PYRIMETHAMINE/SULFADOXINE COMBINATION IN THE TREATMENT OF UNCOMPLICATED FALCIPARUM MALARIA: RELATION BETWEEN DIHYDROPTEROATE SYNTHASE/DIHYDROFOLATE REDUCTASE GENOTYPES, SULFADOXINE PLASMA LEVELS, AND TREATMENT OUTCOME

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Abstract. Several in vitro studies have shown the correlation between mutations in dhfr and dhps genes and resistance to pyrimethamine/sulfadoxine (PYR/SDX) combination. However, the in vivo efficacy of these mutations with PYR/SDX has not been investigated fully. In this study, we assessed the PYR/SDX efficacy in relation to the frequency of dhfr and dhps mutations in 37 Plasmodium falciparum isolates sampled before treatment. Plasma levels of SDX measured at days 0, 3, 7, and 14 were assessed. Point mutations were detected only at codons 51 and 108 of dhfr and codon 436 of dhps. The frequency of dhfr 51/108 and dhps 436 mutations was 79% and 8%, respectively. The plasma levels of SDX indicated adequate drug absorption by all patients. The presence of Ile 51 and Asn 108 mutations among parasites that cleared after treatment indicates that these mutations alone are insufficient to cause in vivo resistance. In all recrudescent parasites, however, the presence of Ile 51/Asn 108 dhfr mutations was coupled with the dhps Ala 436. The findings suggest that the presence of Ile 51/Asn 108 dhfr mutations and Ala 436 dhps confers decreased susceptibility of P. falciparum to PYR/SDX in areas of low endemicity.

INTRODUCTION

Some East and Central African countries have adopted the combination of pyrimethamine and sulfadoxine (PYR/SDX) as a first-line treatment for acute uncomplicated falciparum malaria. Resistance seems to develop soon after wide-scale use of the drug, mirroring events in Southeast Asia in the 1980s. High multiplicity of Plasmodium falciparum infections and the drug’s strong selection pressure for mutant alleles could explain the rapid progression of resistance. In Sudan, PYR/SDX is the second-line treatment. At present, a combination of chloroquine and PYR/SDX or co-trimoxazole is recommended for the treatment of acute uncomplicated malaria. This situation provides a good opportunity to observe the changes in dhfr and dhps genotypes as drug pressure increases.

Point mutations in the coding regions of dhfr and dhps genes have been implicated in in vitro resistance to PYR and SDX. Epidemiology of mutations in these genes is increasingly being studied. Geographic differences have been reported, and the spread of dhps/dhfr mutations has been related to history of PYR/SDX use and resistance in the field. The dhfr Asn-108 mutation has been present in all antifolate resistant forms studied, but no specific set of mutations in dhfr or dhps or both is found to be predictive of therapeutic failure. Prediction of PYR/SDX therapeutic failure is complicated because of the natural variations in host immunity, efficacy of absorption and metabolism of the drugs, serum level of folates, and parasite genotypes and their efficacy in using exogenous folates. More recent studies have indicated that parasite susceptibility to PYR is important in determining clinical outcome to PYR/SDX because synergy between PYR and SDX has been described as the abolition of folate antagonism of SDX inhibition by subinhibitory levels of PYR. The present study assessed the in vivo outcome of treatment with PYR/SDX among patients with acute uncomplicated falciparum malaria in relation to SDX plasma levels (as a marker of PYR/SDX absorption) and dhfr/dhps mutations among pretreatment parasites.

PATIENTS, MATERIALS, AND METHODS

Study area. The study was conducted during November 1996–February 1997 in the outpatient department of Hag Yousif hospital. It was part of a controlled, randomized, double blind, hospital-based clinical trial comparing efficacy and safety of PYR/SDX and co-trimoxazole in patients with acute uncomplicated falciparum malaria. Hag Yousif is an area of unstable malaria in Khartoum (Satti GMH, unpublished data).

In vivo sensitivity test. The in vivo sensitivity test was conducted and evaluated according to 1973 and 1996 WHO guidelines. Inclusion criteria were mono infection with P. falciparum, temperature ≥37.5°C and parasitemia between 1,000 and 80,000 asexual parasites/µl of blood. Pregnant women, patients with severe malaria, patients with concomitant nonmalarial febrile illness, patients allergic to sulfonamide, and patients with glucose-6-phosphate dehydrogenase deficiency were excluded. PYR/SDX (Hoffman La Roche, Basel, Switzerland) was administered orally at a single dose of 25 mg/kg of SDX and 1.25 mg/kg of PYR. Therapeutic responses were evaluated parasitologically and clinically. Parasitologically, treatment outcome is divided into sensitive (no parasites observed at day 7 and no recrudescence observed during the follow-up period) and 3 resistant grades: RI (parasites may disappear but reappeared within the next 28 days), RII (parasite density reduced by ≥75% of the initial parasite density 2 days after therapy), and RIII (parasites continue to rise or reduced by ≤25% 2 days after therapy). Clinically the response is divided into adequate clinical response (ACR) or treatment failures, either early or late treatment failures (ETF and LTF). ETF was defined as positive...
smear with parasite density > day 0 count and fever, positive smear of any density on day 3 and fever, or positive smear on day 3 ≥ 25% of day 0 count. LTIF was defined as parasites in the blood and temperature ≥ 37.5°C. Clinical follow-up and blood films were made daily in the first week and on days 14, 21, and 28, or whenever patients came to the clinic seeking help. Blood samples were taken before treatment; on days 3, 7, and 14; or on recrudescence for dhfr/dhps genotyping and plasma drug measurements.

