Molecular Evidence of Greater Selective Pressure for Drug Resistance Exerted by the Long-Acting Antifolate Pyrimethamine/Sulfadoxine Compared with the Shorter-Acting Chlorproguanil/Dapsone on Kenyan Plasmodium falciparum

Alexis M. Nzila,1,2 Eunice Nduati,1 Edward K. Mberu,1 Carol Hopkins Sibley,1 Stephanie A. Monks,4 Peter A. Winstanley,2 and William M. Watkins1,2

Pyrimethamine (PM) plus sulfadoxine (SD) is the last remaining affordable drug for treating uncomplicated malaria in Africa. The selective pressure exerted by the slowly eliminated combination PM/SD was compared with that exerted by the more rapidly eliminated combination chlorproguanil/dapsone (CPG/Dap) on Kenyan Plasmodium falciparum. Point mutations were analyzed in dihydrofolate reductase and dihydropteroate synthase and in the genetic diversity of 3 genes in isolates collected before and after CPG/Dap and PM/SD treatments. PM/SD was associated strongly with the disappearance of fully drug-sensitive parasites and with a significant increase in the prevalence of resistant parasites in subsequent parastemias. However, this was not a characteristic of treatment with CPG/Dap. Moreover, most of the patients who returned with recrudescent infections were in the PM/SD-treated group. The data predict a longer useful therapeutic life for CPG/Dap than for PM/SD, and, thus, CPG/Dap is a preferable alternative for treatment of chloroquine-resistant falciparum malaria in sub-Saharan Africa.

The antifolate combination of pyrimethamine (PM) and sulfadoxine (SD) is becoming the first-line drug for uncomplicated falciparum malaria in Africa. PM and SD are inhibitors of dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS), respectively. In Southeast Asia, where this combination was used extensively for the first time, it is estimated that its useful therapeutic life (UTL) is only ~5 years [1]. A comparable UTL was observed in South America [2] and is predicted for sub-Saharan Africa [3–6]. Therefore, understanding the mechanism by which antifolate resistance is selected is crucial to the rational use of both available and new antifolate drugs and to operations designed to delay the spread of resistant isolates in Africa.

Several theories have been advanced to explain the short UTL of PM/SD. Uncontrolled drug use and inadequate dosage are cited frequently [7], but the importance of the pharmacokinetic profile of the drug rarely is considered. SD and PM have long elimination half-lives (116 and 81 h, respectively) [8], which initially was thought to be an advantage because of the substantial prophylaxis conferred, but detailed follow-up has shown that, for 15–52 days after PM/SD treatment, there is a strong selective pressure for PM resistance [9]. Our hypothesis is that selection pressure might be minimized if short-acting antifolate drugs were used, despite the disadvantage of a longer dosage regimen [10]. If this proves to be the case, a substantial lengthening of the UTL may be possible. To test this hypothesis, we studied a new short-acting antifolate antimalarial drug combination, chlorproguanil (CPG) and dapsone (Dap). CPG is metabolized in vivo to its active triazine metabolite, chlorcyloguanil (CCG), which, like PM, inhibits the DHFR enzyme. Dap, like SD, inhibits the activity of DHPS. As expected, CCG and Dap act synergistically and have half-lives of ~12–20 and ~20–30 h, respectively [11–14].

The first clinical trial to compare CPG/Dap with PM/SD was performed in Kilifi, Kenya, in 1993–1995, with 511 children enrolled as subjects. The study had 2 major results. First, CPG/Dap given in 3 daily doses was as effective as PM/SD in clearing...
parasitemia. Second, as expected, CPG/Dap did not show the prolonged chemoprophylaxis observed when patients were treated with the long–half-life drug PM/SD [10]. In our study, we compared the selection of drug-resistant *Plasmodium falciparum* in patients treated with PM/SD or CPG/Dap. We addressed 2 hypotheses. First, during the 52-day period after PM/SD treatment, the frequency of DHFR and DHPS alleles that confer antifolate resistance will be greater in parasites that reappear than in those that appeared in the original infection. Second, in contrast, parasites that reappear in the CPG/Dap-treated group are likely to be new infections and should not show a higher proportion of drug-resistant parasites than that observed in the initial infections.

