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Novel antifolate resistant mutations of *Plasmodium falciparum* dihydrofolate reductase selected in *Escherichia coli*

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Abstract

A simple and effective system has been developed from which a number of *Plasmodium falciparum* dihydrofolate reductase (pfDHFR) mutants conferring resistance to antifolates were randomly generated and characterized. The system exploited error-prone PCR to generate random mutations in the pfDHFR. Using the synthetic gene encoding for wild-type and quadruple mutant (N51I + C59R + S108N + I164L) pfDHFRs as templates, mutants resistant to pyrimethamine (Pyr), *m*-Cl analogue of Pyr (SO3) and WR99210 were selected by bacterial complementation system in which the endogenous DHFR activity of bacterial host cells, but not of *Plasmodium*, is selectively inhibited by trimethoprim (Tmp). Mutants conferring resistance to antimalarial antifolates were selected under the condition that inhibited the growth of the wild-type pfDHFR. All obtained Pyr resistant mutants possessed S108 mutation, in combination with common mutations of N51I, C59R and I164L previously found in the field. New Pyr resistant mutants with novel mutations (K27T, N121D, N144K and V213E) not found in the field were also identified. Exposure of the randomly mutated pfDHFR libraries to WR99210 or SO3 resulted in selection of novel single and multiple mutants including D54N, F58L and a combination of C50R, K181R, T219P and K227E, which exhibited 2- to over 2000-fold increase in resistance against antifolates. Kinetic analysis of these mutants suggested that apart from the active site residues that are crucial for DHFR activity, residues remote from the binding pocket also play essential roles in substrate and inhibitor binding.

Keywords: Dihydrofolate reductase; Antifolate-resistant mutants; Malaria

1. Introduction

The dihydrofolate reductase domain (pfDHFR) of *Plasmodium falciparum* bifunctional dihydrofolate reductase–thymidylate synthase (pfDHFR-TS) is a well-defined target of antifolate antimalarial drugs such as pyrimethamine (Pyr) and cycloguanil (Cyc). DHFR catalyzes the NADPH-dependent reduction of dihydrofolate (H_2folate) to regenerate tetrahydrofolate (H_4folate) required for one-carbon transfer reactions and deoxythymidylate synthesis of the parasites. During the past decade, considerable efforts have been devoted toward expression of this important target, with the goal to obtain the three-dimensional structure of the enzyme and rationally to design novel DHFR inhibitors as drug against the drug resistant malaria. Several groups have reported successful expression of the pfDHFR domain of the bifunctional enzyme pfDHFR-TS in *Escherichia coli* [1–4] and in yeast [5]. Evidence available thus far supports the conclusion that the kinetics and inhibition by antifolate inhibitors of the pfDHFR domain and the bifunctional pfDHFR-TS enzyme are comparable [2,3], rendering the former an attractive source for structural studies.

Abbreviations: Cyc, cycloquanil; DHFR-TS, dihydrofolate-thymidylate synthase; IPTG; iso-propyl-β-D-thiogalactopyranoside; MM, minimal media; MTX, methotrexate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PCR, polymerase chain reaction; pfDHFR, *Plasmodium falciparum* dihydrofolate reductase; Pyr, pyrimethamine; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Tmp, trimethoprim.

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Resistance of *P. falciparum* against antifolate antimalarials has previously been shown to be linked with mutations at amino acid positions 16, 51, 59, 108 and 164, the amino acids which are located in, or close to the active site of the enzyme [6–12]. Mutation from Ser108 (AGC) to Asn (AAC) is found in all Pyr-resistant parasites, and more resistant parasites carry additional mutations of Asn51 to Ile (N51I), Cys59 to Arg (C59R) and Ile164 to Leu (I164L). Mutations at residues 51, 59, 108 and 164 of pfDHFR have been reported to be involved in conferring resistance to both Pyr and Cyc, while mutation at residue 16 (Ala16 to Val; A16V) together with Ser108 to Thr (S108T) mutation is responsible for conferring specific resistance to only Cyc [10,11]. The observation that the double (N51I + S108N or C59R + S108N), triple (N51I + C59R + S108N) and quadruple (N51I + C59R + S108N + I164L) mutations are common in many regions of the world, including Southeast Asia where Pyr-sulfadoxine (Sdx) combination has not been extensively utilized [13–15], highlights the importance of these residues in antifolate resistance in malaria. Recent studies attempting to understand the effects of these point mutations on binding of Pyr and Cyc using a molecular model of the enzyme have shown the important role of steric constraints on inhibitor binding in generation of antifolate resistance in malaria [16–18].

In view of the tedious process required for study of the effects of enzyme mutations and their consequences on inhibitor binding, it is desirable to develop a heterologous system expressing pfDHFR for screening for enzyme activity and response to inhibitors. It is furthermore of interest to find out whether such a heterologous system would simulate parasite evolution of drug resistance in nature through mutations. While the natural mutations were shown to reduce the binding affinities of the inhibitors to the enzyme, they also frequently resulted in lower enzyme activities [2,19,20], which could lead to non-viability of the heterologous system. To maximize the opportunity to detect those low activity mutants, a heterologous system with high expression must be exploited. Indeed, it was found that a selection system using yeast complementation yielded mostly different mutant enzymes than those found in the field [21–24].

In this study, we report the development of a simple and powerful bacterial complementation system which allows the selection of pfDHFR mutants whose *dhfr* mutations contribute to antifolate resistance. Using error-prone PCR, we created libraries of randomly mutated *dhfr* genes of *P. falciparum* in transformed *E. coli*, using the synthetic gene for wild-type pfDHFR and its quadruple mutant that carries N51I + C59R + S108N + I164L mutation as templates. We demonstrate that under drug pressure and conditions which promote mutations, mutant pfDHFRs can be selected from these libraries. In addition to mutations found as for *P. falciparum* in the field, a number of other mutations that conferred resistance to antifolates, hitherto not found in nature, were identified. Starting from either wild-type or the quadruple pfDHFR mutant, mutations leading to resistance against two effective compounds, SO3 [16] and WR99210 were identified. Some of these mutant enzymes were expressed, purified and characterized.

