Inhibitors of Multiple Mutants of *Plasmodium falciparum* Dihydrofolate Reductase and Their Antimalarial Activities

Sumalee Kamchonwongpaisan,[†] Rachel Quarrell,[‡] Netnapa Charoensetakul,[§] Rachel Ponsinet,[‡] Tirayut Vilaivan,[§] Jarunee Vanichtanankul,[†] Bongkoch Tarnchompoo,[†] Worachart Sirawaraporn,[||] Gordon Lowe,[‡,⊥] and Yongyuth Yuthavong*[,†]

National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathumthani 12120, Thailand; Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand; Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok 10400, Thailand, and The Dyson Perrins Laboratory, Department of Chemistry, Oxford University, South Parks Road, Oxford, OX1 3QY, U.K.

Received April 10, 2003

Novel analogues of pyrimethamine (Pyr) and cycloguanil (Cyc) have been synthesized and tested as inhibitors of *Plasmodium falciparum* dihydrofolate reductase carrying triple (N51I+C59R+S108N, C59R+S108N+I164L) and quadruple (N51I+C59R+S108N+I164L) mutations responsible for antifolate resistance. The inhibitors were designed to avoid steric clash of the *p*-Cl group of the inhibitors with the side chain of Asn108, augmented by additional mutations of the resistant mutants. Cycloguanil derivatives were also designed to avoid steric clash with the side chain of Val16 in the A16V+S108T mutant. Many compounds have inhibition constants (K_i) at the low nanomolar level against the mutant enzymes and a number have good antimalarial activities against resistant *P. falciparum* parasites bearing multiple mutations in the S108N series and A16V+S108T mutant enzymes. These compounds in the Pyr and Cyc series exhibit low and moderate cytotoxicity to nontumor (Vero) and tumor (KB, BC) cell lines. Some of these inhibitors are therefore potential candidates for further development as antimalarials.

Introduction

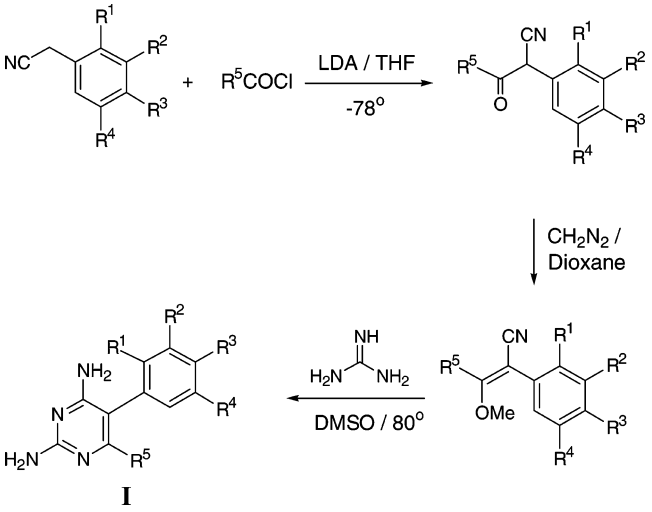
Resistance of *Plasmodium falciparum* (*Pf*) to antifolates is an important problem in antimalarial chemotherapy[1,2] and has been shown to be associated with mutations in the dihydrofolate reductase (DHFR) domain of the bifunctional dihydrofolate reductase-thymidylate synthase (DHFR-TS).[3,4] The level of resistance generally becomes higher as the number of mutations increases.[5−7] A moderate level of resistance found with parasites carrying the single S108N mutation contrasts with higher levels of resistance in parasites carrying the C59R+S108N (double) and N51I+C59R+S108N and C59R+S108N+I164L (triple) mutations and still higher levels in parasites with the N51I+C59R+S108N+I164L (quadruple) mutant enzyme.[5−7] Another double mutant, A16V+S108T, is associated with only cycloguanil (Cyc) resistance.[5] Such mutation-based resistance raises serious questions on the possibility of developing new antifolates with prolonged useful therapeutic life, since new mutations could conceivably arise to compromise the new antifolates. However, the parasites do not have unlimited mutational possibilities, since they need to maintain a functional DHFR.[1,8] Hence, it may be possible to develop inhibitors against which mutations would not be possible, since they would also lead to nonfunctional enzymes.

On the basis of modeling of the wild-type and mutant *Pf*DHFRs, we designed and synthesized a number of Cyc-derived inhibitors effective against both the wild-type and the A16V+S108T mutant[9,10] and pyrimethamine- (Pyr) and Cyc-derived inhibitors effective against S108N and C59R+S108N mutants.[11] The main strategy for obtaining Cyc-derived inhibitors effective against the A16V+S108T mutant *Pf*DHFR was to avoid the steric clash between one of the two 2,2-dimethyl groups and the side chain of Val16.[9,10] Likewise, the main strategy for designing effective Pyr- and Cyc-derived inhibitors against the S108N and C59R+S108N mutant *Pf*DHFRs was to avoid the steric clash between the Cl atom of the *p*-chlorophenyl group of inhibitors and the Asn108 side chain, as previously observed by McKie et al.[12] However, it is not known whether Cyc-derived inhibitors designed against the A16V+S108T mutant would be effective against DHFR mutants in the S108N series, and vice versa, and whether new inhibitors can be designed to avoid steric conflicts resulting from mutations in both series. Effective inhibitors against various mutant DHFRs in both series and against resistant parasites harboring those DHFRs would obviously be desirable as lead antimalarial compounds. In this paper, we show that by employing the strategy of avoiding steric clashes around both residues 16 and 108, a number of Cyc-derived inhibitors can be obtained and shown to be effective against both the A16V+S108T mutant and also multiple mutant *Pf*DHFRs in the S108N series. Pyr-derived inhibitors effective against multiple mutant DHFRs can also likewise be obtained. A number of these inhibitors are shown to have good antimalarial activities against *P.*

* Corresponding author. Phone: 662 564 6700. Fax: 662 644 6707. E-mail: yongyuth@nstda.or.th.
[⊥] Deceased, to whom this work is dedicated.
[†] National Science and Technology Development Agency.
[‡] Oxford University.
[§] Chulalongkorn University.
[||] Mahidol University.

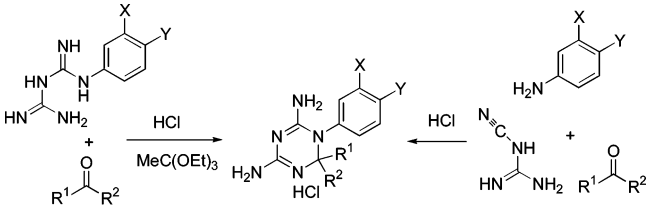
10.1021/jm030165t CCC: $27.50
Published on Web 12/23/2003

Scheme 1. General Procedure for Synthesis of Pyr Analogues



I

Scheme 2. General Procedure for Synthesis of Cyc Analogues



II

falciparum bearing mutant DHFRs with low cytotoxicity to mammalian cells. From our results, the general characteristics of such effective inhibitors with less toxicity can be derived, which might be used for further development of new effective inhibitors.

Results and Discussion

Chemical Syntheses. Syntheses of 2,4-diaminopyrimidine analogues (**I**) were described elsewhere.[11,13] Syntheses of *N*-1-aryl-4,6-diamino-1,2-dihydro-1,3,5-triazines (**II**) were achieved by a one-step acid-catalyzed cyclocondensation between an aromatic amine, dicyandiamide, and a carbonyl compound (3-component syntheses)[14] or in two steps between the preformed arylbiguanide and a carbonyl compound (two-component syntheses).[15,16] General procedures for synthesis of Pyr (Scheme 1) and Cyc (Scheme 2) are as outlined.

