Development of 2,4-Diaminopyrimidines as Antimalarials Based on Inhibition of the S108N and C59R+S108N Mutants of Dihydrofolate Reductase from Pyrimethamine-Resistant *Plasmodium falciparum*

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The reduced binding of pyrimethamine to Ser108Asn (S108N) mutants of parasite dihydrofolate reductase (DHFR), which forms the basis of resistance of *Plasmodium falciparum* to pyrimethamine, is largely due to steric constraint imposed by the bulky side chain of N108 on Cl of the 5-*p*-Cl-phenyl group. This and other S108 mutants with bulky side chains all showed reduced binding to pyrimethamine and cycloguanil. Less effect on binding to some bulky mutants was observed for trimethoprim, with greater flexibility for the 5-substituent. S108N DHFR also binds poorly with other pyrimethamine derivatives with bulky groups in place of the *p*-Cl, and the binding was generally progressively poorer for the double (C59R+S108N) mutant. Removal of the *p*-Cl or replacement with *m*-Cl led to better binding with the mutant DHFRs. Pyrimethamine analogues with unbranched hydrophobic 6-substituents showed generally good binding with the mutant DHFRs. A number of compounds were identified with high affinities for both wild-type and mutant DHFRs, with very low to no affinity to human DHFR. Some of these compounds show good antimalarial activities against pyrimethamine-resistant *P. falciparum* containing the mutant DHFRs with low cytotoxicity to three mammalian cell lines.

Introduction

Falciparum malaria continues to be a major burden on public health, especially in tropical, poor countries of Africa, Asia, and South America.[1] The problem is compounded by the occurrence of resistance to various antimalarial drugs, including pyrimethamine (Pyr), an inhibitor of malarial dihydrofolate reductase (DHFR).[2] Resistance of *Plasmodium falciparum* (pf) to Pyr has been shown to be due to mutations of DHFR, principally Ser108Asn (S108N), which may be accompanied by other mutations, mostly at residues 51, 59, and 164, with subsequent increase in the levels of resistance.[3−6] Understanding of the basis of resistance is important for the development of new effective DHFR inhibitors, which may be used as new effective antimalarials. The prospect of developing such new inhibitors is feasible, considering the fact that the parasite relies on having a critical level of enzyme activity and likely has only a limited number of possible mutation sites from which to produce active mutant enzymes.[7−9]

Understanding of the modes of binding of Pyr to wild-type and mutant DHFRs can lead to identification of the molecular basis for resistance and rational design of new effective inhibitors. Although the structure of *P. falciparum* DHFR, which exists as a bifunctional enzyme linked to thymidylate synthase, has not yet been solved, it can be modeled, based on homologies with prokaryotic and other eukaryotic DHFRs with known structures.[10−13] From one such modeling study, McKie et al.[10] pointed out that the S108N mutation would lead to a steric clash with the *p*-Cl atom of the 5-*p*-chlorophenyl group of Pyr. Analogues of Pyr with *m*-Cl or *m*-methoxy in place of the *p*-Cl show far greater binding affinities with the C59R+S108N mutant DHFR than the parent compound.[10] If this steric constraint is the major basis for the reduction of the binding affinity to DHFR mutants, leading to resistance against Pyr and other 2,4-diaminopyrimidines, new inhibitors should be designed to avoid this constraint. Indeed, Warhurst raised the possibility that to avoid this steric constraint, new effective inhibitors might be based on antifolates with a more flexible 5-substituent than Pyr.[11] It is therefore important to verify the importance of the constraint in the residue 108 area experimentally. In this paper, we have obtained such verification through extensive studies of the binding affinities of various mutant enzymes and inhibitors. Mutant DHFRs with bulky groups at residue 108 were shown to have poor binding with Pyr, and its derivatives with bulky 5-*p*-substituents were shown to have poor binding with S108N and C59R+S108N mutant enzymes. We show further that favorable binding with the mutant DHFRs can be obtained by removing the Cl atom or moving it

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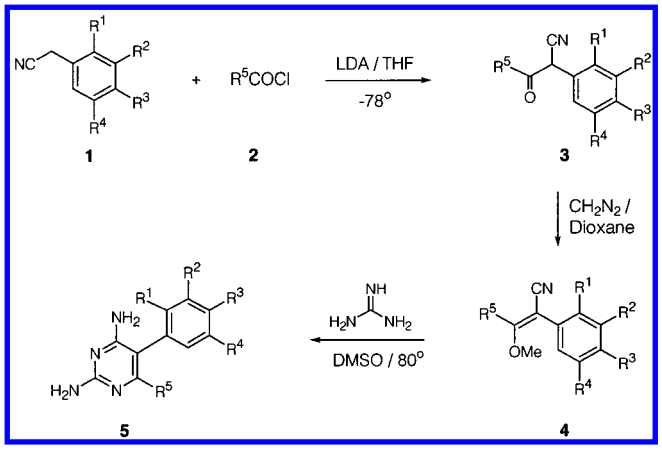
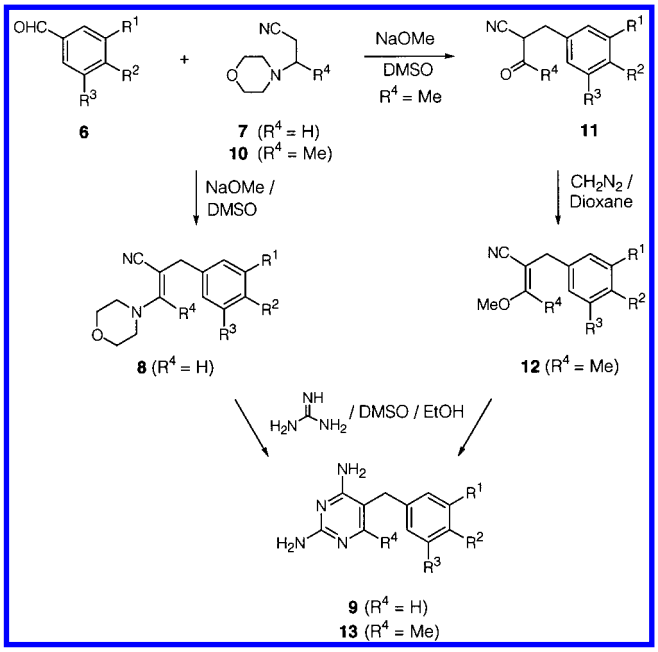
Scheme 1. General Procedure for Synthesis of Pyr Analogues



Scheme 2. General Procedure for Syntheses of Tmp Analogues



from the *p*- to *m*-position. Changing of the general structure of the 5-substituent from the rigid phenyl to a more flexible benzyl group, as in trimethoprim (Tmp) derivatives, can also give rise to compounds that bind effectively with multiple mutant DHFRs. New compounds were consequently identified with very high affinities selectively for the C59R+S108N double mutant DHFR. Some of these compounds show promising activities against both Pyr-sensitive and Pyr-resistant *P. falciparum* containing the double mutant DHFR in culture with good to moderately good safety indices (ratio of cytotoxicity to mammalian cells and the IC_{50} values against the parasite) and warrant further investigation in the search for more effective antimalarials.

