Classical and Nonclassical Furo[2,3-*d*]pyrimidines as Novel Antifolates: Synthesis and Biological Activities[1]

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Classical antifolate analogues containing a novel furo[2,3-*d*]pyrimidine ring system which include *N*-[4-[*N*-[(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)methyl]amino]benzoyl]-L-glutamic acid (1) and its N-9 methyl analogue 2 were synthesized as potential dual inhibitors of thymidylate synthase (TS) and dihydrofolate reductase (DHFR) and as antitumor agents. Four nonclassical antifolates, 2,4-diamino-5-(anilinomethyl)furo[2,3-*d*]pyrimidines **3–6** with 3,4,5-trimethoxy, 3,4,5-trichloro, 3,4-dichloro, and 2,5-dimethoxy substituents, respectively, in the phenyl ring, were also synthesized as potential inhibitors of DHFRs including those from *Pneumocystis carinii* and *Toxoplasma gondii*, which are organisms responsible for opportunistic infections in AIDS patients. The classical and nonclassical analogues were obtained *via* nucleophilic displacements of the key intermediate 2,4-diamino-5-(chloromethyl)furo[2,3-*d*]pyrimidine with the appropriate (*p*-aminobenzoyl)-L-glutamate or substituted aniline. The key intermediate was in turn synthesized from 2,4-diamino-6-hydroxypyrimidine and 1,3-dichloroacetone. The final compounds were tested *in vitro* against rat liver, (recombinant) human, *P. carinii*, *T. gondii*, and *Lactobacillus casei* DHFRs. The classical analogues showed moderate to good DHFR inhibitory activity (IC_{50} 10^{-6}–10^{-8} M) with the N-CH_3 analogue 2 about twice as potent as 1. The nonclassical analogues were inactive with IC_{50}s > 3×10^{-5} M. The classical analogues were also evaluated as inhibitors of TS (*L. casei*, (recombinant) human and human CCRF-CEM), glycinamide ribonucleotide formyltransferase, and 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase and were found to be inactive against these enzymes. The classical analogues (particularly 2) were significantly cytotoxic toward a variety of tumor cell lines in culture. The nonclassical analogues were marginally active. Both classical compounds were good substrates for human folylpolyglutamate synthetase. Further evaluation of the cytotoxicity of 1 and 2 in CCRF-CEM cells and its sublines, having defined mechanisms of methotrexate (MTX) resistance, demonstrated that the analogues utilize the reduced folate/MTX-transport system and primarily inhibit DHFR and that poly-γ-glutamylation was crucial to their mechanism of action. Protection studies in the FaDu squamous cell carcinoma cell line indicated that inhibition was completely reversed by leucovorin or the combination of thymidine plus hypoxanthine. Furthermore, for compounds 1 and 2, in contrast to MTX, the FaDu cells were better protected by thymidine alone than hypoxanthine alone, suggesting a predominantly antithymidylate effect.

Thymidylate synthase (TS) catalyzes the sole *de novo* synthesis of dTMP from dUMP and utilizes 5,10-methylenetetrahydrofolate polyglutamates[5] (CH_2-H_4PteGlu$_n$) as the source of the methyl group as well as the reductant.[2] Many 2-amino-4-oxo classical antifolates inhibit TS. The most notable among these is the *N*-10-propargyl-5,8-dideazafolate (PDDF).[3] During catalysis, CH_2-H_4PteGlu$_n$ species are converted to the corresponding dihydrofolates H_2PteGlu$_n$. Dihydrofolate reductase (DHFR) carries out the first step in the regeneration of CH_2-H_4PteGlu$_n$, *i.e.*, formation of tetrahydrofolates.[4] In contrast to inhibitors of TS, DHFR inhibitors are generally 2,4-diamino antifolates.[5] Antifolate inhibitors of TS and DHFR have found clinical utility as antitumor, antibacterial, and antiprotozoan agents.

Since tumor systems respond to the clinically used antitumor agents 5-fluorouracil (5-FU) and methotrexate (MTX), which are TS and DHFR inhibitors, respectively, it was of interest to combine TS and DHFR inhibitory activity in one molecule. Such dual inhibitors could act at two different sites, *i.e.*, TS and DHFR, and could possess "combination chemotherapy" potential in a single molecule without the pharmacokinetic disadvantages of two separate entities. Hynes *et al.*[6] have reported classical 2,4-diaminoquinazoline analogues as dual inhibitors of DHFR and TS and suggested that the chemotherapeutic utility of such analogues could be possible, provided the affinity for DHFR was similar to that for TS. Synergism of two separate antifolates which inhibit TS and DHFR has been demonstrated in growth inhibitory studies against *Lactobacillus casei*,[7,8] rat hepatoma cells,[9,10] and human lymphoma cells.[8,11,12]

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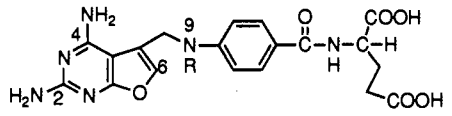

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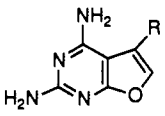
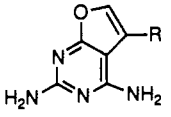
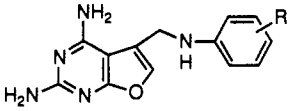
2 R = CH_3



2,4-Diamino Mode



2-Amino-4-Oxo Mode



3 R = 3',4',5'-trimethoxy
4 R = 3',4',5'-trichloro
5 R = 3',4'-dichloro
6 R = 2',5'-dimethoxy

Figure 1.

A molecule that could function as both a 2-amino-4-oxo and a 2,4-diamino-substituted system is 2,4-diaminofuro-[2,3-*d*]pyrimidine (Figure 1). We designed the novel furo-[2,3-*d*]pyrimidines *N*-[4-[*N*-[(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)methyl]amino]benzoyl]-L-glutamic acid (1) and *N*-[4-[*N*-[(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)methyl]methylamino]benzoyl]-L-glutamic acid (2) as potential classical dual inhibitors of TS and DHFR. Binding in the 2,4-diamino mode (Figure 1), similar to MTX,[4] was expected to provide DHFR inhibition. TS inhibition would be possible in the alternate 2-amino-4-oxo mode (Figure 1) which is obtained by a 180° rotation about the 2-NH_2–C_2 bond of the 2,4-diamino mode.

