Synthesis and Biological Evaluation of 2,4-Diamino-6-(arylaminomethyl)pyrido[2,3-*d*]pyrimidines as Inhibitors of *Pneumocystis carinii* and *Toxoplasma gondii* Dihydrofolate Reductase and as Antiopportunistic Infection and Antitumor Agents[1]

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A series of 2,4-diamino-6-(arylaminomethyl)pyrido[2,3-*d*]pyrimidines were synthesized and evaluated as inhibitors of *Pneumocystis carinii* (pc), *Toxoplasma gondii* (tg), and rat liver (rl) dihydrofolate reductase (DHFR) and as inhibitors of the growth of tumor cell lines in culture. Compounds **4**−**15** were designed as part of a continuing effort to examine the effects of substitutions at the 5-position, in the two-atom bridge, and in the side chain phenyl ring on structure−activity/selectivity relationships of 2,4-diaminopyrido[2,3-*d*]pyrimidines against a variety of DHFRs. Reductive amination of the common intermediate 2,4-diaminopyrido[2,3-*d*]pyrimidine-6-carbonitrile **16** with the appropriate anilines afforded the target compounds **4**−**12**. Nucleophilic substitution or reductive methylation afforded the N10-methyl target compounds **13**−**15.** As predicted, compounds **4**−**15** were, in general, less potent against all three DHFRs compared to the corresponding 2,4-diamino-5-methyl analogues previously reported; however, the greater decrease in potency against rlDHFR compared to pcDHFR and tgDHFR resulted in appreciable selectivity toward pathogenic DHFRs from different pathogens. The 2′,5′-dichloro analogue **8** showed selectivity ratios (IC_{50} against rlDHFR/IC_{50} against pcDHFR or tgDHFR) of 15.7 and 23 for pcDHFR and tgDHFR, respectively. Thus, the selectivity of **8** for pcDHFR is higher than the first line clinical agent trimethoprim (TMP). In a *P. carinii* cell culture study, analogue **8** exhibited 88% cell growth inhibition at a concentration of 10 μM and afforded marginal effects in an in vivo study in the *T. gondii* mouse model. Selected compounds were evaluated in the National Cancer Institute (NCI) in vitro preclinical antitumor screening program and inhibited the growth of tumor cells in culture at micromolar to submicromolar concentrations and were selected for evaluation in a NCI in vivo hollow fiber assay.

Important strides have been made in therapies designed to interrupt the viral cell cycle of the human immunodeficiency virus (HIV). Therapeutic agents targeted at opportunistic pathogens, to which immunocompromised patients are susceptible, remain a critical component of the regimen required to maintain quality of life.[2,3] *Pneumocystis carinii* pneumonia (PCP) and toxoplasmosis are two potentially life-threatening infections common in AIDS (acquired immunodeficiency syndrome) patients. Early lead inhibitors of *Pneumocystis carinii* (pc) and *Toxoplasma gondii* (tg) dihydrofolate reductase (DHFR) include trimethoprim (TMP) and pyrimethamine, two modestly selective but weak nonclassical folate inhibitors of pcDHFR and tgDHFR (Figure 1).[4,5] DHFR catalyzes the reduction of folate and 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate (THF), a cofactor involved in the biosynthesis of dTMP. Inhibition of DHFR results in inhibition of cell growth. Several reviews have been published on both nonclassical DHFR inhibitors, which are lipophilic compounds such as TMP that can passively diffuse into cells, and classical inhibitors, such as methotrexate (MTX), which contain a side chain with a polar glutamic acid moiety and require carrier-mediated transport into cells.[6−8] Due to the weak inhibition of DHFR from *T. gondii* and *P. carinii* by TMP, coadministration of sulfonamides is required to provide synergistic effects for clinical utility.[9] Unfortunately, the side effects associated with sulfa drugs often results in cessation of the combination therapy.[10] Trimetrexate (TMQ) and piritrexim (PTX) (Figure 1) are two potent nonclassical inhibitors, neither of which exhibit selectivity for pathogenic DHFR versus mammalian DHFR, and thus cannot be used as single agents due to host toxicity.[11,12] As a result, TMQ, when coadministered with leucovorin (5-formyl-THF), has been approved by the FDA for the treatment of *P. carinii* infections.[13] Leucovorin is a cofactor that can utilize the carrier-mediated transport system present in mammalian cells (but absent in *P. carinii* and *T. gondii* cells) to circumvent DHFR inhibition. Unfortunately, TMQ/leucovorin combination therapy suffers from high cost and variability in the effectiveness of leucovorin rescue of host cells under clinical conditions.[14] Thus, several laboratories continue the search for single agents that

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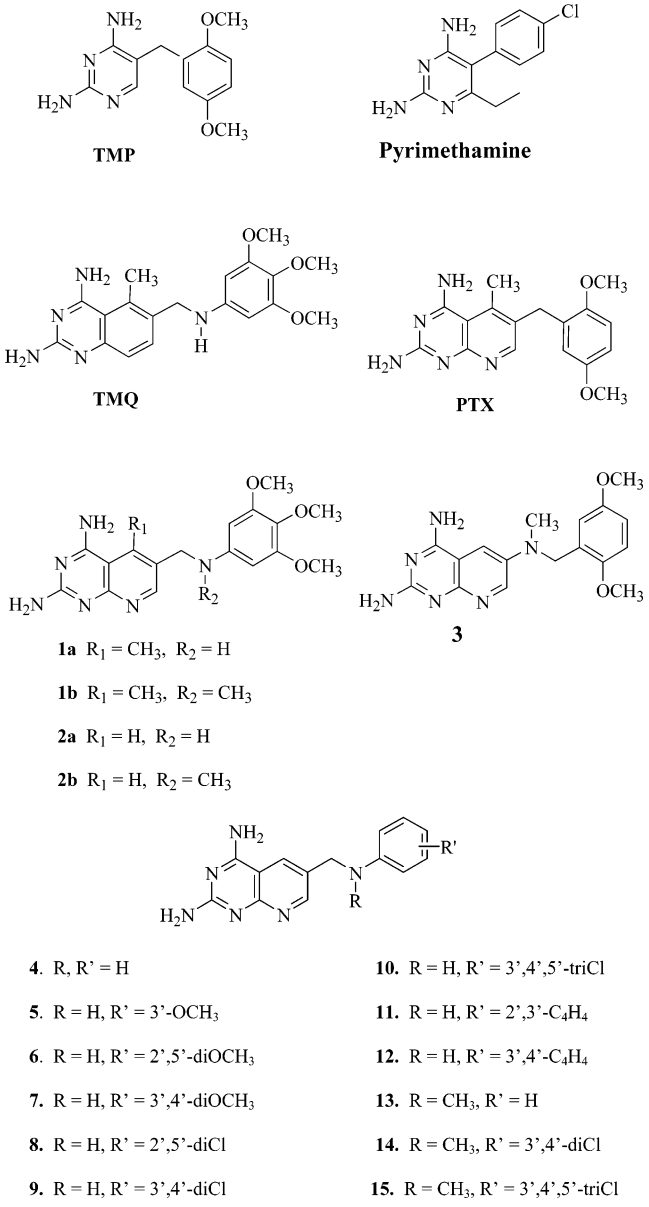


Figure 1. Selected monocyclic and bicyclic nonclassical DHFR inhibitors.

are potent and selective for pcDHFR and/or tgDHFR versus mammalian DHFR.

Gangjee et al.,[15−19] as well as others,[20−24] have been engaged in the investigation and the structure−activity/selectivity relationships of several structural classes of DHFR inhibitors. Variations at the 5-position, the bridge region and the phenyl ring substitutions of the 2,4-diaminopyrido[2,3-*d*]pyrimidine skeleton have afforded several analogues with nanomolar inhibition against tgDHFR and significant selectivity for tgDHFR versus rat liver (rl) or recombinant human (rh) DHFR. Selectivity is quantitated by the ratio of the IC_{50} value of rlDHFR/IC_{50} value of pcDHFR or tgDHFR. Compound **1a** (Figure 1)**,** a pyrido[2,3-*d*]pyrimidine analogue of TMQ, reported by Gangjee et al.[25] had an IC_{50} of 7.4 nM against tgDHFR. N10-methylation in the C9−N10 bridge afforded compound **1b** (Figure 1) and resulted in a 9-fold increase in potency and a 30-fold increase in selectivity for tgDHFR versus rlDHFR. Compound **1b** was 12-fold more potent against tgDHFR, and 30-fold more selective for tgDHFR versus rlDHFR compared to TMQ.

