REVIEW ARTICLE

'FAS't inhibition of malaria

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Malaria, a tropical disease caused by *Plasmodium* sp., has been haunting mankind for ages. Unsuccessful attempts to develop a vaccine, the emergence of resistance against the existing drugs and the increasing mortality rate all call for immediate strategies to treat it. Intense attempts are underway to develop potent analogues of the current antimalarials, as well as a search for novel drug targets in the parasite. The indispensability of apicoplast (plastid) to the survival of the parasite has attracted a lot of attention in the recent past. The present review describes the origin and the essentiality of this relict organelle to the parasite. We also show that among the apicoplast specific pathways, the fatty acid biosynthesis system is an attractive target, because its inhibition

decimates the parasite swiftly unlike the 'delayed death' phenotype exhibited by the inhibition of the other apicoplast processes. As the enzymes of the fatty acid biosynthesis system are present as discrete entities, unlike those of the host, they are amenable to inhibition without impairing the operation of the host-specific pathway. The present review describes the role of these enzymes, the status of their molecular characterization and the current advancements in the area of developing inhibitors against each of the enzymes of the pathway.

Key words: antimalarial, apicoplast, fatty acid biosynthesis pathway, malaria, *Plasmodium falciparum*, triclosan.

"With rings on her fingers and (death) bells on her toes . . ."

Banbury Cross nursery rhyme

Be it in the marshy areas of age-old Rome or in the thickets of Africa and South America, there has always been a battle between malaria and mankind. Afflictions sum up to half a billion, of which a million succumb to death every year [1]. Although man has tried his best to eliminate malaria for some centuries by targeted vector control and changing lifestyles, the disease caused by the apicomplexan protozoan parasite, *Plasmodium*, is seeing a resurgence, and malaria is identified among the top ten killers today [2].

A quick look at the life cycle of the most virulent species of this parasite, *Plasmodium falciparum*, in its two hosts, humans and mosquitoes [3], draws our attention to three facts. The complexity of the life cycle, the short-lived extracellular appearance of the merozoites and the intracellular nature of the other asexual stages in an immune-privileged site makes it extremely difficult for the host to mount an effective immune response. Natural acquired immunity against the parasite is short-lived, requires consistent exposure to infection and is effective only in adults [4]. Additionally, antigenic variation, which results in an evasion of the immune response, and inhibition of T-cell stimulation by the parasite present hurdles in the development of a vaccine.

There are no raging successes today when it comes to currently prescribed antimalarial drugs either. Aminoquinolines and quinines, although they target specifically the disease-causing parasite, *P. falciparum*, act in an as yet unknown manner [5]. Antifolates, artemisinins, and sulphones have known targets, but affect the host biosynthesis machinery too [6–8]. These limitations of current antimalarials, as well as the upsurge of drug-resistant *P. falciparum* strains have fuelled the quest for new antimalarials.

The recent discovery of the apicoplast, the plastid-like organelle in *Plasmodium*, *Toxoplasma* and other apicomplexans offers promise in this regard. In the present review, we focus on the indispensability of the apicoplast to the parasite, particularly with regard to the fatty acid biosynthesis pathway operating within it. We believe that the exploration of this pathway presents us with unique opportunities to tackle *P. falciparum* because of its distinctive organization when compared with that of the host.

FATTY ACID BIOSYNTHESIS

Fatty acid biosynthesis is fundamental to cell growth, differentiation and homeostasis. All living organisms synthesize fatty acids, except for the mycoplasmas, which import them from their surroundings. The production of malonyl-CoA by ACC (acetyl-CoA carboxylase) and the transfer of malonyl group by MCAT [malonyl-CoA:ACP (acyl carrier protein) transacylase] (also known as FabD) to ACP to form malonyl-ACP, which condenses in a reaction catalysed by β -oxoacyl-ACP synthase (β -ketoacyl-ACP synthase; KAS) III (FabH) with an acetyl group from acetyl-CoA or acetyl-ACP set the stage for fatty acid biosynthesis. Subsequently, repeated cycles of elongation each comprising a condensation, a reduction, a dehydration and a reduction step by KAS I and II (FabB/F), β -oxoacyl-ACP reductase (FabG), β -hydroxyacyl-ACP dehydratases (FabZ/FabA) and enoyl-ACP reductase (FabI) respectively yield fatty acids (Scheme 1). The four chemical reactions required to complete successive cycles of fatty acid elongation are catalysed by distinct enzymes encoded by unique genes in bacteria and plants in what is called the Type II or the 'dissociative' pathway. This is in contrast with the Type I or associative pathway in mammals and fungi, where a multifunctional enzyme catalyses all the steps of the pathway.

