Role of *pfmdr1* Amplification and Expression in Induction of Resistance to Artemisinin Derivatives in *Plasmodium falciparum*[†]

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Artemisinin and its derivatives are the most rapidly acting and efficacious antimalarial drugs currently available. Although resistance to these drugs has not been documented, there is growing concern about the potential for resistance to develop. In this paper we report the selection of parasite resistance to artemisinin (AL) and artemisinin (QHS) *in vitro* and the molecular changes that occurred during the selection. Exposure of three *Plasmodium falciparum* lines (W2, D6, and TM91C235) to AL resulted in decreases in parasite susceptibilities to AL and QHS, as well as to mefloquine, quinine, halofantrine, and lumefantrine. The changes in parasite susceptibility were accompanied by increases in the copy number, mRNA expression, and protein expression of the *pfmdr1* gene in the resistant progenies of W2 and TM91C235 parasites but not in those of D6 parasites. No changes were detected in the coding sequences of the *pfmdr1*, *pfcr1*, *pfatp6*, *pfctcp*, and *pfubcth* genes or in the expression levels of *pfatp6* and *pfctcp*. Our data demonstrate that *P. falciparum* lines have the capacity to develop resistance to artemisinin derivatives *in vitro* and that this resistance is achieved by multiple mechanisms, to include amplification and increased expression of *pfmdr1*, a mechanism that also confers resistance to mefloquine. This observation is of practical importance, because artemisinin drugs are often used in combination with mefloquine for the treatment of malaria.

Plasmodium falciparum parasites have developed resistance to conventional antimalarial drugs by various means, including alteration of the enzymes targeted by drugs (8, 23, 24, 32) and mutation or amplification of the genes coding for proteins involved in drug transport (13, 34, 35). One of these proteins, *P. falciparum* multidrug resistance transporter 1 (PfMDR1), or Pgh1, a *P. falciparum* homologue of mammalian P-glycoprotein (15, 45), has been implicated in resistance to several structurally different antimalarial compounds. In early studies, exposure of *P. falciparum* laboratory lines to mefloquine resulted in amplification of the *pfmdr1* gene (45), with concomitant increases in resistance to mefloquine (MQ), quinine (QN), and halofantrine (HF) (7, 30, 31, 45). Amplification of *pfmdr1* was also observed in field isolates from different geographical locations (4, 34, 35, 42). Increased *pfmdr1* copy numbers (CN) in field isolates were associated with higher inhibitory concentrations (IC) of MQ, QN, HF, and artemisinin (QHS) *in vitro* (34, 46) and were linked to the failure of MQ monotherapy and mefloquine-artesunate combination therapy in studies conducted in Thailand and on the Thai-Cambodian border (2, 35). Furthermore, direct evidence of the role of *pfmdr1* in the modulation of parasite susceptibility came from a report where inactivation of 1 of 2 copies of *pfmdr1* in the *P. falciparum* FCB

line led to moderate increases in susceptibilities to artemisinin and arylaminoalcohol drugs (39).

In addition to gene amplification, several polymorphic positions in the *pfmdr1* gene (N86Y, Y184F, S1034C, N1042D, and D1246Y) have been identified in field isolates (14) and have been shown to contribute to altered parasite responses to QN, HF, MQ, chloroquine (CQ), and QHS *in vitro* (10, 28, 33, 36, 39). In particular, the last three mutations (S1034C, N1042D, and D1246Y) are implicated in increased sensitivity to artemisinin over that of the “wild type” (with S, N, and D) (36). Conversely, significant decreases in susceptibilities to QHS, MQ, and HF are observed when the “wild-type” N at position 1042 is restored (39). These findings are consistent with the early observation that the “wild-type” *pfmdr1* allele (with N, Y, S, N, and D) is associated with reduced susceptibilities of the progeny of the genetic cross of the *P. falciparum* 3D7 and HB3 lines to MQ, HF, QHS, and artemether (10).

Although PfMDR1 is clearly implicated in the modulation of parasite responses to antimalarial drugs, including artemisinins, the mechanism of its action is largely unknown. A recent study using heterologous expression of PfMDR1 in *Xenopus laevis* oocytes demonstrated that some drugs, including HF, QN, and CQ, are substrates for PfMDR1 (38). It is not clear whether artemisinins interact with PfMDR1. Several proteins have been shown to interact with artemisinin. The translationally controlled tumor protein (TCTP) binds to radioactively labeled dihydroartemisinin (5) and is overexpressed in rodent *Plasmodium yoelii* parasite lines with decreased susceptibility to artemisinin (44). Another protein that may interact with artemisinins is the sarcoplasmic reticulum Ca²⁺ ATPase 6

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(PfATP6); this enzyme, when expressed in *Xenopus* oocytes, was specifically inhibited by artemisinin derivatives containing an endoperoxide bridge (11). In addition, the activity of the enzyme was greatly influenced by the introduction of several mutations (e.g., L263E) (43). Furthermore, analysis of naturally occurring polymorphisms in PfATP6 in field isolates from French Guiana suggested that a polymorphism at codon 769 may be associated with reduced susceptibility of these isolates to artemether *in vitro* (19). However, subsequent reports failed to detect codon 263 or 769 polymorphisms in the field (12, 27, 48).