Results and Discussion

Chemical Syntheses. Syntheses of Pyr have already been reported by Russell and Hitchings[14] and Barlin.[15] However, a modified method has been employed for the preparation of Pyr analogues in this study. This improved synthetic route, which provided better overall yields of products, is outlined in Scheme 1. Lithiation of *p*-chlorophenylacetonitrile (**1**; $R^1 = R^2 = R^4 = H$, $R^3 = Cl$) with lithium diisopropylamide (LDA) in tetrahydrofuran (THF) at −78 °C followed by acylation with propionyl chloride (**2**; $R^5 = CH_2CH_3$) afforded the corresponding acylphenylacetonitrile **3** ($R^1 = R^2 = R^4 = H$, $R^3 = Cl$, $R^5 = CH_2CH_3$). Upon treatment of **2** with diazomethane in dry dioxane, it provided the corresponding methoxyacrylonitrile **4** ($R^1 = R^2 = R^4 = H$, $R^3 = Cl$, $R^5 = CH_2CH_3$), which was then subjected to the reaction with guanidine in dry dimethyl sulfoxide (DMSO) at 80 °C for 5 min to finally furnish Pyr or 2,4-diamino-5-(4-chlorophenyl)-6-ethylpyrimidine (**5**; $R^1 = R^2 = R^4 = H$, $R^3 = Cl$, $R^5 = CH_2CH_3$).[14]

Tmp analogues, 5-benzyl- and 6-alkyl-2,4-diamino-5-benzylpyrimidines, were synthesized either according to Roth's procedure[16] or by an improved method as outlined in Scheme 2. An aldol condensation reaction of 3′,4′,5′-trimethoxybenzaldehyde (**6**; $R^1 = R^2 = R^3 =$ OMe) and *β*-morpholinopropionitrile (**7**; $R^4 = H$) under NaOMe/DMSO conditions yielded the corresponding acrylonitrile **8** ($R^1 = R^2 = R^3 =$ OMe, $R^4 = H$), which upon treatment with guanidine in DMSO/EtOH under reflux for 18 h gave product **9** (Tmp; $R^1 = R^2 = R^3 =$ OMe, $R^4 = H$). However, the aldol condensation between the aldehyde **6** and the morpholinonitrile **10** ($R^4 = Me$) under a standard method provided only the hydrolyzed product **11** ($R^1 = R^2 = R^3 =$ OMe, $R^4 = Me$) instead of the expected acrylonitrile derivative (i.e., **8**; $R^1 = R^2 = R^3 =$ OMe, $R^4 = H$). Synthesis of Tmp analogue **13** ($R^1 = R^2 = R^3 =$ OMe, $R^4 = Me$) was achieved by the treatment of **11** ($R^1 = R^2 = R^3 =$ OMe, $R^4 = Me$) with diazomethane followed by condensation of the crude methylated product **12** ($R^1 = R^2 = R^3 =$ OMe, $R^4 = Me$) with guanidine to furnish the required compound **13** ($R^1 = R^2 = R^3 =$ OMe, $R^4 = Me$).[17]

Mutational Analysis of Steric Constraint around the Side Chain of Residue 108. Modeling of *P. falciparum* DHFR indicates that in the binding of Pyr with the S108N mutant, there is steric clash between the *p*-Cl moiety and the more bulky group of the side chain.[10−13] Moving the substituent to the *m*-position would relieve the steric constraint. The hypothesis was supported experimentally with Pyr derivatives devoid of other steric interferences and demonstrated that suitable inhibitors may be found with high binding affinities with the mutant enzymes.[10] This interaction model is also consistent with the variation in the K_i values for Pyr and cycloguanil (Cyc) with the nature of the side chains of residue-108 mutant DHFRs.[18] Significant correlations were found between the K_i value and the length and bulk (measured by molecular weight, molar refractivity, volume, and surface area) of the side chain of five (S, N, T, Q, and C) out of seven 108 residues.[11,18] However, limitation of the data available precluded a definitive conclusion from this mutational analysis. Data from Table 1 substantially contribute to the analysis, especially in providing the K_i values for mutants with bulky side chains (N, Q, L, V, and M). Because our preliminary data revealed that compound **20** competitively inhibited the wild-type DHFR (Figure 1) and that Pyr was previously shown to competitively inhibit the Asn108 and Gln108 mutant DHFRs,[18] inhibitions of pfDHFRs by these related analogues were assumed to be competitive. The substantial increase in

Table 1. Steady Kinetic Parameters of Ser108 Mutants of *P. falciparum* DHFR

		kinetic params				inhibition by antifolate, K_i (nM)		
residue 108	expression	k_{cat} (sec^{-1})	K_m H_2folate (μM)	K_m NADPH (μM)	k_{cat}/K_m[a] (M^{-1} sec^{-1}) × 106	Pyr	Cyc	Tmp
Ser (S)	+	68	13 ± 5	5.0 ± 1	5.2	1.5 ± 0.2	2.6 ± 0.3	9.3 ± 1
Asn (N)	+	92	25 ± 9	7 ± 2	3.7	13 ± 4	15 ± 2	115 ± 13.6
Thr (T)	+	36	14 ± 2	17 ± 3	2.6	1.4 ± 0.2	1.6 ± 0.2	12.6 ± 1.6
Gln (Q)	+	37	50 ± 2	14 ± 1	0.7	95 ± 11	158 ± 10	96.4 ± 12
Gly (G)	+	23	20 ± 1	9 ± 1	1.2	7 ± 1	17 ± 2	2.6 ± 0.3
Ala (A)	+	21	14 ± 2	9 ± 1	1.5	4 ± 1	2 ± 0.3	2.4 ± 0.2
Cys (C)	+	12	23 ± 1	9 ± 1	0.5	6 ± 0.1	3 ± 0.4	1.6 ± 0.2
Leu (L)[b]	+	12	8.6 ± 1.2	6.2 ± 0.5	1.4	13.3 ± 1.8	7.8 ± 1.0	3.9 ± 0.5
Val (V)	+	11	6.0 ± 0.8	4.4 ± 0.05	1.8	10.6 ± 0.7	31 ± 2.2	94 ± 8
Met (M)[b]	+	6.3	4.1 ± 0.5	4.6 ± 0.6	1.5	15.6 ± 2.3	254 ± 27	2.3 ± 0.2
others [c]	+							

[a] Calculated from K_m values for H_2folate. [b] Expressed mostly as inclusion bodies. [c] Lys-108 mutant DHFR did not express in *E. coli*; others mutant DHFRs (Ile, Pro, Arg, His, Asp, Glu, Phe, Tyr, and Trp) showed expressed product on sodium dodecyl sulfate polyacrylamide gel electrophoresis, but their DHFR activities were undetectable.

