Inhibition of Glycinamide Ribonucleotide Formyltransferase and Other Folate Enzymes by Homofolate Polyglutamates in Human Lymphoma and Murine Leukemia Cell Extracts


ABSTRACT

In order to determine the biochemical basis for the cytotoxicity of homofolates, poly-γ-glutamyl derivatives of homofolate (H4PteGlu) and tetrahydrohomofolate (H4HptGlu) were synthesized and tested as inhibitors of glycaminide ribonucleotide formyltransferase (GARFT), aminoimidazolecarboxamide ribonucleotide formyltransferase (AICARFT), thymidylate synthase, and serine hydroxymethyltransferase (SHMT) in extracts of Manca human lymphoma and L1210 murine leukemia cells. The most striking inhibitions are that of GARFT by (6R,S)-H4HptGlu and (6R,S)-5-methyl-H4HptGlu at 6 and 8 mM, respectively. Both of these inhibitors of GARFT are potent, selective inhibitors of GARFT in extracts of human and murine lymphoma cells. Our results indicate that both inhibition and polyglutamation are essential for homofolate toxicity.

INTRODUCTION

Homofolates differ from folate only in having an additional methylene group between the C-9 and N-10 positions (Fig. 1). Homofolates increase the survival time of L1210 leukemic mice (1, 2). The antimetabolism of homofolates and methotrexate differ in that (a) homofolates do not inhibit DHF3 reduction (1, 3) and (b) homofolates show enhanced antimetabolism activity against a strain of L1210 that is resistant to the DHF reduction inhibitor methotrexate (1, 2). Homofolates inhibit growth of mammalian tumor cell lines in culture (1, 4-7), an effect that is antagonized by additional purines (4, 6, 7).

MATERIALS AND METHODS

Cell Cultures. Human B-cell lymphoma (Manca, SKD-HLA) (9) and murine leukemia (L1210) cells were grown in RPMI 1640 medium (folate concentration: 2.2 μM) supplemented with 10% horse serum, 2 mM glutamine, 0.01 mM sodium pyruvate, and 0.05 mM 2-mercaptoethanol in a humidified 5% CO2:95% air atmosphere at 37°C (10). A strain of Manca cells was made 180-fold resistant to the TS inhibitor 5,8-dideaza-10-propargylfolic acid (11) by adding the inhibitor at increasing concentrations up to 0.1 mM to the culture medium. For studies of growth inhibition, the original Manca cell strain was grown in RPMI 1640 medium with 2 mM l-glutamine and 10% fetal calf serum (doubling time, 15–17 h). Exponentially growing cells, 7000 in 0.14 ml medium, were transferred to 96-well microplates and incubated for 2 h. Antifolates, dissolved in Hanks' balanced salt solution (GIBCO Laboratories, Grand Island, NY) were added to a total of 0.15 ml. After 72 h (the number of cells per well had reached 180,000), 0.015 ml of 15 mg/ml MTT (Sigma M 2128) was added. Incubation was continued for 2 h and the MTT formazan produced was solubilized by adding 0.17 ml of 0.01 N HCl in isopropanol and quantitated spectrophotometrically (12). To calculate the inhibition of growth to be expected from two agents added simultaneously and acting independently, the fractional inhibition method of Harvey (13) was used. When expressed as the fraction inhibited, the expected effect is the sum of the two separate inhibitory effects minus their product. If the observed inhibition by the combination is greater than expected, the combination is synergistic; if smaller, it is antagonistic. In reversal experiments, inosine (Sigma) was added to 0.1 mM with the inhibitors. Control cultures produced 158 ± 8 mmol MTT formazan (nine determinations) in 2 h.

Cell Exacts. A modification of the method of Caperelli (14) was used for the preparation of cell extracts. Frozen cell pellets were thawed and mixed with two volumes of phosphate buffered saline. The suspension was treated with an equal volume of 10 mm potassium phosphate, pH 7.5, containing (v/v) 25% ethylene glycol, 10% dimethylsulfoxide, 0.25% sucrose, 10 mM 2-mercaptoethanol, 1 mM EDTA, and the following protease inhibitors (Sigma): 25 μg/ml α-1-antipain, 0.12 units/ml aproatin and 1 mM phenylmethylsulfonyl fluoride. After 20 min at 4°C, the cells were disrupted in a glass homogenizer and the solution centrifuged at 11,000 × g for 10 min. The supernatants used for enzyme assays contained 6–9 mg protein/ml as determined by the Lowry method, with bovine serum albumin as standard (15).

