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Substrate Specificity of Mammalian Folylpolyglutamate Synthetase for 5,10-Dideazatetrahydrofolate Analogs

LILLIAN L. HABECK, LAURANE G. MENDELSOHN, CHUAN SHIH, EDWARD C. TAYLOR, PAUL D. COLMAN, LYNN S. GOSSETT, TRACY A. LEITNER, RICHARD M. SCHULTZ, SHERRI L. ANDIS, and RICHARD G. MORAN

Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285 (L.L.H., L.G.M., C.S., L.S.G., T.A.L., R.M.S., S.L.A.), Department of Chemistry, Princeton University, Princeton, New Jersey 08544 (E.C.T.), Scientific Services Group, Los Angeles County Sheriff's Office, Los Angeles, California 90057 (P.D.C.), and Department of Pharmacology and Toxicology and the Massey Cancer Center, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298-0230 (R.G.M.)

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SUMMARY

The metabolism of 5,10-dideazatetrahydrofolate (DDATHF [lometrexol]) to polyglutamate derivatives by folylpoly-γ-glutamate synthetase (FPGS) plays a central role in the activity of this compound as an antineoplastic agent. The availability of a series of DDATHF derivatives differing in structure throughout the molecule has allowed a study of the structural requirements for substrate activity with mouse liver and hog liver FPGS. Kinetics of the polyglutamation reaction *in vitro* have been related to the potency of these compounds as inhibitors of the growth of human CEM leukemic cells. The structure-activity relationships for enzyme from both sources were nearly identical. FPGS from both species showed a broad acceptance for structural changes in the pyridopyrimidine ring, in the phenyl group, and in the intermediate bridge region, with structural changes in these regions being reflected in changes in K_m for FPGS but much more modest alterations in V_{max}. The data suggested that the phenyl ring was not contributing to any π-π hydrophobic interactions. It appeared to function primarily in maintaining a favorable distance between the pyridopyrimidine ring and the glutamate side chain. The lowest K_m values were found for DDATHF analogs in which there were small alterations at the 10 position, e.g., 5-deazatetrahydrofolate, 10-methyl-DDATHF, and 10-formyl-5-deazatetrahydrofolate; the first-order rate constants for these substrates were the highest in this series, an indication of the efficiency of polyglutamation at low substrate concentrations. After correction for the intrinsic inhibitory activity of the parent DDATHF analog as an inhibitor of the target enzyme, the first-order rate constants for FPGS were found to be predictive of the potency of tumor cell growth inhibition for most of the compounds in this structural series.

The usefulness of folate antimetabolites inhibitory to DHFR as cancer therapeutic agents has been recognized for four decades. The widely studied DHFR inhibitor methotrexate has broad-spectrum activity against human cancers and has an established role in the therapy of leukemias, head and neck tumors, and metastatic breast carcinoma. The action of methotrexate is now known to be dependent on activation to poly-γ-glutamate derivatives in tumor cells, a process required *in vivo* for intracellular retention after serum drug levels fall. This metabolism occurs via the enzyme FPGS (EC 6.3.2.17), which catalyzes the addition of several moles of glutamate to the γ-carboxyl group of the naturally occurring folate cofactors. The function of FPGS has been shown to be essential to the survival of proliferating mammalian cells (1, 2) due to the inability of mammalian cells lacking FPGS to retain folate cofactors in the cell (1, 3).

The therapeutic activity of methotrexate stimulated the search for new folate antimetabolites, but the exquisite activity of this drug as an inhibitor of DHFR largely frustrated the search for better DHFR-based therapeutic agents. The subsequent discovery of therapeutic activity of folate-based inhibitors of thymidylate synthase (4, 5) established that folate metabolism overall was a potentially rich source for antitumor drugs and focused efforts on the several other enzymes within the folate pathways. Recognition of the significance of polyglutamation for the cytotoxicity and therapeutic efficacy of methotrexate (6) and the development of the pioneering folate-based inhibitor of thymidylate synthase, CB3717 (7), led to an antifolate drug development strategy for agents that (1) were inhibitors of folate-dependent enzymes other than DHFR and (2) were used efficiently as

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ABBREVIATIONS: DHFR, dihydrofolate reductase; FPGS, folylpoly-γ-glutamate synthetase; GARFT, glycinamide ribonucleotide formyltransferase; rGARFT, human recombinant monofunctional glycinamide ribonucleotide formyltransferase; β-ME, β-mercaptoethanol; IC_{50}, the concentration of a drug that decreased the growth of tumor cells to 50% of untreated controls after 72 hr of continuous exposure; DDATHF, (6R)-5,10-dideazatetrahydrofolate (lometrexol).

substrates for mammalian FPGS. As a result of these efforts, DDATHF (8) and ZD1694 (Tomudex) (9) have been developed and are in clinical trials (10, 11) as prototypical, efficiently polyglutamated inhibitors of GARFT and of thymidylate synthase, respectively. Both compounds have been found to be active against human cancers (10, 11), and these two compounds represent the two most potent folate antimetabolites ever to be administered to humans. The maximal tolerated dose of these compounds has been found to be as low as 6 and 3 mg/m^2, respectively, even with infrequent dosings. Clearly, the process of polyglutamation is central to the characteristics of folate analogs that are antimetabolites of tetrahydrofolate. Thus, activation by FPGS is involved in the potency of tetrahydrofolate antimetabolites, although it remains to be seen whether polyglutamation can be manipulated to achieve or improve antitumor selectivity.

As part of a program for the discovery of novel tetrahydrofolate antimetabolites, we compared the ability of analogs of DDATHF to serve as substrates for mammalian FPGS. In the present report, we describe the surprising promiscuity of mouse liver and hog liver FPGS for structural variation in this series of compounds and the correlation of the potency of GARFT inhibitors assessed by inhibition of tumor cell growth in culture with two parameters: the efficiency of activation to polyglutamates by FPGS and the intrinsic activity of the monoglutamate as an inhibitor of GARFT *in vitro*.

Materials and Methods

Materials. Methotrexate, aminopterin, and acid-washed activated charcoal were obtained from Sigma Chemical Co. (St. Louis, MO). All other folate analogs were synthesized at Lilly Research Laboratories (Indianapolis, IN) or in the laboratory of E. C. Taylor (Department of Chemistry, Princeton University, Princeton, NJ). Enzyme-grade $(NH_4)_2SO_4$ was purchased from Schwarz/Mann Biochemicals (Los Angeles, CA). Human rGARFT was provided by Agouron Pharmaceuticals (San Diego, CA). L-[3,4-^{3}H]-Glutamate and L-[^{14}C(U)]-glutamate were purchased from Dupont-NEN (Boston, MA). The Enzfitter microcomputer package was purchased from Biosoft (Ferguson, MO), and B6D2F1 female mice were obtained from Simonsen Laboratories (Gilroy, CA). Human CEM lymphoblastic leukemia cells were obtained from St. Jude's Children Hospital (Memphis, TN).

