HMG-CoA reductase; this enzyme is the target for the widely used statin class of cholesterol-lowering drugs (14).

In *Plasmodium* species, IPP and DMAPP are produced via an alternative biosynthetic route that does not utilize mevalonate (15, 16). This pathway, also called the MEP (2-C-methyl-D-erythritol 4-phosphate) pathway or DOXP (1-deoxy-D-xylulose 5-

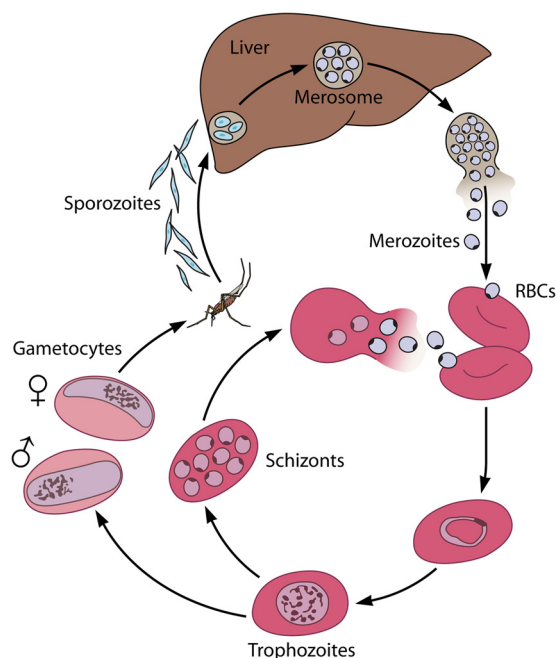
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**FIG 1** Life cycle of *Plasmodium falciparum*. Infection begins with the injection of sporozoites into the host bloodstream by the bite of an *Anopheles* mosquito. Parasites multiply in the liver and are released back into the host bloodstream as merozoites, where they begin the intraerythrocytic developmental cycle (RBCs, red blood cells). Inside the erythrocyte, parasites grow into large trophozoites. They eventually divide to become multinucleate schizonts, which erupt from the host cell and reenter the blood as merozoites. A proportion of these blood-stage parasites become gametocytes and are taken up by the mosquito vector, where they complete sexual replication.

phosphate) pathway, converts glyceraldehyde 3-phosphate and pyruvate to IPP and DMAPP through seven enzymatic steps (Fig. 2C). At least two enzymes of this pathway catalyze rate-limiting steps in IPP production: DOXP synthase (DXS) (EC 2.2.1.7, PlasmoDB identifier [ID] PF3D7\_1337200) converts glyceraldehyde 3-phosphate and pyruvate to DOXP, and DOXP reductoisomerase (DXR) (EC 1.1.1.267, PF3D7\_1467300) converts DOXP to MEP (17, 18). The mechanism by which IPP and DMAPP are exported from the apicoplast for use in the cytoplasm remains unknown.

Given the structural diversity of isoprenoids, it is not surprising that these molecules serve diverse cellular functions. Plants in particular elaborate an incredible range of specialized isoprenoid end products, including pharmacologically active compounds like paclitaxel (originally named taxol) (19) and artemisinin (20), as well as terpenes, volatile isoprenoids that confer the characteristic odors, flavors, and colors of plants. Other roles of isoprenoids include regulation of cell growth and energy production, intracellular signaling, and membrane structural support (15, 21, 22). Recent reviews discuss apicoplast metabolism and, specifically, isoprenoid synthesis, as drug targets in *P. falciparum* (23, 24). Here, we address the key questions in the field: what isoprenoids does the malaria parasite make, and why?

## FOSMIDOMYCIN

An important reagent in the study of the MEP pathway has been the selective MEP pathway inhibitor, fosmidomycin. Fosmidomycin is a small, three-carbon phosphonate compound that was first identified

