Synthesis, Antifolate, and Antitumor Activities of Classical and Nonclassical 2-Amino-4-oxo-5-substituted-pyrrolo[2,3-*d*]pyrimidines

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Classical and nonclassical isosteric C8−N9 bridged analogues of the multitargeted antifolate LY231514 were synthesized as inhibitors of thymidylate synthase (TS), dihydrofolate reductase (DHFR), and as antitumor and antiopportunistic infection agents. The syntheses of the analogues were accomplished by reductive amination of the appropriate anilines with 2-amino-4-oxo-5-cyanopyrrolo[2,3-*d*]pyrimidine (**28**) followed by saponification of the ethyl esters, for the classical analogue **6**. The N9-methyl analogues were obtained from the N9−H precursors by reductive methylation. In general, the nonclassical compounds **7**−**17** were similar in potency to TMP against *Toxoplasma gondii* DHFR, with selectivity ratios greater than 38 and 21 for **11** and **16**, respectively. These compounds were poor inhibitors of *Pneumocystis carinii* DHFR and rat liver DHFR. The nonclassical analogues were also inactive against TS. The classical analogue **6** was a marginal inhibitor of isolated human TS ($IC_{50} = 46\ \mu M$) and of human DHFR ($IC_{50} = 10\ \mu M$), however, it was a potent inhibitor of the growth of two human head and neck squamous cell carcinoma cell lines and of CCRF−CEM human lymphoblastic leukemia cells in culture and was similar to LY231514 against ZR-75-1 human breast carcinoma cell line. Evaluation of **6** against MTX-resistant sublines indicated that DHFR is not the major target of **6**. Metabolite protection studies of the growth inhibitory activity of **6** suggest that TS is a major target of this drug and that polyglutamyl forms of **6** may serve as the intracellular TS inhibitors. These studies also suggest that **6** has a site of action in addition to sites in the folate pathway.

Thymidylate synthase (TS)[1] catalyzes a two-step conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) utilizing the cofactor 5,10-methylenetetrahydrofolate (5,10-CH_2FH_4). This enzyme is unique among those which utilize tetrahydrofolate (FH_4) cofactors in that 5,10-CH_2FH_4 serves the dual function of both a one-carbon donor and a reductant, by concomitant transfer of its methylene group and the 6H atom to the 5-position of dUMP. In the process, the cofactor is oxidized to 7,8-dihydrofolate (FH_2). The enzyme dihydrofolate reductase (DHFR) catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate to regenerate FH_4.[2] This is the sole de novo source of dTMP, and hence, TS and DHFR play a pivotal role in DNA biosynthesis and cell replication. Antifolate inhibitors of TS and DHFR have found clinical utility as antitumor, antibacterial, and antiprotozoan agents.[3] Usually a 2,4-diamino-substituted pyrimidine ring is considered important for potent DHFR inhibitory activity, while a 2-substituted-4-oxopyrimidine ring is considered important for potent TS inhibitory activity.[3−5]

Several 5-substituted-2,4-diaminopyrrolo[2,3-*d*]pyrimidines,[5−7] including **1**, **2**, and the 2-amino-4-oxopyrrolo-[2,3-*d*]pyrimidine[8] (**3**, LY231514) have shown excellent antitumor activity; compound **3** has shown significant promise as an antitumor agent in phase III clinical trials. The strong DHFR inhibitory potency of **1** and **2** was in accord with that reported for 2,4-diamino 6−6 fused ring system analogues such as methotrexate (MTX, **4**). However, the potent DHFR inhibitory activity of **3** (which contains a 2-amino-4-oxopyrimidine ring) in addition to its TS inhibition was unusual, except if one considers a possible alternate mode of DHFR binding, in which the pyrrole NH of compound **3** mimics the 4-NH_2 of the 2,4-diaminopyrimidine ring system of MTX and the pyrrolo[2,3-*d*]pyrimidine ring binds in this alternate mode to DHFR as shown in Figure 1. Gangjee et al.[9] have recently demonstrated that a 4-methyl analogue of **3** does bind in the alternate mode to DHFR. 5-Deazafolic acid **5** synthesized by Piper et al.[10] is a 2-amino-4-oxo classical antifolate that contains a C9−N10 bridge and has appreciable DHFR inhibitory potency. It was thus of interest to determine if replacement of the C8−C9 bridge of LY231514 with a C8−N9 bridge would afford a useful antitumor agent with dual TS-DHFR inhibitory effects. Thus, we synthesized the classical analogue **6**. Dual inhibitors could act at two different sites (TS and DHFR) and could combine dual mechanisms of action in a single agent without the

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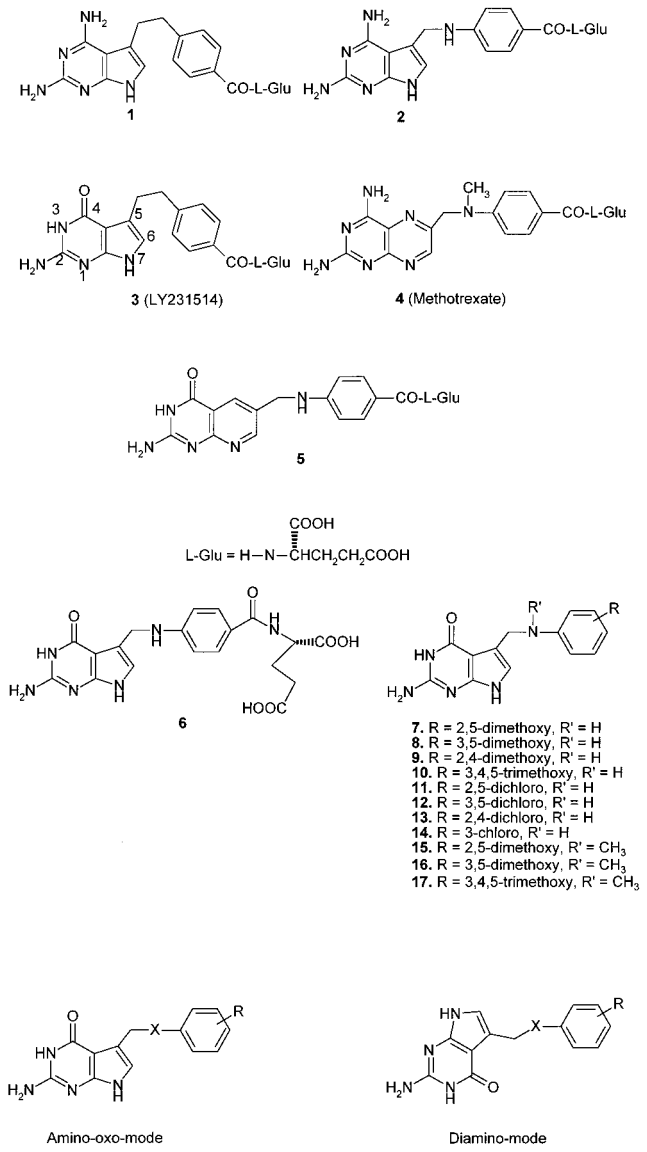


Figure 1.

pharmacokinetic disadvantages of two separate agents. Synergism of the two separate antifolates which inhibit TS and DHFR has been demonstrated in growth inhibitory studies against *Lactobacillus casei*,[11,12] rat hepatoma cells,[13,14] and human lymphoma cells.[12,15,16]

