Design, Synthesis, and Biological Activities of Classical *N*-{4-[2-(2-Amino-4-ethylpyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl}-L-glutamic Acid and Its 6-Methyl Derivative as Potential Dual Inhibitors of Thymidylate Synthase and Dihydrofolate Reductase and as Potential Antitumor Agents[1]

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Two novel analogues, *N*-{2-amino-4-ethyl[(pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl}-L-glutamic acid (**2**) and *N*-{2-amino-4-ethyl-6-methyl[(pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl}-L-glutamic acid (**4**), were designed and synthesized as potent dual inhibitors of thymidylate synthase (TS) and dihydrofolate reductase (DHFR) and as antitumor agents. Compound **2** had inhibitory potency against human DHFR similar to *N*-{4-[2-(amino-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl}-L-glutamic acid (LY231514) and **1**, whereas **4** was inactive against human DHFR. Both **2** and **4** were more potent than LY231514 against *E. coli* TS. Against human TS, **2** was 7-fold less potent than LY231514 and **4** showed similar inhibitory activity as LY231514. In contrast to **2**, which was an efficient substrate of human folypolyglutamate synthetase (FPGS), **4** was a poor substrate of FPGS. Compound **2** showed GI_{50} values in the nanomolar range against more than 18 human tumor cell lines in the standard NCI preclinical in vitro screen.

Introduction

Folate metabolism represents an important and attractive target for cancer chemotherapy because of its crucial role in the biosynthesis of nucleic acid precursors.[2,3] Dihydrofolate reductase (DHFR)[4] catalyzes the reduction of folate or 7,8-dihydrofolate (FH_2) to tetrahydrofolate (FH_4) and couples with thymidylate synthase (TS). TS is a crucial enzyme that catalyzes the reductive methylation of 2′-deoxyuridine 5′-monophosphate (dUMP) to thymidine 5′-monophosphate (dTMP) utilizing 5,10-methylenetetrahydrofolate as a cofactor that functions as the source of the one-carbon group as well as the reductant.[5] This is the sole de novo source of dTMP, and hence, inhibition of DHFR or TS activity leads to "thymineless death". Thus, TS and DHFR have long been recognized as important targets for cancer chemotherapy, and several DHFR and TS inhibitors have found utility as antitumor agents.[6] Usually a 2,4-diamino-substituted pyrimidine ring is considered important for potent DHFR inhibitory activity, while a 2-amino-4-oxopyrimidine or 2-methyl-4-oxopyrimidine ring is considered important for potent TS inhibitory activity.[6−8]

As part of our efforts to design and synthesize dual inhibitors of DHFR and TS that might be capable of providing "combination therapy" in a single agent without the pharmacokinetic disadvantages of two separate agents, Gangjee et al.[9] reported the synthesis of a novel compound *N*-{2-amino-4-methyl[(pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl}-L-glutamic acid **1** as a potent, classical, dual inhibitor of DHFR and TS (Chart 1). Compound **1** was designed to bind in the "2,4-diamino mode" to DHFR and in the "2-amino-4-methyl mode" to TS, and hence function as a dual inhibitor (Figure 1). The biological results showed that **1** was more potent than a structurally related antifolate LY231514 against both TS (*Lactobacillus casei*, *Escherichia coli*, and rat) and DHFR (human, *Toxoplasma gondii*, and *Pneumocystis carinii*). LY231514, a 2-amino-4-oxo-5-substituted pyrrolo[2,3-*d*]pyrimidine, is currently in phases II and III clinical trials as an anticancer agent. Compound **1** also demonstrated remarkable inhibitory potency against the growth of tumor cells in culture ($GI_{50} < 1.00 \times 10^{-8}$ M).[9] The X-ray crystal structure of **1** indicated that in *P. carinii* DHFR, the pyrrolo[2,3-*d*]pyrimidine ring does indeed bind in the "2,4-diamino mode"[9] (Figure 1). In this binding mode, the pyrrole nitrogen mimics the 4-amino moiety of 2,4-diamino pyrimidines. TS binding of **1** was expected to arise from the "2-amino-4-methyl mode" (Figure 1), which is obtained by a 180° rotation about the 2-NH_2−C_2 bond of the "2,4-diamino mode." Molecular modeling studies (SYBYL 6.5[10]) with human DHFR and the conformation of **1** from the X-ray crystal structure of **1** bound to *P. carinii* DHFR[9] shows that the proximity of the 4-methyl group of **1** to Phe31 and Leu22 of human DHFR may be responsible for the increased inhibitory activity of **1** compared with LY231514 (unfavorable interaction of the 4-oxo moiety with Phe31 and Leu22). In addition, molecular modeling of **1** in the crystal structure of the quinazoline CB3717 (PDDF)[11] or ZD1694

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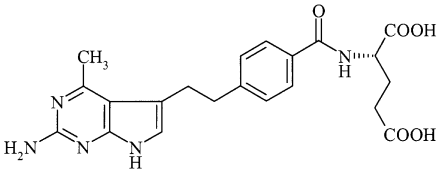
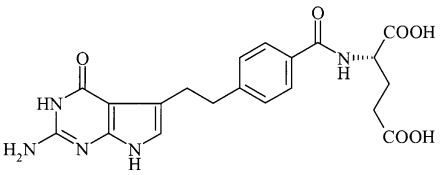
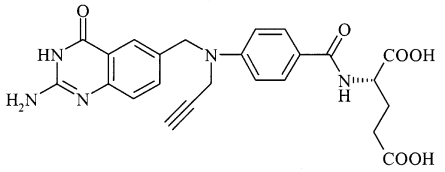
Chart 1



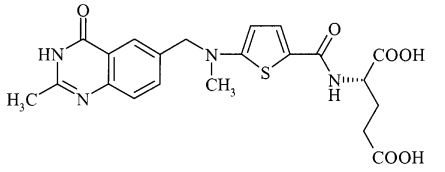
1



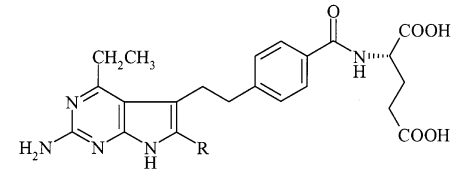
LY231514



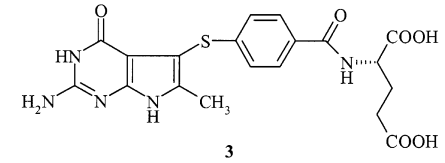
CB3717 (PDDF)



ZD1694 (Tomudex)

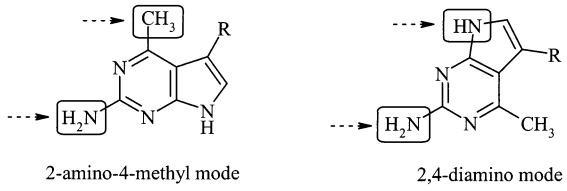


2: R = H
4: R = CH_3



3

(Tomudex)[12] with *E. coli* TS such that the pyrrole nitrogen of **1** is positioned on the 4-oxo moiety of the quinazoline predicts that the 4-methyl group mimics the quinazoline C8 of CB3717 or ZD1694 and can interact with Leu143 (3.42 Å) just as the C7 and C8 positions of the quinazolines. The pyrrole nitrogen with its hydrogen mimics the 4-oxo moiety and could hydrogen-bond with Asp169 and be in contact with the 4C═O of dUMP and Gly173. Further, the stacking interaction of the pyrrolo-[2,3-*d*]pyrimidine of **1** is more appropriately aligned with dUMP than is either CB3717 or ZD1694 and may contribute to the additional binding of **1** compared to LY231514. As a result, compound **1** exhibits greater TS inhibitory activity than LY231514.[9] Further, molecular modeling studies indicated that there was sufficient space in the lipophilic regions of both DHFR and TS to accommodate groups larger than methyl at the 4-position. Since the 4-methyl substitution provided potent inhibition of both DHFR and TS, homologation of the substitution to a 4-ethyl could perhaps further increase potency and provide better antitumor agents. Thus, **2** was designed as a classical dual inhibitor of DHFR and TS.



2-amino-4-methyl mode 2,4-diamino mode

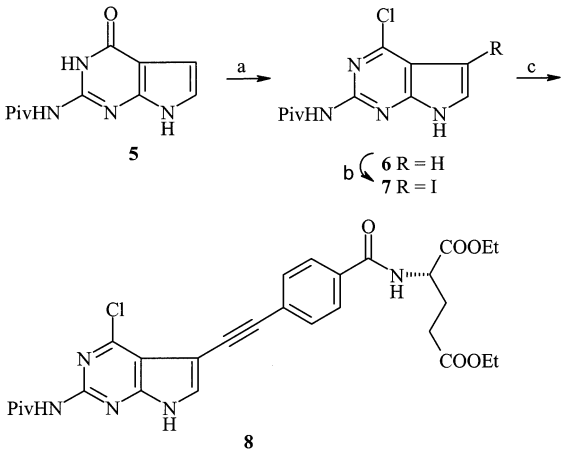
Figure 1.