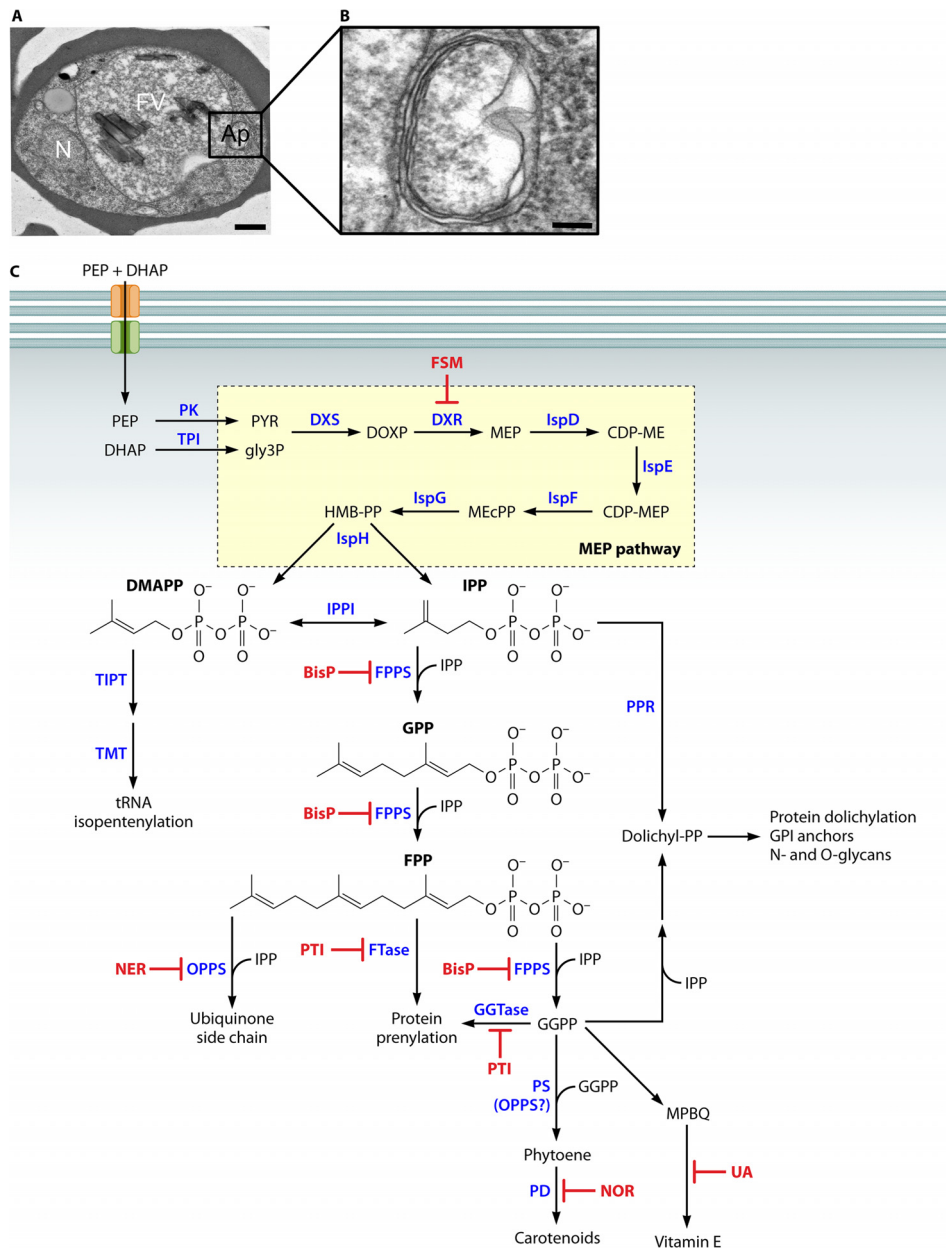
from *Streptomyces lavendulae* by its antibacterial properties (25). Subsequent *in vitro* studies revealed that fosmidomycin competitively inhibits DXR, the first dedicated enzyme of the MEP pathway (26–28). The charged nature of fosmidomycin means that this compound is typically excluded from cells unless actively imported, which has limited its utility against many organisms, including the apicomplexan *Toxoplasma gondii* (29) and the agent of tuberculosis, *Mycobacterium tuberculosis* (30). Intraerythrocytic malaria parasites elaborately remodel the host red blood cell, significantly increasing the cellular uptake of many nutrients (31–33). These so-called new permeability pathways likely facilitate the uptake of fosmidomycin, as fosmidomycin is excluded from uninfected red blood cells but inhibits the growth of *Plasmodium* and a related, tick-borne intraerythrocytic apicomplexan pathogen, *Babesia divergens* (34). It remains unclear what cellular machinery is required for fosmidomycin uptake into *P. falciparum* cells.

Fosmidomycin is well validated as a specific inhibitor of DXR. Analysis of MEP pathway intermediates in bacteria and *P. falciparum* has established that fosmidomycin reduces the intracellular levels of downstream MEP pathway metabolites and isoprenoid products (35–37). In addition, the growth inhibitory effects of fosmidomycin are chemically rescued in bacteria and malaria parasites through supplementation of the medium with IPP or unphosphorylated isoprenols (farnesol and geranylgeraniol). The 50% inhibitory concentration ( $IC_{50}$ ) for fosmidomycin increases 10-fold when the medium is supplemented with farnesol or geranylgeraniol (35, 38). Supplementation of the medium with geranylgeraniol also rescues protein mislocalization and the organelle disruption effects of fosmidomycin treatment (39). Treatment with high concentrations of fosmidomycin is not completely rescued by prenyl alcohol supplementation, perhaps due to the toxicity of these compounds at high concentrations (40).

In asexual parasites, the MEP pathway may be the only essential function of the apicoplast organelle in which it resides. Treating parasites with inhibitors of apicoplast replication forces *P. falciparum* to lose its apicoplast genome and structure. These parasites nonetheless survive when supplemented with exogenous IPP (38).

Small-molecule inhibitors that target apicoplast replication often result in a delayed-death phenotype in *P. falciparum*, in which drug-treated parasites complete the first cell cycle after treatment and arrest in the second (41). In contrast, fosmidomycin treatment inhibits intraerythrocytic growth of *P. falciparum* during the first cell cycle. Interestingly, fosmidomycin-treated parasites develop within the red blood cell, begin hemoglobin digestion, and initiate DNA replication prior to cell cycle arrest as multinucleate schizonts (39). The requirement of the new permeability pathways mentioned above for fosmidomycin import into *P. falciparum* cells may explain this delayed action, as these pathways are not fully developed until the trophozoite stage (31, 32). Liver-stage parasites are also sensitive to fosmidomycin. Treatment of liver-stage *Plasmodium berghei* inhibits the development of the apicoplast and reduces the number of merosomes, the result of liver-stage replication. Thus, the MEP pathway appears to be required for optimal growth in hepatocytes (29). Little is known about isoprenoid synthesis in the gametocyte and mosquito stages of the parasite life cycle, although proteomics studies have identified MEP pathway enzymes expressed in late gametocytes (42).

