DIHYDROFOLATE REDUCTASE AND DIHYDROPTERATE OXIDASE SYNTHASE GENOTYPES ASSOCIATED WITH IN VITRO RESISTANCE OF PLASMODIUM FALCIPARUM TO PYRIMETHAMINE, TRIMETHOPRIM, SULFADOXINE, AND SULFAMETHOXAZOLE

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Abstract. A total of 70 Plasmodium falciparum isolates were tested in vitro against pyrimethamine (PYR), trimethoprim (TRM), sulfadoxine (SDX), and sulfamethoxazole (SMX), and their dihydrofolate reductase (dhfr) and dihydropteroate synthase (dhps) genotypes were determined. dhfr genotypes correlated with PYR and TRM drug responses ($r = 0.93$ and $0.85$). Isolates with wild-type alleles showed mean half inhibitory concentrations ($IC_{50}$) of $0.10 \pm 0.10$ and $0.15 \pm 0.06$ $\mu$g/100 $\mu$l for PYR and TRM. Parasites with mutations at codons 108 and 51 alone or combined with codon 59 have $IC_{50}$ of $11.46 \pm 0.86$ (PYR) and $2.90 \pm 0.59$ $\mu$g/100 $\mu$l (TRM). For both drugs, the differences in the mean $IC_{50}$ between wild and mutant parasites were statistically significant ($P < 0.001$). Isolates with mixed wild and mutant alleles showed an intermediate level of susceptibility. Our data show partial cross-resistance between PYR/TRM and SDX/SMX ($r = 0.85$ and 0.65). Correlation was not observed between different dhps genotypes and the in vitro outcome to SDX and SMX ($r = 0.30$ and 0.34). The lack of correlation could be due to folates and para-aminobenzoic acid in the RPMI medium and the serum used to supplement the cultures.

INTRODUCTION

The antifolate combination pyrimethamine/sulfadoxine (PYR/SDX) currently is used in some African countries as first-line treatment of uncomplicated Plasmodium falciparum malaria.$^1$ Trimethoprim/sulfamethoxazole (TRM/SMX) has been recommended by the World Health Organization for treatment of childhood febrile diseases and for prophylaxis against opportunistic infections for human immunodeficiency virus–infected patients in Africa.$^2$ Many African countries, including the Sudan, are in the process of changing their malaria drug policy, and careful consideration should be given to the potential of parasite populations becoming resistant before introducing antifolates as first-line therapy. Monitoring of antifolate resistance should be done in a systematic and reproducible way. Surveillance and the use of molecular assays must be based on clear understanding of resistance in the context of in vivo and in vitro responses.

In vitro resistance to PYR and SDX is due to point mutations in the conserved regions of dihydrofolate reductase (dhfr) and dihydropteroate synthase (dhps).$^3$–$^6$ Wang et al.$^7$ showed that a gene associated with folate use plays an important role in determining the level of SDX resistance. Although resistance to dhfr inhibitors is drug specific,$^8$–$^9$ wide-spread cross-resistance exists between PYR and cycloguanil,$^9$ which together with chloropropham and TRM are the dhfr inhibitors effective against $P. falciparum$. In contrast, the PYR-resistant Campodian (CAMP) strain is found to be susceptible to TRM.$^{10}$ This finding needs further confirmation with field isolates, however.

The present study was designed to monitor the in vitro sensitivity of $P. falciparum$ to PYR, TRM, SDX, and SMX. Furthermore we want to study cross-resistance between PYR/TRM and SDX/SMX and determine whether point mutations in dhfr and dhps coding regions are correlated with the isolate chemosensitivity to the tested drugs.

MATERIALS AND METHODS

Study populations. The study was conducted during November 1996–February 1997 in an unstable malaria area, Hag Yousif, 11 km east of Khartoum, Sudan. The in vitro sensitivity test was performed in 106 $P. falciparum$ isolates drawn from symptomatic patients (age range, 10–65 years). The parasite density in patients ranged from 1,000–60,000 asexual parasite/$\mu$l blood, and they had no history of antimalarial intake within the last 2 weeks. Ethical clearance was obtained form the National Institutes of Health Khartoum, Sudan, and informed consent was obtained from patients or parents before participation in the study.

