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Review

Delayed Death by Plastid Inhibition in Apicomplexan Parasites

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The discovery of a plastid in apicomplexan parasites was hoped to be a watershed moment in the treatment of parasitic diseases as it revealed drug targets that are implicitly divergent from host molecular processes. Indeed, this organelle, known as the apicoplast, has since been a productive therapeutic target for pharmaceutical interventions against infections by *Plasmodium*, *Toxoplasma*, *Babesia*, and *Theileria*. However, some inhibitors of the apicoplast are restricted in their treatment utility because of their slow-kill kinetics, and this characteristic is called the delayed death effect. Here we review the recent genetic and pharmacological experiments that interrogate the causes of delayed death and explore the foundation of this phenomenon in *Plasmodium* and *Toxoplasma* parasites.

Highlights

Inhibition of apicoplast housekeeping leads to delayed death, while inhibition of apicoplast anabolic pathways leads to rapid death. Some forms of inhibition of apicoplast biogenesis and import also lead to instant rather than delayed death.

Delayed death in *Toxoplasma gondii* relies on the existence of communication (likely in the form of continuities or tubules) between divided parasites, which appears to facilitate sharing of apicoplast-derived metabolites between tachyzoites in the same parasitophorous vacuole.

Delayed death in *T. gondii* (and maybe other apicomplexans) is modulated by the acquisition of metabolites from the host.

Recent published prenylomes in *Plasmodium falciparum* establish a list of candidates to investigate for delayed death actors.

Abnormalities in intracellular trafficking pathways regulated by prenylated proteins underpin the death of *P. falciparum* in the second cycle of delayed death.

Drugging the Apicomplexan Plastid

The phylum Apicomplexa contains some of the most serious human and veterinary parasites (*Plasmodium, Toxoplasma, Cryptosporidium*, and many others). The discovery, 25 years ago, that apicomplexan parasites contain a relic **plastid** (see Glossary) of cyanobacterial origin, the apicoplast (Box 1), gave rise to a burst of drug discovery centred around this bacterium-like organelle. This discovery elicited tremendous excitement about the potential to use selective antibiotics or herbicide-like drugs against these pathogens. Twenty-five years later, many such drugs have been either identified or newly developed, and many are clinically used to treat or prevent human and animal diseases. Some of these compounds had already been used as antiparasitics before the discovery of their target – indeed, one such drug, doxycycline, is a leading malaria prophylactic [1], and another, clindamycin, is an important treatment in combination therapies for toxoplasmosis [2]. However, drugs that inhibit apicoplast housekeeping functions nearly all suffer from the same shortcoming: though they quickly disrupt plastid function, they do not kill parasites instantly, and instead require the parasite to undergo one complete **intracellular developmental cycle (IDC)** before death [3,4]. This slow clearance is known as the **delayed death** phenotype, and this peculiarity creates major limitations for the application of most plastid-blocking drugs, despite their otherwise excellent selectivity and safety profiles.

Although much work has gone into understanding apicoplast function, the direct cause of delayed death was, until recently, unknown. This lack of understanding prevents us from overcoming delayed death or employing delayed-death drugs in appropriate combinations and treatment regimes. Here we review the exploitable bondage between apicoplast and parasite, and recent advances unravelling the still-enigmatic delayed death effect in apicomplexan parasites.

The Delayed Death Effect

The puzzling slow clearance of parasites treated with inhibitors of apicoplast housekeeping functions (DNA replication, transcription, or translation) has been a long-standing mystery in apicomplexan biology. In the late 1940s, the antimalarial properties of the antibiotics chloramphenicol and tetracycline were discovered. Although these antibacterials were clinically efficacious for treating malaria, they required prolonged treatment over several days to clear parasites [5]. Four decades later, Geary and Jensen [6] observed *ex vivo* that tetracycline and erythromycin require treatment over two asexual intraerythrocytic cycles (approximately 96 h) to arrest *Plasmodium falciparum* growth. However, delayed death was best characterised by experiments treating *Toxoplasma gondii* **tachyzoites** with azithromycin, spiramycin, or clindamycin, whereby parasites survived one IDC by dividing several times within a single **parasitophorous vacuole (PV)**, after which they emerged and

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Box 1. The Apicoplast

The apicoplast is a **complex plastid**, found in most members of Apicomplexa, and was acquired by the **endosymbiosis** of an ancient, plastid-bearing red alga. First observed by electron microscopy as a multimembraned spherical body in multiple apicomplexan taxa [92–95], the apicoplast was not initially recognised as an endosymbiont. This changed with the discovery [96] and sequencing of its circular extrachromosomal genome in *Plasmodium* spp. [24], and its subsequent *in situ* localisation to the apicoplast [97,98]. Although the subject of much debate originally, it is now widely accepted that the apicoplast emerged as an apicomplexan organelle when its ancestor engulfed a photosynthetic alga from a red lineage. The nuclear genes of the algal endosymbiont were either lost or transferred to the host nuclear genome [99], thus creating an absolute dependence on its host. Like algae and plants then, modern apicomplexan parasites have three genomes: nuclear, mitochondrial, and plastid.