Below, we detail the isoprenoid products downstream from IPP (Fig. 2C; Table 1) and what is known about their production and/or function in *P. falciparum*.



**FIG 2** Synthesis of isoprenoid products in *P. falciparum*. (A) Electron micrograph of *P. falciparum* cell, with labels showing the red blood cell (RBC), nucleus (N), food vacuole (FV), and apicoplast (Ap). Scale bar represents 500 nm. (B) The *P. falciparum* apicoplast is the site of isoprenoid synthesis by the MEP pathway. It is surrounded by four membranes, indicative of secondary endosymbiotic origins. Scale bar represents 100 nm. (C) Isoprenoid products produced by *P. falciparum*. Abbreviations used: phosphoenol pyruvate (PEP), dihydroxyacetone phosphate (DHAP), pyruvate kinase (PK), triose phosphate isomerase (TPI), 1-deoxy-D-xylulose 5-phosphate synthase (DXS), 1-deoxy-D-xylulose 5-phosphate (DOXP), DOXP reductoisomerase (DXR), fosmidomycin (FSM), 2-C-methyl-D-erythritol 4-phosphate (MEP), MEP cytidyltransferase (IspD), 4-diphosphocytidyl-2-C-methylerythritol (CDP-ME), CDP-ME kinase (IspE), 4-diphosphocytidyl-2-C-methylerythritol 2-phosphate (CDP-MEP), 2-C-methyl-D-erythritol 2,4-cyclopyrophosphate (MEcPP), MEcPP synthase (IspF), (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), HMB-PP synthase (IspG), HMB-PP reductase (IspH), dimethylallyl pyrophosphate (DMAPP), isopentenyl pyrophosphate (IPP), isopentenyl pyrophosphate isomerase (IPPI), tRNA isopentenyltransferase (TIPT), tRNA methylthiolase (TMT), farnesyl pyrophosphate synthase (FPPS), bisphosphate (BisP), geranyl pyrophosphate (GPP), polyprenyl reductase (PPR), farnesyl pyrophosphate (FPP), octaprenyl pyrophosphate synthase (OPPS), nerolidol (NER), farnesyl transferase (FTase), prenyltransferase inhibitors (PTI), geranylgeranyl transferase (GGTase), geranylgeranyl pyrophosphate (GGPP), phytoene synthase (PS), phytoene desaturase (PD), norflurazon (NOR), dolichyl pyrophosphate (dolichyl-PP), 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ), and usnic acid (UA).

**5-CARBON ISOPRENOIDS: DMAPP AND IPP**

The most proximally produced compounds of the MEP pathway are the end products and isoprenoid building blocks, isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP). In bacteria, DMAPP is used as a substrate for tRNA

isopentenylation (43). In this process, an isopentenyl group is added to an adenosine in the tRNA, targeting the tRNA to the ribosome and improving translation fidelity. Evidence suggests that *P. falciparum* produces isopentenylated tRNAs, as its apicoplast genome encodes four tRNAs that represent probable candi-

TABLE 1 Classes of isoprenoid products in *P. falciparum*

Isoprenoid class (no. of carbons)	Present in parasite?	Role(s) in asexual stage	Enzyme(s) (PlasmoDB ID[s])	Notable reference(s)
IPP, DMAPP (5)	Yes	tRNA isopentenylation	tRNA isopentenyltransferase (PF3D7_1207600), isopentenyl-adenosine tRNA methylthiolase (PF3D7_0622200)	
Monoterpenes (10)	No			
Sesquiterpenes, diterpenes (15 and 20)	Yes	Protein prenylation, vitamin E synthesis	FPP-GGPP synthase (PF3D7_1128400), farnesyl transferase (PF3D7_1242600 [ $\alpha$ subunit] and PF3D7_1147500 [ $\beta$ subunit]), geranylgeranyl transferase type I (PF3D7_1242600 [ $\alpha$ subunit] and PF3D7_0602500 [ $\beta$ subunit]), geranylgeranyl transferase type II (PF3D7_1442500 [ $\alpha$ subunit] and PF3D7_1214300 [ $\beta$ subunit]), REP/GDI superfamily members (PF3D7_1242800 and PF3D7_1038100)	35, 49, 63, 64, 68, 78
Sterols (30)	Yes, but taken from host	Membrane stability		86, 87, 89, 90
Carotenoids (40)	Yes	Unknown, possibly response to oxidative stress	Phytoene synthase (PF3D7_0202700)	104
Ubiquinone (40 and 45)	Yes	Electron acceptor in pyrimidine synthesis	Dihydroorotate dehydrogenase (PF3D7_0603300), octaprenyl pyrophosphate synthase (PF3D7_0202700), 4-hydroxybenzoate octaprenyltransferase (PF3D7_0607500)	104, 113, 117, 120
Dolichols (55 and 60)	Yes	Protein modifications: dolichylation, GPI anchors, O- and N-linked glycosylation	Polyprenol reductase (PF3D7_1455900), dolichyldiphosphatase (PF3D7_0805600), GPII (PF3D7_0618900), dolichol phosphate mannose synthase (PF3D7_1141600)	123, 124, 125, 130, 131, 134, 135

dates for isopentenylation (44). *P. falciparum* encodes a homologue of the *Escherichia coli* tRNA isopentenyltransferase MiaA (EC 2.5.1.75, PlasmoDB ID PF3D7\_1207600) (44). *P. falciparum* also possesses a homolog of the isopentenyl-adenosine tRNA methylthiolase MiaB (EC 2.8.4.-, PlasmoDB ID PF3D7\_0622200), an enzyme whose bacterial homologs participate in downstream tRNA isopentenylation steps (44, 45).

