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


Department of Biochemistry, Tufts University Health Science Campus, Boston, Massachusetts 02111; Memorial Sloan-Kettering Cancer Center, New York, New York 10021; Southern Research Institute, Birmingham, Alabama 35225

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 glycinamide ribonucleotide formyltransferase (GARFT), amidinoimidazolecarboxamide 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)-H₄HPteGlu in extracts of Manca human lymphoma and 1.12111 murine leukemia cells.

INTRODUCTION

Homofolate differs 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 antitumor activity of homofolates and methotrexate differ in that (a) homofolates do not inhibit DHFR reductase (1, 3) and (b) homofolates show enhanced antitumor activity against a strain of L1210 that is resistant to the DHF reductase inhibitor methotrexate (1, 2). Homofolates inhibit growth of mammalian tumor cell lines in culture (1, 4–7), an effect that is antagonized by added purines (4, 6, 7). The synthesis of homofolate (6, 7). GARFT, which catalyzes the transfer of the formyl group from 10-formyltetrahydrofolate to GAR, is inhibited by the 5,11-methenyl derivative of H₄HPteGlu in Sarcoma 180 extracts (8). We now show that H₄HPteGlu polyglutamates are potent, selective inhibitors of GARFT in extracts of human and murine lymphoma cells. Our results indicate that both reduction and polyglutamation are essential for homofolate toxicity.

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-propargylglycine (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 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 nmol MTT formazan (nine determinations) in 2 h.

Cell Extracts. A modification of the method of Capelleri (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 mM sucrose, 10 mM 2-mercaptoethanol, 1 mM EDTA, and the following protease inhibitors (Sigma): 25 μg/ml a-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). H₄HPteGlu Derivatives. Homofolic acid, homopteroic acid, and (6R,S)-5-methylH₄HPteGlu 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 homopteroic acid as described (16).
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 HPteGlu 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-m1 fractions were collected and (6S)-H₂HPteGlu (1.5 μmol) was recovered from a peak eluted at 220-250 ml. To obtain H₃HPteGlu and (6R)-H₃HPteGlu, (6R,S)-H₃HPteGlu (1.5 μmol) was incubated with 220 ml of Lactobacillus casei TS in the standard TS assay mixture with increased dUMP (30 μmol) in 30 ml for 5 h at 37°C (19). Sodium chloride (9 mmol) was included to minimize TS inhibition by polyglutamated antifolates (19). 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 to isolate (6R)-H₂HPteGlu by the above procedure, except that a 0.2-0.6 M NaCl gradient was used. Pure (6R)-H₂HPteGlu 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 H₃HPteGlu (17).

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.

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-HCl, pH 7.5, 90 μmol of 2-mercaptoethanol, 54 nmol of (6R)-10-formyltetrahydrofolate and 0.22 μmol of α,β-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)-H₃HPteGlu, (6R)-H₃HPteGlu, 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 assay but at 0.05 M it antagonized the inhibitory effect of H₃HPteGlu. In L1210 extracts, NaCl began to interfere with inhibition at 0.03 M. The preparations of (6R)-H₃HPteGlu and H₃HPteGlu could not be tested at concentrations above 1.5 μmol. The (6S)-H₃HPteGlu preparation was less dilute than the other two samples and could be used at concentrations through 5 μmol 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 aminomimidazolcarboxamide 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-HCl, pH 7.4, 4 μmol MgCl₂, 2.5 μmol HCHO, 0.16 μmol EDTA, 16 μmol 2-mercaptoethanol, 0.13 μmol (6R,S)-tetrahydrofolate, and 16 nmol [5-¹⁴C]dUMP. 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, the reaction was stopped and the mixture released was determined in the charcoal supernatant as described (24). The activity in the control (high TS Manca strain) was 2.6 ± 0.6 μmol/mg protein. SHMT (EC 2.1.2.1) was measured by following the conversion of [³⁻¹⁴C]serine to H¹⁴CH₂COOH. Extracts (0.08 mg protein) were preincubated 5 min at 37°C with 50 μmol potassium phosphate, pH 7.5, 70 nmol (6R,S)-tetrahydrofolate, 50 nmol pyridoxal phosphate and 5 μmol 2-mercaptoethanol 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, H¹⁴CH₂COOH 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 HPteGlu polyglutamate chains took place during enzyme assays, HPteGlu (18 nmol) was incubated with a Manca cell extract (0.1 mg protein), 45 μmol of Tris-HCl, 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). HPteGlu was eluted as a single peak before and after incubation with the cell extract. No species corresponding to HPteGlu-5 was detected.

