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Cycloguanil and Its Parent Compound Proguanil Demonstrate Distinct Activities against *Plasmodium falciparum* Malaria Parasites Transformed with Human Dihydrofolate Reductase

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ABSTRACT

The lack of suitable antimalarial agents to replace chloroquine and pyrimethamine/sulfadoxine threatens efforts to control the spread of drug-resistant strains of the malaria parasite *Plasmodium falciparum*. Here we describe a transformation system, involving WR99210 selection of parasites transformed with either wild-type or methotrexate-resistant human dihydrofolate reductase (DHFR), that has application for the screening of *P. falciparum*-specific DHFR inhibitors that are active against drug-resistant parasites. Using this system, we have found that the prophylactic drug cycloguanil has a mode of pharmacological action distinct from the activity of its parent compound proguanil. Complementation assays demonstrate that cycloguanil acts specifically on *P. falciparum* DHFR and has no other significant target. The target of proguanil itself is separate from DHFR. We propose a strategy of combination chemotherapy incorporating the use of multiple parasite-specific inhibitors that act at the same molecular target and thereby maintain, in combination, their effectiveness against alternative forms of resistance that arise from different sets of point mutations in the target. This approach could be combined with traditional forms of combination chemotherapy in which two or more compounds are used against separate targets.

The rapid spread of *Plasmodium falciparum* strains that are resistant to chloroquine and pyrimethamine/sulfadoxine (Fansidar) underscores the need for pharmacological initiatives to counter the resulting increases in malaria mortality and morbidity rates. New antimalarial agents in such initiatives may be derived as novel compounds or modifications of existing drugs. One prophylactic drug that has undergone a resurgence of interest for use against malaria is the biguanide proguanil (Paludrine); this compound is cyclized by hepatic cytochrome P450 isoenzymes to the active metabolite cycloguanil, which has been reported to act on *P. falciparum* DHFR (EC 1.5.1.3). This enzyme, which in *P. falciparum* is fused with TS as a homodimeric bifunctional protein, catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate, thus providing a source of one-carbon donors for methyl transfer reactions and dTMP synthesis required for parasite survival.

Evidence that cycloguanil acts on *P. falciparum* DHFR comes from the findings of associations between the activity of this metabolite and parasite DHFR point mutations, as well as from more recent inhibition and binding studies with *P. falciparum* DHFR variants expressed in *Escherichia coli* (Foote *et al.*, 1990; Peterson *et al.*, 1990; Sirawaraporn *et al.*, 1997). The possibility that this metabolite may also have activity against a second target has been raised by reports that levels of susceptibility to cycloguanil could vary up to 8-fold among parasite isolates encoding identical DHFR sequences (Foote *et al.*, 1990; Basco *et al.*, 1995). The presence of a secondary target has also been proposed to explain the finding that cycloguanil-induced depletion of dTTP pools was not readily reversed by folinic acid (Yeo *et al.*, 1997). Recently, we found that intraparasitic expression of MTX-resistant human DHFR (which is innately resistant to antimalarial agents and can overcome the metabolic block resulting from inhibition of the parasite DHFR enzyme) resulted in a 10-fold increase in resistance to cycloguanil, as opposed to the >1000-fold increase in resistance to the DHFR inhibitors MTX and WR99210 (Fidock and Wellems, 1997). This discrepancy could have resulted either through the action of cycloguanil on a secondary target whose inhibition could not be complemented by human DHFR or because these assays were performed on a parasite line (FCB) harboring the DHFR mutations Val16 and Thr108, a pair of mutations that render the parasite enzyme resistant to cycloguanil and thus

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ABBREVIATIONS: DHFR, dihydrofolate reductase; TS, thymidylate synthase; MTX, methotrexate; RBC, red blood cell(s); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

might minimize the protective effect of the resistant human enzyme.

Here we report molecular complementation assays that address the role of parasite DHFR in relation to cycloguanil and its parent compound. These studies use a new system for transformation, in which *P. falciparum* parasites expressing either the wild-type or the MTX-resistant L22Y form of human DHFR are selected with the dihydrotriazine WR99210. Results clearly distinguish separate modes of pharmacological action for proguanil and cycloguanil, demonstrate that the only significant action of cycloguanil is against parasite DHFR, and may suggest important avenues of investigation into classes of lead compounds that could be used in combination against these targets.

Materials and Methods

Plasmid constructs. Wild-type, human, full-length *dhfr* cDNA was amplified from the pDS5+wt construct (a kind gift from Raymond Blakley, St. Jude's Hospital, Memphis, TN) using the primers 5′-ccttttttatgcatggttcgctaaactgcatcg and 5′-aatttcaagcttaatcattcttctcatatacttc. After cloning into the pCR2.1 vector (Invitrogen, Carlsbad, CA), this 0.6-kilobase gene was inserted as a *Nsi*I/*Hin*dIII fragment in the place of the luciferase gene in the pHLH-1 construct (Wu *et al.*, 1995), yielding pHDWT. The *dhfr* sequences in both pHDWT and the previously reported construct pHD22Y (Fidock and Wellems, 1997) contain silent mutations at nucleotide positions 90 and 96 (codons 29 and 31; Genbank accession number V00507), which result in loss of an *Eco*RI restriction site. For transfections, plasmid DNA was purified over two cesium gradients or Qiagen Maxiprep columns (Chatsworth, CA) and concentrated on a Centricon-100 column (Amicon, Beverly, MA) in incomplete Cytomix (120 mM KCl, 0.15 mM $CaCl_2$, 2 mM EGTA, 5 mM $MgCl_2$, 10 mM K_2HPO_4/KH_2PO_4, 25 mM HEPES, pH 7.6).