Polyglutamylation *via* folylpolyglutamate synthetase (FPGS) is an important mechanism for trapping classical folates and antifolates within the cell, thus maintaining high intracellular concentrations.[13] The antitumor activity of many classical antifolates is, in part, determined by the ability of the antifolate to function as a substrate for FPGS. In general, the 2-amino-4-oxo-substituted antifolates are superior substrates for FPGS compared to the 2,4-diamino-substituted analogues.[5] The 2,4-diaminofuro[2,3-*d*]pyrimidines 1 and 2 were designed to function as substrates for FPGS in their 2-amino-4-oxo and/or 2,4-diamino mode (Figure 1) which should allow for increased cell retention and antitumor activity.

In addition to classical folate analogues as antitumor agents, we have been extensively involved in the synthesis and biological evaluation of nonclassical antifolates to provide more potent and selective inhibitors of DHFR from *Pneumocystis carinii* and *Toxoplasma gondii* as possible treatments of the often fatal opportunistic infections these organisms cause in patients with AIDS. Our efforts resulted in the synthesis of substituted pyrido-[2,3-*d*]pyrimidines,[14] tetrahydroquinazolines,[15] pyrimidonaphthyridines,[16] and pyrrolo-fused pyrido[2,3-*d*]pyrimidines.[17] Several of these compounds have demonstrated increased potency and/or selectivity against *P. carinii* and *T. gondii* DHFR, compared to the currently used antifolates, trimethoprim, pyrimethamine, and trimetrexate.[14]

2,4-Diamino nonclassical antifolates such as trimetrexate, piritrexim, and our recently synthesized pyrido[2,3-*d*]pyrimidine analogues[14] are much more lipid soluble than the classical antifolates. In addition, these compounds are active against a variety of tumors as well as against *P. carinii* and *T. gondii*. Thus, novel nonclassical 2,4-diaminofuro[2,3-*d*]pyrimidines are of considerable interest both as inhibitors of DHFRs from *P. carinii* and *T. gondii* and as antitumor agents. Previous reports from our laboratory[14] showed that methoxy substituents in the phenyl ring of 2,4-diamino-6-(anilinomethyl)pyrido[2,3-*d*]pyrimidines are conducive to potent and selective inhibition of *P. carinii* and *T. gondii* DHFRs and that chloro substituents in the phenyl ring impart increased inhibition of tumor cell growth in culture and lack the selectivity demonstrated by the methoxy substituents. Thus, we synthesized the nonclassical furo[2,3-*d*]pyrimidine analogues 2,4-diamino-5-[(3',4',5'-trimethoxyanilino)methyl]furo[2,3-*d*]pyrimidine (**3**), 2,4-diamino-5-[(3',4',5'-trichloroanilino)methyl]furo[2,3-*d*]pyrimidine (**4**), 2,4-diamino-5-[(3',4'-dichloranilino)methyl]furo[2,3-*d*]pyrimidine (**5**), and 2,4-diamino-5-[(2',5'-dimethoxyanilino)-methyl]furo[2,3-*d*]pyrimidine (**6**) with either methoxy or chloro substituents in the side-chain phenyl ring.

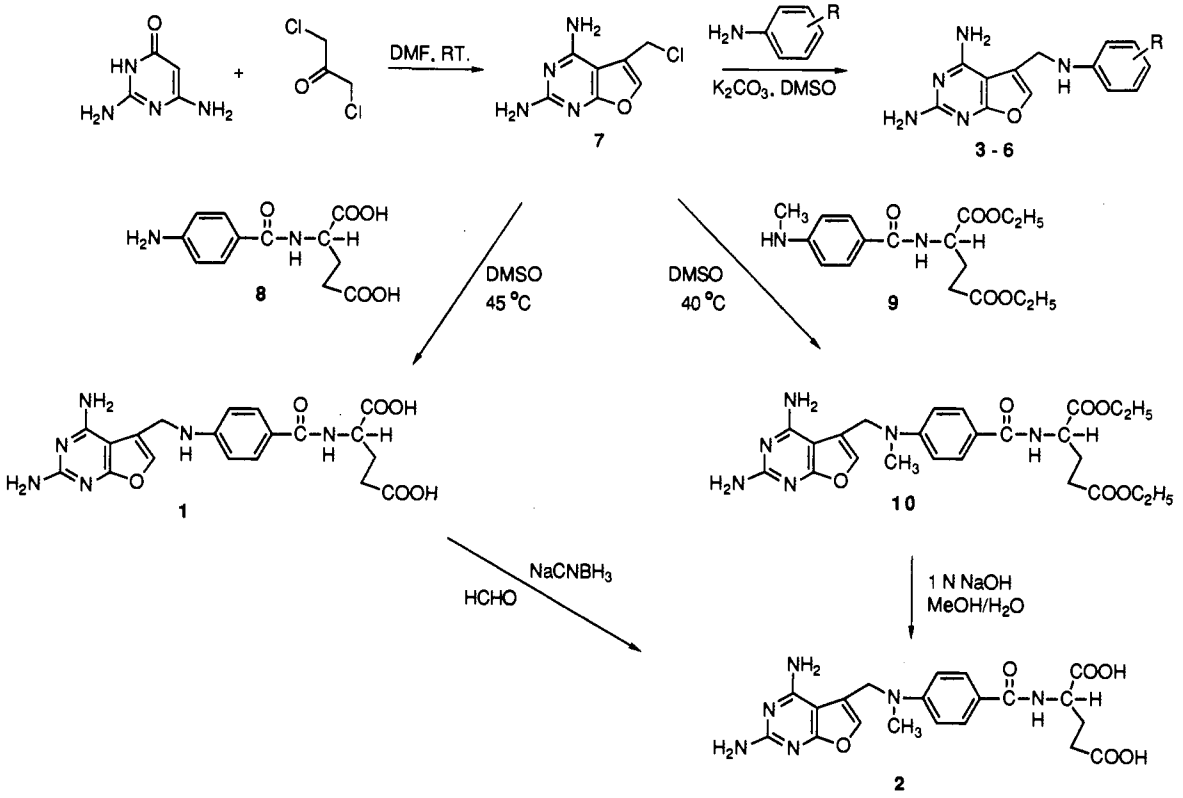
Though the classical and nonclassical furo[2,3-*d*]pyrimidines in this report represent the first in a series of novel furopyrimidine antifolates, other B-ring-contracted analogues as antifolates have been reported in the literature. These include purine analogues by Burchenal *et al.*[18] and Weinstock *et al.*[19] and thienopyrimidine analogues by Roth *et al.*,[20] Elslager *et al.*,[21] and Rosowsky *et al.*[22,23] The recent report by Miwa *et al.*[24] of the potent cytotoxic and DHFR inhibitory effects of classical 2,4-diamino-5-substituted pyrrolo[2,3-*d*]pyrimidines and the reports by Taylor *et al.*[25,26] and Shih and Gosset[27,28] of the TS inhibitory activity and significant cytotoxicity of classical 2-amino-4-oxo-5-substituted pyrrolo[2,3-*d*]pyrimidines and pyrazolo[2,3-*d*]pyrimidines[26] lend further credence to the design of furo[2,3-*d*]pyrimidines as inhibitors of folate-metabolizing enzymes.

