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A Strategy for the Design of Membrane-Permeable Folylpoly-γ-glutamate Synthetase Inhibitors: "Bay-Region"-Substituted 2-Desamino-2-methyl-5,8-dideazafolate Analogs

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SUMMARY

Previous attempts to design inhibitors of mammalian folylpolyglutamate synthetase (FPGS) have resulted in three classes of active compounds, all of which have charged moieties in the side chain, but structural alteration of the rest of the folate molecule has not seemed to be an avenue for drug discovery. However, groups in the side chain of folate analogs that bear charge distributions different from that of glutamic acid appear to prevent efficient transport into mammalian cells on the reduced folate carrier system. We now report that substituents at the 7-, 2′-, or 3′-position of 2-desamino-2-methyl-4-hydroxyquinazoline antifolates decrease or prevent the catalysis of diglutamate formation by FPGS but are compatible with efficient binding to the reduced folate carrier system. Thus, 5,8-dideazafolates with a 3′-alkyl group had a lower V_{max} for FPGS than did the corresponding unsubstituted quinazolines, by a factor of 4–12, but these compounds inhibited the reaction of control FPGS substrates, indicating that the 3′-groups had much larger effects on catalytic activity than on binding to enzyme. A 7-methyl substituent affected the V_{max} of a series of 5,8-dideazafolate compounds by a factor of 2–8, but this decrease in the catalytic rate was also accompanied by an increase in the K_m of the substituted compounds by a factor of 10–100. The extent of the effect of a 7-methyl substituent on V_{max} appeared to be dependent on the size of the substituent at N10. Different substituents at the 2′-position affected the kinetics of the FPGS reaction with one of three patterns, i.e., 1) a 2′-fluoro substituent both increased V_{max} and decreased K_m slightly, 2) either -OH or -NH_2 decreased the V_{max} without affecting the K_m, and 3) 2′-Cl, -CH_3, -CF_3, or -OCH_3 substituents were found to both decrease V_{max} and increase K_m. Substitutions at the 7-, 2′-, or 3′-position had only minor effects on the ability of 2-desamino-2-methyl-4-oxoquinazolines to interfere with the transport of [^{3}H]methotrexate into L1210 cells. Hence, these classes of compounds are likely to be efficiently transported by the reduced folate carrier system. We conclude that the region of the folate molecule bounded by the 7-, 6-, 9-, 10-, 3′-, and 2′-positions, the "bay region," is of major importance both for the binding of folates and folate analogs to FPGS and for the assumption of a conformation of the enzyme-substrate complex compatible with catalysis. We also suggest that appropriate substitutions at these positions would lead to membrane-permeable, specific FPGS inhibitors potent enough to allow evaluation of this enzyme as a target for cancer chemotherapy.

FPGS catalyzes the addition of several moles of L-glutamic acid to folates and folate analogs through an amide linkage between the amino group of the incoming glutamic acid and the γ-COOH of the ω-glutamic acid in the side chain. The products of this reaction are, if anything, more efficient substrates for the folate-dependent enzymes than the corresponding folylmonoglutamates (1) and are retained in mammalian cells against a concentration gradient, in contrast to the facile efflux of the folylmonoglutamates (2–4). The function of FPGS is essential for the survival of proliferating mammalian cells, as evidenced by the fact that a class of somatic cells selected for auxotrophy for thymidine, purines, and glycine were found to lack FPGS activity (5, 6) and were not viable under normal conditions of growth (5). As a result, we (7) and others (8, 9) have advocated that FPGS is an attractive target for cancer chemotherapy. FPGS inhibitors would probably be lethal only to cells that attempted to divide during continued inhibition of FPGS function (7). That is, inhibition of FPGS *per se* would not be cytotoxic but, rather, cytotoxicity would result from the dilution of folate cofactors, due to the distribution of cytoplasm during repeated mitoses, down to a level insufficient to main-

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ABBREVIATIONS: FPGS, folylpoly-γ-glutamate synthetase; FdUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; MTX, methotrexate.

tain DNA and purine synthesis. Hence, FPGS inhibitors would be prototypical antiproliferative agents targeted at cells committed to continued division, i.e., normal and malignant stem cells.

The active site of mammalian FPGS has been probed in several structure-activity studies due to the availability of numerous folate analogs that are substrates for FPGS and a few that have proven to be effective inhibitors. In general, the FPGS active site has proven to be remarkably promiscuous, in that most structural alterations in the ring system of folate analogs equivalent to the pteridine ring of the naturally occurring folates, in the 9,10-bridge region, and in the phenyl ring have been tolerated with retention of substrate activity for FPGS *in vitro* (10–18). With a very few but salient exceptions, structural changes in these regions of the folate molecule have affected only the K_m of the reaction, with minimal concomitant changes in V_{max}, particularly when the mouse liver enzyme has been used as a model system (10, 12, 13, 15, 17). On the other hand, any structural alteration of the glutamic acid side chain of the folate molecule has yielded, for the most part, compounds that are not substrates for FPGS and are usually incapable of binding to the active site (10, 11, 19–23). Three notable exceptions to this generalization have defined three classes of inhibitors, namely, analogs in which the γ-carboxyl group has been replaced by a sulfonic acid group (24, 25) or by a phosphonic acid group (26) or analogs in which the glutamic acid side chain has been replaced by ornithine (11, 27, 28). All three of these classes of inhibitors have been modified, to optimize binding to the active site, by structural changes in the heterocyclic ring of the molecule that are compatible with low K_m values in the corresponding substrates with glutamic acid side chains (29, 30). However, to date, even the best class of these inhibitors, the analogs containing ornithine side chains, have been shown to affect polyglutamation in intact cells only at high concentrations, apparently as a result of inefficient transport by the reduced folate transport system (28). It seems that folate analogs with an unnatural distribution of charge in the side chain at physiological pH are not well accepted by the reduced folate carrier. Thus, the viability of FPGS inhibition as an approach to cancer chemotherapy is still unproven and, as such, continues to be speculative.

We now report structural modifications of 2-desamino-2-methyl-5,8-dideazafolates that can lead to FPGS inhibitors with an intact glutamic acid side chain and, also, to close structural analogs of extremely efficient FPGS substrates that are themselves nearly inactive as substrates for this enzyme. The substituents producing these effects appear to project into the space defined by the angle formed by the position 6 and 7 ring carbons of the heterocyclic ring, the position 9 and 10 bridge atoms, and positions 2′ and 3′ of the phenyl ring, a region of the folate molecule that, in three dimensions, has the appearance of a bay. Although the folate analogs that are discussed herein prove these points, none are themselves particularly potent inhibitors of FPGS. Nevertheless, it appears that folate analogs modified in this region are a lead to the discovery of sufficiently potent, membrane-permeable, FPGS inhibitors to allow testing of the suitability of this enzyme as a target for chemotherapy.

