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[1]

Aleem Gangjee,*,† Ona Adair,† and Sherry F. Queener‡

Division of Medicinal Chemistry, Graduate School of Pharmaceutical Sciences, Duquesne University, Pittsburgh, Pennsylvania 15282, and Department of Pharmacology and Toxicology, Indiana University School of Medicine, Indianapolis, Indiana 46202

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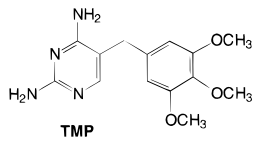
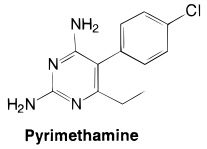
Thirteen 2,4-diamino-5-methyl-6-[(monosubstituted anilino)methyl]pyrido[2,3-*d*]pyrimidines **5**−**17** were synthesized as potential *Pneumocystis carinii* (pc) and *Toxoplasma gondii* (tg) dihydrofolate reductase (DHFR) inhibitors and as antitumor agents. Compounds **5**−**17** were designed to investigate the structure−activity relationship of monomethoxy and monohalide substitution in the phenyl ring and N10-methylation of the C9−N10 bridge. The synthetic route to compounds **5**−**12** involved the reductive amination of a common intermediate, 2,4-diamino-5-methylpyrido[2,3-*d*]pyrimidine-6-carbonitrile (**18**), with the appropriate anilines. N10-Methylation was achieved by reductive methylation. In contrast to previous reports of trimethoprim, the removal of methoxy and chloro groups from the phenyl ring in the 2,4-diamino-5-methyl-6-[(substituted anilino)methyl]pyrido[2,3-*d*]pyrimidine series generally did not decrease DHFR inhibitory activity. The monosubstituted phenyl analogues **5**−**12** were as potent against pcDHFR and tgDHFR as the previously reported disubstituted phenyl analogues. N10-Methylation generally resulted in a marginal increase in potency against both pcDHFR and tgDHFR. Compounds **5**, **7**, and **9** were evaluated and shown to inhibit the growth of *T. gondii* cells in culture at nanomolar concentrations. Compounds **6**−**8**, **9**, **11**, and **16** were selected by the National Cancer Institute for evaluation in an in vitro preclinical antitumor screening program. All six compounds showed GI_{50} values in the 10^{-7}−10^{-9} M range in more than 20 cell lines.

Introduction

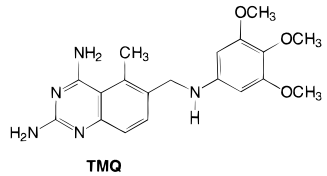
Pneumocystis carinii pneumonia (PCP) and toxoplasmosis are common opportunistic infections resulting in the death of patients with acquired immunodeficiency syndrome (AIDS).[2] Several reviews have been published on the development of lipophilic, nonclassical antifolate inhibitors of *Pneumocystis carinii* (pc) and *Toxoplasma gondii* (tg) dihydrofolate reductase (DHFR).[3−5] However, current therapies are beset with high cost and low selectivity which in many cases result in host toxicity and cessation of therapy.[6,7] Trimethoprim (TMP) and pyrimethamine, reported by Allegra et al.,[8,9] are modestly selective but weak inhibitors of pcDHFR and tgDHFR and require co-administration of sulfonamides to provide synergistic effects for clinical utility. Both the TMP/sulfamethoxazole and pyrimethamine/sulfadiazine regimens currently used against *P. carinii* and *T. gondii* infections, respectively, suffer from side effects attributed to the sulfa drug, which often result in discontinuation of therapy.[10,11] Trimetrexate (TMQ)[12] and piritrexim (PTX)[13] are two potent nonclassical DHFR inhibitors. Nonclassical, lipophilic agents can passively diffuse into cells, in contrast to classical agents, such as methotrexate, which contain side chains with a polar glutamic acid moiety and hence require a carrier-mediated transport system to enter cells.[12] Unfortunately, neither TMQ nor PTX exhibits selectivity for pcDHFR or tgDHFR, which precludes their use as single agents due to host toxicity. As a result, TMQ combined with leucovorin (5-formyltetrahydrofolate) has been approved by the FDA for treatment of *P. carinii* infections.[14] Leucovorin is a classical folate cofactor which is a precursor of 5,10-methylenetetrahydrofolate and utilizes the carrier-mediated transport systems to gain access to host cells, thus circumventing DHFR inhibition. These transport systems are absent in *P. carinii* and *T. gondii* cells. Hence, leucovorin rescue results in decreased host toxicity.[15] Drawbacks of the TMQ/leucovorin combination therapy are the expense as well



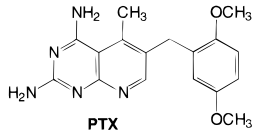
TMP



Pyrimethamine



TMQ



PTX

† Duquesne University.
‡ Indiana University School of Medicine.

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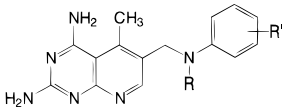
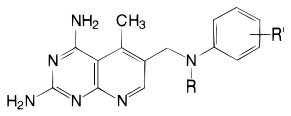
as the inability of leucovorin to rescue host cells under all clinical conditions. Thus, the effort to develop potent and selective inhibitors of pcDHFR and tgDHFR to be used as single agents is currently in progress.

A design approach adopted by Gangjee et al.[16−18] and Piper et al.[19,20] is to combine the pyrido[2,3-*d*]pyrimidine bicyclic skeleton of PTX with the nitrogen-containing two-atom bridge of TMQ containing electron-withdrawing and electron-donating substituents on the side chain phenyl ring to provide antitumor agents and/or selective inhibitors of pcDHFR and tgDHFR. Thus, TMQ−PTX hybrid analogues **1a,b**, **2a,b**, **3a−c**, and **4a,b** were synthesized. Compound **1a**[16] was 2-fold less potent against pcDHFR and equipotent against rat liver (rl) DHFR and tgDHFR compared to TMQ. Compound **1b**,[16] the N10-methylated analogue of **1a**, was 5-fold more potent and 8-fold more selective against pcDHFR compared to TMQ and 12-fold more potent and 31-fold more selective against tgDHFR. Despite its potency and selectivity as an inhibitor of pcDHFR and tgDHFR, compound **1b** showed poor inhibition against the growth of *P. carinii* and *T. gondii* cells in culture. This indicated a possibility of insufficient cell penetration. Compound **2a**,[16] an analogue of **1a** that contains three electron-withdrawing chloro substituents, was equipotent against pcDHFR and tgDHFR compared to TMQ. Compound **2a** was 10-fold less potent against rlDHFR which resulted in a 10-fold increase in selectivity against pcDHFR and tgDHFR compared to TMQ. N10-Methylation of **2a** afforded compound **2b**[16] and resulted in a 2-, 10-, and 3-fold decrease in potency against pcDHFR, rlDHFR, and tgDHFR, respectively, compared to TMQ.