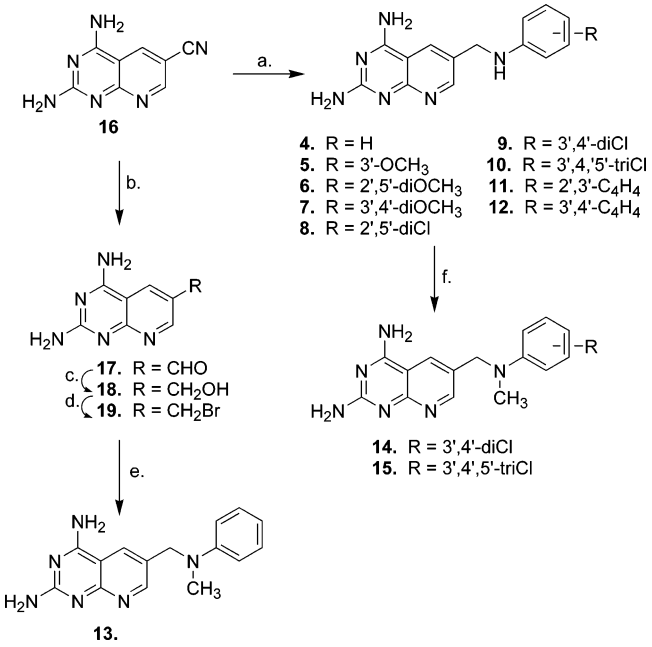
The importance of the 5-methyl group of pyrido[2,3-*d*]pyrimidines for potency against mammalian DHFR has been established. Thus, Gangjee et al.[26] synthesized 5-desmethylpyrido[2,3-*d*]pyrimidine analogues **2a** and **2b** with the expectation that potency against rlDHFR, and possibly pcDHFR and tgDHFR, would decrease compared to compounds **1a** and **1b**. However, a significant increase in selectivity would be realized if the decrease were greater for rlDHFR than pcDHFR or tgDHFR. Compound **2a** showed micromolar inhibitory activity against all three DHFR, and as predicted, the greater decrease in rlDHFR inhibition resulted in an increase in selectivity for both pcDHFR and tgDHFR versus rlDHFR compared to **1a**. The N10-methylated analogue **2b** also showed a decrease in inhibition against all three DHFR compared to **1b**, and as a result, had a selectivity ratio of 31 for tgDHFR, greater than 3-fold higher than **1b.** Consistent with the comparison of compounds **1a** and **1b**, **2b** resulted in a 7- and 3-fold increase in inhibitory potency against pcDHFR and tgDHFR, respectively, compared to **2a**. In addition, **2b** was 5-fold more selective against tgDHFR compared to **2a**. Gangjee et al.[27] also reported the synthesis of a 5-desmethyl reversed bridge 2,4-diaminopyrido[2,3-*d*]-pyrimidine analogue **3**, with a 2,5-dimethoxy substitution on the phenyl ring (Figure 1). Analogue **3** had IC_{50} values of 84 nM and 6.3 nM against pcDHFR and tgDHFR, respectively. When evaluated for selectivity for tgDHFR versus recombinant human (rh) DHFR under different assay conditions,[27] **3** showed a selectivity ratio of 304, the highest selectivity ratio for tgDHFR versus rhDHFR reported for a bicyclic DHFR inhibitor in the literature.

In an attempt to further investigate the potential DHFR inhibitory activity and selectivity of the 2,4-diamino-5-desmethylpyrido[2,3-*d*]pyrimidines, analogues **4**−**15** were synthesized and evaluated as inhibitors of pcDHFR, tgDHFR, rlDHFR, and tumor cell growth. The unsubstituted phenyl, monomethoxyphenyl and dimethoxyphenyl analogues **4**−**7** were designed to study the effect on activity and/or selectivity of increasing electron-donating substituents on the phenyl ring. Additionally, Piper et al.[28] had reported the only 2,4-diamino-5-desmethylpyrido[2,3-*d*]pyrimidine inhibitor with an electron-withdrawing group (*p*-Cl) on the phenyl ring. Thus, analogues **8**−**10** were designed to further examine the effect of electron-withdrawing substituents on the phenyl ring. Analogues **11** and **12** were designed to determine the effect of an increase in the size of the side chain aromatic ring on inhibitory activity and/or selectivity for pcDHFR and tgDHFR. Analogues **13**−**15** were designed to investigate further the effect of N10-methylation in the two-atom bridge on the DHFR inhibitory activity and selectivity of 2,4-diamino-5-desmethylpyrido[2,3-*d*]pyrimidines.

Chemistry

The synthesis of analogues **4**−**15**, outlined in Scheme 1, involved the reductive amination of the common intermediate 2,4-diaminopyrido[2,3-*d*]pyrimidine-6-carbonitrile **16** with the appropriately substituted aniline.

Scheme 1[a]



[a] (a) H_2, atm press., Raney Ni, 80% HOAc, $ArNH_2$; (b) 97% formic acid, Raney Ni; (c) $NaBH_4$, MeOH; (d) 30% hydrogen bromide in acetic acid, acetic acid; (e) NaH, *N*-methylaniline, DMF; (f) 37% formaldehyde, $NaCNBH_3$, 1 N HCl, CH_3CN.

Intermediate **16** was prepared by a procedure reported by Piper et al.[29] and modified by Gangjee et al.[30] Triethyl orthoformate was condensed with malononitrile followed by concentrated hydrochloric acid at 80 °C to afford cyclization to 2-amino-6-chloropyridine-3,5-dicarbonitrile. Reductive dechlorination with hydrogen at 50 psi in the presence of 5% palladium on barium carbonate resulted in 2-aminopyridine-3,5-dicarbonitrile. Cyclization with guanidine afforded the bicyclic intermediate **16**. Reductive amination with the appropriate aniline, hydrogen at atmospheric pressure, and Raney nickel afforded compounds **4**−**12**. The anilines with electron-donating methoxy groups generally resulted in higher yields than those with electron-withdrawing chloro groups. However, the poorest yields were obtained with ortho-substituted anilines, presumably due to steric hindrance.

The N10-methylated compound **13** was synthesized by a modified procedure reported by Piper et al.[31] using 2,4-diaminopyrido[2,3-*d*]pyrimidine-6-methyl bromide **19**, which was obtained in a three-step conversion from **16**, and *N*-methylaniline in a nucleophilic substitution reaction followed by purification by column chromatography. Reduction of intermediate **16** was carried out using 97% formic acid and Raney nickel to yield the carboxaldehyde intermediate **17.** Treatment of **17** with sodium borohydride afforded the hydroxyl intermediate **18.** Bromination of the alcohol was achieved using 37% hydrogen bromide in acetic acid. The resulting bromide **19** was unstable in air even at low temperatures and had to be used immediately without purification. Synthesis of the N10-methylated compounds **14** and **15** was carried out by a modification[30] of a reductive methylation method reported by Borch and Hassid[32] using 37% formaldehyde and $NaCNBH_3$ in acetonitrile adjusted to a pH of 2−3 with 1 N HCl. Compounds **14** and **15** were isolated as analytically pure precipitates without the necessity of further purification.

Table 1. Inhibition Concentrations (IC_{50}, in nM) against DHFR from *P. carinii* (pc), *T. gondii* (tg), and Rat Liver (rl) and Selectivity Ratios[a]

	R	R′	pcDHFR	rlDHFR	rl/pc	tgDHFR	rl/tg
1a			86	2.1	0.02	7.4	0.3
1b			13	7.6	0.6	0.85	8.9
2a			1500	1900	1.3	300	6.3
2b			240	280	1.2	9	31
3			84	57	0.7	6.3	9
4	H	H	2220	2300	1.1	270	8.5
5	H	3′-OMe	520	790	1.5	210	3.8
6	H	2′,5′-diOMe	6100	5000	0.8	450	11
7	H	3′,4′-diOMe	430	560	1.30	330	1.70
8	H	2′,5′-diCl	440	6900	15.7	300	23
9	H	3′,4′-diCl	250	480	1.9	230	2.1
10	H	3′,4′,5′-triCl	350	4600	13	980	4.7
11	H	2′,3′-C_4H_4	70	600	8.6	96	6.3
12	H	3′,4′-C_4H_4	2000	4900	2.5	830	5.90
13	CH_3	H	100000	395000	4	11% @ 31 μM	N/A
14	CH_3	3′,4′-diCl	131	180	1.4	110	1.7
15	CH_3	3′,4′,5′-triCl	196	270	1.4	1980	0.1
TMP[b]			12000	133000	11	2700	49
TMQ[b]			42	3.0	0.07	10	0.3
PTX[b]			31	1.5	0.05	11	0.1

[a] These assays were carried out at 37 °C under saturating conditions of substrate (0.092 mM dihydrofolic acid) and cofactor (0.117 mM NADPH); rlDHFR and tgDHFR were assayed in the presence of 150 mM KCl. [b] These values are from ref 35. Most recent quality control values for rlDHFR are 121400 ± 7500 nM for TMP ($R^2 = 0.9768$), 8.0 ± 0.5 nM ($R^2 = 0.9561$) for TMQ and 4.4 ± 0.4 nM ($R^2 = 0.9386$) for PTX. Most recent quality control values for pcDHFR are 26,820 ± 1700 nM ($R^2 = 0.995$) for TMP, 47 ± 13 nM ($R^2 = 0.923$) for TMQ, and 34.3 ± 10 nM ($R^2 = 0.9876$) for PTX. These quality control assays used recombinant pcDHFR, as did all the assays for experimental compounds shown above. Published data on TMP, TMQ, and PTX used native pcDHFR; side by side comparisons show no difference in drug susceptibility between the two forms of pcDHFR.

