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Molecular basis of differential resistance to cycloguanil and pyrimethamine in *Plasmodium falciparum* malaria

DAVID S. PETERSON*, WILBUR K. MILHOUS†, AND THOMAS E. WELLEMS*

*Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; and †Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, DC 20307-5100

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ABSTRACT **Proguanil and pyrimethamine are antifolate drugs with distinct chemical structures that are used commonly in the prophylaxis and treatment of *Plasmodium falciparum* malaria. Clinical reports and field studies have suggested that some parasites refractory to proguanil can be treated with pyrimethamine, and vice versa. Analysis of the *P. falciparum* dihydrofolate reductase (DHFR) from different parasites reveals the structural basis for differential susceptibility to these antifolate drugs. Parasites harboring a pair of point mutations from Ala-16 to Val-16 and from Ser-108 to Thr-108 are resistant to cycloguanil (the active metabolite of proguanil) but not to pyrimethamine. A single Asn-108 mutation, on the other hand, confers resistance to pyrimethamine with only a moderate decrease in susceptibility to cycloguanil. Significant cross-resistance to both drugs occurs in parasites having mutations that include Ser-108 → Asn-108 and Ile-164 → Leu-164. These results reflect the distinct structures of pyrimethamine and cycloguanil and suggest fine differences in binding within the active site cavity of DHFR. Alternative inhibitors, used alone or in combination, may be effective against some strains of cycloguanil- or pyrimethamine-resistant malaria.**

Proguanil and pyrimethamine, introduced nearly 40 years ago (1, 2), were powerful additions to the spectrum of antimalarial agents. Both drugs are specific inhibitors of the enzyme dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3) and, in combination with sulfa drugs (Fig. 1), have been distributed widely for use against malaria infections. Shortly after the release of each compound, however, prophylactic and therapeutic failures against *Plasmodium falciparum* were reported. Antifolate resistance has subsequently become a major problem in endemic regions throughout the world. The prevalence of resistant strains, and the risk of severe cutaneous reactions from combined administration with sulfa drugs, have limited the clinical utility of pyrimethamine and proguanil in prophylaxis of malaria (3).

Several reports have noted that pyrimethamine and proguanil resistance in *P. falciparum* may occur independently. In Malaya in the early 1950s, where prophylactic use of proguanil was widespread, Wilson and Edeson found no consistent cross-resistance between proguanil and pyrimethamine (4). More recent studies have reported proguanil provides effective protection in some regions where pyrimethamine resistance occurs (5, 6). Since pyrimethamine resistance results from a point mutation to Asn-108 in the DHFR of *P. falciparum* (7–9), the question arises whether proguanil resistance is conferred by different mutations in DHFR. In this report we examine point mutations in several *P. falciparum* clones and lines that exhibit different responses to pyrimethamine and cycloguanil (the active metabolite of proguanil). We show that parasites containing a pair of point mutations (Ser-108 → Thr-108 and Ala-16 → Val-16) are resistant to cycloguanil but not to pyrimethamine. High levels of resistance to both cycloguanil and pyrimethamine are associated with the mutation Ile-164 → Leu-164 in conjunction with Ser-108 → Asn-108.

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MATERIALS AND METHODS

Parasite Cultivation. Parasites used in this work have been described: the clones HB3 (10), 3D7 (11), SL/D6 (12), W2 (12), It.G2.F6 (13), and It.D12 (13); and the lines FCR3 (14), UPA (15, 16), and FCB (17). Clone V1/S was obtained from the mixed isolate V1 (18) after selection by pyrimethamine and cloning by limiting dilution (19). Cultures were maintained *in vitro* by standard methods (20).

Drug Susceptibility Assays. Pyrimethamine and cycloguanil susceptibilities were performed both by microscopic determinations of parasitemia (7) and by [^{3}H]hypoxanthine uptake (21). Assays were conducted independently in two laboratories. Antagonism of drug effect by folic acid and *p*-aminobenzoic acid (PABA) was investigated by varying the concentrations of these compounds in the culture medium (22). IC_{50} values were determined from the drug concentrations at which parasitemias or [^{3}H]hypoxanthine uptake were reduced by 50% compared with control. Pyrimethamine and cycloguanil resistance indices for each line were calculated as ratios of IC_{50} values relative to simultaneous values from the SL/D6 clone. To reduce effects of folate and PABA antagonism, all resistance indices were calculated from IC_{50} values determined in folate/PABA-free RPMI 1640 medium supplemented with 10% normal human plasma.