In vitro test. The in vitro tests for drug sensitivity were conducted in the laboratories of the Biochemistry Department, Faculty of Medicine, University of Khartoum, Sudan. A modified RPMI medium containing the physiologic amounts of folates and para-aminobenzoic acid (PABA) ($10 \mu$g/l and 0.5 $\mu$g/l), 25 mM of HEPES buffer, 4 mM of glutamine, 25 mM of sodium bicarbonate, and 25 $\mu$g/ml of gentamicin was prepared at the laboratories of the Institute of Medical Microbiology and Immunology, University of Copenhagen, Denmark.

The 48-hour in vitro test was performed in 96 well flat-bottomed microculture plates pre-sowed with 10 $\mu$l of either PYR ($0.001–12.8 \mu$g/100 $\mu$l), TRM ($0.05–3.2 \mu$g/100 $\mu$l), SDX ($0.02–240 \mu$g/100 $\mu$l), or SMX ($1–64 \mu$g/100 $\mu$l). The concentrations were chosen to cover the therapeutic levels attained in malaria patients, 0.1–0.4 for TRM, 4.0–10.0 $\mu$g/100 $\mu$l for SMX (A.M. Rønn, personal communication), 0.34–1.80 for PYR, and 3.1–18.6 $\mu$g/100 $\mu$l for SDX. Stock solutions of PYR and TRM were made in 0.5% lactic acid; further dilutions were done in the modified RPMI medium. The final concentration of lactic acid (<0.0005%) had no influence on the growth of $P. falciparum$. $^{11,12}$ SDX and SMX were dissolved in distilled water titrated with sodium hydroxide to pH 10 at 37°C. The pH in the stock solutions did not influence the...
pH of the final culture. Desired dilutions were made in the modified RPMI medium. The plates were kept at 37°C to dry and stored in darkness at 4°C until further use.

From each patient, 400 μl of venous blood was drawn and diluted in 3.6 ml of RPMI medium; 50 μl of the blood/medium mixture was added to each well. The plates were rocked gently to dissolve the drug deposits, then incubated in a candle jar at 37°C for 48 hours. After 48 hours, the medium was removed from individual wells, and thin and thick blood films were performed from the erythrocyte sedimentation. Specimens were stained with 3% Giemsa for 30 minutes. Schizont maturation was assessed for the control and drug wells. Growth was considered successful when schizont maturation in the control well was ≥10%.

**Polymerase chain reaction and the restriction fragment length polymorphism.** DNA was extracted from heparinized whole blood. For the removal of the presumed polymerase chain reaction (PCR) inhibitory effect by heparin, extracted DNA (approximately 300 ng) was incubated with heparinase I (1 U/ng DNA) in 5 mM of TRIS, pH 7.5, and 1 mM of calcium chloride for 2 hours at room temperature. For the amplification of the \textit{dhfr} and \textit{dhps} coding regions, a nested PCR protocol was followed. Mutations at \textit{dhfr} 16, 51, 59, 108, and 164 and \textit{dhps} 436, 437, 540, 581, and 613 were detected by incubating 5 μl of PCR product with the restriction enzymes as recommended by the manufacturers (New England Biolabs, Beverly, MA). Laboratory adapted strains were used as controls for PCR and restriction fragment length polymorphism (RFLP) analysis.

**Data analysis.** Probit/log dose response analysis was used for the determination of the half inhibitory concentrations (IC$_{50}$) quantifiable data expressed as mean ± SD were compared by Mann-Whitney rank-sum test. Cross-resistance and chemosensitivity for the tested drugs and the corresponding \textit{dhfr} alleles were compared by Pearson correlation coefficient test.

**RESULTS**

Of 106 \textit{P. falciparum} isolates, 70 grew successfully \textit{in vitro} for the estimation of sensitivity to PYR, TRM, SDX, and SMX. Sixteen isolates had <20 schizonts/200 asexual parasites in the control wells, whereas 20 failed to show any growth. For the 70 isolates, parasitemia ranged from 1,000–21,600 parasites/μl blood.

**Dihydrofolate reductase and \textit{in vitro} response.** Table 1 presents the chemosensitivity profile and the \textit{dhfr} genotypes at positions 51, 59, and 108. Of the 70 isolates, 13 had wild-type alleles in all \textit{dhfr}-tested codons; 47 isolates had Ile-51 and Asn-108 mutations. Triple mutation (Ile-51, Arg-59, and Asn-108) was detected in 1 isolate. Nine isolates showed mixed \textit{dhfr} alleles (5 isolates at position 51/108, 1 isolate at position 59/108, and 4 showed mixed alleles at position 108 only). None of the isolates had the mutant alleles Val-16, Thr-108, and Ile-164. There was a strong correlation between particular \textit{dhfr} genotype and sensitivity to PYR and TRM (r = 0.93 and 0.85). The increasing number of point mutations was associated with an increasing level of PYR IC$_{50}$ value and to a lesser extent TRM (Table 1). Wild-type profile was associated with lower IC$_{50}$ values ranging from 0.01–0.13 μg/100 μl (0.10 ± 0.11) for PYR and 0.05–0.2 μg/100 μl (0.15 ± 0.06) for TRM (Table 1).

