Fatty acid synthesis and pyruvate metabolism pathways remain active in dihydroartemisinin induced dormant ring stages of *Plasmodium falciparum*

Nanhua Chen[1], Alexis N. LaCrue[2], Franka Teuscher[1, 3], Norman C. Waters[4], Michelle L. Gatton[3,5], Dennis E. Kyle[2] and Qin Cheng[1,3#]

[1]Drug Resistance and Diagnostics, Australian Army Malaria Institute, Brisbane, Australia; [2]Department of Global Health, University of South Florida, Tampa, FL. 33612, USA; [3] Malaria Drug Resistance and Chemotherapy, Queensland Institute of Medical Research, Brisbane, Australia; [4]Experimental Therapeutics, Walter Reed Army Institute of Research, Silver Spring, MD, USA; [5] School of Public Health & Social Work, Queensland University of Technology, Brisbane, Australia.

Corresponding author: Dr Qin Cheng, Drug Resistance and Diagnostics, Australian Army Malaria Institute. Weary Dunlop Drive, Gallipoli Barracks, Enoggera, Qld 4051, Australia.

Tel: +61 7 3332 4834; Fax: +61 7 3332 4800; Email: qin.cheng@defence.gov.au

Running title: Metabolism of artemisinin-indcued dormant parasites

Abstract

Artemisinin (ART) based combination therapy (ACT) is used as the first line treatment of uncomplicated falciparum malaria worldwide. However, despite high potency and rapid action there is a high rate of recrudescence associated with ART monotherapy or ACT long before the recent emergence of ART resistance. ART induced ring stage dormancy and recovery has been implicated as possible cause of recrudescence; however, little is known about the characteristics of dormant parasites including whether dormant parasites are metabolically active. We investigated the transcription of 12 genes encoding key enzymes in various metabolic pathways in *P. falciparum* during dihydroartemisinin (DHA) induced dormancy and recovery. Transcription analysis showed an immediate down regulation for 10 genes following exposure to DHA, but continued transcription of 2 genes encoding apicoplast and mitochondrial proteins. Transcription of several additional genes encoding apicoplast and mitochondrial proteins, particularly genes encoding enzymes in pyruvate metabolism and fatty acid synthesis pathways, were also maintained. Additions of inhibitors for biotin acetyl CoA carbozylase and enoyl-acyl carrier reductase of the fatty acid synthesis pathways delayed the recovery of dormant parasites by 6 and 4 days, respectively following DHA treatment. Our results demonstrate most metabolic pathways are down regulated in DHA induced dormant parasites. In contrast fatty acid and pyruvate metabolic pathways remain active. These findings highlight new targets to interrupt recovery of parasites from ART-induced dormancy and to reduce the rate of recrudescence following ART treatment.

Keywords: *P. falciparum*, artemisinin, dormant, metabolic activity, gene expression, pyruvate metabolism, fatty acid synthesis

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The authors do not have a commercial or other association that might pose a conflict of interest. The opinions expressed herein are those of the authors and do not necessarily reflect those of the Australian Defence Force Joint Health Command or the US Department of Defense. Reprints or correspondence should be addressed to: Dr. Qin Cheng, Department of Drug Resistance and Diagnostics, Australian Army Malaria Institute, Brisbane, Australia. Tel: +61-7-3332 4834; Fax: +61-7-3332 4800; e-mail: qin.cheng@defence.gov.au.

Introduction

Plasmodium falciparum resistance to conventional antimalarial drugs has become a major obstacle in the global effort of malaria control and elimination. To overcome this obstacle the WHO recommended the use of artemisinin (ART)-based combination therapies (ACTs) as first line treatment of uncomplicated falciparum malaria in endemic countries in 2001 (1). The implementation of ACTs has contributed to the significant reduction in the number of malaria cases and in malaria transmission intensity in many countries over the past decade (2).

ART derivatives have high potency and are fast acting against *Plasmodium spp* including parasites that are resistant to conventional antimalarial drugs. However, there is still a high rate of recrudescence (3-50%) that is associated with ART mono-therapy in non-immune patients (3). Increasing the treatment duration from 3 to 7 days reduced, but did not eliminate recrudescence (4, 5). Combining ART with other antimalarial drugs to form ACTs also reduced the rate of recrudescence.

Several lines of evidence have been developed to explain the observed high rate of recrudescence associated with ART monotherapy and the joint action of ACT in reducing recrudescence. Previous studies demonstrate that ring stage parasites are arrested within 6 hours of exposure to an ART derivative *in vitro* and these ring stages transition into a distinctive morphological state, persist without further growth for days, followed by recovery and normal development in a dose dependent manner (6, 7). A mathematical model that incorporates the ring stage dormancy, recovery rates, and dose dependency of ART-induced dormancy predicts clinical and parasitological failures at rates comparable to those reported in the field with ART mono-therapy (8). Dormant parasites similar in morphology to those observed *in vitro* (7) were also observed *in vivo* in a rodent malaria model following ART treatment (9). Importantly, transfer of *in vivo* derived dormant parasites into new hosts established infection in a dose dependent manner, demonstrating these dormant parasites are viable (9). Combined, these results suggest that ART induced dormancy may be a key factor in *P. falciparum* malaria treatment failure of ART therapy.

