Development of a Lead Inhibitor for the A16V+S108T Mutant of Dihydrofolate Reductase from the Cycloguanil-Resistant Strain (T9/94) of *Plasmodium falciparum*†

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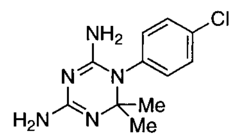
The Ala16Val+Ser108Thr (A16V+S108T) mutant of the *Plasmodium falciparum* dihydrofolate reductase (DHFR) is a key mutant responsible for cycloguanil-resistant malaria due to steric interaction between Val-16 and one of the C-2 methyl groups of cycloguanil. 4,6-Diamino-1,2-dihydrotriazines have been prepared, in which both methyl groups of cycloguanil are replaced by H or by H and an alkyl or phenyl group, and their inhibition constants against wild-type and mutant DHFR determined. The S108T mutation is considered to decrease cycloguanil binding further through the effect on the orientation of the *p*-chlorophenyl group. By moving the *p*-chloro-substituent to the *m*-position in the chlorophenyl group, the activity against the A16V+S108T mutant enzyme is improved, and this effect is reinforced by the *p*-chloro substituent in the 3,4-dichlorophenyl group. A lead compound has been found with inhibitory activity similar to that of cycloguanil against the wild-type DHFR and about 120-fold more effective than cycloguanil against the A16V+S108T mutant enzyme. The activity of this compound against *P. falciparum* clone (T9/94 RC17) which harbors the A16V+S108T DHFR is about 85-fold greater than cycloguanil.

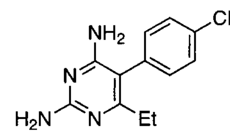
Introduction

Despite continued efforts aimed at complete eradication of malaria, the disease remains a major health threat in many areas of the world, especially in tropical and subtropical countries including Africa.[1] The widespread occurrence of drug-resistant *Plasmodium falciparum* suggests that the effectiveness of the few antimalarials currently in use will have a limited life span and has highlighted the urgent need for the discovery and development of novel antimalarial agents aimed at combating the emerging resistant parasites.

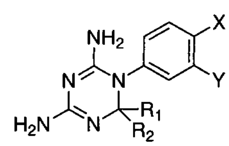
Cycloguanil (Cyc) and its closely related compound pyrimethamine (Pyr) are potent inhibitors of *Plasmodium falciparum* dihydrofolate reductase (pfDHFR), one of a few well-defined drug targets for malaria therapy. Both compounds have been extensively employed, either alone or in combination with sulfa-drugs, as prophylactic agents for the treatment of malaria. Unfortunately, resistance of the malaria parasite to the drugs has rapidly emerged and compromised their clinical utility. Analysis of DHFR sequences of several Pyr- and Cyc-resistant *P. falciparum* isolates from different geographical origins with different drug sensitivities revealed that



Cycloguanil



Pyrimethamine



(A)

resistance to Pyr and Cyc is associated with point mutations in the DHFR.[2−7] The mutations in pfDHFR thus far reported are amino acid residues 16, 51, 59, 108, and 164. Mutant pfDHFRs involving mutations at residues 51, 59, 108, and 164 confer cross-resistance to Pyr and Cyc, while those involving mutation at residue 16 (A16V) are resistant to Cyc but susceptible to Pyr. The importance of residue 16 for binding Cyc has been investigated using the mutants obtained via mutagenesis of a synthetic gene.[8] Recently, a three-dimensional homology model of pfDHFR was constructed to aid understanding of the structural basis of antifolate resistance in malaria.[9] The studies led to a hypothesis which proposed that resistance to Cyc is due to a steric clash for Cyc binding as a result of A16V mutation of the pfDHFR, and that mutation of residue 108 (S108T) further reinforces the steric constraint for Cyc binding through displacement of the *p*-chlorophenyl group of the

† Abbreviations: Cyc, cycloguanil; Pyr, pyrimethamine; DHFR, dihydrofolate reductase; pf, *Plasmodium falciparum*.

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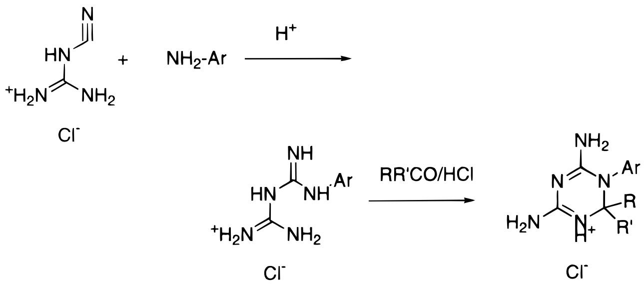
Scheme 1



inhibitor from the nicotinamide ring of the cofactor. Validation of the hypothesis was achieved by testing both the wild-type and A16V+S108T mutant pfDHFRs against Cyc analogues devoid of one or both methyl groups.[9]

In this paper, we describe further modifications to the structure of Cyc in a search for more effective inhibitors of A16V+S108T pfDHFR which are also effective against the wild-type enzyme. Given the important roles of A16V and S108T mutations on Cyc binding, we designed and synthesized a number of Cyc analogues in which the groups at C-2 and N-1 were varied, and we tested them against both wild-type and A16V+S108T pfDHFRs. The studies have led to the discovery of a lead which is as effective as Cyc against the wild-type pfDHFR, but is over 120-fold more effective than Cyc against the A16V+S108T mutant enzyme. The relative effectiveness of the lead was also investigated against the resistant *P. falciparum* strain T9/94, a mutant parasite harboring the A16V+S108T mutant enzyme. Its IC_{50} value is 180-fold lower than that of Cyc, and it retains similar activity to Cyc against the wild-type *P. falciparum*.