Table 2. % Inhibition of Cell Growth at Concentration [μM] of Inhibitor in Culture from *P. carinii* and *T. gondii*[a,b]

	P. carinii cells	*T. gondii* cells
4	ND	0 @ [≤10]
6	ND	<50 @ [10]
8	88 @ [10]	50 @ [4.2]
11	50 @ [50.7]	50 @ [7.8]

[a] *T. gondii* cell culture inhibition was assessed by measuring the incorporation of [^{3}H]uracil by *T. gondii* cells. [b] *P. carinii* cell culture inhibition was assessed by direct counting of organisms in the culture supernatant fluid.

Biological Evaluation and Discussion

Analogues **4**−**15** were evaluated against pcDHFR, tgDHFR, and rlDHFR, and the results are presented in Table 1. Selectivity ratios, expressed as IC_{50} against rlDHFR/IC_{50} against pcDHFR and IC_{50} against rlDHFR/IC_{50} against tgDFHR, are also listed in Table 1. The inhibitory activity and selectivity of TMP, TMQ, and PTX are included for comparison. Selected analogues were also evaluated for inhibition of the growth of *T. gondii* and *P. carinii* cells in culture, and the results are listed in Table 2. The promising inhibitory potency of compound **8** against tgDHFR and against the growth of *T. gondii* and *P. carinii* cells in culture prompted its evaluation in vivo in mice, and the results are shown in Figure 2.

TMP is the most selective inhibitor for pcDHFR and tgDHFR in clinical use today. While several compounds

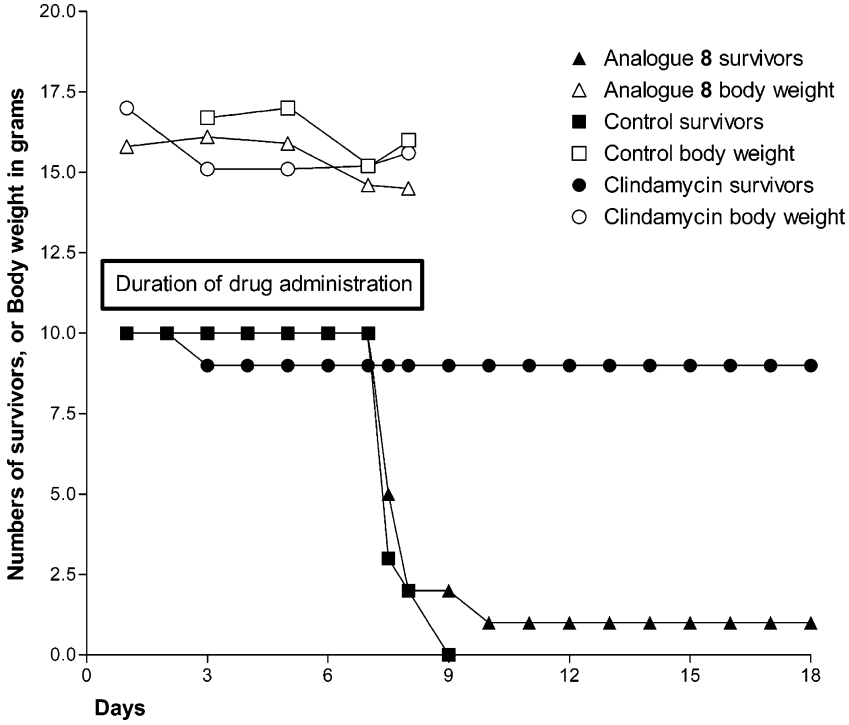


Figure 2. T. Gondii in vivo Study of analogue **8**. Analogue **8** was administered at 50 mg/kg by intraperitoneal injection. Cindamycin phosphate was administered at 24 mg/kg by intraperitoneal injection.

have been identified that are capable of selectively inhibiting tgDHFR with selectivity ratios that surpass that of TMP, very few antifolates have been identified that are capable of selectively inhibiting pcDHFR with selectivity ratios that approach or surpass that of TMP. Recently, Rosowsky et al.[33] reported a 2,4-diaminopteridine analogue that had a pcDHFR selectivity ratio of 21, the highest pcDHFR selectivity ratio for a bicyclic antifolate reported to date. Gangjee et al.[34] reported a 2,4-diaminofuro[2,3-*d*]pyrimidine analogue that had a pcDHFR selectivity ratio of 18.9. Analogues **4**−**12**, **14**, and **15** were more potent than TMP against both pcDHFR and tgDHFR. In addition, analogues **8** and **10** were more selective for pcDHFR versus rlDHFR with selectivity ratios of 15.7 and 13.1, respectively, compared to a pcDHFR selectivity ratio of 11.1 for TMP. Compound **11** showed modest selectivity for pcDHFR with a selectivity ratio of 8.6. However, **11** was the most potent inhibitor of pcDHFR of this series, and of 2,4-diamino-5-desmethylpyrido[2,3-*d*]pyrimidines in general, with an IC_{50} value of 70 nM. Thus, **11** was greater than 2 orders of magnitude more potent against pcDHFR and nearly as selective for pcDHFR versus rlDHFR as TMP. Analogue **13** was essentially inactive against both pcDHFR and rlDHFR, and could not be evaluated at concentrations at which tgDHFR was inhibited by 50% due to its insolubility.

As was demonstrated by the decrease in potency against pcDHFR, tgDHFR, and rlDHFR for compounds **2a** and **2b**, compared to **1a** and **1b**, respectively, the 2,4-diamino-5-desmethylpyrido[2,3-*d*]pyrimidines of this study generally exhibit a decrease in potency of 10- to 100-fold against DHFR compared to the corresponding 5-methyl analogues. The importance of the 5-methyl moiety to DHFR inhibitory potency has been well documented for a number of antifolates. In classical antifolates, Piper et al.[35] reported that aminopterin (AMT) and MTX exhibited decreased inhibition of the growth of L1210 cells in culture due to decreased DHFR inhibition compared to the 5-deaza-5-methyl analogues of AMT and MTX, respectively. In a nonclassical quinazoline series, Bertino et al.[36] reported an 88-fold decrease in DHFR inhibitory potency for a 5-desmethyl quinazoline compared to the 5-methyl congener. Contrary to these reports, Piper et al.[28,38] reported three nonclassical 2,4-diamino-5-desmethylpyrido[2,3-*d*]pyrimidines that showed comparable micromolar DHFR inhibitory activity to the corresponding 5-methyl analogues with identical side chains. Piper et al.[28] noted that the similarity in DHFR inhibitory activity of the 5-methyl and 5-desmethyl analogues reported was the only instance of such similarity in the literature. Thus, moderate micromolar DHFR inhibitory activity for 5-desmethyl analogues **4**−**15** was predicted, and the results confirmed this expectation, with the exception of **11**, which, as mentioned above, approached nanomolar IC_{50} values against both pcDHFR and tgDHFR and **13**, which was inactive. Our intention in this investigation was to explore further the hypothesis that the interaction of the 5-methyl moiety with DHFR that results in greater potency would be more important for mammalian DHFR than pcDHFR or tgDHFR. If the 5-methyl moiety was more crucial to inhibitory activity in rlDHFR than in pcDHFR and/or tgDHFR, the substitution of a hydrogen at the 5-position should result in a greater decrease in potency against rlDHFR compared to pcDHFR or tgDHFR, resulting in a higher selectivity of the 5-desmethyl analogues for pcDHFR or tgDHFR versus rlDHFR. Comparison of **1a** with **2a** shows that the inhibitory potency against pcDHFR, tgDHFR, and rlDHFR decreased by 17-fold, 40-fold, and 900-fold, respectively, resulting in an increase in pcDHFR selectivity of 65-fold and an increase in tgDHFR selectivity of 21-fold for **2a** compared to **1a**. The increase in both

pcDHFR and tgDFHR selectivity was not as pronounced in the N10-methylated analogues **1b** and **2b**. Analogue **2b** showed selectivity ratios that were 2-fold and 3-fold greater for pcDHFR and tgDHFR, respectively, compared to **1b**. However, neither **1a** or **1b**, nor **2a** or **2b**, showed the selectivity exhibited by TMP for either pcDHFR or tgDHFR compared with rlDHFR.

