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EVALUATION OF PTEROYL-*S*-ALKYLHOMOCYSTEINE SULFOXIMINES AS INHIBITORS OF MAMMALIAN FOLYLPOLYGLUTAMATE SYNTHETASE*

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Abstract—The similarity between the reactions catalyzed by folylpoly-γ-glutamate synthetase (FPGS), γ-glutamylcysteine synthetase, and glutamine synthetase, as well as the susceptibility of the latter two enzymes to inhibition by methionine sulfoximine, suggest that folic acid derivatives with methionine sulfoximine or its alkyl homologs in place of the glutamate side chain of folate are good candidates to act as enzyme-generated transition state analog inhibitors of the FPGS reaction. Thus, pteroylmethionine sulfoximine, and the homologous *S*-ethyl-, *S*-propyl-, and *S*-butylhomocysteine sulfoximine derivatives were evaluated as inhibitors of FPGS that was partially purified from mouse liver and from mouse L1210 cells. The related compound, pteroyl-*S*-methylhomocysteine sulfone, which cannot undergo enzyme-mediated activation, was also investigated. Unexpectedly, none of these compounds showed significant inhibition of FPGS from these sources under a variety of conditions. These results, taken together with previously established structure–activity correlations, imply that a negative charge at the γ-position of folate analogs may be required for initial binding to FPGS and thus constitutes a prerequisite for activity of potential mechanism-based inhibitors of this enzyme.

The chemotherapeutic utility of methotrexate and other dihydrofolate reductase inhibitors for the treatment of human cancers has prompted the search for other suitable enzyme targets within the folate pathways and the design of specific inhibitors of these enzymes. Folylpolyglutamate synthetase (FPGS)¶, the enzyme responsible for the biosynthesis of the naturally occurring poly-γ-glutamate conjugates of the tetrahydrofolate cofactors, has been identified as an attractive candidate for such rational cancer chemotherapy [1]. The function of this enzyme appears to be essential for the survival of growing mammalian cell populations: somatic cell mutants deficient in FPGS are not viable under normal conditions of growth [2]. Many folate-dependent enzymes prefer the polyglutamate forms of their folate cofactors [3] and at least one metabolic pathway, the thymidylate biosynthesis cycle, is believed to require polyglutamates of tetrahydrofolate for its efficient operation [4, 5].

Methionine sulfoximine and several of its homologs are tight-binding inhibitors of the closely related enzymes, glutamine synthetase and γ-glutamylcysteine synthetase [6, 7]. Meister and his colleagues [6–10] have characterized these inhibitors extensively and have documented that the enzymes play an active, catalytic role in their own inactivation by these derivatives. The apparent mechanistic similarity between the reactions catalyzed by FPGS and those catalyzed by glutamine synthetase and γ-glutamylcysteine synthetase prompted us [11, 12] to replace the glutamate moiety of folic acid by methionine sulfoximine and some of its homologs to yield a series of potential "enzyme-generated transition state analogs" [13] of FPGS (see Fig. 1). In this paper, we report the evaluation of these comounds as inhibitors of FPGS from mouse liver and from mouse leukemia L1210 cells.

MATERIALS AND METHODS

The syntheses of the pteroyl-*S*-alkyl-D,L-homocysteine sulfoximines and pteroyl-*S*-methyl-D,L-homocysteine sulfone were described previously [12] and reported in part [13] and will be published in detail elsewhere. Briefly, N^{10}-trifluoroacetylpteroic

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¶ Abbreviations: FPGS, folylpolyglutamate synthetase; DHFR, dihydrofolate reductase; PMS, pteroyl-*S*-methylhomocysteine sulfone; PMSI, pteroyl-*S*-methylhomocysteine sulfoximine; PESI, pteroyl-*S*-ethylhomocysteine sulfoximine; PPSI, pteroyl-*S*-propylhomocysteine sulfoximine; PBSI, pteroyl-*S*-butylhomocysteine sulfoximine; PteGlu, folic acid (pteroylglutamic acid); H_2PteGlu, 7,8-dihydrofolic acid; H_4PteGlu, 5,6,7,8-tetrahydrofolic acid; $PteGlu_3$, pteroylglutamyl-γ-glutamyl-γ-glutamic acid (teropterin); 5-CHO-H_4PteGlu, 5-formyl-5,6,7,8-tetrahydrofolic acid; HEPES, *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; and MOPS, 3-(*N*-morpholino)propanesulfonic acid.

