Targeting the apicoplast in malaria

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Abbreviations

aaRS	aminoacyl-tRNA synthetase
ABCF1	ATP-binding cassette protein F1
ACT	Artemisinin-based combination therapy
ATG	Autophagy-related protein
ATrxs	Apicoplast thioredoxins
Clp	Caseinolytic protease
DMT2	Divalent metal transporter 2
EF-G	Elongator factor G
EF-Tu	Elongator factor thermo unstable
FASII	Fatty acid synthesis type II
GGPP	Geranylgeranyl pyrophosphate
IPP	Isopentenyl pyrophosphate
ISC	Iron-Sulfur cluster biosynthesis
MMV	Medicines for Malaria Venture

Abstract

Malaria continues to be one of the leading causes of human mortality in the world, and the therapies available are insufficient for eradication. Malaria is caused by the apicomplexan parasite *Plasmodium.* Apicomplexan parasites, including the *Plasmodium* spp., are descendants of photosynthetic algae, and therefore they possess an essential plastid organelle, named the apicoplast. Since humans and animals have no plastids, the apicoplast is an attractive target for drug development. Indeed, after its discovery, the apicoplast was found to host the target pathways of some known antimalarial drugs, which motivated efforts for further research into its biological functions and biogenesis. Initially, many apicoplast inhibitions were found to result in "delayed death", whereby parasite killing is seen only at the end of one invasion-egress cycle. This slow action is not in line with the current standard for antimalarials, which seeded scepticism about the potential of compounds targeting apicoplast functions as good candidates for drug development. However, recent evidence that highlights apicoplast inhibitors that result in the rapid killing, put this organelle back in the spotlight. We provide an overview of drugs known to inhibit apicoplast pathways, alongside recent findings in apicoplast biology that may provide new avenues for drug development.

Introduction

The Apicomplexa, a phylum of protozoan parasites, places a huge burden on global health and economy. Among these, the human malaria parasite *Plasmodium falciparum* is the most impactful. WHO reported that during 2016 there were 216 million malaria cases and 445,000 deaths globally[1]. Despite intense efforts over the past two centuries to eradicate malaria, it remains a severe threat to human life. Drug resistance poses one of the main limitations to the fight against malaria. For example, for decades chloroquine was the "gold standard" for the treatment of uncomplicated malaria, nearly halving the death rates among children in Africa[2]. However, drug resistance sabotaged this success, as shown by the strong link between the emergence of drug resistance and the resurgence of morbidity and mortality[3]. Nowadays, chloroquine is no longer appropriate for the treatment of malaria caused by *P. falciparum* in nearly all geographic areas. Artemisinin-based combination therapies (ACT) are currently the frontline antimalarials. Artemisinin is unique in being the only drug that can rapidly kill every asexual red blood cell stage[4]. However, an increasingly pronounced trend of delayed clearance time[5] and reports of treatment failure (e.g. [6] and [7]) raise concerns for the future efficacy of ACT[8]. The introduction of new drugs is a necessity, and criteria for their activity and specificity based on this history are outlined by leading programs such as the Medicines for Malaria Venture (MMV)[9]. These include prioritisation of compounds that result in the fast killing of the parasites.

Most apicomplexan parasites, including *P. falciparum*, possess a plastid organelle called the apicoplast, which is a demonstrated source for drug targets[10–13]. The apicoplast originated from a secondary endosymbiosis of a red algal cell, and thus it features some of the essential metabolic pathways that drove that symbiosis[14,15], as

