Synthesis and Biological Activities of Tricyclic Conformationally Restricted Tetrahydropyrido Annulated Furo[2,3-*d*]pyrimidines as Inhibitors of Dihydrofolate Reductases[1]

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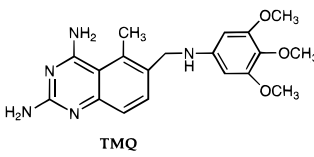
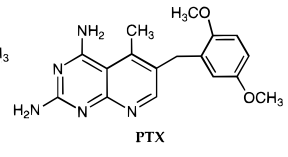
The synthesis of seven 2,4-diamino-5,6,7,8-tetrahydro-7-substituted pyrido[4′,3′:4,5]furo[2,3-*d*]pyrimidines **1**−**6** are reported as nonclassical antifolate inhibitors of dihydrofolate reductase (DHFR) and compound **7** as a classical antifolate inhibitor of tumor cells in culture. The compounds were designed as conformationally restricted analogues of trimetrexate. The synthesis was accomplished from the cyclocondensation of 3-bromo-4-piperidone with 2,4-diamino-6-hydroxypyrimidine to afford regiospecifically 2,4-diamino-5,6,7,8-tetrahydropyrido-[4′,3′:4,5]furo[2,3-*d*]pyrimidine-7-hydrobromide (**16**). This in turn was alkylated with the appropriate benzyl halide to afford the target compounds **1**−**6**. The classical antifolate **7** utilized 4-(chloromethyl)benzoyl-L-glutamic acid diethyl ester (**17**) instead of the benzyl halide for alkylation, followed by saponification to afford **7**. Compounds **1**−**6** showed moderate inhibitory potency against DHFR from *Pneumocystis carinii*, *Toxoplasma gondii*, *Mycobacterium avium*, and rat liver. The classical analogue **7** was 88-fold more potent against *M. avium* DHFR than against rat liver DHFR. The classical analogue was also inhibitory against the growth of tumor cells, CCRF−CEM, and FaDu, in culture.

Infections with *Pneumocystis carinii* and *Toxoplasma gondii* remain the leading cause of death in patients with acquired immunodeficiency syndrome (AIDS).[2] Standard treatment of *P. carinii* pneumonia utilizes pentamidine or sulfonamides with diaminopyrimidine antifolates.[3] Therapy for *T. gondii* infection relies mainly on the antifolate pyrimethamine in combination with sulfonamides.[4] However, all agents currently used to treat *P. carinii* and *T. gondii* infections in AIDS patients cause side effects that are sometimes severe enough to require cessation of therapy.[5,6] The current approach to the treatment of *P. carinii* and *T. gondii* infections with dihydrofolate reductase (DHFR) inhibitors takes advantage of the fact that these organisms are permeable to lipophilic nonclassical antifolates but, unlike mammalian cells, lack a carrier-mediated active transport mechanism for uptake of classical folates with polar glutamate side chains.[7] Thus, in principle, one can selectively protect sensitive host tissues from the toxic effects of nonselective lipophilic antifolates by coadministration (protection) or subsequent administration (rescue) of a reduced folate, leucovorin. Two lipophilic antifolates that have been utilized in the treatment of *P. carinii* and *T. gondii* infections are the potent DHFR inhibitors trimetrexate (TMQ) and piritrexim (PTX).[8−13] A major disadvantage of the therapy of *P. carinii* and *T. gondii* infections with TMQ or PTX is that TMQ and PTX inhibit mammalian DHFR with equal to or greater potency than that displayed toward nonmammalian forms of DHFR.[14] As a result, the use of TMQ is associated with host toxicity including bone marrow depression. Recently approved by the FDA, TMQ along with host protection or rescue by leucovorin has proven to be a viable alternate therapy for *P. carinii* infections in patients with AIDS. However, the high cost of leucovorin, as well as its failure to protect host cells in certain instances, has prompted the search for potent analogues with greater selectivity for *P. carinii* and *T. gondii* DHFR than that of TMQ or PTX.



TMQ



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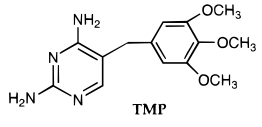
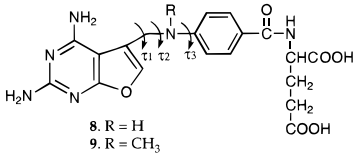
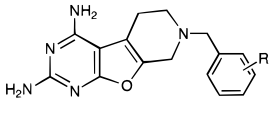


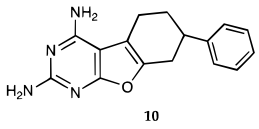
TMP



8. R = H
9. R = CH_3



1. R = H
2. R = 3′,4′,5′ trimethoxy
3. R = 3′,5′ dimethoxy
4. R = 2′,4′ dichloro
5. R = 3′,4′ dichloro
6. R = 2′,6′ dichloro
7. R = 4′-CO-L-glutamic acid



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Efforts in our laboratory have resulted in the synthesis of a variety of bicyclic, lipophilic 6−5 fused furo[2,3-

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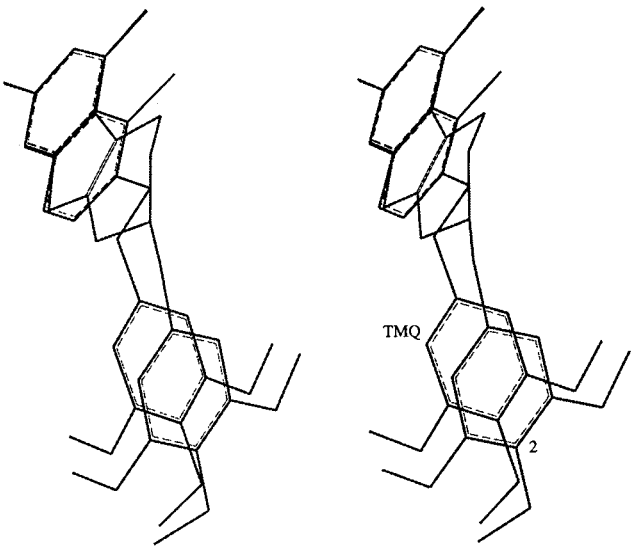


Figure 1. Compound **2** superimposed on hDHFR bound TMQ.

d]pyrimidines,[15] and most recently, the 5-substituted-pyrrolo[2,3-*d*]pyrimidines.[16] These studies strongly suggest that B-ring contracted (6−5 ring systems) analogues are conducive to selectivity for *P. carinii* and *T. gondii* DHFR compared to human DHFR.