Genetic analysis of *P. falciparum* isolates shows that a point mutation from serine to asparagine at codon 108 (S108N) in the DHFR gene increases resistance to PM ~10-fold and that subsequent mutations from asparagine to isoleucine at codon 51 (N51I), from cysteine to arginine at codon 59 (C59R), and from isoleucine to leucine at codon 164 (I164L) progressively enhance PM resistance [15]. Point mutations at codons 436, 437, 540, and 613 of DHPS cause resistance to SD when experiments are done with low-level or folate-free media [16–18]; with physiologic folate, the role of these mutations is less clear [19]. However, these mutations are also observed in field isolates that are resistant to PM/SD [6, 20–22] and, thus, are likely to contribute to the overall response of the parasite to the combination drug [23].

We assessed the selective pressure exerted by PM/SD and CPG/Dap by comparing point mutations at codons 108, 51, and 59 in DHFR and at codons 436, 437, 540, 581, and 613 in DHPS [6, 24] in *P. falciparum* before and after PM/SD and CPG/Dap treatments. We used the size polymorphism variation of merozoite surface protein (MSP)–1 block 2 (MSP1), MSP-2 block 3 (MSP2), and glutamine-rich protein (GLURP) to assess whether parasites that reappeared after PM/SD or CPG/Dap treatment were new or recrudescent [25–27].

Materials and Methods

*P. falciparum isolates and DNA preparation.* The site of our study was Kilifi, which is on the coast of Kenya. In this area, malaria is stable-endemic, with seasonal transmission following 2 periods of heavy rains from April through July and from October through November. After the onset of each rainy season, there is an increase of the number of *Anopheles gambium*, followed by an increase in incidence of malaria. There is a very long period of low/no transmission from February through May. The overall entomologic inoculation rate is ~1.5 per year [28]. We analyzed isolates collected in 1993–1995, during the first clinical trial of CPG/Dap at the Center for Geographic Medicine Research, Coast at Kilifi. Clinical details of this study have been published elsewhere [10]. In brief, 448 children were allocated randomly (double blind) either to a single dose of PM/SD or to 1 of 2 CPG/Dap regimens (a single dose or 3 doses at 24-h intervals), and we measured the incidence of parasitemia in 205 initially aparasitemic children to allow comparison among the 3 treatment groups. The patients and a community surveillance group were followed for 28 days. At the study end point, 31.2% (95% confidence interval [CI], 24.9–38.0) of the community surveillance group subjects were parasitemic, compared with subjects in the treatment groups, whose rates of parasitemia were 40.8% (95% CI, 32.9–49.0; relative risk [RR], 1.31 [0.99–1.73]) after triple-dose CPG/Dap, 19.7% (95% CI, 13.5–27.2; RR, 0.63 [0.43–0.93]) after PM/SD, and 65.6% (95% CI, 57.5–73.0; RR, 2.10 [1.66–2.65]) after single-dose CPG/Dap. PM/SD and triple-dose CPG/Dap were effective treatments.

In our study, patients who were treated either with 1 dose of PM/SD (1.25 and 25 mg/kg, respectively) or with 3 daily doses of CPG/Dap (2.4 mg/kg for both drugs) were followed from day 7 after treatment until day 28 (days 7, 14, 21, and 28). Children who became sick between days 29 and 42 also were included in our study. All patients were aparasitemic at day 7, and new parasitemias were observed from day 14. We collected 1–3 mL of venous blood from each patient before and after treatment for those who became parasitemic and preserved the blood samples at ~20°C until analysis. This blood (50 µL) was spotted onto filter paper, and parasite genomic material was prepared per the methanol fixation protocol described elsewhere [6].

*Parasite DHFR and DHPS analysis.* Point mutations at codons 51, 59, and 59 of DHFR and at codons 436, 437, 540, 581, and 613 of DHPS were detected by allele-specific polymerase chain reaction (PCR) and enzyme digestion as described elsewhere [6, 24]. In our study, we did not routinely include the detection of the point mutation at codon 164 of DHFR, because we showed in a previous study that all isolates collected during the same clinical trial were wild type at codon 164 [29]. Moreover, recent data from Kilifi show that this mutation is absent in parasites that persist to day 7 after PM/SD treatment [6].

*Parasite genetic polymorphisms.* DNA amplification of MSP1, MSP2, and GLURP involved a nested PCR using the primers and the approach described elsewhere [25–27].

*Data analysis.* *P. falciparum* isolates were characterized on the basis of the fragment size of each locus. Because of the size variation of a given DNA fragment in gel electrophoresis, we categorized the DNA fragments into different classes (bins), as described elsewhere [26]. Each bin represents an allele, and comparison of alleles of paired samples was used to investigate whether pre- and posttreatment infections were caused by the same or by different parasites.

