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In Vitro Activities of Quinine and Other Antimalarials and *pfnhe* Polymorphisms in *Plasmodium* Isolates from Kenya▽

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Resistance to the amino alcohol quinine has been associated with polymorphisms in *pfnhe*, a sodium hydrogen exchanger. We investigated the role of this gene in quinine resistance *in vitro* in isolates from Kenya. We analyzed *pfnhe* whole-gene polymorphisms, using capillary sequencing, and *pfcrt* at codon 76 (*pfcrt*-76) and *pfmdr1* at codon 86 (*pfmdr1*-86), using PCR-enzyme restriction methodology, in 29 isolates from Kilifi, Kenya, for association with the *in vitro* activities of quinine and 2 amino alcohols, mefloquine and halofantrine. *In vitro* activity was assessed as the drug concentration that inhibits 50% of parasite growth (IC_{50}). The median IC_{50}s of quinine, halofantrine, and mefloquine were 92, 22, and 18 nM, respectively. The presence of 2 DNNND repeats in microsatellite ms4760 of *pfnhe* was associated with reduced susceptibility to quinine (60 versus 227 nM for 1 and 2 repeats, respectively; $P < 0.05$), while 3 repeats were associated with restoration of susceptibility. The decrease in susceptibility conferred by the 2 DNNND repeats was more pronounced in parasites harboring the *pfmdr1*-86 mutation. No association was found between susceptibility to quinine and the *pfcrt*-76 mutation or between susceptibility to mefloquine or halofantrine and the *pfnhe* gene and the *pfcrt*-76 and *pfmdr1*-86 mutations. Using previously published data on the *in vitro* activities of chloroquine, lumefantrine, piperaquine, and dihydroartemisinin, we investigated the association of their activities with *pfnhe* polymorphism. With the exception of a modulation of the activity of lumefantrine by a mutation at position 1437, *pfnhe* did not modulate their activities. Two DNNND repeats combined with the *pfmdr1*-86 mutation could be used as an indicator of reduced susceptibility to quinine.

The amino alcohol quinine (QN) remains one of the important drugs against malaria. It is the drug of choice for the treatment of severe malaria, and in most African countries, including Kenya, where artemisinin-based combinations (lumefantrine-artemether, amodiaquine-artesunate) are now first-line treatments, 7-day quinine monotherapy has become the second-line treatment for uncomplicated malaria (44). However, there is evidence of selection and spread of QN-resistant parasites or those with reduced susceptibility to QN (1–3, 8, 30, 34). This observation led to the investigation of artesunate (an artemisinin derivative) as an alternative to QN for the treatment of severe malaria (14). However, this option could now be compromised by the emergence of artemisinin resistance (9).

Several studies have been dedicated to understanding the mechanisms of quinine resistance. Polymorphisms in *pfmdr*, a gene associated with chloroquine (CQ) resistance, modulate QN susceptibility (27, 33, 36). A mutation of the CQ resistance gene *pfcrt* at codon 76 (*pfcrt*-76) has been associated with reduced susceptibility to QN *in vitro*, although transfection studies have shown conflicting results (16, 37). A seminal study on the association of polymorphisms in *pfnhe*, a sodium hydrogen exchanger gene, and the *in vitro* activity of QN indicated that an increase in the number of DNNND repeats (1–5) in an ms4760 microsatellite was associated with reduced susceptibility to QN (10), and this finding was confirmed recently (13). Variations in the copy number of this repeat in isolates from areas where QN efficacy is known to be reduced *in vivo* have also been reported (41).

The initial discovery that the microsatellite ms4760 region was associated with modulation of QN activity was based on the sequencing of the *pfnhe* gene of reference strains (71 in total) from several areas where malaria is endemic, including Kenya (10). However, the number of strains from each site of malaria endemicity was relatively small; for instance, only 3 strains from Kenya were analyzed. Subsequent investigations on the role of this gene in QN resistance focused on analysis of only the ms4760 region (13, 41), and additional genetic variations of *Plasmodium falciparum* in local populations may have been overlooked.