The apicoplast DNA replication and protein translation apparatuses are inherently bacterium-like in nature owing to the prokaryotic origin of plastids. The considerable divergence between eukaryotic and prokaryotic lineages has made possible the safe and selective inhibition of apicoplast drug targets. In particular, bacterial antibiotics, such as tetracyclines, have been readily repurposed to treat malaria, toxoplasmosis, and babesiosis. Although early researchers speculated that antibiotics that inhibit bacteria-like protein synthesis might act against the parasite's mitochondrion [100,101], it has since been confirmed that the apicoplast is the primary target of many antibiotics in *Plasmodium* spp. (see review [4]). Indeed, no antibiotic has been shown to specifically inhibit mitochondrial translation in apicomplexans.

infected new host cells, arresting only after invasion in this second IDC [7,8] (Figure 1, Key Figure). The apparently benign effects of these antibacterials in the first treatment cycle, even at concentrations many 1000-fold their observable IC_{50} in the second IDC, suggest that the delayed killing effect is not dependent on dose or exposure. Rather, parasites acquire a hidden defect that lethally manifests only upon establishment of a subsequent IDC, regardless of whether the antibacterial treatment is continued. Indeed, even if clindamycin is removed after only 2 h of treatment, *T. gondii* tachyzoites still potently arrest in the subsequent IDC [9]. A similar delayed death effect was observed for *P. falciparum*, whereby parasites treated with tetracyclines, clindamycin, or azithromycin suffer no initial growth defect and complete one round of intraerythrocytic replication [3,10–13]. The escaped progeny of these parasites invade a new host red blood cell (RBC) and continue to grow, undergoing multiple rounds of karyokinesis, but even when the drug was washed out in the first IDC parasites arrest prior to **schizogony** in this second IDC (Figure 1). Thus, it was widely observed that multiple classes of antibacterials exert a delayed death effect on different genera of apicomplexan species by an unknown mechanism.

A firmer link between the apicoplast and delayed-death-causing drugs came from experiments treating *T. gondii* tachyzoites with ciprofloxacin, a bacterial DNA replication inhibitor. Like many other antibacterials, ciprofloxacin causes delayed death in *T. gondii*. However, it was observed that ciprofloxacin treatment also causes a segregation defect in the apicoplast genome, such that dividing parasites lose apicoplast DNA within the first IDC [14]. A separate study in *P. falciparum* showed that ablation of apicoplast translation using an apicoplast-specific tryptophanyl tRNA synthetase inhibitor also led to delayed death [15]. These effects indicate that drugs targeting plastid housekeeping functions cause delayed death resulting from a hidden defect that blocks inheritance of a functional apicoplast.

Genetic Recapitulation of Delayed Death

Although we know that delayed-death-causing drugs disrupt the inheritance and function of the apicoplast, the molecular mechanisms underlying the resulting parasite death are not understood and appear to differ between apicomplexan genera. Genetic disruption of apicoplast housekeeping functions in *T. gondii* also results in delayed death and has been used to delineate these differences. Delayed death in *T. gondii* can be initiated by inducible knockdown of the class XXII Myosin F (TgMyoF) [16,17] or a dynamin-related protein (TgDrpA) [18], each of which

Glossary

Complex plastid: a plastid with three or more membranes that is derived from the secondary endosymbiosis of a eukaryotic cell that already contained an endosymbiont.
Delayed death: the phenomenon whereby chemical inhibitors of apicoplast housekeeping functions do not kill parasites in the same IDC as treatment but instead kill the progeny of the treated parasites in their subsequent IDC.
Endodyogeny: asexual replication by genesis of two daughter parasites inside the mother cell rather than fission of the mother cell itself.
Endosymbiosis: the evolutionary unification of two organisms whereby one organism (the endosymbiont) is engulfed and assimilated by the other (host) organism. The host organism appropriates the autonomy of the endosymbiont by reprogramming its division to give rise to a heritable symbiosis.
Intracellular developmental cycle (IDC): a complete asexual replicative cycle within a host cell producing many daughter cells. An IDC commences with the invasion of a host cell and terminates with the lytic release of daughter parasites.
Parasitophorous vacuole (PV): a membrane-enclosed compartment produced by some intracellular parasites, inside the host cell but surrounding the parasite. The PV is mainly derived from host cell plasma membrane inverted during invasion; it commences with the invasion of a host cell and terminates with the lytic release of daughter parasites.
Plastid: an autotrophic (often photosynthetic), multimembrane and genome-bearing organelle. Plastids are phylogenetically related, and all ultimately descend from the endosymbiosis of a cyanobacterium.
Prenylation: the post-translational modification of substrate proteins with a lipophilic prenyl group (farnesyl-pyrophosphate or geranylgeranyl-pyrophosphate) at C terminal cysteines.
Schizogony: asexual replication by multiple fission involving repeated nuclear division (mitosis

blocks apicoplast segregation during daughter cell budding in the first IDC. Independent genetic interrogation of delayed death also comes from inadvertent overexpression of a recombinant nuclear-encoded apicoplast-targeting acyl carrier protein fused to GFP and a rhoptry protein (ACP-GFP-mROP1), which serendipitously creates a 'poison protein' that disrupts apicoplast division and segregation [19]. In these parasites, *T. gondii* continues to divide normally by **endodyogeny** to produce many parasites within a single PV. However, the apicoplast fails to transmit between the budding daughter cells, generating progeny where only a lone offspring inherits the single, enlarged, parental apicoplast while all other progeny lack apicoplasts altogether (Figure 1). The parasites lacking an apicoplast do not appear to suffer any gross morphological defect, and initially continue to grow and further divide. However, while these apicoplast-lacking parasites egress normally, and can successfully invade a new host cell, they arrest shortly thereafter. This suggests either that the apicoplast is only essential for the establishment of a new host infection in *T. gondii*, for example, the formation of the PV in which the parasite divides, or that apicoplast-lacking parasites are initially protected from death by one parasite sharing the fruits of the labour from the sole intact plastid.