A major drawback of classical antifolates, such as LY231514, is that they require transport into cells via the reduced folate carrier (RFC) system, which when impaired can lead to drug resistance.[13−15] An additional cause of resistance to classical antifolates is that their antitumor activity is in part determined by their ability to function as substrates for the enzyme folypoly-γ-glutamate synthetase (FPGS).[16,17] Thus, tumor cells with decreased FPGS activity are resistant to agents such as LY231514 that require FPGS for antitumor activity. FPGS catalyzes the formation of folylpoly-γ-glutamates, which are long-acting, noneffluxing forms of the classical antifolates that lead to high intracellular concentrations of these antitumor agents. Though poly-glutamylation of these agents is necessary for cytotoxicity to tumor cells, they have also been implicated as a possible cause of host toxicity, such as renal and hepatic toxicities in the host, since they are polyionic and are retained within normal cells as well.[18] Gangjee et al.[19] reported that compound **3**, bearing a 6-methyl group, was a potent inhibitor of human and bacterial TS. This analogue was neither a substrate nor an inhibitor of human FPGS derived from human CCRF-CEM cells and thus was devoid of the disadvantages mentioned above with respect to FPGS. Therefore, compound **4**, which incorporates a 6-methyl group into compound **2**, was designed and synthesized. Compound **4** was also anticipated to be a dual inhibitor of DHFR and TS and was not expected to be a substrate for FPGS. Further, the 6-methyl group was anticipated to sterically restrict the number of low-energy conformations attainable by the 5-substituted side chain. Such conformational restriction has been reported to enhance TS inhibition by providing conformation(s) more conducive to TS binding.[20] For DHFR inhibition, however, molecular modeling suggests that the 6-methyl group of **4** is expected to lie in a region that contains the backbone carbonyl oxygen of Val115 (hDHFR) and the phenolic hydroxy group of Tyr121 (hDHFR). These unfavorable interactions could result in a decrease in DHFR inhibitory activity of **4** compared to **2**. Thus, it was of interest to determine the effect of the 6-methyl group on DHFR, TS, and FPGS activity as well as its effect on the inhibition of the growth of tumor cells in culture.

Chemistry

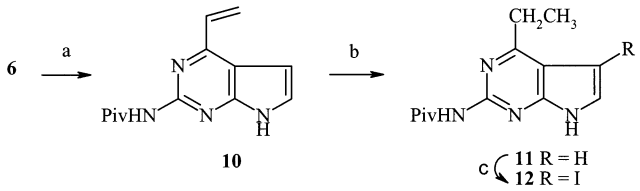
Compound **5**, which was previously reported, was chosen as the starting material (Scheme 1).[21−23] Compound **5**, refluxed with phosphorus oxychloride, afforded

Scheme 1[a]



[a] Reagents and conditions: (a) $POCl_3$, reflux; (b) NIS, DMF, room temp, dark; (c) diethyl *N*-(4-ethynylbenzoyl)-L-glutamate, $Pd(PPh_3)_4$, CuI, NEt_3, DMF.

Scheme 2[a]



[a] Reagents and conditions: (a) tributylvinyltin, $Pd(PPh_3)_4$, toluene, reflux; (b) 5% Pd/C, H_2, 50 psi; (c) NIS, THF, room temp, dark.

the 4-chloro derivative **6**. The 5-position of **6** was regiospecifically iodinated by treatment with *N*-iodosuccinamide (NIS) in the dark for 1 h to afford **7**. Palladium-catalyzed cross-coupling of **7** with diethyl *N*-(4-ethynylbenzoyl)-L-glutamate under Sonogashira conditions gave **8**. Compounds **6**−**8** have been previously reported by Shih et al.[24] However, the synthesis of **6** in this report is different from that of Shih et al.[24] Compounds **7** and **8** were obtained as described by Shih et al.[24] Since the palladium-catalyzed cross-coupling between aryl halides with organostannanes (the Stille reaction[25,26]) has been widely used for the construction of carbon−carbon bonds, coupling of **8** with tributylvinyltin in the presence of $Pd(PPh_3)_4$ was attempted for the synthesis of diethyl *N*-{4-[2-(2-pivaloylamino-4-vinylpyrrolo[2,3-*d*]pyrimidin-5-yl)ethynyl]benzoyl}-L-glutamate (**9**). However, the reaction did not occur even under reflux for 8 h. Changing the solvent from toluene to DMF also did not afford the desired product. Pivaloylation of the 2-amino moiety allows for protection as well as an increase of the organic solubility. An alternative route started from **6** (Scheme 2), which was subjected to Stille coupling with tributylvinyltin in the presence of $Pd(PPh_3)_4$. The reaction was driven to completion by vigorous reflux in toluene. Following reduction of the double bond and regioselective iodination, compound **12** was obtained in a relatively high yield. However, the coupling reaction of **12** with diethyl *N*-(4-ethynylbenzoyl)-L-glutamate under Sonogashira conditions was unsuccessful and did not afford **13**.

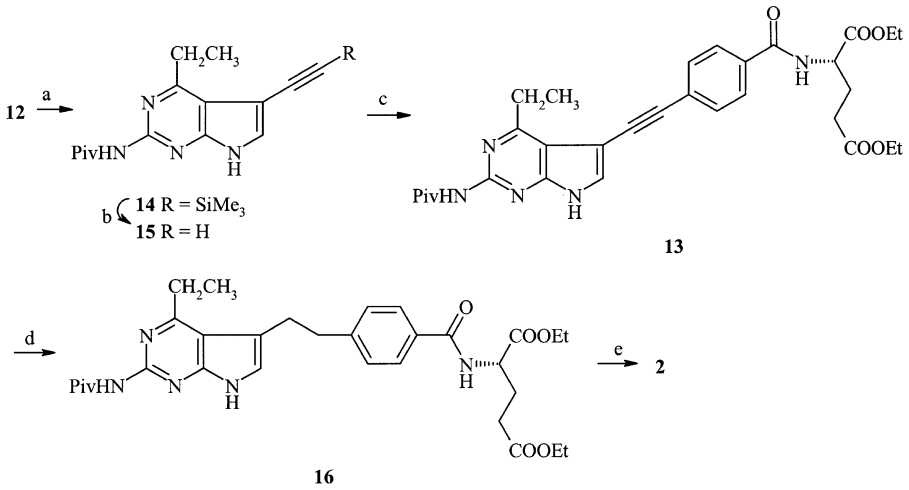
An alternative route for the synthesis of **2** involved the coupling of compound **12** with trimethylsilylacetylene in the presence of $Pd(PPh_3)_4$, CuI, and triethylamine, which afforded **14** in 75% yield (Scheme 3). Desilylation of **14** with fluoride ion gave **15** in 95% yield. Compound **13** was finally obtained by coupling **15** with diethyl *N*-(iodobenzoyl)-L-glutamate. The reaction yield was low and the workup was tedious. Compound **13** was reduced to afford **16** in 91% yield. Treatment of the resulting ethano-bridged intermediate **16** with 1 N NaOH resulted in simultaneous saponification of the ester and removal of the pivaloyl protecting group to afford **2** in 66% yield.