Parasites. *P. falciparum,* asexual, blood-stage parasites, were propagated in leukocyte-free human RBC (at 4% hematocrit) in complete medium [RPMI 1640 with L-glutamine (catalog no. 31800; Life Technologies, Gaithersburg, MD), 50 mg/liter hypoxanthine, 10 mg/liter gentamycin, 25 mM HEPES, 0.225% $NaHCO_3$, and 0.5% Albumax I (Life Technologies)] and were grown at 37° in tissue culture flasks gassed with 5% CO_2/5% O_2/90% N_2. Parasites were transfected using electroporator settings of 0.31 kV and 960 μF. These modified settings result in increased efficiency of transfection, relative to previous high-voltage/low-capacitance settings (Wu *et al.*, 1995), as measured using transient luciferase activity assays (Fidock and Wellems, 1997). Although these newer low-voltage/high-capacitance settings result in greater initial RBC lysis, they consistently result in a 1–2-day reduction in the number of days required to microscopically observe transfected parasites.

Electroporation was routinely performed on parasites 5–7 days after cryopreserved samples had been thawed and placed into culture, to benefit from the period of synchronicity frequently observed after thawing. When a predominance (≥80%) of early ring stages was obtained at 6–8% parasitemia, fresh complete medium was added and parasites were cultured for an additional 3 hr. Samples of 1×10^9 RBC were then washed in incomplete Cytomix and electroporated in 0.2-cm cuvettes with 100 μg of plasmid DNA. Uninfected RBC (3×10^9) and 20 ml of complete medium were added, and cultivation was continued in 75-cm^2 flasks (Corning, Corning, NY). This routinely led to ≥2% parasitemia with few gametocytes at 48 hr after transfection, at which time 10 nM WR99210 was added. The WR99210 concentration was lowered to 5 nM at 96 hr after transfection and was maintained thereafter at that level. Medium was changed daily for the first 6–8 days after transfection, to remove lysed cells and parasite debris, and was then changed every other day until ring-stage parasites could be microscopically detected. At day 10, 30% of each culture was discarded and the remainder was transferred to 25-cm^2 flasks, requiring 5 ml of complete medium.

Continuous culture of pHD22Y-transformed FCB parasites resulted in the slowly growing, episomally transformed parasites being gradually replaced by parasites in which this construct had been integrated into the nuclear genome. Cloning of these "integrant" parasites was initiated at day 145 after transfection by inoculation of 96-well tissue culture plates with 200 μl/well of 2% RBC in complete medium, containing an average of 0.5 infected RBC/ml. Medium was replaced at days 7 and 14, and clones were detected after 19 days using a sensitive parasite-specific lactate dehydrogenase assay (Goodyer and Taraschi, 1997).

Drug assays. MTX (Sigma Chemical, St. Louis, MO), WR99210 (a kind gift from David Jacobus, Jacobus Pharmaceuticals, Princeton, NJ), and cycloguanil and proguanil (kind gifts from Dennis Kyle and Wilbur Milhous, Walter Reed Army Institute of Research, Washington, DC) were maintained at −80° as 10 mg/ml stock solutions in dimethylsulfoxide (Sigma). The structures of these drugs are shown in Fig. 1. For the drug assays, serial 2-fold drug dilutions were made in complete medium modified to contain 2.5 mg/liter hypoxanthine ("low-hypoxanthine medium"). These dilutions were added to 96-well culture plates at 100 μl/well. Parasites were diluted to a 2-fold concentrated stock solution consisting of 0.5–1.0% parasitemia and 4% hematocrit in low-hypoxanthine medium and were added at 100 μl/well. After 48 hr of incubation, 100 μl of culture supernatants were replaced with 100 μl of low-hypoxanthine medium containing [^{3}H]hypoxanthine at a concentration of 7.5 μCi/ml. After an additional 24 hr, supernatants were removed, distilled water was added (200 μl/well), and the plates were frozen and thawed before cells were harvested onto glass fiber filters (Wallac, Turku, Finland). Air-dried filters were placed in sample bags (Wallac) and immersed in scintillation fluid (Ecoscint A; National Diagnostic, Atlanta, GA), and radioactive emissions were counted in a 1205 Betaplate reader (Wallac). Mean emission levels were generally in the range of 20,000–30,000 cpm. Percentage reduction in hypoxanthine uptake (a marker of growth inhibition) was calculated as follows: reduction = 100 × [(geometric mean cpm of no-drug samples) − (mean cpm of test samples)]/(geometric mean cpm of no-drug samples). These percentage reductions were used to plot data as a function of drug concentrations. IC_{50} values were determined by linear regression analyses of the linear segments of the curves.

All assays were performed in duplicate or triplicate on three separate occasions. Within each experiment, standard deviations were routinely <10% of the mean. Differences in stages of parasite development could lead to 15–30% shifts in the IC_{50} values among experiments; however, these differences did not affect the overall relationships among the parasite lines with respect to their different drug responses. In view of the small standard deviations within single experiments, error bars have been omitted from the data presented in Figs. 3–5.

