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Plasmodium falciparum pfmdr1 Amplification, Mefloquine Resistance, and Parasite Fitness▽†

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Mefloquine is widely used in combination with artemisinin derivatives for the treatment of falciparum malaria. Mefloquine resistance in *Plasmodium falciparum* has been related to increased copy numbers of multidrug-resistant gene 1 (*pfmdr1*). We studied the ex vivo dynamics of *pfmdr1* gene amplification in culture-adapted *P. falciparum* in relation to mefloquine resistance and parasite fitness. A Thai *P. falciparum* isolate (isolate TM036) was assessed by the use of multiple genetic markers as a single genotype. Resistance was selected by exposure to stepwise increasing concentrations of mefloquine up to 30 ng/ml in continuous culture. The *pfmdr1* gene copy numbers increased as susceptibility to mefloquine declined ($P = 0.03$). No codon mutations at positions 86, 184, 1034, 1042, and 1246 in the *pfmdr1* gene were detected. Two subclones of selected parasites (average copy numbers, 2.3 and 3.1, respectively) showed a fitness disadvantage when they were grown together with the original parasites containing a single *pfmdr1* gene copy in the absence of mefloquine; the multiplication rates were 6.3% and 8.7% lower, respectively ($P < 0.01$). Modeling of the dynamics of the *pfmdr1* copy numbers over time in relation to the relative fitness of the parasites suggested that net *pfmdr1* gene amplification from one to two copies occurs once in every 10^8 parasites and that amplification from two to three copies occurs once in every 10^3 parasites. *pfmdr1* gene amplification in *P. falciparum* is a frequent event and confers mefloquine resistance. Parasites with multiple copies of the *pfmdr1* gene have decreased survival fitness in the absence of drug pressure.

Whereas a combination of antimalarial drug treatment and vector control were very successful tools for controlling malaria in the mid-20th century and eliminated malaria from most temperate zones in the world, the rates of morbidity and mortality from malaria have worsened over recent decades in many parts of the tropical world. The resurgence of malaria is largely related to the increased resistance of the *Plasmodium falciparum* parasite to many of the available antimalarial drugs, such as chloroquine, pyrimethamine-sulfadoxine, and more recently, mefloquine. Multidrug-resistant *P. falciparum* is widely prevalent in Southeast Asia. This has made the treatment of falciparum malaria increasingly difficult (27). The artemisinin combination treatment artesunate-mefloquine was first introduced in 1994 for the treatment of falciparum malaria on the Thailand-Myanmar (Burma) border and continues to be highly effective (29).

Codon mutations in the gene encoding the *P. falciparum* P-glycoprotein transporter (*pfmdr1*) have been incriminated in resistance to chloroquine and mefloquine (16, 18). A stronger association with mefloquine resistance is the increased expression of the amplified wild-type *pfmdr1* gene (8, 16, 17, 19). In a large study conducted on the Thailand-Myanmar border, where multidrug resistant falciparum malaria is prevalent, an increase in the *pfmdr1* copy number was estimated to account for about two-thirds of the variation in susceptibility to mefloquine (19).

We evaluated the dynamic relationships between mefloquine resistance and *pfmdr1* copy number and mutations in a long-term in vitro culture of a single strain of *P. falciparum* in which resistance was selected by stepwise increases in mefloquine pressure. The stability of the selected genotype containing multiple *pfmdr1* copies was studied, and the relative fitness of parasites with multiple copies of *pfmdr1* was compared to that of the original isolate with a single copy of *pfmdr1*. A mathematical model was constructed to estimate the frequency of the event resulting in *pfmdr1* gene amplification.

MATERIALS AND METHODS

Parasite genotyping and culture. Mefloquine-sensitive Thai *P. falciparum* isolate TM036 was maintained in continuous culture (24). To confirm the presence of a single genotype, the isolate was genotyped with *msp1*, *msp2*, and *glurp* as genetic markers by nested PCR, as described previously (6). To further confirm this, the initial isolate was also genotyped by using 10 microsatellite markers distributed over different chromosomes, including TA1, TAA87, POLY, ARA2, PFPK2, 2490, TAA42, PFG377, TAA60, and TAA81, and fluorescent end-labeled oligonucleotides (3). The gene products were analyzed on an ABI 3100 capillary sequencer, and the alleles were scored by using Genescan and Genotyper software. Pull-up artifacts were prevented by dilution of the PCR product, and a minor allele was identified if its intensity amplitude was >33% of the height of the predominant allele (1).

Parasites were cultured in human blood group O erythrocytes in a total volume of 40 ml to a maximum density of 5% parasitemia at 5% hematocrit. If the level of parasitemia exceeded 5%, the culture was routinely diluted to 0.5 to 1% parasitemia with a standard volume of fresh donor erythrocytes. The culture medium contained RPMI 1640 (ICN Biomedicals, United Kingdom) supplemented with 2 g/liter $NaHCO_3$ (Life Technologies), 5.958 g/liter HEPES

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(Sigma), 1 ml/liter gentamicin sulfate (Government Pharmaceutical Organization, Thailand), 13.6 mg/liter hypoxanthine (Sigma), 4.5 g/liter D-glucose (Sigma), and 0.5% Albumax II (Gibco, New Zealand).

