Title: Exploiting the apicoplast: apicoplast-targeting drugs and malaria vaccine development.

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Abbreviations: ALAS, aminolevulinic acid synthase; CPS, chemoprophylaxis and sporozoite; DCS, D-cycloserine; DMAPP, dimethylallyl pyrophosphate; DOXP, 1-deoxy-D-xylulose 5-phosphate; DSG, 15-deoxyspergualin; FAS, fatty acid synthesis; FC, ferrochelatase; IDC, infection and drug cure; IleRS, isoleucyl-tRNA synthetases; IPP, isopentenyl pyrophosphate; ISC, iron sulfur cluster; MEP, 2-C-methyl-erythritol 4-phosphate; PPM, periplastid membrane; SUF, sulfur utilisation factor;

Abstract

The apicoplast, a relic plastid found in most Apicomplexan parasites, is a notable drug target. Certain antibiotics elicit a delayed death phenotype by targeting this organelle. Here, we review apicoplast targeting drugs and their targets, particularly those that cause delayed death, and highlight its potential uses in malaria vaccine development.

Keywords: apicoplast; malaria; antimalarial drugs; vaccines; delayed death

1. Introduction

The discovery of the apicoplast opened a new avenue for understanding the needs of the malaria parasite, as well as the identification of pathways that could inform further drug and vaccine development. A non-photosynthetic organelle, the apicoplast can be found in all members of the Apicomplexan phylum except for *Cryptosporidium* spp. [1]. It is now known that the apicoplast is a red algae derivative from a secondary endosymbiotic event [2]. Having lost autonomy to its host cell, the apicoplast has a noticeably reduced circular genome due to the transfer of genes to the host genome. Most of the genes left in the apicoplast are necessary for transcription and translation [3, 4], while many other required proteins must be imported into the organelle from the parasite's endoplasmic reticulum (ER) [5].

Research to date has determined that there are several important pathways housed within the apicoplast. Pharmacological targeting and genetic manipulation of the apicoplast has confirmed the importance of the apicoplast to malaria parasite survival. This review will briefly explore the different processes that occur within the apicoplast and the drugs known to affect them, delve into the curious 'delayed death' phenomenon elicited by certain drugs and the applications for such compounds in vaccine development in the context of one of the world's most devastating diseases, malaria.

2. Protein importation to the apicoplast and drug targeting

The apicoplast has a small genome and is comprised of multiple membranes that are indicative of the complex past of the organelle. Sequencing of the 35kb genome has found that it encodes for 30 proteins; these include several ribosomal proteins, a RNA polymerase of eubacterial origin, elongation factor-Tu (EF-Tu), large and small ribosomal RNAs (rRNAs) and several transfer RNAs (tRNAs)[4]. The vast majority of apicoplast proteins, however, are nuclear-encoded and must be imported into the organelle. Amongst the nuclear-encoded proteins are those involved in biosynthetic pathways, as well as enzymes required in housekeeping processes, such as DNA replication, transcription and protein translation [6]. Protein import is complicated with the existence of four membranes surrounding the apicoplast: the inner two membranes pertaining to the double membrane of cyanobacteria, the third membrane (periplastid membrane or PPM) resulting from the first endosymbiotic event and, finally, the outer membrane from the second endosymbiotic event[5]. It is hypothesized that each membrane has an evolved import system reflecting the membranes' origins. These membrane transport systems have been studied most extensively in *Toxoplasma* and *Plasmodium* spp.[7-12].

The transport systems involved in the trafficking of apicoplast proteins to the organelle are an ideal drug target due to the clear majority of proteins needing importation into the organelle. To date, the only known inhibitor of apicoplast protein import is 15-deoxyspergualin (DSG), which has been shown to target nuclear-encoded proteins that are trafficked to the apicoplast [13]. This is believed to occur through the interference of interactions between the transit peptide and the heat shock protein, Hsp70-1, which are involved in organelle targeting and translocation into the apicoplast, respectively [14].