Materials and Methods

Aminopterin, Sephadex G-50 (30–80 μm), formaldehyde, ATP, FdUMP, L-glutamic acid, α-thioglycerol, and β-mercaptoethanol were purchased from Sigma Chemical Co. (St. Louis, MO). One-milliliter syringes were obtained from Becton-Dickinson (Rutherford, NJ). [3,4-^{3}H]Glutamic acid was purchased from E. I. DuPont de Nemours and Co. (Wilmington, DE); [3′,5′,7,9-^{3}H]MTX was purchased from Moravek Radiochemicals (Industry, CA) and before use was determined to be >95% pure by high performance liquid chromatography. The scintillation cocktail was Safety-Solve from Research Products International Corp. (Mount Prospect, IL). The quinazoline analogs studied here were synthesized either at the Institute for Cancer Research or at ICI (Alderley Park, UK) (31–33).[1,2] Those analogs whose synthesis has not yet been fully described in the literature are described in British Patents EP 0 284 338 and EP 0 239 362. (6*S*)-Tetrahydrofolate was prepared by an enzymatic procedure described previously (34).

Preparation of enzymes. Thymidylate synthase was obtained from a strain of *Escherichia coli* bearing a plasmid (35) that allows expression of the *Lactobacillus casei* thymidylate synthase (provided by Dr. Daniel Santi, University of California, San Francisco). It was purified to apparent electrophoretic homogeneity by phosphocellulose chromatography (36) and was stored at −25° in 50% glycerol. Mouse liver FPGS was prepared from 7–10-week-old Swiss Webster female mice. A 0–30% ammonium sulfate precipitate, prepared as described previously (37), was stored at −25° until use and was desalted immediately before use either by Sephadex G-25 chromatography or with centrifugally eluted Sephadex G-50 "spin-columns."

Standard FPGS (charcoal) assay. Compounds were incubated with FPGS for 60 min at 37°. The mixture contained the folate analog (at 0.5–400 μM), 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, in a total volume of either 0.25 or 1 ml (for substrates with low K_m values). The tritium-labeled diglutamyl product was isolated by adsorption onto charcoal, as described previously in detail (37). In the experiments reported here, there were 2–3.6 cpm/pmol of glutamate incorporated into product and the FPGS used had a specific activity of 1.9 ± 1.1 nmol of diglutamate of aminopterin formed/hr × mg of protein. No more than 25% of substrate was consumed at the lowest substrate concentration in these experiments. Under these conditions, reaction rates approximated initial velocities and the reaction being measured was restricted to the formation of diglutamate. Data were analyzed by a weighted nonlinear fit to a rectangular hyperbola, using a standard statistical program for enzyme data (38) to generate estimates of kinetic constants. All experiments were performed at least twice.

FPGS microassay. We adapted a previously described method (39) for the purpose of determining the K_m values of compounds that had low V_{max} values. The assay involves two reactions. In the first reaction, the compound under study competes with (6*S*)-tetrahydrofolate for conversion by FPGS to the ^{3}H-labeled diglutamate product. This was carried out at 37° in a total volume of 10 μl containing 10 μM (6*S*)-tetrahydrofolate, 10–600 μM compound, 5 mM ATP, 10 mM $MgCl_2$, 30 mM KCl, 1 mM L-[^{3}H]glutamic acid, 200 mM Tris·HCl, pH 8.5, and desalted FPGS. In the second reaction, the [^{3}H]tetrahydropteroyldiglutamate formed in the previous step was isolated as a ternary covalent complex in the presence of thymidylate synthase, formaldehyde, and FdUMP. After incubation of the first reaction mixture at 37° for 30 min, 100 μl of thymidylate synthase solution (containing 30 mM Na_2HPO_4, pH 7.2, 8 mM β-mercaptoethanol, 15 mM formaldehyde, 2 μM FdUMP, 0.1 mg/ml bovine serum albumin, and 1 μM *L. casei* thymidylate synthase) were added and incubation was continued for 30 min at 37°. The reaction mixture was then passed through a Sephadex G-50 spin column to isolate the macromolecular product from excess unreacted L-[^{3}H]glutamic acid and was processed for scintillation counting. In the experiments reported here, there were 600–900 cpm/pmol of glutamate incorporated into product. Typical raw data obtained for uninhibited reaction rates were 2800–9000 cpm

[1] F. T. Boyle and L. Hughes, manuscript in preparation.
[2] T. Thornton, manuscript in preparation.

of (6*S*)-tetrahydropteroyldiglutamate formed, indicating that <10% of the total amount of substrate was consumed in the first reaction. The ranges of concentrations for the compounds under study were chosen such that there were at least two points each above and below the IC_{50} values of the compounds. To preclude the possibility that compounds directly inhibited ternary complex formation among the 5,10-methylenetetrahydrofolate, FdUMP, and thymidylate synthase, controls were performed in which the first reaction was carried out in the absence of compound and 2.5 μl of the highest concentration of the compound used in the normal assay were added along with the 100 μl of thymidylate synthase solution. Other controls were included in which tetrahydrofolate was not added, to ensure that the test compounds did not themselves allow formation of a tightly bound complex with thymidylate synthase under the conditions of this assay. Under the conditions of this assay, the compounds studied did not inhibit ternary complex formation from 5,10-methylenetetrahydrofolate, nor did they allow complex formation in the absence of added tetrahydrofolate. In addition, the maximal amount of the diglutamates of the various folate analogs that were products of the first reaction in this microassay was 7 pmol but the thymidylate synthase used in the second reaction was present at reactant levels (200 pmol of binding sites, twice the level of total tetrahydrofolates). Hence, even stoichiometric inhibition of thymidylate synthase by the diglutamate product would not constitute a threat of interference with this assay.

A Dixon plot was used to estimate the K_m of the competitive substrate, by assuming that the reactions of tetrahydrofolate and test compound were mutually exclusive and then using the following relationship: x-intercept $= -K_m(1 + [S]/K_{m,\text{tetrahydrofolate}})$. The K_m of tetrahydrofolate for mouse liver FPGS was taken as 7.1 μM (40).

Transport studies. L1210 cells were grown to a density of 8×10^5 cells/ml in RPMI 1640 medium (supplemented with 10% dialyzed fetal calf serum), harvested by centrifugation at 500 × *g* for 5 min, and washed once with transport buffer (41) (107 mM NaCl, 20 mM Tris·HCl, 26.2 mM $NaHCO_3$, 5.3 mM KCl, 1.9 mM $CaCl_2$, 1 mM $MgCl_2$, 7 mM glucose, at pH 7.4 and 37°). The cells were resuspended in transport buffer at 40×10^6/ml, kept at 37°, and immediately used in transport studies using a rapid sampling procedure (42, 43). Briefly, 250 μl of transport buffer containing 0.4 μM [^{3}H]MTX (3.5 Ci/mmol) and varying concentrations of competing folate analogs were layered onto 600 μl of 1-bromododecane, and the tubes were centrifuged at 10,000 × *g* for 30 sec and prewarmed to 37°. Transport was initiated by forceful addition of 250 μl of cell suspension to the tubes and was terminated by centrifugation in a microcentrifuge for 2 min starting at the end of the timed interval (usually 8 min). The aqueous and organic layers were aspirated and the bottom of the tube containing the cell pellet was cut and incubated with 500 μl of 1 N NaOH at 37° overnight in scintillation vials. The alkali was neutralized with 10 N HCl and radioactivity was determined by scintillation counting. Data were corrected for cell surface adsorption, measured by a 30-sec incubation at 0°. The Michaelis constant for transport (K_t) for MTX was determined by weighted computer fit to a rectangular hyperbola (38) and that for competing folate analogs was determined by a Dixon plot, weighting the data proportionately to the velocity of transport. Each value was estimated at least three times in separate experiments.

