International Journal for Parasitology 44 (2014) 285–289

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

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

Christopher D. Goodman [a,1], Vanessa Mollard [a,1], Theola Louie [b,2], Georgina A. Holloway [b], Keith G. Watson [b], Geoffrey I. McFadden [a,*]

[a] School of Botany, University of Melbourne, Victoria 3010, Australia
[b] The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Victoria 3050, Australia

ARTICLE INFO

Article history:
Received 24 October 2013
Received in revised form 22 December 2013
Accepted 7 January 2014
Available online 25 February 2014

Keywords:
Acetyl Co-A carboxylase
Apicoplast
Fatty acid biosynthesis
Herbicides
Malaria

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.

* Corresponding author. Tel.: +61 414 189 905; fax: +61 3 9347 1071.
E-mail address: gim@unimelb.edu.au (G.I. McFadden).
[1] These authors contributed equally to this work.
[2] Current address: School of Chemistry, University of Melbourne, Parkville, Victoria 3010, Australia.

http://dx.doi.org/10.1016/j.ijpara.2014.01.007
0020-7519/

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).

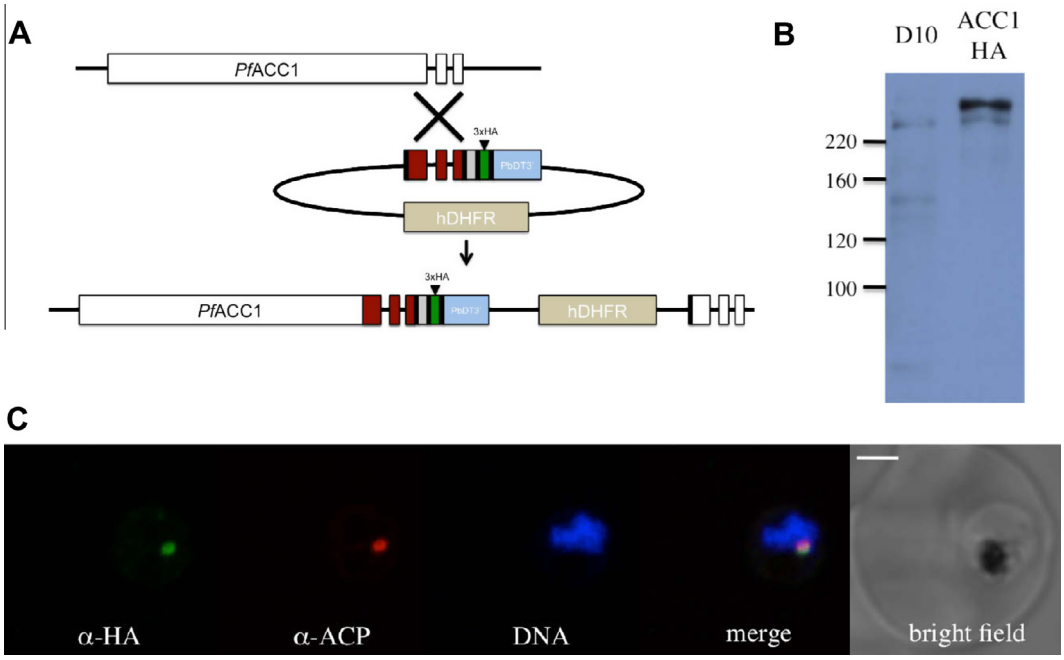


Fig. 1. *Plasmodium falciparum* acetyl CoA carboxylase is targeted to the apicoplast. (A) Single crossover strategy for adding an haemagluttinin tag to the endogenous copy of *P. falciparum* acetyl CoA carboxylase. (B) Western blot probed with anti-haemagluttinin antibody showing a single haemagluttinin 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.

