Conflicting Requirements of *Plasmodium falciparum* Dihydrofolate Reductase Mutations Conferring Resistance to Pyrimethamine-WR99210 Combination[▽]

Deanpen Japrungr, Ubolsree Leartsakulpanich, Sudsanguan Chusacultanachai,
and Yongyuth Yuthavong*

BIOTEC, National Science and Technology Development Agency, 113 Paholyothin Road, Klong 1, Klong Luang,
Pathumthani 12120, Thailand

Received 3 May 2007/Returned for modification 28 July 2007/Accepted 6 September 2007

Plasmodium falciparum strains bearing quadruple mutations of dihydrofolate reductase-thymidylate synthase (PfDHFR-TS) at codons 51, 59, 108, and 164 are highly resistant to pyrimethamine (PYR), a diamino-pyrimidine, but sensitive to WR99210 (WR), a cycloguanil analog, suggesting different enzyme-inhibitor binding interactions. A combination of these inhibitors to delay the onset of antifolate resistance is proposed. Using error-prone PCR, libraries of random mutants of wild-type PfDHFR and PfDHFR-TS were generated and used to transform *Escherichia coli*, and transformants were then selected for PYR or WR resistance. Mutants highly resistant to either PYR or WR were also generated from libraries obtained from further random mutagenesis of quadruple mutants (QM) with mutations in PfDHFR or PfDHFR-TS. For reversion mutants carrying altered residues I51N, N108S, and L164I, a further mutation of D54N was required to achieve resistance against WR, but these mutants regained sensitivity to PYR. When a combination of PYR and WR was used, fewer resistant mutants were generated from both mutant libraries using the QM gene templates. The effectiveness of the drug combination in reducing the appearance of resistance mutations is likely due to conflicting requirements for mutations conferring resistance to the two drugs. Thus, a combination of inhibitors from these two drug classes should be effective in impeding the emergence of *P. falciparum* resistance to antifolates.

The global burden of malaria has worsened due to the widespread resistance of the parasites to almost all currently used antimalarial drugs. *Plasmodium falciparum* has shown increasing resistance to antifolate drugs, such as pyrimethamine (PYR) (13, 14, 16, 19), an inhibitor of parasite dihydrofolate reductase (DHFR) that is normally used in combination with a sulfa drug (3, 5, 12, 18). The mechanism of PYR resistance is due to mutations in the DHFR domain (15, 16, 19) of the bifunctional DHFR-thymidylate synthase (DHFR-TS) enzyme. The most common mutation is S108N, which, when accompanied by the N51I, C59R, and I164L mutations, leads to increasing levels of resistance (1, 6, 10). The X-ray structure of the double-mutant (S108N C59R) *P. falciparum* DHFR-TS (PfDHFR-TS) complexed with PYR provides an explanation: the N108 side chain of the mutant enzyme is in steric conflict with the *p*-Cl atom of the PYR molecule, resulting in decreased affinity of PYR for the mutant enzyme (21). On the other hand, the structure of the quadruple-mutant (QM) (N51I C59R S108N I164L) enzyme complexed with the inhibitor WR99210 (WR), an analog of cycloguanil, shows that the flexible side chain of this inhibitor allows avoidance of a potential steric clash with the N108 side chain, offering an explanation for the effectiveness of WR against PYR-resistant par-

asites (21). Although WR-resistant parasites have not been reported in the field, WR-resistant PfDHFR has been obtained in vitro through screening of random mutant PfDHFR libraries generated from the QM enzyme in a bacterial surrogate system (1). Interestingly, the WR-resistant mutants were mostly found to carry the reversion mutation N108S (1).

It has also been shown previously that PYR and WR exert opposing selection pressures on *Plasmodium vivax* DHFR (7). There are apparently conflicting requirements for the generation of resistant mutants against rigid inhibitors such as PYR and against more-flexible inhibitors such as WR. It is possible that a combination of both types of inhibitors can impede the selection of resistant mutants due to these conflicting requirements. We now show that a combination of PYR and WR can impede the selection of resistant mutants in *Escherichia coli* carrying PfDHFR or PfDHFR-TS libraries with random mutations in the DHFR domain, generated from either wild-type (WT) or QM genes. The combination of a rigid and a flexible inhibitor should be considered in the future deployment of antifolate antimalarials, since this strategy ought to impede the rapid development of resistance.