Results and Discussion

Chemical Syntheses. 4,6-Diamino-1,2-dihydrotriazines, such as Cyc, are generally made in two steps from dicyandiamide and an aniline under acidic conditions to first generate arylbiguanides which are then reacted with an appropriate carbonyl compound in the presence of an acid catalyst to give the 4,6-diamino-1,2-dihydrotriazine substituted at N-1 and C-2.[10,11] The arylbiguanide may be isolated before further reaction, or the two-step procedure may be performed in the same reaction vessel (Scheme 1). This is an attractive feature of the chemistry and lends itself to the techniques of combinatorial chemistry.[12] However, one should be aware that the rate of formation of the biguanides is strongly influenced by the nucleophilicity of the aniline, and for this reason we have chosen to use a parallel synthesis protocol. Previous attempts to prepare the didemethyl analogue of Cyc (**2**) were unsuccessful,[10,11] and we confirm that the standard protocol using formalin or paraformaldehyde does not give the required product. However, when dimethoxymethane is used as the formaldehyde equivalent, the required product is formed in excellent yield. Other Cyc derivatives were prepared according to the literature methods[10,11] without modification, except that in some cases addition of a miscible water scavenger such as triethyl orthoacetate was found to give improved results, although this is rarely necessary when the carbonyl component is an aldehyde.

Inhibition of Wild-Type and A16V+108T pfDHFRs. The data in Table 1 summarize the inhibition constant (K_i) values of Cyc and analogues in which the substituents at C-2 were varied, while the group at N-1 (*p*-chlorophenyl) was unmodified. As reported previously,[8] Cyc (**1**) binds to the wild-type pfDHFR with the K_i value of ~1.5 nM, but it binds approximately 876-fold less tightly to the A16V+S108T mutant enzyme. On the basis of our working hypothesis, we surmise that the poor binding of Cyc to A16V+S108T enzyme might be attributed to one of the methyl substituents at the C-2 position of Cyc. To test this hypothesis, we designed and synthesized a number of Cyc derivatives in which one substituent at C-2 was H and the other varied from H (**2**), methyl (**3**), ethyl (**4**), *n*-propyl (**5**), *n*-butyl (**6**), isopropyl (**7**), *tert*-butyl (**8**), and phenyl (**9**). The effects of the substituents on binding to both wild-type and A16V+S108T DHFRs were assessed by determination of the ratios of the K_i values for the A16V+S108T mutant enzyme and the wild-type enzyme (K_i-mut./K_i-wt) as well as their K_i values relative to Cyc. Table 1 shows that the didemethylcycloguanil (**2**), in which both methyl groups at position C-2 were replaced by H, is ~2-fold more effective against the A16V+S108T pfDHFR than Cyc but inhibited the wild-type enzyme ~16-fold less effectively than Cyc. The monomethyl analogue (**3**) has improved binding affinity (~10-fold) for the A16V+S108T DHFR, but was about 2.7-fold less effective than Cyc for the wild-type enzyme. Since the compound tested was a racemic mixture, if only one enantiomer is active, the relative K_i value of the effective enantiomer could be up to twice that of the value shown. If this is the case, the K_i value of Cyc analogue (**3**) for the wild-type enzyme is within the range reported for Cyc.[8]

Table 1. Inhibition Constants (K_i) of Cyc (**1**) and Its Analogues (A; X = Cl, Y = H) against the Wild-Type and A16V+S108T Mutant DHFRs of *P. falciparum* and Their Growth Inhibition (IC_{50}) against *P. falciparum* Clones with Wild-Type (TM4/8.2) and Mutant (T9/94 RC17) Enzymes

cmpd	R_1	R_2	K_i(wt)[a] (nM)	rel. to Cyc	K_i(mut.)[b] (nM)	rel. to Cyc	K_i(mut.)/K_i(wt)	IC_{50} TM4 (nM)	IC_{50} T9/94 (nM)	IC_{50} ratio T9/94:TM4
1	Me	Me	1.5 ± 0.3[c]	1.0	1314 ± 16[c]	1	876	40 ± 12	2430 ± 571	60.75
2	H	H	24.4 ± 4.3[d]	16	646 ± 77[d]	0.5	26	952	313 ± 18	0.33
3	H	Me	4.1 ± 0.0[d]	2.7	127 ± 14[d]	0.09	31	348 ± 144	347 ± 116	1.00
4	H	Et	3.6 ± 0.0[d]	2.4	189 ± 37[d]	0.14	52	40 ± 2	486 ± 220	12.15
5	H	Pr^n	4.6 ± 0.2	3.1	107 ± 32	0.08	23	65	365 ± 116	5.62
6	H	Bu^n	3.7 ± 0.1	2.5	167 ± 6.0	0.1	45	111	250 ± 109	2.25
7	H	Pr^i	60.5 ± 10.1	40.3	1538 ± 345	1.2	25	2908	2818 ± 438	0.97
8	H	Bu^t	3838 ± 408	2558	82721 ± 9888	63	22	>25000	65386 ± 7516	<2.6
9	H	Ph	4.5 ± 0.2	3.0	49 ± 3	0.04	11	27 ± 11	44 ± 15	1.63

[a] Wild-type pfDHFR. [b] A16V+S108T mutant pfDHFR. [c] Data from ref 8. [d] Data from ref 9.