Selection of mefloquine-resistant parasites. A stock solution of 1 mg/ml mefloquine hydrochloride (Pharmaceutical Technology Department, Institute de Technologia em Farmacos, Italy) dissolved in 0.01 M HCl was prepared in RPMI 1640 medium, stored protected from the light at 4°C, and used within 4 weeks of preparation. The stock solution was then further diluted with RPMI 1640 medium to the required concentrations shortly before each experiment.

Resistance to mefloquine in the TM036 *P. falciparum* strain was selected by exposure of the cultured parasites to stepwise increases in mefloquine concentrations of 15, 20, 25, and 30 ng/ml, after an initial preliminary phase of 127 days of continuous culture with intermittent exposures to very low levels of mefloquine ranging from 1 to 10 ng/ml.

The initial level of parasitemia was 4 to 5%, which corresponds to a total of approximately 10^9 parasites per flask, calculated as follows: parasite count per μl = parasite count per 1,000 erythrocytes × hematocrit (in percent) × 125.6 (23). The parasite culture serving as a control was routinely diluted to 0.5 to 1% parasitemia (1×10^8 to 2×10^8 parasites per flask) in the absence of drug. After the addition of mefloquine, the level of parasitemia in a thin blood film was monitored every 24 h. If the level of parasitemia decreased to 2%, which corresponds to approximately 5×10^8 parasites per flask, the culture medium was replaced by a mefloquine-free medium. The parasites were then allowed to recover until the level of parasitemia was 5% (1.3×10^9 parasites per flask) and the growth rates were comparable to those in the control culture. The recovered parasites were then exposed to the same concentration of mefloquine for a second time. After that, the parasites were exposed to a higher dose of mefloquine and the same cycle of procedures was repeated. DNA samples were taken after removal of the mefloquine drug pressure and after the parasite population was allowed to recover until a level of parasitemia of 50 per 1,000 erythrocytes was reached.

Mefloquine susceptibility assays. Mefloquine susceptibility assays were performed with samples taken from the continuous culture. Susceptibility was measured by assessment of the inhibition of schizont maturation, as described previously (30). In short, parasites were harvested when the level of synchronized ring-stage-infected red blood cell parasitemia reached 5%. The culture was then centrifuged at 800 × *g* at 4°C for 5 min, 100 μl of packed red blood cells was resuspended in culture medium to a 3% hematocrit, and the mixture of medium and cells was added to a microculture plate predosed with mefloquine at fivefold dilutions ranging from 0.064 ng/ml to 1 μg/ml. The parasites were incubated at 37°C with 5% CO_2 for 40 to 44 h, and the assay was terminated when 80% of the mature schizonts (more than six merozoites) presented in the control well containing no drug. Maturation inhibition was defined as the percentage of schizonts in the well containing drug compared to the percentage of schizonts in the control well. The drug concentration that gave 50% inhibition of schizont maturation (IC_{50}) was derived by fitting the dose-maturation inhibition curves (n = 3) by using the statistical program Winnonlin (version 4.1; Pharsight, Mountain View, CA).

Sample preparation and DNA extraction. Parasite DNA was extracted from the parasite-containing red blood cell pellet obtained by centrifugation at 800 × *g* for 5 min at 4°C. The pellet was either frozen at −20°C until further processing for template DNA purification with a QIAamp DNA kit (Qiagen, Hilden, Germany) or 5 μl of the parasite pellet (50% hematocrit) was transferred to filter paper (Whatman, Maidstone, United Kingdom) and the DNA was extracted with PrepMan Ultra reagent (Applied Biosystems, Foster City, CA), followed by precipitation of the supernatant and resuspension in Tris-EDTA buffer (50 μl).

Assessment of *pfmdr1* amplification by real-time PCR. Amplification of the *pfmdr1* gene was assessed by real-time PCR (Rotor Gene 3000; Corbett Research, Australia). The primers and the fluorescence probe used have been described previously (19). The amplification reactions were done as multiplex PCRs in 7.5 μl of a reagent containing the buffer from the Biotool Quantimix easy probe kit (B&M Lab, Spain), $MgCl_2$ (5.5 mmol/liter), forward and reverse primers (each at 300 nmol/liter), a *pfmdr1* probe and a β-tubulin probe (each at 100 nmol/liter), and 2.5 μl of template DNA. Fifty cycles were performed (holds at 95°C for 10 min, followed by cycling of 95°C for 15 min and 58°C for 1 min). Each copy number value was derived from three separate assessments per assay. Estimates were rejected if they did not conform to exponential kinetics. The assay was repeated if one of the following results was obtained: a spread threshold cycle (C_T) value greater than 1.5, a C_T value greater than 35, or a copy number of more than 1.3. The assay was repeated at least twice for validation of consistent results. Both positive and negative control samples were added in each assay.

