Effect of Bridge Region Variation on Antifolate and Antitumor Activity of Classical 5-Substituted 2,4-Diaminofuro[2,3-*d*]pyrimidines

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Variation of the bridge linking the heterocyclic ring and *p*-aminobenzoyl-L-glutamate portions of our previously descibed classical 2,4-diaminofuro[2,3-*d*]pyrimidines **1** and **2** are reported as inhibitors of dihydrofolate reductase (DHFR) and thymidylate synthase (TS) and as antitumor agents. Specifically $-CH_2CH_2-$ and $-CH_2NHCH_2-$ bridged analogues, *N*-[4-[2-(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-L-glutamic acid (**3**) and *N*-[4-[[*N*-[(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)methyl]amino]methyl]benzoyl]-L-glutamic acid (**4**), respectively, were synthesized. Compound **3** was obtained *via* a Wittig reaction of the tributylphosphonium salt of 2,4-diamino-5-(chloromethyl)furo[2,3-*d*]pyrimidine (**5**) and methyl 4-formylbenzoate (**6**) followed by reduction and coupling with the diethyl ester of L-glutamic acid. Compound **4** was synthesized by the nucleophilic displacement of **5** with diethyl *N*-[4-(aminomethyl)benzoyl]-L-glutamate (**15**) and saponification. Both analogues were evaluated *in vitro* as inhibitors of DHFRs from (recombinant) human, human CCRF-CEM cells, and *Lactobacillus casei*. Compound **3** showed moderate activity (IC_{50} 10^{-6}–10^{-7} M). Compound **4** was essentially inactive (IC_{50} 10^{-5} M, CCRF-CEM). The compounds were also evaluated against TS from (recombinant) human and *L. casei* and were of low activity (IC_{50} 10^{-5} M). The three-atom-bridged analogue **4** was somewhat more inhibitory to human TS than methotrexate (MTX). Compound **3** inhibited the growth of tumor cells in culture (IC_{50} 10^{-7} M) while **4** showed a low level of growth inhibitory activity. The inhibition of the growth of leukemia CCRF-CEM cells by both compounds parallels their inhibition of CCRF-CEM DHFR. Analogue **3** was a good substrate for human folylpolyglutamate synthetase (FPGS) derived from CCRF-CEM cells (K_m 8.5 μM). Further evaluation of the growth inhibitory activity of **3** against the MTX-resistant subline of CCRF-CEM cells (R30dm) with decreased FPGS indicated that poly-γ-glutamylation was important for its action. Protection studies with **3** in the FaDu squamous cell carcinoma cell line indicated that inhibition was completely reversed by leucovorin [(6*R*,*S*-5-formyltetrahydrofolate] or by a combination of thymidine and hypoxanthine, suggesting an antifolate effect directed at DHFR.

Folate metabolism represents an important and attractive target for cancer chemotherapy due to its crucial role in the biosynthesis of nucleic acid precursors.[1,2] Tetrahydrofolate, the central component of folate metabolism, in its cofactor forms serves as the main carrier of one-carbon units.[1,3] The cofactor, tetrahydrofolate, is formed by the NADPH-dependent reduction of 7,8-dihydrofolate by the enzyme dihydrofolate reductase (DHFR).[4] DHFR along with thymidylate synthase (TS) forms part of the system responsible for the synthesis of deoxythymidylate (dTMP) from deoxyuridylate (dUMP). TS catalyzes the sole *de novo* synthesis of dTMP from dUMP and utilizes 5,10-methylenetetrahydrofolate as the source of the methyl group as well as the reductant.[5] Thus inhibitors of TS and DHFR have found clinical utility as antitumor, antibacterial, and antiprotozoan agents.

Several classical antifolates have been reported in the literature as inhibitors of TS, DHFR, and antitumor agents.[6] In general these compounds have a 6-6 fused heterocyclic ring system. Prominent examples include methotrexate (MTX)[6] and N^{10}-propargyl-5,8-dideaza folate (PDDF)[7] which are potent inhibitors of DHFR and TS, respectively, and function as antitumor agents. Recently however several groups have reported the synthesis of classical antifolates which contain a 6-5-fused heterocyclic ring system. Miwa *et al.*,[8,9] Taylor *et al.*,[10] and Shih and Gosset[11] have reported the synthesis of classical 2,4-diamino-, 2-amino-4-oxo-, and 2-amino-5-substituted pyrrolo[2,3-*d*]pyrimidines as inhibitors of DHFR and TS and as effective antitumor agents. A classical 2,4-diamino-5-substituted-pyrazolo-[3,4-*d*]pyrimidine antifolate was also shown to possess significant cytotoxicity.[12] Kotake *et al.*[13] have reported the synthesis of classical 2,4-diamino-5-substituted-cyclopenta[*d*]pyrimidines as potent inhibitors of DHFR and as effective antitumor agents. In addition we[14] recently reported the synthesis of novel, classical 2,4-diamino-5-substituted-furo[2,3-*d*]pyrimidines **1** and **2** as potential antifolates and antitumor agents which could bind, in either the antifolate (MTX) orientation or the substrate (folate) orientation, to DHFR. These compounds **1** and **2** were moderate inhibitors of DHFR; however, they were significantly cytotoxic to tumor cells in culture (IC_{50} 0.1–0.01 μM). This cytotoxicity was attributed to the efficient polyglutamylation of **1** and **2** by the enzyme folylpoly-γ-glutamate synthetase (FPGS). Such polyglutamylation *via* FPGS is an important

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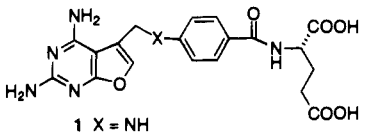
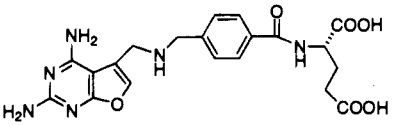
mechanism for trapping classical antifolates within the cell, thus maintaining high intracellular concentrations which allow for increased antitumor activity.[15] In addition, for some folate dependent enzymes such as TS, polyglutamylation also results in enhanced enzyme inhibition.[16,17]



