Structural Features of 5,10-Dideaza-5,6,7,8-tetrahydrofolate That Determine Inhibition of Mammalian Glycinamide Ribonucleotide Formyltransferase†

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ABSTRACT: We have investigated the structural features of 5,10-dideaza-5,6,7,8-tetrahydrofolate (DDATHF) that determine the activity of this compound as an inhibitor of glycinamide ribonucleotide formyltransferase (GARFT) purified from mouse L1210 cells. 5-Deazatetrahydrofolate was as good an inhibitor of GARFT as DDATHF, indicating that isosteric replacement of nitrogen by carbon at the 5-position of tetrahydrofolate is sufficient for inhibition of GARFT. 5,10-Dideazafolic acid, 5,8,10-trideazatetrahydrofolate, and 2-desamino-5,10-dideazatetrahydrofolate were poor inhibitors of GARFT, indicating that a reduced pyridopyrimidine ring, N-8, and the 2-amino group of DDATHF, respectively, play an important role in the binding of tetrahydrofolate analogues to this enzyme. DDATHF analogues in which the phenyl ring was replaced either by a cyclohexyl ring or by methylene groups retained activity as inhibitors. 5,10-Dideazatetrahydrohomofolate was about 6 times more potent as an inhibitor of GARFT than DDATHF, but 5,10-dideazatetrahydronorfolate had about one-fifth of the activity of DDATHF. An analogue of DDATHF in which the glutamic acid side chain was replaced by aspartic acid (which was not a substrate for polyglutamation and was only weakly cytotoxic) was equiactive with DDATHF as an inhibitor of purified GARFT. Surprisingly, 5,10-dideazatetrahydropteroic acid was about as active as DDATHF as an inhibitor of GARFT, an indication that the glutamic acid in the side chain of DDATHF does not play a role in this ligand–enzyme interaction. The polyglutamate derivatives of DDATHF bound up to 100 times tighter to GARFT than DDATHF itself; longer chain polyglutamates conformed to Goldstein's zone B behavior under experimental conditions and were projected to be in zone C, i.e., stoichiometric inhibition, in vivo. We conclude that the presence of carbon at the 5-position of tetrahydrofolate analogues is sufficient for inhibition of GARFT, that N-8 and the 2-amino group are involved in binding of DDATHF to GARFT, probably through hydrogen bonds, and that the structures of the phenyl ring and amino acid side chain of DDATHF analogues are not primary determinants of GARFT inhibition by monoglutamate forms of these compounds. We also conclude that polyglutamation plays a major role in the potent cytotoxicity of DDATHF.

5,10-Dideaza-5,6,7,8-tetrahydrofolate (DDATHF)[1] is a new folate antimetabolite that has virtually no activity as an inhibitor of the two "classical" targets for antifolates, dihydrofolate reductase (EC 1.5.1.3) and thymidylate synthase (EC 2.1.1.45) (Taylor et al., 1985). Unlike the widely used antifolate methotrexate, which is a structural analogue of folic acid, DDATHF is an analogue of tetrahydrofolate, differing from the natural metabolite only by substitutions of carbon for nitrogen at positions 5 and 10. It has been shown that DDATHF prevents the proliferation of tumor cells in culture (Taylor et al., 1985; Beardsley et al., 1989; Moran et al., 1989) and in vivo (Beardsley et al., 1986; Shih et al., 1988), causes purine nucleotide depletion (Beardsley et al., 1989), and inhibits de novo purine synthesis in vivo as a result of a profound effect on glycinamide ribonucleotide formyltransferase (GARFT) (EC 2.1.2.1) (Beardsley et al., 1989; Moran et al., 1989), the first folate-dependent enzyme in the purine biosynthetic pathway.

DDATHF was found to be a substrate for mammalian folylpolyglutamate synthetase (FPGS) in vitro (Taylor et al., 1985; Moran et al., 1989) and has been shown to be metabolized to polyglutamate forms in vivo (Pizzorno et al., 1990). Polyglutamation of folates and folate analogues leads to increased cellular retention and to sustained exposure of intracellular enzymes to drug (Fry et al., 1982; Moran, 1983). While polyglutamation of methotrexate has a small effect on binding affinity for dihydrofolate reductase (Kumar et al., 1986; Appleman et al., 1988), polyglutamation of quinazoline antifolates, such as 10-propargyl-5,8-dideazafolic acid, leads to an ~100-fold increase in affinity for thymidylate synthase (Cheng et al., 1985; Sikora et al., 1988).

DDATHF has shown promise as a chemotherapeutic agent against experimental rodent solid tumors (Beardsley et al., 1986; Shih et al., 1988) and, also, during its first use in patients with cancer (Muggia et al., 1990). As a result, it became of significant interest to understand the basis of the potent antiproliferative and cytotoxic activity of DDATHF and to determine what structural features of the DDATHF molecule are responsible for inhibition of de novo purine synthesis. We have compared the activity of a series of DDATHF analogues as inhibitors of GARFT purified to electrophoretic homoge-

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ncity from mouse L1210 cells. We conclude that the replacement of the nitrogen at the 5-position of tetrahydrofolate by a carbon is sufficient for the inhibition of GARFT but that an amino substituent at the 2-position or a nitrogen at position 8 of a reduced pyridopyrimidine (or the structurally equivalent pyrimidine) is necessary for the inhibition of GARFT by DDATHF analogues, that substantial structural variation at other positions allows retention of activity, and that the polyglutamates of DDATHF are much more active inhibitors of GARFT than is the parent molecule.

Materials and Methods

Materials. Cyanogen bromide activated Sepharose 4B was purchased from Sigma Chemical Co. (St. Louis, MO), ethylenediamine was from Aldrich Chemical Co. (Milwaukee, WI), and RPMI 1640 cell culture medium and fetal calf serum were from GIBCO (Grand Island, NY). All other general chemicals were from Sigma Chemical Co.

(6*S*)- and (6*R*,*S*)-Tetrahydrofolate were prepared by enzymatic reduction of dihydrofolic acid (Moran et al., 1976) and by reduction of folic acid with dimethylamine–borane complex (Martinelli & Chaykovsky, 1980), respectively, and were purified and stored as previously described (Moran et al., 1976). The syntheses of (6*R*,*S*)-DDATHF (Taylor et al., 1985), of (6*R*,*S*)-5-deazatetrahydrofolate (Taylor et al., 1989), and of (6*R*,*S*)-5,8,10-trideazatetrahydrofolate (Rosowsky et al., 1989) have been previously described as has been the separation of the diastereomers of DDATHF by fractional crystallization (Moran et al., 1989). The syntheses of the other deazatetrahydrofolates and of the polyglutamates of DDATHF will be the topic of separate publications. Initially, a sample of 10-formyl-5,8-dideazatetrahydrofolate was generously provided by Dr. Terence R. Jones of Agouron Pharmaceuticals, Inc. (La Jolla, CA); subsequently, this compound was made by a published procedure (Hynes et al., 1977). α,β-Glycinamide ribonucleotide was a gift of Dr. Homer Pearce of Lilly Research Laboratories (Indianapolis, IN) and was prepared by a modification of the procedure of Chettur and Benkovic (1977). (6*S*)-5-Formyltetrahydrofolate was prepared as previously described (Moran & Colman, 1985). (6*R*)-10-Formyltetrahydrofolate was prepared from (6*S*)-5-formyltetrahydrofolate by acidifying a solution to pH 1 for 20 min and then adjusting the pH to 8 for 30 min in the presence of 1% 2-mercaptoethanol (Rabinowitz, 1970).