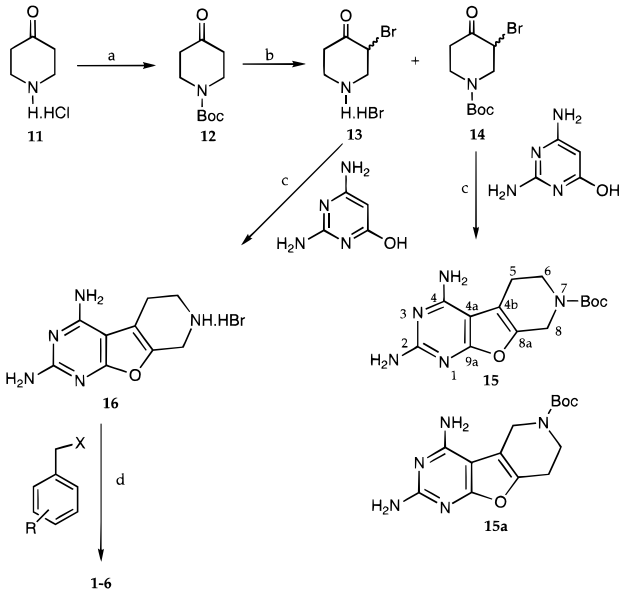
Trimethoprim (TMP) shows selectivity for bacterial DHFR compared to vertebrate DHFR. This selectivity is based, in part, on conformational differences in the DHFR bound orientations of the trimethoxyphenyl side chain of TMP, as elucidated by X-ray crystallographic studies.[17,18,19] The 2,4-diaminopyrimidine ring of TMP binds to *Escherichia coli* DHFR in the same manner as it does to chicken liver DHFR. The trimethoxyphenyl group of TMP, however, occupies a "lower" binding pocket, pointing "downwards" in *E. coli* DHFR, while in chicken liver DHFR, the trimethoxyphenyl group occupies an "upper" binding pocket and is oriented opposite to that in *E. coli* DHFR. The lack of selectivity of TMQ and PTX could arise in part from the conformational flexibility of the side chains which are capable of adopting a variety of different conformations and are thus able to appropriately bind to a variety of DHFR, resulting in potent inhibition without selectivity.

We reasoned that conformational restriction of the C9−N10 bridge of TMQ could result in analogues with a favorable orientation of the lipophilic side chain for selective inhibition of *P. carinii* DHFR and/or *T. gondii* DHFR. Molecular modeling with SYBYL[20] and its SEARCH and MAXIMIN options suggested that analogues of TMQ with the C9−N10 bridge conformationally restricted, by incorporation into a tetrahydropyridine ring fused to a 2,4-diaminofuro[2,3-*d*]pyrimidine, would provide suitably restricted analogues. Superimposition of the energy-minimized conformations of the tetrahydropyrido annulated 2,4-diaminofuro[2,3-*d*]pyrimidines **1**−**6** on the *E. coli* DHFR bound conformation of TMQ[21] with the pyrimidine ring of the target compounds **1**−**6** superimposed on the pyrimidine ring of TMQ showed that the side chains of compounds **1**−**6** overlap reasonably well but are not identical with the side chain of *E. coli* DHFR bound TMQ. This is illustrated for compound **2** in Figure 1. The energy-minimized conformations of **1**−**6**, for the superimposition, were obtained by using the SEARCH and MAXIMIN options of SYBYL with 10° increments of the torsion angles of the flexible side chain. Molecular modeling indicated that the 7-position of attachment of the side chain on the 2,4-diaminotetrahydropyridofuro-[2,3-*d*]pyrimidine, for compounds **1**−**6**, was optimal. Substitutions on the phenyl ring of compounds **1**−**6** were selected on the basis of previous reports from our laboratory[15,16] which showed that methoxy and chloro substituents on the side chain phenyl ring of 2,4-diamino-5-substituted-furo[2,3-*d*]pyrimidines and 5-substituted-pyrrolo[2,3-*d*]pyrimidines are conducive to potent and/or selective inhibition of *P. carinii* and *T. gondii* DHFR. In addition, the clinically used agents TMP and pyrimethamine contain similar substituents. Thus, the design of the conformationally restricted 2,4-diamino-5,6,7,8-tetrahydro-7-(substituted benzyl)pyrido[4',3':4,5]-furo[2,3-*d*]pyrimidines **1**−**6** was based on molecular modeling with the idea that conformational restriction of the bridge would afford orientations of the side chain that are conducive to binding to *P. carinii* and/or *T. gondii* DHFR, which in turn should enhance selectivity.

Gangjee et al.[15] recently synthesized classical antifolate analogues containing a novel furo[2,3-*d*]pyrimidine ring system (**8**, **9**) as inhibitors of DHFR and as antitumor agents. These analogues showed significant inhibitory activities against DHFR from rat liver, (recombinant) human, *P. carinii*, *T. gondii*, and *L. casei*. The most potent inhibition of DHFR was observed with analogue **9** against *P. carinii* DHFR ($IC_{50} = 3.5 \times 10^{-8}$ M). The compounds were also significantly cytotoxic against the growth of a variety of human tumor cell lines in culture. Compound **9** had an $IC_{50} = 4.3 \times 10^{-8}$ M against leukemia CCRF-CEM cells in culture and was about an order of magnitude better than **8**.

