Journal of Medicinal Chemistry

Article

Synthesis of *N*-{4-[(2,4-Diamino-5-methyl-4,7-dihydro-3*H*-pyrrolo[2,3-*d*]pyrimidin-6-yl)thio]benzoyl}-l-glutamic Acid and *N*-{4-[(2-Amino-4-oxo-5-methyl-4,7-dihydro-3*H*-pyrrolo[2,3-*d*]pyrimidin-6-yl)thio]benzoyl}-l-glutamic Acid as Dual Inhibitors of Dihydrofolate Reductase and Thymidylate Synthase and as Potential Antitumor Agents

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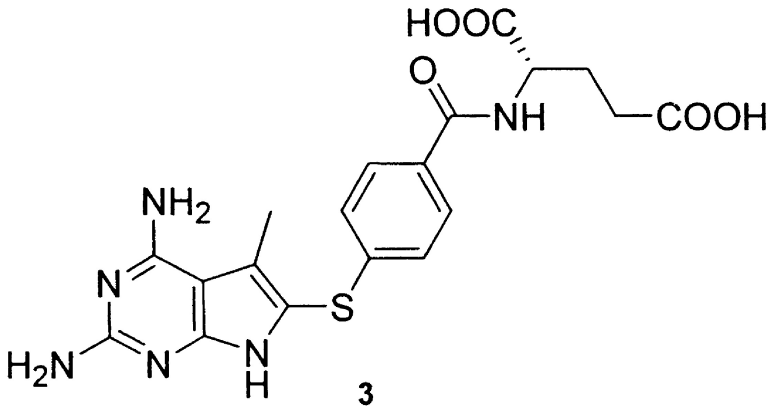

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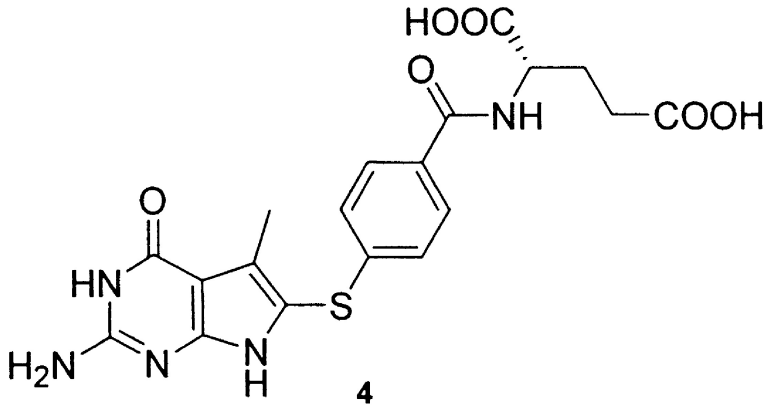

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Synthesis of *N*-{4-[(2,4-Diamino-5-methyl-4,7-dihydro-3*H*-pyrrolo[2,3-*d*]pyrimidin-6-yl)thio]benzoyl}-L-glutamic Acid and *N*-{4-[(2-Amino-4-oxo-5-methyl-4,7-dihydro-3*H*-pyrrolo[2,3-*d*]pyrimidin-6-yl)thio]benzoyl}-L-glutamic Acid as Dual Inhibitors of Dihydrofolate Reductase and Thymidylate Synthase and as Potential Antitumor Agents

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Two novel classical antifolates *N*-{4-[(2,4-diamino-5-methyl-4,7-dihydro-3*H*-pyrrolo[2,3-*d*]-pyrimidin-6-yl)thio]benzoyl}-L-glutamic acid **3** and *N*-{4-[(2-amino-4-oxo-5-methyl-4,7-dihydro-3*H*-pyrrolo[2,3-*d*]pyrimidin-6-yl)thio]benzoyl}-L-glutamic acid **4** were designed, synthesized, and evaluated as antitumor agents. Compounds **3** and **4** were obtained from 2,4-diamino-5-methylpyrrolo[2,3-*d*]pyrimidine **7** and 2-amino-4-oxo-5-methylpyrrolo[2,3-*d*]pyrimidine **12**, respectively, in a concise three-step sequence. Compound **3** is the first example, to our knowledge, of a 2,4-diamino classical antifolate that has potent inhibitory activity against both human dihydrofolate reductase (DHFR) and human thymidylate synthase (TS). Compound **4** was a dual DHFR−TS inhibitor against the bifunctional enzyme derived from *Toxoplasma gondii* (tg). Further evaluation of the mechanism of action of **3** implicated DHFR as its primary intracellular target. Both **3** and **4** were folylpolyglutamate synthetase (FPGS) substrates. Compound **3** also inhibited the growth of several human tumor cell lines in culture with $GI_{50} < 10^{-8}$ M. This study shows that the pyrrolo[2,3-*d*]pyrimidine scaffold is conducive to dual DHFR−TS and tumor inhibitory activity, and the potency is determined by the 4-position substituent.

Introduction

The synthesis of dTMP from deoxyuridylate (dUMP) is catalyzed by thymidylate synthase (TS). During this reductive methylation, 5,10-methylenetetrahydrofolate is converted to dihydrofolate (DHF). DHF is reduced to tetrahydrofolate (THF) via the enzyme dihydrofolate reductase (DHFR).[1] Thus, the important role folate metabolism plays in DNA synthesis and cell proliferation has made it a viable target for cancer chemotherapy.[2] The DHFR inhibitor methotrexate (MTX) (Chart 1) is a mainstay in single agent and combination cancer chemotherapy.[3] Inhibitors of TS such as raltitrexed (ZD1694) (Chart 1)[4] and the recently approved pemetrexed[5] are also clinically important antitumor agents.

It has been observed that when a DHFR inhibitor is used in combination with a TS inhibitor, synergistic growth inhibition can occur against *Lactobacillus casei*,[6,7] rat hepatoma cells,[8,9] and human lymphoma cells.[6,10,11] Therefore, it was of interest to design single agents that could function as dual inhibitors of TS and DHFR. Such inhibitors could circumvent the pharmacokinetic disadvantages of two separate drugs. It has been suggested[12] that the chemotherapeutic utility of such analogues would require that the inhibitory activity against DHFR be similar to that against TS.

