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Mechanisms of *in vitro* resistance to dihydroartemisinin in *Plasmodium falciparum*

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Summary

The recent reports of artemisinin (ART) resistance in the Thai-Cambodian border area raise a serious concern on the long-term efficacy of ARTs. To elucidate the resistance mechanisms, we performed *in vitro* selection with dihydroartemisinin (DHA) and obtained two parasite clones from Dd2 with more than 25-fold decrease in susceptibility to DHA. The DHA-resistant clones were more tolerant of stressful growth conditions and more resistant to several commonly used antimalarial drugs than Dd2. The result is worrisome since many of the drugs are currently used as ART partners in malaria control. This study showed that the DHA resistance is not limited to ring stage, but also occurred in trophozoites and schizonts. Microarray and biochemical analyses revealed *pfmdr1* amplification, elevation of the antioxidant defense network, and increased expression of many chaperones in the DHA-resistant parasites. Without drug pressure, the DHA resistant parasites reverted to sensitive in approximately eight weeks, accompanied by de-amplification of *pfmdr1* and reduced antioxidant activities. The parallel decrease and increase in *pfmdr1* copy number and antioxidant activity and the up and down of DHA sensitivity strongly suggest that *pfmdr1* and antioxidant defense play a role in *in vitro* resistance to DHA, providing potential molecular markers for ART resistance.

Keywords

artemisinin resistance; drug selection; mechanism; *pfmdr1*; antioxidant defense

Introduction

Artemisinin (ART)-based combination therapy (ACT) has been endorsed by World Health Organization (WHO) and adopted by most malaria endemic countries for treating malaria (Bosman & Mendis, 2007). However, the recent report of delayed clearance of parasites after ART treatment in western Cambodia threatens to derail the current malaria control initiatives (Noedl *et al.*, 2008; 2010; Dondorp *et al.*, 2009). To address this urgent issue, WHO is coordinating large-scale risk evaluation and containment efforts to mitigate the spread of resistance (WHO, 2009, 2011). Yet, close surveillance for ART resistance at sentinel sites, an essential step in resistance management, is hindered by the lack of a clear understanding of the molecular mechanism of resistance and molecular markers.

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Although the reports of potential ART resistance have generated interests in surveillance and containment of the resistance, the molecular mechanisms of ART action and resistance in *Plasmodium falciparum* remain largely unknown (Cui & Su, 2009; Ding *et al.*, 2011). In addition, there is a lack of clarity regarding the definition of artemisinin resistance, which makes genetic associations difficult. Further complication arises when prolonged parasite clearance kinetics is used as a basis of defining resistance, since such a phenotype depends on host as well as parasite variables. Some reports suggested potential association of *P. falciparum* multiple drug resistance 1 (*pfmdr1*) and the sarco/endoplasmic reticulum calcium-dependent ATPase (SERCA) homologue *PfATP6* with ART resistance (Cui & Su, 2009; Ding *et al.*, 2011). Point mutations as well as copy number variations in *pfmdr1* have been implicated in altered sensitivity to multiple structurally unrelated antimalarials including ART (Price *et al.*, 1999; Duraisingh *et al.*, 2000; Reed *et al.*, 2000; Pickard *et al.*, 2003; Sidhu *et al.*, 2005). Whereas elevated *pfmdr1* copy number is associated with increased risk for therapy failures of artesunate (ATS)/mefloquine (MQ) in Thailand and Cambodia and is also found in longitudinal studies of children treated with artemether (ATM)/lumefantrine (LUM) (Alker *et al.*, 2007; Shah *et al.*, 2008; Wongsrichanalai & Meshnick, 2008; Lim *et al.*, 2009; Gadalla *et al.*, 2011), recent clinical trials could not confirm a role of *pfmdr1* in ART resistance (Dondorp *et al.*, 2009; Imwong *et al.*, 2010; Noedl *et al.*, 2010). *PfATP6* has been postulated to be an ART target, because ART could inhibit *PfATP6* activity in a heterologous system, and a single amino acid change (L263E) in *PfATP6* could abolish the inhibition (Eckstein-Ludwig *et al.*, 2003; Uhlemann *et al.*, 2005). A number of polymorphisms have been documented in this gene (Dahlstrom *et al.*, 2008; Tanabe *et al.*, 2011), but they do not seem to correlate with altered sensitivity to ART (Dondorp *et al.*, 2009; Imwong *et al.*, 2010). Only one mutation S769N has been linked to reduced ATM sensitivity in *P. falciparum* field isolates from French Guiana (Jambou *et al.*, 2005), but this mutation has not been found in parasites from regions where highest levels of ART selection are expected (Zhang *et al.*, 2008; Imwong *et al.*, 2010). In addition, allelic exchange study did not yield conclusive evidence supporting the involvement of *PfATP6* in ART responsiveness (Valderramos *et al.*, 2010; Cui *et al.*, 2012). In addition, analysis of the progeny of the HB3 × Dd2 genetic cross has identified two additional loci that are associated with the likelihood of acquiring genetically stable, high-level ART resistance (Beez *et al.*, 2011).

In vitro selection for parasites resistant to a drug followed by investigations of phenotypic and genomic changes is an effective laboratory approach to identifying mutations associated with drug resistance. Several studies have attempted to use this approach to select resistance to ART and its derivatives in *P. falciparum in vitro* (Inselburg, 1985; Chavchich *et al.*, 2010; Witkowski *et al.*, 2010; Beez *et al.*, 2011). Results from these studies varied, probably reflecting a multigenetic nature of ART resistance. Ring stage has been postulated to be able to endure high concentrations of ART through a quiescence mechanism (Teuscher *et al.*, 2010; Witkowski *et al.*, 2010), which may partially explain the ART resistance phenotype of delayed parasite clearance occurring in west Cambodia (Nosten, 2010). To further investigate the molecular mechanisms of ART action and resistance, we selected Dd2 parasite with DHA and obtained two parasite clones that were highly resistant to DHA. We show that the response to DHA was associated with increased *pfmdr1* copy number and elevated antioxidant activities, providing potential molecular markers for monitoring the emergence of ART resistance.

Results

Selection for DHA resistance depends on parasite genetic backgrounds

It has been shown that malaria parasites with different genetic backgrounds acquire resistance to novel antimalarials at different frequencies, and certain parasite strains display

a phenotype of accelerated resistance to multiple drugs (ARMD) (Rathod *et al.*, 1997). To investigate the possibility of ARMD phenotype to DHA, we tested five laboratory parasite strains from different geographical origins for *in vitro* sensitivities to DHA (Table 1). All parasite strains had 50% inhibitory concentrations (IC₅₀ values) for DHA in a close range (3.2 – 7.6 nM), although Dd2 and 7G8 had ~two-fold higher IC₅₀ values than 3D7, D10, and HB3. To generate parasites resistant to DHA, we cultured parasites under constant DHA pressure with an initial concentration corresponding to 2×IC₅₀ value of each strain. Parasites surviving this DHA selection were obtained from Dd2, 7G8, HB3, and D10 (except 3D7) in 37 – 70 days. When the selected parasites were subsequently selected using DHA concentrations at 4×IC₅₀ values of the respective strains, only D10 and Dd2 yielded tolerant parasites. Further selection of these two parasite strains under DHA at 8×IC₅₀ values only generated DHA-tolerant parasites from Dd2 (Table 1). These data suggest that field parasite populations may be highly heterogeneous in DHA sensitivity and propensity to develop resistance to DHA, consistent with the presence of an ARMD phenotype.

Using a similar scheme of step-wise selection we subjected Dd2 to DHA selection and obtained parasites that were able to tolerate constant exposure to 320 nM of DHA (Fig. S1A). This population of DHA-resistant parasites was subsequently cloned in the absence of DHA selection, and 26 clones were obtained after culturing for ~35 days. These parasite clones showed dramatically different susceptibilities to DHA, with IC₅₀ values ranging from 3 to 243 nM (Fig. S1B), suggesting that the DHA-resistant parasites consisted of a heterogeneous parasite population and multiple genes might contribute to the resistance. Two clones (DHA1 and DHA2) selected for further characterization showed ~32- and ~25-fold increase in IC₅₀ value (243 and 196 nM, respectively) compared with the wild type Dd2 strain. It is noteworthy that these lines, compared with the original DHA-resistant population which tolerated 320 nM of DHA, had a substantial reduction in resistance after the cloning procedure without DHA selection pressure.

