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Mefloquine Exposure Induces Cell Cycle Delay and Reveals Stage-Specific Expression of the *pfmdr1* Gene

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Drug-resistant *Plasmodium falciparum* malaria is a major public health problem. An elevated *pfmdr1* gene copy number (CN) is known to decrease parasite sensitivity to the commonly used antimalarial mefloquine (MFQ). To understand the relationship between *pfmdr1* CN and mefloquine resistance, we evaluated *pfmdr1* transcript levels in three *P. falciparum* strains with different CNs in the presence and absence of MFQ. Parasite strains with multiple *pfmdr1* gene copies exhibited higher relative transcript levels than single-copy parasites, and MFQ induced *pfmdr1* expression above the levels without treatment in all three strains evaluated. Concomitant morphology analyses of the sampled cultures revealed that MFQ treatment of synchronized ring-stage parasites induced a delay in parasite maturation through the intraerythrocytic cycle. *pfmdr1* expression peaks in the ring stage, and MFQ could be causing increased transcription by delaying parasite maturation. However, pretreatment with mefloquine did not affect the artemisinin *in vitro* half-maximal inhibitory concentration (IC_{50}). These results suggest that MFQ-induced increases in *pfmdr1* expression are the direct result of the maturation delay at the ring stage but that this change in expression does not affect the antimalarial activity of artemisinin.

With 225 million estimated clinical infections that result in 781,000 deaths annually, the protozoan *Plasmodium falciparum* causes the most severe form of malaria (1). Efforts to control malaria have been hampered by the development of resistance to antimalarials such as chloroquine (CQ), sulfadoxine-pyrimethamine, and mefloquine (MFQ). Artemisinin-based combination therapies (ACT) are now the first-line treatment for *P. falciparum* malaria. Unfortunately, resistance to these new drugs is believed to be developing, based on the observation of slower *in vivo* parasite clearance times (2–4). The molecular basis for resistance is unclear; thus, a better understanding of the ACT resistance mechanism is needed.

Early investigations into antimalarial resistance led to the identification of a malaria homolog to the mammalian multidrug resistance gene (5–7). The *P. falciparum* multidrug resistance gene (*pfmdr1*) encodes an ATP-binding cassette protein called P-glycoprotein homolog 1 (Pgh1), which is located on the parasite food vacuole membrane (6) and functions as a transporter (8). The transporter function of Pgh1 couples ATP hydrolysis with solute import into the food vacuole (9). The *pfmdr1* gene has been identified as a possible modulator of resistance to a number of antimalarials (10), and the Pgh1 protein has also been implicated as a specific target of antimalarial drugs, such as MFQ (11). Substantial data support a relationship between the *pfmdr1* gene and MFQ resistance both *in vitro* and *in vivo* (12–21). Specifically, an abundance of *in vitro* and clinical data link higher *pfmdr1* gene copy number and expression with reduced parasite susceptibility to drugs such as quinine (QN), MFQ, and, more recently, artemisinin (9, 12, 13, 16, 20–28).

The control of *pfmdr1* gene expression is only partially understood. MFQ-resistant field isolates with higher *pfmdr1* copy numbers overexpress the transcript compared to isolates with a single copy of *pfmdr1* (8, 29), indicating a direct correlation where the presence of more gene copies results in higher constitutive expression. Expression may also be inducible; a recent study demonstrated higher *pfmdr1* transcript levels in a strain bearing a single *pfmdr1* gene copy after treatment with CQ, MFQ, and QN (30). This suggests that exposure to quinoline drugs can induce *pfmdr1* expression and thereby possibly augment resistance to antimalarials such as artemisinin and its derivatives. This is of particular importance because artesunate-mefloquine is a common ACT. In this study, we sought to expand on these observations to better understand the mechanism of *pfmdr1* induction and how this might affect parasite sensitivity to artemisinins.