A partial solution to this conundrum, whereby tachyzoites lacking an apicoplast survive until establishing a new IDC, is provided by the recent observation that *T. gondii* parasites are able to share resources (potentially proteins and metabolites) following many rounds of cellular division [17]. This is enabled by a tubule that persists after division, which forms a connection between the basal complexes of each tachyzoite within one PV (Figure 1) [17]. The daughter cells are only completely disconnected just prior to egress, immediately preceding a new infection. If the cellular continuity between divided parasites is blocked by genetic knockout of actomyosins required for basal complex formation, a segregation defect in the apicoplast becomes immediately lethal to apicoplast-lacking parasites [17]. These data support the notion that products generated by at least one intact apicoplast are required for the continued division of *T. gondii*, as well as the infection of new host cells. This observation suggests that this product does not readily cross membranes, but instead requires a continuity between cytosols, indicating that the shared mystery apicoplast product is not a structural component of the parasite membrane or PVM, but rather a soluble, membrane-impermeant product available to the cytoplasm.

The scenario in *Plasmodium* is somewhat different. In the stages in which delayed death has been studied, cellular division to produce daughter cells occurs only once, and not until near IDC completion. At that stage, the mother parasite will asynchronously produce multiple nuclei and then bud to form 16–32 merozoites simultaneously in a process known as schizogony. As such, unlike *T. gondii*, inducible knockdown of nuclear-encoded genes involved in apicoplast housekeeping functions, such as *P. falciparum* autophagy-related protein 8 (PfATG8) [20], PfATG18 [21], or *P. falciparum* caseinolytic protease C [22], does not produce parasites lacking apicoplasts until subsequent IDCs. Likewise, all progeny of parasites treated with delayed-death-causing drugs likely survive the first IDC, despite the apicoplast taking on a hidden defect, as daughter cells are still continuous with the cytoplasm that surrounds the original apicoplast until the final moments preceding a new infection.

P. falciparum parasites treated with delayed-death-causing drugs divide their apicoplasts concurrently with merozoite segmentation in the same IDC as treatment, giving rise to daughter cells that each possess an apicoplast that is at least superficially intact [3,10,11] (Figure 1). However, such inherited apicoplasts are defective, with an inability to import nuclear-encoded proteins, branch, or further segregate to daughter cells in the IDC following treatment [3,10,11]. This suggests that apicoplast gene products are not immediately required for the gross maintenance of the existing organelle, but to prepare daughter apicoplasts for the next IDC, and that disrupting this process leads to loss of apicoplast function following segregation. Loss of import and processing of nuclear-encoded proteins to the defective apicoplast is presumably what ultimately dooms the parasite in the IDC following plastid inhibition, as without these enzymes trafficking correctly to the apicoplast, the essential anabolic capacities of the organelle will be crippled.

and karyocytosis) before cellular division (cytokinesis). In *Plasmodium* spp., the cytoplasm of the mother cell is partitioned by inner folds of the plasma membrane, and organelles are mobilised from within the cell to form many daughter cells simultaneously.
Tachyzoite: the asexual and fast-growing life cycle stage of *Toxoplasma gondii* and other coccidia, characterised by successive IDCs with lytic release from host cells.