DHA-resistant lines have a different growth phenotype

When cultures were initiated at a 0.5% ring-stage parasitemia in a drug-free medium with medium changed every other day, all three parasite lines had similar growth curves before reaching peak parasitemias of 8.3, 7.5 and 7.4%, respectively, on day 5 (Fig. 1A). Subsequently, Dd2 parasitemia dropped to 5.5% on day 6, possibly due to increased oxidative stress in the culture (Becker *et al.*, 2004). In comparison, the parasitemias of DHA1 and DHA2 on day 6 and 7 were significantly higher than the respective parasitemias of Dd2 (unpaired *t*-test, *P*<0.017), suggesting that the two resistant lines were more tolerant of oxidative stress. We further determined the intraerythrocytic developmental cycle (IDC) durations of the three parasite lines by monitoring parasite growth every 2 h for 96 h (Fig. S2A). Compared with Dd2 with an IDC duration of 44.5 h (Reilly *et al.*, 2007), DHA1 and DHA2 had significantly longer IDC durations of 47 and 47.5 h, respectively (unpaired *t*-test, *P*<0.017), largely due to more extended schizont development (Fig. S2A, C). The results also suggested that drug selection might have led to some deleterious changes in parasites that survived drug selection.

To determine the stability of the DHA resistance phenotype, DHA1 and DHA2 were cultured without DHA and their sensitivity to DHA was measured at a bi-weekly interval. Without drug pressure, the DHA1 and DHA2 lines gradually lost their resistance, and IC₅₀ values decreased to 20 and 18 nM, respectively, in eight weeks (Fig. 1B). Continuous monitoring of the two parasite lines for two more weeks did not detect a further decrease of DHA sensitivity, although their IC₅₀ values were still significantly higher than that of Dd2 (unpaired *t*-test, *P*<0.003). The loss of DHA resistance was also correlated with the restoration of the IDC duration to levels that were not significantly different from that of Dd2 (Fig. S2B, C). To determine how these parasite lines respond to further DHA selection,

we re-applied the step-wise DHA selection on these revertant parasites. Remarkably, parasites having high DHA IC₅₀ values (244 and 221 nM, respectively) were obtained in ten weeks, as compared to 15 months required for the initial selection of the DHA1 and DHA2 lines. The results suggested that the DHA1 and DHA2 had gained some stable genetic changes that can confer low-level DHA resistance, and these changes are required for acquiring high-level resistance.

Selection for DHA resistance results in reduced susceptibility to other antimalarial drugs

To examine whether selection for DHA resistance also affected sensitivities to other antimalarial drugs, we determined the IC₅₀ values of DHA1 and DHA2 to some commonly used antimalarial drugs (Table 2). Compared with Dd2, DHA1 and DHA2 had significant increases in IC₅₀ values to most drugs tested (unpaired *t*-test, $P < 0.0083$). Specifically, DHA1 and DHA2 had >16-fold increase in IC₅₀ values to ART, but only showed ~5- and ~10-fold increase in IC₅₀ values to the two ART derivatives, ATM and ATS. Also noteworthy, DHA1 and DHA2 were ~10 times more resistant to LUM than Dd2. However, the changes in IC₅₀ values to chloroquine (CQ), amodiaquine (AQ), piperaquine (PQ), MQ and quinine (QN) were modest (2 – 3 folds). In comparison, the DHA-selected lines DHA1 and DHA2 did not show significant changes in sensitivity to atovaquone (ATQ) (unpaired *t*-test, $P > 0.0083$), a drug that acts on the mitochondria (Table 2). Remarkably, *in vitro* sensitivities of Dd2, DHA1, and DHA2 to most of these drugs except for ATQ were significantly correlated (Table S1), suggesting selection of a common resistance mechanism.

The increased resistance to quinoline drugs prompted us to further characterize the resistance phenotype and potential mechanisms. CQ resistance mechanism is by far the best understood; mutations in the *P. falciparum* CQ resistance transporter (PfCRT) are the major determinant of CQ resistance (Fidock *et al.*, 2000). A hallmark of PfCRT-dependent CQ resistance is chemosensitization by verapamil (VP), a Ca²⁺ channel blocker (Martin *et al.*, 1987; Patel *et al.*, 2010). To determine the VP-reversibility of CQ resistance of the two DHA-selected lines, we determined their CQ IC₅₀ values in combination with VP. We found VP could re-sensitize the CQ resistance in all three lines, but the IC₅₀ values in DHA1 and DHA2 had 2.3- and 1.8-fold decreases, from 308 and 270 to 133 and 156 nM, respectively, as compared to a 5.6-fold decrease (from 161 to 28.75 nM) in Dd2 (Fig. S3). This result implies the resulted increase in CQ resistance in DHA1 and DHA2 was mostly not reversible by VP.

The drug sensitivity profiles for DHA-selected parasites revealed that they not only showed significant decreases in sensitivities to all ART related drugs, but also displayed reduced sensitivities to a number of currently used ACT partner drugs. Here we compared the IC₅₀ values of Dd2, DHA1 and DHA2 to two commonly used ACTs: DHA/PQ and ATM/LUM. Compared with Dd2, both DHA1 and DHA2 showed significantly reduced sensitivities to these two ACTs (unpaired *t*-test, $P < 0.003$) (Fig. S4). Specifically, DHA-selected lines had ~5-fold and >2-fold higher IC₅₀ values than Dd2 to DHA/PQ and ATM/LUM, respectively.

DHA resistance is mediated by, but not limited to, ring stage dormancy mechanism

Previous selection for ART resistance using an intermittent drug selection scheme showed that tolerance to ART was mediated by a quiescence mechanism occurring only at the ring stage (Witkowski *et al.*, 2010). This ART-induced temporary growth arrest at ring stage occurs readily in laboratory strains, and the recovery rates depend on genetic backgrounds of the strains (Teuscher *et al.*, 2010). To determine whether DHA resistance in DHA1 and DHA2 also involves the ring-stage quiescence mechanism, we treated the ring-stage cultures with 1 or 3 μM of DHA for 48 h and monitored parasite recovery from drug treatment. After treatment with 1 μM of DHA, DHA1 and DHA2 recovered and reached 10% parasitemia in

seven days, as compared to 16 days for Dd2 (Fig. 2A). When treated with 3 μM of DHA, DHA1 and DHA2 reached 10% parasitemia in 10 and 12 days, respectively, whereas it took 21 days for Dd2 to do so. These results showed that DHA1 and DHA2 could survive and quickly recover from high doses of DHA treatment, possibly reflecting the recrudescence frequently associated with ART monotherapy.

To determine whether this recrudescence mechanism also plays a role in other developmental stages, synchronized parasites at ring, trophozoite and schizont stages were exposed to 1 μM of DHA for 2 h and parasites that survived the treatment were counted 6 h later using flow cytometry (Fig. 2B). Significantly more DHA1 and DHA2 parasites were recovered after DHA treatment from all three stages than Dd2 (unpaired *t*-test, $P < 0.003$). Similar results were obtained when unsynchronized parasites were exposed to 100 nM of DHA for 48 h (Fig. 2C). These results showed that parasite resistance to ART was not limited to ring stage, but also occurred in trophozoite and schizont stages.

DHA resistance does not involve known mutations associated with drug resistance

Reduced susceptibility to ART has been reported to associate with mutations in multiple genes, including *pfmdr1* (Sanchez *et al.*, 2008), *pfcr1*, *pfctcp* (Bhisutthibhan *et al.*, 1998), *pfubcth* (Hunt *et al.*, 2007), putative ABC transporters *G7* and *G49* (Mu *et al.*, 2003; Anderson *et al.*, 2005), *pfmrp* (Raj *et al.*, 2009), and *PfATP6* (Jambou *et al.*, 2005). Sequencing of the coding regions of all these genes in DHA1 and DHA2 did not detect any differences from those in Dd2. These data also verified that DHA1 and DHA2 were derived from Dd2.

Comparison of global gene expression in Dd2 and DHA1

Microarray analysis was performed to determine differences in gene expression at three stages between Dd2 and DHA1 and in response to DHA treatments. Microarray data from the two biological replicates for each treatment were highly correlated ($R^2 > 0.96$, $P < 0.001$). Based on IDC progression pattern in Dd2 and DHA1 (Fig. S2A), we selected three matching time points to compare differences in gene expression in ring, trophozoite and schizont stages between Dd2 and DHA1. Comparison of the microarray results of the parasite lines Dd2 and DHA1 with the predetermined 48 h expression profile of Dd2 (Llinas *et al.*, 2006) revealed that gene expression profiles at three time points from both lines had the best fit with Dd2 gene expression profiles at 14, 27 and 38 h, respectively (Fig. S5, $R^2 > 0.93$, $P < 0.001$). These results strongly indicated that differences between the Dd2 and DHA1 very likely reflected their true biological differences. We first compared differences in gene expression between Dd2 and DHA1 at three stages during the IDC and detected 519 and 213 genes with 2-fold up- and down-regulation in DHA1, respectively (Fig. 3A, Table S2). More genes with 2-fold changes in expression were detected in trophozoite and schizont stages than in ring stage (Fig. 3A). Based on functional annotations of parasite genes in PlasmoDB, we classified the 5333 genes in *P. falciparum* genome into 12 categories. Our results showed that gene ontology (GO) categories of “catalytic/metabolic activity” and “chaperone” were significantly enriched in DHA1 in both up- and down-regulated gene pools (*Z*-test, $P < 0.01$) (Fig. 3B). Strikingly, seven genes annotated as heat shock proteins (HSPs) in the “chaperone” category and at least 22 genes out of 72 in the genome involved in antioxidant defense in the “catalytic/metabolic activity” category were among the up-regulated genes in DHA1 (Fig. 3C). In addition to these two categories, at least 14 genes annotated as “transporters” were also detected in both up- and down-regulated gene pools (Table S2), including >6-fold up-regulation of *pfmdr1*. Several genes potentially involved in transcription such as AP2 domain proteins and chromatin modifiers were also up-regulated in DHA1, which might be responsible for significantly greater number of genes up-regulated in this line. Despite that a large portion of parasite genes in DHA1 showed 2-fold changes

in transcript abundance, only 16 up-regulated and three down-regulated genes were shared among the three stages (Fig. 3A, Table 2). Noticeably, these shared up-regulated genes include *pfmdr1*, five genes associated with antioxidant defense (*PFF1130c*, *PF14_0164*, *PF10925w*, *PF11250w* and *PFL0725w*), and *hsp70*. Up-regulation of *hsp70* is consistent with a recent report of positive selection around the *hsp70* locus in relation to the ATS-treatment delayed parasite clearance phenotype seen in Cambodia (Cheeseman *et al.*, 2012).

