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Pyrimethamine analogs as strong inhibitors of double and quadruple mutants of dihydrofolate reductase in human malaria parasites

Alireza Sardarian,[a] Kenneth T. Douglas,*[a] Martin Read,[b] Paul F. G. Sims,[b] John E. Hyde,[b] Penchit Chitnumsub,[c] Rachada Sirawaraporn[d] and Worachart Sirawaraporn[d]

[a] *School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Manchester, UK M13 9PL*

[b] *Department of Biomolecular Sciences, University of Manchester Institute of Science and Technology (UMIST), Manchester, UK M60 1QD*

[c] *National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Rama VI Rd., Bangkok 10400, Thailand*

[d] *Department of Biochemistry, Faculty of Science, Mahidol University, Rama VI Rd., Bangkok 10400, Thailand*

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Pyrimethamine acts against malarial parasites by selectively inhibiting their dihydrofolate reductase-thymidylate synthase. Resistance to pyrimethamine in *Plasmodium falciparum* is due to point mutations in the DHFR domain, initially at residue 108 (S108N), with additional mutations imparting much greater resistance. Our previous work, the development of a simple rational drug design strategy to overcome such resistance, used suitable *meta*-substituents in the pyrimethamine framework to avoid the unfavorable steric clash with mutant side chains at position 108. Interestingly, the *meta*-chloro analog of pyrimethamine not only overcame the resistance due to S108N, but also that contributed by the more remote mutation, C59R. The present work improves on this by means of other *meta*-substituents. Against wild type DHFR, double mutant types A16V + S108T and C59R + S108T, and the highly pyrimethamine/cycloguanil-resistant quadruple-mutant form N51I + C59R + S108N + I164L, pyrimethamine itself gave K_i values of 1.5, 2.4, 72.3 and 859 nM, respectively. The *meta*-substituted analogs, especially the *meta*-bromo analog, were much more powerful inhibitors of these DHFRs, including the quadruple-mutant form (*meta*-bromo analog, K_i 5.1 nM). For comparison, the dihydropyrazine antifolate, WR99210, gave K_i values of 0.9, 3.2, 0.8 and 0.9 nM, respectively. K_i values were also measured against recombinant human DHFR, as were their activities against the growth of *Plasmodium falciparum* cultures bearing the double mutations (FCB and K1 strains) and quadruple mutation (V1/S) and the wild type (3D7). The *meta*-analogs were highly active against all of these, with the *meta*-bromo again being the strongest, having an IC_{50} of 37 nM against V1/S, compared to >5000 nM for pyrimethamine itself and 1.1 nM for WR99210.

Introduction

Pyrimethamine (**1**) has been used for many years against malaria, initially on its own, but subsequently in combinations such as Maloprim (pyrimethamine–dapsone) and Fansidar (pyrimethamine–sulfadoxine), the latter a widely used formulation for the treatment of chloroquine-resistant malaria. Pyronaridine, a new Chinese drug, has been tested against malaria in combination with pyrimethamine. Pyrimethamine has also been used recently against toxoplasmic encephalitis relapses and *Pneumocystis carinii* pneumonia in HIV-infected patients[1] and atovaquone has been tested against murine toxoplasmosis in combination with pyrimethamine.[2] Pyrimethamine also has antibacterial value. Dapsone–pyrimethamine may prevent mycobacterial disease (*M. tuberculosis*, *M. avium*) in immuno-suppressed patients infected with HIV.[3]

With very few safe, effective and cheap antimalarial drugs, the problem of parasite resistance has enormous economic and social consequences, particularly in Africa, where malaria causes *ca.* two million deaths per year and very high levels of morbidity. As a result, ways are being sought to impede the onset and spread of drug resistance. Resistance to pyrimethamine, first reported shortly after its introduction in the 1950s, is now widespread worldwide. To delay (but not overcome) further malarial resistance to inhibitors such as pyrimethamine, combinations such as those above with sulfonamides or sulfones have been in use for some years. Fansidar, for instance, is currently the first-line drug of choice in a number of African countries, but resistance to both components of this combination is an increasing problem.[4]