MATERIALS AND METHODS

Construction of random mutant libraries of the PfDHFR and PfDHFR-TS genes. pET17b plasmids containing synthetic PfDHFR-WT and PfDHFR-QM genes were used as templates for error prone PCR (1, 9, 20, 22). The reaction mixture contained 10 ng of DNA template, 125 ng of the sense primer (5'-GA AGGAGATATACATATGATGGAACAG-3'), 125 ng of the antisense primer (5'-GATCCGAGCTCGGTACCAAGCTTG-3'), 1 mM dCTP, 1 mM dTTP, 0.2 mM dATP, 0.2 mM dGTP, 1× PCR buffer (20), and 5 U of *Taq* DNA polymer-

* Corresponding author. Mailing address: BIOTEC, National Science and Technology Development Agency, 113 Paholyothin Road, Klong 1, Klong Luang, Pathumthani 12120, Thailand. Phone: 66-2-564-7000, ext. 3487. Fax: 66-2-564-7007. E-mail: yongyuth@biotec.or.th.

[▽] Published ahead of print on 17 September 2007.

ase. PCR was performed for 30 rounds of thermal cycling as follows: 95°C for 1 min for denaturation, 55°C for 1 min for annealing, and 72°C for 1 min for extension, with a final extension step of 72°C for 10 min. The amplified PCR product (approximately 700 bp) was digested with NdeI/HindIII at 37°C for 16 h and ligated into the corresponding sites of the pET17b expression plasmid at 16°C for 12 to 16 h. The products of the ligation reactions were used to transform *E. coli* DH5 α by the heat shock method (8).

Mutant libraries of bifunctional PfDHFR-TS were also constructed using pETpDHFR-TS-WT and pETpDHFR-TS-QM as templates, but mutations were allowed only in the DHFR domain. Error-prone PCR conditions were the same as those described above, except for the sense primer (5'-GCCAGCAAGCTTATGATGGAACAAGTCTGCGACGTT-3') and antisense primer (5'-TC TTGTTCATCATTCTTAAGAGGCATATCATTATTTT-3'), which contained HindIII and AflII restriction sites (underlined), respectively. The resulting products were cloned into the HindIII and AflII sites of the pETpDHFR-TS plasmid from which the DHFR gene had been removed.

Screening for PYR- or WR-resistant mutants. *E. coli* BL21(DE3) was transformed with the DNA libraries and plated onto LB agar containing 100 μ g ml⁻¹ ampicillin. The transformed cells were then cultured before screening as described previously (1, 9). To screen for resistance mutants, cultured cells were selected on minimal medium supplemented with 100 μ g ml⁻¹ ampicillin, 2 μ M trimethoprim, and antifolate at the MIC for a single drug or at half the MIC for drug combinations. In order to minimize the selection of false-positive clones, the MIC used in this study was the minimum antifolate concentration that completely inhibited the growth of bacteria carrying the PfDHFR gene. The MIC of WR and PYR that suppressed cells expressing PfDHFR-WT or PfDHFR-TS-WT was 0.5 μ M. To inhibit PfDHFR-QM cells, 500 μ M PYR and 50 μ M WR were used, and for inhibition of PfDHFR-TS-QM cells, 500 μ M PYR and 5 μ M WR were used. The number of surviving bacterial colonies on each selection plate was counted after overnight incubation. At least three separate experiments were performed. Approximately 30 surviving colonies from each screening were randomly picked for DNA sequencing; in cases where the number of surviving colonies was less than 30, all colonies were analyzed.

Purification and characterization of mutant enzymes. Mutants were expressed in BL21(DE3) and purified using methoxyxate-Sepharose affinity chromatography (1), and the kinetics of DHFR were determined as reported previously (14). The protein concentration was determined as described previously (11). For determination of the K_s of PYR and WR, the standard assay reaction mixture (200 μ l) contained 50 mM *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES; pH 7.0), 7.5 mM 2-mercaptoethanol, 100 μ M dihydrofolate, 100 μ M NADPH, 1 mg/ml of bovine serum albumin, 0.005 U of the enzyme, and varying inhibitor concentrations. The reaction was performed in a 0.5-cm-diameter well and monitored using a microplate spectrophotometer (iEMS reader MF; LabSystems, Finland). K_s s were calculated using a nonlinear least-square fit of the data to a Michaelis-Menten equation, assuming that inhibitors bind competitively to the enzyme active site. The 50% inhibitory concentration (IC₅₀) was determined by initiating the reaction with 0.008 to 0.01 U of enzyme in the presence of varying amounts of inhibitor (9).