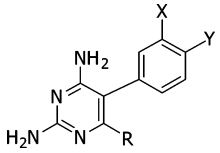
Strategies for Development of Inhibitors of Multiple Mutant PfDHFRs. As previously observed, the S108N mutation probably decreases the affinity of Pyr binding to the enzyme through the steric interaction of the *p*-Cl group with the larger side chain of Asn108.[11,12,17] Modeling studies,[10,12] supported by X-ray diffraction of wild-type and mutant enzymes,[18] showed that both Pyr and Cyc derivatives are locked in the active site with the *p*-Cl group in steric conflict with the Asn108 side chain. Additional mutations, namely N51I, C59R, and I164L, reduce the binding further through unfavorable interaction of the enzyme with other parts of the inhibitor.[10,18] However, the fact that S108N mutation was the first to arise through Pyr and Cyc selection, followed by additional mutations,[17] indicates that these additional mutations cooperate with the S108N mutation to reduce inhibitor binding through enhanced steric constraint at this site. Removal of the *p*-Cl or shifting it to another position should result in inhibitors that retain the binding affinities with the *Pf*DHFR despite multiple mutations. This was shown for the double (C59R+S108N) mutant DHFR.[11,12]

A series of Pyr analogues, some of which have previously been reported to be effective against the C59R+S108N mutant,[11] were examined for their inhibitory effect against multiple mutant enzymes. Table 1 shows that the ratios of K_i(mutant) to K_i(wild-type) for various Pyr derivatives with *p*-Cl (Pyr, **6**, **9**) or other bulky groups in the *p*-position (**1**, **3**) tend to be higher with additional mutation, from N51I+C59R+S108N and C59R+S108N+I164L to N51I+C59R+S108N+I164L, whereas these ratios are not greatly affected by additional mutations for compounds without the substituent (**4**, **7**, **10**, **12**) or those with *m*-Cl substituent (**5**, **8**, **11**, **13**, **15**, **17**, **19**−**22**), indicating the retention of their affinities for the parasite mutant enzymes. In comparing derivatives with similar substituents at C-6, moving the *p*-Cl group (Pyr, **6**, **9**) to the *m*-position (**5**, **8**, **11**) resulted in drastic improvement (85−133-fold) in K_i values of the inhibitors against the quadruple mutant and to a much greater extent than observed for single or double mutants,[11,12] while removing the Cl group (**4**, **7**, **10**) improved K_i against the same mutant by 12−36 times. In general, *m*-Cl derivatives are about 10-fold more effective than their corresponding unsubstituted compounds. Pyr analogue **2**, with two Cl substitutions, at *m*- and *p*-positions, had 3−21-fold better binding affinities than Pyr, implying that the *m*-Cl group may interact favorably with the enzyme active site to enhance the binding and help to reduce the deleterious effect of the *p*-Cl group. The effect is however poorer with the increasing mutations of *Pf*DHFR, probably owing to increased steric constraint imposed by the *p*-Cl group.

The effect of moving the Cl of Cyc derivatives from the *p*- to the *m*-position of the N-1 phenyl substituent on binding to the A16V+S108T mutant DHFR is summarized in Table 2. Shifting the *p*-Cl group of Cyc, **26**, **24**, **28**, and **30** to the *m*-position, giving **23**, **25**, **27**, **29**, and **31**, resulted in reduction of the K_i values by a factor of 4−95, while removing one methyl group at C-2 of Cyc (giving **30**) reduces the K_i value by a factor of 10 as compared to Cyc. Simultaneous removal of one of the C-2 methyl groups and shifting the chloro group at the N-1 from the *p*- to the *m*-position resulted in an analogue (**31**) with a K_i value 30 times lower than Cyc. Moving the *p*-Cl group to the *m*-position improved the binding affinities to the A16V+S108T mutant of other Cyc derivatives with only one substituent at the C-2 position to various extents (**32**−**46**), with smaller effects for derivatives already exhibiting good binding at low nanomolar concentrations (**34**−**46**).

From our results, we can conclude that the binding environments around residues 16 and 108 both contribute to the decrease in the affinities of the mutant enzyme for Cyc, and combined mutations contribute more than the sum of component mutations. As Table 3 shows, relief of the steric effects around both regions contributed to binding enhancement of the derivatives,

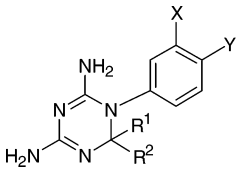
Table 1. Inhibition Constants (K_i) of 2,4-Diaminopyrimidine Derivatives with Various C-5 and C-6 Substituents for Binding with Triple (N51I+C59R+S108N and C59R+S108N+I164L) and Quadruple (N51I+C59R+S108N+I164L) Mutant *Pf*DHFRs



	substituents				N51I+C59R+S108N		C59R+S108N+I164L		N51I+C59R+S108N+I164L	
compd	X	Y	R	K_i(wt)[b] (nM)	K_i(mut)[b] (nM)	K_i(mut)/K_i(wt)	K_i(mut) (nM)	K_i(mut)/K_i(wt)	K_i(mut) (nM)	K_i(mut)/K_i(wt)
Pyr (P1)	H	Cl	Et	0.6 ± 0.2[a]	67.1 ± 4.2	111.8	112 ± 17	186.7	385 ± 163	641.7
1 (P17)	H	Me	Et	0.4 ± 0.0[a]	6.9 ± 1.7	17.2	88 ± 22	220.0	284 ± 121	710.0
2 (P13)	Cl	Cl	Et	1.0 ± 0.3[a]	3.1 ± 0.4	3.1	41 ± 7	41.0	53 ± 10	53.0
3 (P15)	−OCH_2O−		Et	1.1 ± 0.3[a]	8.3 ± 1.8	7.5	121 ± 13	110.0	269 ± 36	244.5
4 (P20)	H	H	Et	2.3 ± 0.3[a]	3.0 ± 0.4	1.3	25 ± 5	10.9	32 ± 8	13.9
5 (P30)	Cl	H	Et	0.8 ± 0.1[a]	2.1 ± 0.2	2.6	2.7 ± 0.4	3.4	3.3 ± 0.4	4.1
6 (P16)	H	Cl	$(CH_2)_3COOMe$	0.3 ± 0.0[a]	29.3 ±1.9	97.7	145 ± 33	483.3	360 ± 100	1200.0
7 (P26)	H	H	$(CH_2)_3COOMe$	0.6 ± 0.0[a]	3.3 ± 0.6	5.5	15 ± 3	25.0	24 ± 2	40.0
8 (P29)	Cl	H	$(CH_2)_3COOMe$	0.5 ± 0.0[a]	0.77 ± 0.2	1.5	1.8 ± 0.4	3.6	2.7 ± 1.2	5.4
9 (P12)	H	Cl	$(CH_2)_3$ Ph	0.7 ± 0.1[a]	15.1 ± 0.6	21.6	63 ± 7.8	90.0	170 ± 33	242.9
10 (P33)	H	H	$(CH_2)_3Ph$	0.5 ± 0.0[a]	1.1 ± 0.1	2.2	1.9 ± 0.1	3.8	4.7 ± 0.9	9.4
11 (P31)	Cl	H	$(CH_2)_3Ph$	1.2 ± 0.2[a]	2.0 ± 0.2	1.7	2.2 ± 0.3	1.8	2.0 ± 0.8	1.7
12 (P45)	H	H	$(CH_2)_3OH$	85 ± 2.8	58.5 ± 13	0.7	121 ± 20	1.4	549 ± 12	6.5
13 (P41)	Cl	H	$(CH_2)_3OH$	9.2 ± 1.3	2.3 ± 0.3	0.25	29 ± 0.7	3.2	57 ± 11	6.1
14 (P46)	H	H	$(CH_2)_3OCOCH_3$	10.6 ± 2.2	5.0 ± 0.8	0.5	18 ± 3.6	1.7	237 ± 2.4	22.3
15 (P42)	Cl	H	$(CH_2)_3OCOCH_3$	3.1 ± 0.7	1.8 ± 0.2	0.6	3.2 ± 0.0	1.0	31.4 ± 4.1	10.1
16 (P47)	H	H	$(CH_2)_3OCOC_6H_5$	3.4 ± 0.4	0.7± 0.1	0.2	2.7 ± 0.4	0.8	14.0 ± 1.4	4.1
17 (P43)	Cl	H	$(CH_2)_3OCOC_6H_5$	1.5 ± 0.4	0.8 ± 0.2	0.5	0.8 ± 0.0	0.5	3.6 ± 0.3	2.4
18 (P39)	H	H	nC_6H_{13}	0.3 ± 0.1[a]	0.4 ± 0.02	1.3	0.7 ± 0.2	2.3	1.4 ± 0.5	4.7
19 (P44)	Cl	H	$(CH_2)_3OCOOCH_2C_6H_5$	1.2 ± 0.1	1.1 ± 0.2	0.9	0.8 ± 0.2	0.7	3.6 ± 0.2	3.0
20 (P38)	Cl	H	Me	1.9 ± 0.5[a]	6.5 ± 1.3	3.4	9.8 ± 2.8	5.2	14 ± 1.3	7.4
21 (P32)	Cl	H	$(CH_2)_3C_6H_4$-(*p*-OMe)	2.2 ± 0.6[a]	1.3 ± 0.1	0.6	2.9 ± 0.3	1.3	2.0 ± 0.4	0.9
22 (P40)	Cl	H	$(CH_2)_2O(CH_2)_3OPh$	0.4 ± 0.2[a]	0.7 ± 0.1	1.7	1.3 ± 0.4	3.2	1.7 ± 0.2	4.2