ART-induced dormancy and an arrest of growth at ring stages of development highlight an interesting physiological state of development that has not been fully characterized. As suggested from the model and accumulated data thus far, ART-induced dormant ring stages are likely the source of parasite biomass that recovers to initiate recrudescent infections. Furthermore, ART induced dormancy has also been shown to be associated with reduced susceptibility to ART (7, 10, 11). Therefore, understanding metabolism of the parasites during dormancy may lead to novel therapeutic options and provide insight into mechanism(s) of ART resistance. One of the first questions to be answered is "Do the dormant ring stages remain metabolically active?" Interestingly, repeated exposure to dihydroartemisinin (DHA) or 24hrs exposure to mefloquine following DHA pulse *in vitro* reduces the overall recovery rate from dormancy by 10 fold (6), suggesting that dormant stages remain partially susceptible to the drugs; these data suggest the rings may be metabolically active.

To investigate the metabolic activities of DHA-induced *P. falciparum* dormant parasites we examined the transcription profiles of genes encoding key enzymes in various metabolic pathways that are important for maintaining parasite viability, growth and development during asexual stage of life cycle (12). These include mitochondrial electron transport chain, glycolysis and tricarbozylic acid metabolism (TCA), folate synthesis, DNA replication, fatty acid syntheses and RNA synthesis. Enzyme activity, ATP content, DNA and protein synthesis were also examined during the dormant recovery period. We found that despite an overall down regulation of most metabolic pathways, two pathways appear to remain active in dormant rings. This finding will have important implications in explaining how companion drugs in ACT work to reduce recrudescence, leading to new approaches to destroy dormant parasites.

Materials and Methods

Cultivation of P. falciparum parasites. Multiple strains of *P. falciparum* that have not been exposed to DHA prior to this experiment, W2, 3D7, HB3 and S55, were cultivated *in vitro* in 3% human erythrocytes suspended in RPMI1640 and 10% human plasma as described by Trager and Jensen (13). Parasite cultures were synchronised at ring stage by using 5%

sorbitol (14) every second day for 2 consecutive life cycles, and again immediately before DHA treatment.

Selection of genes encoding key enzymes in parasite metabolic pathways. Twelve genes encoding key enzymes in several parasite metabolic pathways that have been shown to be active during asexual stage development were selected for transcription analysis and these included electron transport chain, folate synthesis, glycolysis, DNA replication, fatty acid syntheses and RNA synthesis. Forward and reverse primers were designed to amplify a fragment of each gene. Gene names, abbreviations, genome IDs, metabolic pathways and primer sequences are listed in Table 1. Primers were also designed to amplify an additional six apicoplast genes and three mitochondria genes. The complete list of genes, abbreviations, gene IDs and primer sequences are listed in Table 1.

Dynamics of parasitemia and proportions of dormant parasites after DHA treatment. Synchronised W2 ring-stage parasites (at 2% parasitemia) were treated with 200 ng/ml DHA for 6 hrs and then washed with culture medium (6). A parallel parasite culture was treated with 100mM sodium azide for 24 hrs and used as a negative control. All treated parasite cultures were passed through a magnetic column (MACS Miltenyi Biotec) at 24, 48 and 72 hrs post treatment to remove any growing parasites (6). An equal volume of parasite culture was collected each day before (day 0) and after DHA treatment (days 2, 3, 4, 5, 6, 8, 10 and 12) for making blood smears and for RNA isolation. Proportions of dormant rings, dead and other developmental stages of live parasites were determined as described earlier (7, 10). Briefly, dormant ring stage parasites have condensed chromatin surrounded by a small amount of cytoplasm; dead parasites have defuse or degraded chromatin and cytoplasm, whereas viable parasites are present in normal morphology of ring, trophozoite and schizont stages. The entire experiment was repeated three times using three biological replicates.

Reverse transcription and quantitative real-time PCR. Total RNA was isolated from parasite samples using a NucleoSpin RNA II kit (Macherey-Nagel). rRNasin (RNase inhibitor, Promega) was added to each RNA sample before storing at -80^{o}C. cDNA was synthesised using Superscript III reverse transcriptase (Invitrogen) and gene specific primers following the manufacturer's instructions. Quantitative real-time PCR was carried out using Brilliant II

SYBR Green QPCR Master Mix (Stratagene) on a Stratagene MX4000 QPCR Thermal Cycler in triplicate. The entire experiment was repeated three times using three biological replicates.

Analysis of quantitative RT-PCR data. For each gene examined at each time point, triplicate samples from three different cultures (n=3x3=9) were analysed and the average quantification cycle (Cq value) calculated. These values were normalised against the parasite density (including dormant, dead and normal parasites) of the sample determined by microscopy. The relative change of transcripts at each time point was then calculated using the relative transcription level before treatment (day 0) as baseline. Confidence intervals were determined from 3 independent experiments.