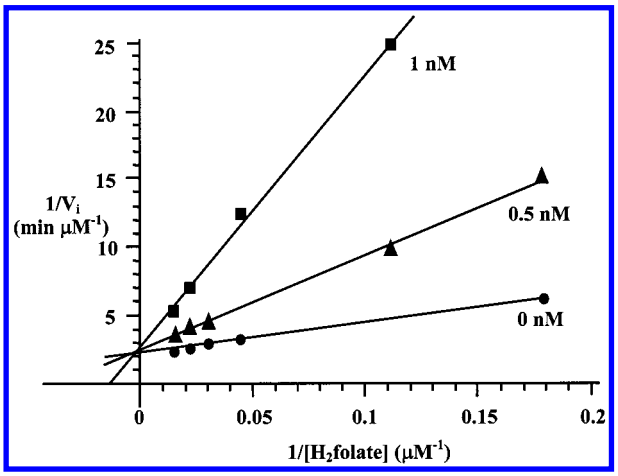


Figure 1. Double reciprocal plot showing competitive inhibition of wild-type pfDHFR by Pyr analogue (compound **20**). The standard assay of DHFR was performed as described in the text. ●, no inhibitor; ▲, 0.5 nM of compound **20**; ■, 1 nM of compound **20**.

K_i values for Pyr and Cyc of the mutants with bulky side chains as compared with the K_i values for the wild-type DHFR confirms the validity of the interaction model proposed. Although the K_i values for Tmp were also found to be increased for some mutants with bulky side chains (N, Q, and V), others (L and M) showed better binding than the wild-type enzyme (Table 1). It is also noteworthy that mutant enzymes G and A, carrying less bulky side chains than the wild-type enzyme, bound more poorly with Pyr and Cyc but more tightly to Tmp as compared to the wild-type enzyme (Table 1). These results indicate that Tmp may be more capable of conformational adjustment to optimize binding with mutant enzymes. This is likely due to the flexible 5-trimethoxybenzyl substituent of Tmp, in comparison with the rigid chlorophenyl substituent of Pyr and Cyc, hence leading to the absence of predictable effect of the size of the side chain in some mutants. Flexible antifolates such as Tmp earlier were predicted to be good candidates for effective inhibition of Pyr-resistant *P. falciparum*, presumably by virtue of their ability to optimize binding with mutant DHFRs.[11]

Inhibition of the Wild-Type and S108N or C59R+S108N pfDHFRs by Pyr Derivatives. To show the validity of this model further, the K_i values for compounds with *p*-substituents of different sizes were determined, for the wild-type, S108N, and C59R+S108N double-mutant DHFRs, which shows cooperativity in mutation effects.[18] The double-mutant parasite is also more commonly found than the single mutant,[8,9] and its corresponding enzyme is therefore a more relevant drug target. If the steric constraint is caused by the unfavorable interaction of the *p*-substituents with the N108 side chain, elimination of this substituent or moving it to another position should lead to relatively better binding with the mutant enzymes, which should be reflected in lower K_i (mut) to K_i (wt) ratios. On the contrary, compounds with bulky substituents should have higher ratios of K_i (mut) for the S108N to K_i (wt) and higher ratios still for the C59R+S108N as compared to the wild-type enzyme. Table 2 shows the K_i values for binding with the wild-type, S108N, and C59R+S108N DHFRs for Pyr with various *p*-substituents. As expected, analogues that lack the *p*-substituent (**19**) led to lower K_i (mut)/K_i (wt) ratios, while those with bulky *p*-substituents such as Br (**14**) or *tert*-butyl (**16**) showed higher K_i (mut)/K_i (wt) ratios. Analogue **17** with a very bulky *p*-phenyl substituent showed very poor binding even with the wild-type DHFR, while those that possess *m*-substituents (**20** and **21**) showed relatively more favorable binding to mutant DHFRs (Table 2), as has been observed previously.[10] Interestingly, the *m*,*p*-Cl_2 derivative showed better binding to the mutant DHFRs than the *p*-Cl parent, indicating that the *m*-substituent also has a significant influence on the binding. It is also noteworthy that although the relative effect of C59R mutation on further reduction in binding was observed, this effect is not critically sensitive to the nature of the *p*-substituent, despite the previously observed cooperative effect of the double mutations.[18]

The nature of the substituent at the 6-position is expected to influence the binding affinity of the inhibitor to both the wild-type and the mutant DHFRs, through various effects such as the effect on the orientation of the H bonds between the D54 side chain carbonyl and the drug. The K_i values for Pyr analogues with various substituents at both the 5-position and the 6-position are given in Table 3. Analogues with 6-ethyl and longer 6-alkyl substituents, both polar and nonpolar, showed more favorable binding to both the wild-type and the mutant enzymes than 6-methyl and unsubstituted

Table 2. K_i Values of Pyr Analogues with Various 5-Phenyl Substituents

compd	5-phenyl substituents	K_i wt[a] (nM)	rel to Pyr	K_i S108N (nM)	rel to Pyr	K_i S108N/ K_i wt	K_i C59R+S108N (nM)	rel to Pyr	K_i C59R+S108N/ K_i wt
				(A) With *p*-Subtitution					
Pyr	*p*-Cl	0.6 ± 0.2	1.0	28.6 ± 2.3	1.0	47.7	53.9 ± 6.5	1.0	89.8
14	*p*-Br	0.3 ± 0.0	0.5	31.5 ± 1.8	1.1	105.0	44.3 ± 3.1	0.8	147.7
15	*p*-Me	0.4 ± 0.0	0.7	10.6 ± 1.3	0.4	26.5	14.7 ± 0.5	0.3	36.8
16	*p*-Bu^t	0.6 ± 0.0	1.0	66.2 ± 10.6	2.3	110.3	126.9 ± 31.0	2.4	211.5
17	*p*-Ph	188.0 ± 18	313.3	421.3 ± 41.7	14.7	2.2	730.1 ± 53.6	13.5	3.9
18	*p*-OMe	0.9 ± 0.1	1.5	39.0 ± 5.9	1.4	43.3	86.0 ± 7.7	1.6	95.6
				(B) With Other Substitutions					
19	H	2.3 ± 0.3	3.8	4.5 ± 0.5	0.2	2.0	8.7 ± 0.2	0.2	3.8
20	*m*-Cl	0.8 ± 0.1	1.3	1.6 ± 0.2	0.06	2.0	1.4 ± 0.0	0.03	1.8
21	*m*,*p*-Cl_2	1.0 ± 0.3	1.7	9.0 ± 0.8	0.3	9.0	12.6 ± 1.6	0.2	12.6
22	*m*,*p*-(−OCH_2O−)	1.1 ± 0.3	1.8	17.2 ± 2.3	0.6	15.6	34.6 ± 3.6	0.6	31.5
23	*o*,*p*-Cl_2	12.6 ± 2.4	21.0	1173.0 ± 206	41.0	93.1	1141.3 ± 73.6	21.2	90.6
24	*m*-OMe,*p*-(2,4-Cl_2-$PhCH_2O$−)	1.0 ± 0.1	1.7	20.9 ± 6.3	0.7	20.9	42.3 ± 2.8	0.8	42.3
25	*m*,*m*-Br_2−*p*-OEt	7.0 ± 1.0	11.7	415.2 ± 45.0	14.5	59.3	534.5 ± 65.2	9.9	76.3

[a] Wild-type pfDHFR.