2. Materials and methods

2.1. Materials

All restriction endonucleases and DNA modifying enzymes were obtained from New England Biolabs. The Qiaprep Spin Miniprep kits were from Qiagen. *E. coli* DH5α strain was used as general host strain for plasmid transformation and manipulation. The expression vector pET-17b and *E. coli* BL21(DE3) were the products from Novagen. Pyr, methotrexate (MTX), and NADPH were purchased from Sigma. WR99210 was a gift from Drs David Jacobus (Jacobus Pharmaceuticals, NJ, USA) and Tirayut Vilaivan, Chulalongkorn University, Thailand. The *m*-Cl Pyr analogue (SO3) was synthesized according to the procedure described previously [16] with some modifications [25]. MTX-Sepharose [26] and H_2folate [27] were prepared and used as described [2]. Oligonucleotide synthesis and DNA sequencing services were provided by BioService Unit, BIOTEC center, National Science and Technology Development Agency (NSTDA), Thailand. All other chemicals were of the highest purity commercially available.

2.2. Construction of residue 164 mutants

Saturated mutagenesis of the codon for amino acid 164 of pfDHFR was performed by 'replacement set' mutagenesis of the synthetic *pfdhfr* gene [2] using an oligonucleotide duplex designed to have degenerate codon (NNG) at residue 164. Briefly, an oligonucleotide pair, 5′-GATCTGATCGTTCTCCTAGGCAAACTGAACTACTACAAATGCTTCATC[NNG]GGTGGATCCGTTGTTTACCAGGAGTTCC-3′ and 5′-TCGAGGAACTCCTGGTAAACAACGGATCCACC[CNN]GATGAAGCATTTGTAGTAGTTC AGTTTGCCTAGGAGAACGATCA-3′, was first denatured by heating at 95 °C for 10 min. After gradual re-annealing at 37 °C, the annealed oligonucleotide duplex was ligated into the *Xho*1–*Bgl*II sites of pET–pfDHFR.

After incubation at 16 °C for approximately 12–16 h, the ligation product was transformed into *E. coli* DH5α and plated onto LB agar plates containing 100 μg ml^{-1} ampicillin. The bacterial colonies grown after incubation at 37 °C for 12–16 h were randomly selected, and the plasmid DNAs were extracted and cleaned up by Qiaprep Spin Miniprep kit according to the procedure recommended by the manufacturer. The DNA sequences of the clones were verified by automated DNA sequencing.

2.3. Random mutagenesis of pfdhfr gene using error-prone PCR approach

Error-prone PCR [28] was performed for 30 cycles. The reaction containing 10 ng of pET–pfDHFR plasmid DNA, 40 pmol of the sense primer (5′-GAAGGAGATATACATATGATGGAACAG-3′), 40 pmol of the antisense primer (5′-GATCCGAGCTCGGTACCAAGCTTG-3′), 1 mM dCTP, 1 mM dTTP, 0.2 mM dATP, 0.2 mM dGTP, 1XPCR buffer [28] and 5 units of *Taq* DNA polymerase. The conditions for the PCR cycle were as follows: 95 °C 1 minute denaturation, 55 °C 1 min annealing and 72 °C 1 min extension, with a final extension cycle of 72 °C for 10 min. The amplified PCR product (approximately 700 bps) was cleaned up, digested with *Nde*I/*Hind*III at 37 °C for 16 h, and ligated into the corresponding sites of pET-17b expression plasmid at 16 °C for 12–16 h. The product of the ligation was used to transform *E. coli* BL21(DE3)pLysS by electroporation.

2.4. Bacterial genetic selection and screening for drug resistant mutants

E. coli BL21(DE3)pLysS was transformed with pET–pfDHFR mutant libraries by electroporation at 2500 V, 25 μF, 200 Ω. The transformed cells were plated on LB agar plates containing 100 μg ml^{-1} ampicillin and 35 μg ml^{-1} chloramphenicol. For each screening, the cells grown on one plate were pooled, inoculated to minimal media (MM) containing 100 μg ml^{-1} ampicillin and 35 μg ml^{-1} chloramphenicol. The bacterial culture was allowed to grow until OD_{600} reached ∼0.6, when aliquots of 100 μl of the 1:10 dilution of the cultured cells were spread on MM agar plates containing 100 μg ml^{-1} ampicillin, 3 μg ml^{-1} chloramphenicol, 0.025 mM IPTG, 2 μM trimethoprim (Tmp) and varying concentrations (15–250 μM) of inhibitors to be screened. Preliminary experiments showed that 2 μM Tmp is sufficient to suppress endogenous DHFR activity completely. Colonies grown overnight at 37 °C were randomly picked. Plasmids were prepared and the DNA sequences were verified by DNA sequencing.

2.5. Expression and purification of mutant pfDHFRs

Fresh overnight culture from a single colony of *E. coli* BL21(DE3) pLysS harboring the selected mutant pET–pfDHFR plasmid was used to inoculate 1–6 l of LB containing 100 μg ml^{-1} ampicillin and 35 μg ml^{-1} chloramphenicol at 1% inoculum. The cultures were grown at 37 °C with shaking until OD_{600} reached ∼0.6, when IPTG was added at a final concentration of 0.4 mM to induce the expression. The cultures were allowed to grow at 16 °C with shaking for an additional 24 h prior to harvesting by centrifugation at $6000 \times g$ for 10 min at 4 °C. The cell pellets were disrupted by subject to two cycles of French press (18000 psi). After centrifugation at 15000 rpm for 1 h at 4 °C to remove cell debris, the clear lysate was applied onto MTX-Sepharose column. Purification of the mutant pfDHFRs was performed according to the procedure previously described [2].

