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# Towards an Understanding of the Mechanism of Pyrimethamine-Sulfadoxine Resistance in *Plasmodium falciparum*: Genotyping of Dihydrofolate Reductase and Dihydropteroate Synthase of Kenyan Parasites

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**The antifolate combination of pyrimethamine (PM) and sulfadoxine (SD) is the last affordable drug combination available for wide-scale treatment of falciparum malaria in Africa. Wherever this combination has been used, drug-resistant parasites have been selected rapidly. A study of PM-SD effectiveness carried out between 1997 and 1999 at Kilifi on the Kenyan coast has shown the emergence of RI and RII resistance to PM-SD (residual parasitemia 7 days after treatment) in 39 out of 240 (16.25%) patients. To understand the mechanism that underlies resistance to PM-SD, we have analyzed the dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) genotypes of 81 patients. Fifty-one samples were obtained, before treatment, from patients who remained parasite free for at least 7 days after treatment. For a further 20 patients, samples were obtained before treatment and again when they returned to the clinic with parasites 7 days after PM-SD treatment. Ten additional isolates were obtained from patients who were parasitemic 7 days after treatment but who were not sampled before treatment. More than 65% of the isolates (30 of 46) in the initial group had wild-type or double mutant DHFR alleles, and all but 7 of the 47 (85%) had wild-type DHPS alleles. In the paired (before and after treatment) samples, the predominant combinations of DHFR and DHPS alleles before treatment were of triple mutant DHFR and double mutant DHPS (41% [7 of 17]) and of double mutant DHFR and double mutant DHPS (29% [5 of 17]). All except one of the posttreatment isolates had triple mutations in DHFR, and most of these were "pure" triple mutants. In these isolates, the combination of a triple mutant DHFR and wild-type DHPS was detected in 6 of 29 cases (20.7%), the combination of a triple mutant DHFR and a single mutant (A437G) DHPS was detected in 4 of 29 cases (13.8%), and the combination of a triple mutant DHFR and a double mutant (A437G, L540E) DHPS was detected in 16 of 29 cases (55.2%). These results demonstrate that the triply mutated allele of DHFR with or without mutant DHPS alleles is associated with RI and RII resistance to PM-SD. The prevalence of the triple mutant DHFR-double mutant DHPS combination may be an operationally useful marker for predicting the effectiveness of PM-SD as a new malaria treatment.**

As chloroquine-resistant parasites have spread throughout Africa (5, 35), pyrimethamine-sulfadoxine (PM-SD) has increasingly become the drug combination of choice for the treatment of uncomplicated malaria. PM is an inhibitor of dihydrofolate reductase (DHFR), which is one component of a bifunctional protein, the other component being thymidylate synthetase (7). SD is thought to act against dihydropteroate synthase (DHPS), a bifunctional protein that combines with hydroxymethypterin pyrophosphokinase (6, 25). The inhibitory activity of each drug is greatly augmented when they are used in combination, but the mechanism of this synergism is not yet understood. Wherever PM-SD has been used, the selection of resistant *Plasmodium falciparum* populations has been rapid (35, 36, 37). The emergence of PM-SD resistance is of immediate concern in Africa since it is the last of the available and affordable antimalarial drugs. This is especially true for Kenya, which only recently replaced chlorquine with PM-SD as the first-line treatment for nonsevere malaria (17).

In vitro analyses have clearly demonstrated that resistance to PM is associated with the mutation of the amino acid Ser to Asn at codon 108 of DHFR. Ancillary point mutations of Asn to Ile at codon 51 and of Cys to Arg at codon 59 are associated with an increase of resistance, and a higher level of PM resistance occurs in the presence of the point mutation Ile to Leu at codon 164 (2, 8, 12, 15, 18, 19, 21, 24, 39). When in vitro analyses are carried out with low-concentration-folate or folate-free medium, a point mutation in DHPS that changes Ala to Gly at codon 437 is associated with SD resistance. Additional changes of Ser to Phe and Ser to Ala at residue 436, Ala to Gly at residue 581, Lys to Glu at residue 540, and Ala to Ser or Ala to Thr at codon 613 are associated with higher levels of resistance (26, 27, 31). However, when the same experiments were carried out with physiological levels of folate, the impact of these point mutations in DHPS on PM-SD susceptibility was less clear (33, 34).

