

Synthesis and evaluation of a classical 2,4-diamino-5-substituted-furo[2,3-*d*]pyrimidine and a 2-amino-4-oxo-6-substituted-pyrrolo[2,3-*d*]pyrimidine as antifolates[☆]

Aleem Gangjee,^{a,*} Jie Yang,^a John J. McGuire^b and Roy L. Kisliuk^c

^a*Division of Medicinal Chemistry, Graduate School of Pharmaceutical Sciences, 600 Forbes Avenue, Duquesne University, Pittsburgh, PA 15282, USA*

^b*Grace Cancer Drug Center, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263, USA*

^c*Department of Biochemistry, Tufts University School of Medicine, Boston, MA 02111, USA*

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Abstract—Two classical antifolates, a 2,4-diamino-5-substituted furo[2,3-*d*]pyrimidine and a 2-amino-4-oxo-6-substituted pyrrolo[2,3-*d*]pyrimidine, were synthesized as potential inhibitors of dihydrofolate reductase (DHFR) and thymidylate synthase (TS). The syntheses were accomplished by condensation of 2,6-diamino-3(*H*)-4-oxo-pyrimidine with α -chloro-ketone **21** to afford two key intermediates **23** and **24**, followed by hydrolysis, coupling with L-glutamate diethyl ester and saponification of the diethyl ester to afford the classical antifolates **13** and **14**. Compounds **13** and **14** with a single carbon atom bridge are both substrates for folypoly- γ -glutamate synthetase (FPGS), the enzyme responsible for forming critical poly- γ -glutamate antifolate metabolites with increased potency and/or increased cell retention. Compound **14** is a highly efficient FPGS substrate demonstrating that 2,4-diamino-5-substituted furo[2,3-*d*]pyrimidines are important lead structures for the design of antifolates with FPGS substrate activity. It retains inhibitory potency for DHFR and TS compared to the two atom bridged analog **5**. Compound **13** is a poor inhibitor of purified DHFR and TS, and both **13** and **14** are poor inhibitors of the growth of CCRF-CEM human leukemia cells in culture, indicating that single carbon bridged compounds in these series though conducive to FPGS substrate activity were not potent inhibitors.

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1. Introduction

Folate metabolism is an attractive chemotherapeutic target since it plays a crucial role in the biosynthesis of nucleic acid precursors.² Tetrahydrofolate (FH₄), the key component of folate metabolism, serves as a cofactor to carry one-carbon units that are important in physiological pathways. FH₄ is formed by the NADPH-dependent reduction of 7,8-dihydrofolate (FH₂) by the enzyme dihydrofolate reductase (DHFR).³ Thymidylate synthase (TS) is a crucial enzyme that catalyzes the conversion of 2'-deoxyuridine-5'-monophosphate (dUMP) to 2'-deoxythymidine-5'-monophosphate (dTMP) utilizing the cofactor 5,10-methylenetetrahydrofolate (5,10-CH₂FH₄) as the source of the one carbon as well as

the reductant. This is the only de novo synthesis of dTMP, and hence, TS and DHFR play a pivotal role in DNA biosynthesis and cell replication.⁴ Antifolate inhibitors of TS and DHFR have found clinical utility as antitumor, antibacterial, and antiprotozoan agents.² Several classical antifolates including *N*¹⁰-propargyl-5,8-dideazafofolate (**1**, PDDF),⁵ methotrexate (**2**, MTX), and pemetrexed (**3**) have been evaluated as antitumor agents.^{5–7} (Fig. 1).

Both **1** PDDF and **2** MTX are 6-6 bicyclic classical antifolates. Recently, some 6-5 bicyclic pyrrolo[2,3-*d*]pyrimidines such as compounds **3** and **4** TNP351^{8,9} were also reported as potent antifolates. Gangjee et al.^{10–12} reported a series of novel furo[2,3-*d*]pyrimidines **5–9** as potent antifolates. A 2,4-diamino-substituted pyrimidine ring is considered important for potent DHFR inhibition, while a 2-substituted-4-oxopyrimidine ring is considered important for TS inhibition.^{2,13,14} Gangjee et al.^{10,11} suggested a dual DHFR–TS inhibitor model and proposed two binding modes for the 2-amino-4-

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[☆] See Ref. 1.

* Corresponding author. Tel.: +1 412 396 6070; fax: +1 412 396 5593; e-mail: gangjee@duq.edu

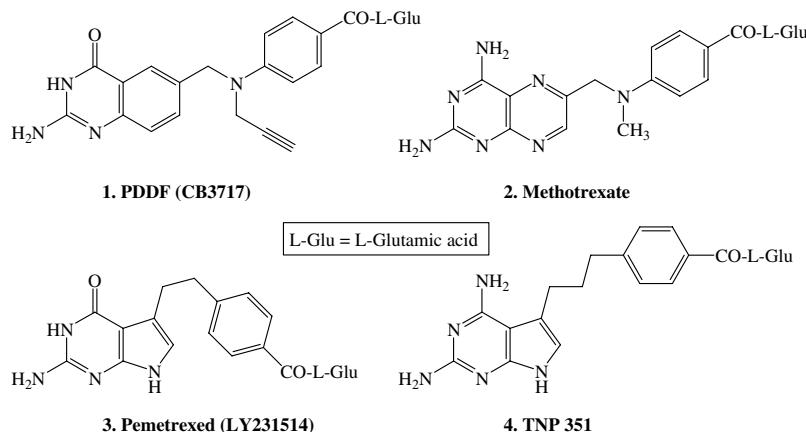


Figure 1.

oxo-pyrrolo[2,3-*d*]pyrimidine system as well as the 2,4-diamino-furo[2,3-*d*]pyrimidine (Fig. 2), Gangjee et al.¹⁵ demonstrated that compound **10**, a 4-methyl analog of compound **3**, binds in the alternate mode to DHFR.