Results

WR99210 selection of *P. falciparum* parasites transformed with human wild-type or MTX-resistant *dhfr* genes. We transformed the *P. falciparum* lines 3D7 (cycloguanil-sensitive) and FCB (cycloguanil-resistant) with plasmids expressing wild-type and MTX-resistant human DHFR, respectively. Plasmids pHDWT and pHD22Y differ only at codon 22 of the human *dhfr* gene, with the former encoding a wild-type leucine and the latter encoding a tyrosine residue responsible for MTX resistance. Expression of human DHFR in these plasmids is under the control of *P. falciparum* regulatory elements, namely the 5′-untranslated region of the *hrp3* gene and the 3′-untranslated region of *hrp2* (Fig. 2).

Human *dhfr*-transformed parasites, selected on the basis of their resistance to the *P. falciparum* DHFR inhibitor WR99210, were observed microscopically 18–22 days after transfection and were cultured for an additional 3 weeks before DNA extraction. Plasmids encoding human *dhfr* were "rescued" by electroporation of 0.1 μg of parasite DNA into *E. coli* (SURE strain; Stratagene, La Jolla, CA) and selection of resistant colonies on ampicillin-selective plates. Restriction mapping of rescued plasmids showed that they had not undergone rearrangement during the periods of parasite and bacterial replication (data not shown). Confirmation that these plasmids were episomally replicated by the parasite and were not merely residual DNA carried along as a result of the initial electroporation was obtained using the approach of Wu *et al.* (1996). Briefly, *P. falciparum* and *E. coli* DNA preparations were incubated with the methylation-dependent restriction enzyme *Dpn*I and analyzed by Southern hybridization. Results confirmed that the episomal DNA was not methylated and therefore had been replicated by *P. falciparum* and not by *E. coli* (data not shown).

Antifolate responses of human *dhfr*-transformed parasites. Parasites episomally transformed with either pHDWT (3D7/pHDWT) or pHD22Y (FCB/pHD22Y) were assayed for their susceptibility to WR99210 and MTX. As controls, we included nontransformed parasites (3D7 and FCB) and also parasites that had been previously derived from the episomally transformed lines and propagated in the absence of drug for 15 generations. This regimen is known to lead to the rapid outgrowth of parasites that have lost episomes (Wu *et al.*, 1996). Plasmid rescue from these cultures (3D7/no pHDWT and FCB/no pHD22Y) revealed >99% reduction in episome numbers, and polymerase chain reaction assays showed no detectable integration of the vector into the genome (data not shown).

Seventy-two-hour drug susceptibility assays showed that intraparasitic expression of either the wild-type or L22Y form of human DHFR conferred very high resistance to WR99210, with 664 nM being required to produce >90% growth inhibition of the transformed parasites. In all nontransformed control parasite lines, comparable inhibition was achieved with drug concentrations as low as 0.65 nM (Fig. 3A). These data confirm that parasite cytosolic expression of the resistant human DHFR enzymes was able to overcome the metabolic block of the parasite DHFR enzyme by WR99210 by generating sufficient tetrahydrofolate levels.

When tested against MTX, human wild-type DHFR-transformed parasites were found to have an IC_{50} of approximately 0.6 μM, compared with an IC_{50} of approximately 0.06 μM for nontransformed controls (Fig. 3B). This 10-fold change contrasted with the >1000-fold increase in the MTX IC_{50} (70 μM) observed in human L22Y DHFR-transformed parasites. The slight decrease in susceptibility to MTX observed in the human wild-type *dhfr*-transformed parasites could be explained by overexpression of the human DHFR enzyme from the strong episomal promoter (in this case, *hrp3*), a phenomenon that has also been observed with DHFR-TS-transformed *Toxoplasma gondii* (Reynolds and Roos, 1998). Indeed, we found that parasite lines episomally expressing *P. falciparum* DHFR-TS from this *hrp3* promoter do show a small decrease in MTX susceptibility (data not shown). Decreased MTX susceptibility of the 3D7/pHDWT line may also result from differences in the MTX affinities of the parasite

