5-Arylthio-Substituted 2-Amino-4-oxo-6-methylpyrrolo[2,3-*d*]pyrimidine Antifolates as Thymidylate Synthase Inhibitors and Antitumor Agents[1]

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Classical antifolate inhibitors of thymidylate synthase (TS) often require the reduced folate uptake system in order to exert their antitumor effects. In addition, these analogues are polyglutamylated *via* the enzyme folylpoly-γ-glutamate synthetase (FPGS), which prevents analogue efflux from the cell and usually increases their inhibitory potency against TS. Impaired function of the reduced folate uptake system and that of FPGS are potential sources of resistance to such antifolates. We designed and synthesized a classical 6–5 ring-fused analogue *N*-[4-[(2-amino-6-methyl-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)thio]-benzoyl]-L-glutamic acid (**5**) and a nonclassical 6–5 ring-fused analogue 2-amino-6-methyl-5-(pyridin-4-ylthio)-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidine (**6**) as TS inhibitors and antitumor agents. The syntheses of analogues **5** and **6** were acheived *via* the oxidative addition of the sodium salt of ethyl 4-mercaptobenzoate or 4-mercaptopyridine to 2-(pivaloylamino)-6-methyl-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidine (**17**) in the presence of iodine. For the synthesis of **5** the ester obtained from the reaction was deprotected and coupled with diethyl L-glutamate followed by saponification. Compound **5** was a potent inhibitor of human and bacterial TS with IC_{50} values of 42 and 21 nM, respectively. Compound **6** was 10-fold less potent than **5** against human TS but more than 4700-fold less potent than **5** against *Lactobacillus casei* TS. The classical analogue **5** was neither a substrate nor an inhibitor of human FPGS derived from CCRF-CEM cells. Compound **5** was cytotoxic to CCRF-CEM and FaDu tumor cell lines as well as to an FPGS-deficient subline of CCRF-CEM. Thymidine protection studies established that TS was the primary target of **5**.

Introduction

Thymidylate synthase (TS) catalyzes the synthesis of deoxythymidylate (dTMP) from deoxyuridylate (dUMP) utilizing 5,10-methylenetetrahydrofolate as the source of the methyl group as well as the reductant.[2] This rate-limiting step constitutes the sole *de novo* synthesis of dTMP which is required for DNA biosynthesis. Several classical antifolates that act by inhibiting TS have been developed as antitumor agents.[3,4] Notable among these are the quinazolines, N^{10}-propargyl-5,8-dideazafolate (PDDF, **1**),[5] and its third-generation analogue ZD1694 (**2**),[6] which is currently in phase III clinical trials. These analogues are structurally similar to the natural folates, *i.e.*, they possess a 6–6 ring-fused system and an *N*-benzoyl-L-glutamic acid side chain appended to the 6-position. In a variation of these conventional 6–6 ring-fused antifolates, Taylor *et al.*[7] reported the synthesis of a B-ring contracted 6–5 ring-fused antifolate **3** as an inhibitor of TS and as an effective antitumor agent; this classical pyrrolo[2,3-*d*]pyrimidine antifolate had a K_i of 340 nM against recombinant mouse TS.[7]

Recently Webber *et al.*[8] reported a series of 5-arylthio-substituted quinazolines as inhibitors of TS. These compounds were designed using a 2.3 Å resolution crystal structure of the ternary complex of *E. coli* TS, 5-FdUMP, and PDDF. The classical analogue **4** was an extremely potent inhibitor of human TS with a K_i of 0.12 nM. Compound **4** has a 6–6 ring-fused system similar to antifolates **1** and **2**; however, the *N*-benzoyl-L-glutamic acid side chain was at the 5-position rather than the normal 6-position. In addition, the methylene-amino bridge of **1** and **2** was replaced with a single sulfur atom and the 6-position contained a methyl group. Molecular modeling of 6–5 ring-fused antifolates superimposed on 6–6 systems shows that 5-substituents of the 6–5 system lie close to the 5-substituent of the 6–6 systems and that 6-substituents of the 6–5 systems lie in between the 6- and 7-substituents of the 6–6 system.[9] The activity of **4** against TS further indicates that the *N*-(4-ethylbenzoyl)-L-glutamic acid side chain of **3** can be replaced with an *N*-(4-mercaptobenzoyl)-L-glutamic acid side chain. Webber *et al.*[8] suggested that the 6-methyl group of **4** not only makes important hydrophobic contacts to the Trp 80 residue in *Escherichia coli* TS but also serves to lock the 5-position side chain into one favorable, low-energy conformation. We have synthesized classical and nonclassical 6–5 fused furo[2,3-*d*]pyrimidine and pyrrolo[2,3-*d*]pyrimidine antifolates and have reported, along with others, that 2,4-diamino 6–5 ring-fused systems are excellent inhibitors of DHFR.[10,11] We reasoned that since the pyrrolo[2,3-*d*]pyrimidine ring can substitute for the quinazoline ring, the incorporation of a methyl group at the 6-position along with the *N*-(4-mercapobenzoyl)-L-glutamic acid in the 5-position of 2-amino-4-oxopyrrolo[2,3-*d*]-pyrimidine might afford a potent TS inhibitor **5**. Structurally, compound **5** is a hybrid of compounds **3** and **4**.

A drawback of classical antifolates is that they may require transport into cells *via* reduced folate uptake systems, which when impaired can lead to drug

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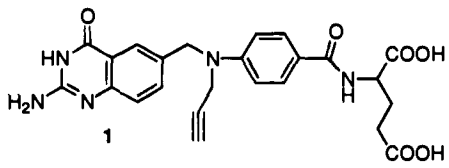
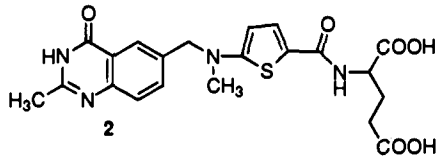
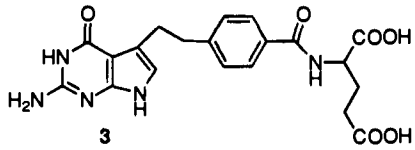
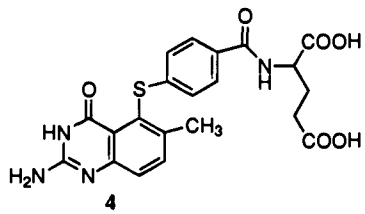
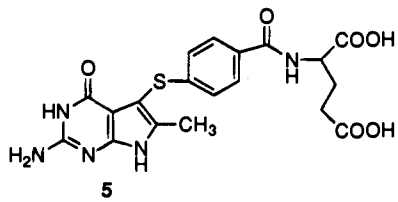
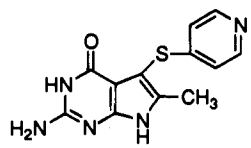


