Benzoyl Ring Halogenated Classical 2-Amino-6-methyl-3,4-dihydro-4-oxo-5-substituted Thiobenzoyl-7*H*-pyrrolo[2,3-*d*]pyrimidine Antifolates as Inhibitors of Thymidylate Synthase and as Antitumor Agents[1]

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In an attempt to circumvent resistance to and toxicity of clinically used folate-based thymidylate synthase (TS) inhibitors that require folylpoly-γ-glutamate synthetase (FPGS) for their antitumor activity, we designed and synthesized two classical 6−5 ring-fused analogues, *N*-[4-[(2-amino-6-methyl-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)thio]-2′-fluorobenzoyl]-L-glutamic acid (**4**) and *N*-[4-[(2-amino-6-methyl-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)thio]-2′-chlorobenzoyl]-L-glutamic acid (**5**), as TS inhibitors and antitumor agents. The key intermediates in the synthesis of these classical analogues were the mercaptans **10** and **11**, which were obtained from the corresponding nitro compounds **6** and **7** respectively, by reduction of the nitro groups followed by diazotization of the amines. The syntheses of analogues **4** and **5** were achieved via the oxidative addition of the sodium salt of ethyl 2-halo-substituted-4-mercaptobenzoate (**16** or **17**) to 2-amino-6-methyl-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidine (**18**) in the presence of iodine. The esters obtained from the reaction were deprotected and coupled with diethyl-L-glutamate followed by saponification. Compounds **4** and **5** were both more potent inhibitors of human TS (IC_{50} values of 54 and 51 nM, respectively) than were PDDF and the clinically used ZD1694 and LY231514. Compounds **4** and **5** were not substrates for human FPGS up to 250 μM. In addition, **4** and **5** were growth inhibitory against CCRF-CEM cells as well as a number of other tumor cell lines in culture, and protection studies established TS as the principal target of these analogues.

Introduction

Due to the involvement of folates in the biosynthesis of nucleic acid precursors, inhibitors of folate metabolism have provided important therapeutic agents useful in cancer chemotherapy.[2,3] Thymidylate synthase (TS) catalyzes the reductive methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) to 2′-deoxythymidine-5′-monophosphate (dTMP) utilizing 5,10-methylenetetrahydrofolate as the source of the methyl group as well as the reductant.[4] This represents the sole de novo source of dTMP; hence, inhibition of TS, in the absence of salvage, leads to "thymineless death". Thus inhibition of TS is an attractive goal for the development of antitumor agents.[3,5] PDDF,[6] ZD1694[7] (Tomudex), and LY231514[8a] (Alimta) are important examples of TS inhibitors (Figure 1). PDDF and ZD1694 are quinazoline analogues and are characterized by the presence of a 6−6 ring-fused system similar to natural folates and have a polar *N*-benzoyl-L-glutamic acid side chain appended to the 6-position. On the other hand, LY231514 is characterized by the presence of a 6−5 ring-fused system and is designated a multitargeted antifolate (MTA). ZD1694 has been approved in several countries for colorectal cancer. LY231514, in combination with cisplatin, has been recently approved for the treatment of malignant pleural mesothelioma.[8b] Some classical antifolates such as ZD1694 and LY231514 have an *N*-benzoyl-L-glutamic acid side chain, which makes them substrates for the enzyme folylpoly-γ-glutamate synthetase (FPGS).[7−12] FPGS catalyzes the formation of poly-γ-glutamates, which leads to high intracellular concentrations of these antitumor agents and in some cases increases TS inhibitory activity for certain antifolates such as ZD1694[7] (60-fold) and LY231514[8a] (130-fold) compared to their monoglutamate forms.[10−13] Although polyglutamylation of certain antifolates is necessary for cytotoxicity, it has also been implicated in toxicity to normal host cells due to cellular retention of the polyanionic poly-γ-glutamate metabolites, which do not efflux host cells.[14] In addition, the problem of resistance in tumors, due to low or defective FPGS, has placed limitations on the use of classical antifolates, which depend on polyglutamylation for their antitumor effects.[15−18] In an attempt to circumvent the potential problems associated with FPGS including resistance, Gangjee et al.[19] and others[20] have designed compounds that are potent inhibitors of isolated human TS in their monoglutamate forms and do not need FPGS for potent TS inhibitory activity. A compound not subject to

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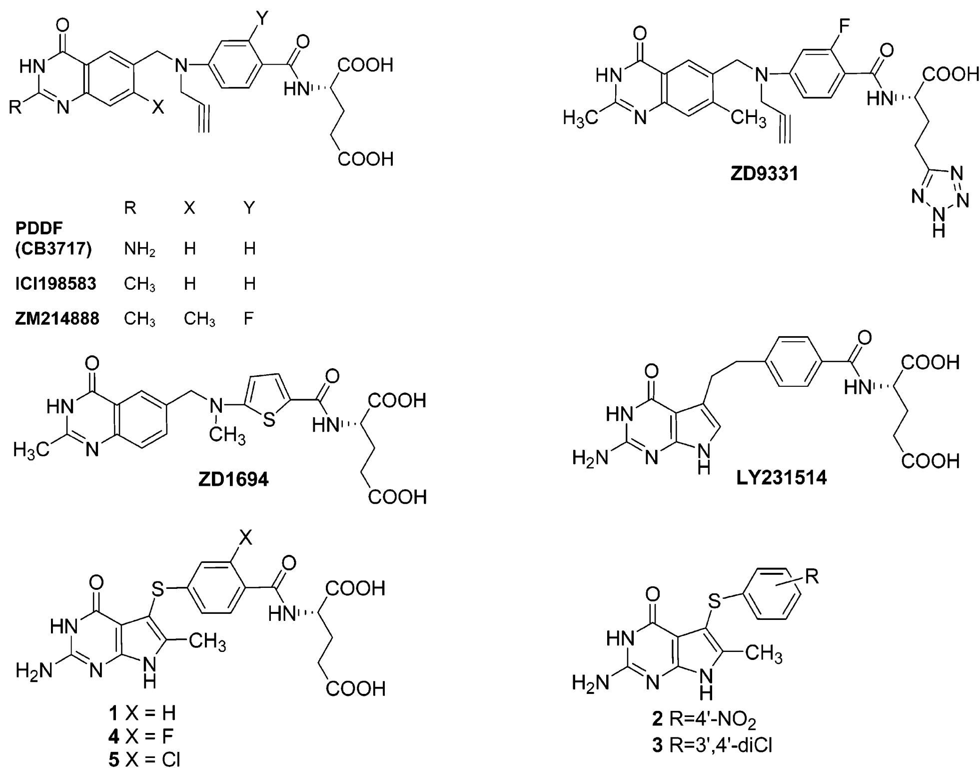


Figure 1.

