Novel 2,4-Diamino-5-Substituted-pyrrolo[2,3-*d*]pyrimidines as Classical and Nonclassical Antifolate Inhibitors of Dihydrofolate Reductases[1]

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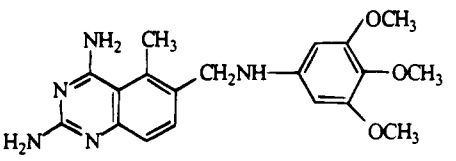
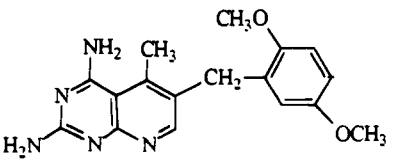
Eight novel, nonclassical, antifolate 2,4-diamino-5-(anilinomethyl)pyrrolo[2,3-*d*]pyrimidines, 1–**8**, with 3′,4′,5′-trimethoxyphenyl, 3′,4′-dimethoxyphenyl, 2′,5′-dimethoxyphenyl, 4′-methoxyphenyl, 2′,5′-diethoxyphenyl, 3′,4′-dichlorophenyl, 1′-naphthyl, and phenyl substituents were synthesized as potential inhibitors of dihydrofolate reductases (DHFRs). The classical analogue *N*-[4-[*N*-[(2,4-diaminopyrrolo[2,3-*d*]pyrimidin-5-yl)methyl]amino]benzoyl]-L-glutamic acid (**9**) was also synthesized as an inhibitor of DHFR and an antitumor agent. The classical and nonclassical analogues were obtained via reductive condensations of the key intermediate 2,4-diamino-5-cyanopyrrolo[2,3-*d*]pyrimidine (**12**) with the appropriate substituted aniline or (*p*-aminobenzoyl)-L-glutamate followed by reduction of the intermediate Schiff bases with $NaCNBH_3$. Compounds **1**–**9** were evaluated *in vitro* as inhibitors of rat liver (rl), *Pneumocystis carinii* (pc), and *Toxoplasma gondii* (tg) DHFRs. The nonclassical analogues were significantly selective against tgDHFR (vs rat liver DHFR), ranging from 7- to 92-fold. The inhibitory activity was lower in pcDHFR and rlDHFR (IC_{50}s > 10^{-5} M) than in tgDHFR (IC_{50}s = 10^{-6} M). The classical analogue had inhibitory activity similar to that of methotrexate (MTX) against the growth of human leukemia CCRF-CEM, A253, and FaDu squamous cell carcinoma (SCC) of the head and neck cell lines. Further evaluation of **9** against CCRF-CEM and its sublines having defined mechanisms of MTX resistance demonstrated that the analogue utilizes the reduced folate/MTX-transport system and primarily inhibits DHFR and poly-γ-glutamylation plays a role in its mechanism of action. Compound **9** was found to be 3-fold more efficient than aminopterin as a substrate for human folylpolyglutamate synthetase.

Opportunistic infections with *Pneumocystis carinii* and *Toxoplasma gondii* associated with loss of cell-mediated immunity remains among the principal causes of morbidity and mortality in patients with acquired immunodeficiency syndrome (AIDS) in the United States.[2] The treatment of *P. carinii* and *T. gondii* infections with antifolates takes advantage of the fact that these organisms are permeable to lipophilic, nonclassical antifolates and, unlike mammalian cells, lack a carrier-mediated active transport mechanism for the uptake of classical folates and antifolates with polar glutamate side chains.[3] Thus, host tissues can be selectively protected from the toxic effects of some nonselective lipophilic antifolates by coadministration of a reduced folate, typically leucovorin, which is taken up only by host cells and reverses toxicity.[3,4] These lipophilic agents can also penetrate into the central nervous system (CNS) where *T. gondii* infections usually occur.

The currently used lipophilic agents trimethoprim (TMP) and pyrimethamine, used for *P. carinii* and *T. gondii* infections, respectively, are weak inhibitors of dihydrofolate reductase (DHFR) from *P. carinii* and *T. gondii* and must be used with sulfonamides to provide synergistic effects.[5] Trimetrexate (TMQ) and piritrexim (PTX), which are 100–10 000 times more potent than TMP or pyrimethamine against DHFRs from *P. carinii* and *T. gondii*, also strongly inhibit DHFRs from mammalian sources.[6] Thus, TMQ needs to be administered in conjunction with leucovorin. The disadvantage of such therapies includes the fact that leucovorin is expensive and the sulfa drugs necessary for augmenting the action of TMP and pyrimethamine are the cause of side effects which quite often necessitate the discontinuation of therapy.[7]



TMQ



PTX

The development of potent analogues with greater selectivity for *P. carinii* and *T. gondii* DHFR than that of TMQ or PTX is a desirable goal. Efforts in our laboratory have resulted in the synthesis of a variety of 6-6 ring-fused analogues[8,9] and, most recently, the 6-5 fused furo[2,3-*d*]pyrimidines[10] which either provided good selectivity for *P. carinii* DHFR (pcDHFR) or *T. gondii* DHFR (tgDHFR) but lacked potency or were potent but lacked significant selectivity.

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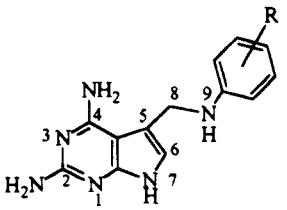
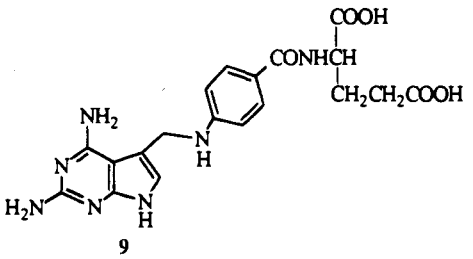


1: R = 3',4',5'-$(OCH_3)_3$
2: R = 3',4'-$(OCH_3)_2$
3: R = 4'-(OCH_3)
4: R = 2',5'-$(OCH_3)_2$
5: R = 2',5'-$(OC_2H_5)_2$
6: R = 3',4'-$(Cl)_2$
7: R = 2',3'-$(CH)_4$
8: R = H



9

Figure 1.

Miwa *et al.*,[11] Taylor *et al.*,[12] and Shih and Gossett[13] have reported classical pyrrolo[2,3-*d*]pyrimidine analogues with significant inhibitory activity against DHFR, thymidylate synthase, and tumor cells in culture. All of the pyrrolo[2,3-*d*]pyrimidine antifolates reported thus far are classical and have a two-, three-, or four-carbon bridge. In addition there have been several reports of other B-ring-contracted analogues as antifolates. These include purine analogues by Burchenal *et al.*[14] and Weinstock *et al.*[15] and thienopyrimidine analogues by Roth *et al.*,[16] Elslager *et al.*,[17] and Rosowsky *et al.*[18,19] To our knowledge, this is the first report of nonclassical analogues of 2,4-diamino-5-(aminomethyl-substituted)-pyrrolo[2,3-*d*]pyrimidines as inhibitors of DHFRs and the first classical analogue with a CH_2–NH bridge. In order to explore the effects of various lipophilic side chains on the 2,4-diaminopyrrolo[2,3-*d*]pyrimidine ring with respect to activity and selectivity against *P. carinii* and *T. gondii* DHFRs, we designed, synthesized, and biologically evaluated compounds **1**–**8** as the first in our series of novel, nonclassical 2,4-diaminopyrrolo[2,3-*d*]-pyrimidines.