Table 3. K_i Values of Pyr Analogues with Various 5- and 6-Substituents

	substituents		K_i wt[a]	rel to	K_i S108N	rel to	K_i S108N/	K_i C59R+S108N	rel to	K_i C59R+S108N/
compd	5-Ph	6-R	(nM)	Pyr	(nM)	Pyr	K_i wt	(nM)	Pyr	K_i wt
26	*p*-Cl	−H	2.4 ± 0.3	4.0	368.7 ± 50.4	12.9	153.6	555.8 ± 34.5	10.3	231.6
27	*p*-Cl	−Me	2.0 ± 0.2	3.3	103.7 ± 10.5	3.6	51.8	142.0 ± 4.3	2.6	71.0
Pyr	*p*-Cl	−Et	0.6 ± 0.2	1.0	28.6 ± 2.3	1.0	47.7	53.9 ± 6.5	1.0	89.8
28	*p*-Cl	−$(CH_2)_3$COOMe	0.3 ± 0.0	0.5	16.9 ± 1.1	0.6	56.3	27.4 ± 3.6	0.5	91.3
29	*p*-Cl	−$(CH_2)_3$Ph	0.7 ± 0.1	1.2	13.9 ± 1.0	0.5	19.9	23.3 ± 2.4	0.4	33.3
30	H	−H	41.9 ± 3.2	69.8	81.0 ± 5.9	2.8	1.9	76.2 ± 5.2	1.4	1.8
31	H	−Me	10.9 ± 1.5	18.2	25.6 ± 2.8	0.9	2.3	36.7 ± 4.3	0.7	3.4
19	H	−Et	2.3 ± 0.3	3.8	4.5 ± 0.5	0.2	1.9	8.7 ± 0.2	0.2	3.8
32	H	−$(CH_2)_3$COOMe	0.6 ± 0.0	1.0	1.8 ± 0.2	0.06	3.0	5.5 ± 0.5	0.1	9.2
33	H	Hex^n	0.3 ± 0.1	0.5	1.2 ± 0.3	0.04	4.0	0.7 ± 0.3	0.01	2.3
34	H	−$(CH_2)_3$Ph	0.5 ± 0.0	0.9	0.9 ± 0.2	0.03	1.8	2.4 ± 0.1	0.04	4.8
35	*m*-Cl	−H	6.3 ± 0.5	10.5	26.3 ± 2.4	0.9	4.2	31.6 ± 10	0.6	5.0
36	*m*-Cl	−Me	1.9 ± 0.5	3.2	3.2 ± 0.4	0.1	1.7	3.1 ± 0.3	0.06	1.6
20	*m*-Cl	−Et	0.8 ± 0.1	1.3	1.6 ± 0.2	0.06	2.0	1.4 ± 0.0	0.03	1.8
37	*m*-Cl	−$(CH_2)_3$COOMe	0.5 ± 0.0	0.8	1.7 ± 0.1	0.06	3.4	2.8 ± 0.2	0.05	5.6
38	*m*-Cl	−$(CH_2)_3$Ph	1.2 ± 0.2	2.0	3.0 ± 0.6	0.1	2.5	4.2 ± 0.2	0.08	3.5
39	*m*-Cl	−$(CH_2)_3$-Ph-(*p*-OMe)	2.2 ± 0.6	3.7	4.0 ± 0.2	0.07	1.8	6.0 ± 0.6	0.2	2.7
40	*m*-Cl	−$(CH_2)_2O(CH_2)_3$OPh	0.4 ± 0.2	0.7	1.8 ± 0.3	0.06	4.5	1.9 ± 0.4	0.03	4.7

[a] Wild-type pfDHFR.

analogues. This tendency is most noticeable for the 5-phenyl series (**19** and **30**−**34**).

Inhibition of the Wild-Type and Mutant pfDHFRs by Tmp Derivatives. 5-Phenylpyrimidines such as Pyr have only a limited possibility for adjustment of the conformation of the side chain owing to the rigidity of the phenyl substituent. In contrast, 5-benzylpyrimidines such as Tmp have greater flexibility for conformational adjustment of the side chain. Such flexibility enabled Tmp to fit into the active site of bacterial DHFR better than phenyl pyrimidines.[19] A similar situation may have arisen with the mutant malarial DHFRs, in which the residue 108 side chain interferes with the 5-substituent. It was suggested[11] that flexible antifolates such as Tmp may be effective in Pyr-resistant *P. falciparum*. Although Tmp is not normally used as an antimalarial, the possibility was raised that its analogues may be found to have good antimalarial activities by virtue of effective inhibition of both wild-type and 108 mutant DHFRs. Table 4 gives the K_i values against the wild-type, S108N, and C59R+S108N mutant DHFRs of a number of Tmp analogues. The K_i (mut) values and the K_i (mut)/K_i (wt) ratios of some of these compounds are significantly smaller than for Pyr and its analogues, indicating their potential antimalarial activities. The rationalization of these activities awaits the elucidation of more definitive enzyme structure and its binding models.

To obtain good antimalarial candidates, we have chosen to investigate compounds that are very effective inhibitors against the wild-type enzyme and also retain good activities against the mutant enzymes. The compounds of special interest are the 4-benzyloxy derivatives of benzyldiaminopyrimidine. A Tmp analogue in this series (**51**) has a K_i value of about 2 nM and a resistance factor (K_i (mut)/K_i (wt) ratios) of 6−7 against both mutant DHFRs used in this study. A few other compounds are also promising inhibitors as judged from their relatively good binding affinities with the mutant enzymes. On the basis of our present results and available evidence from previous studies,[10,11] it is conceivable that compounds with a *p*-benzyloxy substituent on the 5-benzyl group will have favorable binding characteristics. It should be noted that conformationally these side chains are quite similar to the alkyloxy side chain of WR99210. Understanding of the interactions of these groups with the enzyme should aid in the design of more potent inhibitors that bind more tightly and effectively to the mutant enzymes.

In Vitro Antiplasmodial Activity. We have tested the antimalarial activities of our compounds against *P. falciparum* in vitro. The results are summarized in

Table 4. K_i Values of Tmp Analogues

compd	K_i wt[a] (nM)	rel to Tmp	K_i S108N (nM)	rel to Tmp	K_i S108N/ K_i wt	K_i C59R+S108N (nM)	rel to Tmp	K_i C59R+S108N/ K_i wt
Tmp	10.3 ± 0.5	1.0	132.3 ± 11.4	1.0	12.8	242.1 ± 40.1	1.0	23.5
41	1.8 ± 0.4	0.2	26.6 ± 4.9	0.2	14.8	44.2 ± 2.7	0.2	24.5
42	2.2 ± 0.5	0.2	34.9 ± 1.4	0.3	15.9	60.7 ± 8.7	0.3	27.6
43	4.2 ± 0.2	0.4	61.9 ± 3.0	0.5	14.7	71.9 ± 17	0.3	17.1
44	0.9 ± 0.3	0.09	7.1 ± 0.5	0.05	7.9	9.7 ± 1.4	0.04	10.8
45	1.7 ± 0.2	0.2	14.5 ± 2.5	0.1	8.5	60.2 ± 2.6	0.2	35.4
46	8.6 ± 0.4	0.8	51.0 ± 7.2	0.4	5.9	80.0 ± 10.3	0.3	9.3
47	3.7 ± 0.9	0.4	46.4 ± 6.7	0.4	12.5	99.1 ± 17.2	0.4	26.8
48	12.1 ± 1.8	1.2	179.6 ± 29.4	1.4	14.8	502.8 ± 70.7	2.1	41.5
49	1.3 ± 0.2	0.1	3.3 ± 0.7	0.02	2.5	5.2 ± 0.3	0.02	4.0
50	0.6 ± 0.0	0.06	6.2 ± 1.5	0.05	10.3	8.7 ± 1.0	0.04	14.5
51	0.3 ± 0.0	0.03	1.8 ± 0.2	0.01	6.0	2.2 ± 0.1	0.009	7.3
52	0.4 ± 0.2	0.04	1.6 ± 0.8	0.01	4.0	3.5 ± 0.8	0.01	8.8
53	0.4 ± 0.2	0.04	1.7 ± 0.3	0.01	4.3	5.6 ± 0.4	0.02	13.8
54	0.9 ± 0.4	0.09	23.5 ± 4.3	0.2	26.1	39.1 ± 3.1	0.2	43.4

[a] Wild-type pfDHFR.