Pyrimethamine (**1**) inhibits dihydrofolate reductase-thymidylate synthase (DHFR-TS) in the folate biosynthetic pathway (Scheme 1) and resistance to it arises from mutation in the *dhfr* domain of the *dhfr-ts* gene.[5,6]

One of the differences between parasite and host enzymes in this pathway is that the parasite is a bifunctional enzyme with both DHFR and TS active sites encoded by a single gene: the DHFR-TS of *Plasmodium* consists of a DHFR protein chain joined in its primary structure to the TS protein module by a polypeptide chain of *ca.* 90 amino-acid residues.[5] In humans, DHFR and TS are separate gene products, DHFR being monomeric and TS dimeric.

Studies on long-term continuous culture isolates have shown that in the lethal species of the human malaria parasite, *Plasmodium falciparum*, pyrimethamine resistance results in the first instance from an S108N mutation in the DHFR domain, but double and triple mutations impart much higher levels of resistance.[7] Resistance to pyrimethamine and the related antifolate cycloguanil has also been studied in a wide range of field samples of *P. falciparum*,[8] as well as in the long-term continuous culture isolates referred to above. The most common pyrimethamine-resistant strains show the double mutations

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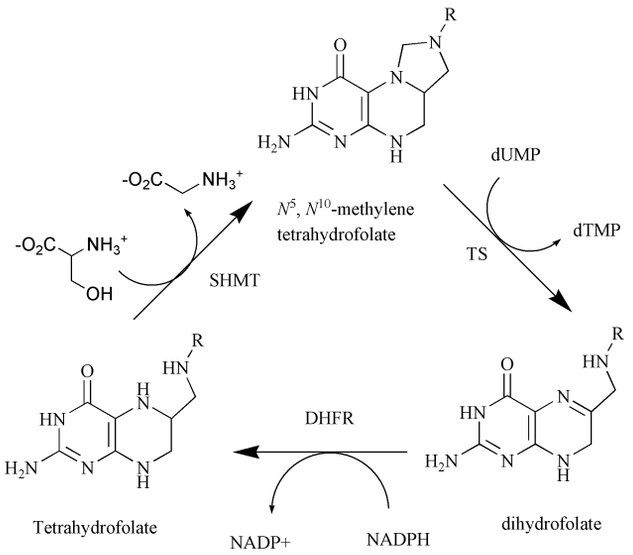


SMHT: serine hydroxymethyltransferase

TS: thymidylate synthase

DHFR: dihydrofolate reductase

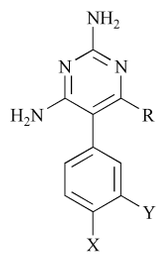
Scheme 1 The thymidylate cycle.

(C59R + S108N) or (N51 + S108N), or the triple combination of these modifications. A different double mutation, (A16V + S108T), increases cycloguanil resistance considerably, but has a much lesser effect on pyrimethamine resistance. However, quadruply-mutated (N51I + C59R + S108N + I164L) parasites, recently emerged in South East Asia, are highly resistant to both pyrimethamine and cycloguanil.[9,10] This is of particular concern, as their spread to African countries, together with the widespread loss of chloroquine efficacy already observed there, would potentially leave those countries with no antimalarial drugs that are both effective and affordable.

The association of antifolate resistance with mutations in the DHFR domain has been directly demonstrated *in vitro via* mutagenesis of synthetic *P. falciparum dhfr* genes,[11,12] as well as by transformation of sensitive parasites with recombinant gene constructs carrying the individual mutations.[13] Further, in multiple mutants there is evidence that these point mutations interact with one another in a cooperative manner.[10] Consequently novel inhibitors of DHFR are being sought.