RESULTS

Selection of mutants from WT PfDHFR and PfDHFR-TS libraries by bacterial complementation. In an effort to mimic the impact of drug pressure on the selection of DHFR mutations in the malaria parasite population, we constructed several libraries of PfDHFR mutants using templates bearing WT and QM sequences of either monofunctional or bifunctional genes. Analyses of 30 randomly selected clones from each library indicated an unbiased distribution of mutations within the DHFR gene with a 0.8% mutation frequency. BL21(DE3) cells transformed with the mutant library generated from PfDHFR-WT were screened on minimal medium supplemented with 2 μ M TMP, a potent bacterial DHFR inhibitor, and in the presence of PYR or WR at the MIC for complete inhibition of the PfDHFR-WT template. Under these conditions, only bacteria possessing antifolate resistance survive. The concentration of TMP used is the minimal necessary, so that it is unlikely that the antimalarial effect of TMP would

TABLE 1. Numbers of antifolate-resistant *E. coli* colonies generated from screening of the PfDHFR-TS-QM and PfDHFR-QM libraries^a

Inhibitor for selection	No. of colonies	
	PfDHFR-TS-QM	PfDHFR-QM
PYR	2,398 $\pm$ 380	19,866 $\pm$ 15,724
WR	3 $\pm$ 2	562 $\pm$ 206
PYR + WR	0	0

^a Libraries were screened against PYR and WR at their MICs and against a combination of PYR and WR at half the MIC of each inhibitor. Values are averages from at least three independent experiments performed in triplicate.

confound the roles of the antifolate antimalarials PYR and WR in the selection process. To confirm the validity of the newly generated libraries and the validity of the bacterial surrogate system, single-drug selection of PYR or WR was used. The PYR-resistant mutants had either a single S108T mutation, double S108T F136S mutations, or quadruple N51I C59R S108N I164L mutations, while the WR-resistant mutants had either a single S108T or C59R mutation, triple C59R S108N F136S mutations, or quintuple E25V N51I C59R S108N I164L mutations. Thus, the results from single-drug selection were consistent with those of our previous study (1) in that the majority of mutants resistant to PYR and WR contained either the natural single mutation of S108T or C59R or multiple mutations of N51I, C59R, S108N, and I164L. In summary, combinations of naturally occurring mutations with novel mutations including E25V and F136S were thus obtained. Natural mutations were also found in selections from the bifunctional PfDHFR-TS-WT library. These observations indicate that this system can simulate the natural selection process.

Selection of mutants from QM random libraries. Because DHFR-TS-QM mutation exhibits the highest degree of antifolate resistance developed in the field and is widespread in many areas of endemicity, a similar selection was performed using PfDHFR-QM and PfDHFR-TS-QM mutant libraries in order to compare mutants selected against either PYR or WR alone and against a combination of the two drugs. Whereas PYR pressure resulted in large numbers of resistant mutants from both QM libraries, WR pressure produced far fewer surviving colonies (3 versus 2,398 for the PfDHFR-TS-QM library and 582 versus 19,866 for the PfDHFR-QM library) (Table 1). It is noteworthy that selection using the PfDHFR-QM library resulted in a much higher number of resistant colonies than that using the PfDHFR-TS-QM library. Selection using a combination of the two drugs resulted in complete suppression of escape mutants (Table 1).

Resistant mutants generated under PYR and WR pressure. Amino acid changes of resistant mutants obtained from the PfDHFR-QM library selected under PYR and WR pressure were determined by sequencing 30 randomly selected surviving colonies. Fifty-six percent (17 out of 30) and 33% (10 out of 30) of the PYR-resistant mutants from the PfDHFR-QM and PfDHFR-TS-QM libraries, respectively, still contained the original four mutations. However, 11 and 7 novel mutants conferring PYR-resistance were found from libraries of PfDHFR-QM and PfDHFR-TS-QM, respectively (Table 2). In addition to the original QM, single mutations of D187A, K96N, C50Y, M92I, K28R, C50N, N144H, N90D, L73F, S22R,

TABLE 2. Identities and frequencies of resistant mutants from the PfDHFR-QM or PfDHFR-TS-QM library screened against PYR and WR at their MICs

