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Apicoplast acetyl Co-A carboxylase of the human malaria parasite is not targeted by cyclohexanedione herbicides

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ABSTRACT

Malaria parasites retain a relict plastid (apicoplast) from a photosynthetic ancestor. The apicoplast is a useful drug target but the specificity of compounds believed to target apicoplast fatty acid biosynthesis has become uncertain, as this pathway is not essential in blood stages of the parasite. Herbicides that inhibit the plastid acetyl Coenzyme A (Co-A) carboxylase of plants also kill *Plasmodium falciparum* in vitro, but their mode of action remains undefined. We characterised the gene for acetyl Co-A carboxylase in *P. falciparum*. The *P. falciparum* acetyl-CoA carboxylase gene product is expressed in blood stage parasites and accumulates in the apicoplast. Ablation of the gene did not render parasites insensitive to herbicides, suggesting that these compounds are acting off-target in blood stages of *P. falciparum*.

1. Introduction

The identification of a fatty acid biosynthesis (FASII) pathway in the vestigial plastid (apicoplast) of the human malaria parasite *Plasmodium falciparum* (Waller et al., 1998) overturned the dogma that malaria parasites are unable to synthesise fatty acids de novo (Holz, 1977; Vial and Ancelin, 1992). Previously, it had been presumed that parasites scavenge all their fatty acid requirements from the host. Indeed, fatty acids are an essential media ingredient for in vitro culture of *P. falciparum* (Mi-Ichi et al., 2006, 2007) and scavenging is likely the principal source of fatty acids. What the apicoplast FASII system contributes to the overall parasite fatty acid budget at each stage of its life cycle is still under investigation (Botté et al. 2013).

After their initial discovery, apicoplast FASII enzymes quickly became the focus of drug discovery strategies to combat malaria. Fatty acid synthesis in the apicoplast utilises enzyme processes that are markedly different from those found in humans, making fatty acid synthesis a potentially attractive target for the development of novel anti-malarial drugs. Whereas human FASI is a multi-activity single protein, in FASII systems of apicoplasts, bacteria and plant plastids, a number of separate enzymes combine with acyl carrier protein (ACP) to form a complex able to extend acyl chains iteratively by two carbon units (Smith, 1994). Various inhibitors of bacterial or plant plastid FASII enzymes (thiolactomycin, triclosan and the aryloxyphenoxypropionate and cyclohexanedione herbicides) were shown to kill malaria parasites, and the presumed mode of action was perturbation of apicoplast fatty acid biosynthesis (Waller et al., 1998, 2003; Zuther et al., 1999; Surolia and Surolia, 2001; Jelenska et al., 2002; Ramya et al., 2007).

Both FASI and FASII are fed by acetyl Coenzyme A (Co-A) carboxylase (ACC), which converts acetyl-CoA and carbonate into malonyl-CoA, the substrate used in acyl chain extension. The ACC for FASI is typically cytosolic in eukaryotes, whereas ACC for FASII is located in the plastids of plants and algae. Malonyl-CoA production by ACC is the first committed step in fatty acid synthesis; ACC activity controls the flux of precursors into the fatty acid synthesis pathway, thereby regulating the rate of fatty acid synthesis (Kim, 1997). The *P. falciparum* genome contains a single copy of ACC (PlasmoDB Gene ID: PF3D7_1469600). The gene encodes a large, multifunctional protein with a biotin carboxylase domain, a biotin carboxy domain and a carboxy transferase domain (Goodman and McFadden, 2007). The multifunctional apicoplast ACC is reminiscent of the equivalent enzymes from the plastids of grasses and algae such as diatoms, which also have a nucleus-encoded multifunctional ACC.

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Grass ACC, which differs from the bacterial multi-subunit ACC of dicotyledonous plants, is the target of two classes of grass-specific herbicides: the aryloxyphenoxypropionates (fops) and the cyclohexanediones (dims). These herbicides are widely used in agriculture to control grassy weeds, and there is a significant body of knowledge regarding their enzymology, structural biology and toxicology (Preston and Powles 2002; Yu et al. 2013). Initial trials using one fop and one dim showed inhibition of *P. falciparum* in vitro growth, but the concentrations were orders of magnitude greater than normally used against grasses (Waller et al., 2003; Ramya et al., 2007). We followed up this initial study with a range of compounds potent against grass ACC to test whether better activity against *P. falciparum* could be obtained. Although more potent compounds with low micromolar half maximal inhibitory concentrations (IC_{50}) values against *P. falciparum* were produced, the structure activity relationships were not consistent with inhibition of parasite ACC (Louie et al., 2010). It appeared possible that these herbicides, although parasiticidal, are off target (Botté et al., 2012).

If drugs are to be targeted against a particular parasite enzyme, ideally the activity of that enzyme should be essential during the particular parasite stage being pursued. The malaria parasite life cycle shifts from mosquitoes to vertebrate by mosquito bite. Parasites injected into mammals by the bite, travel to the liver and initially establish themselves there before multiplying and then shifting into red blood cells. Gene ablation studies in *P. falciparum* and two species of rodent malaria (*Plasmodium yoelii* and *Plasmodium berghei*) to knock down different apicoplast fatty acid biosynthesis (*fab*) genes have demonstrated that apicoplast FASII is not essential for growth of parasites in the blood stage (Yu et al., 2008; Vaughan et al., 2009; Pei et al., 2010). Similarly, deletion of either the E1alpha or E3 subunits of the apicoplast pyruvate dehydrogenase complex (PDH) that generates acetyl-CoA from pyruvate in *P. yoelii*, yield viable blood stage parasites. These gene ablation studies show that FASII is dispensable in blood stages of human and rodent malaria. FASII is also dispensable in rodent insect stages, but vital for liver stage in *P. yoelli* (Vaughan et al., 2009) and important, but not absolutely essential, for liver stage in *P. berghei* (Yu et al., 2008). Surprisingly, a recent report demonstrates that FASII mutants in *P. falciparum* are viable in blood stages but are required for parasite development in the mosquito midgut (van Schaijk et al., 2013), suggesting differences in the metabolic requirements of rodent and human malaria parasites during the insect stage.