MATERIALS AND METHODS

Parasite cultivation. We used *P. falciparum* cultures of three clonal parasite lines obtained from the Malaria Research and Reference Reagent Resource Center (MR4) (Manassas, VA): (i) 3D7, which has one *pfmdr1* copy and is sensitive to CQ and MFQ; (ii) FCB, which has 2 copies and is CQ resistant and MFQ sensitive; and (iii) Dd2, which has 4 *pfmdr1* copies and is resistant to CQ and MFQ. Parasite cultures were maintained at 37°C using the standard Trager-Jenson method for malaria parasite culture (31). Pooled human type O+ serum at 10% and red blood cells (Research Blood Components, LLC, Boston, MA) at 2% hematocrit were used for all cultures and experimental conditions. Cultures were synchronized with 5% sorbitol solution every 48 h for three consecutive life cycles to obtain a uniform culture of parasites at a single stage (32).

Gene expression analysis. Sorbitol-synchronized ring-stage parasites were exposed to 100 ng/ml MFQ for 48 h. Total RNA was isolated from cultures at 0, 6, 12, 24, and 48 h after addition of drug using TriReagent

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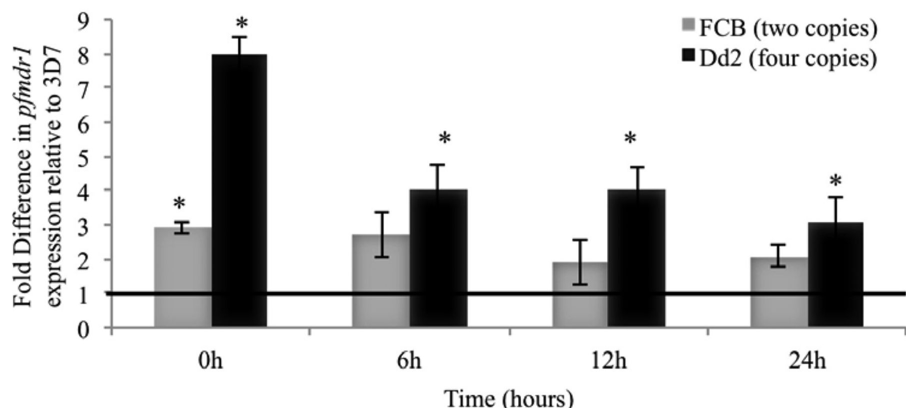

FIG 1 Fold differences in *pfmdr1* gene expression over time in parasite strains with different *pfmdr1* copy numbers. Transcript levels of *pfmdr1* relative to the single-copy *P. falciparum* lactate dehydrogenase gene were evaluated over time following synchronization using real-time RT-PCR. Fold differences in *pfmdr1* expression are relative to 3D7 expression at each time point (not shown), which was set to 1 (black line). Parasite strains with more than one *pfmdr1* copy had higher levels of transcript than single-copy 3D7 parasites. These results were significant for Dd2 through the 24-hour time point. Results are from 6 independent experiments. Statistics were done on the raw data (ΔC_T) at each time point. *, $P < 0.05$ by Tukey's test.

(Molecular Research Center, Inc., Cincinnati, OH), according to the manufacturer's instructions. Real-time reverse transcriptase PCR (RT-PCR) was employed to assess relative *pfmdr1* (the reference identification number for *pfmdr1* from the *Plasmodium* Genomics Database [PlasmoDB] is PFE1150w) mRNA levels between parasite lineages using the $\Delta\Delta C_T$ analysis, as previously described (33). The threshold cycle (C_T) value represents the PCR cycle at which DNA amplification crosses the threshold value. The ΔC_T for each sample is defined as $C_{Tpfmdr1} - C_{Tpfldh}$. Differences in *pfmdr1* gene expression for untreated cultures were compared to expression from single-copy 3D7 untreated cultures. Differences in *pfmdr1* expression for mefloquine-treated cultures were compared to the corresponding untreated culture of the same strain. In experiments involving drug exposure, untreated cultures were used as the negative control, and atovaquone (ATQ) (1 μM)-treated cultures were used to control for effects on gene expression due to the addition to the culture medium of a drug with a known site of action distinct from the *pfmdr1*-encoded protein. Expression of the developmentally regulated trophozoite antigen R45-like gene (PlasmoDB no. PFD1175w) (*tar45*-like gene), which is transcribed during the ring stage only, was assessed using a SYBR green assay as previously described (34). The single-copy *P. falciparum* lactate dehydrogenase gene (PlasmoDB no. PF13-0141), *pfldh*, was used as the reference gene for analysis.