Abbreviations used: ACAT, acyl-CoA:ACP transacylase; ACC, acetyl-CoA carboxylase; ACP, acyl carrier protein; CER, cerulenin; FAS, fatty acid synthase; INH, isoniazid; InhA, enoyl-ACP reductase of *Mycobacterium tuberculosis*; KAS, β -oxoacyl-ACP synthase (β -ketoacyl-ACP synthase); MCAT, malonyl-CoA:ACP transacylase; ORF, open reading frame; PDH, pyruvate dehydrogenase; PEP, phosphoenolpyruvate; Pf, *Plasmodium falciparum*; TLM, thiolactomycin.

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Scheme 1 Type II fatty acid biosynthesis system

Acetyl-CoA (**1**) is converted into malonyl-CoA (**2**) by ACC and then to malonyl-ACP (**3**) by FabD. The resulting malonyl-ACP condenses with another molecule of acetyl-CoA to form β -oxoacyl-ACP (**4**) catalysed by β -oxoacyl ACP synthase III (FabH). This is then converted into β -hydroxyacyl-ACP (**5**) by β -oxoacyl ACP reductase (FabG) and then dehydrated by β -hydroxyacyl ACP dehydratases (FabZ/FabA). The synthesis of unsaturated fatty acids branches out at this step catalysed first by FabA and then by FabB. The dehydrated product, enoyl-ACP (**6**) is then reduced by enoyl-ACP reductase (FabI) to form butyryl-ACP (**7**). This product re-enters the FAS cycle and the growing chain is elongated by two carbon units per cycle. The condensing enzymes involved in elongation are FabB and FabF.

Fatty acid biosynthesis in *P. falciparum*

The identification of nuclear-encoded apicoplast-targeted genes for three enzymes of the fatty acid biosynthesis pathway: ACP, KAS III and β -hydroxyacyl-ACP dehydratase [9] in *P. falciparum* constituted indirect evidence for the existence of a fatty acid biosynthesis pathway in the relict plastid of the parasite. Later, incorporation of ^{14}C -labelled acetate into 10-, 12-, and 14-carbon-long fatty acid chains by *P. falciparum* in culture and that of ^{14}C malonyl-CoA by the cell-free extracts of the parasite confirmed that *P. falciparum* indeed has a *de novo* fatty acid synthesis pathway [10]. This was reinforced by the demonstration of enoyl-ACP reductase activity in the enzyme isolated from *P. falciparum* cultures, demonstration of enzyme activity in purified, recombinant *P. falciparum* FabZ, FabG and FabI proteins expressed in *Escherichia coli* [10–12] and inhibition studies. Triclosan, a broad-spectrum biocide, acts on the Type II fatty acid biosynthesis system [13]. The incorporation of ^{14}C acetate was

inhibited in the presence of triclosan both in the *in vitro* culture of *P. falciparum* and in the cell-free assay of fatty acid synthesis, thus confirming that *P. falciparum* has a functional Type II fatty acid biosynthesis pathway. The cardinal importance of the fatty acid biosynthesis system and the inherent difference between the fatty acid biosynthesis pathways of the parasite (Type II) and the human host (Type I) make it an appealing target for the development of antimalarials.

The two steps involved in synthesizing fatty acids are, broadly, initiation and elongation. The enzymes involved and their inhibitors are described briefly below. Some of their characteristics are also listed in Table 1.

ACC

Fatty acid synthesis is initiated by the carboxylation of acetyl-CoA to malonyl-CoA using bicarbonate as the source of the carboxy group. This step is catalysed by the biotin-containing enzyme,

Table 1 *P. falciparum* FAS enzymes

Enzyme	Molecular mass (kDa)	Subunit composition	Kinetic constants	Inhibitor	Inhibition parameters	Reference
ACC	391*	—	—			
FabH	36†	—	K_m 17.9 μ M (acetyl-CoA) k_{cat} 230 min ⁻¹ K_m 35.7 μ M (butyryl-CoA) K_m 64.9 μ M (malonyl-ACP) k_{cat} 200 min ⁻¹ (butyryl-CoA and malonyl-ACP)	1,2-Dithiole-3-one compounds Thiolactomycin	IC ₅₀ 0.53–10.4 μ M IC ₅₀ > 330 μ M	[22] —
FabD	34.5†	—	—			
FabG	28	—	K_m 75 μ M (acetoacetyl-CoA) V_{max} 0.0054 μ mol/ml per min k_{cat} 0.014 s ⁻¹			[12]
FabZ	17	2	K_m 199 μ M (β -hydroxybutyryl-CoA) k_{cat} 15.99 s ⁻¹ K_m 86 μ M (crotonoyl-CoA) k_{cat} 18.9 s ⁻¹	NAS-21 NAS-91	K_i 1.3, IC ₅₀ 100 μ M K_i 1.5, IC ₅₀ 7.4 μ M	[11]
FabI	41	4	K_m 165 μ M (crotonoyl-CoA) K_m 33 μ M (NADH)	Triclosan 2,2-Dihydroxyphenyl ether	K_i 0.4 mM (with respect to NADH) K_i 0.03 mM (with respect to NAD ⁺) K_i (ternary complex) 96 pM IC ₅₀ 700 nM K_i 0.28 μ M (with respect to NADH) K_i 18 nM (with respect to NAD ⁺)	[37]
FabB/F	45†	2‡				

* Inferred from genomic sequence reported in Sanger Centre, PlasmoDB (<http://plasmodb.org/>).