Isolates with double or triple mutations had IC$_{50}$ values of 9.0–12.8 μg/100 μl (11.46 ± 0.86) for PYR and 0.4–3.2 μg/100 μl (2.90 ± 0.59) for TRM. For both drugs, the differences in mean IC$_{50}$ values of wild and mutant parasites were statistically significant (P < 0.001). For parasites with mutant \textit{dhfr}, the change in the IC$_{50}$ values was >100-fold (PYR) and 16-fold (TRM) compared with the values obtained by \textit{dhfr} wild-type parasites. \textit{P. falciparum} isolates with double or triple mutations were more resistant to PYR than TRM (P < 0.001). The IC$_{50}$ values of isolates with mixed alleles showed an intermediate level of susceptibility varying from 0.01–10.0 μg/100 μl (3.27 ± 3.14) for PYR and 0.4–3.2 μg/100 μl (1.64 ± 1.47) for TRM (Table 1). Our \textit{in vitro} data present evidence of cross-resistance between PYR and TRM because the IC$_{50}$ values for both drugs were highly correlated (r = 0.85, P < 0.01) (Figure 1). The ratio of PYR-to-TRM response among different parasites ranged from 0.7–4.0. This narrow range of

<table>
<thead>
<tr>
<th>\textit{dhfr} genotypes</th>
<th>Range (mean ± SD) of IC$_{50}$ values (μg/100 μl)</th>
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<tbody>
<tr>
<td></td>
<td>Pyrimethamine</td>
</tr>
<tr>
<td>Isolates with wild-type profile (n = 13)</td>
<td>0.01–0.13 (0.10 ± 0.11)*</td>
</tr>
<tr>
<td>Mixed infections at codons 108 or + 51 or 59 (n = 9)</td>
<td>0.01–10.0 (3.27 ± 3.14)</td>
</tr>
<tr>
<td>Mutant at codon 108 and 51 and/or 59 (n = 48)</td>
<td>9.0–12.8 (11.46 ± 0.86)*</td>
</tr>
</tbody>
</table>

* Significant difference in pyrimethamine means IC$_{50}$ of parasites with wild or mutant genotypes (P < 0.001).
† Significant difference in trimethoprim means IC$_{50}$ of parasites with wild or mutant genotypes (P < 0.001).

**FIGURE 1.** Correlation between half inhibitory concentrations (IC$_{50}$) of 70 \textit{P. falciparum} isolates from symptomatic malaria patients tested \textit{in vitro} against the antifolates pyrimethamine and trimethoprim.
PYR-to-TRM ratios is a further confirmation that these 2 compounds are correlated closely in their binding to the mutant dhfr.

Dihydropteroate synthase and in vitro response. For the 70 isolates, amino acid substitutions were seen only in codons 436 (10 isolates) and 437 (3 isolates), 55 isolates showed the wild-type profile, and 2 isolates showed mixed alleles at position 436. IC$_{50}$ values for SDX and SMX shown in Table 2 reflect the high susceptibility of the isolates to both drugs. A statistically insignificant difference was observed between chemosensitivity to SDX and SMX and different dhps genotypes ($r = 0.3$ and 0.34, $P > 0.05$). There was a positive correlation between SDX and SMX IC$_{50}$ ($r = 0.65$, $P < 0.05$), suggesting related but not identical mechanisms of resistance (Figure 2).