1 X = NH

2 X = NCH_3

3 X = CH_2



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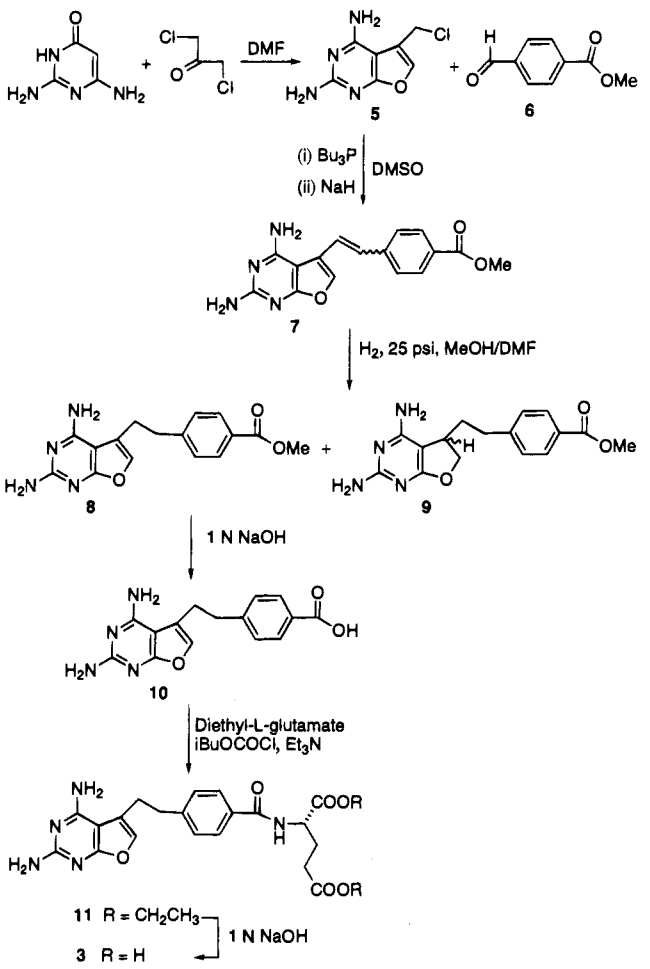
The recently reported classical 6-5 ring fused antifolates[8-13] have a two-, three-, or four-carbon bridge unit connecting the heterocyclic ring system to the benzoylglutamate portion of the molecule. In contrast, our compounds **1** and **2** contain a methyleneamino bridge similar to MTX. In order to allow for direct comparison of the furo[2,3-*d*]pyrimidines with other reported 6-5 ring fused classical antifolates, and as part of a structure–activity relationship study on the nature and length of the bridge unit of classical 2,4-diamino-5-substituted-furo[2,3-*d*]pyrimidine antifolates, we synthesized the 9-deaza analogue of **1**, *N*-[4-[2-(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-L-glutamic acid (**3**), as a classical antifolate and antitumor agent. We also synthesized *N*-[4-[[*N*-[(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)methyl]amino]methyl]benzoyl]-L-glutamic acid (**4**) with a three-atom $-CH_2NHCH_2-$ bridge unit. To our knowledge this bridge unit has not been reported in 6-5 ring fused antifolates. Slavik *et al.*[18] reported the synthesis of isohomoaminopterin as a potential inhibitor of DHFR which utilized this three-atom bridge, and Montgomery *et al.*[19] have reported the synthesis of isohomomethotrexate in which the bridge nitrogen is substituted with a methyl group. Li *et al.*[20] reported the synthesis of isohomo-N^{10}-propargyl-5,8-dideazafolate as an inhibitor of TS, in which the bridge nitrogen is substituted with a propargyl group. Recently Taylor *et al.*[21] reported the synthesis of 5-deaza-5,6,7,8-tetrahydroisohomofolic acid as a potential inhibitor of glycinamide ribonucleotide formyltransferase which also utilized the $-CH_2NHCH_2-$ bridge. The classical furo[2,3-*d*]pyrimidine antifolates **3** and **4** were evaluated as inhibitors of DHFR and TS and as inhibitors of the growth of tumor cells in culture. Since we[14] demonstrated that polyglutamylation is an important component of the mechanism of action of the classical antifolates **1** and **2**, it was of interest to also evaluate compounds **3** and **4** as substrates for FPGS.

Chemistry

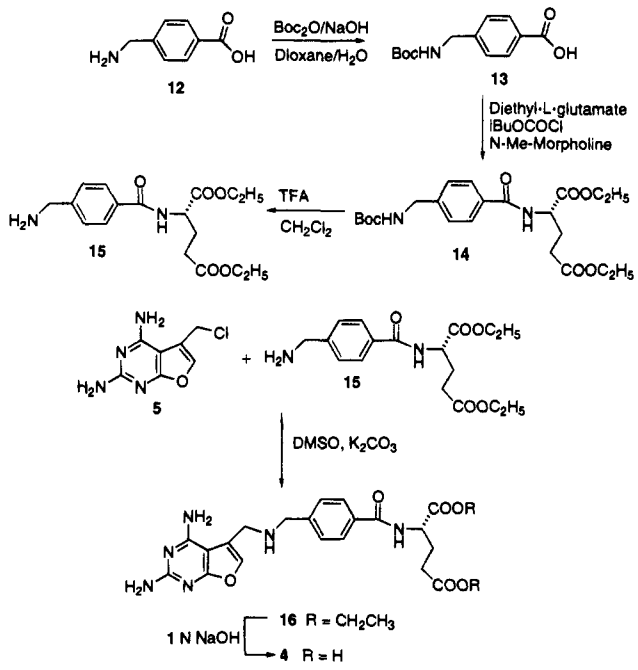
The synthesis of the target molecule **3** is shown in Scheme 1. It was anticipated that a Wittig reaction of a suitable aldehyde with 2,4-diamino-5-(chloromethyl)-furo[2,3-*d*]primidine **5** would furnish the required carbon–carbon bridge in the target analogue **3**. This

Scheme 1



crucial intermediate **5** was synthesized and utilized in the synthesis of the classical antifolates **1** and **2**.[14] Compound **5** was readily obtained by the reaction of 2,4-diamino-6-hydroxypyrimidine and dichloroacetone in *N*,*N*-dimethylformamide at room temperature as previously reported.[14,22] Displacement of the chloride with tributylphosphine occurred smoothly in anhydrous dimethyl sulfoxide at 60 °C. Wittig condensation of this tributylphosphonium salt of **5** with methyl 4-formylbenzoate (**6**) and sodium hydride in anhydrous dimethyl sulfoxide at room temperature afforded the desired olefinic product **7** in 42% yield as an inseparable mixture of the cis and trans isomers in approximately equimolar ratio as determined by the ^1H NMR spectrum. The use of either the triphenylphosphonium salt or sodium methoxide as the base led to lower yields. The ^1H NMR spectrum of **7** in deuterated dimethyl sulfoxide indicated that the chemical shifts of the protons for the trans isomer occurred downfield compared to the cis isomer and was in accord with other reports in the literature for cis–trans mixtures with analogous pteridine and pyrido[2,3-*d*]pyrimidine analogues.[23-25] The furan aromatic proton resonates at 7.11 ppm for the cis isomer, which is 0.71 ppm upfield from the trans isomer (7.82 ppm), due most likely to the shielding effect of the phenyl ring on the 6-aromatic proton. The 8- and 9-olefinic protons of the cis isomer occurred together as a singlet at 6.77 ppm whereas the corresponding pair in the trans isomer occurred as doublets centered at 7.11 and 7.53 ppm with a coupling constant of 16.2 Hz, which confirmed the trans orientation. Catalytic hydrogena-