The primers and fluorescent probes for *pfmdr1* were designed using ABI's Primer Express software and are as follows (5′ to 3′): (i) *pfmdr1* forward primer, TGCCCACAGAATTGCATCTATAA; (ii) *pfmdr1* reverse primer, GACTGTACAAAGGTTCCATTTCGA; and (iii) *pfmdr1* probe, 6-carboxyfluorescein (FAM)-ACGATCAGACAAAATT-MGB. The published primers and fluorescent probes for the *pfldh* and *tar45*-like genes (34) were as follows (5′ to 3′): (i) *pfldh* forward primer, ACGATT TGGCTGGAGCAGAT; (ii) *pfldh* reverse primer, TCTCTATTCCATTCT TTGTCACTCTTTC; (iii) *pfldh* probe, FAM-AGTAATAGTAACAGCTG GATTTACCAAGGCCCCA-6-carboxytetramethylrhodamine (TAMRA); (iv) *tar45*-like gene forward primer, ACGAGCTGACCCACCAAA; and (v) *tar45*-like gene reverse primer, CATTTAAGTCTGTCTTCATTCTAC TTCT. Real-time RT-PCR amplifications were performed in a 96-well plate in the ABI Prism 7700 sequence detection system in a total volume of 30 μl (100 ng RNA plus PCR mixture to reach a total volume of 30 μl). Each real-time RT-PCR amplification was performed in duplicate using a previously reported method that combines cDNA synthesis and real-time PCR in one reaction: 30 min at 48°C for the RT reaction and then 10 min at 94°C, followed by a total of 40 PCR cycles (15 s at 94°C and 1 min at 60°C) (33). During the amplification, the fluorescence of FAM, TAMRA, and ROX (a passive reference dye) was measured by the 7700 sequence detector in each well of the 96-well plate. All experiments were conducted a minimum of 3 times for statistical analysis.

Morphology analysis. Thin blood smears were prepared and stained with Giemsa stain (Sigma-Aldrich Co., St. Louis, MO) at 0, 6, 12, 24, and 48 h after the addition of MFQ. Parasitemia and parasite morphology were assessed via light microscopy. Parasitemia was calculated as the number of parasitized red blood cells per 1,000 total red blood cells. Morphology was examined and the number of parasites at each asexual blood stage was assessed for a minimum of 100 parasites. Parasite morphologies were classified as follows: (i) parasites with a single nucleus and characteristic ring appearance were classified as rings, (ii) parasites containing a single nucleus and hemozoin were considered trophozoites, and (iii) multinucleated parasites with ample hemozoin represented schizonts.

IC_{50} determinations. Parasite drug susceptibility was determined using a modification of a published SYBR green I flow cytometry assay (35). The drug concentration required to inhibit 50% of parasite growth (IC_{50}) was calculated using the sigmoidal dose-response nonlinear regression equation in GraphPad Prism software.

Statistical analysis. Each experiment was conducted a minimum of three times for statistical analysis. All statistical analyses were conducted on the raw data (ΔC_T) for each condition using JMP software (SAS Institute, Inc., Cary, NC). Tukey's test was used to determine significant differences at a P value of ≤ 0.05 between the parasite strains because significance is determined for all possible pairwise comparisons of the three strains. Student's t test was used to determine significant differences at a P value of ≤ 0.05 between untreated and drug-treated expression levels.