Cell culture. Intracellular folate cofactors in L1210 cells were depleted by growth in RPMI 1640 medium formulated without folic acid but with 10% dialyzed fetal calf serum, 5.6 μM thymidine, and 32 μM hypoxanthine (44). Growth inhibition by folate analogs was studied by adding folate-depleted cells to 24-well plates containing drug, 2.3 μM folic acid, and 5.6 μM thymidine in folate-deficient RPMI 1640 medium containing 10% dialyzed fetal calf serum. Culture volumes were 1.5 ml. Culture densities were determined electronically when initiated and after a 60-hr incubation at 37°. For some experiments, cultures were initiated with either folate pool-depleted or folate pool-replete L1210 cells in 13-ml cultures. Densities were determined at intervals and culture densities were maintained at 1–5 × 10^5 cells/ml by daily subculture to a density of 1 × 10^5 cells/ml. All experiments were performed at least twice.

Results

Effects of position 7 substitutions. A 7-methyl substituent was found to greatly decrease the efficiency of utilization of a series of 5,8-dideazafolates studied as substrates by FPGS. Thus, 2-desamino-2-methyl-10-propargyl-5,8-dideazafolic acid (**3**)[3] was an efficient substrate for mouse liver FPGS with a K_m about 2-fold higher and a V_{max} about three fourths of that of the standard compound, aminopterin (Fig. 1A; Table 1). In comparison, the formation of diglutamate from 2-desamino-2,7-dimethyl-10-propargyl-5,8-dideazafolic acid (**4**) was barely detectable, with a V_{max} only 8% of that seen using aminopterin as a substrate (Fig. 1A; Table 1). However, because such low V_{max} values made it difficult to estimate K_m values accurately by an assay that relied on isolation of the diglutamate product, we could not definitively distinguish whether the 7-methyl group was interfering with catalysis or with binding to the active site, using our standard charcoal adsorption-based assay. Hence, we evaluated the ability of **4** to bind to FPGS by simultaneously incubating enzyme with **4** and (6*S*)-tetrahydrofolate and then isolating the diglutamate product formed from tetrahydrofolate as a ternary complex with fluorodeoxyuridylate and pure bacterial thymidylate synthase. When the K_m for FPGS of standard compounds was estimated from interference with the formation of tetrahydropteroyldiglutamate and compared with that measured by direct isolation of product, indistinguishable values were found for substrate K_m and competitive substrate K_m values using either aminopterin or **3** (Table 1). Using this approach, a comparison of **3** and **4** indicated that both K_m and V_{max} were affected by about a factor of 10 by a methyl group at C7, so that the estimated first-order rate constant was decreased by 2 orders of magnitude. The results found with **4** were confirmed using 2-desamino-10-propargyl-2,7,9-trimethyl-5,8-dideazafolic acid (**5**), which was nearly inactive as a substrate for FPGS as a result of substantial changes in both K_m and V_{max} (Table 1).

Our previous studies showed that N10-alkyl substitutions increased the K_m observed with 5,8-dideazafolates and that, with sufficiently large 10-alkyl groups, V_{max} values were lower (12). Given that there would be steric interaction of a 7-methyl group with an N10-alkyl group, we thought it likely that the effect seen with **4** and **5** was due to exacerbation of the structural constraints imposed by bulky groups at position 10. Hence, we studied the interaction of mouse liver FPGS with compounds with a methyl substituent at C7 that had less bulky groups at N10. The 2-desamino-2,10-dimethyl-5,8-dideazafolic acid analog with a 7-methyl group (**2**) had diminished substrate activity, compared with that of the corresponding 7-unsubstituted compound (**1**); both an increase in K_m and a decrease in V_{max} were observed. Interestingly, the effect on K_m seen in the comparison of these latter two compounds was about the same magnitude as seen comparing **4** and **3** (13–30-fold), but the effect of a 7-methyl group on V_{max} was substantially less for the 10-methyl compounds (a 3-fold decrease for **2**, relative to the V_{max} of **1**) than for the compounds with a bulkier propargyl

[3] Compound numbers are shown in bold type and are related to structures given in Tables 1–4.

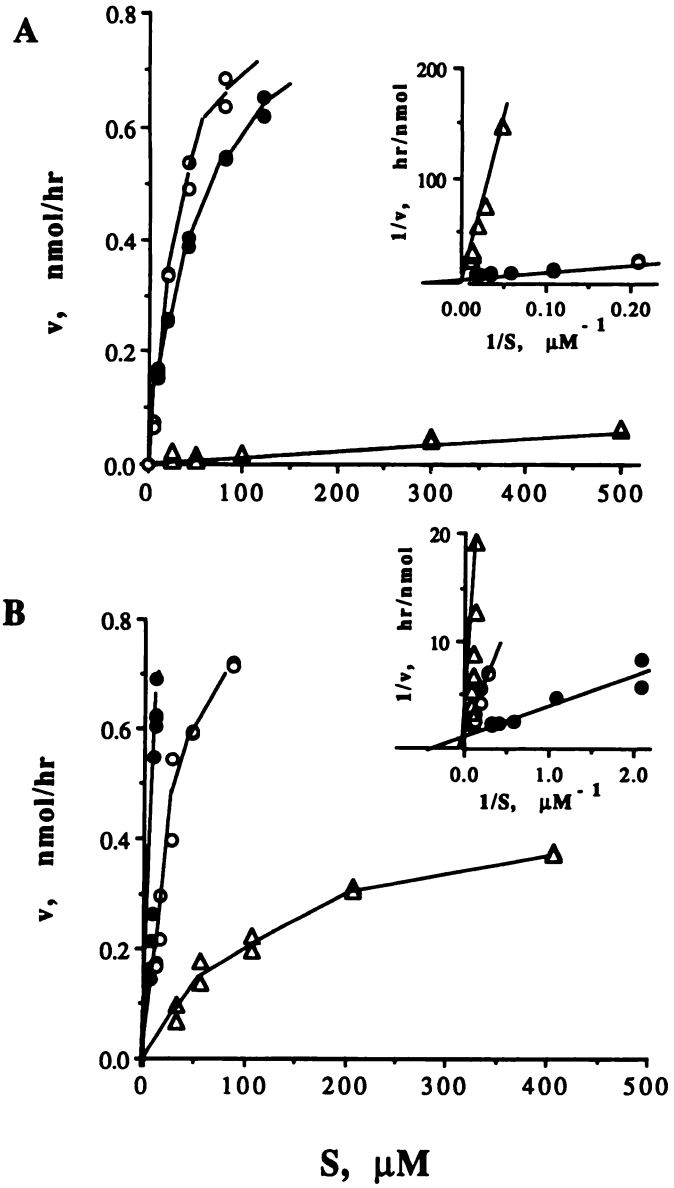


Fig. 1. Effects of a 7-CH_3 group on the substrate activity of 2-desamino-2-methyl-5,8-dideazafolates for FPGS. The indicated concentrations of (A) **3** (●) and its 7-CH_3 analog **4** (△) and of (B) D1694 (●) and its 7-CH_3 analog **6** (△) were incubated with FPGS for 1 hr and the diglutamate product was measured according to the charcoal assay method described in the text. Aminopterin (○) was used as the internal control in both experiments (A and B). *Insets*, double-reciprocal replots of the data in the main figure. Each *point* represents a separate incubation.

group at N10 (an approximately 10-fold lower V_{max} for **4** than for **3**).