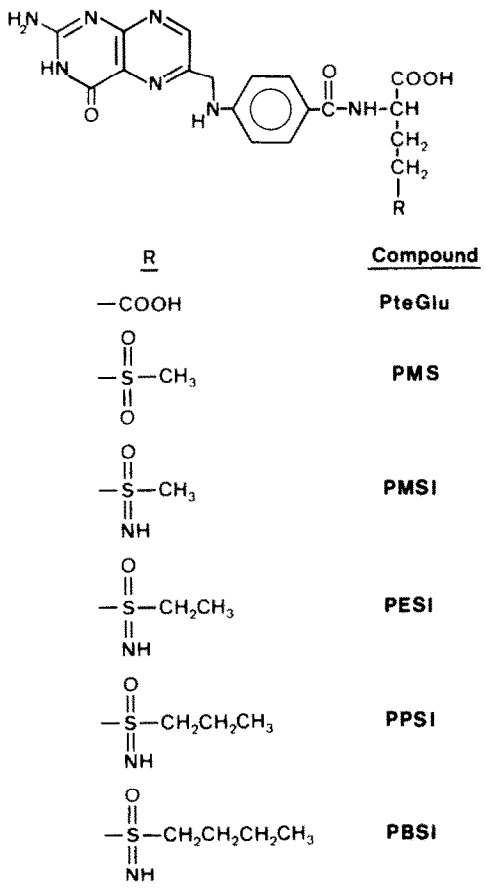


Fig. 1. Comparison of the structures of folic acid (PteGlu), pteroyl-*S*-methylhomocysteine sulfone, and pteroyl-*S*-methyl-, -ethyl-, -propyl- and -butylhomocysteine sulfoximines.

acid was coupled with the respective *S*-alkylhomocysteine sulfoximine ester hydrochlorides using isobutylchloroformate as the condensing agent. The analogs were deprotected with aqueous base and purified by ion-exchange chromatography. All compounds used in the present studies had satisfactory elemental analyses and their structural assignment was confirmed by UV spectra, ^{1}H-NMR and negative ion fast atom bombardment mass spectroscopy. Pteroyl-γ-triglutamate (teropterin; $PteGlu_3$) was obtained from Lederle (Pearl River, NY) and was purified by DEAE cellulose chromatography prior to use [14]. All other chemicals were from the Sigma Chemical Co. (St. Louis, MO) or Schwarz-Mann (Cambridge, MA) and were reagent or enzyme grade.

Mouse leukemia L1210 cells were harvested from the peritoneal cavity of DBA/2 female mice (Simonsen Laboratories, Gilroy, CA) 5–6 days after the i.p. inoculation of 10^6 cells. For the preparation of FPGS, L1210 cells or freshly perfused livers from DBA/2 female mice were suspended in 2 vol. of 20 mM HEPES buffer, pH 7.4, containing 0.25 M sucrose and 50 mM α-thioglycerol, homogenized or sonicated and centrifuged at 160,000 *g* for 1 hr. The supernatant fractions were precipitated with 30% $(NH_4)_2SO_4$ and used as a source of enzyme. These preparations, as previously described [15, 16], had typical specific activities of 1 to 1.5 mol/hr/mg protein and were free of conjugase activity under assay conditions. The reaction catalyzed by such enzyme preparations was shown previously [16] to be linear with time and with protein concentration. The products isolated and quantitated by the assay used have been characterized previously as folyloligoglutamates (chiefly diglutamate) by chromatography on DEAE cellulose, Sephadex G-25, and reverse phase HPLC [15] using preparations similar to those used in these experiments. No more than 15% of the folate derivative used as substrate was converted to product during these experiments. The enzyme assay used was based on the conversion of [^{3}H]glutamate (ICN Biochemicals, Irvine, CA; 4 mCi/mmol) to a charcoal-adsorbable product. In these experiments, there were 2.5 to 3.5 cpm/pmol of product formed. The characteristics of this assay have been described previously [16]. Briefly, enzyme was incubated at 37° with PteGlu (0.5 mM), or other folyl substrates, as noted, [^{3}H]glutamate (1 mM, 4 mCi/mmol), KCl (30 mM), $MgCl_2$ (10 mM), and ATP (5 mM) in 200 mM Tris–HCl buffer, pH 8.5 at 25°, containing 50 mM α-thioglycerol. The total volume of the assay incubation was 0.25 ml. ^{3}H-Labeled product was isolated from incubation mixtures by adsorption onto charcoal (0.5 ml) followed by four washes of the charcoal with 10 mM glutamate containing 10 mM β-mercaptoethanol (first wash, 5 ml, then three 10-ml washes). One milliliter of 3 M NH_4OH in 60% ethanol was used to elute product as described elsewhere [16]. Radioactivity was determined after addition of 10 ml of scintillation fluid (Budget-Solve, Research Products International, Elk Grove Village, IL) in a Beckman Scintillation Counter.