2.6. Kinetic analysis and inhibition of pfDHFRs by antifolates

The activity of pfDHFR was determined spectrophotometrically by monitoring the decrease in absorbance at 340 nm at 25 °C [29]. Briefly, the assay reaction mixture (1 ml; 1-cm cuvette) consisted of 100 μM H_2folate, 100 μM NADPH, 50 mM TES, pH 5.0, 75 mM 2-mercaptoethanol, 1 mg ml^{-1} bovine serum albumin, and 0.006–0.007 units of enzyme. One unit of enzyme is defined as the amount of enzyme required to catalyze the reaction to produce 1 μmol of product min^{-1} at 25 °C. Steady-state kinetic determinations were performed as previously described [2] using Hewlett–Packard UV–VIS Spectrophotometer model 8453. Kinetic parameters were calculated using a non-linear least square fit of the data to the Michaelis–Menten equation, assuming the inhibitors bind competitively to the enzyme active site. The IC_{50} values were determined by initiating the reactions with 0.008–0.01 units of enzyme in the presence of varying amounts of inhibitors as previously described [2].

3. Results

Our aim in the present study is to develop a simple and yet powerful bacterial complementation system whereby pfDHFR mutants resistant to Pyr, WR99210 and SO3 (Fig. 1) could be screened and characterized. The system developed is based on the ability of expressed pfDHFR to complement and rescue *E. coli* cells whose endogenous DHFR was selectively inhibited by Tmp [1]. As a consequence, cell survival depends solely on the activity of heterologously expressed pfDHFR, rendering the system a simple one for selective screen-

ing of mutants expressing resistant pfDHFRs that are catalytically active.

3.1. Saturation mutagenesis of codon 164

As an initial step towards developing this system, we exploited the synthetic gene for pfDHFR [2] as a template for generating all possible mutations at amino acid residue 164. Mutants were selected and characterized for the activity of DHFR present in soluble extract and the ability to complement DHFR deficient BL21(DE3)pLysS in MM agar. The relative DHFR specific activities in crude extract from all 20 mutants are shown in Fig. 2A. Substitution of Ile164 by Ala (I164A) yielded a mutant whose DHFR activity was unperturbed and the enzyme was still as active as the wild-type pfDHFR. The I164V mutant pfDHFR was, however, still active but the DHFR activity was only approximately 60% of the wild-type enzyme. Mutation of I164 to amino acids whose side chains are small uncharged or hydrophobic, i.e. G, L, and S, yielded enzymes that were poorly active, and the enzyme activity was about 2–6% of the wild-type enzyme. The remaining mutants (T, C, M, N, Q, F, Y, W, K, R, H, D, E) yielded enzymes that were either inactive or much less active ($<1\%$) than the wild-type pfDHFR (Fig. 2A).

3.2. Bacterial complementation assay

In order to evaluate the sensitivity of bacterial complementation system in detecting mutants expressing low activity of DHFR, eight selected mutants (V164, G164, R164, H164, D164, M164, E164 and Q164) expressing varying DHFR activities ranging from 100 to 0.003% of the wild-type pfDHFR (Fig. 2A) were streaked on MM plates in the presence of 2 μM Tmp, which preferentially inhibits endogenous DHFR [1]. The ability of the mutants to grow under this selective condition was compared with that of the wild-type pfDHFR (I164). As shown in Fig. 2B, the wild-type (I164) and two mutants (V164 and G164) grew equally well while the remaining mutants (R164, H164, D164, M164, E164 and Q164) could not survive. The observation that the G164 mutant expressing pfDHFR at a level as low as approximately 1.8% of the wild-type clone could still complement the growth of *E. coli* host suggested the potential of the system as a tool for screening mutant pfDHFRs whose activity in the *E. coli* cell was presumably the minimal level required for the host to survive.

The ability to distinguish wild-type and Pyr-resistant pfDHFRs using bacterial complementation system was further evaluated in selective media in which Pyr was added. The *E. coli* BL21(DE3)pLysS cells harboring wild-type pfDHFR (pET–pfDHFR) [2] and Pyr-resistant quadruple mutant pET–pfDHFR (N51I + C59R + S108N + I164L) [19] corresponding to that found in the field, were streaked on selective agar plates with and without inhibitors. Fig. 3 shows the growth of bacteria harboring the three constructs on MM agar plate containing only ampicillin (Fig. 3A), MM agar plate containing 2 μM Tmp to inhibit bacterial DHFR (Fig. 3B), and MM agar plate containing 2 μM Tmp and 15 μM Pyr which is the concentration that was found to completely inhibit the wild-type pfDHFR (Fig. 3C). The results clearly showed that bacteria harboring pET-17b plasmid cannot survive under this selective condition unless there was substantial DHFR activity supplied from expression of the pfDHFR insert (Fig. 3B). The presence of 15 μM Pyr in MM agar plates containing 2 μM Tmp could clearly inhibit the growth of bacteria harboring wild-type pfDHFR, but allow the growth of bacteria harboring the quadruple mutant pET–pfDHFR (N51I + C59R + S108N + I164) (Fig. 3C).

To test whether the mutations generated by random mutagenesis and selected by bacterial complementation system could mimic natural evolution of resistance, Pyr-resistant mutants obtained by transforming *E. coli* BL21(DE3)pLysS with the random libraries of wild-type pfDHFR were selected from MM agar plates supplemented with 2 μM Tmp in the presence of 15 μM Pyr, and the DNA sequences were analyzed. Of 20000 colonies screened, 267 clones were found to be resistant to Pyr. Ten out of 267 clones were randomly selected

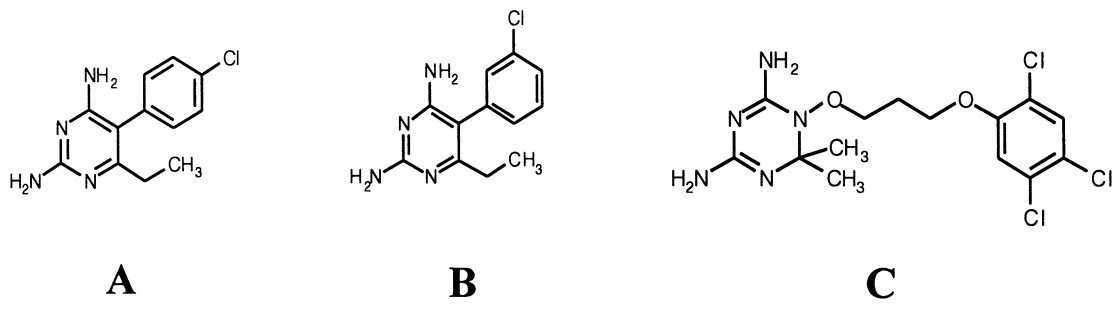

Fig. 1. Structures of antimalarial antifolates and analogues: (A) pyrimethamine; (B) *m*-Cl analogue of pyrimethamine (SO3); (C) WR99210.

