Decreasing *pfmdr1* Copy Number in *Plasmodium falciparum* Malaria Heightens Susceptibility to Mefloquine, Lumefantrine, Halofantrine, Quinine, and Artemisinin

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The global dissemination of drug-resistant *Plasmodium falciparum* is spurring intense efforts to implement artemisinin (ART)–based combination therapies for malaria, including mefloquine (MFQ)–artesunate and lumefantrine (LUM)–artemether. Clinical studies have identified an association between an increased risk of MFQ, MFQ-artesunate, and LUM-artemether treatment failures and *pfmdr1* gene amplification. To directly address the contribution that *pfmdr1* copy number makes to drug resistance, we genetically disrupted 1 of the 2 *pfmdr1* copies in the drug-resistant FCB line, which resulted in reduced *pfmdr1* mRNA and protein expression. These knockdown clones manifested a 3-fold decrease in MFQ IC$_{50}$ values, compared with that for the FCB line, verifying the role played by *pfmdr1* expression levels in mediating resistance to MFQ. These clones also showed increased susceptibility to LUM, halofantrine, quinine, and ART. No change was observed for chloroquine. These results highlight the importance of *pfmdr1* copy number in determining *P. falciparum* susceptibility to multiple agents currently being used to combat malaria caused by multidrug-resistant parasites.

Plasmodium falciparum drug resistance is seriously hindering public health efforts to control infection and is contributing to a global increase in the burden of malaria. In addition to resistance to chloroquine (CQ) and sulfadoxine-pyrimethamine (SP), the former linchpins of malaria treatment, studies have revealed parasite resistance to alternatives, such as mefloquine (MFQ), in

Southeast Asia [1, 2]. In areas where MFQ resistance is prevalent, reduced efficacy can extend to other antimalarial drugs, including lumefantrine (LUM), halofantrine (HF), and quinine (QN), which share variable degrees of cross-resistance [3]. Reliable molecular markers of resistance play a vital, sentinel role in the surveillance of drug efficacy [4]. For example, screening for the *pfcrt* K76T mutation, which is strongly associated with CQ resistance in vitro and with CQ treatment failure in clinical settings, has documented the rapid worldwide dissemination of CQ resistance and highlighted the need for alternative first-line drugs in Africa [5–7]. Surveys of single-nucleotide polymorphisms in the *P. falciparum* dihydropteroate synthase and dihydrofolate reductase genes are similarly vital to monitoring resistance to SP and, together with clinical investigations, have revealed a rapid decrease in SP efficacy [2]. The progression of CQ and SP resistance leaves few alternative treatment strategies that are affordable. Current antimalarial strategies are based on ART-based combination therapies (ACTs), which usu-

ally include an ART (such as artesunate, artemether, or dihydroartemisinin) as a fast-acting component, and MFQ, LUM, a quinoline, or an antifolate as the partner drug [8].

The *P. falciparum* multidrug (MDR) resistance gene (*pfmdr1*) has been implicated in altering parasite susceptibility to a variety of currently available antimalarial drugs. This gene, located on chromosome 5, encodes a predicted 12-transmembrane-domain protein, PfMDR1 (also known as "Pgh-1") [9, 10]. PfMDR1 localizes to the parasite digestive vacuole, which is the site of action of CQ and possibly of other quinoline-based antimalarial drugs, including QN [11–13]. A member of the ABC transporter family, PfMDR1 is a homologue of mammalian P glycoprotein, which is a determinant of MDR resistance in mammalian tumor cells [14]. Point mutations in *pfmdr1* have been associated with changes in parasite susceptibility to CQ, QN, MFQ, and ART derivatives in both laboratory lines and clinical isolates, but these mutations have limited use as molecular markers [1, 14, 15].

Amplification of *pfmdr1* has been implicated in MDR resistance in both in vitro and clinical studies. Early studies on the in vitro selection of MFQ-resistant culture-adapted lines identified increases in *pfmdr1* copy number, which correlated with elevated *pfmdr1* transcript and protein levels [9–11, 16–20]. In vitro selection studies also observed an inverse relationship between MFQ and CQ susceptibility that was associated with changes in *pfmdr1* copy number [19–21]. Analyses of field isolates confirmed the association between *pfmdr1* copy number and parasite susceptibility to MFQ in most studies, although not in all of them [17, 22–26]. Recently, a comprehensive prospective study in Thailand provided compelling evidence that increased *pfmdr1* copy number is a determinant of MFQ treatment failure and also increases the risk of failure of MFQ-artesunate combination therapy [27]—indeed, in multivariate analysis, *pfmdr1* copy number was the most important predictor of failure, and this was not altered by the addition of point-mutation data.