A comparison of the selectivity ratios for analogues **4**−**7** with those of **2a** suggests that a decrease in the number of methoxy groups on the phenyl ring of the side chain in 2,4-diamino[2,3-*d*]pyrimidines does not result in significant increases in selectivity for pcDHFR or tgDHFR.

Comparison of the IC_{50} values of analogues **8**−**10** with those of **4** suggest that the addition of electron-withdrawing chloro substituents results in an increase in pcDHFR inhibitory potency. As was seen in the methoxy analogues, the number and positions of the chloro substituents on the phenyl ring had little effect on the inhibitory potency against tgDHFR. However, the positions of the chloro substituents did affect the potency against rlDHFR. The 2′,5′-dichloro analogue **8** exhibited the lowest potency against rlDHFR of the series, and thus exhibited the highest selectivity for pcDHFR and tgDHFR versus rlDHFR of the series, with selectivity ratios of 15.7 and 23 for pcDHFR and tgDHFR, respectively. The 3′,4′-dichloro analogue **9** is 14-fold more potent against rlDHFR compared to **8**, and thus showed a loss in selectivity for pcDHFR and tgDHFR. The 3′,4′,5′-trichloro compound **10**, showed a decreased potency against rlDHFR and had a selectivity ratio of 13.1 for pcDHFR. Thus, appropriately positioned chloro substituents on the phenyl ring of the side chain of 2,4-diamino-5-desmethylpyrido[2,3-*d*]pyrimidines can decrease the potency against rlDHFR relative to pcDHFR to afford compounds more selective for pcDHFR versus rlDHFR than the clinically utilized agent TMP. In addition, analogues **8** and **10** are 37-fold and 34-fold more potent than TMP, respectively.

Comparison of compounds **11** and **12**, which contain naphthyl substituents, with **4** suggest that a bicyclic aryl group in the side chain is tolerated, and, in the case of **11**, was favorable for potency against pcDHFR, tgDHFR, and rlDHFR. The α-naphthyl analogue **11** was the most potent against pcDHFR and tgDHFR of the series. However, the *β*-naphthyl ring in **12** resulted in a loss of potency against pcDHFR, tgDHFR, and rlDHFR compared to **11**. Analogue **12** did not exhibit significant selectivity for pcDHFR or tgDHFR versus rlDHFR. These results are in contrast to the 2,4-diaminofuro[2,3-*d*]pyrimidine ring where a *β*-naphthyl substituent afforded good selectivity for pcDHFR.[34]

Contrary to the increase in inhibitory potency against both pcDHFR and tgDHFR due to N10-methylation seen in the comparison of **1a** and **1b**, as well as **2a** and **2b**, N10-methylated compounds **13**−**15** showed variable effects on inhibitory potency and selectivity compared to the N10-H analogues.

Analogues **8** and **11** were evaluated as inhibitors of the growth of *P. carinii* cells in culture. Compound **8** inhibited the growth of the *P. carinii* cells in culture by 88% at a concentration of 10 *μ*M, compared to an untreated control. Compound **11** inhibited the growth of the *P. carinii* cells in culture by 50% at a concentration of 50.7 *μ*M compared to a control. Analogues **4**, **6**, **8**, and **11** were selected for in vitro evaluation as inhibitors of the growth of *T. gondii* cell in culture. The unsubstituted analogue **4** showed no inhibition of cell growth at concentrations up to 10 *μ*M. Similarly, analogue **6** inhibited the growth of *T. gondii* cells in culture less than 50% at concentrations up to 10 *μ*M. However, analogues **8** and **11** inhibited the growth of *T. gondii* cells in culture by 50% at concentrations of 4.2 *μ*M and 7.8 *μ*M, respectively.

Table 3. Cytotoxicity Evaluation (GI_{50}, M) against Selected Tumor Cell Lines[38]

	8	**9**	**10**	**12**
leukemia				
K-562	1.6×10^{-6}	3.3×10^{-7}	3.5×10^{-7}	5.0×10^{-7}
Molt-4	2.0×10^{-6}	1.1×10^{-6}	7.1×10^{-7}	2.0×10^{-6}
non-small lung cancer				
EKVX	6.6×10^{-6}	2.4×10^{-6}	8.5×10^{-7}	9.0×10^{-6}
colon cancer				
COLO 205	2.3×10^{-6}	1.7×10^{-6}	3.1×10^{-7}	2.4×10^{-6}
HCC-2998	3.0×10^{-6}	2.7×10^{-6}	7.8×10^{-7}	1.1×10^{-6}
HCT-116	2.1×10^{-6}	6.2×10^{-7}	7.4×10^{-7}	1.9×10^{-6}
CNS cancer				
SF-295	4.0×10^{-6}	3.8×10^{-6}	7.4×10^{-7}	8.2×10^{-6}
melanoma				
LOX IMVI	7.1×10^{-7}	8.2×10^{-7}	3.1×10^{-8}	1.6×10^{-6}
SK-MEL-5	2.2×10^{-6}	1.1×10^{-6}	$3.5 \text{ X } 10^{-7}$	3.2×10^{-6}
breast cancer				
MDA-MB-435	2.4×10^{-6}	1.3×10^{-6}	5.7×10^{-7}	1.0×10^{-5}
MDA-N	3.0×10^{-6}	9.5×10^{-7}	5.0×10^{-7}	4.4×10^{-6}

In light of the in vitro activity against the growth of *P. carinii* and *T. gondii* cells in culture, analogue **8** was evaluated in vivo in a mouse model in which the mice were inoculated with *T. gondii* cells. Ten mice were given a 50 mg/kg dose of analogue **8** for 9 days**.** The results are presented in Figure 2. On day 8, half of the mice died in the treatment group. By the end of the study (18 days), one mouse had survived compared to 9 of 10 mice that had survived after the same dosing schedule of 24 mg/kg of clindamycin.

Analogues **4**, **5**, and **7**−**12** were also evaluated in the preclinical screening program against tumor cell growth by the National Cancer Institute Developmental Therapeutics Program.[38] The analogues that inhibited the growth of a cell line at micromolar or lower concentrations are listed in Table 3. Analogue **10** was especially potent against the growth of a number of tumor cell lines. Due to the potent inhibition of the growth of specific tumor cells, analogues **8**−**10** and **12** were selected for evaluation in an in vivo hollow fiber assay by the National Cancer Institute Developmental Therapeutics Program.[38] Each compound was tested at two different doses against a minimum of twelve human cancer cell lines. However, analogues **8**−**10** and **12** did not attain high enough total score (IP + SC) or cell kill numbers to be considered for additional evaluation. Compounds **8**, **9**, **10**, and **12** had total (IP + SC) scores of 8, 6, 8, and 12 respectively; a score of 20 is necessary for further evaluation.

In summary, the 2,4-diamino-5-desmethylpyrido[2,3-*d*]pyrimidine analogues **4**−**15** were designed to evaluate the possibility of a greater decrease in potency against rlDHFR versus pcDHFR and/or tgDHFR due to the substitution of a hydrogen atom in place of a methyl group at the 5-position to afford greater selectivity for pcDHFR and/or tgDHFR versus rlDHFR. Analogues **8** and **10** showed greater selectivity ratios for pcDHFR

versus rlDHFR than the clinically utilized therapeutic agent TMP. Both **8** and **10** were more potent against pcDHFR and tgDHFR than TMP. Analogue **8** inhibited the growth of *P. carinii* and *T. gondii* cells in culture (Table 2) and showed marginal activity in an in vivo *T. gondii* mouse model for up to 7 days. Analogues **8** and **10** also inhibited the growth of tumor cells at micromolar to submicromolar concentrations; none of the compounds, however, were effective in an in vivo study.

Experimental Section

Thin-layer chromatography (TLC) was performed on silica gel plates with fluorescent indicator purchased from Aldrich Chemical Co., Milwaukee, WI. UV light at 254 and 365 nm was used for visualization. Column chromatography was carried out using silica gel, 200−400 mesh, purchased from Aldrich Chemical Co. Infrared spectra (IR) were recorded on a Perkin-Elmer 1430 spectrophotometer using KBr. ^{1}H NMR spectra were recorded on a Brucker WH-300 (300 MHz) instrument. The chemical shift (δ) values are expressed in part per million (ppm) relative to tetramethylsilane (TMS) as an internal standard: s = singlet, d = doublet, t = triplet, m = multiplet, br = broad peak, exch = exchangeable by addition of D_2O. Elemental analyses were performed by Atlantic Microlabs, Inc., Atlanta, GA. Elemental compositions were within ±0.4% of the calculated value. Fractional moles of solvents in the analytical samples, frequently found in antifolates, could not be prevented despite vigorous drying in vacuo and were confirmed, where possible, by their presence in the NMR spectrum. Melting points were determined on a Fisher-Johns or Thomas-Hoover melting point apparatus and are uncorrected.