Scheme 2



tion of the 8,9-double bond of **7** was carried out in a solution of methanol/*N*,*N*-dimethylformamide under optimized conditions (5% palladium on carbon, 25 psi of hydrogen pressure, 30 min). Column chromatographic separation afforded the desired 9,10-dihydro derivative **8** (51%) along with its overreduced 5,6-dihydrofuro[2,3-*d*]pyrimidine derivative **9** (13%). Compound **9** predominated at higher hydrogen pressures and longer reaction times. The ^{1}H NMR of **9** in deuterated dimethyl sulfoxide confirmed the diastereotopic nature of the 6-CH_2 protons which occurred separately at 4.25 and 4.40 ppm. The ^{1}H NMR of **8** indicated that the 8- and 9-methylene protons were chemically equivalent and occurred together as a singlet at 2.96 ppm. Hydrolysis of **8** with aqueous sodium hydroxide afforded the free acid **10** in 87% yield. Peptide coupling of **10** with the diethyl ester of L-glutamic acid using isobutyl chloroformate as the coupling agent afforded the desired coupled product **11**. The yield of **11** could be substantially increased by one repetition of the activation cycle. Using this methodology the desired diester **11** was obtained in 70% yield. Final saponification with aqueous sodium hydroxide at room temperature followed by acidification to pH 4 afforded the pure target diacid **3** in 96% yield. The ^{1}H NMR of **3** in deuterated dimethyl sulfoxide indicated the characteristic peptide NH as a doublet at 8.53 ppm exchangeable on addition of deuterium oxide.

The synthesis of the three-atom $-CH_2NHCH_2-$ bridged compound **4** is shown in Scheme 2. It was anticipated that a nucleophilic displacement reaction of **5** with a fully functionalized amine **15** followed by saponification would afford the desired target compound **4**. The requisite amine **15** has been used before by Slavik *et al.*[18] and Taylor *et al.*;[21] however, it was not characterized in a pure form. The authors reported the use of **15** as a crude mixture of different products in varying ratios based on paper chromatography.[18] We decided to synthesize and characterize **15** in a pure form using classical peptide synthetic procedures. The amino group of 4-(aminomethyl)benzoic acid (**12**) was protected as its *tert*-butyloxy carbamate in 84% yield by reaction with di-*tert*-butyl dicarbonate and 1 N aqueous sodium hydroxide in a mixture of dioxane/water. Peptide coupling of the Boc-protected amino acid **13** with the diethyl ester of L-glutamic acid in the presence of isobutyl chloroformate and *N*-methylmorpholine afforded **14** in 59% yield after one repetition of the activation cycle. Deprotection of the Boc group with trifluoroacetic acid at room temperature (15 min) gave the amine **15** in 88% yield. Longer reaction time for deprotection resulted in the formation of decomposition products as indicated by TLC. Nucleophilic displacement of the chloro group of **5** with the free amine **15** was most efficient in anhydrous dimethyl sulfoxide and potassium carbonate as the base at 45 °C and afforded the diester **16** in 51% yield. Deprotection of the diethyl ester **16** with aqueous sodium hydroxide at room temperature followed by acidification gave the pure diacid **4** in 88% yield. The ^{1}H NMR of **4** in deuterated dimethyl sulfoxide indicated the presence of the 2- and the 4-amino protons as singlets and the 9-NH as a broad triplet, all exchangeable with the addition of deuterium oxide. The 6-aromatic proton adjacent to the furan oxygen occurred as a singlet at 7.06 ppm for **3** and at 7.43 ppm for **4**.

Table 1. Enzyme Inhibitory Concentrations, IC_{50} in μM

	DHFR			TS	
compd	rec human[a]	*L. casei*[a]	CCRF-CEM[b]	rec human	*L. casei*
3	1.0	0.1	0.25	220	200
4	>200	>200	30.5	63.0	>200
1[c]	0.45	0.56	nd[d]	>200	>200
MTX	0.004	0.006	0.0007	170	

[a] DHFR was assayed using the conditions reported in ref 23. [b] The enzyme was assayed as described in the Experimental Section. [c] IC_{50} values for compound 1 have been taken from ref 14. [d] nd = not determined.

Biological Evaluation and Discussion

The classical analogues **3** and **4** were evaluated as inhibitors of *Lactobacillus casei* DHFR,[26] human recombinant DHFR,[27] and DHFR isolated from human CCRF-CEM leukemic cells. The inhibitory concentration (IC_{50}) values are listed in Table 1 and compared with the previously reported values for the classical furo[2,3-*d*]-pyrimidine antifolate **1**[14] and MTX. The two-carbon-bridged analogue **3** inhibited human recombinant DHFR with an IC_{50} of 1.0 μM and was about one-half as potent as its 9-aza analogue **1**. Compound **3** was 1 order of magnitude more potent against the bacterial enzyme with an IC_{50} of 0.1 μM. The DHFR isolated from CCRF-CEM cells was inhibited by **3** with an IC_{50} of 0.25 μM. In contrast to the two-atom-bridged analogues **1** and **3**, the three-atom $-CH_2NHCH_2-$ bridged analogue **4** was inactive against *L. casei* and human recombinant DHFRs and showed only marginal activity against CCRF-CEM DHFR with an IC_{50} of 30.5 μM. A similar result was reported for isohomoaminopterin (K_i 0.11 μM), which was 25-fold less active than aminopterin against mouse liver DHFR.[18] The 100-fold decrease in the inhibition of CCRF-CEM DHFR by **4** compared to **3** could be attributed to the increase in chain length and/or the addition of a basic, protonatable amine in the bridge which may be detrimental to DHFR binding.

Table 2. Substrate Activity of **3** and **4** for CCRF-CEM Human Leukemia Cell Folylpolyglutamate Synthetase[a]

substrate	K_m, μM	$V_{max(rel)}$	$V_{max}/K_{m(rel)}$
aminopterin	4.8 ± 0.7 (n = 6)	1 (n = 6)	0.21 ± 0.04 (n = 6)
3	8.5 ± 2.1 (n = 3)	0.65 ± 0.01 (n = 3)	0.07 ± 0.02 (n = 3)
4	b	0.6 (n = 5)	

[a] FPGS substrate activity was determined as described in the Experimental Section. Values presented are the average ±SD if $n \geq 3$ and average ± range for $n = 2$. Maximum velocities are calculated relative to aminopterin within the same experiment. [b] K_m could not be estimated because linear substrate activity as a function of time at concentrations below those giving maximal activity (≥50 μM) could not be achieved.