Cell Culture. Mouse L1210 cells were grown in RPMI 1640 medium supplemented with 5–10% fetal calf serum. For enzyme purifications, 20-L batches of cells were grown to a density of (8–10) $\times 10^5$ cells/mL, and cells were harvested by centrifugation. Cultures were initiated from mycoplasma-free frozen stocks at 3-month intervals and were found to be free of mycoplasma by use of a kit (Gene-Probe, San Diego, CA) for the detection of mycobacterial rRNA prior to discarding working stocks.

Enzyme Purification. GARFT was purified by a two-step procedure. Pellets of L1210 cells (15–25 g) were washed once with phosphate-buffered saline, resuspended in 3 volumes of ice-cold PBS, and disrupted by sonication on ice. All subsequent steps were at 0–4 °C. The supernatant from a 1-h 165000*g* centrifugation step was precipitated first with 30% $(NH_4)_2SO_4$ and then with 70% $(NH_4)_2SO_4$. The 30–70% $(NH_4)_2SO_4$ precipitate was resuspended in buffer A [10 mM sodium phosphate, pH 7.5, containing 0.1% Triton X-100, 20 mM deoxyuridylic acid (dUMP), and 10 mM 2-mercaptoethanol] and was dialyzed overnight against buffer A. An affinity column was prepared by linking ethylenediamine to cyanogen bromide activated Sepharose 4B and then linking 10-formyl-5,8-dideazafolic acid to the ethylenediamine with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (Rode et al., 1979); the column was then washed extensively (Young et al., 1984). Dialyzed $(NH_4)_2SO_4$ precipitate was applied to a 1 × 5 cm column of affinity resin at a flow rate of 0.3 mL/min. The column was washed with 5 volumes of buffer A and then with 400 mL of buffer B (0.2 M sodium phosphate, pH 7.5, containing 0.5 M KCl, 10 mM 2-mercaptoethanol, 0.1% Triton X-100, and 20 mM dUMP), a slow overnight wash being used. To elute GARFT, 3 mL of a 3 mM solution of (6*R*)-10-formyltetrahydrofolate was applied to the column, and the column was incubated for 30 min at 4 °C; GARFT was then eluted with this same solution. When the column was subsequently washed with 8 M urea, there was no evidence of residual GARFT elution as judged by SDS–PAGE. This purification scheme was designed to allow preparation of both GARFT (Young et al., 1984) and thymidylate synthase (Rode et al., 1979) from the same sample. For some purifications, the procedure was modified to allow purification of only GARFT by omiting dUMP from buffers A and B.

Enzyme Assays. GARFT activity was determined spectrophotometrically by following the conversion of 10-formyl-5,8-dideazafolic acid to 5,8-dideazafolic acid with $\Delta\epsilon = 18\,900\ M^{-1}\ cm^{-1}$ at A_{295nm} (Smith et al., 1981). Reactions were performed at 25 °C in 75 mM Hepes buffer, pH 7.5, containing 20% glycerol and 45 mM α-thioglycerol. Routine assays were performed with 11 μM 10-formyl-5,8-dideazafolic acid and 10 μM GAR. One unit of enzyme activity represented 1 μmol of product formed per minute. Additions to the reactions were preincubated for 1 min prior to the initiation of reaction with enzyme. (6*R*)-10-Formyltetrahydrofolate was removed from enzyme samples prior to experiments by passage through a 10-mL Sephadex G-50 column.

[1] Abbreviations: GARFT, glycinamide ribonucleotide formyltransferase (EC 2.1.2.1); FPGS, folylpoly-γ-glutamate synthetase; DDATHF, 5,10-dideaza-5,6,7,8-tetrahydrofolate; GAR, α,β-glycinamide ribonucleotide; dUMP, 2′-deoxyuridine 5′-monophosphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The compounds studied are refered to by trivial names in this publication as follows: DDATHF, (±)-*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-desamino-DDATHF, (±)-*N*-[4-[2-(3,4,5,6,7,8-hexahydro-4-oxopyrido-[2,3-*d*]pyrimidin-6-yl)ethyl]benzoyl]-L-glutamic acid; 5-deazatetrahydrofolic acid, (±)-*N*-[4-[[(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)methyl]amino]benzoyl]-L-glutamic acid; 5,8,10-trideazatetrahydrofolic acid, (±)-*N*-[4-[2-(2-amino-3,4,5,6,7,8-hexahydro-4(3*H*)-oxoquinazolin-6-yl)ethyl]benzoyl]-L-glutamic acid; tetrahydrofolic acid, (±)-*N*-[4-[[(2-amino-3,4,5,6,7,8-hexahydro-4-(3*H*)-oxopyrazino[2,3-*d*]pyrimidin-6-yl)methyl]amino]benzoyl]-L-glutamic acid; 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; acyclo-DDATHF, *N*-[4-[4-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)butyl]benzoyl]-L-glutamic acid; nor-DDATHF, (±)-*N*-[4-[(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)methyl]-benzoyl]-L-glutamic acid; 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; abenzyl-DDATHF (n = 2), (±)-*N*-[5-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)-1-oxopentyl]-L-glutamic acid; abenzyl-DDATHF (n = 3), (±)-*N*-[6-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)-1-oxohexyl]-L-glutamic acid; abenzyl-DDATHF (n = 4), (±)-*N*-[7-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)-1-oxoheptyl]-L-glutamic acid; 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; 5,10-dideazatetrahydropteroic acid, (±)-4-[2-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)ethyl]benzoic acid; 5,10-dideazatetrahydropteroylaspartic acid, (±)-*N*-[4-[2-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)ethyl]benzoyl]-DL-aspartic acid.

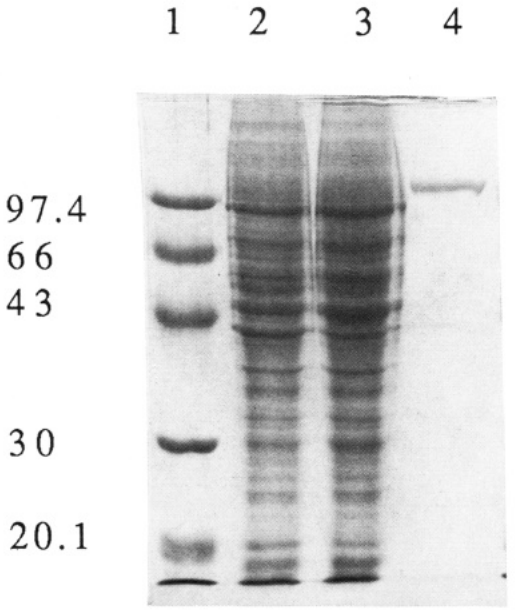


FIGURE 1: SDS–polyacrylamide gel electrophoresis of L1210 GARFT during purification. Aliquots of protein were subjected to SDS–PAGE at each step of the purification of GARFT from L1210 cells. (Lane 1) Molecular weight markers of 97 400, 66 000, 43 000, 30 000, and 20 100. (Lane 2) 30 μg of cell extract. (Lane 3) 30 μg of 30–70% $(NH_4)_2SO_4$ pellet. (Lane 4) 5 μg of GARFT eluted from the affinity resin.

Analogue Concentrations. Analogues were dissolved in GARFT assay buffer and the concentrations determined spectrophotometrically with the extinction coefficients listed in Table I.