activation by polyglutamylation needs to have high intrinsic potency as a TS inhibitor. Gangjee et al.[19] previously reported a classical pyrrolo[2,3-*d*]pyrimidine, compound **1**, as a potent inhibitor of isolated human TS with an IC_{50} value of 42 nM and a reasonable inhibitor of human recombinant DHFR with an IC_{50} value of 2.2 μM in its monoglutamate form, thus potentially providing dual inhibitory activity of TS and DHFR. Compound **1** was equipotent with PDDF and was more potent than the clinically used ZD1694 and LY231514 against isolated human TS in their monoglutamate forms.[19] In addition, compound **1** was a reasonably potent inhibitor of the growth of CCRF-CEM human leukemic cells in culture with an EC_{50} value of 0.45 μM as compared with PDDF (EC_{50} = 0.72 μM). A potential advantage of compound **1** over ZD1694 and LY231514 is that compound **1** was not a substrate for human FPGS from CCRF-CEM cells at concentrations up to 1045 μM.[19] The lack of FPGS substrate activity of **1** was attributed, in part, to the presence of the 6-methyl group on the pyrrolo[2,3-*d*]pyrimidine of **1**.[19] In addition, molecular modeling (SYBYL 6.8)[21] suggested that the 6-methyl group in compound **1** makes important hydrophobic contacts with Trp109 in human TS (Figure 2) and also serves to lock the 5-position side chain into favorable, low-energy conformations. This probably contributes to the high inhibitory activity against TS of compound **1** in its monoglutamate form. The fact that LY231514, a pyrrolo[2,3-*d*]pyrimidine, much like **1**, lacks a 6-methyl group and is a substrate for FPGS lends further credence to the involvement of the 6-methyl moiety in preventing FPGS substrate activity in **1**. In the 6−6 quinazoline analogues of ICI 198583, incorporation of a 7-methyl group was shown to afford compounds with both potent TS (both in vivo and in vitro) inhibitory activity and a lack of FPGS substrate activity.[22] Further, in these series of compounds a 2′-fluoro substituent in the phenyl ring gave a 2−3-fold enhancement in TS inhibitory activity as well as in the growth inhibitory potency against L1210 cells, as exemplified by ZM214888, the 2,7-dimethyl-2′-F benzoyl analogue of PDDF.[23] Further modification of the L-glutamic acid side chain led to ZD9331, which is currently undergoing phase I/II clinical trials as an antitumor agent.[24−26] Similarly, halogen substituents in the benzoyl portion of methotrexate[27] have afforded potent DHFR inhibitors. Recently, Gangjee et al.[28] showed that nonclassical analogues of **1** with electron-withdrawing groups in the phenyl ring of the side chain enhanced TS inhibitory activity. The SAR indicated that analogues with an electron-withdrawing group at both the 3′- and 4′-positions of the phenyl side chain provided optimum inhibitory potency against TS. Some of the nonclassical analogues reported[28] were much more potent than the clinically used classical antitumor agents ZD1694 and LY231514. Electron-donating groups on the phenyl ring of the side chain were found to diminish TS inhibitory activity. Similarly, substitution in the benzoyl ring with electron-donating groups in the classical analogues of ZM214888 were found to diminish TS inhibitory activity.[23] In an attempt to increase the TS inhibitory activity of the classical analogue **1** and to provide potent tumor cell inhibitory activity, electron-withdrawing fluorine and chlorine atoms were incorporated separately at the 2′-position of the benzoyl ring of compound **1** to give compounds **4** and **5**, respectively. The synthesis and biological activities of analogues **4** and **5** are presented in this report.

Chemistry

The method used for the synthesis of the classical compounds **4** and **5** essentially followed a modified

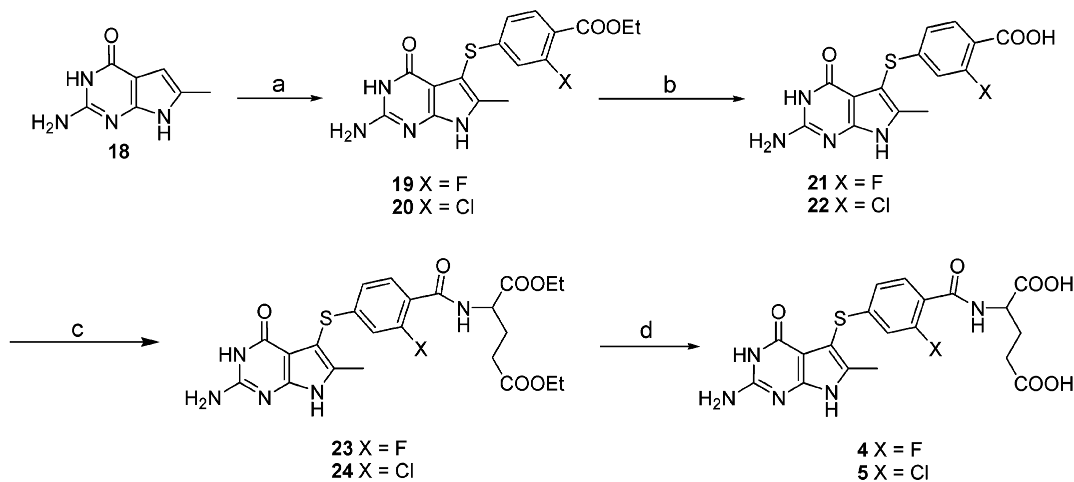
Figure 2. Stereoview compound **1** superimposed on LY231514 (not shown) in human TS (PDB code 1JU6).[50]

Scheme 1[a]

[a] Conditions: (a) 10% Pd−C, H_2, 1 atm, rt; (b) concentrated HCl, 0 °C, $NaNO_2$; (c) potassium xanthogenate, 80 °C; (d) 6 N KOH, reflux; (e) concentrated HCl, 0 °C; (f) 30% H_2O_2, rt; (g) EtI, $NaHCO_3$, rt, 5 days; (h) $NaBH_4$, rt, 30 min.