The classical analogues **3** and **4** were evaluated as inhibitors of *L. casei* TS[28,29] and human recombinant TS[28,29] (Table 1) and were found to be weak inhibitors of these enzymes. The three-atom-bridged analogue **4** had an IC_{50} of 63 μM for inhibition of human recombinant TS and was inactive against *L. casei* TS. The two-atom-bridged compound **3** was poorly active against both the bacterial enzyme (IC_{50} = 200 μM) and the human enzyme (IC_{50} = 220 μM). These results indicated that within the 2,4-diaminofuro[2,3-*d*]pyrimidine series the two-atom $-CH_2NH-$ (**1**) and $-CH_2CH_2-$ (**3**) bridges are not conducive for binding to isolated TS. Interestingly, however, the extension of the bridge length to three atoms, $-CH_2NHCH_2-$ (**4**), moderately increases inhibitory activity against human recombinant TS.

The target compounds **3** and **4** were also evaluated as substrates for human FPGS from CCRF-CEM cells,[30,31] and the results are shown in Table 2. The classical analogue **3** was a good substrate for human FPGS with a K_m of 8.5 μM. The relative first-order rate constant V_{max}/K_m(rel) for **3** was 3 times lower than that of aminopterin (AMT), which is a good substrate for ligation of the first additional glutamic acid.[32] Compound **4** was also a substrate for FPGS; however, linear substrate activity as a function of time at concentrations below those giving maximal activity (≥50 μM) could not be acheived for unknown reasons; because of this unusual kinetic behavior, the K_m could not be determined. Comparison of the activity at saturating AMT and analogue **4** at times where linear activity is observed indicated that **4** has a V_{max} 60% that of AMT. These results indicate that replacement of the $-CH_2NH-$ bridge (**1**, K_m = 1.9 μM[14]) with a $-CH_2CH_2-$ (**3**, K_m = 8.5 μM) bridge in this series leads to decreased affinity for FPGS with a slight increase in catalytic turnover (V_{max}). An analogous replacement of the 10-NH in AMT with a 10-CH_2 to yield 10-deazaAMT also causes a decrease in the relative catalytic efficiency, again primarily as a result of increased K_m.[33] Because of the unusual kinetics displayed by compound **4**, the effect of increasing the bridge length from a $-CH_2NH-$ to a $-CH_2NHCH_2-$ cannot be clearly defined; however, the substitution does not appear to have much effect on the catalytic turnover of this class.

Compounds **3** and **4** were evaluated as inhibitors of the growth of leukemia CCRF-CEM cells in culture during continous exposure (Table 3). The two-carbon-bridged analogue **3** was cytotoxic and had an IC_{50} = 0.29 μM. The three-atom-bridged analogue **4** showed only marginal inhibition with an IC_{50} = 48 μM. The low activity of **4** parallels its poor inhibitory activity against both DHFR and TS. In contrast, the moderate inhibition of DHFR by **3** coupled with its efficient utilization as a substrate for FPGS should allow for the intracellular retention of the polyglutamylated metabolites of **3** which transalates into its better antitumor activity. The classical analogue **3** was further evaluated as an inhibitor of the growth of A253 and FaDu squamous cell carcinoma (Table 3) of the head and neck cell lines in culture, during continous exposure and was found to be cytotoxic having IC_{50} values in the submicromolar range. The high degree of cross-resistance observed for compound **3** with the FPGS-deficient CCRF-CEM subline R30dm[34] (Table 3) indicated that poly-γ-glutamyl metabolites of this analogue were critical for its antitumor activity. These results were consistent with those observed for the previously reported classical furo[2,3-*d*]pyrimidine antifolates **1** and **2**.[14] Comparision of analogue **3** with MTX indicated that although **3** was significantly (200–400 times) less active than MTX in inhibiting isolated DHFRs, it was only 20–40 times less active than MTX in inhibiting the growth of tumor cells in culture. This order of magnitude difference could probably be related to better polyglutamylation and transport properties of **3** into tumor cells compared with MTX.

Metabolite protection studies (Table 4) were carried out with **3** in order to further elucidate its mechanism of action. The results indicated that inhibition of the growth of FaDu cells was completely reversed by addition of 10 μM leucovorin (LV) or by a combination of 40 μM of thymidine (TdR) and 50 μM of hypoxanthine (Hx). Only partial protection was afforded with either TdR or Hx alone. These results are similar to those obtained for MTX (Table 4) and the classical furo[2,3-*d*]pyrimidine antifolates **1** and **2**,[14] indicating that both purine

Table 3. Growth Inhibition of the Human T-Lymphoblastic Leukemia Cell Line CCRF-CEM, Its Methotrexate-Resistant Subline R30dm,[b] Human Squamous Cell Carcinoma FaDu and A253 Cells by **3** and **4** during Continous Exposure (0–120 h)[a]

	EC_{50}, μM			
compd	CCRF-CEM	R30dm[b]	FaDu	A253
3	0.29 ± 0.01 (n = 3)	4.25 ± 0.05 (n = 2)	0.18 ± 0.02 (n = 2)	0.54 ± 0.09 (n = 3)
4	48.0 ± 23.0 (n = 2)	nd[c]	nd	nd
MTX	0.014 ± 0.001 (n = 10)	0.018 ± 0.003 (n = 5)	0.017 ± 0.002 (n = 5)	0.013 ± 0.0008 (n = 3)

[a] Average values are presented ±range for n = 2 and ±SD for $n \geq 3$. [b] 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.[31] [d] nd = not determined.

Table 4. Protection by 10 μM LV, 50 μM Hx, 40 μM TdR, or the Combination of 50 μM Hx + 40 μM TdR against the Growth Inhibition of FaDu cells by MTX and **3**[a]

	relative growth (% of control)				
drug	no addn	LV	Hx	TdR	Hx + TdR
MTX	2 ± 0	96 ± 5	6 ± 1	8 ± 0	98 ± 5
3	7 ± 1	105 ± 2	4 ± 0	14 ± 1	107 ± 3

[a] Protection was determined as described in the Experimental Section. Data are average ± range of duplicate determenations within a single experiment. The entire experiment was repeated with similar results. Hx, TdR, and Hx + TdR at these concentrations had minor effects on cell growth (<10% growth inhibition); data are normalized to an appropriate control treated only with the metabolite.

and pyrimidine biosyntheses are inhibited, thus implicating DHFR as their primary intracellular target.