Miwa *et al.*[24] have suggested that the classical 2,4-diaminopyrrolo[2,3-*d*]pyrimidines could bind to DHFR with the pyrrolo nitrogen positioned in the place of the 4-NH_2. In addition, the recent report[27] that 4-desaminopyrrolo[2,3-*d*]pyrimidine antifolates are excellent DHFR inhibitors supports the contention that these B-ring-contracted analogues may have more than one mode of binding to DHFR. Owing to the novel nature of the ring system, it was also of interest to evaluate the furo[2,3-*d*]pyrimidines 1 and 2 as inhibitors of glycinamide ribonucleotide formyltransferase (GARFT) and 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase (AICARFT), which are folate-cofactor-requiring enzymes in the purine biosynthetic pathway.

Chemistry

Secrist and Liu[29] reported the synthesis of 5-(chloromethyl)-2,4-diaminofuro[2,3-*d*]pyrimidine (**7**). This inter-

Scheme 1



mediate was the obvious choice for the synthesis of the target compounds as shown in Scheme 1. One equivalent each of 2,6-diamino-4-hydroxypyrimidine and 1,3-dichloroacetone in DMF was stirred at room temperature. Initially the reaction mixture was a suspension as the pyrimidine was insoluble in the reaction mixture; however, in 1 h, a clear solution formed and the product began to precipitate within 3 h. The reaction took 24 h for completion at which time column chromatography of the product afforded pure **7**. Compound **7** was not stable for long durations at room temperature and was used immediately in the next step. Nucleophilic displacement of the chloride of **7** was carried out with (*p*-aminobenzoyl)-L-glutamic acid (**8**) in anhydrous dimethyl sulfoxide at 45 °C for 72 h. The product was precipitated by dilution with water and separated from unreacted **8** and impurities by cellulose column chromatography followed by acidification to afford 48% of the desired product **1**. For the synthesis of the classical N-CH_3 analogue **2**, the displacement of **7** was carried out with diethyl *N*-[*p*-(methylamino)benzoyl]glutamate (**9**) which was prepared by the method of Nair *et al.*[30] Deprotection of the resulting diester was accomplished by stirring with 1 N NaOH for 48 h at room temperature followed by acidification to pH 3.5 which gave pure **2**. The N-CH_3 analogue was also obtained by reductive methylation of **1** with formaldehyde and sodium cyanoborohydride at pH 6.5 in a modification of the report of Piper *et al.*[31] Purification was accomplished on a wet cellulose column to afford **2**, after acidification, in 56% yield.

The ^{1}H NMR spectrum in deuterated dimethyl sulfoxide for **1** and **2** indicated the presence of both the 2- and 4-amino protons as singlets, exchangeable with the addition of deuterium oxide. In addition, the ^{1}H NMR spectrum of **2** indicated the absence of the exchangeable proton, corresponding to the 9-NH present in **1** and the collapse of the 8-methylene signal from a doublet in **1** to a singlet in **2**. This confirmed that the reductive methylation had occurred at the 9-position of **1** and not the 2- or 4-position. The 6-aromatic proton adjacent to the furan oxygen occurred at 7.04 ppm in **2** and is 0.3 ppm upfield compared to that in **1** (7.34 ppm). The 6-proton of **1** and **2** also slowly exchanged on standing in deuterated dimethyl sulfoxide containing deuterium oxide.

The syntheses of the nonclassical analogues **3–6** were accomplished (Scheme 1) *via* nucleophilic displacement reactions similar to those described above for the classical analogues. Thus, stirring a mixture of the key intermediate **7** in anhydrous dimethyl sulfoxide with 2 equiv of potassium carbonate and the appropriate aniline for 72 h at room temperature afforded the desired products. Compound **4** was obtained in low yield (<20%) by this procedure, due to the diminished nucleophilicity of 3′,4′,5′-trichloroaniline. Heating the reaction mixture to 40 °C for 72 h, however, increased the yield of **4** to 41%. The isolation of the products was greatly simplified by adding excess water to the reaction mixture and stirring at room temperature for 6–8 h during which time the product separated. Chromatographic purification afforded the target compounds **3–6** in yields ranging from 41% to 53%.

