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Inhibition of Human Liver Folylpolyglutamate Synthetase by Non-γ-glutamylatable Antifolate Analogs

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SUMMARY

Folylpolyglutamate synthetase (FPGS) catalyzes the γ-glutamylation of both folates and folate antagonists and has been found to be essential for the survival of mammalian cells. Twelve analogs of the antifolates aminopterin (AMT) and methotrexate (MTX) having the $-(CH_2)_2COOH$ moiety replaced by $-(CH_2)_nX$, where $X = SO_3H, PO_3H_2$ or NH_2, were evaluated as inhibitors of FPGS isolated from human liver. The AMT analogs were consistently found to be better inhibitors than their MTX counterparts, following the order of K_m values determined for the parent antifolates as FPGS substrates. For the amino and phosphonate (but not for the sulfonate) compounds, inhibitory efficiencies were markedly dependent on the methylene chain length, with the most effective inhibitors having the groups $-(CH_2)_3NH_2$ (K_i = 0.2 μM) and $-(CH_2)_2PO_3H_2$ (K_i = 1.9 μM). Of those compounds exhibiting K_i values <200 μM, six were competitive inhibitors whereas three showed mixed inhibition (K_i' = ~6 K_i) when analyzed using AMT as the variable substrate. This demonstration of mixed inhibition of FPGS is consistent with the binding of inhibitor to a second site on the enzyme. Very similar K_i values (0.2–0.3 μM) were obtained for the $-(CH_2)_3NH_2$ analog of AMT when using folic acid, AMT, MTX, and γ-glutamyl-MTX as variable substrates, suggesting that the same enzymatic site on FPGS is active in the γ-glutamylation of these four folyl derivatives. These findings serve to identify structural features which are important for inhibition of human liver FPGS and may therefore prove useful for the design of new compounds having potential as chemotherapeutic agents.

The importance of polyglutamylation of folate antagonists such as MTX and AMT has become increasingly recognized with the findings that polyglutamylation is necessary for intracellular drug retention (1, 2), that polyglutamates of MTX are at least as inhibitory to dihydrofolate reductase as is MTX itself (3), and that polyglutamates are up to 1000-fold more potent than the corresponding monoglutamates as inhibitors of other folate-dependent enzymes such as 5-aminoimidazole-4-carboxamide ribotide transformylase (4) and thymidylate synthase (5). Polyglutamylation also influences the selectivity of leucovorin rescue (6) and diminished polyglutamylation has been found to contribute to MTX resistance (7–9). Polyglutamylation of natural folates, as well as of antifolates such as MTX and AMT, is catalyzed by the cytosolic enzyme FPGS, which has been purified to homogeneity from bacteria (10, 11) and also has been partially purified from several mammalian sources, including rat (12), mouse (13), beef (14), and human liver.[1]

Mutational deletion of FPGS has been shown to be lethal (16–18), raising the possibility that FPGS itself may be a target enzyme for chemotherapy, and thus prompting the search for specific inhibitors of FPGS (19). One such inhibitor, 4-fluoroglutamate, is metabolized by rat liver FPGS in the presence of MTX to yield MTX-γ-(4-fluoro)glutamate; this MTX derivative is then unable to react further to form higher polyglutamates, probably due to the inductive effect of the fluorine atom (20). γ-FluoroMTX has also been synthesized, but is a poor inhibitor of FPGS (21). Analogs of MTX and AMT, in which the γ-COOH group is replaced by acidic groups that cannot undergo γ-glutamylation, including phosphonate ($-PO_3H_2$) and sulfonate ($-SO_3H$), or in which the glutamate moiety is replaced by ornithine, have been characterized as effective inhibitors of FPGS from mouse liver (22–26) and, more recently, from rat liver and human lymphoblasts (27). These

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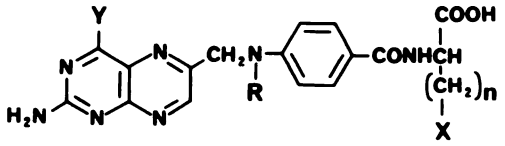
[1] L. Clarke and D. J. Waxman, submitted for publication.