RESULTS

pfmdr1 **expression is proportional to copy number in untreated parasites.** Synchronized parasites from three clonal strains of *P. falciparum* with different *pfmdr1* gene copy numbers were cultured for 24 h and sampled at 0, 6, 12, and 24 h. *pfmdr1* gene expression increased proportionally with the number of *pfmdr1* gene copies present in the parasite at all time points: Dd2 parasites (four *pfmdr1* gene copies) had 3- to 8-times-higher expression levels than 3D7 (one copy), while FCB (two copies) had 2- to 3-fold-higher levels of expression than 3D7, (Fig. 1). The differences between Dd2 and 3D7 are significant ($P < 0.05$) throughout the 24-hour time period; the difference between FCB and 3D7 was significant only at the 0 h time point. The results indicate that

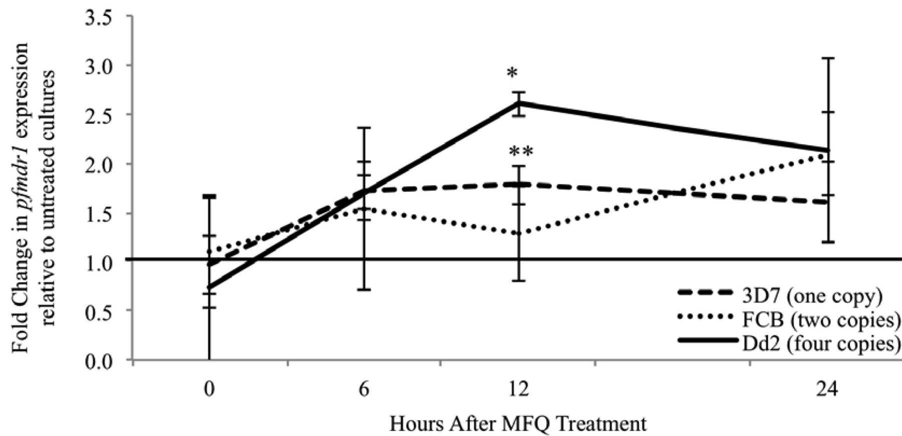

FIG 2 Fold change in *pfmdr1* gene expression in parasites with different *pfmdr1* copy numbers over time following mefloquine exposure. Fold changes in *pfmdr1* expression are relative to that for the corresponding untreated culture for each strain at each time point (not shown), which was set to 1.0 (black line). Significant increases in *pfmdr1* expression upon mefloquine exposure (100 ng/ml) were observed at the 12-hour time point for 3D7 and Dd2 cultures. Results are from 6 independent experiments. Statistics were done on the raw data (ΔC_T) at each time point. *, $P < 0.05$; **, $P < 0.005$ (by Student's *t* test).

there is a direct relationship between *pfmdr1* copy number and expression of the *pfmdr1* transcript.

Mefloquine exposure causes increased *pfmdr1* gene expression. Our results show that, compared with *pfmdr1* transcript levels in the respective untreated culture for each strain, the exposure to mefloquine (MFQ) in all three strains tended to upregulate the expression of *pfmdr1*, with the increase appearing to be an early response that occurred within the first 6 h (Fig. 2). Although we consistently saw higher *pfmdr1* expression in MFQ-exposed parasites than in untreated parasites of the same strain, these results were significant only after synchronized rings of 3D7 with one copy of *pfmdr1* ($P < 0.005$) and of Dd2 with four copies of *pfmdr1* ($P < 0.05$) were exposed to MFQ for 12 h. Gene expression analysis of *pfmdr1* following parasite exposure to the structurally unrelated antimalarial compound atovaquone (ATQ) yielded no significant difference in *pfmdr1* transcript levels compared with those in untreated controls of the same parasite strain (see Fig. S1 in the supplemental material). *pfmdr1* upregulation was observed only after exposure to MFQ not after exposure to ATQ, suggesting that increased *pfmdr1* expression is a specific *P. falciparum* response to MFQ and not a generalized response to antiparasitic drug treatment. Upregulation of *pfmdr1* expression in MFQ-treated parasites compared to untreated parasites was also observed when MFQ was added to synchronized trophozoite stage parasites; however, these results were not significant (see Fig. S2 in the supplemental material).

