Isoprenoid Metabolism in Apicomplexan Parasites

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Abstract Apicomplexan parasites include some of the most prevalent and deadly human pathogens. Novel antiparasitic drugs are urgently needed. Synthesis and metabolism of isoprenoids may present multiple targets for therapeutic intervention. The apicoplast-localized methylerythritol phosphate pathway for isoprenoid precursor biosynthesis is distinct from the mevalonate pathway used by the mammalian host, and this pathway is apparently essential in most Apicomplexa. In this review, we discuss the current field of research on production and metabolic fates of isoprenoids in apicomplexan parasites, including the acquisition of host isoprenoid precursors and downstream products. We describe recent work identifying the first methylerythritol phosphate pathway regulator in apicomplexan parasites, and introduce several promising areas for ongoing research into this well-validated antiparasitic target.

Keywords Apicomplexa · *Plasmodium* · Isoprenoid · Methylerythritol phosphate (MEP) pathway · Fosmidomycin · Metabolism

Introduction

The Apicomplexa are a phylum of protozoan parasites, including some of the most prevalent and deadly human

pathogens. Apicomplexa are distinguished from similar protozoa by a “complex” of structures at the apical end of the parasite, including secretory organelles known as the rhoptries and micronemes, and cytoskeletal features such as the conoid [1, 2]. Apicomplexa include the Gregarina, Cryptosporidia, Coccidia, and Aconoidasida [3]. Apicomplexan parasites infect a diverse range of multi-cellular hosts, including invertebrates; the gregarines exclusively infect invertebrates. The best-studied Apicomplexa, as described briefly below, cause mammalian diseases of importance to global health and agriculture.

The most divergent Apicomplexa, the Cryptosporidia, include parasites of the *Cryptosporidium* genus [4]. Infections with *Cryptosporidium* spp. cause self-limited diarrhea in healthy adults, but cryptosporidiosis can be life threatening in young children and immunocompromised individuals [5]. Recently, *Cryptosporidium* spp. were identified as a major agent of severe diarrhea, a leading cause of child death worldwide [6]. The main human cryptosporidial pathogens are *C. hominis*, which primarily infects humans, and *C. parvum*, which is common among many mammals. Symptoms are caused by several developmental stages that occur within intestinal epithelial cells (as reviewed in [5]). New treatments for cryptosporidiosis are urgently needed, as the only available therapeutic agent, nitazoxanide, is ineffective in immunocompromised individuals and only moderately effective in immunocompetent individuals [7].

The *Coccidia* include many parasites that infect both vertebrates and invertebrates. *Coccidia* of note include *Eimeria* spp., which infect birds, most prominently chickens and other poultry livestock, and *Toxoplasma* spp., which infect a very broad range of hosts, including humans [4, 8, 9]. Toxoplasmosis is generally acquired through ingestion of either tissue cysts (in insufficiently cooked meat) or oocysts (in feces of infected cats, the definitive host species). When acute infection occurs during pregnancy, tachyzoites may infect the fetus,

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leading to severe birth defects or fetal loss [10]. *Toxoplasma gondii* readily infects all nucleated mammalian cells, is easily cultured, and its genetic manipulation is straightforward. For these reasons, *T. gondii* serves as an important model system in studies of apicomplexan biology.

The Aconoidasida, which infect erythrocytes, include the Piroplasmidae and the Hemospororidae [4]. The Piroplasmidae, including *Babesia* and *Theileria* spp., primarily cause economically important diseases in livestock. Babesiosis has recently emerged as a threat to blood transfusion recipients [11]. Hemospororidae include *Plasmodium* spp., which cause malaria in a variety of vertebrates, although each malarial species is typically restricted to a particular host. Five *Plasmodium* species cause malaria in humans: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*. Of these, *P. vivax* is the most common malaria parasite outside of Africa, and *P. falciparum*, the most deadly malaria parasite, contributes to the majority of African cases. *Plasmodium* spp. are estimated to cause 207 million infections and 627,000 human deaths annually; the majority of these deaths occur in African children under the age of 5 years [12]. Resistance to chloroquine and other quinoline-based treatments has become widespread. Artemisinin became the global drug of choice in the 1990s, but resistance has emerged and is spreading [13–15]. The critical need for new antimalarial agents drives research efforts to identify and target essential aspects of parasite biology, in particular those cellular features that distinguish parasites from host. *Plasmodium* infections begin when an infected mosquito injects sporozoites into the mammalian host during a blood meal. Following asymptomatic replication in the liver, the symptoms of malaria occur during the asexual replicative stages in human erythrocytes, as successive generations of parasites develop within red blood cells, which burst to release additional parasites.

The Apicoplast

In addition to the apical organelles from which the phylum derives its name, most Apicomplexa possess an additional unusual plastid organelle, known as the apicoplast. The apicoplast is of similar secondary endosymbiotic origin to the chloroplast of green plants. Although the apicoplast is not photosynthetic, it nonetheless retains several plant-like metabolic pathways [16].

A key process within the apicoplast is the synthesis of the five-carbon isoprenoid precursor molecules, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). All isoprenoids are derived from these two five-carbon molecules and isoprenoids are functionally required in all living cells. These molecules fulfill a variety of cellular roles, including participation in key processes such as N-glycosylation, electron transport (ubiquinone), and protein

prenylation. With the exception of the *Cryptosporidium* spp., which are obligate intracellular pathogens and no longer possess an apicoplast, isoprenoid biosynthesis in apicomplexan parasites occurs via a metabolic pathway housed in the apicoplast, known as the methylerythritol phosphate (MEP) pathway after its first-dedicated metabolite. Because this organelle is cyanobacterial in origin, the MEP pathway is shared by the majority of eubacteria and other plastid-containing eukaryotes, such as plants and algae [16]. In contrast, most other eukaryotes, including mammals, use an independently evolved alternate metabolic route for IPP production, which proceeds through mevalonic acid (MVA).