DNA Extraction and Nucleotide Sequence Analysis. Parasites were obtained from infected erythrocytes by lysis in TSE buffer (100 mM NaCl/50 mM EDTA/20 mM Tris, pH 8.0) containing 0.15% saponin. After centrifugation at 5000 × *g*, the parasites were resuspended in TSE buffer and lysed by addition of sodium lauryl sulfate (SDS) to 1% and $NaClO_4$ to 0.5 M. The lysate was gently mixed at room temperature for 1 hr, extracted twice with phenol equilibrated in TSE buffer (pH 8.0), and finally extracted twice with 1:1 (vol/vol) phenol/chloroform. The solution was brought 0.2 M in sodium acetate, 1.5 volumes of 95% EtOH were added, and the DNA was spooled out of solution.

In *P. falciparum* the DHFR domain is part of the bifunctional enzyme dihydrofolate reductase–thymidylate synthase (DHFR/TS). Oligonucleotide primers were designed from the gene sequence (23) and used in the polymerase chain reaction to amplify the DHFR domain as described (7). Fifty nanograms of *P. falciparum* DNA (representing $\approx 1.5 \times 10^6$ copies of the genome) was used as the starting material. Both strands of amplified DNA were sequenced by a modification of the method reported by Innis *et al.* (24). Two microliters of the polymerase chain reaction product were used in a

Abbreviations: DHFR, dihydrofolate reductase; PABA, *p*-aminobenzoic acid.

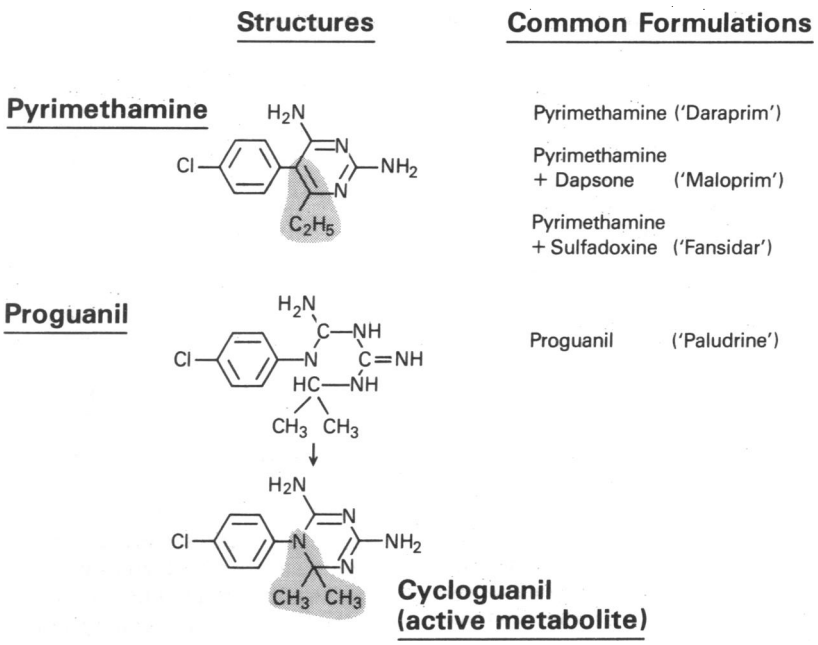

FIG. 1. Structures and common formulations of pyrimethamine and proguanil. Cycloguanil, the biologically active metabolite of proguanil, contains a triazine ring with two substituent methyl groups instead of the pyrimidine ring and ethyl side chain of pyrimethamine (differences indicated by half-tone shading).

second reaction containing a single primer, promoting amplification of just one strand. After precipitation of the single-stranded template in isopropanol, nucleotide sequence data were obtained with sequencing primers SP1-SP6 (7) and the dideoxynucleotide method (Sequenase kit, United States Biochemical).

RESULTS AND DISCUSSION

Pyrimethamine and cycloguanil susceptibilities of 10 *P. falciparum* clones and lines were determined *in vitro* (Table 1). Because relative levels of susceptibility to pyrimethamine and cycloguanil vary with folate and PABA concentrations in the assay medium (22), values were measured in both normal RPMI 1640 medium and folate/PABA-free RPMI 1640 medium. Clones SL/D6 (from a patient treated successfully with pyrimethamine alone) and W2 (from the multidrug-resistant isolate Indo-III) were included in the analysis, as these clones serve in the characterization of drug-resistant malaria strains (25). In folate/PABA-free medium, patient isolates from pyrimethamine/sulfadoxine failures are known to exhibit