Assessment of mutations in the *pfmdr1* gene. The *pfmdr1* gene was amplified from the DNA template obtained from the frozen parasite culture pellets by nested PCR. A PCR-restriction fragment length polymorphism assay was then used to assess whether there were point mutations in the *pfmdr1* gene. Five polymorphic sites have been associated with mefloquine resistance. The polymorphic sites at positions 86, 184, 1034, and 1042 (20) were assessed by using ApoI, DraI, DdeI, and AseI (VspI), respectively, as described before (10). Mutations at another polymorphic site at position 1246 were assessed by gene sequencing (Genetic Analyzer 3100; Applied Biosystems). All restriction enzymes were purchased from New England Biolabs (Hitchen, United Kingdom). Digestion fragments were analyzed on a 3% Seakem LE agarose gel with TBE (Tris-borate-EDTA) buffer under constant power (120 V).

Stability of *pfmdr1* multiple-copy-number genotype in the absence of drug pressure. Three parasite cultures with multiple *pfmdr1* copy numbers selected with stepwise increasing mefloquine concentrations (20, 25, and 30 ng/ml) were used to assess the backmutation frequency and the stability of the multiple-copy-number phenotype. For comparison, two cultures containing parasites with a single copy were also assessed. All five flasks were cultured in a drug-free complete medium for up to a maximum of 6 months. All parasite cultures were kept in 5% hematocrit in a total volume of 5 ml and were routinely diluted to 0.5 to 1% parasitemia to maintain the parasite population at 1×10^7 to 2×10^8 per flask. The *pfmdr1* copy numbers were assessed once per month, as described above. The observations from this experiment were used to estimate an upper limit for the frequency of deamplification (switching down from three to two and from two to one copies of the *pfmdr1* gene).

Relative parasite fitness in relation to *pfmdr1* copy number. The relative fitness of mefloquine-exposed Thai isolate TM036 containing multiple copies of *pfmdr1* was compared with that of the wild-type isolate containing a single copy. For this, 1:1 mixtures of the wild-type culture and the mefloquine-exposed cultures were prepared. Equal volumes of the parasite cultures, both of which were adjusted with fresh erythrocytes to parasitemias of 0.1%, were mixed. It should be noted the accuracies of the levels of parasitemia in this range as assessed by light microscopy are inherently limited. The parasites with average copy numbers of 2.1 and 3.1 were each mixed with a single-copy-number parasite population. Each culture was at 0.1% parasitemia and 5% hematocrit, which corresponds to approximately 3×10^6 parasites per flask. The cultures were then expanded to a total volume of 40 ml, yielding approximately 10^9 parasites. All cultures were kept for up to 4 months. Every 2 to 3 days, parasites were sampled on filter paper for assessment of the *pfmdr1* copy number.

Modeling of parasite fitness. The changes in the *pfmdr1* copy numbers over time in the mixtures of cultures containing single and multiple copies were used to model the relative fitness of the parasites with multiple-copy-number genotypes by using the SPLUS (version 6.0) Professional program (Insightful Corp.). Relative fitness was defined as the multiplication rate of the multiple-copy-number parasites compared to that of the single-copy-number parasites. A model was constructed by assuming that the observed copy number reflected a weighted average value of the copy number for an in vivo mixture of substrains with different copy numbers. It was also assumed that a *pfmdr1* copy number of zero was not viable and that the accuracy of the *pfmdr1* copy number assay was independent of the copy number (13). Another assumption of the model was that the *pfmdr1* copy number of any isolate was stable in the absence of drug pressure, i.e., that there was a balance between the forward and reverse switching of *pfmdr1* copy numbers. This assumption was supported by the results of the other experiments (see Fig. 3). Therefore, the change in the average *pfmdr1* copy number in a mixture of cultures over time represented differences in the fitness of the contributing parasite substrains with different *pfmdr1* copy numbers.

Details on the construction of the model can be found in the supplemental material. The number of parasites with x and y copy numbers at the beginning of the coculture experiment were calculated from the average *pfmdr1* copy numbers before and after they were mixed. The total numbers of parasites with x copies at time t (parasite_x^t) or y copies at time t (parasite_y^t) can be calculated from.

$$\text{parasite}_x^t = \text{parasite}_x^0 \times A^t \quad (1)$$

$$\text{parasite}_y^t = \text{parasite}_y^0 \times B^t \quad (2)$$

where parasite_x^0 and parasite_y^0 are the total numbers of parasites with x and y copy numbers at time zero, respectively, and where A^t and B^t are the multiplication rates of parasites with x and y copy numbers at time t, respectively.

The following formula was used to describe the relationship between the average copy number at time t (av.copy^t), parasite_x^0 and parasite_y^0, and B^t/A^t, which is the ratio of the multiplication rates at time t:

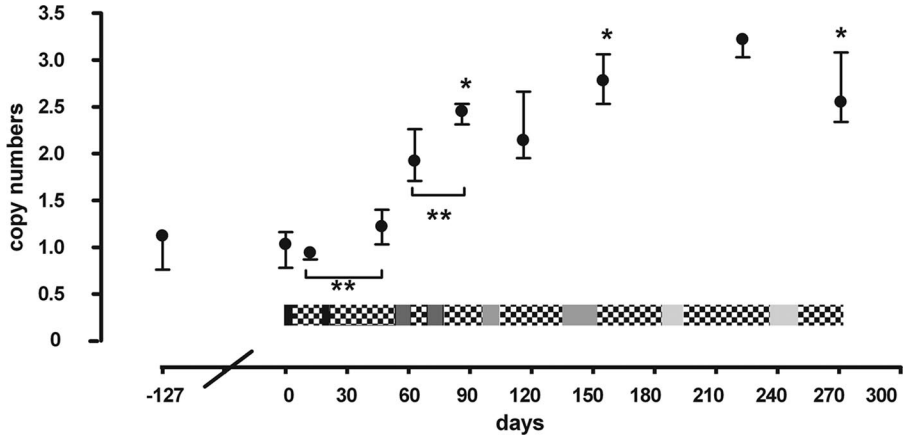

FIG. 1. Change in the median (range) *pfmdr1* gene copy number of parasite strain TM036 over time in relation to exposure to increasing concentrations of mefloquine, as shown on the gray scale, from 15 ng/ml to 20 ng/ml, 25 ng/ml, and 30 ng/ml. Three observations were made at each time point. The checkered bars refer to culture in drug-free medium. *, $P < 0.05$ compared to the copy number at day 0; **, $P < 0.05$ compared to the copy number after the first exposure to the same mefloquine concentration.

$$\text{av.copy}^t = \frac{[x \times (\text{parasite}_x^0 \times 1)] + \left[y \times \left(\text{parasite}_y^0 \times \frac{B^t}{A^t}\right)\right]}{(\text{parasite}_x^0 \times 1) + \left(\text{parasite}_y^0 \times \frac{B^t}{A^t}\right)} \quad (3)$$

Since all parameters except B^t/A^t are measured during the experiment, B^t/A^t can be estimated by finding the value that best fits the curve represented by equation 3 to the observed data of the average copy numbers over time.

Estimation of the event rate of *pfmdr1* gene amplification. In order to derive a quantitative estimate of the net event rate of *pfmdr1* gene amplification, a second mathematical model was constructed (see the supplemental material). The model inputs included the dynamics of the changes in *pfmdr1* copy numbers over time, information on the relative fitness in the absence of drug pressure derived from the first model, and the relative fitness in the presence of drug pressure derived from the assessment of the IC_{50} for mefloquine in relation to the *pfmdr1* copy number. Since the total number of parasites, the average copy number, and the relative multiplication rates of the parasites with different copy numbers are all known over time, the rates of net *pfmdr1* gene amplification events, which denote the balance between the forward switch (from one to two copies and from two to three copies) and the reverse switch (from three to two copies and from two to one copies) can be derived by choosing the values that give the best fit to the observed data.

The equations used can be summarized as follows for time t:

$$\text{parasite}_1^t = [\text{parasite}_1^{t-1} \times \mu_1^c] - [sw_{12} \times \text{parasite}_1^{t-1}] \quad (4)$$

$$\text{parasite}_2^t = [sw_{12} \times \text{parasite}_1^{t-1}] + [\text{parasite}_2^{t-1} \times \mu_2^c] - [sw_{23} \times \text{parasite}_2^{t-1}] \quad (5)$$

$$\text{parasite}_3^t = [sw_{23} \times \text{parasite}_2^{t-1}] + [\text{parasite}_3^{t-1} \times \mu_3^c] \quad (6)$$

$$\text{av.copy}^t = \frac{\text{parasite}_1^t + 2\times \text{parasite}_2^t + 3\times \text{parasite}_3^t}{\text{parasite}_1^t + \text{parasite}_2^t + \text{parasite}_3^t} \quad (7)$$

where parasite_1^t, parasite_2^t, and parasite_3^t are parasite populations with one, two, and three copies at time t (in cycles), respectively; sw_{12} and sw_{23} are the net rates of switching of the copy numbers from one to two and from two to three, respectively. μ_1^c, μ_2^c, and μ_3^c are the multiplication factors for the parasite populations with one, two, and three copies numbers at a specific mefloquine concentration (c), respectively. μ_1^c, μ_2^c, and μ_3^c are derived from μ, which is the observed average multiplication rate of the parasite culture. For details, see the supplemental material.

Statistical analysis. The repeatability of the assessment of copy numbers was assessed by the method of Bland and Altman (5). The repeatability coefficient was calculated as the 95% confidence interval (CI) of the differences between the measurements. Nonparametric data were analyzed by the Kruskal-Wallis, Mann-Whitney U, or Spearman rank test, as appropriate, by using the SPSS statistical program (version 10.0; SPSS Inc., Chicago, IL). Analysis of trends between two nonparametric variables was tested by the np trend test (Stata Inc.). The level of significance in these experiments was set at a P value of <0.05.

RESULTS

Genotyping of *P. falciparum* isolate TM036. Genotyping of the starting isolate with three antigenic markers, *msp1*, *msp2*, and *glurp*, revealed the presence of only a single genotype. In addition, by use of a more sensitive typing system with 10 microsatellite markers, only a single allele was observed.