Library	Mutation(s) of the PYR- or WR-resistant mutant ^a	Frequency ^b
Screening against PYR DHFR-QM	I150V N182I N201D	1
	D187A	1
	K96N	1
	C50Y	1
	M92I	1
	K28R	1
	K76G T85P	1
	C50N	1
	N144H	1
	N90D	1
	L73F	1
	QM	17
	E21D Y35F C50R	3
	S22R	1
	K49R E67G	1
	K114I	1
DHFR-TS-QM	E30G C50R	1
	V45G D91E V151A	1
	E21D Y35F C50R	1
	N121D	1
	QM	10
Screening against WR DHFR-QM	N108S	7
	N29S N108S	3
	N108T	1
	I51N N108S	2
	V8I K117R	2
	E21D	1
	K114E	1
	K19N K117R	1
	N29D	1
	C50S	1
	E30G N89S K117R	3
	N29S	1
	I51N N108S L164I D54N	1
DHFR-TS-QM		

^a Both PYR-resistant and WR-resistant mutants contain QM in addition to the mutations listed. Reversion mutations are boldfaced.

^b From 30 colonies.

and K114I were identified at frequencies of 1 or 2 out of 30. Moreover, novel mutations, I150V N182I N201D and K76G T85P, were obtained from selection of the PfDHFR-QM pool, with a frequency of 1 in 30 for each. In screening of the PfDHFR-TS-QM library under PYR selection, C50R was the dominant mutation, accounting for 16% of the colonies (5 out of 30), and was found in association with E30G, E21D Y35F, and E21D Y35F N121D mutations.

Among WR-resistant mutants, a reversion mutation of N108S was mostly detected from both libraries (13 out of 30 [45%] from the PfDHFR-QM library and 100% from the PfDHFR-TS-QM library) (Table 2). In addition, single N108T, double I51N N108S, and triple I51N N108S L164I reversion mutations were also identified, of which the latter was found in combination with the D54N mutation.

Based on these observations, it can be concluded that different sets of mutations are required to produce resistance to PYR and to WR and that mutations leading to PYR resistance at codons 51, 108, and 164 are incompatible with resistance to

WR. This is in line with the absence of surviving colonies when a combination of PYR and WR was used in the selection process (Table 1).

DHFR kinetic properties. In order to determine the effects of mutations that produced antifolate-resistance on DHFR properties, seven mutants were characterized: six mutants (QM with the N108S reversion mutation, QM with the N108T reversion mutation, QM with the double I51N N108S reversion mutations, QM D187A, QM K96N, and QM I150V N182I N201D) from the PfDHFR-QM library and one mutant (I51N C59R N108S L164I D54N) from the PfDHFR-TS-QM library. The majority of mutant enzymes showed k_{cat} values lower than that of the original QM for both monofunctional and bifunctional enzymes (Table 3). For PfDHFR-TS bearing the I51N C59R N108S L164I D54N mutation, which conferred WR resistance, k_{cat} was 100-fold lower. Because this mutant had very low activity, the K_m for substrates and the K_i of antifolates could not be determined; however, the IC_{50} s of the crude extract against PYR and WR were 42- and 250-fold higher, respectively, than those of the QM. For PfDHFR mutants with single (N108S) and double (N108S I51N) reversion mutations, the K_i values for WR were slightly higher than that of the QM enzyme. Interestingly, these reversion mutants showed K_i values against PYR 50- to 100-fold lower than that of the QM, clearly indicating that the WR resistance mutations could restore sensitivity to PYR.

The kinetic parameters of PYR-resistant mutants were also studied (Table 3). These mutant enzymes exhibited K_m values for DHF and NADPH similar to that of the QM and a K_i for PYR comparable to that of the QM, but these mutants were sensitive to WR.

DISCUSSION

The advantages of antifolate antimalarial chemotherapy are its high safety profile and cost-effectiveness, making it suitable for use in developing countries where the disease is endemic. However, its use has been radically limited by the rapid emergence of antifolate resistance, mainly caused by mutations in the DHFR gene (19). Although PYR and WR act at the same target, PfDHFR-TS, the X-ray structure of PfDHFR-TS revealed that the binding interactions between the inhibitors and the target enzyme are different (21). Also, it has been shown using a *Saccharomyces cerevisiae* surrogate system that PYR and WR exert opposing selection pressures on *P. vivax* DHFR (7), but the effect of drug combination in suppressing resistance has not hitherto been studied. The possibility of antifolate combination therapy to suppress the emergence of resistance had been suggested (4). This notion is now supported experimentally in this study, which shows clearly that a combination of the two drugs retards the selection of resistant mutants.