The alkyloxy and chloro substituents on the side chain phenyl ring were selected on the basis of previous reports from our laboratory[8–10] which indicated that in pyrido[2,3-*d*]pyrimidines and furo[2,3-*d*]pyrimidines these substituents provided for potency and/or selectivity against tgDHFR and to some extent against pcDHFR. In addition, the clinically used agents TMP, pyrimethamine, and TMQ contain similar substituents. The naphthyl side chain substituent was included to increase lipophilicity and enhance penetration of *P. carinii* and *T. gondii* organisms in culture as well as to explore bulk tolerance for pcDHFR and tgDHFR particularly in light of the report by Piper *et al.*[20] that DHFR can accommodate naphthyl replacement of the phenyl ring in classical analogues.

Molecular modeling of 6-5 ring-fused analogues superimposed on 6-6 ring-fused analogues indicates that the 5-substituent almost coincides in both ring systems. However the 6-substituent of the 6-5 system lies in between the 6- and 7-substituents of the 6-6 system. Most 6-6 fused systems, for example, TMQ, which have been found to potently inhibit DHFR are 6-substituted. In the pyrido[2,3-*d*]pyrimidine series, which we have reported previously,[8,9] selectivities for tgDHFR in the realm of 10–15-fold, compared with rat liver DHFR (rlDHFR), was achievable, but the need for increased selectivity was quite evident. We reasoned that 5-substituted 6-5 fused systems with a two-atom chain between the heterocycle and the phenyl ring would provide for a decreased distance compared to the relatively nonselective 6-substituted 6-6 systems. This should allow an exploration of alternate binding residues on DHFRs different from those involved with the 6-6 systems and could perhaps afford analogues with higher selectivity for pcDHFR and/or tgDHFR.

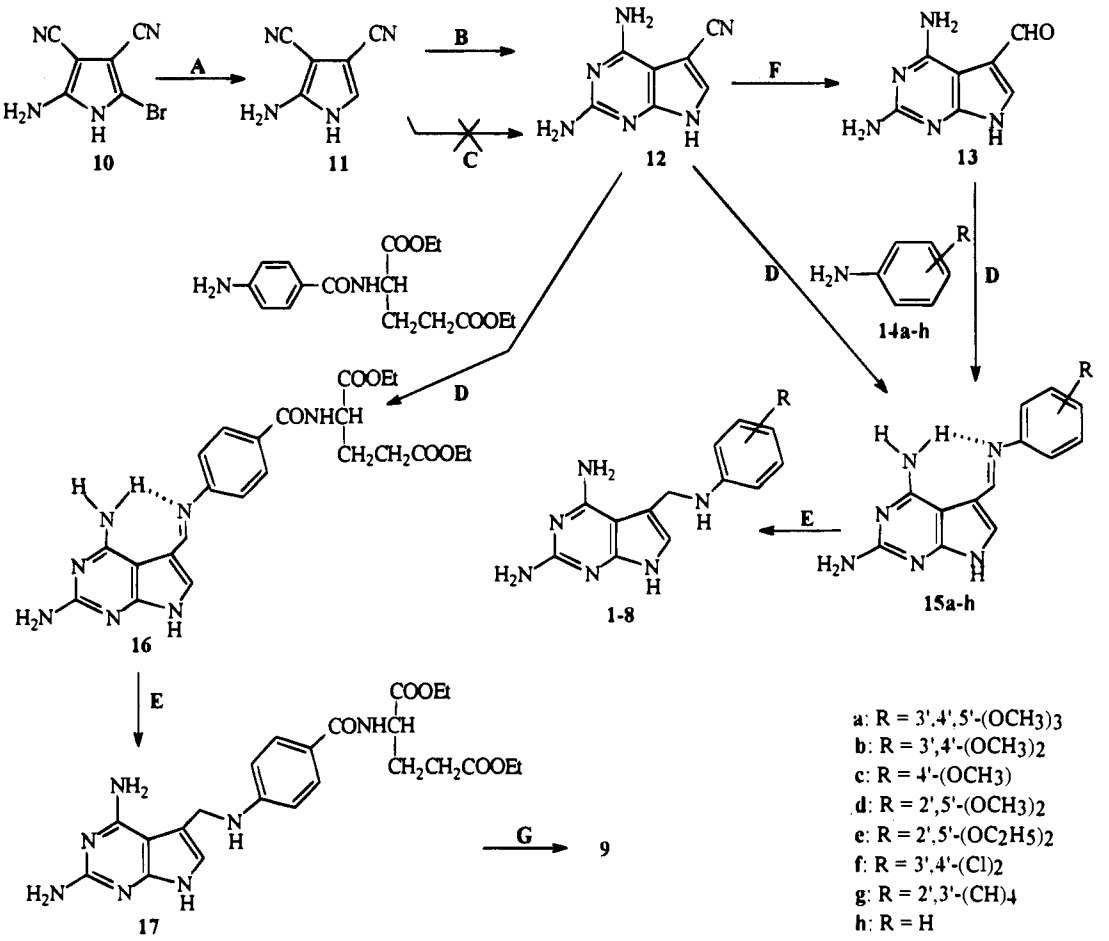
Molecular modeling, using SYBYL[21] and its SEARCH and MAXIMIN options, of the energy-minimized conformations of the 2,4-diaminopyrrolo[2,3-*d*]pyrimidines **1**–**8** on to the DHFR-bound conformation of TMQ[22] with the pyrimidine ring of each ring system superimposed showed that the side chains of the 5-substituted-pyrrolo-[2,3-*d*]pyrimidines fell short of overlapping the side chain of TMQ due to the distance constraints imposed by the contracted B-ring and the 5-position substitution. Thus on the basis of molecular modeling, compounds **1**–**8** were designed to have their side chain substituent oriented differently from that of TMQ to explore alternate conformations and distances between the heterocycle and the phenyl ring in an attempt to improve selectivity and/or potency. We also synthesized the classical analogue **9**, as the first in a series of potential antitumor antifolates, on the basis of the potent antitumor activity reported for similar pyrrolo[2,3-*d*]-pyrimidines.[11]

Chemistry

The previous procedures[11,12] reported for classical antifolates containing pyrrolo[2,3-*d*]pyrimidines utilized total synthesis for each classical analogue. Our goal was to synthesize a key intermediate which could be exploited to afford a variety of classical and nonclassical analogues such that a structure–activity/selectivity relationship could be developed for the 2,4-diamino-pyrrolo[2,3-*d*]pyrimidines. We identified 2,4-diamino-5-cyanopyrrolo[2,3-*d*]pyrimidine, **12**, as the key intermediate, where the 5-cyano moiety could be suitably transformed to afford a variety of 2,4-diamino-5-substituted-pyrrolo[2,3-*d*]pyrimidines in a convergent rather than a total synthesis.