Dihydrofolate reductase (DHFR) was purified from *Lactobacillus casei*/MTX [17] by $(NH_4)_2SO_4$ precipitation, phosphocellulose, and Sephadex G-75 chromatography and was essentially free of NADPH oxidase activity. DHFR reaction rates were determined spectrophotometrically at 340 nm in 100 mM MOPS buffer, pH 6.0, containing 150 mM KCl, 100 μM folate (or 20 μM $H_2PteGlu$), and 50 μM NADPH. Up to 0.09 units (1 unit = 1 μmol $H_2PteGlu$ reduced/min) of enzyme were used for reduction of the pteroyl-*S*-alkylhomocysteine sulfoximines.

RESULTS

Pteroyl-S-alkylhomocysteine sulfoximines as inhibitors of mammalian FPGS. Mouse liver FPGS was incubated with 500 μM folic acid* (PteGlu) and a 500 μM concentration of either pteroyl-*S*-methylhomocysteine sulfone (PMS), pteroyl-*S*-methylhomocysteine sulfoximine (PMSI) or the corresponding pteroyl-*S*-ethyl- (PESI), -propyl- (PPSI) or -butylhomocysteine sulfoximines (PBSI). After 1 hr of incubation with simultaneously added folyl substrate and any of these potential inhibitors in complete reaction mixtures, the amount of pteroyloligoglutamate formed by mouse liver FPGS in the

* Previous studies have established that the apparent K_m for PteGlu under these conditions was 140 ± 47 μM [15].

Table 1. Inactivity of pteroylmethionine sulfoximine and its derivatives as inhibitors of mammalian FPGS*

Inhibitors†	Product formed (nmol/hr) Control (500 μM PteGlu)	Compound alone	PteGlu + compound	% of Control‡
(A) Mouse liver FPGS				
PMS	0.61 ± 0.02	0.01 ± 0.002	0.58 ± 0.008	95
PMSI	0.96 ± 0.02	0 ± 0	0.84 ± 0.01	88 (96)
PESI	0.61 ± 0.02	0 ± 0	0.58 ± 0.02	94 (94)
PPSI	0.61 ± 0.02	0 ± 0	0.59 ± 0.02	96 (85)
PBSI	1.54 ± 0.05	0 ± 0	1.32 ± 0.03	86 (97)§
(B) L1210 mouse leukemia FPGS				
PMS	0.70 ± 0.016	0.069 ± 0.016	0.78 ± 0.003	111 (102)
PMSI	0.70 ± 0.016	0 ± 0	0.85 ± 0.004	122 (114)
PESI	0.70 ± 0.016	0.017 ± 0.000	0.86 ± 0.011	122 (120)
PPSI	0.70 ± 0.016	0.015 ± 0.001	0.76 ± 0.012	108 (97)
PBSI	0.70 ± 0.016	0 ± 0	0.81 ± 0.012	116 (114)

* Partially purified mouse liver (A) or L1210 (B) FPGS was incubated at 37° for 1 hr with either 500 μM PteGlu or with 500 μM compound or with PteGlu and compound, both at 500 μM in a mixture that also contained [^{3}H]glutamate (1 mM, 4 mCi/mmol), KCl (30 mM), $MgCl_2$ (10 mM), ATP (5 mM), and α-thioglycerol (50 mM) in 200 mM Tris–HCl buffer, pH 8.5. Substrate and inhibitor were added simultaneously at time zero. The amount of product formed was determined by adsorption onto charcoal and elution with ethanolic ammonia as previously described [16]. Data are presented as means ± SD for duplicate assays.