Recently, computational docking of commercially available compounds to a homology-built molecular model of the DHFR domain of the DHFR-TS of *P. falciparum*[14] provided two new families of inhibitors, in which the best K_i achieved was 0.54 μM against the recombinant *P. falciparum* DHFR domain (from the drug-sensitive strain 3D7). Using the approximation of the *Leishmania major* DHFR-TS (as a basis for *P. falciparum* DHFR-TS) allowed the design and synthesis of inhibitors able to overcome malarial pyrimethamine (**1**) resistance. The modelling[15] approach, predating the X-ray data for the *Leishmania* enzyme,[16] indicated for the S108N mutation that, if the active-site structure of *Plasmodium* DHFR-TS was similar to that of *Leishmania*, there would be a steric clash of the protein with the *para*-Cl atom of pyrimethamine. Locating a suitable substituent in the adjacent *meta*-position instead should avoid this clash and permit additional interaction with the enzyme. As a result, two analogs of pyrimethamine, **2** (CC83) and **3** (S03), were designed, synthesized and tested.[15]

Compared to pyrimethamine (K_i 1.5 nM) with purified recombinant DHFR from *P. falciparum*, the K_i of the *m*-methoxy analog (**2**) was 1.07 nM, but against the recombinant DHFR bearing the double mutation (C59R + S108N), the K_i values for **1** and **2** were 71.7 and 14.0 nM, respectively.[15] In contrast, the *meta*-Cl compound (**3**) was a stronger inhibitor of both wild type (K_i 0.30 nM) and doubly mutant (K_i 2.40 nM) purified enzymes. Moreover, growth of parasite cultures *in vitro* was also strongly inhibited by these compounds, with 50% inhibition of growth occurring at 3.7 μM (for **2**) and 0.6 μM (for **3**) against the parasite line bearing the double mutation (C59R + S108N), compared to 10.2 μM for pyrimethamine. These inhibitors were also found in preliminary studies to retain curative anti-malarial actions *in vivo* in mice infected with *P. berghei*.[15]



1, R = Et, X = Cl, Y = H; pyrimethamine

2, R = Et, X = H, Y = OMe; CC83

3, R = Et, X = H, Y = Cl; S03

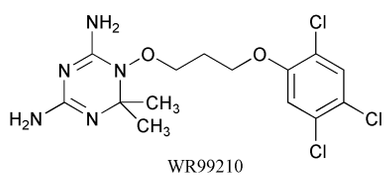
4, R = Et, X = H, Y = CF_3; AR6

5, R = Et, X = H, Y = F; AR4

6, R = Et, X = H, Y = Br; AR3

7, R = Et, X = F, Y = NO_2

We now report further development of this hypothesis to produce leads against other multiple-mutant recombinant enzymes and activity against the growth of (multiply) resistant parasites. Other *meta*-substituents (**4** to **6**) have been found to show strong inhibition of both of the double-mutant, antifolate-resistant enzymes studied (A16N + S108T, cycloguanil-resistant; C59R + S108N, pyrimethamine-resistant), as well as the quadruple-mutant form (N51I + C59R + S108N + I164L, cycloguanil- and pyrimethamine-resistant). These compounds were tested in parallel against *in vitro* cultures of *P. falciparum* lines carrying the same variant forms of DHFR-TS. We also compared the new compounds to the inhibitor WR99210, a powerful antifolate that has activity against mutant forms of *P. falciparum* DHFR in the sub-nM range,[17] but whose development for use in the chemotherapy of human malaria has been limited by its toxicity.[18]



WR99210

Results and discussion

The results of the enzyme inhibition studies and parasite growth assays are summarised in Tables 1 and 2, respectively. In both assays of inhibition of enzyme activity and of parasite growth rates, compounds **4**–**6** were considerably more potent than the parent pyrimethamine against the pyrimethamine-resistant form (C59R + S108N), the cycloguanil-resistant mutant (A16V + S108T) and the pyrimethamine-plus-cycloguanil-resistant quadruple mutant (N51I + C59R + S108N + I164L). In particular, **6** was the most effective of these three potential drugs against the above forms, with K_i and IC_{50} values some two orders of magnitude more favorable than those for pyrimethamine, and approaching those of the dihydrotriazine WR99210.