ABBREVIATIONS: MTX, methotrexate (4-amino-4-deoxy-N^{10}-methylpteroyl-L-glutamic acid); AMT, aminopterin (4-amino-4-deoxypteroyl-L-glutamic acid); γ-fluoroMTX, γ-fluoromethotrexate; MTX-G_2, MTX containing one additional γ-L-glutamyl residue; FPGS, folylpolyglutamate synthetase; mAPA, 4-amino-4-deoxy-N^{10}-methylpteroic acid. The abbreviations used to designate the inhibitors examined in this study are summarized in Table 1.



Scheme 1

compounds have also been shown to retain the inhibitory effect of the parent drugs toward dihydrofolate reductase (22, 23, 25, 27, 28). Recent studies have highlighted small but potentially significant differences in the properties of FPGS isolated from various mammalian species (19),[2] emphasizing the importance of carrying out biochemical studies of FPGS using enzyme isolated from human tissues. In the present study, a series of 12 analogs of MTX and AMT having structural units of the type $—(CH_2)_nX$ in place of $—(CH_2)_2COOH$, where $X = PO_3H_2$, SO_3H, or NH_2, are examined for their inhibitory activity toward FPGS isolated from human liver. Although major similarities are shown to exist in the structure-activity profiles of these compounds with respect to human and rodent FPGS, our data also reveal subtle differences consistent with species variations at the enzyme active site.

Experimental Procedures

Materials. Potential FPGS inhibitors were prepared as described previously (22, 24, 25). L-forms of the amino and sulfonate inhibitors were used throughout; the phosphonate inhibitors used were racemic mixtures. The structures and designations of the compounds evaluated in the current study are summarized in Table 1 and Scheme 1. AMT was obtained from the Southern Research Institute (Birmingham, AL) and MTX from the National Cancer Institute (Bethesda, MD). MTX-G_2 was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO) and folic acid (pteroyl-L-glutamic acid) and ATP from Sigma Chemical Co. (St. Louis, MO).

Enzyme assays. FPGS was purified from human liver by 0–30% ammonium sulfate precipitation of a cytosolic fraction as described previously[2] using sample HL35 (28-year-old male) as the enzyme source. This FPGS preparation has been shown to exhibit biochemical and kinetic properties that are highly similar to preparations from five other individuals. FPGS activity was assayed by the procedure detailed previously.[2] Briefly, standard assay samples contained HL35 FPGS (2 mg of protein/ml), 1 mM L-[^{3}H]glutamate (5 mCi/mmol) (added via 20 μl of 25 mM sodium acetate, pH 5.2), AMT (2.5–25 μM), inhibitor (typically 0–500 μM) and ATP (5 mM) in 0.2 M Tris-Cl (pH 8.3 at 37°) containing 30 mM KCl, 25 mM $MgCl_2$, 30 mM $NaHCO_3$, and 45 mM 2-mercaptoethanol in a final volume of 0.25 ml. Samples were incubated at 37° for 90 min. Radiolabeled product was isolated from reaction mixtures by adsorption on small DEAE-cellulose columns, with extensive washing to remove unreacted L-[^{3}H]glutamate, followed by elution with HCl and scintillation counting.[2] High pressure liquid chromatographic product analyses demonstrated that only a single glutamate residue was incorporated into the antifolate substrate under these assay conditions.

Determination of inhibition constants. All inhibition constants (K_i values) reported refer to apparent K_i values. Preliminary K_i values were determined for each inhibitor from Dixon plots (29) of (FPGS activity)$^{-1}$ versus inhibitor concentration (0–500 μM), with the activity values determined at a fixed concentration of AMT (generally 25 μM). K_i values were then calculated from the equation x-intercept $= -(K_i[1 + S/K_m])$. For those compounds exhibiting a preliminary $K_i > 200$ μM, FPGS activity was determined at 5 μM AMT in the presence of 0–750 μM inhibitor (seven concentrations) and kinetic parameters were then derived from a Dixon plot analyzed by weighted least squares linear regression (weighting factor = velocity^2) as described previously (30). For compounds exhibiting more significant inhibitory activity (preliminary K_i <200 μM), K_i values were redetermined by measuring FPGS activity at 2.5–25 μM AMT in the absence of inhibitor, and in the presence of inhibitor concentrations equivalent to 1 and 3 times the preliminary K_i values. Lineweaver-Burk double reciprocal plots were constructed from these data (three curves, seven points per curve) and analyzed by the least squares method described above. K_i values were determined as the x-intercept of a secondary plot of the slopes of the Lineweaver-Burk curves versus inhibitor concentration. For all inhibitors with K_i <200 μM, FPGS activity was redetermined at inhibitor concentrations equivalent to 0 and 6 times the preliminary K_i in order to identify mixed inhibition (i.e., decreasing V_{max} and increasing K_m with increasing inhibitor concentration). Lineweaver-Burk plots were constructed and secondary plots of the y-intercepts of these curves versus inhibitor concentration yielded K_i' values (see Scheme 2) directly from the x-intercept. Kinetic constants are expressed as ± standard deviation for the best fit determined by the linear regression method described above.