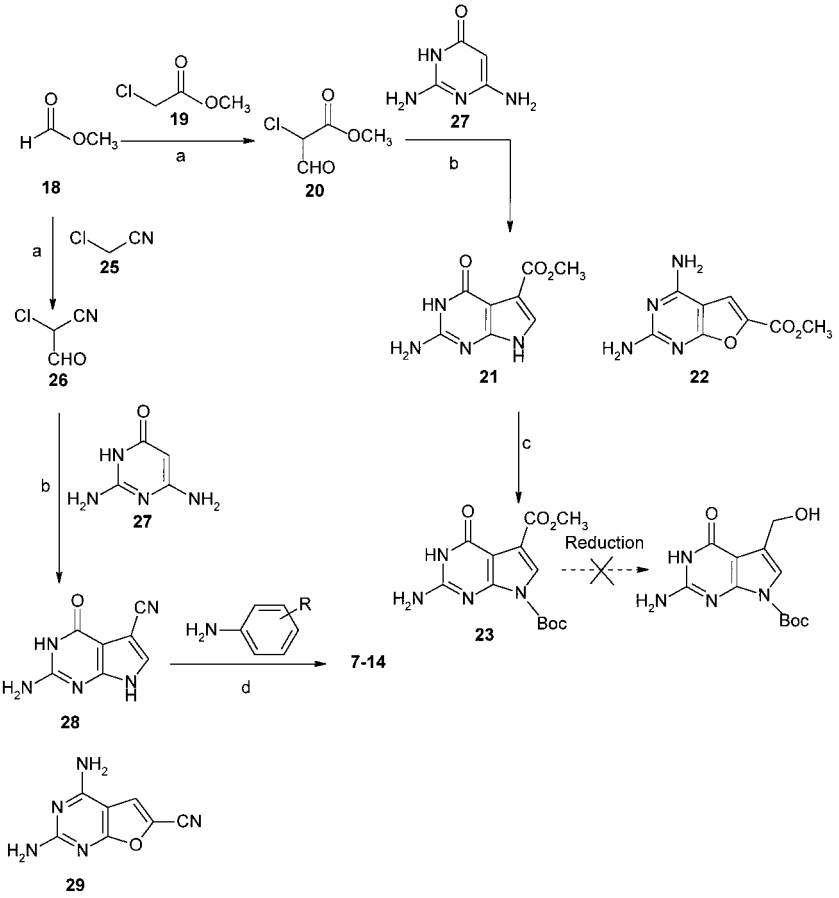
A major drawback of classical antifolates is that they require transport into cells via the reduced folate uptake systems, which when impaired can lead to drug resistance.[17,18] In addition, the antitumor activities of several classical antifolates, particularly TS inhibitors, are in part, determined by their ability to function as substrates for the enzyme folylpolyglutamate synthetase (FPGS).[19,20] Polyglutamylation catalyzed by FPGS produces long-acting, noneffluxing poly-γ-glutamates of classical antifolates that lead to high intracellular concentrations of these antitumor agents. Further, polyglutamylation often provides a significant increase in inhibitory activity against TS compared to the monoglutamate, as has been shown for **3** (LY231514).[21] Though polyglutamylation for many antifolates is necessary for cytotoxcicity to tumor cells, it has also been implicated in adverse effects and toxicity to normal tissues due to retention of polyglutamate metabolites.[22] The problem of tumor resistance to classical antifolates, which is, in part, a result of low or defective FPGS activity, is also a limitation of those classical antifolates that depend on polyglutamylation for their antitumor effects.[23,24] To overcome these disadvantages associated with classical antifolates, lipophilic nonclassical antifolates that lack the glutamate moiety have been developed.[25−28] Thus, in addition to the antitumor activity of **6**, we were also interested in the antitumor activity of the nonclassical analogues **7**−**17**, which could also function as dual inhibitors.

Another facet of our interest in nonclassical dual TS-DHFR inhibitors is the prevalence of opportunistic infections in immune-compromized patients such as those receiving organ transplants or cancer chemotherapy and those with AIDS.[29,30] Two of the most prevalent opportunistic infections which are the major cause of mortality in patients with AIDS are caused by *Pneumocystis carinii* (pc)[31] and *Toxoplasma gondii* (tg).[32] Several nonclassical 2,4-diaminopyrido[2,3-*d*]-pyrimidines,[33] quinazolines,[34] pyrrolo[2,3-*d*]pyrimidines,[5,8,33−35] and furo[2,3-*d*]pyrimidines[36] have been evaluated as inhibitors of *P. carinii* and *T. gondii* DHFR. Some of these have been found to be selective for DHFR from these pathogens. Thus, we were also interested in evaluating the nonclassical compounds **7**−**17** as inhibitors of pcDHFR and tgDHFR. The phenyl ring substitutions in **7**−**17** are based on similar substitutions that have provided potent and/or selective agents.[33−36] The nonclassical analogues **7**−**17** were anticipated to inhibit DHFR and/or TS from *P. carinii* and/or *T. gondii*, and perhaps provide selective inhibitors against these pathogens. *P. carinii* and *T. gondii* lack the transport system(s) required for classical antifolates; however, the lipophilic nonclassical compounds **7**−**17** were anticipated to gain access to the pathogenic cells by passive diffusion.

Chemistry

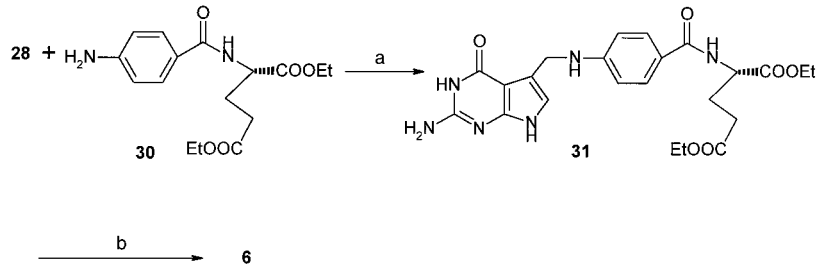
The syntheses of analogues **6**−**17** were initially envisioned through the formation of the key intermediate ester **21**[37] (Scheme 1). Reaction of methyl formate **18** with methyl chloroacetate **19** in toluene using $NaOCH_3$ as a base afforded the chloroaldehyde **20** in quantitative yield. Compound **20** was cyclocondensed with 2,4-diamino-6-hydroxypyrimidine **27** and sodium acetate in water at 100 °C.[38] ^{1}H NMR indicated the regiospecific formation of the methyl 2-amino-4-oxopyrrolo[2,3-*d*]pyrimidine-5-carboxylate (**21**) with no detectable formation of the corresponding 6-substituted-furo[2,3-*d*]pyrimidine **22**. The ^{1}H NMR spectrum showed the characteristic downfield lactam NH signal at 11.64 ppm and the pyrrole NH signal at 10.36 ppm. In addition, the presence of only one amino group at 6.22 ppm and the absence of the pyrimidine 5-proton between 5 and 6 ppm confirmed the structure of the desired intermediate **21**. The ester **21** was insoluble in DMF, methanol, or ethanol at room temperature. To improve the solubility of **21** and to enhance the carbonyl character of the ester (hence improving the chances of its reduction), the pyrrole nitrogen was protected with a Boc group[38] to afford **23**. Attempts to reduce **23** to the alcohol using $LiAlH_4$ or to the aldehyde using DIBAL were unsuccessful.

Scheme 1[a]



[a] (a) toluene, NaOMe; (b) water, sodium acetate, reflux; (c) DMF, triethylamine, $(Boc)_2O$; (d) 15% acetic acid/DMF, Ni/H_2/50 psi.

Scheme 2[a]

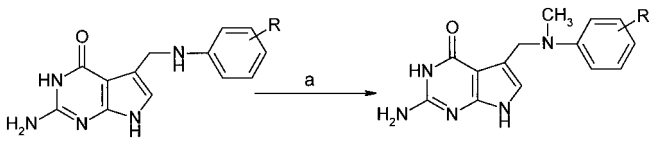


[a] (a) formic acid, Ni/H_2/50 psi; (b) NaOH/MeOH.

Since the reduction of ester **21** was unsuccessful, an alternate route to compounds **6**−**17** was designed. The use of the 2-amino-4-oxo-5-cyanopyrrolo[2,3-*d*]pyrimidine **28** (Scheme 1) instead of the ester **21** appeared to be an attractive method, since similar nitriles have been reductively aminated with a variety of anilines in 6−6 ring systems. Reductive amination of **28** with the appropriate anilines was expected to afford the target analogues. Thus formylation[37] of chloroacetonitrile **25** with methyl formate in toluene using $NaOCH_3$ as base afforded chloro(formyl)acetonitrile **26**, which was used without further purification. Condensation[38] of **26** with **27** in water and sodium acetate at 100 °C afforded regiospecifically the key 5-cyanopyrrolo[2,3-*d*]pyrimidine (**28**), without the formation of the corresponding 6-cyanofuro[2,3-*d*]pyrimidine (**29**). The ^{1}H NMR in DMSO-d_6 of **28** exhibited the 2-amino signal at 6.37 ppm (exchangeable with D_2O), the lactam proton signal at 11.97 ppm, the pyrrole NH signal at 10.69 ppm and the 6-H signal as a doublet at 7.59 ppm, which confirmed the structure of **28**.

Reductive amination of **28** (Scheme 2) in formic acid with ethyl *p*-aminobenzoyl-L-glutamate in the presence of Raney nickel and hydrogen at 50 psi afforded, after saponification, the target **6**. The ^{1}H NMR spectrum of **6** in DMSO-d_6 showed the β- and γ-CH_2 signals at 1.92 and 2.29 ppm, respectively, the 8-CH_2 and the 9-NH signals at 4.31 ppm as a multiplet, the 2-amino signal (exchanges with D_2O) at 6.07 ppm, and the α-CH signal of the glutamic acid at 6.45 ppm. The spectrum also showed the 6-CH signal at 6.49 ppm, the aromatic proton signals as two sets of doublets at 6.65 and 7.65 ppm, and the characteristic lactam NH and pyrrole NH at 10.81 and 10.28 ppm, respectively. For the nonclassical target compounds, reductive amination (Scheme 1) of **28** in 15% acetic acid/DMF with the appropriately

Scheme 3[a]



7. R = 2,5-dimethoxy
8. R = 3,5-dimethoxy
10. R = 3,4,5-trimethoxy

15. R = 2,5-dimethoxy
16. R = 3,5-dimethoxy
17. R = 3,4,5-trimethoxy

[a] (a) HCHO, $NaCNBH_3$, CH_3CN, HCl.