1a	R = H	R' = 3',4',5'-triOCH$_3$	**3a**	R = H	R' = 3',4'-diOCH$_3$
1b	R = CH$_3$	R' = 3',4',5'-triOCH$_3$	**3b**	R = CH$_3$	R' = 3',4'-diOCH$_3$
2a	R = H	R' = 3',4',5'-triCl	**3c**	R = CH$_3$	R' = 2',5'-diOCH$_3$
2b	R = CH$_3$	R' = 3',4',5'-triCl	**4a**	R = H	R' = 3',4'-diCl
			4b	R = CH$_3$	R' = 3',4'-diCl

Baccanari et al.[21] reported that the sequential removal of the methoxy groups of TMP resulted in a significant decrease in the inhibition of bacterial DHFR. Similarly, the DHFR inhibitory activities reported by Gangjee et al.[17] for a series of N10-methylated dimethoxyphenyl-substituted 2,4-diaminopyrido[2,3-*d*]pyrimidines also decreased compared to the corresponding trimethoxyphenyl analogues. In the N10-H series, 3′,4′-dimethoxy compound **3a** was slightly less potent against rlDHFR and equipotent against tgDHFR compared to the trimethoxy compound **1a**. The removal of one methoxy group resulted in a 10- and 3-fold increase in selectivity against pcDHFR and tgDHFR, respectively. However, N10-methylation to afford the 3′,4′-dimethoxy analogue **3b** resulted in a significant decrease in potency against pcDHFR, rlDHFR, and tgDHFR and thus showed a decrease in selectivity compared to **1b**. In addition, the 2′,5′-dimethoxy analogue **3c** showed an improved ability to inhibit the growth of *T. gondii* cells in culture compared to **1b**, indicated by an IC$_{50}$ *T. gondii* culture/IC$_{50}$ tgDHFR ratio of 35 compared to an IC$_{50}$ *T. gondii* culture/IC$_{50}$ tgDHFR ratio of 5529 for compound **1b**. As an extension of our previous efforts to study the structure−activity/selectivity relationships of 6-substituted 2,4-diamino-5-methylpyrido[2,3-*d*]pyrimidines, and to develop potent and selective pcDHFR and/or tgDHFR inhibitors, we synthesized and evaluated compounds **5−17.** Analogues **5−8** were designed to further investigate the effect of removing additional methoxy groups

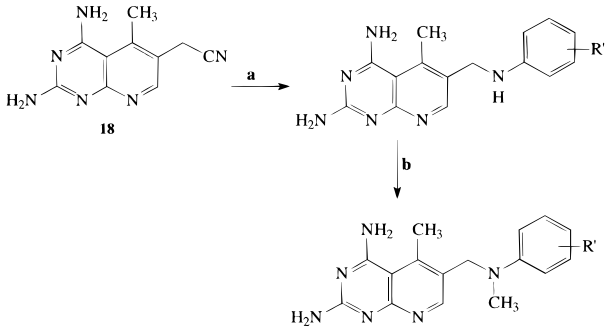


	R	R'		R	R'
5	H	H	**12**	H	4'-Br
6	H	2'-OCH$_3$	**13**	CH$_3$	3'-OCH$_3$
7	H	3'-OCH$_3$	**14**	CH$_3$	4'-OCH$_3$
8	H	4'-OCH$_3$	**15**	CH$_3$	2'-Cl
9	H	2'-Cl	**16**	CH$_3$	4'-Cl
10	H	3'-Cl	**17**	CH$_3$	4'-Br
11	H	4'-Cl			

from the side chain phenyl ring of the pyrido[2,3-*d*]-pyrimidines on DHFR inhibitory potency, selectivity, and cell penetration. Specifically, monosubstituted and unsubstituted phenyl analogues which were not reported in the previous study were necessary to determine if the trend observed in the pyrimidine (TMP) analogues[21] was applicable to the pyrido[2,3-*d*]pyrimidine series. Furthermore, if the expected decrease in activity of the monomethoxy-substituted analogues was greater for rlDHFR, the mammalian standard, than for pcDHFR and/or tgDHFR, the monomethoxy-substituted analogues would afford increased selectivity ratios for pcDHFR and/or tgDHFR. Thus, it was of interest to determine if the unsubstituted or monomethoxy analogues **5−8**, **13**, and **14** would follow this trend and perhaps afford a more favorable culture-to-enzyme inhibitory ratio.

In addition, it was also of interest to determine the activity of monochloro-substituted analogues compared to the trichlorophenyl and dichlorophenyl 6-substituted 2,4-diamino-5-methylpyrido[2,3-*d*]pyrimidines reported by Gangjee et al.[16,22] The 3′,4′,5′-trichloro analogue **2a** showed nanomolar inhibition against pcDHFR, tgDHFR, and rlDHFR and was not selective for the target enzymes. Compared to **2a**, the 3′,4′-dichloro analogue **4a** was 5- and 2-fold less potent against pcDHFR and tgDHFR, respectively, and equipotent against rlDHFR and thus resulted in decreased selectivity ratios. Compound **4b**, the N10-methylated analogue of **4a**, was equipotent against pcDHFR, rlDHFR, and tgDHFR compared to **2b**. In the tri- and dichloro-substituted series, the effect of sequential removal of chloro groups on potency and selectivity did not follow the trends observed in the methoxy series. Thus, compounds **9−11**, **15**, and **16** were designed to investigate the structure−activity/selectivity relationship of monochloro substitution compared to the tri- and dichloro substitution on the phenyl ring of 6-substituted 2,4-diamino-5-methylpyrido[2,3-*d*]pyrimidines, as well as their ability to inhibit the growth of *T. gondii* cells in culture as compared to the monomethoxy or unsubstituted ana-

Scheme 1[a]



[a] (a) H_2, atmospheric pressure, Raney Ni, 80% HOAc, Ar−NH_2; (b) 37% CH_2O, $NaCNBH_3$, 1 N HCl, CH_3CN.

logues. Compounds **12** and **17** were designed to ascertain the biological effect of bromine versus chlorine.