With this in mind, we sequenced the whole *pfnhe* gene of 29 isolates from Kilifi, on the Kenyan coast, and analyzed this gene polymorphism in association with QN activity *in vitro*, along with the activities of the amino alcohols mefloquine (MFQ) and halofantrine (HLF). Isolates we analyzed in the current study were used in a previous study to investigate polymorphisms of *pfcrt* at *pfcrt*-76 and *pfmdr1* at codon 86 (*pfmdr1*-86) for associations with the *in vitro* activities of CQ, the amino alcohol lumefantrine (LM), and the drugs piperaquine (PQ) and dihydroartemisinin (DHA) (19). We included part of these data to establish the impact of *pfnhe* polymorphism on the *in vitro* activities of the aforementioned drugs and the roles of the *pfcrt*-76 and *pfmdr1*-86 genotypes on QN activity *in vitro*.

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MATERIALS AND METHODS

CQ was purchased from Sigma Chemical Company (Poole, Dorset, United Kingdom). QN, MFQ, and HAL were gifts from Steve Ward, Liverpool School of Tropical Medicine, Liverpool, United Kingdom.

Parasite adaptation. *P. falciparum* parasites were collected from malaria patients as parts of several clinical studies that took place between 2005 and 2008 in the Kilifi district of Kenya, and the *in vitro* adaptation of parasites was carried out as reported elsewhere (35). Provision of informed consent for the original studies was assured by the Kenya Medical Research Institute, Nairobi.

Chemosensitivity testing. We employed the *P. falciparum* drug-sensitive reference laboratory strain 3D7. Routine cultures were carried out in RPMI 1640 medium (Gibco BRL, United Kingdom) supplemented with 15% (vol/vol) normal human serum, 25 mM bicarbonate, 2 mM glutamine, 25 mM HEPES buffer, and 3.6 nM para-aminobenzoic acid.

The culture was diluted to 0.5% parasitemia and a final hematocrit of 1.5%, and 200 μl of this culture medium was added to each well of a 96-well microtiter plate containing the various drug concentrations and incubated at 37°C in a gas mixture containing 90% N_2, 5% CO_2, and 5% O_2 for 48 h. Thereafter, 25 μl of 0.5 μCi [^{3}H]hypoxanthine was added, and the mixture was incubated for another 18 h. The culture was then harvested, using a Tomtec Harvester (Tomtec, Hamden, CT), transferred to fiberglass paper (Wallac, Turku, Finland), and air dried. This dry fiberglass paper was wrapped in a sealing paper with scintillation fluid (Perkin Elmer, Norwalk, CT), and the amount of ionizing radiation was determined using a Wallac 1450 MicroBeta counter, (Wallac, Turku, Finland). Results were expressed as the drug concentration required for 50% inhibition of [^{3}H]hypoxanthine incorporation into parasite nucleic acid or the concentration that inhibited 50% of parasite growth (IC_{50}), determined by using nonlinear-regression analysis of the dose-response curve (39).

DNA preparation and sequencing. Parasite DNA was extracted from field isolates adapted *in vitro*, using a QIAamp DNA blood minikit (Qiagen, United Kingdom). *pfnhe* (PF13_0019; PlasmoDB [http://www.plasmodb.org/]) was amplified from 30 parasites, using the primers PFNHE1aF (5′-TGTAGCAACAC TCAGCTCAG-3′) and PFNHE14bR (5′-ACATATCGGTCCCTATTTTG-3′) to generate the PCR product by using a high-fidelity PCR enzyme (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) under the following amplification conditions: 10 cycles of denaturation at 94°C for 2 min, denaturation at 94°C for 15 sec, annealing at 55.8°C for 30 sec, extension at 68°C for 4 min and a further 25 cycles of denaturation at 94°C for 15 sec, annealing at 55.8°C for 30 sec, extension at 68°C for 4 min, and a final extension at 72°C for 7 min. PCR products were purified with a QIAquick PCR purification kit (Qiagen, United Kingdom) and sequenced, using the amplification primers and several internal-sequencing primers, a BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, United Kingdom), and an ABI 3130xl capillary sequencer (Applied Biosystems, United Kingdom). Sequences were assembled and edited, using SeqMan, and aligned, using MegAlign (DNAStar Lasergene 7; Madison, WI) and BioEdit version 7.0.9 to identify single-nucleotide polymorphisms (SNPs) and repeat regions in the gene.

