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Inhibitory Action of 10-Deazaaminopterins and Their Polyglutamates on Human Thymidylate Synthase

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SUMMARY

The action of 10-deazaaminopterin, its 10-alkyl derivatives, and their polyglutamates against thymidylate synthase (TMPS) from human acute myeloblastic leukemia was examined. Comparison of aminopterin with methotrexate showed that the methylation of the N^{10}-position (methotrexate) increased the inhibitory effect of aminopterin on TMPS. In contrast, alkylation of the 10-position of 10-deazaaminopterin decreased inhibition of TMPS, and the 50% inhibitory concentration values were progressively higher, in the order 10,10-dimethyl-, 10-methyl-, and 10-ethyl-derivatives. The addition of γ-glutamyl moieties to both 10-deazaaminopterin and one of its alkylated analogs, 10-ethyl-10-deazaaminopterin, enhanced inhibition. The maximum inhibition was achieved with the addition of three glutamyl moieties to 10-deazaaminopterin and two glutamyl moieties to 10-ethyl-10-deazaaminopterin, respectively. Thus, 10-deazaaminopterin-tetraglutamate was 138-fold and 10-ethyl-10-deazaaminopterin-triglutamate was > 51-fold more active than their respective parental compound. The compounds 10-deazaaminopterin and its polyglutamates, 10-methyl- and 10,10-dimethyl-analogs, inhibited TMPS in a noncompetitive fashion with respect to 5,10-methylene-tetrahydropteroylglutamate. K_i values for the monoglutamates were 220 μM, 310 μM, and 225 μM, respectively. In contrast, 10-ethyl-10-deazaaminopterin and its polyglutamates inhibited TMPS in a competitive fashion with a K_i value of 410 μM for the monoglutamate. With 5,10-methylene-tetrahydropteroylpentaglutamate as a substrate, 10-deazaaminopterin and its polyglutamates behaved as mixed type inhibitors, and 10-ethyl-10-deazaaminopterin, monoglutamate and diglutamate, behaved as noncompetitive inhibitors, whereas its pentaglutamate behaved as a mixed-type inhibitor. These results suggest that the addition of γ-glutamyl moieties to the substrate also caused the change in the mode of inhibitory action of these compounds. These findings also show that both replacement of the N^{10}-position of the 4-aminopteroyl structure with a methylene group and its alkylation caused interesting and unexpected changes in the structure-activity relationships and the mode of action for these 4-aminopteroyl antifolates as inhibitors of TMPS, which may be therapeutically relevant.

The antifolate, MTX, is well known as one of the most effective agents in the treatment of some human malignancies, such as acute leukemia (1) and choriocarcinoma (2), involving the special administration method of high dose MTX with leucovorin rescue (3). A number of attempts have been made to develop new folate analogs with greater antitumor activities and broader spectrum than MTX. In 1974, 10-deazaaminopterin (4) was synthesized and, in 1982, its 10-alkyl analogs (10-methyl-10-deazaaminopterin, 10,10-dimethyl-10-deazaaminopterin, and 10-ethyl-10-deazaaminopterin) (5, 6) were also synthesized (Fig. 1). These analogs were found to be more effective than MTX in some experimental tumor systems (7, 8), and 10-deazaaminopterin and 10-ethyl-10-deazaaminopterin (6) are presently under clinical trial. Recently, it was shown that folate and its analogs could be polyglutamated by folylpolyglutamate synthetase (9–11). The folylpolyglutamates appear to be important as substrates for the folate-dependent enzymes (9), whereas antifolylpolyglutamates have pharmacologic importance either as intracellular retentive forms of the parent compounds (10) or as more potent inhibitors of folate-dependent enzymes (9). 10-Deazaaminopterin and 10-ethyl-10-deazaaminopterin were also demonstrated to be polyglutamated in human cells (11, 12). The superiority of 10-deazaaminopterin and, particularly, its 10-alkyl derivatives may be explained by more efficient transport in tumor cells. Also, the 10-alkyl derivatives showed increased substrate activity for polyglutamation in tumor tissue when compared with MTX (7, 12). An *in vivo* study (7), using tumor-bearing mice, showed that the accumulation of the polyglutamates of these compounds was

