

ROLE OF THE *PFMDR1* GENE IN *PLASMODIUM FALCIPARUM* RESISTANCE TO
ANTIMALARIAL TREATMENT

Elaine Brooks Bohórquez

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Approved by:

Steven R. Meshnick, M.D., Ph.D.

Marcia Hobbs, Ph.D.

Jonathan Juliano, M.D.

Peter Gilligan, Ph.D.

Cornelius Beckers, Ph.D.

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ABSTRACT

ELAINE BROOKS BOHÓRQUEZ: Role of the *pfmdr1* gene in *Plasmodium falciparum*
Resistance to Antimalarial Treatment
(Under the direction of Dr. Steven R. Meshnick)

Plasmodium falciparum is an intracellular protozoan that causes the most severe form of malaria. A major hindrance to disease control efforts is the spread of drug resistant parasite populations, which has been linked to the *Plasmodium falciparum* multidrug resistance (*pfmdr1*) gene. *Pfmdr1* encodes a transporter protein that pumps solutes, including antimalarials, into the parasite food vacuole. Elevated *pfmdr1* gene copy number (CN) is thought to decrease parasite sensitivity to numerous antimalarial compounds, including quinine (QN) and mefloquine (MFQ). Therefore, our central hypothesis was that the efficacy of antimalarials is dependent upon the CN and expression of the *pfmdr1* gene in *P. falciparum*. This hypothesis was addressed in the following objectives: (1) to determine *pfmdr1* expression after MFQ treatment, (2) to determine the frequency of parasite subpopulations with elevated *pfmdr1* CN in patients that fail antimalarial treatment, and (3) to determine the subcellular localization of QN. We found that MFQ exposure resulted in a clear upregulation of *pfmdr1*. Concomitant morphology analyses revealed that MFQ treatment delayed maturation through the normal intraerythrocytic cycle at the ring stage. Our data show that MFQ-induced increases in *pfmdr1* expression are the direct result of the maturation delay and indicate that *pfmdr1* is primarily expressed during the ring stage of development. Next, to

understand the role of elevated *pfmdr1* CN in clinical treatment outcome, we developed a limiting dilution assay to evaluate *pfmdr1* CN in parasite subpopulations within an individual patient. We found that even small proportions of parasites with multiple *pfmdr1* copies could affect the treatment outcome, which suggests that elevated CN has a stronger influence over treatment response than previously reported. Lastly, we explored the effect of multiple *pfmdr1* copies on the subcellular localization of the fluorescent antimalarial compound, QN. We found that, regardless of *pfmdr1* copy number, QN overlapped with hemozoin but did not colocalize with the acidotropic dye. These results suggest that QN localizes to a non-acidic compartment within the food vacuole and that *pfmdr1* CN does not affect QN subcellular localization. Our results reveal novel data on *pfmdr1* gene expression and the degree to which CN can affect clinical treatment outcome.

To My Family, for all of their love and support:

My parents, Janet and Chuck Brooks

My brothers, Charles and Stephen and their families

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LIST OF ABBREVIATIONS

ACT	artemisinin-based combination therapies
AM	Artesunate-mefloquine combination therapy
ATQ	Atovaquone
CN	copy number of a gene
CQ	Chloroquine
DIC	Laser transmitted light differential interference contrast
IC50	Half maximal inhibitory concentration
MFQ	Mefloquine
<i>pfcr</i>	<i>P. falciparum</i> chloroquine resistance transporter gene
<i>pfmdr1</i>	<i>P. falciparum</i> multidrug resistance gene
Pgh1	P-glycoprotein homolog 1
QN	Quinine
SNP	Single nucleotide polymorphism
WHO	World Health Organization

CHAPTER I

Introduction

GLOBAL BURDEN OF MALARIA

The recorded history of malaria dates back to ancient times, including King Tutankhamun's malaria infection at death (circa 1324 BC) and Hippocrates' (460 BC – 370 BC) description of the disease in his book Epidemics (1-3). The adaptability of malaria parasites is evidenced by the antiquity of the disease and its persistence today. In 2009 alone, an estimated 225 million cases and 781,000 deaths worldwide were due to malaria (4). Human malaria is caused by obligate intracellular protozoa of the *Plasmodium* genus, of which there are 4 species: *P. malariae*, *P. ovale*, *P. vivax*, and *P. falciparum*. Although the latter 2 are the most common causes of malaria, particularly in South America and Southeast Asia, *P. falciparum* is responsible for the most severe and fatal form of the disease and accounts for 70% of malaria infections in Sub-Saharan Africa (Figure 1.1) (4, 5).

PATHOGENESIS AND CLINICAL PRESENTATION OF MALARIA

Although several symptoms are typically associated with malaria infection, the clinical presentation can vary substantially depending on the level of parasitemia, species of *Plasmodium*, and the immune status of the infected individual. In high malaria transmission areas, such as sub-Saharan Africa, exposure to malaria-infected mosquito

bites occurs frequently allowing people to gradually develop immunity. Thus, many adults in these areas are infected but show few symptoms. Children under the age of five, however, are particularly at risk of clinical malaria, because they have not yet developed sufficient immunity (4). Alternatively, in low transmission areas, like Southeast Asia, exposure to infected bites is infrequent, resulting in a lack of malaria immunity among the population. In such settings, clinical malaria develops in most people that are infected.

Clinical presentation of malaria can be divided into 2 categories: uncomplicated malaria and severe malaria. Uncomplicated malaria constitutes the vast majority of malaria cases worldwide. The initial symptoms are nonspecific and resemble those of many other febrile illnesses, including headache, chills, malaise, nausea, diarrhea, hepatosplenomegaly, and, most commonly, fever (6-8). The periodicity of fever that is sometimes observed coincides with the rupturing of infected red blood cells and release of parasites during the intraerythrocytic parasite life cycle (Figure 1.2). As infection progresses, rupturing erythrocytes can lead to anemia. Malaria is resolved by treatment with antimalarials or, eventually, by host immune responses.

In some instances, infection may progress to severe malaria with systemic complications. Though high-grade parasitemia (>10% parasitized erythrocytes) likely triggers this progression to severe disease, the specific mechanism is unknown. Severe malaria is a complex multisystem disorder that is characterized by severe anemia, metabolic acidosis, and cerebral complications (9). These complications are predominantly caused by *P. falciparum* and can develop rapidly, progressing to death within a matter of hours or days (10). Malaria-naïve travelers returning from malaria

endemic regions are most likely to suffer life-threatening complications from infection. In fact, the fatality rate in travelers with falciparum malaria may exceed 20% for severe malaria cases (11). In areas with high transmission rates, however, severe malaria is primarily a disease of young, non-immune children. A study of Gambian children with severe malaria showed that 14% of those that presented with cerebral malaria died. Of those that recovered, approximately 12% developed permanent neurological problems, including ataxia, tremors, blindness, and speech disorders (12).

***PLASMODIUM FALCIPARUM* LIFE CYCLE**

There are 2 hosts of the malaria parasite *P. falciparum*: *Anopheles spp.* mosquito and human. A small number of parasites that are circulating in the human bloodstream mature to form male and female gametocytes. These sexual stage parasites are ingested when a female anopheline mosquito takes a blood meal from an infected person. This begins the sexual development cycle and is responsible for malaria transmission (Figure 1.2) (13). The lower body temperature of the mosquito allows the gametocytes to complete their maturation within the mosquito's midgut (14). Here, fertilization results in a motile ookinete which moves through the cells of the stomach wall to form an oocyst. When this oocyst ruptures, the multiple sporozoites inside are released and migrate to the mosquito's salivary glands (6). *Plasmodium* sporozoites are then transmitted to humans through the bite of this infected female *Anopheles* mosquito. These sporozoites migrate to the liver where they multiply inside hepatocytes to produce 10^5 to 10^6 of infective progeny, called merozoites, which are released into the bloodstream (15).

Merozoites invade circulating erythrocytes and initiate the asexual intraerythrocytic cycle, which is responsible for the symptoms and morbidity and mortality associated with malaria disease (13). Once inside the red blood cell, most parasites undergo profound transcriptional and morphological changes during the asexual 48-hour life cycle (16, 17). The parasitophorous vacuole, which encases the parasite within the red blood cell, is formed following merozoite maturation to the ring stage. Rings then develop into metabolically-active trophozoites. During this stage, host hemoglobin is digested, and the toxic heme byproduct is detoxified by biocrystallization to form inert hemozoin crystals (18). The presence of hemozoin is characteristic of trophozoites as well as schizonts, which is the final developmental stage of the intraerythrocytic life cycle. During the schizont stage, DNA replication and mitosis occur to produce multinucleated schizonts that give rise to multiple merozoites. At the end of the 48-hour cycle, schizont-infected erythrocytes rupture and release the merozoites to infect other red blood cells, allowing the cycle to begin again and exponentially increasing parasite burden in the blood; up to 32 new merozoites may be released from each infected erythrocyte (17).

ANTIMALARIAL DRUGS: MODES OF ACTION & MECHANISMS OF RESISTANCE

Quinolines

Quinine

Although no one knows for certain who discovered it, the curative nature of the cinchona tree bark in South America was well-known in Europe in the latter 17th century

and was included in the third edition of the London Pharmacopoeia as *Cortex peruanus* in 1677 (Peruvian fever bark) (19). The first scientific description of this plant came in 1742 by Carl Linnaeus, though it would take another century before two French chemists would isolate from cinchona bark the antimalarial alkaloid quinine (Figure 1.3) (19). Since the mid-1800s, quinine has been used as a monotherapy or in combination with other drugs, like tetracycline, to treat both uncomplicated and severe falciparum malaria (20-23).

Despite its efficacy, treatment with the aryl aminoalcohol quinine has several disadvantages. First, quinine treatment generally requires a relatively long regimen (7 days) for maximum cure, which affects adherence levels to quinine treatment. Second, it is poorly tolerated. Substantial side-effects, including vomiting and blurred vision, are induced by quinine and are mostly experienced during the second half of the treatment course by which time malaria-related symptoms have lessened (24). Such unpleasant side-effects also affect adherence to the treatment and could contribute to premature termination (25). And third, quinine has limited success for post-treatment prophylaxis and thus does not protect against recurrent infections in areas of high malaria transmission (24).

Despite the longevity of its use as an antimalarial, the mechanism of action of quinine remains unknown. Various studies have indicated that transporter proteins located on the food vacuolar membrane modulate sensitivity to quinine, particularly the transporters encoded by the genes *pfmdr1* and *pfcr1* (26-30). The *P. falciparum multidrug resistance* gene, *pfmdr1*, has been extensively studied for its involvement in parasite resistance to a variety of structurally distinct antimalarials (26-29, 31-42). In the case of

quinine, 3 single nucleotide polymorphisms (SNPs) in the *pfmdr1* gene that result in amino acid substitutions (S1034C, N1042D, and D1246Y) have been linked to a quinine-resistant phenotype: the introduction of these 3 SNPs into *pfmdr1* of a quinine-sensitive *P. falciparum* isolate conferred quinine resistance. Removal of these same mutations from a quinine-resistant isolate confers sensitivity (29). However, no correlation between *pfmdr1* SNPs and increases in the half maximal inhibitory concentration (IC₅₀) of quinine has been reported in field studies (27, 28, 30). Increased *pfmdr1* copy number has also been linked with decreased sensitivity to quinine (30, 32). Regarding the *P. falciparum* chloroquine resistance transporter gene, *pfcr1*, an amino acid substitution at position 76 modulates quinine sensitivity in laboratory experiments (43-45). However, this effect is largely dependent upon the background strain; the K76T substitution has been associated with reduced (44), elevated, or unchanged (45) quinine IC₅₀s.

Although decreasing sensitivity to quinine has been observed in Southeast Asia, quinine continues to be recommended for severe malaria (46). Yet, even for severe malaria, its use has dwindled in favor of newer drugs that have far fewer adverse side effects. Recent studies suggest that parenteral artesunate (discussed below) should replace quinine as the treatment of choice for adults and children alike (23, 47, 48).

Chloroquine

Malaria parasites have developed some degree of resistance to nearly all antimalarial therapies in use. After World War II, there was a period of significant reduction in disease burden following the introduction and very successful use of chloroquine as an antimalarial. Chloroquine (CQ) is a synthetic 4-aminoquinoline

compound (Figure 1.3) characterized by its rapid efficacy, availability, low toxicity, and affordability (49). A diprotic weak base, chloroquine can be found in its un-, mono-, and di-protonated forms. Uncharged chloroquine is the only form that freely diffuses into the parasite's acidic food vacuole. Once inside, chloroquine becomes positively charged and accumulates within this organelle. Chloroquine accumulation has been found to be higher in chloroquine-sensitive parasites than in chloroquine-resistant strains (50, 51). In the metabolically-active intracellular trophozoite stage, the food vacuole is the site of hemoglobin digestion, which generates free heme (ferriprotoporphyrin IX) (52). The insoluble heme is toxic to the malaria parasite and thus must be detoxified. Malaria parasites have evolved a mechanism to detoxify heme and precipitate it in the form of the inert crystal, hemozoin (53). This detoxification step is inhibited by chloroquine, which binds to a dimeric form of oxidized heme called hematin. Thus, chloroquine interferes with hemozoin formation, and the chloroquine-heme complex is highly toxic to the parasite (54, 55).

For several decades, chloroquine was the antimalarial drug of choice: highly effective, inexpensive, and well-tolerated; it was ideal for malaria endemic areas where resources are often limited. However, chloroquine resistance eventually developed in Southeast Asia and South America and quickly spread across the globe, rendering chloroquine virtually useless in many areas (56). Initial investigations into chloroquine resistance involved genetic crosses between chloroquine-sensitive and chloroquine-resistant clones and mapped resistance to the *pfcr*t gene on chromosome 7 (57, 58). Like *pfmdr1*, a food vacuolar membrane transporter protein called the *P. falciparum* chloroquine resistance transporter, PfCRT (Figure 1.4a), is encoded by the *pfcr*t gene.

SNPs within this gene modulate sensitivity to chloroquine by reducing accumulation in the food vacuole (44, 59, 60). In particular, a mutation that is ubiquitous in chloroquine-resistant clones encodes an amino acid change at position 76 (K76T), the removal of which results in full restoration of chloroquine sensitivity (45). These *in vitro* results are consistent with *in vivo* findings that report a strong association between the K76T substitution and chloroquine treatment failure (49, 61). Thus, the mutation that causes the K76T substitution is now used as a molecular marker for chloroquine resistance.

Mefloquine

Because of the rapidity with which parasite resistance to antimalarial compounds develops, there is a constant need to develop alternative therapies with plasmocidal activity. In response to the chloroquine resistance encountered during the Vietnam War, the United States Army began to develop new antimalarial drugs to treat infections caused by chloroquine-resistant parasites (62). The synthetic arylaminoalcohol mefloquine (Figure 1.3) was promising as a prophylactic agent, due to its long elimination half-life (10-23 days) (62). Requiring only one dose per week, mefloquine was ideal for people traveling to malaria endemic areas for extended periods of time and is still used by the U.S. Army today.