Results and Discussion

Twelve analogs of the folate antagonists MTX and AMT were evaluated as inhibitors of human liver FPGS. The compounds studied were divided into three groups, according to whether the $—(CH_2)_2COOH$ portion of the L-glutamyl residue of the parent compound was replaced by $—(CH_2)_nSO_3H$ (sulfonate derivatives, designated S), by $—(CH_2)_nPO_3H_2$ (phosphonate derivatives, designated P), or by $—(CH_2)_nNH_2$ (amino derivatives, designated A). A summary of the structures and corresponding abbreviations of these compounds is included in Table 1. Inhibition constants (K_i and K_i') were determined from the slopes and y-intercepts, respectively, of Lineweaver-Burk plots (e.g., Fig. 1), except for the four weakest inhibitors, for which K_i values were determined from the x-intercepts of Dixon plots (26) (e.g., Fig. 2) as described in Experimental Procedures. K_i values for the inhibitors examined ranged from 0.2 μM to about 2.4 mM (Tables 2 and 3). Six of the compounds

TABLE 1
FPGS substrates and inhibitors

Compound[a]	R	Y	n	X
MTX[b]	CH_3	NH_2	2	COOH
AMT[b]	H	NH_2	2	COOH
Folic acid[b]	H	OH	2	COOH
S2m	CH_3	NH_2	2	SO_3H
S2a	H	NH_2	2	SO_3H
S1m	CH_3	NH_2	1	SO_3H
S1a	H	NH_2	1	SO_3H
P2m	CH_3	NH_2	2	PO_3H_2
P2a	H	NH_2	2	PO_3H_2
P1a	H	NH_2	1	PO_3H_2
A4m	CH_3	NH_2	4	NH_2
A3m	CH_3	NH_2	3	NH_2
A2m	CH_3	NH_2	2	NH_2
A1m	CH_3	NH_2	1	NH_2
A3a	H	NH_2	3	NH_2
mAPA[c]	CH_3	NH_2		

[a] Inhibitors are designated as S (sulfonate), P (phosphonate), or A (amino), followed by a number to indicate the number of methylene groups (n = 1–4), followed by letters m or a to identify the pterin moiety as equivalent to that of methotrexate or aminopterin, respectively. See Scheme 1 for definition of R, Y, n, and X.

[b] K_m values determined with HL35 FPGS were as follows: folic acid, 101 ± 16 μM; MTX, 61 ± 5.2 μM; AMT, 4.3 ± 0.5 μM (L. Clarke and D. J. Waxman, submitted for publication).

[c] mAPA lacks the L-glutamyl side chain entirely.

[2] L. Clarke and D. J. Waxman, submitted for publication.

