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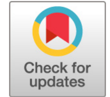

Expansion of a Specific *Plasmodium falciparum* PfMDR1 Haplotype in Southeast Asia with Increased Substrate Transport

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ABSTRACT Artemisinin-based combination therapies (ACTs) have been vital in reducing malaria mortality rates since the 2000s. Their efficacy, however, is threatened by the emergence and spread of artemisinin resistance in Southeast Asia. The *Plasmodium falciparum* multidrug resistance protein 1 (PfMDR1) transporter plays a central role in parasite resistance to ACT partner drugs through gene copy number variations (CNV) and/or single nucleotide polymorphisms (SNPs). Using genomic epidemiology, we show that multiple *pfmdr1* copies encoding the N86 and 184F haplotype are prevalent across Southeast Asia. Applying genome editing tools on the Southeast Asian Dd2 strain and using a surrogate assay to measure transporter activity in infected red blood cells, we demonstrate that parasites harboring multicopy N86/184F PfMDR1 have a higher Fluo-4 transport capacity compared with those expressing the wild-type N86/Y184 haplotype. Multicopy N86/184F PfMDR1 is also associated with decreased parasite susceptibility to lumefantrine. These findings provide evidence of the geographic selection and expansion of specific multicopy PfMDR1 haplotypes associated with multidrug resistance in Southeast Asia.

IMPORTANCE Global efforts to eliminate malaria depend on the continued success of artemisinin-based combination therapies (ACTs) that target *Plasmodium* asexual blood-stage parasites. Resistance to ACTs, however, has emerged, creating the need to define the underlying mechanisms. Mutations in the *P. falciparum* multidrug resistance protein 1 (PfMDR1) transporter constitute an important determinant of resistance. Applying gene editing tools combined with an analysis of a public database containing thousands of parasite genomes, we show geographic selection and expansion of a *pfmdr1* gene amplification encoding the N86/184F haplotype in Southeast Asia. Parasites expressing this PfMDR1 variant possess a higher transport capacity that modulates their responses to antimalarials. These data could help tailor and optimize antimalarial drug usage in different regions where malaria is endemic by taking into account the regional prevalence of *pfmdr1* polymorphisms.

KEYWORDS malaria, *Plasmodium falciparum*, *pfmdr1*, antimalarial drug resistance, copy number variation, Y184F mutation

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In 2018, an estimated 228 million cases of malaria, predominantly in sub-Saharan Africa, resulted in 405,000 deaths worldwide, mostly in children less than five years old. *Plasmodium falciparum* is responsible for most of the global malaria burden. Over the past 2 decades, impressive gains have been made in the reduction of global malaria morbidity and mortality rates, which stood at an estimated one million deaths per year in the early 2000s (1). The widespread adoption of artemisinin (ART)-based combination therapies (ACTs) and increased population-level coverage of insecticide-treated bed nets to protect against infective *Anopheles* mosquito vectors have been important contributors in the fight against malaria (2, 3). Unfortunately, the emergence and spread of ART- and ACT partner drug-resistant *P. falciparum* lineages in the Greater Mekong subregion of Southeast Asia (4–9) and in South America (10, 11) during the past decade pose a significant threat to malaria control and elimination. Efforts are under way to contain the spread of ART resistance into Africa and India (12–14) where it is predicted to have devastating results (15), as happened earlier with the emergence and global spread of chloroquine (CQ) resistance (8, 16, 17).

One important mediator involved in multidrug resistance to ACTs is the ATP-binding cassette (ABC) transporter *P. falciparum* multidrug resistance protein 1 (PfMDR1). PfMDR1 is localized in the membrane of the digestive vacuole (DV) of the parasite and possesses the ability to influx antimalarial drugs toward the lumen of this organelle (18–20). Transport studies using heterologous expression systems have shown that PfMDR1 variants have the ability to modulate the transport of the antimalarial drugs halofantrine and quinine (QN) (21). A surrogate assay of PfMDR1 activity using the fluorochrome Fluo-4 revealed its subcellular distribution and showed that PfMDR1-mediated solute import into the parasite DV is modulated by at least three C-terminal codon variants (S1034C, N1042D, and D1246Y) (22, 23). The *pfmdr1* N-terminal allelic variants N86Y and Y184F have also been implicated in multidrug-resistant phenotypes. In Africa and Asia, the N86 allele is relevant for *in vivo* and *in vitro* parasite antimalarial responses against aryl-amino alcohols such as mefloquine (MFQ) (24, 25) and lumefantrine (LMF) (26–28). In clinical trials of artemether-LMF or artesunate plus amodiaquine (ADQ), an increased risk of posttreatment recrudescent *P. falciparum* infections was associated with parasites harboring the N86 wild-type or 86Y mutant allele, respectively (29). These *in vivo* observations have been supported by recent allelic exchange approaches (30). Mutations in *pfmdr1* have a drug-specific resistance contribution being "pro and con" depending on the antimalarial. Compared to *pfmdr1* N86Y, the impact of the Y184F mutation on parasite responses to ACTs remains less studied (31). This mutation is one of the five canonical PfMDR1 mutations that was earlier considered to be associated with chloroquine resistance (32) and is now widespread in Southeast Asia and Africa (33–36). The capacity of 184F to influence parasite antimalarial responses is thought to be related to alterations in protein structure through allosteric effects that reduce drug binding or alter transport kinetics (20).*pfmdr1* copy number variation (CNV) is another important factor that impacts parasite susceptibility to several antimalarial drugs (18). *P. falciparum* parasites harboring *pfmdr1* amplifications are widely distributed in South America (37) and Southeast Asia (24, 25). Amplification of *pfmdr1* is associated with an increased risk of treatment failure with therapies combining an aryl-amino alcohol such as MFQ or LMF with an ART derivative (25, 38, 39).

Using data from the updated MalariaGEN *Plasmodium falciparum* Community Project (40), we explored the prevalence and distribution of *pfmdr1* copy numbers encoding specific haplotypes. We show that *pfmdr1* gene amplification is especially common in Southeast Asia, with amplifications being selected along with the N86 and 184F alleles. Applying genome editing tools on the 86Y- and Y184-harboring Southeast Asian Dd2 strain, we demonstrate that the N86/184F haplotype in the context of amplified *pfmdr1* imparts a higher transport capacity that directly affects parasite antimalarial responses.

RESULTS

Temporal distribution of PfMDR1 haplotypes and copy number variants in Southeast Asia. We determined the worldwide frequencies of *pfmdr1* N86Y and Y184F mutations and *pfmdr1* copy number using a collection of 7,113 parasite genomes from the MalariaGEN *P. falciparum* Community Project version 6 (https://www.malariagen.net/resource/26) (40). Genomic data were available from 73 countries where malaria is endemic with information on *pfmdr1* N86Y and Y184F mutations, as well as *pfmdr1* copy number, in a subset of 5,003 samples. From that subset, 650 had *pfmdr1* amplifications. Of these, 53 were excluded from further frequency analysis due to the presence of multiple alleles at codon positions 86 and/or 184, indicating the presence of mixed infections (see Table S1 in the supplemental material).*pfmdr1* allele frequency data were extracted from the MalariaGEN Community Project during the period from 2002 to 2015, revealing that the *pfmdr1* N86Y mutation frequency has remained relatively constant in Southeast Asia, with the N86 allele nearly fixed at a frequency of 96 to 98% (N86 allele frequency, 98% between 2002 and 2007, 98% between 2008 and 2010, 99% between 2011 and 2013, and 96% between 2014 and 2015). In Africa, the N86 allele frequency has increased, whereas 86Y has decreased over the same time period (see Fig. S1 in the supplemental material). In contrast to the fixation of the N86 allele in Southeast Asia, the frequency of the 184F variant has increased within the same time frame (21% between 2002 and 2007, 30% between 2008 and 2010, 40% between 2011 and 2013, and 59% between 2014 and 2015), leading to the selection of the N86/184F haplotype in Southeast Asia between 2002 and 2015 (Fig. S1).

The MalariaGEN Community Project data also showed that *pfmdr1* amplifications are the most prevalent in Southeast Asia, with only one African isolate reported in 2009 from Ghana harboring two *pfmdr1* copies encoding a N86/Y184 haplotype (Table S1). In Southeast Asia, infections with *P. falciparum* isolates harboring *pfmdr1* amplifications were most prevalent in Thailand (52%), followed by Cambodia, Myanmar, and Vietnam (15%, 14%, and 6%, respectively) (Fig. 1A). In Cambodia, the frequency of isolates with *pfmdr1* amplifications decreased progressively from 48% (12/25 isolates) during 2002 to 2007 to 25.6% (69/269 isolates) during 2008 to 2010 and 9.6% (55/572 isolates) between 2011 and 2013) (41, 42). In contrast to Cambodia, the frequency of isolates with *pfmdr1* gene amplifications in Thailand showed an increasing trend over the same period (45% [104/229] during 2002 to 2007, 47% [144/306] during 2008 to 2010, and 61% [168/275] during 2011 to 2013). Genomes containing *pfmdr1* amplifications encoding the N86 allele predominated (97.8%), with only three isolates identified in Cambodia carrying amplified copies of the *pfmdr1* 86Y variant (3/136 genomes) (Fig. 1B and Table S1).

In Cambodia, the 184F allele frequency in isolates also carrying multiple copies of the *pfmdr1* N86 allele has increased (25% [3/12] between 2002 and 2007% to 62% [43/69] between 2008 and 2010 and 85% [47/55] between 2011 and 2013). In contrast, in Thailand, the 184F allele frequency has remained stable in isolates containing multicopy *pfmdr1* over the years (8% [8/104] between 2002 and 2007% to 6% [8/144] between 2008 and 2010 and 10% [17/168] between 2011 and 2013). Limited or no genome data were available from Vietnam and Myanmar between 2002 and 2010, precluding the analysis of haplotype selection over time in these countries (Fig. 1B and Table S1).

Together, these results show that *pfmdr1* amplification is especially common in Southeast Asia, with amplifications encoding the N86 and 184F residues being selected.