Table 1. Point mutations and antifolate susceptibility in *P. falciparum*

Clone or isolate		Drug susceptibility, ng/ml: Standard RPMI*		F/P-free RPMI†		Amino acid residue				
Name	Origin	Pyr	Cyc	Pyr	Cyc	16	51	59	108	164
3D7	The Netherlands‡	2	0.26	0.02	0.02	Ala	Asn	Cys	Ser	Ile
SL/D6	Sierra Leone	1	0.06	0.03	0.03	Ala	Asn	Cys	Ser	Ile
FCR3	The Gambia	4	64	0.56	54	Val	Asn	Cys	Thr	Ile
UPA	Uganda	2	32	0.18	18	Val	Asn	Cys	Thr	Ile
FCB	Columbia	2	32	0.41	31	Val	Asn	Cys	Thr	Ile
It.G2.F6	Brazil	2	64	2.1	55	Val	Asn	Cys	Thr	Ile
HB3	Honduras	260	2	22	0.45	Ala	Asn	Cys	Asn	Ile
It.D12	Brazil	1000	16	88	2.4	Ala	Ile	Cys	Asn	Ile
W2	Indochina	4100	64	34	0.68	Ala	Ile	Arg	Asn	Ile
V1/S	Vietnam	2000	1000	84	24	Ala	Ile	Arg	Asn	Leu

Boxes with double lines identify mutations producing resistance to cycloguanil (Cyc) or pyrimethamine (Pyr). Resistance is enhanced by ancillary mutations at positions 51 and 59 (single-line boxes).

*Standard RPMI 1640 medium contains 1 μg of folate and 1 μg of PABA per ml supplemented with 10% undialyzed human serum.

†Folate- and PABA-free RPMI 1640 medium supplemented with 10% undialzyed human serum.

‡Derived from the Amsterdam Airport isolate NF54, thought to be of African origin.

pyrimethamine IC_{50} values of 15–40 ng/ml, 500- to 1500-fold greater than the IC_{50} value of the susceptible SL/D6 standard (26). Tables 1 and 2 present IC_{50} values and corresponding resistance indices for clones and lines in the present study. The resistance indices (Table 2) show that the HB3, It.D12, W2, and V1/S parasites would be expected to be pyrimethamine resistant, while other parasites in the study would be expected to be pyrimethamine susceptible (there exists no evidence that the FCR3, UPA, FCB, and It.G2.F6 indices produce clinical levels of resistance). Cycloguanil IC_{50} values and resistance indices for the parasites are likewise presented in Tables 1 and 2. Five parasites, FCR3, UPA, FCB, It.G2.F6, and V1/S, exhibit high cycloguanil-resistance indices. These indices presumably reflect clinical levels of resistance, assuming that the IC_{50} values of cycloguanil-resistant strains are comparable to those accompanying pyrimethamine resistance.

Comparison of the pyrimethamine and cycloguanil-resistance indices of the 10 *P. falciparum* lines shows that four (FCR3, UPA, FCB, and It.G2.F6) exhibit resistance to cycloguanil but, possessing only slightly increased pyrimethamine indices, would be expected to respond to pyrimethamine *in vivo*. Conversely, three pyrimethamine-resistant lines (HB3, It.D12, and W2) are highly resistant to pyrimethamine, with only a modest decrease in cycloguanil susceptibility. Only V1/S parasites possess high levels of resistance to both pyrimethamine and cycloguanil.

To examine the molecular basis of these different patterns of drug resistance, point mutations in the DHFR domains of the pyrimethamine- and cycloguanil-resistant parasites were determined by direct sequencing of DNA obtained by polymerase chain reaction amplification. Analysis of the cycloguanil-resistant, pyrimethamine-susceptible parasites UPA, FCB, FCR3, and It.G2.F6 revealed paired point mutations (Ala-16 → Val-16 and Ser-108 → Thr-108) associated with cycloguanil resistance. In contrast, cycloguanil-susceptible, pyrimethamine-susceptible lines all contained Ser-108 and Ala-16. No parasites were identified in which the Ser-108 → Thr-108 mutation is not associated with Ala-16 → Val-16.‡

The Asn-108 mutation in DHFR has already been shown to confer resistance to pyrimethamine (7–9). Table 1 confirms the presence of this mutation in all four pyrimethamine-resistant clones. Only a decrease in cycloguanil susceptibility by a factor of 20 is apparent in the HB3 clone containing this mutation. Presence of an ancillary mutation Asn-51 → Ile-51 in the It.D12 clone is associated with an additional decrease by a factor of 5 in response to both drugs.