Treatment of Dd2 with DHA at 1 μ M for 2 h resulted in significantly more genes with 2-fold reduction than those with 2-fold increase in transcript levels (Fig. S6A, Z-test, $P < 0.01$). Interestingly, DHA treatment of Dd2 caused substantial disturbance in gene expression in most GO categories, as compared to only two GO categories in DHA1 (Fig. S6B, Z-test, $P < 0.05$). This divergent response to DHA treatment is further illustrated from comparisons showing insignificant overlaps in up- or down-regulated genes between the two parasite lines (Fig. S7). It is noteworthy that in the “catalytic/metabolic activity” category at least 22 genes involved in antioxidant defense were further up-regulated in DHA1 after DHA treatment (Table S3). In sharp contrast, only two genes involved in antioxidant defense were up-regulated in Dd2 as compared to eight such genes down-regulated after DHA treatment (Table S4). In addition, a tryptophan-rich gene (*PF10_0026*) implicated in tolerance to ATS was also up-regulated in DHA1 in ring stage (Natalang *et al.*, 2008; Deplaine *et al.*, 2011), and upon stimulation with DHA, it had more than 2-fold up-regulation in ring and schizont stages (Table S2–4). Again, this DHA-induced up-regulation of *PF10_0026* was not observed in pre-selected Dd2 parasites.

***Pfmdr1* gene amplification is associated with DHA resistance**

Real-time RT-PCR analysis confirmed over-expression of *pfmdr1* in both DHA1 and DHA2 (Fig. 4A). To elucidate the mechanism of increased *pfmdr1* expression, we quantified the copy numbers of *pfmdr1* in the three parasite lines. Compared with ~4 copies of *pfmdr1* in Dd2, DHA1 and DHA2 had 8.5 and 9.3 copies, respectively (Fig. 4B). Mapping of the amplified region in DHA1 using real-time PCR confirmed further amplification of the same amplicon as in Dd2 (Fig. S8A), which was mapped to the ~880 – 970 k position on chromosome 5 (Gonzales *et al.*, 2008). Microarray data showed that all the 14 genes in this region were up-regulated, but only *pfmdr1* had >6-fold over-expression (Fig. S8B), suggesting that it might be a major contributor to DHA resistance in our selected parasites. To further incriminate *pfmdr1* amplification as a key factor of DHA resistance, we cultured DHA1 and DHA2 in drug-free medium for 10 weeks and monitored the dynamics of *pfmdr1* copy number. Without DHA, *pfmdr1* copy number gradually decreased and reached 4.1 and 4.2 copies by eight weeks, a level similar to that of Dd2 (Fig. 4B, unpaired *t*-test, $P > 0.017$). Concomitantly, IC_{50} values to DHA also gradually declined to 20 and 18 nM, respectively (Fig. 1B). Finally, the revertant parasites had higher IC_{50} values than Dd2 to all drugs tested except ATQ, and for DHA, ART, CQ, MQ, and QN the differences between the revertant populations and Dd2 remained significant (unpaired *t*-test, $P < 0.0083$) (Table 2). These results suggested that *pfmdr1* copy number variation plays a direct and major role in DHA resistance in the selected parasites, but other mechanisms may be responsible for the residual low-level resistance to DHA and other drugs in the revertant parasites.

A second component is required for high-level ART resistance

To further dissect the genetic components contributing to DHA resistance, we applied a similar step-wise selection pressure of DHA and MQ to the two revertant parasite populations. *Pfmdr1* amplification has been associated with MQ resistance and can be selected by *in vitro* MQ treatment (Wilson *et al.*, 1989; Cowman *et al.*, 1994). Under DHA and MQ selection, *pfmdr1* copy numbers in the revertant DHA1 and DHA2 populations were doubled in 8–10 weeks (Fig. S9). DHA re-selection fully restored the DHA-resistant

phenotype in the DHA1 and DHA2 populations with DHA IC₅₀ values of 245 and 221 nM, respectively (Table 2). However, whereas MQ re-selection fully restored the reduced susceptibility to AQ and LUM, it only partially restored DHA resistance with DHA IC₅₀ values of 52 and 65 nM, respectively (Table 2). In addition, the increase in *pfmdr1* copy number from MQ selection did not affect the sensitivity to CQ. These results suggested that some specific components acting in concert with higher-level *pfmdr1* expression are needed in order to confer high-level resistance to DHA, which could not be fully selected by MQ.

Elevated antioxidant activity is correlated with DHA resistance

Microarray and RT-PCR studies detected that the expression of many genes involved in antioxidant pathways was elevated under DHA pressure (Fig. 3C and S10), suggesting that antioxidant defense system might be another component contributing to DHA resistance in DHA1 and DHA2. In malaria parasites, the antioxidant networks include superoxide dismutases (SODs), glutathione (GSH) and thioredoxin (Trx) systems (Muller, 2004; Becker *et al.*, 2005). Both *SOD-1* (cytosolic, Fe-dependent) and *SOD-2* (mitochondrial) were up-regulated in DHA-selected DHA1 (Fig. 3C). In addition, several components of GSH and Trx metabolic pathways were up-regulated after a brief exposure to DHA (Fig. 3C, S10). In agreement, the antioxidant GSH and Trx levels, and intracellular SOD and glutathione-*S*-transferase (GST) activities were significantly higher in DHA1 and DHA2 than in Dd2 (Fig. 5). The increase in the antioxidant defense system is likely due to DHA selection, since the baseline SOD and GST activities between the different parasite strains before DHA selection were not significantly different (Fig. S11). Similar to the *pfmdr1* copy number variation, the DHA1 and DHA2 parasites' intracellular GSH and Trx levels, and SOD and GST enzyme activities gradually declined without DHA pressure; and at week 10, the Trx levels in DHA1 and DHA2 were similar to that in Dd2 (Fig. 5B, unpaired *t*-test, *P*>0.017). However, GSH level, SOD and GST activities at ten weeks were nonetheless significantly higher than those in Dd2 (Fig. 5A, C, and D, unpaired *t*-test, *P*<0.017). The significantly higher SOD and GST activities in the revertant lines than in Dd2 may account for the residual, yet significantly higher DHA and ART IC₅₀ values detected in these revertant lines at week 10 (Table 2). These components were likely the stable changes that allowed parasites to quickly gain high-level resistance to DHA. To corroborate this conclusion, we monitored the SOD and GST activities during re-selection of the revertant parasites by DHA and MQ. We found that DHA re-selection resulted in continuous increases in SOD and GST activities of selected parasites, which reached similar levels of DHA1 and DHA2 at the end of selection. However, MQ re-selection did not result in noticeable increases in SOD and GST activities (unpaired *t*-test, *P*>0.017) (Fig. 5E and F).

We noticed that a brief DHA treatment further raised transcript levels of some antioxidant defense genes including *SODs* and *GST* only in DHA1 but not in Dd2 (Fig. 3C). Likewise, both SOD and GST activities in DHA1 and DHA2 were significantly increased by DHA treatment (Fig. 6A and B, unpaired *t*-test, *P*<0.003). On the contrary, DHA treatment led to a significant reduction in activity of these two enzymes in Dd2 (Fig. 6A and B, *P*<0.017). The overall increase in the antioxidant network in DHA1 and DHA2 suggests that they are more capable of dealing with intracellular reactive oxygen species (ROS). To validate this speculation, we treated the parasites with curcumin and tert-butyl peroxide (TBP) at their respective IC₅₀ values to induce oxidative stress in the parasites (Cui *et al.*, 2007a). Whereas the baseline levels of ROS in Dd2, DHA1 and DHA2 were not significantly different (*P*>0.017), treatment with 50 μM of curcumin or 200 μM of TBP for 4 h resulted in a significant increase of intracellular ROS level in Dd2 (Fig. 6C). However, ROS levels in DHA1 and DHA2 remained relative constant during treatment with these two compounds (unpaired *t*-test, *P*>0.017). This was also reflected from the significantly lower TBP IC₅₀ value in Dd2 (85.3±12.5 μM) than in DHA1 (182.6±25.7 μM) and DHA2 (194.6±12.7

μM). These results suggested that some permanent changes in the ROS response pathways have occurred in the DHA1 and DHA2 parasites.