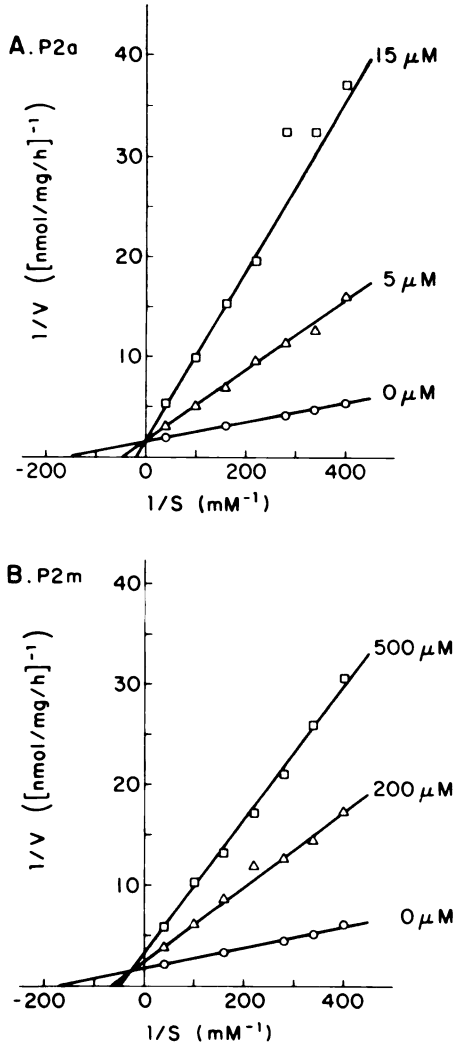


Fig. 1. Inhibition of human liver FPGS by phosphonate analogs of AMT and MTX. FPGS activity was determined under standard assay conditions for samples containing the indicated concentrations of AMT as variable substrate in the presence and absence of the indicated concentrations of P2a (A) or P2m (B). Each panel represents a separate experiment.

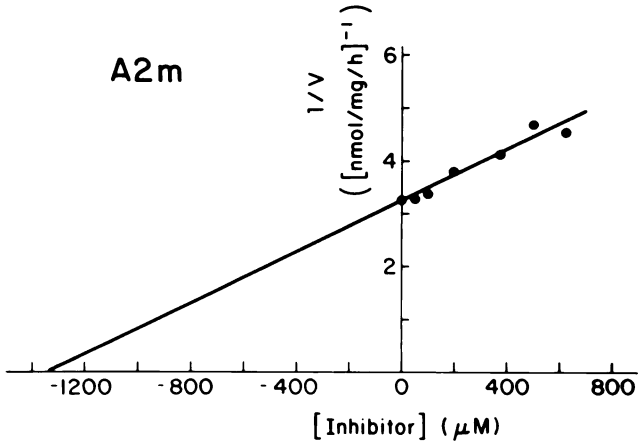


Fig. 2. Inhibition of human liver FPGS by an amino analog of MTX. FPGS activity was determined under standard assay conditions for samples containing 5 μM AMT and the indicated concentrations of A2m.

TABLE 2

Inhibition of human liver FPGS by sulfonate and phosphonate analogs of AMT and MTX

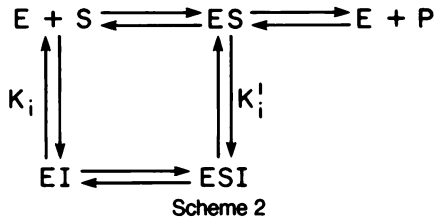
Inhibitor	Inhibition kinetics	K_i[a]	K_i'[b]
		μM	μM
S2m	Competitive	131 ± 6	
S1m	Mixed	173 ± 6	860 ± 90
S2a	Competitive	35 ± 2	
S1a	Mixed	66 ± 5	360 ± 60
P2m	Mixed	83 ± 3	555 ± 70
P2a	Competitive	1.9 ± 0.1	
P1a	–[c]	1500 ± 280[d]	

[a] Values were determined from the slopes of standard Lineweaver-Burk plots using data obtained at concentrations of inhibitor equal to 0, 1, and 3 times the preliminary K_i values (determined with AMT as variable substrate and as described under Experimental Procedures) except where noted.

[b] Inhibitory activity was determined at an inhibitor concentration of $6K_i$. K_i' was then calculated from the apparent V_{max} at that inhibitor concentration relative to the V_{max} determined in the absence of inhibitor. For compounds exhibiting an apparent $K_i' > 30K_i$, a K_i' value is not reported. For compounds where $K_i > 200$ μM, K_i' was not evaluated.

[c] Kinetics of inhibition were not evaluated for compounds exhibiting a $K_i > 200$ μM.

[d] Determined from Dixon plot of $1/V$ versus I (see Experimental Procedures).



Scheme 2

displayed competitive inhibition, as evidenced by a common intercept on the y axis of Lineweaver-Burk double reciprocal plots of data obtained in the presence of three different inhibitor concentrations (e.g., Fig. 1A). This corresponds to the situation represented by Scheme 2 when $K_i' = \infty$. Mixed inhibition was, however, observed for three of the compounds (e.g., Fig. 1B) and in such cases both K_i and K_i' values were determined (Table 2). Mixed inhibition was confirmed by secondary plots of V versus V/S and S/V versus I which clearly showed nonconverging and intersecting lines, respectively (data not shown). The nature of the inhibition effected by four other compounds (the weakest inhibitors; $K_i > 200$ μM) was not examined.