Table 1. Inhibitory Concentrations (IC_{50} μM) and Selectivity Ratios of Analogues **7**−**17** against *P. carinii* (pc) DHFR, *T. gondii* (tg) DHFR, Rat Liver (rl) DHFR

compd	pcDHFR	rlDHFR	tgDHFR	rl/tg
7	47 (10%)[a]	47 (17%)	2.2	>21
8	20 (16%)	20 (7%)	2.6	>8
9	16 (14%)	16 (10%)	7.1	>2.3
10	22 (11%)	22 (3%)	9.3	>2.4
11	25 (42%)	25 (19%)	0.66	>38
12	66.2	63 (28%)	4.1	>15.4
13	15 (15%)	15 (4%)	2.2	>7
14	26 (9%)	26 (3%)	3.5	>7
15	56 (0%)	56 (20%)	10.3	>6
16	87 (8%)	87 (27%)	4.2	>21
17	45 (2%)	45 (14%)	37	>2
TMP	12.0	133.0	2.7	49.0

[a] Values in parentheses are the percent inhibition at the highest concentration tested.

Table 2. Inhibitory Concentration (IC_{50}, M) against Isolated DHFR[39] and TS[40]

DHFR			
compound	rec[a]	rtg[b]	rh[c]
6	2.3×10^{-4}	8.0×10^{-7}	1.0×10^{-5}
LY231514[f]	2.3×10^{-4}	2.3×10^{-7}	2.3×10^{-6}
trimethoprim	1.0×10^{-8}	3.7×10^{-6}	2.0×10^{-4}
trimetrexate	1.8×10^{-8}	5.4×10^{-9}	9.0×10^{-8}
methotrexate	4.4×10^{-9}	1.1×10^{-8}	6.6×10^{-8}

TS			
compound	rec[e]	rpc[d]	rh[d]
6	$>1.2 \times 10^{-4}$ (23)[i]	5.8×10^{-5}	4.6×10^{-5}
LY231514[f]	1.1×10^{-4}	5.7×10^{-6}	5.7×10^{-6}
Tomudex[g]	8.0×10^{-6}		1.0×10^{-6}
PDDF[h]	5.8×10^{-8}	5.0×10^{-8}	1.5×10^{-7}
methotrexate	1.8×10^{-4}		3.6×10^{-5}

[a] Kindly provided by Dr. R. L. Balkley, St. Jude Children's Research Hospital, Memphis, TN. [b] Kindly provided by Dr. D. Borhani, Southern Research Institute, Birmingham, AL. [c] Kindly provided by Dr. J. H. Freisheim, Medical University of Ohio, Toledo, OH. [d] Kindly provided by Dr. D. V. Santi, University of California, San Francisco, CA. [e] Kindly provided by Dr. D. A. Matthews, Agouron Pharmaceuticals, San Diego, CA. [f] MTA, a multitargeted inhibitor, kindly provided by Dr. Chuan Shih, Eli Lilly & Co., Indianapolis, IN. [g] ZD 1694, Raltitrexed, Kindly provided by Dr. Ann Jackman, Institute of Cancer Research, Sutton, Surrey, UK. [h] 5,8-Dideaza-10-propargylfolic acid, CB3717, kindly provided by Dr. M. G. Nair, University of South Alabama, Mobil, AL. [i] Number in parentheses indicates the level of inhibition at the indicated concentration.

substituted anilines in the presence of Raney nickel and hydrogen at 50 psi for 6 h afforded the desired compounds **7**−**14** in 20−62% yield. The N9-methyl analogues **15**−**17** were synthesized (Scheme 3) from the N9−H precursors **7**, **8**, and **10**, respectively, by reductive methylation using formaldehyde and sodium cyanoborohydride in acetonitrile.

Biological Activity and Discussion

The nonclassical analogues **7**−**17** were evaluated as inhibitors of pcDHFR, tgDHFR, and rat liver (rl) DHFR, and the results (IC_{50}) are reported in Table 1. Selectivity ratios (IC_{50} rlDHFR/IC_{50} tgDHFR) for tgDHFR were determined using rlDHFR as the mammalian standard and are also reported in Table 1. The compounds were more potent against tgDHFR than against pcDHFR or rlDHFR. The most potent analogue, the 2,5-dichloro-substituted compound **11**, was 4 times more potent than TMP against tgDHFR. All the analogues were also more selective against tgDHFR than against pcDHFR. In the N9−H series (compounds **7**−**14**), the potency against tgDHFR varied from an IC_{50} of 2.2 to 7.1 μM, with the exception of **11** ($IC_{50} = 0.66$ μM). In the dimethoxy series (**7**−**9**), the position of the methoxy moieties had little effect on the potency. The addition of a third methoxy group (compound **10**) did not change the potency to any significant extent. N9-methylation of **7**, **8**, and **10** to afford **15**, **16**, and **17**, respectively, decreased potency against tgDHFR. This decrease was 4-fold for **17** compared to **10**. Thus, unlike some previously reported heterocyclic 6−6 fused systems where bridge nitrogen methylation substantially increased potency, in this 2-amino-4-oxo-5-substituted-pyrrolo[2,3-*d*]pyrimidine series bridge N-methylation decreased potency. These results underscore the difference in SAR between different heterocyclic systems against different DHFR. The electron-withdrawing chloro-substituted analogues **11**−**14** were similar in potency (except **11**) against tgDHFR. With the exception of compound **11**, the 2,5-dichloro analogue, the position and number of chloro substitution(s) were immaterial to potency against tgDHFR. Clearly the 2,5-dichloro-substituted analogue was the most potent of all of the analogues tested. In addition, similar to the 2,5-dichloro analogue **11**, the 2,5-dimethoxy substitution (compound **7**) in the methoxy series was most conducive to potency against tgDHFR. Since IC_{50} values against pcDHFR and rlDHFR were not obtained (except compound **12** against pcDHFR), construction of a structure−selectivity relationship was not possible for these analogues against pcDHFR or rlDHFR. However, from the data in Table 1, it is clear that the 2-amino-4-oxo-5-substituted-pyrrolo[2,3-*d*]pyrimidines are selective against tgDHFR, and compound **11** could serve as a lead compound for further structural modification to improve selectivity and potency, particularly since rlDHFR, the mammalian standard, was inhibited only 19% at 25 μM by this compound.

Compounds **7**−**14** were also evaluated against TS from *Escherichia coli*, *Lactobacillus casei*, *Pneumocystis carinii*, and human recombinant TS and were essentially inactive with IC_{50} values >160 μM. Thus, analogues **7**−**17** do not exhibit dual inhibition of DHFR and TS anticipated from their structure.

The classical compound **6** was evaluated as an inhibitor of human CCRF−CEM TS, and the results indicated that **6** ($IC_{50} = 94$ μM) was a slightly weaker human TS inhibitor than was MTX ($IC_{50} = 42$ μM). Similar results were obtained with recombinant human TS ($IC_{50} = 46$ and 36 μM for **6** and MTX, respectively, Table 2) and with rpc(recombinant *P. carinii*)TS (Table 2). Compound **6** was much less inhibitory to rec(recombinant *E. coli*)-

Table 3. Inhibition of Growth of A253 and FaDu Human Head and Neck Squamous Cell Carcinoma Cell Lines by MTX, **6**, and **7** in Continuous Exposure[a]

	EC_{50} (nM)	
drug	A253	FaDu
MTX	19 ± 3 (n = 5)	17 ± 4 (n = 5)
6	1000 ± 100 (n = 2)	800 ± 100 (n = 2)
7	>10000 (n = 2)	>10000 (n = 2)

[a] Growth inhibition was assayed as described in Experimental Section. Values presented are average ± SD if $n \geq 3$ and average ± range for $n = 2$.