Purification and determination of kinetic constants for murine liver FPGS. Mouse liver FPGS was prepared from B6D2F1 female mice. A 0–30% ammonium sulfate precipitate was stored at −25° until use and desalted immediately before use with Sephadex G-25 chromatography (12). Folate analogs were incubated with murine liver FPGS for 60 min at 37° in a reaction mix containing 1 mM [^{3}H]-glutamic acid (4 mCi/mmol), 5 mM ATP, 10 mM $MgCl_2$, 30 mM KCl, 20 mM α-thioglycerol, and 200 mM Tris, pH 8.6 (37°), in a total volume of either 0.25 or 1 ml. The tritium-labeled folyldiglutamate product was isolated by adsorption onto charcoal as described previously (12). The reaction being measured was restricted to formation of diglutamate by allowing no more than 25% substrate consumption at the lowest substrate concentration and by limiting the amount of enzyme used for the better substrates.

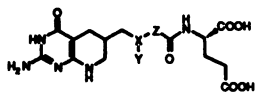
Purification and determination of kinetic constants for hog liver FPGS. Hog liver FPGS was purified through the chromatofocusing step as previously described by Cichowicz and Shane (13). The pooled activity peak was dialyzed against 100 mM Tris·HCl, 50 mM β-ME, and 20% glycerol, pH 8.4, and was placed into aliquots and stored at −70°. An approximate 8000-fold purification was achieved with this procedure for an enzyme-specific activity of 110 nmol/hr × mg protein when assayed with 200 μM methotrexate and 250 μM [^{14}C]-L-glutamate. K_m and V_{max} values were determined with five or six folate analog concentrations in duplicate per experiment. The assay conditions were 100 mM Tris, 10 mM $MgCl_2$, 5 mM ATP, 20 mM KCl, 100 μg/ml bovine serum albumin, 100 mM β-ME, and 1 mM [^{14}C]-L-glutamate (4 mCi/mmol), pH 8.9, in a final volume of 0.25 or 1.0 ml at 37°. The amount of protein added (1–6 μg) and incubation time (1–3 hr) were varied to minimize the formation of higher polyglutamates at a low substrate concentration. The amount of labeled product formed increased as a linear function of protein concentration over the above range. Product formation also was a linear function of the incubation time over 1–3 hr. Blanks consisted of all components of the reaction mix, including enzyme, but did not contain folate analog. Reactions were terminated by the addition of ice-cold 10 mM L-glutamate, pH 7.5. Product was separated from unincorporated [^{14}C]-L-glutamate by binding and elution from Waters Sep-pak Plus C18 cartridges (Milford, MA) as previously described by Jansen *et al.* (14). K_m and V_{max} values were determined by nonlinear fit of the data to a rectangular hyperbola with the Enzfitter microcomputer package.

***In vitro* cytotoxicity and affinity to GARFT.** IC_{50} values against human leukemia CEM cells and K_i values for human rGARFT and murine trifunctional GARFT were determined as described previously (15, 16).

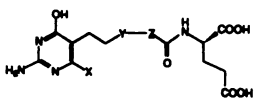
Results

Activity of murine and hog liver FPGS for standard folate substrates. The GARFT inhibitor lometrexol (6R-DDATHF, compound 1; Fig. 1) has been shown to be an excellent substrate for murine liver FPGS (17) and for that from human leukemic cells (17, 18). Because the antifolates evaluated in the present study were analogs of DDATHF and to better facilitate comparison of data from the two enzyme sources, the maximum velocities (V_{max} values) and first-order rate constants (k' values, defined as V_{max}/K_m) of the DDATHF analogs studied were normalized to those of lometrexol. Four additional compounds were useful standards for the comparison of this data set with previously published data and for a comparison of hog liver and mouse liver enzymes, namely, the classic DHFR inhibitors methotrexate and aminopterin and two thymidylate synthase inhibitors of current interest that are substrates for FPGS: LY231514 (19) and ZD1694 (Tomudex) (5, 20). Aminopterin has been reported as a standard in the recent literature on FPGS (21, 22), and methotrexate has been found to rely on metabolism to polyglutamates for therapeutic activity *in vivo* (3, 6). The K_m value for conversion of lometrexol to its diglutamate was 16.4 μM for hog liver FPGS (Table 2), comparable to the value previously found for mouse liver enzyme, 9.3 μM (Tables 1 and 2). Lometrexol was characterized by a first-order rate constant that was 14- and 32-fold higher than that of methotrexate for the hog and mouse liver enzymes, respectively, with most of the difference due to K_m effects.

Aminopterin, which is known to be an efficient substrate for mammalian FPGS (23), was approximately 3-fold less efficient than lometrexol as demonstrated by the k' values for these compounds (Table 1). On the other hand, ZD1694 and LY231514, two of the best known substrates for FPGS (19, 20, 22), had substantially lower K_m values than lometrexol for both the hog and mouse liver enzymes. Over this broad spectrum of standard substrates differing in first-order rate constants by a factor of 120 (hog liver enzyme) to 450 (mouse liver FPGS), the kinetic behaviors of the two species of FPGS were remarkably parallel (Tables 1 and 2), and the major



Compound	X	Y	Z
1 (lometrexol)	CH	H	
2	CH	H	
3	CH	H	
4	CH	H	
5	CH	H	
6	CH	H	
7-9	CH	H	
10	CH	H	
11	CH	H	
12	CH	H	-(CH2)2-
13	CH	H	-(CH2)3-
14	CH	H	-(CH2)4-
15	CH	CH3	
16	CH	CH2OH	
17	N	H	
18	N	CHO	
19	N	COCH3	
20	CHCH2	H	
21	NCH2	H	
22	NCH2	CHO	
23	CHNH	H	



Compound	X	Y	Z
24	NH2	CH2	
25	NH2	CH2CH2	
26	CH3	CH2CH2	
27	NH2	CH2CH2	

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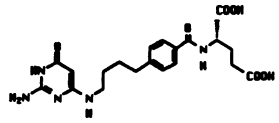

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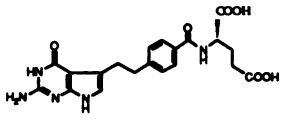
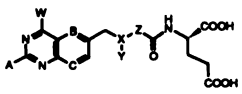




Compound	W	X	Y	Z	A	B	C
30 (methotrexate)	NH2	N	CH3		NH2	N	N
31 (aminopterin)	NH2	N	H		NH2	N	N
32	OH	CH	H		NH2	CH	N
33	OH	CH	H		NH2	CH	N
34 (ZD1694, tomudex)	OH	N	CH3		CH3	CH	CH

