

# Housekeeping and other metabolic functions of the *Plasmodium* plastid

Afreen Haider, Ankit Gupta, Suniti Vaish, Bijay Kumar, Manish Charan, Snober S. Mir, Aiman Tanveer, Ashima Sinha and Saman Habib\*

Division of Molecular and Structural Biology, CSIR–Central Drug Research Institute, Lucknow 226 001, India

The malaria parasite carries a plastid called the apicoplast that has been the subject of intense study in the last 15 years. Having originated from red-algal plastids, the apicoplast has lost its ability to photosynthesize, but carries out other essential functions such as type-II fatty acid synthesis, biosynthesis of haem and isoprenoid synthesis; the DOXP pathway for isoprenoid synthesis has recently been demonstrated to be the only pathway critical for parasite survival in the erythrocytic stage. The apicoplast also has a functional Suf system for assembly of (Fe–S) complexes on target proteins. The organelle has a 35 kb, double-stranded DNA genome that encodes a set of RNAs and proteins, the latter being translated from organellar mRNA by an active translation machinery, a major component of which is encoded by the nucleus. This article reviews current knowledge of housekeeping functions of the *Plasmodium* apicoplast and its (Fe–S) assembly system and discusses these components as sites for drug intervention against malaria.

**Keywords:** Apicoplast, drug intervention, housekeeping functions, malaria parasite.

## Introduction

THE malaria parasite and most of its protist cousins in the group Apicomplexa contain a non-photosynthetic relic plastid called the apicoplast. The organelle was discovered in the mid to late 1990s by Wilson and colleagues, who first identified a genome distinct from the *Plasmodium* mitochondrial DNA<sup>1</sup>. The sequence of this 35 kb circular DNA molecule revealed its close resemblance to plastid genomes and the molecule was subsequently localized to a distinct organelle by *in situ* hybridization<sup>2</sup>. Labelling by a fluorescent apicoplast targeted protein revealed the changing morphology of the apicoplast in the blood stages of the *Plasmodium falciparum* life cycle<sup>3</sup>. The apicoplast exists in close proximity to the mitochondrion of *Plasmodium* and attains an intricate branched structure in the trophozoite stage. Upon

schizogony, each daughter merozoite gets a single apicoplast. The organelle is seen in all stages of the *Plasmodium* cycle and is maternally inherited<sup>4</sup>.

The apicoplast is enclosed by four membranes, suggesting its origin as a secondary endosymbiont. It is now believed that the organelle originated from two endosymbiotic events, the first involving a cyanobacterium-like prokaryote and a primary host, and the second involving an endosymbiotic alga and a eukaryotic host. Despite early disagreements over the green or red algal lineage of the second endosymbiont, there is emerging consensus that it was the latter<sup>5</sup>. This view has been strengthened by the discovery of the photosynthetic, free-living apicomplexan *Chromera velia*, which clearly has a plastid derived from red alga<sup>6</sup>.

The genome of the apicoplast is much reduced in comparison with other plastid genomes and primarily encodes components for transcription and translation<sup>1,7</sup>. There are also ORFs that encode a predicted Ycf24/SufB protein of the (Fe–S) complexation pathway and a Clp protease<sup>1</sup>. The apicoplast is essential to the parasite and inhibition of its housekeeping functions causes parasite death<sup>8</sup>. As its own coding capacity is limited, the organelle imports an array of nuclear-encoded proteins that participate in important metabolic pathways that include type-II fatty acid biosynthesis<sup>3,9</sup>, non-mevalonate pathway of isoprenoid biosynthesis<sup>10</sup> and the synthesis of haem<sup>11,12</sup>. Proteins transported to the apicoplast require a bipartite leader sequence carrying an N-terminal signal and transit peptide, which can be identified by a set of rules<sup>13</sup>. This has helped predict an apicoplast proteome for identification of pathways operative in the organelle<sup>14</sup>.

