

Fatty Acid Synthesis in Protozoan Parasites: Unusual Pathways and Novel Drug Targets

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Abstract: Fatty acid biosynthesis pathways in protozoan parasites are reviewed with a view to targeting this metabolism for drug therapy. The type II fatty acid biosynthesis pathways derived from bacteria in protozoan relict plastids and mitochondria are examined in different groups with emphasis on apicomplexa. The suitability of different enzymes from the type II fatty acid biosynthesis pathway for drug intervention, and the state-of-play with known and potential inhibitors is explored. The type I acid biosynthesis pathways that occur in select protozoan parasites and their potential for inhibition using anti-tumour and obesity management compounds currently in development are also examined. Pathways used by parasites to scavenge and modify host lipids are also described briefly and their potential for therapeutics discussed.

INTRODUCTION

The human suffering inflicted by protozoan parasites is devastating. Reliable estimates of those sickened by this group of organisms range between one and two billion per annum, a number largely unchanged for twenty-five years [1-3]. There is, however, much room for optimism that the scientific and pharmaceutical tools to dramatically reduce the scope of this problem will be available within a relatively short period of time. Scientific advances, particularly in genomics, cell and evolutionary biology, are being combined with a growing recognition of the threat these diseases pose to the entire world to fuel significant growth in the development of novel treatments.

Somewhat perversely, the relative explosion in protozoan research has created an "embarrassment of riches" with many more drug targets identified than can be developed with the available resources. The constraints inherent in developing drugs for populations who, at present, cannot pay for treatment raise the bar in terms of deciding which drug targets are worth developing. Not only must a structure or metabolic pathway meet the usual criteria for drug development – being required for pathogenicity but either not fundamental to host survival or sufficiently different to allow for highly specific inhibition – but there is an almost absolute requirement for pre-existing inhibitory compounds on which to base the drug development process. Given the costs of drug development and inherent failure rate in the process, the more advanced the understanding of the target and its putative inhibitors the greater the chance that development will be a success.

The enzymes involved in fatty acid synthesis have proven to be one of the most fertile grounds for drug discovery in parasitic protozoans. Not only is the pathway not essential for host survival in the time-span of effective treatment [4] but there are fundamental differences in the pathways used by parasites and their human host that allow for highly specific inhibitors [5, 6]. More importantly in the context of diseases of the developing world, there is a large collection of drugs and drug-like compounds that target many components of protozoan fatty acid synthesis [7, 8]. Several of these compounds are currently used clinically or commercially so there is extensive data on safety, efficacy and methods of production. This allows discovery projects in protozoan parasites to be piggybacked on existing knowledge, radically reducing the time and cost involved in developing new drugs.

A DIVERSITY OF FATTY ACID BIOSYNTHESIS PATHWAYS

From an evolutionary perspective, discussing protozoan parasites as a single group is quite misleading (Fig. 1). The apicomplexans parasites, a group including the human parasites *Toxoplasma*, *Cryptosporidium*, and *Plasmodium*, are members of the chromalveolates group and are most closely related to dinoflagellate algae and ciliates [9]. Indeed, one of the defining features of this group of parasites is a relict chloroplast [10] that is known to play an essential role in fatty acid biosynthesis [5, 6]. The evolutionary position of the other major groups of protozoan parasites, the kinetoplastids, which include the causative agents of leishmaniasis and sleeping sickness, and the amitochondriate protozoans, including the trichomonads, *Entamoeba* and *Giardia*, remain somewhat contentious. The most recent evidence suggests that the main groups of protozoa parasites are only distantly related, and many are as evolutionarily distant from each other as they are from their mammalian hosts (Fig. 1, [9]).

From a functional perspective, however, grouping these evolutionarily diverse organisms together can be informative. All share similar environments, being intimately associated with their human hosts. The access these parasites have to the products of host metabolism has led to striking similarities in their general metabolism; specifically a reduction or loss of many of the metabolic pathways required by free living organisms combined with the development of mechanisms to scavenge and recycle the products of their host's metabolism. The evolutionary distance between protozoans and humans also means that there are significant differences in the molecular mechanisms used for basic metabolic processes. These differences provide some of the most promising targets for drug development.

Another functional characteristic shared by protozoan parasites is complex life history. Most have more than one distinct life stage, often with different morphologies and metabolic profiles. The most complex life cycles, found in the apicomplexans and kinetoplastids, include multiple life stages that have radically modified morphology and metabolism suited to survive in hosts as diverse as mammals and insects (Fig. 2). These complex life histories can complicate the drug discovery process, particularly in the early stages. Often only a subset of life stages, generally those involved in human pathogenesis, can be effectively grown in culture. Inhibiting the molecular targets identified from genomic studies may prove ineffective in cultured parasites because the structure or enzyme is not required for that life stage or the products of the pathway are available from the culture media. Therefore, data on the efficacy and specific effects of newly developed inhibitors need to be care-

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fully considered in the context of parasite life cycle to ensure that promising targets and potent inhibitors aren't discarded prematurely.

An understanding of fatty acid synthesis pathways in protozoan parasites is informed by all of the preceding considerations. There are important differences between protozoan parasites and their hosts in their requirements for fatty acids and the molecular means used to obtain them. These differences form the basis for targeting fatty acid synthesis in drug development. The molecular mechanisms of parasite fatty acid synthesis are strongly correlated with their evolutionary history and these evolutionary connections provide some of the most promising leads in drug development, particularly for the apicomplexan parasites [6, 10-12]. Evolutionary

distance has also resulted in the development of a unique approach to fatty acid metabolism among the kinetoplastids [13] that may provide a useful drug target in *Leishmania* and trypanosomes.

HUMAN FATTY ACID SYNTHESIS

The discussion of targeting fatty acid synthesis in the treatment of disease necessarily starts with understanding fatty acid metabolism in the human host. Of prime importance are the molecular mechanisms of synthesis and the impact that disrupting these pathways has on human health. This provides the point of comparison for assessing the value of parasite pathways as specific targets and the potential for piggybacking anti-parasitic drug discovery on existing inhibitors.

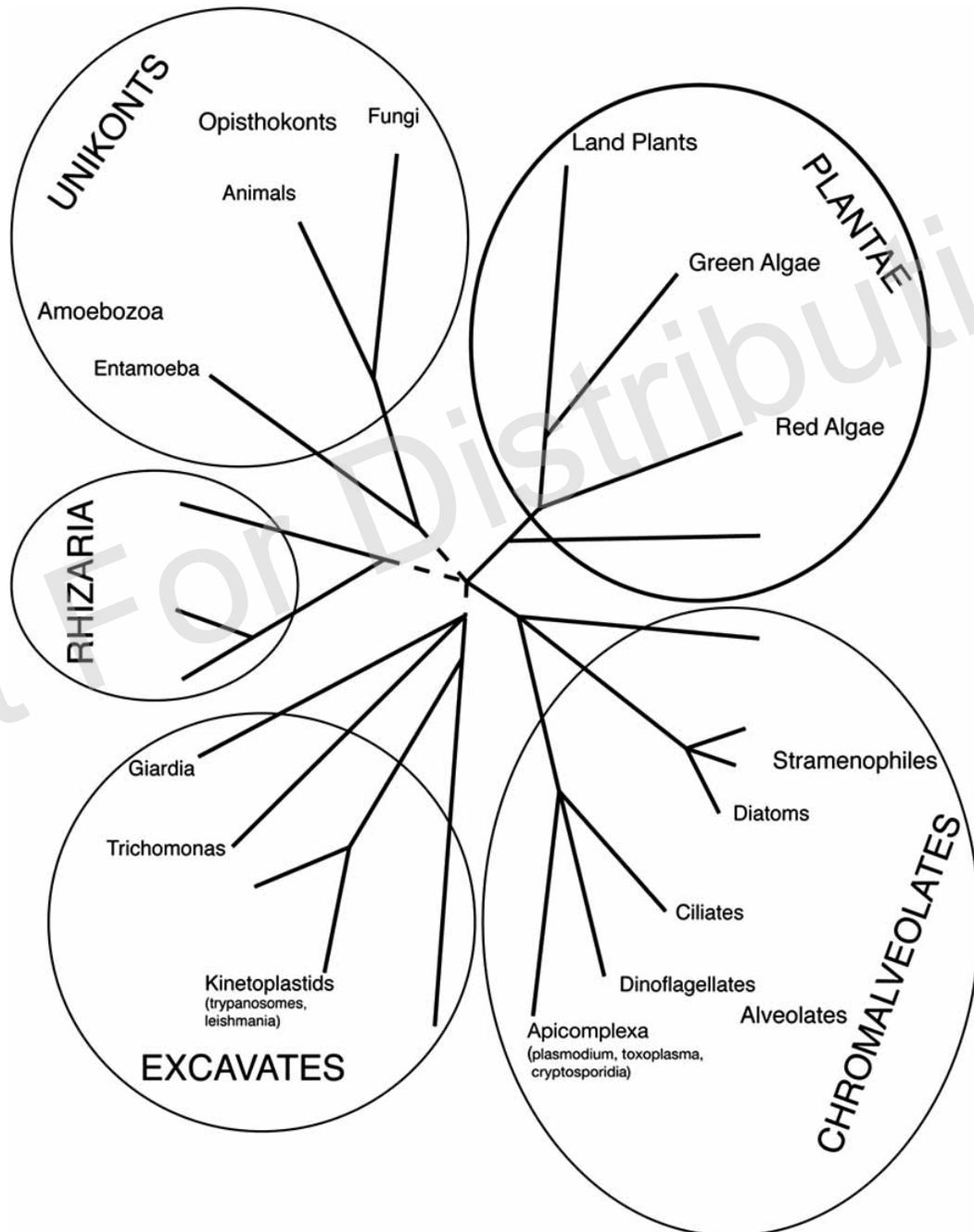


Fig. (1). Simplified phylogenetic tree of the eukaryotes. Phylogenetic tree of the known eukaryotes showing relationships of the most common parasitic protozoa to six major evolutionary groups. Dotted lines represent unresolved relationships between the groups. Adapted from [9].