Although resistance to artemisinins has not been documented in the field, induction of artemisinin resistance *in vitro* may help in the identification of molecular markers and drug target sites as well as in designing strategies for combating artemisinin resistance when it arises.

Several attempts have been made to develop resistance to artemisinin derivatives in *P. falciparum* (18, 20, 47) *in vitro*. Inselburg (18) induced resistance to artemisinin by using mutagens, but these lines are no longer available for study. Other attempts to select resistance with increasing drug pressure have led to various endpoints. Jiang (20) produced a 3-fold decrease in susceptibility to sodium artesunate (AS), but resistance proved unstable. Yang et al. (47) achieved 8.9-fold resistance to AS, although few data are available about the stability of the resistance selected or the methods used. A recent study reported the development of stable resistance to QHS and AS in the rodent parasite *Plasmodium chabaudi chabaudi* (1). Linkage group analysis of the resistant progeny from the same parental parasites identified two nonsynonymous mutations occurring independently in the *P. chabaudi* putative ubiquitin carboxyl-terminal hydrolase gene (*pcubp1*, or *pcubcth*): V739F appeared after selection with artesunate, whereas V770F occurred in progeny selected with CQ (17). No new mutations in PcUBP1 were detected after further selection with QHS. Attempts to develop stable resistance in the *P. falciparum* NF54 and 7G8 parasite lines were unsuccessful; parasites reverted to the sensitive phenotype after cryopreservation (17).

Here we report the selection of resistance to artelinic acid (AL) and to QHS and its derivatives *in vitro* in several *P. falciparum* lines of different genetic backgrounds. We also investigated the possible mechanisms involved in the development of resistance. We present evidence that *pfmdr1* gene amplification and expression are required in order for some, but not all, parasites to withstand high concentrations of AL or QHS *in vitro*.

MATERIALS AND METHODS

Parasite cultures. Two *P. falciparum* laboratory clones, W2 (Indochina) and D6 (Sierra Leone), and a laboratory-adapted field isolate, TM91C235 (Thailand), were grown *in vitro* as previously described (41). Routine cultures were maintained at 1 to 10% parasitemia in complete medium containing 10% heat-inactivated human plasma and in O⁺ human erythrocytes. The three different parasites were chosen in anticipation that differences in the genetic background of the parasite may be important for the success of drug resistance selection.

Development of resistant parasite lines. Resistance to AL in *P. falciparum* lines was induced essentially as described by Oduola et al. (29). The *P. falciparum* W2, D6, and TM91C235 lines were cultured under increasing AL drug pressure in stepwise increments over 28 months. The starting concentrations of AL were 0.5 ng/ml for the D6 and W2 clones and 10 ng/ml for TM91C235. The increments used were 0.5, 1, 2, 4, 10, 20, 30, 40, 50, and 80 ng/ml, with an exposure time of

11 to 93 days at each increment. A routine drug exposure cycle began with the addition of AL to asynchronous cultures at 4 to 8% parasitemia. The medium was changed daily. Drug pressure was removed when parasite morphology degraded and growth was significantly affected. AL pressure was resumed when parasite growth and morphology became normal. A parasite was considered "adapted" when the recovery phase occurred within 2 to 4 days. Several times during adaptation, parasites were frozen, and the process was resumed from cryopreserved stocks. AL was used for the drug resistance selection studies because, at the time these studies began, AL was a preclinical drug candidate in the WRAR program.

When W2 parasites became adapted to growth at 80 ng/ml of AL, these parasites, designated W2AL80, were further subjected to QHS pressure, first at 20 ng/ml and then at 40 ng/ml. In parallel, the bulk culture of W2AL80 parasites was cloned by limiting dilution, and one of the clones was subjected to increasing AL and QHS pressures, up to concentrations of 200 ng/ml. During the adaptation, samples of the cultures were collected and stored for further analysis.

Parasite susceptibility to the drugs was assayed by measuring the inhibition of [³H]hypoxanthine uptake as described previously by Desjardins et al. (9). Data were analyzed using GraphPad Prism software, version 5 (GraphPad Software, Inc.). The concentrations of the drugs that inhibited parasite growth by 50% and 90% (IC₅₀ and IC₉₀) were obtained by nonlinear regression analysis of the inhibition of uptake values and log-transformed concentration values.

Parasite DNA extraction. Genomic DNA was extracted from 1- to 2-ml aliquots of parasite cultures or cultures spotted onto filter paper by using the NucleoSpin tissue kit (Macherey-Nagel, Germany). All of the DNA, RNA, and protein assays were performed with untreated parasites (i.e., no drug pressure).