Table 2. Inhibition Constants (K_i) of Cyc (**1**) and Its Analogues (A; X = Br, Y = H) against the Wild-Type and A16V+S108T Mutant DHFRs of *P. falciparum* and Their Growth Inhibition (IC_{50}) against *P. falciparum* Clones with Wild-Type (TM4/8.2) and Mutant (T9/94 RC17) Enzymes

cmpd	R_1	R_2	K_i(wt)[a] (nM)	rel. to Cyc	K_i(mut.)[b] (nM)	rel. to Cyc	K_i(mut.)/K_i(wt)	IC_{50} TM4 (nM)	IC_{50} T9/94 (nM)	IC_{50} ratio T9/94:TM4
10	Me	Me	1.1 ± 0.2	0.7	1947 ± 366	1.5	1770	31	2759 ± 153	89
11	H	Me	5.7 ± 0.5	3.8	202 ± 17	0.15	35	132	277 ± 32	2.1
12	H	Et	2.7 ± 0.3	1.8	99 ± 7.0	0.08	37	68	220 ± 96	3.24
13	H	Pr^n	2.6 ± 0.8	1.7	127 ± 11	0.1	49	63	250 ± 110	3.97
14	H	Pr^i	30 ± 4.0	20	1195 ± 53	0.9	40	638	725 ± 277	1.14
15	H	Ph	2.9 ± 1.2	1.9	90 ± 11	0.07	31	47 ± 8	180 ± 62	3.86

[a] Wild-type pfDHFR. [b] A16V+S108T mutant pfDHFR.

Table 3. Inhibition Constants (K_i) of Cyc (**1**) and Its Analogues (A; X = Me, Y = H) against the Wild-Type and A16V+S108T Mutant DHFRs of *P. falciparum* and Their Growth Inhibition (IC_{50}) against *P. falciparum* Clones with Wild-Type (TM4/8.2) and Mutant (T9/94 RC17) Enzymes

cmpd	R_1	R_2	K_i(wt)[a] (nM)	rel. to Cyc	K_i(mut.)[b] (nM)	rel. to Cyc	K_i(mut.)/K_i(wt)	IC_{50} TM4 (nM)	IC_{50} T9/94 (nM)	IC_{50} ratio T9/94:TM4
16	Me	Me	1.8 ± 0.2	1.2	1584 ± 210	1.21	880	65	3617 ± 337	55.64
17	H	Me	23.0 ± 1.9	15	185 ± 22	0.14	7.9	462 ± 35	464 ± 208	1.00
18	H	Et	5.9 ± 0.2	3.9	128 ± 4.0	0.10	22	273	517 ± 419	1.89
19	H	Pr^n	13.7 ± 0.8	9.1	188 ± 12	0.14	14	167	152	0.91
20	H	Pr^i	167 ± 11	111	1460 ± 161	1.11	8.7	8223	3446 ± 759	0.42
21	H	Ph	7.7 ± 2.0	5.1	170 ± 14	0.13	22	136 ± 8.3	39 ± 9	0.29

[a] Wild-type pfDHFR. [b] A16V+S108T mutant pfDHFR.

Table 4. Inhibition Constants (K_i) of Cyc (**1**) and Its Analogues (A; $R_1 = R_2$ = R, Y = H) against the Wild-Type and A16V+S108T Mutant DHFRs of *P. falciparum* and Their Growth Inhibition (IC_{50}) against *P. falciparum* Clones with Wild-Type (TM4/8.2) and Mutant (T9/94 RC17) Enzymes

cmpd	R	X	K_i(wt)[a] (nM)	rel. to Cyc	K_i(mut.)[b] (nM)	rel. to Cyc	K_i(mut.)/K_i(wt)	IC_{50} TM4 (nM)	IC_{50} T9/94 (nM)	IC_{50} ratio T9/94:TM4
1	Me	Cl	1.5 ± 0.3[c]	1.0	1314 ± 164[c]	1.0	876	40 ± 12	2430 ± 571	60.75
22	Me	H	20.0 ± 5.2	13	1375 ± 236	1.05	69	546	445 ± 1	0.82
23	Me	F	4.6 ± 0.7	3.0	1633 ± 269	1.20	355	294	1001 ± 466	3.40
2	H	Cl	24.4 ± 4.3	16	646 ± 77	0.5	26	952	313 ± 18	0.33
24	H	H	329 ± 27	220	585 ± 70	0.4	1.8	11632	356 ± 57	0.03
25	H	F	270 ± 28	180	469 ± 71	0.4	1.7	9661	312 ± 36	0.03

[a] Wild-type pfDHFR. [b] A16V+S108T mutant pfDHFR. [c] Data from ref 8.

The K_i values of monoethyl (**4**), mono-*n*-propyl (**5**), and mono-*n*-butyl (**6**) analogues for the wild-type and A16V+S108T pfDHFRs were comparable to that for the monomethyl (**3**) analogue, implying that the C-2 substituents of these analogues did not appreciably influence or improve the binding affinities of the inhibitors compared with analogue **3**. However, as predicted, the K_i values for both wild-type and A16V+S108T pfDHFRs were greatly increased when the C-2 substituents were branched (and therefore bulkier) alkyl groups, as observed with analogues **7** and **8**. Interestingly, analogue **9**, in which one substituent at the C-2 is H and the other a phenyl group, inhibited the A16V+S108T pfDHFR with the K_i value of ~49 nM, a value which is 27-fold lower than that observed for Cyc (**1**). The above results indicate the crucial role of residue 16 for Cyc binding and suggest the importance of the phenyl group at position C-2 for achieving effective inhibition of the A16V+S108T pfDHFR.