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ABBREVIATIONS: MTX, methotrexate; DHFR, dihydrofolate reductase; TMPS, thymidylate synthase; AML, acute myeloblastic leukemia; 5,10-CH_2-H_4-PteGlu, (±)-L-N^5,N^{10}-methylene-5,6,7,8-tetrahydropteroylglutamate.

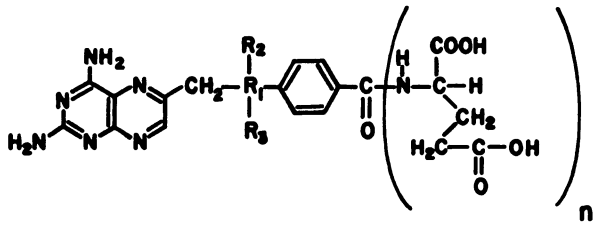

Fig. 1. Chemical structures of 10-deazaaminopterin and its N^{10}-alkyl derivatives as compared with aminopterin and MTX.

	R_1	R_2	R_3
10-Deazaaminopterin (N = 1–6)	C	H	H
10-Methyl-10-deazaaminopterin	C	CH_3	H
10,10-Dimethyl-10-deazaaminopterin	C	CH_3	CH_3
10-Ethyl-10-deazaaminopterin (N = 1–5)	C	C_2H_5	H
Aminopterin	N	H	
MTX	N	CH_3	

higher in some tumor tissues than in normal small intestine. This could suggest a reason for their greater tolerance and improved therapeutic index. DHFR (EC 1.5.1.3) is the primary inhibitory site of MTX. With respect to inhibition, the 10-deazaaminopterin analogs were slightly better than MTX and approximately equivalent to aminopterin against L1210 DHFR (7). Dolnick and Cheng (13) and Allegra *et al.* (14) suggested that TMPS (EC 2.1.1.45), the enzyme which catalyzes the reaction responsible for *de novo* TMP synthesis, is another possible target of MTX. However, the inhibitory action of 10-deazaaminopterin analogs on this enzyme has only been described in one preliminary study, using a microbial enzyme (5). This report contains the precise characterization of the 10-deazaaminopterins and their polyglutamates, with respect to their inhibitory action on TMPS derived from human AML cells. Our findings present not only a quantitative comparison of the inhibitory action on TMPS among these different structures, but also interesting information as to the different modes of TMPS inhibition by these agents.

Materials and Methods

Chemicals. [5-^{3}H]dUMP (22 Ci/mmol) was obtained from Moravek Biochemicals, Inc., Brea, CA. dUMP (Sigma Chemical Co., St. Louis, MO), MTX (Lederle Pharmaceuticals Inc., Pearl River, NY), and aminopterin (Lederle Pharmaceuticals Inc.) were used. (±)-L-5,6,7,8-Tetrahydropteroylglutamate and (±)-L-5,6,7,8-tetrahydropteroylpentaglutamate were prepared by chemical synthesis, leading to a mixture of diastereomers, as described (15, 16).

10-Deazaaminopterin, 10-methyl-10-deazaaminopterin, 10,10-dimethyl-10-deazaaminopterin, and 10-ethyl-10-deazaaminopterin were synthesized as described elsewhere (4, 5). The polyglutamates of both 10-deazaaminopterin and 10-ethyl-10-deazaaminopterin were synthesized by modifications of the procedures previously described for the synthesis of methotrexate polyglutamates (17). Purity of these compounds was established by high pressure liquid chromatography (5, 12) to be greater than 95%, and their concentrations were determined spectrophotometrically.