The requirement of a functional apicoplast for parasite survival as well as the cyanobacterial origin and consequent existence of unique pathways and proteins in the organelle have identified it as an attractive target for anti-malarial drug intervention. However, several important functions and pathways of the organelle are only partly understood. This article presents an overview of the current knowledge of mechanisms of DNA replication and translation in the apicoplast, discusses the Suf system of (Fe–S) cluster biogenesis in the organelle, and briefly reviews the status of the apicoplast as a target for anti-malarials.

\*For correspondence. (e-mail: saman\_habib@cdri.res.in)

### Apicoplast genome organization and replication

Each *Plasmodium* carries a single apicoplast with estimates of apicoplast DNA (pDNA) copy number varying from 1 to 15. The ~35 kb double-stranded pDNA molecule has an A + T content of ~86% (ref. 1). A single pDNA circle is ~12  $\mu\text{m}$  in circumference and several molecules have to be packed into an organelle with a diameter of only ~0.3  $\mu\text{m}$  (ref. 15). pDNA also has to replicate and divide into daughter molecules without getting tangled. This would require the involvement of a DNA-compacting protein in pDNA organization. The *P. falciparum* nuclear-encoded bacterial histone-like protein (*PfHU*) has been identified as the major DNA organization protein in the organelle<sup>16,17</sup>. A gene encoding a HU-ortholog that carries a conserved BHL domain (bacterial histone-like domain) together with a predicted N-terminal apicoplast targeting sequence was identified on Chr 9 of the *P. falciparum* nuclear genome. HU proteins are small basic proteins of prokaryotic origin that are structurally distinct from eukaryotic histones, belong to the DNABII family of DNA-binding proteins, and exhibit hetero- or homo-dimeric DNA binding. HU proteins also have regulatory effects on DNA replication, recombination and transcription. *PfHU* is expressed throughout the intra-erythrocytic phase of parasite growth, exhibits DNA binding and is capable of condensing DNA. The protein has a unique 42aa C-terminal domain which influences its interaction with the DNA<sup>16</sup>. In contrast to bacterial HUs that bend DNA, *PfHU* promotes concatenation of linear DNA and inhibits DNA circularization<sup>16</sup>. Atomic force microscopic study of *PfHU*-DNA complexes shows protein concentration-dependent DNA stiffening, intermolecular bundling and formation of DNA bridges followed by assembly of condensed DNA networks<sup>16</sup>. The bundling of two strands is likely to be mediated by the interaction between two *PfHU* dimers, each of which binds to a single DNA strand; the formation of such tetrameric *PfHU* forms is indicated by chemical cross-linking of the protein in solution. Further, the clustering of long DNA bundles and bridges to form large complexes at high *PfHU* concentration could be mediated by intermolecular interactions between *PfHU* tetramers. The presence of a functional HU-like protein in apicomplexan plastids provides further support for red algal ancestry of the apicoplast; the sequence of *PfHU* is closest to red algal plastid HUs of *Cyanidioschizon merolae* and *Guillardia theta*, and the apicomplexan and red algal HUs cluster with HU proteins of cyanobacteria. The presence of a nuclear gene encoding a HU protein carrying the bipartite signal sequence for apicoplast targeting in the malaria parasite indicates that the gene was acquired by a secondary endosymbiotic event from a red alga with subsequent transfer from the red algal plastid to the host nuclear genome.

Although there is extensive sequence similarity between pDNA of *P. falciparum* and the related apicomplexan, *Toxoplasma gondii*, the two molecules have distinct *in vivo* topology. The former is circular while the latter occurs as an oligomeric series of linear tandem arrays of the 35 kb genome. pDNA of *T. gondii* replicates via the rolling circle mode<sup>18</sup>, while the major replication mode for *P. falciparum* pDNA is via D-loop/bidirectional *ori*<sup>19,20</sup>. pDNA replication proceeds at the late trophozoite-early schizont stage of the intraerythrocytic cycle, and origins of replication (pDNA *ori*) have been localized within the inverted repeat (IR) region of the pDNA molecule<sup>19,20</sup>. The IR region of pDNA comprises genes for small and large subunit rRNAs and some tRNAs. Multiple initiation sites have been identified within each IR sector, with initiation occurring with different frequencies during the replication cycle<sup>21</sup>.