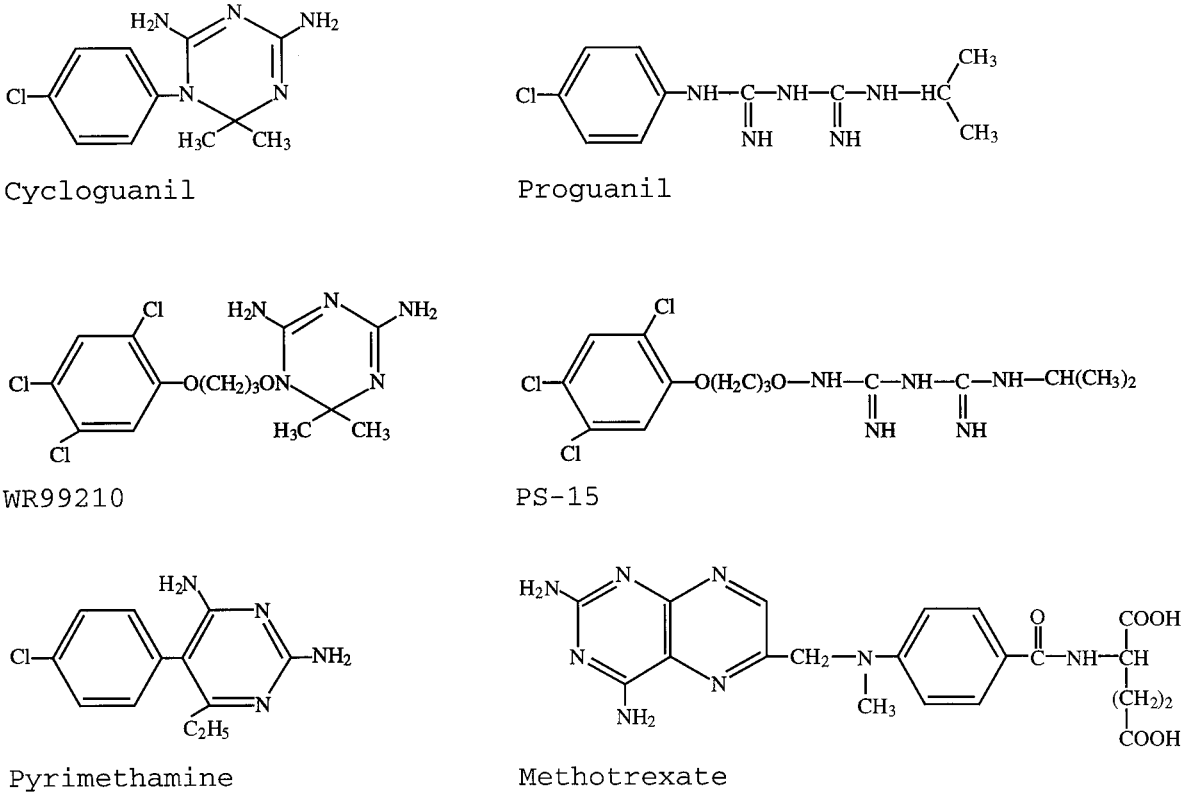

Fig. 1. Structures of agents active against *P. falciparum* and central to this study. Cycloguanil and WR99210 are the active metabolites of the biguanides proguanil and PS-15, respectively.

versus human DHFR enzymes. This would be consistent with findings that MTX is less active against rat DHFR than against DHFR from the rodent parasite *Plasmodium berghei* (Ferone *et al.*, 1969).

To measure the extent to which resistance levels were influenced by expression from episomes (estimated at 5–15 copies in each *P. falciparum* genome) (Crabb *et al.*, 1997), we tested the antifolate susceptibility levels of a cloned parasite expressing a single copy of the L22Y human *dhfr* gene integrated into its nuclear genome. This parasite (FCB/pHD22Y/integrant/A5-C8) demonstrated similar levels of WR99210 and MTX resistance, compared with two independently produced FCB lines in which L22Y DHFR was expressed from multicopy episomes (Fig. 4). This result indicates that resistance levels are dictated more by the structure and function of the human enzyme than by the copy number of this gene introduced into the parasite. We noted that the parasites containing multiple copies of human *dhfr* displayed dose-response curves that increased more gradually, with an evident reduction in hypoxanthine uptake at drug levels that were ineffective against the single-copy human *dhfr* clone. This is likely to reflect increased drug susceptibility of the minor proportion of parasites in the episomal cultures that lack the human *dhfr* construct, as a result of the unequal parsing and distribution of episomes during nuclear division and parasite segmentation. Such a phenomenon is consistent with the persistent presence of a minor proportion of pyknotic parasites in cultures of episomally transformed parasite cultures and the noticeable reduction in overall propagation rates under drug pressure.

Previous studies assessed the extent to which responses to pyrimethamine and cycloguanil are differentially mediated by combinations of point mutations at the DHFR active site (Cowman *et al.*, 1988; Peterson *et al.*, 1988, 1990; Foote *et al.*, 1990). Although WR99210 has been shown to be active against a variety of antifolate-resistant strains (Wooden *et al.*, 1997, and references cited therein), there are no published reports that comprehensively document the effect of DHFR mutations on susceptibility to this agent. We therefore tested the activity of WR99210 against strains representing the observed *dhfr* genotypes that affect residues at positions 16, 51, 59, 108, and 164 (Table 1). For parasites harboring zero to three DHFR mutations, WR99210 IC_{50} values were confined to a range of 0.08–0.71 nM, even though some of these variants could confer an up to 1000-fold decrease in susceptibility to either pyrimethamine or cycloguanil. The presence of the Asn108 variant alone resulted in a slight increase in susceptibility, compared with the wild-type allele, indicating collateral hypersensitivity, as previously noted (Wooden *et al.*, 1997). This hypersensitivity was not found in parasites carrying either the Ile51 plus Asn108 pair