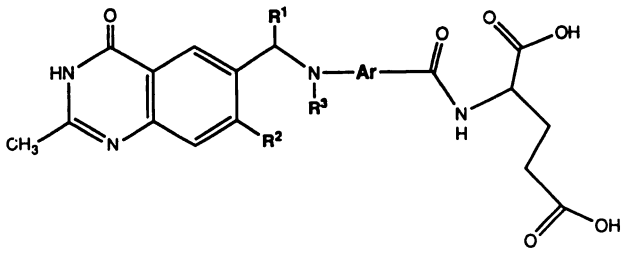
A previous study from our laboratories indicated that the replacement of the phenyl group of 5,8-dideazafolates with some ring systems substantially improved the efficiency of interaction with FPGS (15). This effect is also clear from the comparison of the kinetic parameters of **1** with those of D1694 shown in Table 1, which indicates a 10-fold decrease in K_m and a slight increase in V_{max} as a result of the presence of the thiophene ring. By these criteria, D1694 is one of the most efficient substrates for FPGS yet known. Comparison of the kinetic parameters of the mouse liver FPGS reaction using D1694 with those for its 7-methyl analog, **6**, again indicates that the introduction of a methyl group into the structure of even a highly efficient substrate such as D1694 severely diminishes fit to the enzyme active site (Table 1). The formation of diglutamates of the 7-methyl analog of D1694 (**6**) by mouse liver FPGS was easily measurable (Fig. 1B), but the K_m for this reaction was 100 times higher than that for D1694 and the V_{max} was also lower, by about 60%, for the 7-methyl compound. We concluded that a 7-methyl substitutent negatively affected the acceptance as a substrate for FPGS, with effects on both K_m and V_{max}, and that the magnitude of this effect was determined by the spatial configurations achievable by this region of the folate analog. The overall effect of a 7-methyl substitution on the first-order rate constant of the FPGS reaction was ≥2 orders of magnitude for the three comparisons indicated in Table 1 (**1/2** = 126, **3/4** = 98, and D1694/**6** = 213).

Effects of position 3′ substitutions. A 3′-alkyl substituent was found to have dramatic effects on the substrate properties of 2-desamino-2-methyl-5,8-dideazafolic acid analogs. Whereas 2-desamino-2-methyl-5,8-dideazafolic acid (**7**) was an efficient substrate for FPGS, with a K_m of 6.4 μM and a V_{max} equivalent to that of aminopterin, the analogs of **7** with methyl (**8**), ethyl (**9**), or propyl (**10**) substitutions at the 3′-position had 4-, 5-, and 12-fold lower V_{max} values, respectively (Table 2). For all three 3′-substituted derivatives of **7**, a plot of reaction velocity versus concentration showed a distinct maximum, with higher concentrations resulting in substrate inhibition (Fig. 2A). If enzyme was simultaneously exposed to both 3′-substituted derivatives and either aminopterin (Fig. 2A) or (6*S*)-tetrahydrofolate (Fig. 2B) as a test substrate, the reaction of the test substrate was clearly inhibited. The inhibition of the formation of diglutamate from tetrahydrofolate was used to estimate the K_m values of these 3′-substituted competitive substrates for FPGS, and representative primary data are shown in Fig. 2B. There seemed to be a bimodal effect of 3′-alkyl substituents on K_m; a 3′-methyl-substituted analog (**8**) had a 3.5-fold higher K_m than did the unsubstituted compound, but analogs with longer alkyl groups had lower K_m values and the K_m of a propyl-substituted analog was not different from that of the unsubstituted compound. Two other derivatives were available that confirmed these effects; 3′-ethyl-5,8-dideazafolate (**12**) was a substrate for FPGS but had a substantially lower V_{max} than that of 5,8-dideazafolate (**11**). The K_m values of these two compounds were similar (Table 2). We drew the conclusion that a 3′-aliphatic substituent on 2-desamino-2-methyl-5,8-dideazafolates (and, presumably, other classes of folate analogs) prevented efficient catalysis in the FPGS reaction without having a major effect on binding to the active site.

Effects of position 2′ substitutions. A series of derivatives of 2-desamino-2-methyl-10-propargyl-5,8-dideazafolic acid were available that allowed investigation of the effects of 2′-substituents on the FPGS reaction. One 2′-substituted compound, the 2′-fluoro derivative (**15**), was a better substrate for mouse liver FPGS than was the unsubstituted control (**3**), with slightly superior V_{max} and K_m values (Table 3). All of the other compounds studied that had 2′-substituents were found to be poorer substrates than the unsubstituted compound (Table 3). Compounds substituted with -Cl (**16**), -CH_3 (**17**), -OCH_3 (**18**), and -CF_3 (**19**) were utilized by FPGS with K_m values that were larger than that of the unsubstituted control (**3**) by a factor of 2–8, with the increase in K_m being roughly related to the bulk

TABLE 1

Effect of 7-methyl substituents on the substrate activity of quinazoline antifolates for mouse liver FPGS



R¹	R²	R³	Ar	Compound	K_m[a]		Relative values[b]	
					Isolation of diglutamate	Inhibition of $H_4PteGlu_2$ formation	V_{max}	V_{max}/K_m[c]
					μM			
-H	-H	$-CH_3$		1	13.8 (3.5)	21 (3.8)	1.01 (0.31)	3.8 (0.18)
-H	$-CH_3$	$-CH_3$	〃	2	470 (240)	290 (16)	0.29 (0.04)	0.03 (0.02)
-H	-H	$-CH_2CCH$	〃	3	43 ± 6.2	30 (0.4)	0.76 ± 0.06	0.49 ± 0.25
-H	$-CH_3$	$-CH_2CCH$	〃	4		320 (19)	0.08 (0.03)	0.005[d]
$-CH_3$	$-CH_3$	$-CH_2CCH$	〃	5		420 (45)	0.05 (0.03)	0.002[d]
-H	-H	$-CH_3$		D1694	1.37 ± 0.2		1.26 ± 0.22	19.2 ± 4.7
-H	$-CH_3$	$-CH_3$	〃	6	143 (18)		0.53 (0.01)	0.09 (0.0)
Aminopterin					21 ± 4.8	20.9 ± 4.1	1.0	1.0

[a] The Michaelis constant was either measured directly using isolation of the diglutamate product (charcoal assay) or indirectly inferred from the inhibition of the formation of tetrahydropteroyldiglutamate ($H_4PteGlu_2$) (microassay). If three or more values were averaged, the mean ± standard deviation is shown; if the mean is for two values, the range between values is shown in parentheses.

[b] Relative to V_{max} and V_{max}/K_m values for aminopterin, which are expressed as 1.0. Our previous related papers (e.g., Refs. 10 and 12) expressed these quantities relative to values for folic acid. To convert between these scales, multiply V_{max} relative to aminopterin values by 1.56 and V_{max}/K_m values relative to aminopterin by 10.1 to obtain the corresponding quantities relative to folic acid. Thus, the V_{max} and V_{max}/K_m values for D1694 relative to folic acid would be 2.14 and 194, respectively.

[c] Ratio of V_{max}/K_m of the compound to that of aminopterin in the same experiment, using the charcoal isolation procedure.

[d] The V_{max} values of the compound and of aminopterin were determined in the same experiment, using the charcoal procedure, but the K_m value used in the denominator was derived from a parallel microassay.