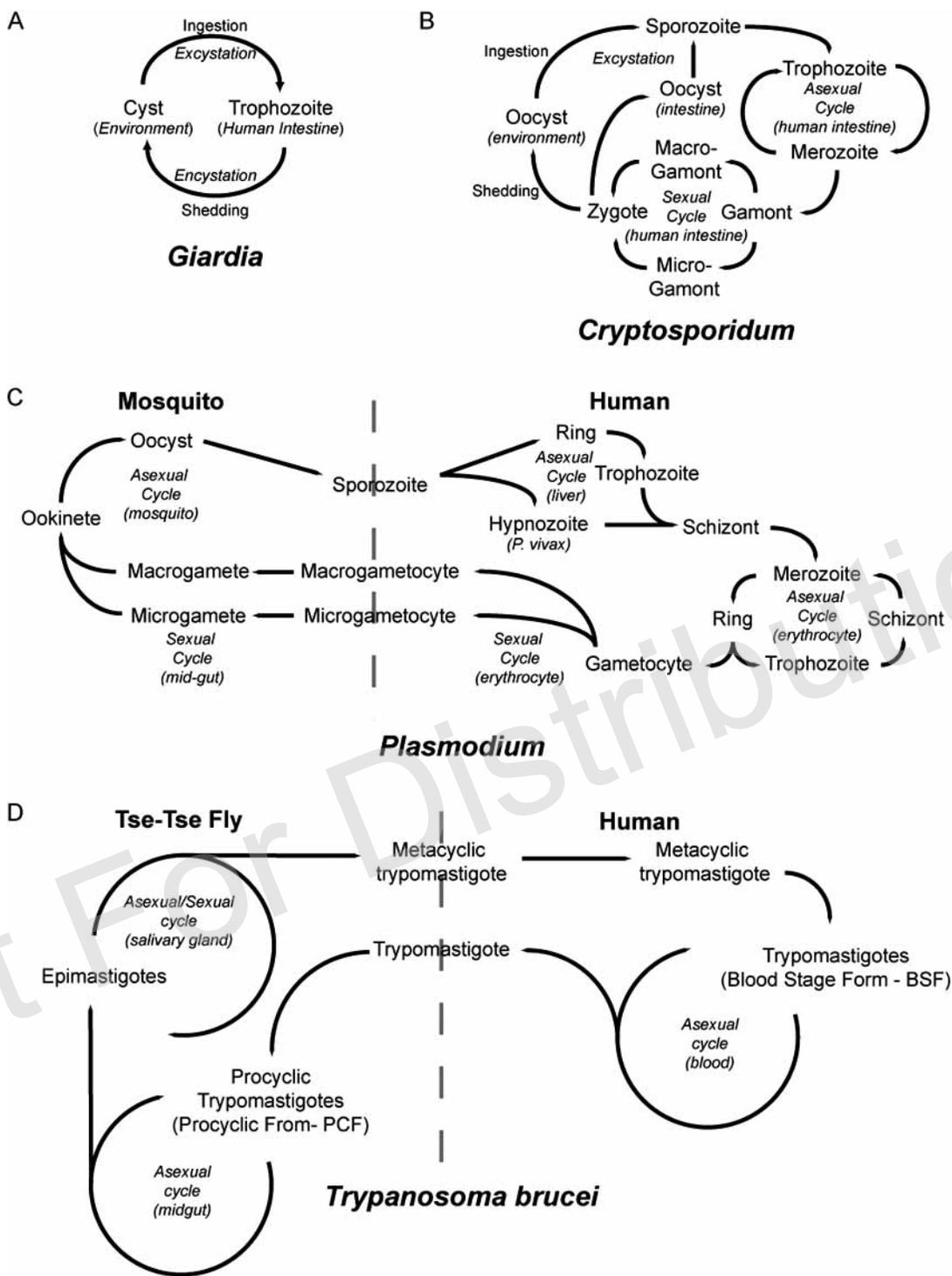


Fig. (2). Protozoan parasite life cycles. Schematic representation of the life cycle of two intestinal parasite – A) *Giardia lamblia*, B) *Cryptosporidium parvum* - and two vector borne parasites – C) *Plasmodium spp.* and D) *Trypanosoma brucei*. Adapted from [3, 138, 139].

In general, *de novo* fatty acid synthesis is relatively unimportant in adult human metabolism. Most fatty acids are obtained from dietary sources and the expression levels of fatty acid synthesis genes are quite low [14]. The minimal role of *de novo* fatty acid synthesis in normal human metabolism is highlighted by the importance this process has in fast growing cancerous tumours. The primary source of fatty acids in many cancerous tissues is *de novo* synthesis and disrupting this pathway effectively limits tumour growth in *in vitro* assays and in animal models [4, 14, 15]. Drugs

inhibiting fatty acid synthesis appear to have very low toxicity to normal tissue or healthy animals even after prolonged exposure [16-19]. The primary effect seen in prolonged inhibition of fatty acid synthesis in animal models is a loss of appetite and reversible weight loss [16]. These effects appear to stem from signalling systems that sense the balance between fatty acid synthesis and degradation [20] and have led to a growing interest in fatty acid synthesis inhibition as a treatment for obesity treatments [17, 21, 22].

Although few side effects of fatty acid synthesis inhibition are seen in adult animals, effects on development appear to be more severe. Homozygous knockout mice that are unable to synthesize fatty acids die as early embryos. Although heterozygous mice retain ~ 35% of fatty acid synthesis activity and appear normal as adults, 60-70% of heterozygotes progeny die *in utero*. The fate of the developing embryos is unaffected by the fatty acid content of the parental diet, indicating that *de novo* fatty acid synthesis is an absolute requirement for mammalian development [23, 24]. While these experiments confirm that a lack of fatty acid synthesis has minimal effect on adult mice, they highlight concerns about the teratogenicity of drugs that inhibit fatty acid synthesis and the need to assess these effects for antiparasitic drugs targeting parasitic fatty acid synthesis.

Human, and indeed, all FAS involve an iterative process in which a series of four reactions repeatedly add two carbons from malonyl-CoA to an acyl chain anchored to an acyl carrier protein (ACP). When the required acyl chain length is reached, a thioesterification reaction transfers the completed acyl chain from ACP to another receptor molecule (Fig. 3A). In humans, two multifunctional enzymes are directly involved in human fatty acid synthesis, acetyl-CoA carboxylase (ACCase) and fatty acid synthase (FAS). Inhibiting either of these enzymes significantly reduces the levels of fatty acid synthesis in mammalian cells [21, 25].

ACCase catalyses a two-step reaction that converts acetyl-CoA to malonyl CoA, the primary substrate of the FAS enzyme. The first step of this reaction uses ATP to power the carboxylation of a biotin moiety. The carboxyl group is then transferred from biotin to acetyl-CoA, forming malonyl-CoA (Fig. 3A). All eukaryotic ACCases are homodimers of a large, multidomain protein. A single dimer is capable of carrying out both steps of the reaction, with the second step of the reaction occurring in highly conserved active sites at the dimer interface [19].

There are two isoforms of ACCase expressed in the human body. ACCase1 is a soluble protein present in fat producing tissue such as the liver and is involved in synthesizing malonyl-CoA for fatty acid synthesis while ACCase2 is associated with the mitochondrial membrane and is involved in regulating fatty acid oxidation [25-27]. Experiments with inhibitors highlight a complex interplay between fatty acid synthesis and oxidation that is important in controlling appetite and fat accumulation [17]. The overall effect of inhibiting ACCase activity in adult mice is weight loss and reduced appetite without significant toxic effects [16, 25, 26]. Trials with ACCase inhibitors and RNAi knockdowns of both ACCase isoforms confirm that adult mammals remain healthy in the absence of ACCase activity [28, 29], but knockout mutants of ACCase1 cause embryo lethality in mice [23].

There are three broad categories of ACCase inhibitor [17]: compounds such as ESP-55016 (Fig. 3B) that compete with acetyl-CoA during the second step of the reaction, those that interfere with the transferase of the carboxyl group to acetyl-CoA by binding near the biotin moiety (eg. CP640186 (Fig. 3B)), and natural compounds such as Soraphen A (Fig. 3B) that allosterically inhibit the activity of the biotin carboxylase reaction. There is a great deal of interest in targeting human ACCases to treat obesity, metabolic syndrome and this has driven the development of many new inhibitors. Several recent reviews offer a complete discussion of these new ACCase inhibitors and their effect on human health [4, 17, 19].

The human genome contains a single fatty acid synthase (FAS) enzyme. This is a 272kDa, soluble protein that contains ACP and all of the enzymatic activity required to convert acetyl-CoA and malonyl-CoA into the C-16 carbon palmitic acid [30]. These large, multifunctional FAS enzymes are termed type I FAS to differentiate them from the prokaryotic fatty acid synthesis pathway (type II). Although each FAS monomer contains all the necessary catalytic domains for fatty acid synthesis human FAS is only functional as a

dimer, with two catalytically active sites present at the dimer interface [31]. As with ACCase, FAS enzyme activity appears to be dispensable for adult mammals but absolutely required for successful embryo development [24].

