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FOLYLPOLYGLUTAMATE SYNTHETASE INHIBITION AND CYTOTOXIC EFFECTS OF METHOTREXATE ANALOGS CONTAINING 2,ω-DIAMINOALKANOIC ACIDS

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Abstract—The properties of a series of methotrexate analogs containing 2,ω-diaminoalkanoic acids have been investigated. The compounds were potent inhibitors of dihydrofolate reductase but, unlike methotrexate, they were also inhibitors of mammalian folylpolyglutamate synthetases. The potency of synthetase and reductase inhibition increased with increasing length of the 2,ω-diaminoalkanoate moiety. The most cytotoxic compound and the most potent inhibitor of both dihydrofolate reductase (I_{50} = 2.5 to 4 nM) and folylpolyglutamate synthetase (K_i *ca.* 4 μM) contained 2,5-diaminopentanoic acid (ornithine). These compounds were 70- to 100-fold less cytotoxic than methotrexate to human leukemia cell lines; however, they retained their potency against sublines resistant to methotrexate via defective transport. Their dual loci of enzyme inhibition and their efficacy against methotrexate transport-defective cell lines indicate that these compounds may be an important new class of antifol.

Methotrexate (MTX†) is an important drug for treatment of many human cancers, including acute lymphoblastic leukemia, choriocarcinoma, osteogenic carcinoma, and squamous cell carcinoma of the head and neck [1]. Use of MTX in the clinic, however, frequently leads to the development of acquired resistance. In addition to acquired resistance, there are classes of tumors (e.g. nonlymphocytic leukemia) which are intrinsically resistant to MTX [2]. Since inhibitors of folate metabolism are usually potent cytotoxic agents, but the efficacy of MTX [as well as other dihydrofolate reductase (DHFR) inhibitors] is limited by intrinsic or acquired resistance, numerous investigators have proposed that inhibitors of other folate-dependent enzymes might be useful. Thymidylate synthase (TS) has been suggested frequently as a target because of its crucial role as the *de novo* source of thymidine nucleotides [3], and because it is thought to be rate-limiting in DNA synthesis [4]. The 2-NH_2-4-OH-quinazolines (2-NH_2-4-OH-5,8-dideazafolates) appear to be specific TS inhibitors, based on cell culture cytotoxicity and isolated enzyme studies [5, 6]. However, these compounds are poorly transported, and their efficacy in animal studies appears to be limited by the high extracellular levels required to achieve cytotoxic intracellular concentrations.‡

Characterization of another important folate enzyme, folylpolyglutamate synthetase (FPGS), has only recently progressed to the point where inhibitors could be reasonably proposed (reviewed in Ref. 7). FPGS catalyzes the general reaction:

$$\text{PteGlu}_n + \text{ATP} + \text{L-Glu} \rightarrow \text{PteGlu}_{n+1} + \text{ADP} + \text{P}_i$$

where a γ-peptide linkage is formed. All natural folate monoglutamates and essentially all classical antifols containing a glutamate moiety are substrates for this enzyme and are converted to polyglutamates intracellularly. Compared to monoglutamates, the folate poly-γ-glutamates are better retained and serve at least as well as co-factors for folate-dependent reactions. Furthermore, formation of folate polyglutamates is essential for cell growth; mutational deletion of FPGS is lethal [8]. Thus, inhibitors of FPGS should be cytotoxic.

Attention has been focused on folate analogs in the search for FPGS inhibitors, since the widespread metabolism of ATP and glutamate would probably render their analogs nonspecific. The stringent structural specificity exhibited by FPGS, however, means that most analogs are either substrates or simply do not bind and thus are not inhibitors [7]. Only two analogs, the MTX derivatives containing 4-fluoroglutamate [9] or homocysteic acid [10, 11] in place of glutamate, are inhibitory to FPGS; neither is very potent. Very recently Shane [12] reported that reduced folates containing ornithine (2,5-diaminopentanoic acid) instead of L-glutamate are potent ($K_i < 1$ μM) inhibitors of mammalian and bacterial

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† Abbreviations: MTX (4-NH_2-10-CH_3PteGlu), methotrexate (4-amino-10-methylpteroylglutamate); DHFR, dihydrofolate reductase (EC 1.5.1.3); TS, thymidylate synthase (EC 2.1.1.45); FPGS, folylpolyglutamate synthetase; and HPLC, high pressure liquid chromatography.