Sequencing of the *pfmdr1*, *pfatp6*, *pfctcp*, *pfprt*, and *pfubcth* genes. The *pfmdr1* (PFE1150w), *pfatp6* (PFA0310c), *pfctcp* (PFE0545c), *pfprt* (MAL7P1.27), and *pfubcth* (PFA0220w) genes were amplified from genomic DNA using PCR. Gene sequences were obtained from PlasmoDB (<http://plasmodb.org/>). The primers used for PCR and sequencing of the genes, as well as the PCR conditions, are shown in Table S1 in the supplemental material. It should be noted that 2 fragments of the *pfmdr1* gene and 9 overlapping fragments of *pfatp6* (3,965 bp) were amplified and sequenced, while a full-length *pfubcth* gene (3,613 bp) was amplified using *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA) and sequenced.

To sequence the gene encoding the chloroquine resistance transporter (*pfprt*), total RNA was isolated, and cDNA was synthesized using the D7 primer (see Table S1 in the supplemental material) as described in "Analysis of *pfmdr1*, *pfatp6*, and *pfctcp* transcription" below. The PCR product corresponding to the mRNA of the gene was amplified using the E1 and D7 primers (see Table S1) and sequenced. All PCR products were sequenced using the BigDye Terminator cycle sequencing kit, version 3.1 (Applied Biosystems, Inc.), and sequences were assembled and analyzed using the GAP4 program of the Staden package (40).

Determination of the *pfmdr1* gene copy number by quantitative real-time PCR. The copy number of the *pfmdr1* gene was estimated by quantitative real-time PCR in an Mx4000 multiplex quantitative PCR system (Stratagene) using a SYBR green-based assay. The single-copy-number lactate dehydrogenase 1 gene (*pfldh1*) was used as a reference (normalizer) gene for estimating the copy number of *pfmdr1* (the target). Primers MDR1-T1F and MDR1-T1R and primers LDH-T1F and LDH-T1R (see Table S1 in the supplemental material) were used to amplify fragments of the *pfmdr1* and *pfldh1* genes, respectively. PCRs were performed in triplicate in 25 µl PCR mixtures containing 1× Absolute QPCR SYBR green mix (ABgene), 100 nM ROX (passive reference dye), 1 µl of DNA template, and 400 nM each primer for either *pfmdr1* or *pfldh1* amplification. Cycling conditions were as follows: 95°C for 15 min, followed by 40 cycles of 95°C for 30 s, 57°C for 1 min, and 72°C for 30 s. Fluorescence data were collected at the end of each annealing and extension step three times and were averaged. Following the amplification cycles, melting curve analysis was performed to confirm that the correct products were synthesized. The text report, containing the threshold cycle (*C_T*) values for every well, was exported into the Excel program (Microsoft Office XP) and analyzed.

The assay was optimized to achieve approximately equal amplification efficiencies for the *pfmdr1* and *pfldh1* fragments within the experimental range of DNA concentrations from 10 pg/µl to 100 ng/µl. Thus, the $\Delta\Delta C_T$ method (3) could be used, and the *pfmdr1* copy number (CN) was calculated as $2^{\Delta\Delta C_T \pm SD}$. $\Delta\Delta C_T$ was calculated as $(C_{Tldh1} - C_{Tmdr1}) - (C_{Tldh1 cal} - C_{Tmdr1 cal})$. SD is a standard deviation, calculated as $\sqrt{(S^2_{mdr1} + S^2_{ldh1} + S^2_{cal})}$, where S_{mdr1} and S_{ldh1} are the standard deviations from the average *C_T* calculated for three or four replicates in the *pfmdr1* and *pfldh1* amplifications, and S_{cal} is an average standard deviation of the ΔC_T values for the calibrator. For every sample, the assay was repeated at least three times. The results are presented as population mean CNs and ranges with 95% confidence. Student's *t* test was applied to evaluate differences between the CN values. The difference was considered significant if the

probability that these values came from the same two underlying populations that have the same mean was <0.05 .

Real-time assays to quantify the *pfatp6* and *pfctcp* copy numbers in the parasite genomes relative to that of the *pfldh1* gene were also developed. Primer sequences and cycling conditions are shown in Table S1 in the supplemental material. The assays were carried out and analyzed as described above.

Analysis of *pfmdr1*, *pfatp6*, and *pfctcp* transcription. For the quantification of the mRNA transcription of the *pfmdr1*, *pfatp6*, and *pfctcp* genes, parasite cultures were synchronized at the ring stage by repeated D-sorbitol-treatment (25) and were allowed to grow for one cycle after the last treatment. Aliquots of parasite cultures were taken every 6 to 8 h, starting from the early ring stage, over a period of 48 h. Total RNA was extracted using NucleoSpin RNA II kits (Macherey-Nagel, Germany) with an additional DNase I (New England Biolabs) treatment at 37°C for ~3 h, followed by a second round of RNA purification using the same kit. To ensure that the total RNA was free from DNA contamination, every preparation of RNA was tested by PCR prior to the first-strand cDNA synthesis.

The first-strand cDNA synthesis was performed in a 20- μ l volume using random hexamer primers (Promega) and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Real-time PCR assays were designed to evaluate the levels of *pfmdr1*, *pfatp6*, and *pfctcp* RNA transcripts relative to those of the seryl-tRNA gene. Each 25- μ l reaction mixture (Absolute QPCR SYBR green mixture; ABgene) contained 1 μ l of cDNA as a template. For the analysis of *pfmdr1*, *pfatp6*, and *pfctcp* RNA expression, the primers were identical to those used in the copy number assays except for the seryl-tRNA (normalizer gene) primers (6). The primer sequences and PCR conditions are shown in Table S1 in the supplemental material. Data were analyzed as described above.