As shown in Figure 1, folate analogues that inhibit TS generally contain a 2-amino-4-oxo or 2-methyl-4-oxo substitution in their pyrimidine ring, for example, PDDF (CB3717) (Chart 1) and raltitrexed.[13,14] In contrast, inhibitors of DHFR generally contain a 2,4-diamino substitution in the pyrimidine ring[13,14] (Figure 1), as typified by MTX (Chart 1). Gangjee et al.[15] hypothesized and demonstrated by crystal structure determination that classical folate analogues with a 6−5 fused ring system could bind to folate-related enzymes in either a "normal" mode (Figure 2), which is similar to the way that natural substrates or inhibitors bind, or a "flipped" mode, which is achieved by rotating the analogue in the normal binding mode by 180° about its NH_2−C2 bond (Figure 2). This could explain, in part, the inhibitory effects of pemetrexed, a 2-amino-4-oxo classical pyrrolo[2,3-*d*]pyrimidine antifolate, against both TS and DHFR. In binding to DHFR in the "flipped" mode, the pyrrole nitrogen of pemetrexed could mimic the 4-amino group of a 2,4-diamino folate analogue such as MTX.

The enzyme folylpolyglutamate synthetase (FPGS) adds L-glutamate moieties in γ-linkage to classical antifolates that contain a L-glutamate.[16] Polyglutamylation of classical antifolates used in cancer chemotherapy has certain advantages. The polyglutamylated

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Chart 1

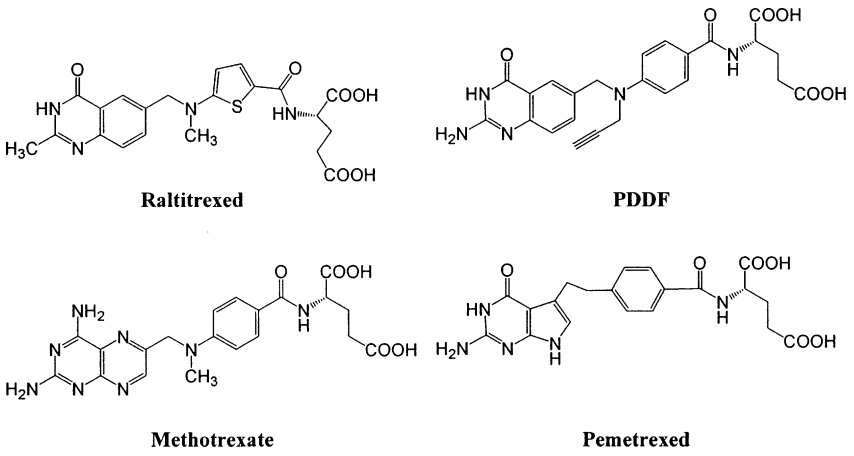
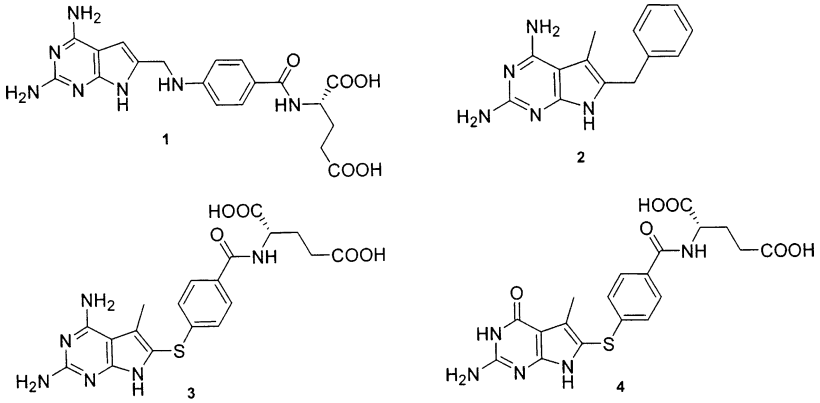


Chart 2



antifolates are retained in cells longer than monoglutamate forms. In addition, some polyglutamylated forms of classical antifolates inhibit the target folate-dependent enzyme(s) to a greater extent than does the monoglutamate forms. There are, however, certain drawbacks to this enzymatic transformation of classical antifolates. For those analogues that require polyglutamylation for activation, either for retention within tumor cells or to increase inhibitory activity against their target folate-dependent enzyme(s), tumor resistance to the antifolates can be manifested by reduction in the level of FPGS activity.[17] In addition, retention of polyglutamate forms of antifolates within normal cells may be a cause of toxicity.[18] Thus, classical antifolates that lack the necessity of polyglutamylation could circumvent resistance resulting from a decrease in the activity of FPGS.

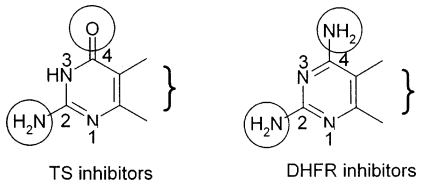


Figure 1.

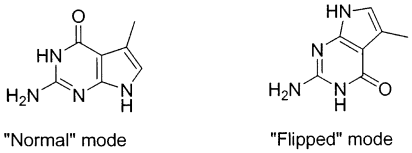
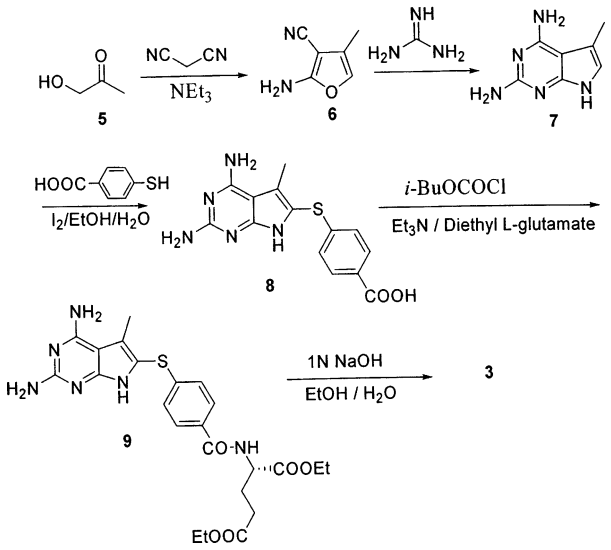


Figure 2.