In the pyrido[2,3-*d*]pyrimidine series, the effect of N10-methylation was found to be dependent on both the electronegativity and the number of phenyl ring substitutions.[16] The N10-H analogue **1a** was 6- and 13-fold less potent than compound **1b** against pcDHFR and tgDHFR, respectively.[16] In addition, compound **1b** was 30-fold more selective against tgDHFR compared to compound **1a**. N10-Methylation in the trichloro-substituted analogues had much less of an effect on potency and selectivity compared to the trimethoxy-substituted analogues. The N10-H analogue **2a** showed a 3-fold increase in potency against tgDHFR and a slight increase in potency against pcDHFR compared to **2b**. The effect on selectivity was marginal. Thus, to investigate the effect of N10-methylation in the monosubstituted series, compounds **7** and **8,** in the methoxy series, and compounds **9**, **11**, and **12**, in the halogenated series, were N10-methylated to afford compounds **13**−**17**.

Several DHFR inhibitors exhibit nanomolar inhibitory potency against the growth of tumor cells in culture and possess therapeutic activity and potential as antitumor agents.[23] It was therefore also of interest to evaluate analogues **5**−**17** as potential antitumor agents.

Compounds **6**−**8**, **9**, **11**, and **16** were selected by the National Cancer Institute (NCI) for evaluation against approximately 50 tumor cell lines in their preclinical in vitro panel of tumor cells. The panel included leukemia, lung, colon, central nervous system (CNS), melanoma, ovarian, renal, prostate, and breast cancer cell lines.

Chemistry

The syntheses of the analogues **5**−**17** are outlined in Scheme 1. The common intermediate, 2,4-diamino-5-methylpyrido[2,3-*d*]pyrimidine-6-carbonitrile (**18**), was prepared by a procedure originally reported by Piper et al.[24] Condensation of malononitrile and triethyl orthoacetate afforded 2-amino-6-chloro-3,5-dicarbonitrilo-4-methylpyridine. Reductive dechlorination by hydrogenation at 50 psi using 5% $Pd/BaCO_3$ afforded 2-amino-3,5-dicarbonitrilo-4-methylpyridine. Cyclization with guanidine afforded **18** in 77% yield. Reductive amination of **18** with the appropriate aniline using hydrogen at atmospheric pressure and Raney nickel afforded compounds **5**−**12**. TLC analyses of the products showed the presence of the desired compounds along with a highly fluorescent spot. This highly fluorescent spot has been previously identified[16] as the alcohol resulting from an overreduction of the intermediate aldehyde. Separation of the desired products from this alcohol in each case was accomplished by column chromatography.

N10-Methylation of compounds **7**−**9**, **11**, and **12** was carried out by a modification[17] of a reductive methylation reported by Borch and Hassid,[25] using 37% formaldehyde and $NaCNBH_3$ in acetonitrile adjusted to a pH of 2−3 (1 N HCl). The N-methylated analogues **13**−**17** precipitated in analytically pure form without the necessity of further purification.

Biological Results and Discussion

Compounds **5**−**17** were evaluated as inhibitors of pcDHFR, tgDHFR, and rlDHFR, and the results are listed in Table 1. The tri- and disubstituted analogues are included for comparison, as are TMP, TMQ, and PTX. In the monomethoxy series, substitution at the meta position resulted in marginally higher potency and selectivity against pcDHFR and tgDHFR compared to substitution at either the ortho or para position. Compound **7** had the highest selectivity ratio (10.8) against tgDHFR in the N10-H monomethoxyphenyl 6-substituted 5-methylpyrido[2,3-*d*]pyrimidine series. Comparing the monomethoxy analogues **6**−**8** with the 3′,4′-dimethoxy analogue **3a** shows that removal of a methoxy group results in significantly decreased inhibition against rlDHFR which affords increased selectivity against pcDHFR and tgDHFR. This is consistent with the effect on selectivity observed in the comparison of **1a** and **3a**. Interestingly, the unsubstituted phenyl analogue **5** showed activity very similar to the monomethoxy analogues. Thus, in the N10-H series of 6-substituted 2,4-diamino-5-methylpyrido[2,3-*d*]pyrimidines, sequential removal of a methoxy group from tri-, di-, or monomethoxy-substituted compounds, with few exceptions, results in minor changes in potency against the pathogenic DHFRs; however, selectivities are increased.

In the N10-methylated series, the 3′,4′-dimethoxy analogue **3b** was 25-, 6-, and 34-fold less potent against pcDHFR, rlDHFR, and tgDHFR, respectively, compared to the 3′,4′,5′-trimethoxy analogue **1b**. This substantial decrease in potency was not observed in the N10-methylated monomethoxy compounds **13** and **14** when compared to **3b**. In fact, compound **13** was more potent against all three DHFRs compared to **3b**. Compound **14** showed similar results. The similarity in the comparison of **13** and **14** to **3b** suggests that the position of the methoxy group that was removed was unimportant. Thus, the trend reported by Baccanari[21] for TMP analogues and seen in the comparison of compounds **1b** and **3b** does not extend to the N10-methylated monomethoxyphenyl 6-substituted 2,4-diamino-5-methylpyrido[2,3-*d*]pyrimidines.

In the monochloro series, substitution at the meta position resulted in higher potency against pcDHFR and rlDHFR than substitution at the ortho or para position. Against tgDHFR, the *o*-chloro-substituted analogue **9** was the most potent (except for compound **13**) and the most selective analogue among the monosubstituted series reported in this study. In contrast to the decrease in inhibitory potency observed against pcDHFR of the 3′,4′-dichloro compound **4a** compared to the 3′,4′,5′-trichloro analogue **2a**, removal of a chloro substituent

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

compd	R	R′	pcDHFR	rlDHFR	rl/pc	tgDHFR	rl/tg
1a	H	3′,4′,5′-triOCH$_3$	86	2.1	0.02	7.4	0.3
1b	CH$_3$	3′,4′,5′-triOCH$_3$	13	7.6	0.6	0.85	8.9
2a	H	3′,4′,5′-triCl	63	33	0.5	12	2.8
2b	CH$_3$	3′,4′,5′-triCl	104.5	36.3	0.4	38.1	1
3a	H	3′,4′-diOCH$_3$	44	7.6	0.2	8.8	0.9
3b	CH$_3$	3′,4′-diOCH$_3$	320	44	0.1	29	1.5
3c	CH$_3$	2′,5′-OCH$_3$	216	407	1.9	30.1	13.5
4a	H	3′,4′-diCl	320	53	0.2	28	1.9
4b	CH$_3$	3′,4′-diCl	100	42	0.4	27	1.6
5	H	H	80	170	2.1	17	10
6	H	2′-OCH$_3$	117	169	1.4	23	7.3
7	H	3′-OCH$_3$	68.9	80.1	1.2	7.4	10.8
8	H	4′-OCH$_3$	95.4	55.6	0.6	12	4.6
9	H	2′-Cl	47	88	1.9	7.1	12.4
10	H	3′-Cl	23	37	1.6	11	3.4
11	H	4′-Cl	55.4	51	0.9	19	2.7
12	H	4′-Br	80.8	34.9	0.4	9.5	3.7
13	CH$_3$	3′-OCH$_3$	30	18	0.6	6.3	2.9
14	CH$_3$	4′-OCH$_3$	35	13	0.4	7.3	1.8
15	CH$_3$	2′-Cl	84	100	1.2	18	5.6
16	CH$_3$	4′-Cl	29	26	0.9	5.4	4.8
17	CH$_3$	4′-Br	37	36	1	30	1.2
TMP[b]			12000	133000	11.1	2700	49
TMQ[b]			42	3.0	0.07	10	0.3
PTX[b]			38	1.5	0.04	11	0.14