**DISCUSSION**

The present study investigated the in vitro susceptibility of *P. falciparum* to PYR, TRM, SDX, and SMX. The in vitro response to PYR and TRM was highly correlated. These results agree with the study of Lyer et al.,$^5$ in which cross-resistance between PYR and TRM has been shown at the molecular level. Our study shows an association between dhfr genotypes and chemosensitivity to PYR and TRM. Isolates with dhfr Asn-108 mutation alone or combined with Ile-51 or Arg-59 or both were less susceptible to PYR and TRM than isolates with a wild-type profile ($P < 0.001$ for both drugs). This finding agrees with previous studies correlating dhfr mutations and chemosensitivity in field samples from Brazil$^{17}$ and Africa.$^{18,19}$ Our results are in contrast to the findings of Jelinek et al.$^{16}$ however, who concluded that Ser-108 plays an important role in TRM resistance because it has been selected for in post-treatment infections of Ugandan children treated with TRM/SMX combination. The highest IC$_{50}$ for PYR and TRM was recorded in an isolate with triple mutation (Asn-180, Ile-51, and Arg-59). A further documentation of the importance of these 3 codons in TRM susceptibility is that this isolate recrudesced in vivo after treatment with TRM/SMX.$^{20}$ Isolates with an intermediate level of susceptibility to PYR showed evidence of both allelic forms at positions 51, 59, or 108. Because the erythrocytic stages of *P. falciparum* are haploid, these isolates presumably contained resistant and sensitive parasites. This is not surprising because polyclonal infections of parasites differing in their susceptibility to drugs commonly are found in natural infections of *P. falciparum*.$^{21}$

The difference in IC$_{50}$ values of isolates with wild or mutant genotype was 16-fold for TRM and 100-fold for PYR. Based on these findings, it seems that additional mutations are needed to confer higher resistance to TRM.

**TABLE 2**

Dihydropteroate synthase (dhps) genotypes and the half-inhibitory concentration (IC$_{50}$) of *P. falciparum* isolates to the sulfonamide sulfadoxine and sulfamethoxazole

<table>
<thead>
<tr>
<th>dhps genotypes</th>
<th>Sulfadoxine (Range (mean ± SD))</th>
<th>Sulfamethoxazole (µg/100 µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type profile (n = 55)</td>
<td>2.3–20.8 (6.3 ± 12.9)</td>
<td>1.0–58.0 (8.9 ± 14.2)</td>
</tr>
<tr>
<td>Mixed infections at codon 436 only (n = 2)</td>
<td>2.9–6.5 (4.7 ± 2.6)</td>
<td>1.9–2.7 (2.3 ± 0.66)</td>
</tr>
<tr>
<td>Isolates with alanine-436 (n = 10)</td>
<td>3.4–22.0 (7.9 ± 5.5)</td>
<td>1.0–64.0 (10.8 ± 16.8)</td>
</tr>
<tr>
<td>Isolates with glycine-437 (n = 2)</td>
<td>2.7–18.3 (8.0 ± 8.9)</td>
<td>2.8–4.5 (2.3 ± 0.66)</td>
</tr>
</tbody>
</table>

*Note: A statistically insignificant difference was observed between chemosensitivity to sulfadoxine and sulfamethoxazole and different dhps genotypes ($P > 0.05$).*

SDX IC$_{50}$ values obtained in this study are comparable to those obtained by Milhous et al.$^{22}$ Chulay et al.$^{23}$ and Watkins et al.$^{24}$ The isolate with IC$_{50}$ of 64.0 µg/100 µl for SMX had alanine-436 dhps mutation and was resistant in vivo to TRM/SMX.$^{20}$ Elevation in its IC$_{50}$ could be due to additional new mutations in the dhps gene or due to the folate effect. It has been shown that use of exogenous folate can play an important role in determining the level of SDX resistance in a given parasite line.$^7$ Currently we are sequencing the dhps and dhfr genes of this isolate for the screening of new mutations.

The poor reproducibility of our in vitro assay in case of the sulfonamides SDX and SMX could be due to the presence of exogenous folate. The major sources of exogenous folate in this study were the serum used to supplement the culture and the culture medium. For reproducible data with large differences between *P. falciparum* isolates with wild or mutant dhps alleles, Wang et al.$^{25}$ developed a new in vitro assay to determine SDX sensitivity of some reference isolates. The assay involved substitution of quality-controlled, lipid-rich albumin for plasma/serum, culturing the parasites first in a low-folate medium, followed by a complete growth cycle of 48 hours in a folate and PABA-free medium before entry into the drug test. Ndounga et al.$^{26}$ evaluated this novel assay system under field conditions with fresh clinical isolates, however, and found that the system needs further improvement to provide relatively reproducible data.

Given the correlation between *P. falciparum* susceptibility to PYR and TRM and dhfr genotypes, PCR-RFLP analysis could be used as a quick and reliable method for epidemiologic surveillance of the dhfr inhibitors used in the field. A simple, standardized and reproducible in vitro system is needed for regular monitoring of sulfonamide resistance in the field.
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