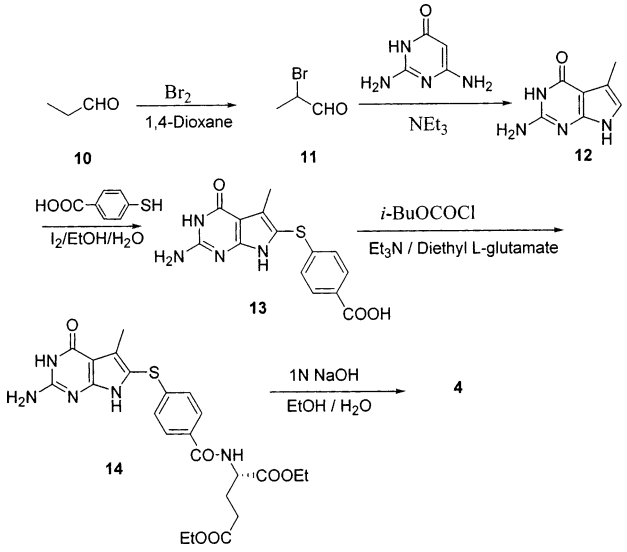
It is known that in some instances the presence of a 6- or 7-substituent prevents FPGS substrate activity in bicyclic antifolates with 6−5[19] or 6−6[20] fused ring systems, respectively. In an attempt to develop compounds that could function as DHFR inhibitors without the necessity of polyglutamylation, Gangjee et al. designed, synthesized, and evaluated the classical antifolate **1** (Chart 2) (data not published) with a 6-substituent. Compound **1**, however, did not afford potent DHFR inhibitory activity.

A 5-methyl group in some 6−6 fused antifolates can increase potency against DHFR. For example, Piper et al.[21] reported that the introduction of a 5-methyl substituent in 5-deaza analogues of aminopterin (AMT) and MTX resulted in a decrease in the K_i (from 3.7 to 2.9 nM) for inhibition of DHFR from L1210 cells, indicating that a methyl group at the 5-position was beneficial to binding to DHFR. Similarly, Hynes et al.[12] reported that 5-methyl-5,8-dideaza-AMT is 4 times as potent as the 5-desmethyl analogue against rat liver DHFR. Molecular modeling using SYBYL 6.8[22] indicated that a 5-methyl substitution in 6−5 fused ring systems, such as a pyrrolo[2,3-*d*]pyrimidine, lies in the same vicinity as a 5-methyl in 6−6 fused ring systems. Thus, a 5-methyl substitution was expected to have hydrophobic interaction with Val115 in human DHFR. Gangjee et al.[23] reported the nonclassical analogue **2** (Chart 2) to be 100-fold more potent than its 5-desmethyl analogue against human DHFR. Hence, the 5-methyl-6-thiolaryl classical analogue **3**[24] (Chart 2) was designed and synthesized as the first in a series of classical antifolate compounds to determine the effect of a 5-methyl sub-

Scheme 1



Scheme 2



stitution on biological activity. In addition to **3**, it was also of interest to synthesize its 2-amino-4-oxo analogue **4** (Chart 2), which, similar to pemetrexed, is a 2-amino-4-oxo classical pyrrolo[2,3-*d*]pyrimidine and was anticipated to perhaps display dual DHFR−TS inhibitory activity by attaching to TS in the normal mode and to DHFR in the flipped mode and hence to also afford antitumor activity.

Chemistry

The synthesis of **3** started from commercially available acetol (hydroxyacetone) **5** (Scheme 1), which was first transformed into the substituted furan **6** with malononitrile in the presence of triethylamine. Subsequent condensation with guanidine afforded compound **7** in a two-step overall yield of 48%.[25] Following a modification of the procedure reported by Gangjee et al.,[26] 4-mercaptobenzoic acid was oxidatively added to the 6-position of compound **7** in the presence of iodine and H_2O/EtOH (1:2) mixture at reflux to give **8** in 95% yield. Peptide coupling of the acid **8** with diethyl L-glutamate using the mixed anhydride method with isobutyl chloroformate and triethylamine followed by chromatographic purification afforded the coupled product **9** in 82% yield. Hydrolysis of the diester **9** with aqueous sodium hydroxide at room temperature, followed by acidification afforded the target compound **3** in 68% yield.

The synthesis of **4** (Scheme 2) started from commercially available propionaldehyde **10**, which was regioselectively α-brominated with bromine in the presence of a catalytic amount of 1,4-dioxane at 0 °C to afford 2-bromopropionaldehyde **11**. Subsequent condensation of **11** with 2,4-diamino-6-oxopyrimidine in DMSO and triethylamine as base afforded 2-amino-4-oxo-5-methylpyrolo[2,3-*d*]pyrimidine **12** after chromatography (silica gel, chloroform/methanol 10:1) in 70% yield. In a procedure similar to the synthesis of **8**, 4-mercaptobenzoic acid was oxidatively added to the 6-position of compound **12** in the presence of iodine in the mixture H_2O/EtOH (1:2) to give compound **13** in 29% yield. Coupling of the acid **13** with diethyl L-glutamate using the mixed anhydride method with isobutyl chloroformate and triethylamine followed by chromatographic purification gave the coupled product **14** in 24% yield. Hydrolysis of the diester **14** with aqueous sodium hydroxide at room temperature followed by careful acidification afforded the target compound **4** in 45% yield.

Biological Evaluation and Discussion

Compounds **3** and **4** were evaluated as inhibitors of human (h), *Escherichia coli* (ec), and *Toxoplasma gondii* (tg) DHFR and TS. The inhibitory potency (IC_{50}) values are compared with PDDF, MTX, raltitrexed, and pemetrexed. Compound **3** is a good inhibitor of hDHFR and hTS with submicromolar IC_{50} values. Thus, compound **3** is a novel dual DHFR−TS inhibitor. To our knowledge this is the first example of a classical 2,4-diamino pyrrolo[2,3-*d*]pyrimidine antifolate that possesses dual DHFR−TS inhibitory activity. Compound **3** was about 10-fold more potent as a hDHFR inhibitor than PDDF and about 300-fold more potent than pemetrexed. Against hTS compound **3** has similar inhibitory potency as PDDF and raltitrexed but was more than an order of magnitude better than pemetrexed.

It is important to note that while the standard compounds PDDF, raltitrexed, pemetrexed, and MTX have good inhibitory activity against one of the enzymes (hDHFR or hTS), only compound **3** has good inhibitory activity against both hDHFR and hTS.

Compound **4** was a poor inhibitor of hDHFR and ecDHFR (Table 1) but showed moderate inhibition against tgDHFR. Compound **4** was a poor inhibitor of hTS and ecTS but was a moderate inhibitor of tgTS. Thus, compound **4**, like compound **3**, is a dual inhibitor of tgDHFR and tgTS.