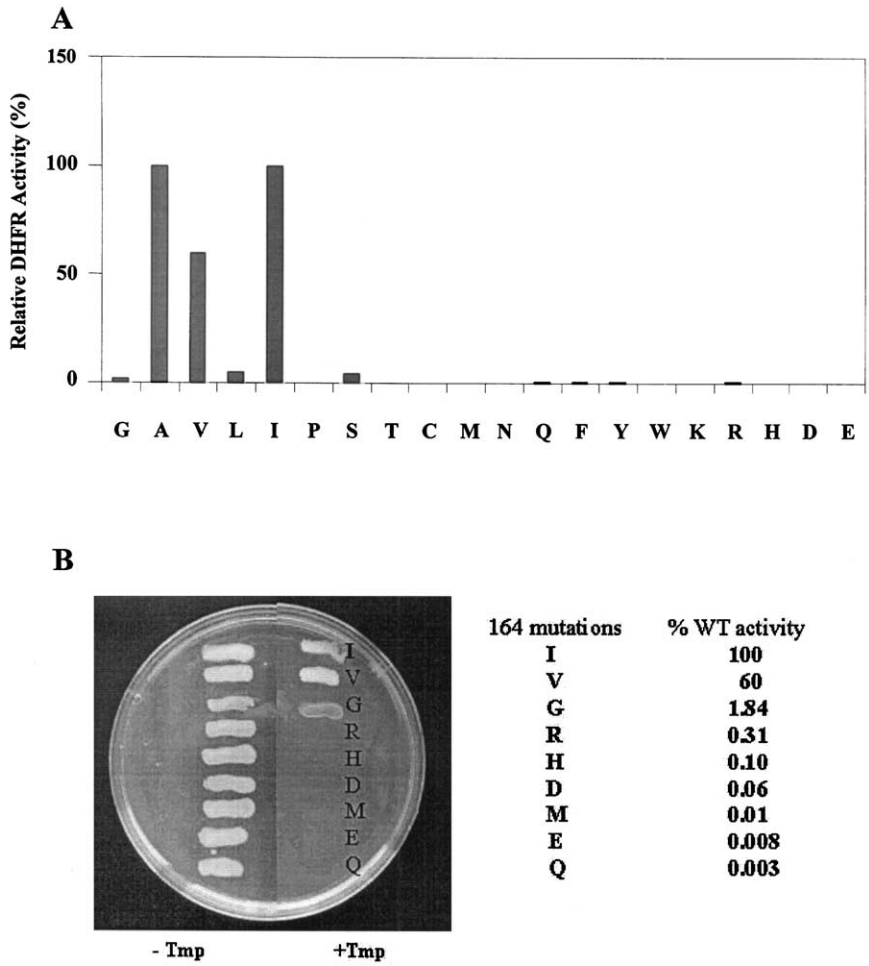

Fig. 2. *E. coli* BL21(DE3)pLysS harboring pET–pfDHFR mutant plasmids carrying all possible mutations at residue 164: (A) relative DHFR activity present in the crude extracts of bacteria; (B) growth of selected mutants (I, V, G, R, H, D, M, E, Q) on minimum agar plate containing 100 μg ml^{-1} ampicillin, 35 μg ml^{-1} chloramphenicol, with and without 2 μM Tmp. The activities of the mutant DHFRs estimated as percentages of wild-type DHFR activity were indicated at the right.

for further characterization. DNA sequence analysis of the 10 selected Pyr resistant mutants showed 1–5 mutations each (Table 1, blue). Interestingly, all ten mutants showed S108 change (Table 1, blue). This observation is remarkably in line with the fact that the S108 mutation plays a critical role in Pyr resistance and supports the theory that Pyr resistance occurred through stepwise mutations starting with S108N [19]. Apart from S108 mutations, a combination of other naturally occurring mutations were also identified. Mutants with N51I, C59R and I164L mutations appeared in the Pyr resistant mutants at frequencies of 2, 4 and 1 out of 10, respectively, as double (C59R + S108N), triple (N51I + C59R + S108N and C59R + S108N + N121D) and quadruple (N51I + C59R + S108N + N144K and N51I + C59R + S108N + I164L) mutants (Table 1, blue). This indicates that the selection system led to an outcome comparable to the natural selection process. In addition to the mutations already found in the field, additional novel mutations including K27T, N121D, N144K and V213E were also identified at frequencies of 3, 1, 1 and 1 out of 10, respectively (Table 1, blue).