In the early 1970s, mefloquine entered initial testing for therapeutic efficacy. While mefloquine was found to be highly effective as an antimalarial therapy (>90% cure rate in initial trials with single-dose regimens), it should be noted that, even when initially introduced, mefloquine was not 100% effective (63, 64). Mefloquine monotherapy was employed in 1976 for chloroquine-resistant malaria in Southeast Asia (63), and, despite

Careful regulation of mefloquine distribution and use, resistance arose within 6 years of use (61). The likely reason for this rapid development of resistance is the long half-life of mefloquine (65). Drugs with long elimination half-lives pose 2 therapeutic benefits for a patient: (1) they require fewer doses for efficacy, and (2) they can provide long-term protection against reinfection. The downside of persistent drug levels is that the residual drug can be a potent selective force for parasites to develop antimalarial drug resistance (66, 67). Thus, what is therapeutically advantageous for an individual is a disadvantage for the population.

Like many other antimalarial compounds, mefloquine was employed in prophylaxis and treatment regimens without the elucidation of its mode of action, which remains unknown. Even before candidate drug resistance genes were identified, mefloquine and chloroquine resistance phenotypes were found to be reciprocally related; mefloquine resistance corresponded to increased chloroquine sensitivity and *vice versa* (68-70). The *P. falciparum* multidrug resistance gene, *pfmdr1*, was initially investigated in the context of chloroquine resistance. Although many subsequent studies demonstrated that *pfmdr1* may be part of a multigenic response for chloroquine resistance (29, 40, 71-73), there is substantial evidence supporting its involvement in the mefloquine resistance phenotype (26-29, 31-33, 37-39, 74-77). The protein encoded by *pfmdr1* (Pgh1) has been implicated as a specific target of antimalarial drugs, such as mefloquine, a known modulator of Pgh1 (78). However, previously conducted studies demonstrate evidence to the contrary (76, 79). Thus, the definitive target of mefloquine is still unknown. Due to the rapid development of mefloquine resistance among *P. falciparum* parasite populations, mefloquine monotherapy was discontinued and new avenues were

investigated for antimalarial therapy (64). Endoperoxides, like artemisinin (discussed below), showed promising plasmocidal activity. Combination therapies revitalized mefloquine use through the addition of artemisinin or its derivatives.

Artemisinin and its derivatives

Multidrug-resistant falciparum malaria is now a serious problem in Southeast Asia, where resistance to chloroquine, quinine, and mefloquine are frequently found (61). This resistance has been curbed to some degree through the use of artemisinin-based combination therapies (ACTs), which are recommended by the WHO as the first-line treatment of uncomplicated falciparum malaria in malaria endemic regions (4). Artemisinin (qinghaosu), originally isolated from the *Artemisia annua* plant in 1972, is a sesquiterpene lactone that contains an endoperoxide bridge (C-O-O-C) essential for antimalarial activity (Figure 1.3) (80). The manner in which this endoperoxide bridge elicits plasmocidal activity is unknown. Two prevailing theories exist: (1) artemisinin interacts with heme via alkylation, generating free radicals that result in cell death (81-84); or (2) artemisinin inhibits falciparum sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase (PfSERCA), which is a protein responsible for maintaining calcium ion concentrations (85-87).

Whereas strong evidence of artemisinin and heme interactions was observed both *in vitro* and in an *in vivo* mouse model of malaria (81, 88, 89), a later study suggested that heme alkylation may not be responsible for artemisinin's antimalarial activity (90). Subsequent studies demonstrated the involvement of PfSERCA proteins in artemisinin activity and also in parasite resistance to artemisinin and its derivatives (85-87, 91, 92).

The only SERCA-type Ca^{2+} -ATPase in *P. falciparum* is PfATP6, which is completely inhibited by artemisinin treatment. This inhibition is highly specific and all other transporters remain unaffected (87). Further support lies in the antagonistic activity of artemisinin and thapsigargin, which inhibits mammalian SERCA. Indeed, the inhibitory profile of artemisinin was exactly superimposable over that of thapsigargin, indicating that both drugs have the same target (86). A single amino acid substitution (L263E) in PfATP6 has been shown to modulate parasite sensitivity to artemisinin (85). Although there are studies suggesting alternative mechanisms for the plasmocidal activity of artemisinin that do not involve PfATP6 (93-95), these experiments were primarily conducted in yeast models and should be explored in malaria parasites to evaluate these alternative targets in the context of malaria infection. With *P. falciparum* parasites in Southeast Asia developing resistance to artemisinin, understanding the mechanism of this resistance is of utmost importance as there are currently no suitably effective alternative drugs (87, 91, 96).

***PFMDR1* AND ITS ROLE IN *P. FALCIPARUM* ANTIMALARIAL RESISTANCE**

Pfmdr1 stands for *P. falciparum* multidrug resistance gene and was identified by Cowman *et al.* (1991) while investigating potential genes involved in malaria chloroquine resistance. *Pfmdr1* is the parasite homolog to a multidrug resistance gene found in mammalian tumor cells (97), making it a good candidate for the broad spectrum resistance to structurally distinct antimalarials (42). Located on chromosome 5, *pfmdr1* encodes a 12 transmembrane-domain protein called P-glycoprotein homolog 1 (Pgh1) (Figure 1.4b), due to its homology to the mammalian ATP-binding glycoprotein (P-glycoprotein, P-gp). Mammalian P-gp is believed to function as an energy-dependent

pump. With an ATP-binding domain on the cytoplasmic side of the tumor plasma membrane, P-gp couples ATP hydrolysis with drug export from the cell thus lowering intracellular levels to sublethal concentrations (98). Unlike P-gp, which is located on the plasma membrane of the mammalian cell, *P. falciparum* Pgh1 was found, using immunoelectron microscopy, to localize to the acidic parasite food vacuole (42) and has been implicated in regulation of intracellular drug concentrations (76, 99). Since Pgh1 resides in the food vacuole membrane, its expression is largely restricted to the asexual metabolically-active trophozoite stage of the erythrocytic cycle (42). Pgh1 is also thought to have a role in active transport of antimalarial drugs; however, based on recent work by Rohrbach *et al.* (2006), transport is suggested to pump drugs into the vacuole rather than out, since the ATP-binding domain faces the cytoplasmic side of the vacuolar membrane (76). Further evidence of transporter function was demonstrated by a heterologous complementation assay of *ste6* (mating factor) in *Saccharomyces cerevisiae*, which showed that *pfmdr1* encodes a functional ABC transporter (100).

Initially, *pfmdr1* was investigated as a possible source for resistance to chloroquine because the chloroquine resistance phenotype resembled that of multidrug resistant mammalian tumor cells; both involved drug expulsion and could be reversed by calcium channel antagonists, like verapamil (101). Though Wellems *et al.* (1990) showed through genetic crosses of chloroquine-resistant and chloroquine-sensitive *P. falciparum* strains that resistance was not linked to the *pfmdr1* locus (102), several subsequent studies linked chloroquine resistance to point mutations within the *pfmdr1* coding region, perhaps as part of a multigenic resistance mechanism (41, 103-106).

Single Nucleotide Polymorphisms

Five polymorphic regions of *pfmdr1*, revealed by initial sequence analyses of chloroquine-resistant lines, result in amino acid substitutions at the following loci: N86Y, Y184F, S1034C, N1042D, and D1246Y (101). The N86Y substitution, found in an isolate from Thailand, is located just before the third hydrophobic transmembrane segment of Pgh1 (101); this is a comparable position to a change in mammalian multidrug resistance gene that alters substrate specificity of P-gp (107). While the N86Y mutation was shown to augment chloroquine resistance in *in vitro* studies, this occurred only when the resistant allele of *pfcr1* (K76T substitution) was present in the same genome (57, 72, 108, 109). This co-selection of the *pfmdr1* N86Y and *pfcr1* K76T substitutions by chloroquine treatment suggests that either these two genes work together to determine chloroquine resistance or deleterious *pfcr1* mutations are compensated for by the *pfmdr1* N86Y (110). Interestingly, N86Y was shown affect parasite response to other antimalarials as well: *in vitro* studies suggest that the mutant allele augments resistance to quinine but increases sensitivity to mefloquine, halofantrine, and artemisinin (35, 110).

A role for the other 4 mutations (Figure 1.4b) has been more difficult to ascertain (56, 77). Position 1042 in *pfmdr1* is homologous to a residue that modulates multidrug resistance on mammalian *mdr3* and was shown to affect *P. falciparum* sensitivity to chloroquine, mefloquine, and artemisinin (34). Both N1042D and S1034C are located within the same transmembrane segment of Pgh1, likely on the hydrophilic side of an amphipathic helix (101). Introducing mutations at *pfmdr1* positions 1034, 1042, and 1246 in a chloroquine-sensitive falciparum strain had no effect on chloroquine response; yet, replacing substituted amino acids with wild-type residues at these positions in a

chloroquine-resistant strain reduced resistance by half (29). These results suggest that these mutations are insufficient to confer resistance in the sensitive strain but that they may provide a cumulative effect with mutations in other genes to confer higher levels of resistance in chloroquine-resistant parasites (29). Interestingly, position 184, while highly polymorphic, does not appear to play a significant role in resistance at all. For instance, although Y184F was found in CQ-resistant parasites along with the other 3 mutations, Y184F was also identified in chloroquine-sensitive parasites, suggesting that this polymorphic position is not involved in chloroquine resistance (101). Additionally, Y184F was not significantly associated with resistance to mefloquine or artemisinin (35).

Pfmdr1 Gene Copy Number

There are many cases in biology, including mosquito genes involved in insecticide resistance and bacterial genes responsible for adaptations to the environment, where phenotypic effects are elicited by gene copy number changes (111-113). Multiple copies in both prokaryotes and eukaryotes generally arise from gene duplication followed by amplification. The initial duplication step is rate-limiting and can occur via one of two mechanisms: (1) homologous recombination involving large regions of homology that employs RecA protein in prokaryotes and RAD52 in eukaryotes, or (2) illegitimate recombination involving short regions of homology without employing either of these proteins. The abundance of monomeric A/T tracts found in the *P. falciparum* genome is consistent with the latter mechanism (114). Subsequent amplification events occur via homologous recombination utilizing the large regions of homology generated by the initial duplication. Notably, these duplications occur more frequently than point

mutations but also tend to revert to a single-copy state more frequently due to unequal recombination (114).

In the case of *P. falciparum*, the most extensive copy number polymorphism, in terms of size and frequency, is located on chromosome 5 and encompasses the *pfmdr1* gene in all strains studied (75, 115). Because the *P. falciparum* genome is extremely A/T-rich (80.6% in the genome of strain 3D7 and up to 90% within introns and intergenic regions (116)) and thus less stable than DNA with higher G/C content, gene duplication events are common. Monomeric A/T tracts found near *pfmdr1* were significantly longer than the genome-wide average, identifying a probable hot spot for gene duplication (114). The *pfmdr1* gene was found to be particularly prone to copy number increases, rendering the parasite resistant to mefloquine and quinine, both *in vitro* and *in vivo* (115). The similarity between drug-selected laboratory strains and evidence of amplification events found in clinical samples indicate that initial duplications likely occur during the mitotic blood stages of the parasite life cycle (69, 114).

Although the mechanism of *pfmdr1*-mediated multidrug resistance is still unknown, increases in *pfmdr1* gene copy number is highly associated with mefloquine, quinine, and artemisinin-based combination therapy (ACT) failure in both *in vitro* and clinical studies (27, 31, 32, 39, 56, 76). A large-scale study in Thailand revealed that *pfmdr1* copy number is highly associated with multidrug resistance; 64% of mefloquine-resistant isolates demonstrated more than 1 copy of *pfmdr1* (37). This was confirmed *in vitro* to show that increased resistance to a number of antimalarials, including mefloquine, quinine, and artemisinin, was the result of multiple *pfmdr1* gene copies (26, 56). These additional copies were found to arise from multiple independent events and

were shown to occur in tandem along the same chromosome, suggesting that *pfmdr1* is under strong selective pressure (42, 117). However, while *pfmdr1* copy number increases occur frequently, there is a fitness cost to parasites with multiple *pfmdr1* copies in the absence of drug pressure; without the drug, these parasites eventually revert to the single-copy state (118).

While the relationship between *pfmdr1* copy number and drug resistance has been well documented both *in vitro* and in clinical studies (27, 31, 32, 36, 39, 114, 118-120), the effect of this change on gene expression is unclear. In order to explain a previously observed increase in *pfmdr1* transcript in drug resistant parasites, two possible mechanisms have been suggested (40, 121, 122): (1) a gene amplification model, and (2) a transcript induction model. The gene amplification model suggests simply that a parasite containing multiple copies of a gene leads to over-expression of the transcript and protein. This is the case for mammalian multidrug resistant (*mdr*) gene (123, 124). Likewise, drug-resistant *P. falciparum* strains selected *in vitro* for mefloquine resistance contain more than one copy of *pfmdr1* and have higher transcript levels (40, 121, 122, 125). Mefloquine-resistant field isolates also had higher *pfmdr1* copy numbers and over-expressed the transcript (56, 76). In contrast, the transcript induction model suggests that, upon drug exposure, gene expression increases above levels seen in the absence of drug. For example, the drug sensitive *P. falciparum* strain, 3D7, was treated with several antimalarials to study how addition of drug influences *pfmdr1* transcript levels. Transcript levels increased 2.5-fold in parasites treated with chloroquine, mefloquine, and quinine, but not with the structurally-unrelated drug, pyrimethamine (126). Thus, adding drug increased the amount of *pfmdr1* transcript present, and this result appeared to be a drug-

specific effect. However, no such induction studies have been performed on laboratory strains already containing multiple copies of *pfmdr1*; such experiments are necessary to ascertain if drug-induced *pfmdr1* transcript increases would be found in parasites already possessing more than one *pfmdr1* copy.

DISSERTATION OBJECTIVES AND RELEVANCE

The focus of this dissertation was to evaluate the role of *pfmdr1* gene copy number on parasite response to antimalarial treatment. There were 3 main objectives that guided this body of work: (1) assess the effect of increased copy number on *pfmdr1* gene expression and response to mefloquine treatment *in vitro*; (2) evaluate whether multiple *pfmdr1* copies affects subcellular localization of the fluorescent antimalarial compound, quinine; and (3) determine the proportion of parasites with increased *pfmdr1* copies within an infected individual and test whether that relates to treatment response.

First, the data outlined in chapter 2 address the question of mefloquine's effect on *pfmdr1* expression in parasites with multiple copies of the *pfmdr1* gene. Understanding how copy number results in the MFQ resistant phenotype will provide insight into possible mechanisms of MFQ action and will be crucial for making future decisions on implementing antimalarial regimens that include MFQ.

Second, the ability of Pgh1, the ABC transporter encoded by *pfmdr1*, to transport compounds across the food vacuolar membrane likely has a role in parasite response to antimalarial treatment. Chapter 3 evaluates the consequence of additional *pfmdr1* gene copies on subcellular localization of quinine, particularly if multiple copies results in

increased trafficking of quinine into the acidic food vacuole. This, coupled with parasite response to quinine, may indicate possible sites of action within the malaria parasite.

Finally, the clinical relevance of increased *pfmdr1* copy number is addressed in chapter 4. The structure of the parasite population within an individual patient will provide great insight into the minimum percentage of parasites with increased *pfmdr1* copies that is necessary to result in clinical treatment failure.

Figure 1.1: Global distribution of endemic *Plasmodium falciparum*. Infection prevalence is indicated by variations in color intensity: light green represents areas with an infection prevalence of $\leq 10\%$; medium green depicts 11-50% prevalence; and dark green represents the highest prevalence, $\geq 50\%$ (127). Figure taken from Snow *et al.*, The global distribution of clinical episodes of *Plasmodium falciparum* malaria. Nature. 2005.