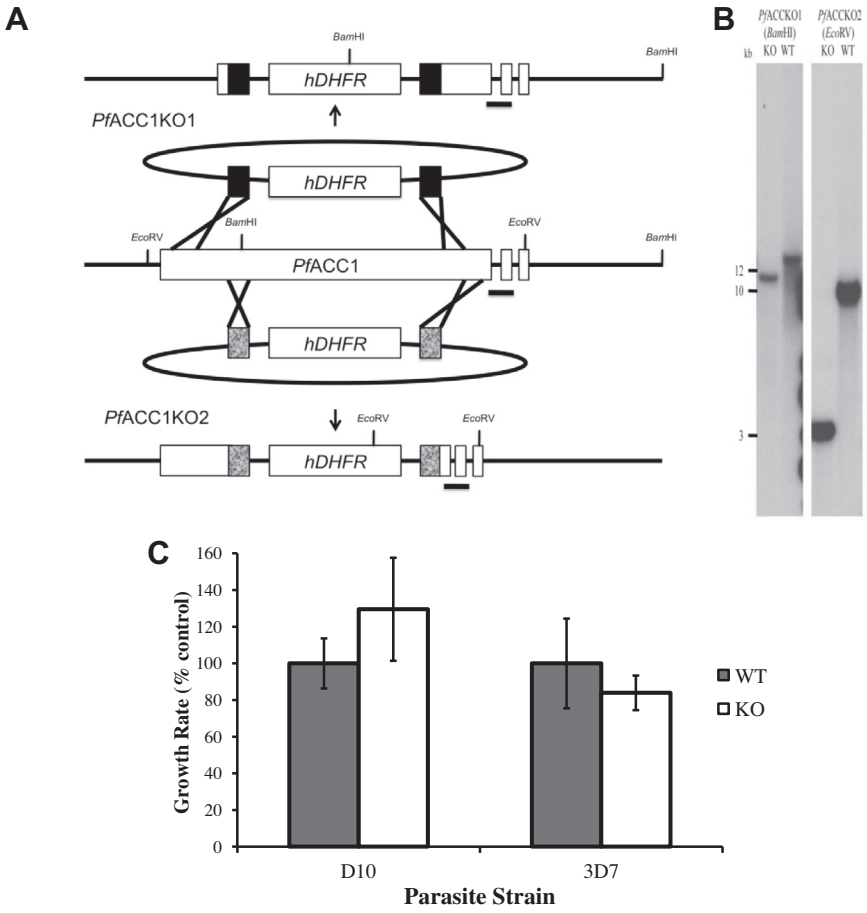


Fig. 2. *Plasmodium falciparum* acetyl CoA carboxylase is not essential in blood stages. (A) Diagram of two independent double crossover constructs used to knockout *P. falciparum* acetyl CoA carboxylase in parasite lines D10 (*Pf*ACCKO1) and 3D7 (*Pf*ACCKO2) showing location of enzymes and probes (black bar) used in Southern blot analysis. Predicted restrictions fragment sizes are listed in Supplementary Table S3. hDHFR, human dihydrofolate reductase. (B) Southern blot analysis confirms insertion of knockout construct into *P. falciparum* acetyl CoA carboxylase. WT, wild type. (C) Chart of parasite growth rate showing no significant difference in growth of knockout lines versus parental (wild type) strain (D10, $P = 0.398$; 3D7, $P = 0.574$, $n = 3$).

Two independent gene disruption constructs were created in plasmid pCC1 (Maier et al., 2006). The 5′ and 3′ flanks of *Pf*ACC (Supplementary Table S2) were PCR amplified from 3D7 genomic DNA using primers listed in Supplementary Table S1. The products were Topo-TA cloned into plasmid pCR2.1 (Invitrogen) and sequenced. The resulting flanks were cloned into pCC-1 to create knockout (KO) mutants *Pf*ACCKO1 and *Pf*ACCKO2 (Fig. 2A).

