Biochemical Pharmacology, Vol. 37, No. 20, pp. 3931–3939, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

STRUCTURAL SPECIFICITY OF INHIBITION OF HUMAN FOLYLPOLYGLUTAMATE SYNTHETASE BY ORNITHINE-CONTAINING FOLATE ANALOGS

JOHN J. MCGUIRE,*† WANDA E. BOLANOWSKA* and JAMES R. PIPER‡

* Grace Cancer Drug Center, Roswell Park Memorial Institute, Buffalo, NY 14263; and ‡ Drug Synthesis Branch, Southern Research Institute, Birmingham, AL 35255, U.S.A.

(*Received* 12 *February* 1988; *accepted* 4 *April* 1988)

Abstract—A series of folate analogs containing ornithine instead of glutamate was synthesized and tested for inhibition of folylpolyglutamate synthetase (FPGS) and other folate-dependent enzymes of human leukemia cell lines. Reduced derivatives of 2-amino-4-oxo-10-methyl-pteroyl-ornithine had dramatically increased inhibitory potency against FPGS compared to the oxidized parent. The aminopterin analog (2,4-diamino-pteroylornithine) was a potent inhibitor of both dihydrofolate reductase and FPGS. It was a much more potent linear competitive inhibitor of human FPGS than the corresponding methotrexate derivative previously described (K_i = 0.15–0.26 and 3 μM respectively). A quinazoline folate analog, 2-amino-4-oxo-5,8-dideazapteroyl-ornithine, was a relatively poor inhibitor of isolated dihydrofolate reductase and thymidylate synthase; however, it is the most potent human FPGS inhibitor identified to date (K_i = 100–150 nM). Because of the lack of appreciable interaction with other folate-dependent enzymes, structures incorporating the 2-amino-4-oxo-5,8-dideazapteroate nucleus may thus lead to selective inhibition of FPGS. Substitution of ornithine for glutamate caused a profound decrease in cytotoxic potency for these analogs; this was apparently the result of poor transport. Together with earlier studies, these data indicate that the potency of FPGS inhibition by an analog containing ornithine closely parallels the relative substrate activity of its glutamate-containing counterpart. The substitution of ornithine apparently does not perturb the pterin specificity of FPGS. The close parallel between substrate and inhibitor specificity may thus allow the use of currently available structure–activity studies on FPGS to design more potent and more selective inhibitors of FPGS.

Folylpolyglutamate synthetase (FPGS)§ catalyzes the formation of poly-gamma-glutamyl metabolites of natural folates and of the classical antifolates [reviewed in Ref. 1]. FPGS catalyzes the general reaction:

$$\text{PteGlu}_n + \text{ATP} + \text{L-Glu} \rightarrow \text{PteGlu}_{n+1} + \text{ADP} + \text{P}_i$$

Available evidence indicates that the folylpolyglutamates are the preferred intracellular co-factors for folate-dependent reactions and aid in retention of this vitamin. Folylpolyglutamates are apparently essential for continued growth and survival, since all organisms synthesize these derivatives of intracellular folates [2], and since mutational deletion of FPGS is a lethal event [3]. Inhibitors of FPGS could thus be useful agents for studying the exact physiological roles of folylpolyglutamates and could potentially be useful as cytotoxic agents.

The strict structural requirements for binding to FPGS by folate-like substrates have severely limited the possible structures for inhibitors [1]. In general, structural changes to mono-amino acid structures either alter substrate activity and inhibitory potency in parallel (as for pterin and pABA modifications) or abolish both inhibitory and substrate activity (as for glutamate modifications). Shane and co-workers [4] were the first to report that reduced folates containing ornithine (2,5-diaminopentanoic acid) in place of glutamate are potent (K_i = 0.2 μM) inhibitors of the FPGS from both mammalian and bacterial sources. Subsequent observations from this [5, 6] and other laboratories [7–9] confirmed this general observation and extended it by showing both that replacement of glutamate by ornithine in folate analogs such as methotrexate ([5, 6, 8]; Fig. 1C) or aminopterin ([8]; Fig. 1B) also yields inhibitors of FPGS and that ornithine is the optimal member of a homologous series of 2,ω-diaminoalkanoic acids to use as the replacement.

We have now synthesized and characterized several new series of ornithine-containing folates and folate analogs in order to develop more potent and/or more specific inhibitors of FPGS. These analogs were tested for cytotoxic effects against cultured human leukemia cell lines and as inhibitors of dihydrofolate reductase, thymidylate synthase, and FPGS isolated from these cell lines.

† Correspondence: John J. McGuire, Ph.D., Grace Cancer Drug Center, Roswell Park Memorial Institute, 666 Elm St., Buffalo, NY 14263.

§ Abbreviations: FPGS, folylpolyglutamate synthetase; DHFR, dihydrofolate reductase (EC 1.5.1.3); TS, thymidylate synthase (EC 2.1.1.45); pABA, *p*-aminobenzoic acid; MTX, methotrexate; BOC, butyloxy carbonyl; Pte, pteroic acid (4-[((2-amino-4-(3H)-oxopteridin-6-yl)-methyl)amino]-benzoic acid). Analogs may be derived from this moiety by the N-acylation of amino acids, e.g. PteOrn or PteGlu. A subscript integer following Glu indicates that additional glutamates are attached in gamma-linkage. An H_2, prefix indicates the 7,8-dihydro form of any pteroate; an H_4 prefix indicates the 5,6,7,8-tetrahydro form of any pteroate.