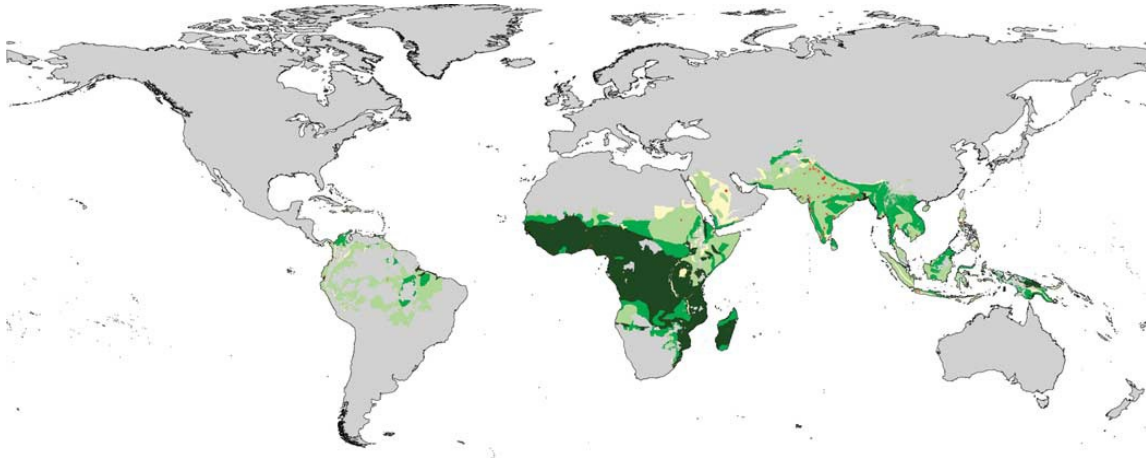


Figure 1.2: *Plasmodium* life cycle. *Plasmodium* parasites have a complex life cycle that spans 2 hosts and 3 stages: (1) sexual stage in the *Anopheles* mosquito host which is important for malaria transmission; (2) asymptomatic stage in the human liver wherein parasite burden amplifies; and (3) asexual blood stage in the human, responsible for malaria symptoms. The morphologies depicted here are for *P. falciparum*, except for the hypnozoite, which only occurs in *P. ovale* and *P. vivax* (6). Figure taken from Griffith *et al.*, Treatment of malaria in the United States: a systematic review. JAMA. 2007.

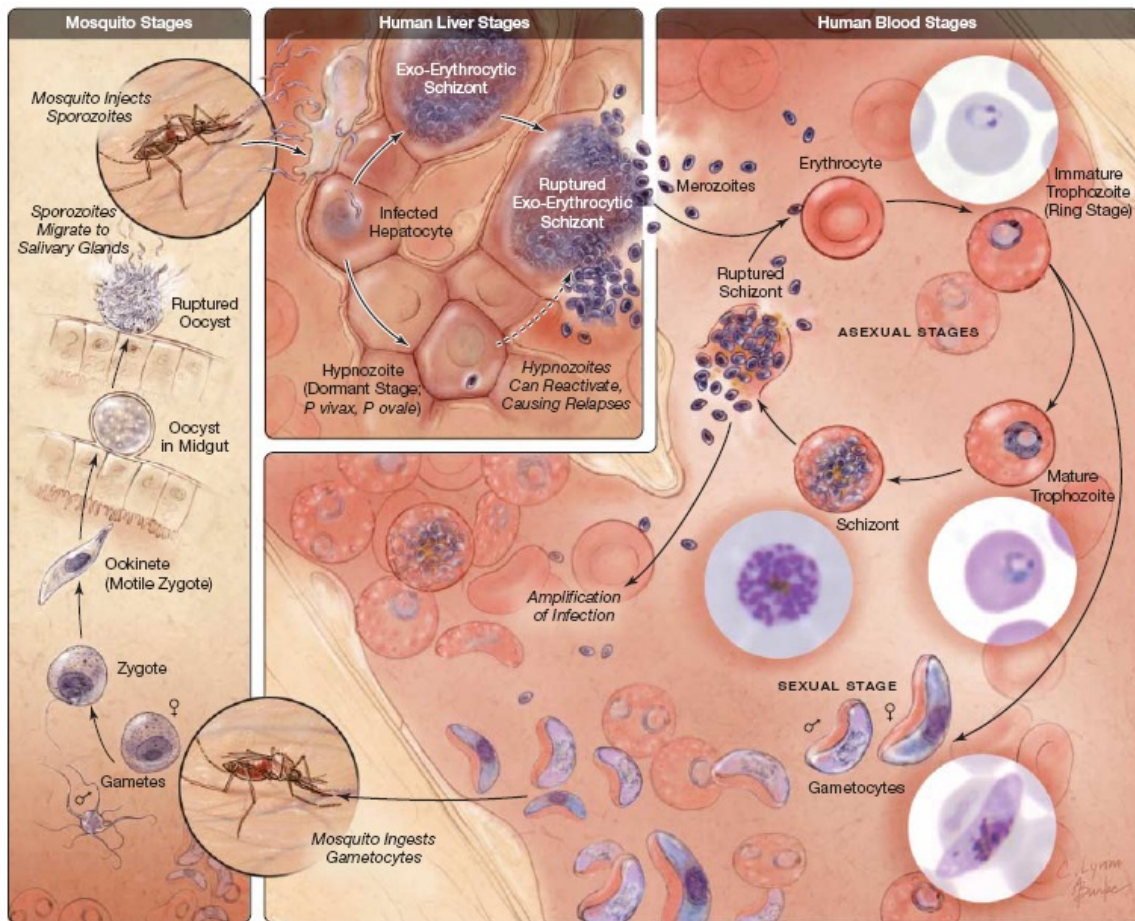
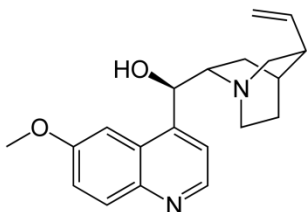
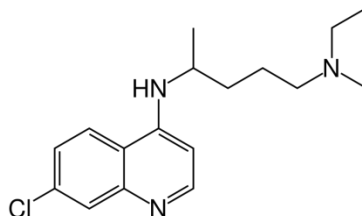


Figure 1.3: Chemical structures of antimalarial compounds. (a) Quinine; (b) Chloroquine; (c) Mefloquine; (d) Artemisinin. Structurally-related quinolines are shown in (a), (b), and (c). Artemisinin (d) is structurally distinct and is derived from the wormwood herb, *Artemisia annua*.

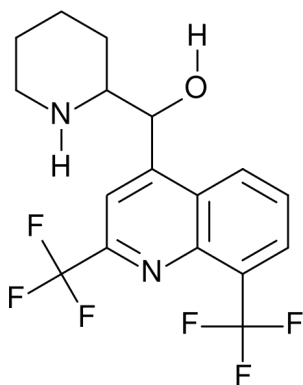
(a) Quinine



(b) Chloroquine



(c) Mefloquine



(d) Artemisinin

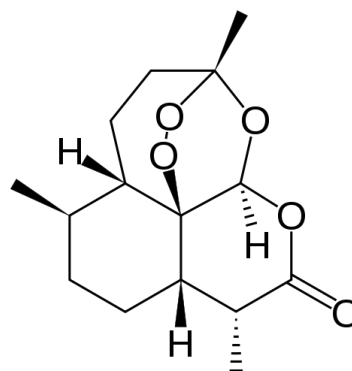
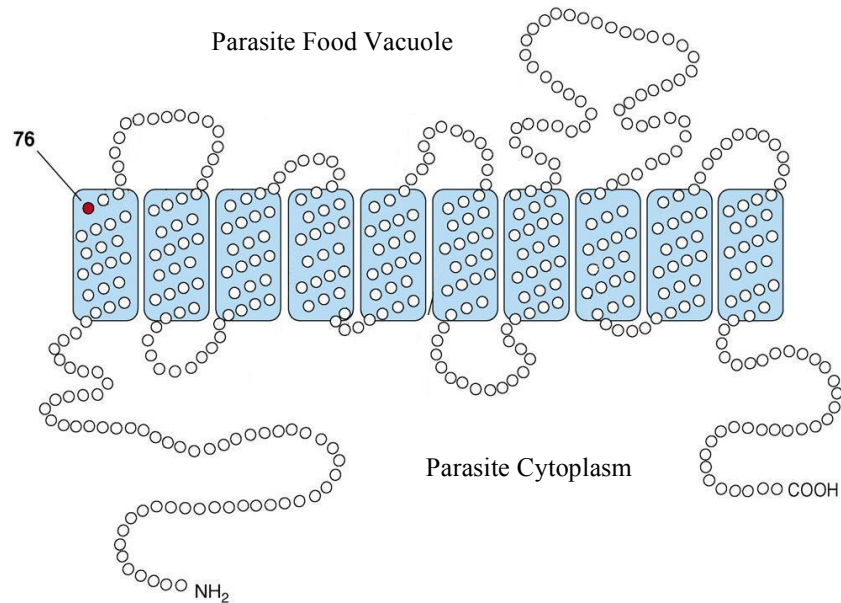
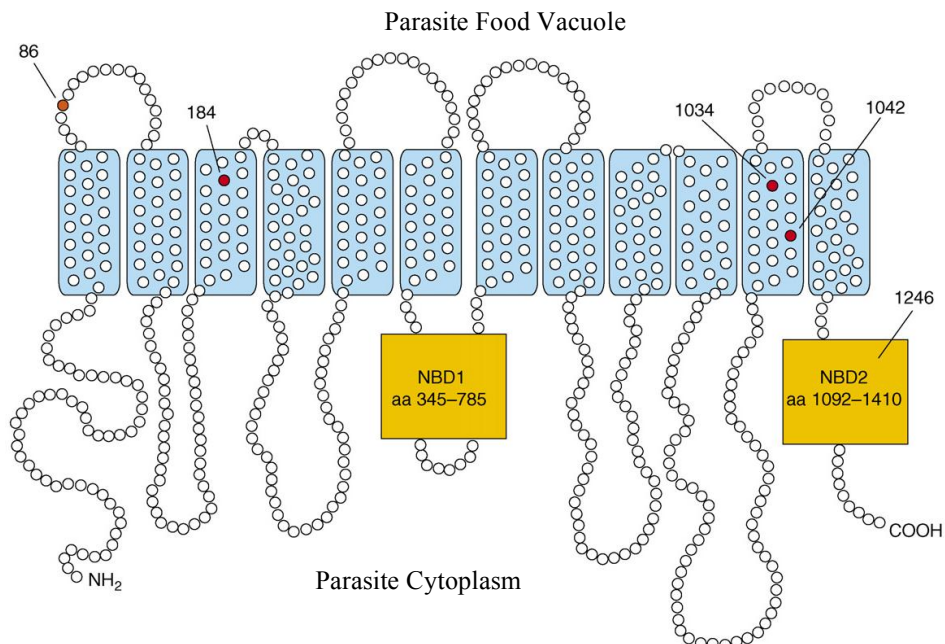


Figure 1.4: Predicted topologies of *P. falciparum* Chloroquine Resistance Transporter (PfCRT) encoded by *pfCRT* and P-glycoprotein homolog 1 (Pgh1) encoded by *pfmdr1*. (a) PfCRT has 10 predicted transmembrane domains. The K76T mutation found in all chloroquine-resistant strains is indicated in red. (b) Pgh1 has 12 transmembrane domains and 2 nucleotide-binding domains (NBD). Single nucleotide polymorphisms that encode amino acid changes associated with antimalarial resistance are indicated. Figure from Valderramos and Fidock. Transporters involved in resistance to antimalarial drugs. Trends Pharmacol Sci. 2006 (56).

(a)



(b)



CHAPTER II

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

INTRODUCTION

With 225 million estimated clinical infections that result in 781,000 deaths annually, the protozoan *Plasmodium falciparum* causes the most severe form of malaria, (4). Efforts to control malaria have been hampered by the development of resistance to antimalarials such as chloroquine, sulfadoxine-pyrimethamine, and mefloquine (MFQ). Artemisinin-based combination therapies (ACT) are now the first-line treatment for falciparum malaria. Unfortunately, resistance to these new drugs is believed to be developing based on the observation of slower *in vivo* parasite clearance times (61, 91, 128). 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 (42, 101, 103). 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 (42) and functions as a transporter (76). The transporter function of Pgh1 couples ATP hydrolysis with solute import into the food vacuole (129). The *pfmdr1* gene has been identified as a possible modulator of resistance to a number of antimalarials (97), and the Pgh1 protein has also been implicated as a specific target of antimalarial

drugs, such as MFQ (78). Substantial data support a relationship between the *pfmdr1* gene and MFQ resistance both *in vitro* and *in vivo* (27, 31, 33, 37-39, 41, 74, 75, 118). 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 (27, 31, 32, 39, 41, 114, 118, 120, 129-133).

The control of *pfmdr1* gene expression is only partially understood. MFQ-resistant field isolates with higher *pfmdr1* copy numbers over-express the transcript compared to isolates with a single copy of *pfmdr1* (56, 76), indicating a direct correlation wherein 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 (126). 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, MR4¹: (1) 3D7: one *pfmdr1* copy, sensitive to CQ and MFQ, (2) FCB: 2 copies, CQ-resistant and MFQ-sensitive, and (3) Dd2: 4 *pfmdr1* copies, resistant to CQ and MFQ. Parasite cultures were maintained at

¹ MR4: Manassas, VA

37°C using the standard Trager-Jenson method for malaria parasite culture (134). Pooled human type O+ serum at 10% and red blood cells² at 2% hematocrit were used for all cultures and experimental conditions. Cultures were synchronized with 5% sorbitol solution every 48 hours for three consecutive life cycles to obtain a uniform culture of parasites at a single stage (135).

Gene expression analysis

Sorbitol-synchronized ring stage parasites were exposed to 100 ng/mL MFQ for 48 hours. Total RNA was isolated from cultures at 0, 6, 12, 24, and 48 hours after addition of drug using TriReagent³, according to the manufacturer's instructions. Real-time reverse transcriptase (RT)-PCR was employed to assess relative *pfmdr1* (reference ID 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 (136). The C_t value represents the PCR cycle at which DNA amplification crosses the threshold value. The ΔC_t for each sample is defined as $C_{t_{pfmdr1}} - C_{t_{PjLDH}}$. 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)-treated (1 μ M) cultures were used to control for effects on gene expression due to the addition to the culture media of a drug with a known site of action distinct from the *pfmdr1*-encoded protein. Expression of the developmentally-

² Research Blood Components, LLC: Boston, MA

³ Molecular Research Center, Inc.: Cincinnati, OH

regulated *trophozoite antigen R45-like* (PlasmoDB: PFD1175w), *tar45-like*, gene, which is transcribed during the ring stage only, was assessed using a SYBR Green assay as previously described (137). Single-copy *P. falciparum lactate dehydrogenase* (PlasmoDB: PF13-0141) gene, *pfl dh*, 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'): (1) *pfmdr1* forward primer: TGCCACAGAATTGCATCTATAA, (2) *pfmdr1* reverse primer: GACTGTACAAAGGTTCCATTTCGA, and (3) *pfmdr1* probe: FAM-ACGATCAGACAAAATT-MGB. The published primers and fluorescent probes for the *pfl dh* and *tar45-like* genes (137) were used and are as follows (5' to 3'): (1) *pfl dh* forward primer: ACGATTTGGCTGGAGCAGAT, (2) *pfl dh* reverse primer: TCTCTATTCCATTCTTTGTCACTCTTTC, (3) *pfl dh* probe: FAM-AGTAATAGTAACAGCTGGATTTACCAAGGCCCA-TAMRA, (4) *tar45-like* forward primer: ACGAGCTGACCCACCAAA, and (5) *tar45-like* reverse primer: CATTTAAGTCTGTCTTCATTCTACTTCT. 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 30µL: 100ng RNA plus PCR reaction mixture to reach a total volume of 30µL. 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, then 10 min. at 94°C, followed by a total of 40 PCR cycles (15 sec. at 94°C and 1 min. at 60°C) (136). 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 stain⁴ at 0, 6, 12, 24, and 48 hours 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: (1) parasites with a single nucleus and characteristic ring appearance were classified as rings; (2) parasites containing a single nucleus and hemozoin were considered trophozoites; and (3) multinucleated parasites with ample hemozoin represented schizonts.