Compound **4** was synthesized from intermediate **20**. The scheme employed for the synthesis of **20** was a modification of reports by Taylor et al.[27] 4-Hydroxy-1-pentene **17** was reacted with methyl 4-bromobenzoate via palladium acetate catalyzed coupling in the presence of tetra-*n*-butylammonium chloride, lithium acetate, and lithium chloride to afford **18** in 80% yield (Scheme 4).[27a] Heating **18** under reflux in 2-methoxyethanol with 1 equiv of 2-amino-4-oxo-6-hydrazinopyrimidine[27a] afforded the requisite hydrazone **19** in 85% yield as a brown solid. The subsequent cyclocondensation was first attempted by thermolysis in refluxing tetralin (bp 207 °C); however, no reaction occurred. The reaction was eventually accomplished by using diphenyl ether (bp 256 °C) as a solvent, under nitrogen, to afford **20** in 47% yield. The purification of **20** was carried out by column chromatography, which was different from that reported by Taylor et al.[27b] To increase the solubility of **20** in organic solvents and to improve the yield of the subsequent 4-chlorination, the 2-amino group was pivaloylated using pivaloyl chloride and pyridine, which afforded **21** in high yield (96%) as a pale-yellow solid. Compound **21** was then refluxed for 2 h with phosphorus oxychloride to afford **22** in 90% yield. Compound **22** was coupled with tributylvinyltin in the presence of $Pd(PPh_3)_4$ to afford **23**. The reaction was refluxed in toluene and went to completion after 5 h. Unfortunately, the isolated yield, after several attempts, was always very low (5−10%). Brathe et al.[28] reported similar low yields in the synthesis of 6-alkenylpurines. It was mentioned in their work that 6-vinylpurine was prone to decomposition on extensive drying under high vacuum or storage. Thus, **23** was isolated by flash chromatography and immediately subjected to catalytic hydrogenation using palladium/charcoal to afford **24** in 62% yield (over two steps). Saponification of **24** with aqueous sodium hydroxide afforded the free acid **25** in 70% yield. Peptide coupling of **25** with the diethyl ester of L-glutamic acid afforded **26** in 76% yield. It was noted that the number of equivalents of the diethyl ester of L-glutamic acid required for the coupling was more than that of **25** and the reaction was repeated by at least one additional activation cycle (second cycle of addition of isobutyl chloroformate, diethyl ester of L-glutamic acid, and triethylamine). Final saponification with aqueous sodium hydroxide at room temperature followed by acidification to pH 4 in an ice bath afforded the diacid **4** in 52% yield.

Biological Evaluation and Discussion

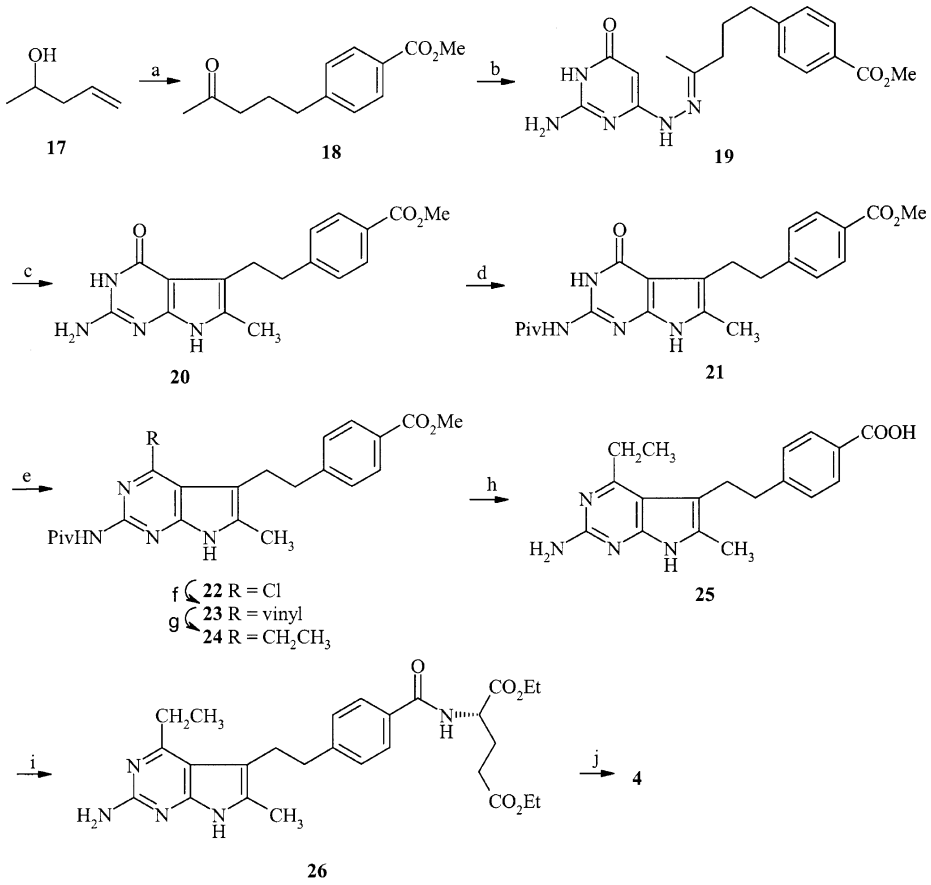
Compounds **2** and **4** were evaluated as inhibitors of *L. casei*, *E. coli*, and human DHFR and compared to **1**, LY231514, methotrexate (MTX), and trimethoprim (TMP) (Table 1). Compound **2** showed significant inhibitory

Scheme 3[a]



[a] Reagents and conditions: (a) trimethylsilylacetylene, $Pd(PPh_3)_4$, CuI, NEt_3, THF; (b) n-Bu_4NF, THF, room temp; (c) diethyl 4-iodobenzoyl-L-glutamate, $Pd(PPh_3)_4$, CuI, NEt_3, THF; (d) 5% Pd/C, H_2, 50 psi; (e) 1 N NaOH, then 1 N HCl.

Scheme 4[a]



[a] Reagents and conditions: (a) methyl 4-bromobenzoate, $Pd(OAc)_2$, n-Bu_4NCl, LiOAc, LiCl; (b) 2-amino-6-hydrazino-4-oxopyrimidine, 2-methoxyethanol, reflux; (c) diphenyl ether, reflux; (d) PivCl, pyridine, 120−130 °C; (e) $POCl_3$, reflux; (f) tributylvinyltin, $Pd(PPh_3)_4$, toluene, reflux; (g) 5% Pd/C, H_2, 50 psi; (h) 1 N NaOH, then 1 N HCl; (i) diethyl L-glutamate hydrochloride, isobutyl chloroformate, NEt_3; (j) 1 N NaOH, then 1 N HCl.

activity against *E. coli* DHFR and was 250-fold and 2-fold more potent than LY231514 and **1**, respectively, but was much less potent than MTX and TMP. Compound **2** had inhibitory activity against human DHFR similar to that of LY231514 and **1**, although it was less potent than MTX. While **4** was 10-fold more potent than LY231514 against *E. coli* DHFR, it was significantly less potent than **1** against *L. casei* DHFR (3000-fold) and *E. coli* DHFR (12-fold) (Table 1). Further, compound **4** was inactive against human DHFR, indicating that the 6-methyl moiety in this system all but abolishes human DHFR inhibitory activity. This decrease in activity against human DHFR of **4** compared to **2** is probably due to the unfavorable interaction of the 6-methyl moiety of **4** with the carbonyl oxygen of Val115 and the phenolic hydroxyl of Tyr121 of human DHFR.

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

compd	IC_{50} (M)		
	L. casei	*E. coli*[a]	human[b]
1	2.4×10^{-8}	1.8×10^{-6}	1.2×10^{-6}
2	*e*	9.2×10^{-7}	9.2×10^{-7}
4	7.0×10^{-5}	2.2×10^{-5}	2.2×10^{-5} (14[c])
LY231514[d]	2.3×10^{-4}	2.3×10^{-4}	1.4×10^{-6}
methotrexate	2.2×10^{-8}	7.0×10^{-9}	2.2×10^{-8}
trimethoprim	6.2×10^{-7}	1.5×10^{-8}	3.4×10^{-6}

[a] Kindly provided by Dr. R. L. Blakley, St. Jude Children's Research Hospital, Memphis, TN. [b] Kindly provided by Dr. J. H. Freisheim, Medical College of Ohio, Toledo, OH. [c] Percent inhibition. [d] Kindly provided by Dr. Chuan Shih, Eli Lilly & Co., Indianapolis, IN. [e] Not determined.

Table 2. Inhibitory Concentrations (IC_{50}) against Isolated TS

compd	IC_{50} (M)		
	L. casei	*E. coli*[a]	human[b]
1	9.0×10^{-6}	2.0×10^{-6}	1.0×10^{-6}
2	*f*	8.0×10^{-5}	8.0×10^{-5}
4	$>1.0 \times 10^{-4}$ (23[b])	1.8×10^{-5}	9.0×10^{-6}
LY231514[c]	2.0×10^{-5}	1.1×10^{-4}	1.1×10^{-5}
ZD1694[d]	8.0×10^{-6}	1.0×10^{-5}	1.0×10^{-6}
PDDF[e]	5.5×10^{-8}	1.0×10^{-7}	1.5×10^{-7}

[a] Kindly provided by Dr. F. Maley, New York State Department of Health, Albany, NY. [b] Percent inhibition. [c] Kindly provided by Dr. Chuan Shih, Eli Lilly & Co., Indianapolis, IN. [d] Kindly provided by Dr. Ann Jackman, Institute of Cancer Research, Sutton, Surrey, U.K. [e] Kindly provided by Dr. M. G. Nair, University of South Alabama, Mobile, AL. [f] Not determined.