35

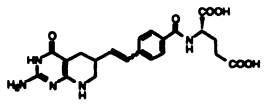


Fig. 1. Chemical structures of 5,10-dideaza-5,6,7,8-tetrahydrofolate analogs and other standard antifolate compounds. The trivial and full chemical names of each of the compounds are as follows: (1) 6*R*-DDATHF (lometrexol), (6*R*)-*N*-[4-[2-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)ethyl]benzoyl]-L-glutamic acid; (2) (6*R,S*)-2′-*F*-phenyl-DDATHF, (±)-*N*-[4-[2-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)ethyl]-2′-fluorobenzoyl-]L-glutamic acid; (3) (6*R,S*)-3′-*F*-phenyl-DDATHF, (±)-*N*-[4-[2-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)ethyl]-3′-fluorobenzoyl]-L-glutamic acid; (4) (6*R,S*)-3′,5′-diF-phenyl-DDATHF, (±)-*N*-[4-[2-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)-ethyl]-3′,5′-difluorobenzoyl]-L-glutamic acid; (5) (6*R,S*)-2′-Cl-phenyl-DDATHF, (±)-*N*-[4-[2-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)ethyl]-2′-chlorobenzoyl]-L-glutamic acid; (6) (6*R,S*)-*cis*-1′,4′-cyclohexyl-DDATHF, (±)-*N*-[[4-[2-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)ethyl]-cyclohexyl]carbonyl]-L-glutamic acid; (7) (6*R,S*)-2′,5′-thienyl-DDATHF, (±)-*N*-[[5′-[2-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)ethyl]-2′-thienyl]carbonyl]-L-glutamic acid; (8) (6*S*)-2′,5′-thienyl-DDATHF, (6*S*)-*N*-[[5′-[2-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido-[2,3-*d*]pyrimidin-6-yl)ethyl]-2′-thienyl]carbonyl]-L-glutamic acid; (9) (6*R*)-2′,5′-thienyl-DDATHF, (6*R*)-*N*-[[5′-[2-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)ethyl]-2′-thienyl]carbonyl]-L-glutamic acid; (10) (6*R,S*)-2′,5′-furyl-DDATHF, (±)-*N*-[[5′-[2-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)ethyl]-2′-furyl]carbonyl]-L-glutamic acid; (11) (6*R,S*)-*m*-DDATHF, (±)-*N*-[3-[2-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido-[2,3-*d*]pyrimidin-6-yl)ethyl]-benzoyl]-L-glutamic acid; (12) (6*R,S*)-abenzyl-DDATHF (*n* = 2), (±)-*N*-[5-[2-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)-1-oxo-pentyl]-L-glutamic acid; (13) (6*R,S*)-abenzyl-DDATHF (*n* = 3), (±)-*N*-[6-[2-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)-1-oxo-hexyl]-L-glutamic acid; (14) (6*R,S*)-abenzyl-DDATHF (*n* = 4), (±)-*N*-[7-[2-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)-1-oxo-heptyl]-L-glutamic acid; (15) (6*R,S*)-10-methyl-DDATHF, (±)-*N*-[4-[2-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)-1-methylethyl]benzoyl]-L-glutamic acid; (16) (6*R,S*)-10-hydroxymethyl-DDATHF, (±)-*N*-[4-[2-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]-pyrimidin-6-yl)-1-hydroxymethylethyl]benzoyl]-L-glutamic acid; (17) (6*R,S*)-5-DATHF, (±)-*N*-[4-[[2-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]-

TABLE 1

Substrate activity of folate analogs for murine liver FPGS

Compound no.	Compound name	K_m (μM)[a]	rel V_{max}[b]	V_{max}/K_m[c]
Standard compounds				
1	DDATHF (lometrexol)	9.3 ± 1.6 (3)	1.0	1.0
29	LY231514	0.80 ± 0.11 (3)	0.63 ± 0.18	13.7
34	ZD1694 (Tomudex)	1.37 ± 0.11 (3)	1.0 ± 0.19	6.8
31	Aminopterin	23.6 ± 1.2 (18)	0.80 ± 0.01	0.35
30	Methotrexate	166.0 ± 14 (12)	0.50 ± 0.09	0.031
Fused ring analogs				
17	5-Deazatetrahydrofolate (5-DATHF)	0.95 ± 0.10 (3)	0.63 ± 0.02	6.6
28	5-Desmethylene-DDATHF	13.3 ± 2.2 (3)	0.58 ± 0.18	0.62
25	7-Desmethylene-DDATHF	68.0 ± 6.0 (2)	0.76 ± 0.06	0.12
26	7-Desmethylenetrideazatetrahydrofolate	820.0 ± 80 (2)	1.28 ± 0.15	0.02
27	7-Desmethylene-thiophene-DDATHF	72.0 ± 3.2 (2)	0.59 ± 0.04	0.11
32	5,10-Dideazafolic acid	157.0 ± 5.0 (2)	0.45 ± 0.06	0.046
33	Thiophene-5,10-dideazafolic acid	175.0 ± 2.2 (2)	0.45 ± 0.01	0.023
Bridge region modifications				
35	5,10-Dideaza-9,10-dehydro-folic acid	255.0 ± 1.0 (2)	0.10 ± 0.01	0.007
20	Homo-DDATHF	3.8 ± 0.1 (2)	1.04 ± 0.13	2.4
23	Homo-5-deazatetrahydrofolate	24.0 ± 2.1 (3)	1.00 ± 0.44	0.34
21	Isohomo-5-deazatetrahydrofolate	39.0 ± 2.4 (3)	0.59 ± 0.25	0.33
18	10-Formyl-5-deazatetrahydrofolate	1.44 ± 0.31 (5)	0.50 ± 0.07	4.0
19	10-Acetyl-5-deazatetrahydrofolate	10.9 ± 1.1 (2)	1.14 ± 0.08	1.1
22	10-Formyl-isohomo-5-deazatetrahydrofolate	3.2 ± 0.7 (3)	0.40 ± 0.05	1.3
15	10-Methyl-DDATHF	0.91 ± 0.12 (2)	0.64 ± 0.02	6.3
16	10-Hydroxymethyl-DDATHF	0.43 (1)	0.50	13.0
Replacement of the 1′,4′-phenyl ring of DDATHF with:				
11	1′,3′-phenyl-(meta-DDATHF)	No detectable substrate activity		
6	*cis*-1′,4′-Cyclohexyl	7.9 ± 0.8 (2)	0.61 ± 0.02	0.87
7	2′,5′-Thienyl-	3.9 ± 0.7 (2)	0.55 ± 0.04	1.3
12	-$(CH_2)_2$-	40.0 ± 2.5 (2)	0.55 ± 0.05	0.18
13	-$(CH_2)_3$-	10.6 ± 2.3 (2)	0.46 ± 0.04	0.57
14	-$(CH_2)_4$-	28.0 ± 0.3 (2)	0.55 ± 0.07	0.26
2	(6-*R,S*)-2′-*F*-Phenyl-	4.6 ± 1.1 (2)	0.69 ± 0.08	1.40
	(6-*R*)-2′-*F*-Phenyl-	4.6 ± 0.6 (2)	0.75 ± 0.16	1.36
3	3′-*F*-Phenyl-	6.5 ± 2.0 (2)	0.93 ± 0.14	1.30
4	3′,5′-di*F*-Phenyl-	82.0 ± 6.9 (2)	0.33 ± 0.08	0.040
5	2′-Cl-Phenyl-	21.0 ± 0.4 (2)	0.46 ± 0.02	0.23