well as a complex multi-compartment structure reflecting its multiple origins[16–18]. The metabolic roles of the apicoplast in *P. falciparum* include the biosynthesis of isoprenoid precursors (IPP), iron-sulfur clusters (ISC), fatty acids (FASII) and haem intermediates[15,19,20]. Genetics studies have found that these pathways are essential for different *Plasmodium* life stages. Specifically, IPP and ISC production are required during the asexual stages[21,22], while the genes encoding the FASII enzyme fabB and the haem biosynthesis enzyme ferrochelatase, are only essential during the parasite liver[23–25] and mosquito[24,25] development stages. The importance of these functions is further supported by their drug sensitivity (see Fig. 1 for a summary of drugs targeting apicoplast functions active during *P. falciparum* asexual development). As an example, IPP biosynthesis is inhibited by fosmidomycin, which demonstrated effective *in vivo* antimalarial activity when conjugated with clindamycin[26,27]. Additionally, ISC synthesis is affected by D-cycloserine[28], while FASII is inhibited by thiolactomycin, cerulenin, triclosan, and fops[29].

Due to these essential synthesis pathways being housed there, apicoplast maintenance and biogenesis are also essential for parasite survival. Studies describing the importance of proteins involved in ISC synthesis[22] and of apicoplast proteases[30,31] in *Plasmodium* provide examples for this principle. Similarly, studies in the related parasites *Toxoplasma gondii*, whose apicoplast shares the same metabolic pathways, highlight further examples[32–40]. Additionally, due to its complex evolution, different apicoplast biogenesis pathways are of different evolutionary origins. Thus, in addition to being essential, many housekeeping processes have their origin in the prokaryotic ancestry of the algal plastid or were uniquely evolved with the apicoplast acquisition, hence they have no parallels in humans. These essential and divergent apicoplast biogenesis

pathways are already proven drug targets or have the potential to be exploited for drug development. We review here the advances made to understand this potential.

Housekeeping pathways hosted in the apicoplast stroma

Many housekeeping pathways hosted in the apicoplast stroma retain a prokaryotic origin from the algal plastid. Therefore, apicoplast biogenesis is susceptible to the action of a series of antibiotics. Fluoroquinolones and aminocoumarins typically interfere with prokaryotic gyrase activity during DNA replication and have proven antimalarial activity[41,42]. Rifampicin is a selective inhibitor of RNA polymerase and was also demonstrated to have antimalarial effects both *in vitro* and in rodent models[43]. The antiparasitic kinetics of these drugs have been termed "delayed death"[44] because parasites start dying only after the first round of egress and re-invasion following the introduction of the drug.

Apicoplast translation provides a rich source for drug and antibiotic targets

Like many Apicomplexa, *Plasmodium* spp. possess three translationally active compartments. The cytosol operates eukaryotic translation machinery while the apicoplast and the mitochondrion have translation systems of prokaryotic origin. Each system consists of a ribosome, tRNAs, aminoacyl tRNA synthetases (aaRS), initiation factors and elongation factors. The apicoplast genome encodes the 23S and 16S rRNA, 35 tRNAs, 17 ribosomal proteins and the thermo-unstable translation elongation factor (EF-Tu)[45]. Other components are encoded in the nuclear genome and imported to the organelle post-translationally. 15 of the 36 nuclear-encoded aaRSs are exclusively apicoplast-targeted and four are shared with the cytosol[46,47]. Additionally, the apicoplast GluRS binds glutamate to both $tRNA^{Glu}$ and $tRNA^{Gln}$, with the latter being processed by an apicoplast amidotransferase to generate $Gln\text{-}tRNA^{Gln}$[48]. Together, 21

aminoacylated tRNAs can form in the apicoplast. The apicoplast prokaryotic-derived aaRSs are attractive drug targets due to their structural divergence and weak cross-recognition with eukaryotic aaRSs. Common aaRS inhibitors are the amino acid analogues, which compete with an amino acid in binding to the aaRS catalytic site, blocking prokaryotic translation. Examples include the isoleucine analogues, cispentacin and icofungipen; the phenylalanine analogue, ochratoxin A; and the tryptophan analogues, chaungxinmycin and indolmycin[49] (see Table 1). The inhibitory power and specificity of indolmycin for the apicoplast TrpRS was recently demonstrated[50]. Similarly, the isoleucine analogue mupirocin has antiplasmodial activity due to its inhibition of IleRS. Similarly to indolmycin, mupirocin activity causes delayed death, which suggests apicoplast inhibition[51]. Further confirmation to mupirocin specificity is provided by resistant parasites carrying mutations in the apicoplast IleRS gene[51]. Other inhibitors with antiparasitic activity include the proline analogue halofuginone[52] and the lysyl-tRNA synthetase inhibitors cladosporin[53], borrelidin and febrifugine[54]. However, the uncommon immediate, rather than delayed, effect of these compounds may infer the presence of non-apicoplast targets (see Table 1 for a list of apicoplast-specific aaRS inhibitors).