The only difference between **3** and **4** is the 4-substitution. Compound **4** is a 2-amino-4-oxo analogue, which should bind to TS in the "normal" mode. The poor activity of compound **4** against human TS was surprising and suggests that for classical 2-amino-4-oxo pyrrolo[2,3-*d*]pyrimidines the 6-aryl substitution may be detrimental to potent hTS inhibitory activity (compare **4** with **3** and pemetrexed). However, like **3** and pemetrexed, compound **4** is a dual DHFR−TS inhibitor but was much less potent than **3**. These results suggest that the pyrrolo[2,3-*d*]pyrimidine scaffold is conducive to

Table 1. Inhibitory Concentrations (IC_{50}, M) of DHFR and TS[a]

	DHFR			TS		
compd	human[b]	*E. coli*[c]	*T. gondii*[d]	human[e]	*E. coli*[e]	*T. gondii*[d]
3	2.1×10^{-7}	1.6×10^{-8}	1.7×10^{-7}	5.4×10^{-7}	$>1.8 \times 10^{-4}$	1.8×10^{-6}
4	$>2.4 \times 10^{-4}$	$>2.4 \times 10^{-4}$	2.2×10^{-6}	1.0×10^{-4}	$>1.8 \times 10^{-4}$	3.6×10^{-6}
PDDF[f]	1.9×10^{-6}	2.3×10^{-5}	2.2×10^{-7}	1.0×10^{-7}	7.2×10^{-8}	1.8×10^{-8}
methotrexate	2.2×10^{-8}	6.6×10^{-9}	1.1×10^{-8}	2.9×10^{-5}	9.0×10^{-5}	1.8×10^{-5}
raltitrexed[g]	2.1×10^{-5}	2.3×10^{-5}	2.3×10^{-6}	3.8×10^{-7}	5.7×10^{-6}	9.0×10^{-7}
pemetrexed[h]	1.5×10^{-6}	2.3×10^{-4}	4.6×10^{-7}	9.5×10^{-6}	7.6×10^{-5}	2.8×10^{-6}

[a] The percent inhibition was determined at a minimum of four inhibitor concentrations within 20% of the 50% point. The standard deviations for determination of 50% points were within ±10% of the value given. [b] Kindly provided by Dr. J. H. Freisheim, Medical College of Ohio, Toledo, OH. [c] Kindly provided by Dr. R. L. Blakley, St. Jude Children's Hospital, Memphis, TN. [d] Kindly provided by Dr. K. Anderson, Yale University. [e] Kindly provided by Dr. F. Maley, New York State Department of Health, Albany, NY. [f] Kindly provided by Dr. M. G. Nair, University of South Alabama. [g] Kindly provided by Dr. A. Jackman, Institute of Cancer Research, Sutton, Surrey, U.K. [h] Kindly provided by Dr. C. Shih, Eli Lilly.

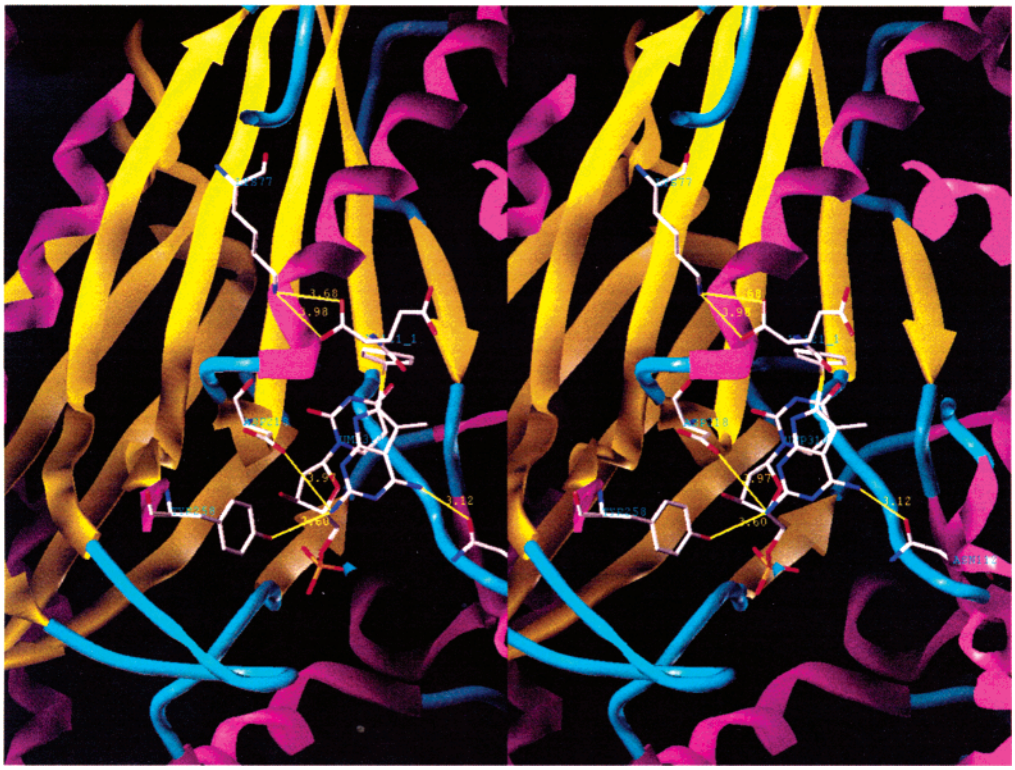


Figure 3. Stereoview of compound **3** modeled in human TS.[27]

dual inhibition of DHFR and TS and that for 6-aryl-substituted analogues potency is determined by the 4-substituent.

Compound **3** was subsequently modeled into the binding site of human TS using SYBYL 6.8[22] as shown in Figure 3. The coordinates of hTS were extracted from the coordinates of the hTS−dUMP−pemetrexed ternary complex[27] in the Protein Data Bank (Protein Data Bank code 1JU6). The binding pocket of human TS is capable of accommodating **3** when it binds in the flipped mode. In this binding mode, the 2-NH_2 hydrogen-bonds with both Tyr258 and Asp218, while the 4-NH_2 hydrogen-bonds with Asn112. The Glu α-carboxyl group of **3** interacts with Lys77. In addition, there is a stacking effect between the pyrrole ring of **3** and the pyrimidine ring of dUMP.

Compounds **3** and **4** were also evaluated as inhibitors of the growth of CCRF-CEM human leukemia cells[28] in culture during continuous exposure (Table 2).[29,30] Compound **3** was a potent inhibitor of CCRF-CEM cell[28] growth with an EC_{50} value that is only 15-fold greater than MTX. Compound **4** did not inhibit the growth of CCRF-CEM at 20 μM. These results were consistent with the enzyme inhibitory potencies of the compounds.