[a] Values listed are mean ± standard error for $n \geq 3$ or ± ½ range for $n = 2$ replicate experiments.
[b] The ratio of V_{max} for a substrate to the V_{max} for lometrexol with murine liver FPGS.
[c] The V_{max} of a substrate relative to lometrexol divided by the K_m of a substrate relative to lometrexol. The kinetics of a standard compound was measured in each experiment to allow accurate comparisons among substrates.

contribution to the differences among substrates for either enzyme was due to differences in K_m. This pattern remained for the series of compounds studied (Tables 1 and 2), and differences in V_{max} among substrates varied only by 4- and 8-fold for mouse and hog liver enzymes, respectively.

Substrate activity of DDATHF analogs modified in the phenyl moiety. Analogs of DDATHF were available in which the phenyl ring was modified or replaced. A comparison of the activity of DDATHF analogs bearing a 2′- or 3′-fluoro group (compounds **2** and **3**) indicated a modest increase in the first-order rate constant (k') relative to that of lometrexol (Table 1). A 2′-Cl substitution (compound **5**) resulted in a 4-fold decrease in k', with an increased K_m and a decreased V_{max}. Very similar effects of 2′-F and -Cl groups on the FPGS reaction have been reported for 5,8-dideazafolic acid analogs (22). Substitution of fluorine at both the 3′ and 5′ positions of the phenyl ring (compound **4**) substantially decreased the substrate activity of the dideazatetrahydrofolate analog, with a 25-fold decrease in first-order rate constant.

pyrimidin-6-yl)methyl]amino]benzoyl]-L-glutamic acid; (**18**) (6*R,S*)-10-formyl-5-DATHF, (±)-*N*-[4-[[[2-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]-pyrimidin-6-yl)methyl]formyl]amino]benzoyl]-L-glutamic acid; (**19**) (6*R,S*)-10-acetyl-5-DATHF, (±)-*N*-[4-[[[2-(2-amino-3,4,5,6,7,8-hexahydro-4-oxo-pyrido[2,3-*d*]pyrimidin-6-yl)methyl]acetyl]amino]benzoyl]-L-glutamic acid; (**20**) (6*R,S*)-homo-DDATHF, (±)-*N*-[4-[3-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)propyl]benzoyl]-L-glutamic acid; (**21**) (6*R,S*)-isohomo-5-DATHF, (±)-*N*-[4-[[2-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)methyl]aminomethyl]benzoyl]-L-glutamic acid; (**22**) (6*R,S*)-10-formyl-isohomo-5-DATHF, isohomo-DDATHF; (±)-*N*-[4-[[2-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)methyl]amino(formyl)methyl]benzoyl]-L-glutamic acid; (**23**) (6*R,S*)-homo-5-DATHF, (±)-*N*-[4-[[[2-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)ethyl]amino-]benzoyl]-L-glutamic acid; (**24**) 7-desmethylene-nor-DDATHF, *N*-[4-[4-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)propyl]benzoyl]-L-glutamic acid; (**25**) 7-desmethylene-DDATHF, *N*-[4-[4-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)butyl]benzoyl]-L-glutamic acid; (**26**) 7-desmethylene-trideazatetrahydrofolic acid, *N*-[4-[4-(2-diamino-4-methyl-1,6-dihydro-6-oxo-5-pyrimidinyl)butyl]benzoyl]-L-glutamic acid; (**27**) 7-desmethylene-2′,5′-thienyl-DDATHF, *N*-[5′-[4-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)butyl]-2′-thienyl]-L-glutamic acid; (**28**) 5-desmethylene-DDATHF; *N*-[4-[4-(2amino-1,6-dihydro-6-oxo-4-pyrimidinyl)aminobutyl]benzoyl]-L-glutamic acid; (**29**) LY231514, *N*-[4-[2-(2-amino-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-L-glutamic acid; (**30**) methotrexate; (**31**) aminopterin; (**32**) 5,10-dideazafolic acid, *N*-[4-[2-(2-amino-3,4-dihydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)ethyl]benzoyl]-L-glutamic acid; (**33**) thiophene-5,10-dideazafolic acid, *N*-[5′-[2-(2-amino-3,4-dihydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)ethyl]2′-thienyl]-L-glutamic acid; (**34**) ZD1694 (tomudex); (**35**) 5,10-dideaza-9,10-dehydro-folic acid, *N*-[4-[2-(2-amino-3,4-dihydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)ethenyl]benzoyl]-L-glutamic acid.

The 1′,4′-phenyl group of DDATHF could be replaced by other ring systems or by much simpler aliphatic moieties with retention of activity as a substrate for mammalian FPGS. Thus, replacement of this group with a 2′,5′-thiophene (compounds **7–9**) barely changed the kinetic characteristics of the FPGS reaction with either enzyme source. As had been previously found with the diastereomers of DDATHF differing in chirality about carbon 6 (17), both isomers of the thiophene derivative of DDATHF were used as substrates by FPGS; the 6*S* isomer was a somewhat more efficient substrate as a result of its lower K_m (Table 2). Remarkably, replacing the phenyl group of DDATHF with a *cis*-1′,4′-cyclohexyl group (compound **6**) allowed almost the same substrate activity as observed for lometrexol for either source of FPGS. The phenyl ring of lometrexol could be replaced by an aliphatic linker of two, three, or four methylene units (compounds **12–14**, Table 1) with retention of substrate activity for FPGS. The kinetic characteristics observed for this homologous series suggested that this presumptive space-filling role of the phenyl ring of DDATHF was best modeled by an aliphatic chain that was three methylene units in length. Despite this degree of tolerance for structural variation in this region of the DDATHF molecule, two structural modifications in this region were found to disrupt substrate activity: (1) replacement of the 1′,4′-phenyl group by a 2′,5′-furan (compound **10**, Table 2) resulted in an 11-fold decrease in first-order rate constant due to a 2.4-fold increase in K_m and a 4.8-fold decrease in V_{max}, and (2) *in vitro* polyglutamation was undetectable when the substituents on the phenyl ring were changed from the 1′,4′-*para* to the 1′,3′-*meta* configuration (compound **11**, Tables 1 and 2). A similar lack of FPGS substrate activity had been observed with the *meta* derivative of aminopterin (24).