Discussion

We successfully selected two *P. falciparum* parasite lines that were able to grow under continuous exposure to 320 nM of DHA, the highest fold increase in IC_{50} value obtained so far from *in vitro* induced ART resistance. We have shown that the resistance to ART is not limited to ring-stage dormancy, but also occurs in trophozoites and schizonts. Using molecular and biochemical approaches, we have provided strong evidence showing that sensitivity to DHA is dynamically correlated with *pfmdr1* copy number and the antioxidant activity. To our knowledge, this study, for the first time, integrates *pfmdr1* gene amplification and enhanced antioxidant defense as the mechanism of ART resistance. This finding has significant implications for development of ART resistance in Southeast Asia.

In vitro selection for antimalarial drug resistance is an effective approach to studying drug resistance mechanisms. It is noteworthy that the DHA selection protocol used here differs from the way that the parasites are exposed to ACTs in patients, which may explain the differences between *in vitro* selected parasites and parasites isolated from clinical resistance manifested as delayed parasite clearance. Nevertheless, the *in vitro* selected DHA resistance revealed several important points. First, although ART, ART derivatives and synthetic trioxanes have been proposed to act by a similar mechanism, DHA selection resulted in strikingly distinctive responses to ART family drugs, revealing surprising structural specificity to the selection of resistance phenotype. The selected DHA-resistant lines displayed a >25 and >16 fold decrease in sensitivity to DHA and ART, respectively, but the increase in resistance to other ART derivatives was modest (~5 and ~10 fold reduction in ATM and ATS sensitivity, respectively). In parallel, the revertant parasites still maintained residual, yet significant resistance to DHA and ART. On one hand, since DHA is less stable under aqueous conditions *in vitro* (Haynes *et al.*, 2007), the selected phenotype may reflect the increased ability of the parasites to decompose DHA (and ART) compared with other more stable derivatives. On the other hand, this result may imply that a specific resistance mechanism selected by DHA (and potentially ART) is yet to be discovered. Therefore, this structure-related finding and especially the distinction of ATM underscores that future studies should be directed to address the structure-specific resistance mechanisms. Second, DHA selection resulted in general elevation of resistance to a number of commonly used antimalarials, suggesting of common potential resistance mechanisms. Together with the results from MQ re-selection, this study pointed out that *pfmdr1* amplification largely explains the increased resistance to MQ, LUM and QN. However, the divergent responses between MQ and LUM suggest the involvement of different components in addition to *pfmdr1*. Third, the results also present a troubling clinical outlook since many of these tested drugs are ACT partner drugs. This further emphasizes that selection of ART partner drugs needs to be carefully evaluated to avoid cross resistance. In this study, DHA selection did not lead to noticeable changes in sensitivity to ATQ, a drug targeting the mitochondria. This may suggest that drugs targeting parasite's mitochondria and apicoplast deserve future considerations in ACTs.

ART monotherapy of *P. falciparum* malaria is often associated with recrudescence (Li *et al.*, 1984), and there are clear indications that the recrudescence rates associated with ART treatments have increased in regions of Southeast Asia where ART and derivatives have been used extensively (WHO, 2010). The recently confirmed ART resistance in the Thai-Cambodian border area is manifested as delayed parasite clearance (Noedl *et al.*, 2008; Dondorp *et al.*, 2009). A ring-stage dormancy mechanism has been invoked to explain this phenomenon (Teuscher *et al.*, 2010; Witkowski *et al.*, 2010). In cultured parasite strains,

ART-induced temporary arrest of growth can occur readily at the ring stage (Teuscher *et al.*, 2010), and after selection, ring stage parasites can tolerate high-dose ART pressure (Witkowski *et al.*, 2010). Ring-stage tolerance to ART is likely associated with low-level hemoglobin digestion at this stage, since hemoglobin digestion is required for the action of ARTs (Klonis *et al.*, 2011). However, our study showed that increased tolerance to DHA is not limited to ring stage. Throughout the IDC, the DHA-resistant parasites displayed much faster recovery rates and drastically higher survivorship after exposure to high doses of DHA. A recent genome-wide association study has identified a selective sweep on chromosome 13 that shows strong association with reduced parasite clearance rates (Cheeseman *et al.*, 2012). Interestingly, *hsp70* gene is located in the region under positive selection and shows increased expression, which is consistent with our *in vitro* DHA selection result. Furthermore, among genes up-regulated in DHA1, many encode chaperones including HSPs and HSP-like proteins. Although the roles of HSPs in drug resistance in *P. falciparum* are mostly unexplored, an analogy drawn from other eukaryotic systems suggests that up-regulation of HSPs may contribute to the overall increase in resistance to multiple antimalarial drugs.

Pfmdr1 mutations have been associated with altered susceptibilities to multiple antimalarial drugs (Reed *et al.*, 2000; Duraisingh & Cowman, 2005) and many more non-antimalarial drugs (Yuan *et al.*, 2009; 2011). In particular, *pfmdr1* gene amplification is associated with increased resistance to ARTs in both field parasite isolates and *in vitro* selected ART-resistant parasites (Wilson *et al.*, 1993; Price *et al.*, 2004; Chavchich *et al.*, 2010; Rodrigues *et al.*, 2010; Borges *et al.*, 2011). By following the dynamics of *pfmdr1* copy number and corresponding DHA IC₅₀ values during *in vitro* culture and selection, our study conclusively showed that DHA resistance is dynamically associated with the *pfmdr1* copy number. Furthermore, we have shown that the *pfmdr1* up-regulation in selected parasite DHA1 is unique among the 14 genes in the amplified fragment, and further *pfmdr1* up-regulation can be induced by DHA treatment. Since *pfmdr1* amplification has been shown to affect the parasite's fitness (Hayward *et al.*, 2005), parasites reverted to the original *pfmdr1* copy number in the absence of drug selection, which was accompanied by a gradual loss of the *pfmdr1* amplification. This observation might explain the findings that parasites obtained from areas where low-grade ART resistance has been found from clinical studies do not exhibit such resistance when they are tested *in vitro*. The low-grade resistance could have been reverted during *in vitro* culture without drug selection pressure.

Our study has pinpointed elevated antioxidant defense in the parasite as another major mechanism of ART resistance. Although increased antioxidant defense activity and up-regulation of some antioxidant genes in *Plasmodium* in response to ARTs and several antimalarial drugs such as CQ have been reported (Ginsburg & Golenser, 1999; Ginsburg *et al.*, 1999; Natalang *et al.*, 2008; Radfar *et al.*, 2008; Nogueira *et al.*, 2010; Chandra *et al.*, 2011), our study showed that up-regulation of many genes of the antioxidant network was critical for high-level resistance to DHA. Not only are these genes highly enriched among up-regulated genes in DHA1, they were further up-regulated in response to DHA treatment. In contrast, up-regulation of antioxidant defense genes was not apparent in Dd2. In agreement, the intracellular antioxidants GSH and Trx, as well as two important enzymes involved in redox metabolism SOD and GST, were markedly augmented in the selected parasites. Moreover, by following the dynamics of GSH, Trx, SOD and GST during culture of the parasite without drug pressure, we conclusively showed that gradual decreases of the antioxidant levels and enzymatic activities were highly correlated with gradual reduction of the DHA IC₅₀ values. Also noteworthy is that re-selection of the revertant parasite lines with DHA fully restored the SOD and GST activities, but MQ selection had only negligible effect on the activities of these enzymes (Fig. 5E and F). These data further underline the significant contribution of elevated antioxidant defense in DHA resistance. These findings

are consistent with the proposed mode of action of ART family drugs because these drugs were shown to increase oxidative stress and reduce the levels of GSH in the parasites (Krungrai & Yuthavong, 1987; Meshnick, 2002; Hartwig *et al.*, 2009). Enhanced antioxidant defense in selected parasites is well reflected in their increased capability of tolerating stressful culturing conditions under high parasitemia and dealing with induced ROS. The enhanced antioxidant activities in DHA-resistant parasites DHA1 and DHA2 may partially explain the overall decrease in sensitivity to many antimalarial drugs. MQ selection normally leads to *pfmdr1* amplification and increased CQ sensitivity, although it does not fully revert the parasites to wild-type CQ sensitivity. DHA selection, however, resulted in *pfmdr1* amplification but increased CQ resistance, which suggests a direct link of the DHA-selected CQ resistance with elevated antioxidant defense (Ginsburg & Golenser, 2003). Collectively, this study indicates that antioxidant defense system in the malaria parasites might be another important component for induced ART resistance.