† See Fig. 1 for structures of inhibitors.

‡ Values shown in parentheses are the results from a second experiment.

§ 350 μM concentration of PBSI was used in the repeat experiment.

presence of these compounds was not different from control (Table 1). When mouse L1210 cells were used as a source of FPGS, slightly more product was formed in the presence of PMSI, PESI, and PBSI than in control reactions (Table 1). Reproducible, significant levels of inhibition were not observed with any of these compounds with FPGS of either source. Because the natural substrates for FPGS *in vivo* are most likely reduced folates and are often oligoglutamyl folate derivatives, similar experiments were performed using either 5-formyltetrahydrofolate (5-CHO-H_4PteGlu) or $PteGlu_3$ as a substrate for mouse liver FPGS (Table 2). However, no inhibitory activity was seen with any of the pteroyl-S-alkylhomocysteine sulfoximines when these alternative substrates were used. It should be noted that no significant decomposition of PBSI could be detected by TLC during incubation at pH 8.5 for several hours (data not shown).

Inhibitory activity following preincubation with FPGS. Mouse liver FPGS was precincubated with each of these pteroyl-S-alkylhomocysteine sulfoximines under FPGS reaction conditions lacking various components. When folyl substrate was omitted from the preincubation and reaction was started by the addition of PteGlu, inhibition of FPGS activity was minimal in spite of 2- or 10-min exposure of enzyme in the absence of PteGlu, and a consistent, time-dependent increase in inhibitory activity was not observed in repetititive experiments. In other experiments, mouse liver FPGS was precincubated with 500 μM PBSI for 10 min under reaction conditions in the absence of folate and glutamate and also in the absence of folate, glutamate, and ATP. Under either condition, PBSI did not cause significant inhibition of FPGS activity during a subsequent 1-hr incubation initiated by the addition of the missing components (data not shown). In those experiments in which ATP was present during preincubation, mouse liver FPGS activity declined slowly during preincubation (Table 3). In contrast, when ATP was absent during precincubation, enzyme activity decreased >40% in 10 min in the

Table 2. Inhibition of mouse liver FPGS reaction by pteroyl-S-alkylhomocysteine sulfoximines using alternative folyl substrates*

Inhibitor†	Substrate	Reaction rate (nmol/hr)	% of Control
Control	$PteGlu_3$	1.15 ± 0.008	100
PMSI	$PteGlu_3$	1.19 ± 0.011	104
PESI	$PteGlu_3$	1.27 ± 0.018	111
PPSI	$PteGlu_3$	1.27 ± 0.005	111
PBSI	$PteGlu_3$	1.29 ± 0.015	113
Control	5-CHO-H_4PteGlu	0.79 ± 0.016	100
PMSI	5-CHO-H_4PteGlu	0.67 ± 0.001	88
PESI	5-CHO-H_4PteGlu	0.69 ± 0.008	88
PPSI	5-CHO-H_4PteGlu	0.67 ± 0.013	85
PBSI	5-CHO-H_4PteGlu	0.69 ± 0.006	86

* Folyl substrate and inhibitor were added simultaneously to enzyme at zero time and incubation was at 37° for 1 hr. Inhibitors were present at 500 μM and folyl substrates at either 500 μM (for $PteGlu_3$) or 20 μM (for 5-CHO-H_4PteGlu, which was the mixture of 6-*R*,*S* diastereomers). All other incubation conditions were as described for Table 1. Reaction rates are listed as means ± SD for duplicate assays.

† See Fig. 1 for structures of inhibitors.

