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Polymorphism in PfMRP1 (*Plasmodium falciparum* Multidrug Resistance Protein 1) Amino Acid 1466 Associated with Resistance to Sulfadoxine-Pyrimethamine Treatment▽

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Sulfadoxine-pyrimethamine (SP) remains widely recommended for intermittent preventive treatment against *Plasmodium falciparum* malaria for pregnant women and infants in Africa. Resistance to SP is increasing and associated primarily with mutations in the *P. falciparum dhfr* (*Pfdhfr*) and *Pfdhps* genes. This study aimed to explore the hypothetical association of genetic alterations in the *P. falciparum* multidrug resistance protein gene (*Pfmrp1*) with the in vivo response to SP by detecting the selection of single nucleotide polymorphisms (SNPs) following standard single-dose treatment administered to children with acute uncomplicated *P. falciparum* malaria in Tanzania. We detected significant selection of parasites carrying the *Pfmrp1* 1466K allele in samples from children with recrudescent infections, with 12 (100%) of 12 such samples being positive for this allele, compared to 52 (67.5%) of 77 baseline samples ($P = 0.017$), in parallel with the selection of the *Pfdhfr Pfdhps* quintuple mutant haplotype in cases of recrudescence ($P = 0.001$). There was no association between the 1466K SNP and the *Pfdhfr Pfdhps* quintuple mutation, indicating independent selections. Our data point for the first time to a role for a *P. falciparum* multidrug resistance protein homologue in the antimalarial activity of SP. Moreover, they add to the growing evidence of the potential importance of *Pfmrp1* in antimalarial drug resistance.

The antifolate drug formulation sulfadoxine-pyrimethamine (SP) is still globally used, either as monotherapy or in combination with other antimalarials (http://www.who.int/malaria/treatmentpolicies.html). Importantly, SP remains the treatment of choice for intermittent preventive treatment of pregnant women and infants in Africa, both groups being at major risk for severe disease manifestations of *Plasmodium falciparum* malaria (14). Understanding the detailed basis of resistance to this drug is hence of noteworthy importance.

P. falciparum in vivo and in vitro resistance to pyrimethamine and sulfadoxine has been well documented to be associated with point mutations in the respective target genes, i.e., the *P. falciparum* dihydrofolate reductase gene (*Pfdhfr*) and the *P. falciparum* dihydropteroate synthase gene (*Pfdhps*). In particular, a quintuple mutant (with *Pfdhfr* mutations S108N, N51I, and C59R and *Pfdhps* mutations A437G and K540E) has been strongly associated with SP treatment failure (9, 12, 19) on the African continent. However, these genes do not seem to explain the full mechanism of resistance to SP (23, 24).

P. falciparum has an endogenous folate biosynthesis pathway, but most strains can also use exogenous folate through the folate salvage pathway (17, 23). The influx of exogenous folate (23) and the proteins responsible for this process (8, 16, 18), as well as efflux systems able to pump antifolate drugs out of the parasite intracellular space (16), have been proposed to further influence antifolate resistance.

In mammalian cells, resistance to antifolates can be mediated through the influx and efflux of folates and antifolates (1). Multidrug resistance proteins (MRPs) have a central role in folate and antifolate transport (2). These proteins are membrane transporters that pump xenobiotics and other substances, e.g., folate (27), out of cells. MRPs have been shown to mediate antifolate resistance by alteration in the efflux of drugs, e.g., methotrexate, and folate (10, 21, 27).

In *P. falciparum*, two MRP homologues, PfMRP1 and PfMRP2, have been identified (4, 11). PfMRP1 (identification no. PFA0590w in PlasmoDB [http://plasmodb.org]) has been suggested previously to have a role in the parasite response to several antimalarial drugs, including chloroquine and quinine, through drug efflux (20).

In this work, we hypothesized that an MRP may contribute to *P. falciparum* resistance to SP in vivo. This possibility was tested by examining the genotypes associated with the I876V and K1466R mutations, previously identified as the most frequent *Pfmrp1* polymorphisms in Africa (6), in blood samples collected from patients pre- and posttreatment in a clinical efficacy study conducted in Tanzania. The selection of a *Pfmrp1* single nucleotide polymorphism (SNP) upon SP treatment would suggest an involvement of the encoded protein in the parasite in vivo response to antimalarial drugs. Polymorphisms in *Pfdhfr* and *Pfdhps* were analyzed as part of a surveillance study for the SP drug, with the results also serving as a

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TABLE 1. Primers for *Pfmrp1* PCR and pyrosequencing