The role played by type I FAS in the growth of human cancer cells has driven the development of many novel inhibitors. C-75 (Fig. 3B) was among the first FAS inhibitors used to treat cancer [32, 33]. A derivative of the natural product cerulenin (Fig. 3B), C-75 has multiple targets in the FAS enzyme, inhibiting the beta ketoacyl synthase, enoyl reductase and thioesterase activity of human FAS [34]. Pronounced weight-loss is a by-product of C-75 treatment, and this characteristic is now being exploited to develop treatments for obesity [16, 18, 21, 22]. Other recently reported inhibitors of human FAS include derivatives of the bacterial fatty acid synthesis inhibitor thiolactomycin [35], inhibitors of thioesterase such as orlistat [36] and naturally occurring flavonoids [37]. Several reviews are available that fully explore recent developments in this area [14, 20, 22].

PROTOZOAN PARASITES SCAVENGE MOST OF THEIR FATTY ACIDS

For protozoan parasites residing in their human host, the primary source of fatty acids is the host organism. Studies tracing the uptake of radio-labelled fatty acids clearly demonstrate that all of these parasites can take up fatty acids from their environment [38-41]. This reliance on an outside source for this cellular component is not surprising. There is an abundant supply of fatty acids or fatty acid components in the parasite's environment; be it the intestinal tract for *Giardia*, *Entamoeba* and the apicomplexan parasite *Cryptosporidium*, or the cells and human serum that provide a home to the kinetoplastids, *Plasmodium*, *Toxoplasma* and *Trichomonas*. These scavenged compounds provide most, if not all, of the fatty acid requirements of many parasites.

To get the most out of host supplied fatty acids, parasites encode many pathways for customizing them for parasite-specific uses. *Cryptosporidium parvum* provides an excellent example of the unusual adaptations to utilize fatty acids from the environment. This intestinal parasite has apparently lost the ability to synthesize fatty acids but requires specific fatty acids not available from its environment [42]. To lengthen scavenged fatty acids, *C. parvum* utilizes a both a typical long chain fatty acid elongase or ELO [43] and, more strikingly, an unusual type I FAS enzyme [44]. The *C. parvum* type I FAS, unlike type I FAS in humans, is not capable of *de novo* fatty acid synthesis but instead, plays a role in elongating existing 16 to 22 carbon fatty acid chains [44]. To date the functional significance of this enzyme is not clear.

Plasmodium appears to have adopted a different strategy, incorporating a wide range of scavenged fatty acids into their metabolic processes with a minimum of modification [41]. The malaria parasite retains some ability to modify host fatty acids, but the modification pathways are limited [45]. Instead of manipulating host fatty acids, it appears that *Plasmodium* has adapted downstream function so that small variations in fatty acid structure can be accommodated [41, 45]. The lack of modification pathways likely reflects the constant environment in which plasmodium exists during the infection of its human host. The parasite can rely on the host to maintain a consistent supply of fatty acids, so there is little need to manufacture or modify fatty acids in these stages of the life cycle, particularly if downstream pathways are able to adapt to slight variations in host fatty acid content stemming from changes in host health or diet.

For *Cryptosporidium*, *Giardia*, *Trichomonas*, and *Entamoeba* the salvage pathway appears to be the sole source of fatty acids. A substantial body of biochemical and genomic evidence supports the conclusion that these parasites lack *de novo* fatty acid synthesis, altogether [40, 42]. While fatty acid salvage and modification

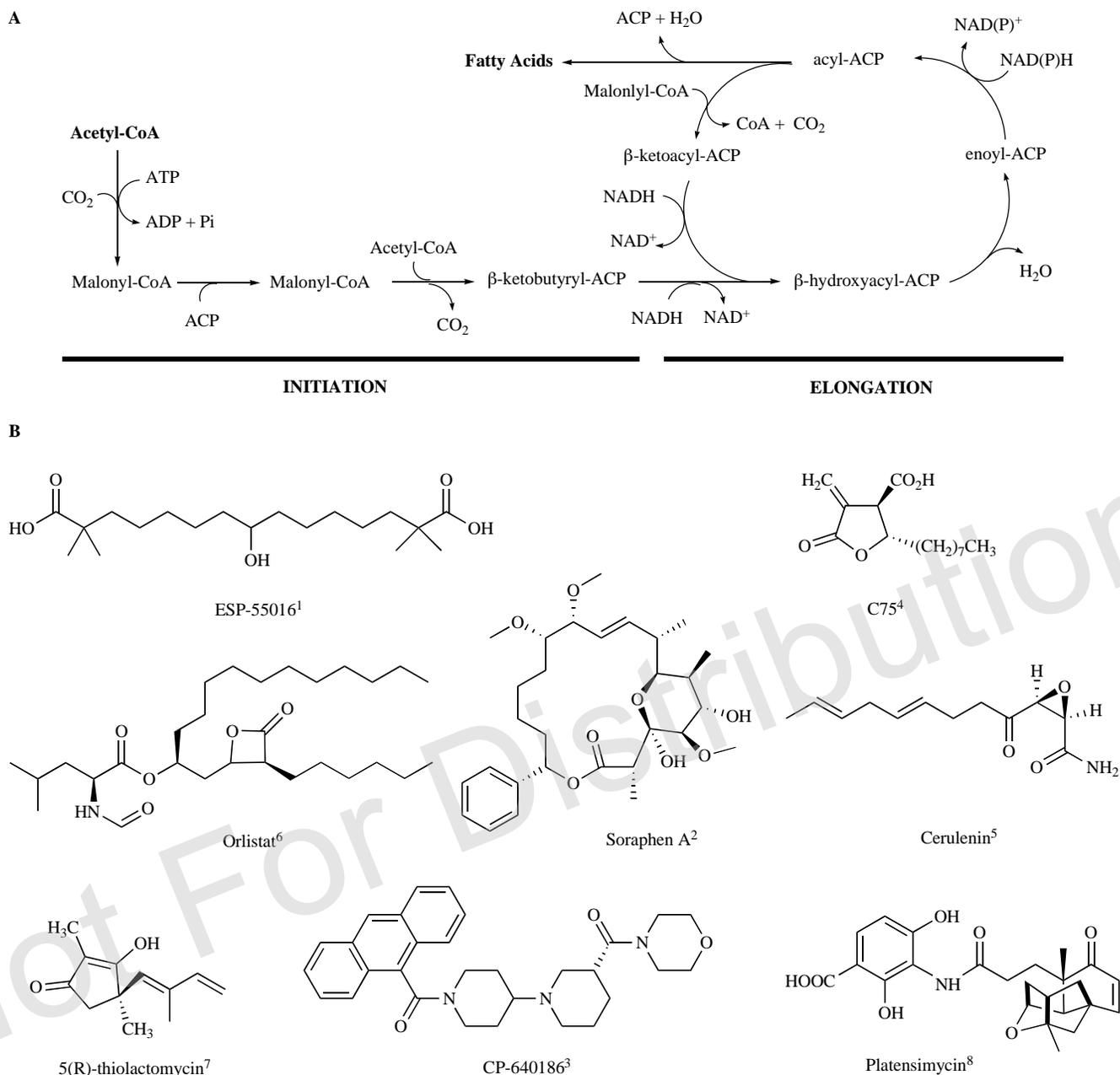


Fig. (3). Fatty acid synthesis and its inhibitors. A) Schematic of the enzymatic reactions involved in type I and II fatty acid synthesis. **B**) Chemical structures of selected ACCase and FAS inhibitors. ¹[140], ²[141], ³[28], ⁴[33], ⁵[142], ⁶[36], ⁷[35], ⁸[93].

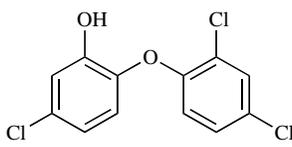
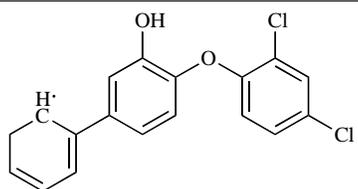
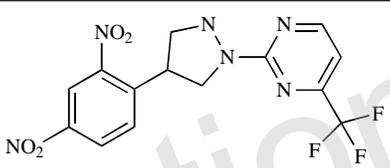
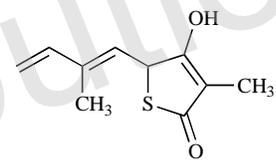
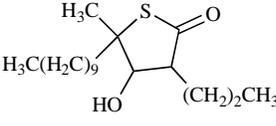
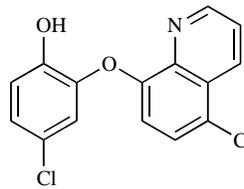
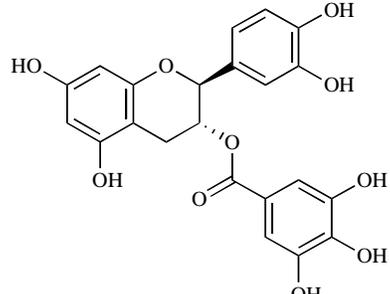
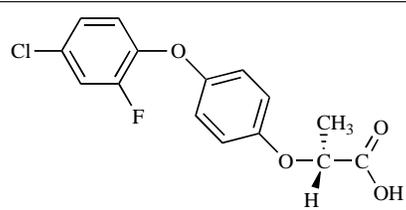
pathways present unique metabolic processes that are possible drug targets, a thorough discussion of this topic is available in several recent reviews [40, 42, 46] and will, therefore, not be included here. Rather, this review will focus on the two recently described *de novo* fatty acid synthesis pathways that have formed the basis of a large body of drug development research.