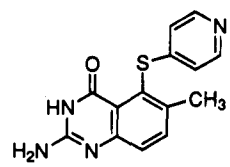








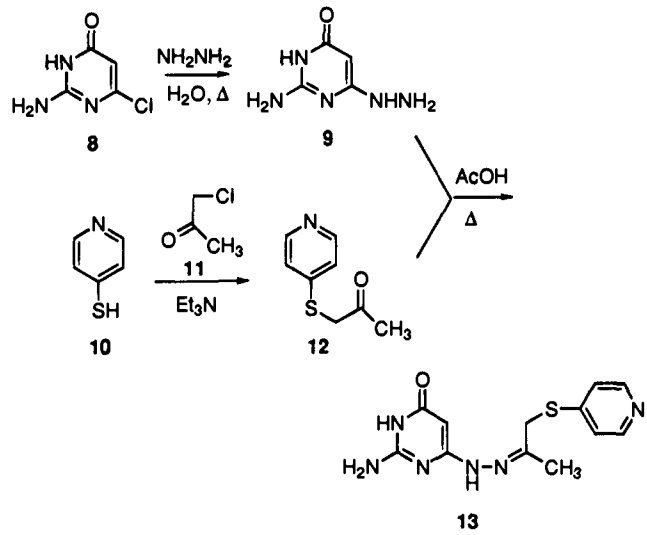




resistance.[12-14] In addition, the antitumor activities of several classical antifolates, particularly TS inhibitors, are in part determined by their ability to function as substrates for the enzyme folylpoly-γ-glutamate synthetase (FPGS).[6,7,15-18] Polyglutamylation catalyzed by FPGS produces long-acting, noneffluxing poly-γ-glutamates of classical antifolates that lead to high intracellular concentrations of these antitumor agents. Further, polyglutamylation provides for a significant increase in inhibitory activity against TS compared to the monoglutamates.[6,7,16-19] It has been shown for both ZD1694 (**2**),[6] and for the pyrrolo[2,3-*d*]pyrimidine antifolate **3**,[7] that the conversion of the monoglutamate form of these compounds to their pentaglutamates produces a 60-fold (for **2**) to 130-fold (for **3**) increase in TS inhibition. Though polyglutamylation for many antifolates is necessary for cytotoxicity to tumor cells, it has also been implicated in adverse effects on normal tissues due to retention of poly-γ-glutamate metabolites.[20] The problem of resistance in tumors, which is a result of low or defective FPGS, is also a potential limitation of the use of such classical antifolates, which depend on polyglutamylation for their antitumor effects.[21-24]

To overcome these disadvantages associated with classical antifolates we designed nonclassical, lipophilic inhibitors of TS that lack the L-glutamic acid found in classical TS inhibitors, allowing for passive uptake independent of the folate transport systems.[25,26] The target molecule **6** was designed based on the 5-arylthio-substituted quinazoline **7** which is a nonclassical inhibitor of TS.[8] Compound **7** is a potent inhibitor of human TS with a K_i of 15 nM, and is currently under clinical study as an antitumor agent, as its dihydrochloride salt, AG-337.[27] The lipophilic pyrrolo[2,3-*d*]pyrimidine antifolate **6** was designed as a structural hybrid of compounds **3** and **7**. This report consists of the synthesis of the classical and lipophilic pyrrolo[2,3-*d*]pyrimidine antifolates **5** and **6** and their evaluation against isolated TS and as inhibitors of the growth of tumor cells in culture. The classical antifolate **5** was also evaluated as a substrate for human FPGS.

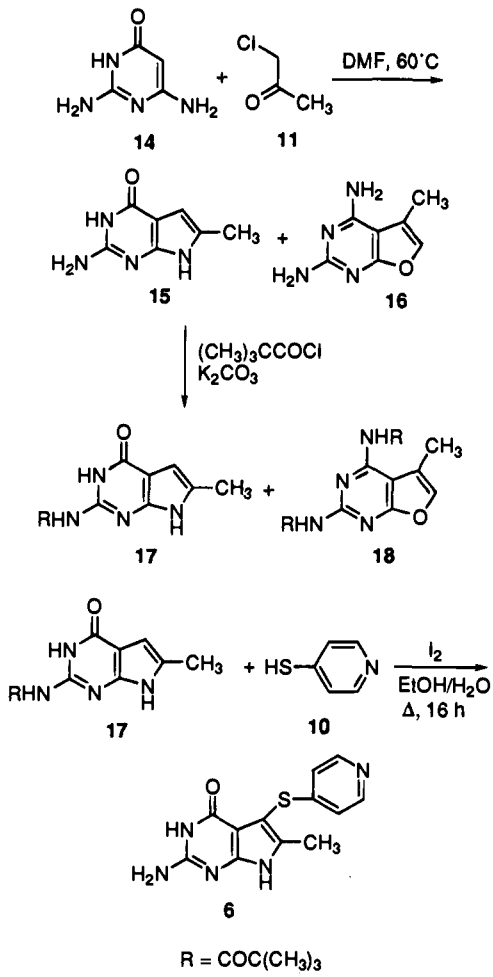
Scheme 1



Chemistry

Our initial attempt at the synthesis of **6** involved a Fischer-indole type cyclization of a suitable pyrimidinyl 6-hydrazone[28,29] to afford the pyrrolo[2,3-*d*]pyrimidine ring system. 2-Amino-4-oxo-6-hydrazinopyrimidine (**9**) was synthesized in 90% yield by refluxing a mixture of the 6-chloro compound **8** with hydrazine hydrate in water (Scheme 1).[30] Alkylation of 4-mercaptopyridine (**10**) with chloroacetone (**11**) in the presence of triethylamine afforded the desired ketone **12** in 75% yield. Hydrazone formation was carried out by heating a mixture of the hydrazine **9** and the ketone **12** in glacial acetic acid, which afforded the hydrazone **13** in 72% yield. Fischer-indole cyclization of the hydrazone **13** was expected to afford a mixture of regioisomeric products resulting from the attack of the 5-position of the pyrimidine on the methyl group, to afford the undesired isomer, or the attack of the 5-position on the methylene unit, to afford the desired compound **6**. All attempts at effecting this cyclization failed to afford the desired product(s). Thermal cyclization in ethylene glycol or diphenyl ether at temperatures greater than 150 °C resulted in significant decomposition of the starting hydrazone **13**. Acidic methods of cyclization including methanolic hydrochloric acid or refluxing acetic acid resulted in minor decomposition and a significant amount of the starting material was recovered unreacted.