Since dual inhibitors can act two different sites (TS and DHFR), such inhibitors would afford dual mechanism of action in a single agent without the pharmacokinetic disadvantages of two separate agents. Thus, it was of interest to develop additional dual DHFR–TS inhibitors as antitumor agents. Gangjee et al.^{10–12} reported the synthesis of novel, classical 2,4-diamino-5-substituted-furo[2,3-*d*]pyrimidines **5–9** as antifolates which have a two-, or three-atom bridge connecting the furo[2,3-*d*]pyrimidine ring system to the benzoyl-L-glutamic acid (L-Glu). Compounds **5–8** showed moderate to high potency against DHFR ($IC_{50} = 1.0 \times 10^{-6}$ to 1.0×10^{-8} M), whereas compound **9** was less active against DHFR ($IC_{50} = 1.0 \times 10^{-5}$ M). Compound **10** however showed excellent dual DHFR–TS inhibitory activities.¹⁵

Some of these analogs were also significantly cytotoxic to the growth of tumor cells in culture ($EC_{50} = 1.0 \times 10^{-7}$ to 1.0×10^{-8} M). This cytotoxicity was attributed to the efficient poly(γ -glutamyl)ation by the enzyme folypoly- γ -glutamate synthetase (FPGS). Polyglutamyl)ation via FPGS is an important mechanism for trapping folates and classical antifolates within the cell, thus maintaining high intracellular concentra-

tions which allow for increased antitumor activity. In addition, for some folate-dependent enzymes such as TS, polyglutamyl)ation can also afford enhanced enzyme inhibition.¹⁶

Classical single-carbon bridged 2,4-diamino-5-substituted-furo[2,3-*d*]pyrimidines have not been explored in the literature as potential antifolates. Several single-atom bridged antifolates such as pyrrolo[2,3-*d*]pyrimidines (**11** and **12**) (Fig. 3) have been reported as potent inhibitors of TS and DHFR.^{17,18} These analogs demonstrated that a one-atom bridge in 6-5 bicyclic systems can provide potent inhibitory activity. In order to allow for a direct comparison of 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 of classical 2,4-diamino-5-substituted-furo[2,3-*d*]pyrimidine antifolates, we designed and synthesized single-carbon atom bridged classical 2,4-diamino-5-substituted furo[2,3-*d*]pyrimidine **14** (Fig. 3). Compound **14** is a truncated analog of the classical two-carbon bridged 2,4-diamino-5-substituted-furo[2,3-*d*]pyrimidine parent compound **5**.

The synthetic methodology adopted for compound **14** also afforded the 2-amino-4-oxo-6-substituted-pyrrolo[2,3-*d*]pyrimidine intermediate for the synthesis of compound **13**. Thus it was also of interest to synthesize the classical one-carbon bridged 2-amino-4-oxo-

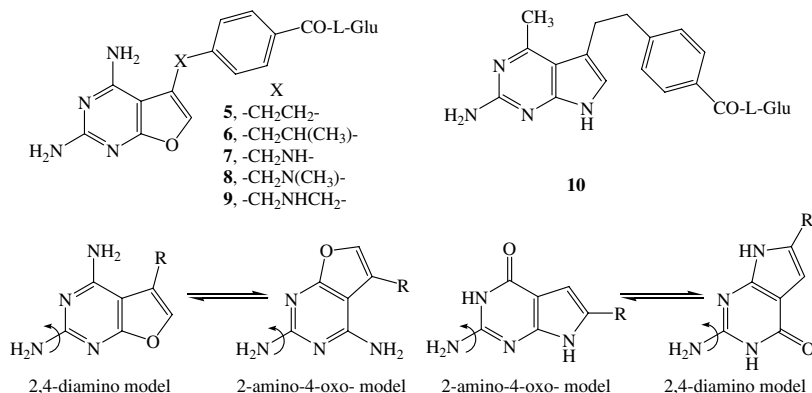


Figure 2.

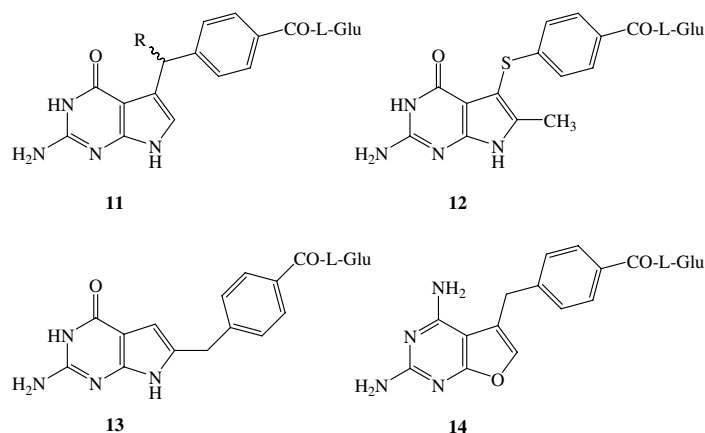


Figure 3.