Structural alterations of the bridge region. The addition of another methylene group in the bridge region of DDATHF (compound **20**, homo-DDATHF) increased the first-order rate constant relative to lometrexol marginally (1.6- to 2.4-fold for hog and murine FPGS, respectively) due to an effect on K_m (Tables 1 and 2). Replacement of either the central bridge methylene of homo-DDATHF (compound **21**) or the 11 position methylene (compound **23**) with nitrogen resulted in analogs that were less active substrates than DDATHF by a factor of 3 and approximately 7-fold less active than the analogous compound with a three carbon methylene bridge, i.e., homo-DDATHF, compound **20**. These differences reflected mainly higher K_m values of the heterosubstituted bridge compounds.

Four DDATHF analogs were as active as substrates for FPGS as the thymidylate synthase inhibitors ZD1694 and LY231514: (1) either a methyl or a hydroxymethyl substitution at carbon 10 of DDATHF (compounds **15** and **16**) resulted in a major decrease in K_m, relative to that of DDATHF and an overall 6.3- and 13-fold increase in first-order rate constant for the FPGS reaction, respectively; (2) replacement of carbon 10 of DDATHF with nitrogen (5-DATHF, compound **17**) had a similar effect; and (3) a 10-formyl substitution of 5-DATHF (compound **18**) did not alter the favorable K_m or V_{max} values. The similarity of the use of 5-DATHF and its 10-formyl derivative agrees with the equivalent acceptance of tetrahydrofolate and 10-formyltetrahydrofolate by mammalian liver FPGS (25–27). An additional methylene group between the *N*-formyl group of compound **18** and the phenyl ring, i.e., compound **22**, had the same effect on the kinetic pattern observed from a comparison of 5-DATHF with isohomo-5-DATHF (compound **21**, Table 1). An acetyl group at the 10 position of 5-DATHF (compound **19**) decreased the efficiency of polyglutamation of this analog due to a 10-fold increase in K_m relative to 5-DATHF, presumably an indication of a limited bulk tolerance at this position, similar to that observed with a series of *N*-10 alkylated 5,8-dideazafolic acid analogs (28).

Structural alteration of the pyridopyrimidine ring. The thiophene analog of DDATHF in which the pyridopyrimidine ring is not reduced (compound **33**) displayed a substantially higher (45-fold) K_m than its reduced analog (compound **7**) and a lower first-order rate constant, which reflected this difference (Table 1). This was similar to the poor substrate activity previously noted for folic acid (25–27) and for 5,10-dideazafolic acid (28). The kinetics of the mouse liver FPGS reaction with an analog in which the flexibility of the bridge region was decreased by a 9,10 double bond (compound **35**) were remarkably similar to the corresponding control compound **32**. Acyclic analogs of DDATHF (compounds **25** and **28**) or its thiophene analog (compound **27**)

TABLE 2
Substrate activity of folate analogs for hog liver FPGS

Compound no.	Compound name	K_m	rel V_{max}[b]	rel V_{max}/K_m[c]
		μM		
Standard compounds				
1	DDATHF (lometrexol)	16.4 ± 1.0 (5)	1.0	1.0
29	LY231514	1.9 ± 0.5 (3)	0.74 ± 0.10	6.40
34	ZD1694 (Tomudex)	2.7 ± 0.4 (2)	1.37 ± 0.16	8.31
30	Methotrexate	116.0 ± 14 (3)	0.51 ± 0.08	0.07
DDATHF analogs				
6	Cyclohexyl-DDATHF	9.8 ± 1.0 (3)	0.51 ± 0.05	0.85
7	(6-*R,S*)-Thienyl-DDATHF	9.2 ± 0.8 (4)	0.46 ± 0.03	0.82
8	(6*S*)-Thienyl-DDATHF	6.0 ± 0.5 (4)	0.44 ± 0.03	1.19
9	(6*R*)-Thienyl-DDATHF	15.8 ± 2.5 (5)	0.70 ± 0.12	0.73
10	2′,5′-Furyl-DDATHF	38.9 ± 10.2 (3)	0.21 ± 0.02	0.09
11	Meta-DDATHF	No detectable substrate activity		
20	Homo-DDATHF	10.0 ± 1.6 (4)	0.97 ± 0.08	1.60
24	7-Desmethylene-norDDATHF	49.5 ± 2.9 (2)	0.18 ± 0.01	0.06

[a] Values listed are mean ± standard error for $n \geq 3$ or ± ½ range for $n = 2$ replicate experiments.
[b] The ratio of V_{max} for a substrate to the V_{max} for lometrexol (977 ± 128 nmol/hr·mg, $n = 5$) with hog liver FPGS.
[c] The V_{max} of a substrate relative to lometrexol divided by the K_m of a substrate relative to lometrexol.

TABLE 3

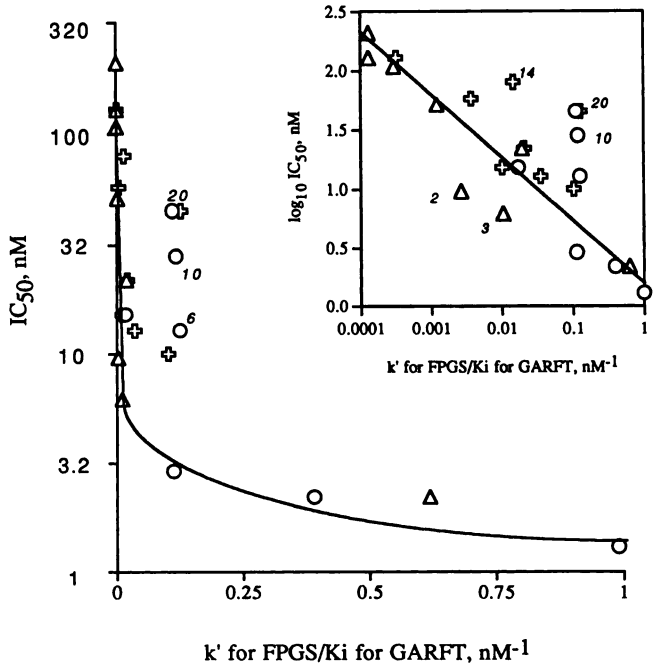
Affinity for human rGARFT, relative first-order rate constant for FPGS, and *in vitro* cytotoxicity for inhibitors of GARFT