Sulfonate inhibitors. The four sulfonates studied were all moderately good inhibitors of human liver FPGS (Table 2). Comparison of the K_i value of S2m with that of its AMT analog S2a and of S1m with S1a revealed that in both cases the MTX derivative is a poorer inhibitor than the corresponding AMT derivative (3.7-fold and 2.6-fold higher K_i values, respectively). MTX itself is a poorer substrate for human liver FPGS than is AMT (14-fold lower K_m; see Table 1, Footnote *b*). Decreasing the length of the sulfonate-bearing hydrocarbon chain from 2 to 1 methylene units resulted in a small (1.3- to 1.9-fold) increase in K_i for both the MTX and AMT derivatives, suggesting that the shorter chain compounds bind somewhat less tightly to the enzyme. Although K_i values of similar magnitude to those of Table 2 have been reported for the sulfonate compounds with mouse liver FPGS, in that system compounds having only 1 methylene unit were better inhibitors than their counterparts containing 2 units (22), further highlighting the existence of interspecies differences with respect to FPGS. These findings are surprising in light of the reported strict

requirement of FPGS for the L-glutamate hydrocarbon chain length (19).

Phosphonate inhibitors. Several phosphonic acid derivatives were then evaluated for their effectiveness as inhibitors of human liver FPGS (Table 2). In this series as well, the AMT derivative, P2a, was a more potent inhibitor than the MTX derivative, P2m. Thus, as judged by their K_i values, these inhibitors exhibit relative binding affinities for human liver FPGS that are comparable to those of the parent substrates folic acid, MTX, and AMT, respectively. Removal of one methylene group from the phosphonate inhibitor P2a was accompanied by a very large increase in K_i [K_i(P1a)/K_i(P2a) = ~800], one that is much more striking than that observed for the analogous sulfonate inhibitors [K_i(S1a)/K_i(S2a) = 1.9] (Table 2).

Amino inhibitors. The findings described above prompted us to investigate further the effect of methylene chain length on FPGS inhibition. In these experiments the inhibitory activity of amino derivatives of MTX having 1–4 methylene units was studied. Initial experiments established that the amino compound A3m is a good inhibitor of FPGS, with 93% inhibition of FPGS activity effected by 500 μM inhibitor in the presence of 10 μM AMT. By contrast, amino compounds having either one more (A4m) or one less (A2m) methylene unit were much less inhibitory under comparable assay conditions (Fig. 3). Surprisingly, though, compound A1m (with two less methylenes than A3m) was moderately inhibitory. This contrasted with the low activity of this compound against enzyme from mouse liver (25) and other sources (27). K_i values determined in more detailed kinetic experiments (Table 3) confirmed the relative effectiveness of these amino derivatives and established that the K_i values increase sharply (50- to 120-fold) on either side of the optimal chain length of $n = 3$, but that the K_i for A1m is 4.2-fold lower than that for A2m. A similar chain length effect has been observed with mouse liver FPGS using a series of potential FPGS substrates (based on MTX) where the L-glutamate moiety was replaced by groups of the general structure —NHCH(COOH)$(CH_2)_n$COOH (19). In those experiments, FPGS activity was found to be ≤1% relative to MTX ($n = 2$) when $n = 1$, 3, or 5, but ~20% relative to MTX when $n = 4$. As would be expected, the compound mAPA, which lacks the L-glutamate residue entirely, is an exceedingly poor inhibitor of human liver FPGS (Table 3). In contrast, with FPGS from rat liver or from human lymphoblasts (K562 and CCRF-CEM cells), although A3m was also the most potent of the amino analogs, A2m was a 10- to 100-fold better inhibitor than A1m (27). Thus, for inhibitors with a terminal amino group, the rank order of potency against human liver FPGS is more nearly like the order against mouse liver enzyme than rat liver enzyme. Interestingly, the K_i of 110 μM reported for A2m inhibition of FPGS from K562 cells (27) is about 6-fold lower than the value obtained with human liver enzyme (Table 3). This suggests that there may be important differences in the binding characteristics of FPGS from transformed versus normal cells, and that it might be possible to design FPGS inhibitors that will selectively interfere with reduced folate polyglutamylation in tumors as compared with non-neoplastic tissue.