[a] Data from ref 11. [b] K_i(wt) is the K_i value for the wild-type *Pf*DHFR, and K_i(mut) is the K_i value for the mutant.

Table 2. Inhibition Constants (K_i) for Binding with Wild-Type and Mutant (A16V+S108T) *Pf*DHFR of 1,3,5-Dihydrotriazine Derivatives with Various N-1 (X, Y) and C-2 (R^1, R^2) Substituents



compd	X	Y	R^1	R^2	K_i(wt)[a] (nM)	K_i(mut)[a] (nM)	K_i(mut)/K_i(wt)
Cyc[b] (C21)	H	Cl	Me	Me	1.5 ± 0.3	1314 ± 165	876
23[b] (C97)	Cl	H	Me	Me	3.0 ± 0.2	308 ± 53	103
24 (C66)	H	Cl	Me	*n*Pr	3.5 ± 0.4	9229 ± 547	2637
25 (C433)	Cl	H	Me	*i*Pr	25.5 ± 2.8	2177 ± 348	85
26 (C22)	H	Cl	Me	*i*Pr	36.5 ± 4.1	44791 ± 5872	1227
27 (C434)	Cl	H	Me	*n*Pr	4.6 ± 0.4	471 ± 95	102
28 (C71)	H	Cl	Me	*n*Hex	0.6 ± 0.1	955 ± 77	1592
29 (C435)	Cl	H	Me	*n*Hex	2.4 ± 0.2	101 ± 9	41.9
30[b] (C17)	H	Cl	H	Me	4.1 ± 0.0	127 ± 14	31.0
31[b] (C248)	Cl	H	H	Me	10.2 ± 0.6	39 ± 2.9	3.8
32[b] (C53)	H	Cl	H	C_6H_5	4.5 ± 0.2	49.3 ± 3.3	11.0
33[b] (C96)	Cl	H	H	C_6H_5	11.7 ± 2.5	10.5 ± 7.4	0.9
34 (C121)	H	Cl	H	4-$C_6H_5OC_6H_4$	0.4 ± 0.0	3.8 ± 0.4	9.5
35 (C138)	Cl	H	H	4-$C_6H_5OC_6H_4$	0.7 ± 0.0	2.7 ± 0.4	3.9
36 (C133)	H	Cl	H	3-$C_6H_5OC_6H_4$	0.5 ± 0.0	2.7 ± 0.3	5.4
37 (C110)	Cl	H	H	3-$C_6H_5OC_6H_4$	1.1 ± 0.1	2.5 ± 0.3	2.3
38 (C111)	H	Cl	H	3-$C_6H_5CH_2OC_6H_4$	0.7 ± 0.0	6.2 ± 0.5	8.9
39 (C185)	Cl	H	H	3-$C_6H_5CH_2OC_6H_4$	2.3 ± 0.5	3.2 ± 0.3	1.4
40 (C188)	H	Cl	H	3-(4-$ClC_6H_4O)C_6H_4$	1.4 ± 0.3	7.5 ± 0.5	5.4
41 (C143)	Cl	H	H	3-(4-$ClC_6H_4O)C_6H_4$	1.3 ± 0.0	3.5 ± 0.5	2.7
42 (C372)	Cl	H	H	nC_7H_{15}	2.7 ± 0.1	3.8 ± 0.1	1.4
43 (C229)	Cl	H	H	4-$PrOC_6H_4$	3.3 ± 0.4	9.3 ± 0.6	2.8
44 (C186)	Cl	H	H	3-(3,5-$Cl_2C_6H_3O)C_6H_4$	1.8 ± 0.6	4.7 ± 0.4	2.6
45 (C313)	Cl	H	H	3-[2,4,5-$Cl_3C_6H_2O(CH_2)_3O]C_6H_4$	4.0 ± 2.2	2.6 ± 0.1	0.7
46 (C299)	Cl	H	H	3-(3-$CF_3C_6H_4O)C_6H_4$	2.7 ± 0.6	6.5 ± 0.5	2.4

[a] K_i(wt) is the K_i value for the wild-type *Pf*DHFR, and K_i(mut) is the K_i value for the A16V+S108T mutant. [b] Data from ref 9.

Table 3. Inhibition Constants (K_i) of 1,3,5-Dihydrotriazine Derivatives in Binding with Wild-Type and Multiple Mutant *Pf*DHFRs