‡ McGuire *et al.*, manuscript in preparation.

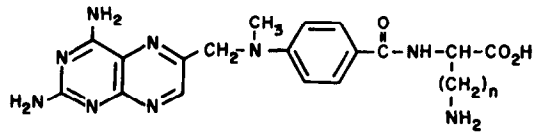

Fig. 1. Structure of methotrexate analogs containing 2,ω-diaminoalkanoic acids instead of L-glutamate. Analogs containing n = 1–3 were tested. The ornithine-containing derivative is n = 3.

FPGS. Based on these observations, we have investigated the effects of a homologous series (Fig. 1) of methotrexate analogs containing 2,ω-diaminoalkanoic acids, including ornithine, as inhibitors of DHFR, FPGS, and cell growth.

MATERIALS AND METHODS

MTX was obtained from the Division of Cancer Treatment, National Cancer Institute. The MTX derivative containing one additional glutamate was obtained from Dr. J. K. Coward, Department of Chemistry, Rensselaer Polytechnic Institute. The MTX derivatives containing two or three additional glutamates were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. Aminopterin (4-NH_2-PteGlu) was a gift of the Lederle Drug Co. The 2,ω-diaminoalkanoic acid containing MTX analogs (n = 1–3; Fig. 1) were synthesized as previously described [13].

Enzyme assays. DHFR activity was assayed spectrophotometrically (37°) essentially according to Osborn and Huennekens [14]. Each assay (1 ml) contained 100 μmoles Tris–Cl, pH 7.0, 150 μmoles KCl, 20 nmoles dihydrofolate, 50 nmoles NADPH, and DHFR. I_{50} values (concentration required for 50% inhibition) for DHFR inhibitors were obtained from graphs of log drug concentration versus activity. For measurement of inhibition, standard activity of 1.3×10^{-3} I.U. (K562) or 1.6×10^{-3} I.U. (CCRF-CEM) of purified DHFR from human leukemia cell lines was used. FPGS was assayed using L-[^{3}H]glutamate (New England Nuclear, Boston, MA) as a substrate [15, 16], and DEAE-cellulose minicolumns were used to separate free [^{3}H]glutamate from the ligated to a folate substrate. Standard assay mixtures (0.25 ml, pH 8.4) which contained Tris–Cl (0.1 M), ATP (5 mM), $MgCl_2$ (10 mM), KCl (20 mM), 2-mercaptoethanol (100 mM), 4 mM [^{3}H]glutamate (2×10^6 cpm/μmole), either 35 μM (6-*R*,*S*)-tetrahydrofolate or an appropriate concentration of a folate analog, and enzyme were incubated at 37°. Assays contained 530 units of rat liver FPGS, 310 units of CCRF-CEM cell FPGS, or 240 units of K562 cell FPGS. One unit of enzyme activity is defined as the incorporation of 1 pmole [^{3}H]-glutamate/hr under standard conditions. Kinetic data were obtained over a 10-fold concentration range of substrate in the presence or absence of inhibitor. Incorporation of radiolabel was shown to be linear with respect to time under all conditions. Slopes of the lines generated in a double-reciprocal plot of these data were plotted versus inhibitor concentration to obtain K_{is} values [17]. Since FPGS gives multiple products with some monoglutamate substrates (e.g. tetrahydrofolate), kinetic studies were done only with a substrate (either MTX or aminopterin) which gave essentially only the diglutamate during the incubation period under standard conditions. Michaelis–Menten conditions were thus maintained. With both human leukemia cell line FPGS enzymes, MTX was a relatively poor substrate with low affinity and a low maximum velocity, whereas aminopterin was a better substrate than even the best natural folate substrate, tetrahydrofolate (Fig. 2). Thus, aminopterin was used as the substrate for the human leukemia cell line FPGS. MTX and aminopterin are essentially equal as rat liver FPGS substrates, and MTX was used as a substrate for this enzyme for its kinetic analyses.