In the present study, we sought to define the role played by *pfmdr1* copy number in *P. falciparum* resistance to MFQ and to extend this analysis to other drugs currently being used to treat malaria caused by CQ- and SP-resistant parasites. To do this, we genetically disrupted 1 of the 2 copies of *pfmdr1* present in the drug-resistant *P. falciparum* FCB line and assessed the subsequent alterations in drug susceptibility. The data from our experiments—and their implications for ACT—are presented below.

MATERIALS AND METHODS

Parasites and transfection. The *P. falciparum* FCB line was cultured and transfected as described elsewhere [28]. Episomally transfected parasites were selected with 2.5 μg/mL blasticidin HCl (Invitrogen). Plasmid integration into the endogenous *pfmdr1* locus was detected by polymerase chain reaction (PCR) and was confirmed by Southern blot analysis (see below). Recombinant parasites were cloned by limiting dilution and identified by their expression of parasite lactate dehydrogenase, as described elsewhere [7].

DNA constructs. A 1.6-kb *pfmdr1* coding-sequence fragment (nt 1372–2983 of the 4.3-kb *pfmdr1* gene; PlasmoDB identification no. PFE1150w [available at: http://www.plasmoDB.org/]) was amplified by PCR from FCB genomic DNA with the primers 5'-AAGGATCCGGAGTTGTTAGTCAAGATC-CAT-3' (*Bam*HI site underscored) and 5'-AAGCGGCCGCATG-CATATTATAAAATGCTTCCTGT-3' (*Not*I site underscored). This fragment was subcloned into *Bam*HI/*Not*I-digested pcamBSD [28]. This transfection plasmid expresses the blasticidin-S-deaminase *(bsd)* selectable marker, which is under the control of a 0.6-kb *P. falciparum* calmodulin 5' untranslated region (UTR) and a 0.6-kb *hrp2* 3' UTR. The resulting 6.1-kb plasmid was designated "pcamBSD$^{KD/mdr}$."

DNA analysis. *P. falciparum* DNA was purified from saponin-released trophozoite pellets by use of DNeasy tissue kits (QIAGEN). PCR-based detection of integration (figure 1) used the *pfmdr1*-specific primers P1 (5'-TTAGAACAAGTGAGTTCA-GGAAT-3') and P4 (5'-AATTTTCCAGCATAACTACCAGT-3') and the pBluescript-specific primers P2 (5'-CAATTAACCCTCA-CTAAAGGG-3') and P3 (5'-GCGTAATACGACTCACTATAG-GGC-3'). For Southern blot analysis, 1–2 μg of DNA was digested by use of *Bam*HI, electrophoresed, and transferred onto nylon membranes. Hybridizations were performed with a hexamer-primed [^{32}P]-labeled probe that was prepared from full-length *bsd*. Plasmid rescue was performed by electroporating *Escherichia coli* (DH5α strain) with 100 ng of parasite DNA. The rescue efficiency, calculated as the number of colony-forming units (cfu) per microgram of genomic DNA, is a measure of the number of episomally replicating plasmids in individual lines [29].

RNA preparation and quantitative real-time reverse-transcription (RT)–PCR assays. Parasites were tightly synchronized using consecutive rounds of sorbitol lysis performed on ring-stage parasites. Samples were collected from 5 time points over the following generation, corresponding to early rings, mid rings, late rings/early trophozoites, mid-late trophozoites, and schizonts (harvested at ~10, 18, 26, 34, and 42 h after invasion). RNA was prepared after Trizol treatment (Invitrogen) of saponin-lysed parasite pellets and was treated with 2 rounds of DNaseI (Ambion), to remove contaminating genomic DNA (confirmed with intron-spanning *pfcrt* and β-tubulin gene primers; data not shown). cDNA was prepared from purified RNA by reverse transcription with oligo-dT primers. Quantitative reactions to determine relative transcript levels between lines were performed using real-time PCR [27]. Primers and probes for *pfmdr1* and the β-tubulin gene (PlasmoDB identi-