Comparison of transcription levels in different strains. Synchronised ring-stage parasite cultures of W2, 3D7, HB3 and S55 with 2% parasitemia were treated with DHA (200 ng/ml) for 6 hrs. A sample was collected from each culture 48 hrs post treatment. Transcription levels of seryl-tRNA synthetase (*sars*) and biotin carboxylase subunit of acetyl CoA carboxylase (*bc*) were measured by quantitative RT-PCR. The experiment was repeated 3 times.

Lactate dehydrogenase (LDH) assay and cellular ATP assay. A *P. falciparum* LDH enzyme activity assay was performed in triplicate using the LDH based In Vitro Toxicology Assay Kit (Sigma-Aldrich) following the manufacturer's instructions. Cellular ATP content was detected in triplicate using CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega) following the manufacturer's instructions.

Detection of DNA and protein synthesis. Synchronised ring-stage parasites of W2 were treated with 20ng/ml DHA to induce dormancy (6). Parasite samples were collected before (day 0) and after treatment (day 3). For measuring DNA synthesis, [^{3}H] hypoxanthine was added to parasite culture 24 hrs post treatment, incubated for 72 hrs, harvested and analysed (15). For measuring protein synthesis, [^{3}H] isoleucine (0.25mCi) was added to the parasite culture which was then maintained in isoleucine-free media and incubated for 72 hrs. Parasites were then harvested and analysed on SDS PAGE.

Effect of inhibitors on dormancy recovery. Three sets of experiments were carried out to assess the effect of inhibitors on dormancy recovery. In each set of experiments, parallel

synchronised ring-stage parasite cultures of W2 (2% parasitemia) were treated with one of four treatment options: 1) SHAM (untreated); 2) DHA (200ng/ml for 6 hrs); 3) an inhibitor (IC_{50} for 48 hrs); 4) DHA (200ng/ml for 6 hrs) followed by an inhibitor (IC_{50} for 48 hrs). Haloxyfob (Sigma-Aldrich), an inhibitor of biotin acetyl CoA carboxylase (ACC), and triclosan (Sigma-Aldrich), an inhibitor of enoyl-acyl carrier reductase (FABI) (16), were used as an inhibitor in the first and second set of experiments, respectively, while pyrimethamine was used in the third experiment as a control. IC_{50} values of these inhibitors were determined by inhibition of ^{3}H-hypoxanthine uptake as described by Desjardins et al (15). Parasitemias in treated cultures were estimated daily by microscopy until the parasitemia reached 10%. The effect of inhibitors on the recovery of dormant parasites was quantified by comparing the days required to reach 10% parasitemia in cultures with and without inhibitors. Each assay was carried out in triplicate.

Results

Dynamics of parasite density and proportion of dormant parasites following 6 hr DHA treatment of W2 P. falciparum parasites. Parasite density and parasite classification (dormant, dead and viable) were determined microscopically by examining smears made before (day 0) and after DHA treatment (days 2 - 12) in three independent experiments. Parasites were highly synchronised rings on day 0. On day 2 after DHA treatment, the proportion of parasites classified as dormant averaged 51.2%, with the remaining parasites classified as dead based on their morphology. The average proportion of dormant parasites decreased from day 2, with only 13.6% being dormant rings on day 8 (Fig. 1). The proportion of parasites classified as dead increased as the proportion of dormant parasites decreased. Therefore, the parasite population collected on day 2 had the highest prevalence of dormant, yet still viable parasites for investigating gene transcription. The average parasite density, including both dormant and dead parasites, remained stable until day 6. A small number of healthy growing parasites was observed on day 8 and their numbers increased rapidly on days 10 and 12 (Fig. 1). In control cultures where parasites were treated with sodium azide all parasites were dead on day 2 (not shown) and cultures lysed by day 6 without recovery of parasite growth.

Transcription of genes encoding key enzymes in major metabolic pathways during dormancy and recovery. Transcription levels of 12 genes in major metabolic pathways were determined in dormant parasites and compared to the pre-treatment levels (day 0) (Figure 2). On day 2 (48 hrs post treatment), transcription levels in 7 genes including cytochrome b (*cyt b*), *ldh, sars,* 1-deoxy-D-xylulose 5-phosphate synthase (*doxp*), deoxyuridine 5'-triphosphate nucleotidohydrolase (*dUTPase*), dihydrofolate reductase (*dhfr*) and dihydropteroate synthetase (*dhps*), decreased to below 40% of the day 0 transcription levels, suggesting a marked down regulation in electron transport chain, glycolysis, and DNA, tRNA and folate synthesis pathways. Transcription levels of two genes, DNA polymerase alpha (*DNA pol α*) and malate dehydrogenase (*mdh*), were moderately down regulated to 50-60% of day 0 levels (Fig. 2). Interestingly, genes encoding two apicoplast enzymes, pyruvate kinase 2 (*pykii*) and long chain fatty acid elongation enzyme (*lcfaee*), and cytochrome c oxidase subunit II (*coxii*) of the mitochondrial electron transport chain maintained transcription levels comparable to or higher than that of untreated ring parasites (day 0). This suggests that apicoplast and mitochondria pathways are active in dormant parasites of *P. falciparum* 48 hrs after DHA treatment. By contrast, transcription levels of all 12 selected genes in the azide treated control decreased to 0 to 0.01% of pre-treatment levels on day 2, and to below detectable levels by day 6 .