Compounds **8** and **9** were both substrates for human folylpolyglutamate synthase (FPGS) from CCRF-CEM cells, and their first-order rate constants were as good as aminopterin (AMT), which is a good substrate for the ligation of the first glutamic acid. The *N*-CH_3 analogue **9** was a more potent inhibitor of DHFR as well as of tumor cells in culture than its *N*-desmethyl analogue **8**. A possible explanation for this difference in activities is that, for the desmethyl analogue **8**, there is greater flexibility in the side chain about τ_1,τ_2, and τ_3, and the benzoyl glutamate moiety could be oriented in a conformation that may not be conducive to optimum binding. In contrast, in the *N*-methyl analogue **9**, the methyl group induces some constraint on the flexibility of τ_2 and τ_3 which may result in a more favorable conformation of the benzoyl glutamate side chain. In addition, the N-9 methyl moiety is known to increase potency against DHFR depending on the nature of the heterocycle as well as the substituents on the side chain phenyl ring.[15] These studies provided the impetus for the design and synthesis of the classical tricyclic compound **7**, as a conformationally restricted analogue of **8** and **9** and as a potential antitumor agent. Molecular modeling studies using SYBYL[20] showed that in tricyclic rigid analogues such as compound **10** (which we have previously synthesized)[22] the orientation of the phenyl side chain is dictated entirely by the conformation of the C ring, and this orientation was not conducive to DHFR binding. Thus we incorporated an additional methylene bridge between the nitrogen in the C ring

Scheme 1[a]



[a] (a) Di-*tert*-butyl dicarbonate, DMF, triethylamine; (b) chloroform/bromine/room temperature; (c) DMF/room temperature; (d) DMSO/potassium carbonate/room temperature.

and the side chain to allow for an appropriate orientation of the *p*-aminobenzoyl-L-glutamic acid in compound **7** as a semirigid analogue of compounds **8** and **9**, in which part of the side chain was incorporated in a third ring thus restricting τ_1 and τ_2 of **8** and **9**.

Chemistry

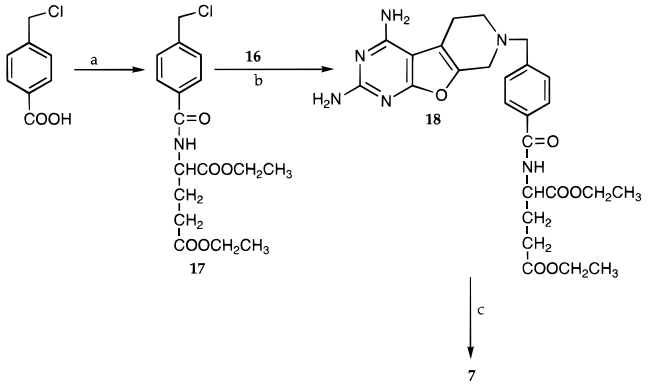
The synthesis of the tricyclic ring system was envisioned via a one-step cyclocondensation of a biselectrophile derived from an appropriate piperidone and 2,4-diamino-6-hydroxypyrimidine, similar to that employed for the synthesis of the bicyclic furo[2,3-*d*]pyrimidines.[15] The synthesis of compounds **1**−**6** is shown in Scheme 1. Protection of the commercially available 4-piperidone hydrochloride **11** using di-*tert*-butyl dicarbonate in dimethylformamide (DMF) at room temperature afforded the *N*-Boc-4-piperidone **12** in 86% yield. Bromination of **12** with 1 equiv of bromine in chloroform at room temperature resulted in a mixture of the 3-bromo-4-piperidone hydrobromide (**13**) as a white precipitate (82% yield) and about 10% of the *N*-Boc brominated product **14**. Condensation of **13** or **14** with 2,6-diamino-4-hydroxypyrimidine in anhydrous DMF at room temperature for 48 h afforded **16** in 58% yield and **15** in 33% yield, respectively. Since this cyclocondensation reaction could occur to afford the tetrahydropyrido annulated 2-amino-4-oxopyrrolo[2,3-*d*]pyrimidine or the tetrahydropyrido annulated 2,4-diaminofuro[2,3-*d*]pyrimidine, it was essential to establish the structure of the product. The reaction in each case afforded a single product. The ^{1}H NMR spectrum in deuterated dimethyl sulfoxide of **15** and **16** indicated the presence of both the 2- and 4-amino protons as singlets, exchangeable with deuterium oxide. This along with the absence of the H5 proton of the pyrimidine and the presence of the methylene protons of the C ring confirmed that the condensation of **13** and **14** with 2,6-diamino-4-hydroxypyrimidine was regiospecific and afforded exclusively the tetrahydropyrido annulated 2,4-diaminofuro[2,3-*d*]-pyrimidines **15** and **16** without any of the 2-amino-4-oxopyrrolo[2,3-*d*]pyrimidines as has been observed for other bicyclic systems.[23] The fact that the furo[2,3-*d*]-pyrimidine had formed rather than the pyrrolo[2,3-*d*]-pyrimidine was supported by ^{1}H NMR data indicated above as well as the observation of Secrist and Liu[23] who established that cyclic α-halo ketones such as α-chlorocyclohexanone regiospecifically afford the 2,4-diaminofuro[2,3-*d*]pyrimidine when cyclocondensed with 2,4-diamino-6-hydroxypyrimidine. In addition, the absence of the characteristic downfield (9−12 ppm) pyrrole NH proton in the ^{1}H NMR along with the presence of the 2- and 4-amino protons confirms that the product was exclusively the 2,4-diamino-5,6,7,8-tetrahydropyridofuro[2,3-*d*]pyrimidine rather than the pyrrolo[2,3-*d*]pyrimidine.

Two positional isomers of the tricyclic pyridofuro[2,3-*d*]pyrimidine are possible in the cyclocondensation of **14** (or **13**) with 2,4-diamino-6-hydroxypyrimidine to afford the desired *N*-Boc-2,4-diamino-5,6,7,8-tetrahydropyrido[4′,3′:4,5]furo[2,3-*d*]pyrimidine regioisomer **15** or the alternate *N*-Boc-2,4-diamino-5,6,7,8-tetrahydropyrido[3′,4′:4,5]furo[2,3-*d*]pyrimidine regioisomer **15a**. Using the ^{13}C chemical shift prediction software,[24] two distinct ^{13}C NMR were predicted for the two isomers **15** and **15a**. The critical differences were that for compound **15** the chemical shift of the 4b carbon was predicted at 109.16 ppm while for **15a** it was predicted at 100.07 ppm, and the ^{13}C shift for the 8a carbon was predicted at 139.03 for **15** and 148.84 for **15a**. In the ^{13}C NMR of the product obtained, the ^{13}C chemical shift for the 4b carbon was at 110.08 ppm, and there was no signal around 100.07 ppm; the next high field signal was at 91.90 ppm. Further, the 8a carbon was observed at 140.31 ppm in accord with the predicted value of 139.03 ppm for **15**, and there was no signal in the vicinity of 148.84 ppm predicted for **15a**. This supports the structure of the product as **15** rather than **15a**.

