Pfmdr1 amplification associated with clinical resistance to mefloquine in West Africa: implication in artemisinin combination therapies efficacy

Benoit Witkowski,1,2,§ Xavier Iriart,1,3,§ Patrice Njomnang Soh,1,2 Sandie Menard,1,3 Muriel Alvarez,4 Veronique Naneix-Laroche,4 Bruno Marchou,4 Jean-François Magnaval,3 Françoise Benoit-Vical,1,2,‡ Antoine Berry1,3,‡

Running head: mefloquine and pfmdr1 in Africa

1 Service de Parasitologie-Mycologie, Centre Hospitalier Universitaire de Toulouse, Université de Toulouse and Faculté de Médecine de Rangueil, Université de Toulouse III, Toulouse, France ; 2 CNRS; Laboratoire de Chimie de Coordination, UPR8241, Toulouse, France, and Université de Toulouse III, Toulouse, France ; 3 UMR3 MD-UM-UPS, Université Paul Sabatier Toulouse III, France ; 4 Service des Maladies Infectieuses et Tropicales, Centre Hospitalier Universitaire de Toulouse, Toulouse, France.

§ Authors B.W. and X.I. contributed equally to this manuscript.
‡ Authors F.B.V. and A.B contributed equally to this manuscript.

Keywords: mefloquine, ACT, resistance, Pfmdr1 amplification, Africa

*Correspondence: Service de Parasitologie-Mycologie, Centre Hospitalier Universitaire de Toulouse, Hôpital Rangueil, TSA 50032, 31059 Toulouse Cedex 9, France. Phone: 33 5 61 32 28 92. Fax: 33 5 61 32 20 96. E-mail: berry.a@chu-toulouse.fr
Abstract
We describe here a clinical failure in the treatment with mefloquine of acute falciparum malaria contracted in Africa associated with in vitro mefloquine-resistance and pfmdr1 copy number amplification. This case raises the question of the presence and the evolution of this genotype in Africa that is also known to alter the artemisinin combination therapies (ACT) susceptibility.

Case report
On March 30th, 2004, a 36-year-old man was admitted to the Tropical and Infectious Disease Unit of the Toulouse University hospital (France) with a 1-day history of a fever associated with shaking chills and nausea.

This patient went to Benin in August 2003 for humanitarian purposes and returned to France on February 10th, 2004 (Fig. 1). In the month preceding his arrival in Benin, he had travelled in Burkina Faso, Senegal, Mauritania, and Mali. He had never taken any malaria chemoprophylaxis. A few days before his return to France on February 10th, the patient reported an acute clinical malaria crisis, without parasitological diagnostic evidence, treated in Benin with artesunate 200 mg orally for the first day, and 100 mg/d for the following four days. On February 17th, a new P. falciparum malaria attack was diagnosed in Tarbes Hospital (France) by a thin blood smear (0.1 % parasitemia) and by a Core Malaria Pf® test (Core Diagnostics), an immunochromatographic rapid diagnostic test (RTD) detecting the P. falciparum-specific antigen histidine-rich protein 2 (2). Blood tests showed a slight anemia (124 g/L) and a thrombocytopenia (116 G/L). He was treated with mefloquine (MQ) (750 mg, 500 mg, and 250 mg oral doses spaced out 8 hours apart) which was well tolerated and seemed efficient. On March 13th, in the face of the same clinical signs of malaria again, the
patient took a further antimalarial treatment with MQ on his own initiative and using the same
protocol.