TYPE II FATTY ACID SYNTHESIS

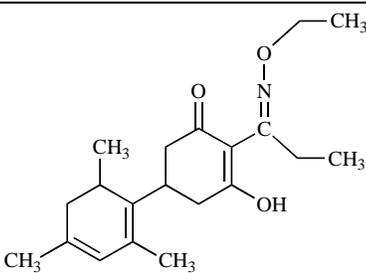
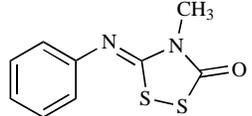
The identification and development of fatty acid synthesis as an important drug target in *Plasmodium falciparum*, is one of the success stories of post-genomic malaria research. The discovery that *Plasmodium falciparum* and *Toxoplasma gondii* carry a relict plastid, or apicoplast, [10] immediately preceded the publication of the complete sequence of the malaria genome [12]. By combining sequence information with predictions of apicoplast targeting, a com-

plete map of the metabolic pathways of the apicoplast was generated [6, 47, 48]. This approach identified a prokaryotic type II fatty acid synthesis pathway localized to the *Plasmodium falciparum* apicoplast [6]. The presence of type II FAS was immediately recognized as an excellent drug target because it is enzymatically different from the type I FAS in the human host and susceptible to several pre-existing compounds targeting type II FAS in bacteria (Table 1). Apicoplast localized type II FAS is also present in *Toxoplasma gondii* [49, 50], but appears to have been lost in several apicomplexans; even some, such as *Theileria* and *Babesia*, that retain the apicoplast [51-53]. Blocking the apicomplexan type II fatty acid synthesis pathway with existing inhibitors of bacterial type II FAS has proven an effective way to kill both *Plasmodium* and *Toxoplasma* [49, 54, 55] and many of the enzymes involved has been targeted for drug development.

Table 1. Apicomplexan Type II FAS Enzymes and Their Inhibitors

Enzyme	Reaction Product	Inhibitors	Enzyme IC ₅₀ (μM)	Parasite IC ₅₀ (μM)	Structure
ENR	Acyl-ACP	Triclosan ¹	Pf 0.07, Tg 0.2	Pf 2.8	
		Triclosan analogue 15 ²	Pf 0.4	Pf 1.6	
		Genz 8575 ³	Not reported	Pf 11	
KASI/II	β-ketoacyl-ACP	Thiolactomycin ⁴		Pf 50, Tg 100	
		Thiolactomycin analogue 7j ⁵	Not Reported	Pf 10, (Tc 56, Tb 21) ⁶	
HAD	Enoyl-ACP	NAS-91 ⁷	Pf K _i 1.5 ⁸	Pf 7.4	
KAR	β-hydroxyacyl-ACP	(-) – catechin gallate ⁹	Pf 0.4	Pf 3.2	
ACCase	Malonyl-CoA	Clodinafop ¹⁰	Tg 20	Pf 100, Tg 10	

(Table 1) contd...

Enzyme	Reaction Product	Inhibitors	Enzyme IC ₅₀ (μM)	Parasite IC ₅₀ (μM)	Structure
ACCase	Malonyl-CoA	Tralkoxydim ¹¹	Not reported	Pf 181	
KASIII	β-isobutyryl-ACP	Thiolactomycin analogue HR19 ¹²	Pf 5.31	Pf 9	

¹ [5, 70, 143]; ² [65]; ³ [11]; ⁴ [6, 54]; ⁵ [90]; ⁶ The target in *T. cruzi* and *T. brucei* are not known. ⁷ [102]. ⁸ The IC₅₀ of enzymatic activity was not reported. ⁹ [76] Also inhibits PfENR and PfHAD; ¹⁰ [50, 55]; ¹¹ [55]; ¹² One of several thiolactomycin derivatives reported in [116].

The kinetoplastids also encode an organellar type II FAS but it is localized to the mitochondria. This pathway is responsible for generating precursors for lipoic acid synthesis and longer chain fatty acids, and also appears to be targeted by existing bacterial inhibitors [56]. The kinetoplastid type II FAS has not received the attention given to the apicomplast version and it is only very recently that the pathway from the mitochondria of *Trypanosoma brucei* was fully characterized [57]. Therefore, our primary focus will be on the apicomplexans, mentioning the kinetoplastids where work has identified specific inhibitors of type II FAS in these parasites.

The prokaryotic type II FAS generates fatty acids using essentially the same enzymatic reactions as the type I FAS in humans (Fig. 3A). The growing acyl chain is anchored to acyl carrier protein and a cycle of four reactions extends the chain by two carbons per cycle. However, rather than having all the required enzymatic activity fused into one large protein, type II FAS involves a collection of individual proteins that combine to catalyse the reaction cycle. Despite similarities in function, there are significant differences between the enzymes of the two pathways and these have been exploited to develop specific inhibitors of the type II FAS pathway, particularly to be used as antibacterials.

The first evidence that the *Plasmodium* type II fatty acid synthesis pathway is essential for blood stage growth was inhibition of malaria growth in culture by the type II FAS targeting anti-bacterial thiolactomycin [6]. This finding was confirmed and expanded using another bacterial type II fatty acid inhibitor, triclosan, to eliminate parasite growth in culture and in the mouse model *Plasmodium berghei* [5]. Metabolic labelling confirms that *P. falciparum* synthesizes fatty acids *de novo*, although these endogenous fatty acids were generally 2-6 carbons shorter than those commonly found in parasite membranes. Triclosan inhibits the synthesis of these fatty acids and does so by binding to and inhibiting the activity of the type II FAS enzyme enoyl reductase (ENR) [5, 58, 59]. Subsequently, inhibitors of several type II FAS enzymes have been reported (Table 1) confirming the essential role of fatty acid synthesis in *Plasmodium* and *Toxoplasma* and validating apicomplast type II FAS as an important drug target.

ENR is an attractive drug target because of its regulatory role in type II FAS. ENR catalyses the final step in each cycle of FAS – the reduction of enoyl-ACP to acyl-ACP (Fig. 3A). This reaction regulates the flux through the FAS cycle by depleting the pool of enoyl-ACP and thereby driving the otherwise unfavourable upstream reduction of β-hydroxyl-acyl by β-hydroxy-acyl-dehydratase (HAD) [60]. ENR is also a site of FAS regulation in bacteria,

with its activity being inhibited by long-chain acyl-ACP [61]. There is a single copy of the ENR gene in both *Plasmodium* and *Toxoplasma* [62], it is predicted to be targeted to the apicomplast [6] and its homologue is the bacterial ENR (FabI) that is sensitive to triclosan.

Triclosan is one of the most widely used antibacterial compounds. This 2-hydroxy diphenylether compound (Table 1) has long been used as an antibacterial component in commercial products such as soap, toothpaste and plastics but its specificity for bacterial ENR was only described fairly recently [58]. Surprisingly for a widely used inhibitor targeting a single bacterial enzyme, very few cases of natural resistance to triclosan have been reported [63]. This has led to the suggestion that triclosan activity involves multiple targets but there is little published evidence to support this. Triclosan has significant antiparasitic effects on cultures of *Trypanosoma brucei*, killing both life stages of the parasites at concentrations <15 μM [64]. Whether triclosan specifically targets the mitochondrial ENR or, as has been suggested, exerts its effects through non-specific membrane interactions [64] remains to be determined.

ENR is the most thoroughly researched enzyme of type II FAS in apicomplexans and is the focus of several ongoing drug discovery programs for the treatment of malaria [65-67]. Sequence comparisons of the ENRs from *Toxoplasma* and *Plasmodium* with the bacterial and plant ENR proteins reveal a highly conserved enzyme, with more similarity to the plant plastid ENR than bacterial forms [68]. Crystal structures of both *P. falciparum* [69] and *T. gondii* [68] enzymes confirm the close relationship between the parasite and plant plastid ENRs. The apicomplast/plastid ENRs share two conserved inserts, one of which forms a groove around these enzymes in their natural, tetrameric conformation [68]. At present the significance of these conserved inserts and the groove is not clear. Enzymatically, the triclosan sensitivity of both *Toxoplasma* and *Plasmodium* ENR, and the preference for of these enzymes for NADH over NADPH as a cofactor [5, 69, 70], support the grouping of PfENR and TgENR with the *Brassica napus*, *Mycobacterium tuberculosis* and *Escherichia coli* enzymes. The similar enzymology is reflected in the crystal structures of these enzymes in complex with triclosan and NAD⁺, which shows a general conservation of important residues of the active site and for subunit interaction [5, 65, 69].

Triclosan inhibits ENR by forming a ternary complex with the enzyme and NAD⁺. Complex formation is reversible, but the tight binding and very slow dissociation rate of the ternary complex give

triclosan its potency [71, 72]. Crystal structures show a strong conservation of the binding interaction between triclosan and bacterial, plant and apicomplexan ENRs [65, 69] with a few notable exceptions. Currently the most interesting of these is a larger inhibitor-binding pocket in the parasite ENR [65]. This feature has been exploited in the synthesis of novel triclosan derivatives with side groups that better fill this pocket and show slight improvement in inhibition of both the enzyme and parasite growth (Table 1) [65]. This is, however, one of the few success stories in the development of triclosan derivatives as anti-parasitic drugs.

Although triclosan inhibits *in vitro* parasite growth at concentrations between 1 and 5 μM [5, 65, 70] and is effective *in vivo* in a mouse malaria model, it is not orally bioavailable [5] and cannot yet be used in treatment. Despite significant efforts, there are no published reports of novel triclosan derivatives with an efficacious combination of favourable pharmacokinetic and anti-parasitic properties. Studies into the structure-function relationship of PfENR continue and are beginning to provide important clues in the development of more effective triclosan derivatives.