Biological Evaluation and Discussion

The classical and nonclassical analogues were evaluated as inhibitors of *P. carinii*, *T. gondii*, rat liver (RL),[32,33] *L. casei*,[34] and human recombinant DHFR.[35] The inhibitory concentrations (IC_{50}) are listed in Table 1. Both classical analogues **1** and **2** were moderate inhibitors of DHFR from mammalian, bacterial, and protozoal sources with the N-CH_3 analogue **2** about twice as potent as **1**. The most potent inhibition of DHFR was seen with **2** against *P. carinii* DHFR (IC_{50} = 3.5 × 10^{-8} M). Surprisingly, the nonclassical analogues **3–6** were essentially inactive with IC_{50} values of >3 × 10^{-5} M. A possible explanation for

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

	DHFR					TS			
compd no.	rec human	rat liver	*L. casei*	*P. carini*	*T. gondii*	human	*L. casei*	GARFT *L. casei*	AICARFT *L. casei*
1	0.45	1.3	0.56	0.90	0.70	>200	>200	>30	>30
2	0.22	0.43	0.22	0.035	19.8	>200	>200	>30	>30
3	>26	>37.0	>26	>4.0	>4				
4	>25	25.6	>25	8.3	>3.9				
5	>15	35.2	>3.0	>35	89.3				
6	>15	>21	>31	>21	>21				
MTX	0.004	0.003	0.006	0.001	0.014	170			

Table 2. IC_{50}, in μM, against Tumor Cell Growth in Culture[a]

	CCRF-CEM	A-CHN	SK-5
1	0.59	>100[b]	>100[b]
2	0.043	0.837[b]	14.0[b]
3	110.77	>130	>130
4	10.55	40.94	47.65
5	40.89	>150	>150
6	132.49	>160	>160

[a] IC_{50} = concentration of drug required to decrease cell viability as measured by MTA (MTT assay) by 50% after 3 days of treatment. [b] IG_{50} from National Cancer Institute Preclinical Screening Program.[45]

Table 3. Growth Inhibitory Concentrations, IG_{50}, in μM, of 1 and 2 against Tumor Cells in Culture[45]

	1	2
leukemia		
HL-60(TB)	0.02	<0.01
K-562	0.06	0.024
non-small-cell lung		
NCI-H460	3.75	0.15
colon cancer		
HCT-116	0.09	0.02
CNS cancer		
SF-268	1.77	0.59
U-251	1.97	0.75
melanoma		
LOX-IMVI	1.23	0.04
ovarian cancer		
1GROV-1	>100	0.73
renal cancer		
796-0	0.91	0.07
prostate cancer		
PC-3	0.70	<0.01
breast cancer		
MDA-MB-435	45.80	0.09
MDA-N	1.73	0.065
MCF-7	6.31	0.08

the lack of DHFR inhibitory activity of the nonclassical analogues is that the two-atom C8–N9 bridge between the furo[2,3-*d*]pyrimidine and the substituted phenyl side chain is too short and does not permit interaction of the substituted phenyl ring with appropriate portions of the DHFR enzyme.[4,36] Alternatively, the side chain could be held in a conformation that is not conducive to optimum binding.[4] Why then were the classical analogues moderately inhibitory to DHFR? The answer probably lies in the α-carboxylic acid moiety of the glutamate of 1 and 2 which should make appropriate ionic interactions with DHFR.[4] Thus, the classical analogues have additional binding sites which orient the side chain appropriately (like MTX) and translate into better inhibitory activity. The syntheses of one- to four-atom-bridged analogues, in both the classical and nonclassical series, are currently underway to determine the effect of bridge length and conformational flexibility of the side chain on biological activity. In addition, the X-ray crystal structures of the classical and nonclassical analogues with various DHFRs are in progress, some of which will be reported in the near future.[37]

Table 4. Activity of 1 and 2 as Substrates for CCRF-CEM Human Leukemia Cell Folylpolyglutamate Synthetase[a]

substrate	K_m, μM	$V_{max,rel}$	$V_{max}/K_{m(rel)}$	n
aminopterin	6.8 ± 0.6	1	0.15	5
1	1.9 ± 0.6	0.45 ± 0.02	0.24	3
2	4.65 ± 0.05	0.75 ± 0.02	0.16	2

[a] FPGS substrate activity was determined as described in the Experimental Section. Values presented are average ± SD if $n > 3$ and average ± range for $n = 2$.

The classical analogues 1 and 2 were also evaluated as inhibitors of TS from *L. casei*[38,39] and recombinant human sources[38,39] (Table 1) and were found to be essentially inactive. They were also essentially inactive ($IC_{50} > 1.6 \times 10^{-4}$ M) against TS isolated from the human CCRF-CEM cell line. This indicated that the 2-amino-4-oxo mode of binding (Figure 1) was probably not possible in the monoglutamate forms of this series of furo[2,3-*d*]pyrimidines. In addition, both classical analogues were inactive against both GARFT[40] and AICARFT[41] at 3×10^{-5} M.

The target compounds were evaluated as antitumor agents against leukemia CCRF-CEM, kidney ACHN, and melanoma SK-5 tumor cells in culture (Table 2).[42–44] The nonclassical analogues were marginally active. The classical analogues 1 and 2, however, were more cytotoxic against these tumor cells in culture. The N-9 methyl analogue 2 had an $IC_{50} = 4.3 \times 10^{-8}$ M against leukemia CCRF-CEM cells in culture and was about an order of magnitude better than 1. In addition, both compounds 1 and 2 were evaluated in the National Cancer Institute preclinical *in vitro* tumor screening program.[45] As shown in Table 3, the N-9 methyl analogue 2 was again more inhibitory than 1.