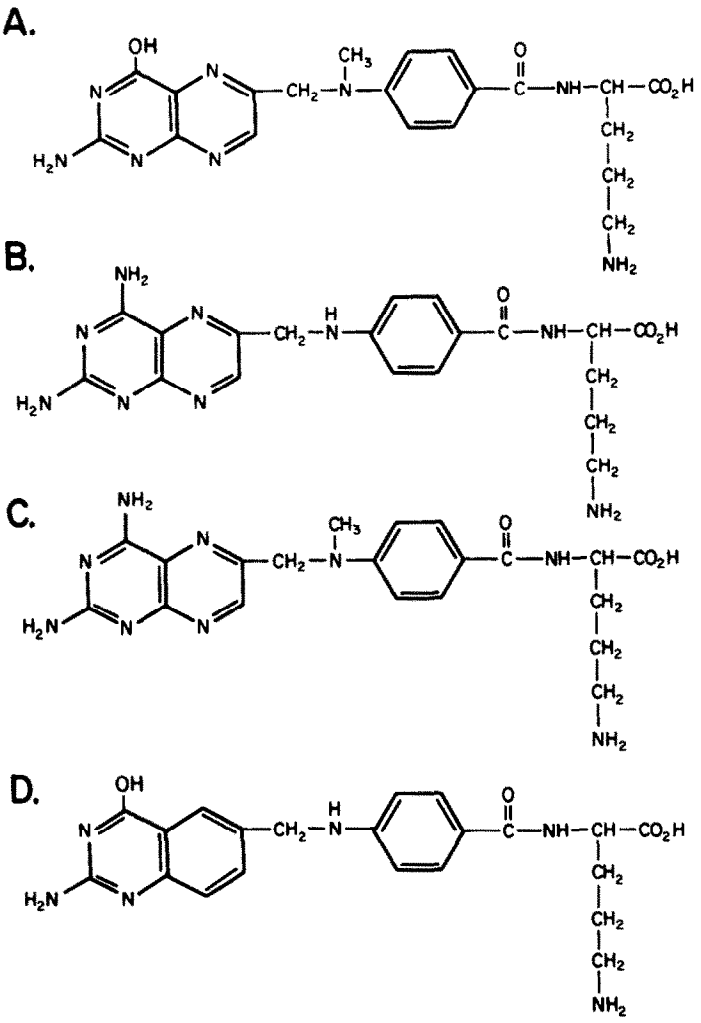

Fig. 1. Structures of ornithine-containing folate analogs. (A) 10-Methyl-pteroyl-ornithine; (B) 2,4-diamino-pteroyl-ornithine, the ornithine analog of aminopterin; (C) 2,4-diamino-10-methyl-pteroyl-ornithine, the previously described [5] ornithine analog of methotrexate; and (D) 5,8-dideazapteroyl-ornithine; the lysine-containing structure contains one additional methylene group in the 2,ω-diamino-alkanoate residue.

MATERIALS AND METHODS

MTX was obtained from the Division of Cancer Treatment, National Cancer Institute. Aminopterin was a product of the Sigma Chemical Co. (St. Louis, MO). The analog of MTX containing ornithine was synthesized as previously described [10]. The analog of aminopterin containing ornithine was synthesized by a similar procedure except that the appropriately blocked para-aminobenzoylornithine, rather than its N-methylated derivative, was coupled to 2,4-diamino-6-bromomethyl-pterin. N^2-(10-Methyl-pteroyl)ornithine was prepared in two steps from the reported N^5-BOC-protected precursor to the ornithine-containing MTX analog: (a) standard hydrolytic deamination (refluxing 1 N NaOH) to replace the 4-amino group, and then (b) removal of the BOC group by trifluoroacetic acid. N^2-(5,8-Dideazapteroyl)ornithine and the corresponding lysine compound were prepared via alkylation of the N^2-(4-aminobenzoyl) derivatives of N^5-BOC-ornithine and N^6-BOC-lysine by 2-amino-6-bromomethyl-4-hydroxyquinazoline hydrobromide [11, 12]. The products were purified by preparative thin-layer chromatography and then deprotected by treatment with trifluoroacetic acid. Final products, isolated as bis(trifluoroacetate) salts, gave satisfactory elemental analysis results (for C, H and N), and the mass spectra (field-desorption mode) gave the expected molecular ions corresponding to the free bases. All other chemicals were of the highest grade available.

Synthesis of reduced folate analogs. Synthesis of 10-methyl-dihydropteroyl-ornithine was performed essentially according to Hayman *et al.* [13]. Approximately 3 mg of 10-methyl-pteroyl-ornithine was dissolved in 1% Na_2CO_3 (0.5 ml). Following addition of 0.5 ml sodium-ascorbate, pH 6.0, and 40 mg of $Na_2O_4S_2$, the reduction mixture was incubated at ambient temperature for 5 min in the dark. The

mixture was placed on ice and titrated to pH 1.2–1.9 with HCl. Unlike dihydrofolate, this dihydro derivative did not precipitate readily under these conditions, and 70–120 min were required for complete precipitation. The precipitate was collected by centrifugation and washed three times with iced 10 mM HCl. The precipitate was dissolved in 1 ml of 250 (or 50) mM Tris–HCl, pH 7.5, containing 5 mM dithiothreitol. Any trace insoluble material was removed by centrifugation, and the UV-spectrum of the clear supernatant fraction was determined. The literature values for the spectrum of 10-methyl-dihydrofolate ([14]; $\lambda_{max} = 284$ nm ($E_M = 30.0 \times 10^3$) and 305 nm ($E_M = 30.3 \times 10^3$), ratio 284/305 = 0.99) were used to determine the concentration of the final product (observed $\lambda_{max} = 282$ and 306 nm; ratio 282/306 = 1.16). For storage, solutions were made 200 mM with respect to 2-mercaptoethanol, sparged with N_2, and placed at −70°.

10-Methyl-tetrahydropteroyl-ornithine was synthesized enzymatically from the dihydro derivative using excess *Lactobacillus casei* DHFR (New England Enzyme Center). The reaction mixture (1 ml) contained (final concentration) 50 mM Tris–HCl, pH 7.0, 100 μM NADPH, and 100 μM dihydro compound. The mixture was incubated at 37° for 4 min during which time the absorbance at 340 nm was measured. Following the initiation of the reaction by the addition of 0.2 to 0.32 I.U. of *L. casei* DHFR in 30 μl, the decrease in absorption at 340 nm was followed until no further decrease occurred (4–10 min). An incubation mixture containing all components except dihydro derivative served as the control. No decrease in absorbance was observed in this sample. The final concentration of tetrahydro derivative was calculated based on the change in absorbance at 340 nm and the millimolar extinction coefficient of 12.0 [15]. The average conversion under these conditions was 81 ± 2% (N = 7) and was identical to the yield when dihydrofolate was used as the substrate. Attempts to purify the tetrahydro derivative on either DEAE-cellulose or CM-cellulose were unsuccessful as neither matrix adsorbed the product. As a consequence, inhibitory potency of the tetrahydro derivative against FPGS was assessed using the reduction mixture. The reduction mixture lacking dihydro derivative (above) was added to FPGS reactions in the same proportion as a control for the effects of the reduction mixture itself; this mixture had no effect on FPGS activity.