Although many factors such as *pfmdr1* amplification, increased antioxidant activity, and mutations in *PfATP6* and other genes have been implicated in *P. falciparum* response to ART (Cui & Su, 2009; Ding *et al.*, 2011), our carefully designed experiments and extensive data allow us to dissect relationship of these factors and to propose a potential DHA resistance mechanism for the first time (Fig. 7). It is clear that increased *pfmdr1* copy number and elevated expression of genes in many antioxidant pathways are required for high-level resistance to DHA. Selection of DHA will lead to some complex stable changes in SOD and GST pathways, which may take times, and reversible changes in the Trx pathway. These changes in antioxidant pathways only lead to low-grade DHA resistance (also possibly to many other antimalarials). Selection with MQ leads to increased *pfmdr1* copy number and medium-level resistance to DHA, consistent with previous reports of shared components in resistance to MQ and DHA (Sidhu *et al.*, 2005; Sidhu *et al.*, 2006). In addition, enhanced production of antioxidants will facilitate the removal of free radicals, ROS, as well as heme, thus increasing parasite's capacity to tolerate oxidative stress from drugs. Upon the changes in antioxidant pathways, increased copy number of *pfmdr1* can result in high-level resistance to DHA. Based on a current view suggesting that PfMDR1 likely imports drugs into rather than pumps drugs out of the food vacuole (Sanchez *et al.*, 2011), increased *pfmdr1* expression potentially enhances transport of DHA into the food vacuole, which may sequester the drug away from its sites of activity. Yet, a recent study suggested that the food vacuole might be an initial site of antimalarial activity of endoperoxides (del Pilar Crespo *et al.*, 2008). Therefore, the mechanism by which *pfmdr1* amplification leads to increased resistance to DHA requires further investigations.

Our observations have significant implications for the emerging ART resistance in the Thai-Cambodian area of the Greater Mekong Subregion (GMS). Our results indicate that selection for resistance to ART is dependent on genetic changes in the parasite genome, particularly those of antioxidant pathways. Among the five laboratory strains tested, Dd2 displayed a much higher propensity to develop resistance to DHA, which is reminiscent of the "mutator" ARMD phenotype showing that certain parasite isolates are predisposed to develop resistance to multiple antimalarial drugs (Rathod *et al.*, 1997). This ARMD phenotype has been verified in the MQ-selected Dd2 line and its parental strain W2 from Indochina (Oduola *et al.*, 1988; Rathod *et al.*, 1997). While this phenotype may be attributed to defective DNA mismatch repair in these parasites (Trotta *et al.*, 2004; Castellini *et al.*, 2011), we also noticed that among the five strains tested only Dd2 has more than one copy of *pfmdr1*, suggesting that the *pfmdr1* amplification background may favor development of resistance to ART family drugs. In Thailand and Cambodia, MQ alone or in combination with ATS has been used extensively to treat uncomplicated falciparum malaria, and MQ resistance was evident (Mockenhaupt, 1995). Increased *pfmdr1* gene copy number, the main cause of MQ resistance, has been found prevalent in this region (Price *et al.*, 2004).

Although *pfmdr1* amplification was not associated with the clinical ART-resistant isolates (Imwong *et al.*, 2010; Mok *et al.*, 2011), it is possible that the resistance phenotype is unstable and resistant parasites could rapidly revert to ART sensitivity. However, we showed that parasites with some specific genetic backgrounds (mutations or elevated expression in antioxidant pathways) could regain ART resistance at much accelerated rates. This highlights the significance of extensive surveillance at sentinel sites in this area of the GMS. Furthermore, we speculate that parasites originated from the GMS might also have elevated antioxidant levels, which again favor the development of drug resistance. In the GMS, glucose-6-phosphate dehydrogenase (G6PD) deficiency, which confers resistance against malaria, is very common (Cappellini & Fiorelli, 2008). In G6PD-deficient red cells, defense against oxidative damage is reduced. Malaria parasite growing in G6PD-deficient erythrocytes displays enhanced, coordinated expression of antioxidant enzymes and HSPs (Akide-Ndunge *et al.*, 2009), which may inevitably reduce the parasite's sensitivity to antimalarial drugs. Taken together, our study has unified two potential pathways for the development of ART resistance in malaria parasite. The genetic backgrounds of the parasite in the GMS with high-frequency *pfmdr1* amplification and elevated antioxidant defense shaped by drug history and host genetic factor, respectively, underline the high likelihood of emergence of ART resistance in this region. Further studies are necessary to dissect the molecular events in the antioxidant pathways leading to stable increased resistance to DHA.

Experimental procedures

Parasite culture, drug selection and assays

Laboratory *P. falciparum* strains (3D7, Dd2, 7G8, HB3, and D10) were obtained from MR4. Parasite culture and synchronization were done as described previously (Miao *et al.*, 2010). To investigate the possibility of ARMD phenotype to DHA, we subjected five parasite strains (3D7, Dd2, 7G8, HB3, and D10) with different genetic backgrounds to DHA selection. Ten milliliters of cultures of parasites were maintained in 25 cm² flask at 5% hematocrit. For each strain, 5×10^8 parasites at 5% parasitemia were continuously exposed to a stepwise increase of DHA concentrations starting at two-fold IC₅₀ values of the respective parasite strains. The time when parasites reappeared in culture and reached 1% parasitemia was recorded for each parasite strain. Afterwards, selection was continued with 2-fold increase of the DHA concentrations, and the highest DHA concentrations used were 8-fold IC₅₀ values of the respective parasite strains. Meanwhile, DHA selection was performed on Dd2 with increasing DHA pressure in stepwise two-fold increments from 10 to 320 nM. Each time when the parasite appeared and reached 2% parasitemia, a two-fold increase of the drug pressure was re-applied. Once the parasites could grow steadily under 320 nM of DHA, they were cloned by limiting dilution (Rosario, 1981). Two parasites clones DHA1 and DHA2 with highest IC₅₀ values of DHA were routinely cultured under 100 nM of DHA.

For drug assays, parasites were synchronized and zero time point of the IDC was set at 1 h post invasion. IC₅₀ values of antimalarials were determined by using the SYBR-Green I method (Smilkstein *et al.*, 2004). A 2-fold serial dilution was used with a starting concentration of 1000 nM for DHA, ATM, ATS, ART, AQ, PQ and MQ, 2000 nM for CQ, 20 nM for ATQ, and 3000 nM for LUM and QN. For the two ACTs, the stock concentrations of DHA/PQ and ATM/LUM were 500 nM/1100 nM and 500 nM/1500 nM, respectively, according to the commercial formulations of Duocotexcin® and Coartem®, respectively. To determine VP-reversibility of CQ resistance in DHA1 and DHA2, IC₅₀ values of CQ were measured together with VP at a starting concentration of 2/25 μM of the combination (Patel *et al.*, 2010). Each drug or drug combination was tested in three technical replicates and three biological replicates. IC₅₀ values were calculated using the program GraphPad Prism V5 (La Jolla, CA, USA) by constructing a dose-response curve.

Phenotype analysis

To measure parasite proliferation, cultures of synchronized Dd2, DHA1, and DHA2 parasites were initiated in 24-well plates with 0.5% rings and 5% hematocrit. Medium was changed every 48 h and parasitemia was monitored daily for eight days. Cell cycle duration of each parasite line was precisely measured using Giemsa-stained smears every 2 h for two cycles and determined as the interval between the peak trophozoite parasitemias of two cycles (Reilly *et al.*, 2007).

To determine the responses of the parasites to brief or more extended treatment of ARTs, we measured the survivorship of the parasites after DHA treatment. For brief treatments, synchronized parasites at 12, 24 and 36 h were treated with 1 μ M of DHA for 2 h. After removal of the drug, parasites were let to recover in a drug-free medium for 6 h, and viability of the parasites was determined by flow cytometry as described (Witkowski *et al.*, 2010). To determine the potential involvement of ring-specific dormancy in ART resistance, synchronized ring-stage parasites were treated with 1 or 3 μ M DHA for 48 h. Subsequently, cultures were maintained in drug-free medium and examined daily using microscopy to determine parasitemias. Medium was changed daily and the amount of time needed for the each parasite culture to reach 10% parasitemia was recorded.