Scheme 2



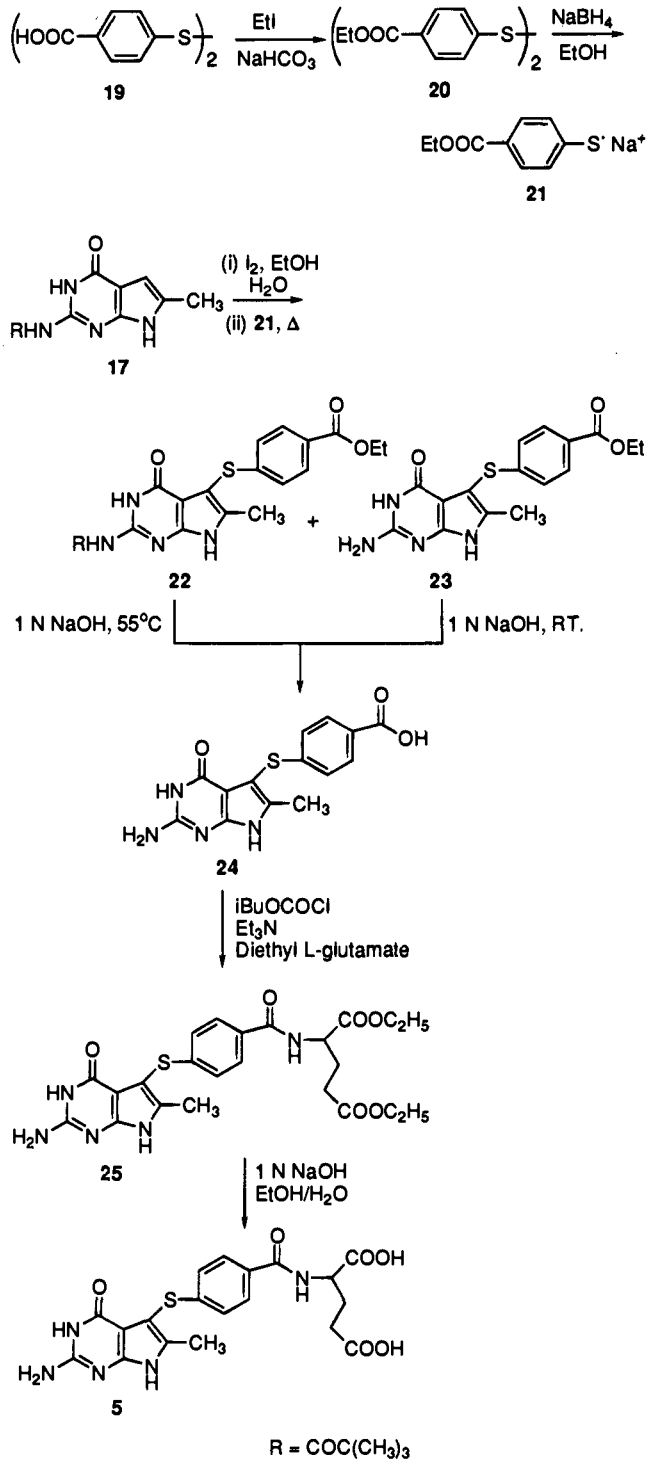
Failure of the Fischer-indole methodology led us to pursue a strategy where the bicyclic ring system would be synthesized prior to attachment of the 5-thioaryl substituent. Following a literature method[31] the desired 2-amino-4-oxo-6-methylpyrrolo[2,3-*d*]pyrimidine (**15**) was constructed in a single step by the reaction of 2,6-diamino-4-oxopyrimidine (**14**) with chloroacetone (**11**) in DMF at 60 °C (Scheme 2). This reaction also produced the undesired 2,4-diamino-5-methylfuro[2,3-*d*]pyrimidine (**16**). Although the desired compound **15** could be separated from **16** by column chromatography, it was more convenient to convert the mixture of the products **15** and **16**, without isolation, to their more soluble pivaloyl protected derivatives. This increased solubility was expected to provide easier purification of these and subsequent derivatives. Following reaction with pivaloyl chloride and potassium carbonate, the desired 2-pivaloyl-protected compound **17** was isolated as an ethyl acetate insoluble solid. TLC analysis indicated that the more lipophilic 2,4-bis(pivaloylamino)furo[2,3-*d*]pyrimidine derivative **18** was present in the ethyl acetate soluble fraction; however, it was not isolated.