Enzymes. DHFR was obtained from a crude extract of the CCRF-CEM cell line. Cells were lysed by three cycles of freeze–thawing (dry ice–methanol) in 0.1 M Tris–HCl, pH 8.85, and centrifuged at 27,000 *g* for 30 min to obtain the crude supernatant fraction. Under standard assay conditions, this extract contained negligible NADPH oxidase activity. These extracts were also the source of TS activity. Human leukemia cell folylpolyglutamate synthetases from cell lines [5] were partially purified as previously described. These preparations contained no detectable gamma-glutamyl hydrolase (conjugase) activity under standard FPGS assay conditions. Rabbit liver aldehyde oxidase was purified 46-fold according to our modification [16] of the procedure of Johns *et al.* [17].

Enzyme assays. DHFR activity was assayed spectrophotometrically (37°) essentially according to Osborn and Huennekens [18]. Each assay (1 ml) contained 100 μmol Tris–HCl, pH 7.0, 150 μmol KCl, 20 nmol dihydrofolate, 50 nmol NADPH, and DHFR. The IC_{50} values (concentration required for 50% inhibition) for DHFR inhibitors were obtained from graphs of activity versus the logarithm of drug concentration. For measurement of inhibition, standard activity of 0.0017 I.U. of CCRF-CEM DHFR was used. Thymidylate synthase was assayed by the displacement of tritium from [5-^{3}H]dUMP (Moravek Biochemicals, Inc., Brea, CA) into $[^3H]H_2O$ [19, 20]. One unit of TS activity synthesizes 1 nmol thymidylate/min. FPGS was assayed using L-[^{3}H]glutamate (New England Nuclear, Boston, MA) as a substrate [21, 22], and DEAE-cellulose mini-columns were used to separate free [^{3}H]glutamate from that ligated to a folate substrate. Standard assay mixtures (0.25 ml, pH 8.4, at 37°) that contained Tris–HCl (0.1 M), ATP (5 mM), $MgCl_2$ (10 mM), KCl (20 mM), 2-mercaptoethanol (100 mM), 4 mM [^{3}H]glutamate (2×10^6 cpm/μmol), either aminopterin (50 μM) or an appropriate concentration of a folate analog, and enzyme were incubated at 37°. Assays contained 600 units of CCRF-CEM FPGS or 400 units of K562 FPGS. One unit of activity is defined as the incorporation of 1 pmol of [^{3}H]glutamate/hr under standard conditions. Apparent K_m values for natural folates were determined in experiments where activity was measured as a function of substrate concentration. It should be stressed that, at low initial concentrations, some substrates (e.g. tetrahydrofolate) allow multiple additions of glutamate during reasonable incubation periods, and true Michaelis–Menten conditions are not maintained. Thus, apparent K_m values for any FPGS substrate should be viewed cautiously and with consideration of whether multiple products are formed. Aminopterin was used as the variable folate substrate in kinetic analyses with inhibitors in order to obtain high activity and maintain Michaelis–Menten conditions [5]. During the course of this work, two lots of aminopterin were used; both were about the same degree of purity by HPLC. The older lot gave K_m values with CCRF-CEM FPGS of 14 ± 2.4 μM (N = 6), while the newer lot gave 7.5 ± 1.8 (N = 8). Only the newer lot was used with K562 enzyme. K_i values for inhibitors were the same regardless of which aminopterin was used. All kinetic data were obtained over a 10-fold concentration range of substrate in the presence or absence of inhibitor. Activity was shown to be linear with respect to time at the highest and lowest substrate concentrations both in the presence and absence of inhibitor. Slopes of the lines and $1/V$ intercepts generated in a double-reciprocal plot of these data were plotted versus inhibitor concentration to obtain K_{is} and K_{ii} respectively [23].

Rabbit liver aldehyde oxidase was routinely assayed at room temperature by measuring the change in absorbance at 341 nm as MTX was converted to its 7-hydroxylated form [17]. Standard reaction mixtures contained 70 mM potassium phosphate, pH 7.8, 0.007% Na_2EDTA, 250 mM $(NH_4)_2SO_4$, 100 μM MTX, and aldehyde oxidase.

The amount of enzyme used caused a change in absorbance/min at 341 nm of 0.030 to 0.038 under standard conditions. In cases where the absorption spectrum of a putative reaction product was not known, the reaction was followed by examining the complete absorption spectrum (230–370 nm) as a function of time. To increase the sensitivity of this assay, an amount of aldehyde oxidase which would cause a change in absorbance at 341 nm of 0.16/min (under standard conditions) was used. Under these conditions, 40 μM MTX was quantitatively converted to 7-OH-MTX in $\leq$5 min. Spectra of 40 μM 5,8-dideazapteroylornithine were taken at intervals up to 30 min at which time the mixture and blank were adjusted to pH 1.0 with HCl and the acid spectrum was determined. Control experiments with MTX demonstrated the validity of this entire procedure.

[^{3}H]*MTX transport and metabolism*. Transport studies were performed by a micro method utilizing repeated iced saline washes to remove extracellular drug [24]. [^{3}H]MTX metabolites were measured by HPLC following incubation of cells with [^{3}H]MTX [25].

