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HUMAN THYMIDYLATE SYNTHETASE—III

EFFECTS OF METHOTREXATE AND FOLATE ANALOGS

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Abstract—The structure–activity relationship of human thymidylate synthetase (EC 2.1.1.45) was studied with two groups of folate analogs: (1) methotrexate (MTX) analogs modified at the glutamate residue and N^{10}; and (2) tetrahydrofolate (H_4PteGlu) analogs modified at N^5 and N^{10}. With respect to MTX analogs, it was found that: (1) substitution of the glutamate side chain by α-aminoadipic acid, α-aminopimelic acid or β-aminoglutaric acid slightly affects its K_i; (2) a free α-carboxyl group on the amino acid side chain of MTX, or any free carboxyl group in that vicinity plays an important role in the inhibitory potency of MTX analogs to the enzyme; (3) esterification or amidation of the α-carboxyl group of MTX decreases the inhibitory potency; and (4) free aspartyl or glutamyl conjugation through a peptide linkage to the γ-carboxyl group of the glutamate side chain decreases its K_i to the enzyme by 5- and 8-fold respectively. Tetrahydrofolate analogs formed by inserting an ethylene, iminyl or a carbonyl bridge between the nitrogen at N^5 and N^{10} or by substitution at the N^5 position were found to be poor inhibitors under our assay conditions.

Thymidylate synthetase (EC 2.1.1.45), an enzyme which catalyzes the *de novo* biosynthesis of dTMP, has been purified in this laboratory [1] from blast cells of patients with acute myelocytic leukemia. The significance of inhibiting this enzyme in cancer chemotherapy, as compared to dihydrofolate reductase, has been reported by Borsa and Whitmore [2]. Partially purified thymidylate synthetases from calf thymus [3], Ehrlich ascites carcinoma [4] and pig thymus [5] have been reported and studied with various folate derivatives as substrates and inhibitors. However, it has been shown previously in this laboratory [1, 6] that purified human thymidylate synthetase may differ qualitatively from thymidylate synthetase derived from other sources, with regard to certain kinetic and physical parameters. Subsequently, the effects of modification of the pteridine portion of folate, aminopterin and MTX* with respect to modes and potency of inhibition were studied [6]. Modifications of the glutamate side chain of folate were attempted in order to investigate the effects of γ-glutamyl conjugation on the activity of H_2PteGlu and PteGlu as inhibitors, and of 5,10-CH_2-H_4PteGlu as a substrate for human thymidylate synthetase. Our results were in agreement with those reported by Kisliuk *et al.* [7] and Friedkin *et al.* [8], that lengthening of the γ-glutamyl chain in folate derivatives potentiates the binding affinity of these derivatives to thymidylate synthetase, thus enhancing both cofactor activity and inhibitory activity. On the basis of data obtained from multiple inhibitor studies, Dolnick and Cheng [6] suggested the existence of two types of folate binding sites, A and B, on human thymidylate synthetase. Site A was designated for the binding of a competitive inhibitor, such as 10-CH_3-PteGlu, and site B was designated for the binding of a non-competitive inhibitor, such as methotrexate. The addition of 4 γ-glutamyl residues to the substrate, 5,10-CH_2-H_4PteGlu, decreases the K_m value at site A from 31 ± 8.3 μM to 2.2. ± 0.1 μM. PteGlu, a competitive inhibitor with respect to 5,10-CH_2-H_4PteGlu, showed a surprising qualitative change in the mode of inhibition with γ-glutamyl conjugation. Inhibitory potency was enhanced enormously even though there was a change in the mode of inhibition from competitive to non-competitive. In light of these data, it seems reasonable to study the potency of MTX analogs with glutamyl or aspartyl residues conjugated at the γ-carboxyl group.

As an extension of our study on the inhibitory specificity of MTX analogs to human thymidylate synthetase, several regions of the MTX molecule were modified and studied. Monoester, monoamide, diester and bisamide derivatives of MTX were tested in order

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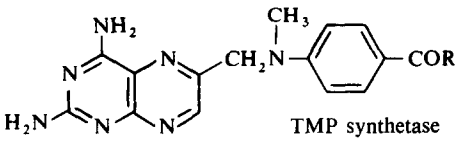
* The abbreviations used are: MTX, methotrexate; 5,10-CH_2-H_4-PteGlu, (±)-N^5,N^{10}-methylenetetrahydrofolic acid; and PteGlu, folate. The number of glutamate residues on the various folate derivatives is designated by a numerical subscript unless only one glutamate is present. The α-aminoadipate, α-aminopimelate and β-aminoglutarate compounds all have the D,L-configuration.