The MEP Pathway

The MEP pathway (see Fig. 1) commences with synthesis of 1-deoxy-D-xylulose 5-phosphate (DXP) from two glycolytic intermediates, pyruvate and glyceraldehyde-3-phosphate, and proceeds through seven enzymatic steps to production of IPP. The initial reaction is catalyzed by deoxyxylulose 5-phosphate synthase (DXS). In most organisms, DXS is not considered to be a “committed” member of the MEP pathway because its product, DXP, also participates in thiamine (vitamin B₁) biosynthesis and/or pyridoxine (vitamin B₆) synthesis. DXP-dependent pyridoxine synthesis is specific to γ -proteobacteria, but DXP is required for *de novo* thiamine biosynthesis in diverse bacteria. Some downstream synthesis and salvage enzymes are conserved in *P. falciparum* [27], but not other apicomplexan parasites. It is unclear whether thiamine biosynthesis is required, but thiamine use is essential in

Fig. 1 Isoprenoid metabolism in apicomplexan parasites. Some enzymes and processes are not conserved in all Apicomplexa; see text and Table 1. In *Plasmodium* and *Toxoplasma* spp., FPP and GGPP are synthesized by a single bifunctional enzyme; in *Cryptosporidium* spp., NPPPS (nonspecific polyprenyl pyrophosphate synthase) synthesizes products with a wide range of chain lengths [20–22]. Abbreviations: G3P, glyceraldehyde-3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; CDP-ME, 4-diphosphocytidyl-2-C-methyl-D-erythritol; CDP-MEP, 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate; MEcPP, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; HMBPP, 1-hydroxy-2-methyl-2-buten-4-yl 4-diphosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; FPPS, farnesyl pyrophosphate synthase; GGPPS, geranylgeranyl pyrophosphate synthase; FT, protein farnesyltransferase; GGT1, type I protein geranylgeranyltransferase; GGT2, type II (Rab) protein geranylgeranyltransferase; OPP, octaprenyl pyrophosphate; OPPS, octaprenyl pyrophosphate synthase; Q8, ubiquinone-8; cis-IPtase, cis-isopentenyltransferase; polyprenyl-PP, polyprenyl pyrophosphate; dol-P, dolichol phosphate; DPM1, dolichol phosphate mannosyltransferase; GPT, dolichol phosphate N-acetylglucosamine-1-phosphotransferase; dol-P mannose, dolichol phosphate mannose; dol-PP-GlcNAc, dolichol pyrophosphate N-acetylglucosamine; OST, oligosaccharyltransferase; dol-PP, dolichol pyrophosphate

Table 1 Presence of isoprenoid metabolic genes in the genomes of apicomplexan parasites. Available genome data was searched for homologs to known enzymes using default settings on NCBI BLAST; EuPath DB annotations were also consulted. Bold entries represent enzymes whose activities have been experimentally verified. Accurate assignment of enzyme homologs in *Eimeria tenella* was limited by the quality of currently available genome data. Entries represent the most likely identified candidates. PfHad1 paralogs were identified based on sequence homology to PF3D7_1033400 (NCBI BLAST) and membership in the IIb subfamily of HAD-superfamily hydrolases (Interpro IPR006379). X denotes no homolog found; * denotes no definite homolog found but activity is expected. Proteins in **bold** have been characterized *in vitro*

Enzyme	EC number	<i>Plasmodium falciparum</i> 3D7	<i>Toxoplasma gondii</i> GT-1	<i>Eimeria tenella</i>	<i>Babesia microti</i> RI	<i>Theileria parva</i> Mugaga	<i>Cryptosporidium parvum</i> Iowa II
MEP pathway enzymes							
DXS	2.2.1.7	PF3D7_1337200	TGGT1_20820	ETH_00003770	BBM_III00540	TP01_0516	X
DXR	1.1.1.267	PF3D7_1467300 [17]	TGGT1_214850 [18]	ETH_00017440	BBM_III02665	TP02_0073	X
IspD	2.7.7.60	PF3D7_0106900	TGGT1_306260	*	BBM_III02480	TP01_0057	X
IspE	2.7.1.148	PF3D7_0503100	TGGT1_306550	*	BBM_III01890	TP02_0581	X
IspF	4.6.1.12	PF3D7_0209300 [19]	TGGT1_255690	ETH_00030180	BBM_III05000	TP03_0365	X
IspG	1.17.7.1	PF3D7_1022800	TGGT1_262430	ETH_00001880	BBM_III01825	TP01_0667	X
IspH	1.17.1.2	PF3D7_0104400	TGGT1_227420	ETH_00020795	BBM_III05255	TP03_0674	X
Prenyl synthases							
FPPS	2.5.1.10	PF3D7_1128400 [20] ^a	TGGT1_224490 [21] ^b	ETH_00019475	BBM_III00130	TP03_0857	cgd4_2550 [22] ^c
OPPS	2.5.1.90	PF3D7_0202700 [23] ^d	TGGT1_269430	ETH_00035470	BBM_III01090	TP03_0238	cgd7_3730
Cis-IPase	2.5.1.87	PF3D7_0826400	TGGT1_316770	ETH_00037555	BBM_III01680	TP03_0421	cgd4_1510
Ubiquinone synthesis							
Coq2	2.5.1.39	PF3D7_0607500	TGGT1_259130	*	BBM_III09587	TP03_0802	*
Coq3	2.1.1.64	PF3D7_0724300	TGGT1_266850	ETH_00031320	BBM_III02105	TP02_0197	cgd2_2830
tRNA							
MiaA	2.5.1.75	PF3D7_1207600	TGGT1_312520	ETH_00042745	BBM_III01495	TP01_0445	Cgd6_2540
N- glycosylation							
GPT	2.7.8.15	PF3D7_0321200	TGGT1_244520	ETH_00020690	BBM_III00105	TP01_0118 ^e	cgd5_2240
OST, Stt3p subunit	2.4.99.18	PF3D7_1116600	TGGT1_231430	ETH_00007235	Homolog found only in other <i>Babesia</i> spp.	X	cgd6_2040
Protein prenylation							
DPM1	2.4.1.83	PF3D7_1141600	TGGT1_277970	*(cite Theil)	BBM_III00170	TP02_0741	cgd5_2040
FT, GGT1, GGT2	2.5.1.58 2.5.1.59	Although phylogenetic searches identified multiple proteins with homology to protein prenyltransferases in each species examined, it was not possible to distinguish between types of prenyltransferases (e.g., GGT-1 vs. FT-1) based on homology alone. However, evidence of protein prenylation has been demonstrated in several apicomplexans.					
PfHad1 paralogs		PF3D7_1033400 (PfHad1) [24•], PF3D7_1226300, PF3D7_1226100, PF3D7_1017400, PF3D7_1118400	TGGT1_243910, TGGT1_239710, TGGT1_229320, TGGT1_297720, TGGT1_229330	ETH_00014830, ETH_00010345, ETH_00027645	BBM_III03770, BBM_III01380	TP02_0864, TP01_1081, TP01_1077, TP01_1076, TP01_1075, TP01_1074, TP01_0861, TP01_0785	cgd4_960, cgd1_3340