To further establish the structure of **15** as distinct from **15a**, a NOESY was performed between the 4-NH_2 and the 5-CH_2. In structure **15a** this NOESY would be expected to occur with the most downfield CH_2 moiety which would be the 5-CH_2. However, in **15** this 5-CH_2 would not be the most downfield CH_2. The NOESY for the product obtained shows a cross peak between the 4-NH_2 and the CH_2 (3.58 ppm) which is not the most downfield CH_2 thus establishing **15** rather than **15a** as the structure of the regiospecific product. In addition, a CH correlation via long-range coupling (or COLOC) experiment was performed which indicates CH relationships through both two and three bonds ($^2J_{CH}$ and $^3J_{CH}$) applicable to quaternary carbon atoms. Such a COLOC was observed with the 4b carbon at 110.08 and all three CH_2 moieties of the tetrahydropyrido ring, indicating a two or three bond relationship. Such a relationship for all three CH_2s would be absent for the structure **15a** since the 7-CH_2 protons would be four bonds away and would not afford a COLOC with C4b, thus confirming the structure of the product as **15**.

Having established the structure of **15** and hence the regiospecific nature of the cyclocondensation of **14** (and hence **13**) with 2,4-diamino-6-hydroxypyrimidine, we turned our attention to the synthesis of the target compounds. Stirring **16** in anhydrous DMSO with 2

Scheme 2[a]



[a] (a) DMF, isobutyl chloroformate, triethylamine, L-glutamic acid diethyl ester; (b) DMSO/potassium carbonate/room temperature; (c) methoxyethanol, 1 N NaOH.

equiv of anhydrous potassium carbonate and the appropriate benzyl halide for 48−72 h at room temperature afforded the desired products **1**−**6**. The classical analogue was synthesized in a manner similar to that employed for the synthesis of **1**−**6** using 4-(chloromethyl)benzoyl-L-glutamic acid diethyl ester **17** instead of the benzyl halide (Scheme 2). The isolation of the products was greatly simplified by adding an excess of water to the reaction mixture and stirring at room temperature for 1−3 h, which allows the products to separate. Chromatographic purification afforded analytically pure target compounds **1**−**7** in yields ranging from 38 to 52%. The diester **17** was in turn prepared by coupling 4-(chloromethyl)benzoic acid with glutamic acid diethyl ester using the mixed anhydride method and triethylamine as the base.[15]

Biological Evaluation and Discussion

Nonclassical analogues **1**−**6** were evaluated as inhibitors of DHFR from *P. carinii* (pcDHFR), *T. gondii* (tgDHFR), and rat liver (rlDHFR).[25,26] The inhibitory potencies (IC_{50}) are listed in Table 1. Surprisingly, analogues **1**−**6** were weak inhibitors of these DHFR with IC_{50} values ranging from 1.4 to >35 μM. There are three possible explanations for the lack of DHFR inhibitory activity of theses analogues: first, that protonation of the basic alicyclic nitrogen in the C ring, at physiological pH, may result in a positive charge on the nitrogen which is not conducive to DHFR binding; second, the tricyclic ring system may be too rigid and/or bulky to allow for optimum orientation of the phenyl side chain upon binding to DHFR; third, the distance between the lipophilic side chain and the 5-position of the bicyclic furo[2,3-*d*]pyrimidine ring system may be one carbon atom too long, which does not permit proper interaction of the substituted side chain with appropriate portions of DHFR. A study involving the synthesis of additional analogues is currently underway to circumvent the above possible reasons for the inactivity of compounds **1**−**6**. The classical analogue **7** also showed weak inhibitory activity against pcDHFR and tgDHFR. Compound **7** did however display selectivity ratios of 7.9 and 4 against pcDHFR and tgDHFR, respectively, versus rlDHFR.

Compounds **1**−**7** were also evaluated against DHFR from *Mycobacterium avium*, an agent of opportunistic infection in HIV-infected patients. Compound **7** showed significant inhibitory activity against *M. avium* DHFR and has a selectivity ratio of 88 versus rlDHFR. This encouraging result suggested that such tricyclic analogues may be selective agents against *M. avium*. However, the nonclassical analogues **1**−**6** were poor inhibitors of *M. avium* DHFR. Nevertheless, the significant selectivity of **7** for *M. avium* DHFR allows it to function as a lead compound for structural modifications, which are currently underway.

The ability of **7** to inhibit human CCRF-CEM DHFR was also determined. Compound **7** (IC_{50} = 11.8 ± 3.3 μM) was more than 10^4-fold less potent than methotrexate (MTX) (IC_{50} = 0.7 ± 1 nM) against CCRF-CEM DHFR.

The activity of the classical analogue **7** was compared to MTX as inhibitors of the growth of CCRF-CEM human leukemia and FaDu squamous cell carcinoma of the head and neck cell lines during continuous exposure in vitro (Table 2). Both parent cell lines are similarly sensitive to MTX under these conditions. Compound **7** was 200-fold less potent than MTX as a growth inhibitor of CCRF-CEM cells and >5000-fold less potent than MTX against FaDu.

Compound **7** was also tested as a growth inhibitor of two CCRF-CEM sublines with defined mechanisms of MTX resistance (Table 2). R30dm[27] has 1% of the parental FPGS level; it is not MTX resistant in continuous exposure (resistance is only seen in intermittent exposure) because the nonpolyglutamylated parent drug is a tight-binding DHFR inhibitor and transport is sufficient to maintain drug in excess of DHFR. However, on continuous exposure to **7**, R30dm is highly cross resistant, indicating that polyglutamylation must be essential to its mechanism of action. It is well documented that some classical antifolates that inhibit thymidylate synthase (TS) have significant cross resistance to R30dm.[28] This result is attributed to the necessity of these classical antifolates to be polyglutamylated in order to exert their antitumor activity. R1,[29] with amplified DHFR expression, displayed 40-fold resistance to MTX under continuous exposure. R1 is only about 3-fold cross resistant to **7**. Thus DHFR does not appear to be the primary target of **7** despite its 2,4-diamino structure, since it is a weak DHFR inhibitor and a DHFR-amplified cell line is only minimally cross resistant to this agent.

