The apicoplast as an antimalarial drug target

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Abstract Resistance to commonly used malaria drugs is spreading and new drugs are required urgently. The recent identification of a relict chloroplast (apicoplast) in malaria and related parasites offers numerous new targets for drug therapy using well-characterized compounds. The apicoplast contains a range of metabolic pathways and housekeeping processes that differ radically to those of the host thereby presenting ideal strategies for drug therapy. Indeed, many compounds targeting these plastid pathways are antimalarial and have favourable profiles based on extensive knowledge from their use as antibacterials.

INTRODUCTION

Malaria is a major global health problem. Three main strategies are presently attempting to control the disease: vaccination, vector control, and parasiticidal drugs. Of these, parasiticidal drugs are currently the main line of disease control until vaccination or mosquito control can be implemented more successfully. There is however a deepening crisis with emerging resistance among malaria parasites to the existing drugs. For these reasons it is imperative that new lines of drugs be explored before existing drugs lose too much efficacy.

The recent identification of a relict chloroplast in malaria parasites has profound implications for drug therapies to combat this important disease. Chloroplasts are chlorophyll-containing organelles found in plants and algae. Their key function is photosynthesis and they come in red, brown and even colourless, non-photosynthetic versions. Strictly speaking the term chloroplast should only be used to describe green versions of the organelle; plastid is the more generic term. Plastids (of all colours) originate from endosymbiotic cyanobacteria.[1] In other words a plastid is a modified photosynthetic bacterium living inside a eukaryotic cell. These endosymbionts are semi-autonomous, living (to a limited extent) a life of their own within the host. The original acquisition of plastids presumably provided the host with the ability to photosynthesise. However, creating food from CO_2 and sunshine is not the only task plastids perform for their hosts. Plastids are also the site of a number of anabolic processes whose products are supplied to the surrounding host cell. These metabolic services make plastids indispensable to plant and algal cells, even non-photosynthetic ones.

The apicomplexan plastid, which we refer to as the apicoplast, ultimately arose from the same endosymbiosis as all other plastids.[2] The apicoplast also appears to service its host with multiple functions, but the apicoplast apparently lacks the capacity to photosynthesise. Several biosynthetic pathways similar to those found in plant and algal chloroplasts are beginning to be characterized in apicoplasts. It is not yet clear what the key function of the apicoplast is but the organelle is clearly indispensable.[3,4] Curiously though, parasites cured of their apicoplasts do not die immediately. Rather, they fail to invade new host cells successfully.[4] This suggests that apicoplasts provide some component essential to invasion and or establishment of the parasitophorous vacuole in the host cell. Current efforts to identify this unknown component centre around unravelling the various biosynthetic pathways of the apicoplasts. As an adjunct of this research many parasiticidal compounds are being identified.

For drug purposes, we can think of the apicoplast as a minibacterium living inside the malaria parasite. Inside this 'cell'-within-a-cell occur all the familiar cellular processes such as DNA replication, transcription, translation, post-translational modification, catabolism and anabolism. Each of these processes is bacterial in nature and potentially a drug target because they differ to the host processes, which are fundamentally eukaryotic. In this review we will examine each of these apicoplast metabolic categories, and their drug potential, in turn.

DNA REPLICATION

The genome of the parasite plastid is circular[5] (like most bacterial chromosomes) and a bacterial-type DNA gyrase is thought to be required for replication of the apicoplast genome in *P. falciparum.*[3] In support of this, the fluoroquinolone ciprofloxacin has been shown to inhibit apicoplast (prokaryotic) DNA replication but not nuclear (eukaryotic) replication in *P. falciparum*.[3] Additionally, Fichera and Roos[3] demonstrated that ciprofloxacin inhibits apicoplast DNA replication in another apicomplexan parasite, *Toxoplasma gondii.* Various derivatives of quinolones and fluoroquinolones have been tested for their ability to inhibit apicoplast DNA replication and models for their mode of action proposed.[6] These findings validate DNA replication as a viable therapeutic target in the apicoplast and point towards ciprofloxacin as a potential antimalarial.

Ciprofloxacin blocks prokaryotic DNA replication by inhibiting DNA gyrase, a prokaryotic type II topoisomerase involved in untangling DNA during replication. DNA gyrase works by cutting a double-stranded DNA molecule, passing the tangled lengths of DNA through the cut, then resealing the cut to restore the circular architecture of prokaryotic DNA. A prokaryotic DNA gyrase has not yet been identified in *P. falciparum* but there is evidence of *gyrA* and *gyrB* like sequences (the genes encoding DNA gyrase) in the unpublished *P. falciparum* genome data.