It should be acknowledged that while the molecular modelling approach we used was reasonably expected to allow us to overcome the resistance caused by the S108 mutations, it was a surprise that it also overcame resistance to the C59 alteration. Residue C59 is some distance away from the S108 and not a primary contact residue for the ligands. The effectiveness of these new compounds against the other double and quadruple mutants again would not have been predicted from an essentially static molecular model of the 3-dimensional structure of DHFR. Fig. 1 shows the location of the mutation sites in the

Table 1 Inhibition of DHFR activity by *meta*-pyrimethamine analogs of recombinant wild type (WT) and mutant DHFR domains of bifunctional DHFR-TS from *P. falciparum* assayed as described in the experimental section

		PfDHFR (K_i/nM)				
		Mutant				
Compound		WT	A16V + S108T	C59R + S108N	N51I + C59R + S108N + I64L	hDHFR (K_i, nM)
1	Pyrimethamine	1.5 ± 0.2[a]	2.4 ± 0.2[a]	72 ± 3[a]	859 ± 117[a]	58 ± 3.3
	Cycloguanil	2.6 ± 0.3[a]	1,314 ± 165[a]	82 ± 4[a]	730 ± 19[a]	85 ± 14.5
	WR99210	0.9 ± 0.16	3.2 ± 0.5	0.8 ± 0.1	0.9 ± 0.1	8.1 ± 0.8
2	CC83(*m*-OMe)	1.07 ± 0.10[b]	nd	14.0 ± 2.6[b]	nd	nd
3	S03 (*m*-Cl)	0.3 ± 0.03[b]	nd	2.4 ± 0.26[b]	nd	nd
4	AR6 (*m*-CF_3)	3.9 ± 0.1	3.6 ± 0.2	3.3 ± 0.5	12 ± 0.7	17 ± 1.2
5	AR4 (*m*-F)	2.0 ± 0.5	3.7 ± 0.5	3.6 ± 0.6	28.8 ± 2.6	21 ± 4.0
6	AR3 (*m*-Br)	0.7 ± 0.1	2.5 ± 0.2	1.1 ± 0.1	5.1 ± 0.4	12 ± 1.0

[a] Data from ref. 10. [b] Data from ref. 15. nd = Not determined.

Table 2 Susceptibility of different parasite lines to inhibitors, as measured by growth inhibition relative to controls lacking drug

		Parasite susceptibility to inhibitor (IC_{50}/nM)			
		Mutant			
Compound		3D7 (WT)	FCB (A16V + S108T)	K1 (C59R + S108N)	V1/S (N51I + C59R + S108N + I64L)
1	Pyrimethamine	2	37	617	>5000
	Cycloguanil	nd	nd	nd	nd
	WR99210	<0.05	0.08	0.03	1.1
2	CC83 (*m*-OMe)	nd	nd	nd	nd
3	S03 (*m*-Cl)	nd	nd	nd	nd
4	AR6 (*m*-CF_3)	21	5.4	7.2	312
5	AR4 (*m*-F)	1.4	3.2	95	57
6	AR3 (*m*-Br)	0.7	4.2	5	37