Table 4. Growth Inhibition of Parental CCRF−CEM and Sublines with Single, Defined Mechanisms of MTX Resistance during Continuous (0−120 h) Exposure[a]

	EC_{50}, nM			
drug	CCRF−CEM	R1[b] (↑ DHFR)	R2[c] (↓ uptake)	R30dm[d] (↓ Glu_n)
MTX	14 ± 1 (n = 7)	600 ± 110 (n = 5)	1900 ± 510 (n = 5)	15 ± 1 (n = 5)
6	80 ± 11 (n = 3)	200 ± 20 (n = 2)	310 ± 30 (n = 2)	120 ± 10 (n = 2)
7	230 ± 60 (n = 4)	420 ± 50 (n = 3)	1530 ± 240 (n = 3)	460 ± 115 (n = 3)

[a] Average values are presented ± range for $n = 2$ and ± SD for $n \geq 3$. [b] CCRF−CEM subline resistant to MTX solely as a result of a 20-fold increase in wild-type DHFR protein and activity.[45] [c] CCRF−CEM subline resistant as a result of decreased uptake of MTX.[46] [d] CCRF−CEM subline resistant to MTX 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.[41]

TS (Table 2). In contrast, LY231514, as the monoglutamate, is 10 times more potent than **6** against TS for both rh(recombinant human)TS and rpcTS (Table 2). The low inhibitory potency of **6** suggests either that this drug does not target TS or that polyglutamylation enhances its TS inhibitory activity (see below). Compound **6** was also tested as an inhibitor of rh-, rtg-(recombinant *T. gondii*)-, and recDHFR (Table 2). The IC_{50} for rhDHFR was 10 μM and as with rhTS, LY231514 was 10 times more inhibitory than compound **6**. tgDHFR was the most sensitive DHFR tested (IC_{50} = 0.8 μM) and recDHFR the least sensitive (IC_{50} = 230 μM).

Classical compound **6** and the lipophilic analogue **7** were evaluated as inhibitors of in vitro human tumor cell growth. Growth inhibition of two human head and neck squamous cell carcinoma cell lines, A253 and FaDu, were assessed in continuous (120 h) exposure (Table 3). Compound **6** was ≈0.50-fold less potent than MTX as a growth inhibitor of each cell line. However, the potency of **6** is similar to that observed for LY231514 against the ZR-75-1 human breast carcinoma cell line.[21] The lipophilic analogue **7** was not cytotoxic at 10 μM, the highest concentration that could be tested without solvent (DMSO) toxicity.

The growth inhibitory potency of classical compound **6** and lipophilic **7** was also compared to that of MTX in continuous exposure against CCRF−CEM human lymphoblastic leukemia cells in culture (Table 4). In addition, the compounds **6** and **7** were also evaluated against a series of MTX-resistant sublines to determine the mechanism of action (Table 4). Compound **6** was only 6-fold less potent than MTX against parental CCRF−CEM cells; this is similar to the differential potency of MTX and LY231514 in this cell line.[21] DHFR-overexpressing cells (R1) were 43-fold resistant to MTX, but only 2.5-fold resistant to **6**, suggesting that DHFR is not a major target of this agent, in contrast to LY231514.[21] This is consistent with the low level of rhDHFR inhibition observed with **6** (Table 2). MTX transport-defective cells (R2) are 135-fold resistant to MTX, but less than 4-fold resistant to **6**. This suggests either that the mutation decreasing the V_{max} of the reduced folate/MTX carrier system (RFC) in this cell line does not affect **6** in a similar manner or that **6** primarily uses a separate transport pathway. LY231514 has been suggested to use the RFC on the basis of similar low (4-fold) cross-resistance data.[21] Polyglutamylation of tight-binding enzyme inhibitors, like MTX, is not required in continuous exposure; this accounts for the lack of resistance of polyglutamylation-defective R30dm to MTX. However, for inhibitors where polyglutamylation is required for potent target inhibition (e.g., ZD1694), resistance may become apparent for polyglutamylation-defective cell lines such as R30dm, even in continuous exposure. The low cross-resistance of R30dm to **6** suggests either that the parent drug is a potent inhibitor of its target or that polyglutamylation of this drug is extremely efficient. LY231514 exhibits cross-resistance in a similar polyglutamylation-deficient cell line.[21] Lipophilic **7** was less potent than the classical analogue at inhibiting the growth of CCRF−CEM cells in culture and all the sublines; however, the degree of cross-resistance was similar for the two drugs. The source of the low-level cross-resistance to **7** of the subline with impaired MTX transport (R2) is unclear, since this analogue presumably does not require active transport. The slope of the concentration-dependence curve for **7** with all the CCRF−CEM lines was shallow; it required a 0.3 log range of drug to decrease cell growth from 100% to <10% (compared to 3-fold change for MTX; data not shown).

Table 5. Metabolite Protection of FaDu Cells by Leucovorin (LV), Hypoxanthine (Hx), and/or Thymidine (TdR) from Growth Inhibition Induced by MTX or **6**[a]

	relative growth				
drug	no addn	10 μM LV	+50 μM Hx	+40 μM TdR	+(Hx+TdR)
MTX	1 ± 0	100 ± 1	1 ± 0	4 ± 0	110 ± 2
6	5 ± 0	64 ± 0	5 ± 0	74 ± 2	79 ± 2

[a] Cells were exposed to drug concentrations previously shown to inhibit growth by ≥95% (50 nM MTX, 9 μM **6**); the indicated concentrations of metabolites were present simultaneously with drug. Values are the average growth relative to a control culture treated only with metabolite (see Experimental Section); the indicated error is the range of the two values used to determine the average. LV and TdR/Hx protection were measured in separate experiments that gave essentially identical growth inhibition data (no addition); each experiment was repeated with comparable results. MTX was fully protected by ≥0.1 μM LV, but **6** also showed incomplete protection at lower levels (36 ± 1% relative growth at 0.1 μM LV and 59 ± 2% relative growth at 1 μM LV; data not shown).

Metabolite protection studies of the growth inhibition of compound **6** were carried out in order to further elucidate its mechanism of action. FaDu cells were fully protected by leucovorin (LV) against growth inhibition by MTX (Table 5), even at LV levels as low as 0.1 μM

(data not shown). In contrast, although LV provided some protection against **6**, complete protection could not be achieved even at 10 μM. This is in contrast to LY231514, against which LV is fully protective.[41] Protection by the purine hypoxanthine (Hx) and by thymidine (TdR) was also assessed (Table 4). TdR alone, but not Hx alone, protected against MTX to a low degree, but full protection required both Hx and TdR. Hx alone offered no protection against **6**, while TdR alone was only partially protective; the combination of Hx and TdR typically offered only a slightly higher level of protection against **6** than TdR alone. It is to be noted that the lack of full protection cannot be a result of insufficient metabolite concentration, since their levels are sufficient to fully protect against MTX. This suggests that **6** has a site of action in addition to sites in the folate pathway.

Our cellular and biochemical studies offer some clues as to the mechanism of action of compound **6**. The low level of cross-resistance of the DHFR-amplified cell line, along with the lack of enhanced protection by the combination of TdR + Hx relative to TdR alone, is inconsistent with DHFR as a target of this drug. Similarly, the lack of protection by Hx alone suggests that purine synthesis is not a target. Substantial protection against **6** by TdR alone suggests that thymidylate synthesis, and hence TS itself, is a major target of this drug. In contrast to these results with whole cells, **6** is a weak inhibitor of isolated TS. Since TS shows substantial increase in inhibitor potency for polyglutamyl relative to monoglutamyl forms,[42] these observations suggest that polyglutamyl forms of **6** may serve as the actual intracellular TS inhibitors. Since the polyglutamylation-deficient subline R30dm is not cross-resistant to **6** in continuous exposure, the data further suggest that polyglutamylation may be a very efficient process for **6**. This hypothesis will be evaluated in further studies. We have already shown that **7** is, as expected, not a human FPGS substrate or inhibitor up to 50 μM. Compound **7** did not inhibit human FPGS at 50 μM.

Experimental Section

Melting points were determined on a Fisher-Johns melting point apparatus or a Mel Temp apparatus and are uncorrected. Infrared spectra (IR) were recorded with a Perkin-Elmer Model 1430, in Nujol mulls. Nuclear magnetic resonance spectra for ^{1}H NMR were recorded on a Bruker WH-300 (300 MHz). The data were accumulated by 16K size with 0.5 s delay time and 70° tip angle with internal standard TMS; s = singlet, br = broad, d = doublet, t = triplet, q = quartet, m = multiplet. Thin-layer chromatography was performed on silica gel plates with fluorescent indicator and were visualized at 254 and 366 nm, unless indicated otherwise. Column chromatography was performed with 230−400 mesh silica gel purchased from Aldrich Chemical Co., Milwaukee, WI. All solvents were purchased from Aldrich Chemical Co. and were used without further purification. Samples for microanalysis were dried in vacuo over P_2O_5 at 70 or 110 °C. Elemental analysis were performed by Atlantic Microlabs, Norcross, GA. Despite drying, fractions of solvents could not be removed from some of the samples and were confirmed, where possible, from the ^{1}H NMR.