In summary two classical furo[2,3-*d*]pyrimidine antifolates **3** and **4** modified in the bridge region were synthesized. The biological results indicated that the three-atom $-CH_2NHCH_2-$ bridged analogue **4** had poor activity against isolated DHFR and TS and was also marginally active against the growth of CCRF-CEM leukemic cells in culture. This low activity is probably due to the presence of the basic, protonatable, secondary nitrogen atom in the three-atom bridge of **4** since analogues in the 2,4-diaminopyrrolo[2,3-*d*]pyrimidine series[8] and the dihydrocyclopenta[*d*]pyrimidine series[13] with a three-carbon $-CH_2CH_2CH_2-$ bridge were exceptionally potent inhibitors of DHFR. However the length of the chain in the furo[2,3-*d*]pyrimidine series cannot be ruled out as being responsible for the low activity. The two-atom $-CH_2CH_2-$ bridged furo[2,3-*d*]pyrimidine **3** was a good inhibitor of the growth of tumor cells in culture and a moderate inhibitor of isolated DHFR. Its activity was comparable to that of analogue **1** which has a two-atom $-CH_2NH-$ bridge. We are currently in the process of synthesizing the three- and four-atom-bridged analogues (without a protonatable nitrogen) of the furo[2,3-*d*]pyrimidines which should shed further light on the relation of bridge length and biological activity in this series.

Experimental Section

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). The chemical shift values are expressed in δ values (parts per million) relative to tetramethylsilane as an internal standard: s = singlet, d = doublet, t = triplet, m = multiplet, and dd = doublet of doublet. Mass spectra were recorded on a VG-7070 double-focusing mass spectrometer in the electron ionization (EI) mode or chemical ionization (CI) mode. Fast atom bombardment mass spectra (FABMS) and peak match FABMS were obtained on a Kratos MS50 spectrometer. Thin-layer chromatography (TLC) was performed on silica gel plates and cellulose plates with flourescent indicator that were visualized with light at 254 or 366 nm. Proportions of solvents used for TLC are by volume. Column chromatography was performed on 230–400 mesh silica gel purchased from Aldrich, Milwaukee, WI. Elemental analysis were performed by Atlantic Microlab, Inc., Norcross, GA. Element compositions are within ±0.4% of the calculated values. Fractional moles of water or organic solvents frequently found in some analytical samples of antifolates could not be removed in spite of drying *in vacuo* and were confirmed by their presence in the ^{1}H NMR spectrum.

Methyl 4-[2-(2,4-Diaminofuro[2,3-*d*]pyrimidin-5-yl)ethenyl]benzoate (7). To a solution of 2,4-diamino-5-(chloromethyl)furo[2,3-*d*]pyrimidine (**5**) (0.79 g, 4 mmol) in anhydrous DMSO (10 mL) was added tributylphosphine (92%, 2.64 g, 12 mmol), and the resulting mixture was stirred at 60 °C for 2 h under nitrogen to form the phosphonium salt. The deep orange solution was then cooled to room temperature. To this solution was added sodium hydride (60% dispersion in mineral oil, 0.35 g, 8.8 mmol), followed by methyl 4-formylbenzoate (**6**) (0.72 g, 4.4 mmol). The reaction mixture was stirred for 24 h at room temperature. TLC (silica gel, $CHCl_3$/MeOH, 5:1) indicated the disappearance of starting material **5** at R_f 0.55 and formation of a major product spot at R_f 0.66. The DMSO was removed by vacuum distillation. Ethyl ether (50 mL) was added to the residue and the supernatant decanted after 10 min. Another portion of ethyl ether (50 mL) was added, the sticky residue stirred for a period of 1 h, and the supernatant carefully decanted. This process was repeated three more times, and finally the mixture was stored at 0 °C with ethyl ether (50 mL). After 18 h, the mixture was ultrasonicated for 2 h and cooled to 0 °C for a period of 10 h. The orange solid thus formed was filtered, washed with ethyl ether, and air-dried. The solid was then washed well with water and air-dried. The crude solid was suspended in hot MeOH (250 mL) and some undissolved material filtered. Silica gel (3 g) was added to the filtrate, and the suspension was evaporated to dryness under reduced pressure. The silica gel plug was loaded on a dry silica gel column (2.4 × 20 cm) and flushed initially with $CHCl_3$ (500 mL). The column was then eluted sequentially with 100 mL portions of 1–8% MeOH in $CHCl_3$. Fractions which showed the major spot at R_f 0.66 along with a minor trailing impurity were pooled and evaporated to dryness to afford a residue. This residue was dissolved in glacial acetic acid and evaporated to dryness. This oily residue was redissolved in hot MeOH and the solution stored at 0 °C for 72 h. The solid that formed was filtered, washed with ether, and dried to afford 0.52 g (42%) of **7** as an orange solid, homogeneous on TLC. The ^{1}H NMR of this solid indicated it to be a mixture of the cis and trans isomers, across the 8,9 double bond, in a ratio of 1.3 to 1, respectively: mp 211–215 °C; TLC R_f 0.66 ($CHCl_3$/MeOH, 5:1, silica gel); ^{1}H NMR (DMSO-d_6) for the cis isomer δ 3.82 (s, $COOCH_3$), 6.08 (s, 4-NH_2), 6.29 (s, 2-NH_2), 6.77 (s, 8- and 9-CH), 7.11 (s, 6-CH), 7.45 (d, 3′-, 5′-CH, J = 8.4 Hz), 7.85 (d, 2′-, 6′-CH, J = 8.4 Hz), for the trans isomer δ 3.85 (s, $COOCH_3$), 6.08 (s, 4-NH_2), 6.78 (s, 2-NH_2), 7.11 (d, 8- or 9-CH, J = 16.2 Hz), 7.53 (d, 8- or 9-CH, J = 16.2 Hz), 7.79 (d, 3′-, 5′-CH, J = 8.4 Hz), 7.82 (s, 6-CH), 7.94 (d, 2′-, 6′-CH, J = 8.4 Hz); MS (FAB) m/z 311 (MH^+). Anal. Calcd for ($C_{16}H_{14}N_4O_3 \cdot 0.5CH_3COOH$) C, H, N.

Methyl 4-[2-(2,4-Diaminofuro[2,3-*d*]pyrimidin-5-yl)ethyl]benzoate (8) and (±)-Methyl 4-[2-(2,4-Diamino-5,6-dihydrofuro[2,3-*d*]pyrimidin-5-yl)ethyl]benzoate (9). To a solution of **7** (0.155 g, 0.5 mmol) in a mixture of MeOH/DMF (1/1, 20 mL) was added 5% palladium on carbon (0.31 g), and the suspension was hydrogenated in a Parr apparaus at room temperature and 25 psi of hydrogen pressure for 30 min. The TLC ($CHCl_3$/MeOH, 5:1, silica gel) at this time indicated the formation of two spots at R_f 0.63 and R_f 0.52 and a faint starting material spot at R_f 0.66. The reaction mixture was filtered through Celite, and the catalyst was washed with MeOH/DMF (1:1, 30 mL). The filtrate was evaporated to dryness under reduced pressure, and the residue was dissolved in MeOH (100 mL). To this solution was added silica gel (500 mg) and the suspension evaporated to dryness. This silica gel plug was loaded on a dry silica gel column (2.4 × 16 cm) and flushed with $CHCl_3$ (500 mL). The column was then eluted with a gradient of 1–9% MeOH in $CHCl_3$, collecting 15 mL fractions. Early fractions showing a single spot at R_f 0.63 were pooled and evaporated to dryness, and the residue was stirred in ether, filtered, and dried to afford 0.08 g (51%) of **8** as a light pink solid: mp 265–268 °C; ^{1}H NMR (DMSO-d_6) δ 2.96 (s, 4 H, 8- and 9-CH_2), 3.83 (s, 3 H, $COOCH_3$), 5.97 (s, 2 H, 4-NH_2), 6.46 (s, 2 H, 2-NH_2), 7.05 (s, 1 H, 6-CH), 7.40 (d, 2 H, 3′-, 5′-CH, J = 8.1 Hz), 7.87 (d, 2 H, 2′-, 6′-CH, J = 8.1 Hz); MS (FAB) m/z 313 (MH^+). Anal. Calcd for ($C_{16}H_{16}N_4O_3 \cdot 0.7H_2O$) C, H, N.