Cell culture and cytotoxicity. Cells were cultured using standard sterile technique. The nonlymphocytic leukemia cell line K562 [26] was grown in RPMI 1640 (GIBCO) containing 10% fetal bovine serum (GIBCO). The lymphoblastic cell line CCRF-CEM [27] was cultured in RPMI 1640 containing 10% horse serum (GIBCO). To measure cytotoxicity, logarithmically growing cells were seeded at 1×10^4 cells/ml in the absence or presence of at least five concentrations of drug. Following 120 hr (CCRF-CEM) or 96 hr (K562) of continuous exposure to the drug, a cell count was performed using a model ZB 1 Coulter Counter. Control samples (containing no drug) grew logarithmically during this entire period. The EC_{50} values (drug concentration inhibiting cell growth by 50%) were determined from plots of percent of control cell growth versus the logarithm of drug concentration.

Miscellaneous. All substrate concentrations were determined from absorption spectra and published extinction coefficients [28, 29], assuming that the substitution of ornithine or lysine for glutamate did not affect the molar extinction.

RESULTS

Inhibition of human folate-requiring enzymes by 2,4-diamino folate analogs containing ornithine. The aminopterin analog containing ornithine (Fig. 1B) was a potent inhibitor of human leukemia cell DHFR, although not as potent as aminopterin itself (Table 1). In addition, the inhibition curve for 2,4-diamino-pteroyl-ornithine was shallower than that of aminopterin (data not shown), also indicating that the analog may not be tight-binding. The difference in inhibitory potency was about the same as was previously observed for MTX and its ornithine-containing analog ([15] and Table 1). Thus, substitution of ornithine for glutamate has a slight effect on binding of analogs to DHFR.

Inhibition of thymidylate synthase by this general class of analogs was also assessed (Table 2). Substitution of ornithine for glutamate did not change the character of the inhibition, which remained linear noncompetitive. This substitution, however, did increase the inhibition constants for both the MTX and aminopterin analogs to such an extent that inhibition at this site in cells would not be expected to be significant.

Table 1. Inhibition of dihydrofolate reductase from human leukemia cells (CCRF-CEM) by analogs containing ornithine instead of glutamate

Inhibitor	IC_{50} (nM)
4-NH_2-10-CH_3-PteGlu [MTX]	1
4-NH_2-10-CH_3-PteOrn*	(2.5)
4-NH_2-PteGlu [aminopterin]	0.6
4-NH_2-PteOrn	1.5
5,8-Dideaza-PteGlu†	(50)
5,8-Dideaza-PteOrn‡	150
5,8-Dideaza-PteLys‡	150

Inhibition of human DHFR was determined as described in Materials and Methods.

* Data from Ref. 5 for comparison.

† Data from Ref. 30; obtained with DHFR from another human cell line, HCT-8, which shows identical sensitivity to inhibition by MTX.

‡ Using the DHFR from K562 cells, the IC_{50} values were 350 and 220 nM, respectively, for the ornithine and lysine analogs (one determination), while for MTX it was 0.9 nM.

The ornithine analog of aminopterin was a potent inhibitor of human leukemia cell FPGS (Table 3) and was not a substrate. It was at least 10-fold more potent an inhibitor of FPGS than the previously described ornithine analog of MTX ([5]; Table 3). It was still a linear competitive inhibitor with respect to the aminopterin substrate. This analog also inhibited activity with tetrahydrofolate as the substrate with similar potency (data not shown). The relative inhibitory potency of the ornithine-containing analogs of MTX and aminopterin paralleled the relative substrate activity of MTX and aminopterin for the human FPGS (Table 3).

Interaction of human folate-requiring enzymes with oxidized and reduced derivatives of 10-methyl-pteroyl-ornithine (Fig. 1A). The dihydro-derivative of 10-methyl-pteroyl-ornithine was a substrate for DHFR from *L. casei* (K_m = 13 μM; relative V_{max} = 0.2), although it was used at a much lower efficiency than dihydrofolate (K_m = 2.2 μM; relative V_{max} = 1.0). The 10-methyl-dihydropteroyl-ornithine derivative was a substrate for DHFR from K562 human leukemia cells, although the maximum velocity achieved was only 60% that of dihydrofolate. The K_m values for both substrates were $\ll$ 10 μM, which is below the sensitivity of our spectrophotometric assay.

The inhibitory potency of 10-methyl-pteroyl-ornithine analogs for human FPGS (Table 3) increased with increasing reduction state, with the greatest effect seen between the fully oxidized and the dihydro levels. Further reduction to the tetrahydro level increased potency only marginally. All three compounds were linear competitive inhibitors with respect to aminopterin, the pteroyl substrate used. None of the derivatives was a substrate for human

Table 2. Inhibitors of thymidylate synthase from human leukemia cells (CCRF-CEM)

Inhibitor	K_{is} (μM)	K_{ii} (μM)	Type
4-NH_2-10-CH_3-PteGlu [MTX]	52	67	L,NC
4-NH_2-10-CH_3-PteOrn	170	480	L,NC
4,NH_2-PteGlu [aminopterin]	142	188	L,NC
4-NH_2-PteOrn	>370	>740	L,NC
5,8-Dideaza-PteOrn	2.7	21	L,NC
5,8-Dideaza-PteLys	2.1	20	L,NC

Thymidylate synthase was assayed by measuring the release of [^{3}H]H_2O from [5-^{3}H]dUMP as described in Materials and Methods. The [^{3}H]dUMP concentration was fixed at a saturating level (110 μM) and the (6*R,S*)-methylenetetrahydrofolate was varied from 50 to 500 μM (K_m = 50 ± 15 μM, N = 8). Type: L = linear, NC = noncompetitive. Values are averages of duplicate determinations.

FPGS. Increasing state of reduction had a similar relative effect on FPGS substrate activity of the homologous natural folate derivatives which contain glutamate instead of ornithine (Table 3). Again, the greatest effect occurred between the fully oxidized and the dihydro levels, and there was only a minor effect on increasing reduction to the tetrahydro level.