[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.[4] [b] These values are from ref 19. Most recent quality control values for rlDHFR are 121 400 ± 7 500 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.

from **4a** to afford the monochloro-substituted analogues **9**−**11** resulted in a 6−14-fold increase in potency against pcDHFR. The removal of one chloro group had a variable effect on the potency against rlDHFR, and only **9** showed a significant increase in potency and selectivity against tgDHFR compared to **4a**. Replacement of chlorine with bromine at the para position resulted in minimal effect on potency as well as on the selectivity for pcDHFR and tgDHFR.

In the N10-methylated monohalo series, the 2′-chloro compound **15** was equipotent against pcDHFR and tgDHFR and less potent against rlDHFR compared to the 3′,4′-dichloro **4b** and was more selective than **4b** against pcDHFR and tgDHFR. Compound **16** was more potent against all three DHFRs compared to compound **4b**. In both the N10-H and N10-methylated chloro series, comparison of the inhibitory activity of the di- and monochloro-substituted compounds showed that the removal of one chloro group increased potency against pcDHFR and tgDHFR and had little effect on rlDHFR. Thus, the monosubstituted compounds **9**−**11** and **15**−**16** had higher selectivity ratios compared to compounds **4a** and **4b**, respectively.

In light of their significant tgDHFR inhibitory potencies and selectivities, compounds **5**, **7**, and **9** were evaluated as inhibitors of the growth of *T. gondii* cells in culture,[19] and the results are reported in Table 2. Compounds **1b** and **3c** are also included for comparison. The N10-methylated 3′,4′,5′-trimethoxy compound **1b** was 35-fold more potent against tgDHFR compared to the N10-methylated 2′,5′-dimethoxy compound **3c**. However, **3c** was 4-fold more potent against the growth of *T. gondii* cells in culture compared to compound **1b**. Thus, compound **3c** had a much lower culture-to-enzyme inhibitory ratio than compound **1b**. It was hypothesized

Table 2. Inhibition Concentrations (IC_{50}, nM) of DHFR and Cell Growth in Culture from *T. gondii*, Culture versus Enzyme Ratios, and Calculated Log $k_{o/w}$ for Selected Analogues[a,b]

compd	tgDHFR	tg cell culture	culture/DHFR	log $k_{o/w}$
1b	0.85	4,700	5529	1.76
3c	30.1	1,050	35	2.42
5	17	317	19	1.79
7	7.4	371	50	2.14
9	7.1	58	8	2.35

[a] *T. gondii* cell culture inhibition was assessed by measuring the incorporation of [^{3}H]uracil by *T. gondii* cells.[19] [b] Log $K_{o/w}$ values were calculated using the LOGKOW Program, Syracuse Research Corp., Syracuse, NY 13210.[26]

that **1b**, a subnanomolar enzyme inhibitor, was perhaps not penetrating the cell membrane and thus was not an effective inhibitor of *T. gondii* cell growth in culture. The lipophilicity of each compound was calculated using log $K_{o/w}$ (Table 2). Compound **3c** had a higher log $K_{o/w}$ value compared to **1b** indicating a higher degree of lipophilicity. The increased lipophilicity of compound **3c** may have increased cell penetration to afford a lower IC_{50} value against the growth of *T. gondii* cells in culture and hence lowered the culture-to-enzyme IC_{50} ratio compared to **1b**. Compound **7** had a calculated log $K_{o/w}$ value and culture-to-enzyme ratio similar to that of compound **3c**. However, compound **5**, the unsubstituted phenyl analogue, despite having a calculated log $K_{o/w}$ value similar to that of **1b**, showed a 300-fold lower culture-to-enzyme ratio compared to **1b**. In addition, the monochloro-substituted analogue **9**, which was the most potent of the series, had an IC_{50} against *T. gondii* cells in culture of 58 nM. The log $K_{o/w}$ for **9** was 2.35, only slightly higher than that of compound **7**, yet the IC_{50} *T. gondii* culture/IC_{50} tgDHFR ratio for **9** was 8, 6-fold lower than that for **7**. Compound **9** exhibited the highest

Table 3. Cytotoxicity Evaluation (GI_{50}, M) against Selected Tumor CellLines[36]