Key Figure

Forms of Delayed Death in Apicomplexan Parasites

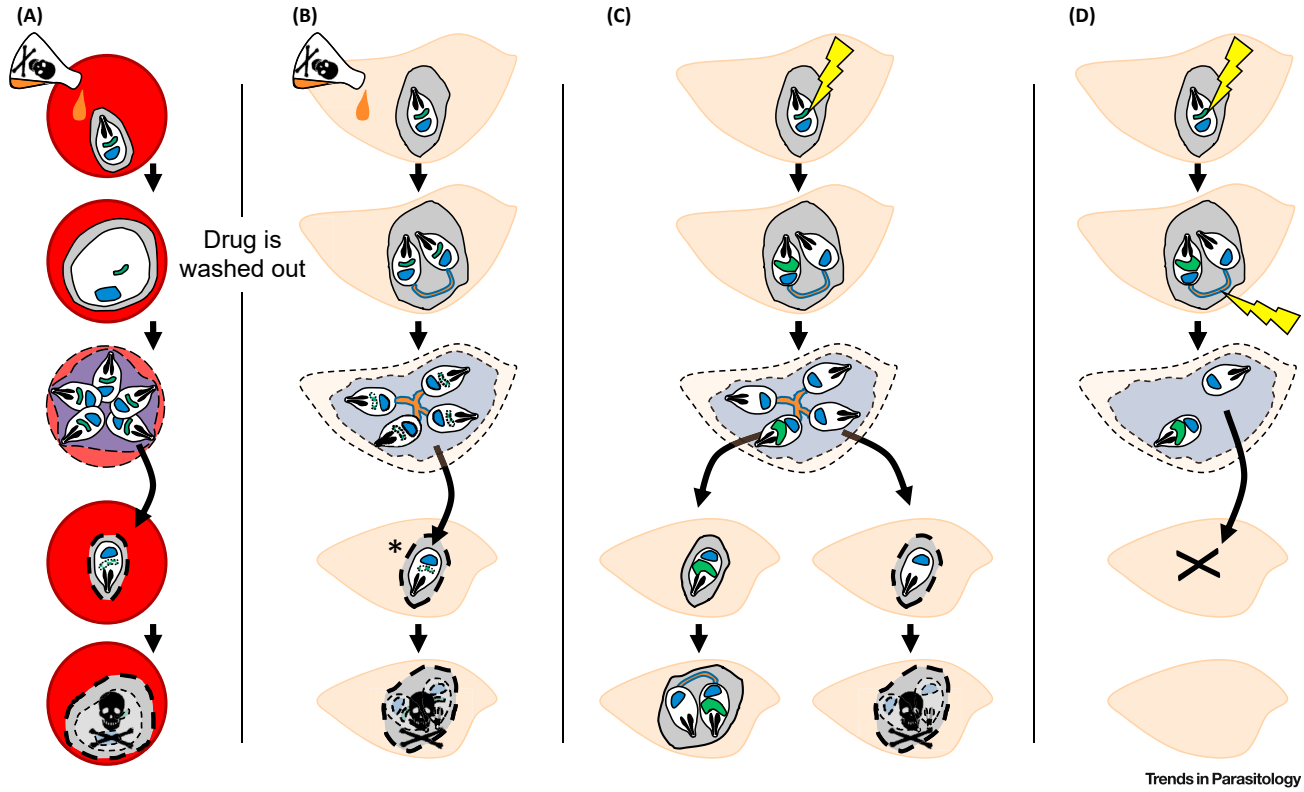

Figure 1. (A) Treatment of *Plasmodium* intraerythrocytic asexual parasites with an inhibitor of apicoplast housekeeping function has no immediate antiparasitic activity. The treated parasites divide and produce progeny with defective apicoplasts, which can reinvade new red blood cells (RBCs) but die before further egress [6]. (B) Treatment of *Toxoplasma gondii* tachyzoites with an inhibitor of apicoplast housekeeping functions does not immediately arrest parasite growth but allows tachyzoites to continue dividing. These daughter tachyzoites can egress and reinvade a new host cell, where they divide again but die before further egress [9]. (C) Genetic disruption of apicoplast segregation in *Toxoplasma* tachyzoites generates a single tachyzoite with a large nondividing apicoplast, and numerous apicoplast-lacking sisters. Each of these tachyzoites can continue to divide and egress. Apicoplast-containing progeny go on to form normal new infections, whereas apicoplast-lacking progeny can invade and divide, but die before further egress [19]. (D) *Toxoplasma* tachyzoites subject to disruption of apicoplast function that would otherwise result in delayed death are instead killed quickly when additionally subject to genetic disruption of tubular connections between divided tachyzoites. Presumably, sharing of apicoplast-derived metabolites through these continuities temporarily protects apicoplast-lacking parasites when apicoplast segregation is blocked [17].

An Essential Anabolic Organelle

If delayed death results from damage that renders apicoplasts unable to produce some essential metabolite, what exactly is that metabolite? The clue to answering this question comes partially from insights from chloroplasts grown in the dark. While apicomplexan plastids have lost their photosynthetic capacity and lack pigments or thylakoids, they have retained pathways that persist in plastids found in plant tissues hidden from sunlight, including biosynthetic pathways for isoprenoids, fatty acids, iron–sulfur clusters, and haem [23]. Unlike proteins for genome replication and protein translation, enzymes for these pathways are encoded in the nucleus and imported into the apicoplast, (with the exception of a single apicoplast-encoded component for iron–sulfur cluster biosynthesis [24]).

Not all apicomplexan apicoplasts house each of these enzymes, with isoprenoid biosynthesis appearing to be the only consistently retained pathway in all known apicoplasts [25–27]. Additionally, some pathways are only essential in selected parasite life cycle stages; fatty acid biosynthesis via the type II pathway and haem biosynthesis are indispensable for the growth of *T. gondii* tachyzoites [28,29] as well as mosquito and liver stages of *P. falciparum* [30–32], but are dispensable for *ex vivo* growth of blood-stage *P. falciparum* [31–33]. *Babesia* and *Theileria* parasites lack these enzymes needed for type II fatty acid and haem biosynthesis entirely [25,27]. Apicoplast-localised isoprenoid biosynthesis is thought to be essential for the progression through all life cycle stages of *P. falciparum* [33–35]. In many eukaryotes, isoprenoids are synthesised by the cytosolic mevalonate pathway to produce the universal isoprenoid precursor, isopentenyl pyrophosphate (IPP). Plants also possess the plastid-localised methylerythritol phosphate (MEP)/1-deoxy-D-xylulose 5-phosphate (DOXP) pathway to synthesise IPP [36]. Apicomplexans have lost the mevalonate pathway and depend on their apicoplast MEP/DOXP pathway for IPP biosynthesis [23,37,38].

Exogenous Addition of Isoprenoids Rescues Delayed Death

In a seminal paper, Yeh and DeRisi [33] demonstrated that, in *ex vivo* cultured *P. falciparum*, isoprenoid biosynthesis by the MEP/DOXP pathway is the sole essential function of the apicoplast during the blood stage. They showed that chemical inhibition resulting in loss of apicoplast inheritance and delayed death in *P. falciparum*, can be protected against by providing an abundant external source of the isoprenoid precursor IPP [33]. These IPP-rescued parasites continue to grow indefinitely without an apicoplast, demonstrating that other products of this organelle are dispensable in this life stage under *ex vivo* culture, or that parasites are capable of scavenging other needed metabolites from their surroundings. Vesicle-like structures containing apicoplast-targeted proteins persist in the cytoplasm of parasites that lack an apicoplast [33]. However, it is unlikely for metabolic pathways of the apicoplast to be reconstituted in these vesicles without apicoplast-mediated processing (cleavage and folding) of the apicoplast-targeted proteins. In *P. falciparum* then, the metabolic cause of delayed death is a loss of isoprenoid precursor biosynthesis.