Table 2. Growth Inhibition of Parental CCRF-CEM Human Leukemia Cells and Sublines with Single, Defined Mechanisms of MTX Resistance during Continuous (0−120 h) Exposure to MTX or **3**

	EC_{50},[a] nM			
compd	CCRF-CEM	R1[b]	R2[c]	R30dm[d]
3	190 ± 10	8300 ± 800	7750 ± 0.5	515 ± 15
4	>20000	>20000	>20000	>20000
MTX	12.5 ± 0.5	615 ± 35	1500 ± 0	14.5 ± 0.5

[a] Values presented are the average ± range. [b] CCRF-CEM subline resistant to MTX solely as a result of a 20-fold increase in wild-type DHFR protein and activity.[30] [c] CCRF-CEM subline resistant as a result of decreased uptake of MTX.[31] [d] CCRF-CEM subline resistant to MTX solely as a result of decreased polyglutamylation.[28] This cell line has 1% of the FPGS specific activity (measured with MTX as the folate substrate) of parental CCRF-CEM.

To further elucidate the mechanism of action of compound **3**, it was evaluated as an inhibitor of the

Table 3. Activities of **3** and **4** as Substrates for Human FPGS

substrate	K_m (μM)	V_{max}(rel)	V_{max}(rel)/K_m	n
(a) Activity of **3** as a Substrate for Human FPGS[a]				
AMT	4.6	1.0	0.22	1
3	4.1 ± 1	0.17	0.04	2
(b) Activity of **4** as a Substrate for Human FPGS[a]				
AMT	6 ± 1	1.0	0.17	2
4	5.1 ± 0.8	0.29 ± 0	0.06	2

[a] FPGS substrate activity was determined as previously described.[32] Values presented are the average ± range. V_{max} is calculated relative to AMT within the same experiment.

Table 4. Protection by Leucovorin (LV) against the Growth Inhibition of CCRF-CEM Human Leukemia Cells

		growth relative to untreated control (%)			
compd	[compd] (nM)	[LV] = 0 μM	[LV] = 0.1 μM	[LV] = 1 μM	[LV] = 10 μM
3	1000	5	69	88	93
MTX	20	5	90	96	95

growth of three CCRF-CEM sublines with defined mechanisms of resistance to MTX and the results were compared with those of compound **4** and MTX (Table 2). DHFR overexpressing line R1[31] was 44-fold cross-resistant to **3**, which was similar to the cross-resistance of R1 to MTX (49-fold), indicating that DHFR is likely the primary target of compound **3**. The MTX-transport resistant subline R2,[32] which does not express functional reduced folate carrier (RFC), is 41-fold cross-resistant to **3**, while it is 120-fold cross-resistant to MTX. The data suggest that **3** utilizes the RFC as its primary means of transport. The difference in resistance levels probably reflects the fact that at the higher concentration required by **3** compared to MTX, passive diffusion contributes to its uptake. The R30dm subline[29] expressing low levels of FPGS is about 3-fold cross-resistant to **3** under continuous exposure conditions, suggesting that polyglutamates of **3** must be considered as part of its mechanism of action.

The cross-resistance data for FPGS-deficient subline R30dm suggest that **3** may be a human FPGS substrate. In this connection, the activities of **3** and **4** were evaluated in vitro with recombinant human FPGS[33] and compared to that of AMT, a good substrate for FPGS. The data (Table 3) show that both **3** and **4** were human FPGS substrates but they were 5- and 3-fold less efficient than AMT, respectively. These data again support the notion that metabolism to polyglutamates may be important in the mechanism of action of **3.** The contribution of polyglutamylation cannot be discerned for **4** because of its inactivity in the growth inhibition assays. These results of **3** and **4** as FPGS substrates are in contrast to previous findings[19] that the presence of a 6-substituent in 6−5 fused ring systems prevents FPGS substrate activity in antifolates.

Metabolite protection studies were preformed to further elucidate the mechanism of action of **3**. At concentrations of drug that inhibited growth of CCRF-CEM cells by more than 90%, leucovorin at 0.1 μM was able to fully protect against the effects of both **3** and MTX (Table 4). This is consistent with an antifolate mechanism of action for **3**.

Further studies against CCRF-CEM cells in culture examined the ability of thymidine (TdR) and hypoxanthine (Hx) to protect against growth inhibition of **3** (Table 5). These metabolites can be salvaged to produce dTTP and the purine dNTPs required for DNA synthesis and thus bypass the MTX blockade.[34] In T-lymphoblast cell lines such as CCRF-CEM, TdR can only be tested in the presence of dCyd, which reverses its toxic effects; however, dCyd has no protective effect on MTX either alone or in paired combination with either Hx or TdR. The data (Table 5) show that for both MTX and **3**, TdR alone protected poorly and Hx alone did not protect. Both metabolites were required to achieve substantial protection. This indicates that both purine and thymidylate synthesis are inhibited and is consistent with DHFR being the primary target of **3**.

Table 5. Protection of CCRF-CEM Human Leukemia Cells against the Growth Inhibitory Effects of MTX and **3** by 10 μM Hypoxanthine (Hx), 5 μM Thymidine (TdR), and Their Combination

		relative growth (%)[b]			
compd	concn (nM)	no addition	5 μM TdR	10 μM Hx	Hx + TdR
3	4000	12 ± 0	20 ± 0	11 ± 0	71 ± 0
MTX	40	12 ± 1	18 ± 0	18 ± 3	68 ± 1

[a] Growth is expressed relative to quadruplicate cultures not treated with either drug or metabolite and is the average ± range for duplicate treated samples. The experiment was repeated with similar results. [b] Deoxycytidine (dCyd, 10 μM) was present in all the above cultures to prevent the inhibition of growth caused in T-cell leukemias such as CCRF-CEM by TdR. In typical results, dCyd alone had no effect on CCRF-CEM growth (96−3% of control) and did not protect against growth inhibition by either drug (data not shown). Hx alone (95 ± 2% of control) or in the presence of dCyd (97 ± 2% of control) did not affect CCRF-CEM growth and did not protect against MTX-induced growth inhibition (table above). TdR alone inhibited growth of CCRF-CEM (31 ± 1% of control), but TdR + dCyd was essentially not growth inhibitory (93 ± 3% of control), and neither protected against MTX-induced growth inhibition (table above). Similarly, Hx + TdR + dCyd did not appreciably inhibit growth of CCRF-CEM (90 ± 2% of control).