Transcription levels of all 12 genes were reduced from day 3 onwards and reached the lowest expression levels on days 5 and 6. From day 8 onwards, transcription levels of all 12 genes began to increase during recovery of dormant parasites and the subsequent increase in parasitemia. Transcription levels of five genes (*dhfr*, *DNA pol α, pykii, coxii and lcfaee*) recovered more quickly with levels at or above the baseline level by day 8. These 5 genes were transcribed at much higher levels in the recovered parasites on days 10 and 12 compared to pre-treatment parasites after normalising against parasite density (Fig. 2). The over expression of these 5 genes after recovery is likely a result of a mixture of rings, trophozoites and schizonts in cultures on days 10 and 12 and higher transcription levels of these genes in trophzoites and schizonts as shown in PlasmoDB (17).

LDH activity during dormancy and recovery. On day one (24 hrs after DHA treatment) LDH enzyme activity decreased to only 10% of pre-treatment enzyme activity. LDH activity was below detectable levels between days 2 and 8 after DHA treatment, but increased on days

10 and 12 (Fig. 3). This result is in agreement with the *ldh* transcription profile suggesting that glycolysis pathway was severely down regulated in dormant parasites and recovered when normal parasite growth resumed.

Cellular ATP levels during dormancy and recovery. ATP levels mirrored the trend of LDH levels (Fig. 3). Since glycolysis is the main source of ATP production in blood stages of malaria parasites (18), reduction in ATP levels are expected when glycolysis has been suspended.

Transcription of genes encoding apicoplast pyruvate metabolism and fatty acid synthesis pathways (FASII) during dormancy and recovery. We found that two enzyme encoding genes (*pykii* and *lcfaee*) in the apicoplast maintained high levels of transcription during DHA induced dormancy. To further investigate metabolism in the apicoplast during dormancy and recovery, transcription of six additional genes encoding enzymes in pyruvate metabolism and fatty acid synthesis (FASII) pathways were examined (Fig. 4). Similar to *pykii*, triose phosphate transporter (*itpt*) and pyruvate dehydrogenase E1 beta subunit (*pdh e1β*) of the pyruvate metabolism pathway maintained their baseline transcription levels on day 2, suggesting this pathway is active in dormant parasites following DHA treatment.

Transcription of genes encoding enzymes in FASII pathway and lipoyl synthesis pathway downstream of FASII including *acc*, *fabi* and lipoyl synthase (*lipA*) was also examined. Both subunits of ACC, biotin acetyl CoA carboxylase (*acc*) and *bc,* had transcription levels on day 2 that were 1.83 and 3.72 fold higher, respectively, than untreated rings (Fig. 4). Their transcription levels remained above 30% of baseline level throughout the dormancy recovery experiment. Transcriptions of *fabi* and *lipA* were also up-regulated on day 2 by approximately 2 to 3 fold respectively, compared to baseline (Fig. 4). Interestingly, transcription levels of *lipA* were maintained at levels comparable to, or higher than, untreated rings throughout the entire dormancy recovery period. It is worth noting that lipoic acid produced by the lipoyl metabolism pathway is a cofactor of the E2 subunit of pyruvate dehydrogenase (PDH) (19), thus it may have a positive feedback effect on PDH in the upstream pyruvate metabolism pathway. Transcription levels of *Lcfaee* in the apicoplast fatty acid synthesis pathway were equivalent on day 2 with pre-treatment parasites (Fig. 2). In contrast to these results, transcription of *doxp*, a key enzyme of isoprenoids metabolism

pathway, was markedly down-regulated in dormant parasites (Fig. 2). These results suggest that the apicoplast fatty acid synthesis pathway is important for survival of dormant parasites, while other metabolic pathways in the apicoplast such as isoprenoid metabolism are not.

Effect of fatty acid synthesis inhibitors on dormancy recovery. To confirm the importance of FASII in the survival of DHA induced dormant parasites, the effects of haloxyfop and triclosan on dormancy recovery profile in W2 were examined. Haloxyfob is an inhibitor of ACC while triclosan is an inhibitor of FABI (16). As shown in Figure 5A, haloxyfob treatment (IC_{50} =440μM for W2) alone for 48 hrs delayed parasite growth by 2 days, while the same treatment of haloxyfob after 6 hrs treatment with DHA delayed dormant parasite recovery to 10% parasitemia by 6 days when compared to DHA alone (day 20 compared to day 14). Triclosan (IC_{50}=5μM for W2) delayed both parasite growth and dormant parasite recovery to 10% parasitemia by 4 days (Fig. 5B). In contrast, pyrimethamine (IC_{50}=27μM for W2), a DHFR inhibitor, delayed parasite growth by 2 days when used alone, but had no effect on the recovery of dormant parasites (Fig. 5C). These results demonstrate both haloxyfob and triclosan had an inhibitory effect on dormant parasites and were in good agreement with findings of the transcription component of this study where *acc* and *fabi* were found to be up-regulated, while *dhfr* was down-regulated in dormant parasites. As both haloxyfob and triclosan are inhibitors of enzymes in the apicoplast FASII pathway, the results suggest that FASII is active during DHA induced dormancy and is important for the survival and recovery of dormant parasites.