A. pcamBSD$^{KD/mdr}$
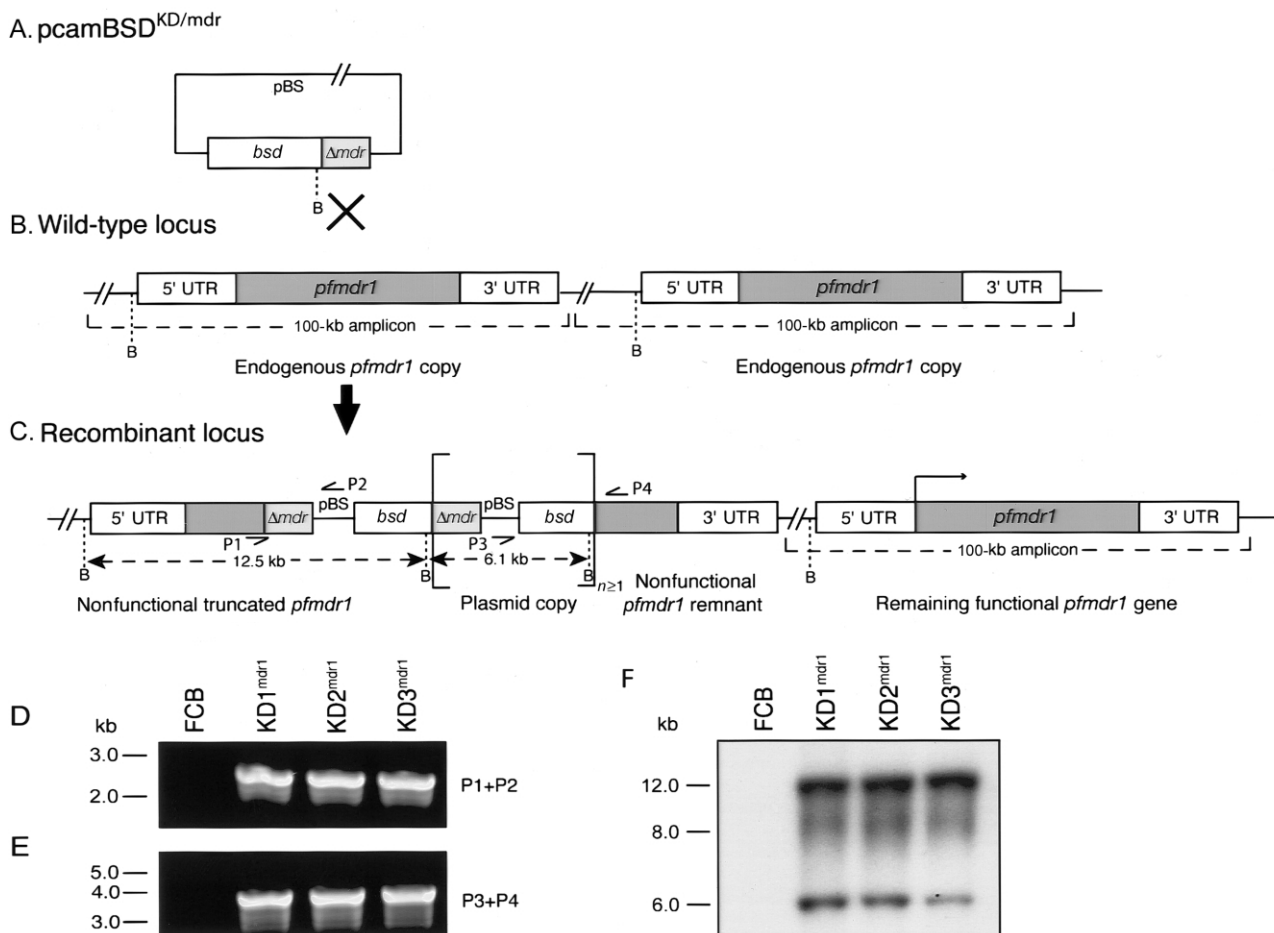
B. Wild-type locus

C. Recombinant locus

D

E

F

Figure 1. *pfmdr1* knockdown strategy and molecular characterization of clones. The transfection plasmid pcamBSD$^{KD/mdr}$ contains a 1.6-kb *pfmdr1* fragment (Δ*mdr*) centrally located in the coding sequence and a blasticidin-S-deaminase *(bsd)* selectable marker cassette *(A)*. Single-site crossover between the plasmid and 1 of the 2 endogenous *pfmdr1* copies results in inactivation of the copy, leaving 1 functional *pfmdr1* copy remaining (*B* and *C*). The disrupted locus contains an upstream *pfmdr1* fragment lacking the 3′ end of the gene and the 3′ untranslated region (UTR) as well as a downstream *pfmdr1* fragment lacking the 5′ UTR and the 5′ start of the gene, with these 2 fragments separated by the *bsd* selectable marker. Polymerase chain reaction (PCR) primers (P1–P4) and *Bam*HI (B) fragment sizes are indicated. Square brackets delineate the plasmid sequence that can integrate as tandem linear copies ($n \geqslant 1$). PCR analyses of the parental FCB line and the knockdown clones (KD1^{mdr1}, KD2^{mdr1}, and KD3^{mdr1}) with primers specific for either the upstream truncated locus (P1+P2) or the downstream remnant (P3+P4) confirmed the disruption of 1 copy of *pfmdr1* (*D* and *E*). Southern blot hybridization of *Bam*HI-digested genomic DNA samples with a *bsd* probe revealed 12.5-kb and 6.1-kb bands in the recombinants only, which is consistent with integration of tandem plasmid copies into the *pfmdr1* locus *(F)*. The no. of plasmid copies that integrated in tandem was estimated by densitometry to be 3 for KD1^{mdr1} and KD2^{mdr1} and 2 for KD3^{mdr1}, indicating that the integration event that occurred in KD3^{mdr1} was distinct from that which occurred in the 2 other clones. pBS, pBluescript.

