AAC Journals.ASM.org

pfmdr1 Amplification Is Related to Increased *Plasmodium falciparum In Vitro* Sensitivity to the Bisquinoline Piperaquine

M. I. Veiga,[a,b,i] P. E. Ferreira,[a,c] M. Malmberg,[a] L. Jörnhagen,[a] A. Björkman,[a] F. Nosten,[d,e,f] and J. P. Gil[c,g,h]

Malaria Research Lab, Department of Medicine, Karolinska Institutet, Stockholm, Sweden[a]; Microbiology and Infection Research Domain, Life and Health Sciences Research Institute, School of Health Sciences, University of Minho, Braga, Portugal[b]; Drug Resistance and Pharmacogenetics Group, Institute of Biotechnology and Bioengineering (IBB), Centre of Molecular and Structural Biomedicine (CBME), University of Algarve, Faro, Portugal[c]; Shoklo Malaria Research Unit, Mae Sot Tak, Thailand[d]; Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand[e]; Centre for Clinical Vaccinology and Tropical Medicine, Nuffield Department of Clinical Medicine, University of Oxford, Oxford, United Kingdom[f]; Drug Resistance Unit, Division of Pharmacogenetics, Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden[g]; Laboratory of Molecular Anthropology and Health, Department of Anthropology, Binghamton University, Binghamton, New York, USA[h]; and Department of Microbiology and Immunology, Columbia University College of Physicians and Surgeons, New York, New York, USA[i]

The 4-aminoquinoline bisquinoline piperaquine is an important partner drug in one of the presently recommended artemisinin combination therapies. Recent clinical trials have confirmed its high efficacy in combination with dihydroartemisinin. Resistance to piperaquine alone has, however, been documented. Amplification in copy number of the *Plasmodium falciparum* multidrug resistance locus on chromosome 5, containing the *pfmdr1* gene, has been shown to confer resistance to structurally unrelated antimalarials. Through the determination of the 50% inhibitory concentrations (IC_{50}s) and IC_{90}s for piperaquine and chloroquine in a set of 46 adapted *P. falciparum* cultures originating from the Thai-Burmese border, we have characterized the regions around the *pfmdr1* gene and identified a significant association between the presence of *pfmdr1* duplications and enhanced sensitivity to piperaquine ($P = 0.005$ for IC_{50} and $P = 0.002$ for IC_{90}) and chloroquine, reaching statistical significance at IC_{90}s ($P = 0.026$). These results substantiate the potential importance of *pfmdr1* copy number amplifications in the efficacy of the combination therapy piperaquine-dihydroartemisinin. It supports the rational use of 4-aminoquinolines and artemisinin-based compounds, as they independently select for mutually incompatible combinations of mutations.

Artemisinin combination therapy (ACT) is instrumental in the global decrease of *Plasmodium falciparum* malaria in recent years. However, *P. falciparum* resistance to several partner drugs, namely, artesunate-mefloquine (25), artemether-lumefantrine (26, 31), and artesunate-amodiaquine (15), has been documented. These early signs have been associated with decreased parasite responses to the slowly eliminated partner drugs, a phenomenon believed to be accelerated in circumstances of high transmission due to the exposure of reinfecting parasites to subtherapeutic concentrations (14).

A more recent ACT, dihydroartemisinin-piperaquine (DHA-PPQ), has been widely used in Southeast Asia and is now ready to be launched in sub-Saharan Africa. PPQ is a bisquinoline, structurally a 4-aminoquinoline-based antimalarial like chloroquine (CQ). Piperaquine is not a recent newcomer, having been extensively used in monotherapy regimens in the southern regions of China in the 1970s and 1980s, as a response to the rise of CQ resistance. Later, it was adopted by the national Vietnamese malaria control program in several formulations (9). The most recent development of PPQ-based ACT is commercially known as Artekin (Hollekyn Pharmaceuticals, China) or Eurartesim (Sigma-Tau, Italy), each tablet containing 40 mg dihydroartemisinin plus 320 mg piperaquine phosphate.