On the other hand, the majority of apicoplast drug targets tend to be intraorganellar proteins involved in both housekeeping and non-housekeeping functions within the organelle. Effective anti-malaria drugs targeting these processes must have the ability to transverse

multiple membranes to reach their target; those targeting the apicoplast must also pass through the infected host cell membrane, parasitophorous vacuolar membrane and parasite plasma membrane in addition to the organelle's four membranes to access their targets. This may be achieved by diffusion across the lipid bilayer, membrane transporters or a mixture of both [15]. In theory, drug development should consider the many barriers in designing a suitable candidate; however, as highlighted in a review by Botté et al.(2011), there are some drugs that do not necessarily conform to ideal drug characteristics; however, these are efficacious against malaria [16].

3. Antibiotics and housekeeping functions of the apicoplast

Given the prokaryotic nature of the apicoplast and its components the ability to use readily available antibiotics for treatment of malaria has since been largely attributed to targeting of the apicoplast. Families of antibiotics with anti-malarial activity include tetracyclines, lincosamides, macrolides, and ketolides (for an extensive list of apicoplast targeting drugs and associated IC₅₀s please refer to [17]). These drugs act to disrupt DNA replication, RNA transcription and protein translation [18]. Most housekeeping targeting drugs mentioned in this section invoke a delayed death phenotype, which will be examined later in this review.

DNA replication of the apicoplast genome is achieved through the D-loop/bidirectional *ori* model, with most enzymes requiring import into the apicoplast [19]. Fluoroquinolones and aminocoumarins, such as ciprofloxacin and novobiocin, specifically target and inhibit prokaryotic DNA gyrases, which are involved in the unravelling of DNA in preparation for replication. These antibiotics inhibit the growth of *P. falciparum* in *in vitro* cultures [18, 20].

RNA transcription is dependent upon an RNA polymerase ($\alpha\beta\beta'\omega$) that is similar to those found in eubacteria [21]. The plastid genome encodes for the β and β' subunits required

for this RNA polymerase. Rifampicin (also known as Rifampin), a selective inhibitor of the β subunit of bacterial polymerases, has been shown to have anti-malarial effects [18, 22]. Inhibition of apicoplast protein translation can also indirectly affect RNA transcription; studies using thiostrepton and doxycycline observed a decrease in expression of the genes encoding for the β and β' subunits (RpoB and RpoC) [23, 24].

The machinery required for apicoplast protein translation is largely encoded by the circular plastid genome, although several other components, such as aminoacyl-tRNA synthetases, are imported into the organelle [25]. Many antibiotics have been shown to be effective in targeting the apicoplast via its translational machineries – of particular note is doxycycline, which targets the 30S rRNA subunit and is currently used worldwide in prophylactic and combination chemotherapy against malaria [26]. Other anti-malaria drugs, such as clindamycin, azithromycin and chloramphenicol, exert their effect by targeting the 50S rRNA subunit [26, 18]. Most recently, indolmycin and mupirocin, inhibitors which specifically inhibit apicoplast bacterial aminoacyl-tRNA synthetases that are involved in the addition of amino acids onto tRNAs during protein translation, inhibit *P. falciparum* growth *in vitro* [27, 28].

4. Drugs affecting the biosynthetic pathways of the apicoplast

The detection of the apicoplast genome and subsequent realization of its origins as a red algae plastid lacking photosynthetic ability prompted investigation into other possible functions harbored by the apicoplast within the Apicomplexan parasite. Four biosynthetic pathways have since been found to be associated with the apicoplast: heme synthesis, iron-sulfur complex synthesis, fatty acid biosynthesis and isoprenoid precursor synthesis (Fig. 1).

4.1 Heme synthesis

Heme is used by the malaria parasite as a prosthetic group for cytochromes [29]. While the parasite can import hemoglobin during the erythrocytic stages of infection, there is

a pathway which allows for *de novo* synthesis. This is the resulting hybrid of two separate pathways inherited by the mitochondrion and apicoplast, with the current pathway spanning between the two organelles and the cytoplasmic compartment [30, 31].