*Statistical analyses.* Because of the small sample size, the data were analyzed by 2 exact tests, the unconditional pooled Z test [30] and the conditional χ² test [31]. The conditional χ² test was performed with SAS software (version 6.12; SAS Institute, Cary, NC), and the unconditional pooled Z test was performed with program XUN2X2, available at http://www.stat.ncsu.edu/~berger/tables.html.

Results

For a clear analysis of our data, we categorized the parasite DHFR genotypes into 5 groups. The first group comprised wild-type, fully pyrimethamine-sensitive parasites. The second
Parasites with the DHFR genotype of S108N

tional

statistically signiﬁcant difference in this distribution (condi-

tion samples. Treatment with CPG/Dap did not produce a

distribution of genotypes was different in the pre- and posttreat-

were compared in 3 ways. First, we analyzed whether the dis-

oberved in comparison with the treatment received. The data

C59R.

1

1 wild-type allele at any of these 3 codons formed the

mixed allele at either of the 2 mutated codons.

Table 1 summarizes the proportion of each DHFR genotype

observed in comparison with the treatment received. The data

were compared in 3 ways. First, we analyzed whether the dis-

gregation of genotypes was different in the pre- and posttreat-

ments that carried only triple-mutant parasites in the PM/SD-

in other comparisons. There was a signiﬁcant increase in iso-

ence ( ). Second, a 1-sided unconditional pooled

Treatment samples in the PM/SD group showed such a differ-

2 of 28 before and 5 of 28 after treatment; ). Overall,

the trend toward elimination of the PM-sensitive parasites and

retention of the triple-mutant DHFR alleles after PM/SD treat-

ment was clear. This contrasted sharply with the lack of such a

trend after CPG/Dap treatment.

There is not complete agreement on the role of particular

point mutations in the DHPS gene in conferring resistance to

sulfa drugs. It has been proposed that a point mutation from

alanine to glycine at codon 437 (A437G) is a precondition for

resistance to SD [17, 18], but there is an exception to that

suggestion [32]. Subsequent surveys [21, 22] and our data [6]

support the importance of the initial A437G change in sulfa

resistance. On the basis of that information, we classiﬁed an

isolate that harbored only 1 point mutation other than A437G

as sulfa sensitive. The DHPS genotyping is summarized in table

2. Most of the isolates were either sulfa sensitive or double

mutants at codon 437 (A437G) and at codon 540 (from Lys to

Glu [K540E]). Eight isolates had a mutation at codon 436

alone (Ala and/or Phe, mixed or not with Ser), and 5 isolates

were mutated at codon 613 alone (Thr or Ser, mixed or not

with Ala); these were included in the sulfa-sensitive group.

Three isolates had a mutation both at codon 436 (from Ser to

Phe) and at codon 613 (from Ala to Ser), and 5 were mutant

at A437G + K540E. Both groups were classiﬁed as sulfa re-

sistant. As shown in table 2, the proportion of mutant alleles

after treatment (7 of 28) with CPG/Dap was not signiﬁcantly

greater than the proportion before treatment (5 of 28; uncon-

ditional pooled Z test, ). In contrast, in the PM/SD

group, there was a signiﬁcant reduction of wild-type isolates

(19 before and 9 after treatment) and a corresponding increase

in sulfa-resistant alleles (5 before and 15 after treatment, P =

.0018).

Association between DHFR/DHPS genotypes. Mutations

in DHPS (sulfa resistant) were observed in 26% of the isolates

that carried doubly mutant DHFR alleles and in 44% of the

isolates with the triple-mutant DHFR (table 3). In contrast, 15

of 17 parasites that harbored wild-type genotypes for DHFR

also had wild-type genotypes for DHPS (sulfa sensitive). This

suggests that selection for resistance to PM/SD is focused pre-

dominantly on DHFR and that mutations in DHPS are selected

Table 2. Proportion of dihydropteroate (DHPS) genotypes (ex-

pressed as sulfa sensitive or sulfa resistant) of Plasmodium falciparum

before and after chlorproguanil/dapsone (CPG/Dap) and pyrimeth-

amine/sulfadoxine (PM/SD) treatment.

% (n) DHFR genotypes

<table>
<thead>
<tr>
<th>Treatment (n)</th>
<th>Wild type</th>
<th>Single + double mutants</th>
<th>Mixed triple mutants</th>
<th>Pure triple mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPG/Dap</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before (28)</td>
<td>17.86 (5)</td>
<td>53.57 (15)</td>
<td>21.43 (6)</td>
<td>7.14 (2)</td>
</tr>
<tr>
<td>After (28)</td>
<td>21.43 (6)</td>
<td>50.00 (14)</td>
<td>10.71 (3)</td>
<td>17.86 (5)</td>
</tr>
<tr>
<td>PM/SD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before (24)</td>
<td>25.00 (6)</td>
<td>29.17 (7)</td>
<td>33.33 (8)</td>
<td>12.50 (3)</td>
</tr>
<tr>
<td>After (24)</td>
<td>0</td>
<td>41.67 (10)</td>
<td>20.83 (5)</td>
<td>37.50 (9)</td>
</tr>
</tbody>
</table>

NOTE. Parasites were collected within 42 days after each treatment, and
24 and 28 pairs of PM/SD and CPG/Dap were analyzed, respectively.