H4PteGlu Derivatives. Homofolic acid, homopteroic acid, and (6R,S)-5-methyl-H4HptGlu were provided by the Drug Development Branch of the National Cancer Institute through the courtesy of Dr. J. A. R. Mead. Poly-γ-glutamyl derivatives of H4PteGlu with 1–5 additional glutamates were prepared from homofolate acid as described (16).
ENZYME INHIBITION BY HOMOFOLATE POLYGLUTAMATES

Tetrahydro derivatives, (6R,S)-H4HPteGlu and its polyglutamates, were prepared from the corresponding homofolates by catalytic reduction (17). The 6S diastereomer of H2HPteGlu was prepared by enzymatic reduction of H4HPteGlu as described for the preparation of (6S)-tetrahydrofolate from folic acid (18). The product, (6S)-H2HPteGlu, was purified by chromatography on a DEAE-cellulose column, using a linear 0.2-1.0 M NaCl gradient as described (18), except that the pH of the buffer was 7.5 and the temperature, 25°C. Five-mI fractions were collected and (6S)-H2HPteGlu (1.5 μmol) was recovered from a peak eluted at 220-250 ml. To obtain H3H2HPteGlu and (6R)-H2HPteGlu, (6R,S)-H2HPteGlu (1.5 μmol) was incubated with 220 milliunits of Lactobacillus casei TS in the standard TS assay mixture with increased sodium chloride (9 M NaCl). The sample was diluted with 30 ml water and chromatographed on DEAE-cellulose. H2HPteGlu and the unreacted 6R form of H2HPteGlu were eluted from the peak extending from 280 through 350 ml. Pure H2HPteGlu was recovered at 325-350 ml. A mixture of H2HPteGlu and (6R)-H2HPteGlu was recovered at 280-320 ml. To resolve the mixture, fractions were pooled, diluted with an equal volume of NaCl-free elution buffer, and rechromatographed on DEAE-cellulose as described (18), except that the pH of the buffer was 7.5, 70 nmol (6R,S)-tetrahydrofolate, 50 nmol pyridoxal phosphate, pH 7.5, 70 mmol HCHO, 2.5 mmol MgCl2, 0.16 Mmol EDTA, 16 mmol 2-mercaptoethanol, and 0.13 μmol (6R,S)-tetrahydrofolate, and 16 mmol 5'-H2UMP, 10 μCi/μmol, in 0.2 ml for 10 min at 30°C. The tritium released was determined in the charcoal supernatant as described (24). The activity in the control (high TS Manca strain) was 2.6 ± 0.6 μg/mg protein. SHMT (EC 2.1.2.1) was measured by following the conversion of [3-14C]serine to H[14C]HO. Extracts (0.08 mg protein) were preincubated 5 min at 37°C with 50 μmol potassium phosphate, pH 7.5, 70 mmol (6R,S)-tetrahydrofolate, 50 nmol pyridoxal phosphate and 5 μmol 2-mercaptopethanol in 0.19 ml. The reaction was begun by the addition of 0.01 ml of 0.25 M [3-14C]serine (0.012 μCi/μmol). After incubation for 15 min at 37°C, H[14C]HO was determined as the inhibition assay but at 0.05 M it antagonized the inhibitory effect of H4HPteGlu. In L1210 extracts, NaCl began to interfere with inhibition at 0.03 M. The preparations of (6R)-H4HPteGlu and H2HPteGlu could not be tested at concentrations above 1.5 μM. The (6S)-H2HPteGlu preparation was less dilute than the other two samples and could be used at concentrations through 5 μM without interference by NaCl.