Ciprofloxacin interferes with the resealing step and results in linearization of the circular DNA, thus causing death of prokaryotic organisms. Because DNA replication in mammalian cells does not involve a prokaryotic DNA gyrase, treatment of malaria with ciprofloxacin would be expected to result in few side effects. However, bacteria resistant to ciprofloxacin have been reported,[7,8] which raises the spectre that resistant strains of *P. falciparum* may also emerge if ciprofloxacin is pursued as an antimalarial. Ciprofloxacin

resistance in bacteria appears to result from single point mutations in *gyrA*.[7,8] The observed mutations conferring ciprofloxacin resistance involve residues between 83 and 94.[7,8] This suggests that residues in this region of *gyrA* are close to, or are part of, the substrate binding site, indicating that resistance is caused by a reduced binding affinity of ciprofloxacin to DNA gyrase. Thus, there may be a danger that use of ciprofloxacin as an antimalarial could lead to drug resistance by mutation of the malarial DNA gyrase. It is therefore paramount to analyze the likelihood of whether similar point mutations could arise in *P. falciparum* treated with ciprofloxacin; the presence of *gyrA* in the malaria parasite needs to be confirmed and the sequence examined for potential resistant variants. Putatively resistant versions can be constructed by genetic engineering and tested in laboratory cultures. This information should be obtained prior to further development of ciprofloxacin as an antimalarial.

TRANSCRIPTION

Transcription in plastids utilizes an RNA polymerase homologous to that of cyanobacteria and other eubacteria, the so-called α_2, β, β' DNA-dependent RNA polymerase.[9] Just as it does in bacteria, this polymerase recognises -10, -35 promoters courtesy of the σ factor and transcribes polycistronic RNAs from plastid DNA operons. The apicoplast genome encodes the β and β' subunits of the polymerase (*rpoB*, *rpoC*1, *rpoC*2 genes) strongly suggesting it uses a similar transcription system.[5] The α subunit (*rpoA*) and the sigma factor (*rpoD*) are not encoded by the apicoplast DNA so these genes are likely to be encoded by the nucleus and the products targeted into the plastid, as occurs in plants and algae for the *rpoD* gene. The α_2, β, β' polymerase of bacteria and plastids is highly sensitive to rifampicin, and antimalarial activity of rifampicin suggests this drug blocks apicoplast transcription.[5] Plant plastids also use a second phage-like RNA polymerase that is related to the RNA polymerase used by most mitochondria.[9] A phage-like RNA polymerase was recently identified in *Plasmodium falciparum* but it is believed to be the mitochondrial polymerase.[10] No drugs specific to phage type polymerase are known to us.

TRANSLATION

Numerous antibacterial agents work by inhibiting protein translation. There is currently no direct proof of translation in the apicoplast (despite the efforts of many laboratories), but, based on indirect evidence, apicoplast genes are almost certainly translated. For example, the apicoplast genome contains genes for ribosomal RNAs predicted to fold into proper ribosomal subunits, open reading frames predicted to encode many ribosomal proteins, translation components such as elongation factor-Tu (*tufA*), and a full set of tRNAs.[5] Persistence of these and other genes provides strong evidence for a translation system. Moreover, ribosome-like particles of bacterial size are visible in the apicoplast[11-15] and polysomes containing plastid rRNA and mRNA can be partially purified from erythrocyte stages of the parasite.[16] Finally, several drugs that block prokaryotic translation systems are parasiticidal.[14] Some of these drugs (including doxycycline, clindamycin and spiramycin) are used clinically for the treatment of malaria and toxoplasmosis, so it is vital that we understand how (or whether?) they affect the apicoplast.

Lincosamides (lincomycin and clindamycin) and macrolides (erythromycin and azithromycin) block protein synthesis by interacting with the peptidyl transferase domain of bacterial 23S rRNA.[17] It has long been known that these antibiotics inhibit growth of apicomplexan parasites such as *P. falciparum*, *T. gondii*, *C. parvum* and *Babesia microti* (cited in Refs 2, 18, 19)[1] and in *T. gondii* there is strong circumstantial evidence implicating blockage of apicoplast translation.[20-23]

Two thiopeptide antibacterials (thiostrepton and micrococcin) are potent inhibitors of *P. falciparum* growth in vitro[24-26] and in vivo (Sullivan et al. 2000). Thiostrepton appears to function by binding to guanosine triphosphatase (GTPase) binding domain of the large subunit rRNA blocking apicoplast translation.[24-27] Only the plastid rRNA has the thiostrepton-susceptible genotype in *P. falciparum*, so this drug is unlikely to affect cytosolic or mitochondrial rRNAs. The apicoplast rRNA of *T. gondii* does not have the susceptible genotype, and *T. gondii* growth is not inhibited by the drug (Roos unpublished).