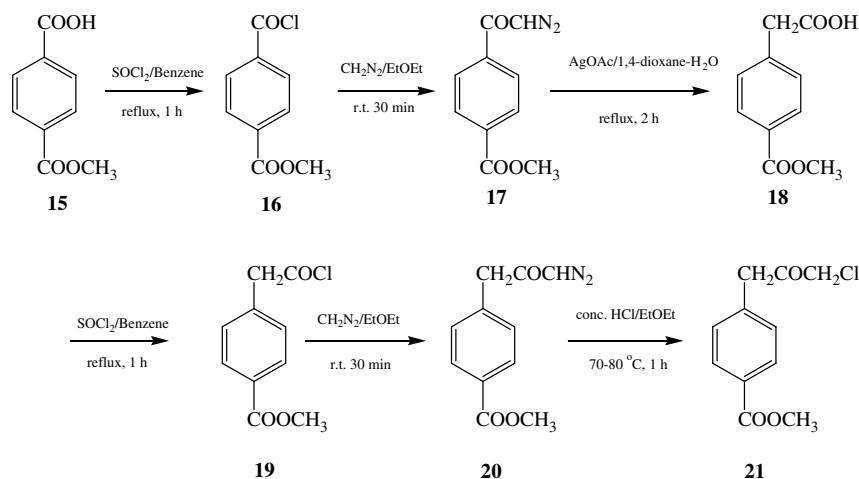
substituted pyrrolo[2,3-*d*]pyrimidine analog, compound **13** (Fig. 3), to determine the effect on biological activity of moving the substituent from the 5-position as in **11** to the 6-position in **13**.

2. Results and discussion

It was anticipated that condensation of 2,6-diamino-3(*H*)-4-oxo-pyrimidine **22** with an appropriate α -chloro ketone **21** could form both the 2-amino-4-oxo-6-substituted pyrrolo[2,3-*d*]pyrimidine **23** and the 2,4-diamino-5-substituted-furo[2,3-*d*]pyrimidine **24** in one step.¹⁹ For the synthesis of the α -halo ketone **21** from the corresponding α -diazomethane,²⁰ the corresponding [4-(methoxycarbonyl)phenyl]acetic acid **18** was required. The literature provides several methods for the synthesis of **18**.^{21,22} We elected to use the Arndt-Eistert rearrangement to homologate the carbon chain from the diazoketone **17** to **18** starting from commercially available *mono*-methyl terephthalate **15**.²³ Thus **15** was refluxed in thionyl chloride and benzene to give the acid chloride **16**, which was converted to diazoketone **17** after being treated with ethereal diazome-

thane.²⁴ Compound **17** was converted to the acid **18** via an Arndt-Eistert rearrangement. Conversion of the acid **18** to the corresponding acid chloride **19** followed by treatment with ethereal diazomethane gave the diazoketone **20**, which was immediately treated with concentrated HCl to afford the α -chloro ketone **21** (Scheme 1).²⁵

With key intermediate **21** in hand, several variations were attempted to optimize the annulation of **21** with **22** using different solvents and temperature conditions both with and without bases (Scheme 2). The reaction was finally optimized at 50–60 °C for 2 days (Scheme 2). Compound **21** condensed with 2,6-diamino-3(*H*)-4-oxo-6-pyrimidine **22** to afford a mixture of two products: 2-amino-3(*H*)-4-oxo-6-substituted-pyrrolo[2,3-*d*]pyrimidine **23** and 2,4-diamino-5-substituted-furo[2,3-*d*]pyrimidine **24**. The mixture was separated via column chromatography to give 33% and 27% yields of **23** and **24**, respectively. Hydrolysis of **23** and **24** afforded the corresponding acids **25** and **26**, respectively, which were coupled with diethyl L-glutamate using isobutyl chloroformate to form the corresponding amides **27**



Scheme 1.

and **28** in 65% and 60% yield, respectively. Hydrolysis of compounds **27** and **28** at 0 °C with 1 N NaOH gave the target compounds **13** (56%) and **14** (60%) (Scheme 2).²⁶

Compounds **13** and **14** were evaluated as inhibitors of *Escherichia coli* (ec), *Toxoplasma gondii* (tg), and recombinant human (rh) DHFR, and also as inhibitors of ecTS and rhTS. The inhibitory potencies (IC₅₀) are reported in Table 1 and compared with previously reported values for compounds **5–8**, MTX and PDDF, a first generation classical TS inhibitor. Compound **14** had reasonably potent DHFR and TS inhibitory activity compared with the parent two-carbon bridged furo[2,3-*d*]pyrimidine **5**. However, the 6-substituted single-carbon bridged pyrrolo[2,3-*d*]pyrimidine **13** was devoid of DHFR or TS inhibitory activity. This indicated that the 5-substituted single-atom bridged 2,4-diamino-furo[2,3-*d*]pyrimidine retains inhibitory activity for DHFR at similar levels comparable with other two-atom bridged 2,4-diamino-furo[2,3-*d*]pyrimidines. However, the 6-substituted single-atom bridged 2-amino-4-oxo-pyrrolo[2,3-*d*]pyrimidine is not conducive to DHFR or TS inhibition.