Compounds 1 and 2 were both significantly more active as inhibitors of tumor cell lines than as inhibitors of DHFR, TS, GARFT, or AICARFT. This indicated that the molecules were acting at additional sites and/or that metabolism was necessary for cytotoxicity. Since many classical antifolates owe their tumor inhibitory activity to their ability to form polyglutamates *via* FPGS,[46,47] it was possible that polyglutamylation of 1 and 2 was responsible for the significant activity against tumor cells in culture. Compounds 1 and 2 were therefore evaluated as substrates for human FPGS from CCRF-CEM cells, and the results are shown in Table 4. Both analogues were substrates for human FPGS, and their relative first-order rate constant $V_{max}/K_{m(rel)}$, which is a measure of catalytic efficiency, was as good or slightly better than that of aminopterin, which is a good substrate for ligation of the first additional glutamic acid.[48] The efficient utilization of these classical 2,4-diaminofuro[2,3-*d*]pyrimidine analogues as substrates for human FPGS suggested that polyglutamylation must be considered as a component of their therapeutic mechanism of action. In addition, these findings extend our knowledge regarding the specificity of FPGS for the

Table 5. Growth Inhibition of the Human T-Lymphoblastic Leukemia Cell Line CCRF-CEM, Its Methotrexate-Resistant Sublines, and the Human Squamous Cell Carcinoma FaDu by 1 and 2 during Continuous Exposure

cell line	resistance mechanism	EC_{50}, nM MTX	1	2
CCRF-CEM	sensitive	16 ± 1 (n = 3)	142 ± 11 (n = 4)	47 ± 7 (n = 4)
CEM/MTX	decr influx	2760 ± 43 (n = 3)	3167 ± 450 (n = 3)	2267 ± 262 (n = 3)
R1	incr DHFR	720 ± 59 (n = 3)	1523 ± 196 (n = 3)	807 ± 68 (n = 3)
R30dm	decr FPGS	18 ± 1 (n = 2)	663 ± 66 (n = 3)	1030 ± 510 (n = 3)
FaDu	sensitive	19 ± 5 (n = 2)	126 ± 34 (n = 2)	55 ± 15 (n = 2)

B-ring of pterin-like substrates.[5] It was previously known that other 6-6-fused systems including 5,8-dideaza (quinazoline)[49-51] and 5-deaza[52] derivatives could be substituted for the pyrazine ring of folates with retention of FPGS activity. Acyclic des(C-7) analogues[53,54] which do not contain the intact B-ring are also substrates. The data on the furo[2,3-d]pyrimidine analogues 1 and 2 indicate that B-ring contraction was well tolerated by human FPGS. That both binding affinity and catalytic rate are high indicated that FPGS has flexibility in its requirement for positioning the two portions of the substrate molecule. This conclusion is consistent with preliminary data reported for analogous pyrrolo[2,3-d]-pyrimidine derivatives.[55,56] A further important point was evident from the relative FPGS substrate activity of 1 and 2 compared to that of aminopterin and MTX, respectively. Methylation of the N-10 of aminopterin (producing MTX) dramatically reduces its efficiency as an FPGS substrate.[57] However, methylation of the N-9 of 1 does not have a detrimental effect on its catalytic efficiency. This result indicated that substitutions in the bridge region must be evaluated for each new series of analogues.

In an attempt to further elucidate the mechanism of action of 1 and 2, they were compared as inhibitors of the growth of the human T-lymphoblastic cell line CCRF-CEM[58] and its sublines, having defined mechanisms of MTX resistance, *viz.*, CEM/MTX[59] (decreased influx), R1[60] (increased DHFR), and R30dm[61] (decreased FPGS), and the results are shown in Table 5. The high degree of cross-resistance of the MTX transport-defective CEM/MTX subline and the subline with elevated DHFR (R1) indicated that these analogues enter the cell primarily *via* the reduced folate/MTX-transport system and that DHFR is their primary intracellular target. Cross-resistance of the FPGS-deficient subline R30dm to these agents indicated that poly(γ-glutamyl) metabolites of these analogues were critical to their mechanism of action even under continuous exposure conditions. These results are in keeping with other literature reports that glutamylation of classical antifolates can increase DHFR inhibitory effects as compared with their monoglutamate forms.[63,64] However, the difference in substrate efficiency between 1 and 2 resembles the 2-desamino-2-methyl[64] rather than the 2,4-diamino antifolates. DHFR was also suggested as the locus of action of 1 and 2 by studies with the FaDu squamous cell carcinoma cell line. FaDu was about as sensitive to MTX as was CCRF-CEM during continuous exposure, and the relative sensitivity of FaDu to 1 or 2 was also similar to that of CCRF-CEM (Table 5). Metabolite protection studies (Table 6) showed that growth inhibition of FaDu at EC_{85-97} levels of either MTX, 1, or 2 was completely reversed by 10^{-5} M leucovorin (LV) or the combination of 40 μM thymidine (TdR) and 50 μM hypoxanthine (Hx) but only partially by either TdR or Hx alone. MTX tended to be better protected partially by Hx alone, while both 1 and 2 were better protected partially by TdR alone. This may suggest a shift in these cells from a predominantly antipurine effect for MTX to a predominantly antithymidylate effect for 1 and 2; further study would be required to substantiate this hypothesis. Compound 2 is currently undergoing further biochemical evaluations which should provide further insight to the possible dual mechanism of action of these classical furo-[2,3-d]pyrimidines.

Table 6. Protection of FaDu Cells from the Growth Inhibitory Effects of MTX, 1, and 2 by 10 μM LV, 50 μM Hx, 40 μM TdR, or the Combination of 50 μM Hx + 40 μM TdR[a]

drug	relative growth (% of control) no addn	LV	Hx	TdR	Hx + TdR
MTX	2.5 ± 0	114 ± 3	11 ± 0.5	7.3 ± 0.4	98 ± 6
1	4.9 ± 0.3	110 ± 1	4.5 ± 0.2	18.4 ± 0.7	82 ± 4
2	6.6 ± 0.5	106 ± 2	1.9 ± 0	17.1 ± 1	80 ± 2

[a] Protection was determined as described in the Experimental Section. Data are average ± range of duplicate determinations within a single experiment. The entire experiment was repeated with similar results. Metabolites themselves had no effect on cell growth.

Experimental Section

Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Nuclear magnetic resonance for proton (^{1}H NMR) were recorded on a Bruker WH-300 (300 MHz). The data were accumulated by 16K size with 1-s delay time and 70° tip angle. 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 obtained on a VG-7070 double-focusing mass spectrometer in chemical ionization (CI) mode. Thin-layer chromatography (TLC) was pefrormed on silica gel plates and cellulose plates with fluorescent indicator that were visualized with light at 254 and 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, and microcrystalline cellulose, Avicel, from FMC Corporation, Newark, DE. Elemental analyses 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 were not prevented in spite of 24–48 h of drying *in vacuo* and were confirmed by their presence in the ^{1}H NMR spectrum.