		N51I+C59R +S108N		C59R+S108N+I164L		N51I+C59R+S108N+I164L	
compd	K_i(wt)[b] (nM)	K_i(mut)[b] (nM)	K_i(mut)/ K_i(wt)	K_i(mut) (nM)	K_i(mut)/ K_i(wt)	K_i(mut) (nM)	K_i(mut)/ K_i(wt)
Cyc (C21)	1.5 ± 0.3[a]	31.4 ± 1.2	20.9	285 ± 19	190	420 ± 130	280
23 (C97)	3.0 ± 0.2[a]	2.2 ± 0.2	0.7	14 ± 1	4.7	25 ± 5	8.3
24 (C66)	3.5 ± 0.4	560 ± 83.6	160	3654 ± 1037	1044	4432 ± 355	1266
25 (C433)	25.5 ± 2.8	196 ± 24.4	7.7	213 ± 22	8.4	472 ± 41	18.1
26 (C22)	36.5 ± 4.1	1367 ± 381.3	37.5	4622 ± 1065	127	>10000	>274
27 (C434)	4.6 ± 0.4	9.7 ± 1.2	2.1	33.1 ± 2.7	7.2	39.4± 4.6	8.53
28 (C71)	0.6 ± 0.1	11.3 ± 2.54	18.8	41.8 ± 7.3	69.6	69.2 ± 11.7	115
29 (C435)	2.4 ± 0.2	5.5 ± 0.4	2.3	12.1 ± 0.8	5.0	15.9 ± 1.0	6.58
30 (C17)	4.1 ± 0.0[a]	134 ± 13.5	32.7	1582 ± 556	386	2427 ± 201	592
31 (C248)	10.2 ± 0.6[a]	4.2 ± 0.6	0.4	9.9 ± 3.3	1.0	14.3 ± 4.6	1.4
32 (C53)	4.5 ± 0.2[a]	196 ± 30	43.5	1866 ± 231	415	2436 ± 1257	541
33 (C96)	11.7 ± 2.5[a]	111 ± 32	9.5	217 ± 39	18.5	984 ± 139	84.1
34 (C121)	0.4 ± 0.0	29.3 ± 4.6	73.1	145 ± 19	363	222 ± 112	555
35 (C138)	0.7 ± 0.0	0.9 ± 0.2	1.2	0.3 ± 0.03	0.5	1.3 ± 0.05	1.9
36 (C133)	0.5 ± 0.0	23.6 ± 7.9	47.1	132 ± 23	264	239 ± 26	478
37 (C110)	1.1 ± 0.1	2.5 ± 0.5	2.3	3 ± 1	2.7	5.8 ± 1.8	5.3
38 (C111)	0.7 ± 0.0	220 ± 23	315	771 ± 11	1101	638 ± 139	911
39 (C185)	2.3 ± 0.5	3.2 ± 0.4	1.4	3.6 ± 1.0	1.6	7.7 ± 2.8	3.3
40 (C188)	1.4 ± 0.3	85.5 ± 12	61.1	151 ± 31	108	121 ± 13	86.4
41 (C143)	1.3 ± 0.0	1.9 ± 0.3	1.5	5.1 ± 0.5	3.9	8 ± 2	6.1
42 (C372)	2.7 ± 0.1	1.0 ± 0.1	0.4	0.3 ± 0.0	0.1	0.8 ± 0.0	0.3
43 (C229)	3.3 ± 0.4	2.1 ± 0.6	0.6	0.5 ± 0.1	0.2	2.7 ± 0.2	0.8
44 (C186)	1.8 ± 0.6	1.0 ± 0.1	0.6	0.6 ± 0.2	0.3	2.7 ± 0.4	1.5
45 (C313)	4.0 ± 2.2	2.0 ± 0.2	0.5	2.2 ± 0.2	0.6	4.5 ± 0.5	1.1
46 (C299)	2.7 ± 0.6	2.0 ± 0.2	0.7	0.6 ± 0.0	0.2	4.8 ± 0.4	1.8

[a] Data from ref 9. [b] K_i(wt) is the K_i value for the wild-type *Pf*DHFR, and K_i(mut) is the K_i value for the mutant.

although the combined effects also include further contribution to binding of the C-2 -substituents to the mutant enzyme, complicating the synergy between the two sites. Table 3 shows drastic changes in K_i values for the triple and quadruple mutants of DHFR in the series of cycloguanil derivatives, where moving the *p*-Cl group to the *m*-position caused 15−170-fold decreases in the K_i to the quadruple mutant (Cyc, **23**−**41**). The binding affinity for the quadruple mutant enzyme was marginally improved when only one Me substituent was present at the 2-position of the dihydrotriazine ring (compound **31** compared to **23**), indicating that the linkage between the steric constraints around residue 108 and residue 16 may not be as strong in these multiple mutant DHFRs as in the A16V+S108T mutant. This result is not surprising, considering that residue 16 in these mutants is still the original Ala, with a relatively small side chain. Indeed, the C-2-monosubstituted derivatives with the *N*-1-*p*-chlorophenyl group (**30**, **32**, **34**, **36**, **38**, **40**) have poorer or only marginally better binding with the mutiple mutant enzymes than Cyc, while the C-2-monosubstituted analogues with the *N*-1-*m*-chlorophenyl group (**31**, **33**, **35**, **37**, **39**, **41**−**46**) were very effective against these multiple mutants, with K_i values in the low nanomolar region for both the triple and quadruple mutant enzymes. The strong affinities of these derivatives for the mutant enzymes in the S108N series are probably due to the contributions of the C-2-substituents, as observed for the A16V+S108T mutant.

In Vitro Antiplasmodial Activity. The effectiveness of Pyr and Cyc analogues against multiple mutant *Pf*DHFRs was determined against *P. falciparum* carrying the corresponding mutant enzymes. The data in Table 4 (Pyr series) show that some of the compounds have good in vitro antiplasmodial activities (IC_{50} values) against resistant strains of *P. falciparum*. Most of these inhibitors have been shown to be effective against

Table 4. Antiplasmodial Activities (IC_{50}) of 2,4-Diaminopyrimidine Inhibitors against *P. falciparum* with Wild-Type (TM4/8.2) and the Mutant Enzymes: N51I+C59R+S108N (W2), C59R+S108N+I164L (Csl-2), and N51I+C59R+S108N+I164L (V1/S)

	IC_{50} (μM)			
compd	TM4/8.2	W2	Csl-2	V1/S
Pyr (P1)	0.08 ± 0.01[a]	73.5 ± 8.5	41.7 ± 14.8	>100
5 (P30)	0.42 ± 0.1[a]	3.9 ± 0.8	2.5 ± 0.4	9.1 ± 2.8
10 (P33)	0.21 ± 0.06[a]	2.7 ± 0.6	2.7 ± 0.7	8.6 ± 2.4
11 (P31)	0.67 ± 0.06[a]	4.3 ± 0.7	3.6 ± 0.4	5.7 ± 2.5
13 (P41)	1.1 ± 0.2	4.5 ± 1.7	1.9 ± 0.7	6.2 ± 1.0
15 (P42)	1.6 ± 0.7	2.1 ± 0.4	1.8 ± 0.3	5.3 ± 1.0
17 (P43)	2.1 ± 1.0	2.0 ± 0.6	1.9 ± 0.4	7.5 ± 1.1
18 (P39)	0.06 ± 0.02[a]	2.3 ± 0.7	3.0 ± 1.1	6.4 ± 2.9
19 (P44)	1.9 ± 0.7	4.1 ± 2.0	1.9 ± 0.8	5.9 ± 1.2
21 (P32)	0.41 ± 0.05[a]	4.0 ± 0.3	2.7 ± 0.3	4.7 ± 0.6
22 (P40)	0.35 ± 0.08[a]	4.5 ± 0.4	2.9 ± 0.4	4.6 ± 0.2

[a] Data from ref 11.

double mutants with low toxicity to mammalian cell lines.[11] Pyr analogues bearing a *m*-Cl-phenyl (**5**, **11**, **13**, **15**, **17**, **19**, **21**, **22**) and an unsubstituted C-5-phenyl group (**4**, **7**, **10**, **16**, **18**) exhibit IC_{50} values at the low micromolar level against the resistant parasite carrying DHFR with triple (W2 and Csl-2) and quadruple (V1/S) mutations, which are about 10−25 times more effective than their parent compound. The IC_{50} ratios for the resistant strains to the wild-type (TM4/8.2) parasite are also far lower than the parent compounds.