2.2. Parasite culture and transfection

Parasites were grown as previously described (Trager and Jensen, 1976). The allelic replacement construct was transfected into the D10 parasite line and the gene disruption constructs were transfected into both D10 and 3D7 parasites. All transfections were performed as previously described (Crabb and Cowman, 1996; Tonkin et al., 2004) and yielded drug resistant parasites within 20–30 days. The ACC3′HA lines were subjected to three cycles of alternating drug selection to enrich for lines carrying the integrated tag. Lines carrying the KO constructs were negatively selected with 1 μM 5-Fluorocytosine to eliminate episomal copies (Maier et al., 2006).

2.3. Southern and Western blotting

Integration of gene disruption constructs was confirmed by Southern blot analysis performed as previously described (Crabb et al., 1997; Kalanon et al., 2009). Genomic DNA purified by phenol–chloroform extraction of saponin lysed parasites was digested and probed as shown in Fig. 2A. The sizes of the expected fragments are detailed in Supplementary Table S3. DNA probes were

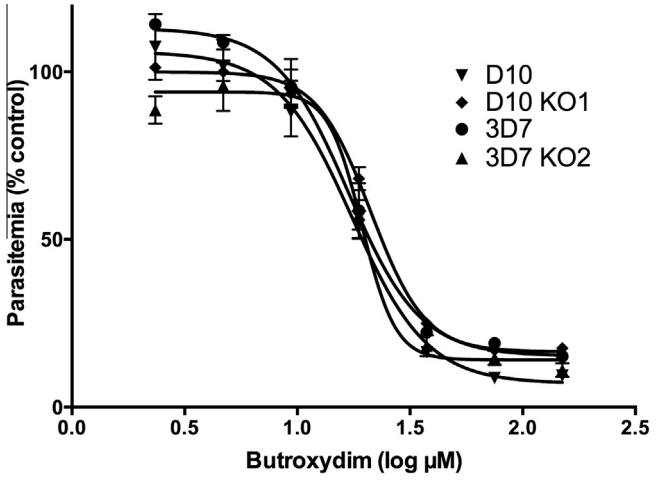
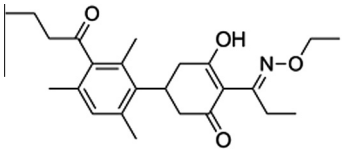
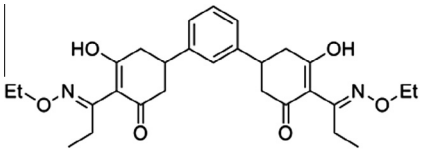


Fig. 3. *Plasmodium falciparum* acetyl CoA carboxylase is not the target of ACC inhibiting herbicide, butroxydim. Dose–response curves for inhibition of *P. falciparum* acetyl CoA carboxylase knockout and D10 and 3D7 parental strains by the grass ACC inhibiting herbicide, butroxydim ($n = 3$).

Table 1
Comparison of acetyl CoA carboxylase inhibitor effects on *Plasmodium falciparum* acetyl CoA carboxylase knockout parasites.

Inhibitor	Strain	IC_{50} (μM)	*P* value
Butroxydim 	D10	17.5	0.45
	D10 KO1	21.9	
	3D7	16.8	0.69
	3D7 KO2	19.4	
Dimeric cyclohexane-1,3-dione oxime 11 	3D7	3.0	0.86
	3D7 KO2	4.4	

generated by PCR amplification and labelled with α-[^{32}P] dATP using the Prime-a-Gene® system (Promega). Western blots were performed as previously described (Mullin et al., 2006). Mouse anti-HA primary antibody (Roche, Australia) was diluted 1/500, and anti-mouse horseradish peroxidase (HRP) secondary antibody (Thermo Fisher Scientific, Australia) was diluted 1/5,000.