Apicoplast ribosome

Many of the antibiotics active against *Plasmodium* inhibit the apicoplast ribosome (See the enlarged section in Fig. 1 and Table 1). While their mechanisms of action in bacteria are known, little mechanistic insight is currently available regarding the parasites. Pactamycin is a translation initiation inhibitor in bacteria that has *in vitro* antiplasmodial activity comparable to artemisinin[55]. However, pactamycin is not likely to be apicoplast-specific since it has a high affinity to *P. falciparum* cytosolic ribosome[56]. Tetracycline, macrolides, and lincosamides represent three classes of

antibiotics that are approved for the treatment of several apicomplexan diseases[57] and that inhibit translation elongation. Tetracyclines bind to multiple sites of the 30S subunit of the prokaryotic ribosome and inhibit the delivery of aa-tRNAs to the ribosomal A-site. The insurgence of drug resistance promoted the development of second (doxycycline) and third (glycylcyclines) generations of compounds[58]. Doxycycline is an antibiotic commonly used for antimalarial prophylaxis and has a direct effect on the apicoplast[59]. The tetracycline tigecycline (a glycylcycline) is also an antiplasmodial. Tigecycline is more effective than its tetracycline predecessors and a strong candidate for combination with chloroquine[60]. The macrolide azithromycin and the lincosamide derivative clindamycin were the first antibiotics functionally characterised to have an antiplasmodial effect by targeting the apicoplast ribosome. Studies performed in both *Toxoplasma*[61,62] and *Plasmodium*[63] showed that resistant parasites have ribosome mutations mimicking those occurring in resistant bacteria. The power of macrolides and associated antibiotics for malaria treatment was recently reviewed in detail[64]. Chloramphenicol is a broad spectrum antibiotic affecting both Gram-positive and Gram-negative bacteria, docking to the A-site of the ribosome and stabilising the binding of tRNA to the P-site[65]. Chloramphenicol selectively targets *P. falciparum* apicoplast *in vitro*[66]. Among the few known antibiotics that affect termination and ribosome recycling in bacteria, fusidic acid was reported to have an *in vitro* inhibitory effect on *P. falciparum* and other Apicomplexa[67–69]. In bacteria, fusidic acid stabilises the bond between elongation factor-G (EF-G) and the ribosome, preventing ribosome recycling. Fusidic acid activity on *P. falciparum* was proposed to be specific for the apicoplast EF-G, because the mitochondrial EF-G contains a GVG motif that provids resistance to this drug[67]. Surprisingly, fusidic acid has an immediate death effect on *P. falciparum*[70,71], providing evidence for the existence of an off-

apicoplast target. IPP supplementation studies (explant in the next section) provided further support to this hypothesis in *P. falciparum*[71]. Whether fusidic acid causes parasite death by targeting the mitochondrial EF-G, by synergically affecting both apicoplast and mitochondrial EF-G or by blocking the apicoplast EG-F and a yet unknown third target, remains to be discovered. The antibiotic thiostrepton also hampers EF-G activity by preventing the docking of this and other factors to the bacterial ribosome[65]. Thiostrepton is active against *P. falciparum* but may primarily target the proteasome with residual activity on apicoplast and mitochondrial translation[72,73].