† Molecular mass from S. Sharma, S. K. Sharma, A. Misra and A. Suroliya, unpublished work.

‡ Subunit composition from S. Sharma, S. K. Sharma, A. Misra and A. Suroliya, unpublished work.

ACC. As this enzyme is responsible for initiating the FAS (fatty acid synthase) pathway and thus regulating the metabolic flux through the pathway, its inhibition should significantly affect the fatty acid production and thereby affect the growth of the organism. Indeed, the apicoplast ACC of *Toxoplasma gondii*, being a multidomain protein like the ACC of grasses, is sensitive to fops, a class of aryloxyphenoxypropionate herbicides [14] (Figure 1). Fops such as fenoxaprop and diclofop are also known to inhibit *P. falciparum*, at comparable concentrations [15]. Unlike the enzymes involved in fatty acid elongation, the ACC is eukaryotic in nature. However, human ACC is a part of a multi-functional enzyme complex, whereas the ACC of *P. falciparum* is a discrete multidomain enzyme. This feature confers the selectivity of the herbicides to the apicoplast ACC, thus making it a promising target for the development of antimalarials.

MCAT (FabD)

This enzyme transfers the malonyl moiety from malonyl-CoA to ACP through a Ping Pong mechanism of catalysis. Mutation of the FabD enzyme has been shown to be lethal in many pathogenic organisms [16–18]. The sequence is known [19,20] and the crystal structure of *E. coli* FabD has been solved [21]. Recently, Prigge et al. [22] reported the purification and expression of MCAT of *P. falciparum*. Although the enzyme has been implicated in the survival of the organism, it is hitherto unexploited as a target for antimalarials.

KAS III (FabH)

This is the first enzyme of the elongation pathway, which condenses acetyl-CoA and malonyl-ACP to form acetoacetyl-ACP. KAS III enzymes can have two activities. They can transfer the acetyl moiety from CoA to ACP [ACAT (acyl-CoA:ACP transacylase)] or condense malonyl-ACP and acetyl-CoA [23]. To compare these two activities of KAS III in *P. falciparum*,

¹⁴C-labelled acetyl-CoA was used along with *Pf*ACP (*P. falciparum* ACP) and malonyl-*Pf*ACP, and it was shown that KAS activity dominates well over the ACAT activity. The specificity for butyryl-CoA as a substrate over isobutyryl-CoA points to the fact that *P. falciparum* may not synthesize branched chain lipids.

TLM (thiolactomycin) (Figure 1) proved to be a poor inhibitor of KAS III with an IC₅₀ of > 330 μ M. Since the IC₅₀ of TLM is < 50 μ M in cultures of *P. falciparum* in human red blood cells, it probably inhibits the other condensing enzymes of the pathway such as KAS I/II. However, three compounds, 1,2-dithiole-3-ones, structurally related to TLM were shown to inhibit KAS III with IC₅₀ values below 10 μ M. These compounds also inhibit *P. falciparum* cultures, both sensitive and resistant to chloroquine [22]. CER (cerulenin) (Figure 1), an inhibitor of the other condensing enzymes, FabB and FabF, does not inhibit FabH.

β -Oxoacyl-ACP reductase (FabG)

The resulting condensed product, acetoacetyl-ACP, is reduced by β -oxoacyl-ACP reductase (FabG) in a NADPH-dependent manner to produce β -hydroxyacyl-ACP. Following the characterization of the enzyme in *E. coli* and *Brassica napus*, it has been characterized in *P. falciparum* [12,24,25]. The distinctive feature of this enzyme is that, thus far, only one isoform of FabG is identified. This target remained unexploited until Zhang and Rock [26] assessed the inhibitory activity of plant polyphenols against FabG and FabH, which, although less potent than TLM, inhibit more than one enzyme in the pathway, definitely a preferred feature for an inhibitor.

β -Hydroxyacyl-ACP dehydratases (FabZ/FabA)

The β -hydroxyacyl-ACP so produced is dehydrated in the next step to form enoyl-ACP. In *E. coli*, there are two dehydratases that can perform this function: FabZ and FabA. Although both have dehydratase activity, FabA is a bifunctional enzyme which

ACC:

Fenoxaprop (Fop)

Diclofop (Fop)

FabA: *cis*-3-decynoyl-NAC

NAS-21

NAS-91

FabI:

Triclosan

Hexachlorophene

Isoniazid

Genz-8575

Genz-10850

Condensing enzymes FabB/F:

Cerulenin

Thiolactomycin

Figure 1 Structures of the inhibitors of different enzymes of the Type II fatty acid biosynthetic pathway

also catalyses the isomerization of *trans*-2-decenoyl-ACP to *cis*-3-decenoyl-ACP in addition to dehydration. This *cis*-3-decenoyl-ACP is then used by FabB, and thus the flux is diverted towards the production of unsaturated fatty acids. However, overexpression of FabA does not result in overproduction of unsaturated fatty acids [27], because FabA cannot dehydrate *cis*-unsaturated β -hydroxyacyl-ACP since its active site is too small to accommodate the bent unsaturated fatty acid. Thus, in further cycles of elongation of unsaturated fatty acids, FabZ plays an important role. Also, FabZ is found to be more active than FabA on both

long-chain and short-chain saturated acyl-ACPs, crediting FabZ to be the primary dehydratase [28].