Amplified *pfmdr1*-edited parasites at codons 86 and 184. We developed a targeted gene editing approach to study the impact of haplotype-specific increases in *pfmdr1* copy number on *P. falciparum*. Parasite lines derived from Southeast Asian *P. falciparum* isolates from the 1980s, like Dd2, characteristically contain the 86Y allele. To investigate the advantage of the predominant genotypes observed in Southeast Asia described above (*pfmdr1* amplifications containing haplotypes N86/184F or N86/Y184), we performed gene editing in all *pfmdr1* gene copies present in Dd2 parasites. Our Dd2 line represents a MFQ-resistant clone carrying four *pfmdr1* copies isolated from the

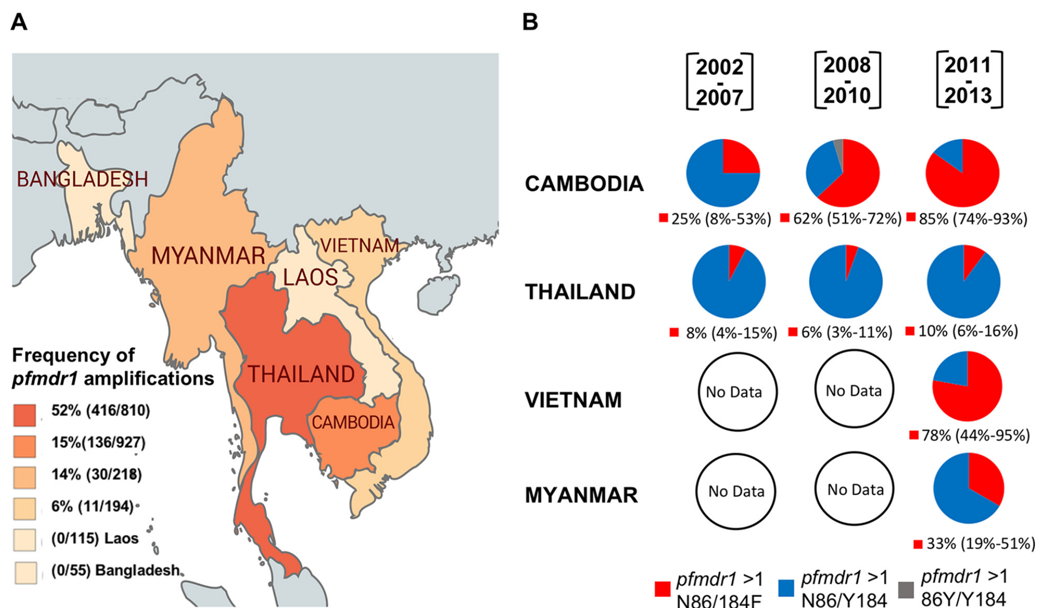

FIG 1 Geographical distribution of amplified *pfmdr1* gene copies and haplotype selection in Southeast Asia. Genetic analysis used data generated by the recently updated MalariaGEN *Plasmodium falciparum* Community Project (version 6; https://www.malariagen.net/resource/26) (40), comprising genomes with information on *pfmdr1* copy number, single nucleotide polymorphisms (SNPs), and no evidence of mixed infections. (A) Country shadings show the frequency of genomes with amplified *pfmdr1* copies (number of amplified *pfmdr1* copies/total number of genomes) collected between 2002 and 2015. (B) Pie charts represent different PfMDR1 haplotypes present in genomes containing multiple *pfmdr1* gene copies (per country data are shown in Table S1 in the supplemental material) and stratified into three intervals of time (2002 to 2007, 2008 to 2010, and 2011 to 2013). No samples were available for the years 2014 and 2015. The N86/184F haplotype frequency is shown with 95% confidence intervals in parentheses below each pie chart. Empty pie charts represent time periods in countries where no data were available.

W2mef line, originally derived from the Southeast Asian CQ-resistant parasite strain W2 (harboring a single copy of *pfmdr1*) upon extended culture with MFQ for 96 weeks (43). Dd2 and W2 parasites express the PfMDR1 86Y/Y184 haplotype and the CQ resistance-conferring CVIET haplotype of the *P. falciparum* chloroquine resistance transporter (PfCRT) (Table 1) (44, 45).

Using the previously described zinc-finger nuclease (ZFN) strategy to engineer *pfmdr1* mutations at codons 86 and 184, we successfully generated the Dd2 edited lines, NF^{Dd2} and NY^{Dd2}, each maintaining four copies of *pfmdr1* encoding the N86/184F and N86/Y184 haplotypes, respectively (Table 1 and Fig. 2A and B). These edited lines represent the two major geographic variants of *pfmdr1* present in Southeast Asia and include gene amplifications. The presence of pure N86/184F and N86/Y184 haplotypes in NF^{Dd2} and NY^{Dd2} lines, respectively, was confirmed by Sanger sequencing (Fig. 2C). Quantitative PCR analysis demonstrated that the edited NF^{Dd2} and NY^{Dd2} lines maintained four copies of *pfmdr1* as present in the unedited Dd2 parental strain (Fig. 2D). Attempts to engineer parasites expressing the 86Y/184F haplotype failed. We verified that this haplotype combination was not found among the 597 parasite genomes harboring amplified copies of *pfmdr1* in the MalariaGEN Community Project data set

TABLE 1 *pfmdr1* and *pfcrt* polymorphisms of gene-edited and parental *P. falciparum* laboratory strains

		pfmdr1 polymorphisms						*pfcrt* polymorphisms				
Parasite	Plasmid	CNV	86	184	1034	1042	1246	72	73	74	75	76
NF^{Dd2}	$pmdr1^{NFa}$	4	N	F	S	N	D	C	V	I	E	T
NY^{Dd2}	$pmdr1^{NYa}$	4	N	Y	S	N	D	C	V	I	E	T
Dd2		4	Y	Y	S	N	D	C	V	I	E	T
W2		1	Y	Y	S	N	D	C	V	I	E	T

[a]Plasmids were previously constructed (30). CNV, copy number variation.

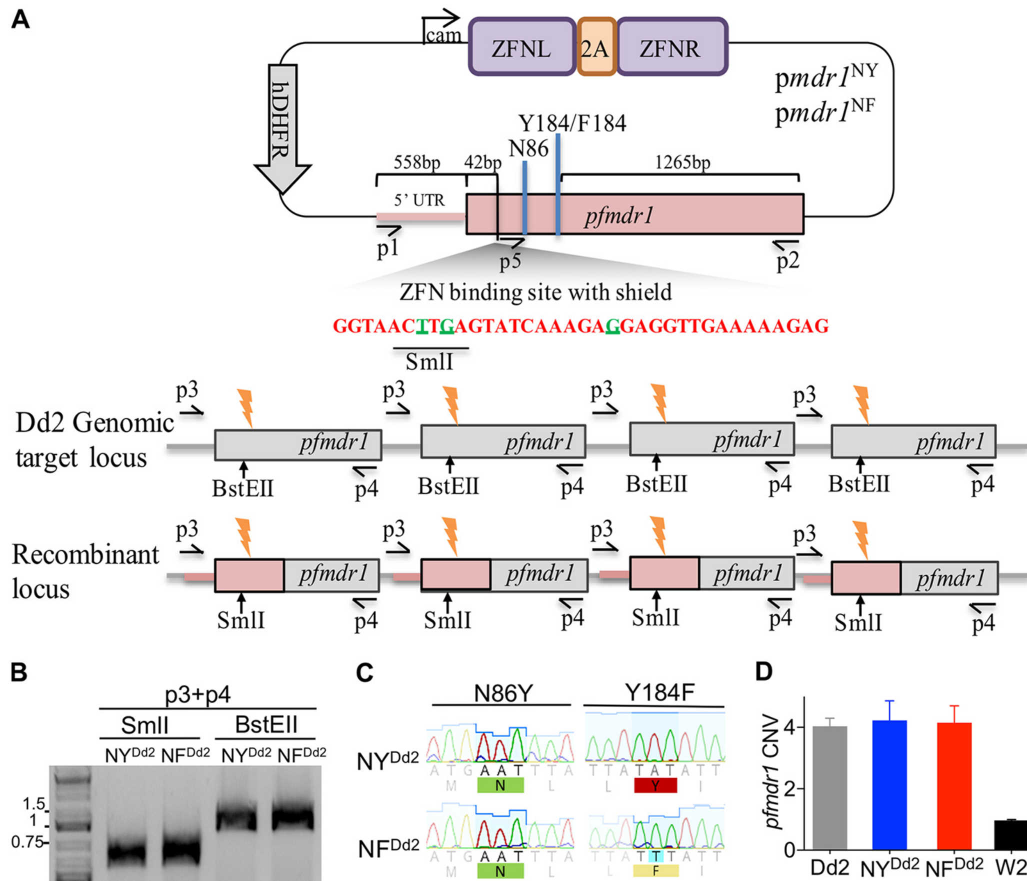

FIG 2 Schematic representation of the ZFN-based *pfmdr1* editing strategy. (A) The plasmids p*mdr1*NY and p*mdr1*NF (30) harbor a pair of *pfmdr1*-specific ZFNs (ZFN left [ZFN L] and ZFN right [ZFN R]) separated by a 2A skip peptide that drives their polycistronic expression from the calmodulin promoter (cam), and the human dhfr (hdhfr) selectable marker. The ZFNs induce a double-strand break (orange thunderbolt) 42 bp downstream of the *pfmdr1* start codon, which is repaired by homologous recombination with the 2.4-kb *pfmdr1* donor sequence present on the plasmid. The *pfmdr1* donor sequence from p*mdr1*NY and p*mdr1*NF plasmids contains the N86/Y184 or N86/184F genotypes, respectively, and carries three synonymous mutations (nucleotides colored in green) at the ZFN binding site to prevent ZFNs from cleaving the plasmids or the *pfmdr1*-edited sequences. Two of the three synonymous mutations create a SmlI restriction site and abolish a BstEII restriction site present in the nonedited (genomic) template. (B) PCR-RFLP genotyping confirming the presence of *pfmdr1*-edited lines NYDd2 and NFDd2. The silent mutations introduced into the donor sequence created a SmlI restriction site, which was used to screen PCR products (yielding a 1.3-kb band with P3 and P4 primers, which formed a doublet at 667/669 bp in edited parasites upon SmlI digestion). BstEII cuts the P3 and P4 PCR product in nonedited parasites and was used as a control; the observed 1.3-kb band was not cut, indicating the presence of correctly edited parasites. (C) Sanger sequencing chromatograms of DNA samples from the NYDd2 and NFDd2 edited lines at the *pfmdr1* locus, confirming correct editing at codons 86 and 184. (D) Plot showing means plus standard errors of the means (SEM) (error bars) from three independent assays of *pfmdr1* copy number, analyzed through quantitative PCR using β-tubulin as a control gene and the 3D7 strain as a calibrator with a single *pfmdr1* copy.

(Table S1). These findings suggest that the presence of the 86Y/184F haplotype in amplified *pfmdr1* copies might render the parasites nonviable or might confer a survival disadvantage to the parasite population.

Fluo-4 transport into the parasite DV is altered by amplified PfMDR1 haplotypes. We evaluated the influence of *pfmdr1* polymorphisms at codons 86 and 184 on PfMDR1 functional activity by measuring the accumulation of Fluo-4 in the DVs (22) of the gene-edited parasite lines (NF^{Dd2} and NY^{Dd2}) compared to the Dd2 parental control strain.

Accumulation of Fluo-4 in the DVs of NF^{Dd2}, NY^{Dd2}, and Dd2 parasites was observed by fluorescence microscopy and measured by flow cytometry (Fig. 3). Fluo-4 accumulation in the DV of the parasite was detectable after 120 min of incubation, prior to which time the probe was distributed throughout the infected red blood cell (RBC) and parasite cytosol (Fig. S2).