T. gondii tachyzoites are also refractory to genetic disruption that blocks plastid isoprenoid biosynthesis, indicating the essentially of this pathway [39]. Nonetheless, *T. gondii* is thought to be able to scavenge some IPP from its host cell [40]. Indeed, chemical inhibition of host mevalonate isoprenoid biosynthesis works synergistically with plastid inhibitors and sensitised *T. gondii* tachyzoites such that they arrest in the first IDC, rather than via delayed death [41]. This suggests that apicoplast-lacking tachyzoites are able to survive provided they can share resources with at least one parasite with an intact apicoplast in the same PV, as well as scavenge IPP from their host cells. Unlike blood-stage *Plasmodium*, isoprenoid biosynthesis is not thought to be the only important product of the apicoplast in *T. gondii*. At a minimum, fatty acid biosynthesis is also required [28]. Unfortunately, IPP rescue as an investigatory tool is not experimentally possible for *T. gondii*, perhaps because sufficient concentrations of this polar metabolite cannot be achieved inside the PV. By comparison, IPP uptake by *P. falciparum*-infected RBCs is likely facilitated through parasite-dependent new permeation pathways present in the RBC plasma membrane [42].

Inhibitors of Apicoplast Metabolism

In contrast to most inhibitors of plastid housekeeping functions, genetic or chemical disruption of essential apicoplast metabolic pathways leads to immediate parasite arrest, without requiring apicoplast loss [43,44]. Triclosan, a well characterised chemical inhibitor of the type II fatty acid biosynthesis (FASII) enzyme enoyl ACP-reductase (a.k.a. FabI) [45], arrests *T. gondii* proliferation within a single IDC, leading to defects in addition of parasite-derived membranes [46]. Although loss of FASII by chemical or genetic disruption does cause an inheritance defect in the *T. gondii* apicoplast [28,46], suggesting a requirement for *de novo* synthesised fatty acid in apicoplast biogenesis, it also causes a defect in tachyzoite cytokinesis during endodyogeny [46], indicating that fatty

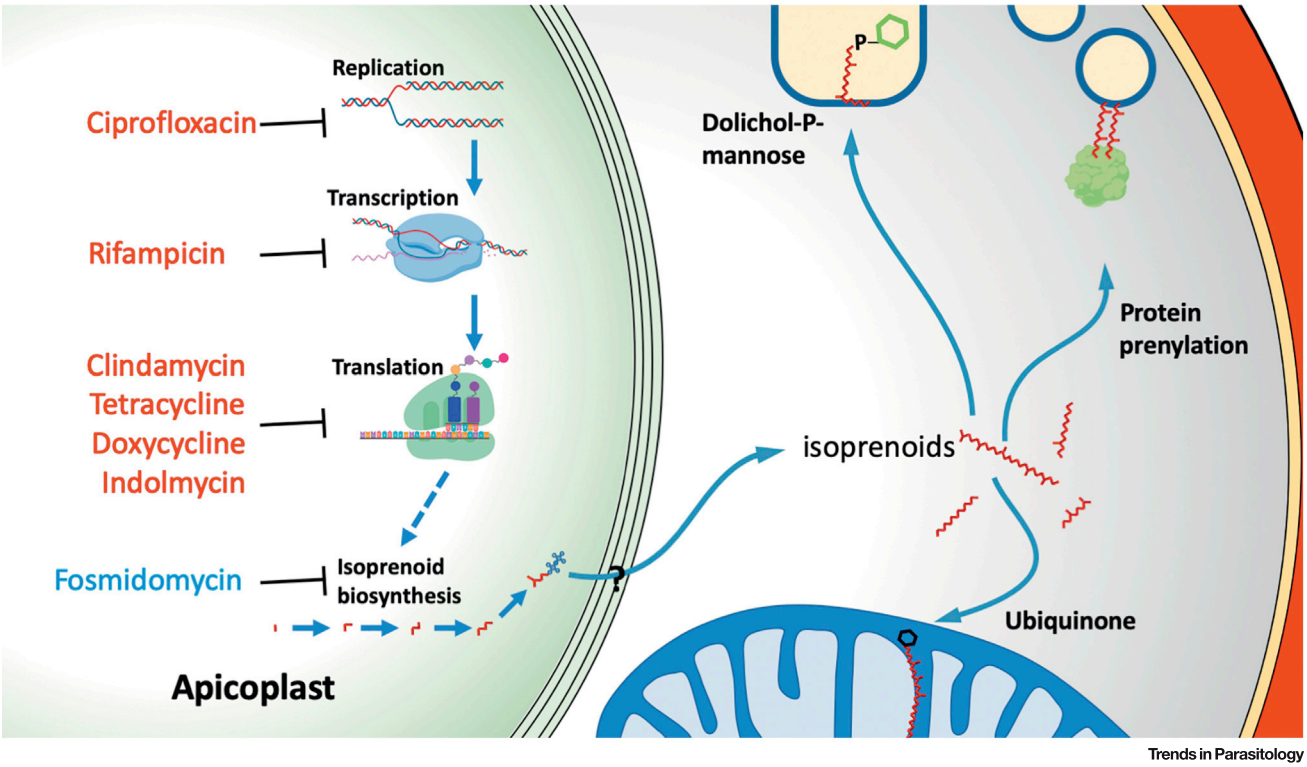

Figure 2. Inhibition of Apicoplast Housekeeping Functions Leads to Delayed Death.
Inhibitors of replication, transcription, or translation of the apicoplast genome lead to defects in apicoplast biogenesis and segregation. Treated parasites can temporarily continue some metabolic functions (particularly isoprenoid biosynthesis in the case of *Plasmodium* spp.) but divide to form progeny with an absent or defective apicoplast that is unable to support normal metabolism. By contrast, direct attenuation of *Plasmodium* isoprenoid biosynthesis (e.g., by fosmidomycin) leads to immediate growth inhibition, indicating that apicoplast-supplied isoprenoids must be necessary elsewhere in the *Plasmodium* cell. The three plausible isoprenoid endpoints are protein prenylation, dolichol for glycosylphosphatidylinositol (GPI) biosynthesis in the endoplasmic reticulum, and the ubiquinone side-chain in the mitochondrion.