Table 3. Inhibition of mouse liver FPGS following preincubation with pteroyl-S-alkylhomocysteine sulfoximines in the absence of folyl substrate*,†

Inhibitor‡	Product formed (nmol/hr)		
	No preincubation	2-Min preincubation	10-Min preincubation
No inhibitor	1.54 ± 0.05§	1.46 ± 0.04	1.17 ± 0.03
+ PBSI	1.32 ± 0.03	1.37 ± 0.03	1.07 ± 0.03
% Inhibition	14%	6.2%	8.6%
No inhibitor	0.96 ± 0.02	0.95 ± 0.01	0.87 ± 0.01
+ PMSI	0.84 ± 0.01	0.89 ± 0.01	0.80 ± 0.02
% Inhibition	13%	6.3%	8.1%
No inhibitor	0.46 ± 0.003	0.50 ± 0.003	0.45 ± 0.004
+ PESI		0.45 ± 0.01	0.42 ± 0.001
+ PPSI		0.44 ± 0.005	0.40 ± 0.005
% Inhibition		10%, 12%	6.7%, 11%

* Each inhibitor was incubated at a concentration of 500 μM with mouse liver FPGS, ATP (5 mM), KCl (30 mM), $MgCl_2$ (10 mM), α-thioglycerol (50 mM), and glutamate (1 mM) in reaction mixtures lacking only folyl substrate and buffered with Tris–HCl at pH 8.5. After 0, 2 or 10 min of incubation at 37°, reaction was begun by the addition of 500 μM PteGlu. The product was isolated after an additional 60-min incubation at 37°.

† Repeat experiments with each compound showed similarly low inhibition.

‡ See Fig. 1 for structures of inhibitors.

§ Values listed are means ± SD of duplicate assays.

absence of inhibitors. Stabilization of mammalian FPGS by ATP has been documented previously [15, 18].

Inhibitory activity of enzymatically reduced pteroyl-S-alkylhomocysteine sulfoximines. All of the folate analogs in Table 1 were found to be substrates for *L. casei*/MTX dihydrofolate reductase*. This enzyme accepted PteGlu as a substrate at pH 6.0, although maximal reaction velocities were seventy times lower than those found using H_2PteGlu as a substrate, in agreement with the work of others, [19, 20]. Using high levels of *L. casei*/MTX dihydrofolate reductase, PMSI, PESI, PPSI, PBSI, and PMS (at 100 μM) were reduced at rates of 50, 90, 93, 100, and 110%, respectively, of that observed with equimolar PteGlu.

Our previous experience with inhibitors of FPGS has indicated that reduction of the pteridine ring to the 5,6,7,8-tetrahydro structure increases the activity of compounds that are substrates for FPGS by a factor of 10–30 [21, 22]. Hence, we evaluated the pteroyl-S-alkylhomocysteine sulfoximines as inhibitors of mouse liver FPGS after preincubation with NADPH and DHFR. The amount of DHFR used in these experiments was approximately three times that found necessary to reduce completely 100 μM PteGlu in 1 hr. As an internal control in these experiments, PteGlu was added to some tubes at a concentration (20 μM) well below the K_m of FPGS for PteGlu, but close to saturation† if this substrate was converted to H_4PteGlu. As expected, the amount of FPGS product formed in 1 hr from 20 μM PteGlu substantially increased after preincubation with DHFR (Table 4). As was the case using PteGlu (Table 1) and either 5-CHO-H_4PteGlu or $PteGlu_3$ (Table 2), as test substrates, none of the compounds tested was inhibitory to the enzymatic reaction using aminopterin as a substrate for mouse liver FPGS (Table 4). In addition, enzymatic reduction of the pteroyl-S-alkylhomocysteine sulfoximines with DHFR to the corresponding tetrahydro-derivatives did not make these analogs inhibitory to FPGS.

DISCUSSION

The reaction catalyzed by FPGS has a clear similarity to those catalyzed by glutamine synthetase and γ-glutamylcysteine synthetase (Fig. 2). All three enzymes use the metabolic energy of ATP to form an amide bond between an (substituted) amine and the γ-carboxylic acid group of a (N-substituted) glutamic acid. On the basis of this analogy, it is reasonable to consider that all of these enzymes operate by similar mechanisms. Substantial evidence exists indicating that the reactions catalyzed by both glutamine synthetase and γ-glutamylcysteine synthetase proceed through an intermediate of glutamyl-γ-phosphate that is tightly bound to these enzymes (reviewed in Refs. 6–8). Similarly, it has been postulated that the FPGS reaction involves the transient formation of enzyme-bound pteroylglutamyl-γ-phosphate [23, 24]. Meister and his colleages [6–10] have shown that methionine sulfoximine is phospho-

* PBSI was shown previously [12] to serve as a good substrate for bovine dihydrofolate reductase although only the L-isomer of the racemic PBSI was reduced to the corresponding H_4PteGlu analog. This latter compound can replace H_4PteGlu as a cofactor for serine hydroxymethyltransferase and thymidylate synthase in permeabilized L1210 cells [12].