Abbreviations: FPPS, farnesyl pyrophosphate synthase; OPPS, octaprenyl pyrophosphate synthase; cis-IPase, cis-isoprenyltransferase; GPT, dolichol phosphate N-acetylglucosamine-1-phosphotransferase; OST, oligosaccharyltransferase; DPM1, dolichol phosphate mannose transferase; FT, protein farnesyltransferase; GGT1, type I protein farnesyltransferase; GGT2, type II (Rab) protein farnesyltransferase

^a PF3D7_1128400 is a bifunctional FPP/GGPP synthase

^b The bifunctional *T. gondii* protein, TGGT1_224490 (E.C. 2.5.1.29), carries FPP/GGPP synthase activity but is more homologous to GGPPS proteins

^c cgd4_2550 demonstrates nonspecific polyprenyl pyrophosphate synthase activity

^d PF3D7_0202700 also has phytoene synthase activity [25]

^e *Theileria* spp. were not expected to encode GPT activity [26]

P. falciparum [28]. The subsequent enzyme of the MEP pathway, IspC/DXR, which is bifunctional, catalyzes the isomerization and the NADPH-dependent reduction of DXP to form 2-C-methyl-D-erythritol 4-phosphate (MEP). IspD and IspE activate MEP for cyclization by IspF. IspD transfers a cytidyl group to MEP, and the resulting 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME) is phosphorylated by IspE to form 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate (CDP-MEP). IspF cyclizes CDP-MEP, resulting in 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MEcPP). The remaining two steps of the pathway are catalyzed by two [4Fe-4S] cluster enzymes, IspG and IspH. IspG opens the MEcPP ring and performs a reduction, resulting in HMBPP (1-hydroxy-2-methyl-2-buten-4-yl 4-diphosphate). Finally, IspH reduces HMBPP, producing IPP and DMAPP products [29].

Validation of the MEP Pathway

Both genetic and chemical evidence strongly suggest that the MEP pathway is essential in Apicomplexa. In *P. falciparum*, the IspC/DXR locus is resistant to disruption in erythrocytic-stage parasites [27]. Similarly, in *T. gondii*, IspC/DXR and IspH disruptions do not result in viable parasites, and parasites forced to turn off IspH expression do not survive [30]. Because these results agree with similar studies in other MEP pathway containing organisms, including bacteria, genetic studies alone give strong support to the essential nature of the MEP pathway in Apicomplexa [31–35].

In addition, a major pharmacological tool for study of the MEP pathway in apicomplexan parasites has been a well-defined chemical inhibitor of the pathway, fosmidomycin. Fosmidomycin is a phosphonic acid antibiotic that is a substrate mimic and direct inhibitor of the first dedicated MEP pathway enzyme, IspC/DXR [36, 37]. Fosmidomycin (and its analog, FR-9000098) inhibit growth of cultured *P. falciparum*, *Babesia bovis*, and *B. bigemina*, providing some of the earliest evidence that this pathway is essential in Apicomplexa [38, 39]. Subsequent studies have established that the antimicrobial effects of fosmidomycin in malaria parasites are mediated exclusively through inhibition of the MEP pathway [40, 41]. Metabolic profiling of fosmidomycin-treated *P. falciparum* demonstrates decreased cellular levels of downstream MEP pathway metabolites, confirming that fosmidomycin affects isoprenoid metabolism [41]. In addition, the growth inhibitory effects of fosmidomycin are reduced upon media supplementation with IPP or downstream isoprenols, establishing that there are no significant off-target effects of fosmidomycin treatment. Because IPP-supplemented *P. falciparum* can survive in the absence of the apicoplast organellar genome and structure, these apicoplast-null strains have been used to suggest that the MEP pathway is the only essential function of the apicoplast [40]. Ongoing studies will be required to confirm

whether other nuclear-encoded metabolic pathways, such as heme biosynthesis, might remain functional in these cells, even in the absence of a well-defined apicoplast structure.