AICARFT (EC 2.1.2.3) was assayed in Manca extracts as described for GARFT except that 22 μmol of KCl were included. GAR was replaced by 50 nmol of aminomimidazolecarboxamide ribonucleotide, and incubation was at 37°C (23). The activity of control extracts was 1.5 ± 0.1 μmol/mg protein. To assay for TS (EC 2.1.1.45), the cell extract (0.1 mg protein) was incubated with 8 μmol of Tris-Cl, pH 7.4, 4 μmol MgCl2, 2.5 μmol HCHO, 0.16 μmol EDTA, 16 μmol 2-mercaptoethanol, 0.13 μmol (6R)-H4HPteGlu, and 16 mmol 5'-H2UMP, 10 μCi/μmol, in 0.2 ml for 5 min at 30°C. The 5-formyl compound was formed from (6S)-tetrahydrofolate (18) and formic acid (21). TMTX was supplied by Warner-Lambert Co, Ann Arbor, MI.

Enzyme Assays. Extracts of Manca and L1210 cells were used for enzyme assays except for TS. Extracts of 5,8-dideaza-10-propargylfolate-resistant cells containing 10 times the TS activity of extracts of the original strain were used as the source of TS. Reaction rates were linear with time and enzyme concentration. All assays were begun by addition of enzyme unless otherwise specified. Blanks lacking enzyme or one of the substrates gave negligible activity. The specific activity is expressed in milliunits, or nanomoles of product formed/min per mg protein ± SD for four extracts. GARFT (EC 2.1.2.2) was assayed by a modification of a continuous spectrophotometric method (22). Cell extracts (0.1 mg protein) were incubated with 45 μmol of Tris-Cl, pH 7.5, 90 μmol of 2-mercaptoethanol, 54 nmol of (6R)-10-formyltetrahydrofolate and 0.22 μmol of a,b-GAR in 0.9 ml for 5 min at 30°C. Incubation mixtures were flushed with argon before initiating the reaction. The formation of tetrahydrofolate was measured by following the increase in absorbance at 298 nm using a molar absorbance of 19,700. Activities in the controls lacking inhibitors were 2.9 ± 0.7 and 7.9 ± 1.2 μmol/mg protein for Manca and L1210, respectively. Since the inhibitors (6S)-H4HPteGlu, (6R)-H4HPteGlu, and H2HPteGlu were purified on NaCl gradients, it was necessary to test the effect of NaCl on GARFT assays. In Manca extracts, NaCl at 0.01-0.04 M did not interfere with the inhibition but at 0.05 M it antagonized the inhibitory effect of H4HPteGlu. In L1210 extracts, NaCl began to interfere with inhibition at 0.03 M. The preparations of (6R)-H4HPteGlu and H2HPteGlu could not be tested at concentrations above 1.5 μM. The (6S)-H2HPteGlu preparation was less dilute than the other two samples and could be used at concentrations through 5 μM without interference by NaCl.

AICARFT (EC 2.1.2.3) was assayed in Manca extracts as described for GARFT except that 22 μmol of KCl were included, GAR was replaced by 50 nmol of aminomimidazolecarboxamide ribonucleotide, and incubation was at 37°C (23). The activity of control extracts was 1.5 ± 0.1 μmol/mg protein. To assay for TS (EC 2.1.1.45), the cell extract (0.1 mg protein) was incubated with 8 μmol of Tris-Cl, pH 7.4, 4 μmol MgCl2, 2.5 μmol HCHO, 0.16 μmol EDTA, 16 μmol 2-mercaptoethanol, 0.13 μmol (6R)-H4HPteGlu, and 16 mmol 5'-H2UMP, 10 μCi/μmol, in 0.2 ml for 10 min at 30°C. The tritium released was determined in the charcoal supernatant as described (24). The activity in the control (high TS Manca strain) was 2.6 ± 0.6 μg/mg protein. SHMT (EC 2.1.2.1) was measured by following the conversion of [3-14C]serine to H[14C]HO. Extracts (0.08 mg protein) were preincubated 5 min at 37°C with 50 μmol potassium phosphate, pH 7.5, 70 mmol (6R,S)-tetrahydrofolate, 50 nmol pyridoxal phosphate and 5 μmol 2-mercaptopethanol in 0.19 ml. The reaction was begun by the addition of 0.01 ml of 0.25 M [3-14C]serine (0.012 μCi/μmol). After incubation for 15 min at 37°C, H[14C]HO was determined as the dimedon derivative (25, 26). Activities were 5.9 ± 0.4 and 20.0 ± 5.7 μmol/mg protein in Manca and L1210 extracts, respectively.