Table 1 Apicoplast-specific inhibitors of aaRS and ribosome components with details on the parasite death effect

aaRS inhibitors				
Target	**PDB GeneID**	**Drug**	**Effect**	**Reference**
isoleucine--tRNA ligase, putative	PF3D7_1225100	Mupirocin	Delayed death	46,67
lysine--tRNA ligase, putative	PF3D7_1416800	M-33; M-37	Delayed death	68
tryptophan--tRNA ligase	PF3D7_1251700	Indolmycin	Delayed death	45
leucine--tRNA ligase, putative	PF3D7_0828200	AN2729	Delayed death	69
tyrosine--tRNA ligase	PF3D7_1117500	TCMDC-141232	Unspecified	70
glutamate--tRNA ligase	PF3D7_1357200	Glu-SA	Enzyme inhibition	43
Apicoplast ribosome inhibitors				
Target	**PDB GeneID**	**Drug**	**Effect**	**Reference**
Small subunit ribosomal RNA	PF3D7_API05700	Tetracycline	Delayed death	Tetracycline 71, Doxycycline 54,72 Tigecycline 55,73
Large subunit ribosomal RNA	PF3D7_API04900	Lincosamides	Delayed death	Clindamycin 37,57
		Macrolides	Delayed death	Azithromycin 37,58 Erythromycin 74 Clarithromycin 75
		Chloramphenicol	Delayed death	61
Elongation factor G	PF3D7_0602400	Fusidic Acid	Rapid death*	62
Elongation factor Tu	PF3D7_API02900	Kirromycin, GE2270A, Amythiamicin A Enacyloxin IIa	Rapid death	62,76

*IPP supplemented *P. falciparum* proved to be still susceptible to fusidic acid activity[71]. This provides evidence for the existence of a second target for this compound, which may putatively be the mitochondrial EF-G[70].

Novel apicoplast targets identified and drugs confirmed via IPP supplementation

Yeh and DeRisi demonstrated that IPP biosynthesis is the only required apicoplast metabolic pathway for culturing *P. falciparum* asexual stages[21]. In this study, the authors succeeded to chemically ablate the apicoplast in *P. falciparum* cultures supplemented with IPP, obtaining viable IPP-dependant offspring with no plastid[21] (referred to from here onwards as API-minus). This procedure became a powerful tool to study apicoplast biogenesis and to characterise organelle-specific drug targets and compounds (summarised in Fig. 1). In one example, this method allowed the characterisation of the autophagy-related proteins (ATG) as regulators of apicoplast vesicle transport[74] and apicoplast segregation[75]. The proteins ATG8 and ATG18 proved to be involved in apicoplast biogenesis in both *P. falciparum* and *T. gondii*[34,76,77], while a similar function for ATG4 was described in *T. gondii*[78]. These studies clarified the role of autophagy enzymes in light of the unclear nature of autophagy in Apicomplexa and highlighted this pathway as a possible target for novel antimalarials.

IPP supplementation also helped to identify the apicoplast segregation role played by the caseinolytic proteases ClpC and ClpP[30]. Moreover, the analysis of *P. falciparum* dominant negative mutants for the sufC gene revealed apicoplast loss when parasites were rescued with IPP[22]. The observed apicoplast loss suggests that ISC synthesis plays a role in organelle biogenesis. This observation calls for a re-evaluation of the previously suggested exclusive role of ISC synthesis in sustaining the IPP biosynthesis pathway itself.

Recently, several uncharacterised proteins were assigned apicoplast localization via proximity-tagging proteomics in *P. falciparum* and through genetic screens in *P. berghei*[79,80]. Among these, the ATP-binding cassette protein ABCF1 and the membrane transporter DMT2 were described as essential components of *P. falciparum*

apicoplast, as confirmed by conditional knockdown and IPP rescue of the mutants[79,80]. These studies provided new important entries to the limited list of currently known apicoplast proteins and especially apicoplast transporters.