	8	**11**	**16**
		Leukemia	
CCRF-CEM	5.61×10^{-7}	9.83×10^{-8}	3.01×10^{-7}
HL-60(TB)	$>1.00 \times 10^{-4}$	1.94×10^{-5}	1.10×10^{-7}
K-562	5.63×10^{-8}	$<1.00 \times 10^{-8}$ (65)[b]	2.47×10^{-8}
MOLT-4	4.34×10^{-7}	6.65×10^{-8}	4.22×10^{-7}
SR	8.27×10^{-7}	5.81×10^{-8}	1.39×10^{-7}
		Non-Small-Cell Lung Cancer	
A549/ATCC	2.19×10^{-7}	$<1.00 \times 10^{-8}$ (56)[b]	$<1.00 \times 10^{-8}$ (52)[b]
NCI-H23	8.50×10^{-7}	1.07×10^{-7}	8.04×10^{-7}
NCI-H460	2.78×10^{-7}	NT[a]	8.87×10^{-8}
		Colon Cancer	
COLO 205	9.94×10^{-6}	6.53×10^{-7}	4.77×10^{-8}
HCT-116	4.11×10^{-8}	$<1.00 \times 10^{-8}$ (87)[b]	8.18×10^{-6}
HCT-15	2.19×10^{-7}	2.73×10^{-8}	1.64×10^{-7}
HT29	4.30×10^{-7}	3.93×10^{-8}	1.10×10^{-7}
KM12	1.29×10^{-5}	7.66×10^{-7}	7.52×10^{-7}
SW-620	1.04×10^{-6}	7.00×10^{-8}	1.88×10^{-7}
		CNS Cancer	
SF-268	6.25×10^{-7}	8.58×10^{-8}	4.41×10^{-7}
SF-295	4.37×10^{-7}	2.48×10^{-8}	NT[a]
U251	5.57×10^{-7}	6.36×10^{-8}	2.75×10^{-7}
		Melanoma	
LOX IMVI	6.76×10^{-7}	7.95×10^{-8}	2.06×10^{-7}
M14	4.48×10^{-6}	1.18×10^{-6}	1.13×10^{-6}
SK-MEL-5	3.38×10^{-7}	6.83×10^{-8}	2.77×10^{-7}
UACC-62	3.27×10^{-7}	3.29×10^{-8}	3.53×10^{-7}
		Ovarian Cancer	
IGROV1	6.02×10^{-7}	3.50×10^{-7}	4.80×10^{-7}
OVCAR-8	7.79×10^{-7}	6.22×10^{-8}	5.61×10^{-7}
		Renal Cancer	
786-0	1.74×10^{-7}	2.76×10^{-8}	3.62×10^{-7}
ACHN	4.44×10^{-7}	5.46×10^{-8}	3.33×10^{-7}
SN12C	2.24×10^{-5}	NT[a]	1.99×10^{-6}
		Prostate Cancer	
PC-3	9.84×10^{-7}	1.19×10^{-7}	6.71×10^{-8}
		Breast Cancer	
MDA-MB-231/ATCC	1.21×10^{-7}	$<1.00 \times 10^{-8}$ (64)[b]	7.16×10^{-8}
MDA-MB-435	6.34×10^{-7}	4.32×10^{-8}	1.34×10^{-7}
MDA-N	7.26×10^{-8}	$<1.00 \times 10^{-8}$ (68)[b]	1.18×10^{-5}

[a] NT, not tested. [b] Numbers in parentheses are percent inhibition at 1.00×10^{-8} M.

inhibitory activity against the growth of *T. gondii* cells in culture in this series. The fact that **5** resulted in greater inhibition of the growth of *T. gondii* cells in culture than the more lipophilic compounds **7** and **3c** and that the monochloro-substituted analogue **9,** which was not the most lipophilic, showed the greatest inhibition of the growth of *T. gondii* cells in culture suggests that the calculated log $K_{o/w}$ does not correlate with inhibition of *T. gondii* cells in culture for the 6-substituted 2,4-diamino-5-methylpyrido[2,3-*d*]pyrimidines. The potent inhibitory activity and selectivity against tgDHFR and the low IC_{50} *T. gondii* culture/IC_{50} tgDHFR ratio of compounds **5** and **9** have prompted the selection of **5** and **9** in an in vivo *T. gondii* therapy study in mice.

Compounds **6**−**9**, **11**, and **16** were selected by the NCI[27] for evaluation in an in vitro preclinical antitumor screening program. The ability of these analogues to inhibit the growth of 58 tumor cell lines was measured as GI_{50} values, the concentration required to inhibit the growth of tumor cells in culture by 50% as compared to a control. All of the compounds showed GI_{50} values in the 10^{-7}−10^{-9} M range in at least 20 cell lines. Data for compounds **8**, **11**, and **16** are shown in Table 3. Compound **8**, with a *p*-methoxy substituent on the side chain phenyl ring, was inhibitory at submicromolar concentrations in 24 cell lines. However, compounds containing halogens were better inhibitors of cell growth in specific cell lines in culture. For example, compound **11**, with a *p*-chloro substituent on the side chain phenyl ring, inhibited cell growth by 50% in submicromolar concentrations in 26 cell lines, similar to compound **8**. However, in five cell lines, namely, K-562, A549/ATCC, HCT-116, MDA-MB-231-ATCC, and MDA-N, compound **11** had a GI_{50} value in the nanomolar range. N10-Methylation of compound **11** to afford compound **16** resulted in a decrease in the inhibition of tumor cells in culture.

In summary, this study demonstrates that simple molecular modifications such as removal of a methoxy group, varying the position of the methoxy group on the phenyl ring, replacing an electron-donating methoxy group with an electron-withdrawing chloro group, or adding a N10-methyl moiety have varying effects on the activities and selectivities in the 6-substituted 2,4-diamino-5-methylpyrido[2,3-*d*]pyrimidines. No substantial change in inhibitory potency against pcDHFR or tgDHFR was seen for the monosubstituted analogues as a result of the removal of a methoxy or chloro group from the disubstituted analogues previously reported. In fact, monosubstituted analogues **7** and **9** both showed increased potency against tgDHFR compared to the disubstituted analogues. Compounds **5** and **7** also exhibited higher inhibitory potency against the growth of

T. gondii cells in culture compared to the dimethoxy and trimethoxy analogues reported previously. However, the monochloro-substituted compound **9** (IC_{50} = 58 nM) was the most potent inhibitor of the growth of *T. gondii* cells in culture in this study. Thus, single modifications in the side chain have varying effects on the biological activity, and no predictions of the effect of a single modification on inhibitory potency and/or selectivity can be made. This result is in contrast to that observed for the sequential removal of methoxy groups in TMP analogues.[21] Interestingly, all of the compounds synthesized were more selective than either TMQ or PTX.

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) was 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., Norcross, 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.

Procedure A: General Procedure for the Synthesis of Compounds 5−12. To a solution of the substituted aniline dissolved in 70−80% acetic acid were added the intermediate **18** and Raney Ni. The mixture was hydrogenated at atmospheric pressure and room temperature for 6 h. TLC analysis using solvent A (4:1:3 drops $CHCl_3$:MeOH:NH_4OH) or solvent B (6:1 EtOAc: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 overreduction of the nitrile **18**. 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 a pH of 8 using 1 N Na_2CO_3 and the resulting crude precipitate filtered. The crude product was stirred in hot methanol, the undissolved solids were 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 (gradient: 100% $CHCl_3$ to 80:20 $CHCl_3$:MeOH) or solvent D (3:1 EtOAc:MeOH). Fractions containing pure product (TLC) were pooled and evaporated to afford analytically pure compounds **5**−**12**.

Procedure B: General Procedure for the Synthesis of Compounds 13−17. To a suspension of the 2,4-diamino-5-methyl-6-[(substituted anilino)methyl]pyrido[2,3-*d*]pyrimidine in acetonitrile were added 37% formaldehyde and $NaCNBH_3$. The pH of the mixture was adjusted to 3 using 1 N HCl. After approximately 10 min, a precipitate began to form. TLC analysis of the reaction mixture using solvent A or E (2:1:3 drops $CHCl_3$:MeOH:NH_4OH) showed the presence of starting material and a new product spot. The reaction mixture was stirred for 3 h, followed by overnight refrigeration. The light-yellow precipitate that formed was filtered and washed with H_2O, EtOH, and Et_2O to yield analytically pure compounds **13**−**17**.