**Molecular analysis.** DNA was extracted from heparinized blood samples using organic extraction. For the removal of the polymerase chain reaction (PCR) inhibitory effect of heparin, extracted samples were treated with heparinase as described. Nested PCR and restriction fragment-length polymorphism analysis (RFLP) were used to detect mutations in dhfr and dhps genes as described by Duraisingh et al. Electrophoresis of 10 μL of the restriction digest was carried out on 2.0–2.5% agarose gels, depending on the size of the digested product. To size the bands, 100 base pairs DNA ladder marker (MBI, Fermenta, Lithuania) were used. DNA samples from established laboratory strains of *P. falciparum* were used as controls for PCR and RFLP.

**Diagnostic PCR.** Diagnostic specific PCR was performed for all post-treatment samples to confirm the rapid parasite clearance time obtained in this study.

**Determination of SDX plasma levels.** SDX plasma levels were measured before and after treatment with PYR/SDX to screen for the degree of drug usage among the study population and to confirm PYR/SDX absorption and metabolism. Plasma samples were extracted by slight modifications of a previously described method. Plasma samples, 200 μL were added to 100 μL of the internal standard (sulfamethoxazole) and vortexed for 1 minute, then 500 μL of zinc sulfate (1M) was added to deproteinize the sample. The mixture was shaken for 15 minutes. After centrifugation (10 minutes at 1,000 × G), 100 μL of the supernatant was injected into the chromatogram.

**Statistical analysis.** Fisher exact test, 2-tailed, was used to test the association between different dhfr and dhps genotypes and treatment failure. Normally distributed data were compared by Student *t*-test, and non–normally distributed parameters were compared with the Mann-Whitney test with a significance level of 5%. One-way analysis of variance was used for serial drug analysis.

**Ethical review.** Ethical clearance to conduct the clinical trial was obtained from the National Institutes of Health (NIH), Khartoum, Sudan. Patients or guardians were asked to give a verbal consent to participate in the study.

**RESULTS**

There were 37 symptomatic malaria patients recruited. The mean age was 27.6 years (SD = 4.6; range = 10–65 years), and the median parasite density was 1,000–40,100 asexual parasites/μL blood (median ± 25%; 75th: 6,240 ± 3,260; 13,170). No serious side effects were recorded in any of the patients after PYR/SDX therapy. The clinical response showed that 91.8% of the patients had ACR; 5.4%, LTR; and 2.7%, ETF. Parasitologically, there were 91.8% sensitive, 5.4% RI, and 2.4% RII. The prevalence of PYR/SDX clinical and parasitologic failure was 8.1%. The mean parasite clearance time in patients with ACR was 1.4 days (SD = 0.98). In all patients, the clinical outcome matched the parasitologic response, and all PYR/SDX-resistant infections were treated successfully with a single dose of mefloquine (25 mg/kg). The mean age of patients with ACR or treatment failure was comparable (*P* > 0.05). Pretreatment parasitemia was significantly higher (*P* = 0.004) in patients with resistant infections (median ± 25th; 75th: 13.760 ± 2,930, 9,643 versus 6,100 ± 2,107; 4,690 in patients with susceptible infections). With the exception of the patient with RII infection, parasite density on recrudescence was lower than before treatment.

The rapid *in vivo* parasite clearance, 1.4 days, was confirmed further by diagnostic PCR. On day 3, parasite DNA was detected in 10 of 37 samples. On days 7, 14, 21, and 28 post-therapy, DNA was detected only in the blood samples of the 3 patients with recurring infections.

**Drug concentrations.** SDX was not detected in the plasma samples of any of the patients before therapy. Figure 1 shows SDX plasma concentration in patients classified as being infected with sensitive or resistant *P. falciparum* isolates *in vivo*. Full compliance was shown in all patients. In all patients, SDX plasma concentration ranged from 89–264 μg/ml 3 days post-therapy.

**Genotyping of dhfr/dhps and treatment outcome.** Pretreatment genotyping is presented in Figure 2. *dhfr* Asn 108 mutation was seen in 31 of 37 of the tested isolates; 6 isolates carried the wild type Ser 108, whereas 5 isolates were mixed wild/mutant genotype infections. None of the isolates had the Thr 108 mutation, which is associated with resistance to cycloguanil. At codon 51, 29 isolates had the mutant type Ile, 8 isolates had the wild type Asn, and 1 isolate was mixed genotype. All isolates showed the wild-type alleles in positions 16, 59, and 164.