Microarray analysis

To determine genome-wide transcriptional changes in the parasites, microarray analysis was performed for Dd2 and DHA1. A custom-designed expression microarray was developed by Roche NimbleGen (Madison, WI) based on the *P. falciparum* 3D7 genomic sequence. A total of 35809 45–60-mer probes were replicated and arranged on the array slides using ArrayScribe array design software (NimbleGen). For transcripts of <299 bp, 300–3999 bp and >4000 bp in length, 3, 6 and 11 probes per transcript were selected, respectively. No 3' bias was used during probe selection and the melting temperatures of the probes are distributed between 60.9 and 77.8. For microarray analysis, 3×10^9 tightly synchronized Dd2 and DHA1 parasites were treated for 2 h with or without 1 μ M DHA at approximately equivalent developmental stages (14, 27 and 38 h for Dd2; 15, 29, and 41 h for DHA1). Total RNA was isolated from untreated control or treated parasites using Trizol Reagent, and treated with RNase-free DNase I to remove contamination of genomic DNA. The quality of the RNA samples was assessed using RNA Nano Chips on the Agilent Bioanalyzer (Agilent, Santa Clara, CA). RNA labeling and array hybridization followed the manufacturer's instructions (Nimblegen). Slides were scanned using Axon GenePix 4000B. Signals were normalized using the RMA (Robust Microarray Analysis) method. Signal values for each chip were adjusted using RMA background correction and data across all chips were normalized using the quantile method to adjust the probe intensity values to achieve a uniform distribution. Finally, data was summarized into expression level signal values using RMA's median polish summarization. The transformed microarray data (\log_2 values) were generated using the ArrayStar 4 software (DNASTAR, Madison, WI). Data were further filtered to remove spots with standard deviations of ≥ 1 from two independent experiments. The correlations of the data between Dd2 and DHA1 and between our data and the 48-h gene expression profiles in Dd2 (Llinas *et al.*, 2006) were compared to ensure the selected parasites were from similar developmental time points. The data between untreated parasites of Dd2 and DHA1 and between drug treated and untreated control of same line were further compared. The genes with two-fold changes were used for gene ontology (GO) analysis based on data from the PlasmoDB (plasmodb.org).

Analysis of candidate genes associated with drug resistance

Genes that are associated with drug resistance in *P. falciparum* including *pfmdr1* (PFE1150w), *pfatp6* (PFA0310c), *pftctp* (PFE0545c), *pfubctb* (PFA0220w), *pfcr1*

(MAL7P1.27), *G7* (PF13_0271), *G49* (PF08_0078), and ABC transporter *pfmrp* (PFA0590w) were analyzed for variation in copy number and sequence. Copy number was determined by real-time PCR analysis as previously described using the single-copy gene *seryl-tRNA synthetase* (*PF07_0073*) as an internal reference (Cui *et al.*, 2007b). The coding regions of these candidate genes were amplified from the genomic DNA, sequenced, and compared with Dd2 sequences to identify mutations.

Quantitative analysis of redox metabolism

To measure ROS levels in the parasites, cultures were maintained in drug-free medium for three cycles, and late trophozoites were labeled with 2',7'-dichlorofluorescein-diacetate A (50 mM) at 37°C for 15 min and treated with 50 μM curcumin or 200 μM TBP for 4 h. ROS levels, represented by the fluorescence intensity, were measured using flow cytometry (Cui *et al.*, 2007a). To determine the cellular levels of major antioxidants GSH and Trx, and the activities of GST and SOD, 3×10⁹ parasites were harvested from Dd2, DHA1 and DHA2, and at a biweekly interval during the ten-week culture period of these lines in the absence of selective drug pressure. Cell lysates were prepared after sonication and centrifugation, and protein concentrations were determined using a protein assay kit (Bio-Rad, Hercules, CA). GSH level, GST and SOD enzyme activities in protein extract were determined using respective assay kits (Cayman Chemical, Ann Arbor, MI). The unit activity of GST was defined as the amount of 1-chloro-2,4-dinitrobenzene (mM) reduced GSH/min/mg protein. The SOD unit activity is defined as the amount of SOD needed for 50% dismutation of the superoxide radical. The levels of Trxs were determined using the Thioredoxin Assay kit (Redoxica, Little Rock, AR).

Statistical analysis

The differences of IC₅₀ values among parasite lines were compared by unpaired *t*-tests as previously described (Cui *et al.*, 2012). We used the Bonferroni correction to control for multiple tests; differences were considered significant when $P < 0.05/n$ (n =test number). Cross resistance to different drugs was evaluated using Pearson's correlation coefficient. Differences between percent representations of functional categories from microarray experiments were compared using the *Z*-test; differences were considered significant at $P < 0.05$ and $P < 0.01$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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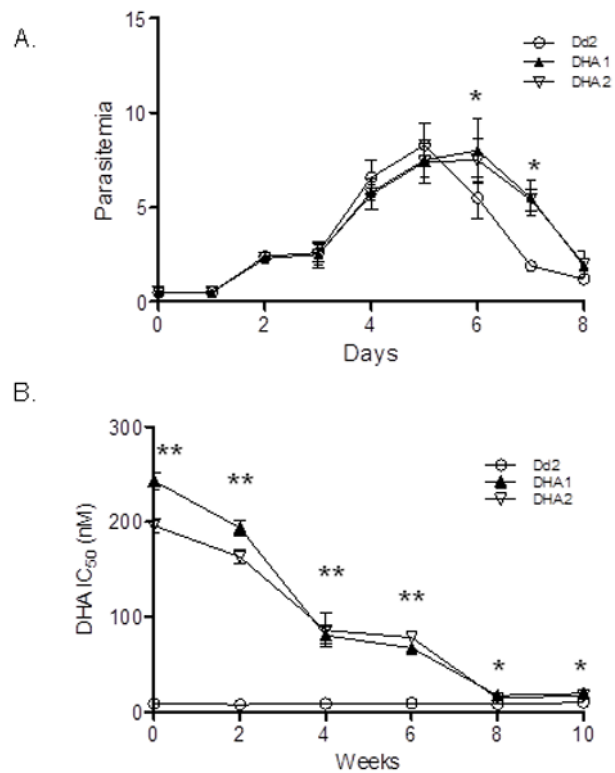


Figure 1. Growth phenotypes

A. Growth curves of parasite clones DHA1, DHA2 and Dd2. Synchronized ring-stage parasites were seeded into 24-well plate, and parasitemias of each line were counted daily for four IDCs. Medium was changed every 48 h. The asterisk (*) denotes significant difference in parasitemia between Dd2 and DHA selected parasite lines DHA1 and DHA2 (unpaired *t*-test, $P < 0.003$). **B.** IC₅₀ values of Dd2, DHA1 and DHA2 to DHA during in vitro culture without drug. Asterisk (*) and asterisks (**) indicate significant difference between Dd2 and either DHA1 or DHA2 at $P=0.017$ and $P=0.003$ level (unpaired *t*-test).

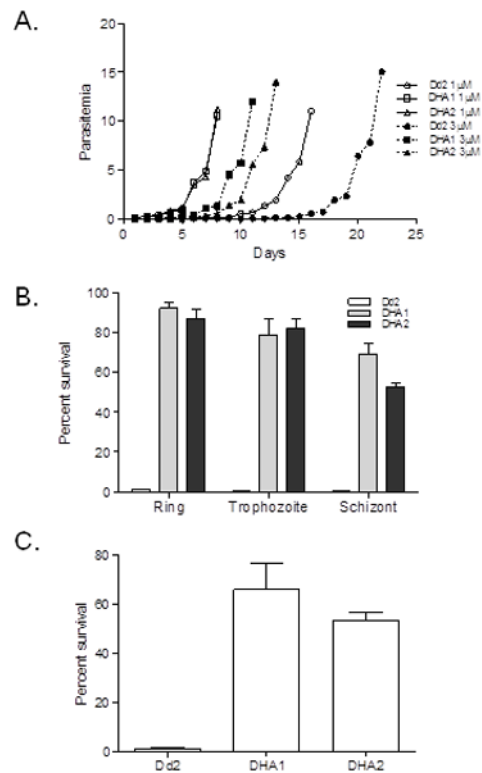


Figure 2. Survivorship and growth recovery of Dd2, DHA1 and DHA2 after DHA treatment
A. Recovery of growth in Dd2, DHA1 and DHA2 after DHA treatment. Synchronized parasites of DHA1, DHA2, and Dd2 were treated at ring stage with 1 or 3 μM DHA for 48 h. After treatment, drug-free medium was changed daily, and parasitemias were counted daily. **B.** Survival of parasites after DHA treatment. Synchronized parasites at ring, trophozoite and schizont stages were treated with 1 μM of DHA for 2 h and percentages of survived parasite were determined 6 h later by flow cytometry as the rhodamine 123/To-Pro III ratios. **C.** Unsynchronized parasites were treated with 100 nM of DHA for 48 h and survivorship of the parasites was determined by flow cytometry. All results from DHA1 and DHA2 in **B** and **C** are significantly different from those with Dd2 (unpaired *t*-test, $P < 0.003$)