DHA-PPQ has shown excellent efficacy in recent clinical trials in Africa (1, 4, 17), making it a promising fixed-dose formulation for malaria treatment on the continent. Nevertheless, the PPQ long elimination half-life of >4 weeks after the standard 3-day course (2.25 mg/kg [of body weight] DHA and 18 mg/kg of piperaquine phosphate per day) raises concerns about the long-term sustainability of its efficacy in African high-transmission settings (22). Furthermore, *P. falciparum* resistance to PPQ has long been documented, albeit in the context of monotherapy (9). In this scenario, the search for molecular markers of early detection of PPQ resistance is increasingly important. Interestingly, the *pfmdr1* (multidrug resistance 1) N86Y and *pfcrt* (chloroquine resistance transporter) K76T CQ resistance markers, known to confer different degrees of resistance to the common 4-aminoquinolines, have not been consistently associated with parasite response to PPQ either *in vitro* (5) or *in vivo* (32).

Copy number variation (CNV) polymorphism at the multidrug resistance locus *pfmdr1* in chromosome 5 is well known in Southeast Asian settings to be associated with susceptibility to antimalarial drugs both *in vitro* (2, 7, 36) and *in vivo* (24–26). CNV events in this chromosomal region normally involve more than the *pfmdr1* gene. In fact, the amplification includes a genomic fragment that can reach 100 kb, encompassing multiple genes (12, 34). These amplified genome regions (amplicons) have been structurally characterized in field samples from the Thailand-Burma border (21), although the precise importance and impact of their diversity in the development of drug resistance are yet to be investigated.

Recently, it has been reported that the use of PPQ in Vietnam is

Received 13 December 2011 **Returned for modification** 26 February 2012
Accepted 9 April 2012
Published ahead of print 16 April 2012
Address correspondence to M. I. Veiga, maria.isabel.veiga@gmail.com.
Supplemental material for this article may be found at http://aac.asm.org/.

doi:10.1128/AAC.06350-11

associated with a significant decrease in the prevalence of *pfmdr1* gene duplications in the parasite population of this region (16). Trying to identify the genetic basis of PPQ resistance, a recent study showed that continuous *in vitro* exposure leads to a deamplification of the amplicon encompassing the *pfmdr1* gene and amplification of a neighboring upstream region on chromosome 5 (11). This, associated with previous observations of decreased copy number in parasites long exposed to chloroquine (2), led us to hypothesize that gene copy number amplifications in *pfmdr1* and its amplicon size might lead to an increased sensitivity to PPQ.

MATERIALS AND METHODS

Parasite adaptation and *in vitro* drug susceptibility assays. We analyzed 46 strains previously culture adapted from the region of Mae Sot, in the Thai-Myanmar border, from clinical cases occurring between 2002 and 2008, specifically, 1 strain from 2002, 4 strains from 2007, and 41 strains from 2008 (36). The study was ethically cleared by the relevant institutions, the blood samples having been obtained upon informed consent provided in the local language.

The maintenance medium for the parasite cultures contained 5% hematocrit (O^+ erythrocytes) in RPMI 1640 (GIBCO BRL; Invitrogen 42402-010) supplemented with 1× L-glutamine (GIBCO BRL; Invitrogen 25030-032), 25 μg/ml gentamicin (GIBCO BRL; Invitrogen 15750-037), and 10% human serum. Piperaquine monophosphate (molecular weight [MW], 633.5) was obtained from AvaChem (San Antonio, TX), and chloroquine diphosphate (MW, 515.86) was purchased from Sigma-Aldrich (St. Louis, MO). The inhibitory concentrations (ICs) were determined through the histidine-rich protein 2-based double-site sandwich enzyme-linked immunosorbent assay (ELISA) (23). Briefly, 200 μl of synchronized culture at ring stage containing 0.05% parasitemia and 1.5% hematocrit was precoated in 96-well culture plates (titration of 1/2), with a PPQ concentration in row 8 of 200 nM and a CQ concentration of 2 μM. Cultures were incubated at 37°C in a candle jar (33) for 72 h, followed by the lysis of the cells by freeze-thawing for ELISA analysis. Four independent assays were performed for each field strain, and the 3D7 reference strain 50% IC (IC_{50}) was measured for drug quality and efficacy control. The 3D7 strain was kindly provided by the late D. Walliker (Department of Animal and Population Genetics, University of Edinburgh, United Kingdom).

Molecular analysis. Extraction of genomic DNA (gDNA) of the 46 Thai strains and its molecular characterization, including *pfmdr1* N86Y, *pfcrt* K76T single nucleotide polymorphisms (SNPs), and *pfmdr1* gene copy number variation, were previously performed (36).