To investigate the significance of the *p*-chloro group on the N-1 substituent of Cyc, two series of Cyc analogues were synthesized in which the N-1 *p*-chlorophenyl group is replaced by *p*-bromophenyl and *p*-tolyl groups. Their K_i values against the wild-type and the mutant pfDHFRs are shown in Table 2 and Table 3. Inhibition constants for the wild-type and mutant enzymes by *p*-bromophenyl (**10** to **15**) and *p*-tolyl (**16** to **21**) analogues showed a trend similar to those where the N-1 substituent is *p*-chlorophenyl (Table 1). However, as the *p*-substituent changes in polarity and size, the monophenyl compounds became progressively less effective against the A16V+S108T mutant enzyme; the K_i values for the mutant enzyme of analogues **9**, **15**, and **21** being 49, 90, and 170 nM, respectively.

The contribution of the *p*-chloro substituent toward the binding affinities of the inhibitors to pfDHFRs was investigated. The C-2 substituents of Cyc were either kept unmodified as dimethyl groups or removed (substituted with H), and the *p*-chloro substituent of *p*-chlorophenyl group at N-1 in Cyc was replaced with either H or F. The analogues were then tested against the wild-type and the A16V+S108T mutant pfDHFRs (Table 4). The results in Table 4 revealed that replacement of the *p*-chloro group with H (analogues **22** and **24**) did not affect the K_i values for the A16V+S108T mutant DHFR but substantially increased the K_i values for the wild-type enzyme. Replacement of the chlorine by fluorine, however, resulted in a 3−14-fold decrease in the binding affinities of the inhibitors to the wild-type enzyme (analogues **23** and **25**, Table 4). The data imply that the *p*-chloro substituent of the *p*-chlorophenyl group is important for binding to the wild-type enzyme and that replacement of the chlorine by a smaller group has relatively little effect on the binding of the inhibitor to the A16V+S108T pfDHFR.

We next investigated the Cyc analogues in which an additional chlorine atom was placed at the *m*-position of the *p*-chlorophenyl substituent. The importance of

Table 5. Inhibition Constants (K_i) of Cyc (**1**) and Its Analogues (A; Y = Cl) against the Wild-Type and A16V+S108T Mutant DHFRs of *P. falciparum* and Their Growth Inhibition (IC_{50}) against *P. falciparum* Clones with Wild-Type (TM4/8.2) and Mutant (T9/94 RC17) Enzymes

cmpd	R_1	R_2	X	K_i(wt)[a] (nM)	rel. to Cyc	K_i(mut.)[b] (nM)	rel. to Cyc	K_i(mut.)/K_i(wt)	IC_{50} TM4 (nM)	IC_{50} T9/94 (nM)	IC_{50} ratio T9/94:TM4
26	Me	Me	H	3.7 ± 0.6	2.5	340 ± 28	0.3	92	60 ± 13	298 ± 54	4.97
27	Me	Me	Cl	1.1 ± 0.4	0.73	130.7 ± 13.4	0.1	119	4 ± 1	307 ± 99	76.75
28	H	Me	H	10.2 ± 0.6	6.8	38.7 ± 2.9	0.03	3.8	ND[c]	28 ± 4	ND[c]
29	H	Me	Cl	1.4 ± 0.2	0.9	17.8 ± 0.8	0.014	12.7	35	19 ± 8	0.54
30	H	Ph	H	11.7 ± 2.5	7.8	10 ± 7	0.008	0.9	455 ± 228	24 ± 8	0.05
31	H	Ph	Cl	1.6 ± 0.2	1.1	11 ± 1.8	0.008	6.9	40 ± 25	29 ± 2	0.72

[a] Wild-type pfDHFR. [b] A16V+S108T mutant pfDHFR. [c] Not determined.

m-chloro substituent was recently shown in Pyr to improve the effectiveness against the C59R+S108N mutant pfDHFR.[13] As shown in Table 5, the Cyc analogue with *m*-chlorophenyl group (**26**) inhibited the wild-type pfDHFR with the K_i value ~2.5 times higher than that of Cyc (**1**), but it was about 3-fold more effective than Cyc against the A16V+S108T mutant enzyme. Replacement of the *p*-chlorophenyl group of Cyc with the 3,4-dichlorophenyl substituent yielded analogue **27,** which was about as effective as Cyc against the wild-type DHFR but inhibited the A16V+S108T mutant enzyme about 10 times better than Cyc. Indeed, analogue **27**, also known as chlorocycloguanil, is a potent inhibitor of wild-type pfDHFR and has been used as an effective agent for the treatment of malaria.[14] Replacing one of the C-2 methyl groups of the *m*-chlorophenyl analogue by H yielded analogue **28** which showed a significant decrease in the K_i value for the A16V+S108T pfDHFR, but the analogue inhibited the wild-type enzyme ~7-fold less effectively than Cyc (**1**). Addition of a chloro group to analogue **28** gave the monomethyl analogue with the 3,4-dichlorophenyl group at position N-1 (**29**). While the K_i value for analogue **29** was similar to that of Cyc (**1**) against the wild-type pfDHFR, the binding affinity for the A16V+S108T enzyme was dramatically improved, being about 73-fold more effective than Cyc.

The effects of *m*-chlorophenyl and 3,4-dichlorophenyl substituents at N-1 were further tested in the most promising lead compound in which the groups at C-2 were H and phenyl (**9**) (Table 1). Analogues **30** and **31** were over 120-fold more effective than Cyc against the A16V+S108T mutant pfDHFR. While analogue **30** was about 8-fold less effective than Cyc against the wild-type enzyme, analogue **31** was about as effective as Cyc (Table 5).