The apicoplast genome lacks genes encoding a protein for DNA replication. Thus, all major protein components involved in pDNA replication must be nuclear-encoded and transported to the organelle. Some components of the pDNA replication machinery have been identified. A ~220 kDa multi-domain polypeptide (*PfPREX*) that contains DNA polymerase as well as DNA primase, DNA helicase and 3'-5' exonuclease activities has been proposed as a key enzyme for pDNA replication<sup>22</sup>. The kinetic parameters and fidelity of the DNA polymerase domain of *PfPREX* have been reported recently<sup>23</sup>. An apicoplast-targeted, single-strand, DNA-binding protein that interacts with pDNA has also been identified<sup>24</sup>. Genes encoding putative apicoplast-targeted gyrase A and B have been located on Chr12 of *P. falciparum*, and gyrase B has been functionally characterized<sup>25,26</sup>. Ciprofloxacin and novobiocin, that target bacterial DNA gyrase A and B subunits respectively specifically inhibit replication of *P. falciparum* pDNA and also reduce parasite growth in culture<sup>2,8,26</sup>. Ciprofloxacin has been shown to specifically increase the number of double-strand breaks in *P. falciparum* apicoplast DNA, but not affect nuclear DNA<sup>27</sup>. *Plasmodium* apicoplast DNA replication is thus a validated drug target.

### Protein synthesis in *Plasmodium* organelles

The pathway of protein translation in *Plasmodium* organelles, particularly the apicoplast, is of interest in the search for new drug targets. Most genes within the apicoplast genome encode players involved in translation. These include 18 ribosomal proteins, large and small subunit rRNA, elongation factor Tu and a minimal set of 25 tRNAs sufficient for successful synthesis of proteins within the compartment<sup>1</sup>. The parasite plastid is now known to be an active site for synthesis of proteins, evidence for which was provided by the detection and organellar localization of apicoplast-encoded protein EF-

Tu by Western blot and immunofluorescence<sup>28</sup>. Prior indirect evidences for apicoplast as a translation-competent organelle were the detection of 70S ribosome-like particles by electron microscopy<sup>29</sup>, and polysomes containing plastid-type rRNA and mRNA<sup>30</sup>.

Considering the endosymbiotic descent of the plastid, it is assumed that the mechanism and constituents of the process will be essentially prokaryotic. The apicoplast ribosomal complex is predicted to include ~35 ribosomal proteins, all of which are not translated in the plastid. Sparing the 18 that are plastid-encoded, the rest are predicted to be nuclear-encoded and transported to the apicoplast. The tRNA repertoire in the apicoplast genome is sufficient for carrying the 20 amino acids needed for translation of proteins encoded by the organelle<sup>31</sup>, whereas the mitochondrial genome lacks any genes for tRNAs<sup>32</sup>. A significant fraction of the parasite proteome is dedicated to aminoacyl-tRNA synthetases (aaRSs), which catalyse the charging of tRNAs with the cognate amino acid<sup>33</sup>. Computational analyses have revealed that out of 37 aaRS encoded by the nuclear genome, 32% is apicoplast-targeted and 38% is cytosolic<sup>33</sup>. While very few *Plasmodium* aaRSs are predicted to be mitochondria-targeted and there is also an indication of dual targeting of aaRSs between organelles in *Plasmodium*<sup>33</sup>, a study in *T. gondii* has revealed that aaRSs are altogether absent in mitochondria, thus suggesting that aminoacylated tRNAs are imported into the organelle<sup>34</sup>.

