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The F423Y Mutation in the *pfmdr2* Gene and Mutations N51I, C59R, and S108N in the *pfdhfr* Gene Are Independently Associated with Pyrimethamine Resistance in *Plasmodium falciparum* Isolates

Sébastien Briolant,[a] Hervé Bogreau,[a] Marine Gil,[a] Housem Bouchiba,[a] Eric Baret,[a,b] Rémy Amalvict,[a,b] Christophe Rogier,[a,c] and Bruno Pradines[a,b]

Unité de Recherche en Biologie et Epidémiologie Parasitaires, Institut de Recherche Biomédicale des Armées—Antenne de Marseille, Marseille, France[a]; Centre National de Référence du Paludisme, Marseille, France[b]; and Institut Pasteur de Madagascar, Antananarivo, Madagascar[c]

Screening for *in vitro* susceptibility to pyrimethamine and sequencing of the *pfmdr2* and *pfdhfr* genes were performed in 140 *Plasmodium falciparum* isolates. The risk of *in vitro* resistance to pyrimethamine was analyzed with a logistic regression model. The mutation F423Y in *pfmdr2* (odds ratio [OR] = 2.12 [confidence interval {CI}, 1.02 to 4.59]; $P = 0.0489$) and the mutation N51I, C59R, or S108N in *pfdhfr* (OR = 42.34 [CI, 5.52 to 324.61]; $P = 0.0003$) were independently associated with *in vitro* resistance to pyrimethamine.

Sulfadoxine and pyrimethamine (Pyr) (SP) were widely used as first-line drugs for the treatment of malaria until the spread of resistance in *Plasmodium falciparum* isolates all over the world. SP is still recommended in combination with artesunate or chloroquine as a second-line therapy for uncomplicated *P. falciparum* and *P. vivax* malaria (7, 17, 19). SP is also still widely used in Africa for the intermittent preventive treatment (IPT) of malaria in infants (6), children (3), and pregnant women (8). SP resistance has been well characterized at the molecular level. Mutations involving residues 51, 59, 108, and 164 of *P. falciparum* dihydrofolate reductase conferred cross-resistance to the antifolates pyrimethamine and cycloguanil, whereas a mutation in residue 16 specifically conferred resistance to cycloguanil (16). The accumulation of mutations in the enzyme dihydropteroate synthase (in which Gly437 proved to be a key residue) was associated with sulfadoxine resistance in *P. falciparum* (18). Mutations involving other residues in dihydrofolate reductase and dihydropteroate synthase have also been linked to SP resistance in *P. vivax* isolates (1, 9). More recently, other mechanisms of resistance to SP have been proposed. Dahlström et al. (4) have suggested that mutations (specifically, I876V and K1466R) in multidrug resistance protein 1 may mediate an alternative mechanism of SP resistance in *P. falciparum*, and Martinelli et al. (10) have identified a mutation (K392Q) in the *mdr2* gene in a *P. chabaudi* clone that was genetically linked to sulfadoxine resistance.

pfmdr2 is an ABC transporter gene that encodes a P-glycoprotein similar to that encoded by *pfmdr1* (13). Although Ekong et al. (5) reported increased transcription of *pfmdr2* in chloroquine-resistant parasites, other studies have demonstrated neither amplification nor overexpression of *pfmdr2* (15, 21). To date, there appears to be no evidence to suggest that *pfmdr2* is involved in drug resistance in *P. falciparum*.

In this paper, we assessed the association between single nucleotide polymorphisms in the sequence of *pfmdr2* and *in vitro* resistance to pyrimethamine (Pyr) in *P. falciparum* isolates.

Between 2004 and 2010, 140 *P. falciparum* isolates (55 from Comoros, 18 from Cameroon, 18 from Republic of Ivory Coast, 11 from Senegal, 7 from Burkina Faso, 2 from Madagascar, 1 from French Guyana, 1 from Mayotte, and 27 from 13 other African countries) were collected from patients with uncomplicated malaria who were hospitalized in different French hospitals, and the isolates were sent to the French National Reference Center for Malaria. *In vitro* susceptibility to Pyr was evaluated as described in reference 11, and a threshold of 2,000 nM was applied to determine the *in vitro* resistance of *P. falciparum* isolates as defined by Basco et al. (2). A 562-bp coding region of *pfdhfr* was amplified by PCR and sequenced as described in reference 12; three mutations, including a Ser→Asn change at codon 108, an Asn→Ile change at codon 51 and a Cys→Arg change at codon 59, were analyzed. Copy numbers and single nucleotide polymorphisms in the *pfmdr2* gene were evaluated as described in reference 20. All statistical analyses were performed using the R software package, version 2.10.1.