Kinetic Analysis. The estimation of K_i values was made from experiments in which 10-formyl-5,8-dideazafolate concentrations were varied in the presence of three or four concentrations of inhibitor. Data were fitted to a rectangular hyperbola with the program of Cleland (1967) and K_i values determined from the intercept of a replot of slope vs analogue concentration. All experiments were performed at least twice with duplicate points at five concentrations of 10-formyl-5,8-dideazafolate. It was calculated from simple binding equilibria that a Lineweaver–Burk analysis would be legitimate (less than 15% of total inhibitor bound to enzyme at $E = I$) for inhibitors with a K_i value of ≥ 8 nM by use of a total enzyme concentration of 20 nM in 1-mL cuvettes. This limit could have been extended to somewhat lower K_i values by use of high competing substrate concentrations. However, for more potent inhibitors, K_i values were derived either by use of the Henderson relationship or by fitting data to the Morrison equation (eq 1) (see Results) with the ENZFITTER microcomputer package (Elsevier Science Publishers, Amsterdam) with proportional weighting of the data. This allowed estimation of the K_{iapp}, while correcting for depletion of free inhibitor due to binding to enzyme and, also, specification of total enzyme levels as an invariant parameter. For these experiments, enzyme concentrations were calculated from uninhibited reaction velocities with a specific activity of 1.2 units/mg of protein when assayed with 10 μM GAR and 11 μM 10-formyl-5,8-dideazafolic acid. These values of K_{iapp} were then converted to estimates of K_i by assuming a competitive interaction between folate substrate and inhibitor and by correcting for the K_m of the substrate as indicated in eq 2 (see Results).

RESULTS

Purification of Glycinamide Ribonucleotide Formyltransferase (GARFT). GARFT was purified from mouse L1210 cells by affinity chromatography with 10-formyl-5,8-dideazafolate as a ligand. Enzyme was purified 435-fold from a 160000g supernatant to a specific activity of 0.8 IU/mg of protein when assayed at 25 °C with 75 μM 10-formyl-5,8-dideazafolate and 200 μM GAR[2] as substrates. The specific elution of GARFT from this affinity column was accomplished with (6R)-10-formyltetrahydrofolate in place of the 2 M urea wash used by others (Daubner & Benkovic, 1985). This was similar to the use of 10-formyl-5,8-dideazafolic acid to elute L5178Y GARFT from a 10-formyl-5,8-dideazafolic acid affinity column (Caperelli, 1985). A single band corresponding to a M_r of 110 000 was observed on denaturing SDS gel electrophoresis (Figure 1) for affinity-purified enzyme. Of the enzyme activity in the 160000g supernatant fraction, 60–80% was recovered as purified enzyme. GARFT prepared according to this procedure was stable to storage at −25 °C for at least 2 months, and proteolytic degradation was not detectable on SDS gels after up to 4 h at 37 °C.

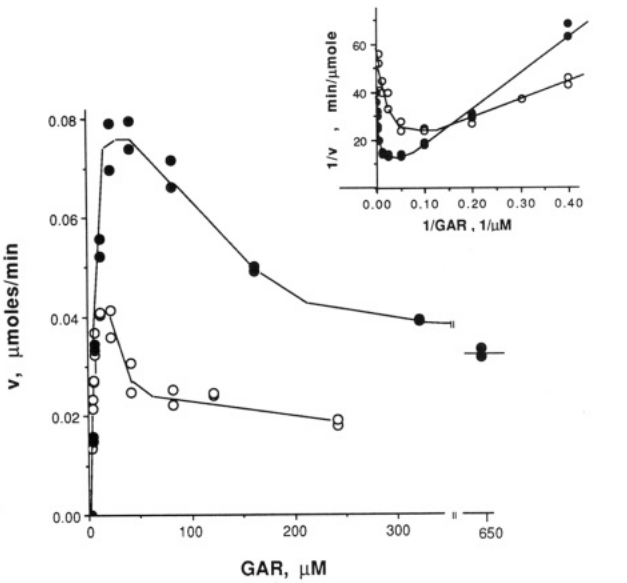


FIGURE 2: Substrate inhibition of L1210 GARFT by GAR. The rate of reaction was measured in the presence of the indicated concentrations of GAR and either 11 μM 10-CHO-5,8-dideazafolic acid (○) or 60 μM (6S)-10-formyltetrahydrofolate (●). The inset is a replot of $1/v$ (min/μmol) versus 1/GAR (1/μM).

During initial studies with L1210 GARFT, it was found that even highly (but incompletely) purified enzyme was readily proteolyzed to lower molecular weight fragments, several of which had catalytic activity (data not shown), an observation also reported by others (Daubner & Benkovic, 1985). Smaller fragments not only had GARFT activity, but at least one such fragment was a more efficient catalyst with a higher turnover number than that of the 110000-dalton enzyme.[3] Presumably, some of the other characteristics of these active fragments will also prove different from those of cellular enzyme. Hence, it was concluded that GARFT preparations for this type of study must be highly purified and stable during incubations. The procedure described for purification sufficed these requirements, so long as enzyme was quickly prepared from harvested L1210 cells.

[2] The assay conditions used during purification procedures were based on the high K_ms reported in the earlier literature (Daubner & Benkovic, 1985; Caperelli, 1985) for mouse leukemic cells. These concentrations of GAR result in significant substrate inhibition (Figure 2) and an underestimate of the specific activity. In more recent literature on mouse leukemic enzyme (Caperelli, 1989) K_m values agree with those reported in this paper.

[3] S. W. Baldwin and R. G. Moran, unpublished observations.

Table I: Activity of DDATHF Analogues as Inhibitors of GARFT[a]