Southern blot hybridization. Parasite chromosomal DNA was prepared in agarose blocks (21) and was digested with the BglI and SmaI enzymes. DNA fragments were separated by pulsed-field gel electrophoresis (PFGE) in a 1% SeaKem LE agarose gel and 0.5 $\times$ Tris-borate-EDTA buffer (TBE) using a CHEF-DR III system (Bio-Rad Laboratories, Hercules, CA) at 6 V/cm, with a 25- to 50-s pulse ramp for 16.5 h for BglI-digested DNA and a 70- to 130-s pulse ramp for 22 h for SmaI digestion; then they were transferred to a nylon membrane (Roche Diagnostics Pty, Ltd., Castle Hill, Australia) by capillary blotting. Digoxigenin (DIG)-labeled *pfmdr1*- and fructose biphosphate aldolase (PF14_0425)-specific probes were generated by PCR using the MDR01-MDR02 and AldoF-AldoR primer pairs, respectively (see Table S1 in the supplemental material), and were purified using a PCR purification kit (Macherey-Nagel, Germany).

Southern blot hybridizations were performed using EasyHyb hybridization buffer (Roche Diagnostics Pty, Ltd., Castle Hill, Australia), alkaline phosphatase-conjugated anti-digoxigenin Fab fragments, CDP star substrate, and the DIG Wash and Block buffer set (Roche Diagnostics Pty, Ltd., Castle Hill, Australia) according to the manufacturer's instructions.

Band intensity was quantified by ImageQuant software (Molecular Dynamics). The ratio of the band intensity for resistant parasites (e.g., W2AL80) to that for control parasites (e.g., W2) was calculated for the hybridization with the *pfmdr1* probe and normalized to that with the aldolase probe.

Analysis of PfMDR1 protein expression by Western blotting. Synchronized parasite cultures at 5 to 7% parasitemia were harvested at the late-trophozoite-early-schizont stage, lysed in 0.1% saponin, and stored at -80°C. For Western blot analysis, parasite pellets containing approximately 10^6 parasites were resuspended in 1 $\times$ phosphate-buffered saline (PBS) supplemented with protease inhibitor cocktail (Roche Diagnostics, Australia) and were lysed by the addition of sodium dodecyl sulfate (SDS) to a final concentration of 1%, followed by incubation on ice for 10 to 15 min. Lysates were cleared by centrifugation at $13,000 \times g$ and 4°C for 10 min. Supernatants were mixed with 6 $\times$ sample loading buffer (containing 10% SDS, 100 mM dithiothreitol [DTT], Tris-HCl [pH 6.8], glycerol, and bromophenol blue) and were boiled prior to being loaded onto a 7% SDS-polyacrylamide gel electrophoresis (PAGE) gel for subsequent Western blot analysis.

For PfMDR1 detection, a 1/50 dilution of affinity-purified polyclonal sera against the C terminus of Pgh1 (α 1435; kindly provided by A. Cowman) was used. Monoclonal anti-RAP2 antibodies (Ab) (6C1; kindly provided by M. Hogarth) were used (at a 1:2,000 dilution) as an internal control. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (dilution, 1:5,000 or 1:10,000) was used to detect PfMDR1-bound Ab, and HRP-conjugated anti-mouse IgG (1:5,000) was used for RAP2 detection with an ECL detection kit (Amersham, United Kingdom). Band intensity was quantified using ImageQuant software (Molecular Dynamics).

TABLE 1. *In vitro* susceptibilities of *Plasmodium falciparum* lines to artemisinin acid

Parasite line	Mean IC (nM) $\pm$ SD ^a	
	50%	90%
W2	4.73 $\pm$ 0.19	7.45 $\pm$ 0.40
W2AL50	23.30 $\pm$ 3.95	51.31 $\pm$ 1.31
W2AL80	22.75 $\pm$ 5.37	56.80 $\pm$ 5.44
D6	9.81 $\pm$ 1.41	21.83 $\pm$ 3.04
D6AL50	34.00 $\pm$ 3.14	81.26 $\pm$ 8.67
D6AL80	23.64 $\pm$ 3.71	75.89 $\pm$ 2.60
TM91C235	11.08 $\pm$ 1.68	22.99 $\pm$ 2.60
TM91C235AL50	33.72 $\pm$ 1.64	53.51 $\pm$ 1.12
TM91C235AL80	24.55 $\pm$ 4.13	75.89 $\pm$ 6.8

^a $n \geq 3$.