In order to better understand the importance for drug resistance of the amplified regions at chromosome 5, all strains carrying an increased *pfmdr1* copy number (24 strains), as well as the laboratory strains FCB, FCR3, F32, and Dd2, known to carry multiple copies of this gene, were further characterized for their approximate amplicon size through real-time PCR of the *pfmdr1*-adjacent regions.

The software program Primer Express 2.0 (Applied Biosystems, CA) was used for the design of 20 sets of TaqMan probes and primers to quantify the CNV at relevant chromosome 5 regions. This scanning approach covered a ca. 157-kb fragment containing the *pfmdr1* gene (see Table S1 in the supplemental material).

The β-tubulin gene (PF10_0084) was used as the single-copy endogenous control as previously described (25). We estimated the copy number of the target sequence in relation to a standard calibrator lab strain genome (3D7; single *pfmdr1* copy) by using the cycle threshold ($\Delta\Delta CT$) method.

All assays were performed at least in triplicate on 96-well plates using the ABI PRISM 7000 sequence detection system (Applied Biosystems, Fresno, CA). The detection threshold was set above the mean baseline value for the first 6 to 15 cycles.

Following the previous findings that *in vitro* exposure of PPQ in strain Dd2 leads to an amplification of the fragment (spanning from PFE1010w to the PFE1085w gene) identified by Eastman et al. at chromosome 5, we scrutinized all Thai samples ($n = 46$) for CNV polymorphism in the PFE1010w and PFE1085W loci. Primers and probes as well as the real-time PCR conditions were as Eastman et al. described (11).

Statistical analysis. Piperaquine and chloroquine 50% and 90% inhibitory concentrations were calculated by nonlinear regression analysis (http://malaria.farch.net). Statistical analysis was carried out using SigmaPlot for Windows, version 11.0. Pearson correlation was used to assess linear relations of PPQ and CQ. The associations between the *in vitro* PPQ susceptibility values (IC_{50} and IC_{90}) and the *pfmdr1* gene copy numbers were tested through the performance of the *t* test, and when the normality test failed (Shapiro-Wilk), the Mann-Whitney rank sum test was applied.

RESULTS

The 50% and 90% inhibitory concentrations (IC_{50}s and IC_{90}s) were successfully determined for PPQ and CQ in the 46 *P. falciparum* strains. A large range of sensitivities was recorded for PPQ, with a median IC_{50} of 39.4 nM, from 13.8 to 108.2 nM (see Table S2 in the supplemental material). All strains were found to be highly resistant to CQ, with an IC_{50} of >450 nM. The interaction between PPQ and CQ ICs was tested with Pearson correlation analysis. It was agonistic both for IC_{50}s (correlation coefficient of 0.37; $P = 0.01$) and IC_{90}s (correlation coefficient = 0.40; $P = 0.006$).

Out of the 46 strains analyzed, 24 carried increased *pfmdr1* copy numbers. Specifically, 16 strains carried two copies, 6 strains carried three copies, and 2 strains harbored four copies of the *pfmdr1* gene. A significant association was found between the *pfmdr1* gene copy number amplifications and a decrease in the IC_{50}s for PPQ (geometric mean of 42.9 nM for strains with *pfmdr1* CNV of 1 versus geometric mean of 29.2 nM for the strains with *pfmdr1* CNV of >1; $P = 0.002$). The same pattern was observed for the IC_{90}s for CQ ($P = 0.026$) (Fig. 1). Stratifying the strains with >1 *pfmdr1* copy (2, 3, or 4 copies) did not, however, reveal any trend of progressive enhanced PPQ or CQ sensitivity.

In order to find any association with the *in vitro* drug outcome phenotype and the type of amplified fragment containing the *pfmdr1* gene, the approximate amplicon size and structure (i.e., which loci were involved) were characterized in the 24 Thai strains and in the reference strains FCB, FCR3, F32, and Dd2. Six different types of amplicons were found in the Thai samples (types a to f [Fig. 2]). Two amplicon types were found in the reference lab strains, with amplicon size and position on chromosome 5 illustrated as vertical pink bars in Fig. 2. The amplicon varied in size from approximately 14 to 100 kb, spanning different genes. All the characterized amplicons in the Thai strains were found to be smaller than the structure present in the analyzed reference strains.