IC50 Determinations

Parasite drug susceptibility was determined using a modification of a published SYBR Green I flow cytometry assay (138). Drug concentration required to inhibit 50% of parasite growth 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

⁴ Sigma-Aldrich Co.: St. Louis, MO

JMP Software⁵. Tukey's test was used to determine significant differences at $P \leq 0.05$ between the parasite strains because significance is determined for all possible pairwise comparisons of the three strains. The student's *t* test was used to determine significant differences at $P \leq 0.05$ between untreated and drug-treated expression levels.

RESULTS

Pfmdr1 expression was proportional to copy number

Synchronized parasites from three clonal strains of *P. falciparum* with different *pfmdr1* gene copy numbers were used. Total RNA was isolated at several time points during the 48-hour asexual blood stage life cycle. In untreated parasites, *pfmdr1* gene expression was directly proportional to the number of *pfmdr1* gene copies present in the parasite: Dd2 parasites (four *pfmdr1* gene copies) had higher expression of *pfmdr1* transcript than 3D7 (one copy) or FCB parasites (two copies) (Figure 2.1). These differences are significant ($P < 0.05$) for Dd2 parasites through the 24-hour time point. The results indicate that there is a direct relationship between *pfmdr1* copy number and expression of the *pfmdr1* transcript.

Mefloquine exposure caused 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 hours (Figure 2.2). Although we consistently saw higher *pfmdr1* expression in MFQ-exposed parasites compared with untreated parasites

⁵ SAS Institute, Inc.: Cary, NC

of the same strain, these results were significant only after synchronized rings of 3D7 with one copy of *pfmdr1* ($P < 0.005$) and Dd2 with four copies of *pfmdr1* ($P < 0.05$) were exposed to MFQ for 12 hours. *Pfmdr1* upregulation was observed only after exposure to MFQ but not to atovaquone (ATQ), suggesting that increased *pfmdr1* expression is a specific *P. falciparum* response to MFQ and not a generalized response to antiparasitic drug treatment (data not shown). Similar trends, though not significant, were seen when drugs were added to synchronized parasites at the trophozoite stage.

Mefloquine induced P. falciparum cell cycle delay

Parasite maturation through the asexual blood stages was evaluated by thin blood smears using light microscopy. We found that the addition of MFQ to synchronized ring-stage parasites delayed parasite maturation at the ring stage (Figure 2.3). In all strains tested, delay in maturation began at 6 hours post-treatment 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 2.4 shows that when MFQ was added to synchronized trophozoites, maturation proceeded normally until the ring stage, at which point maturation again stalled. Thus, MFQ appears to have a selective effect on ring-stage parasites.

MFQ treatment resulted in increased expression of ring stage genes

To confirm the effects of MFQ on parasite development, we used real-time PCR to test the expression of the developmentally-regulated *trophozoite antigen R45-like* (*tar45-like*) gene (PlasmoDB: PFD1175w), which is transcribed during the ring stage

only (137). The fold difference in *tar45-like* gene expression relative to *pfl dh* was higher 12 hours after treatment with MFQ (Table 2.1), whereas *tar45-like* gene expression was not affected by ATQ. These data confirm that MFQ-treated parasites remained in the ring stage for a longer period of time than untreated parasites.

Pfmdr1 was primarily expressed 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 (Figure 2.5). 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.

MFQ-induced maturation delay had no effect on artesunate IC50

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 hours to induce the maturation delay. The half maximal inhibitory concentration (IC50) of artesunate was assessed on MFQ-exposed parasites. A SYBR Green I flow cytometry assay was used to quantify parasite growth inhibition by artesunate, as previously reported (138). We found no difference in artesunate IC50 values between untreated and MFQ-treated parasites (Table 2.2).

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 specific role of *pfmdr1* on MFQ resistance.

Upregulation of *pfmdr1* following MFQ treatment was consistently observed in all strains. However, the results were statistically significant only for 3D7 and Dd2 parasites at the 12-hour time point. Although the reason for this is unclear, subsequent analysis of parasite morphology on the sampled cultures to correlate our *pfmdr1* expression data with parasite life cycle revealed an interesting phenotype. In agreement with a recent report that studied MFQ exposure on synchronized rings only (139), 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 caused the parasites to stall but only after the parasites matured through the intraerythrocytic cycle to the ring stage. This indicates that MFQ only affected the parasites' ability to proceed through the asexual intraerythrocytic cycle when ring stage parasites were exposed to the drug.

Two theories can explain the increase in *pfmdr1* expression in response to MFQ exposure: (1) direct induction of *pfmdr1* gene expression by MFQ; or (2) an indirect upregulation due to the maturation delay. At each time point assessed, a direct relationship was observed between *pfmdr1* expression levels and the proportion of ring-stage parasites in the culture. In one interval, between 12 and 24 hours, 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 *tar45-like* also increased, peaking at 12 hours after the addition of MFQ. This increase was not observed in untreated or atovaquone-treated cultures, which also did not exhibit 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 (140-143). Recently, such delays have been described in *P. falciparum* (137, 139, 144, 145). Drug-induced delay in parasite maturation through the intraerythrocytic cycle was seen after short-term MFQ exposure in MFQ-sensitive strains (139). Similarly, we showed that treatment of synchronized ring-stage parasites with MFQ induced a delay in parasite maturation in both the MFQ-sensitive (3D7, 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. MFQ-induced maturation delay occurred as early as 6 hours post-treatment and was observed after MFQ treatment but not after ATQ treatment, which indicates that the delay is 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 effect of other quinoline compounds, like 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: (1) the presence of MFQ exerts an effect on the parasites' ability to progress through the asexual cell cycle, or (2) the maturation delay is an active response by the parasite to the presence of MFQ. 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, like cyclohexamide 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 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 (146, 147). 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 G1 phase following 5-FU exposure, thus preventing the drug from engaging its S-phase target (146). These delays are directly linked to the treatment dose and duration, and the removal of the therapy restores 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 (139). This would explain the maturation delay if MFQ activity is in one of the later intraerythrocytic stages.

A second, and perhaps more interesting, explanation for the maturation delay and expression increase could be that the delay is an initial response that allows time to increase expression of drug transporters to evade destruction. Delay at the ring stage results in an upregulation of ring stage genes including *pfmdr1* that would result in greater transport capacity due to the presence of more transporter proteins. For *P. falciparum* parasites that possess multiple copies of *pfmdr1*, this delay-induced expression increase would result in many more transporter proteins than single-copy strains and may displace sufficient amounts of MFQ to allow the parasite to survive treatment. Our observations of MFQ-induced maturation delay and the ring stage-specific transcription of *pfmdr1* support this hypothesis and could explain why multiple *pfmdr1*

copies are linked with a resistant phenotype even though all strains exhibit the maturation delay.

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 (148-151), whereas other evidence indicates activity on all blood stages (151-153). If the former is true, then MFQ should be 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. We found no differences in the artesunate IC₅₀ after MFQ exposure, which suggests that it is neither synergistic nor antagonistic. Thus, no evidence was found against combining artesunate and mefloquine in malaria treatment.

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 (27, 31, 32, 39, 114, 118, 120, 128, 133). 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. Further studies are needed to fully understand the mechanism by which MFQ slows cell cycle

progression and if 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 (155-161), would be worthwhile to determine if MFQ delays maturation by interfering with one or more cell cycle enzymes.

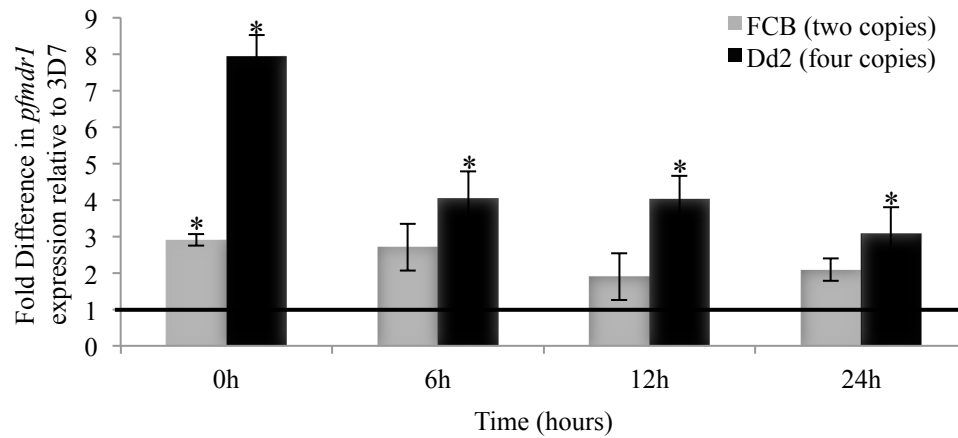


Figure 2.1: Fold differences in *pfmdr1* gene expression over time in parasite strains with different *pfmdr1* copy numbers. Transcript levels of *pfmdr1* relative to the single-copy *P. falciparum* lactate dehydrogenase gene were evaluated over time following synchronization using real-time RT-PCR. 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. * $P < 0.05$, Tukey's test.

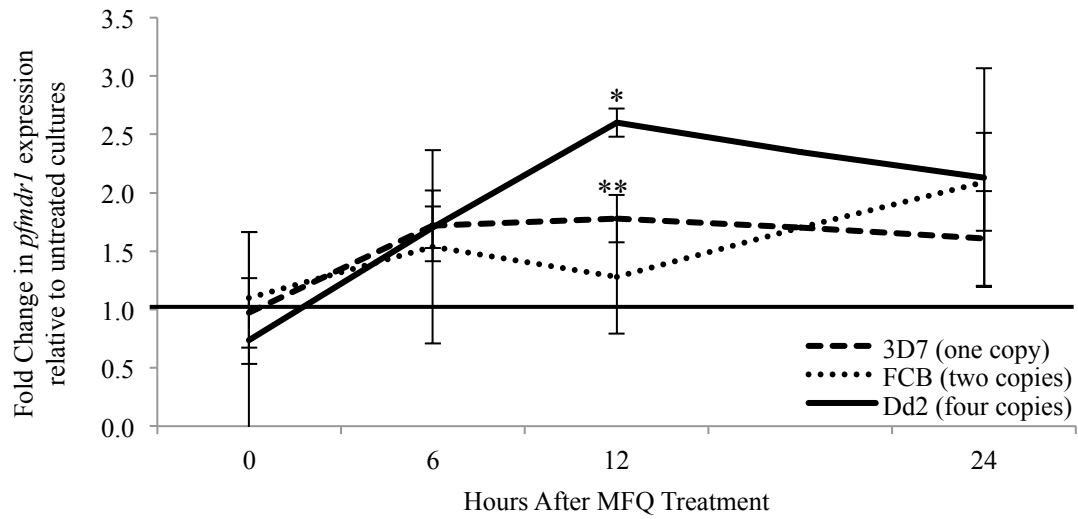


Figure 2.2: Fold change in *pfmdr1* gene expression in parasites with different *pfmdr1* copy numbers over time following mefloquine exposure. Fold changes in *pfmdr1* expression are relative to the corresponding untreated culture for each strain at each time point (not shown), which were set to 1.0 (black line). Significant increases in *pfmdr1* expression upon mefloquine exposure 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 (ΔCt) at each time point. * $P < 0.05$ and ** $P < 0.005$, Student's *t* test.

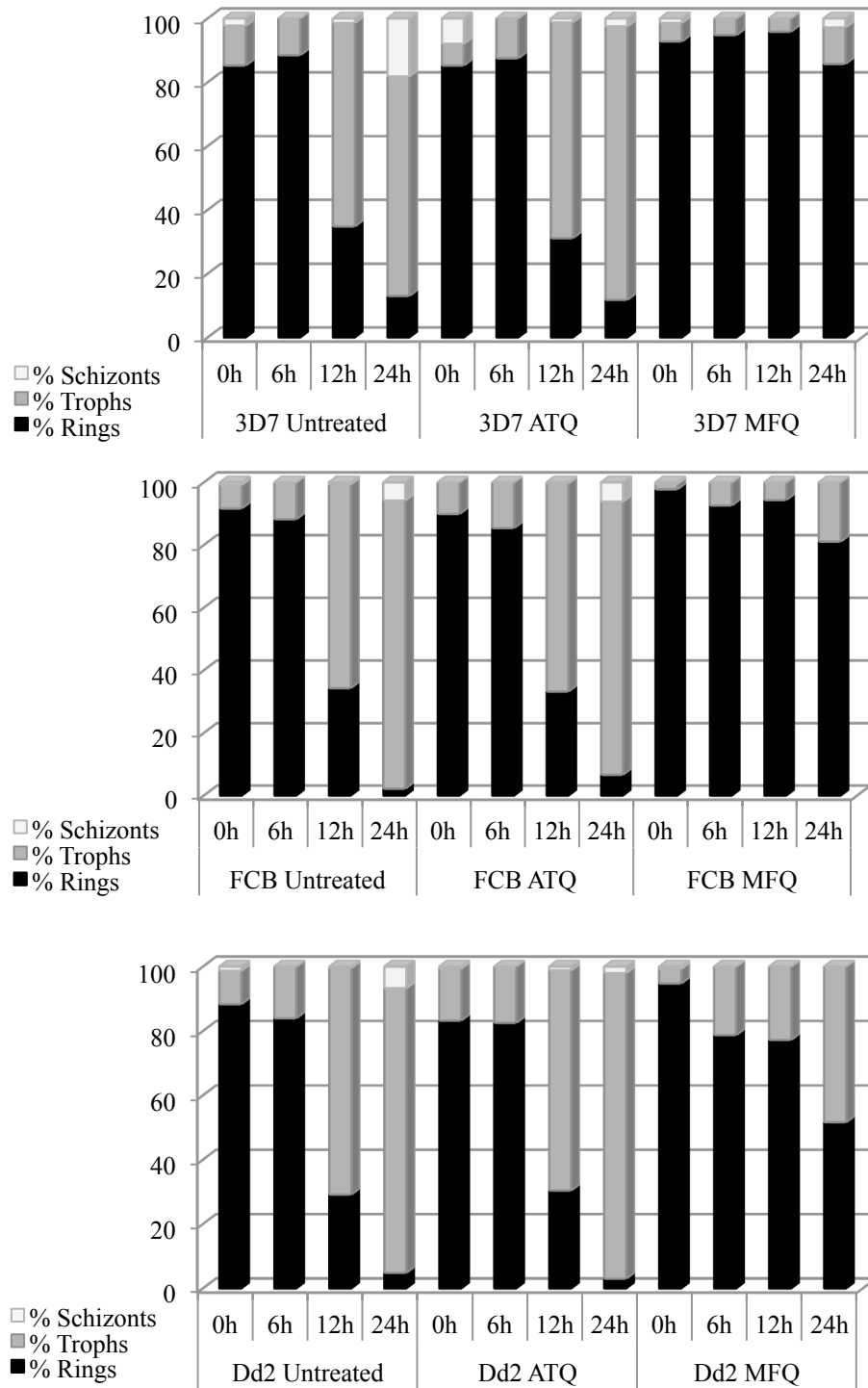


Figure 2.3: Morphology stage development of untreated and drug-treated parasites. Blood stages of 3D7 (1 *pfmdr1* copy), FCB (2 copies), and Dd2 (4 copies) parasites were monitored in untreated, atovaquone (ATQ)-treated, and mefloquine (MFQ)-treated parasites over time. Untreated and ATQ-treated cultures progressed through the normal asexual cell cycle. MFQ treatment caused a delay in parasite maturation, resulting in a persistence of ring-stage parasites. All strains exhibited this delay.