Association between level of mefloquine resistance and *pfmdr1* gene amplification and mutations. In *P. falciparum* mefloquine-sensitive isolate TM036, mefloquine resistance could be selected in vitro by use of a stepwise increase in the mefloquine concentration to which it was exposed to up to 30 ng/ml. The selection of tolerance was illustrated by the increased exposure time tolerated (defined as the ability to maintain a level of parasitemia above 2%) during the second exposure compared to that during the first exposure with the same mefloquine concentration. For instance, after 3 days of exposure to 15 ng/ml mefloquine, the level of parasitemia dropped from 5% to 2%; but after recovery of the parasite numbers, a second exposure to the same mefloquine concentration was tolerated for 7 days. This corresponded to an increase in the IC_{50} of mefloquine for the parasite population (see below).

Over time, there was a stepwise increase in the median *pfmdr1* copy number with exposure to increasing mefloquine concentrations, as shown in Fig. 1. The initial exposure for 127 days to intermittent low doses of mefloquine (1 to 10 ng/ml) in the early phase of the study did not affect the *pfmdr1* copy number. The median *pfmdr1* copy number of the original strain was 1.03 (range, 0.78 to 1.16). The first increase in the *pfmdr1* copy number to a median value of 1.22 (range, 1.03 to 1.40; $P < 0.05$) was observed after the second exposure to mefloquine at a concentration of 15 ng/ml. After the mefloquine concentration was increased to 20 ng/ml, the copy number increased sharply to 1.92 (range, 1.71 to 2.26; $P < 0.05$), and there was a subsequent further significant increase in copy

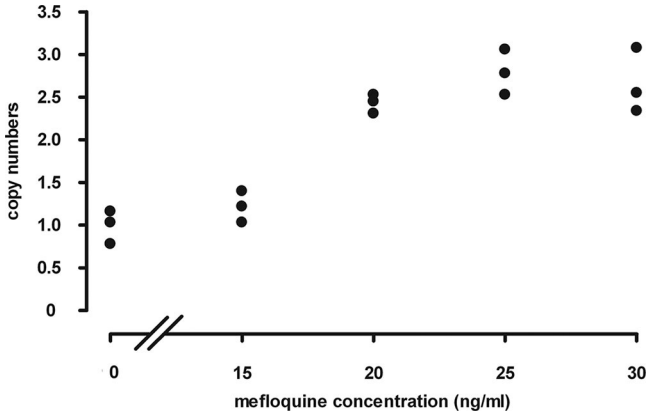

FIG. 2. Correlation between *pfmdr1* copy number and mefloquine exposure.

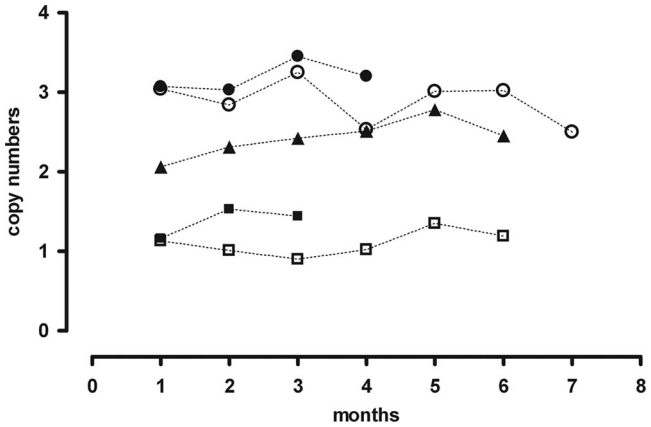

FIG. 3. Stability of *pfmdr1* copy number genotype in drug-free medium over time in four parasite populations with different *pfmdr1* copy numbers. □, control wild-type parasite (one copy); ■, copy number after exposure to mefloquine at 15 ng/ml; ▲, copy number after exposure to mefloquine at 20 ng/ml; ○, 25 ng/ml; ●, copy number after exposure to mefloquine at 30 ng/ml.

number after the second exposure to the same dose to 2.45 (range, 2.31 to 2.53). After this, exposure to higher mefloquine concentrations of 25 and 30 ng/ml did not significantly increase the median *pfmdr1* copy number after either the first or the second exposure, with the exception of the *pfmdr1* copy number after the first exposure to 30 ng/ml (median, 3.22; range, 3.03 to 3.22). Figure 2 shows the relationship between the mefloquine drug exposure and the *pfmdr1* copy number (values after the second exposures). There was a significant increase in the *pfmdr1* copy number with increasing mefloquine concentrations (test for trend across ordered groups, $P < 0.001$).

To confirm that tolerance to increasing concentrations of mefloquine corresponded to increased resistance to the drug, the IC_{50}s for parasites containing an average of 1.0, 2.1, and 3.0 copies of the *pfmdr1* gene were assessed (in triplicate). The median IC_{50}s of mefloquine for parasites with 2.1 and 3.0 copies were 7.90 ng/ml (range, 3.99 to 9.04 ng/ml) and 9.29 ng/ml (range, 6.62 to 13.29 ng/ml), respectively, whereas the median IC_{50} was 1.47 ng/ml (range, 0.62 to 1.88 ng/ml) for parasites with a single copy ($P = 0.03$) (Table 1). The IC_{50}s of mefloquine correlated positively with the *pfmdr1* copy number (Spearman's rho = 0.87; $P = 0.001$).

No codon mutations in the *pfmdr1* gene at positions 84, 184, 1034, 1042, and 1246 were detected in any of the samples.