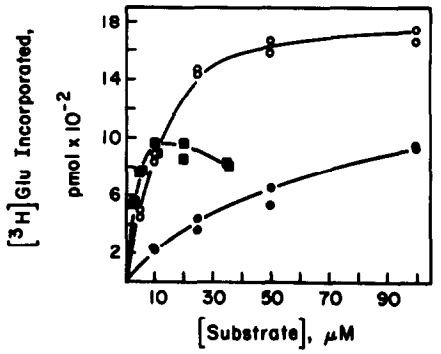

Fig. 2. Concentration dependence of CCRF-CEM folylpolyglutamate synthetase substrate activity of methotrexate, aminopterin, and (6-*R*,*S*)-tetrahydrofolate. Standard folylpolyglutamate synthetase reaction mixtures contained either MTX (●), aminopterin (○), or (6-*R*,*S*)-tetrahydrofolate (■) at the indicated concentration. Activity was measured as described in Materials and Methods. Results with K562 cell FPGS were similar.

Rabbit liver aldehyde oxidase was assayed at room temperature by measuring the change in absorbance at 341 nm as MTX (or its analog) was converted to its 7-hydroxylated derivative [18]. Standard reaction mixtures contained 70 mM potassium-phosphate, pH 7.8, 0.007% Na_2-EDTA, 250 mM $(NH_4)_2SO_4$, 100 μM MTX, and aldehyde oxidase. The amount of enzyme used caused a ΔA_{341}/min of 0.03 under standard conditions.

Enzymes. The purified K562 and CCRF-CEM cell line DHFR, prepared by MTX-Sepharose affinity chromatography, were supplied by A. R. Cashmore and W. Whyte-Bauer, respectively, of this laboratory. Rat liver FPGS was partially purified as previously described [15]. This preparation contains no γ-glutamyl hydrolase activity. The FPGS from the K562 and the CCRF-CEM human leukemia cell lines were prepared by fractionating freeze-thaw lysates (in 0.1 M Tris–Cl, pH 8.85, and 0.1 M 2-mercaptoethanol) with $(NH_4)_2SO_4$ (0–40% saturation pellet) followed by BioGel A-0.5 M chromatography. The active fractions (in 20 mM potassium-phosphate, pH 7.5, with 500 mM KCl and 50 mM 2-mercaptoethanol) were concentrated, made 20% (v/v) glycerol, and stored at −90° where

Table 1. Inhibition of dihydrofolate reductase and folylpolyglutamate synthetase by 2,ω-diaminoalkanoic acid analogs of MTX

Enzyme source	Inhibitor 4-NH_2-10-CH_3Pte-R	DHFR inhibition IC_{50} (nM)	FPGS inhibition K_{is}* (μM)	FPGS inhibition Type†
	R =			
K562	2,5-Diaminopentanoate	4	4.1	LC
	2,4-Diaminobutanoate	7.5	110	C
	2,3-Diaminopropanoate	18	>2000	C
	Glutamate (MTX)	1		
CCRF-CEM	2,5-Diaminopentanoate	2.5	3.2	C
	2,4-Diaminobutanoate	5.7	330	C
	2,3-Diaminopropanoate	17		
	Glutamate (MTX)	1		
Rat liver	2,5-Diaminopentanoate		3.8	LC
	2,5-Diaminopentanoate		3.2‡	C
	2,4-Diaminobutanoate		200	LC
	2,3-Diaminopropanoate		>1000	C

Named compounds were tested as inhibitors of DHFR and FPGS as described in Materials and Methods.

* K_{is} values were obtained from replots of slope (derived from double-reciprocal plots) versus inhibitor concentration.

† All inhibitors were of the competitive type (Type = C), and in some cases plots of $(\text{velocity})^{-1}$ versus inhibitor concentration at fixed substrate established that this was of the linear competitive type (Type = LC).

‡ The diglutamate of MTX, 4-NH_2-10-CH_3PteGlu$_2$, was used as a substrate (K_m = 25 μM).

they were stable indefinitely. Rabbit liver aldehyde oxidase was purified 46-fold according to our modification [19] of the procedure of Johns *et al.* [18].