We found no significant associations between the types of amplicon (types a to f) and the determined ICs for PPQ and CQ. Considering the previously determined phenotypes (36), significance difference was detected where parasites carrying amplicon type a are associated with significantly higher mefloquine IC_{50}s ($P < 0.01$) (Table 1). A trend in increased IC_{50} is also detected for the same type of amplicon with artemisinin (ART) and lumefantrine (LUM), although statistically nonsignificant (Table 1).

A region from the PFE1010w gene to the PFE1085w locus, located ca. 72 kbp upstream of the *pfmdr1* gene (Fig. 2, dashed green box), has been previously associated with *in vitro* PPQ resistance (11). However, no increase in copy number for the

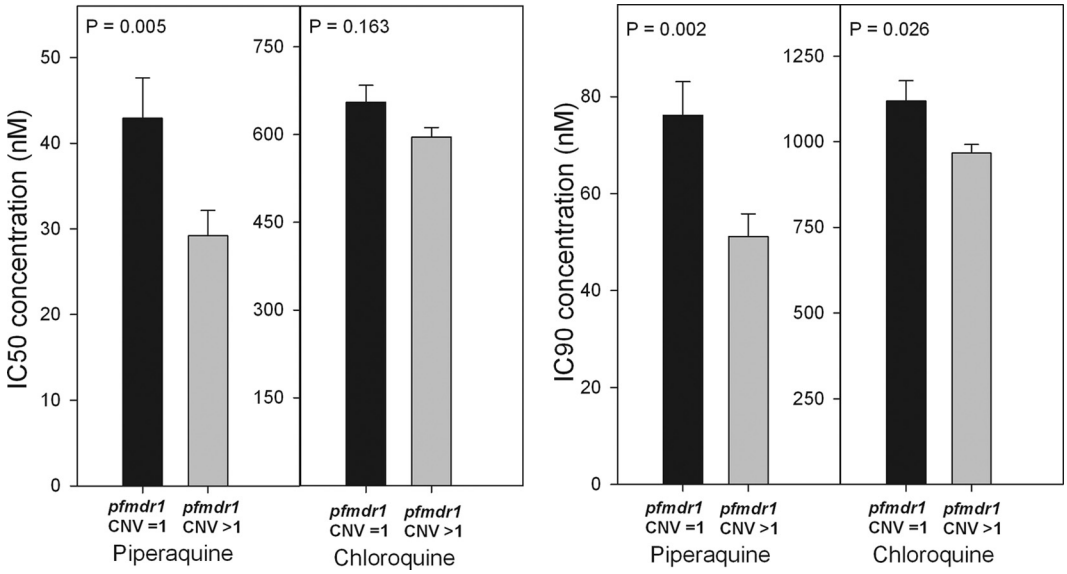

FIG 1 *pfmdr1* copy number variation (CNV) and *in vitro* IC_{50}s and IC_{90}s of PPQ and CQ. Shown is the association between the geometric mean of *in vitro* PPQ and CQ ICs and the *pfmdr1* CNV polymorphism. Data are described in Table S2 in the supplemental material. Black bars, single copy of the *pfmdr1* gene (22 strains); gray bars, more than 1 *pfmdr1* gene copy (24 strains). The *t* test was applied to determine significant difference. Error bars represent standard errors of the means.