Compounds **2** and **4** were also evaluated as inhibitors of *L. casei*, *E. coli*, and human TS (Table 2) along with LY231514, ZD1694, and PDDF. Compounds **2** and **4** were slightly more potent than LY231514 against *E. coli* TS. However compound **2** was 7-fold less potent than LY231514 against human TS, while compound **4** showed inhibitory activity similar to LY231514. Interestingly, both **2** and **4** were less potent than **1** against *E. coli* TS (9-fold and 40-fold, respectively) and human TS (9-fold and 80-fold, respectively). In contrast to DHFR, compound **4** was more potent than **2** against both *E. coli* TS (5-fold) and human TS (8-fold), indicating that the 6-methyl moiety was conducive to TS inhibitory activity. Thus, to some extent, analogues **2** and **4** do exhibit dual inhibition of DHFR and TS, as anticipated from their structural resemblance to **1**, and the 6-methyl moiety was conducive to TS inhibition but detrimental to DHFR inhibitory activity. Both **2** and **4** were less potent than ZD1694 and PDDF against TS.

The growth inhibitory potencies of **2** and **4** were compared to the growth inhibitory potencies of **1** and MTX in continuous exposure against CCRF-CEM[29] human leukemia cells in culture (Table 3). Compound **2** was highly growth inhibitory with an EC_{50} of 15 nM, which is similar to the potency of MTX and **1.** Interestingly, compound **4** was devoid of inhibitory activity against the growth of CCRF-CEM cells in culture. This indicates that 4-alkylation (as in **1** and **2**) is highly conducive to tumor growth inhibition in culture; however, minor changes (such as a 6-methyl group) in this structure can profoundly affect the inhibitory activity. CCRF-CEM cells treated with levels of **2** (50 and 70 nM) inhibiting growth by 94−97% could be fully protected by leucovorin at ≥ 0.1 μM (data not shown); this was similar to the pattern of protection against MTX-induced growth inhibition. This indicates an antifolate mechanism of action of **2**. Since **4** did not inhibit growth of CCRF-CEM cells even at 10 μM, the effect of leucovorin was not tested.

Table 3. Growth Inhibition of CCRF-CEM Human Leukemia Cells: Its MTX-Resistant Sublines with Defined Mechanism during Continuous Exposure (0−120 h)

drug	EC_{50},[a] nM			
	CCRF-CEM	R1[b] (↑DHFR)	R2[c] (↓uptake)	R30dm[d] (↓Glu_n)
MTX	13.7 ± 0.5 ($n = 7$)	985 ± 215 ($n = 4$)	2630 ± 95 ($n = 3$)	12.8 ± 3.2 ($n = 3$)
1[e]	12.5 ± 2.1 ($n = 4$)	158 ± 8 ($n = 2$)	575 ± 25 ($n = 2$)	18.5 ± 0.5 ($n = 2$)
2	15.0 ± 1.0 ($n = 2$)	695 ± 25 ($n = 2$)	750 ± 160 ($n = 2$)	18.5 ± 1.5 ($n = 2$)
4	>10000 ($n = 2$)	>20000 ($n = 2$)	>10000 ($n = 2$)	>20000 ($n = 2$)

[a] EC_{50} = concentration of drug required to decrease cell growth by 50% after 5 days of treatment. [b] CCRF-CEM subline resistant to MTX solely as a result of a 20-fold increase in wild-type DHFR protein and activity. [c] CCRF-CEM subline resistant as a result of decreased uptake of MTX. [d] 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. [e] Data from ref 9.

Since compound **2** was 42-fold less potent than MTX against isolated hDHFR but equipotent against the growth of tumor cells (CCRF-CEM) in culture, it was of interest to further define its mechanism of action. Compound **2** was thus evaluated in culture as an inhibitor of the growth of three CCRF-CEM sublines with defined MTX resistance mechanisms. A subline with increased DHFR expression,[30] R1, is 72-fold resistant to MTX but only 13-fold cross-resistant to **2**. A subline with decreased MTX uptake,[31] R2, is 179-fold resistant to MTX but only 46-fold cross-resistant to **2**. The subline with diminished ability to polyglutamylate MTX,[17] R30dm, is not MTX-resistant in continuous exposure (resistance is only seen in intermittent exposure) because nonpolyglutamylated MTX is a tight-binding DHFR inhibitor and transport is sufficient to maintain the drug in excess of DHFR. R30dm displayed only a 1.2-fold cross-resistance to **2** in continuous exposure. The cross-resistance patterns of the CCRF-CEM sublines are consistent with DHFR being the primary target of **2**. Cross-resistance of the MTX transport deficient subline R2 to **2** indicates that it uses the MTX/reduced folate carrier (RFC) for uptake. However, the degree of cross-resistance to **2** was much less than to MTX. This could indicate that the drug is more efficiently transported and/or polyglutamylated than is MTX and the higher intracellular levels attained are able to inhibit DHFR. The low level of cross-resistance of the polyglutamylation-deficient cell line R30dm could suggest that polyglutamylation of the analogue is not required for potent target inhibition by **2** or that the analogue is an extremely efficient FPGS substrate (see below).

Since polyglutamylation can markedly enhance the binding properties of substrate and inhibitors to most folate-dependent enzymes,[32] polyglutamates of **2** may be responsible for the inhibition of DHFR and TS. Both **2** and **4** were also tested as substrates for human FPGS to determine if polyglutamylation may be part of their mechanism of action (Table 4). The data indicate that

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

substrate	K_m, μM	$V_{max,rel}$	V_{max}/K_m	n
MTX[d]	49	0.82	0.02	
aminopterin	4.9 ± 1.0	1	0.21	4
1[b]	<1	0.17 ± 0.02	>0.17	2
2[c]	<2	0.23	>0.11	2
4	96 ± 19	0.10 ± 0.01	0.001 ± 0.0001	2

[a] FPGS substrate activity was determined as described in Experimental Section. Values presented are the average ± SD if $n \geq 3$ and are the average ± range for $n = 2$. [b] Data from ref 9. [c] Substrate activity was maximal at 5 μM. Slight substrate inhibition occurred at 5−50 μM. $V_{max,rel}$ is estimated on the basis of maximum activity of substrate relative to the maximum for aminopterin within the same experiment. [d] Data from ref 38.

Table 5. Cytotoxicity Evaluation against Selected Tumor Cell Lines[35]

	GI_{50}, M		
	1	**2**	**4**
	Leukemia		
HL-60	$<1.00 \times 10^{-8}$	$<1.00 \times 10^{-8}$	$<1.00 \times 10^{-8}$
MOLT-4	$<1.00 \times 10^{-8}$	$>1.00 \times 10^{-4}$	1.00×10^{-7}
K-562	1.47×10^{-5}	$<1.00 \times 10^{-8}$	1.43×10^{-7}
	Non-Small-Cell Lung Cancer		
A549/ATCC		$<1.00 \times 10^{-8}$	$>1.00 \times 10^{-4}$
NCI−H226	6.14×10^{-8}	$<1.00 \times 10^{-8}$	$>1.00 \times 10^{-4}$
NCI−H460	$<1.00 \times 10^{-8}$	$<1.00 \times 10^{-8}$	$>1.00 \times 10^{-4}$
	Colon Cancer		
HCT-15	$>1.00 \times 10^{-4}$	$<1.00 \times 10^{-8}$	$>1.00 \times 10^{-4}$
HT29	$>1.00 \times 10^{-4}$	$<1.00 \times 10^{-8}$	$>1.00 \times 10^{-4}$
SW-620	$>1.00 \times 10^{-4}$	$<1.00 \times 10^{-8}$	$>1.00 \times 10^{-4}$
	CNS Cancer		
SF-539	$<1.00 \times 10^{-8}$	$<1.00 \times 10^{-8}$	$>1.00 \times 10^{-4}$
	Melanoma		
LOX IMVI	1.15×10^{-5}	$<1.00 \times 10^{-8}$	9.86×10^{-5}
UACC-257	$>1.00 \times 10^{-4}$	$<1.00 \times 10^{-8}$	$>1.00 \times 10^{-4}$
UACC-62	$>1.00 \times 10^{-4}$	$<1.00 \times 10^{-8}$	$>1.00 \times 10^{-4}$
	Ovarian Cancer		
OVCAR-4	$>1.00 \times 10^{-4}$	$<1.00 \times 10^{-8}$	$>1.00 \times 10^{-4}$
	Renal Cancer		
ACHN	$<1.00 \times 10^{-8}$	1.69×10^{-7}	$>1.00 \times 10^{-4}$
SN12C	$>1.00 \times 10^{-4}$	$<1.00 \times 10^{-8}$	$>1.00 \times 10^{-4}$
	Breast Cancer		
MCF7	1.69×10^{-7}	$<1.00 \times 10^{-8}$	$>1.00 \times 10^{-4}$
MDA-MB-435	$>1.00 \times 10^{-4}$	$<1.00 \times 10^{-8}$	$>1.00 \times 10^{-4}$
MAD-N	$>1.00 \times 10^{-4}$	$<1.00 \times 10^{-8}$	$>1.00 \times 10^{-4}$

analogue **2** is nearly as efficient as aminopterin (AMT) as an FPGS substrate; its low K_m value and low V_{max} precluded determination of these kinetic constants. Analogue **2** also has a 19-fold lower K_m and greater than 220-fold higher substrate efficiency compared to analogue **4**. These results support the previous hypothesis[33] that the existence of a 6-methyl group in both **3** and **4** contributes to their poorer FPGS substrate activity.