2.1. In vitro human tumor cell growth inhibition

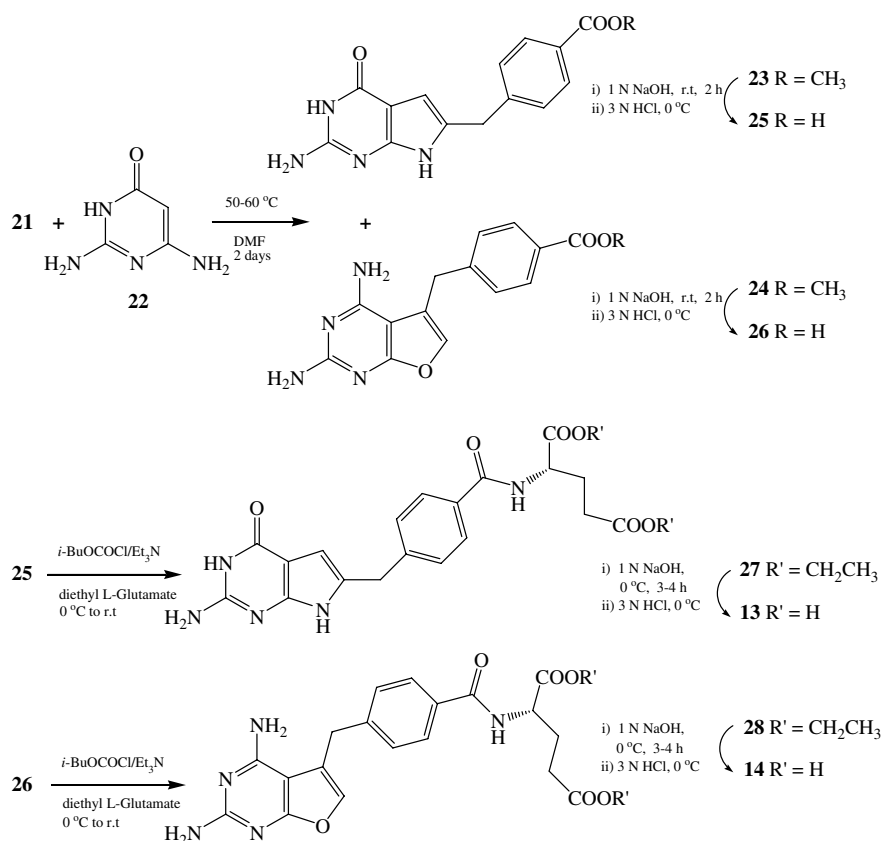
Growth inhibitory potency of **13** and **14** were compared to that of MTX in a continuous exposure against the CCRF-CEM human lymphoblastic leukemia and a ser-

ies of MTX-resistant sublines in culture during continuous exposure (Table 2). Compounds **13** and **14** were both at least 1250-fold less potent than MTX as growth inhibitors of CCRF-CEM. The MTX-resistant sublines also showed low potency of these two drugs demonstrating that none of the common mechanisms of MTX-resistance show collateral sensitivity to either agent.

2.2. FPGS substrate activity

Since these structures contain the intrinsic glutamic acid residue of ‘classical’ antifolates, they were evaluated in vitro as substrates for recombinant human folypolyglutamate synthetase (FPGS) and compared to AMT, a good substrate for FPGS. It is possible that poor metabolism to polyglutamate metabolites contributes to their low growth inhibitory potency. The data (Table 3) show that both **13** and **14** are substrates for human FPGS, however they differ markedly in their efficiency. Compound **14** has both a lower K_m and V_{max} than AMT, but is overall about 2-fold more efficient than AMT. In contrast, although the K_m of **13** is near that of AMT, it has a very diminished V_{max} and is 10-fold less efficient a substrate than AMT. Thus, the low growth inhibitory potency of **13** may be attributed, in part, to low metabolism to polyglutamates, but this is an unlikely explanation for the low activity of **14**.

Compound **14** is the one carbon-bridge analog of the two carbon-bridge compound **5** described previously.¹¹



Scheme 2.

Table 1. Inhibitory concentration (IC₅₀ in μ M) against isolated DHFR and TS^a

Compound	DHFR			TS	
	rh	ec	tg	ec	rh
5 ^b	1.00	nd	nd	nd	220
6 ^b	0.42	1.10	2.10	100	>360
7 ^b	0.45	nd	0.70	nd	63.0
8 ^b	0.22	nd	19.80	nd	>200
13	0% at 22	0% at 22	37% at 22	190	190
14	2.80	9.20	2.80	190	>190
MTX	0.022	0.009	0.022		
PDDF				0.10	0.15

^a rh, ec and tg DHFR kindly supplied by J. H. Freisheim, R. L. Blakley and D. V. Santi, respectively; ec and rh TS kindly supplied by F. Maley.

^b Data derived from Refs. 11 and 12. nd, not determined.

Table 2. Growth inhibition of parental CCRF-CEM human leukemia cells and sub-lines with single, defined mechanisms of MTX resistance during continuous (0–120 h) exposure to MTX, **13**, or **14**

Drug	EC ₅₀ (nM)			
	CCRF-CEM	R1 ^a (\uparrow DHFR)	R2 ^b (\downarrow Uptake)	R30dm ^c (\downarrow Glu _n)
MTX	12.7 \pm 3.3	915 \pm 285	2600 \pm 100	13.1 \pm 3.4
13	\approx 18,000	\geq 15,000	>10,000	>20,000
14	\approx 16,000	>20,000	>10,000	\approx 20,000

Values presented are average \pm SD for $n > 2$ or average \pm range for $n = 2$.