We have proposed a model for the mechanism of antifolate resistance in *P. falciparum* which ascribes a primary role to

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TABLE 1. DHFR and DHPS genotypes of *P. falciparum* isolates collected before PM-SD treatment in Kilifi, Kenya, between 1997 and 1998[a]

| No. of isolates | Genotype[b] at indicated codon in: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | DHFR | | | | DHPS | | | | |
| | 108 | 51 | 59 | 164 | 436 | 437 | 540 | 581 | 613 |
| 4 | ND | ND | ND | ND | — | — | — | — | — |
| 1 | ND | ND | ND | ND | Mix | — | — | — | — |
| 7 | — | — | — | — | — | — | — | — | — |
| 1 | — | — | — | — | Mut | — | — | — | — |
| 1 | — | — | — | — | — | — | — | — | — |
| 1 | — | — | — | — | ND | ND | ND | ND | ND |
| 2 | Mut | — | — | — | — | — | — | — | — |
| 6 | Mut | Mut | — | — | — | — | — | — | — |
| 1 | Mix | Mut | — | — | — | — | — | — | — |
| 1 | Mut | Mix | — | — | — | — | — | — | — |
| 1 | Mut | Mix | — | — | — | Mix | Mut | — | — |
| 1 | Mut | Mut | — | — | — | Mut | Mut | — | — |
| 3 | Mut | Mut | — | — | ND | ND | ND | ND | ND |
| 1 | Mix | — | Mix | — | Mut | — | — | — | — |
| 1 | Mix | — | Mix | — | — | — | — | — | — |
| 2 | Mut | — | Mut | — | — | — | — | — | — |
| 1 | Mut | — | Mut | — | Mut | — | — | — | — |
| 1 | Mix | Mut | Mix | — | — | — | — | — | — |
| 1 | Mut | Mut | Mix | — | — | — | — | — | — |
| 1 | Mut | Mut | Mix | — | Mut | — | — | — | — |
| 2 | Mut | Mix | Mut | — | — | — | — | — | — |
| 11 | Mut | Mut | Mut | — | — | — | — | — | — |

[a] All isolates were collected from patients who were aparasitemic 7 days after treatment.
[b] Mut, mutated; Mix, mixed; —, wild type; ND, not determined. At position 436 of DHPS, the mutated codon is Ala and the mixed codons are Ala and Ser.

mutations in the parasite gene which encodes DHFR-thymidylate synthetase and considers mutations in the parasite DHPS to be of secondary importance (34). This model predicts that populations comprised principally of parasites with triple mutant alleles of DHFR begin to accumulate mutations in DHPS as well. If this is the case, then the DHPS mutations would augment the already significant resistance to PM-SD (22, 23, 32).

It is now common at our study site in Kilifi, Kenya, for 10 to 20% of patients treated with PM-SD to remain parasitemic 7 days after treatment. To understand the mechanism that underlies resistance to PM-SD, we analyzed the DHFR and DHPS alleles in these PM-SD-resistant parasites and correlated the clinical and parasitological data with molecular markers to better define the alleles in each gene associated with resistance to PM-SD.