General Procedure for the Synthesis of Compounds 4−12. To a solution of the substituted aniline in 70−80% acetic acid were added **16** and Raney Ni. The mixture was hydrogenated in a Parr hydrogenation apparatus at atmospheric pressure and room temperature for 6 h. TLC analysis using solvent A (5:1:0.1 $CHCl_3$:MeOH:NH_4OH) or solvent B (6:1 $CHCl_3$:MeOH) showed the disappearance of the starting material and the appearance of a product spot along with a spot for the alcohol which resulted from an over-reduction of the nitrile **16**. The mixture was treated with Norit and filtered through Celite. To the acidic filtrate was added 1−3 g of silica gel, and the solvent was evaporated to afford a silica gel plug. Alternatively, the acidic filtrate was evaporated and the residue dissolved in 50 mL of warm ethanol. The solution was adjusted to pH 8 using 1 N Na_2CO_3 and the resulting crude precipitate filtered. The crude product was stirred in hot methanol and filtered, to the filtrate was added 1−3 g of silica gel, and the solvent was evaporated to afford a silica gel plug. The resulting plug was applied to a 2.2 × 24 cm silica gel column and eluted with solvent C (6:1:0.1$CHCl_3$:MeOH;NH_4-OH) or solvent D (5:1 $CHCl_3$:MeOH). Fractions containing pure product (TLC) were pooled and evaporated to afford analytically pure compounds **4**−**12**.

2,4-Diamino-6-[(anilino)methyl]pyrido[2,3-*d*]pyrimidine (4). Compound **4** was synthesized from intermediate **16** (0.50 g, 2.7 mmol), aniline (1.2 mL, 13 mmol), and Raney Ni (2.5 g) in 100 mL of 80% acetic acid and purified by column chromatography using solvent A to afford a light yellow solid (0.08 g, 11%): mp > 247 °C dec; TLC R_f 0.31 in solvent A; ^{1}H NMR (DMSO-d_6) δ 4.21 (d, 2 H, CH_2), 6.09 (t, 1 H, NH, exch), 6.28 (br, 2 H, 4-NH_2, exch), 6.51−6.63 (m, 3 H, 2′,4′,6′-H), 7.04−7.09 (t, 2 H, 3′,5′-H), 7.49 (br, 2 H, 2-NH_2, exch), 8.37 (d, 1 H, 5-H), 8.63 (d, 1 H, 7-H). Anal. ($C_{14}H_{14}N_6$), C, H, N.

2,4-Diamino-6-[(3′-methoxyanilino)methyl]pyrido[2,3-*d*]pyrimidine (5). Compound **5** was synthesized from intermediate **16** (0.30 g, 1.6 mmol), *m*-anisidine (0.9 mL, 8.0 mmol), and Raney Ni (2.0 g) in 50 mL of 80% acetic acid and purified by column chromatography using solvent A, followed by recrystallization from MeOH/$CHCl_3$ with a few drops of concd HCl to afford a yellow solid (0.13 g, 16%): mp > 235 °C dec; TLC R_f 0.37 in solvent A; ^{1}H NMR (DMSO-d_6) δ 3.61 (s, 3 H, OCH_3), 4.17 (d, 2 H, CH_2), 6.09−6.17 (m, 3 H, 2′,6′-H, NH, exch), 6.19 (d, 1 H, 4′-H), 6.27 (br, 2 H, 4-NH_2, exch), 6.90−6.95 (t, 1 H, 5′-H), 7.46 (br, 2 H, 2-NH_2, exch), 8.33 (s, 1 H, 5-H), 8.60 (1 H, 7-H). Anal. ($C_{15}H_{16}N_6O\cdot0.6H_2O\cdot0.2HCl$), C, H, N.

2,4-Diamino-6-[(2′,5′-dimethoxyanilino)methyl]pyrido-[2,3-*d*]pyrimidine (6). Compound **6** was synthesized from intermediate **16** (0.50 g, 2.7 mmol), 2,5-dimethoxyaniline (0.45 g, 2.9 mmol), and Raney Ni (5.0 g) in 100 mL of 80% acetic acid and purified by column chromatography using solvent C to afford a yellow solid (0.03 g, 3%): mp > 263 °C dec; TLC R_f 0.50 in solvent B; ^{1}H NMR (DMSO-d_6) δ 3.58 (s, 3 H, 2′-OCH_3), 3.73 (s 3 H, 5′-OCH_3), 4.28 (d, 2 H, CH_2), 5.51−5.55 (t, 1 H, NH), 6.05−6.08 (m, 2 H, 4′,6′-H), 6.37 (s, 2 H, 4-NH_2, exch), 6.69 (d, 1 H, 3′-H), 7.57 (br, 2 H, 2-NH_2, exch), 8.37 (d, 1 H, 5-H), 8.63 (d, 1 H, 7-H). Anal. ($C_{16}H_{18}N_6O_2\cdot2H_2O\cdot0.3CH_3OH$), C, H, N.

2,4-Diamino-6-[(3′,4′-dimethoxyanilino)methyl]pyrido-[2,3-*d*]pyrimidine (7). Compound **7** was synthesized from intermediate **16** (0.50 g, 2.7 mmol), 4-aminoveratrole (2.1 g, 13.4 mmol), and Raney Ni (2.0 g) in 100 mL of 80% acetic acid and purified by column chromatography using solvent A to afford a yellow solid (0.09 g, 10%): mp > 268 °C dec; TLC R_f 0.42 in solvent A; ^{1}H NMR (DMSO-d_6) δ 3.60 (s, 3 H, 4′-OCH_3), 3.67 (s, 3 H, 3′-OCH_3), 4.17 (d, 2 H, CH_2), 5.70−5.74 (t, 1 H, NH, exch), 6.07−6.10 (m, 1 H, 6′-H), 6.28 (s, 2 H, 4-NH_2), 6.34 (d, 1 H, 2′-H), 6.69 (d, 1 H, 5′-H), 7.47 (br, 2 H, 2-NH_2, exch), 8.36 (d, 1 H, 5-H), 8.63 (d, 1 H, 7-H). Anal. ($C_{16}H_{18}N_6O_2\cdot0.2H_2O$), C, H, N.

2,4-Diamino-6-[(2′,5′-dichloroanilino)methyl]pyrido-[2,3-*d*]pyrimidine (8). Compound **8** was synthesized from intermediate **16** (0.50 g, 2.7 mmol), 2,5-dichloroaniline (0.48 g, 3.0 mmol), and Raney Ni (2.0 g) in 100 mL of 80% acetic acid and purified by column chromatography using solvent D to afford a yellow solid (0.06 g, 6%): mp > 232 °C dec; TLC R_f 0.2 in solvent D; ^{1}H NMR (DMSO-d_6) δ 4.37 (d, 2 H, CH_2), 6.26−6.33 (m, 3 H, 2-NH_2 and NH, exch), 6.56 (m, 2 H, 2′,4′-H), 7.24 (t, 1 H 3′-H), 7.49 (br, 2 H, 4-NH_2, exch), 8.49 (s, 1 H, 5-H), 8.58 (d, 1 H, 7-H). Anal. ($C_{14}H_{12}N_6Cl_2$), C, H, N, Cl.

2,4-Diamino-6-[(3′,4′-dichloroanilino)methyl]pyrido-[2,3-*d*]pyrimidine (9). Compound **9** was synthesized from intermediate **16** (1.00 g, 5.4 mmol), 3,4-dichloroaniline (0.97 g, 6.0 mmol), and Raney Ni (5.0 g) in 200 mL of 80% acetic acid and purified by column chromatography using solvent A to afford a yellow solid (0.17 g, 9%): mp > 235 °C dec; TLC R_f 0.25 in solvent A; ^{1}H NMR (DMSO-d_6) δ 4.20 (d, 2 H, CH_2), 6.33 (br, 2 H, NH_2, exch), 6.55−6.59 (m, 2 H, 6′-H and NH, exch), 6.76 (d, 1 H, 2′-H), 7.23 (d, 1 H, 5′-H), 7.51 (br, 2 H, 2-NH_2, exch), 8.33 (d, 1 H, 5-H), 8.59 (d, 1 H, 7-H). Anal. ($C_{14}H_{12}N_6Cl_2$), C, H, N, Cl.

2,4-Diamino −6-[(3′,4′,5′-trichloroanilino)methyl]pyrido[2,3-*d*]pyrimidine (10). Compound **10** was synthesized from intermediate **16** (0.38 g, 1.9 mmol), 3,4,5-trichloroaniline (0.18 g, 1.0 mmol), and Raney Ni (1.5 g) in 100 mL of 80% acetic acid and purified by column chromatography using solvent A to afford a yellow solid (0.0.06 g, 9%): mp > 267 °C dec; TLC R_f 0.23 in solvent A; ^{1}H NMR (DMSO-d_6) δ 4.22 (d, 2 H, CH_2), 6.31 (br, 2 H, NH_2, exch), 6.78−6.82 (m, 3 H, 2′,6′H and NH, exch), 7.49 (br, 2 H, 2-NH_2, exch), 8.30 (d, 1 H, 5-H), 8.58 (d, 1 H, 7-H). Anal. ($C_{14}H_{11}N_6Cl_3$), C, H, N, Cl.