Using assay values obtained in normal RPMI 1640 medium, we previously found parasites containing three mutations (Asn-108, Ile-51, and Arg-59) to be 16- to 32-fold more pyrimethamine resistant than parasites containing the Asn-108 mutation alone (7). However, Table 1 shows that the W2 parasite, which contains all three of these mutations, exhibits different relative levels of resistance depending upon the PABA and folate concentrations in the culture medium. Assays of the W2 clone in folate/PABA-free RPMI 1640 medium yielded markedly lower IC_{50} values than did assays performed in standard RPMI 1640 medium (1 μg of folate and 1 μg of PABA per ml). Antagonistic effects of exogenous PABA and folate on the activity of antifolate drugs have been described (22). Therefore, to explore the effects of folate and PABA on the relative drug susceptibilities, we compared the responses of the W2 and SL/D6 clones at various concentrations of these metabolites. These studies revealed the drug response of clone W2 to be dramatically affected by PABA when compared with the SL/D6 response (Fig. 2*A*). Folate produced a similar, although less pronounced, effect (Fig. 2*B*). We note that serum level ranges of PABA and folate *in vivo* are ≈0.5–1 ng/ml and 5–20 ng/ml, respectively (27, 28). At these levels of PABA and folate, enhanced antagonism of drug action on clone W2 may significantly affect antifolate resistance.

‡Our previous report (7) of Ala-16 in the It.G2.F6 sequence was in error. The correct residue, Val-16, is indicated in Table 1.

Table 2. Pyrimethamine and cycloguanil resistance indices of *P. falciparum* parasites

Clone or isolate	Resistance index	
	Pyrimethamine	Cycloguanil
3D7	0.7	0.7
SL/D6	1.0	1.0
FCR3	19	1800
UPA	6	600
FCB	14	1000
It.G2.F6	70	1800
HB3	730	15
It.D12	2900	80
W2	1100	23
V1/S	2800	800

Parasite line V1/S contains the unique mutation Ile-164 → Leu-164 in addition to Ile-51, Arg-59, and Asn-108. High resistance to both cycloguanil and pyrimethamine is associated with this cluster of mutations. That the V1/S and W2 enzymes differ only by the Ile-164 → Leu-164 mutation suggests this mutation predominantly affects cycloguanil binding.

Three arguments indicate that it is the point mutations in DHFR that confer drug resistance in the lines we have examined. First, analysis of two genetic crosses has now shown linkage of drug resistance to the mutations. Our

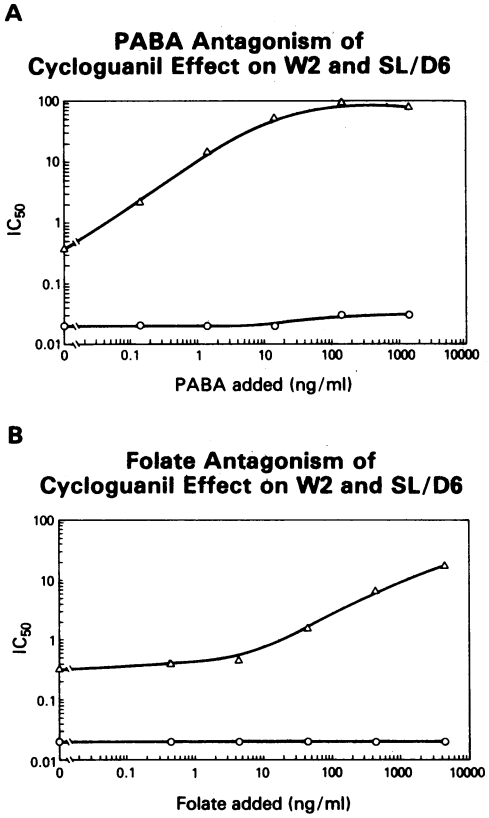

FIG. 2. PABA and folate antagonism of the cycloguanil effect on the SL/D6 (○) and W2 (△) parasites. Assays were performed in folate/PABA-free RPMI 1640 medium supplemented with 10% pooled human plasma and the indicated amounts of folate or PABA.