acids provided by the apicoplast to the rest of the cell are needed for continued proliferation. Triclosan also inhibits *P. falciparum* growth; however, enoyl ACP-reductase is genetically dispensable in blood stages of *P. falciparum*, and these knockout parasites remain sensitive to triclosan, demonstrating that triclosan's inhibitory effect is off-target in this case [47].

Fosmidomycin is a promising inhibitor of isoprenoid biosynthesis that competitively inhibits deoxyxylulose 5-phosphate reductoisomerase (DXR), the enzyme that carries out the first dedicated step of the MEP/DOXP pathway [39], and the downstream enzyme methylerythritol phosphate cytidyltransferase (IspD) [48]. The blockade of isoprenoid precursor biosynthesis immediately inhibits the growth of *Plasmodium* spp. and *Babesia* spp., and is rescuable by providing an external source of isoprenoids [33,38,49,50]. The potent first-cycle growth inhibition by apicoplast metabolic inhibitors indicates that anabolic products of the apicoplast are required for the immediate and continued survival of parasites [39].

Isoprenoids: Fate and Function

Isoprenoid precursor biosynthesis is the most conserved function of the apicoplast throughout apicomplexans, and delayed death in *P. falciparum* is unequivocally a result of an isoprenoid depletion caused by loss of apicoplast inheritance (Figure 2). However, multiple downstream fates for IPP in cellular processes are possible in apicomplexan parasites, and these fates are still to be extensively characterised. Isoprenoids are a large family of branched hydrocarbon products that are assembled

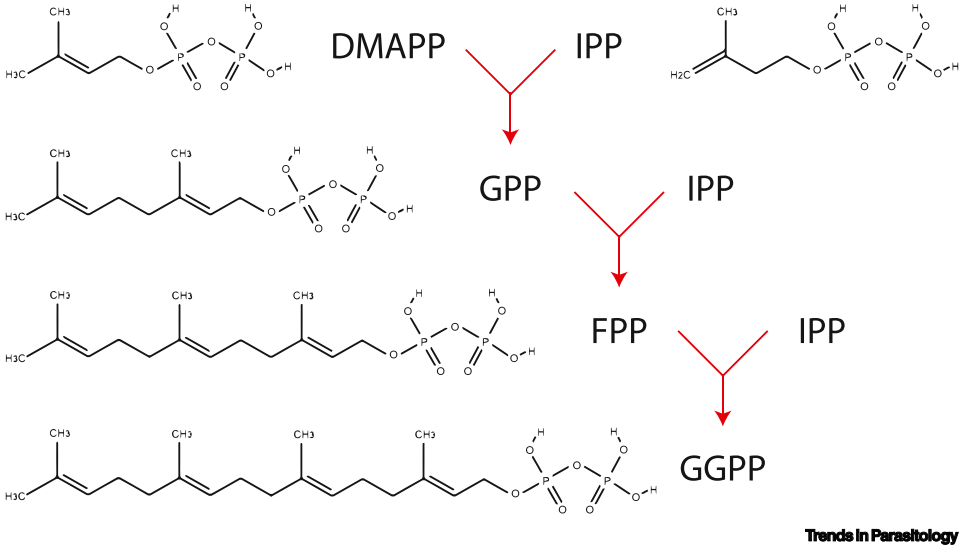

Figure 3. Isoprenoid Biosynthesis.
Isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) are the terminal products of the methylerythritol phosphate (MEP)/1-deoxy-D-xylulose 5-phosphate (DOXP) pathway in plastids. Geranyl pyrophosphate (GPP) is synthesised from IPP and DMAPP and is further lengthened to form the prenyl substrates farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) by successive addition of IPP by farnesyl/geranylgeranyl diphosphate synthase.

from repeating subunits of the 5-carbon apicoplast-derived precursor, IPP, and its isomer dimethylallyl diphosphate (DMAPP) [37] (Figure 3). The varying isoprene chain lengths are further modified or incorporated into proteins and lipids to generate a variety of isoprenoid products, such as carotenoids used for pigment in plants and bacteria [51], sterols abundant in membranes [52], lipophilic groups to modify protein [53], the isoprene sidechain of ubiquinone needed for electron transport [54], isopentenyl modification of tRNA in bacteria [55], and dolichols that mediate glycosylation and synthesis of glycosylphosphatidylinositol (GPI) [37]. For the purpose of this review, we will discuss isoprenoids known to be used during the blood stages of *P. falciparum*, that is, lipophilic prenyl groups, dolichols, and the isoprene side-chain of ubiquinone, which therefore likely contribute to delayed death (Figure 2). For a comprehensive review of all isoprenoids in *P. falciparum* see [37].

Prenyl Groups

Prenylation is a form of post-translational protein modification that attaches a lipophilic isoprenoid, called a prenyl group, to C terminal cysteines of protein substrates with an appropriate amino acid motif (see review [53]). Prenyl groups are synthesised by polymerisation of IPP into the short isoprenoid chains farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) (Figure 3), and then covalently attached to their protein targets by prenyl-transferase enzymes [56].