Inhibition of human folate-requiring enzymes by quinazoline folate analogs (5,8-dideazafolates) containing ornithine or lysine. 5,8-Dideazapteroyl-ornithine and -lysine were weak inhibitors of human leukemia cell DHFR (Table 1), as might be anticipated based on their 2-amino-4-oxo structure (Fig. 1D). In addition, the slope of the inhibition curve for each of these analogs was very shallow, indicating that they are not tight-binding inhibitors of DHFR (data not shown). Since 2-amino-4-oxo-quinazoline antifols are inhibitors of cellular thymidylate synthase [30–32], both new analogs were tested as TS inhibitors (Table 2). Both analogs had about the same moderate potency against this human leukemia cell (CCRF-CEM) TS. The K_i values were in the range of values obtained with closely related quinazolines containing glutamate [30, 32]. Thus, this human TS seems to be relatively insensitive to changes in the amino acid substituent.

Folate analogs containing 2,ω-diaminoalkanoic acid substitutions shorter (C_3 and C_4) than ornithine (2,5-diaminopentanoic acid) were demonstrated previously to be weak inhibitors of human FPGS [5]. The two 5,8-dideazafolic acid analogs containing ornithine or lysine, the C_6 homolog, were assessed as FPGS inhibitors to determine the effect of increased length of the amino acid substitution (Table 3).

Table 3. Inhibition of folylpolyglutamate synthetase from human leukemia cell lines by folate analogs containing ornithine instead of glutamate and the substrate activity of the glutamate-containing homolog

Pteroate structure (R-)	R-Orn inhibitory activity		R-Glu substrate activity*		
	CCRF-CEM	K562			
	K_{is} (μM)		$K_{m,app}$ (μM)	Rel V_{max}	V_{max}/K_m
10-CH_3-Pte-	94 ± 41	78	59.5 ± 2.2	0.73	0.012
10-CH_3-H_2Pte-	3.2	1.9	1.73 ± 0.10	1.15	0.66
10-CH_3-H_4-Pte-	1.9 ± 0.6	1.5	1.65 ± 0.17	1.0	0.61
4-NH_2-10-CH_3-Pte-	3.2† (13, 17.1)‡	4.1†	74 ± 44	0.97 ± 0.04	0.013
4-NH_2-Pte-	0.26	0.15	7.5 ± 1.8	1.46	0.195
5,8-Dideaza-Pte-	0.14 (0.27, 0.54)‡	0.087			
	64§	48§			

Inhibition constants were determined using aminopterin as the variable substrate (see Materials and Methods). Values listed with standard deviation were determined using at least three values. Other values are averages of closely agreeing duplicate determinations.

* Values are for CCRF-CEM FPGS. K_m values are for PteGlu, H_2PteGlu, and (6*R,S*)-H_4PteGlu since their 10-methyl substituted derivatives were not available. V_{max} is relative to (6*R,S*)-H_4PteGlu measured in the same experiment. For those substrates where K562 FPGS was also tested, the apparent K_m values were similar (e.g. aminopterin K_m = 5.9 ± 1.2 μM, N = 12; MTX K_m = 49 μM).

† Data from Ref. 5.

‡ K_{is} and K_{ii}, respectively, with glutamate as the variable substrate; K_m = 0.67 ± 0.14 (N = 3). Inhibition was noncompetitive with respect to glutamate.

§ Values for 5,8-dideazapteroyl-lysine.

Neither analog was a substrate for human FPGS. The ornithine-containing analog was a very potent inhibitor of human FPGS, the most potent identified to date. The lysine analog, however, was about 500-fold less potent. The ornithine analog was a linear competitive inhibitor with respect to the pterin substrate, aminopterin. Inhibition by the lysine analog was linear and best described as competitive, although there may have been a small effect on V_{max}. Since the K_i was so high and the solubility of this compound was exceeded, further characterization could not be carried out. Preincubation of 5,8-dideazapteroyl-ornithine (or 2,4-diamino-pteroyl-ornithine) with FPGS for 1 hr at 37° in the presence of all reactants, except aminopterin, did not lead to enhanced inhibition, indicating that this inhibitor is not tight-binding. FPGS activity with tetrahydrofolate as the substrate was inhibited with similar potency (IC_{50}) by these analogs (data not shown); however, K_i values could not be determined because of complex kinetics with this substrate [21]. The 5,8-dideazapteroyl-ornithine analog was a non-competitive inhibitor of the CCRF-CEM FPGS with respect to the variable substrate glutamate (Table 3), as was the ornithine-containing MTX analog. Rat liver FPGS (data not shown) was inhibited by each analog to an extent similar to that shown in Table 3 for the human enzymes.

Cytotoxic effects of ornithine-containing analogs in human leukemia cell lines. Both the CCRF-CEM and K562 cell lines were much less sensitive to the ornithine-containing analog of aminopterin than to aminopterin itself (Table 4). The 10-CH_3-pteroyl-ornithine analog was non-toxic even at 200 μM. The 5,8-dideazapteroate analogs were essentially non-toxic to either cell line at 50 μM, the highest concentration tested.

Transport of 5,8-dideazapteroyl-ornithine. Since radiolabeled material was not available, transport of 5,8-dideazapteroyl-ornithine was examined indirectly by looking at its effect on [^{3}H]MTX transport in CCRF-CEM cells. Inclusion of 40 μM 5,8-dideazapteroyl-ornithine in the transport medium affected neither the initial velocity (0–5 min, linear uptake) of 2 μM MTX nor the plateau level of radiolabel reached in 30 min. Under otherwise identical conditions, 20 μM aminopterin, which shares the MTX transport system, decreased the initial rate of MTX uptake by 84% and the plateau level by 87%.