sequence previously reported[19] for compound **1**. The key intermediates in the synthesis of **4** and **5** were the appropriately substituted aryl thiols, **10** and **11** (Scheme 1). There was no reported synthesis for **10** and **11** in the literature. However, methods[29,30] were reported for the synthesis of aryl thiols from the corresponding amines. The anilino precursors **8** and **9** for the synthesis of **10** and **11,** respectively, were not commercially available. The corresponding nitro compounds **6** and **7** were used as the logical starting materials for the synthesis of the aryl thiols **10** and **11**. Thus, reduction[23] of the nitro group in the commercially available 2-halo-substituted 4-nitrobenzoic acids **6** and **7** with 10% Pd−C and H_2 at 1 atm pressure afforded the corresponding 2-halo-substituted 4-aminobenzoic acids **8** and **9** in 100% and 98% yields, respectively. The amines were then converted to the corresponding 2-halo-substituted-4-mercaptobenzoic acids **10** and **11** using the standard diazotization method.[29,30] This involved diazotization of **8** and **9** with sodium nitrite in aqueous acid, followed by nucleophilic displacement of the diazo moiety with ethyl xanthic acid potassium salt and subsequent hydrolysis of the resulting xanthate esters with potassium hydroxide (Scheme 1). Acidification of the potassium salt of xanthic acid so obtained resulted in the evolution of carbon oxysulfide and the formation of an oil, which was extracted into ether. The crude oil obtained on evaporation of the solvent afforded the desired 2-halo-substituted 4-mercaptobenzoic acids, **10** and **11**. Oxidation[31] of **10** and **11** with 30% H_2O_2 in ethanol gave the corresponding substituted aryl disulfides **12** and **13** in 35% and 25% yield, respectively. Oxidation with 30% H_2O_2 at room temperature was found to give better yields than oxidation with iodine in ethanol. The

Scheme 2[a]



[a] Conditions: (a) I_2, **16** or **17**, EtOH/H_2O (2:1), 100−110 °C; (b) 1 N NaOH, 80 °C; (c) ^{i}BuOCOCl, NEt_3, diethyl-L-glutamate hydrochloride, 0 °C to rt; (d) 1 N NaOH, 0 °C.

substituted aryl disulfides **12** and **13** were then alkylated[19] with ethyl iodide to afford the corresponding diethyl 4,4′-dithio-2,2′-dihalo-substituted-bis(benzoate) **14** and **15**, respectively, as oils, which were used without further purification. Reduction[19] of **14** and **15** to the corresponding sodium salt of ethyl 2-halo-substituted-4-thiobenzoates **16** and **17** was achieved using sodium borohydride in ethanol as reported previously.[19] Reaction of the sodium salts of the mercaptans **16** and **17** with the pyrrolo[2,3-*d*]pyrimidine **18**[28] gave the corresponding esters **19** and **20** (Scheme 2) in reasonably good yields without the necessity of protecting the 2-amino group of **18**, as reported previously.[19] Compound **19** was obtained in 41% yield, while **20** was obtained in 52% yield. This method had the advantage over our previously reported method[19] of shorter reaction times (4 h vs 16 h) and that it can be carried out without protecting and deprotecting the 2-amino group, which occurs in poor yields (20−30%) over two steps. The structures of **19** and **20** were established by ^{1}H NMR, where the 3′-aromatic proton of **19** coupled with the adjacent 2′-fluorine to afford the expected triplet at 7.68−7.74 ppm. This, along with the disappearance of the 5-aryl proton at 5.83 ppm, present in **18**, and the presence of the requisite protons of the side chain confirmed the structure of **19**. Similarly, the splitting pattern of the aromatic protons in **20** along with the disappearance of the 5-aryl proton, present in **18**, confirmed the structure of **20**. Ester hydrolysis of **19** and **20** with 1 N NaOH at 80 °C afforded the corresponding acids **21** and **22** in 85% and 92% yields, respectively.[19] Peptide coupling of the acids **21** and **22** with diethyl-L-glutamate using the mixed anhydride method[19] with isobutyl chloroformate and triethylamine afforded **23** and **24** in yields of 20% and 30%, respectively. The ^{1}H NMR spectrum of **23** and **24** in deuterated dimethyl sulfoxide revealed the expected peptide NH doublet at 8.49 and 8.74−8.76 ppm, respectively, which exchange on addition of D_2O. Hydrolysis[19] of the diesters **23** and **24** with aqueous sodium hydroxide at room temperature, followed by acidification with 3 N HCl in the cold, afforded the desired target compounds **4** and **5** in 60% and 68% yields, respectively.

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

compd	TS IC_{50} (nM)		DHFR IC_{50} (μM)	
	human[a]	*E. coli*[a]	human[b]	*E. coli*[c]
1	54	270	2.1	21
4	54	720	2.1	21
5	51	5100	11	40
PDDF[d]	72	72	ND	ND
ZD1694[e] (Tomudex)	380	5700	ND	ND
LY231514[f] (Alimta)	9500	76000	6.6	230
MTX	ND	ND	0.009	0.032

[a] Kindly provided by Dr. Frank Maley, New York State Department of Health. [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. M. G. Nair, University of South Alabama. [e] Kindly provided by Dr. Ann Jackman, Institute of Cancer Research, Sutton, Surrey, UK. [f] Kindly provided by Dr. Chuan Shih, Eli Lilly and Co.

Biological Evaluation and Discussion

The classical analogues **4** and **5** were evaluated as inhibitors of TS[32] and DHFR[33] from human and *Escherichia coli*. The inhibitory potencies (IC_{50}) are listed in Table 1 along with those of PDDF, ZD1694, LY231514, and methotrexate (MTX). The classical analogues **4** and **5** were both excellent inhibitors of human TS. Both **4** and **5** were equipotent with the previously reported compound **1** and PDDF. Interestingly, **4** and **5** were remarkably more potent than ZD1694 (7-fold) and LY231514 (176-fold). Similar results were obtained with *E. coli* TS. Both **4** (13-fold) and **5** (100-fold) were more potent in inhibiting human TS than *E. coli* TS, indicating a significant species difference. Compound **4** was 10-fold less while **5** was 71-fold less potent in inhibiting *E. coli* TS than PDDF. The inhibitory data for compound **4** against human TS was unexpected, in that it was essentially the same as that for the unhalogenated compound **1** and did not result in an increase in TS inhibitory activity. Jackman et al.[23] have reported that benzoyl ring substitution with an electron-withdrawing fluorine atom at the 2′-position results in a 2−5-fold increase in TS inhibitory activity in their series of compounds. A plausible explanation for the observed increase in TS inhibition was that the 2′-fluorine atom

Table 2. Growth Inhibition of Parental CCRF-CEM Human Leukemia Cells and Sublines with Single, Defined Meachanisms of MTX Resistance during Continous Exposure (0–120 h) to MTX, **4**, or **5**[e]

	EC_{50} (nM)			
drug	CCRF-CEM	R1[a] (↑ DHFR)	R2[b] (↓ uptake)	R30dm[c] (↓ Glu_n)
MTX	17.5 ± 0.5	680 ± 30	2100 ± 290	16.5 ± 0.5
4	215 ± 25	505 ± 15	2450 ± 150	305 ± 5
5	690 ± 110	1900 ± 100	12000[d]	1200 ± 0

[a] CCRF-CEM subline resistant to MTX solely as a result of a 20-fold increase in wild-type DHFR protein and activity.[44] [b] CCRF-CEM subline resistant as a result of decrease uptake of MTX.[45] [c] CCRF-CEM subline 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.[17] [d] The first experiment with this line indicated an EC_{50} > 10 000 nM. The value presented is from a second experiment covering a higher concentration range. [e] Values presented are average ± range for $n = 2$.