RESULTS

Growth Inhibition

Homofolates were tested as inhibitors of the growth of cultured Manca cells. HPteGlu and 5-methyl-(6R,S)-HPteGlu inhibit growth 50% at 6 and 8 μM, respectively, as shown in Fig. 2 (each value represents the mean of three determinations,
were grown as described for the simulations of HPteGlu: added alone (O), 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 (A). The IC₅₀ values used for comparisons of inhibitory potency are values derived from the inhibition curves are shown in Table 1.

With H₄HPteGlu₂-₆, 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 Glu₁ 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 H₄HPteGlu₁₆ is between that of HPteGlu and (6R,S)-H₄HPteGlu₆ (Fig. 3). Higher concentrations of H₄HPteGlu₁₆ were not tested because NaCl in this preparation interfered with inhibition assays.

The effect of the stereochemical configuration at carbon 6 of H₄HPteGlu₁₆ 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 H₄HPteGlu₁₆ were chosen for further studies because Glu₁₆ is the most potent (6R,S)-H₄HPteGlu derivative against L1210 GARFT.

We had observed that (6R)-H₄HPteGlu₁₆ was eluted from DEAE-cellulose more slowly than the (6S) form. During preparation of (6R)-H₄HPteGlu₁₆, the (6R,S) mixture is incubated with formaldehyde in the TS assay to form 5,11-methylene-H₄HPteGlu₁₆. It is therefore possible that formation of 5,11-methylene-H₄HPteGlu₁₆ alters inhibitory potency, (6R,S)-H₄HPteGlu₁₆ (1.6 mM) was incubated with 12 mM HCHO in 0.05 M Tris-Cl 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)-H₄HPteGlu₁₆.
ENZYME INHIBITION BY HOMOFOLATE POLYGLUTAMATES

Fig. 4. Effect of diastereomers of H₄HPteGlu₂ on GARFT activity in cell extracts. A, Manca; B, L1210. S, natural diastereomer; R, unnatural diastereomer; RS, equimolar mixture of diastereomers. 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

Inhibition of enzyme activities by homofolates

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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 Glu₂ 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 derivatives of (6R,S)-H₄HPteGlu are 3–5-fold less inhibitory than the corresponding HPteGlu derivatives. The inhibition curve for (6R,S)-H₄HPteGlu₂ (not shown) was nearly identical to that for Glu₂. As with the unreduced series, a marked increase in the potency of (6R,S)-H₄HPteGlu occurs between Glu₂ and Glu₄, with no further increase with Glu₆, 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 Glu₁ and Glu₄ polyglutamates of HPteGlu are slightly inhibitory (Table 1 and Fig. 6). The curve for HPteGlu₂ (not shown) is nearly identical to that for Glu₂. 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 HPteGlu₁ and (6R,S)-H₄HPteGlu₁ 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
The in vivo reduction reaction is growth by inosine indicates that a step in purine biosynthesis is trexate (1, 31). The reduction of H2HPteGlu to HJHPteGlu they are diminished when animals are pre-treated with methotrexate. 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 μM) while the 6S form acts as a growth factor (43). The (6R) form of H2HPteGlu5 is more potent (IC50, 0.1 μM) than the (6S) form (IC50, 5 μM) 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 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 HPteGlu 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.

Folic acid polyglutamates inhibit avian GARFT and their activity in L1210 leukemic mice. Polyglutamation of HPteGlu is demethylated, since it is a substrate for N-5-methyltetrahydrofolate-homocysteine methyltransferase (44). Polyglutamation of homofolates is likely to occur in vivo, since homofolates are substrates for mammalian folylpolyglutamate 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 folates as GARFT substrates and inhibitors; studies of the avian enzyme show that the ratio of Vmax to the Km 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 H2HPteGlu6 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 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.

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REFERENCES


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