Effects of 5,8-dideazapteroyl-ornithine on [^{3}H]MTX metabolism by CCRF-CEM cells. Logarithmically growing cells were exposed to 10 μM [^{3}H]MTX for 4 hr in the presence or absence of 50 μM 5,8-dideazapteroyl-ornithine. The total MTX accumulated over 4 hr was similar under both conditions (76.8 and 75.9 pmol/10^7 cells in the presence and absence of ornithine analog respectively). The total level of MTX polyglutamates was slightly greater in the absence than in the presence of the analog (58.6 and 48.3 pmol/10^7 cells respectively); however, the product distributions (proportion of lengths between di- and pentaglutamate) were similar. A separate experiment examined metabolism of MTX in the absence of potential competition for uptake by extracellular drug. Cells were exposed to 10 μM [^{3}H]MTX for 4 hr to allow accumulation of MTX polyglutamates, washed free of extracellular radiolabel, and placed in medium containing or lacking 50 μM 5,8-dideazapteroyl-ornithine. The efflux of unmetabolized MTX was unaffected at 1 and 4 hr of incubation. The elongation of [^{3}H]MTX polyglutamates was inhibited only modestly at 4 hr even under these forcing conditions (tetra- and pentaglutamates increased from 2.23 to 5.77 pmol/10^7 cells in untreated cells, but only from 2.23 to 3.94 pmol/10^7 cells in treated cells).

Substrate activity of ornithine-containing folate analogs for rabbit liver aldehyde oxidase. Under conditions where complete conversion of MTX to 7-hydroxy-MTX occurred in less than 5 min, there was no change in the UV absorption spectrum of 5,8-dideazapteroyl-ornithine over 30 min at either pH 7.8 or 1.0. Assuming that the 7-hydroxylation of 2-amino-4-oxo-quinazoline structures would cause a pronounced spectral change, as it does on MTX-like structures, the 5,8-dideazapteroyl-ornithine is apparently not a significant substrate for aldehyde oxidase.

Table 4. Cytotoxic effects of aminopterin, 5,8-dideazafolate, and their 2,ω-diaminoalkanoic acid analogs on human leukemia cell lines

	EC_{50} (μM)	
	Cell line	
	CCRF-CEM	K562
4-NH_2-Pte-		
-Glu (aminopterin)	0.004	0.0017
-ornithine	0.62	0.4
10-CH_3-Pte-ornithine	>200	>200
5,8-dideaza-Pte-		
-Glu	1.45*	ND†
-Orn	⩾50	⩾50
-Lys	⩾50	⩾50

* Data were from CCRF-CEM cells, but for a related quinazoline [33]. This quinazoline, 5,8-dideaza-isoPteGlu, has a potency similar to that of 5,8-dideaza-PteGlu against many human cell lines [30, 34].

† Not determined.

DISCUSSION

Workers in a number of laboratories have attempted to design and characterize inhibitors of FPGS. Early substitutions of other acidic amino acids for glutamate in classical folate analogs had only limited success. For example, the substitution of 4-fluoroglutamate led to a structure which is quite useful as a probe of polyglutamate function but which is only a weak inhibitor, suggesting low affinity for FPGS [35]. Homocysteic acid substitution in a tetrahydrofolate analog [4, 7] results in an inhibitor whose K_i (43 μM) is 6-fold higher than the K_m of the natural substrate. Substitution of homocysteic acid or cysteic acid [36] in MTX or aminopterin yields analogs which again are not substrates, but whose affinities as inhibitors are poorer (aminopterin) or only the same (MTX; not much poorer as we erroneously stated in a previous publication [5]) as the affinity of the corresponding substrate. The absolute values for the

inhibitor constants, however, are ≥43 μM. The discovery by Shane and co-workers that the substitution of the basic amino acid ornithine for glutamate led to a potent FPGS inhibitor, viz. tetrahydropteroyl-ornithine, was the first lead to compounds with high potency [4]. Work from our laboratory ([5]; Table 3) and others [8, 9] has demonstrated that, among the substitutions of 2,ω-diaminoalkanoic acids for glutamate, the FPGS inhibition is very specific for ornithine (2,5-diaminopentanoic acid). This specificity carries across species lines in mammals (mice and rats to humans).

The data derived from characterization of the interaction of new series of structurally divergent analogs (Fig. 1) with human FPGS suggest a useful relation concerning substrate specificity and inhibitor potency of ornithine-containing antifols (Table 3). In each series, the potency of inhibition by the ornithine-containing analog paralleled the apparent K_m of the glutamate-containing substrate. Thus, the structural specificity of FPGS for the pteridine moiety was not altered by the substitution of ornithine. Also, in many cases the K_i value was lower (by as much as 10-fold) than the K_m of the corresponding substrate. This relation holds true whether FPGS from human lymphoid or non-lymphoid cells was used. For example, as the state of reduction of the pyrazine ring of 10-methyl-pteroyl-ornithine was increased, there was a large decrease in the K_i. At the point where the greatest change in K_m for substrates occurred (on formation of the dihydro derivative), there was also the greatest change in the inhibitory potency. Inhibition of rat liver FPGS by 10-methyl-pteroyl-ornithine shows a similar dependence of linear competitive inhibition on reduction state [6]. At least in the case of ornithine-containing inhibitors, this relation between K_m (or perhaps V_{max}/K_m) and K_i allows the design of more potent inhibitors based on the substrate activity of the glutamate-containing species. Since there is now a substantial body of such substrate data in the literature [7, 16, 21, 30, 37], and since a very large number of classical (i.e. glutamate-containing) folate analogs have already been synthesized, new inhibitors may be rationally designed either based on the literature or by determining the relative substrate activity of an existing classical compound. The data from other studies of such analogs are consistent with this relation [4, 7–9].

Our observations (Table 3; [5, 16]) on substrate activity with FPGS demonstrate that the human FPGS displays broad specificity for pterins, similar to other mammalian FPGS [1]. The data accumulated also confirm the findings of Rosowsky *et al.* [36] with mouse liver enzyme that 10-methyl substitution may be deleterious to FPGS inhibitory potency, as well as substrate activity, and extend them to the human leukemia enzyme. A comparison of the aminopterin and methotrexate analogs (Table 3) shows that the 10-methyl group decreased the potency by more than 10-fold. The aminopterin analog was quite a potent inhibitor of human FPGS, as was shown previously with the mouse liver enzyme [8]. Assuming that the human and hog liver FPGS behave similarly, the 10-methyl effect can also be seen in the decreased potency of 10-methyl-tetrahydropteroyl-ornithine (1.5 to 1.9 μM; Table 3) as compared to tetrahydropteroyl-ornithine (K_i = 0.2 μM; [4]).