IPP rescue also provided an efficient tool to analyse apicoplast functions in response to drug exposure. An interesting example is provided by the study of artemisinin-induced dormancy in *P. falciparum*[81]. Recrudescence from this dormant stage to complete recovery occurs only in parasites with a functional apicoplast. Parasites exposed to fosmidomycin followed by induction of dormancy can also be rescued but only upon supplementation with either IPP or its derivative geranylgeranyl pyrophosphate (GGPP). While GGPP can be used to temporarily maintain an API-minus culture, the recrudescence of dormant API-minus parasites is only achievable by IPP supplementation[81]. These observations, indicate the possible involvement of IPP in the recovery mechanism, possibly through influencing mitochondrion activity[81,82].

In another study, an IPP rescue-based drug screen confirmed that the compound MMV008138 shows similar kinetics of parasite killing to fosmidomycin and that the effect is reversible by IPP rescue[83,84]. Further studies identified the enzyme IspD as the target of MMV008138[85], encouraging the development of new analogues for both this compound[86] and for the benzoisothiazolones[87]. Although both fosmidomycin and MMV008138 do not seem to have an effect on gametocytes, parasites lacking an apicoplast are unable to reach gametocyte maturity unless IPP was supplemented prior to stage III-IV[88]. This may suggest that while a functional apicoplast is required for gametocyte survival, these drugs show different pharmacokinetics at this stage. This limitation was overcome by the development of the MEPicide RCB-185, which affects both gametocyte and asexual *P. falciparum*, as confirmed by IPP rescue[89]. Another IPP based drug-screen identified the membrane metalloprotease, FtsH1, as the target of

the apicoplast biogenesis inhibitor actinonin. Interestingly, as opposed to most other apicoplast targeting drugs, actinonin has a rapid-death effect on the parasites[31]. A recent screen, testing the apicoplast-specific effect of multiple antibiotics on API-minus parasites, provided more support for the immediate death effect of actinonin on *P. falciparum* when compared with the effect of other antibiotics[71]. Both studies raise the possibility that the inhibition of specific apicoplast proteins could generate apicoplast-defect-dependent rapid parasite death. This hypothesis brings the apicoplast back to the focus of antimalarial development as a powerful source for targets causing immediate parasite death.

Sabotaging redox regulation in the apicoplast as a new strategy for drug development

Redox is the name given to the sum of reducing and oxidizing powers in a compartment. The carefully maintained balance between oxidising and reducing agents provides a suitable environment for all cellular functions and is essential for the survival of all cells. *Plasmodium* parasites are constantly exposed to signals and molecules causing changes to their cellular redox balance. For example, during the intraerythrocytic cycle, the parasites experience oxidative stresses generated exogenously by the host immune system, and endogenously by the activity of their own mitochondrial metabolism and from haemoglobin degradation[90,91]. The ability to maintain redox balance is thus a potential 'Achilles heel' for *Plasmodium* and other Apicomplexa. In support of this, tipping the parasite redox balance by elevation of exogenous oxidative stress *in vitro* results in parasite death for both asexual stages[92] and sexual gametocyte stages[93]. Moreover, recent evidence suggests that elevation of redox stress *in vivo* is also detrimental to the parasites[94]. In this study, which was aimed to evaluate the effect of animal diet on susceptibility to malaria, elevated oxidation in the liver was identified

as a cause of impaired liver infection[94]. Sabotaging the parasites' ability to maintain redox balance is, therefore, emerging as a killing mechanism that could be exploited for drug development[32].