3.3. Selection for SO3 and WR99210 resistant mutants

In naturally occurring Pyr- and Cyc-resistance, the mutant enzymes carried sets of mutations, which would confer resistance to different but closely related antifolates. As a consequence, cross-resistance to one or more antifolates occurred. In order to test whether mutations generated under selective pressure of different antifolates can lead to mutants that are cross-resistant against newly developed effective antifolates, and whether the quadruple mutant already found in nature would be prone to further mutations leading to mutants that are highly resistant to antifolate drugs, the wild-type pfDHFR and the quadruple mutant carrying N51I + C59R + S108N + I164L with high resistance were used as templates to generate mutated pfDHFR libraries. The libraries were transformed into *E. coli*

BL21(DE3)pLysS host cells and plated on minimal media containing 2 μM Tmp and either WR99210 or SO3. To minimize selecting the false positive clones, the concentration of each drug required for complete inhibition of the wild-type and quadruple mutant pfDHFRs was separately determined and used in the screening. The concentration of WR99210 found to be effective against the growth of *E. coli* expressing wild-type and quadruple mutant pfDHFRs was 15 μM. While SO3 at a final concentration of 15 μM was needed to inhibit the growth of bacteria expressing the wild-type pfDHFR effectively, up to 250 μM of the inhibitor was required in order to inhibit the quadruple mutant enzyme completely. Under the selective condition established, we screened mutants from both wild-type and quadruple-mutant pools. Of the total 20000 clones screened, 392 clones of WR99210-resistant mutants (256 from wild-type pool and 136 from quadruple-mutant pool) and 469 clones of SO3-resistant mutants (236 from wild-type pool and 233 from quadruple-mutant pool) were obtained. Twenty WR99210-resistant mutants (ten from wild-type pool and ten from quadruple-mutant pool) and twenty SO3-resistant mutants (ten from wild-type pool and ten from quadruple-mutant pool) were randomly selected for further analysis and characterization.

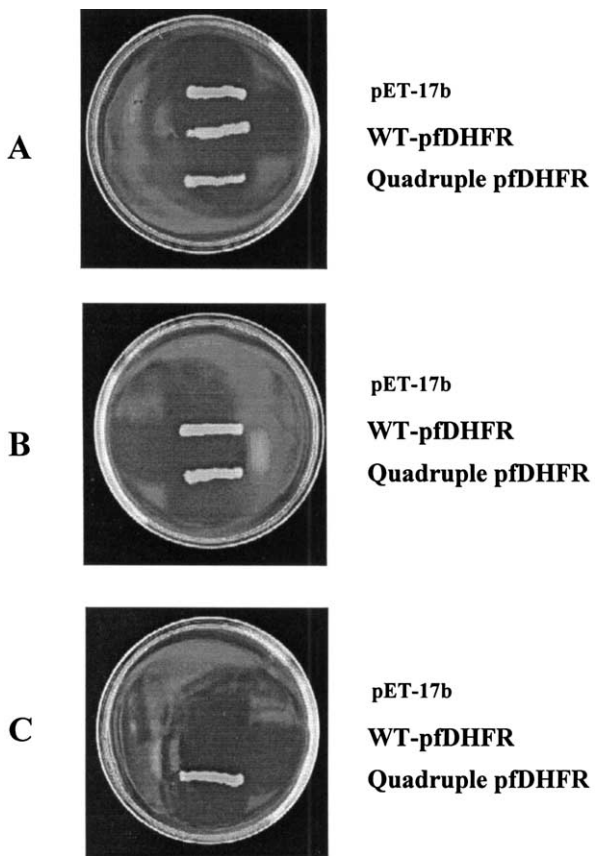

Fig. 3. Genetic complementation showing the growth of transformed bacteria on minimum agar. *E. coli* BL21(DE3)pLysS harboring pET-17b, wild-type pET–pfDHFR and quadruple mutant pET–pfDHFR having N51I + C59R + S108N + I164L were streaked on: (A) minimal agar plates containing 100 μg ml^{-1} ampicillin, 35 μg ml^{-1} chloramphenicol; (B) supplemented with 2 μM Tmp; (C) supplemented with 2 μM Tmp and 15 μM Pyr. Plates were incubated at 37 °C for 16 h before the photographs were taken.

Results from DNA sequencing analysis revealed that all SO3-resistant mutants selected from the wild-type pool contained C59 mutation (Table 1, red). Surprisingly, substitution of C59 was not restricted only to the naturally found mutation C59R, but amino acids with aromatic side chains including Y, F and W (Table 1, red) were also identified. Different combinations of C59 mutations with novel and known mutations including T63S, D87G, S108N and N121D, were also observed (Table 1, red).

Among ten SO3-resistant mutants selected from the quadruple mutant pool, C50R was a preferred mutation (eight out of ten, Table 1, violet). Five out of ten mutations in this set were combinations of C50R, K181R, T219P and K227E (Table 1, violet), in addition to four mutations already carried by the quadruple mutant template. Although one mutant with a single mutation of C50R was obtained as a SO3 resistant mutant selected from the quadruple mutant pool, most selected mutants in this set carried multiple changes of 2–4 mutations (Table 1, violet). Interestingly, two mutants from the quadruple-mutant pool carried a reverse mutation of N108 in the resistant mutant template to T. In both cases, the reversal N108T mutation occurred together with additional F58L mutation (Table 1, violet).

Among WR99210-resistant mutants selected from wild-type pool, D54N was a preferred mutation (five out of ten, Table 1, green). Apart from D54N, other mutations including C50R, F58L, C59R, N82D, N144D and V169G were identified at frequencies of 1–2 out of ten (Table 1, green). Unlike most of the mutants selected from the wild-type pool, seven out of ten of WR99210-resistant mutants selected from the quadruple-mutant pool carried F58 mutations (Table 1, teal). Another novel mutation, K115N, was also detected among this set of mutants (three out of ten, Table 1, teal). Surprisingly, there was a significantly high frequency (eight out of ten) of a reverse mutation of N108 to either S or T (Table 1, teal). It is worth pointing out that the D54N mutation, which was a preferred mutation for WR99210-resistant mutants selected from the wild-type pool, was not detected in any of the WR99210-resistant mutants selected from the quadruple-mutant pool.