Prenylated proteins have roles in cellular signalling and vesicular trafficking, as their farnesyl or geranylgernanyl moieties facilitate their association and anchoring to lipid membranes. Recent analyses identified 15–20 prenylated proteins in *P. falciparum*, predominantly Rab GTPases, with roles in coordinating vesicular budding, motility, and fusion [57,58]. Among other functions, prenylated Rab homologues in *T. gondii* are important for secretion and biogenesis of invasion organelles, which suggests some repurposing of Rabs in Apicomplexa compared with model eukaryotes [59]. Prenylated Rab GTPases in *P. falciparum* are suggested to have roles in coordinating haemoglobin transport [60], DV biogenesis [61], inner-membrane complex assembly [62], and

endoplasmic reticulum (ER)-Golgi sorting [63]. The Ykt6 SNARE protein, a fusogen in *P. falciparum* that mediates vesicle fusion, has been shown to be farnesylated and dependent on its CaaX motif for prenylation and membrane targeting [64]. Congruent with a role in delayed death, inhibition of isoprenoid biosynthesis with fosmidomycin decreases global *P. falciparum* prenylation and this coincides with the mislocalisation of at least two prenylated proteins: PfRab5a [61] and PfFCP [58], demonstrating that their prenyl modification is a determinant of localisation and function. Depletion of isoprenoids, and hence prenyl groups, could contribute to delayed death by disrupting the function of prenylated proteins that regulate vesicle trafficking. Indeed, we have recently shown that protein prenylation is reduced in delayed death parasites and that this corresponds to the mislocalisation of at least one normally vesicle-bound prenylated protein (PfRab5a), abnormal digestive vacuole biogenesis, and disrupted haemoglobin uptake [65]. Parasites could be protected from delayed death effects by supplementation with geranylgeraniol (GGOH), an analogue of the prenyl substrate GGPP, which allows for the prenylation of proteins [65]. However, in contrast to the ongoing rescue from delayed death provided by IPP supplementation [33], GGOH only rescues parasites during the second intraerythrocytic cycle after treatment with an inhibitor of apicoplast maintenance functions [65]. The temporary protection from delayed death by GGOH supplementation suggests that disruption to prenylation-mediated vesicle trafficking is the primary cause of delayed death, but that other isoprenoid pathways must also be essential for the continued survival of parasites.

Isoprene Side-Chain of Ubiquinone

Ubiquinone (also known as Co-enzyme Q) is an essential electron carrier required for electron transport in mitochondria. Ubiquinone uses a lipophilic isoprenoid side-chain to embed in the inner mitochondrial membrane, where it accepts electrons and passes them into the electron transport chain. In both *P. falciparum and T. gondii*, ubiquinone is required for the oxidation of dihydroorotate dehydrogenase (DHODH) [66,67], which is itself required for pyrimidine biosynthesis, an essential pathway in *Plasmodium* spp. and *T. gondii* [68]. It is therefore possible that blocking isoprenoid biosynthesis through apicoplast inhibition contributes to delayed death by disrupting the production of ubiquinone. Indeed, nerolidol, a sesquiterpene alcohol, inhibits the synthesis of the ubiquinone's isoprenoid side-chain and also has antiparasitic activity in the same IDC [69–71], although it is unclear if nerolidol has other targets in the parasite.

It is possible to uncouple electron transport from DHODH oxidation in *P. falciparum* or *T. gondii* by using a transgenic yeast DHODH protein that uses cytosolic fumarate rather than ubiquinone as an electron acceptor [66,67]. In *P. falciparum*, these yDHODH transgenic parasites remain sensitive to direct inhibition of isoprenoid biosynthesis by fosmidomycin treatment [33] and isoprenoid depletion by delayed death [65], indicating that there are other essential roles for isoprenoids such as the requirement for prenyl groups used for protein prenylation (see above).

Dolichols

Dolichols are large isoprenoid chains consisting of many isoprene units that principally serve as sugar donors to facilitate protein glycosylation, GPI-anchor biosynthesis, and lipidation of proteins (see review [37]). Dolichols of 11 and 12 isoprene chain lengths, derived from FPP and GGPP, have been identified in *P. falciparum* [72]. GPI-anchors are the most abundant type of glycosylation present in *P. falciparum*, forming the major glycan constituent on the surface of merozoites [73]. Mannosylation of the nascent GPI occurs using dolichol-phosphate-mannose (Dol-P-Man) as a sugar donor of mannose residues. *P. falciparum* synthesises many essential GPI-anchored proteins, which are predominantly found on the surface of merozoites and have roles in host attachment and invasion [74,75]. Inhibition of GPI anchor biosynthesis using sugar analogues inhibits *P. falciparum* growth [76]. However, the rescue of delayed death parasites by GGOH indicates that dolichol depletion is not itself a cause of delayed death [65]. Nonetheless, GGOH-rescued and ubiquinone-uncoupled parasites do eventually die after apicoplast loss, suggesting that dolichols (required for GPI biosynthesis) are among the essential fates of apicoplast-synthesised isoprenoids [65].