In eukaryotic cells, each compartment has its unique redox state, suitable for the pathways it hosts. Compartmental redox regulatory networks have two arms: (1) pathways responsible to maintain a redox state that is suited to the biochemical conditions required in the organelle, e.g. via the action of antioxidants; (2) pathways that modulate the function of proteins in response to changes in the redox state in a specific compartment. For example, thioredoxins mediate protein folding, which in turn affects both their trafficking through compartments and translocation complexes, as well as their catalytic function. The thioredoxin potential to induce folding is directly controlled by the redox environment in its corresponding compartment. Due to its multi-compartmental structure and complex evolutionary origin, the apicoplast is expected to rely on an elaborate network of redox regulatory pathways[95,96]. This prediction is based on the different redox conditions found in the origins of the different apicoplast compartments (e.g. the outermost compartment is likely to be highly reducing, like the ER; while the PPC is likely less reducing, like the cytosol). This prediction is further based on the expectation that some apicoplast proteins must be kept in a state of folding that is suitable for traversing several compartments, and that all proteins should fold to their catalytic form in their resident compartment. While the information about these pathways is sparse, evidence points to apicoplast redox regulation as a promising target for drug development. For example, our recent work characterized the function of *Toxoplasma* apicoplast thioredoxins (ATrxs) and demonstrated their role in apicoplast protein folding and sorting[32]. This study showed that the deletion of a single redox regulation component is sufficient to cause parasite

death. It further identified ATrx2 as a parasite- and algal-specific protein and generated an *in vitro* activity assay that can now be used to screen for inhibitors. Two different genetic screens[97,98] and our own unpublished data points to the essentiality of ATrx2 also in *P. falciparum.* ATrx2 is localised in one of the outer apicoplast compartments (the periplastid compartment[32,99], see Fig 1 for representation). Because of its localization, inhibitors of ATrx2 need to cross two membranes fewer than inhibitors of targets in the apicoplast stroma. Likewise, due to its role in organelle biogenesis, ATrx2 inhibitors also have the potential to provide fast killing like actinonin[31] and like a recently reported putative inhibitor of apicoplast protein translocation[102]. Together these features highlight ATrx2 as an attractive target for drug development. A proximity-tagging based analysis[79] has expanded the number of apicoplast proteins predicted with high-confident in *P. falciparum.* This allows an evaluation of the number of potential ATrx substrates and their predicted functions (Table 2). The catalytic activity of thioredoxins, including ATrxs[32], consists of disulfide exchange with their substrate, which takes place via a typical double cysteine (CXXC) motif. While target cysteines on the substrate could be found anywhere, they are commonly located in CXXC motifs, which can provide a predictive tool in the search for putative substrates. Table 2 summarizes the proteins found in the proximity tagging screen that also possess CXXC motifs, and are thus candidate ATrx substrates. CXXC motifs in known thioredoxin substrates could be found anywhere in the protein sequence where their reduction or oxidation could affect folding. We would predict that in the case of apicoplast proteins, CXXC found in the targeting signal are not likely to affect protein folding once in the organelle and thus those proteins are not included. Further analysis of these new apicoplast proteins may present new target candidates for drugs inhibiting apicoplast redox regulation.

297 **Table 2. Apicoplast-targeted proteins predicted by Boucher *et al.* (2018)[79] that contain double**
298 **cysteines that may serve as ATrx regulation domains**