## MATERIALS AND METHODS

*P. falciparum* isolates were collected during a monitoring exercise to establish the extent of PM-SD resistance at the Kenya Medical Research Institute unit within the Kilifi District Hospital on the Kenya coast between July 1997 and December 1998. Febrile children aged 3 to 71 months with uncomplicated malaria were recruited if they were well enough for outpatient management and fulfilled the following criteria: (i) a capillary hemoglobin level of 5 g% or greater and (ii) a *P. falciparum* parasitemia level above 2,000 but below 250,000 per μl of blood. In our study, we were interested only in those who received PM-SD (PM, 1.25 mg/kg of body weight; SD, 25 mg/kg; single dose). Isolates from 51 patients who were aparasitemic after 7 days and 30 patients who remained parasitemic 7 days after treatment were analyzed. Fifty-microliter blood samples before and 7 days after PM-SD treatment were spotted onto filter paper, dried, and kept at ambient temperature until analyzed. Parasite genomic material was prepared according to the methanol fixation protocol (13) adapted for *P. falciparum* parasites by J. Cortese (personal communication). Briefly, a small piece of blood-impregnated filter paper was snipped and put in a 0.5-ml microcentrifuge tube containing 50 μl of absolute methanol for 15 min at ambient temperature. Thereafter, the methanol was poured off and the paper was allowed to dry so that all of the methanol evaporated. Fifty microliters of sterilized water was then added to the tube, which was heated at 95°C for 15 min in a PCR machine, and tubes were vortexed every 3 min during this incubation. Following centrifugation (15,000 × *g*, 2 min), 2.5 to 5 μl of the solution was used as the template for PCR analysis.

Point mutations in codons 108, 51, 59, and 164 of DHFR were detected by the techniques of allele-specific PCR and enzyme digestion (11, 18) after the nested amplification of the DHFR domain as described elsewhere (21). Point mutations in codons 436, 437, 540, 581, and 613 of DHPS were analyzed by PCR amplification and restriction enzyme digestion (11). To increase the sensitivity and specificity of the DHPS amplification, we designed a new set of long primers for the first round of PCR; the primer designations and sequences are as follows: 209 (sense), 5′ AACCTAAACGTCCTGTTCAAAGAATGTTTGAAATGTAAA TGAAGG 3′, and 210 (antisense), 5′ CCTCATGTAATTCATCTGAAACAT CCAATTGTGTGATTTGTCCAC 3′. These primers were used at a concentration of 0.2 μM in the presence of 200 μM each deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, and 1.5 U of *Taq* polymerase (Promega, Southampton, United Kingdom) under the following conditions: preincubation at 92°C for 45 s; cycling at 92°C for 30 s, 55°C for 45 s, and 72°C for 45 s; and a final extension at 72°C for 5 min. The initial amplification was set up in 25 μl for 25 cycles. From this reaction, 1 μl was used for the secondary amplification (11), for which the primer concentration was increased to 1 μM and the number of cycles was 35. All the other amplification parameters were identical to those of the initial step. Restriction enzyme digestions were carried out as described elsewhere (11).

The Epi Info (Atlanta, Ga.) version 6 package was used for statistical analyses.

## RESULTS

Between July 1997 and January 1999, 272 children with uncomplicated falciparum malaria were treated with PM-SD in the outpatient clinic at the Kilifi District Hospital. Eleven children were withdrawn because of vomiting of the drug. Of the 261 remaining, 240 returned to the clinic at day 7 for follow-up. Of these 240 children, 39 (16.25%) were parasitemic at day 7. These 39 children had a mean of 17,498 parasites/μl of blood before treatment and 638 parasites/μl 7 days after PM-SD treatment, which is a clear indication of RI or RII resistance according to the World Health Organization classification for chloroquine resistance (38).

Table 1 summarizes the initial DHFR and DHPS genotypes of 51 isolates from children who remained aparasitemic at day 7. Thirty out of 46 isolates (65%) had either wild-type DHFR, single mutant DHFR (S108N), or double mutant DHFR

TABLE 2. DHFR and DHPS genotypes of *P. falciparum* isolates collected before and after PM-SD treatment in Kilifi, Kenya, between 1997 and 1999