2,4-Diamino-5-methyl-6-[(anilino)methyl]pyrido[2,3-*d*]-pyrimidine (5). Compound **5** was synthesized from intermediate **18** (1.00 g, 5.0 mmol), aniline (0.72 g, 7.7 mmol), and Raney Ni (5.0 g) in 250 mL of 75% acetic acid using procedure A and purified by column chromatography using solvent D to afford a light-yellow solid (0.21 g, 14%): mp > 255 °C dec; TLC R_f 0.48 in solvent A; ^{1}H NMR (DMSO-d_6) δ 2.73 (s, 3 H, 5-CH_3), 4.26 (d, 2 H, CH_2), 5.94 (t, 1 H, NH, exch), 6.29 (br, 2 H, 4-NH_2, exch), 6.62 (t, 1 H, 4′-H), 6.70 (d, 2 H, 2′-H and 6′-H), 7.06 (br, 2 H, 2-NH_2, exch), 7.15 (t, 2 H, 3′-H and 5′-H), 8.54 (s, 1 H, 7-H). Anal. ($C_{15}H_{16}N_6$·0.35H_2O) C, H, N.

2,4-Diamino-5-methyl-6-[(2′-methoxyanilino)methyl]-pyrido[2,3-*d*]pyrimidine (6). Compound **6** was synthesized from intermediate **18** (3.00 g, 15.0 mmol), *o*-anisidine (2.73 g, 22 mmol), and Raney Ni (10.0 g) in 500 mL of 75% acetic acid using procedure A and purified by column chromatography using solvent C to afford a yellow solid (0.42 g, 9%): mp > 245 °C dec; TLC R_f 0.41 in solvent A; ^{1}H NMR (DMSO-d_6) δ 2.66 (s, 3 H, 5-CH_3), 3.76 (s, 3 H, OCH_3), 4.27 (d, 2 H, CH_2), 5.09 (t, 1 H, NH, exch), 6.29 (br, 2 H, 4-NH_2, exch), 6.47 (d, 1 H, C_6H_4), 6.53 (t, 1 H, C_6H_4), 6.68 (t, 1 H, C_6H_4) 6.74 (d, 1 H, C_6H_4), 7.01 (br, 2 H, 2-NH_2, exch), 8.42 (s, 1 H, 7-H). Anal. ($C_{16}H_{18}N_6O$·0.5H_2O·0.4MeOH) C, H, N.

2,4-Diamino-5-methyl-6-[(3′-methoxyanilino)methyl]-pyrido[2,3-*d*]pyrimidine (7). Compound **7** was synthesized from intermediate **18** (3.00 g, 15.0 mmol), *m*-anisidine (2.74 g, 22 mmol), and Raney Ni (10.0 g) in 500 mL of 75% acetic acid using procedure A and purified by column chromatography using solvent C to afford a yellow solid (0.23 g, 5%): mp > 278 °C dec; TLC R_f 0.55 in solvent A; IR (KBr) cm^{-1} 3338 (NH_2), 1212, 1163 (Ar−O−C); ^{1}H NMR (DMSO-d_6) δ 2.64 (s, 3 H, 5-CH_3), 3.64 (s, 3 H, OCH_3), 4.17 (d, 2 H, CH_2), 5.90 (t, 1 H, NH, exch), 6.12 (m, 2 H, 4-NH_2, exch and 3 H, C_6H_4), 6.95 (m, 2 H, 2-NH_2, exch and 1 H, C_6H_4), 8.44 (s, 1 H, 7-H). Anal. ($C_{16}H_{18}N_6O$·0.1H_2O·0.2MeOH) C, H, N.

2,4-Diamino-5-methyl-6-[(4′-methoxyanilino)methyl]-pyrido[2,3-*d*]pyrimidine (8). Compound **8** was synthesized from intermediate **18** (3.00 g, 15.0 mmol), *p*-anisidine (2.71 g, 22 mmol), and Raney Ni (10.0 g) in 500 mL of 75% acetic acid using procedure A and purified by column chromatography using solvent D to afford a yellow solid (0.74 g, 16%): mp > 278 °C dec; TLC R_f 0.48 in solvent A; ^{1}H NMR (DMSO-d_6) δ 2.66 (s, 3 H, 5-CH_3), 3.63 (s, 3 H, OCH_3), 4.17 (s, 2 H, CH_2), 5.47 (br, 1 H, NH, exch), 6.20 (d, 2 H, 4-NH_2, exch), 6.66 (dd, 4 H, C_6H_4), 6.98 (br, 2 H, 2-NH_2, exch), 8.44 (s, 1 H, 7-H). Anal. ($C_{16}H_{18}N_6O$·0.6MeOH) C, H, N.

2,4-Diamino-5-methyl-6-[(2′-chloroanilino)methyl]pyrido[2,3-*d*]pyrimidine (9). Compound **9** was synthesized from intermediate **18** (1.50 g, 7.5 mmol), 2-chloroaniline (1.46 g, 11.4 mmol), and Raney Ni (5.0 g) in 250 mL of 80% acetic acid using procedure A and purified by column chromatography using solvent C to afford a yellow solid (0.09 g, 4%): mp > 241 °C dec; TLC R_f 0.37 in solvent A; ^{1}H NMR (DMSO-d_6) δ 2.68 (s, 3 H, 5-CH_3), 4.38 (s, 2 H, CH_2), 5.68 (t, 1 H, NH, exch), 6.22 (m, 2 H, 4-NH_2, exch), 6.69 (d, 1 H, C_6H_4), 6.98 (br, 2 H, 2-NH_2, exch), 7.10 (t, 1 H, C_6H_4), 7.26 (d, 1 H, C_6H_4), 8.43 (s, 1 H, 7-H). Anal. ($C_{15}H_{15}N_6Cl$·0.1H_2O) C, H, N, Cl.

2,4-Diamino-5-methyl-6-[(3′-chloroanilino)methyl]pyrido[2,3-*d*]pyrimidine (10). Compound **10** was synthesized from intermediate **18** (1.00 g, 5.0 mmol), 3-chloroaniline (0.94 g, 7.4 mmol), and Raney Ni (5.0 g) in 250 mL of 80% acetic acid using procedure A and purified by column chromatography using solvent C to afford a yellow solid (0.18 g, 8%): mp > 235 °C dec; TLC R_f 0.48 in solvent A; ^{1}H NMR (DMSO-d_6) δ 2.65 (s, 3 H, 5-CH_3), 4.20 (d, 2 H, CH_2), 6.24 (br, 1 H, NH, exch and 4-NH_2, exch), 6.55 (m, 2 H, C_6H_4), 6.61 (s, 1 H, 2′−H), 7.01 (br, 2 H, 2-NH_2, exch), 7.08 (t, 1 H, 5′-H), 8.47 (s, 1 H, 7-H). Anal. ($C_{15}H_{15}N_6Cl$·0.1H_2O·0.1HCl) C, H, N, Cl.