Cell culture and cytotoxicity. Cells were cultured using a standard sterile technique. The myeloblastic leukemia cell line K562 [20] was grown in RPMI 1640 (GIBCO) containing 10% filtered fetal bovine serum (GIBCO). The lymphoblastic cell line CCRF-CEM [21] was cultured in RPMI 1640 containing 10% filtered horse serum (GIBCO). A subline of CCRF-CEM, resistant to MTX because of defective MTX transport [22], was cultured in the same medium containing 1 μM MTX. These cells were grown in the absence of MTX for 48 hr prior to their use in cytotoxicity studies.

Synthesis of MTX polyglutamates was examined by incubating cells for the required time in [^{3}H]MTX in the presence or absence of inhibitors. Intracellular labeled drug was isolated and analyzed as described [23].

To measure EC_{50} values (drug concentration inhibiting growth by 50%), logarithmically growing cells were seeded at 1×10^4 cells/ml in the absence of presence of various concentrations of drug. Following 120 hr of continuous exposure to the drug, a cell count was performed using a model ZB1 Coulter counter. Control samples (containing no drug) grew logarithmically during this entire period. The EC_{50} values were obtained from plots of log drug concentration versus percent of control growth.

High pressure liquid chromatography. HPLC was performed on a microparticulate anion exchange column (Whatman Partisil 10 SAX) as previously described [16].

Miscellaneous. All substrate concentrations were determined from absorption spectra and published extinction coefficients [24]. The extinction coefficients of MTX were used for its 2,ω-diaminoalkanoic acid analogs.

RESULTS

Inhibition of human DHFR by MTX analogs derived from 2,ω-diaminoalkanoic acids. The 2,ω-diaminoalkanoic acid containing derivatives were potent inhibitors of DHFR from two human leukemia cell lines, although none was as potent as MTX (Table 1). The DHFR from lymphoblastic (CCRF-CEM) and myeloblastic (K562) cell types showed essentially the same sensitivity to inhibition by each compound. The degree of inhibition increased as the length of the side chain increased to pentanoate.

Inhibition of rat liver and human FPGS. The 2,ω-diaminoalkanoic acid containing analogs of MTX lacked FPGS substrate activity but were inhibitory towards FPGS isolated from two human leukemia cell lines and rat liver (Table 1). As with DHFR inhibition, potency increased in this series with increasing length of side chain, the most inhibitory again being the 2,5-diaminopentanoate (ornithine) containing derivative with a K_i near 4 μM for all three enzymes. The dependence of inhibition on side chain length was more apparent with FPGS than DHFR, however. Thus, the change from butanoate to pentanoate caused only a 2-fold increase in DHFR inhibitory potency but increased FPGS inhibition by 30- to 100-fold. Inhibition by these compounds was

Table 2. Effect of 4-NH_2-10-CH_3 Pte-ornithine on synthesis of methotrexate polyglutamates (4-NH_2-10-CH_3PteGlu$_n$) by purified rat liver FPGS

Concn of 4-NH_2-10-CH_3-Pte-ornithine (μM)	4-NH_2-10-CH_3PteGlu$_n$ forms (pmoles)			
	n = 1	2	3	4
0	1441	820	118	3.6
1	1508	780	97	2.4
5	1785	353	28	0

Standard reaction mixtures contained 10 μM [^{3}H]methotrexate (235 cpm/pmole), the indicated concentration of inhibitor, and partially purified rat liver FPGS. The products were separated by HPLC, and the individual components were quantitated by liquid scintillation counting.

competitive in all cases and appeared to be of the linear type based on the secondary replots of slope versus inhibitor concentration. This was verified for several compounds in separate experiments where the substrate concentration was fixed while the inhibitor concentration was varied over a 10-fold range. Plots of 1/velocity versus inhibitor concentration were linear in all these cases (data not shown). FPGS activity with tetrahydrofolate as the substrate was inhibited with similar potency by these MTX analogs (data not shown) but, because of the complex kinetics with this substrate [15], K_i values could not be determined.

Preincubation of the pentanoate derivative with rat liver FPGS in reaction mixtures lacking only MTX did not enhance inhibition, indicating that this analog is not tight-binding. In addition, inhibition by the pentanoate derivative was only additive with the inhibition caused by β,γ-methylene-ATP when the two inhibitors were present simultaneously.