2.4. Immunofluorescence assays and microscopy

Immunofluorescence assays were performed as previously described (Tonkin et al., 2004). Rat anti-HA primary antibody (Roche) was diluted 1/200. Goat anti-rat antibody conjugated to Alexafluor 488 (Molecular Probes, Australia) was diluted 1/1,000. Rabbit anti-*Pf*ACP antibody (Waller et al., 2000) was diluted 1/1,000, as was anti-rabbit antibody conjugated to Alexafluor 546 (Molecular Probes). Nuclei were stained using Hoechst 33258. Images were collected with a Leica SP2 confocal microscope, adjusted for brightness and contrast, merged and assembled into panels using Adobe Photoshop.

2.5. Parasite growth assays and drug trials

Analysis of parasite growth rates was performed using Giemsa stained parasites prepared as previously described (Goodman et al., 2007). Tightly synchronised parasites were started at low (0.1–0.2%) parasitemia and grown for 96 h. Total parasitemia was assessed at 24 h intervals. Statistical comparisons of growth rates were carried out using Excel software (Microsoft, USA).

The ACC inhibitors butroxydim and dimeric cyclohexane-1,3-diome oxime 11 (Louie et al., 2010) were dissolved in DMSO at 1,000× the maximum concentration and diluted with growth media to working concentrations. Parasite growth rates were assessed using a modified version of the SYBRgreen assay (Smilkstein et al., 2004; Goodman et al., 2007). Calculation of IC_{50} values and statistical comparison between strains (Mann–Whitney test) were carried out using GraphPad Prism Software.

3. Results and discussion

3.1. PfACC localises to the apicoplast

The *Pf*ACC protein was tagged at the C-terminus with HA peptide by allelic replacement via a single crossover to modify the 3′ end of the gene. Integration was confirmed by Western blotting of parasite proteins with anti-HA sera, which identified a band with molecular mass consistent with the predicted mass of 395 kDa (Fig 1A). Immunofluorescence assays using anti-HA sera on early trophozoite stage parasites expressing HA-tagged *Pf*ACC labelled a small structure in the parasites that is also positive for the apicoplast marker ACP (Fig 1B). Negative control immunofluorescence assays on wild type D10 parasites did not label the apicoplast with anti-HA sera (data not shown). The *Pf*ACC gene thus encodes an apicoplast-localised protein of approximately the expected mass in blood stages, confirming a prediction of apicoplast targeting based on the presence of a bipartite N-terminal leader (Ralph et al., 2004). This also confirms that *Pf*ACC is expressed at detectable levels during the blood stage, suggesting possible activity.

3.2. Deletion of PfACC yields viable blood stage parasites

Several genes of the apicoplast FASII pathway are dispensable in the blood stages of *Plasmodium* spp. infections (Yu et al., 2008; Vaughan et al., 2009; Pei et al., 2010), suggesting that de novo fatty acid synthesis is not essential for successful erythrocytic infection. The only predicted role for *Pf*ACC is providing precursors for FASII. To test whether *Pf*ACC is also dispensable in blood stages we constructed two independent deletions of *Pf*ACC in both 3D7 and D10 parasite strains. In each line, integration of the construct deleted more than 6 kb of the *Pf*ACC gene (Fig 2A). Following negative selection with 5-FC to eliminate episomal copies of the construct, Southern blotting confirmed the integration of the gene deletion construct and the clonal nature of the parasite line (Fig. 2B). Growth rates based on total parasitemia were not observably different between the parental and the knockout strains (Fig 2C) confirming that in the red blood cell stages, *Pf*ACC is dispensable. This suggests that this protein does not have critical roles in the blood stage beyond the provision of malonyl-CoA for apicoplast localised fatty acid synthesis.

The role of ACC beyond the asexual stage in red blood cells remains unexplored. In rodent parasites there is a common phenotype seen in parasites lacking either the PDH (Pei et al., 2010), which provides substrates for ACC, or the downstream FASII genes (Yu et al., 2008; Vaughan et al., 2009). Therefore, it would be surprising if ACC KOs yield a different phenotype and this would suggest that ACC has a function beyond fatty apicoplast fatty acid synthesis in the mosquito stage. In *P. falciparum* it would be expected that, similar to the FASII KOs (van Schaijk et al., 2013), ACC KO parasites would fail to complete the mosquito stage. Again, any novel phenotype in the stages prior to sporozoite development would suggest alternative functions for ACC.