All Cyc analogues with monosubstitution at the C-2-position of the dihydrotriazine ring, especially designed for the A16V+S108T mutant, were highly effective against the T9/94RC17 parasite carrying A16V+S108T DHFR mutant with IC_{50} values in the nanomolar levels (Table 5). These C-2-monosubstituted Cyc derivatives with a *m*-chlorophenyl group at N-1 were also effective against the malaria parasites with triple and quadruple mutations at low micromolar level. Surprisingly, some *N*-1-*p*-chlorophenyl derivatives (**28**, **34**, **36**, **40**) also have

Table 5. Antiplasmodial Activities (IC_{50}) of Cyc and Its Analogues against *P. falciparum* with Wild-Type (TM4/8.2) and the Mutant DHFR Enzymes: T9/94RC17 (A16V+S108T), C59R+S108N (K1CB1), N51I+C59R+S108N (W2), C59R+S108N+I164L (Csl-2), and N51I+C59R+S108N+I164L (V1/S)

	IC_{50} (μM)					
compd	TM4/8.2	T9/94RC17	K1CB1	W2	Csl-2	V1/S
Cyc (C21)	0.04 ± 0.01[a]	3.7 ± 1.7[a]	2.4 ± 1.1	5.8 ± 1.9	37.9 ± 14.6	>100
23 (C97)	0.05 ± 0.01[a]	0.33 ± 0.1[a]	0.22 ± 0.05	0.22 ± 0.05	0.52 ± 0.17	1.3 ± 0.5
27 (C434)	0.18 ± 0.05	ND[b]	0.18 ± 0.06	0.28 ± 0.09	0.29 ± 0.05	1.1 ± 0.4
28 (C71)	0.02 ± 0.006	1.9 ± 0.5	2.4 ± 0.9	3.3 ± 1.0	4.1 ± 0.7	6.4 ± 0.7
29 (C435)	0.06 ± 0.02	ND[b]	0.48 ± 0.22	0.51 ± 0.27	0.49 ± 0.17	3.2 ± 1.1
33 (C96)	0.56 ± 0.06	0.02 ± 0.007	0.31 ± 0.09	0.20 ± 0.05	0.25 ± 0.07	0.80 ± 0.1
34 (C121)	0.12 ± 0.05	0.04 ± 0.01	3.1 ± 0.9	3.2 ± 1.1	4.8 ± 0.6	6.9 ± 2.3
35 (C138)	0.65 ± 0.30	0.004 ± 0.002	3.1 ± 0.7	0.74 ± 0.2	3.3 ± 0.4	3.3 ± 0.4
37 (C110)	2.2 ± 0.7	0.06 ± 0.02	2.7 ± 0.1	3.5 ± 1.1	3.4 ± 0.3	3.6 ± 0.4
39 (C185)	4.0 ± 1.0	0.19 ± 0.08	4.9 ± 1.7	3.3 ± 1.1	7.5 ± 3.3	8.2 ± 3.5
40 (C188)	2.8 ± 1.0	0.42 ± 0.02	3.3 ± 0.5	4.0 ± 2.1	4.0 ± 0.3	3.8 ± 0.1
41 (C143)	3.9 ± 1.1	0.39 ± 0.15	2.6 ± 0.6	2.0 ± 0.7	3.1 ± 0.4	3.2 ± 0.4
42 (C372)	0.04 ± 0.01	0.004 ± 0.002	0.26 ± 0.1	0.33 ± 0.04	0.14 ± 0.07	2.5 ± 1.1
43 (C229)	0.25 ± 0.1	0.004 ± 0.001	1.0 ± 0.4	0.38 ± 0.2	2.9 ± 0.9	2.9 ± 1.2
44 (C186)	5.5 ± 2.7	0.05 ± 0.02	3.1 ± 0.2	3.4 ± 0.9	3.9 ± 0.1	3.9 ± 0.9
45 (C313)	3.0 ± 0.9	0.37 ± 0.08	7.4 ± 4.1	3.2 ± 0.9	5.2 ± 2.9	8.6 ± 2.1
46 (C299)	3.2 ± 0.4	0.18 ± 0.08	2.8 ± 0.8	2.8 ± 0.5	3.4 ± 0.1	3.2 ± 0.4

[a] Data from ref 9. [b] Not determined.

Table 6. Cytotoxicity and Safety Ratio of Some Pyr and Cyc Analogues to Mammalian Cells

	cytotoxicity to Vero cells				cytotoxicity to KB cells				cytotoxicity to BC cells			
		safety ratio[a]				safety ratio[a]				safety ratio[a]		
compd	IC_{50} (μM)	TM4/8.2	Csl-2	V1/S	IC_{50} (μM)	TM4/8.2	Csl-2	V1/S	IC_{50} (μM)	TM4/8.2	Csl-2	V1/S
Pyrimethamine Analogues												
Pyr	32[b]	400	0.8	<0.3	109[b]	1362	2.6	<1.1	40	500	1.0	<0.4
10 (P33)	>50[b]	>238	>18.5	>5.8	150	714	55.6	17.4	250	1191	92.6	29.1
11 (P31)	2.1	3.1	0.6	0.4	1.6	2.4	0.4	0.3	1.6	2.4	0.4	0.3
13 (P41)	22.3	20.3	11.7	3.6	10.1	9.2	5.3	1.6	15	13.6	7.9	2.4
15 (P42)	50	31.3	27.8	9.4	17	10.6	9.4	3.2	10	6.3	5.6	1.9
17 (P43)	61	29.0	32.1	8.1	8.4	4.0	4.4	1.1	9.5	4.5	5.0	1.3
18 (P39)	55[b]	917	18.3	8.6	113[b]	1883	37.7	17.7	268[b]	4467	89.3	41.9
19 (P44)	5.2	2.7	2.7	0.9	1.6	0.8	0.8	0.3	3.6	1.9	1.9	0.6
21 (P32)	3[b]	7.3	1.1	0.6	15[b]	36.6	5.6	3.2	3	7.3	1.1	0.6
22 (P40)	15[b]	42.9	5.2	3.3	6.4	18.3	2.2	1.4	4.9	14	1.7	1.1
Cycloguanil Analogues												
Cyc	>50	>1250	>1.3	-	41	1025.0	1.1	<0.4	>50	>1250	>1.3	-
23 (C97)	49	980	94.2	37.7	4	80.0	7.7	3.1	8.9	178	17.1	6.8
27 (C434)	>500	>2778	>1724	454	110	611	379	1.1	64	356	221	58.2
29 (C435)	230	3833	469	71.9	4	66.7	8.2	1.3	2.2	36.7	4.5	0.7
33 (C96)	250	446	1000	312	87	155	348	109	168	300	672	210
34 (C121)	>50	>417	>10.4	>7.2	7	58.3	1.5	1.0	8.6	71.7	1.8	1.2
35 (C138)	>50	>76.9	>15.2	>15.2	4	6.2	1.2	1.2	5.3	8.2	1.6	1.6
37 (C110)	>50	>22.7	>14.7	>13.9	4	1.8	1.2	1.1	8.4	3.8	2.5	2.3
39 (C185)	>50	>12.5	>6.7	>6.1	8	2.0	1.1	1.0	22	5.5	2.9	2.7
40 (C188)	31	11.1	7.8	8.2	4	1.4	1.0	1.1	7	2.5	1.8	1.9
41 (C143)	31	7.9	10.0	9.7	7	1.8	2.3	2.2	10	2.6	3.2	3.1
42 (C372)	>50	>1250	>357	>20	1	25	7.1	0.4	1.8	45	12.9	0.7
43 (C229)	>50	>200	>17.2	>17.2	6	24	2.1	2.1	30	120	10.3	10.3
44 (C186)	28	5.1	7.2	7.2	6	1.1	1.5	1.5	10	1.8	2.6	2.6
45 (C313)	18	6.0	3.5	2.1	7	2.3	1.3	0.8	10	3.3	1.9	1.2
46 (C299)	30	9.4	8.8	9.4	6	1.9	1.8	1.9	6.4	2.0	1.9	2.0

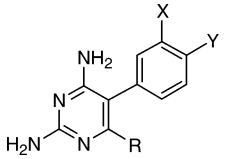
[a] Cytotoxicity/antiplasmodial activity. [b] Data from ref 11.

comparably good antimalarial activities, although not as good as the corresponding *m*-derivatives, which may be due to the favorable effect of substituents at the C-2 position.