Mefloquine induces *P. falciparum* cell cycle delay. Parasite maturation through the asexual blood stages was evaluated by thin blood smears using light microscopy. For all 3 strains, untreated parasite populations progressed from predominantly rings to predominantly trophozoites over 24 h. In contrast, the addition of MFQ to synchronized ring-stage parasites delayed parasite maturation at the ring stage (Fig. 3). In all strains tested, the delay in maturation began at 6 h posttreatment and became more pronounced by the 12-hour time point. This phenotype occurred following exposure to MFQ only. The addition of ATQ did not result in the same delay in maturation. Figure 4 shows that when MFQ was added to cultures containing predominantly synchronized trophozoites, maturation proceeded normally until the ring stage (24 h), at which point maturation again stalled. Thus, MFQ appears to have a selective effect on ring-stage parasites.

MFQ treatment results in increased expression of ring-stage genes. To confirm the effects of MFQ on parasite development, we measured, in duplicate, the expression of the developmentally regulated trophozoite antigen R45-like (*tar45*-like) gene (PlasmoDB

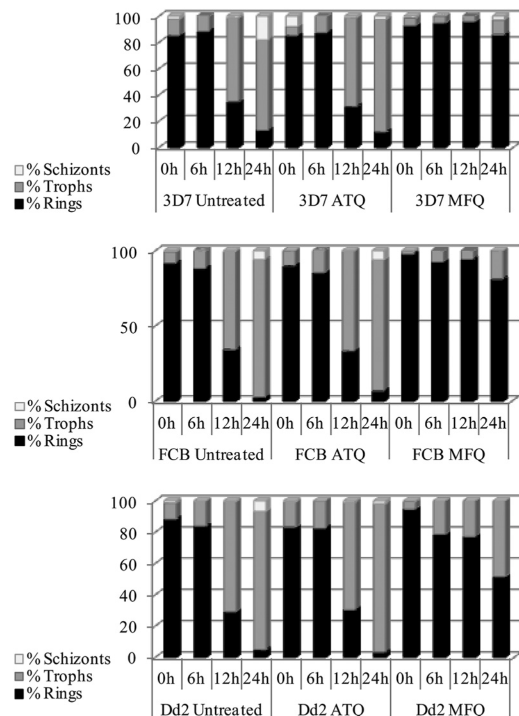

FIG 3 Morphology stage development of untreated and drug-treated ring-stage parasites. Synchronized ring-stage 3D7 (1 *pfmdr1* copy), FCB (2 copies), and Dd2 (4 copies) parasites that were untreated or treated with atovaquone (ATQ) (1 μM) or mefloquine (MFQ) (100 ng/ml) were monitored over time. Untreated and ATQ-treated cultures progressed through the normal asexual cell cycle. MFQ treatment caused a delay in parasite maturation, resulting in a persistence of ring stages. All strains exhibited this delay.

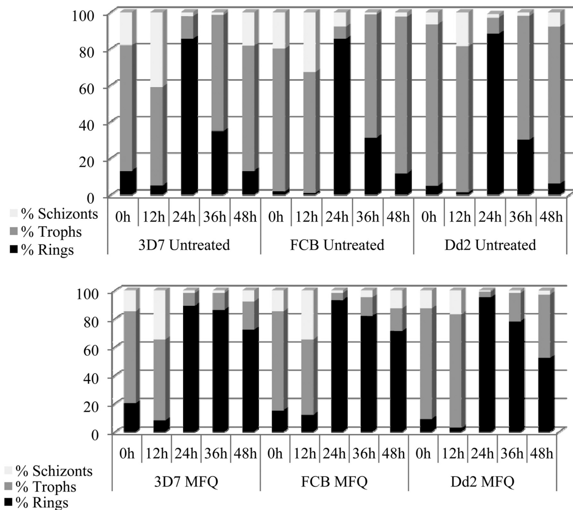

FIG 4 Morphology stage development of untreated and MFQ-treated synchronized trophozoite parasites. Synchronized trophozoites of 3D7 (1 *pfmdr1* copy), FCB (2 copies), and Dd2 (4 copies) parasites that were untreated or mefloquine (MFQ) (100 ng/ml) treated were monitored over time. Untreated cultures (top graph) progressed through the normal asexual cell cycle. MFQ treatment caused a delay in parasite maturation at the ring stage (bottom graph), resulting in a persistence of ring-stage parasites. All strains exhibited this delay.

no. PFD1175w), which is transcribed during the ring stage only (34). Compared with *tar45*-like gene expression in untreated controls of each strain (which was set to 1.00), the fold difference in *tar45*-like gene expression relative to that of *pfldh* was higher for all three strains at 12 h after MFQ treatment (3D7, 5.73; FCB, 6.14; Dd2, 6.48). Expression of the *tar45*-like gene was not affected by ATQ (3D7, 1.31; FCB, 1.02; Dd2, 1.47). These data confirm that MFQ-treated parasites remained in the ring stage for a longer period of time than untreated parasites.