3.3. Herbicide trials with ACC minus parasites

Wild type (D10 and 3D7) and corresponding *Pf*ACC minus parasite lines (D10 KO1 and 3D7 KO2) were exposed to the ACC

inhibitor butroxydim during in vitro growth assays (Fig. 3). There was no observable difference in the growth response of the knockout parasites when compared with the parental strains. The observed IC_{50} values of the deletion parasites (Table 1) were in agreement with those reported previously (Louie et al., 2010) and there was no significant difference in the IC_{50} values found between the parasite lines carrying the gene deletion and wild type strains for either compound. Similar results were found for when the inhibitor dimeric cyclohexane-1,3-dione oxime 11 (Louie et al., 2010) was tested against the 3D7 and 3D7 KO2 parasite lines (Table 1) with no significant differences between the IC_{50} values for the two lines. We conclude that ACC is not the target of these compounds and that their parasiticidal activity is off target.

Acknowledgments

The work was supported by a National Health and Medical Research Council of Australia Program Grant to GIM. GIM was supported by an Australian Research Council Federation Fellow and a Howard Hughes Medical Institute, USA, International Research Scholar Award. We thank the Australian Red Cross for blood.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2014.01.007.

References

Botté, C., Dubar, F., McFadden, G.I., Marechal, E., Biot, C., 2012. *Plasmodium falciparum* apicoplast drugs: targets or off-targets? Chem. Rev. 112, 1269–1283.

Botté, C., Yamaryo-Botté, Y., Rupasinghe, T., Mullin, K.A., MacRae, J., Spurck, T., Kalanon, M., Shears, M., Coppel, R., Crellin, P., Maréchal, E., McConville, M., McFadden, G., 2013. Atypical lipid composition in the purified relict plastid (apicoplast) of malaria parasites. Proc. Natl. Acad. Sci. U.S.A. 110, 7506–7511.

Crabb, B.S., Cowman, A.F., 1996. Characterization of promoters and stable transfection by homologous and nonhomologous recombination in *Plasmodium falciparum*. Proc. Natl. Acad. Sci. U.S.A. 93, 7289–7294.

Crabb, B.S., Cooke, B.M., Reeder, J.C., Waller, R.F., Caruana, S.R., Davern, K.M., Wickham, M.E., Brown, G.V., Coppel, R.L., Cowman, A.F., 1997. Targeted gene disruption shows that knobs enable malaria-infected red cells to cytoadhere under physiological shear stress. Cell 89, 287–296.

Fidock, D.A., Wellems, T.E., 1997. Transformation with human dihydrofolate reductase renders malaria parasites insensitive to WR99210 but does not affect the intrinsic activity of proguanil. Proc. Natl. Acad. Sci. U.S.A. 94, 10931–10936.

Goodman, C.D., McFadden, G.I., 2007. Fatty acid biosynthesis as a drug target in apicomplexan parasites. Curr. Drug Targets 8, 15–30.

Goodman, C.D., Su, V., McFadden, G.I., 2007. The effects of anti-bacterials on the malaria parasite *Plasmodium falciparum*. Mol. Biochem. Parasitol. 152, 181–191.

Holz, G.G., 1977. Lipids and the malarial parasite. Bull. WHO 55, 237–248.

Jelenska, J., Sirikhachornkit, A., Haselkorn, R., Gornicki, P., 2002. The carboxyltransferase activity of the apicoplast acetyl-CoA carboxylase of *Toxoplasma gondii* is the target of aryloxyphenoxypropionate inhibitors. J. Biol. Chem. 277, 23208–23215.