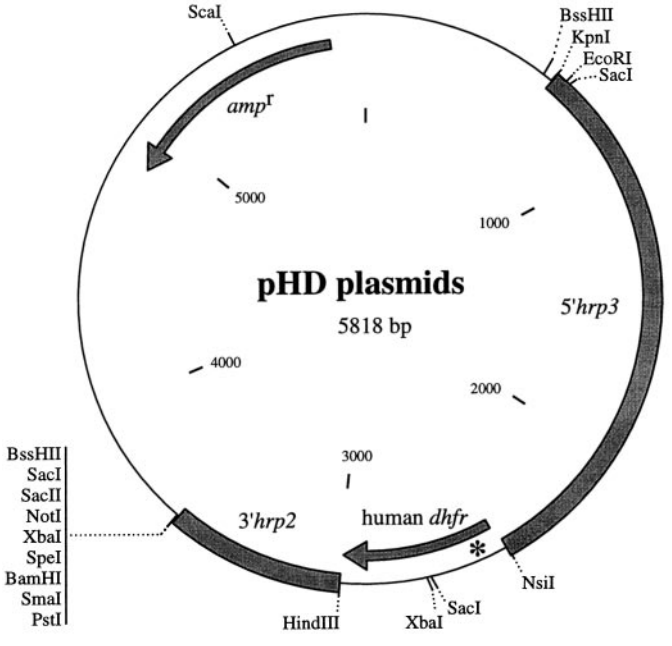

Fig. 2. Map of the human *dhfr* vectors pHDWT and pHD22Y used for transfection of *P. falciparum*. The L22Y mutation (*) that distinguishes these two vectors is located within the folate binding pocket and results in a >3000-fold decrease in MTX affinity in the presence of dihydrofolate and NADPH, while leading to only minimal decreases in catalytic efficiency at physiological pH (Lewis *et al.*, 1995).

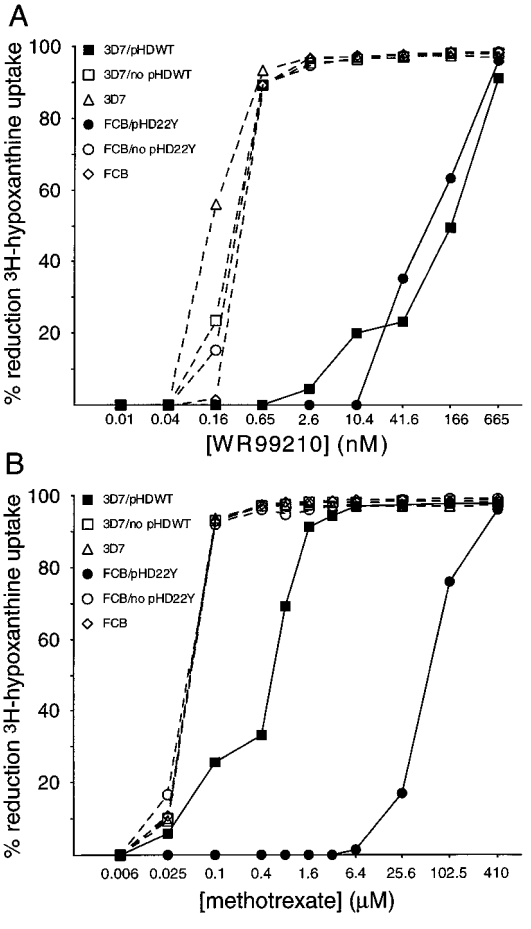

Fig. 3. Inhibition levels of *P. falciparum* lines transformed with human wild-type or L22Y *dhfr* plasmids (pHDWT or pHD22Y), measured as the percentage reduction of hypoxanthine uptake as a function of WR99210 (A) and MTX (B) concentrations, shown on a logarithmic scale (log_4). The episomally transformed lines 3D7/pHDWT and FCB/pHD22Y were maintained under continuous drug pressure for 2 months after DNA electroporation. Nontransformed parasites and lines cured of episomes (3D7/no pHDWT and FCB/no pHD22Y) were included as controls. Results presented in Figs. 3–5 were obtained in single representative experiments. In repeated experiments, variations of up to 30% were observed in absolute IC_{50} values, without affecting the relative values for the different parasite lines (see Materials and Methods). At the single WR99210 drug concentration of 0.16 nM, the nontransformed and cured 3D7 lines showed variation that was not observed in repeated experiments or in the response to MTX.