***pfmdr1* is expressed primarily during the ring stage of the intraerythrocytic cycle.** Untreated cultures of each parasite strain were assessed for morphology and *pfmdr1* gene expression as described above. A direct relationship was observed between the proportion of ring-stage parasites in the culture and the fold difference in *pfmdr1* transcript levels (Fig. 5). These data suggest that ring-stage parasites express higher levels of the *pfmdr1* transcript than later stages and that the MFQ-induced increase in *pfmdr1* expression could be due to the maturation delay.

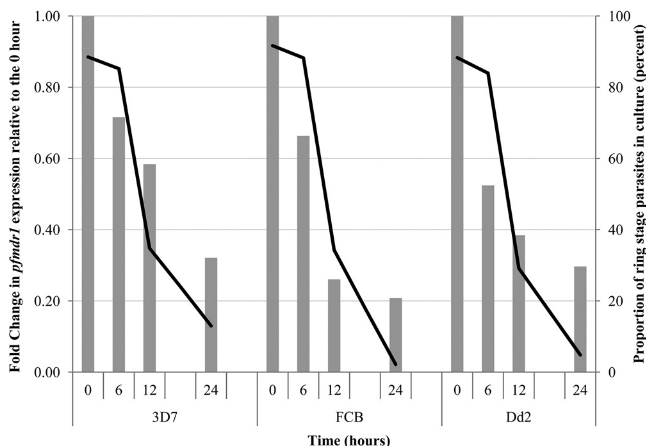

FIG 5 Ring-stage-specific expression of *pfmdr1* in untreated parasite strains containing different *pfmdr1* copy numbers. Untreated cultures of each parasite strain were assessed for morphology (lines) and for *pfmdr1* gene expression (bars) relative to that of the *P. falciparum* lactate dehydrogenase gene. A direct relationship between the proportion of ring-stage parasites in the culture and the relative expression of *pfmdr1* was observed.

MFQ-induced maturation delay has no effect on artesunate IC_{50}. Because MFQ-treated parasites expressed more *pfmdr1*, we tested to see whether MFQ treatment might antagonize its common partner drug artesunate. Parasites were exposed to MFQ for 12 h to induce the maturation delay. The half-maximal inhibitory concentration (IC_{50}) of artesunate was assessed with MFQ-exposed parasites. A SYBR green I flow cytometry assay was used to quantify parasite growth inhibition by artesunate, as previously reported (35). We found no difference in artesunate IC_{50} values between untreated and MFQ-treated parasites (Table 1).

DISCUSSION

The objective of this study was to determine how mefloquine (MFQ) treatment affects *pfmdr1* gene expression in parasites with different *pfmdr1* gene copy numbers. We found that the expression of *pfmdr1* was upregulated following MFQ treatment, and parasites with four *pfmdr1* copies expressed higher transcript levels than single-copy parasites. Interestingly, cultures of MFQ-sensitive FCB parasites (two *pfmdr1* gene copies) did not exhibit a significant increase in *pfmdr1* expression following MFQ treatment compared with single-copy 3D7 parasites at any of the time points assessed. One possible explanation is that there may be a threshold for the number of *pfmdr1* gene copies required for a parasite to exhibit the MFQ-resistant phenotype. Because these parasite strains are not isogenic, it is also possible that other genes could exert an effect on the parasite response to MFQ. Studies in which the expression from the additional *pfmdr1* gene copies is reduced or eliminated in Dd2 parasites would be useful in evaluating the possibility of a threshold number of *pfmdr1* gene copies being necessary to cause the Dd2 MFQ-resistant phenotype.