2,4-Diamino-5-(chloromethyl)furo[2,3-*d*]pyrimidine (7). To a suspension of 2,6-diamino-4-hydroxypyrimidine (1.26 g, 10 mmol) in anhydrous DMF (12 mL) was added 1,3-dichloroacetone (1.27 g, 10 mmol), and the mixture was stirred at room temperature for 24 h. The DMF was removed under reduced pressure. The residue was dissolved in hot MeOH, and silica gel (5 g) was added to this and the suspension evaporated to dryness. The silica gel plug was poured on top of a dry silica gel column (2.4 cm × 20 cm) and eluted with 9:1 $CHCl_3$/MeOH. Fractions corresponding to the product were pooled and evaporated to dryness under reduced pressure. The residue was stirred in ether and filtered to obtain 1.35 g (68%) of 7: mp 179–184 °C (lit.[29] mp 178–188 °C); TLC R_f 0.30 ($CHCl_3$/MeOH 9:1, silica gel); ^{1}H NMR (DMSO-d_6) δ 4.90 (s, 2H, 8-CH_2), 6.10 (s, 2H, 4-NH_2), 6.57 (s, 2H, 2-NH_2), 7.46 (s, 1H, 6-CH).

2,4-Diamino-5-[(3′,4′,5′-trimethoxyanilino)methyl]furo-[2,3-*d*]pyrimidine (3). To a solution of 7 (0.149 g, 0.75 mmol) in anhydrous DMSO (3 mL) was added 3,4,5-trimethoxyaniline (0.275 g, 1.5 mmol) followed by anhydrous K_2CO_3 (0.207 g, 1.5 mmol), and the reaction mixture was stoppered and stirred at

room temperature for 72 h. The reaction mixture was then diluted with water (30 mL) and stirred for 4 h. The resulting precipitate was collected by filtration, washed with water, and air-dried and then washed with ether. The crude solid was dissolved in boiling methanol with drops of CF_3COOH, 1 g of silica gel was added, and the solution was evaporated to dryness. This silica gel plug was placed on top of a dry silica gel column (10 cm × 2.4 cm). The column was flushed with 1% MeOH in $CHCl_3$ (200 mL) and then eluted with 5% MeOH in $CHCl_3$. The fractions showing a single spot on TLC of the desired product were pooled and evaporated to dryness under reduced pressure. The resulting solid was recrystallized from hot MeOH to afford 0.129 g (50%) of a yellow solid, **3**: mp 213–215 °C; TLC R_f 0.56 ($CHCl_3$/MeOH 5:1, silica gel); ^{1}H NMR (DMSO-d_6) δ 3.53 (s, 3H, 4′-OCH_3), 3.69 (s, 6H, 3′-, 5′-OCH_3), 4.18 (s, 2H, 8-CH_2), 5.89 (br s, 1H, 9-NH), 6.08 (s, 2H, 2′-, 5′-CH), 6.21 (br s, 2H, 4-NH_2), 6.78 (br s, 2H, 2-NH_2), 7.44 (s, 1H, 6-CH). Anal. Calcd for ($C_{16}H_{19}N_5O_4$·0.20CF_3-COOH·0.5H_2O) C, H, N.

2,4-Diamino-5-[(3′,4′,5′-trichloroanilino)methyl]furo[2,3-*d*]pyrimidine (4). This compound was prepared in a manner similar to that described above for **3** except that the reaction was carried out at 40 °C using 3,4,5-trichloroaniline to afford 0.110 g (41%) of **4** as an orange solid: mp >240 °C dec; TLC R_f 0.54 ($CHCl_3$/MeOH 5:1, silica gel); ^{1}H NMR (DMSO-d_6) δ 4.28 (d, 2H, 8-CH_2), 6.06 (s, 2H, 4-NH_2), 6.44 (s, 2H, 2-NH_2), 6.70 (t, 1H, 9-NH), 6.93 (s, 2H, 2′-, 5′-CH), 7.34 (s, 1H, 6-CH). Anal. Calcd for ($C_{13}H_{10}N_5OCl_3$·0.25CF_3COOH·0.5H_2O) C, H, N.

2,4-Diamino-5-[(3′,4′-dichloroanilino)methyl]furo[2,3-*d*]pyrimidine (5). To a solution of **2** (0.149 g, 0.75 mmol) and 3,4-dichloroaniline (243 g, 1.5 mmol) in anhydrous DMSO (3 mL) was added anhydrous K_2CO_3 (0.207 g, 1.5 mmol), and the resulting mixture was stirred at room temperature for 72 h. Water (30 mL) was added and the reaction mixture stirred for 4 h at room temperature. The resulting precipitate was collected by filtration, washed with water, and air-dried and then washed with ether to remove unreacted aniline. The solid was dissolved in hot MeOH, 1.2 g of silica gel was added, and the solution was evaporated to dryness under reduced pressure. The silica gel plug was placed on top of a dry silica gel column (1.5 cm × 10 cm) and eluted with 5% MeOH in $CHCl_3$. Fractions showing a single spot on TLC of the desired product were pooled and evaporated under reduced pressure to dryness. The resulting solid was recrystallized from hot MeOH to afford 0.104 g (43%) of **5** as a white solid: mp 250–252 °C; TLC R_f 0.56 ($CHCl_3$/MeOH 5:1, silica gel); ^{1}H NMR (DMSO-d_6) δ 4.24 (d, 2H, 8-CH_2), 6.06 (s, 2H, 4-NH_2), 6.47–6.53 (3H, 2-NH_2, 9-NH), 6.70 (dd, 1H, 6′-CH), 6.91 (d, 1H, 2′-CH), 7.30 (d, 1H, 5′-CH), 7.35 (s, 1H, 6-CH). Anal. Calcd for ($C_{13}H_{11}N_5OCl_2$·0.25H_2O) C, H, N.