2,4-Diamino-6-[(α-naphthylanilino)methyl]pyrido[2,3-*d*]pyrimidine (11). Compound **11** was synthesized from intermediate **16** (0.50 g, 2.7 mmol), α-aminonaphthalene (1.92 g, 13.4 mmol), and Raney Ni (2.5 g) in 100 mL of 80% acetic acid and purified by column chromatography using solvent A to yield a yellow solid (0.03 g, 3%): mp > 224 °C dec; TLC R_f 0.42 in solvent A; ^{1}H NMR (DMSO-d_6) δ 4.45 (d, 2 H, CH_2), 6.27 (s, 2 H, 4-NH_2, exch), 6.47 (d, 1 H, Ar-H), 6.78 (t, 1 H NH), 7.09 (d, 1 H, Ar-H), 7.22 (t, 1 H, Ar-H), 7.40−7.49 (m, 4 H, Ar-H_2, 2′-NH_2, exch), 7.74 (m, 1 H, Ar-H), 8.22 (t, 1 H, Ar-H), 8.41 (d, 1 H, 5-H), 8.69 (d, 1 H, 7-H). Anal. ($C_{18}H_{16}N_6$), C, H, N.

2,4-Diamino-6-[(*β*-naphthylanilino)methyl]pyrido[2,3-*d*]pyrimidine (12). Compound **12** was synthesized from

intermediate **16** (0.46 g, 2.5 mmol), β-aminonaphthalene (1.06 g, 7.4 mmol), and Raney Ni (2.0 g) in 100 mL of 80% acetic acid and purified by column chromatography using solvent A to yield a yellow solid (0.06 g, 7%): mp > 220 °C dec; TLC R_f 0.44 in solvent A; ^{1}H NMR (DMSO-d_6) δ 4.32 (d, 2 H, CH_2), 6.30 (s, 2 H, 4-NH_2, exch), 6.43 (t, 1 H, NH), 6.78 (d, 1 H Ar-H), 7.02−7.12 (m, 2 H, Ar-H_2), 7.28 (t, 1 H, Ar-H), 7.52−7.64 (m, 5 H, Ar-H_3, 2′-NH_2, exch), 8.42 (d, 1 H, 5-H), 8.69 (d, 1 H, 7-H). Anal. ($C_{18}H_{16}N_6$·0.4H_2O), C, H, N.

2,4-Diamino-6-[(*N*-methylanilino)methyl]pyrido[2,3-*d*]-pyrimidine (13). NaH (60% dispersion in mineral oil, 0.12 g, 3.0 mmol) was added to a cooled solution (0−5 °C) of *N*-methylaniline (0.22 mL, 2.0 mmol) in 50 mL of DMF and stirred under N_2 for 15 min. To this mixture was added freshly prepared intermediate **19** (0.50 g). The reaction mixture was allowed to warm to room temperature and stirred under N_2 for 24 h. DMF was evaporated, and the residue was stirred in H_2O at 0−5 °C. The resulting yellow solid was filtered and washed with EtOH and Et_2O. The solid was dissolved in hot methanol and purified by column chromatography using solvent A to afford a yellow solid (0.17 g, 30%): mp > 251 °C dec; TLC R_f 0.34 in solvent A; ^{1}H NMR (DMSO-d_6) δ 3.01 (s, 3 H, CH_3), 4.53 (s, 2 H, CH_2), 6.29 (br s, 2 H, 4-NH_2), 6.60−6.65 (t, 1 H, 4′-H), 6.76−6.78 (d, 2 H, 2′,6′-H), 7.12−7.18 (t, 2 H, 3′,5′-H), 7.51 (br s, 2 H, 2-NH_2), 8.25−8.26 (d, 1 H, 5-H), 8.52−8.53 (d, 1 H, 7-H). Anal. ($C_{15}H_{16}N_6$·0.4H_2O), C, H, N.

2,4-Diamino-6-[(*N*-methyl-3′,4′-dichloroanilino)methyl]-pyrido[2,3-*d*]pyrimidine (14). To a suspension of **9** (0.095 g, 0.28 mmol) in 10 mL of acetonitrile were added 0.10 mL of 37% formaldehyde and $NaCNBH_3$ (0.053 g, 0.85 mmol). The pH of the mixture was adjusted to 2−3 by dropwise addition of 1 N HCl. After 24 h, the precipitate obtained was filtered and washed successively with H_2O, EtOH, and Et_2O to afford an analytically pure yellow solid (0.028 g, 27%): mp > 227 °C dec; TLC R_f 0.31 in solvent A; ^{1}H NMR (DMSO-d_6) δ 3.07 (s, 3 H, CH_3), 4.64 (s, 2 H, CH_2), 6.70−6.74 (m, 1 H, 6′-H), 6.90−6.91 (d, 1 H, 2′-H), 7.31−7.34 (d, 1 H, 5′-H), 8.31 (s, 1 H, 5-H), 8.60 (s, 1 H, 7-H). Anal. ($C_{15}H_{14}N_6Cl_2$), C, H, N, Cl.

2,4-Diamino-6-[(*N*-methyl-3′,4′,5′-trichloroanilino)methyl]pyrido[2,3-*d*]pyrimidine (15). To a suspension of **10** (0.30 g, 0.81 mmol) in 10 mL of acetonitrile were added 0.31 mL of 37% formaldehyde and $NaCNBH_3$ (0.14 g, 2.23 mmol). The pH of the mixture was adjusted to 2−3 by dropwise addition of 1 N HCl. After 24 h, the precipitate obtained was filtered and washed successively with H_2O, EtOH, and Et_2O to afford an analytically pure yellow solid (0.065 g, 21%): mp > 243 °C dec; TLC R_f 0.26 in solvent A; ^{1}H NMR (DMSO-d_6) δ 3.06 (s, 3 H, CH_3), 4.62 (s, 2 H, CH_2), 6.31 (br s, 2 H, 4-NH_2), 6.93 (s, 2 H, 2′,6′-H), 7.53 (br s, 2 H, 2-NH_2), 8.12−8.13 (d, 1 H, 5-H), 8.52−8.53 (d, 1 H, 7-H). Anal. ($C_{15}H_{14}N_6Cl_2$·0.3H_2O), C, H, N, Cl.

Dihydrofolate Reductase (DHFR) Assay. The spectrophotometric assay for DHFR was modified to optimize for temperature, substrate concentration, and cofactor concentration for each enzyme form assayed. The standard assay contained sodium phosphate buffer pH 7.4 (40.7 mM), 2-mercaptoethanol (8.9 mM), NADPH (0.117 mM), 1 to 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 2.63-fold, respectively. KCl was omitted from assays of P. *carinii* DHFR because no stimulation was produced with high salt. The first three reagents were combined in a disposable cuvette and brought to 37 °C. Drug dilutions were added at this stage. The enzyme was added 30 s before the reaction was initiated with dihydrofolic acid. The reaction was followed for 5 min with continuous recording. Activity under these conditions of assay was linear with enzyme concentration over at least a 4-fold range. Background activity measured with no added dihydrofolic acid was zero with the enzyme obtained from cultured *T. gondii* and near zero for other forms of DHFR. All DHFR inhibitors were tested against rat liver DHFR as well as against pathogen DHFR to allow assessment of selectivity.

Determination of IC_{50} Values. DHFR was assayed without inhibitor and with a series of concentrations of inhibitors to produce 10 to 90% inhibition. At least three concentrations were required for calculation. Semilogarithmic plots of the data yielded normal sigmoidal curves for most inhibitors. The 50% inhibitory concentration (IC_{50}) was calculated from these curves using Prism 3.0.

Source of *T. gondii*. A clinical isolate of *T. gondii* was obtained from the Department of Pathology, Indiana University School of Medicine, after a single passage in a female BALB/c mouse (Harlan Industries, Indianapolis). The organisms were passaged in mice twice more, increasing the number of mice at each passage. After the final passage, the 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 to 15 h. Stocks were stored in liquid nitrogen.

Culture of *T. gondii* for Enzyme Production. By using a chinese hamster ovary cell line that lacks DHFR (American Type Culture Collection, 3952 CL, CHO/dhfr−), *T. gondii* cells were grown and maintained in Iscove's Modified Eagle's Medium with 10% fetal calf serum, 1% penicillin/streptomycin, 100 mM hypoxanthine, and 10 mM thymidine. To each 75 cm^2 tissue culture flask containing the monolayer of cells was added an inoculum of approximately 10^7 organisms. Organisms (4×10^8) were harvested from each flask, within 6 to 8 days.