^a CCRF-CEM subline resistant to MTX solely as a result of a 20-fold increase in wild-type DHFR protein and activity.²⁷

^b CCRF-CEM subline resistant as a result of decreased uptake of MTX.²⁸

^c CCRF-CEM subline resistant to MTX solely as a result of decreased polyglutamylolation; this cell line has 1% of the FPGS specific activity (measured with MTX as the folate substrate) of parental CCRF-CEM.²⁹

Table 3. Activity of folate analogs as substrates for recombinant human FPGS^a

Substrate	K_m (μ M)	$V_{max,rel}$ ^b	$V_{max,rel}/K_m$	n
AMT	4.4 \pm 1.1	1.00	0.23	6
13	4.7 \pm 0.4	0.11 \pm 0	0.023	3
14	1.6 \pm 0.2	0.74 \pm 0	0.48	2

^a FPGS substrate activity was determined as described in Section 3 at 2 mM L-[³H]glutamate. Values presented are the average \pm SD if $n \geq 3$ and are average \pm range for $n = 2$.

^b $V_{max,rel}$ is calculated based on the apparent V_{max} of a substrate relative to the apparent V_{max} of AMT within the same experiment.

Although polyglutamates of **5** are clearly involved in its mechanism of action, **14**, which contains the shorter bridge, is a 7-fold more efficient human FPGS substrate than **5**. This indicates that the shorter length of the bridge is more conducive to FPGS substrate activity. However, this activity does not translate into greater tumor growth inhibitory potency since **5** is >34-fold more potent as an inhibitor of CCRF-CEM cell growth in continuous exposure. The mechanism of action of **5** appears to involve DHFR inhibition;¹¹ because of its low potency, however, the target of **14** could not be elucidated. The biological activity data with **13** and **14** suggest that one-carbon bridges are too short to allow one or more key determinants of antitumor activity in these two classes of agents.

In summary, truncation of the two-carbon bridge of 2,4-diamino-furo[2,3-*d*]pyrimidine to a single carbon leads to an enhanced efficiency for FPGS, a slight decrease

in DHFR and TS inhibitory activities but a significant loss of cytotoxicity to CCRF-CEM cells in culture compared to the two-carbon bridged analog, thus indicating that the distance between the pyrimidine ring and the side chain L-glutamic acid in furo[2,3-*d*]pyrimidines, though not detrimental for FPGS activity, is important for activity against the growth of tumor cells in culture. In addition, 6-substituted pyrrolo[2,3-*d*]pyrimidines with a one-carbon atom bridge are substrates for FPGS, they are essentially inactive, however, as antifolates compared to their 5-substituted regioisomers indicating that the position of attachment to the heterocycle is also important for TS and/or DHFR inhibitory activity.

3. Experimental

Melting points were determined on a Mel-Temp II melting point apparatus with FLUKE 51 K/J electronic thermometer and are uncorrected. Nuclear magnetic resonance spectra for proton (¹H) were recorded on a Bruker WH-300 (300 MHz) spectrometer. Chemical shift values are expressed in ppm (parts per million) relative to tetramethylsilane as internal standard; s = singlet, d = double, t = triplet, q = quartet, m = multiplet, br = broad singlet. The relative integrals of peak areas agreed with those expected for the assigned structures. High-resolution mass spectra (HRMS), using Electron Impact (EI), were recorded on a VG AUTOSPEC (Fisons Instruments) micromass (EBE Geometry) double focusing mass spectrometer. Thin-layer chromatography (TLC) was performed on POLYGRAM Sil G/UV254 silica gel plates with fluorescent indicator,

and the spots were visualized under 254 and 366 nm illumination. Proportions of solvents used for TLC were by volume. Column chromatography was performed on 230–400 mesh silica gel purchased from Aldrich Chemical Co., Milwaukee, WI. 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 at 75–110 °C. Elemental analysis was performed by Atlantic Microlabs, Norcross, GA. Element compositions are within $\pm 0.4\%$ of calculated values. Fractional moles of water or organic solvents frequently found in some analytical samples could not be prevented despite 24–48 h of drying in vacuo and were confirmed where possible by their presence in the 1H NMR spectra. All solvents and chemicals were purchased from Aldrich Chemical Co. and Fisher Scientific and were used as received.

3.1. [4-(Methoxycarbonyl)phenyl]acetic acid (**18**)

