pfmdr1 amplification is related to increased Plasmodium falciparum in vitro sensitivity to the bisquinoline piperaquine

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The 4-aminoquinoline bisquinoline piperaquine is an important partner drug in one of 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 IC$_{50}$ and IC$_{90}$ values 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$_{90}$ levels (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.
Introduction

Artemisinin combination therapy (ACT) is instrumental for the global decrease of *Plasmodium falciparum* malaria in recent years. However, *P. falciparum* resistance has been documented to several partner drugs, namely artesunate-mefloquine (25), artemether-lumefantrine (26, 31) and artesunate-amodiaquine (15). 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 re-infecting parasites to sub-therapeutic concentrations (14).

A more recent ACT, dihydroartemisinin-piperaquine (DHA-PPQ) has been widely used in South-east Asia and is now ready to be launched in sub-Saharan Africa. Piperaquine (PPQ) is a bisquinoline, structurally a 4-aminoquinoline based antimalarial like chloroquine (CQ). Piperaquine is not a recent newcomer, having been extensively used in mono-therapy regimens in the Southern regions of China in the 1970’s and 1980’s, 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 + 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 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 been long 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 neither *in vitro* (5) nor *in vivo* (32).

Copy number variation (CNV) polymorphism at the multidrug resistant locus *pfmdr1* in chromosome 5 is well known in South East 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 characterised in field samples from the Thailand-Burma border (21), although the precise importance and impact of their diversity in the development of drug resistance is yet to be investigated.

Recently, it has been reported that the use of PPQ in Vietnam is 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 de-amplification of the amplicon encompassing *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 of the parasite cultures contained 5% hematocrit (O+ erythrocytes) in RPMI 1640 (GIBCO BRL, Invitrogen 42402-010) supplemented with 1x L-Glutamine (GIBCO BRL, Invitrogen 25030-032), 25µg/mL Gentamicine (GIBCO BRL, Invitrogen 15750-037) and 10% human serum. Piperaquine monophosphate (633.5 MW) was obtained from AvaChem (San Antonio, TX, USA) and chloroquine diphosphate (515.86 MW), was purchased from Sigma-Aldrich (St Louis, MO, USA). The ICs values were determined through the Histidine-Rich Protein 2 based Double-Site Sandwich Enzyme-Linked Immunosorbent Assay (23). Briefly, 200μl of synchronized culture at ring stage containing 0.05% parasitaemia and 1.5% hematocrit were pre-coated in 96-well culture plates (titration of ½), with a PPQ concentration in row 8 of 200 nM and CQ of 2µM. Cultures were incubated at 37°C in a candle jar (33) for 72 hours, followed by the lysis of the cells by freeze-thawing for ELISA analysis. Four independent assays were performed for each field strain and 3D7 reference strain IC_{50} measured for drug quality/efficacy control. The 3D7 strain was kindly provided by the late Prof. D. Walliker (Department of Animal and population genetics, University of Edinburgh, UK).

Molecular analysis
Extraction of gDNA of the 46 Thai strains and its molecular characterization including \textit{pfmdr1} N86Y, \textit{pfcrt} K76T SNPs and \textit{pfmdr1} gene copy number variation was previously performed (36). In order to better understand the importance for drug resistance of the amplified regions at chromosome 5, all strains carrying \textit{pfmdr1} increased copy number (#24 strains), as well as the laboratory strains FCB, FCR3, F32 and Dd2 known to carry multiple amplification of this gene, were further characterized for their approximate amplicon size through real-time PCR of the \textit{pfmdr1} adjacent regions.

The software program Primer Express 2.0. (Applied Biosystems, CA, USA) 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 \textit{ca.} 157 Kb fragment containing the \textit{pfmdr1} gene (supplementary table 1). The \(\beta\)-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 \textit{pfmdr1} copy) by using the \(\Delta\Delta Ct\) method. All assays were performed at least in triplicate on 96-well plates ABI PRISM® 7000 Sequence Detection System (Applied Biosystems™, Fresno, CA, USA). The detection threshold was set above the mean baseline value for the first 6–15 cycles.