RESULTS

Development of *P. falciparum* lines resistant to artemisinin acid and other artemisinin derivatives *in vitro*. Before drug exposure, the IC₉₀ values of AL for the parental parasite lines W2, D6, and TM91C235 ranged from 7.45 to 22.99 nM (Table 1). As the parasites became adapted to gradually increased concentrations of AL, their susceptibilities to AL and other antimalarial drugs changed markedly (Fig. 1). All three lines became less susceptible to AL, QHS, dihydroartemisinin (DHA), and MQ (Fig. 1) but more susceptible to CQ. Three different adaptation patterns were observed. For the W2 clone, marked reductions in sensitivities to AL and QHS were observed when parasites became adapted to 40-ng/ml AL drug pressure, and the same trend continued up to the 80-ng/ml AL level (Fig. 1A). The susceptibility of the TM91C235 line to AL (Fig. 1B) decreased and reached a plateau following exposure to 30 ng/ml, while the susceptibility of D6 decreased immediately after exposure to 30 ng/ml of AL and did not decrease further after exposure to higher drug concentrations (Fig. 1C). Interestingly, in the absence of drug pressure, the selected progeny progressed normally through the erythrocytic stages of the life cycle, with no substantial differences in growth from the parental lines.

Acquisition of resistant phenotypes is independent of polymorphism in the *pfprt*, *pfmdr1*, *pfatp6*, and *pfubth* genes. The key polymorphic regions of the *pfmdr1* gene, containing codons 86, 184, 1034, 1042, and 1246, were sequenced in the parental and resistant progenies. The amino acids at these positions are shown in Table 2. Mutations N86Y and Y184F were observed in the parental W2 and TM91C235 parasite lines, respectively, while D6 parasites had "wild-type" PfMDR1. No changes in the gene coding sequences were detected in the resistant progenies D6AL80, TM91C235AL80, W2AL200, W2QHS200, and W2AS80 (Table 2).

The entire genes coding for TCTP, PfATP6, and PfUCTH, including introns of the two latter genes, were sequenced in the parental parasites and their resistant progenies D6AL80, TM91C235AL80, W2AL200, W2QHS200, and W2AS80. In all parental and resistant lines, the sequence of the *pfctcp* gene was identical to that of isolate 3D7 (PlasmoDB). The *pfatp6* DNA sequences in the D6, W2, and TM91C235 parental lines were identical to that of isolate K1 (GenBank accession no. X71765) with the following exceptions. A nucleotide change from G to

FIG. 1. Changes in the index of resistance (ratio of the IC₅₀ for the resistant progeny to the IC₅₀ for the parental line) to various drugs during the adaptation of the *Plasmodium falciparum* lines W2 (A), TM91C235 (B), and D6 (C) to artelinic acid (AL).

A at position 3376 (numbering as in GenBank accession no. X71765) in the *pfatp6* genes of the parental TM91C235 and D6 parasites, as well as in their respective resistant progenies, resulted in an amino acid change from D to G in the region between the M6 and M7 transmembrane domains (22). In addition, a different number of microsatellite (AT) repeats was

observed in the 2nd intron sequence of the parental lines. The previously reported polymorphisms (19) at position 414, corresponding to codon 89I or codon 769, were not observed in any of the sequences. Importantly, no changes in the *pfatp6* sequence were observed between the parental lines and their corresponding resistant progenies.

No mutations corresponding to those in the putative *pcubcth* gene found in artemisinin-resistant *P. chabaudi* parasites (V739F and V770F [17]) were detected in the *pfubcth* genes amplified from these parasite lines. Differences in sequence between parasites of different origins were detected: the repetitive region between codons 670 and 737 (based on the PfUBCTH sequence of 3D7) differed in the number and types of repeat units (see Table S2 in the supplemental material). However, there was no difference in sequences between the parent and progenies of the same lineage (see Table S2). The part of the *pfcr1* gene coding sequence (excluding introns) corresponding to codons 36 to 380 was also sequenced. No differences were observed in the DNA sequences of the *pfcr1* gene between the W2, D6, and TM91C235 parental parasites and their respective progeny.

TABLE 2. *pfmdr1* allelic types in parental and resistant *Plasmodium falciparum* lines

Parasite line	Amino acid at position:				
	86	184	1034	1042	1246
D6	N	Y	S	N	D
D6AL80	N	Y	S	N	D
TM91C235	N	F	S	N	D
TM91C235AL80	N	F	S	N	D
W2	Y	Y	S	N	D
W2AL200	Y	Y	S	N	D
W2QHS200	Y	Y	S	N	D
W2AS80	Y	Y	S	N	D

FIG. 2. *pfmdr1* copy numbers in parental parasites and resistant progenies.

Amplification of the *pfmdr1* gene is associated with the acquisition of the resistant phenotype, and the copy number is proportionate to the level of resistance in W2 and TM91C235, but not in the D6 line. The copy number of *pfmdr1* was assessed in samples taken from parental lines and their corresponding progeny during adaptation to AL and QHS by using quantitative real-time PCR. The parental parasite W2 contained a single copy of the *pfmdr1* gene (CN, 0.93; 95% confidence interval [95% CI], 0.83 to 1.04). A single copy of *pfmdr1* was also detected in samples taken from W2 cultures exposed to 10 to 30 ng/ml of AL (CN, 1.02; 95% CI, 0.99 to 1.06) (Fig. 2). However, a change from 1 to 3 copies (CN, 3.01; 95% CI, 2.83 to 3.19 [P , <0.001]) was observed when the AL concentration was increased to 50 ng/ml. A further increase in the AL drug pressure to 80 ng/ml did not result in any detectable change in the *pfmdr1* copy number (P , 0.53), since W2AL80 parasites appeared to retain 3 copies of *pfmdr1* per genome (CN, 3.15; 95% CI, 3.01 to 3.30) (Fig. 2).