The Exceptions That Prove the Rule

Several genetic and chemical modulators of apicoplast function give rise to immediate growth inhibition rather than delayed death. These generally fall into three categories: direct disruptors of apicoplast biogenesis; inhibitors with additional targets; and inhibitors of apicoplast metabolism such as fosmidomycin, as mentioned above. In the former category, genetic disruption and chemical inhibition of apicoplast trafficking or division, in some cases, results in death in the first IDC. Actinonin is a canonical inhibitor of the peptide deformylase required for prokaryotic/plastid post-translational processing of the initiator formyl-methionine, but in *P. falciparum* and *T. gondii* it appears to preferentially inhibit the metalloprotease, FtsH [77]. Apicomplexan FtsH, as in some other eukaryotes, is involved in organellar division [77,78], and FtsH inhibition or disruption leads to apicoplast defects, and is rescuable by IPP [44,77], consistent with an essential role in the apicoplast. However, FtsH inhibition has antiparasitic activity against *P. falciparum* in the first rather than second IDC [44,77], suggesting that failure to divide an apicoplast can also lead to rapid death. Strangely, comparable inhibition in *T. gondii* results in a typical delayed death effect [77]. Another rapid phenotype is observed in parasites with induced blockages in apicoplast trafficking. Boucher and colleagues [79] used an inducible-folding cargo to block trafficking of an apicoplast-targeted protein, which appeared to gum up the apicoplast protein import apparatus and lead to rapid death of *P. falciparum* parasites. In neither of these cases is it clear why delayed death was circumvented, but the rapidity of death might result from a failure to proceed past an unknown cell-cycle checkpoint, or from catastrophic trafficking defects that impact other trafficking pathways (though why this is not seen in parasites that completely lose their apicoplast is a mystery). Interestingly, genetic defects in the components of the autophagy-related ATG system, which is thought to direct apicoplast membrane trafficking as opposed to protein trafficking, lead to delayed death in *P. falciparum* and *T. gondii* [20,21,80].

A number of compounds also have slight or undependable induction of delayed death due to inhibition of multiple targets inside and outside the apicoplast. One such drug is rifampicin, an inhibitor of rpoB, the β-subunit of the apicoplast RNA polymerase [81,82]. Though there is good evidence that rifampicin targets the apicoplast [81], it does not display the same characteristic delayed-death phenotype as typical apicoplast translation inhibitors. Although most reports describe immediate growth inhibition in *P. falciparum* [3,10,44,83], instances of delayed death have been noted, albeit exhibiting a narrow window between the first and second IDC IC_{50}s [12,84]. Fusidic acid is another inhibitor with similar properties. Though it blocks the apicoplast translation elongation factor G (EF-G), it arrests parasite growth in the first IDC [85,86]. Both rifampicin- and fusidic acid-treated *P. falciparum* parasites are not rescuable by IPP [44], suggesting an additional target outside the apicoplast, which, in the case of fusidic acid, is likely to be the mitochondrial EF-G [44,86].

Other delayed death drugs that have a narrow window between reported first and second IDC IC_{50}s include doxycycline, an apicoplast translation inhibitor [3,11], and ciprofloxacin, an inhibitor of plastid DNA gyrase [3,87]. Although there are conflicting reports as to whether ciprofloxacin causes immediate parasite death in *P. falciparum* [10,88], ciprofloxacin-treated *T. gondii* parasites exhibit a clear delayed death phenotype [14]. A possible explanation for the two-pronged lethal effects of doxycycline and ciprofloxacin may be that these drugs have an additional target outside the apicoplast. However, unlike fusidic acid, parasites treated with doxycycline or ciprofloxacin can be rescued by IPP, demonstrating that the apicoplast is their primary target in *P. falciparum* [33,44].

In some cases, understanding the basis for circumventing delayed death is likely to be trivial (e.g., an unknown off-target activity), but in others may illuminate the basis of first versus second IDC killing. The absence of delayed death in cases of inhibition of FtsH [77] (which may act in processing of imported proteins) or in direct inhibition of protein import [79] suggests that parasites die when apicoplast protein import fails. A number of other studies have also interrogated components of apicoplast import [89,90], but the inducible knockdown systems in these cases did not allow an

Outstanding Questions

Why does specific inhibition of apicoplast import or biogenesis not cause delayed death, in contrast to translation inhibitors that also cause biogenesis defects?

What is the basis of differences between *Toxoplasma* and *Plasmodium* in genetically- and chemically-induced delayed death?

Do other parasite stages have delayed death, and is delayed death found in other apicomplexans (particularly *Babesia*, *Theileria*, *Eimeria*)?

unequivocal dissection of rapid versus delayed death, and such import phenotypes deserve further investigation.

Concluding Remarks

Although much remains unknown about delayed death in apicomplexan parasites (see Outstanding Questions), the past decade has seen significant advances furthering our understanding of this puzzling phenomenon. In general, we now know that compounds that interfere specifically with the expression of apicoplast genes do not lead to rapid death but generate superficially healthy progeny with a defective apicoplast. This defect may include loss of the apicoplast genome and/or loss of apicoplast translation apparatus. Some apicoplast-encoded gene products, such as the apicoplast-encoded Clp subunit (note this gene, PF3D7_API03600, has been variously referred to as PfClpC and PfClpM, and is distinct from the aforementioned nuclear-encoded PfClpC, PF3D7_1406600), are presumably necessary for protein import [91], and in their absence, nuclear-encoded enzymes necessary for apicoplast metabolism fail to import. This leads to a collapse in apicoplast metabolism in the second IDC and parasite death. In *Plasmodium* intraerythrocytic stages, collapse of isoprenoid biosynthesis may be sufficient to cause delayed death, but in other parasites other metabolites are also involved. The resultant phenotypes are likely to vary according to which important metabolites are exhausted, and in which order, and this may explain some of the delayed death differences observed between parasites.

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