Growth inhibition of CCRF-CEM cells in culture by MTX (EC_{96}) and **7** (EC_{91}) are completely protected by the simultaneous presence of 10 μM leucovorin (data not shown). This indicates that the inhibitory effects of **7** appear to be antifolate in nature.

Most classical antifolates (which contain a *p*-aminobenzoyl-L-glutamate) are substrates for human folylpolyglutamate synthetase (FPGS), although their relative substrate activity is a function of their structure. Gangjee et al.[15,30] have shown that furo[2,3-*d*]pyrimidine analogues containing CH_2NH, $CH_2N(CH_3)$, CH_2-CH_2, and CH_2NHCH_2 bridges from the 5-position of the furo[2,3-d]pyrimidine to the *p*-benzoylglutamate moiety serve as efficient FPGS substrates. Since compound **7** showed significant cross resistance to R30dm cell line (Table 3), its activity as a substrate for human FPGS was evaluated and compared to that of aminop-

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

compd	*P. carinii*	rat liver	rl/pc	*T. gondii*	rl/tg	*M. avium*	rl/ma
1	>9.0	15.0	ND	1.4	>10.0	>15.0	ND
2	>35.0	>35.0	ND	>35.0	ND	12.1	>2.9
3	>22.8	22.8	ND	>20.4	ND	>20.0	ND
4	22.6	50.9	2.3	13.1	3.9	>50.0	ND
5	24.1	20.6	0.9	22.3	ND	>20.0	ND
6	40.5	81.2	2.0	31.7	2.5	>81.2	ND
7[a]	10.9	85.8	7.9	21.5	4.0	0.97	88.0
8[b]	0.90	1.3	1.4	0.70	1.9	NT	
9[b]	0.035	0.43	12.2	19.8	0.021	NT	
TMP	12.0[c]	133.0[c]	11.1	2.7[d]	49.0		
TMQ	0.042[c]	0.003[d]	0.01	0.01d	0.30		
MTX	0.001[c]	0.003[d]	0.014[d]	0.014	0.21		

[a] IC_{50} against CCRF-CEM DHFR = 11.8 μM. [b] Data from ref 15. [c] Data from ref 25. [d] Data from ref 26. ND = not done, NT = not tested.

Table 2. Growth Inhibition (EC_{50}, nM) of Parental CCRF−CEM, Sublines with Single, Defined Mechanism of MTX Resistance, and FaDu Human Squamous Cell Carcinoma of the Head and Neck during Continuous (0−120 h) Exposure to MTX and **7**[a]

drug	CCRF-CEM	CEM/R30dm[b] ↓(Glu_n)	CEM/R1c[c] ↑(DHFR)	FaDu
MTX	14.3 ± 1.11 (n = 12)	8.0 ± 2.7 (n = 5)	567 ± 29 (n = 3)	17.2 ± 1.5 (n = 5)
7	3600 ± 1200 (n = 7)	>50000 (n = 2)	10300 ± 5400 (n = 3)	>100000 (n = 2)

[a] Average values are presented ± range for n = 2 and ± SD for $n \geq 3$. [b] CCRF-CEM sublines resistant to MTX solely as a result of decreased polyglutamylation; this cell line has 1% of the FPGS specific activity (measured with MTX as the folate substrate) of parental CCRF-CEM.[27] [c] CCRF-CEM subline resistant to MTX solely as a result of a 20-fold increase in wild-type DHFR protein and activity.[28]

terin (AMT), which is a good substrate for ligation of the first γ-glutamic acid (Table 3). Although compound **7** is a less efficient FPGS substrate than AMT, polyglutamylation must still be considered as a potential component of its mechanism of action. The current findings show that replacement of simple bridges (CH_2-NH, $CH_2N(CH_3)$, CH_2CH_2, and CH_2NHCH_2) from the 5-position of furo[2,3-*d*]pyrimidine with a constrained annular bridge involving the 5- and 6-positions allows the analogue to retain FPGS substrate activity with a K_m similar to those of the open chain bicyclic analogues and only a slight decrease in V_{max}. These results show that the substrate binding site of FPGS tolerates significant bulk in the so-called C-region,[31] as has been suggested based on the excellent substrate activity of BW1843U89.[32] The importance of polyglutamate metabolism of compound **7** to its mechanism is underscored by the cross resistance of a cell line with decreased FPGS activity (Table 2); in this regard it is similar to our earlier reported furo[2,3-*d*]pyrimidine analogues.[15]

Experimental Section

Melting points were determined on a Mel-Temp II melting point apparatus and are uncorrected. Nuclear magnetic resonance spectra for ^{1}H NMR were recorded on a Bruker WH-300 (300 MHz) spectrometer, and the chemical shifts are presented relative to TMS as the internal standard. Chemical shifts listed for multiplets were measured from the approximate centers, and relative integrals of peak areas agreed with those expected for the assigned structures. Thin-layer chromatography (TLC) was performed on POLYGRAM Sil G/UV254 silica gel plates with fluorescent indicator, and the spots were visualized under 254 and 366 nm illumination. Proportions of solvents used for TLC are by volume. Elemental analyses were performed by Atlantic Microlabs Inc., Norcross, GA. Analytical results indicated by an element symbol are within ±0.4% of the calculated values. Fractional moles of water frequently found in some analytical samples of antifolates were not removed despite 24−48 h of drying in vacuum and were confirmed, where possible, by their presence in the ^{1}H NMR spectrum.

Table 3. Activity of **7** as Substrate for CCRF-CEM Human Leukemia Cell Folylpolyglutamate Synthetase[a]

substrate	K_m, μM	$V_{max(rel)}$	$V_{max(rel)}/K_m$	n
aminopterin	4.8 ± 0.7	1	0.21	6
7	6.2 ± 1.4	0.29 ± 0.05	0.05	2

[a] FPGS substrate activity was determined as described in the Experimental Section. Values presented are average ± SD if $n \geq 3$ and average ± range for n = 2. V_{max} are calculated relative to aminopterin within the same experiment.