Table 2.1: Fold differences in *tar45-like* gene expression after 12 hours of drug exposure in parasites with different *pfmdr1* copy numbers

Parasite Strain^a	Atovaquone-treated^b	Mefloquine-treated^b
3D7	1.31	5.73
FCB	1.02	6.14
Dd2	1.47	6.48

^a*Pfmdr1* copy number of each parasite strain is as follows: 3D7 has one *pfmdr1* gene copy, FCB has two copies, and Dd2 has four copies.

^bFold differences in *tar45-like* expression levels are relative to untreated levels for each strain at the 12 hour time point, which were set to 1.00. Results are from two samples for each condition.

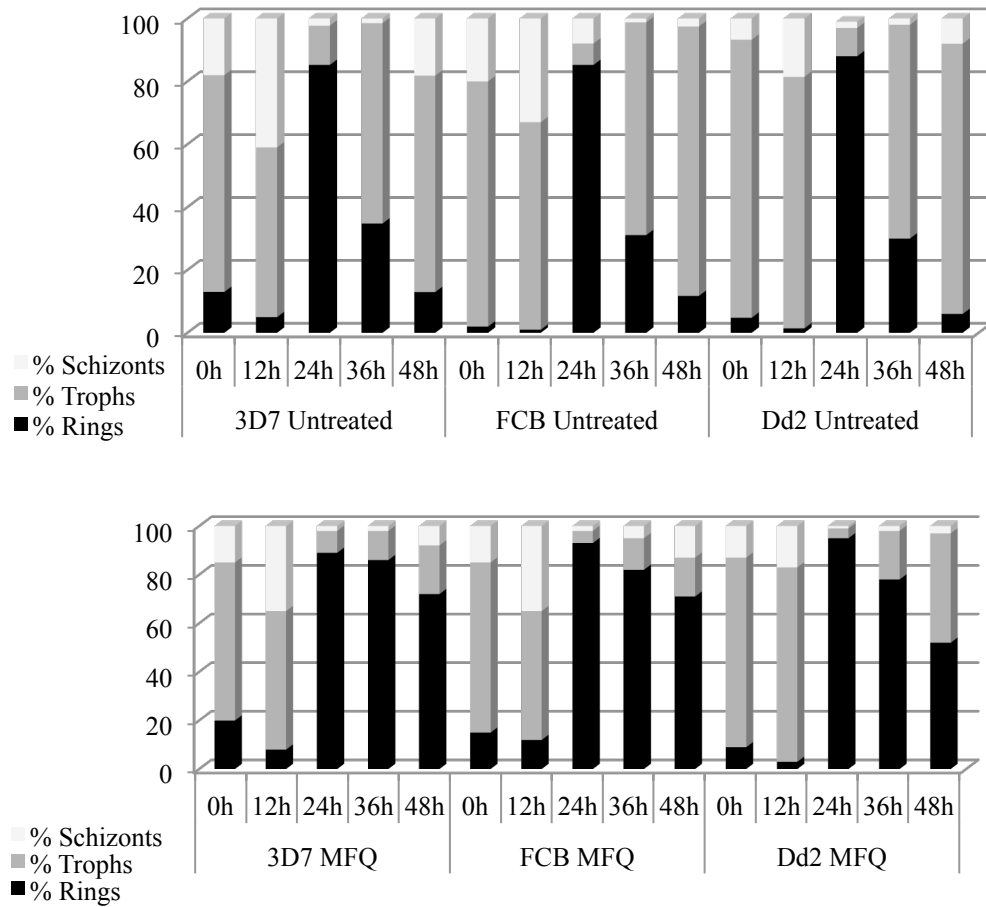


Figure 2.4: Morphology stage development of untreated and MFQ-treated synchronized trophozoite parasites. Synchronized trophozoites of 3D7 (1 *pfmdr1* copy), FCB (2 copies), and Dd2 (4 copies) parasites were monitored in untreated and mefloquine (MFQ)-treated parasites over time. Untreated cultures (top 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.

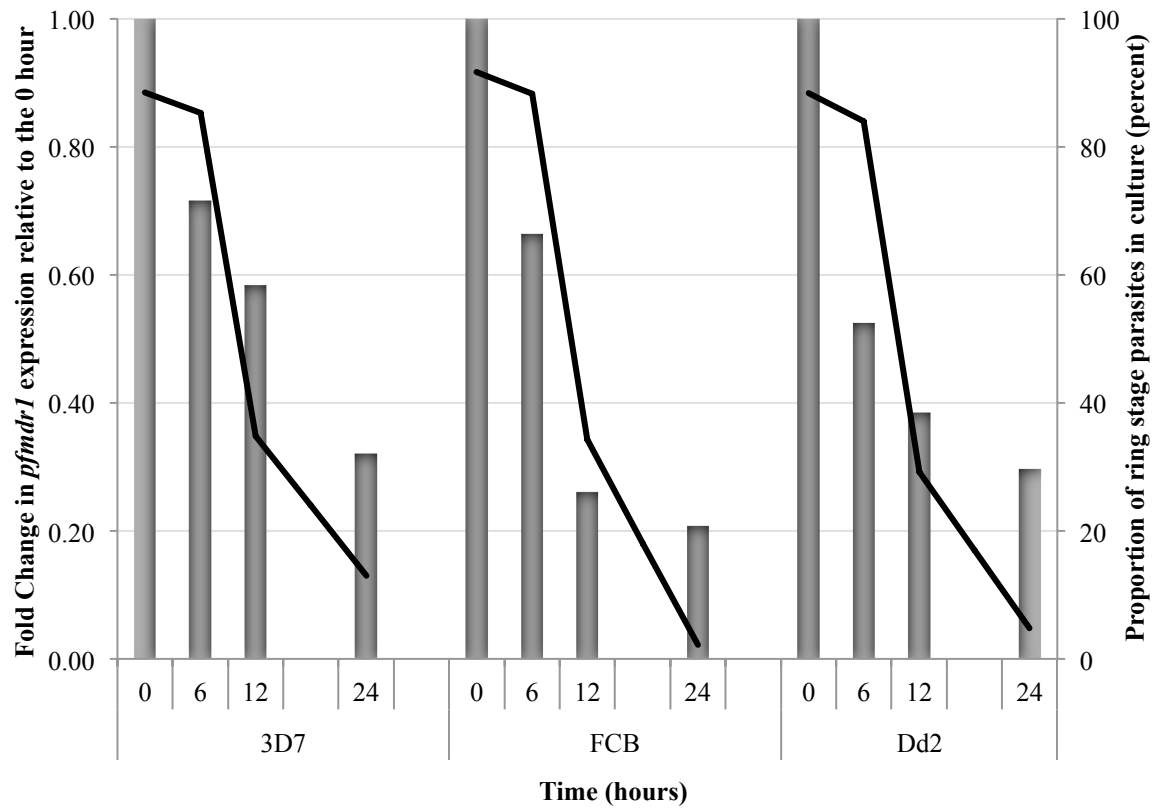


Figure 2.5: Ring stage-specific expression of *pfmdr1* in untreated parasite strains containing different *pfmdr1* copy numbers. Untreated cultures of each parasite strain were assessed for morphology (lines) and *pfmdr1* gene expression (bars) relative to *P. falciparum* lactate dehydrogenase. A direct relationship between the proportion of ring stage parasites in the culture and the relative expression of *pfmdr1* was observed.

Table 2.2: Half maximal inhibitory concentration (IC50) of artesunate in parasite cultures before and after exposure to mefloquine (MFQ).

Parasite Strain	Untreated	Mefloquine-treated ^c
3D7 ^a	0.61 ± 0.04nM	0.62 ± 0.07nM
Dd2 ^b	0.39 ± 0.05nM	0.40 ± 0.02nM

^a3D7 parasites contain one *pfmdr1* gene copy.

^bDd2 parasites contain four *pfmdr1* gene copies.

^cMFQ-treated cultures were exposed for 12 hours to MFQ prior to IC50 analysis.

CHAPTER III

Novel Limiting Dilution Real-Time PCR Assay for the Detection of Subpopulations of Parasites within Individual Patients Infected with *Plasmodium falciparum*

INTRODUCTION

One of the major complications to malaria control efforts is the spread of drug resistant parasites. Drug-resistant *Plasmodium falciparum* is now a serious problem in Southeast Asia, where resistance to a number of antimalarials is common (61, 162). Western Cambodia has been an epicenter for the emergence of antimalarial resistance, where chloroquine resistance was first reported in the late 1950s (163). As chloroquine-resistant parasites pervaded the malaria population, mefloquine (MFQ) was adopted in 1976 as the first-line treatment for *P. falciparum* (64). Although its use was carefully regulated, significant resistance to MFQ monotherapy rapidly developed (27). As a result, combination therapies involving artemisinin derivatives, specifically artesunate-mefloquine (AM), were introduced in 1995 (64). While these are more effective at parasite clearance than MFQ alone, clinical treatment failure, or recrudescence, to AM has emerged (31, 91, 130). The declining efficacy in recent years of AM therapy confirms the need to fully explore the parasite populations of this region in order to understand the resistance mechanisms and spread of drug-resistant parasites.

As resistance to new antimalarial therapies arises, understanding how mutations evolve is needed. Resistance to MFQ *in vitro* is linked to both single nucleotide

polymorphisms (SNPs) and gene copy number in the *P. falciparum multidrug resistance* gene (27, 31, 32, 118, 120, 122, 130, 132, 133). Elevated *pfmdr1* copy number has also been linked to increased resistance to both MFQ monotherapy and AM *in vivo*.

Individual patients are frequently infected with multiple genetic variants of malaria parasites at once, often with a mixture of both drug-sensitive and drug-resistant variants. Theoretically, someone infected with predominantly drug-sensitive parasites might fail therapy because of a low level of drug-resistant parasites. The ability to detect minority variants with higher *pfmdr1* copy numbers might be able to better predict *in vivo* antimalarial treatment failure. *Pfmdr1* copy number has traditionally been measured using real time PCR and measures the average copy number of all parasites within the individual. In order to be able to study the proportion of parasites with elevated *pfmdr1* copy number within an individual patient, we developed a limiting dilution real-time PCR assay to assess *pfmdr1* copy number of parasite subpopulations present in a mixed infection. The purpose of this study was to evaluate the distribution of subpopulations with different *pfmdr1* copy numbers in patients before and after artesunate-mefloquine treatment.

MATERIALS AND METHODS

***P. falciparum* genomic DNA**

Genomic DNA from two clonal *P. falciparum* strains was obtained from the Malaria Research and Reference Reagent Resource Center (MR4): 3D7 (MRA-102G) contains 1 *pfmdr1* copy and Dd2 (MRA-150G) contains 4 *pfmdr1* copies. Paired enrollment (pre-treatment) and recurrent parasitemias from five randomly selected

participants were obtained from a clinical trial conducted in the Chumkiri District, Kampot Province, in southern Cambodia between August 2006 and February 2008. The details of the study have been published elsewhere (130). In brief, subjects with uncomplicated mono-infection by *P. falciparum* with parasitemias between 1,000 and 100,000 parasites/mm³ were included in this study. Parasitemias were determined by counting parasites per 1,000 red blood cells in Giemsa-stained blood films and multiplying by standard estimates of red blood cell counts (4,000,000 RBC/mm³) (130). Subjects with mixed *Plasmodium* infection, an unwillingness to remain at the clinic for directly observed therapy or to return for follow-up visits, or any sign of severe malaria according to World Health Organization criteria were excluded from this study. One hundred fifty-one subjects received directly observed therapy with 12 mg/kg artesunate (over three days) and 25 mg/kg mefloquine (two doses, eight hours apart), up to a maximum dose of 600 mg artesunate/1,000 mg mefloquine (130). Subjects were followed for 42 days or until recurrent parasitemia was observed. Blood was collected for examination on days 0, 1, 2, 3, 7, 14, 21, 28, 35, and 42, and at any time when the subject reported to the clinic with fever or other malaria-related symptoms.

Development of TaqMan[®] Real-time PCR Limiting Dilution Assay

Limiting dilution was used to obtain single copies of the *P. falciparum* genome (165, 166). Because all isolates that have been studied thus far show that any additional copies of *pfmdr1* occur in tandem along chromosome 5 (117), we chose the single-copy *RNA helicase-1* gene as the reference gene because it is also located on chromosome 5

but is outside of the size polymorphism responsible for the *pfmdr1* gene amplification (PlasmoDB) (115).

The *pfmdr1* (PlasmoDB: PFE1150w) and *RNA helicase-1* (PlasmoDB: PFE1390w) primers and fluorescent probes were designed using ABI's Primer Express software and are as follows (5' to 3'): (1) *pfmdr1* forward primer: TGCCACAGAATTGCATCTATAA, (2) *pfmdr1* reverse primer: GACTGTACAAAGGTTCCATTTCGA, (3) *pfmdr1* probe: FAM-ACGATCAGACAAAATT-MGB, (4) *RNA helicase-1* forward primer: TGATATCCCTGCACCAATAAAAAA, (5) *RNA helicase-1* reverse primer: CCACTACCGGTAAACGCTATACCT, and (6) *RNA helicase-1* probe: VIC-AAGAAGCCAACACAAATTCAAATGCAAGGA-TAMRA. Each duplex real-time PCR reaction contained the following: 1μL of gDNA, ABI's TaqMan[®] Gene Expression MasterMix (2X)⁶, 300nM each *pfmdr1* primer, 200nM *pfmdr1* probe, 900nM each *RNA helicase-1* primer, 250nM *RNA helicase-1* probe, and nuclease-free water to reach a total reaction volume of 20μL. We elected to use the TaqMan[®] Gene Expression MasterMix because it has been optimized for the amplification of small quantities of DNA⁷. The real-time PCR reactions were carried out using the 384-well block on an Applied Biosystems 7900HT Fast Real-Time PCR System¹ under the following conditions: 50°C for 2 minutes, 95°C for 10 min., and 45 cycles of 95°C for 15 seconds and 60°C for 1 min. Nuclease-free water and human gDNA extracted from whole blood were used as negative controls. *P. falciparum* 3D7 and Dd2 gDNA from MR4 at the following concentrations were used as positive controls: 10ng/μL and 1ng/μL. Although we

⁶ Applied Biosystems, Inc.: Carlsbad, CA

⁷ Personal communication with Chris Miller, Field Application Specialist, Applied Biosystems, Inc.

conducted the same calculations while correcting for differences in primer/probe efficiencies using a previously described method (31), we found that the slight alterations did not significantly affect the results. Therefore, the results presented were calculated using the ideal PCR cycle efficiency of 2, which states that the DNA template doubles at each PCR cycle.