Synthesis of heme does not appear to be essential to malaria parasites during blood-stage infection, with the converse being true of liver and mosquito stages [32, 33]. This was demonstrated by knocking out aminolevulinic acid synthase (ALAS) and ferrochelatase (FC), enzymes of the heme pathway, resulting in no significant changes to parasite growth during *in vitro* and *in vivo* blood-stage infection; however, oocyst development and sporozoite formation was abolished in mosquitoes [32, 33].

Drugs affecting the heme pathway include succinylacetone, which acts through inhibition of aminolevulinic acid dehydratase (ALAD) [32]. Although succinylacetone has been reported to be lethal to the malaria parasite at concentrations of 1-2mM, this is believed to be due to off-target inhibition rather than complete inhibition of heme synthesis [32]. Herbicides have additionally been established as affecting heme synthesis through the inhibition of protoporphyrinogen IX oxidase [34].

3.2 Iron-sulfur (Fe-S) complex synthesis

The malaria parasite harbors genes for two distinct Fe-S synthetic pathways (iron-sulfur cluster (ISC) and sulfur utilization factor (SUF) pathways) inherited by the mitochondrion and apicoplast, respectively. It is unknown if components of each pathway intermingle to form a hybrid pathway, as with heme synthesis, although evidence to date suggests that they remain separate [35].

Fe-S complexes are known to play a role in electron transport and act as co-factors to enzymes in a variety of pathways, including fatty acid and isoprenoid biosynthesis. Interruption of this pathway may, therefore, also fatally impact on the parasite [30]. This was demonstrated when disruption of the apicoplast Fe-S cluster synthesis pathway and

subsequent chemical rescue with an isoprenoid byproduct was able to rescue parasites from death, indicating its importance to isoprenoid biosynthesis [35]. Fe-S cluster synthesis was also found to be critical to *P. berghei* sexual stages when genetic knockouts of a cysteine desulfurase involved in the SUF pathway, SufS, were found to have defective sporozoite development in oocysts [36].

D-cycloserine (DCS) is the only drug reported to have an anti-malarial effect mediated through inhibition of SufS in the apicoplast SUF pathway in *P. falciparum* [37].

3.3 Fatty acid biosynthesis

In the apicoplast, fatty acid synthesis (FAS) is via the Type II pathway, which involves several enzymes as opposed to a main enzymatic complex. This metabolic path differs from that of animals which derive their fatty acids via the Type I pathway [31]. The ability to make fatty acids *de novo* was initially thought to be non-existent in *Plasmodium* spp. with fatty acids obtained only via scavenging mechanisms [38]. It was soon realized, with the discovery of genes pertaining to the FAS II pathway, that *Plasmodia* parasites could obtain fatty acids via both methods [8, 6, 30].

Studies have since indicated that this pathway is essential to liver-stage but not blood-stage parasites, with the latter dependent upon scavenging exogenous fatty acids [39]. Disruption to the FAS II pathway resulted in no changes to growth in *P. falciparum* *in vitro*, and *in vivo* for *P. yoelii* and *P. berghei*, although investigation in the rodent models found decreased infectivity of sporozoites and arrested development during the liver-stage [40, 41, 39].

Multiple inhibitory compounds have been described as acting through disruption of the FAS pathway and appear to affect *P. falciparum* growth *in vitro*. Of note are thiolactomycin [40, 42, 43], triclosan [44, 45] and cerulenin [45, 46], each targeting different enzymes associated with fatty acid synthesis and resulting in immediate parasite death. The

FAS II pathway appears to be an ideal target for drug development due to the differences in parasite and host FAS pathways.