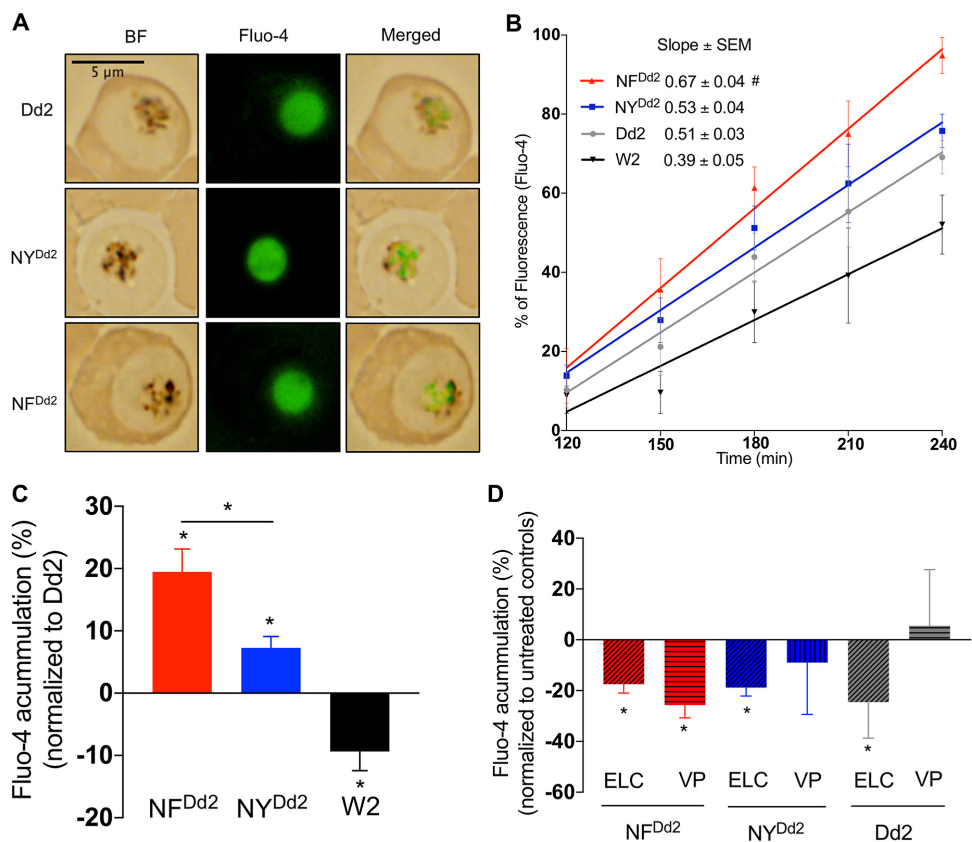

FIG 3 Evaluation of Fluo-4 accumulation. (A) Representative images of Dd2 and *pfmdr1*-edited lines (NY^{Dd2} and NF^{Dd2}) stained with Fluo-4 AM (green). BF, Bright field. The bar measures 5 μm. (B) PfMDR1-associated dynamic transport between the strains Dd2, NF^{Dd2}, NY^{Dd2}, and W2, as determined by linear regression of the Fluo-4 probe signal over time. Data are shown as comparative mean ± SEM (error bars) (as a percentage) from four independent assays. Linear regression and slopes extracted from each equation. # indicates a significant difference in the rate of Fluo-4 accumulation in NF^{Dd2} parasites compared with the rates obtained in NY^{Dd2} and Dd2 parasites ($P = 0.04$ and $P = 0.007$, respectively). (C) Normalized Fluo-4 AM intensities (as a percentage) in all parasite strains measured at 240 min. The fluorescence intensities exhibited by NF^{Dd2} and NY^{Dd2} and the W2 strain were normalized to the parental Dd2 parasite strain (indicated in the graph as the baseline [$y = 0$]). Values below or greater than zero indicate a decrease or increase, respectively, in the percentage of accumulated fluorescence compared with Dd2 parasites. Mann-Whitney U tests were used to assess statistically significant differences between the NF^{Dd2}, NY^{Dd2}, Dd2, and W2 parasites in panels B and C (*, $P < 0.05$). (D) Normalized Fluo-4 intensities (as a percentage) in the absence (untreated control) and presence of elacridar (ELC, 0.1 μM) and verapamil (VP, 0.8 μM). Results were normalized to untreated controls (indicated in the graph as the baseline [$y = 0$]). Values below or greater than zero indicate a decrease or increase, respectively, in the percentage of accumulated fluorescence compared with untreated controls. The means plus SEM of four independent measurements are shown. Mann-Whitney U tests were used to assess statistically significant differences between untreated and treated parasites (*, $P < 0.05$).

Time variances in Fluo-4 transport associated with the PfMDR1 N86/184F and N86/Y184 haplotypes in the edited parasite lines were measured by applying a linear regression model from 120 min to 240 min (Fig. 3B). We observed a higher positive slope in the edited NF^{Dd2} parasites that express the 184F variant (slope = 0.67 ± 0.04), compared with NY^{Dd2} ($P = 0.04$) or the Dd2 parental strain (86Y/Y184) ($P = 0.007$) (slope = 0.53 ± 0.04 and 0.51 ± 0.03, respectively), suggesting a nonnegligible influence of amino acid position 184 in PfMDR1 transport capacity (Fig. 3B).

To determine whether there were PfMDR1 haplotype-specific differences in the accumulation of Fluo-4, we measured the Fluo-4 intensity of the *pfmdr1*-edited lines and the W2 comparator strain after normalizing to the fluorescence intensity of Dd2 parasites after 240 min. The results showed that the N86 allele has an important role in the modulation of the PfMDR1 transporter since N86 edited parasites accumulated more Fluo-4 compared with the Dd2 parental strain containing the 86Y allele ($P = 0.03$). Furthermore, edited NF^{Dd2} parasites containing the N86/184F haplotype accumulated

more Fluo-4 in their DVs than edited NY^{Dd2} parasites (haplotype N86/Y184) ($P = 0.03$). W2 parasites, which harbor a single genomic copy of the *pfmdr1* gene with the 86Y/Y184 haplotype, accumulated less Fluo-4 ($P = 0.03$) relative to the parental Dd2 control strain that expresses four copies with the same 86Y/Y184 haplotype (Fig. 3C). The influence of *pfmdr1* copy number on the import of Fluo-4 into the parasite DV observed herein confirms previous reports where a parasite strain harboring only one *pfmdr1* copy accumulated less Fluo-4 compared with parasites expressing two copies (31).

To determine whether the changes in Fluo-4 accumulation were related to the PfMDR1 haplotype, we used elacridar (ELC) as a tool, since it is a well-established inhibitor of P-glycoprotein type ABC transporters (46). We measured the fluorescence intensity of Fluo-4 in NF^{Dd2}, NY^{Dd2}, or Dd2 parasites in the presence of ELC. Data were normalized to the respective untreated controls (NF^{Dd2}, NY^{Dd2}, or Dd2; indicated in Fig. 3D as the baseline [$y = 0$]). We observed a significantly decreased Fluo-4 accumulation in ELC-treated parasites compared with untreated controls ($P = 0.03$). The addition of verapamil (VP), a calcium ion channel blocker that reverses parasite resistance to CQ, led to a significant reduction of Fluo-4 accumulation only in the edited NF^{Dd2} line compared to untreated NF^{Dd2} ($P = 0.03$), suggesting a possible interplay between the PfMDR1 and PfCRT transporters in 184F-expressing parasites (Fig. 3D). The addition of VP produced a trend toward more Fluo-4 accumulation in NY^{Dd2} and Y184-harboring Dd2 parasites compared with NF^{Dd2}. Nevertheless, despite having a different trend, the quite large standard error of the mean (SEM) made these results inconclusive.

Amplified *pfmdr1* with the N86 allele decreases parasite susceptibility to antimalarials. We determined the impact of the N86 allele in the genomic context of multicopy *pfmdr1* on parasite susceptibility to antimalarial drugs. Replacement of the 86Y variant present in Dd2 with N86 in each of the four *pfmdr1* copies (edited parasite line NY^{Dd2}) induced a significant increase in the half-maximal *in vitro* growth inhibitory concentration (IC_{50}) values for MFQ (2.5-fold, $P = 0.02$) and LMF (2.4-fold, $P = 0.02$), as well as for dihydroartemisinin (DHA) (2.3-fold, $P = 0.05$) (Fig. 4 and Table 2). A reduction in the IC_{50} values for MFQ (6.1-fold, $P = 0.05$) in W2 (a single *pfmdr1* copy strain with the 86Y/Y184 haplotype) compared with Dd2 parasites was observed. These data show a clear role of *pfmdr1* gene amplifications, particularly those encoding the N86 allelic variant, in the multidrug resistance phenotype.

In the case of QN, the replacement of 86Y to N86 in multicopy *pfmdr1* resulted in a decrease of IC_{50} values in the NF^{Dd2} and NY^{Dd2} lines (1.6-fold, $P = 0.02$) relative to the parental Dd2 strain.

Our assays revealed no differences in susceptibilities to the quinoline drugs CQ, monodesethyl-ADQ (md-ADQ), piperaquine (PPQ), and pyronaridine (PND) in the context of multicopy *pfmdr1* containing N86.

The N86/184F PfMDR1 haplotype decreases parasite susceptibility to LMF. Analysis of the MalariaGEN *P. falciparum* Community Project data predicted a prevalence of the *pfmdr1* 184F allele in Africa of 58.6% (184F, 1,438/2,454 genomes sampled between 2002 and 2015) and 36% (184F, 858/2,403 between 2002 and 2015) in Southeast Asia, regardless of the number of *pfmdr1* copies (Table S1 and Fig. S1). The frequency of the 184F allele increased worldwide over the years analyzed (Table S1 and Fig. S1). Here, in the context of amplified *pfmdr1* gene copies, we observed up to 1.7-fold ($P = 0.002$) decreased susceptibility to LMF in edited parasites expressing the N86/184F haplotype compared with isogenic parasites expressing the N86/Y184 haplotype (Fig. 4 and Table 2). This suggests a specific effect of the 184F allele in conferring resistance to LMF in parasites that harbor amplified copies of *pfmdr1*.

PfMDR1-mediated resistance phenotypes are modulated by VP and ELC. We investigated the potential interplay between PfMDR1 and PfCRT transporters in modulating parasite susceptibility to antimalarials using the PfCRT and P-type ATPase transporter inhibitors VP and ELC, respectively. All the parasite strains used in this study express the CVIET variant at amino acid positions 72 to 76 of PfCRT (Table 1). In the

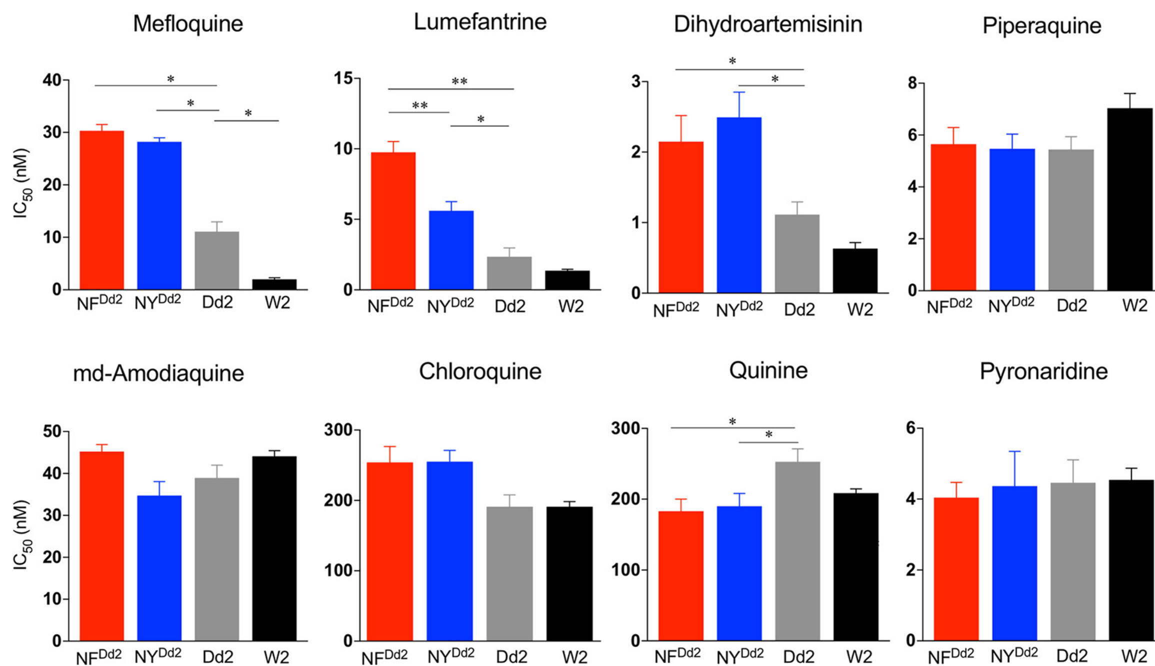

FIG 4 *In vitro* IC_{50} antimalarial response of NF^{Dd2}, NY^{Dd2}, Dd2, and W2 parasite strains. The antimalarial drugs (mefloquine [MFQ], lumefantrine [LMF], dihydroartemisinin [DHA], piperaquine [PPQ], monodesethyl-amodiaquine [md-ADQ], chloroquine [CQ], quinine [QN], and pyronaridine [PND]) were serially diluted, and after 72 h, the parasitemias were determined by flow cytometry after staining the parasites with Mito Tracker Deep Red FM and SYBR green. IC_{50} values were calculated using nonlinear regression analysis. Data are means plus SEM of at least three independent assays. Mann-Whitney U tests were used to assess statistically differences between Dd2 parental strain and NF^{Dd2}, NY^{Dd2}, and W2 strains. *, $P < 0.05$; **, $P < 0.01$.