Methyl Chloro(formyl)acetate (20). Methyl chloroacetate **19** (3.2 g, 30.0 mmol) and methyl formate **18** (1.8 g, 30.0 mmol) were dissolved in toluene volume, and the mixture was cooled to 0 °C using an ice bath. $NaOCH_3$ (95%) (2.0 g, 35.0 mmol) was added and the reaction mixture was stirred at 0 °C for 5 h. Water (100 mL) was added and the mixture was extracted with 100 mL of toluene and ethyl ether. The aqueous layer was separated, cooled to 0 °C, and acidified to pH 4 using 5 N HCl. The aqueous layer was then extracted with 2 × 100 mL of EtOAc. The combined organic layer was dried over $MgSO_4$ and concentrated to afford 3.6 g (72%) of a white foam that was used without further purification. TLC on silica gel indicated two spots (cis and trans enol forms) R_f = 0.36 and 0.38, Hex:EtOAc 1:1.

Methyl 2-Amino-4-oxopyrrolo[2,3-*d*]pyrimidine-5-carboxylate (21). 2,4-Diamino-6-hydroxypyrimidine (**27**) (1.0 g, 7.9 mmol) was suspended in water along with NaOAc (0.33 g, 3.9 mmol). The mixture was heated to 100 °C and at that time all the material dissolved. A suspension of methyl chloro(formyl)acetate (**20**) (1.7 g, 11.5 mmol) in water was added followed by the addition of NaOAc (0.17 g, 2.0 mmol) in 5 mL of water. The reaction mixture was stirred at 100 °C for 2 h, during which time a white precipitate was formed. The reaction mixture was cooled to room temperature and filtered. The precipitate was washed with water, acetone, and ethyl ether to afford 4.7 g (62%) of a white solid: mp >295 °C; TLC, silica gel R_f 0.21 $CHCl_3$:MeOH 3:1; ^{1}H NMR (DMSO-d_6) δ 3.72 (s, 3 H, OCH_3), 6.22 (s, 2 H, 2-NH_2), 7.43 (d, 1 H, 6-H), 10.36 (s, 1 H, 7-H), 11.64 (s, 1 H, 3-H). Anal. Calcd for ($C_8H_8N_4O_3$): C, H, N.

Methyl 2-Amino-4-oxo-7-(tert-butoxycarbonyl)pyrrolo-[2,3-*d*]pyrimidine-5-carboxylate (23). Compound **21** (1.0 g, 4.8 mmol) was dissolved in 50 mL of DMF at 100 °C. The solution was cooled to room temperature and treated with triethylamine (0.48 g, 4.8 mmol) and $(BOC)_2O$ (1.65 g, 7.6 mmol). The reaction was stirred at room temperature for 24 h. The solvent was removed under reduced pressure. The residue was dissolve in 2 mL of MeOH and placed on a dry silica gel column (1.5 × 10 cm) and eluted with 10:1 $CHCl_3$:MeOH to afford 1.2 g (93%) of **23**: mp >250 °C; TLC, silica gel R_f 0.61 $CHCl_3$:MeOH 3:1; ^{1}H NMR (DMSO-d_6) δ 1.56 (s, 9 H, Boc), 3.72 (s, 3 H, OCH_3), 6.58 (s, 2 H, 2-NH_2), 7.53 (s, 1 H, 6-H), 10.75 (s, 1 H, 3-H). Anal. Calcd for ($C_{13}H_{16}N_4O_5$): C, H, N.

Chloro(formyl)acetonitrile (26). While the temperature was maintained between 0 and 5 °C, methyl formate **18** (4.3 g, 77.0 mmol) was added to a stirred mixture of $NaOCH_3$ (3.57 g, 66.0 mmol) in 100 mL of toluene. This was followed by dropwise addition of chloroacetonitrile **25** (5.0 g, 66.0 mmol) over a period of 1 h. The mixture was allowed to stir for an additional 3 h, 100 mL of water was added, and the mixture was extracted with 2 × 100 mL of toluene. The aqueous layer was cooled to 0 °C and acidified to pH 4 using 5 N HCl. The aqueous layer was extracted with 3 × 100 mL EtOAc, and the combined organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to afford 5.1 g (65%) of **26**, which was used without further purification.

2-Amino-5-cyanopyrrolo[2,3-*d*]pyrimidin-4-one (28). NaOAc (1.4 g, 11.0 mmol) was dissolved in 20 mL of distilled water. 2-Diamino-6-hydroxypyrimidine (**27**) (0.66 g, 5.28 mmol) was added, and the mixture was heated to 100 °C, at which time 0.65 g (6.3 mmol) of **26** in 10 mL of water was added. The reaction mixture was refluxed for 5 h and cooled to room temperature, and the precipitate that formed was filtered and washed with copious amounts of water and acetone to yield 0.65 g (72%) of **28** as the only product: mp >360 °C; ^{1}H NMR (DMSO-d_6) δ 6.37 (s, 2 H, 2-NH_2), 7.59 (d,1 H, 6-H), 10.69 (s, 1 H, 7-H), 11.97 (s, 1 H, 3-H).

N-[4-[N-[(2-Amino-4-oxopyrrolo[2,3-*d*]pyrimidin-5-yl)-methyl]amino]benzoyl]-L-glutamic Acid (6). 2-Amino-5-cyanopyrrolo[2,3-*d*]pyrimidin-4-one (**28**, 0.3 g, 1.7 mmol) was dissolved in 10 mL of formic acid in a hydrogenator bottle. To the solution was added *N*-(4-aminobenzoyl)-L-glutamic acid diethyl ester (**30**) (0.640 g, 2.0 mmol) and Raney Ni. The reaction mixture was hydrogenated at 50 psi for 3 h. The reaction mixture was filtered and concentrated under reduced pressure. The residue was dissolve in 2 mL of MeOH and placed on a dry silica gel column (1.5 × 10 cm) and eluted with 10:1 $CHCl_3$:MeOH to afford 0.63 g (75%) of the diester **31** as a white solid: mp >250 °C; TLC, silica gel R_f 0.51 $CHCl_3$:MeOH

3:1; ^{1}H NMR (DMSO-d_6) δ 1.16 (2t, 6 H, CH_3), 2.03 (m, 2 H, β-CH_2), 2.39 (m, 2 H, γ-CH_2), 4.01 (m, 4 H, CH_2CH$_3$), 4.31 (m, 3 H, 8-CH_2 and 9-NH), 6.08 (s, 2 H, 2-NH_2), 6.46 (m, 1 H, α-CH), 6.50 (d, 1 H, 6-CH), 6.0 (d, 2 H, Ph), 7.62 (d, 2 H, Ph), 8.23 (d, 1 H, glu-NH), 10.35 (s, 1 H, 7-NH), 10.81 (s, 1 H, 3-NH). Anal. Calcd for ($C_{23}H_{28}N_6O_6$): C, H, N.

The diester **31** (0.12 g, 0.25 mmol) was dissolved in 5 mL of MeOH. To the solution was added 5 mL of 1 N NaOH, and the mixture was stirred at room temperature for 24 h. The solvent was removed under reduced pressure and the residue was dissolved in 10 mL of water and acidified to pH 4 using concentrated HCl. The mixture was then extracted with 2 × 50 mL EtOAc and the combined organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was dissolved in 2 mL of MeOH and treated with 10 mL of ethyl ether. The precipitate formed was collected by filtration to afford 0.1 g (94%) of **6:** ^{1}H NMR (DMSO-d_6) δ 1.92 (m, 2 H, β-CH_2), 2.29 (m, 2 H, γ-CH_2), 4.31 (m, 3 H, 8-CH_2 and 9-NH), 6.07 (s, 2 H, 2-NH_2), 6.45 (m, 1 H, α-CH), 6.49 (d, 2 H, 6-CH), 6.65 (d, 2 H, Ph), 7.65 (d, 2 H, Ph), 8.10 (d, 1 H, glu-NH), 10.28 (s, 1 H, 7-NH), 10.81 (s, 1 H, 3-NH), 12.31 (b s, 1 H, COOH). Anal. Calcd for ($C_{19}H_{20}N_6O_6$): C, H, N.

General Procedure for the Synthesis of Compounds 7−14. The nitrile 25 (0.5 g, 2.8 mmol) and the appropriate aniline (3.4 mmol) were dissolved in 100 mL of 15% AcOH in DMF with heating. To this solution was added 1.0 g of Raney nickel and the mixture was hydrogenated at 50 psi for a period of 6−8 h. The progress of the reaction was followed by TLC (7:2:0.5, $CHCl_3$:MeOH, NH_4OH). After completion of the reaction, the mixture was filtered through a Celite pad and washed with DMF. Silica gel (1.0 g) was added to the filterate and the solvent was evaporated to afford a dry plug. This plug was placed on a top of 1.05 in. × 23 in. silica gel column and eluted with $CHCl_3$:MeOH (a gradient elution, 95:5 to 85:15). Fractions containing the product were pooled and evaporated to afford analytically pure compound.