Table 1.



TMP synthetase

R	% Inhibition*	K_i^+ (μM)
—OH	13 ± 4	(306)
H, —N—CH, $(CH_2)_2$—CO_2H	65	30
H, —N—CH, CH_2—CO_2H, CH_2—CO_2H	53	40
—— CO_2H, H, —N—CH, $(CH_2)_3$—CO_2H	41	62
H, —N—CH, $(CH_2)_4$—CO_2H	54	40

* The concentrations of [5-^{3}H]dUMP (0.43 mCi/μmole), inhibitors and 5,10-CH_2-H_4PteGlu were 25, 50 and 90 μM respectively. Incubations were for 30 min at 37° with 0.0013 to 0.0046 units of purified human TMP synthetase. One unit of enzyme is defined as the amount of enzyme needed to convert 1 nmole dUMP/min in the condition used.

+ The concentration of 5,10-CH_2-H_4PteGlu was varied from 225 to 90 μM. The concentration of [5-^{3}H]dUMP (0.43 mCi/μmole) was 25 μM. The concentrations of inhibitors were varied accordingly to the potencies of the inhibitors. Incubations were for 45 min at 37° with 0.0046 to 0.0058 units of purified human TMP synthetase. K_i values in parentheses were estimated from the per cent inhibition by using the following equation:

$$V/v = 1 + I/K_i$$

where V = velocity without inhibitor; v = velocity with inhibitor; I = inhibitor concentration; and K_i = inhibitor constant.

The derivation of the equation is based on the following reasoning. All MTX analogs are non-competitive inhibitors, and the values of K_i slopes and K_i intercepts determined for various analogs were equal or approximately equal. Therefore, we assume all the analogs kinetically behave the same; then the equation for the non-competitive inhibitor could be deduced to the above equation.

to determine which carboxyl groups contribute more to the inhibitory potency of the inhibitor for the enzyme. MTX α-L-glutamic acid, MTX α-L-aspartic acid and 4-amino-4-deoxy-N^{10}-methylpteroyl-D,L-β-aminoglutaric acid were used to probe the inhibitory binding site of the enzyme to the α-carboxyl group. Similarly, two higher homologs of MTX were used to determine the inhibitory effects of the γ positional isomers. Finally, H_4PteGlu analogs were studied to evaluate the effects of modifications at the N^5 and N^{10} positions with respect to inhibition.

MATERIAL AND METHODS

Chemicals. dUMP was obtained from the Sigma Chemical Co. (St. Louis, MO). [5-^{3}H]dUMP (14 Ci/m-mole) was purchased from the Amersham Corp. (Arlington Heights, IL). Methotrexate, obtained from Nutritional Biochemicals (Cleveland, OH) and found to be > 96 per cent pure, was used without further purification. H_4PteGlu was prepared as described by Dolnick and Cheng [6]. Tris(hydroxymethyl)aminomethane and DEAE-cellulose were obtained from the Eastman Kodak Co. Rochester, N.Y. All other chemicals are of reagent grade. The MTX analogs were synthesized by Rosowsky *et al.* [9–12], and Montgomery.*

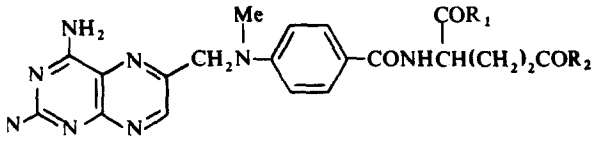
Enzyme preparation and assay. Thymidylate synthetase was purified from blast cells of patients with acute myelocytic leukemia, as described by Dolnick and Cheng [1]. The enzyme was assayed by the tritium release procedure of Roberts [13] as described [1] but with the following modification. The assay reaction had a final volume of 50 μl and the concentrations of 5,10-CH_2-H_4PteGlu and dUMP were 90 and 50 μM respectively. One unit of enzyme is defined as the amount of enzyme needed to convert 1 nmole dUMP/min at 37° under the assay condition used. All assays were conducted in duplicate and repeated at least once with similar results. No esterase activity was apparent in the enzyme preparation. For K_i determination, the lines drawn through the data points were fitted by eye. The reaction velocity was linear with time and enzyme concentration under the conditions employed. The concentration of each inhibitor was 50 μM in all assays of per cent inhibition studies.