Compounds **2** and **4** were selected by the National Cancer Institute for evaluation as antitumor agents in the preclinical in vitro screening program.[34] The ability of compounds **2** and **4** 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% compared to a control (Table 5). Compound **2** showed potent antitumor activity and displayed a much broader spectrum of activity (18 cell lines) than **1** (6 cell lines) with IC_{50} values below 1.00×10^{-8} M. Compared with the 4-methyl group in compound **1**, the 4-ethyl group in compound **2** is clearly responsible for this increased spectrum of activity. In contrast, compound **4** had an IC_{50} value below 1.00×10^{-8} M in only one cell line (HL-60).

In summary, 2-amino-4-ethyl 5-substituted pyrrolo[2,3-*d*]pyrimidine **2** and its 6-methyl analogue **4** were designed and synthesized as classical antifolates. The biological results show that, as an analogue of compound **1**, compound **2** retained similar activity against both DHFR and TS and compound **4** exhibited relatively low activity against DHFR but was a better inhibitor of TS than **2**. It is noteworthy that extending the 4-substituent from methyl to ethyl in compound **2** dramatically increased the inhibitory activity and the spectrum against the growth of several tumor cells in culture. Similar to compound **1**, compound **2** was an excellent substrate for FPGS. The poor FPGS activity of compound **4** demonstrated that a 6-methyl group, as anticipated from previous reports, is detrimental to FPGS substrate activity.

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 and ethanol at reflux. 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 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, exch = protons exchangeable by addition of D_2O. 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. and Fisher Scientific and were used as received except anhydrous solvents, which were freshly dried in the laboratory.

2-Pivaloylamino-4-chloropyrrolo[2,3-*d*]pyrimidine (6). A mixture of **5**[21] (0.45 g, 1.92 mmol) and phosphorus oxychloride (4.5 mL) was heated under reflux for 2 h. Excess phosphorus oxychloride was removed under vacuum. The gummy residue was stirred vigorously with crushed ice (5 g), and the pH was adjusted to 4 with concentrated ammonium hydroxide. The resulting precipitate was filtered, washed with cold water (10 mL × 2), and dried. The crude solid was dissolved in methanol, silica gel (5 g) was added, and the solvent was evaporated to form a plug, which was dried, loaded on top of a silica gel column, and eluted with 5:2 hexanes/ethyl acetate. Fractions containing the product (TLC) were pooled, and the solvent evaporated to afford 0.20 g (41%) of **6** as a white solid: TLC $R_f = 0.58$ ($CHCl_3$/MeOH, 9:1); mp 212 °C, dec (lit.[24] mp 217−220 °C, dec); ^{1}H NMR (DMSO-d_6) δ 1.22 (s, 9 H, $C(CH_3)_3$), 6.52 (d, 1 H, 5-H), 7.54 (t, 1 H, 6-H), 10.05 (br, 1 H, 2-N*H*Piv, exch), 12.19 (br, 1 H, 7-NH, exch).

2-Pivaloylamino-4-vinylpyrrolo[2,3-*d*]pyrimidine (10). A solution of **6** (0.10 g, 0.40 mmol) and tributylvinyltin (0.38

g, 1.20 mmol) in toluene (10 mL) was stirred under nitrogen for 30 min, followed by the addition of tetrakis(triphenylphosphine)palladium(0) (0.05 g, 0.04 mmol). The reaction mixture was then refluxed for 3 h, cooled to room temperature, and filtered through a Celite pad. The solvent was removed in vacuo to afford the crude product, which was further purified by flash chromatography on silica gel and eluted with 1.5% MeOH in $CHCl_3$. Fractions containing the product (TLC) were pooled, and the solvent was evaporated to afford 0.07 g (70%) of **10** as a yellow solid: TLC $R_f = 0.37$ ($CHCl_3$/MeOH, 19:1); mp 188−190 °C; ^{1}H NMR (DMSO-d_6) δ 1.23 (s, 9 H, $C(CH_3)_3$), 5.75 (dd, 1 H, $J = 10.8$ Hz, $J = 1.0$ Hz, CH=CH_2), 6.53 (dd, 1 H, $J = 17.45$ Hz, $J = 1.0$ Hz, CH=CH_2), 6.59 (d, 1 H, $J = 2.1$ Hz, 5-H), 7.13 (dd, 1 H, $J = 10.8$ Hz, $J = 17.45$ Hz, CH=CH_2), 7.48 (d, 1 H, $J = 2.9$ Hz, 6-H), 9.67 (s, 1 H, 2-NHPiv, exch), 11.95 (s, 1 H, 7-NH, exch); HRMS (EI) calcd for $C_{13}H_{16}N_4O$ 244.1324, found 244.1334.

2-Pivaloylamino-4-ethylpyrrolo[2,3-*d*]pyrimidine (11). To a Parr hydrogenation bottle was added **10** (0.72 g, 2.95 mmol) dissolved in a mixture of THF (15 mL) and methylene chloride (15 mL), followed by the addition of 5% Pd/C (0.90 g). This mixture was hydrogenated at 50 psi for 18 h. After the catalyst was filtered and washed thoroughly with hot methanol (40 mL), the filtrate was concentrated in vacuo. The crude residue was then flash-chromatographed on silica gel and eluted with a gradient of 0−1.5% MeOH in $CHCl_3$. Fractions containing the product (TLC) were pooled and the solvent was evaporated to afford 0.65 g (91%) of **11** as a yellow foam: TLC $R_f = 0.37$ ($CHCl_3$/MeOH, 19:1); ^{1}H NMR (DMSO-d_6) δ 1.23 (m, 12 H, CH_2CH_3 and $C(CH_3)_3$), 2.92 (q, 2 H, $J = 7.5$ Hz, CH_2CH$_3$), 6.58 (d, 1 H, $J = 2.0$ Hz, 5-H), 7.37 (d, 1 H, $J = 2.0$ Hz, 6-H), 9.67 (s, 1 H, 2-NHPiv, exch), 11.85 (s, 1 H, 7-NH, exch).

2-Pivaloylamino-4-ethyl-5-iodopyrrolo[2,3-*d*]pyrimidine (12). To a 25 mL round-bottomed flask, protected from light with aluminum foil, were added **11** (0.41 g, 1.67 mmol) and *N*-iodosuccinimide (0.41 g, 1.84 mmol) dissolved in anhydrous THF (10 mL). The reaction mixture was stirred under nitrogen for 1 h. The solvent was removed in vacuo, and the residue was dissolved in methylene chloride (100 mL). The reaction mixture was washed with brine, and the organic layer was separated, dried over Na_2SO_4, and concentrated in vacuo. The crude residue was flash-chromatographed on silica gel and eluted with 0.5% MeOH in $CHCl_3$. Fractions containing the desired product (TLC) were pooled and evaporated to afford 0.35 g (56%) of **12** as a pale-yellow solid: TLC $R_f = 0.33$ (MeOH/$CHCl_3$, 1:19); mp 206−208 °C; ^{1}H NMR (DMSO-d_6) δ 1.27 (s, 9 H, $C(CH_3)_3$), 1.32 (t, 3 H, $J = 7.5$ Hz, CH_2CH_3), 3.13 (q, 2 H, $J = 7.5$ Hz, CH_2CH_3), 7.62 (s, 1 H, 6-H), 9.75 (s, 1 H, 2-NHPiv, exch), 12.28 (s, 1 H, 7-NH, exch); HRMS (EI) calcd for $C_{13}H_{17}N_4O$ 372.0447, found 372.0437.