Serial dilutions of sample DNA were made and subjected to real-time PCR for *RNA helicase-1*. Dilutions were chosen in which amplification was observed in ~67% of the total number of replicate PCR wells. Assuming the Poisson distribution, approximately one-third of the replicates at the selected dilution would not contain any chromosome 5 DNA, one-third would contain one copy of chromosome 5, ~18% would contain two copies, and the rest would contain three or more copies of chromosome 5.

Ct values (cycle at which probe fluorescence from DNA amplification crosses a threshold value) were determined for *pfmdr1* and *RNA helicase-1* in each individual replicate well. *Pfmdr1* copy number was calculated using the Pfaffl $\Delta\Delta C_t$ method (167). The wells in which the *RNA helicase-1* Ct value was highest (*i.e.* the DNA product accumulates and exceeds a threshold value at a later PCR cycle, which suggests that there was less DNA in the well at the initiation of the PCR run) were categorized as wells containing one chromosome 5 at the outset. A decrease in *RNA helicase-1* Ct value of 1.0 indicated that there was twice as much initial DNA. These wells were categorized as having two initial copies of chromosome 5. For these wells, a three-fold difference in *pfmdr1* copy number relative to the two copies of *RNA helicase-1* was categorized as one variant with two *pfmdr1* copies and one variant with one *pfmdr1* copy. Figure 3.1 shows

the distribution of wells with *RNA helicase-I* amplification (Ct) that were used in the limiting dilution assay analysis for Patient A.

Wells with Ct values below 37 were excluded from the analysis since they were likely to contain three or more copies of chromosome 5.

Mock Clinical Samples

Live cultured *P. falciparum* parasites from the following MR4 strains were used: 3D7 and Dd2. Parasites were combined with fresh type O+ human whole blood and spotted on sterile Whatman filter paper to mimic clinical samples with parasitemias of 135 parasites/ μ L, 45 parasites/ μ L, and 15 parasites/ μ L. The blood spots were dried for 24 hours at room temperature and then placed in plastic bags with a desiccant. QIAmp DNA Blood Mini Kits were used to extract gDNA from the entire 50 μ L blood spot. Extracted DNA was used to evaluate the assay sensitivity in the presence of human DNA under the real-time PCR conditions noted above.

Use of Limiting Dilution Assay to assess pfmdr1 copy number in clinical samples

QIAmp DNA Blood Mini Kits⁸ were used to extract genomic DNA from dried blood spots of patients in Chumkiri, Cambodia (164), where malaria multidrug resistance is currently an issue. Five random patients with recurrent parasitemia following antimalarial treatment were selected for analysis. Blood spots from these patients were prepared from blood collected at two times during the study: (1) enrollment (*i.e.* before antimalarial treatment) and (2) at parasitemia recurrence following treatment. Real-time

⁸ Qiagen, Inc.: Valencia, CA

PCR of preliminary dilution tests was performed to determine the appropriate dilution to achieve approximately one amplifiable chromosome 5 per well. Once the dilution factor was identified, real-time PCR was performed on multiple replicates of each sample at the selected gDNA dilution in a 384-well PCR plate, as described above. The $\Delta\Delta C_t$ method (167) was used to evaluate *pfmdr1* gene copy number relative to the single-copy *RNA helicase-1* gene. Below is the formula for determining gene copy number from C_t values:

$$\text{Copy Number} = \frac{2^{C_t_{RNA\ helicase-1}}}{2^{C_t_{pfmdr1}}}$$

RESULTS

Validation of RNA helicase-1 as the control gene for the limiting dilution assay

The objective of these experiments was to evaluate *RNA helicase-1* as the control gene for our assay. Traditionally, *P. falciparum* lactate dehydrogenase or β -tubulin genes have been used for copy number analysis. Because the objective of this assay is to serially dilute the gDNA to obtain one amplifiable chromosome per well, we used *RNA helicase-1* because it is located on the same chromosome as *pfmdr1*. Duplex PCR reactions were used so that each well was evaluated for amplification of the gene of interest (*pfmdr1*) and the reference gene (*RNA helicase-1*). Our results show that this assay can reproducibly detect difference in *pfmdr1* copy number. At each serial dilution tested, we detected approximately four *pfmdr1* copies in Dd2 parasites and one *pfmdr1* copy in 3D7 parasites (data not shown). These results validate the use of single-copy *RNA helicase-1* as the reference gene of copy number analysis.

In order to determine the sensitivity of this new assay for DNA extracted from clinical samples, we determined *pfmdr1* copy number from mock blood spots at known

parasitemia levels. Dried blood spots made from cultured 3D7 and Dd2 parasites mixed with whole human blood were used to mimic the clinical samples. Parasite strains in blood spots were as follows: 3D7 only, Dd2 only, and 3D7 + Dd2 (50:50, 3D7:Dd2). These were used to assess the ability of the assay to detect copy number changes in the presence of human DNA. Differences in *pfmdr1* copies were reproducibly detected at blood spots from the lowest parasitemia tested (15 parasites/ μ L) (Figure 3.2).

Comparison of pfmdr1 copy number profiles of initial and recurrent infections

The objective of these experiments was to evaluate *pfmdr1* copy number in parasite subpopulations within an individual patient. Our limiting dilution assay was applied to genomic DNA extracted from dried blood spots from western Cambodia. Table 3.1 compares the median *pfmdr1* copy numbers for each patient at enrollment and recurrence determined by our limiting dilution real-time PCR assay with the previously published results for these patients (130). Figure 3.3 depicts the percentage of wells with different *pfmdr1* copy numbers for each patient before (gray bars) and after (black bars) AM treatment. For patients shown in Figure 3.3A and 3.3B, the recurrent samples were composed of parasites with higher numbers of *pfmdr1* copies compared with their enrollment sample. Patients shown in Figure 3.3C and 3.3D had similar *pfmdr1* copy numbers at enrollment and recurrence. Genotyping of the parasite genes *msh1*, *msh2*, and *glurp* (130) revealed that the recurrent parasitemias from these four patients (A-D) were recrudescences, or treatment failures. The patient shown in Figure 3.3E had the opposite result, with higher *pfmdr1* copy numbers at enrollment compared with recurrence. Genotyping of this patient showed that recurrent parasitemia was the result of a

reinfection (130). The *pfmdr1* copy number trends for these patients are consistent with the previous report of the average *pfmdr1* copy number trend for this patient at enrollment and recurrence (130).

DISCUSSION

Clinical failures of mefloquine monotherapy and of AM combination therapy have been linked to elevated copy number of the *P. falciparum multidrug resistance (pfmdr1)* gene in multiple studies. However, not everyone with high copy numbers fail and not all failures are associated with high copy numbers (27, 31-33, 114, 130, 164). Conventional copy number assays provide an average number of gene copies for the entire parasite population within an individual patient. It is possible that patients might have a low average copy number but recrudesce because of a small subpopulation of high copy number parasites. Thus, we attempted to develop a method of identifying minority variants with high *pfmdr1* copy numbers.

We first developed a new *pfmdr1* copy number assay. Since serial dilutions of DNA, not parasites, were being made, we needed a reference gene that was located on the same chromosome as *pfmdr1*. *RNA helicase-1* is also located on chromosome 5 but is outside of the size polymorphism responsible for *pfmdr1* copy number increases (PlasmoDB). Copy numbers were assessed using the Pfaffl $\Delta\Delta C_t$ method (167), which compares crossover threshold (C_t) values of the target gene (*pfmdr1*) and the reference gene (*RNA helicase-1*). In mock clinical samples, this assay was able to reproducibly detect the correct relationship between *pfmdr1* copy number and single-copy *RNA helicase-1* (Figure 3.2). For patient samples (Table 3.1), our median *pfmdr1* copy number

determinations were very similar to those published previously using conventional methods (130).

Blood spots from five patients were used to assess the efficacy of this assay on clinical samples from a region where drug resistant malaria is present. Four of the patients (A-D) were classified as recrudescence (treatment failures) based on *msp1*, *msp2*, and *glurp* genotyping (130). Of these four paired isolates, two showed higher *pfmdr1* copy numbers from recurrent gDNA compared with enrollment (day 0) gDNA (Figure 3.3A-3.3B) and two showed similar copy numbers at both enrollment and recurrence (Figure 3.3C-3.3D). These data suggest that elevated *pfmdr1* copy number is sometimes selected by drug treatment but is sometimes not. Thus, treatment failure could be due to other parasite mutations or to host factors (178-179). One paired isolate (Patient E) was from a patient who was cured and reinfected (130). The *pfmdr1* copy numbers were lower at follow-up (day 21). Particularly interesting for patient E was the presence of recurrent parasites with single copies of *pfmdr1* that were not found at enrollment. This indicates that drug-sensitive strains were introduced after this patient completed treatment (*i.e.* reinfection).

Our limiting dilution copy number assay provides new information about the percentage of parasites with multiple *pfmdr1* gene copies that are present in an individual patient and how that frequency changes after antimalarial treatment. Although this assay is labor intensive, clinical blood spots can be easily collected and stored in desiccant at 4°C to be analyzed at a later date. Further analysis of clinical isolates from multidrug resistant malaria regions using this assay would be worthwhile to gain insight into the

minimum frequency of parasites with elevated *pfmdr1* copy numbers required to cause recrudescence.

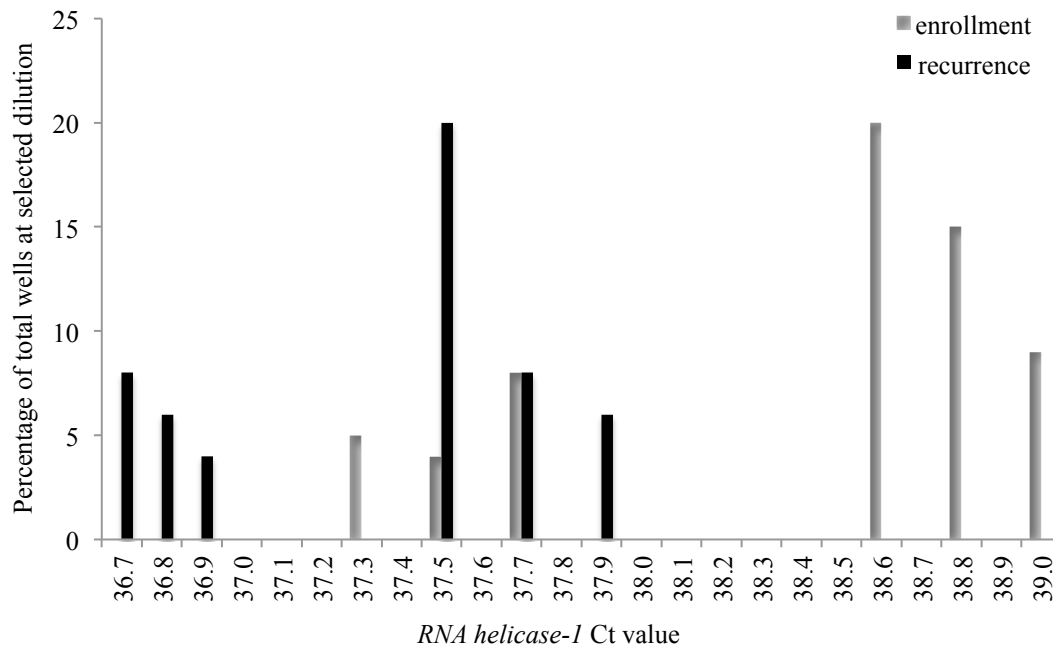


Figure 3.1: Distribution of *RNA helicase-1* Ct values used in *pfmdr1* copy number analysis by the limiting dilution real-time PCR assay. These data show the distribution (percentage of total PCR-positive wells) of *RNA helicase-1* Ct values for patient A at enrollment (gray bars) and recurrence (black bars). As described in the methods section, the *RNA helicase-1* Ct value was used to determine the number of amplifiable copies of chromosome 5 that were present in a well. Assuming the Poisson distribution, the ideal distribution would show ~33% of wells with an *RNA helicase-1* Ct value of 38 (one copy of chromosome 5) and ~18% of wells with an *RNA helicase-1* Ct value of 37 (two copies of chromosome 5). For the enrollment sample depicted above, *RNA helicase-1* Ct values of 38.6-39.0 contain one chromosome 5 copy and Ct values of 37.3-37.7 contain two chromosome 5 copies. For the recurrence sample, Ct values of 37.5-37.9 contain one chromosome 5 copy and Ct values of 36.7-36.9 contain two chromosome 5 copies. The differences in Ct values for the wells containing a single copy of chromosome 5 between the two samples is likely due to normal inter-plate run differences in real-time PCR.

Pfmdr1 Copy Number

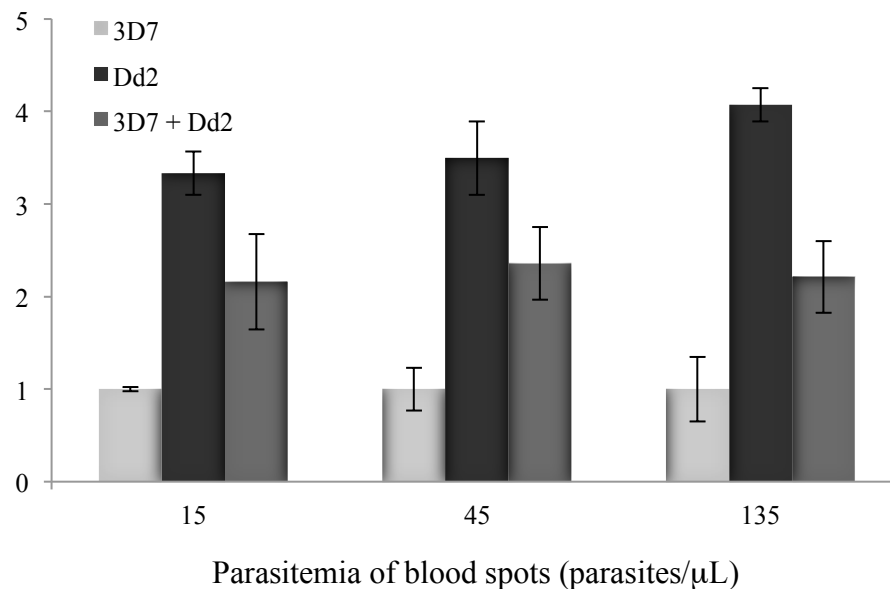


Figure 3.2: *Pfmdr1* copy number relative to single-copy *RNA helicase-1* assessed from mock blood spots. Cultured 3D7 and Dd2 parasites were added to whole human blood at serial 1:3 dilutions and spotted onto filter paper to mimic clinical samples. Equal numbers of 3D7 and Dd2 parasites were added to whole blood for the 3D7+Dd2 blood spots. Genomic DNA was isolated from each blood spot and used in the real-time PCR assay to confirm the validity of the assay on parasite DNA in the presence of human DNA. As anticipated, the fold difference in *pfmdr1* relative to single-copy *RNA helicase-1* for the combined (3D7+Dd2) blood spots reproducibly amplified halfway between those for each strain alone.