2,4-Diamino-5-[(2′,5-dimethoxyanilino)methyl]furo[2,3-*d*]pyrimidine (6). This compound was prepared in a manner similar to that described for **5** using 2,5-dimethoxyaniline instead of 3,4-dichloroaniline to afford 0.126 g (53%) of **6** as a light brown solid: mp 227–229 °C; TLC R_f 0.59 ($CHCl_3$/MeOH 5:1, silica gel); ^{1}H NMR (DMSO-d_6) δ 3.61 (s, 3H, 2′-OCH_3), 3.72 (s, 3H, 5′-OCH_3), 4.23 (s, 2H, 8-CH_2), 5.53 (br, 1H, 9-NH), 6.0 (s, 2H, 4-NH_2), 6.13 (dd, 1H, 4′-CH), 6.31 (d, 1H, 6′-CH), 6.65 (s, 2H, 2-NH_2), 6.71 (d, 1H, 3′-CH), 7.38 (s, 1H, 6-CH). Anal. Calcd for ($C_{15}H_{17}N_5O_3$) C, H, N.

***N*-[4-[*N*-[(2,4-Diaminofuro[2,3-*d*]pyrimidin-5-yl)methyl]-amino]benzoyl]-L-glutamic Acid (1).** A solution of **7** (0.980 g, 0.5 mmol) and (*p*-aminobenzoyl)-L-glutamic acid (**8**) (0.133 g, 0.5 mmol) in 3 mL of anhydrous DMSO was stirred at 45 °C under nitrogen for 24 h. An additional 0.133 g (0.5 mmol) of **8** was added to the mixture, and the reaction was continued for a further 48 h at 45 °C. The reaction mixture was cooled and diluted with water (40 mL). The resulting suspension (pH ≃ 3.5) was stirred at room temperature for 4 h and then left at 5 °C for 14 h. The precipitate obtained was filtered, washed with cold water (10 mL), dissolved in 0.2 N NaOH (4 mL), and applied to a cellulose column (2.4 cm × 18 cm) packed in 0.25% NH_4HCO_3. The column was eluted with 0.25% NH_4HCO_3; 4-mL fractions were collected. Fractions showing a single spot on TLC corresponding to the product were pooled, and the pH was adjusted to 3.5 *via* dropwise addition of 1 N HCl. The suspension was left at 5 °C overnight, and the precipitate was collected by filtration, washed sequentially with water, acetone, and ether, and air-dried to afford 0.102 g (48%) of a brown solid: mp >185 °C dec; TLC R_f 0.50 (3% NH_4HCO_3, cellulose); ^{1}H NMR (DMSO-d_6) δ 1.93–2.08 (m, 2H, Glu β-CH_2), 2.33 (t, 2H, Glu γ-CH_2), 4.30–4.40 (overlapping m, 3H, 8-CH_2, Glu α-CH), 6.04 (s, 2H, 4-NH_2), 6.47 (s, 2H, 2-NH_2), 6.61 (t, 1H, 9-NH), 6.73 (d, 2H, 3′-, 5′-CH), 7.34 (s, 1H, 6-CH), 7.69 (d, 2H, 2′-, 6′-CH), 8.16 (d, 1H, CONH), 11.8–12.7 (br s, α-, γ-COOH). Anal. Calcd for ($C_{19}H_{20}N_6O_6$·0.75H_2O) C, H, N.

Diethyl *N*-[4-[*N*-[(2,4-Diaminofuro[2,3-*d*]pyrimidin-5-yl)-methyl]methylamino]benzoyl]-L-glutamate (10). To a solution of **7** (0.149 g, 0.75 mmol) in 3 mL of anhydrous DMSO were added diethyl [*p*-(methylamino)benzoyl]-L-glutamate (**9**) (0.252 g, 0.75 mmol) and anhydrous K_2CO_3 (0.207 g, 1.5 mmol), and the reaction mixture was stirred at room temperature under nitrogen for 24 h. An additional 0.252 g of **9** was added to the mixture and stirring continued at 40 °C under nitrogen for an additional 48 h. The reaction mixture was then diluted with 40 mL of water and stirred at room temperature for 6 h. The solid that separated was filtered, washed with water, air-dried, and dissolved in MeOH followed by the addition of 1.2 g of silica gel. The mixture was evaporated to dryness under reduced pressure. This plug was placed on top of a dry silica gel column (2.4 cm × 18 cm) and eluted with a gradient of 1% MeOH in $CHCl_3$ to 7% MeOH in $CHCl_3$. Fractions corresponding to the product (TLC) were pooled and evaporated under reduced pressure to dryness. The residue was triturated in cold ether and filtered to afford 0.255 g (68%) of **10** as a pale yellow solid: mp 135–136 °C; TLC R_f 0.66 ($CHCl_3$/MeOH 5:1, silica gel); ^{1}H NMR (DMSO-d_6) δ 1.17 (m, 6H, CH_2CH_3), 1.95–2.10 (m, 2H, Glu β-CH_2), 2.42 (t, 2H, Glu γ-CH_2), 2.99 (s, 3H, NCH_3), 4.0–4.12 (m, 4H, CH_2-CH_3), 4.40 (m, 1H, Glu α-CH), 4.63 (s, 2H, 8-CH_2), 6.06 (s, 2H, 4-NH_2), 6.43 (s, 2H, 2-NH_2), 6.88 (d, 2H, 3′-, 5′-CH), 7.03 (s, 1H, 6-CH), 7.77 (d, 2H, 2′-, 6′-CH), 8.38 (d, 1H, CONH); mass spectrum CI (isobutane) *m/e* 499 (M + H^+).