3.4 Isoprenoid precursor synthesis (MEP/DOXP pathway)

Synthesis of isoprenoid precursors is exclusive to the apicoplast (for a comprehensive review of isoprenoid biosynthesis, refer to [47]). Precursors are produced via the MEP/DOXP (non-mevalonate pathway), which is primarily used by bacteria and chloroplasts, as opposed to the mevalonate pathway used in animals. The lack of commonality between the pathways makes the DOXP pathway a suitable target for anti-malarial drugs.

Both pathways produce isomeric compounds - isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). These precursors produce a diverse range of isoprenoids (e.g. sterols, ubiquinone and dolichol) that are involved in several parasite functions such as the regulation of erythrocyte invasion and RNA translation (reviewed extensively in [47, 48]). They also attach as prosthetic groups to enzymes and contribute to prenylation of proteins and tRNAs [30, 49].

Inhibitors of the second enzyme of the pathway, DOXP reductoisomerase, target and successfully inhibit isoprenoid synthesis and subsequently cause parasite death. Fosmidomycin and its derivative, FR-900098, are well-known highly specific inhibitors of DOXP reductoisomerase [50]. Fosmidomycin has been shown to suppress growth of *P. falciparum* *in vitro*; and when administered to mice infected with *P. vinckei*, resulted in clearance of the parasites [50]. Additionally, studies with human volunteers have shown it to be effective as an anti-malarial drug when given in conjunction with clindamycin [51, 52].

Isoprenoid synthesis is essential to the blood-stage malaria parasite. This was demonstrated in a series of experiments conducted by Yeh & DeRisi (2011), wherein *P. falciparum* parasites treated fosmidomycin could be rescued with IPP [53]. This was not observed with DMAPP, or alcohol analogs of both IPP and DMAPP, indicating that the

production of IPP by the apicoplast is essential to the parasite's survival. Additionally, continued culturing of *P. falciparum* parasites with doxycycline and IPP supplementation found that parasites eventually lose the apicoplast genome, as well as protein import function, becoming entirely dependent on exogenous IPP, thus indicating the importance of this pathway to parasite survival [53].

Isoprenoids have additionally been shown to be important during gametocytogenesis. Adopting the method established by Yeh & DeRisi, gametocytes were subjected to chemical attenuation and rescue to generate stage V gametocytes without apicoplasts [54]. As seen with erythrocytic parasites, these gametocytes were dependent upon IPP supplementation. Mosquitoes infected with apicoplast negative gametocytes had impacted oocyst development and no detectable sporozoites in their salivary glands, indicating that the apicoplast has an important role in parasite development within mosquitoes [54].

The role of isoprenoid synthesis in liver-stage malaria parasites has been less clear. Studies treating liver-stage *P. berghei* with fosmidomycin did not see any significant effect upon parasite development within hepatocytes, though modification of fosmidomycin to improve drug uptake consequently improved growth inhibition [55].

As mentioned previously, fosmidomycin is the most well-known and described inhibitor of the DOXP/MEP pathway. There are several other compounds (e.g. bisphosphonates, nerolidol, prenyltransferase inhibitors) which can exert inhibitory effects downstream of IPP and DMAPP production (reviewed in [47]).

5. A slow, slow demise...the delayed death phenotype

Delayed death refers to a phenomenon in which treated parasites appear to grow normally; however, the effects of drug treatment are observed in the progeny of the treated parasite. This occurrence is still observed even with the removal of the drug prior to

egression and continuation into the second cycle after treatment, a finding that has been observed in both *Toxoplasma* and *Plasmodia*[49].

While the exact mechanism for this delayed death phenotype is still currently unknown, the treated parasite appears to initially uphold metabolic pathways; however, daughter cells inherit a dysfunctional apicoplast that is unable to sustain critical biological processes [53, 23].