2-Amino-4-oxo-5-[(2′,5′-dimethoxyanilino)methyl]pyrrolo[2,3-*d*]pyrimidine (7). Compound **7** was synthesized from 2-amino-4-oxo-5-cyanopyrrolo[2,3-*d*]pyrimidine (**25**) (0.5 g, 2.85 mmol) and 2,5-dimethoxyaniline (0.52 g, 3.42 mmol) using the general procedure described above to afford after purification 0.3 g (33%) as a brownish solid: mp >230 °C; TLC R_f 0.43 ($CHCl_3$:MeOH:NH_4OH, 7:3:0.5); ^{1}H NMR (DMSO-d_6) δ 3.58 (s, 3 H, OCH_3), 3.63 (s, 3 H, OCH_3), 4.20 (d, 2 H, 8-CH_2), 5.46 (t, 1 H, 9-NH), 6.01 (d, 1 H, 6-CH), 6.04 (s, 2 H, 2-NH_2), 6.24 (d, 1 H, Ar), 6.56 (d, 1 H, Ar), 6.64 (s, 1 H, Ar), 10.31 (s, 1 H, 7-NH), 10.75 (s, 1 H, 3-NH). Anal. Calcd for ($C_{15}H_{17}N_5O_3$): C, H, N.

2-Amino-4-oxo-5-[(3′,5′-dimethoxyanilino)methyl]pyrrolo[2,3-*d*]pyrimidine (8). Compound **8** was synthesized from 2-amino-4-oxo-5-cyanopyrrolo[2,3-*d*]pyrimidine (**25**) (0.5 g, 2.85 mmol) and 3,5-dimethoxyaniline (0.52 g, 3.42 mmol) using the general procedure described above to afford after purification 0.18 g (20%) as a brown solid: mp 241 °C; TLC R_f 0.45 ($CHCl_3$: MeOH: NH_4OH, 7:3:0.5); ^{1}H NMR (DMSO-d_6) δ 3.62 (s, 6 H, 3,5-OCH_3), 4.17 (d, 2 H, 8-CH_2), 5.61 (s, 1 H, Ar), 5.70 (d, 2 H, Ar), 5.79 (t, 1 H, 9-NH), 6.05 (s, 2 H, 2-NH_2), 6.53 (d, 1 H, Ar), 10.30 (s, 1 H, 7-NH), 10.77 (s, 1 H, 3-NH). Anal. Calcd for ($C_{15}H_{17}N_5O_3$·0.2H_2O): C, H, N.

2-Amino-4-oxo-5-[(2′,4′-dimethoxyanilino)methyl]pyrrolo[2,3-*d*]pyrimidine (9). Compound **9** was synthesized from 2-amino-4-oxo-5-cyanopyrrolo[2,3-*d*]pyrimidine (**25**) (0.5 g, 2.85 mmol) and 2,4-dimethoxyaniline (0.52 g, 3.42 mmol) using the general procedure described above to afford after purification 0.22 g (24%) as a brown solid: mp 252−256 °C; TLC R_f 0.43 ($CHCl_3$:MeOH:NH_4OH, 7:3:0.5); ^{1}H NMR (DMSO-*d6*) δ 3.58 (s, 3 H, OCH_3), 3.66 (s, 3 H, OCH3), 4.15 (d, 2 H, 8-CH_2), 5.55 (t, 1 H, 9-NH), 6.09 (t, 3 H, Ar, 2-NH_2), 6.53 (s, 1 H, Ar), 6.67 (d, 1 H, Ar), 10.33 (s, 1 H, 7-NH), 10.75 (s, 1 H, 3-NH). Anal. Calcd for ($C_{15}H_{17}N_5O_3$): C, H, N.

2-Amino-4-oxo-5-[(3′,4′,5′-trimethoxyanilino)methyl]-pyrrolo[2,3-*d*]pyrimidine (10). Compound **10** was synthesized from 2-amino-4-oxo-5-cyanopyrrolo[2,3-*d*]pyrimidine (**25**) (0.5 g, 2.85 mmol) and 3,4,5-dimethoxyaniline (0.62 g, 3.42 mmol) using the general procedure described above to afford after purification 0.18 g (18%) as a brown solid: mp 262−268 °C; TLC R_f 0.41 ($CHCl_3$:MeOH:NH_4OH, 7:3:0.5); ^{1}H NMR (DMSO-*d6*) δ 3.49 (s, 3 H, OCH_3), 3.67 (s, 6 H, OCH_3), 4.18 (d, 2 H, 8-CH_2), 5.71 (t, 1 H, 9-NH), 5.91 (s, 2 H, Ar), 6.05 (s, 2 H, 2-NH_2), 10.31 (s, 1 H, 7-NH), 10.77 (s, 1 H, 3-NH). Anal. Calcd for ($C_{16}H_{19}N_5O_4$): C, H, N.

2-Amino-4-oxo-5-[(2′,5′-dichloroanilino)methyl]pyrrolo-[2,3-*d*]pyrimidine (11). Compound **11** was synthesized from 2-amino-4-oxo-5-cyanopyrrolo[2,3-*d*]pyrimidine (**25**) (0.5 g, 2.85 mmol) and 2,5-dichloroaniline (0.55 g, 3.42 mmol) using the general procedure described above to afford after purification 0.17 g (18%) as a white solid: mp 282−285 °C; TLC R_f 0.40 ($CHCl_3$:MeOH:NH_4OH, 7:3:0.5); ^{1}H NMR (DMSO-d_6) δ 4.29 (d, 2 H, 8-CH_2), 6.09 (s, 2 H, 2-NH_2), 6.27 (t, 1 H, 9-NH), 6.57 (m, 2 H, Ar), 6.87 (d, 1 H, Ar), 7.22 (d, 1 H, Ar), 10.30 (s, 1 H, 7-NH), 10.82 (s, 1 H, 3-NH). Anal. Calcd for ($C_{13}H_{11}N_5OCl_2$): C, H, N, Cl.

2-Amino-4-oxo-5-[(3′,5′-dichloroanilino)methyl]pyrrolo-[2,3-*d*]pyrimidine (12). Compound **12** was synthesized from 2-amino-4-oxo-5-cyanopyrrolo[2,3-*d*]pyrimidine (**25**) (0.5 g, 2.85 mmol) and 3,5-dichloroaniline (0.55 g, 3.42 mmol) using the general procedure described above to afford after purification 0.16 g (17%) as a white solid: mp 270−275 °C; TLC R_f 0.39 ($CHCl_3$:MeOH:NH_4OH, 7:3:0.5); ^{1}H NMR (DMSO-d_6) δ 4.22 (d, 2 H, 8-CH_2), 5.55 (t, 1 H, 9-NH), 6.07 (t, 3 H, Ar, 2-NH_2), 6.53 (s, 1 H, Ar), 6.67 (d, 1 H, Ar), 10.33 (s, 1 H, 7-NH), 10.75 (s, 1 H, 3-NH). Anal. Calcd for ($C_{13}H_{11}N_5OCl_2$): C, H, N. Cl.

2-Amino-4-oxo-5-[(2′,4′-dichloroanilino)methyl]pyrrolo-[2,3-*d*]pyrimidine (13). Compound **13** was synthesized from 2-amino-4-oxo-5-cyanopyrrolo[2,3-*d*]pyrimidine (**25**) (0.5 g, 2.85 mmol) and 2,4-dichloroaniline (0.55 g, 3.42 mmol) using the general procedure described above to afford after purification 0.18 g (19%) as a white solid: mp >280 °C; TLC R_f 0.41 ($CHCl_3$:MeOH:NH_4OH, 7:3:0.5); ^{1}H NMR (DMSO-d_6) δ 4.28 (d, 2 H, 8-CH_2), 6.07 (s, 2 H, 2-NH_2), 6.57 (t, 1 H, 9-NH), 6.85 (s, 1 H, Ar), 7.10 (d, 1 H, Ar), 7.13 (d, 1 H, Ar), 10.37(s, 1 H, 7-NH), 10.79 (s, 1 H, 3-NH). Anal. Calcd for ($C_{13}H_{11}N_5OCl_2$): C, H, N, Cl.