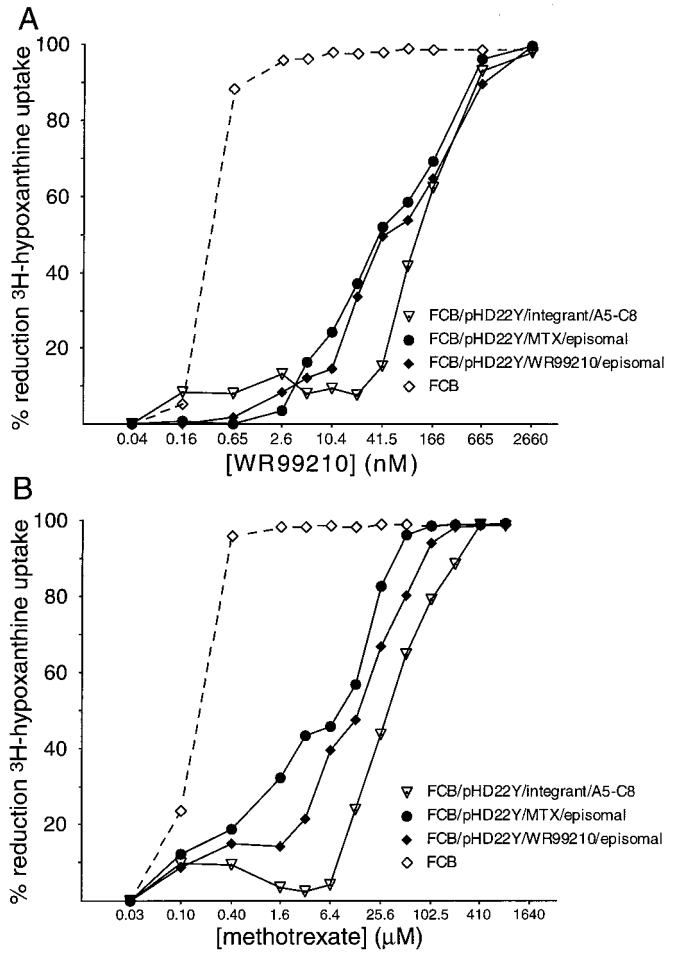

Fig. 4. Inhibition levels of parasites expressing human DHFR from genome-integrated, single-copy genes or episomally based, multicopy genes, as a function of WR99210 (A) and MTX (B) concentrations, shown on a logarithmic scale ($\log_4$). The clone FCB/pHD22Y/integrant/A5-C8 expresses a single copy of the L22Y human *dhfr* gene from a chromosomal location, after recombination and integration of pHD22Y into the nuclear genome. Polymerase chain reaction and Southern hybridization assays demonstrated the absence of episomally replicating plasmids in this clone. Drug responses were tested in parallel in the independently generated control lines FCB/pHD22Y/MTX/episomal and FCB/pHD22Y/WR99210/episomal, which had been transfected with the pHD22Y vector 1 month earlier and selected using MTX and WR99210, respectively. These cultures were confirmed to be episomally replicating the human *dhfr* construct, as shown by plasmid rescue and Southern hybridization assays (data not shown).

or the highly cycloguanil-resistant pair of Val16 plus Thr108. We note that a 5–6-fold decrease in susceptibility was observed in parasites harboring the Arg59 variant residue, a mutation that is found in combination with the Asn108 variant (Cowman *et al.*, 1988; Peterson *et al.*, 1988). Studies of mutant DHFR forms in *E. coli* and *T. gondii* indicate that this Arg59 variant retains high catalytic efficiency and significantly enhances the resistance to pyrimethamine or cycloguanil of moderately resistant alleles, although by itself it confers no resistance to these two antifolates (Sirawaraporn *et al.*, 1997; Reynolds and Roos, 1998). For the V1/S strain, which expresses a quadruple-mutant DHFR including the Leu164 mutation, the WR99210 IC_{50} was increased 35–40-fold, compared with the wild-type allele. We note, however, that this increase is much less than the 400-4000-fold increase in IC_{50} observed with pyrimethamine and cycloguanil (Foote *et al.*, 1990; Peterson *et al.*, 1990; Sirawaraporn *et al.*, 1997).

With respect to the transformation of *P. falciparum*, these data suggest that WR99210 selection (routinely initiated at 10 nM) should be widely applicable for parasite strains, regardless of native drug resistance profiles. Even for the rare quadruple mutant, human *dhfr* transformation should be possible, inasmuch as we have found selection to be successful using 25 nM WR99210. In theory, this new transformation system may also be applicable to parasites rendered pyrimethamine-resistant through prior genetic manipulation with constructs encoding protozoan pyrimethamine-resistant DHFR-TS enzymes (Crabb and Cowman, 1996; Wu *et al.*, 1996). The low MTX IC_{50} observed with pHDWT-transformed parasites may even make it possible to subsequently transform these parasites with pHD22Y-based constructs selected using high MTX levels, which would create significant flexibility for parasite studies requiring multiple sequential genetic manipulations.

Cycloguanil, but not proguanil, targets *P. falciparum* DHFR. We tested the effect of the metabolite cycloguanil and its parent compound proguanil on human DHFR-transformed 3D7 parasites. Results showed that, whereas 3D7 and the control line 3D7/no pHDWT (transformed but subsequently cured of its episomes) were sensitive to cycloguanil, with IC_{50} values of 2 nM, the IC_{50} of the human *dhfr*-transformed 3D7 line was 7 μM (an increase of 3500-fold) (Fig. 5A). This indicates a very high degree of protection

TABLE 1

WR99210 susceptibility of *P. falciparum* lines differing in their DHFR sequences and antifolate phenotypes

Values are mean ± standard deviation. Data shown are from a representative experiment that was performed in triplicate. Results were reproduced in two additional experiments. Three degrees of susceptibility to WR99210 were observed, namely the highly susceptible group of 3D7, HB3, 7G8, and FCB, the less susceptible group of K1 and Dd2 (characterized by the presence of the Arg59 residue), and the least susceptible, quadruple-mutant, parasite V1/S.

Strains[a]	DHFR amino acid residue					WR99210 susceptibility	
	16	51	59	108	164	IC_{50}	IC_{90}
						nM	
3D7	Ala	Asn	Cys	Ser	Ile	0.12 ± 0.01	0.19 ± 0.01
HB3 (PYR-R)	Ala	Asn	Cys	**Asn**	Ile	0.08 ± 0.01	0.12 ± 0.01
7G8 (PYR-R)	Ala	**Ile**	Cys	**Asn**	Ile	0.28 ± 0.02	0.62 ± 0.06
FCB (CYC-R)	**Val**	Asn	Cys	**Thr**	Ile	0.23 ± 0.03	0.62 ± 0.08
K1 (PYR-R)	Ala	Asn	**Arg**	**Asn**	Ile	0.71 ± 0.04	1.30 ± 0.06
Dd2 (PYR-R, CYC-R)	Ala	**Ile**	**Arg**	**Asn**	Ile	0.64 ± 0.04	1.39 ± 0.03
V1/S (PYR-R, CYC-R)	Ala	**Ile**	**Arg**	**Asn**	**Leu**	4.66 ± 0.08	14.32 ± 0.46