3.4. Kinetic properties of the selected mutants

To further elucidate and characterize the effect of the mutations on enzyme properties, a number of mutants

Table 1
Mutations selected from screening of pfDHFR libraries against Pyr, SO3 and WR99210

Clone	Amino acid #																				
	27	50	51	54	58	59	63	82	87	108	115	121	128	144	164	169	181	213	219	221	227
WT	K	C	N	D	F	C	T	N	D	S	K	N	S	N	I	V	K	V	T	L	K
w1						R				N		D									
w2										N											
w3	T									T											
w4	T									T											
w5										T								E			
w6	T									T											
w8						R				N											
w9										T											
w10			I			R				N				K							
w11			I			R				N					L						
w12						R				N		D									
w13						R															
w14						Y	S														
w15						R															
w16						F			G												
w17						R															
w18						R															
w19						W															
w20						R															
w21						R															
w22		R																			
w23						R		D													
w25					L																
w29				N																	
w30				N																	
w31				N												G					
w32				N										D							
w33				N																	
w34		R				R															
w35					L																
QM	K	C	I	D	F	R	T	N	D	N	K	N	S	N	L	V	K	V	T	L	K
q2		R															R		P		E
q3		R											W							V	
q4					L					T											
q8		R															R		P		E
q28					L					T											
q34		R																			
q35		R															R		P		E
q45		R																			
q58		R															R		P		E
q100		R															R		P		E
q9					L					T											
q12					L																
q15					C																
q18										S	N										
q21					L					S											
q24					L					S											
q27										S	N										
q32					L					T											
q38										S	N										
q41					L					T											

WT, wild-type pfDHFR, QM, quadruple mutant pfDHFR. Blue set represents mutants selected from screening of libraries of wild-type template against Pyr. Red set represents mutants selected from screening of libraries of wild-type template against SO3. Green set represents mutants selected from screening of libraries of wild-type template against WR99210. Violet set represents mutants selected from screening of libraries of quadruple mutant template against SO3. Teal set represents mutants selected from screening of libraries of quadruple mutant template against WR99210.

were selected based on their novelty and frequencies. Seven mutants were chosen; four (w1, w4, w16 and w30) from wild-type pool and three (q2, q12 and q15) from quadruple mutant pool.

Table 2 summarizes the kinetic properties of mutants selected from the wild-type and quadruple-mutant pools. All mutant enzymes showed decreased k_{cat} values, representing only 5–10% of the wild-type enzyme.

The mutant w1 carrying triple C59R + S108N + N121D mutation had K_m value for H_2folate that was comparable to that of quadruple mutant and wild-type pfDHFR. However, its k_{cat} was dramatically reduced (about eight folds), resulting in a remarkable decrease in the k_{cat}/K_m value of the enzyme. The mutant w4, carrying double K27T + S108T mutation, also exhibited k_{cat} value that was within the same range as that of w1 mutant, but the K_m value for H_2folate of the mutant was about 4-fold lower than that of the wild-type enzyme, rendering a slight increase in the k_{cat}/K_m value. The mutants w16 (C59F + D87G) and q2 (C50R + K181R + T219P + K227E) had the poorest k_{cat} values among the four mutants selected for study. The K_m values for H_2folate of both w16 and q2 were 3- and 5-fold, respectively, higher than the wild-type enzyme with very poor k_{cat}/K_m values (Table 2).

The effects of mutations on drug sensitivity were investigated and the results are also summarized in Table 2. The triple mutant w1 (C59R + S108N + N121D) showed approximately 6-fold higher K_i value for Pyr as compared to the wild-type enzyme, but had comparable K_i values for SO3 and WR99210 that were within the range observed for the wild-type pfDHFR. Mutant w4, however, was slightly resistant to Pyr, with K_i value that was approximately 3-fold higher than that of the wild-type pfDHFR, and there was no significant alteration in the K_i values for both SO3 and WR99210 as compared to the wild-type enzyme. Interestingly, while the mutants w16 and q2 exhibited K_i values for SO3 that were about 7- and 20-fold, respectively, higher than the wild-type enzyme, the enzymes conferred considerably higher resistance to Pyr; the K_i values for Pyr of the mutant w16 and q2 pfDHFRs were about 10- and 580-fold higher than that of the wild-type enzyme (Table 2). All four mutants selected showed either unchanged or moderate elevation of K_i values (2- to 5-fold) for WR99210.

Three additional single mutants, i.e. w30 (D54N), q12 (F58L) and q15 (F58C), selected against WR99210 were characterized. The DHFR activities from these three mutants were extremely poor and it was impractical to obtain purified enzymes from these mutants. Therefore, the IC_{50} values for Pyr, SO3 and WR99210 of the mutant enzymes were determined from the crude extracts of the mutants and the data are summarized in Table 3. The w30, q12 and q15 pfHDFRs exhibited a range of 40- to over 2000-fold increase in IC_{50} values for all drugs tested indicating the mutations were crucial for inhibitor binding and hence conferring high to very high resistance to antifolates.

4. Discussion

The availability of a system capable of expressing sufficient amounts of catalytically active pfDHFR and amenable to screening novel mutations linked to antifolate resistance would aid a better understanding of the functional role of the mutated residues in causing antifolate resistance. In the present study, we have developed a simple bacteria-based screening system which

Table 2
Kinetic properties and inhibition by Pyr, SO3 and WR99210 of purified pfDHFRs from Pyr, and SO3 resistant mutants selected by genetic complementation

Clone	Mutation	Selected against	Kinetic parameters				Inhibition constant, K_i (nM)		
			k_{cat} (s^{-1})	K_m H_2folate (μM)	K_m NADPH (μM)	k_{cat}/K_m ($M^{-1}\,s^{-1}$) × 10^{6a}	Pyr	SO3	WR99210
WT	–	–	88[b]	13 ± 5[b]	5 ± 1[b]	6.8[b]	1.5 ± 0.2[b]	0.6 ± 0.2	0.5 ± 0.1
QM	N51I+C59R +S108N +I164L	Pyr	15[b]	14 ± 1[b]	25 ± 6[b]	1.1[b]	859 ± 117[b]	3.3 ± 0.4	1.9 ± 0.8
w1	C59R+S108N +N121D	Pyr	12	15 ± 3	5.8 ± 0.8	1.4	95 ± 9	0.7 ± 0.05	0.6 ± 0.05
w4	K27T+S108T	Pyr	8.3	3.0 ± 0.4	6.2 ± 0.7	2.7	4.5 ± 0.4	0.4 ± 0.02	0.3 ± 0.01
w16	C59F+D87G	SO3	5.0	30 ± 1	9 ± 1	0.16	16.6 ± 2	4.5 ± 0.8	1.2 ± 0.1
q2	C50R +K181R +T219P +K227E	SO3	5.6	64 ± 12	6 ± 0.4	0.08	867 ± 91	12.4 ± 2	2.5 ± 0.2

WT, wild-type pfDHFR; QM, quadruple mutant pfDHFR; clones w1, w4 and w16 were selected from wild-type template; clone q2 was selected from quadruple mutant template.