Although there are multiple mutations in the DHFR domain resulting in antifolate resistance, mutations in the TS domain have not been identified from field isolates. It is assumed that resistance mutations would occur mostly in the DHFR domain, and thus, only the DHFR region of the DHFR-TS gene was subjected to random mutagenesis. Libraries for mono- and bifunctional enzymes generated from the WT DHFR template were comparable to that of our previous study (1). Most of the

TABLE 3. Kinetic parameters and inhibition constants of purified PfDHFR and PfDHFR-TS

Resistance type ^a	Mutations	IC ₅₀ (μM) ^b		<i>k</i> _{cat} (1/s)	<i>K</i> _m (μM)		<i>K</i> _i (nM)	
		PYR	WR		DHF	NADPH	WR	PYR
PfDHFR-TS-QM template	N51I C59R S108N I164L	0.17 ± 0.04 (1)	0.16 ± 0.06 (1)	72	13.32 ± 1.39	6.43 ± 1.2	2.1 ± 0.3	209.3 ± 19.2
WR resist A	I51N C59R N108S L164I D54N	7.2 ± 2.3 (42)	40 ± 7.1 (250)	0.78	ND ^c	ND	ND	ND
PfDHFR-QM template	N51I C59R S108N I164L	0.42 ± 0.29 (1)	0.05 ± 0.005 (1)	44.5	14 ± 1 ^d	25 ± 6 ^d	1.9 ± 0.8 ^d	859 ± 117 ^d
WR resist 1	I51N C59R N108S I164L	0.09 ± 0.03 (0.2)	0.20 ± 0.05 (4.0)	0.2	8.3 ± 2.1	26.7 ± 4.15	3.57 ± 0.23	8.09 ± 1.9
WR resist 2	N51I C59R N108S I164L	0.01 ± 0.006 (0.02)	0.06 ± 0.02 (1.2)	1.5	5.2 ± 0.8	52.0 ± 3.7	7.26 ± 1.2	17.6 ± 1.7
WR resist 3	N51I C59R N108T I164L	1.82 ± 0.17 (4.3)	0.68 ± 0.09 (13.6)	1.9	5.0 ± 0.77	5.95 ± 1.6	15.5 ± 3.2	72.4 ± 21.4
PYR resist 1	N51I C59R S108N I164L D187A	0.87 ± 0.45 (2.1)	0.05 ± 0.002 (1.0)	13.6	14.53 ± 0.7	6.5 ± 1.2	1.24 ± 0.1	514.4 ± 51.2
PYR resist 2	N51I C59R S108N I164L K96N	2.48 ± 0.69 (5.9)	0.09 ± 0.02 (1.8)	28.3	8.1 ± 1.5	7.2 ± 1.0	0.85 ± 0.21	292.3 ± 74.7
PYR resist 3	N51I C59R S108N I164L I150V N182I N201D	3.47 ± 0.79 (8.3)	0.92 ± 0.12 (18.4)	11.4	14.65 ± 4.3	11.26 ± 1.17	1.07 ± 0.42	146.8 ± 22.7

^a The mutant with the WR resist A resistance type was selected against WR from screening of the PfDHFR-TS-QM library. Mutants with resistance types WR resist 1-3 and PYR resist 1-3 were selected against WR and PYR, respectively, from screening of the PfDHFR-QM library.

^b Numbers in parentheses are values (*n*-fold) relative to the value for the QM template.

^c ND, not determined due to low enzyme activity.

^d Data from Chusacultanaichai et al. (1).

mutants (S108T, C59R, and N51I C59R S108N I164L mutants) identified in PYR-resistant parasites from the field were also obtained in this study. Additionally, novel mutations, E25V and F136S, were selected in association with naturally occurring mutations.

Interestingly, WR-resistant mutants with the reversion mutations I51N, N108S, and L164I, separately or together, were often present in combination with novel mutations. All four mutations of the QM are located in the WR binding site (21) and are likely to change binding characteristics from that of the WT enzyme. In QM DHFR, WR is oriented in such a way as to avoid a steric clash with the side chain of N108 but still allows effective binding with the mutant enzyme. Nevertheless, in these reversion mutants, the WT sequences together with the new mutations could reduce the affinity for WR sufficiently to generate resistance. On the other hand, the N108S reversion should restore the affinity of PYR for the active site, since the steric conflict is reduced. Thus, it can be predicted that reversion mutations will restore sensitivity to PYR but do not exert a substantial effect on WR binding, except in the presence of novel mutations. It should also be noted that although the reversion at codon 59 has not been previously observed, this reversion is not unexpected, since the mutation of C59 to R probably serves mainly to enhance the binding of the substrate (21) so as to maintain enzyme activity that has been affected by the other mutations.