As previously described, supplementation of medium with farnesol or geranylgeraniol rescues fosmidomycin treatment of malaria parasites. These isoprenols are presumed to be phosphorylated intracellularly by nonspecific kinases to generate their cognate diphosphates, farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). Fosmidomycin-treated parasites supplemented with farnesol (15-carbon) or geranylgeraniol (20-carbon) therefore do not have a known source of 5- or 10-carbon isoprenoids but are still capable of intraerythrocytic growth (35). These studies cannot rule out a role for tRNA isopentenylation in maintaining apicoplast function, but they do suggest there may not be additional roles for tRNA isopentenylation outside the maintenance of isoprenoid biosynthesis within the apicoplast.

## 10-, 15-, AND 20-CARBON ISOPRENOIDS

### MONOTERPENES

Condensation of two isoprene units produces the 10-carbon isoprenoid geranyl pyrophosphate (GPP). GPP is utilized by monoterpene synthases and monoterpene cyclases to produce 10-carbon monoterpenes. Monoterpenes are the most abundant compounds found in plant essential oils. Terpene mixtures such as citronella

(citronella) and citral (lemon) are produced by plants to deter herbivores (46). Some monoterpenes have been found to have antimicrobial properties; thymol, a monoterpene component of the essential oil in thyme, has been shown to decrease counts of *Salmonella enterica* serovar Typhimurium and *Staphylococcus aureus* (47). To date, no studies have identified evidence of monoterpene synthesis in *P. falciparum*. Homology-based searches do not identify potential monoterpene synthases or monoterpene cyclases in the *P. falciparum* genome, although this class of enzymes is remarkably diverse (48).

### SESQUITERPENES AND DITERPENES

FPP and GGPP are also used to produce sesquiterpenes and diterpenes. In *P. falciparum*, the condensation of GPP and IPP (to produce FPP) and of FPP and IPP (to produce geranylgeranyl diphosphate [GGPP]) appears to be catalyzed by a bifunctional FPP-GGPP synthase (PlasmoDB ID PF3D7\_1128400) (49). The crystal structure of the *P. vivax* enzyme has been solved (50, 51). This bifunctional enzyme is sensitive to bisphosphonates, such as zoledronate and risedronate (49). These compounds bind to bone minerals and are traditionally used to inhibit bone resorption in the treatment of diseases like osteoporosis (52). Metabolic-labeling studies using [ $^{14}$ C]mevalonate, [ $^{14}$ C]IPP, and [ $^{14}$ C]DMAPP validate FPP synthesis as the target of bisphosphonate inhibition (53). Bisphosphonates, as well as their analogs, have been shown to bind FPP-GGPP synthase in the active site of both the human and parasite enzymes (51). Bisphosphonates compete with GPP for binding, and their efficacy is enhanced by IPP stabilization of the enzyme inhibitor complex (54, 55). Treatment with bisphosphonates inhibits parasite growth and decreases protein prenyla-

tion (50, 51, 56–58). Partial rescue of parasite growth can be achieved by the addition of FPP or GGPP to the culture medium, indicating that bisphosphonates target FPP and GGPP synthesis in *P. falciparum* (56).

*P. falciparum* utilizes sesquiterpenes and diterpenes for protein prenylation. In protein prenylation, lipophilic farnesyl (15-carbon) and geranylgeranyl groups (20-carbon) are attached to C-terminal cysteines, which results in protein association with membranes. Prenylation is crucial for the function of a variety of membrane-bound enzymes, such as the Ras, Rho, and Rab families of small GTPases. Farnesyl transferase (EC 2.5.1.58, PlasmoDB IDs PF3D7\_1242600 [ $\alpha$  subunit] and PF3D7\_1147500 [ $\beta$  subunit]) and geranylgeranyl transferase type I (EC 2.5.1.59, PlasmoDB IDs PF3D7\_1242600 [ $\alpha$  subunit] and PF3D7\_0602500 [ $\beta$  subunit]) transfer FPP and GGPP moieties to the target protein via recognition of a C-terminal CaaX motif (59). This motif is composed of a cysteine (C), two aliphatic amino acids (aa), and the C-terminal amino acid (X). These two prenyltransferases share an  $\alpha$  subunit but have distinct  $\beta$  subunits (60). Geranylgeranyl transferase type II (EC 2.5.1.60, Rab geranylgeranyltransferase, PlasmoDB IDs PF3D7\_1442500 [ $\alpha$  subunit] and PF3D7\_1214300 [ $\beta$  subunit]) utilizes a different mechanism of substrate recognition, which requires a Rab escort protein (REP) (61). The REP binds Rab proteins, facilitates their prenylation, and delivers them to their target membrane. REPs are part of the REP/GDI superfamily, which also includes GDP dissociation inhibitor (GDI) proteins. GDI proteins are involved in cycling the Rab between membranes and the cytosol (61). Two members of the REP/GDI superfamily are found in the *P. falciparum* genome (PlasmoDB IDs PF3D7\_1242800 and PF3D7\_1038100) (62). Further studies are required to determine if these proteins function as REPs or GDIs.