fication no. PF10_0084) have been reported elsewhere [27]. Note that the *pfmdr1* real-time RT-PCR is specific for the 3′ end of this gene and detects full-length transcript but not the truncated sequence present in the upstream, nonfunctional locus in the knockdown clones (figure 1). For the 13-exon *pfcrt* gene (PlasmoDB identification no. MAL7P1.27), the primers used were 5′-AATATAAAAAATGGTTTCGCATGTTTA-3′ (exon 7) and 5′-AGAAGGAAAACAATGCGAAGGTT-3′ (exon 9), resulting in amplification of nt 844–973 of the *pfcrt* coding sequence, and the FAM-labeled internal probe was 5′-TCCATGCTCCGTCACAATCATCACAT-3′ (exon 8). *pfmdr1*

and *pfcrt* transcript levels were normalized against that of the single-copy *β*-tubulin gene. All probes had a TAMRA (6-carboxytetramethylrhodamine) label at their 3′ end. Values were calibrated by performing parallel assays with DNA from the reference 3D7 line, which has a single copy of *pfmdr1*. Data were quantified using the $2^{-\Delta\Delta Ct}$ method [27].

Protein analysis. Protein extracts were prepared from sodium deoxycholate–treated, sorbitol-synchronized trophozoite-stage parasites, as described elsewhere [28]. For each sample, protein from $\sim 1 \times 10^{6}$ parasites was loaded per well, electrophoresed on 10% SDS-PAGE gel, and transferred onto poly-

vinylidene difluoride membranes (Bio-Rad). Membranes were probed with rabbit anti-PfMDR1 antibodies (diluted 1:500), which were raised against a C-terminal fragment containing the last 168 aa [11], followed by incubation with horseradish peroxidase–conjugated donkey anti–rabbit IgG (diluted 1:5000; Santa Cruz Biotech). Rabbit anti–PfERD2 antibodies (MR4, ATCC; http://www.malaria.MR4.org/) were used at a 1:500 dilution [30]. Bands were visualized using the ECL Western blot analysis system (Amersham Biosciences). Protein levels were quantified by densitometric analysis of autoradiograph data, using NIH Image (version 1.6.2; available at: http://rsb.info.nih.gov/nih-image/). PfMDR1 band intensities were normalized against the PfERD2 bands, to correct for minor differences in protein loading.

In vitro antimalarial drug assays. MFQ, LUM, and HF were provided by W. Ellis and W. Milhous (Walter Reed Army Institute of Research, Silver Spring, MD). ART, QN, and CQ were purchased from Sigma. Parasite susceptibilities to antimalarial drugs were measured in vitro by [³H]-hypoxanthine assays [31]. Briefly, predominantly ring-stage cultures that had been sorbitol synchronized were seeded in duplicate in 96-well plates at a parasitemia and hematocrit of 0.4% and 1.6%, respectively. [³H]-hypoxanthine (0.5 μCi/well) was added after 48 h, and cells were harvested after another 24 h. IC_{50} values were determined as described elsewhere [31]. For statistical comparisons between lines, 1-way analysis of variance tests with Bonferroni posttests were performed for each drug.

RESULTS

Generation of knockdown clones containing 1 less copy of pfmdr1. To study the contribution that *pfmdr1* amplification makes to *P. falciparum* resistance to antimalarial drugs, we applied a gene-disruption strategy to the *P. falciparum* FCB line. This line has two 100-kb amplicons that each contain 1 copy of *pfmdr1* (expressing the haplotype 86Y/184Y/1034S/1042N/1246D, which is frequently found in Southeast Asia) and has low and moderately high levels of resistance to MFQ and CQ, respectively (mean ± SE IC_{50} values of 22.1 ± 0.6 and 216.8 ± 34.6 nmol/L, respectively) (table 1) [32, 33]. The genetic strategy is outlined in figure 1. Briefly, we constructed the 6.1-kb pcamBSD$^{KD/mdr}$ plasmid to contain an internal 1.6-kb *pfmdr1* fragment from the central region of this gene and a *bsd* selectable marker cassette (figure 1*A*). Single-site crossover between this plasmid insert and the homologous *pfmdr1* sequence in one of the tandem endogenous copies was predicted to disrupt a single copy, leaving a single functional locus (figure 1*B* and 1*C*).