The mean 50% inhibitory concentration (IC_{50}) of Pyr for the 140 isolates was 3,093 nM (95% confidence interval [CI], 2,527 to 3,660; minimum and maximum, 9.43 and 19,654 nM). Of these isolates, 69 had a Pyr $IC_{50} > 2{,}000$ nM (mean Pyr $IC_{50} = 5{,}687$ nM; 95% CI, 4,941 to 6,432) and were considered resistant; 71 isolates had a Pyr $IC_{50} < 2{,}000$ nM (mean Pyr $IC_{50} = 573.3$ nM; 95% CI, 425.2 to 721.4) and were considered susceptible to Pyr. Regarding the *pfdhfr* gene polymorphism, 74% (104/140) of the isolates had a point mutation at codon 51 (N51I); 73% (102/140) had a mutation at codon 59 (C59R), and 79% (110/140) had a mutation at codon 108 (S108N). Further, 69% (97/140) of the isolates had a triple mutation in *pfdhfr*. All 140 isolates possessed only one copy of the *pfmdr2* gene. The analysis of the sequence of *pfmdr2* gene revealed three points of mutation: S208N, F423Y, and K924E. There was no significant association between the S208N or K924E mutation and *in vitro* resistance to Pyr ($P = 1$;

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Address correspondence to Bruno Pradines, bruno.pradines@free.fr.
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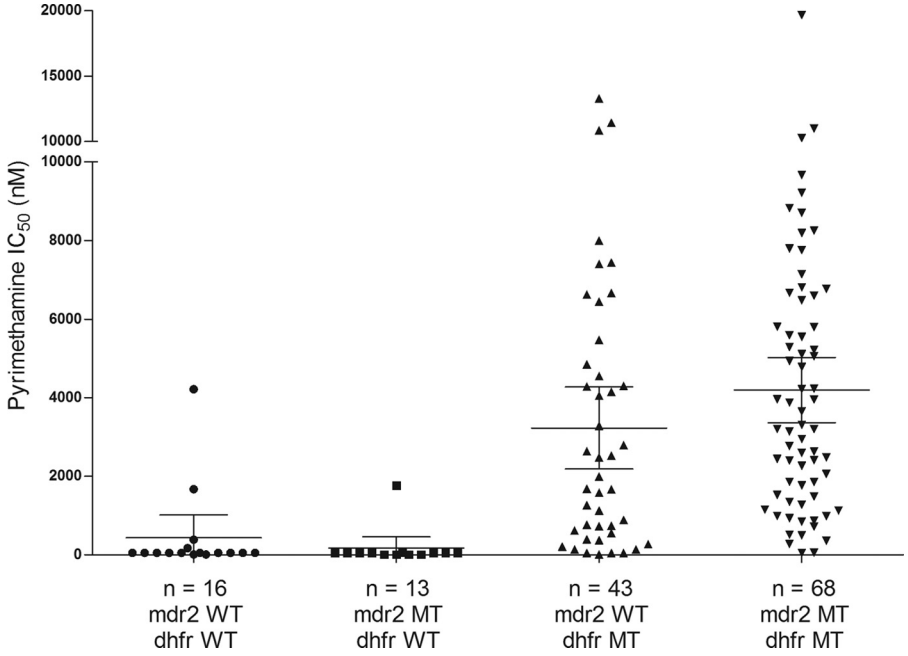

FIG 1 Pyrimethamine IC_{50}s (in nanomoles) of the 140 *Plasmodium falciparum* isolates according to their genotypes.

Fisher's exact test). The difference between the median of the Pyr IC_{50} (2,598 nM) for isolates harboring the Y substitution at codon 423 ($n = 81$) and the median of the Pyr IC_{50} (890 nM) for wild-type isolates ($n = 59$) was statistically significant ($P < 0.02$; Mann-Whitney test).