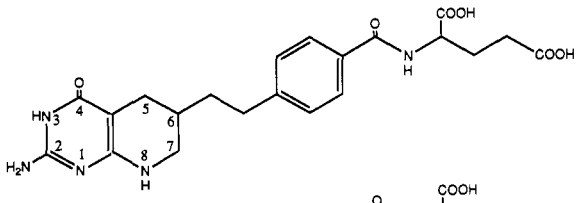
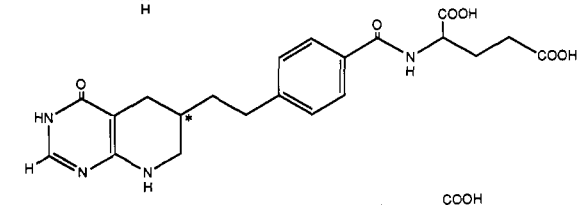
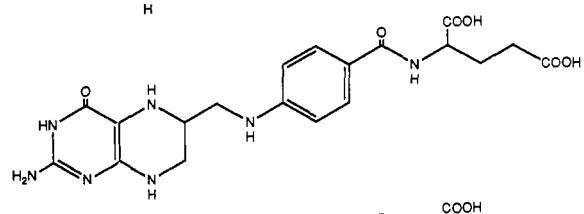
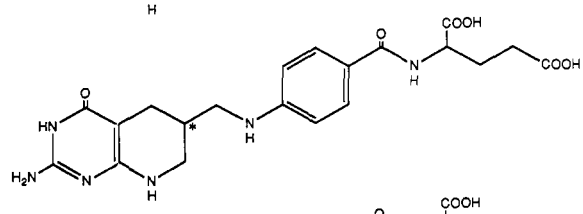
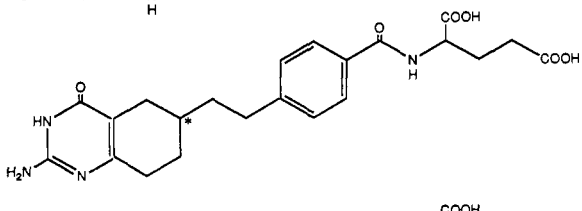
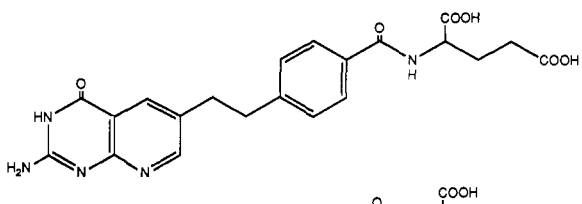
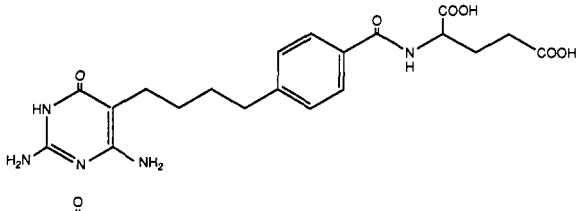
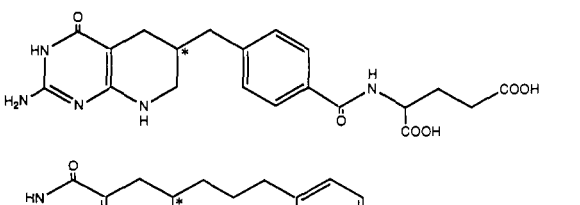
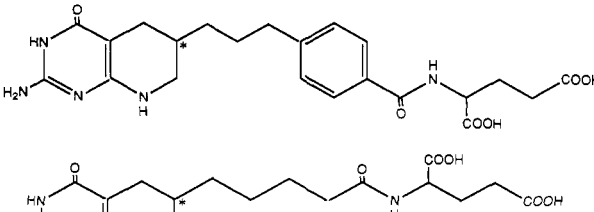
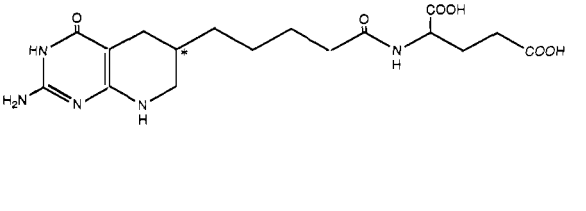
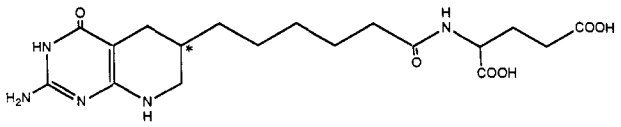
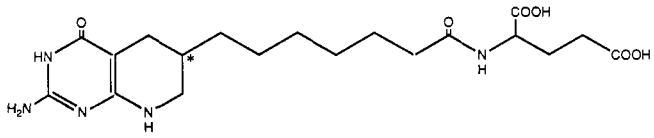
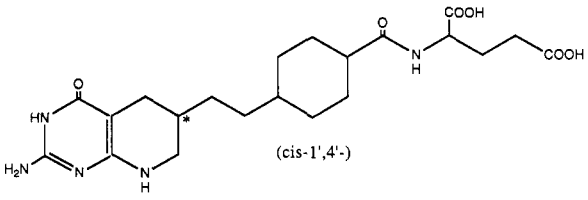
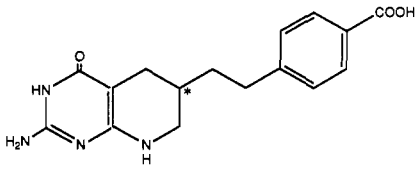
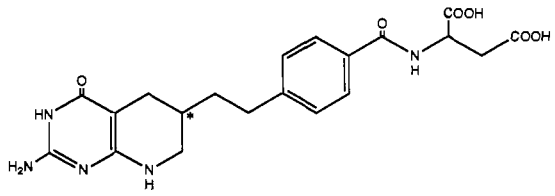
structure	compound	ϵ (cm^{-1} M^{-1}) (λ_{max}, nm)[b]	K_i (μM)
	(6*R*,*S*)-DDATHF	11 700 (272)	0.12 ± 0.02
	(6*R*)-DDATHF	11 700 (272)	0.10 ± 0.02
	(6*S*)-DDATHF	11 700 (272)	0.029 ± 0.012
	2-desamino-DDATHF	9000 (269)	27 (23, 31)
	(6*R*,*S*)-tetrahydrofolic acid	9200 (292)	6.0 (5.8, 6.1)
	(6*S*)-tetrahydrofolic acid	9200 (292)	6.3 (5.0, 7.6)
	5-deazatetrahydrofolic acid	23 800 (278)	0.065 (0.064, 0.066)
	5,8,10-trideazatetrahydrofolic acid	21 700 (237) 8300 (269)	12 (9.0, 15)
	5,10-dideazafolic acid	6400 (335)	13 (12, 14)
	acyclo-DDATHF	12 300 (273)	0.38 (0.47, 0.29)
	nor-DDATHF	10 970 (272)	0.63 (0.72, 0.54)
	homo-DDATHF	12 000 (272)	0.019 ± 0.009
	abenzyl-DDATHF (n = 2)	10 400 (274)	0.049 (0.061, 0.036)

Table I (Continued)

structure	compound	ϵ (cm^{-1} M^{-1}) (λ_{max}, nm)[b]	K_i (μM)
	abenzyl-DDATHF (n = 3)	10400 (274)	0.028 (0.035, 0.021)
	abenzyl-DDATHF (n = 4)	10400 (274)	0.018 ± 0.006
	cyclohexyl-DDATHF	10400 (274)	0.025 (0.022, 0.027)
	5,10-dideazatetraydropteroic acid	11700 (272)	0.15 (0.16, 0.14)
	5,10-dideazatetrahydropteroylaspartic acid	11700 (273)	0.047 (0.032, 0.062)

[a] Mixture of 6*R* and 6*S* diastereomers is indicated by an asterisk in the structure. Kinetic constants were obtained from replots of the slope of Lineweaver–Burk plots versus inhibitor concentrations with at least three concentrations of inhibitor. Kinetic constants and slopes were determined with the program of Cleland (1967). The values listed are the means of three or more determinations (±SD) or the means of two experiments with individual values in parentheses. [b] Extinction coefficients were measured in 0.1 N NaOH.

The Michaelis constants observed for the reaction using (6*R*)-10-formyltetrahydrofolate and α,β-GAR as substrates were 6.1 ± 1.1 μM (n = 3) and 18.5 ± 2.3 μM (n = 3), respectively. For the reaction using 10-formyl-5,8-dideazafolate and GAR, K_m values[4] were 1.3 ± 0.2 μM (n = 16) and 4.3 ± 0.4 μM (n = 8), respectively. Substate inhibition was observed as a function of GAR when either 10-formyl-5,8-dideazafolate or (6*R*)-10-formyltetrahydrofolate was used as a cosubstrate (Figure 2). Maximum reaction rates (V_{max}s) obtained by extrapolation of the straight-line portions of plots such as Figure 2 were 1.2 and 2.8 units/mg of protein when measured with saturating concentrations of 10-formyl-5,8-dideazafolate and (6*R*)-10-formyltetrahydrofolate, respectively.