Statistical analysis. All statistical analyses were performed with Stata 11 software (StataCorp LP, College Station, TX). We reported IC_{50}s as median values. The nonparametric Kruskal-Wallis test was used to compare differences in IC_{50}s for different molecular markers (microsatellite variations, codon polymorphisms, and *pfcrt*/*pfmdr-1* genotypes) and antimalarial responses. Statistical significance was defined at the 5% level ($P < 0.05$).

RESULTS AND DISCUSSION

We analyzed the *in vitro* activities of 29 field isolates against the amino alcohols QN, MFQ, and HLF (Fig. 1). QN, MFQ, and HLF median IC_{50}s were 92 nM with a 95% confidence interval (CI) of 55 to 216 nM, 22 nM with a 95% CI of 18 to 37 nM, and 18 nM with a 95% CI of 10 to 34 nM, respectively. To date, the QN resistance cutoff point has not been defined. Parasites for which the QN IC_{50} was >800 nM have been proposed to be resistant; however, lower IC_{50} values (>500 nM) have been suggested as well (26, 40). Based on the lower threshold, all but 2 of our isolates are sensitive *in vitro*, and based on the high cutoff point, none of the tested isolates is QN resistant; thus, QN is active against isolates from this part of Kenya. Interestingly, studies carried out almost 20 years ago in the coastal region of Kenya (including our study site, Kilifi) showed a similar QN activity range against field isolates, with IC_{50}s of <500 nM (12, 25, 43). Our data are in line with reports from another part of Kenya (Western region) and other African countries (4, 20, 22, 28, 32, 40), although QN-resistant parasites *in vitro* have also been reported in Africa (6, 15, 18, 26). *In vivo*, QN is used every 8 h for 7 days, and lack of compliance with this regimen is common (11). Several reports point to a decrease in QN efficacy (1, 3, 17, 24, 30, 31, 34); however, most of these results are based on effectiveness studies. Thus, the observed reduced efficacy could be associated with lack of compliance. In the absence of clear resistance *in vivo*, it is difficult to define a cutoff point.

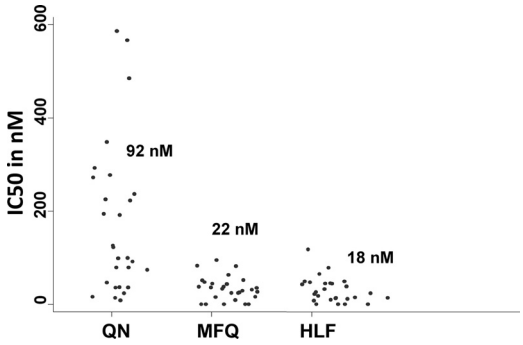

FIG. 1. *In vitro* activities of the amino alcohols quinine (QN), mefloquine (MFQ), and halofantrine (HLF). Values on the *y* axis represent drug concentrations that inhibit 50% of parasite growth (IC_{50}s), in nanomoles. Median IC_{50}s for each drug are also represented.

To establish the role of *pfnhe* in QN susceptibility, we sequenced the whole *pfnhe* gene from 30 parasites, including the gene of the reference strain 3D7 (GenBank accession numbers HM210746 to HM210771). Information on the gene's structure and polymorphism is summarized in Table 1 and Fig. 2. We identified SNPs (nonsynonymous) at codons 834, (AAT to AAA), 950 (GGG to GTG), and 1437 (TAT to TTT) (Table 1). The first 2 SNPs, at codons 834 and 950, were reported previously (10).