Compound no.	rGARFT[a] K_i (nM)		V_{max}/K_m[b] (relative to DDATHF)	CCRF-CEM[c] IC_{50} (nM)	
1	59.7 ± 7.6	(2)	1.0	15.2 ± 2.6	(3)
2	530 ± 20	(2)	1.40	9.6 ± 2.2	(7)
3	130	(1)	1.30	6.2 ± 1.0	(2)
4	310	(1)	0.04	209	(1)
5	780	(1)	0.23	108 ± 23	(2)
6	6.8	(1)	0.85, 0.87	12.8 ± 4.3	(2)
7	2.1 ± 0.2	(5)	0.82, 1.30	2.2	(1)
8	1.2 ± 0.1	(3)	1.19	1.3	(1)
9	6.5 ± 1.1	(3)	0.73	2.9	(1)
10	0.77 ± 0.04	(2)	0.09	28.2 ± 3.0	(3)
11	1.5	(1)	No activity	71.3 ± 21.3	(3)
13	30	(1)	0.57	22.0 ± 3.4	(2)
18	3400	(1)	4.0	51.5 ± 5.4	(3)
20	14.6 ± 0.7	(2)	1.60, 2.4	45.2	(1)
25	630	(1)	0.12	129 ± 4	(2)

[a] K_i for human recombinant monofunctional GARFT. Values listed are mean ± standard error for $n \geq 3$ or ± ½ range for $n = 2$ experiments. Values >100 nM were determined with the Michaelis-Menten equation, and values <100 nM were determined with the Morrison equation for tight-binding inhibitors as described previously (15).

[b] First-order rate constants for conversion to diglutamate relative to lometrexol (compound 1). When two rate constants are listed, the first is for hog liver FPGS and the second is for murine liver FPGS.

[c] Values listed are mean ± standard error for $n \geq 3$ or ± ½ range for $n = 2$ experiments. The 72-hr *in vitro* cytotoxicity assays were performed as described previously (15).



k' for FPGS/Ki for GARFT, nM^{-1}

Fig. 2. Correlation of the activity of DDATHF analogs as inhibitors of the growth of CEM cells with the efficiency as substrates for FPGS and as inhibitors of mammalian GARFT. Each symbol represents one compound. The ratio of k' for FPGS to K_i for GARFT was calculated using data obtained using human rGARFT and hog liver FPGS (○), using human rGARFT and mouse liver FPGS (△), and using mouse L1210 cell trifunctional GARFT (16) and mouse liver FPGS (✣).

displayed higher K_m values (13–72 μM, Table 1), and an acylic analog of 5,8,10-trideazatetrahydrofolic acid (compound **26**) had an even higher K_m value. Remarkably, these compounds were still accepted as substrates for mammalian FPGS.

Inhibition of GARFT and of the growth of human leukemic cells. The usefulness of data on the kinetics of activation by FPGS and on inhibition of human rGARFT to predict the potency of DDATHF analogs as inhibitors of the growth of human leukemic cells was determined (Table 3). In the series studied, most compounds that had even a moderate affinity for human rGARFT (K_i<150 nM) but high relative first-order rate constants for FPGS (>0.70) were potent inhibitors (IC_{50}<20 nM) of the growth of CEM cells (compounds **1**, **3**, **6–9**). As expected, inhibitors with moderate or low affinity for human rGARFT (K_i>150 nM) and low relative first-order rate constants for FPGS (k'<0.30) were relatively poor (IC_{50}>100 nM) inhibitors of tumor cell growth (compounds **4**, **5**, and **25**). When correlations among the potency of inhibition of CEM cells, the first-order rate constant for FPGS, and the K_i for human or murine GARFT were examined, a correlation was apparent between the potency of DDATHF analogs as tumor cell growth inhibitors and the ratio of k' for FPGS to K_i for the target enzyme, GARFT. This suggests that both processes, namely, efficient FPGS activation and intrinsic binding of the monoglutamate to the target enzyme, were necessary prerequisites for efficient antitumor activity in this series (Fig. 2). Perhaps more interesting than this correlation were the compounds that constituted outlying data points in this analysis: the 2'- and 3'-F derivatives of DDATHF (compounds **2** and **3**) were more active as growth inhibitors than expected from the values of these two parameters, whereas three compounds with structural modifications in the bridge or phenyl region (**14**~**10**>**20**) were less growth inhibitory than expected from this ratio. Compound **14** had a K_i value of 18 nM for murine trifunctional GARFT (16) and an IC_{50} value of 80 nM against human CEM cells (Fig. 2). We speculate that the fluorinated DDATHF analogs may be more facilely transported than DDATHF or have inhibitory activity on some other folate-dependent process, whereas the latter compounds (compounds **14**, **10**, and **20**) may be poorly transported or poorly metabolized to longer chain length polyglutamates.

Discussion

The present study was initiated several years ago to aid in the development of second-generation DDATHF analogs. On the basis of what was known about lometrexol, as well as ZD1694, CB3717, and methotrexate, it was clear that metabolism of folate analogs to long chain polyglutamates would enhance intracellular trapping of antifolate metabolites. Polyglutamate derivatives of thymidylate synthase inhibitors and antifolate inhibitors to GARFT are so much more potent inhibitors of their target enzymes (15, 16, 29, 30) that we have previously proposed that the polyglutamate metabolites are the active forms of these compounds and that parent compounds are actually prodrugs (31). Therefore, the original concept of this work was to identify structural motifs that would enhance the use of analogs by mammalian FPGS. Armed with this information, we sought to design potent folate antimetabolites capable of rapid accumulation and activation to polyglutamates in tumor cells *in vivo*. Should the ongoing clinical studies on lometrexol indicate that GARFT inhibitors that are better or poorer FPGS substrates would be expected to have a therapeutic index superior to that of

DDATHF, compounds with such characteristics could be identified on the basis of these studies (Tables 1 and 2). At this point in the study of these compounds, it is not clear whether efficiently polyglutamated folate analogs would necessarily lead to selective chemotherapy of cancer or under what circumstances (if any) that should be expected. It is important to recognize that transport mechanisms such as folate receptor isotype and the reduced folate carrier may play significant roles in modulating selectivity as well. Nevertheless, in the present study, we identified several compounds inhibitory to GARFT that would be expected to accumulate in tumors cells expressing FPGS more readily than DDATHF, namely, 5-deazatetrahydrofolate, 10-formyl-5-deazatetrahydrofolate, 10-hydroxymethyl-DDATHF, and 10-methyl-DDATHF.