presence of VP, the multicopy *pfmdr1* Dd2 strain and the edited parasites lines NF^{Dd2} and NY^{Dd2} exhibited increased susceptibility to the 4-aminoquinolines CQ and md-ADQ compared with the respective untreated parasites, with 5-fold and 2- to 3-fold changes, respectively. Increases in susceptibility were also obtained with QN and MFQ in the presence of VP compared with untreated parasites, with N86 producing higher fold changes than 86Y (QN, NF^{Dd2} = 2.8-fold, NY^{Dd2} = 3.1-fold, and Dd2 = 2.2-fold; MFQ, NF^{Dd2} = 4.1-fold, NY^{Dd2} = 5.0-fold, and Dd2 = 2.9-fold) (Table 2). The differences in fold changes observed in the presence of VP can be explained by the differential basal levels of susceptibility to QN and MFQ in the absence of VP in NF^{Dd2}, NY^{Dd2}, and Dd2 parasites (Fig. 4). For LMF, VP sensitized NF^{Dd2} and NY^{Dd2} parasites up to twofold with no effect observed on unedited Dd2 parasites compared with untreated parasites (Table 2).

Comparing the IC_{50} values between the three haplotypes in the presence of VP, a significant increase in QN susceptibility was observed in NF^{Dd2} and NY^{Dd2} parasites compared with the Dd2 parental control (NY^{Dd2} IC_{50} = 60.6 nM versus Dd2 IC_{50} = 111.5 nM, $P = 0.01$; NF^{Dd2} IC_{50} = 65.3 nM versus Dd2 IC_{50} = 111.5 nM, $P = 0.02$). For MFQ and LMF, there was an increase in IC_{50} values for NF^{Dd2} in the presence of VP compared with VP-treated Dd2 parasites (MFQ NF^{Dd2} IC_{50} = 7.5 nM versus Dd2 IC_{50} = 4.2 nM, $P = 0.005$; LMF NF^{Dd2} IC_{50} = 4.4 nM versus Dd2 IC_{50} = 2.5 nM, $P = 0.01$).

In the presence of the P-type ATPase transporter inhibitor ELC, the parasite lines NF^{Dd2} and NY^{Dd2} exhibited 2.8-fold and 2.5-fold increased susceptibility to MFQ, respectively, compared with the corresponding untreated parasites. It is interesting to observe that ELC treatment reverted the MFQ IC_{50} values of NF^{Dd2} and NY^{Dd2} parasites to the same level as that of Dd2 in the absence of ELC, validating the impact of the N86 *pfmdr1* variant. ELC treatment did not significantly impact the susceptibility of Dd2, NY^{Dd2}, or NF^{Dd2} parasites to any of the other aryl-amino alcohols tested (i.e., QN and LMF) or the 4-aminoquinolines (CQ and md-ADQ).

TABLE 2 Parasite responses to antimalarials in the presence of verapamil or elacridar[a]

Antimalarial drug(s)	NF^{Dd2}		NY^{Dd2}		Dd2	
	Mean IC_{50} (± SEM)	*P* value	Mean IC_{50} (± SEM)	*P* value	Mean IC_{50} (± SEM)	*P* value
MFQ	30.3 (±1.1)		28.2 (±1.0)		11.1 (±1.8)	
MFQ + VP	7.5 (±0.4)	0.03	5.6 (±0.8)	0.03	4.2 (±0.1)	0.03
MFQ + ELC	10.9 (±0.7)	0.03	11.1 (±1.5)	0.03	7.7 (±0.8)	NS
LMF	9.7 (±0.8)		5.6 (±0.6)		2.3 (±0.6)	
LMF + VP	4.4 (±0.3)	0.01	3.0 (±0.2)	0.008	2.5 (±0.3)	NS
LMF + ELC	6.8 (±1.7)	NS	5.5 (±0.8)	NS	3.6 (±0.4)	NS
DHA	2.1 (±0.4)		2.5 (±0.4)		1.1 (±0.2)	
DHA + VP	1.2 (±0.1)	NS	1.3 (±0.1)	NS	1.0 (±0.1)	NS
DHA + ELC	1.5 (±0.1)	NS	1.8 (±0.09)	NS	1.2 (±0.1)	NS
PPQ	5.7 (±0.6)		5.5 (±0.6)		5.4 (±0.5)	
PPQ + VP	4.8 (±0.9)	NS	4.7 (±0.2)	NS	4.9 (±0.5)	NS
PPQ + ELC	5.9 (±1.2)	NS	5.1 (±0.5)	NS	5.0 (±0.7)	NS
md-ADQ	45.2 (±1.7)		34.7 (±3.4)		38.9 (±3.0)	
md-ADQ + VP	21.7 (±1.0)	0.05	15.0 (±2.8)	0.05	14.3 (±1.2)	0.05
md-ADQ + ELC	39.6 (±3.9)	NS	35.4 (±3.2)	NS	43.3 (±1.4)	NS
CQ	254.1 (±15.8)		255.1 (±22.6)		191.2 (±16.6)	
CQ + VP	45.8 (±2.7)	0.008	47.2 (±2.8)	0.004	37.1 (±2.6)	0.004
CQ + ELC	211.9 (±18.4)	NS	224.7 (±19.2)	NS	169.2 (±6.6)	NS
QN	183.1 (±16.9)		190.0 (±18.1)		252.8 (±18.3)	
QN + VP	65.3 (±22.8)	0.003	60.6 (±33.3)	0.003	111.5 (±3.3)	0.003
QN + ELC	198.1 (±39.6)	NS	211.2 (±57.6)	NS	263.2 (±19.5)	NS
PND	4.1 (±0.4)		4.4 (±0.9)		4.4 (±0.6)	
PND + VP	2.5 (±0.5)	NS	3.4 (±0.2)	NS	3.0 (±0.3)	NS
PND + ELC	2.6 (±0.6)	NS	2.7 (±0.3)	NS	2.6 (±0.2)	0.05

[a]NF^{Dd2}, NY^{Dd2}, and Dd2 parasites were incubated with mefloquine (MFQ), lumefantrine (LMF), dihydroartemisinin (DHA), piperaquine (PPQ), monodesethyl-amodiaquine (md-ADQ), chloroquine (CQ), quinine (QN), and pyronaridine (PND) in the presence or absence of verapamil (VP) or elacridar (ELC). When incubated with verapamil or elacridar, the antimalarial drugs used in study were serially diluted in the presence of 0.8 μM VP or 0.1 μM ELC for the 72-h *in vitro* growth inhibition assays. Parasitemias were determined by flow cytometry after staining the parasites with Mito Tracker Deep Red FM and SYBR green. Mean ± SEM IC_{50} values (nanomolar) were derived from three to five independent drug assays. Mann-Whitney U tests were used to assess statistically significant differences between the untreated control parasites (Dd2, NY^{Dd2}, and NF^{Dd2}) and VP- or ELC-treated parasites. NS, not significant.

DISCUSSION

The propensity of *P. falciparum* to develop antimalarial drug resistance underscores the importance of characterizing its genetic basis. The amplification of *pfmdr1* was the first candidate mechanism implicated in CQ resistance (CQR) (47). Although we now know that PfCRT is the primary determinant of CQR (48), *pfmdr1* can modulate the degree of resistance (49, 50). Several other studies have also shown that increased *pfmdr1* copy number decreases the parasite's susceptibility to other antimalarials such as MFQ, LMF, and ART (25, 38, 39). The worldwide spread of the *pfmdr1* N-terminal 86Y and 184F mutations have also been linked with differential responses to multiple quinoline-based antimalarials and ART, both *in vitro* and *in vivo* (33, 36, 51–54). The conjugation of these two types of polymorphisms (gene copy number and specific sequence haplotypes) could modulate the *P. falciparum* response to these antimalarials.

Using data from the MalariaGEN *P. falciparum* Community Project, we determined the worldwide prevalence and temporal changes of *pfmdr1* copy number with specific *pfmdr1* N-terminal mutations. Furthermore, we measured the impact of these polymorphisms on solute kinetics of this transporter. We note that the MalariaGEN data from a given period (2002 to 2015 in our case) relates to samples whose locations were dependent on which partner studies were operative at the time (40). Temporal trends in aggregated data should therefore be interpreted with caution. From 73 countries where malaria is endemic with information on *pfmdr1* amplifications and mutations at amino acid positions 86 and 184, between 2002 and 2015, *pfmdr1* amplification was found almost exclusively in Southeast Asia, where we also observed a near fixation of the N86 allele (Fig. 1; see also Table S1 and Fig. S1 in the supplemental material). Considering the previous strong association of *pfmdr1* amplifications and the N86 allele with treatment failures that included MFQ or LMF (24, 25, 39), the frequencies observed could be explained by the extensive use of artesunate plus MFQ and artemether plus LMF in this region during that time period. Thailand, for example, is the country with

the highest frequency of *pfmdr1* amplifications, possibly reflecting the extensive use of artesunate-MFQ as a first-line antimalarial treatment. Its failure during the following years led to changes in treatment policy, with the adoption of dihydroartemisinin (DHA)-PPQ as first-line therapy in 2008 (1, 2). This combination was associated with a rapid decline in *pfmdr1* copy number (55, 56), which could have resulted from PPQ-mediated selection against multicopy *pfmdr1* and/or selective pressure against multicopy *pfmdr1* parasites that show some loss of fitness and can therefore be outcompeted by single-copy *pfmdr1* parasites (2, 57, 58). In Cambodia, the loss of DHA-PPQ efficacy led to the resumption of artesunate-MFQ use beginning in 2011, and this combination retains good efficacy with *pfmdr1* copy number generally remaining at one (59).

In contrast, the MalariaGEN data illustrate *pfmdr1* amplifications as a rare event in Africa. The substantial use of CQ in the past and the current use of the ACT combination artesunate-ADQ possibly contribute to this fact by selecting for deamplifications (60, 61). Furthermore, although artemether-LMF has been used for many years in Africa (62), the lack of MFQ usage could partially explain the lack of *pfmdr1* amplifications. Nevertheless, it is conceivable that *pfmdr1* amplifications may increase in prevalence in Africa due to the widespread use of artemether-LMF (63, 64). The presence of the 86Y allele in Africa could also contribute to the low prevalence of *pfmdr1* amplifications as this allele is present at a very low frequency worldwide when combined with *pfmdr1* amplifications (6/597 genomes). Another factor could be related to the known fitness cost associated with *pfmdr1* amplifications, which manifests as decreased parasite survival in the absence of drug pressure (58, 65). This feature is of particular relevance in high transmission areas such as in sub-Saharan Africa. An interesting observation is that within the MalariaGEN data, *pfmdr1* amplifications with the haplotype 86Y/184F were not present and despite several attempts to engineer this specific parasite line, we were unsuccessful, possibly related to a genetic background that is detrimental for survival.