Several other inhibitors of bacterial translation (including tetracycline and doxycycline) are also parasiticidal and may inhibit plastid translation (Table 1), although the precise target of these drugs is uncertain.[14,28]

FATTY ACID SYNTHESIS

Bacteria and chloroplasts synthesize fatty acids using a so-called Type II FAS (fatty acid synthase), which utilizes several distinct enzymes (proteins) for the steps of the fatty acid synthase biosynthetic pathway.[29] Conversely, humans and other plastid-lacking eukaryotes possess a single multi-functional protein (termed Type I) which appears to be derived from a gene fusion event incorporating several type II proteins into a single multifunctional protein.[30] Malaria parasites have long been held to be unable to synthesize fatty acids *de novo* but emerging evidence indicates that the apicoplast harbours a Type II FAS machinery. This anabolic pathway could be involved in synthesis of lipids required by the apicoplast. It has been suggested that *P. falciparum* synthesises fatty acids for formation of the parasitophorous vacuole (PV) during invasion of host erythrocytes and the requirement for apicoplast activity for successful infection is consistent with this postulate.[14,31]

The first breakthrough in the identification of a fatty acid biosynthesis pathway was the identification of genes encoding apicoplast-targeted homologues of several bacterial type II fatty acid biosynthesis sub-units.[31] One of these sub-units, ß-ketoacyl-ACP synthase (FabH), is the target for the antibiotic thiolactomycin, and thiolactomycin displays in vitro inhibition of *Plasmodium*.[31] Another type II FAS sub-unit, enoyl-ACP reductase (FabI), has also been identified in the *P. falciparum* apicoplast.[32] FabI catalyses the NADH-dependent reduction of enoyl-ACP into a saturated acyl chain.[33] FabI is the target of the broad spectrum antibacterial triclosan[34] and it was recently shown that triclosan inhibits the purified *P. falciparum* enoyl-ACP reductase, as well as inhibiting parasite growth

Table 1 **Drugs with possible targets in the malaria plastid**

Metabolic activity	Drug/herbicide	Putative target	IC_{50}	Comments	Refs
DNA Replication	Ciprofloxacin	plastid DNA topoisomerase II (*gyrA*)	*Pf* 50 μM [b] *Tg* 30 μM *Cp* 80 μg/ml	Confirmed by direct observation of apicoplast DNA in *Tg*	[6, 65–67]
RNA Transcription	Rifampicin	plastid RNA polymerase ß-subunit (*rpoB*)	*Pf* 3 μM *Tg* 3 μM [b]	Causes delayed death but no hard evidence for mode of action	[68, 69]
Protein translation	Clindamycin	plastid 23S rRNA (*rrnL*)	*Pf* 20 n*M* [b] *Tg* 10 n*M* [b] *Cp* 20 μM	Causes delayed death but no hard evidence for mode of action	[3, 22, 23, 65]
	Erythromycin		*Cp* ~40 μM	Causes delayed death but no hard evidence for mode of action	[65]
	Azithromycin		*Pf* 2 μM [b] *Tg* 2 μM *Cp* 90 μM	Causes delayed death but no hard evidence for mode of action	[21–23, 65]
	Spiramycin		*Tg* 40 ng/ml	Causes delayed death but no hard evidence for mode of action	[22, 23]
	Thiostrepton		*Pf* 2 μM *Tg* – NA	Strong indirect evidence for activity in plastid	[24, 25, 70]
	Micrococcin		*Pf* 35 n*M*	Strong indirect evidence for activity in plastid	[26]
	Chloramphenicol		*Pf* 10 μM [b] *Tg* 5 μM [b] *Cp* 300 μM	Causes delayed death but no hard evidence for mode of action	[21, 23, 28, 65]
	Doxycycline	plastid 16S rRNA (*rrnS*)	*Pf* 100 μM *Cp* 200 μM	May also target mitochondrial protein synthesis	[28, 65]
	Tetracycline		*Pf* 10 μM *Tg* 20 μM *Cp* 100 μM	May also target mitochondrial protein synthesis	[21, 28, 65]
	Amythiamicin	elongation factor Tu (*tufA*)	*Pf* 10 n*M*	Implied in *Pf* by observation of polysome formations	[71]
Amino acid biosynthesis	Glyphosate	5-enopyruvyl shikimate 3-phosphate synthase (*aroA*)	*Pf* 3 m*M* *Tg* 2 m*M* *Cp* 6 m*M*	Target enzyme characterised but pathway shown to be cytosolic rather than plastidic	[54, 55, 56]
IPP biosynthesis	Fosmidomycin	DOXP reductoisomerase	*Pf* 350 nM	Confirmed by inhibition of recombinant *Pf* enzyme	[51]
Fatty acid biosynthesis	Thiolactomycin	ß-ketoacyl-ACP synthase III (*fabH*)	*pf* 50 μM *Tg* 100 μM [b]	No direct evidence for mechanism of parasiticidal activity	[31]
	Clodinafop	acetyl-CoA carboxylase (ACC)	*Tg* 10 μM	Implied by growth inhibition in *Tg*	[48]
	Quizalofop		*Tg* 100 μM	Implied by growth inhibition in *Tg*	[48]
	Haloxyfop		*Tg* 100 μM	Implied by growth inhibition in *Tg*	[48]
	Triclosan	enoyl-ACP reductase (*fabI*)		Confirmed in vitro and in vivo in *Pf*	[32]