In contrast to *Plasmodium* and *Babesia* spp., fosmidomycin is ineffective against other apicomplexan parasites, including *Theileria parva*, *Eimeria tenella*, *T. gondii*, and *Cryptosporidium* spp. [42, 43]. Resistance in *Cryptosporidium* spp. was not unexpected, because these organisms (which lack an apicoplast) do not express MEP pathway enzymes. Fosmidomycin is a highly charged molecule that is excluded from uninfected erythrocytes but accumulates during *Plasmodium* and *Babesia* infections, suggesting that active transport through hemsporidian-specific permeability pathways is required for drug uptake [44]. This cellular exclusion appears to be the mechanism by which *T. gondii* parasites (and likely other Apicomplexa) are naturally fosmidomycin resistant. In an elegant series of experiments, Nair et al. demonstrated that expression of a bacterial glycerol-3-phosphate transporter (GlpT), which also allows import of fosfomycin (a drug related to fosmidomycin), confers fosmidomycin sensitivity to cultured *T. gondii*. Thus, the MEP pathway for isoprenoid biosynthesis is essential in *T. gondii*, and likely required for development of the remaining fosmidomycin-insensitive apicomplexan parasites [30].

Host Isoprenoid Scavenging

Most apicomplexan parasites spend all or part of their life cycle within a metazoan host cell. This particular environmental niche therefore offers the possibility of scavenging host cell components, including isoprenoid precursors and downstream isoprenoid products. While, in many cases, the extent to which this occurs is not yet fully characterized, evidence to date indicates distinct differences between apicomplexan parasite species and likely between developmental stages of each individual parasite.

Mammalian cells generate isoprenoids through the MVA pathway, although the overall flux is dependent upon cell type. For example, because the liver is a major site of sterol production, hepatocytes have a high capacity for isoprenoid biosynthesis. In contrast, proteomic studies of human erythrocytes suggest that host isoprenoid biosynthesis is absent in these cells [45, 46]. MVA-dependent isoprenoid biosynthesis in the host is sensitive to inhibition by the statin class of therapeutics, which potently inhibits the rate-limiting step of this pathway, HMG-CoA reductase [47].

Scavenging in *Cryptosporidium*

Cryptosporidium spp. are unique among the parasitic Apicomplexa. As obligate intracellular parasites, these organisms have lost the apicoplast organelle and do not produce the machinery for *de novo* isoprenoid biosynthesis. Because isoprenoids are required for cellular growth, these parasites

are therefore expected to depend entirely on host precursor biosynthesis. Indeed, MVA pathway inhibitors such as itavastatin effectively inhibit *C. parvum* growth *in vitro*. Exogenous IPP partially rescues statin-mediated growth inhibition, supporting that the antiparasitic action of these compounds is exerted through their effects on host isoprenoid biosynthesis [48••]. The mechanism by which *Cryptosporidium* spp. acquire isoprenoid precursors and/or products from the host has not been determined, but *C. parvum* and *C. hominis* are predicted to encode enzymes for N-glycosylation, ubiquinone biosynthesis, and protein prenylation, suggesting that the parasite still modifies downstream isoprenoids [48••].

Scavenging in *Plasmodium* and *Babesia*

In studies prior to discovery of the MEP pathway, *Plasmodium* and *Babesia* spp. were found to be sensitive to treatment with high doses of statins, including simvastatin, lovastatin, and mevastatin [49, 50]. Subsequent studies have indicated that the growth inhibitory effects of statins are not mediated through inhibition of isoprenoid biosynthesis. While statin-treated mammalian cells are rescued with exogenous mevalonate supplementation, similar rescue of parasites treated with simvastatin, lovastatin [49], or mevastatin [50] was not observed. It seems likely that the intra-erythrocytic stages of the Hemospororidae may be unusual in their independence from host isoprenoid biosynthesis. MVA pathway enzymes are only present in erythrocytes at very low levels, and isoprenoid levels in serum are also very low [45, 46, 51, 52]. Thus, availability of isoprenoid precursors or products seems likely to play only a minor role, at least in the asexual erythrocytic stages. As described below, other apicomplexan parasites, which reside within more metabolically active host cells, may have increased reliance on host isoprenoid synthesis.

In contrast to the metabolically restricted erythrocyte, mammalian hepatocytes produce large quantities of isoprenoid metabolites, including membrane sterols (e.g., cholesterol). Fosmidomycin is also detrimental to the growth of liver-stage parasites in cell culture [30], suggesting that even in these relatively isoprenoid-rich cells, *de novo* synthesis through the MEP pathway is important for parasite development.

Scavenging in *Toxoplasma*

In contrast to the Hemospororidae, *T. gondii* infect nucleated mammalian cells. To assess the reliance of *Toxoplasma* spp. on host isoprenoids, Li et al. recently generated a farnesyl pyrophosphate synthase (FPPS)-null strain of *T. gondii* [53••]. The *T. gondii* FPPS is a bifunctional enzyme that generates both FPP and GGPP from IPP and DMAPP [21]. Viability of the FPPS mutant strain varied with host cell type, but extracellular incubation of FPPS mutants depleted intracellular adenosine

triphosphate (ATP) stores and disrupted the mitochondrial membrane potential. Although many downstream isoprenoids are likely essential, this FPPS-null phenotype suggests a shortage of ubiquinone to support ATP production through the electron transport chain (ETC). The parasite appears to use both host and endogenous isoprenoids for downstream isoprenoid synthesis, and is able to increase the host contribution when parasite-derived short-chain isoprenoids (<C₂₀, GGPP) are unavailable. Consistent with this suggestion, FPPS-null or -knockdown parasites were more sensitive to inhibition of host isoprenoid biosynthesis by statins. In summary, although the MEP pathway is essential, *T. gondii* appears to derive short-chain isoprenoids from both endogenous and host synthases [53••]. The possibility that a subset of precursors (IPP and DMAPP) may also be host derived has not yet been excluded.