A bulk culture of W2AL80 parasites was cloned by limiting dilution, and one of the clones with 3 copies of the *pfmdr1* gene (CN, 2.99; 95% CI, 2.74 to 3.26) was used in further experiments. No detectable increase (P , 0.44) in the *pfmdr1* copy number (CN, 2.94; 95% CI, 2.77 to 3.12) was observed when the AL concentration reached 120 ng/ml. However, at 200 ng/ml, the *pfmdr1* copy number rose to 3.65 (95% CI, 3.19 to 4.17) (Fig. 2). Still, the difference in CN between W2AL120 and W2AL200 was not significant (P , 0.07).

During the study, W2AL80 parasites (bulk culture) were also exposed to QHS pressure at increasing concentrations, starting with 20 ng/ml and reaching 200 ng/ml. The adaptation

of parasites to 40 ng/ml of QHS was associated with a decrease in the *pfmdr1* copy number from 3 to 2 (CN, 2.2; 95% CI, 2.1 to 2.4) (Fig. 2). These parasites, referred to as W2QHS40, were cloned by limiting dilution. A clone carrying 2 copies of *pfmdr1* was subjected to further QHS pressure, yielding parasites able to withstand a QHS concentration of 200 ng/ml. The *pfmdr1* copy number remained at ~2 (CN, 1.99; 95% CI, 1.94 to 2.03) (Fig. 2).

The adaptation of TM91C235 parasites to AL also resulted in an increase in the *pfmdr1* copy number (Fig. 2). Although the uncloned parental line had a single copy of *pfmdr1* at the start of the selection studies, TM91C235 was isolated from a patient with mefloquine treatment failure and originally had multiple copies of *pfmdr1* (unpublished results). During long-term culture in the lab, the line had reverted to having only 1 copy. Exposure of these parasites to AL pressure resulted in an immediate increase in the *pfmdr1* copy number from 1 to 2 (CN, 2.05; 95% CI, 1.93 to 2.08) at 10 to 50 ng/ml of AL and a further increase to 3 copies (CN, 2.94; 95% CI, 2.6 to 3.2) when the AL concentration was increased from 50 to 80 ng/ml. The difference in the CN was significant (P , <0.001).

The D6 parental parasites contained 1 copy of the *pfmdr1* gene (CN, 0.97; 95% CI, 0.90 to 1.15). However, when these parasites had adapted to AL pressure (up to 80 ng/ml), the *pfmdr1* copy number remained stable, with 1 copy detected in all samples taken from resistant D6 progeny during the course of the adaptation (P , 0.61) (Fig. 2).

A Southern blot hybridization technique was employed to analyze the chromosomal rearrangements, as well as to confirm the changes in the *pfmdr1* copy number, in the resistant

FIG. 3. Southern blot hybridization of *P. falciparum* chromosomes digested with *Sma*I (left) and *Bgl*II (right) and hybridized with probes derived from the *pfmdr1* (A) and aldolase (B) genes.

progenies. The *Sma*I DNA fragment containing *pfmdr1* in W2AL80 parasites is approximately 300 kb longer than that in W2 parasites. The intensity of this band was ~3.6 times greater than that in W2 parasites after the DNA loading was normalized to the hybridization signal obtained for the aldolase gene

probe, indicating an amplification of the segment of chromosome 5 containing the *pfmdr1* gene (Fig. 3). A faint band was also observed in the *Sma*I digest of the W2AL80 parasites, indicating the presence of a minor population of parasites with a smaller version of chromosome 5 containing *pfmdr1*.

FIG. 4. Transcription of the *pfmdr1* gene in the W2, W2AL80, W2QHS40, TM91C235, TM91C235AL80, D6, and D6AL80 lines of *P. falciparum*.

It is apparent from the blots that the W2QHS40 parasite progeny consisted of two parasite populations: those with a larger *Sma*I fragment, equal in size to that in W2AL80 parasites, and those with a smaller version of the *Sma*I fragment of the chromosome hybridizing to the *pfmdr1* probe (Fig. 3). The intensities of these bands were 1.4- and 2-fold (for the upper and lower bands, respectively) greater than those for W2 parasites after correction for equal loading, indicating the presence of multiple copies of the *pfmdr1* gene. In contrast to *Sma*I, the *Bgl*II enzyme cuts between the amplicons, resulting in an apparently single band, similar in size to that for 3D7 parasites but with increased intensity. From the 3D7 genome sequence data (www.plasmodb.org/), the size of the *Bgl*II fragment containing the *pfmdr1* gene is 111,139 bp. In the 3D7 genome, this fragment is located between bp 903371 and 1014509 of chromosome 5 (www.plasmodb.org/) and contains 21 genes; *pfmdr1* is in the middle, at bp 957885 to 962144. Thus, it appears that chromosome 5 in W2AL80 or W2QHS40 parasites contains three- or two-times-amplified segments of ~111 kb, respectively.