In Vitro Antiplasmodial Activity. The activities of the Cyc analogues against *P. falciparum* were tested in vitro, both in the wild-type clone (TM4/8.2) and the Cyc-resistant clone, which harbors the A16V+S108T pfDHFR (T9/94 RC17). The data in Table 1 shows that some of the 2-monosubstituted analogues of Cyc, namely ethyl, *n*-propyl, and phenyl, have IC_{50} values against the wild-type parasite which are comparable to that of the parent compound. Furthermore, all the compounds have relatively low resistance factors (ratios of IC_{50} for T9/94 to TM4) in comparison to Cyc, some with factors of 1 or less. All compounds except for isopropyl and *tert*-butyl derivatives are much more effective than Cyc against the resistant parasite. The most notable compound in Table 1 is the phenyl derivative, which is almost twice as effective against the wild-type parasite and is over 50 times more effective than Cyc against the resistant parasite. The data in Tables 2 and 3 show the same trend as in Table 1, in that the resistance factors for the 2-monosubstituted derivatives are all lower than those for the 2,2-dimethyl parent compounds. All the 2-monosubstituted compounds in Tables 2 and 3 are more effective against the resistant parasite than the parent compounds. However, none of the compounds in Tables 2 and 3 are as effective as the parent compounds against the wild-type parasite.

The Cyc analogues in Table 4, in which the *p*-chloro substituents had been replaced by sterically less demanding groups, have higher IC_{50} values against the wild-type parasite than those for the parent compounds, generally reflecting the higher K_i values. The low values of resistance factors reflect the poor activities against the wild-type parasite rather than relatively high activities against the resistant parasite.

Table 5 shows the effect on the antiplasmodial activities of Cyc analogues in which dimethyl is changed to a 2-monosubstituted group and the *p*-chloro is replaced by an *m*-chloro or 3,4-dichloro group. All the 2-monosubstituted compounds show excellent activities against the resistant parasite, with IC_{50} values approximately 100 times lower than that of Cyc. Both the 2-monosubstituted and the 3,4-dichlorophenyl derivatives (**29** and **31**) have comparable activities to Cyc against the wild-type parasite.

Preliminary results on human DHFR with some 2-monosubstituted analogues of Cyc show relatively high K_i values (data not shown), suggesting that these compounds are probably of low toxicity and therefore might be suitable for further investigation as lead compounds in the search for new effective antimalarials against antifolate-resistant *P. falciparum*.

Conclusions

1-(3′,4′-Dichlorophenyl)-2-monosubstituted-4,6-diamino-1,2-dihydro-1,3,5-triazines (**29** and **31**) are useful lead compounds, being at least as effective against the mutant resistant *P. falciparum* strain (T9/94 RC17) as against the wild-type strain (TM4/8.2). They are as effective as Cyc against the wild-type strain of *P. falciparum* and approximately 100-fold more effective than Cyc against the resistant *P. falciparum* strain (T9/94 RC17).

Experimental Section

Methods and Materials. *m*-Chloroaniline was distilled under reduced pressure before use. All other chemicals were obtained from Sigma-Aldrich Ltd., Lancaster, Avocado Ltd., BDH, or other standard suppliers, and they were used without further purification. Reagent grade acetone and absolute alcohol were used.

Table 6. Tabulated Elemental, Mass, and NMR Analysis Data for Cyc Analogues **1**−**31**