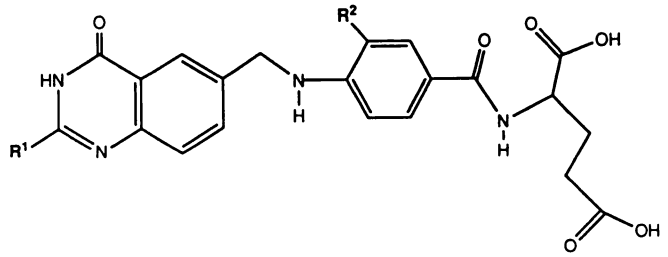
of the 2′-substituent. These compounds also were characterized by lower V_{max} values, compared with that of **3**, by a factor of 3 (for the 2′-Cl compound **16**) to >76 (for the 2′-OCH_3 compound **18**). Two compounds of this series (**13** and **14**) (Table 3) with 2′-substituents (-OH and $-NH_2$, respectively) had K_m values indistinguishable from that of the corresponding 2′-unsubstituted 5,8-dideazafolate (**3**), but the V_{max} of the FPGS reaction was decreased by a factor of 4 and 7.5, respectively, compared with that for the 2′-unsubstituted substrate (**3**). We concluded that the placement of groups at the 2′-position of these analogs could interfere with catalysis by this enzyme and that the ability of folate analogs to bind to the active site might also be affected by such substitutions.

Binding of "bay-region" analogs to the reduced folate transporter. The ability of bay-region-substituted 2-methyl-4-oxoquinazoline antifolates to interfere with the transport of [^{3}H]MTX into L1210 cells was used as an index of the binding of these compounds to the membrane protein involved in the reduced folate transport system. Under the conditions used for these experiments, the uninhibited transport of MTX was linear with time for about 12 min (data not shown). Transport of MTX over an 8-min interval was saturable, with a Michaelis constant of 2.9 ± 0.36 μM and a maximum rate of transport of 5.1 ± 0.7 pmol/min × 10^7 cells (n = 3); these values were comparable to results of previous investigations on the reduced folate transport system in these cells (41, 42, 45, 46). In agreement with other published experiments (45), CB 3717 was a weak inhibitor of this transport route, with a K_i value >10-fold higher than the K_t for MTX, whereas the 2-desamino-2-methyl analog of CB 3717 (**3**) interfered with the transport of MTX at concentrations in the low micromolar range (Table 4). The 2-methyl-4-oxoquinazoline folate analogs with a methyl group at position 7 (**4**), a hydroxyl group at position 2′ (**13**), or a propyl group at position 3′ (**10**) all inhibited the transport of [^{3}H]MTX into these cells very efficiently, with K_i values that differed from that of the appropriate unsubstituted control compounds by <3-fold (Table 4). Thus, we concluded that these bay-region substitutions did not adversely affect the affinity of 2-methyl-4-oxoquinazoline folate analogs for the reduced folate carrier protein and that these compounds have the potential to enter mammalian cells using this transport carrier system.

Inhibition of cell growth by bay-region FPGS inhibitors. Inhibition of FPGS would not be expected to be growth inhibitory by itself and would result in antiproliferative effects only when folate pools are decreased either by dilution due to successive mitoses (7) or by the turnover of folylpolyglutamates

TABLE 2

Effect of 3′-alkyl substituents on the substrate activity of quinazoline antifolates for mouse liver FPGS



R^1	R^2	Compound	K_m[a]		Relative values[b]	
			Isolation of diglutamate	Inhibition of $H_4PteGlu_2$ formation	V_{max}[c]	V_{max}/K_m[d]
				μM		
$-CH_3$	-H	**7**	6.4 (0.6)	4.4 (3.5)	0.97 ± 0.13	4.1 (0.3)
$-CH_3$	$-CH_3$	**8**		21 (0.9)	0.22 ± 0.03	0.22[e]
$-CH_3$	$-CH_2CH_3$	**9**		13.2 ± 3.9	0.18 (0.09)	0.29[e]
$-CH_3$	$-CH_2CH_2CH_3$	**10**		6.6 ± 1.8	0.08 (0.0)	0.25[e]
$-NH_2$	-H	**11**	6.4 (2.5)		0.8 (0.18)	2.9 (0.6)
$-NH_2$	$-CH_2CH_3$	**12**		9.9 (0.4)	0.09 (0.08)	0.19[e]

[a] The Michaelis constant was either measured directly using isolation of the diglutamate product (charcoal assay) or indirectly inferred from the inhibition of the formation of tetrahydropteroyldiglutamate ($H_4PteGlu_2$) (microassay). If three or more values were averaged, the mean ± standard deviation is shown; if the mean is for two values, the range between values is shown in parentheses.

[b] Relative to V_{max} and V_{max}/K_m values for aminopterin, which are expressed as 1.0.

[c] V_{max} values for compounds **8**, **9**, **10**, and **12** were not computer-estimated extrapolations but rather were the maximum velocity observed (see Fig. 2A).

[d] Ratio of V_{max}/K_m of the compound to that of aminopterin in the same experiment, using the charcoal isolation procedure.

[e] The V_{max} values of the compound and of aminopterin were determined in the same experiment, using the charcoal procedure, but the K_m value used in the denominator was derived from a parallel microassay.

(47). To model the complete inhibition of the flow of folates into intracellularly retained polyglutamate pools, the inhibition of cell growth was monitored in cultures in which L1210 cells had been transferred to folate-free RPMI 1640 medium containing dialyzed fetal calf serum. Under these conditions, there was virtually no inhibition of cell growth for 2–3 generation times, with initially identical growth curves seen for cells transferred into folate-deficient medium or into folate-replete medium (Fig. 3). Thereafter, cell growth progressively decreased in cultures exposed to folate-deficient conditions (Fig. 3). The fact that the delay in growth inhibition was due to preexisting intracellular pools in the cells used to inoculate these cultures was shown by the lack of such a lag phase in cultures initiated using cells that had been previously depleted of intracellular folates (Fig. 3). Thus, cells containing folate pools would continue to grow for a substantial time even under the condition of complete cessation of the *de novo* formation of folylpolyglutamate pools, but folate-deficient cells would be immediately growth inhibited if simultaneously exposed to a membrane-permeable FPGS inhibitor and a folate source.

In view of this concept, the growth of folate-depleted L1210 cells was studied after simultaneous exposure to some of the most promising bay-region-modified folate analogs and to folic acid. Because several of these compounds had previously been found to be moderately potent inhibitors of thymidylate synthase, thymidine was included in the medium, so that any such effects would not result in inhibition of growth. [Thymidine did not alter the pattern of growth of folate-depleted cells in either folate-replete or folate-deficient medium (Fig. 3).] The results of these experiments (Table 5) indicated that both 2′- and 3′-substituted 2-methyl-4-oxoquinazoline folate analogs were inhibitors of the growth of L1210 cells under conditions in which growth was dependent on the influx of folic acid into intracellular cofactor pools and that the activity of these compounds as growth inhibitors correlated well with their activity as inhibitors of FPGS. Likewise, all of the excellent substrates for FPGS that were studied as controls in these experiments were also inhibitors of cell growth, suggesting that antiproliferative effects can be manifested under these conditions simply due to competition for influx with extracellular folate. It should be noted that these results do not allow distinction to be made between inhibition of cell growth due to competitive inhibition of FPGS and inhibition of cell growth due to inhibition of membrane transport. Such distinction will require more extensive investigations.