We identified 10 ms4760 sequences, as shown in Fig. 2. The majority of the isolates had a single repeat of microsatellites msR1 (TCDNNMPNNNMSNNN) and ms3580 (NIH), while the rest had no repeats (Table 1). Twenty percent (6 out of 29), 76% (22 out 29), and 3% (1 out 29) of isolates had 1, 2, and 3 DDNHNDNHNND repeats (in ms4760), respectively. DNNND (in ms4760) was distributed as follows: of 29 isolates, 17 and 6 isolates had 2 and 1 repeats, respectively, and the remaining 6 isolates had 3 repeats. The *pfnhe* genetic profile of reference strain 3D7 is identical to one described by Ferdig et al. (10), and overall, the distribution of microsatellites in our field isolates is in line with that of previous reports (10, 13). Interestingly, however, we found repeat variations in the microsatellite SDNN, with the number of repeats changing from 1 to 4 and the majority of isolates (more than 52%) harboring 4 repeats (Table 1).

No association was found between the IC_{50} of QN and SNPs, the repeat number variation in msR1, ms3580, and DDNHNDHHNND (in ms4067), and the new repeat, SDNN. Parasites with 1 DNNND or DDNHNDHHNND repeat (in ms4067) were more susceptible to QN than those with >1

TABLE 1. IC_{50}s of quinine and *pfnhe* polymorphisms in *P. falciparum*

Isolate	IC_{50} (nM)	Codon polymorphisms			Microsatellite polymorphisms			
		894	950	1437	No. of SDNN repeats	msR1	ms3580	ms4760
3D7	27.1	AAT	GTG	TAT	4	Absent	NIH	ms4760-2
AK127	569.5	AAT	GGG	TAT	4	TCDNNMPNNNMSNNN	NIH	ms4760-1
AK182	549.2	AAT	GGG	TTT	2	TCDNNMPNNNMSNNN	NIH	ms4760-1
AK227	495.3	AAT	GGG	TAT	4	TCDNNMPNNNMSNNN	NIH	ms4760-1
CDA-1553	333.3	AAT	GGG	TTT	4	TCDNNMPNNNMSNNN	NIH	ms4760-1
AK150 D-0	296.6	AAT	GGG	TAT	3	Absent	NIH	ms4760-1
AK249	270.4	AAT	GTG	TAT	3	Absent	NIH	ms4760-1
AK121	270.2	AAT	GGG	TAT	4	TCDNNMPNNNMSNNN	NIH	ms4760-21
AK158	234.0	AAT	GGG	TAT	2	TCDNNMPNNNMSNNN	NIH	ms4760-1
AK167	227.0	AAT	GGG	TAT	4	TCDNNMPNNNMSNNN	NIH	ms4760-1
AK222	210.5	AAT	GGG	TAT	2	TCDNNMPNNNMSNNN	NIH	ms4760-1
AK152 D-56	207.7	AAA	GTG	TAT	2	Absent	NIH	ms4760-6
AK062	181.5	AAT	GTG	TAT	4	TCDNNMPNNNMSNNN	NIH	ms4760-1
AK144	145.8	AAT	GTG	TTT	4	Absent	NIH	ms4760-23
AK018	141.1	AAT	GGG	TTT	2	Absent	NIH	ms4760-20
AK150 D-42	91.6	AAT	GGG	TAT	2	Absent	NIH	ms4760-1
9067	80.1	AAT	GTG	TAT	4	TCDNNMPNNNMSNNN	NIH	ms4760-3
8885	78.2	AAT	GGG	TAT	3	Absent	NIH	ms4760-22
AK183	73.9	AAT	GGG	TTT	4	TCDNNMPNNNMSNNN	Absent	ms4760-20
6816	73.3	AAT	GGG	TAT	1	TCDNNMPNNNMSNNN	NIH	ms4760-18
8966	55.8	AAT	GTG	TAT	4	TCDNNMPNNNMSNNN	Absent	ms4760-1
AK033 D-49	54.1	AAT	GGG	TAT	3	TCDNNMPNNNMSNNN	NIH	ms4760-7
AK022D-0	42.4	AAT	GGG	TAT	4	TCDNNMPNNNMSNNN	NIH	ms4760-22
9070	41.5	AAT	GGG	TAT	4	TCDNNMPNNNMSNNN	NIH	ms4760-22
8977	35.9	AAT	GGG	TTT	3	Absent	NIH	ms4760-7
AK033 D-0	32.8	AAT	GGG	TAT	4	Absent	NIH	ms4760-1
8968	30.6	AAA	GGG	TAT	4	Absent	NIH	ms4760-2
8948	27.8	AAA	GGG	TAT	4	Absent	NIH	ms4760-7
AK022 D-28	22.0	AAT	GGG	TAT	4	TCDNNMPNNNMSNNN	NIH	ms4760-1
8973	20.0	AAT	GGG	TAT	4	TCDNNMPNNNMSNNN	NIH	ms4760-7