Table 6. Cytotoxicity Evaluation (GI_{50}, M) of Compound **3** against Selected Tumor Cell Lines[35]

cell line	GI_{50} of **3** (M)
leukemia	
CCRF-CEM	2.21×10^{-7}
HL-60 (TB)	4.35×10^{-8}
K-562	2.95×10^{-8}
MOLT-4	$<1.00 \times 10^{-8}$
RPMI-8226	$<1.00 \times 10^{-8}$
nonsmall cell lung cancer	
NCI-H460	6.31×10^{-7}
colon cancer	
HT29	1.60×10^{-7}
SW-620	1.24×10^{-7}
central nervous system cancer	
U251	8.24×10^{-7}
melanoma	
LOX IMVI	1.71×10^{-7}
prostate cancer	
PC-3	4.36×10^{-8}

Compound **3** was selected by the National Cancer Institute (NCI) for evaluation in its in vitro preclinical antitumor screening program.[35] The ability of compound **3** to inhibit the growth of tumor cells was measured as GI_{50} values, the concentration required to inhibit the growth of tumor cells in culture by 50% compared to a control. In 11 of the 60 tumor cell lines evaluated, compound **3** showed GI_{50} values of $\leq 10^{-7}$ M (Table 6). It is noteworthy that compound **3** was not a general cell poison but showed selectivity both within a type of

tumor cell line and across different tumor cell lines, with inhibitory values which in some instances differed by 10000-fold. In all but one leukemia cell line (CCRF-CEM) and the prostate cancer cell line PC-3, compound **3** displayed GI_{50} values of $\leq 10^{-8}$ M, which was more potent than either its human DHFR or human TS inhibitory activity alone and could be the result of a synergistic effect of dual inhibitory activities against TS and DHFR and/or that polyglutamylation increases inhibitory activity against TS and/or DHFR in cell systems. Compound **3** is currently under further evaluation by the NCI as an antitumor agent.

Experimental Section

All evaporations were carried out in vacuo with a rotary evaporator. Analytical samples were dried in vaccuo (0.2 mmHg) in a CHEM-DRY drying apparatus over P_2O_5 at 70 °C. Thin-layer chromatography (TLC) was performed on silica gel plates with fluorescent indicator. Spots were visualized by UV light (254 and 365 nm). All analytical samples were homogeneous on TLC in at least two different solvent systems. Purification by column and flash chromatography was carried out using Merck silica gel 60 (200−400 mesh). The amount (weight) of silica gel for column chromatography was in the range of 50−100 times the amount (weight) of the crude compounds being separated. Columns were dry-packed unless specified otherwise. Solvent systems are reported as volume percent of mixture. Melting points were determined on a MEL-TEMP II melting point apparatus with a FLUKE 51 K/J electronic thermometer and are uncorrected. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker WH-300 (300 MHz) spectrometer. The chemical shift (δ) values are reported as parts per million (ppm) relative to tetramethylsilane as internal standard; s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad singlet. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA. Elemental compositions were 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 removed despite 24 h of drying in vacuo and were confirmed, where possible, by their presence in the ^{1}H NMR spectrum. All solvents and chemicals were purchased from Aldrich Chemical Co. or Fisher Scientific and were used as received.

4-(2,4-Diamino-5-methylpyrrolo[2,3-*d*]pyrimidin-6-ylthio)benzoic Acid (8). A solution of 2,4-diamino-5-methylpyrrolo[2,3-*d*]pyrimidine **7** (210 mg, 1.3 mmol) and 4-mercaptabenzoic acid (0.4 g, 2.6 mmol) in a 250 mL round-bottom flask and 60 mL of a mixture of ethyl alcohol and water (v/v 2:1) was heated to reflux, and iodine (0.66 g, 2.6 mmol) was then added to the solution at reflux. The reaction mixture was maintained at reflux for 2 h and then allowed to cool to room temperature. The precipitate obtained was filtered, and the filtrate was evaporated under reduced pressure, washed with ethyl acetate, and filtered. The residue was washed with a mixture of ethyl acetate and ethyl alcohol (v/v 2:1) to afford **8** (390 mg, 95%) as a light-yellow solid: mp >250 °C (dec); TLC R_f = 0.40 ($CHCl_3$/MeOH, 5:1, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 2.31 (s, 3H, 5-CH_3), 6.00 (br, 2H, NH_2), 6.66 (s, 2H, NH_2), 7.06 (d, 2H, C_6H_4), 7.82(d, 2H, C_6H_4), 12.15 (s, 1H, N7−H). HRMS (EI) *m/e* Calcd for $C_{14}H_{13}N_5O_2S$: 315.078997. Found: (M^+) 315.078647.

Diethyl *N*-[4-[(2,4-Diamino-5-methyl-3,4-dihydro-7*H*-pyrrolo[2,3-*d*]pyrimidin-6-yl)thio]benzoyl]-L-glutamate (9). To a suspension of the acid **8** (0.2 g, 0.63 mmol) in anhydrous DMF under N_2 was added triethylamine (260 μL, 1.88 mmol). The solution was cooled to 0 °C, and isobutyl chloroformate (160 μL, 1.23 mmol) was added, followed, 15 min later, by diethyl L-glutamate hydrochloride (0.23 g, 0.94 mmol) and immediately followed by triethylamine (260 μL, 1.88 mmol). The reaction mixture was warmed slowly to room temperature and stirred for 12 h. To the reaction mixture was added 500 mg of silica gel and the solvent evaporated under reduced pressure (oil pump). The resulting silica gel plug was loaded on a dry silica gel column, and the column was flushed with $CHCl_3$ and eluted with 2% MeOH in $CHCl_3$. The fractions showing a single spot (TLC) were pooled and evaporated to afford 0.25 g (82%) of **9**: mp 204.8−207.3 °C (dec); TLC R_f = 0.65 ($CHCl_3$/MeOH, 5:1, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 1.15 (m, 6H, CH_2CH_3), 1.98−2.08 (m, 2H, Gluβ-CH_2), 2.31 (s, 3H, 5-CH_3), 2.42 (t, 2H, Gluγ-CH_2), 4.08 (m, 4H, CH_2CH_3), 4.40−4.44 (br, 1H, Gluα-CH), 5.65 (s, 2H, NH_2), 6.31 (s, 2H, NH_2), 7.04 (d, 2H, 3′-, 5′-CH), 7.76 (d, 2H, 2′-, 6′-CH), 8.64 (d, 1H, CONH), 11.01 (s, 1H, N7−H). HRMS (EI) *m/e* Calcd for $C_{23}H_{28}N_6O_5S$: 500.184190. Found: (M^+) 500.185676.