The antiplasmodial activities of these compounds against pyrimethamine-resistant parasites, while in line with their high affinities for the mutant enzymes, are generally poorer than expected from the K_i values. This may be due to a number of reasons, including the possibility that the resistant parasites may have additional mechanisms for resistance to mutations of the DHFR-TS, such as increased drug efflux or increased enzyme expression.

Cytotoxicity. Cytotoxicity of compounds in the Pyr and Cyc series against three mammalian cell lines, African green monkey kidney fibroblast (Vero cells), human epidermoid carcinoma (KB), and human breast cancer (BC), is shown in Table 6. Analogues **10**, **18** (previously named as compounds **34** and **33** in ref 11), and **33** have low cytotoxicity to these mammalian cells with an acceptable safety index for the three mammalian cells. Analogues **10** and **18** are Pyr analogues with no Cl substitution on the *N*-1-phenyl, while **33** is a *N*-1-*m*-chlorophenyl Cyc derivative. The safety ratios to Vero cells of analogues **15** and **17** in the Pyr series and those in the Cyc series in reference to quadruple

Table 7. Data for Pyr Analogues



compd	X	Y	R	% yield	mp (°C)	formula	anal.
Pyr	H	Cl	CH_2CH_3	94	235−236	$C_{12}H_{13}ClN_4$	CHN
12 (P45)	H	H	$(CH_2)_3OH$	55	168−170	$C_{13}H_{16}N_4O{\cdot}0.3H_2O$	CHN
13 (P41)	Cl	H	$(CH_2)_3OH$	60	169−171	$C_{13}H_{15}N_4OCl$	dec[a]
14 (P46)	H	H	$(CH_2)_3OCOCH_3$	38	151−153	$C_{15}H_{18}N_4O_2{\cdot}0.15AcOH$	CHN
15 (P42)	Cl	H	$(CH_2)_3OCOCH_3$	60	153−155	$C_{15}H_{17}N_4O_2Cl$	CHN
16 (P47)	H	H	$(CH_2)_3OCOC_6H_5$	31	155−156	$C_{20}H_{20}N_4O_2{\cdot}0.2CH_3OH$	CHN
17 (P43)	Cl	H	$(CH_2)_3OCOC_6H_5$	84	124−126	$C_{20}H_{19}N_4O_2Cl$	CHN
19 (P44)	Cl	H	$(CH_2)_3OCOOCH_2C_6H_5$	57	dec	$C_{21}H_{21}N_4O_3Cl$	dec

[a] Decomposed.

mutant parasite were in a satisfactory range (>10). Some of the Cyc analogues were quite toxic to two cancer cells, KB and BC cells. The effectiveness of these Pyr and Cyc analogues against multiple mutants with their selective toxicity to the antifolate-resistant malarial parasites, based mainly on their C-6 and C-2 substitution, respectively, makes them worthy of further development.

Conclusions

Steric constraints on inhibitor binding play a dominant role in loss of affinity of mutant *Pf*DHFRs for antifolate drugs such as Pyr and Cyc, significantly accounting for the parasite resistance to these drugs.[9−12] A major strategy for the development of new antifolates effective against multiple mutant DHFRs is, therefore, to avoid such steric constraints on binding with the enzymes. The steric constraint caused by the S108N mutation[11,12] can be relieved by moving the Cl of *p*-Cl-phenyl to the *m*-position. This strategy has been shown here to be especially effective for multiple mutants (Tables 1−3), in which the steric constraint around the residue 108 side chain has been further augmented by further mutations.

We have previously shown that steric constraint around the side chain of residue 16 is a significant factor in the loss of binding affinity of A16V+S108T *Pf*DHFR to Cyc, but not to Pyr.[9,10] Unlike Pyr, which has an aromatic diaminopyrimidine ring and only one substituent at the equivalent position, Cyc has two Me substituents at the tetrahedral 2-position. One of the methyl groups is in steric conflict with the side chain of V16, and as shown previously [9,10] and further in this paper (Table 2), Cyc derivatives with only one 2-substituent generally bind to A16V+S108T DHFR with higher affinities than the disubstituted derivatives. Linkage between the two subsites, around residue 16 and residue 108, is shown from the fact that Cyc derivatives with only one C-2 -substituent bind with high affinities to A16V+S108T mutant enzyme, especially with *m*-Cl-phenyl substituent at N-1 (Table 2). A major strategy for developing new effective antifolates against multiple mutant *Pf*DHFRs is therefore to exploit the linkage between binding environment at different subsites to enable increased combined binding enhancements, resulting in K_i values in the low nanomolar range. This strategy is in line with the previous finding that synergy often exists between the effects of combined mutations in the reduction of binding affinities of inhibitors.[19]

In addition to avoidance of steric effects due to mutations, we also employed substitutions that contributed further to the binding. Further modification of our previously identified lead compound has led to a series of Cyc analogues that is much more effective against these multiple mutants[9,11] (Tables 1−3). As seen from Tables 4−6, a number of the compounds synthesized through this strategy show good antiplasmodial activities with relatively low toxicities. The high binding affinities with multiple mutant *Pf*DHFRs and the antiplasmodial activities demonstrate the feasibility of developing compounds directed against the mutated targets and provide an impetus for a further search for more active antifolate antimalarials.

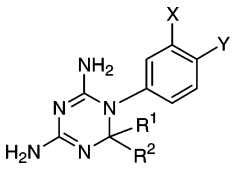
Experimental Section

Methods and Materials. Chemicals and solvents were purchased from Fluka AG, Aldrich Chemical Co., Rathburn Chemicals Ltd., Avocado Research Chemicals Ltd., and Lancaster Synthesis Ltd. Deionized water was produced using a linked Elgastat Prima (reverse osmosis) and Elga Maxima (deionization) purification system. ^{1}H NMR spectra were acquired on a Varian Gemini 200FT (200 MHz), Bruker ACX200 (200 MHz), Bruker AM400 (400 MHz), or Bruker AX500 (500 MHz) spectrometer, internally referenced to standard residual protonated solvent references, and chemical shifts are given in ppm quoted relative to tetramethylsilane. Microanalysis was performed either at Chulalongkorn Research Equipment Centre, the University of Oxford Inorganic Chemistry Laboratory or by Elemental Microanalysis Limited (Devon, UK). APCI mass spectra were obtained using a Fisons Instruments VG Platform spectrometer. Mass spectra were recorded on a Micromass LCT using the electrospray ionization technique and a MALDI-TOF Bruker Biflex spectrometer at the National Centre for Genetic Engineering and Biotechnology and Chulalongkorn University.