Preparation of Enzymes from *T. gondii*. *T. gondii* organisms are minimally contaminated with mammalian host cells when harvested from tissue culture, without detectable mammalian DHFR activity.[39] When prepared as noted above, DHFR from cultured *T. gondii* has been shown to yield IC_{50} values similar to those reported in the literature.[39] The kinetics for cofactor and substrate are also similar to reported values in the literature.[39]

Uracil Incorporation by Cultured *T. gondii*. For *T. gondii* grown in culture, uracil is incorporated into nucleic acid, but mammalian cells do not. Thus incorporation of uracil is used as an index of growth of *T. gondii* on host cells.[39] *T. gondii* is grown on HEL (human embryonic lung) cells with Minimum Essential Medium (MEM) supplemented with glutamine (2mM), penicillin/streptomycin (100 units/mL and 100 μg/mL, respectively), and fetal bovine serum (10%). The experiment was carried out as described previously.[39]

In Vivo Testing of Drugs against *T. gondii*. Female BALB/c mice (18−20 g) were injected intraperitoneally with 5×10^3 trophozoites of *T. gondii* from culture. Drug treatment started immediately if the drugs were given in drinking water of food; if the drugs were injected, treatment started 4 h after inoculation. Survival or counts of *T. gondii* present in peritoneal exudates or liver were monitored as an index of drug efficacy.

Isolation of *P. carinii* DHFR. Recombinant *P. carinii* DHFR was produced for enzyme assays as previously described.[40]

Culture of *P. carinii* for Drug Testing. With inocula from *P. carinii*-infected rat lung and cell cultures of human embryonic lung fibroblasts (HEL cells)[40] the compounds were evaluated in short-term culture. Tissue cultures were prepared using 24-well plates in which HEL cells had been grown to confluency in Minimum Essential Medium (MEM) containing 10% fetal calf serum and were inoculated with 7×10^5 viable *P. carinii* trophozoites per mL.

Each drug concentration to be tested was incorporated into the medium of four wells on each of four plates. For positive control, each plate also contained four wells without drug that were inoculated with *P. carinii*. If numbers of organisms in these wells failed to increase more than 3-fold over 7 days, the experiments were discarded. Plates were incubated in a gaseous mixture of 5% O_2, 10% CO_2, and balance N_2 at 35 °C.

***P. carinii* Cell Growth Inhibition Evaluated by Morphology.** For determination of numbers of organisms by morphology, cultures were sampled by washing medium over cells with Pasteur pipets and removing 10 μL samples of the

supernate to 1 cm sq areas that had been etched on slides. Slides were air-dried, fixed in methanol, and stained with Giemsa. Two individuals counted numbers of trophozoites, cysts, and cells in 10 oil immersion fields of each slide, and mean values were plotted.

Human Tumor Cell Hollow Fiber Assay.[38] Human tumor cells were cultivated in polyvinylidene fluoride (PVDF) hollow fibers and samples of the cells were implanted into two physiologic compartments of each of mouse. The mice were treated by the intraperitoneal route with the analogues over time, the fibers were harvested the day following the last treatment, and the degree of cell kill was compared to controls was measured by optical density. Each compound was tested at two different doses against a minimum of 12 human cancer cell lines.