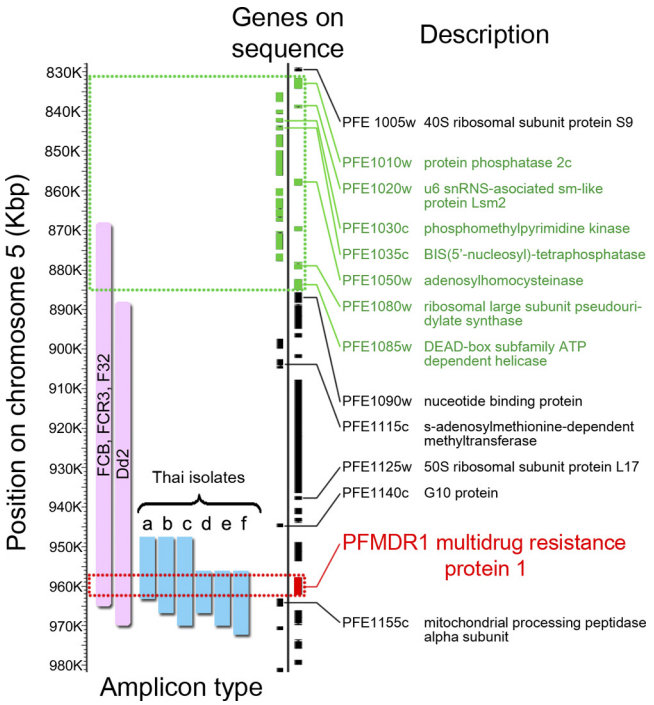

FIG 2 Genomic characterization of amplified regions. Vertical bars characterize amplicon size and position on chromosome 5 (left *y* axis), including information for genes in the sequence (right *y* axis). Pink bars illustrate amplicon type for the lab strains, and blue bars different amplicons found in the Thai strains ($n = 24$) all carrying *pfmdr1* copy number amplifications (gene highlighted in red). The green box highlights a genomic region previously found to be associated with decreased susceptibility to PPQ (11) which is not found in the Thai strains. The left *y* axis shows the position on chromosome 5, and the right *y* axis shows the gene annotation relative to the 3D7 genome sequence. Genes with known or putative function described in the depicted genomic position were acquired from NCBI Map viewer at http://www.ncbi.nlm.nih.gov. The nonannotated genes represent hypothetical proteins with unknown function.

PFE1010w and PFE1085w genes was found among our 46 field strains.

DISCUSSION

The ACT DHA-PPQ has shown excellent efficacy in field clinical trials, making it a promising fixed-dose formulation for the control of malaria. The recent detection of ART resistance in Southeast Asia (10), in regions where the partner drug (mefloquine) is failing (6), supports the importance of maintaining the integrity of the efficacy of the long-standing partner drug. With its very long half-life, PPQ is potentially at risk in settings of high transmission, due to the possibility of a large posttreatment drug resistance selection window, akin to what has been seen with other ACTs (15, 31). This, associated with the knowledge that PPQ resistance has already emerged in the past (13), commands urgency regarding the research of its molecular mechanisms. Additionally, it should be noted that emerging drug resistance has even been suggested as an explanation for an unexpected low cure rate of the DHA-PPQ ACT in a clinical trial conducted in Papua New Guinea (18).

In the present study, a large range of sensitivities was registered for PPQ, covering almost 1 log of IC_{50}s. According to the previous genotype data for the herein-analyzed parasites (36), this large range of sensitivities cannot be explained by the monomorphic *pfcrt* gene, nor by the SNPs found in the *pfmdr1* and *pfmrp1* genes. The noninvolvement of *pfmdr1* and *pfmrp1* SNPs in the parasite's response to PPQ mirrors recent similar studies conducted in Kenyan isolates (19) as well as other studies *in vitro* (5) and *in vivo* (32).

On the other hand, we have found for the first time evidence that increased copy number of *pfmdr1* sensitizes the parasite to PPQ. The P-glycoprotein homologue 1 (Pgh1) encoded by *pfmdr1* is almost exclusively located in the parasite food vacuole (FV) (8), facing the lumen of the organelle (29), consistent with the presently accepted model of this transporter being an importer of sol-

TABLE 1 Association between the type of amplicon and the determined IC_{50}s[a]

pfmdr1 CNV or amplicon type[b]	No. of Thai strains	IC_{50}, nM (SE)					
		ART[c]	DHA[c]	MQ[c]	LUM[c]	PPQ	CQ
pfmdr1 CNV							
= 1	22	4.4 (0.6)[d]	1.3 (0.3)	49.1 (19.7)[d]	8.6 (1.8)[d]	49.7 (7.8)	676.1 (40.9)
>1	24	11.0 (1.1)	1.5 (0.5)	159.1 (26.6)	18.9 (2.7)	32.2 (5.2)	601.0 (25.8)
Amplicon type							
a	3	14.8 (4.7)	2.4 (1.2)	243.6 (16.5)[e]	26.0 (6.8)	35.5 (8.7)	585.3 (17.2)
b	6	8.1 (2.1)	0.9 (0.2)	113.3 (31.6)	13.6 (2.7)	28.2 (4.8)	569.2 (23.6)
c	2	11.3 (7.0)	1.0 (0.4)	130.4 (23.6)	21.1 (5.7)	35.3 (8.6)	592.9 (12.7)
d	3	6.7 (1.9)	1.0 (0.3)	135.2 (34.7)	10.4 (1.7)	32.5 (4.5)	588.7 (9.5)
e	8	12.7 (1.1)	1.8 (0.5)	170.9 (13.8)	21.9 (1.4)	35.0 (5.9)	638.8 (28.3)
f	2	12.9 (5.1)	2.6 (1.5)	186.4 (4.7)	22.8 (2.8)	25.1 (2.2)	594.9 (109.9)