[a] PYR-R, pyrimethamine-resistant; CYC-R, cycloguanil-resistant.

afforded by the human enzyme against the metabolic target of this drug; therefore, by inference, cycloguanil specifically targets *P. falciparum* DHFR to the exclusion of any other major target. Parallel tests on FCB parasites carrying the Val16 and Thr108 DHFR mutations, which correlate with cycloguanil resistance, revealed an IC_{50} of 0.5–0.6 μM in nontransformed controls, compared with an IC_{50} of 2.6 μM in human *dhfr*-transformed FCB parasites. This indicates that the FCB mutant DHFR enzyme is almost as resistant to cycloguanil as its human counterpart.

In contrast to the dramatic change in the cycloguanil response observed for 3D7 parasites transformed with human *dhfr*, no changes in susceptibility to proguanil were detected after transformation of 3D7 or FCB parasites (Fig. 5B). Indeed, the proguanil IC_{50} values for transformed and nontransformed lines were restricted to a range of 27–42 μM and 60–71 μM for 3D7 and FCB parasites, respectively. We note that these values are 10–40-fold higher than the IC_{50} values previously reported on the basis of hypoxanthine assays in culture medium depleted of folic acid and *p*-aminobenzoic acid (Canfield *et al.*, 1993).

These data establish the dual nature of proguanil activity *in vitro*, with the metabolite cycloguanil acting on *P. falciparum* DHFR and the parent compound acting on a unique target. Our finding that transformed parasites showed no changes in their levels of response to proguanil but acquired high-level resistance to cycloguanil argues that proguanil activity *in vitro* did not result from low-level conversion to cycloguanil. This agrees with earlier findings of a lack of metabolism of proguanil to cycloguanil *in vitro* (Watkins *et al.*, 1984). Dissociation of parasite responses to these two drugs was also evidenced here by the finding that FCB and 3D7 parasites showed 250- and 2-fold differences, respectively, in their susceptibilities to cycloguanil and proguanil.

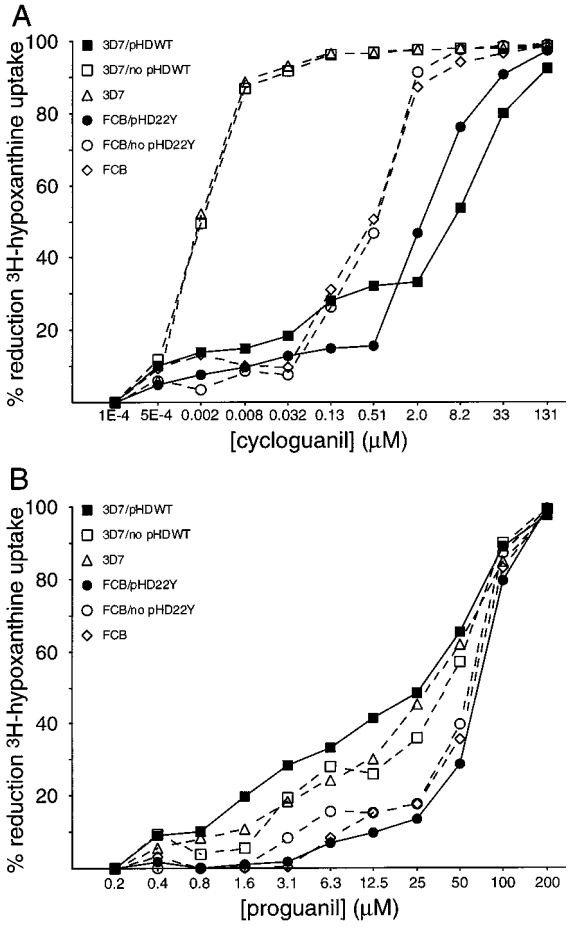

Fig. 5. Inhibition levels of cycloguanil-sensitive (3D7) versus -resistant (FCB) *P. falciparum* lines episomally transformed with human *dhfr*, as a function of cycloguanil (A) and proguanil (B) concentrations, shown on a logarithmic scale (log_4 and log_2, respectively). Control lines are the same as those described in the legend to Fig. 3.

Discussion

Using complementation assays based on expression of human DHFR in drug-sensitive malaria parasites, we show that the only significant antimalarial target of cycloguanil is parasite DHFR. These assays indicate a separate mode of action for the parent compound proguanil. Evidence in favor of a pharmacologically important role for the parent compound comes from early findings that it was considerably more effective than its cycloguanil metabolite in treating malaria in humans or monkeys, with subsequent work demonstrating that this was not attributable to pharmacokinetic differences (Schmidt *et al.*, 1952; Robertson, 1957; Smith *et al.*, 1961). This is confirmed by reports that the use of proguanil for antimalarial prophylaxis or treatment can be equally effective in individuals who do not metabolize proguanil to cycloguanil (Ward *et al.*, 1989; Mutabingwa *et al.*, 1993; Mberu *et al.*, 1995).