E. coli FabA is shown to be inhibited by 3-decynoyl-*N*-acetylcysteamine (Figure 1), an analogue of *cis*-3-decenoyl-ACP [29]. This is remarkable in that this is the first suicide inhibitor or mechanism-based inhibitor described. This is also supported by the recently solved crystal structure of FabA with the inhibitor [30]. Despite the importance of FabZ in fatty acid synthesis, it has not yet been exploited in the development of drugs. Only recently has the FabZ of *P. falciparum* been cloned, expressed and

Figure 2 NAS-91 and NAS-21 docked with homology-modelled FabZ from *P. falciparum*

PfFabZ being a dimer, has two active sites, which, hence, can house either two molecules of NAS-21 or NAS-91, or one of each, simultaneously. In this Figure, however, for clarity, only one of the active sites has been demonstrated. While NAS-21 occludes the entry to the active site, NAS-91 sits in the active site and inhibits FabZ.

characterized, and inhibitors identified which happen to be the first of their kind [11]. Homology modelling of *PfFabZ* was performed with *E. coli* FabA because of the 70% amino acid similarity. The major contributing factor is the identity of residues between 60 and 90, which accounts for 21% identity. Based on this, docking studies were carried out, and the rational synthesis of the two inhibitors, NAS91 and NAS21 (Figure 1) accomplished. Both inhibitors were competitive for crotonoyl-CoA and β -hydroxybutyryl-CoA. Also, both of them inhibited fatty acid synthesis in the cell-free extracts. Moreover, the mode of binding of these inhibitors was also investigated which should stimulate further research in this area. Figure 2 shows NAS-91 and NAS-21 docked with homology-modelled *PfFabZ*. Also, recently, *PfFabZ* has been crystallized [31]. Solution of its structure should augment the process of designing rational inhibitors.

Enoyl-ACP reductase (FabI)

Enoyl-ACP reductase, which catalyses the reduction of the double bond in the dehydrated product, enoyl-ACP, catalyses the rate-determining step in fatty acid synthesis, and so is an appealing target not only for antimalarials, but also for antibacterials. Its quintessential nature is well evident from the fact that mutant FabI (Ts) temperature-sensitive strain is also sensitive to antibiotics, and leakage of cytoplasmic contents occurs due to membrane perturbations at non-permissive temperatures [32]. Research on the mechanism of action of diazaborines led to the discovery of the gene encoding the NADH-dependent enoyl-ACP reductase [33]. This enzyme appears to be the target of many broad-spectrum antimicrobial biocides [34], one of which is triclosan, possessing broad-spectrum antibacterial action, and it is used in many consumer products (Figure 1).

Contradicting the earlier views that triclosan inhibits growth by disrupting the cell membrane, McMurry et al. [13] showed that triclosan inhibited the lipid synthesis in bacteria. Heath et al. [35] reported that triclosan and other 2-hydroxydiphenyl ethers inhibit FabI activity. Triclosan has IC_{50} values varying from 0.2 to $1.2 \mu\text{M}$ for different *P. falciparum* strains, and shows differential inhibition for the different stages of the parasite, with the trophozoite and ring stage showing the greatest inhibition [10,15].

A homology model of *Plasmodium* FabI complexed with its cofactor NAD^+ and triclosan built by Suguna et al. [36] showed that FabI of *P. falciparum* prefers NAD^+ to NADP^+ . It also accounted for the 1000-fold increased affinity of *Plasmodium* FabI for triclosan when compared with 2,2'-dihydroxydiphenyl ether, because the chlorine at the 2' position of the former makes favourable contacts with *PfFabI* and NAD^+ .

The characterization of *Plasmodium* FabI in *E. coli* and inhibition studies carried out by Kapoor et al. [37] showed that triclosan is competitive with respect to NADH and shows uncompetitive inhibition with NAD^+ . In fact, the binding of NAD^+ to the enzyme promotes the binding of triclosan. Kinetic studies on the binding of triclosan with FabI show that the inhibition is faster when NAD^+ , the product of the reaction, is added. Pre-incubation of the enzyme with NAD^+ increases the degree of inhibition further. This accounts for the slow-binding nature of triclosan. Although the binding is reversible, it is exceptionally tight given the very low dissociation rate of triclosan from its ternary complex [38]. In continuation with these efforts, time-dependent inhibition of FabI by triclosan was investigated using steady-state kinetics, and it was shown that triclosan is a slow tight inhibitor of FabI [39]. Surface plasmon resonance studies conducted with immobilized FabI reveal that, while NAD^+ binding to FabI is not detectable, in the presence of triclosan, the binding constant is $6.5 \times 10^4 \text{ M}^{-1}$. Similarly, triclosan binding to FabI is increased