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References

(1) Presented in part at the 213th American Chemical Society National Meeting, San Francisco, CA, April 1997; MEDI 245.
(2) Klepser, M. E.; Lepser, T. B. Drug Treatment of HIV-related Opportunistic Infections. *Drugs* **1997**, *53*, 40−73.
(3) Sparano, J. A.; Sara, C. Infection Prophylaxis and Antiretroviral Therapy in Patients with HIV Infection and Malignancy. *Curr. Opin. Oncol.* **1996**, *8*, 392−399.
(4) Allegra, C. J.; Kovacs, J. A.; Drake, J. C.; Swan, J. C.; Chabner, B. A.; Masur, H. Activity of Antifolates Against *Pneumocystis carinii* Dihydrofolate Reductase and Identification of a Potent New Agent. *J. Exp. Med.* **1987**, *165*, 926−931.
(5) Queener, S. F.; Bartlett, M. S.; Jay, M. A.; Durkin, M. M.; Smith, J. W. Activity of Lipid-Soluble Inhibitors of Dihydrofolate Reductase Against *Pneumocystis carinii* in Culture and in a Rat Model of Infection. *Antimicrob. Agents Chemother.* **1987**, *31*, 1323−1327.
(6) Gangjee, A.; Elzein, E. Kothare, M.; Vasudevan, A. Classical and Nonclassical Antifolates as Potential Antitumor, Antipneumocystis and Antitoxoplasma Agents. *Curr. Pharm. Des.* **1996**, *2*, 263−280.
(7) Broughton, M. C.; Queener, S. F. *Pneumocystis carinii* Dihydrofolate Reductase Used to Screen Potential Antipneumocystis Drugs. *Antimicrob. Agents Chemother.* **1991**, *35*, 1348−1355.
(8) Chio, L.-C.; Queener, S. F. Identification of Highly Potent and Selective Inhibitors of *Toxoplasma gondii* Dihydrofolate Reductase. *Antimicrob. Agents Chemother.* **1993**, *37*, 1914−1923.
(9) Podzamczer, D.; Salazar, A.; Jimenes, J.; Santin, M.; Consiglio, E.; Casanova, A.; Rufi, G.; Gudiol, F. Intermittent Trimethoprim-Sulfathoxazole compared with Dapsone-Pyrimethamine for the Simultaneous Prophylaxis of *Pneumocystis* Pneumonia and Toxoplasmosis in Patients Infected with HIV. *Ann. Intern. Med.* **1995**, *122*, 755−761.
(10) Roudier, C.; Caumes, E.; Rogeauz, O.; Bricaire, F.; Gentilini, M. Adverse Cutaneous Reactions to Trimethoprim-Sulfamethoxazole in Patients with the Aquired Immunodeficiency Syndrome and *Pneumocystis carinii* Pneumonia. *Arch. Dermatol.* 1994, *30*, 1383−1386.
(11) Allegra, C. J.; Kovacs, J. A.; Drake, J. C.; Chabner, B. A.; Masur, H. Potent *In Vitro* and In Vivo Antitoxoplasma Activity of the Lipid-Soluble Antifolate Trimetrexate. *J. Clin. Invest.* **1987**, *79*, 478−482.
(12) Kovacs, J. A.; Allegra, C. J.; Swan, J. C.; Parillo, J. E.; Chabner, B. A.; Masur, H. Potent Antipneumocystis and Antitoxoplasma Activities of Piritrexim, a Lipid Soluble Antifolate. *Antimicrob. Agents Chemother.* **1988**, *32*, 430−433.
(13) News. FDA Approves Trimetrexate as Second line Therapy of *Pneumocystis carinii* Pneumonia. *Am. J. Hosp. Pharm.* **1994**, *51*, 591−592.
(14) Masur, H.; Polis, M. A.; Tuazon, C. U.; Ogata-Arakaki, D.; Kovacs, J. A.; Katz, D.; Hilt, D.; Simmons, T.; Feurerstein, I.; Lundgren, B.; Lane, H. C.; Chabner, B. A.; Allegra, C. J. Salvage Trial of Trimetrexate-Leucovorin for the Treatment of Cerebral Toxoplasmosis. *J. Infect. Dis.* **1993**, *167*, 1422−1426.
(15) Gangjee, A.; Yu, J.; McGuire, J. J.; Cody, V.; Galitsky, N.; Kisliuk, R. L.; Queener, S. F. Design, Synthesis and X-ray Crystal Structure of a Potent Dual Inhibitor of Thymidylate Synthase and Dihydrofolate Reductase as an Antitumor Agent. *J. Med. Chem.* **2000**, *43*, 3837−3851.
(16) Gangjee, A.; Zeng, Y.; McGuire, J. J.; Kisliuk, R. L. Effect of C-9 Methyl Substitution and C8−C9 Conformational Restriction on Antifolate and Antitumor Activity of Classical 5-Substituted 2,4-Diaminofuro[2, 3-*d*]pyrimidines. *J. Med. Chem.* **2000**, *43*, 3125−3133.
(17) Gangjee, A.; Adair, O.; Queener, S. F. *Pneumocystis carinii* and *Toxoplasma gondii* Dihydrofolate Reductase Inhibitors and Antitumor Agents: Synthesis and Biological Activities of 2,4-Diamino-5-methyl-6-[(monosubstituted anilino)methyl]-pyrido-[2,3-*d*]pyrimidines. *J. Med. Chem.* **1999**, *42*, 2447−2455.
(18) Gangjee, A.; Zhu, Y.; Queener, S. F. 6-Substituted 2,4-Diaminopyrido[3,2-*d*]pyrimidine Analogues of Piritrexim as Inhibitors of Dihydrofolate Reductase from Rat Liver, *Pneumocystis carinii*, and *Toxoplasma gondii* and as Antitumor Agents. *J. Med. Chem.* **1998**, *41*, 4533−4541.
(19) Gangjee, A.; Vidwans, A. P.; Vasudevan, A.; Queener, S. F.; Kisliuk, R. L.; Cody, V.; Li, R.; Galitsky, N.; Luft, J. R.; Pangborn, W. Structure-Based Design and Synthesis of Lipophilic 2,4-Diamino-6-Substituted Quinazolines and Their Evaluation as Inhibitors of Dihydrofolate Reductases and Potential Antitumor Agents. *J. Med. Chem.* **1998**, *41*, 3426−3434.
(20) Rosowsky, A.; Papoulis, A. T.; Forsch, R. A.; Queener, S. F. Synthesis and Antiparasitic and Antitumor Activity of 2,4-Diamino-6-(arylmethyl)-5,6,7,8-tetrahydroquinazoline Analogues of Piritrexim. *J. Med. Chem.* **1999**, *42*, 1007−1017.
(21) Robson, C.; Meek, M. A.; Grunwaldt, J.-D.; Lambert, P. A.; Queener, S. F.; Schmidt, D.; Griffin, R. J. Nonclassical 2,4-Diamino-5-aryl-6-ethylpyrimidine Antifolates: Activity as Inhibitors of Dihydrofolate Reductase from *Pneumocystis carinii* and *Toxoplasma gondii* and as Antitumor Agents. *J. Med. Chem.* **1997**, *40*, 3040−3048.
(22) Stevens, M. F. G.; Phillip, K. S.; Rathbone, D. L.; O'Shea, D. M.; Queener, S. F.; Schwalbe, C. H.; Lambert, P. A. Structural Studies on Bioactive Compounds. 28. Selective Activity of Triazenyl-Substituted Pyrimethamine Derivatives against *Pneumocystis carinii* Dihydrofolate Reductase. *J. Med. Chem.* **1997**, *40*, 1886−1893.
(23) Piper, J. R.; Ramamurthy, B.; Johnson, C. A.; Otter, G. M.; Sirotnak, F. M. Analogues of 10-Deazaaminopterin and 5-Alkyl-5, 10-dideazaaminopterin with the 4-Substituted 1-Naphthoyl Group in the Place of 4-Substituted Benzoyl. *J. Med. Chem.* **1996**, *39*, 614−618.
(24) Kuyper, L. F.; Baccanari, D. P.; Jones, M. L.; Hunter, R. N.; Tansik, R. L.; Joyner, S. S.; Boytos, C. M.; Rudolph, S. K.; Knick, B.; Wilson, H. R.; Caddell, J. M.; Friedman, H. S.; Comley, J. C. W.; Stables, J. N. High-Affinity Inhibitors of Dihydrofolate Reductase: Antimicrobial and Anticancer Activities of 7,8-Dialkyl-1,3-diaminopyrrolo[3,2-*f*]quinazolines with Small Molecular Size. *J. Med. Chem.* **1996**, *39*, 892−903.
(25) Gangjee, A.; Shi, J.; Queener, S. F.; Barrows, L. R.; Kisliuk, R. L. Synthesis of 5-Methyl-5-deaza Nonclassical Antifolates as Inhibitors of Dihydrofolate Reductases and as Potential Antipneumocystis, Antitoxoplasma, and Antitumor Agents. *J. Med. Chem.* **1993**, *36*, 3437−3443.
(26) Gangjee, A.; Devraj, R.; Queener, S. F. Synthesis and Dihydrofolate Reductase Inhibitory Activities of 2,4-Diamino-5-deaza and 2,4-Diamino-5, 10-dideaza Lipophilic Antifolates. *J. Med. Chem.* **1997**, *40*, 470−478.
(27) Gangjee, A.; Vasudevan, A.; Queener, S. F.; Kisliuk, R. L. 2,4-Diamino-5-deaza-6-substituted Pyrido[2, 3-*d*]pyrimidines as Potent and Selective Nonclassical Inhibitors of Dihydrofolate Reductases. *J. Med. Chem.* **1996**, *39*, 1438−1446.
(28) Piper, J. R.; Johnson, C. A.; Krauth, C. A.; Carter, R. L.; Hosmer, C. A.; Queener, S. F.; Borotz, S. E.; Pfefferkorn, E. R. Lipophilic Antifolates as Agents against Opportunistic Infections. 1. Agents Superior to Trimetrexate and Piritrexim against *T. gondii* and *P. carinii* in In Vitro Evaluations. *J. Med. Chem.* **1996**, *39*, 1271−1280.
(29) Piper, J. R.; McCaleb, G. S.; Montgomery, J. A.; Kisliuk, R. L.; Gaumont, Y.; Sirotnak, F. M. Synthesis and Antifolate Activity of 5-Methyl-5-deaza Analogues of Aminopterin, Methotrexate, Folic Acid and N10-Methylfolic Acid. *J. Med. Chem.* **1986,** *29*, 1080−1087.
(30) Gangjee, A.; Vasudevan, A.; Queener, S. F.; Kisliuk, R. L. 6-Substituted 2,4-Diamino-5-methylpyrido[2, 3-*d*]pyrimidines as Inhibitors of Dihydrofolate Reductases from *Pneumocystis carinii* and *Toxoplasma gondii* and as Antitumor Agents. *J. Med. Chem.* **1995**, *38*, 1778−1785.
(31) Piper, J. R.; Johnson, C. A.; Maddry, J. A.; Malik, N. D.; McGuire, J. J.; Otter, G. M.; Sirotnak, F. M. Studies on Analogues of Classical Antifolates Bearing the Naphthoyl Group in Place of Benzoyl in the Side Chain. *J. Med. Chem.* **1993**, *36*, 4161−4171.
(32) Borch, R. F.; Hassid, A. I. A New Method for the Methylation of Amines. *J. Org. Chem.* **1972**, *37*, 1673−1674.

(33) Rosowsky, A.; Cody, V.; Galitsky, N.; Fu, H.; Papoulis, A. T.; Queener, S. F. Structure-Based Design of Selective Inhibitors of Dihydrofolate Reductase: Synthesis and Antiparasitic Activity of 2,4-Diaminopteridine Analogues with a Bridged Diarylamine Side Chain. *J. Med. Chem.* **1999**, *42*, 4853−4860.
(34) Gangjee, A.; Guo, X.; Queener, S. F.; Cody, V.; Galitsky, N.; Luft, J. R.; Panghorn, W. Selective *Pneumocystis carinii* Dihydrofolate Reductase Inhibitors: Design, Synthesis and Biological Evaluation of New 2,4-Diamino-5-substituted-furo[2,3-*d*]pyrimidines. *J. Med. Chem* **1998**, *41*, 1263−1271.
(35) Piper, J. R.; McCaleb, G. S.; Montgomery, J. A.; Kisliuk, R. L.; Gaumont, Y.; Sirotnak, F. M. Synthesis and Antifolate Activity of 5-Methyl-5-Deaza Analogues of Aminopterin, Methotrexate, Folic Acid and N-10 Methyl Folic Acid. *J. Med. Chem.* **1986**, *29*, 1080−1086.
(36) Schornagel, J. H.; Chang, P. K.; Sciarini, L. J.; Moroson, B. A.; Mini, E.; cashmore, A. R.; Bertino, J. Synthesis and Evaluation of 2,4-Diamino Quinazoline Antifolates with Activity Against Methotrexate Resistant Human Tumor Cells. *Biochem. Pharmacol.* **1984**, *33*, 3251−3255.
(37) Piper, J. R.; Johnson, C. A.; Hosmer, C. A.; Carter, R. L.; Pfefferkorn, E. R.; Borotz, S. E.; Queener, S. F. Lipophilic Antifolates as Candidates Against Opportunistic Infections. *Adv. Exp. Med. Biol.* **1993**, *338*, 429−433.
(38) We thank the Developmental Therapeutics Program of the National Cancer Institute for performing the in vitro and in vivo anticancer evaluations.
(39) Chio, L. C. and Queener, S. F. Identification of Highly Potent and Selective Inhibitors of *Toxoplasma gondii* Dihydrofolate Reductase. *Antimicrob. Agents Chemother.* **1993**, *37*, 1914−1923.
(40) Bartlett, M. S.; Shaw, M.; Navaran, P.; Smith, J. W. and Queener, S. F. Evaluation of Potent Inhibitors of Dihydrofolate Reductase in a Culture Model for Growth of *Pneumocystis carinii*. *Antimicrob. Agents Chemother.* **1995**, *39*, 2436−2441.

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