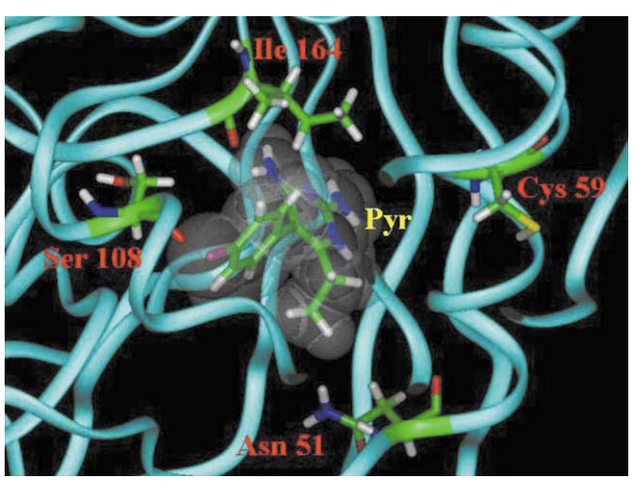


Fig. 1 Molecular graphics representation of the locations of mutations N51I, C59R, S108N and I164L in the DHFR domain of *Plasmodium falciparum* dihydrofolate reductase-thymidylate synthase based on the similarity-modelled coordinates from Rastelli *et al.*[30]

quadruple-resistance mutant (N51I + 59R + S108N + I164L) with respect to the docked position of pyrimethamine using the model of the plasmodial DHFR domain by Rastelli *et al.*[30]

From Table 1, pyrimethamine and cycloguanil are both severely compromised in their inhibition of resistance-mutant plasmodial DHFRs with their K_i values increasing by 572- and 281-fold, respectively, for the quadruple mutant *versus* the wild type. To obtain a therapeutically promising lead one must calibrate inhibitor improvement against the quadruple-resistance mutant form of PfDHFR. The data in Table 1 for WR99210 indicate it to be as effective against the quadruple mutant as against the wild type. We do not have data with the quadruple mutant for CC83 and S03, but of the other *meta*-analogs of pyrimethamine in Table 1, the *meta*-bromo analog (**6**) has a K_i of 5.1 nM for the quadruple mutant, only 5.7-fold weaker than WR99210. It is also a powerful inhibitor of the two double-mutants in Table 1, with low-nM K_i values. The K_i values measured against the human DHFR serve to give an indication of species selectivity for inhibition of the target. They do not give a guide to the therapeutic indices that would be obtained for such compounds as pharmacokinetics, delivery *etc.* become important at the whole organism level. However, there is a 2.4-fold weaker inhibition of the human DHFR than of the quadruple mutant DHFR for the *meta*-bromo analog (**6**), compared with a 9-fold difference for WR99210. The K_i values in Table 1 show that inhibition by **6** is somewhat weaker for human DHFR than is WR99210, but both are strong inhibitors, so it is not clear what the therapeutic index in humans of **6** would be compared to WR99210. Replacing the 6-ethyl group in the heterocyclic ring of **6** by a CF_3 group weakens inhibition across the board for the enzymes in Table 1 (with K_i values of 648 ± 86, 397 ± 28 and 594 ± 127 nM, for WT, (A16V + S108T) and (C59R + S1089N), respectively, with a value too high to determine for the quadruple mutant enzyme), so such variants were not followed up.

Looking at the most complete data sets (WT and (C59R + S108N)), simple correlation with the electronic effect of the substituent (*e.g.* measured by Hammett σ constants), the size (measured by E_s), or its hydrophobicity (measured by the π substituent constant) does not offer any explanation of trends in K_i for either class of DHFR. This is to be expected as some of the mutations which clearly affect the K_i values for the series in Table 1 are located at sites remote from the immediate region forming the binding locus of the *para*- to *meta*- change in the pyrimethamine skeleton. The design stage moved the *p*-Cl group of pyrimethamine to ablate the anti-binding repulsion caused by an S108X mutation. With **2**, and especially with **3**, the simple *meta*-re-location not only overcame the S108T problem, but unexpectedly also the C59R mutant problem.[15] With the extended data of Table 1, the *meta*-substituents clearly also