Many other classes of ENR inhibitors have been reported. Isoniazid, an ENR inhibitor used in tuberculosis treatment since 1952, has been tested as an antiparasitic but has little inhibitory effect, presumably because it is a pro-drug that is non-reactive until activated by the mycobacterial catalase peroxidase KatG [73]. Other drugs originally developed to target *Mycobacterium* ENR have, however, shown anti-parasitic properties. The most successful reported to date are Genz-8575 and 10850, which inhibit *Plasmodium* growth in *in vitro* cultures at concentrations of 10-32 μM (Table 1). Other classes of ENR inhibitors with anti-malarial properties have been identified in a wide variety of drug screens. These include rhodanines [66], substituted pyrazoles, diphenylamines [69], amide substituted indoles [74], and various natural plant products [75-77]. Virtual library screening has also identified several inhibitory compounds that appear to mimic triclosan in their mechanism of binding to ENR [78].

An unfortunate shared characteristic of all of these inhibitors is an inability to improve on the anti-parasitic properties of triclosan. Clearly the goal to identify inhibitors that have greater potency and bioavailability than triclosan remains unmet, but the rapid developments in the search for inhibitors of bacterial ENR are providing a growing source of potential anti-parasitic compounds.

The *Mycobacterium* ENR remains an important target for anti-tuberculosis drugs, and several compounds identified in anti-tuberculosis screening programs also have anti-parasitic activity [74]. Type II FAS has also been identified as an important bacterial drug target because methicillin-resistant *Staphylococcus aureus* (MRSA) is sensitive to ENR inhibitors [79]. MRSA is one of the main sources of multidrug resistant bacterial infections in hospitals and recently has become a problem for healthy populations in general. To exploit the susceptibility of MRSA to ENR inhibition, several high-throughput screening projects have been undertaken and these have identified several new classes of ENR inhibitor [79]. Hopefully, the ongoing hunt for bacterial ENR inhibitors will uncover a wealth of potential anti-parasitic drugs.

While ENR and its inhibitors have been at the forefront of drug targets in apicomplexan type II FAS, the three other enzymes of the cycle have also been identified as potential drug targets. The product of the ENR reaction, β -acyl-ACP is elongated through a condensation reaction that adds two carbons from malonyl-ACP by the enzyme β -ketoacyl-ACP synthase (KAS I/II). In *E. coli*, KASI is the primary synthase [80] with KASII controlling fatty acid composition in a temperature sensitive manner [81, 82]. KASI and KASII are quite similar in protein sequence, structure and function making it difficult to discern to which enzyme the β -ketoacyl-ACP synthases in other organisms are most closely related. Therefore, the β -ketoacyl-ACP synthases from other organisms, including parasites, are referred to as KASI/II.

The genomes of *P. falciparum* [12], *P. yoelii* [83], *P. berghei* [84] and *T. gondii* [85] each encode a single copy of KASI/II and prediction programs suggest that the *P. falciparum* enzyme is targeted to the apicoplast [48]. KASI/II has been cloned and expressed as part of an effort to create a cell-free system for assaying *P. falciparum* type II FAS [86]. This *in vitro* system can be inhibited by cerulenin (Fig. 3B), a known inhibitor of condensation reaction in fatty acid synthesis [87], resulting in the build up of products consistent with blocking fatty acid synthesis at the β -ketoacyl-ACP synthase step [86]. Cerulenin also inhibits *P. falciparum* growth in culture [5], and this lends strong support to the conclusion that PfKASI/II is involved in apicoplast localized type II FAS in apicomplexans.

KASI/II has only recently begun to be exploited as a drug target. Although there is significant data for the structural and functional basis of cerulenin activity [8], little progress has been reported in developing cerulenin as an antibacterial. This may reflect the inhibitory effect that this compound has on type I FAS [33] and the issues this raises for selectivity. Thiolactomycin, a natural product known to selectively inhibit KASI/II in bacteria inhibits *T. brucei* type II FAS in cell free assays [88]. Thiolactomycin has also shown efficacy against *Plasmodium*, *Trypanosoma* and *Leishmania* cultures *in vitro* [89]. There have been two reports of thiolactomycin analogues with IC₅₀s for *P. falciparum* as low as 1 μM [90, 91]. These compounds also showed similar potency against trypanosomes and *Leishmania*, although the specific target of these inhibitors has not been determined [90]. Thiolactomycin analogues are also the subject of recent investigations as inhibitors of fatty acid synthesis in humans [35] and as drugs to combat tuberculosis [92]. Some of these analogues target the β -ketoacyl synthase activity of type I FAS, so they may not be suitable as type II FAS inhibitors. They could, however, provide important sources for compounds affecting the type I FAS found in *Cryptosporidium* and *Toxoplasma*.

Targeting KASI/II in bacteria has been the focus of several recent screening projects, again because of the need for new drugs to combat MRSA. The greatest success in discovering KASI/II inhibitors with anti-bacterial activity has come from screens of natural product extracts. Platensimycins (Fig. 3B) are the most effective class of KASI/II inhibitors reported [93], being potent inhibitors of *S. aureus* and other Gram-positive bacteria [79]. The discovery of platensimycins was followed by the development or discovery of several related KASI/II inhibitors [79] and there are also reports of unrelated compounds that inhibit this enzyme [94]. It is intriguing to note that many of these new compounds inhibit both KASI/II and the related type II FAS enzyme KASIII (see below). This raises the tantalizing possibility of slowing the rise of drug resistance by targeting two proteins with a single compound. Unfortunately, there are no reports detailing the efficacy of these new inhibitors against apicomplexans or other parasitic protozoa but these compounds clearly represent another novel source of potential anti-parasitic drugs.

β -Ketoacyl-ACP reductase (KAR) is the next enzyme in the type II FAS elongation cycle (Fig. 3A). This enzyme reduces the β -ketoacyl-ACP produced by the KASI/II to β -hydroxyacyl-ACP. Like ENR, KAR is a member of the short-chain dehydrogenase/reductase (SDR) superfamily of enzymes and has been identified as a possible drug target because it essential for fatty acid synthesis and exists as a single isoform in most bacteria and in apicomplexan parasites [95, 96]. KAR appears to be highly conserved in structure and function in bacteria, plants and *P. falciparum* [96-98] suggesting that inhibitors of bacterial KAR could provide lead compounds for the development of drugs against apicomplexan parasites.

To date, however, there are few reports of inhibitors of KAR from bacteria or parasites. Plant products from the catechin gallate family appear to be the most promising inhibitors of KAR. These compounds have significant anti-malarial effects in *in vitro* cell

culture and can inhibit both KAR and ENR reductases in *P. falciparum* and bacteria (Zhang and Rock 2004). Cross-inhibition of ENR and KAR has been observed with the anti-tuberculosis drug isoniazid [99] although, in this case, the inhibition of the *Mycobacterium tuberculosis* KAR homologue has negligible impact on overall growth of *M. tuberculosis* [100]. Work using the cell free type II FAS from *P. falciparum* indicates that KAR is the primary target of (-) -catechin gallate and that the effect on other enzymes is less important [86]. It remains to be seen if results from the cell-free system can be extrapolated to the whole parasite, but these experiments clearly link KAR inhibition with anti-plasmodial activity.

One other KAR inhibitor has been reported. That is the anti-helminthic compound hexachlorophene. This compound inhibits *P. falciparum* KAR and parasite growth in culture at the low μM range [98]. The structure of hexachlorophene is quite similar to triclosan and this compound may be acting on both KAR and ENR.

The relative lack of progress in identifying KAR inhibitors is most likely a reflection of how this enzyme is integrated into the type II FAS pathway. In *E. coli* KAR activity is not limiting [60] nor is it a point of fatty acid synthesis regulation [97]. Given these characteristics, it appears that KAR activity will have to be almost completely eliminated in order to inhibit fatty acid synthesis. If KAR can be comprehensively inhibited, as reported in the cell free FAS assay [86], then it clearly blocks the synthesis process. Given these limitations, a much clearer picture of the function and regulation of apicomplexan KAR will be needed before serious efforts to target this enzyme are undertaken.

The two reductases KAR and ENR are linked by the activity of β -hydroxyacyl-ACP dehydratase (HAD) (Fig. 3A). The single HAD enzyme found in apicomplexans is homologous to the most common bacterial and plant HAD isoform, FabZ [6, 101, 102]. Like FabZ, *P. falciparum* HAD has the unusual characteristic of being more efficient at catalysing the hydration of enoyl-ACP to β -hydroxyacyl-ACP rather than the dehydration reaction required for FAS activity [60, 102]. Type II FAS is only functional because the activity of ENR depletes the pool of enoyl-ACP, thereby driving the HAD dehydration reaction [60]. Somewhat surprisingly, given its kinetic properties, HAD has emerged as an important drug target.

Research on HAD inhibitors as novel drugs has occurred primarily in the apicomplexan parasites. The difficulty in targeting bacterial HADs results from the presence of more than one HAD isoform in many bacteria. Indeed, one of the first bacterial HAD inhibitors described was 3 decynoyl-NAC, a "suicide" inhibitor that binds irreversibly to the HAD active site and blocks enzyme activity [103]. This compound has good antibacterial activity and is not toxic to mammals [104] but it is only effective against one bacterial HAD isoform, FabA. It does not inhibit the most common HAD isoform, FabZ, and therefore, has extremely limited potential as an anti-bacterial [8]. Intriguingly, 3 decynoyl-NAC can inhibit *P. falciparum* HAD activity *in vitro* and appears to be covalently bound to the enzyme [105]. Whether 3 decynoyl-NAC has anti-malarial activity is not known.