To determine if any degradation of H4HPteGlu polyglutamate chains took place during enzyme assays, H4HPteGlu (18 nmol) was incubated with a Manca cell extract (0.1 mg protein), 45 μmol of Tris-Cl, pH 7.5, and 90 μmol 2-mercaptoethanol in 0.9 ml for 5 min at 30°C. Protein was removed by precipitation with 0.09 ml of 2 N perchloric acid at 4°C and samples were neutralized with 0.02 ml of 10 N KOH. Zero-time samples were used as controls. Aliquots of the resulting supernatant (2.5 nmol in 0.05 ml) were analyzed by reversed-phase high-performance liquid chromatography (27). H4HPteGlu was eluted as a single peak before and after incubation with the cell extract. No species corresponding to H4HPteGlu-5 was detected.

RESULTS

Growth Inhibition

Homofolates were tested as inhibitors of the growth of cultured Manca cells. H4HPteGlu and 5-methyl-(6R,S)-H4HPteGlu inhibit growth 50% at 6 and 8 μM, respectively, as shown in Fig. 2 (each value represents the mean of three determinations,
ENZYME INHIBITION BY HOMOFOLATE POLYGLUTAMATES

Fig. 2. Effect of combinations of homofolate (HPteGlu) or (6R,S)-5-methylH4HPteGlu with TMTX on growth of cultured Manca cells. A, cultures were grown for 72 h in 96-well microtiter plates at 7000 cells per well and growth was estimated by MTT reduction. Cultures were treated with varying concentrations of HPteGlu: added alone (△), with 10 nM TMTX (□), with 0.1 mM inosine (▲), and with 10 nM TMTX and 0.1 mM inosine (▲). Dashed line, additive effects expected for each combination of agents, calculated from the results of TMTX (A), and with 10 nM TMTX and 0.1 mM inosine (▲). Fig. 2A shows that inhibition by HPteGlu derivatives is reversed >90% by the effect of the DHF reductase inhibitor TMTX and the potency of HPteGlu derivatives is similar in Manca cell extracts. The potency of (6R,S)-H4HPteGlu is similar in both extracts. In Manca extracts, inhibition by (6R,S)-H4HPteGlu is competitive with (6R)-10-formyltetrahydrofolate, with a Kᵢ value (29) of 0.3 μM (data not shown). Polyglutamates of HPteGlu are weak inhibitors of GARFT and the potency of H2HPteGlu is between that of HPteGlu and (6R,S)-H4HPteGlu (Fig. 3). Higher concentrations of H2HPteGlu were not tested because NaCl in this preparation interfered with inhibition assays.

Enzyme Inhibition

GARFT. The influence of the following factors on the inhibition of GARFT by HPteGlu derivatives was studied: (a) polyglutamate chain length; (b) the extent of reduction of the pyrazine ring; (c) stereochemical configuration at C6; and (d) enzyme source. The inhibition curves for GARFT are shown in Fig. 3. Based on a minimum of three trials, the standard deviations of the plotted values were within 15%. The IC₅₀ values derived from the inhibition curves are shown in Table 1. The IC₅₀ values used for comparisons of inhibitory potency are valid for these experiments because none of the inhibitors bind tightly, that is, with Kᵢ values in the 5–60 μM range (28). The IC₅₀ values obtained for the Manca extracts can be compared with those of L1210 because assay conditions are identical and both extracts have similar Kᵢ values for (6R)-formyltetrahydrofolate (25 and 35 μM, respectively) and for GAR (29 and 40 μM).

With H₂HPteGlu₁₋₆, increasing polyglutamate chain length enhances inhibition in both Manca and L1210 cell extracts. In Manca extracts, (6R,S)-H₂HPteGlu₂ is 12 times more inhibitory than Glu₁. As more Glu residues are added to (6R,S)-H₂HPteGlu, little further change occurs until Glu₅, which is 50 times more inhibitory than Glu₁. The inhibitory pattern is similar in L1210 extracts, except that Glu₁ is 1/6 as active as in Manca cell extracts. The potency of (6R,S)-H₂HPteGlu₆ is similar in both extracts. In Manca extracts, inhibition by (6R,S)-H₂HPteGlu₆ is competitive with (6R)-10-formyltetrahydrofolate, with a Kᵢ value (29) of 0.3 μM (data not shown). Polyglutamates of HPteGlu are weak inhibitors of GARFT and the potency of H₂HPteGlu₆ is between that of HPteGlu and (6R,S)-H₂HPteGlu (Fig. 3). Higher concentrations of H₂HPteGlu₆ were not tested because NaCl in this preparation interfered with inhibition assays.