All other chemicals used were of reagent grade or higher.

Enzyme purification and assays. TMPS was purified approximately 1350-fold to a specific activity of 17.4 units/mg of protein by procedures described previously (13) from blast cells (obtained by leukophoresis) from an untreated patient with AML. These were provided by North Carolina Memorial Hospital, associated with this university. The enzyme assay was performed by the tritium release procedure of Roberts (18), as reported by Dolnick and Cheng (13), except that 28 μM [5-^{3}H]dUMP (1.07 mCi/μmol) was used. One unit of enzyme activity was defined as the conversion of 1 nmol of substrate (dUMP) per min. The reaction was started by the addition of the enzyme and the incubation time was 45 or 60 min. All assays were conducted in duplicate or triplicate and repeated at least once with similar results. K_i determinations were performed by assaying the enzyme at varying levels of 5,10-CH_2-H_4PteGlu or 5,10-CH_2-H_4PteGlu$_5$[1] in the presence of several fixed concentrations of each inhibitor. K_i values for competitive inhibitors were determined from slope replots of Lineweaver-Burk analysis and those for noncompetitive and mixed type inhibitors were determined from both slope and intercept replots.

Results and Discussion

Inhibition of TMPS from human AML blast cells. Aminopterin and 10-deazaaminopterin had similar IC_{50} values (Fig. 2, a and b). However, whereas methylation (MTX) of the aminopterin enhanced the inhibitory action (Fig. 2a), alkylation of the C^{10}-position of 10-deazaaminopterin decreased the inhibitory action. Measured IC_{50} values were in the order 10-deazaaminopterin < 10,10-dimethyl-10-deazaaminopterin < 10-methyl-10-deazaaminopterin < 10-ethyl-10-deazaaminopterin (Fig. 2b). Since other folate compounds, including pteroylglutamic acid, 7,8-dihydrofolate, and 5,8-dideazafolate, as well as aminopterin, were previously found to show increased inhibitory activity as a result of substitution of the hydrogen with a methyl group in the N^{10}-position (16, 19), the present results were somewhat unexpected. These results were different from those obtained with L1210 cell DHFR, where studies showed no substantial difference among 10-deazaaminopterin and its analogs in their inhibition of this enzyme (5). These were also different from that of a preliminary study on inhibition of a microbial TMPS. In that study the IC_{50} for these compounds were almost equivalent to each other (5). An exact comparison between these two enzymes, however, is difficult because experimental conditions were quite different. It should also be pointed out, that the higher IC_{50} values for 10-methyl- and 10-ethyl-10-deazaaminopterin compared to 10,10-dimethyl-10-deazaaminopterin may relate to the fact that they are racemic mixtures. In view of the asymmetric carbon at

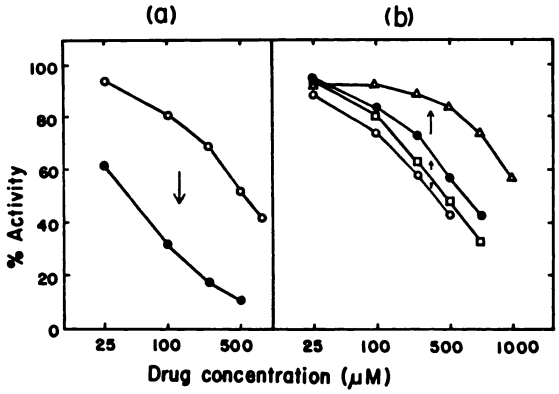

Fig. 2. Inhibitory activities of 4-amino-pteroylglutamic acid derivatives (a) and 10-deazaaminopterin derivatives (b) on TMPS. The concentration of TMPS used in each assay was 0.18 unit/ml. The mean values of two experiments with duplicate determination are shown. a. Aminopterin (○), MTX (●). b. 10-Deazaaminopterin (○), 10-methyl-10-deazaaminopterin (●), 10,10-dimethyl-10-deazaaminopterin (□), and 10-ethyl-10-deazaaminopterin (△).