Discussion

In these studies, we demonstrate that 1) alkyl substituents at position 3′ in the phenyl ring of 2-desamino-2-methyl-5,8-dideazafolate analogs decrease V_{max} for substrate utilization by FPGS, with only minor effects on K_m, 2) a methyl group at position 7 increases K_m by a large factor and, if a sufficiently bulky group is present at N10, can also lead to a substantial decrease in V_{max} for this enzyme, and 3) substituents of even moderate bulk at position 2′ decrease the possible V_{max} without necessarily increasing K_m. These results serendipitously arose from experiments aimed at optimizing the utilization of 5,8-dideazafolate inhibitors of thymidylate synthase as substrates for FPGS (15). They certainly indicate a series of structural alterations that cannot be made without dramatically decreasing FPGS substrate activity. In addition, our results lead to suggestions for inhibitors of FPGS and also indicate that the surfaces of the active site opposing these regions of the folate molecule are very important both for binding to enzyme and

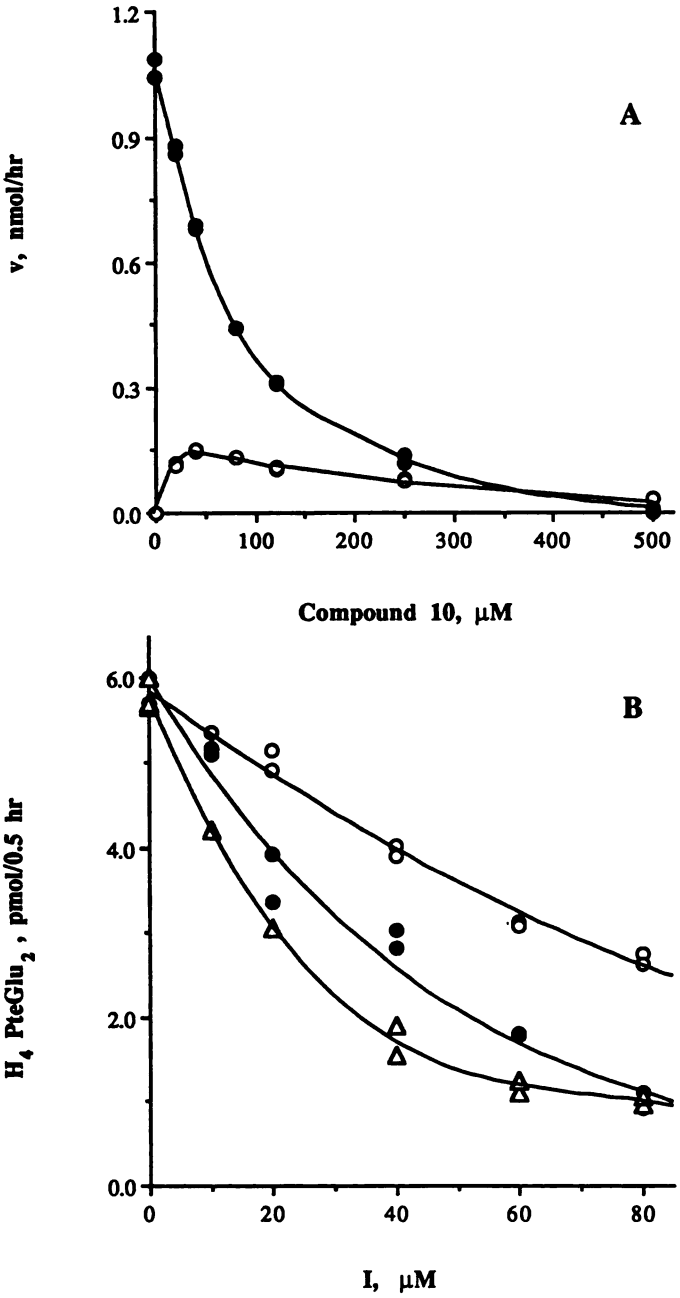


Fig. 2. Substrate activity of 2-desamino-2-methyl-3′-propyl-5,8-dideazafolic acid (**10**) for FPGS. A, The indicated concentrations of **10** (structure shown in Table 2) alone (○) and with 40 μM aminopterin (●) were incubated with FPGS for 1 hr, and the diglutamate product was isolated by adsorption onto charcoal and quantitated as described in the text. Each *point* represents a single reaction, although single points represent superimposed data. B, The indicated concentrations of aminopterin (○), **7** (△), and **10** (●) were incubated with FPGS in the presence of 10 μM (6*S*)-tetrahydrofolate for 30 min. (6*S*)-Tetrahydropteroyldiglutamate (H_4PteGlu$_2$) was quantitated by the microassay procedure described in the text. Each *point* represents a single reaction.

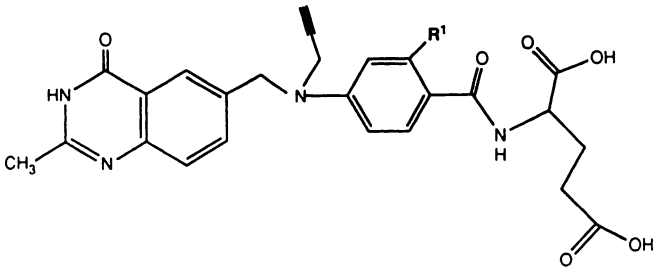
for the attainment of a catalytically competent enzyme-substrate complex.

The effect of a 7-methyl group was surprising. Previous experience with folate analogs modified in the heterocyclic or phenyl rings indicated a large degree of tolerance to deletion of either ring system (12–14, 17), and other series have indicated substantial tolerance to lengthening of the bridge between the heterocycle and the phenyl ring (10, 16). We (10) and others (48) previously reported that 7-hydroxymethotrexate was a substrate for mammalian FPGS, although, in retrospect, in our studies this compound had a V_{max} that was 3-fold lower than that of aminopterin and 2-fold lower than that of MTX. It was reported several years ago that 7-methylaminopterin and 7-methylmethotrexate were not active against intact tumor cells *in vitro* or *in vivo* (49, 50). Viewed in light of the data in Table 1, this fact retrospectively suggests inactivity of these 7-methyl-2,4-diaminopteridines as substrates for FPGS. The inactivation of polyglutamation by a 7-methyl group is so striking that one of our laboratories (51) has adopted the use of the 7-methyl derivative of D1694, i.e., **6**, as a negative control to indicate the role of polyglutamation in the potent biological activity of D1694. Compound **6**, while retaining activity against isolated thymidylate synthase, has poor activity against intact tumor cells, particularly after brief exposures, compared with the potent activity of D1694 (51).