Protein prenylation appears to be an essential use of isoprenoids in *P. falciparum*, as the parasite is sensitive to chemical inhibition of protein prenylation. Prenyltransferase inhibitors, major candidates for anticancer therapy, have shown potent anti-malarial activity (63–65). These include a number of peptidomimetics of the CaaX motif (63, 66, 67). Additionally, certain monoterpenes have been shown to inhibit the growth of *P. falciparum* via inhibition of prenylation (40, 68). Limonene has been shown to inhibit the prenylation of 21- to 26-kDa proteins in mammalian cell culture and *in vitro* (69–71). *P. falciparum* parasites treated with limonene are unable to progress from the ring to the trophozoite stage (68).

Feeding *P. falciparum* with labeled [ $^3$ H]FPP and [ $^3$ H]GPP identifies 21- to 24-kDa and 50-kDa prenylation target proteins that are differentially labeled by FPP and GPP (68). Overall, it appears that the majority of prenylated proteins are small and preferentially geranylgeranylated *in vivo*, with the exception of a single 50-kDa protein (63). Bioinformatic methods have been used to compile a list of predicted prenylation targets in *P. falciparum* (60). These include a number of GTP-binding proteins, such as Rab2 and Rab11a.

A number of studies have identified specific prenylation targets in *P. falciparum*. The localization of the small GTPase Rab7 to endosomal vesicles was shown to be prenylation dependent. These vesicles are predicted to participate in endosomal trafficking (72). The SNARE protein Ykt6.1 of *P. falciparum* (PYkt6.1) has been shown to be a farnesyltransferase substrate *in vitro*, and its localization is disrupted when lacking a CaaX motif, suggesting it is also

a prenylation target *in vivo* (73). Similarly, the *P. falciparum* tyrosine phosphatase PfPRL is a substrate for farnesylation *in vitro* (74).

Inhibition of isoprenoid synthesis by fosmidomycin produces prenylation phenotypes in *P. falciparum*. Similar to metabolic labeling, probing for prenylation using a prenylation-specific antibody identifies proteins of 21 to 24 kDa and 50 kDa (39, 68). Prenylation of these targets is reduced in fosmidomycin-treated parasites, confirming that prenyl groups are indeed products of the MEP pathway. The *P. falciparum* geranylgeranyltransferase substrates PfRab5a and PfRab5c mislocalize from hemoglobin-containing vesicles to the host cell membrane upon treatment with fosmidomycin, and this mislocalization correlates with changes to food vacuole morphology and integrity. Proper localization is restored by geranylgeraniol supplementation (39). Geranylgeraniol supplementation also substantially rescues growth inhibition by fosmidomycin, suggesting that geranylgeranylation may be the only essential form of protein prenylation in *P. falciparum* (35, 38).

The group of compounds collectively known as vitamin E (tocopherols and tocotrienols) function as antioxidants and membrane stabilizers (75–77). Recently, a metabolic labeling study using [ $^3$ H]FPP and [ $^3$ H]GPP identified *de novo* vitamin E synthesis by *P. falciparum*. Parasite growth is sensitive to usnic acid, an inhibitor of vitamin E biosynthesis. Growth is partially rescued by  $\alpha$ -tocopherol, indicating that vitamin E synthesis is essential in malaria parasites (78).

In plants, the homogentisic acid head group of vitamin E is synthesized via the shikimate pathway. This head group is then prenylated with phytyl diphosphate (20-carbon) or geranylgeranyl diphosphate to generate 2-methyl-6-phytyl-1,4-benzoquinol, the first committed intermediate for the synthesis of tocopherols and tocotrienols. This prenylation is catalyzed by homogentisate prenyltransferases (EC 2.5.1.115 and EC 2.5.1.116) (79). As no obvious homogentisate prenyltransferase homologs exist in the *P. falciparum* genome, further work will be required to understand the mechanism by which the parasite synthesizes vitamin E.

## STEROLS

Sterols are 30-carbon isoprenoids that are ubiquitous among eukaryotes and are utilized for a variety of cellular functions. In particular, cholesterol is essential for membrane architecture in eukaryotes, and its production is tightly regulated (80, 81). Cholesterol is also a precursor for signaling molecules, such as sex steroids and mineralocorticoids in mammals and brassinosteroids in plants. Squalene synthase (EC 2.5.1.21) commits the isoprenoid pathway to sterol biosynthesis by converting two molecules of FPP to squalene; squalene then serves as the backbone for subsequent modifications (82).

The animal host synthesizes cholesterol *de novo* and is also able to import it from dietary sources (83). Radioactive labeling experiments show no evidence for cholesterol biosynthesis in *P. falciparum* (84, 85). Homology searches do not identify a squalene synthase in the *P. falciparum* genome. Instead, *Plasmodium* spp. appear to obtain cholesterol from the host cell. *P. knowlesi* was shown to import host-derived  $^{14}$ C-labeled cholesterol, and cellular uptake by the host cell itself was also increased upon infection with malaria parasites (86–88). *P. falciparum* import of cholesterol has been studied within hepatocytes, a site of high cholesterol synthesis and parasite replication early in infection. Inhibition of

host cell isoprenoid synthesis decreases sterol levels in the liver-stage parasite (89). While cholesterol is essential for the maintenance of parasite membrane stability (90), these studies suggest that cholesterol synthesis does not occur in *P. falciparum* and is therefore not an essential function of *de novo* isoprenoid synthesis by the parasite.