***N*-(Butoxycarbonyl)-4-piperidone (12).** 4-Piperidone hydrochloride monohydrate **11** (2.0 g, 13.0 mmol) was dissolved in 30 mL of *N*,*N*-dimethylformamide (DMF) at 110−115 °C. The solution was cooled to room temperature, and to it was added triethylamine (2.60 g, 26.0 mmol) and a solution of di-*tert*-butyl dicarbonate (3.06 g, 14.0 mmol) in 10 mL of DMF. The mixture was stirred for 24 h at room temperature. The DMF was removed under reduced pressure. Water (100 mL) was added to the residue, the mixture was extracted with 2 × 100 mL of ethyl ether, the organic layer was dried ($MgSO_4$) and filtered, and ether was removed under reduced pressure to afford 2.33 g (90%) of **12** as a white powder: mp 113−115 °C; ^{1}H NMR ($CDCl_3$) δ 1.48 (s, 9 H, Boc), 2.41 (t, 4 H, 2-CH_2, 6-CH_2), 3.67 (t, 4 H, 3-CH_2, 5-CH_2).

3-Bromo-4-piperidone Hydrobromide (13). *N*-(Butoxycarbonyl)-4-piperidone **12** (2.60 g, 13.0 mmol) was dissolved in 70 mL of chloroform. To it was added Br_2 (2.08 g, 13.0 mmol) over a period of 30 min. The reaction was continued for 2 h at room temperature during which time a white precipitate of **13** was formed. The reaction mixture was filtered and the solid washed with chloroform and ether to afford 2.76 g (82%) of **13**: mp 200−201 °C; ^{1}H NMR (Me_2SO-d_6) spectrum was complex due to interference from DMSO-d_6 and water (due to the hygroscopic nature of the product). However, the proton α to the carbonyl and the bromine was observed at δ 5.09 ppm. In addition, the spectrum showed the absence of the Boc group. This compound was unstable for long durations at room temperature and was used in the next step without further purification.

2,4-Diamino-5,6,7,8-tetrahydropyrido[4′,3′:4,5]furo[2,3-*d*]pyrimidine-7-Hydrobromide (16). To a suspension of 2,4-diamino-6-hydroxypyrimidine (0.50 g, 4.0 mmol) in 3 mL of anhydrous DMF under nitrogen was added dropwise a solution of **13** (1.83 g, 4.0 mmol) in 10 mL of anhydrous DMF. The reaction became a clear solution after 1 h and was left at room temperature for 48 h. The white precipitate which formed was collected by filtration and air-dried to afford 0.66 g (58%) of **16**: mp 302−304 °C; ^{1}H NMR (Me_2SO-d_6) spectrum indicated a mixture of two hydrobromide salts, δ 2.73−2.97

(overlapped m, 4 H, 6-CH_2), 3.40, (overlapped m, 4 H, 5-CH_2), 4.18, 4.29 (overlapped m, 4 H, 8-CH_2), 6.24 (br s, 2 H, 4-NH_2), 8.11 (br s, 2 H, 4-NH_2), 8.90 (br s, 2 H, 2-NH_2), 9.34 (br s, 2 H, 2-NH_2), 10.42 (s, 1 H, N−H), 11.09 (s, H, N−H). Anal. Calcd for ($C_9H_{11}N_5O$·1.5HBr·0.3H_2O) C, H, N, Br.

2,4-Diamino-5,6,7,8-tetrahydro-7-(butoxycarbonyl)pyrido[4′,3′:4,5]furo[2,3-*d*]pyrimidine (15). The filtrate obtained in the synthesis of **13** was diluted with 150 mL of chloroform and washed with water, saturated sodium bicarbonate, and then brine. The organic layer was dried over anhydrous $MgSO_4$ and filtered and the chloroform removed under reduced pressure to afford a viscous brown oil. The residue was immediately dissolved in 5 mL of anhydrous DMF and added to a suspension of 2,4-diamino-6-hydroxypyrimidine (0.25 g, 2.0 mmol) in anhydrous DMF. The mixture was stirred for 48 h at room temperature. The DMF was removed under reduced pressure, the residue was dissolved in 50 mL of methanol, 1.70 g of silica gel was added, and the mixture was evaporated to dryness under reduced pressure. Ethyl ether (50 mL) was added to the silica gel plug and the plug collected after filtration was air-dried and placed on top of a dry silica gel column (1.5 × 10 cm) and eluted with $CHCl_3$/MeOH (15:1). Fractions containing the desired product were pooled and evaporated to dryness under reduced pressure to afford 0.03 g (33%) of **15**: mp 246−248 °C; TLC R_f 0.63 ($CHCl_3$/MeOH, 9:1, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 1.43 (s, 9 H, Boc), 2.67, (t, 2 H, 6-CH_2), 3.58 (t, 2 H, 5-CH_2), 4.37 (s, 2 H, 8-CH_2), 5.99 (s, 2 H, 4-NH_2), 6.43 (s, 2 H, 2-NH_2); ^{13}C NMR (125 MHz, Me_2SO-d_6) δ 21.5 (C5), 28.0 (Boc CH_3), 41.7 (C8 or C6), 42.9 (C6 or C8), 79.5 (Boc C), 91.9 (C4a), 110.1 (C4b), 140.3 (C8a), 155.0 (C4), 158.4 (C2 or Boc-CO), 160 (Boc-CO or C2), 168 (C9a). Anal. Calcd for ($C_{14}H_{19}N_5O_3$) C, H, N.

2,4-Diamino-5,6,7,8-tetrahydro-7-benzylpyrido[4′,3′:4,5]furo[2,3-*d*]pyrimidine (1). Compound **16** (0.46 g, 1.40 mmol) was suspended in 5 mL of anhydrous DMSO. To it was added anhydrous potassium carbonate (0.48 g, 3.50 mmol) and benzyl bromide (0.24 g, 1.40 mmol) and the mixture stirred for 24 h at room temperature. The mixture was then diluted with water (30 mL) and stirred for an additional 24 h at room temperature. The resulting precipitate was collected by filtration, washed with water, acetone, and ether, and air-dried. The crude solid was dissolved in a mixture of DMF:MeOH (1:5), 1.20 g of silica gel was added, and the mixture was evaporated to dryness under reduced pressure. The resulting silica gel plug was placed on the top of a dry silica gel column (1.5 × 10 cm) and eluted with MeOH in $CHCl_3$. The fractions containing the desired product (TLC) were pooled and evaporated to dryness under reduced pressure. The resulting solid was triturated with ether and filtered to afford 0.16 g (38%) of **1** as a white powder: mp 233−235 °C; TLC R_f 0.55 ($CHCl_3$/MeOH, 2:1, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 2.70 (overlapped m, 4 H, 5-CH_2, 6-CH_2), 3.43 (s, 2 H, 8-CH_2), 3.70 (s, 2 H, benzylic CH_2), 5.91 (s, 2 H, 4-NH_2), 6.35 (s, 2H, 2-NH_2); 7.29 (m, 5 H, phenyl). Anal. Calcd for ($C_{16}H_{17}N_5O$) C, H, N.