2-Amino-4-oxo-5-[(3′-chloroanilino)methyl]pyrrolo[2,3-*d*]pyrimidine (14). Compound **14** was synthesized from 2-amino-4-oxo-5-cyanopyrrolo[2,3-*d*]pyrimidine (**25**) (0.5 g, 2.85 mmol) and 3-chloroaniline (0.43 g, 3.42 mmol) using the general procedure described above to afford after purification 0.19 g (23%) as a white solid: mp 267−270 °C; TLC R_f 0.43 ($CHCl_3$:MeOH:NH_4OH, 7:3:0.5); ^{1}H NMR (DMSO-d_6) δ 4.21 (d, 2 H, 8-CH_2), 6.05 (s, 2 H, 2-NH_2), 6.48 (t, 1 H, 9-NH), 6.53 (m, 4 H, Ar), 7.05 (m, 1 H, Ar), 10.30 (s, 1 H, 7-NH), 10.79 (s, 1 H, 3-NH). Anal. Calcd for ($C_{13}H_{12}N_5OCl$): C, H, N, Cl.

General Procedure for the Synthesis of Compounds 15−17.

To 2-amino-4-oxo-5-[(substituted anilino)methyl]pyrrolo[2,3-*d*]pyrimidine (0.10 g) in 25 mL of acetonitrile was added formaldehyde (1.1 equiv) with constant stirring. To this suspension was added sodium cyanoborohydride, and then the pH of the mixture was adjusted to 2−3 using concentrated HCl. The starting material initially went into solution at pH 2−3, and after 5 min a bright yellow precipitate was formed. TLC ($CHCl_3$:CH_3OH:NH_4OH; 7:3:1 drop) showed the presence of a new spot with the disappearance of the starting material. The acetonitrile was evaporated under reduced pressure and the residue obtained was suspended in 10 mL of water and neutralized using NH_4OH to afford the desired analogues **15−17**.

2-Amino-4-oxo-5-[(2′,5′-dimethoxy-N-methylanilino)-methyl]pyrrolo[2,3-*d*]pyrimidine (15). Compound **15** was obtained as a white solid in 86% yield using the general procedure described above: mp 250−252 °C; TLC R_f 0.43 ($CHCl_3$:MeOH:NH_4OH, 7:3:0.5); ^{1}H NMR (DMSO-d_6) δ 2.85 (s, 3 H, N−CH_3), 3.61 (s, 3 H, OCH_3), 3.70 (s, 3 H, −OCH_3), 4.48 (s, 2 H, 8-CH_2), 6.02 (s, 2 H, 2-NH2), 6.21 (m, 1 H, Ar), 6.31 (s, 1 H, 2-Ar), 6.60 (s, 1 H, Ar), 6.74 (d, 1 H, Ar) 10.77 (s, 1 H, 3-NH), 10.76 (s, 1 H, 3-NH). Anal. Calcd for ($C_{16}H_{19}N_5O_3$·1.0 H_2O): C, H, N.

2-Amino-4-oxo-5-[(3′,5′-dimethoxy-N-methylanilino)-methyl]pyrrolo[2,3-*d*]pyrimidine (16). Compound **16** was obtained as a white solid in 81% yield using the general procedure described above: mp 250−252 °C; TLC R_f 0.46 ($CHCl_3$:MeOH:NH_4OH, 7:3:0.5); ^{1}H NMR (DMSO-d_6) δ 2.95 (s, 3 H, N−CH_3), 3.45 (s, 3 H, OCH_3), 3.67 (s, 3 H, −OCH_3), 4.55 (d, 2 H, 8-CH_2), 5.77 (s, 1 H, Ar), 5.90 (d, 2 H, Ar), 6.21 (s, 2 H, 2-NH_2), 6.46 (s, 1 H, Ar), 10.30 (s, 1 H, 7-NH), 10.76 (s, 1 H, 3-NH). Anal. Calcd for ($C_{16}H_{19}N_5O_3C$·0.75H_2O): C, H, N.

2-Amino-4-oxo-5-[(3′,4′,5′-trimethoxy-N-methylanilino)-methyl]pyrrolo[2,3-*d*]pyrimidine (17). Compound **17** was obtained as a brown solid in 86% yield using the general procedure described above: mp 265−270 °C; TLC R_f 0.42 ($CHCl_3$: MeOH: NH_4OH, 7:3:0.5); ^{1}H NMR (DMSO-d_6) δ 2.92 (s, 3 H, N−CH_3), 3.48 (s, 3 H, OCH_3), 3.70 (s, 6 H, 3,5-diOCH_3), 4.53 (d, 2 H, 8-CH_2), 5.19 (s, 1 H, Ar), 6.04 (s, 1 H, Ar), 6.09 (s, 2 H, 2-NH_2), 6.39 (s, 1 H, Ar), 10.81 (s, 1 H, 3-NH). Anal. Calcd for ($C_{17}H_{21}N_5O_4$·H_2O): C, H, N.

Methods for Biological Evaluations.

Drugs. Drug solutions were standardized using extinction coefficients. Extinction coefficients were determined for **6** [pH 1, λ_{max} 252 nm (sh; 23 100); pH 7, λ_{max} 287 nm (43 000); pH 13, λ_{max} 282 nm (43 500)] and for **7** (pH 1, λ_{max} 262 nm (10 300); pH 7, λ_{max} 289 nm (10 800); pH 13, λ_{max} 272 nm (sh; 10 300)]. Extinction coefficients for methotrexate (MTX), a gift of Immunex (Seattle, WA), were from the literature.[56]

Cell Lines and Methods for Measuring Growth Inhibitory Potency. All cell lines were verified to be negative for mycoplasma contamination using the GenProbe (San Diego, CA) test kit. The human T-lymphoblastic leukemia cell line CCRF−CEM[44] and its MTX-resistant sublines R1,[45] R2,[46] and R30dm[47] were cultured as described.[47] R1 expresses 20-fold elevated levels of DHFR, the target enzyme of MTX. R2 has dramatically reduced MTX uptake, but normal levels of MTX-sensitive DHFR. R30dm expresses 1% of the folylpolyglutamate synthetase (FPGS) activity of CCRF−CEM and is resistant to short-term, but not continuous, MTX exposure; however, R30dm is generally cross-resistant in continuous exposure to antifolates requiring polyglutamylation to form potent inhibitors. The A253 and FaDu human head and neck squamous cell carcinoma cell lines, obtained from ATCC (Manassas, VA), were propagated in RPMI 1640/10% fetal calf serum (FCS); however, growth inhibition was measured in medium containing 5% FCS[48] to minimize the levels of purine and pyrimidines introduced from FCS. Growth inhibition of all cell lines by continuous drug exposure was assayed as described.[47,48] Compound **7** was soluble only in neat DMSO; appropriate vehicle controls were included in all growth experiments, and only data where the vehicle was nontoxic were analyzed. EC_{50} values were determined visually from plots of percent control growth versus the logarithm of drug concentration.

Protection of FaDu cells against growth inhibition was assayed by including metabolites simultaneously with a concentration of drug previously determined to inhibit growth by ≥95%; the remainder of the assay was as described. The metabolites included were leucovorin (LV, 0.1−10 μM) or thymidine (TdR, 40 μM) or hypoxanthine (Hx, 50 μM) or a combination of TdR and Hx at the same individual concentrations. Growth inhibition was measured relative to the appropriate metabolite-treated control; LV had no effect on growth and 40 μM TdR or 50 μM Hx caused ≤10% growth inhibition, while the TdR + Hx combination caused ≤30% growth inhibition.

Enzyme Assays:

Enzyme Inhibition. Partial purification of human CCRF−CEM thymidylate synthase (TMPS) and measurement of its inhibition were performed as previously described.[43]

Dihydrofolate Reductase (DHFR). The spectrophotometric assay for DHFR was modified to optimize for temperature, substrate concentration, and cofactor concentration for each enzyme form assayed.[49,50] The standard assay performed at 37 °C contained sodium phosphate buffer pH 7.4 (40.7 mM), 2-mercaptoethanol (8.9 mM), NADPH (0.117 mM), 1−3.7 IU of enzyme activity (1 IU = 0.005 OD units/min), and dihydrofolic acid (0.092 mM). KCl (150 mM) was included in the assay for *T. gondii* and rat liver DHFR, because it stimulated the enzymes 1.4- and 2.6-fold, respectively. KCl was omitted from assays of *P. carinii* DHFR, because no stimulation was produced with high salt. Background activity measured with no added dihydrofolic acid ranged from 4% of signal for the enzyme obtained from cultured *T. gondii* to 15% of signal for *M. avium* DHFR. All DHFR inhibitors were tested against rat liver DHFR as well as against pathogen DHFR to allow assessment of selectivity.

Stock solutions of inhibitors were prepared in DMSO and then diluted in water to provide a range of concentrations suitable for testing in the enzyme assay. Carry-over of DMSO into the DHFR assay was kept below 1%, because higher concentrations caused inhibition. Solutions of drug were stored at −70 °C in the dark for short periods.