Chemical Syntheses of Pyr Analogues. Synthesis (Scheme 1) and chemical data of compounds **1**−**11**, **18** and **20**−**22** have been previously described.[11] Synthesis of compounds **12**−**17**, **19** was accomplished using a variant of this method.[13] See Table 7 for data for Pyr analogues.

Chemical Syntheses of Cyc Analogues. An ethanolic suspension of arylbiguanide hydrochloride or a mixture of aromatic amine and dicyandiamide was refluxed with an

Table 8. Data for Cyc Analogues



compd	X	Y	R^1	R^2	% yield	formula	anal.
Cyc[a] (C21)	H	Cl	Me	Me	69	$C_{11}H_{15}Cl_2N_5$	CHN
23[a] (C97)	Cl	H	Me	Me	79	$C_{11}H_{15}Cl_2N_5 \cdot 0.4H_2O$	CHN
24 (C66)	H	Cl	Me	*n*Pr	68	$C_{13}H_{19}Cl_2N_5$	CHN
25 (C433)	Cl	H	Me	*i*Pr	55	$C_{13}H_{19}Cl_2N_5$	CHN
26 (C22)	H	Cl	Me	*i*Pr	38	$C_{13}H_{19}Cl_2N_5$	CHN
27 (C434)	Cl	H	Me	*n*Pr	68	$C_{13}H_{19}Cl_2N_5$	CHN
28 (C71)	H	Cl	Me	*n*Hex	85	$C_{16}H_{25}Cl_2N_5$	CHN
29 (C435)	Cl	H	Me	*n*Hex	37	$C_{16}H_{25}N_5Cl_2$	CHN
30[a] (C17)	H	Cl	H	Me	49	$C_{10}H_{13}Cl_2N_5$	CHN
31[a] (C248)	Cl	H	H	Me	76	$C_{10}H_{13}Cl_2N_5$	CHN
32 (C53)	H	Cl	H	C_6H_5	85	$C_{15}H_{15}Cl_2N_5$	CHN
33 (C96)	Cl	H	H	C_6H_5	99	$C_{15}H_{15}Cl_2N_5 \cdot H_2O$	HN[b]
34 (C121)	H	Cl	H	4-$C_6H_5OC_6H_4$	31	$C_{21}H_{19}N_5OCl_2$	CHN
35 (C138)	Cl	H	H	4-$C_6H_5OC_6H_4$	24	$C_{21}H_{19}N_5OCl_2$	CHN
36 (C133)	H	Cl	H	3-$C_6H_5OC_6H_4$	40	$C_{21}H_{19}N_5OCl_2$	CHN
37 (C110)	Cl	H	H	3-$C_6H_5OC_6H_4$	42	$C_{21}H_{19}N_5OCl_2$	CHN
38 (C111)	H	Cl	H	3-$C_6H_5CH_2OC_6H_4$	73	$C_{22}H_{21}N_5OCl_2$	CHN
39 (C185)	Cl	H	H	3-$C_6H_5CH_2OC_6H_4$	19	$C_{22}H_{21}N_5OCl_2$	CHN
40 (C188)	H	Cl	H	3-(4-ClC_6H_4O)C_6H_4	53	$C_{21}H_{18}N_5OCl_3$	CHN
41 (C143)	Cl	H	H	3-(4-ClC_6H_4O)C_6H_4	15	$C_{21}H_{18}N_5OCl_3$	CHN
42 (C372)	Cl	H	H	nC_7H_{15}	39	$C_{16}H_{24}ClN_5 \cdot 1.5HCl \cdot 1.5H_2O$	CH[c]
43 (C229)	Cl	H	H	4-$PrOC_6H_4$	8	$C_{18}H_{21}N_5OCl_2$	CHN
44 (C186)	Cl	H	H	3-(3,5-$Cl_2C_6H_3O$)C_6H_4	4	$C_{21}H_{17}N_5OCl_4$	CHN
45 (C313)	Cl	H	H	3-[2,4,5-$Cl_3C_6H_2O(CH_2)_3O$]C_6H_4	16	$C_{24}H_{22}Cl_5N_5O_2$	CHN
46 (C299)	Cl	H	H	3-(3-$CF_3C_6H_4O$)C_6H_4	17	$C_{22}H_{18}N_5OCl_2F_3 \cdot H_2O \cdot 0.5HCl$	CHN

[a] Data from ref 9. [b] Anal. Calcd for $C_{15}H_{15}Cl_2N_5 \cdot H_2O$: C, 46.4; H, 4.2; N, 18.0. Found: C, 46.9; H, 4.4; N, 18.0; HRMS Calcd for $C_{15}H_{14}ClN_5 + H^+$: 300.1016. Found: 300.1006. [c] Anal. Calcd for $C_{16}H_{24}ClN_5 \cdot 1.5HCl \cdot 1.5H_2O$: C, 47.62; H, 7.12; N, 17.35. Found: C, 47.23; H, 6.99; N, 17.75; HRMS Calcd for $C_{16}H_{24}ClN_5 + H^+$: 322.1798. Found: 322.1792.

excess of carbonyl compound in the presence of concentrated HCl, or optionally in the presence of triethyl orthoacetate as a water scavenger according to the method previously described (Scheme 2).[14−16] The products were isolated as hydrochloride salts and purified by recrystallization. See Table 8 for data for Cyc analogues.

Enzyme Preparation. The wild-type and mutant *Pf*DHFR enzymes were expressed in *Escherichia coli* BL21(DE3)pLysS according to the procedure previously described.[9] After disruption of the bacterial cells by a French pressure cell at 18 000 psi, the crude extract in 20 mM potassium phosphate buffer at pH 7.0, 0.1 mM EDTA, 10 mM DTT, 50 mM KCl, and 20% v/v glycerol was applied to Methotrexate-Sepharose column, and the *Pf*DHFR was purified according to the procedure described.[11,19,20]

Enzyme Assays and Inhibition by Antifolates and Derivatives. The methods used for determination of *Pf*DHFR activities and for the study of inhibition by antifolates and derivatives were as previously described[9,11] Calculation of K_i values was based upon the assumption that the inhibitors compete for substrate binding to the active site of the enzyme.

Parasite Culture and Antimalarial Testing in Vitro. Six *P. falciparum* strains were used in this study. Strains TM4/8.2 (wild-type DHFR), T9/94RC17 (A16V+S108T), and K1CB1 (C59R+S108N) were generous gifts from S. Thaithong, Department of Biology, Faculty of Science, Chulalongkorn University; Csl-2 (C59R+S108N+I164L) was from A. Cowman WEHI, Australia; and W2 (N51I+C59R+S108N) and V1/S (N51I+C59R+S108N+I164L) were from D. Kyle through MR4. These parasites were maintained continuously in human erythrocytes at 37 °C under 3% CO_2 in RPMI 1640 culture media supplemented with 25 mM HEPES, pH 7.4, 0.2% $NaHCO_3$, 40 μg/mL gentamicin, and 10% human serum.[21] In vitro antimalarial activity was determined by using the [^{3}H]hypoxanthine incorporation method[22] as described in detail elsewhere.[9,11]

Cytotoxicity tests of selected analogues against African green monkey kidney fibroblast (Vero cells), human epidermoid carcinoma (KB) cells, and human breast cancer (BC) cells were performed at the Bioassay Research Facility of the BIOTEC Center, NSTDA, according to the protocol described by Skehan et al.[23]

Acknowledgment. We thank Supannee Taweechai, Nungruthai Saesaengseerung, and Deanpen Japrung for their technical assistance. This research was supported by grants to Y.Y. (Wellcome Trust, TDR and MMV) and to W.S. (TDR/WHO) and by grants from the European Union (INCO-DC IC18CT970223 and INCO-DEV) to Y.Y. and G.L. and from TDR/WHO and Thailand-TDR (T-2) Program to S.K.