Figure 3 Superposition of the binary complex of FabI-NADH and the ternary complex of FabI-NAD⁺-triclosan

Superposition of the binary complex of FabI-NADH (black) and the ternary complex of FabI-NAD⁺-triclosan (grey). Movement of residues 318–324 of the substrate-binding loop and the nicotinamide ring of NAD⁺ result in increased van der Waals contacts, explaining the increased affinity of NAD⁺ and triclosan for FabI in the presence of triclosan and NAD⁺ respectively. TCL, triclosan.

300-fold in the presence of NAD⁺ [40,41]. The crystal structure of *P. falciparum* enoyl-ACP reductase (FabI) as a binary complex with NADPH and as a ternary complex with triclosan and NAD⁺ have been solved, and it was demonstrated that stacking interactions, hydrogen bonds and van der Waals interactions aid in stabilizing the ternary complex. This was also similar to the mode of binding of diazaborine and *E. coli* FabI, suggesting that all 2-hydroxydiphenyl ethers plausibly have similar mode of binding to FabI. It has also been shown that the outward movement of the loop (residues 318–324) plays a major role in inhibitor binding and substrate recognition [40,41]. This outward movement of the loop increases the affinity and rapidity of the interaction for binding of both triclosan and NAD⁺, and thus promotes the formation of the ternary complex (Figure 3). These results contribute to our understanding of the inhibition of FabI by triclosan, and should help in synthesizing more potent analogues.

Studies of Suguna et al. [36] had averred that the replacement of a methionine by alanine in the malarial enzyme leaves enough room for introducing bulkier substituents in the B-ring of triclosan. Indeed, attempts have already been made to test some derivatives of triclosan, such as naphthalene derivatives, with this intent. Hexachlorophene, an antimicrobial compound used in scrubs and detergents was also tested against *E. coli* FabI (Figure 1). But it was found to be less effective compared with triclosan due to its inability to form ternary complexes [42]. 2,9-Di-substituted 1,2,3,4-tetrahydropyrido[3,4-*b*]indoles [43], aminopyridines [44], indole naphthyridinones [45] and 1,4-disubstituted imidazoles [46] were tested against FabI of *E. coli*. Although these are found to be less effective than triclosan, such studies help to underscore the importance of groups responsible for the stabilization of triclosan thereby helping us retain those characteristics in further syntheses of efficacious analogues.

The other important inhibitor of enoyl-ACP reductase is INH (isoniazid), a frontline drug used for the treatment of tuberculosis

(Figure 1). It has been shown that triclosan is an inhibitor of InhA (enoyl-ACP reductase of *Mycobacterium tuberculosis*) [47]. This was confirmed by the crystal structure of InhA with triclosan. It provides additional evidence that triclosan binds to a site different from INH [48]. Two other novel inhibitors were found, namely Genz-8575 and Genz-10850 (Figure 1), but these showed IC₅₀s of 32 μ M and 18 μ M respectively, far above that of triclosan. It is surprising that, although triclosan, which has been shown to be an antimalarial, has been found to inhibit InhA, the activity of the celebrated antitubercular drug, INH, on *P. falciparum* enoyl-ACP reductase has not yet been investigated. Nevertheless, this would require activating the drug INH into its acyl radical in the same way as it is activated by an enzyme, KatG, for exhibiting its inhibitory action on *M. tuberculosis*. Nonetheless, a combination of INH with rifampicin and chloroquine has already been shown to constitute an effective antidote for malaria in mice [49]. However, the ease with which INH resistance develops in *M. tuberculosis*, and the fact that many individuals in the developing world, and now even in the developed world, are already hosting this organism, call for caution in using INH in any formulation for treating malaria.

KAS I and II (FabB/F)

The fatty acids synthesized in the previous step are either transferred to glycerol phosphate by the acyl transferase system to be incorporated into phospholipids or elongated by two enzymes KAS I (FabB) or II (FabF). FabB is required in a critical step for the elongation of unsaturated fatty acids [50,51], and FabF is required for the thermal regulation of fatty acid composition. Both contain the His-His-Cys catalytic triad in their active site [52]. To date, two inhibitors of these enzymes are known. CER, synthesized by the fungus *Cephalosporium ceruleans* irreversibly inhibits FabB and FabF by binding to the active-site cysteine residue [53–56]. But, as β -oxoacyl ACP synthase III (FabH) has an aspartate instead of a histidine residue in its active site, CER is found to be inactive against it [57]. These residues are found to be conserved in *P. falciparum* FabB and FabF. CER was also found to act synergistically with triclosan on *P. falciparum* culture [10]. However, the disadvantage is that CER can be accommodated in the Type I FAS active site too, thereby limiting its use as an antimalarial. However, analogues of CER can be synthesized which would be specific only to the Type II FAS system.