At this stage, iodination of the 5-position of **17**, followed by an Ullmann type coupling with a suitable arylthiol, was anticipated to afford the target molecule. A literature search revealed, however, that arylthiols could be oxidatively added directly to the indole 3-position in the presence of iodine. Beveridge and Harris[32] reported that the addition of several substituted thiophenols in the presence of iodine in a H_2O/EtOH mixture afforded the desired 3-substituted indoles in moderate to good yields. In a recent publication, Gubin *et al.*[33] further showed that the reaction works equally well with 2-isopropylindole. Extension of this strategy to a pyrrolo[2,3-*d*]pyrimidine ring system would afford the desired 5-substituted derivative in a single step. Reaction of **17** with 4-mercaptopyridine (**10**) in a mixture of ethanol/water with 2 equiv of iodine at 80 °C for a period of 16 h afforded the desired 5-substituted target compound **6** in 42% yield (Scheme 2) with concomitant deprotection of the 2-position. The ^{1}H NMR spectrum of **6** in deuterated dimethyl sulfoxide revealed the presence of two doublets at 6.93 and 8.26 ppm with a coupling constant of J = 5.8 Hz, corresponding to the pyridine aromatic protons, and the presence of a deuterium oxide exchangeable singlet at 6.13 ppm, corresponding to the 2-amino group. A closer analysis of the progress of the reaction by TLC revealed that the substitution reaction preceded amide deprotection. The hydrogen iodide released during the course of the reaction probably caused the acidic deprotection of the 2-amide group. Extension of this strategy to the synthesis of the classical analogue **5** required the synthesis of the corresponding thiol **21** (Scheme 3). 4,4′-Dithiobis(benzoic acid) (**19**) was alkylated with ethyl iodide to afford the desired diethyl ester **20** in 81% yield. This disulfide was reduced to the mercaptan **21** with sodium borohydride and was used without further purification. Addition of an ethanolic solution of the mercaptan **20** to a solution of **17** and iodine afforded an easily separable mixture of the 2-pivaloyl-protected compound **22** (39%) and the 2-deprotected compound **23** (19%). The ^{1}H NMR spectra of **22** and **23** indicated the characteristic AA′BB′ pattern for the 5-aryl protons and the disappearance of the 5-vinyl proton at 6.07 ppm present in **17**. Both **22** and **23** were hydrolyzed to the acid **24** with aqueous sodium hydroxide. Deprotection of the ester and the amide of **22** was achieved by carrying out the reaction at 55 °C for 18 h. Peptide coupling of the acid **24** with diethyl L-glutamate using the mixed anhydride method with isobutyl chloroformate and triethylamine, with a repeated cycle of activation (Scheme 3), followed by chromatographic purification afforded the coupled product **25** in 61% yield. The ^{1}H NMR spectrum of **25** in deuterated dimethyl sulfoxide revealed the newly formed peptide NH proton at 8.60 ppm as a doublet with a coupling constant J = 7.5 Hz. Hydrolysis of the diester **25** with aqueous sodium hydroxide at room temperature, followed by acidification gave the desired compound **5** in 89% yield.

Biological Evaluation and Discussion

The classical and nonclassical analogues **5** and **6** were evaluated as inhibitors of TS[35] from *Lactobacillus casei*[34] and recombinant human sources.[34] The inhibitory potencies (IC_{50}) are listed in Table 1 along with that of PDDF (**1**). The classical analogue **5** was a good inhibitor of human TS with an IC_{50} of 42 nM and was equipotent with PDDF (36 nM). Compound **5** was twice as potent in inhibiting bacterial TS than human TS. The lipophilic analogue **6** was also active against human TS with an IC_{50} of 340 nM. Interestingly compound **6** was 300-fold more potent against human TS than against *L. casei* TS, indicating a significant species difference. The nonclassical analogue **6** was also a poor inhibitor

Scheme 3



of *S. faecium* TS (IC_{50} = 29 μM) and *E. coli* TS (30% at 29 μM) (data not shown).

The target compounds **5** and **6** were also evaluated as inhibitors of *L. casei*,[36] human recombinant DHFR,[37] and DHFR isolated from the human CCRF-CEM leukemic cell line (Table 1). Although the nonclassical analogue **6** was inactive (IC_{50} > 69 μM) against both *L. casei* and human DHFR, the classical analogue **5** was a moderate inhibitor of both DHFRs with an IC_{50} = 2.2 μM. Compound **5** was also a good inhibitor of CCRF-CEM DHFR with an IC_{50} = 0.124 μM. It is well known that both the interaction of the 4-amino protons and the protonated N1 of 2,4-diaminopyrimidine-containing antifolates such as methotrexate (MTX) with DHFR, are

Table 1. Inhibitory Concentrations (IC_{50}) against Isolated TS and DHFR

	TS (IC_{50}) in nM		DHFR (IC_{50}) in mM		
compd	human	*L. casei*	rec human	*L. casei*	CCRF-CEM
5	42	21	2.2	2.2	0.124
6	340	100000	>69	>69	–
1 (PDDF)	36	36			

Table 2. Growth Inhibition of CCRF-CEM Human Leukemic Cells, Its Methotrexate-Resistant[a] Subline Having Decreased FPGS (R30dm), and the Human Squamous Cell Carcinomas FaDu and A253 Cells by **5**[b] during Continuous Exposure

	EC_{50}, μM			
compd	CCRF-CEM	R30dm[a]	Fadu	A253
5	0.45 ± 0.10 (n = 4)	0.81 ± 0.05 (n = 3)	0.53 ± 0.16 (n = 3)	3.0 ± 1.2 (n = 2)
MTX	0.014 ± 0.001 (n = 10)	0.018 ± 0.003 (n = 5)	0.017 ± 0.002 (n = 5)	0.013 ± 0.0 (n = 3)
1 (PDDF)	0.72[c]			

[a] CCRF-CEM subline resistant to intermittent MTX exposure solely as a result of decreased polyglutamylation; this cell line has 1% of the FPGS specific activity (measured with MTX as the folate substrate) of parental CCRF-CEM. [b] Average values are presented ±range for n = 2 and ±SD for $n \geq 3$. [c] The EC_{50} value for 1 has been taken from ref 20.

essential for tight binding.[38] In compound **5** the substrate orientation may allow the pyrrole nitrogen and the lactam 3-NH proton to be positioned similar to the 4-NH_2 and the protonated N1 of MTX, thereby providing for DHFR inhibition. Such dual binding orientations of pyrrolo[2,3-*d*]pyrimidine antifolates has been proposed by Miwa *et al.*[39] and is further supported by the potent DHFR inhibitory activity reported for the 4-desaminopyrrolo[2,3-*d*]pyrimidine antifolates.[40,41]