Putative apicoplast proteins containing potential dicysteine domains			
Reference	**Annotation**	**CxxC domain(s)**	**Essentiality***
PF3D7_0107700	conserved membrane protein, unknown function	CilC	Dispensable
PF3D7_0313800	conserved protein, unknown function	CrdC, CpqC	Essential
PF3D7_0313900	conserved protein, unknown function	CriC	Dispensable
PF3D7_0414100	conserved membrane protein, unknown function	CvkC	Dispensable
PF3D7_0529000	conserved protein, unknown function	CnyCC	Essential
PF3D7_0619100	conserved protein, unknown function	CilC	Essential
PF3D7_0715200	conserved protein, unknown function	CglCC	Dispensable
PF3D7_0721100	conserved protein, unknown function	CnlC	Essential
PF3D7_0820600	conserved membrane protein, unknown function	CneC, ChtC, CirC	Dispensable
PF3D7_0916200	conserved protein, unknown function	CknC	Essential
PF3D7_0918400	conserved protein, unknown function	CinC	Dispensable
PF3D7_1023300	conserved protein, unknown function	CgtCtaC, CaaC, CtaC	Essential
PF3D7_1242000	conserved protein, unknown function	CpaC, CqnC, CknC	Essential
PF3D7_1351800	conserved protein, unknown function	CalC, CqyC	Essential
PF3D7_1352000	conserved protein, unknown function	CvgC, CkrC	Dispensable
PF3D7_1436100	conserved membrane protein, unknown function	CekC	Essential
PF3D7_1437100	conserved protein, unknown function	CakC, CpnC	Essential
PF3D7_1457300	conserved protein, unknown function	CplC	Essential
Apicoplast proteins with potential redox functions			
Reference	**Annotation**	**Function**	**Essentiality***
PF3D7_0529100	thioredoxin-like protein, putative (ATrx2)	Redox	Essential
PF3D7_0604700	glyoxalase I (GILP)	Glyoxalase System	Essential
PF3D7_0623200	ferredoxin--NADP reductase	Redox	Essential
PF3D7_0623500	superoxide dismutase [Fe] (SOD2)	Redox	Essential
PF3D7_0716600	cysteine desulfurase	Fe-S cluster	Essential
PF3D7_0723700	metallo-hydrolase/oxidoreductase, putative	Unknown	Essential
PF3D7_0729200	1-cys peroxiredoxin (AOP)	Redox	Dispensable
PF3D7_0815900	dihydrolipoyl dehydrogenase, apicoplast	PDC component	Dispensable
PF3D7_0823600	lipoate-protein ligase B	Lipoic acid synthesis	Dispensable
PF3D7_0914300	met-10+ like protein, putative	Unknown	Dispensable
PF3D7_1020800	dihydrolipoamide acyltransferase component E2	PDC component	Essential
PF3D7_1114800	glycerol-3-phosphate dehydrogenase, putative	Carbon metabolism	Essential
PF3D7_1124500	pyruvate dehydrogenase E1 component subunit α	PDC component	Essential
PF3D7_1205700	targeted glyoxalase II	Glyoxalase system	Dispensable
PF3D7_1212000	glutathione peroxidase-like thioredoxin peroxidase	Redox	Essential
PF3D7_1318100	ferredoxin, putative	Redox	Essential
PF3D7_1364600	aldehyde reductase, putative reducing enzyme	Unknown	Essential
PF3D7_1409100	aldo-keto reductase, putative	Unknown	Essential
PF3D7_1419200	thioredoxin-like protein, putative (ATrx1)	Redox	Dispensable
PF3D7_1419800	glutathione reductase	Redox	Essential
PF3D7_1430700	NADP-specific glutamate dehydrogenase	Carbon metabolism	Essential
PF3D7_1437200	ribonucleoside-diphosphate reductase large subunit, putative	DNA replication	Essential
PF3D7_1446400	pyruvate dehydrogenase E1 component subunit β	PDC component	Dispensable
PF3D7_1455900	polyprenol reductase, putative	Unknown	Essential

*The indication of gene essentiality is according to the high-throughput *piggyBac* transposon mutagenesis screen performed by Zhang *et al.* (2018) on *P. falciparum*[97]. In this study, libraries of parasite genomes containing a single *piggyBac* insertion were used to identify essential genes by Illumina-based sequencing. This approach identified 2042 genes that are dispensable for *P. falciparum* intraerythrocytic development.