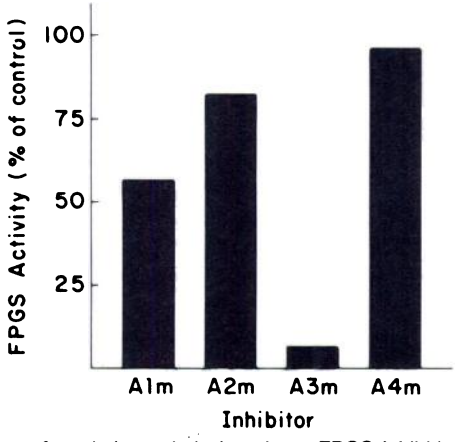


Fig. 3. Effect of methylene chain length on FPGS inhibition by amino analogs of MTX. FPGS activity was determined under standard assay conditions for samples containing 10 μM AMT and 500 μM inhibitor and is expressed as a percentage of the activity determined in the absence of inhibitor. Values graphed represent the average of duplicate determinations (±5–7%).

TABLE 3
Inhibition of human liver FPGS by amino analogs of MTX and AMT

Inhibitor	Inhibition kinetics	K_i[a]	Substrate
		μM	
mAPA	–[b]	2360 ± 170[c]	AMT
A1m	Competitive	165 ± 36	AMT
A2m	–	690 ± 80[c]	AMT
A3m	Competitive	13.4 ± 0.1	AMT
A4m	–	1580 ± 280[c]	AMT
A3a	Competitive	0.23 ± 0.01	AMT
A3a	Competitive	0.18 ± 0.02[d]	Folic acid
A3a	Competitive	0.30 ± 0.01[d]	MTX
A3a	Competitive	0.17 ± 0.01[d]	MTX-G_2

[a] See Table 2, Footnote *a*.
[b] Kinetics of inhibition were not evaluated for compounds exhibiting a $K_i > 200$ μM.
[c] Determined from Dixon plot of 1/*V* versus *I* (see Experimental Procedures).
[d] Determined as described in Table 2, Footnote *a*, except that the variable substrate was as indicated in the last column.

Since the AMT analogs were consistently better inhibitors of FPGS than the corresponding MTX derivatives, more detailed kinetic studies were carried out using the AMT analog of A3m, designated A3a. This compound was found to inhibit human liver FPGS competitively and with a K_i of 0.23 μM (Table 3). This K_i value is almost 60-fold lower than that of the MTX analog A3m, and is about 20-fold lower than the K_m exhibited by human liver FPGS for AMT.[3] Essentially the same K_i values were observed for A3a when using either AMT, MTX, folic acid, or MTX-G_2 as the folyl co-substrate for FPGS (Table 3); these observations are consistent with the presence of a single enzymatic site in our FPGS preparation that is active in the γ-glutamylation of folic acid, AMT, and MTX as well as the diglutamate MTX-G_2.

Kinetics of inhibition. Several of the FPGS inhibitors characterized in the present study have been previously studied for their inhibition of mouse liver FPGS and were shown to be competitive inhibitors (22–26). In the present study of human liver FPGS, however, mixed inhibition (Scheme 2) with $K_i'/K_i = 5$–7 was observed for three of the compounds (S1a, S1m, and P2m) (Table 2), suggesting potential important species differences with respect to their inhibition of FPGS. It should be emphasized, however, that kinetic data consistent with mixed inhibition might *appear* to indicate competitive inhibition (i.e., small shifts in V_{max} might be overlooked), unless the analyses

[3] L. Clarke and D. J. Waxman, submitted for publication.

are carried out at a suitably high inhibitor concentration.[4] Substrate inhibition has been observed for human liver FPGS with AMT and folinic acid[5] and also for the mouse kidney enzyme with folic acid (19), possibly reflecting the binding of substrate at a second site on the enzyme. Similarly, the mixed inhibition of human liver FPGS by compounds S1a, S1m, and P2m (Table 2) may reflect the binding of these inhibitors at such a second site.