alleviate the weakened binding introduced by other remote mutations, the *meta*-bromo (**6**) consistently being the strongest inhibitor. One explanation of this is that the mutations actually cause structural changes in the DHFR, extensive enough to be monitored by the *meta*-analog K_i values, but as yet X-ray data have not been published for WT or mutant DHFRs for *Plasmodium*. Another explanation is that plasmodial DHFR shows conformational flexibility, and that this is a factor in the interaction with the *meta*-analogs of pyrimethamine. Of course, both explanations may contribute in reality. Conformational flexibility has been detected for DHFR from other species. For example, the binding of trimethoprim to DHFR is cooperatively influenced by the NADPH cofactor and the degree of cooperativity varies with species, being much reduced for mammalian *versus* bacterial enzymes.[19] Moreover, the native DHFR-TS enzyme, isolated from *P. falciparum*, is known to be somewhat labile.[20] Dynamic processes in ligand binding by DHFRs have been extensively probed by NMR methods (reviewed in 21). Variant pyrimethamines have also been studied by NMR and compound **7**, with a *meta*-NO_2 group, has two detectable bound species with *Lactobacillus casei* DHFR (binding to the enzyme produces two rotational isomers, differing by 180° in terms of the heterocycle–phenyl bond).[22,23,24] Whilst the S108X mutation would prevent a full 180° difference for bound rotamers of the *meta*-analogs of Table 1, partial opening of the active site in this region for some of the multiple mutants could permit (multiple) rotamer-binding and thus could contribute to the magnitude of the measured K_i values. For such reasons it would be naive to expect a simple, generic explanation for all the mutant forms of DHFR in Table 1 and their handling of the *meta*-analogs of pyrimethamine. There is experimental evidence that point mutations in multiple mutants interact with one another in a cooperative manner,[10] and the kinetic data we report in Table 1 are consistent with this.

It is not possible to compare anti-parasite data directly for **2** (CC83) or **3** (SO3) with that for **4**–**6** as the parasite culture conditions for the two studies were different. However, against HB3 culture, the S108N single mutant parasite, **2** was 6 times more powerful than pyrimethamine itself, and **3** was 30 times more potent. Against the double-mutant parasite culture K1 (C59R + S108N), **2** was 2.8-fold, and **3** was 17-fold more potent than pyrimethamine.[15] These values are consistent with the data in Table 2 for the other *meta*-analogs of pyrimethamine, with **6** being 123-fold, **4** being 86-fold and **5** being 6.5-fold more potent against the K1 culture in this set of experiments. The pattern of activity against the various multiple-mutant resistant cultures depends on the particular mutant line and so features additional to the tightness of binding to the DHFR target active site are also important in determining biological efficacy. However, in general we observed a reasonable correlation between the inhibitory properties of the new pyrimethamine derivatives against the DHFR activity of the purified recombinant enzymes on the one hand, and the growth rates of the live parasites on the other. The most important feature of Table 2 is that **6** is highly active against the clinically important quadruple mutant, and is at least 135-fold more potent against this mutant than pyrimethamine. Moreover, it is only 34-fold weaker in its control of resistant parasite growth than the internal standard, WR99210. As the pharmacokinetics and other therapeutically important aspects of **6** have yet to be determined, one cannot extrapolate and assess a potential therapeutic window for **6** compared to WR 99210.

Conclusion

The simple *meta*-analogs of pyrimethamine are active not only against the S108N mutation of *P. falciparum* DHFR, the resistance of which they were designed to circumvent, but also against other double and quadruple mutants, which are clinically more important origins of resistance to therapy, as such multiply-mutant forms are now common in areas such as East Africa and S. E. Asia. The best of these inhibitors to date has been found to be the *meta*-bromo analog, **6**, which is a strong inhibitor of the recombinant DHFR bearing the four mutations, and also a strong inhibitor of the growth of parasite cultures carrying such a mutation pattern.