Features of DDATHF Structure Essential for GARFT Inhibition. Several deazafolates that are structurally related to DDATHF were found to inhibit L1210 GARFT (Table I). Tetrahydrofolate itself was a weak inhibitor of the GARFT reaction; inhibition was competitive with the 10-formyl-5,8-dideazafolate substrate. The tetrahydrofolate diastereomer with the 6*S* configuration was as inhibitory as the 6*R*,*S* mixture. (6*R*,*S*)-DDATHF, which has been shown to be a potent inhibitor of de novo purine synthesis in intact tumor cells (Beardsley et al., 1989), was a moderately potent inhibitor of GARFT. Because DDATHF has near-isosteric replacement of both N-5 and N-10 of tetrahydrofolate with carbon atoms, it was of interest to determine whether one of these modifications was sufficient for inhibition of GARFT. In order to address this question, 5-deazatetrahydrofolate was compared with DDATHF as an inhibitor of GARFT. (6*R*,*S*)-5-Deazatetrahydrofolate not only was an inhibitor of GARFT but was somewhat more potent than DDATHF in this regard (Table I). With DDATHF, as with all of the analogues described in Table I, inhibition of GARFT was competitive with the folate substrate, as exemplified by the data for homo-DDATHF in Figure 3A. With GAR as the variable substrate, inhibition was compatible with a noncompetitive model (Figure 3B). This kinetic behavior agreed with the ordered sequential binding mechanism described by Caparelli (1989). Interestingly, (6*R*,*S*)-5,8,10-trideazatetrahydrofolate was 100 times less potent than DDATHF as an inhibitor of L1210 GARFT as was 5,10-dideazafolate (Table I). In spite of the fact that these specific alterations in the structure of the tetrahydropyridine ring of DDATHF virtually eliminated GARFT inhibitory activity, an open-chain analogue equivalent to DDATHF with the 7-carbon removed was as active as DDATHF as an inhibitor of GARFT (Table I). In previous studies on a series of quinazoline analogues inhibitory to thymidylate synthase (Jones et al., 1989), it was found the 2-desamino analogues of CB 3717 were only slightly less active than the corresponding 2-amino compounds. In contrast, the 2-desamino analogue of DDATHF was >200-fold less active than DDATHF itself against GARFT (Table I). This is reminescent of the inactivity of 2-desaminomethotrexate and

[4] It appears (Caperelli, 1989) that $K_{ia}/K_A \sim 1$ and, therefore, that these values approximate true K_m values.

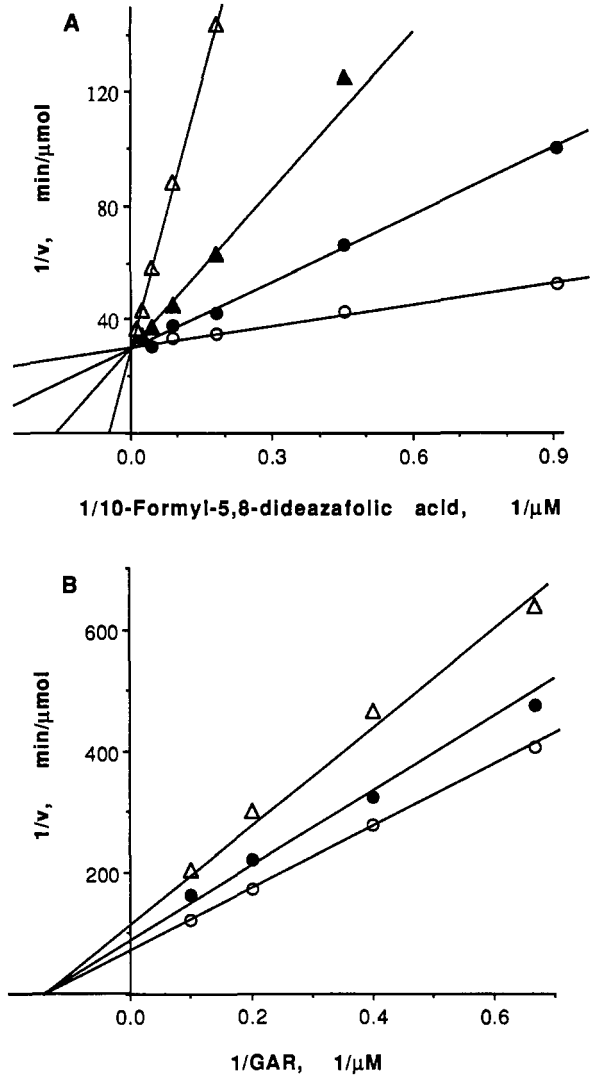


FIGURE 3: Kinetics of inhibition of GARFT activity by DDATHF analogues. (A) Lineweaver–Burk plot of the rate of the GARFT reaction in the presence of increasing concentrations of 10-formyl-5,8-dideazafolic acid and homo-DDATHF [at 0.014 (●), 0.036 (▲), and 0.17 μM (△)]. The GAR concentration was 10 μM. (B) Lineweaver–Burk plot for reaction rates observed in the presence of 0.035 μM (●) and 0.12 μM (△) homo-DDATHF at various concentrations of GAR. The 10-formyl-5,8-dideazafolic acid concentration was 11 μm. Uninhibited reactions are indicated by (○) in both panels.

2-desaminopterin as inhibitors of dihydrofolate reductase (Rosowsky et al., 1989a), which reflects the involvement of a hydrogen bond between the 2-amino group of methotrexate and an active site aspartate or glutamate as seen by X-ray crystallography (Matthews et al., 1978; Bolin et al., 1982).

Inhibition of GARFT by DDATHF Analogues Modified in the Bridge and Phenyl Ring Regions. The above results raised the question of which of the other regions of the DDATHF molecule contribute to the interaction of the drug with the protein. A series of analogues of DDATHF were available in which either the length of the methylene bridge between the pyridopyrimidine ring and the phenyl ring was changed or the phenyl ring was replaced by a methylene bridge of variable length or by a saturated ring system. These analogues served as probes for the binding of the central portion of the DDATHF molecule to GARFT. 5,10-Dideazatetrahydrohomofolate, the analogue of DDATHF with one more methylene unit between the heterocycle and the phenyl ring, was 6 times more potent as an inhibitor of GARFT than the parent compound (Table I). However, 5,10-dideazatetrahydronorfolate, the DDATHF analogue with only one methylene group between the heterocycle and the phenyl ring, inhibited GARFT only at 5-fold higher concentrations than DDATHF. Replacement of the phenyl ring of DDATHF by either two, three, or four methylene units did not abrogate activity against GARFT. There was a clear increase in binding affinity of analogues to enzyme as the methylene chain was extended either in the bridge region between the heterocycle and phenyl ring [compare the K_i values measured for 5,10-dideazatetrahydronorfolate (0.62 μM), DDATHF (0.12 μM), and 5,10-dideazatetrahydrohomofolate (0.018 μM)] or in place of the phenyl ring (Table I). An analogue of DDATHF in which the phenyl ring was replaced by a cyclohexyl group was a moderate inhibitor of GARFT, with a K_i lower than that of DDATHF. It was concluded that π–π interactions with the aromatic system of the phenyl ring are unimportant to binding and that the spatial requirements for the bridge region/phenyl ring region were not rigid.