Earlier studies [30–32] demonstrated that folate analogs containing the 5,8-dideazafolate (2-amino-4-hydroxy-quinazoline) structure have thymidylate synthase as their sole locus of action. The monoglutamates are weak inhibitors of TS; however, these quinazolines, which are excellent substrates for FPGS [30, 38], are converted intracellularly to polyglutamate forms which are potent TS inhibitors. The high affinity of the quinazolines for FPGS and the essential requirement for polyglutamate synthesis to achieve TS inhibition suggested that 5,8-dideazapteroyl-ornithine, and related structures, might still bind tightly to and inhibit FPGS, but would not inhibit TS since no polyglutamates could be formed. Thus, these inhibitors might be specific for FPGS. The results demonstrated that the named compound is only a weak inhibitor of DHFR (weaker than a related compound, H-388, which is known not to inhibit intracellular DHFR to any significant extent [30]) and TS. This compound is, however, the most potent human FPGS inhibitor yet described. It is also the first FPGS-specific inhibitor which is not a potential substrate for other folate-dependent enzymes, as are Pte-Orn and its derivatives [4]. Further studies of this interesting class of folate analogs may lead to more selective and more potent inhibitors of FPGS.

All of the ornithine-containing analogs were only weakly inhibitory to human leukemia cell growth (Table 4). The most potent analogs were of the 2,4-diamino variety, but these inhibited cell growth at concentrations nearly 100-fold higher than the corresponding glutamate-containing species. The 5,8-dideazafolates are not very potent cytotoxic compounds [30], but their ornithine analog is even poorer. This low cytotoxic activity was described previously for the MTX [5] and aminopterin [8] analogs. This has been attributed to poor transport because of the basic amino acid residue [5, 8]. This explanation is further substantiated by our data on inhibition by 5,8-dideazapteroyl-ornithine of the transport and glutamylation of [^{3}H]MTX by CCRF-CEM cells. The lack of effect of a 20-fold excess of 5,8-dideazapteroyl-ornithine on [^{3}H]MTX influx suggests that 5,8-dideazapteroyl-ornithine either has a very low affinity for the MTX influx system or that it uses an alternate pathway. However, the lack of appreciable inhibition of [^{3}H]MTX polyglutamate formation by this potent FPGS inhibitor, particularly under conditions where it is the sole extracellular agent, argues for low affinity. Thus, although more potent and more specific inhibitors of FPGS may be developed based on the relation described above, the chemotherapeutic utility of such inhibitors cannot be evaluated until cellular uptake can be increased by further structural modification or alternate strategies.

Acknowledgements—This research was supported by Grants CA 43500 and CA 24538 (J. J. M.) and CA 25236 (J. R. P.) from the National Cancer Insitute, DHHS. John J. McGuire is a Scholar of the Leukemia Society of America. The authors gratefully acknowledge William

Haile for performing all cytotoxicity testing and Mae Brown for secretarial assistance.