2-Pivaloylamino-4-ethyl-5-trimethylsilylethynylpyrrolo-[2,3-*d*]pyrimidine (14). To a 50 mL round-bottomed flask, protected from light with aluminum foil, were added **12** (0.29 g, 0.77 mmol), copper(I) iodide (0.057 g, 0.30 mmol), trimethylsilylacetylene (0.45 g, 4.6 mmol), and tetrakis(triphenylphosphine)palladium(0) (0.088 g, 0.077 mmol) dissolved in anhydrous THF (15 mL), anhydrous DMF (5 mL), and triethylamine (0.1 mL). The solution was stirred at room temperature for 4 h under nitrogen. The volatiles were removed in vacuo, and the residue was dissolved in dichloromethane (100 mL). The reaction mixture was washed with water, and the organic layer was separated, dried over Na_2SO_4, and concentrated in vacuo. The crude residue was then flash-chromatographed on silica gel and eluted with 1% MeOH in $CHCl_3$. Fractions containing the desired product (TLC) were pooled and evaporated to afford 0.20 g (75%) of **14** as a white solid: TLC $R_f =$ 0.42 (MeOH/$CHCl_3$, 1:19); mp 261−262.5 °C; ^{1}H NMR (DMSO-d_6) δ 0.23 (s, 9 H, $Si(CH_3)_3$), 1.17 (s, 9 H, $C(CH_3)_3$), 1.29 (t, 3 H, $J = 7.5$ Hz, CH_2CH_3), 3.14 (q, 2 H, $J = 7.5$ Hz, CH_2CH_3), 7.76 (d, 1 H, $J = 2.0$ Hz, 6-H), 9.75 (s, 1 H, 2-NHPiv, exch), 12.27 (s, 1 H, 7-NH, exch); HRMS (EI) calcd for $C_{18}H_{26}N_4OSi$ 342.1875, found 342.1885.

2-Pivaloylamino-4-ethyl-5-ethynylpyrrolo[2,3-*d*]pyrimidine (15). To a 25-mL round-bottomed flask were added **14** (0.17 g, 0.50 mmol) and tetrabutylammonium fluoride (1 mL) dissolved in THF (10 mL). The reaction mixture was heated at 50 °C under nitrogen for 30 min. The reaction was then quenched by pouring the mixture into chloroform (80 mL) and washing it with brine. The organic layer was separated, dried over Na_2SO_4, and concentrated in vacuo. The residue was then flash-chromatographed on silica gel and eluted with 3% MeOH in $CHCl_3$. Fractions containing the desired product (TLC) were pooled and evaporated to afford 0.13 g (95%) of **15** as a pale-yellow solid: TLC $R_f = 0.40$ (MeOH/$CHCl_3$, 1:19); mp 200 °C, dec; ^{1}H NMR (DMSO-d_6) δ 1.23 (s, 9 H, $C(CH_3)_3$), 1.32 (t, 3 H, $J = 7.5$ Hz, CH_2CH_3), 3.13 (q, 2 H, $J = 7.5$ Hz, CH_2CH_3), 4.19 (s, 1 H, CH), 7.77 (s, 1 H, 6-H), 9.75 (s, 1 H, 2-NHPiv, exch), 12.26 (s, 1 H, 7-NH, exch); HRMS (EI) calcd for $C_{15}H_{18}N_4O$ 270.1481, found 270.1484.

Diethyl *N*-{4-[2-(2-Pivaloylamino-4-ethylpyrrolo[2,3-*d*]-pyrimidin-5-yl)ethynyl]benzoyl}-L-glutamate (13). To a 25 mL round-bottomed flask, protected from light with aluminum foil, were added **15** (0.06 g, 0.22 mmol), copper(I) iodide (0.05 g, 0.03 mmol), diethyl 4-iodobenzoyl-L-glutamate (0.29 g, 0.66 mmol), and tetrakis(triphenylphosphine)palladium(0) (0.04 g, 0.035 mmol) dissolved in anhydrous THF (10 mL) and triethylamine (0.5 mL). The solution was stirred at room temperature for 4 h under nitrogen. The volatiles were removed in vacuo, and the residue was dissolved in methylene chloride (100 mL). The reaction mixture was washed with brine, and the organic layer was separated, dried over Na_2SO_4, and concentrated in vacuo. The crude residue was then flash-chromatographed on silica gel and eluted with a gradient of 0−1% MeOH in $CHCl_3$. Fractions containing the desired product (TLC) were pooled and evaporated to afford 0.03 g (24%) of **13** as a pale-yellow solid: TLC $R_f = 0.40$ (MeOH/$CHCl_3$, 1:19); mp 223−225 °C; ^{1}H NMR (DMSO-d_6) δ 1.21 (m, 6 H, α- and γ-$COOCH_2CH_3$), 1.24 (s, 9 H, $C(CH_3)_3$), 1.38 (t, 3 H, $J = 7.4$ Hz, CH_2CH_3), 2.10 (m, 2 H, β-CH_2), 2.45 (t, 2 H, $J = 7.5$ Hz, γ-CH_2), 3.23 (q, 2 H, $J = 7.4$ Hz, CH_2CH$_3$), 4.06 (m, 4 H, α- and γ-$COOCH_2CH_3$), 4.15 (m, 1 H, α-CH), 7.62 (d, 2 H, $J = 8.4$ Hz, C_6H_4), 7.89 (d, 1 H, $J = 2.2$ Hz, 6-H), 7.94 (d, 2 H, $J = 8.4$ Hz, C_6H_4), 8.83 (d, 1 H, $J = 7.4$ Hz, CONH, exch), 9.81 (s, 1 H, 2-NHPiv, exch), 12.43 (s, 1 H, 7-NH, exch); HRMS (EI) calcd for $C_{31}H_{37}N_5O_6$ 575.2744, found 575.2749.

Diethyl *N*-{4-[2-(2-Pivaloylamino-4-ethylpyrrolo[2,3-*d*]-pyrimidin-5-yl)ethyl]benzoyl}-L-glutamate (16). To a Parr hydrogenation bottle was added **13** (0.115 g, 0.20 mmol), dissolved in methanol (15 mL) and methylene chloride (15 mL), followed by the addition of 5% Pd/C (0.15 g). This mixture was hydrogenated at 50 psi for 2 days. After the catalyst was filtered and washed thoroughly with hot methanol (30 mL), the filtrate was concentrated in vacuo. The crude residue was then flash-chromatographed on silica gel and eluted with 1:19 MeOH/$CHCl_3$. Fractions containing the desired product (TLC) were pooled and evaporated to afford 0.109 g (91%) of **16** as a white solid: TLC $R_f = 0.32$ (MeOH/$CHCl_3$, 1:19); mp 160.5−162.5 °C; ^{1}H NMR (DMSO-d_6) δ 1.17 (m, 6 H, α- and γ-$COOCH_2CH_3$), 1.23 (s, 9 H, $C(CH_3)_3$), 1.28 (t, 3 H, $J = 7.5$ Hz, 4-CH_2CH_3), 2.09 (m, 2 H, β-CH_2), 2.44 (t, 2 H, $J = 7.5$ Hz, γ-CH_2), 2.95 (q, 2 H, $J = 7.5$ Hz, 4-CH_2CH$_3$), 3.06 (m, 4 H, CH_2CH_2), 4.09 (m, 4 H, α- and γ-$COOCH_2CH_3$), 4.43 (m, 1 H, α-CH), 7.12 (s, 1 H, 6-H), 7.36 (d, 2 H, $J = 7.9$ Hz, C_6H_4), 7.81 (d, 2 H, $J = 7.9$ Hz, C_6H_4), 8.66 (d, 1 H, $J = 7.4$ Hz, CONH, exch), 9.62 (s, 1 H, 2-NHPiv, exch), 11.59 (s, 1 H, 7-NH, exch); HRMS (EI) calcd for $C_{31}H_{41}N_5O_6$ 579.3075, found 579.3057.

***N*-{4-[2-(2-Amino-4-ethylpyrrolo[2,3-*d*]pyrimidin-5-yl)-ethyl]benzoyl}-L-glutamic Acid (2).** To a 15 mL round-bottomed flask was added **16** (0.09 g, 0.16 mmol) suspended in 1.0 N NaOH (2 mL). The reaction mixture was stirred at 50 °C for 3 days. The resulting solution was cooled in an ice bath and acidified with 1.0 N HCl to pH 4, and the precipitate was filtered, washed with water, and dried in vacuo to give 0.05 g (66%) of **2** as a white solid: mp 248−250 °C, dec; ^{1}H NMR (DMSO-d_6) δ 1.25 (t, 3 H, $J = 7.2$ Hz, CH_2CH_3), 1.95−2.02 (m, 2 H, β-CH_2), 2.37 (t, 2 H, $J = 7.0$ Hz, γ-CH_2), 2.85 (q, 2 H, $J = 7.2$ Hz, CH_2CH$_3$), 2.97 (m, 4 H, CH_2CH_2), 4.27 (m, 1 H, α-CH), 5.91 (s, 2 H, 2-NH_2, exch), 6.68 (d, 1 H, 6-H), 7.34

(d, 2 H, J = 8.0 Hz, C_6H_4), 7.80 (d, 2 H, J = 8.0 Hz, C_6H_4), 8.52 (d, 1 H, J = 7.4 Hz, CONH, exch), 10.75 (s, 1 H, 7-NH, exch), 12.35 (br, 1 H, COOH, exch). Anal. ($C_{22}H_{25}N_5O_5$) C, H, N.