The effect of the ornithine (2,5-diaminopentanoate) containing MTX analog on the distribution of MTX polyglutamate products synthesized by isolated rat liver FPGS was examined (Table 2). Although synthesis of derivatives longer than diglutamate was limited, a decline in all products was evident which was dependent on inhibitor concentration. Each step in elongation appeared to be sensitive to inhibition, although part of the decline in each length might be attributable to the decrease in its immediate precursor. Consistent with each step being inhibited is the finding (Table 1) that the ornithine analog was equally inhibitory whether MTX or its diglutamate form was the substrate (K_i *ca.* 3 μM) for rat liver FPGS.

The effect of the ornithine containing MTX analog on MTX polyglutamate synthesis by intact CCRF-CEM cells was also examined (Table 3). During simultaneous exposure to 10 μM [^{3}H]MTX and 50 μM analog, there was a pronounced decrease in both total drug and total polyglutamate forms. All classes of polyglutamate derivatives were decreased. The total percentage of drug present as polyglutamates was about 75% in both cases, however. The ornithine analog only very weakly inhibited [^{3}H]MTX transport (data not shown), arguing that the decreased intracellular drug is not due to competition for transport. Inhibition of MTX polyglutamate elongation was observed when MTX polyglutamates were allowed to accumulate, extracellular MTX was removed, and efflux was allowed to occur in the presence or absence of ornithine analog (Table

Table 3. Effect of 4-NH_2-10-CH_3Pte-ornithine on MTX polyglutamate (4-NH_2-10-CH_3PteGlu$_n$) synthesis by CCRF-CEM cells

Conc 4-NH_2-10-CH_3-Pte-ornithine (μM)	Efflux time	Intracellular 4-NH_2-10-CH_3PteGlu$_n$ forms (pmoles/10^7 cells)						
		n = 1	2	3	4	5	Total n ≥ 2	n = 1–5
0	0	18.2	38.0	18.4	2.1	0.13	58.6	76.8
50	0	11.5	20.4	10.0	1.2	0.10	31.7	43.2
0	1	1.8	23.5	18.0	2.9	0.71	45.1	46.9
50	1	1.3	20.1	15.6	2.7	0.46	38.9	40.2
0	4	1.1	8.2	17.6	4.9	0.87	31.6	32.7
50	4	0.7	6.7	16.5	4.3	0.87	28.4	29.1

Logarithmically growing CCRF-CEM cells were harvested and resuspended at 5.9×10^5 cells/ml in RPMI 1640 containing 10% filtered horse serum (37°) in spinner flasks. [^{3}H]MTX (110 cpm/pmole; 10 μM final concentration) was added along with its ornithine analog. Incubation was continued for 4 hr at which time aliquots were removed, and the intracellular [^{3}H] pool was analyzed as described in Materials and Methods. An aliquot of the [^{3}H]MTX alone reaction was centrifuged, washed once with medium, and resuspended at approximately the same cell density to study efflux. These cells were divided equally and one portion received the ornithine analog while one did not. At 1 and 4 hr of efflux, aliquots were removed from each, and the intracellular [^{3}H] pool was analyzed.

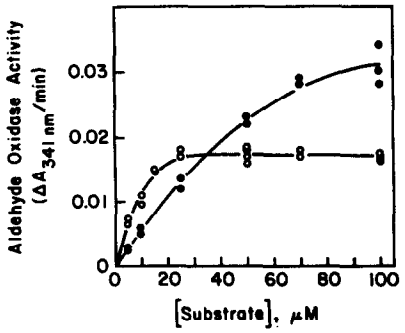

Fig. 3. Concentration dependence of rabbit liver aldehyde oxidase substrate activity of methotrexate (4-NH_2-10-CH_3PteGlu) and 4-NH_2-10-CH_3Pte-(2,5-diaminopentanoic acid). Partially purified rabbit liver aldehyde oxidase was assayed as described in Materials and Methods. The concentration of methotrexate (●) or 4-NH_2-10-CH_3Pte-(2,5-diaminopentanoate) (○) was varied, as indicated.