Table 3.1: Comparison of *pfmdr1* copy numbers (CN) for clinical samples at enrollment and at recurrent parasitemias between our limiting dilution assay and the published results for these patients (130)

Patient ID	<i>pfmdr1</i> CN at enrollment ^a		<i>pfmdr1</i> CN at recurrence ^b		day of recurrent parasitemia
	published CN	limiting dilution CN ^d	published CN	limiting dilution CN ^d	
A	1.3	1 (69)	2.3	2 (62)	21
B	2.9	2.5 (69)	4.3	4 (21)	35
C	1.9	1 (53)	2.1	2 (17)	21
D	1.1	2 (36)	1.1	2 (18)	28
E ^c	3.7	5.5 (56)	3.1	4 (11)	21

^aEnrollment refers to the sample that was collected at the beginning of the study.

^bRecurrence refers to the sample that was collected at the final follow-up time point for patients that were parasitemic.

^cThe study from which these samples came was published in the following article: Rogers WO, *et al.* Failure of artesunate-mefloquine combination therapy for uncomplicated *Plasmodium falciparum* malaria in southern Cambodia. Malaria Journal. 2009; 8:10.

^dLimiting dilution copy numbers were not normally distributed. Therefore, the median value is listed here. The number of wells used to calculate each median is given in parenthesis.

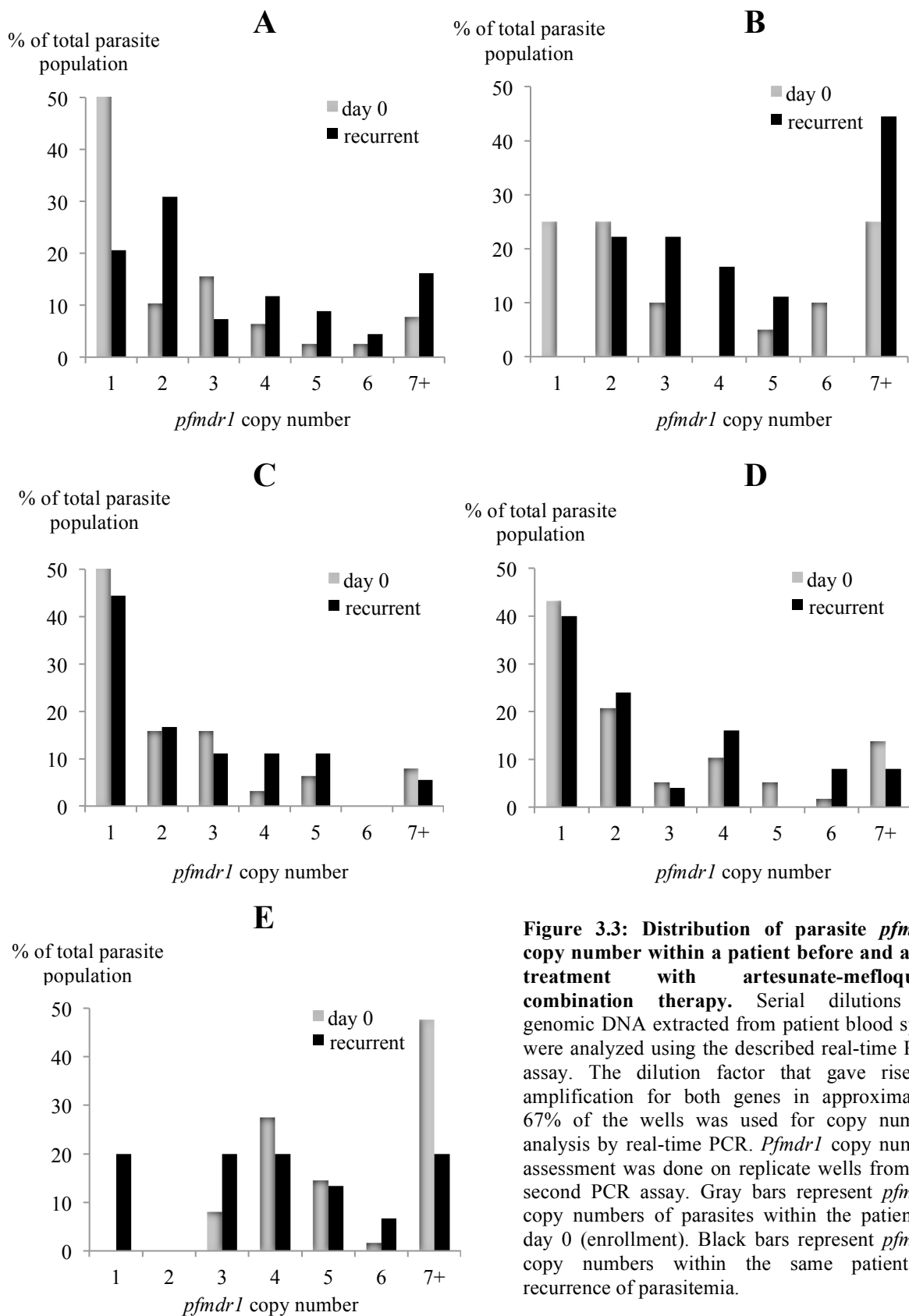


Figure 3.3: Distribution of parasite *pfmdr1* copy number within a patient before and after treatment with artesunate-mefloquine combination therapy. Serial dilutions of genomic DNA extracted from patient blood spots were analyzed using the described real-time PCR assay. The dilution factor that gave rise to amplification for both genes in approximately 67% of the wells was used for copy number analysis by real-time PCR. *Pfmdr1* copy number assessment was done on replicate wells from the second PCR assay. Gray bars represent *pfmdr1* copy numbers of parasites within the patient at day 0 (enrollment). Black bars represent *pfmdr1* copy numbers within the same patient at recurrence of parasitemia.

CHAPTER IV

Quinine Localizes to a Non-Acidic Compartment within the Food Vacuole of the Malaria Parasite *Plasmodium falciparum*

INTRODUCTION

Quinine (QN) was isolated from the bark of the Peruvian cinchona tree in the mid-1800s (19) and quickly became the treatment of choice for intermittent fever worldwide (24). Although efficacious, QN use as an antimalarial requires a relatively long treatment regimen and has substantial side effects. As a result, artemisinin-based combination therapies (ACT) have now been implemented as first-line treatment regimens due to drug efficacy and better patient tolerance. Because ACTs are prohibitively expensive or unavailable in regions where resources are limited, QN remains an important therapy to treat uncomplicated and severe malaria infections (4, 46, 168). Despite the longevity of its use as an antimalarial, the mechanism of action of quinine has not yet been fully resolved.

Early investigations into the antimalarial activity of QN demonstrated the ability to inhibit chloroquine-induced clumping of hemozoin in the parasite food vacuole (169). Subsequent evidence found that, like chloroquine, QN interferes with heme polymerization (170, 171), indicating that quinine acts in the food vacuole. In recent years, parasite resistance to QN has been reported in Africa and Southeast Asia, prompting investigations into resistance mechanisms with a focus on the food vacuole

(26, 29, 32, 168, 172, 173). Decreased QN sensitivity has been strongly linked to a protein on the food vacuolar membrane, encoded by the *Plasmodium falciparum* multidrug resistance (*pfmdr1*) gene (26, 32, 39, 110, 173, 174). Whereas some studies have found that *pfmdr1* mutations exert a significant effect on *in vitro* quinine sensitivity (26, 29, 110), other studies have found no association between *pfmdr1* mutations and quinine sensitivity (27, 28, 175). Elevated *pfmdr1* gene copy number, however, has been linked to reduced parasite susceptibility to quinine in both *in vitro* and clinical studies (27, 28, 30, 32, 174). Thus, *pfmdr1* has an effect on QN activity.

Quinine is an aromatic alkaloid compound that naturally fluoresces under ultraviolet (UV) light. Because of its relatively constant fluorescence quantum yield, quinine is commonly used in photochemistry as a fluorescence standard (176). In this study, we exploited the fluorescent properties of quinine to evaluate subcellular localization of the drug by fluorescence microscopy in parasites containing different *pfmdr1* copy numbers to determine if copy number of the gene affects drug localization.

MATERIALS AND METHODS

Parasite cultivation

We used *P. falciparum* cultures of two clonal parasite strains (3D7 and Dd2) with different *pfmdr1* copy numbers and quinine (QN) sensitivity. The 3D7 strain has one *pfmdr1* copy and is sensitive to QN. The Dd2 strain has four *pfmdr1* copies and is resistant to QN. Cultures were maintained at 37°C under the Trager-Jenson method for

malaria parasite culturing (134). Type O+ serum at 10% and red blood cells⁹ at 2% hematocrit were used in culturing and experimental conditions.

Imaging of parasites using fluorescence microscopy

Live asynchronous cultures were incubated with 1 μ M of QN and imaged over an 11-hour period using fluorescence microscopy. Time-lapse imaging of multiple parasites was carried out with a Prior Scientific¹⁰ motorized x-y stage. An incubator box maintained the microscope work area including the objective at 37°C. LysoTracker DND Red¹¹ (50nM) was used to stain the food vacuole and DRAQ5¹² (1 μ M) was used to stain DNA. Fluorescence images were acquired using an Olympus FV1000 inverted IX81 microscope with a 63x 1.42 NA oil immersion objective at the UNC-Chapel Hill Michael Hooker Microscopy Core Facility. Serial sections with step sizes of 0.20 or 0.25 μ m were gathered using sequential scanning at 405nm (quinine), 559nm (LysoTracker Red) & 635nm (DRAQ5) excitation, and 435/50nm, 595/50nm & 705/100nm emission respectively. Confocal pinhole was set to maximum (800 μ m, ~8 Airy units). Laser transmitted light differential interference contrast (DIC) images were collected at an optimum z-level separately. Image stacks were deconvolved using the iterative blind deconvolution method with Volocity Image Analysis software, version 5.52¹³. Occasionally, Tetraspec 0.1 μ m beads (Molecular Probes, OR) were added to the sample and used for deconvolution with measured point spread functions. 3-D opacity volume rendering and 3-D colocalization analysis was carried out with Volocity.

⁹ Research Blood Components, LLC: Boston, MA

¹⁰ Prior Scientific: Rockland, MA

¹¹ Invitrogen: Carlsbad, CA

¹² Biostatus Limited: Leicestershire, United Kingdom

¹³ Perkin Elmer: Waltham, MA

RESULTS

Quinine localizes to the parasite food vacuole

Two-dimensional (2-D) fluorescence and DIC images of both 3D7 (one *pfmdr1* copy) and Dd2 (four *pfmdr1* copies) parasites showed that quinine (QN) colocalizes with hemozoin, which suggests localization in the parasite food vacuole (Figure 4.1). The colocalization was highly repeatable and seen in four fields in at least ten experiments. Parasites were also imaged at earlier time points following the addition of QN (beginning at one hour of exposure through 11 hours). QN fluorescence was found in the food vacuole as early as one hour of exposure and remained there for the duration of the experiment (Figure 4.2). This effect appears to be independent of *pfmdr1* copy number as experiments in both strains rendered similar results. The same pattern was seen regardless of whether the infected red cells were maintained on the heated microscope stage or in a candle jar, indicating that photobleaching was not a factor.

To further characterize subcellular localization of QN, we determined whether it colocalized with either LysoTracker Red, which localizes to acidic compartments, or DRAQ5, which localizes to the nucleus, using three-dimensional (3-D) reconstruction of z-stack fluorescence images. As expected, no colocalization with DRAQ5 was seen. However, QN was found to localize in a separate but adjacent compartment from that in which LysoTracker Red was localized (Figure 4.3). This 3-D imaging method does not allow for hemozoin to be visualized. The pattern of colocalization was the same for 3D7 and Dd2 parasites, despite their differences in *pfmdr1* copy number. These observations suggest that QN localizes to a distinct, non-acidic compartment within the parasite food

vacuole, possibly overlapping with hemozoin in parasites with both low and high *pfmdr1* copy number.

DISCUSSION

Although quinine was the first therapeutic compound used to treat malaria infection (19), its mechanism of action has never been fully resolved (169-171). Some evidence suggests that parasite resistance to quinine is associated with mutations and/or elevated copy number of the *pfmdr1* gene, which encodes for a transporter protein found in the membrane of the parasite food vacuole (26, 27, 28, 29, 30, 32, 110, 172, 174). Here, we exploit the natural fluorescent properties of quinine to obtain insight into the mechanism of action of the drug. Although knowledge of quinine's fluorescent properties has been around since the late-1800s (177), this is the first study to employ the quinine's fluorescence for imaging in the malaria parasite.

We employed fluorescence microscopy to image quinine subcellular localization in two *P. falciparum* strains that contained different *pfmdr1* copy numbers. Quinine consistently overlapped with the hemozoin crystals in both strains when evaluated by two-dimensional microscopy (Figure 4.1). However, upon 3-D reconstruction of serial z-stack images, we found the quinine resides in a distinct compartment, which is contiguous to, but separate from, the compartment stained by LysoTracker Red. The lack of colocalization with the acidotropic dye suggests that quinine resides in a non-acidic compartment within the food vacuole, possibly the same one occupied by hemozoin. This would be consistent with previous reports that it interacts with hemozoin crystals directly or with enzymes involved in the hemozoin crystallization process, as previously reported

(170, 171). In summary, our findings suggest that QN is localized in the food vacuole but outside of the acidic compartment.

This study underscores the importance of utilizing the 3-D reconstruction software in imaging studies, since the localization of quinine into this novel compartment would not have been detected otherwise. Although there was no apparent difference in localization in strains containing different *pfmdr1* copy numbers, we cannot rule out the possibility that the *pfmdr1* gene has a role. Single nucleotide polymorphisms in *pfmdr1* have also been associated with decreased sensitivity to quinine (26, 29, 110). Because the protein encoded by *pfmdr1* is a membrane transporter that pumps solutes into the food vacuole, it is possible that mutations within the *pfmdr1* gene could affect the transporter function of the protein.

In summary, this study is novel because it is the first to exploit QN fluorescence to study the intracellular distribution of the drug. Here, we demonstrate that QN enters a distinct, non-acidic compartment inside the parasite food vacuole. These results are important because they provide visual support for the hypothesis that QN interferes with hemozoin production, which could guide future studies to investigate a possible interaction between QN and enzymes involved in the hemozoin formation process.