RESULTS

Positional isomers and MTX homologs. The parent compound, 4-amino-4-deoxy-N^{10}-methylpteroic acid, containing no glutamyl group, showed low inhibitory activity against thymidylate synthetase (Table 1). This indicated that the amino acid side chain of MTX is essential for the inhibition. The positional isomer, 4-amino-4-deoxy-N^{10}-methylpteroyl-D,L-β-aminoglutaric acid, was slightly less active than MTX (Table 1). The minor decrease in activity resulting from the increase in distance between the α-carboxyl group and the amide nitrogen indicates that the spatial orientation of the enzyme for this group is not stringent. A slight drop in activity was observed with the α-aminopimelate homolog and a further decrease in activity was observed with the α-aminoadpiate homolog. The inconsistent variation in activity with the increase of distance between the

* J. A. Montgomery, unpublished observations.

Table 2. Effects of MTX monoester, diester, monoamide and bisamide derivatives on human dTMP synthetase



		TMP synthetase	
R_1	R_2	% Inhibition*	K_i† (μM)
—OH	—OH	65	30
—NH_2	—OH	27	(130)
—OC_4H_9	—OH	18	(220)
—OH	—NH_2	63	(30)
—OH	—$NHCH_3$	60	(33)
—OH	—$N(CH_3)_2$	62	(30)
—OH	—$NH(C_5H_{11}$-n)	55	(40)
—OH	—OC_4H_9-n)	54	(40)
—OC_2H_5	—OC_2H_5	20	(200)
—OC_4H_9-n	—OC_4H_9-n	2	
—OC_6H_{13}-n	—OC_6H_{13}-n	0	
—OC_8H_{17}-n	—OC_8H_{17}-n	1	
—$OCH_2C_6H_5$	—$OCH_2C_6H_5$	0	
—NHC_3H_7-n	—NHC_3H_7-n	8	(575)
—$NHCH_2C_6H_5$	—$NHCH_2C_6H_5$	11	(405)
—NH—	—NH—	11	(405)
—N	—N	14	(307)

* See first footnote of Table 1.
† See second footnote of Table 1.

γ-carboxylate and the amide nitrogen is not as yet understood. All the inhibitors here and below were non-competitive inhibitors from K_i intercept equal to K_i slope.

Methotrexate monoester, diester, monoamide and bisamide derivatives. The importance of a free α-carboxyl group on the glutamate moiety was demonstrated by the drastic loss of inhibitory activity on esterification or amidation of the α-carboxyl group. A 4- to 7-fold increase in K_i was observed with MTX α-monoamide and MTX α-monobutyl ester (Table 2). However, derivatization of the γ-carboxylate of the glutamate side chain results in little change in K_i value. MTX γ-monoamide, MTX γ-monomethylamide and MTX γ-monopentylamide, which is the amide with the longest alkyl substituent, showed a 1.3-fold increase of K_i value. A similar decrease in activity was observed with MTX γ-monobutyl ester. With the exception of MTX diethyl ester, none of the MTX diesters showed potent activity against human thymidylate synthetase. The bis(propylamide), bis(benzylamide), bis(cyclohexylamide) and bis(pyrrolidineamide) were approximately one order of magnitude less potent than MTX with the assay system used. This indicates that the free α-carboxyl group of the glutamate side chain is a strong binding point of the MTX molecule to the enzyme. A fairly large bulk tolerance of the enzyme in the γ-carboxyl region is indicated by the small change in activity when the γ-amides are varied from primary to tertiary and by only a 1.3-fold increase in K_i when a 5-carbon secondary amide is substituted for the primary amide.

Peptide derivatives and N^{10}-analog of MTX. As shown in Table 3, attachment of a free aspartate or glutamate residue to the γ-carboxyl group of MTX via a peptide bond decreased the K_i value of MTX to the enzyme by 5-fold ($K_i = 6$ μM) and 8-fold ($K_i = 4$ μM) respectively. In contrast, the α-glutamyl derivative of MTX gave the same level of inhibition as MTX. However, the α-aspartyl showed a 2-fold increase in K_i value ($K_i = 68$ μM). Conjugation of diethyl glutamate at the α-carboxyl of MTX results in a drastic loss of activity ($K_i = 450$ μM). On the other hand, conjugation of diethyl glutamate at the γ-carboxyl group results only in a small loss of inhibitory potency ($K_i = 67$ μM). Conjugation of diethyl glutamate at both the α- and γ-carboxyl of MTX results in a drastic loss of activity ($K_i = 940$ μM). A minimal bulk tolerance of thymidylate synthetase for substituents at the N^{10} position of MTX is demonstrated by the lack of inhibitory activity of N^{10}-benzylaminopterin.