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

\textbf{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 Sigma-Plot 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$_{50}$, IC$_{90}$) and the *pfmdr1* gene copy numbers were tested through the performance of t-test and when normality test failed (Shapiro-Wilk), Mann-Whitney Rank Sum Test was applied.
Results

The 50% and 90% inhibitory concentration (IC₅₀ and IC₉₀) values 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 IC₅₀ of 39.4 nM, from 13.8 to 108.2 nM (supplementary table 2). All strains were found to be highly resistant to CQ with IC₅₀ > 450 nM. The interaction between PPQ and CQ ICs was tested with Pearson correlation analysis. It was agonistic both for IC₅₀ values (correlation coefficient of 0.37, P=0.01) and IC₉₀ (correlation coefficient = 0.40; P =0.006).

Out of the 46 strains analyzed, 24 carried increased *pfmdr1* copy number. Specifically, 16 strains carried two copies, 6 strains three copies, and 2 strains harboured four copies of the *pfmdr1* gene. A significant association was found between the *pfmdr1* gene copy number amplifications and a decrease in the IC₅₀ values for PPQ (geometric mean of 42.9 nM for strains with *pfmdr1* CNV =1 vs geometric mean of 29.2 nM for the strains with *pfmdr1* CNV >1, P=0.002). The same pattern was observed for the IC₉₀ values for CQ (P=0.026) (figure 1).

Stratifying the strains with *pfmdr1* >1 copies (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 *pfmdr1* gene, the approximate amplicon size and structure (i.e. which loci 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”, see figure 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 figure 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 from the structure present in the analyzed reference strains.
We found no significant associations between the types of amplicon ("a" to "f") and the determined ICs for PPQ and CQ. Considering the previously determined phenotypes (36), significance difference was detected where parasites carrying the amplicon type “a” are associated with significantly higher mefloquine IC_{50} values (p<0.01)(table 1). A trend in increased IC_{50} is also detected for the same type of amplicon with the ART and LUM drugs, although statistically non-significant (table 1).

A region from the PFE1010w gene to the PFE1085w locus, located ca. 72Kbp upstream of the pfmdr1 gene (Figure 2, dashed green box), has been previously associated with in vitro PPQ resistance (11). However, no increase in copy number for the PFE1010w and PFE1085w genes was found among our 46 field strains.
The ACT DHA-PPQ has shown excellent efficacy in field clinical trials, making it a promising fixed dose formulation for the control of the disease. The recent detection of ART resistance in SE Asia (10), in regions where the partner drug (mefloquine) is failing (6) supports the importance of maintaining the integrity of the long-standing partner drug efficacy. With its very long half-life, PPQ is potentially at risk in settings of high transmission, due to the possibility of a large post-treatment drug resistance selection window, akin to what have been seen with other ACTs (15, 31). This, associated with the knowledge that PPQ resistance has already emerged in the past (13), commands urgency on the research of its molecular mechanisms. Additionally, it is to note that emerging drug resistance has been even 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 one log of IC50 values. According to the previous genotyped data in the herein analyzed parasites (36), this large range of sensitivities cannot be explained by the monomorphic pfcr1 gene, neither by the SNPs found in pfmdrl and pfmrpl gene. The non-involvement of pfmdrl and pfmrpl 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 pfmdrl increased copy number sensitizes the parasite to PPQ. The P-glycoprotein homologue 1 (Pgh1) coded by pfmdrl 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 solutes towards the FV (28). Such function implicates that the presence of increased pfmdrl copy (expected to lead to an increase of Pgh1 load in the FV membrane) is
associated to 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 ICs 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 a.a.
position 76, are a central factor for CQ resistance. The pfcrt 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 IC₅₀ > 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 have higher ICs values, reaching
statistical significance at IC₅₀ levels (Figure 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 only
visible in certain genomic environments (e.g. possibly the presence of the pfmdr1 86N allele).

It has been long known that the duplication events involving the 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 influences drug
susceptibility phenotypes. Overall the field strains were found to carry smaller amplicons, as
compared with lab strains (Figure 2), possibly due to a fitness cost, consistent with previous
reports of intra-host 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 the continuous exposure to PPQ was able to select parasites with IC$_{50}$ values $>$100-fold greater than 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 figure 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 are 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, mefloquine) (25, 26, 30). Such effects, which mirror the previously observed for the $pfmdr1$ N86Y SNP (15, 31) supports the rational use of 4-aminoquinoline and aminoalcohol 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.
References:


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Figures:

Figure 1: pfmdr1 copy number variation (CNV) and in vitro IC₅₀ and IC₉₀ of PPQ and CQ.

Association between the geometric mean of in vitro PPQ and CQ IC₅₀ and the pfmdr1 CNV polymorphism. Data described in supplementary table 2. Black bars: single copy of pfmdr1 gene (#22 strains). Grey bars: more than 1 pfmdr1 gene copies (#24 strains). T-test statistics was applied to see significant difference. Error bars represent standard error of the mean.