To investigate the essentiality of ACC, which feeds carbons to FASII, we deleted the gene in blood stage *P. falciparum* parasites and successfully recovered viable parasites. We then tested herbicides against these ACC minus parasites to determine whether ACC is the target of these inhibitors.

2. Materials and methods

2.1. Creation of allelic replacement and gene disruption constructs

Allelic replacement constructs designed to introduce an haemagluttinin (HA) tag were created using the MultiSite Gateway system™ (Invitrogen, Australia), as previously described (van Dooren et al., 2005). A 1,055 bp portion of the 3′ end of the *P. falciparum* ACC (*Pf*ACC) gene (PlasmoDB gene ID: PF3D7_1469600) was amplified from parasite line 3D7 genomic DNA using the primers D137 and D138, adding the *Att*B4 and *Att*B1 sites to the 5′ and 3′ end of the PCR product, respectively (Supplementary Table S1). The *Pf*ACC3′ product was TA cloned into a pGEM-T vector (Promega, Australia) and sequenced. *Pf*ACC3′HA was linearised with *Cla*I restriction enzyme digestion and recombined into pDONR P4-P1 according to the manufacturer's instructions, creating *Pf*ACC3′-P4P1. The final construct, *Pf*ACC3′HA (Fig. 1A), was created by recombining *Pf*ACC3′-P4P1 with a 3xHA tag carrying a stop codon in pDONR221, a filler construct (3xHA tag in pDONRP2P3), and a human dihydrofolate reductase (hDHFR) selection cassette (Fidock and Wellems, 1997) in pCHD3/4 via the Gateway multisite LR™ reaction (Invitrogen, Australia).

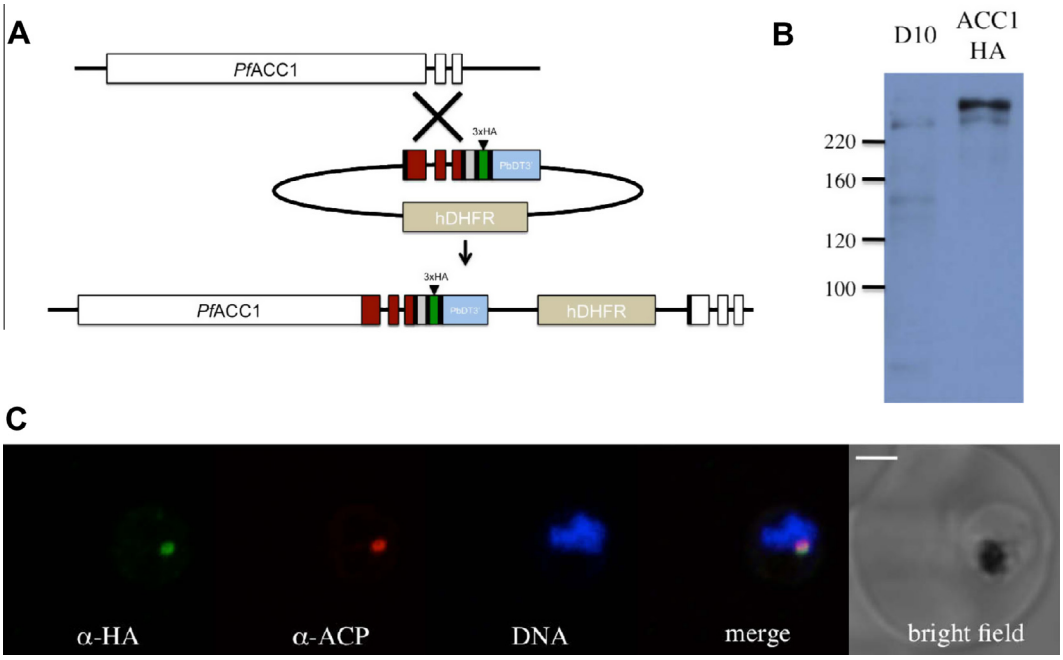

Fig. 1. *Plasmodium falciparum* acetyl CoA carboxylase is targeted to the apicoplast. (A) Single crossover strategy for adding an haemagluttinin tag to the endogenous copy of *P. falciparum* acetyl CoA carboxylase. (B) Western blot probed with anti-haemagluttinin antibody showing a single haemagluttinin specific band at >220 kDa. (C) Indirect immunofluorescence assay of *P. falciparum* acetyl CoA carboxylase-haemagluttinin transgenic parasites showing localisation of *P. falciparum* acetyl CoA carboxylase in the apicoplast (green, *P. falciparum* acetyl CoA carboxylase-haemagluttinin labelled with anti-haemagluttinin antibody; red, apicoplast labelled with anti-*Pf* acyl carrier protein; blue, DNA stained with Hoechst 33342). Bar = 1 μm.