Stability of parasite isolates containing multiple *pfmdr1* copies in the absence of drug pressure. The stability of the genotypes of parasites containing different *pfmdr1* copy numbers in the absence of mefloquine was evaluated with four samples of parasites, which were cultured for periods that varied from 2 to 6 months. Up to the 90th successive generation in a mefloquine-free culture medium, the increased *pfmdr1* copy numbers in the selected parasite populations remained unchanged (*t* test for slope, $P > 0.05$) (Fig. 3). This stability of the culture containing parasites with multiple copies was used to define a lower limit of the reverse switch rates (i.e., three to two and two to one *pfmdr1* copies). From the total numbers of parasites in the experiment, a reverse switch rate from two to one copies to a more fit parasite subclone with a lower *pfmdr1* copy number cannot be more frequent than the forward switch rate. Since the switch rate from one to two *pfmdr1* copies occurs in approximately 1 in 10^8 parasites, it can be assumed that the switch-back rate has to be less frequent than 1 in 10^8, since any value lower than this would result in a measurable decrease in the mean copy numbers. Similarly, the switch-back rate from three to two copy numbers cannot exceed 1 in 5×10^3 asexual divisions.

TABLE 1. Mefloquine IC_{50}s for parasites with different copy numbers

Copy no.[a]	Median (range) IC_{50} (ng/ml)	*P* value
1	1.47 (0.62–1.88)	
2	7.90 (3.99–9.04)	0.03[b]
3	9.29 (6.62–13.29)	0.03[b]

[a] Integer value.
[b] Mann-Whitney, $P < 0.05$.

Relative fitness of parasites with multiple *pfmdr1* copies compared to that of parasites with a single copy. Coculturing of parasite subclones containing a single *pfmdr1* copy and multiple *pfmdr1* copies in the absence of mefloquine drug pressure resulted in a gradual decrease in the weighted average copy number, indicating the preferential survival of parasites with single-copy-number genotypes over time (Fig. 4). Parasite populations containing apparently single subclones with one or multiple copies remained unchanged over time. In contrast, in a coculture containing parasite populations with 1 and 2.3 *pfmdr1* copies, the copy number decreased from an average of 2.0 to 1.1 in 21 days (10 generations); in the mix containing 1.0 and 3.1 copies, the number declined from 1.8 to 1.2 in 33 days (16 generations) (Fig. 4). All assessments of *pfmdr1* copy numbers were made in triplicate assays.

The results of this experiment were used to model the relative fitness of the parasites containing multiple copies of the *pfmdr1* gene compared to that of the wild-type isolate.

Modeling of parasite fitness. The curve describing the reduction in the average copy number over time starting with an initial equal mix of parasites containing 1.0 and 2.3 copies numbers was fitted by using equation 3 in a nonlinear regression model. The best fit was achieved when the ratio of the

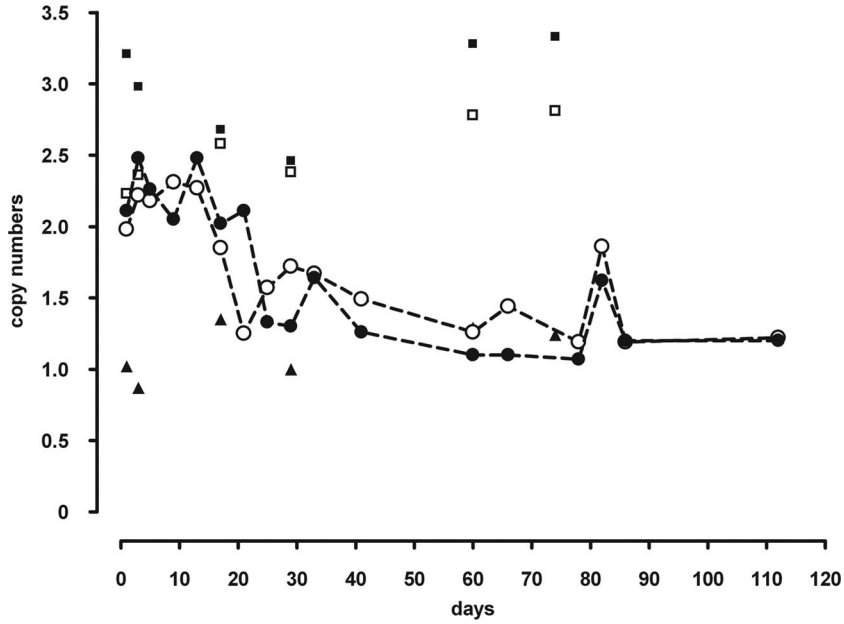

FIG. 4. Change in average *pfmdr1* copy number over time in cocultures of equal mixes of *P. falciparum* with a median of 1.0 and 2.3 copies (○) and 1.0 and 3.1 copy numbers (●). For comparison, the average copy numbers of a monoculture with one copy (▲), two copies (□), and three copies (■) are also shown.

multiplication rate of the isolates with multiple copies (2.3 copies) to the multiplication rate of the isolate with a single copy (*B*/*A*) was 0.937, with a residual sum of squares of 0.83 with 12 degrees of freedom (see Fig. SA1 in the supplemental material). Thus, compared to the fitness of parasites with a single-copy-number genotype, the relative fitness disadvantage of the parasites with an average copy number of 2.3 was estimated to be 6.3% (95% CI = 3.9 to 8.9%).