The effect of the stereochemical configuration at carbon 6 of H₂HPteGlu₆ on GARFT inhibition is shown in Fig. 4 and Table 1. In Manca extracts the natural (6S) form is about four times more inhibitory than the unnatural (6R) form. The results differ in L1210 extracts, the (6R) form being slightly more inhibitory than the (6S) form. The (6R,S) mixture gives inhibition curves similar to those calculated for the equimolar mixture by the fractional inhibition method (13) in both Manca and L1210 extracts. The individual diastereomers of H₂HPteGlu₆ were chosen for further studies because Glu₆ is the most potent (6R,S)-H₂HPteGlu derivative against L1210 GARFT.

We had observed that (6R)-H₂HPteGlu₆ was eluted from DEAE-cellulose more slowly than the (6S) form. During preparation of (6R)-H₂HPteGlu₆, the (6R,S) mixture is incubated with formaldehyde in the TS assay to form 5,11-methylene H₂HPteGlu₆. It is therefore possible that (6R)-H₂HPteGlu₆ is recovered as its 5,11-methylene derivative after chromatography on DEAE-cellulose. To exclude the possibility that formation of 5,11-methylene-H₂HPteGlu₆ alters inhibitory potency, (6R,S)-H₂HPteGlu₆ (1.6 mM) was incubated with 12 mM HCHO in 0.05 M Tris-HCl and 0.2 μM 2-mercaptoethanol, pH 7.5, for 5 min at 37°C before adding to the GARFT assay mixture. This treatment had no effect on the potency of (6R,S)-H₂HPteGlu₆.
ENZYME INHIBITION BY HOMOFOLATE POLYGLUTAMATES

Fig. 4. Effect of diastereomers of H₄HPteGlu₆ on GARFT activity in cell extracts. A, Manca; B, L1210. S, natural diastereomer (S); R, unnatural diastereomer (R); RS, equimolar mixture of diastereomers (A). In A and B, the dashed line represents the additive values expected for an equimolar RS mixture, calculated from lines R and S by the fractional inhibition method (13).

Table 1 Inhibition of enzyme activities by homofolates

Concentration causing 50% inhibition of enzyme activity. IC₅₀ values interpolated from curves in Figs. 3–6.

<table>
<thead>
<tr>
<th>IC₅₀ (μM)</th>
<th>GARFT</th>
<th>AICARFT</th>
<th>TS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Manca</td>
<td>L1210</td>
<td>Manca</td>
</tr>
<tr>
<td>HPteGlu₆</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>HPteGlu₄</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>HPteGlu₃</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>HPteGlu₂</td>
<td>&gt;20</td>
<td>2.5</td>
<td>&gt;20</td>
</tr>
<tr>
<td>HPteGlu</td>
<td>&gt;20</td>
<td>2.1</td>
<td>8.0</td>
</tr>
<tr>
<td>HPteGlu₆</td>
<td>&gt;20</td>
<td>2.1</td>
<td>8.8</td>
</tr>
<tr>
<td>(6R,S)-HPteGlu₆</td>
<td>&gt;15</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>(6R,S)-HPteGlu₅</td>
<td>1.3</td>
<td>8.5</td>
<td>&gt;20</td>
</tr>
<tr>
<td>(6R,S)-HPteGlu₄</td>
<td>2.1</td>
<td>2.9</td>
<td>&gt;20</td>
</tr>
<tr>
<td>(6R,S)-HPteGlu₃</td>
<td>1.2</td>
<td>1.3</td>
<td>7.3</td>
</tr>
<tr>
<td>(6R,S)-HPteGlu₂</td>
<td>0.3</td>
<td>1.1</td>
<td>6.2</td>
</tr>
<tr>
<td>(6R,S)-HPteGlu</td>
<td>0.51</td>
<td>0.60</td>
<td>10</td>
</tr>
<tr>
<td>(6S)-HPteGlu₆</td>
<td>0.37</td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td>(6R)-HPteGlu₆</td>
<td>1.5</td>
<td>0.33</td>
<td></td>
</tr>
</tbody>
</table>