The use of structure-activity relationships to probe enzyme-active sites is a classic approach, albeit one that only slowly and tentatively furnishes data on the shape of the enzyme surfaces involved in substrate binding and catalysis. In agreement with previous work on the substrate specificity of mammalian FPGS, these studies indicate that substantial structural modification of the DDATHF molecule is compatible with substrate activity. In addition, the kinetic evaluation of the FPGS reaction with the series studied herein has given some new information about the active site of mouse and hog liver FPGS: 1) the active sites of the mouse and hog liver enzymes appear very similar on the basis of probing with the spectrum of compounds compared in this study; 2) the phenyl ring typical of folates apparently is not involved in anchoring the DDATHF structure in the active site by any π-π hydrophobic interactions, as demonstrated by the activity of the DDATHF analog in which the phenyl ring is replaced by a cyclohexyl group; 3) instead, the phenyl ring appears to be fulfilling a space-filling role equivalent to the length of a three carbon aliphatic chain so that both pyridopyrimidine ring and the glutamic acid side chain are in positions within the active site compatible with substrate binding and catalysis; 4) the requirements of the folate binding domain of FPGS were not rigorous with respect to the tetrahydropyridine ring found in DDATHF; analogs in which this ring was replaced by aliphatic connecting groups bound to FPGS and were used as substrates; 5) likewise, considerable structural modification in the bridge region between the pyridopyrimidine and the phenyl ring was compatible with substrate activity; 6) some compounds that were close structural homologs to the DDATHF molecule were substantially better substrates for FPGS while maintaining GARFT as a target for growth inhibition, for example, 5-deazatetrahydrofolate, 10-formyl-5-deazatetrahydrofolate, and 10-methyl-DDATHF; 7) other structures related to DDATHF that resulted in increases in FPGS substrate activity did so at the expense of potency to inhibit GARFT or in some cases, specificity for GARFT e.g., LY231514 (19).

Several comparisons that could be drawn on the basis of these data, like the previous SAR studies published on this enzyme, appear to be incompatible with any single detailed model for the active site of FPGS. As one example, homo-DDATHF is an apparently superior substrate for both mouse liver FPGS (Table 1) and for hog liver FPGS (Table 2). Yet, all other homofolate derivatives of the naturally occurring folate cofactors are worse substrates for mouse liver FPGS than the corresponding folate compounds, sometimes by a substantial factor (32). Indeed, homo-5-deazatetrahydrofolate is much less readily accepted by mouse liver FPGS than is 5-deazatetrahydrofolate itself (Table 1). Therefore, although one can now deduce some general rules regarding which folate substrates will be accepted by the active site of this enzyme, it is not yet possible to predict quantitative estimates of how well new compounds would be activated by this enzyme. In addition, others have found that some compounds that are efficiently metabolized to a diglutamate by FPGS are poorly metabolized to longer chain polyglutamates (33). Under these circumstances, it is clear that the predictive accuracy of *in vitro* enzyme assays that measure the conversion of parent antifolylmonoglutamate to the corresponding diglutamate will only imperfectly predict metabolism of potential new drugs to intracellularly retained, long chain polyglutamates *in vivo*. With the recent publication of the cDNA sequence of human FPGS (34), it would be expected that recombinant FPGS would soon become available in pure form, allowing alternative and more direct approaches to these questions.

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References

1. McBurney, M. W., and G. F. Whitmore. Isolation and biochemical characterization of folate deficient mutants of Chinese hamster cells. *Cell* **2**:173–182 (1974).
2. Taylor, R. T., and M. L. Hanna. Folate dependent enzymes in cultured Chinese hamster cells: Folylpolyglutamate synthetase and its absence in mutants auxotrophic for glycine + adenosine + thymidine. *Arch. Biochem. Biophys.* **181**:331–344 (1977).
3. McGuire, J. J., and J. R. Bertino. Enzymatic synthesis and function of folylpolyglutamates. *Mol. Cell. Biochem.* **39**:19–48 (1981).
4. Jones, T. R., A. H. Calvert, A. L. Jackman, S. J. Brown, M. Jones, and K. R. Harrap. A potent antitumor quinazoline inhibitor of thymidylate synthetase: synthesis, biological properties, and therapeutic results in mice. *Eur. J. Cancer* **17**:11–19 (1981).
5. Jackman, A. L., G. A. Taylor, W. Gibson, R. Kimball, M. Brown, A. H. Calvert. and I. R. Judson. ICI D1694, a quinazoline antifolate thymidylate synthase inhibitor that is a potent inhibitor of L1210 tumor cell growth *in vitro* and *in vivo*: a new agent for clinical study. *Cancer Res.* **51**:5579–5586 (1991).
6. Fry, D. W., J. C. Yalowich, and I. D. Goldman. Rapid formation of poly-γ-glutamyl derivatives of methotrexate and their association with dihydrofolate reductase as assessed by high-pressure liquid chromatography in the Ehrlich ascites tumor cell *in vitro*. *J. Biol. Chem.* **257**:1890–1896 (1982).
7. Calvert, A. H., D. L. Alison, S. J. Harland, B. A. Robinson, A. L. Jackman, T. R. Jones, D. R. Newall, Z. H. Siddick, E. Wiltshaw, T. J. McElwain, I. E. Smith, and K. R. Harrup. A phase I evaluation of the quinazoline antifolate thymidylate synthase inhibitor, N^{10}-propargyl-5,8- dideazafolic acid. *J. Clin. Oncol.* **4**:1245–1252 (1986).
8. Beardsley, G. P., E. C. Taylor, B. A. Moroson, and R. G. Moran. 5,10-Dideaza-5,6,7,8-tetrahydrofolate, an exceptionally potent inhibitor of *de novo* purine synthesis. *J. Biol. Chem.* **264**:328–333 (1989).
9. Clarke, S. J., A. L. Jackman, and I. R. Judson. The history of the development and clinical use of CB3717 and D1694. *Adv. Exp. Med. Biol.* **339**:277–287 (1993).
10. Ray, M., F. M. Muggia, G. C. Leichman, S. G. Grunberg, R. Dyke, and R. G. Moran. Phase I study of (6R) 5,10-dideaza-5,6,7,8-tetrahydrofolate (Lometrexol): a folate antimetabolite inhibitory to *de novo* purine synthesis. *J. Nat. Cancer Inst.* **85**:1154–1159 (1993).
11. Clarke, S., W. Ward, A. Planting, J. Spiers, R. Smith, J. Verweij, and I. R. Judson. Phase I trial of ICI D1694: a novel thymidylate synthase inhibitor. *Proc. Am. Assoc. Cancer Res.* **33**:406 (1992).
12. Moran, R. G., and P. D. Colman. Measurement of folylpolyglutamate synthetase in mammalian tissue. *Anal. Biochem.* **140**:326–342 (1984).
13. Cichowicz, D. J., and B. Shane. Mammalian folylpoly-γ-glutamate synthetase. 1: purification and general properties of the hog liver enzyme. *Biochemistry* **26**:504–512 (1987).
14. Jansen, G., J. H. Schornagel, I. Kathmann, G. R. Westerhof, G. J. Hordijk, and B. van der Laan. Measurement of folylpolyglutamate synthetase activity in head and neck squamous carcinoma cell lines and clinical samples using a new rapid separation procedure. *Oncol. Res.* **7**:299–305 (1992).
15. Habeck, L. L., T. A. Leitner, K. A. Shackelford, L. S. Gossett, R. M. Schultz,

S. L. Andis, C. Shih, G. B. Grindey, and L. G. Mendelsohn. A novel class of monoglutamated antifolates exhibits tight-binding inhibition of human glycinamide ribonucleotide formyltransferase and potent activity against solid tumors. *Cancer Res.* **54**:1021–1026 (1994).