The syntheses of the target compounds **1**–**9**, which represent the first in a series of transformations of the 5-cyano moiety of **12**, were achieved using the general procedure illustrated in Scheme 1. 3,4-Dicyano-2-aminopyrrole, **11**, was obtained by reductive debromination of the pyrrole **10** which in turn was prepared from tetracyanoethylene utilizing the method reported by Townsend *et al.*[23] The debromination reaction, of a solution of the pyrrole in a mixture of DMF/MeOH, went to completion within 3 h under H_2 (50 psi) in the presence of a Pd catalyst. The type of Pd catalyst used

Scheme 1[a]



[a] (A) Pd–$BaCO_3$/DMF/MeOH; (B) chlorformamidine/Dowtherm-A; (C) guanidine HCl/NaOEt/Δ; (D) Raney Ni/80% AcOH/MeOH; (E) $NaCNBH_3$/MeOH/AcOH; (F) HCOOH/Raney Ni;Δ; (G) 1 N NaOH/MeOH/rt.

was important, 5% Pd–$BaCO_3$ afforded a higher yield (64%) than 10% Pd–C (51%). Unlike the pyrido[2,3-*d*]-pyrimidines,[8] condensation of the pyrrole *o*-aminonitrile **11** with guanidine failed to yield the desired product **12**. However, the key intermediate 2,4-diamino-5-cyanopyrrolo[2,3-*d*]pyrimidine, **12**, was first obtained, in 45–50% yield, by a high-temperature (160–170 °C) fusion reaction of an intimate mixture of **11** and chlorformamidine hydrochloride.[24] The fusion reaction was more efficient, providing a significant increase in yield (79%), when a uniformly stirred suspension of the mixture, in Dowtherm-A, was heated at 160–170 °C for 48 h. The product obtained from this reaction was sufficiently pure to utilize in subsequent reactions without further purification. Reductive condensation of the nitrile **12** with the appropriate anilines **14a–h** or diethyl (*p*-aminobenzoyl)-L-glutamate in 70–80% HOAc and Raney Ni as the catalyst was performed utilizing the procedure we have reported for the pyrido-[2,3-*d*]pyrimidines.[8] The reaction, however, did not afford the expected target compounds but rather the intermediate Schiff bases **15a–h** and **16**. Prolonged hydrogenation times, from 24–72 h, and/or increased hydrogenation pressures, from atmospheric up to 55 psi, consistently afforded the Schiff bases. Replacement of Raney Ni with 10% Pd–C did not afford any condensation product. The ^{1}H NMR spectrum for the Schiff bases **15a–h** and **16** showed the presence of a singlet between 8.40 and 8.55 ppm which was assigned to the 8-C*H*. In addition, the ^{1}H NMR spectrum showed a splitting of the 4-NH_2 protons into two distinct peaks at 6.52 and 8.88 ppm, an indication of hydrogen bonding with the 9-N as shown in Scheme 1. Further the absence of both the 8-CH_2 doublet and the 9-NH triplet, which were present in the final compounds **1–9**, and the downfield shift of the 7-NH and 6-CH compared to the target compounds confirmed the structures of the Schiff bases which were further substantiated by elemental analysis and for **15a,e** by mass spectra as well.

An alternate route to the target compounds via the aldehyde **13** was also carried out. Compound **13** was obtained by heating a mixture of **12**, HCOOH, and Raney Ni at 80 °C for 2 h. The ^{1}H NMR spectrum showed the presence of the aldehydic proton at 9.23 ppm. The IR spectrum indicated the absence of the nitrile peak, at 2160–2180 cm^{-1}, and the presence of an intense aldehydic peak between 1600 and 1650 cm^{-1}. Compound **13** was unstable at room temperature for long durations and was used immediately, without purification, in the next step. Reductive condensation of **13** with 2,5-diethoxyaniline in 80% HOAc, hydrogen, and Raney Ni as catalyst, again, afforded only the intermediate Schiff base **15e**.

Reduction of the Schiff bases, obtained from the nitrile or the aldehyde and the appropriate aniline, proceeded smoothly within 4 h, on stirring a solution of the Schiff bases in MeOH at room temperature with $NaCNBH_3$ as the reducing agent; 50% methanolic HCl or glacial HOAc was used to maintain the pH of the reaction mixture at 2. The target compounds **1–8** and the

Table 1. Inhibitory Concentrations (IC_{50}, μM) against DHFRs and Selectivity Ratios[25,26]

compd	*P. carinii*	rl	selectivity ratio rl/pc	*T. gondii*	selectivity ratio rl/tg
1	>23	56.3	<2.4	8.1	7.0
2	119.0	116.0	1.0	4.3	27.0
3	279.0	63.0	0.23	6.0	10.5
4	45.7	156.0	3.4	1.7	92.0
5	>21	70.0	<3.3	5.3	13.2
6	35.3	14.4	0.4	1.4	10.3
7	307.0	59.3	0.2	1.1	53.9
8	252.0	>252	<1	3.9	>65
9[a]	0.038	0.044	1.2	0.21	0.21
TMQ	0.042[b]	0.003[b]	0.07	0.01[c]	0.30
PTX	0.038	0.001	0.04	0.01	0.14
TMP	12.0[b]	133.0[b]	11.1	2.7	49.0
MTX[d]	0.001[b]	0.003[b]	3.0	0.014[c]	0.21

[a] IC_{50} against CCRF-CEM DHFR: 0.0034 μM. [b] Data from ref 25. [c] Data from ref 26. [d] IC_{50} against CCRF-CEM DHFR: 0.00055 μM.

precursor **17** were purified either by silica gel column chromatography or by dissolution of the crude compounds in MeOH, filtration, evaporation of the filtrate, and trituration of the residue in anhydrous diethyl ether to afford analytically pure compounds in yields ranging from 41% to 63%.

The classical analogue **9** was obtained from **17** on hydrolysis of the diethyl ester by stirring a solution of **9** in 1 N NaOH–MeOH (1:1) at room temperature for 72 h followed by acidification to pH 2 with glacial HOAc to afford analytically pure **9** in 89% yield.

Biological Evaluation and Discussion

The nonclassical analogues **1–8** were evaluated as inhibitors of pcDHFR, tgDHFR, and rlDHFR.[25,26] The inhibitory concentrations (IC_{50}) and the selectivity ratios vs rlDHFR are listed in Table 1. The nonclassical analogues were significantly selective against tgDHFR, ranging from 7- to 92-fold. The 3′,4′,5′-trimethoxy analogue **1** had better selectivity than TMQ and was somewhat less potent than TMP against tgDHFR. Removal of one *m*-methoxy group (compound **2**) afforded an increase in both potency and selectivity against tgDHFR. Compound **4**, the 2′,5′-dimethoxy positional isomer of **2** was more active against both pcDHFR and tgDHFR and was the most selective (92-fold) tgDHFR inhibitor. This analogue, **4**, was almost twice as potent and selective against tgDHFR as TMP. Compound **4** was also the most selective and potent (except **6**) in the nonclassical series against pcDHFR. Replacement of the dimethoxy groups with the larger 2′,5′-diethoxy groups, as in compound **5**, decreased selectivity and potency against tgDHFR, indicating a lack of tolerance for the ethyl moiety. Removal of the 3′- and 5′-methoxy moieties of **1** afforded the 4′-OCH_3 analogue **3** which produced little change in tgDHFR activity. Substituting the electron-donating 3′,4′-dimethoxy groups of **2** with electron-withdrawing 3′,4′-dichloro moieties as in **6** increased activity against all three DHFRs with loss of selectivity for both pcDHFR and tgDHFR, indicating that electron-withdrawing groups increase potency against rlDHFR to a greater extent than for pcDHFR or tgDHFR. Replacement of the substituted phenyl ring with a naphthyl ring or an unsubstituted phenyl afforded compounds **7** and **8**, respectively. Significantly, both these compounds, **7** and **8**, were reasonably potent and highly selective against tgDHFR. Replacement of the phenyl ring with a naphthyl allowed for increased potency against both rlDHFR and tgDHFR, indicating that for these two enzymes, in contrast to pcDHFR, the naphthyl moiety was conducive to inhibitory activity. Comparing compound **8** with **1–3** indicates that the methoxy moieties in the 3′-, 4′-, and 5′-positions are not important for tgDHFR inhibition; however, they are for rlDHFR inhibition. Thus both potency and selectivity against pcDHFR and tgDHFR for the nonclassical 5-substituted-2,4-diaminopyrrolo[2,3-*d*]pyrimidines are dictated by both the nature and position of the substitution on the phenyl ring.