AICARFT. The effect of HPteGlu polyglutamate chain length and reduction of the pyrazine ring on the inhibition of AICARFT was studied in extracts of Manca cells. With HPteGlu₆, inhibitory potency increases with polyglutamate chain length, a sharp increase occurring between Glu₃ and Glu₄, with little change thereafter (Table 1, Fig. 5A). Conversion to the corresponding (6R,S)-H₄HPteGlu forms weakens inhibition (Table 1, Fig. 5B); the Glu₆ derivatives of (6R,S)-H₄HPteGlu are 3–5-fold less inhibitory than the corresponding HPteGlu derivatives. The inhibition curve for (6R,S)-H₄HPteGlu₄ (not shown) was nearly identical to that for Glu₄. As with the unreduced series, a marked increase in the potency of (6R,S)-H₄HPteGlu occurs between Glu₃ and Glu₄, with no further increase with Glu₅, and the effect of Glu₆ is weaker than that of Glu₄.

TS. Inhibitory potency of HPteGlu increases with the length of the polyglutamate chain, but effects are weaker for TS than for AICARFT. The Glu₃ and Glu₄ polyglutamates of HPteGlu are slightly inhibitory (Table 1 and Fig. 6). The curve for HPteGlu₆ (not shown) is nearly identical to that for Glu₆. If the concentration of (6R,S)-tetrahydrofolate is decreased from 650 to 180 μM and that of dUMP from 0.08 to 0.04 μM, the IC₅₀ of HPteGlu₆ is lowered from 9 μM to the previously reported value, 2 μM (30). Conversion of HPteGlu derivatives to their tetrahydro derivatives reduces their inhibitory potency against TS (Table 1).

SHMT. In extracts of Manca and L1210 cells, SHMT activity was inhibited less than 50% by HPteGlu₄ and (6R,S)-H₄HPteGlu₄ at 20 μM.

DISCUSSION

The results show that HPteGlu inhibits growth of cultured Manca cells, and that this effect is antagonized by the DHF reductase inhibitor, TMTX. In contrast, inhibition of Manca cell growth by a tetrahydro derivative of HPteGlu, 5-methyl-H₄HPteGlu, is not antagonized by TMTX. This supports the hypothesis that the mechanism for growth inhibition by HPteGlu involves reduction to H₄HPteGlu. Our findings are consistent with results of earlier studies with L1210 leukemic mice: (a) the antitumor effects of homofolates are increased if
animals are inoculated with a methotrexate-resistant strain of L1210 with elevated levels of DHF reductase (1, 2), and (b) they are diminished when animals are pre-treated with methotrexate (1, 31). The reduction of H2HPteGlu to HJHPteGlu occurs in tissues of normal and leukemic mice in vivo and in cell-free extracts (3, 32). The in vivo reduction reaction is blocked in tissues of animals pretreated with methotrexate (32).

Reversal of the inhibitory effects of HPteGlu on Manca cell growth by inosine indicates that a step in purine biosynthesis is involved. Previous studies have shown that synthesis of formylGAR is inhibited by homofolates in sarcoma-180 (7). We have now demonstrated that polyglutamates of H2HPteGlu are potent inhibitors of GARFT activity in cell-free extracts of Manca and L1210 tumor cells. Polyglutamates of HPteGlu are considerably less inhibitory. This supports the hypothesis that a reduction step is required. Since GARFT is inhibited by the natural diastereomer of H2HPteGlu, growth inhibition by HPteGlu could be explained by its conversion to a polyglutamate of (6S)-H2HPteGlu. Polyglutamation of homofolates is likely to occur in vivo, since homofolates are substrates for mammalian folypolyglutamate synthetase (33, 34). Other antifolates have been shown to be converted into polyglutamated forms with increased potency as inhibitors of DHF reductase, TS, and AICARFT (10, 35–39).

Polyglutamate chain length also regulates the activity of folic acid as GARFT substrates and inhibitors; studies of the avian enzyme show that the ratio of \( V_{\text{max}} \) to \( K_a \) for polyglutamates of the 10-formyltetrahydrofolate precursor, 5,10-methenyltetrahydrofolate, increases with chain length from Glu1 to Glu6 (40). Folic acid polyglutamates inhibit avian GARFT and their potency increases from Glu1 to Glu6 (40).