Transcription of genes encoding mitochondrial electron transport chain during dormancy and recovery. Transcription of two genes encoding proteins in the mitochondrial electron transport chain, *cytb* and *coxii,* were included in the initial 12 genes studied. While transcription of *cytb* (complex iii) reduced significantly in dormant parasites on day 2, *coxii* (complex iv) maintained its transcription level comparable to the baseline level (Fig. 2). To further investigate the activity of the electron transport chain in dormant parasites, transcription of genes encoding NADH: ubiquinone oxidoreductase II (*ndh2,* complex i), flavoprotein subunit of succinate dehydrogenase (*sdha,* complex ii) and ubiquinnol-cytochrome c reductase iron-sulfur subunit (*uqcr*, complex iii) in this transport chain were investigated. Similar to *coxii*, the three additional genes maintained their transcription at

baseline levels on day 2 (Fig. 6). All except *cyt b* (encoded by the mitochondria genome) are nuclear genes encoding enzymes in the mitochondria. It is possible that transcription of these nuclear genes accumulates, preparing for recovery from dormancy, and that *cyt b* serves as a control point of the pathway. As soon as *cyt b* transcription in mitochondria is resumed, the pathway will be fully functional immediately.

Comparison of transcription levels in different parasite strains. Transcription of two genes that were either up (*bc*) and down (*sars*) regulated in the W2 strain following DHA treatment, were compared in 3 additional *P. falciparum* strains with different genetic background (in samples collected on day 2 following DHA exposure). Transcription levels of *sars* in all strains were low, ranging from 3.6 to 8.6% of those in pre-treatment rings. While the transcription levels of *bc* in 3D7, HB3 and S55 strains were not up-regulated as in W2, they remained above 43% of those in untreated rings, and considerably higher than those of *sars* (data not shown).

DNA and protein synthesis in dormant parasites. [^{3}H] hypoxanthine uptake in dormant parasites was analysed and compared with normal ring stage parasites as well as red blood cell controls. There was no detectable [^{3}H] hypoxanthine uptake in dormant parasites, indicating that DNA synthesis was suspended (data not shown). Similarly, [^{3}H] isoleucine uptake was not detected in dormant parasites (data not shown), suggesting that protein synthesis also was suspended or at levels below detection in dormant parasites after DHA treatment.

Discussion

A proportion of ring stage *P. falciparum* parasites are capable of arresting their development after a short exposure to DHA *in vitro* and resuming growth several days later. This DHA-induced dormancy phenomenon shares similarities to environmentally induced microbial persistence that causes recurrent infections after therapy (reviewed by Cohen et al) (20). To better understand the mechanisms underlying this phenomenon it is important to first determine whether the arrested parasites are metabolically active. In a previous study, we investigated DHA induced dormancy in *P. falciparum in vitro* and observed that treatment of dormant parasites with mefloquine or repeating DHA treatment delayed

parasite recovery markedly (6). The observation suggested that DHA induced dormant parasites were susceptible to other drugs, meaning some metabolic pathways are active.

In the current study, we have investigated metabolism in DHA-induced dormant parasites by using several different approaches, including quantifying transcription levels for genes encoding key enzymes, measuring LDH activity, ATP content, DNA/protein syntheses and examining the effect of enzyme inhibitors during dormancy recovery. The outcome of these investigations provide direct and indirect evidence for metabolic activities in dormant parasites. Overall, dormant parasites appear to be metabolically inactive, as DNA and protein synthesis were not detected, and LDH activity and ATP content were also not detectable. These results indicate that glycolysis, the main ATP producing pathway is suspended in dormant parasites; thus most cellular activities that require energy would be expected to be reduced significantly. However, ATP can also be produced within the apicoplast by the pyruvate metabolism pathway (19), and within mitochondria by the electron transport chain (18). Our transcription analysis demonstrated up-regulation of the pyruvate pathway, suggesting that ATP may be produced by the pyruvate metabolism pathway to power other metabolic pathways within the apicoplast and mitochondria (e.g., FASII and electron transport chain). If ATP is used as it is produced, the overall ATP level would remain in steady state in dormant parasites, and could be measured as low or undetectable.

Gene transcription activities are important biomarkers for metabolism. The results of qRT-PCR indicate that all 12 genes selected from major metabolic pathways were still transcribed at some level in dormant parasites (day 2 after DHA treatment). Among these genes, those encoding enzymes in pyruvate and fatty acid metabolism pathways in the apicoplast (*pykii* and *lcfaee*) were most interesting as their transcription in dormant parasites was maintained at comparable or even higher level than those in corresponding untreated ring stage parasites. Further examination of transcription of other nuclear genes encoding enzymes in pyruvate metabolism, fatty acid synthesis (FASII) and lipoyl metabolism in apicoplast demonstrates that they also maintained comparable or elevated transcription levels. In the apicoplast, pyruvate metabolism pathway is upstream of the fatty acid synthesis pathway, while lipoyl metabolism is downstream (19). Pyruvate metabolism produces ATP and acetyl CoA for fatty acid synthesis (19, 21), whereas Lipoyl metabolism

pathway provides cofactor for PDH to facilitate pyruvate metabolism. It is very interesting to see that all the examined genes encoding enzymes on these pathways were transcriptionally active. Therefore transcription analysis suggests that this extended fatty acid synthesis pathway in the apicoplast is still active in dormant parasites. It should be noted that although the 12 genes selected for transcription analysis encode key enzymes in several important metabolic pathways they may not reflect all metabolic activities of dormant parasites. A comprehensive transcriptional profiling and metabolomics analysis of dormant parasites may identify more active pathways.