An examination of the 12 FPGS inhibitors characterized in the present study reveals that for each class of compounds (sulfonates, phosphonates, and amino compounds) the AMT analog is a more effective inhibitor than the corresponding MTX analog. These differences follow the order of the K_m values of the parent compounds, although the ratio of K_i values for the AMT versus MTX analogs varies for each class of inhibitor. For instance, K_i(P2m)/K_i(P2a) = 44 as compared to K_i(S2m)/K_i(S2a) = 3.7 [c.f. K_m(MTX)/K_m(AMT) = 14]. Similar effects have also been observed for the compounds P2m, P2a, and S2m, S2a with mouse liver FPGS (24).

Previous studies have established that changes in the L-glutamyl portion of an FPGS substrate can result in greatly diminished substrate activity (19). However, effective inhibition of human liver FPGS was observed in the current study with a number of compounds altered in this portion of the molecule. Thus, FPGS inhibitory activity does not necessarily follow the strict structural requirements observed for FPGS substrates. A marked dependence of inhibition upon methylene chain length was, however, observed for the amino and phosphonate inhibitors (e.g., Fig. 3). This finding is reminiscent of the reported effect of potential FPGS substrates of the type —$(CH_2)_n$COOH (where n = 2 corresponds to MTX), where a decrease by 1 methylene unit results in greatly reduced substrate activity (19). By contrast, the sulfonate inhibitors S1a and S1m have K_i values only ~1.5-fold greater than those of S2a and S2m. The most effective inhibitor examined in the present study, A3a (K_i = 0.23 μM), differs from its parent compound AMT by replacement of the γ-COOH with —CH_2NH_2. In this compound the amino group occupies a position equivalent to that of the α-NH_2 group of the incoming glutamic acid when it forms an amide bond to yield MTX-G_2. The phosphonate inhibitor P2a (K_i = 1.9 μM) is also a good inhibitor of human liver FPGS, whereas the corresponding sulfonic acid analog, S2a (K_i = 35 μM), is somewhat less effective. Thus, both acidic and basic compounds are effective inhibitors of human liver FPGS. MTX analogs with neutral groups in place of the L-glutamate γ-COOH have been reported to be inactive as inhibitors of mouse liver FPGS (19), suggesting that a charged group may be required for binding to the enzyme.

The inhibitors discussed above are all unable to act as FPGS substrates due to replacement of the γ-COOH group. An alternative approach to abolishing FPGS substrate activity has been demonstrated using γ-fluoroMTX, where the presence of a fluorine atom adjacent to the γ-COOH prevents glutamylation by its inductive effect (21). A K_i value of ~350 μM can be estimated from the published data (21), indicating that γ-fluoroMTX is a poorer inhibitor than many of the compounds evaluated in this study.

The ability of the MTX and AMT analogs used in this study to inhibit not only FPGS but also dihydrofolate reductase (22, 23, 27, 28) ("dual action" inhibition) is of potential therapeutic usefulness, since perturbation of reduced folate polyglutamate cofactor pools in a cell should render the cell more sensitive to the action of an antifolate, particularly if the latter is one whose activity is independent of it being polyglutamylated itself (e.g., trimetrexate). It is important to note, however, that the K_i values for FPGS inhibition by the compounds examined to date are considerably higher than their K_i values as dihydrofolate reductase inhibitors. Thus, unless a level of free drug in the cell can be reached which is well in excess of the amount needed to saturate dihydrofolate reductase, this dual action mechanism will not be operative. Cytotoxicity data available (22, 23, 27, 28) indicate that none of these compounds is as potent as MTX or AMT in preventing cell growth in culture. It is not known whether this merely reflects poor cellular penetration or whether it reflects the fact that, in contrast to MTX and AMT, polyglutamates that would inhibit 5-aminoimidazole-4-carboxamide ribotide transformylase and/or thymidylate synthase (4, 5) cannot form from these compounds because they lack a γ-carboxyl group. The best of the inhibitors examined in this study, A3a, is at least 10 times more potent against FPGS than any of the other inhibitors studied and is clearly superior to γ-fluoroMTX (21). However, its low cytotoxicity (25, 27, 28) makes it unlikely to be of therapeutic value unless means are found to improve its delivery into the cell. Studies directed toward this goal would be of interest.

[4] Thus, for K_i'/K_i = 6, the V_{max} determined when the inhibitor is present at a concentration equal to its K_i would be equal to 86% of the V_{max} determined in the absence of the inhibitor.

[5] L. Clarke, [illegible]. J. Waxman, submitted for publication.

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