Apart from ribosomal particles and tRNA, mRNA translation depends upon vital protein translation factors that catalyse each step in the process providing kinetic stability to the intermediate complexes. Of these, except for elongation factor Tu, none is encoded by the plastid genome. Hence, it is obvious that the rest will have to be translocated from the cytosol to complement the resident translation machinery. The apicoplast elongation factor Tu is a 47 kDa protein belonging to the family of guanine nucleotide-binding proteins. EF-Tu mainly serves as a carrier for aminoacylated elongator-tRNAs during the elongation phase. Recombinant EF-Tu shows GTP hydrolysing activity and also an ancillary chaperone-related disulphide reductase function. Recently, another elongation factor, EF-Ts, necessary for the turnover of GTP on EF-Tu for continuous elongation cycles, has been localized to the plastid using immunofluorescence of N-terminal GFP tagged leader peptide of *Pf*EF-Ts. The factor has also been demonstrated to interact with *Pf*EF-Tu to mediate GDP release from the latter, although the rate of this turnover is lower when compared to the bacterial system, which is understandable considering the less amount of translation needed in the organelle<sup>35</sup>. Interestingly, a mitochondrial EF-Ts is not identified on the *Plasmodium* genome, suggesting that since only three genes need to be translated the mitochondria can do without a EF-Ts catalysed turnover of the guanine nucleotide on EF-Tu<sup>35</sup>. Elongation factor EF-G that aids translocation of the pep-

tidyl tRNA from the ribosomal A site to the P site in prokaryotes is nuclear-encoded in *Plasmodium* species. Localization of two EF-Gs predicted to be targeted to the apicoplast and mitochondrion, has been confirmed by immunofluorescence analyses of parasites expressing C-terminal GFP and 3XHA tagged EF-Gs respectively<sup>36</sup>.

Although considerable attention has been directed towards elongation factors involved in apicoplast protein synthesis, the initiation and release factors targeted to the organelle have not been characterized. Prokaryotic translation initiation involves the participation of three initiation factors IF1, IF2 and IF3 (ref. 37), all of which are predicted to be nuclear-encoded and transported to the apicoplast in *Plasmodium*. In bacteria, IF3 is known as the anti-association factor as it binds at the contact point of 50S and 30S ribosome subunits for keeping the ribosome in a split state<sup>38-40</sup>. It thus helps in maintaining the pool of free ribosome subunits for unhindered translation initiation via the 30S pre-initiation complex. IF1 is a small protein which mainly binds at the A site in the ribosome of the initiation complex and prevents premature binding of elongator tRNA<sup>41,42</sup>, but also shows 70S ribosome dissociation activity<sup>38</sup>. While IF1 and IF3 are predominantly involved in ribosomal splitting, IF2, an essential GTPase, is responsible for recruitment of specific initiator tRNA to the mRNA start codon<sup>43</sup>. The apicoplast DNA contains a gene for the probable initiator Met<sub>i</sub>-tRNA which may be aminoacylated by N-formylmethionine and be carried by IF2 to the initiation complex in accordance to its prokaryotic counterpart. The aminoacylation and formylation reactions are thought to be catalysed by putative methionyl-tRNA synthetase (MRS) and methionine-tRNA formyltransferase (FMT) respectively, both of which are nuclear-encoded proteins and contain positive targeting signals for apicoplast compartmentalization<sup>44</sup>. A putative nuclear-encoded peptide deformylase (PDF) which removes formyl group from nascent polypeptides is also predicted to be apicoplast localized, indicating the use of formylmethionine as the initiating amino acid in apicoplast-translated proteins<sup>44</sup>. Both FMT and PDF in *Toxoplasma* are targeted uniquely to the apicoplast and are absent from the mitochondria<sup>34</sup>. The absence of these enzymes in the mitochondria either means use of an unformylated initiator Met-tRNA, or a possible transport of formylated Met-tRNA from the plastid.