There is more interest in HAD as a drug target in apicomplexans because the genomes of *T. gondii* and *P. falciparum* each carry a single copy of the HAD gene [6, 102]. This indicates that HAD inhibitors are more likely to block fatty acid synthesis in these parasites. Homology modelling of PfHAD facilitated the identification of two compounds that inhibited enzyme activity at concentrations of $<1.5 \mu\text{M}$. One of these compounds, the diaryl-ether NAS-91 (Table 1), killed *in vitro* malaria cultures at drug concentrations below $10 \mu\text{M}$ [102]. The specificity of NAS-91 for PfHAD was confirmed by its ability to inhibit the dehydratase reaction in a *P. falciparum*, cell-free fatty acid synthesis assay [86]. The (-) -catechin gallates that were identified in screens of natural products are competitive inhibitors of PfHAD at $<5 \mu\text{M}$ and showed inhibition of *in vitro* malaria cultures at similar concentrations [76]. As

noted above, cell free fatty acid synthesis assays with (-) -catechin gallate suggest that the primary target is the KAR enzyme, however this is yet to be confirmed in whole parasites. The success in blocking *P. falciparum* fatty acid synthesis by inhibiting the HAD enzyme confirms the validity of this enzyme as a drug target and further structure function studies using the recently crystallized PfHAD enzyme [105, 106] should greatly enhance drug development in this area.

An exciting aspect in research on the inhibitors of the type II FAS pathway is the possibility of developing a single inhibitor targeting multiple enzymes. The acyl-ACP substrates for the KAR, HAD and ENR reactions are structurally very similar and the example of the (-) -catechin gallates and other flavonoids (Perozzo, Sharma) suggest that a single small molecule can interfere with the activity of all three enzymes at roughly the same concentrations [75, 76, 86]. If this type of multi-enzyme inhibition can be achieved by in whole parasites, and if the inhibition of any one enzyme alone is sufficient to kill the parasite, it would be extremely difficult for parasites to develop resistance *via* simple mutations. Given the rapid rise in resistance to new anti-malaria treatments [107], the development of this type of "resistance-resistant" inhibitor would be invaluable for fighting parasitic disease.

OTHER DRUG TARGETS IN TYPE II FAS

Upstream of the type II FAS elongation cycle there are several enzymatic reactions required to initiate the fatty acid synthesis cycle. Before any synthesis reactions can take place the malonyl-CoA precursor must be synthesized from acetyl-CoA, ACP must be converted from apo-ACP to the holo-ACP conformation that can accept the acyl chain, and malonyl-CoA must be attached to the active ACP. The first synthesis reaction, and the final step in initiation, is the condensation of acetyl-CoA with malonyl-ACP to produce β -ketoacyl-ACP, the starting point for the type II elongation cycle discussed in the preceding section (Fig. 3A). While each of the enzymes catalysing the initiation steps is required for fatty acid synthesis, two - ACCase and KASIII - have the molecular and biological characteristics that make them extremely promising targets for inhibitors of fatty acid synthesis in apicomplexan parasites.

Malonyl-CoA is the primary substrate in fatty acid synthesis, providing the two carbons needed for each cycle of fatty acyl chain extension. The synthesis of malonyl-CoA from acetyl-CoA and bicarbonate is the first committed step in both type I and type II fatty acid synthesis and represents an important regulator of fatty acid production in the cell. Long acyl chains act as negative regulators of malonyl-CoA synthesis [19] and, thereby, inhibit the metabolic flux through the entire fatty acid synthesis pathway [108]. Thus, as a point of control in the entire pathway, malonyl-CoA synthesis is very attractive as a drug target.

As in type I FAS, the malonyl-CoA for the type II pathway is obtained from acetyl-CoA and carbonic acid through the activity of an acetyl-CoA carboxylase enzyme. The differences between these enzymes in eukaryotes and prokaryotes are reminiscent of the differences between type I and type II FAS, but with an important twist. Eukaryotic ACCase is a large, multifunctional enzyme but in prokaryotes and most endosymbiotic organelles (i.e. plastids and mitochondria) the ACCase enzyme is composed of four individual proteins - biotin carboxylase carrier protein (BCCP) carries the biotin cofactor, the biotin carboxylase (BC) protein catalyses the carboxylation of biotin, and two proteins make up the carboxyl-transferase enzyme that transfers the carboxyl group from the biotin to acetyl-CoA [17].

Although ACCases in most plastids follow the prokaryotic model, the grasses (family Poaceae) encode an unusual plastid ACCase. Similar to the eukaryotic, cytosolic ACCases, the protein subunits of the grass enzyme have fused to form a multidomain ACCase. Despite their structural similarities, there are fundamental

differences between the true eukaryotic ACCase and the grass plastid ACCase [109] and these have been exploited to create two classes of grass-specific herbicide – the aryloxyphenoxypropionates (fops) and the cyclohexanediones (dims) – that target this unique ACCase (Table 1). Somewhat surprisingly, apicomplexan parasites also use a single multidomain ACCase in their plastid – an unusual feature that marks the apicomplexan plastid ACCases as a potential target for drug development based on existing herbicides.

In assays of antiparasitic activity, grass specific herbicides of both the fop and dim classes were shown to have some activity. The ACCase isolated from the *T. gondii* apicoplast is inhibited by several fops at concentrations down to 10 μ M (Table 1) and whole parasites growing in human foreskin fibroblast (HFF) cells show a similar sensitivity to these herbicides [50, 110]. These herbicides had little effect on non-parasitised HFF cells at 40 fold higher herbicide concentrations, confirming the specificity of these compounds for the parasite ACCase [50]. Surprising, and somewhat disappointing, was the complete resistance to dims seen in *T. gondii* [110] and the high concentrations (~100 μ M) of both fops and dims required to kill *P. falciparum* (Table 1) [55]. It is important to note that these experiments sampled just a small fraction of the thousands of fops and dims that have been created in the course of herbicide discovery, so a large pool of prospective inhibitors remains unexamined.

Differences in the susceptibility of apicomplexan ACCase to the fops and dims stem, at least in part, from sequence differences in the carboxyltransferase (CT) active site. Multidomain ACCases function as dimers, with the active site located at the interface between the two proteins. Fops and dims act by binding at the active site of the CT domain and initiating a conformational change that creates a hydrophobic pocket [111]. The herbicide induced conformational change both enhances herbicide binding and competitively inhibits acetyl-CoA binding [111]. Examination of the residues at the CT active site reveal features of the apicomplexan enzymes that are not conserved in either grass plastidic or eukaryotic cytosolic ACCases [7]. In particular there are two isoleucine residues conserved in the CT domain active site of the grass ACCases that can confer resistance to fops and dims when either is mutated [112–114]. In apicomplexans, the upstream residue is not isoleucine but leucine, a residue conserved in human and yeast cytosolic ACCases and associated with resistance to fops and dims in the grasses [112–114]. In contrast, the more C-terminal isoleucine is conserved between apicomplexan and grass ACCases but is a valine in human and yeast [111, 113]. So it appears that in its active site, the apicomplexans share characteristics with both classes of multidomain ACCases.

The unique nature of the apicomplexan ACCases is further highlighted by sequence differences along the dimer interface. Proteins in this region also play a role in determining susceptibility to fops and dims. In this region, the apicomplexan ACCases share several important residues with the resistant cytoplasmic proteins but they also encode a number amino acids that are unique to the apicomplexans [111, 114, 115]. Determining the functional role of these sequence differences will play a vital role in optimising ACCase inhibitor for the apicomplexan enzyme. Comparisons between the three groups of multidomain ACCases will also provide valuable insight into the structure function relationships of ACCases in general.

Given the relatively poor inhibition of ACCases seen with the fops and dims already tested, the value of ACCases as drug targets might appear doubtful. There are, however, three considerations that highlight the possibilities inherent in targeting this enzyme as an anti-parasitic drug. The first is the cost of producing ACCase inhibitors, particularly those based on herbicides. Commercial herbicide synthesis takes place at the scale of multiple tonnes and the final retail cost of a few dollars per kilogram reflects the simple

chemistry and low cost of goods for their manufacture. The ability to produce anti-parasitic drugs at such low cost is vital if they are to be widely distributed in the developing world where, at present, resources available for the purchase of medicines are severely limited.

A valuable by-product of the wide spread commercial use of herbicides targeting grass ACCases is the available body of research into their effects on human health. These herbicides generally have low toxicity because of the differences between the grass CT domain and that found in humans [7] and because inhibiting ACCases in humans seems to be relatively non-toxic, at least in adults [16]. Other, off target effects on human health have generally been tested during the approval process for herbicide use and this provides a wealth of information to screen out specific inhibitors classes with problematic toxicity profiles. Pre-existing safety data should both speed inhibitor development and reduce the risk of safety related failures during clinical trials.

The large, and growing, collection of ACCase inhibitors provides an invaluable resource in the search for anti-parasitic drugs. The huge market for grass ACCase inhibitors drives industrial research programs aimed at developing more potent and safer herbicides as cheaply and efficiently as possible. Simultaneously, the growing focus on the human ACCases enzymes as targets for treatments of obesity and metabolic syndrome [17] are providing both new inhibitory compounds [4, 19] and a wealth of structure function data [4] that will greatly enhance the discovery and optimisation of ACCase inhibitors that specifically target the apicomplexan enzyme. Taken together, the potential for successful drug development based on ACCase inhibitors warrants further investigation of these enzymes as parasitic drug targets.