<table>
<thead>
<tr>
<th>Treatment (n)</th>
<th>Wild type</th>
<th>Single + double mutants</th>
<th>Mixed triple mutants</th>
<th>Pure triple mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPG/Dap</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before (28)</td>
<td>17.86 (5)</td>
<td>53.57 (15)</td>
<td>21.43 (6)</td>
<td>7.14 (2)</td>
</tr>
<tr>
<td>After (28)</td>
<td>21.43 (6)</td>
<td>50.00 (14)</td>
<td>10.71 (3)</td>
<td>17.86 (5)</td>
</tr>
<tr>
<td>PM/SD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before (24)</td>
<td>25.00 (6)</td>
<td>29.17 (7)</td>
<td>33.33 (8)</td>
<td>12.50 (3)</td>
</tr>
<tr>
<td>After (24)</td>
<td>0</td>
<td>41.67 (10)</td>
<td>20.83 (5)</td>
<td>37.50 (9)</td>
</tr>
</tbody>
</table>

NOTE. Parasites were collected within 42 days after each treatment, and
24 and 28 pairs of PM/SD and CPG/Dap were analyzed, respectively.
and C59R) and wild-type DHPS. The recrudescent isolate in the PM/SD-treated group and 1 of 28 in the CPG/Dap-treated group had the same alleles at all 3 polymorphic loci before and after treatment; these were classified as recrudescent parasites. Three of the 5 patients with recrudescent parasites in the PM/SD-treated group were triple-mutant DHFR and double-mutant DHPS (A437G + N51I), 1 was a triple-mutant DHFR and wild-type DHPS, and the fifth was a double-mutant DHFR (S108N and C59R) and wild-type DHPS. The recrudescent isolate in the CPG/Dap group was a double-mutant (S108N + N51I) and wild-type DHPS. PM/SD is both a less-potent drug and more persistent than CPG/Dap. One would predict that recrudescent infections would be more common than new infections in the PM/SD group than they are in the CPG/Dap group; that trend was observed in our study.

### Discussion

This report provides evidence at the molecular level that the long-acting antifolate PM/SD exerts a stronger selective pressure for resistance than does the short-acting antifolate CPG/Dap. We genotyped DHFR and DHPS loci, both of which are expected to be under selection, and 3 polymorphic loci that encode cell surface proteins and that are not expected to be subject to drug selection. Our data show that, within the 42 days following PM/SD treatment, all isolates were composed of parasites that had undergone at least 1 point mutation associated with PM resistance in DHFR and that there was a highly significant increase in mutants in the DHPS gene. In contrast, the CPG/Dap-treated group showed prevalences of the wild-type DHFR and DHPS alleles that were not different from those observed in the population before treatment. For up to 52 days after PM/SD treatment, physiologic drug concentrations are adequate to clear new infections of sensitive parasites [9], and, when introduced into clinical use, PM/SD chemoprophyaxis was a potential advantage. However, it is now apparent that PM/SD protects persons from new infections during this period only if the parasites are drug sensitive. In the 2-year study period (1993–1995), ~80% of the parasites carried DHFR genotypes with at least 1 mutation, and 30%–40% of the isolates carried at least some triple-mutant alleles [29]. Under these circumstances, the advantage of extended chemoprophyaxis disappears rapidly.

Our study provides additional insights on the mechanism that underlies resistance to PM/SD. PM/SD exerts a selective pressure on DHPS, as reported elsewhere [33]; however, the selection for mutations in DHPS seems to occur only when most parasites in the population also carry at least a double-mutant, and usually a triple-mutant, allele of DHFR. This asymmetry in the selection pattern of DHFR and DHPS is illustrated by in vitro chemosensitivity analyses. In a longitudinal study of parasites from Kilifi, isolates with increased resistance to SD were observed only after a substantial fraction of the population had been selected for PM resistance [34]. Reports from Mali show that parasites that carry a wild-type allele of DHFR can harbor mutations in DHPS; however, these mutations did not appear to be selected by PM/SD treatment and may have been selected by the use of sulfa drugs alone in that region [22, 35]. On the basis of these data, we propose that the triple-mutant DHFR genotype is a reliable marker for monitoring changes in PM/SD susceptibility in populations where PM/SD has been implemented recently. The data also suggest that monitoring both the DHFR and DHPS genes will be necessary to give a more complete picture of what goes on “later” in the progression of resistance, once triple-mutant parasites predominate in the population.