Termination of translation is supported by three release factors RF1, RF2 and RF3, in prokaryotes. In bacteria, class-I release factors RF1 and RF2 recognize the stop codons UAA/UAG and UAA/UGA respectively<sup>45</sup>, and by mimicking aminoacylated elongator-tRNAs in the ribosomal A-site promote peptide chain release from the last elongator tRNA in the P-site<sup>45-47</sup>. UAA is the most commonly encountered stop codon in *P. falciparum* apicoplast genes in the plasmoDB database. Class II-release factor RF3 is a GTPase which functions in recycling of RF1 or RF2, and their removal from the ribosomal A site

after hydrolysis of the peptidyl-tRNA bond<sup>48</sup>. While there are putative candidates for nuclear-encoded RF1 and RF2 targeted to the plastid, a similar homologue for the RF3 seems to be missing<sup>44</sup>. Various candidate genes that can serve as possible apicoplast and mitochondrial homologues of bacterial translation initiation factors and release factors have been shortlisted after bioinformatic analyses of the *Plasmodium* genome database, as described in Jackson *et al.*<sup>44</sup>.

### Assembly of (Fe–S) complexes on apicoplast proteins

Iron-sulphur (Fe–S) complexes assembled on target proteins are known to play critical roles in electron transfer as well as redox and non-redox catalysis. They participate in important biological functions by serving as catalytic centres in electron transfer enzymes<sup>49,50</sup>. Fe ions in the complex are coordinated by cysteines from the protein and are linked to each other through sulphide bridges. These clusters are found in all living organisms and are most commonly assembled as (2Fe–2S), (3Fe–4S) and (4Fe–4S). Three distinct operons, the *nif*, *isc* and *suf* operons, encode proteins required for (Fe–S) cluster biosynthesis in prokaryotes<sup>51–53</sup>. There is evidence that in bacteria, NIF (NifS, NifU required for maturation of the nitrogenase enzyme) and ISC (iron-sulphur cluster formation) are the housekeeping (Fe–S) assembly systems<sup>54</sup>, while SUF (sulphur mobilization, SufA, B, C, D, S, E) is adapted to assemble (Fe–S) clusters under environmental conditions such as iron starvation or oxidative stress<sup>55,56</sup>. The ISC system is present in bacteria and most eukarya, and the SUF system is found in archaea, bacteria, plants and protozoa. Plant chloroplasts use the SUF system for (Fe–S) biogenesis.

In general, several proteins act to assemble (Fe–S) clusters on target proteins. A cysteine desulphurase (NifS, IscS and SufS) is used by the three systems for the utilization of L-cysteine as a source of sulphur. A scaffold protein (NifU, NFU, IscU/ISU, IscA/ISA, SufU and SufA) then mobilizes sulphur atoms from a cysteine desulphurase and iron atoms from an iron donor to synthesize an (Fe–S) cluster. Subsequently, the cluster is transferred to an apoprotein target to yield the holoprotein<sup>57</sup>. In addition to SufS, U and A, the SUF system is comprised of the SufB, C, D and E proteins. Three of these (SufBCD) form a tight complex<sup>58</sup>. SufC exhibits ATPase activity which is enhanced by SufB, as shown in *Thermotoga maritima*<sup>59,60</sup>. It has been proposed that the SufBCD complex may also function as the assembling centre for (Fe–S) clusters<sup>61</sup>. Bacterial SufE interacts with SufS, and the cysteine desulphurase activity of SufSE is stimulated by the SufBCD complex<sup>61,62</sup>.

The existence of a (Fe–S) cluster biogenesis pathway in both the apicoplast and mitochondria of *P. falciparum*