[1] The numbers of glutamyl moieties on the various folate derivatives are designated by a numerical subscript unless only one glutamyl moiety is present.

position 10 in these structures, their synthesis results in a mixture of both L- and D-diastereoisomers. In fact, it appears that TMPS does exhibit stereospecificity at carbon 10 of these structures, at least in the case of 10-ethyl-10-deazaaminopterin. Our experiment, which compared both L and D forms, gave IC_{50} values of 5.0 mM and 1.4 mM, respectively. The slope of the dose-response curve for the L form was similar to that for the D form (data not shown). This suggests a similar inhibitory mode of the DL form of 10-ethyl-10-deazaaminopterin and 10-methyl-10-deazaaminopterin on TMPS. The K_i values of these compounds having diastereomers, as shown in the following section, should only be considered as the apparent value. The relationship of these values should be:

$$\frac{1}{K_i^{app}} = \frac{1}{2}\left(\frac{1}{K_i^{L}} + \frac{1}{K_i^{D}}\right)$$

where K_i^{app} is the apparent K_i value of the DL form, K_i^{L} is the K_i value of the L form, and K_i^{D} is the K_i value of the D form, provided the amount of L form is about the same as that of D form (20). Similar results which showed the different values between the D and L forms were also obtained (21) with L1210 DHFR but not for membrane transport which did not exhibit stereospecificity at this position. In both 10-deazaaminopterin (Fig. 3a) and 10-ethyl-10-deazaaminopterin (Fig. 3b), the addition of γ-glutamyl moieties enhanced the inhibitory activity. The addition of three glutamyl moieties to 10-deazaaminopterin and two glutamyl moieties to 10-ethyl-10-deazaaminopterin gave the maximal effect. Thus, 10-deazaaminopterin-tetraglutamate (addition of three glutamates) was 138-fold, and 10-ethyl-10-deazaaminopterin-triglutamate (addition of two glutamates) was greater than 51-fold more active than their respective parental compounds. Further addition of γ-glutamyl moieties did not show a significant increase in the inhibitory action of these compounds.

Kinetic studies. With respect to 5,10-CH_2-H_4PteGlu, as the substrate of TMPS, 10-deazaaminopterin (Fig. 4a), 10-methyl-10-deazaaminopterin, and 10,10-dimethyl-10-deazaaminopterin behaved as noncompetitive inhibitors, whereas 10-ethyl-10-deazaaminopterin behaved as a competitive inhibitor (Fig. 4b). When the inhibitory actions of the polyglutamated derivatives of 10-deazaaminopterin and 10-ethyl-10-deazaaminopterin were examined, they showed the same behavior as the respective monoglutamates. In earlier studies Dolnick and Cheng (16) suggested the existence of two types of folate-binding sites, A (designated for the binding of a competitive inhibitor) and B (designated for the binding of a noncompetitive inhibitor). The difference in mode of action between 10-deazaaminopterin and 10-ethyl-10-deazaaminopterin might be due to the binding of each drug to a different site on the enzyme. However, another explanation may also be possible (16, 22). Further studies will be required to clarify this issue, since the suggestion is based on the kinetic studies.

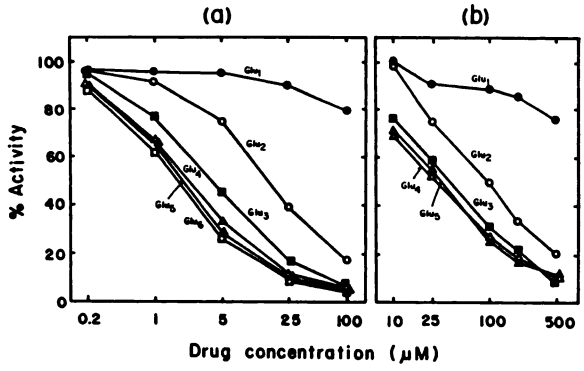

Fig. 3. The inhibitory effect on TMPS of 10-deazaaminopterin and its polyglutamates (a) and 10-ethyl-10-deazaaminopterin and its polyglutamates (b). Conditions are the same as in Fig. 2.