[a] PPQ, piperaquine; ART, artemisinin; DHA, dihydroartemisinin; MQ, mefloquine; LUM, lumefantrine; CQ, chloroquine. Pearson correlations (correlation coefficients [*r*]) with the PPQ IC_{50} were as follows: for ART, $r = 0.03$ ($P > 0.05$); for DHA, $r = 0.21$ ($P > 0.05$); for MQ, $r = -0.11$ ($P > 0.05$); for LUM, $r = 0.14$ ($P > 0.05$), and for CQ, $r = 0.37$ ($P = 0.01$). For pairs with *P* values greater than 0.05, there is no significant relationship between the two variables.

[b] Amplicon type is described in detail in Fig. 2, with the characterization of amplicon size and position of the amplified fragment carrying the *pfmdr1* gene.

[c] Inhibitory concentrations previously published (36).

[d] $P < 0.001$ (*t* test). Results previously published (36).

[e] $P = 0.005$ (unpaired *t* test between amplicon type a and amplicon type other than a).

utes toward the FV (28). Such function implies that the presence of increased *pfmdr1* copy numbers (expected to lead to an increase of Pgh1 load in the FV membrane) is associated with an enhanced accumulation of PPQ in the organelle. The increased susceptibility to PPQ hence suggests that the target of this drug is located in the FV, as in the case of CQ, another 4-aminoquinoline. This hypothesis is supported by the positive correlation we found in the parasite IC response to PPQ and CQ. It is, however, important to note that although PPQ is structurally related to CQ, these two drugs must be associated with other relevant mechanisms of resistance, as PPQ remains active against CQ-resistant parasites (3).

As for CQ, all strains were highly resistant to this drug. *pfcrt* mutations, in particular at amino acid position 76, are a central factor for CQ resistance. The *pfcrt* gene in this set of Thai samples was found to be totally monomorphic of the Dd2 type, which explains the fact that all the parasites were highly resistant to this drug (i.e., $IC_{50} > 100$ nM threshold) but does not explain the large variation that was observed between them. Interestingly, we could see a trend where decreased *pfmdr1* copy number polymorphism tends to coincide with higher ICs, reaching statistical significance at IC_{90}s (Fig. 1). The inverse relation between CQ resistance and *pfmdr1* copy number has previously been documented *in vitro* (2) but not observed in highly controlled gene knockdown experiments (30), possibly indicative of an effect visible only in certain genomic environments (e.g., possibly the presence of the *pfmdr1* 86N allele).

It has long been known that the duplication events involving *pfmdr1* vary in size while spanning different genes in different parasites (12, 20, 27, 34). We tried to further investigate if the type (i.e., which genes are included) and size of the amplicon influence drug susceptibility phenotypes. Overall, the field strains were found to carry smaller amplicons than were lab strains (Fig. 2), possibly due to a fitness cost, consistent with previous reports of intrahost population dynamics (35). Selection of parasites with smaller amplicons may be favored due to the reduced costs associated with the lower number of genes and/or replication of unnecessary DNA (20). This penalty might also be the underlying reason for the trend observed for the amplicon type a to be associated with significant decreased susceptibility to mefloquine and the observed trend for ART and LUM (Table 1). Further studies are warranted to explore the relationship between amplicon size and drug activity.

To elucidate potential determinants of resistance to PPQ, a recent work based on continuous exposure to PPQ was able to select parasites with IC_{50}s >100-fold greater than that for the parent line (Dd2). Applying comparative genome hybridization procedures on these parasites allowed the detection of a new amplification fragment neighboring the amplicon containing the *pfmdr1* gene (11) (see Fig. 2, green dashed box). The Thai samples herein analyzed did not show events of genomic amplification in this region. It is possible that this has to do with the fact that PPQ has not been widely used in western Thailand, where these parasites originated. It is of particular interest to analyze this genomic region on parasites from locations where PPQ has been used in national malaria control programs—like Vietnam or Papua, Indonesia—in order to identify relevant DNA changes.