Similarly starting with an equal mix of parasites with 1.0 and 3.1 *pfmdr1* copies, the fitted model-derived ratio of multiplication rates (*B*/*A*) was 0.913, with a residual sum of squares of 1.03 with 16 degrees of freedom, which corresponds to a fitness disadvantage of 8.7% (95% CI = 5.9 to 11.5%) in parasites with an average of 3.1 *pfmdr1* copies per parasite compared to the fitness of parasites with an average copy number of 1 (see the supplemental material). The incremental reduction in the fitness of parasites with 3.1 *pfmdr1* copies (compared to the fitness of those with 2.3 copies) was thus smaller (2.4%) than the reduction in the fitness of parasites with 2.3 copies (6.3%) compared to the fitness of those with 1 copy.

Modeling the rate of *pfmdr1* amplification. By fitting the observed increase in the *pfmdr1* copy numbers over time in the presence of mefloquine drug pressure by using the model described above and further detailed in the supplemental material, the rate of *pfmdr1* amplification events was estimated. The relative fitness of parasites with one, two, or three copies in the presence of various mefloquine concentrations was derived from the in vitro dose-maturation inhibition curves. With increasing mefloquine concentrations in the culture medium, the relative fitness of parasites with lower numbers of copies decreased compared to the fitness of those with three copies (which was set equal to 100%): at a concentration of 15 ng/ml mefloquine, the relative levels of fitness of parasites with one and two copies were 24.0% and 93.9%, respectively; at a concentration of 20 ng/ml mefloquine, the relative levels of fitness of parasites with one and two copies were 22.2% and 92.9%, respectively; at a concentration of 25 ng/ml mefloquine, the relative levels of fitness of parasites with one and two copies were 21.1% and 92.3%, respectively; and at a concentration of 30 ng/ml mefloquine, the relative levels of fitness of parasites with one and two copies were 20.3% and 91.8%, respectively. The observed multiplication rates (μ) assessed from the growth curves and total parasite numbers varied greatly over time (range, 0.04 to 10.84), with the level of mefloquine exposure being the main determinant. By application of the model, net switch rates of 1 in 10^8 for amplification from one to two copies and 1 in 5×10^3 for amplification from two to three copies gave the best fit of the model to the observed data (see Fig. SA3 in the supplemental material). The residual sum of squares was 20.15. It should be noted that these values describe the net switch rate, which is the difference between the amplification rate and the reverse switch rate or deamplification (to a lower *pfmdr1* copy number).

DISCUSSION

When mefloquine was deployed in 1984 as treatment for uncomplicated falciparum malaria on the Thailand-Myanmar border, high-grade resistance developed rapidly over a 6-year period, despite the use of close controls that limited prescription of the drug for the treatment of symptomatic falciparum malaria (14). By mid-1994, 50% of infections treated with high-dose mefloquine monotherapy recrudesced and in 10% of infections parasites were not cleared at all from patients who lived in that region. After the drug was combined with a 3-day course of artesunate, the efficacy of the combination restored

the cure rate to >90% and the in vitro susceptibility of *P. falciparum* to mefloquine initially improved (1).

In several studies, mefloquine resistance has been associated with an increase in the parasite *pfmdr1* copy number (9, 16, 19). Increased *pfmdr1* copy numbers were found to be highly predictive of a subsequent recrudescence of the infection in both patients treated with mefloquine monotherapy and those treated with a combination of artesunate with mefloquine. The *pfmdr1* copy number also correlated with in vitro susceptibility to mefloquine (19, 22). The results of in vivo studies suggested that changes in the *pfmdr1* copy number were frequent and were certainly much more frequent than mutations in the gene (26) and could be observed within a single infection (25).

To explore the relationships between *pfmdr1* gene amplification and mefloquine resistance further, we studied the dynamics of the changes in *pfmdr1* copy number under mefloquine drug pressure in relation to mefloquine sensitivity. The results showed that *pfmdr1* gene amplification could be selected in vitro by exposing the parasites to stepwise increasing concentrations of mefloquine and that, indeed, amplification of the *pfmdr1* gene was a frequent event. A weakness of the current study is that the data came from a single, albeit protracted and labor-intensive, experiment. The fresh parasite isolate used in the experiment was also not cloned, although extensive genotyping with both conventional genetic markers (*msp1*, *msp2*, and *glurp*) and 10 microsatellite markers revealed only a single genotype. The possibility that minute subpopulations of genotypically different clones were present cannot be ruled out entirely, but this is unlikely, since no new genotype was detected over several months of continuous culture. Any putative minority clone could not have contributed numerically to the estimates obtained in this study; otherwise, it would have been detected. Parasites with increased copy numbers exhibited increased tolerance to mefloquine in the culture medium, corresponding to a significant increase in the IC_{50} for mefloquine in the maturation inhibition assay. Other factors may also have contributed to the reduced mefloquine susceptibility, including transcriptional and translational modification of the *pfmdr1* product.