The steady state in the transcription level is generally determined by the balance between gene transcription and degradation of mRNA molecules. Therefore mRNA with a shorter half-life will have lower mRNA level when gene transcription suspends. In the genes we examined, mRNA of *ldh* and *sars* have been shown to have a relatively long half-lives of 17 and 21.4 hr, respectively, at ring stage (PlasmoDB, (22)), while *lcfaee*, *pykii* and *coxii* have relatively shorter half-lives of 7.4 hr, 12.2 hr and 9.1 hr at ring stage, respectively (PlasmoDB, (22)). Since three genes with shorter half-lives had higher transcription levels detected in dormant rings compared to those for the two genes with longer half-lives, we believe the difference in transcription levels of the genes examined was mainly due to the difference in levels of gene transcription, rather than speed of mRNA degradation. It is also unlikely that the up-regulation of genes in two pathways was due to parasites arrested at ring stage leading to accumulation of transcripts because only 3 of the 12 genes examined were found up-regulated while the remaining 9 genes were found down regulated.

Transcription profiles for genes of interest are usually normalised against the transcription of a house keeping gene. For *P. falciparum*, *sars* is one of the house keeping genes that is transcribed consistently throughout blood stage schizongony and often is used as a normaliser (23-25). However, in the current study total parasitemia of each sample was used as a normaliser because transcription of *sars* could be affected during DHA-induced dormancy. Indeed, transcription of *sars* was severely down regulated from the beginning of dormancy and was among the slowest genes to recover to its baseline level. To minimize the effect of dead parasites, transcription levels for each gene was primarily assessed on day 2 after treatment when over 50% of parasites were dormant so the effect of dead parasites was similar for each gene measured. Importantly, the relative proportion of dormant to

dead parasites was identical for each gene measured at the same time point for each gene transcript. In addition, since our main finding was the up-regulation of genes encoding enzymes in two pathways, it is likely that transcription levels of these genes were underestimated due to the effect of dead parasites.

Further evidence for the fatty acid synthesis pathway being active in dormant rings came from inhibitor experiments where the effect of inhibitors of these pathways, haloxyfob (inhibitor of ACC) and triclosan (inhibitor of FABI), on parasite recovery from dormancy was assessed. Both inhibitors significantly delayed the recovery of DHA-induced dormant parasites when added after DHA treatment for 48 hrs while pyrimethamine, added as a negative control to inhibit the down regulated folate synthesis pathway, failed to do so. This suggests that ACC and FABI were active in dormant rings, and that by blocking their activities for a period of 48 hrs during dormancy the recovery of dormant parasites could be impacted. In contrast, DHFR was not active in dormant parasites judging from a strong down regulation of its transcription level and the cessation of DNA synthesis, therefore it is not unexpected that dormant parasites were not susceptible to pyrimethamine making it a good negative control. Haloxyfob is a known inhibitor of plant plastid ACC (26) and has been shown to inhibit ACC of *Toxoplasma gondii* (27). In the current study it is likely that haloxyfob inhibited ACC of dormant parasites and the downstream fatty acid synthesis pathway, delaying their recovery by 6 days. Triclosan is an inhibitor of FABI in bacteria (28, 29) and was initially shown to inhibit *P. falciparum* growth (30, 31). However, triclosan also has unknown off-target(s) in *P. falciparum* since parasites with a genetic knockout of FabI remains susceptible to triclosan (32). Therefore, we could not rule out an off-target effect of triclosan as impacting on the recovery of dormant parasites.

The FASII pathway is present in all bacteria and in the plastid of plants, algae and apicomplexan parasites. Its presence in *P. falciparum* was discovered in the late 1990s (33, 34) and was targeted for antimalarial drug development. Recent knockout studies show that FASII is essential for the development of the liver stage of the parasite, but not for blood stage parasites *in vitro* (32, 35). This is in agreement with earlier reports that blood stage parasites could scavenge fatty acid (36, 37) and lipoic acid (38) from the host. However, FASII may play a role *in vivo* and when parasites are under stress. Indeed, genes encoding FASII enzymes were reported to have been up-regulated in 43 patients in Senegal

(39), especially in a cluster of parasite isolates with transcription profile matching starvation response. Exposure to artemisinin drugs such as DHA also poses a strong stress to malaria ring stage parasites as evidenced by the abrupt arrest of parasite development and immediate killing effect. Although mechanisms underlying dormancy recovery remain to be elucidated, the apicoplast fatty acid synthesis pathway is likely important for the survival and/or recovery of dormant parasites.