The lower DHFR inhibitory potency compared to similar 6-6-fused ring systems (TMQ and PTX) in the nonclassical series can be attributed to contraction of the B-ring and the 5-position (rather than 6-position) substitution which decreases the distance between the heterocyclic and side chain phenyl rings. However, these same modifications provide for a substantial increase in selectivity for tgDHFR compared to TMP, PTX, and the corresponding pyrido[2,3-*d*]pyrimidines.[8] Thus the rationale for the design of compounds **1–8** was partially realized, and highly selective tgDHFR inhibitors compared to TMP, PTX, TMQ, and the pyrido[2,3-*d*]pyrimidines[8] were obtained, particularly compounds **4**, **7**, and **8**. This study along with our previous report on similar 6-5 furo[2,3-*d*]pyrimidines[10] strongly suggests that B-ring-contracted analogues are conducive to selectivity for pcDHFR and tgDHFR compared to similar 6-6-fused ring systems which lack selectivity. In addition the 5-substituted-2,4-diaminofuran[2,3-*d*]pyrimidines were more selective toward pcDHFR,[10] while the 5-substituted-2,4-diaminopyrrolo[2,3-*d*]pyrimidines of this report were more selective toward tgDHFR. Thus, in addition to the side chain, the heterocyclic ring system also plays a crucial role in selectivity for the two DHFRs.

The classical analogue **9** was evaluated as an inhibitor of DHFR from *P. carinii*, *T. gondii*, rat liver (rl), and CCRF-CEM, and the results are indicated in Table 1. Compound **9** was more potent than the nonclassical analogues. Against pcDHFR, **9** was 1000-fold more potent than **6**, the most potent of the nonclassical analogues, and 327 times more potent against rlDHFR than **6** but only 5 times more potent against tgDHFR, compared to **7**, the most potent analogue against tgDHFR of the nonclassical series. The different extents of enhancement of inhibitory activity, against pcDHFR, tgDHFR, and rlDHFR, of the classical analogue compared to the nonclassical analogues further support the premise that significant differences exist in the interactions of these analogues with these DHFRs which include the (*p*-aminobenzoyl)-L-glutamate binding sites as well as the heterocyclic binding sites on these DHFRs.

The classical analogue **9** was a more potent inhibitor of all the DHFRs tested compared to the corresponding classical furo[2,3-*d*]pyrimidine analogue which we reported earlier (rlDHFR IC_{50} = 1.3 μM).[10] Compound **9** had a significant IC_{50} of 3.4 nM against CCRF-CEM DHFR which prompted its evaluation as an antitumor agent against the growth of human leukemia CCRF-CEM and A253 and FaDu squamous cell carcinoma (SCC) of the head and neck cell lines during continuous exposure *in vitro* (Tables 2 and 3). All three cell lines

Table 2. Growth Inhibition of Parental CCRF-CEM and Sublines with Single, Defined Mechanisms of MTX Resistance during Continuous (0−120 h) Exposure to MTX and **9**[a] (EC_{50}, nM)

drug	CCRF-CEM	R30dm[b] (↓ Glu_n)	R1[c] (↑ DHFR)	R_2[d] (↓ uptake)
MTX	14.5 ± 0.4 (n = 5)	14.5 ± 0.5 (n = 2)	595 ± 5 (n = 2)	3100 ± 100 (n = 2)
9	12.8 ± 2.2 (n = 5)	36 ± 1 (n = 2)	515 ± 25 (n = 2)	1650 ± 200 (n = 2)

[a] Average values are presented ±range for n = 2 and ±SD for $n \geq 3$. [b] CCRF-CEM subline resistant to intermittent MTX exposure solely as a result of decreased polyglutamylation; this cell line has 1% of the FPGS specific activity (measured with MTX as the folate substrate) of parental CCRF-CEM. [c] CCRF-CEM subline resistant to MTX solely as a result of a 20-fold increase in wild-type DHFR protein and activity. [d] CCRF-CEM subline resistant to MTX as a result of defective uptake.

Table 3. Growth Inhibition of A253 and FaDu Human Squamous Carcinoma Cell Lines following Continuous (120 h) Exposure to MTX and **9** (EC_{50}, nM)[a]

compd	A253	FaDu
MTX	17 ± 1	31 ± 2
9	46 ± 4	22 ± 4

[a] All values are average ± range for duplicate determinations.

Table 4. Activity of Aminopterin and **9** as Substrates for CCRF-CEM Human Leukemia Cell Folylpolyglutamate Synthetase[a]

substrate	K_m, μM	$V_{max,rel}$	$V_{max}/K_{m(rel)}$	n
aminopterin	4.3 ± 0.2	1	0.23 ± 0.01	3
9	<1	0.72 ± 0.07	>0.72	4

[a] FPGS substrate activity was determined as described in the Experimental Section. Values presented are average ± SD.

were similarly sensitive to methotrexate (MTX) under continuous exposure conditions. Compound **9** had similar inhibitory activity to MTX against the growth of these tumor cells in culture.

Compound **9** was also evaluated as an inhibitor of the growth of three CCRF-CEM sublines with defined mechanisms of MTX resistance (Table 2) to further define its mechanism of action. The R30dm subline,[27] which has 1% of the wild type level of folylpolyglutamate synthetase (FPGS), is not resistant to MTX on continuous exposure (resistance is only seen in intermittent exposure) because the nonpolyglutamylated parent drug MTX is a tight-binding inhibitor of DHFR and transport is sufficient to maintain drug in excess of DHFR. The R30dm subline displayed a low level of cross-resistance to continuous exposure to **9**, indicating that polyglutamylation may play a limited role in its mechanism of action even in continuous exposure. The R1 subline,[28] with amplified DHFR expression, displayed resistance (41-fold) to MTX under continuous exposure as does the MTX-transport deficient R2 (214-fold).[29] These cell lines displayed similar high cross-resistance to compound **9**.