cpd	X	Y	R_1	R_2	yd%	anal.	formula	$MH^{+\ a}$	NMR details (200 MHz unless otherwise specified)
1	Cl	H	Me	Me	62	CHN	$C_{11}H_{15}N_5Cl_2$	252 (iii)	^{1}H (D_2O, 400 MHz) 1.38 (6H, s, 2 × Me), 7.35 and 7.54 (2 × 2H, AB doublet, J = 8 Hz, aromatic C−H)
2	Cl	H	H	H	79	CHN	$C_9H_{11}N_5Cl_2$	224 (i)	^{1}H (DMSO-d_6) 4.71 (2H, s, CH_2), 6.98 (1H, s br ex, NH), 7.48 (4H, dd, J_{AB} = 8, Ar-C-H), 7.65 (2H, s, NH_2), 7.85 (1H, s br ex, NH), 8.74 (1H, s, NH^+)
3	Cl	H	H	Me	49	CHN	$C_{10}H_{13}N_5Cl_2$	238 (i)	^{1}H (D_2O) 1.20 (3H, d, J = 6 Hz, Me-2), 5.06 (1H, q, J = 6 Hz, H-2), 7.22 and 7.40 (2 × 2H, AB doublet, J = 8 Hz, Ar-C−H)
4	Cl	H	H	Et	92	CHN	$C_{11}H_{15}N_5Cl_2$	252 (ii)	^{1}H (D_2O) 0.94 (3H, J = 6.5 Hz, CH_3), 1.52 (2H, m, CH_2), 4.84 (1H, dd, J = 6.5, 4 Hz, H-2), 7.22 and 7.38 (2 × 2H, AB doublet, J = 8 Hz, Ar-C-H)
5	Cl	H	H	Pr^n	86	HN^b	$C_{21}H_{17}N_5Cl_2$· H_2O	266 (ii)	^{1}H (D_2O) 0.65 (3H, t, CH_3), 1.15 (2H, m, CH_2), 1.55 (2H, m, CH_2), 4.94 (1H, dd, J = 6, 4 Hz, H-2), 7.25 and 7.42 (2 × 2H, AB doublet, J = 8 Hz, aromatic C-H)
6	Cl	H	H	Bu^n	89	CHN	$C_{13}H_{19}N_5Cl_2$· $3.5H_2O$	280 (iii)	^{1}H (D_2O) 0.60 (3H, t J=6.5 Hz, Me), 1.08 (4H, m, 2 × CH_2), 1.55 (2H, m, CH_2), 4.96 (1H, dd J = 6.5, 4 Hz, H-2) 7.25, 7.40 (2 × 2H, AB doublet, J = 8 Hz, Ar-C-H)
7	Cl	H	H	Pr^i	59	CHN	$C_{12}H_{17}N_5Cl_2$	266 (i)	^{1}H (D_2O) 0.65 and 0.75 (6H, 2 × d J = 6.8 Hz, 2 × Me), 1.88 (1H, m, CHMe$_2$), 4.74 (1H, d J = 2.8 Hz, H-2), 7.24, 7.40 (2 × 2H, AB doublet, J = 8 Hz, Ar-C-H)
8	C	H	H	Bu^t	31	CHN	$C_{13}H_{19}N_5Cl_2$	280 (i)	^{1}H (D_2O) 0.66 (9H, s, 3 × Me), 4.65 (1H, s, H-2), 7.31 (4H, m, aromatic C-H)
9	C	H	H	Ph	55	CHN	$C_{15}H_{15}N_5Cl_2$	300 (iii)	^{1}H (D_2O) 5.84 (1H, s, H-2), 6.90 (2H, part of AB doublet, J = 8 Hz, Ar-C-H), 7.05 (7H, m, Ar-C-H)
10	Br	H	Me	Me	78	CHN	$C_{11}H_{15}N_5BrCl$	246 (iii)	^{1}H (D_2O) 1.28 (6H, s, 2 × Me), 7.16 and 7.58 (2 × 2H, AB doublet, J = 8 Hz, aromatic C-H)
11	Br	H	H	Me	53	CHN	$C_{10}H_{13}N_5BrCl$	282, 284 (iii)	^{1}H (D_2O) 1.16 (3H, d, J = 6 Hz, Me-2), 5.00 (1H, q, J = 6 Hz, H-2), 7.14 and 7.55 (2 × 2H, AB doublet, J = 8 Hz, aromatic C-H)
12	Br	H	H	Et	93	CHN	$C_{11}H_{15}N_5BrCl$	296, 298 (iii)	^{1}H (D_2O) 0.74 (3H, t, J = 6.5 Hz, CH_3), 1.50 (2H, m, CH_2), 4.92 (1H, dd, J = 6.5, 4 Hz, H-2), 7.20 and 7.50 (2 × 2H, AB doublet, J = 8 Hz, aromatic C-H)
13	Br	H	H	Pr^n	74	CHN	$C_{12}H_{17}N_5BrCl$· H_2O	310, 312 (iii)	^{1}H (D_2O) 0.68 (3H, t, J = 6 Hz, CH_3), 1.18 (2H, m, CH_2), 1.60 (2H, m, CH_2), 4.92 (1H, dd, J = 6, 4 Hz, H-2), 7.20 and 7.50 (2 × 2H, AB doublet, J = 8 Hz, aromatic C-H)
14	Br	H	H	Pr^i	71	CHN	$C_{12}H_{17}N_5BrCl$	310, 312 (iii)	^{1}H (D_2O) 0.66 and 0.76 (6H, 2 × d, J = 7 Hz, 2 × Me), 1.88 (1H, m, CHMe$_2$), 4.78 (1H, d, J = 3 Hz, H-2), 7.18 and 7.56 (2 × 2H, AB doublet, J = 8 Hz, aromatic C-H)
15	Br	H	H	Ph	93	CHN	$C_{15}H_{15}N_5BrCl$	344, 346 (iii)	^{1}H (D_2O) 5.88 (1H, s, H-2), 6.88 and 7.34 (2 × 2H, AB doublet, J = 8 Hz, aromatic C-H), 7.16 (5H, m, aromatic C-H)
16	Me	H	Me	Me	59	CHN	$C_{12}H_{20}N_5OCl$	232 (iii)	^{1}H (D_2O) 1.26 (6H, s, 2 × Me), 2.20 (3H, s, 4′-Me), 7.08 and 7.25 (2 × 2H, AB doublet, J = 8 Hz, Ar-C-H)
17	Me	H	H	Me	27	CHN	$C_{11}H_{16}N_5Cl$· $HCl·0.7H_2O$	218 (iii)	^{1}H (D_2O) 1.18 (3H, d, J = 6.5 Hz, Me-2), 2.15 (3H, s, Me-4′), 5.05 (1H, q, J = 6.5 Hz, H-2), 7.08 and 7.20 (2 × 2H, AB doublet, J = 8 Hz, aromatic C-H)
18	Me	H	H	Et	77	CHN	$C_{12}H_{18}N_5Cl$	232 (iii)	^{1}H (D_2O) 0.72 (3H, t, J = 6 Hz, CH_3), 1.65 (2H, m, CH_2), 2.18 (3H, s, Me-4′), 4.94 (1H, dd, J = 6, 5 Hz, H-2), 7.15 and 7.25 (2 × 2H, AB doublet, J = 8 Hz, aromatic C-H)
19	Me	H	H	Pr^n	87	CHN	$C_{13}H_{20}N_5Cl$· HCl	246 (iii)	^{1}H (D_2O) 0.64 (3H, t, J = 7 Hz, CH_3), 1.15 (2H, m, CH_2CH$_3$), 1.60 (2H, m, CH_2CH$_2$), 2.18 (3H, s, Me-4′), 4.94 (1H, dd, J = 6, 4 Hz, H-2), 7.12 and 7.22 (2 × 2H, AB doublet, J = 8 Hz, aromatic C-H)
20	Me	H	H	Pr^i	85	HN^c	$C_{13}H_{20}N_5Cl·HCl·$ CH_3OH	246 (iii)	^{1}H (D_2O) 0.65 and 0.75 (6H, 2 × d, J = 7 Hz, 2 × Me), 1.88 (1H, m, CHMe$_2$), 2.18 (3H, s, Me-4′), 4.82 (1H, d, J = 3 Hz, H-2), 7.12 and 7.22 (2 × 2H, AB doublet, J = 8 Hz, aromatic C-H)
21	Me	H	H	Ph	58	CHN	$C_{16}H_{18}N_5Cl$	280 (iii)	^{1}H (D_2O) 2.16 (3H, s, Me-4′), 5.96 (1H, s, H-2), 6.94 and 7.12 (2 × 2H, AB doublet, J = 8 Hz, aromatic C-H), 7.30 (5H, m, aromatic C-H)
22	H	H	Me	Me	67	CN^d	$C_{11}H_{16}N_5Cl$	218 (iii)	^{1}H (D_2O, 400 MHz) 1.45 (6H, s, 2 × Me), 2.25 (2H, q, J = 7 Hz, CH_2), 7.40, 7.55 (2 × m, 5H, Ar-C-H)
23	F	H	Me	Me	93	CHN	$C_{11}H_{15}N_5FCl$	235 (i)	^{1}H (DMSO-d_6) 1.31 (6H, s, 2 × Me), 6.42 (1H, s br ex, NH), 7.29−7.47 (6H, m, Ar-C-H and NH_2), 7.67 (1H, s br ex, NH), 9.01 (1H, s, NH^+). ^{13}C (DMSO-d_6) 27.6 (2C, 2 × Me), 70.1 (1C, CMe$_2$), 117.4 + 117.9 (2C, Ar-C-F ortho), 131.7 (1C, CN_3), 133.0 + 133.2 (2C, Ar-C-F meta), 158.24 (1C, Ar-C-F ipso), 160.1 (1C, CN_3), 165.6 (1C, Ar-C-N). ^{19}F (DMSO-d_6, 250 MHz) −112.5 (1F, Ar-F)
24	H	H	H	H	85	CHN	$C_9H_{12}N_5Cl$	190 (i)	^{1}H (DMSO-d_6) 4.78 (2H, s, CH_2), 6.89 (1H, s br ex, NH), 7.34−7.58 (m, 7H, Ar-C-H and NH_2), 7.80 (1H, s br ex, NH) 8.58 (1H, s, NH^+)
25	F	H	H	H	95	CHN	$C_9H_{11}N_5FCl$	208 (i)	^{1}H (DMSO-d_6) 1.31 (6H, s, 2 × Me), 6.42 (1H, s br ex, NH), 7.29−7.47 (6H, m, Ar-C-H and NH_2), 7.67 (1H, s br ex, NH), 9.01 (1H, s, NH^+). ^{13}C (DMSO-d_6) 27.6 (2C, 2 × Me), 70.1 (1C, CMe$_2$), 117.4 + 117.9 (2C, Ar-C-F ortho), 131.7 (1C, CN_3), 133.0 + 133.2 (2C, Ar-C-F meta), 158.24 (1C, Ar-C-F ipso), 160.1 (1C, CN_3), 165.6 (1C, Ar-C-N). ^{19}F (DMSO-d_6, 250 MHz) −112.5 (1F, Ar-F)
26	H	Cl	Me	Me	60	CHN	$C_{11}H_{15}N_5Cl_2$· $0.4H_2O$	252 (iii)	^{1}H (D_2O) 1.30 (6H, s, 2 × Me), 7.10−7.50 (m, 4H, Ar-C-H)
27	Cl	Cl	Me	Me	71	CHN	$C_{11}H_{14}N_5Cl_3$	286 (iii)	^{1}H (D_2O, 400 MHz) 1.38 (6H, s, 2 × Me), 7.26 (1H, dd, J = 8.5, 2.6 Hz, Ar-CH), 7.60 (1H, d, J= 2.6 Hz, Ar-CH), 7.65 (d, 1H, J = 8.5 Hz, Ar-C-H)