3). Rapid efflux of unmetabolized MTX and a slower efflux of its diglutamate form occurred both in the presence and absence of the ornithine derivative. The ornithine analog thus did not interfere with the efflux process. Relatively little elongation of polyglutamates occurred during the efflux period, but some inhibition of this process was apparent in the cultures treated with the ornithine-containing compound.

Substrate activity for rabbit liver aldehyde oxidase. MTX, and other classical 2,4-diamino antifolates, are known to be metabolized significantly by aldehyde oxidase to their 7-hydroxylated forms in man [25]. The aldehyde oxidase substrate activity of the 2,5-diaminopentanoate containing analog was compared to that of MTX (Fig. 3). The analog exhibited a K_m strikingly lower than that of MTX (9 and 164 μM respectively), although its relative V_{max} was only 22% that of MTX. The catalytic effectiveness of the new analog, as measured by V_{max}/K_m [26], was thus about 4-fold greater than MTX. The u.v. absorption spectrum of the product of the pentanoate analog was identical with that of authentic 7-hydroxymethotrexate.

Cytotoxicity to human leukemia cell lines. The cytotoxic effects of the 2,ω-diaminoalkanoic acid containing analogs on K562 and CCRF-CEM cells were determined (Table 4). With the K562 cell line, there were small, but reproducible differences in cytotoxicity of the three analogs with the 2,5-diaminopentanoate derivative again being the most potent. All derivatives were greater than 100-fold less potent than MTX. The higher EC_{50} values were not the result of degradation or decomposition of the drug in the culture medium, as determined by DHFR inhibition studies (data not shown). The CCRF-CEM lymphoblastic line was similarly less sensitive (70-fold) to the 2,5-diaminopentanoate derivative than to MTX. However, a subline of CCRF-CEM, which is 250-fold resistant to MTX via defective MTX transport, was only 5-fold less sensitive to the 2,5-diaminopentanoate derivative than was the parent (Table 2).

The metabolite protection profile of the 2,5-diaminopentanoate derivative was essentially the same as that for MTX with the K562 cell line (data not shown). Hypoxanthine up to 10^{-5} M offered no protection, whereas thymidine alone at that concentration protected 10% and the combination of the two protected 30%. Higher concentrations of thymidine were toxic. At equitoxic doses, EC_{90}, for MTX (2×10^{-8} M) or the pentanoate analog (3×10^{-6} M), leucovorin (5-formyltetrahydrofolate) at 5×10^{-8} M was completely protective.

DISCUSSION

The 2,ω-diaminoalkanoic acid series of MTX analogs were potent inhibitors of human leukemia cell DHFR in agreement with previous findings with murine DHFR [13]. The 2,5-diaminopentanoate (ornithine) analog of MTX has been prepared previously as a DHFR inhibitor [27, 28] and was shown to be a slightly less potent inhibitor than MTX.

Our studies showed that 2,ω-diaminoalkanoate containing analogs of MTX were also competitive inhibitors of mammalian FPGS, a property not previously tested. The most potent analog against three different FPGS was the 2,5-diaminopentanoate

Table 4. Cytotoxic effects on K562 and CCRF-CEM human leukemia cell lines of 2,ω-diaminoalkanoic acid analogs of MTX

Cell line	Inhibitor 4-NH_2-10-CH_3Pte-R	EC_{50} (μM)
	R =	
K562	2,5-Diaminopentanoate	1.7
	2,4-Diaminobutanoate	2.3
	2,3-Diaminopropanoate	3.0
	Glutamate (MTX)	0.0175
CCRF-CEM	2,5-Diaminopentanoate	0.74
	Glutamate (MTX)	0.011
CCRF-CEM(MTX^R)*	2,5-Diaminopentanoate	3.7
	Glutamate (MTX)	2.7

Cytotoxicity was measured by outgrowth following a 120-hr continuous exposure to inhibitor according to procedures in Materials and Methods.
* Cell line resistant to MTX by virtue of defective MTX transport [22].