† The apparent K_m values for PteGlu and H_4PteGlu for this enzyme were reported previously to be 140 ± 47 and 7 ± 1.4 μM respectively [15, 21].

Table 4. Inhibition of mouse liver FPGS by pteroyl-S-alkylhomocysteine sulfoximines enzymatically reduced *in situ**

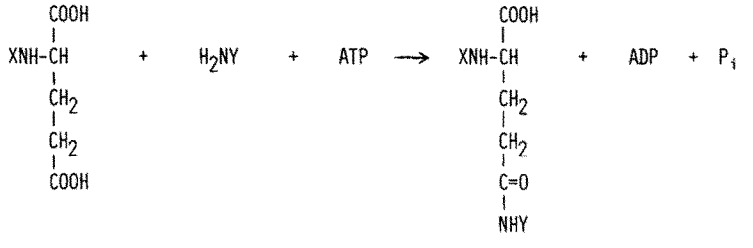
	Product formed (nmol/hr)	
	−DHFR	+DHFR
Aminopterin, 50 μM	0.72 ± 0.005 (100)†	0.71 ± 0.03 (100)†
PteGlu, 20 μM	0.088 ± 0.005 –	0.55 ± 0.015 –
Aminopterin + PMSI, 100 μM	0.69 ± 0.005 (95)	0.67 ± 0.005 (94)
Aminopterin + PESI, 100 μM	0.72 ± 0.02 (100)	0.64 ± 0.05 (90)
Aminopterin + PPSI, 100 μM	0.69 ± 0.03 (97)	0.68 ± 0.05 (95)
Aminopterin + PBSI, 100 μM	0.75 ± 0.03 (104)	0.77 ± 0.06 (108)
Aminopterin + PMS, 100 μM	0.68 ± 0.04 (94)	0.68 ± 0.05 (95)

* PteGlu, PMSI, PESI, PPSI, PBSI or PMS was incubated with NADPH (2.7 mM), KCl (100 mM), α-thioglycerol (33 mM) and *L. casei* DHFR (0.09 I.U.) at 37° for 60 min in a total volume of 38 μl of 67 mM MOPS buffer, pH 6. Other assay tubes (−DHFR) contained the same constituents but an equal volume of 50% glycerol was added in place of DHFR. Following this preincubation, mouse liver FPGS and the other components of the FPGS reaction [glutamic acid (to a final concentration of 1 mM), KCl (30 mM), $MgCl_2$ (10 mM), ATP (5 mM), and α-thioglycerol (50 mM) in 200 mM Tris–HCl, pH 8.5] were added to a total volume of 250 μl, and incubation was continued for an additional 60 min at 37°. During the folyl polyglutamate synthesis reaction, aminopterin (50 μM) was used as a folyl substrate, except where noted. The concentrations noted in the table refer to those present during the incubation with FPGS. Data are presented as means ± SD of duplicate assays.

† Values in parentheses list the reaction rates observed as percent of those in the uninhibited control incubations.

rylated by both glutamine synthetase and γ-glutamylcysteine synthetase and that the enzyme-generated methionine sulfoximine phosphate is an exceedingly tightly bound inhibitor of both enzymes. The ethyl, propyl, and butyl homologs of methionine sulfoximine are even better inhibitors of γ-glutamylcysteine synthetase, but the longer side chains decrease the effectiveness of these analogs against glutamine synthetase [8, 9]. It could be predicted from this analogy that the two classes of compounds most likely to act as transition state analogs of FPGS are structural analogs of pteroylglutamyl-γ-phosphate [23] and the pteroyl-S-alkylhomocysteine sulfoximines [9]. The structural similarity between the proposed transition state for FPGS and the N-phosphorylated form of PBSI is shown in Fig. 3. It has been reported recently that ^{18}O is transferred from the γ-position of methotrexate to inorganic phosphate during the reaction catalyzed by hog liver FPGS [25]. This is strong evidence that the FPGS reaction involves a pteroylglutamyl-γ-phosphate intermediate.