2,4-Diamino-5-methyl-6-[(4′-chloroanilino)methyl]pyrido[2,3-*d*]pyrimidine (11). Compound **10** was synthesized from intermediate **18** (1.50 g, 7.5 mmol), 4-chloroaniline (4.85 g, 38.0 mmol), and Raney Ni (5.0 g) in 250 mL of 80% acetic acid using procedure A and purified by column chromatography using solvent C to afford a yellow solid (0.20 g, 9%): mp

> 224 °C dec; TLC R_f 0.40 in solvent B; ^{1}H NMR (DMSO-d_6) δ 2.65 (s, 3 H, 5-CH_3), 4.18 (d, 2 H, CH_2), 6.11 (t, 1 H, NH, exch), 6.23 (br, 2 H, 4-NH_2, exch), 6.64 (d, 2 H, 3′-H and 5′-H), 7.00 (br, 2 H, 2-NH_2, exch), 7.10 (d, 2 H, 2′-H and 6′-H), 8.46 (s, 1 H, 7-H). Anal. ($C_{15}H_{15}N_6Cl\cdot0.5H_2O\cdot0.5AcOH$) C, H, N, Cl.

2,4-Diamino-5-methyl-6-[(4′-bromoanilino)methyl]pyrido[2,3-*d*]pyrimidine (12). Compound **12** was synthesized from intermediate **18** (1.50 g, 7.5 mmol), 4-bromoaniline (1.93 g, 11.2 mmol), and Raney Ni (5.0 g) in 300 mL of 80% acetic acid using procedure A and purified by column chromatography using solvent C to yield a yellow solid (0.11 g, 4%): mp > 220 °C dec; TLC R_f 0.44 in solvent B; ^{1}H NMR (DMSO-d_6) δ 2.64 (s, 3 H, 5-CH_3), 4.18 (d, 2 H, CH_2), 6.13 (t, 1 H, NH, exch), 6.21 (br, 2 H, 4-NH_2, exch), 6.59 (d, 2 H, 3′-H and 5′-H), 6.98 (br, 2 H, 2-NH_2, exch), 7.20 (d, 2 H, 2′-H and 6′-H), 8.46 (s, 1 H, 7-H). Anal. ($C_{15}H_{15}N_6Br$) C, H, N, Br.

2,4-Diamino-5-methyl-6-[(*N*-methyl-3′-methoxyanilino)methyl]pyrido[2,3-*d*]pyrimidine (13). Using procedure B, compound **13** was synthesized from compound **7** (0.10 g, 0.32 mmol), 0.1 mL of 37% formaldehyde, and 0.08 g (1.3 mmol) of $NaCNBH_3$ in 5 mL of acetonitrile to yield a light-yellow solid (0.04 g, 41%): mp > 249 °C dec; TLC R_f 0.52 in solvent E; ^{1}H NMR δ 2.65 (s, 3 H, 5-CH_3), 2.94 (s, 3 H, N10-CH_3), 3.69 (s, 3 H, OCH_3), 4.57 (s, 2 H, CH_2), 6.28 (d, 2 H, C_6H_4), 6.36 (d, 1 H, C_6H_4), 7.10 (m, 2 H, 4-NH_2, exch and 1 H, 2′-H), 7.83 (br, 2 H, 2-NH_2, exch), 8.18 (s, 1 H, 7-H). Anal. ($C_{17}H_{20}N_6O\cdot1.0H_2O\cdot0.4CH_3CN$) C, H, N.

2,4-Diamino-5-methyl-6-[(*N*-methyl-4′-methoxyanilino)methyl]pyrido[2,3-*d*]pyrimidine (14). Compound **14** was synthesized from compound **8** (0.10 g, 0.32 mmol), 0.1 mL of 37% formaldehyde, and 0.08 g (1.3 mmol) of $NaCNBH_3$ in 5 mL of acetonitrile using procedure B to yield a light-yellow solid (0.03 g, 32%): mp > 249 °C dec; TLC R_f 0.54 in solvent A; ^{1}H NMR δ 2.67 (s, 3 H, 5-CH_3), 2.82 (s, 3 H, N10-CH_3), 3.66 (s, 3 H, OCH_3), 4.43 (s, 2 H, CH_2), 6.80 (q, 4 H, C_6H_4), 7.14 (br, 2 H, 4-NH_2, exch), 7.88 (br, 2 H, 2-NH_2, exch), 8.20 (s, 1 H, 7-H). Anal. ($C_{17}H_{20}N_6O\cdot0.8H_2O\cdot0.8CH_3CN$) C, H, N.

2,4-Diamino-5-methyl-6-[(*N*-methyl-2′-chloroanilino)methyl]pyrido[2,3-*d*]pyrimidine (15). Compound **15** was synthesized from crude compound **9** (1.43 g), 1.4 mL of 37% formaldehyde, and 0.5 g (8 mmol) of $NaCNBH_3$ in 100 mL of acetonitrile using procedure B to yield a light-yellow solid (0.06 g): mp > 215 °C dec; TLC R_f 0.52 in solvent A; ^{1}H NMR δ 2.62 (s, 3 H, 5-CH_3), 2.67 (s, 3 H, N10-CH_3), 4.24 (s, 2 H, CH_2), 7.03−7.08 (t, 1 H, C_6H_4), 7.15 (br, 2 H, 4-NH_2, exch), 7.27−7.42 (m, 3 H, C_6H_4), 7.84 (br, 2 H, 2-NH_2, exch), 8.56 (s, 1 H, 7-H). Anal. ($C_{16}H_{17}N_6Cl\cdot0.1H_2O\cdot0.6HCl$) C, H, N, Cl.