Table 5A,B. Some Pyr analogues, bearing *m*-Cl and unsubstituted 5-phenyl group together with long 6-alkyl substituents (Table 5A) and Tmp analogues (Table 5B), exhibit IC_{50} values against the resistant parasite (K1CB1, with C59R+S108N mutations in the DHFR) at low micromolar level, the concentrations that are about 10−25 times more effective than their corresponding parent compounds. The IC_{50} ratios for the resistant strain to the wild-type (TM4) parasite are also far lower than their parent compounds. Particularly noteworthy are the antimalarial activities of the Pyr analogues **19**, **33**, **34**, and **39** and the Tmp analogues **51** and **52**.

Toxicity tests of some of these compounds to evaluate the selective inhibition against human DHFR (Table 6A) and three mammalian cell lines, African green monkey kidney fibroblast (vero cells), human epidermoid carcinoma (KB), and human breast cancer (BC) (Table 6B), showed that the compounds are relatively selective to pfDHFR and malaria parasites. Indeed, the results obtained from the three cell lines are in a similar figure and within the range observed for Pyr. Analogues with unsubstituted at 5-Ph, **19**, **33**, and **34**, were less toxic than Pyr and those with 5-*m*ClPh (**20**, **39**, and **40**). These suggest that there is some general toxicity of some of these compounds and that care should be taken in further design for 2,4-diaminopyrimidine analogues with and without substitution at the *meta*-position of the 5-Ph ring. The approach of modeling, design, and selected syntheses should yield understanding and lead to development of more active compounds with more selective toxicity against the antifolate-resistant parasites.

Conclusion. The 2,4-diaminopyrimidine analogues bearing a *m*-Cl and an unsubstituted 5-phenyl group together with long 6-alkyl substituents and some 4-benzyloxy derivatives of benzyldiaminopyrimidine show high binding affinity with the wild-type, S108N, and C59R+S108N DHFRs. These compounds also exhibit IC_{50} values against the resistant parasite (K1, with C59R+S108N mutations in the DHFR) at low micromolar level, about 10−25 times more effective than their corresponding parent compounds. Unsubstituted 5-phenyl analogues of 2,4-diaminopyrimidine give low to no toxicity in mammalian cells.

Table 5. Antiplasmodial Activities (IC_{50}) of (A) Pyr and (B) Tmp Analogues against *P. falciparum* Clones with Wild-Type (TM4/8.2) and the Mutant Enzyme C59R+S108N-pfDHFR (K1CB1)

A

compd	IC_{50} TM4/ 8.2 (μM)	rel to Pyr	IC_{50} K1CB1 (μM)	rel to Pyr	IC_{50} ratio K1CB1:TM4
Pyr	0.08 ± 0.011	1.0	30.9 ± 8.4	1.0	386.3
14	0.05 ± 0.026	0.6	25.0 ± 3.9	0.8	500.0
15	0.10 ± 0.03	1.2	12.7 ± 4.2	0.4	127.0
16	0.6 ± 0.25	7.5	3.9 ± 1.3	0.1	6.5
17	13.1 ± 1.7	163.8	4.4 ± 1.3	0.1	0.3
18	0.3 ± 0.1	3.8	>25	> 0.8	> 84
19	0.7 ± 0.24	8.7	3.3 ± 0.4	0.1	4.7
20	0.4 ± 0.1	5.0	1.3 ± 0.5	0.04	3.3
21	0.06 ± 0.02	0.8	17.4 ± 3.5	0.6	290.0
22	0.4 ± 0.1	5.0	21.5 ± 1.9	0.7	53.8
23	11.3 ± 4.2	141.3	22.5 ± 7.4	0.7	2.0
24	3.7 ± 1.6	46.3	>50	> 1.6	> 14
25	3.7 ± 0.8	46.3	17.4 ± 3.1	0.6	4.7
26	2.3 ± 0.5	28.8	26.2 ± 5.2	0.8	11.4
27	0.5 ± 0.02	6.3	60.1 ± 15.2	1.9	120.2
28	0.04 ± 0.013	0.4	22.3 ± 6.1	0.7	557.5
29	0.05 ± 0.014	0.6	10.6 ± 1.7	0.3	212.0
30	20.9 ± 3.9	261.3	19.4 ± 1.9	0.6	0.9
31	7.4 ± 2.6	92.5	30.8 ± 6.5	1.0	4.2
32	0.4 ± 0.06	5.0	31.3 ± 5.5	1.0	78.3
33	0.06 ± 0.015	0.8	1.3 ± 0.1	0.04	21.7
34	0.2 ± 0.06	2.5	2.3 ± 0.6	0.07	11.5
35	6.0 ± 2.5	75.0	8.8 ± 2.6	0.3	1.5
36	2.4 ± 1.1	30.0	4.8 ± 0.7	0.2	2.0
37	0.8 ± 0.10	10.0	36.3 ± 6.1	1.17	45.4
38	0.7 ± 0.06	8.8	3.0 ± 0.8	0.10	4.3
39	0.4 ± 0.06	5.0	2.7 ± 0.6	0.09	6.8
40	0.4 ± 0.08	5.0	3.3 ± 0.1	0.1	8.3

B

compd	IC_{50} TM4/ 8.2 (μM)	rel to Tmp	IC_{50} K1CB1 (μM)	rel to Tmp	IC_{50} ratio K1CB1:TM4
Tmp	6.7 ± 1.4	1.0	136.5 ± 27.1	1.0	20.4
41	3.0 ± 0.5	0.4	11.7 ± 3.9	0.09	3.9
42	4.7 ± 1.7	0.7	>100	> 74	> 22
43	4.2 ± 0.9	0.6	30.1 ± 9.3	0.2	7.1
44	0.5 ± 0.1	0.07	16.6 ± 1.8	0.1	33.2
45	3.1 ± 0.6	0.5	14.6 ± 2.3	0.1	4.7
46	4.5 ± 0.3	0.7	4.4 ± 1.7	0.03	1.0
47	3.6 ± 0.5	0.5	4.2 ± 1.5	0.03	1.2
48	19.0 ± 7.2	2.8	17.5 ± 3.0	0.1	0.9
49	0.7 ± 0.2	0.1	19.9 ± 3.8	0.1	28.4
50	0.3 ± 0.06	0.04	27.8 ± 10.6	0.2	92.7
51	0.07 ± 0.02	0.01	5.0 ± 1.5	0.04	71.4
52	0.15 ± 0.05	0.3	4.3 ± 0.9	0.03	21.5
53	0.44 ± 0.2	0.6	10.1 ± 4.2	0.07	25.3
54	3.0 ± 0.4	0.4	3.5 ± 0.3	0.03	1.2