Later fractions, from the column described above, showing a single spot at R_f 0.52 were pooled and evaporated to dryness under reduced pressure, and the residue obtained was stirred in ether, filtered, and dried to afford 0.02 g (13%) of **9** as an off-white solid: mp 215–217 °C; TLC R_f 0.52 ($CHCl_3$/MeOH, 5:1, silica gel); ^{1}H NMR (DMSO-d_6) δ 1.53–1.66 (m, 1 H, 8-*H*CH), 1.90–2.02 (m, 1 H, 8-HC*H*), 2.59 (m, 2 H, 9-CH_2), 3.29 (m, 1 H, 5-CH), 3.82 (s, 3 H, $COOCH_3$), 4.25 (dd, 1 H, 6-*H*CH, J = 4.0, 9.0 Hz), 4.40 (t, 1 H, 6-HC*H*, J = 9.0 Hz), 5.79 (s, 2 H, 4-NH_2), 6.08 (s, 2 H, 2-NH_2), 7.36 (d, 2 H, 3′-, 5′-CH, J = 8.1 Hz), 7.86 (d, 2 H, 2′-, 6′-CH, J = 8.1 Hz); MS (FAB) m/z 315 (MH^+); high-resolution FABMS calcd MH^+ 315.3543, found 315.3554.

4-[2-(2,4-Diaminofuro[2,3-*d*]pyrimidin-5-yl)ethyl]benzoic Acid (10). To a solution of **8** (0.065 g, 0.21 mmol) in a mixture of MeOH/DMSO (2:1, 10 mL) was added 1 N NaOH (1.5 mL) and the mixture stirred at room temperature for 18 h. The reaction mixture was then evaporated to dryness under reduced pressure (oil pump). The residue was dissolved in

water (5 mL), and 1 N HCl was added dropwise to bring the pH of the solution to 5.5. The resulting suspension was cooled to 5 °C for 12 h and filtered. The residue was washed sequentially with water, acetone, and ether and dried to afford 0.054 g (87%) of **10** as a pink brown solid: mp >300 °C; TLC R_f 0.15 ($CHCl_3$/MeOH, 5:1, silica gel). This compound was used directly in the next step.

***N*-[4-[2-(2,4-Diaminofuro[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-L-glutamic Acid (3).** To a suspension of **10** (0.047 g, 0.16 mmol) in anhydrous DMF (3 mL) was added triethylamine (45 μL, 0.32 mmol) and the mixture stirred under nitrogen at room temperature for 5 min. The resulting solution was cooled to 0 °C, isobutyl chloroformate (42 μL, 0.32 mmol) was added, and the mixture was stirred at 0 °C for 30 min. At this time TLC ($CHCl_3$/MeOH, 5:1, silica gel) indicated the formation of the activated intermediate at R_f 0.27 and a faint starting material spot at R_f 0.15. Diethyl L-glutamate hydrochloride (0.077 g, 0.32 mmol) was added to the reaction mixture, followed immediately by triethylamine (45 μL, 0.32 mmol). The mixture was slowly allowed to warm to room temperature and stirred under nitrogen for a period of 18 h. TLC at this time indicated the formation of a major spot at R_f 0.58 and also some starting material (R_f 0.15). The reaction mixture was then subjected to another cycle of activation using one-half the quantities listed above. Thus triethylamine (23 μL, 0.16 mmol) was added and the reaction mixture cooled again to 0 °C. Isobutyl chloroformate (21 μL, 0.16 mmol) was added, and after 30 min at 0 °C diethyl L-glutamate hydrochloride (0.038 g, 0.16 mmol) was added, followed immediately by triethylamine (23 μL, 0.16 mmol). The reaction mixture was warmed to room temperature and stirred for a further 24 h and evaporated to dryness under reduced pressure. The residue was dissolved in a minimum amount of $CHCl_3$/MeOH, 4:1, and chromatographed on a silica gel column (2.4 × 15 cm, packed with $CHCl_3$/MeOH, 24:1), eluting with 24:1 $CHCl_3$/MeOH. Fractions showing a single spot were pooled and evaporated to dryness. The residue was stirred in cold anhydrous ether and filtered to obtain 0.054 g (70%) of the diethyl ester **11** as an off-white solid: TLC R_f 0.58 ($CHCl_3$/MeOH, 5:1, silica gel).

To a solution of the diester **11** (0.052 g, 0.107 mmol) in MeOH (5 mL) was added 1 N NaOH (1 mL) and the solution stirred at room temperature for 24 h. The MeOH was evaporated under reduced pressure, the residue was dissolved in water (5 mL), and stirring was continued for an additional 24 h. The pH of the solution was then adjusted to 4.0 by dropwise addition of 1 N HCl. The resulting suspension was stored at 5 °C for 12 h and filtered. The residue was washed well with water and acetone and dried to afford 0.044 g (96%) of **3** as a light pink solid: mp 162–164 °C; TLC R_f 0.59 (3% NH_4HCO_3, cellulose); UV λ_{max} (pH 1) 249 nm (ε 22 650), 302 (7900); λ_{max} (pH 7) 249 nm (ε 21 280); λ_{max} (pH 13) 249 (ε 21 640); ^{1}H NMR (DMSO-d_6) δ 1.90–2.11 (m, 2 H, Gluβ-CH_2), 2.35 (t, 2 H, Gluγ-CH_2, J = 7.0 Hz), 2.95 (s, 4 H, 8- and 9-CH_2), 4.38 (m, 1 H, Gluα-CH), 6.0 (s, 2 H, 4-NH_2), 6.49 (s, 2 H, 2-NH_2), 7.06 (s, 1 H, 6-CH), 7.34 (d, 2 H, 3′-, 5′-CH, J = 7.5 Hz), 7.80 (d, 2 H, 2′-, 6′-CH, J = 7.5 Hz), 8.53 (d, 1 H, CONH, J = 7.5 Hz), 12.0–12.6 (br s, COOH); HPLC t_R = 38.3 min (C_{18} silica gel, 8% CH_3CN in 0.1 M NaOAc, pH 5.5, flow rate 1.0 mL/min). Anal. Calcd for ($C_{20}H_{21}N_5O_6$·1.25H_2O) C, H, N.