FCB parasites transfected with the pcamBSD$^{KD/mdr}$ plasmid were screened for integration into the *pfmdr1* locus by PCR, and knockdown clones were subsequently obtained by limiting dilution. These clones were named "KD1^{mdr1}," "KD2^{mdr1}," and

"KD3^{mdr1}." Plasmid rescue of genomic DNA prepared from these clones confirmed that they no longer harbored episomes (rescue efficiencies of <100 cfu/μg of genomic DNA for these clones vs. efficiencies of 1×10^4–1×10^5 cfu/μg of genomic DNA for episomally transfected control lines; data not shown). The disruption of 1 *pfmdr1* copy was demonstrated by PCR with primer pairs specific for the upstream and downstream truncated fragments, which produced the expected 2.6-kb and 3.5-kb bands with the primer pair P1 and P2 and the primer pair P3 and P4, respectively (figure 1*D*). Disruption of this second endogenous *pfmdr1* copy was confirmed by Southern blot analysis. Hybridization of *Bam*HI-digested genomic DNA samples with a *bsd* probe revealed 12.5-kb and 6.1-kb bands in the recombinant clones (figure 1*F*), as was expected for single-site crossover into this locus. The presence of the 6.1-kb plasmid band in the knockdown clones provided evidence that the plasmid had integrated into the endogenous locus as multiple tandem copies. The numbers of integrated copies differed between KD3^{mdr1} and the 2 other clones, indicating independent integration events.

Reduced transcript and protein expression in pfmdr1 knockdown clones. The effect that the genetic disruption of 1 *pfmdr1* copy had was determined at both the transcript level and the protein level. To compare transcript levels, cultures of FCB parasites and the knockdown clones were tightly synchronized and used to prepare RNA from 5 time points over a single 48-h generation. cDNA was then prepared and subjected to quantitative PCR analysis. *pfmdr1* transcript levels for the FCB parasites and the knockdown clones were normalized against those for the single-copy β-tubulin gene and the reference 3D7 line, which carries a single copy of *pfmdr1* [27]. For comparison, quantitative multiplex PCRs were also performed with primers for *pfcrt*, whose transcription profile is similar to that of *pfmdr1* (see http://www.plasmoDB.org/). *pfcrt* transcript levels were also normalized against β-tubulin and 3D7. For both *pfmdr1* and *pfcrt*, the highest normalized transcript levels were observed in early ring stages and revealed an ~80% reduction in *pfmdr1* transcript levels for the KD1^{mdr1} and KD2^{mdr1} clones, compared with that for the parental FCB line. In contrast, *pfcrt* transcript levels remained unchanged for the *pfmdr1* knockdown clones, compared with those for the FCB line (figure 2).

To compare PfMDR1 expression levels between the recombinant clones and the parental line, Western blot analysis was performed using polyclonal anti-PfMDR1 antibodies. This de-

Table 1. Antimalarial drug IC_{50} values for the parental FCB line and the *pfmdr1* knockdown clones.

The table is available in its entirety in the online edition of the *Journal of Infectious Diseases.*
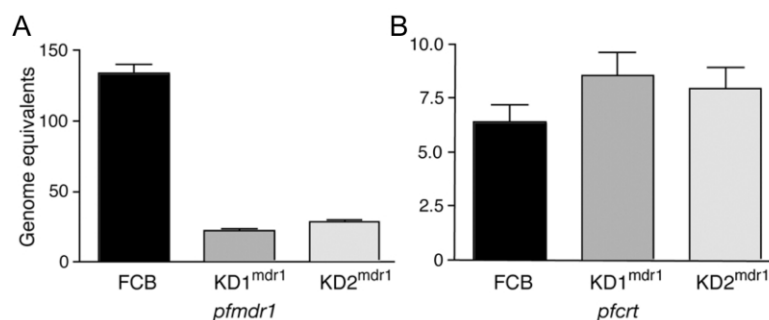
Figure 2. Quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) analysis of the parental FCB line and the *pfmdr1* knockdown clones. The effect that disruption of 1 copy of *pfmdr1* had on transcript levels was determined by quantitative real-time PCR using cDNA prepared from tightly synchronized parasites. *pfmdr1* transcript levels for each are presented as mean ± SE normalized genome equivalents [27]. Panel A shows an ~80% reduction in *pfmdr1* transcript levels for the clones KD1^{mdr1} and KD2^{mdr1}, compared with that for the FCB line, as determined from early ring-stage preparations (when transcription is maximal). Results for KD3^{mdr1} were excluded, because of low real-time PCR yields. Panel B shows the results for parallel quantitative real-time RT-PCRs, which revealed no significant change in *pfcrt* transcript levels between the parental line and the knockdown clones.

tected an ~160-kDa protein, as has been previously reported [11]. These same protein samples were also probed with control polyclonal antibodies to PfERD2, a *P. falciparum* ER-Golgi transport protein [30]. Comparison of PfMDR1 signal intensities (normalized against these PfERD2 results) revealed a reduction in PfMDR1 expression levels in the order of 40%–60% for the knockdown clones, which is consistent with expression of 1 of the 2 original gene copies (figure 3).