previous paper demonstrated linkage of the Asn-108 point mutation to pyrimethamine resistance in the 3D7 × HB3 cross (7). Analysis of a second cross (involving a clone derived from W2) has shown further that high levels of resistance at elevated PABA and folate concentrations are linked to the Ile-51 and Arg-59 mutations in the DHFR locus (unpublished results). A second argument that drug resistance is from alterations in the DHFR domain is provided from the data in Table 1. This table lists the mutations of 10 independent parasite lines from geographically distant regions. Of the 9 that do not contain the Leu-164 mutation, 4 are cycloguanil resistant and 5 are cycloguanil susceptible in the absence of folate and PABA antagonism. Because of the innumerable crossover events separating these strains, the chance of a random correlation between the Thr-108/Val-16 mutations and cycloguanil resistance is minimal (on the order of $0.5^9 \approx 0.2\%$). Finally, a third argument derives from alignment of the mutations with known structures from other organisms (7, 23, 29). All mutations align with residues known to occur at the active site cavity of DHFR. Since pyrimethamine and cycloguanil bind in this cavity, the mutations occur where they would be expected. Given the linkage data already obtained from the crosses and numerous independent isolates, the conclusion that the point mutations are responsible for resistance in natural strains of *P. falciparum* is compelling.

The various mutations in the DHFR enzyme appear to have arisen in response to the different ways cycloguanil and pyrimethamine bind to DHFR. Alignment of the *P. falciparum* sequence with DHFR sequences from other organisms shows that all of these mutations occur in conserved regions that border the active site cavity of the enzyme (Fig. 3). Residue 108 occurs in the C α-helix of the enzyme; residues 51 and 59 align in or near the B α-helix at the back of the cavity; and residues 16 and 164 are located in the A β-strand and E β-strand, respectively. Residues at or near these positions have been shown to be involved in the binding of various inhibitors to avian, bacterial, and mammalian DHFRs (31–34).

It is interesting that Ser-108 → Thr-108 and Ala-16 → Val-16 occur together in cycloguanil-resistant pyrimethamine-susceptible parasites. With the exception of *Plasmodium*, Thr-108 has been reported at the analogous position of the C α-helix for all organisms studied to date (35). Ser-108, however, is apparently the wild-type residue in *P. falciparum*. The role of Thr-108 and its relation to Val-16 need to be clarified by the ability to study these mutations independently, perhaps by means of stable transfection or heterologous expression.

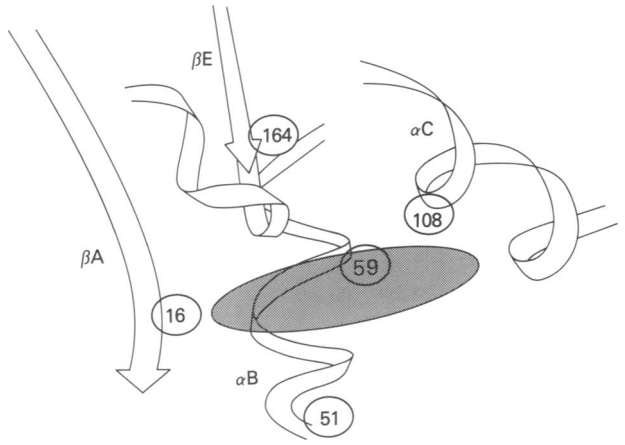

FIG. 3. Schematic depiction of point mutations in the active site cavity of *P. falciparum* DHFR. The mutations in cycloguanil-resistant, pyrimethamine-susceptible forms are located in the A β-strand (Ala-16 → Val-16) and the C α-helix (Ser-108 → Thr-108). Pyrimethamine-resistant forms contain the Asn-108 mutation. Parasites resistant to both pyrimethamine and cycloguanil have an additional mutation in the E β-strand (Ile-164 → Leu-164). Ancillary mutations Asn-51 → Ile-51 and Cys-59 → Arg-59 are associated with enhanced levels of pyrimethamine and cycloguanil resistance. The diagram is based on an adaptation from the work of Matthews *et al.* (30).

The results of this work support clinical observations that alternative anti-DHFR agents can be effective against some strains of pyrimethamine- or cycloguanil-resistant malaria. In addition to cycloguanil and pyrimethamine, known inhibitors of DHFR include other biguanil derivatives, triazines, and quinazoline analogs. Point mutations affecting one inhibitor might be countered in some cases by alternative antifolate drugs capable of acting against the mutant enzyme. However, it is already clear that multiply-resistant malaria strains are likely to spread with exposure to different antifolate drugs. Future chemotherapy with antifolate drugs will require knowledge of the distribution of resistant strains and discovery of new inhibitors that are effective against them.

Independent but concurrent work by Foote *et al.* (36), using different lines of *P. falciparum*, has shown comparable linkage of the DHFR point mutations to cycloguanil and pyrimethamine resistance.

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