[a] Calculated from K_m values for H_2folate.

[b] Data from Ref. [19].

Table 3
IC_{50} values of Pyr, SO3 and WR99210 determined using crude extracts prepared from mutants selected against WR99210

Clone	Mutation	Selected against	IC_{50} (nM)		
			Pyr	SO3	WR99210
WT	–	–	5.0 ± 0.5	3.1 ± 0.6	2.0 ± 0.15
w30[a]	D54N	WR99210	>10 000	2340 ± 330	101 ± 8.1
QM	N51I + C59R + S108N + I164L	–	2280 ± 389	10.6 ± 0.8	2.1 ± 0.3
q12[b]	F58L	WR99210	>10 000	153 ± 19	81 ± 11
q15[b]	F58C	WR99210	>10 000	160 ± 20	73.3 ± 11

WT, wild-type pfDHFR; QM, quadruple mutant pfDHFR.
[a] Selected from wild-type template.
[b] Selected from quadruple mutant template.

allows identification of novel pfDHFR mutants with increased resistance to common antifolates and their analogues from diverse *pfdhfr* libraries. In addition to its simplicity and efficacy, the bacterial complementation system is applicable to any *E. coli* host strain as the successful complementation relies upon the availability of expressed pfDHFR to complement DHFR function and rescue *E. coli* host whose endogenous DHFR activity is selectively inhibited.

Our approach was first tested by making combinatorial mutations at residue 164 of the pfDHFR domain. Residue 164 was chosen because it is an important residue in the active site of pfDHFR. Alignment of DHFR sequences of a number of organisms [30–34] revealed that the amino acid is considerably conserved and is clustered among other important residues responsible for substrate and antifolate binding [17,18,35]. Further, I164L is a commonly found mutation known to be associated with highly Pyr- and Cyc-resistant *P. falciparum* malaria [6–15]. Our studies selected five single mutations (I164V, I164A, I164G, I164L, and I164S) with detectable DHFR activity. Of five mutations identified, two (I164A and I164V) yielded active DHFR activity of comparable level to or at least near the level of the wild-type pfDHFR (Fig. 2A), while the remaining three mutants exhibited relatively poor activity. This is not surprising considering the fact that residue I164 is a conserved amino acid for *P. falciparum* DHFR, and that amino acids A and V also present in the analogous position of *L. casei* DHFR [30] and human or chicken DHFRs [31,33,36], respectively. It is noteworthy that the bacterial complementation system we developed was able to detect mutants that expressed low DHFR activity, such as in the case of I164G mutant which expressed pfHDFR at a level as low as about 2% of the wild-type DHFR activity (Fig. 2B). Based on the previously reported data on genetic complementation of TS in TS-deficient *E. coli* which estimated the necessary amounts of TS activity to be about 1% of the activity of wild-type construct [37], it is conceivable that our bacterial complementation system for identifying pfDHFR mutants was sufficiently sensitive to detect mutants expressing the lowest level of DHFR activity required for bacterial survival.

We have optimized and adopted the bacterial complementation system to identify a number of mutations that are critical for DHFR function as well as capable of conferring resistance to antifolates. In essence, we have clearly shown the ability of the system to distinguish between mutations from originally Pyr sensitive wild-type and those from already Pyr-resistant pfDHFR carrying quadruple mutations (Fig. 3). Starting from the wild-type pfDHFR, all known mutations (N51I, C59R, S108N and I164L) previously identified in Pyr-resistant parasites from the field were identified by our system (Table 1, blue).

The fact that mutations known from the field to be associated with antifolate resistance in parasites could be identified by our bacterial complementation system implies that our system simulates natural process of evolution of resistance. This is different to what was previously obtained from the yeast complementation system [23,24], from which only mutations not previously observed in natural samples, and not located within the vicinity of the enzyme active site were preferentially identified. One reason for the difference in results may be difference in levels of enzyme expression; another may be difference in host viability in the presence of inhibitors.

Among ten randomly picked clones from wild-type *pfdhfr* mutant pool selected by Pyr, there were two single, four double, two triple, and two quadruple mutants (Table 1, blue). All carry mutations at residue 108 (S108N or S108T). It is interesting that we were able to select the w9 mutant carrying a single S108T mutation that is rare in nature [38]. Among the four double mutants selected, three were found to carry the same K27T + S108T mutation, while another mutant had S108T + V213E mutation. Although V213E was previously identified in the yeast system [23,24], neither mutation has ever been found in Pyr-resistant parasite. Surprisingly, with approximately 10-fold reduction in the k_{cat} value and the slight elevation (about 3-fold) of

K_i for Pyr compared to the wild-type enzyme, the affinity for binding to H_2folate substrate of the enzyme from K27T + S108T mutant was found to be approximately 4-fold higher than that of the wild-type pfDHFR. Since the S108T single mutant was previously reported to have similar K_m for substrate and with about 2-fold decrease in the k_{cat} value, it is likely that the changes found in K27T + S108T could be attributed mainly to the K27T mutation.

One triple mutant (C59R + S108N + N121D) was identified. Its kinetic data showed approximately 3-fold better binding to H_2folate substrate and about 7.5-fold higher k_{cat}/K_m value compared to the C59R + S108N mutant pfDHFR previously reported [19]. Of particular interest was the fact that the quadruple mutations (N51I + C59R + S108N + I164L) found in highly resistant *P. falciparum* were identified in one of ten Pyr-resistant clones selected. Another novel quadruple mutant (N51I + C59R + S108N + N144K) was also identified. It remains to be seen whether the novel single or multiple mutations will be found in the field in the future.