has been proposed<sup>63,64</sup>, and several organellar proteins that participate in anabolic pathways are predicted to be modified by (Fe–S) prosthetic groups. Ferredoxin (Fd), IspG, IspH (involved in isoprenoid biosynthesis), LipA (lipoic acid synthase) and MiaB (tRNA methyltransferase) are apicoplast-targeted proteins which require (Fe–S) clusters; mitochondrial electron transport chain members such as the Rieske protein, a subunit of succinate dehydrogenase, aconitase/IRP and class-I fumarate hydratase also require (Fe–S) assembly<sup>65,66</sup>. The (Fe–S) biogenesis pathway is likely to exist independently in the two organelles. Nuclear-encoded proteins of *P. falciparum* that exhibit homology to some ISC and SUF system components, which also carry predicted organelle targeting sequences have been identified<sup>63,67</sup>. The machinery for mitochondrial (Fe–S) cluster assembly includes homologs of the cysteine desulphurase NFS and scaffold proteins ISU and ISA<sup>68,69</sup>. Other mitochondrial proteins such as Fd and Fd reductase, chaperones Hsp70, DnaJ and GrpE may also be involved<sup>70</sup>. Proteins predicted to be involved in apicoplast (Fe–S) biogenesis are primarily of the SUF system. SufC, D, E and S are nuclear-encoded and a SufB analog (also called ORF470, Ycf24) is encoded by the apicoplast genome. The nuclear-encoded putative SUF components carry predicted N-terminal bipartite apicoplast targeting elements<sup>13</sup>. Only one scaffold protein (NifU) exhibiting high homology with the cyanobacterium *Synechocystis* NifU is predicted to be apicoplast-targeted.

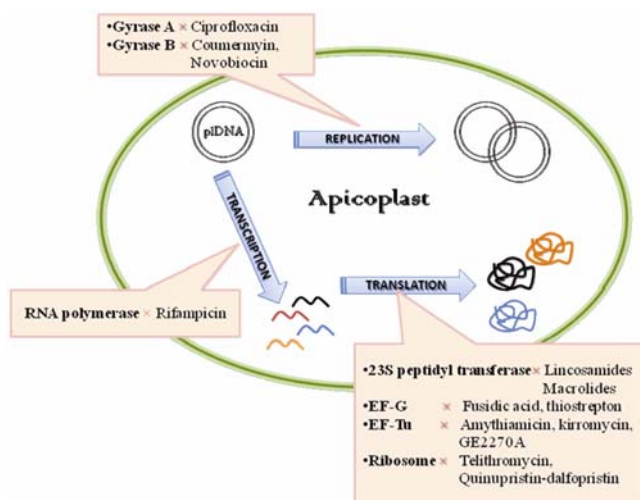
Recent biochemical characterization of the *P. falciparum* apicoplast-targeted SufC and its interaction with apicoplast-encoded SufB has provided functional evidence for the existence of the SUF (Fe–S) complexation pathway in the apicoplast<sup>69</sup>. SufC exhibits ATPase activity and localizes to the organelle. The two recombinant proteins interact *in vitro* and column-bound recombinant SufC co-elutes SufB from the *P. falciparum* lysate. Investigation of other components of the pathway is complicated by the difficulty of expressing functional recombinant proteins. The absence of the SUF pathway from the human host suggests the possibility of investigating it as a target for antimalarial compounds<sup>5</sup>. However, the absence of any known inhibitors for Sufs makes it difficult to test the hypothesis pharmacologically and conditional knockout mutants would be required to confirm the essential nature of the (Fe–S) pathway in *Plasmodium*.

### Apicoplast housekeeping pathways as targets for antimalarials

Early studies revealing the requirement of a functional apicoplast for parasite survival used the fluoroquinolone ciprofloxacin, a selective-inhibitor of DNA gyrase A (Figure 1). Ciprofloxacin was shown to inhibit apicoplast

DNA replication in *P. falciparum* without affecting the replication of the parasite nuclear DNA<sup>2</sup>. The drug has been reported to cause parasite death both in the first infection cycle as well as in a delayed-death manner<sup>71,72</sup>, and chemical derivatives of ciprofloxacin that enhance its antimalarial activity have been reported<sup>73</sup>. The coumerin drugs, coumermycin and novobiocin, inhibit GyrB and also exhibit anti-plasmodial activity. The intrinsic ATPase activity of the 45 kDa GyrB domain of *P. vivax* was shown to be sensitive to coumermycin, although at a higher  $K_i$  than reported for *E. coli* GyrB (ref. 74) and novobiocin was shown to inhibit the ATPase activity of *Pf*GyrB with a  $K_i$  of 1.24  $\mu\text{M}$ , a value  $\sim 3.5$ -fold higher than that reported for full-length *E. coli* GyrB (0.35  $\mu\text{M}$ )<sup>26</sup>. Specific reduction in apicoplast/nuclear DNA ratios compared to mitochondrial/nuclear DNA ratios was observed upon novobiocin treatment in *P. falciparum*, indicating that the drug inhibited apicoplast DNA replication<sup>26</sup>. The elucidation of activity and structures of other DNA replication proteins such as the DNA polymerase subunit should aid in the identification of newer sites for drug design and intervention.