hydrogen bonds with the amide hydrogen atom, which restricts this part of the molecule in a more favorable conformation for binding to TS than in the analogue without the 2′-fluorine. A spin–spin coupling constant of 6.4 Hz between these two atoms was observed in the ^{1}H NMR of an analogue of ICI198583 (Figure 1), where Y = F instead of H.[23] However, in the ^{1}H NMR of compound **4**, no such coupling was observed between the 2′-fluoro and the amide hydrogen atom and may account at least in part for the lack of increase in TS inhibitory activity. Compounds **4** and **5** were inhibitors of both human DHFR and *E. coli* DHFR (Table 1) and were 10- and 2-fold more potent, respectively, in inhibiting human DHFR than *E. coli* DHFR. It is well documented that both the interaction of the 4-amino protons and the protonated N1 of 2,4-diaminopyrimidine-containing antifolates such as MTX are essential for tight binding to DHFR.[34] In compounds **4** and **5**, this binding orientation can be achieved by rotating the bond between the C2 and 2-NH_2 by 180° such that the pyrrole NH mimics the 4-amino group while the 3-NH proton mimics the protonated N1 of MTX, thereby allowing for DHFR inhibition. Such dual binding orientations of pyrrolo[2,3-*d*]pyrimidine antifolates has been proposed for the 2,4-diaminopyrrolo[2,3-*d*]pyrimidines by Miwa et al.[35] and is further supported by the potent DHFR inhibitory activity reported for the 4-desaminopyrrolo-[2,3-*d*]pyrimidine antifolates.[36,37]

Growth inhibitory potency of **4** and **5** were compared to that of MTX in continuous exposure against CCRF-CEM human lymphoblatic leukemia and a series of MTX-resistant sublines (Table 2). Compound **4** was only 12-fold less potent than MTX, while **5** was 39-fold less potent than MTX. The DHFR overexpressing cell line R1 was <3-fold cross-resistant to both **4** and **5**, suggesting that DHFR is probably not the primary target of these analogues. The MTX-resistant transport-deficient subline R2, that does not express functional reduced folate carrier[38] (RFC), is >17-fold cross-resistant to **5** and 11-fold cross-resistant to **4**, while it is 120-fold resistant to MTX. The data suggest that both **4** and **5** utilize the RFC as their primary means of transport, but at high extracellular levels, they are able to diffuse through the plasma membrane. A subline (R30dm) expressing low levels of folylpolyglutamate synthetase (FPGS) is only marginally cross-resistant to both analogues under continuous exposure conditions, suggesting that polyglutamate forms of **4** and **5** are not essential to their mechanisms of action.

Table 3. Protection of CCRF-CEM Human Leukemia Cells against the Growth Inhibitory Effects of MTX, **4**, and **5** by 10 μM Hypoxanthine (Hx), 5 μM Thymidine (TdR), and Their Combination

	relative growth (%)[a,b]			
drug	no addition	5 μM TdR	10 μM Hx	Hx + TdR
MTX (40 nM)	10 ± 1	10 ± 1	11 ± 1	100 ± 2
4 (5000 nM)	10 ± 1	96 ± 4	11 ± 0	103 ± 1
5 (2400 nM)	11 ± 0	92 ± 3	12 ± 1	96 ± 5

[a] Deoxycytidine (dCyd; 10 μM) was present in all the above cultures to prevent the inhibition of growth caused by TdR in T-cell leukemia cell lines such as CCRF-CEM (see the methods). In control results from the experiment above, dCyd alone had no effect on CCRF-CEM growth (107 ± 1% of control) and did not protect against growth inhibition by any of these drugs (data not shown). Hx alone (101 ± 0% of control) or in the presence of dCyd (110 ± 1% 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 (40 ± 1% of control), but TdR+dCyd was not growth inhibitory (106 ± 2% of control) and neither protected against MTX-induced growth inhibition (table above). Similarly, Hx + TdR + dCyd did not inhibit growth of CCRF-CEM (110 ± 3% of control). [b] 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.

Metabolite protection studies were performed to further elucidate the mechanism of action of **4** and **5**. At a concentration of MTX that inhibited growth of CCRF-CEM cells by 95%, leucovorin at 0.1 mM was able to fully protect against the effects of MTX (6 ± 4% growth inhibition). In contrast, at concentrations of either **4** or **5** that inhibited growth by 95%, even 10 mM leucovorin only afforded marginal protection (90 ± 3% growth inhibition). Although this might suggest that these analogues are not antifolates, some validated antifolates (e.g., BW1843U89[39]) are poorly protected by leucovorin. These data do suggest, however, that leucovorin "rescue", as used clinically with high-dose MTX,[40] would not be successful with these analogues. Further studies in CCRF-CEM cells examined the ability of thymidine (TdR) and/or hypoxanthine (Hx) to protect against growth inhibition. These metabolites can be salvaged to produce dTTP and the purine dNTPs required for DNA synthesis and thus bypass the MTX blockade.[41] As described in the Experimental Section, in T-lymphoblast cell lines such as CCRF-CEM, TdR can only be tested in the presence of deoxycytidine (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 (Table 3, footnote *a*). The data (Table 3) show that for **4** and **5**, TdR alone protects against growth inhibition, while Hx alone does not; addition of Hx to TdR does not affect the protection. These data suggest that **4** and **5** inhibit only thymidylate synthesis, indicating that TS is the target of these drugs. Thus, these halogenated analogues have the same mechanism of action as their unhalogenated parent **1**.

The substrate activity of **4** and **5** was evaluated in vitro with recombinant human FPGS and compared to that of AMT, a good substrate for FPGS. The data (Table 4) showed that neither **4** nor **5** is a substrate for human

Table 4. Activity of Folate Analog as Substrates for Recombinant Human FPGS[a]

substrate	K_m, μM	$V_{max,rel}$[b]	$V_{max,rel}/K_m$	n
AMT	4.3 ± 0.6	1.00	0.23	4
4	inactive ≤ 250 μM	0	0	2
5	inactive ≤ 250 μM	0	0	2

[a] FPGS substrate activity was determined as described in Experimental section. Values presented are the average ± SD. [b] $V_{max,rel}$ is calculated based on the apparent V_{max} of a substrate relative to the apparent V_{max} of AMT within the same experiment.