It is very difficult to assign a structural basis to these striking effects in the absence of detailed information on the three-dimensional coordinates for mammalian FPGS. Nevertheless, there were substantial decreases in V_{max} that resulted from the introduction of substituents at position 7 for 10-propargyl-5,8-dideazafolate derivatives (Fig. 1; Table 1). In addition, substituents at position 3′ also had major effects on V_{max} of the FPGS reaction, with little or no effect on K_m (Fig. 2; Table 2). We interpret these observations as suggesting either that perturbation of the opposing enzyme surface in turn disturbs the catalytic pocket or that the conformation of the folate molecule needed to properly place the γ-carboxyl within the active site is disallowed by 3′- and 7-substituents. We also note that compounds with 3′-alkyl groups appeared to gain new hydrophobic interactions with active site residues, as judged by decreases in K_m with increasing size of the 3′-alkyl substituent. The effects of 2′-substituents are clearly still more complex but may shed structural light on two previous observations, i.e., the inability of MTX analogs that do not have an α-carboxyl group to bind to this enzyme and the inability of the aminopterin analog in which the amide carboxyl group is replaced by a methylene unit to bind to FPGS (10, 20). We previously proposed that the α-carbonyl group of the glutamic acid side chain forms either a salt bridge or a hydrogen bond with an active site residue that is essential for positioning of the glutamic acid side chain in a tight-fitting catalytic cleft on the enzyme (20). However, the fastidiousness of the active site for the exact shape of glutamic acid as a side chain in folate analogs (10, 11, 21, 22) was difficult to explain completely on this basis, and the complete inactivity as a substrate and lack of binding of deoxoaminopterin (10) (which differs from aminopterin by replacement of the amide carbonyl by a methylene group) was still unexplained. For instance, the inactivity of folate analogs that have 3-aminoglutaric acid side chains (10) would require an alignment mechanism based on a two (or more)-point attachment. If one hypothesized an active site hydrogen bond to the amide oxygen atom as well as a bridge to the α-carboxyl group (Fig. 4A), both of which were essential for binding and fit to the catalytically involved residues, then the rigid requirements of the active site for glutamic acid side chains could be explained, as could the results shown in Table 3. Thus, the lower V_{max} values observed with the 2′-Cl-, -CH_3-, -OCH_3-, and -CF_3-substituted compounds would reflect improper positioning

TABLE 3

Effect of 2′-substituents on the substrate activity of 2-desamino-2-methyl-10-propargyl-5,8-dideazafolic acid for mouse liver FPGS



R¹	Compound	K_m[a]		Relative values[b]	
		Isolation of diglutamate	Inhibition of $H_4PteGlu_2$ formation	V_{max}	V_{max}/K_m[c]
		μM			
-H	3	43 ± 6.2	30 (0.4)	0.76 ± 0.06	0.49 ± 0.25
-OH	13	42 (20)	30 (8)	0.19 (0.07)	0.15 (0.08)
$-NH_2$	14	43 (29)	54 (2.1)	0.1 (0.02)	0.09 (0.05)
-F	15	23.3 (11)		0.92 ± 0.11	1.08 (0.09)
-Cl	16	86 (3)	121 (20)	0.27 (0.02)	0.1 (0.02)
$-CH_3$	17	132 (4)	113 (24)	0.3 (0.0)	0.09 (0.01)
$-OCH_3$	18		180 ± 27	<0.01 (0.01)	<0.001[d]
$-CF_3$	19		250 ± 15	0.05 (0.03)	0.004[d]

[a] The Michaelis constant was either measured directly using isolation of the diglutamate product (charcoal assay) or indirectly inferred from the inhibition of the formation of tetrahydropteroyldiglutamate ($H_4PteGlu_2$) (microassay). If three or more values were averaged, the mean ± standard deviation is shown; if the mean is for two values, the range of values is shown in parentheses.

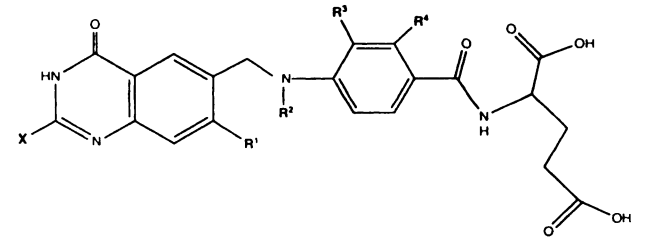
[b] Relative to V_{max} and V_{max}/K_m values for aminopterin, which are expressed as 1.0.

[c] Ratio of V_{max}/K_m of the compound to that of aminopterin in the same experiment, using the charcoal isolation procedure.

[d] The V_{max} value of the compound and of aminopterin were determined in the same experiment, using the charcoal procedure, but the K_m value used in the denominator was derived from a parallel microassay.

TABLE 4

Effect of bay-region substituents on the affinity of quinazoline folates for the reduced folate carrier



Compound	X	R¹	R²	R³	R⁴	K_i[a]	K_{ratio}[b]
						μM	
MTX						2.90 ± 0.36	
CB 3717	$-NH_2$	-H	$-CH_2CCH$	-H	-H	33.5 ± 2.40	
3	$-CH_3$	-H	$-CH_2CCH$	-H	-H	1.78 ± 0.17	
4	$-CH_3$	$-CH_3$	$-CH_2CCH$	-H	-H	4.80 ± 0.36	2.7
13	$-CH_3$	-H	$-CH_2CCH$	-H	-OH	1.30 ± 0.49	0.73
7	$-CH_3$	-H	-H	-H	-H	0.85 ± 0.14	
10	$-CH_3$	-H	-H	$-CH_2CH_2CH_3$	-H	0.41 ± 0.19	0.48

[a] Each value is reported as the mean ± standard deviation obtained from three experiments. See Materials and Methods for the detailed procedures.

[b] Values obtained by dividing the K_i values of the compound by that of an appropriate control, for example, K_{ratio} for 10 = K_i of 10/K_i of 7.

because of a distortion of the hydrogen bond usually present with the amide oxo group (see Fig. 4A). This hydrogen bond might also be disturbed for the 2′-NH_2- and -OH-substituted compounds, but the K_m was not changed because of the possibility of a replacement hydrogen bond between the same enzyme residue and the 2′-group, as shown in Fig. 4B for the 2′-OH-substituted compound. A similar structure can be drawn for a 2′-NH_2 substitution. Alternatively, the formation of an intramolecular hydrogen bond, as shown in Fig. 4C, would allow the maintenance of the proposed bond with the amide oxygen. This latter intramolecularly hydrogen-bonded structure might be responsible for the partial substrate activity observed for the 2′-OH and -NH_2 compounds. This concept is also supported by the superior substrate activity of the 2′-fluoro compound, which has been shown by NMR studies (52) to exist as an intramolecularly hydrogen-bonded species (Fig. 4D). Additional support for this concept is provided by the superb FPGS substrate activity of BW 1843U89, in which the 2′-carbon is covalently linked to the amide nitrogen through a