Drug response of* pfmdr1 *knockdown clones. IC$_{50}$ values for these parasite clones were obtained for MFQ, LUM, HF, QN, ART, and CQ and are shown in figure 4 (numerical values are provided in table 1). Of note, the disruption of 1 copy of *pfmdr1* resulted in a 3-fold decrease in MFQ IC$_{50}$ values, which decreased from a mean of 22 nnol/L for the parental FCB line to 6–7 nmol/L for the knockdown clones ($P<.0001$). The response to LUM was also pronounced, with the knockdown parasites displaying a 4–5-fold decrease in IC$_{50}$ values, which decreased from 90 nmol/L for the FCB line to 18–24 nmol/L for the knockdown clones ($P<.001$). A decrease was also observed for HF, with IC$_{50}$ values reduced from 1.2 nmol/L for the FCB line to 0.5–0.6 nmol/L for the knockdown clones ($P<.0001$).

Significant decreases in IC$_{50}$ values were also observed for ART (decreasing from 34 nmol/L for the FCB line to 19–22 nmol/L for the knockdown clones; $P<.05$) and for QN (decreasing from 419 nmol/L for the FCB line to 216–239 nmol/L for the knockdown clones; $P<.001$). These results indicate that amplification of *pfmdr1* directly contributes to reduced in vitro susceptibility to these antimalarial drugs. There were no significant changes in CQ IC$_{50}$ values for the knockdown clones, compared with those for the FCB line (all values ranged from 204 to 242 nmol/L).

DISCUSSION

pfmdr1 copy number has a pronounced effect on the response of *P. falciparum* to a number of drugs currently being used to treat malaria caused by CQ- and SP-resistant parasites. In the present study, ablation of a second functional copy of *pfmdr1* in the MDR-resistant FCB line and phenotypic comparison of the resulting knockdown clones with the parental line has dem-
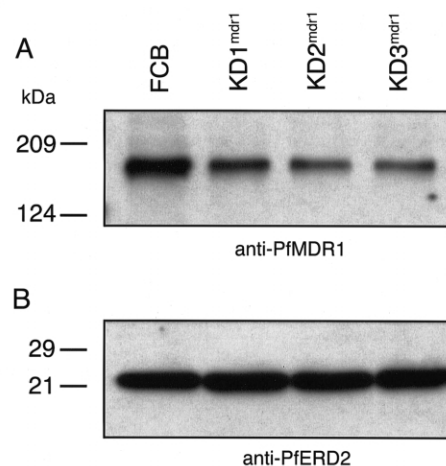