Table 5. Cytotoxicity Evaluation against Selected Tumor Cell Lines[40]

cell line	GI_{50} (M)	
	4	**5**
leukemia		
CCRF-CEM	7.12×10^{-7}	ND
non-small-cell lung cancer		
EKVX	6.83×10^{-6}	7.58×10^{-7}
colon cancer		
HCC-2998	$<1.00 \times 10^{-8}$	1.48×10^{-6}
CNS cancer		
SF-268	3.47×10^{-7}	7.44×10^{-6}
breast cancer		
NCI/ADR-RES	3.83×10^{-7}	8.54×10^{-6}
BT-549	2.21×10^{-6}	$<1.00 \times 10^{-8}$

FPGS at up to 250 mM. The lack of FPGS substrate activity is consistent with the minimal cross-resistance of the FPGS-deficient subline R30dm (above) to both **4** and **5** and underscores the absence of polygluatamylation for the cytotoxicity of **4** and **5**. Marsham et al.[20] have reported a 7-CH_3 analogue of 2-desamino-2-methyl PDDF that was not a substrate for human FPGS. This further substantiates the idea that methyl substituents in the 6-position of classical pyrrolo[2,3-*d*]pyrimidines and in the 7-position of quinazolines prevent FPGS substrate activity as a consequence of bulk and/or hydrophobicity, which is not tolerated by human FPGS.

Compounds **4** and **5** were selected by the National Cancer Institute[42] for evaluation as antitumor agents in the preclinical in vitro screening program. The ability of **4** and **5** to inhibit the growth of different tumor cell lines was measured as GI_{50} values, the concentration required to inhibit the growth of tumor cells in culture by 50%. Compounds **4** and **5** displayed potent antitumor activity with GI_{50} values in the 8.54×10^{-6} to $<1.00 \times 10^{-8}$ M range in six cell lines. GI_{50} values for compounds **4** and **5** are shown in Table 5.

In summary, benzoyl ring modification of the 2-amino-4-oxo-5-thiobenzoyl-6-methylpyrrolo[2,3-*d*]pyrimidine with electron-withdrawing halogens such as chlorine and fluorine has modest effects on biological activity as compared to the parent unhalogenated compound. Analogues **4** and **5** were comparable to the unhalogenated analogue **1** in inhibiting human TS. This result indicates that benzoyl ring modification of classical pyrrolo[2,3-*d*]pyrimidine with halogen substitution in the 2′-position has little or no effect on human TS inhibitory activity. The unhalogenated derivative, compound **1**, is slightly more potent than the 2′-chloro analogue **5** and slightly less potent than the 2′-fluoro analogue **4** as an inhibitor of CCRF-CEM cell growth. Cross-resistance data corroborate a similar mechanism of action for **1**, **4**, and **5**. On the basis of metabolite protection studies that TdR alone is able to protect against the growth inhibitory effects of compounds **1**, **4**, and **5**, all three target TS directly. Interestingly, leucovorin cannot provide complete protection for **1**, **4**, or **5**. None of the three analogues is a substrate for human FPGS and thus polyglutamates are not critical to their mechanism of action. This lack of substrate activity of compounds **4** and **5** could be an important attribute to overcome potential resistance to classical TS inhibitors such as ZD1694 and LY231514, which depend on intracellular polyglutamylation to exert their cytotoxic effects.

Experimental Section

All evaporations were carried out in vacuo with a rotary evaporator. Analytical samples were dried in vacuo (0.2 mmHg) in an Abderhalden drying apparatus over P_2O_5. Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Nuclear magnetic resonance spectra for proton (^{1}H NMR) were recorded on a Bruker WH-300 (300 MHz) spectrometer. Data were accumulated by 16K size with a 0.5 s delay time and 70° tip angle. The chemical shift values are expressed in ppm (parts per million) relative to tetramethylsilane as internal standard; s = singlet, d = doublet, dd = doublet of doublet, t = triplet, q = quartet, m = multiplet, bs = broad singlet. The relative integrals of peak areas agreed with those expected for the assigned structures. Mass spectra were recorded on a VG-7070 double-focusing mass spectrometer or in a LKB-9000 instrument in the electron ionization (EI) mode. Thin-layer chromatography (TLC) was performed on POLYGRAM Sil G/UV_{254} 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., Norcoss, GA. Analytical results indicated by element symbols 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 removed, despite 24−48 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. and Fisher Scientific and were used as received.

4-Amino-2-fluorobenzoic Acid (8). To a solution of 2-fluoro-4-nitrobenzoic acid, **6** (5 g, 27 mmol), in ethyl acetate in a Parr hydrogenation bottle was added 1 g of 10% Pd−C. The resulting mixture was hydrogenated at 1 atm of pressure until complete reduction was indicated by TLC (ca. 4 h), following which the mixture was filtered through Celite and the Celite was washed with excess ethyl acetate until the washings did not give a spot on TLC. The combined washings were then evaporated under reduced pressure to afford 4.17 g (ca 100%) of **8** as a light cream powder: mp 211.3-214 °C; TLC R_f = 0.54 ($CHCl_3$/MeOH 5:1); ^{1}H NMR (DMSO-d_6) δ 6.16 (s, 2 H, 4-NH_2), 6.24−6.29 (d, 1 H, 5-CH), 6.35−6.37 (d, 1 H, 6-CH), 7.51−7.57 (t, 1 H, 3-CH), 12.15 (br s, 1 H, COOH); HRMS calcd for $C_7H_6NO_2F$ *m/z* = 155.0382, found *m/z* = 155.0375.

4-Amino-2-chlorobenzoic Acid (9). To a solution of 2-chloro-4-nitrobenzoic acid, **7** (5 g, 24.8 mmol), in ethyl acetate in a Parr hydrogenation bottle was added 1 g of 10% Pd−C. The resulting solution was hydrogenated at 1 atm of pressure until complete reduction was indicated by TLC (ca. 4.5 h), following which the mixture was filtered through Celite and the Celite was washed with excess ethyl acetate until the washings did not give a spot on TLC. The combined washings were then evaporated under reduced pressure to afford 4.25 g (ca 98%) of **9** as a light green powder: mp 208−210 °C (lit.[43] mp 205−207 °C); TLC R_f = 0.52 ($CHCl_3$/MeOH 5:1); ^{1}H NMR (DMSO-d_6) δ 6.07 (s, 2 H, 4-NH_2), 6.47−6.51 (d, 1 H, 5-CH), 6.61 (s, 1 H, 3-CH), 7.62−7.65 (d, 1 H, 6-CH).