***N*-[4-[*N*-[(2,4-Diaminofuro[2,3-*d*]pyrimidin-5-yl)methyl]-methylamino]benzoyl]-L-glutamic Acid (2). Method A, from 10.** To a solution of **10** (0.174 g, 0.35 mmol) in 10 mL of MeOH was added 1.1 mL of 1 N NaOH, and the solution was stirred at room temperature for 24 h. The MeOH was then evaporated under reduced pressure, the residue was dissolved in water (10 mL), and stirring was continued for an additional 24 h. The solution was then cooled in an ice bath and the pH adjusted to 3.5 *via* dropwise addition of 1 N HCl. The precipitate formed was collected by filtration, washed with water, acetone, and ether, and air-dried to obtain 0.152 g (99%) of a pale yellow solid, **2**: mp 199–202 °C; TLC R_f 0.68 (3% NH_4HCO_3, cellulose); ^{1}H NMR (DMSO-d_6) δ 1.94–2.08 (m, 2H, Glu β-CH_2), 2.34 (t, 2H, Glu γ-CH_2), 2.99 (s, 3H, NCH_3), 4.37 (m, 1H, Glu α-CH), 4.63 (s, 2H, 8-CH_2), 6.07 (s, 2H, 4-NH_2), 6.43 (s, 2H, 2-NH_2), 6.88 (d, 2H, 3′-, 5′-CH), 7.04 (s, 1H, 6-CH), 7.78 (d, 2H, 2′-, 6′-CH), 8.25 (d, 1H, CONH), 12.0–12.7 (br s, α-, γ-COOH). Anal. Calcd for ($C_{20}H_{22}N_6O_6$·1.0H_2O) C, H, N.

Method B, from 1. A suspension of **1** (0.086 g, 0.2 mmol) in water (6 mL, previously bubbled with nitrogen for 30 min) was dissolved by dropwise addition of 1 N NaOH. The pH was then adjusted to 6.5 by dropwise addition of 1 N HCl. To this solution under nitrogen was added a solution of 46% HCHO in water (0.163 g, 2.0 mmol) followed by $NaBH_3CN$ (0.019 g, 0.3 mmol). The pH of the solution was maintained at 6.5 by the addition of 1 N HCl as required for the next 1.5 h. The reaction was allowed to continue for a further 24 h at room temperature under nitrogen, at which time the TLC (cellulose, 3% NH_4HCO_3) indicated that **1** (R_f 0.50) had disappeared and **2** (R_f 0.68) had formed. The pH of the solution was adjusted to 3.5 by dropwise addition of 1 N HCl, and the precipitate that formed was filtered and washed with water. This crude solid was dissolved in 0.2 N NaOH (3 mL) and chromatographed on a cellulose column (1.5 cm × 15 cm) packed in 0.10% NH_4HCO_3. Following elution with the same solvent, the appropriate fractions (TLC) were pooled, and the pH was adjusted to 3.5 with 1 N HCl. The suspension was left at 5 °C overnight and filtered. The precipitate was washed with water, acetone, and ether and air-dried to afford 0.050 g (56%) of **2**, identical in all respects with the sample prepared as described above under method A.

Folylpolyglutamate Synthetase (Table 4). FPGS was partially purified from CCRF-CEM human leukemia cells by

ammonium sulfate fractionation and size-exclusion chromatography as previously described.[46] In some experiments, FPGS which had been further purified by phosphocellulose chromatography was used. Briefly, the enzyme from the size-exclusion column was concentrated over a YM-30 membrane (Amicon) and dialyzed exhaustively against 20 mM K-phosphate, pH 7.5, containing both 50 mM 2-mercaptoethanol and 2.5 mM benzamidine-HCl, pH 7.5 (Calbiochem). After removal of insoluble protein by centrifugation (10 min, 14600g), the enzyme was applied to a phosphocellulose column (Whatman P-11) equilibrated with the same buffer, and the column was washed with equilibration buffer until the absorbance of fractions at 280 nm was low and constant. The column was eluted with a linear gradient from 0–250 mM KCl in equilibration buffer. Fractions containing FPGS activity were pooled, adjusted to 500 mM KCl, concentrated as above, made 20% (v/v) in glycerol, and stored at −100 °C. There was no difference between the kinetic constants obtained with these two preparations when aminopterin was the substrate (data not shown). FPGS activity was assayed as described previously.[47] It was determined that each parent compound was quantitatively recovered during the standard assay procedure, thus ensuring that polyglutamate products would also be quantitatively recovered. Kinetic constants were determined over at least a 10-fold range of substrate concentrations, and activity was linear with respect to time at the highest and lowest concentration tested. Kinetic constants were determined using the hyperbolic curve-fitting subprogram of SigmaPlot (Jandel).

Cell Lines and Methods for Continuous Exposure (Table 5). Human T-lymphoblastic leukemia cell line CCRF-CEM[58] and its methotrexate-resistant sublines R30dm,[61] R1,[60] and CEM/MTX[59] used in these studies were cultured as described.[61] R30dm expresses only 1% of the FPGS activity of CCRF-CEM. R1 expresses high levels of DHFR, the target enzyme of MTX, and CEM/MTX is deficient in MTX transport. The FaDu (human squamous cell carcinoma of the pharynx) monolayer cell line was obtained as a frozen stock (ATCC), cultured in RPMI 1640/10% fetal calf serum in 100-mm cell culture dishes (Falcon) essentially as described.[62] FaDu exhibited an initial decline in cell number of 25–35%, independent of inoculum, after replating. After a 24–72-h lag (depending on inoculum density), log growth resumed with a generation time of about 19 h. All cell lines were verified to be negative for mycoplasma contamination using the GenProbe test kit.

Inhibition of the growth of CCRF-CEM and its MTX-resistant sublines in continuous drug exposure was measured as described.[61] FaDu cells for growth inhibition studies were plated at 2×10^4/60-mm dish using RPMI 1640/5% fetal calf serum; there was no difference in cell growth or the potency of MTX at the lower serum concentration. The cell count at $t = 48$ or 72 h (dependent on serum lot) was used as the "initial" density to avoid complications resulting from the initial growth lag. Growth was measured at 120 h after the "initial" time; growth was logarithmic during that period. EC_{50} values were determined from plots of percent control growth versus logarithm of drug concentration. Protection by metabolites against growth inhibitory effects in the FaDu line was assayed by including metabolites simultaneously with a concentration of drug previously determined to yield growth inhibition of about 90%; the remainder of the assay was as described above. Metabolites tested were 10^{-5} M LV, 40 μM TdR, 50 μM Hx, or 40 μM TdR + 50 μM Hx.

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