Over time in Southeast Asia, we observed a clear selection of the 184F allele in combination with *pfmdr1* amplifications containing the N86 allele. Cambodia was the country with the highest observed selection (25% [3/12] between 2002 and 2007% to 62% [43/69] between 2008 and 2010 and 85% [47/55] between 2011 and 2013) (Fig. 1). It is important to note that at the same time that this takeover of the 184F occurs, the prevalence of *pfmdr1* duplications was actually shrinking due to the switch in first-line treatment to DHA-PPQ. To better understand this selection of the N86/184F haplotype in the context of amplified *pfmdr1*, we generated two edited lines representing the two major geographic variants from Southeast Asia and studied the impact of the specific haplotypes on antimalarial responses. The impact of these polymorphisms on PfMDR1 functional transport was also measured through accumulation of Fluo-4 in the DV of the parasite (Fig. 3). This approach has been previously used for *pfmdr1* variants and revealed an important impact of the C-terminal amino acid 1042 on Fluo-4 transport (22, 23). In these studies, although they used strains encompassing different haplotypes in regard to the *pfmdr1* N-terminal mutations, their direct impact was not evident. In our Fluo-4 accumulation study with the Dd2 strain and isogenic lines differing only in their *pfmdr1* N-terminal residues at positions 86 and 184, we observed that parasites expressing the mutations 86Y and 184F were capable of transporting Fluo-4 into the DV (Fig. 3A). The N86 allele by itself impacted Fluo-4 transport capacity, with significant differences obtained between the parental Dd2 strain and NY^{Dd2} (Fig. 3C). Transport studies in Xenopus oocytes have also provided evidence of the impact of polymorphisms at position 86 on the ability of PfMDR1 to transport different drugs. A single amino acid alteration from N86 to 86Y in PfMDR1 resulted in loss of the ability to transport QN and CQ (21). Consistent with this observation, the presence of the N86 allele induced a significant impact on antimalarial responses (Fig. 4). We were able to confirm the involvement of residue 86, specifically in the context of multicopy *pfmdr1*, in modulating parasite susceptibility to multiple antimalarials. Gene-edited parasites with amplified *pfmdr1* harboring the N86 allele displayed decreased susceptibility to

MFQ, LMF, and DHA as well as increased susceptibility to QN, compared to the unedited Dd2 parental line possessing the 86Y allele (Fig. 4). These findings are consistent with our previous allelic exchange work that used ZFN-edited parasites expressing the 86Y variant in the context of single-copy *pfmdr1* (30). This observation explains the selection of the N86 allele after treatment with artemether-LMF (26–28) and corroborates with N86 being a risk factor for recrudescence following treatment with artemether-LMF compared with *P. falciparum* infections containing 86Y (29).

The *pfmdr1* 184F mutation adds to the role of polymorphisms at position 86 in modulating parasite drug resistance and transport. We found that edited parasites harboring the N86/184F haplotype accumulated more Fluo-4 inside the DV compared to edited parasites with the N86/Y184 haplotype, accompanied by faster kinetics of PfMDR1-mediated transport activity (Fig. 3B). A previous in silico PfMDR1 model proposed that the 184F allele alters the parasite's response by an allosteric effect on transport kinetics, independent from drug-binding capacity (20). Our gene-edited parasites expressing amplified *pfmdr1* with the N86/184F haplotype displayed a mild but significant decreased susceptibility to LMF (1.7-fold) compared to those harboring the PfMDR1 N86/Y184 haplotype or to the 86Y/Y184-bearing parental Dd2 parasites (Fig. 4). The mild but significant fold difference herein observed was not detected in our previous allelic exchange work for the same haplotype, possibly due to the single *pfmdr1* copy present in the NF10 and KC5 strains precluding sufficient expression to display this phenotype (30). *In vivo* selection of the 184F allele has been reported, typically along with other *pfmdr1* alleles, after artemether-LMF treatment. Most notably, the N86/184F haplotype was selected upon the regular six-dose therapy with artemether-LMF (26, 27). In their analysis of two clinical trials after artemether-LMF treatment, Malmberg et al. demonstrated that recrudescent infections harboring the PfMDR1 N86/184F haplotype, in the context of a single *pfmdr1* copy, were able to endure higher blood LMF concentrations (28). Together with our results that examine the role of copy number, these *in vivo* data suggest that although the *pfmdr1* 184F allele might not be the primary determinant for parasite resistance, it could provide a complementary genetic background that facilitates the acquisition of multidrug-resistant phenotypes, possibly leading to the specific haplotype selection observed in Southeast Asia (Fig. 1).

The interplay between PfMDR1 and PfCRT transporters in modulating parasite susceptibility to antimalarials was also evident in this work (Table 2). Heightened susceptibilities of NF^{Dd2}, NY^{Dd2}, and Dd2 *P. falciparum* lines to MFQ, md-ADQ, CQ, and QN were affected by VP as previously observed in other genetic backgrounds (48, 66). The role of PfMDR1 in the parasite's response to QN remains unclear, but mutant PfCRT is known to contribute to QN resistance (67). Our results with VP also suggest a role for PfCRT. We note that VP increased susceptibility to LMF only in the edited parasites and not in the unedited Dd2 control. Prior data showed selection of the *pfcrt* CQ-sensitive wild-type K76 allele occurs upon artemether-LMF treatment, as confirmed *in vitro* using allelic exchange (68). In the case of LMF, VP might have a greater effect on PfMDR1 in the context of N86, since we observed that VP decreased Fluo-4 accumulation in the parasite's DV in N86 parasites (Fig. 3D). This observation would explain the increased parasite susceptibility to LMF upon VP exposure.

In the presence of ELC, decreased MFQ IC_{50} values were observed in NF^{Dd2} and NY^{Dd2} edited parasites, reinforcing the hypothesis that not only are *pfmdr1* amplifications associated with MFQ resistance (24, 25, 39) but also that the *pfmdr1* N86 allele is a key mediator of MFQ susceptibility. These findings are in line with the MalariaGEN data showing that almost all parasites that contain *pfmdr1* amplifications are in the context of the *pfmdr1* N86 allele.

In conclusion, our *in vitro* parasite drug susceptibility data using *pfmdr1* gene-edited parasites reinforce the role of both *pfmdr1* CNV and haplotype variation in modulating parasite responses to multiple first-line antimalarial drugs. Our investigation highlights the steady geographic expansion of a parasite population harboring a *pfmdr1* gene amplification with the N86/184F haplotype in Southeast Asia, which we show has a

proven higher DV transport efficacy. These data could help tailor and optimize antimalarial drug usage in different areas where malaria is endemic by taking into account the regional prevalence of *pfmdr1* polymorphisms.

MATERIALS AND METHODS

Geographical distribution of *P. falciparum* isolates carrying *pfmdr1* copy number amplifications and the mutations N86Y and Y184F. Genome analysis used data generated by the Wellcome Sanger Institute as part of the MalariaGEN *Plasmodium falciparum* Community Project version 6 (https://www.malariagen.net/resource/26) (40). Sample metadata were obtained from https://www.malariagen.net/resource/26 and were combined with the genotype data for *pfmdr1* polymorphisms at codons 86 and 184. *pfmdr1* copy number was determined using a combination of a coverage-based approach and a method based on position and orientation of reads near discovered duplication breakpoints as described elsewhere (41). The map presented in Fig. 1 was created using mapchart.net, geo-referencing the *pfmdr1* gene copy number prevalence in the region.

Parasite culture. The Dd2 strain (MRA-156, MR4-Malaria Resources) was maintained at ~4% hematocrit with human red blood cells in RPMI 1640 medium supplemented with 2 mM l-glutamine, 200 μM hypoxanthine, 0.25 μg/ml gentamicin, 25 mM HEPES, 0.2% NaHCO3, and 0.25% Albumax II (Invitrogen; ThermoFisher Scientific). Red blood cells were isolated either from whole blood or from buffy coat samples provided by healthy donors of all blood types. Parasite cultures were maintained at 37°C under a humidified controlled atmosphere of 5% O2/5% CO2/90% N2. Parasite growth was monitored by inspecting Giemsa-stained blood smears. Parasite synchronization was performed with 5% sorbitol for 15 min at 37°C. To obtain highly synchronous cultures, sorbitol was added to the culture to eliminate trophozoites. This was repeated 20 h and 44 h after the initial treatment to obtain 4-h postinvasion ring stages that then mature into trophozoites, which were used to measure Fluo-4 fluorescence.

***P. falciparum* transfections.** The previously designed plasmids p*mdr1*^{NF} and p*mdr1*^{NY} (30) were used to transfect Dd2 parasites. Briefly, we introduced the desired mutations into the endogenous *pfmdr1* locus of Dd2 parasites using ZFN-mediated genome editing. These customized ZFNs bind on opposite strands of *pfmdr1*, producing a double-stranded break 42 bp downstream of the start codon. DNA repair proceeds via homologous recombination, as *P. falciparum* lacks the nonhomologous end-joining pathway (69). Our homology-driven repair template consisted of a 2.4-kb *pfmdr1* fragment that encompassed codons 86 and 184 (30).

Ring-stage cultures at 5% parasitemia were electroporated with ~50 μg of plasmid DNA diluted in Cytomix (120 mM KCl, 0.15 mM $CaCl_2$, 2 mM EGTA, 5 mM $MgCl_2$, 10 mM K_2HPO_4/KH_2PO_4, 25 mM HEPES [pH 7.6]). The plasmids express the human dihydrofolate reductase (hDHFR) selectable marker, which confers resistance to the antifolate drug WR99210. To allow transient expression of the ZFNs, WR99210 selection was applied to transfected cultures beginning 24 h postelectroporation and maintained for 6 days to select for edited parasites. Parasites were visible microscopically 6 weeks postelectroporation and screened for editing events via blood PCR using the primers p3 and p4. Positive bulk cultures were cloned by limiting dilution in 96-well plates. After 15 days, cloning plates were screened and parasite-positive wells were screened for ZFN-mediated editing by blood PCR amplification using a supreme NZYTaq II 2× Green Master Mix (NZYTech, Lisbon, Portugal) and the p3 and p4 primers (see Table S2 in the supplemental material). The donor sequence includes silent mutations that create a SmlI restriction site, which was used to screen the PCR products by restriction fragment length polymorphism (RFLP) analysis (a 1.3-kb band obtained with primers P3 plus P4 [Table S2] that forms a doublet at 667/669 bp with SmlI in edited parasites). The BstEII restriction site is present only in the nonedited genomic *pfmdr1* sequence and was therefore used as a control in the PCR-RFLP assay (Fig. 2B). Editing events at codons 86 and 184 were confirmed by Sanger sequencing using the p5 primer (Table S2). Successfully edited parasite lines (NF^{Dd2} and NY^{Dd2}) were expanded and cryopreserved.

In this work, we have compared the edited NF^{Dd2} and NY^{Dd2} lines to an unedited Dd2 parental control harboring the 86Y/Y184 haplotype. An edited Dd2 control was not performed in this study, since the plasmids used herein to edit Dd2 parasites were the same as those used previously by our group (30). In that study, we demonstrated that gene-edited control parasites carrying only silent ZFN binding site mutations did not alter the parasite's response to antimalarial drugs compared with unedited parental lines.