2,2′-Difluoro-4,4′-dithiobis(benzoic acid) (12). To a stirred solution of 12.5 mL of concentrated HCl and the same amount of water was added the substituted aniline **8** (5 g, 32 mmol)

and the suspension was warmed to 70 °C. After this, the solution was cooled to 0 °C and diazotized with $NaNO_2$ (2.2 g, 32 mmol) over 20 min. This solution was then added dropwise over 1 h to potassium xanthogenate (19 g, 117 mmol) solution kept at 80 °C, after which the oil that separated was extracted with diethyl ether, dried over $MgSO_4$ for 2 h, and then evaporated under low pressure to give a brownish oil. The oil that was obtained was dissolved in 50 mL of ethanol to which 10 g of KOH in ethanol was added slowly over 30 min and the solution was heated at 80 °C for 8 h. The ethanol was removed under reduced pressure and the residue that was obtained was dissolved in water and slowly neutralized with concentrated HCl at 0 °C to pH 5 and then extracted with ethyl acetate. The ethyl acetate was then dried over $MgSO_4$ and removed under low pressure to give the aryl thiol **10** as a solid with strong thiol odor. The thiol **10** that was obtained was suspended in ethanol and to this suspension was added 30% H_2O_2 (3.8 mL) and the mixture stirred at room temperature under N_2 for 24 h. The solvent was then removed and the residue that was obtained was chromatographed on silica gel with 1−3% MeOH in $CHCl_3$ to give 1.95 g (35%) of the disulfide **12** as a light cream solid: mp >245 °C (dec); TLC R_f = 0.76 ($CHCl_3$/MeOH 5:1); ^1H NMR (DMSO-d_6) δ 7.39−7.46 (t, 2 H, 5-CH and 6-CH), 7.78−7.83 (t, 1 H, 3-CH); HRMS calcd for $C_{14}H_8O_4S_2F_2$ m/z = 341.9832, found m/z = 341.9835.

2,2′-Dichloro-4,4′-dithiobis(benzoic acid) (13). To a stirred solution of 12.5 mL of concentrated HCl and the same amount of water was added the substituted aniline **9** (5 g, 29 mmol) and the suspension was warmed to 70 °C. After this, the solution was cooled to 0 °C and diazotized with $NaNO_2$ (2 g, 29 mmol) over 20 min. This solution was then added dropwise over 1 h to potassium xanthogenate (19 g, 117 mmol) solution (10 mL) kept at 80 °C, after which the oil that separated was extracted with diethyl ether, dried over $MgSO_4$ for 2 h, and then evaporated under low pressure to give a brownish oil. The oil that was obtained was dissolved in 50 mL of ethanol to which 10 g of KOH in ethanol was added slowly over 30 min and the solution was heated at 80 °C for 8 h. The ethanol was removed under reduced pressure and the residue that was obtained was dissolved in water and slowly neutralized with concentrated HCl at 0 °C to pH 5 and then extracted with ethyl acetate. The ethyl acetate was then dried over $MgSO_4$ and removed under low pressure to give the substituted aryl thiol **11** as a solid with a strong thiol odor. The thiol **11** that was obtained was suspended in ethanol, to this suspension was added 30% H_2O_2 (3.8 mL), and the mixture was stirred at room temperature under N_2 for 24 h. The solvent was then removed and the residue that was obtained was chromatographed on silica gel with 1−3% MeOH to give 1.34 g (25%) of the disulfide **13** as a light yellow solid: mp >185 °C (dec); TLC R_f = 0.74 ($CHCl_3$/MeOH 5:1); ^1H NMR (DMSO-d_6) δ 7.60 (d, 1 H, 5-CH), 7.69 (s, 1 H, 3-CH), 7.79 (d, 1 H, 6-CH).

Ethyl 4-[(2-Amino-6-methyl-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidine-5-yl)sulfanyl]-2′-fluorobenzoate (19). To a solution of the disulfide **12** (1.05 g, 3.0 mmol) in anhydrous *N*,*N*-dimethylacetamide (10 mL) was added powdered $NaHCO_3$ (0.9 g, 11 mmol) followed by ethyl iodide (1.72 g, 11 mmol), and the reaction mixture was stirred under N_2 for 5 days. The mixture was then diluted with water (25 mL) and extracted with ethyl acetate (3 × 25 mL). The combined organic layer was 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 and eluted with CH_2Cl_2. Fractions containing the desired spot (TLC) were pooled and evaporated to dryness to afford **14** as a tan oil that was used immediately in the next step. To a solution of **14** (0.8 g, 2 mmol) in absolute ethanol (10 mL) was added $NaBH_4$ (0.075 g, 2 mmol) all at once and the mixture stirred under N_2 for 30 min. To this ethanolic solution of the sodium salt of ethyl 2-fluoro-4-mercaptobenzoate, **16**, was added pyrrolopyrimidine **18** (0.33 g, 2 mmol), the ratio of ethanol/water was adjusted to 2/1 (40 mL), and the resulting suspension was heated to 100 °C. At this point, iodine (1.0 g, 4 mmol) was added and the resulting solution was refluxed for 4 h, when TLC indicated the disappearance of starting material at R_f 0.33 and the formation of a major spot at R_f 0.41 ($CHCl_3$/MeOH 5:1). To the resulting solution was added excess $Na_2S_2O_3$ and the reaction mixture was evaporated to dryness. To the resulting residue were added silica gel (5 g) and methanol, and the solvent was evaporated under low pressure to give a plug which was chromatograhed on silica gel using a gradient of 1−5% MeOH in $CHCl_3$. Fractions containing the desired spot (TLC) were pooled and evaporated to dryness to afford 300 mg (41%) of **19** as a light cream solid: mp 297−303 °C; TLC R_f = 0.49 ($CHCl_3$/MeOH 5:1); ^1H NMR (DMSO-d_6) δ 1.24−1.29 (t, 3 H, CH_2CH_3), 2.17 (s, 3 H, 6-CH_3), 4.22−4.29 (q, 2 H, CH_2-CH_3), 6.16 (s, 2 H, 2-NH_2), 6.77−6.81 (d, 1 H, C_6H_3), 6.99−6.93 (d, 1 H, C_6H_3), 7.68−7.71 (t, 1 H, C_6H_3), 10.27 (s, 1 H, 7NH), 11.55 (s, 1 H, 3NH). Anal. Calcd for ($C_{16}H_{15}N_4SO_3F$·$0.1H_2O$) C, H, N, S, F.