From these studies, we propose that proguanil acts as an antimalarial agent in its native form as well as in the form of its active metabolite cycloguanil. This dual activity helps to explain why the proguanil/atovaquone (Malarone) combination provides almost 100% efficacy in treating *P. falciparum* malaria in areas in which cycloguanil resistance can be high and administration of atovaquone alone results in very rapid selection of resistant parasites (Blanchard *et al.*, 1994; Looareesuwan *et al.*, 1996; Radloff *et al.*, 1996; Lell *et al.*, 1998). Such a "triple-drug" effect of proguanil/atovaquone may account for its usefulness against malaria in regions that already show a high prevalence of drug-resistant strains. Furthermore, the structure and dual activities of proguanil might serve as a valuable starting point for the development of new antimalarial agents that are effective as both therapeutic and prophylactic agents.

The WR99210/human *dhfr* transformation system described here has several advantages over previous methods of genetically modifying *P. falciparum* parasites, which have relied on the use of pyrimethamine or MTX to select parasites expressing pyrimethamine-resistant DHFR enzymes of protozoan origin or human MTX-resistant DHFR (Crabb and Cowman, 1996; Wu *et al.*, 1996; Fidock and Wellems, 1997). The pyrimethamine system is restricted, in that it cannot be used in selection schemes with parasites from field isolates and laboratory lines that are already resistant to this drug. MTX is highly toxic to mammalian cells (Huennekens, 1994) and can select nontransformed MTX-resistant *P. falciparum* mutants, even at high drug concentrations. In contrast, WR99210 remains effective against parasites having a range of DHFR point mutations associated with high-level resistance to pyrimethamine and/or cycloguanil. Furthermore, this drug has not selected resistant mutants in 15 independent transformation experiments (using the lines 3D7, FCB,

HB3, and Dd2, which differ at the *dhfr* locus). WR99210 also shows potential for *in vivo* selection studies. Indeed, we recently used this agent to select human *dhfr*-transformed rodent *P. berghei* parasites *in vivo* (de Koning-Ward T and Fidock D, unpublished observations). More generally, the proven efficacy of WR99210 or its prodrug PS-15 against the opportunistic pathogens *T. gondii*, *Pneumocystis carinii*, and *Mycobacterium avium* complex (Hughes *et al.*, 1993; Meyer *et al.*, 1995; Brun-Pascaud *et al.*, 1996) raises the possibility that this WR99210/human *dhfr* system might be broadly applicable to genetic manipulation of infectious microorganisms that are susceptible to this agent.

The transformation system described here provides a new approach for screening antimalarial compounds. Assays using this system could be used to identify lead DHFR inhibitors, whose specificity for parasite DHFR would be indicated by the relative drug activities against nontransformed versus human *dhfr*-transformed parasite lines. This would provide valuable preliminary information on the therapeutic value of these compounds. Use of a set of *P. falciparum* lines encompassing the known range of *dhfr* variants would also allow measurements of activity against drug-resistant strains. These assays have the advantage of directly testing compounds against parasites expressing native DFHR and enable comparisons of activities against parasite and human DHFR enzymes in the same host cell. This provides an alternative to systems that promote screening using recombinant DHFR enzymes expressed in *E. coli* or *P. falciparum dhfr*-transformed yeast (Brobey *et al.*, 1996; Wooden *et al.*, 1997).

For combination chemotherapy, we propose that one promising strategy is to combine multiple parasite-specific DHFR inhibitors that interact with different residues surrounding the active site, to form multiple hits on the same target enzyme. This approach would complement traditional combination chemotherapeutic strategies that advocate the use of two or more compounds that act on separate targets to produce additive or (preferably) synergistic effects. The rationale for combining multiple compounds that act against *P. falciparum* DHFR comes from the findings that different patterns of resistance to individual DHFR inhibitors arise from separate sets of mutations affecting the folate substrate pocket (Cowman *et al.*, 1988; Peterson *et al.*, 1988, 1990; Foote *et al.*, 1990; Sirawaraporn *et al.*, 1993, 1997). As a consequence, certain combinations of DHFR inhibitors (for example, WR99210 and cycloguanil) display little or no cross-resistance (Toyoda *et al.*, 1997; Wooden *et al.*, 1997). Furthermore, it is known that particular combinations of drug-resistant point mutations can totally ablate parasite DHFR enzyme function *in vitro* (Sirawaraporn *et al.*, 1997; Reynolds and Roos, 1998), suggesting that these combinations would be excluded from natural parasite populations. Thus, the combination of multiple DHFR inhibitors such as cycloguanil and WR99210 (or improved derivatives) might be an effective strategy to counter the spread of drug-resistant *P. falciparum* by preventing the selection of multidrug-resistant DHFR enzymes. This approach might be particularly effective in Africa, where cycloguanil-resistant *dhfr* genotypes have not been found in extensive molecular epidemiological investigations (Basco *et al.*, 1995; Plowe *et al.*, 1997; Wang *et al.*, 1997). Such a strategy could be incorporated into a combination chemotherapeutic approach that attacks multiple targets, including the target of proguanil and DHFR or other enzymes involved in the folate biosynthetic pathway, in a manner analogous to that of triple- or quadruple-drug combinations now being used to treat human immunodeficiency virus or tuberculosis infection (Lipsky, 1996; Reichman, 1996). This calls for a change in thinking about the treatment of malaria in regions harboring multidrug-resistant *P. falciparum* strains.

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