Net amplification of the *pfmdr1* gene from one to two copies in this in vitro study occurred in approximately in $1{:}10^8$ parasites per asexual cycle. Since the total number of parasites in an adult patient with symptomatic malaria varies between approximately 10^8 and 10^{13} parasites, *pfmdr1* gene amplification can be expected to occur in most clinical cases. This finding is concordant with clinical observations (25) and emphasizes the importance of using combination antimalarial treatment strategies to reduce the chances of selecting the resistant phenotype. The survival and expansion of parasites with multiple-copy-number genotypes depend on the presence of subtherapeutic and selective blood concentrations of mefloquine, which compensate for any intrinsic impairment of fitness and which provide a net fitness advantage for this genotype. These findings are in line with the rapid development of drug resistance observed when mefloquine, which has a long terminal half-life, was deployed as monotherapy.

According to our model, the amplification from two to three copies was an even more frequent event, occurring in a genetic background of two copies per genome in every 5,000 parasites per cycle. Although this seems to be a very high rate, it is biologically plausible because the initial duplication event, which is the rate-limiting step, creates regions of homology in the genome that facilitate further amplification (13). A possible mechanism in P-glycoprotein gene amplification is a so-called breakage-fusion bridge cycle, as has been shown in a Chinese hamster cell line. Chromosome breakage at a major fragile site was shown to be associated with P-glycoprotein gene amplification in multidrug-resistant CHO cells (11). In the present study, a stepwise increase in copy number was observed. It is unclear whether gene deamplification also occurs in a stepwise pattern.

Gene amplification is likely to be a random event, as is codon mutation, but the latter is much less frequent: for example, mutations in the mitochondrial DNA conferring high-grade resistance to atovaquone emerge in vivo at a frequency of approximately 1 in 10^{12} parasites (28) and codon mutations at positions 46 and 108 of pyrimethamine-resistant parasites occur at less than 1 in 2.5×10^9 mutations/*dhfr* gene/replication (15). Since codon mutations in the *pfmdr1* gene occur at a much lower frequency ($1{:}10^{14}$) (28), this explains why codon mutations at any of the putative resistance loci (positions 84, 184, 1034, 1042, and 1246) were not detected in this study. This does not exclude the possibility that codon mutations in the *pfmdr1* gene associated with mefloquine resistance happen in nature, but it shows that these are not essential for the development of mefloquine resistance. Indeed, epidemiological studies suggest that *pfmdr1* amplification occurs more readily in parasites bearing the wild-type gene. In a field study at the Thailand-Myanmar border, no significant association between codon mutations at positions 86, 1034, and 1042 and the recrudescence of parasites was found (19). Other studies have shown an association between the deamplification of *pfmdr1* and an increase in mefloquine sensitivity (4). However, some investigators claim that, on the basis of data from both in vitro studies (12) and in vivo studies (7), amplification of the *pfmdr1* gene is not a prerequisite for mefloquine resistance.

Our study directly assessed the fitness disadvantage of the parasites with multiple numbers of copies of the *pfmdr1* gene compared to the fitness of parasites with a single copy. The decreases in fitness were 6.3% for parasites with two copies and 8.7% for parasites with three copies. Since the *pfmdr1* gene product is an ATP-consuming P-glycoprotein pump, this could be explained by the fact that in the absence of drug pressure, an increase in the expression of the *pfmdr1* gene product will give a less favorable metabolic balance. Moreover, given that amplified regions of chromosomes may exceed 100 kb (7) and contain multiple genes, it can be expected that there will be significant metabolic costs associated with replication. It should be noted that in our study, parasite populations with increases in the average *pfmdr1* copy number were compared; the observed differences in fitness thus apply strictly to these particular populations containing mixtures of parasites with different copy numbers. Concurrent additional changes in the genome apart from the *pfmdr1* gene also cannot be excluded to play a role in the observed differences in fitness. The magnitude of fitness loss associated with *pfmdr1* amplification is on the same order as that observed for parasites with other mutations conferring resistance to antimalarial drugs, like atovaquone (9%) (21) and chloroquine (7 to 13%) (2).

In the absence of drugs, no reversal of the *pfmdr1* copy

numbers in the genome of the parasite was observed over a period of up to 6 months, and we estimated from this experiment that the reverse switch rate from two to one *pfmdr1* copies was less than 1 in 10^8 parasites. Since parasite populations with multiple copies are still likely to contain a small fraction of parasites with a single copy, which will outcompete the parasites with multiple copies in the absence of drug pressure, it is possible that a longer observation period would have shown a decrease in the *pfmdr1* copy number. Competition between parasite clones with different *pfmdr1* copy numbers is more likely to occur in areas in sub-Saharan Africa with high rates of transmission, where infections with multiple clones are the rule, than in areas like Southeast Asia, where the rates of transmission are low. This could be a factor contributing to the delay of antimalarial drug resistance in settings with high rates of transmission.

In conclusion, the current study shows that *pfmdr1* gene amplification is a frequent event and is associated directly with mefloquine susceptibility in an in vitro culture system. In the absence of drug, the presence of multiple *pfmdr1* copies confers a significant fitness disadvantage.

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