repeat (60 versus 182 nM and 58 versus 141 nM for DNNND and DDNHNDHHNND, respectively), but the difference was not significant. Previous work clearly indicates that the increase in DNNND repeats is associated with reduced susceptibility to QN (10, 13). However, detailed observation of our data shows that the increase in the QN IC_{50} is observed only for parasites with 2 DNNND repeats. Indeed, the QN IC_{50} rose from 60 to 227 nM for parasites harboring 1 and 2 repeats, respectively ($P < 0.05$), and the latter value dropped to 45 nM for parasites with 3 repeats ($P < 0.01$) (Fig. 3A). To investigate this further, we reused data from the study of Ferdig et al. (10). As shown in Fig. 3B, the increase from 1 to 2 repeats was associated with decreasing susceptibility to QN (IC_{90}s from 157 to 471 nM; $P < 0.01$), and the change from 2 to 3 repeats rendered parasites more susceptible (IC_{90}s from 471 to 125 nM; $P < 0.01$). Thus, these data indicate that the presence of

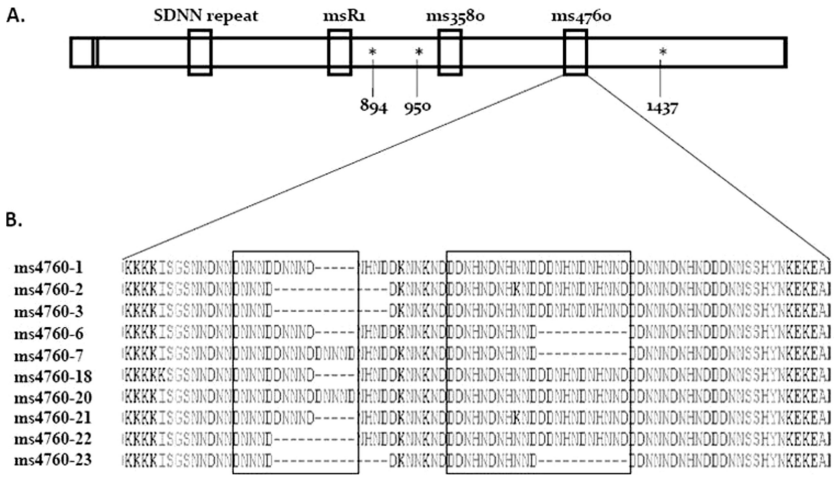

FIG. 2. Diagram of *pfnhe-1* gene (A) and ms4760 microsatellite sequence profiles (B). (A) Asterisks indicate polymorphic codons (894, 950, and 1437). (B) ms4760 profiles. Profiles 1 to 18 were previously described (10, 13), and we identified 4 new profiles, 20 to 23.