Primer name	Sequence (5′→3′)
A2626G First fw	AATATTCCATTCAATGAAAATTAC
A2626G First rev	CAACGTACTTTTATTCATTGAGA
A2626G Nest fw	Biotin-TATTCCATTCAATGAAAATTACCT
A2626G Nest rev	TATGGAAGGATCTAAAGATGTAAA
A2626G Seq rev	GGAAGGATCTAAAGATGTAA
A4397G First fw	AATAAAGAACATTCAGACACAAT
A4397G First rev	TGATTTTCCTACTATCCCAATT
A4397G Nest fw	TGGATACTGTATATCGTTTTCTGC
A4397G Nest rev	Biotin-CCCAATTTTTTGATTTTTTAAAGC
A4397G Seq fw	TGATTATACTCACATAGAAA

control for in vivo SP-driven SNP selection in the present study. The present work is an integrated part of an ongoing global molecular epidemiology project on the association of *Pfmrp1* diversity and in vivo responses to antimalarial drugs.

MATERIALS AND METHODS

Study subjects. We analyzed *P. falciparum*-positive blood samples from patients participating in a clinical trial conducted in Fukayosi, Bagamoyo District, Coast Region, Tanzania, comparing SP (Fansidar; Roche) with artemether-lumefantrine (Coartem; Novartis, Basel, Switzerland) during the period from April to July 2004 (13). In brief, 106 children with microscopically confirmed acute uncomplicated *P. falciparum* malaria were enrolled and randomly allocated to receive standard treatment with SP ($n = 56$) or artemether-lumefantrine ($n = 50$) according to body weight. After enrolment, the children were followed up with and checked for parasitemia routinely on days 1, 2, 3, 7, 14, 21, 28, 35, and 42 of the study or on any day of recurrent illness. Blood samples were collected on filter paper (3MM; Whatman) for molecular genotyping. Informed consent was obtained from the enrolled children's parents or guardians. The study was approved by the ethics committees at the Muhimbili University College of Health Sciences, Tanzania, and Karolinska Institutet, Sweden.

For the present study, samples from the entire baseline population prior to drug administration (day 0 [D_0]; $n = 106$) and the 40 children in the SP arm of the study with recurrent infections detected were analyzed. The analysis was based on previous *Pfmsp2* and *Pfmsp1* stepwise genotyping to define the recurrent parasitemias as either reinfections (new infections) or recrudescences (treatment failures) (13). A recurrent infection was classified as a recrudescence if there was at least one allelic band for both genetic markers matching with that in the corresponding D_0 sample or as a reinfection if there was no matching allelic band for at least one genetic marker (13).

DNA extraction. *P. falciparum* genomic DNA from the clinical trial patients was extracted from filter paper using an ABI PRISM 6100 nucleic acid prep station (Applied Biosystems, Fresno, CA) as described previously (5) and frozen at −20°C until use.

***Pfmrp1* I876V and K1466R SNP pyrosequencing.** The polymorphic nucleotide positions 2626 and 4397 (corresponding to amino acid positions 876 and 1466, respectively) in *Pfmrp1* were analyzed by pyrosequencing. The pyrosequencing protocol and primers were designed with Pyrosequencing assay design software, version 1.0 (Biotage AB, Uppsala, Sweden). A first PCR amplification was performed for nucleotide 2626 with the primers A2626G First fw and A2626G First rev, resulting in a product of 208 bp, and for nucleotide 4397 with A4397G First fw and A4397G First rev, resulting in a product of 1,176 bp (Table 1). The nested PCR amplification was performed with primers A2626G Nest fw and A2626G Nest rev for 2626, giving a 104-bp product, and A4397G Nest fw and A4397G Nest rev for 4397, giving a 436-bp product. Streptavidin Sepharose beads (Amersham Biosciences, Little Chalfont, United Kingdom) were added to the PCR products, and single-stranded biotinylated PCR products were obtained with a pyrosequencing vacuum prep workstation (Biotage AB, Uppsala, Sweden). Pyrosequencing reactions with the primers A2626G Seq rev for nucleotide position 2626 and A4397G Seq fw for position 4397 were performed using the PSQ 96 SNP reagent kit and a PyroMark ID instrument according to the recommendations of the manufacturer (Biotage AB, Uppsala, Sweden). The nucleotide dispensation orders were GATACTGAT for 2626 and CGACGATGT for 4397.

***Pfdhfr* and *Pfdhps* genotyping.** The genotypes for *Pfdhfr* N51I, C59R, and S108N/T SNPs were determined through PCR-restriction fragment length polymorphism analysis as described by Veiga and colleagues (26), while the analysis of G437A and K540E SNPs in *Pfdhps* was performed according to the method of Duraisingh and colleagues (7) with minor modifications. All restricted products were visualized on 2% agarose gels (with 0.1 μg/ml ethidium bromide) and analyzed under UV light by using the Bio-Rad Gel Doc 2000 system. The algorithm from Kublin and colleagues (12) was used to define the *Pfdhfr* and *Pfdhps* haplotypes.