Requirement for the Glutamic Acid Side Chain for GARFT Inhibitory Activity. 5,10-Dideaza-5,6,7,8-tetrahydropteroic acid was found to be about as potent an inhibitor of GARFT as DDATHF (Table I), a clear indication that interaction of the side chain of the monoglutamate form of DDATHF with active sites residues does not contribute to the association of inhibitor with GARFT. Likewise, 5,10-dideaza-5,6,7,8-tetrahydropteroylaspartate was equivalent to DDATHF as an inhibitor of GARFT, and again, inhibition was competitive with the folate substrate. It was of interest that the K_i for the complex of GARFT with the analogue bearing an aspartate side chain was identical with that of DDATHF, in spite of the fact that 5,10-dideazatetrahydropteroylaspartate was 30-fold less inhibitory to the growth of intact tumor cells than DDATHF (Moran et al., 1989).

Interaction of DDATHF Polyglutamates with GARFT. DDATHF and most of the deazafolates listed in Table I have been shown to be efficient substrates for mammalian FPGS (Taylor et al., 1989; Moran et al., 1989, 1990; Rosowsky et al., 1989a). This suggested that the accumulation of polyglutamate forms of DDATHF was involved in the cytotoxicity of this compound and could account for the discrepancy between the relative cytotoxicities and GARFT inhibitory activities of DDATHF and 5,10-dideazatetrahydropteroylaspartate [Table I and Moran et al. (1989)]. The activity of polyglutamate forms of DDATHF as inhibitors of GARFT was examined in order to directly determine the potential for involvement of these metabolites in the cytotoxicity of DDATHF. When the association of GARFT and polyglutamate derivatives of DDATHF was initially estimated with Dixon plots, it was found that the concentration of compound needed to inhibit GARFT sharply decreased as the side-chain length increased from one to five residues (Table II), so that K_i values less than 1 nM were calculated for the pentaglutamate derivative. Given that the amount of GARFT used in these experiments was in the range of 2–4 nM, any kinetic assessment of inhibitors of that potency that was based on the assumption that enzyme-bound inhibitor is a negligible fraction of the total inhibitor (such as Lineweaver–Burk or Dixon analysis) would be invalid. Therefore, kinetic data were fit to the nonlinear equation described by Morrison (1969):

$$v_i = (v_o/2E_t)\{E_t - K_{iapp} - I_t + [(E_t - K_{iapp} - I_t)^2 + 4K_{iapp}E_t]^{1/2}\} \quad (1)$$

where v_o and v_i are the uninhibited and inhibited reaction rates, respectively, E_t and I_t are the total concentrations of enzyme and inhibitor, and K_{iapp} is an apparent dissociation constant whose meaning depends on the mode of interaction of inhibitor

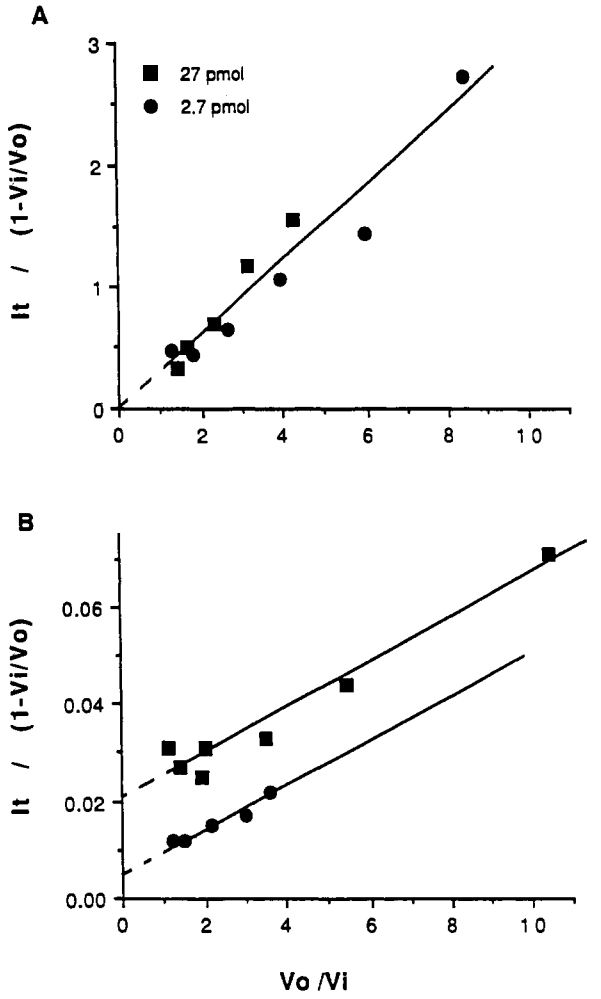


FIGURE 4: Henderson plot of the relationship between total inhibitor concentration (I_t) and inhibited reaction velocity (v_i) at different total enzyme concentrations for (6*R*,*S*)-DDATHF (B) and the pentaglutamate derivative of (6*R*,*S*)-DDATHF (A). Data on the GARFT reaction rate observed in the presence of 2.7 and 27 pmol of GARFT per 1-mL assay at varying concentrations of inhibitors were replotted to fit the Henderson equation (see text).

with the enzyme. K_i values were derived from the computer-fit estimates of K_{iapp}, assuming that inhibition was competitive with the folate substrate, using the relationship

$$K_{iapp} = K_i(1 + S/K_m) \quad (2)$$

Data were also fit to the relationship derived by Henderson (1972):

$$\frac{I_t}{1 - (v_i/v_o)} = K_{iapp}(v_o/v_i) + E_t \quad (3)$$

which allows graphical estimation of the strength of tight-binding interactions.

Analysis of the inhibition of L1210 GARFT by the monoglutamate form of DDATHF using eq 3 for total enzyme concentrations of 2.7 and 27 nM indicated zone A behavior (Figure 4) and allowed estimation of a K_i value (Table II) somewhat lower than the K_i for this compound estimated from Lineweaver–Burk analysis[5] (Table I). However, the pentaglutamate form of DDATHF gave parallel lines of finite slope. This indicates that the K_i of the pentaglutamate form of DDATHF was too low to allow for zone A behavior but was in a range less than 1 nM. Solution of the Henderson equation for these data sets allowed estimates of 33 and 0.40 nM for the K_is of the interactions of the monoglutamate and pentaglutamate forms of drug, respectively. However, because the Henderson relationship is not a function, statistical limits on these estimates could not be set by this technique. Use of an iterative computer fit to the nonlinear Morrison equation allowed the K_i estimates for the polyglutamated derivatives listed in Table II and also allowed estimation of the goodness of fit of the data to these estimates.

[5] The K_i value derived from Lineweaver–Burk analysis seems more accurate, given that it was based on data drawn from a larger set of substrate–inhibitor reaction rate values.

