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


Department of Biochemistry, Tufts University Health Sciences Campus, Boston, Massachusetts 02111 [J. T., Y. G., R. L. K.]; Memorial Sloan-Kettering Cancer Center, New York, New York 10021 [F. M. S.]; Department of Biochemistry, University of South Alabama, Mobile, Alabama 36689 [B. R. M., M. G. N.]; and Drug Synthesis Section, Southern Research Institute, Birmingham, Alabama 35255 [J. R. P.]

ABSTRACT

In order to determine the biochemical basis for the cytotoxicity of homofolates, poly-γ-glutamyl derivatives of homofolate (HPteGlu) and tetrahydrohomofolate (H₄HPteGlu) were synthesized and tested as inhibitors of glycaminamide 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,5R)-H₄HPteGlu polyglutamates are weak inhibitors of human AICARFT (IC₅₀, 6–10 μM). Polyglutamates of HPteGlu, however, are more inhibitory to AICARFT, with HPteGlu having IC₅₀ values close to 2 μM. Polyglutamates of HPteGlu and of H₄HPteGlu are weaker inhibitors of thymidylate synthase (IC₅₀, 8 μM for HPteGlu and >20 μM for HPteGlu), respectively. Both of these effects are reversed by 0.1 mM inosine. Trimeretate at a subinhibitory concentration, 10 nm, antagonizes growth inhibition by HPteGlu, raising the IC₅₀ from 6 to 64 μM, but enhances inhibition by (6R,5S)-5-methyl-H₄HPteGlu from 8 to 5 μM. Our results support the view that homofolates become toxic after conversion to H₄HPteGlu polyglutamates which block GARFT, a step in purine biosynthesis.

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 M 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-propargylfolate (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 vol of 0.015 ml of 15 mg/ml MTX (Sigma M 2128) was added. Incubation was continued for 2 h and the MTX formazan produced was solubilized by adding 0.17 ml of 0.01 M 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 nmol MTT formazan (nine determinations) in 2 h.

Cell Extracts. 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-antitrypsin, 0.12 units/ml aprotinin 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). HPteGlu Derivatives. Homofolic acid, homoperoxic acid, and (6R,5S)-5-methyl-H₄HPteGlu 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 HPteGlu with 1–5 additional glutamates were prepared from homoperoxic acid as described (16).

Received 6/24/88; revised 9/20/88; accepted 10/5/88.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by NCI Grants CA 10914 (R. L. K.), CA 22764, CA 10843, and MA 26136 (J. R. P.) from the National Cancer Institute, USPHS/Department of Health and Human Services. 2 To whom requests for reprints should be addressed, at Department of Biochemistry, Tufts University Health Sciences Campus, Boston MA 02111.

The abbreviations used are: DHF, dihydrofolate; HPteGlu, homofolate; H₄HPteGlu, tetrahydrohomofolate; TMTX, trimeretate; GARFT, glycaminamide ribonucleotide formyltransferase; AICARFT, aminoimidazolecarboxamide ribonucleotide formyltransferase; TS, thymidylate synthase; SHMT, serine hydroxymethyltransferase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IC₅₀, the inhibitor concentration causing 50% inhibition; mu, milliunits.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Tetrahydro derivatives, (6R,S)-H₄HPteGlu and its polyglutamates, were prepared from the corresponding homofolates by catalytic reduction (17). The 6S diastereomer of H₄HPteGlu was prepared by enzymatic reduction of HPteGlu as described for the preparation of (6S)-tetrahydrofolate from folic acid (18). The product, (6S)-H₄HPteGlu₆, 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-milliliter fractions were collected and (6S)-H₄HPteGlu₆ (1.5 μmol) was recovered from a peak extending from 280 to 250 ml. To obtain H₂HPteGlu₆ and (6R)-H₄HPteGlu₆, the unreacted 6R form of H₄HPteGlu₆ was eluted from the peak extending from 280 to 350 ml. Pure H₂HPteGlu₆ was recovered at 325–350 ml. A mixture of H₂HPteGlu₆ and (6R)-H₄HPteGlu₆ 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 a DEAE-cellulose column, using a linear 0.2–0.6 M NaCl gradient. Pure (6R)-H₂HPteGlu₆ was recovered at 600 ml. All homofolate derivatives were identified and quantitated spectrophotometrically. The molar absorbance coefficients used were 19,400 at 281 nm (pH 13) for HPteGlu, 19,700 at 282 nm (pH 7) for H₂HPteGlu, and 20,500 at 295 nm (pH 7) for H₄HPteGlu.

**Other Materials.** To prepare the natural (6R) diastereomer of 10-formyl-tetrahydrofolate, (6S)-5-formyl-tetrahydrofolate was incubated in 0.1 N HCl for 1 h, followed by neutralization (20). The 5-formyl compound was formed from (6S)-tetrahydrofolate (18) and formic acid (21). TMTX was supplied by Warner-Lambert Co, Ann Arbor, MI.

**RESULTS**

**Growth Inhibition**

Homofolates were tested as inhibitors of the growth of cultured Manca cells. HPteGlu 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, ± S.D.).
except that HPteGlu was replaced by (6R,S)-5-methylH4HPteGlu.

and HPteGlu when added singly (13). B, cultures were grown as described for A, expected for each combination of agents, calculated from the results of TMTX (A), and with 10 nM TMTX and 0.1 μM inosine (Δ). Dashed line, additive effects of HPteGlu: added alone (O), with 10 nM TMTX (•), with 0.1 μM inosine (△), and with 10 nM TMTX and 0.1 μM inosine (△). Expected results were obtained for each combination of agents, calculated from the results of TMTX and HPteGlu when added singly (13). B, cultures were grown as described for A, except that HPteGlu was replaced by (6R,S)-5-methylH4HPteGlu.