Mono-methyl terephthalate **15** (2 g, 5 mmol) was dissolved in benzene (10 mL) and thionyl chloride (10 mL) was added. The resulting reaction mixture was refluxed for 1 h. After removing the volatiles, the crude acyl acid chloride **16** was dissolved in anhydrous ethyl ether, and then added dropwise at 0 °C to a solution of diazomethane (made from *N*-methyl-*N*-nitrosourea) and triethyl amine (1.5 mL) in ether. The reaction was maintained for 16 h at room temperature, excess diazomethane was decomposed with 1 mL of acetic acid, and the salt was filtered and the solution evaporated to dryness to afford **17**. To a stirred suspension of silver acetate (1.2 g) in 30 mL of water was added a solution of the diazo compound **17** in 30 mL of 1,4-dioxane. The reaction mixture was heated to reflux for 1.5 h, and then 1.2 g of sodium carbonate was added. The solid obtained was filtered and extracted with chloroform (3 \times 25 mL). The combined chloroform extract was dried (Na_2SO_4), filtered, and the solvent was evaporated under reduced pressure. The residue was recrystallized from ethyl acetate to afford white needles of **18** (910 mg). The yield over three steps was 43%; mp: 107–110 °C (lit. 110–113 °C);²¹ TLC: R_f = 0.29 (hexane/EtOAc = 3:1); 1H NMR (DMSO- d_6): δ 3.69 (s, 2H, CH_2), 3.82 (s, 3H, OCH_3), and 7.32–8.11 (dd, 4H, Ar-H). This compound was used directly for the next step without further purification.

3.2. Methyl 4-(3-chloro-2-oxopropyl)benzoate (**21**)

A solution of 4-methoxycarbonylphenyl acetic acid **18** (1.0 g, 5 mmol) in 5 mL of benzene and 5 mL of thionyl chloride was refluxed for 1 h and the solvents evaporated under reduced pressure. The resulting acid chloride **19** was dissolved in 8 mL of dry ether and added dropwise to 30–40 mL of ethereal diazomethane (about 13 mmol, from 20 mmol of *N*-methyl-*N*-nitrosourea) at 0–5 °C. After 1 h at room temperature, concentrated HCl was added dropwise to the solution and then heated to reflux at 70–80 °C for 1 h. After cooling to room temperature, the ether layer was separated out. The organic layer was washed with water, saturated sodium bicarbonate solution, water, and dried (Na_2SO_4). The dried

solvent was evaporated under reduced pressure and the residue was loaded on a silica gel column (15 \times 150 mm) and eluted with hexane/EtOAc = 5:1. The desired fraction was pooled and evaporated to afford **21** (640 mg), yield over three steps 57%; mp: 67.5–70 °C; TLC: R_f = 0.69 (hexane/EtOAc = 3:1); 1H NMR (DMSO- d_6): δ 3.84 (s, 3H, OCH_3), 3.99 (s, 2H, CH_2), 4.66 (s, 2H, CH_2), and 7.23–7.99 (dd, 4H, Ar-H). This compound was used directly for the next step without further purification.

3.3. Methyl 4-[(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-*d*]pyrimidin-6-yl)methyl]benzoate (**23**) and methyl 4-[(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)methyl]benzoate (**24**)

Methyl 4-(3-chloro-2-oxopropyl)benzoate **21** (610 mg, 1.7 mmol) was placed in a 50 mL flask, and 2,6-diaminopyrimidin-4-one **22** (214 mg, 1.7 mmol) and 5 mL of DMF were added. The reaction mixture was stirred at 50–60 °C for 2 days. TLC showed two major products: R_f = 0.88 and 0.78 ($CHCl_3$ /MeOH = 5:1). To the reaction mixture was added 1 g of silica gel and evaporated to dryness under reduced pressure to form a plug. This plug was loaded on the top of a silica gel column (45 \times 150 mm) and eluted with 4% methanol in chloroform. The desired fractions containing the product (R_f = 0.88) were pooled and evaporated to afford 220 mg of an off-white solid **24** (27%); mp: 200–222 °C. 1H NMR (DMSO- d_6): δ 3.82 (s, 3H, OCH_3), 4.09 (s, 2H, CH_2), 6.02 (s, 2H, NH_2), 6.25 (s, 2H, NH_2), 7.07 (s, 1H, CH), and 7.39–7.91 (dd, 4H, Ar-H). Anal. Calcd for $C_{15}H_{14}N_4O_3$: C, 60.40; H, 4.73; N, 18.78. Found: C, 60.32; H, 4.85; N, 18.57.

Changing the eluent to 5% methanol in chloroform afforded fractions containing the desired product (R_f = 0.78). These fractions were pooled and evaporated to afford 270 mg of an off-yellow solid **23** (33%); mp: 237–241 °C; 1H NMR (DMSO- d_6): δ 3.90 (s, 3H, OCH_3), 3.96 (s, 2H, CH_2), 5.91 (s, 1H, CH), 6.01 (s, 2H, NH_2), 7.35–7.89 (dd, 4H, Ar-H), 10.18 (s, 1H, NH) and 10.97 (s, 1H, NH). Anal. Calcd for $C_{15}H_{14}N_4O_3 \cdot 0.5H_2O$: C, 58.63; H, 4.92; N, 18.23. Found: C, 58.30; H, 4.65; N, 18.03.

4. General procedure for the synthesis of **25** and **26**

To a solution of **23** or **24** (250 mg, 0.8 mmol) in 10 mL of ethanol was added aqueous 1 N NaOH and the mixture stirred at 80 °C for 10 h. TLC ($CHCl_3$ /MeOH = 5:1) showed the disappearance of the starting material (R_f = 0.78 or 0.88) and formation of one major spot at the origin. The solution was evaporated to dryness under reduced pressure, and the sodium salt was dissolved in 10 mL of water and carefully acidified to pH 4 with dropwise addition of 3 N HCl. The resulting suspension was left at 0 °C for 2 h and filtered. The residue was washed with cold water, acetone, and ether and dried in vacuum to afford the free acid.