***N*-[4-[(2,4-Diamino-5-methyl-3,4-dihydro-7*H*-pyrrolo[2,3-*d*]pyrimidin-6-yl)thio]benzoyl]-L-glutamic Acid (3).** To a solution of **9** (0.04 g, 0.08 mmol) in EtOH 95 mL was added 1 N NaOH (1 mL), and the solution was stirred at room temperature for 24 h. The EtOH 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 the dropwise addition of 3 N HCl. This suspension was chilled to −78 °C and was kept refrigerated overnight and filtered. The residue was washed well with water and dried over P_2O_5/vacuum to afford 0.025 g (68%) of **3** as a white solid: mp >236.3 °C (dec); TLC R_f = 0.82 ($CHCl_3$/MeOH, 5:1, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 1.92−2.05 (m, 2H, Gluβ-CH_3), 2.31 (m, 5H, 5-CH_3 and Gluγ-CH_2), 4.36 (br, 1H, Gluα-CH), 6.16 (s, 2H, NH_2), 6.83 (s, 2H, NH_2), 7.04 (d, 2H, 3′-, 5′-CH), 7.77 (d, 2H, 2′-, 6′-CH), 8.55 (d, 1H, CONH), 11.37 (s, 1H, N7−H), 12.30 (br, 2H, COOH). Anal. ($C_{19}H_{20}N_6O_5S\cdot 1.0H_2O\cdot 0.6HCl$) C, H, N, Cl, S.

2-Bromopropanal (11). To a 250 mL round-bottom flask was added propionaldehyde (40 mL, 0.53 mol) and 5 mL of 1,4-dioxane. The reaction mixture was kept at 0 °C, and bromine (27 mL, 0.53 mol) was added dropwise within 1 h. The reaction was allowed to continue for an additional 10 min till the reaction mixture became colorless. 2-Bromopropanal (30 mL, 62%) was obtained as a colorless oil following distillation of the reaction mixture under reduced pressure.

2-Amino-4-oxo-5-methylpyrrolo[2,3-*d*]pyrimidine (12). To a solution of 2,4-diamino-6-oxopyrimidine (5 g, 39 mmol) in 30 mL of DMSO was added 2-bromopropanal (5 mL, 48 mmol) and 10 mL of triethylamine. The reaction mixture was stirred vigorously at room temperature for 1 h and then loaded on a dry silica gel column. The column was eluted with 1.5 L of $CHCl_3$ and then with 1.5 L of $CHCl_3$/MeOH (10:1). The fractions containing the product (TLC) were pooled and $CHCl_3$ was evaporated to afford 4.6 g (71%) of compound **12**: TLC R_f = 0.45 ($CHCl_3$/CH_3OH 5:1); ^{1}H NMR (Me_2SO-d_6) δ 2.12 (m, 3H, 5-Me), 5.94 (s, 2H, 2-NH_2), 6.28 (s, 1H, 6-H), 10.09 (s, 1H, NH), 10.56 (s, 1H, NH).

4-(2-Amino-4-oxo-5-methylpyrrolo[2,3-*d*]pyrimidin-6-ylthio)benzoic Acid (13). To a 250 mL round-bottom flask were added 2,4-diamino-5-methylpyrrolo[2,3-*d*]pyrimidine **12** (0.5 g, 3.47 mmol) and 4-mercaptabenzoic acid (0.94 g, 6.1 mmol). Both reactants dissolved on the addition of 60 mL of a mixture of EtOH and H_2O (v/v 2:1). The reaction mixture was heated to reflux, and iodine (1.55 g, 6.1 mmol) was then added to the solution at reflux. The reaction mixture was kept at reflux for an additional 2 h. Following this, the reaction mixture was allowed to cool to room temperature and the precipitate obtained was filtered. To the filtrate was added a solution of 60 mL of 1 N sodium thiosulfate in water. The reaction mixture was kept at room temperature for 12 h, and the resulting precipitate was filtered to afford 280 mg (29% yield) of compound **13** as a light-brown solid: TLC R_f = 0.42 ($CHCl_3$/MeOH, 5:1, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 2.25 (s, 3H, 5-CH_3), 6.38 (s, 2H, NH_2), 7.14 (d, 2H, C_6H_4), 7.82 (d, 2H, C_6H_4), 10.53 (s, 1H, NH), 11.46 (s, 1H, NH).

Diethyl *N*-[4-[(2-Amino-4-oxo-5-methyl-3,4-dihydro-7*H*-pyrrolo[2,3-*d*]pyrimidin-6-yl)thio]benzoyl]-L-glutamate (14). To a suspension of the acid **13** (0.2 g, 0.62 mmol) in anhydrous DMF under N_2 was added triethylamine (260

μL, 1.88 mmol). The solution was cooled to 0 °C, and isobutyl chloroformate (160 μL, 1.23 mmol) was added, followed 15 min later by diethyl L-glutamate hydrochloride (0.23 g, 0.94 mmol) and immediately followed by triethylamine (260 μL, 1.88 mmol). The reaction mixture was warmed slowly to room temperature and stirred for 12 h. To the reaction mixture was added 500 mg of silica gel, and the solvent was evaporated under reduced pressure (oil pump). The resulting silica gel plug was loaded on a dry silica gel column, and the column was flushed with $CHCl_3$ and eluted with 2% MeOH in $CHCl_3$. The fractions showing a single spot (TLC) were pooled and evaporated to afford 0.075 g (24%) of **14**: mp 167.8−171.8 °C (dec); TLC R_f = 0.60 ($CHCl_3$/MeOH, 5:1, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 1.15 (t, 6H, CH_2CH_3), 1.92−2.15 (m, 2H, Gluβ-CH_2), 2.22 (s, 3H, 5-CH_3), 2.42(t, 2H, Gluγ-CH_2), 4.04 (q, 4H, CH_2CH_3), 4.40 (s, 1H, Gluα-CH), 6.22 (s, 2H, NH_2), 7.04 (d, 2H, 3′-, 5′-CH), 7.76 (d, 2H, 2′-, 6′-CH), 8.64 (d, 1H, CONH), 10.33 (s, 1H, NH), 11.31 (s, 1H, NH). HRMS (EI) *m/e* Calcd for $C_{23}H_{27}N_5O_6S$: 501.168206. Found: (M^+) 501.168611.