| Isolates | Genotype[a] of indicated codon before treatment | | | | | | | | | Genotype of indicated codon after treatment | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | DHFR | | | | DHPS | | | | | DHFR | | | | DHPS | | | | |
| | 108 | 51 | 59 | 164 | 436 | 437 | 540 | 581 | 613 | 108 | 51 | 59 | 164 | 436 | 437 | 540 | 581 | 613 |
| −/Pf001 | NA | NA | NA | NA | NA | NA | NA | NA | NA | Mut | Mut | — | — | — | Mut | Mut | — | — |
| −/Pf014 | NA | NA | NA | NA | NA | NA | NA | NA | NA | Mut | Mut | Mut | — | — | Mut | Mut | — | — |
| −/Pf024 | NA | NA | NA | NA | NA | NA | NA | NA | NA | Mut | Mut | Mut | — | — | — | — | — | |
| −/Pf026 | NA | NA | NA | NA | NA | NA | NA | NA | NA | Mut | Mut | Mut | — | — | — | — | — | — |
| −/pg128[b] | NA | NA | NA | NA | NA | NA | NA | NA | NA | Mut | Mut | Mut | — | — | — | — | — | — |
| −/Pf039 | NA | NA | NA | NA | NA | NA | NA | NA | NA | Mut | Mut | Mut | — | Mix | — | — | — | — |
| −/107F | NA | NA | NA | NA | NA | NA | NA | NA | NA | Mut | Mix | Mut | — | — | Mix | — | — | — |
| −/142[c] | NA | NA | NA | NA | NA | NA | NA | NA | NA | Mut | Mut | Mut | — | — | Mut | — | — | — |
| −/L030 | NA | NA | NA | NA | NA | NA | NA | NA | NA | Mut | Mut | Mut | — | — | Mut | — | — | — |
| −/154F | NA | NA | NA | NA | NA | NA | NA | NA | NA | Mut | Mut | Mix | — | — | Mut | Mut | — | — |
| 269/Pf017 | Mut | — | Mut | — | — | Mut | Mut | — | — | Mut | Mut | Mut | — | — | Mut | Mut | — | — |
| 15/Pf021[d] | Mut | Mix | Mut | — | — | — | — | — | — | Mut | Mut | Mut | — | — | Mut | Mix | — | — |
| 47/Pf042 | Mut | Mix | Mix | — | ND | ND | ND | ND | ND | Mix | Mix | Mix | — | — | — | — | — | — |
| L19/L19PM | Mut | Mix | Mix | — | — | Mut | Mut | — | — | Mut | Mix | Mix | — | — | Mut | Mut | — | — |
| L20/L20PM | Mut | Mut | — | — | — | Mut | Mix | — | — | Mut | Mut | Mut | — | — | Mut | Mut | — | — |
| L21/L21PM | Mut | Mut | Mix | — | — | Mut | Mut | — | — | Mut | Mut | Mut | — | — | Mut | Mut | — | — |
| L22/L22DPM | ND | ND | ND | ND | Mut | Mut | — | — | — | ND | ND | ND | ND | — | Mut | Mut | — | — |
| L23/L23PM | ND | ND | ND | ND | — | Mut | Mix | — | — | Mut | Mut | Mut | — | — | — | — | — | — |
| L24/L24PM | Mut | Mix | — | — | | Mix | Mix | — | — | Mut | Mut | Mut | — | Mut | Mut | — | — | — |
| L25/L25PM | Mut | Mut | — | — | — | Mut | Mut | — | — | Mut | Mut | Mut | — | — | Mut | Mut | — | — |
| 300/210F | Mut | Mix | Mix | — | — | Mut | — | — | — | Mut | Mut | Mut | — | — | Mut | Mut | — | — |
| 206/206F | Mut | Mix | Mix | — | — | Mut | Mut | — | — | Mut | Mix | Mix | — | — | Mut | Mut | — | — |
| 214/214F | Mut | Mut | Mut | — | — | Mut | Mut | — | — | Mut | Mut | Mut | — | — | Mut | — | — | — |
| 286/286F | Mut | Mix | Mix | — | — | Mut | Mut | — | — | Mut | Mut | Mut | — | — | Mut | Mut | — | — |
| 280/280F | Mut | Mix | Mix | — | — | Mut | — | — | — | Mut | Mut | Mut | — | — | Mut | Mut | — | — |
| 285/285F | Mut | Mut | — | — | — | Mut | Mut | — | — | Mut | Mut | Mut | — | — | Mut | Mut | — | — |
| 292/292F | Mut | Mut | Mix | — | — | Mut | Mut | — | — | Mut | Mut | Mut | — | — | Mut | Mut | — | — |
| 281/281F | Mut | Mut | Mut | — | — | Mut | Mut | — | — | Mut | Mut | Mut | — | — | Mut | Mut | — | — |
| 275/275F | Mut | Mut | — | — | — | — | — | — | — | Mut | Mut | Mut | — | — | — | — | — | — |
| 219/219F | ND | ND | ND | ND | — | Mut | Mut | — | — | Mut | Mut | Mut | — | — | Mut | Mut | — | — |