Methyl 4-[2-(2-Amino-4-oxo-6-methylpyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoate (20). A mixture of **19** (0.40 g, 1.16 mmol) in diphenyl ether (20 mL) was stirred under nitrogen and heated under reflux for 5 h. After the mixture was cooled to room temperature, hexanes (50 mL) were added and the precipitated solid was collected by filtration. The residue was suspended in methanol (50 mL), silica gel (10 g) was added, and the solvent was evaporated to afford a plug. The silica gel plug obtained was loaded onto a silica gel column and eluted with a gradient of 5−10% MeOH in $CHCl_3$. Fractions containing the product (TLC) were pooled and the solvent was evaporated to afford 0.18 g (47%) of **20** as a pale-yellow solid: TLC R_f = 0.41 ($CHCl_3$/MeOH, 5:1); mp 260 °C, dec (lit.[27] 260 °C ,dec); ^{1}H NMR (DMSO-d_6) δ 1.82 (s, 3 H, CH_3, 2.76 (t, 2 H, J = 6.5 Hz, CH_2), 2.90 (t, 2 H, J = 6.5 Hz, CH_2), 3.82 (s, 3 H, OCH_3), 5.94 (br, 2 H, 2-NH_2, exch), 7.26 (d, 2 H, J = 7.7 Hz, C_6H_4), 7.83 (d, 2 H, J = 7.7 Hz, C_6H_4), 10.07 (br, 1 H, 3-NH, exch), 10.48 (br, 1 H, 7-NH, exch). Anal. ($C_{17}H_{18}N_4O_3$·0.5H_2O) C, H, N.

Methyl 4-[2-(2-Pivaloyamino-4-oxo-6-methylpyrrolo-[2,3-*d*]pyrimidin-5-yl)ethyl]benzoate (21). To a 50 mL round-bottomed flask was added **20** (0.15 g, 0.43 mmol) in anhydrous pyridine (5 mL). To this solution was added pivaloyl chloride (0.27 mL, 2.14 mmol). The reaction mixture was heated to reflux for 3.5 h under nitrogen. The solvents were removed in vacuo, and the residue was dissolved in MeOH (50 mL). Silica gel (10 g) was added to this solution and the solvent was evaporated to afford a plug that was flash-chromatographed on a silica gel column and that eluted with 0.5% MeOH in $CHCl_3$ to afford 0.18 g (96%) of **21** as a pale-yellow solid: TLC R_f = 0.31 ($CHCl_3$/MeOH, 19:1); mp 246−248 °C; ^{1}H NMR (DMSO-d_6) δ 1.24 (s, 9 H, $C(CH_3)_3$), 1.91 (s, 3 H, 6-CH_3), 2.87 (m, 4 H, CH_2CH_2), 3.82 (s, 3 H, OCH_3), 7.26 (d, 2 H, J = 7.8 Hz, C_6H_4), 7.83 (d, 2 H, J = 7.8 Hz, C_6H_4), 10.74 (s, 1 H, 2-N*H*Piv or 3-NH, exch), 11.05 (s, 1 H, 2-N*H*Piv or 3-NH, exch), 11.75 (s, 1 H, 7-NH, exch). Anal. ($C_{22}H_{27}N_4O_3$) C, H, N.

Methyl 4-[2-(2-Pivaloyamino-4-chloro-6-methylpyrrolo-[2,3-*d*]pyrimidin-5-yl)ethyl]benzoate (22). A solution of **21** (0.18 g, 0.44 mmol) and phosphorus oxychloride (5 mL) was refluxed at 120 °C for 2.5 h. The excess $POCl_3$ was evaporated, and the residue was neutralized with NH_4OH to pH 4. The solution was diluted by addition of methylene chloride (100 mL). The organic layer was washed with brine (50 mL × 2), separated, dried over Na_2SO_4, and concentrated in vacuo. To this solution were added methylene chloride (20 mL) and silica gel (10 g), and the solvent was then evaporated. The silica gel plug obtained was loaded onto a silica gel column and eluted with 0.5% MeOH in $CHCl_3$. Fractions containing the product (TLC) were pooled and the solvent evaporated to afford 0.17 g (90%) of **22** as a white solid: TLC R_f = 0.38 ($CHCl_3$/MeOH, 19:1); mp 202−204 °C; ^{1}H NMR (DMSO-d_6) δ 1.23 (s, 9 H, $C(CH_3)_3$), 2.02 (s, 3 H, 6-CH_3), 2.94 (t, 2 H, J = 6.7 Hz, CH_2CH_2), 3.01 (t, 2 H, J = 6.7 Hz, CH_2CH_2), 3.83 (s, 3 H, OCH_3), 7.26 (d, 2 H, J = 7.4 Hz, C_6H_4), 7.85 (d, 2 H, J = 7.4 Hz, C_6H_4), 9.95 (s, 1 H, 2-N*H*Piv, exch), 11.95 (s, 1 H, 7-NH, exch). Anal. ($C_{22}H_{25}N_4O_3Cl$) C, H, N, Cl.

Methyl 4-[2-(2-Pivaloyamino-4-vinyl-6-methylpyrrolo-[2,3-*d*]pyrimidin-5-yl)ethyl]benzoate (23). A solution of **22** (0.18 g, 0.42 mmol) and tributylvinyltin (0.40 g, 1.26 mmol) in toluene (15 mL) was stirred under nitrogen for 30 min, followed by the addition of $Pd(PPh_3)_2Cl_2$ (0.06 g, 0.084 mmol). The reaction mixture was then refluxed for 4 h, cooled to room temperature, and filtered through a Celite pad. The filtrate was evaporated in vacuo to afford the crude product that was further purified by flash chromatography on silica gel and eluted with 1% MeOH in $CHCl_3$. Fractions containing the product (TLC) were pooled and the solvent was evaporated to afford 0.12 g (67%) of **23** as a yellow sticky foam: TLC R_f = 0.37 ($CHCl_3$/MeOH, 19:1); ^{1}H NMR (DMSO-d_6) δ 1.22 (s, 9 H, $C(CH_3)_3$), 1.96 (s, 3 H, 6-CH_3), 2.70−3.30 (m, 4 H, CH_2CH_2), 3.82 (s, 3 H, OCH_3), 5.72 (dd, 1 H, J = 10.8 Hz, J = 1.0 Hz, CH=CH_2), 6.57 (dd, 1 H, J = 17.4 Hz, J = 1.0 Hz, CH=CH_2), 7.29 (d, 2 H, J = 7.7 Hz, 5-H), 7.32 (dd, 1 H, J = 10.8 Hz, J = 17.4 Hz, C*H*=CH_2), 7.84 (d, 2 H, J = 7.7 Hz, C_6H_4), 9.56 (s, 1 H, 2-N*H*Piv, exch), 11.52 (s, 1 H, 7-NH, exch).

Methyl 4-[2-(2-Pivaloyamino-4-ethyl-6-methylpyrrolo-[2,3-*d*]pyrimidin-5-yl)ethyl]benzoate (24). To a Parr hydrogenation bottle was added **23** (0.12 g, 0.35 mmol) dissolved in a mixture of THF (10 mL) and methylene chloride (10 mL), followed by the addition of 5% Pd/C (0.10 g). This mixture was hydrogenated at 50 psi for 18 h. After the catalyst was filtered and washed thoroughly with methanol (30 mL), the filtrate was concentrated in vacuo. The crude residue was then flash-chromatographed on silica gel and eluted with a gradient of 0−1% MeOH in $CHCl_3$. Fractions containing the product (TLC) were pooled and the solvent was evaporated to afford 0.11 g (92%) of **24** as a yellow sticky solid: TLC R_f = 0.42 ($CHCl_3$/MeOH, 19:1); mp 72−74 °C; ^{1}H NMR (DMSO-d_6) δ 1.22−1.29 (m, 12 H, $C(CH_3)_3$ and 4-CH_2CH_3), 1.99 (s, 3 H, 6-CH_3), 2.80−3.20 (m, 6 H, CH_2CH_2 and 4-CH_2CH_3), 3.83 (s, 3 H, OCH_3), 7.25 (d, 2 H, J = 7.4 Hz, C_6H_4), 7.85 (d, 2H, J = 7.4 Hz, C_6H_4), 9.55 (s, 1 H, 2-N*H*Piv, exch), 11.50 (s, 1 H, 7-NH, exch); HRMS (EI) calcd for $C_{24}H_{30}N_4O_3$ 422.2321, found 422.2318.