Derivatives of tetrahydrofolic acid. Since N^5,N^{10}-methylenetetrahydrofolic acid is one of the substrates of thymidylate synthetase, a number of analogs of this compound were evaluated (Table 4). None of these compounds showed potent inhibitory activity under our assay conditions.

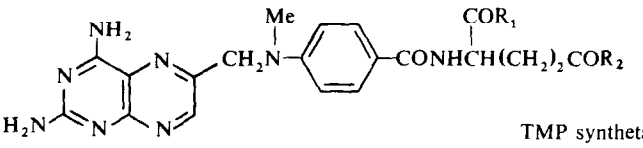
DISCUSSION

The structure–activity relationships of MTX and tetrahydrofolate analogs as inhibitors of human thymidylate synthetase have been investigated. The non-competitive mode of inhibition of MTX was unaltered by modification of the glutamate side chain, in contrast to our earlier finding with PteGlu [6]. All MTX analogs studied were found to be equal to, or less potent than, MTX except the γ-glutamic acid and γ-aspartic acid conjugates of MTX. We observed previously that addition of γ-glutamyl residues to folate decreased the K_i values of PteGlu and H_2PteGlu analogs by approximately one order of magnitude. A similar effect was observed with MTX regardless of whether the additional conjugated residue was glutamate or aspartate. The enhancement of inhibitory potency of MTX to the enzyme by the addition of an γ-glutamyl residue to MTX is important with regard to the mode of action of MTX since the formation of MTX polyglutamate was demonstrated in cells treated with MTX.

In addition, our results also indicate that for the enhancement of inhibitory potency of MTX to the enzyme by conjugation at a γ-carboxyl group, it did not have to be glutamate; it could be aspartate. This raised the possibility that other amino acid conjugation may also enhance the inhibitory potency of MTX.

Recently, the γ-glycinate derivative of MTX was prepared by J. R. Piper and J. A. Montgomery, and it will be evaluated as an inhibitor of thymidylate synthetase. The other advantages of having other amino acids instead of glutamate conjugated to a γ-carboxyl group of MTX are: (1) they may not subject to the action of conjugase which could degrade polyglutamate of folate; and (2) they may be carried through cell membrane by a different carrier mechanism which could overcome the clinic MTX resistance due to the lack of transport of MTX into cells [14–17]. Recently, the γ-glutamyl and

Table 3. Effects of peptide derivatives of MTX on human dTMP synthetase



TMP synthetase

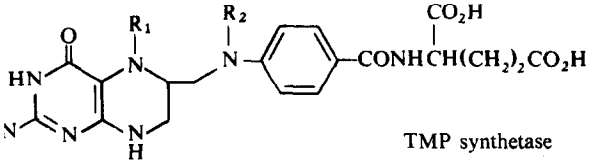
R_1	R_2	% Inhibition *	K_i (μM)
—OH	—OH	65	30
H CO_2H —N—CH $(CH_2)_2$—CO_2H	—OH	66	30
H CO_2H —N—CH CH_2—CO_2H	—OH	32	68
H $CO_2C_2H_5$ —N—CH $(CH_2)_2$—$CO_2C_2H_5$	—OH	11	(450)
—OH	H CO_2H —N—CH $(CH_2)_2$—CO_2H	95	4
—OH	H CO_2H —N—CH CH_2—CO_2H	89	6
—OH	H $CO_2C_2H_5$ —N—CH $(CH_2)_2$—$CO_2C_2H_5$	43	(67)
H $CO_2C_2H_5$ —N—CH $(CH_2)_2CO_2C_2H_5$	H $CO_2C_2H_5$ —N—C $(CH_2)_2$—$CO_2C_2H_5$	5	(940)

* See first footnote of Table 1.
+ See second footnote of Table 1.

γ-aspartate derivative of folic acid was found to be resistant to the action of carboxylpeptidase from human liver [18] and the γ-aspartate but not γ-glutamate derivative of methotrexate was found to be resistant to cleavage by L1210 cells [19].