[a]abbreviations.

[b]David Roos unpublished.

[c]The apparent lack of an apicoplast in the related apicomplexan parasite *Cryptosporidium parvum* suggests drugs that inhibit both *C. parvum* and *P. falciparum* may additionally possess extra-plastidic targets. Indeed, where antibiotic drugs inhibit *C. parvum* at similar levels to apicoplast-possessing parasites, the target organelle may likely be the mitochondria.

in vitro and in vivo.[32] Furthermore, in vivo experiments using 40 mg/kg triclosan on blood stage malaria parasites showed no evidence of side effects,[32] suggesting that triclosan is nontoxic to human erythrocytes. Other evidence for nontoxicity comes from the safe, widespread use of triclosan in consumer products such as toothpastes, deodorants, soaps, detergents, and anti-acne preparations.[35] Taken together, these observations point towards triclosan as a promising antimalarial.

In *E. coli*, triclosan competes with enoyl-ACP for the FabI substrate site.[36] Once bound to the site, triclosan blocks oxidation of NADH and promotes binding of NAD^+ to FabI.[36] This results in formation of a noncovalent FabI-NAD^+-triclosan ternary complex which effectively removes FabI protein from the catalytic cycle and, in doing so, inhibits fatty acid biosynthesis.[36] However, McMurry et al.[37] have found that *E. coli* harbouring the point mutation Gly93 to Val in the *fabI* gene are resistant to triclosan. Analysis of the crystal structure of *E. coli* revealed that Gly93 lines the cleft of the substrate binding pocket,[37] indicating that substitution of Gly93 with Val interferes with triclosan binding. Subsequent studies have found that Val, a bulkier amino acid than Gly, protrudes into the binding pocket and occupies the same space as triclosan.[38] Thus, it has been concluded that the Val93 mutation results in triclosan resistance because of steric interference at the substrate binding pocket,[38] which ultimately prevents the formation of the FabI-NAD^+-triclosan ternary complex.[36] Additionally, Heath et al.[39] have found that the Val93 mutation and similar mutations in the region of the substrate binding pocket confer resistance to triclosan analogues and other FabI inhibitors. This implies that *E. coli* exhibiting resistance to one FabI inhibitor may exhibit cross resistance to additional FabI inhibitors.

Could triclosan resistance arise in *P. falciparum*? Similarities between the FabI proteins of *E. coli* and *P. falciparum* suggest that a similar mutation might result in triclosan resistance in *P. falciparum* compromising the usefulness of triclosan as an antimalarial. To determine whether the Val93 mutation confers triclosan resistance in *P. falciparum*, we are in the process of mutating the *fabI* gene of *P. falciparum*. The result of this study will indicate whether triclosan resistance in *P. falciparum* can arise from a point mutation and will impact on how development of triclosan as an antimalarial should be pursued.