2,4-Diamino-5-methyl-6-[(*N*-methyl-4′-chloroanilino)methyl]pyrido[2,3-*d*]pyrimidine (16). Compound **16** was synthesized from compound **11** (0.08 g, 0.26 mmol), 0.1 mL of 37% formaldehyde, and 0.05 g (0.8 mmol) of $NaCNBH_3$ in 5 mL of acetonitrile using procedure B to yield a light-yellow solid (0.04 g, 32%): mp > 249 °C dec; TLC R_f 0.49 in solvent A; ^{1}H NMR δ 2.64 (s, 3 H, 5-CH_3), 2.96 (s, 3 H, N10-CH_3), 4.59 (s, 2 H, CH_2), 6.23 (br, 2 H, 4-NH_2, exch), 6.75 (d, 2 H, C_6H_4), 7.11 (br, 2 H, 2-NH_2, exch), 7.18 (d, 2 H, C_6H_4), 8.16 (s, 1 H, 7-H). Anal. ($C_{16}H_{17}N_6Cl\cdot0.45HCl$) C, H, N, Cl.

2,4-Diamino-5-methyl-6-[(*N*-methyl-4′-bromoanilino)-methyl]pyrido[2,3-*d*]pyrimidine (17). Using procedure B, compound **17** was synthesized from compound **12** (0.25 g, 0.7 mmol), 0.23 mL of 37% formaldehyde, and 0.14 g (2.1 mmol) of $NaCNBH_3$ in 15 mL of acetonitrile to yield a light-yellow solid (0.09 g, 28%): mp > 223 °C dec; TLC R_f 0.64 in solvent E; ^{1}H NMR δ 2.67 (s, 3 H, 5-CH_3), 2.97 (s, 3 H, N10-CH_3), 4.63 (s, 2 H, CH_2), 6.70 (d, 2 H, C_6H_4), 7.30 (d, 2 H, C_6H_4), 8.12 (br, 2 H, 4-NH_2, exch), 8.17 (s, 1 H, 7-H), 9.26 (br, 2 H, 2-NH_2, exch). Anal. ($C_{16}H_{17}N_6Br\cdot0.6H_2O$) C, H, N, Br.

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.[4,5] The standard assay 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 tgDHFR and rlDHFR because it stimulated the enzymes 1.4- and 2.63-fold, respectively. KCl was omitted from assays of pcDHFR 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 rlDHFR 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 10−90% 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, 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.

A clinical isolate of *T. gondii* was obtained 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 displayed kinetics of growth in culture that are more advantageous for production of enzymes and is now a standard source of DHFR from *T. gondii*. The material was 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−).[5] Cells were 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 were 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 combined and sonicated, and the 100000*g* supernate was assayed for DHFR; no DHFR activity was 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), phenylmethanesulfonyl fluoride (9 μg/mL), soybean trypsin inhibitor (50 μg/mL), aprotinin (50 μg/mL), and 20 mM 2-mercaptoethanol. This buffer released the cytoplasmic contents of *T. gondii*. The suspension was centrifuged at 100000*g*. The 100000*g* supernates from *T. gondii* prepared in culture contained both DHFR and dihydropteroate synthase (DHPS) activity. The presence of DHPS, which is not found in mam-

malian cells, and the absence of DHFR in the specific host cell line used for culture support the conclusion that the DHFR activity measured arose from *T. gondii*. The preparation was stored in liquid nitrogen without appreciable loss of activity. The yield of DHFR from *T. gondii* cultures was approximately 40 IU/flask.

DHFR from cultured *T. gondii* was 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 agreed closely with the reported values of 0.021 and 0.76 μM.[9] The IC_{50} value for pyrimethamine with DHFR prepared from *T. gondii* harvested from mice was 0.39 μM, a value in close 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.[9]

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 conditions of assay that differed from the Michaelis−Menten model.[27] Curves suggested substrate inhibition at concentrations above that used in the standard assay. The kinetic parameters of rlDHFR and tgDHFR were similar, with half-maximal rates at 11−13 μM dihydrofolic acid, respectively. Others have also suggested that substrate kinetics of the two forms of DHFR are similar.[28] 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*.[28] These kinetic studies and studies using known inhibitors of tgDHFR confirmed that the enzyme from cultured *T. gondii* was similar to preparations previously reported. On the basis of these studies with DHFR, cultured *T. gondii* was used as a standard source for enzyme preparations.

Uracil Incorporation by Cultured *T. gondii*. Uracil is incorporated into nucleic acid by *T. gondii* grown in culture.[29] Because mammalian cells do not avidly incorporate uracil, the incorporation of uracil can be used as an index of growth of *T. gondii* on host cells. For these experiments *T. gondii* was grown on HEL (human embryonic lung) cells with minimum essential medium (MEM) supplemented with glutamine (2 mM), penicillin/streptomycin (100 units/mL and 100 μg/mL, respectively), and fetal bovine serum (10%). HEL cells were grown to confluency in 24-well tissue culture plates using MEM as described above. To inoculate, old medium was removed and replaced with 0.5 mL of fresh medium containing 5×10^4 *T. gondii* tachyzoites obtained from mouse peritoneal fluid or from tissue culture. Four hours after addition of the inoculum, an additional 0.5 mL of medium containing drug or control diluent was added to the wells. Twenty-four hours after inoculation, 1 μCi of tritiated uracil (New England Nuclear) was added to each well and incubation continued for an additional 24 h. At the end of this period, the medium was sampled to determine remaining counts in the supernatant. The cells were dislodged, suspended by agitation, and filtered through glass fiber filters (Whatman) using a well-washing apparatus with a 24-place filter manifold. Each well was washed with cold isotonic saline and added to the filter. The filters were washed with approximately 20 mL of cold isotonic saline, dried overnight, removed to scintillation vials, and counted with Ultima Gold scintillant (Packard).

Isolation of *P. carinii* DHFR. Recombinant pcDHFR was also produced for enzyme assays. The gene sequence was identical to that previously reported.[30] The expression system used pET8C, which employs the T7 RNA polymerase.[31] Host *Escherichia 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} reached 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 pcDHFR has been the standard enzyme used in the screen under contract NO1-Al-35171. Studies with [^{35}S]methionine incorporation have shown this preparation to contain predominately one strong band on autoradiography at a molecular weight corresponding to that of DHFR.

Culture of *P. carinii* for Drug Testing. Compounds were evaluated in short-term culture using inocula from *P. carinii*-infected rat lung and cell cultures of HEL fibroblasts as described.[32−35] Briefly, tissue cultures were prepared using 24-well plates in which HEL cells had been grown to confluency in MEM containing 10% fetal calf serum. They were inoculated with 7×10^5 viable *P. carinii* trophozoites/mL. Inoculum was prepared by grinding *P. carinii*-infected rat lung in MEM, centrifuging at 250*g* to remove fragments of tissue, and counting numbers of organisms in 10-μL samples of supernate with Giemsa staining and fluorescein diacetate/ethidium bromide viability stain. On the basis of these counts, the number of organisms/mL was adjusted by adding MEM to achieve the desired concentration.

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

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