The use of drugs, such as azithromycin, clindamycin and doxycycline, with *P. falciparum* parasites *in vitro* demonstrated that clinically available antibiotics exerted a delayed death effect on asexual blood-stage parasites [18]. These treated parasites continued to develop through the first erythrocytic cycle without any ill effects and were observed to invade new erythrocytes [18, 23]. However, the parasite's progeny were unable to complete a second cycle with development arresting in the schizont stage with a single abnormal apicoplast being observed [18]. This indicated that the apicoplast genome was unable to replicate and segregate during division. Further, it was observed that delayed death-causing drugs were most effective when treating late trophozoite and early schizont blood-stage parasites [18].

Many observations of the delayed death phenotype have been made with drugs involved in inhibition of housekeeping processes within the apicoplast (Table 1), whereas those that target non-housekeeping functions result in rapid death [46, 18]. Reports of the inducement of delayed death with ciprofloxacin and rifampicin, which act upon DNA replication and transcription, have been conflicting within the literature [18, 46, 56, 57]. However, most anti-malarial drugs which affect protein translation (e.g. doxycycline), to date, exhibit the delayed death phenomenon [16, 18, 46]. New antibiotics that are known to act by targeting bacterial ribosomes, such as telithromycin and quinupristin-dalfopristin, have shown similar anti-malarial delayed death effects [58]. 15-deoxyspergualin, the only known

inhibitor of apicoplast protein import, is also reported to display the delayed death phenotype [13].

6. Utilization of the delayed death phenotype in vaccine development

The uniqueness of the apicoplast and the vital biosynthetic functions it provides have identified it as an attractive target for crippling the malaria parasite. The observation of delayed death during antibiotic treatment and confirmation of its link to the apicoplast raises the question as to how can one utilize this characteristic to our advantage? The obvious answer is to apply it as screening criteria for new anti-malarials and this has already been used in such efforts [59, 60]. However, can this interesting phenotype be utilized for purposes other than anti-malarial drug screening?

While malaria vaccine development has been extensive over the past half century, it has been fraught with little success. At present, the sub-unit vaccine, RTS,S/AS01 (Mosquirix™), is the first and only candidate to recently complete Phase III clinical trials and as of 2018, will be available in select countries within the framework of a WHO Pilot Implementation Program [61]. Even so, Phase III results revealed modest vaccine efficacy of 26-37% and 18-28% in children and infants with or without a fourth booster dose; efficacy additionally declined with time, and those receiving the vaccine had an increased associated risk of developing severe malaria [62]. As such, there is renewed interest in developing a whole parasite vaccine to combat the limited protective efficacy seen with sub-unit vaccines.

There are several attenuation approaches utilized in whole malaria parasite vaccine development, of note is the ability to attenuate the parasite using chemical treatment. This has produced encouraging results, with homologous and heterologous protection observed in rodent models and cell-mediated responses in non-human primates [63-66]. How then can we exploit the delayed death phenotype for vaccine development?

6.1 In vitro chemical attenuation

The generation of apicoplast negative parasites can be considered as a possible whole parasite vaccine candidate [53, 67]. Through combined treatment with a delayed death drug (doxycycline) and chemical rescue with IPP, both blood-stage and sexual-stage malaria parasites have successfully been able to be grown *in vitro* without apicoplasts [53, 54]. *In vitro* observations indicate that these chemically treated parasites would develop normally under IPP supplementation but would arrest in latter stages of development when IPP supplementation is removed [53]. This would allow for increased persistence and exposure to different asexual stage antigens. Apicoplast negative parasites could, theoretically, be grown in bulk under IPP supplementation and administered as a vaccine. Following administration and with the loss of IPP supplementation, these parasites persist throughout one cycle before dying [53]. A benefit of this approach lies in the unlikelihood of reversion to wild-type as the loss of the apicoplast is not reversible; however, while very uncommon, it is not without possibility for doxycycline resistance to develop in *P. falciparum* [26]. In spite of this, there are no reports of resistant strains cultivated *in vitro* and only one *in vivo* study reported *P. berghei* resistance under drug pressure with the tetracycline, minocycline [68].