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

References

(1) Yuthavong, Y. Basis for Antifolate Action and Resistance in Malaria. *Microb. Infect.* **2002**, *4*, 175−182.
(2) Cowman, A. F. The Molecular Basis of Resistance to The Sulfones, Sulfonamides, and Dihydrofolate Reductase Inhibitors. In *Malaria: Parasite Biology, Pathogenesis, and Protection.*; Sherman, I. W., Ed.; ASM Press: Washington, DC, 1998; Chapter 22, pp 317−330.
(3) Wang, P.; Lee, C.-S.; Bayoumi, R.; Djimde, A.; Doumbo, O.; Swedberg, G.; Dao, L. D.; Mshinda, H.; Tanner, M.; Watkins, W. M.; Sims, P. F.; Hyde, J. E. Resistance to Antifolates in

Plasmodium falciparum Monitored by Sequence Analysis of Dihydropteroate Synthetase and Dihydrofolate Reductase Alleles in a Large Number of Field Samples of Diverse Origins. *Mol. Biochem. Parasitol.* **1997**, *89*, 161−177.

(4) Plowe, C. V.; Kublin, J. G.; Doumbo, O. K. *Plasmodium falciparum* Dihydrofolate Reductase and Dihydropteroate Synthase Mutations: Epidemiology and Role in Clinical Resistance to Antifolates. *Drug Resistance Updates* **1998**, *1*, 389−396.

(5) Peterson, D. S.; Milhous, W. K.; Wellems, T. E. Molecular Basis of Differential Resistance to Cycloguanil and Pyrimethamine in *Plasmodium falciparum* Malaria. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 3018−3022.

(6) Nzila-Mounda, A.; Mberu, E. K.; Sibley, C. H.; Plowe, C. V., Winstanley, P. A., Watkins, W. M. Kenyan *Plasmodium falciparum* Field Isolates: Correlation Between Pyrimethamine and Chlorcycloguanil Activity in Vitro and Point Mutations in the Dihydrofolate Reductase Domain. *Antimicrob. Agents Chemother.* **1998**, *42*, 164−169.

(7) Basco, L. K.; Ringwald, P. Molecular Epidemiology of Malaria in Yaounde, Cameroon. VI. Sequence Variations in the *Plasmodium falciparum* Dihydrofolate Reductase-Thymidylate Synthase Gene and In Vitro Resistance to Pyrimethamine and Cycloguanil. *Am. J. Trop. Med. Hyg.* **2000**, *62*, 271−276.

(8) Chusacultanachai, S.; Thiensathit, P.; Tarnchopoo, B.; Sirawaraporn W.; Yuthavong, Y. Novel Antifolate Resistant Mutations of *Plasmodium falciparum* Dihydrofolate Reductase Selected in *Escherichia coli*. *Mol. Biochem. Parasitol.* **2002**, *120*, 61−72.

(9) Yuthavong, Y.; Vilaivan, T.; Chareonsethakul, N.; Kamchonwongpaisan, S.; Sirawaraporn, W.; Quarrell, R.; Lowe, G. Development of A Lead Inhibitor for The A16V+S108T Mutant of Dihydrofolate Reductase from the Cycloguanil-Resistant Strain (T9/94) of *Plasmodium falciparum*. *J. Med. Chem.* **2000**, *43*, 2738−2744.

(10) Rastelli, G.; Sirawaraporn, W.; Sompornpisut, P.; Vilaivan, T.; Kamchonwongpaisan, S.; Quarrell, R.; Lowe, G.; Thebtaranonth, Y.; Yuthavong, Y. Interaction of Pyrimethamine, Cycloguanil, WR99210 and Their Analogues with *Plasmodium falciparum* Dihydrofolate Reductase: Structural Basis of Antifolate Resistance. *Bioorg. Med. Chem.* **2000**, *8*, 1117−1128.

(11) Tarnchompoo, B.; Sirichaiwat, C.; Phupong, W.; Intaraudom, C.; Sirawaraporn, W.; Kamchonwongpaisan, S.; Vanichtanankul, J.; Thebtaranonth, Y.; Yuthavong, Y. 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 J. Med. Chem.* **2002**, *45*, 1244−1252.

(12) McKie, J. H.; Douglas, K. T.; Chan, C.; Roser, S. A.; Yates, R.; Read, M.; Hyde, J. E.; Dascombe, M. J.; Yuthavong, Y.; Sirawaraporn, W. Rational Drug Design Approach for Overcoming Drug Resistance: Application to Pyrimethamine Resistance in Malaria. *J. Med. Chem.* **1998**, *41*, 1367−1370.

(13) Lowe, G.; Ponsinet, R.; Quarrell, R. An Efficient Synthetic Route to 2,4-Diaminopyrimidine Analogues of the Antimalarial Pyrimethamine. Submitted.

(14) Modest, E. J. Chemical and Biological Studies on 1,2-Dihydro-s-triazines II. Three-Component Synthesis. *J. Org. Chem.* **1956**, *21*, 1−13.

(15) Modest, E. J.; Levine, P. Chemical and Biological Studies on 1,2-Dihydro-s-triazines. III. Two-Component Synthesis. *J. Org. Chem.* **1956**, *21*, 14−21.

(16) Saesaengseerung, N.; Thebtaranonth, Y.; Vilaivan, T. An Efficient Synthesis of 1-Aryl-4,6-Diamino-1,2-Dihydro-1,3,5-Triazines. *Synth. Commun.* **2002**, *32*, 2089−2100.

(17) Warhurst, D. C. Antimalarial Drug Discovery: Development of Inhibitors of Dihydrofolate Reductase Active in Drug Resistance. *Drug Discovery Today* **1998**, *3*, 538−546.

(18) Yuvaniyama, J.; Chitnumsub, P.; Kamchonwongpaisan, S.; Vanichtanankul, J.; Sirawaraporn, W.; Taylor, P.; Walkinshaw, M.; Yuthavong, Y. Insights into Antifolate Resistance from Malarial DHFR-TS Structures. *Nature Struct. Biol.* **2003**, *10*, 357−365.

(19) Sirawaraporn, W.; Sathitkul, T.; Sirawaraporn, R.; Yuthavong, Y.; Santi, D. V. Antifolate-Resistant Mutants of *Plasmodium falciparum* Dihydrofolate Reductase. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 1124−1129.

(20) Sirawaraporn, W.; Prapunwattana, P.; Sirawaraporn, R.; Yuthavong, Y.; Santi, D. V. The Dihydrofolate Reductase Domain of *Plasmodium falciparum* Thymidylate Synthase-Dihydrofolate Reductase: Gene Synthesis, Expression, and Anti-Folate Resistant Mutants. *J. Biol. Chem.* **1993**, *268*, 21637−21644.

(21) Trager, W.; Jensen, J. B. Human Malaria Parasites in Continuous Culture. *Science* **1976**, *193*, 673−675.

(22) Desjardins, R. E.; Canfield, C. J.; Haynes, J. D.; Chulay, J. D. Quantitative Assessment of Antimalarial Activity in Vitro by a Semiautomated Microdilution Techniques. *Antimicrob. Agents Chemother.* **1979**, *16*, 710−718.

(23) Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. New Colorimetric Cytotoxicity Assay for Anticancer-Drug Screening. *J. Natl. Cancer Inst.* **1990**, *82*, 1107−1112.

JM030165T