2,4-Diamino-5,6,7,8-tetrahydro-7-(3′,4′,5′-trimethoxybenzyl)pyrido[4′,3′:4,5]furo[2,3-*d*]pyrimidine (2). This compound was prepared and purified in a manner similar to that described for **1** using 3′,4′,5′- trimethoxybenzyl chloride, instead of benzyl bromide, to afford 0.12 g (44%) of **2** as a yellow solid: mp 268−270 °C; TLC R_f 0.60 ($CHCl_3$/MeOH, 2:1, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 2.70 (overlapped m, 4 H, 5-CH_2, 6-CH_2), 3.47 (s, 2 H, 8-CH_2), 3.65 (overlapped m, 5 H, benzylic-CH_2, 4′-OCH_3), 3.76 (s, 6 H, 3′,5′-OCH_3), 5.91(s, 2 H, 4-NH_2), 6.35 (s, 2 H, 2-NH_2), 6.66 (s, 2 H, phenyl). Anal. Calcd for ($C_{19}H_{23}N_5O_4$·0.3H_2O) C, H, N.

2,4-Diamino-5,6,7,8-tetrahydro-7-(3′,5′-dimethoxybenzyl)pyrido[4′,3′:4,5] furo[2,3-*d*]pyrimidine (3). This compound was prepared and purified in a manner similar to that described for **1** using 3′,5′-dimethoxybenzyl chloride, instead of benzyl bromide, to afford 0.13 g (52%) of **3**: mp 228−230 °C; TLC R_f 0.48 ($CHCl_3$/MeOH, 4:1, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 2.69 (overlapped m, 4 H, 5-CH_2, 6-CH_2), 3.45 (s, 2 H, 8-CH_2), 3.63 (s, 2 H, benzylic CH_2), 3.73 (s, 6H, 3′,5′-OCH_3), 5.93 (s, 2 H, 4-NH_2), 6.40 (m, 3 H, 2-NH_2, 4′-H), 6.52 (d, 2 H, 2′,6′-H). Anal. Calcd for ($C_{18}H_{21}N_5O_3$·0.06H_2O) C, H, N.

2,4-Diamino-5,6,7,8-tetrahydro-7-(2′,4′-dichloro-benzyl)-pyrido-[4′,3′:4,5]furo[2,3-*d*]pyrimidine (4). This compound was prepared and purified in a manner similar to that described for **1** using 2′,4′-dichlorobenzyl chloride, instead of benzyl bromide, to afford 0.15 g (48%) of **4**: mp 265−267 °C; TLC R_f 0.66 ($CHCl_3$/MeOH, 2:1, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 2.74 (overlapped m, 4 H, 5-CH_2, 6-CH_2), 3.52 (s, 2 H, 8-CH_2), 3.78 (s, 2 H, benzylic-CH_2), 5.93 (s, 2 H, 4-NH_2), 6.38 (s, 2 H, 2-NH_2), 7.44 (m, 1 H, 6′-H), 7.57 (m, 2 H, 3′-H, 5′-H). Anal. Calcd for ($C_{16}H_{15}N_5O$ Cl_2) C, H, N, Cl.

2,4-Diamino-5,6,7,8-tetrahydro-7-(3′,4′-dichlorobenzyl)-pyrido[4′,3′:4,5]furo[2,3-*d*]pyrimidine (5). This compound was prepared and purified in a manner similar to that described for **1** using 3′,4′-dichlorobenzyl chloride, instead of benzyl bromide, to afford 0.13 g (45%) of **5**: mp 275−278 °C; TLC R_f 0.64 ($CHCl_3$/MeOH, 2:1, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 2.71 (overlapped m, 4 H, 5-CH_2, 6-CH_2), 3.32 (s, 2 H, 8-CH_2), 3.71 (s, 2 H, benzylic CH_2), 5.93 (s, 2 H, 4-NH_2), 6.37 (s, 2 H, 2-NH_2), 7.36 (d, 1 H, 6′-H), 7.59 (m, 2 H, 2′-H, 5′-H). Anal. Calcd for ($C_{16}H_{15}N_5O$ Cl_2) C, H, N, Cl.

2,4-Diamino-5,6,7,8-tetrahydro-7-(2′,6′-dichlorobenzyl)-pyrido[4′,3′:4,5]furo[2,3-*d*]pyrimidine (6). This compound was prepared and purified in a manner similar to that described for **1** using 2′,6′-dichlorobenzyl chloride, instead of benzyl bromide, to afford 0.10 g (39%) of **6**: mp 280−282 °C; TLC R_f ($CHCl_3$/MeOH, 2:1, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 2.71 (overlapped m, 4 H, 5-CH_2, 6-CH_2), 3.43 (s, 2 H, 8-CH_2), 3.71 (s, 2 H, benzylic CH_2), 5.93 (s, 2 H, 4-NH_2), 6.37 (s, 2 H, 2-NH_2), 7.38 (m, 1 H, 4′-H), 7.61 (m, 2 H, 3′,5′-H). Anal. Calcd for ($C_{16}H_{15}N_5OCl_2$·0.1H_2O) C, H, N, Cl.