Bacillus subtilis and *Staphylococcus aureus* also exhibit triclosan resistance.[38,40] However, unlike *E. coli*, resistance in these bacteria is not the result of mutated FabI. Rather, resistance in these bacteria results from the presence of additional genes, *fabL* in *B. subtilis*[38] and *fabK* in *S. aureus*,[40] that are able to replace FabI in FAB. FabL and FabK are not inhibited by triclosan, hence the resistance of these organisms to triclosan.[38,40] To further justify the development of triclosan as an antimalarial, it should be determined whether *P. falciparum* possesses FabL and/or FabK homologues. These caveats notwithstanding, FabI is an exciting new drug target for malaria and perhaps other infectious diseases. For instance, the mycobacterial homologue of FabI is the target of the important anti-tuberculosis drug isoniazid.[41,42] Hence, it is important that the FAB pathway of the apicoplast, and the fate of the lipids produced, be further investigated. We note, for instance, that the related parasite *Cryptosporidium* is thiolactomycin-insensitive[43] and possesses a Type I FAS. *Cryptosporidium* may lack an apicoplast[44] so the link between drug sensitivity and a type II apicoplast FAB pathway appears valid but much still remains to be learned, particularly if effective drug strategies are to be put in place.

The rate-limiting step of fatty acid synthesis in plants is the supply of malonyl-CoA by the chloroplast acetyl-CoA carboxylase (ACC), an enzyme targeted by the arloxyphenoxypropionate class of anti-graminicidal herbicides.[45,46] While the chloroplast ACC is a single large polypeptide in the grasses, in other plants it is made up of four separate sub-units that are insensitive to arloxyphenoxyproprionates. *T. gondii* possesses an apicoplast-targetted ACC carboxylase similar to the multi-domain chloroplast enzyme found in diatom algae and grasses,[47] and *Toxoplasma* is likewise sensitive to arloxyphenoxyproprionate herbicides.[48] Several of these herbicides also inhibited *T. gondii* growth in human fibroblasts.[48] A likely apicoplast-targeted ACC is also found in *P. falciparum*.[47] Some apicomplexan parasites also appear to possess cytosolic isoforms of the enzyme, which, as with those isoforms in plants, are expected to be herbicide-insensitive.[47,48] Another class of herbicides targeting the ACC of grass, the cyclohexanediones (see [45] for review), did not inhibit the *T. gondii* ACC.[48] Progress is being made towards an understanding of the specific interaction between herbicides and ACC,[49] which may pave the way for the rational design of more potent ACC inhibitors.

ISOPENTENYL DIPHOSPHATE SYNTHESIS

Isopentenyl diphosphate (IPP) is the precursor for the structurally diverse isoprenoid class of compounds. In animals and plants, IPP is synthesized via the classical mevalonate pathway. An alternate pathway proceeding via 1-deoxy-D-xylulose 5-phosphate (DOXP) has recently been elucidated in chloroplasts and bacteria,[50] and two enzymes from this pathway have been identified in *P. falciparum*.[51] One of these enzymes, DOXP reductoisomerase, is the target of the antibiotic fosmidomycin, and recombinant *P. falciparum* DOXP reductoisomerase is inhibited by fosmidomycin.[51] Fosmidomycin also inhibits the growth of *P. falciparum* in culture, and cures malaria in a mouse model.[51] Assays to test potential inhibitors of DOXP enzymes in bacterial systems[52] and in plant test systems[53] have already exposed new inhibitors of DOXP synthase (the first step of IPP synthesis). It remains to be seen if these compounds act against the *P. falciparum* DOXP synthase and if so, whether they inhibit parasite growth.

AROMATIC AMINO ACID SYNTHESIS

Aromatic (essential) amino acids are synthesized by the shikimate pathway, which is located in the plastid in plants and algae. Animals lack a shikimate pathway, which is why aromatic amino acids are essential in their diet. *Plasmodium* has been presumed to be auxotrophic for aromatic amino acids, but this paradigm has recently been called into question with the discovery of a shikimate pathway in *Plasmodium*, *Cryptosporidium* and *Toxoplasma*.[54] This might explain the parasiticidal activity of glyphosate, a common herbicide (RoundUp™, Zero™, Tumbleweed™), which blocks the

activity of 5-enopyruvyl shikimate 3-phosphate synthase.[54] Although the shikimate pathway may provide a useful target for parasiticidal drug design, these enzymes appear to be located in the parasite cytosol[55,56] (as they are in fungi) and not in the apicoplast as initially implied.