Scavenging of Membrane Sterols

While isoprenoid precursors appear to be scavenged by *Cryptosporidium* and *Toxoplasma* spp., little is known about which downstream isoprenoids may be scavenged by apicomplexan parasites. One exception is the use of host cholesterol. Cholesterol appears to be essential for growth of Apicomplexa, but there is no bioinformatic or biochemical evidence for *de novo* sterol synthesis in this phylum. Rather, evidence suggests that apicomplexan parasites scavenge cholesterol from various host sources (reviewed in [54]). Typically, cholesterol is transported in the plasma as low-density lipoprotein (LDL) and high-density lipoprotein (HDL). Host cells obtain cholesterol by either receptor-mediated endocytosis of LDL or by *de novo* synthesis via the MVA pathway. Under normal circumstances, HDL delivers cholesterol back to the liver. In *Plasmodium* spp.-infected erythrocytes, HDL particles can provide cholesterol to the parasite through an unknown mechanism [55]. The malaria parasite also may receive cholesterol from the erythrocyte membrane, possibly via contacts with the parasite vacuolar membrane (PVM) [56]. In the liver, *Plasmodium* parasites receive cholesterol both from LDL particles and from *de novo* synthesis by the host [57]. The parasitophorous vacuole associates both with the host ER [58], which stores cholesterol-enriched lipid bodies, and with cholesterol-enriched late endosomes from the host [59].

Toxoplasma gondii mainly receives exogenous cholesterol from LDL [60]. As in *Plasmodium*, cholesterol-filled host lysosomes are closely associated with the PVM [61]. Finally, as parasites that infect intestinal epithelial cells, *Cryptosporidium* spp. can obtain host cholesterol through LDL uptake, host *de novo* synthesis, or through absorption of dietary cholesterol from intestinal micelles. Evidence suggests that the parasite relies upon all three of these sources for optimal growth [62].

Fatty acid esterification permits cholesterol storage without excessive perturbation of membrane fluidity. Although *Plasmodium* spp. do not encode the enzymes necessary for

cholesterol storage [63], this process appears to be essential in *Toxoplasma* [64].

Cellular Functions of Isoprenoids in Apicomplexa

tRNA Isoprenylation

Transfer RNAs (tRNAs) are required for ribosomal protein synthesis. A common tRNA modification is methylthioisoprenylation, in which tRNAs are modified at adenine 37. These modifications stabilize binding between tRNAs and the mRNA/ribosome complex and promote proper codon-anticodon interactions, protecting against premature stops and frame-shift mistakes during translation [65]. tRNA dimethylallyl transferase (MiaA) and MiaB perform the isoprenylation and the thio/methylation reactions, respectively. Although tRNA modification has not been extensively studied in Apicomplexa, most species appear to encode annotated homologs of MiaA and MiaB, suggesting that these processes do occur (see Table 1). In *Theileria* and *Plasmodium* spp., MiaA and MiaB are predicted to be apicoplast localized and are therefore expected to modify apicoplast tRNAs, but the biological implications of this modification have yet to be explored [42, 65].

Prenyl Synthases

Other than tRNA isoprenylation, the majority of cellular functions require isoprenoids of at least 15 carbons (C_{15} ; 3 isoprene units). Elongation of 5-carbon (C_5) precursors requires iterative addition of IPP (C_5) units to a DMAPP (C_5) seed molecule, producing geranyl pyrophosphate (GPP; C_{10}), farnesyl pyrophosphate (FPP; C_{15}), and geranylgeranyl pyrophosphate (GGPP; C_{20}), in succession. In most organisms, the first two reactions (GPP and FPP synthesis) are catalyzed by a single farnesyl pyrophosphate synthase (FPPS), while a second enzyme, geranylgeranyl pyrophosphate synthase (GGPPS) adds an additional IPP unit. However, in *Plasmodium* and *Toxoplasma* spp., a single bifunctional FPPS/GGPPS performs each of these reactions [20, 21, 66]. Further elongation may be performed by downstream prenyl synthases, such as the *P. falciparum* octaprenyl pyrophosphate synthase (OPPS), which has been characterized *in vitro* [23]. In *C. parvum*, a unique nonspecific polyprenyl pyrophosphate synthase produces a remarkable range of products, from C_{15} to greater than C_{40} [22]. These 10-, 15-, and 20-carbon pyrophosphate products are necessary for synthesis of essential downstream isoprenoids, such as ubiquinone. For this reason, Apicomplexan prenyl synthases are expected to be essential, unless these compounds can be scavenged from the host.

Protein Prenylation

Proteins may be modified post-translationally by isoprenylation. Such protein prenylation provides a membrane anchor, typically essential for proper localization and/or function of the modified protein substrate. Prenyltransferases recognize specific motifs at the C-termini of proteins, so-called CaaX motifs (cysteine, followed by two aliphatic residues, followed by any residue). Type I protein farnesyltransferases (FT) and protein geranylgeranyltransferases (GGT1) recognize this CaaX motif, and the identity of the fourth residue can determine whether the protein is farnesylated or geranylgeranylated. Rab proteins, which help regulate vesicular trafficking, additionally require an escort protein for CaaX recognition by Type II geranylgeranyltransferases (GGT2; Rab GGT) (reviewed in [67]).