The lipophilic analogue **6** was selected for evaluation by the National Cancer Institute in its preclinical *in vitro* tumor screening program[42,43] and was found to be moderately cytotoxic with a growth inhibitory concentration GI_{50} = 9.9 μM against human CCRF-CEM leukemic cells and a GI_{50} = 4.8 μM against SF-539 CNS cancer cells. The classical analogue **5** was however more cytotoxic and the EC_{50} values against CCRF-CEM cells and the human squamous cell carcinoma lines A253 and FaDu are listed in Table 2. The target antifolate inhibited CCRF-CEM cells with an EC_{50} = 0.451 μM and showed similar inhibition of the other two cell lines, with the A253 cells being slightly less sensitive. Since the antitumor activities of several classical antifolates, particularly TS inhibitors, are predicated on their ability to form polyglutamates *via* FPGS, compound **5** was evaluated as a substrate for human FPGS from CCRF-CEM cells and was found to be inactive as a substrate at concentrations up to 1045 μM. It was also only a weak inhibitor of this enzyme (<15% inhibition at 100 μM). The minimal cross-resistance of the FPGS deficient subline R30dm[23] to compound **5** (Table 2) is consistent with the results against isolated FPGS and underscores the absence of polyglutamylation as part of the mechanism of the cytotoxicity of **5**. Marsham *et al.*[44] have recently reported a 7-CH_3 analogue of 2-desamino-2-methyl PDDF which was not a substrate for human FPGS. This corroborates our results with **5** and indicates that methyl substituents in the 6-position of classical pyrrolo[2,3-*d*]pyrimidines and in the 7-position of quinazolines are devoid of FPGS

Table 3. Protection by 10 μM LV, 50 μM Hx, 40 μM TdR, or the combination of 50 μM Hx + 40 μM Hx against the Growth Inhibition of FaDu Cells by MTX and **5**

	relative growth (% of control)				
drug	no addn	LV	Hx	TdR	Hx + TdR
MTX	2.0	114 ± 5	18 ± 0	11 ± 1	95 ± 2
5	5 ± 1	7 ± 1	4 ± 1	83 ± 14	78 ± 5

substrate activity as a result of bulk and/or hydrophobicity which is not tolerated by human FPGS.

Since the classical analogue **5** was active against both isolated TS and DHFR, further studies were warranted to elucidate its mechanism of action. Metabolite protection studies (Table 3) with FaDu squamous cell carcinoma cells indicated that the growth inhibitory activity of **5** was not reversed with the addition of 10 μM of leucovorin, in contrast to MTX. A similar result was obtained using human CCRF-CEM leukemic cells (data not shown). In addition the inhibition of the growth of FaDu cells in culture (Table 3) by **5** was completely reversed by the addition of 40 μM of thymidine (TdR) or the combination of 40 μM of TdR and 50 μM of hypoxanthine (Hx) but not by Hx alone. These results indicated that the primary intracellular target of **5** is TS and not DHFR. This is probably a consequence of the greater inhibitory activity of compound **5** against TS than against DHFR.

In summary, a new 5,6-disubstituted pyrrolo[2,3-*d*]-pyrimidine classical antifolate **5** was synthesized and found to be a potent inhibitor of TS and a moderate inhibitor of DHFR. In contrast to several other classical antifolates (e.g. **2** and **3**) that inhibit TS, compound **5** was not a substrate for FPGS. This lack of substrate activity of **5** could be an important feature to overcome potential resistance to classical TS inhibitors such as **2** and **3** which depend on intracellular polyglutamylation to exert their cytotoxic effects. Compound **5** is a potent nonpolyglutamatable TS inhibitor which is active against tumors expressing low or defective FPGS and is the first example of a 6–5 ring-fused antifolate with this attribute. It is currently undergoing further evaluations. The pyrrolo[2,3-*d*]pyrimidine antifolate **6** is the first example of a 6–5 ring-fused nonclassical lipophilic antifolate that is an inhibitor of TS. The excellent selectivity of compound **6** against the human enzyme suggests that differences in mammalian and bacterial TS can be exploited with nonclassical TS inhibitors. We are in the process of developing other nonclassical TS inhibitors with potential selectivity toward nonmammalian TSs and also as antitumor agents.

Experimental Section

Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Infrared spectra (IR) were recorded with a Perkin-Elmer Model 1430, in Nujol mulls. Nuclear magnetic resonance spectra for protons (^{1}H NMR) were recorded on a Varian EM-360 (60 MHz) or a Bruker WH-300 (300 MHz). Chemical shift values are expressed in δ values (parts per million) relative to tetramethylsilane as an internal standard: s = singlet, d = doublet, t = triplet, m = multiplet, and dd = doublet of doublets. Mass spectra were recorded on a VG-7070 double-focusing mass spectrometer or in a LKB-9000 instrument in the electron ionization (EI) mode or chemical ionization (CI) mode. Fast atom bombardment mass spectra (FABMS) were obtained on a Kratos MS50 spectrometer. High-resolution mass spectra for FABMS were obtained by peak-matched FABMS on a Kratos MS50 spectrometer. Thin-layer chromatography (TLC) was performed on silica gel plates with a fluorescent indicator that was visualized with light at 254 or 366 nm. Proportions of solvents used for TLC are by volume. Column chromatography was performed on 230–400 mesh silica gel purchased from Aldrich (Milwaukee, WI). Solvents routinely used for reactions and purification were purchased from Aldrich (Milwaukee, WI) or Fischer Scientific (Pittsburgh, PA) and were used without further purification. Samples for microanalysis were dried *in vacuo* over phosphorus pentoxide at 70 or 110 °C. Elemental analysis were performed by Atlantic Microlabs, Inc., Norcross, GA. Elemental compositions are within ±0.4% of the calculated values. Fractional moles of water or organic solvents, frequently found in some analytical samples of antifolates, could not be prevented in spite of drying *in vacuo* and were confirmed by their presence in the ^{1}H NMR spectrum.

2-Amino-6-hydrazino-4-oxopyrimidine (9). To a suspension of 2-amino-6-chloropyrimidin-4-ol (**8**) (1.46 g, 10 mmol) in water (70 mL) was added hydrazine hydrate (55% aqueous solution, 1.17 g, 20 mmol) and the mixture was refluxed for 2 h. The reaction mixture was then cooled to room temperature and filtered. The residue was washed with water (500 mL) and air-dried to afford 1.28 g (90%) of **9** as a white powder: mp 293–294 °C (lit.[30] mp 292–293 °C); TLC R_f 0.56 (EtOAc/MeOH 1:1 with two drops of acetic acid).