Experimental

The compounds in Table 1 were synthesized as described.[15,25,26,27,28] For synthetic procedures anhydrous reactants and dry solvents were used. Aluminium oxide (active neutral, Brockmann Grade 1) was purchased from BDH Chemicals Ltd. TLC was carried out on alumina and silica plates. Spots were made visible by UV and light or exposure to iodine vapour. Melting points, taken on a Gallenkamp apparatus with digital thermometer, are uncorrected. NMR spectra were recorded on a JEOL JNM Ex 270 spectrometer operating at 270 MHz for ^{1}H-NMR and 68 MHz for ^{13}C-NMR. Chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane. Splitting patterns are abbreviated as: s, singlet; d, doublet; t, triplet; q, quartet; and m, unresolved multiplet. Mass spectra were taken by means of a Kratos MS20 Instrument coupled to a DS55 data system for precise mass determination. Elemental analyses were performed on an EA 1108-Elemental Analyser (Carlo Erba Instruments) in the Department of Chemistry of the University of Manchester.

Synthesis

5-(3-Bromophenyl)-6-ethylpyrimidine-2,4-diamine (6)

To a solution prepared by dissolving sodium (0.32 g) in absolute ethanol (50 ml) were added in succession guanidine nitrate (1.68 g, 13.8 mmol) and 3-isobutoxy-2-*m*-bromophenylpent-2-enonitrile[28] (3.54 g, 11.5 mmol). The mixture was refluxed for 18 h, cooled to room temperature and the solvent evaporated by rotary evaporator. A mixture of water and ether (50 ml, 1 : 1 v/v) was added to the residue and the mixture stirred for 10 min. The precipitate was filtered off, washed with water (2 x 15 ml), dried under vacuum and recrystallized from ethanol to yield 2,4-diamino-5-*m*-bromophenyl-6-ethylpyrimidine (**6**) (1.54 g, 45.8% yield) as colorless, flattened needles, mp 198–200 °C (uncorr.). TLC R_f 0.56 in ethyl acetate–methanol (10 : 1, v/v). Anal. calc.for $C_{12}H_{13}BrN_4$: C, 49.2; H, 4.5; Br, 27.3; N, 19.1. Found: C, 49.1; H, 4.5; Br, 27.4; N, 19.2%. ^{1}H-NMR (DMSO): δ 0.96 (3H, t, CH_3), 2.08 (2H, q, CH_2), 5.69 (2H, br s, NH_2), 5.92 (2H, br s, NH_2), 7.14–7.22 (1H, d, ArH), 7.32–7.43 (2H, m, ArH), 7.49–7.56 (1H, d, ArH). ^{13}C-NMR (DMSO): δ 12.94 (CH_3), 27.35 (CH_2), 105.2 (C_5), 121.9 ($C_{3'}$), 129.8 ($C_{6'}$), 129.8 ($C_{2'}$), 130.8 ($C_{5'}$), 133.1 ($C_{4'}$), 138.6 ($C_{1'}$), 161.8 (C_2), 162.1 (C_4), 166.3 (C_6). ES^+ mass spectrum, calc. 292.16; found 293.

Compounds **4** and **5** were synthesized by similar procedures. 6-Ethyl-5-(3-trifluoromethylphenyl)pyrimidine-2,4-diamine (**4**) was isolated as a white precipitate, which was crystallized from ethanol as colorless, flattened needles (25.4% yield), mp 202–204 °C (uncorr.).[26] TLC R_f 0.5 in ethyl acetate–methanol (4 : 1). Anal.calc. for $C_{13}H_{13}F_3N_4$: C, 55.3; H, 4.6; F, 20.2; N, 19.9. Found: C, 55.0; H, 4.7; F, 20.0; N, 19.9%. ^{1}H-NMR (DMSO): δ 0.92 (3H, t, CH_3), 2.05 (2H, q, CH_2), 5.66 (2H, br s, NH_2), 5.92 (2H, br s, NH_2), 7.43–7.52 (2H, m, ArH), 7.63–7.72 (2H, m, ArH). ^{13}C-NMR (DMSO): δ 12.90 (CH_3), 27.39 (CH_2), 105.1 (C_5), 123.8 ($C_{2'}$, d, ^3J_F 3.7 Hz), 124.2 (CF_3, q, ^1J_F 272.2 Hz), 127.1 ($C_{4'}$, d, ^3J_F 3.6 Hz), 129.5 ($C_{3'}$, q, ^2J_F 31.7), 129.8 ($C_{5'}$), 134.9 ($C_{6'}$), 137.2 ($C_{1'}$), 161.8 (C_4), 162.2 (C_2), 166.5 (C_6). ES^+ mass spectrum, calc. 282.26; found 283.