Copy number analysis of *pfmdr1*. Genomic DNA from parental control Dd2 and *pfmdr1*-edited parasites (NY^{Dd2} and NF^{Dd2}) were extracted using the NZY Blood gDNA isolation kit (NZYTech, Lisbon, Portugal) with RNase treatment. Copy numbers of the *pfmdr1* gene were determined using a NZYSpeedy Green Master Mix (2×) (NZYTech, Lisbon, Portugal). The amplification reactions were carried out in 20-μl volumes in a 96-well plate using 10 ng of genomic DNA, 400 nM each forward and reverse primer, and the master mix. The *P. falciparum* β-tubulin gene was used as the housekeeping control gene. The *pfmdr1* and pfβ-tubulin forward and reverse primers (Table S2) were previously designed (70). For each run, 3D7 was used as a single-copy-number control for *pfmdr1*. Thermal cycling was performed at 98°C for 3 min, followed by 44 cycles with 1 cycle consisting of 98°C for 15 s, 59°C for 30 s, and 72°C for 25 s using the Bio-Rad CFX96 real-time system C1000 thermal cycler. Results were analyzed by the $2 - \Delta\Delta Ct$ (threshold cycle) method of relative quantification. The ΔΔCt calculation was used as follows $\Delta\Delta Ct =$ (*Ct* of *pfmdr1* – *Ct* of Pfβ-tubulin)χ – (*Ct* of *pfmdr1* – *Ct* of Pfβ-tubulin)y, where χ is sample and *y* is *P. falciparum* 3D7 strain. The average gene copy number was calculated from three biological replicates, each with technical replicates run in duplicate.

Live-cell imaging of Fluo-4 accumulation in the DV. Fluorescence microscopy was used to evaluate the accumulation site of Fluo-4 AM (Invitrogen; ThermoFisher Scientific) in different parasite strains in trophozoite stages. Synchronized trophozoite cultures, at ~2% parasitemia and 2% hematocrit, were washed with RPMI 1640 medium and loaded with 5 μM Fluo-4 diluted in RPMI 1640 medium. Parasites were incubated for 240 min at 37°C in a humidified controlled atmosphere of 5% O2/5% CO2/90% N2. After 240 min, the parasites were washed twice with 1× phosphate-buffered saline (PBS) and transferred onto a slide. Fluorescence microscopy was performed using an Olympus BX61 microscope equipped with a visible light laser, and the images were recorded with a digital camera (DP70). The parasites incubated with Fluo-4 were excited at 488 nm with the emission in the green channel (505-nm filter), and an exposure time of 457 ms was applied to obtain the images. Single images were obtained using a 100× objective lens. Regions of interest within the infected RBC, including the parasite cytosol and the parasite DV, were recorded with Cell^P software (Electro optics, UK) and the image overlays were obtained using Image J software version 1.52a.

Quantification of Fluo-4 accumulation by flow cytometry. Flow cytometry was used to quantify Fluo-4 accumulation in NF^{Dd2}, NY^{Dd2}, Dd2, and W2 parasites. Parasite labeling and evaluation of Fluo-4 accumulation were performed as described previously (22) with minor modifications. Briefly, synchronized trophozoites were incubated with 5 μM Fluo-4 AM (Invitrogen; ThermoFisher Scientific) for 240 min at 37°C in an airtight environment flushed with 5% O2/5% CO2/90% N2. The level of fluorescence in infected RBCs was recorded by flow cytometry every 15 min until 120 min, and every 30 min thereafter up to a total incubation period of 240 min (see Fig. S2 in the supplemental material). This assay was followed by live-cell imaging at all time points described above to visualize when the Fluo-4 probe accumulated in the parasite DV (Fig. S2). Fluo-4 accumulation in the DV, presented in Fig. 3, was confirmed by fluorescence microscopy before flow cytometry. Thirty minutes before the incubation ended, parasites were loaded with 1.6 μM Mitotracker Deep Red FM (Invitrogen; ThermoFisher Scientific). For flow cytometry, labeled parasites were excited at 488 nm and 633 nm to detect Fluo-4 and Mitotracker Deep Red FM, respectively. Forward (FSC) and side scatterplots (SSC) were used to define the RBC populations followed by gating double-positive populations for Fluo-4 and Mitotracker Deep Red FM. Approximately 200,000 events were read per experimental condition, and the mean Fluo-4 intensity was calculated for each parasite strain. Due to intrinsic variability observed in the different replicate assays, the fluorescence intensity of NF^{Dd2}, NY^{Dd2}, and W2 was normalized to the Dd2 parental control (i.e., Dd2 corresponding to 100%; indicated in the graph as the baseline [$y = 0$]). Values below zero indicate a decrease in the percentage of accumulated fluorescence compared with Dd2 parasites, whereas values above zero indicate an increase.

Fluo-4 accumulation in NF^{Dd2}, NY^{Dd2}, and Dd2 parasites was also measured in the presence or absence of ELC, an inhibitor of P-type ATPase transporters, including PfMDR1 (and others, e.g., ABCI3), or VP, a calcium channel blocker that functions as a CQ resistance reversal agent. Briefly, synchronized trophozoites were washed with RPMI 1640 medium and preincubated for 10 min with ELC (0.1 μM) or VP (0.8 μM). Sample preparation and flow cytometry were performed as described above. The fluorescence intensities of NF^{Dd2}, NY^{Dd2}, and Dd2 in the presence of VP or ELC were normalized to the respective untreated controls (indicated in the graph as the baseline [$y = 0$]). Values below zero indicate a decrease in the percentage of accumulated fluorescence compared with untreated controls, while values above zero indicate an increase.

***In vitro* antimalarial drug assays.** Drug susceptibility assays using CQ, DHA, MFQ, QN, LMF, md-ADQ, PPQ, and PND in the absence or presence of ELC (0.1 μM) or VP (0.8 μM) (48) were run for the edited parasite lines NF^{Dd2} and NY^{Dd2}, the parental control strain Dd2, and the W2 strain. These assays were performed using a published flow cytometry-based method (30). Briefly, synchronized ring-stage parasites at 0.2% starting parasitemia and 1% hematocrit were incubated in the presence of different concentrations of drug (across a dilution range of twofold; 12-point dilution series). After 72 h of incubation at 37°C, samples were stained with 1.6 μM Mitotracker Deep Red FM (Invitrogen; ThermoFisher Scientific) and 2× SYBR green (for DNA staining) (Invitrogen; ThermoFisher Scientific) in 1× PBS for 30 min and analyzed by flow cytometry to determine the parasite growth rates. Approximately 100,000 events were captured per well. *In vitro* IC_{50} values were calculated using nonlinear regression analysis performed with GraphPad Prism 6 software.

Statistical analysis. Nonparametric, two-tailed Mann-Whitney U tests were used to assess IC_{50} antimalarial responses in the presence or absence of VP or ELC and Fluo-4 accumulation differences between the NF^{Dd2} and NY^{Dd2}, Dd2, and W2 parasite strains (normal distribution not assumed; performed with GraphPad Prism Software).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 0.7 MB.

FIG S2, TIF file, 0.4 MB.

TABLE S1, XLSX file, 0.02 MB.

TABLE S2, XLSX file, 0.01 MB.

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REFERENCES

1. WHO. 2019. World Malaria Report 2019. World Health Organization, Geneva, Switzerland. https://www.who.int/malaria/publications/world-malaria-report-2019/en/.
2. Blasco B, Leroy D, Fidock DA. 2017. Antimalarial drug resistance: linking Plasmodium falciparum parasite biology to the clinic. Nat Med 23:917–928. https://doi.org/10.1038/nm.4381.
3. Gething PW, Casey DC, Weiss DJ, Bisanzio D, Bhatt S, Cameron E, Battle KE, Dalrymple U, Rozier J, Rao PC, Kutz MJ, Barber RM, Huynh C, Shackelford KA, Coates MM, Nguyen G, Fraser MS, Kulikoff R, Wang H, Naghavi M, Smith DL, Murray CJ, Hay SI, Lim SS. 2016. Mapping Plasmodium falciparum mortality in Africa between 1990 and 2015. N Engl J Med 375:2435–2445. https://doi.org/10.1056/NEJMoa1606701.
4. Dhingra SK, Small-Saunders JL, Menard D, Fidock DA. 2019. Plasmodium falciparum resistance to piperaquine driven by PfCRT. Lancet Infect Dis 19:1168–1169. https://doi.org/10.1016/S1473-3099(19)30543-2.
5. Hamilton WL, Amato R, van der Pluijm RW, Jacob CG, Quang HH, Thuy-Nhien NT, Hien TT, Hongvanthong B, Chindavongsa K, Mayxay M, Huy R, Leang R, Huch C, Dysoley L, Amaratunga C, Suon S, Fairhurst RM, Tripura R, Peto TJ, Sovann Y, Jittamala P, Hanboonkunupakarn B, Pukrittayakamee S, Chau NH, Imwong M, Dhorda M, Vongpromek R, Chan XHS, Maude RJ, Pearson RD, Nguyen T, Rockett K, Drury E, Goncalves S, White NJ, Day NP, Kwiatkowski DP, Dondorp AM, Miotto O. 2019. Evolution and expansion of multidrug-resistant malaria in southeast Asia: a genomic epidemiology study. Lancet Infect Dis 19:943–951. https://doi.org/10.1016/S1473-3099(19)30392-5.
6. van der Pluijm RW, Imwong M, Chau NH, Hoa NT, Thuy-Nhien NT, Thanh NV, Jittamala P, Hanboonkunupakarn B, Chutasmit K, Saelow C, Runjarern R, Kaewmok W, Tripura R, Peto TJ, Yok S, Suon S, Sreng S, Mao S, Oun S, Yen S, Amaratunga C, Lek D, Huy R, Dhorda M, Chotivanich K, Ashley EA, Mukaka M, Waithira N, Cheah PY, Maude RJ, Amato R, Pearson RD, Goncalves S, Jacob CG, Hamilton WL, Fairhurst RM, Tarning J, Winterberg M, Kwiatkowski DP, Pukrittayakamee S, Hien TT, Day NP, Miotto O, White NJ, Dondorp AM. 2019. Determinants of dihydroartemisinin-piperaquine treatment failure in Plasmodium falciparum malaria in Cambodia, Thailand, and Vietnam: a prospective clinical, pharmacological, and genetic study. Lancet Infect Dis 19:952–961. https://doi.org/10.1016/S1473-3099(19)30391-3.
7. Menard D, Fidock DA. 2019. Accelerated evolution and spread of multidrug-resistant Plasmodium falciparum takes down the latest first-line antimalarial drug in southeast Asia. Lancet Infect Dis 19:916–917. https://doi.org/10.1016/S1473-3099(19)30394-9.
8. Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, Lwin KM, Ariey F, Hanpithakpong W, Lee SJ, Ringwald P, Silamut K, Imwong M, Chotivanich K, Lim P, Herdman T, An SS, Yeung S, Singhasivanon P, Day NP, Lindegardh N, Socheat D, White NJ. 2009. Artemisinin resistance in Plasmodium falciparum malaria. N Engl J Med 361:455–467. https://doi.org/10.1056/NEJMoa0808859.
9. Amaratunga C, Sreng S, Suon S, Phelps ES, Stepniewska K, Lim P, Zhou C, Mao S, Anderson JM, Lindegardh N, Jiang H, Song J, Su XZ, White NJ, Dondorp AM, Anderson TJ, Fay MP, Mu J, Duong S, Fairhurst RM. 2012. Artemisinin-resistant Plasmodium falciparum in Pursat province, western Cambodia: a parasite clearance rate study. Lancet Infect Dis 12:851–858. https://doi.org/10.1016/S1473-3099(12)70181-0.
10. Cortese JF, Caraballo A, Contreras CE, Plowe CV. 2002. Origin and dissemination of Plasmodium falciparum drug-resistance mutations in South America. J Infect Dis 186:999–1006. https://doi.org/10.1086/342946.
11. Chenet SM, Akinyi Okoth S, Huber CS, Chandrabose J, Lucchi NW, Talundzic E, Krishnalall K, Ceron N, Musset L, Macedo de Oliveira A, Venkatesan M, Rahman R, Barnwell JW, Udhayakumar V. 2016. Independent emergence of the Plasmodium falciparum kelch propeller domain mutant allele C580Y in Guyana. J Infect Dis 213:1472–1475. https://doi.org/10.1093/infdis/jiv752.
12. Silva M, Ferreira PE, Otienoburu SD, Calcada C, Ngasala B, Bjorkman A, Martensson A, Gil JP, Veiga MI. 2019. Plasmodium falciparum K13 expression associated with parasite clearance during artemisinin-based combination therapy. J Antimicrob Chemother 74:1890–1893. https://doi.org/10.1093/jac/dkz098.
13. Das S, Saha B, Hati AK, Roy S. 2018. Evidence of artemisinin-resistant Plasmodium falciparum malaria in eastern India. N Engl J Med 379:1962–1964. https://doi.org/10.1056/NEJMc1713777.
14. Uwimana A, Legrand E, Stokes BH, Ndikumana JM, Warsame M, Umulisa N, Ngamije D, Munyaneza T, Mazarati JB, Munguti K, Campagne P, Criscuolo A, Ariey F, Murindahabi M, Ringwald P, Fidock DA, Mbituyumuremyi A, Menard D. 2020. Emergence and clonal expansion of in vitro artemisinin-resistant Plasmodium falciparum kelch13 R561H mutant parasites in Rwanda. Nat Med 26:1602–1608. https://doi.org/10.1038/s41591-020-1005-2.
15. Conrad MD, Rosenthal PJ. 2019. Antimalarial drug resistance in Africa: the calm before the storm? Lancet Infect Dis 19:e338–e351. https://doi.org/10.1016/S1473-3099(19)30261-0.
16. Mita T, Tanabe K, Kita K. 2009. Spread and evolution of Plasmodium falciparum drug resistance. Parasitol Int 58:201–209. https://doi.org/10.1016/j.parint.2009.04.004.
17. Miotto O, Amato R, Ashley EA, MacInnis B, Almagro-Garcia J, Amaratunga C, Lim P, Mead D, Oyola SO, Dhorda M, Imwong M, Woodrow C,