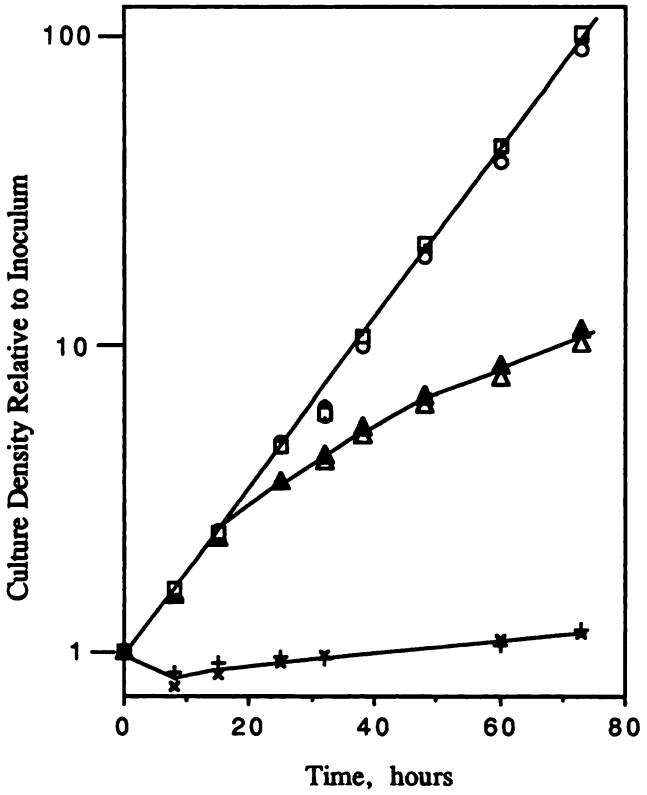


Fig. 3. Inhibition of the growth of L1210 cells in folate-deficient medium is not immediate, due to the effect of preexisting folate cofactor pools. Intracellular folate cofactor pools were depleted in a culture of L1210 cells by continuous exponential growth for 1 week in medium formulated without folic acid but supplemented with 5.6 μM thymidine, 32 μM hypoxanthine, and 10% dialyzed fetal calf serum. Cells that were growing in standard medium (RPMI 1640 medium supplemented with dialyzed fetal calf serum) or folate-depleted cells were washed once with phosphate-buffered saline and distributed into either standard medium or folate-deficient medium, and growth was followed as a function of time. Medium was added as needed to keep the cultures at densities compatible with exponential growth. *Symbols*, density (relative to the inoculum) of cultures initiated either with cells initially containing folate cofactor pools (□, transferred to standard medium; △, transferred to folate-deficient medium; ▲, transferred to folate-deficient medium containing 5.6 μM thymidine) or with folate-depleted cells (○, transferred to standard medium; +, transferred to folate-deficient medium; ×, transferred to folate-deficient medium containing thymidine). Growth of cultures initiated either with cells initially containing standard folate pools or with folate-depleted cells and then transferred to standard medium containing 5.6 μM thymidine was identical to that of controls (□, ○) and is not separately indicated, for clarity.

TABLE 5

Inhibition of L1210 cell growth dependent on entry of folic acid into polyglutamate pools

Compound	K_m for FPGS[a]	Growth inhibition IC_{50}[b]
	μM	μM
3	30	1.38 (0.31)
13	30	1.33 (0.24)
14	54	2.89 (0.88)
18	180	28 (1.3)
19	250	17.3 (17.4)
D1694	1.4	0.2 (0.2)
12	9.9	2.64 (1.52)
10	6.6	1.75 (0.69)

[a] K_m values are taken from Tables 1, 2, and 3.
[b] Each value is the mean of two experiments, with the range shown in parentheses. See Materials and Methods for details.

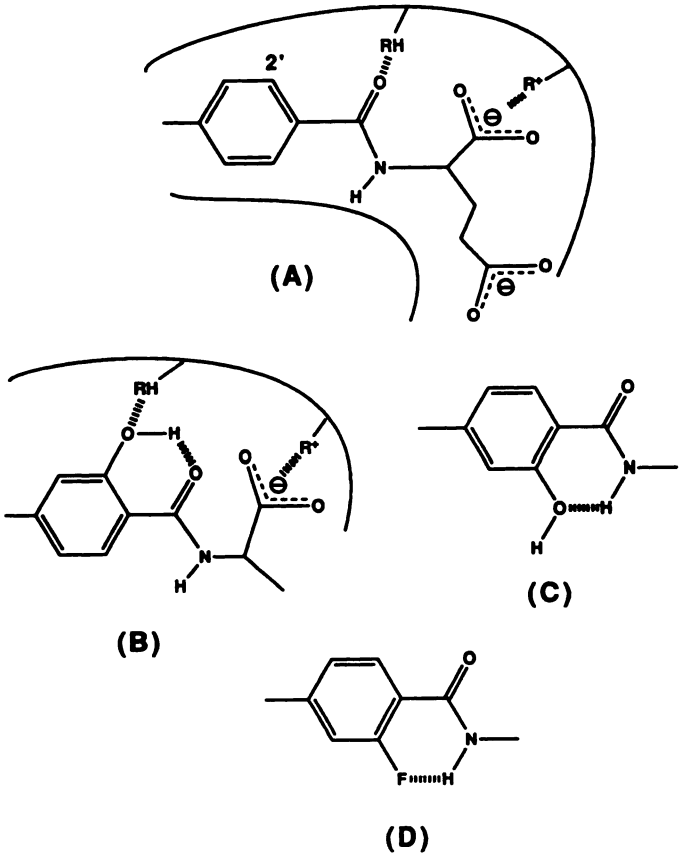


Fig. 4. Hydrogen and ionic bonds postulated to be involved in positioning of folate analogs in the active site of FPGS. The hydrogen bond involving an active site residue and the amide carbonyl (A) would be subject to steric effects of 2′-substituents but could be replaced by hydrogen bonds to 2′-OH or -NH_2 groups (B). For C and D, see text.

methylene bridge and which is perhaps the best substrate for FPGS yet known (53).

A continuing complication of the stringency of the domain of the FPGS active site responsible for aligning the glutamic acid side chain has been the difficulty of designing inhibitors for this enzyme. The only potent inhibitors yet designed have had either ornithine, homocysteic acid, or 2-amino-4-phosphonobutanoic acid side chains (24–30). However, all of these three groups differ from the usual glutamate side chain in two ways, i.e., 1) they have different orbital geometry at the δ-carbon of the side chain and 2) they have different charge distribution, compared with that characteristic of glutamic acid at physiological pH. This seems to interfere with transport into tumor cells via the reduced folate carrier system and, hence, some very potent enzyme inhibitors are nearly inactive against intact tumor cells. Our results indicate an alternative approach to the development of FPGS inhibitors that would leave the glutamic acid side chain intact. At first glance, analogs substituted at position 3′ seem to be the most promising, given that K_m values (and, we presume, K_d values) are virtually unchanged, compared with the corresponding 3′-unsubstituted control substrate (Table 2). Substitutions at position 7 seem inappropriate for development of inhibitors, at least as a single structural change (Table 1). However, we suggest that inhibitors could also be obtained by the use of 2′-groups that disrupt the putative essential hydrogen bond with the amide oxygen while

satisfying the other important binding domains. Now that the sequence of human FPGS has been published (54), we presume that structural information on this fascinating protein will soon become available to confirm our tentative structural conclusions and guide the development of FPGS inhibitors that are potent enough to allow an assessment of FPGS as a target for chemotherapy.

We note that a recent report suggested that different species of FPGS were expressed in normal adult intestinal stem cells and some experimental tumors (55). Although this concept is not yet conclusively proven, such a scenario could lead to rationally designed therapeutics based on selective inhibition of either the enzyme expressed in tumor or that expressed in normal stem cells. Thus, an inhibitor specific for the enzyme expressed in normal cells would prevent the accumulation of potent antifolates such as 5,10-dideazatetrahydrofolate or D1694 in normal cells while allowing cytotoxicity to tumor cells. On the other hand, folate antimetabolites that are specific substrates for tumor-derived FPGS, but not for FPGS in normal stem cells, would be drugs with very high therapeutic indices.

Acknowledgments

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