Bioinformatic and statistical analyses. The SNP software PSQTM96MA (Biotage AB, Uppsala, Sweden) was used to analyze the output from the PyroMark ID instrument (Biotage AB, Uppsala, Sweden). Fisher's two-tailed test was used to evaluate the difference in genotype frequencies between baseline samples and samples from the times of infection recurrence. Statistical significance was defined by a P value of <0.05. To test for an association between the quintuple mutant haplotype and variation in amino acid 1466, the χ^2 test was used. Samples with a negative result for either or both of the parameters were excluded. Mixed genotypes for position 1466 were counted as a K-encoding genotype. These statistical analyses were performed with GraphPad QuickCalcs (http://www.graphpad.com). Associations between genotype in baseline samples and recrudescent outcome were evaluated using odds ratios (OR), and the analyses were performed with JavaStat (http://statpages.org/ctab2x2.html).

RESULTS

Forty (71%) of 56 children in the SP arm had recurrent parasitemia during follow-up (13). *Pfmsp2* and *Pfmsp1* diversity analysis of D_0 samples versus samples from the times of infection recurrence identified 15 recrudescences, 23 reinfections, and 2 inconclusive outcomes among the 40 recurrences. To test if *Pfdhfr*, *Pfdhps*, and *Pfmrp1* were under SP selection pressure, the frequencies of SNPs in these genes at D_0 and at the times of the recrudescences and reinfections were compared. For *Pfmrp1*, there was statistically significant selection of the pure 1466K allele among the samples from children with recrudescences, of which 12 of 12 (100%) carried the allele, compared to 52 (67.5%) of 77 baseline samples ($P = 0.017$; Fisher's two-tailed test), when samples from subjects with mixed infections were excluded. This selection was confirmed by including the mixed infections, counted as corresponding to either 1466K ($P = 0.038$; Fisher's two-tailed test) or 1466R ($P = 0.020$; Fisher's two-tailed test) (Table 2). Consequently, the pure 1466R allele that was found in 25 (32.5%) of 77 baseline samples was not detectable in the samples from patients with recrudescences. Among the samples from children with reinfections, there were no statistically significant changes in the frequency of the 1466K allele. There was an association between the carriage of the 1466K genotype at baseline and the recrudescence outcome (OR = 5.50; 95% confidence interval, 0.81 to 35.66), although it was not statistically significant. There were no statistically significant changes in the frequency of the I876I/V SNP (Table 2).

At the baseline, the *Pfdhfr Pfdhps* quintuple mutant haplotype was found in 20 of 96 samples, giving a prevalence of 20.8%. Upon SP therapy, statistically significant selection of this haplotype, among both 14 (93.3%) of 15 subjects with recrudescences ($P = 0.001$; Fisher's two-tailed test) and 17 (77.3%) of 22 subjects with reinfections ($P = 0.002$; Fisher's two-tailed test), was observed (Table 2). The presence of the quintuple mutant haplotype at baseline was associated with treatment failure (OR = 8.25; 95% confidence interval, 2.21 to 31.00).

No association between the quintuple mutation and 1466K either in the recurrent infections ($\chi^2 = 2.210$; degrees of freedom = 3; $P = 0.5301$) or at baseline ($\chi^2 = 0.004$; degrees of

TABLE 2. Prevalence of *Pfmrp1* genotypes for amino acid positions 876 and 1466 and *Pfdhfr Pfdhps* haplotypes in *P. falciparum* infections at baseline (D_0) and at times of recurrent parasitemia after SP treatment

Gene(s) and amino acid encoded or haplotype description	No. of positive samples/total no. of samples successfully tested (ratio)[a] at:		
	D_0[d]	Time of recrudescence	Time of reinfection
Pfmrp1			
1466K	52/101 (0.515)	12/14 (0.857)*	14/21 (0.667)
1466R	25/101 (0.248)	0/14 (0.000)*	2/21 (0.095)
1466K/R	24/101 (0.238)	2/14 (0.143)	5/21 (0.238)
876I	75/104 (0.721)	12/13 (0.923)	18/23 (0.783)
876V	13/104 (0.125)	1/13 (0.077)	2/23 (0.087)
876I/V	16/104 (0.154)	0/13 (0.000)	3/23 (0.130)
Pfdhfr and *Pfdhps*[b]			
Wild type	3/96 (0.031)	0/15 (0.000)	0/22 (0.000)
Single mutant	1/96 (0.010)	0/15 (0.000)	0/22 (0.000)
Double mutant	21/96 (0.219)	0/15 (0.000)	1/22 (0.046)
Triple mutant	36/96 (0.375)	1/15 (0.067)	0/22 (0.048)
Quadruple mutant	15/96 (0.157)	0/15 (0.000)	4/22 (0.182)
Quintuple mutant[c]	20 (3 + 17)/96 (0.208)	14 (0 + 14)/15 (0.933)**	17 (3 + 14)/22 (0.773)*