While TLM specifically targets FabB [58], it is also an inhibitor of the other condensing enzymes, FabF and FabH [59,60]. Unlike CER, which mimics the transition state of the condensation reaction, TLM mimics malonyl-ACP. The sensitivity of TLM to the condensing enzymes is in the order FabF > FabB $\gg$ FabH. The isoprenoid moiety fits into the hydrophobic pocket of FabB/F and is stabilized by stacking interactions [57]. These studies also showed that the hydrophobic pocket is not completely filled with the side chain of TLM, leaving space for further substitutions on it for a better fitting molecule. These are confirmed by analysing TLM analogues, wherein increased side-chain length and increased unsaturation enhanced their potencies. TLM has been shown to inhibit the growth of *P. falciparum* [61]. Furthermore, *P. falciparum* FabB/F exhibits significant homology with *E. coli* FabB. This draws our attention to their potential as an antimalarial target.

Stage-specific expression of FAS enzymes

High-density oligonucleotide arrays have been used to generate expression profiles of the human and mosquito stages of the

Figure 4 Pictorial representation of the origin of the apicoplast

(A) The photosynthetic cyanobacterium (green) is engulfed by a primary eukaryote (light blue) and some of the genes from the primary endosymbiont are transferred to the host nucleus (dark blue). Subsequently, this eukaryote containing the endosymbiont is engulfed further by a secondary eukaryote (buff). Here again, some genes are transferred to the host nucleus (red) reducing the apicoplast genome to the bare minimum. (B) During evolution, most of the genes were transferred from the plastid to the nucleus. Some of these genes code for proteins destined to the apicoplast (APM, apicoplast membrane). These proteins carry a signal peptide (SP; shown in pink) followed by a transit peptide (TP; shown in blue). The SP directs the protein into the endoplasmic reticulum where it is cleaved by a signal peptidase I. The resultant protein carries the TP, which directs its entry into the apicoplast. The TP is then cleaved by the plastid peptidase. Thus the nuclear-encoded proteins enter the apicoplast by a bipartite targeting signal.

malarial parasite's life cycle [62,63]. The transcriptome of the enzymes involved in the fatty acid biosynthesis pathway reveals that FabZ mRNA is expressed in all stages of the malarial parasite to almost similar levels. However, while FabB/F and FabH are expressed in the late trophozoite and schizont stages, and FabG in the trophozoite stage, expression profiles of these enzymes, as well as those of FabD and FabI, are associated with low confidence levels. Triclosan and TLM inhibition data demonstrate that the stages most affected by inhibition of fatty acid biosynthesis are the late ring and the trophozoite stages [9,10], suggesting perhaps that the fatty acid biosynthesis enzymes are expressed maximally at these stages. More rigorous data on the expression profiles of

these enzymes would be required to resolve this issue, which will help in deciding a stratagem for the therapeutic use of inhibitors towards various enzymes.

Apicoplast

The multifunctional enzyme of the type I pathway has the advantage that substrates for consecutive reactions are channelled from one domain of the enzyme to another without diffusion of substrates limiting the rate of the reaction. *Plasmodium*, which has the Type II fatty acid biosynthesis pathway, has perhaps got around this problem by confining all the reactions of fatty acid

biosynthesis to a small organelle: the apicoplast. This unique, apically positioned, plastid-like organelle, which has been implicated in several other biological processes, such as haem biosynthesis, protein synthesis and developmental processes [10,64], owes its discovery to its 35 kb genome. First identified in the avian malarial parasite, *Plasmodium lophurae* and attributed to the mitochondria [65], this extrachromosomal DNA was suggested to be plastid in origin after analysis of the rRNA genes which are arranged in an inverted repeat form, like in chloroplasts. Immunogold labelling and *in situ* hybridization experiments confirmed the presence of the relict plastid both in *T. gondii* [66] and in *Plasmodium* sp.

Evidence such as the presence of four membranes around the organelle, susceptibility of malarial parasites to rifampicin, a drug that inhibits prokaryotic RNA polymerase, but not eukaryotic RNA polymerase, and comprehensive analysis of genes suggests that, like plant plastids, the apicoplast arose by secondary endosymbiosis probably of a red alga [67] (Figure 4A). It is assumed that biochemical exchanges during this process resulted in the endosymbionts (cyanobacterial-like prokaryote) losing genes that were no longer required in its new environment, and transferring other genes that are essential for endosymbiont functions to the host cell nucleus [68–70] (Figure 4A). Although a few proteins involved in apicoplast function, such as protein synthesis, are encoded by the reduced apicoplast genome itself, most are nuclear encoded and targeted to the plastid with the help of a transit peptide via the endomembrane system [64,71] (Figure 4B). An apicoplast-encoded chaperone, ClpC, renders assistance in their entry into the organelle [72].