## CAROTENOIDS

Carotenoids are 40-carbon isoprenoids derived from the condensation of two GGPP molecules by phytoene synthase (EC 2.5.1.32, PlasmoDB ID PF3D7\_0202700). Carotenoids are synthesized by plants and algae, as well as some bacteria and fungi. In plants, algae, and photosynthetic bacteria, carotenoids like carotene, lycopene, xanthophyll, and lutein function in photosynthesis and protect against free radical damage (91–95). In plants, carotenoid synthesis occurs in the chloroplast (96, 97). Fungi also utilize carotenoid pigments for protection against free radicals (98, 99). In animals, which cannot synthesize carotenoids, dietary carotenoids are used for the synthesis of vitamin A (91). An exception is found in insects that have acquired carotenoid synthesis from fungi through lateral gene transfer (100, 101). Commercial synthesis of carotenoids is of interest for their use as nutraceuticals, dietary supplements, and pigments (102, 103).

Carotenoids have recently been detected in the intraerythrocytic stages of *P. falciparum*; schizonts contain the highest concentrations, indicating that carotenoid synthesis begins in the ring stage and builds during the schizont stage (104). Geranyl pyrophosphate serves as a substrate for carotenoid synthesis by phytoene synthase (EC 2.5.1.32, PlasmoDB ID PF3D7\_0202700). Phytoene is then converted to carotenoid products by phytoene desaturase (EC 1.3.99.30, locus unknown). *P. falciparum* is sensitive to the small molecular herbicide norflurazon, which inhibits phytoene desaturase. Norflurazon treatment causes an accumulation of phytoene and a decrease in carotenoid content. Inhibition by norflurazon can be partially rescued with lycopene (104). While carotenoids serve important functions in plants, algae, bacteria, and fungi, it is not yet known what physiological role they play in *Plasmodium*. As in other organisms, they may play a role in the cellular response to oxidative stress.

In plants, the phytohormone abscisic acid is also produced from carotenoid intermediates (105). The *Plasmodium* relative and apicomplexan parasite, *Toxoplasma gondii*, has been shown to produce abscisic acid to control calcium signaling for processes like protein secretion and parasite egress. The abscisic acid response genes identified in *T. gondii* are conserved in *P. falciparum*, but it is not known whether *P. falciparum* also synthesizes this isoprenoid product. The route for abscisic acid synthesis from isoprenoid precursors in apicomplexan parasites remains unknown, as no clear biosynthetic route is readily identified bioinformatically (106).

## COENZYME Q (UBIQUINONE)

In most eukaryotes, mitochondria are the site of energy generation through oxidative phosphorylation. Within the mitochondrial matrix, the tricarboxylic acid cycle uses 2-carbon metabolites generated from the breakdown of glucose, amino acids, and fatty acids to produce high-energy electron carriers. In the inner mitochondrial membrane, the electron transport chain uses high-energy electrons to harness energy in the form of ATP. In the mitochondria of asexual *Plasmodium* parasites, however, the electron

transport chain is not a primary source of ATP and the parasite instead relies on glycolysis for most of its ATP production (107). Indeed, little of the parasitic glucose supply is completely oxidized, and glucose is instead excreted as lactic acid (108, 109). Additionally, the parasites show relatively little oxygen consumption, consistent with minimal respiration (110). However, ATP generation by the electron transport chain may be essential for parasite stages within the mosquito host, where extracellular glucose levels are lower and the parasite cannot rely solely on glycolysis for ATP production (111, 112).

In asexual-stage parasites, the electron transport chain operates to provide a continuous supply of reduced coenzyme Q. Coenzyme Q, or ubiquinone, typically functions as an electron acceptor in the electron transport chain. It is maintained in asexual *Plasmodium* parasites as an electron acceptor for dihydroorotate dehydrogenase (DHODH) (EC 1.3.98.1, PlasmoDB ID PF3D7\_0603300), an enzyme required for pyrimidine synthesis. DHODH is essential for survival, as the parasite is incapable of pyrimidine salvage, and small molecules targeting DHODH have potent antimalarial activity (39, 113–116). Ubiquinone levels have been shown to peak at the beginning of schizogony and are sensitive to fosmidomycin treatment (36).

Synthesis of coenzyme Q requires the addition of an isoprenyl side chain to a benzoquinone ring. The parasite possesses an octaprenyl pyrophosphate synthase (EC 2.5.1.90, PlasmoDB ID PF3D7\_0202700) that is capable of synthesizing these side chains. This multifunctional enzyme produces 40-carbon, 45-carbon, and 55-carbon isoprenoid products and has been shown to also have phytoene synthase activity (104, 117). The addition of these isoprenoids to 4-hydroxybenzoate is performed by 4-hydroxybenzoate octaprenyltransferase (EC 2.5.1.39, PlasmoDB ID PF3D7\_0607500) (118, 119). Labeling studies in *P. falciparum* identify coenzyme Q isoforms coenzyme Q8 and Q9, which have 8 and 9 isoprene units (40-carbon and 45-carbon), respectively, in their side chains (120, 121). Incorporation of labeled FPP results in the detection of coenzyme Q8, and incorporation of labeled GGPP detects coenzyme Q9 (120). In another study using labeled *p*-hydrobenzoic acid, coenzyme Q8 was found to be the dominant form of coenzyme Q (121).