Figure 2: Genomic characterization of amplified regions.

Vertical bar charts characterize amplicons size and position on chromosome 5 (left y axis) including information of gene on sequence (right y axis). Pink bars illustrate amplicon type for the lab strains and blue bars different amplicons found in the Thai strains (#24) all carrying pfmdr1 copy number amplification (gene highlighted in red). Green box give prominence to a genomic region previously found to be associated with PPQ decrease susceptibility (11) which is not found in the Thai strains. The left y axis shows the position (kilo base pairs) on chromosome 5 and right y axis the gene annotation relative to 3D7 genome sequence. Genes with known or putative function described in the depicted genomic position was acquired from NCBI Map viewer at http://www.ncbi.nlm.nih.gov. The non-annotated genes in the figure are hypothetical proteins with unknown function.
### Table 1: Association between the type of amplicon and the determined IC50s.

<table>
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<tr>
<th># Thai strains</th>
<th>ART&lt;sup&gt;b&lt;/sup&gt; IC50(SE)</th>
<th>DHA&lt;sup&gt;b&lt;/sup&gt; IC50(SE)</th>
<th>MQ&lt;sup&gt;b&lt;/sup&gt; IC50(SE)</th>
<th>LUM&lt;sup&gt;b&lt;/sup&gt; IC50(SE)</th>
<th>PPQ IC50(SE)</th>
<th>CQ IC50(SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pfmdr1 CNV = 1</td>
<td>4.4(0.6)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.3(0.5)</td>
<td>49.1(19.7)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.6(1.8)</td>
<td>49.7(7.8)</td>
<td>676.1(40.9)</td>
</tr>
<tr>
<td></td>
<td>11.0(1.1)</td>
<td>15.0(5.5)</td>
<td>159.1(26.6)</td>
<td>18.9(2.7)</td>
<td>32.2(5.2)</td>
<td>601.0(25.8)</td>
</tr>
<tr>
<td>a CNV &gt; 1</td>
<td>14.8(4.7)</td>
<td>2.4(1.2)</td>
<td>243.6(16.5)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>26.0(6.8)</td>
<td>35.8(8.7)</td>
<td>585.3(17.2)</td>
</tr>
<tr>
<td></td>
<td>8.1(2.1)</td>
<td>0.9(0.2)</td>
<td>113.3(31.6)</td>
<td>13.6(2.7)</td>
<td>28.2(4.8)</td>
<td>509.2(23.6)</td>
</tr>
<tr>
<td>Amplicon type</td>
<td>3 CNV = 1</td>
<td>11.3(7.0)</td>
<td>1.0(0.4)</td>
<td>130.4(23.6)</td>
<td>21.1(5.7)</td>
<td>35.3(8.6)</td>
</tr>
<tr>
<td></td>
<td>6.5(1.9)</td>
<td>1.0(0.3)</td>
<td>135.2(34.7)</td>
<td>10.4(1.7)</td>
<td>32.5(4.5)</td>
<td>588.7(9.5)</td>
</tr>
<tr>
<td></td>
<td>2 CNV &gt; 1</td>
<td>12.7(1.1)</td>
<td>1.8(0.5)</td>
<td>170.9(13.8)</td>
<td>21.9(1.4)</td>
<td>35.0(5.9)</td>
</tr>
<tr>
<td></td>
<td>8.2 CNV = 1</td>
<td>12.9(4.6)</td>
<td>2.6(1.5)</td>
<td>186.4(4.7)</td>
<td>22.8(2.8)</td>
<td>25.1(2.2)</td>
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Pearson correlation with PPQ IC50

<table>
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<tr>
<th>46</th>
<th>r=0.37</th>
<th>P&lt;0.01</th>
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<sup>a</sup>: Amplicon type is described in detail in Figure 2 with the characterization of amplicon size and position of the amplified fragment carrying the pfmdr1 gene.

<sup>b</sup>: Inhibition concentration values previously published (36). SE: Standard error of the mean.

<sup>c</sup>: P value < 0.001 (T-test). Results previously published (36).

<sup>d</sup>: P value = 0.005. Unpair T-test between amplicon type “a” and amplicon type not “a”.

<sup>e</sup>: For pairs with P values greater than 0.05, there is no significant relationship between the two variables. r: correlation coefficient.