The phylogenetic relationship of FabI, a nucleus-encoded, plastid-targeted protein with those of other organisms is depicted as a representative example of the proteins of FAS II pathway of the parasite in Figure 5. Consistent with the fact that apicoplasts have many characteristics of plant plastids, the enoyl-ACP reductase was similar to FabI found in the plastid of the plant, *B. napus*.

Apicoplast and fatty acid synthesis

The apicoplast membrane is impermeable to acetyl-CoA, the substrate of the FAS system, akin to the mitochondrial membrane. In mitochondria, where acetyl-CoA is the precursor of the TCA (tricarboxylic acid) cycle, the glycolytic end product, pyruvate, is transported into the mitochondria by a pyruvate- H^+ symport and is converted into acetyl-CoA inside the organelle. It is possible that in the apicoplast too, a similar mechanism exists wherein pyruvate is formed from PEP (phosphoenolpyruvate), which is transported from the cytosol into the apicoplast [73]. This is corroborated by the findings of Kubis and Rawsthorne [74] that PEP transporters play an important role in providing resources for the fatty acid synthesis system in plant plastids. Assuming that pyruvate is transported across the apicoplast membrane, a PDH (pyruvate dehydrogenase) complex would be required to convert the pyruvate, inside the apicoplast, into acetyl-CoA. To explore this, the genome sequence of *P. falciparum* at PlasmoDB (<http://plasmodb.org/>) and at the Sanger Institute *P. falciparum* Genome Project (<http://www.sanger.ac.uk>) were searched for the genes coding for the subunits of PDH complex (E1 α , E1 β , E2 and E3). Indeed, four ORFs (open reading frames) corresponding to the four subunits of the PDH complex are found in the *P. falciparum* genome (accession codes PF11_0256, PF14_0441, PF10_0407 and PF08_0066), implicating the possibility of a PDH in the apicoplast. We find it compelling to hypothesize that pyruvate, either transported directly or generated from PEP, acts as a source of the substrate, acetyl-CoA, for fatty acid synthesis in the apicoplast. Nevertheless, one cannot rule out the

Figure 5 Phylogenetic relationship of FabI of *P. falciparum* with those of other organisms

The values shown represent those with confidence levels above 50%.

other possibility that acetyl-CoA can be derived from acetate by the action of acetyl-CoA synthetase. But, although there are three annotated ORFs (accession codes PFF1350c, MAL6P1.150 and PF14_0357) in the *P. falciparum* genome sequence, which could have acetyl-CoA synthetase activity, neither is there an apicoplast-targeting signal in any of these ORFs, nor has the enzyme activity been demonstrated in their expressed protein products. Whether it is the PDH complex or the acetyl-CoA synthetase that generates acetyl-CoA in the apicoplast has yet to be experimentally demonstrated.

Apicoplast and delayed death

Its indispensability for the survival of the parasite and the presence of parasite-specific metabolic pathways of the prokaryotic type make the apicoplast a suitable drug target. The fatty acid biosynthesis system appears to be appealing as a potential target due to the dependence of the parasite on fatty acids for survival and infection. What makes FAS score over the other pathways that are present in the apicoplast and are thus unique to the parasite? It has been shown that antibiotics such as ciprofloxacin and clindamycin target apicoplast replication by inhibiting DNA replication and protein synthesis respectively [75,76]. But the kinetics of inhibition by these drugs is peculiar in the sense that, although they are parasitocidal, there is a delay in the death after the administration of the drug. For instance, in the case of *T. gondii*, when DNA replication in the apicoplast is affected by clindamycin, the drug has its effect only on the second replication cycle when the tachyzoites re-infect the host [77].

We observed that clindamycin and chloramphenicol invoked delayed death in the cultures of *P. falciparum*. The result was the same when the cultures were incubated for the entire duration of the experiment or only for the first 48 h, asserting that delayed death is the cause of the anti-parasitic activity of these drugs (Figures 6 and 7). In an attempt to understand the rationale behind the delayed-death phenomenon, it was hypothesized that, in the case of *T. gondii*, the apicoplast was required for the establishment of the parasitophorous vacuole, inside which the parasite grows and divides, in the second host [78]. One of the hypotheses suggests that the specific fatty acids and lipids synthesized in the

Figure 6 Giemsa-stained blood smears of the *Plasmodium* culture

Cultures were treated with various reagents as indicated and were incubated for 48 or 96 hours.

apicoplast are required for the successful establishment of the parasitophorous vacuole [77,79]. If this is true, then the drugs inhibiting the fatty acid synthesis pathway should lead to a delayed-death phenotype. But, on the other hand, if the fatty acid biosynthesis pathway was essential to the survival of the parasite in the first cell itself, then the drugs targeting it, such as triclosan, should effect immediate death, unlike the inhibition of other processes of the apicoplast-like DNA replication, which cause only a delayed death. This is of paramount importance in treating cases of cerebral malaria, where delay in the action of drug is synonymous with death of the individual.