Statistically significant upregulation of *pfmdr1* following MFQ treatment was observed in 3D7 and Dd2 parasites at the 12-hour time point. Although the reason for this upregulation in 3D7 and Dd2 but not FCB is unclear, subsequent analysis of parasite morphology on the sampled cultures to correlate our *pfmdr1* expression data with the parasite life cycle revealed an interesting phenotype. In agreement with a recent study that examined MFQ exposure on synchronized rings only (36), our results revealed that MFQ treatment of synchronized ring-stage parasites resulted in a delay in parasite maturation at the ring stage of development. When we analyzed the morphology of synchronized trophozoites, we observed that MFQ exposure at the trophozoite stage also

TABLE 1 Half-maximal inhibitory concentration of artesunate in parasite cultures before and after exposure to mefloquine

Parasite strain	Mean IC_{50} (nM) ± SD in:	
	Untreated cultures	MFQ-treated cultures[c]
3D7[a]	0.61 ± 0.04	0.62 ± 0.07
Dd2[b]	0.39 ± 0.05	0.40 ± 0.02

[a] 3D7 parasites contain one *pfmdr1* gene copy.
[b] Dd2 parasites contain four *pfmdr1* gene copies.
[c] MFQ-treated cultures were exposed for 12 h to 100 ng/ml MFQ prior to IC_{50} analysis.

caused the parasites to stall but only after the parasites matured through the intraerythrocytic cycle to the ring stage. This indicates that MFQ affected the parasites' ability to proceed through the asexual intraerythrocytic cycle only when ring-stage parasites were exposed to the drug.

Two theories can explain the increase in *pfmdr1* expression in response to MFQ exposure: (i) direct induction of *pfmdr1* gene expression by MFQ or (ii) an indirect upregulation due to the maturation delay. At each time point assessed, a direct relationship between *pfmdr1* expression levels and the proportion of ring-stage parasites in the culture was observed. In one interval, between 12 and 24 h, the change in *pfmdr1* expression was consistently greater than the change in the proportion of ring-stage parasites. This could be due to the difficulty in distinguishing late rings and early trophozoites by light microscopy.

To demonstrate that MFQ-treated parasites were stalled at the ring stage and were not dying parasites, we investigated the expression levels of the developmentally regulated trophozoite antigen R45-like (*tar45*-like) gene, which is normally expressed in the ring stage. Transcripts of the *tar45*-like gene also increased, peaking at 12 h after the addition of MFQ. This increase was not observed in untreated or atovaquone-treated cultures, which also did not exhibit a maturation delay. Thus, the observed increases in *pfmdr1* transcript levels in the persistent rings found in MFQ-treated cultures are likely due to an overall increase in the expression of ring-stage genes. Ring-stage-dependent transcription of *pfmdr1* would explain the apparent induction of expression after treatment with a drug that slows the cell cycle at the ring stage.

Cell cycle delay upon drug exposure is a common phenomenon, most often seen in cancer cells (37–40). Recently, such delays have been described for *P. falciparum* treated with other drugs (34, 36, 41, 42). A drug-induced delay in parasite maturation through the intraerythrocytic cycle was seen after short-term MFQ exposure in MFQ-sensitive strains (36). Similarly, we showed that treatment of synchronized ring-stage parasites with MFQ induced a delay in parasite maturation in both the MFQ-sensitive (3D7 and FCB) and MFQ-resistant (Dd2) *P. falciparum* strains tested. Our data revealed that this delay was only slightly altered in Dd2 parasites, which have four *pfmdr1* copies. The MFQ-induced maturation delay occurred as early as 6 h posttreatment, and the delay was observed after MFQ treatment but not after ATQ treatment, which indicates that it not a generalized response to the presence of any antimalarial compound. Because decreased sensitivity to other quinoline drugs has also been linked to elevated *pfmdr1* copy number, studies to evaluate the effects of other quinoline compounds, such as quinine, on parasite maturation would be useful to understand if this delay is due to a certain structural class of antimalarial drugs.