Polyglutamylation allows the cellular retention of a number of classical antifolates and has been implicated in the mechanism of action of a variety of antifolates.[30,31] Since compound **9** showed that polyglutamylation may play a role in its mechanism of inhibition of tumor cells, it was of interest to determine its ability to function as a substrate for human FPGS (Table 4). The analogue is a substrate for human FPGS with a very low K_m indicating that polyglutamylation must be considered as a component of the mechanism of action of this agent. An exact K_m value could be not be determined, however, because linear substrate activity as a function of time could not be maintained at the required low substrate concentrations. Compound **9** is at least 3-fold more efficient as a substrate than is aminopterin (AMT), which is a good substrate for ligation of the first additional glutamic acid.[32]

The biochemical and biological results showed that the classical 2,4-diaminopyrrolo[2,3-*d*]pyrimidine **9** is a potent tumor cell growth inhibitor that shares determinants of response with MTX. It is similar in potency to MTX as an inhibitor of the growth of human leukemia and SCC cell lines in culture. DHFR is suggested as the target of **9** which is supported by the data showing relatively potent DHFR inhibition *in vitro* and the cross-resistance to **9** of a human cell line having amplified expression of DHFR. Compound **9** also uses the MTX/reduced folate-transport protein for uptake as evidenced by the cross-resistance of a human cell line in which this transport system is defective. It is an excellent substrate for human FPGS with a very low K_m. The slight cross-resistance to continuous exposure of the cell line having low levels of FPGS suggests that polyglutamate metabolites may play a role in growth inhibition even under these conditions; this low level of cross-resistance contrasts with the high level of cross-resistance observed in this cell line with the 2,4-diaminofuro[2,3-*d*]pyrimidine analogue of **9** which we reported previously.[10]

The data on compound **9** extend our current knowledge on the FPGS substrate activity of previously described 2,4-diaminopyrrolo[2,3-*d*]pyrimidines which contained ethyl or propyl bridge regions[11] and indicate that isosteric replacement of the 9-CH_2 by an NH in the ethyl bridge region maintains excellent substrate activity. Compound **9** has been selected by the National Cancer Institute for evaluation in their preclinical *in vitro* screening program.

On the basis of the biological results of compounds **1**−**9**, as inhibitors of a variety of DHFRs and as antitumor agents, in particular the high selectivity for tgDHFR, we are currently in the process of synthesizing other classical and nonclassical analogues using the key intermediate nitrile **12** and the aldehyde **13** which allow for variations in the 8-9 bridge region and the phenyl ring, the results of which will be reported in a future communication.

Experimental Section

Melting points were determined on a Fischer-John melting point apparatus and are uncorrected. IR spectra were obtained on a Perkin-Elmer Model 781 double-beam recording spectrophotometer. Nuclear magnetic resonance spectra for proton (^{1}H NMR) were obtained in Me_2SO-d_6 on a Bruker WH-300 (300 MHz) spectrometer, and the chemical shifts are presented relative to TMS as the internal standard. Chemical shifts (δ) listed for multiplets were measured from the approximate centers, and relative integrals of peak areas agreed with those expected for the assigned structures. Mass spectra (MS) were recorded on a Varian MATCH-311A mass spectrometer in the electron-impact (EI^+) mode. Thin-layer chromatography (TLC) was performed on POLYGRAM Sil G/UV_{254} silica gel plates and cellulose plates with fluorescent indicator, and the spots were visualized under 254 and 366 nm illumination. Proportions of solvents used for TLC are by volume. Elemental analyses were performed by Atlantic Microlabs Inc., Norcross, GA. Analytical results indicated by element symbols are within ±0.4% of the calculated values. Fractional moles

of water or organic solvents frequently found in some analytical samples of antifolates were not removed in spite of 24–48 h of drying *in vacuo* and were confirmed, where possible, by their presence in the ^{1}H NMR spectrum.

2-Amino-3,4-dicyanopyrrole (11). A mixture of **10** (4.0 g) and 5% Pd on $BaCO_3$ (4.0 g) in DMF (15 mL) and MeOH (25 mL) was hydrogenated at 50 psi for 3 h. The mixture was filtered through Celite and the filtrate concentrated under reduced pressure to 10 mL. Cold water (200 mL) was added to the concentrate, and the light brown solid was collected by filtration to yield 1.60 g (64%) of 11: mp >220 °C dec; TLC R_f 0.48 ($CHCl_3$/MeOH/NH_4OH, 10:3:0.1, silica gel); IR (Nujol) 2160, 2180 cm^{-1}; ^{1}H NMR (Me_2SO-d_6) δ 5.50–6.70 (br s, 2H, 2-NH_2), 7.11 (s, 1H, 5-CH), 11.08 (br s, 1H, 1-NH). Anal. Calcd for ($C_6H_4N_4$·0.2H_2O) C, H, N.

2,4-Diamino-5-cyanopyrrolo[2,3-*d*]pyrimidine (12). An intimate mixture of 11 (2.63 g, 19.94 mmol) and chlorformamidine hydrochloride[24] (2.50 g, 21.93 mmol) in Dowtherm-A (50 mL) was heated at 160–170 °C for 48 h until 11 could not be detected on TLC. The mixture was cooled to room temperature, and to it was added Et_2O (50 mL). The greenish brown solid obtained was filtered and washed with Et_2O to afford 3.0 g (79%) of crude **12** which was used without further purification in all subsequent reactions. An analytically pure sample was obtained by dissolving crude **12** in MeOH and treating this solution with silica gel followed by evaporation of the MeOH to give a dry silica gel plug. The plug was then loaded onto a silica gel column (2.4 cm × 20 cm) and eluted with a gradient of 10% MeOH in $CHCl_3$ (containing 0.2% NH_4-OH) to 50% MeOH in $CHCl_3$ (containing 0.2% NH_4OH). Fractions corresponding to the product (TLC) were pooled and evaporated under reduced pressure to a small volume and neutralized with glacial HOAc. The neutral solution was evaporated to dryness, triturated with Et_2O, and filtered to yield analytically pure **12**: mp >300 °C; TLC R_f 0.43 ($CHCl_3$/MeOH/NH_4OH, 10:3:0.1, silica gel); IR (Nujol) 2180 cm^{-1}; ^{1}H NMR (Me_2SO-d_6) δ 5.89 (br s, 2H, 2-NH_2), 6.16 (br s, 2H, 4-NH_2), 7.71 (s, 1H, 6-CH), 11.81 (br s, 1H, 7-NH). Anal. Calcd for ($C_7H_6N_6$·0.3H_2O·0.1CH_3COOH) C, H, N.

2,4-Diaminopyrrolo[2,3-*d*]pyrimidine-5-carboxaldehyde (13). To a stirred solution of **12** (2.0 g, 11.40 mmol) in HCOOH (50 mL) was added Raney Ni (6.0 g). The mixture was heated to 80 °C for 2 h until no more starting material could be detected (TLC). The mixture was then cooled to room temperature and filtered through Celite. The filtrate was evaporated under reduced pressure azeotroping with MeOH to remove traces of HCOOH. The residue was dissolved in hot water (25 mL), treated with Norit, and filtered through Celite and the filtrate neutralized with NH_4OH. The light brown precipitate was filtered and dried to yield 1.40 g (63%) of **13** which was immediately used in subsequent reactions without further purification.