Table 6. (A) K_i Values of Some Pyr and Tmp Analogues against Human DHFR and (B) Cytotoxicity of Some Pyr and Tmp Analogues to Mammalian Cells

A

compd	K_i human DHFR (μM)	K_i human/ K_i wt ratio	K_i human/K_i C59R+S108N ratio
Pyr	0.008	13	0.15
20	not determined		
33	15	50 000	21 500
34	no inhibition		
39	>5	>2300	>830
Tmp	no inhibition		
51	no inhibition		
52	no inhibition		

B

	cytotoxicity to vero cells			cytotoxicity to KB cells			cytotoxicity to BC cells		
compd	IC_{50} (μM)	safety ratio[a]	safety ratio[b]	IC_{50} (μM)	safety ratio[a]	safety ratio[b]	IC_{50} (μM)	safety ratio[a]	safety ratio[b]
Pyr	32	400	1.03	109	1362.5	3.5	>250	>3125	>8.1
16	100	172	26	87	145	22	56	93.3	14.4
19	320	444	96	>500	>714	>151	>500	>714	>151
20	7	13	5.4	46	115	35.4	25	62.5	19.2
33	55	857	41	113	1883	87	268	4466	206
34	> 50	>250	>22	>500	>2500	>217	>500	>2500	>217
39	3	7.3	1.1	15	37	5	>500	1250	185
40	15	38	4.5	8.1	20	2.5	6.1	15	1.8
Tmp	>50	>7.5	>0.4	>250	>37	>1.8	>250	>37	>1.8
41	200	67	17	72	24	6	32	11	2.7
44	>250	>532	>15	47	94	2.8	100	200	6
49	>250	>368	>13	>250	>357	>13	>250	>357	>13
51	34	485	6.8	102	1457	20	139	1986	28
52	13	87	3.0	180	1200	42	56	373	13
53	>50	>114	>5	>50	>114	>5	16	36	1.6
54	45	15	13	>50	>17	>14	>50	>17	>14

[a] Cytotoxicity/IC_{50} TM4 ratio. [b] Cytotoxicity/IC_{50} K1 ratio.

Experimental Section

Methods and Materials. For the synthesis of Pyr and Tmp analoques, solvents (THF, DMSO, ethanol, and dioxane) were dried according to standard methods, and reagents were purchased from Fluka and were distilled before use. For enzyme studies, chemicals were obtained from Sigma-Aldrich Ltd., Merck, and BDH and were used without further purification. Melting points were determined by an Electrothermal 9100 melting point apparatus and were uncorrected. Nuclear magnetic resonance (NMR) spectra were recorded in DMSO-d_6 on a Bruker DRX 400 spectrometer; chemical shifts are reported in parts per million (δ). Mass spectra were recorded on a Micromass LCT using the electrospray ionization technique. Elemental analyses were carried out using a Perkin-Elmer Elemental Analyzer PE2400 Series II and a Perkin-Elmer Elemental Analyzer 2400.

Chemical Syntheses of Pyr Analogues. An improved method providing better yield of Pyr analogues has been employed as follows. Several Pyr analogues were prepared, and the results are summarized in Table 7A.

α-(4-Chlorophenyl)-α-propionylacetonitrile (3; $R^1 = R^2 = R^4 = H$, $R^3 = Cl$, $R^5 = CH_2CH_3$). To a solution of LDA (20 mmol, 1 equiv) in THF (30 mL) at −78 °C was added slowly a solution of *p*-chlorophenylacetonitrile (**1**; $R^1 = R^2 = R^4 = H$, $R^3 = Cl$) (3.0 g, 20 mmol, 1 equiv) in THF (30 mL), and the reaction mixture was left stirring at −78 °C for 10 min. A solution of propionyl chloride (**2**, $R^5 = CH_2CH_3$) (2 mL, 20 mmol, 1 equiv) in THF (5 mL) was added into the reaction at −78 °C. After the mixture was quenched with saturated aqueous ammonium chloride solution, the crude mixture was extracted three times with CH_2Cl_2 (3 × 150 mL). The combined CH_2Cl_2 extracts were successively washed with water and saturated aqueous sodium chloride solution, dried over $MgSO_4$, filtered, and evaporated to dryness under reduced pressure. Purification of the crude product by column chromatography (silica gel, 70% CH_2Cl_2/hexane as developing solvent) gave α-(4-chlorophenyl)-α-propionylacetonitrile (**3**; $R^1 = R^2 = R^4 = H$, $R^3 = Cl$, $R^5 = CH_2CH_3$) as white crystals (2.58 g, 62%, mp 54−55 °C, crystallized from a mixture of CH_2Cl_2 and hexane).

α-(4-Chlorophenyl)-β-methoxy-α-propionylacrylonitrile (4; $R^1 = R^2 = R^4 = H$, $R^3 = Cl$, $R^5 = CH_2CH_3$). Diazomethane gas, generated from a reaction of diazald (1.58 g, 7.5 mmol) and sodium hydroxide solution,[20] was passed into a solution of **3** ($R^1 = R^2 = R^4 = H$, $R^3 = Cl$, $R^5 = CH_2CH_3$) (1.04 g, 5 mmol) in cold dioxane (10 mL). The reaction mixture was left at room temperature overnight and evaporated to dryness under reduced pressure to give **4** ($R^1 = R^2 = R^4 = H$, $R^3 = Cl$, $R^5 = CH_2CH_3$) as a yellow oil in quantitative yield. The crude product was used in the next step without purification.

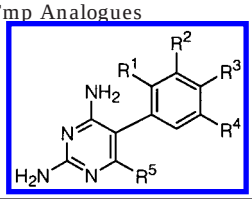
2,4-Diamino-5-(4-chlorophenyl)-6-ethylpyrimidine (5, Pyr; $R^1 = R^2 = R^4 = H$, $R^3 = Cl$, $R^5 = CH_2CH_3$). A mixture of **4** ($R^1 = R^2 = R^4 = H$, $R^3 = Cl$, $R^5 = CH_2CH_3$) (0.66 g, 3 mmol) and guanidine (0.30 g, 5 mmol) in dry DMSO (5 mL) was heated at 80 °C for 5 min under N_2. The reaction mixture was poured into CH_2Cl_2 (100 mL) and was successively washed with water and saturated aqueous sodium chloride solution, dried over $MgSO_4$, filtered, and evaporated to dryness under reduced pressure. Compound **5** ($R^1 = R^2 = R^4 = H$, $R^3 = Cl$, $R^5 = CH_2CH_3$) was crystallized from methanol to afford white crystals (0.70 g, 94%, mp 235−236 °C).[14]

Chemical Syntheses of Tmp Analogues. Tmp analogues were synthesized either by the reported procedure[16] or by an improved method outlined in Scheme 2. Several Tmp analogues were prepared mostly by the latter modified procedure, and the results are summarized in Table 7B.