The D54N mutation in combination with C59R obtained from the PfDHFR-TS-QM library led to WR resistance but gave a very low enzyme activity. The D54N mutation has been observed in combination with A16V, S108T, and F223S (17). Combination of the D54E mutation with the A16V, S108T, and F223S mutations gives rise to high PYR resistance, but the D54N and D54N F223S mutants do not produce catalytically active enzymes (16). The D54N mutation has previously been

identified alone or in combination with other mutations during the screening of a PfDHFR-WT library for resistance to WR (1). Our results and those from earlier reports can be explained by the fact that the carboxyl group of D54 is important for both substrate and inhibitor binding and its function can be served, albeit much less well, by the amide group of N54 or the carboxylic group of E54.

PYR-resistant mutants from screening of a random QM library retained the quadruple mutations with additional mutations at amino acids remote from the active site, namely, QM D187A, QM K96N, and QM M92I. Some mutations, such as K49R, occurred in the predicted substrate-channeling groove of PfDHFR-TS (21). Although this amino acid may not directly interact with PYR, mutation at this position may enhance substrate transfer, thereby relieving the inhibitory effect of PYR binding.

The C50 mutation, found in our previous study (1) and in field-caught parasites (2), was also identified from the QM pool selected against the single drug PYR or WR. However, PYR-resistant mutants acquired diverse amino acids with larger side chains (R, Y, and N) at this position, whereas an amino acid with a similar size (S) was selected for the WR-resistant mutant, confirming the hypothesis of conflicting requirements for mutations conferring resistance to PYR and to WR.

It is noted that some selected mutants were different from those in the previous study (1), which is possibly due to the different concentrations of antifolates used in the selection process. Another point is the possibility that not every mutation acquired is directly responsible for the antifolate resistance property. Further studies to elucidate the role of an individual mutation are necessary before any conclusion can be drawn. Nevertheless, the finding of a prevalent mutation may indicate that the mutation occurs at an early cycle of an error-

prone PCR process. For example, in the PfDHFR-QM library resistant to WR, where N108S was commonly found, this change presumably occurred first and was then followed by I51N.

Additionally, when the overall composition of resistant mutations from each selection process was examined, selections using the PfDHFR monofunctional template gave rise to far more diverse mutations than those using the natural bifunctional template PfDHFR-TS. This indicates that the TS domain of the bifunctional molecule may restrict the conformation of its DHFR counterpart, making the latter less able to accommodate diverse changes.

In the screening of both WT and QM libraries, we consistently obtained a larger number of escape PYR mutants than of WR mutants. This may be due to the flexibility of the WR inhibitor, making it more difficult for the parasite to make steric adjustments in the enzyme in order to reduce the binding affinity to a flexible molecule. In other words, WR with its flexible side chain can accommodate itself to maintain tight binding affinity toward a number of DHFR mutants, whereas a more rigid compound such as PYR is unable to do so. This rationale is in line with the observation that the MIC of PYR determined in this study was much higher than that of WR.

Our study not only showed an absence of resistance mutants under selection by the PYR and WR combination but also found that there were different sets of mutations for resistance to each drug. This finding indicates that PfDHFR, either as a monofunctional or a bifunctional enzyme, has limited possibilities for generating mutations against a combination of inhibitors with different structures, each of which requires a specific set of mutations. Thus, the use of a suitable combination of antifolates, such as a rigid inhibitor (PYR) together with a more flexible compound (WR), should be effective against the development of resistance by the parasite and accordingly should be more effective in inhibiting the emergence of antifolate-resistant parasites. This strategy may be applicable to antifolate drug combinations other than PYR and WR as demonstrated in this study and should generally be valid for screening using other drug targets.

ACKNOWLEDGMENTS

This work was supported by MMV and the Wellcome Trust.

We thank Worachart Sirawaraporn and Jarunee Vanichthanankul for the gifts of the pETpfDHFR and pETpfDHFR-TS plasmids and Sumalee Kamchonwongpaisan for helpful suggestions. We gratefully acknowledge Tirayut Vilaivan for the gift of WR99210. We are also grateful to Darin Kongkasuriyachai and Prapon Wilairat for critical reading of the manuscript.

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