Table 6. Continued

cpd	X	Y	R_1	R_2	yd%	anal.	formula	MH^{+} [a]	NMR details (200 MHz unless otherwise specified)
28	H	Cl	H	Me	18	CHN	$C_{10}H_{13}N_5Cl_2$	238 (iii)	^{1}H (D_2O) 1.18 (3H, d, $J = 6$ Hz, Me-2), 4.98 (1H, q, $J = 6$ Hz, H-2), 7.20, 7.35 (m, 4H, Ar-C-H)
29	Cl	Cl	H	Me	76	CHN	$C_{10}H_{12}N_5Cl_3\cdot 2HCl\cdot H_2O$	272 (iii)	^{1}H (D_2O) 1.18 (3H, d, $J = 6.5$ Hz, Me), 5.04 (1H, q, $J = 6.5$ Hz, H-2), 7.15 (1H, d, $J = 8.5$, 2.6 Hz, Ar-C-H), 7.50 (2H, m, Ar-C-H)
30	H	Cl	H	Ph	90	HN[e]	$C_{15}H_{15}N_5Cl_2\cdot H_2O$	300 (iii)	^{1}H (D_2O) 5.95 (1H, s, H-2), 6.90−7.30 (m, 9H, Ar-C-H)
31	Cl	Cl	H	Ph	63	CHN	$C_{11}H_{14}N_5Cl_3$	334 (iii)	^{1}H (D_2O) 5.92 (1H, s, H-2), 6.90 (1H, dd, $J = 8.0$, 2.5 Hz, Ar-CH), 7.10−7.30 (7H, m, Ar-CH)

[a] Mass spectrometric methods are (i) APCI+, (ii) ESI, (iii) MALDI-TOF. [b] C calcd 45.0; found 45.6. [c] C calcd 48.0; found 47.5. [d] H calcd 6.4; found 7.0. [e] C calcd 46.4; found 46.9.