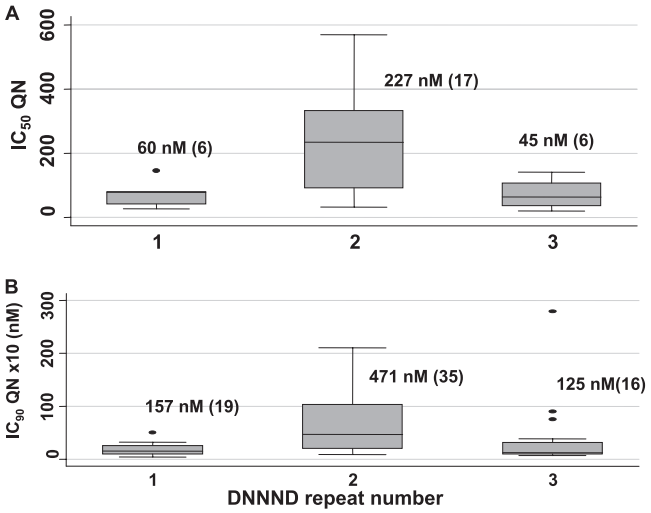

FIG. 3. Association between the number of DNNND repeats in *pfnhe* and the quinine concentration that inhibits 50% (A) or 90% (B) of parasite growth. Isolate numbers are in brackets. (A) Our current data from Kilifi. (B) Data from Ferdig et al. (10) (from Table 1). In both graphs, differences between 1 and 2 and between 2 and 3 repeats were significant ($P < 0.0\ 5$), while those between 1 and 3 repeats were not ($P > 0.05$). In the work of Fergid et al. (10), 1 isolate had 4 copies (IC_{90} = 582 nM) and was not included.

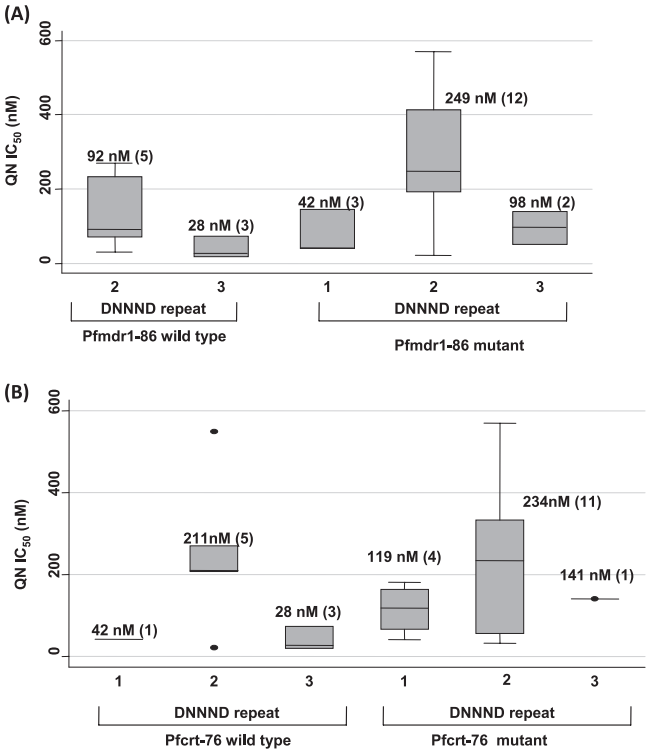

FIG. 4. Relationship between drug concentrations that inhibit 50% of parasite growth (IC_{50}s) and the genotype combination of DNNND repeat number and *pfmdr1-86* (A) or *pfcrt-76* (B). On a backdrop of 2 repeats of DNNND, the *pfcrt*-76 genotype did not affect QN activity, while a 3-fold increase in the IC_{50} was observed for the *pfmdr1*-86 mutants.

3 repeats of DNNND restores susceptibility to QN. However, more studies are needed to confirm this observation, since a recent study shows no decrease in QN activity in parasites with 3 DNNND repeats (13).

Early reports demonstrated *in vitro* cross-resistance between QN and its related drugs, such as CQ, LM and MFQ, and HLF (5, 7, 38, 42), an indication that some of the mechanisms of resistance to these drugs may be common. We therefore investigated the *in vitro* activities of the quinoline CQ, PQ, the amino alcohol LM, MFQ, and the sesquiterpene DHA in association with *pfnhe* polymorphism. Detailed information on the *in vitro* activities of CQ, LM, PQ, and DHA in relation to polymorphisms in *pfcrt*-76 and *pfmdr1*-86 was reported elsewhere (19). We reused these data in the current study.