Nerolidol, a sesquiterpene alcohol, was found to inhibit the synthesis of the isoprenyl side chain destined for coenzyme Q, likely because of its structural similarity to FPP. Treatment with nerolidol inhibits the intraerythrocytic development of *P. falciparum* (117, 120).

## DOLICHOLS

Dolichols are long-chain hydrocarbon compounds made of various numbers of isoprene units. In the form of dolichyl phosphate or pyrophosphate, dolichols are essential for the transfer of sugars onto proteins, i.e., dolichylation, O-linked glycosylation, N-linked glycosylation, and the production of glycosylphosphatidylinositol (GPI) anchors, which are essential for successful infection (122).

Multiple studies have demonstrated the presence of dolichols and their intermediates in *P. falciparum*, specifically, those composed of 11 and 12 isoprene units (55 and 60 carbons). Labeling experiments demonstrate that these dolichols are formed from FPP and GPP, respectively (123, 124). As expected, dolichol synthesis is also sensitive to fosmidomycin treatment (37).

Dolichyl pyrophosphate is produced from IPP by a polyprenol

reductase (EC 1.3.1.94) and from GGPP via a dehydrodolichol pyrophosphate intermediate. Dolichyl pyrophosphate is then converted to dolichyl phosphate by dolichyldiphosphatase (EC 3.6.1.43). Dolichyl phosphate is utilized for glycosylation and synthesis of GPI anchors. *P. falciparum* possesses homologs of both polyprenol reductase (PlasmoDB ID PF3D7\_1455900) and dolichyldiphosphatase (PlasmoDB ID PF3D7\_0805600).

Posttranslational addition of dolichols to proteins has been demonstrated in *P. falciparum*. Labeling using [<sup>3</sup>H]FPP and [<sup>3</sup>H]GGPP identified a dolichol with 11 isoprene units attached to 21- to 28-kDa protein(s). The target proteins and enzyme(s) responsible for dolichylation of proteins in *P. falciparum* remain unknown (124).

*P. falciparum* synthesizes GPI anchors for protein modification (125). The parasite is sensitive to known inhibitors of GPI synthesis, such as the mannose analogue 2-deoxyglucose (126, 127). Studies have identified a number of parasite proteins as targets for GPI addition, including merozoite surface antigens, a serine protease, and a heat shock 70 protein (128, 129). Merozoite surface antigens appear to be the primary targets of GPI anchor addition and are of great interest as antigens for vaccine development (130). The enzyme GPI1 (EC 2.4.1.198, PlasmoDB ID PF3D7\_0618900) transfers *N*-acetylglucosamine to phosphatidylinositol in GPI biosynthesis. The *P. falciparum* GPI1 homolog was shown to complement a yeast *gpi1* mutant, confirming its function (131). Dolichol phosphate mannose synthase (EC 2.4.1.83, PlasmoDB ID PF3D7\_1141600) catalyzes the addition of sugar moieties in both GPI anchor glycosylations and N-linked glycosylations. The *P. falciparum* enzyme has been shown to be of a novel clade distinct from animal or yeast synthases of the same type (132).

While GPI anchors appear to constitute most of the glycosylation in *P. falciparum*, there is also evidence for O- and N-linked glycosylation of proteins (133–135). The presence of N-linked glycosylation has long been debated (136, 137). However, the parasite is sensitive to tunicamycin, an inhibitor of N-linked glycosylation (135), and parasite proteins have been shown to be capable substrates of N-linked glycosylation when expressed in heterologous systems (138).

## REGULATION OF ISOPRENOID SYNTHESIS

MEP pathway regulation has been thoroughly studied in plants, which utilize a large variety of isoprenoids for signaling and environmental interactions (139–141). However, little is known about regulation of the MEP pathway in *P. falciparum*. Given the variety and essentiality of isoprenoid products in *P. falciparum*, it is likely that isoprenoid synthesis by the MEP pathway is regulated to control the product availability of IPP and DMAPP. Two ATPs and 3 NADPHs are consumed for the production of each IPP molecule from glucose (142). The cell likely regulates this pathway to optimize energy consumption. A better understanding of MEP pathway regulation in the parasite will facilitate new strategies to inhibit the MEP pathway and may contribute an improved understanding of MEP pathway regulation in other plastid-possessing eukaryotic pathogens.

Regulation of the MEP pathway may operate at the level of gene expression, protein activity, or metabolite availability. Studies in *Arabidopsis thaliana* have identified an RNA processing protein, Rif10, which posttranscriptionally regulates the levels of MEP pathway enzymes (143). However, studies in *P. falciparum* have indicated that the transcript levels of MEP pathway enzymes

do not change significantly upon chemical inhibition of the MEP pathway, suggesting that modulating transcript levels is likely not a primary mechanism of MEP pathway regulation in the parasite (37). In the asexual intraerythrocytic cycle, the transcript levels of MEP pathway genes peak in the late trophozoite stage (144). It is possible that the expression levels of MEP pathway genes may respond to other environmental and cellular cues, but other perturbation studies have yet to uncover such regulation (145).