Parallel synthesis reactions were performed in a Radley multiple synthesis block of 56 wells, equipped with a "Big Bill" rotating shaker, a water-cooled condensing stage, and a J-KEM programmable thermocouple and controller. Reagents were heated and refluxed in 4 mL capacity ReactiVials equipped with 10 cm condensing tubes inserted into Teflon-coated seals and caps. Reactions involving highly volatile solvents were contained under SubaSeals vented using balloon leaks.

Positive-ion chemical ionization mass spectrometry was carried out as APCI in 1:1 methanol/dichloromethane as carrier solvent (Dyson Perrins Laboratory, University of Oxford). MALDI-TOF mass spectra were recorded on a Bruker Biflex mass spectrometer (Institute of Genetic Engineering and Biotechnology, Chulalongkorn University, Bangkok). ESI spectra were obtained using a Fisons Instrument Trio 2000 mass spectrometer (Department of Chemistry, Chulalongkorn University, Bangkok) operating in ESI mode. Masses given are molecular ions and major fragments only, and they are quoted as m/z unless otherwise stated. Elemental analysis (C, H, N) was performed by the Oxford University Inorganic Laboratory service and by Ms. A. Ungpakornkaew on a Perkin-Elmer CHN analyzer model PE2400 series II (Chulalongkorn Research Equipment Centre, Bangkok). Routine ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini 200 spectrometer, a Bruker AMX250 spectrometer (both Dyson Perrins Laboratory, Oxford), or a Bruker ACF200 (Chulalongkorn University, Bangkok) operating at 200 MHz (^{1}H) and 50.28 MHz (^{13}C). High field NMR experiments were performed on a Bruker DRX400 (400 MHz) (National Science and Technology Development Agency, Bangkok). ^{1}H and ^{13}C chemical shifts are quoted in ppm relative to tetramethylsilane and were internally referenced to the residual protonated solvent signal.

Chemical Syntheses of 4,6-Diamino-1,2-dihydrotriazine Analogues. The Cyc derivatives bearing gem-dimethyl groups at the C-2 position were prepared by a three-component condensation reaction between an aromatic amine, dicyandiamide, and acetone in the presence of concentrated aqueous HCl as described in the literature (Scheme 1).[10] When the carbonyl compound is an aldehyde, a two-component condensation between the carbonyl compound (1−2 equiv or as solvent) and an aryl biguanide,[11,15,16] obtained from a reaction between an aromatic amine and dicyandiamide in the presence of HCl as catalyst, or a one-pot reaction in which the biguanide was preformed before reacting with the carbonyl compound was sometimes found to be superior. Derivatives of formaldehyde were prepared in the same way as other aldehydes, except that dimethoxymethane was used as a source of formaldehyde. In most cases the desired products precipitated from the reaction medium (usually ethanol) as the crystalline hydrochloride salt. In cases where the product did not crystallize from the reaction, the solvent was completely removed and the product was precipitated by addition of lithium picrate. The picrate salt so obtained was converted back to the hydrochloride salt by treatment with a strongly basic anion-exchange resin Amberlite IRA400 (Cl^- form). The products were recrystallized from ethanol, methanol, or aqueous alcohols. All Cyc derivatives were characterized by ^{1}H NMR, mass spectra (APCI, ESI, or MALDI-TOF), and elemental analysis (CHN). The full detailed analysis and the data of the Cyc analogues are shown in Table 6.

Enzyme Assays and Inhibition by Cyc Analogues. The activities of wild-type and A16V+S108T mutant pfDHFRs were determined spectrophotometrically according to the method previously described.[17] 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_2 folate and cofactor NADPH, and an appropriate amount (0.001−0.005 units) of the affinity-purified enzymes. Inhibition of the enzymes by Cyc and its analogues was carried out by determination of the K_i values of the inhibitors for the enzymes by fitting to the equation $IC_{50} = K_i\,(1 + ([S]/K_m))$,[18] where IC_{50} is the concentration of inhibitor which inhibits 50% of the enzyme activity under the standard assay condition and K_m is the Michaelis constant for the substrate H_2/folate. The resistance factors which determine the effectiveness of the inhibitor against the mutant pfDHFR over the wild-type enzyme were assessed from the values of the ratios of the K_i for the A16V+S108T mutant enzyme and the wild-type enzyme (K_i-mut/K_i-wt).

Drug Screening against *Plasmodium falciparum* in Vitro. Two clones of *P. falciparum*, TM4/8.2 (wild-type DHFR) and T9/94 RC17 (A16V+S108T DHFR),[19] from diverse sources (generous gifts from the Malaria Research Unit, Chulalongkorn University, Bangkok, Thailand) 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.[20] In vitro antimalarial activity was determined by using the [^{3}H]-hypoxanthine incorporation method.[21]

The drugs were initially dissolved in DMSO and diluted with the culture media. Aliquots (25 μL) of the drug having different concentrations were dispensed into 96-well plates, and 1.5% cell suspension of parasitized erythrocytes with 1−2% parasitemia (200 μL) were added. The final concentration of DMSO (0.1%) did not affect the growth of the parasite. 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, and the parasite cultures were further incubated under the same conditions for 18−24 h prior to harvesting the parasite DNA onto 96-well microplates with built-in glass fiber filters (Unifilter TM plates, Packard, USA). The filters in the plates were air-dried, and then 22 μL of 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 which inhibits 50% of the parasite growth (IC_{50}) was determined from the sigmoidal curve obtained by plotting the percentages of [^{3}H]-hypoxanthine incorporation against the concentrations of the drug used.

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