2,4-Diamino-5-[[*N*-(3′,4′,5′-trimethoxyphenyl)imino]methyl]pyrrolo[2,3-*d*]pyrimidine (15a). A solution of **12** (1.30 g, 7.50 mmol) and 3,4,5-trimethoxyaniline, **14a** (2.06 g, 11.25 mmol), in 70% HOAc (75 mL) containing damp Raney nickel (6.50 g) was hydrogenated at atmospheric pressure for 24 h at room temperature. The mixture was treated with Norit and filtered through Celite and the solvent removed from the filtrate by evaporation under reduced pressure. Cold water (15 mL) was added to the residue, and the suspension was added to a stirred, cold, saturated solution of $NaHCO_3$ (100 mL). The mixture was stirred for 10 min and refrigerated for 6 h. A brown precipitate formed which was collected, washed with water, and dried. The crude product containing **14a** was washed repeatedly with Et_2O until no aniline was detected (TLC) in the washings. The residue was then dissolved in MeOH (100 mL) and filtered and the filtrate evaporated under reduced pressure to near dryness. Et_2O (50 mL) was added to this solution and the precipitate filtered to afford 1.20 g (47%) of **15a** as a tan solid: mp >240 °C dec; TLC R_f 0.59 ($CHCl_3$/MeOH/NH_4OH, 10:3:0.1, silica gel); MS *m/z* 342 (M^+); ^{1}H NMR (Me_2SO-d_6) δ 3.65 (s, 3H, 4′-OCH_3), 3.81 (s, 6H, 3′-, 5′-OCH_3), 5.68 (br s, 2H, 2-NH_2), 6.52 (s, 3H, 2′-, 6′-CH, 4-NH_2), 7.46 (s, 1H, 6-CH), 8.46 (s, 1H, 8-CH), 8.88 (br s, 1H, 4-NH_2), 11.44 (br s, 1H, 7-NH). Anal. Calcd for ($C_{16}H_{18}N_6O_3$·1.1H_2O·0.2HCl) C, H, N.

2,4-Diamino-5-[(3′,4′,5′-trimethoxyanilino)methyl]pyrrolo[2,3-*d*]pyrimidine (1). $NaCNBH_3$ (0.05 g, 0.83 mmol) was added to a solution of **15a** (0.20 g, 0.60 mmol) in MeOH (25 mL), the pH was adjusted to 2 with 50% MeOH/HCl, and stirring was continued at room temperature for 4 h. The solvent was evaporated to dryness, and cold water was added to the residue which was neutralized with NH_4OH. The precipitate obtained was filtered, dried, and dissolved in $CHCl_3$/MeOH (9:1). This was applied to a silica gel column (2.4 cm × 20 cm) packed in $CHCl_3$. The column was eluted with a gradient of 1% MeOH in $CHCl_3$ to 15% MeOH in $CHCl_3$. Fractions corresponding to the product (TLC) were pooled and evaporated to dryness under reduced press. The residue was triturated in cold Et_2O and filtered to afford 0.10 g (49%) of 1 as a tan solid: mp >230 °C dec; TLC R_f 0.57 ($CHCl_3$/MeOH/NH_4OH, 10:3:0.1, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 3.53 (s, 3H, 4′-OCH_3), 3.68 (s, 6H, 3′-, 5′-OCH_3), 4.15 (d, 2H, 8-CH_2), 5.42 (br s, 2H, 2-NH_2), 5.69 (t, 1H, 9-NH), 6.11 (s, 2H, 2′-, 6′-CH), 6.24 (br s, 2H, 4-NH_2), 6.71 (s, 1H, 6-CH), 10.55 (br s, 1H, 7-NH). Anal. Calcd for ($C_{16}H_{20}N_6O_3$·0.7H_2O) C, H, N.

2,4-Diamino-5-[(3′,4′-dimethoxyanilino)methyl]pyrrolo[2,3-*d*]pyrimidine (2). The Schiff base was prepared as described for **15a** except that the reaction was carried out in 80% HOAc using 3,4-dimethoxyaniline to afford 1.20 g (67%) of **15b** as a tan solid: mp >275 °C dec; TLC R_f 0.59 ($CHCl_3$/MeOH/NH_4OH, 10:3:0.1, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 3.76 (s, 3H, 4′-OCH_3), 3.80 (s, 3H, 3′-OCH_3), 5.58 (s, 2H, 2-NH_2), 6.62 (br s, 1H, 4-NH_2), 6.76 (dd, 1H, 6′-CH), 6.88 (d, 1H, 2′-CH), 6.96 (dd, 1H, 5′-CH), 7.43 (s, 1H, 6-CH), 8.47 (s, 1H, 8-CH), 9.00 (br s, 1H, 4-NH_2), 11.35 (br s, 1H, 7-NH).

Reduction of **15b** was carried out as described for 1. The crude product was dissolved in MeOH and filtered. The filtrate was evaporated under reduced pressure to dryness. The residue was triturated in cold Et_2O and filtered to afford 0.51 g (51%) of **2** as a light brown powder: mp >230 °C dec; TLC R_f 0.55 ($CHCl_3$/MeOH/NH_4OH, 10:3:0.1, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 3.63 (s, 3H, 4′-OCH_3), 3.66 (s, 3H, 3′-OCH_3), 4.11 (d, 2H, 8-CH_2), 5.50 (br s, 3H, 9-NH, 2-NH_2), 6.30 (dd, 1H, 6′-CH), 6.41 (br s, 2H, 4-NH_2), 6.50 (d, 1H, 2′-CH), 6.72 (m, 3H, 9-NH, 5′-, 6-CH), 10.56 (br s, 1H, 7-NH). Anal. Calcd for ($C_{15}H_{18}N_6O_2$·1.3HCl) C, H, N.

2,4-Diamino-5-[(4′-methoxyanilino)methyl]pyrrolo[2,3-*d*]pyrimidine (3). The Schiff base was prepared as described above for **15b** using 4-methoxyaniline to afford 0.32 g (51%) of **15c**. Reduction of **15c** was carried out as described for **15b** to afford **3**: mp >300 °C; TLC R_f 0.53 ($CHCl_3$/MeOH/NH_4OH, 10:3:0.1, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 3.65 (s, 3H, 4′-OCH_3), 4.09 (d, 2H, 8-CH_2), 5.40 (br s, 2H, 2-NH_2), 5.52 (t, 1H, 9-NH), 6.36 (br s, 2H, 4-NH_2), 6.68 (s, 1H, 6-CH), 6.75 (s, 4H, 2′-, 3′-, 5′-, 6′-CH), 10.50 (br s, 1H, 7-NH). Anal. Calcd for ($C_{14}H_{18}N_6O$·0.6HCl) C, H, N.

2,4-Diamino-5-[(2′,5′-dimethoxyanilino)methyl]pyrrolo[2,3-*d*]pyrimidine (4). The Schiff base was prepared as described above for **15b** using 2,5-dimethoxyaniline to afford 0.90 g (50%) of **15d** as a tan solid: mp >250 °C dec; TLC R_f 0.61 ($CHCl_3$/MeOH/NH_4OH, 10:3:0.1, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 3.15 (s, 3H, 2′-OCH_3), 3.74 (s, 3H, 5′-OCH_3), 5.61 (br s, 2H, 2-NH_2), 6.66 (s, 1H, 4-NH_2), 6.73 (dd, 1H, 4′-CH), 6.81 (d, 1H, 6′-CH), 6.95 (dd, 1H, 3′-CH), 7.43 (s, 1H, 6-CH), 8.48 (s, 1H, 8-CH), 9.27 (br s, 1H, 4-NH_2), 11.34 (br s, 1H, 7-NH).