[a] NA, not available; Mut, mutated; —, wild type; Mix, mixed; ND, not determined. For trimorphic codon 436 of DHPS, the mutated codon is Phe and the mixed codons are Ala and Ser.
[b] Isolate collected after 28 days.
[c] Isolate collected after 14 days.
[d] Isolate collected after 16 days.

(S108N paired with N51I or C59R). Sixteen of 46 isolates (35%) carried a triple mutant DHFR genotype (S108N, N51I, and C59R). Some of these mutants were from mixed infections that included wild-type DHFRs from parasites as well. Forty-two of 47 (89%) of these isolates exhibited the wild-type DHPS genotype at positions 437 and 540, but 5 carried a mutant allele at position 436. In these isolates, no I164L mutation in DHFR or A581G or A613S mutation in DHPS was observed.

Thirty children who remained parasitemic after treatment with PM-SD were identified. Twenty isolates were collected both before and 7 days after treatment for DHFR and DHPS genotype analysis (three additional isolates were collected 14, 16, and 28 days after treatment). Samples from an additional 10 children were analyzed upon their return at 7 days, but their day 0 samples were not available for analysis. The results are summarized in Table 2. Before treatment, 11 of 17 isolates contained triple mutant DHFR alleles (S108N, N51I, and C59R), predominantly mixed with wild-type codons at positions 51 and/or 59, and 6 of 17 contained double mutant DHFR alleles (S108N paired with N51I or C59R); no isolate with wild-type DHFR was found. The analysis of DHPS alleles showed that only 2 isolates among 19 had wild-type DHPS alleles, 2 had single mutant alleles (A437G), and 14 had double mutant alleles (A436F and K540E). One isolate had point mutations that encode A436F and A437G. In this group, the predominant combinations of DHFR and DHPS in the pretreatment isolates were triple mutant DHFR with double mutant DHPS alleles (43.75% [7 of 16]) and double mutant DHFR with double mutant DHPS alleles (31.25% [5 of 16]).

Examination of the pretreatment isolates from the two groups allows two important conclusions. First, the triple mutant DHFR allele is now extremely common in this population. The patients who remained parasitemic were more likely to carry parasites with this genotype than those who cleared their infection for at least 7 days (relative risk, 2.44; confidence interval, 1.03 to 5.78). Second, if one examines both DHFR and DHPS genotypes, a striking difference is observed: patients who carried parasites with triple or double mutations in DHFR and double mutations in DHPS were far more likely to remain parasitemic than those with wild-type DHPS (relative risk, 12.0; confidence interval, 3.1 to 46.4). Thus, the combination of these two mutant genotypes is a very clear risk factor for drug failure.

The most pronounced difference between the pre- and post-treatment samples was observed in the DHFR genotype. After treatment, all parasites except one carried triple mutant DHFR alleles and isolates that had carried mixed populations with wild-type DHFR alleles were often rendered "pure" triple mutants (PCR analysis showed only parasites with mutant codons at positions 108, 51, and 59) by PM-SD treatment. The

remaining isolate contained a double mutant (S108N-N51I) DHFR allele. The change in DHPS genotype as a function of PM-SD treatment was far less pronounced. Most initial isolates that were doubly mutant in DHPS retained the same genotype after treatment. Two A437G DHPS mutants and one A436F-A437G DHPS mutant exhibited the double mutation A437G K540E after treatment. One isolate, L23, exhibited mutations at both 437 and 540 before treatment but reversed to the wild-type genotype after treatment, and one A436F-A437G double mutant (isolate 214) became an A437G single mutant after treatment. Because 10 of the pretreatment isolates were unavailable for analysis, we cannot directly examine changes in the full data set, but overall, changes in the DHPS genotypes did not suggest any strong trends of selection during treatment. However, the isolates are presented chronologically, and the A437G-K540E double mutant genotype is more common in isolates collected at the end of the period. In addition, there was a tendency for some mixed genotypes in the pretreated group to become pure genotypes after treatment.