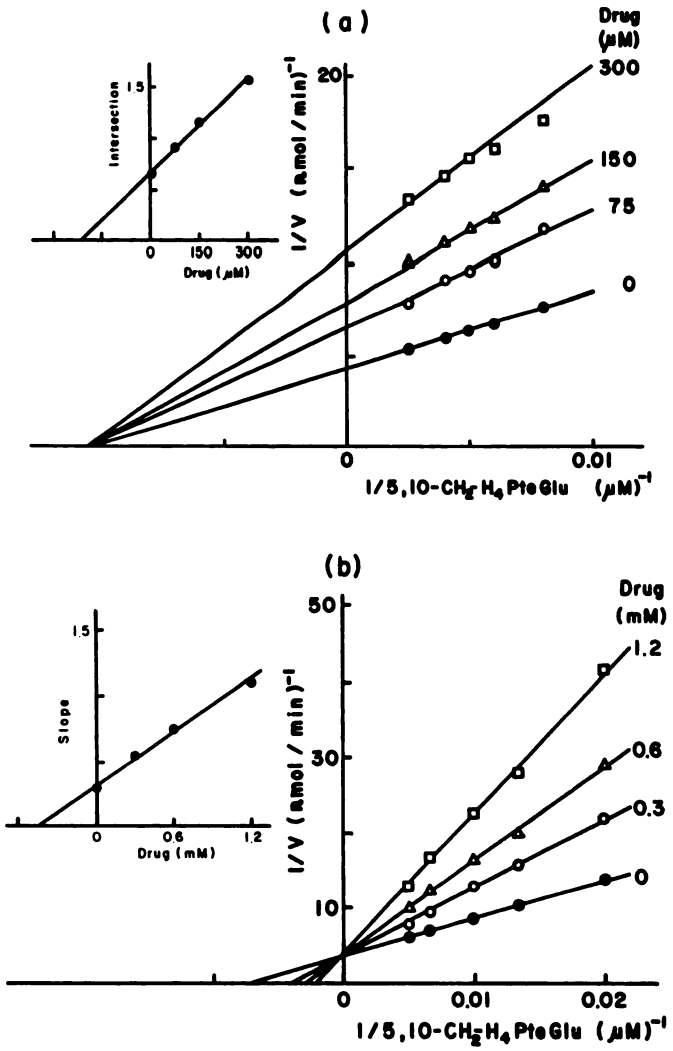

Fig. 4. Kinetic properties of 10-deazaaminopterin (a) and 10-ethyl-10-deazaaminopterin (b) with respect to 5,10-CH_2-H_4PteGlu. The concentration of the enzyme was 0.18 unit/ml. *v* is expressed as nmol of TMP formed/min/ml of the enzyme.

The inhibitory behaviors of 10-deazaaminopterin and its derivatives including polyglutamates are summarized in Table 1. 10-Ethyl-10-deazaaminopterin and its polyglutamates had significantly higher K_i values with respect to 5,10-CH_2-H_4PteGlu than did 10-deazaaminopterin and its polyglutamates. The K_i values of 10-deazaaminopterin and its polyglutamates, which behaved as noncompetitive inhibitors, were substantially equal to the IC_{50} values, whereas the K_i values of 10-ethyl-10-deazaaminopterin and its polyglutamates, which behaved as competitive inhibitors, were not, which is in agreement with the relationship between K_i and IC_{50}, described previously (23).