2-Oxo-3-(4-pyridylthio)propane (12). To a mixture of 4-mercaptopyridine (**10**) (2.44 g, 90%, 20 mmol) and triethylamine (2.78 mL, 20 mmol) in CH_2Cl_2 (30 mL) was added chloroacetone (**11**) (1.85 g, 96%, 20 mmol) and the reaction mixture stirred at room temperature for 14 h. TLC (EtOAc) indicated that the starting material **10** (R_f 0.06) had almost disappeared and a new spot (R_f 0.29) had formed. The reaction mixture was diluted with CH_2Cl_2 (70 mL) and washed with water (100 mL) and saturated NaCl (100 mL). The organic layer was dried (Na_2SO_4) and filtered. The filtrate was chromatographed on a silica gel column (2.4 x 18 cm, packed with EtOAc) and eluted with EtOAc. Fractions showing a single spot were pooled and evaporated to dryness under reduced pressure to afford 2.51 g (75%) of **12** as yellow needles: TLC R_f 0.29 (EtOAc, silica gel); IR (Nujol) 3040, 1740, 1570 cm^{-1}; MS (EI) m/z 167 (M^+).

2-[(2-Amino-4-oxopyrimidin-6-yl)hydrazono]-3-(4-pyridylthio)propane (13). A solution of 2-amino-6-hydrazino-4-oxopyrimidine (**9**) (0.49 g, 3.5 mmol) and **12** (0.59 g, 3.5 mmol) in glacial acetic acid (5 mL) was placed in a preheated oil bath at 85 °C and stirred at this temperature for 20 min under nitrogen. The solution was then evaporated to dryness under reduced pressure and coevaporated with absolute ethanol (3 × 30 mL). The residue was stirred in ethanol (100 mL) and filtered to remove any insoluble solid. Silica gel (1.2 g) was added to the filtrate and the suspension evaporated to dryness. This plug was placed on a dry silica gel column (2.4 × 20 cm) and eluted with a gradient of 10% to 20% MeOH in $CHCl_3$. Fractions showing a single spot were pooled and evaporated to dryness. The residue was stirred in anhydrous ether, filtered, and dried to afford 0.73 g (72%) of **13** as an off-white solid: mp 219–220 °C dec; TLC R_f 0.35 ($CHCl_3$/MeOH 4:1, with two drops of NH_4OH); ^{1}H NMR (DMSO-d_6) δ 1.96 (s, 3 H, CH_3), 3.96 (s, 2 H, CH_2S), 5.15 (s, 1 H, 5-CH), 6.20 (s, 2 H, 2-NH_2), 7.37 (dd, 2 H, 3′-, 5′-CH, J = 1.5, 5.1 Hz), 8.34 (dd, 2 H, 2′-, 6′-CH, J = 1.5, 5.1 Hz), 9.05 (s, 1 H, 6-NH), 10.01 (s, 1 H, 3-NH). Anal. Calcd for ($C_{12}H_{14}N_6OS\cdot0.4H_2O$) C, H, N.

2-(Pivaloylamino)-6-methyl-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidine (17). To a suspension of 2,6-diamino-4-hydroxypyrimidine (**14**) (5.04 g, 40 mmol) in anhydrous DMF (70 mL) was added chloroacetone (**11**) (4.44 g, 48 mmol) and the mixture stirred at 60 °C for 48 h under nitrogen. TLC ($CHCl_3$/MeOH 4:1) indicated the formation of two new spots at R_f 0.36 (corresponding to **15**) and R_f 0.56 (corresponding to **16**).[31] At this time powdered anhydrous potassium carbonate (27.64 g, 200 mmol) was added to the reaction mixture followed by pivaloyl chloride (24.12 g, 200 mmol) and the suspension was stirred under nitrogen for an additional 10 h. The reaction mixture was filtered and the filtrate evaporated to dryness

under reduced pressure (oil pump). The residue was dissolved in CH_2Cl_2 (300 mL) and washed with water (2 × 150 mL). The organic layer was dried ($MgSO_4$) and filtered. After evaporation of the filtrate, the residue was stirred in boiling EtOAc (300 mL) and filtered. The residue was washed with a fresh portion of hot EtOAc (100 mL) and dried to afford 3.5 g (35%) of **17** as an off-white solid. An analytical sample was recrystallized from a mixture of DMF and water: mp 290–294 °C (dec); TLC R_f 0.54 ($CHCl_3$/MeOH 9:1 with 2 drops of concentrated NH_4OH); ^1H NMR (DMSO-d_6) δ 1.23 (s, 9 H, $C(CH_3)_3$), 2.25 (s, 3 H, 6-CH_3), 6.07 (s, 1 H, 5-CH), 10.75 (s, 1 H, 7-NH), 11.35 (s, 1 H, 3-NH), 11.78 (s, 1 H, CONH); MS (FAB) m/z 249 (MH^+); high-resolution FABMS calcd MH^+ 249.2944, found 249.2935.

2-Amino-6-methyl-5-(pyridin-4-ylthio)-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidine (6). To a solution of **17** (0.99 g, 4 mmol) in a mixture of ethanol/water (6/4, 30 mL) was added iodine (1.02 g, 4 mmol) followed by 4-mercaptopyridine (**10**) (0.55 g, 5 mmol) and the reaction mixture refluxed for 6 h. At this time an additional 4 mmol of iodine and 3 mmol of **10** was added to the mixture and the reaction continued at 80 °C for 16 h. The mixture was left at 0 °C for 2 h and filtered. The residue was suspended in water and the pH adjusted to 8 with concentrated NH_4OH with stirring. This suspension was left at 5° C for 6 h and filtered. The residue was washed well with water and dried to afford 0.45 g (42%) of **6** as tan solid. An analytical sample was recrystallized from a mixture of DMSO and water: mp >250 °C (dec); TLC R_f 0.26 ($CHCl_3$/MeOH 5:1 with 2 drops of concentrated NH_4OH); ^1H NMR (DMSO-d_6) δ 2.17 (s, 3 H, 6-CH_3), 6.14 (s, 2 H, 2-NH_2), 6.93 (d, 2 H, 3′-, 5′-CH, J = 5.8 Hz), 8.26 (d, 2 H, 2′-, 6′-CH, J = 5.8 Hz), 10.26 (s, 1 H, 7-NH), 11.53 (s, 1 H, 3-NH). Anal. Calcd for ($C_{12}H_{11}N_5OS \cdot H_2O$) C, H, N, S.