HEME SYNTHESIS

Animals synthesize heme from glycine and succinyl CoA using the Shemin pathway, but plastids (and their cyanobacterial forebears) use an unusual pathway starting with glutamate ligated to tRNA-Glu.[57] The Shemin pathway apparently occurs in *P. falciparum*[58] – presumably in the mitochondrion and cytosol – but there may also be a cyanobacterial-type heme pathway in the apicoplast. For instance, a likely apicoplast-targeted dehydratase (a key enzyme of heme synthesis) has been identified in *P. falciparum* that groups with the predominantly Mg^{2+} binding plastid dehydratases, rather than the Zn^{2+} binding mitochondrial equivalents.[59] However, another heme synthesis enzyme has been identified which bears an apparent mitochondrial-targeting signal.[60] Moreover, Bonday and colleagues[61] have speculated that *P. falciparum* uses an additional dehydratase imported from the erythrocyte cytosol. Locallization of the enzymes recognised to-date, and the characterization of the remaining unidentified enzymes of the heme synthesis pathway should help clarify this currently confusing issue. Heme synthesis is an established target for herbicides,[62] and given *P. falciparum* possesses at least one plant-like heme synthesis enzyme, the elucidation of the full pathway should illuminate further potential drug targets.

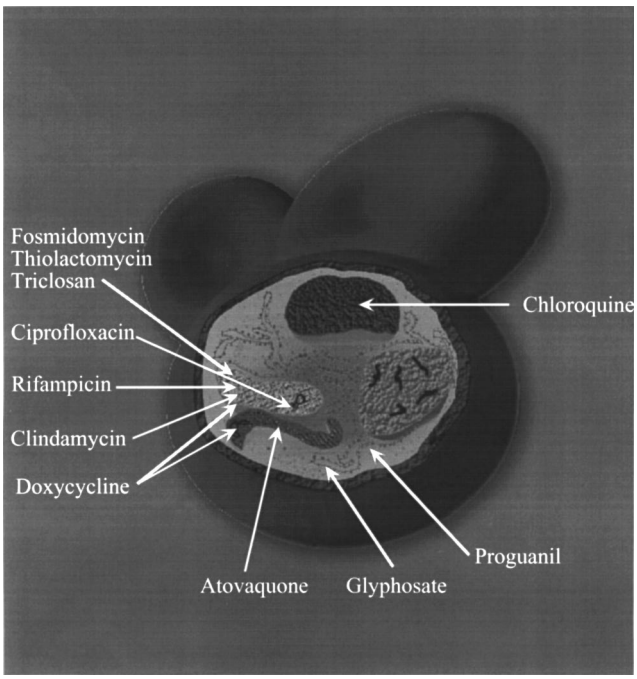

Fig. 1 **Anti-malarials inhibit essential processes located in several parasite sub-cellular compartments. The image depicts three erythrocytes, one of which (cut away) is infected with a malarial parasite. Among the commonly used anti-malarials, proguanil targets the cytosolic folate biosynthetic pathway, chloroquine interferes with the process of haem detoxification in the food-vacuole (brown), and atovaquone inhibits the mitochondrial electron transport chain (For reviews see 63, 64). Triclosan and thiolactomycin inhibit apicoplast (green) fatty acid synthesis, while fosmidomycin targets the apicoplast-based DOXP pathway. Ciprofloxacin prevents the replication of the apicoplast genome and rifampicin acts upon apicoplast RNA transcription. Clindamycin seems to act upon apicoplast protein translation, while Doxycycline appears to inhibit both apicoplast and mitochondrial protein translation. Conversely, glyphosate inhibits a shikimate pathway that appears to be cytosolic, rather than plastidic.**

CONCLUSION

The malaria plastid presents a broad range of potential new chemotherapeutic options to combat the disease (Fig. 1). After only a few years of research, a relatively large number of antimalarial compounds with alleged targets in the apicoplast have already been identified (Table 1). If these targets turn out to be viable in therapy, an enormous head start for drug development is already in hand. Progress has been rapid thanks to the availability of genome data to identify genes encoding these targets. Since the mode of action, and potential mechanisms of resistance, for many of these lead compounds is already established in bacterial systems, we can anticipate relatively rapid progress towards utility. This array of new targets complements the existing collection of targets in other subcellular compartments of the parasite (Fig. 1) and offers increasing hope for management of the disease.

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