On this basis, a homologous series of PteGlu analogs, pteroyl-S-methyl, -ethyl, -propyl, and -butylhomocysteine sulfoximines were prepared [11, 12] and tested as inhibitors of mammalian FPGS.



Enzyme	X	Y
FPGS	Pteroyl	-CH(COOH)CH_2CH_2COOH
Glutamine Synthetase	H	H
Glutamylcysteine Synthetase	H	-CH(COOH)CH_2SH

Fig. 2. Similarity of the reactions catalyzed by FPGS, glutamine synthetase and γ-glutamylcysteine synthetase.

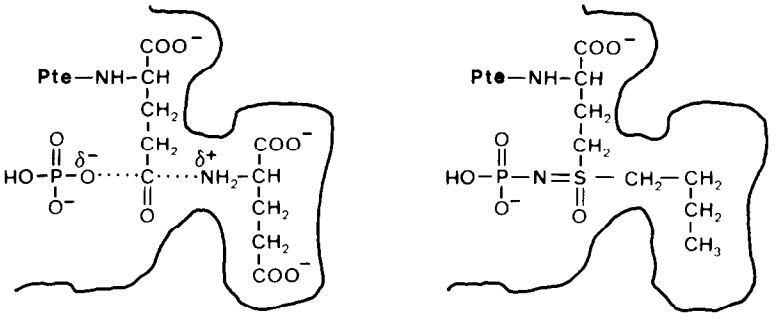


Fig. 3. Structures of the putative transition state of the FPGS reaction (left) and of the N-phosphorylated derivative of PBSI (right).

Contrary to our expectations, these compounds were not inhibitory to either mouse liver or L1210 FPGS under standard assay conditions. Inhibition was also not observed following preincubation with enzyme in the presence or absence of ATP or in incubations using alternative folyl substrates. Likewise, *in situ* reduction of these analogs to the corresponding tetrahydropteroyl-*S*-alkylhomocysteine sulfoximines did not result in any significant inhibition of FPGS.

We interpret our results as an indication that the pteroyl-*S*-alkylhomocysteine sulfoximines may not bind strongly to the enzyme either because of the absence of a negative charge at the γ-position and/or because of low bulk tolerance about the γ-position. In the course of our previous studies and those from other laboratories, the only* inhibitors of FPGS found to date that are competitive with the folyl substrate have ω-sulfonic acid [26–28], ω-phosphonic acid [29] or ω-carboxylic acid [21, 28,†] functional groups and, hence, bear negative charges at or close to the position equivalent to that of the γ-carboxyl group of PteGlu. In addition, we have also noticed that mouse liver FPGS seems to have a limited spatial tolerance around the amino acid side chain so that a small degree of steric interference results in the exclusion of an analog from the active site [21,†].

Although the present study seems to rule out the pteroyl-*S*-alkylhomocysteine sulfoximines as mechanism-based inhibitors of FPGS, it does not necessarily invalidate the original working hypothesis which led to the development of these analogs. We believe that our results indicate a tight fit of the active site of FPGS about the γ-carboxylic acid of folate compounds and the requirement of a negative change at or near this position. These structural requirements must be taken into account for the design of effective mechanism-based inhibitors of mammalian FPGS.

* This generalization excluded the cases of the ornithine analogs of PteGlu, H_2PteGlu and H_4PteGlu [28] and of methotrexate and aminopterin [30–32] which, we believe, represent bisubstrate analogs spatially similar to the folyl substrate and the α-amino group of the incoming glutamic acid and of pteroylglutamyl-γ-amide [28, 32] which may be considered a product analog.

† Also, R. G. Moran, unpublished observations.