Since the determination of the nature of selection exerted by the combination of PM and SD is one goal of our study, the combinations of DHFR and DHPS alleles are of particular interest. After treatment, the combination of a triple mutant DHFR and a double mutant (A437G-L540E) DHPS was the most common, being detected in 16 of 29 cases (55.17%). Six of 29 isolates (20.68%) had a triple mutant DHFR and a wild-type DHPS, and 4 of 29 isolates (13.80%) had a triple mutant DHFR and single mutant (A437G) DHPS. One triple mutant DHFR isolate had the S436F-A437G DHPS allele, and another had a single mutation at S436A but mixed with the wild-type codon. The isolate that had a double mutant DHFR allele also had a double mutant DHPS allele (A437G-K540E). Even after selection, none of the analyzed isolates had alleles with point mutations at codon 164 of DHFR or at codons 581 and 613 of DHPS. Overall, the patterns show a strong trend toward selection of triply mutated DHFR alleles and some tendency toward DHPS alleles with additional mutations as well.

## DISCUSSION

In vitro analyses have clearly shown that polymorphisms in DHFR and DHPS are associated with resistance to PM and SD (when tests are carried out in low-concentration-folate or folate-free medium), but markers associated with the emergence of in vivo resistance to the combination PM-SD are yet to be clearly defined. Our results demonstrate a clear genotype pattern associated with the emergence of RI or RII resistance to PM-SD in vivo: parasites that carried a triple mutant allele of DHFR and a double mutant allele of DHPS were the most likely to escape drug action. Indeed, more than 96% of the parasites observed 7 days after treatment had triple mutations in DHFR, and more than 75% of these also had a single mutation (A437G) or a double mutation (A437G and K540E) in DHPS. Our previous data (33) and those published by Basco and coworkers (3) show that three mutants in the DHFR domain can be used as a marker to predict the clinical outcome of PM-SD treatment in situations where PM-SD has been used only sparingly. Under these conditions, the prevalence of the triple DHFR allele is relatively low and the parasites that carry these alleles are clearly at a selective advantage. However, when triple mutant DHFR becomes the most common allele in the *P. falciparum* population, then the status of DHPS is also relevant to the prediction of PM-SD failure, as we have observed here.

Even the combination of both genetic markers in a parasite or a patient is unlikely to be a perfect predictor of clinical outcome in an individual patient. For example, among those patients that retained parasites after PM-SD treatment, we found one isolate (47/Pf042) that exhibited wild-type DHFR (although this allele occurred together with double and/or triple mutant DHFR) and another isolate with only a double mutant DHFR genotype. If the triple mutant DHFR is necessary for the occurrence of PM-SD resistance, these parasites should have been sensitive to PM-SD. Such discrepancies have also been described elsewhere (16). These discrepancies presumably reflect the variation in the many parameters that can affect response to treatment: absorption and metabolism of the drug, immunological status, and, most importantly, the level of folate. Indeed, it has been shown that folate supplementation is associated with a decrease of PM-SD effectiveness in vivo (28; also unpublished data from Kilifi and Entasopia, Kenya). In addition, *P. falciparum* isolates differ in their abilities to use exogenous folate (30). These factors complicate the prediction of treatment outcome on the basis of parasite DHFR genotype for a single patient but do not (from our data) significantly reduce the usefulness of the triple mutation in DHFR with the double mutation in DHPS as a predictor of escape from PM-SD treatment. Surveillance of the DHFR and DHPS genotypes in *P. falciparum* populations can provide a potent tool to predict the efficacy of PM-SD in a local region.