When we analyzed samples from Kilifi taken during 1997–1998, we found that almost 20% of patients failed to clear parasites by 7 days after PM/SD treatment. Most of these persistent parasites carried the triple-mutant allele of DHFR with or without mutations in DHPS [6]. This change most likely reflected the rapid increase in the prevalence of triple mutants in the general population, compared with 1993–1995, when isolates for our study were collected [29]. However, the experience in Southeast Asia and South America suggests that the continued use of PM/SD is likely to select quickly for additional mutations in DHFR and DHPS that will render PM/SD completely ineffective. For example, the mutation at codon 164 in DHFR certainly would have this effect. Once this occurs, CPG/Dap also will become ineffective as a short-course treatment for malaria [19].

The effectiveness and short half-life of CPG/Dap make it a drug with great potential utility. However, resistance still can arise through the selection of a few mutations, and, thus, CPG/Dap is likely to have a short UTL. The experience with atoquaone monotherapy is a case in point [36, 37]. The most practicable solution to this dilemma is to combine drugs that have different mechanisms of action (combination therapy), rather than to rely on monotherapy, and to make frequent changes as the single agents become useless. The principle of combination therapy recently was described in detail elsewhere [38], but the underlying concept is simple: the probability that a single parasite will carry mutations that confer resistance to DHFR genotypes.

<table>
<thead>
<tr>
<th>DHFR genotypes (n)</th>
<th>Sulfadoxine sensitive</th>
<th>Sulfadoxine resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (17)</td>
<td>88.23 (15)</td>
<td>11.76 (02)</td>
</tr>
<tr>
<td>Double + single mutant (46)</td>
<td>73.91 (34)</td>
<td>26.08 (121)</td>
</tr>
<tr>
<td>Triple mutant (41)</td>
<td>56.09 (23)</td>
<td>43.90 (18)</td>
</tr>
</tbody>
</table>

Table 3. Association between the dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) genotypes (expressed as sulfa sensitive or sulfa resistant) of Kenyan isolates.

Recrudescence versus new parasitemia and the association with DHFR genotypes. Five of 24 children in the PM/SD-treated group and 1 of 28 in the CPG/Dap-treated group had the same alleles at all 3 polymorphic loci before and after treatment; these were classified as recrudescent parasites. Three of the 5 patients with recrudescent parasites in the PM/SD-treated group were triple-mutant DHFR and double-mutant DHPS (A437G + K540E), 1 was a triple-mutant DHFR and wild-type DHPS, and the fifth was a double-mutant DHFR (S108N and C59R) and wild-type DHPS. The recrudescent isolate in the CPG/Dap group was a double-mutant (S108N + N51I) and wild-type DHPS. PM/SD is a weaker drug and more persistent than CPG/Dap. One would predict that recrudescent infections would be more common than new infections in the PM/SD group than they are in the CPG/Dap group; that trend was observed in our study.
The combination of CPG/Dap and artesunate approaches this combination to be affordable for people living in endemic areas.

How can this idea be applied to the possible introduction of CPG/Dap? In Africa, one of the proposed combinations is PM/SD with an artemisinin derivative (artesunate). Artesunate is a very short-acting antimalarial drug with a half-life that does not exceed 1 h [39]. When given as a 3-day course with single-dose PM/SD, the combination most likely will clear PM/SD-resistant parasites (DHFR triple mutants) and thus extend the UTL of PM/SD. However, as our data show, during new infections the long-acting property of PM/SD most likely will concentrate parasites with resistant DHFR and DHPS alleles. We therefore suggest that a combination of drugs with short elimination profiles may have the longest UTL in most African countries. An ideal combination of antimalarial drugs would be an association of ≥2 short-acting drugs that have ≥2 different modes of action. It would be important for such a combination to be affordable for people living in endemic areas. The combination of CPG/Dap and artesunate approaches this description, and we suggest that this combination be developed as a matter of highest urgency.

Acknowledgment

We thank the director of the Kenya Medical Research Institute for permission to publish these data.

References

28. Mbogo CN, Snow RW, Kamala CP, et al. Relationships between Plasmodium falciparum transmission by vector populations and the incidence of