Kalanon, M., Tonkin, C.J., McFadden, G.I., 2009. Characterization of two putative protein translocation components in the apicoplast of *Plasmodium falciparum*. Eukaryot. Cell 8, 1146–1154.

Kim, K.H., 1997. Regulation of mammalian acetyl-coenzyme A carboxylase. Annu. Rev. Nutr. 17, 77–99.

Louie, T., Goodman, C.D., Holloway, G.A., McFadden, G.I., Mollard, V., Watson, K.G., 2010. Dimeric cyclohexane-1,3-dione oximes inhibit wheat acetyl-CoA carboxylase and show anti-malarial activity. Bioorg. Med. Chem. Lett. 20, 4611–4613.

Maier, A.G., Braks, J.A., Waters, A.P., Cowman, A.F., 2006. Negative selection using yeast cytosine deaminase/uracil phosphoribosyl transferase in *Plasmodium falciparum* for targeted gene deletion by double crossover recombination. Mol. Biochem. Parasitol. 150, 118–121.

Mi-Ichi, F., Kita, K., Mitamura, T., 2006. Intraerythrocytic *Plasmodium falciparum* utilize a broad range of serum-derived fatty acids with limited modification for their growth. Parasitology 133, 399–410.

Mi-Ichi, F., Kano, S., Mitamura, T., 2007. Oleic acid is indispensable for intraerythrocytic proliferation of *Plasmodium falciparum*. Parasitology 134, 1671–1677.

Mullin, K.A., Lim, L., Ralph, S.A., Spurck, T.P., Handman, E., McFadden, G.I., 2006. Membrane transporters in the relict plastid of malaria parasites. Proc. Natl. Acad. Sci. U.S.A. 103, 9572–9577.

Pei, Y., Tarun, A.S., Vaughan, A.M., Herman, R.W., Soliman, J.M., Erickson-Wayman, A., Kappe, S.H., 2010. *Plasmodium* pyruvate dehydrogenase activity is only essential for the parasite's progression from liver infection to blood infection. Mol. Microbiol. 75, 957–971.

Preston, C., Powles, S.B., 2002. Evolution of herbicide resistance in weeds: initial frequency of target site-based resistance to acetolactate synthase-inhibiting herbicides in *Lolium rigidum*. Heredity 88, 8–13.

Ralph, S.A., van Dooren, G.G., Waller, R.F., Crawford, M.J., Fraunholz, M.J., Foth, B.J., Tonkin, C.J., Roos, D.S., McFadden, G.I., 2004. Metabolic maps and functions of the *Plasmodium falciparum* apicoplast. Nat. Rev. Microbiol. 2, 203–216.

Ramya, T.N., Mishra, S., Karmodiya, K., Surolia, N., Surolia, A., 2007. Inhibitors of nonhousekeeping functions of the apicoplast defy delayed death in *Plasmodium falciparum*. Antimicrob. Agents Chemother. 51, 307–316.

Smilkstein, M., Sriwilaijaroen, N., Kelly, J.X., Wilairat, P., Riscoe, M., 2004. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. Antimicrob. Agents Chemother. 48, 1803–1806.

Smith, S., 1994. The animal fatty acid synthase: one gene, one polypeptide, seven enzymes. FASEB J. 8, 1248–1259.

Surolia, N., Surolia, A., 2001. Triclosan offers protection against blood stages of malaria by inhibiting enoyl-ACP reductase of *Plasmodium falciparum*. Nat. Med. 7, 167–173.

Tonkin, C.J., Van Dooren, G.G., Spurck, T.P., Struck, N.S., Good, R.T., Handman, E., Cowman, A.F., McFadden, G.I., 2004. Localization of organellar proteins in *Plasmodium falciparum* using a novel set of transfection vectors and a new immunofluorescence fixation method. Mol. Biochem. Parasitol. 137, 13–21.