Malaria parasites are capable of protein prenylation, and prenyltransferase inhibitors inhibit parasite growth [68–73]. Protein prenylation is likely to be one of the essential functions of isoprenoids in malaria parasites, because inhibition of isoprenoid biosynthesis mislocalizes a putative prenyltransferase substrate (Rab5) and results in trafficking defects consistent with loss of Rab5 function [74]. Other prenylated *Plasmodium* proteins include a tyrosine phosphatase, PfPRL, and the Ykt6 SNARE protein [75, 76]. Addition of C_{55} dolichyl and C_{60} isoprenyl chains to *P. falciparum* proteins has also been observed, but the biological functions of these modifications have not been explored [77].

Protein prenylation has been observed in *T. gondii*. This activity is inhibited by certain synthetic heptapeptides [78]. The presence of protein prenyltransferases has been predicted bioinformatically in *Cryptosporidia*, but not yet experimentally confirmed [67].

Quinones

The most prominent cellular function of molecules such as ubiquinone and menaquinone is as intermediates in the ETC, allowing generation of the mitochondrial proton gradient. This gradient provides an energy source for ATP generation and active transport. During ubiquinone biosynthesis, polyisoprenylation of the redox-active benzoquinone group, which allows mitochondrial membrane localization, is catalyzed by 4-hydroxybenzoate polyprenyltransferase (Coq2). This step is followed by several additional modifications to the benzoquinone moiety, including methylation by Coq3 (reviewed in [79]). Both Coq2 and Coq3 functions appear to be conserved among apicomplexan parasites (Table 1).

Within the mitochondrial ETC, multiple dehydrogenases play important roles in reducing ubiquinone (coenzyme Q) to generate ubiquinol, which then passes electrons to complex III, the cytochrome bc1 complex. In mammals, the most prominent ubiquinone reductases are complex I (type I NADH

dehydrogenase; NDH1) or complex II (succinate dehydrogenase; SDH). In various Apicomplexa, ubiquinone can accept electrons from several dehydrogenases, including glycerol-3-phosphate dehydrogenase, malate-quinone oxidoreductase, dihydroorotate dehydrogenase (DHODH), SDH, and type II NADH dehydrogenase (NDH2). Apicomplexa have not retained NDH1. After electron transfer from ubiquinol to complex III, electrons are transferred to cytochrome c, and finally to complex IV. Protons are pumped across the membrane throughout this process (reviewed in [80]).

Ubiquinone biosynthesis and function have been best studied in *P. falciparum*, in which parasites modulate ubiquinone:menaquinone ratios according to oxygen levels. Menaquinone can substitute for ubiquinone in the ETC, but how these ratios are modulated is unknown [81]. Biosynthesis of the isoprenyl sidechain of ubiquinone in *P. falciparum*, which contains eight or nine isoprenyl units, was first described in 2002 [82]. In cultured erythrocytic *P. falciparum*, expression of yeast dihydroorotate dehydrogenase (DHODH), which, in contrast to the native *P. falciparum* enzyme, does not require ubiquinone as an electron acceptor, reduces sensitivity to inhibition by the Complex III inhibitor, atovaquone. Thus, the essential function of the *Plasmodium* ubiquinone, at least in the asexual stages, is to allow pyrimidine synthesis by acting as an electron sink for the essential pyrimidine biosynthesis enzyme DHODH [83]. Because expression of yeast DHODH does not confer resistance to fosmidomycin, it is clear that in intra-erythrocytic parasites, pyrimidine biosynthesis is not the only essential process in malaria parasites that requires isoprenoid synthesis [74].

In contrast, mosquito-stage *Plasmodium* parasites appear to depend upon oxidative phosphorylation and ubiquinone for ATP generation. *Plasmodium berghei* parasites lacking functional SDH or NDH2 fail to form functional oocysts in mosquitoes, although they are still capable of asexual replication [84, 85].

Like *Plasmodium*, *T. gondii* does not rely heavily on the ETC for ATP generation, but is nonetheless sensitive to disruption of the ETC by atovaquone, which inhibits complex III. DHODH is also essential in *T. gondii* [86]. Single disruptions of either of two ubiquinone-reducing NDH2 isoforms are possible, but confer growth defects; a double knockout was unable to be generated [87].

Cryptosporidium spp. are distinguished from other apicomplexan parasites by the presence of a mitochondrion-like organelle, the mitosome. Based on *in silico* analysis, the mitosome of the rodent intestinal parasite, *C. murum*, harbors a complete TCA cycle, simplified ETC, and intact ATP synthase, in contrast to *C. hominis* and *C. parvum*. In the human pathogens, the only TCA enzyme is a truncated malate:quinone oxidoreductase (MQO) homolog (reviewed in [88]). This simplified *Cryptosporidium* ETC lacks complexes III and IV; rather, an alternative oxidase (AOX) allows electron transfer between ubiquinol (probably generated via MQO) and O₂ [88]. Recombinantly produced *C. parvum*

AOX was verified to have ubiquinol oxidase activity. This enzyme is sensitive to both ascofuranone, an inhibitor of *Trypanosoma brucei* AOX, and to salicylhydroxamic acid (SHAM), a known AOX inhibitor [89]. Treatment with SHAM and 8-hydroxyquinoline, another known AOX inhibitor, inhibit growth of *C. parvum*, *T. gondii*, and *P. falciparum* in culture, although homology-based identification of specific AOX candidates from *T. gondii* or *P. falciparum* has not been successful [90]. Because ubiquinol oxidase activity appears to be essential, ubiquinone synthesis or salvage is likely essential as well.