Drug induced persistence is common in microbes. Its underlying mechanism had been linked to metabolic dormancy because antibiotics kill growing microbes by interrupting various metabolic pathways. However, increasing evidence suggest that persistence is not a passive dormancy as some metabolic pathways, such as global regulators, amino acid synthesis, DNA repair (40) and nucleotide metabolism (41), are active in microbe persisters. Recently, it has been proposed that persistence is an active process microbes use for survival (42). Our current study on DHA induced-dormancy provides supporting evidence for this mechanism in *P. falciparum*. Our results show that dormant ring stages down regulated gene transcription in glycolysis, TCA, DNA and protein syntheses pathways after exposure to DHA. These dormant parasites are not metabolically quiescent yet they have stopped growing and dividing. Genes in the pyruvate and FASII pathway continue to be transcribed in dormant parasites and furthermore, inhibitors of enzymes of the FASII pathway delayed the recovery of dormant parasites. The results suggest that the extended FASII pathway is active during dormancy and is important for the survival and recovery of dormant parasites. In summary, these data highlight new avenues to interrupt dormancy recovery that will help to reduce the rate of recrudescence following ART treatment. Successful reduction in survival of dormant parasites will reduce the reliance and consequential pressure on companion drugs in ACT, especially where resistance to the companion drug already exists.

These observations shed new light on the biology of ART induced dormant ring stages of *P. falciparum*, yet the precise role of dormancy as it relates to the emergence of ART resistance remains to be elucidated. Importantly, the metabolic data obtained in this study were from parasites that expressed the previously described, morphologically distinct dormant rings (6, 7) rather than a quiescent stage with normal ring stage morphology (11). Our current understanding of the biology of dormant ring stages is that ART-induced dormancy is a phenotype expressed in both susceptible and ART-resistant *P. falciparum* (6,

7) and therefore could confound the underlying biology of emerging ART resistance. Secondly a proportion of dormant parasites remain viable and this viability is linked to up-regulation of two key pathways, fatty acid synthesis and pyruvate metabolism. Based upon accumulated evidence, ART-induced dormancy therefore enhances survival of parasites following treatment with ART therapy and is a likely mechanism for the high rate of recrudescence observed even prior to ART-resistance emergence. The proportion of ring stages that survive dormancy following treatment should enhance the potential acquisition of secondary resistance traits that would allow the parasite to continue to develop in the presence of increasing drug concentrations (43). More detailed studies are required to assess on a global scale if additional pathways are important in the biology of dormant parasites and to determine if these same pathways are up-regulated in ART-resistant parasites following exposure to drug.

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Table 1. Description of gene and primer sequences for genes encoding key enzymes in various pathways (* Genes included in the initial screening).

Cellular Compartment	Pathway	Gene ID	Gene name (abbreviation)	Forward primer (5' to 3')	Reverse primer (5' to 3')
Cytosol	Glycolysis	PF3D7_1324900	L-lactate dehydrogenase (*ldh*)*	AGGACAATATGGACACTCCGAT	TTTCAGCTATGGCTTCATCAAA
	Folate metabolism	PF3D7_0417200	dihydrofolate reductase (*dhfr*)*	ACCTGCGCAGTTCATACACG	TGTTGGGAATGGATAGGGTATTCTGT
	Folate metabolism	PF3D7_0810800	dihydropteroate synthetase (*dhps*)*	AGGTATTTTTGTTGAACCTAAACGTGC	AGGACCAGAGGATTCTCCACCT
	Aminoacyl tRNA synthesis	PF3D7_0717700	seryl-tRNA synthetase (*sars*)*	AAGTAGCAGGTCATCGTG	CGGCACATTCTTCCATA
Nucleus	DNA replication	PF3D7_0411900	DNA polymerase alpha (*DNA pol α*)*	GCCAAGCCAACCAACCAAACC	TGTGGCTGTTTGTGGATGCAA
	Nucleotide metabolism	PF3D7_1127100	deoxyuridine 5'-triphosphate nucleotidohydrolase (*dUTPase*)*	GCCGCCTTGGATAATACTAGTGACC	TCCAAAACCTCCTTCTCCTCTGGA
Apicoplast	Pyruvate metabolism	PF3D7_0530200	triose phosphate transporter (*itpt*)	CACATGCTGTTGCAAGCACAGTTA	AGAGGAACCAAGTCCACCAAGGA
	Pyruvate metabolism	PF3D7_1037100	pyruvate kinase 2 (*pykii*)*	TGGGTGATATTCAAGGGCCT	ACCCTGTTTTGGTTACCTAATGAA
	Pyruvate metabolism	PF3D7_1446400	pyruvate dehydrogenase E1 beta subunit (*pdh e1β*)	TCCGAAGCAGCAAAAGAATTAACGA	CCCAAAACCAGCTGACTCATCCA
	Isoprenoids metabolism	PF3D7_1337200	1-deoxy-D-xylulose 5-phosphate synthase (*doxp*) *	CCCGCTATGTTAGGAGGATCAGG	TGCCATAGCTGCTGCGAAAGT
	Fatty acid synthesis	PF3D7_1026900	biotin acetyl CoA carboxylase subunit (*acc*)	TGAGTATCTCGATTCCACACAACA	CGTTCTTGTAAGTATCTCGTGTTCC
	Fatty acid synthesis	PF3D7_1469600	biotin carboxylase subunit of acetyl CoA carboxylase (*bc*)	TGCAGTATGGCCTGGATGGG	TCCATAACATTACCAGTTGGACCT
	Fatty acid synthesis	PF3D7_0615100	enoyl-acyl carrier reductase (*fabI*)	TTTTCGGTATTTGGCCTCCT	CAAAAGAAGCGTCAAAGGGT
	Fatty acid synthesis	PF3D7_0920000	long chain fatty acid elongation enzyme (*lcfaee*)*	CATCAACAAATATTATTGGACACCTCA	TCATGTCCATTTCTTTTCTCTTTTTCA
	Lipoic acid	PF3D7_1344600	lipoyl synthase (*lipA*)	TGCATTTTGGTATCCCATCC	TGTATGAACAGGTTCGTTTCT