The size of the *Sma*I fragment containing the *pfmdr1* gene in TM91C235AL80 parasites did not differ significantly from that for either TM91C235, W2, or 3D7 parasites. Based on the results of *Bgl*II blotting, the *pfmdr1*-containing *Bgl*II fragment is estimated to be ~10 to 20 kb larger than that in the parental TM91C235 and 3D7 parasites. The intensity of the *pfmdr1* gene-containing band is 2-fold higher for *Sma*I-digested TM91C235AL80 DNA than for *Sma*I-digested TM91C235 DNA. Thus, the size of the amplicon containing the *pfmdr1* gene in TM91C235AL80 parasites is different from that in W2AL80 parasites. No rearrangements were detected around the *pfmdr1* gene in D6AL80 parasites relative to D6 parasites, and the bands were of similar intensities (calculated ratio, 1.3).

***pfmdr1* mRNA expression and PfMDR1 protein expression are proportionate to the copy number of the *pfmdr1* gene.** The RNA transcription levels of the *pfmdr1* gene were assessed using a quantitative real-time reverse transcription-PCR (RT-PCR) assay. The transcription of the *pfmdr1* gene was measured relative to that of the seryl-tRNA synthetase gene (PF07-0073) every 6 to 8 h, starting with rings (0 h) and ending with mature schizonts. When these values for the parental parasites and their resistant progeny were compared (Fig. 4), *pfmdr1* transcription was found to be 3- to 5-fold higher in W2AL80 parasites and 2.2- to 2.4-fold higher in W2QHS40 parasites

than in the parental W2 parasites. A similar increase of 2.1-fold in transcription was observed for TM91C235AL80 parasites relative to their parental parasites. No difference in *pfmdr1* transcription was observed between D6AL80 parasites and the parental D6 parasites (Fig. 4).

Western blot analysis of PfMDR1 protein expression (Fig. 5) revealed that the amounts of the MDR1 protein detected in the W2AL80, W2QHS40, and TM91C235AL80 lines were 3.1-, 2.2-, and 3.8-fold higher than those in their corresponding parental lines. The amount of MDR1 protein in the D6AL80 parasites was 2.5-fold higher than that in D6 parasites (Fig. 5).

***pfatp6* and *tctp* transcription in parental and resistant parasite lines.** The transcription levels of two other genes of interest, *pfatp6* and *pfictp*, at the different time points of the parasite life cycle were also analyzed using real-time PCR assays. No significant changes in the transcription profiles of these genes between the resistant parasites and their respective parental parasites were detected (data not shown).

DISCUSSION

The potential emergence of artemisinin resistance is a major concern given the global reliance on artemisinin combination therapy as the major control measure. In this study, resistance to artemisinin derivatives has been induced in several clones and lines of *P. falciparum* *in vitro*. This *in vitro* resistance to AL is different from resistance to other conventional antimalarial drugs, such as CQ, in that AL-resistant parasites could not grow permanently under the AL pressure. It is confirmed by the ability of the parasites to survive and grow with increasing concentrations of artemisinin derivatives for many generations and by major increases in the IC₅₀. The discontinuous drug pressure selection scheme may have been the key to success in selecting the resistant lines. Upon adaptation, parasites were able to tolerate as much as 200 ng/ml of QHS or AL and grew rapidly under the pressure for many asexual cycles. These concentrations are comparable to those achieved in plasma obtained from malaria patients treated with QHS. Furthermore, when *in vitro* resistance to one artemisinin derivative developed, there was a significant degree of cross-resistance to various other artemisinin derivatives, including AL, AS, and DHA, as well as to MQ. These artemisinin-resistant parasites provide a unique resource and opportunity for the study of the artemisinin resistance mechanism(s) and modes of action. For

FIG. 5. MDR1 protein expression in resistant and parental *Plasmodium falciparum* lines. Western blot hybridization was performed with anti-Pgh1 (α 1435; left side in panels A and B) and anti-RAP2 (6C1; right side in panels A and B) antibodies.

example, the drug-selected lines were used to demonstrate that deamplification of the *pfmdr1* amplicon only partially restores susceptibility to AL (6a).

In two of the three parasite lines (W2 and TM91C235) used in the present study, the decrease in parasite susceptibility to the drugs was associated with a concomitant increase in the *pfmdr1* copy number and a proportional increase in the levels of *pfmdr1* transcription and protein expression. These findings indicate a clear involvement of *pfmdr1* in the AL- and QHS-resistant phenotypes. Notably, a switch from AL to QHS resulted in a shift from a parasite population with 3 copies of *pfmdr1* to a parasite population that predominantly had 2 copies of the *pfmdr1* gene. This indicates that the parasite population with 2 *pfmdr1* copies had an advantage over that with 3 copies under QHS pressure. Whether this is due to a difference in the mode of action between AL and QHS or to a difference in the mechanisms of resistance to the two artemisinin derivatives remains to be investigated.