Dolichols

Dolichols are long isoprene chains with saturated isoprenic units at the alpha position. Chain lengths vary by species. These molecules are required both for N-glycosylation of proteins and for glycosylphosphatidylinositol (GPI) anchor biosynthesis. During N-glycosylation, dolichol serves as a membrane anchor for the growing glycan chain, which is eventually transferred from the dolichol to the target protein. Mannose residues are added during the latter phases of glycan chain elongation; dolichol phosphate mannose serves as the donor for these reactions. Dolichol phosphate mannose is also required as a donor during GPI anchor synthesis (reviewed in [91]).

Both protein N-glycosylation and GPI anchor biosynthesis appear to be active processes in Apicomplexa. GPIs play important roles in the biology of *Plasmodium*, *Toxoplasma*, and *Cryptosporidium* spp. (reviewed in [92, 93]). Although *T. gondii* performs N-glycosylation [94], it was unclear for some time whether these modifications were absent, or simply rare, in *Plasmodium* spp. It now appears that *Plasmodium* spp. produce a small number of N-glycosylated proteins [95], with unusually short N-glycan chains. In fact, many apicomplexan genomes encode “incomplete” protein N-glycosylation pathways, which result in truncated N-glycans [96]. For example, *Theileria* is reported to lack N-glycosylation machinery altogether [26]. Tunicamycin, which inhibits transfer of the first GlcNAc residue onto the dolichol anchor, during N-glycan synthesis, is toxic to both *P. falciparum* and *T. gondii*, suggesting that this process is required for parasite survival [94, 97].

Other Isoprenoids

Carotenoids are tetraterpene (eight isoprene units) pigments, often with antioxidant activity. Typically, two GGPP molecules are combined to form phytoene, from which subsequent carotenoids are derived [98]. Several carotenoids, including all-*trans*- β -carotene and all-*trans*-lutein, have been identified in cultured *P. falciparum*, but not uninfected control cultures [25]. No clear homologs of known carotenoid biosynthesis enzymes are apparent in *Plasmodium* or *Toxoplasma* genomes, but a previously identified OPPS showed synthesis of phytoene and some downstream carotenoids *in vitro*. Inhibition of carotenoid

biosynthesis sensitized *P. falciparum* parasites to high environmental oxygen concentrations, suggesting that carotenoids may function as antioxidants in malaria parasites [25].

In plants, abscisic acid, another carotenoid, acts as a signaling molecule. Signal transduction involves stimulation of intracellular calcium release [99]. In *T. gondii*, exogenous abscisic acid also triggers release of intracellular calcium stores. While the biosynthetic enzymes to produce abscisic acid are not readily identified bioinformatically, abscisic acid was detected in parasite lysates and reduced after treatment with fluridone, a carotenoid biosynthesis inhibitor. Fluridone treatment also prevented parasite egress, suggesting that abscisic acid signaling plays a crucial role in this process [100].

Vitamin E (α - and γ -tocopherol) was recently identified in *P. falciparum* extracts. Growth inhibition by usnic acid, which inhibits vitamin E synthesis, was accompanied by a decline in vitamin E concentrations, but only partially rescued by addition of α -tocopherol. α -Tocopherol synthesis increased by 40 % under high (20 %) oxygen, suggesting a role for vitamin E in protection from oxidative stress [101].

Regulation of the MEP Pathway

Because the MEP pathway is considered to be a promising target for anti-parasitic drug development, regulation of the pathway is of great interest to the field. However, very little is known about pathway regulation in Apicomplexa. As in other metabolic pathways, regulation of the MEP pathway is typically at the level of so-called rate-limiting enzymes. In many plants and bacterial species, DXS has been identified as a rate-limiting enzyme of the MEP pathway. In addition, DXR and IspF have also been identified as rate limiting in some cases (reviewed in [102]).

Several studies have identified transcript-level regulation of MEP pathway genes in plants (reviewed in [102]). Furthermore, Sauret-Güeto et al. found that fosmidomycin resistance in *Arabidopsis thaliana* is due to impaired translation of plastome mRNAs. This resistance mechanism results in increased levels of IspC/DXR protein, which is encoded in the nucleus; DXS, IspG, and IspH protein levels also increase. The precise regulatory mechanism by which MEP enzyme expression responds to plastome expression has not been identified, but it is possible that similar post-transcriptional regulation may occur in Apicomplexa [103].

Post-translational regulation of MEP pathway enzymes has also been described in several organisms, and may also be present in apicomplexan parasites. For example, in *Francisella tularensis*, phosphorylation of either IspC/DXR or IspD at conserved sites down-regulates enzyme activity. *Francisella tularensis* IspC/DXR is phosphorylated at Ser177; phosphorylation of *F. tularensis* IspD occurs at

Thr141 [104, 105]. The IspC/DXR Ser177 residue appears to be conserved in most Apicomplexa; the IspD Thr141 position is generally either a threonine or a serine, either of which could be phosphorylated.

For many MEP enzymes, metabolite binding also appears to regulate enzymatic function, at least *in vitro*. First, the *Populus trichocarpa* (black cottonwood tree) DXS enzyme is inhibited by high concentrations of IPP, suggesting feedback inhibition may occur [106•]. Second, the enzyme IspF may be a target for feed-forward regulation; the upstream metabolite MEP stabilizes activity of purified recombinant *E. coli* IspF, and this stabilization is inhibited by co-incubation with the downstream metabolite FPP [107•]. Finally, IspF monomers form a very stable trimer, which is assumed to be required for activity. A hydrophobic cavity at this trimer interface is conserved in most organisms, including *P. falciparum* [108] (A *P. vivax* IspF structure has been deposited but not published; PDB: 3B6N). Multiple structural studies have identified IPP, GPP, or FPP bound at this interface, suggesting a potential role in feedback regulation [109–111].