The other important control point in fatty acid synthesis is the initial condensation of malonyl-ACP and acetyl-CoA catalysed by β -ketoacyl synthase III (KASIII). *Plasmodium falciparum* KASIII (*Pf*KASIII) was among the earliest fatty acid synthesis enzymes to be identified as a potential drug target with inhibitors of this enzyme blocking *in vitro* growth of *P. falciparum* at concentration <10 μ M [116] *Pf*KASIII shares closest homology with the plastid and cyanobacterial KASIII [6] and enzyme activity requires the canonical KASIII cysteine-asparagine-histidine catalytic triad [117, 118]. Malonyl-ACP and acetyl-CoA are the preferred substrates for *Pf*KASIII and the reaction occurs at a similar rate to that seen in bacterial KASIII [116]. Longer chain fatty acids are not good substrates and, unlike many of its bacterial homologues, *Pf*KASIII shows negligible activity towards unsaturated fatty acid precursors or acetyl-CoA alone [116]. These structural and enzymatic features confirm that the primary function of *Pf*KASIII is the initiation of straight-chain fatty acid synthesis and distinguish *Pf*KASIII from *Pf*KASI/II, the enzyme catalysing the condensation reaction in the type II FAS elongation cycle.

There are two significant structural differences between the KASIII and the KASI/II enzymes. The catalytic triads of KASI/II replaces the central asparagine with a histidine and the substrate binding pocket in the KASI/II enzymes is much larger, a modification made necessary by the need to accommodate the growing acyl chain during repeated cycles of fatty acid elongation [97]. These structural differences have important implication for drug development. The structural differences make most bacterial KASIII enzymes resistant to the common KASI/II inhibitor thiolactomycin [8]. However, thiolactomycin analogues that are a better fit for the active site of KASIII (Table 1) have proved effective as inhibitors of bacterial and *P. falciparum* growth [116, 119].

Inhibitors of *Pf*KASIII have been pursued less vigorously than other targets in the apicomplexan type II FAS pathway, but they are of great interest as antibacterials. As is the case with the enzymes involved in fatty acid elongation, the structural and functional similarities between the apicomplexan and bacterial enzymes means

that the growing number of bacterial KASIII inhibitors will provide an important resource in developing PfKASIII as a drug target. Such antibacterials include thiolactomycin derivatives [120], and compounds based on benzoylamino benzoic acid [121], 1,2-dithiole-3-one [119] and indole [122] backbones. Several natural products have also been reported as inhibitors of bacterial KASIII with antibacterial properties. The most intriguing of these is plastencin, an inhibitor targeting both KASIII and KASI/II [123]. As discussed above, this type of multi-target inhibitor offers important advantages in slowing the rise of drug resistance and, therefore, makes these inhibitors extremely attractive drugs.

TYPE I FAS IN APICOMPLEXANS

In addition to the organellar type II FAS, *Cryptosporidium* and *Toxoplasma* express an unusual type I FAS. These enzymes are made up of several modules, each of which contains all the subunits found in human type I FAS [44, 124]. The presence of repeated synthesis modules is reminiscent of bacterial polyketide synthases (PKS), an observation supported by phylogenetic data that groups *Cryptosporidium* type I FAS with other polyketide synthase rather than with eukaryotic type I FAS [44]. Both *Cryptosporidium* and *Toxoplasma* encode PKS enzymes in addition to the unusual type I FAS [42, 124] but the activity and biological function of these enzymes have not been investigated.

The role of type I FAS in overall apicomplexan fatty acid synthesis remains ill defined. In *Cryptosporidium parvum*, the type I FAS appears to be integrated into the fatty acid elongation system designed to modify lipids scavenged from the host. Complete *C. parvum* type I FAS activity has been reconstituted *in vitro*, and studies on this enzyme system clearly demonstrate that it acts as a fatty acid elongase rather than in *de novo* synthesis [44] and that it acts on fatty acid chains with at least 16 carbons. This would be consistent with its phylogenetic relationship to polyketide synthases, enzymes that modify existing acyl-CoA chains to produce a variety of secondary compounds in bacteria and fungi. It is not entirely clear how the activity or the type I FAS interacts with the activity of the more typical long chain fatty acid elongase (LCE) that is also present in this parasite [43]. Differences in the substrate specificity of these two enzymes suggests they could act in concert to convert C14 fatty acid to much longer C22 chains, but this has not been explored experimentally.

The role of the type I FAS identified in the *Toxoplasma* genome has not been investigated. Given the sequence similarity between the *T. gondii* and *C. parvum* enzymes it seems likely that these two enzymes have similar functions. Incorporation of endogenous acetate into *T. gondii* fatty acids is sensitive to cerulenin, an inhibitor of type I FAS (and possibly of the LCE), but is resistant to the type II FAS specific inhibitor thiolactomycin. This suggests that *T. gondii* type I FAS is responsible for either fatty acid elongation or *de novo* synthesis, or both. Given the unusual structures and the lack of information on the role that type I FAS enzymes play in parasite fatty acid synthesis, the apicomplexan type I FAS are not being actively investigated as drug targets.

In general, the biological role that *de novo* fatty acid synthesis plays in parasite metabolism is an important question that remains largely unaddressed. The impact that fatty acid inhibitors have on parasite growth [49] confirms that this pathway is absolutely required for the survival of parasites that retain type II FAS. However, the type II FAS has been dispensed with by three apicomplexan parasites: *Cryptosporidium*, which has eliminated the apicoplast altogether [125], and *Theileria* and *Babesia*, related cattle parasites that retain the apicoplast but encode neither type I nor type II FAS [51-53]. A correlation has been noted between the presence of a parasitophorous vacuole (PV - the membrane surrounding the parasite and separating it from the cytoplasm of the host cell) and the retention of some form of FAS pathway [7]. Two hypotheses have been put forward to explain this correlation. It has been sug-

gested that the PV acts as a barrier to the import of specific fatty acid products. Parasites without a PV can obtain the essential fatty acids from the host and can, therefore, eliminate their fatty acid synthesis pathways [126]. An alternative suggestion is that the maintenance of the PV requires products from the *de novo* synthesis pathway, so FAS activity is essential for successful parasite development in those parasites retaining the PV [7]. Unfortunately, the data available to date is insufficient to resolve this question.

The most complete investigation of apicomplexan type II fatty acid synthesis to date used a conditional knockout of the apicoplast localized ACP to assess the role of type II FAS in *T. gondii* [49]. Disrupting apicoplast FAS was lethal but it did not appear to interfere with bulk labelling of parasite fatty acids, suggesting that type II FAS makes only a minor contribution to the fatty acid content of the cell. However, technical difficulties in the experiment precluded the investigators from ruling out any contribution of type II FAS to the fatty acid content of the parasite at all. The generation of lipoic acid precursors was found to be an important role for apicoplast generated fatty acids, with the knockout parasites being unable to lipoylate the apicoplast localized pyruvate dehydrogenase (PDH). Without active PDH, the apicoplast cannot generate acetyl-CoA and this may be the ultimate cause of parasite death [49]. However, the only known role of the apicoplast PDH is to generate acetyl-CoA and the only metabolic pathway known to require acetyl-CoA is fatty acid synthesis, so it seems unlikely that this pathway has been maintained by evolution merely as a self-contained loop. Rather, it seems clear that one or all of the PDH, lipoic acid, acetyl-CoA or newly synthesized fatty acids are required for some other metabolic purpose.

The kinetics of parasite death in response to the loss of ACP or treatment with the type II FAS inhibitor thiolactomycin are consistent with the conclusion that the primary effect of type II FAS inhibition is on the apicoplast itself. This conclusion is supported by the microscopic inspection of the knockout cells, which showed that defects in the apicoplast preceded more general effects on the parasite [49]. Thus, parasite death appears to be a secondary effect following on from a general loss of apicoplast function. This type of delayed death is in stark contrast to the immediate impact type II FAS inhibitors have on *P. falciparum* and suggests that, in contrast to *T. gondii*, the products of *Plasmodium* type II FAS are involved in pathways beyond the apicoplast membrane.

In *P. falciparum*, type II FAS is limited to synthesis of acyl chains between 10 and 14 carbons [5]. These are much shorter than the fatty acyl chains predominating in the parasite membranes [41] and it appears that *Plasmodium* lacks the enzymes needed efficiently to elongate these short fatty acid chains [41, 45]. These findings lend support to the idea that providing C-8 fatty acids for lipoic acid synthesis is a major destination for the products of type II FAS. However, the recovery of fatty acids 10-14 carbons in length suggests other roles for these fatty acids. One intriguing hypothesis is that type II FAS contributes shorter chain fatty acid substrates for sphingolipid synthesis [86], a process that requires C-10 to C-14 fatty acids [127] that are generally unavailable from the host [41]. Unfortunately, the role that inhibiting fatty acid synthesis has on sphingolipid synthesis has not been investigated. With so many open questions remaining there is a clear need for further work to clarify the biology underlying fatty acid synthesis. A better understanding of what fatty acids do is vital to the understanding of the implications of using fatty acid inhibitors to treat parasitic disease.

A NOVEL FATTY ACID SYNTHESIS PATHWAY IN KINETOPLASTIDS

Recently, a novel fatty acid synthesis pathway was described in trypanosomes and *Leishmania*. *Trypanosoma brucei* has adapted the endoplasmic reticulum (ER)-based elongases (ELOs), enzymes more commonly involved in fatty acid elongation, to synthesize fatty acids *de novo* [13]. Most eukaryotes, including apicomplex-

ans, *Giardia*, and *Entamoeba* have multiple elongases [88, 128, 129], typically localized in the membrane of the ER [130]. As their name implies, these enzymes are involved in extending the length of existing fatty acids to form long and very long chain fatty acids. Four proteins are required for the elongation reaction, and the mechanism is remarkably similar to the type II FAS (Fig. 4). The primary differences are that the enzymes are integral membrane proteins and the acyl chain is linked to CoA, not ACP [130]. The enzyme responsible for the condensing reaction (similar to KAS/II) appears to be the rate-limiting step in most organisms [130]. These condensing enzymes are, somewhat confusingly, referred to as elongases (ELO or sometimes LCE), a nomenclature that has arisen because multiple elongases can share common reductase and dehydratase enzymes, with the ELO condensing enzyme conferring substrate specificity [130]. For clarity we will use ELO to refer to the condensing enzyme itself and ER-based elongase to refer to each elongation complex.