Reduction of **15d** was carried out as described for **15b** to afford 0.25 g (41%) of **4** as a tan powder: mp 177–180 °C; TLC R_f 0.60 ($CHCl_3$/MeOH/NH_4OH, 10:3:0.1, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 3.62 (s, 3H, 2′-OCH_3), 3.71 (s, 3H, 5′-OCH_3), 4.22 (d, 2H, 8-CH_2), 5.08 (t, 1H, 9-NH), 5.39 (br s, 2H, 2-NH_2), 6.12 (m, 2H, 4′-, 6-CH), 6.22 (br s, 2H, 4-NH_2), 6.38 (s, 1H, 6′-CH), 6.71 (m, 1H, 3′-CH), 10.53 (br s, 1H, 7-NH). Anal. Calcd for ($C_{15}H_{18}N_6O_2$) C, H, N.

2,4-Diamino-5-[[(2′,5′-diethoxyphenyl)imino]methyl]pyrrolo[2,3-*d*]pyrimidine (15e). **Method A, from 13.** A solution of **13** (1.15 g, 6.49 mmol) and 2,5-diethoxyaniline, **14e** (1.76 g, 9.74 mmol), in 70% HOAc (75 mL) containing damp Raney nickel (5.75 g) was hydrogenated at 55 psi for 12 h at room temperature. The mixture was filtered through Celite

and the filtrate evaporated under reduced pressure. Cold water (15 mL) was added to the residue, and this suspension was added to a stirred, cold, saturated solution of $NaHCO_3$ (100 mL). The mixture was stirred for an additional 10 min and refrigerated for 4 h. A brown precipitate formed which was collected, washed with water, and dried. The crude product containing **14e** was washed repeatedly with Et_2O until no aniline could be detected (TLC) in the washings. The residue was then dissolved in MeOH (100 mL) and filtered and the filtrate evaporated to dryness under reduced pressure. Et_2O (20 mL) was added to this solution, and the precipitate obtained was filtered to afford 1.20 g (54%) of **15e** as a tan solid: mp 165–170 °C; TLC R_f 0.63 ($CHCl_3$/MeOH/NH_4OH, 10:3:0.1, silica gel); MS *m/z* 340 (M^+); ^1H NMR (Me_2SO-d_6) δ 1.32 (t, 6H, OCH_2CH_3), 3.98 (q, 4H, OCH_2CH_3), 6.01 (br s, 2H, 2-NH_2), 6.69 (dd, 1H, 4′-CH), 6.80 (d, 1H, 6′-CH), 6.93 (dd, 1H, 3′-CH), 6.99 (br s, 1H, 4-NH_2), 7.48 (s, 1H, 6-CH), 8.52 (s, 1H, 8-CH), 9.70 (br s, 1H, 4-NH_2), 11.60 (br s, 1H, 7-NH). Anal. Calcd for ($C_{17}H_{20}N_6O_2$·0.05H_2O·0.8CH_3COOH) C, H, N.

Method B, from 12. The Schiff base was prepared as described for **15b** using 2,5-diethoxyaniline to afford 0.90 g (50%) of **15e** which was identical in all respects with the sample prepared as described above under method A.

2,4-Diamino-5-[(2′,5′-diethoxyanilino)methyl]pyrrolo[2,3-*d*]pyrimidine (5). Reduction of **15e** was carried out as described for **15b** to afford 0.36 g (51%) of a tan solid, **5**: mp 185–190 °C; TLC, R_f 0.54 ($CHCl_3$/MeOH/NH_4OH, 10:3:0.1, silica gel); ^1H NMR (Me_2SO-d_6) δ 1.27 (t, 6H, OCH_2CH_3), 3.89 (q, 4H, OCH_2CH_3), 4.23 (d, 2H, 8-CH_2), 4.98 (t, 1H, 9-NH), 5.41 (br s, 2H, 2-NH_2), 6.11 (dd, 1H, 4′-CH), 6.23 (br s, 2H, 4-NH_2), 6.37 (d, 1H, 6′-CH), 6.68 (m, 2H, 3′-, 6-CH), 10.53 (br s, 1H, 7-NH). Anal. Calcd for ($C_{17}H_{22}N_6O_2$·0.5H_2O) C, H, N.

2,4-Diamino-5-[(3′,4′-dichloroanilino)methyl]pyrrolo[2,3-*d*]pyrimidine (6). The Schiff base was prepared as described above for **15b** using 3,4-dichloroaniline to afford 1.0 g (54%) of a tan solid, **15f**. Reduction of **15f** was carried out as described for **15b** to afford 0.34 g (54%) of an off-white powder, **6**: mp 230 °C; TLC R_f 0.48 ($CHCl_3$/MeOH/NH_4OH, 10:3:0.1, silica gel); ^1H NMR (Me_2SO-d_6) δ 4.25 (d, 2H, 8-CH_2), 5.49 (br s, 2H, 2-NH_2), 6.11 (br s, 2H, 4-NH_2), 6.45 (t, 1H, 9-NH), 6.75 (m, 1H, 6′-CH), 6.78 (s, 1H, 6-CH), 6.97 (d, 1H, 2′-CH), 7.32 (d, 1H, 5′-CH), 10.62 (br s, 1H, 7-NH). Anal. Calcd for ($C_{13}H_{12}N_6Cl_2$·0.3H_2O) C, H, N, Cl.

2,4-Diamino-5-[(1′-naphthylamino)methyl]pyrrolo[2,3-*d*]pyrimidine (7). The Schiff base was prepared as described above for **15b** except that the reaction was carried out at 30 psi for 12 h at room temperature using 1-aminonaphthalene to afford 0.92 g (53%) of a brown solid, **15g**: mp >275 °C dec; TLC R_f 0.60 ($CHCl_3$/MeOH/NH_4OH, 10:3:0.1, silica gel); ^1H NMR (Me_2SO-d_6) δ 5.73 (s, 2H, 2-NH_2), 6.84 (br s, 1H, 4-NH_2), 7.17 (s, 1H, 6-CH), 7.56 (m, 4H, $C_{10}H_7$), 7.74 (m, 1H, $C_{10}H_7$), 7.93 (m, 1H, $C_{10}H_7$), 8.13 (m, 1H, $C_{10}H_7$), 8.49 (s, 1H, 8-CH), 8.97 (br s, 1H, 4-NH_2), 11.53 (br s, 1H, 7-NH).