6.2 *In vivo* chemical attenuation

In addition to traditional methods of *in vitro* attenuation, apicoplast targeting drugs may also be used for *in vivo* attenuation. Chemoprophylaxis immunisation involves the administration of infectious parasites to individuals under prophylactic treatment, as opposed to receiving an attenuated or weakened form of the parasite. Most studies to date have focused on the use of infectious sporozoites and thus refer to this as the chemoprophylaxis and sporozoite (CPS) model [69]. In conjunction, chloroquine treatment is administered to target blood-stage, but not liver-stage, parasites. Sporozoites are, therefore, allowed to infect and develop normally within hepatocytes, with emerging merozoites invading erythrocytes and being eliminated by chloroquine.

Protection, using the CPS model, has been shown in rodent, non-human primate and human experimental models [69-78]. Rodent studies demonstrated protection against homologous challenge can be induced following immunisation with *P. berghei*, *P. chabaudi* and *P. yoelii* sporozoites [70-74]. Most recently the CPS model was demonstrated with *P. knowlesi* in rhesus monkeys with two out of four monkeys showing complete protection from sporozoite challenge [75]. Sterile homologous protection was observed in human volunteers in which volunteers received 3 rounds of immunisation with *P. falciparum* sporozoites combined with chloroquine treatment [69, 76-78]. Volunteers previously vaccinated and challenged with *P. falciparum* NF54 were additionally re-challenged 14 months later with a heterologous strain of *P. falciparum* and demonstrated partial heterologous protection[79]. Cross-stage protection has been observed in rodent models [74, 71], however little to no blood-stage protection has been noted in humans [77].

Similar to the CPS model, administration of low levels of infective whole blood-stage parasites followed by drug treatment has been investigated, although less extensively, in rodents and humans [80-82]. In these studies, treatment with malarone or chloroquine commenced in rodents immediately with infection [82] or after 48 hours (allowing 2 cycles of parasite replication [81]) and after 8 days in humans (allowing 4 cycles of replication [80]). Cross-stage protection (sporozoites) [82] and homologous [81, 80] and heterologous [81] blood-stage immunity/protection was induced in these studies. In all instances, multiple doses of drug were required following infection, with drug treatment generally being initiated after a specific period following infection. The feasibility of the infection and drug cure as an immunisation approach is impacted by the delayed and repeated administration of treatment, requiring individuals to return to the clinic to initiate treatment.

To combat the issue of postponed treatment, a delayed death inducing anti-malarial drug might be used. Delayed death drugs, such as doxycycline, are currently used in

combinational therapy with fast acting drugs (e.g. quinine), as well as for prophylaxis, due to their safety profile and lack of reported resistance [26]. An infection and drug cure model utilising the delayed death characteristic would allow for both the infection and drug treatment to be administered simultaneously. Ideally, the delayed death-causing nature of the drug would allow treated parasites to undergo at least one full cycle of replication with daughter cells arresting, promoting persistence of the parasite and exposure to a variety of antigens expressed across the blood-stage of the lifecycle.

Currently, only one laboratory group has published studies which apply this concept. Mice were immunized with *P. berghei* sporozoites under a three-day treatment regimen with clindamycin or azithromycin and were protected from sporozoite challenge [83]. A combination therapy of azithromycin and chloroquine also produced similar results, however no blood-stage immunity was observed in both studies [84, 83].

Our laboratory group has also adapted the CPS model for infection and drug cure (IDC) with blood-stage parasites and antibiotics. We have found that IDCs with a delayed death inducing drug were able to induce strong homologous and heterologous protective immunity against blood-stage challenge. We believe that the prolonged persistence allowed by the effects of delayed death permits for the establishment of a protective immune response (Low et al, unpublished).

7. Conclusion

Since its characterization, the apicoplast has offered interesting insights into the *Plasmodium* parasite. While much research is still needed in elucidating all of the apicoplasts secrets, the identification of key synthetic processes and the revelation that delayed death-causing drugs specifically target this organelle offer an Achilles heel to an otherwise resilient organism. Mechanisms of delayed death remain largely unclear. However, this feature can be exploited in drug discovery, as well as in vaccine development.