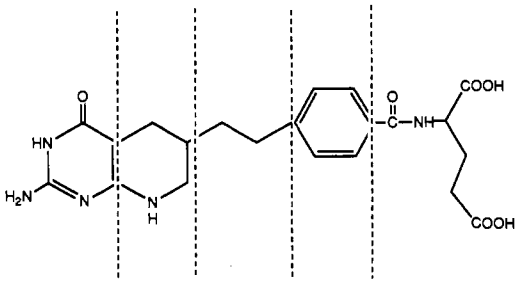
Table II: Inhibition of GARFT by (6*R*,*S*)-DDATHF Polyglutamates

no. of glutamates	K_i (nM)		
	Dixon plot[a]	Henderson plot[b]	Morrison eq[c]
1[d]	39 ± 4 (n = 6)	33	37 ± 5 (n = 6)
2	5.8 (5.6, 6.0)		6.1 (5.9, 6.2)
3	2.7 ± 1.6 (n = 3)		3.4 ± 1.5 (n = 3)
4	1.4 (1.0, 1.7)		1.2 (1.1, 1.3)
5	0.39 ± 0.15 (n = 5)	0.40	0.47 ± 0.01 (n = 5)

[a] Values listed are the mean (±SD) of n replicate experiments or the mean of two experiments with individual values in parentheses. [b] K_i was estimated with eqs 2 and 3. [c] Data were fitted to eq 1 with ENZFITTER to determine the K_{iapp}, and the K_i was calculated from eq 2. Values listed are the mean (±SD) of n replicate experiments or the average of two experiments with the invidual values in parentheses. The goodness of fit of the data to eq 1 was indicated by the average SE of deviation from the function after the residuals were minimized; this value was 5.1, 0.7, 0.46, 0.22, and 0.04 when applied to the K_i values for the mono- to pentaglutamate derivatives, respectively. [d] Lineweaver–Burk analysis indicated a K_i value of 0.12 μM for the monoglutamate of DDATHF (see Table I).

Discussion

A comparison of the structures of the compounds we have studied with their activities as inhibitors of GARFT allowed some deductions to be drawn about the mode of binding of DDATHF to the active site of this enzyme. These compounds are probably best viewed as analogues of the tetrahydrofolate product formed by the GARFT reaction rather than of the 10-formyltetrahydrofolate substrate, given that tetrahydrofolate itself is an inhibitor of the forward reaction (Table I). There are several observations we have made that suggest a model for the binding interactions of DDATHF with GARFT:



Pyrimidine Ring. Because replacement of the 2-amino function with a hydrogen virtually eliminated inhibitory activity, we postulate that the 2-amino group participates in one or more hydrogen bonds to active site residues or to bridging water molecules.

Tetrahydropyridine Ring. Some changes in the structure of the tetrahydropyridine ring of DDATHF had a major effect on binding of reduced analogues to GARFT. The near-isosteric replacement of N-5 of tetrahydrofolate with carbon created a very potent inhibitor of GARFT: the difference in the association constants for the binding of 5-deazatetra-

hydrofolate and of tetrahydrofolate to GARFT was equivalent to 2.7 kcal/mol. It appears that the modification of N-5 of tetrahydrofolate rather than the replacement of N-10 of the naturally occurring compound by carbon was responsible for the activity of DDATHF as an inhibitor of GARFT. Likewise, replacement of N-8 of DDATHF by a carbon atom decreased the strength of binding to GARFT by almost 3 kcal/mol. Although any of several factors could be at the root of the tighter binding of compounds with carbon at the 5-position, the poorer binding with removal of N-8 seems to indicate that a hydrogen bond between the N-8 hydrogen and an active site residue on GARFT is essential for the tight binding of DDATHF. This concept would be in accord with the fact that 5,10-dideazafolate (in which N-8 would not be available for hydrogen bonding) bound poorly to GARFT (Table I).

Bridge Region and Phenyl Ring. The effects of changing the length of the bridge region of DDATHF on the binding to GARFT were dramatic, with a one methylene group extension increasing activity 6-fold and a one methylene unit shorter analogue about 5-fold less active than DDATHF (Table I). We would speculate that DDATHF and its analogues bind to GARFT in an extended "L"-shape, in which the bridge phenyl ring is at an obtuse angle to the heterocycle similar to the configuration assumed in aqueous solution. The lowest energy configuration of DDATHF in free solution has been calculated to be one in which the plane which contains the pyridopyrimidine ring is at an angle of about 90° to the plane that contains the phenyl ring (Shih et al., 1990). The structure assumed by 10-propargyl-5,8-dideazafolic acid bound to thymidylate synthase has been shown to have this type of configuration (Jones et al., 1990).

We have previously reported that the two diastereomers of DDATHF differing in chirality about the 6-carbon were nearly equiactive inhibitors of both L1210 GARFT and the growth of leukemic cells (Moran et al., 1989). Given the considerable tolerance of L1210 GARFT for changes in the structure of the bridge and phenyl regions and the fact that the first glutamic acid of the side chain does not seem to participate in binding, it is no longer surprising that both diastereomers of DDATHF are inhibitory. In addition, given that both diastereomers are equivalent substrates for FPGS (Moran et al., 1989), the equivalent cytotoxicity of (6*S*)- and (6*R*)-DDATHF for tumor cells suggests that the flexibility of the bridge and phenyl regions may allow proper orientation of polyglutamates of DDATHF on GARFT for compounds regardless of their configuration about C-6. A similar explanation of the low stereoselectivity of FPGS for the diastereomers of DDATHF has previously been proposed (Shih et al., 1990).

Side Chain. The activity of 5,10-dideazatetrahydropteroic acid as an inhibitor of GARFT was unexpected. Nevertheless, it is a clear indication that the side chain of the monoglutamate form of DDATHF does not add significantly to the strength of binding to GARFT. However, given the tighter binding of polyglutamate forms of DDATHF to GARFT (Table II), the glutamic acid residues added to DDATHF in vivo (Pizzorno et al., 1990) must participate in ionic interactions with enzyme that result in substantial decreases in binding energy.

The question arises of how tightly an inhibitor must bind to an enzyme to act as an effective cytotoxic agent to mammalian tumor cells and what the relationship would be between the K_i of a GARFT inhibitor, cellular GARFT content, and the potency of inhibition of de novo purine synthesis. In this regard, the theoretical studies of Strauss and Goldstein (1943) were first brought to a practical application by Werkheiser (1961), who defined that, when the magnitude of the dissociation constant for a freely dissociable drug–enzyme complex was much less than the enzyme concentration, "stoichiometric" inhibition would be observed. The significance of the concept of stoichiometric inhibition for therapeutics is that, under these conditions, for any total inhibitor concentration less than the cellular concentration of enzyme [the cellular equivalent of the zone C inhibition of Strauss and Goldstein (1943)] essentially all of the inhibitor is enzyme-bound, or viewed somewhat differently, there is no free inhibitor. As a result, one would expect effective titration of enzyme activity even by a reversibly bound inhibitor and a slow loss of inhibitor from the cell due to protein binding alone. From the specific activity of our pure enzyme and the GARFT activity found in leukemic cells, it can be calculated that the concentration of GARFT in L1210 cells would be 0.3–0.6 μM [assuming uniform distribution of GARFT in total intracellular water and a value of 0.67 μL of water per 10^6 L1210 cells (Jackson & Harrap, 1973)]. Hence, DDATHF itself would not be a stoichiometric inhibitor of tumor cell GARFT (K_i/E_t = 0.15–0.3), whereas the pentaglutamate derivative of DDATHF would be expected to behave in this manner (K_i/E_t = 0.0005–0.001). Presumably, the interaction of the polyglutamate forms of DDATHF with GARFT is competitive with the folate substrate as was the case for the monoglutamate forms of DDATHF and its analogues (Figure 3). If this is the case in vivo, then the effective K_i would be a function of the available substrate concentration, i.e., K_i $(1 + S/K_m)$, and any change in 10-formyltetrahydrofolate polyglutamate concentrations secondary to GARFT inhibition would affect the suppression of de novo purine synthesis. Such an accumulation of substrate of the dihydrofolate reductase reaction is substantial after exposure to competitive inhibitors (Priest et al., 1989; Allegra et al., 1986; Matherly et al., 1986), and computer modeling studies (Jackson & Harrap, 1973; White, 1979) have indicated that substrate accumulation plays an essential role in the recovery of DNA synthesis after exposure of cells to methotrexate. However, if the 5,10-methenyltetrahydrofolate cyclohydrolase reaction is reversible in vivo, one would not expect accumulation of 10-formyltetrahydrofolate polyglutamates in DDATHF-inhibited cells but rather accumulation of glycinamide ribonucleotide, which would intensify binding of DDATHF analogues to GARFT.