4.1. 4-[(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)methyl]benzoic acid (25)

This compound was synthesized in 91% yield using the general procedure starting from **23**; mp: 270 °C (dec); ¹H NMR (DMSO-*d*₆): δ 3.89 (s, 2H, CH₂), 5.90 (s, 1H, CH), 6.02 (s, 2H, NH₂), 7.33–7.87 (dd, 4H, Ar-H), 10.18 (s, 1H, NH), 10.96 (s, 1H, NH) and 12.85 (s, 1H, COOH). This compound was used directly for the next step without further purification.

4.2. 4-[(2, 4-Diaminofuro[2,3-*d*]pyrimidin-5-yl)methyl]benzoic acid (26)

This compound was synthesized in 85% yield using the general procedure starting from **24**; mp: >250 °C (dec); ¹H NMR (DMSO-*d*₆): δ 4.08 (s, 2H, CH₂), 6.05 (s, 2H, NH₂), 6.28 (s, 2H, NH₂), 7.07 (s, 1H, CH), 7.36–8.00 (dd, 4H, Ar-H) and 12.90 (s, 1H, COOH). This compound was used directly for the next step without further purification.

5. General procedure for the synthesis of 27 and 28

To a suspension of the acid **25** or **26** (150 mg, 0.5 mmol) in anhydrous DMF (10 mL) under nitrogen was added triethylamine (0.21 mL, 1.5 mmol) and the suspension heated to form a solution. The solution was cooled to 0 °C and isobutyl chloroformate (0.13 mL, 1 mmol) was added, followed 15 min later by diethyl L-glutamate hydrochloride (180 mg, 0.75 mmol) and immediately followed by triethylamine (0.21 mL, 1.5 mmol). The reaction mixture was warmed slowly to room temperature and stirred for 12 h. At this time the reaction mixture was cooled to 0 °C and the activation steps described above were repeated using triethylamine (0.11 mL, 0.75 mmol), followed by isobutyl chloroformate (0.065 mL, 0.5 mmol). After stirring for 15 min at 0 °C, diethyl L-glutamate hydrochloride (90 mg, 0.38 mmol) was added followed immediately by triethylamine (0.11 mL, 0.75 mmol). The reaction mixture was stirred for 24 h at room temperature. TLC (CHCl₃/MeOH = 5:1) showed a new major spot was formed. The reaction mixture was evaporated to dryness under reduced pressure. The residue was suspended in water and the pH adjusted to 8 with ammonium hydroxide and stirred for 30 min. The suspension was filtered and the residue washed well with water, air dried, and dissolved in methanol. To this solution was added 500 mg of silica gel, the solvent was evaporated and the residue dried under reduced pressure to form a plug. The dry plug was loaded on a silica gel column (15 × 150 mm) and eluted using 2% methanol in chloroform. The fractions containing the desired product were pooled and the solvent evaporated to give the desired compounds.

5.1. Diethyl N-{4-[(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-*d*]pyrimidin-6-yl)methyl] benzoyl}-L-glutamate (27)

This compound was synthesized in 65% yield using the general procedure starting from **25**; mp: 156–158 °C;

TLC: *R*_f = 0.48 (CHCl₃/MeOH = 5:1); ¹H NMR (DMSO-*d*₆): δ 1.12–1.32 (m, 6H, 2 × OCH₂CH₃), 2.00–2.44 (m, 4H, 2 × CH₂), 3.88 (s, 2H, CH₂), 3.99–4.12 (m, 4H, 2 × OCH₂CH₃), 4.41–4.50 (m, 1H, CH), 5.86 (s, 1H, CH), 6.06 (s, 2H, NH₂), 7.32–7.81 (dd, 4H, Ar-H), 8.65 (d, 1H, NH), 10.21 (s, 1H, NH) and 10.96 (s, 1H, NH₂). Anal. Calcd for C₂₃H₂₇N₅O₆: C, 58.84; H, 5.80; N, 14.92. Found: C, 58.49; H, 6.02; N, 15.05.

5.2. Diethyl N-{4-[(2,4-diaminofuro[2,3-*d*]pyrimidin-5-yl)methyl]benzoyl}-L-glutamate (28)

This compound was synthesized in 60% yield using the general procedure starting from **26**; mp: 140–142 °C. *R*_f = 0.59 (CHCl₃/MeOH = 5:1); ¹H NMR (DMSO-*d*₆): δ 1.13–1.21 (m, 6H, 2 × OCH₂CH₃), 2.00–2.50 (m, 4H, 2 × CH₂), 3.69 (s, 2H, CH₂), 4.02–4.10 (m, 4H, 2 × OCH₂CH₃), 4.34–4.45 (m, 1H, CH), 6.04 (s, 2H, NH₂), 6.21 (s, 2H, NH₂), 7.05 (s, 1H, CH), 7.35–7.84 (dd, 4H, Ar-H) and 8.69 (d, 1H, NH). Anal. Calcd for C₂₃H₂₇N₅O₆·0.3H₂O: C, 58.18; H, 5.75; N, 14.75. Found: C, 58.25; H, 5.93; N, 15.02.