***N*-[4-[(2-Amino-4-oxo-5-methyl-3,4-dihydro-7*H*-pyrrolo-[2,3-*d*]pyrimidin-6-yl)thio]benzoyl]-L-glutamic Acid (4).** Compound **4** (0.025 g, 45%) was obtained from **14** (0.06 g, 0.12 mmol) using the procedure described above for the synthesis of **3**: mp 242.0−244.5 °C (dec); TLC R_f = 0.80 ($CHCl_3$/MeOH, 5:1, silica gel); ^{1}H NMR (Me_2SO-d_6) δ 1.92−2.10 (m, 2H, Gluβ-CH_2), 2.22 (s, 3H, 5-CH_3), 2.33 (t, 2H, Gluγ-CH_2), 4.39 (t, 1H, Gluα-CH), 6.22 (s, 2H, NH_2), 7.04 (d, 2H, 3′-, 5′-CH), 7.78 (d, 2H, 2′-, 6′-CH), 8.52 (d, 1H, CONH), 10.32 (s, 1H, NH), 11.31 (s, 1H, NH), 12.30 (br, 2H, COOH). Anal. ($C_{19}H_{19}N_5O_6S\cdot 0.5H_2O$) C, H, N, S.

Dihydrofolate Reductase (DHFR) Assay.[36] All enzymes were assayed spectrophotometrically in a solution containing 50 μM dihydrofolate, 80 μM NADPH, 0.05 M Tris-HCl, 0.001 M 2-mercaptoethanol, and 0.001 M EDTA at pH 7.4 and 30 °C. The reaction was initiated with an amount of enzyme yielding a change in optical density at 340 nm of 0.015/min.

Thymidylate Synthase (TS) Assay. TS was assayed spectrophotometrically at 30 °C and pH 7.4 in a mixture containing 0.1 M 2-mercaptoethanol, 0.0003 M (6*R*,*S*)-tetrahydrofolate, 0.012 M formaldehyde, 0.02 M $MgCl_2$, 0.001 M dUMP, 0.04 M Tris-HCl, and 0.000 75 M NaEDTA. This was the assay described by Wahba and Friedkin[37] except that the dUMP concentration was increased 25-fold according to the method of Davisson et al.[38] The reaction was initiated by the addition of an amount of enzyme yielding a change in absorbance at 340 nm of 0.016/min in the absence of inhibitor.

Cell Lines and Methods for Measuring Growth Inhibitory Potency. Drug solutions were standardized using extinction coefficients. Extinction coefficients were determined for **3** [pH 1, λ_{max} = 235 nm (24 900) and λ_{max} = 277 nm (20 000); pH 7, λ_{max} = 274 nm (20 200); pH 13, λ_{max} 275 nm (20 100)] and for **4** [pH 1, λ_{max-1} = 278 nm (29 200); pH 7, λ_{max} = 278 nm (33 100); pH 13, λ_{max} = 290 nm (29 000)]. Extinction coefficients for MTX, a gift of Immunex (Seattle, WA), were from the literature.[39] AMT, Hx, TdR, and dCyd were purchased from Sigma Chemical Co. (St. Louis, MO). Calcium leucovorin (LV) was purchased from Schircks Laboratories (Jona, Switzerland). Other chemicals and reagents were reagent grade or higher.

Cell lines were verified to be negative for mycoplasma contamination (Mycoplasma Plus PCR primers, Stratagene, La Jolla, CA). The human T-lymphoblastic leukemia cell line CCRF-CEM[28] and its MTX-resistant sublines R1,[31] R2,[32] and R30dm[29] were cultured as described.[29] R1 expresses 20-fold elevated levels of DHFR, the target enzyme of MTX. R2 has dramatically reduced MTX uptake but normal levels of MTX-sensitive DHFR. R30dm expresses 1% of the folylpolyglutamate synthetase (FPGS) activity of CCRF-CEM and is resistant to short-term, but not continuous, MTX exposure; however, R30dm is cross-resistant in continuous exposure to antifolates that require polyglutamylation to form potent inhibitors. Growth inhibition of all cell lines by continuous drug exposure was assayed as described.[29,30] EC_{50} values (drug concentration effective at inhibiting cell growth by 50%) were determined visually from plots of percent growth relative to a solvent-treated control culture versus the logarithm of drug concentration.

Protection against growth inhibition of CCRF-CEM cells was assayed by including LV at 0.1−10 μM with a concentration of drug previously determined to inhibit growth by 90−95%; the remainder of the assay was as described. Growth inhibition was measured relative to the appropriate leucovorin-treated control; leucovorin, even at 10 μM, caused no growth inhibition in the absence of drug, however. Protection against growth inhibition of CCRF-CEM cells was assayed by including Hx (10 μM), TdR (5 μM), or dCyd (10 μM) individually, in pairs (Hx + dCyd, TdR + dCyd), or all together (Hx + TdR + dCyd) with concentrations of MTX and **3** that would inhibit growth by ∼90−95% over a growth period of ∼72 h. The growth period was limited because beyond 72 h CCRF-CEM cells deplete TdR in the growth media and drug effects are no longer protected. dCyd is added only to alleviate the growth inhibitory effects of 5 μM TdR against CCRF-CEM cells.[40] Controls with metabolites alone (no drug) in the combinations described above (in duplicate), controls with drug alone with no metabolites (in duplicate), and untreated controls with neither drugs nor metabolites (in quadruplicate) were used. Growth inhibition was measured as percent growth relative to untreated control cells (absence of drugs and metabolites).

Folylpolyglutamate Synthetase (FPGS) Purification and Assay. Recombinant human cytosolic FPGS was purified and assayed as described previously. Whereas **3** was essentially quantitatively recovered during the standard assay procedure, **4** was nearly quantitatively recovered during a modified assay procedure in which a total of 4 mL of 0.1 N HCl, rather than the standard 3 mL, was used to elute the assay columns. The quantitative recovery of the drugs ensured that their polyglutamate products would also be quantitatively recovered. Kinetic constants were determined by the hyperbolic curve-fitting subroutine of SigmaPlot (Jandel) or Kaleidagraph (Synergy Software) using a ≥10-fold range of substrate concentration. Activity was linear with respect to time at the highest and lowest substrate concentrations tested. Assays contained ∼400 units of FPGS activity; 1 unit of FPGS catalyzes incorporation of 1 pmol of [^{3}H]glutamate/h.

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Supporting Information Available: Results from elemental analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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