The SO3, a *m*-Cl analogue of Pyr, has previously been shown to be effective against Pyr-resistant malaria parasites and in binding with the mutant enzymes [16]. Our data clearly show that mutations leading to resistance to SO3 are possible (Table 1, red, violet). Interestingly, all resistant mutants obtained here had mutation at residue C59 to R, Y, F and W, while some mutants were associated with T63S, D87G, S108N or N121D (Table 1, red). Although the single C59R mutation of pfDHFR has not been found naturally in malaria parasite, its association with S108N is known to be among key residues responsible for increasing resistance to Pyr and Cyc [7,8,10,11,39]. It is important to note that C59R mutation is already present in the quadruple mutant template and might be, at least in part, responsible for moderate resistance against SO3 exhibited by a natural quadruple mutants (Table 2).

We have also shown that mutations leading to WR99210 resistance are also possible (Table 1, green and teal). It is interesting to note that the mutants obtained from selecting the wild-type pool against both SO3 and WR99210 were not the same as those selected from the quadruple-mutant pool (Table 1, red, violet, green and teal). This is exemplified by the mutations at residues 59 and 50 preferentially identified when SO3 was used (Table 1 red and violet), and mutations at residue 54 and 58 identified from WR99210 plates, using wild-type and quadruple mutant pool, respectively (Table 1, green and teal).

The residue 54 is known to be critical for DHFR activity and drug binding. Mutant enzymes with D54N such as mutant w30 (Table 1, green), exhibited a severely impaired DHFR function with a broad resistance to any antifolate tested. Crystal structures of DHFR from various organisms revealed that, the amino acid equivalent to D54 of pfDHFR is an essential determinant of one end of the H_2folate binding pocket and specifically interacts with the substrate and inhibitors by making a H-bonding between its carboxyl group and N1 and 2-amino of the heterocyclic ring. [40–42]. Therefore it is very likely that changing D to N results in perturbation of the H-bonding between the enzyme and the substrate or the inhibitor, resulting in marked reduction of binding affinities. In *P. falciparum*, D54N was previously identified from selection of Pyr-resistant parasites in a prolonged parasite culture under Pyr pressure [43]. The mutated D54N was found to occur together with F223S, an apparently compensating mutation for the low DHFR activity caused by D54N (Sirawaraporn et al., in preparation). Interestingly, we have identified D54N single mutation and two double mutants having D54N paired with N144D and V169G. Based on homology models of pfDHFR previously reported [17,18,35], while the residue D54 is located on the edge of H_2folate binding site forming one end of the substrate-cofactor binding pocket, residues N144 and V169 are at the opposite end of the H_2folate-NADPH binding site, closing the NADPH binding pocket. It is possible that significant changes in H_2folate-NADPH pocket caused by D54 mutation on one end could perturb and displace the position of N114 and V169 on the other end of the active site. The observation that D54N mutation was completely absent from mutants selected from quadruple mutant pool under WR99210 pressure could be due to the combination of critical point mutations in the *pfdhfr* gene resulting in insufficient DHFR activity required for the survival of the bacterial host cells.

An additional F58 mutation was preferentially identified from the quadruple mutant pool selected against WR99210. This residue is highly conserved among DHFRs from different organisms. The phenyl ring of F30 in bacterial DHFR, equivalent to F58 is known to be involved in interaction with both the ring of the inhibitor and the H_2 folate [44]. Mutation of F58 of pfDHFR would have caused reduced binding of both H_2folate and the inhibitor molecule, leading to an impaired DHFR function and severe drug resistance. The F58 mutation of pfDHFR does not exist in nature, but an equivalent mutation F57L was identified in antifolate-resistant *P. vivax* [45]. Characterization of *P. vivax* F57L mutants showed markedly decreased DHFR activity and inhibitor binding, which led to broad resistance to antifolate [46]. It is important to point out that, in most quadruple mutants carrying additional F58 mutation, the residue N108 originally carried by the quadruple mutant was reverted to the wild-type S or T. In a previous report [19], it was shown that a single mutation at residue 108 (S108N) causes about 50% reduction of k_{cat}/K_m value and rendered the

enzyme to be moderately resistant to Pyr [19]. Additional mutations (N51I, C59R and I 164L) in the quadruple mutant caused a further reduction in enzyme activity and inhibitor binding [19]. The presence of the additional F58L in the quadruple mutant might contribute too much deleterious effect on enzyme activity to sustain the growth of bacteria. This correlates well with our observation that *E. coli* carrying the revertant N108S mutation with N51I, F58L, C59R and I164L grew relatively well compared to the mutant with N51I, F58L, C59R, S108N and I164L in the selective agar plates. Our data also suggest that the malaria parasite might encounter limitation in possibility to generate mutations to overcome drug pressure and yet to still maintain sufficient DHFR activity for the survival. Therefore, identification and characterization of amino acid residues essential for the maintenance of sufficient enzyme activity for parasite survival not only provides further information on enzyme function, but is also critical for antifolate antimalarial development. It is noteworthy that all the pfDHFR mutants identified through random mutagenesis exhibit significant reduced k_{cat} values and unfavorable kinetic properties compared to the wild-type enzyme. The mutations required for the parasite to confer antifolate resistance needs to be balanced by the availability of sufficient DHFR activity to allow the survival.

Our bacterial complementation system not only provides a rapid and simple screening of both naturally occurring and naturally unfound mutations of pfDHFR, but also provides insight into understanding of interactions between inhibitors and the active site of pfDHFR. Previous works [2,3,19,20] have led to a general notion that mutations present in naturally occurring antifolate resistant mutants are clustered around the enzyme active site and cause a marked reduction in DHFR activity. The results from the present study suggest that the pfDHFR activity may be governed not only by amino acids in the active site, but also by those remote from the binding pocket, including those which are in the regions with no homology to other DHFRs [17]. The system we report here thus presents a powerful tool to identify novel amino acids potentially involved in conferring antifolate resistance, which should be advantageous for the rational design of new effective antimalarial drugs targeted against antifolate-resistant parasites.

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