Reduction of **15g** was carried out as described for **15b** except that glacial HOAc was used to adjust the pH at 2. Crude **7** was dissolved in a mixture of $CHCl_3$/MeOH (9:1), and this was loaded on a silica gel column (2.4 cm × 20 cm) packed in $CHCl_3$. The column was eluted with a gradient of 1% MeOH in $CHCl_3$ to 5% MeOH in $CHCl_3$. Fractions corresponding to the product (TLC) were pooled and evaporated under reduced pressure to dryness. The residue was triturated in cold Et_2O and the suspension filtered to yield 0.24 g (48%) of a tan powder, **7**: mp >240 °C dec; TLC R_f 0.61 ($CHCl_3$/MeOH/NH_4OH, 10:3:0.1, silica gel); ^1H NMR (Me_2SO-d_6) δ 4.40 (d, 2H, 8-CH_2), 5.39 (s, 2H, 2-NH_2), 6.31 (s, 2H, 4-NH_2), 6.53 (t, 1H, 9-NH), 6.79 (m, 2H, $C_{10}H_7$), 7.21 (m, 2H, $C_{10}H_7$, 6-CH), 7.41 (m, 2H, $C_{10}H_7$), 7.74 (d, 1H, $C_{10}H_7$), 8.06 (d, 1H, $C_{10}H_7$), 10.54 (s, 1H, 7-NH). Anal. Calcd for ($C_{17}H_{18}N_6$·0.3CH_3COOH) C, H, N.

2,4-Diamino-5-(anilinomethyl)pyrrolo[2,3-*d*]pyrimidine (8). The Schiff base was prepared as described above for **15b** using aniline to afford 0.69 g (67%) of a tan solid, **15h**: mp >275 °C dec; TLC R_f 0.50 ($CHCl_3$/MeOH/NH_4OH, 10:3:0.1, silica gel); ^1H NMR (Me_2SO-d_6) δ 5.69 (s, 2H, 2-NH_2), 6.75 (s, 1H, 4-NH_2), 7.23 (d, 2H, 2′-, 6′-CH), 7.40 (m, 3H, 3′-, 4′-, 5′-CH), 7.51 (s, 1H, 6-CH), 8.45 (s, 1H, 8-CH), 11.46 (br s, 1H, 7-NH).

Reduction of **15h** was carried out as described for **15b** except that glacial HOAc was used to adjust the pH at 2. This yielded 0.19 g (63%) of a tan powder, **8**: mp >180 °C dec; TLC R_f 0.51 ($CHCl_3$/MeOH/NH_4OH, 10:3:0.1, silica gel); ^1H NMR (Me_2SO-d_6) δ 4.15 (d, 2H, 8-CH_2), 5.40 (br s, 2H, 2-NH_2), 5.89 (t, 1H, 9-NH), 6.20 (br s, 2H, 4-NH_2), 6.61 (m, 1H, 4′-CH), 6.69 (s, 1H, 6-CH), 6.66 (d, 2H, 2′-, 6′-CH), 7.09 (m, 2H, 3′-, 5′-CH), 10.51 (s, 1H, 7-NH). Anal. Calcd for ($C_{13}H_{14}N_6$·0.2CH_3COOH) C, H, N.

***N*-[4-[*N*-[(2,4-Diaminopyrrolo[2,3-*d*]pyrimidin-5-yl)methyl]amino]benzoyl]-L-glutamic Acid (9).** The Schiff base was prepared as described above for **15b** using diethyl (*p*-aminobenzoyl)-L-glutamate to afford 1.39 g (50%) of the diethyl ester **16** as a yellow solid. Reduction of **16** was carried as described for **15b** to yield 0.44 g (50%) of **17** as a light yellow powder: mp >275 °C dec; ^1H NMR (Me_2SO-d_6) δ 1.16 (t, 6H, OCH_2CH_3), 1.91 (m, 2H, Glu β-CH_2), 2.41 (t, 2H, Glu γ-CH_2), 4.05 (q, 4H, OCH_2CH_3), 4.29 (d, 2H, 8-CH_2), 4.49 (m, 1H, Glu α-CH), 5.56 (br s, 2H, 2-NH_2), 6.20 (br s, 2H, 4-NH_2), 6.55 (t, 1H, 9-NH), 6.74 (m, 3H, 3′-, 5′-, 6-CH), 7.66 (d, 2H, 2′-, 6′-CH), 8.33 (d, 1H, CONH), 10.67 (s, 1H, 7-NH).

Hydrolysis of the esters was carried out by stirring a solution of **17** (0.30 g, 0.62 mmol) in 1 N NaOH (10 mL) and MeOH (10 mL) for 72 h at room temperature. The solvent was evaporated to 5 mL, and the mixture was carefully acidified with glacial HOAc in an ice bath. The tan precipitate was filtered, washed with water, and dried to yield 0.23 g (88.5%) of **9**: mp >235 °C dec; TLC R_f 0.72 (3% NH_4HCO_3 cellulose); ^1H NMR (Me_2SO-d_6) δ 1.91 (m, 1H, Glu β-CH_2), 2.05 (m, 1H, Glu β-CH_2), 2.32 (m, 2H, Glu γ-CH_2), 4.33 (m, 3H, 8-CH_2, Glu α-CH), 6.47 (t, 1H, 9-NH), 6.53 (s, 2H, 2-NH_2), 6.74 (d, 2H, 3′-, 5′-CH), 6.86 (s, 1H, 6-CH), 7.11 (s, 2H, 4-NH_2), 7.68 (d, 2H, 2′-, 6′-CH), 8.16 (d, 1H, CONH), 11.18 (br s, 1H, 7-NH). Anal. Calcd for ($C_{19}H_{21}N_7O_5$·1.0H_2O) C, H, N.

Cell Lines and Methods for Continuous Exposure (Tables 2 and 3). The human T-lymphoblastic leukemia cell line CCRF-CEM[34] and its methotrexate-resistant sublines R30dm,[27] R1,[28] and R2[29] were cultured as described.[30] R30dm expresses only 1% of the FPGS activity of CCRF-CEM. R1 expresses 20-fold elevated levels of DHFR, the target enzyme of MTX. R2 is defective in MTX uptake. The A253 and FaDu human squamous cell carcinoma monolayer cell lines were subcultured in RPMI 1640/10% fetal calf serum in 100 nm cell culture dishes (Falcon) as described.[10] Inhibition of growth of CCRF-CEM and its MTX-resistant sublines as well as A253 and FaDu in continuous drug exposure was measured as described.[10,27] EC_{50} values were determined visually from plots of percent control growth versus the logarithm of drug concentration. All cell lines were verified to be negative for mycoplasma contamination using the GenProbe test kit during the course of these studies.

Folylpolyglutamate Synthetase (Table 4) and CCRF-CEM DHFR. FPGS was partially purified from CCRF-CEM human leukemia cells by ammonium sulfate fractionation and by gel sieving and phosphocellulose chromatography as previously described.[10] FPGS activity was assayed as described previously.[31] It was verified that compound **9** was quantitatively recovered during the standard assay procedure, thus ensuring that polyglutamate products would also be quantitatively recovered. Kinetic constants were determined using the hyperbolic curve-fitting subprogram of SigmaPlot (Jandel) using at least a 10-fold range of substrate concentrations. Assays contained approximately 400 units of FPGS activity; 1 unit of FPGS catalyzes the incorporation of 1 pmol of [^{3}H]-Glu/h. DHFR from CCRF-CEM cells was partially purified and assayed as previously described.[33] DHFR inhibitory potency was measured by adding graded amounts of drugs to standard assays; the drug concentration required to reduce activity by 50% of the control (IC_{50}) was determined graphically from plots of residual activity versus drug concentration. All DHFR assays contained 1.6×10^{-3} units of DHFR activity; 1 unit of DHFR can reduce 1 μmol of dihydrofolate/min under standard conditions.

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