REFERENCES

1. R. G. Moran, in *Folyl and Antifolyl Polyglutamates* (Eds. I. D. Goldman, B. A. Chabner and J. R. Bertino), p. 327. Plenum Press, New York (1983).
2. M. W. McBurney and G. F. Whitmore, *Cell* **2**, 173 (1974).
3. R. G. Matthews, Y-Z. Lu, J. M. Green and R. E. MacKenzie, in *Proceedings of the Second Workshop on Folyl and Antifolyl Polyglutamates* (Ed. I. D. Goldman), p. 65. Praeger Publishers, New York (1985).
4. T. I. Kalman and M. C. Hsiao, *Proc. Am. Ass. Cancer Res.* **26**, 235 (1985).
5. T. I. Kalman, in *Chemistry and Biology of Pteridines 1986* (Eds. B. A. Cooper and V. M. Whitehead), p. 763. Walter de Gruyter, Berlin (1986).
6. A. Meister, in *The Enzymes* (Ed. P. Boyer), Vol. 10, p. 671. Academic Press, New York (1974).
7. A. Meister, in *The Enzymes* (Ed. P. Boyer), Vol. 10, p. 699. Academic Press, New York (1974).
8. A. Meister, in *Enzyme-Activated Irreversible Inhibitors* (Eds. N. Seiler, M. J. Jung and J. Koch-Weser), p. 187. Elsevier/North Holland, New York (1978).
9. O. W. Griffith, M. E. Anderson and A. Meister, *J. biol. Chem.* **254**, 1205 (1979).
10. R. A. Ronzio, W. B. Rowe and A. Meister, *Biochemistry* **8**, 1066 (1969).
11. T. I. Kalman, *Acta pharm. suecica* (Suppl. 2), 297 (1985).
12. P. J. Harvison, *Ph.D. Dissertation*. State University of New York at Buffalo (1983).
13. T. I. Kalman and P. J. Harvison, *Proc. Am. Ass. Cancer Res.* **24**, 278 (1983).
14. R. G. Moran, W. C. Werkheiser and S. F. Zakrzewski, *J. biol. Chem.* **251**, 3569 (1976).
15. R. G. Moran and P. D. Colman, *Biochemistry* **23**, 4580 (1984).
16. R. G. Moran and P. D. Colman, *Analyt. Biochem.* **140**, 326 (1984).
17. R. B. Dunlap, N. G. L. Harding and F. M. Huennekens, *Biochemistry* **10**, 88 (1971).
18. R. T. Taylor and M. L. Hanna, *Archs Biochem. Biophys.* **181**, 331 (1977).
19. L. J. McIntyre and N. G. L. Harding, *Biochim. biophys. Acta* **482**, 261 (1977).
20. R. G. Matthews, J. Ross, C. M. Baugh, J. D. Cook and L. Davis, *Biochemistry* **21**, 1230 (1982).
21. R. G. Moran, P. D. Colman, A. Rosowsky, R. A. Forsch and K. K. Chan, *Molec. Pharmac.* **27**, 156 (1985).
22. E. C. Taylor, P. J. Harrington, S. R. Fletcher, G. P. Beardsley and R. G. Moran, *J. med. Chem.* **28**, 914 (1985).

23. J. K. Coward, in *Drug Action and Design: Mechanism-Based Enzyme Inhibitors* (Ed. T. I. Kalman), p. 13. Elsevier/North Holland, New York (1979).
24. B. Shane, *J. biol. Chem.* **255**, 5663 (1980).
25. R. Banerjee, J. J. McGuire, B. Shane and J. K. Coward, *Fedn Proc.* **45**, 1609 (1986).
26. A. Rosowsky, R. G. Moran, R. Forsch, P. Colman and M. Wick, *Biochem. Pharmac.* **33**, 155 (1984).
27. A. Rosowsky, R. A. Forsch, J. H. Freisheim, R. G. Moran and M. Wick, *J. med. Chem.* **27**, 600 (1984).
28. S. George, D. J. Cichowicz and B. Shane, *Biochemistry* **26**, 522 (1987).
29. A. Rosowsky, R. G. Moran, R. Forsch, M. Radike-Smith, P. D. Colman, M. M. Wick and J. H. Freisheim, *Biochem. Pharmac.* **35**, 3327 (1986).
30. A. Rosowsky, J. H. Freisheim, R. G. Moran, V. C. Solan, H. Bader, J. E. Wright and M. Radike-Smith, *J. med. Chem.* **29**, 655 (1986).
31. J. J. McGuire, P. Hsieh, C. T. Franco and J. R. Piper, *Biochem. Pharmac.* **35**, 1607 (1986).
32. D. Cichowicz, J. Cook, S. George and B. Shane, in *Proceedings of the Second Workshop on Folyl and Antifolyl Polylglutamates* (Ed. I. D. Goldman), p. 7. Praeger Publishers, New York (1985).