There is evidence for regulation of the MEP pathway by MEP pathway metabolites. A recent study identified a feed-forward mechanism of MEP pathway regulation in *E. coli*. The MEP pathway enzyme 2-*C*-methyl-*D*-erythritol 2,4-cyclodiphosphate synthase (EC 4.6.1.12, PlasmoDB ID PF3D7\_0209300) is activated by the upstream intermediate MEP (146). Additionally, IPP and DMAPP have been shown to cause feedback inhibition of the rate-limiting enzyme DXS in *Populus trichocarpa* (147). Further study is required to determine whether similar regulatory mechanisms exist in *P. falciparum*.

A recent study has identified a novel regulator of the MEP pathway in *P. falciparum*. Its HAD1 (PfHAD1) (PlasmoDB ID PF3D7\_1033400), a sugar phosphatase member of the haloacid dehalogenase-like hydrolase (HAD) superfamily, was shown to be a negative regulator that acts upstream from the MEP pathway. Loss of PfHAD1 results in resistance to fosmidomycin and increased levels of MEP pathway metabolites, primarily DOXP. PfHAD1 appears to utilize cellular sugar phosphates upstream from the MEP pathway. PfHAD1 is predicted to restrict the availability of precursors to the apicoplast-localized MEP pathway (148).

## CONCLUSION

While gaps remain in our understanding of isoprenoid biology in *P. falciparum*, it is clear that isoprenoids are essential and diverse in the malaria parasite. The efficacy of fosmidomycin and the recent screening for apicoplast inhibitors demonstrates that apicoplast biology and, specifically, isoprenoid synthesis are promising, druggable targets for the development of new antimalarials (149). Therefore, developing a complete understanding of the biology and regulation of the MEP pathway should be a priority for the field. This may identify additional drug targets and will inform future antimalarial development. For example, targeting MEP pathway regulation may prove synergistic in combination therapies with direct MEP pathway enzyme inhibitors, such as fosmidomycin.

The discovery of PfHAD1 as a negative regulator of isoprenoid precursor synthesis has begun to expand our understanding of the regulation of this essential pathway. Whether PfHAD1 itself will be a useful antimalarial target will depend on whether PfHAD1 is required for parasite growth during human infection, which remains unknown. Parasite strains selected for fosmidomycin resistance lose PfHAD1 function, and this loss is necessary for resistance. However, the loss of PfHAD1 is not the only genetic change found in these strains, and other changes may be required for parasites to tolerate the loss of PfHAD1 (148). Alternatively, PfHAD1 function may be dispensable under laboratory culture conditions but necessary for development *in vivo*. The strong sequence conservation of PfHAD1 in *P. falciparum* field isolates suggests that it likely plays an important role in the cell, but further studies are required to demonstrate whether this is the case. Inactivating alleles of PfHAD1 were readily obtained during *in vitro*



culture with fosmidomycin. If PfHAD1 is not essential during natural infection, this locus may represent an important biomarker for clinical resistance to fosmidomycin, its analogs, or other MEP pathway inhibitors as they are developed.

PfHAD1 belongs to the Cof-like hydrolase subfamily (InterPro accession number IPR000150) of the haloacid dehalogenase-like hydrolase (HAD) superfamily (150). Two additional members of this subfamily exist in the *P. falciparum* genome, PfHAD2 (PlasmoDB ID PF3D7\_1226300) and PfHAD3 (PlasmoDB ID PF3D7\_1226100), whose functions are unknown. PfHAD2 and PfHAD3 protein sequences possess 25 to 30% identity and approximately 50% similarity with PfHAD1, which is typical for enzymes within this subfamily. Sequence homology predicts that, like PfHAD1, they also utilize sugar phosphates as substrates. Ongoing studies are needed to evaluate whether these PfHAD1 homologs also function as regulators of isoprenoid synthesis or regulators of other essential biosynthetic processes in *P. falciparum*.

The malaria parasite, *Plasmodium falciparum*, is relatively slow growing and difficult to genetically manipulate. Classical genetics in this organism have been hampered by the regulatory and ethical challenges raised by the need for primate infection. The rise in modern next-generation sequencing technologies has been an enormous technical advance in genetic studies of nonmodel organisms, including *P. falciparum*. The presence of PfHAD1 homologs in other organisms that utilize the MEP pathway, such as bacteria and plants, demonstrates that *P. falciparum* can be a useful model eukaryote for study of the isoprenoid metabolism. Since PfHAD1 homologs are closely conserved in organisms containing the MEP pathway (eubacteria, algae, and plants) but are absent in organisms that do not utilize the MEP pathway (archaea, fungi, and animals), HAD homologs may be regulators of the MEP pathway in multiple systems (148). Perhaps sugar phosphatase members of the HAD superfamily coevolved alongside the energetically expensive MEP pathway, in order to regulate substrate availability and control energy consumption when downstream products are not required.

Other MEP pathway-utilizing and HAD-containing organisms are used to produce commercially important isoprenoids. These products include pharmaceuticals (such as paclitaxel and artemisinin), food additives (such as lycopene), fragrances, and biofuel precursors (151–155). Because complex isoprenoids are challenging to synthesize chemically, there is great interest in increasing and optimizing bioproduction of these compounds. Antimalarial drug development is naturally of critical importance in the malaria field. However, as we advance our understanding of isoprenoid biology and regulation in malaria parasites, we may also gain insight into fundamental aspects of MEP pathway biology that will have impacts on the diverse disciplines touched by this ancient cellular process.

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