Determination of IC_{50} Values. DHFR was assayed without inhibitor and with a series of concentrations of inhibitors to produce 10−90% inhibition. At least three concentrations were required for calculation. Semilogarithmic plots of the data yielded normal sigmoidal curves for most inhibitors. The data were converted from percent inhibition to probit values, which were plotted versus the log of the drug concentration The resultant straight lines were analyzed by least-squares linear regression. The 50% inhibitory concentration (IC_{50}) is the concentration at which the probit value is 5.0.

Source of *T. gondii*. A frozen sample of the RH strain of *T. gondii* was obtained from the Centers for Disease Control, Atlanta, GA, and inoculated intraperitoneally into female ICR mice (Harlan Industries, Indianapolis, IN). Peritoneal exudate was collected 7 days later and found to contain numerous *T. gondii* organisms, as well as many host cells, some of which were infected with *T. gondii*. Approximately 2×10^6 *T. gondii* were inoculated intraperitoneally into new host mice. The organisms grew more rapidly on second passage in mice and were harvested within 4 days after inoculation. Harvests were scaled up to 20 mice and peritoneal exudate was pooled and centrifuged, and the organisms were resuspended in RPMI medium containing 10% fetal calf serum. Frozen stocks were prepared by adding 5% DMSO to the medium and freezing slowly over 8−15 h. Stocks were stored in liquid nitrogen.

Four years ago we obtained a clinical isolate of *T. gondii* from the Department of Pathology, Indiana University School of Medicine. This strain was handled as described above for the RH strain and stocks were prepared in liquid nitrogen. The clinical isolate displays kinetics of growth in culture that are more advantageous for production of enzymes and is now our standard source of DHFR from *T. gondii*. The material is maintained as described in the previous paragraph for the RH strain.

Culture of *T. gondii* for Enzyme Production. *T. gondii* was grown on a Chinese hamster ovary cell line that lacks DHFR (American Type Culture Collection, 3952 CL, CHO/dhfr$^-$).[50] Cells are maintained in Iscove's Modified Eagle's Medium with 10% fetal calf serum, 1% penicillin/streptomycin, 100 mM hypoxanthine, and 10 mM thymidine. An inoculum of approximately 10^7 organisms was added to each 75 cm^2 tissue culture flask containing the monolayer of cells. Within 6−8 days, 4×10^8 organisms can be harvested from each flask.

Preparation of Enzymes from *T. gondii*. When harvested from tissue culture, *T. gondii* organisms were minimally contaminated with mammalian host cells, which in any case should contain no DHFR activity. To confirm this property, uninoculated monolayer cultures from three flasks were harvested to prepare a 100000*g* supernatant from host cells; no activity of DHFR could be detected. Organisms from culture were washed in phosphate-buffered saline containing 10 mM citrate and resuspended in 50 mM phosphate buffer (pH 7.0) containing leupeptin (20 μg/mL), phenylmethylsulfonyl fluoride (9 μg/mL), soybean trypsin inhibitor (50 μg/mL), aprotinin (50 μg/mL), and 20 mM 2-mercaptoethanol. This buffer lysed the *T. gondii*. The suspension was centrifuged at 100000*g*. The supernate contained both DHFR and dihydropteroate synthase

activity. The preparation was stored in liquid nitrogen without appreciable loss of activity. The yield of DHFR from *T. gondii* cultures has been about 40 IU per flask.

The 100000*g* supernates from *T. gondii* prepared in culture contain both DHFR and dihydropteroate synthase (DHPS) activity. The presence of DHPS, which is not found in mammalian cells, and the absence of DHFR in the specific host cell line used for culture supports the conclusion that the DHFR activity being measured arises from *T. gondii*.

DHFR from Cultured *T. gondii* Tested with Known Inhibitors of the Enzyme. IC_{50} values calculated for methotrexate and pyrimethamine were 0.014 and 0.24 μM, respectively; the IC_{50} values agree closely with the reported values of 0.021 and 0.76 μM. The IC_{50} value for pyrimethamine with DHFR prepared from *T. gondii* harvested from mice was 0.39 μM, a value closely in agreement with the IC_{50} for the enzyme from cultured *T. gondii*. The IC_{50} value for trimethoprim was 1.8, 2.9, and 3.5 μM in three independent trials with enzyme from cultured *T. gondii* but was reported by others to be 14.5 μM for the enzyme prepared from organisms harvested from mice.[51]

Kinetics for cofactor and substrate were determined for DHFR from rat liver and *T. gondii*. Both forms of DHFR displayed kinetics for dihydrofolic acid under our conditions of assay that differed from the Michaelis−Menten model.[52] Curves suggested substrate inhibition at concentrations above that used in the standard assay. The kinetic parameters of rat liver DHFR and *T. gondii* DHFR were similar, with half-maximal rates at 11−13 μM dihydrofolic acid. Others have also suggested that substrate kinetics of the two forms of DHFR are similar.[53] Kinetics for NADPH followed the Michaelis−Menten model and yielded linear double reciprocal plots; K_m values of 11 and 23 μM were determined for DHFR from *T. gondii* and rat liver, respectively. Others reported a K_m of 6.7 μM for NADPH with DHFR from *T. gondii*.[53] These kinetic studies and studies using known inhibitors of *T. gondii* DHFR confirmed that the enzyme from cultured *T. gondii* was similar to preparations previously reported. On the basis of these studies with DHFR, we have designated cultured *T. gondii* as our standard source for enzyme preparations.

Isolation of *P. carinii DHFR*. Cytosolic enzymes of *P. carinii* were isolated as previously described for DHFR.[49] Isolated organisms were resuspended in 50 mM phosphate buffer (pH 7.0) containing leupeptin (20 μg/mL), phenylmethylsulfonyl fluoride (9 μg/mL), soybean trypsin inhibitor (50 μg/mL), aprotinin (50 μg/mL), and 20 mM 2-mercaptoethanol, sonicated, and centrifuged at 100000*g*. The supernate, which contained both DHFR and DHPS activity, was stored in liquid nitrogen.

Recombinant *P. carinii* DHFR was also produced for enzyme assays. The gene sequence was identical to that previously reported. The expression system used pET8C, which employs the T7 RNA polymerase.[55] Host *E. coli* containing the appropriate plasmid construction was grown in Luria broth culture with 75 μg/mL kanamycin at 37 °C on a rotary shaker. The culture was transferred to fresh medium and OD_{590} was monitored. When the OD_{590} reaches 0.4, the culture was shifted to 42 °C for 30 min to induce the gene for T7 RNA polymerase. Rifampin (200 μg/mL) was added to suppress *E. coli* RNA polymerase. After 30 min, the culture was shifted back to 37 °C for 90 min. Cells were harvested by centrifugation, washed, and suspended in appropriate buffer containing protease inhibitors and 2-mercaptoethanol as described above. Bacterial cells were ruptured by sonication. The 100000*g* supernate containing recombinant *P. carinii* DHFR has been the standard enzyme used in the screen under contract NO1-AI-35171. Studies with [^{35}S]methionine incorporation have shown this preparation to contain predominantly one heavy band on autoradiography at a molecular weight corresponding to DHFR.

Recombinant *P. carinii* DHFR can also be purified from these 100000*g* supemates. We currently produce about 4 mg of material suitable for X-ray crystallography from each purification. The first step is an ammonium sulfate precipitation to yield a concentrated 45−90% cut, which consistently contains about 72% of the original activity. This material is loaded onto a P-100 column equilibrated with 100 mM sodium phosphate buffer pH 7.4 containing 20 mM 2-mercaptoethanol and eluted with the same buffer. Most of the protein is eluted in the void volume of the column, but DHFR activity elutes in a broad peak at about 1.3 times the void volume. Fractions containing significant DHFR activity are combined and loaded directly onto an affinity column.

The affinity column is prepared from folate-Sepharose (Sigma). The resin is poured into an appropriately sized column and washed sequentially with 100 mg/mL ascorbate, 5 mg/mL sodium metabisulfite, and 10 mM phosphate buffer pH 7 with 20 mM 2-mercaptoethanol (ME). This treatment reduces the folate on the column to dihydrofolate, the substrate for DHFR. After washing to remove all traces of sodium metabisulfite, the sample containing DHFR is slowly loaded onto the column. When the entire sample has been loaded, the column is washed with ME until protein in the eluate becomes undetectable. Elution continues with stepwise washings of about 2 column volumes of ME containing 0.1 M NaCl, ME with 0.5 M NaCl, and ME with 1 M NaCl and 3 mM folate. The activity of DHFR from affinity columns can be maintained with folate and with glycerol. These materials are used during the concentration steps that follow the affinity column. Glycerol is added to all samples and they are frozen at −70 °C. Aliquots are analyzed for protein content, DHFR activity, and purity by SDS−polyacrylamide gel electrophoresis (SDS−PAGE).

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Supporting Information Available: Analysis data for **6**−**17**, **21**, **23**, and **31**. This material is available free of charge via the Internet at http/::pubs.acs.org.

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