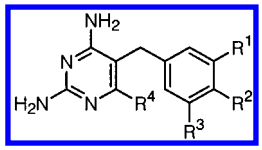
3-Anilino-2-(3′,4′,5′-trimethoxybenzyl)acrylonitrile (8; $R^1 = R^2 = R^3 = OMe$, $R^4 = H$). Compound **8** ($R^1 = R^2 = R^3 =$ OMe, $R^4 = H$) was prepared from β-morpholinopropionitrile (**7**, $R^4 = H$) and 3′,4′,5′-trimethoxybenzaldehyde (**6**; $R^1 = R^2 = R^3 = OMe$) according to the published procedure.[16]

2,4-Diamino-5-(3′,4′,5′-trimethoxybenzyl)pyrimidine (9, Tmp; $R^1 = R^2 = R^3 = OMe$, $R^4 = H$). A mixture of **8** ($R^1 = R^2 = R^3 =$ OMe, $R^4 = H$) (1.01 g, 3.2 mmol) in DMSO (1.5 mL) and guanidine (0.47 g, 8 mmol) in EtOH (5 mL) was heated

Table 7. (A) Data of Pyr Analogues and (B) Data of Tmp Analogues



compd	R^1	R^2	R^3	R^4	R^5	yield (%)	mp (°C)	formula	anal
Pyr[a]	H	H	Cl	H	Et	94	235−236	$C_{12}H_{13}ClN_4$	CHN
14[a]	H	H	Br	H	Et	92	236−237	$C_{12}H_{13}BrN_4$	CHN
15	H	H	Me	H	Et	94	228.5−229	$C_{13}H_{16}N_4$	CHN
16	H	H	Bu^t	H	Et	96	237−237.5	$C_{16}H_{22}N_4$	CHN
17	H	H	Ph	H	Et	94	210−210.5	$C_{18}H_{18}N_4$	CHN
18[b]	H	H	OMe	H	Et	99	259−260	$C_{13}H_{16}N_4O$	CHN
19[a]	H	H	H	H	Et	86	245−246	$C_{12}H_{14}N_4$	CHN
20[b]	H	Cl	H	H	Et	82	211−212	$C_{12}H_{13}ClN_4$	CHN
21[a]	H	Cl	Cl	H	Et	64	216−218	$C_{12}H_{12}Cl_2N_4$	CHN
22[c]	H		OCH_2O	H	Et	87	241−242	$C_{13}H_{14}N_4O_2$	CHN
23	Cl	H	Cl	H	Et	88	219−220	$C_{12}H_{12}Cl_2N_4$	CHN
24	H	OMe	(2,4-Cl_2Ph)CH_2O	H	Et	98	228−229	$C_{20}H_{20}Cl_2N_4O_2$	CHN
25	H	Br	OEt	Br	Et	99	179.5−180	$C_{14}H_{16}Br_2N_4O$	CHN
26[a]	H	H	Cl	H	H	76	199−200	$C_{10}H_9ClN_4$	CHN
27[a]	H	H	Cl	H	Me	96	274−274.5	$C_{11}H_{11}ClN_4$	CHN
28	H	H	Cl	H	$CH_2CH_2CH_2CO_2Me$	84	203−204.5	$C_{15}H_{17}ClN_4O_2$	CHN
29	H	H	Cl	H	$CH_2CH_2CH_2Ph$	82	171.5−172	$C_{19}H_{19}ClN_4$	CHN
30[a]	H	H	H	H	H	62	165−166	$C_{10}H_{10}N_4$	CHN
31[a]	H	H	H	H	Me	92	249−251	$C_{11}H_{12}N_4$	CHN
32	H	H	H	H	$CH_2CH_2CH_2CO_2Me$	94	174−175	$C_{15}H_{18}N_4O_2$	CHN
33	H	H	H	H	hex^n	88	155−156	$C_{16}H_{22}N_4$	CHN
34	H	H	H	H	$CH_2CH_2CH_2Ph$	94	154−154.5	$C_{19}H_{20}N_4$	CHN
35[a]	H	Cl	H	H	H	90	210−211	$C_{10}H_9ClN_4$	CHN
36[b]	H	Cl	H	H	Me	91	221−223	$C_{11}H_{11}ClN_4$	CHN
37	H	Cl	H	H	$CH_2CH_2CH_2CO_2Me$	88	187.5−188	$C_{15}H_{17}ClN_4O_2$	CHN
38	H	Cl	H	H	$CH_2CH_2CH_2Ph$	81	140−141	$C_{19}H_{19}ClN_4$	CHN
39	H	Cl	H	H	$CH_2CH_2CH_2Ph$(MeO-*p*)	90	111−111.5	$C_{20}H_{21}ClN_4O$	CHN
40	H	Cl	H	H	$CH_2CH_2OCH_2CH_2CH_2OPh$	56	93−94	$C_{21}H_{23}ClN_4O_2$	CHN



compd	R^1	R^2	R^3	R^4	yield (%)	mp (°C)	formula	anal
Tmp[d]	OMe	OMe	OMe	H	84	200−201	$C_{14}H_{18}N_4O_3$	CHN
41	OMe	$OCH_2CH_2CH_2Ph$	H	H	80	134.5−135.5	$C_{21}H_{24}N_4O_2$	CHN
42	OMe	OCH_2Ph	H	H	84	162.5−163	$C_{19}H_{20}N_4O_2$	CH[e]
43	OMe	OCH_2Ph	OMe	H	78	167.5−168	$C_{20}H_{22}N_4O_3$	CHN
44	OEt	OCH_2Ph	H	H	65	173.5−174	$C_{20}H_{22}N_4O_2$	CHN
45	OCH_2Ph	OCH_2Ph	H	H	63	137−137.5	$C_{25}H_{24}N_4O_2$	N[f]
46	OCH_2Ph	OCH_2Ph	H	Me	92	152.5−153.5	$C_{26}H_{26}N_4O_2$	N[g]
47	OCH_2Ph	OCH_2Ph	H	Et	91	114.5−115	$C_{27}H_{28}N_4O_2$	CN[h]
48	OCH_2-[3,4,5-$(OMe)_3$]Ph	OCH_2Ph	H	H	80	223.5−224.5	$C_{28}H_{30}N_4O_5$	CHN
49	H	OCH_2-[3,4,5-$(OMe)_3$]Ph	H	H	89	174−174.5	$C_{21}H_{24}N_4O_4$	CHN
50	OMe	OCH_2-[3,4,5-$(OMe)_3$]Ph	H	H	90	155.5−156	$C_{22}H_{26}N_4O_5$	H[i]
51	OEt	OCH_2-[3,4,5-$(OMe)_3$]Ph	H	H	83	153−154	$C_{23}H_{28}N_4O_5$	CN[j]
52	OPr^n	OCH_2-[3,4,5-$(OMe)_3$]Ph	H	H	65	143.5−144.5	$C_{24}H_{30}N_4O_5$	CN[k]
53	OBu^n	OCH_2-[3,4,5-$(OMe)_3$]Ph	H	H	87	154.5−155.5	$C_{25}H_{32}N_4O_5$	C[l]
54	H	$OCH_2CH_2CH_2O$-(2,4,5-Cl_3)Ph	H	H	87	165−166	$C_{20}H_{19}N_4O_2Cl_3$	C[m]