4-[2-(2-Amino-4-ethyl-6-methylpyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoic Acid (25). To a solution of **24** (0.12 g, 0.35 mmol) in MeOH (10 mL) was added 1 N NaOH (2 mL), and the mixture was stirred under nitrogen at room temperature for 2 days to complete the reaction. The reaction mixture was concentrated in vacuo and cooled in an ice bath, and the residual aqueous solution was acidified with 1 N HCl to pH 4. The resulting suspension was filtered, washed with a small amount of cold water and ethyl ether, and dried in vacuo to afford 0.08 g (70%) of **25** as a buff-colored solid: mp 190 °C, dec; ^{1}H NMR (DMSO-d_6) δ 1.24 (t, 3 H, J = 7.3 Hz, 4-CH_2CH_3), 1.90 (s, 3 H, CH_3), 2.82 (m, 6 H, CH_2CH_2 and 4-CH_2CH_3), 5.79 (s, 2 H, 2-NH_2, exch), 7.22 (d, 2 H, J = 7.4 Hz, C_6H_4), 7.82 (d, 2 H, J = 7.4 Hz, C_6H_4), 10.66 (s, 1 H, 7-NH, exch), 12.70 (br, 1 H, COOH, exch); HRMS (EI) calcd for $C_{18}H_{20}N_4O_2$ 324.1586, found 324.1585.

Diethyl *N*-{4-[2-(2-Amino-4-ethyl-6-methylpyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl}-L-glutamate (26). To a solution of **25** (0.10 g, 0.31 mmol) in anhydrous DMF (5 mL) was added triethylamine (0.13 mL), and the mixture was stirred under nitrogen at room temperature for 5 min. The resulting solution was cooled to 0 °C, isobutyl chloroformate (0.13 mL, 0.96 mmol) was added, and the mixture was stirred at 0 °C for 30 min. At this time TLC ($CHCl_3$/MeOH, 5:1) indicated the formation of the activated intermediate at R_f = 0.55 and the disappearance of the starting acid (R_f = 0.35). Diethyl L-glutamate hydrochloride (0.24 g, 0.96 mmol) was added to the reaction mixture followed immediately by triethylamine (0.13 mL, 0.96 mmol). The reaction mixture was slowly warmed to room temperature and was stirred under nitrogen for 6 h. The reaction mixture was then subjected to another cycle of activation and coupling using half the quantities listed above. The reaction mixture was slowly warmed to room temperature and was stirred for an additional 24 h. The reaction mixture was then subjected to a third round of activation and coupling using the same quantities as the second round and was stirred for an additional 24 h. TLC showed the formation of one major spot at R_f = 0.52 ($CHCl_3$/MeOH, 5:1). The reaction mixture was evaporated to dryness under reduced pressure. The residue was dissolved in MeOH (5 mL), silica gel (10 g) was added, and the solvent was removed to form a plug. The silica gel plug obtained was loaded onto a silica gel column and eluted with a gradient of 0.5−5% methanol in chloroform. Fractions containing the product (TLC) were pooled and the solvent was evaporated to afford 0.12 g (76%) of **26** as a yellow solid: R_f = 0.52 ($CHCl_3$/MeOH, 5:1); mp 125.5−127.5 °C; ^{1}H NMR (DMSO-d_6) δ 1.20 (m, 9 H, 4-CH_2CH_3, α- and γ-$COOCH_2CH_3$), 1.64 (m, 2 H, β-CH_2), 1.92 (s, 5 H, 6-CH_3 and γ-CH_2), 2.40 (m, 5H, 4-CH_2CH_3) 2.84 (m, 4 H, CH_2CH_2), 4.08 (m, 4 H, α- and γ-$COOCH_2CH_3$), 4.43 (m, 1 H, α-CH), 5.77 (br, 2 H, 2-NH_2, exch), 7.28 (d, 2 H, J = 7.4

Hz, C_6H_4), 7.79 (d, 2H, J = 7.4 Hz, C_6H_4), 8.65 (d, 1 H, J = 7.1 Hz, CONH, exch), 10.66 (s, 1 H, 7-NH, exch); HRMS (EI) calcd for $C_{27}H_{35}N_5O_5$ 509.2638, found 509.2627.

***N*-{4-[2-(2-Amino-4-ethyl-6-methylpyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl}-L-glutamic Acid (4).** To a solution of **26** (0.10 g, 0.20 mmol) in methanol (10 mL) was added 1 N NaOH (2 mL). After 12 h of being stirred at room temperature, the reaction mixture was concentrated under reduced pressure, and the residual aqueous solution, cooled in an ice bath, was acidified to pH 4 with 1 N HCl. The precipitated solid was collected by filtration, washed with brine, and dried in vacuo to afford 0.05 g (51%) of **4** as an off-white solid: mp 238−240 °C; ^{1}H NMR (DMSO-d_6) δ 1.26 (t, 3 H, J = 6.9 Hz, 4-CH_2CH_3), 1.92 (m, 5 H, 6-CH_3 and β-CH_2), 2.09 (m, 2 H, γ-CH_2), 2.32 (t, 2 H, J = 6.9 Hz, 4-CH_2CH_3), 2.85 (m, 4 H, CH_2CH_2), 4.38 (m, 1 H, α-CH), 6.19 (bs, 2 H, 2-NH_2, exch), 7.23 (d, 2 H, J = 7.2 Hz, C_6H_4), 7.84 (d, 2 H, J = 7.2 Hz, C_6H_4), 8.54 (d, 1 H, J = 7.2 Hz, CONH, exch), 10.97 (s, 1 H, 7-NH, exch), 12.48 (br, 1 H, COOH, exch). Anal. ($C_{23}H_{27}N_5O_5$) C, H, N.

Folylpolyglutamate Synthetase (FPGS) Purification and Assay. A BaculoGold transfection kit (Pharmingen, San Diego, CA) was used to cotransfect *Sf*9 insect cells with pVL1392/cFPGS shuttle vector (a generous gift from Dr. B. Shane, UC Berkeley), and BaculoGold DNA was used according to manufacturer's instructions to yield recombinant human cytosolic FPGS-encoding baculovirus. After titering, recombinant virus was used to infect (MOI = 4) *Sf*9 monolayers and cells were harvested at 72 h. FPGS activity was extracted[36] and purified by $(NH_4)_2SO_4$ fractionation and BioGel A-0.5M chromatography (specific activity 6.4 × 10^6 pmol [^{3}H]Glu h^{-1} mg^{-1} protein). K_m values for AMT and MTX as substrates are the same for this recombinant FPGS as those reported for human CCRF-CEM FPGS.[37] FPGS was assayed as previously described.[37] Drug solutions used in FPGS assays and cell culture studies (below) were standardized using extinction coefficients. For **2**: pH 1, 241 nm (33 200 cms^{-1} m^{-1}); pH 7, 237 nm (31 500 cms^{-1} m^{-1}); pH 13, 236 nm (32 000 cms^{-1} m^{-1}). For **4**: pH 1, 243 nm (35 600 cms^{-1} m^{-1}); pH 7, 239 nm (35 500 cms^{-1} m^{-1}); pH 13, 239 nm (35 700 cms^{-1} m^{-1}). Extinction coefficients for methotrexate (MTX), a generous gift of Immunex (Seattle, WA), and aminopterin were from the literature.[35]

Cell Culture. Human T-lymphoblastic leukemia cell line CCRF-CEM and its MTX transport deficient subline R2, their culture, and inhibition of their growth in continuous (120 h) drug exposure are as described in ref 17. Cell lines were negative for mycoplasma (Mycoplasma Plus PCR primers, Stratagene, La Jolla, CA). EC_{50} values were interpolated from plots of percent growth relative to solvent-treated control versus logarithm of the drug concentration. Protection against growth inhibition of CCRF-CEM cells was assayed by including leucovorin (0.1−10 μM) with a concentration of drug previously determined to inhibit growth by 90−95%; the remainder of assay was as described. Growth inhibition was measured relative to the appropriate leucovorin-treated control; leucovorin caused no growth inhibition in the absence of drug, however.

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