Transcription of apicoplast genes utilizes a eubacterial ( $\alpha_2\beta\beta'$ ) system of DNA-dependent RNA polymerases that was inherited with the cyanobacterial endosymbiont<sup>75</sup>. The apicoplast genome encodes part of this system ( $\beta$  subunit by *rpoB*, and  $\beta'$  subunit by *rpoC1* and *rpoC2*), while the remainder ( $\alpha$  subunit, as well as sigma factor) are apparently encoded in the nucleus and targeted back to the apicoplast. The RNA polymerase of plastids is highly sensitive to rifampicin, and the *in vitro* and *in vivo* antimalarial activity of rifampicin suggests that the drug blocks apicoplast transcription<sup>76,77</sup> (Figure 1). Trials with rifampicin in patients infected with *P. vivax* showed that although the drug, when given alone, cleared fever and reduced parasitaemia initially, it was not curative.



**Figure 1.** Antimalarial checkpoints in housekeeping pathways of the *Plasmodium* apicoplast.

Although all patients treated with the rifampin–primaquine combination cleared both fever and parasitaemia, the therapeutic responses were slower than those following treatment with the chloroquine–primaquine combination<sup>76</sup>. Initial studies using a fixed combination of cotrimoxazole, rifampicin and isoniazid (Cotrifazid) had shown efficacy against resistant strains of *P. falciparum* in animal models and in small-scale human studies. However, a multicentric trial to test the safety and efficacy of Cotrifazid concluded that the combination, in the formulation and regimen used, was not an attractive alternative combination therapy for malaria<sup>78</sup>.

Antibiotics that target prokaryotic translation have been demonstrated to exhibit antimalarial activity (Figure 1), although their inhibitory effect on parasitic growth is often slow. Despite this slow action, clinical studies have demonstrated good malaria cure rates with the translation inhibitors tetracycline and clindamycin when used in combination with a faster acting drug<sup>79,80</sup>, and doxycycline is recommended by WHO as a prophylactic for travellers to endemic regions<sup>81</sup>. Investigations in *T. gondii* facilitated our understanding of antimalarial activities of these antibiotics and led to the observation of the ‘delayed-death’ phenomenon wherein the progeny of the parasite treated with the drug were more severely affected than the parasite itself<sup>2</sup>. Similar phenotypes were observed in *P. falciparum* treated with tetracycline, clindamycin and azithromycin<sup>71,72,82</sup>. These antibiotics affected the formation of functional merozoites in the second cycle, such that the abnormal merozoites were incapable of rupturing the host cell to establish fresh infection<sup>83</sup>.

Translation inhibitory antibiotics act on different components of the prokaryotic translation machinery. For instance, lincosamides (lincomycin and clindamycin) and macrolides (erythromycin and azithromycin) block protein synthesis by interacting with the peptidyl transferase domain of bacterial 23S rRNA. Clindamycin and chloramphenicol both act by blocking translocation of peptidyl-tRNAs and interfere with peptidyl transferase activity<sup>84</sup>. Tetracyclins interrupt translation by blocking the binding of the peptidyl-tRNA to the acceptor on the ribosomal small subunit<sup>85</sup>. Azithromycin has been shown to interact with domains IV and V of the 23S rRNA and the ribosomal proteins L4 and L22 (ref. 86). Thiostrepton and micrococin also inhibit parasite growth. Thiostrepton binds to 23S rRNA and inhibits the activity of EF-G by blocking Pi release after GTP hydrolysis. Thiostrepton has been reported to inhibit total *P. falciparum* protein synthesis at an  $\text{IC}_{50}$  that is greater than that for inhibition of parasite growth, suggesting that the principal target of the drug is different from cytoplasmic protein synthesis<sup>87,88</sup>. The observation that the GTPase domain of the *P. falciparum* apicoplast LSU rRNA contains adenine nucleotide at two positions and is thus the likely target molecule for thiostrepton supported the apicoplast as the