6-Ethyl-5-(3-fluorophenyl)pyrimidine-2,4-diamine (**5**) was isolated as a white precipitate, which was recrystallized from

ethanol as colorless flattened needles (35%), mp 220–224 °C (uncorr.). TLC R_f in ethyl acetate–methanol (10 : 1, v/v). Anal. calc. for $C_{12}H_{13}FN_4$: C, 62.1; H, 5.6; F, 8.2; N, 24.1. Found: C, 62.0; H, 5.71; F, 8.3; N, 24.5%. ^{1}H-NMR (DMSO): δ 0.95 (3H, t, CH_3), 2.10 (2H, q, CH_2), 5.60 (2H, br s, NH_2), 5.91 (2H, br s, NH_2), 6.97–7.05 (2H, dd, ArH), 7.12–7.22 (1H, m, ArH), 7.42–7.52 (1H, m, ArH). ^{13}C-NMR (DMSO): δ 12.97 (CH_3), 27.33 (CH_2), 105.4 (C_5), 113.8 ($C_{2'}$, d, ^2J_F 20.7 Hz), 117.7 ($C_{4'}$, d, ^2J_F 19.6 Hz), 126.8 ($C_{6'}$, d, ^4J_F 2.5 Hz), 130.6 ($C_{5'}$, d, ^3J_F 8.5 Hz), 138.5 ($C_{1'}$, d, ^3J_F 7.4 Hz), 161.7 (C_4), 162.1 (C_2), 162.3 ($C_{3'}$, d, ^1J_F 244.2 Hz), 166.3 (C_6). ES$^+$ mass spectrum, calc. 232.26; found 233.

Enzyme assays and inhibition studies

The DHFR activities of both recombinant *P. falciparum*[10] and human DHFRs (the construct from which the recombinant enzyme was expressed and purified was a gift from Dr D. V. Santi) was determined spectrophotometrically according to the method previously described.[11,15] The reactions, performed in 1 mL cuvettes, contained 1 × DHFR buffer (50 mM TES [tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid], pH 7.0, 75 mM 2-mercaptoethanol, 1 mg mL^{-1} Bovine Serum Albumin), 100 μM each of the substrate H_2 folate and cofactor NADPH, and an appropriate amount (~0.005 units) of the affinity-purified enzymes. The kinetic reaction was followed by monitoring the decrease in absorbance at 340 nm using a Hewlett Packard model 8453. The K_i values of the inhibitors for the enzymes were determined by fitting to the equation $IC_{50} = K_i (1 + ([S]/K_m))$.[29]

In vitro parasite cultures

IC_{50} values for inhibition of parasite growth were derived as described[15] with some modification. Duplicate asynchronous cultures (200 μL), with a starting parasitemia of 0.5%, were treated with the appropriate drug concentration (1 μL of pre-diluted drug in 50% DMSO–50% phosphate-buffered saline) and the culture medium plus drug renewed at 24 h and 48 h. Final drug concentrations were varied from 50 pM to 5 μM in 10-fold steps. Final parasitemias were recorded at 72 h and mean values were used to calculate percentage growth inhibition relative to controls with no drug.

List of abbreviations

Cyc = cycloguanil, DHFR-TS = dihydrofolate reductase-thymidylate synthase, DMSO = dimethyl sulfoxide, PfDHFR = dihydrofolate reductase from *Plasmodium falciparum*, pyr = pyrimethamine, WT = wild type.

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