Ferone et al. (1990) have reported that a kinetically determined K_i value for a GARFT inhibitor was different when measured against (6*R*)-10-formyltetrahydrofolate and against (6*R*)-10-formyltetrahydrofolate pentaglutamate in spite of the fact that inhibition seemed to be competitive with the folate substrate. Similar results have been reported for the aminoimidazole carboxamide ribonucleotide formyltransferase reaction (Allegra et al., 1985). This seems to indicate a more complex interaction among the inhibitor, the substrate, and the enzyme catalyzing these reactions than is implied by the mutually exclusive binding of a simple competitive inhibition pattern. We have not investigated this point in this kinetic study but rather are pursuing thermodynamic criteria for interaction of the binding of these substrates and inhibitors to GARFT.

Available evidence indicates that the polyglutamate metabolites of DDATHF are the principal, if not the only, species causative of cell growth inhibition and cytotoxicity in DDATHF-treated tumor cells and that DDATHF itself may have minor cytotoxic activity in and of itself. Thus, the pentaglutamate of DDATHF bound to GARFT about 100 times more tightly than DDATHF (Table II). In this respect, the interaction of GARFT with these compounds resembles that of thymidylate synthase with 10-propargyl-5,8-dideazafolate polyglutamates (which are as much as 100 times more

potent inhibitors than the monoglutamate form of this compound) (Cheng et al., 1985; Sikora et al., 1988) but differs from that of dihydrofolate reductase with methotrexate polyglutamates, which are only 2-5-fold better inhibitors than methotrexate itself (Kumar et al., 1986; Appleman et al., 1988). DDATHF was recognized as a very efficient substrate for FPGS when we first reported its synthesis (Taylor et al., 1985) and has subsequently been shown (Pizzorno et al., 1990) to be efficiently metabolized to long-chain polyglutamates in leukemic cells. The equivalent activity of DDATHF and its aspartate analogue as inhibitors of GARFT (Table I) is in contrast to the 30-fold lower potency of the aspartate compound as a tumor cell growth inhibitor (Moran et al., 1989). However, the lack of activity of the aspartate analogue as a substrate for FPGS (Moran et al., 1989) is sufficient to explain the discrepancy. Presumably, polyglutamation of DDATHF analogues not only substantially increases their affinity for GARFT but also causes poor efflux of inhibitory species from the cell.

In a separate paper, we compare the activities of the compounds in Table I as inhibitors of tumor cell growth in culture and as substrates for FPGS. Among this series, some analogues were potent GARFT inhibitors but poor substrates for FPGS; others were efficient FPGS substrates and poor GARFT inhibitors; both of these patterns resulted in poor inhibition of tumor cell growth. We conclude from these studies that both intrinsic inhibitory activity against GARFT and polyglutamation are essential factors in the antitumor activity of this class of antimetabolites.

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*Nae*I Endonuclease Binding to pBR322 DNA Induces Looping†

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ABSTRACT: Previous work has demonstrated the existence of both resistant and cleavable *Nae*I sites. Cleavable sites introduced on exogenous DNA can act in trans to increase the catalysis of *Nae*I endonuclease cleavage at resistant sites without affecting the apparent binding affinity of the enzyme for the resistant site [Conrad, M., & Topal, M. D. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9707-9711]. This activation suggests allosteric regulation of *Nae*I cleavage by distant cis- and trans-acting sites in DNAs containing both resistant and cleavable sites. Plasmid pBR322 contains four *Nae*I sites, at least one of which is resistant to cleavage. Electron microscopy is used here to demonstrate that *Nae*I endonuclease simultaneously binds to multiple recognition sites in pBR322 DNA to form loops with *Nae*I protein bound at the loop's base. The maximum number of loops formed with a common base suggests four binding sites per enzyme molecule. Looping was inhibited by addition of enzyme-saturating amounts of double-stranded oligonucleotide containing an *Nae*I site, whereas another double-strand oligonucleotide without the *Nae*I site had no effect. The number of loops seen was not above background when double-stranded M13 DNA, which contains only a single *Nae*I recognition site, was used as substrate.

Examples of one DNA site affecting an event at another distant site are known in transcription, replication, and recombination systems in both prokaryotes and eukaryotes (Majumdar & Adhya, 1984; Dynan & Tjian, 1985; Ptashne, 1986; Gellert & Nash, 1987; Moitoso de Vargas et al., 1988; Schleif, 1988). A number of protein-driven mechanisms have been proposed to account for communication between cis(intramolecular)-acting sites on DNA. These mechanisms include protein sliding, cooperative binding of protein to span the distance between sites, and looping-out of intervening DNA to bring the sites in contact with a common protein structure [see Ptashne (1986) for a review]. It is probable that looping-out of intervening DNA may be a general mechanism used for communication between distant cis-acting sites (Griffith et al., 1986; Kramer et al., 1987; Theveny et al., 1987; Amouyal et al., 1988; Chattoraj, 1988; Mukherjee et al., 1988).

In the *lac* repressor system, *lac* repressor monomers bind to sites on the same face of the helix; dimerization then causes the DNA between the sites to loop-out (Ptashne, 1986; Griffith et al., 1986). In the *Nae*I restriction endonuclease system, *Nae*I endonuclease cleavage of resistant sites has been shown to be activated in trans (intermolecular) by the addition of a separate cleavable site either on supercoiled plasmid DNAs or on small DNA fragments (Conrad & Topal, 1989). Trans-activation strongly suggests that *Nae*I endonuclease might be subject to cis-activation by DNA looping when cleavable and resistant sites reside on the same DNA molecule. This looping should be visible with the aid of an electron microscope.

The activation of *Nae*I endonuclease catalysis of DNA cleavage, according to the kinetic models of Monod et al. (1965) and Koshland et al. (1966), suggests an allosteric change in the protein upon activator binding to perhaps correctly position the catalytic site. Thus, *Nae*I endonuclease regulation by DNA provides a paradigm for regulation of enzyme activity by allosteric modification of the enzyme by trans- and cis-acting sites. In this study, electron microscopy was used to demonstrate that in a DNA containing both cleavable and resistant *Nae*I recognition sites, *Nae*I protein induces the formation of a high frequency of protein–DNA structures in which DNA is held stably in loops by *Nae*I protein bound at the loop's base. In contrast, DNAs containing a single recognition site exhibited very low levels of looping, and looping within the multiple-site DNA was reduced to background by competition for enzyme binding sites by short duplexes containing the cognate site.

Materials and Methods

Materials. *Nae*I, *Nde*I, and *Pvu*II endonucleases and bacteriophage T4 DNA polymerase were purchased from New England Biolabs (Beverly, MA). Biotinylated dCTP was

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