Previously, we have observed a difference in the value of K^i slope and K_i intercept with $Pte(Glu)_3$ (K_i slope = 3 μM, K_i intercept = 14 μM) and $Pte(Glu)_5$ (K_i slope = 2 μM, K_i intercept = 13 μM) [6]. The K_i slope in each case was about 5- to 6-fold lower than the K_i intercept, indicating greater affinity of $Pte(Glu)_3$ and $Pte(Glu)_5$ for the enzyme–dUMP complex than its

Table 4. Effects of H_4PteGlu derivatives on human dTMP synthetase*



R_1	R_2	% Inhibition†	K_i‡ (μM)
Me	Me	16	(260)
—CH_2—CH_2—		12	(370)
—C(=O)—		0	
—C(=NH)—		0	
$PhCH_2$—	H	10	(450)
$ClCH_2CH_2NHCO$	H	0	
H_2NCO	H	0	
EtNHCS	H	0	
PhNHCS	H	0	
HO_2CCH_2	H	0	

* The K_m value for 5,10-CH_2-H_4PteGlu is 31 μM.
† See first footnote of Table 1.
‡ See second footnote of Table 1.

ternary complex [6]. However, no difference between K_i intercept and K_i slope was observed with MTX γ-glutamate and MTX γ-aspartate. This indicates that the affinity of MTX γ-glutamate and MTX γ-aspartate for the enzyme–dUMP complex and the ternary complex is the same. Slavik and Zakrzewski [20] reported that amidation of both glutamate carboxyls markedly decreases the substrate activity of 5,10-CH_2-H_4PteGlu and that elimination of the glutamate side chain from tetrahydroaminopterin also decreases the inhibitory activity. Elimination of the glutamate side chain from MTX increased the K_i value by about one order of magnitude. Only esterification and amidation of the α-carboxylate of MTX had a markedly negative effect on the inhibitory activity. This suggests that the α-carboxyl group is one of the main points of attachment to the enzyme.

The distance of the α-carboxyl group from the amide nitrogen was proved not to be stringent. Replacement of the α-carboxyl group of the glutamate side chain by the carboxyl of β-aminoglutaric acid decreases the binding affinity by approximately 1.3-fold. With this modification, the new carboxyl group is one carbon further from the amide nitrogen than the original α-carboxyl group of the glutamate side chain. Furthermore, MTX α-monobutyl ester and MTX α-L-glutamate diethyl ester showed low activity (K_i = 220 and 450 μM, respectively), whereas the inhibitory potency of MTX α-L-glutamate was comparable to MTX. α-Glutamate conjugation of MTX introduces a free carboxyl group which is approximately three bonds lengths farther away from the amide nitrogen than the α-carboxyl group in a normal glutamate side chain. All this evidence suggests that a free carboxyl group on the α position is not absolutely required for the inhibition. The absolute requirement for inhibition is the presence of a free carboxyl group in the approximate vicinity of the α-carboxyl of the glutamate side chain. It will be of interest to test the specificity of human thymidylate synthetase by replacing this carboxyl group with another acidic moiety. To evaluate this approach, the same acidic moiety substitution can also be applied to the γ-carboxyl group of MTX. In addition, the γ-carboxyl group appears not to be essential for the inhibition since blockage of this group by amidation or esterification results in little change in inhibitory activity. Furthermore, increasing the distance between the two carboxyl groups by replacement of the glutamate side chain with α-aminoadipate and α-aminopimelate increased K_i within a 2-fold range. Some degree of bulk tolerance in the region of the γ-carboxyl group was demonstrated by the minimal drop in activity as the amide was varied from primary to tertiary, and when the γ-carboxyl group was conjugated to diethyl L-glutamate.

Minimal bulk tolerance at N^5 and N^{10} was demonstrated by the low inhibitory activity of the N^5-substituted H_4PteGlu derivatives as well as the lack of activity of 10-benzylaminopterin. Isosteric replacement of the methylene bridge of 5,10-CH_2-H_4PteGlu by a polar bridge, such as an imidyl or a carbonyl, was detrimental to its action. This indicates that the N^5 and 10^{10} positions are sensitive to both electronic and steric effects. For optimum inhibitory activity, N^{10}-methyl substitution was clearly the best choice of modification at N^5 and N^{10} of PteGlu analogs, as suggested previously [6].

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