[a] *, $P < 0.05$;**, $P \leq 0.001$. Fisher's exact two-tailed test was used to evaluate if there was a difference in SNP prevalence between the baseline and the recrudescence and reinfection samples.
[b] *Pfdhfr Pfdhps* haplotypes were defined according to the nomenclature proposed by Kublin and colleagues (12).
[c] The numbers of samples with mixed and pure quintuple mutants (mixed + pure), respectively, are shown.
[d] The frequencies prior to the administration of the drug at D_0 are based on samples from all of the patients enrolled in the study, independent of which treatment arm they were allocated to.

freedom = 3; $P = 0.9999$) was seen, suggesting independent selections.

DISCUSSION

SP is still widely used for the treatment of uncomplicated *P. falciparum* malaria, particularly in combination with artesunate or amodiaquine. A remaining key indication for this drug is intermittent preventive treatment for pregnant women and infants in Africa. Improved understanding of the molecular basis of parasite resistance to antifolate drugs is therefore of major importance.

Point mutations in the genes coding for *P. falciparum* dihydrofolate reductase (PfDHFR) and dihydropteroate synthase have long been known to be a main mechanism of *P. falciparum* resistance against SP. In accordance, we have observed significant selection of the *Pfdhfr Pfdhps* quintuple mutant haplotype in recrudescences and reinfections after SP treatment. Furthermore, there was a significant association between the presence of the quintuple mutant haplotype at baseline and subsequent treatment failure, consistent with data in previous reports (9, 12, 19), pointing to the value of using this haplotype as an in vivo marker of SP resistance.

However, the likelihood that SP action and resistance involve factors beyond *Pfdhfr* and *Pfdhps* has been discussed previously (23, 24). Herein, we describe the selection of PfMRP1 1466K among parasites in recrudescent infections after SP treatment. Importantly, there was no association between the 1466K SNP and the *Pfdhfr Pfdhps* quintuple mutation, indicating that 1466K is selected independently of the quintuple mutant haplotype. Our data suggest that the 1466 genotype at baseline may be related to treatment outcome, although the results of the analysis were not statistically significant, due probably to the small sample size, which was reflected in the large confidence intervals.

MRPs are frequently involved in the efflux of therapeutic drugs in various organisms, e.g., chloroquine and quinine in *P. falciparum* (20) and the antifolate methotrexate in mammals (10, 27). However, this mechanism is unlikely to explain the herein-described selection by SP since these particular antifolates are not organic anions, the typical MRP substrates, and hence probably not transported by MRPs. Instead, PfMRP1 may affect intracellular folate homeostasis in parasites, as proposed previously for higher eukaryotes (2, 3). In *P. falciparum*, the intracellular folate concentration is important for the activities of antifolates. This concentration depends on input from the de novo synthesis of folate and influx through the folate salvage pathway and output through folate efflux from the cell. It has been shown previously that increasing folate levels can impair the effects of antifolate drugs on *P. falciparum* in vitro (15, 23) and in vivo (22). These observations are applied in vitro to modify protocols for the determination of parasite sensitivity to SP (25). Further studies have shown that probenecid can reverse *P. falciparum* resistance to several antifolates, including sulfadoxine and pyrimethamine, due to the reduction of folate influx into both red blood cells and parasites (8, 16, 18).

In mammalian cells, MRPs have a role in folate homeostasis and have been shown to transport folate out of the cell (3, 27). The Chinese hamster ovary cell line selected for pyrimethamine resistance has impaired folate export due to the repression of MRP genes, resulting in a significant increase in the intracellular concentrations of folates (21). An expansion of the folate pool results in the accumulation of dihydrofolate, the natural substrate of DHFR, if the enzyme is inhibited by pyrimethamine. Dihydrofolate will compete with pyrimethamine in binding to DHFR, decreasing the inhibition of DHFR and resulting in pyrimethamine resistance (2). In this context, we suggest that PfMRP1 is a putative parasite folate efflux mechanism, with the 1466R-carrying protein hypothetically being more effective in this function. In a population

exposed to SP, this enhanced capacity would turn out to be a disadvantage by reducing the intraparasite folate pool and, consequently, decreasing the competition with the incoming drugs for their targets. The less active 1466K-harboring proteins would better counteract the actions of the drugs than the 1466R-harboring proteins by allowing increased intracellular accumulation of folates, which may explain the selection of the 1466K allele upon SP exposure.

In conclusion, our data (i) provide, for the first time, clinical evidence for the possibility that a *P. falciparum* MRP homologue contributes to antifolate resistance and (ii) support the previously reported importance of the *Pfdhfr Pfdhps* quintuple mutation in *P. falciparum* resistance to SP treatment.

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