Our data reinforce the notion that *pfmdr1* copy number amplifications are selected out by the use of 4-aminoquinolines, in contrast with what is observed with amino alcohol quinolines (lumefantrine and mefloquine) (25, 26, 30). Such effects, which mirror those previously observed for the *pfmdr1* N86Y SNP (15, 31), support the rational use of 4-aminoquinoline and amino alcohol quinoline-based ACTs as alternative first- and second-line chemotherapies in malaria control programs, as potentially they independently select for mutually incompatible combinations of mutations.

ACKNOWLEDGMENTS

We declare no commercial or other association that might pose a conflict of interest.

This work was supported by project grants from Swedish Development Cooperation Agency-Department for Research Cooperation (SWE 2005-0017, SWE 2005-4596, SWE-2007-174, and SWE-2005-4027). M.I.V. was supported by a postdoctoral grant from Fundação para a Ciência e Tecnologia (FCT)/Ministerio da Ciência e Ensino Superior, Portugal—MCES (SFRH/BPD/76614/2011). The Shoklo Malaria Research

Unit is part of the Mahidol Oxford University Tropical Medicine Research Unit, supported by the Wellcome Trust of Great Britain.

REFERENCES

1. **Adam I, et al.** 2010. Dihydroartemisinin-piperaquine versus artemether-lumefantrine, in the treatment of uncomplicated Plasmodium falciparum malaria in central Sudan. Ann. Trop. Med. Parasitol. **104**:319–326.
2. **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.
3. **Basco LK, Ringwald P.** 2003. In vitro activities of piperaquine and other 4-aminoquinolines against clinical isolates of Plasmodium falciparum in Cameroon. Antimicrob. Agents Chemother. **47**:1391–1394.
4. **Bassat Q, et al.** 2009. Dihydroartemisinin-piperaquine and artemether-lumefantrine for treating uncomplicated malaria in African children: a randomised, non-inferiority trial. PLoS One **4**:e7871. doi:10.1371/journal.pone.0007871.
5. **Briolant S, et al.** 2010. Absence of association between piperaquine in vitro responses and polymorphisms in the pfcrt, pfmdr1, pfmrp, and pfnhe genes in Plasmodium falciparum. Antimicrob. Agents Chemother. **54**:3537–3544.
6. **Carrara VI, et al.** 2009. Changes in the treatment responses to artesunate-mefloquine on the northwestern border of Thailand during 13 years of continuous deployment. PLoS One **4**:e4551. doi:10.1371/journal.pone.0004551.
7. **Cowman AF, Galatis D, Thompson JK.** 1994. Selection for mefloquine resistance in Plasmodium falciparum is linked to amplification of the pfmdr1 gene and cross-resistance to halofantrine and quinine. Proc. Natl. Acad. Sci. U. S. A. **91**:1143–1147.
8. **Cowman AF, Karcz S, Galatis D, Culvenor JG.** 1991. A P-glycoprotein homologue of Plasmodium falciparum is localized on the digestive vacuole. J. Cell Biol. **113**:1033–1042.
9. **Davis TM, Hung TY, Sim IK, Karunajeewa HA, Ilett KF.** 2005. Piperaquine: a resurgent antimalarial drug. Drugs **65**:75–87.
10. **Dondorp AM, et al.** 2010. Artemisinin resistance: current status and scenarios for containment. Nat. Rev. Microbiol. **8**:272–280.
11. **Eastman RT, Dharia NV, Winzeler EA, Fidock DA.** 2011. Piperaquine resistance is associated with a copy number variation on chromosome 5 in drug-pressured Plasmodium falciparum parasites. Antimicrob. Agents Chemother. **55**:3908–3916.
12. **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.
13. **Gargano N, Cenci F, Bassat Q.** 2011. Antimalarial efficacy of piperaquine-based antimalarial combination therapies: facts and uncertainties. Trop. Med. Int. Health **16**:1466–1473.
14. **Hastings IM, Ward SA.** 2005. Coartem (artemether-lumefantrine) in Africa: the beginning of the end? J. Infect. Dis. **192**:1303–1304. (Author reply, **192**:1304–1305.)
15. **Holmgren G, et al.** 2007. Selection of pfmdr1 mutations after amodiaquine monotherapy and amodiaquine plus artemisinin combination therapy in East Africa. Infect. Genet. Evol. **7**:562–569.