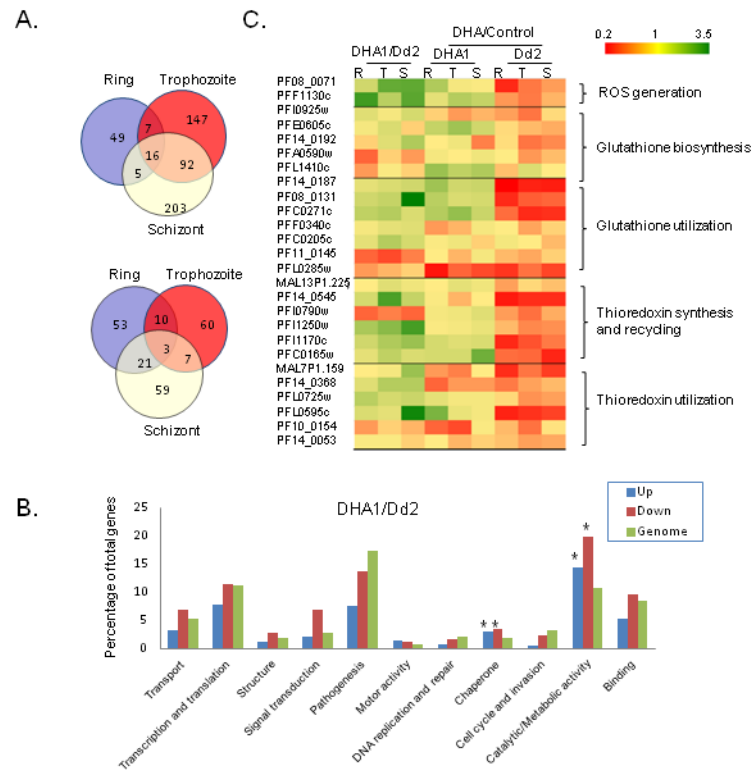


Figure 3. Microarray comparison of gene expression at three developmental stages

A. Venn diagrams show the overlap of genes with 2-fold up-regulation (upper panel) and down-regulation (lower panel) in DHA1 versus Dd2 at three stages. **B.** Representations (percentages) of the number of genes from different functional categories in the gene pools with 2-fold up-(blue) and down-regulation (red) are compared to the overall representations of the functional categories in the genome (green). Genes encoding hypothetical proteins are not shown. Asterisk (*) denotes categories significantly enriched in up- or down-regulated gene pools as compared to genomic representations (Z -test, $P < 0.05$). **C.** Expression changes of genes involved in antioxidant defense in Dd2 and selected DHA-resistant clone DHA1 with or without DHA treatment. R, T, and S denote ring, trophozoite, and schizont stages, respectively.

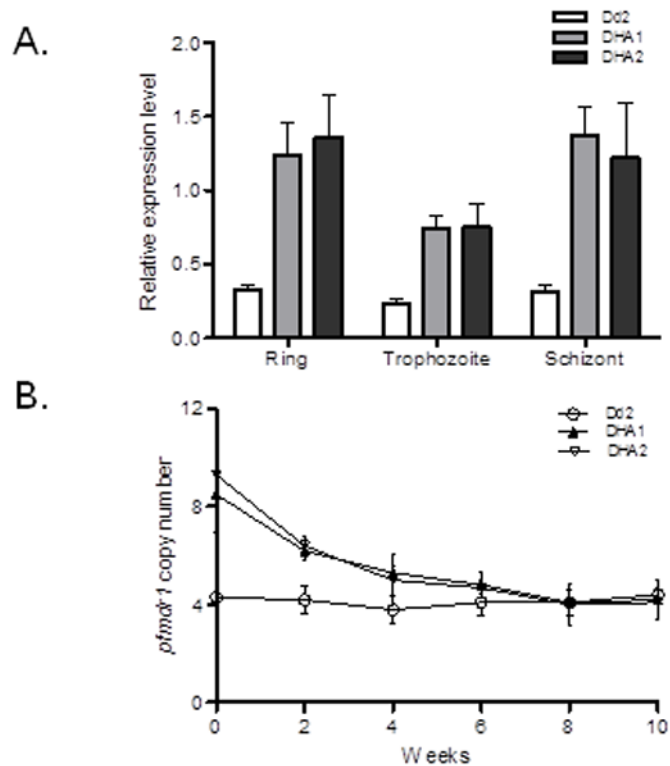


Figure 4. *Pfmdr1* amplification and correlation with DHA resistance in DHA1 and DHA2
A. Confirmation of elevated *Pfmdr1* expression in Dd2 and two DHA-selected lines DHA1 and DHA2. *Pfmdr1* mRNA levels in rings (14 h), trophozoites (27 h) and schizonts (38 h) were determined by real-time RT-PCR with the constitutively expressed *seryl-tRNA synthetase* as an internal reference gene. **B.** *Pfmdr1* copy numbers in Dd2, DHA1 and DHA2. *Pfmdr1* copy numbers were determined biweekly by real-time PCR in the three parasite lines during *in vitro* culture without drug.

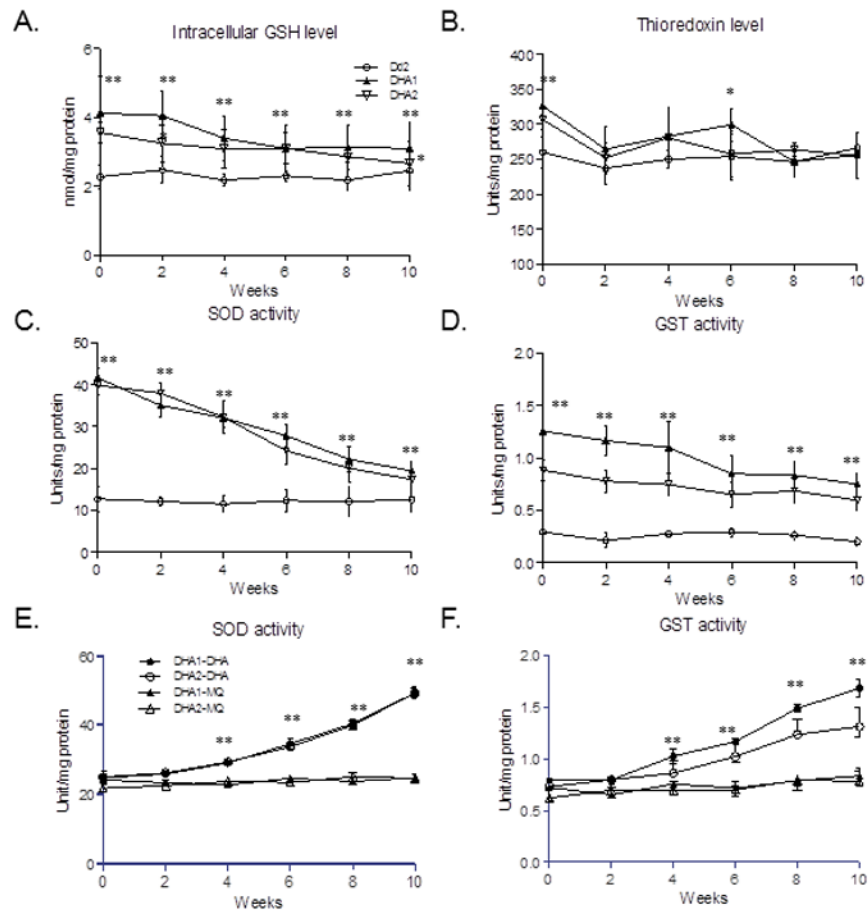


Figure 5. Cellular levels or activities of major components of the antioxidant defense system
A. Glutathione (GSH). **B.** Thioredoxins. **C.** Superoxide dismutase (SOD). **D.** Glutathione *S*-transferase (GST). **E.** SOD activity in the DHA resistance-reverted DHA1 and DHA2 parasites during re-selection with DHA (DHA1-DHA and DHA2-DHA) and MQ (DHA1-MQ and DHA2-MQ). **F.** GST activity in the DHA resistance-reverted DHA1 and DHA2 parasites during re-selection with DHA (DHA1-DHA and DHA2-DHA) and MQ (DHA1-MQ and DHA2-MQ). Cellular levels of GSH and thioredoxins, enzymatic activities of GST and SOD were measured at a bi-weekly interval during 10 weeks of culture of the DHA-resistant clones DHA1 and DHA2 without DHA (**A–D**), and during DHA and MQ re-selection (**E–F**). Statistical analysis was performed at each time point by comparing the differences (unpaired *t*-test). Asterisk (*) and asterisks (**) indicate significant difference at $P=0.017$ and $P=0.003$, respectively, between Dd2 and either DHA1 or DHA2 (**A–D**), and between DHA re-selected and MQ re-selected parasites (**E–F**).