4-[[*N*-(*tert*-Butyloxycarbonyl)amino]methyl]benzoic Acid (13). To a solution of 4-(aminomethyl)benzoic acid (**12**) (1.51 g, 10 mmol) in dioxane/water (1:1, 20 mL) was added 1 N NaOH (10 mL). Di-*tert*-butyl dicarbonate (2.62 g, 12 mmol) was added to this solution and the mixture stirred at room temperature for 12 h. The reaction mixture was evaporated to half its original volume under reduced pressure. The pH of this solution was adjusted to 3 by dropwise addition of 50% aqueous HCl while maintaining the temperature below 10 °C with an ice bath. The resulting suspension was diluted with water (70 mL) and extracted with ethyl acetate (3 × 50 mL). The combined organic layers were washed with saturated NaCl (50 mL), dried ($MgSO_4$), and filtered. The filtrate was evaporated to dryness under reduced pressure and the residue recrystallized from a mixture of ethyl acetate/hexanes to afford 2.10 g (84%) of **13** as a white solid: mp 164–166 °C; TLC R_f 0.74 ($CHCl_3$/MeOH, 4:1, silica gel); ^{1}H NMR ($CDCl_3$) δ 1.46 (s, 9 H, C(CH_3)$_3$), 4.36 (d, 2 H, CH_2, J = 6.0 Hz), 5.21 (br s, 1 H, NH), 7.35 (d, 2 H, 3′-, 5′-CH, J = 8.1 Hz), 8.02 (d, 2 H, 2′-, 6′-CH, J = 8.1 Hz); MS (EI) m/z 251 (M^+). Anal. Calcd for ($C_{13}H_{17}NO_4$) C, H, N.

Diethyl *N*-[4-[[*N*-(*tert*-Butyloxycarbonyl)amino]methyl]benzoyl]-L-glutamate (14). A solution of **13** (1.26 g, 5 mmol) in anhydrous DMF (20 mL) under nitrogen was cooled in an ice–salt bath. To the cooled solution was added *N*-methylmorpholine (0.55 mL, 5 mmol) followed 5 min later by isobutyl chloroformate (0.65 mL, 5 mmol). After stirring for 20 min diethyl L-glutamate hydrochloride (1.20 g, 5 mmol) was added, followed immediately by *N*-methylmorpholine (0.55 mL, 5 mmol). The reaction mixture was warmed to room temperature and stirred for 12 h. At this time the activation cycle was repeated using one-half the amount of reagents indicated above, after which the reaction mixture was warmed to room temperature and stirred for an additional 12 h. The solvents were removed under reduced pressure, and the residue was dissolved in CH_2Cl_2 (100 mL) and washed with water (75 mL), 0.1 N HCl (50 mL), and saturated NaCl (50 mL). The organic layers were dried ($MgSO_4$) and filtered. The filtrate was evaporated under reduced pressure and the residue flash chromatographed on silica gel (2.4 × 24 cm), eluting first with CH_2Cl_2 and then with 1% MeOH in CH_2Cl_2. Fractions showing a single spot corresponding to the product were pooled and evaporated under reduced pressure to afford 1.29 g (59%) of **14** as a shiny white solid: mp 130–132 °C; TLC R_f 0.87 ($CHCl_3$/MeOH, 4:1, silica gel); ^{1}H NMR ($CDCl_3$) δ 1.23 (t, 3 H, CH_2CH_3, J = 7.2 Hz), 1.31 (t, 3 H, CH_2CH_3, J = 7.2 Hz), 1.46 (s, 9 H, C(CH_3)$_3$), 2.08–2.26 (m, 1 H, Gluβ-HCH), 2.30–2.37 (m, 1 H, Gluβ-HCH), 2.40–2.55 (m, 2 H, Gluγ-CH_2), 4.08 (q, 2 H, CH_2CH_3, J = 7.2 Hz), 4.24 (q, 2 H, CH_2CH_3, J = 7.2 Hz), 4.36 (d, 2 H, $NHCH_2$, J = 6.0 Hz), 4.75–4.82 (m, 1 H, Gluα-CH), 4.92 (br s, 1 H, NH), 7.03 (d, 1 H, CONH, J = 7.2 Hz), 7.36 (d, 2 H, 3′-, 5′-CH, J = 8.2 Hz), 7.79 (d, 2 H, 2′-, 6′-CH, J = 8.2 Hz); MS (EI) m/z 436 (M^+). Anal. Calcd for ($C_{22}H_{32}N_2O_7$) C, H, N.

Diethyl *N*-[4-(Aminomethyl)benzoyl]-L-glutamate (15). Trifluoroacetic acid (1.8 mL, 23 mmol) was added dropwise to a stirred solution of **14** (1.0 g, 2.3 mmol) in CH_2Cl_2 (20 mL). The mixture was stirred at room temperature for 15 min, evaporated to dryness under reduced pressure, and coevaporated twice with absolute ethanol (30 mL). The residue was then subjected to column chromatography on silica gel (1.5 × 15 cm), eluting with a gradient of 5–10% MeOH in $CHCl_3$ to afford 0.68 g (88%) of **15** as a white solid: mp 111–114 °C; TLC R_f 0.26 ($CHCl_3$/MeOH, 4:1, silica gel); ^{1}H NMR (DMSO-d_6) δ 1.14–1.24 (2 overlapping t, 6 H, CH_2CH_3, J = 7.0 Hz), 1.94–2.15 (m, 2 H, Gluβ-CH_2), 2.45 (t, 2 H, Glu γ-CH_2, J = 7.2 Hz), 4.0 (s, 2 H, $NHCH_2$), 4.01–4.14 (2 overlapping q, 4 H, CH_2CH_3, J = 7.0 Hz), 4.43 (m, 1 H, Gluα-CH), 6.87 (br s, 2 H, NH_2), 7.42 and 7.52 (2d, 2 H, 3′-, 5′-CH, J = 8.2 Hz), 7.84 and 7.89 (2d, 2 H, 2′-, 6′-CH, J = 8.2 Hz), 8.67 and 8.74 (2d, 1 H, CONH, J = 7.2 Hz); MS (EI) m/z 336 (M^+). Anal. Calcd for ($C_{17}H_{24}N_2O_5$·0.5CF_3COOH·0.5H_2O) C, H, N.