Figure 3. Western blot analysis of the parental FCB line and the *pfmdr1* knockdown clones. Probing of sorbitol-synchronized trophozoite-stage proteins with anti-PfMDR1 antibodies revealed an ~160-kDa band in both the parental line and the clones *(A)*. Densitometric analysis revealed that PfMDR1 expression levels (normalized to PfERD2 expression levels) were reduced by 38%, 61%, and 52% for the KD1^{mdr1}, KD2^{mdr1}, and KD3^{mdr1} clones, respectively. Densitometric analysis of these PfERD2 bands was indicative of essentially equivalent protein loadings across the parental FCB line and the knockdown clones, with the clones displaying signals that were 85%–106% of those of the FCB line *(B)*.
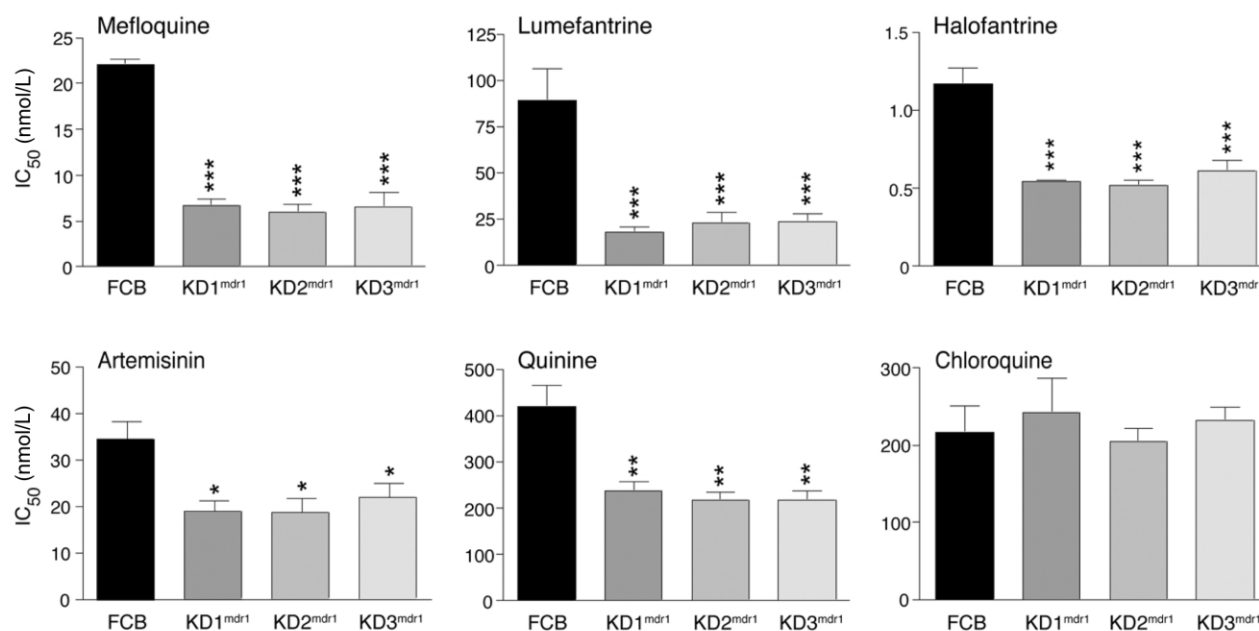
Figure 4. In vitro antimalarial drug response of the *pfmdr1* knockdown clones. In vitro [^{3}H]-hypoxanthine incorporation assays (72 h) were performed with the knockdown clones and the parental FCB line, which were tested in duplicate against each antimalarial drug on 3–5 separate occasions. IC$_{50}$ values (shown as means ± SEs) were derived by regression analysis. Numerical values are listed in table 1. For statistical comparisons, 1-way analysis of variance tests with Bonferroni posttests were performed. $*P < .05$, $**P < .01$, and $***P < .001$, compared with the FCB parental line.

onstrated that *pfmdr1* gene amplification significantly reduces parasite susceptibility to MFQ, LUM, HF, ART, and QN. Our data on MFQ complement those of a recent clinical study that found an association between increased *pfmdr1* copy number and increased risk of failure of MFQ monotherapy or MFQ-artesunate combination therapy [27]. That study observed a gene dose effect, whereby incremental increases in copy number translated into increasing risks of treatment failure; for both treatment regimens, however, the transition from 1 to 2 copies of *pfmdr1* signaled the highest increase in the risk of treatment failure as well as the largest shift in IC$_{50}$ values in the sampled Thai parasite populations [27]. The present results for LUM also agree with those from a separate clinical trial in Thailand [34], which found that increased *pfmdr1* copy number was associated with parasite recrudescence after a low-dose course of LUM-artemether. That effect, however, was not detected with an increased LUM-artemether dose regimen [34]. In the present study, the 3- and 5-fold decreases in IC$_{50}$ values observed for MFQ and LUM, respectively, underscore the marked effect that *pfmdr1* copy number has on their in vitro potency and highlight the importance of surveying for *pfmdr1* copy number to monitor the spread of resistance to these agents.

Both MFQ and LUM are important partners of ACTs. The present study revealed a 2-fold increase in ART potency after genetic ablation of a second copy of *pfmdr1*, confirming earlier reports of an in vitro association between *pfmdr1* copy number and ART susceptibility [24, 26, 27]. This association, however, does not extend to clinical resistance, which to date has not been observed with the ART family of endoperoxide antimalarial drugs [8, 35]. Our data suggest that PfMDR1 expression levels might modulate the disposition of ART in the parasites and thereby alter in vitro susceptibility.

Our phenotypic data on the arylaminoalcohol antimalarial drugs MFQ, LUM, and HF are consistent with previous reports of cross-resistance between these drugs in both field isolates and laboratory lines [17, 19, 20, 27, 36, 37]. These findings confirm that *pfmdr1* copy number contributes to their cross-resistance patterns. Other factors that could play a role in resistance to these drugs in vitro include the presence of other genetic polymorphisms in *pfmdr1*, as evidenced, for example, by allelic-exchange studies in culture-adapted lines that demonstrated that the C-terminal S1034C, N1042D, and D1246Y mutations had an effect on in vitro parasite responses to MFQ, HF, QN, and ART [28, 38]. Field studies supporting this include one that was recently conducted in Zanzibar and that indicated that late treatment failures for LUM-artemether were associated with selection for parasites harboring the *pfmdr1* 86N mutation [39, 40]. *pfcrt* mutations can also alter in vitro susceptibilities to MFQ and HF, as has been demonstrated in allelic-exchange and drug-selection experiments [7, 41, 42]. Additional deter-

minants may exist, as suggested by a study in Thailand that identified in vitro MFQ resistance in 16 of the 85 isolates harboring a single copy of *pfmdr1* [27]. High-level MFQ resistance observed in vitro may also involve separate genes, as seen in the laboratory-adapted MFQ-resistant FAC8 and W2mef lines (each of which contained 3 copies of *pfmdr1*), which, on selection for high-level resistance, showed no further *pfmdr1* amplification [19, 43].