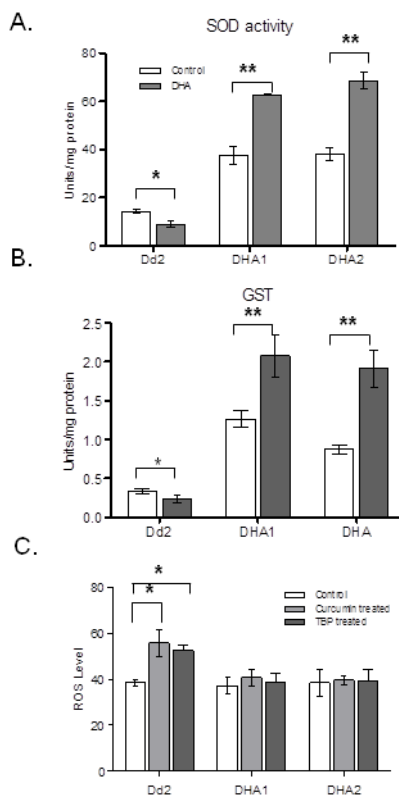


Figure 6. Antioxidant activities in Dd2, DHA1 and DHA2 after treatment with DHA, curcumin, or tert-butyl peroxide (TBP)

A. SOD levels in parasites treated with or without (control) 100 nM of DHA for 2 h. **B.** GST levels in parasites treated with or without 100 nM DHA for 2 h. The result represents Mean \pm SD from three replications. **C.** ROS generation in Dd2, DHA1 and DHA2. The parasites Dd2, DHA1 and DHA2 at trophozoite stage were treated with 50 μ M of curcumin or 200 μ M of TBP for 4 h and intracellular ROS levels were determined. Asterisk (*) and asterisks (**) indicate significant difference between drug-treated and control parasites at $P=0.05$ and $P=0.003$, respectively (unpaired t -test).

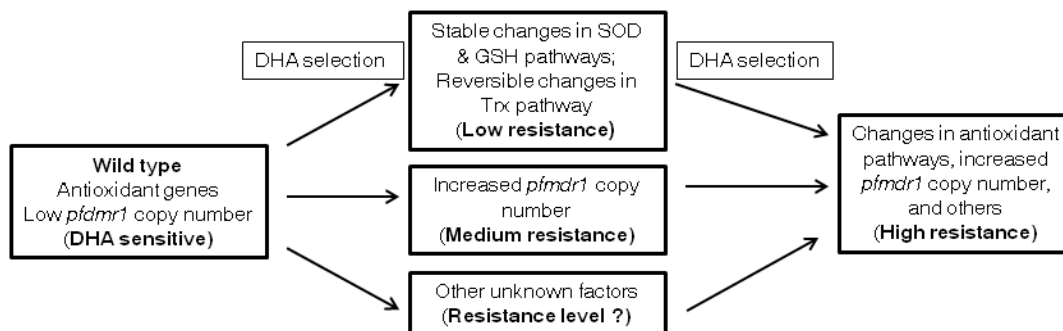


Figure 7. A proposed mechanism for the in vitro DHA resistance in *P. falciparum*

DHA selection can lead to stable changes in some antioxidant pathways (such as the SOD and GSH pathways) and reversible changes in the Trx pathway, which enable the parasites to more effectively remove heme and neutralize free radicals generated from the action of the drug, resulting in low-level resistance to DHA. DHA selection can also lead to *pfmdr1* amplification, resulting in medium-level resistance to DHA. In parasites with the background of elevated antioxidant defense, selection of *pfmdr1* amplification results in high-level resistance to DHA. Other unknown factors may contribute to additional resistance to DHA.

Table 1

Selection of different parasite strains for reduced sensitivity to DHA.

Strains	Origin	IC ₅₀ to DHA (nM) [*]	Days needed to reach 1% parasitemia [#]		
			2 × IC ₅₀	4 × IC ₅₀	8 × IC ₅₀
3D7	Africa	3.2±1.1	–	–	–
D10	PNG	3.5±0.4	37	52	–
HB3	Honduras	3.9±1.4	53	–	–
Dd2	Indochina	7.6±2.1	43	60	72
7G8	Brazil	6.2±0.8	70	–	–

^{*} IC₅₀ values of the parasite lines to DHA (mean ± standard deviation).

[#] Parasite strains were subject to sequential DHA selection at a DHA concentration of 2 ×, 4 ×, and 8 × IC₅₀ values of the respective parasite strains and days needed for the parasites to reach 1% parasitemia under the specific drug concentrations were recorded. –, parasite growth was not detected.

Table 2

In vitro responses of Dd2, two DHA-selected strains (DHA1 and DHA2), their respective revertant lines DHA1-R and DHA2-R, and reselected lines by DHA (DHA1-R-DHA and DHA2-R-DHA) and MQ (DHA1-R-MQ and DHA2-R-MQ) to different antimalarial drugs (nM)*

Strains	DHA	ATM	ATS	ART	CQ	AQ	PQ	MQ	LUM	QN	ATQ
Dd2	7.6±2.1	7.0±1.8	9.8±2.2	5.5±1.4	161.1±27.5	25.7±3.4	14.8±1.1	40.2±8.6	37.6±6.3	307.2±54.7	1.4±0.2
DHA1	197.9±27.5*	38.3±10.0*	86.5±17.7*	93.4±12.6*	307.5±97.2*	65.3±5.6*	37.0±6.5*	102.6±17.8*	398.3±87.0*	467.6±120.8	1.2±0.4
DHA2	167.5±14.5*	30.1±7.7*	102.0±24.2*	91.5±26.0*	270.3±79.0*	40.2±12.3*	40.7±10.5*	146.7±24.3*	487.9±78.8*	578.2±54.3*	1.6±0.5
DHA1-R	19.8±3.7*	11.1±3.7	12.9±2.2	15.1±1.7*	231.3±30.4*	43.5±11.7*	15.8±5.1	54.5±3.8*	46.5±7.8	464.5±87.4*	2.2±0.3
DHA2-R	17.6±4.1*	12.8±4.0*	17.6±4.2*	18.2±4.3*	290.6±16.2*	39.4±7.8*	19.0±3.8	60.2±6.4*	58.3±8.0*	449.8±95.1*	1.7±0.1
DHA1-R-DHA	244.6±19.4*	26.4±3.6*	99.6±22.8*	120.5±23.0*	409.7±107.3*	49.6±14.1*	45.2±13.0*	140.2±25.0*	214.5±54.5*	420.9±104.7	1.5±0.3
DHA2-R-DHA	221.4±45.2*	58.3±23.6*	79.9±7.6*	116.7±24.7*	307.5±97.2*	45.3±3.4*	35.1±2.2*	122.5±12.1*	236.3±37.3*	567.2±52.5*	1.4±0.5
DHA1-R-MQ	52.4±3.6*	9.6±1.3	16.5±3.0*	37.5±8.8*	148.5±20.6	56.3±12.5*	26.7±6.9*	156.2±24.3*	435.1±76.6*	372.5±62.7	2.2±0.7
DHA2-R-MQ	65.0±3.2*	11.4±2.9*	10.3±2.2	29.7±11.2*	156.3±13.6	61.1±10.5*	30.3±1.8*	147.0±8.8*	411.3±52.8*	422.9±38.3*	1.3±0.2

* Numbers represent the IC₅₀ values (means ± standard deviations) in nM from at least six independent determinations. For each drug, significant differences between Dd2 and each of the re-selected strains were marked with * indicate significance at $P=0.0083$ (unpaired *t*-test), respectively.

DHA, dihydroartemisinin; ATM, artemether; ATS, artesunate; ART, artemisinin; CQ, chloroquine; AQ, amodiaquine; PQ, piperaquine; MQ, mefloquine; LUM, lumefantrine; QN, quinine; ATQ, atovaquone.

Table 3

Up and down regulated genes in selected DHA-resistant clone DHA1 during IDC.

Gene ID	Gene description	Fold change (DHA1/Dd2)		
		Ring	Trophozoite	Schizont
PF10_0336	Conserved <i>Plasmodium</i> protein, unknown function	9.2	6.4	5.0
PF14_0164	NADP-specific glutamate dehydrogenase	3.1	3.0	2.4
PF14_0463	Chloroquine resistance marker protein	3.0	2.8	3.4
PFE1150w	Multidrug resistance protein 1 (pfmdr1)	6.2	8.6	9.8
PFF1130c	Superoxide dismutase	4.8	2.5	2.6
PFI0925w	Gamma-glutamylcysteine synthetase	2.5	2.8	3.1
PFI1250w	Thioredoxin-like protein 2	2.8	3.4	4.7
PFL0725w	Thioredoxin peroxidase 2	2.5	2.0	2.2
PF08_0054	Heat shock protein 70	2.1	2.1	2.1
PFI1780w	Plasmodium exported protein (PHISTc),	66.0	21.4	5.1
MAL7P1.58	Pfmc-2TM	8.9	62.0	13.0
PFB0100c	Knob-associated histidine-rich protein	8.7	3.1	2.2
PFI1805w	Rifin	15.7	3.0	2.0
PFF0280w	Conserved <i>Plasmodium</i> protein, unknown function	2.2	2.5	2.3
PF13_0235	Transcription factor with AP2 domain(s), putative	3.5	4.2	2.8
PF0150w	Conserved Plasmodium protein, unknown function	4.1	2.0	2.4
PF0295c	Apical sushi protein	-8.2	-2.0	-4.4
PF10_0331a	Conserved Plasmodium protein, unknown function	-2.0	-2.4	-2.5
PFC0345w	Conserved Plasmodium protein, unknown function	-7.7	-4.1	-9.4