Manske M, Stalker J, Drury E, Campino S, Amenga-Etego L, Thanh TN, Tran HT, Ringwald P, Bethell D, Nosten F, Phyo AP, Pukrittayakamee S, Chotivanich K, Chuor CM, Nguon C, Suon S, Sreng S, Newton PN, Mayxay M, Khanthavong M, Hongvanthong B, Htut Y, Han KT, Kyaw MP, Faiz MA, Fanello CI, Onyamboko M, Mokuolu OA, Jacob CG, Takala-Harrison S, Plowe CV, Day NP, Dondorp AM, Spencer CC, McVean G, Fairhurst RM, White NJ, Kwiatkowski DP. 2015. Genetic architecture of artemisinin-resistant Plasmodium falciparum. Nat Genet 47:226–234. https://doi.org/10.1038/ng.3189.

18. Gil JP, Krishna S. 2017. pfmdr1 (Plasmodium falciparum multidrug drug resistance gene 1): a pivotal factor in malaria resistance to artemisinin combination therapies. Expert Rev Anti Infect Ther 15:527–543. https://doi.org/10.1080/14787210.2017.1313703.
19. Valderramos SG, Fidock DA. 2006. Transporters involved in resistance to antimalarial drugs. Trends Pharmacol Sci 27:594–601. https://doi.org/10.1016/j.tips.2006.09.005.
20. Ferreira PE, Holmgren G, Veiga MI, Uhlen P, Kaneko A, Gil JP. 2011. PfMDR1: mechanisms of transport modulation by functional polymorphisms. PLoS One 6:e23875. https://doi.org/10.1371/journal.pone.0023875.
21. Sanchez CP, Rotmann A, Stein WD, Lanzer M. 2008. Polymorphisms within PfMDR1 alter the substrate specificity for anti-malarial drugs in Plasmodium falciparum. Mol Microbiol 70:786–798. https://doi.org/10.1111/j.1365-2958.2008.06413.x.
22. Rohrbach P, Sanchez CP, Hayton K, Friedrich O, Patel J, Sidhu AB, Ferdig MT, Fidock DA, Lanzer M. 2006. Genetic linkage of pfmdr1 with food vacuolar solute import in Plasmodium falciparum. EMBO J 25:3000–3011. https://doi.org/10.1038/sj.emboj.7601203.
23. Reiling SJ, Rohrbach P. 2015. Monitoring PfMDR1 transport in Plasmodium falciparum. Malar J 14:270. https://doi.org/10.1186/s12936-015-0791-3.
24. Price RN, Cassar C, Brockman A, Duraisingh M, van Vugt M, White NJ, Nosten F, Krishna S. 1999. The pfmdr1 gene is associated with a multidrug-resistant phenotype in Plasmodium falciparum from the western border of Thailand. Antimicrob Agents Chemother 43:2943–2949. https://doi.org/10.1128/AAC.43.12.2943.
25. Price RN, Uhlemann AC, Brockman A, McGready R, Ashley E, Phaipun L, Patel R, Laing K, Looareesuwan S, White NJ, Nosten F, Krishna S. 2004. Mefloquine resistance in Plasmodium falciparum and increased pfmdr1 gene copy number. Lancet 364:438–447. https://doi.org/10.1016/S0140-6736(04)16767-6.
26. Sisowath C, Stromberg J, Martensson A, Msellem M, Obondo C, Bjorkman A, Gil JP. 2005. In vivo selection of Plasmodium falciparum pfmdr1 86N coding alleles by artemether-lumefantrine (Coartem). J Infect Dis 191:1014–1017. https://doi.org/10.1086/427997.
27. Sisowath C, Ferreira PE, Bustamante LY, Dahlstrom S, Martensson A, Bjorkman A, Krishna S, Gil JP. 2007. The role of pfmdr1 in Plasmodium falciparum tolerance to artemether-lumefantrine in Africa. Trop Med Int Health 12:736–742. https://doi.org/10.1111/j.1365-3156.2007.01843.x.
28. Malmberg M, Ferreira PE, Tarning J, Ursing J, Ngasala B, Bjorkman A, Martensson A, Gil JP. 2013. Plasmodium falciparum drug resistance phenotype as assessed by patient antimalarial drug levels and its association with pfmdr1 polymorphisms. J Infect Dis 207:842–847. https://doi.org/10.1093/infdis/jis747.
29. Venkatesan M, Gadalla NB, Stepniewska K, Dahal P, Nsanzabana C, Moriera C, Price RN, Martensson A, Rosenthal PJ, Dorsey G, Sutherland CJ, Guerin P, Davis TME, Menard D, Adam I, Ademowo G, Arze C, Baliraine FN, Berens-Riha N, Bjorkman A, Borrmann S, Checchi F, Desai M, Dhorda M, Djimde AA, El-Sayed BB, Eshetu T, Eyase F, Falade C, Faucher JF, Froberg G, Grivoyannis A, Hamour S, Houze S, Johnson J, Kamugisha E, Kariuki S, Kiechel JR, Kironde F, Kofoed PE, LeBras J, Malmberg M, Mwai L, Ngasala B, Nosten F, Nsobya SL, Nzila A, Oguike M, Otienoburu SD, Ogutu B, Ouédraogo J-B, et al. 2014. Polymorphisms in Plasmodium falciparum chloroquine resistance transporter and multidrug resistance 1 genes: parasite risk factors that affect treatment outcomes for P. falciparum malaria after artemether-lumefantrine and artesunate-amodiaquine. Am J Trop Med Hyg 91:833–843. https://doi.org/10.4269/ajtmh.14-0031.
30. Veiga MI, Dhingra SK, Henrich PP, Straimer J, Gnadig N, Uhlemann AC, Martin RE, Lehane AM, Fidock DA. 2016. Globally prevalent PfMDR1 mutations modulate Plasmodium falciparum susceptibility to artemisinin-based combination therapies. Nat Commun 7:11553. https://doi.org/10.1038/ncomms11553.
31. Vinayak S, Alam MT, Sem R, Shah NK, Susanti AI, Lim P, Muth S, Maguire JD, Rogers WO, Fandeur T, Barnwell JW, Escalante AA, Wongsrichanalai C, Ariey F, Meshnick SR, Udhayakumar V. 2010. Multiple genetic backgrounds of the amplified Plasmodium falciparum multidrug resistance (pfmdr1) gene and selective sweep of 184F mutation in Cambodia. J Infect Dis 201:1551–1560. https://doi.org/10.1086/651949.
32. Foote SJ, Kyle DE, Martin RK, Oduola AM, Forsyth K, Kemp DJ, Cowman AF. 1990. Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in Plasmodium falciparum. Nature 345:255–258. https://doi.org/10.1038/345255a0.
33. Wurtz N, Fall B, Pascual A, Fall M, Baret E, Camara C, Nakoulima A, Diatta B, Fall KB, Mbaye PS, Dieme Y, Bercion R, Wade B, Pradines B. 2014. Role of Pfmdr1 in in vitro Plasmodium falciparum susceptibility to chloroquine, quinine, monodesethylamodiaquine, mefloquine, lumefantrine, and dihydroartemisinin. Antimicrob Agents Chemother 58:7032–7040. https://doi.org/10.1128/AAC.03494-14.
34. Khim N, Bouchier C, Ekala MT, Incardona S, Lim P, Legrand E, Jambou R, Doung S, Puijalon OM, Fandeur T. 2005. Countrywide survey shows very high prevalence of Plasmodium falciparum multilocus resistance genotypes in Cambodia. Antimicrob Agents Chemother 49:3147–3152. https://doi.org/10.1128/AAC.49.8.3147-3152.2005.
35. Dokomajilar C, Nsobya SL, Greenhouse B, Rosenthal PJ, Dorsey G. 2006. Selection of Plasmodium falciparum pfmdr1 alleles following therapy with artemether-lumefantrine in an area of Uganda where malaria is highly endemic. Antimicrob Agents Chemother 50:1893–1895. https://doi.org/10.1128/AAC.50.5.1893-1895.2006.
36. Pickard AL, Wongsrichanalai C, Purfield A, Kamwendo D, Emery K, Zalewski C, Kawamoto F, Miller RS, Meshnick SR. 2003. Resistance to antimalarials in Southeast Asia and genetic polymorphisms in pfmdr1. Antimicrob Agents Chemother 47:2418–2423. https://doi.org/10.1128/AAC.47.8.2418-2423.2003.
37. Pacheco C, Moreno J, Herrera F. 2019. A high number of pfmdr1 gene copies in P falciparum from Venezuela. Parasitol Res 118:3085–3089. https://doi.org/10.1007/s00436-019-06409-4.
38. Sidhu AB, Uhlemann AC, Valderramos SG, Valderramos JC, Krishna S, Fidock DA. 2006. Decreasing pfmdr1 copy number in Plasmodium falciparum malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin. J Infect Dis 194:528–535. https://doi.org/10.1086/507115.
39. Price RN, Uhlemann AC, van Vugt M, Brockman A, Hutagalung R, Nair S, Nash D, Singhasivanon P, Anderson TJ, Krishna S, White NJ, Nosten F. 2006. Molecular and pharmacological determinants of the therapeutic response to artemether-lumefantrine in multidrug-resistant Plasmodium falciparum malaria. Clin Infect Dis 42:1570–1577. https://doi.org/10.1086/503423.
40. Pearson RD, Amato R, Kwiatkowski DP. 2019. An open dataset of Plasmodium falciparum genome variation in 7,000 worldwide samples. bioRxiv https://doi.org/10.1101/824730.
41. Amato R, Pearson RD, Almagro-Garcia J, Amaratunga C, Lim P, Suon S, Sreng S, Drury E, Stalker J, Miotto O, Fairhurst RM, Kwiatkowski DP. 2018. Origins of the current outbreak of multidrug-resistant malaria in southeast Asia: a retrospective genetic study. Lancet Infect Dis 18:337–345. https://doi.org/10.1016/S1473-3099(18)30068-9.
42. Imwong M, Dondorp AM, Nosten F, Yi P, Mungthin M, Hanchana S, Das D, Phyo AP, Lwin KM, Pukrittayakamee S, Lee SJ, Saisung S, Koecharoen K, Nguon C, Day NP, Socheat D, White NJ. 2010. Exploring the contribution of candidate genes to artemisinin resistance in Plasmodium falciparum. Antimicrob Agents Chemother 54:2886–2892. https://doi.org/10.1128/AAC.00032-10.
43. Oduola AM, Milhous WK, Weatherly NF, Bowdre JH, Desjardins RE. 1988. Plasmodium falciparum: induction of resistance to mefloquine in cloned strains by continuous drug exposure in vitro. Exp Parasitol 67:354–360. https://doi.org/10.1016/0014-4894(88)90082-3.
44. Wootton JC, Feng X, Ferdig MT, Cooper RA, Mu J, Baruch DI, Magill AJ, Su XZ. 2002. Genetic diversity and chloroquine selective sweeps in Plasmodium falciparum. Nature 418:320–323. https://doi.org/10.1038/nature00813.
45. Ariey F, Fandeur T, Durand R, Randrianarivelojosia M, Jambou R, Legrand E, Ekala MT, Bouchier C, Cojean S, Duchemin JB, Robert V, Le Bras J, Mercereau-Puijalon O. 2006. Invasion of Africa by a single pfcrt allele of South East Asian type. Malar J 5:34. https://doi.org/10.1186/1475-2875-5-34.
46. Mollazadeh S, Sahebkar A, Hadizadeh F, Behravan J, Arabzadeh S. 2018. Structural and functional aspects of P-glycoprotein and its inhibitors. Life Sci 214:118–123. https://doi.org/10.1016/j.lfs.2018.10.048.
47. Foote SJ, Thompson JK, Cowman AF, Kemp DJ. 1989. Amplification of the