With regard to *dhps*, all parasites carried the wild type at positions 437/540/581/613. Variations were seen only in codon 436, where Ala 436 mutation was detected in 3 isolates. These isolates were resistant to PYR/SDX *in vivo*.

Out of the 29 isolates with *dhfr* mutations at codon 51/108, 26 were cleared successfully with PYR/SDX. When this set of mutations coupled with *dhps* Ala 436, parasites recrudesced. The association of Ala 436 with treatment failure, although seen throughout the follow-up period. No additional *dhfr/* dhps mutations were detected on recrudescence.

**DISCUSSION**

Although the first case of PYR/SDX resistance in Sudan was reported in 1991, studies of the prevalence of *dhfr* and *dhps* mutations are lacking, and this is the first study to address the prevalence of these mutations in the country and their contribution to the *in vivo* outcome.

Our *in vivo* results show the effectiveness of PYR/SDX
combination in the study area. SDX plasma concentrations reported from patients infected with resistant isolates do not suggest inadequate absorption, abnormal SDX metabolism, or noncompliance, but point to parasite resistance and increased pretreatment parasitemia as causes of treatment failure.

Transfection studies have implicated Asn 108 mutation as playing a central role in pyrimethamine resistance. Transfection studies have implicated Asn 108 mutation as playing a central role in pyrimethamine resistance. Several other studies have shown that parasites that carry this mutation alone were cleared by PYR/SDX, even in semi-immune patients. These findings are in agreement with our results. The high prevalence of this mutation in our study area should not be ignored, however, taking into consideration that dhfr mutations occur in a stepwise fashion, and initial mutations conferring low-level PYR resistance must be present for high-level resistance mutations to appear. Thr 108, Val 16, and Leu 164, which are associated with resistance to PYR and cycloguanil, were not detected, confirming the rarity of these genotypes in the field isolates. The dhfr 164 mutation has been found in areas of South East Asia and South America, where resistance to PYR/SDX is well established. The dominance of Ile 51 and the absence of Arg 59 mutation in the study isolates might suggest the earlier appearance of this mutation or that PYR/SDX exerts more selective pressure for the 51 codon. The mutations at dhfr 51 and 108 could be selected for by the related antifolate cotrimoxazole, which is used frequently for the treatment of acute respiratory tract infections and other infectious diseases in the study area (Satti GMH, unpublished data). The genotype distribution of dhfr/dhps in this study is similar to that in the Middle East, where parasites are generally wild type in dhps, but doubly mutated in dhfr. Triple mutant alleles of dhfr are now common in Tanzania, Kenya, and Uganda, where PYR/SDX has been used for some time. The quadruple mutant allele has not been reported previously from Africa, but it has been present in South East Asia and South America for many years, and in these regions, neither PYR/SDX nor chlorproguanil/dapsone is clinically effective.

Concerning dhps, only the Ala 436 was detected in 3 isolates. Ala 436 mutation is common in areas of less PYR/SDX use and resistance and has been proposed to provide a selective advantage under low drug pressure, by precluding the progression of additional mutations associated with SDX resistance.

The ability of certain individuals to clear parasites with resistance-related dhfr/dhps alleles is due to the interaction of many factors, among which acquired immunity is presumably important. Immunity to malaria develops over years of repeated infections, and in malaria-endemic areas, spontaneous elimination of parasitemia and treatment responses were found to improve with age. The association of dhps Ala 436 with treatment failure in this study is significant, but the number is too low to permit valid conclusions. Such an association in a population with little immunity is important, however, and suggests dhps mutations are important in individuals without underlying immunity when combined with dhfr 51/108 mutations but potentially masked in individuals with a degree of immunity. Our finding is in agreement with a study carried out in Kenya in 2 areas of different endemicities. In the epidemic area, where the level of immunity is low, mutations in dhps and dhfr were found to be important, and the occurrence of ≥1 mutation in dhps predicted PYR/SDX treatment failure, whereas in the holoendemic area, potentially resistant parasites, especially those with mutations in dhps only, were cleared by a combination of drug response and acquired immunity. In such areas, triple mutant dhfr (Ile 51/Arg 59/Asn 108) with or without mutant dhps at positions 437/540 could be the main genetic determinants of treatment failure. Further studies are needed to address the importance of dhps mutations as predictive markers of PYR/SDX treatment in areas of low transmission and in non-immune subjects.
Figure 2. Prevalence of dihydrofolate reductase and dihydropteroate synthase genotypes in pretreatment samples of patients treated with PYR/SDX and classified as in vivo sensitive (n = 34) or resistant (n = 3), Khartoum, Sudan. First column represents the in vivo response after therapy with PYR/SDX. White cells, wild genotypes; black cells, mutant genotypes; horizontal lines, mixed infections.

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