Diethyl *N*-[4-[*N*-[(2,4-Diaminofuro[2,3-*d*]pyrimidin-5-yl)methyl]amino]methyl]benzoyl]-L-glutamate (16). To a solution of 2,4-diamino-5-(chloromethyl)furo[2,3-*d*]pyrimidine (**5**) (0.2 g, 1 mmol) in anhydrous DMSO (3 mL) were added anhydrous K_2CO_3 (0.28 g, 2 mmol) and **15** (0.67 g, 2 mmol), and the reaction mixture was stirred under nitrogen at room temperature for 24 h. The temperature was then raised to 45 °C and the reaction continued for an additional 48 h. The reaction mixture was then cooled to room temperature, diluted with water (50 mL), and stirred for 8 h. The solid that separated was filtered, washed with water, air-dried, and dissolved in MeOH. Silica gel (1 g) was added to the solution and the suspension evaporated to dryness under reduced pressure. This plug was loaded on a dry silica gel column (2.4 × 17 cm) and eluted with a gradient of 1–7% MeOH in $CHCl_3$. Fractions corresponding to the product were pooled and evaporated to dryness. The residue was triturated with cold anhydrous ether to afford 0.25 g (51%) of **16** as a glassy solid: mp 127–128 °C; TLC R_f 0.60 ($CHCl_3$/MeOH, 4:1, silica gel); ^{1}H NMR (DMSO-d_6) δ 1.17 (m, 6 H, CH_2CH_3), 1.99–2.14 (m,

2 H, Gluβ-CH_2), 2.44 (t, 2 H, Gluγ-CH_2, J = 7.2 Hz), 4.0–4.14 (m, 4 H, CH_2CH_3), 4.27 (d, 2 H, 8- or 10-CH_2, J = 6.0 Hz), 4.39–4.46 (m, 1 H, Gluα-CH), 5.13 (s, 2 H, 8- or 10-CH_2), 6.17 (s, 2 H, 4-NH_2), 6.62 (s, 2 H, 2-NH_2), 7.35 (d, 2 H, 3'-, 5'-CH, J = 8.1 Hz), 7.46 (s, 1 H, 6-CH), 7.83 (d, 2 H, 2'-, 6'-CH, J = 8.1 Hz), 8.0 (t, 1 H, 9-NH, J = 6.0 Hz), 8.68 (d, 1 H, CONH, J = 7.2 Hz); MS (CI) m/z 499 (MH^+).

***N*-[4-[[*N*-[(2,4-Diaminofuro[2,3-*d*]pyrimidin-5-yl)methyl]-amino]methyl]benzoyl]-L-glutamic Acid (4).** To a solution of **16** (0.1 g, 0.20 mmol) in MeOH/THF (2:1, 10 mL) was added 1 N NaOH (1 mL) and the mixture stirred at room temperature for 24 h. The volatiles were removed under reduced pressure, and the residue was dissolved in water (5 mL) and stirred for an additional 24 h. The solution was then cooled in an ice bath and the pH adjusted carefully to 4.0 by dropwise addition of 1 N HCl. The precipitate that formed was collected by filtration, washed well with water and ether, and immediately dried under high vacuum to afford 0.08 g (88%) of **4** as a white solid: mp 154–157 °C; TLC R_f 0.76 (MeOH, silica gel); UV λ_{max} (pH 1) 247 nm (ϵ 20 490), 300 (7730); λ_{max} (pH 7) 249 nm (ϵ 18 690); λ_{max} (pH 13) 249 (ϵ 18 660); ^{1}H NMR (DMSO-d_6) δ 1.90–2.20 (m, 2 H, Gluβ-CH_2), 2.35 (t, 2 H, Gluγ-CH_2, J = 7.2 Hz), 4.27 (d, 2 H, 8- or 10-CH_2, J = 5.4 Hz), 4.40 (m, 1 H, Gluα-CH), 5.12 (s, 2 H, 8- or 10-CH_2), 6.06 (s, 2 H, 4-NH_2), 6.49 (s, 2 H, 2-NH_2), 7.34 (d, 2 H, 3'-, 5'-CH, J = 7.7 Hz), 7.43 (s, 1 H, 6-CH), 7.83 (d, 2 H, 2'-, 6'-CH, J = 7.7 Hz), 7.97 (br t, 1 H, 9-NH), 8.51 (d, 1 H, CONH, J = 7.5 Hz), 12.1–12.7 (br s, COOH); HPLC t_R = 21.4 min (C_{18} silica gel, 8% CH_3CN in 0.1 M NaOAc, pH 5.5, flow rate 1.0 mL/min). Anal. Calcd for ($C_{20}H_{22}N_6O_6$·0.75 HCl·0.25H_2O), C, H, N.

Enzymes and Enzyme Assays. FPGS was partially purified from CCRF-CEM human leukemia cells by ammonium sulfate fractionation and by gel seiving and phosphocellulose chromatography as previously described.[14] FPGS activity was assayed as described previously.[31] It was verified that each parent drug was quantitatively recovered during the standard assay procedure, thus ensuring that polyglutamate products would also be quantitatively recovered. Kinetic constants were determined using the hyperbolic curve fitting subprogram of Sigmaplot (Jandel) using at least a 10-fold range of substrate concentrations; activity was linear with respect to time at the highest and lowest concentration tested. Assays contained ≈ 400 units of FPGS activity; one unit of FPGS catalyzes the incorporation of 1 pmol of [^{3}H]Glu/h. DHFR from CCRF-CEM cells was partially purified and assayed as previously described.[35] DHFR (CCRF-CEM) inhibitory potency was measured by adding graded amounts of drugs to standard assays; the drug concentration required to reduce activity to 50% of control (IC_{50}) was determined graphically from plots of residual activity versus drug concentration. All DHFR (CCRF-CEM) assays contained 1.8×10^{-3} units of DHFR activity; one unit of DHFR can reduce 1 μmol of dihydrofolate/min under standard conditions.

Cell Lines and Methods for Measuring Growth Inhibitory Potency. The human T-lymphoblastic leukemia cell line CCRF-CEM[36] and its MTX-resistant subline R30dm[34] were cultured as described.[34] R30dm expresses only 1% of the FPGS activity of CCRF-CEM. The A253 and FaDu human squamous cell carcinoma monolayer cell lines were subcultured in RPMI 1640/10% fetal calf serum in 100 mm cell culture dishes (Falcon) as described.[14] Inhibition of growth of CCRF-CEM and its MTX-resistant subline R30dm, as well as A253 and FaDu, under continous drug exposure was measured as described.[14,34] EC_{50} values were determined visually from plots of percent control growth versus the logarithm of drug concentration. Protection by metabolites against growth inhibitory effects was assayed by including metabolites simultaneously with a concentration of drug previously determined to yield growth inhibition of 90–99%; the remainder of the assay was as described above. Metabolites tested were 10 μM LV, 40 μM TdR, 50 μM Hx, or 40 μM TdR + 50 μM Hx. All cell lines were verified to be negative for Mycoplasma contamination using the GenProbe test kit during the course of these studies.

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