There was no statistically significant correlation between the F423Y mutation in *pfmdr2* and the mutation in *pfdhfr* ($P = 0.14$; Fisher's exact test). As illustrated in Fig. 1, 16 isolates were wild type for both the *pfmdr2* and *pfdhfr* genes; the mean Pyr IC_{50} for these isolates was 434.6 nM (95% CI, 0 to 1,014; minimum and maximum, 10 and 4,214 nM). A total of 13 isolates had the F423Y mutation in *pfmdr2* and were wild type for *pfdhfr*; the mean Pyr IC_{50} for these isolates was 170.1 nM (95% CI, 0 to 459.3; minimum and maximum, 10 and 1,762 nM). A total of 43 isolates were wild type for *pfmdr2* and mutant type for *pfdhfr* (with at least one of the mutations N51I, C59R, and S108N); the mean Pyr IC_{50} for these isolates was 3,229 nM (95% CI, 2,186 to 4,272; minimum and maximum, 9.43 and 13,294 nM). A total of 68 isolates were mutant type for both the *pfmdr2* and *pfdhfr* genes; the mean Pyr IC_{50} for these isolates was 4,192 nM (95% CI, 3,362 to 5,022; minimum and maximum, 50 and 19,654 nM). There was no statistically significant association between the median of the Pyr IC_{50} (50 nM) for isolates with both *pfmdr2* and the *pfdhfr* wild type and the median of the Pyr IC_{50} (50 nM) for isolates with the mutant type for *pfmdr2* and the wild type for *pfdhfr* ($P = 0.25$; Mann-Whitney test). The difference between the median of the Pyr IC_{50} (1,993 nM) of *pfmdr2* wild-type and *pfdhfr* mutant-type isolates and the median of the Pyr IC_{50} (3,255 nM) for isolates harboring the Y substitution at codon 423 and a *pfdhfr* mutation was statistically significant ($P = 0.0438$; Mann-Whitney test).

The *in vitro* resistance to Pyr (Pyr $IC_{50} > 2{,}000$ nM) was analyzed using a logistic regression model (Table 1). The mutation F423Y in *pfmdr2* (OR = 2.12 [95% CI, 1.02 to 4.59]; $P = 0.0489$) and the mutation N51I, C59R, or S108N in *pfdhfr* (OR = 42.34 [95% CI, 5.52 to 324.61]; $P = 0.0003$) (Hosmer-Lemeshow goodness-of-fit test; $P = 0.77$) were independently associated with *in vitro* resistance to Pyr.

MDR2 is associated with heavy metal ion efflux and resistance to cadmium (14). MDR2 may be involved in the transport of organic anions, such as folate or pABA, as has been suggested for

TABLE 1 Multivariate logistic regression model

Genotype	No. (%) of Pyr-susceptible isolates	No. (%) of Pyr-resistant isolates	Crude OR (95% CI)[a]	*P* value[b]	Adjusted OR (95% CI)	*P* value[c]
pfmdr2						
F423	37 (26.4)	22 (15.7)	1 (reference)		1 (reference)	
Y423	34 (24.3)	47 (33.6)	2.31 (1.11–4.91)	0.017	2.12 (1.02–4.59)	0.0489
pfdhfr						
Wild type	28 (20)	1 (0.7)	1 (reference)		1 (reference)	
Mutant type	43 (30.7)	68 (48.6)	43.36 (6.67–1,819.1)	<0.0001	42.34 (5.52–324.61)	0.0003

[a] OR, odds ratio; CI, 95% confidence interval.
[b] Fisher's exact test.
[c] Multivariate logistic regression model.

MRP1 (4); it may be also implicated in the transport of sulfadoxine, as suggested by Martinelli et al. (10). Interestingly, in the latter study, an association between the *in vivo* resistance of *Plasmodium chabaudi* to sulfadoxine and a mutation in the *mdr2* gene has been shown. The same investigation performed with *Plasmodium falciparum* would be really interesting, but no laboratory is able to evaluate the sulfadoxine IC_{50} with sufficient reproducibility. As MDR2 (like MDR1, which is well known to be an efflux pump involved in *Plasmodium falciparum* resistance to mefloquine) belongs to the ABC transporter family, the F423Y mutation in MDR2 might be associated with the efflux of pyrimethamine from outside the parasite. This AA modification concerns a cytoplasmic loop of the MDR2 protein and does not represent a significant alteration in terms of size and charge characteristics of the side chain, as phenylalanine and tyrosine are both hydrophobic. But this tyrosine could be a phosphorylation site, and it is known that protein structure and activity can be modulated by phosphorylation of tyrosine residues. The mechanisms of *P. falciparum* resistance to SP are probably multigenic; it is important for all of these underlying mechanisms to be elucidated, because SP is still used for the IPT of malaria in Africa. The results of the present study are of interest from this point of view. But further investigations would be necessary to confirm these data with field isolates in particular and also to evaluate the potential association between this *pfmdr2* mutation and the resistance of *P. falciparum* to sulfadoxine.

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