At the time of admission (March 30th) to the University hospital, the patient felt tired but was
afebrile without nausea or vomiting. The clinical examination (weight 75 kg) was normal
without hepatosplenomegaly. All the biological parameters (haemoglobin: 134 g/L, platelet
count: 158 G/L) were in a normal range. The thin blood smear revealed 0.8% P. falciparum
parasitemia. The species identification was confirmed both by a positive histidine-rich protein
2 RDT, the Core Malaria Pf® test (Core Diagnostics) and by a P. falciparum-specific PCR
which amplifies a 422 base pairs fragment of the mitochondrial Cox 1 gene (primers
sequences: Pf1: 5′-GGAATGTTATTGCTAACAC-3′ and Pf2: 5′-
AATGAAGAGCTGTATC -3′ (9). As the treatment with MQ was clinically ineffective,
oral treatment with quinine was initiated, 500 mg (one tablet every 8 hours). Quinine was
switched after one day to chloroquine (CQ) for 4 days given the wild-type K76 haplotype on
the pfcrt gene (indicating chloroquine-sensitive parasites) which was determined by qPCR
(4). The CQ treatment was well tolerated and efficient. The sensitivity to chloroquine and to
artemisinin was determined in vitro whereas these parasites were shown resistant to MQ and
quinine (Table 1). This in vitro radioisotopic chemo-susceptibility analysis was based on
parasite growth estimated by [3H] hypoxanthine incorporation. The IC₅₀ values were
determined with GraphPad software by plotting the drug concentration versus the percentage
of parasite growth inhibition at 48 h of incubation (3). The determination of the Pfmdr1 copy
number was carried out by qPCR on a LightCycler480® with the SYB Green I Master kit
(Roche Diagnostics) as described by Witkowski et al (25). Primers used were from Price et al
(18), pfmdr1-1F: 5′-TGCATCTATAAAACGATCAGACAAA-3′ and pfmdr1-R: 5′-
TCGTTGTTCCATGTGACTGT-3′ for pfmdr1 gene; β-tubulin-1F: 5′-
TGATGTCGCAAGTGTCC-3′ and β-tubulin-1R: 5′-
TCCTTTGTGGACATTCTTCCTC-3’ for β-tubulin gene. Briefly, β-tubulin was used as one copy reference gene. In each experiment, DNA from the laboratory strains of FcM29-Cameroon (1 pfmdr1 copy) and Dd2 (2-3 pfmdr1 copies) were used as controls. The patient’s sample was analyzed in triplicate and was checked twice. The P. falciparum isolate from the patient showed between 3-4 copies of pfmdr1 (copy number value at 3.6 ± 0.2). The sequencing of pfmdr1 was performed on a 3100 Genetic Analyzer (Applied Biosystems). Primers used were Pfmdr1-nest-F: TGTATGTGCTGTATTATCAGGA and Pfmdr1-nest-R: CTCTTCTATAATGGACATGGTA for the analysis of codons 86 and 184; Pfmdr2-nest-F: GAATTATTGTAAATGCAGCTTTA and Pfmdr2-nest-R: GCAGCAAACTTACTAACAG for the analysis of codon 1264. Experimentations revealed wild-type N86 and D1264 alleles and a mutated Y184F allele (Table 2).

Discussion
To our knowledge, this case is the first observation showing a link between this pfmdr1 genotype and MQ clinical resistance to falciparum malaria in Africa. This P. falciparum isolate harbored the molecular markers associated with MQ-resistance: a high copy number of pfmdr1 (3.6 ± 0.2) with wild-type N86 and D1264 alleles, that could explain in part the very high IC_{50} values for MQ, quinine, the low IC_{50} value for CQ and the MQ clinical failure. Mefloquine-resistant malaria is well known in Southeast Asia and particularly in some parts of Thailand, Burma and Cambodia. This resistance is directly linked to the extensive use of MQ, an aryl-amino-alcohol, as the first line treatment for falciparum malaria since the mid 1980s (18). In Africa, mefloquine-resistant isolates exist for a long time even when MQ was not used (6, 15) but only one clinical failure with MQ in a curative treatment regimen has previously been described in 1990 (10). Moreover, MQ-susceptibility is usually inversely correlated to CQ-susceptibility as observed in our case (8). Thus, the very low number of MQ
resistant isolates is possibly related, first to the low use of MQ in this area and secondly to the
high prevalence of CQ-resistant isolates due to massive CQ-pressure in the whole of Africa
for decades.

Amplification of the \textit{pfmdr1} gene is the most relevant molecular marker associated with
resistance to MQ in the field (18). In Africa, \textit{pfmdr1} amplification was rarely reported. Only
11 isolates (3 in Ivory Cost, 1 in Togo, 1 in Burkina Faso, 5 in Gabon and 1 in Kenya) from
1289 samples tested have been found (1, 11, 24, 25). An increase in the \textit{pfmdr1} copy number
is also associated with an \textit{in vitro} decrease in sensitivity to other aryl-amino-alcohols, in
particular lumefantrine, the partner of artemether in one of the major ACT used worldwide
(13, 19). Similarly, a positive correlation between the decrease in susceptibility to artemisinin
derivatives and \textit{pfmdr1} amplification has also been observed (5, 13). Interestingly, clones with
\textit{pfmdr1} amplification nearly all had the wild-type N86 \textit{pfmdr1} allele (16, 18, 19) since only 2
clones in 2 different studies have been described with the mutated N86Y allele (1, 24). \textit{In
vitro}, the N86 and D1264 alleles, independently of the \textit{pfmdr1} copy number, are associated
with a lower susceptibility to MQ and to lumefantrine (8, 18, 21). In summary, \textit{pfmdr1}
amplification and the N86 allele pose not only the problem of resistance to MQ but also
reduced susceptibility to ACT.