SD < 5%). Inhibition by these agents is reversed >90% by the simultaneous addition of inosine, indicating a block in purine biosynthesis. The effects of HPteGlu and 5-methylH4HPteGlu are also completely reversed by (6S)-5-formyltetrahydrofolate at 10 μM (not shown). To determine if the inhibitory action of HPteGlu requires a reduction step mediated by DHF reductase, the effect of the DHF reductase inhibitor TMTX was tested. In the presence of 10 nM TMTX, inhibition by HPteGlu is diminished 10-fold (Fig. 2A). When added alone at 10 μM, TMTX inhibits cell growth less than 5%. With 5-methyl-H4HPteGlu, the reduction step is by-passed and no antagonism by TMTX would be expected. Fig. 2B shows that inhibition by 5-methyl-H4HPteGlu is not antagonized but enhanced by TMTX.

**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 Km values for (6S)-formyltetrahydrofolate (25 and 35 μM, respectively) and for GAR (29 and 40 μM).

With H4HPteGlu6, increasing polyglutamate chain length enhances inhibition in both Manca and L1210 cell extracts. In Manca extracts, (6R,S)-H4HPteGlu2 is 12 times more inhibitory than Glu1. As more Glu residues are added to (6R,S)-H4HPteGlu, little further change occurs until Glu5, which is 50 times more inhibitory than Glu1. The inhibitory pattern is similar in L1210 extracts, except that Glu5 is 1/6 as active as in Manca cell extracts. The potency of (6R,S)-H4HPteGlu6 is similar in both extracts. In Manca extracts, inhibition by (6R,S)-H4HPteGlu6 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)-H4HPteGlu6 (Fig. 2). 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 H4HPteGlu6 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 H4HPteGlu6 were chosen for further studies because Glu6 is the most potent (6R,S)-H4HPteGlu6 derivative against L1210 GARFT.

We had observed that (6R)-H4HPteGlu6 was eluted from DEAE-cellulose more slowly than the (6S) form. During preparation of (6R)-H4HPteGlu6, the (6R,S) mixture is incubated with formaldehyde in the TS assay to form 5,11-methylene (6R,S)-H4HPteGlu6. It is therefore possible that (6R)-H4HPteGlu6 is recovered as its 5,11-methylene derivative after chromatography on DEAE-cellulose. To exclude the possibility that formation of 5,11-methylene-H4HPteGlu6 alters inhibitory potency, (6R,S)-H4HPteGlu6 (1.6 mM) was incubated with 12 mM HCHO in 0.05 M Tris-HCl and 0.2 M 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)-H4HPteGlu6.
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 R:S mixture, calculated from lines R and S by the fractional inhibition method (13).

Table 1 Inhibition of enzyme activities by homofolates

<table>
<thead>
<tr>
<th>Compound</th>
<th>GARFT Manca</th>
<th>L1210</th>
<th>AICARFT Manca</th>
<th>TS Manca</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPteGlu</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>HPteGlu2</td>
<td>&gt;20</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>
<td>&gt;20</td>
</tr>
<tr>
<td>HPteGlu4</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>HPteGlu5</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>2.5</td>
<td>&gt;20</td>
</tr>
<tr>
<td>HPteGlu6</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>2.1</td>
<td>8.0</td>
</tr>
<tr>
<td>(6R,S)-H4HPteGlu</td>
<td>&gt;15</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>(6R,S)-H4HPteGlu2</td>
<td>1.3</td>
<td>8.5</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>(6R,S)-H4HPteGlu3</td>
<td>2.1</td>
<td>2.9</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td>(6R,S)-H4HPteGlu4</td>
<td>1.2</td>
<td>1.3</td>
<td>7.3</td>
<td>18</td>
</tr>
<tr>
<td>(6R,S)-H4HPteGlu5</td>
<td>0.3</td>
<td>1.1</td>
<td>6.2</td>
<td>&gt;20</td>
</tr>
<tr>
<td>(6R,S)-H4HPteGlu6</td>
<td>0.51</td>
<td>0.60</td>
<td>10</td>
<td>&gt;20</td>
</tr>
<tr>
<td>(6S)-H4HPteGlu6</td>
<td>0.37</td>
<td>0.74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(6R)-H4HPteGlu6</td>
<td>1.5</td>
<td>0.33</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 5. Effect of polyglutamate chain length on inhibition of AICARFT by homofolates in extracts of Manca cells. The total number of glutamate residues is indicated on each curve. A, HPteGlu; B, H4HPteGlu.

Fig. 6. Effect of polyglutamate chain length on inhibition of TS by HPteGlu in extracts of Manca cells. The total number of glutamate residues is indicated on each curve.

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 HPteGlu, 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 Glu4-6 derivatives of (6R,S)-H4HPteGlu are 3–5-fold less inhibitory than the corresponding HPteGlu derivatives. The inhibition curve for (6R,S)-H4HPteGlu (not shown) is nearly identical to that for Glu5. 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 HPteGlu 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...
Fig. 7. Potential metabolites and inhibitory sites of homofolates.

ENZYME INHIBITION BY HOMOFOLATE POLYGLUTAMATES

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 H4HPteGlu polyglutamates. Since GARFT is the only reaction found to be sensitive to H4HPteGlu in cell extracts, it is the most likely target in vivo. With 5-methyl-H4HPteGlu, no reduction step is required. It is possible that 5-methyl-H4HPteGlu is demethylated, since it is a substrate for N-5-methylerthyrodryfolate-homocysteine methyltransferase (44). Conversion of HPteGlu to folate coenzyme analogues such as 5-methyl or 11-formyl-H4HPteGlu may also occur, since H4HPteGlu 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-H4HPteGlu 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 H4HPteGlu 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


enzymine inhibition by homofolate polyglutamates


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