Ethyl 4-[(2-Amino-6-methyl-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidine-5-yl)sulfanyl]-2′-chlorobenzoate (20). To a solution of the disulfide **13** (1 g, 2.66 mmol) in anhydrous *N*,*N*-dimethylacetamide (10 mL) was added powdered $NaHCO_3$ (0.9 g, 11 mmol) followed by ethyl iodide (1.72 g, 11 mmol), and the reaction mixture was stirred under N_2 for 5 days. The mixture was then diluted with water (25 mL) and extracted with ethyl acetate (3 × 25 mL). The combined organic layer was 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 and eluted with CH_2Cl_2. Fractions containing the desired spot (TLC) were pooled and evaporated to dryness to afford a tan oil which slowly crystallized to afford an solid that was used immediately in the next step. To a solution of crude **15** (1.00 g, 2.3 mmol) in absolute ethanol (10 mL) was added $NaBH_4$ (0.087 g, 2.3 mmol) all at once and the mixture stirred under N_2 for 30 min. To this ethanolic solution of the sodium salt of ethyl 2-chloro-4-mercaptobenzoate was added pyrrolopyrimidine **18** (0.38 g, 2.3 mmol), the ratio of ethanol/water was adjusted to 2/1 (40 mL), and the resulting suspension was heated to 100 °C. At this point, iodine (1.16 g, 4.6 mmol) was added and the resulting solution was refluxed for 4 h, when TLC indicated the disappearance of starting material at R_f 0.33 and the formation of a major spot at R_f 0.44 ($CHCl_3$/MeOH 5:1). To the resulting solution was added excess $Na_2S_2O_3$ and the reaction mixture was evaporated to dryness. To the resulting residue were added silica gel (5 g) and methanol, and the solvent was evaporated under low pressure to give a plug which was chromatograhed on silica gel using a gradient of 1−5% MeOH in $CHCl_3$. Fractions containing the desired spot (TLC) were pooled and evaporated to dryness to afford 460 mg (52%) of **20** as a light cream solid: mp 295−300 °C; TLC R_f = 0.47 ($CHCl_3$/MeOH 5:1); ^1H NMR (DMSO-d_6) δ 1.25−1.30 (t, 3 H, CH_2CH_3), 2.18 (s, 3 H, 6-CH_3), 4.25−4.30 (q, 2 H, CH_2CH_3), 6.16 (s, 2 H, 2-NH_2), 7.02−7.04 (m, 2 H, C_6H_3), 7.65−7.68 (d, 1 H, C_6H_3), 10.27 (s, 1 H, 7NH), 11.54 (s, 1 H, 3NH). Anal. Calcd for ($C_{16}H_{15}N_4SO_3Cl$·$0.6H_2O$) C, H, N, S, Cl.

4-[(2-Amino-6-methyl-3,4-dihydro-4-oxo-7*H*-pyrrolo-[2,3-*d*]pyrimidin-5-yl)thio]-2′-fluorobenzoic Acid (21). To a solution of **19** (800 mg, 2.33 mmol) in ethanol (80 mL) was added aqueous 1 N NaOH and the reaction mixture stirred at room temperature for 24 h. The solution was evaporated to dryness, and the sodium salt was dissolved in water (40 mL) and carefully acidified to pH 4 by dropwise addition of 3 N HCl. The resulting suspension was left at 0 °C for 24 h and filtered. The residue was washed with water, acetone, and diethyl ether and dried over P_2O_5 at 78 °C to afford 662 mg (85%) of the free acid **21** as a light brown solid: mp >320 °C (dec); TLC R_f 0.32 ($CHCl_3$/MeOH 5:1); ^1H NMR (DMSO-d_6) δ 2.17 (s, 3 H, 6-CH_3), 6.23 (s, 2 H, 2-NH_2), 6.74−6.78 (d, 1 H, C_6H_3), 6.87−6.89 (d, 1 H, C_6H_3), 7.67−7.72 (t, 1 H, C_6H_3), 10.34 (s, 1 H, 7NH), 11.56 (s,1 H, 3NH); HRMS calcd for $C_{14}H_{11}N_4O_3SF$ m/z = 334.05359, found m/z = 334.05326.

4-[(2-Amino-6-methyl-3,4-dihydro-4-oxo-7*H*-pyrrolo-[2,3-*d*]pyrimidin-5-yl)thio]-2′-chlorobenzoic Acid (22). To a solution of **20** (110 mg, 0.29 mmol) in ethanol (10 mL) was added aqueous 1 N NaOH and the reaction mixture stirred at room temperature for 24 h. The solution was evaporated to dryness, and the sodium salt was dissolved in water (8 mL) and carefully acidified to pH 4.0 by dropwise addition of 3 N HCl. The resulting suspension was left at 5 °C for 24 h and filtered. The residue was washed with water, acetone, and diethyl ether and dried over P_2O_5 at 78 °C to afford 94 mg (92%) of the free acid **22** as a light brown solid: mp 290−295 °C; TLC R_f = 0.34 ($CHCl_3$/MeOH 5:1); ^1H NMR (DMSO-d_6) δ 2.18 (s, 3 H, 6-CH_3), 6.18 (s, 2 H, 2-NH_2), 6.98−7.01 (d, 2 H, C_6H_3), 7.65−7.68 (d, 1 H, C_6H_3), 10.29 (s, 1 H, 7NH), 11.54 (s, 1 H, 3NH) 13.08 (bs, 1 H, COOH); HRMS calcd for $C_{14}H_{11}N_4O_3SCl$ m/z = 305.0263, found m/z = 305.0257 (M^- COOH)$^+$.

Diethyl *N*-[4-[(2-Amino-6-methyl-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)thio]-2′-fluorobenzoyl]-L-glutamate (23). To a suspension of the acid **21** (535 mg, 1.6 mmol) in anhydrous DMF (21 mL) under N_2 was added triethylamine (675 μL, 4.8 mmol) and the suspension was heated to 80 °C to form a solution. This solution was cooled to 0 °C and isobutyl chloroformate (632 μL, 4.8 mmol) was added, followed 15 min later by diethyl-L-glutamate hydrochloride (1.15 g, 4.8 mmol) and immediately followed by triethylamine (675 μL, 4.8 mmol). The reaction mixture was slowly allowed to warm to room temperature and stirred for 24 h. The DMF was evaporated using an oil pump at room temperature. To the resulting residue were added 25 mL of methanol and silica gel (4 g), and the suspension was evaporated to dryness. The silica gel plug was loaded on a wet ($CHCl_3$) silica gel column and eluted with a gradient of 1−5% MeOH in $CHCl_3$. Fractions containing the desired spot (TLC) were pooled and evaporated to dryness under vaccum to give 170 mg (20%) of **23** as a light cream solid: mp 132−134 °C; TLC R_f = 0.62 ($CHCl_3$/MeOH 5:1); ^1H NMR (DMSO-d_6) δ 1.14−1.21 (m, 6 H, CH_2CH_3), 1.87−2.03 (m, 2 H, Glu β-CH_2), 2.25 (s, 3 H, 6-CH_3), 2.40 (t, 2 H, Glu γ-CH_2), 4.00−4.10 (q, 4 H, CH_2CH_3), 4.39 (m, 1 H, Glu α-CH), 6.13 (s, 2 H, 2-NH_2), 6.75−6.90 (m, 2 H, C_6H_3), 7.43−7.46 (t, 1 H, C_6H_3), 8.50 (d, 1 H, CONH), 10.25 (s, 1 H, 7NH), 11.51 (s, 1 H, 3NH). Anal. Calcd for ($C_{23}H_{26}N_5SO_6F{\cdot}0.3H_2O$) C, H, N, S, F.