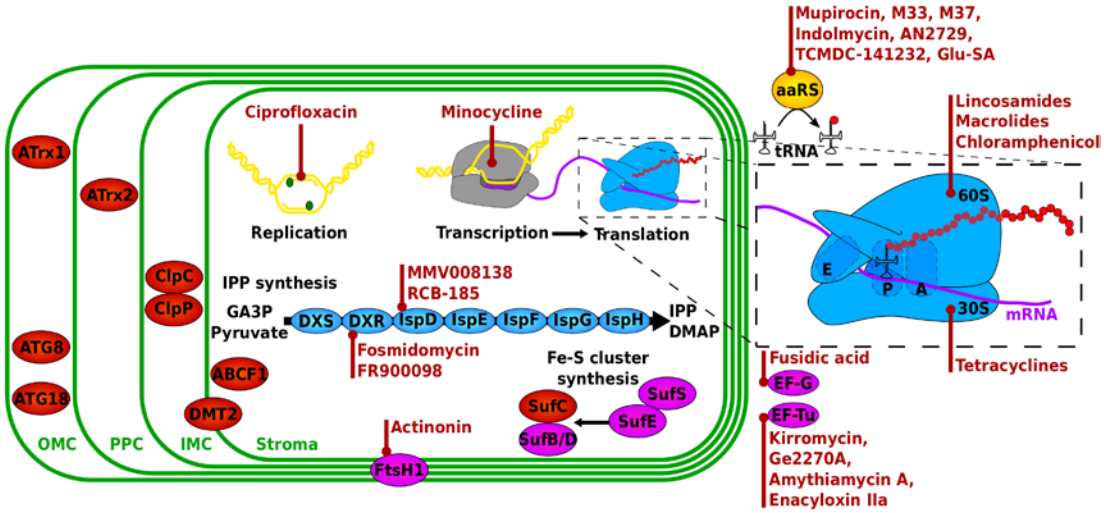

Figure 1 Schematic representation of the apicoplast with confirmed and potential drug targets.

The figure represents the various apicoplast compartments delimited by four green membranes. The most relevant apicoplast functions sustaining *Plasmodium* spp. intraerythrocytic development are represented. Antibiotics having a specific effect on the organelle are indicated by names in red. Apicoplast potential drug targets that have been confirmed by IPP supplementation are depicted in red circles and are placed in their hypothetical organelle position. The big variety of antibiotics active against the translation machinery are listed on the right panel, representing an enlargement of the ribosome including the elongation factors EF-G, EF-Tu and a circle representing the apicoplast tRNA synthetases.

Abbreviations: **aaRS**: aminacyl-tRNA synthetase; **ABCF1**: ATP-binding cassette protein F1; **ATG8/18**: autophagy related protein 8/18; **ATrx1/2**: apicoplast-thioredoxin 1/2; **ClpC/P**: caseinolytic protease C/P; **DMAP**: dimethylallyl-pyrophosphate; **DMT2**: divalent metal transporter 2; **DXS**: 1-deoxy-D-xylulose 5-phosphate synthase; **DXR**, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; **EF-G**: elongation factor-G; **EF-Tu**: elongation factor thermo unstable; **GA3P**: glyceraldehyde 3-phosphate; **IMC**: innermost compartment; **IPP**: isopentenyl pyrophosphate; **IspD**, 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase; **IspE**, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; **IspF**, 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; **IspG**, 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase; **IspH**, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; **PPC**: periplastid compartment; **OMC**: outermost compartment.

Perspectives

- Malaria continues to be one of the major global killers. Upon its discovery, the apicoplast was proposed as a prime target for antimalarials. However, the "delayed death" phenotype, characterized by parasite killing only in the second generation after the introduction of drugs inhibiting apicoplast targets, does not align with the killing dynamics proposed for the new generation of antimalarials. This resulted in scepticism about apicoplast functions providing good targets for new drugs within the malaria drug research and development community.

- Several recent studies highlight that there are apicoplast functions whose inhibition could result in the rapid killing of *Plasmodium* spp.[31,72,102] and the related *Toxoplasma gondii*[100]. Likewise, studies started pointing to potential apicoplast essential functions *in vivo*. For example, while IPP complementation suggests IPP synthesis as the only essential apicoplast function in asexual stages, experiments point to other crucial apicoplast functions that sustain parasite growth when fever response is simulated in culture[101]. The same may well be true for growth under the pressure of oxidative stress generated by the immune system.
- These observations should provide motivation to deepen our understanding of apicoplast biology, and our knowledge of the proteins that take part in its functions. In the context of drug development, it is a high priority to improve our appreciation of the role of the apicoplast in different parasites life stages *in vivo.*

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