Inhibition of AICARFT by homofolates may also be involved in the inhibition of cell growth, since polyglutamates of unreduced HPteGlu inhibit AICARFT. These effects, however, are more modest than the inhibition of GARFT by H2HPteGlu derivatives. TS is an unlikely target enzyme since inhibition of cell growth by homofolates is completely reversed by inosine in the absence of thymidine.

The results show a lack of stereospecificity for the inhibition of GARFT in mammalian systems. Both the 6S and 6R diastereomers of H2HPteGlu show inhibitory effects of the same magnitude against GARFT. This is consistent with the observation that both (6S) and (6R)-H2HPteGlu have antitumor activity in L1210 leukemic mice (41). Similarly, both diastereomers at C-6 of the GARFT inhibitor 5,10-dideazatetrahydrofolate are active against L1210 cell growth (42). With L. casei, by contrast, (6R)-H2HPteGlu inhibits growth (IC50, 0.018 \( \mu \)M) while the 6S form acts as a growth factor (43). The (6R) form of H2HPteGlu is more potent (IC50, 0.1 \( \mu \)M) than the (6S) form (IC50, 5 \( \mu \)M) as an inhibitor of GARFT activity in extracts of L. casei. It is of interest that avian GARFT is inhibited by the unnatural diastereomer of its substrate, 10-formyltetrahydrofolate (22).

The target enzyme for HPteGlu in vivo would depend on relative rates of reduction and polyglutamation of this agent. As shown in Fig. 7, the balance among these reactions could determine whether GARFT, AICARFT, or TS is the principal site of inhibition. Our results suggest that HPteGlu is converted to H2HPteGlu polyglutamates. Since GARFT is the only reaction found to be sensitive to H2HPteGlu in cell extracts, it is the most likely target in vivo. With 5-methyl-H2HPteGlu, no reduction step is required. It is possible that 5-methyl-H2HPteGlu is demethylated, since it is a substrate for N-5-methyltetrahydrofolate-homocysteine methyltransferase (44). Conversion of HPteGlu to folate coenzyme analogues such as 5-methyl or 11-formyl-H2HPteGlu may also occur, since H2HPteGlu is a substrate for 10-formyltetrahydrofolate synthetase and other folate-dependent enzymes (5, 44). The potency of polyglutamates of these coenzyme analogues as enzyme inhibitors is as yet undetermined. The intracellular distribution of polyglutamated folate substrates may also play a role since the degree of polyglutamation can regulate enzyme reaction rates and sensitivities to antifolates (23, 35–40). Conversely, these folate pools may be altered by homofolate coenzyme analogues competing for the same enzyme sites.

The ability of TMTX to enhance the potency of 5-methyl-H2HPteGlu as a growth inhibitor is similar to effects of combining an inhibitor of DHF reductase with 5,10-dideazatetrahydrofolate (45, 46). It is unlikely that subtoxic levels of TMTX inhibit GARFT by causing depletion of the GARFT substrate 10-formyltetrahydrofolate (47). TMTX may block the AICARFT reaction indirectly instead by causing the accumulation of dihydrofolate polyglutamates, since it is known that these compounds inhibit AICARFT (36, 47). It is also possible that polyglutamates of both HPteGlu and H2HPteGlu are formed in the presence of low concentrations of TMTX, blocking both AICARFT and GARFT. It has been suggested that homofolates become inhibitors of purine biosynthesis after conversion to polyglutamate forms (8, 48). Our studies provide direct evidence for this view and point to GARFT as the most likely target enzyme.

**ACKNOWLEDGMENTS**

The authors wish to thank Dr. Jacob Selhub of the U. S. Department of Agriculture Human Nutrition Research Center on Aging, Tufts University, Boston, MA, for performing high-performance liquid chromatography analyses of homofolate polyglutamates.

**REFERENCES**

3. Plante, L. T., Crawford, E. J., and Friedkin, M. Enzyme studies with new...
Inhibition of Glycinamide Ribonucleotide Formyltransferase and Other Folate Enzymes by Homofolate Polyglutamates in Human Lymphoma and Murine Leukemia Cell Extracts


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/49/1/158