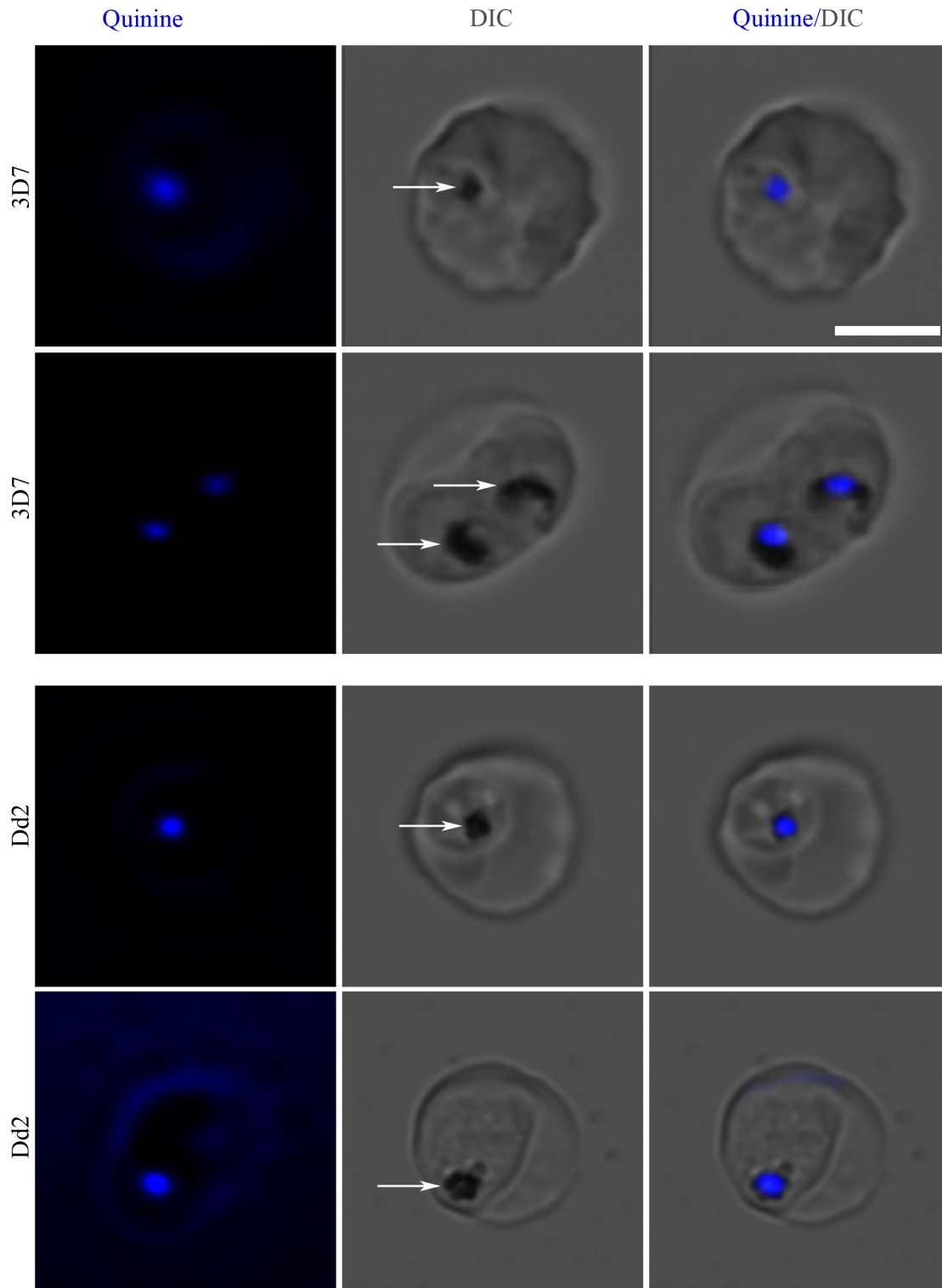


Figure 4.1: Quinine localizes with hemozoin in the parasite food vacuole. Trophozoites of *P. falciparum* strains 3D7 (one *pfmdr1* copy) and Dd2 (four copies) were incubated with QN to determine its subcellular localization. The scale bar represents 5 μ m. White arrows point to hemozoin crystals in the food vacuole. Quinine overlapped with hemozoin in both strains and thus localized to the food vacuole.

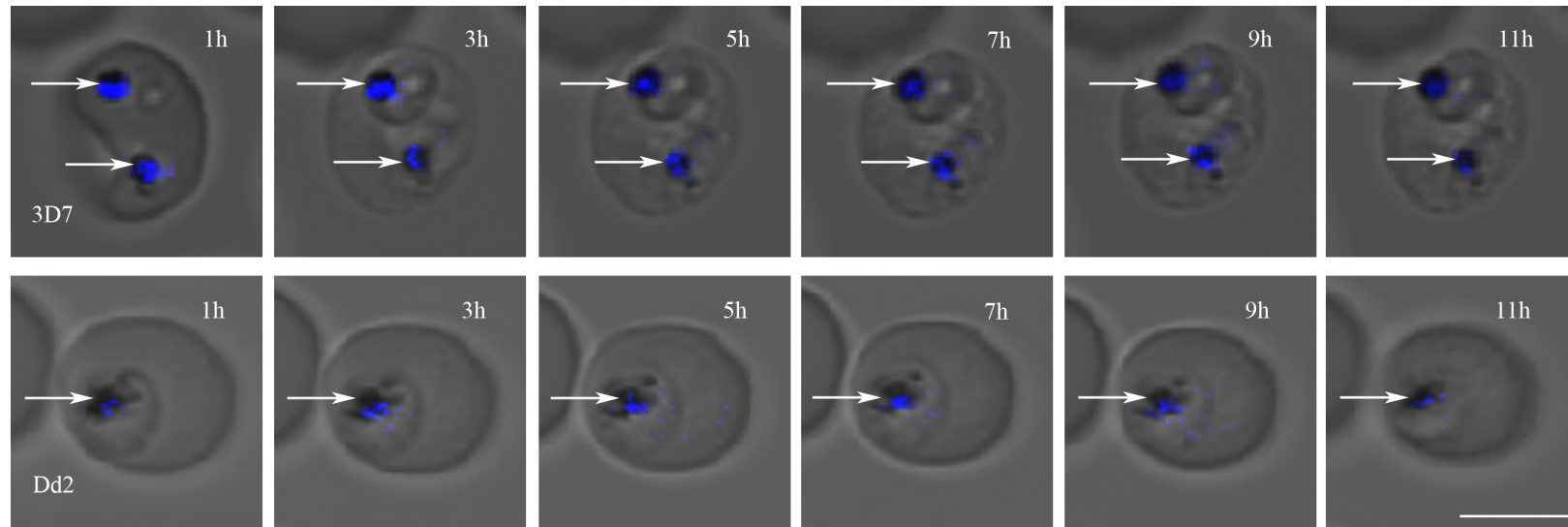


Figure 4.2: Quinine localization over time in *Plasmodium falciparum*. Trophozoites of *P. falciparum* strains were incubated with quinine (blue) to determine subcellular localization of the drug over an 11-hour period. White arrows point to hemozoin crystals in the food vacuole. Quinine overlapped with hemozoin crystals at all time points assessed. The scale bar represents 5µm.

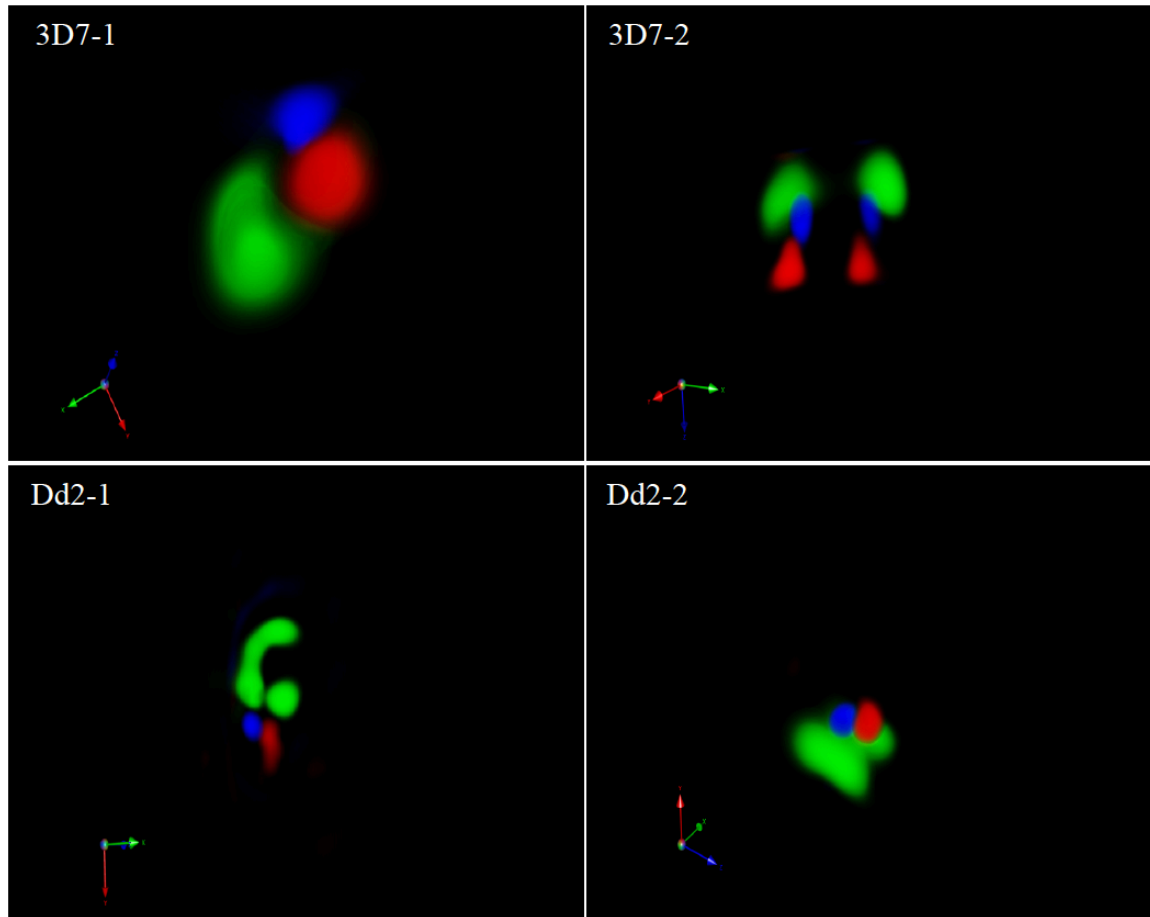


Figure 4.3: 3-D reconstruction of *Plasmodium falciparum* parasites. Z-stack images of each parasite shown in Figure 4.2 were used for 3-D volume rendering and colocalization analysis. No quinine colocalization with LysoTracker Red was observed in either the 3D7 (left) or the Dd2 (right) strain. Because LysoTracker Red is highly specific for acidic organelles, quinine appears to be in a separate and non-acidic compartment of the food vacuole.

CHAPTER V

Dissertation Summary and Future Directions

SUMMARY

The focus of this dissertation was to evaluate the molecular aspects and clinical relevance of the *pfmdr1* gene in the resistance to antimalarial drugs. This is important because numerous *in vitro* and clinical studies have linked elevated *pfmdr1* copy number to decreased sensitivity several different antimalarial compounds. Future studies on ways to circumvent *pfmdr1*-mediated resistance would benefit from a better understanding the interplay between *pfmdr1* and drug treatment.

First, we explored the effect of MFQ treatment on *pfmdr1* gene expression in parasite strains containing different *pfmdr1* copy numbers: 3D7 (1 *pfmdr1* copy), FCB (2 copies), and Dd2 (4 copies). Parasites with multiple *pfmdr1* gene copies exhibited higher relative transcript levels than single-copy parasites, and MFQ induced *pfmdr1* expression above untreated levels in all three strains evaluated. Concomitant morphology analyses of the sampled cultures revealed an interesting phenomenon. Our data demonstrated that the MFQ treatment of synchronized ring-stage parasites induced a delay in parasite maturation through the intraerythrocytic cell cycle. This delay was specific to MFQ treatment and was observed in both MFQ-sensitive (3D7, FCB) and MFQ-resistant (Dd2) *P. falciparum* strains. Together, our expression and morphology data showed that peak

pfmdr1 expression levels were observed when the cultures had the highest percentage of ring-stage parasites, revealing stage-specific *pfmdr1* expression. The benefit of prolonging the ring stage for multi-copy parasites could be to allow time for the increased expression of ring stage genes like *pfmdr1*. More *pfmdr1*-encoded transporters may displace sufficient amounts of MFQ to allow the parasite to survive treatment in strains with multiple *pfmdr1* copies.

Next, we developed a limiting dilution assay that evaluated *pfmdr1* copy number in subpopulations of parasites within patients that failed artesunate-mefloquine (AM) treatment. The purpose of this study was to evaluate the distribution of subpopulations with elevated *pfmdr1* copy numbers before AM treatment and in recurrent infections following AM failure. The output of conventional copy number assays is an average copy number for the entire parasite population. Our assay is the first to assess copy number on subpopulations. We found that parasites with several *pfmdr1* copies, even if present in smaller relative quantities, have a greater influence over the patient's response to treatment than the more abundant single-copy parasites. Moreover, our limiting dilution assay provides new information about the frequency of parasites with elevated *pfmdr1* gene copies that are present in an individual patient and how that frequency changes after antimalarial treatment. The ability to detect minority variants with increases in *pfmdr1* copy number might be able to better predict *in vivo* antimalarial treatment failure. This assay can be utilized on samples from multidrug resistant regions to determine the minimum frequency of parasites containing multiple *pfmdr1* copies within a patient that is required to cause clinical treatment failure.

Finally, we imaged malaria parasites with fluorescence microscopy to determine the subcellular localization of the oldest antimalarial compound quinine (QN). The goal of this study was to utilize the naturally fluorescent properties of QN to determine its subcellular localization in malaria parasites with different *pfmdr1* copy numbers. Decreased QN sensitivity has been linked in clinical studies and *in vitro* to elevated *pfmdr1* copy number. Our results showed that QN consistently overlapped with hemozoin crystals in the parasite food vacuole. We observed apparent colocalization of QN and LysoTracker Red in the two-dimensional (2-D) images of multi-copy Dd2 parasites but not single-copy 3D7 parasites. This suggested that the extra *pfmdr1* gene copies in Dd2 result in an increased pumping of QN into the food vacuole by the *pfmdr1*-encoded transporter. However, upon three-dimensional (3-D) reconstruction of serial z-stack images, we found the QN exhibits no colocalization with LysoTracker Red in either 3D7 or Dd2 parasites and thus resides in a distinct, non-acidic compartment within the food vacuole. These results demonstrate the importance of utilizing 3-D rendering software and illustrate the possibility of drawing inaccurate or incomplete conclusions when relying solely on 2-D imaging. These findings are novel because they are the first visual evidence of QN subcellular localization within the malaria parasite and serve as the foundation for future studies of QN activity within the parasite food vacuole.

Taken together, the findings of this dissertation accomplished our objective to more fully evaluate the role of the *pfmdr1* gene in drug resistance in the malaria parasite. First, we identified that *pfmdr1* is primarily transcribed during the ring stage of the parasite intraerythrocytic cycle. Even though MFQ induced a maturation delay at the ring stage in all strains, this ring stage-dependent *pfmdr1* expression would explain how

parasites with multiple *pfmdr1* gene copies evade destruction by producing more *pfmdr1*-encoded transporters than single-copy parasites. Second, our limiting dilution assay revealed that some patients that fail antimalarial treatment are infected with parasite subpopulations with multiple *pfmdr1* copy numbers at a higher frequency than those that do not fail. We found that there is a greater percentage of subpopulations containing more than five *pfmdr1* copies than previously believed, based on conventional copy number assays that measure the average gene copies for the entire population. And third, we show the first visual data for subcellular localization of QN within the malaria parasite. This is important because it reveals a novel putative site of QN activity within the food vacuole. Exploiting the natural fluorescence in QN by fluorescence microscopy is the foundation for future studies that address differences in subcellular localization between QN-sensitive and QN-resistant parasites. Because of the vast amount of data that links *pfmdr1* to antimalarial resistance, understanding the expression of this gene and the degree of its influence over parasite response to drug exposure can aid in the development of therapies that circumvent such broad antimalarial resistance.

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