primary site of action of the drug. Mitochondrial and nuclear LSU rRNAs have altered residues at one or both of these positions, and are thus unlikely to interact with the drug<sup>89</sup>. However, thiostrepton inhibits erythrocytic *Plasmodium* growth in both the first and second cycles of infection (countering the 'delayed death' phenomenon observed for apicoplast-specific drug action), and it has been recently suggested to additionally act on the parasite proteasome as well as affect the mitochondrion<sup>90,91</sup>. Antibiotics that interact with the translation factor EF-Tu (amythiamicin, GE2270A, and kirromycin) also exhibit antimalarial activity in blood culture<sup>92</sup> and kirromycin affects apicoplast EF-Tu activity *in vitro*<sup>35</sup>. The steroid antibiotic fusidic acid targets EF-G and experiments in our laboratory have demonstrated the effect of this drug on apicoplast translation as well as interaction of apicoplast EF-G with the ribosome (Gupta *et al.* unpublished results). In addition, ribosome-blocking antibiotics telithromycin and quinupristin-dalfopristin have also been shown to inhibit the growth of *P. falciparum* in a delayed-death manner<sup>93</sup>, suggesting their action on apicoplast translation.

There is accumulating evidence that components of the apicoplast translation apparatus are the actual targets of antibiotics that exhibit antimalarial effects. Apicoplast-specific effects such as disruption of protein import into the organelle by clindamycin and tetracycline<sup>71</sup> and doxycycline-mediated block in expression of the apicoplast genome resulting in the distribution of non-functional apicoplasts during erythrocytic schizogony<sup>83</sup> have been implicated in the antimalarial action of these drugs. 15-Deoxyspergualin that binds Hsp70 also shows apicoplast-specific inhibition leading to delayed death of parasites by blocking the import of nuclear-encoded proteins into the plastid<sup>94</sup>. Azithromycin resistance generated in parasite lines has been attributed to a point mutation in the *P. falciparum* LSU rRNA as well as the apicoplast-encoded ribosomal protein subunit<sup>82</sup> Rpl4, and a point mutation in the LSU rRNA gene of the *T. gondii* apicoplast has been shown to confer resistance to clindamycin *in vitro*<sup>84</sup>. Live microscopy on *Plasmodium berghei* has shown that microbial translation inhibitors also block development of the apicoplast during liver-stage schizogony and lead to impaired parasite maturation<sup>95</sup>. Identification of additional targets in the organellar protein translation apparatus may offer new sites for drug intervention and contribute to enhancing the drug repertoire against resistant strains.

Although the unique pathways and proteins of the apicoplast offer opportunities for identification of novel drug targets, the evaluation of the precise target-specific activities of lead compounds remains a challenge. For instance, it is possible that inhibitors believed to target the apicoplast translation process may also have off-targets at other locations in the parasite, including the other organelle of prokaryotic origin – the mitochondrion.

A very recent development that will aid in confirming the apicoplast as the site of action of specific antimalarials is the chemical rescue of apicoplast minus *P. falciparum* parasites in asexual blood stages by isopentenyl pyrophosphate (IPP), which is the product of the non-mevalonate isoprenoid biosynthesis in the apicoplast<sup>95</sup>. Maintaining IPP supply seems to be the only essential function of the relic plastid. Thus, apicoplast-specific action of an active compound can be assayed by rescue of the treated blood-stage parasites on supplementation with IPP<sup>96</sup>. In addition, attention should also be focused on the role of the apicoplast in the liver stages of the parasite cycle, where its metabolic activities may be different from those in the blood stages.

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