(ornithine) derivative. Based on the work of Shane and co-workers [12] with reduced pteroyl-ornithine, it was expected that this analog would be inhibitory. However, our results showed for the first time that MTX analogs containing shorter 2,ω-diaminoalkanoate homologs are much less inhibitory to FPGS (Table 1). The effect on FPGS inhibition of increasing the length of side chain in the MTX analog to 2,6-diaminohexanoic acid (lysine) is not known. However, FPGS studies with a series of structurally related compounds containing either ornithine or lysine indicated that ornithine was the optimal length for inhibition (McGuire *et al.*, unpublished observations). The lysine containing MTX analog is a more potent inhibitor of DHFR than is the ornithine derivative, but both are less potent than MTX [28].

The cytotoxic effect of these compounds also decreased as the 2,ω-diaminoalkanoic chain length decreased from C_5. The degree of change in cytotoxicity more closely paralleled the change in DHFR inhibition rather than the FPGS inhibitory potency. However, the relatively low K_i of the ornithine-containing derivative for FPGS indicated that inhibition of this enzyme may also play a role in its cytotoxicity. The protection afforded against cytotoxicity by thymidine and hypoxanthine was expected for either DHFR or FPGS inhibitors in RMPI 1640 medium (which contains the other end products of folate metabolism) and thus did not distinguish between the importance of inhibition at each site. The most cytotoxic of these compounds, 4-NH-10-CH_3pteroyl-ornithine, was 70- to 100-fold less cytotoxic than MTX itself. This could be the result of several factors, including the weaker inhibition of DHFR by this compound (Table 1). Another factor may be that polyglutamates of this derivative cannot be formed and MTX polyglutamates may be important in cytotoxicity even under conditions of continuous exposure [9]. Finally, transport of this compound may be limiting. The ornithine-containing MTX analog apparently does not enter cells via the MTX/reduced folate active transport system. This conclusion is based on the similar potency of this analog against parental CCRF-CEM cells and a resistant subline which is defective in MTX transport (Table 2) and the observation that 20 μM analog did not affect the initial uptake of 2 μM [^{3}H]MTX into parental CCRF-CEM cells (data not shown).

The molecular basis for the potent inhibition of FPGS by reduced pteroyl-ornithine [12] and the ornithine containing MTX analog is puzzling at present. FPGS generally displays very strict specificity for L-glutamate as the residue in the acceptor position on the folate substrate (reviewed in Ref. 7). Changes in either the enantiomeric configuration or the carbon skeleton length lead to loss of both substrate activity and binding affinity. In addition, substrate activity is lost if the acceptor gamma-carboxyl is eliminated [29] or another acidic group is substitued as in MTX analogs containing either homocysteic acid [10, 11] or 4-fluoroglutamate [9]. Binding affinity is not eliminated completely by the latter two substitutions, but is decreased greatly. This apparent requirement of FPGS for an acid function with pK_a close to that of a γ-carboxyl which is in a specific steric and geometric configuration contrasts sharply with the high affinity for the enzyme of the basic ornithine-containing analog. A large increase in binding energy contributed by ornithine is indicated by the fact that the K_i of the ornithine-MTX analog for rat liver FPGS (4 μM; Table 1) is considerably less than the K_m for substrate activity of MTX itself (32 μM; Fig. 2).

Regardless of its detailed mechanism of FPGS inhibition, the interesting properties of the ornithine-containing MTX analog suggest it should be investigated in *in vivo* tumor models. This was not reported in earlier work with this compound [13]. The lack of glutamylation of this compound may be advantageous if MTX polyglutamates contribute more to host toxicity than to tumor cytotoxicity. Also, lack of glutamylation could lead to efflux of the analog from cells as plasma drug levels decline, unlike MTX whose polyglutamates are retained under these conditions [9]. This may, in turn, allow its use at higher doses and/or longer times without causing host toxicity; under these conditions, the inhibition of FPGS might contribute to cytotoxicity by preventing synthesis of the folylpolyglutamates which are essential for cell survival [8]. A potential complicating factor in *in vivo* experiments is the high aldehyde oxidase substrate activity of the ornithine-containing analog (Fig. 3). This could lead to rapid inactivation of the drug and loss of efficacy [19]. This property might be exploited, however, in arterial infusions into liver which has high levels of aldehyde oxidase activity. Thus, infusion might allow high levels of drug to be obtained locally around hepatic tumors but most drug would be inactivated before returning to systemic circulation, and thus toxicity to other organs would be reduced.

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