To date, a single regulator has been described for the MEP pathway in Apicomplexa. *P. falciparum* Had1 (PfHad1) is a sugar phosphatase and a member of the haloacid dehalogenase superfamily. PfHad1 cleaves phosphate groups from a variety of substrates, including MEP pathway intermediates and glycolytic intermediates upstream of the MEP pathway. Loss-of-function mutations in PfHad1 confer partial resistance to fosmidomycin, likely as a result of increased substrate availability [24••]. Had1 homologs in other apicomplexan parasites may also be negative regulators of MEP pathway activity (Table 1).

Conclusion

Apicomplexan parasites include several human pathogens of global importance. Current treatments for these diseases are inadequate and novel drugs are urgently needed, particularly for the treatment of cryptosporidial diarrhea and malaria. Isoprenoids appear to be essential in all organisms, and apicomplexan parasites acquire isoprenoids via scavenging or the apicoplast-localized MEP pathway. Therefore, it is important to understand the fundamental biology and regulation of isoprenoid biosynthesis in apicomplexan parasites, en route to discovery of novel therapeutic agents with parasite-specific mechanisms of action.

Several aspects of apicomplexan isoprenoid metabolism have yet to be fully elucidated. To begin, most isoprenoid precursors and early isoprenoid products are highly charged and likely to require active transport across membranes. For example, plastidic phosphate translocators (pPT family) on the apicoplast membranes import glycolytic intermediates from which the MEP precursors, pyruvate and glyceraldehyde-3-

phosphate, are generated [112, 113]. However, IPP and DMAPP products must ultimately exit the apicoplast, because downstream metabolism occurs outside this organelle. The molecular identity of these isoprenyl pyrophosphate transporters is yet unknown, but these proteins are expected to be required for parasite viability. In addition, while current evidence strongly suggests that many apicomplexan parasites scavenge isoprenoid precursors and components from host cells, the molecular mechanisms and transporters that support this scavenging are unclear. In particular, simple host isoprenoids (e.g., IPP, FPP, and GGPP) are necessary for development of *C. parvum*, and *T. gondii* [48•, 53•], but whether these molecules are accessed directly through transport or through endocytosis of host cytoplasm is unknown. While some crucial steps of cholesterol scavenging have been identified in *Toxoplasma* and *Cryptosporidium* spp., the process is not yet fully understood, especially in *Plasmodium* spp. It is possible that further complex or longer-chain isoprenoid products may also be obtained from the host. The mechanisms of host scavenging are not likely to have close human or mammalian homologs. Therefore, a deeper understanding of this process is a promising avenue for the identification of additional drug targets and for the use of well-characterized inhibitors of host isoprenoid biosynthesis (e.g., statins) as adjunctive therapeutic agents for apicomplexan diseases.

Improving our understanding of MEP pathway regulation is also likely to identify new therapeutic targets. For example, the recent discovery of the first regulator of apicomplexan MEP metabolism, PfHAD1, has raised several questions about the normal function of Had1 and its homologs [24•]. PfHAD1 activity exerts a strong effect on MEP pathway function. Potential mechanisms for regulation of PfHAD1 activity, or stimuli to which PfHAD1 may respond, have yet to be identified. PfHAD1 has very close homologs in all other apicomplexan parasites, and, in fact, *P. falciparum* itself encodes four additional HAD paralogs (Table 1). The close sequence conservation within this enzyme family strongly suggests that HAD proteins have important biological functions under normal physiological conditions. For example, given its diverse substrate profile, PfHAD1 may regulate additional metabolic pathways in the cell, in addition to the MEP pathway. Future studies are required to elucidate the functional significance of HAD homologs in other apicomplexan parasites.

Because the MEP pathway is energetically expensive, requiring both nucleotides and reducing power, HAD homologs are not likely to be the only mechanism by which parasite cells regulate MEP pathway flux. This is particularly likely in organisms other than blood-stage *P. falciparum*, in which the host cell does not produce IPP. Because most other apicomplexan parasites depend upon both host and *de novo* isoprenoid metabolism, these species are likely to adjust their own biosynthesis of isoprenoid precursors in response to available host supplies. The isoprenoid pyrophosphate-binding cavity at the core of the IspF trimer, which is

conserved in *Plasmodium* spp. and is likely present in additional apicomplexan parasites, suggests one possible mechanism for such feedback regulation [108].

Finally, it is likely that future studies will result in the identification of additional isoprenoid-using enzymes and their products in apicomplexan parasites. Bioinformatic strategies have not conclusively identified the enzymes responsible for synthesis of many known isoprenoid metabolites, such as abscisic acid [100]. This likely reflects both the diversity of isoprenoid products and the evolutionary distance between apicomplexan parasites and other MEP-pathway-using organisms. Furthermore, given the apparent substrate flexibility of many of the enzymes involved in isoprenoid metabolism, it is clear that phylogenetic prediction alone will be insufficient to elucidate the reactions catalyzed by specific proteins. For example, the *P. falciparum* OPPS, which elongates an FPP precursor to a C₄₀ or C₄₅ chain by repeated addition of IPP units, also unexpectedly catalyzes synthesis of phytoene (C₄₀) from two GGPP precursors. The enzyme also appears to derivatize phytoene into several additional carotenoid products [25]. Altogether, the evolutionary distance and the ongoing challenges of functional annotations in apicomplexan parasites will ultimately require directed study of particular enzymes and their functions as they are discovered.

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Compliance with Ethics Guidelines

Conflict of Interest Leah Imlay and Audrey Odom declare that they have no conflict of interests.

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