Diethyl 4,4′-Dithiobis(benzoate) (20). To a solution of 4,4′-dithiobis(benzoic acid) (**19**) (4.59 g, 15 mmol) in anhydrous *N*,*N*-dimethylacetamide (25 mL) was added powdered sodium bicarbonate (5.04 g, 60 mmol) followed by ethyl iodide (9.36 g, 60 mmol), and the reaction mixture was stirred under nitrogen for 5 days. The mixture was then diluted with water (50 mL) and extracted with EtOAc (3 × 60 mL). The combined organic layers were washed with water (50 mL) and brine (50 mL) and then dried ($MgSO_4$) and filtered. The filtrate was evaporated to a dark brown oil under reduced pressure. This oil was chromatographed on silica gel (2.8 × 35 cm, packed with CH_2Cl_2) and eluted with CH_2Cl_2. Fractions showing a single spot were pooled and evaporated to dryness to afford a tan oil which slowly crystallized to afford 4.39 g (81%) of **20** as a tan solid: mp 55–57 °C (lit.[45] mp 53–56 °C); TLC R_f 0.95 (EtOAc/toluene 1:4, silica gel); ^1H NMR ($CDCl_3$) δ 1.29 (t, 6 H, CH_2CH_3, J = 7.0 Hz), 4.27 (q, 4 H, CH_2CH_3, J = 7.0 Hz), 7.42 (d, 4 H, 3′-, 5′-CH, J = 8.4 Hz), 7.87 (d, 4 H, 2′-, 6′-CH, J = 8.4 Hz); MS (CI) m/z 363 (MH^+).

Ethyl 4-[(2-(Pivaloylamino)-6-methyl-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)sulfanyl]benzoate (22) and Ethyl 4-[(2-Amino-6-methyl-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)sulfanyl]benzoate (23). To a solution of diethyl 4,4′-dithiobis(benzoate) (**20**) (1.09 g, 3 mmol) in absolute ethanol (10 mL) was added sodium borohydride (0.113 g, 3 mmol) all at once and the mixture stirred under nitrogen for 30 min. This ethanolic solution of the sodium salt of ethyl 4-mercaptobenzoate (**21**) was added to a solution of **17** (0.99 g, 4 mmol) and iodine (2.04 g, 8 mmol) in a mixture of ethanol/water (5/4, 40 mL) maintained at 80 °C. The temperature was increased to 90 °C and the solution refluxed for 1.5 h, when TLC indicated the disappearance of starting material at R_f 0.54 ($CHCl_3$/MeOH 9:1 with 2 drops of concentrated NH_4OH) and the formation of two spots at R_f 0.62 and R_f 0.24. The reaction mixture was cooled to 5 °C and filtered. The residue was washed with acetone (30 mL) and cold methanol (20 mL) and then air-dried. The residue was recrystallized from a mixture of DMSO and water to afford 0.26 g (19%) of **23** as a light brown solid: mp >300 °C; TLC R_f 0.24 ($CHCl_3$/MeOH 9:1 with 2 drops of concentrated NH_4OH); ^1H NMR (DMSO-d_6) δ 1.28 (t, 3 H, CH_2CH_3, J = 7.0 Hz), 2.18 (s, 3 H, 6-CH_3), 4.27 (q, 2 H, CH_2CH_3, J = 7.0 Hz), 6.17 (br s, 2 H, 2-NH_2), 7.07 (d, 2 H, 3′-, 5′-CH, J = 8.4 Hz), 7.77 (d, 2 H, 2′-, 6′-CH, J = 8.4 Hz), 10.33 (s, 1 H, 7-NH), 11.53 (s, 1 H, 3-NH); MS (FAB) m/z 345 (MH^+). Anal. Calcd for ($C_{16}H_{16}N_4O_3S \cdot 0.9H_2O \cdot 0.1$DMSO) C, H, N, S.

The filtrate and washings obtained above were combined and evaporated to dryness under reduced pressure. The residue was diluted with water (150 mL) and extracted with $CHCl_3$ (3 × 75 mL). The $CHCl_3$ layers were dried ($MgSO_4$) and filtered. Silica gel (2 g) was added to the filtrate and this suspension was evaporated to dryness. The silica gel plug was placed on top of a dry silica gel column (2.4 × 16 cm). The column was flushed with $CHCl_3$ (500 mL) and eluted with a gradient of 1% to 4% MeOH in $CHCl_3$. Fractions showing a single spot were pooled and evaporated to dryness to afford 0.62 g (36%) of **22** as a white solid: mp 270–273 °C; TLC R_f 0.62 ($CHCl_3$/MeOH 9:1 with 2 drops of concentrated NH_4OH); ^1H NMR (DMSO-d_6) δ 1.24–1.30 (overlapping s and t, 12 H, $C(CH_3)_3$ and CH_2CH_3, J = 7.2 Hz), 2.28 (s, 3 H, 6-CH_3), 4.26 (q, 2 H, CH_2CH_3, J = 7.2 Hz), 7.09 (d, 2 H, 3′-, 5′-CH, J = 8.4 Hz), 7.78 (d, 2 H, 2′-, 6′-CH, J = 8.4 Hz); MS (FAB) m/z 429 (MH^+). Anal. Calcd for ($C_{21}H_{24}N_4O_4S \cdot 0.25H_2O$) C, H, N, S.

Diethyl *N*-[4-[(2-amino-6-methyl-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)thio]benzoyl]-L-glutamate (25). To a solution of **22** (0.25 g, 0.58 mmol) in ethanol (10 mL) was added aqueous 1 N NaOH and the reaction mixture stirred at 55 °C for 18 h. The solution was evaporated to dryness, and the sodium salt was dissolved in water (8 mL) and carefully acidified to pH 4 with dropwise addition of 3 N HCl. The resulting suspension was left at 0 °C for 2 h and filtered. The residue was washed with water (10 mL), acetone, and ethyl ether and dried over P_2O_5/vacuum at 78 °C to afford 0.16 g (87%) of the depivaloylated free acid **24** as a light brown solid.

In a similar manner **23** (0.10 g, 0.29 mmol) was deprotected with 1 N NaOH at room temperature to afford the free acid **24** in 95% yield (0.09 g).