2,4-Diamino-5,6,7,8-tetrahydro-7-(4′-benzoyl-L-glutamic acid diethyl ester)pyrido[4′,3′:4,5]furo[2,3-*d*]pyrimidine (18). This compound was prepared and purified in a manner similar to that described for **1** using 4′-(chloromethyl)-benzoylglutamic acid diethyl ester **17** instead of benzyl bromide to afford 0.12 g (47%) of **18**: mp 280−282 °C; TLC R_f 0.62 ($CHCl_3$/MeOH, 2:1, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 1.14 (m, 6 H, CH_3), 2.08 (m, 2 H, Gluβ-CH_2), 2.42 (m, 2 H, Gluγ-CH_2), 2.70 (m, 4 H, 5-CH_2, 6-CH_2), 3.46 (s, 2 H, 8-CH_2), 3.76 (s, 2 H, benzyl-CH_2), 4.01 (overlap m, 4 H, CH_2), 4.43 (m, 1 H, Gluα-CH), 5.91 (s, 2 H, 4-NH_2), 6.35 (s, 2 H, 2-NH_2), 7.45 (d, 2 H, 3′-,5′-CH), 7.84 (d, 2 H, 2′,6′-CH), 8.68 (d, 1H, NH). Anal. Calcd for ($C_{26}H_{32}N_6O_6$) C, H, N.

2,4-Diamino-5,6,7,8-tetrahydro-7-(4′-benzoyl-L-glutamic acid)-pyrido [4′,3′:4,5]furo[2,3-*d*]pyrimidine (7). To a solution of **18** (0.18 g, 0.35 mmol) in methoxyethanol (10 mL) was added 1.5 mL of 1 N NaOH, and the solution was stirred at room temperature for 24 h. The methoxyethanol was evaporated under reduced pressure, the residue was dissolved in water (10 mL), and stirring was continued for an additional 24 h. The solution was then cooled in an ice bath and the pH adjusted to 3.5 via the dropwise addition of 1 N HCl. The precipitate that formed was collected by filtration, washed with water, acetone, and ether, and air-dried to afford 0.16 g (99%) of **7** as a pale yellow solid: mp >280 °C; TLC R_f 0.70 (3% NH_4HCO_3, cellulose); ^{1}H NMR (Me_2SO-d_6) δ 1.96 (m, 2 H, Gluβ-CH_2), 2.36 (m, 2 H, Gluγ-CH_2), 2.70 (m, 4 H, 5-CH_2, 6-CH_2), 3.46 (s, 2 H, 8-CH_2), 4.01 (s, 2 H, benzyl-CH_2), 4.43 (m, 1 H, Gluα-CH), 5.91 (s, 2 H, 4-NH_2), 6.35 (s, 2 H, 2-NH_2), 7.45 (d, 2 H, 3′-,5′-CH), 7.84 (d, 2 H, 2′-,6′-CH), 8.68 (d, 1 H, CONH), 12.37 (br s, 2H, COOH). Anal. Calcd for ($C_{22}H_{24}N_6O_6$·0.5H_2O) C, H, N.

4-(Chloromethyl)benzoyl-L-glutamic Acid Diethyl Ester (17). 4-(Chloromethyl)benzoic acid (2.0 g, 11.70 mmol) was dissolved in anhydrous DMF and stirred under nitrogen at 0 °C for 10 min. To this solution was added triethylamine (2.38 g, 23.40 mmol), and the mixture was stirred for an additional 15 min. A solution of glutamic acid diethyl ester (2.80 g, 11.70 mmol) in DMF (10 mL) was neutralized with triethylamine (1.19 g, 11.70 mmol) and immediately added to the reaction mixure. The reaction was stirred for 2 h at 0 °C, after which the DMF was removed under reduced pressure.

The residue was extracted with a bilayer of ethyl ether and water (100 mL; 50 mL), and the organic layer was washed with 0.5 N HCl and sodium bicarbonate and dried over $MgSO_4$. The ether was evaporated under reduced pressure to afford pure **17,** 2.75 g (66%): TLC R_f 0.46 (hexane/ethyl acetate, 1:1, silica gel); ^{1}H NMR ($CDCl_3$) δ 1.20 (t, 3 H, CH_3), 1.28 (t, 3 H, CH_3), 2.13−2.51 (m, 4 H, Gluβ and Gluγ-CH_2), 4.07 (q, 2 H, CH_2), 4.20 (q, 2 H, CH_2), 4.61 (s, 2 H, benzyl-CH_2), 4.43 (m, 1 H, Gluα-CH), 7.06 (d, 1 H, CONH), 7.45 (d, 2 H, 3′-,5′-CH), 7.80 (d, 2 H, 2′-,6′-CH). Anal. Calcd for ($C_{17}H_{22}NO_5Cl$) C, H, N, Cl.

Enzymes and Enzymes Assays. Folylpolyglutamate synthetase (FPGS) was partially purified from CCRF-CEM human leukemia cells by $(NH_4)_2SO_4$ fractionation, gel sieving, and phosphocellulose chromatography.[15] FPGS activity was assayed as described;[33] compound **7** was quantitatively (=98%) recovered during the standard assay procedure, thus ensuring that polyglutamate products would also be quantitatively recovered. Kinetic constants were determined by the hyperbolic curve fitting subprogram of SigmaPlot (Jandel) using a >10-fold range of substrate concentration; activity was linear with respect to time at the highest and lowest concentrations tested. Assays contained ~400 units of FPGS activity; one unit of FPGS catalyzes incorporation of 1 pmol of [^{3}H]Glu/h. CCRF-CEM dihydrofolate reductase was partially purified and assayed as described.[34] DHFR inhibitory potency as measured by adding drug to standard assays; the drug concentration required to reduce activity to 50% of control (IC_{50}) was determined graphically from the plots of residual activity versus drug concentration. Assays contained 1.8×10^{-3} units of DHFR activity; one unit of DHFR reduces 1 μmol dihydrofolate/min under standard conditions.

Cell Lines and Methods for Measuring Growth Inhibitory Potency. The human T-lymphocytes leukemia cell line CCRF-CEM[35] and its MTX-resistant sublines R30dm[27] and R1[29] were cultured as described.[27] R30dm expresses 1% of the FPGS activity of CCRF-CEM. R1 expresses 20-fold elevated levels of DHFR, the target enzyme of MTX. The FaDu human squamous cell carcinoma monolayer cell line was cultured in RPMI 1640/10% fetal calf serum in 100 mm cell culture dishes (Falcon).[15] Growth inhibition of all lines by continuous drug exposure was measured as described.[15,27] EC_{50} values were determined visually from plots of percent control growth versus the logarithm of drug concentration. Protection against growth inhibition was assayed by including 10 μM leucovorin (LV) simultaneously with a concentration of drug previously determined to yield growth inhibition of 90−99%; the remainder of the assay was as described above. All lines were verified to be negative for Mycoplasma contamination using the GenProbe test kit.

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