The median IC_{50}s of CQ, PQ, LM, and DHA for the 29 field isolates we used were 56 nM with a 95% CI of 26 to 88 nM, 56 nM with a 95% CI of 43 to 69 nM, 58 nM with a 95% CI of 49 to 881 nM, and 1 nM with a 95% CI of 0.7 to 1 nM, respectively. Neither *pfnhe* SNPs nor microsatellite repeats modulated susceptibility to these 4 drugs, except that LM activity decreased from 52 to 104 nM ($P < 0.005$) in the presence of an SNP at position 1437 (TAT to TTT). However, more studies are needed to confirm whether *pfnhe*, in addition to *pfcrt*-76 and *pfmdr1*-86, contributes to a decrease in the median LM susceptibility value (19).

We also investigated the role of the *pfcrt*-76 and *pfmdr1*-86 mutations in QN resistance (*pfcrt* and *pfmdr1* are the 2 genes associated with CQ resistance). The *pfcrt*-76 mutation did not affect QN activity (IC_{50}s were 164 [n = 16] and 142 nM [n = 9] for the mutant and wild type, respectively; $P > 0.05$). However, a trend toward a decrease in QN activity in parasites carrying the *pfmdr1*-86 mutation was observed, although the difference was not significant (74 [n = 16] versus 208 nM [n = 9] for the wild type and mutant, respectively; $P > 0.05$). Transfection studies showed that polymorphisms in *pfmdr1*, mainly those at codons N1042D and D1246Y, modulate QN activity (16, 33). We limited our studies to codon 86 of *pfmdr1*; thus, this mutation may have been selected with those at N1042D and D1246Y, though these mutations are predominantly found in South America (8). Interestingly, the presence of wild-type *pfmdr1*-86 has been associated with reduced susceptibility to QN but in association with an increase in the number of *pfmdr1* copies (8). However, the isolates we analyzed in the present study have 1 *pfmdr1* copy (a finding from our previous work [19]); thus, *pfmdr1* point mutations also modulate QN activity, as previously reported (16, 21, 33).

Our data indicate that *pfcrt*-76 does not affect quinine activity, which is in line with a recent report (23), although modulation of QN activity by *pfcrt* has been supported by transfection studies (16, 37).

We also sought to establish the contributions of *pfmdr1*-86 and *pfcrt*-76 in the context of DNNND polymorphisms in QN resistance. As shown in Fig. 4A, parasites with 2 DNNND repeats on a background of wild-type *pfmdr1*-86 have QN IC_{50}s of 92 nM, while this value rises by almost 3-fold in the *pfmdr1-86* mutant parasites (QN IC_{50} of 249 nM). In contrast, no difference was found with regard to *pfcrt*-76 (211 versus 234 nM) (Fig. 4B). These data indicate that *pfmdr1* is an important contributing factor in QN resistance. Thus, mon-

itoring QN resistance should involve both the *pfnhe* and *pfmdr1* genes.

Our isolates were sensitive to MFQ and HLF, with IC_{50}s of <25. These drugs are not commonly used in Africa because of their relatively high cost and the reported potential side effects, mainly cardiotoxicity and neuropsychiatric complications for HLF and MFQ, respectively. *pfcrt*-76, *pfmdr1*-86, and *pfnhe* did not modulate their *in vitro* activities. In Southeast Asia, where MFQ has been used extensively, resistance has been associated with an increase in *pfmdr1* copy number (29). All our isolates have 1 copy of the *pfmdr1* gene (19), explaining the susceptibility of our parasites to MFQ.

In summary, the presence of 2 repeats of DNNND in ms4760 of the *pfnhe* and *pfmdr1*-86 mutants is associated with a decrease in *in vitro* QN susceptibility, and an increase to 3 repeats of DNNND may restore QN activity, although this observation needs to be confirmed. Thus, *pfnhe* and *pfmdr1* could be used as markers to monitor QN resistance.

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The authors declare no conflict of interest.

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