To a suspension of the acid **24** (0.16 g, 0.5 mmol) in anhydrous DMF (10 mL) under nitrogen was added triethylamine (209 μL, 1.5 mmol) and the suspension heated to 80 °C to form a solution. This solution was cooled to 0 °C and isobutyl chloroformate (130 μL, 1 mmol) was added, followed 15 min later by diethyl L-glutamate hydrochloride (0.18 g, 0.75 mmol) and immediately followed by triethylamine (209 μL, 1.5 mmol). The reaction mixture was warmed slowly to room temperature and stirred for 12 h. At this time the reaction mixture was cooled to 0 °C and the activation steps described above were repeated using triethylamine (105 μL, 0.75 mmol), followed by isobutyl chloroformate (65 μL, 0.5 mmol). After stirring for 15 min at 0 °C diethyl L-glutamate hydrochloride (0.09 g, 0.38 mmol) was added followed immediately by triethylamine (105 μL, 0.75 mmol). The reaction mixture was stirred for another 24 h at room temperature and evaporated to dryness under reduced pressure (oil pump). The residue was suspended in water (10 mL) and the pH brought to 8 with concentrated NH_4OH and stirred for 30 min. This suspension was filtered and the residue washed well with water, air-dried, and dissolved in MeOH (100 mL). To this solution was added silica gel (1.2 g) and the suspension evaporated to dryness. The silica gel plug was loaded on a dry silica gel column (2.4 × 14 cm), and the column was flushed with $CHCl_3$ (300 mL) and eluted stepwise with 200 mL of 1%, 2%, to 5% MeOH in $CHCl_3$. The fractions showing a single spot (TLC) were pooled and evaporated to afford 0.15 g (61%) of **25** as an off-white solid: mp 200–203 °C; TLC R_f 0.55 ($CHCl_3$/MeOH 4:1 with 2 drops of concentrated NH_4OH); ^1H NMR (DMSO-d_6) δ 1.16 (m, 6 H, CH_2CH_3), 1.93–2.11 (m, 2 H, Gluβ-CH_2), 2.18 (s, 3 H, 6-CH_3), 2.41 (t, 2 H, Gluγ-CH_2, J = 7.2 Hz), 4.06 (m, 4 H, CH_2CH_3), 4.35–4.43 (m, 1 H, Gluα-CH), 6.19 (br s, 2 H, 2-NH_2), 7.04 (d, 2 H, 3′-, 5′-CH, J = 8.1 Hz), 7.70 (d, 2 H, 2′-, 6′-CH, J = 8.1 Hz), 8.60 (d, 1 H, CONH, J = 7.5 Hz), 10.29 (s, 1 H, 7-NH), 11.51 (s, 1 H, 3-NH); MS (FAB) m/z 501 (M^+), high-resolution FABMS calcd M^+ 501.5667, found 501.5686.

***N*-[4-[(2-Amino-6-methyl-3,4-dihydro-4-oxo-7*H*-pyrrolo-[2,3-*d*]pyrimidin-5-yl)thio]benzoyl]-L-glutamic Acid (5).** To a solution of **25** (0.10 g, 0.20 mmol) in ethanol (5 mL) was added 1 N NaOH (1 mL) and the solution stirred at room temperature for 24 h. The ethanol was evaporated under

reduced pressure, the residue was dissolved in water (5 mL), and the solution was stirred for a further 24 h. The solution was then cooled in an ice-bath and acidified carefully to pH 4.0 with dropwise addition of 3 N HCl. This suspension was left at 5 °C for 24 h and filtered. The residue was washed well with water and ether and dried over P_2O_5/vacuum to afford 0.08 g (89%) of **5** as a white solid: mp 217–220 °C; TLC R_f 0.87 ($CHCl_3$/MeOH/NH_4OH 3:9:1); UV λ_{max} (pH 1) 280 nm (ϵ 24 100); Λ_{max} (pH 7) 277 nm (ϵ 24 200); Λ_{max} (pH 13) 280 nm (ϵ 24 100); ^{1}H NMR (DMSO-d_6) δ 1.89–2.10 (m, 2 H, Gluβ-CH_2), 2.18 (s, 3 H, 6-CH_3), 2.33 (t, 2 H, Gluγ-CH_2, J = 7.2 Hz), 4.33–4.40 (m, 1 H, Gluα-CH), 6.10 (s, 2 H, 2-NH_2), 7.03 (d, 2 H, 3'-, 5'-CH, J = 8.4 Hz), 7.70 (d, 2 H, 2'-, 6'-CH, J = 8.4 Hz), 8.42 (d, 1 H, CONH, J = 7.5 Hz), 10.21 (s, 1 H, 7-NH), 11.44 (s, 1 H, 3-NH), 12.33 (br s, COOH); HPLC: t_R = 27.6 min (C_{18} silica gel, 8% CH_3CN in 0.1 M NaOAc, pH 5.5, flow rate 1.0 mL/min). Anal. Calcd for ($C_{19}H_{19}N_5O_6S$·0.25H_2O) C, H, N.

Enzymes and Enzyme Assays. FPGS was partially purified from CCRF-CEM human leukemia cells by ammonium sulfate fractionation and by gel sieving and phosphocellulose chromatography as previously described.[10] FPGS activity was assayed as described previously.[46] Assays contained ≈400 units of FPGS activity; one unit of FPGS catalyzes the incorporation of 1 pmol of [^{3}H]Glu/h. DHFR from CCRF-CEM cells was partially purified and assayed as described.[47] All DHFR (CCRF-CEM) assays contained 1.8×10^{-3} units of DHFR activity; one unit of DHFR can reduce 1 μmol of dihydrofolate/min under standard conditions.

Cell Lines and Methods for Measuring Growth Inhibitory Potency. The human T-lymphoblastic leukemic cell line CCRF-CEM[48] and its MTX-resistant subline R30dm[23] were cultured as described.[23] R30dm expresses only 1% of the FPGS activity of CCRF-CEM. The A253 and FaDu human squamous cell carcinoma monolayer cell lines were subcultured in RPMI 1640/10% fetal calf serum in 100 mm cell culture dishes (Falcon) as described.[10] Inhibition of growth of these cell lines during continous drug exposure was measured as described.[10,23] EC_{50} values were determined visually from plots of percent control growth versus the logarithm of drug concentration. Protection by metabolites against growth inhibitory effects was assayed by including metabolites (LV, TdR, Hx) 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 cell lines were verified to be negative for Mycoplasma contamination using the GenProbe test kit during the course of these studies.

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