REFERENCES

1. McGuire JJ and Coward JK, Pteroylpolyglutamates. Biosynthesis, degradation and function. In: *Folates and Pterins* (Eds. Blakley RL and Benkovic SJ), Vol. 1, pp. 135–190. John Wiley, New York, 1984.
2. Cossins E, Folates in biological materials. In: *Folates and Pterins* (Eds. Blakley RL and Benkovic SJ), Vol. 1, pp. 1–59. John Wiley, New York, 1984.
3. McBurney MW and Whitmore GF, Isolation and biochemical characterization of folate deficient mutants of Chinese hamster cells. *Cell* **2**: 173–182, 1974.
4. Cichowicz D, Cook J, George S and Shane B, Hog liver folylpolyglutamate synthetase: Substrate specificity and regulation. In: *Proceedings of the Second Workshop on Folyl and Antifolyl Polyglutamates* (Ed. Goldman ID), pp. 7–13. Praeger, New York, 1985.
5. McGuire JJ, Hsieh P, Franco CT and Piper JR, Folylpolyglutamate synthetase inhibition and cytotoxic effects of methotrexate analogs containing 2,ω-diaminoalkanoic acids. *Biochem Pharmacol* **35**: 2607–2613, 1986.
6. McGuire JJ and Piper JR, Cytotoxic effects and inhibition of folylpolyglutamate synthetase by folate analogs containing 2,omega-diaminoalkanoic acids. In: *Chemistry and Biology of Pteridines* (Eds. Cooper BA and Whitehead VM), pp. 729–732. Walter de Gruyter, Berlin, 1986.
7. George S, Cichowicz DJ and Shane B, Mammalian folylpoly-γ-glutamate synthetase. 3. Specificity for folate analogues. *Biochemistry* **26**: 522–529, 1987.
8. Rosowsky A, Freisheim JH, Moran RG, Solan VC, Bader H, Wright JE and Radike-Smith M, Methotrexate analogues 26. Inhibition of dihydrofolate reductase and folylpolyglutamate synthetase activity and *in vitro* tumor cell growth by methotrexate and aminopterin analogues containing a basic amino acid side chain. *J Med Chem* **29**: 655–660, 1986.
9. Clarke L, Rosowsky A and Waxman DJ, Inhibition of human liver folylpolyglutamate synthetase by non-γ-glutamylatable antifolate analogs. *Mol Pharmacol* **31**: 122–127, 1987.
10. Piper JR, McCaleb GS, Montgomery JA, Schmid FA and Sirotnak FM, Syntheses and evaluation as antifolates of methotrexate analogues derived from 2,ω-diaminoalkanoic acids. *J Med Chem* **28**: 1016–1025, 1985.
11. Acharya SP and Hynes JB, A new synthetic route to quinazoline analogs of folic acid. *J Heterocyclic Chem* **12**: 1283–1286, 1975.
12. Calvert AH, Jones TR, Jackman AL, Brown SJ and Harrup KR, An approach to the design of antimetabolites active against cells resistant to conventional agents illustrated by quinazoline antifolates with N-10 substitutions. In: *Advances in Tumour Prevention, Detection, and Characterization* (Eds. Davis W, Harrup KR and Stathopoulos G), Vol. 5, pp. 272–283. Excerpta Medica, Amsterdam, 1980.
13. Hayman R, McGready R and Van der Weyden M, A rapid radiometric assay for dihydrofolate reductase. *Anal Biochem* **87**: 460–465, 1978.
14. Gupta VS and Huennekins FM, Preparation and properties of crystalline 5-methyltetrahydrofolate and related compounds. *Arch Biochem Biophys* **120**: 712–718, 1967.
15. Mathews CK and Huennekens FM, Further studies on dihydrofolic reductase. *J Biol Chem* **238**: 3436–3442, 1963.
16. McGuire JJ, Hsieh P and Bertino JR, Enzymatic synthesis of polyglutamate derivatives of 7-hydroxymethotrexate. *Biochem Pharmacol* **33**: 1355–1361, 1984.
17. Johns DG, Iannoti AJ, Sartorelli AC and Bertino JR, The relative toxicities of methotrexate and aminopterin. *Biochem Pharmacol* **15**: 555–561, 1966.
18. Osborn MJ and Huennekens FM, Enzymatic reduction of dihydrofolic acid. *J Biol Chem* **233**: 969–974, 1958.
19. Lomax MIS and Greenberg GR, A new assay of thymidylate synthetase activity based on the release of tritium from deoxyuridylate-5-^{3}H. *J Biol Chem* **242**: 109–113, 1967.
20. Dolnick BJ and Cheng Y-C, Human thymidylate synthetase derived from blast cells of patients with acute myelocytic leukemia. Purification and characterization. *J Biol Chem* **252**: 7697–7703, 1977.
21. McGuire JJ, Hsieh P, Coward JK and Bertino JR, Enzymatic synthesis of folylpolyglutamates. Characterization of the reaction and its products. *J Biol Chem* **255**: 5776–5788, 1980.
22. McGuire JJ, Hsieh P, Coward JK and Bertino JR, *In vitro* methotrexate polyglutamate synthesis by rat liver folylpolyglutamate synthetase and inhibition by bromosulfophthalein. *Adv Exp Med Biol* **163**: 199–214, 1983.
23. Segel IH, *Enzyme Kinetics*. Wiley Interscience, New York, 1975.
24. Kamen BA, Cashmore AR, Dreyer RN, Moroson BA, Hsieh P and Bertino JR, Effect of [^{3}H]methotrexate impurities on apparent transport of methotrexate by a sensitive and resistant L1210 cell line. *J Biol Chem* **255**: 3254–3257, 1980.
25. McGuire JJ, Mini E, Hsieh P and Bertino JR, Role of methotrexate polyglutamates in methotrexate and sequential methotrexate-5-fluorouracil-mediated cell kill. *Cancer Res.* **45**: 6395–6400, 1985.
26. Lozzio CB and Lozzio BB, Human chronic myelogenous leukemia cell line with positive Philadelphia chromosome. *Blood* **45**: 321–334, 1975.
27. Foley GE, Lazarus H, Farber S, Uzman BG, Boone BA and McCarthy RE, Continuous culture of lymphoblasts from peripheral blood of a child with acute leukemia. *Cancer* **18**: 522–529, 1965.
28. Blakley RL, *The Biochemistry of Folic Acid and Related Pteridines*. North Holland, Amsterdam, 1969.
29. Davoll J and Johnson AM, Quinazoline analogues of folic acid. *J Chem Soc* C997–1002, 1970.
30. McGuire JJ, Sobrero AF, Hynes JB and Bertino JR, Mechanism of action of 5,8-dideazaisofolic acid and other quinazoline antifols in human colon carcinoma cells. *Cancer Res* **47**: 5975–5981, 1987.
31. Jones TR, Calvert AH, Jackman AL, Brown SJ, Jones M and Harrup KR, A potent antitumour quinazoline inhibition of thymidylate synthetase: Synthesis, biological properties and therapeutic results in mice. *Eur J Cancer* **17**: 11–19, 1981.
32. Fernandes DJ, Bertino JR and Hynes JB, Biochemical and antitumor effects of 5,8-dideazoisopteroylglutamate, a unique quinazoline inhibitor of thymidylate synthase. *Cancer Res* **43**: 1117–1123, 1983.
33. Mini E, Moroson BA, Franco CT and Bertino JR, Cytotoxic effects of folate antagonists against methotrexate-resistant human leukemic lymphoblast CCRF-CEM cell lines. *Cancer Res* **45**: 325–330, 1985.
34. Hynes JB, Yang YCS, McGill JE, Harmon SJ and Washtien WL, Improved synthesis and antitumor evaluation of 5,8-dideazaisofolic acid (IAHQ) and closely related analogs. *J Med Chem* **27**: 232–235, 1984.
35. Galivan J, Inglese J, McGuire JJ, Nimec Z and Coward JK, Gamma-fluoromethotrexate: Synthesis and biological activity of a potent inhibitor of dihydrofolate reductase with greatly diminished ability to form poly-

gamma-glutamates. *Proc Natl Acad Sci USA* **82**: 2598–2602, 1985.
36. Rosowsky A, Forsch RA, Freisheim JH, Moran RG and Wick M, Methotrexate analogs. 19. Replacement of the glutamate side chain in classical antifolates by L-homocysteic acid. Effect on enzyme inhibition and antitumor activity. *J Med Chem* **27**: 600–604, 1984.
37. Moran RG, Colman PD, Rosowsky A, Forsch RA and Chan KK, Structural features of 4-amino antifolates required for substrate activity with mammalian folylpolyglutamate synthetase. *Molec Pharmacol* **27**: 156–166, 1985.
38. McGuire JJ, Mini E, Hsieh P and Bertino JR, Folylpolyglutamate synthetase: Relation to methotrexate action and as a target for new drug development. In: *The Development of Target Oriented Anticancer Drugs* (Eds. Cheng Y-C, Gaz B and Minkoff M), pp. 97–106. Raven Press, New York, 1983.