There are two possible explanations for the observed effects of MFQ on parasite maturation: (i) the presence of MFQ exerts an effect on the parasites' ability to progress through the asexual cell cycle, or (ii) the maturation delay is an active response by the parasite to adverse conditions caused by mefloquine. If the maturation delay is caused by MFQ, then it would likely be a nonspecific response, perhaps due to a disruption in the availability of nutrients or energy for the parasite to be able to continue through its normal asexual cycle. The addition of chemical agents that inhibit protein synthesis, such as cycloheximide, for example, could shed light on this as a possibility. However, if the maturation delay is an active response by the parasite, one possible consequence of the disruption in the parasite cell cycle is to allow time for parasites to survive peak serum drug concentrations by preventing the maturation to intraerythrocytic stages that express the drug target. Such is the case for certain chemoresistant or radioresistant phenotypes observed in cancer cells (43, 44). For example, 5-fluorouracil (5-FU) is a chemotherapeutic agent that specifically targets the S phase of the eukaryotic cell cycle. Cancer cells resistant to 5-FU are known to arrest at the G_1 phase following 5-FU exposure, thus preventing the drug from engaging its S-phase target (43). These delays are directly linked to the treatment dose and duration, and the removal of the therapy restores the normal cell cycle. In *P. falciparum*, MFQ-induced cell cycle delay is also dose dependent, and removal of MFQ drug pressure restores cell cycle proliferation (36). This would explain the maturation delay if MFQ activity is in one of the later intraerythrocytic stages.

A second explanation for the maturation delay and expression increase could be that the delay is an initial response to the adverse conditions caused by mefloquine but not by atovaquone. Parasites could have evolved this delay mechanism to allow them to survive a specific type of stress. All in all, further work is needed to understand the etiology of this response.

Despite the rapid evolution of resistance to MFQ, its use has gained new life in combination with artemisinin derivatives. Some evidence suggests that the activity of artemisinin and its derivatives is stage specific, with greater plasmocidal activity on late-stage rings and trophozoites than on schizonts or early rings (45–48), whereas other evidence indicates activity on all blood stages (48–50). If the former is true, then MFQ should be additive or synergistic with artesunate. On the other hand, elevated *pfmdr1* copy numbers have been implicated as a cause of artesunate resistance as well. Thus, if MFQ leads to increased *pfmdr1* expression, then it could be antagonistic with artesunate. Whereas our artesunate IC_{50} values for 3D7 fell within the range of reported artesunate sensitivity levels (3), our IC_{50} for Dd2 was slightly lower than reported values. However, we found no differences in the artesunate IC_{50} for untreated parasites compared with parasites of the same strain that had been exposed to MFQ. This suggests that (i) artesunate is neither synergistic nor antagonistic and (ii) the effect of MFQ on parasite maturation does not alter its sensitivity to artesunate. Thus, no evidence against combining artesunate and mefloquine in malaria treatment was found.

An abundance of *in vitro* and clinical evidence links copy number increases in the *P. falciparum* multidrug resistance gene with resistance to several antimalarial compounds (3, 12, 16, 20–22, 24, 25, 27). Our results on the maturation delay and the stage-dependent transcription of *pfmdr1* provide new insight into possible resistance mechanisms mediated by copy number changes. The slowing of the parasite intraerythrocytic cell cycle in response to MFQ appears to be a general survival response by all *P. falciparum* strains tested. This delay could function to alleviate time constraints by prolonging the ring stage, providing the opportunity for higher production of drug transporter proteins in parasites containing multiple *pfmdr1* gene copies. This study was limited to the investigation of *pfmdr1*. Experiments to explore the expression of other important drug resistance transporter genes, such as *pfcrt*, would be worthwhile. Additionally, further studies are needed to fully understand the mechanism by which MFQ slows cell cycle progression and whether this does increase the relative amount of *pfmdr1*-encoded transporter expressed in each parasite. Particularly, investigation into whether or not MFQ has an effect on cell

cycle regulators, such as *P. falciparum* homologs for cyclin-dependent kinases (51–57), would be worthwhile to determine if MFQ delays maturation by interfering with one or more cell cycle enzymes.

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