Very few studies have assessed the relation between polymorphism in DHFR and DHPS and the clinical outcome of PM-SD treatment in Africa. The first analysis was done with Tanzanian *P. falciparum* populations (9, 14, 29). Similar studies have been carried out in Cameroon (3) and Mali (10), although the number of PM-SD-resistant isolates was very low in these two West African sites. Our report is the first of its kind to assess Kenyan isolates. All of these studies show that the triple mutant DHFR allele can be paired with wild-type, single mutant (A437), or double mutant (A437G-K540E or A437G-A581G) DHPS in patients who exhibit an RI or RII level of resistance to PM-SD. One interpretation of these data is that the selection of PM-SD resistance is a stepwise process. We propose that selection of mutations in DHFR gradually decreases the sensitivity of the parasites to the PM component of PM-SD. Once the marginally PM-sensitive triple mutant alleles of DHFR are well established in the population, selection of mutations in DHPS occurs and sensitivity to SD diminishes as well (E. Mberu, M. Mosobo, M. A. Nzila, G. Kokwaro, C. H. Sibley, and W. M. Watkins, unpublished data; M. A. Nzila et al., unpublished data). The result is an inexorable decrease in the efficacy of PM-SD, and when three or more mutations in both DHFR and DHPS are common in the population, clinical failure of the drug results.

The most frequent point mutation in DHPS in our isolates was A437G. This mutation is also the predominant allele described previously for Kenya (29) and other locations throughout the world (4, 6, 10, 20, 25, 26, 29). It occurs alone or in combination with other point mutations. In vitro analyses have shown that this single mutation can lead to a 10-fold increase in the $K_i$ (26) and 50% inhibitory concentrations (31) for SD and can confer on transfected parasites a 5-fold increase in SD resistance (27) in low-concentration-folate or folate-free medium. These observations led Triglia et al. to propose that this mutation is a necessary first step that allows the other changes to be selected, a situation similar to the apparent requirement for the S108N change before subsequent mutations are selected in the DHFR allele. Our results are in agreement with this hypothesis. We have described for the first time two PM-SD-resistant isolates with the S436F-A437G DHPS genotype. One of two isolates was collected at day 0, and it showed a

reversal to the wild-type genotype at codon 436 after PM-SD treatment. By relating PM-SD clinical outcome and DHPS genotype, the same reversal of the S436A codon to the wild-type genotype was reported for isolates after PM-SD treatment in Tanzania (9, 14), in South America (20), and in Mali (10). These observations support the hypothesis that polymorphism at codon 436, especially the Ala codon, may be considered an alternative wild-type codon (4). Our data confirm the absence of mutations at codon 613 (A613S and A613T) in DHPS in areas where PM-SD resistance is common. These alterations have also been found in only three reference isolates, D6, W2, and V1S, and recently in Mali (10) (an area where PM-SD is still highly effective). Despite the high resistance level conferred by the presence of these codons (in the isolates V1S and W2, which have A437G), the presence of point mutations at this codon may be associated in some way with reduced fitness of the parasite, explaining their absence in field isolates, as previously proposed (27).

An alternative antifolate drug, chlorproguanil-dapsone, is currently in clinical trials (1). As we predicted (33), PM-SD-resistant parasites (those with a triple mutation in DHFR but with or without mutations in DHPS) are sensitive to this combination (H. Dayo, J. Sulo, and P. Winstanley, unpublished data). However, the continued use of PM-SD will likely select for DHFR quadruple mutations (N51I, C59R, S108N, and I164L) and additional mutations in DHPS which are likely to be associated with RIII PM-SD resistance and which may render even chlorproguanil-dapsone ineffective (34). Recognition of the rapid selection of resistance to PM-SD offers the opportunity to choose alternatives other than PM-SD when chloroquine is replaced. Monitoring mutations in *P. falciparum* DHFR and DHPS by molecular analysis is one tool for understanding the dynamics of the selection for resistance to antifolate drugs and designing optimal strategies for their therapeutic use.

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