Diethyl *N*-[4-[(2-Amino-6-methyl-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)thio]-2′-chlorobenzoyl]-L-glutamate (24). To a suspension of the acid **22** (400 mg, 1.14 mmol) in anhydrous DMF (21 mL) under N_2 was added triethylamine (481 μL, 3.42 mmol) and the suspension was heated to 80 °C to form a solution. This solution was cooled to 0 °C and isobutyl chloroformate (450 μL, 3.42 mmoL) was added, followed 15 min later by diethyl-L-glutamate hydrochloride (820 mg, 3.42 mmoL) and immediately followed by triethylamine (481 μL, 3.42 mmol). The reaction mixture was slowly allowed to warm to room temperature and stirred for 24 h. The DMF was evaporated using an oil pump at room temperature. To the resulting residue were added 15 mL of methanol and silica gel (2 g), and the suspension evaporated to dryness. The silica gel plug was loaded on a wet silica gel column and eluted with a gradient of 1−5% MeOH in $CHCl_3$. Fractions containing the desired spot (TLC) were pooled and evaporated to dryness under vaccum to give 185 mg (30%) of **24** as a light cream solid: mp 145−155 °C; TLC R_f = 0.61 ($CHCl_3$/MeOH 5:1); ^1H NMR (DMSO-d_6) δ 1.14−1.21 (m, 6 H, CH_2CH_3), 1.87−2.03 (m, 2 H, Glu β-CH_2), 2.18 (s, 3 H, 6-CH_3), 2.42 (t, 2 H, Glu γ-CH_2), 4.03−4.11 (q, 4 H, CH_2CH_3), 4.35 (m, 1 H, Glu α-CH), 6.15 (s, 2 H, 2-NH_2), 6.98−7.02 (m, 2 H, C_6H_3), 7.24−7.26 (d, 1 H, C_6H_3), 8.73−8.76 (d, 1 H, CONH), 10.26 (s, 1 H, 7NH), 11.51 (s, 1 H, 3NH). Anal. Calcd for ($C_{23}H_{26}N_5SO_6Cl{\cdot}0.2H_2O$) C, H, N, S, Cl.

***N*-[4-[(2-Amino-6-methyl-3,4-dihydro-4-oxo-7*H*-pyrrolo-[2,3-*d*]pyrimidin-5-yl)thio]-2′-fluorobenzoyl]-L-glutamic Acid (4).** To a solution of **23** (200 mg, 0.38 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 110 mg (60%) of **4** as a light cream solid: mp 192−200 °C; TLC R_f = 0.58 ($CHCl_3$/MeOH/NH_4OH 3:9:1); ^1H NMR (DMSO-d_6) δ 1.88−2.04 (m, 2 H, Glu β-CH_2), 2.19 (s, 3 H, 6-CH_3), 2.30−2.32 (t, 2 H, Glu γ-CH_2), 4.35 (m, 1 H, Glu α-CH), 6.17 (s, 2 H, 2-NH_2), 6.75−6.79 (d, 1 H, 5′-CH), 6.88−6.91 (d, 1 H, 6′-CH), 7.44−7.49(t, 1 H, 3′-CH), 8.36 (d, 1 H, CONH), 10.28 (s, 1 H, 7NH), 11.53 (s, 1 H, 3NH), 12.50 (br s, COOH). Anal. Calcd for ($C_{19}H_{18}N_5SO_6F{\cdot}0.6H_2O$) C, H, N, S, F.

***N*-[4-[(2-Amino-6-methyl-3,4-dihydro-4-oxo-7*H*-pyrrolo-[2,3-*d*]pyrimidin-5-yl)thio]-2′-chlorobenzoyl]-L-glutamic Acid (5).** To a solution of **24** (120 mg, 0.22 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 77 mg (68%) of **5** as a light brown solid: mp 221−225 °C; TLC R_f = 0.56 ($CHCl_3$/MeOH/NH_4OH 3:9:1); ^1H NMR (DMSO-d_6) δ 1.90−1.94 (m, 2 H, Glu β-CH_2), 2.19 (s, 3 H, 6-CH_3), 2.30 (t, 2 H, Glu γ-CH_2), 4.28 (m, 1 H, Glu α-CH), 6.23 (s, 2 H, 2-NH_2), 6.97−6.98 (m, 2 H, C_6H_3), 7.28−7.30 (d, 1 H, C_6H_3), 8.31 (d, 1 H, CONH), 10.43 (s, 1 H, 7NH), 11.50 (s, 1 H, 3NH). Anal. Calcd for ($C_{19}H_{18}N_5SO_6Cl{\cdot}1.6H_2O{\cdot}0.1HCl$) C, H, N, S, Cl.

Drugs and Chemicals. Drug solutions were standardized using extinction coefficients. Extinction coefficients were determined for **5** [pH 1, λ_{max-1} 264 nm (22 600); pH 7, λ_{max} 264 nm (22 700); pH 13, λ_{max} 268 (24 300)] and for **4** [pH 1, λ_{max} 272 nm (22 300); pH 7, λ_{max} 271 nm (23 000); pH 13, λ_{max} 277 nm (24 900)]. Extinction coefficients for methotrexate (MTX), a gift of Immunex (Seattle, WA), were from the literature.[44] Aminopterin was purchased from Sigma Chemical Co. (St. Louis, MO). Calcium leucovorin (LV) was purchased from Schircks Laboratoties (Jona, Switzerland). Hypoxanthine (Hx), thymidine (TdR), and deoxycytidine (dCyd) were purchased from Sigma Chemical Co, (St. Louis, MO). Other chemicals and reagents were reagent grade or higher.

Cell Lines and Methods for Measuring Growth Inhibitory Potency. 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[45] and its MTX-resistant sublines R1,[46] R2,[47] and R30dm[17] were cultured as described.[17] R1 expresses 20-fold elevated levels of dihydrofolate reductase (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.[17,39] 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 leucovorin ((6*R*,*S*)-5-formyltetrahydrofolate) at 0.1−10 mM 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 mM, caused no growth inhibition in the absence of drug, however. Protection against growth inhibition of CCRF-CEM cells was also 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, **4**, or **5** that would inhibit growth by >80% over a growth period of ≈72 h; horse serum was decreased to 5% (normally 10%) in these studies to reduce its contribution of metabolites. 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.[48] 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 performed. 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.[49] Both **4** (93% recovery) and **5** (86% recovery) were themselves nearly quantitatively recovered during the assay procedure, thus ensuring that their polyglutamate products would also be quantitatively recovered. Kinetic constants for AMT 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 AMT concentrations tested. Assays contained ≈400 units of FPGS activity; one unit of FPGS catalyzes incorporation of 1 pmol of [^{3}H]glutamate/h.

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