16. **Imwong M, et al.** 2010. Exploring the contribution of candidate genes to artemisinin resistance in Plasmodium falciparum. Antimicrob. Agents Chemother. **54**:2886–2892.
17. **Kamya MR, et al.** 2007. Artemether-lumefantrine versus dihydroartemisinin-piperaquine for treatment of malaria: a randomized trial. PLoS Clin. Trials **2**:e20. doi:10.1371/journal.pctr.0020020.
18. **Karunajeewa HA, et al.** 2008. Pharmacokinetics and efficacy of piperaquine and chloroquine in Melanesian children with uncomplicated malaria. Antimicrob. Agents Chemother. **52**:237–243.
19. **Mwai L, et al.** 2009. In vitro activities of piperaquine, lumefantrine, and dihydroartemisinin in Kenyan Plasmodium falciparum isolates and polymorphisms in pfcrt and pfmdr1. Antimicrob. Agents Chemother. **53**:5069–5073.
20. **Nair S, et al.** 2007. Recurrent gene amplification and soft selective sweeps during evolution of multidrug resistance in malaria parasites. Mol. Biol. Evol. **24**:562–573.
21. **Nair S, et al.** 2003. A selective sweep driven by pyrimethamine treatment in Southeast Asian malaria parasites. Mol. Biol. Evol. **20**:1526–1536.
22. **Nguyen DV, et al.** 2009. Pharmacokinetics and ex vivo pharmacodynamic antimalarial activity of dihydroartemisinin-piperaquine in patients with uncomplicated falciparum malaria in Vietnam. Antimicrob. Agents Chemother. **53**:3534–3537.
23. **Noedl H, et al.** 2005. Simple histidine-rich protein 2 double-site sandwich enzyme-linked immunosorbent assay for use in malaria drug sensitivity testing. Antimicrob. Agents Chemother. **49**:3575–3577.
24. **Price RN, et al.** 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.
25. **Price RN, et al.** 2004. Mefloquine resistance in Plasmodium falciparum and increased pfmdr1 gene copy number. Lancet **364**:438–447.
26. **Price RN, et al.** 2006. Molecular and pharmacological determinants of the therapeutic response to artemether-lumefantrine in multidrug-resistant Plasmodium falciparum malaria. Clin. Infect. Dis. **42**:1570–1577.
27. **Ribacke U, et al.** 2007. Genome wide gene amplifications and deletions in Plasmodium falciparum. Mol. Biochem. Parasitol. **155**:33–44.
28. **Rohrbach P, et al.** 2006. Genetic linkage of pfmdr1 with food vacuolar solute import in Plasmodium falciparum. EMBO J. **25**:3000–3011.
29. **Sanchez CP, Dave A, Stein WD, Lanzer M.** 2010. Transporters as mediators of drug resistance in Plasmodium falciparum. Int. J. Parasitol. **40**:1109–1118.
30. **Sidhu AB, et al.** 2006. Decreasing pfmdr1 copy number in Plasmodium falciparum malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin. J. Infect. Dis. **194**:528–535.
31. **Sisowath C, et al.** 2005. In vivo selection of Plasmodium falciparum pfmdr1 86N coding alleles by artemether-lumefantrine (Coartem). J. Infect. Dis. **191**:1014–1017.
32. **Somé AF, et al.** 2010. Selection of known Plasmodium falciparum resistance-mediating polymorphisms by artemether-lumefantrine and amodiaquine-sulfadoxine-pyrimethamine but not dihydroartemisinin-piperaquine in Burkina Faso. Antimicrob. Agents Chemother. **54**:1949–1954.
33. **Trager W, Jensen JB.** 1976. Human malaria parasites in continuous culture. Science **193**:673–675.
34. **Triglia T, Foote SJ, Kemp DJ, Cowman AF.** 1991. Amplification of the multidrug resistance gene pfmdr1 in Plasmodium falciparum has arisen as multiple independent events. Mol. Cell. Biol. **11**:5244–5250.
35. **Uhlemann AC, et al.** 2007. Intrahost selection of Plasmodium falciparum pfmdr1 alleles after antimalarial treatment on the northwestern border of Thailand. J. Infect. Dis. **195**:134–141.
36. **Veiga MI, et al.** 2011. Novel polymorphisms in Plasmodium falciparum ABC transporter genes are associated with major ACT antimalarial drug resistance. PLoS One **6**:e20212. doi:10.1371/journal.pone.0020212.