Trager, W., Jensen, J.B., 1976. Human malaria parasites in continuous culture. Science 193, 673–675.

van Dooren, G.G., Marti, M., Tonkin, C.J., Stimmler, L.M., Cowman, A.F., McFadden, G.I., 2005. Development of the endoplasmic reticulum, mitochondrion and apicoplast during the asexual life cycle of *Plasmodium falciparum*. Mol. Microbiol. 57, 405–419.

van Schaijk, B.C., Kumar, T.R., Vos, M.W., Richman, A., van Gemert, G.J., Li, T., Eappen, A.G., Williamson, K.C., Morahan, B.J., Fishbaugher, M., Kennedy, M., Camargo, N., Khan, S.M., Janse, C.J., Sim, K.L., Hoffman, S.L., Kappe, S.H., Sauerwein, R.W., Fidock, D.A., Vaughan, A.M., 2013. Type II fatty acid biosynthesis is essential for *Plasmodium falciparum* sporozoite development in the midgut of *Anopheles* mosquitoes. Eukaryot. Cell. http://dx.doi.org/10.1128/EC.00264-13.

Vaughan, A.M., O'Neill, M.T., Tarun, A.S., Camargo, N., Phuong, T.M., Aly, A.S., Cowman, A.F., Kappe, S.H., 2009. Type II fatty acid synthesis is essential only for malaria parasite late liver stage development. Cell. Microbiol. 11, 506–520.

Vial, H., Ancelin, M., 1992. Malarial lipids, an overview. In: Avila, J., Harris, J. (Eds.), Subcellular Biochemistry. Plenum Press, NY, Intracellular Parasites, pp. 259–306.

Waller, R.F., Keeling, P.J., Donald, R.G.K., Striepen, B., Handman, E., Lang-Unnasch, N., Cowman, A.F., Besra, G.S., Roos, D.S., McFadden, G.I., 1998. Nuclear-encoded proteins target to the plastid in *Toxoplasma gondii* and *Plasmodium falciparum*. Proc. Natl. Acad. Sci. U.S.A. 95, 12352–12357.

Waller, R.F., Cowman, A.F., Reed, M.B., McFadden, G.I., 2000. Protein trafficking to the plastid in *Plasmodium falciparum* is via the secretory pathway. EMBO J. 19, 1794–1802.

Waller, R.F., Ralph, S.A., Reed, M.B., Su, V., Douglas, J.D., Minnikin, D.E., Cowman, A.F., Besra, G.S., McFadden, G.I., 2003. A type II pathway for fatty acid biosynthesis presents drug targets in *Plasmodium falciparum*. Antimicrob. Agents Chemother. 47, 297–301.

Yu, M., Kumar, T.R., Nkrumah, L.J., Coppi, A., Retzlaff, S., Li, C.D., Kelly, B.J., Moura, P.A., Lakshmanan, V., Freundlich, J.S., Valderramos, J.C., Vilcheze, C., Siedner, M., Tsai, J.H., Falkard, B., Sidhu, A.B., Purcell, L.A., Gratraud, P., Kremer, L., Waters, A.P., Schiehser, G., Jacobus, D.P., Janse, C.J., Ager, A., Jacobs Jr., W.R., Sacchettini, J.C., Heussler, V., Sinnis, P., Fidock, D.A., 2008. The fatty acid biosynthesis enzyme FabI plays a key role in the development of liver-stage malarial parasites. Cell Host Microbe 4, 567–578.

Yu, Q., Ahmad-Hamdani, M.S., Han, H., Christoffers, M.J., Powles, S.B., 2013. Herbicide resistance-endowing ACCase gene mutations in hexaploid wild oat (*Avena fatua*): insights into resistance evolution in a hexaploid species. Heredity 110, 220–231.

Zuther, E., Johnson, J.J., Haselkorn, R., McLeod, R., Gornicki, P., 1999. Growth of *Toxoplasma gondii* is inhibited by aryloxyphenoxypropionate herbicides targeting acetyl-CoA carboxylase. Proc. Natl. Acad. Sci. U.S.A. 96, 13387–13392.