	metabolism				
Mitochondria	TCA cycle	PF3D7_0618500	malate dehydrogenase (*mdh*)*	AGGGGGCACATCCAGTTGAA	AGTCGAAAGCTTTTTGTGTGTTGCT
	Electron transport chain	PF3D7_0915000	NADH:ubiquinone oxidoreductase II (*ndh2*)	GTTCAGGAAATGTGGACAAGC	ACCACCCCATCCTGAACCTA
	Electron transport chain	PF3D7_1034400	flavoprotein subunit of succinate dehydrogenase (*sdha*)	GGTTCAGATTGGCTTGGGGA	TGTTCTTGAAAACGGGAGTCCA
	Electron transport chain	PF3D7_1439400	ubiquinnol-cytochrome c reductase iron-sulfur subunit (*uqcr*)	ACCTAGGTTGTGTTCCAGCTC	AGGTGCAGGTCCTTGTCTGA
	Electron transport chain	mal_mito_3	cytochrome b (*cyt b*)*	AGCAAGTCGATATACACCAGATGTT	GAGAAGCACCTGTTGCGTGC
	Electron transport chain	PF3D7_1430900	cytochrome c oxidase subunit II (*coxii*)*	GCTATTCCGGGGCGCTTACA	TGCCTCTGGTGAGACAGCCT

Figure legends

Figure 1. Percentage of parasite density (including normal, dormant and dead parasites) and dormant rings prior to and post DHA treatment. Data (mean with 95% confidence intervals) were obtained from 3 independent experiments.

Figure 2. Transcription levels of 12 genes encoding enzymes of several metabolic pathways in DHA treated rings relative to pre-treatment rings (fold ratio ± 95% confidence intervals). Relative transcription levels measured for each of the 12 genes are represented with different colour bars. For each gene, relative transcription levels measured on days 2 (outlined in black), 3, 4, 5, 6, 8, 10 and 12 post DHA treatment (normalised against overall parasitemia including dormant, dead and normal parasites) are grouped together from left to right. Transcription level measured on day 0 (pre-treatment) is set as 1 for each gene. Data (mean with 95% confidence intervals) were obtained from samples in triplicate and three independent experiments. Note: lcfaee could also be active in the endoplasmic reticulum.

Figure 3. LDH activity and cellular ATP level relative to pre-treatment during DHA induced dormancy recovery period. Pre-treatment levels are set as 1. Data (mean and standard error) were obtained from triplicate samples.

Figure 4. Transcription levels of 8 genes encoding enzymes of apicoplast pyruvate metabolism and fatty acid synthesis pathways in DHA treated rings relative to pre-treatment rings (fold ratio ± 95% confidence intervals). Relative transcription levels measured for each of the 8 genes are represented with different colour bars. For each gene, relative transcription levels measured on days 2 (outlined in black), 3, 4, 5, 6, 8, 10 and 12 post DHA

treatment are grouped together from left to right. Transcription level measured on day 0 (pre-treatment) is set as 1 for each gene. Data (mean with 95% confidence intervals) were obtained from samples in triplicate and three independent experiments. Note: lcfaee could also be active in the endoplasmic reticulum.

Figure 5. Effect of haloxyfob (A), triclosan (B) and pyrimethamine (C) on recovery of DHA induced dormant parasite. Parasitemia of viable parasites on various days is shown in the figure. Data (mean with 95% confidence intervals) were obtained from samples in triplicate and three independent experiments.

Figure 6. Transcription levels of 5 genes encoding enzymes of mitochondria electron transport chain in DHA treated rings relative to pre-treatment rings (ratio ± confidence intervals). Relative transcription levels measured for each of the 5 genes are represented with different colour bars. For each gene, relative transcription levels measured on days 2 (outlined in black), 3, 4, 5, 6, 8, 10 and 12 post DHA treatment are grouped together from left to right. Transcription level measured on day 0 (pre-treatment) is set as 1 for each gene. Data (mean with 95% confidence intervals) were obtained from samples in triplicate and three independent experiments.