16. Baldwin, S. W., A. Tse, L. S. Gossett, E. C. Taylor, A. Rosowsky, C. Shih, and R. G. Moran. Structural features of 5,10-dideaza-5,6,7,8- tetrahydrofolate that determine inhibition of mammalian glycinamide ribonucleotide formyltransferase. *Biochemistry* **30**:1997–2006 (1991).
17. Moran, R. G., S. W. Baldwin, E. C. Taylor, and C. Shih. The 6-*S* and 6-*R* diastereomers of 5,10-dideaza-5,6,7,8-tetrahydrofolate are equiactive inhibitors of *de novo* purine synthesis. *J. Biol. Chem.* **264**:21047–21051 (1989).
18. Pizzorno, G., B. A. Moroson, A. R. Cashmore, O. Rusello, J. R. Mayer, J. Galivan, M. A. Bunni, D. G. Priest, and G. P. Beardsley. Multifactorial resistance to 5,10-dideazatetrahydrofolic acid in cell lines derived from human lymphoblastic leukemia CCRF-CEM. *Cancer Res.* **55**:566–573 (1995).
19. Taylor, E. C., D. Kuhnt, C. Shih, S. M. Rinzel, G. B. Grindey, J. Barredo, M. Jannatipour, and R. G. Moran. A dideazatetrahydrofolate analog lacking a chiral center at C-6, N-[4]-[2-(2-amino-4(3H)-oxo-7H-pyrrolo[2,3-d]-pyrimidin-5yl)ethyl]benzoyl−L-glutamic acid, is an inhibitor of thymidylate synthase. *J. Med. Chem.* **35**:4450–4454 (1992).
20. Jackman, A. L., P. R. Marsham, R. G. Moran, R. Kimball, B. M. O'Connor, L. R. Hughes, and A. H. Calvert. Thymidylate synthase inhibitors: the *in vitro* activity of a series of heterocyclic benzoyl ring modified 2-desamino-2-methyl-N10-substituted-5,8-dideazafolates. *Adv. Enzym. Regul.* **29**:161–179 (1991).
21. McGuire, J. J., C. A. Russell, W. E. Bolanowska, C. M. Freitag, C. S. Jones, and T. I. Kalman. Biochemical and growth inhibition studies of methotrexate and aminopterin analogs containing a tetrazole ring in place of the γ-carboxyl group. *Cancer Res.* **50**:1726–1731 (1990).
22. Sanghani, P. C., V. R. Evans, T. Thornton, T. Boyle, A. H. Calvert, A. L. Jackman, and R. G. Moran. A strategy for the design of membrane-permeable folylpoly-γ-glutamate synthetase inhibitors: "bay region"-substituted 2-desamino-2-methyl-5,8-dideazafolate analogs. *Mol. Pharmacol.* **45**:341–351 (1994).
23. Moran, R. G., P. D. Colman, A. Rosowsky, R. Forsch, and K. K. Chan. Structural features of 4-amino-antifolates required for substrate activity with mammalian folylpolyglutamate synthetase. *Mol. Pharmacol.* **27**:156–166 (1985).
24. Rosowsky, A., H. Bader, R. A. Forsch, R. G. Moran, and J. H. Freisheim. Methotrexate analogs. 31: meta and ortho isomers of aminopterin, compounds with a double bond in the side chain and a novel analog modified at the α-carbon, chemical and *in vitro* biological studies. *J. Med. Chem.* **31**:763–768 (1988).
25. Moran, R. G., and P. D. Colman. Mammalian folylpolyglutamate synthetase: partial purification and properties of the mouse liver enzyme. *Biochemistry* **23**:4580–4589 (1984).
26. George, S., D. J. Cichowicz, and B. Shane. Mammalian folylpoly-γ-glutamate synthetase. 3: specificity for folate analogs. *Biochemistry* **26**: 522–529 (1987).
27. McGuire, J. J., P. Hsieh, J. K. Coward, and J. R. Bertino. Enzymatic synthesis of folylpolyglutamate synthetase: characterization of the reaction and its products. *J. Biol. Chem.* **255**:5776–5788 (1980).
28. Moran, R. G., P. D. Colman, and T. R. Jones. The relative substrate activities of structurally related pteridine, quinazoline, and pyrimidine analogs for mouse liver folylpolyglutamate synthetase. *Mol. Pharmacol.* **36**:736–743 (1989).
29. Taylor, E. C., P. J. Harrington, S. R. Fletcher, G. P. Beardsley, and R. G. Moran. Synthesis of the antileukemic agents 5,10-dideazaaminopterin and 5,10-dideaza-5,6,7,8-tetrahydroaminopterin. *J. Med. Chem.* **28**:914–921 (1985).
30. Ward, W. H. J., R. Kimbell, and A. L. Jackman. Kinetic characteristics of ICI D1694: a quinazoline antifolate which inhibits thymidylate synthase. *Biochem. Pharmacol.* **43**:2029–2031 (1992).
31. Moran, R. G., C. Shih, E. C. Taylor, and G. B. Grindey. Are classical antifolates directed at thymidylate and purine synthesis prodrugs? in *National Cancer Institute-European Organization For Research and Treatment of Cancer Symposium Abstracts* **7**:91 (1992).
32. Moran, R. G., A. Rosowsky, and R. Forsch. A new folate antimetabolite specifically inhibitory to *de novo* purine synthesis. *Proc. Am. Assoc. Cancer Res.* **25**:311 (1984).
33. Dev, I. K., W. S. Dallas, R. Ferone, M. Hanlon, D. D. McGee, and B. B. Yates. Mode of binding of folate analogs to thymidylate synthase: evidence for two asymmetric but interactive substrate binding sites. *J. Biol. Chem.* **269**:1873–1882 (1994).
34. Garrow, T. A., A. Admon, and B. Shane. Expression cloning of a human cDNA encoding folylpoly(γ-glutamate) synthetase and determination of its primary structure. *Proc. Natl. Acad. Sci. USA* **89**:9151–9155 (1992).

Send reprint requests to: Dr. Laurane G. Mendelsohn, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46285.