Earlier studies of MFQ selection produced resistant lines that were found to have undergone sizeable amplifications of chromosome 5 segments harboring the *pfmdr1* locus [19, 20]. Mapping of amplicon breakpoints from these lines and other culture-adapted field isolates has identified multiple origins of amplification, with amplicons ranging in size from 20 to 200 kb [16, 23, 32]. The present data imply that the alteration in *pfmdr1* copy number was the causal factor accounting for changes in response to MFQ. Interestingly, several selection studies have also found that amplification or deamplification of this chromosome 5 region affected CQ response, such that increased *pfmdr1* copy number was associated with increased CQ susceptibility [19–21]. This association has been observed in some, although not all, studies conducted with field isolates [24, 27]. Our knockdown clones, however, showed no significant difference in response to CQ. We suggest that this may reflect either a strain-specific contribution of *pfmdr1* copy number to CQ resistance or an attenuation of its effect by the combined contribution of point mutations in *pfcrt* and *pfmdr1*. Of note, the FCB line used in our experiments carries the Asian/African *pfcrt* allele that confers CQ resistance as well as the *pfmdr1* N86Y mutation that has been associated with resistance to CQ and with reduced MFQ IC_{50} values in some, but not all, studies [5, 7, 14, 27, 33]. This N86Y mutation has been found very rarely in field isolates harboring multiple *pfmdr1* copies in studies conducted to date [17, 24, 26, 27, 34], suggesting that parasites harboring multicopy 86Y alleles fare poorly in natural infection. Attempts to transfect the MFQ-resistant Tm91C235 Thai isolate (which harbors 2 copies of wild-type *pfmdr1* [44]) with the knockdown transfection plasmid used here for the FCB line have, to date, proven to be unsuccessful (data not shown).

Our present findings also imply a role for *pfmdr1* copy number in in vitro QN resistance (defined as an IC_{50} >450 nmol/L [38]), which is in agreement with the findings of some, although not all, studies that have tested for this association in vitro [17, 19, 23]. QN resistance, which is relatively rare and is mostly restricted to pockets of Southeast Asia [2], is nevertheless clearly multifactorial. Point mutations in *pfmdr1* and *pfcrt* [7, 42, 45] also contribute, as does a locus on chromosome 13 that was identified via analysis of a genetic cross and that may correspond to the parasite sodium-proton exchanger *pfnhe* (although this needs to be confirmed experimentally [46]).

Interestingly, an earlier study in West Africa identified MFQ resistance in areas where this drug had not been used and suggested that this may have resulted from QN use and cross-resistance [47]. The present findings suggest that *pfmdr1* copy number may play a role and highlight the potential utility of assessing *pfmdr1* copy number in areas where ACTs involving MFQ or LUM are being considered for widescale use against malaria caused by MDR-resistant parasites. This will be particularly important in areas such as Southeast Asia, where increased *pfmdr1* copy number is frequently observed. Even though increased *pfmdr1* copy number is rare in Africa at present, it will be useful to monitor its emergence there prospectively, especially given that increased copy number has been detected after MFQ selection pressure in Gabon [48].

The World Health Organization has endorsed ACTs, which pair highly potent and fast-acting ART derivatives with a partner drug, as the preferred policy for treatment of malaria caused by CQ-resistant *P. falciparum* [49]. Current ACT regimens include MFQ-artesunate, LUM-artemether, dihydroartemisinin-piperaquine (Artekin, which combines the active metabolite of ART with a bisquinoline that is active against CQ-resistant malarial parasites), SP-artesunate, and amodiaquine-artesunate. These regimens, although costly, benefit from the very rapid reductions in parasite biomass afforded by ART derivatives. Nevertheless, ART derivatives have relatively short plasma elimination half-lives in vivo, and their success also depends on the efficacy of the longer-acting partner [8]. Our genetic evidence indicating that *pfmdr1* copy number reduces in vitro susceptibility to MFQ, ART, and LUM emphasizes the importance of monitoring this marker of reduced drug susceptibility as efforts rapidly proceed to implement ACTs globally for the treatment of malaria.

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