6. General procedure for the synthesis of 13 and 14

To a solution of **27** or **28** (120 mg, 0.25 mmol) in methanol (5 mL) was added 1 N NaOH (2 mL) at 0 °C and mixture stirred at room temperature for 3–4 h. TLC (CHCl₃/MeOH = 5:1) showed the disappearance of the starting material (*R*_f 0.48 or 0.59) and the formation of one major spot at the origin. The methanol was evaporated under reduced pressure, the residue was dissolved in water (5 mL), and the solution was cooled to 0 °C and acidified carefully to pH 4 with dropwise addition of 3 N HCl. The suspension was left at 0 °C for 24 h and filtered. The residue was washed well with cold water. The solid was recrystallized from methanol, filtered, washed with ether, and dried well in vacuum.

6.1. N-{4-[(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-*d*]pyrimidin-6-yl)methyl]benzoyl}-L-glutamic acid (13)

This compound was synthesized in 56% yield using the general procedure starting from **27**; mp: 192–194.5 °C; ¹H NMR (DMSO-*d*₆): δ 1.94–2.49 (m, 4H, 2 × CH₂), 3.88 (s, 2H, CH₂), 4.37 (s, 1H, CH), 5.87 (s, 1H, CH), 6.00 (s, 2H, NH₂), 7.32–7.81 (dd, 4H, Ar-H), 8.55 (d, 1H, NH), 10.16 (s, 1H, NH), 10.96 (s, 1H, NH) and 12.41 (s, br, 2H, 2 × COOH). Anal. Calcd for C₁₉H₁₉N₅O₆·1.8H₂O: C, 51.19; H, 5.11; N, 15.71. Found: C, 51.02; H, 4.82; N, 15.75.

6.2. N-{4-[(2,4-Diaminofuro[2,3-*d*]pyrimidin-5-yl)methyl]benzoyl}-L-glutamic acid (14)

This compound was synthesized from **28** in 60% yield; mp: 167.5–170.5 °C; ¹H NMR (DMSO-*d*₆): δ 1.89–2.36 (m, 4H, 2 × CH₂), 4.07 (s, 2H, CH₂), 4.38 (m, 1H, CH), 6.06 (s, 2H, NH₂), 6.29 (s, 2H, NH₂), 7.04 (s, 1H, CH), 7.35–7.83 (dd, 4H, Ar-H), 8.56 (1H, NH), 12.35 (s, br, 2H, 2 × COOH). Anal. Calcd for

C₁₉H₁₉N₅O₆·H₂O·0.2CH₃OH: C, 52.67; H, 5.02; N, 16.00. Found: C, 52.84; H, 4.77; N, 15.64.

6.2.1. Drug preparation. Drug solutions were standardized using extinction coefficients. Extinction coefficients were determined for **13** (pH 1, λ_{\max} 227 nm (22,900); pH 7, λ_{\max} 255 nm (19,200); pH 13, λ_{\max} 248 nm (19,300)) and for **14** (pH 1, $\lambda_{\max-1}$ 248 nm (20,300), $\lambda_{\max-2}$ 301 nm (7,900); pH 7, λ_{\max} 247 nm (20,400); pH 13, λ_{\max} 248 nm (20,200)) and the extinction coefficients for methotrexate (MTX), a gift of Immunex (Seattle, WA), were from the literature.³⁰ Aminopterin was purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals and reagents were of reagent grade or higher.

6.2.2. Cell lines and methods for measuring growth inhibitory potency. Cell lines were verified to be negative for Mycoplasma contamination (Mycoplasma Plus PCR primers, Stratagene, La Jolla, CA). The human T-lymphoblastic leukemia cell line CCRF-CEM³¹ and its MTX-resistant sublines R1,²⁷ R2,²⁸ and R30dm²⁹ were cultured as described.²⁹ R1 expresses 20-fold elevated levels of dihydrofolate reductase (DHFR), the target enzyme of MTX. R2 has dramatically reduced MTX uptake, but normal levels of MTX-sensitive DHFR. R30dm expresses 1% of the folylpolyglutamate synthetase (FPGS) activity of CCRF-CEM and is resistant to short-term, but not continuous, MTX exposure; however, R30dm is cross-resistant in continuous exposure to antifolates that require polyglutamylation to form potent inhibitors. Growth inhibition of all cell lines by continuous drug exposure was assayed as described.^{29,32} EC₅₀ values (drug concentration effective at inhibiting cell growth by 50%) were determined visually from plots of percent growth relative to a solvent-treated control culture versus the logarithm of drug concentration.

6.2.3. Folylpolyglutamate synthetase (FPGS) purification and assay. Recombinant human cytosolic FPGS was purified and assayed as described previously.³³ Both **13** (80% recovery) and **14** (89% recovery) were themselves recovered during the standard assay procedure, thus suggesting that their polyglutamate products would be quantitatively recovered. Kinetic constants were determined by the hyperbolic curve fitting subroutine of SigmaPlot (Jandel) or Kaleidagraph (Synergy Software) using a ≥ 10 -fold range of substrate concentration. Activity was linear with respect to time at the highest and lowest substrate concentrations tested. Assays contained ≈ 400 U of FPGS activity; one unit of FPGS catalyzes incorporation of 1 pmol of [³H]glutamate/h. Because V_{\max}/K_m values for **13** and **14** were low, the assays to determine kinetic constants were modified to include 2 mM L-[³H]glutamate, instead of the standard 4 mM. The resulting lower background allowed quantitation at lower levels of product synthesis. The K_m value for AMT was the same whether 2 or 4 mM glutamate was used (data not shown).

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