The amplification of the *pfmdr1* gene was shown to be a random, independent event (42). The size differences in the *pfmdr1* amplicons observed for the TM91C235 and W2 resistant progenies in this study provide further support for this observation. These rearrangements involve large regions (20 to 110 kb) of the chromosomes, with amplification of as many as 19 genes, including the *pfmdr1* gene (Chen et al., submitted). None of these genes except *pfmdr1* had a known modulation effect on antimalarial drugs. A recent study by Gonzales et al. identified this region of chromosome 5 as one of the most prominent expression regulatory loci, which influenced the expression of 269 genes located on other chromosomes (16). Although we could not rule out the possibility that the amplification of genes other than

pfmdr1 may play a part in the development of resistance, our results, taken together with those of Sidhu et al. (39), suggest that overexpression of *pfmdr1* plays the most significant role in the drug susceptibility changes compared to the overall effect of the amplification of other genes contained within the *pfmdr1* amplicon.

While our data demonstrate the involvement of *pfmdr1* expression in the resistance phenotype in two of the three parasite lines, it is also apparent that amplification of *pfmdr1* is not the only possible mechanism of sustaining resistance, since no *pfmdr1* amplification was observed in D6 parasites exposed to similar drug levels. The reason for this difference is not clear, though it does suggest that amplification of *pfmdr1* is not a general stress response for *P. falciparum*. D6 is the only parental parasite line that harbors a “wild-type” *pfmdr1* allele, while the other two parental parasite lines carry “mutant-type” *pfmdr1* alleles. The “wild-type” *pfmdr1* allele is intrinsically associated with decreased susceptibilities to MQ, HF, and the artemisinin drugs. Whether a “wild-type” *pfmdr1* allele is associated with a different resistance mechanism remains to be investigated. D6 also is the only line from Africa used in this study. Perhaps there is a genetic basis for *pfmdr1* amplification, since most reports of this CN variant, both in lab-pressured mutants and in field isolates, are from Asia.

Although it is clear that *pfmdr1* plays a significant role in resistance, some studies also demonstrated that changes in susceptibility to MQ or HF could be achieved without changes in the expression level or allelic type of *pfmdr1* (26, 37), suggesting a possible involvement of other genes and/or mechanisms of sustaining resistance in these parasites, including transient changes in gene expression, which may account for the loss of resistance after cryopreservation of parasites observed

by Hunt et al. (17). Importantly, the parasites produced in this study retain the resistance phenotype, as defined by the ability to survive exposure to high drug concentrations, even after cryopreservation.

Notably, the IC₉₀ values of AL and QHS for all three parasite lines after adaptation were lower than the concentrations the parasites could endure *in vitro*. Moreover, although parasites became adapted to 80 ng of AL/ml, their IC₅₀ and IC₉₀ values were not different from those obtained for AL50 parasites. This suggests that the artemisinin resistance phenotype may be underestimated by the commonly used *in vitro* resistance measurements, such as IC₅₀ and IC₉₀, obtained by the standard 48- to 72-h drug susceptibility assay. These data also suggest that the standard drug assays may not be appropriate for assessment of the artemisinin resistance phenotype in the field.

Several recent publications have suggested that mutations in *pfatp6*, encoding the sarco-endoplasmic reticulum Ca²⁺ ATPase, are associated with resistance to artemisinin compounds. In particular, the S769N mutation, found in the *pfatp6* genes of field isolates collected in French Guiana, was associated with higher IC₅₀ of artemether *in vitro* (19). In our study, no amino acid changes in the *pfatp6* product, and no changes in its expression, were detected. No changes in the expression or DNA sequence of the *pfctcp1* gene were detected in the *P. falciparum* parasite lines used in our study, in contrast to a previously reported increase in *tctp* expression in artemisinin-resistant rodent parasites (44), but in agreement with findings reported by Hunt et al. (17).

The identification of putative molecular markers of resistance to artemisinin drugs is of immense importance. The development of artemisinin-resistant parasites *in vitro* provides a powerful framework for studies that need not focus on candidate genes. Our data suggest that among all the proposed candidate genes, only *pfmdr1* amplification is associated with the artemisinin resistance phenotype, yet clearly this is not the most critical determinant for resistance to artemisinin. In the drug-selected lines, mutations in the candidate resistance genes have no role in the resistance phenotype, yet the possibility of single nucleotide polymorphisms (SNPs) in other transporters or novel CN variants cannot be ruled out. Therefore, the selected lines are being used for whole-genome sequencing to identify CN variants or SNPs associated with the resistance phenotype.

At present, artemisinin combinations are the most effective antimalarial drugs. However, our data clearly suggest that parasites can tolerate increasing concentrations of artemisinin drugs by amplifying the *pfmdr1* gene. Since amplification of *pfmdr1* is also a mechanism of resistance to MQ, consideration should be given when combinations such as artesunate-mefloquine are used in areas with high levels of MQ resistance. Finally, it is clear that *pfmdr1* amplification is not the central determinant of artemisinin resistance, but it is possibly an important event that may enhance artemisinin resistance, especially in Southeast Asia. The artemisinin-resistant mutants produced in this study will be an important resource in the search for molecular markers of artemisinin resistance.

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