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Table 1: Delayed death causing drugs.

Drug name	Housekeeping function	Apicoplast Specific Target	References
Macrolides (e.g. azithromycin, solithromycin, erythromycin)	Protein translation	50S ribosomal subunit	[57]
Ketolides (e.g. telithromycin)			[58]
Quinupristin-dalfopristin			[58]
Chloramphenicol			[46]
Tetracyclines (e.g. doxycycline, minocycline)		30S ribosomal subunit	[18, 46, 57]
Indolmycin		Tryptophanyl-tRNA synthetases	[27]
Mupirocin		Isoleucyl-tRNA synthetase	[28]
Fluoroquinolones (e.g. ciprofloxacin, norfloxacin)	DNA replication	DNA gyrase	[18, 57]
Novobiocin			[20]
Rifampicin	RNA transcription	RNA polymerase (β subunit)	[18, 46, 57]
15-Deoxyspergualin (DGS)	Protein import	Transit peptide/Hsp70-1	[13, 14]

Figure 1. Summary of functions housed by the apicoplast and drug targets. A)

Apicoplast housekeeping functions. B) 15-Deoxyspergualin (DSG) interferes with protein import of nuclear encoded proteins targeted to the apicoplast [14, 13]. C) The Fe-S cluster biosynthesis pathway (highlighted in purple). SufS and SufE aid in sulfur mobilization, releasing sulfur from cysteine. SufB/SufD/SufC complex aids in assembly of sulfur with iron and are transferred to carrier proteins, Nfu and SufA, for transfer to appropriate proteins [36, 37]. Phosphoenol pyruvate (PEP) is imported into the apicoplast and converted to pyruvate by pyruvate kinase II (PKII). Pyruvate is subsequently used in fatty acid (FAS, highlighted in orange) or isoprenoid synthesis (highlighted in yellow). D) PDH, pyruvate dehydrogenase; ACC, acetyl-CoA carboxylase; FabD, malonyl-CoA transacylase; FabH, β -ketoacyl-ACP synthase III; FabB/F, β -ketoacyl-ACP synthase I/II; FabG, β -ketoacyl-ACP reductase; FabZ, β -hydroxyacyl-ACP dehydratase; FabI, enoyl-ACP reductase [85]. E) G3P, glyceraldehyde-3-phosphate; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; DOXP, 1-deoxy-D-xylulose 5-phosphate; DXR, DOXP reductoisomerase; MEP, 2-C-methyl-D-erythritol 4-phosphate; IspD, MEP cytidyltransferase; CDP-ME, 4-diphosphocytidyl-2-C-methylerythritol; IspE, CDP-ME kinase; CDP-MEP, 4-diphosphocytidyl-2-C-methylerythritol 2-phosphate; IspF, MeCPP synthase; MEcPP, 2-C-methyl-D-erythritol 2,4-cyclopyrophosphate; HMBPP, 4-hydroxy-3-methyl-but-2-enyl pyrophosphate; IspG, HMBPP synthase; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; IspH, HMBPP reductase [47]. F) Heme biosynthesis pathway (highlighted in blue). ALA, aminolevulinic acid; ALAS, aminolevulinic acid synthase; PBG, porphobilinogen; ALAD, aminolevulinic acid dehydratase; HMB, hydroxymethylbilane; PBGD, porphobilinogen deaminase; Urogen III, uroporphyrinogen III; UROS, uroporphyrinogen III synthase; Coprogen III, coproporphyrinogen III; UROD, uroporphyrinogen decarboxylase; Protopogen IX, protoporphyrinogen IX; CPO, coproporphyrinogen oxidase; PPIX, protoporphyrin IX; PPO,

protoporphyrinogen oxidase; FC, ferrochelatase [33]. Compounds are bolded black, enzymes in red, inhibitors in green.