The addition of four γ-glutamyl residues to the substrate, 5,10-CH_2-H_4PteGlu, decreased the K_m value from 117 ± 19 μM to 13.3 ± 3.7 μM. With respect to 5,10-CH_2-H_4PteGlu$_5$ as the substrate of TMPS, 10-deazaaminopterin and its polygluta-

TABLE 1

Kinetic properties of 10-deazaaminopterin derivatives and their polyglutamates in TMPS assay

Experimental conditions were the same as those for Fig. 4. K_m values for 5,10-CH_2-H_4PteGlu and for 5,10-CH_2-H_4PteGlu$_5$ were 117 ± 19 μM and 13.3 ± 3.7 μM, respectively.

Inhibitors	5,10-CH_2-H_4PteGlu			5,10-CH_2-H_4PteGlu$_5$		
	IC_{50}[a]	K_{is}[b]	Inhibition	K_{is}	K_{ii}[c]	Inhibition
	μM	μM		μM	μM	
deazaAMT[d]-Glu	310 ± 85	220 ± 8.7[e]	NC[f]	1200 ± 420	130 ± 28	Mixed[g]
deazaAMT-Glu$_2$	16.5 ± 0.5	11.7 ± 1.7[e]	NC	24.1 ± 4.4	3.8 ± 0.6	Mixed
deazaAMT-Glu$_3$	3.83 ± 0.04	3.5 ± 0.8[e]	NC			
deazaAMT-Glu$_4$	2.25 ± 0.64					
deazaAMT-Glu$_5$	1.93 ± 0.03	1.97 ± 0.09[e]	NC	4.4 ± 1.7	0.80 ± 0.29	Mixed
deazaAMT-Glu$_6$	1.73 ± 0.11					
methyl-deazaAMT[h]-Glu	705 ± 78	310 ± 14.1[e]	NC			
dimethyl-deazaAMT[i]-Glu	455 ± 64	225 ± 7.1[e]	NC			
ethyl-deazaAMT[j]-Glu	>2000	410 ± 99	C	1300 ± 0.1	1300 ± 0.1	NC
ethyl-deazaAMT-Glu$_2$	110 ± 42	26 ± 5.7	C	76 ± 34	76 ± 34	NC
ethyl-deazaAMT-Glu$_3$	39 ± 2.8	12.5 ± 0.7	C			
ethyl-deazaAMT-Glu$_4$	27 ± 11					
ethyl-deazaAMT-Glu$_5$	31 ± 3.5	6.5 ± 2.1	C	68 ± 23	17 ± 2.6	Mixed

[a] Values are means ± standard deviations. Standard deviation was determined by $\sqrt{\frac{\sum x^2 - n\bar{x}^2}{n-1}}$ using values from a minimum of two experiments with samples done in duplicate.
[b] K_{is}, K_i slope.
[c] K_{ii}, K_i intercept.
[d] deazaAMT, 10-deazaaminopterin.
[e] K_{is} was equal to K_{ii} for these compounds.
[f] NC, noncompetitive inhibition.
[g] Mixed-type inhibition.
[h] methyl-deazaaminopterin.
[i] dimethyl-deazaAMT, 10,10-dimethyl-10-deazaaminopterin.
[j] ethyl-deazaAMT, 10-ethyl-10-deazaaminopterin.