multidrug resistance gene in some chloroquine-resistant isolates of P. falciparum. Cell 57:921–930. https://doi.org/10.1016/0092-8674(89)90330-9.
48. Sidhu AB, Verdier-Pinard D, Fidock DA. 2002. Chloroquine resistance in Plasmodium falciparum malaria parasites conferred by pfcrt mutations. Science 298:210–213. https://doi.org/10.1126/science.1074045.
49. Patel JJ, Thacker D, Tan JC, Pleeter P, Checkley L, Gonzales JM, Deng B, Roepe PD, Cooper RA, Ferdig MT. 2010. Chloroquine susceptibility and reversibility in a Plasmodium falciparum genetic cross. Mol Microbiol 78:770–787. https://doi.org/10.1111/j.1365-2958.2010.07366.x.
50. Sa JM, Twu O, Hayton K, Reyes S, Fay MP, Ringwald P, Wellems TE. 2009. Geographic patterns of Plasmodium falciparum drug resistance distinguished by differential responses to amodiaquine and chloroquine. Proc Natl Acad Sci U S A 106:18883–18889. https://doi.org/10.1073/pnas.0911317106.
51. Veiga MI, Ferreira PE, Jornhagen L, Malmberg M, Kone A, Schmidt BA, Petzold M, Bjorkman A, Nosten F, Gil JP. 2011. Novel polymorphisms in Plasmodium falciparum ABC transporter genes are associated with major ACT antimalarial drug resistance. PLoS One 6:e20212. https://doi.org/10.1371/journal.pone.0020212.
52. Duraisingh MT, Roper C, Walliker D, Warhurst DC. 2000. Increased sensitivity to the antimalarials mefloquine and artemisinin is conferred by mutations in the pfmdr1 gene of Plasmodium falciparum. Mol Microbiol 36:955–961. https://doi.org/10.1046/j.1365-2958.2000.01914.x.
53. Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF. 2000. Pgh1 modulates sensitivity and resistance to multiple antimalarials in Plasmodium falciparum. Nature 403:906–909. https://doi.org/10.1038/35002615.
54. Ngo T, Duraisingh M, Reed M, Hipgrave D, Biggs B, Cowman AF. 2003. Analysis of pfcrt, pfmdr1, dhfr, and dhps mutations and drug sensitivities in Plasmodium falciparum isolates from patients in Vietnam before and after treatment with artemisinin. Am J Trop Med Hyg 68:350–356. https://doi.org/10.4269/ajtmh.2003.68.350.
55. Amato R, Lim P, Miotto O, Amaratunga C, Dek D, Pearson RD, Almagro-Garcia J, Neal AT, Sreng S, Suon S, Drury E, Jyothi D, Stalker J, Kwiatkowski DP, Fairhurst RM. 2017. Genetic markers associated with dihydroartemisinin-piperaquine failure in Plasmodium falciparum malaria in Cambodia: a genotype-phenotype association study. Lancet Infect Dis 17:164–173. https://doi.org/10.1016/S1473-3099(16)30409-1.
56. Witkowski B, Duru V, Khim N, Ross LS, Saintpierre B, Beghain J, Chy S, Kim S, Ke S, Kloeung N, Eam R, Khean C, Ken M, Loch K, Bouillon A, Domergue A, Ma L, Bouchier C, Leang R, Huy R, Nuel G, Barale JC, Legrand E, Ringwald P, Fidock DA, Mercereau-Puijalon O, Ariey F, Menard D. 2017. A surrogate marker of piperaquine-resistant Plasmodium falciparum malaria: a phenotype-genotype association study. Lancet Infect Dis 17:174–183. https://doi.org/10.1016/S1473-3099(16)30415-7.
57. Silva M, Calcada C, Teixeira M, Veiga MI, Ferreira PE. 2020. Multigenic architecture of piperaquine resistance trait in Plasmodium falciparum. Lancet Infect Dis 20:26–27. https://doi.org/10.1016/S1473-3099(19)30689-9.
58. Preechapornkul P, Imwong M, Chotivanich K, Pongtavornpinyo W, Dondorp AM, Day NP, White NJ, Pukrittayakamee S. 2009. Plasmodium falciparum pfmdr1 amplification, mefloquine resistance, and parasite fitness. Antimicrob Agents Chemother 53:1509–1515. https://doi.org/10.1128/AAC.00241-08.
59. Imwong M, Dhorda M, Myo Tun K, Thu AM, Phyo AP, Proux S, Suwannasin K, Kunasol C, Srisutham S, Duanguppama J, Vongpromek R, Promnarate C, Saejeng A, Khantikul N, Sugaram R, Thanapongpichat S, Sawangjaroen N, Sutawong K, Han KT, Htut Y, Linn K, Win AA, Hlaing TM, van der Pluijm RW, Mayxay M, Pongvongsa T, Phommasone K, Tripura R, Peto TJ, von Seidlein L, Nguon C, Lek D, Chan XHS, Rekol H, Leang R, Huch C, Kwiatkowski DP, Miotto O, Ashley EA, Kyaw MP, Pukrittayakamee S, Day NPJ, Dondorp AM, Smithuis FM, Nosten FH, White NJ. 2020. Molecular epidemiology of resistance to antimalarial drugs in the Greater Mekong subregion: an observational study. Lancet Infect Dis https://doi.org/10.1016/S1473-3099(20)30228-0.
60. Barnes DA, Foote SJ, Galatis D, Kemp DJ, Cowman AF. 1992. Selection for high-level chloroquine resistance results in deamplification of the pfmdr1 gene and increased sensitivity to mefloquine in Plasmodium falciparum. EMBO J 11:3067–3075. https://doi.org/10.1002/j.1460-2075.1992.tb05378.x.
61. Veiga MI, Ferreira PE, Malmberg M, Jornhagen L, Bjorkman A, Nosten F, Gil JP. 2012. pfmdr1 amplification is related to increased Plasmodium falciparum in vitro sensitivity to the bisquinoline piperaquine. Antimicrob Agents Chemother 56:3615–3619. https://doi.org/10.1128/AAC.06350-11.
62. WHO. 2015. World Malaria Report 2015. World Health Organization, Geneva, Switzerland. https://www.who.int/malaria/publications/world-malaria-report-2015/report/en/.
63. Kiaco K, Teixeira J, Machado M, do Rosario V, Lopes D. 2015. Evaluation of artemether-lumefantrine efficacy in the treatment of uncomplicated malaria and its association with pfmdr1, pfatpase6 and K13-propeller polymorphisms in Luanda, Angola. Malar J 14:504. https://doi.org/10.1186/s12936-015-1018-3.
64. Pascual A, Fall B, Wurtz N, Fall M, Camara C, Nakoulima A, Baret E, Diatta B, Wade B, Briolant S, Pradines B. 2013. Plasmodium falciparum with multidrug resistance 1 gene duplications, Senegal. Emerg Infect Dis 19:814–815. https://doi.org/10.3201/eid1905.121603.
65. Rosenthal PJ. 2013. The interplay between drug resistance and fitness in malaria parasites. Mol Microbiol 89:1025–1038. https://doi.org/10.1111/mmi.12349.
66. Lakshmanan V, Bray PG, Verdier-Pinard D, Johnson DJ, Horrocks P, Muhle RA, Alakpa GE, Hughes RH, Ward SA, Krogstad DJ, Sidhu AB, Fidock DA. 2005. A critical role for PfCRT K76T in Plasmodium falciparum verapamil-reversible chloroquine resistance. EMBO J 24:2294–2305. https://doi.org/10.1038/sj.emboj.7600681.
67. Sanchez CP, Mayer S, Nurhasanah A, Stein WD, Lanzer M. 2011. Genetic linkage analyses redefine the roles of PfCRT and PfMDR1 in drug accumulation and susceptibility in Plasmodium falciparum. Mol Microbiol 82:865–878. https://doi.org/10.1111/j.1365-2958.2011.07855.x.
68. Sisowath C, Petersen I, Veiga MI, Martensson A, Premji Z, Bjorkman A, Fidock DA, Gil JP. 2009. In vivo selection of Plasmodium falciparum parasites carrying the chloroquine-susceptible pfcrt K76 allele after treatment with artemether-lumefantrine in Africa. J Infect Dis 199:750–757. https://doi.org/10.1086/596738.
69. Lee AH, Symington LS, Fidock DA. 2014. DNA repair mechanisms and their biological roles in the malaria parasite Plasmodium falciparum. Microbiol Mol Biol Rev 78:469–486. https://doi.org/10.1128/MMBR.00059-13.
70. Bopp S, Magistrado P, Wong W, Schaffner SF, Mukherjee A, Lim P, Dhorda M, Amaratunga C, Woodrow CJ, Ashley EA, White NJ, Dondorp AM, Fairhurst RM, Ariey F, Menard D, Wirth DF, Volkman SK. 2018. Plasmepsin II-III copy number accounts for bimodal piperaquine resistance among Cambodian Plasmodium falciparum. Nat Commun 9:1769. https://doi.org/10.1038/s41467-018-04104-z.