A recent phylogenetic analysis of ELOs from 56 eukaryotic species found that these enzymes fall into two subfamilies that are defined by substrate specificity. One clade contains ELOs that act on polyunsaturated fatty acids and the other clade unites enzymes that have monounsaturated and saturated fatty acids (MUFAs) as substrates [129]. The ELOs in the MUFA-specific subfamily can be further separated into two groups: one consisting primarily of plant and fungal enzymes, and the other containing the ELOs from animals, apicomplexans and kinetoplastids. Different ELO proteins from different species are generally spread across this group of "animal-like" enzymes, but the kinetoplastid ELOs cluster together to form a distinct group [88, 129, 131]. Given the close correlation of phylogeny and activity seen in the ELOs [129], this grouping supports the idea that the kinetoplastid ELOs have a unique enzymatic function.

The unusual enzymatic activity of the kinetoplastid ELOs was demonstrated when Lee *et al.* [13] used RNAi knockdowns of the three *T. brucei* ELOs to dissect the function of these enzymes. Inhibiting ELO1 in this way abolishes fatty acid synthesis in a *T. brucei* cell free assay. Enzymatic analysis demonstrates that the elongase pathway involving ELO1 is responsible for converting the C4 butyryl-CoA to a C10 fatty acyl chain. ELO2 and its related enzymes can extend C10 to C14 (myristate) and the pathway including ELO3 extends myristate to C18 (Fig. 4), the most common

fatty acid in the procyclic form of the parasite [88]. Starting with a butyryl-CoA precursor, these three enzyme complexes can synthesize all the fatty acids required for growth in both life stages. The modular nature of the elongation pathway also creates an elegant system for regulating fatty acid content. By simply altering the levels of ELO3, fatty acid synthesis can be directed towards producing either myristate or the longer C18 fatty acids depending on the needs in different parasite life stages (Fig. 4).

An unresolved aspect of this novel type of *de novo* fatty acid synthesis is how it is initiated. The ER-based elongase pathway uses butyryl-CoA as a primer rather than the standard acetyl-CoA [13, 89] but the source of this butyryl-CoA remains unexplored. The *T. brucei* genome lacks the typical eukaryotic type I FAS but encodes a mitochondrial type II FAS [132]. Prior to the discovery of the ER-based elongase synthesis pathway, this organellar pathway was assumed to be the source of all *de novo* fatty acids in the parasite [89]. The elongation cycle (Fig. 3A) of this mitochondrial fatty acid synthesis pathway has been characterized and is responsible for producing lipoic acid and C16 fatty acids [57]. The enzymes and pathways required for initiation of this mitochondrial type II FAS are not yet clearly defined. It is clear that the mitochondrial pathway does not use butyryl-CoA as a primer [57] but, instead, it seems likely that the *T. brucei* type II FAS will follow the model of other type II FAS that use acetyl-CoA as the primer for this pathway [97]. This presents the possibility that the mitochondrial type II FAS synthesizes the butyryl-CoA precursor needed for the ER-based elongase pathway, a finding consistent with the dramatic effect that knockdowns of mitochondrial-ACP have on total fatty acid content [57]. Disrupting ACP can have multiple effects, however, so the exact source of the butyryl-CoA primer needed for the ER-based elongase pathway remains a mystery.

In common with *T. brucei*, *Leishmania* can synthesize fatty acids *de novo* [88] and appears to lack a type I FAS [133]. These characteristics clearly implicate the ER-based elongase pathway as the source of fatty acid synthesis in this parasite, an idea supported by the presence of homologs of *T. brucei* ELO1-3 in the *L. major* genome [88, 129, 131, 133]. The unusual function and distinct phylogenetic position of the kinetoplastid ELOs suggest that they could provide a specific and effective drug target. Evidence suggests that some of the common FAS inhibitors also inhibit the ER-based elongase pathway in *T. brucei* [64, 90] so it may be possible

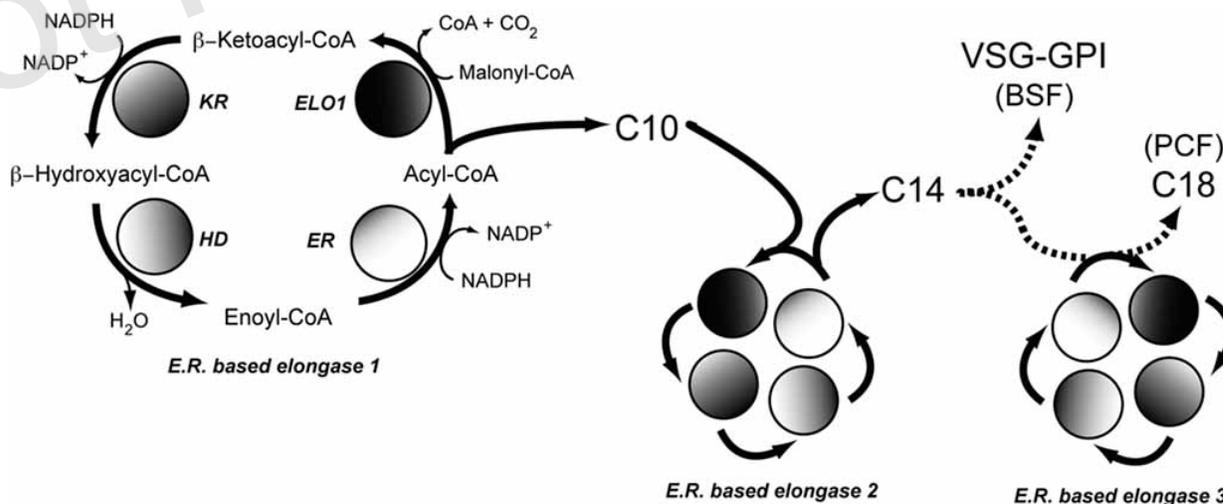


Fig. (4). Fatty acid synthesis by endoplasmic reticulum-based elongases. Schematic of the proposed fatty acid synthesis pathway in *T. brucei*. The initial substrate is proposed to be a four carbon short chain fatty acid that is converted to a ten carbon chain by three reaction cycles of the ER-based elongase 1, a complex of four enzymes—*ELO1*— β -ketoacyl-CoA synthase, *KR*— β -ketoacyl-CoA reductase, *HD*— β -hydroxyacyl-CoA dehydratase and *ER*—enoyl-CoA reductase. The ten carbon product is elongated to fourteen carbon myristate by two reaction cycles of ER based elongase 2. The length of the final fatty acid chain is regulated by the activity of the ER based elongase 3 (dashed lines). In the blood stage forms (BSF) the bulk of fatty acids remain as myristate and contribute to the synthesis of the variable surface glycoprotein (VSG-GPI). In the procyclic form (PCF), most fatty acids are elongated to C18. Adapted from [43, 88].

to tap into the vast collection of bacterial FAS inhibitors to develop specific ER-based elongase pathway inhibitors. A clear understanding of the structure, enzymology and inhibition of the kinetoplastid ELOs will go a long way to determining if they can fulfil their potential as drug targets.

The long chain fatty acid elongase pathway also presents an intriguing potential drug target for *Giardia* and *Entamoeba*. The genomes of both of these organisms encode enzymes predicted to be involved in long-chain fatty acid elongation [128, 134] but preliminary BLAST comparisons suggest that these elongases are members of the plant fatty acid elongase (FAE) family of enzymes. The FAE enzymes are structurally distinct from the ELOs found in mammals, yeast and kinetoplastids [130]. Although these enzymes are not involved in *de novo* fatty acid synthesis, their function is essential for normal growth and development in *Arabidopsis thaliana* [135]. The requirement for FAE activity has led to the development of herbicides, such as flufenacet, that target plant fatty acid elongases [136, 137]. The similarity between plant fatty acid elongases and those of *Giardia* and *Entamoeba* suggests that herbicides may be able to inhibit protozoan FAE-type pathways and be a potential starting point for the development of novel drugs against these protozoan parasites.

CONCLUSIONS

Fatty acid synthesis in protozoan parasites holds much promise as a drug target. Even though the parasites acquire the bulk of their lipid building blocks from their hosts, almost all retain systems for either *de novo* synthesis and/or remodelling of scavenged lipids. These systems are potentially excellent targets and early exploration has identified some interesting lead compounds. Access to the genomes for many parasitic protozoa has allowed us to peer into their metabolisms and has informed our strategies and choices of pathways against which to intervene, many of them previously unknown. A striking insight is the diversity of fatty acid activities carried out by the different parasitic protozoa. Although it was not surprising to learn that distantly related parasites such as *Giardia*, kinetoplastids and apicomplexa have extremely different strategies for fulfilling their fatty acid requirements, it is genuinely remarkable that such a spectrum of fatty acid metabolisms exists within a relatively uniform group of parasites such as the apicomplexa. Indeed, it appears that each type of apicomplexan parasite has a unique repertoire of fatty acid metabolism as befits their slightly different modes of parasitising their specific hosts. Nevertheless, even though this diversity means that there will be no universal, one-drug-fits-all solution to combating apicomplexa using fatty acid synthesis as a target, we are now well placed to pursue specific strategies against the different parasites. Importantly, the ongoing research into inhibitors of fatty acid metabolism as antibacterials, anti-cancer agents, and to reduce obesity is constantly unearthing compounds and information that can be enlisted into the anti-parasite arsenal. Hopefully these better-funded areas will spin off efficacious drugs into the less well-supported area of protozoan parasites.

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