[a] Data from ref 14. [b] Data from ref 25. [c] Data from ref 15. [d] Data from ref 17. [e] N calcd, 16.66; found, 16.09. [f] C calcd, 72.78; found, 72.09. H calcd, 5.87; found, 6.91. [g] C calcd, 73.16; found, 72.95. H calcd, 6.15; found, 6.64. [h] H calcd, 6.41; found, 6.92. [i] C calcd, 61.94; found, 61.42. N calcd, 13.14; found, 12.63. [j] H calcd, 6.41; found, 5.75. [k] H calcd, 6.65; found, 5.78. [l] H calcd, 6.89; found, 5.96. N calcd, 11.97; found, 11.32. [m] H calcd, 4.22; found, 4.89. N calcd, 12.35; found, 11.69.

under reflux for 18 h. Ethanol was removed by distillation. The mixture was cooled to 5 °C, diluted with H_2O (20 mL), and further stirred at 5 °C for 2 h. The precipitate obtained was collected by filtration and washed successively with H_2O and cold acetone to yield partially pure Tmp ($R^1 = R^2 = R^3 =$ OMe, $R^4 =$ H) as a yellow solid (0.64 g, 84%).[17] White crystals of Tmp were obtained after recrystallization from MeOH.

3-Methoxy-2-(3′,4′,5′-trimethoxybenzyl)crotononitrile (12; $R^1 = R^2 = R^3 =$ OMe, $R^4 =$ Me). Compound **11** ($R^1 = R^2 = R^3 =$ OMe, $R^4 =$ Me) was prepared from **6** ($R^1 = R^2 = R^3 =$ OMe) and **10** ($R^4 =$ Me) according to the published procedure.[16] Diazomethane gas, generated from a reaction of diazald (2.7 g, 12.6 mmol) and sodium hydroxide solution,[20] was passed into a solution of **11** ($R^1 = R^2 = R^3 =$ OMe, $R^4 =$ Me) (1.11 g, 4.2 mmol) in cold dioxane (2 mL). The reaction mixture was left at room temperature overnight and evaporated to dryness. The crude product was crystallized from MeOH to obtain white crystals of **12** ($R^1 = R^2 = R^3 =$ OMe, $R^4 =$ Me).

2,4-Diamino-6-methyl-5-(3′,4′,5′-trimethoxybenzyl)pyrimidine (13; $R^1 = R^2 = R^3 =$ OMe, $R^4 =$ Me). Compound **13** ($R^1 = R^2 = R^3 =$ OMe, $R^4 =$ Me) (white crystals) was

prepared from the enol ether **12** ($R^1 = R^2 = R^3 = OMe$, $R^4 = Me$) and guanidine according to the procedure given for **9**, followed by recrystallization from MeOH.

Mutants Preparation. The pfDHFR mutants with all possible amino acids at residue 108 were prepared by cassette mutagenesis using pUC−pfDHFR, which carries the wild-type synthetic *dhfr* gene of *P. falciparum* as a template and were those previously described.[18] Expression in *E. coli* BL21(DE3)-pLysS of the mutant pfDHFRs was achieved by IPTG induction as previously described[18] with slight modification in which the induction temperature was reduced to 20 °C and the induction time was increased to 20 h. The cell extract obtained after disruption of the bacterial cells by a French pressure cell at 18 000 psi was assayed for DHFR activities. The pfDHFR mutants with active and/or detectable DHFR activities were further purified by affinity chromatography using Methotrexate-Sepharose resin.

Enzyme Assays and Inhibition by Antifolates and Derivatives. The activity of pfDHFR was determined spectrophotometrically at 25 °C according to the method previously described[6] using a Hewlette Packard UV−vis spectrophotometer (HP 8453). The reaction (1 mL) contained 1×DHFR buffer (50 mM TES, pH 7.0, 75 mM β-mercaptoethanol, 1 mg/mL bovine serum albumin), 100 μM each of the substrate H_2folate and cofactor, reduced nicotinamide adenine dinucleotide phosphate (NADPH), and an appropriate amount (0.001−0.005 units in phosphate buffer containing 50 mM KCl) of affinity-purified enzyme to initiate the reaction. The final concentration of KCl present in the assay reaction (~0.5 mM) did not affect the activity of the enzyme.[6] One unit of enzyme is defined as the amount of enzyme required to produce 1 μmol of product/min at 25 °C. The inhibition of the enzymes with Pyr and its substituted derivatives was investigated in a 96 well plate with 200 μL reaction of the above mixture, in the presence of antifolate. Verification of the mode of inhibition of the enzyme by selected analogues was performed by determining the DHFR activities at different concentrations of inhibitors in the presence of varying concentration of substrate H_2folate using the standard condition as described above, and the reciprocal of initial velocity data was plotted against the reciprocal of substrate concentrations. For the determination of kinetic parameters, the kinetic reactions at 340 nm were followed using a microplate reader (Labsystems, Finland). The K_i values of the inhibitors for the wild-type and mutant enzymes were determined by fitting to the equation $IC_{50} = K_i(1 + ([S]/K_m))$,[21] where IC_{50} is the concentration of inhibitor that inhibits 50% of the enzyme activity under the standard assay condition and K_m is the Michaelis constant for the substrate H_2folate. This equation assumes competitive inhibition.

Parasite Culture and Antimalarial Testing In Vitro. Two *P. falciparum* clones, TM4/8.2 (Wild-type DHFR) and K1CB1 (C59R+S108N-DHFR), were kindly provided by S. Thaithong, Department of Biology, Faculty of Science, Chulalongkorn University.[22] The parasites were maintained continuously in human erythrocytes at 37 °C under 3% CO_2 in RPMI 1640 culture media supplemented with 25 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid, pH 7.4, 0.2% $NaHCO_3$, 40 μg/mL gentamicin, and 10% human serum.[23] In vitro antimalarial activity was determined by using the [^{3}H]-hypoxanthine incorporation method.[24]

The drugs were initially dissolved in DMSO and diluted with the culture media. Aliquots (25 μL) of the drug of different concentrations were dispensed in 96 well plates, and 200 μL of 1.5% cell suspension of parasitized erythrocytes containing 1−2% parasitemia was added. The final concentration of DMSO (0.1%) did not affect the parasite growth. The mixtures were incubated in a 3% CO_2 incubator at 37 °C. After 24 h of incubation, 25 μL (0.25 μCi) of [^{3}H]-hypoxanthine was added to each well. The parasite cultures were further incubated under the same conditions for 18−24 h. DNA of parasites was harvested onto glass filter paper (Unifilter, Packard, USA). The filters were air-dried, and 20 μL liquid scintillation fluid (Microscint, Packard) was added. The radioactivity on the filters was then measured using a microplate scintillation counter (Topcount, Packard, USA). The concentration of inhibitor that inhibited 50% of the parasite growth (IC_{50}) was determined from the sigmoidal curve obtained by plotting the percentages of [^{3}H]-hypoxanthine incorporation against drug concentrations. Cytotoxicity tests of some analogues against vero cells, KB cells, and BC cells were performed according to the protocol described by Skehan et al. (1990).[26]

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Supporting Information Available: Additional experimental data (^{1}H NMR) of all compounds not listed in the Experimental Section. This material is available free charge via the Internet at http://pubs.acs.org.

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