Nevertheless a selection of mefloquine resistant clones directly intra-host (23) is also quite
plausible here since this patient was treated with MQ for nearly 2 months (Fig. 1). Indeed,
\textit{Pfmdr1} duplication or amplification from 2 to 3 copies, are frequent genetic events
(respectively $1/10^8$ or $1/5 \times 10^3$ parasites per asexual cycles) (17).

The conditions for the emergence of \textit{pfmdr1} amplification in Southeast Asia such as the high
pressure of MQ monotherapy or low level of transmission should never occur in Africa
suggesting a low risk of this genotype to develop. Nevertheless, the rapid abandonment of CQ
in the treatment of \textit{P. falciparum} malaria in all African areas, replaced by ACT, will allow the
reappearance of genotypes such as the N86 haplotype on pfmdr1 (12, 22) that are favorable to the decreasing effectiveness of aryl-amino-alcohols. This could occur faster than observed in Southeast Asia as the non-falciparum species that are still treated with CQ are less present in Africa (< 20 %) than in Asia (50 %). Furthermore lumefantrine, widely prescribed in Africa with artemether, is well known to also select the pfmdr1 genotype leading to a lesser susceptibility to aryl-amino-alcohols (7, 22). Added to this, prevalence of pfmdr1 amplification may be increased by some combined formulations such as MQ+artesunate and MQ+sulfadoxine-pyrimethamine, used in several African countries although not supported by official health policies (14, 20).

The global switch from CQ to aryl-amino-alcohol-based ACTs would be a critical conjunction for the emergence of clones with drug resistance related to the pfmdr1 genotype. As the emergence of this genotype could announce the decline of ACT efficacy, the molecular monitoring of the pfmdr1 genotype is therefore now warranted to optimize the management of antimalarial policy and thus to ensure a longer period of efficacy for the ACT treatments.

Acknowledgments

The authors gratefully acknowledge John Woodley for the English revision of the manuscript.
References


artemether-lumefantrine in an area of Uganda where malaria is highly endemic.


therapy by health professionals in urban health facilities in Yaounde, central province, Cameroon. Malar J 8:176.


Figure and legend

Legend Figure 1:

Summary of the patient's malaria attacks and treatment. The periods of treatment (dark grey areas) and mean half-life (pale grey areas) of artesunate (ATS), mefloquine (MQ) are shown. The % of parasitemia are in brackets.
Table 1: IC$_{50}$ value of this *P. falciparum* isolate

<table>
<thead>
<tr>
<th>Drugs</th>
<th>IC$_{50}$ (nM)</th>
<th>Resistance thresholds (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MQ</td>
<td>910</td>
<td>30-40</td>
</tr>
<tr>
<td>CQ</td>
<td>7</td>
<td>100</td>
</tr>
<tr>
<td>Q</td>
<td>2500</td>
<td>800</td>
</tr>
<tr>
<td>ART</td>
<td>10</td>
<td>NA</td>
</tr>
</tbody>
</table>

IC$_{50}$ value of mefloquine (MQ), chloroquine (CQ), quinine (Q) and artemisinin (ART) obtained with this isolate by the radioactive micro-method.

NA: data not available

Table 2: *Pfmdr1* genotype of this *P. falciparum* isolate

<table>
<thead>
<tr>
<th>Molecular markers</th>
<th>Alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>pfmdr1</em> copy number</td>
<td>3.6±0.2</td>
</tr>
<tr>
<td>codon 86</td>
<td>N86</td>
</tr>
<tr>
<td>codon 184</td>
<td>Y184F</td>
</tr>
<tr>
<td>codon 1264</td>
<td>D1264</td>
</tr>
</tbody>
</table>