To investigate the parasitocidal effect of triclosan and compare with the other drugs that invoke delayed death, *P. falciparum* cultures were incubated with clindamycin or triclosan, and assessed using standard smear staining and hypoxanthine-incorporation assay. We noted that, in the case of clindamycin, the parasitaemia decreases sharply only after 84 h, corresponding to the end of the second asexual cycle, and the surviving trophozoites

showed enlarged vacuoles in the cytoplasm characteristic of dying parasites (Figure 6). On the other hand, triclosan showed a rapid parasitocidal effect ablating young trophozoites within 24 h of incubation (Figure 7a). To confirm that the death is not of delayed type, quantitative hybridization analysis with probes specific for single-copy genes in nucleus (FabI) and plastid [EF-Tu (elongation factor Tu)] genomes were performed. In a normal parasite, the apicoplast genome is present at approx. 1.75 copies per haploid nuclear genome. We observed a specific reduction in the plastid genome copy number to approx. 1.2 copies after 48 h and then approx. 0.7 copies after 96 h on treatment with clindamycin. On the contrary, in the case of triclosan, the nuclear and plastid genome copies decreased in parallel as indicated by their constant ratio (T. N. C. Ramya, N. Surolia and A. Surolia, unpublished work). Thus triclosan does not cause delayed death, but rather leads to a rapid inhibition (depicted in Figure 7b), underpinning the fact that fatty acid synthesis in *P. falciparum* is of prime importance for the survival of the parasite.

Figure 7 Death kinetics invoked by apicoplast-targeting drugs: triclosan and clindamycin

(A) Chloroquine (Q), triclosan (T) and CER (results not shown) ablate the parasites within the first cycle of asexual reproduction, unlike clindamycin (C) and chloramphenicol (H) which invoke parasite death only after 84 h (towards the end of the second cycle). Parasitaemia remained high in the untreated culture (N, no drug control). Parasite growth was monitored by making Giemsa-stained smears and counting number of infected cells per total number of cells, as well as by hypoxanthine incorporation (results not shown). Results are means $\pm$ S.E.M. The statistical significance of changes in parasitaemia was confirmed using the two-tailed Student's *t* test ($P < 0.05$). (B) Cartoon depicting the effect of the action of clindamycin and triclosan on the culture of *P. falciparum*. In the case of clindamycin, the effect is felt only in the second asexual cycle leading to the delayed-death phenotype, whereas triclosan effects immediate death by inhibiting the first asexual cycle itself.

Future drug targets

The complete genome of *P. falciparum* sequenced by Gardner et al. [71] has provided a plethora of information on the genes encoded by the parasite, particularly those which are apicoplast-encoded or -targeted [71]. Taking the leads, many enzymes of the fatty acid biosynthetic pathway have been cloned, characterized and purified. High-throughput assays devised to test the inhibitory activity of many putative drug candidates and *in vitro* testing of

the same against the cell culture *P. falciparum* with [3 H]hypoxanthine-uptake studies has helped identify a few putative drug molecules, which are described earlier in the present review. However, inhibition of many of the enzymes, including regulatory enzymes, is unexplored, opening new and rich avenues for research. Also interesting are the condensing enzymes. A common inhibitor of the condensing enzymes would affect more than one step of the FAS pathway. The development of resistance in such cases is difficult, making the drug more effective.

The elucidation of mechanism of inhibition by crystallizing the enzymes with the inhibitors would help us to synthesize more potent analogues of such molecules while retaining the basic interactions that are important for the inhibition. This is also aided by computational tools such as modelling and docking studies of the enzymes with the potential drug candidates. QSAR (quantitative structure–activity relationship) studies have been employed recently where model pharmacophores are built using the structure and activity of a set of lead compounds. These identify the interacting sites of the compounds in the training set and develop a model pharmacophore, which can be used as a paradigm for design and synthesis of drugs. One such pharmacophore model has been built recently based on the structure–activity relationship of trypanthrin (indolo[2,1-*b*]quinazoline-6,12-dione) [80]. The pharmacophore was found to map well with existing potent antimalarials, although the target enzyme is yet to be found. Such studies can also be extended to develop pharmacophores against key enzymes in the FAS pathway.

Apart from the distinctive nature of FAS enzymes of the parasite from those of its host, what makes it all the more appealing is that inhibiting fatty acid synthesis rapidly compromises the growth of the parasite, unlike apicoplast DNA replication, transcription and translation. Biochemical approaches, together with computational approaches, should provide us with the required thrust to identify many compounds, which may become potent drugs in the future and help us put an end to the sound of the ringing death bells.

The work outlined in the present review is supported by a grant from the Department of Biotechnology, Government of India and partially from the Council of Scientific and Industrial Research (CSIR), India, to N.S. and A.S. T.N.C.R. is a CSIR senior research fellow. We thank I. Surolia for editorial assistance, and G. Kumar and P.L. Mukhi for Figures 2 and 3.

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Received 22 June 2004/4 August 2004; accepted 18 August 2004

Published as BJ Immediate Publication 18 August 2004, DOI 10.1042/BJ20041051