mates behaved as mixed-type inhibitors. Regarding 10-ethyl-10-deazaaminopterin, both the monoglutamate and diglutamate behaved as noncompetitive inhibitors, but its pentaglutamate behaved as a mixed-type inhibitor (Table 1). The K_i slope values of both compounds were higher than those with respect to 5,10-CH_2-H_4PteGlu as the substrate, and the K_i intercept values of 10-deazaaminopterin and its polyglutamates were lower. The change in the mode of inhibitory action of 10-ethyl-10-deazaaminopterin and its polyglutamates suggested an opening of the other binding site of the enzyme, which could be an advantage for its inhibitory action, although the K_i values were higher than the K_m value in this combination. These results suggest that the addition of four γ-glutamyl residues to the substrate not only affected the affinity of the inhibitors to the enzyme, but also altered the enzyme conformation, causing changes in the mode of action. Previously, N^{10}-methylfolate (16), MTX (14, 16) and MTX-polyglutamates (14) were reported to behave in the same fashion with both 5,10-CH_2-H_4PteGlu and 5,10-CH_2-H_4PteGlu$_5$ as substrates, although the inhibition pattern of MTX was different in the former case (noncompetitive), using TMPS from AML cells (16), compared to the latter (uncompetitive), using TMPS from a human breast cancer cell line (14). However, Kisliuk *et al.* (24) reported the change of the inhibitory pattern of pteroylglutamic acid and its triglutamate from a noncompetitive fashion with L-5,6,7,8-tetrahydrofolate as a substrate to a competitive fashion with L-5,6,7,8-tetrahydrofolatetriglutamate as a substrate using TMPS from *Lactobacillus casei*, although only the natural diastereomers were used in their study. Another fact to be considered is that, since both 5,10-CH_2-H_4PteGlu and 5,10-CH_2-H_4PteGlu$_5$ were prepared by chemical synthesis as described in Materials and Methods, they contain the unnatural diastereomers of these compounds which are inhibitors of *L. casei* thymidylate synthase (25, 26). Therefore, it would be interesting to examine whether the kinetic data presented might be altered if pure L form diastereomers of these compounds are used. Thus, it has now been shown that both replacement of the N^{10}-position of the 4-aminopteroyl structure with a methylene group and its alkylation appear to cause interesting changes in the structure-activity relationship and the mode of action of 4-aminopteroyl antifolates as inhibitors of TMPS. The results so derived may be useful for the design of new antifolate analogs, with improved therapeutic properties.

Purified TMPS from the blast cells of an AML patient had a K_m value for 5,10-CH_2-H_4PteGlu of 117 ± 19 μM. This value was different from that of 31 ± 8.3 μM which we reported previously (13). We had already shown the different kinetic pattern of human TMPS between leukemic cells and cultured solid tumor cells (KB and HeLa cells) regarding the K_m value and inhibitory action of N^{10}-propargyl-5,8-dideazafolate on this enzyme and had suggested the possibility of the presence of a TMPS isozyme (27). The TMPS used during this study behaved very similarly to KB or HeLa cells not only with respect to the K_m values, but also with respect to the inhibitory action by 10-deazaaminopterin (data not shown). Although the difference of K_m values between two leukemic cells might be due to the difficulty of determining the K_m values of 5,10-CH_2-H_4PteGlu for TMPS and/or to the different purity of the two AML-derived preparations, the present data might also suggest the possibility that there are various forms of TMPS among the malignant hematopoietic cells that have been studied. This possibility is still under investigation in our laboratory. Sirot-

nak *et al.* (7) reported that 10-ethyl-10-deazaaminopterin was usually more extensively polyglutamated in tumor cells than 10-deazaaminopterin, but both were polyglutamated to a somewhat similar extent in mouse small intestine. Our results showed that polyglutamates of 10-ethyl-10-deazaaminopterin acted as stronger inhibitors than 10-ethyl-10-deazaaminopterin, itself, and that 10-ethyl-10-deazaaminopterin and its polyglutamates behaved as competitive inhibitors, with respect to 5,10-CH_2-H_4PteGlu as a substrate, but as noncompetitive, or mixed-type inhibitors with respect to 5,10-CH_2-H_4PteGlu$_5$ as a substrate. Since, in cells, most of the folate cofactors exist as polyglutamate forms, it is likely that the mode of inhibition by 10-ethyl-10-deazaaminopterin in cells against TMPS is noncompetitive or mixed type, with respect to its cofactor. The selectivity in cytotoxicity of 10-ethyl-10-deazaaminopterin against different tissues could, to a large extent, be dependent on the different extent of polyglutamation in these tissues.

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